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Characterisation of the Role of Two Two-Component Signal Transduction Systems and a Putative Zinc Metalloprotease In the Virulence of *Streptococcus pneumoniae*

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A thesis submitted to the University of Glasgow for the degree of Ph.D.

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Declaration

This thesis is the original work of the author unless stated otherwise

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Abstract

Streptococcus pneumoniae (the pneumococcus) is an important human pathogen responsible for life threatening invasive diseases such as pneumonia, septicaemia and meningitis, as well as less severe but highly prevalent infections. Despite the availability of antibiotics and a vaccine, pneumococcal infection contributes significantly to human mortality worldwide. There is a need for a greater understanding of the molecular mechanisms of pathogenicity of this pathogen, which will enable the development of novel therapeutic targets and vaccine candidates.

This thesis aimed to evaluate the contribution of several pneumococcal two-component systems to virulence by analysis of null mutants in a murine model of infection. In addition, a putative zinc metalloprotease, ZmpB, located immediately downstream of one of the TCS, was analysed for its role in virulence.

Data indicated that one of the systems studied, TCS08, does not contribute significantly to virulence in serotype 2 pneumococcus, but may have a slightly more important role in a serotype 3 background. A second two-component system, TCS09, was found to be essential for virulence in a serotype 2. Despite the completely avirulent phenotype of the mutant, no difference in expression of many of the previously identified pneumococcal virulence-associated genes was detected in the mutant compared to its isogenic parental strain. Microarray analysis indicated that in serotype 2, TCS09 may be involved in nutrient perception. TCS09 was found to be required for full virulence in a serotype 3 strain. In this strain, mutants appeared to be impaired in their ability to disseminate from the lungs to the blood in a pneumonia model of infection, but were not attenuated in virulence following direct inoculation into the systemic circulation. These data provide evidence that virulence determinants can behave differently based on the genetic background of the parental strain.

ZmpB was found to contribute significantly to pneumococcal virulence in a serotype 2 strain. Further analysis of the contribution of this protein to infection found that ZmpB appears to have a role in promoting inflammation. Thus this work has identified ZmpB as being a novel pneumococcal virulence factor. The role of this protein in inflammation is being investigated further.

This thesis has thus identified several genes important in the virulence of *S. pneumoniae* and work is currently ongoing to assess the potential of these genes as future vaccine or drug candidates. Data presented within this work also provides evidence that virulence determinants can behave differently based on the genetic background of the parent bacterial strain. This important observation could have significant implications for the future characterisation of pneumococcal virulence factors and may apply to other bacterial pathogens.

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Abbreviations

AIDS	Acquired immunodeficiency syndrome
ABC	ATP-binding cassette
AIP	Auto-inducing peptide
AOM	Acute otitis media
APS	Ammonium persulphate
ATP	Adenine tri-phosphate
BAB	Blood agar base
BALF	Bronchi alveolar lavage fluid
BALT	Bronchus-associated lymphoid tissue
BHI	Brain heart infusion media
BLAST	Basic Local Alignment Search Tool
bp	Base pair
BSA	Bovine serum albumin
CAP	Community-acquired pneumonia
Cbp	Choline binding protein
cDNA	Complementary DNA
CNS	Central nervous system
CFU	Colony Forming Units
CHP	Conserved hypothetical protein
CR	Complement receptor
CRP	C-reactive protein
CSP	Competence stimulating peptide
^{o} C	Degrees Celsius
d	Day(s)
DFI	Differential fluorescence induction
dH_2O	Distilled water
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphates
DOC	Deoxycholate

DTT	Dithiothreitol
ECM	Extracellular matrix
EDTA	Diaminoethanetetra-acetic acid disodium salt
ELISA	Enzyme-linked immunosorbent assay
ery	Erythromycin
FACS	Fluorescence-activated cell sorting
FBS	Foetal bovine serum
FCA	Freunds complete adjuvant
FIA	Freunds incomplete adjuvant
gDNA	Genomic DNA
g	Gram(s) or g-force (when referring to centrifugation speeds)
GSK	GlaxoSmithKline
HEXXH/E	Motif for a zinc metalloprotease
HK / *hk*	Histidine kinase
HP	Hypothetical protein
hr	Hour(s)
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
i.v	Intravenous
i.n.	Intranasal
i.p.	Intraperitoneal
IPTG	Isopropyl-1-thio-β-D-galactopyranoside
IU	International units
Kb	Kilobase
K.Da	Kilodaltons
KPSI	Kilo pounds per square inch
L	Litre(s)
LB	Luria broth
LPXTH	Cell anchor motif
LRT	Lower respiratory tract

LytA	major pneumococcal autolysin
mRNA	Messenger ribonucleic acid
MsrA	Methionine sulphoxide reductase
µg	Microgram(s)
µl	Microlitre(s)
mg	Milligram(s)
ml	Millilitre(s)
mm	Millimeter(s)
mM	Millimolar
min	Minute(s)
M	Molar
MCP	Methyl-accepting chemotaxis protein
MHC	Major histocompatability complex
MLST	Multilocus sequence typing
MMP	Matrix metalloprotease (proteinase)
mRNA	Messenger RNA
MRSA	Multi [methicillin] resistant *Staphylococcus aureus*
n	Population size (for statistical analysis)
nM	Nanomolar
ND	Not done
NO	Nitric oxide
OD	Optical density
OMP	Outer membrane protein / porin
O/N	Overnight
%	Percent
PAF	Platelet activating factor
PAGE	Polyacrylamide gel electrophoresis
PBP	Penicillin binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pg	Picogram

Pln	Pneumolysin
PMNs	Polymorphonuclear leukocytes
PTS	phospho-transferase system
rpm	Revolutions per minute
RNA	Ribonucleic acid
RR / *rr*	Response regulator
RT	Room temperature
RT-PCR	Reverse transcription polymerase chain reaction
RTI	Respiratory tract infection
sec	Second(s)
SDS	Sodium-dodecyl sulphate
SEM	Standard error of the mean
spp.	Bacterial species
STM	Signature-tagged mutagenesis
TCS	Two-component system
TIGR	The Institute of Genomic Research
TEMED	N',N',N',N'-tetramethylethylenediamine
TNF	Tumour necrosis factor
tRNA	Transfer RNA
U	Units
URT	Upper respiratory tract
UV	Ultraviolet
V	Volt(s)
Wk	Week(s)
WT	wild-type
ZmpB	Zinc metalloprotease B
Δ	Designates an isogenic mutant strain
2D	2-dimensional
-/-	Knock-out / deficient strain (in reference to murine models)

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Chapter 1

Introduction

Part 1: *Streptococcus pneumoniae*

1.1 Overview of *Streptococcus pneumoniae*

Streptococcus pneumoniae (the pneumococcus) is a Gram-positive, facultatively anaerobic bacterium, that is an important human pathogen. The bacterium was first identified in 1880 independently by Pasteur and Sternberg (Davis *et al.*, 1990), and since has been studied extensively which has led to important scientific discoveries. The principle of transformation was first identified in the pneumococcus and this led to the discovery of nucleic acid as the hereditary material (Avery *et al.*, 1944). Initial studies with penicillin and bacterial polysaccharide capsules were also carried out with the pneumococcus (Alonso De Velasco *et al.*, 1995).

There are several tests available for identifying *S. pneumoniae* (Gillespie, 1994 and Kellogg *et al.*, 2001). When grown on blood agar, colonies display a characteristic draughtsman-like appearance and produce a zone of α-haemolysis. Staining with Gram's stain reveals dark, coccoid cells that are present singly, in pairs or in short chains. Sensitivity of *S. pneumoniae* to optochin (hydrocupreine hydrochloride) can be used to distinguish the pneumococcus from other viridans Streptococcus (Gardam and Miller, 1998). Solubility in bile / deoxycholate and several biochemical tests are often used in diagnostic laboratories for the identification of *S. pneumoniae*. Generally, several tests are used in combination as individual tests may result in erroneous identification. For example, optochin sensitivity is thought to be over 98 % sensitive with a specificity of 100 % (Kellogg *et al.*, 2001), but atypical isolates have been identified that are resistant to optochin (Borek *et al.*, 1997). PCR using known *S. pneumoniae* genes, such as pneumolysin, may be used, but pneumolysin has been identified in other Streptococcal species (Kearns *et al.*, 2000).

Over 90 different serotypes of *S. pneumoniae* exist and these are classified by differences in the chemical structure of the polysaccharide capsule that surrounds the organism (Kalin, 1998). The capsule serotype can have a large influence on the virulence of the organism, and certain serotypes are more frequently associated with clinical disease than others. Thus, it is necessary to be able to distinguish between serotypes. The Quellung reaction is a simple test that involves mixing serotype-specific anti-capsule antisera with bacterial cultures. The presence of capsule is detected by swelling and clumping of organisms which is attributed to the binding of capsule-specific antibody. Recently, multilocus sequence typing (MLST) has been applied to the typing of *S. pneumoniae* (Enright and Spratt, 1998). This technique involves sequencing seven selected

housekeeping genes and analysing results using a database to obtain an allelic profile. The method can distinguish between pneumococcal strains and has been useful for identifying virulent and antibiotic resistant clones of the pneumococcus (Enright and Spratt, 1999).

1.2 *S. pneumoniae* as a human pathogen

1.2.1 Carriage

S. pneumoniae is primarily a human pathogen, although horses may also become infected (Benson and Sweeney, 1984). The situation with equine infection is interesting as, unlike in human infection, all isolates are serotype 3 and appear to form a clonal group. These isolates are virtually identical to each other but genetically distinguishable from human type 3 isolates which suggests that they may have been acquired from humans relatively recently (Whatmore *et al.*, 1999). Furthermore, equine isolates appear to lack pneumolysin and autolysin activities, two virulence factors thought to be important in human pneumococcal infection (Whatmore *et al.*, 1999). It is not known if equine isolates can infect humans, and vice versa, but studies have shown that farmworkers are usually colonised with pneumococcal serotypes distinct from that found in horses, suggesting that cross-infection does not occur (Burrell *et al.*, 1986).

In humans, *S. pneumoniae* asymptomatically colonises the upper respiratory tract (URT) of healthy individuals. Virtually all individuals are thought to be colonised at some stage and carriage rates may exceed 70 % in some areas. Carriage rates are higher in young children or in environments where crowding is present (Obaro and Adegbola, 2002). Carriage generally results in an antibody-mediated immune response that is capable of eliminating the organism, and this response is serotype-specific. However, in some individuals, carriage can result in infection and this is thought to be due to a recently acquired serotype rather than one that has been present asymptomatically for some time (Obaro and Adegbola, 2002). Infection and carriage is spread by aerosols and is acquired via the respiratory tract.

1.2.2 Human disease

S. pneumoniae can cause a range of illnesses and these can be grouped into upper respiratory tract infections (URTI), lower respiratory tract infections (LRTI) and disseminated / invasive infection. Of these otitis media, pneumonia, septicaemia and meningitis are the most prevalent. Table 1.1 lists other disease states that have been attributed to pneumococcal infection. Many of

these result from complications following systemic infection and although they are generally rare they can dramatically increase the seriousness of the disease. Nevertheless, 4 % of all cases of septic arthritis and osteomyelitis in children are attributed to *S. pneumoniae* (Tan, 2000).

Infection	Disease state	Ref.
URT	Otitis media	Tuomanen, 2001
	Sinusitis	Conrad and Jenson, 2002
LRT	Pneumonia	Kayhty and Eskola, 1996
Invasive	Septicaemia	Kayhty and Eskola, 1996
	Meningitis	Meli *et al.*, 2002
	Haemolytic uremic syndrome	Brandt *et al.*, 2002
	Osteomyelitis	Tan, 2000
	Cellulitis	Parada and Maslow, 1999
	Pericarditis	Tan, 2000
	Endocarditis	Tan, 2000
	Septic arthritis	Peters *et al.*, 2000

Table 1.1 Diseases caused by *S. pneumoniae*

Pneumonia, otitis media, septicaemia and meningitis are the most common infections caused *by S. pneumoniae*. Other infections generally arise following bloodstream infection and often occur as isolated cases.

1.2.3 Factors predisposing to infection

Invasive pneumococcal disease is rare in immunocompetent individuals. The immune system is very efficient in clearing pneumococcal infection, so those most at risk have underlying conditions that weaken the immune system. Infants under the age of 2 years and elderly persons over 65 years of age have an increased risk of pneumococcal infection and these are the groups in which pneumococcal disease is most prevalent (figure 1.1). Other at-risk groups include splenectomised patients or those with a spleen defect, HIV-infected patients and other immunocompromised individuals (Parsons and Dockrell, 2002). Patients with sickle-cell anaemia are particularly susceptible to pneumococcal infections, possibly due to the accumulation of sickle cells in the liver and spleen, which may inhibit normal functioning

(Kumar and Clark, 1998). IgG2 deficiencies are frequently associated with infections with encapsulated bacteria, such as *S. pneumoniae* and *Haemophilus influenzae* as this is the predominant antibody class produced in response to polysaccharide antigens (Escobar-Pérez *et al.*, 2000). Alcoholism and smoking may also predispose to pneumococcal infection. There also appears to be a genetic basis for susceptibility to infection as some ethnic groups such as black Americans, Alaskan natives and certain American-Indian populations are at higher risk than others for developing pneumococcal disease (Schoenmaker *et al.*, 2002). However, this could also be due to socio-economic factors such as alcoholism, low education/health care, higher incidence of AIDS, sickle-cell disease.

A recent episode of viral infection, particularly with adenovirus or influenza virus, is another factor predisposing to disease. In such circumstances it is thought that prior infection exposes hosts ligands that can enhance adhesion of the pneumococcus (Hakansson *et al.*, 1994).

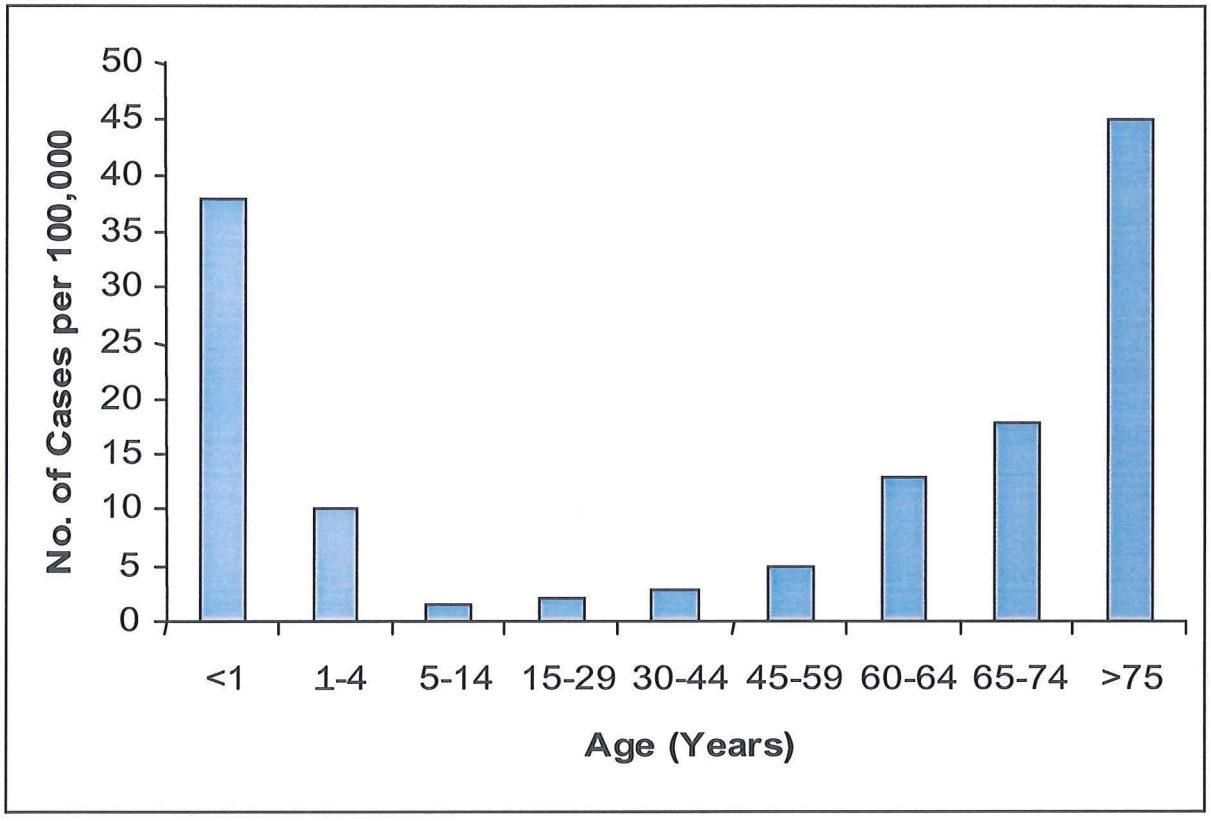

Figure 1.1 Incidence of pneumococcal invasive disease with age

The figure shows that the incidence of invasive disease is highest at the extremes of age. Data is taken from mean annual incidence in England and Wales between 1995 and 1997. Modified from Sleeman *et al.*, 2001.

1.2.4 Epidemiology

Epidemiology of pneumococcal disease is complicated, varying both geographically and within populations. Exact figures for disease may depend on differences in identification and reporting strategies and is further complicated by the range of other pathogens with the ability to induce similar symptoms to those observed in pneumococcal infection and the difficulty in obtaining appropriate samples for microbiological diagnosis.

S. pneumoniae causes significant morbidity and mortality world wide, particularly among young children and the elderly. Disease is largely endemic, although epidemics may occur in conditions of crowding and institutional settings, such as military camps (Gray *et al.*, 1999). The pneumococcus is the major cause of acute bacterial pneumonia, otitis media and sinusitis, and is a significant cause of meningitis (Kalin, 1998). It is estimated that over 1 million deaths occur each year in children under the age of 5 due to *S. pneumoniae* (Hausdorff *et al.*, 2000a). A large proportion of these deaths occur in developing countries. In the United States, pneumococcal infection causes approximately 40,000 deaths each year, making it one of the top 10 causes of death. A significant proportion of these deaths are thought to be preventable by the 23-valent vaccine. Meningitis and bacteraemia have the highest case fatality rates, especially in those most susceptible to disease. Pneumococcal meningitis, when not fatal, can result in serious neurological sequelae such as loss of hearing in up to 50 % of survivors (Meli *et al.*, 2002). Although non-invasive infections are associated with lower mortality, these infections still contribute significantly to morbidity and are a huge burden for health trusts due to the costs involved in treating such infections. It is estimated that 30 % of all children under the age of 5 years are seen annually by general practitioners in the UK, with the majority of these infections being caused by *S. pneumoniae* (Choo and Finn, 2001).

Within the UK, case fatality may be as high as 38 % (with pneumococcal meningitis), despite the use of appropriate antimicrobial agents (Kyaw *et al.*, 2002a). In Scotland an increase in the annual incidence of invasive disease was reported between 1988 and 1999, together with an increase in the prevalence of antibiotic non-susceptible isolates (Kyaw *et al.*, 2002a). This highlights the clinical impact of this human pathogen, despite the availability of antibiotics and vaccines.

Although most, if not all, capsular serotypes have been associated with clinical infection, some serotypes are more commonly associated with infection than others. Furthermore, serotypes prevalent in invasive disease vary geographically, may depend on age, and also have been shown to change over time. Studies have shown that types 1, 2 and 3 accounted for up to 75 % of bacteraemic cases in the US and Europe during the beginning of the 20th century (Kalin, 1998). However, serotypes 1 and 2 are now rarely isolated from clinical infections, with type 2 being uncommon in all geographical regions for several decades. The reason for alterations in serotype prevalence over time are not fully understood but could be due to different socioeconomic circumstances, availability of antimicrobials, underlying medical conditions or the prevalence of other pathogens. Capsule switching between strains has also been reported and could contribute to infections with strains previously thought to have low incidence of infection (Coffey *et al.*, 1998). In the United States, types 6B, 14, 18C and 19F are prevalent in young children, but serotype distribution in adults tends to be more evenly spread and includes a greater number of serotypes (Kalin, 1998). Despite having similar populations, the serotypes causing disease in the UK differ to those prevalent in the United States. A wider range of serotypes causing disease is present in developing countries than in developed countries. The virulent serotype 3 that is often found to be associated with fatal illness predominates in Spain and the United States (Wuorimaa and Kayhty, 2002). One study even highlights the contribution of specific serotypes to different disease manifestations (Haustdorff *et al.*, 2000b).

1.3 Vaccines

1.3.1 Polysaccharide vaccine

The pneumococcal polysaccharide capsule is a major virulence determinant and anti-capsular immunoglobulin (Ig) G2 antibodies are an important host response in clearing pneumococcal infection (Catterall, 1999). The immunogenicity of a vaccine preparation depends on the antibody titres and antibody affinity induced, although the levels of antibody required for protection against pneumococcal disease are yet to be determined. As only a small proportion of the 90 pneumococcal serotypes cause disease in humans, current vaccines contain preparations of capsular material from disease-associated serotypes. A pneumococcal vaccine comprising purified polysaccharide preparations from 14 serotypes was first licensed in the United States in 1977. This was soon replaced, in 1983, with the 23-valent polysaccharide vaccine that is now

widely in use (Pneumovax II, Pnu-Immune 23). This vaccine contains purified polysaccharide antigens from 23 serotypes implicated in the majority of invasive disease in the developed world (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F). It is recommended for those at risk of pneumococcal infection and has been shown to be highly cost effective when used for vaccinating elderly persons (Ament *et al.*, 2000). Protection conferred is serotype specific so it does not protect against serotypes not included in the vaccine. Although effective in adults, this vaccine fails to induce a good response in children younger than 2 years, the age group with the highest incidence of invasive and non-invasive infection (Wuorimaa and Kayhty, 2002). This is because polysaccharide cannot associate with major histocompatability complex (MHC) class II on antigen presenting cells and is thus classed as a T-cell independent antigen. The mechanism by which polysaccharide does induce an immune response (via induction of interferon-gamma [IFN-γ] which can enhance antibody production by B cells and induce class switching) is not fully developed in infants. This may explain why T-independent antigens fail to induce an immune response, immunological memory and are unable to prime for subsequent boosting in this high-risk group. 20 % of the elderly population also respond poorly to polysaccharide antigen due to a weak IgG response (Wuorimaa and Kayhty, 2002). The polysaccharide vaccine does not provide any protection against carriage of the organism and does not protect against mucosal infections or prevent the spread of invasive or resistant strains (Wright *et al.*, 1981; Obaro and Adegbola, 2002). Protection is severely reduced in other groups at high risk from pneumococcal infection, including those who are HIV-positive.

1.3.2 Conjugate vaccine

Due to the ineffectiveness of the polysaccharide vaccines in infants, conjugate vaccines were developed. These vaccines covalently link polysaccharide antigens to a carrier protein to convert the immune response to a thymus-dependent one. The protein component in such preparations has the ability to elicit a T-cell dependent response and induce B cell memory, so that immunity is longer lasting. However T-cell memory is not induced. A conjugate vaccine developed against *Haemophilus influenzae* (Hib) has been hugely successful and is routinely used in many countries (Scheifele, 2001). Several conjugate vaccines have been developed and are in various stages of testing. One of these preparations, a 7-valent conjugate (Prevnar™ / Prevenar®, Wyeth Lederle Vaccines), was licensed in the United States in February, 2000 and since, over 17

million doses have been administered. The polysaccharides included in this vaccine comprise serotypes 4, 6B, 9V, 14, 18C, 19F and 23F. These are conjugated to the non-toxic CRM197 diphtheria carrier protein. Other conjugate vaccines in trials include 9- and 11-valent preparations. Studies with Prevnar™ have shown it to be protective against otitis media, pneumonia and invasive disease caused by vaccine serotypes (Black *et al.*, 2000; Eskola *et al.*, 2001). Serogroup cross protection may also be achieved. The vaccine has been shown to be immunogenic in infants under 2 years of age and other high-risk groups. The vaccine has also been shown to induce mucosal IgA responses in infants (Choo *et al.*, 2000). The conjugate has not yet been licensed for use in the UK.

Despite the success of the 7-valent vaccine, conjugate vaccines have several drawbacks. The manufacturing process involved means they are considerably more expensive than the 23-valent polysaccharide vaccine and that fewer serotypes can be included within each preparation. This will have a huge impact on the suitability of the vaccine to provide protection for all those who require it. Natural changes in serotype distribution, which have been discussed above, may have important implications for the use of pneumococcal conjugate vaccines. Although the 7 serotypes included in the vaccine currently account for over 80 % of invasive pneumococcal disease in the United States, during the beginning of the 20th century the same 7 serotypes accounted for as little as 15 % and 53 % of disease in adults and children, respectively (Feikin and Klugman, 2002). The use of the vaccine itself may also result in serotype replacement, through its ability to reduce colonisation of vaccine serotypes. Alternatively other pathogens may be able to colonise the URT resulting in new or increased infections due to such organisms. Indeed *S. pneumoniae* has been shown to inhibit the growth of *H. influenzae*, another respiratory tract pathogen) through production of hydrogen peroxide (Pericone *et al.*, 2000) and can desialylate the lipopolysaccharide of *H. influenzae* and *Neisseria meningitidis* (Shakhnovich *et al.*, 2002). Furthermore, although providing high coverage against disease in North America and some European countries, the vaccine will not provide sufficient protection for those in Africa, Asia and Oceania where a different set of serotypes are responsible for serious pneumococcal infections (Wuorimaa and Kayhty, 2002). One study in Finland demonstrated that the vaccine reduced 57 % of pneumococcal acute otitis media (AOM) due to vaccine-related serotypes (Eskola *et al.*, 2001). This is in contrast to the predicted 84 % protection against AOM in the United States with the same vaccine (Joloba *et al.*, 2001). The former study also reported an

increase in AOM due to non-vaccine serotypes (Eskola *et al.*, 2001), eluding to the risk of serotype replacement. This could be a potentially serious problem with the conjugate vaccines as it is not desirable to have a vaccine that increases the risk of infection against other organisms or strains.

1.3.3 Future vaccines

The inefficiency of the 23-valent vaccine in infants and the problems with cost and serotype coverage discussed with the conjugate vaccines increase the need for a new pneumococcal vaccine with increased serotype coverage and at a lower cost. Intense epidemiological investigations may increase our understanding of disease causing serotypes and their geographical prevalence but it would not be practical to tailor-make conjugate vaccines to suit individual populations, and those in developing countries would not be able to afford such vaccines. Much research is currently focussed upon identifying suitable candidates for a new pneumococcal vaccine. An ideal vaccine would induce IgA and IgG antibodies with high affinity and would ideally contain a pneumococcal protein with the ability to induce a good antibody response that is cross protective against the majority of serotypes. Currently there is debate as to whether an ideal vaccine would reduce / prevent carriage of the organism. A vaccine able to prevent carriage would have the advantage of interfering with infection at the earliest possible stage, could reduce the spread of highly virulent and resistant organisms and could eventually induce herd immunity. Such a vaccine could, however, allow for the colonisation and subsequent infection by non-vaccine serotypes, although the inclusion of a pneumococcal protein antigen should help prevent this. Elimination of *S. pneumoniae* from the nasopharynx could, however, allow other pathogens to occupy this niche. A vaccine that was to include several protein antigens important at various stages of infection would significantly increase immunogenicity and efficacy and could remove the need to include any polysaccharide antigens. Several pneumococcal proteins, including pneumolysin and pneumococcal surface protein A (PspA), are currently being investigated as potential vaccine candidates (Alexander *et al.*, 1994; Paton 2001). Many new techniques have been applied to the identification of virulence factors and putative vaccine candidates of *S. pneumoniae*, including signature tagged mutagenesis (STM), differential fluorescence induction (DFI) and motif searches for surface expressed molecules (Pearce *et al*, 1993; Polissi, *et al.*, 1998; Lau *et al*, 2001; Wizemann *et al*, 2001; Hava and Camilli, 2002;

Marra *et al.*, 2002). These studies have identified a huge number of genes that are potential candidates, although detailed analysis of these must be performed to assess their suitability for inclusion in a future vaccine.

1.4 Antibiotics and resistance

At the beginning of the 20th Century, Dr. William Osler, an influential physician, described the pneumococcus as the 'captain of the men of death', due to its prevalence and the high mortality associated with infection. This situation changed dramatically with the introduction of sulphonamides in the late 1930s, followed by penicillin in 1945. The use of antibiotics at this time was so successful in treating pneumococcal infection that vaccine development was halted for many years. The first report of a pneumococcal strain showing resistance to penicillin was in Papua, New Guinea in 1969 (Tomasz, 1999). An editorial at the time evaluated this case and described the possibility of the spread of resistant *S. pneumoniae* as being extremely remote. This editorial could not have been further from the truth. Reports of penicillin non-susceptible strains in Spain were seen with increasing frequency and this progressed to an increase in resistant strains worldwide. Since the late 1980s, penicillin resistant strains have been increasingly isolated from infections in the United States (Figure 1.2) and other countries, and may be present in up to 80 % of isolates (Low 2000; Normark *et al.*, 2001). A recent study found that 61 % of clinical strains isolated in Scotland between 1992 and 1999 were not susceptible to penicillin. In the same study 14 % of isolates were resistant to erythromycin (Kyaw *et al.*, 2002b). The highly resistant penicillin isolates are also invariably resistant to other β-lactam antibiotics, including the third generation cephalosporins, cefotaxime and ceftriaxone (Linares *et al.*, 1992). Vancomycin tolerance in *S. pneumoniae* has also been reported (Novak *et al.*, 1999b; Normark *et al.*, 2001). Prescribing of antibiotics is thought to have played a major role in antibiotic resistance, although other factors may also be involved. Some serotypes are more frequently associated with antimicrobial resistance than others and include 6A, 6B, 9V, 14, 19F and 23F (Wuorimaa and Kayhty, 2002). These serotypes are included in the Prevnar™ 7-valent conjugate vaccine. Capsular switch has also been reported in highly resistant clones and may facilitate the spread of multi-resistant determinants to other serotypes (Tomasz, 1999).

Resistance to antibiotics may be achieved through several mechanisms and is thought to be transferred between bacterial strains by horizontal gene transfer and homologous recombination

(Dowson *et al.*, 1997). This transfer is largely facilitated by the natural transformability of the pneumococcus. The alarming increase in multi-resistant strains, together with the problems associated with the vaccines, requires the design of new therapeutic agents or the introduction of a more effective vaccine to prevent pneumococcal infection.

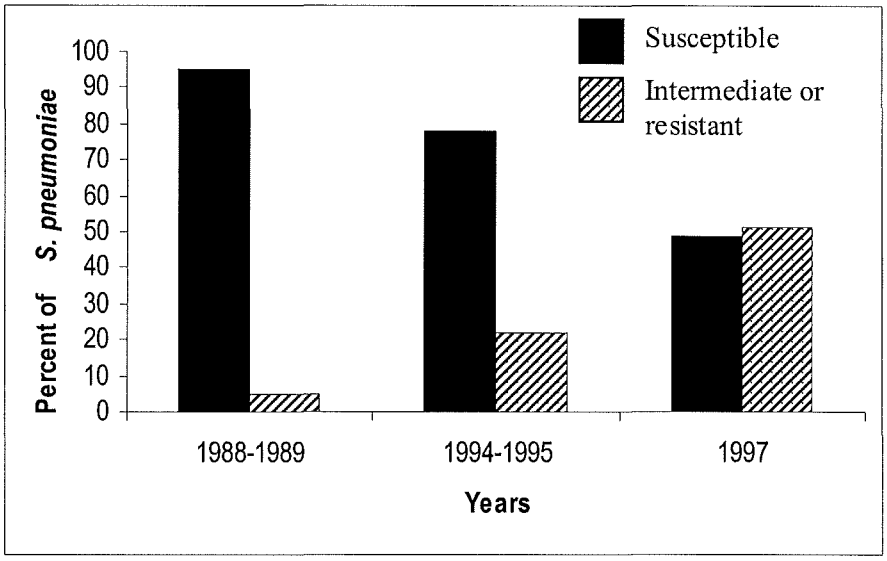

Figure 1.2: Emergence of penicillin resistance in *S. pneumoniae* in the United States, 1988-1997
The figure shows the increase of penicillin intermediate or resistant isolates in the United States over 10 years. Data was compiled by the Centre for Disease Control and Prevention and is modified from a presentation by The National Foundation for Infectious Diseases and the National Coalition for Adult Immunisation (http://www.nfid.org/library/pneumococcal).

1.5 Host response to disease

1.5.1 Defences in the upper airways

The immunocompetent host has a series of innate defences that are highly effective in protecting against *S. pneumoniae* and other respiratory pathogens. Airway defences include intact epithelial membranes, mucociliary clearance, antimicrobial peptides, local IgA production and the cough and sneeze reflexes. IgA is able to agglutinate microbial particles to promote mucociliary clearance but has no opsonic activity (Canto *et al.*, 1994). However, its role in preventing pneumococcal infection is unclear as human IgA deficiencies do not appear to have increased

susceptibility to infection (Kaliner, 1992). Small amounts of IgG may be present in the upper airways, although concentrations of this immunoglobulin are greater in the lower airways and lung tissue and also increase following changes in vascular permeability. IgG is a good opsonin and can thus fix complement. Lymphoid tissue in the URT includes tonsils, adenoids and bronchus-associated lymphoid tissue (BALT). Ig-bearing cells may be sampled by BALT and migrate to the lamina propria in the respiratory tract to produce local antibody for the mucosal surface.

1.5.2 Defences within the lung

The human lungs are usually sterile, so bacterial infection in this environment requires a more vigorous immune response to control microbial invasion and promote clearance. Resident alveolar macrophages are abundant in the lung environment and have an important role in clearing pneumococci (Gordon *et al.*, 2001). These cells are the first line of phagocytic defence and are also efficient immune effector cells, having the ability to release a range of mediators. Released mediators include pro-inflammatory cytokines interleukin 1-beta (IL-1β), interleukin 6 (IL-6), IFN-γ and tumour necrosis factor-alpha (TNF-α), enzymes (including lysozyme), biologically active lipids, oxygen metabolites and proteins (Roitt *et al.*, 1996a). The release of chemotactic factors attracts polymorphonuclear leukocytes (PMN)s, such as neutrophils, to the site of infection. Neutrophils are a major defence against microbial invasion via phagocytosis. They have cell surface receptors for IgG Fc region. Following phagocytosis, microbes are killed by the respiratory burst and through release of potent enzymes within the neutrophil. Certain complement components can also attract neutrophils.

Within the lung environment, the invading microbes may be coated with opsonins, such as surfactant (secreted by Type II pneumocytes), complement components (generated by the classical and alternative complement pathways) or antibody. This opsonisation greatly facilitates phagocytosis (Gordon *et al.*, 2000). Cytokines produced by T cells within the lung can enhance the phagocytic and bactericidal capabilities of macrophages. Figure 1.3 summarises some of the major host defences present within the lung environment.

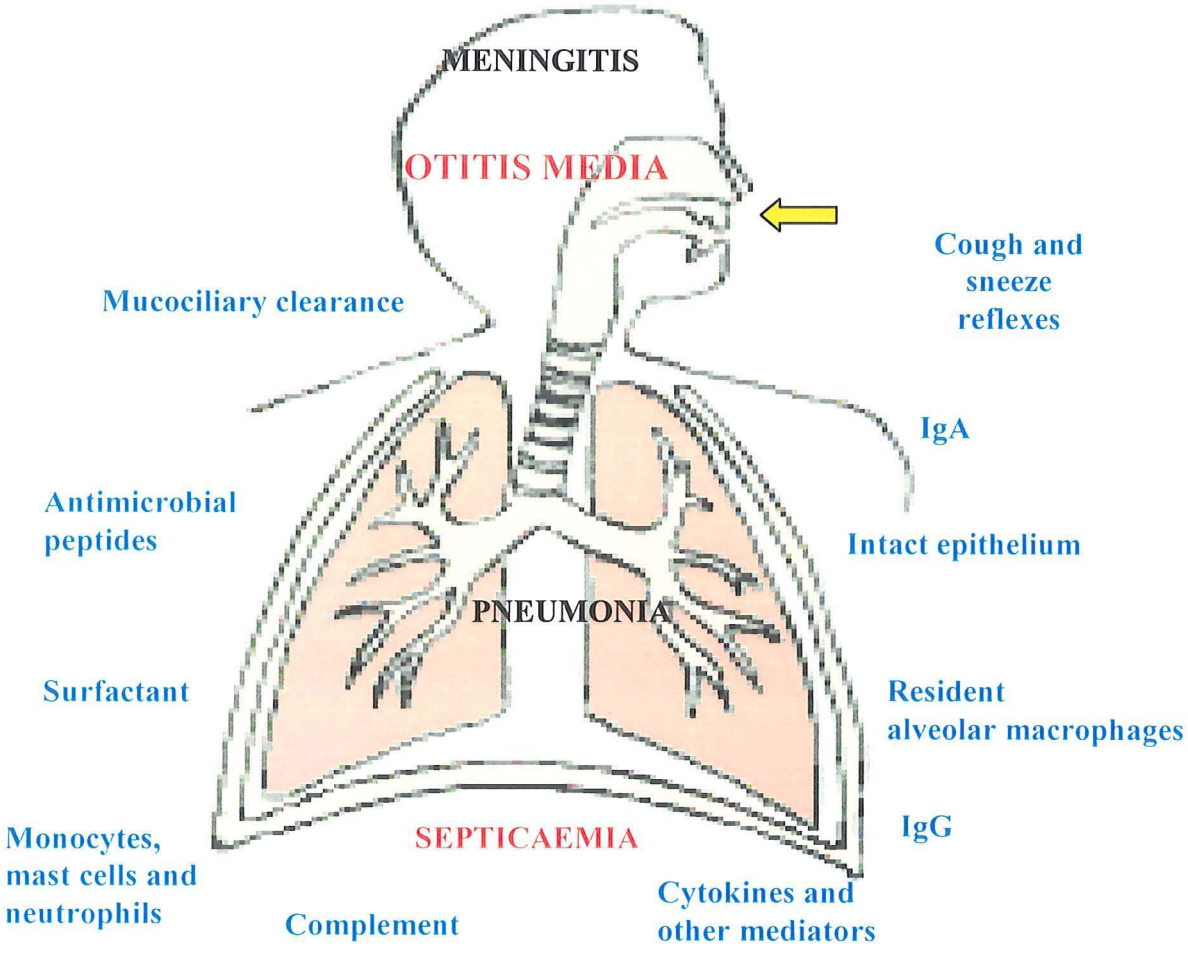

Figure 1.3: Host mechanisms involved in clearing pneumococcal infection

Some of the mechanisms detailed are specific for certain regions of the respiratory tract only. For example IgA tends to predominate in the upper airways whilst IgG is found in higher concentrations in the lower airways. Mucociliary clearance operates in the upper respiratory tract and intact epithelial barriers are important throughout the respiratory tract. Complement, cytokines, IgG and neutrophils are also important in systemic infection. Different aspects of the host immune response may be required at different sites of infection and for different disease states. The arrow indicates the general route of infection.

1.5.3 The inflammatory response

If the mechanical and immune defence barriers fail, an appropriate inflammatory response is generated, the initiation of which involves the release of cytokines and other mediators described above. The nature of this response is complex, multifactorial and requires careful regulation to prevent potentially damaging effects. A massive inflammatory response is a key element of certain pneumococcal infections (meningitis and pneumonia) and has been associated with the tissue destruction and pathology that results from such infection (Meli *et al.*, 2002). Cell wall components and other bacteria products, such as pneumolysin, have been identified as important pneumococcal inflammatory mediators (Tuomanen, 2000). Several key cytokines involved in the response following infection with *S. pneumoniae* have been studied and are discussed below. The cytokines described represent only a small proportion of the mediators likely to be involved in the host inflammatory response to pneumococcal infection, but represent those best studied.

1.5.3.1 Cytokines

Cytokines are soluble molecules produced by a range of cell types that mediate interactions between cells. They are usually small proteins with low molecular mass, and help regulate many biological processes such as cell growth, cell activation, immunity, inflammation and tissue repair. Members of the cytokine family include interleukins (IL), interferons (IFN), tumour necrosis factors (TNF) and colony stimulating factors (CSF). Cytokines are extremely potent molecules, often capable of exerting their effect at femtomolar concentrations. Locally produced cytokines can act in autocrine and paracrine fashion, integrating signals over short cellular distances. Cytokines rarely, if ever, act in isolation and cytokine networks are dynamic and complex, with individual cytokines being pleiotropic in their functions.

Cytokines can broadly be subdivided into those that are pro-inflammatory and those that are able to down regulate host responses or anti-inflammatory, and the balance between such molecules is crucial in determining the outcome of infection. Perturbation of this balance, either by microbial products and interactions, or intervention using immunomodulatory therapies, could have serious consequences for the ability of the host to cope with infections.

1.5.3.2 Pro-inflammatory cytokines and pneumococcal infection

IL-1β

IL-1β is a pro-inflammatory cytokine secreted by many cells, although most abundantly by macrophages. Virtually all cells on the body have receptors for IL-1β, but the main targets are T and B cells where it induces inflammatory responses. During pneumococcal infection, IL-1 β does not appear to be important early in infection but in an experimental model was found to be important for survival after 5 days (Benton *et al.*, 1998). The presence of pneumococci within the lung tissue causes alveolar macrophages to secrete TNF-α and IL-1 β, and this is the start of the host inflammatory response.

IL-6

Although generally classed as pro-inflammatory, IL-6 can have both pro and anti-inflammatory activities. Within the lung environment, interstitial fibroblasts, alveolar macrophages, endothelial cells and bronchial epithelial cells produce IL-6. Serving an inflammatory role, IL-6 is able to activate monocytes and can increase the respiratory burst of neutrophils in combination with TNF-α (Mullen *et al.*, 1995). However, several studies have indicated that IL-6 serves to down-regulate the inflammatory response within the lung environment. It has been found to inhibit IL-1 and TNF production from macrophages (Ulich *et al.*, 1991). One study found that IL-6 plays an anti-inflammatory role in lipoteichoic acid-induced pulmonary inflammation but had a pro-inflammatory role in lung inflammation induced by peptidoglycan from the Gram positive organism *S. aureus* (Leemans *et al.*, 2002). IL-6 deficient mice (IL-6-/-) are more susceptible to intranasal (i.n.). challenge with pneumococcus, displaying a more pronounced inflammatory response when compared to control mice. Within the lung environment, these IL-6-/- mice had elevated levels of TNF-α, IL-1β, IFN-γ and IL-10 (van der Poll *et al.*, 1997). Such data indicates a regulatory role for IL-6 in the control of the inflammatory response induced by pneumococcal infection. IL-6 levels have been shown to increase in children with septic shock and are predictive for the isolation of bacteria, including *S. pneumoniae*, from the blood (Saladino *et al.*, 1992). Stimulation of whole blood from infants and adults with live and heat-killed *S. pneumoniae* and other pathogens results in increased levels of IL-6 but stimulation of whole blood from pre-term infants was shown to result in significantly lower levels of IL-6 (Yachie *et al.*, 1992). As pre-term infants are more susceptible to bacterial infections, these findings suggest

that IL-6 may have a role in protection against infection. However, the immune response as a whole will be underdeveloped in pre-term infants and susceptibility to infection in this group should not be attributed to a single cytokine. In contrast, a study in pigs showed that high levels of IL-6 in the blood resulted in increased mortality from pneumococcal infection, suggesting IL-6 is non-beneficial (Ziegler-Heitbrock *et al.*, 1992). Other published data have indicated that IL-6 concentrations increase during pneumococcal infection. For example, serum levels of IL-6 were significantly higher during episodes of pneumococcal AOM than AOM caused by other bacteria (Heikkinen *et al.*, 1998). Increased levels of IL-6 in the serum have also been shown to be associated with community acquired pneumonia (CAP) caused by the pneumococcus compared with infections caused by *Mycoplasma pneumoniae* (Lieberman *et al.*, 1997). These studies illustrate that AOM, CAP and invasive infection with *S. pneumoniae* all result in increased serum concentrations of IL-6, although the role that this cytokine has during infection is unclear.

TNF-α

TNF- α and IL-1β have multiple overlapping activities. TNF- α is a potent pro-inflammatory cytokine that has both beneficial and toxic effects. TNF-α can have detrimental effects during infection. Increased levels of TNF-α have been detected in the central nervous system (CNS) during infections such as meningitis and brain injury, and high levels in the CSF during bacterial meningitis are predictive of poor outcome. TNF-α, however, has also been shown to be a critical inflammatory mediator in the control of numerous microbial infections using experimental animal models (Blanchard *et al.*, 1988; Mastroeni *et al.*, 1991).

Many groups have studied the role of TNF-α in pneumococcal infection *in vivo*. Following intranasal challenge, the levels of TNF-α in lung tissue increase over a period of 48 hours and can reach very high levels (Bergeron *et al.*, 1998). In bronchi-alveolar lavage fluid (BALF), TNF-α concentration increases up to 12 hours post-infection, after which time concentrations return to normal levels, despite progression of the infection. Serum TNF-α levels also increase, albeit at a later stage (2-3 days post infection) and at lower levels than those present in the lung. These increases in lung and serum TNF-α concentrations appear to correlate with bacterial loads in the lung tissue and migration of bacteria into the bloodstream respectively (Takashima *et al.*, 1997; Bergeron *et al.*, 1998). Several studies have shown the importance of the TNF-receptor-1 (TNFR1) in pneumococcal infection and the benefits of early production of TNF-α in protection

(O' Brien *et al.*, 1999; Kerr *et al.*, 2002). Kerr and colleagues also showed that neutralisation of TNF- α with polyclonal anti-TNF antibody significantly increased bacterial loads in the BALF and lung tissue of MF1 mice following intranasal infection, although this had no effect on levels of bacteraemia (Kerr *et al*, 2002). Intraperitoneal administration of anti-TNF-α antibodies have also been shown to produce higher levels of bacteraemia, lower counts of neutrophils in the blood and acceleration of death in CBA/J mice challenged with type 19 pneumococcus (Takashima *et al.*, 1997). As neutrophils are crucial for the phagocytosis of *S. pneumoniae*, it was suggested that this decrease in neutrophil count was allowing for accelerated access of bacteria into the bloodstream. Furthermore, as TNF-α can potentiate the bactericidal activities of neutrophils, any of these cells present during infection may not be as efficient at phagocytosing bacteria in the absence of TNF-α. However, O'Brien and colleagues found the number of neutrophils recruited to the site of infection over 3 days post infection did not differ from control mice, despite a more severe infection with TNF-α receptor knock out mice (O'Brien *et al.*, 1999). This could illustrate differences in the effects of TNF-α at different sites of infection or between different mouse strains and supports the idea that neutrophils were less bactericidal. Indeed work within our own laboratory has implicated TNF-α in protection against bacterial challenge. A reduced ability to induce rapid TNF-α within the airways of CBA/Ca mice was proposed to render such mice susceptible to pneumococcal challenge with type 2, D39, when compared to the resistant BALB/c mouse model (Kerr *et al.*, 2002).

The mechanisms by which TNF- α is able to elicit protection may be the same mechanisms that could be non-beneficial to the host. TNF- α is thought to attract neutrophils by up-regulating vascular and neutrophil adhesion molecules. It can also up-regulate the release of chemokines, such as macrophage inflammatory potentiator 1-alpha (MIP-1α) and MIP-2, which are chemoattractant for inflammatory cells (Standiford *et al.*, 1996). Once at the infection site, neutrophils can be activated by TNF- α by direct stimulation of the respiratory burst and modulation of lysosomal enzyme release. Such activities are likely to be protective against infection at low levels but at higher levels could cause injury to host tissue and subsequently provoke a detrimental inflammatory response. TNF- α and IL-1β have been shown to stimulate nitric oxide (NO) production, which can also be protective, but overproduction has been associated with a high histopathology score and rapid death in pneumococcal models of infection. Blocking NO is able to delay such mortality (Bergeron *et al.*, 1998).

TNF- α is one of the first inflammatory mediators detected early in pneumococcal infection in both BALF and lung tissue (Bergeron *et al.*, 1998), and this early release is thought to be necessary to afford protection (O'Brien *et al.*, 1999 and Kerr *et al.*, 2002). It is possible that mast cells within the lung environment are responsible for this early appearance of TNF- α, being the only cells to possess the cytokine pre-synthesised (Gordon *et al.*, 1990). Indeed a correlation between increased numbers of mast cells and TNF- α levels has been shown (Kerr *et al.*, 2002). Following this initial release, TNF- α concentrations may increase through its release from other stimulated cells within the site of infection, as well as cells recruited to the infection site. TNF- α can up-regulate its own expression resulting in a vigorous immune response which must then be controlled through anti-inflammatory cytokines and other regulatory factors to prevent it becoming detrimental to the host.

The role of TNF- α depends on its location and when present in the systemic circulation, has been associated with sepsis. High levels of TNF- α are detected in the blood of patients suffering Gram-negative sepsis, although elevated TNF- α levels are also detected during infection with Gram-positive organisms, including *S. pneumoniae* (Cohen and Abraham, 1999). Although sepsis is often initiated by microbial infection, the resulting tissue injury and organ failure is due to host mediators rather than bacterial products (Sriskandan and Cohen, 1995). TNF- α is central in this injury and can cause a range of systemic effects. When recombinant human TNF- α was injected intravenously into experimental animals, shock, tissue injury, capillary leakage, hypoxia, pulmonary oedema and multiple organ failure associated with high mortality ensued (Tracey and Cerami, 1994). Thus, TNF- α may have both beneficial and detrimental effects both in human and experimental murine infection, depending on where it is produced and the levels present.

IFN-γ

IFN-γ is a potent pro-inflammatory cytokine that contributes to immunity by inducing a range of physiologically significant responses (Shtrichman and Samuel, 2001). It is the main T cell product that activates macrophages. Although there is much data on the importance of this cytokine in the innate immune response to many microbial pathogens, the evidence of its role in pneumococcal infection is conflicting. IFN-γ -/- mice have been used in two independent studies, but resulting data are conflicting. Rubins and Pomeroy (1997) used IFNγ-/- mice on a C57BL/6

background and found that such mice were more susceptible to intranasal challenge with type 2 pneumococcus compared to wild type C57/Bl mice. Rijneveld and co-workers however, found no difference in mortality of IFNγ-/- mice on a BALB/c background when challenged intranasally with type 3 pneumococcus (Rijneveld *et al.*, 2002). Evidence indicates that T cells from C57BL/6 mice produce significantly higher amounts of IFN-γ in response to pathogens than lymphocytes from other murine models, including BALB/c (Shtrichman and Samuel, 2001). This illustrates how genetic background of both mouse and bacterial strains used in such studies can influence the data obtained and that care must be taken when interpreting and extrapolating data from experiments.

1.5.3.3 Anti-inflammatory cytokines and pneumococcal infection

IL-10

IL-10 is a potent regulatory cytokine that down-regulates the synthesis of pro-inflammatory cytokines by macrophages, neutrophils and mast cells. It also suppresses free oxygen radical release and NO-dependent microbicidal activity of macrophages. Various cell types, including T cells, B cells and macrophages can produce IL-10. Following challenge with S. *pneumoniae*, IL-10 levels within lung tissue increase and remain sustained throughout infection (van der Poll *et al.*, 1996). Administration of anti-IL-10 antibody has demonstrated that IL-10 can reduce the pro-inflammatory response during experimental pneumococcal pneumonia. Abolishing IL-10 resulted in increased levels of TNF-α and IFN-γ in lungs and blood and caused early lethality (van der Poll *et al.*, 1996). Similarily, in patients with sepsis, a high TNF-α to IL-10 ratio has been associated with fatal outcome (van Dissel *et al.*, 1998).

1.5.4 Defence in the systemic circulation

Antibody or complement mediated phagocytosis provide the major clearance mechanisms for pneumococcal infection and the spleen is the major organ involved. The IgG2 class of antibody is the predominant form produced in response to the pneumococcal polysaccharide capsule (Escobar-Pérez *et al.*, 2000). Deficiencies in this class of antibody can predispose to infection with *S. pneumoniae* and other encapsulated bacteria such as *H. influenzae* (Escobar-Pérez *et al.*, 2000). Complement is a series of over 30 serum proteins that have multiple roles in host defence. It is part of the innate immune system and can be activated by antibody/antigen complexes

(classical pathway) or by the deposition of complement component C3b on the surface of invading microbes (alternative pathway). Complement components may also be produced as acute phase proteins by the liver. Complement can result in bacterial killing either directly by its ability to form pores in bacteria membranes (membrane attack complex) or indirectly by opsonising bacterial surfaces (C3 & C3b) and targeting them for phagocytosis. Some complement components are also chemotactic or have the ability to activate lymphocytes or increase vascular permeability.

The complement membrane-attack complex is unable to penetrate the rigid cell wall of *S. pneumoniae* so deposition of opsonic C3b on the surface is the principle mediator of pneumococcal clearance in the non-immune host (Hostetter 2000). C3 can bind to the polysaccharide capsule and to teichoic acid within the cell wall. In the immune host, anti-capsular antibodies recognise and bind to the capsule and this further facilitates the deposition of complement C3b. Bound C3b can be cleaved by serum proteases to the smaller fragments iC3b and C3d. These are recognised by receptors on phagocytes and the organisms are subsequently rapidly cleared. The importance of complement in the host response to pneumococcal infection can be demonstrated by the use of complement knock-out mice which are highly susceptible to infection compared to their isogenic parental strains (our laboratory, unpublished data). The Fc gamma receptors also have an important role in phagocytosis of pneumococcal cells. Antibody mediated phagocytosis by FcγRIIa can occur even in the absence of complement, although opsonisation by C3 greatly enhances phagocytosis. In children and adults, activity of FcγRIIa largely depends on anti-capsular IgG2 antibody. However, IgG2 levels are slow to mature and children under the age of 2 years are unable to produce this immunoglobulin subclass.

It is known that some pneumococcal serotypes are more virulent than others and this has often been attributed to differences in the chemical structure of the capsule. Different cleavage patterns of deposited C3b by pneumococcal serotypes can explain why some are more resistant to opsonophagocytosis than others. Studies have shown that with serotypes 3 and 4, which are resistant to phagocytosis but potent immunogens, C3d is the predominant C3 fragment on the surface. This component is recognised by CR2 receptors on B cells but not by phagocyte receptors. In contrast, serotypes 6A and 14 display iC3b on their surface and this is recognised by CR3 on phagocytes but not by B cell receptors. The reasons for these differences are unclear. These serotypes are poor immunogens but readily phagocytosed (Hostetter, 2000). Surface-

bound C-reactive protein (CRP) is also able to induce opsonic activity, possibly through its ability to activate complement by the classical pathway (Volanakis and Kaplan, 1974), although macrophages themselves have surface receptors with the ability to bind CRP (Tebo and Mortensen, 1990). This would enhance phagocytosis of opsonised bacteria.

S. pneumoniae is capable of phase variation whereby two variants, opaque and transparent are found. Opaque variants have increased amounts of capsule and decreased levels of several surface components such as autolysin (LytA), choline binding protein A (CbpA) and teichoic acid compared to transparent variants. The opaque variants tend to predominate in systemic infection, whilst transparent variants are more adapted for colonisation (Weiser, 2000). One study has demonstrated that more C-reactive protein (CRP) binds to the transparent form of pneumococci than the opaque form and that opsonophagocytosis occurs only in the former (Kim *et al.*, 1999). This may explain why the transparent variants are less virulent in systemic infection.

1.6 Pneumococcal pathogenicity and virulence factors

As with any bacterial pathogen, the ability of *S. pneumoniae* to cause disease depends on several important stages, namely colonisation of host surfaces, damage to host tissue and evasion of the host immune response. Although a variety of pneumococcal virulence factors have been identified that contribute to each stage of infection, the molecular details of pathogenicity are not yet fully understood. Some of the known mechanisms that contribute to virulence are described below. Although these will be discussed with particular reference to pneumococcal pneumonia, many will apply to other disease states caused by *S. pneumoniae*, including meningitis. The following section does not aim to discuss all of the *S. pneumoniae* virulence factors identified to date, as these are too numerous to include in this thesis. Instead, it aims to discuss several virulence factors associated with different aspects of infection to provide an overview of the intricate nature of bacterial pathogenicity and gene regulation. Some virulence factors, such as pneumolysin, may play a role in many stages of infection, such as adhesion, tissue damage, nutrient acquisition and evasion of the host response. Again, this demonstrates the complex issues involved in studying bacterial pathogens.

1.6.1 Colonisation and adhesion

The first step in pneumococcal infection is colonisation of the nasopharynx. This site is the natural niche of the pneumococcus and colonisation is generally asymptomatic. However, colonisation with a newly acquired isolate may result in infection if the host is immunocompromised or at high-risk. The success of *S. pneumoniae* to inhabit this niche may be partly due to its ability to inhibit other URT bacteria such as *H. influenzae*, *Moraxella catarrhalis* and *N. meningitidis*, via the production of extracellular products such as hydrogen peroxide (Pericone *et al.*, 2000). The neuraminidase (NanA) from *S. pneumoniae* can also desialylate the lipopolysaccharide from *H. influenzae* and *N. meningitidis* (Shakhnovich *et al.*, 2002). *S. pneumoniae* can bind to sugar receptors on resting host cells and host cell ligands that are induced through activation with cytokines, for example (Tuomanen, 1997). On resting cells, bacteria are able to adhere to nasopharyngeal cells through recognition of at least 2 specific host disaccharides (Anderson *et al.*, 1983). These sugars include *N*-acetyl-$_D$-galactosamine β1-3 galactose (GalNAcβ1-3Gal) and *N*-acetyl-$_D$-galactosamine β1-4 galactose (GalNAcβ1-4Gal) (Cundell *et al.*, 1995b). *S. pneumoniae* has also been shown to bind to the human polymeric immunoglobulin receptor found in the URT (Zhang *et al.*, 2000) although this appears to be strain-specific (Brock *et al.*, 2002). It has been proposed that this binding may facilitate translocation of bacteria across nasopharyngeal epithelial cells. Surface-located CbpA has a role in this binding (Zhang *et al.*, 2000).

Spread to the lungs probably occurs by aspiration and is enhanced by impaired defences such as the cough reflex or mucociliary activity. Pneumococci have been shown to adhere to and invade human or other bronchial epithelial cells (Talbot *et al.*, 1996; Adamou *et al.*, 1998; Kadioglu *et al.*, 2001). Platelet activating factor receptor (PAFr), on cytokine-activated cells, has been shown to act as a host ligand for this interaction and it is thought that this binds phosphorylcholine present on the pneumococcal cell wall (Cundell *et al.*, 1995a). Phosphorylcholine also serves to anchor the Cbps to the pneumococcal cell wall. Cbp comprise a set of at least 12 surface-located proteins that have a variety of functions, many of which are thought to be important in adhesion and colonisation (Paton, 1998; Gosink *et al.*, 2000). CbpA, discussed above for its role in nasopharangeal adhesion, also appears to be able to bind to cytokine-activated type II pneumocytes and endothelial cells (Paton 1998). Due to its conservation across pneumococcal serotypes and its role in adhesion, this protein may be a good vaccine candidate. Inhibition of

choline mediated adhesion by dimyristoylphosphatidlcholine (DMPC) was demonstrated to reduce binding of several pneumococcal strains to lung cells by up to 80 % (Bérubé *et al.*, 1999). This could be a potential therapeutic approach to treating early stages of pneumococcal infection. *S. pneumoniae* has also been shown to bind to another glycoconjugate receptor that is expressed on human epithelial cells following stimulation with pro-inflammatory cytokines (Cundell *et al.*, 1995b).

Pneumococcal phase variation also influences adhesion and colonisation. The transparent pneumococcal variants (see section 1.5.4) are thought to be better adapted for colonisation compared to the opaque variants (Weiser, 2000). This is attributed to different expression of surface proteins between the variants, where transparent forms have increased amounts of CbpA, a pneumococcal protein previously shown to be involved in adhesion to cytokine-stimulated type II pneumocytes (Rosenow *et al.*, 1997; Weiser, 2000). Furthermore, transparent variants demonstrate increased production of hydrogen peroxide (Overweg *et al.*, 2000b) which has been shown to inhibit the growth of other pathogens that may be present in the nasopharynx (Pericone *et al.*, 2000). This could confer a competitive advantage upon pneumococcal cells, allowing for increased colonisation of this niche.

Adhesion / colonisation is a complex process and involves many other pneumococcal components too numerous to discuss here e.g. pneumolysin (pln) and PavA (Rubins *et al.*, 1998; Holmes *et al.*, 2001). Environmental factors may influence this process, such as previous viral infection and / or activation of host cells by cytokines and other mediators. These may serve to reveal new or up-regulate existing host ligands that the pneumococcal can than adhere to. Cigarette smoking has also been shown to increase susceptibility to respiratory pathogens by inhibiting mucociliary clearance and coating the airways with smoke components which may facilitate bacterial adhesion (Ahmer *et al.*, 1999).

1.6.2 Environmental sensing and expression of virulence factors

Regulation of bacterial genes in response to environmental signals is an essential yet complex mechanism used by bacteria to adapt to their surroundings. The ability to induce or repress gene expression at different sites and in response to a whole range of stimuli has allowed bacteria to evolve as highly successful pathogens, both in their ability to colonise and damage host tissue and in the evasion of host immune responses. Many bacterial virulence factors are likely to be

tightly regulated according to environmental signals. A single regulatory element may control the expression of numerous, unrelated genes (global regulation) (Finlay and Falkow, 1997). Examples include the *Bordetella pertussis* BvgA/S and *Staphylococcus. aureus* AgrC/A two-component systems (TCS) (Uhl and Miller, 1996; Lyon *et al.*, 2000). mRNA transcripts for several known pneumococcal virulence factors have been shown to be up-regulated during *in vivo* infection (Orihuela *et al.*, 2001; Ogunniyi *et al.*, 2002). Further studies, including microarray analysis, should allow the identification of a whole range of virulence genes that are differentially regulated at different stages *of S. pneumoniae* infection.

Two-component systems (TCS) have a central role in the sensing of environmental conditions and in subsequent gene regulation. These systems are ubiquitous among bacteria where they control a whole range of cellular functions. Thirteen of these systems have been identified in the pneumococcus and these are discussed in further detail in section 1.14.

Other components that may be involved in gene regulation include DNA topology, alternate sigma factors including heat shock proteins, and other transcriptional regulators such as the Fur protein involved in gene regulation in response to iron (Finlay and Falkow, 1997).

1.6.3 Damage to host tissue and dissemination of infection

Many pneumococcal factors are thought to play a role in tissue destruction. Direct destruction may be achieved through the action of enzymes or toxins. Pln, for example, is a potent toxin released upon pneumococcal cell lysis. It has multiple activities at a range of different sites and many studies have identified it as an important virulence factor directly involved in the pathogenesis of pneumococcal infections (Berry *et al.*, 1989; Canvin *et al.*, 1995; Paton, 1996). This toxin is able to lyse many eukaryotic cells with cholesterol in their membrane *in vitro* by the formation of transmembrane pores (Alonso De Velasco *et al.*, 1995). A second haemolysin has also been identified in *S. pneumoniae* (Canvin *et al.*, 1997). Both haemolysins could contribute to tissue destruction.

Two enzymes, neuraminidase and hyaluronidase may also contribute to tissue damage. Neuraminidase cleaves terminal sialic acid residues from a variety of cell surfaces (Paton *et al.*, 1993). This could also expose host ligands that can be utilised by the invading bacteria for attachment. It is thought that more than one form of the neuraminidase enzyme exists in pneumococcal strains (Berry *et al.*, 1996). Hyaluronidase is secreted by *S. pneumoniae* during

exponential growth and is able to break down hyaluronic acid (Paton *et al.*, 1993), a component of mammalian connective tissue. This could facilitate bacterial dissemination in host tissues.

Indirect tissue destruction may occur though the ability of pneumococcal components to induce a vigorous inflammatory response within host tissue. The natural response to invading organisms within lung tissue will result in the release of inflammatory cytokines from resident alveolar macrophages and / or mast cells or other sources. However several pneumococcal components are known to potentiate this response. Pln has been shown to increase the production of pro-inflammatory cytokines TNF-α and IL-1β from monocytes (Houldwsorth *et al.*, 1994) and to induce nitric oxide production from macrophages (Braun *et al.*, 1999). The cell wall is also a potent inducer of inflammation, via the activation of complement and the induction of pro-inflammatory cytokines (Catterall, 1999). Peptidoglycan and teichoic acids are the active components in the cell wall.

A range of uncharacterised surface molecules, secreted or intracellular bacterial components, such as CpG motifs on DNA, may also contribute to the inflammatory response. Autolysin, is an enzyme that is involved in maintaining cell surface integrity and can induce lysis of pneumococci *in vitro*. This enzyme may be indirectly involved in activating the host inflammatory response by its ability to release pro-inflammatory cell wall components and intracellular pneumolysin into the host tissue.

Damage to host tissue may also occur through the action of bacterial proteases. Several proteases have been identified in *S. pneumoniae* and many of these are thought to be involved in virulence. Pneumococcal strains possess at least one IgA1 protease, thought to be involved in cleaving secretory IgA (discussed below). Proteases may contribute to the dissemination of bacteria or expose potential host ligands for binding / colonisation. An pneumococcal enolase, capable of binding and activating plasminogen, may facilitate degradation of the extracellular matrix and enhance dissemination of bacteria through host tissues. Two serine proteases have been identified in the pneumococcus, HtrA, shown to be important in nasopharyngeal colonisation (Sebert *et al.*, 2002) and PrtA, shown to be important in systemic infection (Bethe *et al.*, 2001), although the exact function of these proteases is unknown.

Within this work, a putative zinc metalloprotease has been characterised. This protease is >5.5Kb (approximately 1800 amino acids) and has a molecular weight of ~210 KDa. This protein was identified in a search for putative adhesion molecules and was designated ZmpB to distinguish it

from a second zinc metalloprotease in the pneumococcus (Novak *et al.*, 2000). It was characterised as a putative zinc metalloprotease based on a HEXXH-E motif that is conserved in other zinc metalloproteases (Rawlings and Barrett, 1995). A mutation in the *zmpB* gene demonstrated a marked *in vitro* phenotype, leading the authors to propose an involvement of ZmpB in the translocation of certain proteins to the bacterial cell surface via a proteolysis based mechanism (Novak *et al.*, 2000). However, a second study could not reproduce any of the phenotypes observed by Novak and colleagues, and subsequently demonstrated that the *zmpB* mutant used within the first study was a not *S. pneumoniae* but was one of the viridans group of Streptococcus (Bergé *et al.*, 2001). Thus the role of ZmpB remains unknown.

ZmpB is one of the few proteins in *S. pneumoniae* which demonstrates extensive sequence variation between the serotypes for which a sequence has been published. There is a region of approximately 300-400 amino acids at the start of the protein which are approximately 99 % homologous between serotypes. This region contains a potential signal peptidase site, followed by two hydrophobic regions of alpha helices and an LPXTG motif (Bergé *et al.*, 2001). The remainder of the protein is highly variable, with the TIGR4 strain showing 40 % and 41 % identity with the R6 and G54 sequences respectively. The G54 and R6 sequences are more closely related but still only show 67 % identity within the variable region. Although the HEXXH-E motif is situated within the variable region, this motif is conserved between serotypes for which sequence data are available. Figure 1.4 is a schematic diagram of the ZmpB protein. Sequence alignments of this protein from TIGR4, G54 and R6 are provided in the appendix (appendix 6a and 6b).

The LPXTG motif is one often found in proteins of Gram positive bacteria, where it serves to act as a cell-surface anchor, through the action of sortase enzymes (Pallen *et al.*, 2001). However, the position of this LPXTG motif is usually found near the C-terminus end of the protein. As the LPXTG motif lies near the N-terminus region of ZmpB, it is not known if it serves as a true surface anchor. The cellular location of ZmpB is thus unknown.

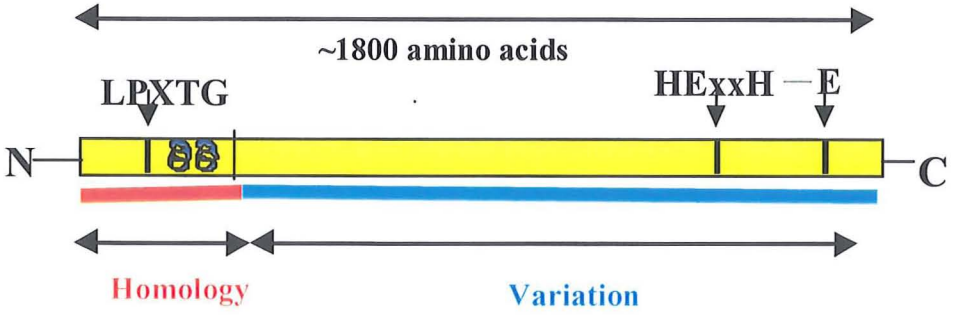

Figure 1.4 Schematic diagram of ZmpB

The figure illustrates the 1800 amino acid ZmpB protein of *S. pneumoniae*. The LPXTG motif, followed by the hydrophobic region (represented by dark swirling lines) at the N-terminal region of the protein are shown. The putative signal peptidase site is situated before the LPXTG motif (not shown on the diagram).This region appears to be ~99 % homologous between serotypes (amino acids 1-300, shown by the red bar). The remainder of the protein sequence is highly variable between the serotypes for which sequence data are available (shown by the blue bar). The HEXXH-E motif, characteristic of zinc metalloproteases is found near the C-terminus. This motif is also conserved between serotypes. The entire protein has a molecular weight of approximately 210 KDa.

1.6.4 Acquisition of nutrients and growth factors

The ability to acquire essential nutrients and growth factors in host tissue is crucial for bacterial pathogens, so mechanisms involved in this process may also be classed as virulence factors. The virulence factors discussed above that result in damage to host tissue may also serve to release nutrients required for growth of invading bacteria. Other factors may also be used in combination with the above to obtain specific nutrients. For example, iron, an essential co-factor required for growth of most bacterial pathogens, is not freely available in the host and must be sequestered from mammalian iron-binding or haem-containing proteins. Several putative mechanisms for obtaining iron in the human host have been identified in the pneumococcus. PspA, a choline binding protein, has been shown to bind human lactoferrin, an iron-binding protein found in mucosal secretions (Hakansson *et al.*, 2001). However in iron-deficient medium, lactoferrin does not support the growth of *S. pneumoniae* (Brown and Holden, 2002), although this could indicate that environmental factors specific to the infected host may be required to induce certain iron uptake mechanisms. One group has shown that haemin binds to the pneumococcus *in vitro* and

used affinity chromatography to identify a putative haemin binding protein (Tai *et al.*, 1997). A *S. pneumoniae* pathogenicity island has been identified that has 2 genetic loci encoding ATP-binding cassette (ABC) transporters required for iron uptake (Brown *et al.*, 2001a). Mutations in either of these transporters only moderately affected the ability of the bacteria to use haemoglobin as an iron source. A double mutation in both ABC transporters markedly reduced growth in iron deficient media and was highly attenuated in a murine model of pneumonia and septicaemia (Brown *et al.*, 2001a). Recombinant proteins from these transporters were shown to elicit protective immunity against sepsis when used to immunise mice (Brown *et al.*, 2001b). These data also suggested that loss of one locus could be compensated for by the other functional locus. A third ABC transporter involved in iron uptake has also been identified (Brown *et al.*, 2002). The identification of several loci utilising different mechanisms for the acquisition of iron in *S. pneumoniae* suggests that these systems could be important at different sites of infection and are likely to be regulated accordingly. Indeed TCS may be involved in the regulation of some of these systems. Siderophores, microbial iron transporters, have not been identified in *S. pneumoniae.*

1.6.5 Evasion of the host immune response

The pneumococcus has many strategies for evading the host responses to infection. In the upper airways, pneumolysin is able to inhibit the ciliary beat, which is an important mechanism in preventing bacteria from reaching the LRT. *S. pneumoniae* also possesses an IgA1 protease that is able to cleave human, but not murine, IgA1 (Poulsen *et al.*, 1996; Wani *et al.*, 1996), and could prevent clearance by this immunoglobulin. A pneumococcal protein, CbpA is also able to bind the secretory component of IgA that may serve to sequester this immunoglobulin (Hammerschmidt *et al.*, 1997). However, the role of IgA in protecting against pneumococcal infection is unclear as human IgA-deficiencies do not appear to result in increased susceptibility to infection.

Phagocytosis is a major mechanism involved in clearing pneumococcal infection, both within the lungs and in the blood. This host defense mechanism is enhanced through opsonisation by complement, antibody or other components. Several pneumococcal proteins are thought to play a role in modulating host phagocytic and opsonisation functions. Pneumolysin is able to inhibit the bactericidal activity of PMNs, inhibit lymphocyte proliferation and inhibit antibody synthesis

(Paton *et al.*, 1993; Mitchell and Andrew, 2000). Pneumolysin has been reported to have complement activating activity (Paton *et al.*, 1993 ; Alcantara *et al.*, 2001). It is thought that continuous activation of complement at a site distant from the invading bacteria may serve to deplete complement resources and remove opsonic complement components away from intact bacterial surfaces, thus reducing opsonophagocytosis (Alcantara *et al.*, 2001). Other pneumococcal proteins, such as PspC and PspA, have the ability to bind complement components and this may inhibit complement activity (Alonso De Velasco *et al.*, 1995; Janulczyk *et al.*, 2000; Duthy *et al.*, 2002).

The polysaccharide capsule surrounding pneumococcal cells is essential for the virulence of *S. pneumoniae*. The capsular material itself is inert and has no inflammatory or tissue-destroying activities but it has a major role in the ability of pneumococci to resist phagocytosis. The amount of polysaccharide capsule surrounding invading organisms increases greatly following transition from the respiratory tract to the bloodstream and this is thought to promote survival of the bacteria systemically where phagocytosis is of major importance in controlling infection. This process of phase variation also involves differential expression of other surface-located components thought to have roles at different sites of infection (Overweg *et al.*, 2000). Some capsular serotypes are more virulent than others. This has generally been attributed to differences in chemical structures resulting in variation in the ability to resist phagocytosis and / or opsonisation, or having different levels of immunogenicity (Hostetter, 2000). However it is now becoming apparent that genetic background may also have an important role in how virulent any particular serotype is (Kadioglu *et al.*, 2002). The ability of the capsule to resist opsonisation by complement components has been discussed elsewhere (section 1.5.4).

1.7 Manipulation of the pneumococcal genome

The genomes of 3 pneumococcal serotypes (2, 4 and 19F) (Dopazo *et al.*, 2001; Hoskins *et al.*, 2001; Tettelin *et al.*, 2001) have been sequenced. The genomes for type 2 and 4 have been completely annotated and are publicly available (http://www.tirg.org/). The 19F sequence has not yet been assembled and annotated Availability of this sequence information has been of enormous benefit to those involved in pneumococcal research as it has enabled the development of sophisticated techniques such as microarray analysis. Furthermore, large scale techniques to identify putative new virulence factors have made use of the availability of annotated sequence

data to provide lists of previously uncharacterised genes that are involved in virulence (Pearce *et al*, 1993; Polissi, *et al.*1998; Lau *et al*, 2001; Wizemann *et al*, 2001; Hava and Camilli, 2002; Marra *et al.*, 2002).

Although microarray analysis and other techniques have the potential to generate vast amounts of data previously unattainable using conventional techniques, genes of interest must still be analysed in further detail to determine their role within the pneumococcus or for their suitability as vaccine candidates / drug targets. There are currently several techniques available for creating mutations within target genes, some of which are discussed below. All of these techniques make use of the natural ability of *S. pneumoniae* to take up DNA from its environment and incorporate it into its genome. By using a peptide (competence stimulating peptide, CSP) to stimulate this DNA uptake (competence), target genes which have been manipulated or mutated within plasmids or other constructs can be introduced into the bacterium with relative ease. Regions of homology within the introduced DNA combine with homologous sequences within the chromosome and can result in a stable genomic mutation. Sequence information has allowed the refinement of mutagenesis techniques such that single amino acids can now be mutated within genes of interest.

1.7.1 Insertion-duplication mutagenesis

This was one of the first techniques used to create mutations in pneumococcal genes. A small region with homology to the target gene is cloned into a suicide plasmid containing an antibiotic resistance marker and introduced into the target cell by transformation (Lee *et al.*, 1998). The region of homology combines with the homologous sequence in the target gene via a single cross-over event and the whole plasmid integrates into the bacterial chromosome. During the process, the homologous region is duplicated, hence the name insertion-duplication. Potential mutants are selected in media or agar containing the appropriate antibiotic marker. The plasmid vector used is such that it can replicate in *Escherichia coli* (for propagation) but is unable to replicate within the pneumococcal cell and will be eliminated unless it is able to insert into the genome. This process is illustrated schematically in figure 1.5, and is based on the vector, pAS1, used within this work to create insertion-duplication mutants. The problems associated with this technique include the integration of a large amount of foreign DNA into the gene of interest that could have polar effects on downstream genes. Furthermore, the homologous region should be

designed to be at the start of the gene to prevent translation of a truncated protein that could have some functional activity. Plasmid excision and potential reversion back to wild-type has also been demonstrated with this form of mutagenesis (Yother, 2000).

1.7.2 Allelic replacement mutagenesis

This technique is similar to insertion duplication mutagenesis but an antibiotic cassette is used to replaces the entire gene or a large region of the target gene using a double cross-over event. This prevents the integration of large regions of 'foreign' DNA and ensures that the whole gene is replaced. No gene duplication occurs and mutants can be selected using antibiotics as described above. Mutants created using this technique are usually more stable than those made by insertion duplication, although care must be taken to ensure no polar effects result. Mutations are easily confirmed by using PCR across the whole mutated region and sequencing the PCR product to ensure the gene is absent and to check for polar effects. The process of creating mutants using this technique is given in figure 1.6.

1.7.3 *Mariner* mutagenesis

This technique uses transposon mutagenesis (Akerley *et al.*, 1998; Bergé *et al.*, 2001). The PCR product of the target gene is mutated *in vitro* by the random insertion of the *mariner* minitransposon encoding antibiotic resistance. Transposition products are used as donor DNA in the transformation of *S. pneumoniae* and double cross-over events occur between DNA regions flanking the transposon integration as described for allelic replacement mutagenesis. As above, mutants are selected by their ability to grow in the presence of antibiotic and are confirmed by PCR. The technique has the advantage that it can create a series of integrations along the length of the gene and these can be analysed simultaneously. This may help identify regions of importance, and may be especially useful for large genes.

1.7.4 Gene replacement through negative selection (Janus)

This is a new mutagenesis technique based on the Janus cassette constructed by Sung and co-workers (Sung *et al.*, 2001). Using two transformations, this cassette can be used to insert DNA of arbitrary sequence at any chosen site. The technique is extremely versatile, allowing complete inactivation of genes by the insertion of stop codons, or more subtle mutations such as amino

acid changes, or epitope tagging. The resultant mutations do not possess any antibiotic-selection markers that may interfere with further investigation. This also allows for the construction of multiple mutations in different genes without the need for various antibiotic selection markers. The technique allows for the transfer of large regions of DNA between different strains. Furthermore, polar effects on downstream genes are avoided.

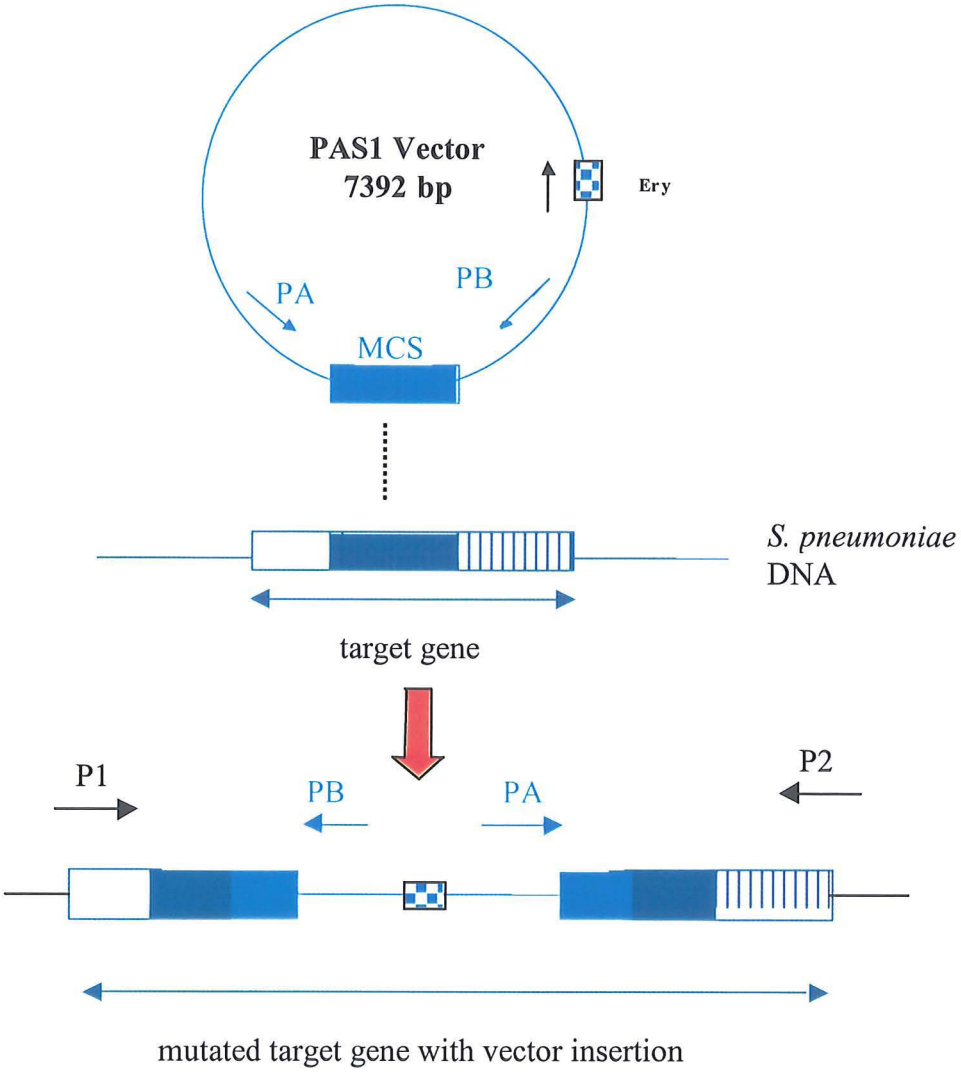

Figure 1.5: Insertion-duplication mutagenesis

A short region homologous to the target gene is cloned into a suicide vector, PAS1, within the multiple cloning site (MCS). The vector, containing a gene conferring erythromycin resistance (ery) is transformed into *S. pneumoniae*. Unable to replicate in the pneumococcal cells, the plasmid (shown in light blue) is eliminated or integrates into the genome via homologous recombination with the target gene (shown in dark blue). This integration results in a duplication of the homologous region and mutants can be selected for their growth on erythromycin. Successful integration into the target gene can be determined by PCR across the chromosome / plasmid junctions. Primer pairs P1 + PB and PA + P2 would confirm the 5' and 3' integration sites, respectively, where one primer of each set is specific for genomic DNA (P1 and P2) and one primer for each set is specific for the integrated plasmid DNA (PA and PB).

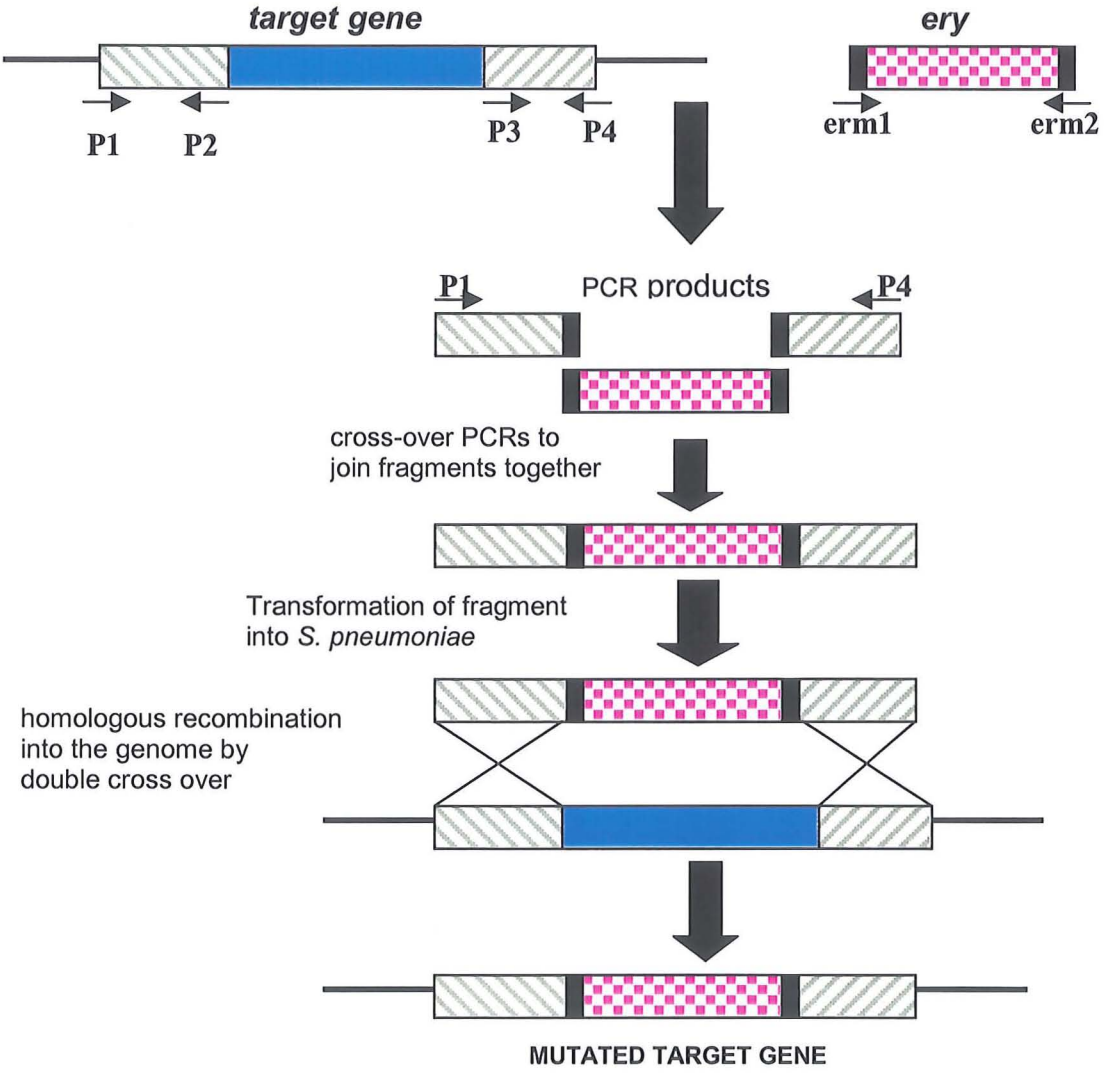

Figure 1.6: Allelic-replacement mutagenesis

Null mutations are made in target genes by replacing the gene with an cassette encoding antibiotic resistance. Primer pairs P1 + P2 and P3 + P4 are used to create PCR products flanking the gene of interest. Primers erm1 and erm2 are used to amplify up the erythromycin resistance (ery) cassette. Primers P2 and P3 have short sequences at the 5' end that are homologous to the 5' regions of erm1 and erm2, respectively. The two flanking regions are mixed with the ery cassette in a cross over PCR to generate an ery cassette with flanking regions homologous to the target gene or regions immediately outwith the target gene. When transformed into *S. pneumoniae*, double cross-over events occur at each flanking region and the cassette replaces the target gene. Mutants can be selected on erythromycin and confirmed using primers P1 and P4. Figure modified from Throup *et al.*, 2000.

Chapter 1

Introduction

Part 2. Bacterial Two Component Signal Transduction Systems (TCS)

1.8 Overview of two component systems (TCS)

The TCS of bacteria generally comprise two protein modules; a transmembrane receptor also known as a sensor protein, or histidine protein kinase (HK / *hk*), due to its ability to autophosphorylate a histidine residue, and its cognate, intracellular signalling component commonly known as a response regulator (RR / *rr*). The various histidine kinases and response regulators can be organised in a number of different ways to assemble a variety of signal transduction circuits. For instance, such couples, including hybrid histidine kinases that contain additional response regulators or histidine-containing phosphotransfer domains can be conjugated to constitute a complex phosphorelay pathway (Dutta *et al.*, 1999). Bacterial TCS were first described in the mid-late eighties when the amino acid sequence of CheY, a response regulator involved in chemotaxis in *E. coli*, and other Gram-negative bacteria, was shown to be related to regulatory proteins of other cellular processes (Stock *et al.*, 1985). The dual component nature of such systems was then recognised (Nixon *et al.*, 1986) and since, these systems have been found to be ubiquitous in bacteria. Indeed, over three hundred have been identified in approximately one hundred different bacterial genera (Goudreau and Stock, 1998). The only bacteria studied to date in which multiple TCS have not been identified are *Borrelia burgdorferi* and *Mycoplasma genitalium* (Barrett *et al.*, 1998). The small genome and limited metabolic capacities of the two pathogens suggest they may not require or be able to respond to their environment via gene regulation. TCS have been shown to respond to a very wide range of environmental signals (such as pH, temperature, nutrient availability and oxygen/carbon dioxide levels) and control a range of activities, including the regulation of virulence factors, sporulation, chemotaxis and competence. Table 1.2 illustrates the diversity of cellular processes controlled by bacterial TCS in a large range of different bacteria. This table represents a range of bacteria, including Gram-positive and Gram-negative organisms, human and plant pathogens, swarming bacteria, photosynthetic bacteria and spores-forming organisms. Most bacteria will contain multiple TCS and these can be involved in the regulation of a large number of genes or sets of genes. Indeed any one TCS may interact with one or more other TCS, and activation of a single TCS can induce both negative and positive regulation of different sets of genes. The *S. aureus agr* system and other TCS involved in regulation of virulence genes in this organism are examples of the complex nature of bacterial gene regulation.

Study of the control of virulence factors in response to environmental stimulus by a pathogenic organism is obviously an important area in terms of understanding bacterial pathogenicity and in the development of novel therapies (Magasanik, 1995). Many bacterial TCS are recognised for their role in the regulation of genes important for different stages of the infection process. This regulation of virulence genes varies greatly and includes control of secreted toxins and toxic products, alteration of membrane / cell wall proteins, control of capsule synthesis, motility and nutrient utilisation. The contribution to virulence includes damage to host tissue, evasion of the immune response and resistance or tolerance to antibiotics. Some of the TCS recognised to be important in the control of virulence factors in bacterial and plant pathogens are listed in table 1.3. Only selected TCS from individual organisms are shown and often homologues of the described systems are present in other bacteria. TCS important in the virulence of *S. pneumoniae* are discussed under a separate section (introduction, section 1.14.5). The list of TCS represented in tables 1.2 and 1.3 is by no means exclusive. Indeed most bacteria will possess more than one TCS and therefore can detect and respond to several different environmental stimuli. At least thirty TCS have been identified so far in *E. coli* on the basis of sequence homology. Mutants in all of these have recently been analysed using microarrays (Oshima *et al.*, 2002). Given the large number of TCS in individual organisms, interactions between individual TCS within a cell ('cross-talk') are likely to occur. Indeed several TCS interact in *S. aureus* to regulate different sets of virulence factors (Lyon *et al.*, 2000; Fournier *et al.*, 2001; Yarwood *et al.*, 2001). One of these systems, Agr, is very well characterised and acts as a global regulator capable of inducing and repressing different groups of virulence-associated genes depending on the site of infection (Lyon *et al.*, 2000).

Organism	TCS (HK/RR)	Function	Reference
B. subtilis	VanS/VanR	Vancomycin tolerance	Evers and Courvalin, 1996
B. subtilis	KinA /Spo0F	Sporulation regulation	Wang *et al.*, 2001
B. subtilis	ComP/ComA	Competence regulation	Weinrauch *et al.*, 1990
B. subtilis	DegS/DegU	Regulation of many cellular processes e.g. exoprotease production, catabolism, competence development and motility	Ogura *et al.*, 2001
C. crescentus	CckA/CtrA	Co-ordination of cell cycle progression and polar morphogenesis	Jacobs *et al*, 1999 Wheeler and Shapiro, 1999
E. coli	CheA/CheY,B	Chemotaxis	Hess *et al.*, 1988
E. coli	EnvZ/OmpR	Adaptation to environmental osmotic changes by controlling expression of major outer membrane proteins	Egger *et al.*, 1997
Enteric bacteria	PhoR/PhoB	Phosphate regulation	Lee *et al.*, 1989
R. sphaeroides	PrrA/PrrB	Expression of photosynthesis and Calvin cycle CO_2 fixing operons	Emmerich *et al*, 2000
Rhizobium spp.	FixL/FixJ	Nitrogen fixation	Weinstein *et al.*, 1992
S. aureus	SrhS/SrhR	Regulation of energy transduction in response to changes in oxygen availability	Throup *et al.*, 2001
S. aureus	YycF/YycG	Regulation of bacterial cell wall / membrane composition.	Martin *et al.*, 1999
S. coelicolor	AbsA1/AbsA2	Antibiotic synthesis	Brian *et al*, 1996 Anderson *et al*, 2001
V. fischeri	LuxI/LuxR	Quorum sensing and induction of bioluminescent genes	Salmond *et al.*, 1995

Table 1.2 Diversity of bacterial processes controlled by TCS

Legend on following page

Table 1.2 Diversity of bacterial processes controlled by TCS

Bacillus subtilis is a sporulating Gram-positive organism. *Caulobacter crescentus* represents a swarming bacterium. *Escherichia coli* is a commensal of humans and mammals and can also be pathogenic, causing a range of diseases. The *Enterobacteriaceae* are Gram-negative commensals of the digestive tract, although they can be important pathogens. *Rhodobacter sphaeroides* is a purple photosynthetic, symbiotic bacteria. *Rhizobium* spp. are Gram-negative bacterium common in soil and can induce nodule formation for nitrogen fixation in leguminous plants. *Staphylococcus aureus* is an important pathogen of humans and animals. *Streptomyces coelicolor* is a Gram-positive bacterium found within the environment and commonly associated with antibiotic production. *Vibrio fischeri* is a Gram-negative aquatic organism capable of bioluminescence.

Organism	TCS (HK/RR)	Function	Reference
A. tumefaciens	VirA/VirG	Crown gall tumour formation	Dziejman and Mekalanos, 1995
B. pertussis	BvgS/BvgA	Activation and repression of a range of virulence factors	Akerley *et al.*, 1992
E. faecium	VanR/VanS	Vancomycin resistance	Arthur *et al.*, 1992
K. pneumoniae	NtrA/NtrC	Urease production	Collins *et al.*, 1993
M. tuberculosis	PrrB/PrrA	Involved in early intracellular multiplication during macrophage infection	Ewann *et al.*, 2002
N. gonorrhoeae	PilA/PilB	Pilus production	Taha *et al.*, 1991
P aeruginosa	PirS/PirR PilS/PilR FleS/FleR AlgR2/AlgR1	Iron acquisition Pilus production Adhesion Alginate synthesis	Rumbaugh *et al.*, 1999 Rodrigue *et al.*, 2000 Dziejman and Mekalanos, 1995
S. aureus	AgrC/AgrA ArlS/ArlR	Expression of a range of extracellular proteins and toxins involved in various aspects of virulence (global expression) Interacts with other systems to modulate virulence factor regulation	Balaban *et al.*, 1998 Yarwood *et al.*, 2001 Fournier *et al.*, 2001
S. pyogenes	CsrS/CsrR	Negative regulation of hyaluronic acid production and several toxins	Bernish and Rijn, 1999 Engleberg *et al.*, 2001
Salmonella spp.	PhoQ/PhoP	Required for survival in macrophages	Groisman 2001
Salmonella, E. coli, Shigella	EnvZ/OmpR	Outer membrane protein expression for nutrient acquisition	Bernardini *et al.*, 1989 Barrett and Hoch, 1998
V. cholerae	ToxS/ToxR	Expression of virulence factors e.g. pilus and toxins	DiRita, 1992

Table 1.3: Bacterial TCS involved in the regulation of virulence determinants

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Table 1.3: Bacterial TCS involved in the regulation of virulence determinants

The examples given in the table generally represent pathogens important in human infection, although *Agrobacterium tumefaciens* is a plant pathogen able to induce tumour formation in its host. The human pathogens are responsible for a range of disease processes, some capable of causing many different infections. *Bordetella pertussis* is the causative agent of whooping cough. *Enterobacter faecium*, *Klebsiella pneumoniae*, *Salmonella*, *Escherichia* and *Shigella* are all members of the *Enterobacteriaceae* (see table 1.2). *Mycobacterium tuberculosis* causes tuberculosis. *Pseudomonas aeruginosa* is a prominent pathogen in cystic fibrosis patients. *Staphylococcus aureus* and *Streptococcus pyogenes* are both Gram-positive organisms that can cause a range of diseases at different sites within the body. *S. aureus* has become a pathogen of particular significance due to strains that are now resistant to virtually all licensed antibiotics (MRSA). *Vibrio cholerae* is the causative agent of cholera.

1.9 Signal Transduction

The mechanism of converting an extracellular signal into gene expression in bacterial TCS occurs via reversible protein phosphorylation (Wurgler-Murphy and Saito, 1997), hence the reason such systems are also classified as phosphorelay systems (figure 1.7). It is believed that on sensing its stimulus the histidine kinase undergoes a conformation change that results in auto-phosphorylation within a single, conserved histidine residue. The γ-phosphate of ATP is utilised as the high-energy phosphate source. Subsequently the phosphoryl group is then transferred to an aspartate residue on a cognate response regulator, resulting in activation of the regulator in question (Perego and Hoch, 1996). Frequently histidine kinases and response regulators are organised within an operon and often, though not exclusively, these proteins are members of a cognate pair.

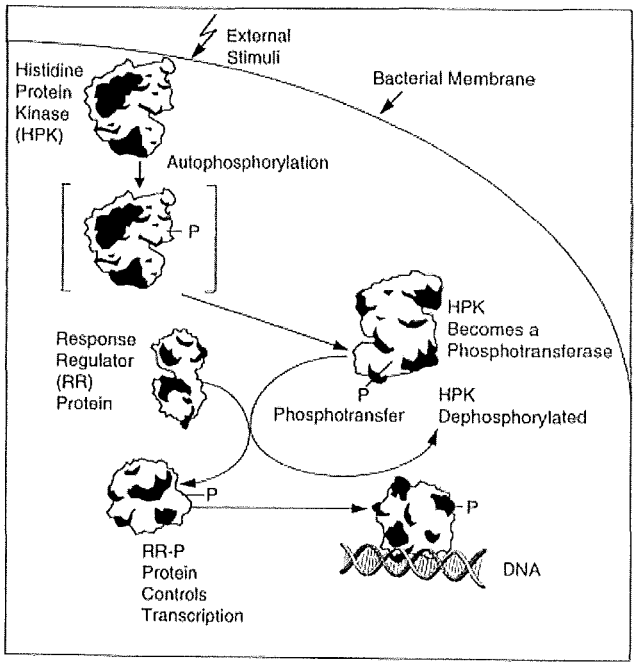

Figure 1.7: Overview of bacterial TCS

The figure shows the phosphoryl transfer process occurring between the histidine kinase (HPK) and its cognate response regulator (RR) (reproduced from Barrett and Hoch, 1998). Following detection of its environmental stilulus, the HPK autophosphorylates on a histidine residue. The phosphoryl group is transferred from the HPK to its cognate RR protein, found in the cytoplasm. The phosphorylated RR protein can control gene regulation by binding to promoter regions upstream of its target genes. .

The majority or regulators to date appear to act as transcriptional activators of a gene or set of genes although phosphorylated response regulators that also act to repress genes are a common feature. Such systems have also been described as sensory-recognition and signal transduction systems, although the term 'two-component systems' will be used throughout this report for convenience.

1.10 Histidine kinases

Histidine kinases are generally made up of an extracellular sensing domain, two or more hydrophobic transmembrane helices traversing the cell membrane, and a cytoplasmic domain containing the histidine residue that is autophosphorylated. Several conserved regions or boxes, which are used for identifying members of the histidine protein kinase superfamily, are also located within the cytoplasmic domain (Grebe and Stock, 1999). Such conserved sequences extend for approximately 200 amino acid residues and are located at the C-terminal end of the protein. All histidine kinases share 20–30 % sequence homology within this region (Hakenbeck and Stock, 1996). The hydrophobic, transmembrane regions are situated toward the N-terminus of the protein (Stock *et al.*, 1995). Histidine kinases can thus be divided into two different functionally and structurally distinct regions or domains, the extracellular portion that senses stimuli, and the cytoplasmic domain involved in autophosphorylation and phosphotransfer to the cognate response regulator. Due to the range of stimuli that can be detected by histidine kinase proteins, it is not surprising that the regions involved in sensing show the greatest diversity within the family. In contrast the functionally conserved boxes are associated with autophosphorylation and phosphoryl transfer. Histidine kinase sensing proteins tend to be found as homodimers that operate in *trans*, with the kinase domain of one subunit catalysing the phosphorylation of a second subunit (Stock *et al.*, 1995; Lau *et al.*, 1997; Dutta *et al.*, 1999; Surette *et al.*, 1999). Histidine kinases may also be assigned into either of two classes (ClassI or ClassII), depending on various structural differences (Bilwes *et al.*, 1999). Figure 1.8 shows a schematic diagram of a typical histidine kinase EnvZ.

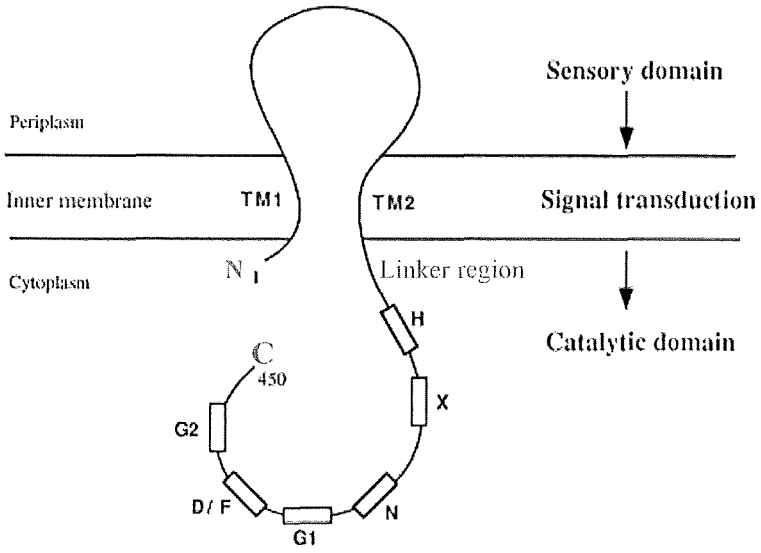

Figure 1.8: Schematic representation of a histidine kinase, EnvZ, from *E. coli*

The figure shows the various regions associated with bacterial histidine kinase sensors. This particular sensor senses osmolarity and regulates porin expression accordingly. Homology boxes (H, X, N, G1, D/F, G2) and their relative positions to one another in the primary amino acid sequence are illustrated. Only one member of the homodimer pair is shown for convenience and the periplasm would be covered by a further membrane in *E. coli* and other Gram-negative cells (reproduced from Hsing *et al.*, 1998).

Initially it was believed that the sensory region of histidine kinase sensors was an essential component, being the region that sensed the environmental signal (Stock *et al.*, 1990). However, one study found no change in the ability of the EnvZ histidine kinase to regulate the expression of the outer membrane porins, OmpF and OmpC following deletion or replacement of the periplasmic sensing domain (Leonardo and Forst, 1996). Following these observations, the authors proposed that osmolarity changes are sensed directly through the transmembrane regions of EnvZ and that the periplasmic domain may be required for correct positioning of the transmembrane helices for signalling or could detect some other, as yet unidentified, signal. This may not be the case, however, for all histidine kinase proteins.

Although it is rare to have kinases that naturally lack the periplasmic / extracellular sensing domain, several have been identified, one example being the chemotaxis kinase CheA (Grebe and Stock, 1999), which senses chemical gradients using a distinct class of chemoreceptors known as methyl-accepting chemotaxis proteins (MCPs). This HK protein is a cytoplasmic protein. Other cytoplasmic HK proteins have also been identified, the NtrB nitrogen-regulating kinase of *E. coli* being an example (Stock *et al.*, 2000). Genomic sequencing projects may assist in identifying other examples where sensing modules can be found to deviate from the general model.

A role for the transmembrane spanning regions beyond surface anchoring is not yet clear although it has been proposed that they undergo a conformational change following activation, which results in autophosphorylation within the cytoplasmic domain. It is possible that, in *E. coli* EnvZ and similar systems, one transmembrane helix moves relative to the other during signalling (Hugson and Hazelbauer, 1996), but different systems may not exhibit such movement. Not all histidine kinases possess two transmembrane domains as described for the *E. coli* EnvZ sensor. The sensor kinase KdpD of *E. coli* has four predicted transmembrane helices (Zimmann *et al.*, 1995) and at least eight transmembrane regions are present in UhpB, a histidine kinase within the same organism. The histidine kinases ComD (*S. pneumoniae*) and AgrC (*S. aureus*), which show homology to TCS13 of *S. pneumoniae*, have 5-7 potential transmembrane segments that are characteristic of this group. Although most members of the histidine kinase protein family do display transmembrane stretches of hydrophobic amino acids, thought to be essential for correct functioning (Stock *et al.*, 1990), this region may be indispensable in some members. Several soluble histidine kinases have been identified and were discussed above. Otterman and Koshland demonstrated that signalling via the Tar receptor (a signalling molecule involved in *E. coli* chemotaxis) was still functional when the transmembrane domains were deleted and the periplasmic and cytosolic domains were directly linked, thus creating a soluble protein (Ottermann and Koshland, 1997).

1.10.1 Homology Boxes

Members of the histidine kinase superfamily can be identified based on five conserved regions/motifs of similarity, named the H, N, G1, F (D) and G2 boxes respectively (figure 1.8), (Alex and Simon, 1994; Grebe and Stock, 1999). These cytoplasmic sequences are arranged in the same order but variably spaced within kinases (Parkinson and Kofoid, 1992). As these motifs

display highly conserved residues they are presumed to play crucial roles in substrate binding, catalysis and/or structure, and have also been used to classify the histidine kinases into subfamilies. In some bacteria, most of the histidine kinase proteins belong to the same family but other bacteria have histidine kinases that represent members of many families (Foussard *et al.*, 2001). From the descriptions below it can be seen that most of the conserved sequences play a role in nucleotide binding, implying that they are in close proximity to one another within the tertiary structure of the mature protein.

H box

This region contains the site of histidine phosphorylation and is the most variable of the five motifs. It can be found within the N-terminus of the cytoplasmic region of the histidine kinase and the phosphorylated histidine is usually located approximately 110 amino acids N-proximal to the N-box (Stock *et al.*, 1995). Although generally present in most histidine kinase members, there are examples where the phospho-accepting histidine is replaced by other residues, namely aspartate, tyrosine or arginine. Wu and colleagues have shown experimentally that a tyrosine residue in a *Caulobacter crescentus* histidine kinase, DivL, is phosphorylated (Wu *et al.*, 1999). However, in most systems, if this phospho-accepting histidine residue is substituted, autophosphorylation and phosphatase activity are both lost (Kanamaru, 1990), although the region surrounding the conserved histidine residue is also likely to influence the ratio of these two activities.

N box

Mutations within the N region of *E. coli* EnvZ suggest it is critical for kinase activity. A mutation within this region did not bind ATP as well as the wild type, which infers that the N region has an involvement in nucleotide binding (Hsing *et al.*, 1998).

G1 and G2 boxes

These glycine-rich regions and are also thought to be involved in nucleotide binding as they form an ATP-binding cavity, along with the N and F boxes (Foussard *et al.*, 2001). They may also be associated with autokinase activity. Substitution of certain glycine residues in G1 and G2 with alanine in the CheA HK of *E. coli* resulted in a reduced affinity for ATP and no detectable autokinase activity (Stewart *et al.*, 1998). Kinase and phosphatase activities may also be

associated with the G boxes as they play a critical role in phosphotransfer (Stewart *et al.*, 1998). The G boxes, if both present, are separated by a variable sequence in which block F (also known in some of the literature as block D) is located. Nucleotide binding may still occur, however, with only one of the G boxes present, as some functional transmitters have been identified that lack one of these blocks.

F(D) box

This motif is part of the nucleotide binding domain and probably interacts with the α-group of the adenine ring (Grebe and Stock, 1999), although its exact function is as yet unknown. Hsing *et al.*, 1998, created a mutant, in EnvZ, within this region that resulted in a porin negative phenotype. In eukaryotic kinases, a conserved aspartic acid/phenylalanine/glycine (DFG) motif is believed to be involved in chelating the Mg^{2+} that bridges the β and γ phosphates of ATP. This helps to orient the phosphate moiety facilitating its transfer (Taylor *et al.*, 1992). Thus, F(D) and G boxes are thought to be important in positioning of ATP for optimal phosphoryl transfer.

X box

One further region of potential importance in the cytoplasmic domain of histidine kinases was recently identified through various mutations that affected kinase or phosphatase activity (Hsing *et al.*, 1998). Most of these mutations mapped to the aforementioned regions, but a novel motif, weakly conserved in other bacterial TCSs, was also discovered. Mutations within this region were found to alter the conformation of EnvZ, resulting in a significant decrease in phosphatase activity. This region was found to be N-terminal to the H-box and appears to be important in the de-phosphorylation of OmpR~P (the cognate response regulator) in *E. coli*.

Linker Region

The region between the transmembrane hydrophobic segments and the cytoplasmic signalling domain of histidine kinases may also be classed as an important domain in its own right (Goudreau and Stock, 1998). A role for this cytoplasmic linker region has been implicated in signalling in the EnvZ/OmpR pathway of *E. coli* (Park and Inouye, 1997), and has been shown to adopt a closer juxtapositioning between subunits of a histidine protein kinase sensor upon binding of a ligand (Chen and Koshland Jr, 1997). In histidine kinases the linker regions are not usually well conserved (Lange *et al.*, 1999). Lange *et al.*, 1999, identified a region of low, but

significant, level of sequence similarity within the linker region in five of the histidine kinases identified in *S. pneumoniae*. This was termed the 'E box' since in all of the sequences a glutamate residue ('E' in the one-letter amino acid code) was present. This motif was used to screen databases, and several histidine kinases, including EnvZ, MCPs were found to have homologous regions.

1.10.2 Unorthodox bacterial histidine kinases

Several bacterial HK exist that do not phosphorylate on a single histidine residue as described above. Instead, such sensor proteins have 3 phosphorylation sites and are thus referred to as tripartite or unorthodox. An example of such a sensor is the TorS kinase of *E. coli* (Ansaldi *et al.*, 2001). The HK detects thimethylamine *N*-oxide and once activated, its cognate RR activates transcription of the genes encoding the thimethylamine *N*-oxide anaerobic system. This HK has two histidine phosphorylation sites and one aspartate phosphorylation site and the multi-step phosphorelay progresses from His(HK)-Asp(HK)-His(HK)-Asp(RR). The reason for the existence of such systems is unclear but it may enable additional regulation of expression. Other unorthodox sensors include the HK BvgS of *Bordetella* species.

1.10.3 Structure of histidine kinases

The three-dimensional structures of several response regulator proteins have now been elucidated but, being membrane-located proteins, the structures for histidine kinases have been harder to characterise. Recently, the 2.6 Å resolution crystal structure of part of the CheA histidine kinase of *Thermotoga maritima* has been determined, CheAΔ289, (Bilwes *et al.*, 1999), which possesses a chemotaxis system similar to that found in mesophilic bacteria. The two subunits of the CheA dimer were each found to contain separate dimerisation, kinase and regulatory domains with no contact occurring between the latter two (figure 1.9)

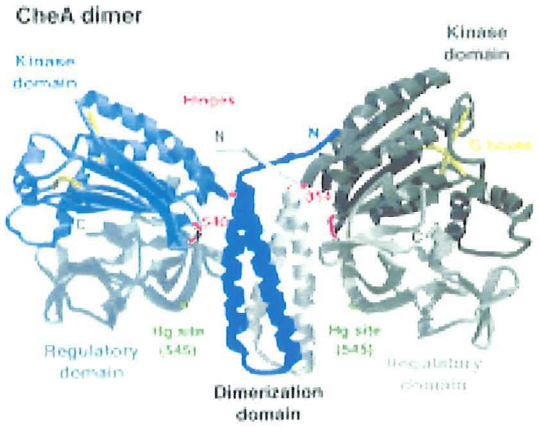

Figure 1.9: Structure of part of the CheA dimer from *T. maritima* (CheAΔ289)

The figure shows the three functionally different domains; the kinase domain, regulatory domain and dimerisation domain. Two monomers of CheA associate via a centrally located four-helix bundle which places the two ATP-binding sites (G-boxes) 90 Å away from each other to form a dimer (taken from Bilwes *et al.*, 1999).

The domain showing kinase activity is a two-layered α-β sandwich with a five-stranded β sheet and six α-helices, which produces a cavity presumed to be the ATP-binding site. This catalytic domain was found to have completely distinct topology to that of serine/threonine or tyrosine kinases, but did show similarity to two functionally different ATPases; the N-terminal of gyrase B (a type II topoisomerase) and the N-terminal of Hsp90 (a chaperone protein). The NMR structure of EnvZ of *E. coli* ,a class I kinase, was also found to show similarity to gyrase B and Hsp90 (Tanaka *et al.*, 1998). However, CheA belongs to the class II group of histidine kinases (Bilwes *et al.*, 1999) which show structural differences to those in class I (the latter of which most of the bacterial TCS concerning us are grouped), and variation in three-dimensional structure may thus be expected. Through sequence alignment around the H box, Bilwes and co-workers proposed that class I histidine kinases are likely to share a similar dimerisation motif with CheA. This demonstrated several conserved hydrophobic residues indicative of two amphipathic helices preceding the kinase domain. The phosphorylated histidine residue may be located within this dimerisation motif for class I but is at a distant region for class II histidine

kinases. Dimerisation may be important for the regulation of signalling and would therefore be critical for histidine kinase activity.

Hinge regions within histidine protein kinase structures may allow for movement and transphosphorylation to the phospho-accepting histidine residue. In multi-domain histidine kinases, relative movement between the domains is likely to be an essential feature of signalling, as mutation of a hinge residue in the CheA histidine kinase of *E. coli* (Arg354-Cys) reduces autophosphorylation and suppresses chemotaxis. In class I histidine kinases a hinge region would be required to allow the kinase domain to reach the histidine substrate on the dimer interface and then to release this region to allow for subsequent transfer of the phosphoryl group from the His~P to an aspartate residue on the response regulator. The availability of the crystal structure for CheAΔ289 provides an opportunity for further study into the interactions between domains/modules within histidine kinases and also provides a focus for drug design.

1.11 Response regulators

Response regulators are generally cytoplasmic proteins that associate with a cognate histidine kinase sensor by an unknown mechanism. This protein family share a conserved N-terminus sequence of approximately 125 amino acids, termed the 'receiver' domain which contains the aspartic acid residue that accepts the phosphoryl group from the phosphorylated histidine kinase in a Mg^{2+}-dependent reaction (Goudreau and Stock, 1998). Like the C-terminus of histidine kinases, the conserved N-terminus of any response regulator exhibits 20-30 % amino acid sequence homology to other response regulators, and also contains several invariant residues. These include an aspartate residue near the N-terminus border of the domain, one near the centre and a lysine residue near the C-terminus border. These conserved residues cluster within an acidic pocket that serves as the site for aspartyl phosphorylation. The gain of a phosphate group within the receiver domain is assumed to activate the DNA-binding/transcriptional regulatory domain located at the C-terminus, which consequently results in activation and/or (Lange *et al.*, 1999) repression of a given set of genes (Hakenbeck and Stock, 1996). A schematic structure of a response regulator is shown in figure 1.10.

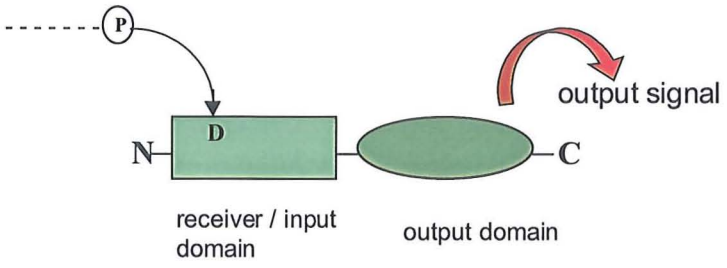

Figure 1.10: Schematic diagram of a typical bacterial response regulator

The response regulator is phosphorylated on an aspartate (D) residue within the receiver / input domain by its cognate histidine kinase (not shown). Phosphorylation is thought to result in a conformation change allowing the activation or repression of genes by the output domain located at the C-terminus region of the response regulator.

The majority of response regulators are multi-domain proteins that are very diverse, with the exception of the receiver domain (Volz, 1995). These proteins have been divided into subfamilies based on various similarities in their C-terminal sequences, and the families are often named by their founder member, for example, OmpR, AraC, CheY, NtrC and FixJ (Stock *et al.*, 1990; Egger *et al.*, 1997). Within each subfamily members associate with a different set of promoters, and almost identical response regulators can regulate different responses in different species (Hakenbeck and Stock, 1996). The response regulator of *S. pneumoniae* TCS08 belongs to the OmpR family of response regulators (Lange *et al.*, 1999) which contain a conserved DNA-binding domain of approximately 150 amino acids, linked to the C-terminus of a receiver domain. Members are able to activate and/or repress target genes depending on features of the promoters with which they interact. OmpR proteins appear to function through direct contact with the C-terminus of the α-subunit of RNA polymerase (Pratt *et al.*, 1994). When these response regulators are not in the phosphorylated state the N-terminus receiver domains appear to interfere with DNA binding and/or transcriptional control by blocking the access of the C-terminus domain. It has been proposed that phosphorylation of the receiver domain could then result in a conformational change which allows the active site to be exposed and thus reverses the inhibitory effect. This is just one possible mechanism of action of response regulators.

Phosphorylated receiver domains have also been shown to act in a positive manner in the activation process, by providing additional surfaces for intermolecular interactions (Djordjevic *et al.*, 1998), or to act to enhance oligomerisation between response regulators, which in turn could enhance DNA binding properties. Indeed, when purified OmpR is phosphorylated *in vitro*, it dimerises and shows increased ability to bind specific target DNA sequences (Nakashima *et al.*, 1991).

1.11.1 Structure of response regulators

CheY of *E. coli* was the first member of the response regulator family for which a three-dimensional, X-ray crystallographic structure was determined (Stock *et al.*, 1989) and from which further studies helped to elucidate mechanisms of phosphoryl transfer and identify important residues in this process (Volz and Matsumura, 1991; Stock *et al.*, 1993). In this particular response regulator it was found that the signalling ability is modulated by the movement of the phenolic side chain of tyrosine, position 106, between the inside and outside positions within the CheY structure (Zhu *et al.*, 1997). Within the receiver domain residues D-13, D-57 and K-109 (aspartic acid and lysine residues respectively) have been implicated as having important roles in the phosphorylation active site (Lukat *et al.*, 1990). Within this site, the presence of a hydroxyl containing amino acid (serine or threonine) is thought to be crucial for proper functioning. All members of the CheY family appear to share a doubly-wound, five-stranded, α/β motif with a hydrophobic core. Until recently it was assumed that all response regulator domains shared this α/β structure (Guillet *et al.*, 2002; Im *et al.*, 2002), but the crystal structure of the OmpR DNA binding domains defined a new structure for transcriptional activators which have been termed 'winged helix-turn-helix' motif (Martinez-Hackert and Stock, 1997). These helix-turn-helix consensus sequences have been identified in many members of the OmpR families, and function in both the binding of specific DNA sequences and in the interaction with RNA polymerases. This provides evidence that response regulators of other families could have different structural features that are, as yet, unidentified. Over half of the response regulators identified in *S. pneumoniae* belong to this OmpR family, and the ones studied so far contain the helix-turn-helix consensus.

A crystal structure is also available for a member of the NarL family of response regulators bound to DNA (Maris *et al.*, 2002).

1.11.2 Phosphorylation reactions

The chemistry of phosphorylation at histidine and aspartate residues that predominates as the signal transducing mechanism in bacteria is very distinct from eukaryotic phosphorylation which generally occurs at serine, threonine and tyrosine side chains (Stock *et al.*, 1990).

The intrinsic free energies of phosphorylation of amino acids alone often differs from when these amino acids are residues within a protein. When isolated histidine and aspartate amino acids are phosphorylated, they are high-energy molecules with the equilibrium well in favour of the un-phosphorylated state (figure 1.11).

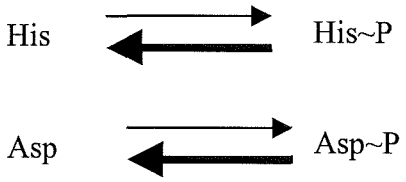

Figure 1.11: Phosphorylation reaction

The phosphorylation of histidine and aspartate residues of HK and RR, respectively, lies with the equilibrium in favour of the un-phosphorylated form.

When a phospho-histidine residue (his~P) within a protein is de-phosphorylated, the energy released is very similar to the isolated amino acid, which indicates that de-phosphorylation of this particular amino acid does not depend on the protein of which it is a part. This is important in the context of histidine-aspartate phospho transfer signal transduction where the phosphorylated histidine residue functions as a catalytic phosphate donor with the ability to drive phosphorylation of its substrate. However, the free energy of phosphorylation of aspartate within a protein depends on the rest of the protein structure and explains why phosphorylation of this residue may trigger a conformational change within the protein or response regulator in which it is located (Stock *et al.*, 1990).

Response regulators have been shown to autophosphorylate themselves *in vitro* using small, low molecular weight phosphodonors, including phosphoramidate or acetyl phosphate (Parkinson and Kofoid, 1992), so a small amount of phosphorylation of these modules may occur within the cell without input from the cognate histidine kinase. However, the *in vivo* significance of this

phenomenon is unclear and, until recently (Throup *et al.*, 2000), no evidence has been found in which a response regulator exists independently of a cognate histidine kinase.

The phosphorylated receiver of response regulators is unstable so is readily converted to its un-phosphorylated form. This de-phosphorylation process may be autocatalytic, although it has been proposed, and generally accepted, that the cognate histidine kinase protein plays a major role in this action (Hakenbeck and Stock, 1996; Mizuno, 1998). Thus the absolute level of phosphorylation of a response regulator may be determined by the ratio of kinase to phosphatase activities of its kinase and the autophosphatase activity of the response regulator. Systems have been identified whereby the kinase does not function as a phosphatase and this role is served by a completely distinct protein. This occurs in the Che system of chemotaxis in *E. coli*, where de-phosphorylation of CheY~P response regulator is catalysed, independently of the CheA kinase, by the CheZ protein. In EnvZ, a histidine kinase involved in sensing osmolarity in *E. coli*, the G2 box has been implicated as having a role in phosphatase activity (Zhu and Inouye, 2002). However, in NtrB, a histidine kinase involved in nitrogen regulation in *E. coli*, the phosphatase activity has been attributed to the H-box (Weiss *et al.*, 2002).

The half life of the phosphorylated receiver can vary from only a few seconds, as in the case of phospho-CheY, to several hours, as in the case of phospho-OmpR and this may play an important role in the type of response generated. In the case of CheY phosphorylation, which is involved in the chemotactic response of *E. coli*, a very short half life is necessary to allow the bacteria to respond quickly to changes in chemotactic gradients which could occur over a very short time period. Such systems may thus be harder to study and elucidate than those in which the phosphorylated response regulator persists for longer time periods.

1.11.3 Orphan response regulators

Although response regulators are usually part of a TCS with a cognate HK protein, there are examples of 'orphan' *rr* genes found in the absence of a *hk* gene. Examples or organisms having unpaired response regulators in their genomes include *S. pneumoniae*, *Listeria monocytogenes*, *Helicobacter pylori*, *Streptococcus agalactiae*, *Streptococcus suis* and *Campylobacter jejuni* (Throup *et al.*, 2000; Glaser *et al*, 2001; Delany *et al.*, 2002; Glaser *et al.*, 2002; de Greeff *et al.*, 2002). Most of these *rr* retain the conserved active-site residues of other *rr* genes, with the exception of the orphan regulators in *H. pylori* (Delany *et al.*, 2002). Two studies in *Streptococcus* species have demonstrated that these RR proteins are active (Throup *et al.*, 2000;

de Greeff *et al.*, 2002). The pneumococcal unpaired *rr* gene (RR14) will be described elsewhere (introduction, section 1.14) and a null mutation in this gene was found to be highly attenuated in its ability to colonise the lungs in a murine model of pneumonia (Throup *et al.*, 2000). A second unpaired *rr* gene has been described in *S. suis*, a pig pathogen, and a mutation within this gene was also found to be impaired in virulence compared to wild-type (de Greeff *et al.*, 2002). *H. pylori* has 2 orphan response regulators, which is surprising considering that only three complete TCS have been identified in the genome of this Gram-negative pathogen. One of these RR proteins has been shown to bind to specific DNA sequences in response to growth phase. Phosphorylation of this RR is not a prerequisite for its activity. This RR appears to be autoregulatory and it is thought that this protein is tightly regulated at the post-transcriptional level. Interestingly, analysis of phosphotransfer reactions between purified proteins has illustrated that one of the complete TCS of this organism comprises *hk* and *rr* genes but these are encoded at distant loci (Beier and Frank, 2000).

1.11.4 Autoinduction of bacterial TCS

Auto-induction of the genes involved in regulation is a common feature in many bacterial TCS, whereby expression of the histidine kinase and response regulator genes is increased upon activation of the response regulator. Examples include the BvgA/S system of *Bordetella* species and the PhoPQ system of *Salmonella* species. Where auto-induction is present, genes other than the regulatory components are also regulated. Hoffer and colleagues describe an auto-amplification system in genes of the *phoR/phoB* TCS of *E. coli* and hypothesise that such a mechanism can help induce a more rapid response when sensing the same conditions on subsequent occasions (Hoffer *et al.*, 2001). Autoinduction is often a feature in bacterial quorum sensing systems to ensure that gene regulation only occurs at a certain quorum or cell density of bacteria.

1.11.5 Cross regulation in bacterial TCS

Cross regulation has been described by several researchers and is likely to play an important role in global control (co-ordination of several systems), linking response regulators of TCS to one another and/or to other general regulatory systems. The complex issues of cross regulation *in vivo* in *E. coli* and *Salmonella typhimurium* have been raised (Wanner, 1992). It appears that there are three separate TCS that act on the phosphate (PHO) regulon in *E. coli*. The histidine

kinase sensor, PhoR and the response regulator, PhoB, pair sense extracellular inorganic phosphate levels and respond accordingly. Usually, extracellular phosphate is the preferred phosphate source for the PHO system and is transported into the cell via the PstSCAB transporter. In contrast two other controls associated with expression of the PHO regulon act independently of inorganic phosphate. These involve metabolic pathways and are regulated by carbon source in different ways. The CreC sensor can be involved in regulating PhoB and is induced by growth on glucose while the other sensor is induced during growth on pyruvate but not on glucose, and is CreC independent. This regulation occurs between non-cognate partners / proteins not part of the same TCS.

A recent study used microarray analysis to examine gene expression *in vitro* in mutants in all TCSs in *E. coli* strain K-12. This study indicated that cross regulation and cascade regulation have important roles in cell physiology for co-operative functioning between multiple TCSs (Oshima *et al.*, 2002). Other studies have also provided evidence that sensor proteins may have the ability to phosphorylate non-partner regulators as well as their cognate partners. However, much larger amounts of the non-partner protein are required for this level of regulation, and the phosphorylation rates between non-partner proteins are much slower than that which occurs between partner proteins. For instance, Fisher *et al* produced evidence, again involving the PhoB response regulator of *E. coli*, whereby *in vitro* and *in vivo* activation of this response regulator was achieved using the cytoplasmic domain of the VanS histidine kinase from *E. faecium* (Fisher *et al.*, 1995). This was shown to occur in the absence of the cognate kinase of PhoB (PhoR), CreC or acetyl phosphate synthesis, which were eliminated by mutation. The VanS/VanR TCS of *E. faecium* (histidine kinase and response regulator respectively) is involved in activating genes that are responsible for vancomycin resistance in this Gram-positive organism. The resistance is achieved through activation, by VanR, of several structural genes that encode enzymes involved in modifying the peptidoglycan structure that ultimately results in a one thousand-fold decrease in the affinity for the glycopeptide antibiotic. The activation levels of the non-cognate response regulator, PhoB, by VanS were high with approximately ninety percent transfer of the phosphate group to PhoB within five minutes. However, the rate of transfer was at least one hundred-fold lower than that occurring between VanS and VanR. Similar studies have also reported on cross-regulation between non-cognate components of TCS, often demonstrating phosphorylation between proteins of different genera as illustrated above, demonstrating that histidine kinases and response regulators may be involved in very complex pathways.

The ability to induce gene expression by a response regulator in the absence of its cognate histidine kinase is demonstrated by the study of Ogura and colleagues. Plasmids expressing RR protein were introduced into cells where the cognate HK was deleted, yet expression of the target genes could still be induced (Ogura *et al.*, 2001). The mechanism behind this is not known but could indicate that the RR proteins were being phosphorylated by other, non-cognate HK proteins. It has also been observed that a deletion in a *hk* gene often does not result in a phenotypic change that is as pronounced as when the cognate *rr* gene is deleted. For example a null mutation in *S. pneumoniae* RR02 is lethal, whereas a null mutation in the cognate HK gene is not lethal (Throup *et al.*, 2000). This suggests that RR proteins may still be able to function in the absence of their HK partner, possibly through cross-regulation by other HK proteins.

1.12 Eukaryotic signalling systems

The two-component signal transduction systems described above are abundant in, and originally thought to be unique to, prokaryotes. However, recent studies have reported the existence of homologous systems in eukaryotes. Similarly eukaryotic-type signalling domains involving serine, threonine and tyrosine kinase cascades have been found in bacteria through sequence similarity (Goudreau and Stock, 1998). However, bacterial TCS homologues have not been identified in mammals or other vertebrates.

1.12.1 Eukaryotic-type signalling systems in bacteria

Eukaryote-type systems in bacteria are classed as those that do not conform to the general mechanism of two-component signalling as described above, and/or involve phosphorylation of residues other than histidine. Several such systems have now been recognised, including a system in the Gram-negative gliding bacterium, *Myxococcus xanthus,* which uses soluble and transmembrane serine and threonine kinases combined with changes in tyrosine phosphorylation for some developmental processes (Hanlon *et al.*, 1997). A second example occurs in the complex signalling cascades involved in sporulation in *B. subtilis*. Here, phosphorylation of a serine residue within the regulator for sigma factor σ^F occurs together with de-phosphorylation performed by a protein phosphatase, SpoIIE, that is a member of one of the subfamilies of eukaryotic serine/threonine phosphatases (Adler *et al.*, 1997).

1.12.2 Prokaryote-type systems in eukaryotic organisms

One of the first discoveries of a 'bacterial' histidine kinase within a eukaryotic organism occurred when Popov and colleagues cloned a kinase gene from rat mitochondria and found that the closest homologues to this enzyme were within the protein histidine kinase family of bacteria (Popov *et al.*, 1992). Although this protein has a putative catalytic histidine residue, it appears to phosphorylate its substrate on a pair of serine residues rather than on an aspartate residue, as is the case for 'typical' prokaryotic signal transduction. Subsequently, systems resembling bacterial TCS have been discovered in other eukaryotes, including the fungus, *Neurospora crassa* (Alex *et al.*, 1996), and the yeast, *Saccharomyces cerevisiae* (Maeda *et al.*, 1994). These results raise intriguing evolutionary questions as to how similar protein kinases and/or protein phosphatases occurred within different domains of life. For instance it is interesting to speculate on whether they were inherited directly from a universal ancestor or acquired through gene transfer.

The discovery of prokaryote-like signalling proteins in eukaryotes, and vice versa, provides further data to support the concept that within different systems there appears to be extensive variation and ways in which different components of signalling systems are arranged and operate to create complex information processing pathways. Even within the prokaryotes themselves, a variety of signalling mechanisms may occur where periplasmic, transmembrane and cytoplasmic domains are all required for a response, or a combination of these can suffice. Sensor dimers and conformational changes may or may not be required.

1.13 Targeting TCS for therapeutic purposes

Bacterial TCS are attractive targets for anti-microbial therapy for a number of reasons. Firstly, multiple TCS have been identified in most bacteria. TCS are often essential for viability (Volz 1995; Azoulay-Dupuis *et al.*, 1998; Lange *et al.*, 1999) and / or virulence (Roychoudhury *et al.*, 1993; Dziejman and Mekalanos 1995; Rumbaugh *et al.*, 1999 and table 1.1). Homology exists between TCS of different bacteria, which could facilitate the design of a broad-spectrum drug. Similarily, regions unique to individual TCS could be targeted to produce drugs specific for a given bacterium. Furthermore, homologous systems have not been identified in vertebrates so any drug specific for TCS should show low toxicity to the host cells (Hilliard *et al.*, 1999). A drug targeting several different regions within TCS would have the additional benefit of overcoming the 'target-alteration' mechanism utilised by microbes to confer resistance in

response to antibiotics that possess a very specific mechanism of action against one target only. The emergence of resistance to such drugs could also be minimised as most bacteria will contain multiple TCS with different functions, and the ability to mutate one of these to develop resistance will still leave the remaining systems susceptible to the drug in question. The crystal structure for several response regulators and the CheA histidine kinase have now been elucidated (Bilwes *et al.*, 1999; Guillet *et al.*, 2002; Maris *et al.*, 2002), providing a framework for rational drug design. Furthermore, with the increasing plethora of genome sequences that are now available in public databases, it is now possible to identify most/all TCS within an organism. Mutants can then be created within each individual system to evaluate their putative role in the viability / pathogenicity of the bacterium in question.

The therapeutic approach may not necessarily need to kill the pathogenic agent to be effective, as a bacteriostatic effect may be desirable and easier to achieve. *S. pneumoniae* infection is a prime example where it may be desirable to inhibit growth and virulence whilst avoiding death of the organism, as the latter would release the inflammation inducing cell wall products and the potent intracellular toxin, pneumolysin. Furthermore, this would block disease rather than carriage and would prevent niche replacement by other potential pathogens.

1.13.1 Potential sites for targeting TCS

Various sites of TCS could be targeted for intervention depending on whether the drug was required to be broad-spectrum and thus active against both Gram-positive and Gram-negative organisms, or if the therapy was to be targeted against a specific pathogen. In the former case, sequences conserved against all TCS in bacteria would be investigated. These sequences are found within the autophosphorylating sites of histidine kinases and the phosphoryl-accepting regions of response regulators. Residues specific to a single TCS within a bacterium are located in the sensory region involved in stimuli detection of histidine protein kinases, and the C-terminal of response regulators responsible for DNA binding and gene activation and/or repression.

Ideally it would be desirable to target drugs against surface components, i.e. the histidine kinase sensory region to avoid difficulties of drug entry into cells. In that regard, one possible obstacle in targeting TCS is that the histidine kinases are generally situated in the cell membrane. In Gram-positive bacteria this membrane will be covered by a thick layer of peptidoglycan that must first be penetrated. Gram-negative organisms have a thin layer of peptidoglycan but the

presence of an additional outer membrane. Cross regulation may also pose a problem as any particular set of genes controlled by a specific TCS could also be under the control of other systems, and thus the effect of inhibiting a given component may not be observed if the functions of this system can be complemented by other systems.

1.13.2 Natural inhibitors of TCS

Several natural inhibitors of TCS have been described. Strauch and co-workers reported that oleic acid, an unsaturated fatty acid, acted to inhibit the phosphorylation of a histidine kinase in *B. subtilis* (Strauch *et al.*, 1992). This inhibition was non-competitive, occurred independently of the presence/absence of the cognate response regulator and did not inhibit bacterial growth. Bromoacetosyringone, another natural compound, was shown to completely inhibit expression of genes required for crown-gall tumour formation in the pathogenic plant bacterium *A. tumefaciens*. These genes are under the control of a two-component system and could be completely inhibited at concentrations that did not affect bacterial growth (Barrett and Hoch 1998).

1.13.3 Chemical inhibitors of TCS

Roychoudhury and colleagues screened approximately 25,000 natural and synthetic compounds for their ability to inhibit the TCS which partially controls alginate production in the Gram-negative bacterium *P. aeruginosa*, an important pathogen in cystic fibrosis (Roychoudhury *et al.*, 1993). Alginate is thought to promote adherence of the organism and help resist phagocytosis. Out of all the compounds screened, fifteen were identified as inhibitory to *in vivo* alginate production in whole cells. Four of these chemicals showing significant inhibition were studied further.

Two of the chemicals (A and A') inhibited phosphorylation of the response regulator AlgR1, involved in alginate production, and had significant inhibitory effect on autophosphorylation of the cognate histidine kinase, AlgR2, above a concentration threshold of ~ 0.5 $\mu g\ ml^{-1}$. They also affected histidine kinase autophosphatase activity. Chemicals A and A' had no significant effect on the DNA-binding properties of the response regulator, AlgR1, but chemical B did inhibit DNA binding of this protein. These compounds appear to act at different stages in the signalling pathway that partially controls alginate production and so could be used in combination to help avoid the selection of resistant strains. However, alginate synthesis is a complex process

involving many genes that could be under the control of multiple systems with the ability to compensate for the loss of one.

The effect of these compounds on other bacterial histidine kinases; CheA & NRII involved in bacterial chemotaxis and nitrogen assimilation respectively in *E. coli*, and the KinA histidine kinase involved in *B. subtilis* sporulation, were investigated. Chemicals B and B' appeared to show significant to potent inhibition of all the kinase activities tested at low concentrations and could therefore be general inhibitors of many bacterial kinases, whilst compounds A and A' showed varying degrees of inhibition depending on the system being studied. This was one of the first reports of inhibitors of prokaryotic kinases or phosphatases.

A series of diphenol-methane compounds were found to inhibit two-component systems in Gram-positive organisms, through high-throughput screening, with MICs of 1-4 $\mu g\ ml^{-1}$. (Domagala *et al.*, 1997). Several other chemical groups with inhibitory activity against TCS, and in particular histidine kinases, have also been reported. These include benzoxazines, benzimidazoles, bis-phenols, cyclohexenes, trityls, salicylanilides and a family of hydrophobic tyramines termed RWJ-49815 (Barrett *et al.*, 1998; Hlasta *et al.*, 1998; Hilliard *et al.*, 1999; Kanojia *et al.*, 1999), the latter of which were found to inhibit growth of MRSA, vancomycin-resistance *E. faecium* and penicillin-resistant *S. pneumoniae*. Like many of the other compounds described with 'anti-kinase' activity, RWJ-49815 was found to be a potent inhibitor of the autophosphorylation reaction of the histidine kinase and is competitive with respect to ATP, causing a bactericidal effect. This suggests that the compound is active against the ATP binding site common to all two-component kinases. RWJ-49815 has also been reported to have antifungal properties, although the mode of action in this case appears to be independent of HK inhibition (Deschenes *et al.*, 1999). An inhibitor of bacterial HK-phosphorylation has also been shown to have an inhibitory effect on the succinyl Co-A synthase enzyme of the parasite *Trypanosoma brucei* (Hunger-Glaser *et al.*, 1999), and illustrates the potential of therapeutic compounds against a range of microbial infections. Some studies have examined the mechanisms of action of some of the inhibitory compounds described above in further detail (Barrett *et al.*, 1998; Stephenson *et al.*, 2000). These compounds may also be of interest as basic research tools to probe the mechanisms of TCS signalling.

1.13.4 Potential problems in targeting TCS

The chemical agents described above are generally inhibitory to Gram-positive bacteria, with MICs ranging from 0.5-16 μg ml^{-1}. However, at higher concentrations than the MIC, many of the compounds tested affected the integrity of the bacterial cell membrane, indicating that inhibition by some, or all, of the chemicals may not be specific against the two-component systems themselves. The toxicological properties of most of the agents described have also yet to be established, and several that have been analysed in studies were shown to have acute *in vivo* toxicity in mice when given alone (Melton *et al.*, 1997). Many known inhibitors are also very hydrophobic compounds so are not suitable as therapeutic agents (Barrett *et al.*, 1998). Furthermore, many of the chemicals examined were not effective against Gram-negative bacteria, unless these organisms were first treated with a membrane-permeabilising compound, highlighting the problem posed by the Gram-negative outer membrane. Many of the chemicals described seem to target the ATP-binding site of histidine protein kinases and this type of site has been found in the serine protein kinases of anti-σ factors in *B. subtilis* (Min *et al.*, 1993). As a family of serine protein kinases possessing similar characteristics have been found in mitochondria (Harris *et al.*, 1997), consideration must therefore be given to the potential inhibition of these mitochondrial enzymes by such chemicals.

1.13.5 Global inhibitors of TCS

Global regulators of virulence factors have been identified in several bacterial pathogens. The *S. aureus* AgrC/AgrA system is such an example of global regulation which is activated in a quorum-sensing fashion through binding of secreted autoinducing peptide (AIP) to the HK AgrC protein (Ji *et al.*, 1995). Activation of this system ultimately results in the expression of RNAIII, which in turn is able to activate the expression of several secreted virulence factors and down-regulate expression of some surface proteins. Inhibition of a single global regulator thus has the potential to disrupt many cellular processes that could be important for survival or virulence. Several inhibitors of the *S. aureus* Agr system have been described (Matsushita and Janda, 2002). However there is much sequence diversity between the AIP/AgrC receptor pair between strains, such that AIP structures that activate the AgrC HK of some strains may inhibit that of other strain (Lyon *et al.*, 2000; Dufour *et al.*, 2002). A similar phenomenon has been found with inhibitors of the Agr system (Matsushita and Janda, 2002). Similarly an *agr*-like locus is present in *S. epidermidis* and a synthetic AIP from this organism has been found to inhibit virulence

factor expression from the *S. aureus* Agr (van Wamel, 1998; Otto *et al.*, 1999). Vaccination with the peptide autoinducer of the *S. aureus* Agr system has been shown to protect mice from *S. aureus* pathology (Balaban *et al.*, 1998), and could be an alternative approach for targeting similar TCS in other pathogenic bacteria.

A second TCS, the *sae* locus, demonstrating global regulation of *S. aureus* virulence genes has been described (Giraudo *et al.*, 1997). It is thought this system controls expression of α and β haemolysins and coagulase independently of the agr system, although the latter system is also thought to be involved in the control of these genes. Another global regulation system in *S. aureus*, also mediated by a RR/HH pair (SrrA/SrrB) has recently been identified (Yarwood *et al.*, 2001). This system appears to repress virulence factor expression under low oxygen conditions and it is thought that regulation of this system is partly mediated by the *agr* system. Several other less well characterised systems are also involved in regulating *S. aureus* virulence factors (Fournier *et al.*, 2001), illustrating the highly complex nature of bacterial gene regulation in response to environmental stimuli.

1.13.6 Non-bactericidal therapy

As already mentioned, a potential therapy against bacterial TCS may not necessarily have to be bactericidal to be effective. Many TCS have been described that play some role in the emergence of antibiotic resistance or tolerance, and inhibition of these systems without killing the host cell may render the bacterium sensitive to antibiotics again. *S. pneumoniae*, for example, has several such systems, including a TCS involved in vancomycin tolerance and one with a role in penicillin resistance. The former system, VncS/VncR, is required in a fully functional form to render the pneumococcus susceptible to the antibiotic vancomycin. The antibiotic is the stimulus for the histidine kinase (VncS) and resulting activation of this sensor results in de-phosphorylation of the cognate response regulator (compared to other histidine kinases which tend to phosphorylate their cognate response regulator when activated). This de-phosphorylation reaction undoes the repression of autolysin activity controlled by the phosphorylated response regulator. As a result, this enzyme is free to partake in the disruption of the cell wall, leading to eventual cell death due to the presence of vancomycin. In *S. pneumoniae*, insertion-duplication mutagenesis to create a knock-out mutant in VncS resulted in tolerance both to vancomycin and other classes of antibiotic, including beta-lactams, cephalosporins, aminoglycosides and quinolones (Novak *et al.*, 1999b). Activation of this system could therefore help in the

susceptibility of the pneumococcus to vancomycin. This is in contrast to the situation in *E. faecalis* where high level vancomycin resistance is regulated by the VanR/VanS TCS, the inhibition of which should render a resistant organism sensitive to vancomycin again.

RR	TIGR4 gene name	R6 gene name	Common name	Possible function	Tigr4/R6 protein homology	Orientation	Reference
RR01 / 480	SP1633	Spr1474		Unknown	100%	RR/HK (-)	Lange *et al* Throup *et al*
RR02 / 492	SP1227	Spr1107	MicA	Redox / energy sensing. Essential for viability. Competence? Homologous to *B. subtilis* yycFG	100%	RR/HK (-)	Lange *et al* Throup *et al* Echenique *et al*, 2001
RR03 / 474	SP0387	Spr0344		Homologous to *B. subtilis* yvqCE	100%	HK/RR (+)	Lange *et al* Throup *et al*
RR04 / 481	SP2082	Spr1893	PnpR	Phosphate metabolism ? Homologous to *B. subtilis* phoPR	100%	RR/HK (+)	Novak *et al* (1999a)
RR05 / 494	SP0798	Spr0707	CiaR	Involved in competence and penicillin susceptibility	100%	RR/HK (+)	Giammarinaro *et al*, 1999
RR06 / 478	SP2193	Spr1998		CbpA regulation?	99%	RR/HK (-)	Our laboratory, unpublished data
RR07 / 539	SP0156	Spr0154		Unknown	98%	HK/RR (+)	Lange *et al* Throup *et al*
RR08 / 484	SP0083	Spr0076		Unknown	99%	RR/HK (+)	Lange *et al* Throup *et al*
RR09 / 488	SP0661	Spr0578		Role in virulence. Nutrient perception?	100%	RR/HK (+)	This work
RR10 / 491	SP0603	Spr0528	VncR	Vancomycin tolerance	100%	RR/HK (+)	Novak *et al* (199b)
RR11 / 479	SP2000	Spr1814		Homologous to *B. subtilis* yvfTU	93%	HK/RR (-)	Lange *et al* Throup *et al*
RR12 / 498	SP2235	Spr2041	ComE	Competence regulation / quorum sensing	100%	HK/RR (-)	Lacks and Greenberg, 2001

RR13 / 486	SP0526	Spr0463	BlpR / SpiR	Bacteriocin production / quorum sensing	97%	RR/HK (+)	De Saizieu *et al.*, 2000 Reichmann and Hakenbeck, 2000
RR14	SP0376	Spr0336		Unknown	99%	RR (+)	Throup *et al*

Table 1.4: Response regulators of *S. pneumoniae*

RR detailed are those detected in the TIGR4 sequence of *S. pneumoniae* identified by Hoffmann-La-Roche and SmithKline-Beecham (Lange *et al.*, 1999, Throup *et al.*, 2000). The nomenclature TCS01-TCS13 (designated by Lange *et al.*, 1999) by which the TCS are referred to in this work are given in the first column. The corresponding names given by Throup *et al.*, are also provided in this column. The official gene names as given by the two sequencing projects for strains TIGR4 (serotype 4) and R6 (serotype 2) are provided for reference. Several of the systems have been studied previously and thus are known by more common names, often with reference to the processes the systems control. These are also provided. Putative functions still to be confirmed are followed by a question mark. Homology between the RR proteins of TIGR4 and R6 sequences is indicated. The orientation of the HK/RR pairs on the bacterial chromosome are given. A plus sign (+) indicates genes encoded on the positive strand. The minus (-) symbol represents gene pairs encoded on the negative/complementary strand.

1.14 TCS in *Streptococcus pneumoniae*

Groups at Hoffmann-La-Roche and SmithKline-Beecham (now GlaxoSmithKline) have used genomic analysis to identify all the TCS in a virulent serotype 4 pneumococcus (Lange *et al.*, 1999; Throup *et al.*, 2000), of which thirteen HK/RR pairs were found. Throup *et al* identified a further, unpaired response regulator (Throup *et al.*, 2000). All thirteen HK/RR pairs have been identified in all the pneumococcal genomes that have been sequenced to date (Dopazo *et al.* 2001; Hoskins *et al.*, 2001; Tettelin *et al.*, 2001). All TIGR4 TCS genes had a median G + C content of approximately 42 %, with the exception of TCS12 (ComD/E) which had a G+C content of 31 %, which is comparatively low. The median G+C content for *S. pneumoniae* is 42.2 % (TIGR4) / 39.1 % (R6) (http://www.tigr.org/), suggesting that the ComD/E system may have been acquired from an external source. Indeed the *comC/D/E* operon is flanked by 2 tRNA loci which are the preferred integration sites for other bacterial pathogenicity islands (Ohlsen *et al.*, 1999). The coding sequences of individual gene pairs of pneumococcal TCS were found to overlap or were spaced by no more than 11 base pairs suggesting that, for many systems, the histidine kinase/response regulator genes are transcribed as a single transcriptional unit. Table 1.4 summarises the pneumococcal TCS.

1.14.1 Response regulators of *S. pneumoniae*

Fourteen putative response regulator genes were identified in the pneumococcus, thirteen of which are found paired to a histidine kinase gene. The receiver domains of the pneumococcal response regulators were all shown to contain the three invariant residues corresponding to D-13, D-57 and K-109 of the CheY phosphorylation / active site (Lukat *et al.*, 1990), together with a conserved threonine thought to be important for function. All thirteen RR also display hydrophobic regions that correspond to the β-sheets 1, 3 and 4 present in the hydrophobic inner core of CheY. This indicates that the *S. pneumoniae* receiver domains adopt a similar tertiary fold to the CheY response regulator in *S. typhimurium*. Alignments of the receiver domain sequences of the response regulators studied in detail in this work, RR08 and RR09, compared to CheY are shown in figure 1.12. Lange *et al.*, (Lange *et al.*, 1999) provide alignments of other pneumococcal RRs.

Figure 1.12: Alignments of pneumococcal RR08 and RR09 receiver domains compared to *S. typhimurium* CheY

Sequences from TIGR4 and R6 genomes are given. Homologous amino acids are highlighted. The three conserved aspartic acid/lysine residues important in the receiver domain active site are indicated with an asterix (*). The conserved threonine residue important for function is indicated by ψ.

The response regulators of *S. pneumoniae* fall into four major families (OmpR/PhoB, AraC/XylS, LuxR/FixJ and ComE/ArgA) when grouped on both similarities within the receiver domain and homologies within the output domain. Three-dimensional crystal structures are available for members of the families with the exception of ComE/ArgA. The two response regulators in the AraC family (RR07 & RR09) are the first response regulator representatives of this family to be identified. The AraC-type DNA binding domain of this family comprises seventeen highly conserved positions extending over 99 amino-acids, and has a different three-dimensional structure to other response regulators previously described. Seven of the thirteen response regulators, including TCS08, were identified as being in the OmpR family, where the

output domain comprises three α-helices flanked on either side by anti-parallel β-sheets and two C-terminal β-sheets. This forms the 'winged-helix-turn-helix' motif described previously (Martinez-Hackertand and Stock, 1997). TCS13 has been grouped, along with the previously characterised response regulator ComE, into the ArgA/ComE class of DNA binding proteins. Although no structural data are available on this family, a cluster of basic amino-acid residues has been identified within the C-terminal (Pestova *et al.*, 1996). This charged region may have a role in DNA binding.

1.14.2 Histidine kinases of *S. pneumoniae*

The histidine kinases of *S. pneumoniae* were identified on the basis of their paired association to response regulators on the chromosome and on the homology boxes. All of the histidine kinases were found to have the linker region (E box) and H, N and G boxes, although histidine kinases from TCS03, TCS07 and TCS11 were found to have unusual G boxes that were different to the typical GXGL motif (see Figure 1.13). HK06, HK10, HK12 and HK13 all lack a D box. HK03, HK07 and HK09-13 do not possess an F-box, suggesting that D and F boxes are not essential for activity in these systems.

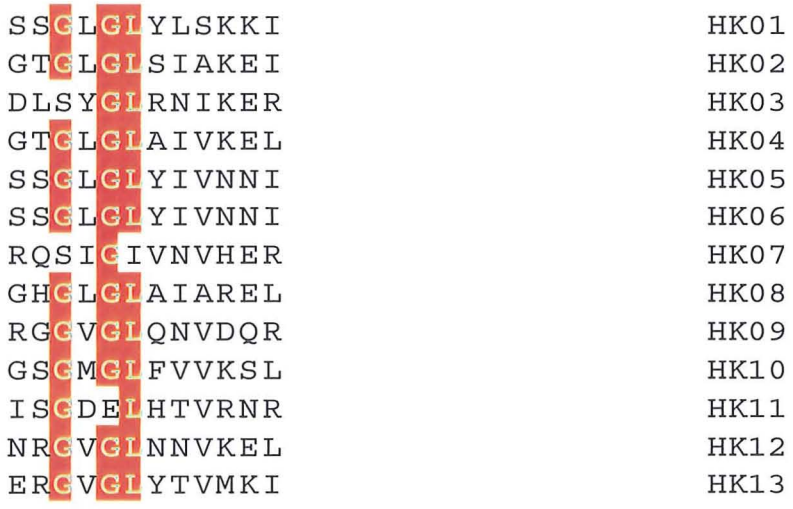

Figure 1.13: Alignment of the G-box sequences of *S. pneumoniae* histidine kinases

Sequences were taken from the published sequence for a serotype 4 pneumococcus (Tettelin *et al.*, 2001). The GxGL motif characteristic of the G-box is conserved in all but three of the pneumococcal HKs; HK03, HK07 and HK11.

Transmembrane segments within the N-terminus of the histidine kinases were predicted and results revealed 1, 5 or 7 potential transmembrane segments. All the histidine kinases are thus predicted to be membrane-bound. The pairs of histidine kinases and response regulators were predominantly arranged with the response regulator gene first, followed by the histidine kinase gene (TCS01, TCS02, TCS04, TCS05, TCS06, TCS08, TCS09, TCS10, TCS13), although in some cases the kinase gene occurred first (TCS03, TCS07, TCS11, TCS12).

1.14.3 Functions of TCS in *S. pneumoniae*

Several of the pneumococcal two-component systems identified have been characterised previously and their functions are known. Of these, the ComD/E system (TCS12) involved in the regulation of competence is probably the best characterised. Competence refers to the ability of certain bacteria to take up DNA from their external environment. In *S. pneumoniae*, DNA uptake is regulated, in part, by a quorum sensing mechanism. The regulation of the early competence genes is thought to be in response to oxygen availability (Echenique and Trombe, 2001). The bacteria secrete a competence stimulating peptide (CSP) (Havarstein *et al.*, 1995) which, at a certain cell density, serves to activate a membrane bound HK, ComD. HK~P subsequently transfers its phosphoryl group to its cognate RR, ComE. Activated ComE serves to regulate genes involved in the uptake of DNA. The ComD/E TCS is also an example of an auto-regulatory system and the process is summarised in figure 1.14. Creation of a null mutant within *comE* results in bacteria that are unable to respond to CSP and a subsequent loss in competence (Pestova *et al.*, 1996). However, one study created a single amino-acid change in the C-terminal region of *comE* that resulted in cells becoming constitutively competent, even in the absence of CSP (Lacks and Greenberg, 2001). Other systems thought to play a role in the regulation of competence in the pneumococcus are PnpS/R (TCS04), CiaR/H (TCS05) and MicA/B (TCS02) (Guenzi *et al.*, 1994; Giammarinaro *et al.*, 1999; Novak *et al.*, 1999; Echenique *et al.*, 2000, Martin *et al.*, 2000; Echenique and Trombe, 2001). The PnpS/R system has recently been described as a potential phosphate-sensing system with a possible role in competence (Novak *et al*, 1999). MicB/A (TCS02) is a TCS that may be involved in the negative regulation of competence under limiting oxygen concentrations (Echenique and Trombe, 2001). The *hk* and *rr* genes of TCS02 form a single transcriptional unit, together with a third gene encoding a putative protein of unknown function (Wagner *et al.*, 2002). It is the only TCS in *S. pneumoniae* that contains a PAS domain. PAS domains are widely distributed and can monitor changes in light,

redox potential, oxygen and the overall energy levels of a cell (Taylor and Zhulin, 1999). PAS domains are commonly involved in sensing intracellular signals, so the activating signal for TCS02 may reside within the bacterial cell (Wagner *et al.*, 2002). Several groups have reported the inability to create mutations in the response regulator gene of this TCS, indicating that the system is essential for viability, and presumably regulates cellular functions other than competence (Lange *et al.*, 1999; Throup *et al.*, 2000; Echenique and Trombe, 2001). Furthermore, homologues of this system exist in other Gram-positive bacteria, including *B. subtilis* and *S, aureus*, where the systems also appear to be essential for cell viability (Fabret and Hoch, 1998; Wagner *et al.*, 2002), although the exact function of these systems is unknown. A mutation in the *hk02* gene was found to have a 400-fold reduction in transformation frequency compared to the parental wild-type strain (Wagner *et al.*, 2002). A mutation over-expressing HK02 was shown to result in longer murine survival times compared to wild-type parental strain when administered by intraperitoneal injection, suggesting the system may have a role in virulence (Wagner *et al.*, 2002).

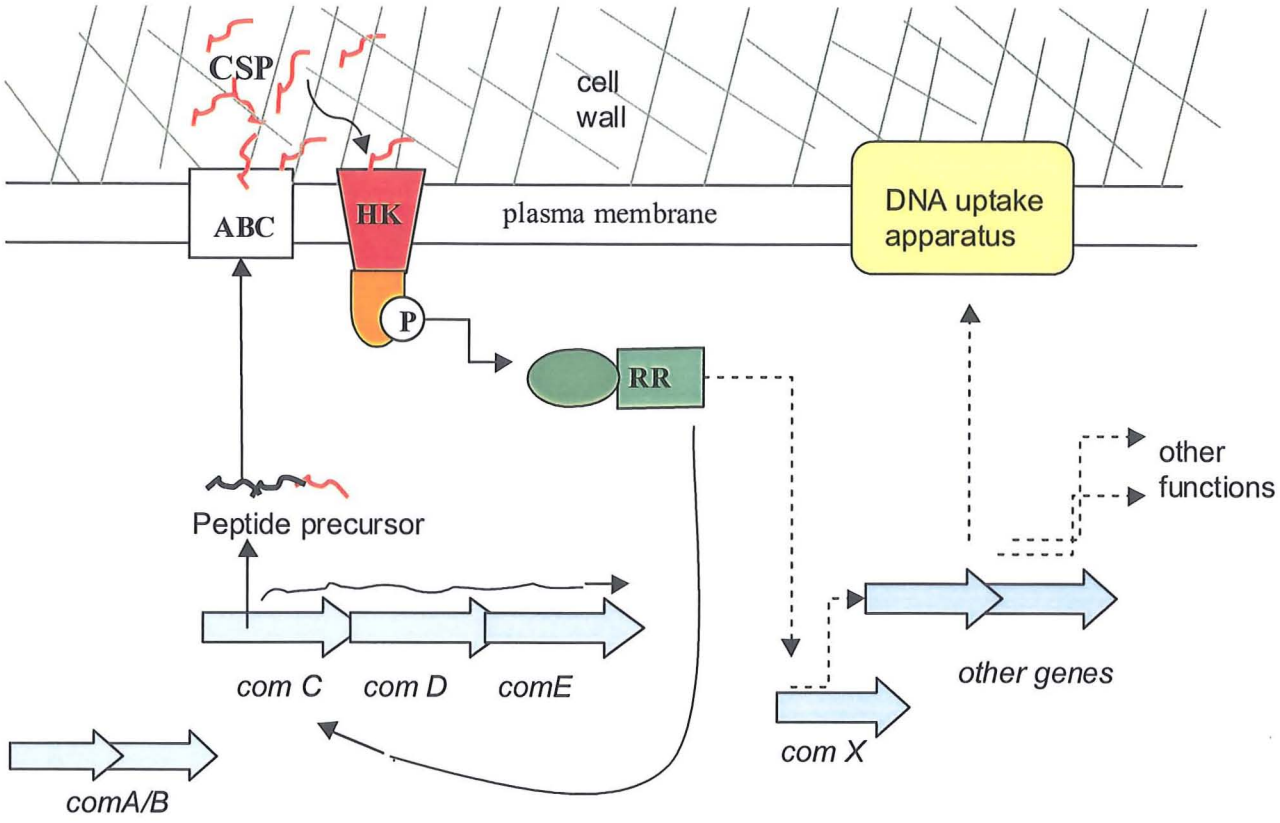

Figure 1.14: Competence regulation in *S. pneumoniae*

Competence is a complex series of events that eventually lead to the uptake of DNA from the external environment. It is mediated by CSP, which is encoded, as a pre-cursor, by the *comC* gene. The peptide precursor is cleaved during export through the ATP-binding cassette (ABC), encoded by *comA/B*. Mature, secreted CSP accumulates in the external medium and, upon reaching a critical cell density, is able to bind to and activate the membrane-located HK protein, encoded by *comD*. Phosphoryl transfer proceeds from the activated HK protein to the intracellular RR protein, encoded by *comE*. Phosphorylated RR can auto-regulate expression of the *comC/D/E* and *comA/B* operons and also activates transcription of comX / early competence genes which encode a SigH-like transcription factor (not shown). This then serves to activate genes involved in DNA uptake apparatus, together with other genes involved in competence (late competence genes). Development of competence is extremely complex, involving activation and repression of different genes or sets of genes at different stages of the process and this diagram represents a simplified overview of the events. Indeed other pneumococcal TCS, CiaH/R (TCS05) and TCS02 are also involved in the mechanism (Giammarinaro, et al, 1999,and Echenique and Trombe, 2001). The *comC/D/E* system is autoregulated. Genes other than the *comC/D/E* operon are also regulated by activated RR (RR~P). (Lee and Morrison, 1999, Lacks and Greenberg, 2001 and Berge *et al.*, 2002). Phosphorylated ComE~P may also regulate expression of the comAB operon as a ComE binding site upstream of this operon has been identified (Martin *et al.*, 2000).

A further system involved in competence regulation is the CiaRH system (TCS05). This system appears to function as a pleiotropic regulator, controlling several diverse functions within the cell. Much of the characterisation of this system to date has resulted from experiments into the mechanism of β-lactam resistance in the pneumococcus. Until recently it was thought that resistance to this class of antibiotics was solely attributed to the alteration of penicillin binding proteins (PBPs), cell wall enzymes involved in peptidoglycan synthesis. These proteins are the cellular targets for β-lactams and accumulations of point mutations in their structure can result in decreased affinity for the binding of β-lactam antibiotics. However, laboratory experiments designed to isolate mutations that resulted in increased resistance to antibiotics detected a point mutation in *ciaR* that resulted in resistance to cefotaxime (a third generation cephalosporin / β-lactam) in the absence of any alteration in PBPs (Guenzi *et al.*, 1994). This mutation (CiaH*) appears to result in constitutive expression of *ciaRH* through the inability of the CiaH* mutant to de-phosphorylated activated CiaR. The kinase activity of CiaH* was not affected by the mutation. This mutant could not be transformed and it was demonstrated that mutants were unable to express CSP, indicating that activation of the CiaRH system is involved in repressing the early stages of competence in pneumococcal cells. Regulation of expression of competence-related genes other than CSP were suspected as the addition of exogenous CSP failed to abolish the defective transformation phenotype (Guenzi *et al.*, 1994). Null mutations in the *ciaH* and *ciaR* genes were created and were not found to be attenuated in transformation ability. Of these mutants, only the *ciaH* null mutation (and CiaH*) was resistant to cefotaxime indicating that the CiaR protein is required for resistance to this β-lactam, but this requirement is independent of the phosphorylation state of the response regulator (Guenzi *et al.*, 1994). Alternatively, in the absence of the CiaH histidine kinase, CiaH may be phosphorylated by other means. Another study showed that inactivation of *ciaR* affects expression of the *comCDE* operon more drastically than inactivation of *ciaH*, providing further evidence that the non-phosphorylated response regulator / phosphorylation by other proteins can repress competence (Martin *et al.*, 2000). It is not known if the CiaRH system controls the competence-related genes directly or if the negative regulation of transformation is indirect. A 24 bp long sequence within the promotor region of *comCDE* and *ciaRH* has been shown to exhibit 83 % identity, indicating that CiaR~P could directly regulate expression of the *comCDE* operon. Other phenotypes that were attributed to the CiaH* mutant included resistance to lysis by deoxycholate (DOC), penicillin and several other antibiotics, indicating a role for this system in regulation of cell wall biosynthesis

(Giammarinaro *et al*, 1999). Indeed one group have reported preliminary data demonstrating that the CiaRH system controls the level of the lipid carrier, bactoprenol, involved in cell wall / peptidoglycan synthesis (Hakenbeck *et al.*, 1999). The link between the regulation of competence and resistance to β-lactams is not understood, the system may sense disruption of cell wall integrity, which is important for both competence and resistance to β-lactam antibiotics. Several studies have eluded to the possible sensory signals to which the CiaRH system responds. The development of competence requires several cations, including Ca^{2+}, Mg^{2+} and Zn^{2+}, phosphate, oxygen and other environmental factors. One group has demonstrated that low Ca^{2+} activates transcription of the *ciaRH* operon and that this activation is also increased upon increasing phosphate concentration. It was thus proposed that low $Ca^{2+}$was required for CiaH activation and that the addition of phosphate served to decrease Ca^{2+} levels further by reacting with this cation to remove it from the system (Giammarinaro *et al.*, 1999). A second study, examining the requirement of oxygen for development of competence, isolated mutants in *ciaH* that enabled transformation when cells were grown in low oxygen environments and thus proposed that competence development in aerobic cultures was a result of the lifting of CiaRH-mediated repression of competence genes (Echenique *et al.*, 2000). Thus low oxygen concentrations may also activate the CiaRH system.

Several studies have indicated a role for the CiaRH system in pneumococcal virulence. A *ciaRH* mutant was highly attenuated in a systemic model of infection (Marra *et al.*, 2002) and had reduced counts in the lungs following intranasal infection (Throup *et al.*, 2000). In another study, mutations in *ciaRH* were significantly impaired in their ability to colonise the nasopharynx using an infant rat model of colonisation (Sebert *et al.*, 2002). This study subsequently used microarray analysis to compare the gene expression between the *ciaRH* mutant and its isogenic parental strain and found 24 genes that had significantly altered regulation in the mutant strain. These genes were specific to the CiaRH TCS and not altered in other pneumococcal TCS mutants. The study identified one gene in particular, *htrA*, that had a 27-fold reduction in expression in the *ciaRH* mutant compared to the wild-type strain. A null mutation was created in this gene and the resulting mutant was significantly impaired in colonisation (Sebert *et al.*, 2002). The *htrA* gene is thought to encode a serine protease and homologues of this gene have been identified in other bacteria. Of the genes with altered expression levels in the *ciaRH* mutant, 12 were significantly

down-regulated and 12 were significantly up-regulated. Many of these genes were not linked to competence or any of the other phenotypes thought to be associated with this system. This highlights the complex nature of gene regulation by the CiaRH system and indicates that the system may be involved in regulating a plethora of cellular functions not yet identified. Figure 1.15 summarises the data available on the CiaRH TCS.

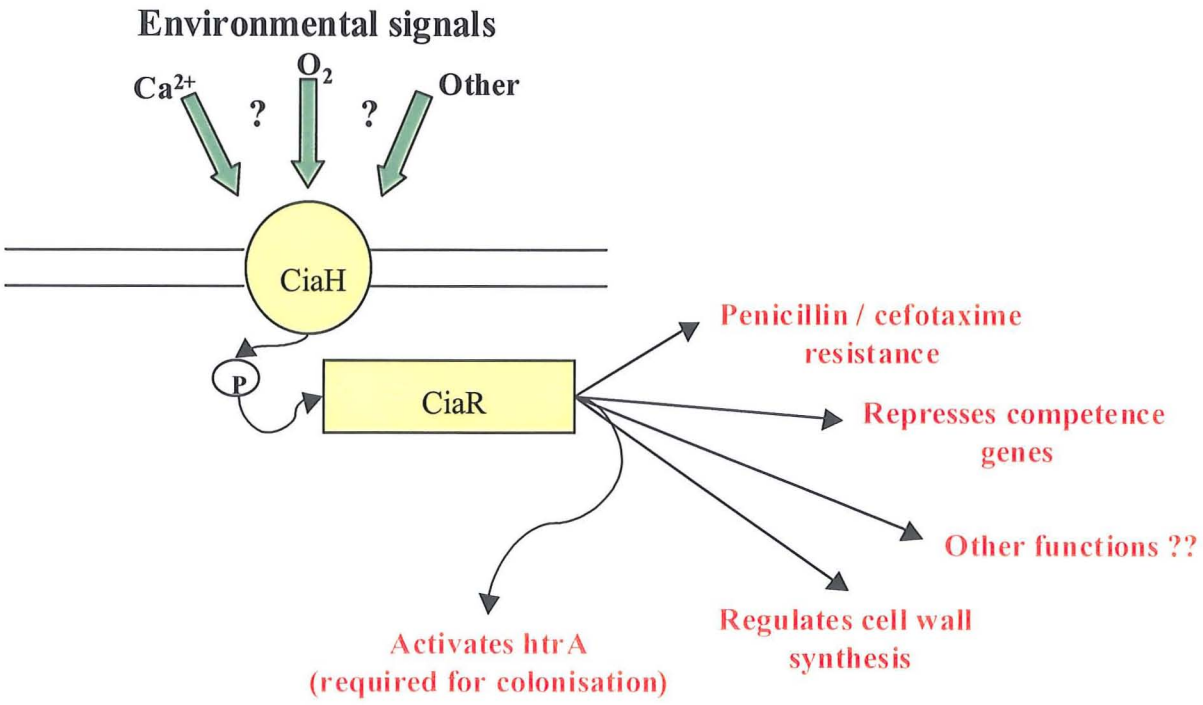

Figure 1.15 Summary of the *S. pneumoniae* CiaRH system (TCS05)

The signals to which the CiaH protein responds are not well understood, but low Ca 2+ and low oxygen may both have a role in activating this system. Other environmental factors may also be involved. The activated system has been shown to result in resistance to β-lactam antibiotics in laboratory mutants and its role in repressing the development of competence is well recognised. The system appears to regulate cell wall synthesis and is required for nasopharyngeal colonisation in an animal model. Microarray data also implies a role for this system in the regulation of many other genes. The figure is based on published data discussed within the text.

Several pneumococcal TCS have been implied to have a role in resistance or tolerance to antibiotics. CiaR/H, as discussed above has a role in penicillin / β-lactam resistance. VncS/R (TCS10) has been proposed to have a role in vancomycin tolerance (Novak *et al.*, 1999b; Novak, *et al.*, 2000) and has been described above (section 1.13.6).

One other pneumococcal TCS that has been characterised is TCS13. This system has been shown to be a quorum sensing system activated by a auto-induced peptide and it appears to regulate the production of an ABC transporter and a set 16 of bacteriocin-like genes (de Saizieu *et al.*, 2000; Reichmann and Hakenbeck, 2000). Bacteriocins are potent proteins produced by bacteria that can inhibit the growth or kill closely related bacterial species. They have been well characterised in many lactic acid bacteria so their presence in *S. pneumoniae* is not surprising. However, bactericidal activity for these putative bacteriocins has not been identified to date.

Some of the systems also showed homology to the yvfTU, yycFG, yvqCE and phoPR TCS pairs of *B. subtilis*. Of these, only the phoPR and yycFG systems have been previously studied and are responsible for phosphate regulation and *in vitro* growth respectively. Both Lange and co-workers and Throup and co-workers (Lange *et al.*, 1999; Throup *et al.*, 2000) attempted to created a mutant in the response regulator that showed homology to yycFG (TCS02), but were not successful. This response regulator thus appears to be essential for growth and viability of *S. pneumoniae* and is a possible drug target. Only three other bacterial RR had previously been identified as being essential for viability, the *B. subtilis* yycF with the other two being in *Caulobacter crescentus* (Hecht *et al.*, 1995; Quon et *al.*, 1996). Homologues of the yycFG system have been identified in many Gram-positive bacteria through genome data base analysis, which suggests that this TCS may have an essential role in such bacteria (Throup *et al.*, 2000).

1.14.4 *S. pneumoniae* TCS studied in this work

TCS08

This system was first identified by researchers at Hoffmann-La-Roche (Lange *et al.*, 1999) and later by SmithKline Beecham (Throup *et al.*, 2000). The environmental signal to which this TCS responds is unknown and the function of this system has not been determined, although it may have a role in virulence (discussed in section 1.14.5). Hoffmann-La-Roche have done preliminary array experiments with this TCS using a custom designed Affymetrix array based on the TIGR4 sequence. This work indicated that the activated response regulator appears to induce

the expression of a single surface protein, designated Sp0082, which is located immediately upstream of TCS08 (unpublished data) (figure 1.16). This clear-cut correlation between a TCS and a surface protein was one of the reasons we chose to study this system.

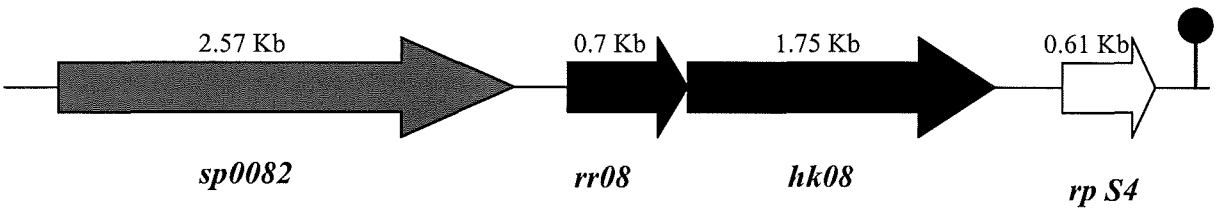

Figure 1.16: *S. pneumoniae* TCS08 locus and surrounding genes

rr08 and *hk08* designate genes for the response regulator and histidine kinase proteins respectively. *sp0082* encodes a surface protein and the *rpS4* gene encodes ribosomal protein S4. Genes are not drawn to scale but represent approximate relations in size between genes shown. The closed circle represents a rho-independent transcriptional terminator.

The response regulator of TCS08 belongs to the OmpR family of regulators, and levels within the cell have been shown to vary from between 100-200 molecules per cell. *RR08* expression is thought to be auto-regulated (Hoffmann-La-Roche, personal communication). The histidine kinase possesses all the conserved regions/boxes characteristic of other HK proteins and is predicted to have 2 transmembrane helices (Lange *et al.*, 1999).

Mature Sp0082 is a 83 KD protein with 857 amino acids, and its expression appears to be induced in stationary phase or when pneumococcal cells are grown as a biofilm (Lange *et al.*, 1999). The *sp0082* gene codes for a typical surface protein precursor with a LPXTG cell wall anchor motif at the C-terminus region. The N-terminal has a unique domain of ~100 amino-acids. The function of the protein is unknown and it shows homology to other bacterial proteins including those in *S. agalactiae*, *Lactococcus lactis* and *S. aureus*. A knock-out in the *sp0082* gene does not affect the expression of TCS08 (Lange *et al.*, 1999).

TCS09

As for TCS08, this system was identified based on sequence homology to other bacterial TCS (Lange *et al.*, 1999; Throup *et al.*, 2000). The function of this system is unknown, although Lange and colleagues proposed a role for nutrient perception. This was on the basis of some

homology between HK09 and two proteins involved in nutrient sensing and chemotaxis in *B. subtilis* (McpA and McpB). No further data exist to provide evidence for this putative role. The TCS09 locus and surrounding genes are illustrated in figure 1.17.

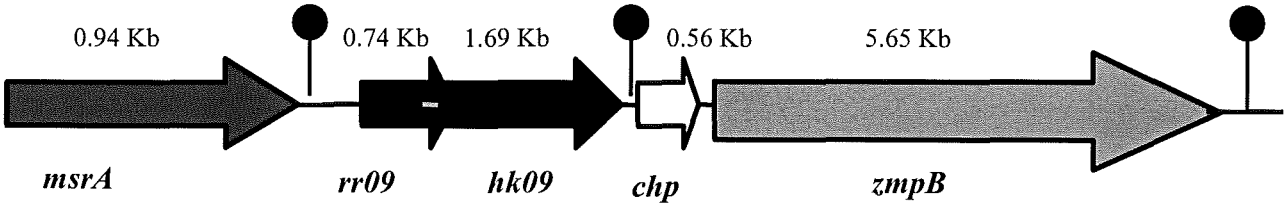

Figure 1.17: *S. pneumoniae* TCS09 locus and surrounding genes

The *rr/hk* genes make up *S. pneumoniae tcs09*. Downstream of the *tcs09* locus is a conserved hypothetical protein (*chp*) and a gene, *zmpB*, encoding a putative zinc metalloprotease (Berge *et al.*, 2001; Novak *et al.*, 2000). Immediately upstream of *tcs09* is the *msrA* gene, encoding a methionine sulphoxide reductase, also important in virulence (Wizemann *et al.*, 1996). The closed circles represent rho-independent transcriptional terminators.

Although this TCS has been referred to as ZmpR/ZmpS in recent studies (Novak *et al.*, 2000; Hava and Camilli, 2002), it is not known if any of the genes surrounding *S. pneumoniae tcs09* are actually regulated by this system. One group investigated this hypothesis but did not obtain a similar phenotype in a knock-out mutation of this system compared to a *zmpB*-deficient mutant (Novak *et al.*, 2000). This, however, does not eliminate the possibility of regulation of *zmpB*, as mutating one single TCS may not be sufficient to expose any regulatory links. The same group found a single transcript for the *chp* and *zmpB* genes, indicating that these genes form an operon (Novak *et al.*, 2000), as indicated by the transcriptional terminators shown on figure 1.17.

1.14.5 *In vivo* role of TCS in *S. pneumoniae*

Lange and colleagues and Throup and colleagues created mutant *S. pneumoniae* strains by inactivating each of the 12 response regulators that would not result in cell death (Lange *et al.*, 1999; Throup *et al.*, 2000). Lange and colleagues used insertional mutagenesis to disrupt all *rr* genes. Throup and colleagues used the technique of allelic replacement to delete either the *rr* genes or the whole *hk/rr* pair. The former group inoculated mice intraperitoneally with each of

these mutants and found no significant difference in the ability to cause bacteraemia *in vivo* compared to wild-type. The latter group, however, used intranasal infection of mice and enumerated bacterial load from lung tissue samples. Eight response regulator mutants showed a dramatic decrease in *in vivo* lung counts 48 hr post infection compared to wild-type (RR01, RR04, RR05-08, RR13 and the fourteenth unpaired response regulator identified by this group [RR14]). However such mutants had little detrimental effect on *in vitro* bacterial growth in nutrient-replete conditions. (RR12 was not studied *in vivo*). A time-course study followed the growth of mutants within the lung tissue at pre-selected time points. This identified three TCS that had similar counts to wild-type parental strain at earlier time points but a significant reduction was observed at later time points (TCS06, TCS13 and the un-paired RR14, Throup *et al*, 2000). This indicates that TCS may contribute differently to various stages of infection.

The eight TCS that showed reduced virulence in the lung in the work by Throup and colleagues were analysed in a separate study for their ability to colonise the nasopharynx in an infant rat model (Sebert *et al.*, 2002). Only one system (CiaH/R / TCS05) was found to have reduced colonisation in this model of infection (the system was determined to be essential for colonisation), indicating the importance of TCS for sensing different environments and expressing their genes accordingly.

Other studies using techniques for the large-scale identification of pneumococcal virulence factors have identified several pneumococcal TCS as having a role in virulence (Lau *et al.*, 2001; Marra *et al.*, 2002; Hava and Camilli, 2002). Three groups have used signature tagged mutagenesis (STM) screens using serotype 19, 3 and 4 pneumococcus, respectively (Polissi *et al.*, 1998; Lau *et al.*, 2001; Hava and Camilli, 2002). The first STM study identified 126 different genes that were attenuated in virulence and were subsequently analysed individually in a murine model. However, none of the TCS genes were included in the detailed analysis, although not all attenuated genes were listed (Polissi *et al.*, 1998). Lau and colleagues used the same serotype 3 strain (0100993) used for the identification of all pneumococcal TCS and their virulence studies within the lungs (Throup *et al.*, 2000) and found HK07 to be highly attenuated in a competitive model of pneumonia (lung counts 48-72 hr post i.n. challenge). ComD/HK12 was also attenuated in a competitive model of pneumonia and bacteraemia (bacterial load in the spleen 16-24 hr post intraperitoneal [i.p.] challenge) (Lau *et al.*, 2001). The most recent STM screen used a derivative of the TIGR4 sequenced pneumococcal strain and found no detectable hybridisation signal for the output pools of *rr01*, *rr07*, *rr09* and *hk12* mutants following i.n. challenge with subsequent

recovery of pools from lung tissue 44 hr post challenge (compared to output pools) (Hava and Camilli, 2002). A BlpA mutant, a transporter thought to be linked to the quorum sensing TCS13, also failed to produce a detectable output signal in this pneumonia model. RR01, RR07 and RR09 were analysed further in competition studies in combination with WT parental strain using three models of infection (pneumonia, bacteraemia and nasopharyngeal colonisation). The RR07 mutant was not attenuated in any of the models tested. This does not agree with the attenuated phenotype seen in the lung tissues by Throup and co-workers (Throup *et al.*, 2000) but does agree with the bacteraemia model described by Lange and co-workers (Lange *et al.*, 1999) where no reduction in virulence following i.p. challenge was demonstrated. RR01 was found to be attenuated in all three models which is in agreement with Throup and colleagues but not with the data presented by Lange and colleagues. The RR09 mutant was found to be highly attenuated in lung counts only following i.n. infection. This is in agreement with the bacteraemia data of Lange and others, who found no difference for any of the TCS mutants following i.p. challenge. Throup and co-workers did find that the mean lung count of the RR09 mutant was lower than that of WT but this was not deemed to be 'highly attenuated'. This final discrepancy could merely highlight differences in the way data is interpreted between studies.

Differential fluorescence induction (DFI) has also been used to identify promoters that are induced over two-fold under a variety of environmental conditions (Marra *et al.*, 2002). This study used a serotype 2 pneumococcus (D39) and found a 4.8 fold increase in expression of TCS05 (CiaH/R) and a mutation in this operon resulted in attenuation in systemic infection (i.p., LD50 dose) but not in a RTI model (lung counts and bacteraemia following i.n. challenge). These data are in complete disagreement to published data from other studies (Lange *et al.*, 1999 and Throup *et al.*, 2000). TCS04 (PnpS/R) was identified in a RTI model as having increased expression but attempts at making a mutation within this system were unsuccessful. Genes within *tcs03* were induced greater than two-fold under conditions where temperature was altered, although the role in virulence of this system was not determined.

The differences seen in the *in vivo* experiments described may be attributed to different routes of administration, different pneumococcal strains/ serotypes or use of different animal models. Alternatively the different methods of mutagenesis used to create the mutants (insertional duplication, allelic replacement and STM) may contribute to the differences in observed phenotypes. It was also noted that disruption of the *hk* gene often resulted in substantially less attenuation compared to disruption of the cognate *rr* gene (Throup *et al*, 2000). This could

indicate that cross-regulation could be occurring, where the HK of one system is able to compensate for the loss of a HK sensor in a different system. Furthermore individual studies define their parameters of virulence differently, some using single analysis, others using competitive studies, so the significance of results may be interpreted in various ways. The definitions of infection models also vary. For example, separate studies defined bacteraemia as counts in the blood (Hava and Camilli, 2002) or spleen following i.p. challenge (Lau *et al.*, 2001) and another study defined it as the challenge dose that resulted in 50 % mortality of animals following i.p. injection (Marra *et al.*, 2002). Bacteraemia may also refer to counts in the blood following i.n. challenge (Kerr *et al.*, 2002). Caution must therefore be used when comparing data from individual studies.

A response regulator, RevS, that appears to have a role in virulence in the pig pathogen *S. suis* has been shown have 61 % similarity to pneumococcal RR13, a system proposed to have a role in regulating a set of bacteriocin-like genes (Lange *et al.*, 2000; Reichmann and Hakenbeck, 2000; de Greeff *et al.*, 2002; de Greeff, personal communication). Upstream of *revS* is an incomplete open reading frame that shows homology to a conserved hypothetical protein of several organisms, including *S. pneumoniae*. In the pneumococcal genome, this conserved hypothetical protein does not appear to be in close proximity to any of the TCSs or the unpaired RR14. The *S. suis* RevS protein was initially selected under iron-restricted conditions (Smith *et al.*, 2001). A mutation in the *revS* gene had identical *in vitro* growth rate compared to the parental strain, but infected less organs and had lower counts in organs that were infected in a piglet model of infection (de Greeff *et al.*, 2002).

The contribution of various TCS to the virulence of *S. pneumoniae* to date has been summarised in tables 1.5. Data for RR08 and RR09, the two systems studied in this work, are displayed in a separate table to the other systems (table 1.6).

RR	Attenuation	Ref
RR01	Reduced lung counts, 48 hr (i.n.) Highly attenuated in nasopharyngeal and lung counts (i.n.). Highly attenuated in bacteraemia (i.p) (Using competitive studies)	a d
RR04	Reduced lung counts, 48 hr (i.n.) Gene expression induced > 2 fold in RTI but attempt to construct mutant was unsuccessful (unlike [a])	a c
RR05	Reduced lung counts, 48 hr (i.n.) Attenuated in systemic infection (i.p., LD_{50}) but not in RTI (lung and bacteraemia i.n., 24 & 48 hr) Impaired in ability to colonise nasopharynx	a c e
RR06	Reduced lung counts, 48 hr (i.n.)	a
RR07	Reduced lung counts, 48 hr (i.n.) Highly attenuated in lung counts (i.n.) (competitive studies). No attenuation in bacteraemia (I/P) Attenuated in lung counts (i.n.) (but no attenuation when examined using a competitive study)	a b d
RR11	Putative attenuation in lung infection and bacteraemia (i.n. & i.p.) (based on mutation in upstream gene that may have polar effects)	d
RR12	Attenuated in lung counts (i.n.) and bacteraemia (i.p.) using competitive studies Attenuated in lung counts (HK) (i.n.) (not tested in competitive studies)	b d
RR13	Reduced lung counts, 48 hr (i.n.) A transporter linked to TCS13, BlpA, was attenuated in lung counts	a d
RR14	Reduced lung counts, 48 hr (i.n.)	a

Table 1.5 *S. pneumoniae* response regulators involved in virulence

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Table 1.5: *S. pneumoniae* response regulators involved in virulence

The table gives details of TCS (in particular RR) found to be important in virulence in several published studies (a; Throup *et al.*, 2000, b; Lau *et al.*, 2001, c; Marra *et al*, 2002 d; Hava and Camilli, 2002 e; Sebert *et al.*, 2002). RR03 and RR10 have not been reported to have any role in virulence to date and RR02 is essential for viability, so these have been removed from the analysis. RR08 and RR09 have been summarised in a separate table (6). Three of the published studies (b; Lau *et al.*, 2001, c; Marra *et al*, 2002 d; Hava and Camilli, 2002), involved various screening techniques which would not identify every gene involved in virulence. Thus the absence of data for individual TCS is not evidence that they are not involved in virulence in any particular study. Differences in mutagenesis technique, bacterial strains, challenge doses and animal models may account for any discrepancies seen in the data between individual studies. Routes of infection were either intranasal (i.n.) or intraperitoneal (i.p). LD_{50} represents the dose that can kill 50 % of infected animals. Where studies examined virulence using competitive infections with wild-type parental strain and infections with single strains, the conditions in which attenuation was found are indicated. Individual studies have different ways of determining the significance of an attenuated phenotype and data included within this table are based on the definitions provided in the literature reviewed, not on my own interpretations. Lange *et al.*, 1999, created mutants in all *rr* genes and found no attenuation in virulence following i.p. challenge.

Reference	Strain / serotype of bacteria	Mutagenesis technique	Murine / animal strain	Route of infection	Inoculum	Measure of virulence	Role in Virulence
TCS08							
Lange *et al.*, 1999	Sp.1 (type 22)	Insertion duplication	C57BL6/J (I) Females	i.p. (NP)	4×10^6	Mean survival time	None
Lange *et al.*, 1999	Sp.1711 (type 3)	Insertion duplication	C57BL6/J (I) Females	i.p. (NP)	1×10^3	Mean survival time	None
Throup *et al.*, 2000	0100993 (type 3)	Allelic replacement	CBA/J (I) Males, 5 wk	i.n.(NP)	1×10^7	Mean lung counts, 48 hr	> 1000 fold decrease in lung counts compared to WT
Sebert *et al.*, 2002	0100993 (type 3)	Allelic replacement	Sprague-Dawley infant rats (O)	i.n.	4×10^6 – 1.6×10^7	Colonisation of nasopharynx	None
TCS09							
Lange *et al.*, 1999	Sp.1 (type 22)	Insertion duplication	C57BL6/J (I) Females	i.p. (NP)	4×10^6	Mean survival time	None
Lange *et al.*, 1999	Sp.1711 (type 3)	Insertion duplication	C57BL6/J (I) Females	i.p. (NP)	1×10^3	Mean survival time	None
Throup *et al.*, 2000	0100993 (type 3)	Allelic replacement	CBA/J (I) Males, 5 wk	i.n. (NP)	1×10^7	Mean lung counts, 48 hr	10-100 fold decrease in lung counts compared to WT, not significant
Hava and Camilli, 2002	TIGR4 (type 4) (streptomycin resistant derivative)	STM	Swiss Webster mice, 6-10 wk, males (O)	i.n. (NP) i.p. (NP)	1×10^7 5×10^5	Lung counts, 44 hr Bacteraemia, 20 hr (competition experiments)	Highly attenuated in lung counts but not bacteraemia

Table 1.6 Role of *S. pneumoniae* TCS08 and TCS09 in virulence

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Table 1.6 Role of *S. pneumoniae* TCS08 and TCS09 in virulence

The table gives details of several published studies examining the contribution of TCS08 or TCS09 to virulence. All mutations were created in the response regulator component. The techniques used for creating mutants are given, where signature-tagged mutagenesis is represented as STM. The murine strain used is provided, together with details of whether the strain was inbred (I) or outbred (O). Routes of infection were either intranasal (i.n) or intraperitoneal (i.p). Wild-type is represented as WT. NP designates strains that were non-passeged.

1.14.6 Identification of genes regulated by pneumococcal TCS

For many of the TCS identified in *S. pneumoniae* and other bacteria, the signals that they respond to and the genes they regulate are largely unknown. Until recently, creating null mutations in the genes and looking for possible phenotypes in a range of assays or animal models was perhaps the only way to assign putative functions. However, the availability of several *S. pneumoniae* genome sequences and the introduction of exciting technologies such as microarrays, proteomics and DNA footprinting should now enable researchers to perform detailed analyses of microbial TCS (Throup *et al.*, 2001; Dietz *et al.*, 2002).

Microarrays

Microarrays are powerful tools for studying gene regulation or to compare genomes. Microarray chips comprise gene-specific probes immobilised on a nylon membrane, glass or silicon platform (chip). Chips can contain probes to detect as many as 60,000 different genes (Affymetrix Human Genome U95 set, Affymetrix) on an area the size of a microscope slide. Nucleotide probes synthesised onto the chip are hybridised to sample cDNA labelled with fluorescent dyes. The microarray is then scanned to measure the fluorescent intensity of individual spots and data is analysed using appropriate software.

Arrays may allow the discrimination between genes that are up to 90 % identical and technology is progressing such that very high sensitivity and specificity are possible with microarray experiments. Many arrays are available commercially, although researches can choose to have arrays custom made to contain desirable genes or sets of genes. Microarrays are excellent research tools for many scientific disciplines and microbiology has benefited greatly from such technology. Whole microbial genomes can be printed onto a single array allowing gene expression to be characterised using a variety of different parameters. For examples, microbial arrays have been used to study microbial response to stress and environmental change, cell cycle-associated gene expression and characterisation of regulatory pathways (Ye *et al.*, 2000; Lucchini *et al.*, 2001; Stephens, 2001; Yarwood *et al*, 2002). Certain arrays (e.g. Affymetrix oligonucleotide arrays) can also be used to detect point mutations or polymorphisms, deletions and insertions, to serotype bacteria and to compare gene profiles between bacterial strains (Chizhikov *et al.*, 2001; Murray *et al*, 2001). The effects of chemical agents and antibiotics on microbial gene expression can also be characterised. Human or murine arrays are also of huge

benefit to microbiology research, allowing the study of the expression of host genes in response to microbes or microbial products. They are also useful for the identification of drug or vaccine targets (Belcher *et al.*, 2000; Detweiler *et al.*, 2001; Diehn and Relman, 2001; Yowe *et al.*, 2001; Grifantini *et al.*, 2002). Microarrays are useful to TCS research as they can aid in the characterisation of complex processes expected to involve many genes.

The role of a single gene in inducing gene expression can be studied through the use of null or over-expression mutations, and this is where microarrays are useful, and have been applied, for the study of bacterial TCS (Ye *et al.*, 2000; Detweiler *et al.*, 2001; Ogura *et al.*, 2001; Sebert *et al.*, 2002). This method is particularly useful for target gene identification where the signals required for TCS induction are unknown, or hard to simulate. One such study has been used to confirm known, and identify unknown, targets for three response regulator genes of *B. subtilis* (Ogura *et al.*, 2001). IPTG-inducible *rr* genes cloned into plasmids were introduced into cells and gene expression compared in bacterial cells with and without IPTG induction. Experiments were performed in bacterial strains where the cognate *hk* gene had been mutated, indicating that the identified candidate genes were expressed in the absence of signal transduction, and that the over-expressed regulators were behaving as if phosphorylated. Many genes previously identified as being regulated by the RRs used in this study were detected by microarray analysis. This helps validate the identification of genes previously unknown to be under the control of individual TCSs. Many genes previously unknown to be regulated by the individual RR proteins were also identified. A recent study used microarray analysis to examine gene expression in mutations in all TCSs *of E. coli*, strain K-12 (> 30 TCS). Gene expression at one time point during *in vitro* growth was studied and showed that over half the mutants had significant alterations in a small number of genes. Unknown functions of several TCSs were discovered, along with evidence for the control of several genes by other TCSs in a cascade-type regulation. Furthermore, this analysis showed that cross regulation plays an important role in cell-physiology of *E. coli* (Oshima *et al.*, 2002). Microarray analysis has also been used to show that a response regulator in group A Streptococcus, CovR, can influence the transcription of over 250 genes (Graham *et al.*, 2002). Although direct regulation of all of the identified genes is unlikely, this data indicates how powerful microarrays can be at identifying complex gene regulatory networks. Indeed, some of the genes that had altered expression in a *covR* mutant were regulators themselves (Graham *et al.*, 2002).

In *S. pneumoniae*, microarrays have been used in two studies to identify genes involved in competence (Peterson *et al.*, 2000; Rimini *et al.*, 2000). Many genes are known to play a role in this complex mechanism of DNA uptake that proceeds via several stages, although the timing of their expression is largely uncharacterised. The process is under the control of a TCS, ComD/E (TCS12), although several other TCS may play a role, and is induced by a peptide, CSP, in a cell-density dependant manner. The system can be analysed in a variety of ways using microarrays. Expression of groups of genes at different stages of competence, via the induction of CSP, can be studied using time-scale experiments (Peterson *et al.*, 2000 and Rimini *et al.*, 2000). Alternatively, individual genes can be mutated to determine their influence on other genes (Peterson *et al.*, 2000). The arrays used for both studies were not full genome arrays. Peterson and colleagues used 3 mini arrays of 40, 68 and 99 genes respectively. Rimini and co-workers spotted 4301 clones from a pneumococcal library onto a nylon membrane and used this array for subsequent analysis. This array was thought to represent most of the genome of a type 19F(G54) strain.

The above studies identified known and unknown genes induced by CSP and provided data on the timing of expression of certain sets of genes. One further study with the CiaH/R (TCS05) system has also identified genes involved in competence and is discussed below (Sebert *et al.*, 2002).

Microarrays have been used with TCS13 (BlpH/R or SpiH/R) to identify a group of bacteriocin-like genes and their immunity proteins that appear to be regulated by the system in response to a peptide signal. This system shares many features with the ComD/E system. A time course analysis was performed to monitor the expression of genes over time in cultures treated with the synthetic peptide. 16 genes were found to be up-regulated over eight-fold. Most of these genes were located in close proximity to the TCS locus itself. The microarray analysis also indicated that expression of the system is auto-induced, as is the case for ComD/E. A deletion in this system was found to have reduced growth in the lung environment (Throup *et al.*, 2000), suggesting it is important in virulence. However, as the lungs are normally sterile, the requirement for bacteriocins that inhibit the growth of closely related bacteria in this environment is doubtful. These pore-forming toxins may thus function to damage host cells in some way and could constitute the second, as yet, unidentified haemolysin of *S. pneumoniae* (Canvin *et al.*, 1997). Alternatively the bacteriocins could act on inhabitants of the nasopharynx,

the site of initial pneumococcal colonisation, although a recent study has demonstrated that a mutation within this system is not impaired in its ability to colonise the nasopharynx in an infant rat model (Sebert *et al.*, 2002). However colonisation could be a human-specific effect.

Recently another pneumococcal TCS, CiaH/R (TCS05) has been analysed using microarrays (Sebert *et al.*, 2002). A mutation within this system was found to have greatly reduced virulence in an infant rat model of nasopharyngeal colonisation and was studied using microarrays to determine genes that could contribute to this phenotype. Expression of genes in the *ciaH/R* mutant were compared with those of wild type from *in vitro* cultures grown to an $OD_{600\ nm}$ of 0.3. The mutation resulted in the up-regulation of genes involved in the later stages of competence (the contribution of this TCS to competence has been discussed elsewhere in this chapter) but the down-regulation of two other genes *htrA* and *spoJ*. Mutations were made in both of these genes and tested for their ability to colonise the nasopharynx. Only a mutation in *htrA* was impaired in colonisation. This appears to encode a serine protease, although its substrate has not been identified. *HtrA* is located in close proximity to the *comC/D/E* locus so could too be involved in competence.

1.15 TCS Summary

Two component systems are ubiquitous in bacteria where they control a wide range of processes in response to various environmental stimuli. Research into these systems is revealing the complex nature of gene regulation in bacteria in response to their environment. These systems, therefore, are unlikely to be solely 'two-components' as their name suggests, but rather two-components functioning as part of a more global and highly intricate network of systems capable of a range of functions. A vast amount of work remains to be done before we can begin to comprehend how these systems function in the context of the whole bacterial cell and in combination with other systems. Advanced technology, such as microarrays and proteomics should help to determine genes regulated by these systems, and structure function studies will be of benefit in the identification of active sites and design of therapeutic targets.

Thirteen HK/RR pairs have been identified in *S. pneumoniae*, together with a single, unpaired RR. One of these systems appears to be essential and many seem to have a role in virulence, albeit at different stages or at different sites of infection. More comprehensive analysis of

virulence will enable researchers to identify the precise roles of these systems in infection. However, bacterial TCS may respond to signals present solely in the human host that may require more sophisticated analyses to enable complete characterisation. This project aims to characterise two pneumococcal TCS and putative related genes for their role in virulence using a well-characterised murine model of infection.

Chapter 2

Materials and Methods

2. Materials and methods

Prior to use, all heat stable reagents were sterilised by autoclaving.

A table of all strains and mutants used in this work is given in the appendix (A1)

Details of vectors (supplied by Hoffman-La-Roche) used for creating insertion-duplication mutants in TCS08 are provided in A2

Details and sequences of all primers used in this thesis are given in A3. All sequences for *S. pneumoniae* specific primers were blasted against the TIGR4 and R6 pneumococcal genome sequences (http://www.tigr.org/) to ensure binding only to the required region.

Preparation of common solutions and buffers is detailed in A4.

2.1 Confirming organisms as *Streptococcus pneumoniae*

2.1.1 Bacterial cell culture

All *S. pneumoniae* strains were grown on Blood Agar Base, number 2 (BAB, Oxoid Ltd. Basingstoke) supplemented with 5 % defibrinated horse blood (E&O Laboratories, Bonnybridge, Scotland), or maintained in Brain Heart Infusion media (BHI, Oxoid Ltd., Basingstoke). Mutants were maintained with appropriate selection: erythromycin (1.0 μg ml^{-1}) or spectinomycin (200 μg ml^{-1}). Plates /cultures were incubated statically at 37 ^{o} C and colonies were examined for their appearance and production of α-haemolysis on blood agar. These conditions were maintained throughout experimental procedures, unless stated otherwise.

2.1.2 General confirmation of strains

All strains used within this work were confirmed by colony morphology, production of α-haemolysis on blood agar, Grams stain and optochin sensitivity (5 μg optochin/ethylhydrocupreine hydrochloride). Quellung reaction was used to confirm capsular serotype. Multilocus sequence typing (MLST) was also performed to confirm strains. Analysis was performed at the Scottish Meningococcal and Pneumococcal Reference Laboratory

(SMPRL), Stobhill Hospital, Glasgow, Scotland and clarified using the MLST data-base (http://fi-srvmlst1-ide.sm.med.ic.ac.uk/new/).

2.1.3 PCR for pneumolysin gene

Following preparation of genomic DNA from bacterial strains (see below), primers specific for pneumolysin (PlyF & PlyR, A3) were used to confirm the presence of pneumolysin. PCR reaction mixtures were as follows (in a 100 µl reaction); 65 - 150 µg ml^{-1} genomic DNA (gDNA) template, 1 µM of each primer (desalted, Sigma Genosys, UK), 10 µl buffer (10 x, no Mg^{2+}), 1-2 mM $MgSO_4$, 50 µM dNTP's and 1-2 units Taq polymerase (all reagents from Promega). PCR conditions; Stage 1: 94 ^{0} C (5 min), repeat x 1. Stage 2: 94 ^{0} C (30 sec), 62 ^{0} C (30 sec), 72 ^{0} C (1 min, 30 sec), repeated x 30. Stage 3: 72 ^{0} C (10 min), repeated x 1. Products were electrophoresed on a 1 % agarose gel (Gibco BRL Life Technologies, Scotland, UK) containing ethidium bromide (0.5 µg ml^{-1}, Sigma-Aldrich, UK) in TAE buffer. DNA was visualised using an UV transilluminator (Spectroline, TVC-312A) and images were captured using a UVP GelDoc and analysis system (UVP Laboratories).

Conditions for all PCRs were optimised for each new primer pair using a temperature gradient (50-65 ^{o} C) and a range of Mg^{2+} concentrations (1-4 mM).

2.1.4 Glycerol stock preparation

Glycerol stocks of all confirmed *S. pneumoniae* strains and mutants were prepared by growing a bacterial culture from a single, pure colony to mid-exponential phase (OD_{600} nm 0.5-0.6). Glycerol was added to a final concentration of 12 % (v/v). 1 ml aliquots were prepared and frozen at - 70 ^{o} C in cryotubes. Viability of stocks was determined 24 hr after freezing by the method described below (section 2.4.1).

2.2 Construction of mutants

2.2.1 Construction of mutants by insertion-duplication mutagenesis

2.2.1.1 Transformation of plasmid constructs into E. coli competent cells

Plasmid constructs used to create mutants were based on the pAS1 vector and were supplied by R. Lange, Hoffmann-La-Roche (see A2). Plasmids were transformed into NovaBlue Singles ™ competent cells (Novagen), following manufacturer's instructions. Following overnight incubation, transformed cells were selected by their ability to grow on erythromycin selection (1mg ml^{-1}). Single colonies were inoculated into LB media supplemented with erythromycin, grown to an optical density (OD_{600} nm) of 0.5-0.6 and used for the preparation of glycerol stocks (section 2.1.7). Preparations were stored at - 70 ^{o} C. Plasmid pVA838 (a kind gift from J. Paton), a positive control for pneumococcal transformation was also transformed into NovaBlue Single cells as described above. This plasmid also carries a gene encoding erythromycin resistance.

2.2.1.2 Plasmid isolation and purification

Purified plasmid constructs were isolated from transformed NovaBlue Single cells using a commercially available plasmid isolation kit, according to manufacturer's instructions (QIAprep® Spin Miniprep Kit, Qiagen). This technique uses alkaline lysis of bacterial cells. Released DNA is adsorbed onto a silica gel from which plasmid DNA can be eluted in a low salt buffer. Plasmid DNA concentration and purity was determined by OD_{260} nm and OD_{280} nm readings (Unicam UV2, UV/Vis spectrometer), where an OD_{260} nm of 1.0 corresponds to 50 μg ml^{-1} DNA and OD_{260} nm/OD_{280} nm is 1.8 for pure DNA.

2.2.1.3 Confirmation of isolated plasmid by restriction digest

Purified plasmid constructs based on the pAS1 vector were cut using EcoR1 and BamH1 restriction enzymes (Promega, 10 U μl-1). Each mixture comprised 10 U of each enzyme in Multicore buffer (Promega, 10 x) and 3 μl plasmid DNA in a 10 μl reaction. Samples were incubated for 2-12 hr at 37 ^{o} C. DNA loading buffer was added and samples were electrophoresed on a 1 % agarose gel as described in section 2.1.3. This yielded bands of ~ 0.3 Kb and 7.4 Kb, representing the cloned region with homology to the target gene and empty vector respectively. Plasmid pVA838 (Macrina et al., 1983) was also cut using EcoR1 and

BamH1 enzymes as described above to yield expected bands of approximately 2.3 Kb and 6.9 Kb.

2.2.1.4 Transformation of S. pneumoniae

A transformation protocol based on previously described methods was developed to enable the construction of mutants in *S. pneumoniae*. 50 µl of thawed pneumococcal stock was used to inoculate 30 ml BHI supplemented with 1 mM $CaCl_2$ (Sigma-Aldrich, UK). Cultures were grown to an OD_{600} nm of 0.1-0.3. For each transformation 1 ml of culture was added to a sterile bijoux. CSP-1 (Havarstein *et al*, 1995) (Sigma Genosys, UK) was added to a final concentration of 100 ng ml^{-1}, and samples were incubated at 37 ^{o} C for 15 min. Plasmid/sample DNA was added to a final concentration of 8-10 µg ml^{-1}. Total volume of DNA added was kept to a maximum of 50 µl. For each transformation, a positive (pVA838) and a negative (no sample DNA) control was set up. Samples were incubated for 75 min at 37 ^{o} C. Following incubation, 450 µl aliquots of transformation mixture were plated onto blood agar plates containing appropriate antibiotic selection. Plates were air dried under a bunsen burner and incubated O/N at 37 ^{o} C. Potential transformants were selected by their ability to grow on antibiotic-selective plates. Single colonies were sub-cultured into BHI containing antibiotic, from which glycerol stocks were prepared as described above (section 2.1.4). pVA838 is able to replicate in *S. pneumoniae* and thus does not require chromosomal integration. The efficiency of transformation using this plasmid is therefore greater than using a suicide vector or PCR fragment (used to create mutants) which require chromosomal integration to prevent elimination.

2.2.2 Construction of mutants by allelic-replacement and *Mariner* mutagenesis.

2.2.2.1a Allelic replacement; amplification of DNA from existing mutants

Our laboratory was supplied with mutants in RR and HK genes in a serotype 3 background (M.K. Burnham, GlaxoSmithKleine). To create isogenic mutants in serotype 2 D39, PCR was used to amplify DNA encoding desired mutation from the type 3 mutants. This was carried out using PCR with primers designed 5' and 3' to the mutated region (primers described below and in A3). PCR reactions were carried out and DNA was visualised as described above (section 2.1.3) replacing Taq polymerase with Vent polymerase (New England Biolabs®, Inc.) for

proofreading activity. Primers used and optimised conditions for PCR are provided in the table below.

Mutation	Primers (forward, reverse)	Annealing / elongation conditions
Δrr08	SKB484P1 SKB484P4	58 ° C, 30 sec 72 ° C, 1 min 30 sec
Δrr09	SKB488P1 SKB488P4	62 ° C, 30 sec 72 ° C, 1 min 20 sec

2.2.2.1b Allelic replacement; DNA purification

Following PCR, the desired DNA product was excised from the agarose gel and purified using a commercially available kit (QIAquick® Gel Extraction Kit, Qiagen). The purified product was re-analysed on an agarose gel and then transformed into D39 as described below.

2.2.2.2 Mariner mutagenesis

PCR products, designed to create two mutations in the *zmpB* gene, were supplied by Jean-Pierre Claverys (Toulouse, France). Mutation *Δzmp739* results in a more truncated version of the *Δzmp738* mutation. Both mutants were created by *Mariner* cassette insertion into the *zmpB* gene (Bergé *et. al*, 2001).

2.2.2.3 Transformation of D39

S. pneumoniae, strain D39, was transformed using the purified *tcs08*, *tcs09* and *zmpB* PCR products as described in section 2.2.1.4. For mutants created by allelic replacement, potential transformants containing the desired mutation were selected on erythromycin plates (1 μg ml^{-1}). Mutations in *zmpB* were selected on spectinomycin plates (200 μg ml^{-1}).

2.3 Confirming mutant genotypes

2.3.1 Confirming knock-out mutants created by insertion-duplication mutagenesis

2.3.1.1 Preparation of Genomic DNA

A sweep of 4-5 bacterial colonies from a blood plate was used to inoculate 10 ml BHI. Following O/N incubation at 37 ° C, cultures were centrifuged for 15 min, 5,000 rpm at RT (Hereus Sepatech medifuge). The pellet was re-suspended in 1 ml extraction buffer and incubated for 1 hr at 37 ° C. Proteinase K (Sigma-Aldrich, UK) was added to a final concentration of 100 μg ml^{-1} and the mixture was incubated for 3 hr at 50 ° C. RNase (Sigma-Aldrich, UK) was added to a final concentration of 20 μg ml^{-1} and incubated at 37 ° C for 30 min. Resultant mixture was gently mixed with an equal volume of phenol/chloroform/isoamylalcohol (25:24:1 v/v, BDH & Fisher Scientific UK. Ltd.) and centrifuged at 13,000 rpm for 3 min (Sigma 113 microfuge). The aqueous layer was removed and added to 0.2 volumes of 10 M ammonium acetate (Sigma-Aldrich, UK). Ethanol (100 %) was added to precipitate the DNA and the mixture was centrifuged at13,000 rpm for 30 min. The DNA pellet was left to air dry for 10 min, re-suspended in TE buffer, pH 7.4, and stored at 4 ° C or - 20 ° C. DNA concentration and purity was determined as described above (section 2.2.1.2).

2.3.1.2a PCR to confirm chromosome/ plasmid junctions

PCR was used to confirm the plasmid integrations for mutants created by insertion duplication mutagenesis. For each mutation, a primer specific for the inserted DNA was used in combination with a primer specific for the D39 genomic DNA. The resulting PCR fragment thus spanned the chromosome / plasmid junction. All PCR reactions were carried out in 100 μl volumes as described in section 2.1.3. PCR mixtures were denatured at 94 ° C, 5 min., followed by 30 cycles of denaturation (94 ° C, 30 sec.), annealing and elongation (conditions vary, see table below). Samples were then held at 72 ° C for 10 min. The table below shows conditions, optimised for individual reactions. Genomic DNA prepared from strains as described in section 2.3.1.1 was used for PCR reactions.

Mutation	Primers (forward, reverse)	Annealing / elongation conditions
ΔpRPL7, 5' region	HK8PFW, RPL22	53 ° C, 1 min 72 ° C, 45 sec
ΔpRPL7, 3' region	AP70, HK8PR	54 ° C, 30 sec 72 ° C, 1 min
ΔpAS1, 5' region	URR8F, RPl22	54 ° C, 30 sec 72 ° C, 1 min
ΔpAS1, 3' region	AP70, RR08R	55 ° C, 30 sec, 72 ° C, 1 min
ΔpRPL50, 5' region	RPL102, RPL22	56 ° C, 1.5 min, 72 ° C, 30 sec
ΔpRPL50, 3' region	AP70, RPL109	56 ° C, 1.5 min, 72 ° C, 30 sec

2.3.1.2b Sequencing to confirm insertions

Several PCR fragments from the PCRs described above were selected for sequencing to further ensure mutations were correct. Sequencing was performed in-house (MBSU DNA Sequencing Service, University of Glasgow, UK) using primers specific for individual PCR reactions described above. Primers were used at 3.2 μM for sequencing reactions. Sequences were analysed using Edit View ASI Automated DNA Sequence Viewer (ABI Prism™) and DNASTAR software. Sequence homology searches were performed to confirm mutations using BLAST (Altschul *et al.*, 1990) and The Institute of Genomic Research's Comprehensive Microbial Resource (TIRG CMR, http://www.tigr.org/).

2.3.1.2c PCR to confirm absence of wild-type gene in mutants

Primers were also designed flanking the target genes. PCR was performed using these primers to demonstrate the absence of the wild-type gene in mutant DNA (the inserted plasmids were too big to give a detectable PCR product for mutants using the primers described and under the

conditions stated). This experiment also enabled checking for any gene duplications that may have been present on the chromosome.

Mutation	Primers (forward, reverse)	Annealing / elongation conditions
ΔpAS1	RR08F RR08R	51 ° C, 30 sec 72 ° C, 1 min
ΔpRPL7	HK08F HK08R	51.4 ° C, 30 sec 72 ° C, 1 min
ΔpRPL50	Sp0644F Sp0644R	53 ° C, 30 sec 72 ° C, 3 min, 30 sec

2.3.2 Confirming mutants created by allelic-replacement

2.3.2.1 PCR

PCR using primers out-with the mutated region was performed using the primers and PCR conditions described in section 2.2.2.1a. Reactions were set up as previously described, section 2.1.3., using Vent polymerase (New England Biolabs® Inc.) to provide proofreading activity to ensure high fidelity. 3' adenine overhangs were subsequently added onto PCR products by the addition of 1 U Taq polymerase to the reaction mixture and further incubation at 72 ° C for 10 min. This enabled direct cloning using the TOPO TA cloning Kit at a later stage (described below). PCR products were electrophoresed on a 1 % agarose gel as described in section 2.1.3. Genomic DNA prepared from strains as described in section 2.3.1.1 was used for PCR reactions. Insertion of mutated DNA resulted in a detectable increase in PCR product size for mutants when compared to wild type DNA.

2.3.2.2 Cloning of PCR fragments into a plasmid vector (TOPO) for sequencing

PCR products were excised from the agarose gel and purified using a commercially available DNA purification kit (QIAquick® Gel Extraction Kit, Qiagen) according to manufacturer's instructions. The size of the purified PCR product was confirmed by electrophoresis and cloned

into the vector pCR®4-TOPO® (Invitrogen™ life technologies). This was then used to transform TOP10 chemically competent *E.coli* cells (Invitrogen™ life technologies) according to manufacturer's instructions. Transformation mixture was plated onto LB plates containing ampicillin (75 μg ml^{-1}), for selection of cells containing pCR®4-TOPO®.

2.3.2.3 Restriction digest to confirm insert in TOPO

pCR®4-TOPO® containing insert was purified using a commercially available plasmid isolation kit (QIAprep® Spin Miniprep Kit, Qiagen) according to manufacturer's instructions. Isolated plasmid vector was digested with EcoR1 (Promega, 10 U μl^{-1}) as described previously (section 2.2.1.3). Products were electrophoresed on a 1 % agarose as described previously.

2.3.2.4. Sequencing of cloned products

Following confirmation of DNA insert into pCR®4-TOPO®, purified plasmid was sequenced in-house as described in section 2.3.1.2b, using primers T3 and T7 specific for the pCR®4-TOPO® vector. This confirmed replacement of the target gene with an erythromycin cassette.

2.3.3 Confirming mutants created by *mariner* mutagenesis

Mutants were confirmed by PCR as described in section 2.1.3. Due to the large size of the *zmpB* gene, expand polymerase (Expand Long Template PCR System, Roche Diagnostics), a polymerase for the amplification of large DNA fragments, was used in replacement of Taq polymerase. This polymerase has proof-reading activity. Primers used and optimum PCR conditions are shown below.

Mutation	Primers (forward, reverse)	Annealing / elongation conditions
Both *Δzmp* mutants	ZmpUp, ZmpDo	55 ° C, 30 sec 72 ° C, 10 min
Both *Δzmp* mutants	ZmpUp, MP128	55 ° C, 30 sec 72 ° C, 10 min

2.4 *In vitro* characterisation of bacterial strains

2.4.1 Determining number of viable organisms

To enumerate number of CFU in frozen stocks, an aliquot of culture was rapidly thawed in a 37 ° C water bath. 900 µl was removed and cells pelleted by centrifugation, 13,000 rpm. (Sigma 113 microfuge). Bacterial pellets were re-suspended in 900 µl PBS. Serial 10-fold dilutions were carried out in a 96-well round-bottomed tissue culture plate (Nunclon™ surface, Nalge Nunc International™) to a final concentration of 10^{-6} in PBS. 6 x 20 µl of each dilution were plated onto blood agar and plates were incubated for at least 16 hr, 37 ° C. Following incubation the dilution of culture giving counts of 60 – 200 CFU per 20 µl spot were counted. CFU ml^{-1} was calculated. This method is based on that of Miles and Misra (1938).

2.4.2 Growth curves

15 ml BHI was inoculated with 1.0 x 10^{6} CFU bacterial strains and incubated at 37 ° C. OD_{600} nm readings were taken at 2-hourly intervals for at least 14 hr post inoculation. Growth of mutant strains was monitored in both selective and non-selective media.

2.4.3 Viability curves

The viability of bacterial strains was monitored during the growth described above. At 2-hourly intervals, samples removed for OD_{600} nm data were used for viable cell enumeration as described in section 2.4.1.

2.4.4 Lysis with deoxycholate

15 ml BHI, without antibiotic selection, was inoculated with 1.0 x 10^{6} CFU bacterial strains. Cultures were incubated at 37 ° C until mid-log phase ($OD_{600\ nm}$ 0.5-0.6) was reached. A 10 % (w/v) solution of deoxycholate (DOC) (Sigma-Aldrich, UK) was prepared in PBS and added to 5 ml aliquots of each culture to a final concentration of 0.04 % (w/v). Samples were removed for viable cell enumeration immediately prior to and following treatment with DOC at pre-determined time points for up to 60 min. Viability counts of untreated culture were used as a control.

2.4.5 Stability of mutants

To assess the ability of mutant strains to retain antibiotic resistance, cultures were grown in the absence of erythromycin or spectinomycin selection. BHI was inoculated with 1 x 10^6 CFU bacterial strains and incubated at 37 ^{o} C. At predetermined time points viability was determined using BAB plates both with and without appropriate antibiotic selection.

2.4.6 Haemolytic assay

Bacterial strains were grown to mid log phase ($OD_{600\ nm}$ 0.5-0.6). Samples were removed and treated for 5 minutes with 0.04 % (w/v) deoxycholate (Sigma-Aldrich, UK) to lyse all cells and release haemolysins. Lysed cultures were diluted two-fold across a 96-well round bottomed plate (Nunclon™ surface, Nalge Nunc International™) in PBS in a final volume of 50 µl. Control wells were set up containing PBS alone, cultures of *Δpln* (a pneumolysin null mutant created by allelic replacement in our laboratory, unpublished data), BHI with 0.04 % deoxycholate and non-deoxycholate-lysed bacterial cultures. 50 µl of a 2 % (w/v) suspension of sheep red blood cells (E&O Laboratories, Bonnybridge, UK) was added to each well. Plates were incubated at 37 ^{o} C for 30 min then room temperature for 30 min. Haemolysis of sheep red blood cells was assessed visually by the absence of a compact pellet following incubation. Partial haemolysis was recorded when a very small pellet was visible.

2.4.7. Transformation efficiency

To determine the transformation efficiency of strains, the transformation procedure was carried out, as described in section 2.2.1.4. pVA838 plasmid was used as a marker for erythromycin resistance to determine transformation rates in strains not possessing erythromycin resistance. For strains already resistant to erythromycin, *S. pneumoniae* chromosomal DNA containing a marker for streptomycin resistance (rpsL41 allele with a change from lysine (AAA) to threonine (ACA) at position 56 of the S12 protein, Salles *et al.*, 1992) was used to determine the transformation efficiency. The transformation mix was plated out on BAB both with and without antibiotic selection (erythromycin , 1 µg ml^{-1} or streptomycin, 150 µg ml^{-1}) for viable cell enumeration. Efficiency was calculated based on number of transformants per µg plasmid/chromosomal DNA.

2.4.8 Carbohydrate utilisation

Thioglycollate medium (without dextrose or indicator) (4.8 g) containing purple broth base (3.2 g) (both from Difco/Becton Dickinson), was made up in 200 ml dH_2O and autoclaved. Carbohydrate solutions (10 % [w/v], all from Sigma-Aldrich, UK) were made up in dH_2O and filter sterilised using 0.2 μM filters (Sartorius Minisart, Germany). Carbohydrate was added to the sterilised thioglycollate/purple media to a final concentration of 1 % (w/v). Bacterial cultures were grown in Todd-Hewitt broth (Oxoid Ltd., Basingstoke, UK) O/N at 37 ° C. 75 μl of carbohydrate solution was mixed with 25 μl of overnight culture in a 96-well, flat bottomed microtitre plate (Nunclon™ surface, Nalge Nunc International™), placed into an anaerobic bag (BBL™ GasPak Pouch™, Becton Dickinson, USA) and incubated at 37 ° C for 24 hr. Addition of 25 μl Todd-Hewitt broth alone to the carbohydrate solution was set up as a negative control. Ability to ferment the carbohydrate was demonstrated by a colour change from purple to yellow, due to the production of acid, and was determined by visual inspection.

2.5 *In vivo* experiments

All *S. pneumoniae* strains used were mouse passaged (intraperitoneally, see below) prior to use in the animal model, unless stated otherwise. This enhances virulence of the bacterial strains and is a technique commonly applied to many *S. pneumoniae* studies.

2.5.1 Animal models

Adult female MF1 mice aged 9-13 wk and weighing 28-35 g were used as the standard model of pneumococcal pneumonia and sepsis. Male CBA/J mice, aged 5 wk and weighing 14-16 g were used to repeat the work of Throup *et. al.* and were housed in filter top cages to reduce environmental infection. Female CBA/J mice were purchased for breeding purposes and were also housed in filter top cages. Complement studies were carried out using C3-/- mice (Wessels *et al.*, 1995), bred in-house. Adult male and female C57/Bl mice, aged 9-13 wk were purchased as wild type controls for the C3-/- experiments. All mice, with the exception of C3-/- mice, were purchased from Harlan Olac, Bicester, UK. All mice were provided with sterile pelleted food (B&K Universal, North Humberside, England) and water *ad libitum* throughout the experiments. Mice were housed at the Central Research Facility, University of Glasgow, UK. All animal work was carried out under appropriate licensing and with approval from the Home Office and the

University of Glasgow. CBA/Js with endemic infections were treated with Borgal (a sulphonamide), to a final concentration of 0.01 % in drinking water.

2.5.2 Preparation of standard inoculum / intraperitoneal challenge

Bacterial strains were re-suspended in sterile PBS to give approximately 1.0 x 10^{6} CFU ml^{-1}. 200 µl was injected into the peritoneal cavity of MF1 mice using a 1 ml insulin syringe (Micro-fine, 12,7 mm, Becton Dickinson). 24 hr following injection, mice were sacrificed by cervical dislocation and the chest cavity opened. Blood was collected from the heart using a 23 gauge needle inserted into the right ventricle. 50 µl of removed blood was inoculated into 10 ml BHI and grown O/N, statically at 37 ^{o} C. Bacteria were harvested by centrifugation at 3,000 rpm. (Heraeus Sepatech medifuge) and re-suspended in 1 ml BHI. 100 µl of this suspension was used to inoculate 20 ml BHI containing 20 % (v/v) heat inactivated FBS (Gibco BRL, Life Technologies, U.K.) and then incubated at 37 ^{o} C until mid-exponential phase was reached (OD_{600} nm 0.5 – 0.6). Culture was stored at $^{-}$ 70 ^{o} C in 1 ml aliquots until required. Viability of stocks was determined 24 hr following freezing as described in section 2.4.1. Appropriate antibiotic selection was added to BHI to sustain the growth of all mutant strains.

2.5.3 Intranasal challenge

900 µl of standard inoculum were rapidly thawed and centrifuged at 13,000 rpm. for 3 min (Sigma 113 microfuge). The pellet was re-suspended in 900 µl PBS and diluted to give 2.0 x 10^{7} CFU ml^{-1} (unless stated otherwise). Mice were lightly anaesthetised with 2.5 % (v/v) halothane (Zeneca Pharmaceuticals, Macclesfield, UK.) over oxygen (1.5 L min^{-1}) using a calibrated vaporiser. Confirmation of anaesthesia was determined when mice failed to produce a reflex reaction when gently pinched. 50 µl of bacterial suspension was administered, drop-wise, to the nares using a Proline® Pipette (Biohit). Individual mice were thus given 1.0 x 10^{6} CFU. Mice were laid on their backs until they recovered. Viable counts of the bacterial suspensions were determined prior to and immediately after challenge, by viable cell enumeration, as described in section 2.4.1.

2.5.4 Intravenous challenge

900 µl of standard inoculum was rapidly thawed and centrifuged at 13,000 rpm. for 3 min (Sigma 113 microfuge). The pellet was re-suspended in 900 µl PBS and diluted to give 2.0 x 10^6 CFU ml^{-1} (unless stated otherwise). Mice were placed in a ventilated, heated perspex box for 5 min and restrained in appropriate apparatus. 100 µl of bacterial suspension was administered directly into the bloodstream via the lateral tail vein using a 1 ml insulin syringe (Micro-fine, 12,7 mm, Becton Dickinson). Blood was sampled from a different tail vein 1 min following challenge to ensure all mice were administered equal bacterial counts. At pre-determined time points, blood was sampled from the lateral tail vein as described above and used for viable cell enumeration, as described in section 2.4.1. Viable counts of the bacterial suspensions were determined prior to and immediately after challenge.

2.5.5 Determining levels of bacteraemia

Blood was sampled at pre-determined intervals following challenge via removal of blood from peripheral veins. Mice were placed in a ventilated, heated perspex box for 5 min and restrained in appropriate apparatus. A 1 ml insulin syringe (Micro-fine, 12,7 mm, Becton Dickinson) was used to remove a maximum of 100 µl blood from the lateral tail vein. Blood was immediately diluted 1:10 in PBS and stored on ice until viability counts were carried out, as described in section 2.4.1.

2.5.6 Determining bacterial loads in lung tissue

At pre-determined time points following intranasal challenge, as described in section 2.5.3, mice were culled by cervical dislocation and the thoracic cavity opened up to expose the lungs. Lungs were aseptically removed, rinsed with dH_2O and placed in 5 ml sterile PBS. Weight of the lungs was recorded. Lungs were homogenised for 10 sec using an electric hand-held tissue homogeniser set at high speed (IKA®-Ultra-Turrax® T25, Janke Kunkel Laboratories). Samples of homogenised lung tissue were used for viable cell enumeration, as described section 2.4.1.

2.5.7. Preparation of lung tissue for cytokine analysis

At pre-determined time points following intranasal challenge, as described in section 2.5.3, mice were culled by cervical dislocation and the thoracic cavity opened up to expose the lungs. Lungs

were aseptically removed, wrapped in aluminium foil and snap frozen in liquid nitrogen. Prior to use, lungs were weighed and homogenised as described in section 2.5.6. Based on the protocol by van der Poll *et al* (van der Poll *et al.* 1996) homogenates were centrifuged at 1600 g for 30 min at 4 ^{o} C. to pellet cell debris. Supernatants were then centrifuged at 5000 g for 30 min at 4 ^{o} C (Sigma 4K15 centrifuge). The supernatant was removed and filter sterilised through 0.20 μM filters (Sartorius Minisart, Germany). Cell free supernatant was aliquoted into cryovials and stored at - 70 ^{o} C until required for cytokine analysis.

2.5.8 Bronchi-alveolar lavage

Mice were killed by cervical dislocation. The skin and muscles surrounding the trachea were exposed and the thoracic cavity opened up to allow expansion of the lungs. The trachea was clamped using Spencer-Wells forceps (Fisher Scientific Ltd. UK) to prevent backflow of fluid up the trachea. A 16-gauge non-pyrogenic angiocath (F. Baker Scientific) was inserted into the trachea immediately above a ring of cartilage to hold the catheter in place. Lavage was performed using 2 x 1 ml volumes of PBS. Recovery volume was between 1.6 – 1.8 ml per mouse. A small aliquot of BALF was retained for viable cell enumeration and the remainder was snap frozen in liquid N_2 in cryovials for cytokine analysis. BALF was stored at - 70 ^{o} C until required.

2.5.9 Murine survival and pain scoring

Mice were monitored frequently throughout the infection by a pain scoring system devised within this laboratory. Symptoms progressed from normal to severe with mice showing starry coat/piloerection, hunched posture and lethargy. Severe infection resulted in the mice eventually becoming moribund. Mice were sacrificed, by cervical dislocation, when showing signs of severe lethargy or upon becoming moribund. Where animals were actively culled, the time of reaching a moribund state was taken as the time of death ('mortality') / experimental end point for survival data. Mice that survived the course of infection (7 days, unless stated otherwise) were assigned an arbitrary survival time of 168 hr for statistical analysis.

2.5.10 Stability of mutants

On removing blood samples for bacterial load determination, viable counts for all the mutant strains were plated onto BAB plates with and without antibiotic selection to ensure the mutation conferring antibiotic resistance was maintained throughout infection. Viable cell enumeration was determined as described in section 2.4.1.

2.5.11 Confirming mutants following *in vivo* infection

DNA was isolated from all mutants retrieved from the blood of infected mice at least 24 hours post challenge as described in section 2.3.1.1. PCR as detailed in previous sections was then used to re-confirm the individual mutations.

2.6 Immunological techniques

For ELISAs, standards were run in duplicate and samples in triplicate. A set of standards was included on every plate. Measurements were determined from the standard curve specific for individual ELISAs using Biolinx 2.21™ software.

2.6.1 Detection of TNF-α by ELISA

TNF-α was detected using an OptEIA™ Mouse TNF-α Set (Pharmingen, USA), optimised prior to use. Batch-specific recombinant mouse TNF- α standard was re-suspended in dH_20 as directed and stored in aliquots at ¯ 70 ° C. Plastic, flat bottomed 96-well maxisorb™ plates (Nalge Nunc International™) were coated O/N at 4 ° C with anti-mouse TNF- α capture antibody, diluted 1:350 in coating buffer. Wells were washed x 3 in wash buffer, and blocked with 200 µl assay diluent for 1 hr at RT. Standards were prepared in assay diluent, using 1:2 dilutions to produce a standard curve ranging from 15.6 – 1000 pg ml^{-1}. Assay diluent served as the zero standard. Wells were washed, as above. 100 µl samples and standards were added to wells and plates were incubated, sealed, for 2 hr at RT. Wells were washed (x 5) in wash buffer and 100 µl working detector was added (biotinylated anti-mouse TNF-α detection antibody 1:350). Plates were sealed and incubated for 1 hr at RT. Wells were washed (x7) in wash buffer and 100 µl substrate solution was added. Plates were incubated, unsealed, in the dark for 30 min. Reaction was stopped with 50 µl stop solution and absorbance read at A_{450} nm with A_{570} nm correction using a Dynatech MRX ELISA reader and Biolinx 2.21™ software.

2.6.2 Detection of IL-10 by ELISA

IL-10 was detected using an OptEIA™ Mouse IL-10 Set (Pharmingen, USA), following the protocol described above for TNF-α. Capture antibody, biotinylated anti-mouse IL-10 detection antibody and avidin horseradish peroxidase conjugate were all used at 1:250 dilutions. Standard curve values ranged from 32.25-2000 pg ml^{-1}.

2.6.3 Detection of IL-6 by ELISA

IL-6 was detected using an OptEIA™ Mouse IL-6 Set (Pharmingen, USA), optimised prior to use and following the protocol described above for TNF-α. Capture antibody and avidin horseradish peroxidase conjugate were used at 1:350 dilutions. Biotinylated anti-mouse IL-6 detection antibody was diluted 1:500 in assay diluent prior to use. Standard curve values ranged from 32.25-2000 pg ml^{-1}.

2.6.4 Detection of IFN-γ by ELISA

IFN-γ was detected using an OptEIA™ Mouse IFN-γ Set (Pharmingen, USA), optimised prior to use and following the protocol described for TNF-α. Capture antibody and avidin horseradish peroxidase conjugate were all used at 1:350 dilutions. Biotinylated anti-mouse IFN-γ detection antibody was diluted1:1000. Standard curve values ranged from 32.25-2000 pg ml^{-1}.

2.7 Tissue culture

2.7.1 Recovery of L929 cells from liquid nitrogen storage

The L929 mouse fibroblast cell line was purchased from the European Collection of Animal Cell Cultures (ECACC no. 85011425). A vial of L929 cells was removed from liquid nitrogen storage. The lid was loosened and cells were thawed rapidly by immersion in a 37 ^{o} C water bath. The tube was swabbed with 70 % ethanol to ensure sterility. Cells were re-suspended in 10 ml growth media and centrifuged at 1000 rpm for 10 min (Sigma 4K15 centrifuge). The pellet was re-suspended in 5 ml growth media, transferred to a 25 cm^2 tissue culture flask (IWAKI, Scitech) and incubated at 37 ^{o} C with 5 % CO_2.

2.7.2 Passage of L929 cells

Upon reaching confluency, cells were left for a further 24 hr. The growth media was removed and the cell layer was gently washed with 10 ml sterile PBS. The PBS was removed and 1 ml trypsin/EDTA (Gibco, Life Technologies) was added to digest junctions between cells. Flasks were incubated at 37 ° C with 5 % CO_2 for 5 min, or until the cell layer had become detached from the bottom of the flask. Cells were re-suspended in fresh medium and mixed by gentle pipetting to separate cell clumps. 2 ml of the resulting suspension was used to inoculate 18 ml fresh medium. Caps were loosely replaced and flasks were incubated at 37 ° C with 5 % CO_2.

2.7.3 TNF bioassay

This assay was optimised by Dr. A.R. Kerr within our laboratory, based on a procedure run by AstraZeneca pharmaceuticals and published cytotoxicity assays. The bioassay measures levels of active TNF (both TNF-α and TNF-β) through its ability to induce fibroblast cytotoxicity. Resulting cell death is measured by the inability of fibroblast mitochondria to breakdown MTT (3-[4,5-cimethylthiazol-2yl]-2-5, diphenyltetrazolium bromide), a yellow substrate to formazan product (dark blue). MTT cleavage is detected by colorimetrically following solubilisation of formazan product by addition of DMSO (dimethyl-sulphoxide) + HCl. TNF activity within the sample is determined using a standard curve.

During late afternoon (to prevent over-proliferation of cells), cells were trypsinised as described above. Live cells were counted in a haemocytometer using trypan blue staining (500 µl trypan blue (Sigma-Aldrich, UK), 300 µl medium and 200 µl re-suspended cells). The number of live cells were adjusted to 3.0 x 10^5 ml^{-1} in growth medium and 200 µl of this suspension was added to each well of a 96-well, flat-bottomed tissue culture plate (Nunclon™ surface, Nalge Nunc International™). Following O/N incubation at 37 ° C with 5 % CO_2, medium was removed from wells and replaced with 50 µl of a 20 µl ml^{-1} solution of Actinomycin D (Sigma-Aldrich, UK) in PBS, to prevent further multiplication of cells. TNF-α standard (Sigma-Aldrich, UK) was diluted in medium to produce concentrations ranging from 0.014 – 2500 U ml^{-1}. 25 µl of standard and samples were added in triplicate to appropriate wells. Samples were applied neat and in 1 in 3, 1 in 6 and 1 in 9 dilutions. Medium alone was used as a 0 % kill control.

Following O/N incubation at 37 ° C with 5 % CO_2, 100 µl MTT in PBS (1 mg ml^{-1}) was added to each well. Plates were incubated for 4 hr at 37 ° C with 5 % CO_2. 100 µl of DMSO/0.04N HCl was added to wells to solubilise any formazan product. Plates were tapped gently to mix contents

and A_{540} nm read with the correction set at A_{630} nm, using a Dynatech MRX plate reader and Biolinx 2.21™ software. A logarithmic standard curve was used to determine the TNF activity in samples using 50 % kill measurements.

2.8 ZmpB protein expression

2.8.1 PCR to amplify desired regions of *zmpB* for expression

2.8.1.1 Primer design

Primers were designed to amplify up the central and 3' regions of *zmpB* (ZmpB itself is thought to be too large to express whole [>5 Kb]). Primers were designed with restriction sites (BamHI and SacI) to enable subsequent in-frame cloning of the PCR product into pET33b (see below) downstream of the T7 promoter. The reverse primer for the central region amplification was also designed to contain a stop codon to prevent read-through during transcription. 6 random bp were added onto the 5' ends of all primers to aid restriction enzyme digestion and enable direct cloning into an expression vector.

2.8.1.2 PCR and restriction digest of fragments for cloning and expression

PCR reactions were set up as previously described, section 2.1.3., using Vent polymerase (New England Biolabs® Inc.) to provide proofreading activity.

Region of ZmpB for expression	Primers (forward, reverse)	Annealing / elongation conditions
Central (600 amino acids)	ForBamZmp RevSacZmp	62 ° C, 30 sec 72 ° C, 1min 40 sec
C-terminal (530 amino acids)	ForBamZmp2 RevSacZmp2	62 ° C, 30 sec 72 ° C, 1min 40 sec

PCR products were electrophoresed on a 1 % agarose as previously described. D39 WT genomic DNA prepared as described in section 2.3.1.1 was used for PCR reactions. PCR products were excised from the agarose gel and purified using a commercially available DNA purification kit

(QIAquick® Gel Extraction Kit, Qiagen) according to manufacturer's instructions. PCR product was digested with BamHI and SacI (Promega, 10 U μl^{-1}) as described in section 2.2.1.3.

2.8.2 Cloning into pET33b and transformation into expression host

2.8.2.1 Vector preparation

3 µl of pET33b expression vector (Novagen, UK) was digested with BamHI and SacI as described in section 2.3.1.1. 2 µl of digested product was electrophoresed on a 1 % agarose gel to check vector had linearised. The vector preparation was heated at 65 ° C for 10 min to inactivate enzymes. Vector was incubated at 37 ° C for 4 hr with alkaline phosphatase (Promega 1 U μl^{-1}) and 10 x buffer (Promega) to prevent ligation of the removed fragment back into the vector (1 µl alkaline phosphatase per 10 ul reaction mixture). The vector preparation was purified using a commercially available gel extraction column (QIAquick® Gel Extraction Kit, Qiagen), without prior electrophoresis on a gel.

2.8.2.2 Ligation of PCR product into pET vector

For each *zmpB* insert, 7 µl PCR product was mixed with 1 µl prepared pET33b vector, 1 µl T4 ligase (Promega, 3 U μl^{-1}) and 1 µl 10 x ligation buffer (Promega). The ligation reaction was allowed to proceed O/N at 16 ° C. A control ligation was set up without insert.

2.8.2.3 Transformation into DH5α cells for sequencing

E. coli DH5α cells were used to enable sequencing of the insert prior to transformation into *E. coli* BL21 cells, due to the increased transformation efficiency of the former. One vial of competent DH5α-Library efficiency cells (Invitrogen™ Life Technologies) was thawed on ice and divided into 3 x 100 µl aliquots. 3 µl of ligation mixture was added to each (one transformation for each zmpB ligation and one control to check for self ligation). Cells were incubated on ice for 30 min then heat shocked at 42 ° C for 30 sec. Tubes were placed back on ice for a further 2 min. 450 µl SOC media was added and cells were incubated for 1 hr at 37 ° C, with shaking. Transformation mixtures were spread onto LB agar plates containing kanamycin (30 µg ml^{-1}) to select for pET33b. Plates were incubated O/N at 37 ° C. Several colonies from each transformation were selected and grown to $OD_{600\,nm}$ 0.5-0.6. Glycerol stocks were made as described in section 2.1.4.

2.8.2.4 Restriction digest and sequencing to confirm inserts

pET33b vector was purified from DH5α stocks using a commercially available plasmid isolation kit (QIAprep® Spin Miniprep Kit, Qiagen) according to manufacturer's instructions. Isolated plasmid vector was digested with BamHI and SacI as described above. Products were electrophoresed on a 1 % agarose gel. Confirmation of the insert was indicated by two DNA bands of 1.6-1.85 Kb and 5.4 Kb. These bands represented insert and empty vector respectively. Following confirmation of DNA insert into pET33b, purified plasmid was sequenced in-house as described in section 2.3.1.2b. T7 promoter and T7 terminator primers, specific for pET33b were used for sequencing. Following confirmation that insert was present and in frame, the middle region of the cloned fragment was sequenced using primers specific for *zmpB* (ZmpseqgF, ZmpseqgR) to ensure sequence homology with the R6 genome sequence. The sequence of the entire insert could thus be checked for amino acid changes.

2.8.2.5 Transformation of BL21 cells for zmpB expression

Following confirmation of *zmpB* insert in pET33b by sequencing, purified vector was used to transform BL21 (DE3) competent cells (Stratagene) according to manufacturer's instructions. Transformants were selected on kanamycin plates (30 μg ml^{-1}) and glycerol stocks were prepared as described previously.

2.8.3 ZmpB protein expression

2.8.3.1 Protein expression

BL21 (DE3) cells (Stratagene) were used for the expression of protein as these cells have the T7 polymerase and its *lacUV5* promoter on a bacteriophage, DE3. Addition of IPTG induces T7 polymerase transcription from *lacUV5*. T7 polymerase then acts to induce protein expression from the T7 promoter on pET33b. BL21 (DE3) cells transformed with pET33b vector containing *zmpB* insert were streaked onto an L-agar plate containing kanamycin (30 μg ml^{-1}) and plates were incubated O/N at 37 ° C. Single colonies were used to inoculate 100 ml LB media supplemented with kanamycin. Cultures were incubated O/N at 37 ° C with shaking. 10 ml of overnight culture was used to inoculate 1 L fresh media containing kanamycin selection. Cultures were incubated at 37 ° C, with shaking, until an $OD_{600\ nm}$ of 0.6 was reached. Protein

expression from the T7 promoter was induced by the addition of 1 ml of 1M IPTG (Melford Laboratories Ltd, Ipswich, UK). Cultures were placed back into the shaking incubator for a further 3 hr to allow protein expression to proceed. Cells were then pelleted by centrifugation at 5000 rpm for 15 min (Sigma 4K15 centrifuge). The pellet was re-suspended in 10 ml PBS and lysed as described below.

2.8.3.2 Cell disruption to release protein

Re-suspended cell pellets were passed (x 2) through a One Shot Benchtop Cell Disrupter (Constant Systems Ltd., Warwick, UK) at 12 KPSI to ensure complete disruption. A small aliquot was retained for analysis on a SDS-PAGE gel (described below in section 2.9.5). The remainder of the lysed cell pellet was centrifuged at 14,000 rpm for 1 hr (Sigma 4K15 centrifuge). A small aliquot of the supernatant was retained for analysis on a SDS-PAGE gel and the remainder was discarded. The pellet was frozen at - 70 ^{o} C until required.

2.8.3.3 Detection of expressed protein by SDS-PAGE

Detection of expressed protein in soluble and insoluble cell fractions was determined by SDS-PAGE with Coomassie staining, as described in section 2.9.5.

2.8.3.4 Preparation of protein from inclusion bodies

The majority of expressed protein was in the insoluble fraction and had to be released from the inclusion bodies. The frozen cell pellet from section 2.8.3.2 was thawed at room temperature and re-suspended in PBS containing 30 % (w/v) sucrose and 10 mM EDTA (Fisher Scientific). An electronic hand-held tissue homogeniser set at high speed (IKA®-Ultra-Turrax® T25, Janke Kunkel Laboratories) was used to aid re-suspension of the hard cell pellet. The resulting suspension was centrifuged at 14,000 rpm for 1 hr. The pellet was washed in PBS containing 1 % Triton-X-100 (Sigma-Aldrich, UK) and centrifuged as described above. Following each centrifugation stage, a small aliquot of the supernatant was retained for gel analysis and the remainder discarded. The pellet was re-suspended in 100 ml of 7 M urea (Sigma-Aldrich, UK) made up in PBS to re-solubilise the released protein. Mixtures were kept on ice for 2 hr, with frequent mixing. The re-solubilised protein solution was poured into dialysis tubing with a molecular weight cut off of between 6-8 KDa (Spectra/Por 1®, Fisher Scientific) and dialysed

against PBS for 1 hr at 4 ^{o} C. Used PBS was discarded and replaced with fresh PBS, and dialysis was continued for 4 hr, changing the PBS once during this time. Finally the protein solution was dialysed O/N at 4 ^{o} C in fresh PBS. Following O/N dialysis, large precipitates of protein were formed within the tubing. This precipitate was retained and the supernatant passed down a nickel column (Ni-NTA superflow column, Qiagen) using a Pharmacia FPLC machine (purification step performed by Crystallography Department, University of Glasgow). The column was washed in PBS with 4 mM imidazole (Sigma-Aldrich, UK). His-tagged protein bound to the column was eluted with PBS containing 250 mM imidazole. Eluted protein was electrophoresed on a 10 % SDS PAGE gel as described above.

The protein precipitate from the overnight dialysis was re-solubilised in 100 ml of 7 M urea in order to measure the protein concentration. Re-solubilised protein was scanned using a Unicam UV2, UV/Vis spectrophotometer (Sigma) between $A_{220\ nm}$ and $A_{320\ nm}$. Absorbance was determined and used to estimate the total protein concentration using the equation C=A/E.L, where C = concentration, A = absorbance E = extinction co-efficient and L = pathlength of cell used to read absorbance. E was calculated using the ExPASy molecular biology server with the ProtParam programme (http://ca.expasy.org/tools/protparam.html). The protein was precipitated once more, following the dialysis procedure described above. The protein concentration in the supernatant was calculated in order to determine the total protein concentration in the remaining precipitate. This precipitate was suspended in sterile, LPS-free NaCl to give a final concentration of 2.2 mg ml^{-1} and used as the source of crude ZmpB protein for vaccination. The suspension was aliquoted and stored at - 20 ^{o} C until required.

2.9 ZmpB antibody production

2.9.1 Preparation of ZmpB protein for vaccination

For each vaccination with Freunds complete and incomplete adjuvant (FCA and FIA respectively, Sigma-Aldrich, UK), frozen protein aliquots, see above, were thawed on ice. Sterile, LPS-free NaCl was then added to give a final concentration of 1 mg ml^{-1} protein. Protein suspension was added to an equal volume of FCA / FIA and the mixture was vortexed vigorously for 15-25 min, or until a thick emulsion was formed.

2.9.2 Vaccination schedule

The vaccination schedule was as follows:

Day -1: Pre-vaccination bleed

Day 1: Vaccinate subcutaneously with 50 μg ml^{-1} ZmpB in FCA

Day 13: Pre-boost bleed 1

Day 14: Boost # 1, vaccinate subcutaneously with 50 μg ml^{-1} ZmpB in FIA

Day 27: Pre-boost #2 bleed

Day 28: Boost # 2, vaccinate subcutaneously with 50 μg ml^{-1} ZmpB in FIA

Day 41: Pre-boost #3 bleed

Day 42: Boost # 3, vaccinate intravenously with 50 μg ml^{-1} ZmpB in sterile NaCl

Day 44: Terminal bleed

Vaccinations were given in 100 μl volumes.

2.9.3 Vaccination of mice

A small group of MF1 mice were vaccinated subcutaneously with 50 μg ml^{-1} of ZmpB (central region) protein using a 21 gauge needle. Mice were scruffed and held firmly to restrict movement. 100 μl of protein emulsified in FCA / FIA was injected into the layer of skin around the neck. Successful vaccination was evident by the formation of a granuloma beneath the top layer of skin, 24 hours post vaccination. Mice were monitored frequently to ensure granulomas did not become exposed to the surface or cause the mice any evident distress. A control vaccination of one mouse was set up using PBS with FCA/FIA.

2.9.4 Bleeds to obtain sera

Blood was taken from mice as described in section 2.5.5. 100 μl was removed and placed on ice. Blood was allowed to clot O/N at 4 ^{o} C. Clotted blood was pelleted by centrifugation at 10,000 rpm and the sera was decanted off and stored at 4 ^{o} C or - 20 ^{o} C until required. For the terminal bleeds, mice were sacrificed by cervical dislocation and the thoracic cavity opened up to expose the heart and lungs. A 23-gauge needle was inserted into the right ventricle of the heart and blood was collected into an attached syringe. Blood was processed as described for the tail bleeds.

2.9.5 Analysis of immune sera for antibodies by Western blot

2.9.5.1 SDS-PAGE

A 12 % SDS polyacrylamide gel was prepared as described below using standard protein gel apparatus (Bio-Rad). An aliquot of frozen ZmpB protein was thawed at RT and mixed with the appropriate volume of 5 x sample buffer. Protein was denatured by heating to 100 ° C for 2 –3 min. 25 µl of sample was added to wells and the gel was electrophoresed at 200 V for 1 hr 30 min. A standard was included with each gel (Kaleidoscope Pre-stained Standards, Bio-Rad). Total concentration of protein loaded per well was 44 µg. For each group of immune sera to be analysed, duplicate gels were set up. One of each gel was used for Western blot and the other was stained with Coomassie Blue to ensure presence of ZmpB protein.

Separating gel:

Stock solution	Volume added for 12 % gel
1.5M Tris-HCl, pH 8.8 (Sigma-Aldrich, UK),	2.5 ml
10 % SDS (Fisher Scientific UK. Ltd.),	100 µl
30 % acrylamide-bis (Bio-Rad Laboratories)	4.0 ml
10 APS (Sigma-Aldrich, UK)	50 µl
TEMED (Sigma-Aldrich, UK)	5 µl
dH_2O	3.35 ml

Stacking gel:

Stock solution	Volume added for 10 % gel
0.5M Tris-HCl, pH 8.8 (Sigma-Aldrich, UK),	1.25 ml
10 % SDS (Fisher Scientific UK. Ltd.),	50 µl
30 % acrylamide-bis (Bio-Rad Laboratories)	665 µl
10 APS (Sigma-Aldrich, UK)	25 µl
TEMED (Sigma-Aldrich, UK)	5 µl
dH_2O	3.05 ml

2.9.5.2 Coomassie Blue staining

Gels were immersed in Coomassie Blue stain for 1-3 hr on a shaking platform (Edmund Bühler KM-2 shaker). Gels were subsequently rinsed several times in dH_2O, immersed in destain for 1-2 hr, rinsed in dH_2O and dried between cellophane support sheets on a GelAir dryer (BioRad).

2.9.5.3 Western Blot

Transfer buffer was cooled to 4 ° C prior to use. The gel was placed in the cooled transfer buffer for 30 min and set up with Hybond™-C super membrane (Nitrocellulose, 0.45 μM, Amersham Life Science) with filter paper and fibre pads within a standard Western blot gel tank (BioRad Mini Protein N™). The tank was filled with cold transfer buffer and the gel blotted at 30 V O/N. The nitrocellulose membrane was subsequently blocked with Tris NaCl, pH 7.4, containing skimmed milk (3 % w/v, Marvel) for 1 hr. Primary antibody (mouse sera) was diluted 1:1000 in Tris NaCl, pH 7.4, containing 3 % skimmed milk, and incubated with the membrane with gentle shaking at 37 ° C for 3 hr. The membrane was washed several times in Tris NaCl, pH 7.4, and transferred to Tris NaCl, pH 7.4, containing 3 % skimmed milk and secondary antibody (sheep anti-mouse, horseradish peroxidase-linked whole antibody, Amersham Life Science) diluted 1:1000. Following incubation with gentle shaking at 37 ° C for 1 hr, the membrane was washed thoroughly in Tris NaCl, pH7.4, and developed in the dark using 4-chloro-1-napthol. Sera samples that were positive for antibody production were pooled, aliquoted and stored at - 20 ° C.

2.10 Expression analysis

Powder-free gloves were worn at all times when preparing RNA or working with microarrays to prevent RNA degradation by RNases present on the skin and to avoid contamination of arrays with dust particles. All glassware used for RNA / microarray work was washed thoroughly with diethyl pyrocarbonate (DEPC)-treated dH20 prior to use and filter tips were used for pipetting all liquids during this work.

2.10.1 Culture of bacterial strains for RNA isolation

Bacterial strains were grown in BHI, in the absence of antibiotic selection, to mid-log phase ($OD_{600\ nm}$ 0.6-0.7). 2 ml aliquots were removed and rapidly frozen in liquid N2. This was to preserve

the cellular RNA profile as quickly as possible. Frozen cultures were stored at - 70 ^{o} C until required.

2.10.2 Isolation of bacterial RNA

Bacterial RNA was isolated using a commercially available kit (Rneasy® Mini Kit, Qiagen) with minor modifications to manufacturer's instructions. All reagents described in this section were provided with the kit, unless stated otherwise.

Aliquots of frozen bacterial cultures (described above) were removed and 8 ml sterile, RNase-free dH_2O was added to each. Once the bacterial stock had thawed in the dH_2O, suspensions were centrifuged at 5000 rpm for 10 min (Sigma 4K15 centrifuge). The supernatant was gently removed and tubes were inverted on a paper towel to remove all final traces of supernatant. 200 µl of freshly-prepared TE buffer, pH 8.0 (see appendix, A4) containing 15 mg ml^{-1} lysozyme (Sigma-Aldrich, UK) was used to re-suspend the bacterial pellet. The suspension was gently mixed by pipetting for 1 min. The resultant mixture was incubated at RT for 10 min with brief vortexing every 2 min. 700 µl of Buffer RLT containing β-mercaptoethanol (10 µl β-mercaptoethanol per 1 ml buffer, added immediately prior to use) was added to lyse the bacteria and mixed by vortexing vigorously. 500 µl of 100 % ethanol was added and mixed by pipetting. Solutions were applied to RNeasy® mini spin columns and centrifuged at 10,000 rpm for 15 sec (Sigma 113 microfuge). 350 µl wash Buffer RW1 was applied to each column and tubes were centrifuged at 10,000 rpm for 15 sec. The flow-through was discarded. DNA was removed by DNase treatment (70 µl Buffer RDD with 10 µl DnaseI stock per column Qiagen). DNase was applied directly onto the RNeasy® membrane and left to incubate at RT for 15 min. The membrane was washed by the addition of 350 µl Buffer RW1 followed by centrifugation of columns at 10,000 rpm for 15 sec. The RNeasy® columns were transferred to new collection tube. 500 µl Buffer RPE was added and columns centrifuged at 10,000 rpm for 15 sec. The flow-through was discarded. 500 µl Buffer RPE was added and columns centrifuged at 10,000 rpm for 2 min. The flow-through was discarded and columns were centrifuged at 10,000 rpm for an extra 1 minute. Columns were transferred to new RNase-free collection tubes and RNA was eluted by the addition of 50 µl sterile, RNase-free dH_20 followed by centrifugation at 10,000 for 1 min.

2.10.3 Determination of RNA concentration

The concentration of isolated bacterial RNA was quantified by electrophoresing 5 µl RNA sample with 1 µl DNA sample buffer on a 1 % agarose gel containing ethidium bromide as described previously. Commercially available RNA of a known concentration (*E. coli* 16S and 23S RNA, 4 U μl^{-1}, Boehringer) was diluted as appropriate and used as a reference for RNA concentration.

2.10.4 Printing of *S. pneumoniae* microarrays

The array was designed based on the 2 pneumococcal genome sequences currently available publicly (TIGR4 and R6, http://www.tigr.org/). Probes were initially designed based on the TIGR4 sequence (as part of a collaboration between T.J. Mitchell, J. Hinds and J. Wells). The 2 genomes were compared to each other using the BLAST algorithm to identify genes present in R6 but absent in TIGR4. These genes were also included on the array to produce a composite array representative of both genomes (2238 TIGR4 genes, 116 R6 genes). Probes were designed using the Primer 3 software. One probe per gene was used and probes were designed to be specific to target genes and to have hybridisation / annealing temperatures as close as possible. This resulted in probes of different lengths, binding to random regions of the target genes. All oligonucleotide sequences were analysed for specificity using the BLAST algorithm.

S. pneumoniae microarrays were printed by the Bacterial Microarray Group at St. Georges Hospital (BµG@S, http://www.sghms.ac.uk/depts/medmicro/bugs/
London, UK).

2.10.5 Hybridisation of RNA to arrays

cDNA hybridisations were performed following the protocols optimised at BµG@S.

2.10.5.1 Cy3/Cy5 labelling of cDNA

One Cy3 and one Cy5 labelled cDNA sample were prepared per microarray slide.

RNA samples were adjusted to equal concentrations by the addition of sterile, RNase-free dH_20 where appropriate. RNA samples containing less than 7 µg μl^{-1} were not used for microarray hybridisation.

10 µl each RNA sample was mixed with 2 µl random primers (3 µg μl^{-1}, Gibco BRL, Life Technologies), heated at 95 ° C, snap cooled on ice and briefly centrifuged. The following were added to each reaction; 5 µl 5 x First Strand Buffer (Gibco BRL, Life Technologies), 2.5 µl DTT (100 mM, Buffer, Gibco BRL, Life Technologies), 2.3 µl dNTPs (5 mM dA/G/TTP, 2 mM dCTP, Gibco BRL, Life Technologies), 1.7 µl Cy3 OR Cy5 (dCTP Fluorolink, Amersham Pharmacia Biotech) and 2.5 µl Superscript II Reverse Transcriptase (200 U μl^{-1}, Gibco BRL, Life Technologies). Reactions were incubated at 25 ° C, for 10 min, followed by 42 ° C for 90 min. Both incubations were carried out in the dark.

2.10.5.2 Pre-hybridisation

The pre-hybridisation solution was equilibrated at 65 ° C in a Coplin jar (Fisher Scientific) during the labelling reaction described above. The microarray slide was incubated in the pre-heated pre-hybridisation solution at 65 ° C for 20 min. The slide was then rinsed in 400 ml dH_20 for 1 min, followed by a rinse in 400 ml propan-2-ol (BDH Laboratories) for 1 min (using slide staining troughs and racks, Fisher Scientific). The slide was placed into a 50 ml falcon tube and dried by centrifugation at 1500 rpm for 5 min.

2.10.5.3 Purification of Cy3/Cy5 labelled cDNA

Following labelling of cDNA with Cy3/Cy5 as described above, reaction mixes were combined into a singe tube and purified using a commercially available kit (MinElute PCR Purification Kit, Qiagen) according to manufacturer's instructions. Labelled cDNA was eluted in 15 µl sterile, RNase-free dH_20.

2.10.5.4 Wash preparation

Wash A was placed in a sealed Duran and incubated O/N at 65 ° C, together with an empty staining trough (Fisher Scientific).

2.10.5.5 Microarray hybridisation with Cy3/Cy5 labelled cDNA

The freshly prepared microarray slide was placed into a hybridisation cassette (Telechem International). 15 µl aliquots of water were added to the wells at either end of the cassette. 4.6 µl of filter sterilised 20 x SSC (0.2 µM filters, Sartorius Minisart, Germany) and 3.5 µl filter

sterilised SDS (20 %) was added to the purified Cy3/Cy5 labelled cDNA. The mixture was heated at 95 ^{o} C for 2 min, allowed to cool slightly and briefly centrifuged. The solution was gently pipetted onto the array and covered with a glass coverslip (BDH Laboratories). The hybridisation cassette was sealed and immersed in a water bath at 65 ^{o} C in the dark for 16-20 hr.

2.10.5.6 Final washing of arrays

Following hybridisation, the microarray slide was removed for the hybridisation cassette and immersed into pre-heated Wash A to remove the coverslip. The array was then washed for 2 min by gentle agitation in Wash A using the staining troughs and slide racks (Fisher Scientific). Arrays were transferred to new racks and washed in Wash B (x2) for 2 min each. The slide was placed into a 50 ml Falcon tube and dried by centrifugation at 1500 rpm for 5 min. Array slides were stored in a dark, dust-free box.

2.10.6 Scanning microarrays

Arrays were scanned using a dual-laser scanner (Affymetrix). Individual arrays were scanned x 2.

2.10.7 Data analysis

Scanned images were imported into Imagene™ (BioDiscovery) for examination and optimisation of raw data. Data analysis was performed using Genespring™ version 4.2.1 (Silicon Genetics) with the *S. pneumoniae* array template installed. Each gene's measured intensity was divided by its control channel value in each sample.

2.10.8 Normalisation of data

The Genespring™ default settings were used for normalisation for the array experiment performed in this thesis.

2.10.9 RT-PCR

2.10.9.1 cDNA synthesis

Reverse transcription reactions were carried out using the ThermoScript™ RT-PCR System (Invitrogen Life Technologies). This system utilises a reverse transcriptase that has high thermal

stability and can increase specificity of reactions. Prior to use, RNA was treated with DNase (RQ1, Promega) according to manufacturer's specifications. cDNA synthesis using random hexamers was performed using the recommended Thermoscript ™ RT-PCR System protocol. This proved more efficient than using gene-specific primers. Control reactions were performed in the absence of reverse transcriptase. Resulting cDNA was treated with 1 µl RNase H (*E. coli*, 2 U μl^{-1}, Invitrogen Life Technologies) at 37 ° C for 20 min, and stored at - 20 ° C until required.

2.10.9.2 PCR

PCR reactions were performed using 2 µl of the cDNA from the reverse transcription reaction described above. This was incorporated into a general PCR reaction as described in section 2.1.6 using PCR conditions optimised to suit individual primer pairs. Negative control reactions were set up using cDNA synthesis reactions carried out in the absence of reverse transcriptase and PCR reactions where cDNA was substituted with dH_2O. A genomic DNA PCR was included as a positive control. Details of all primers used for RT-PCR analysis are provided in the appendix (A3).

2.11 Statistics

2.11.1 Overview

Much of the data presented in this work is based on *in vivo* experiments using a murine model of pneumococcal infection, where the behaviour of selected mutants is compared to that of isogenic parental strain. The model used for the majority of this data is an outbred strain of mouse and thus variation is expected. Statistical analysis is used to determine if any differences observed are significant. This section aims to highlight the parameters that have been applied to allow for the appropriate presentation of data and analysis of the significance of such data. A data set comprising bacterial counts in the blood of mice 24 hours post challenge with a virulent, type 2 strain of *S. pneumoniae* (D39) was selected to demonstrate the variation typically seen and how this was analysed. The data set comprises 50 mice selected at random from the experiments performed during the course of this project and is representative of all data sets obtained in this work. The time point selected represents one in which mice would be expected to be showing symptoms and have detectable bacterial counts within the blood.

2.11.2 Normally distributed measurements

A data set has normal / parametric distribution when a symmetrical histogram is produced. The tip of this plot should correlate approximately with the mean value and +/- 1 SD around the mean should encompass 68 % of the population. Normal data sets can be analysed statistically using tests that take into account mean values, such as t-tests. A histogram-based analysis was applied to 50 MF1s challenged intranasally with 10^6 CFU D39 (following intraperitoneal challenge), to determine if the *in vivo* data sets produced in this work had normal distribution. Mice included in this analysis all became bacteraemic during the course of infection.

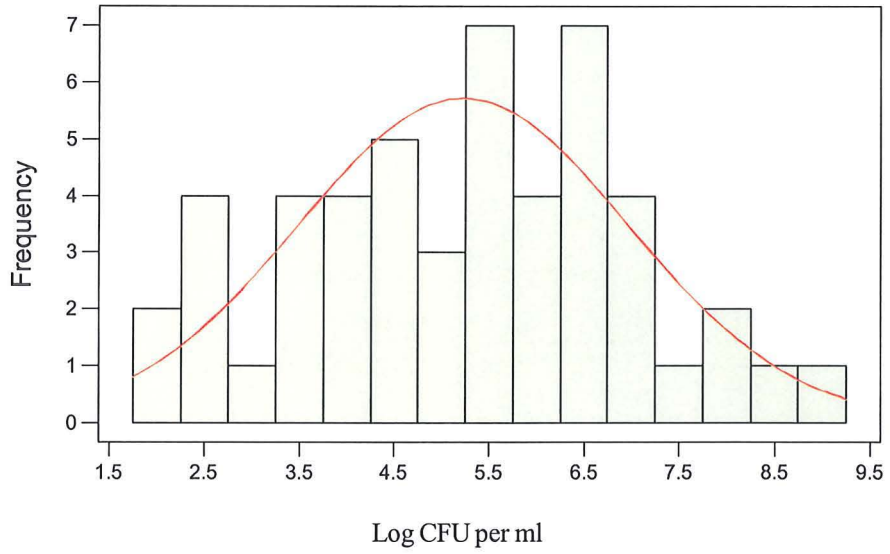


Figure 2.1: D39 bacteraemia, 24 hr post challenge

Histogram showing the spread of bacterial counts in the bloodstream of mice following intranasal challenge with 10^6 CFU D39 bacteria. A normal distribution curve has been superimposed onto the data set using Minitab Software, Release 13.1.

The mean value of Log_{10} 5.4 CFU ml^{-1}, as calculated using basic statistical tests, lies within the peak of the normal distribution curve. 1 +/- SD of the mean (Log_{10} 1.78) includes 69 % of the population, indicating normal distribution. To determine if these data were representative of a normal distribution set a specific test for normality was applied. This test converts the bell shaped normal distribution curve into a straight line. The data points can be observed visually for exactness of fit to this line. Alternatively, and more accurately, a statistical test can be applied to determine if the individual points are significantly different from the normal curve ($P<0.05$). The vertical axis of this plot represents a probability scale and the horizontal axis represents the data scale. A reading of 0.5 on the probability scale should correspond to the mean value of the data set if the data has normal distribution.

Several variations of these normality tests are available and these depend on the shape of the histogram described above. For example, the Ryan-Joiner test is based on curved distributions

whilst a second test, the Anderson-Darling test, is based on a flatter distribution. As the D39 data shown above (figure 2.1) appears to represent a curved distribution, it was analysed for normality using the Ryan-Joiner test. The results of this normality plot are shown below (figure 2.2).

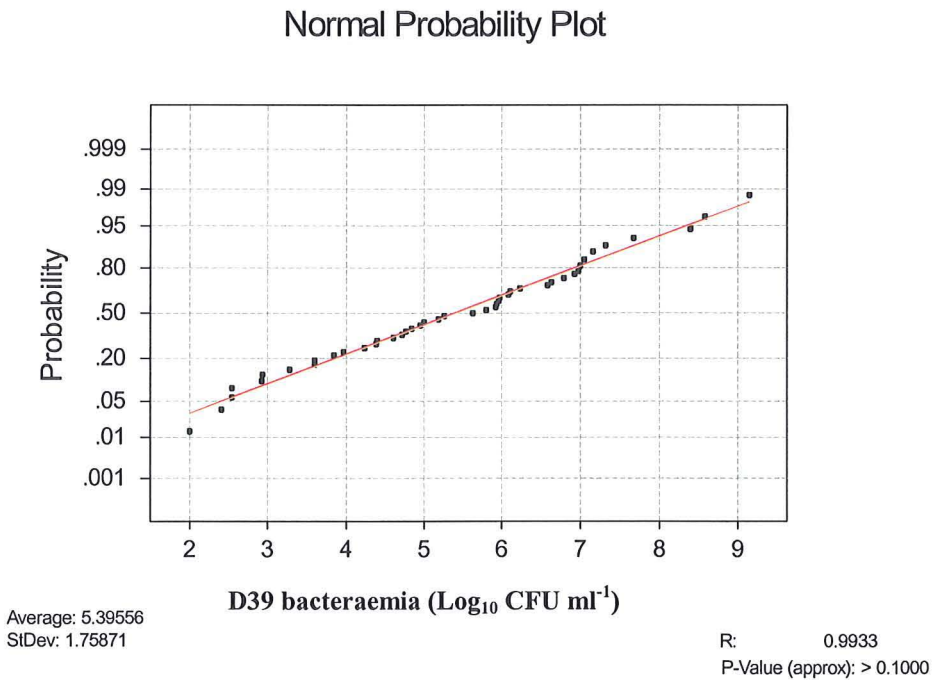


Figure 2.2: The Ryan-Joiner normality test

This test was applied to the D39 data set described in the text to determine normality. Spots represent counts from the blood of individual mice, 24 hr post challenge with 10^6 CFU bacteria. The data is normally distributed.

The Ryan-Joiner normality test showed that the D39 data set was normally distributed as the P value was greater than 0.05 (a P value of <0.05 is indicative of data that are not normally distributed). Furthermore, a value of 0.5 on the probability scale correlated to a value of $\log_{10}$ ~5.3-5.4 CFU ml^{-1} which is in agreement with the mean value of the data set ($\log_{10}$ 5.40 CFU ml^{-1}). This provides further evidence that this data set is normally distributed. This data set also demonstrated normal distribution using the Anderson-Darling test (data not shown).

2.11.3 Non-parametric measurements

The normality test used above demonstrated that the D39 data sets have normal distribution and that mean values can be used to represent these data. However, the data set included only mice that became bacteraemic, and working with an outbred murine model means that this may not always be the case. Outliers in data sets can distort the results, especially when working with small populations (n=5-10) and being an outbred, biological system, outliers are to be expected with our model. Similarily, in comparing two groups of data such as wild-type verses mutant data sets there may be significant differences in the standard deviations between the two groups, making t-tests invalid (this tests relies on the standard deviations between groups being very similar). Non-parametric statistical analysis can overcome these differences by using the median value as the measure of central tendancy. The Mann-Whitney Test is a non-parametric statistical test that can be used to examine differences between 2 random populations. It does not require that the data have a normal distribution or that populations have similar standard deviations. It is based on the null hypothesis that the medians of the two populations are equal. Even if data do show normal distribution, this test of statistical significance can be applied to provide a more stringent basis for detecting differences.

2.11.4 Summary

Since non-parametric analysis is a more stringent determination of significant difference between 2 populations, this statistical analysis will be applied to all *in vivo* data sets used in this work, where mutant behaviour is compared to that of wild type. Mann Whitney Tests will be performed for all data sets and a P value of <0.05, represented by a single asterix (*) will be considered statistically significant. A P value of <0.005 will be considered highly significant and will be respresented with a double asterix (**). Graphical presentation of all data (*in vitro* and *in vivo*) will be based on mean values for all experiments with error bars representing +/- 1 standard error of the mean (SEM). Where logarithmic values are involved the geometric mean will be used to represent data. This will allow the reader to appreciate the error associated with experiments as median values do not have the equivalent of a SD or SEM as measures of variability[1]. This

[1] SEM refers to the estimation of the significance in the deviation of the mean of a sample from the true mean of the population. SD is a measure of variance in a sample. Approximately 68 % of the sample population should lie within +/- 1 SD of the mean

method of presentation should represent results accurately as median values were rarely found to differ substantially from mean values during the course of this work. However, should mean and median values differ for any particular set of data, such that the chosen form of presentation does not accurately represent the results, this will be highlighted and discussed as appropriate[2]. Furthermore, mean values are represented to allow for comparison of results obtained within this thesis concerning the *rr08* and *rr09* mutants with that of published data which also used mean values (Throup *et al.*, 2000).

All statistics were performed using Minitab™ Statistical Software (Release 13, Minitab Inc.). Mann-Whitney Tests were used for the analysis of all *in vivo* data. For all animal work, a minimum of 5 mice were used for all bacterial strains for each time point / measurement examined and experiments were repeated on at least 2 separate occasions. Therefore n = at least 10 for each data point displayed for animal work. Measurements of bacteraemia were continuous, using the same group of infected mice for successive time points. Such mice were also used for analysis of survival. Analysis of bacterial loads in the lung tissue or BALF required that the mice be culled first, and such measurements at different time points are thus from different mice.

2-sample t-tests were used to analyse *in vitro* data and for all microarray analysis of statistical significance. For all *in vitro* work (growth curves, transformation efficiencies etc) assays were performed in replicates of at least 2, and repeated on at least one separate occasion, unless stated otherwise. Therefore n = at least 4 for all *in vitro* analysis.

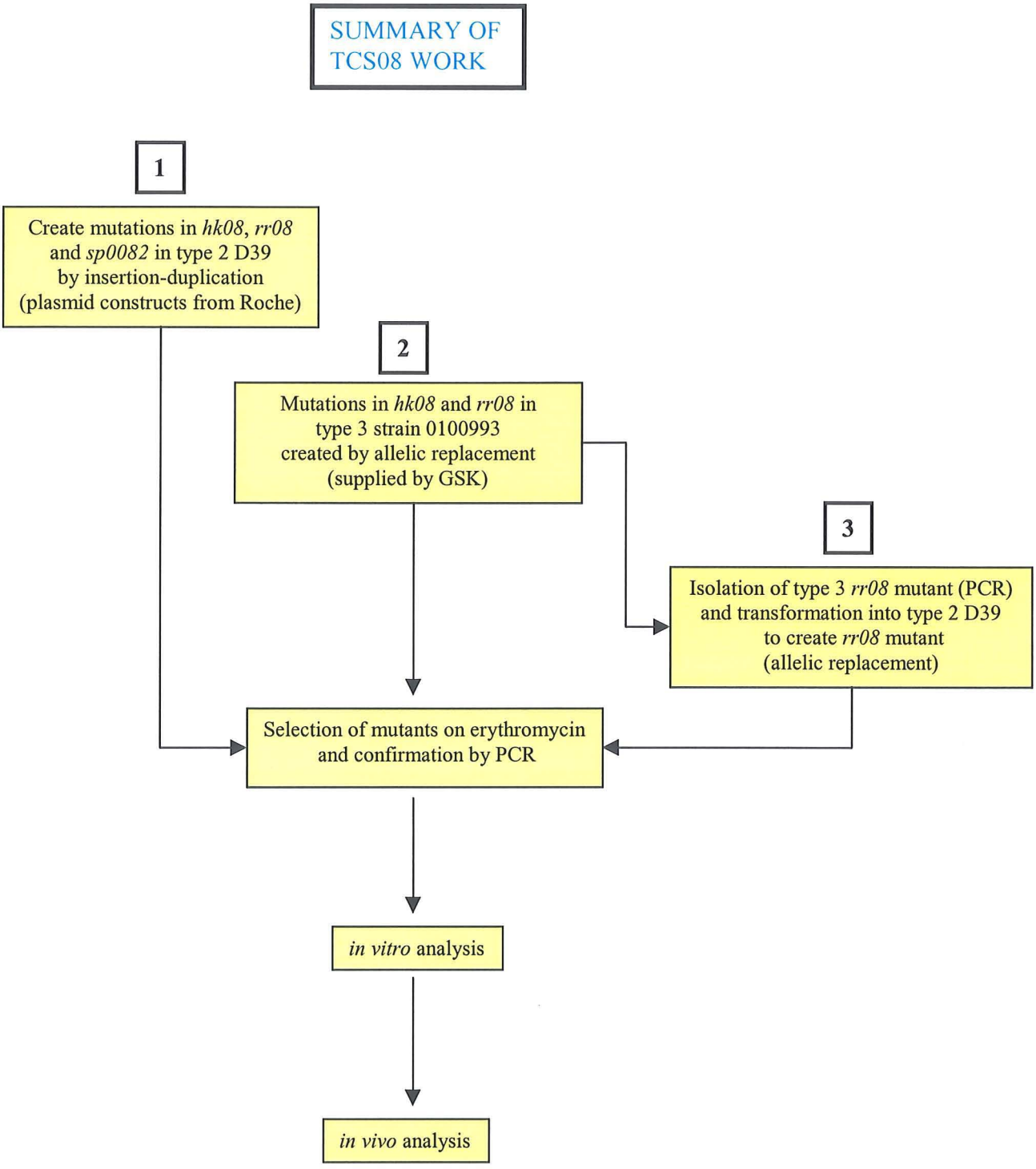
Data points on individual figures represent the mean value of all repetitions and error bars represent 1+/- SEM, unless stated otherwise.

[2] Evaluation of statistical tests and methods discussed in this section was obtained from 'Practical Statistics for Experimental Biologists' by A. C. Wardlaw, John Wiley & Sons Ltd, Second Edition

Chapter 3

Results

S. pneumoniae TCS08


SUMMARY OF TCS08 WORK
1
Create mutations in *hk08*, *rr08* and *sp0082* in type 2 D39 by insertion-duplication (plasmid constructs from Roche)
2
Mutations in *hk08* and *rr08* in type 3 strain 0100993 created by allelic replacement (supplied by GSK)
3
Isolation of type 3 *rr08* mutant (PCR) and transformation into type 2 D39 to create *rr08* mutant (allelic replacement)
Selection of mutants on erythromycin and confirmation by PCR
in vitro analysis
in vivo analysis

3 Results; *S. pneumoniae* TCS08

The study of this system for its role in virulence of *S. pneumoniae* was chosen based on preliminary data that suggested that the system may be important in respiratory tract infection (Hoffmann-La-Roche, personal communication, Throup *et al.*, 2000). Furthermore, expression analysis indicated that the system controls the expression of a single gene of unknown function, *sp0082*, located immediately upstream of the *rr/hk08* locus, indicative of a relatively simple system of gene regulation (Hoffmann-La-Roche, personal communication). Thus the aim of this section was to create mutations in the *sp0082*, *hk08* and *rr08* genes and to analyse the mutants individually for virulence using a murine model of infection. Mutations in the *rr08* gene in 2 different serotypes were created and analysed. The effect of the mutagenesis technique on phenotype was compared.

3.1 Transformation of *S. pneumoniae* and its effect on virulence

The natural ability of the pneumococcus to obtain DNA from the extracellular environment and incorporate it into its genome (transformation or competence), and the availability of several pneumococcal genome sequences can be exploited to create mutations in virtually any gene of choice in *S. pneumoniae*. Much of the experimental data presented in this thesis are based on the construction of different mutations using several mutagenesis techniques, all of which rely on the transformation of parental *S. pneumoniae* cells with different DNA constructs. Resulting mutants are compared with isogenic parental strains to characterise any differences in phenotypes, both *in vitro* and *in vivo*. To ensure that any differences observed in the mutant strains are due to the mutation itself and not the transformation procedure, the virulent, encapsulated serotype 2 pneumococcal strain D39 was transformed with a control plasmid pVA838 and compared to a non-transformed strain for *in vitro* growth and virulence. pVA838 contains an erythromycin-resistance gene, which acts as a selection marker for transformants, and is able to replicate in *S. pneumoniae*.

3.1.1 *In vitro* growth

Growth of the pVA838-transformed D39 strain (tD39) in BHI was compared to that of non-transformed D39 (D39) for a period of 20 hr. During this time, no differences in growth were observed between the 2 strains. The tD39 strain also retained its erythromycin resistance when grown in culture in the absence of antibiotic selection for up to 20 hr (data not shown).

3.1.2 *In vivo* characterisation

Mice were challenged intranasally with 10^6 CFU of both tD39 and D39 and monitored for survival. Levels of bacteraemia in the blood were measured at pre-determined time points following challenge. No significant differences were observed between tD39 and D39 for murine survival or levels of bacteraemia in the blood at any of the time points studied, see figure 3.1.

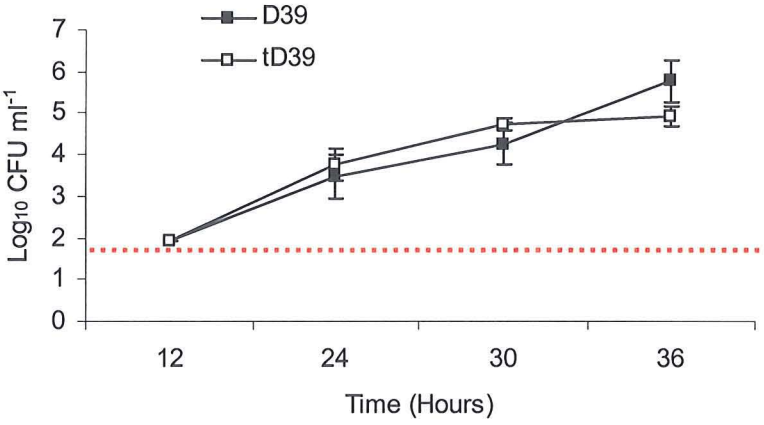


Figure 3.1 Bacteraemia following intranasal challenge with D39 and tD39

Mice were challenged intranasally with 10^6 CFU D39 wild-type and tD39, a strain that has been transformed with plasmid pVA838. Bacterial counts in the blood of infected mice were monitored at pre-determined time points. The broken line represents the limit of detection.

To ensure that the bacteria counted in the blood for the tD39 strain were not wild-type D39 revertants that had lost the plasmid during growth, the blood collected at the 36 hr time point was plated onto blood plates in the presence and absence of erythromycin. Viability counts were compared and this illustrated that there was no reduction in bacterial counts when the tD39 strain was counted in the presence or absence of antibiotic (figure 3.2). Thus tD39 retains its pVA838 plasmid for up to 36 hr following intranasal challenge and is not impaired in virulence compared

to wild-type D39. This provides evidence that the transformation protocol used to produce the mutants discussed in this thesis does not result in attenuated virulence.

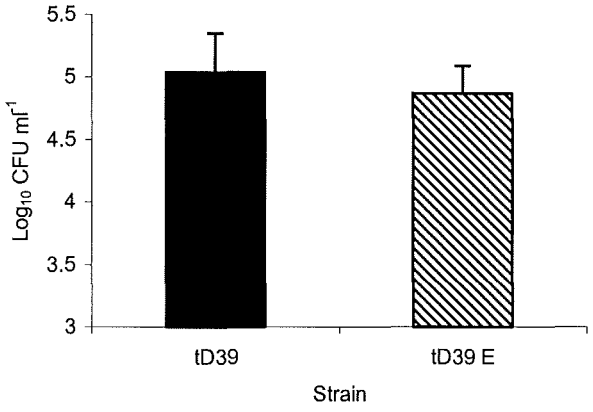


Figure 3.2 Stability of tD39 following intranasal challenge

Mice were challenged intranasally with 10^6 tD39. At 36 hr post infection cell viability counts were performed in the presence (tD39 E) and absence (tD39) of erythromycin (1 μg ml^{-1}). No significant difference in bacterial counts were found between the 2 strains.

3.2 Insertion-duplication mutagenesis; Basic *in vitro* analysis

3.2.1 Creating and confirming mutants in *hk08*, *rr08* and *sp0082*

Plasmid vectors containing a region of DNA homologous to the target gene for mutagenesis were constructed by Lange *et al* (Hoffmann-La-Roche) and supplied to our laboratory (see appendix). Upon transformation into pneumococcal cells the plasmids integrate into the target gene by homologous recombination. The constructs contain a gene encoding erythromycin resistance, which acts as a selective marker for chromosomal integration of the plasmid. The plasmid constructs are suicide vectors that are unable to replicate in *S. pneumoniae* and are eliminated from the cell unless they integrate into the chromosome.

Constructs designed to integrate into the *hk08*, *rr08* and *sp0082* genes were transformed into the virulent serotype 2 D39 strain of *S. pneumoniae*. Transformants were selected on erythromycin and each was confirmed using PCR. The resulting mutants were named based on the constructs provided by Hoffmann-La-Roche to create the mutants (see appendix). Thus the *hk08*, *rr08* and *sp0082* mutants will be referred to as *ΔpRPL7*, *ΔpAS1* and *ΔpRPL50*, respectively in all subsequent work.

To confirm the individual mutants, the 5' and 3' plasmid integration sites into the target genes were confirmed using a chromosome-specific primer and a plasmid-specific primer for each PCR (figure 3.3). This resulted in a band present in the mutant but absent in the wild-type parental strain for each integration site. For each mutant, the PCR product confirming the 5' integration site was sequenced. The chromosome-specific primers, or other primers flanking the target gene, can also be used together to amplify a band from the wild-type strain that is absent in the mutant (due to the large amount of plasmid DNA inserted between the primer sites) if PCR conditions are optimised (data not shown). This PCR also confirms that a lack of band in the wild-type strain when confirming the 5' and 3' integrations is not due to poor genomic DNA template.

All insertion-duplication mutants were confirmed using the PCRs described above. Figure 3.4 shows confirmation of the *ΔpRPL7* mutant only, using PCR to confirm the 5' and 3' integration sites only. All other mutants were confirmed in the same way.

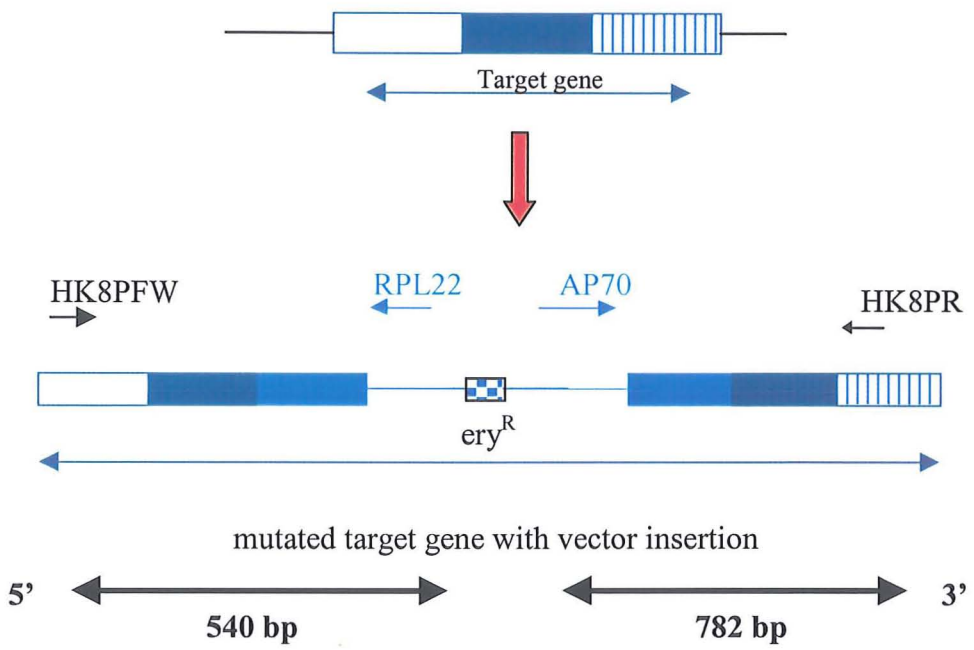


Figure 3.3 Confirmation of insertion-duplication mutants

The figure illustrates the insertion of an entire plasmid construct (shown in light blue) into the target gene (dark blue) by insertion-duplication mutagenesis. The diagram is modified from figure 1.4 (introduction, section 1.7), which should be referred to for a more detailed description of the mutagenesis technique. To ensure the integration of the construct into the target site, PCR is used to confirm the 5' and 3' plasmid / chromosome junctions. The figure shows the primers used to confirm the *ΔpRPL7* mutant (mutation within *hk08*), although the same principle was used to confirm all insertion-duplication mutants. Primers HK8PF and RPL22 were used to confirm the 5' integration site, with an expected product size of ~ 540 bp. Primers AP70 and HK8PR were used to confirm the 3' integration site, with an expected product size of ~782 bp. Plasmid-specific primers (RPL2 and AP70) are shown in light blue and chromosome-specific primers (HK8PF and RPL22) are shown in black.

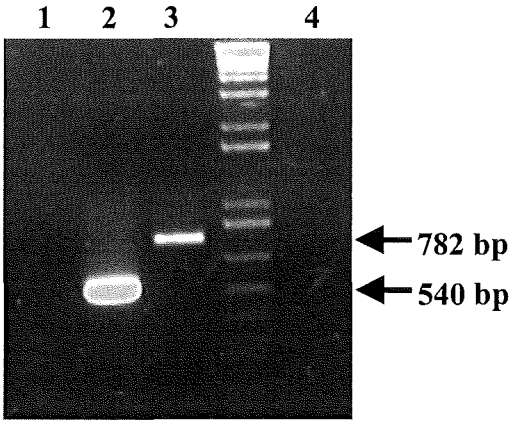


Figure 3.4 Confirmation of the *ΔpRPL7* mutant by PCR

PCR was used to confirm the 5' and 3' integration sites of the *ΔpRPL7* mutant. The expected PCR fragments for the 5' and 3' integration sites using the primer sets described in figure 3.3 are 540 bp and 782 bp, respectively. The absence of these bands in the wild-type genomic DNA is shown (lanes 1 and 4, respectively). Lane 1; D39 wild-type with primers specific for 5' integration site. Lane 2; *ΔpRPL7* mutant with primers specific for 5' integration site. Lane 3; *ΔpRPL7* mutant with primers specific for 3' integration site. Lane 4; D39 wild-type with primers specific for 3' integration site.

3.2.2 Growth and autolysis *in vitro*

All strains were subject to growth and autolysis determination using a combination of viable cell enumeration and optical density readings. None of the mutants displayed growth or autolysis characteristics that were significantly different from the wild-type parental strain. Furthermore, all mutants retained their erythromycin resistance when grown in culture in the absence of antibiotic selection for up to 24 hr, demonstrating that the plasmid insertion is stable under these conditions (data not shown). Growth of the *ΔpAS1* (*rr08*) mutant only, as determined by viable cell counting, is shown in figure 3.5, and is representative of growth of all the other mutants. The stages of growth characteristic of all pneumococcal strains used in this thesis are illustrated in figure 3.5. Phases characteristic of *S. pneumoniae* growth *in vitro* include exponential phase which represents rapid growth where the bacteria divide logarithmically and stationary phase where the growth rate is balanced by the number of dying cells. Following stationary phase, pneumococci autolyse through activation of the major autolysin LytA (autolysis).

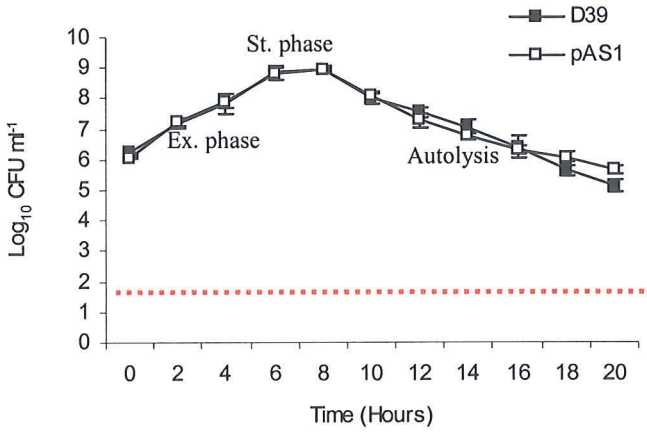


Figure 3.5 *In vitro* growth of *ΔpAS1*

15 ml BHI was inoculated with 1.0 x 10^6 CFU D39 or *ΔpAS1* mutant. Viable cell enumeration was performed every 2 hr for 20 hr. The mutant strain was grown in the absence of erythromycin selection, being shown to be stable under such conditions in a previous experiment. No difference in growth or autolysis was observed with the mutant compared to D39 parental strain. Exponential phase (Ex. phase), stationary phase (St. phase), and autolysis are represented on the curve. The broken line represents the limit of detection.

3.3 Insertion-duplication mutagenesis; *In vivo* characterisation

Prior to all subsequent *in vivo* work, all bacterial strains were mouse-passaged by intraperitoneal injection.

3.3.1 Survival and bacteraemia following intranasal challenge

Strains were analysed for their ability to induce disease in a murine model of pneumonia. Outbred MF1 mice were challenged intranasally with 10^6 CFU. Following challenge, none of the mutants were significantly attenuated in virulence compared to the isogenic parental D39 strain, as determined by survival and bacterial counts in the blood. Median survival times of mice infected with all mutants were all very similar, with the exception of the *ΔpRPL7* mutant, which had a median survival time of 95 hr compared to 54 hr with D39 wild-type (figure 3.6A). However, this value was not found to be statistically different to that of wild-type parental strain. The median counts within the blood of mice challenged with the *ΔpRPL7* mutant were also consistently lower than those of mice challenged with wild-type bacteria, being > 100-fold lower than wild-type at 36 hr post infection (figure 3.6B). Again, these differences were not found to be statistically different.

Following challenge with the *ΔpAS1* and *ΔpRPL50* mutants, the median survival times and counts in the blood were no different to those seen with wild-type D39 challenge. Survival and bacteraemia for these mutants compared to the isogenic parental strain are shown in figures 3.7 and 3.8.

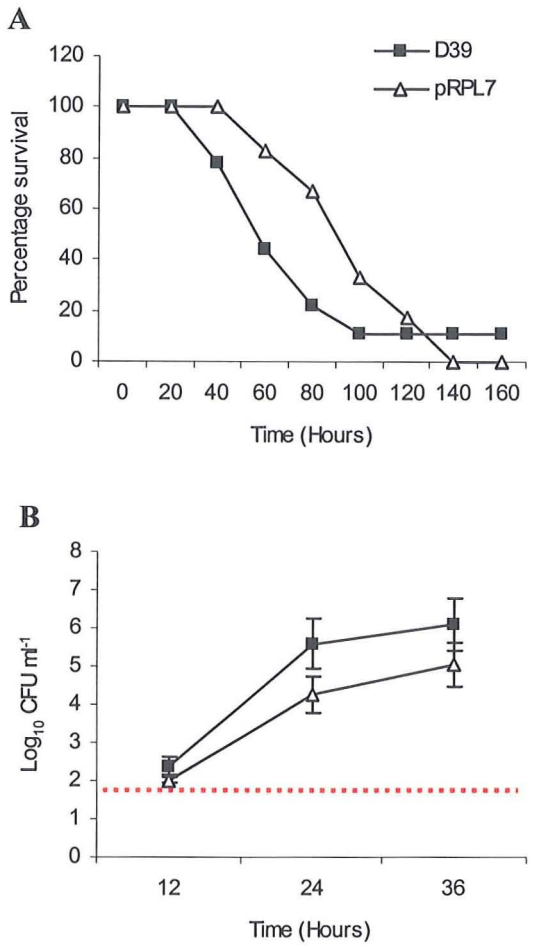


Figure 3.6 Survival and bacteraemia following intranasal challenge with *ΔpRPL7*

Mice were challenged intranasally with 1.0 x 10^6 CFU bacterial strains. Figure 3.6A shows survival of the mice. Figure 3.6B shows bacteraemia at 12 hr, 24 hr and 36 hr post infection. No significant differences were observed between survival and bacteraemia with the *ΔpRPL7* mutant compared to mice challenged with D39 wild-type. The broken line represents the limit of detection for determination of bacteraemia.

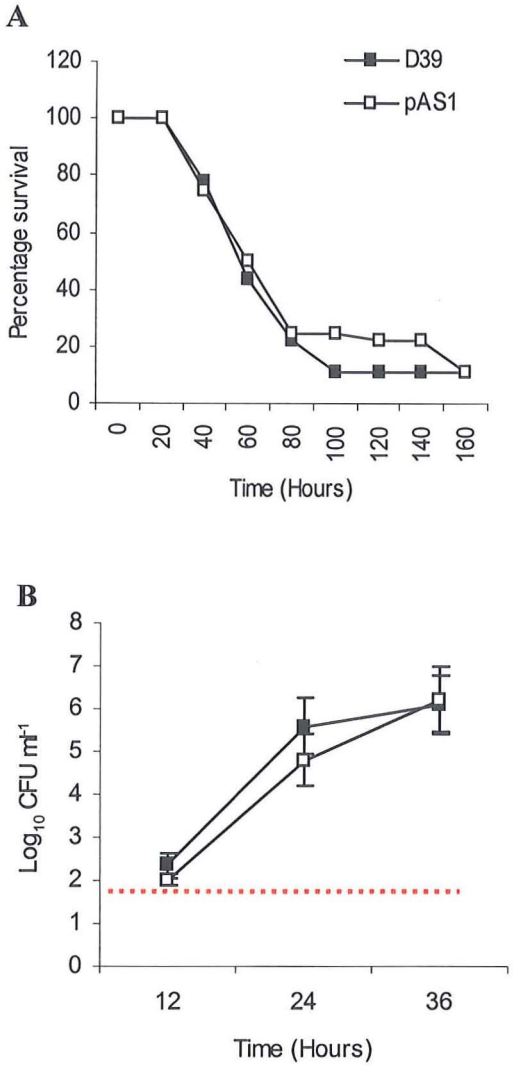


Figure 3.7 Survival and bacteraemia following intranasal challenge with *ΔpAS1*

Mice were challenged intranasally with 1.0 x 10^6 CFU bacterial strains. Figure 3.7A shows survival of the mice. Figure 3.7B shows bacteraemia at 12 hr, 24 hr and 36 hr post infection. No significant differences were observed between survival and bacteraemia with the *ΔpAS1* mutant compared to mice challenged with D39 wild-type. The broken line represents the limit of detection for determination of bacteraemia.

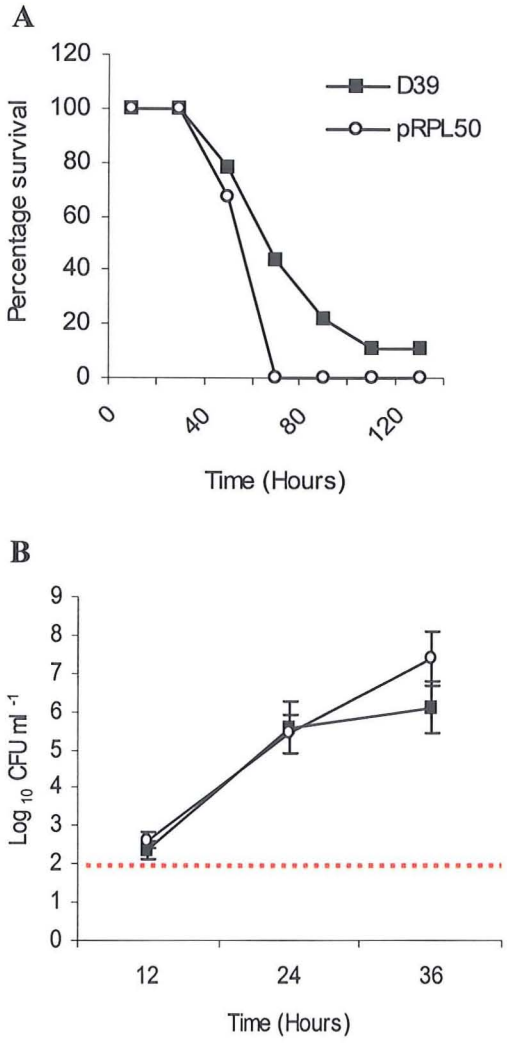


Figure 3.8 Survival and bacteraemia following intranasal challenge with *ΔpRPL50*

Mice were challenged intranasally with 1.0 x 10^6 CFU bacterial strains. Figure 3.8A shows survival of the mice. Figure 3.8B shows bacteraemia at 12 hr, 24 hr and 36 hr post infection. No significant differences were observed between survival and bacteraemia with the *ΔpRPL50* mutant compared to mice challenged with D39 wild-type. The broken line represents the limit of detection for determination of bacteraemia.

3.3.2 Lung counts following intranasal challenge

Bacterial counts within the lung tissue of mice challenged intranasally with 10^6 CFU were determined 24 hr post challenge. No differences were see between bacterial loads in mice infected with all mutants compared to the isogenic parental strain.

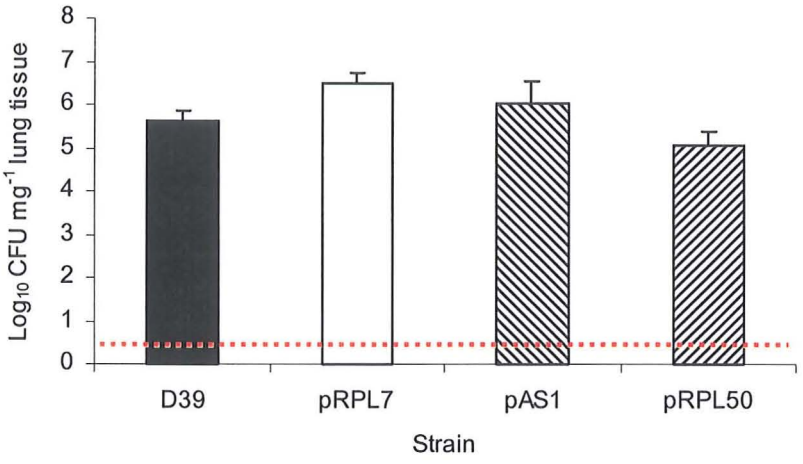


Figure 3.9 Bacterial load in the lung tissue 24 hr post intranasal infection

Mice were challenged intranasally with 1.0 x 10^6 CFU with bacterial strains. 24 hr post infection, bacterial counts in the lung tissue were determined. No significant difference was observed in counts of mice challenged with any of the mutants compared to D39 wild-type. The broken line represents the limit of detection.

3.3.3 Intranasal challenge with increased challenge dose; Survival and bacteraemia

Mice were challenged intranasally with 1.0 x 10^7 CFU, a 10-fold increase in bacterial dose administered compared to previous challenges, to determine if this would show any differences in virulence of the mutants compared to D39 wild-type. Mice were monitored for survival and bacteraemia as described above.

Following intranasal challenge with the *ΔpRPL7* mutant, mice survived significantly longer than those challenged with the wild-type parental strain ($P<0.05$). However, no difference in levels of bacteraemia were observed between mice infected with wild-type or mutant strains. No differences in survival or bacteraemia of the *ΔpAS1* or *ΔpRPL50* mutants were observed compared to wild-type D39 following intranasal challenge with 10^7 CFU. Comparison of the *ΔpRPL7* and *ΔpRPL50* mutants to D39 wild-type at 36 hr was not possible as many of the mice challenged with wild-type bacteria had succumbed to infection by this stage. Similarly, some

mice challenged with the *ΔpAS1* mutant had also succumbed to infection by 36 hr. Although the median counts in the blood of mice challenged with *ΔpRPL50* at 36 hr post infection were almost 2 orders of magnitude higher than bacterial counts in the blood of mice challenged with the *ΔpRPL7* mutant at the same time point, this difference was not found to be statistically significant. Table 3.1 summarises the data from this set of challenges.

	Median survival (Hours)	Median counts in the blood, 12 hr (Log_{10} CFU ml^{-1})	Median counts in the blood, 24 hr (Log_{10} CFU ml^{-1})	Median counts in the blood, 36 hr (Log_{10} CFU ml^{-1})
D39	48	2.88	6.52	N/D
ΔpRPL7	65*	2.28	5.74	5.86
ΔpAS1	49	2.17	6.0	N/D
ΔpRPL50	48	1.92	5.4	7.64

Table 3.1 Survival and bacteraemia following intranasal challenge with 10^7 CFU

Mice were challenged intranasally with 1.0 x 10^7 CFU bacterial strains. The table shows median survival of mice and median bacterial counts in the blood at pre-determined time points following infection. A statistical difference in survival was found for the *ΔpRPL7* mutant only, when compared to wild-type D39 ($P<0.05$*). No significant differences were found for bacteraemia in mice challenged with any of the mutant bacteria compared to mice challenged with D39 wild-type at any time point examined. Counts in the blood for wild-type D39 and the *ΔpAS1* mutant at 36 hr post challenge have not been included in the table as some mice had succumbed to infection by this stage (ND).

3.4 Allelic-replacement mutagenesis; Type 3 mutants

Data published by during the course of this work indicated that a mutation in the *rr08* gene in a serotype 3 pneumococcus resulted in an attenuated phenotype following intranasal challenge (Throup *et al.*, 2000, GlaxoSmithKline). The mutants in this study were created using allelic-replacement mutagenesis (see introduction, section 1.7.2 for a more detailed description of this mutagenesis technique) and were selected using erythromycin. The study used type 3 parental strain 0100993, and administered an intranasal challenge dose of 1.0 x 10^7 to 5 wk old, male CBA/J mice. Following this challenge, CBA/J mice infected with the type 3 *rr08* mutant (*Δ488rr*) had mean counts in the lung tissue that were >1000-fold lower than counts in the lung

tissue of mice challenged with wild-type bacteria (Throup *et al.*, 2000). The mutant and parental strains used for the above study, together with a mutation in the *hk08* gene (*Δ488hk*) were obtained from GlaxoSmithKline for further analysis in our laboratory, and to determine if the reported attenuation in virulence could be repeated using our model of infection. Growth, autolysis and stability were determined prior to any animal work, and both mutants did not differ from the wild-type parental strain for any of these parameters (data not shown).

3.4.1 Intranasal challenge with type 3 mutants; Survival and bacteraemia

The parental 0100993 type 3 strain had not been previously assessed in our model of infection so a dose response curve was set up to determine the optimum challenge dose for subsequent infections. Groups of mice were challenged with 10^5, 10^6 and 10^7 of passaged 0100993 parental strain and monitored for bacteraemia and survival (data not shown). Only the latter dose resulted in 100 % mortality of mice, and this was the dose chosen for all subsequent *in vivo* work using the type 3 strains. Thus the dose administered to mice in this thesis matches that used in the work by Throup *et al.*

MF1 mice were infected intranasally with 10^7 CFU 0100993 wild-type and the *Δ488hk* and *Δ488rr* mutants, and monitored for survival and bacteraemia. Following challenge, mice infected with both mutants had highly significantly increased survival times compared to mice infected with wild-type 0100993 ($P<0.005$ for both mutants). Mice challenged with 0100993 parental strain had a median survival time of 71 hr. Mice challenged with the *Δ488hk* and *Δ488rr* mutants had median survival times of 99 and 156 hr respectively. Figure 3.10 shows the *in vivo* data for all type 3 strains examined in this work.

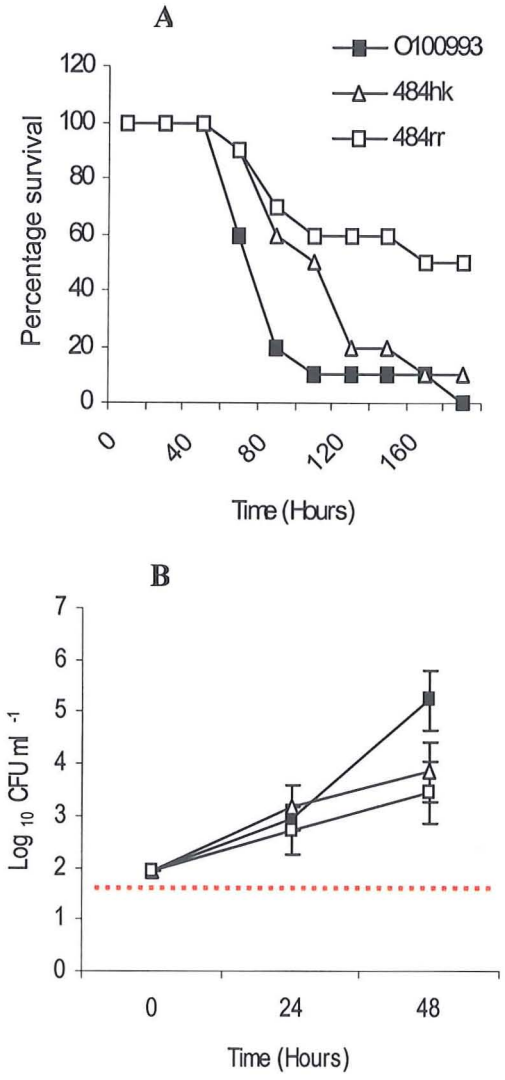


Figure 3.10 Survival and bacteraemia following intranasal challenge with type 3 *Δ484hk* and *Δ484rr* mutants

Mice were challenged intranasally with 1.0 x 10^7 CFU bacterial strains. Figure 3.10A shows survival of the mice. Both mutants resulted in highly significant increases in murine survival time compared to infection with the isogenic 0100993 parental strain ($P<0.005$). Figure 3.10B shows bacteraemia at 24 hr and 48 hr post challenge. No significant differences were observed with bacteraemia in mice challenged with mutant bacteria compared to mice challenged with 0100993 wild-type. The broken line represents the limit of detection for determination of bacteraemia.

3.4.2 Intranasal challenge with type 3 mutants; Lung counts

Following intranasal challenge with 10^7 CFU type 3 strains, bacterial counts within the lung tissue were determined 48 hr post challenge. Although the bacterial counts in the lung for the *Δ488hk* and *Δ488rr* mutants were shown to be approximately 10-100-fold lower than those of mice infected with wild-type bacteria, these differences were not found to be statistically significant (figure 3.11).

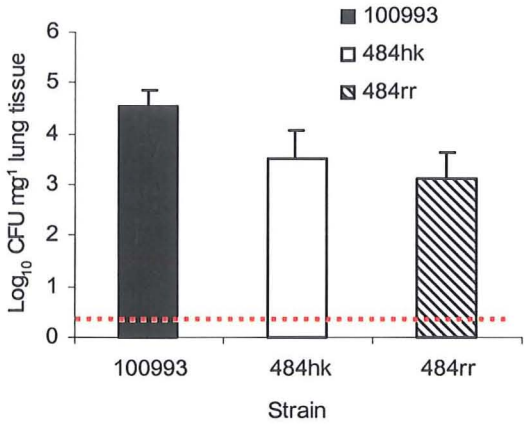


Figure 3.11 Bacterial load in the lung tissue 48 hr post intranasal infection with type 3 strains
Mice were challenged intranasally with 1.0 x 10^7 CFU with bacterial strains. 48 hr post infection, bacterial counts in the lung tissue were determined. No significant difference was observed in counts of mice challenged with either of the mutants compared to mice infected with the 0100993 wild-type. The broken line represents the limit of detection.

In the study by Throup and colleagues, intranasal challenge with 10^7 CFU of the *Δ488rr* mutant resulted in mean counts in the lung tissue that were >1000-fold lower than those found in the lungs of mice challenged with the wild-type parental strain, 0100993 (Throup *et al.*, 2000, no P values to determine statistical significance were provided). In this thesis, intranasal challenged with the same strains used by Throup and colleagues resulted in mean counts in the lung that were only 20-fold lower than those in the lungs of mice challenged with wild-type bacteria. This difference was not statistically significant.

3.4.3 Intranasal challenge with type 3 mutants; CBA/J mice

Data presented in this thesis indicate a role in virulence for RR08 in a type 3 pneumococcal background, as shown by increased survival times of mice infected with the mutant compared to wild type bacteria. Lower counts of mutant bacteria in lung tissue were also found 48 hr post challenge, compared to wild-type although the degree of attenuation did not match that reported by Throup and co-workers (Throup *et al.*, 2000), despite using the same bacterial strains and the same challenge dose. Thus, the only other factor that was different between the work of Throup and co-workers. and the work presented in this thesis was the murine model of infection used. To determine if the different strain / age / sex of mice used were responsible for the differences observed, CBA/J mice (age and sex matched to those used by Throup *et al.*, 2000) were used to repeat the work described above. Unfortunately, the colony of mice from which this strain was derived had respiratory tract infections with *Proteus spp.*, which would have interfered with the proposed experiments. Attempts were made to breed a stock of CBA/Js from *Proteus*-infected parents that were treated with antibiotic (Borgal® 0.01 % final concentration) to clear the infecting organisms, but the second-generation offspring still showed evidence of swarming bacteria representative of *Proteus spp.* when the lungs were homogenised and plated onto blood plates. This work was subsequently abandoned.

3.5 Allelic-replacement mutagenesis; Type 2 mutants

3.5.1 Creating and confirming mutants

A mutation in the *rr08* gene was constructed in serotype 2 D39 by allelic-replacement to determine if the technique used to create mutants altered the phenotype of the resulting mutants. Primers flanking the type 3 *Δ488rr* mutant (484P1 and 484P4) were used to transfer the mutated gene from a type 3 background to the D39 type 2 background, by transformation. Potential transformants were selected on erythromycin. PCR, using primers 484P1 and 484P4 flanking *Δ488rr*, were used to confirm the new type 2 mutant. The resulting mutant, where the *rr08* gene was replaced with a cassette encoding erythromycin resistance, produced a PCR product ~ 300 bp larger than that of wild-type D39 as expected, see figure 3.12. The PCR product was sequenced to further confirm replacement of the *rr08* gene This mutation will be referred to as *Δrr08* from this point onwards.

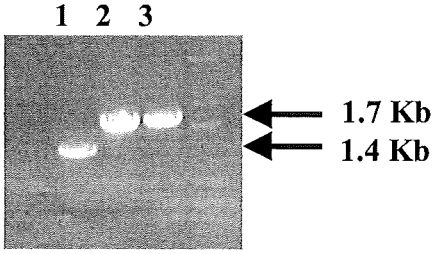


Figure 3.12 Confirmation of the *Δrr08* mutation by PCR

Primers 484P1 and 484P4 were used to confirm the replacement of the *rr08* gene in D39 with a gene encoding erythromycin resistance. Replacement of the target gene resulted in a PCR product ~ 300 bp larger in the mutant than that of the wild-type strain. The figure shows the expected band sizes of 1.7 Kb and 1.4 Kb for the mutant and wild-type strains, respectively. Lane 1, genomic DNA from wild-type D39. Lane 2, genomic DNA from *Δrr08* mutant. Lane 3, genomic DNA from the type 3 *Δ484* mutant (used as a control).

3.5.2 Intranasal challenge; Survival and bacteraemia

MF1 mice were challenged intranasally with 10^6 CFU of D39 and the confirmed *Δrr08* mutant and monitored for their ability to survive infection. Bacteraemia at pre-determined time points was also monitored. Following challenge, mice infected with the *Δrr08* mutant showed increased survival (> 2-fold) compared to mice infected with wild-type D39 (median survival times 89 hr and 41 hr, respectively), although this difference was not found to be statistically significant (figure 3.13A). No significant difference was observed in bacterial counts in the blood between the mutant and its isogenic parental strain at 12 hr, 24 hr or 36 hr post infection (figure 3.13B)

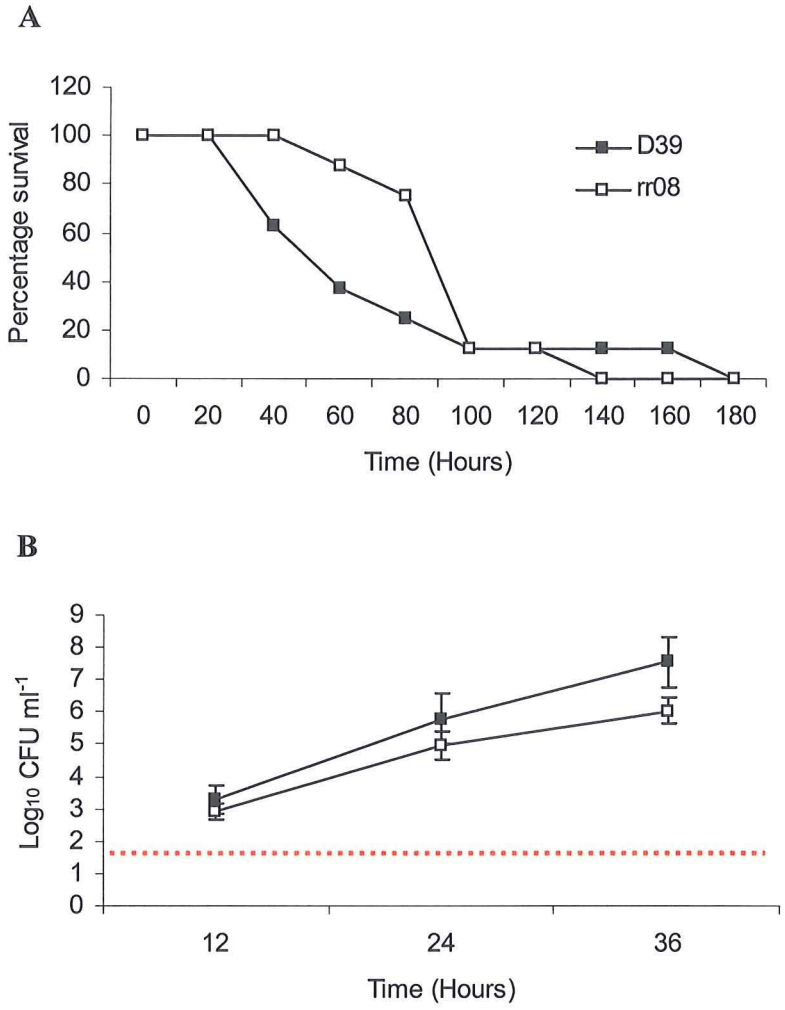


Figure 3.13 Survival and bacteraemia following intranasal challenge with *Δrr08*

Mice were challenged intranasally with 1.0 x 10^6 CFU *Δrr08* and D39 wild-type. Figure 3.13A shows survival of mice. Figure 3.13B shows bacteraemia at 12 hr, 24 hr and 36 hr post challenge. No significant differences were observed between survival or bacteraemia in mice challenged with mutant bacteria compared to mice challenged with D39 wild-type. The broken line represents the limit of detection for determination of bacteraemia.

3.6 Summary of TCS08 results

3.6.1 Effect of transformation on virulence

D39, the parental strain used for much of the work in this section, was transformed with a control plasmid, pVA838, conferring erythromycin resistance. The resultant strain, tD39, was not impaired in growth in BHI *in vitro*, nor was it impaired in virulence following intranasal challenge, as determined by bacteraemia at 12 hr, 24 hr and 36 hr post challenge, or murine survival. Furthermore, viable counts from tD39 obtained from the blood of infected mice, 36 hr post challenge showed that the erythromycin resistance was retained at this time point.

3.6.2 Creating mutants and *in vitro* analysis

Initially mutations in the genes encoding the HK and RR of TCS08, together with the upstream putative surface protein, Sp0082, were created by insertion duplication mutagenesis, using D39 serotype 2 as the parental strain. Mutations in the *hk08* and *rr08* genes, created by allelic replacement mutagenesis in a serotype 3 parental strain, 0100993, were also supplied to our laboratory by GlaxoSmithKline. Subsequently a new mutation in the *rr08* gene of D39 was created by allelic-replacement, by moving the serotype 3 mutant into the serotype 2 D39 by transformation. All mutants were selected on erythromycin and confirmed by PCR and sequencing. All mutants had no growth or autolysis defects when grown *in vitro* in BHI compared to the isogenic parental strains (data shown for *ΔpAS1* mutant only). Furthermore, mutants were stable and retained their erythromycin resistance in the absence of antibiotic selection for up to 12 hr (data not shown).

3.6.3 *In vivo* analysis of virulence; Bacteraemia and survival

All mutants were analysed in a murine model of infection for bacteraemia and survival following intranasal challenge. Some of the mutants were also analysed for their ability to colonise lung tissue. Table 3.2 summarises some of the main observations from this work.

Gene	Parental strain	Mutagenesis technique	Challenge Dose	Median survival (WT)	Attenuation in virulence
RR08 (pAS1)	D39 (type 2)	Insertion-duplication	10^6 CFU	60 hr (54 hr) 1.1-fold longer than wild-type	No significant difference for bacteraemia (12, 24 & 36 hr) or survival No significant difference in lung counts at 24 hr
HK08 (pRPL7)	D39 (type 2)	Insertion-duplication	10^6 CFU	95 hr (54 hr) 1.8 fold longer than wild-type	No significant difference for bacteraemia (12, 24 & 36 hr) or survival No significant difference in lung counts at 24 hr
sp0082	D39 (type 2)	Insertion-duplication	10^6 CFU	45 hr (54 hr) < wild-type	No significant difference for bacteraemia (12, 24 & 36 hr) or survival No significant difference in lung counts at 24 hr
RR08 (pAS1)	D39 (type 2)	Insertion-duplication	10^7 CFU	49 hr (48 hr) 1.02 fold longer than wild-type	No significant difference for bacteraemia (12, 24 & 36 hr) or survival Lung counts not done
HK08 (pRPL7)	D39 (type 2)	Insertion-duplication	10^7 CFU	65 hr (48 hr) 1.4 fold longer than wild-type	No significant difference for bacteraemia (12, 24 & 36 hr) **Significant difference in survival *** Lung counts not done
sp0080	D39 (type 2)	Insertion-duplication	10^7 CFU	48 hr (48 hr) No different to wild-type	No significant difference for bacteraemia (12, 24 & 36 hr) or survival Lung counts not done
RR08 (*Δ484rr*)	0100993 (type 3)	Allelic-replacement	10^7 CFU	156 hr (71 hr) 2.2 fold longer than wild-type	No significant differences in bacteraemia (24 & 48 hr) **Significant difference in survival **** No significant difference in lung counts at 48 hr
HK08 (*Δ484hk*)	0100993 (type 3)	Allelic-replacement	10^7 CFU	99 hr (71 hr) 1.4 fold longer than wild-type	No significant differences in bacteraemia (24 & 48 hr) **Significant difference in survival **** No significant difference in lung counts at 48 hr
RR08 (*Δrr08*)	D39 (type 2)	Allelic-replacement	10^6 CFU	89 hr (41 hr) 2.2 fold longer than wild-type	No significant difference in bacteraemia (12, 24 & 36 hr) or survival No significant difference in lung counts at 24 hr

Table 3.2 Summary of *in vivo* work with all TCS08 mutants

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Table 3.2 Summary of *in vivo* work with all TCS08 mutants

The table summarises the main conclusions following intranasal challenge with the TCS08 mutants. The common gene name is provided along with the name to which mutants are referred within this thesis, and the parental strain in which the mutant is created. The method of mutagenesis and challenge dose are shown in columns 3 and 4. Column 5 illustrates the median survival time of mice infected with the mutants, with the survival time of mice challenged with isogenic parental given in brackets for comparison. Although mutants *ΔpRPL7*, *ΔpAS1* and *ΔpRPL50* have the same parental strain as mutant *Δrr08*, the latter has a slightly different median survival time (using the same challenge dose) due to variation between experiments, different batches of mice etc. The difference in survival between mice challenged with mutants compared to mice challenged with the wild-type strain is shown in bold red typescript. Significant differences are given in bold black typescript, where $P<0.05$ is represented by a single asterix (*) and $P<0.005$ is represented by a double asterix (**). The final column summarises the virulence of mutant strains compared with their isogenic parental strains. Significant alterations in virulence are highlighted in bold typescript.

Table 3.2 highlights that the mutants did not appear to be highly attenuated in virulence, as determined by levels of bacteria within the blood and lungs at various time points post infection. Survival of mice following challenge with most of the mutants (7 out of 9 challenges) increased compared to survival of mice infected with the isogenic wild-type strains, although was only significant for the 3 of the mutants.

Intranasal challenge with 10^6 CFU of the insertion-duplication mutants did not result in significant differences for any of the virulence parameters examined. When the challenge dose was increased to 10^7 CFU, in an attempt to reduce variation and identify possible differences, the survival of mice challenged with the *ΔpRPL7* mutant was found to be significantly greater than those infected with wild type ($P<0.05$). The remaining 2 insertion-duplication mutants did not differ significantly from wild-type D39 in any of the challenges.

The type 3 allelic-replacement mutants in the *hk08* and *rr08* genes had significantly longer survival than wild-type 0100993 following intranasal challenge with ($P<0.005$), but did not show reduced bacterial counts in the blood.

The type 2 allelic-replacement did not show any significant difference in survival or bacteraemia.

3.6.4 *In vivo* analysis of virulence; Lung counts

Of the challenges where bacterial counts within the lung tissue were examined, only mice infected with the type 3, allelic-replacement mutants showed lower counts compared to mice infected with the wild-type strain. These differences were not significant, however, and do not match the high degree of attenuation found by Throup and colleagues using the same challenge dose and the same strains in a different model of infection (Throup *et al.*, 2000). Attempts to evaluate these differences using the same model of infection as Throup *et al.* (CBA/Js) were unsuccessful due endemic *Proteus* infection in the CBA/J colony.

In summary, none of the mutants were highly attenuated in virulence in the MF1 model of infection although some did result in significantly longer survival times in infected mice. In a type 2 background a mutation in the *hk08* gene appears to be more attenuated than a mutation in the *rr08* gene (insertion-duplication mutants). Mutations in a type 3 background for TCS08 may be slightly more attenuated than those in a type 2 background.

Chapter 4

Discussion

S. pneumoniae TCS08

Chapter 4 Discussion; *S. pneumoniae* TCS08

4.1 Creating mutants in *S. pneumoniae*

The publication of 2 pneumococcal genomes (R6 and TIGR4) combined with the natural ability of *S. pneumoniae* to take up DNA from the extracellular environment (transformation / competence) provides excellent capacity to create mutations in virtually any gene of choice in the pneumococcus. Several mutagenesis techniques are available for use, two of which (insertion-duplication and allelic-replacement) have been used for this section of work. Both techniques rely on transformation of *S. pneumoniae* cultures with donor DNA (plasmid or PCR product respectively) to create mutations in the target gene. The donor DNA carries a detectable marker, usually a gene encoding antibiotic resistance, allowing for selection of potential transformants. As plasmid donors used for this purpose are suicide vectors, failure to integrate into the chromosome will result in loss of the plasmid from the cell. Homologous sequences from the target gene itself or from regions flanking the target gene are included in the donor DNA to promote insertion at the required site. Thus the donor DNA must gain entry into the pneumococcal cell and integrate into the bacterial chromosome. Unless this integration results in a non-viable phenotype, mutants can be isolated using appropriate antibiotic selection and characterised further in comparison with the isogenic parental strain.

The transformation protocol to create the mutants used in this thesis used a 9.2 Kb plasmid vector, pVA838, as a positive control for transformation. This vector is not a suicide vector and is able to replicate in *S. pneumoniae*, conferring erythromycin resistance upon host cells (Macrina *et al.*, 1983). As integration into the chromosome is not required for the erythromycin-resistance phenotype, the transformation efficiency using this plasmid can be up to several hundred-fold greater than using donor DNA for mutant construction (which must integrate into the chromosome). Thus the protocol for transformation of strain D39 was optimised to allow for the construction of the mutants described in this thesis (data not shown).

It is possible that the transformation procedure itself could result in mutants that have altered phenotype. Indeed, of the opaque and transparent pneumococcal variants that have been described previously (introduction, section 1.5.4), it is thought that the transparent variants have increased transformation efficiency. Due to the low levels of transformation obtained using donor DNA, it is possible that transparent variants, possessing less capsular material and thus

having higher transformation efficiency (Weiser and Kapoor, 1999), are preferentially selected for when creating mutants. These variants have reduced virulence in systemic infection (Kim and Weiser, 1998) and could thus indicate an attenuated phenotype that is due to the procedure used to create the mutant rather than the effects of disrupting the target gene. To investigate this possibility, strain D39, used as the parental strain for many of the mutants characterised in this thesis, was transformed with control plasmid pVA838, and analysed for *in vitro* growth, virulence and stability, compared to a non-transformed D39 parental strain. Data showed that the tD39 was not impaired in growth both *in vitro* and *in vivo* and that bacteria retrieved from the blood of infected mice at the 36 hr time point still retained their erythromycin resistance. This indicates that the presence of this 9.2 Kb plasmid does not exert a large enough metabolic burden on host cells to result in rapid elimination. Thus virulence was not compromised by the transformation procedure in the murine model of infection used within this thesis.

Although the aims of this thesis were to identify genes important in the virulence of the pneumococcus, all mutants were analysed for their ability to grow and retain their antibiotic selection in culture prior to any *in vivo* characterisation. Growth curve experiments are important to ensure that any differences seen in virulence are not due to general growth defects that are also present *in vitro*. Furthermore these basic *in vitro* characterisations can be used to determine any effect antibiotic selection has on the growth and autolysis of mutants, and to determine the stability of the mutants in the absence of antibiotic selection (although this must also be checked following *in vivo* infection). None of the TCS08 mutants were found to be impaired in *in vitro* growth or stability.

4.2 Analysis of virulence of TCS08 mutants using a murine model of infection

Outbred adult MF1 mice were the murine model used throughout this thesis. This is a well characterised model of pneumococcal infection, especially with the serotype 2 D39 strain used to make many of the mutants described within this work (Canvin *et al.*, 1995; Kadioglu *et al.*, 2000). MF1s can be infected through a variety of challenge routes, including intranasal (to study nasopharyngeal / airway / lung colonisation, pneumonia and septicaemia), intraperitoneal (to passage strains and to study septicaemia) and intravenous (to study septicaemia). Several parameters of virulence can subsequently be analysed, such as bacterial counts in different tissues, survival of mice following challenge and the host inflammatory response to infection /

tissue histology. Being an outbred strain of mouse, this model can be highly variable but has been used previously to successfully identify and characterise pneumococcal virulence factors (Canvin *et al.*, 1995; Overweg *et al.*, 2000; this thesis, chapters 5 and 7).

Insertion-duplication TCS08 mutants

Mutations in the genes encoding HK08, RR08 and Sp0082 were created by insertion-duplication mutagenesis, where an entire plasmid construct (> 7 Kb) was inserted into the target gene, creating mutants *pRPL7*, *pAS1* and *pRPL50*, respectively. Normal gene function is disrupted due to the integration of such a large piece of DNA, although none of the coding sequence of the target gene is removed. Insertion towards the 5' end of the gene should ensure that any region of DNA prior to the insert that could result in translation of a truncated protein with some functional activity is avoided. Alternatively, a tmRNA tag targeting any translated protein for degradation can be included within the plasmid construct, and this technique was used for creating the *ΔpRPL50* (*sp0082* gene) mutant (Hoffmann-La-Roche, personal communication). tmRNAs can enter a ribosome when it is stalled on a damaged mRNA / mRNA lacking a stop codon. Upon entry of the tmRNA molecule, the ribosome can switch from the mRNA to the tmRNA and continue translating a short peptide sequence encoded by the latter. This short sequence encodes a tag that is a target for proteolysis and subsequent degradation of the damaged peptide (Keiler *et al.*, 1996; Molnos *et al.*, 2000).

Following intranasal challenge with 10^6 CFU, no significant differences were observed in the behaviour of any of the TCS08 insertion-duplication mutants compared to wild-type in any parameter of virulence tested (bacteraemia, lung counts or murine survival). All values were very similar for the *ΔpAS1* and *ΔpRPL50* mutants compared to isogenic wild-type. The *ΔpRPL50* mutation (*sp0082* gene) even showed slightly increased bacterial counts in the blood at 36 hr post infection and mice challenged with this mutant reached the experimental end point prior to those challenged with wild-type D39. The *ΔpRPL7* mutant, however had lower median counts in the blood by at least one order of magnitude at 24 and 36 hr post infection, and resulted in 1.8-fold increased murine survival time compared to infection with isogenic parental strain. These differences however, were not found to be significant, although this could be due to the variation present using the MF1 model of infection. Lung counts at 24 hr post infection were all very similar in all the mutants compared to the D39 wild-type strain.

In an attempt to decrease the variation seen with the above infections, mice were challenged intranasally with a 10-fold increased dose of bacterial strains, and virulence assessed as

described above, with the exception of lung counts which were not performed. No difference in bacterial counts were observed in the blood of mice challenged with any of the mutants compared to wild-type, although infection with the *ΔpRPL7* mutant again resulted in decreased counts by at least one order of magnitude at 24 hr post infection. The difference in survival with this mutant was found to be significant compared to infection with D39 wild-type ($P<0.05$). No difference in survival of the remaining 2 mutants was observed. This data suggests that for the TCS08 mutants created by insertion-duplication mutagenesis, the only one that appears to be attenuated in virulence is the mutant with a disruption in the *hk08* gene. This finding is unusual as it is generally assumed that mutations in the *rr* genes of bacteria TCS are more attenuated than a mutation in the cognate *hk* gene (Throup *et al.*, 2000). The *ΔpAS1* mutant (*rr08* gene) did not show any signs of even being slightly attenuated in the infection model used for this work. This data indicates that HK08 could have a role in regulating genes or TCS other than *rr08* and that it is the effect on other genes that is resulting in the slightly attenuated phenotype described above for the *hk08* mutant.

Allelic-replacement TCS08 mutants

During the course of this work, data were published showing that a *rr08* mutant in a serotype 3 strain of the pneumococcus was highly attenuated in virulence as assessed by bacterial counts in the lung tissue of CBA/J mice, 48 hr post intranasal challenge (Throup *et al.*, 2000). This mutant (*Δ484rr*) was created by allelic-replacement mutagenesis. Mean counts in the lungs of mice challenged with the *Δ484rr* mutant were shown to be >1000-fold lower than counts in the lungs of mice challenged with the isogenic parental strain (Throup *et al.*, 2000). Such an attenuated phenotype was not observed using the insertion-duplication *ΔpAS1* mutant during the work described above, so parental and mutant strains used in the study by Throup *et al.* were sent to us for further characterisation in our MF1 model of infection. The strains (0100993, *Δ484hk* and *Δ484rr*) were used in an intranasal infection of MF1 mice and virulence was assessed by monitoring bacteraemia, survival and lung counts 48 hr post infection. Mice challenged with both mutants had significantly increased survival times ($P<0.005$ for both mutants) compared to mice challenged with the isogenic parental strain, although no difference in bacteraemia at any of the time points studied was found. Counts within the lung tissue were lower for mice challenged with mutant bacteria, being approximately 10-fold lower for the *Δ484rr* mutant compared to the wild-type 0100993 strain. However, such differences were not statistically significant. This data

indicates that the mutants are attenuated following intranasal infection, but not as severely attenuated as indicated by Throup and colleagues (Throup *et al.*, 2000). These differences could be attributed to the use of different murine models for the study of virulence. Analysis of the type 3 mutants in the CBA/J strain of mice used by Throup *et al.*, was to form a component of this thesis but work had to be stopped due to endemic Gram-negative bacterial infection within the mouse colony. Significant variation in the susceptibility of 9 different inbred mouse strains to pneumococcal infection has been documented previously (Gingles *et al.*, 2001), so it would not be surprising to find that mutants behave differently with different murine genetic backgrounds. The *Δ484rr* mutation was transferred into D39, serotype 2 to create the *Δrr08* mutant in this parental strain by allelic replacement, to allow for comparison of this mutant with the insertion-duplication mutant *ΔpAS1*. In agreement with the data presented following infection with the *ΔpAS1* mutant, intranasal challenge with the *Δrr08* mutant did not result in significant differences in bacteraemia or survival of infected mice compared to the D39 parental strain. This suggests that the techniques used to create mutants in the work presented within this section result in the same phenotype of the *rr08* mutants.

The variation present using a biological system, such as the MF1 murine model of infection described within this thesis, can have a significant impact on the final interpretation of data. For example, in the infections described above with all of the TCS08 mutants, statistically significant differences were found for survival of mice following intranasal challenge with the type 3 *Δ484hk* / *Δ484rr* mutants and the *ΔpRPL7* mutant only. A significant difference in murine survival was found for the latter mutant only when the challenge dose of mutant and wild-type was increased 10-fold (from 10^6-10^7 CFU). However, the fold-difference in survival time of mice challenged with mutant bacteria compared to that of challenge with the isogenic parental strain for all mutants (highlighted in table 3.2), shows that challenges with the *ΔpRPL7* and *Δrr08* mutants (both with a challenge dose of 10^6 CFU) result in differences in survival comparable to those of the *Δ484hk* / *Δ484rr* and *ΔpRPL7* (10^7 CFU), but these differences were not found to be statistically significant. The variation present in the model is also illustrated by the median survival times of the D39 wild-type strain. The challenges using D39 wild-type with the insertion-duplication mutants (*ΔpRPL7*, *ΔpAS1* and *ΔpRPL50*) resulted in a median survival time of 54 hr for mice challenged with D39 wild-type. However, challenges with D39 and the *Δrr08* allelic-replacement mutant, gave a median survival time of 41 hr for mice challenged with

D39, wild-type. Although the D39 parental strain was the same for both studies, the differences in median survival times illustrate the variation that can occur between experiments and when using different batches of mice.

In an attempt to eliminate some of the variation present and to retrieve statistical differences between mutants and their wild-type parental strains, several methods were tried. Increasing the challenge dose 10-fold did result in a slight reduction in variation, and this allowed significant differences in survival following challenge with the *ΔpRPL7* mutant to be detected. An inbred strain of mouse (C57/Bl) was also tested using some of the insertion-duplication mutants to assess if this could be a suitable alternative to the MF1 model, but results were just as variable using this inbred strain of mouse as with the outbred MF1 mice (data not shown). This was also demonstrated in a later study (Gingles *et al.*, 2001).

Competition experiments, where individual mice are challenged with a 1:1 mix of mutant and wild-type bacteria within the same inoculum, are currently being developed in our laboratory in an attempt to overcome some of the variation in murine infections highlighted above. This technique may be useful for detecting more subtle differences between strains that can not be detected using the conventional infection procedures and will also reduce the number of animal models required for individual data sets. Other laboratories have successfully used these competition experiments for *in vivo* studies of pneumococcal mutants (Brown *et al.*, 2001a; Hava and Camilli, 2002; Sebert *et al.*, 2002).

4.3 Summary; involvement of TCS08 in virulence

This section of work used mutations in genes associated with *S. pneumoniae* TCS08 in 2 different serotypes to determine the contribution of this TCS to virulence in a murine model of infection. The data suggest the a mutation within the *hk08* gene is slightly attenuated in virulence following intranasal infection in both type 2 and type 3 parental strains. Mutation of the *rr08* gene appears to be attenuated in virulence in a type 3 strain only, although increased murine survival times were observed following challenge with a *rr08* mutant created in type 2 by allelic-replacement, but not in a type 2 *rr08* insertion-duplication mutant (differences were not significant). Whether this represents a true difference between these mutants or variation within the MF1 model used for the analysis of virulence remains to be determined. Further work is required to determine if the differences in virulence described with the TCS08 mutants are significant. Many of the TCS08 mutants described above will be analysed in the competitive

experiments described above to further characterise the role of TCS08 in virulence. Alternatively, other models of infection could be utilised to examine the aforementioned mutants. For example, the nematode *Caenorhabditis elegans* has been used to assess the virulence of many bacterial pathogens (Garsin *et al.*, 2001; Couillault and Ewbank, 2002; Gan *et al.*, 2002). Furthermore, virulence factors important for mammalian pathogenesis have also been shown to be required for efficient killing of *C. elegans* (Garsin *et al.*, 2001), indicating that this system can be used to identify important virulence factors of pathogens. This model has been shown to be susceptible to killing by *S. pneumoniae*, and *zmpB* and pneumolysin mutants from our laboratory are currently being analysed in a *C. elegans* model of infection (Wouter Jansen, CX Utrecht, the Netherlands). Such non-vertebrate systems may offer cheaper and simpler alternatives to the murine models more commonly used for virulence studies, or could be used in combination with other systems to aid in the detection and characterisation of attenuated phenotypes. They may also allow for the identification of host factors that contribute to infection, as powerful genetic tools are available for some of these models.

In summary several of the mutants within the *hk08 / rr08* genes of type 2 and 3 used within this work demonstrated attenuated virulence compared to their isogenic parental strains in a murine model of infection. The exact role of the TCS08 genes in virulence is unknown. The contribution of TCS08 to virulence may depend on the genetic background of the bacterial strain, as differences between the virulence of type 2 and type 3 mutants were observed in this work, although variation in the animal model used may also explain such differences. Further work is required to determine the role of this TCS in pneumococcal virulence.

Table 4.1 summarises all current data on the study of *S. pneumoniae* TCS08 for its role in virulence, including data presented in this thesis.

Reference	Strain / serotype of bacteria	Mutagenesis technique	Murine Strain	Route of infection	Inoculum	Measure of virulence	Role in virulence / attenuation
Lange *et al*, 1999	Sp.1 (type 22)	Insertion duplication	C57BL6/J (I) Females	i.p. (NP)	4×10^6	Mean survival time	No attenuation
Lange *et al*, 1999	Sp.1711 (type 3)	Insertion duplication	C57BL6/J (I) Females	i.p. (NP)	1×10^3	Mean survival time	No attenuation
Throup *et al.*, 2000	0100993 (type 3)	Allelic replacement	CBA/J (I) Males, 5 wk	i.n. (NP)	1×10^7	Mean lung counts at 48 hr	> 1000 fold decrease in lung counts
Sebert *et al.*, 2002	0100993 (type 3)	Allelic replacement	Sprague-Dawley infant rats (O)	i.n. (NP)	4×10^6 – 1.6×10^7	Colonisation of nasopharynx	No attenuation
This work	D39 (type 2)	Insertion duplication	MF1 (O) Females, 9-13 wk	i.n. (P)	1×10^6	Bacteraemia, survival and lung counts at 24 hr	No attenuation
This work	D39 (type 2)	Allelic replacement	MF1 (O) Females, 9-13 wk	i.n. (P)	1×10^7	Bacteraemia and survival	No attenuation
This work	0100993 (type 3)	Allelic replacement	MF1 (O) Females, 9-13 wk	i.n. (P)	1×10^7	Bacteraemia, survival and lung counts at 48 hr	No attenuation in bacteraemia Significant increase in murine survival 10-fold decrease in lung counts (N/S)
This work	D39 (type 2)	Allelic replacement	MF1 (O) Females, 9-13 wk	i.n. (P)	1×10^6	Bacteraemia and survival	2-fold increase in murine survival (N/S)

Table 4.1 Summary of all *in vivo* analysis of *rr08* mutants

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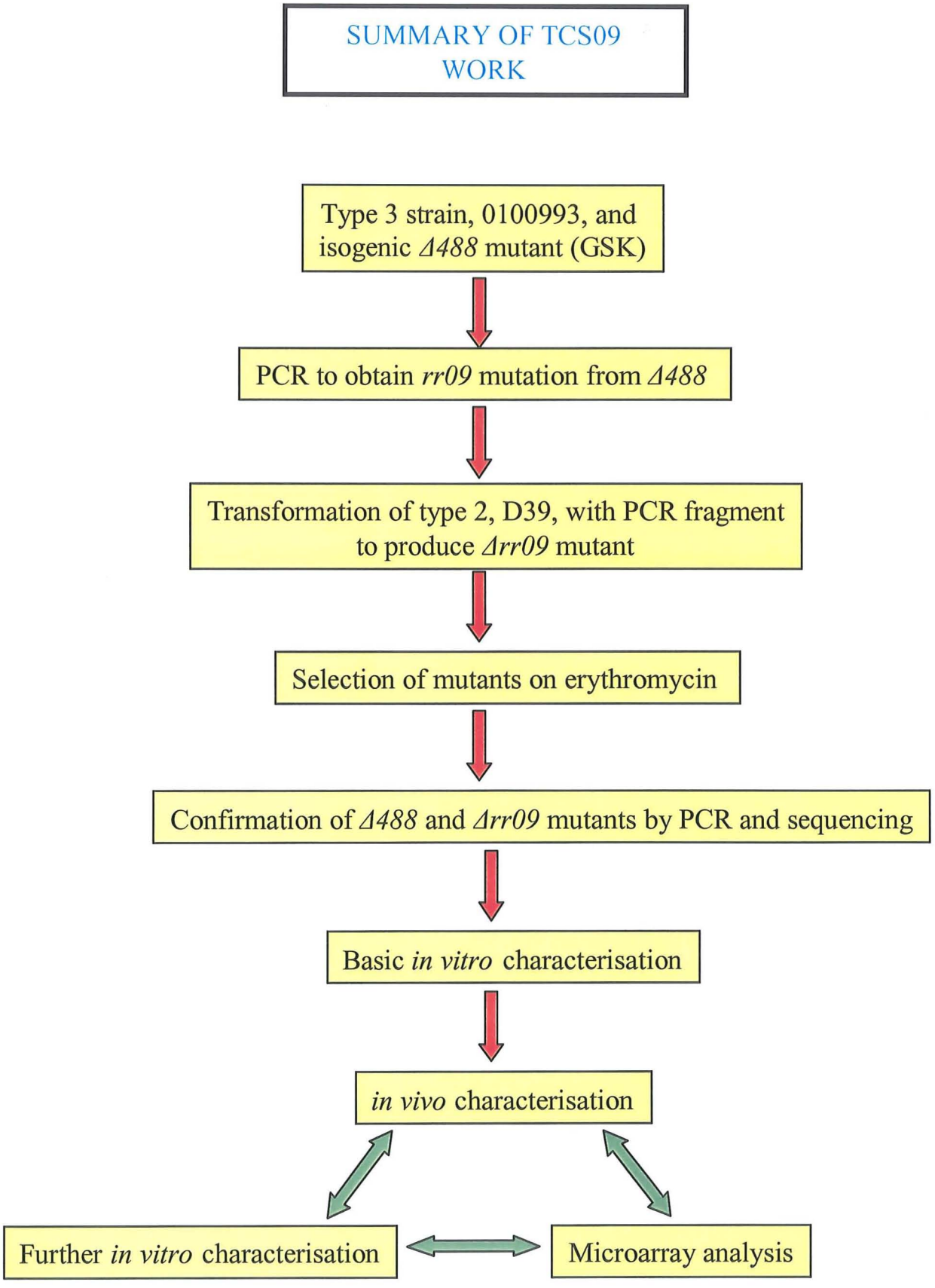
Table 4.1 Summary of all *in vivo* analysis of *rr08* mutants

The table summarises all *in vivo* work available to date with *S. pneumoniae* TCS08. (I) / (O) designates inbred or outbred strains of mice respectively. Route of challenge includes intranasal (i.n.) or intraperitoneal (i.p.). Strains passaged or non-passaged prior to challenge are represented by (P) and (NP), respectively. Role in virulence is in comparison with the isogenic wild-type parental strain. (NS) designates data that was not found to be statistically significant. The table is modified from that in the introduction (table 1.6) by the addition of work detailed in this thesis.

Chapter 5

Results

S. pneumoniae TCS09


SUMMARY OF TCS09 WORK
Type 3 strain, 0100993, and isogenic Δ488 mutant (GSK)
PCR to obtain rr09 mutation from Δ488
Transformation of type 2, D39, with PCR fragment to produce Δrr09 mutant
Selection of mutants on erythromycin
Confirmation of Δ488 and Δrr09 mutants by PCR and sequencing
Basic in vitro characterisation
in vivo characterisation
Further in vitro characterisation
Microarray analysis

5.1 TCS09 basic *in vitro* analysis

5.1.1 Creating and confirming mutants

TCS09 was characterised within this thesis based on its close proximity to several genes of interest. These include a methionine sulphoxide reductase previously shown to be involved in virulence (Wizemann *et al.*, 1996), and a putative zinc metalloprotease, previously not extensively characterised for its role in virulence (Novak *et al.*, 2000; Bergé *et al.*, 2001). It was speculated that TCS09 could be involved in the regulation of these genes.

S. pneumoniae strain 0100993 (serotype 3) and an isogenic strain with a null mutation in the *rr09* gene (strain *Δ488 / 488*) were provided by M. K. Burnham, GlaxoSmithKline (Throup *et al.*, 2000). The mutation was created by allelic-replacement mutagenesis as described in section 1.7.2, and figure 1.5 (introduction). The *rr09* gene was replaced with the constitutively expressed *ermAM* gene cassette and care was taken to ensure that insertion of the cassette would still allow for the expression of the downstream *hk09* gene that appears to be co-transcribed with *rr09* (http://www.tigr.org/). Furthermore, to prevent polar effects, the transcription termination signals were removed from this cassette prior to insertion. Replacement of the *rr09* gene with the *ermAM* cassette results in a PCR fragment approximately 200 bp larger than wild-type gene when PCR with primers flanking the region is performed (figure 5.1).

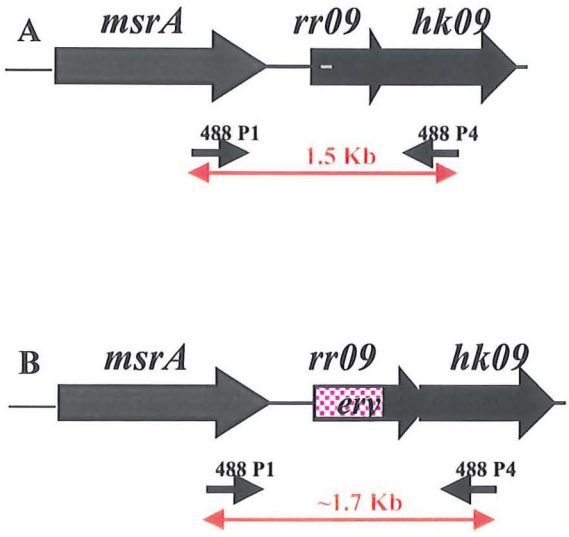


Figure 5.1: Allelic-replacement mutagenesis to create a *rr09* null mutant

A cassette encoding erythromycin-resistance was used to replace the *rr09* gene as described in the text and in section1.7.2. Primers 488P1 and 488P4 flanking the region were used to confirm the mutation, whereby the mutated gene results in a slightly larger PCR product compared to wild type. Expression of *hk09* should not be affected by this method of mutagenesis. Figure 5.1A shows the wild-type *tcs09* locus. Figure 5.1B illustrates the *tcs09* locus after replacement of the *rr09* gene with a cassette encoding erythromycin resistance.

Primers 488P1 and 488P4 were used to amplify the mutated *rr09* gene from strain *Δ488*. The PCR product was excised from the agarose gel, purified and used to transform strain D39 (serotype 2). Potential mutants were selected by their ability to grow in the presence of erythromycin and PCR, as described above, was used to confirm potential transformants (figure 5.2). Resulting PCR fragments were sequenced to further ensure cassette insertion into the correct target gene. The *rr09* null mutation in strain D39 has been designated *Δrr09 / rr09*.

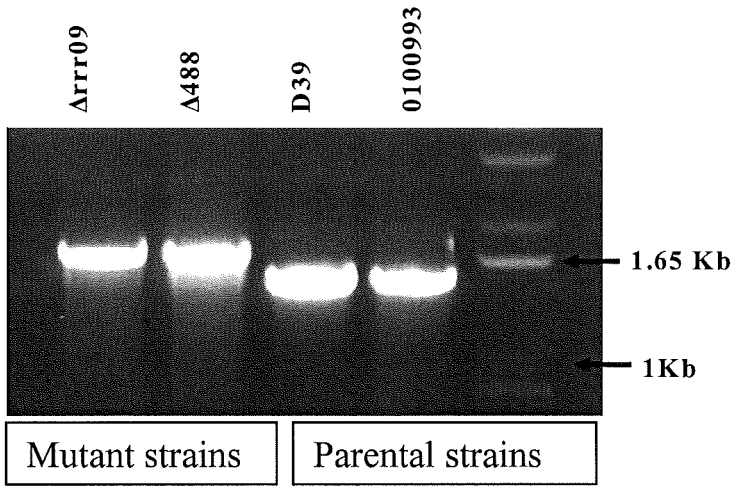


Figure 5.2: Confirmation of *Δrr09* null mutations by PCR

Primers 488P1 and 488P2 (see figure 5.1) were used to confirm the *Δrr09* mutation in strains D39 and 0100993. Replacement of the target gene with the *ermAM* cassette produces a PCR product in the mutant strain ~ 200 bp larger than that of the wild type parental strains (PCR bands 1.7 Kb and 1.5 Kb respectively).

5.1.2 Confirmation of *tcs09* locus and surrounding genes in strains used within this work

The organisation of *tcs09* and surrounding genes, as described in section 1.14.4, figure 1.15 (introduction) is based on the sequenced R6 (type 2) and TIGR4 (type 4) genomes (http://www.tigr.org/). The TCS09 system was identified in both these pneumococcal genomes. To determine if the gene organisation in the strains used within this work is the same as the sequenced genomes, PCR was performed. Primers were designed against the *rr09* (RR09R), *hk09* (ZmpUp), *msrA* (MsrAF) and *zmpB* (ZmpR) genes. Three regions were examined, including the whole region spanning from the *msrA* gene to the *zmpB* gene (primers MsrAF & ZmpR), region 'A' from *msrA* to *rr09* (primers MsrAF & RR09R) and region 'B' from *hk09* to *zmpB* (primers ZmpUp & ZmpR). The relative location of primers and expected fragment sizes based on the sequenced genomes is illustrated in figure 5.3.

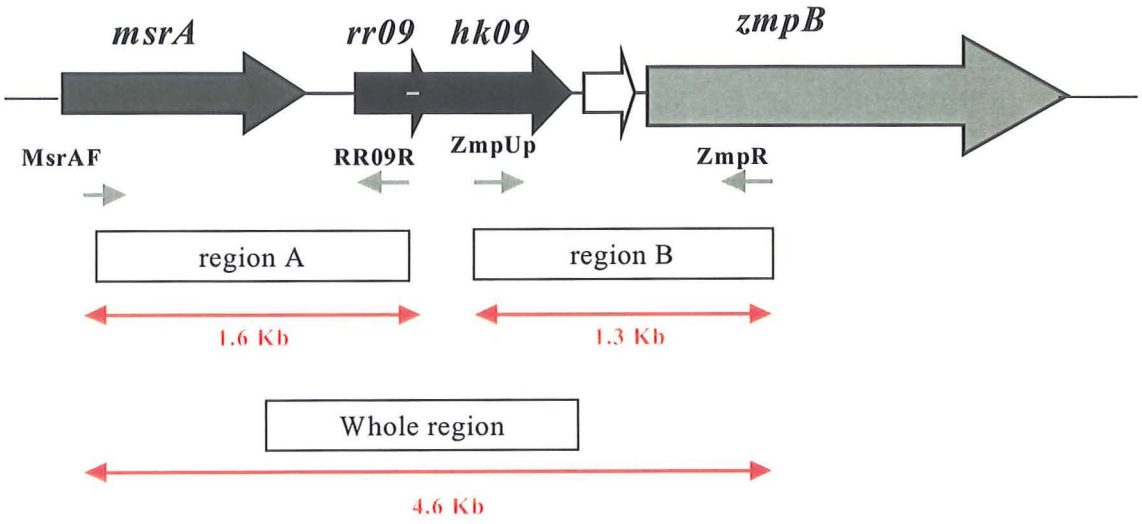


Figure 5.3: Gene organisation around the *tcs09* locus and primers used to examine this region in strains D39 and 0100993

The organisation of *tcs09* and surrounding genes shown is based on that of the sequenced strains R6 and TIGR4. Primers were designed to bind specifically to all of the indicated genes with the exception of that encoding a conserved hypothetical protein situated between *hk09* and *zmpB*. Three PCR reactions were performed to amplify up the whole region and regions A and B using genomic DNA isolated from strains R6, TIGR4, D39 and 0100993. The expected product size, based on the sequenced genomes is shown (not to scale).

ZmpB has been shown to exhibit considerable sequence variation between several serotypes with homology existing only within the N' terminal region of the gene (Bérge *et al.*, 2000). Thus primer ZmpR was designed within the homologous region of *zmpB* to help ensure detection of this gene in the strains examined.

PCR using the primers described above resulted in bands of equal size for all three regions examined in all strains (figure 5.4). Although this does not indicate sequence homology it shows that the organisation of *tcs09* and the surrounding genes in strains D39 and 0100993 used within this work is the same as that in the sequenced strains (including G54 / type 19F). The protein sequences of the 5 genes shown in figure 5.3 are at least 99 % homologous between the R6, TIGR4 and G54 genomes based on sequences obtained from PubMed and TIGR, using a BLAST alignment tool, [http://www.ncbi.nlm.nih.gov/entrez/query.fcgi and http://www. ncbi.nlm.nih. gov:80/BLAST/]).

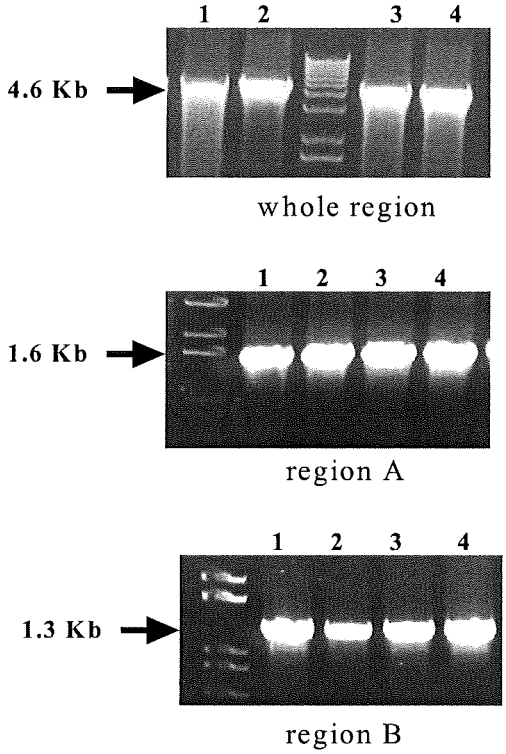


Figure 5.4: Confirmation of *tcs09* and surrounding genes in strains D39 and 0100993

PCR using the primers described in the text and figure 5.3 was used to confirm that the *tcs09* locus and surrounding genes had the same organisation in the strains used in this work compared to the sequenced strains R6 and TIGR4. Three regions were examined, the whole region; upper gel, region A; middle gel and region B; lower gel. The expected product sizes for each region based on the published genome sequences are shown. For each gel, lane 1; R6, lane 2; TIGR4, lane 3; D39 and lane 4; 0100993.

The PCR reactions described above were performed using several other strains, including type 1, type 19F and a different type 4 strain. All strains tested produced bands of the same size as R6 and TIGR4 for all three regions, suggesting the organisation of this region may be conserved throughout all pneumococcal serotypes.

5.1.3 Growth and autolysis *in vitro*

All strains were subject to growth and autolysis determination using a combination of viable cell enumeration and optical density (OD 600 nm) readings over time. The effect of erythromycin on the growth and autolysis of mutant strains was also examined. Figure 5.5 illustrates the growth of strain D39 and its isogenic *Δrr09* mutant.

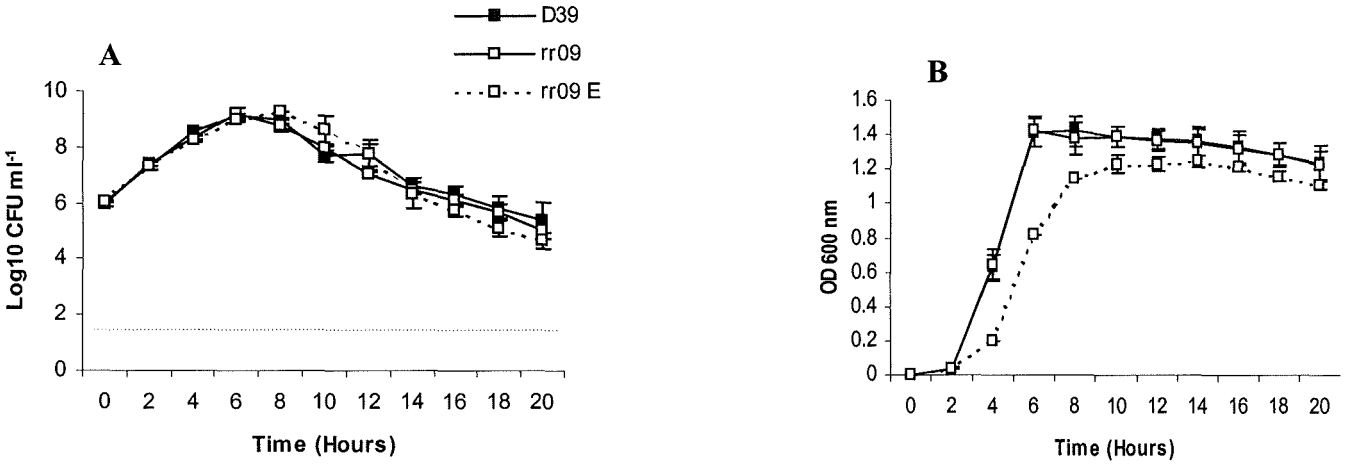


Figure 5.5: *In vitro* growth and autolysis of D39 and *Δrr09*

15 ml BHI was inoculated with 1.0 x 10^6 CFU bacterial strains. Viable cell enumeration (figure 5.5A) and OD 600 nm readings (figure 5.5B) were performed every 2 hr for a period of 20 hr. Mutant strains were grown in BHI with (rr09E) and without (rr09) erythromycin (1 μg ml^{-1}). The broken line represents the limit of detection for viable cell counts.

In the absence of erythromycin, no difference was observed in the growth rate or autolysis of the *Δrr09* mutant compared to its parental strain, D39, as determined by viable cell enumeration or $OD_{600\ nm}$. Erythromycin did not have any effect on the number of viable cells detected, but did appear to result in slightly lower $OD_{600\ nm}$ readings for strain *Δrr09* (figure 5.5B). In the absence of antibiotic selection, $OD_{600\ nm}$ is representative of growth up to stationary phase (approximately 7 hours after inoculation of cultures), as judged by viable cell counts. After this point readings do not appear to decrease to the same extent as indicated by the viable cell counts.

The growth and autolysis of type 3 strain 0100993 and its isogenic mutant *Δ488* is illustrated in figure 5.6.

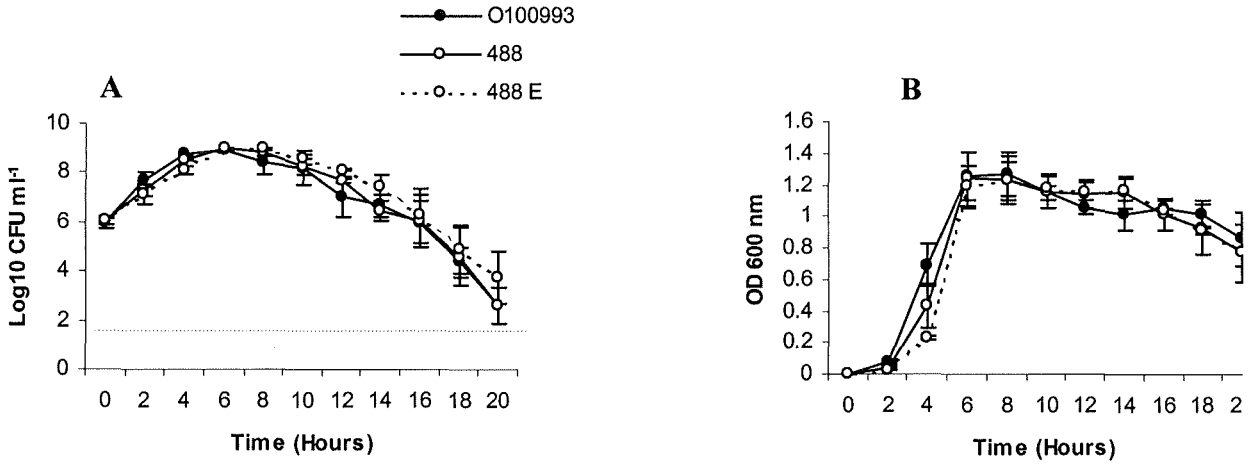


Figure 5.6: *In vitro* growth and autolysis of strains 0100993 and *Δ488*

15 ml BHI was inoculated with 1.0 x 10^6 CFU bacterial strains. Viable cell enumeration (figure 5.6A) and OD 600 nm readings (figure 5.6B) were performed every 2 hr for a period of 20 hr. Mutant strains were grown in BHI with (488E) and without (488) erythromycin (1 μg ml^{-1}). The broken line represents the limit of detection for viable cell counts.

Data for the serotype 3 strains shows that there was no difference in growth of mutant *Δ488* compared to its isogenic parental strain 0100993. The presence of erythromycin in the media did not alter the viable counts or OD $_{600\ nm}$ readings of the mutant. As discussed above for the type 2 strains, OD $_{600\ nm}$ is representative of growth up to stationary phase (approximately 7 hours after inoculation of cultures), after which readings do not appear to decrease to the same extent as the viable cell counts.

Figure 5.7 combines the growth data for wild type D39 and 0100993 strains to illustrate any differences observed between the 2 serotypes used for this work. The figure shows that the 2 strains have similar growth as determined by both viable cell enumeration and OD $_{600\ nm}$ readings during exponential phase. Upon reaching stationary growth phase both strains appear to autolyse at similar rates as shown by the viable cell counts in figure 5.5B, although strain 0100993 shows slightly accelerated autolysis between 16 and 20 hour compared to D39. OD $_{600\ nm}$ readings for

cells undergoing autolysis however, are slightly lower for serotype 3 strain 0100993 compared to D39 (figure 5.7B). This is possibly due to differences in the relative thickness of the polysaccharide capsules surrounding the organisms. Type 3 strains characteristically produce a lot of capsular material (Waite *et al.*, 2001) that does not appear to alter OD $_{600\ nm}$ readings of live cells but may alter the readings of dead or dying cells.

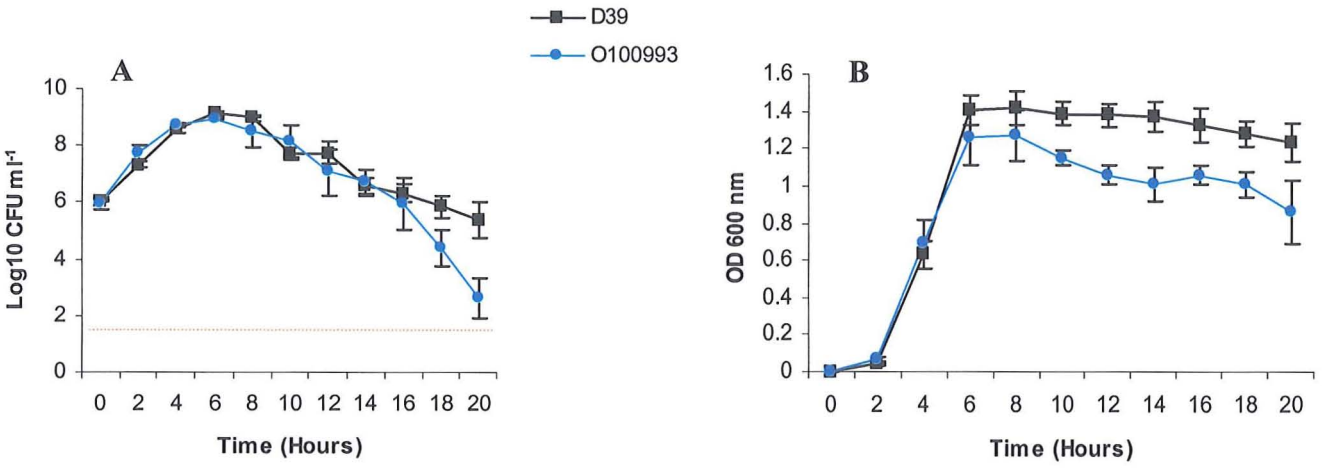


Figure 5.7: Comparison of *in vitro* growth and autolysis of strains D39 and 0100993

15 ml BHI was inoculated with 1.0 x 10^6 CFU bacterial strains. Viable cell enumeration (figure 5.7A) and OD 600 nm readings (figure 5.7B) were performed every 2 hr for a period of 20 hr. The broken line represents the limit of detection for viable cell counts.

The data in figure 5.7 illustrates that cultures of all strains in this work harvested at the same OD $_{600\ nm}$ reading (during exponential growth phase) will result in equal numbers of viable cells. This observation is useful for the design of further experiments where it may be necessary to use equal numbers of cells for comparison purposes, but impractical to wait over 16 hr for the results of viable cell enumeration analysis. In such instances, cells can be harvested upon reaching a set OD 600 nm and used for appropriate experiments.

5.1.4 Stability of *rr09* mutants *in vitro*

To determine the ability of the *rr09* mutants to retain the *ermAM* cassette during *in vitro* culture, strains were grown in BHI in the absence of antibiotic selection. At pre-determined time points the number of viable cells on agar plates without erythromycin and agar plates supplemented with antibiotic was compared for each mutant. Mutants *Δrr09* and *Δ488* did not lose their ability to grow in the presence of antibiotic up to 20 hr following culture inoculation (figure 5.8). Time points post 20 hr were not examined as pneumococci exhibit autolysis during *in vitro* growth and few CFU can be detected in cultures grown for over 20 hr.

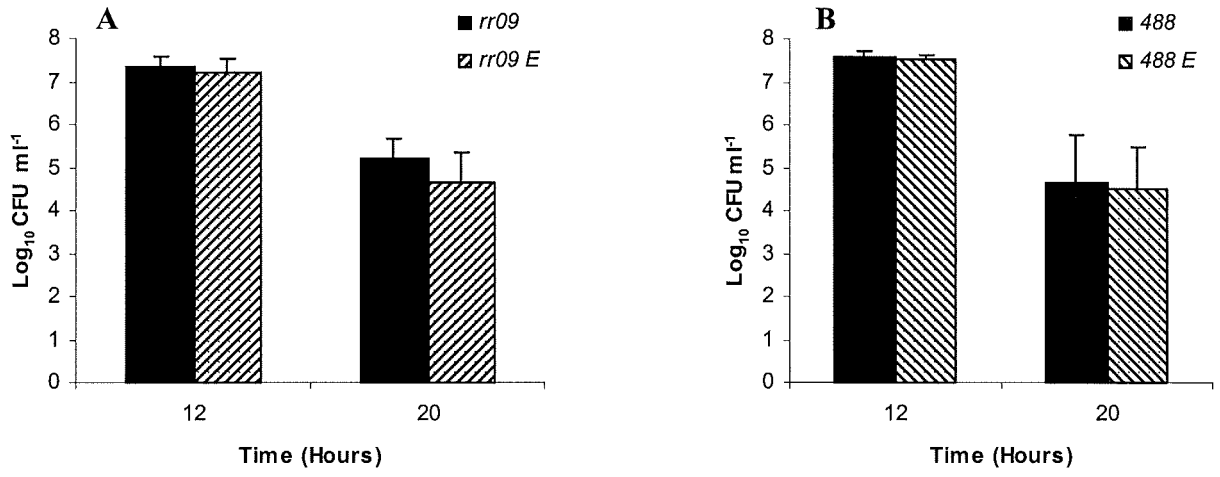


Figure 5.8 Stability of *rr09* mutants *in vitro*

BHI was inoculated with 1.0 x 10^6 CFU mutant strains in the absence of erythromycin selection. At 12 hr and 20 hr post inoculation cell viability counts were performed to determine if the mutants had lost their erythromycin resistance. Figure 5.8A shows the stability of the type 2 mutant *Δrr09*. Figure 5.8B shows the stability of the type 3 mutant *Δ488*. Both *Δrr09* and *Δ488* were stable for up to 20 hr during *in vitro* growth.

Figure 5.8 indicates that in the absence of erythromycin mutants remained stable under the conditions used. Furthermore, the growth curves illustrated in figures 5.5 and 5.6 show that the mutants are not impaired in growth in the presence or absence of erythromycin. Thus for all further work the *rr09* mutants were grown in the absence of erythromycin selection unless stated otherwise.

5.1.5 Expression of *rr09 in vitro*

RT-PCR using primers specific for the *rr09* gene was used to determine if it is expressed during *in vitro* growth. RNA was isolated from D39 wild type grown to mid-log phase of growth in BHI media. RT-PCR analysis was performed on two independent RNA preparations. Figure 5.9 shows the *in vitro* expression of *rr09* transcript from strain D39.

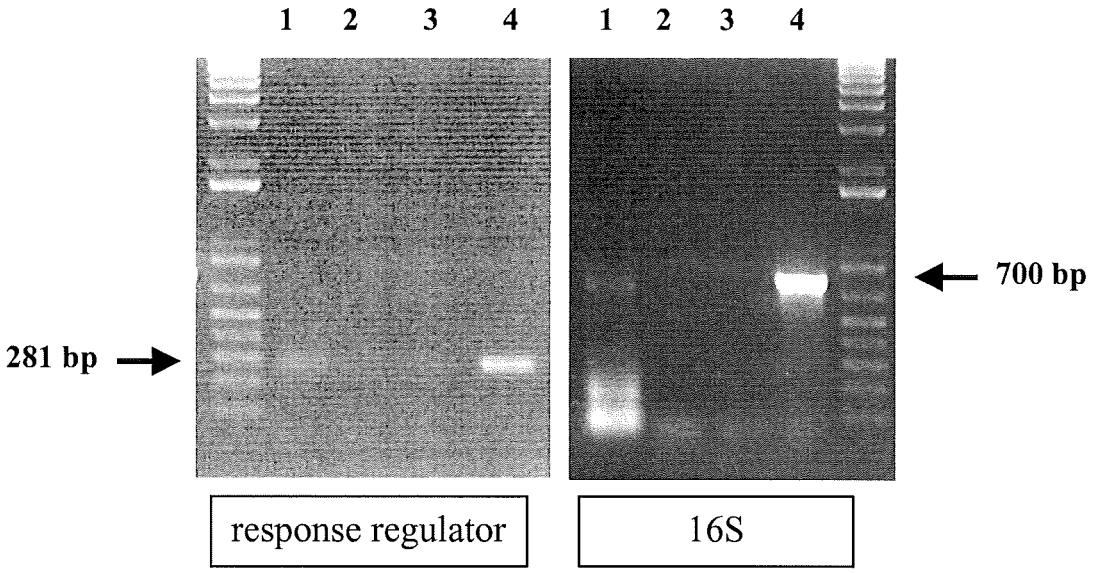


Figure 5.9: RT-PCR showing expression of *rr09* during *in vitro* growth of D39

RNA isolated from cells grown to mid-log phase was used to determine the presence of *rr09* transcript in D39 during *in vitro* growth. Primers RR09F & RR09R designed within the *rr09* gene gave an expected band size of 281 bp. Primers 16SF & 16SR specific for 16S RNA were used as a positive control for RNA detection and resulted in a band of ~700 bp. For both gels, lane 1; PCR using cDNA template from RNA prepared from strain D39, lane 2 PCR using cDNA template prepared from RNA isolated from D39 in the absence of reverse transcriptase, lane 3; PCR with no DNA template, lane 4; PCR using D39 genomic DNA template. The figure shows that a band of the expected size was present in RNA from D39 using primers for *rr09* (lane 1). This band was present in the genomic DNA positive control (lane 4) but absent in the absence of reverse transcriptase and the no template negative controls (lane 2 and 3 respectively).

5.2 *In vivo* characterisation; Role of RR09 in virulence

5.2.1 Intraperitoneal challenge

Prior to any *in vivo* analysis with our murine model of infection, bacteria are passaged via intraperitoneal (i.p.) injection. This generally results in high levels of bacteraemia 24 hr post injection. Bacteria are recovered from the blood and used to produce standard inoculum stocks for subsequent analysis of virulence. 1 x 10^5 CFU of all bacterial strains used for the TCS09 characterisation were given to MF1 mice via i.p. challenge. Survival of mice was monitored as described in section 2.5.9 (figure 5.10).

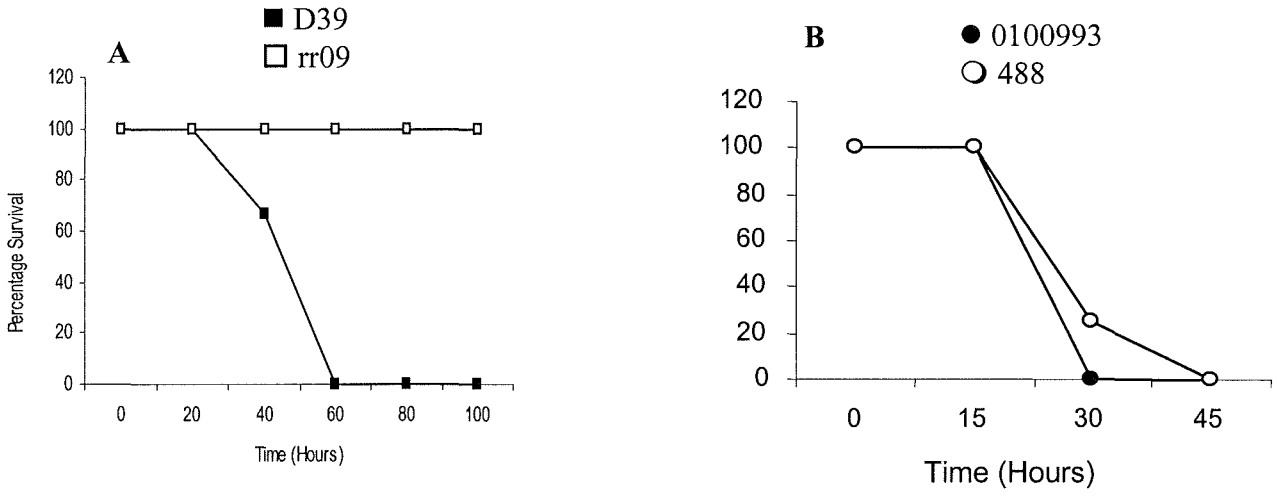


Figure 5.10: Survival of MF1 mice following intraperitoneal challenge

Mice were challenged with 1.0 x 10^5 CFU bacterial strains. Survival was monitored for 1 week. Figure 5.10A shows survival data for serotype 2 strains. $P<0.05$ for survival time of mice challenged with *Δrr09* compared to D39 parental strain. Figure 5.10B shows survival data for serotype 3 strains. No significant difference in survival was seen for the *Δ488* mutant compared to 0100993 parental strain.

Intraperitoneal injection resulted in the appearance of moderate to severe disease symptoms in mice 24 hr post infection, with high levels of bacteraemia (>log 5 CFU ml^{-1} blood) for strains D39, 0100993 and *Δ488*. Such mice all succumbed to infection within 1-3 days and median survival times were 24 hr, 28 hr and 28 hr, respectively. However, i.p. injection with *Δrr09* failed to result in detectable levels of bacteraemia at any time point following infection and all mice survived for over 5 days without showing any symptoms of disease ($P<0.05$), (Figure 5.10A). The challenge dose of *Δrr09* given to mice was increased up to 1000-fold, but mice remained healthy and did not become bacteraemic at any point during the course of infection (data not shown). Several independent *rr09* null mutations in different D39 stocks (confirmed by PCR and sequencing) were evaluated for their virulence following i.p. challenge but none resulted in bacteraemia or symptoms of disease. This indicated that RR09 could be essential in the virulence of D39 in our model of infection.

For all subsequent challenges, passaged bacteria were used for serotype 3 strains 0100993 and *Δ488* only. Non-passaged glycerol stocks were used for all subsequent challenges with D39 and *Δrr09* mutant.

5.2.2 Intranasal challenge

5.2.2.1 Intranasal challenge; Survival and bacteraemia

Strains were analysed for their ability to cause disease in a pneumonia model of infection. Mice were given 1.0×10^7 CFU intranasally and monitored for their ability to survive infection. The doses for serotype 3 were based on previous analysis of optimum bacterial counts required to induce 100 % mortality by intranasal (i.n.) challenge, discussed in section 3.4 1, chapter 3. A 10-fold increase from the usual challenge dose was used for the serotype 2 strains to attempt to overcome the decrease in virulence due to the absence of prior i.p. passage discussed above. Following challenge with D39, 60 % of mice succumbed to infection by 120 hr (median survival time 96 hours), but mice infected with *Δrr09* did not succumb to infection and all these mice survived the entire course of the experiment ($P<0.05$ for survival) (Figure 5.11A). These observations were matched by the levels of bacteraemia at predetermined time points following infection. Levels of bacteria in the blood increased over time with D39 infected mice but no bacteraemia was detected in mice infected with strain *Δrr09* at any of the time points examined (Figure 5.11B). Differences in bacteraemia between wild type and isogenic mutant were

statistically significant for all time points after 0 hr ($P<0.05$). The 100 % mortality usually seen with wild-type D39 infection in our laboratory was not obtained in this study and this could be attributed to the absence of i.p. passage of the bacteria prior to infection.

Intranasal infection of mice with strains in a type 3 background resulted in 100 % mortality with the 0100993 strain and 40 % mortality with the isogenic *Δ488* mutant ($P<0.05$ for survival of *Δ488* compared to parental strain) (Figure 5.11C). Median survival times of mice were 66 hr and 168 hr, respectively (based on mice that survived the entire course of infection being assigned an arbitrary survival time of 168 hr). Blood counts were similar to those seen for type 2 infection, where a steady increase in counts for strain 0100993 was observed but levels of *Δ488* mutant remained very low up to 60 hr post challenge (Figure 5.11D). However, some mice did succumb to infection with *Δ488* so bacterial levels within the blood of such mice must have increased after the time points selected for this experiment (when moribund, these mice had counts of 10^7-10^9 CFU ml^{-1} in their blood).

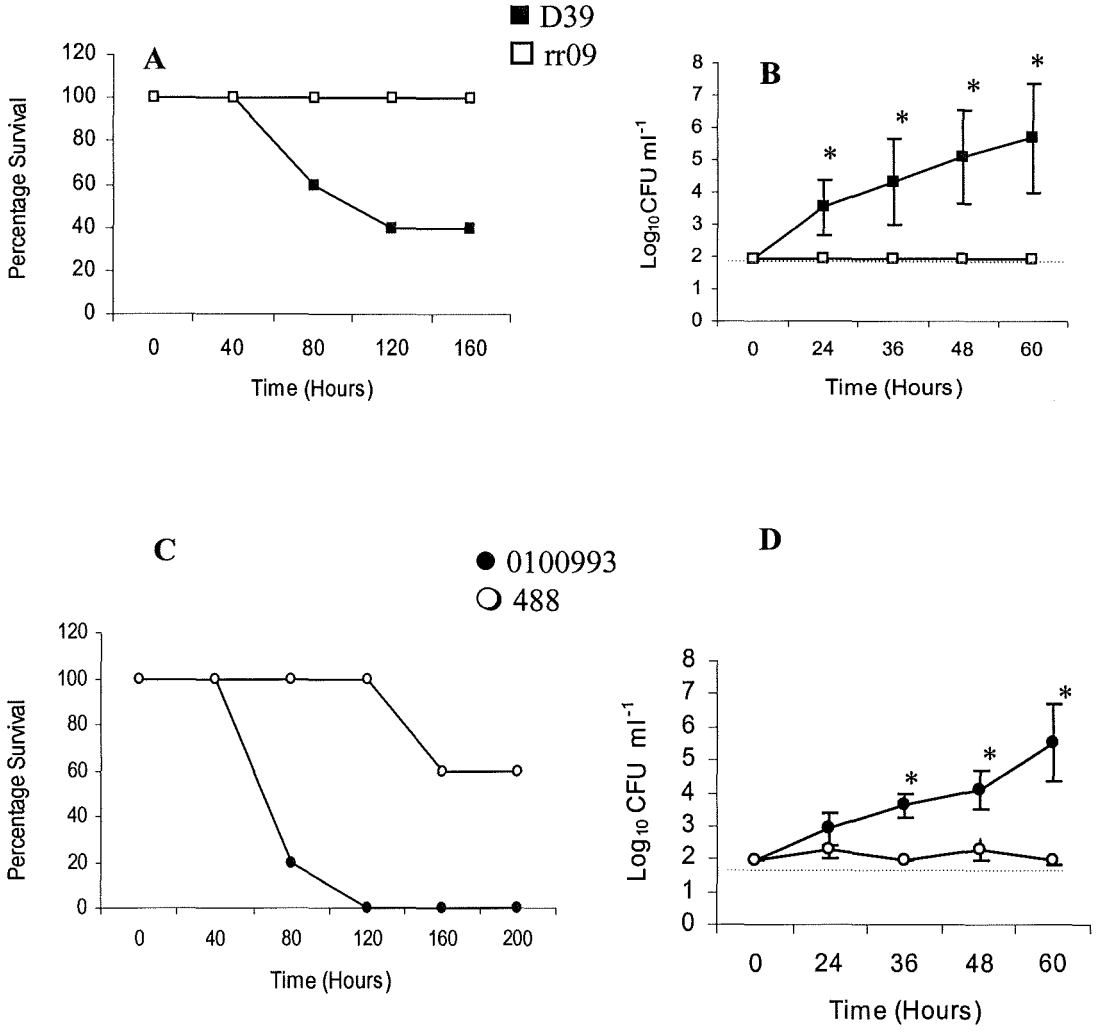


Figure 5.11 Survival and bacteraemia following intranasal challenge with *rr09* mutants and parental strains

Mice were challenged intranasally with 1.0 x 10^7 CFU bacterial strains. Figure 5.11A shows survival of mice following challenge with type 2 strains. P<0.05 for survival of mice infected with *Δrr09* compared to D39 parental strain. Figure 5.11B shows bacteraemia in mice challenged with type 2 strains. P<0.05 (*) for bacteraemia with *Δrr09* compared to D39 parental strain at all time points after 0 hr. Figure 5.11C shows survival of mice following challenge with type 3 strains. Figure 5.11D shows bacteraemia in mice challenged with type 3 strains. P<0.05(*) for survival and bacteraemia at all time points after 24 hr with *Δ488* compared to 0100993 parental strain. The broken line represents the limit of detection.

5.2.2.2 Intranasal challenge; Lung counts

Bacterial loads within the lung tissue were determined 24 and 48 hours post infection with 1 x 10^7 CFU intranasally. Challenge with type 2 strains resulted in low numbers of bacteria within lung tissue both 24 and 48 hr post infection, with median counts of *Δrr09* being below the detection limit (Log_{10} 0.40 CFU mg^{-1} lung tissue) (Figure 5.12A). The difference in counts between wild type D39 and isogenic *Δrr09* mutant at 24 hr and 48 hr were statistically significant ($P<0.05$). Counts in lung tissue following challenge with type 3 strains were relatively high at both time points, showing an increase between 24 hr and 48 hr post infection (Figure 5.12B). This increase in counts at 48 hours compared to 24 hours was only statistically significant for wild type bacteria ($P<0.05$). No significant difference was observed between counts of 0100993 and *Δ488* in the lung tissue at either time point studied.

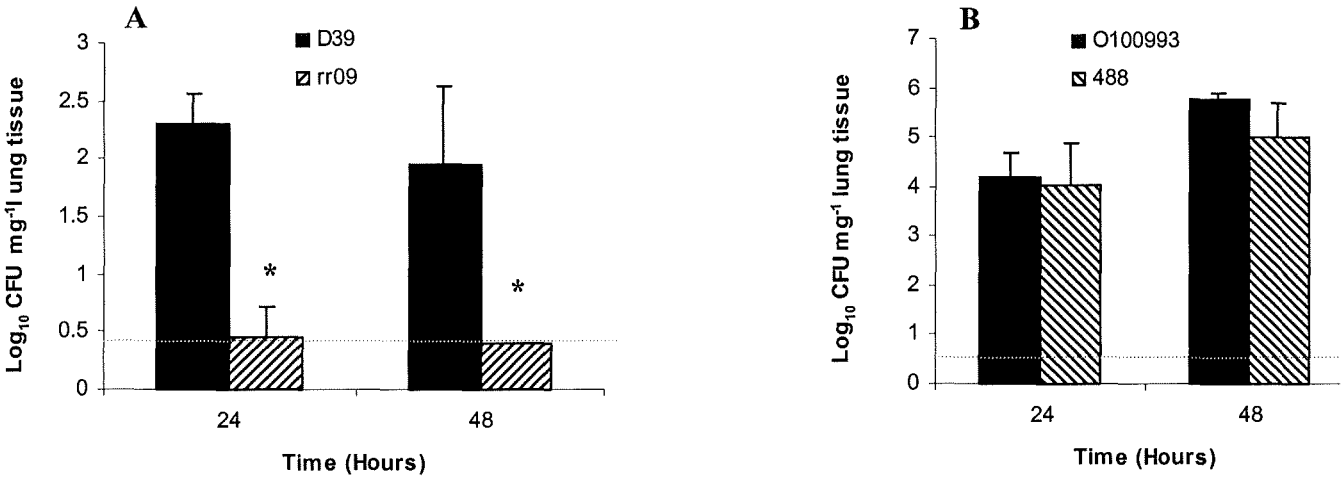


Figure 5.12: Bacterial loads in the lung tissue following intranasal challenge with *rr09* mutants and parental strains

Mice were challenged intranasally with 1.0 x 10^7 CFU. Bacterial loads within the lung tissue was determined 24 hr and 48 hr post infection. Figure 5.12A represents strains on a type 2 background, where filled bars represent D39 wild type and hatched bars represent *Δrr09* mutant. $P<0.05$(*) for wild type values compared to mutant at both time points. Figure 5.12B represents strains on a type 3 background, where filled bars represent 0100993 wild type and hatched bars represent *Δ488* mutant. No differences were found between wild type and mutant lung counts at either time point studied. The broken line represents the limit of detection. The lower counts in the lungs for the D39 parental strain compared to the 0100993 parental strain are likely to be due to absence of passaging in the former strain.

5.2.3 Intravenous challenge

Bacterial strains were inoculated directly into the veins of mice to determine their ability to grow in the blood. Counts at 0 hr confirmed the bacterial dose administered and subsequent levels of bacteraemia were examined at pre-determined time points. Mice were also monitored for survival. 40 % of mice given D39 survived the infection, while all mice injected with *Δrr09* survived (Figure 5.13A). Median survival time for mice given D39 was 84hr. The experiment was allowed to proceed for 7 days, after which all surviving animals were given arbitrary survival times of 168 hr. On this basis, the difference in survival between D39 wild type and isogenic *Δrr09* was highly significant ($P<0.005$). 8 hr post infection, counts in the blood of mice administered D39 and *Δrr09* had fallen significantly. Levels of D39 began to increase after this time point, albeit slowly, whilst *Δrr09* counts remained below the detection limit (Log_{10} 1.92 CFU ml^{-1}) at all time points studied (Figure 5.13B). The initial decrease observed in bacteraemia is likely to be due to the absence of bacterial passaging prior to infection as passaged wild-type D39 bacteria do not display this decrease in the blood following i.v. challenge (chapter 7, section 7.3.2). Counts of D39 presumably increased within the blood at time points later than those examined as 60 % of mice eventually succumbed to infection, and when moribund, these mice had counts of 10^7-10^9 CFU ml^{-1} in their blood.

All mice infected with strains 0100993 and *Δ488* succumbed to infection very quickly and both had median survival times of 36 hr (Figure 5.13C). Bacterial counts in the blood of mice administered 0100993 and *Δ488* were very similar at all time points examined (Figure 5.13D). No significant differences were observed between mutant and wild type for bacteraemia or murine survival. After 36 hr, no further time points were studied for type 3 strains as many of the mice had become moribund by this stage and had to be culled.

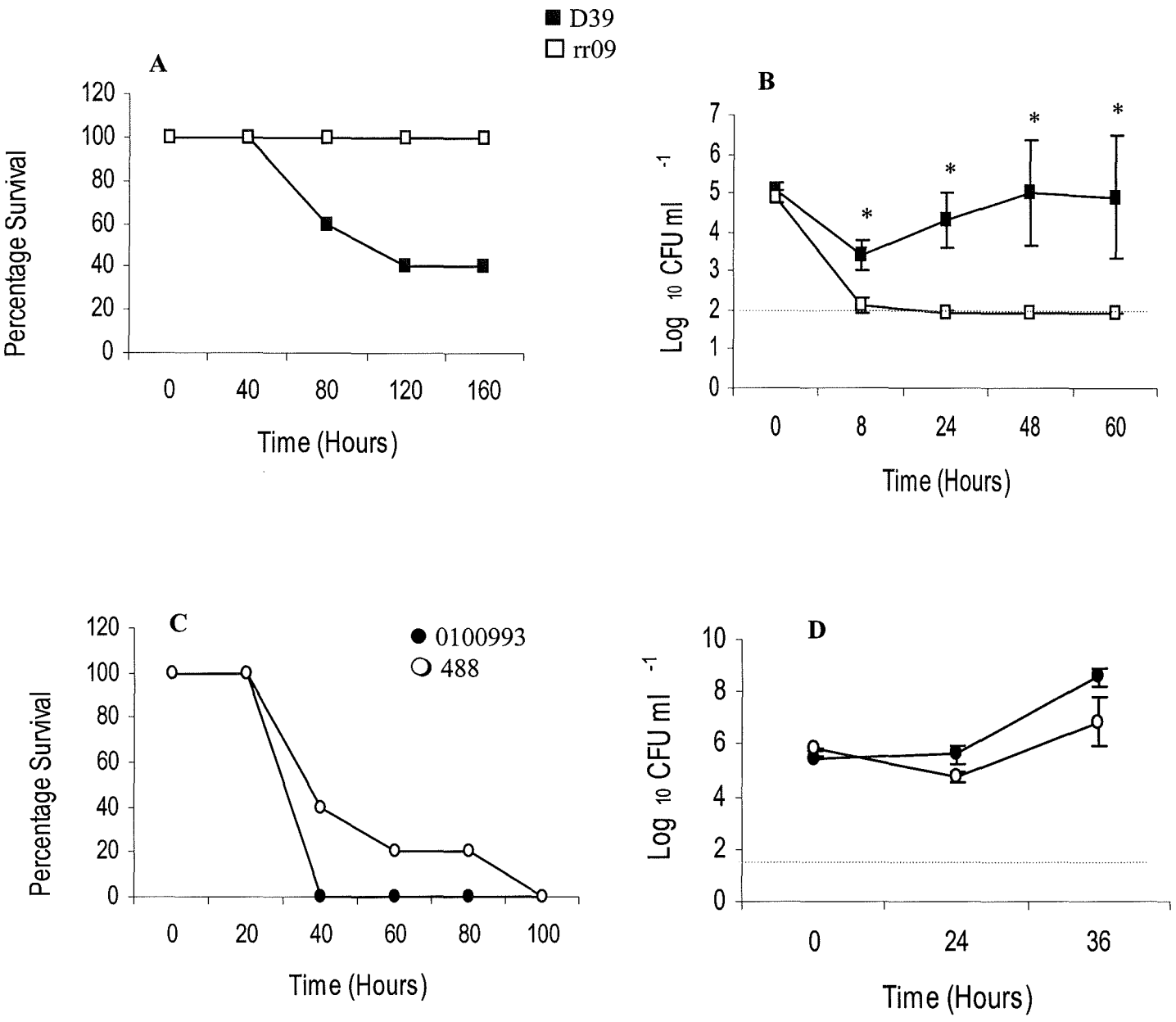


Figure 5.13: Survival and bacteraemia following intravenous challenge with *rr09* mutants and parental strains

Mice were challenged intranasally with 1.0 x 10^6 CFU bacterial strains. Figure 5.12A shows survival of mice following challenge with type 2 strains. P<0.005 for survival of mice infected with *Δrr09* compared to D39 parental strain. Figure 5.13B shows bacteraemia in mice challenged with type 2 strains. P<0.05(*) for bacteraemia of mice infected with *Δrr09* compared to D39 parental strain at all points after 0 hr. Figure 5.13C shows survival of mice following challenge with type 3 strains. Figure 5.13D shows bacteraemia in mice challenged with type 3 strains. No differences were seen in survival or levels of bacteraemia between mice infected with 0100993 or *Δ488* mutant. The broken line represents the limit of detection.

5.3 Further *in vitro* characterisation

Several assays were performed in an attempt to determine why the *rr09* mutants had reduced virulence.

5.3.1 Haemolytic activity

All strains were characterised for their ability to induce lysis of sheep red blood cells. Complete haemolysis was not observed with any of the negative controls, including the pneumolysin null mutant, *Δpln*, treated with deoxycholate (DOC), indicating that any lysis observed was largely due to pneumolysin and not other pneumococcal haemolysins. All deoxycholate-lysed cultures, including mutants, showed complete haemolysis of sheep red blood cells up to a 1:8 dilution. No haemolysis was seen when lysed cultures were diluted 1:32 (table 5.1). The dilutions ranged from neat (un-dilute) to 1:128. These data indicate that strains D39, *Δrr09*, 0100993 and *Δ488* all express similar amounts of active pneumolysin.

	Dilution of culture resulting in haemolysis		
	Complete lysis	**Partial lysis**	**no lysis**
All negative controls	No haemolysis	No haemolysis	All dilutions
D39 (- DOC)	No haemolysis	No haemolysis	All dilutions
D39 (+ DOC)	Neat-8-fold	8-16-fold	16-32-fold
Δrr09 (- DOC)	No haemolysis	No haemolysis	All dilutions
Δrr09 (+ DOC)	Neat-8-fold	8-16-fold	16-32-fold
01009993 (- DOC)	No haemolysis	No haemolysis	All dilutions
0100993 (+ DOC)	Neat-8-fold	8-16-fold	16-32-fold
Δ488 (- DOC)	No haemolysis	No haemolysis	All dilutions
Δ488 (+ DOC)	Neat-8-fold	8-16-fold	16-32-fold

Table 5.1 Haemolytic activity of cultures

All bacterial strains were analysed for their ability to induce lysis of sheep red blood cells without (- DOC) and with (+ DOC) treatment with 0.04 % deoxycholate. Negative controls included a pneumolysin null mutant, PBS alone, media alone and media with 0.04 % deoxycholate.

5.3.2 Transformation efficiency

Strains were analysed for their ability to take up DNA from their surroundings using a standard transformation protocol. No significant differences were found in the transformation efficiencies of either mutant when compared to its parental strain, although the transformation efficiency of *Δrr09* was lower than its parental stain (table 5.2). A >100-fold increase was seen in the ability of encapsulated type 2 strains to take up extracellular DNA compared to encapsulated type 3 strains (median transformation efficiencies of 69.4 and 0.42 transformants per μg DNA respectively). This is likely to be due to the thick mucoid capsule characteristic of the serotype 3 strain used, as presence of any capsule is known to greatly reduce transformation efficiency in *S. pneumoniae* (Yother *et al.*, 1986).

	Transformation Efficiency (Total transformants per ug DNA)		
	mean	med	SEM
D39	69.4	69.4	32.3
Δrr09	38.7	38.7	24.3
0100993	0.42	0.04	0.388
Δ488	0.36	0.04	0.322

Table 5.2 Transformation efficiency of bacterial strains

Bacterial cultures were grown to OD 600 nm 0.1 and transformed with chromosomal DNA encoding streptomycin resistance in the presence of CSP1. Transformants were selected on streptomycin (150 μg ml^{-1}). All cultures had equal total cell counts following transformation as determined by viable cell counting. The table shows mean and median values for experiments performed in duplicate on 3 separate occasions.

5.4 Expression analysis

5.4.1 Optimisation of RNA preparation from bacterial strains

For all expression analyses, RNA was isolated from cultures grown to mid log phase (OD 600 nm 0.6-0.7) in BHI media at 37 ° C. Mutant *Δrr09* was grown in the absence of erythromycin to prevent detection of differentially expressed genes due to the presence of antibiotic alone. Previous experiments have shown that the *Δrr09* mutant phenotype is stable in the absence of erythromycin selection (section 5.1.4). Viable cell counts from cultures from which RNA was isolated were determined, although previous experiments comparing *in vitro* growth of strains D39, *Δrr09*, 0100993 and *Δ488* indicated that at any given optical density during exponential growth would result in equal cell counts (section 5.1.3). Concentration of isolated RNA was determined by agarose gel electrophoresis using an RNA standard.

The isolation of pure, intact RNA is crucial to the success of gene expression analysis. Bacterial RNA is known to be less stable than eukaryotic RNA making isolation relatively more difficult. Furthermore, *S. pneumoniae*, being a Gram-positive organism, has a thick cell wall that must be broken down as quickly as possible to avoid RNA degradation. Several commercially available kits are available for isolation of bacterial RNA and these include reagents, such as RNA Protect® (Qiagen) which has been formulated to help prevent degradation of RNA immediately upon harvesting bacterial cultures. However, using this reagent significantly decreased the final yield of RNA obtained from individual preparations up to 10-fold (figure 5.14). The reason for this loss of RNA yield is unknown.

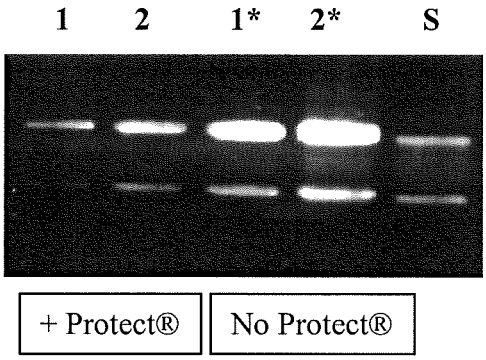


Figure 5.14 Effect of RNA Protect® on pneumococcal RNA yields

RNA was prepared from 2 independent cultures (1 & 2) of D39 grown to mid-exponential phase in the presence and absence of RNA Protect®. Each culture was divided into 2 x 5 ml volumes, where RNA from one x 5 ml from each was isolated in the presence of RNA Protect® according to manufacturer's instructions (lanes 1 and 2 respectively). RNA was also isolated from the remaining 2 x 5 ml in the absence of RNA Protect® (lanes 1* and 2* respectively). The gel shows the greatly increased RNA yield obtained in the absence of RNA Protect®. Lane S represents RNA standard from *E. coli*, with a total of 1.6 µg of RNA loaded onto the gel. The 2 discrete bands seen for each sample represent 23S (upper band) and 16S (lower band) RNA.

For microarray hybridisations a good RNA yield is required to allow sufficient data analysis with appropriate repetitions from each RNA preparation. Isolation of RNA using RNA Protect® or other commercially available stabilising reagents was thus avoided. Immediate snap freezing of cultures prior to harvesting bacterial cells may help preserve the RNA from degradation, although could also have an effect on the final yield of RNA obtained. RNA preparations from cultures frozen slowly at - 70 $^{\circ}$ C were compared with cultures flash frozen in liquid nitrogen (figure 5.15). No difference was observed in RNA yields between the methods of freezing, so an RNA isolation protocol was developed using cultures that were flash frozen in liquid nitrogen prior to isolation, with the aim of preserving RNA from degradation as much as possible.

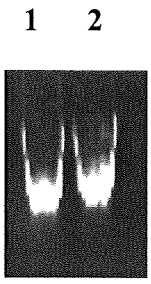


Figure 5.15: Effect of freezing methods on RNA yields

RNA was prepared from a culture of D39 grown to mid-exponential phase. The culture was divided into 2 x 5 ml volumes, with 1 x 5 ml being frozen slowly at - 70 ° C and the other being immersed immediately in liquid nitrogen. RNA was isolated from both cultures using the same protocol. Lane 1 represents RNA isolated from culture frozen at - 70 ° C and lane 2 represents RNA isolated from cultures flash frozen in liquid nitrogen. No difference in final yield of RNA was observed.

5.4.2 Microarray analysis

Microarray analysis was performed using RNA isolated only from D39 and *Δrr09* cultures grown to mid-exponential phase in BHI. Hybridisations were performed using RNA from the same preparation but with reverse labelling with Cy3 and Cy5. Thus each RNA preparation was analysed in duplicate. Figure 5.16 summarises the protocol used for microarray analysis.

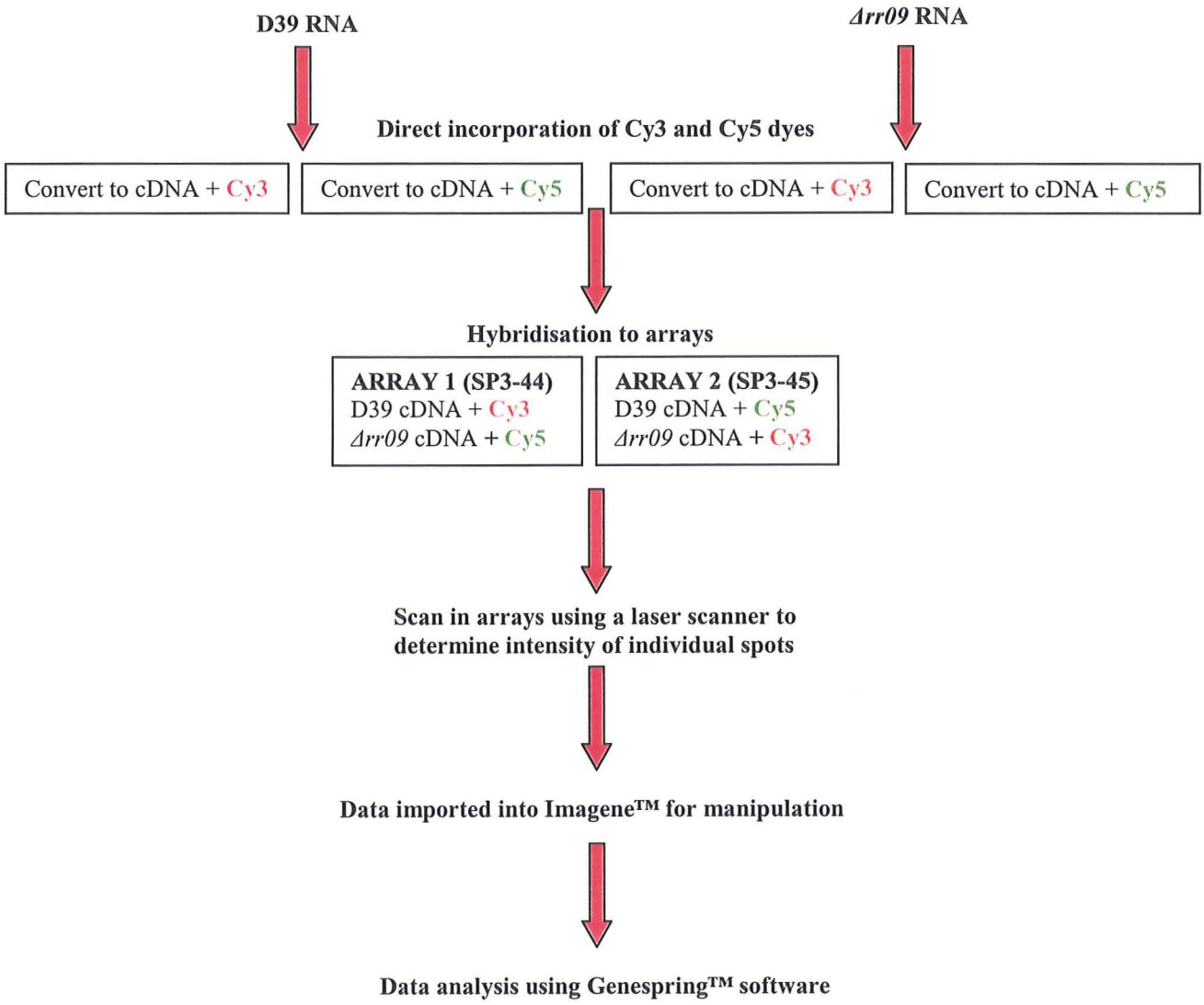


Figure 5.16: Summary of procedure used for mircoarray analysis of D39 and *Δrr09*

Prior to analysis, data were imported into Imagene™ (BioDiscovery) to visually inspect the computer-generated grid and spot alignment. During this inspection it was noted that many of the spots / genes (between 10-20 %) were very poorly expressed or completely absent, although there was no way of determining if this was due to lack of expression of absence / loss of RNA transcript during preparation. Data were analysed using Genespring™ software. This software determines the relative amounts of all gene transcripts from the strains by measuring the individual signal intensities of the Cy3 and Cy5 dyes and calculating signal ratios. Data was imported into Genespring™ such that a ratio of 1.0 indicates that the intensity of signal is equal for both samples, a ratio of <1.0 indicates lower signal intensity in the mutant strain and a ratio of >1.0 indicates increased signal intensity in the mutant strain, relative to wild-type. The software was also set up to allow spot intensities to be colour co-ordinated to help identify differential gene expression. Using this system, gene transcripts present in equal concentrations in D39 and *Δrr09* (a ratio of 1.0) result in a yellow spot. Blue spots represent genes that have reduced expression in the mutant strain (a ratio of <1.0), while red spots represent genes that have increased expression in the mutant strain compared to wild-type (a ratio of >1.0). Each gene is present on the microarray in duplicate and the intensity of individual replicates of genes for both wild-type and mutant can be determined.

The complete data set generated from one of the microarray experiments is represented in figure 5.17 as a scatter plot. Wild type genes (x-axis) were plotted against mutant genes (y-axis) and this was used to examine the quality of data generated and to identify genes differentially expressed in the *Δrr09* mutant D39 compared to wild-type D39. Most genes can be seen to cluster together around the central green diagonal line which represents a ratio of 1.0, indicating that the hybridisations worked well and most genes detected are expressed the same in both strains. Diagonal green lines above and below this central line represent two-fold increased and decreased gene expression respectively in *Δrr09* compared to isogenic D39 parental strain. During analysis, genes with an intensity of below 500 were considered too low for reliable analysis and intermediate intensities of 500-1000 were considered but with caution. For most genes present, the spot intensity was above 1000 which illustrates reliable expression data. Only genes with altered expression in both arrays, and those with combined signal intensities of > 500 were considered for further analysis.

Within the data presented as a scatter plot, 2 spots were observed that had greatly reduced expression in the mutant compared to D39 wild-type. These spots were coloured blue and were located away from the rest of the data and outwith the line representing a two-fold decrease in expression. Furthermore, these spots were identified as having reduced expression by > 2-fold in both arrays. Other genes with altered expression in the mutant compared to wild-type will be discussed below and included in later figures.

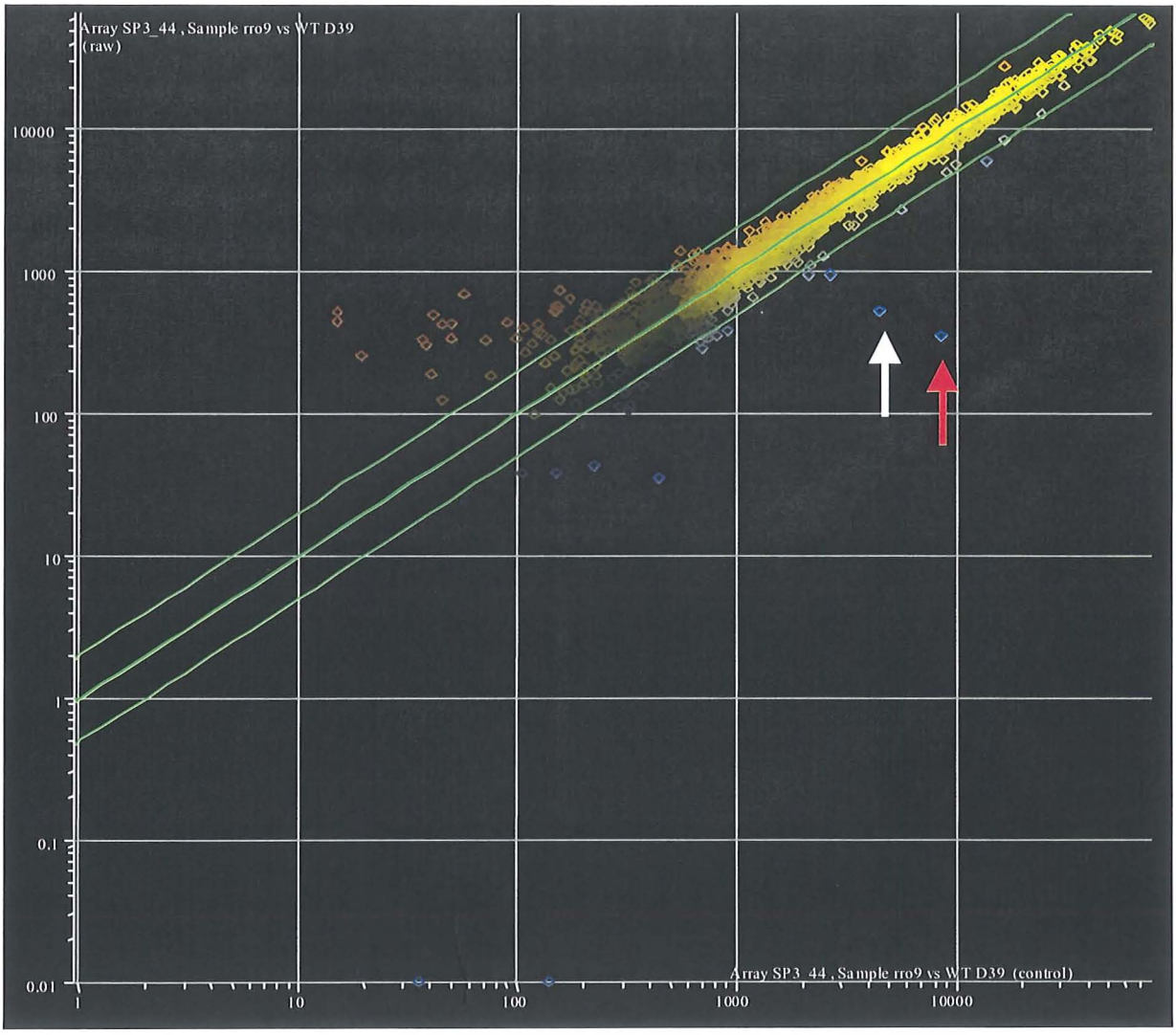


Figure 5.17 Microarray data comparing gene expression in *Δrr09* mutant with isogenic D39 parental strain

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RNA was prepared from bacterial cultures grown to mid-exponential phase in BHI media at 37 ° C. cDNA was prepared from the RNA extracts and labelled directly with Cy3 (*Δrr09*) or Cy5 (D39). Labelled cDNA preparations were combined and hybridised to the array overnight. The scatter plot illustrates the spot intensities of all individual genes detected. D39 data (x-axis) is plotted against *Δrr09* data (y-axis). Each spot represents the combined intensities of labelled cDNA from mutant and wild-type for any given gene. Yellow spots indicate similar expression of individual genes on both bacterial strains, with a ratio of ~ 1.0. These genes lie close to the central, diagonal line. Blue spots represent genes with decreased expression in the mutant compared to wild type and red spots are genes with increased expression in *Δrr09*. If spots lie outside the upper and lower diagonal lines they represent genes expressed in *Δrr09*greater than or less than 2-fold expression, respectively, compared to wild-type expression[1]. The further away from the lines, the bigger the difference in expression. The scatter plot illustrates that the hybridisations worked well as most spots lie within the diagonal lines and within the upper region of the plot, so have reliable spot intensities of >1000. Most of the genes detected have similar levels of expression in both bacterial strains. The arrows represent genes of interest that have decreased expression in *Δrr09* compared to D39. The red arrow represents the *rr09* gene, further confirming the null mutation within this gene for strain *Δrr09*. The white arrow represents the gene encoding a subunit (IIB) of a bacterial phosphotransferase (PTS) system previously not associated with TCS09. Several other genes lying on or immediately outside the lower diagonal line were also characterised and these are discussed in the text. Only genes with altered expression in both arrays, and those with combined signal intensities of > 500 were considered for analysis.

[1] As no preliminary experiments were performed to assess the variation in signal intensity associated with a single cDNA preparation, the lines used to represent 2-fold increase / decrease in variation are only an approximate representation of these values. Thus data lying close to these lines should be regarded with caution.

Further analysis of the 2 spots showing decreased expression in *Δrr09* indicated that one corresponded to the *rr09* gene itself (sp0661), further confirming the null mutation. Furthermore, this served as a true negative control for subsequent analysis. The second spot was identified as a component (IIB) of a bacterial phosphotransferase (PTS) system (sp0061). Within the published R6 and TIGR4 sequences, this gene lies within a cluster of other genes encoding PTS components or related genes (figure 5.18). In the sequenced TIGR4 genome, this gene was annotated as a component of a PTS involved in sorbose uptake (Tettelin *et al.*, 2001).

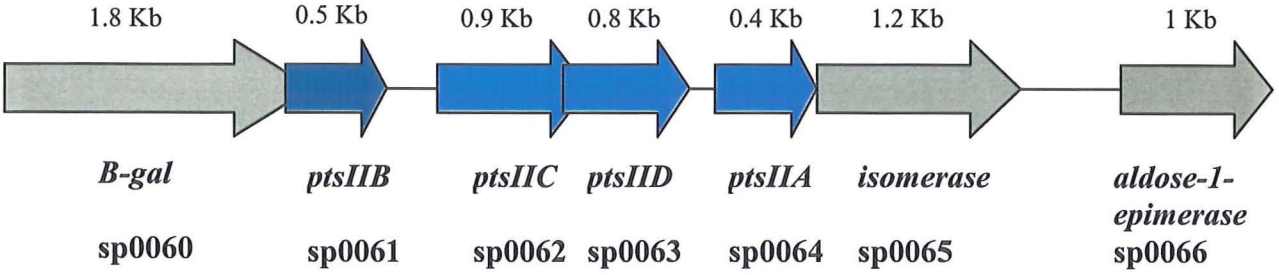

Figure 5.18 PTS locus and surrounding genes

The figure illustrates the *ptsIIB* gene (in dark blue) found to have significantly decreased expression in *Δrr09* compared to D39. Other genes that are part of the PTS system are shown (light blue), together with surrounding genes *β-galactosidase*, *sugar isomerase domain agaS* and *aldose-1-epimerase* (all in grey). The gene names corresponding to the TIGR4 nomenclature are given in bold and approximate gene lengths are given. The *β-gal* & *ptsIIB* genes overlap by 3 bp, *ptsIIC* & *ptsIID* overlap by 13 bp and *ptsIIA* & *isomerase* overlap by 1 bp. The regions between genes *ptsIIB* & *ptsIIC*, *ptsIID* & *ptsIIA* and *isomerase* and *aldose-1-epimerase* are 28, 7 and1116 bp, respectively, indicating that the genes may form an operon. All gene annotations and lengths are based on those of the TIGR4 genome (http://www.tigr.org). No rho-independent terminator sites were found following any of the genes shown.

The ratio values (figure 5.20) and expression levels (figure 5.21) of genes shown in figure 5.18 were determined and all showed decreased expression in the mutant compared to wild type (ratio <0.1), although the expression levels of some of the genes was below the 500 limit cut off. No expression of *ptsIID* was detected in either D39 or *Δrr09*, so no data for this gene are available. Spots that lay on or just below the lower line, representing a 2-fold decrease in expression in *Δrr09* were also analysed in further detail[2]. This highlighted decreased expression of several other genes that are located close to one another on the TIGR4 genome sequence and may form an operon or functionally related group of genes (figure 5.19). The function of many of these genes is unknown, although one has been annotated as a putative general stress protein (sp1804).

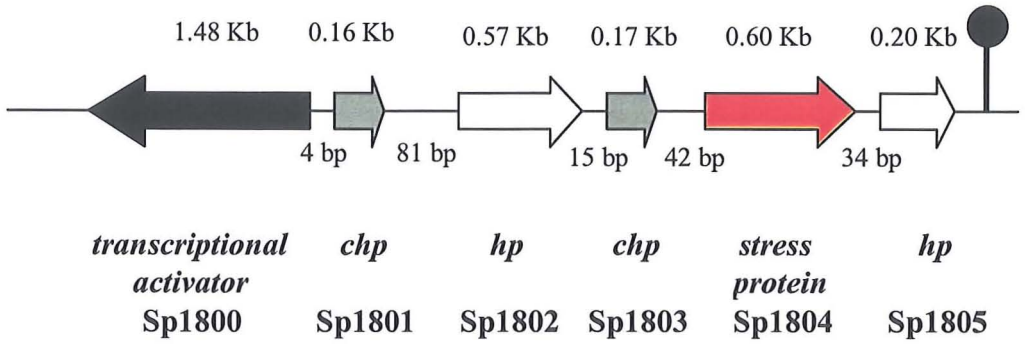

Figure 5.19 Group of genes of unknown function with decreased expression in *Δrr09* compared to D39

The figure illustrates a putative operon comprising largely of genes with unknown function that have lower levels of expression in the *Δrr09* mutant compared to isogenic parental strain (see figures 5.20 and 5.21). Conserved hypothetical proteins (chp) are shown in grey and hypothetical proteins (hp) are shown in white. The gene annotated as a putative general stress protein is shown in red. A putative transcriptional activator, which may or may not be involved in regulating the illustrated genes is shown in black. This is the only gene shown that is encoded on the complementary DNA strand. The gene names corresponding to the TIGR4 nomenclature are given in bold and approximate gene lengths are given. None of the encoding regions of the genes overlap, although the regions between genes are relatively small and are provided beneath the gene layout. All gene annotations and lengths are based on those of the TIGR4 genome (http://www.tigr.org). The closed circle represents a rho-independent termination site.

[2] See footnote, p217

The ratio values and expression levels of all the genes described above was combined and analysed statistically to determine if the genes were expressed at lower levels in the *Δrr09* mutant compared to D39 wild-type. Data for all the identified pneumococcal response regulator genes was also collected and analysed. The ratio values for each gene are shown in figure 5.20. Values close to 1.0 illustrate genes that are expressed at similar levels between wild-type and mutant. Expression levels for several genes were too low to be considered so data is not available for these (*ptsIID*, *rr07*, *rr11*, *rr12*, *sp1800*, *sp1803* and *sp1806*). Genes that had ratio values of <1.0 are shown in table 5.3. The 16S and 23S genes also had ratios of significantly <1.0, although the mean ratios for both these genes is >0.9 so these genes were not included in the table. Of particular interest is the *rr14* gene which had a ratio of >1.0 (P=0.01), indicating it had significantly increased expression in the *Δrr09* mutant compared to D39 wild-type. However, when intensity values were compared, this increase was not found to be statistically significant (figure 5.22).

Comparisons of intensity between mutant and wild-type cDNA preparations, for all the genes discussed above, is provided in figure 5.21. To allow differences in expression of the above genes to be appreciated, the expression intensity for each of the response regulator genes is shown in a separate figure (figure 5.22). This is due to the comparatively high levels of expression of several of the response regulator genes (*rr02* and *rr05*) that are outwith the scale used for figure 5.20. All of the genes discussed above (table 5.3) as having a ratio of <1.0, had lower levels of intensity of expression in the mutant compared to wild-type (figure 5.21). Statistically significant differences in expression were only observed for *rr09* (P=0.008), *ptsIIB* (P=0.001), *isomerase agaS* (P=0.016), *sp1801* (P=0.048), *sp1804* (P=0.001) and *sp1805* (P=0.037). For other genes the mean intensity levels may be too low to obtain accurate results or the variation between individual values too high to result in statistical significance. None of the other pneumococcal response regulator genes have significant differences in expression between wild-type and mutant strains. It is interesting that *rr07*, *rr11* and *rr12* all have expression intensities below the 500 threshold in both strains and that the intensity for *rr03* is only just above this threshold. For the figures displaying expression intensity, data for 16S and 23S genes have been omitted as these genes gave very high levels of expression, as expected, (mean wild-

type values of ~50,000 and 71,000 respectively), which would have been over the scales represented.

Genes surrounding the *rr09* gene (*msrA*, *hk09*, *chp* and *zmpB*) do not appear to have reduced expression in the mutant compared to wild-type, although the *hk09* and *zmpB* genes have mean intensities only just above the 500 threshold limit. This data indicates that *rr09* does not regulate genes immediately upstream or downstream of *tcs09*. Further data and discussion alluding to expression of the *zmpB* gene by TCS09 are presented in chapters 7 and 8.

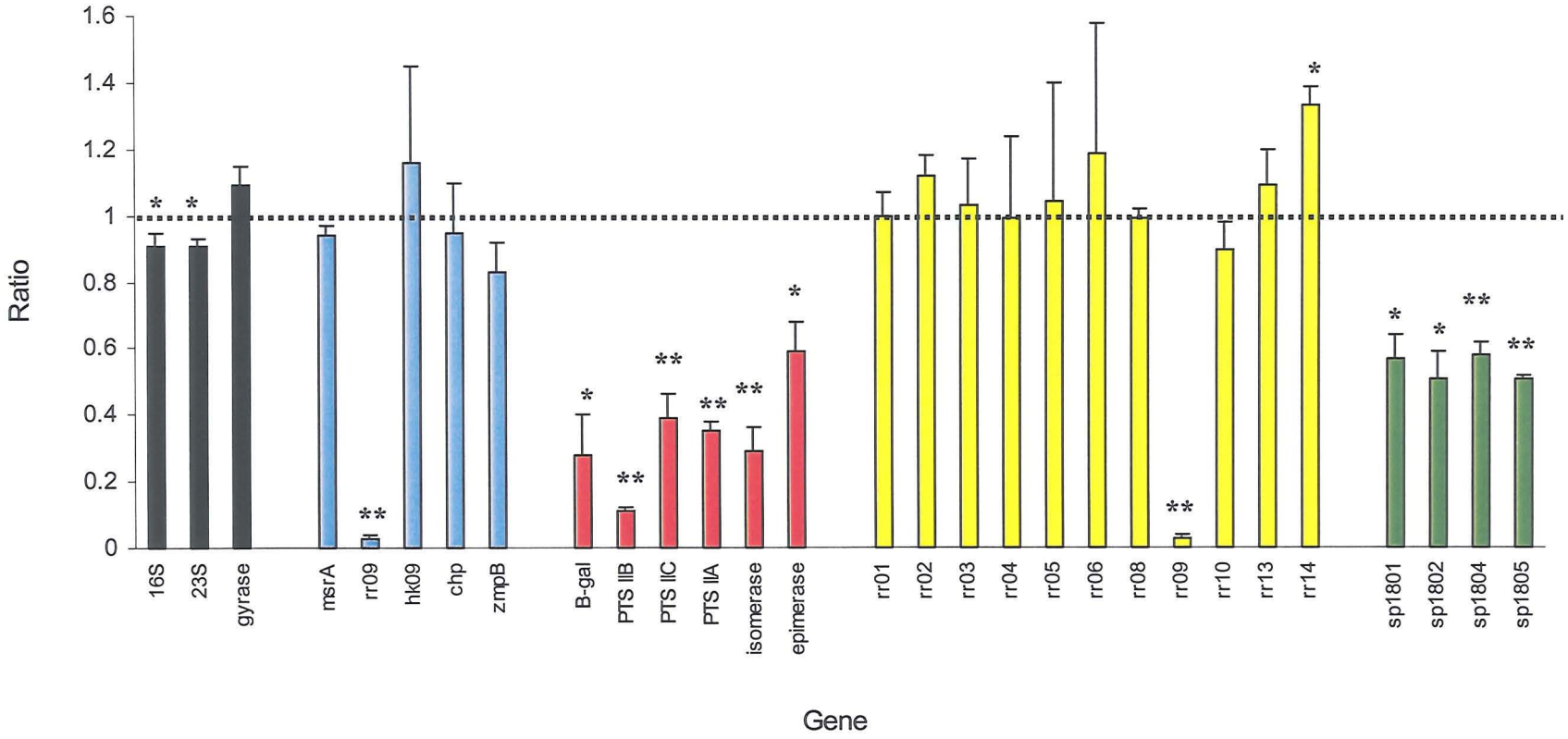

Figure 5.20: Ratio of selected genes in *rr09* compared to D39

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Figure 5.20: Ratio of selected genes in *Δrr09* compared to D39

RNA prepared from bacterial strains grown to mid-exponential phase in BHI media was converted to cDNA with the direct incorporation of either of the fluorophores Cy3 or Cy5. D39 cDNA labelled with Cy3 was hybridised on one array with *Δrr09* cDNA labelled with Cy5. Hybridisation to a second array was also set up using reverse labelling of cDNA (D39 cDNA-Cy5, *Δrr09* cDNA-Cy3). Arrays were scanned using a laser scanner and data was analysed using Genespring™ version software. The figure represents the ratios obtained. A ratio of 1.0 indicates that genes had similar levels of expression in D39 and *Δrr09*. A ratio of <1.0 illustrates genes that are expressed at lower levels in the mutant, and the lower the value the greater the difference in expression. A ratio of >1.0 highlights genes that have increased expression in the *Δrr09* mutant compared to D39 parental strain. The dashed line represents a ratio of 1.0. Genes that have a ratio significantly different from 1.0, as determined by a 2-sample t-test, are indicated by a single asterix (*, $P<0.05$) or a double asterix ($P<0.005$)[3]. Genes are grouped into colour co-ordinated sets. Those in black represent positive controls for gene expression (16S RNA, 23S RNA and DNA gyrase). Genes of the TCS09 locus and genes immediately surrounding this region are given in blue. The PTS and surrounding genes are shown in red. All response regulator genes (including *rr09* for completeness) are coloured yellow. Genes from *sp1801-sp1805* are shown in green. Genes *ptsIID*, *rr07*, *rr11*, *rr12*, *sp1800*, *sp1803* and *sp1806* are absent as expression levels were too low to obtain reliable data.

[3] See footnote p217

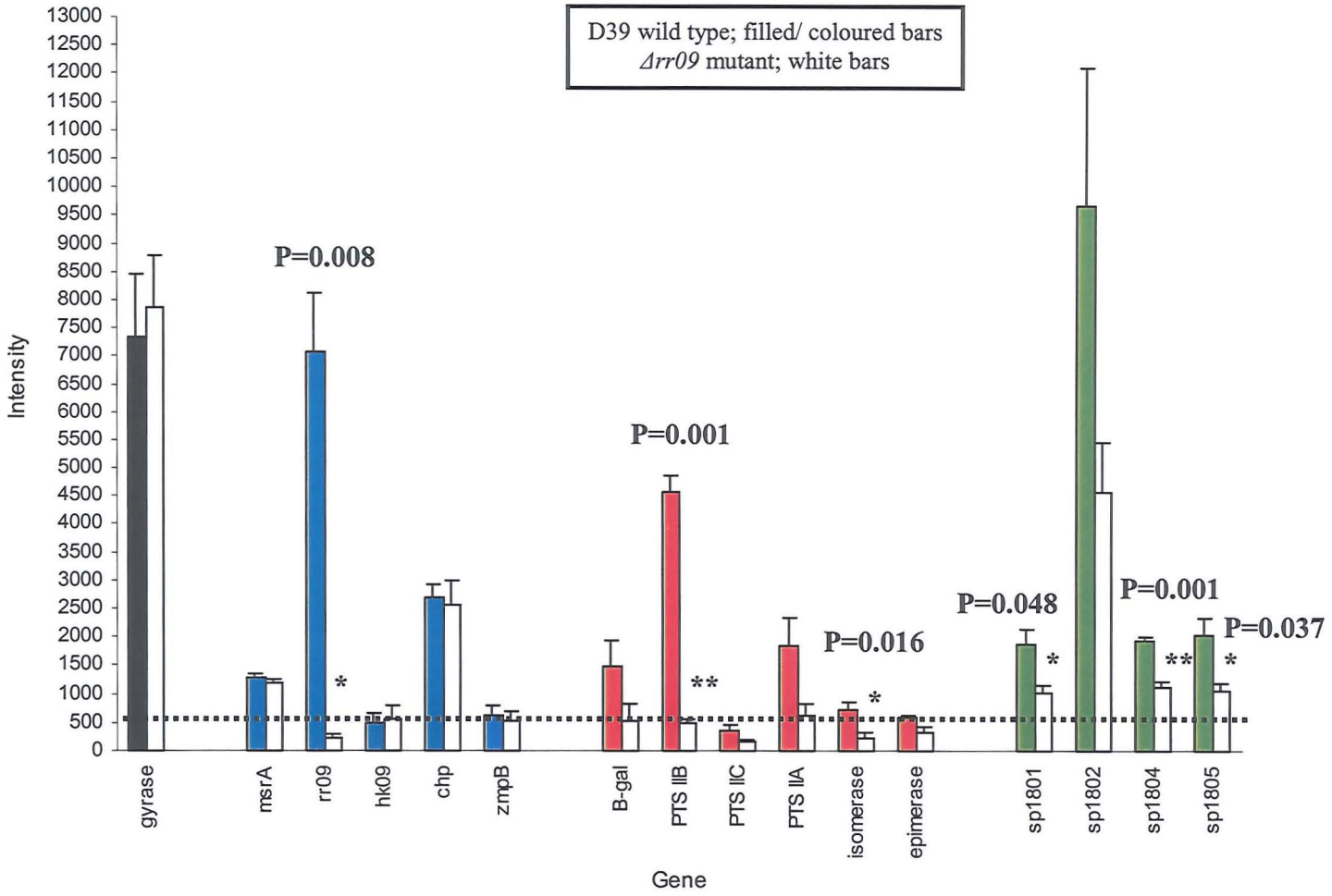

Figure 5.21: Expression of selected genes in *rr09* compared to D39

Legend is provided on the following page

Figure5.21: Expression of selected genes in *Δrr09* compared to D39

RNA prepared from bacterial strains grown to mid-exponential phase in BHI media was converted to cDNA with the direct incorporation of either of the fluorophores Cy3 or Cy5. D39 cDNA labelled with Cy3 was hybridised on one array with *Δrr09* cDNA labelled with Cy5. Hybridisation to a second array was also set up using reverse labelling of cDNA (D39 cDNA-Cy5, *Δrr09* cDNA-Cy3). Arrays were scanned using a laser scanner and data was analysed using Genespring™ version software. The figure represents the expression intensity of selected genes discussed in the text[4]. The dashed line represents an intensity of 500. Expression below this limit is regarded as too low for accurate data analysis. Genes in the *Δrr09* mutant that have expression intensities that are significantly different from D39 wild-type, as determined by a 2-sample t-test, are indicated by a single asterix (*, $P<0.05$) or a double asterix ($P<0.005$). Wild-type genes are grouped into colour co-ordinated sets. All mutant genes are shown in white but are displayed adjacent to the corresponding wild-type gene. DNA gyrase is shown in black to represent a positive control for gene expression. Genes of the TCS09 locus and genes immediately surrounding this region are given in blue. The PTS and surrounding genes are shown in red. Genes from sp1801-sp1805 are shown in green. Genes *ptsIID*, *sp1800*, *sp1803* and *sp1806* are absent as expression levels were too low to obtain reliable data. Data for 16S and 23S genes have been omitted as these genes gave very high levels of expression, as expected, (mean wild-type values of ~50,000 and 71,000 respectively), which would have been over the scale represented.

[4] See footnote p217

Genes that had ratios of < 1.0 in the *Δrr09* mutant compared to D39 are summarised in table 5.3.

TIGR4 name	Other name / gene description	P value
Sp0661	RR09 component of TCS09, function unknown	0.000**
Sp0060	B-galactosidase enzyme, hydrolyses lactose	0.009*
Sp0061	PTSIIB, component of putative sorbose PTS	0.000**
Sp0062	PTSIIC, component of putative sorbose PTS	0.003**
Sp0064	PTSIIA, component of putative sorbose PTS	0.000**
Sp0065	Isomerase agaS enzyme, catalyses isomerisations	0.003**
Sp0066	Aldose-1epimerase enzyme, cataslyses isomerisations	0.019*
Sp1801	None, homology to proteins of unknown function in *L. lactis*, *E. faecalis*, *S.pyogenes* and *B. subtilis*	0.009*
Sp1802	None, hypothetical protein	0.009*
Sp1804	Stress protein	0.002**
Sp1805	None, hypothetical protein	0.000**

Table 5.3 Genes having a ratio of <1.0

With the exception of the *rr09* gene, all genes that had ratios of <1.0, indicating reduced expression in the *Δrr09* mutant were clustered at 2 regions of the genome. The first set of genes appears to be components or genes related to the functioning of a PTS system for sugar (sorbose) uptake. The remaining genes are largely of unknown function, although one encodes a putative stress protein. Gene identifications are based on the TIGR4 genome sequence (Tettelin *et al.*, 2001 and http://www.tigr,org/). The P value, as determined by 2-sample t-tests, indicates the statistical significance of each gene having a ratio of <1.0 (P<0.05 is represented by a single asterix, *. P<0.005 is represented by a double asterix, **).

Genes that had lower levels of expression in the *Δrr09* mutant compared to D39 are summarised in table 5.4.

TIGR4 name	Other name	Difference in expression (represented as fold decrease)	P value
Sp0661	RR09	31.0-fold	0.008*
Sp0060	B-gal	2.8-fold	0.140
Sp0061	PTSIIB	9.0-fold	0.001**
Sp0062	PTSIIC	2.4-fold	0.107
Sp0064	PTSIIA	2.9-fold	0.117
Sp0065	Isomerase	3.0-fold	0.016*
Sp0066	Epimerase	1.7-fold	0.066
Sp1801	Conserved hypothetical	1.7-fold	0.048*
Sp1802	Hypothetical	1.9-fold	0.145
Sp1804	Stress protein	2.0-fold	0.001**
Sp1805	Hypothetical	1.9-fold	0.037*

Table 5.4: Genes having lower expression intensities in the *Δrr09* mutant compared to D39 wild-type
Genes with expression intensities at least 1.5-fold lower in the mutant strain compared to wild type are shown in the table. The P value, as determined by 2-sample t-tests, represents statistical significance in expression intensities. A P value of <0.05 is represented by a single asterix (*). Highly significant P values of <0.005 are represented by a double asterix (**). Values are based on mean intensity levels. The mean expression of *rr09* is based on a reduction in intensity when compared against background values (as this gene is absent in the mutant).

The table above shows that the *ptsIIB* and the putative stress protein gene both have expression levels that are highly significantly lower in the *Δrr09* strain compared to D39 (9-fold and 2-fold decreases, respectively). Other genes that have significantly lower expression include *isomerase*, *epimerase*, *sp1801* and *sp1805*. *B-gal*, *ptsIIC* and *ptsIIA* all demonstrated decreased expression levels of >2-fold, but these differences were not found to be statistically significant.

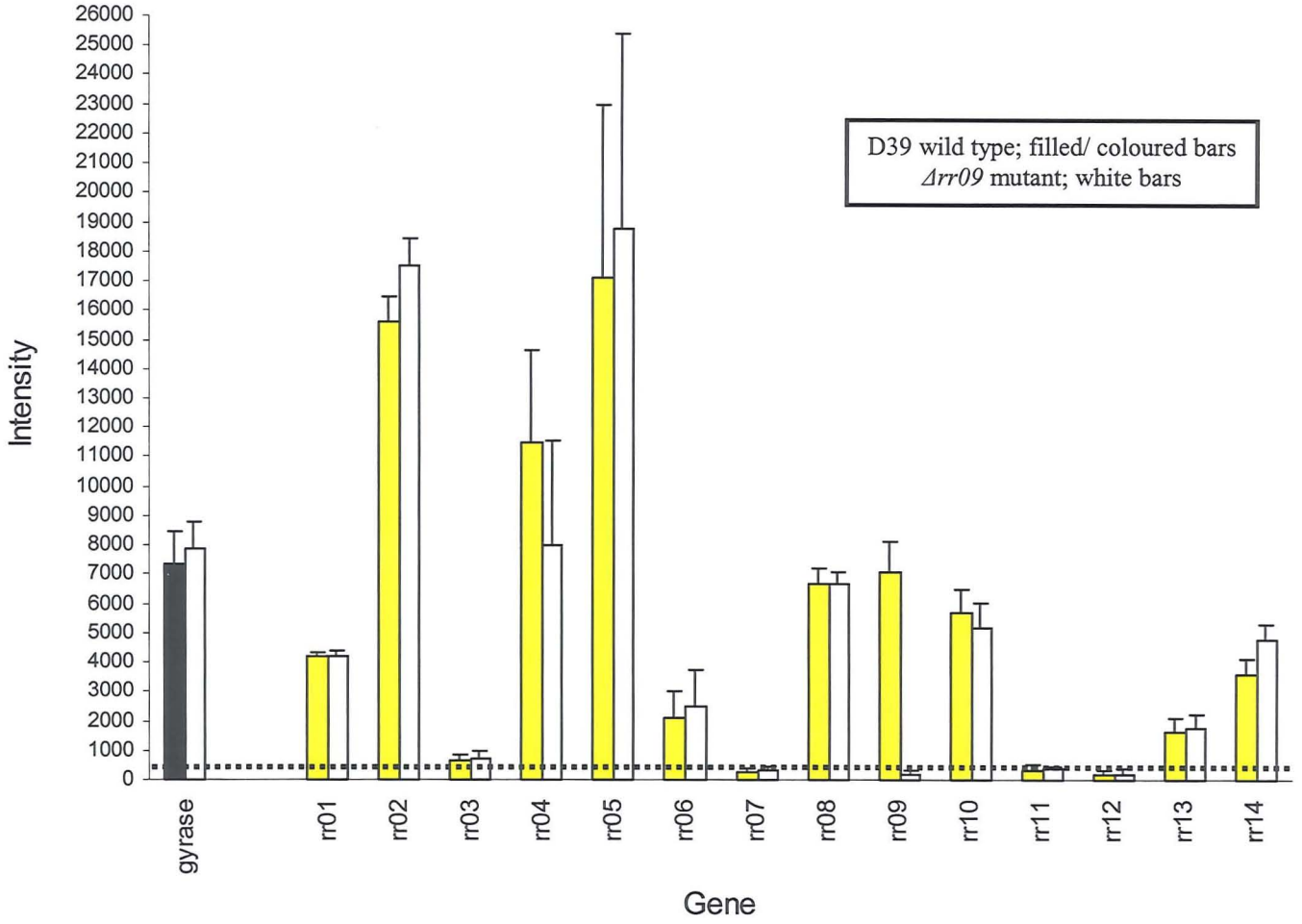

Figure 5.22: Expression of all response regulator genes in *rr09* compared to D39

Legend is provided on the following page

Figure 5.22: Expression of all response regulator genes in *Δrr09* compared to D39

The figure represents the expression intensity of all 14 pneumococcal response regulator genes in the *Δrr09* mutant compared to D39 wild-type. The dashed line represents an intensity of 500. Expression below this limit is regarded as too low for accurate data analysis. Wild-type genes are coloured yellow and mutant genes are shown in white. None of the response regulators had significantly different expression intensities in the mutant compared to the isogenic parental strain, with the exception of *rr09*. DNA gyrase is shown in black as a positive control for gene expression.

Detection of expression of the sorbose-family PTS during *in vitro* growth of wild-type D39 in defined media is somewhat surprising given that such systems would be expected to be expressed only when commonly-used sugar sources are limiting. Thus, expression of components specific to each individual pneumococcal PTS (EII components) in D39 grown to mid-exponential phase were compared (see figure 5.23). This illustrated that of 21 individual PTS systems, only 6 were expressed at intensities above the threshold limit of 500, and of these the putative sorbose system had greatest expression.

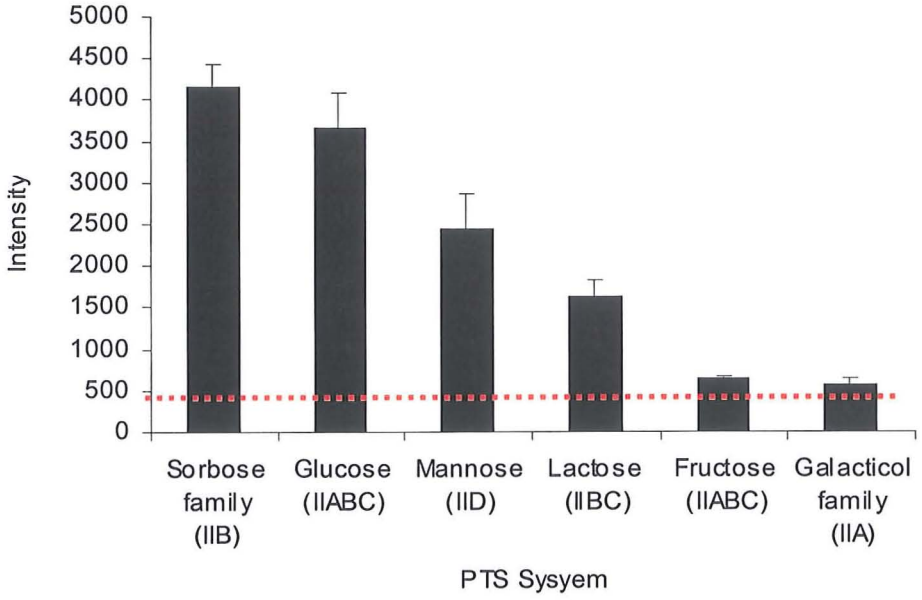

Figure 5.23 Expression of PTS systems in D39 wild-type during *in vitro* growth

Following microarray analysis of RNA isolated from wild-type D39 grown to mid-exponential phase in BHI media, the expression of EII components, specific to 21 individual pneumococcal PTS systems were compared. The figure shows the 6 PTS systems that had expression intensities greater than the cut-off threshold of 500. The component of each PTS used to examine gene expression in individual systems is shown in brackets. The broken line represents the set limit of detection for gene expression.

As the *Δrr09* mutant was avirulent, expression of several known virulence factors was compared between D39 wild-type and the mutant and illustrated in table 5.5.

Gene	**D39 (gene intensity)**	***Δrr09* (gene intensity)**
LytA (sp1937)	22954	25058
Pln (sp1923)	9550	10573
Cps2L (SPR0320)	4134	5064
HtrA (sp2239)	3820	5302
PspC / CbpA (spr1995)	2025	4719 *
PrtA (sp0641)	1854	2590
PspA (sp0117)	1409	1343
CpsO (SPR0323)	1111	1819
Cps2P	941	1012
Hya (sp0314)	473	469

Table 5.5 Expression of several known virulence factors in the Δrr09 mutant compared to D39 wild-type

The figure shows the mean expression intensity of several genes selected to represent known virulence factors *in S. pneumoniae*. These are the major autolysin (LytA), the pneumolysin toxin (Pln), 3 genes associated with capsule synthesis (Cps2L, CpsO and Cps2P), 2 surface proteins, pneumococcal surface protein C (PspC, also known as choline-binding protein A / CbpA) and pneumococcal surface protein A (PspA), 2 putative serine proteases (PrtA and HtrA) and the enzyme hyaluronidase (Hya). The genes are arranged in descending order of expression intensity. The TIGR4 gene names are given in brackets. Where genes are specific to R6 due to absence in TIGR4 (capsule genes) or sequence variation (PspC), the R6 gene names are given. The only significant difference in expression between the wild-type and mutant was with PspC, where significantly higher levels were detected in the mutant ($P<0.05$, *).

The data in table 5.5 is in agreement with previous experimental analysis that found no difference in autolysis (due to LytA) or haemolytic activity (due to Pln) of the mutant compared to wild-type. At least 9 capsule-associated genes were analysed (CpsH-CpsP), and of these, only 3 had expression levels above the 500 threshold intensity. Only these 3 genes (Cps2L, CpsO and Cps2P) are shown in the table. These capsule genes comprise genes specific to R6. PspC is the only gene identified in the entire microarray analysis that is significantly up-regulated in the *Δrr09* mutant.

5.5 Further analysis

5.5.1 Carbohydrate utilisation assays

Preliminary microarray analysis identified genes of a putative sorbose-specific PTS system that had reduced expression in the *Δrr09* mutant compared to D39 wild-type. Previously TCS09 was suggested to have a role in nutrient perception based on homology of this system with chemotaxis-associated sensors in *B. subtilis* (Lange *et al.*, 1999). To investigate the role of this system in nutrient sensing or uptake an assay was set up (based on a protocol by Ruoff *et al.*, 1999) to examine the ability of pneumococcal strains to ferment a selection of sugars, including the L- and D- forms of sorbose. Bacterial strains were grown overnight in nutrient-replete (THB) culture media then transferred to nutrient-deplete media (thioglycollate/purple broth) containing a single, defined carbon source. Ability to ferment this carbon source and subsequent acid production results in a colour change from purple to yellow due to an indicator present in the media. Figure 5.24 shows the ability of parental strains (D39 and 0100993) and mutants (*Δrr09* and *Δ488*) to ferment 7 different sugars. These sugars represent a selection of monosaccharides (glucose, sorbose, mannose and galactose), disaccharides (maltose and sucrose) and a trisaccharide (raffinose). All sugars used were the naturally occurring D-forms. Both L- and D-forms of sorbose were included due to the putative association of TCS09 with this sugar. THB medium alone was used as a negative control. In a previous experiment bacterial cultures grown in THB were added to thioglycollate/purple broth medium in the absence of carbohydrate to show that carbohydrate or acid from the THB medium was not carried over to detectable levels in the assay.

All of the strains, including the mutants, were able to ferment all the carbohydrates tested with the exception of sorbose (both L and D forms).

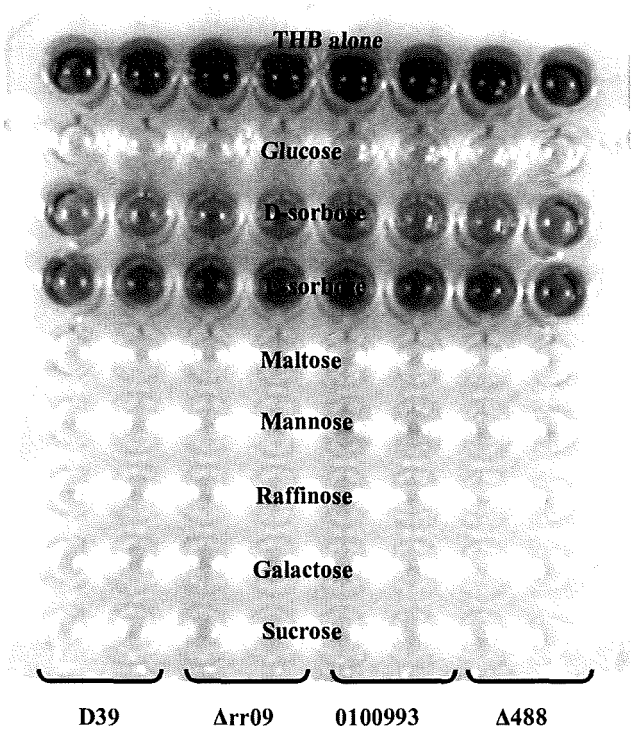

Figure 5.24 Carbohydrate fermentation by bacterial strains

Bacteria strains D39, 0100993, *rr09* and *488* were grown overnight in THB media. Small volumes of overnight culture were added to thioglycollate/purple broth media containing various carbon sources (1 % final concentration). Following anaerobic growth at 37 ° C for 24 hr, strains capable of fermenting individual carbon sources were indicated by a colour change in the media from purple to yellow, due to acid production. The figure shows the fermentation of all sugars (shown by clear wells) with the exception of D- and L-sorbose in all strains. The darker wells (THB control and L/D-sorbose wells) indicate absence of fermentation and hence no colour change. Each strain was tested in duplicate for individual sugars and the experiment was repeated twice.

5.6 Summary of TCS09 Results

Discussion of mutant phenotypes is in relation to the isogenic parental strains, unless stated otherwise

5.6.1 Basic *in vitro* analysis

Mutations in the *rr09* gene of 2 pneumococcal strains (D39, serotype 2 and 0100993, serotype 3) were constructed by allelic replacement and confirmed by PCR and sequencing. PCR was also used to confirm that the organisation of *tcs09* and surrounding genes in strains D39 and 0100993 was the same as that of the sequenced strains R6 (type 2) and TIGR4 (type 4)

Mutants were not impaired in growth in nutrient-replete BHI media, although RT-PCR indicated that *rr09* is expressed in mid-exponential phase of growth in D39. Mutants were stable (retain erythromycin resistance) *in vitro* for up to 20 hr in the absence of erythromycin.

5.6.2 *In vivo* characterisation

Intraperitoneal challenge

Following intraperitoneal injection, the mutation in a serotype 2 background (*Δrr09*) was completely attenuated ($P<0.05$). All mice survived infection with no symptoms or bacteraemia. The mutation in a serotype 3 background (*Δ488*) was not impaired in virulence.

Intranasal challenge

Following intranasal challenge *Δrr09* was completely attenuated. All mice survived with no symptoms of infection or evidence of bacteraemia. Counts in the lung tissue at 24 and 48 hr post challenge were below the detection limit for most mice challenged with this strain. $P<0.05$ for survival, bacteraemia and lung counts. The *Δ488* mutant was also attenuated in murine survival and bacteraemia ($P<0.05$) but not in lung counts.

Intravenous challenge

Following intravenous challenge *Δrr09* was again completely attenuated. All mice survived, with no symptoms of infection or evidence of bacteraemia after 8 hr post injection. $P<0.05$ for survival and bacteraemia. The *Δ488* mutant was not impaired in survival or bacteraemia following intravenous injection.

These data implies that in our model of infection, *rr09* is important in virulence in both strains but has a different influence depending on the genetic background of the parental strain. In serotype 2 D39 it is essential for virulence in all studied routes of infection. In serotype 3, 0100993, it is not required for virulence following intraperitoneal or intravenous challenge or in lung counts following intranasal challenge, but the *rr09* mutant is impaired in bacteraemia and murine survival following intranasal challenge.

5.6.3 Further *in vitro* characterisation

The mutants were not significantly impaired in their ability to take up and incorporate extracellular DNA during transformation and appear to have equal amounts of functional pneumolysin

5.6.4 Expression analysis

Microarray analysis was used to compare gene expression in D39 and the *Δrr09* mutant (serotype 2). This identified decreased expression of two groups of genes in the mutant strain. One set of genes encodes for various components of a PTS sugar transport system, with a possible link with the monosaccharide sorbose. This PTS has the highest levels of expression of 21 individual systems examined in D39 grown in BHI. The second set of genes are clustered together on the genome but their function is unknown, with the exception of one that is a putative stress protein. The *rr09* gene itself was also identified as being absent in the mutant. Expression of all other response regulator genes was not significantly affected in the *rr09* mutant. Expression of several known virulence was not altered between mutant and wild-type, with the exception of PspC, which was up-regulated in the mutant strain.

5.6.5 Further analysis

The ability of all strains to ferment several carbohydrates was investigated in an attempt to demonstrate a link between TCS09 and sugar fermentation. All strains, including the mutants, fermented all sugars tested with the exception of sorbose, which was not fermented by any of the strains.

Chapter 6

Discussion

S. pneumoniae TCS09

6.1 Mutant construction and basic *in vitro* analysis

Null mutants were made in the *rr09* gene of *S. pneumoniae* TCS09 in strains D39 (serotype 2) and 0100993 (serotype 3). Initially, allelic replacement was used to create the mutation in the type 3 background (mutant *Δ488*). This mutation was subsequently isolated by PCR and the product used to transform D39 to create the same mutation in a different strain / serotype (type 2) to create the mutant *Δrr09*. All mutations were confirmed by PCR and sequencing. Prior studies using D39 transformed with plasmid pVA838, which does not integrate into the chromosome, have shown that virulence in this strain is not compromised by the transformation procedure used to create the mutants (chapter 3, section 3.1.2). This observation confirms that published by another group (Kadioglu *et al.*, 2001), and illustrates that any phenotypes observed for the mutant strains are due to the null mutation and not artefacts of the transformation procedure used to create the mutants.

The 0100993 and D39 strains used for the analysis in the previous chapter have not been sequenced, although a sequence for another serotype 2 pneumococcal strain (R6) does exist and has been published (Hoskins *et al.*, 2001, http://www.tigr.org/). R6 is a strain derived from D39 so should be very closely related. The gene organisation of *tcs09* and surrounding genes in strain R6 is in agreement with that of a second published and annotated sequence, TIGR4 (serotype 4) and with the G54 (serotype 19F) sequence (publicly available but not annotated). Furthermore, the MsrA, RR09, HK09 and Chp proteins are at least 99 % homologous between R6, TIGR4 and G54 (data not shown), indicating that these genes are highly conserved between serotypes / strains. PCR using a series of primers designed against genes of *tcs09* and the surrounding genes (based on the R6 and TIGR4 sequences) were designed and used to confirm that the gene organisation for all genes studied was the same in the D39 and 0100993 parental strains used in this work as in the R6 and TIGR4 strains. These genes were also found to be present in several other strains examined in this work, including serotype 19F, serotype 1 and one other serotype 4 strain. This indicates that the organisation of the *tcs09* region and surrounding genes is conserved between serotypes. Sequencing the PCR products would determine if the protein sequence is conserved between the 2 strains used in this work. It would be particularly useful to compare the *rr09* gene sequences in strains D39 and 0100993, given the differences seen in virulence (discussed below).

In the absence of erythromycin, the mutant strains were not impaired in growth in BHI media and autolysed at the same rate as the isogenic parental strains, indicating that the *rr09* gene is not an essential gene for the growth of D39 and 0100993 under the conditions used. However, RT-PCR was used to show that the *rr09* gene is expressed during the mid-exponential growth of wild-type D39 in BHI. This indicates that the gene may be constitutively expressed or switched on at the stage of growth tested, although is not required for growth under the conditions used. Expression of the gene at different stages of growth or using different growth media could be examined to provide further insight into this finding. Expression of *rr09* in strain 0100993 *in vitro* has yet to be examined.

The presence of erythromycin did not affect the growth or optical density readings of the *Δ488* mutant. However, the presence of erythromycin resulted in lower optical density readings at all time points with the *Δrr09* mutant, compared to D39 wild-type. As viability counts did not differ between the mutant and wild-type strains at any time point, the reduction in optical density suggests that there could be differences in capsule expression which subsequently results in different light-scattering ability of the strains without altering viability. This could also contribute to the differences seen in virulence, both between the *Δrr09* mutant and its D39 parental strain, and in the virulence of *rr09* mutants in different pneumococcal serotypes.

The analysis of *in vitro* growth demonstrated that optical density during the exponential phase of growth could be used as an accurate measurement of cell viability for all the strains examined in the absence of erythromycin. This is useful for further experiments where cultures of different strains and serotypes can be grown to a set OD $_{600\ nm}$ reading and harvested for experimental analysis, with the knowledge that total cell counts isolated should be very similar. The ability of mutants to grow well in the absence of antibiotic selection, without losing the selection marker and hence their null mutation, is also useful for further work. Cultures required for RNA preparation, for example, could be grown in the absence of antibiotic that could otherwise alter gene expression, with the knowledge that the mutant genotype will still be retained.

After progression into stationary phase optical density does not appear to accurately reflect viable cell counts so should not be used for such a purpose. Furthermore, the optical density between serotypes differed from 8 hour after culture inoculation, such that OD $_{600\ nm}$ values were lower for serotype 3, strain 0100993 when compared to D39 (figure 5.7B). This could be related

to the presence of the relatively thick mucoid capsule present on type 3 strains, which may dissociate from dying or lysed cells and affect the optical density accordingly.

6.2 Role of RR09 in virulence

Bacterial strains were initially administered to mice as an intraperitoneal injection. Administration of *S. pneumoniae* via this route generally results in high levels of bacteraemia (>10^5 CFU ml^{-1}) within 24 hours of injection, even with very low challenge doses of several CFU (Aaberge *et al.*, 1995). Bacteraemia generally correlates with the observation of moderate to severe symptoms (hunched stance, piloerection, lethargy) which progress until the mice become moribund. This pattern of infection was observed consistently when mice were given strains D39, 0100993 and *Δ488*, but intraperitoneal challenge with the *Δrr09* mutant did not result in any detectable bacteraemia following infection and mice did not display any signs of infection. The mutation was created and confirmed in at least two further, independent experiments, using different D39 stocks and groups of mice. However, none of the mutants were virulent, even when the challenge dose was increased 1000-fold compared to the initial dose of 10^5 CFU. These data indicated that *rr09* was important for virulence in a serotype 2 background but not in a serotype 3 background when given to MF1 mice via intraperitoneal injection. In a study by Lange and colleagues, knock out mutations in the *rr09* gene of a type 22 and type 3 pneumococcus were not found to be impaired in virulence compared to the wild type strains when given to C57/Bl mice i.p. (Lange *et al.*, 1999). Their data are in agreement with the results reported in this thesis for the *Δ488* mutant which is also serotype 3, although of a different strain. However, the findings of Lange and co-workers do not agree with the data in this thesis for a *rr09* knock-out in a serotype 2 background, giving the first indication that genetic differences between bacteria strains and / or serotypes could exist for the role of RR09 in infection.

Mice were subsequently challenged with mutants and their parental strains via an intranasal route of infection that allows the evaluation of the virulence of pneumococcal strains at several important stages of the infection process. Fully virulent strains that can cause mortality of mice via this route must be able to grow within the lung tissue, disseminate into the systemic circulation and survive and multiply in the blood, whilst avoiding elimination by the host's immune system. Initially, virulence was assessed by murine survival and bacterial load in the blood following intranasal challenge. Mice given 10^7 CFU D39 showed significantly increased

levels of bacteraemia at all time points examined, post infection, compared with those challenged with the isogenic *Δrr09* mutant. Furthermore, while 60 % of infected mice did not survive challenge with D39, all mice given 10^7 CFU *Δrr09* mutant survived the experiment without showing any symptoms of infection or bacteraemia. Similarily, with the strains on a type 3 background, challenge with the wild type 0100993 resulted in levels of bacteraemia comparable to those with D39 infection, resulting in 100 % mortality of infected mice by 120 hours post infection. Infection with the *Δ488* mutant did not result in significant levels of bacteraemia at any of the time points examined, although 40 % of mice did eventually succumb to infection. These data indicates that *rr09* gene is important in virulence via an intranasal route of infection in our murine model of pneumonia in both serotype 2 and 3 pneumococcus, with significant differences seen between wild type strains and their isogenic mutants in both survival and bacteraemia.

Prior to intranasal challenge, our laboratory and other groups routinely use i.p. passage to obtain a standard inoculum of bacteria that has been prepared directly from the blood of infected mice. This increases the virulence of the bacterial strains such that subsequent intranasal challenge with a suitable dose of standard inoculum (determined experimentally) will generally result in bacteraemia and mortality in all mice challenged intranasally within 48-60 hr post infection. However, the D39 and its isogenic Δ*rr09* mutant used for analysis in the previous chapter were not passaged prior to challenge due to the inability to isolate Δ*rr09* from the blood of mice after i.p. injection and the less virulent glycerol stock preparation was used. To compensate for this, the dose administered by all routes of infection was increased 10-fold to that usually used in our laboratory for D39 standard inoculum challenges. Despite this dose increase, 40 % of mice challenged both i.n. and i.v. with wild-type D39 survived the course of infection. This illustrates the importance of passaging *S. pneumoniae* prior to experiments using our model of infection. Several studies have examined gene or protein expression following murine peritoneal culture with *S. pneumoniae*. One study used a type 3 pneumococcus (strain WU2) to demonstrate that i.p. culture resulted in increased gene transcripts for pneumolysin and an enzyme involved in capsule biosynthesis compared to *in vitro* growth (Orihuela *et al.*, 2000). Both of these are known pneumococcal virulence factors. 2D gel electrophoresis also identified many proteins that were unique to *in vivo* conditions (Orihuela *et al.*, 2000). A second study used quantitative RT-PCR to compare mRNA levels of 5 known pneumococcal virulence factors (pneumolysin, PspA, PsaA, CbpA and capsule genes) from type 2 strains harvested from the blood of infected mice

(i.p.) with strains grown *in vitro*. All virulence factors had increased expression *in vivo* with the exception of CbpA (Ogunniyi *et al.*, 2002). CbpA is thought to be important in adhesion (Rosenow *et al.*, 1997) so expression of this gene may not be altered following i.p. challenge but may be expected to increase should a colonisation model of infection be used.

The use of non-passaged bacterial stocks for the determination of virulence of certain mutants may also have its advantages, as passaging may allow the role of the mutated gene to be compensated for by other bacteria factors. For example, with TCS mutants, cross regulation may occur and other systems may be able to take over the role of the absent component during passage. However, as demonstrated in this work, large bacterial challenge doses may have to be given to obtain satisfactory wild-type infection against which mutants can be compared.

Bacterial loads in the lung tissue following intranasal challenge were determined to see if mutant strains were impaired in their ability to grow in the lungs. Significant differences were seen between the counts of D39 and the *Δrr09* mutant at 24 hours and 48 hours post challenge, where the lung tissue of mice challenged with the Δ*rr09* mutant failed to yield detectable bacterial counts. Nevertheless, bacterial counts in the lung tissue of mice challenged with wild-type D39 were very low compared to the counts seen using standard inoculum D39 stocks (chapter 7, section 7.3.1.3) or the counts seen for the type 3 strains. Again, this is likely to be due to the absence of passaging prior to challenge. Furthermore, the median counts in the lungs of mice challenged with type 2 D39 wild-type bacteria decrease between 24 and 48 hr, whereas median counts in the lungs of mice following challenge with type 3 wild-type 0100993 increase between 24 and 48 hr. This indicates differences in growth kinetics between the strains used and requires further investigation. As the lung environment is one of the first to be encountered by bacteria administered to mice intranasally, the inability to detect the Δ*rr09* mutant in lung tissue may indicate clearance by defence mechanisms within this environment. Thus assays could be set up to compare the efficiency of alveolar macrophages at phagocytosing both D39 and Δ*rr09* mutant strains, or to evaluate the cytokine profile of these macrophages following stimulation with bacterial strains. However, the Δ*rr09* mutant is also unable to survive following direct inoculation into the bloodstream, suggesting a mechanism of host clearance that is not specific to the lungs.

No significant difference was seen in the lung counts of mice challenged i.n. with type 3 strains. This suggests that *rr09* null mutants in a type 2 background are unable to colonise lung tissue of MF1 mice, while mutants in a type 3 background are not impaired in their ability to grow in the lungs. The data using type 3 pneumococcus is in agreement with that of Throup and colleagues, who examined lung counts at 48 hours post infection, using the same type 3 strains but in the CBA/J inbred strain of mouse (Throup *et al.*, 2000). This group found a reduction in bacterial counts in the lung 48 hour post challenge with the *Δ488* mutant compared to isogenic parental strain (approximately reduction of Log_{10} 1.5 CFU), but the difference was not statistically significant (Throup *et al.,* 2000). In this work, a decrease in lung counts of approximately Log_{10} 0.5 CFU was observed for the *Δ488* mutant, and this was not statistically different from counts with wild-type. As the *Δ488* mutant used in this work is the same strain used in the study by Throup and co-workers, the slight difference may be due to experimental variation or the use of a different murine model (see table 6.1). Indeed the mean lung counts at 48 hour post infection with the wild type strain, 0100993, which is also the same strain used by Throup and colleagues were approximately Log_{10} 2 CFU lower in this work compared to the published study. An attempt was made to repeat the lung count data using the CBA/J strain of mice used by Throup and co-workers, but problems were encountered with the sterility of the lungs in these mice so work had to be abandoned (chapter 3, section 3.4.3). As no significant differences were seen in lung counts between type 3 wild-type and the *Δ488* mutant, it may be useful to examine cytokine profiles in the lungs of infected mice to determine if there are any differences in host inflammation between strains. Nasopharyngeal colonisation and airway counts could also be determined for all strains using the MF1 model of infection.

Intravenous injection of strains directly into the bloodstream was performed to assess the ability of the mutants to grow in the blood. This experiment suggested that the *Δrr09* mutant in a serotype 2 background was unable to sustain itself in the blood, with bacterial counts falling to below the limit of detection within 8 hours of infection and remaining at this level throughout the course of the experiment. Subsequently, mice challenged intravenously with *Δrr09* all survived, whilst 60 % of mice given parental D39 bacteria succumbed to infection. This difference in survival was statistically significant. Mice challenged with the type 3 mutant, *Δ488* did not show

any statistical difference in survival or bacteraemia from those infected with wild type 0100993 strain.

Overall, the *in vivo* infections suggest that in our murine model of infection RR09 is important in virulence in both serotypes examined, but its role in virulence differs between serotypes/strains. In a type 2 background RR09 is essential for virulence with null mutants being unable to survive all routes of infection examined. These mutants are unable to grow in lung tissue and are cleared very rapidly from the blood. As these mutants are not impaired in their ability to grow in culture media *in vitro*, it appears that the loss of the RR09 protein is preventing survival in the blood and lungs. It has not been determined if this is due to an increased ability of the host immune system to clear mutant bacteria or the inability of the mutants to obtain essential nutrients and growth factors required for survival. Further experiments are required to determine this. Classical *in vitro* phagocyte killing assays could be used to determine if the mutants are more susceptible to phagocytosis. These assays use freshly isolated PMN cells, which are incubated with bacteria in the presence of complement. Following incubation, bacterial cell viability is analysed and the susceptibility of the pneumococci to phagocytosis determined (Jansen *et al.*, 2001). Should increased levels of phagocytosis be shown for the Δ*rr09* mutant, Western blot could be used to compare the ability of wild-type and mutant strains to deposit host components that promote or inhibit opsonophagocytosis onto the bacterial cell surface. Such assays have been performed previously to demonstrate the binding of complement factor H and other components to the surface of bacteria (Janulczyk *et al.*, 2000; Pandiripally *et al.*, 2002). Alternatively, isolated whole human and / or murine blood could be used to determine if this supports the growth of the *Δrr09* mutant. This could help determine if a component within the blood, or the lack of certain nutrients in the blood is preventing the growth of mutant strains, or if this growth defect is due to a host component not present in isolated blood. Similar experiments using whole sheep blood have been used to examine the virulence of a mutant in a *S. suis* response regulator, revS (de Greeff *et al.*, 2002). Growth of the bacterial strains in nutrient deplete broth could help identify if wild-type D39 bacteria are able to grow more efficiently in such conditions compared to the *Δrr09* mutant strain. This is discussed further with respect to the microarray data below.

Loss of RR09 in a type 3 background does not result in a loss of virulence in an i.p. route of infection and mutants are not impaired in their ability to grow in the blood or within lung tissue.

However, following intranasal challenge much lower counts in the blood are observed compared to the parental strain and survival times of mice are increased. It thus appears that the 0100993 mutant is impaired in its ability to disseminate from the lung tissue into the bloodstream or that transition from the lungs to the blood results in the mutants being less well adapted for survival. This could result from the inability to detect certain environmental signals that are required for the regulation of genes or sets of genes required for systemic virulence and survival following transition from the lung tissue into the blood. Certain aspects of the host immune system may be activated during this transition, via tissue damage or the inflammatory response, and the mutants may have impaired ability to resist such defence mechanisms compared to wild-type bacteria. Whatever the cause, the defect appears to be responsible for the lower levels of bacteraemia seen at the selected time points and subsequently the difference in survival time of mice following intranasal challenge. However, such mutants can induce mortality in 40 % of infected mice, albeit at a later stage. This suggests that if TCS09 is involved in dissemination from the lungs in serotype 3 pneumococcus and / or bloodstream survival, it is not the sole mediator of this process as *rr09* null mutants can eventually gain access to the bloodstream, multiply and result in murine death. This could also indicate that another TCS could be compensating for the loss of *rr09* in the mutant on a type 3 background.

In an effort to identify possible differences in the mutants that could account for the altered virulence profiles seen, or to identify the role of RR09 in virulence, several aspects of virulence were studied experimentally. Firstly a haemolytic assay was performed to determine the presence of functional pneumolysin, an important pneumococcal virulence factor with multiple activities thought to be detrimental to the host (Mitchell and Andrew, 2000; Cockeran *et al.*, 2002). No differences were seen in the *in vitro* haemolytic activities for any of the strains, although this does not allow us to make inferences to the situation during the infection process. This observation was also found when microarray analysis was used to compare the expression of several known virulence factors, including pneumolysin, between the mutant and wild-type strains (discussed later).

The ability to take up DNA from the extracellular environment is also thought to be important for virulence. This is highlighted by mutations in components of the competence TCS (ComD/E, TCS12) being identified in several genome wide screens for virulence factors (Lau *et al.*, 2000; Camilli and Hava, 2002). Thus the transformation efficiency of the *Δrr09* mutants compared with their parental strains was examined. The *Δrr09* mutant had a transformation efficiency 56 % that of D39 wild-type. Although this was not significantly different using a 2-sample t-test, Lange and colleagues (Hoffmann-La-Roche) found that a mutation in the *rr09* gene in a serotype 2 strain (R6) also had decreased transformation efficiency of 60 %, relative to the parental strain (R. Lange, personal communication). This consistency in reduced competence in the *rr09* mutant in 2 different serotype 2 strains used in this work and an independent study may indicate that this is a phenotype to which further investigation could prove interesting.

No difference in the transformation efficiency of the serotype 3 *Δ488* mutant compared to its isogenic parental strain was observed, although the efficiency of both strains was extremely low compared to the type 2 strains. No data on transformation of this serotype is available from Lange and colleagues. Both type 3 strains had a decrease in mean transformation efficiency of greater than 100-fold, when compared to D39. This is likely to be related to the thickness of the capsule as serotype 3 pneumococci produce a thicker, more mucoid capsule generally not found in other serotypes (Yother *et al.*, 1986). Thus a difference in transformation efficiency was found between serotypes. Although this may have an effect on virulence it unlikely to account for the completely avirulent phenotype of the *Δrr09* mutant.

The capsule has been recognised as an important virulence factor of *S. pneumoniae* for many years now, with un-encapsulated strains being avirulent in humans and the murine model. The exact contribution of the capsule to virulence is not fully understood, although it is thought to have a role in allowing organisms to avoid phagocytosis, possibly due to the highly charged surface. The ability of different capsule structures to avoid complement deposition on the bacterial cell surface has been alluded to previously (section 1.6.5, introduction). Serotype 3 strains produce a much thicker capsule than type 2 strains, and this can be seen by growth on blood agar plates, with the former producing large, extremely mucoid colonies (Waite *et al.*, 2001). Thickness of the capsule may influence virulence to some extent but other factors are also important. For example serotypes 3 and 37 both have very thick capsules, but only the former is

virulent for both humans and laboratory animals (Paton *et al.*, 2000). Furthermore, the virulence of wild-type type 2 and type 3 standard inoculum preparations are very similar in our model of infection, using similar doses, suggesting that capsule composition differences should not account for the differences seen between the *rr09* mutants of serotype 2 and 3.

Although polysaccharide capsular material of most pneumococcal serotypes are generally thought to be innocuous and will not induce any inflammation or symptoms of disease if given as a cell-free preparation (Berry *et al.*, 1996), the capsules of both serotype 2 and serotype 3 pneumococci have been shown to induce inflammation. This was shown in a study that measured leukocyte influx and protein concentration following intra-tracheal installation of capsular material into rabbits (Tuomanen *et al.*, 1987).

In relation to the importance of capsule to pneumococcal infection it is possible that the *Δrr09* mutant could be producing less (or no capsule) capsule compared to wild type and this is reducing its virulence. The expression of several capsule-associated genes were compared in the *Δrr09* mutant and D39 wild-type following microarray analysis (see table 5.5) and none were significantly different. However, this does not allow inferences to be made on the expression of such genes during *in vivo* infection. It is doubtful that this phenomenon is occurring in the *Δ488* mutant as the capsule of type 3 colonies is clearly visible when cultures are plated onto blood agar plates and a reduction in capsule of the type 3 mutant is not observed upon visual inspection. This does not, however, confirm that the amount of capsule in the type 3 strains is equal. Quellung reaction was performed on all strains to determine if a reduction in capsule could be detected, but results were hard to interpret, not reproducible and not quantitative (data not shown). A flow cytometry assay is thus being optimised to quantitate the amount of capsule present in pneumococcal strains. This procedure involves incubating pneumococcal cells with anti-capsular antibody and detecting bound antibody with a secondary antibody conjugated to FITC. Cells are sorted by FACS® analysis to detect polysaccharide capsular material. Studies using this technique for the purpose of work presented in this thesis are still preliminary (data not shown), although cell sorting has been successfully used by other groups to serotype pneumococci (Park *et al.*, 2000). ELISA may also prove a useful technique for capsule quantification and has been used in a previous study for this purpose (Weiser *et al.*, 1994).

In a recently published STM screen, Hava and Camilli (2002) identified a *rr09* mutant in a serotype 4 background as being highly attenuated in a pneumonia model based on counts within lung tissue 44 hours post challenge with 1 x 10^7 CFU. The same mutant was not highly attenuated in a model of bacteraemia on the basis of bacterial counts in the blood 20 hours post i.p. challenge (5 x 10^5 CFU). The challenge doses given in this study were representative of the doses used in our work, although Hava and Camilli used a different strain of mice (Swiss Webster) and a different pneumococcal serotype. The results of Hava and Camilli is in agreement with our findings that RR09 is important in the virulence of the pneumococcus, but its exact role in virulence depends on the genetic background of the parental strain and site of infection.

The contribution of the capsule to pneumococcal infection has been studied for many years. Although it is generally accepted that virulence of pneumococcal strains in both human and mouse differs with capsular serotype (Briles *et al.*, 1992; Kelly *et al.*, 1994), the reasons for this are largely unknown. The biochemical composition of the capsule can affect phagocytosis and the production of antibodies, and thus can play a role in virulence of a given serotype. For example serotypes 3 and 4, which are often isolated from adult infections, are very resistant to phagocytosis yet highly immunogenic. In contrast serotypes 6A and 14 are easily phagocytosed but are not immunogenic (Hostetter, 2000). However, research by several groups has highlighted the importance of both the capsular serotype and the genetic background of the bacterial strain. Kelly and colleagues (Kelly *et al.*, 1994) transformed strains of type 2, 5 and 6B pneumococcus with genes specific for type 3 capsule production. The resulting strains all grew as thick mucoid colonies, characteristic of type 3 pneumococcus, but were otherwise isogenic. The strains were analysed for virulence in murine models of infection. The original type 6B strain, which was relatively avirulent, increased in virulence when it was expressing the type 3 capsule. In contrast, the otherwise highly virulent serotype 5 strain became essentially avirulent with a type 3 capsule. Interestingly, the serotype 2 strain did not differ significantly in virulence when it was expressing a type 3 capsule. Although serotypes 2 and 3 generally behave very similarly in murine infection models, the structure of their capsules differ largely (Kelly *et al.*, 1994). This indicates that capsule and genetic background play equally important roles in determining strain virulence. A second study found that not only were capsule type and background important in virulence, but

the site of infection also determined the virulence of strains (Kadiouglu *et al.*, 2002). This study, by Kadioglu and colleagues, used the same serotype 2 pneumococcal strain (D39) as the previous study by Kelly and colleagues, and also made a chimeric mutant that expressed a type 3 capsule. This chimeric mutant, however, was significantly less virulent than the D39 parental strain in nasopharyngeal colonisation and lung colonisation and was essentially avirulent with respect to survival of mice following intranasal infection. These data highlights that there is an optimal combination of capsule type with other factors.

Very little data have been published highlighting differences in the role of pneumococcal virulence determinants in different strains or serotypes. CbpA of pneumococcal strain R6x was proposed to bind to the human polymeric immunoglobulin receptor and use this as a mechanism of translocation across the nasopharyngeal epithelium (Zhang *et al.*, 2000). However the strain used in this study is an avirulent, non-encapsulated strain and a further study has shown that this binding effect was specific only to strain R6x when other non-encapsulated laboratory strains and encapsulated clinical isolates were examined (Brock *et al.*, 2002). Tomasz and colleagues created mutants in the gene for the major autolysin gene, *lytA*, in serotypes 3 and 6 and found no difference in virulence for either mutant when compared to its parental strain (intraperitoneal challenge with 3 different challenge doses) (Tomasz *et al.*, 1988). In contrast, another group also created a *lytA* mutant in a serotype 3 strain and showed a significant decrease in virulence following intraperitoneal challenge (Berry *et al.*, 1992). The strain of type 3 pneumococcus used in both these studies was different, which could suggest that strain differences could exist alongside serotype differences. However, as the groups used different mutagenesis techniques and different strains of mice an accurate assessment of the reasons for the differences seen cannot be made. Thus the data presented in this thesis are the first study in a properly controlled manner, as far as we are aware, where the same mutation introduced into the same gene of two pneumococcal serotypes has resulted in large differences in virulence between the serotypes. PCR has been used to show that the *rr09/hk09* locus and surrounding genes have the same organisation in the strains used in this work to the sequenced strains, TIGR4 and R6. Although the serotype 3 sequence is not publicly available, the *rr09* genes of strains R6, TIGR4 and G54 are homologous, so this hypothesis assumes that the *rr09* gene does not show significant sequence variation between type 2 and 3 and that this does not account for the differences in virulence.

The data presented in this thesis, combined with that of a recently published study (Hava and Camilli, 2002), strongly suggest differences in the way RR09 behaves in different pneumococcal strains/serotypes. These data highlight a crucial issue in terms of identification of putative vaccine candidates and drug targets, as a cellular component essential for virulence in one strain may not have such an important role in virulence in another strain. Additional investigations are required to examine the role of the capsule combined with other genetic factors when analysing the contribution of certain components to virulence. To characterise this further and determine if differences are serotype or strain specific, mutations in the *rr09* genes of different serotypes and / or strains need to be created using the same mutagenesis techniques and subject to the same degree of comprehensive analysis in the same models of infection. Currently a *rr09* mutation is being constructed in the TIGR4 (serotype 4) strain within our laboratory and will be used for *in vivo* analysis and microarray experiments. Sequencing of the *rr09* genes in all the strains examined will ensure that any differences observed are due to the genetic background of the strains and not subtle differences in RR09 that could result in different functions. It would be interesting to create *Δrr09* mutations in a D39 background but using a strain of D39 that has been transformed with the capsule genes of strain 0100993. Such a technique has been used in a previous group as described above (Kelly *et al.*, 1994), and mutants would easily be identified by the presence of a thick capsule which results in large, mucoid colonies on blood agar plates. Alternatively, the D39 *Δrr09* mutation could be reverted / complemented with the wild-type *rr09* gene from D39 and 0100993 and virulence of the revertant assessed as described previously. Expression analysis using microarrays with *rr09* mutations in different strains / serotypes could produce data that may help explain the differences in the contribution of RR09 virulence between pneumococcal strains. This is explained in detail below.

Table 6.1 summarises all *in vivo* analysis, including that from this thesis, available to date for the virulence of *S. pneumoniae* TCS09.

Reference	Strain / serotype of bacteria	Mutagenesis technique	Murine Strain	Route of infection	Inoculum	Measure of virulence	Role in Virulence
Lange *et al.*, 1999	Sp.1 (type 22)	Insertion duplication	C57BL6/J (I) Females	i.p. (NP)	4×10^6	Mean survival time	Fully virulent
Lange *et al.*, 1999	Sp.1711 (type 3)	Insertion duplication	C57BL6/J (I) Females	i.p. (NP)	1×10^3	Mean survival time	Fully virulent
Throup *et al.*, 2000	0100993 (type 3)	Allelic replacement	CBA/J (I) Males, 5 wk	i.n. (NP)	1×10^7	Mean lung counts, 48 hr	10-100 fold decrease in lung counts compared to WT, not statistically significant
Hava and Camilli, 2002	TIGR4 (type 4) (streptomycin-resistant derivative)	STM	Swiss Webster (O) 6-10 wk, males	i.n. (NP) i.p. (NP)	2×10^7 5×10^5	Lung counts, 44 hr (competative index) Bacteraemia, 20 hr (competative index)	Highly attenuated in lung counts Not attenuated in bacteraemia
This work	D39 (type 2)	Allelic replacement	MF1 (O) Females, 9-13 wk	i.p. (NP) i.n. (NP) i.n. (NP) i.v. (NP)	1×10^5 1×10^7 1×10^7 1×10^6	Survival Bacteraemia and survival Lung counts Bacteraemia and survival	Avirulent; all mice survived Avirulent; all mice survived, no bacteraemia Avirulent; no counts in lungs 24 hr and 48 hr Avirulent; all mice survived, no bacteraemia after 8 hr
This work	0100993 (type 3)	Allelic replacement	MF1 (O) Females, 9 – 13 wk	i.p. (P) i.n. (P) i.n. (P) i.v. (P)	1×10^5 1×10^7 1×10^7 1×10^6	Survival Bacteraemia and survival Lung counts Bacteraemia and survival	Fully virulent Attenuated in bacteraemia and survival Fully virulent Fully virulent

Table 6.1 *In vivo* analysis of *tcs09* mutants

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Table 6.1: *In vivo* analysis of *tcs09* mutants

The table summarises all *in vivo* work available to date with *S. pneumoniae* TCS09. (I) / (O) designates inbred or outbred strains of mice respectively. Route of challenge includes intranasal (i.n.), intraperitoneal (i.p.) or intravenous (i.v.). (NP) and (P) illustrate strains that are non-passaged or passaged, prior to challenge respectively. Role in virulence is compared to the parental wild-type strain. STM; signature tagged mutagenesis. The table is modified from that in the introduction (table 1.6) by the addition of work detailed in this thesis

6.3 Expression analysis

mRNA expression profiles in D39 and *Δrr09* only were compared using microarray analysis due to time restrictions. The hybridisations worked well and both array replicates identified the *rr09* gene as being significantly down-regulated / absent in the mutant compared to wild-type. Several other genes were expressed at significantly lower levels in the mutant. These clustered around 2 different regions of the genome. The first region is associated with a bacterial phosphotransferase system (PTS). These sensory systems transport a large number of carbohydrates into cells with concomitant phosphorylation of substrate, and tend to be associated with fermentative organisms (Reizer, 1989). The PTS is a phosphorelay system where a phosphoryl group is transferred from phosphoenolpyruvate to the incoming sugar via a series of protein intermediates. At least 3 proteins are required for the PTS systems, enzyme I (EI), histidine protein (HPr) and enzyme II (EII). EI and HPr are usually soluble proteins that phosphorylate all PTS carbohydrates of an organism and only one of each of these enzymes is generally present in a bacterial cell (Postma *et al.*, 1993). Substrate specificity of any particular chain resides in the membrane-associated EII enzymes of which many different EII are present in a bacterial cell. The EII components may be present as a single membrane-bound protein with 3 domains (A, B and C) or as 2 or more proteins, at least one of which is membrane bound (EIIB and EIIC) and one of which is soluble (EIIA). Of the membrane bound domains, EIIC is generally thought to be embedded within the membrane and EIIB may be associated with the membrane but not embedded within it. EIID forms a second membrane bound protein component of some PTSs (Postma *et al.*, 1993). It is a group of these EII enzymes that have significantly lower expression in the *Δrr09* mutant compared to D39 wild-type. The sequencing project using the TIGR4 strain identified 21 PTS systems in *S. pneumoniae* which is more than twice as many as any other sequenced bacterium to date, relative to genome size (Tettelin *et al.*, 2001). The authors of this published sequence annotated the PTS EII enzymes showing lower expression in the *Δrr09* mutant as belonging to a sorbose-family transport system (Tettelin *et al.*, 2001, supplementary information). This was based on an identification alignment with a *Klebsiella pneumoniae* sorbose PTS system (Wehmeier and Lengeler, 1994). Sorbose, an intermediate of vitamin C, is a ketose sugar with 6 carbon atoms and is a monosaccharide (Stryer, 1988). The EII components of this particular pneumococcal PTS have homology to sorbose systems in other bacteria, including *Lactobacillus casei* and *E. coli* (from the 'gene versus alignment' tool at http://www.tigr.org/ website).

Furthermore, when the protein sequence of the PTSIIB component (sp0061) is subjected to a BLAST search (http://www.ncbi.nlm.nih.gov:80/BLAST/) which can identify conserved domains, it was identified as having a conserved sorbose domain. The PTSIIB component was the gene identified as having highly significantly reduced expression in the *Δrr09* mutant compared to the isogenic D39 parental strain, as indicated in figure 5.17. The PTSIIB proteins of the sequenced R6 and TIGR4 strains show 99.4 % similarity and identity (http://www.tigr.org/).

Following identification of decreased expression of this putative sorbose transport system a carbohydrate fermentation assay was used to determine if the *rr09* mutants in both serotype 2 and 3 had reduced ability to ferment this sugar. Although most sugars occur naturally as the D-isomer, the L- form of sorbose is transported and metabolised by *K. pneumoniae* and *E. coli* (Wöhrl and Lengeler, 1990), although the reason for this is unknown. For this reason, both L- and D-forms of sorbose were included in the assay together with several other sugars, selected to represent monosaccharides, disaccharides and a trisaccharide. All strains, including the *rr09* mutants fermented all sugars to acid under anaerobic conditions with the exception of both sorbose isomers, which were not fermented by any of the strains, including wild-type D39 and 0100993. There are several possible explanations for this. Firstly, sorbose fermentation may be specific to certain strains and absent in the 2 strains used for this work. Indeed, although all strains of *K. pneumoniae* have been found to transport sorbose, only 30 % *of E. coli strains* can transport this sugar (Wöhrl and Lengeler, 1990). This could be tested by subjecting a range of pneumococcal strains, including the sequenced TIGR4, to the sorbose utilisation assay. The media and growth conditions used can also affect bacterial metabolism (Neijssel *et al.*, 1997), so the strains could be tested using several different culture media formulations and using different growth conditions, such as pH. Should these assays indicate that none of the strains tested ferment sorbose then it could be possible that sorbose is transported but not fermented in *S. pneumoniae*. This could be tested by radiolabelled-sorbose uptake assays. Fermentation of sugars by streptococci will only occur in anaerobic conditions (Neijssel *et al.*, 1997) and presumably the pneumococcus will encounter aerobic environments during infection so other sugars may be taken up by PTS but not fermented. As more data becomes available on bacterial PTSs, it is clear that they may have roles distinct from sugar transport, including gene regulation so it is possible that the role of this specific PTS system in *S. pneumoniae* is not solely as a sugar transporter.

Alternatively, the identified PTS may function as a sugar transporter but for a sugar other than sorbose. Indeed, the PTSIIB component (sp0061) of *S. pneumoniae* also shows identity to non-sorbose PTS systems, including a mannose-specific PTS in *E. faecalis* and a fructose-specific transporter in *L. monocytogenes* (BLAST alignment tool, http://www.ncbi.nlm.nih.Gov:80/BLAST/). Although annotated by TIGR as being part of the PTS-sorbose subfamily, this family is also known as the mannose/fructose/sorbose family as its permease members exhibit broad specificity for a range of sugars including mannose, fructose, sorbose and N-acetylglucosamine (http://www.togr.org/tigr-scripts/CMR2/). Of these sugars, all pneumococcal strains used in this work have been shown to ferment mannose. The remaining sugars, together with other sugars not previously tested could be assayed using the carbohydrate assay described above to determine if this system is involved in transport of alternative sugars. It is possible that TCS09 could be involved in nutrient perception as Lange and colleagues found that the HK09 sensing domain is related to the extracellular regions of the *B. subtilis* McpA and McpB proteins (Lange *et al.*, 1999). These proteins are involved in the control of chemotaxis via sensing environmental nutrient concentration (Garrity *et al.*, 1998). Indeed, of the 3 upper respiratory tract pathogens for which genome sequences have been published (*S. pneumoniae*, *H. influenzae* and *N. meningitidis*), *S. pneumoniae* has the widest substrate utilisation range for sugars (Tettelin *et al.*, 2001). Over 30 % of the pneumococcal transporters are thought to be for sugars, representing the highest percentage observed to date in any sequenced prokaryote (Tettelin *et al.*, 2001). It would not be surprising to find that genes required for nutrient acquisition were under the control of bacterial TCS as nutrient uptake places a huge metabolic burden on bacterial cells (Neijssel *et al.*, 1997). Thus, expression of genes required for nutrient acquisition is likely to be tightly regulated. Infection at different sites may require that bacteria can utilise a range of carbohydrates depending on the availability, and streptococci are known to exhibit metabolic flexibility (Neijssel *et al.*, 1997). TCS could be involved in sensing nutrient availability at various sites of infection. Furthermore, if TCS09 is involved in nutrient perception, then the differences presented between the *in vivo* role of the RR09 protein in type 2 and type 3 could be attributed to different nutrient requirements between strains / serotypes. Synthesis of the polysaccharide capsule may require uptake of different sugars. Indeed the capsules of type 2 and type 3 pneumococci are composed of different sugars, D-glucuronic acid, D-glucose and L-rhamnose for type 2 (Ianelli *et al.*, 1999) and cellobiuronic acid (a disaccharide of D-glucuronic

acid and D-glucose) for type 3 (Garcia *et al.*, 2000). Thus these sugars are obvious candidates for the carbohydrate fermentation assay. Alternatively, the structure or thickness of the capsule may inhibit the uptake of some sugars. Further work needs to be carried out to determine which sugars, if any, the PTS system described transports and if such sugars, or products of these, are related to the sugars required for capsule synthesis of the individual strains. It could also be possible that in conditions of severe nutrient deprivation, the bacteria could use their own capsule polysaccharide as a carbohydrate source, and that the TCS could control this function to prevent it become disadvantageous to the bacterial cell. In this situation, a thicker capsule may confer an advantage on the invading organism, and could be an explanation for the ability of the *rr09* mutant in a serotype 3 strain to be more virulent than a serotype 2 mutant due to the thicker capsule of the former. Creating null mutations in the pneumococcal PTSIIB (sp0061) component (or other EII PTS components) and comparing the effect of these in different serotypes could provide important data to analyse the effect of nutrient acquisition between pneumococcal serotypes, if this is indeed the reason for the differences seen in virulence between type 2 and type 3.

Incorrect annotation may also explain the reason that a PTS system in *S. pneumoniae* has been identified as a sorbose uptake system if the bacteria does not ferment sorbose. Such mistakes are known to occur, such an example being in *S. suis* whereby a conserved hypothetical protein had homology with a protein in *Pseudomonas aeruginosa* that was annotated as a macrophage infectivity potentiator (mip). However, the *P. aeruginosa* 'mip' protein was found to be very distinct from other, better characterised mip proteins and thus appears to be mis-annotated (King *et al.*, 2001). This highlights the caution that must be involved when identifying unknown sequences on previously annotated proteins.

It is intriguing why a putative sorbose uptake system would be expressed during growth of wild-type bacteria in BHI. The expression of this system was thus compared with expression of other PTS. Of 21 individual PTS examined, only 6 had expression levels above the set threshold limit of 500, 2 of which had expression intensities of <1000. This observation is intriguing as the BHI medium used to grow the bacteria used for expression analysis contains glucose (0.2 %, see appendix). As a glucose-specific PTS is expressed by D39 during *in vitro* growth, the function of the remaining PTS is unknown. Similarily, of the 4 PTS with mean expression intensities of >500, the sorbose-family PTS identified in this work as being significantly down-regulated in the

Δrr09 mutant, had the highest expression. This is further evidence that it could transport sugars other than sorbose or has an entirely different role in bacterial cell function. None of the 6 PTS that were expressed during *in vitro* growth in BHI were identified as being essential pneumococcal genes in a recent study by Thanassi and co-workers (Thanassi *et al.*, 2002). This study used a bioinformatics approach to identify pneumococcal genes that had 40 % global amino acid sequence identity with genes of several other pathogens, on the basis that such genes could be targeted for broad-spectrum anti-microbial therapy. All candidate genes were mutated and 113 essential genes were subsequently identified (Thanassi *et al.*, 2002). However, this study did not aim to identify all essential genes in the pneumococcal genome, only those that were conserved between certain bacterial species, so some of the PTS described above may be essential for growth and this would require further investigation.

Given the apparent importance of sugar utilisation in *S. pneumoniae* discussed above, it is possible that pneumococcal PTS could become the target of future therapies or vaccines. Although many individual PTS have been identified, there is only one copy of the EI and HPr genes (Tettelin *et al.*, 2001). As these proteins perform roles central to and essential for all of the pneumococcal PTS, it would be interesting to create null mutations in either of, or both of these genes for further *in vivo* analysis. Furthermore, this work could have significance for other bacterial pathogens. Several of the pneumococcal PTS have been identified in genome-wide approaches for the identification of virulence factors, illustrating that they are important in infection (Polissi *et al.*, 1998; Lau *et al.*, 2001; Marra *et al.*, 2002; Hava and Camilli, 2002). Indeed, a study using STM to identify *S. pneumoniae* virulence factors in a type 4 strain pulled out components of 8 different sugar PTS, where mutations in such systems were highly attenuated in lung infections, including the EIID component of the sorbose-family PTS discussed above (Hava and Camilli, 2002).

Immediately upstream of the PTS EII components that were all down-regulated in the *Δrr09* mutant is a gene encoding β-galactosidase, an enzyme often involved in breaking down lactose. The expression of this gene was decreased in the *Δrr09* mutant compared to the D39 parental strain. The association of the *β-galactosidase* gene with the described PTS system is unknown, as none of the BLAST alignments of the PTSIIB component (sp0061) had homology to systems involved in lactose transport. However, the *β-galactosidase* and *ptsIIB* genes overlap, indicating

that they may form a single transcriptional unit and thus may have related functions. Within the R6 and TIGR4 genomes, 2 β-galactosidase genes have been identified, together with 2 6-phospho-β-galactosidase genes (table 6.2)(http://www.tigr.org/). The latter enzymes are likely to be responsible for cleaving intracellular lactose-6-phosphate and are both located next to putative lactose PTSs. The β-galactosidase genes are also located close to putative PTS (of which the sorbose-family PTS described above is one), although the specific sugars associated with these systems is yet to be determined. Table 6.2 illustrates the β-galactosidase and 6-phospho-β-galactosidase genes found in the sequenced, annotated genomes. As demonstrated in the table, the corresponding genes between the different genomes are at least 99 % homologous. However, the 2 individual β-galactosidase genes within each genome do not show any significant homology to each other, while the 2 individual 6-phospho-β-galactosidase genes within each genome are approximately 47 % identical (68 % similar).

This thesis has identified sp0060 (R6 spr0059), one of the genes annotated as a β-galactosidase, as having lower expression in the *Δrr09* mutant. However, the function of this gene as a true β-galactosidase can be questioned based on a study by Zahner and Hakenbeck (Zahner and Hakenbeck, 2000). This study created a disruption within the larger β-galactosidase gene (R6 spr0565) in strain R6 using allelic replacement, and found that this resulted in a complete loss of β-galactosidase activity (Zahner and Hakenbeck, 2000). Surprisingly this gene did not appear to be involved in lactose metabolism as growth of the mutant in defined media containing lactose as the only carbon source did not result in decreased generation time compared to the parental wild-type strain (Zahner and Hakenbeck, 2000). As gene *spr0565* has a LPXTG motif, used for anchoring many Gram positive proteins to the cell surface, it was proposed that this enzyme has a role in interactions with host cells rather than for lactose metabolism. Indeed β-galactosidases are able to cleave β-1,4-glycosidic bonds and Galβ1-4GlcNAc bonds which are present within many polysaccharides on the surface of eukaryote cells (Zeleny *et al.*, 1997). Cleavage of such bonds may release sugars for further utilisation by the bacteria. The lack of homology between the 2 annotated β-galactosidases genes of R6 indicates that the study by Zahner and Hakenbeck would indeed have disrupted the larger gene only, without having any effect on the β-galactosidase gene identified within this work. This indicates that the smaller β-galactosidase gene identified in this work may not have β-galactosidase activity. However, assay conditions used to detect β-galactosidase activity could affect the ability to detect other β-galactosidases that

may have, for example, different optimum pH for activity, and modification of the assay may indeed allow β-galactosidase activity of other genes to be analysed. Indeed, a second neuraminidase enzyme (NanB) present in the pneumococcus was identified and found to function at a different pH optimum to that of the known, previously characterised neuraminidase (NanA) (Berry *et al.*, 1996). A null mutation in NanA resulted in apparent complete loss of neuramidase activity pH6.5, but when the pH was lowered to 4.5, neuraminidase activity due to NanB could be detected (Berry *et al.*, 1996). Assays are available to test for both β-galactosidase and phospho-β-galactosidase activity (Zahner and Hakenbeck, 2000 and Chen *et al.*, 2002 respectively). Mutations in the 4 β-galactosidase-associated genes could thus be created and individual mutants analysed for their ability to cleave the substrates for the respective enzymes, using several different assay conditions. This could help determine the role of the putative β-galactosidase gene identified in this work. Alternatively, the ability of the *Δrr09* mutant to cleave the β-galactosidase substrate 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-gal) from a colourless substrate to a blue product could be compared to that of the isogenic parental strain, D39 (Nicholl, 1994).

The larger β-galactosidase gene, Spr0565 has an LPXTG motif, and this cell-surface anchor motif is absent in spr0059 / sp0060. Thus it may be possible that there are 2 functional β-galactosidase genes, one functioning outside the cell and the other acting intracellularly, and that the study by Zahner and Hakenbeck only detected activity of the former. Alternatively, the smaller spr0059 / sp0060 gene may be mis-annotated and could have other functions that may or may not be related to sugar uptake and utilisation. This concept could be investigated further by using mutations in the assays described above.

R6 genome				Homology between corresponding R6 / TIGR4 gene identity (similarity)	TIGR 4 genome			
Name	**Gene symbol**	**Common name**	**Length of protein (aa)**		**Name**	**Gene symbol**	**Common name**	**Length of protein (aa)**
Spr0059	bga	beta-galactosidase	595	99.3 % (99.7 %)	Sp0060	bga	beta-galactosidase	595
Spr0565	bgaA	beta-galactosidase	2228	98.2 % (99.2 %)	Sp0648	bgaA	beta-galactosidase	2233
Spr0424	lacG	6-phospho-beta-galactosidase	470	99.8 % (99.8 %)	Sp0477	lacG	6-phospho-beta-galactosidase	470
Spr1069	lacG	6-phospho-beta-galactosidase	468	98.9 % (99.6)	Sp1184	lacG	6-phospho-beta-galactosidase	468

Table 6.2 β-galactosidase-associated genes in R6 and TIGR4 genomes

Each of the sequenced genomes possess 2 genes annotated as β-galactosidase and 2 genes annotated as 6-phospho-β-galactosidase. The latter gene products are likely to use phosphorylated lactose as their substrate and are located close to lactose-specific PTSs. The function of the β-galactosidase genes has not been fully determined, although the larger gene (spr0565 / sp0648) has been proposed to act on the cell surface to cleave host sugars (Zahner and Hakenbeck, 2000). The corresponding genes between genomes are highly homologous (>99 %).

Immediately downstream of the *pts* genes are 2 enzymes, isomerase and aldose-1-epimerase that also show reduced expression in the *Δrr09* mutant. Isomerases catalyse isomerisations / intramolecular rearrangements and this group of enzymes includes epimerases. All genes discussed so far are located in relative close proximity to each other suggesting that they may form an operon or functional unit. Furthermore, a rho-independent transcription termination site was not identified in the region comprising any of the described genes, providing further evidence that the genes form an operon (see figure 5.18). If the genes do form an operon, the different levels of expression of the genes within this operon seen with both D39 wild-type and the mutant strain could indicate post-transcriptional regulation or the presence of rho-dependent transcriptional terminator sites between the genes. Although annotated as a sorbose system, sorbose is a ketose sugar (has a ketone group in its structure) and the epimerase within this cluster of genes is annotated as an aldose-1-epimerase, indicating that it is specific for aldose sugars (which contain an aldehyde group in their structure). This provides further evidence suggesting that this PTS is involved in the transport of a sugar other than sorbose. Furthermore, as discussed above, the link with *β-galactosidase* is intriguing. As the phospho-β-galactosidase genes are found close to lactose PTSs, it is unlikely that a third uptake system for lactose exists in the genome. Lactose is a disaccharide of galactose and glucose, both of which are aldose sugars and both of which were fermented by all the strains used within this work. The sugar that is transported and metabolised by these genes therefore remains to be determined.

The second cluster of genes identified as having lower expression in the *Δrr09* mutant [1](*sp1800-sp1805*) do not have known functions, with the exception of one, *sp1804*, which has been annotated as a putative stress-related protein (http://www.tigr.org/). This annotation is based on some homology with general stress proteins in several other bacteria, although the type of stress to which these proteins respond has not been identified. The TIGR4 *sp1804* gene is 100 % homologous to the equivalent gene in R6. Genes upstream of *sp1800* did not appear to be related to this cluster and had normal expression levels in the mutant. Downstream genes *sp1806-sp1808* had expression levels in both strains that were too low to be analysed. As for the pts-related genes, all of the above genes are situated within close proximity of each other on the chromosome, although none of the coding regions overlap. However, only one rho-independent

[1] See footnote p217

transcription terminator was identified within this set of genes, and was located in the region downstream of *sp0085*. This indicates that the genes may indeed form an operon. As with the sorbose PTS-related genes described above, if the genes do form an operon, the different levels of expression of the genes within this operon seen with both D39 wild-type and the mutant strain (figure 5.21) could indicate post-transcriptional regulation or the presence of rho-dependent transcriptional terminator sites between the genes. All genes are encoded on the coding strand, with the exception of *sp1800*, a transcriptional activator, which is found on the complementary strand (figure 5.19). This does not, however, exclude the possibility that this activator is involved in regulating genes *sp1801-1805*, as a regulator in the pneumococcus termed RirA has recently been shown to regulate 6 divergently transcribed genes (Hava and Camilli, 2002).

Reduced expression of the genes discussed does not prove that such genes are directly regulated by TCS09. It is likely that some genes could be affected indirectly through other regulatory networks. Indeed, varying levels of expression of genes that could form an operon and be transcribed together (figure 5.21) could indicate a role for TCS09 in post-transcriptional regulation of certain genes. DNase I footprinting could be used to identify promoter regions to which the active RR09 binds. This technique is used to characterise the targets of sequence-specific DNA-binding proteins (Galas *et al.*, 1978). Labelled DNA containing a potential binding site is mixed with the purified protein or the protein's DNA binding region, and the complex is then treated with DNase I. When compared with a control reaction, target sites on the DNA to which the protein has bound will be protected from DNaseI treatment. These sites can be identified with subsequent sequence determination. This method has successfully been applied to other bacterial TCSs to identify potential regulator-binding sites (Bernish and van de Rijn, 1999; Kovacikova and Skorupski, 2001; Dietz *et al.*, 2002), although it depends on the response regulator being in its phosphorylated form. If applied to *S. pneumoniae* RR09, resulting data could be used to determine which of the identified genes or sets of genes are controlled directly by TCS09. Should a promoter sequence to which RR09 binds to be identified, then this sequence can be used to search the genome for other genes potentially regulated by the system that may not identified by microarray analysis.

The array analysis was performed using only one RNA preparation taken from a single time point during *in vitro* growth. Thus, the array work needs to be repeated in duplicate at least one

further time using a different RNA preparation, but from the same stage of bacterial growth to confirm the data discussed above. Furthermore, the genes discussed above do not provide an exhaustive list of all of the genes potentially regulated by *S. pneumoniae* TCS09 in strain D39 due to expression analysis using a single time point only. During *in vitro* growth alone, different sets of genes are regulated at different stages of growth and the growth conditions are also likely to affect gene expression. Indeed, a study using *E. coli* estimated that 200 genes are induced upon entry into stationary phase during *in vitro* culture (Tani *et al.*, 2002). During *in vivo* infection, gene expression profiles are likely to change rapidly and frequently as the bacteria encounter many different and mixed environmental signals. As an infection progresses, the host immune response at various sites of infection will change over time and this is also likely to alter bacterial gene expression. It would thus be ideal to compare gene expression in pneumococcal strains isolated from different sites of murine infection over a series of time points using TCS mutants, as this could help identify differential regulation of virulence-associated genes by TCSs. However, several problems may be encountered during such a study. Firstly it is difficult, although not impossible, to isolate sufficient concentrations of RNA from *in vivo* samples for microarray analysis. The isolated sample must contain high bacterial loads be processed rapidly to prevent significant alteration of bacterial mRNA transcripts. Biological systems are also extremely variable so RNA preparations from several animal models must be compared for each time point examined with each individual tissue specimen to allow for variation. Problems will be encountered where it is desirable to compare gene expression levels in a mutant impaired in virulence with its isogenic parental strain, as it will be difficult to isolate sufficient RNA from tissue samples containing mutant bacteria due to significantly lower bacterial counts. This is highlighted by the *in vivo* data described for the *Δrr09* mutant, where bacterial load in all tissues following several routes of infection was generally so low that it was barely detectable. Finally, being a human pathogen, many of the environmental signals that are sensed by pneumococcal and other bacteria TCSs may be specific to the human host. Therefore, the use of murine models of infection may never reveal some of the more important genes regulated by such systems.

With respect to the decrease in transformation efficiency of the *Δrr09* mutant compared to D39 wild-type, microarray analysis did not reveal any genes involved in competence that had reduced expression in the mutant. As expected for the stage of growth from which RNA was isolated, the

TCS system largely associated with development of competence (ComD/E) had very low expression intensities (<200), so could not be analysed. One possible way of investigating gene expression differences related to transformation efficiency would be to grow cultures in the presence of CSP1 to induce the genes involved in competence and compare expression of competence-related genes in the mutant and wild type.

Expression data for all of the pneumococcal response regulator genes was included in the microarray analysis. None of these genes showed decreased expression in the *Δrr09* mutant compared to D39 wild-type, indicating that under the conditions used, RR09 does not regulate any of the other systems, or if it does, its function can be taken over by another system. A ratio of >1.0 was found for the expression of *rr14* in the *Δrr09* mutant compared to D39, indicating that this response regulator could be up-regulated in the mutant. This increase was statistically significant ($P<0.05$). The expression intensity of this gene, however, was not found to be significantly different to that of wild-type. To determine if the differences seen with *rr14* are genuine or due to experimental variation, these experiments need to be repeated.

Figure 5.23 compares the expression intensities of all response regulator genes in both D39 and *Δrr09*. Genes for *rr02* and *rr05* resulted in very high mean expression intensities indicating that the genes are required in high concentrations for cell function, at least during *in vitro* growth. TCS02 has already been identified as being essential for *S. pneumoniae* viability (Lange *et al.*, 1999; Throup *et al.*, 2002; Wagner *et al.*, 2002) and homologues of this system in other bacteria are also essential (Fabret and Hoch, 1998; Martin *et al.*, 1999). The multifunctional role of TCS05/CiaRH has been discussed previously (introduction, section 1.14.3). Many of the response regulator genes had intensities that were below the set threshold limit. Whether these represent systems that genuinely do have very low expression in pneumococcal growth in culture media or whether the RNA isolated for these systems was of poor quality or was rapidly degraded is not known. One of the TCS for which expression could not be detected is TCS12 / ComD/E involved in transformation. It is of interest that TCS05 / CiaR/H, which negatively regulates the comC/D/E operon (Echenique *et al.*, 2000), has very high expression when expression of the comD/E system is too low to detect. Thus comparison of expression levels of these two systems following treatment of cultures with CSP-1, which induces expression of the comD/E system, would be expected to result in increased expression of the ComD/E system and

possibly reduced expression of the CiaR/H system. However, caution must be taken when comparing expression levels of different genes, as different probe lengths combined with problems encountered in direct labelling with Cy dyes may result in inaccurate data interpretation. Problems associated with isolation / detection of RNA transcripts and comparing expression intensities in microarray analysis are discussed below.

As the *Δrr09* mutant was completely avirulent following all routes of infection, the expression of 7 known virulence factors, together with several capsule-related genes, was compared between the wild-type and mutant strains. None of the genes had reduced expression in the mutant compared to D39 wild-type (table 5.5). The genes for the major autolysin, LytA, and pneumolysin had the greatest levels of expression, which may be expected as the RNA analysis was performed on cultures in mid-late exponential phase, where the autolysin is known to become active and lyse cells, releasing the toxin pneumolysin. At least 9 capsule-associated genes were compared but only 3 of these had expression intensities above the set threshold limit of detection (500). One of these capsule-associated genes is a UDP-glucose dehydrogenase and the remaining 2 are dTDP-rhamnose biosynthesis genes (rhamnose is one of the 3 sugars required for type 2 capsule biosynthesis) (Ianelli *et al.*, 1999). The finding that a completely avirulent phenotype is not associated with decreased expression of many of the main pneumococcal virulence factors identified to date, illustrates the complex nature of virulence in this pathogen and highlights that there are important *S. pneumoniae* virulence factors still to be characterised. Expression of one virulence factor, PspC (also known as CbpA, Hic, SpsA and PbcA), was shown to be significantly increased in the *Δrr09* mutant compared to D39 wild-type. This pneumococcal surface protein has several properties thought to be important in virulence. It is an adhesin and promotes invasion of epithelial cells (Rosenow *et al.*, 1997; Zhang *et al.*, 2000), can bind components of complement, including factor H (Janulczyk *et al.*, 2000; Dave *et al.*, 2001; Duthy *et al.*, 2002), binds the secretory component of IgA (Hammerschmidt *et al.*, 1997) and has been shown to be a good vaccine candidate (Briles *et al.*, 2000). Furthermore, PspC is one of the few pneumococcal genes that displays extensive sequence variation between serotypes (Ianelli *et al.*, 2002). The significance of the up-regulation of this important virulence gene in the *Δrr09* mutant, which is completely avirulent, is not understood and needs to be characterised further. Further microarray work with the *Δrr09* mutant should help determine if

this observation is a true result of the null mutation in *rr09*. Microarray analysis with mutations in the *rr09* gene in other serotypes would be useful to determine if this increased expression of PspC is specific to certain pneumococcal strains / serotypes. Furthermore, PspC was not identified as being significantly up-regulated in the *Δrr09* mutant by examination of the Genespring™ scatterplot, and this indicates that analysis of gene expression with microarrays should not depend on one method of analysis and that caution must be taken when presenting data from such work.

A more detailed study based upon microarray analysis is required to enable a full discussion of the genes regulated by TCS09 and to correlate these with the role of the system in virulence. The experiments done with D39 and the *Δrr09* mutant must be repeated to ensure that the genes described above are significantly and consistently down regulated in the mutant. Experiments must be repeated using RNA isolated under the same conditions from the type 3 strains, 0100993 and *Δ488*. Should any differences in gene expression be found between *Δrr09* and *Δ488*, this could provide important clues as to why the RR09 proteins in type 2 and type 3 pneumococci have different roles in virulence in our model of infection. Examination of mutations in *rr09* genes of other strains / serotypes using *in vivo* characterisation combined with expression analysis may provide further insight into the role of TCS09. Following detailed analysis, genes of interest must be investigated further by RT-PCR, Northern blot and / or real time-RT-PCR. Indeed, one study has shown that TaqMan RT-PCR (real-time RT-PCR) was more efficient than microarrays at detecting low-abundance transcripts (Graham *et al.*, 2002). Where possible, functional assays should be performed as gene transcription does not necessarily correlate with protein expression. A study by Kothapalli and colleagues, examining gene expression in leukaemia patients, found that 3 genes of interest that were down-regulated according to microarray analysis could not be confirmed as having differential expression by Northern blot. Similarly, analysis of the up-regulated genes in leukaemia patients compared to healthy controls found that only 47 % of those identified by microarray experiments could be confirmed by Northern blot (Kothapalli *et al.*, 2002). This study highlights the importance of clarifying all data obtained through microarray work. The carbohydrate utilisation and β-galactosidase assays described above are examples where altered levels of gene transcripts may be correlated with functional protein expression.

The microarray analysis discussed above was very preliminary and the need for repeat experiments has been highlighted. Despite this, several issues regarding microarray analysis should be discussed so that future work can be interpreted correctly. Although the hybridisations worked well, many gene spots on the array did not show up. The exact number of genes absent following hydridisation was not accurately determined, but a visual inspection indicated at least 10 % of genes were missing. This could indicate a genuine absence of expression under the conditions used, or could be due to several other causes. For example, the loss of gene transcripts during RNA isolation, the detection limit of the arrays or the failure of certain probes to detect cDNA transcripts. Bacterial mRNA is generally regarded to be less stable that eukaryotic RNA so samples must be processed rapidly during RNA preparations to prevent degradation of transcripts. This procedure is further complicated by the thick cell wall of bacteria, especially Gram-positive species, such as *S. pneumoniae* that must be disrupted by rigorous vortexing or under high pressure in the presence of lysozyme. Several commercially available reagents have been developed to help 'protect' RNA immediately upon harvesting cells, but use of one of these reagents was found to significantly reduce the RNA yield obtained. To try and overcome this, cell cultures were flash frozen in liquid nitrogen prior to RNA isolation.

High density oligonucleotide arrays have the ability to detect gene transcripts as low as one copy (Lockhart and Winzeler, 2000). The array probes used for this thesis were obtained by spotting PCR products onto a microarray slide and the detection limit for this type of array will not be as sensitive as the high density oligonucleotide arrays. For this work, the limit of detection for RNA transcripts was not determined but is an important consideration for future work. The intensity value of 500 as a threshold for considering gene expression was recommended by the Bacterial Microarray Group at St. Georges (http://www.sghms.ac.uk/depts/medmicro/bugs/). However, it would be useful to select several genes that have expression levels equal to or below the intensity threshold of 500, and use RT-PCR (with the same RNA preparation used for array analysis) to determine if RNA transcript can be detected and to assess the accuracy of the assigned threshold value. The detection limit for microarray analysis could also be determined by spiking cDNA preparations with known concentrations of cDNA for the human genes (β-actin and GAPDH) included on the array as negative controls. This would help determine the background levels of intensity and allow analysis of the minimum concentrations of RNA expected to be detected by

array analysis so a more accurate limit of detection could be set. Such controls should be included with all subsequent array analysis to take into account variation between arrays, and would have been included had time permitted.

Probe design and cDNA labelling may also affect the detection of RNA transcripts. For the array used, the probes were designed using software that places the probes at regions within the gene that will result in high specificity to avoid cross hybridisation. The probes were also designed to have similar annealing temperatures. This resulted in probes of different lengths designed against different regions of pneumococcal genes. Only one probe per gene was used. Random primers were used to produce cDNA from the RNA preparations so all probes should lie within the cDNA molecule (probes that lie outwith cDNA may result in false negatives). Cy3 / Cy5 were incorporated directly into the cDNA in the same procedure. The Cy dyes used are coupled to dCTP, although non Cy-coupled dCTP is also included in the reaction mixture, albeit at lower concentration than the other dNTPs. Thus it may be possible that probes having a higher guanine content will have greater dye incorporation. Being large structures, the Cy dyes are not incorporated into the growing cDNA as easily as the dCTP molecules and the incorporation of such large molecules results in relatively short cDNA molecules that do not usually exceed 300 bp. Uneven incorporation of the Cy fluorophores also occurs, with Cy3 incorporating easier than Cy5. Following incorporation, Cy3 and Cy5 do not fluoresce to the same extent, whereby Cy3 molecules in close proximity to one another enhance fluorescence, but Cy5 molecules in close proximity to one another quench fluorescence (Molecular Genetics NoAb Diagnostics, Inc; http://bfx.kribb.re.kr/gene-array2/0509.html). Cy5 is also susceptible to bleaching so arrays must be protected from light and scanned as soon as possible following hybridisations, with Cy5 scanning performed first. These problems highlight the amount of error that can be introduced into the transcription expression profiles solely by using direct incorporation of Cy3 and Cy5 dyes. Reverse labelling of cDNA is an option that will help overcome some of this error, and this procedure was used for the data presented in this thesis. However, aminoallyl indirect labelling may be more suitable for future work. This method involves the production of cDNA using aminoallyl dNTP in the absence of fluorescent dyes. Cy3 / Cy5 is coupled to the resulting aminoallyl-cDNA as a separate stage in the protocol. This results in longer cDNA molecules (500-1000 bp) which results in a greater signal intensity, allowing detection of transcripts that may only be present in low copy number. Longer cDNAs also increase the chances of the

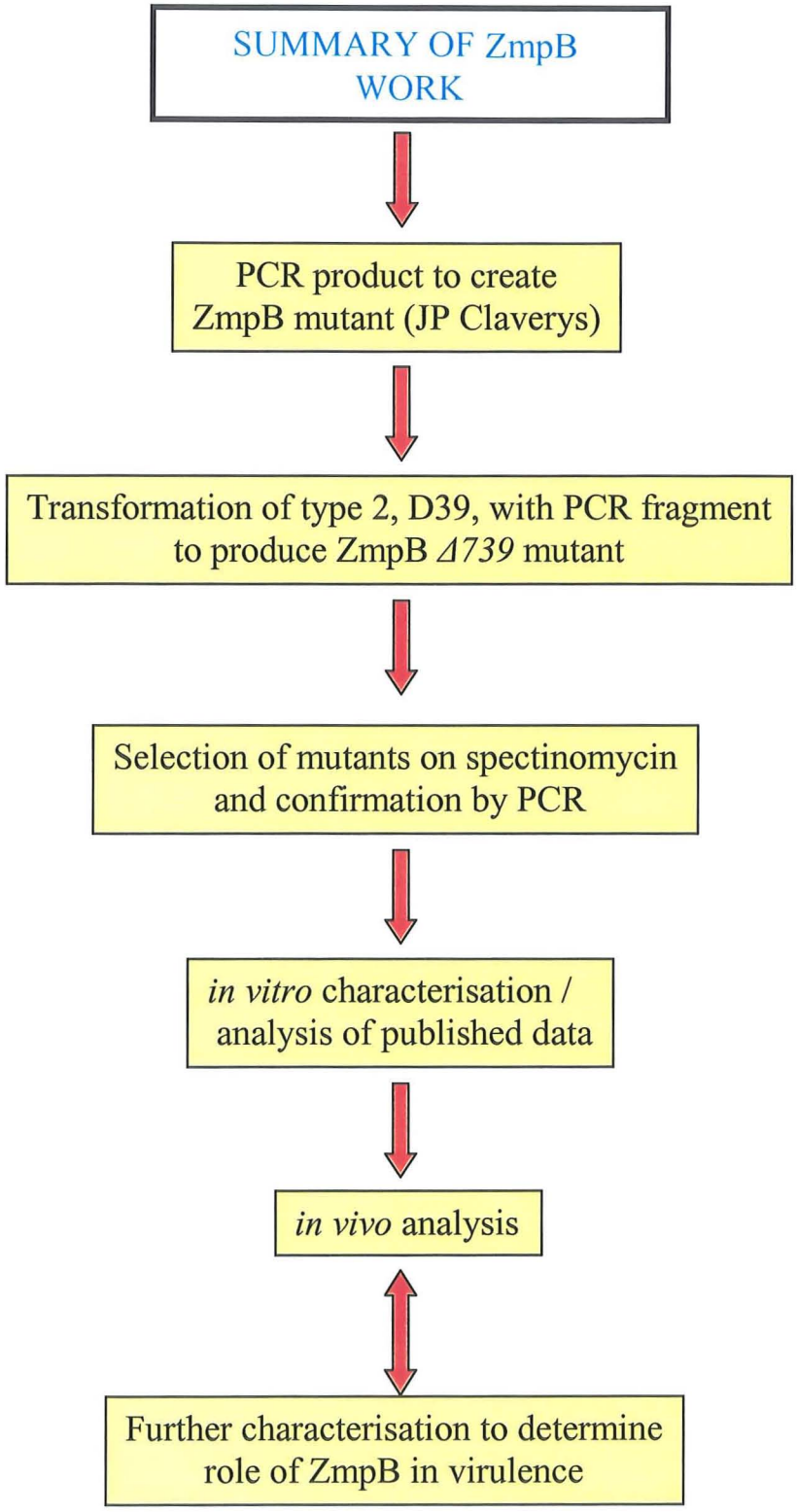
sequences complementary to the array probe being present. Incorporation of the Cy3 / Cy5 dyes is more uniform and a smaller amount of starting RNA is required. This latter advantage could allow RNA preparations to be performed in the presence of RNA Protect™, which results in lower yields but could protect more of the RNA transcripts from degradation. Combined with the advantages of increased signal intensity obtained using indirect labelling, these techniques could greatly increase the number of spots detected on the arrays and would help determine the differences between low expression and absence of expression. One study has demonstrated an improvement in results using indirect labelling compared to direct labelling, where the former produced consistently higher intensity hybridisations with low background signal (Yu *et al.*, 2002).

In summary, microarray experiments can yield large volumes of exciting data previously unattainable using conventional techniques. However due to the complex nature of microarray design, array printing, batch variation, sample preparation / hybridisation, labelling methods and data analysis / normalisation there are numerous stages at which error may be introduced resulting in inaccurate conclusions and interpretation of data (Hess *et al.*, 2001). Care must be taken that all stages and protocols are optimised and strictly adhered to, appropriate controls are included in every experiment and that data is normalised and analysed adequately.

Chapter 7

Results

S. pneumoniae ZmpB


SUMMARY OF ZmpB WORK
PCR product to create ZmpB mutant (JP Claverys)
Transformation of type 2, D39, with PCR fragment to produce ZmpB Δ739 mutant
Selection of mutants on spectinomycin and confirmation by PCR
in vitro characterisation / analysis of published data
in vivo analysis
Further characterisation to determine role of ZmpB in virulence

7 Results: *S. pneumoniae* ZmpB

Based on the observation that the *zmpB* gene was located in close proximity to TCS09, it was hypothesised that this particular TCS could control expression of *zmpB*. Furthermore as *zmpB* has not been extensively characterised previously for its role in pneumococcal virulence, it was the aim of this work to create a mutant in this gene, analyse its role in virulence and determine if its expression was influenced by TCS09.

7.1 ZmpB basic *in vitro* analysis

7.1.1 Creating and confirming mutants

A series of interruptions along the *zmpB* gene were created by M. Bergé / JP. Claverys (Toulouse, France) using *mariner* mutagenesis (Bergé *et al.*, 2001). Briefly, this mutagenesis technique is based upon a mini-transposon, *mariner*, which integrates at random sites resulting in a series of truncations within the target gene, as described in chapter 1, section 1.7.3. The *mariner* cassette includes a gene encoding spectinomycin resistance, which acts as a selective marker for mutants. The mutations were originally made in *S. pneumoniae* strain R6, an un-encapsulated, avirulent derivative of D39 (Ianelli *et al.*, 1999). PCR products from 2 independent R6 *zmpB* integration mutants (zmpB738 and zmpB739) were sent to our laboratory and used to transform the virulent, type 2, pneumococcal strain, D39. Potential transformants were selected on spectinomycin plates and confirmed by PCR. The insertion of the mariner cassette into the *zmpB* gene results in a PCR product approximately 1 Kb larger than wild-type *zmpB* when using primers ZmpUp and ZmpDo flanking the entire *zmpB* gene (figure 7.1). Mariner insertion into *zmpB* was further confirmed using a primer specific to the *mariner* cassette (MP128) in combination with a primer designed against the chromosomal region flanking the insertion site, ZmpUp (figure 7.1). The latter PCR will result in a detectable product for the mutants but not for wild-type D39, which does not contain a binding site for the MP128 primer. PCR with this primer also determines the site of integration within the *zmpB* gene. Both zmpB738 and zmpB739 mutants were created and confirmed this way. Mutant zmpB739 has a *mariner* integration approximately 600 bp from the start of the gene and is thus more truncated than the zmpB738 mutation where the *mariner* cassette has inserted approximately 1-1.5 Kb from the start of the gene. Both mutants were analysed in all *in vitro* and *in vivo* experiments presented in this chapter, with the exception of the IL-6, IL-10 and IFN-γ ELISA work which was performed

using zmpB739 alone. As the mutations behaved similarly in all experiments, only data for the more truncated zmpB739 mutant is presented. This mutant will be referred to as *Δ739* or *739* from this point onwards, with D39 representing the isogenic wild-type parental strain. PCR data confirming this mutation is shown in figure 7.2.

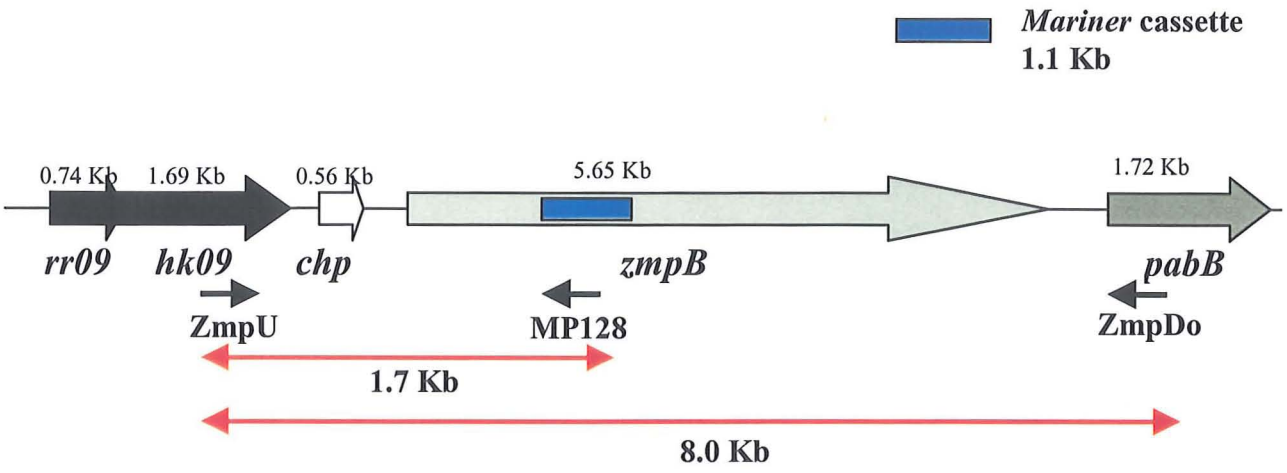


Figure 7.1 Mariner mutagenesis to create the Δ739 mutant

A *mariner* mini-transposon cassette (shown as a blue bar), containing a gene for spectinomycin resistance, was used to create integrations within the *zmpB* gene. For mutant *Δ739* shown in the diagram, the cassette integrated approximately 600 bp from the start of the gene, resulting in a PCR product 1.1Kb larger in the mutant compared to wild-type when primers ZmpUp and ZmpDo flanking the entire *zmpB* gene were used. The integration was also confirmed using a *mariner*-specific primer (MP128) with a primer ZmpUp upstream of the insertion site. This results in a 1.7 Kb band for the *Δ739* mutant that is absent in the wild-type strain. PCRs confirming the integration for *Δ739* are shown in the following figure (figure 7.2). The distance between the ZmpUp primer and the start of the *zmpB* gene is 1.1 Kb. As MP128 binds to the start of the *mariner* cassette, the 1.7 Kb PCR product using these primers indicates that the mini-transposon has integrated approximately 600 bp from the start of the *zmpB* gene. Sizes of *zmpB* and the surrounding genes are indicated above the illustration and are based on the TIGR4 genome sequence (http://www.tigr.org). Rho-independent transcription termination sites have been indicated in a previous figure (introduction, figure 1.17).

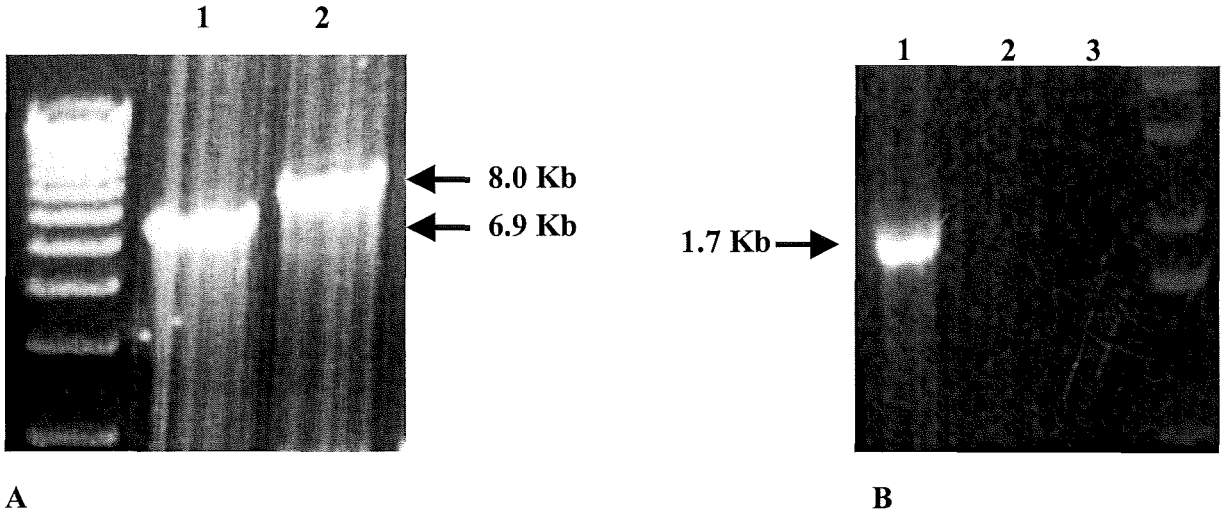


Figure 7.2 Confirming *Δ739* mutant by PCR

Primers ZmpUp and ZmpDo flanking the entire *zmpB* gene (see figure 7.1) were used to confirm the *Δ739* mutation in strain D39. Insertion of the mariner cassette into *zmpB* results in a PCR product 1.1 Kb larger in the mutant compared to wild-type (figure 7.2A). Figure 7.2A shows the expected 6.9 Kb band for D39 wild-type (lane 1) and the larger 8.0 Kb band with the *Δ739* mutant (lane 2). To further confirm the presence and position of the *mariner* cassette, primer MP128, specific for mariner was used with primer ZmpUp (figure 7.2B). Lane 1; *Δ739* mutant. Lane 2; D39 wild-type. Lane 3; negative control for PCR, set up in the absence of template DNA. A band would not be expected using D39 DNA due the absence of the *mariner*-specific binding site for primer MP128.

7.1.2 Growth and autolysis *in vitro*

Strains were subject to growth and autolysis determination using viable cell enumeration and optical density readings (figure 7.4). Comparison of the *Δ739* mutant grown in the presence and absence of spectinomycin indicated that the antibiotic did not adversely affect growth (data not shown). The mutants were also shown to be stable for up to 12 hours during *in vitro* growth in the absence of spectinomycin (figure 7.3). Time points after 12 hour were not examined as the *Δ739* mutant strains rapidly autolyse and lose viability from 10 hours post inoculation (figure 7.4).

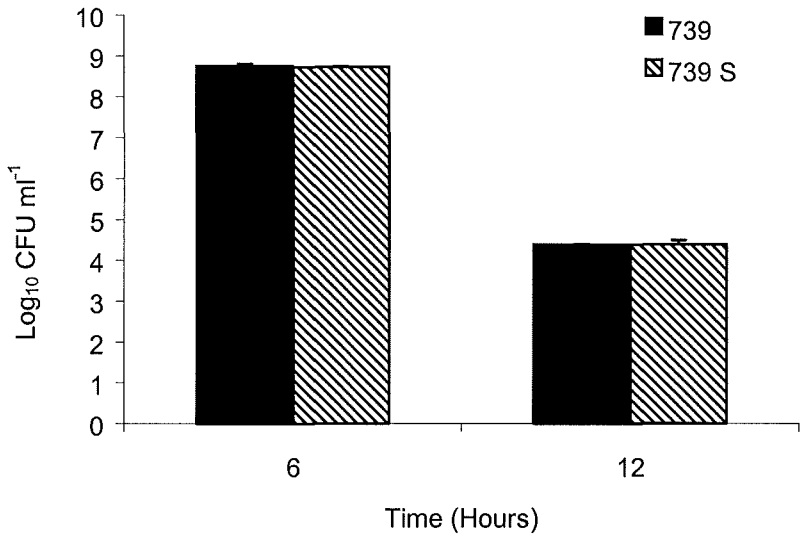


Figure 7.3 Stability of *Δ739 in vitro*

15 ml BHI was inoculated with 1.0 x 10^6 CFU mutant strains in the absence of spectinomycin selection. At 6 hr and 12 hr post inoculation, cell viability counts were performed to determine if the mutants had lost their antibiotic resistance. Counts were performed in the presence (739S) and absence (739) of spectinomycin (200 μg ml^{-1}). The figure illustrates that the mutant is stable for up to 12 hours during *in vitro* growth.

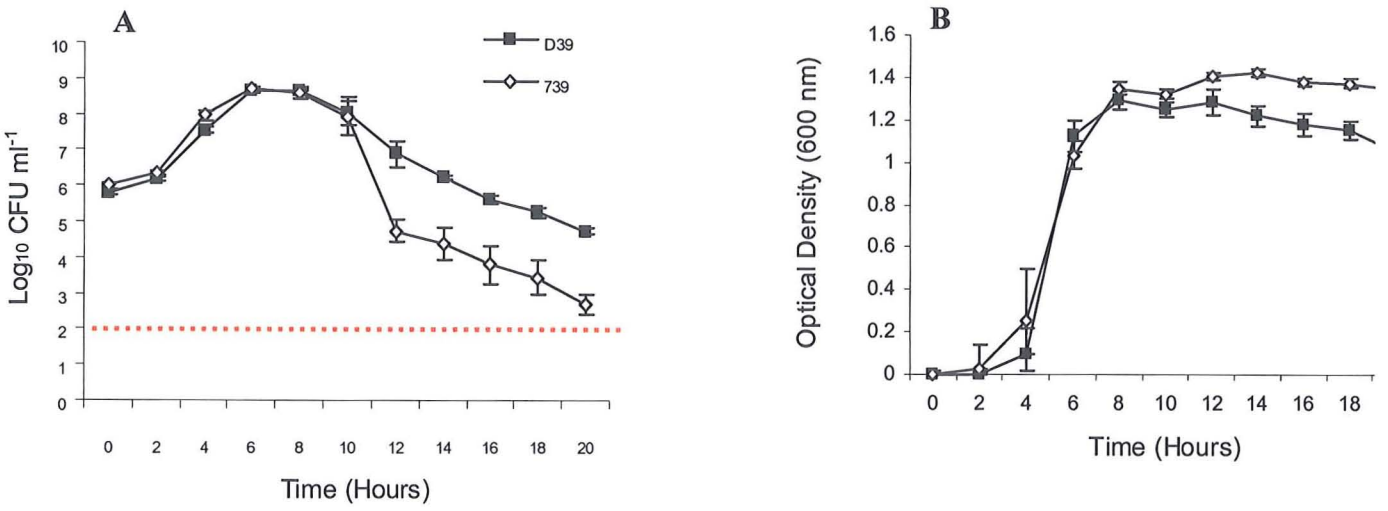


Figure 7.4 *In vitro* growth and autolysis of *Δ739*

15 ml BHI was inoculated with 1.0 x 10^6 bacterial strains. Viable cell enumeration (figure 7.4A) and $OD_{600\ nm}$ readings (figure 7.4B) were performed every 2 hour for a period of 20 hour. Significantly lower counts were found for the mutant compared to wild-type from 12 hr onwards. The broken line represents the limit of detection for viable cell counts.

No difference in growth was observed for the mutant compared to D39 parental strain up to 10 hours following culture inoculation, as measured by viable cell enumeration or optical density. However, between 10 and 12 hours, the mutant showed a significant increase in autolysis rate compared to wild-type ($P<0.05$). This resulted in lower viability counts for *Δ739* at all subsequent time points. Between 12 and 20 hours of growth, the strains appeared to lyse at similar rates, despite the lower viable counts for the mutant at each time point, as the autolysis curves became parallel (figure 7.4A). The optical density readings appeared to match viable cell counts up to 10 hours, but as described in previous sections, this was not representative of cell counts following entry into stationary phase (figure 7.4B). Furthermore, although viable counts for the mutant were lower that the wild-type during the autolysis phase, the $OD_{600\ nm}$ readings for the mutant were higher than D39 and remained constant for the remainder of the experiment (figure 7.4).

7.1.3 Expression of *zmpB* by D39 *in vitro*

RT-PCR was used to determine if *zmpB* is expressed by D39 during *in vitro* growth. RNA was isolated from wild-type D39 grown in BHI media to mid-exponential phase. Primers were designed against 2 different regions of *zmpB* to improve the chances of detecting mRNA. RT-PCR analysis was performed on 2 independent D39 RNA preparations. Expression of *zmp*B in D39 could be detected during *in vitro* growth using this technique (figure 7.5).

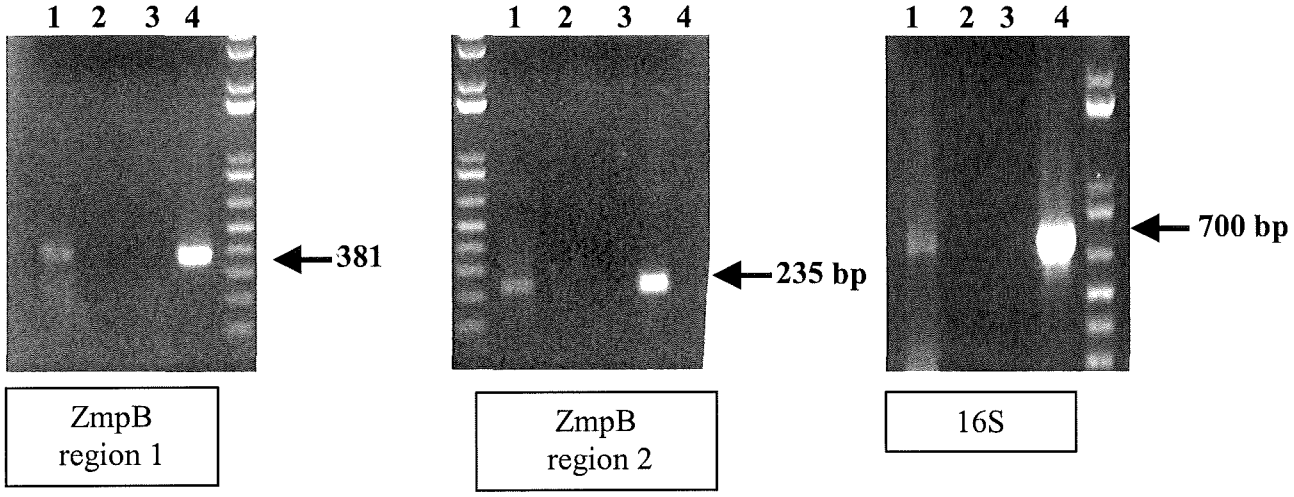


Figure 7.5: RT-PCR showing expression of *zmpB* during *in vitro* growth of D39

RNA isolated from cells grown to mid-log phase was used to determine the presence of *zmpB* transcript in wild-type D39 during *in vitro* growth. Primers ZmpIntF1 & ZmpIntR1 designed to amplify a region approximately 1 Kb downstream from the start of the gene (region 1; bp 1062-1442) produced an expected band size of 381 bp (left hand side gel). Primers ZmpIntF2 & ZmpIntR2 were designed towards the 3' end of the gene (region 2; bp 4411-4644) and produced an expected band size of 233 bp (central gel). Primers 16SF & 16SR specific for 16S RNA were used a positive control for RNA detection and resulted in a band of 700 bp (right hand side gel). For all gels, lane 1; cDNA template from D39, lane 2 D39 cDNA template prepared in the absence of reverse transcriptase, lane 3; No DNA template, lane 4; D39 genomic DNA template. Details of primers are provided in the appendix (A3). A band of the expected size was observed in lanes 1 and 4 only using both ZmpB primer sets and the 16S primers. No bands were observed for the negative controls (lanes 2 and 3).

7.2 *In vitro* analysis; Re-evaluation of published data

Two independent groups have characterised mutations in *zmpB* in different pneumococcal serotypes, and have found conflicting results (Novak *et al.*, 2000 and Bergé *et al.*, 2001). On the basis of this, several of the phenotypes were re-evaluated as part of this work.

7.2.1 Cell morphology

Microscopic examination by Gram's staining was used to determine if the *Δ739* mutant showed abnormal chain formation during *in vitro* growth. As shown in figure 7.6, the *Δ739* mutant grows in pairs or short chains, which is indistinguishable from the appearance of wild-type D39, and characteristic of all pneumococcal growth *in vitro*. Cultures at different stages of growth were examined to determine if chain formation was specific to a certain phase of growth, but no differences were detected (data not shown), suggesting that the mutant strain was able to undergo normal separation of daughter cells.

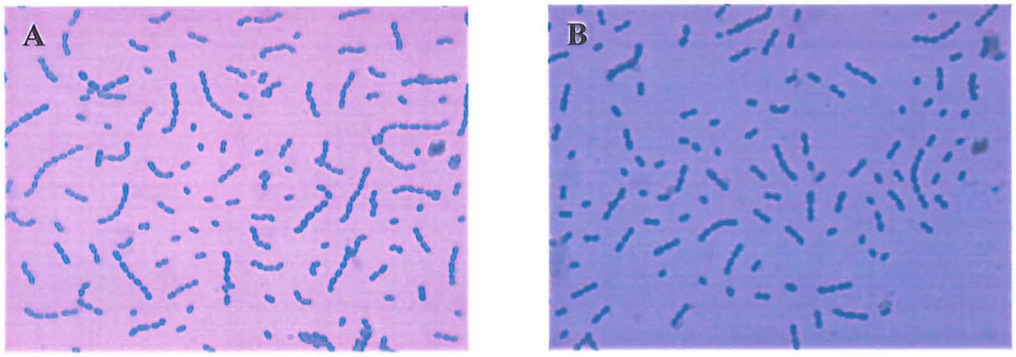


Figure 7.6 Gram's stain of *Δ739* and D39 wild-type

Bacterial strains were grown in BHI media and examined microscopically at various stages of growth. Grams staining revealed dark purple, Gram-positive cocci which occurred singly, in pairs and in short chains for both wild-type (figure 7.6A) and mutant (figure 7.6B) strains. The figure illustrates samples examined at mid-exponential phase of growth. No difference in cell morphology was observed at other stages of growth.

7.2.2 DOC-induced autolysis of *Δ739* mutant

The data shown above (figure 7.4A) illustrates that during *in vitro* growth the *Δ739* mutant is able to autolyse following entry into stationary phase, demonstrating that the mutant has a functional LytA enzyme, which is required for pneumococcal lysis (Tomasz *et al.*, 1988). To further confirm the presence of active LytA, the effect of DOC (an allosteric activator of LytA) on the viability of cultures was determined. Following addition of DOC to bacterial cultures grown to mid-exponential phase, a rapid reduction in viable cell counts was observed for both D39 and *Δ739*, with counts being below the detection limit 15 minutes post DOC treatment. This provides further evidence that the LytA enzyme responsible for autolysis is fully functional in the *Δ739* mutant.

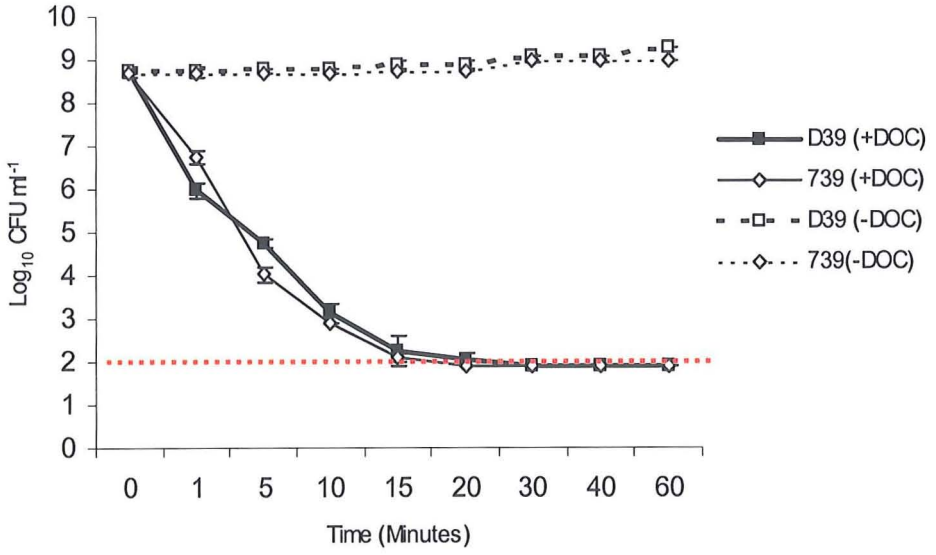


Figure 7.7 Autolysis following treatment with 0.04 % DOC

D39 and *Δ739* cultures were grown to mid-log phase in BHI. Each culture was divided into two equal volumes and DOC (0.04 %, final concentration) was added to an aliquot of each strain. Untreated aliqouts of cultures were used as controls. Viability counts on all cultures were performed at pre-determined intervals for 60 min post DOC treatment. The black broken / dashed lines represent untreated cultures and solid lines represent DOC-treated cultures. The broken line represents the limit of detection.

7.2.3 Transformation efficiency of *Δ739* mutant

To assess the ability of the *Δ739* mutant to take up DNA from the surrounding environment, bacterial strains were transformed with pVA838, a plasmid conferring erythromycin resistance with the ability to replicate in *S. pneumoniae* (Macrina *et al.*, 1982). The transformation efficiency of strains was determined by calculating the mean number of transformants (colonies exhibiting erythromycin resistance) per μg of sample DNA (pVA838). Although transformation efficiencies varied greatly between experiments, the number of transformants obtained for the *Δ739* mutant was consistently greater than for the D39 parent. This suggests that the mutant is not impaired in its ability to acquire DNA from the external environment and may even have increased transformation efficiency compared to the isogenic wild-type strain. Table 7.1 shows the mean transformation efficiencies of both strains from replicates of a single experiment. The comparisons between strains in the table are representative of all other experiments.

	Transformation Efficiency (Total transformants per ug DNA)	
	mean	SEM
D39	2207	327
739	2428	398

Table 7.1 Transformation efficiency of *Δ739* mutant

Bacterial cultures were grown to an $OD_{600\ nm}$ of 0.1 in BHI and induced to undergo transformation by the addition of CSP1. Plasmid pVA838 (conferring erythromycin resistance) was used to transform cells. Potential transformants were selected by their ability to grow on erythromycin-supplemented blood agar (1 μg ml^{-1}). The transformation efficiency was calculated based on mean number of transformants per μg test DNA (pVA838).

7.2.4 Haemolytic activity of *Δ739* mutant

Although haemolytic activity was not determined in any of the published work with the *zmpB* mutants, D39 and the *Δ739* mutant were characterised as part of this thesis for their ability to induce lysis of sheep red blood cells. Haemolysis was not observed with any of the negative controls, including the pneumolysin null mutant, *Δpln*, treated with deoxycholate (DOC), indicating that any lysis observed was largely due to pneumolysin and not other pneumococcal haemolysins. All deoxycholate-lysed cultures (with the exception of the *Δpln* mutant) showed complete haemolysis of sheep red blood cells up to a 1:8 dilution. The final dilutions tested ranged from neat (undilute) to 1:128. No haemolysis was seen when lysed cultures were diluted 1:32 and above (table 7.2). These data indicate that the *Δ739* mutant expresses similar amounts of active pneumolysin compared to D39 wild-type during *in vitro* growth.

	Dilution of culture resulting in haemolysis		
	Complete lysis	**Partial lysis**	**no lysis**
All negative controls	No haemolysis	No haemolysis	All dilutions
D39 (- DOC)	No haemolysis	No haemolysis	All dilutions
D39 (+ DOC)	neat-8-fold	8-16-fold	16-32-fold
Δ739 (- DOC)	No haemolysis	No haemolysis	All dilutions
Δ739 (+ DOC)	neat-8-fold	8-16-fold	16-32-fold

Table 7.2 Haemolytic activity of *Δ739* mutant

Bacterial strains grown to mid-exponential phase were analysed for their ability to induce lysis of sheep red blood cells without (- DOC) and with (+ DOC) treatment with 0.04 % deoxycholate. Negative controls included a pneumolysin null mutant, PBS alone, media alone and media with 0.04 % deoxycholate.

7.3 *In vivo* characterisation

Prior to all subsequent *in vivo* work, bacterial strains were passaged by intraperitoneal injection.

7.3.1 Intranasal challenge

7.3.1.1 Intranasal challenge; Survival and bacteraemia

Strains were analysed for their ability to induce disease in a murine infection model of pneumonia. Outbred MF1 mice were given 10^6 CFU intranasally and monitored for their ability to survive infection. All mice challenged with wild-type D39 had succumbed to infection within 100 hr following challenge, and had a median survival time of 45 hr. Although 90 % of mice challenged with the *Δ739* mutant succumbed to infection, the progression of infection was much slower than wild-type infection with a median murine survival time of 127 hr, nearly 3 times longer than survival with D39 challenge. Indeed at 60 hour post infection only 1 of 10 mice challenged with D39 had survived the challenge, but 9 of 10 mice infected with *Δ739* remained healthy and were not showing any signs of infection (figure 7.8A). The difference between survival of the two groups of mice was highly significant ($P<0.005$).

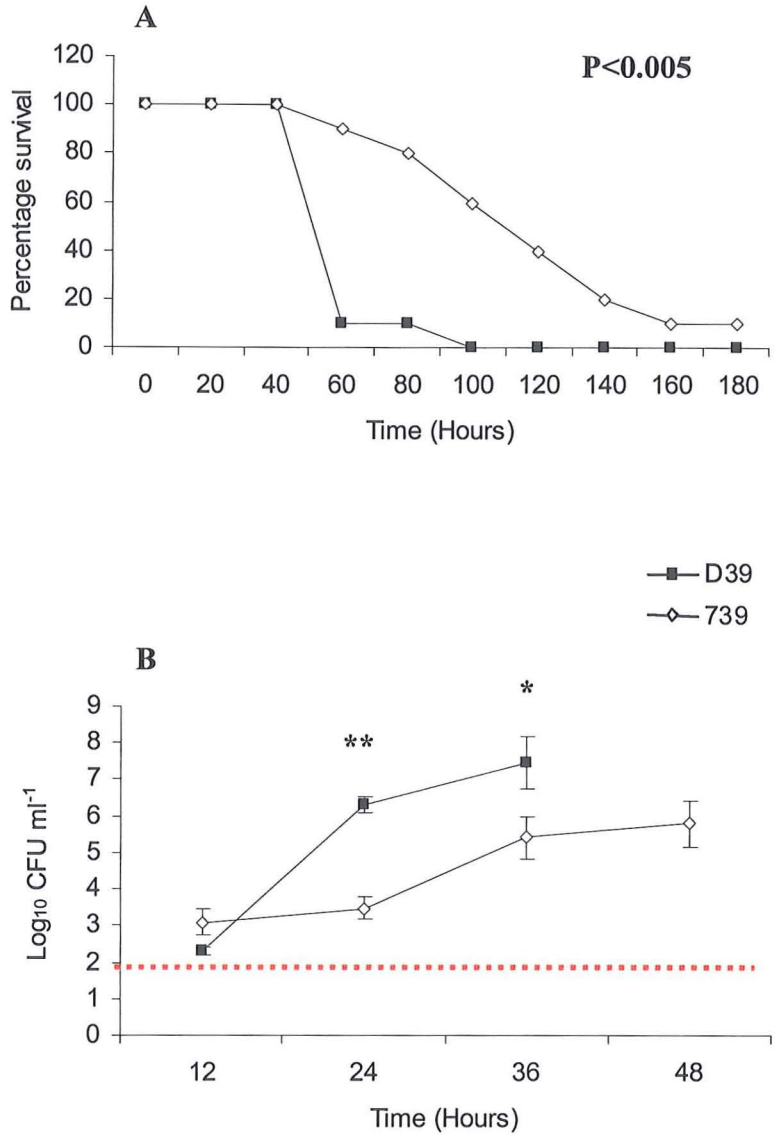


Figure 7.8 Survival and bacteraemia following intranasal challenge

Mice were challenged intranasally with 1.0 x 10^6 CFU bacterial strains. Figure 7.8A shows survival of mice. P<0.005 for survival of mice infected with *Δ739* compared to D39 parental strain. Figure 7.8B shows bacteraemia. Levels of bacteraemia for *Δ739* compared to D39 were statistically lower at 24 hour (P<0.005**) and 36 hour (P<0.05*) post infection. Counts for wild-type bacteria were not included at 48 hr as some of the mice had to be culled prior to this time point due to severity of infection. The broken line represents the limit of detection for determination of bacteraemia.

Bacterial counts for both D39 and the *Δ739* mutant appear in the bloodstream 12 hours post infection, with the mutant having slightly higher counts than wild-type (not significantly different). However, for all time points studied after 12 hours, mice challenged with wild-type bacteria had significantly higher counts in the blood compared to mice challenged with mutant bacteria ($P<0.005$ for 24 hour and $P<0.05$ for 36 hour, figure 7.8B). Indeed at 24 hr post challenge, the observed median bacterial count in the blood of mice infected with mutant bacteria is 0.5 % of that seen with wild-type infection. Bacteraemia in the blood of mice infected with D39 48 hours post challenge was not determined as over 40 % of mice had been culled by this time due to severity of infection. It thus appears that during the early stages of infection the mutant bacteria can gain entry into the bloodstream, but are then impaired in their ability to grow in the blood showing no significant increase in numbers between 12 and 24 hours. This is in contrast to D39 growth in the blood, which increases from $\log_{10}$ 2.9 CFU ml^{-1} at 12 hour to $\log_{10}$ 6.3 CFU ml^{-1} blood at 24 hours post infection (median values), an increase of over 3 orders of magnitude. This increase is statistically significant ($P<0.005$). However, between 24 and 36 hours, the mutant shows a similar rate of growth in the blood compared to wild-type during the same time period, displaying an increase in median counts of over 2 orders of magnitude, although this does plateau off slightly after 36 hours for infection with the *Δ739* mutant (figure 7.8B). Despite the slower growth of the mutant strain in the bloodstream, counts do eventually increase to a level which result in the appearance of severe symptoms and eventual mortality of mice as mice in a moribund state have counts of 10^7-10^9 CFU ml^{-1} blood which is similar to levels in moribund mice infected with D39 wild-type.

An unusual feature observed following intranasal infection with the *Δ739* mutant was the late onset of symptoms which subsequently appeared to persist in the milder stages (starey coat and slightly hunched stance) for longer periods of time compared to the progression of symptoms characteristic of wild-type D39 infection. A more quantitative analysis of this observation was performed following intravenous challenge, see section 7.3.2, table 7.3. Furthermore, mice challenged with mutant bacteria often showed mild to moderate signs of infection for up to 5 days, after which the mice often appeared to recover and be free of symptoms. Following this recovery a rapid relapse was frequently observed, where mice showed moderate to severe signs of infection within 6-12 hr of appearing to be completely healthy. Such mice had to be sacrificed soon after the development of the latter symptoms. These observations are unusual for pneumococcal infection of MF1 mice with D39, where, following infection and the onset of mild

symptoms, the mice progress through several stages of infection displaying moderate (intense piloerection and hunched stance) then severe (lethargy) symptoms of disease until they become severely lethargic and eventually moribund. The moribund state is usually reached within 24-48 hr of the mice showing initial signs of infection, and recovery from even mild symptoms is a rare occurrence.

7.3.1.2 Intranasal challenge; Stability of Δ739

At 36 hour post challenge, counts in the blood of mice were used to determine if the *Δ739* mutant retained its spectinomycin selection marker as a measure of the stability of the *mariner* mutation during *in vivo* infection. Figure 7.9 shows that the mutant was stable for up to 36 hour in the blood of infected mice. This is in agreement with *in vitro* stability, shown in figure 7.3. Furthermore, 10 single colonies that grew on spectinomycin plates following isolation from the blood of infected mice at 36 hour were used to re-confirm the mutation using the PCR reactions described above. The *mariner* cassette was detected within the *zmpB* gene of all 10 isolated colonies (data not shown). Following this observation, viability counts of the mutant strain were determined in the absence of antibiotic selection for all subsequent work.

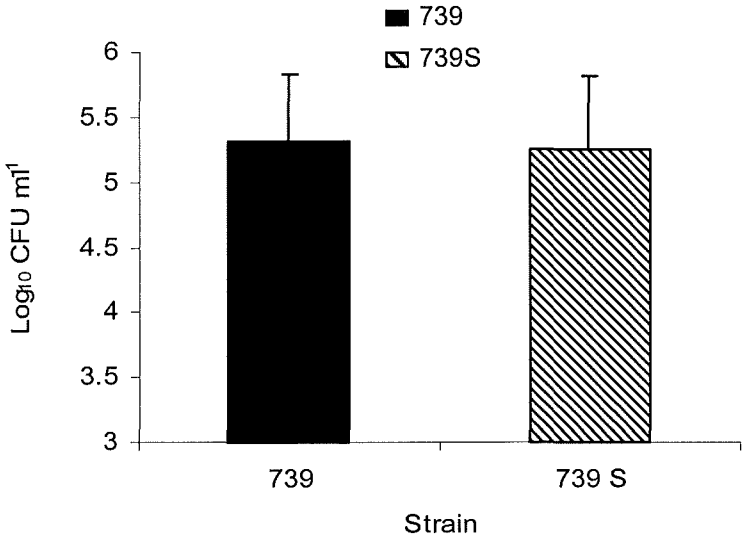


Figure 7.9 Stability of *Δ739* mutant *in vivo*

Blood taken from mice infected intranasally with 10^6 CFU *Δ739*, 36 hr post challenge was used to determine mutant stability *in vivo*. Viable cell counts were performed in the presence (739S) and absence (739) of spectinomycin (200 μg ml^{-1}). The figure illustrates that the mutants was stable and had not lost the *mariner* insertion.

7.3.1.3 Intranasal challenge; Lung counts

Bacterial loads within the lung tissue were determined at 12, 24 and 36 hr post challenge. No difference in lung counts between mice infected with D39 wild-type and *Δ739* mutant were observed at any of the time points, indicating that the mutant can grow in murine lung tissue as efficiently as wild-type (figure 7.10).

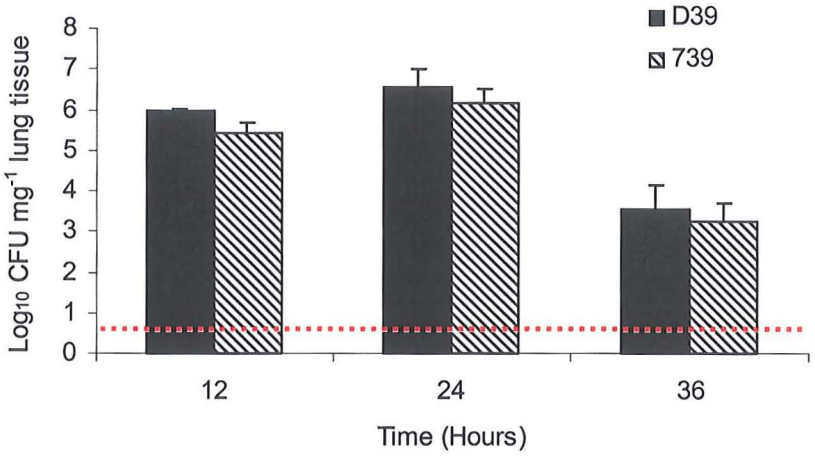


Figure 7.10 Bacterial loads in the lung tissue

Mice were challenged intranasally with *Δ739* mutant and D39 parental strain. Bacterial loads within the lung tissue were determined 12, 24 and 36 hr post infection. The broken line represents the limit of detection.

7.3.1.4 Intranasal challenge; Airway counts

To determine the number of bacteria in the murine airways, mice were challenged intranasally as described above, and lavage fluid removed at pre-determined time points. A small aliquot of this fluid was used for viable cell enumeration and the remainder was snap frozen for cytokine analysis (see later). Figure 7.11 illustrates that counts in the airways for mice challenged with wild-type and mutant strains remained equal at all time points with the exception of 36 hr, where significantly higher counts were found in the airways of mice infected with D39 wild-type ($P<0.05$).

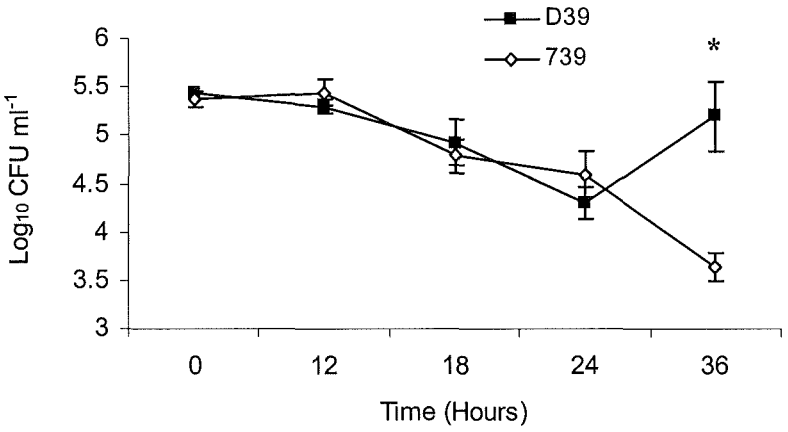


Figure 7.11 Bacterial loads in the BALF following challenge with *Δ739* mutant

Mice were challenged intranasally with 10^6 CFU *Δ739* mutant and D39 parental strain. Bacterial loads within the airways / BALF were determined at 0, 12, 18, 24 and 36 hr post infection. A significant difference between counts of mice infected with mutant compared to wild-type were observed at 36 hr post infection ($P<0.05$*). The limit of detection ($\log_{10}$ 1.92 CFU ml^{-1}) is below the scale used for this figure so is not represented.

7.3.2 Intravenous challenge

To by-pass the respiratory system, bacterial strains were inoculated directly into the veins of mice to determine their ability to grow in the blood. Counts at 0 hr confirmed the bacterial dose administered and subsequent levels of bacteraemia were measured at pre-determined time intervals. Mice were also monitored for survival. All mice challenged intravenously with D39 and *Δ739* succumbed to infection. As described for the intranasal challenges, mice challenged with the *Δ739* mutant survived much longer than those infected with the D39 parental strain ($P<0.05$) with median survival times of 97 hr and 46 hr respectively (figure 7.12A). This is >2-fold greater survival for mice challenged with mutant bacteria. Bacterial counts in the blood also showed a similar pattern to that described with intranasal challenge where counts of D39 increased by 1.5 orders of magnitude within the first 12 hour following infection, and continued to increase at a steady rate for all time points examined. However, counts within the blood of mice challenged with *Δ739* mutant only increased slightly between 0-30 hr post injection. A significant increase in bacteraemia compared to the 0 hr time point for the mutant strain was only found at 30 hr post challenge, after which counts decreased again by 36 hour post challenge (figure 7.12B). Bacterial counts in the blood of mice challenged with mutant bacteria were significantly lower at 24, 30 and 36 hours compared to counts of mice administered wild-type

bacteria (figure 7.12B). At 24 hr counts within the blood of mice infected with the mutant were 3 % of those in the blood of mice challenged with wild-type D39.

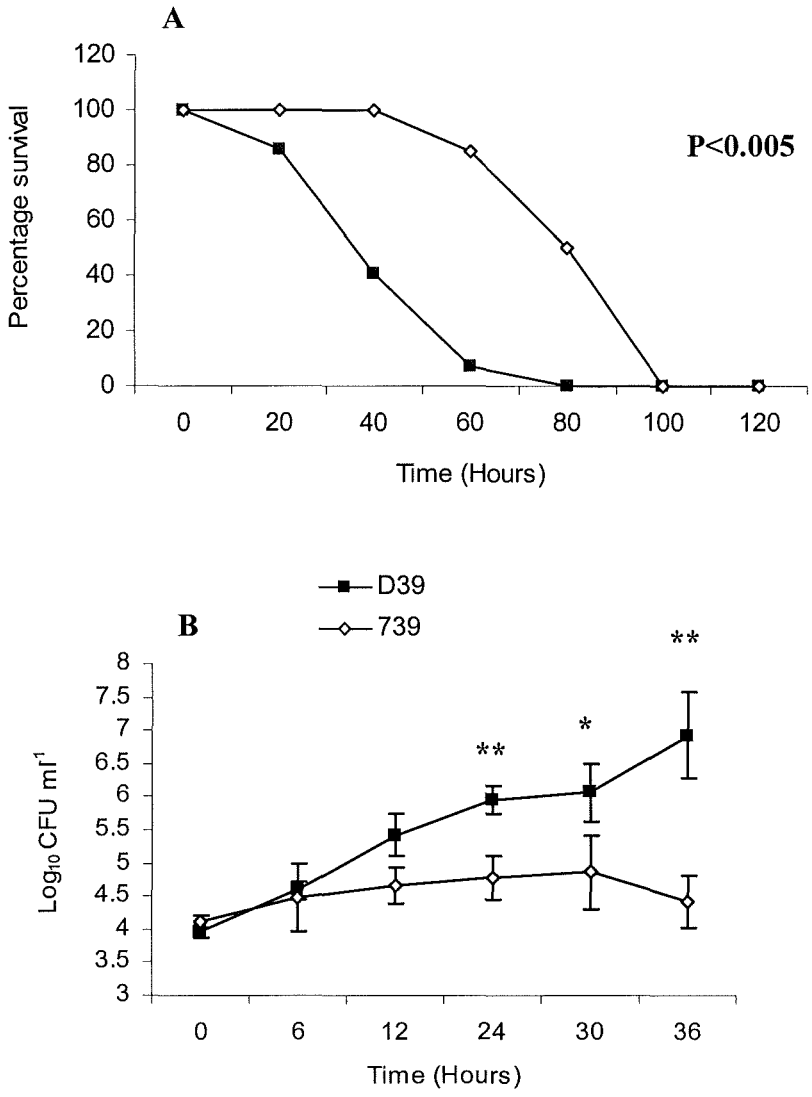


Figure 7.12 Survival and bacteraemia following intravenous challenge with *Δ739* mutant

Mice were challenged intravenously with 1.0 x 10^4 CFU bacterial strains. Figure 7.12A shows survival of mice. P<0.005 for survival of mice infected with *Δ739* compared to D39 parental strain. Figure 7.8B shows bacteraemia following challenge. Levels of bacteraemia for *Δ739* compared to D39 were statistically significant at 24 hr (P<0.005**), 30 hr (P<0.05*) and 36 hr (P<0.005**) post infection. The limit of detection (log_{10} 1.92 CFU ml^{-1}) is below the scale used for this figure so is not represented.

Despite showing low counts within the blood at the time points studied the levels of *Δ739* in the blood appear to have increased after the 36 hr time point, as some of the mice started to show symptoms between 60 and 80 hr post infection. Furthermore, all mice challenged with the mutant had been sacrificed by 100 hr post challenge, and when moribund these mice had high counts, representative of wild-type infection, within their blood (10^7-10^9 CFU ml^{-1} blood). Again, this suggests that upon entry into the blood the *Δ739* is poorly adapted for growth and survival in this environment, although given time it is able to cause lethal infection.

During intranasal challenge, it was noted that the mice challenged with the mutant strains showed signs of infection for longer periods of time before finally succumbing to the terminal stages of the infection and being sacrificed. This observation was recorded for the intravenous challenges and table 7.3 below shows mean and median times from the first appearance of symptoms of infection (starey coat and slightly hunched stance) to the terminal stages of infection (extreme lethargy and moribund state) in mice infected intravenously. The table clearly illustrates that, even when survival is measured from the onset of visible symptoms, the mice challenged with mutant bacteria can survive the infection > 2-fold longer than those challenged with D39 wild-type.

	n	mean	median	SEM
D39	21	19.6	17.5	1.88
Δ739	16	47.0**	48.5**	4.9

Table 7.3 Time between appearance of symptoms of infection and mortality in mice following intravenous challenge

Mice were challenged intravenously with 10^4 CFU *Δ739* and D39 strains and monitored carefully for the appearance of mild symptoms (starey coat and slightly hunched stance). The time interval between the onset of these symptoms and terminal stages of infection (mice extremely lethargic or moribund) where mice were actively culled was recorded. The column labelled 'n' provides the group sizes for the experiment. The table shows both mean and median values, to indicate that even with relatively large group sizes, these two parameters of measurement do not differ greatly. The error of the mean value is also provided (SEM). The time from showing symptoms to succumbing to infection was greater in the mice challenged with mutant bacteria compared to wild-type infection, and this increase was highly significant ($P<0.005$**).

7.3.3 Role of ZmpB in virulence in complement deficient mice

The data presented above indicated that ZmpB has a role in the virulence of strain D39 in our model of infection. Thus it was the objective of the work discussed below to characterise this protein further to gain insight into the possible mechanism of action of ZmpB within the pneumococcal cell. As complement is an important host defence mechanism in pneumococcal infection, and a region of ZmpB shows homology with another pneumococcal protein able to interact with complement, the *Δ739* mutation was characterised in complement-deficient mice.

7.3.3.1 Survival and bacteraemia following intranasal challenge

A BLAST search of the protein sequence from the TIGR4 strain revealed some homology to *S. pneumoniae* surface protein PspC / complement factor H binding inhibitor (see Appendix 6c). Based on this homology, the behaviour of the *Δ739* mutant in complement deficient / C3 knock-out mice (C3-/- , C57/Bl background, Wessels *et al.*, 1995) was characterised to determine if ZmpB was able to exert an effect on complement or components of the complement cascade. Should interference with normal complement function be a major function of active ZmpB, then the virulence of wild-type and mutant bacterial strains in C3-/- mice should not be significantly different. C3-/- mice were challenged intranasally with D39 and *Δ739* strains and monitored for survival and bacteraemia. Figure 7.13 illustrates that, as described above for challenges with MF1 mice, infection of C3-/- with the *Δ739*mutant resulted in greater murine survival times ($P<0.005$, figure 7.13A) and lower levels of bacteraemia at 24 and 36 hr post infection ($P<0.05$, figure 7.13B) compared to infection with wild-type bacteria. Median survival time for C3-/- mice infected with the *Δ739* mutant was 73 hr. Infection of complement-sufficient, wild-type control mice (C3+/+), challenge with the *Δ739* mutant did not result in bacteraemia at any of the time points studied, and all mice survived the challenge.

Following challenge with D39 parental strain, C3-/- mice quickly succumbed to infection, with mice having a median survival time of 36 hr, and only 10 % surviving the challenge by 40 hr post infection (figure 7.13A). The counts of D39 in the blood increased rapidly, as expected, and reached very high median counts of $\log_{10}$ 9.54 CFU ml^{-1} at 36 hr post challenge (figure 7.13B). In C3+/+, infection with D39 bacteria only results in 20 % of the mice succumbing to infection, with median counts in the blood barely increasing above the limit of detection (figure 7.13A&B). This illustrates the importance of complement in preventing pneumococcal infection in this murine model of infection.

The data above indicate that the *Δ739* mutant is also impaired in virulence in the C3-/- mice compared to D39 wild-type. This suggests that interference with the host's complement system does not appear to be a major function of active ZmpB in strain D39 when assessed in C3-/- mice on a C57/Bl background.

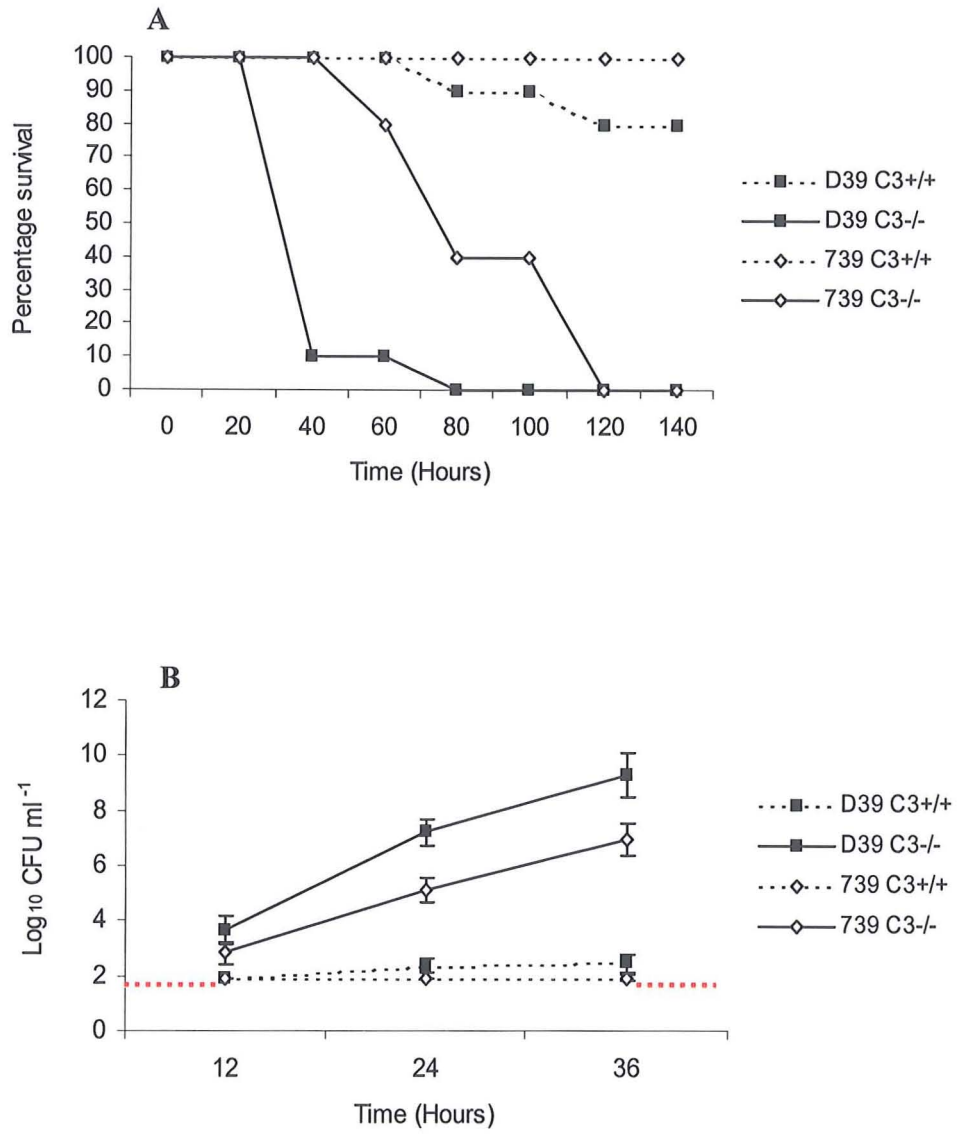


Figure 7.13 Virulence of *Δ739* in C3-/- mice

Complement-sufficient (C3+/+, black dotted lines) and complement knock-out (C3 -/-, black unbroken lines) mice were challenged intranasally with 10^6 CFU D39 and *Δ739* mutant bacterial strains. Mice were monitored for survival (figure 7.13A) and bacteraemia at pre-determined time points (figure 7.13B). No difference was observed between bacterial strains in the survival or bacterial counts in the blood of C3+/+ mice. In the C3-/- mice, those challenged with mutant bacteria survived longer than mice challenged with D39 parental strain, and this difference was highly significant ($P<0.005$). Similarily, for bacterial counts in the blood of mice, no difference was seen between bacterial strains in wild-type C3+/+ mice, but in C3-/- mice lower bacterial counts were observed at all time points examined following challenge with mutant bacteria compared to D39 parental strain. This difference was statistically significant for 24 and 36 hr post infection ($P<0.05$, significance not shown on the figure). The broken red line represents the limit of detection for bacterial counts in the blood and has been interrupted to allow visualisation of the low bacterial counts in wild-type C3+/+ mice.

7.3.3.2 Comparison of survival between murine challenges

With all murine challenges described so far in this chapter, mice infected with mutant *Δ739* bacteria all survived longer than mice infected with the isogenic parental strain, D39. This difference was found to be highly significant in all experiments ($P<0.005$). Direct comparison of survival time between experiments is not possible as mice were challenged with different doses of bacteria, so survival of all mice at 60 hr was compared. This time point was chosen to represent the mean halfway point for the individual experiments. (Mice were monitored for a maximum of 2 weeks [336 hr], but individual experiment end points were based on the point at which all mice that succumbed to infection had to be culled). Analysis of this time point showed that despite the route of infection or the strain of mice used, at least 90 % of mice challenged with wild-type bacteria had been culled 60 hr post infection, while only 10 % of mice infected with the *Δ739* mutant had succumbed to infection (figure 7.14).

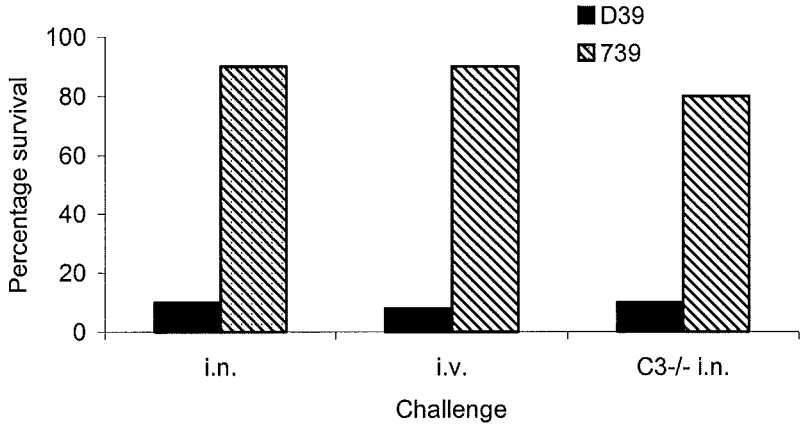


Figure 7.14 Comparison of murine survival at 60 hr following three individual challenges

The survival of infected mice at the mean halfway point (60 hr post challenge) of three individual experiments was compared. Experiments included intranasal (i.n.) and intravenous (i.v.) challenges of MF1 mice and intranasal challenge of C3-/- mice (C3-/- i.n.). Mice were infected with 10^6, 10^4 and 10^6 CFU, respectively. The figure illustrates the difference in survival times between mice infected with the *Δ739* mutant and its isogenic parental strain, D39, which is consistent across different challenges.

7.4 Role of ZmpB in inflammation

The work with the C3-/- mice presented above indicates that ZmpB does not have a large influence on complement function. Another host defence mechanism important in both clearing pneumococcal infection, and also being implied in the pathology of infection is the host inflammatory response. It was thus decided to characterise the inflammatory response within the respiratory system to determine if ZmpB could act within this environment to either promote or inhibit inflammation. The cytokine profiles of several key mediators and regulators previously shown to be involved in pneumococcal infection was studied.

The data presented in figures 7.10 and 7.11 showed that there was no difference in the ability of the *Δ739* mutant to colonise the airways or lung tissue of mice at the time points studied, with the exception of 36 hr BALF counts, where higher counts were found for strain D39 (figure 7.11). Thus any differences observed in cytokine profiles with these samples can be attributed to the *zmpB* mutation and not differences in bacterial loads.

7.4.1 TNF production

TNF-α, being an important mediator of inflammation, was the first cytokine to be characterised in this work. A bioassay that measures the activity of both TNF-α and TNF-β was used for BALF and lung tissue sampled over a range of pre-determine time points following intranasal infection. The assay indicated that increased levels of TNF were present in lung tissue isolated from mice infected with D39 parental strain compared to infection with the *Δ739* mutant at 12 and 24 hr post infection. This difference was statistically significant for the 24 hr time point only ($P<0.05$) Figure 7.15A shows the mean values obtained for TNF in the lung tissue 24 hr post infection using the bioassay to measure TNF activity. However, this experiment was an exception from all other data discussed in this thesis because the mean and median values for the data do not correlate well. The mean and median values for lung tissue isolated from mice 24 hr post infection with wild-type D39 are 487 U and 200 U units respectively. Similarly for lungs isolated from mice infected with the *Δ739* mutant, mean and median counts were 41 U and 0.6 U respectively. Thus the median values for both lung samples from both bacterial strains are actually significantly lower that those shown in the figure. Nevertheless, statistical analysis based on both mean (student t-tests) and median (Mann-Whitney tests) values for the data sets obtained indicated that the differences observed between D39 and the *Δ739* mutant are statistically

significant ($P<0.05$). No TNF within the BALF at any time point studied was detected using the bioassay.

To confirm the results from the bioassay, an ELISA specific for TNF-α was used. This assay confirmed the increased concentrations of TNF-α within lung tissue at 24 hr post infection with D39 wild-type compared to the *Δ739* mutant ($P<0.05$). In contrast to the bioassay, mean and median values for the ELISA data were in agreement with each other (304 & 295 pg ml^{-1} for D39 infection, and 94 & 95 pg ml^{-1} for the mutant, respectively). Thus for all subsequent analysis and discussion, ELISA values will be used as a measure of TNF-α activity in lung tissue. This method is also more suited to TNF-α measurement as the bioassay measures combined activities of TNF- α and TNF-β. As for the TNF bioassay, the ELISA did not detect TNF- α in the BALF at any of the time points studied (12, 18, 24 or 36hr). Figure 7.15 shows the increased TNF- α concentration in lung tissue following challenge with D39, as determined by both bioassay (figure 7.15A) and ELISA (figure 7.15B).

Controls were set up for all bioassays and cytokine ELISAs using lung and BALF samples taken from uninfected mice and from mice immediately following challenge with both D39 and *Δ739.* Subsequent analysis showed that there was no significant differences between the cytokine concentrations in uninfected mice and infected mice immediately following challenge (data not shown), so all subsequent cytokine data sets will show the uninfected value as the control. This control therefore represents both a 0 hr time point and un-infected mice.

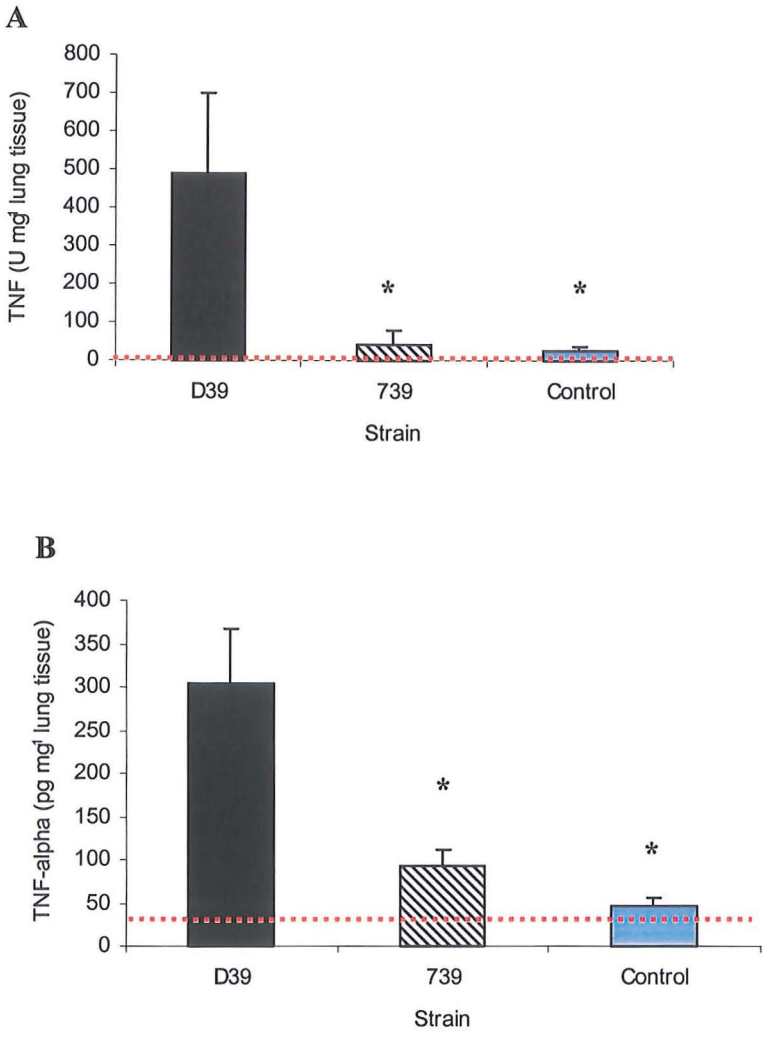


Figure 7.15 TNF in lung tissue, 24 hr post challenge

Total TNF activity in lung tissue 24 hr post intranasal infection with D39 and the *Δ739* mutant was determined by a bioassay. This assay measures the specific killing of L929 fibroblasts due to TNF. The bioassay measures both TNF-α and TNF-β activity (figure 7.15A). Total TNF-α concentration was determined by ELISA, which is specific for TNF-α (figure 7.15B). Both assays showed that mice challenged with D39 had significantly higher levels of TNF(α) compared to mice infected with the *Δ739* mutant ($P<0.05$*). The difference between the D39-infected samples and the 0 hr control was also significant ($P<0.05$*). The levels of TNF(α) in the lung tissue from mice challenged with *Δ739* were only just above the limit of detection for both assays and were not significantly different from the 0 hr control values. The broken line represents the limit of detection for both assays.

7.4.2 IFN-γ production in BALF and lung tissue

The concentration of the pro-inflammatory cytokine, IFN-γ, in murine BALF and lung samples was determined by ELISA. In the lung tissue, IFN-γ concentrations increased with each time point studied for both bacterial strains (figure 7.16B). No significant difference between IFN-γ concentrations in lung tissue from mice challenged with wild-type and mutant bacteria was observed at any of the time points examined.

In the BALF, IFN-γ concentrations increased at each time point with airway washes from mice challenged with D39. Following intranasal challenge with *Δ739,* IFN-γ concentrations also increased but with a peak at 24 hr post challenge. Concentrations at 36 hr post infection with the mutant were similar to those seen at 18 hr (figure 7.16A). The only significant difference between IFN-γ concentration in the BALF of mice challenged with wild-type and mutant bacteria was at 24 hr post challenge where challenge with the *Δ739* mutant resulted in higher IFN-γ ($P<0.05$). The IFN-γ concentrations detected in the BALF were much lower than those present in the lung tissue at all time points for both bacterial strains.

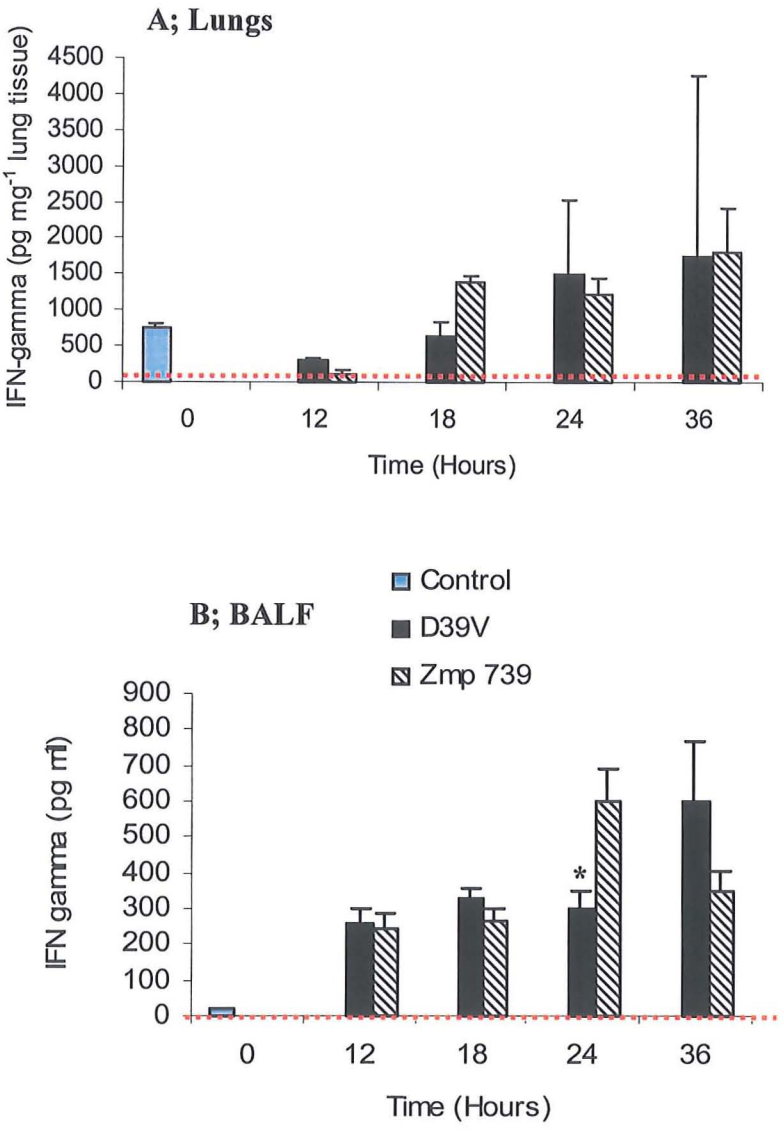


Figure 7.16 IFN-γ concentrations in lung tissue and BALF following intranasal challenge

ELISA was used to determine the concentration of IFN-γ in murine lung tissue (7.16A) and BALF (7.16B) following intranasal challenge with 10^6 CFU D39 and the *Δ739* mutant. The only significant difference observed between mutant and wild-type IFN-γ levels was within the BALF, 24 hr post challenge, where mice infected with the *Δ739* mutant had increased IFN-γ ($P<0.05$*). The broken line represents the limit of detection for the assays

7.4.3 IL-6 production in BALF and lung tissue

The concentration of IL-6 in lung tissue and BALF was determined by ELISA. The IL-6 concentration increased in both the lung tissue and BALF as the infection progressed following challenge with both D39 and the *Δ739* mutant (figure 7.17). No significant difference was observed in IL-6 levels between D39 and *Δ739*-infected tissues at any time point.

7.4.4 IL-10 production in BALF and lung tissue

The concentration of IL-10 in lung tissue and BALF was determined by ELISA. The IL-10 concentrations increased in both the lung tissue and BALF as the infection progressed following challenge with both D39 and the *Δ739* mutant, with the exception of BALF from mice infected with D39 wild-type which showed a peak in IL-10 concentration at 18 hr post challenge (figure 7.17). However, concentrations of IL-10 detected in the BALF at all time points were lower than those of the control. This could indicate that infection with both strains results in suppression of IL-10 at the time points studied. No significant difference was observed in IL-10 levels between D39 and *Δ739*-infected tissues at any time point.

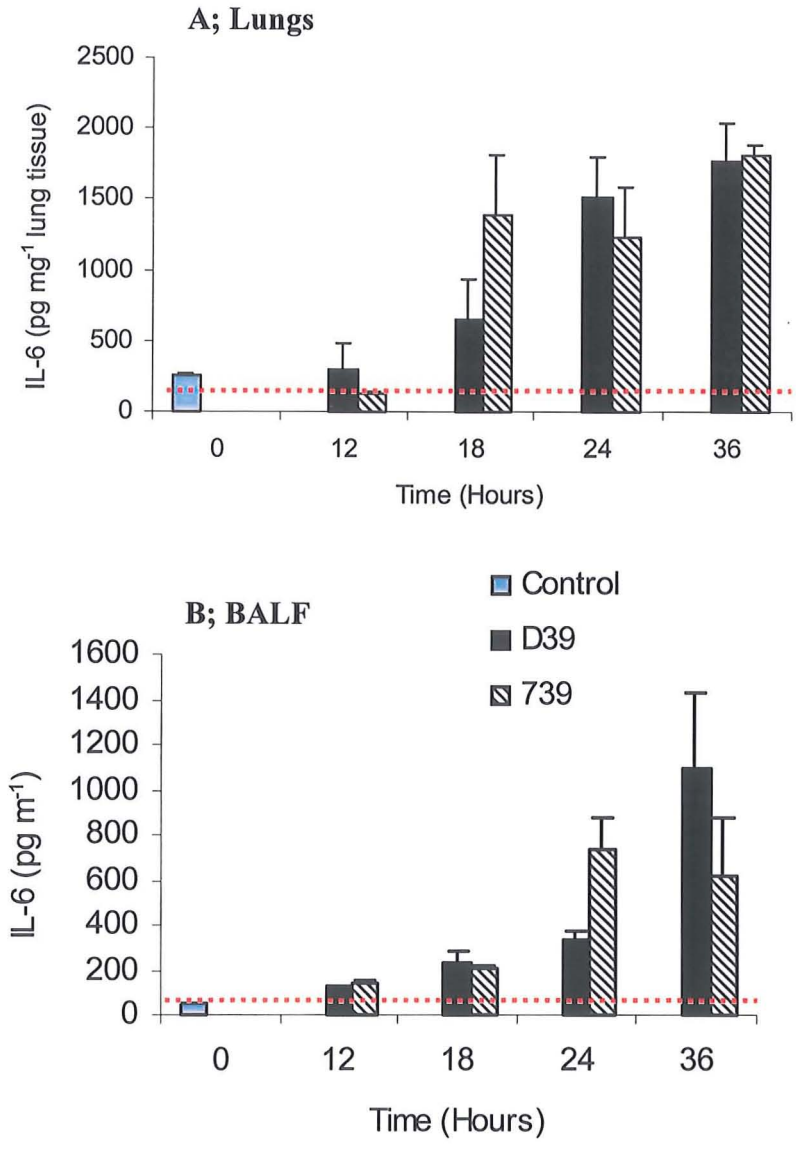


Figure 7.17 IL-6 concentrations in lung tissue and BALF following intranasal challenge

ELISA was used to determine the concentration of IL-6 in murine lung tissue (7.17A) and BALF (7.17B) following intranasal challenge with 10^6 CFU D39 and the *Δ739* mutant. No significant difference between IL-6 concentration in lung or BALF from mice challenged with D39 or the *Δ739* mutant was observed at any time point examined. The broken line represents the limit of detection for the assays.

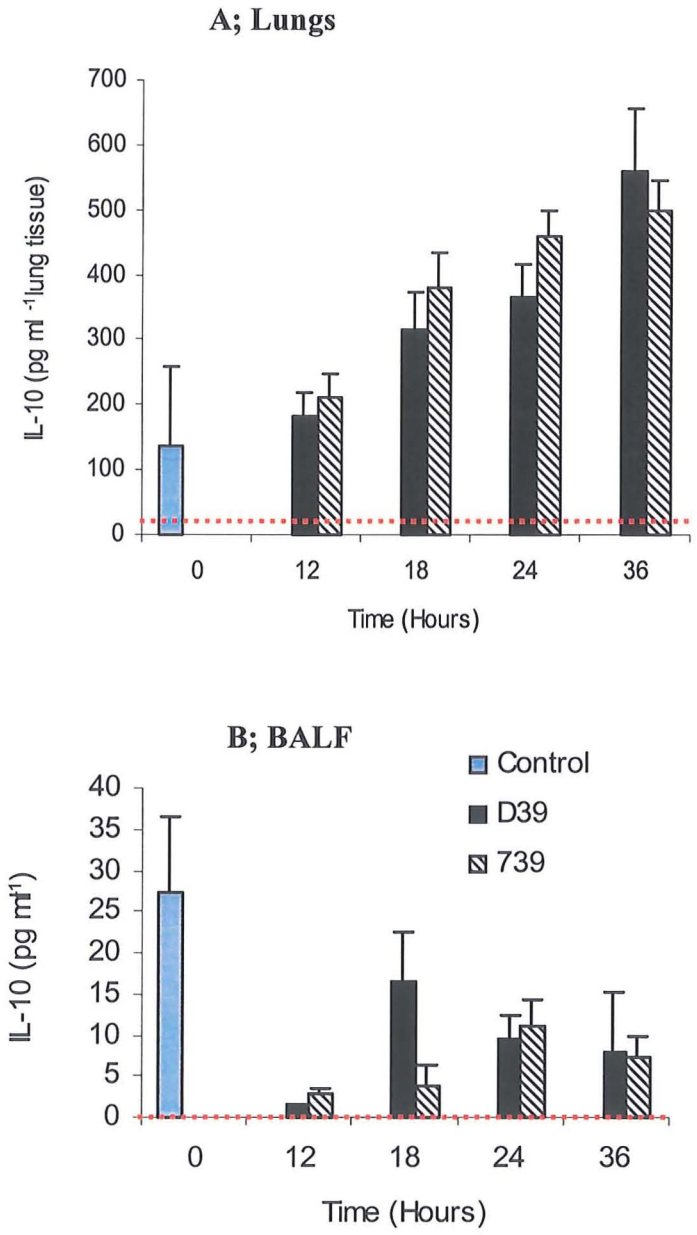


Figure 7.18 IL-10 concentrations in lung tissue and BALF following intranasal challenge

ELISA was used to determine the concentration of IL-10 in murine lung tissue (7.18A) and BALF (7.18B) following intranasal challenge with 10^6 CFU D39 and the *Δ739* mutant. No significant difference between IL-10 concentration in lung or BALF from mice challenged with D39 or the *Δ739* mutant was observed at any time point examined. The broken line represents the limit of detection for the assays.

7.5 Expression of ZmpB / antibody production

The availability of pure ZmpB protein would be useful for further characterisation of this virulence factor, especially should high affinity antibodies against the protein be available. The aim of the following section was to express a region of ZmpB in a pure form and use this protein to obtain anti-ZmpB antibodies which could be used in a range of experiments summarised in figure 7.19.

7.5.1 Expression of ZmpB protein

As ZmpB is such a large gene (>5.5 Kb), it was envisaged that attempts to express the whole protein would be difficult, if not impossible. To overcome this, the gene was expressed in 2 fragments, which would exclude the hydrophobic region at the N-terminal end of the protein which could have caused problems during expression. Primers containing BamHI and SacI restriction sites were designed to amplify up the central and C-terminal regions of the gene, as illustrated in figure 7.19. The reverse primer for amplification of the central region was designed to contain a stop codon to prevent read-through into the downstream sequence once cloned. PCR was used to amplify up these regions from *zmpB* in strain D39 (serotype 2). PCR products were cloned into the expression vector pET-33b, transformed into DH5α competent cells and checked by restriction digest. Positive clones were selected on kanamycin. The insertions in pET-33b were sequenced to ensure that the *zmpB* regions were in-frame with the T7 promoter for expression. PET-33b containing the confirmed *zmpB* insert was subsequently used to transform BL21 cells for protein expression. Cultures of transformed BL21s were grown to mid-exponential phase and expression of the T7 polymerase was induced by the addition of IPTG. Protein expression was determined by SDS-PAGE with Coomassie staining. Only the clones containing the central region of ZmpB resulted in satisfactory levels of protein expression, as judged by SDS-PAGE, so this region was used for further work to obtain protein for vaccination. Following induction of protein expression using the central region of ZmpB, most of the protein was within inclusion bodies in the insoluble fraction (figure 7.20). Preparation of protein from the inclusion bodies involved disrupting the cells mechanically followed by a series of washes in sucrose and Triton-X-100, and re-solubilisation in urea. The protein solution was dialysed to remove all traces of urea, but following this procedure, a precipitate was formed, with very little protein remaining in solution. A decision was made to vaccinate with this precipitate instead of soluble protein as had originally been intended. The ZmpB-central region precipitate was re-

solubilised in urea to determine the protein concentration and the dialysis procedure was repeated. The resulting precipitate was suspended in sterile saline for vaccination studies. Figure 7.19 summarises the process described above to obtain semi-purified ZmpB protein for vaccination. Figure 7.20 illustrates the individual stages of protein purification from the inclusion bodies.

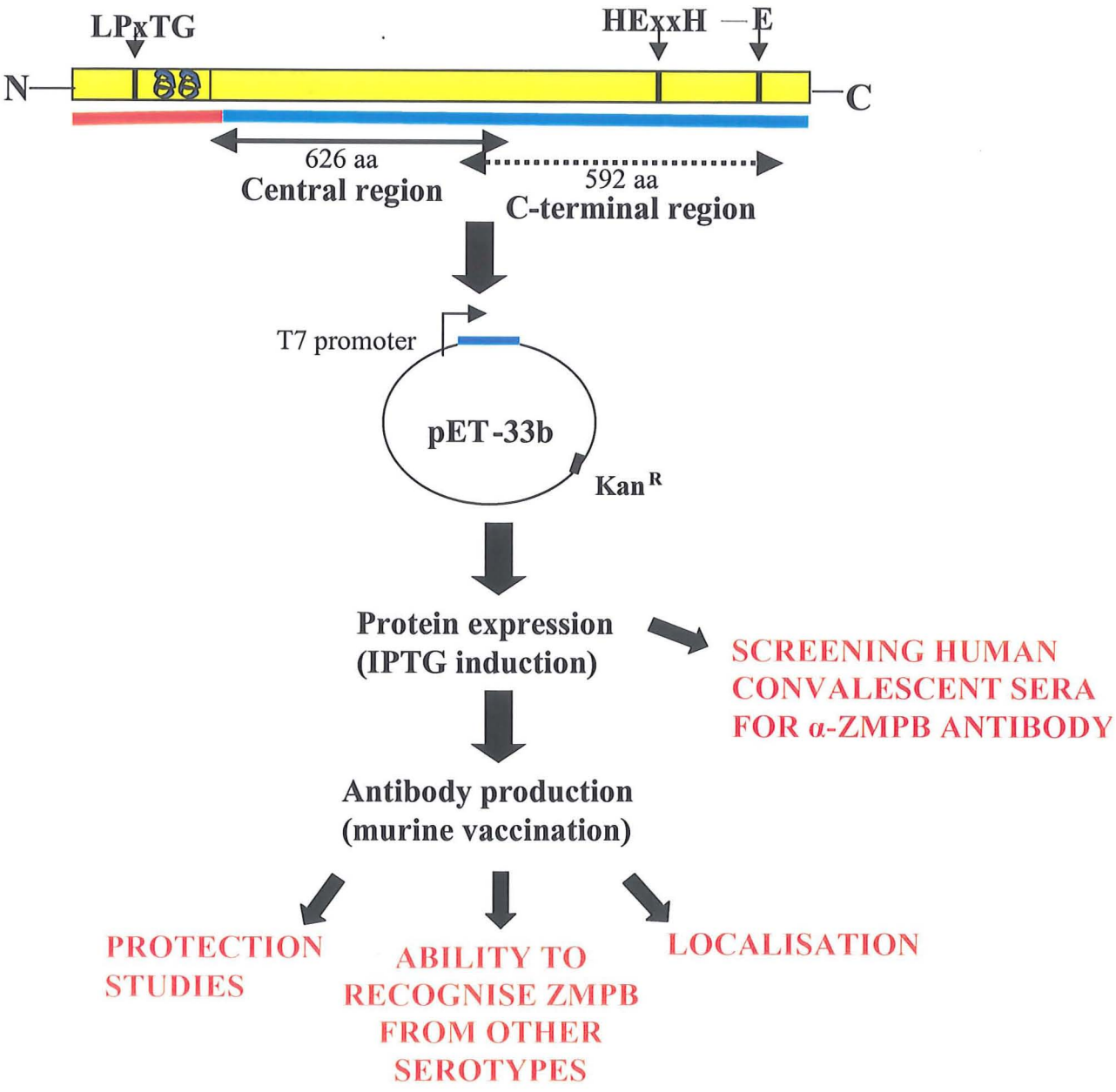


Figure 7.19 Overview of the production of ZmpB protein for vaccination

Two regions of *zmpB* were cloned into expression vector pET-33b and used to transform BL21 cells. Expression of the protein was induced by the addition of IPTG to growing cultures. SDS-PAGE indicated that the clones expressing the central region of *zmpB* produced the best levels of protein expression. The protein was purified from the insoluble inclusion bodies and used to vaccinate MF1 mice together with Freund's adjuvant. Upon completion of the vaccination schedule, Western blot was used to confirm the presence of anti-ZmpB antibody, and sera from positive mice were pooled. The red labels on the diagram represent possible future work using the purified protein or the murine immune sera.

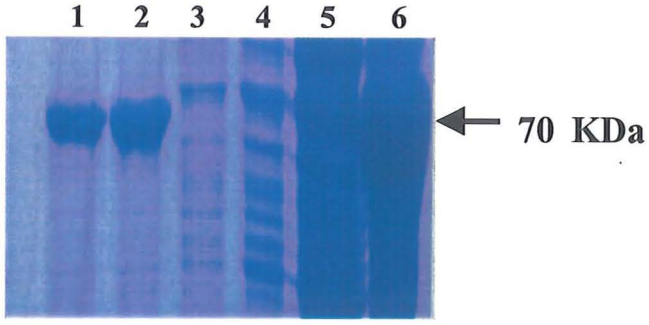


Figure 7.20 Purification of ZmpB-central region from inclusion bodies

The gel shows the 70 KDa ZmpB-central region protein at various stages during purification. Lane 6 represents the total cell pellet following induction of expression by IPTG, with lane 5 showing the soluble fraction. Although it is not clear from the figure, most of the expressed protein was in insoluble inclusion bodies. These were disrupted mechanically and washed in 30 %sucrose and Triton-X-100 (lanes 4 and 3 respectively represent the supernatants from these washes), then solubilised twice in 7M urea (lanes 1 and 2). The figure shows that very little protein was present in the supernatant following the washed with sucrose and Triton-x-100. Resolubilisation in urea resulted in a substantial band representing crude ZmpB-central region protein.

Several mice were vaccinated following the schedule outlined in chapter 2, section 2.92. Prior to each vaccination, pre-bleeds were taken, and Western blot was used to determine the presence of detectable anti-zmpB antibody at each stage of the vaccination. Antibody could be detected after the first boost in some mice, although detection improved with subsequent boosts and antibody was detected in the terminal bleed of all mice vaccinated (data not shown). The amount of antibody produced by individual mice appeared to vary substantially (data not shown). The immune sera from all mice was pooled. Figure 7.21 illustrates the ability of this pooled sera to recognise the purified ZmpB central region using a Western blot technique. This immune sera will be used for a variety of applications, discussed in the following chapter.

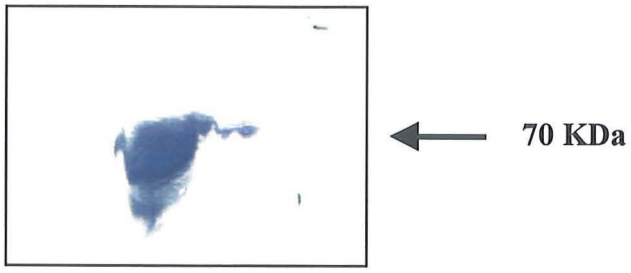


Figure 7.21 Westerm blot showing the ability of mouse immune sera to recognise ZmpB
Mice were vaccinated with partially purified ZmpB protein (central region) following a vaccination schedule described previously. Sera from individual mice was tested for anti-ZmpB antibodies and all positive samples were pooled. The figure shows the recognition of ZmpB (central region) by this pooled sera, with the band being the expected size of 70 KDa. A total of 44 µg of ZmpB protein was used for detection. A marker was used to determine protein size but is not clearly visible from this figure.

7.5 Regulation of *zmpB* by TCS09

Microarray analysis was used to characterise the expression profile of the *Δrr09* mutant compared to isogenic parental strain (see chapter 5, section 5.4.2). Although the ratio of *zmpB* expression in D39 wild-type compared to the *Δrr09* mutant was close to 1.0 (figure 5.20), indicating that the gene is expressed the same in both strains, the actual intensity of expression was very close to the threshold for detection of expression (figure 5.21). This low level of expression is likely to be due to problems in the design of the probe for the *zmpB* gene. As discussed in the introduction (section 1.6.3), the nucleotide sequence for *zmpB* between different serotypes only shows significant homology within the first 1000 bp, at the 5' end of the gene. The only probe on the *S. pneumoniae* microarray designed against *zmpB* includes only a very small section of the homologous region and is thus unlikely to hybridise well to sequences that are significantly different to the TIGR4 *zmpB* sequence. Figure 7.22 illustrates the sequence variation between the TIGR4 and R6 *zmpB* genes, together with the position of the *zmpB* array probe, as analysed using the Artemis Comparison Tool (ACT) programme (The Wellcome Trust Sanger Institute, http://www.sanger.ac.uk/Software/ACT/). Figure 7.23 illustrates the BLAST alignment of the TIGR4 probe with the corresponding region on the R6 genome sequence. The latter figure shows that the probe on the array has very little homology with the R6 sequence,

although low levels of hybridisation may still be possible. The regions of D39 that were sequenced as part of ZmpB expression were homologous with the R6 sequence, indicating that significant variation may exist between the *zmpB* gene of D39 and the TIGR4 strain. Thus it can be assumed that *zmpB* expression in D39 may not be detected by the *S. pneumoniae* array. This further highlights some of the problems, discussed in chapter 6, that may be encountered using microarrays to study gene expression. To overcome this problem, the *Δrr09* mutation is currently being moved into the TIGR4 parental strain in our laboratory to enable further characterisation of this mutant and the genes it regulates.

However, RT-PCR analysis with primers designed within the *zmpB* gene, indicated that *zmpB* was expressed during *in vitro* growth in the *Δrr09* mutant. This indicates that TCS09 does not prevent expression of *zmpB*, but does not confirm that TCS09 does not influence *zmpB* regulation.

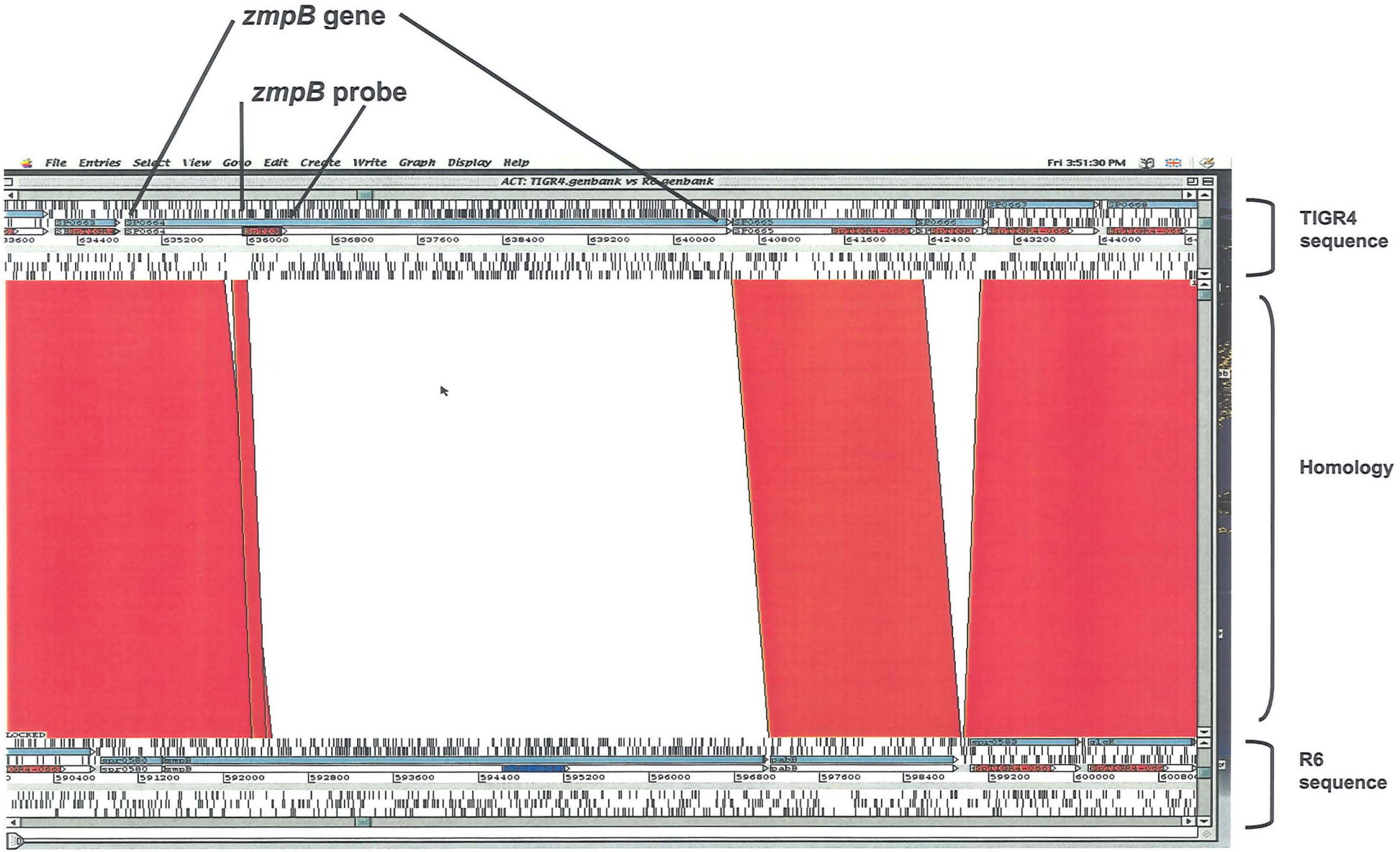

zmpB gene
zmpB probe
File Entries Select View Go to Edit Create Write Graph Display Help
Fri 3:51:30 PM
ACT: TIGR4.genbank vs R6.genbank
TIGR4 sequence
Homology
R6 sequence

Figure 7.22 Artemis comparison of the *zmpB* gene sequences in *S. pneumoniae* strains TIGR4 and R6

Artemis Comparison Tool (ACT) allows comparison of the sequences of genomes. The figure illustrates a comparison between the region of the *S. pneumoniae* R6 and TIGR4 genomes encoding the gene for *zmpB* (R6 spr0580, TIGR4 sp0644). Genes / open reading frames within individual genome sequences are shown in turquoise / bright blue. The TIGR4 sequence is shown along the top half of the figure and the R6 sequence is along the bottom. Red colouring inbetween the 2 genome sequences represent conserved regions. Sequence variation (or absence of sequence) is shown by the white regions. The diagram highlights the lack of homology between the *zmpB* genes of R6 and TIGR4, with the only homologous region being found at the start of the gene only. The ZmpB probe on the array, designed against the TIGR4 sequence, is shown as a red bar beneath the TIGR4 *zmpB* gene, and the figure demonstrates that this probe lies mostly within the heterologous region.

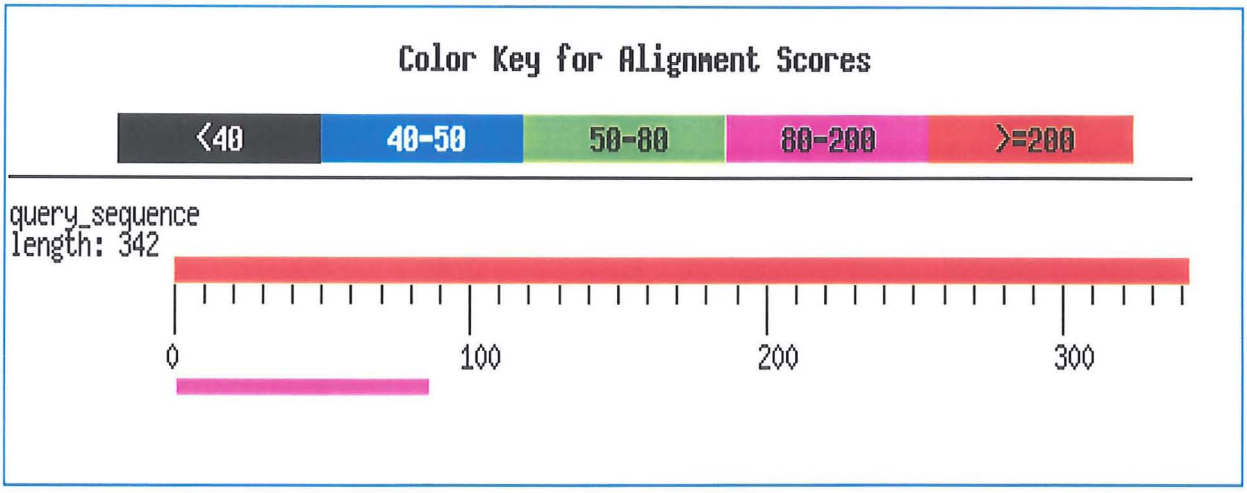


Figure 7.23 BLAST alignment of the TIGR4 ZmpB probe with the R6 *zmpB* gene

The 342 bp probe (shown as a red bar along the top of the figure), designed using the *zmpB* sequence from the type 4 TIGR4 strain was aligned against the R6 genome using the BLAST programme. The figure illustrates that the only region of homology of the probe (shown as a short pink bar) with the R6 gene is within the first quarter (80 bp) of the probe only, which showed 68 % identity. This low homology may be sufficient to detect some expression of *zmpB* in R6 / D39, but will not result in efficient detection.

7.7 Summary of ZmpB Results

Discussion of mutant phenotypes is in relation to the isogenic parental strain, unless stated otherwise.

7.7.1 Basic *in vitro* analysis and analysis of published data

Two interruptions at different points within the *zmpB* gene of a serotype 2 pneumococcus, D39, were created by mariner transposon mutagenesis, resulting in 2 independent mutants. The mutants were confirmed by PCR. Both mutants were characterised *in vitro* and *in vivo* for this work, and both demonstrated similar phenotypes in all experiments. Thus results for only one mutant, *Δ739* (the more truncated mutation) were presented in this chapter and are summarised in this section.

The mutant was not impaired in growth in nutrient-replete BHI media, although demonstrated an increased initial rate of autolysis upon entry into stationary phase. The mutant was stable and retained the spectinomycin resistant phenotype *in vitro* for up to 12 hr.

Published data by 2 independent groups found conflicting phenotypes for mutations within the pneumococcal *zmpB* gene. One study found that the *zmpB* mutant grew in abnormally long chains, was unable to autolyse, had significantly decreased transformation efficiency and was unable to transport certain proteins to the cell surface (Novak *et al.*, 2000). A second study failed to confirm any of these distinct phenotypes (Bergé *et al.*, 2001). Several of the reported phenotypes were re-examined as part of this work and found to be in complete agreement with those reported by Bergé *et al* (2001). The mutant did not demonstrate abnormal colony morphology in culture, was able to autolyse in the presence and absence of DOC, an allosteric activator of the LytA autolysin enzyme, and did not demonstrate a reduction in transformation efficiency. This work also examined the ability of the mutant to lyse sheep red blood cells *in vitro* during mid-exponential growth and found no alteration in the haemolytic activity compared to isogenic D39 wild-type.

7.7.2 *In vivo* characterisation

The mutant was virulent following i.p. challenge and all subsequent *in vivo* work was performed using bacterial strains that had initially been mouse-passaged. The mutant was found to be attenuated in virulence following both i.n. and i.v. challenge, demonstrating significantly lower levels of bacteraemia and increased murine survival times. Although showing decreased virulence in both models of infection, the reduced virulence of the *Δ739* mutant appeared to be more attenuated following intranasal infection compared to direct inoculation of the bacteria into the bloodstream. This is based on differences in bacteraemia at selected time points and survival times following infection in comparison to wild-type values. Table 7.4 summarises some of the key observations from the *in vivo* work carried out with the *Δ739* mutant.

Observation	**Intranasal challenge**	**Intravenous challenge**
Percentage survival	No D39 infected mice survived 10% *Δ739* infected mice survived	No mice survived infection with D39 or *Δ739*
Experiment endpoint	100 hr for mice infected with D39 160 hr for mice infected with *Δ739* **60 % longer than wild-type**	80 hr for mice infected with D39 100 hr for mice infected with *Δ739* **25 % longer than wild-type**
Median survival time	45 hr for mice infected with D39 127 hr for mice infected with *Δ739* **2.8-fold longer than wild-type**	46 hr for mice infected with D39 97 hr for mice infected with *Δ739* **2.1-fold longer than wild-type**
Survival at the 60 hr halfway point	10 % for mice infected with D39 90 % for mice infected with *Δ739*	10 % for mice infected with D39 90 % for mice infected with *Δ739*
Bacteraemia at 24 hr (Log10 median values)	6.28 for mice infected with D39 3.93 hr for mice infected with *Δ739* **0.5 % that of wild-type**	6.39 for mice infected with D39 4.91 hr for mice infected with *Δ739* **3 % that of wild-type**

Table 7.4 Comparison of virulence of *Δ739* following intranasal and intravenous challenge

MF1 mice were challenged intranasally or intravenously with 10^6 or 10^4 CFU, respectively. Bacteraemia and survival was monitored. The table highlights some of the main differences seen between mutant and wild-type D39 strains for each challenge, and illustrates the more attenuated phenotype of the mutant following intranasal challenge compared to intravenous infection (highlighted in bold, red typescript). The experimental endpoint is the point at which all mice have been actively culled or the point where infection does not progress further (ie. mice remain healthy for the remainder of the experiment).

Mutants were stable / retained their spectinomycin resistance up to 36 hr post challenge. No significant difference was seen in the ability of the *Δ739* mutant to colonise lung tissue at 12, 24 or 36 hr post intranasal challenge, compared to the D39 parental strain. Bacterial counts in the BALF were also similar for mice challenged with mutant compared to wild-type bacteria at 0, 12, 18 and 24 hr post intranasal challenge. Significantly higher counts of D39 were found in the BALF at 36 hr post infection compared to mutant counts ($P<0.05$).

Mice challenged, both intranasally and intravenously with the *Δ739* mutant often displayed an unusual response to infection, where they showed mild signs of infection for up to 5 days but then seemed to recover and have a healthy appearance. However, following this recovery phase, the mice often relapsed and began to show severe signs of infection, having to be actively culled quickly after the re-appearance of symptoms. This prolonged display of symptoms followed by recovery with or without relapse is not a common observation following infection with wild-type D39 in our murine model.

7.7.3 Role of ZmpB in complement deficient mice

A short region within the *zmpB* sequence showed some homology to other pneumococcal proteins previously shown to interfere with complement. Complement deficient mice were thus challenged with wild-type and *Δ739* mutant bacteria to determine if ZmpB is able to interfere with complement. Mutants retained their attenuated phenotype in this model of infection compared to D39 wild-type, indicating that ZmpB does not interact with complement in any way that significantly impairs the host (murine) response to pneumococcal infection.

7.7.4 Role of ZmpB in inflammation

The study of selected cytokines in the BALF and lung tissue following intranasal infection with bacterial strains indicated that there were no significant alterations in the cytokine profiles of IFN-γ, IL-6 and IL-10 between infection with D39 wild-type or *Δ739* mutant bacteria. However, significantly higher concentrations of the pro-inflammatory cytokine TNF-α were found in the lung tissue of mice infected with wild-type parental strain compared to mice infected with the mutant, 24 hr post challenge. This indicates that active ZmpB could have a role in inducing inflammation.

7.7.5 Expression of ZmpB for vaccination and antibody production

The central region of the *zmpB* gene was expressed, semi-purified and used to make anti-ZmpB antibodies. Immune sera generated from the vaccination of mice recognised the semi-purified ZmpB protein using a Western blot technique and will thus be useful for future applications.

7.7.6 Regulation of *zmpB* by TCS09

RT-PCR data indicates that TCS09 does not prevent expression of *zmpB in vitro*. Microarray analysis could not confirm this observation as the probe on the array does not have sufficient homology with the *zmpB* gene found in strain D39 used for this analysis.

Chapter 8

Discussion

S. pneumoniae ZmpB

Chapter 8; *S. pneumoniae* ZmpB discussion

Zinc metalloproteases have previously been shown to have important roles in the virulence of several pathogens (Hase and Finkelstein, 1993). The elasatase of *Pseudomonas aeruginosa* is a zinc metalloprotease (Wretlind and Pavlovskis, 1983), as are the tetanus and botulinum neurotoxins of Clostridia species (Schiavo *et al.*, 1992). Bacterial zinc metalloproteases cleave a range of substrates and contribute differently to the infection process. *S. pneumoniae* ZmpB is a large gene (>5.5 Kb) and has been detected in several genome-wide approaches for identifying potential pneumococcal virulence factors. Such approaches have included differential fluorescence induction to identify genes up-regulated *in vivo* (Marra *et al.*, 2002) and searches based on known cell surface anchoring motifs (S. Koenig, personal communication, based on Wizemann *et al.*, 2001). In the latter study, ZmpB was identified through its LPXTG motif (see later). However, the role of ZmpB in virulence has not been investigated in any detail. *ZmpB* is one of the few pneumococcal genes that displays extensive sequence variation between serotypes. This suggests that the gene could be important in human infection and that immune pressure exists from the host against this gene. It was thus decided to examine the role of this putative metalloprotease as part of this thesis and to determine if its expression is regulated by TCS09.

8.1 Mutant construction and *in vitro* analysis

Mutations were created in the *zmpB* gene of *S. pneumoniae* strain D39 by *mariner* mutagenesis and confirmed by PCR. Although 2 independent mutations were created and analysed throughout this work, only data for one of these is presented and will be discussed, as both mutants had very similar phenotypes in comparison with one another.

The mutant, *Δ739,* was not impaired in growth *in vitro* indicating that ZmpB is not essential for growth in BHI media. However, RT-PCR was used to show that the *zmpB* gene is expressed by D39 during the mid-exponential phase of growth in BHI. This indicates that the gene may be constitutively expressed or switched on at the stage of growth tested

Upon initial entry into the autolysis phase of growth, the mutant appeared to demonstrate a rapid reduction in viable cell counts between 10 and 12 hr (figure 7.4A). Although this stage of growth is generally attributed to autolysis of pneumococcal cells, it is possible that the mutants may be dying without cell lysis. This possibility could also explain why, following stationary phase, the

optical density readings of the *Δ739* mutant do not decrease and remain above those of D39 wild-type, despite the mutant having lower viable cell counts than the parental strain. As cell lysis is thought to release the haemolytic toxin pneumolysin, a simple haemolytic assay could determine if the mutant bacteria are lysing faster than wild-type D39, and thus releasing more pneumolysin into the culture media at this stage of growth. A haemolytic assay was performed as part this work and did not indicate greater levels of pneumolysin (described later), but this assay was performed at one time point only, where cell viability counts were equal between mutant and wild-type bacteria. Furthermore, all cultures were completely lysed in the presence of DOC. The haemolytic assay would thus have to be repeated at various time points after the stationary phase of growth is reached, and in the absence of DOC. The significance of increased autolysis to *in vivo* infection is not known. Firstly *in vivo* infection is not likely to represent the batch culture growth conditions present during *in vitro* growth, and consequently it is not known if batches of pneumococcal cells undergo autolysis during infection. If, however, pneumococci do autolyse during infection, an increased rate of autolysis in the *Δ739* mutant could result in a slower overall increase in bacterial load and could thus contribute to the attenuated phenotype described for the mutant. However, based on the observation that wild-type bacteria induce greater inflammation in the lungs early in infection (described later), it is doubtful whether increased autolysis of the *Δ739* mutant is occurring *in vivo*, as this would presumably result in increased release of pneumolysin which is itself a potent mediator of inflammation (Mitchell and Andrew, 2000).
The *Δ739* mutant retained its spectinomycin resistance for up to 12 hr in the absence of antibiotic selection, demonstrating that the *mariner* cassette remains integrated within the genome during this period of time.

Two studies have published data on the *in vitro* phenotypes of mutations in the pneumococcal *zmpB* gene, although the findings from these studies are conflicting. In one study, a *zmpB* knock-out mutation was created in a virulent, encapsulated serotype 4 TIGR4 strain using insertion-duplication mutagenesis, and resulting mutants were found to possess a distinct phenotype (Novak *et al.*, 2000). The *zmpB* insertion-duplication mutant grew in very long chains of 2000-3000 cells in culture, had lost the ability to express several choline binding proteins on the cell surface, was unable to autolyse in the presence of DOC and penicillin and had significantly reduced transformation ability (99 % reduction compared to wild-type). The

mutants were also deficient in the translocation of competence proteins CinA and RecA to the cell membrane. This led the authors to propose that active ZmpB regulates translocation of Cbps, CinA and RecA to the surface of the cell where they can perform functions such as lysis / separation of daughter cells and transformation.

A second study created a series of integrations in *zmpB* in the avirulent, un-encapsulated serotype 2 R6 strain, using *mariner* mutagenesis (Bergé *et al.*, 2001, described in the introduction, section 1.7.3). None of the phenotypes found by Novak *et al* could be confirmed using the *zmpB* mutants created in the second study. Table 8.1 summarises the conflicting data published from the 2 studies.

Novak *et al.*, 2000, serotype 4 (TIGR4)	**Bérge *et al.*, 2001, serotype 2 (R6)**
Abnormal chain formation	Normal chain formation
Autolysis defect (even with excess deoxycholate and penicillin)	Retained ability to autolyse in the presence of deoxycholate
LytA trapped in cytoplasm complexed with CinA; No trafficking of CinA and RecA to the cell surface	LytA and CinA complexes not detected. Individual proteins detected by Western blot
99 % decrease in transformation efficiency	Transformed at normal frequency
Proposed a role for ZmpB in the control of translocation of Cbp to the cell surface	Found no evidence for the role of ZmpB in controlling translocation of Cbps to the cell surface

Table 8.1 Conflicting data published on ZmpB

Two independent studies have characterised the *in vitro* phenotype of *zmpB* mutants, with conflicting results. The table summarises some of the phenotypes of the mutants found by the individual studies.

Due to the contrasting sets of data published with respect to *zmpB* mutants, several of the phenotypes above were re-examined as part of this thesis and were in complete agreement with the data of Bergé and co-workers (Bergé *et al.*, 2001). The *Δ739* mutant exhibited cell morphology identical to that of the wild-type parental strain, with cells growing singly, in pairs or in very short chains. This indicates the presence of functional LytB, a Cbp required for daughter cell separation. Upon treatment with DOC, an allosteric activator of the LytA autolytic enzyme, all strains were completely lysed within 15 min of treatment, indicating the presence of functional LytA, also a Cbp. Western blot, performed within the laboratory, was used to detect a single band of the expected size for LytA alone, indicating that this protein was not complexed with any other proteins (data not shown). Furthermore, the mutant transformed at the same frequency as wild-type. These data highlights that the absence of capsule in the R6 strain used in the study by Bergé and colleagues (Bergé *et al.*, 2001) does not account for the differences observed in the mutant strains. The contribution of different genetic backgrounds of the strains used for the above studies could indeed result in very distinct phenotypes, a phenomenon that was discussed in chapter 6. However, the mutant strain used in the study by Novak and colleagues (Novak *et al.*, 2000) was independently analysed in an identification laboratory and, in contrast to the wild-type parental strain, was subsequently classified as *Streptococcus viridans* based on its resistance to optochin and the absence of a capsule. Thus it appears that contamination occurred during the work of Novak and co-workers (Novak *et al.*, 2000) that resulted in the unusual phenotype documented for the *zmpB* mutant strain used in this study. This highlights the requirement for continuous evaluation of all pneumococcal strains used during any study. Furthermore, several different identification techniques should be employed to allow for accurate *S. pneumoniae* identification, as pneumococcal colony morphology and haemolysis on blood plates is often indistinguishable from that of other Streptococcal species.

8.2 Role of ZmpB in virulence

To assess if ZmpB contributed to the virulence of *S. pneumoniae*, mice were initially administered the *Δ739* mutant and its isogenic parental strain intranasally. This infection resulted in significantly longer survival times and lower bacterial counts in the blood of mice 24 and 36 hr post challenge with the mutant compared to D39 wild-type, demonstrating that ZmpB is required for virulence. To assess the stages of virulence where ZmpB has a significant influence,

bacterial counts in the lung tissue post challenge were determined. This indicated that the *Δ739* mutant was not impaired in its ability to colonise the lungs at any of the time points studied. Mutant bacteria were detected in the bloodstream of mice 12 hr post infection and the counts detected were slightly higher than those of wild-type bacteria, indicating that the *Δ739* mutant is not impaired in its ability to disseminate from the lungs to the bloodstream, early in the infectious process. However, once in the blood, the bacterial counts for the mutant remain significantly lower than those for wild-type strain. This reduction in levels of bacteraemia is also observed when the strains are inoculated directly into the bloodstream of mice and demonstrates that the mutants are less adapted for survival within this environment. The data from infections with the *Δ739* mutant, summarised in table 7.4, indicates that intranasal challenge may result in a more attenuated phenotype than intravenous challenge, as the former results in increased murine survival times and a greater difference in bacterial counts in the blood 24 hr post infection compared to wild type. This could indicate that at later time points (post 12 hr) wild-type bacteria can disseminate more efficiently from the lungs to the bloodstream, or that during the transition from the lungs to the blood, the mutants are altered in such a way that reduces their ability to survive or replicate in the blood. Regardless of the challenge route, the *Δ739* mutant does not start showing a significant increase in bacterial counts until at least 12 hr after its entry into the blood. This is in contrast to the wild-type D39 strain, which demonstrates increased bacterial counts as soon as it enters the systemic circulation. The *Δ739* mutant is not, however, essential for virulence as mice do eventually succumb to infection with this strain. Furthermore, when moribund, such mice have high levels of bacterial counts in their blood, which are indistinguishable from counts in moribund mice following challenge with D39 wild-type. The mutant was shown to be stable for up to 36 hr post challenge and bacteria isolated from selected mice infected with *Δ739* at the time of culling (ie. when moribund) were also shown to retain the mariner insertion within the *zmpB* gene as determined by PCR (data not shown). This illustrates that the high bacterial counts in moribund mice are due to the *Δ739* and not due to reversion to wild-type. The attenuated phenotype with regards to survival in the blood can thus be overcome eventually.

The levels of bacteraemia in the blood of mice infected with the mutant appear to correlate with the unusual progression of symptoms observed with such mice. During the earlier stages of infection, bacteria are able to gain entry to the systemic circulation and this correlates with the

appearance of mild symptoms. Following challenge with the *Δ739* mutant, bacterial counts remain low, particularly following intravenous infection, at several subsequent time points. This could explain the prolonged time of mice displaying milder symptoms, and the apparent recovery of some mice. However, bacterial counts are known to increase at time points later than those selected, due to the high counts present in moribund mice. This delayed increase in bacteraemia may explain the sudden relapse seen with some mice, where mice showing very mild symptoms, or appear to have recovered, quickly progress to more severe and terminal stages of infection. This could suggest that early in infection the immune system is more effective at controlling infection with the mutant strain but is unable to completely eliminate infection in the majority of infected mice. During the later stages of infection, mutant bacteria are able to overcome their attenuation, possibly through reaching high enough numbers to resist the host immune response and subsequently can cause severe infection and mortality of the infected mice. Host defence mechanisms that may have a role in this response are discussed below.

8.3 Interaction of ZmpB with complement

The *in vivo* data discussed above suggested that the ZmpB mutant was highly attenuated within the systemic circulation, but was able to grow in the lung tissue. This indicates that components of the blood are able to prevent growth of the *Δ739* mutant. During systemic infection with *S. pneumoniae*, splenic phagocytic cells play an important role in clearing the invading microbes. Complement plays a major role in this clearance by acting as an opsonin, coating the surface of the bacteria which enhances phagocytosis (Hostetter, 2000). A BLAST search (http://www.ncbi.nlm.nih.gov:80/BLAST/) of the ZmpB protein of TIGR4 and R6 revealed a region that had 35 % identity (47 % positive residues) with another pneumococcal protein, Hic (also known as PspC and CbpA). This protein has been shown to bind complement factor H (Neeleman *et al.*, 1999; Janulczyk *et al.*, 2000; Jarva *et al.*, 2002), which is a host protein that acts to inhibit complement activation. The binding of factor H to pneumococcal cells is thought to inihbit the activation of complement near the cell surface and prevent subsequent deposition of opsonic complement components. The region of ZmpB showing homology to Hic/PspC is found within the first 400 amino acid residues, which comprises the homologous region between pneumococcal serotypes. An alignment showing the homology of ZmpB from strains R6, TIGR4 and G54 with Hic/PspC is provided in the appendix (A6c). Hic/PspC itself displays extensive

sequence variation between serotypes (Ianelli *et al.*, 2000), although the region found to bind factor H is situated towards the more conserved N-terminus part of the protein (Janulczyk *et al.*, 2000). A fragment of Hic that covered this homologous region was cloned and expressed and was shown to bind factor H, within the short consensus repeats 8-11, and to inhibit the binding of this component to the pneumococcal cell surface (Janulczyk *et al.*, 2000; Jarva *et al.*, 2002). The region encoding this fragment (Hic residues 39-261) is located immediately upstream of the region found to have homology with ZmpB (Hic residues 268-526), indicating that ZmpB itself may also be capable of binding small amounts of factor H. On the basis of this homology and the importance of complement in clearing pneumococcal infection, it was hypothesised that ZmpB had a role in inhibiting complement activity during murine infection and that loss of this function results in the attenuated phenotype described. To test this, the *Δ739* mutant and its isogenic parental strain were analysed in complement deficient (C3-/-) mice (C57Bl/6 background, Wessels *et al.*, 1995). These mice are unable to produce complement component C3, a crucial part of both the alternative and classical complement pathways, and are thus unable to mount any complement-associated response to infection. Wild-type C57Bl/6 control mice (C3+/+) of equal age and gender were also infected with the same dose of bacteria. C3-/- mice challenged with wild-type bacteria succumbed to infection quicker and had significantly higher levels of bacteraemia at pre-selected time points compared to C3-/- mice challenged with *Δ739* mutant. When compared to wild type bacteria, the *ΔzmpB* mutants showed the same attenuated phenotype in C3-/- as observed in MF1 mice suggesting that complement is important in clearing both D39 wild-type and the *Δ739* mutant. Thus a factor other than complement appears to be responsible for the impaired growth in the blood and increased murine survival following challenge with such mutants. If the hypothesis of ZmpB interacting significantly with complement had been correct, similar levels of virulence between the wild-type and mutant bacteria in C3-/- mice would have been observed. The indication from these data that ZmpB does not bind factor H could be confirmed by Western blot using anti-factor H antibody, and comparing the binding of factor H to D39 wild-type and the *Δ739* mutant (Pandiripally *et al.*, 2002).

Although ZmpB was not shown to interact with complement, the work with the C3-/- mice highlighted the importance of complement in the response to pneumococcal infection in C57/Bl mice. Following intranasal challenge with wild-type bacteria, 80 % of complement-sufficient

C3+/+ mice survived the challenge and did not have any detectable bacterial counts in the blood at all time points examined. However, in the absence of complement, all mice (C3-/-) infected with D39 quickly succumbed to infection and had extremely high bacterial counts in their blood within 24 - 36 hr post infection. By 40 hr post infection, 90 % of C3-/- mice infected with D39 had been culled due to the severity of their symptoms. Complement is known to be important in the human host defence system against *S. pneumoniae*, as complement deficiencies predispose patients to pneumococcal infection (Figueroa and Densen, 1991). Thus this is an example where the use of animal models can be highly relevant to understanding the infection process in the human host.

8.4 Role of ZmpB in inflammation

Pathology of pneumococcal pneumonia and meningitis is thought to result from an exaggerated host inflammatory response to bacterial components (Tuomanen *et al.*, 1995). Overwhelming inflammation can contribute to tissue injury and shock. This is supported by the observation that death may occur days after the initiation of antibiotic therapy when tissue culture is sterile but a strong inflammatory response still persists (Bergeron *et al.*, 1998). The ability of pneumococcal cell wall and toxin pneumolysin to induce inflammation has been described (introduction 1.6.3), but little is known about other bacterial factors that could influence the inflammatory response.

The host inflammatory response to pneumococcal pneumonia is complex and involves a range of pro and anti-inflammatory cytokines, the balance of which is crucial in determining outcome of infection. TNF- α is a well-established early mediator of inflammation and is critical for the ability of the host to control microbial infections (Blanchard *et al.*, 1988; Steinshamn *et al.*, 1996; Lima *et al.*, 1997 and). Many groups have identified a protective role for TNF-α in pneumococcal infection *in vivo* (Takashima *et al.*, 1997; O'Brien *et al.*, 1999; Kerr *et al.*, 2002). However, TNF-α can also be detrimental by inducing tissue injury within the lung. Such injury could impair tissue integrity and enhance passage of bacteria from the lungs to the bloodstream (Bergeron *et al.* 1998).

The reduced levels of bacteraemia in the blood of mice challenged with the *Δ739* mutant compared to wild-type from 24 hr post challenge could indicate that wild-type are more able to survive in the blood. Alternatively, it could suggest that at later time points more wild-type bacteria are able to disseminate from the lung tissue into the blood stream. As the inflammatory

response is thought to result in tissue damage (Bergeron *et al.*, 1998), it was hypothesised that a more vigorous host response in the lungs tissue during wild-type infection could impair tissue integrity and allow bacteria to gain further entry into the bloodstream. To test this hypothesis, levels of TNF-α within the respiratory tract were compared following challenge with wild-type and mutant bacteria. TNF-α was chosen based on its role as a potent pro-inflammatory cytokine that has been characterised previously with regards to its role in pneumococcal pneumonia. It is also one of the first inflammatory mediators to be produced following pneumococcal infection (O'Brien *et al.*, 1999).

Significantly increased levels of TNF-α were found in the lung tissue 24 hr post challenge with the wild-type bacteria compared to the Δ739 mutant. TNF-α, at this time point, was only detected in the lungs of 20 % of mice infected with the mutant, compared to 90 % of lungs following challenged with wild-type D39 (data not shown). When detected in the lung tissue of mice following challenge with the *Δ739* mutant, TNF-α concentrations were extremely low and did not differ significantly from those in the uninfected / control mice. TNF-α could not be detected significantly within the lungs at any other time point studied, nor could it be detected in the BALF. However, visual inspection of the bioassay, using lung tissue 12 hr post infection, indicated that TNF-α was present in the lung tissue of mice infected with D39 wild-type but absent in mice infected with the mutant (data not shown). In this assay, the presence of TNF kills the L929 fibroblasts, and the dead cells are subsequently unable to metabolise added MTT to its purple-coloured formazan product. The absence of a colour change was visible by eye for the 90 % of the lung samples from mice challenged with wild-type bacteria, although the levels of TNF were too low to be detected using the conventional measurements of the assay. No colour change was seen for any of the lung samples taken from mice infected with the *Δ739* mutant, indicating the absence of detectable TNF-α. These data indicated that ZmpB is involved in generating a host inflammatory response through the production of TNF-α, 12-24 hr into the infection.

To further analyse the inflammatory response in the respiratory tract of mice, the profiles of several other cytokines were studied. These cytokines were chosen based on previous data that indicated a role for such cytokines during pneumococcal infection (see introduction, section 1.5.3.2). IFN-γ and IL-6 were chosen to represent further pro-inflammatory cytokines and IL-10 was chosen to represent an anti-inflammatory cytokine.

The concentration of the above cytokines was determined at 12, 18, 24 and 36 hr post infection in the BALF and the lung tissue. Bacterial counts from the BALF samples were also determined to ensure that any differences in cytokine concentration were not due to different bacterial counts. These data showed that as infection progressed, bacterial counts in the BALF decreased, indicating that infection from the airways was being eliminated by the host response or had progressed into the lower respiratory tract. No difference was observed for BALF counts between the wild-type and mutant strain, with the exception of the 36 hr time point, where mice challenged with wild-type bacteria had increased bacterial counts in the BALF. These counts were significantly higher than those seen in mice infected with the *Δ739* mutant. It is not known if this increase is characteristic of D39 infection as previous studies of cytokine profiles following pneumococcal infection have used different mouse models. To assess this further, the experiment would have to be repeated as challenges for cytokine determination were performed with 5 mice only for each time point examined (with the exception of the TNF-α studies where n=10 and where experiments were performed on two separate occasions). Bacterial counts in the lung tissue samples used for cytokine determination could not be assessed because the snap freezing procedure used to preserve the cytokines from degradation in the lung tissue would kill any bacteria present. However, previous data measuring the bacterial loads in lung tissue at 12, 24 and 36 hr post infection did not detect any significant difference in counts between wild-type and the *Δ739* mutant, indicating that the bacterial counts in the lung tissue used for cytokine analysis should also remain the same.

The only significant difference in cytokine profile between the wild-type and mutant bacterial strains was for IFN-γ, in the BALF, 24 hr post infection, where higher levels of this cytokine were present in mice infected with mutant bacteria. The relevance of this finding to the infection is not clear as IFN-γ is a pro-inflammatory cytokine and thus would indicate more inflammation in the airways of mice challenged with the *Δ739* mutant compared to wild-type infection. This would not be in agreement with the findings based on TNF-α levels within the lung tissue at the same time point. A similar increase in the level of IFN-γ is not observed in the lung tissue at 24 hr post infection. Furthermore, the level of IFN-γ in the BALF at this time point is much higher than any of the other time points observed following infection with *Δ739,* suggesting that abnormally high levels of this cytokine in the airways of one or two of the mice may have skewed the data for this experiment. The concentration of cytokines within the BALF tissue was

consistently much lower than concentrations in the lung tissue, indicating that the latter environment is more important in terms of generating an inflammatory response, and further discussion will thus only be based on cytokine profiles in the lung tissue. One point of interest with respect to the cytokine profile in the BALF is the observation that lower IL-10 levels are seen in the lung tissue following infection with both bacterial strains compared to the uninfected / control mice. This could indicate that infection with both strains results in inhibition of IL-10 production, although the mechanism by which this occurs is unknown.

Within the lung tissue the concentrations of all cytokines appeared to increase over time, and this may be attributed to the increase in bacterial load up to 24 hr post infection (see figure 7.10). However, at 36 hr post infection, bacterial counts in the lung tissue were much lower than previous time points for both the wild-type and the mutant. This decrease in bacterial counts was not parallelled by a decrease in cytokine levels, as median concentrations of IFN-γ, IL-6 and IL-10 all increased between the 24 hr and 36 hr time points. The increase in IFN-γ and IL-6 is in agreement with published data suggesting that the inflammatory response can continue long after bacterial infection has been eliminated (Bergeron *et al.*, 1998). Indeed concentrations of the pro-inflammatory cytokine IFN-γ are the highest of the cytokines studied at all time points examined, following by IL-6, also proposed to have pro-inflammatory activities. The increase in the concentration of the pro-inflammatory cytokines is matched by an increase in the anti-inflammatory cytokine IL-10. Presumably as inflammation progresses, higher levels of this cytokine are required to control the host response and prevent it from becoming detrimental. It is interesting to note that levels of IL-10 in the lung tissue of mice challenged with mutant bacteria are consistently higher than the lung tissue of mice challenged with D39 wild-type, up to and including 24 hr post challenge, although this difference is not statistically significant. At 36 hr, levels of IL-10 are not higher following infection with the mutant.

Due to the pleiotropic effects of TNF-α and the complex nature of the host inflammatory response, the level of many cytokines and other mediators may be altered between infection with the mutant and wild-type bacteria. For example, it has been shown that TNF-α can induce the production of IFN-γ from immune cells (Zhang and Tracey, 1998). This would suggest that the decreased levels of TNF-α following challenge with mutant bacteria would similarly result in decreased levels of IFN-γ compared to infection with wild-type bacteria. Although median levels of IFN-γ in the lungs of mice challenged with mutant bacteria were lower than levels in the lung

tissue of mice challenged with wild-type bacteria at 24 hr, this difference was only slight and not statistically significant. However IFN-γ production can be induced by other factors, including IL-12, and it can also stimulate its own production (Roitt *et al.*, 1996b). This highlights the caution that must be exerted when analysing host immune responses mediated by cytokines and other factors. Furthermore different immune cell types will be recruited to or activated at different stages of infection, and the presence of such cells will have a huge influence on the immune response generated. Thus further characterisation of the immune response following challenge with mutant and wild-type bacteria is required, and will be discussed later in this chapter.

8.5 Proposed series of events following intranasal challenge

Based on the data presented above, it appears that ZmpB has a role in inducing an inflammatory response and this confers an advantage upon wild-type bacteria during the earlier-middle stages of infection. The proposed series of events following intranasal infection are highlighted below and are based.

0-12 hr

During this time an inflammatory response is initiated through the activity of TNF-α, induced by wild-type bacteria only (as determined by eye). This inflammation is not strong enough to induce tissue damage at this very early stage of infection and both wild-type and mutant strains are able to breach the lung tissue and gain access to the blood at this point independent of the intensity of inflammation.

12-36 hr

Although inflammation is present in the lungs following challenge with the *Δ739* mutant, as evident by the increased concentrations of IFN-γ and IL-6, the inflammatory response appears to be less severe than that in the lungs of mice infected with D39 wild-type. This is based on significantly lower levels of TNF-α and slightly increased levels of IL-10. The increased inflammatory response proposed during D39 infection could impair tissue integrity, allowing more bacteria into the bloodstream and this would enhance the ability of bacteria to reach sufficient counts to evade the immune response. Furthermore, the balance of cytokines at this stage could have a significant influence on the host immune response at later stages. As a result

of less inflammation, fewer numbers of mutant bacteria would gain access to the bloodstream, allowing host defences to deal with the decreased numbers of mutant bacteria already present in the systemic circulation.

36 hr onwards

By this stage, inflammation within the lung tissue appears to have reached similar levels in mice infected with both the mutant and wild-type strains. Consequently more mutant bacteria will have gained access to the bloodstream, and bacterial counts within the blood will have increased. This increased bacterial load will eventually reach a level where the immune system is unable to control the infection any further and mice become sick, quickly leading to the terminal stages of infection relatively quickly, as evident by the sudden relapse of such mice.

The hypothesis highlighted above is based on the inflammation induced by ZmpB leading to tissue destruction and subsequently increasing access to the bloodstream. However, if this hypothesis is true, then ZmpB must have other functions important in virulence as direct inoculation of the *Δ739* mutant into the blood also results in lower bacterial counts and increased murine survival. This suggests that active ZmpB also functions to evade the host immune response or to help bacteria survive in the blood, possibly through the sequestration of essential nutrients.

These data suggests that the role of ZmpB in virulence is multifactorial. Being such a large protein, it is possible that ZmpB, like pneumolysin, has separate regions within the protein that contribute differently to virulence. Further experiments to examine the role of ZmpB in pneumococcal infection, and to provide evidence for the hypothesis described above are suggested below.

With respect to the phenotype of the *Δ739* mutant discussed above, a similar phenotype has been described for mutants in *Mycobacterium tuberculosis* which lacked the alternative sigma factor, SigH (Kaushal *et al.*, 2002). Such mutants persisted within murine lung tissue but had a delayed inflammatory response (Kaushal *et al.*, 2002). Thus further insight into the basis of the *Δ739* phenotype could also be relevant to other microbial pathogens.

8.6 Further determination of the role of ZmpB in virulence

8.6.1 Inflammation

To further characterise the inflammatory response other inflammatory mediators could be examined, including other cytokines or nitric oxide (NO). The latter is a potent molecule produced in response to pneumococcal infection and can be induced by TNF-α (Coleman, 2002). NO, like TNF-α, is thought to be beneficial in small amounts but can cause tissue damage when present at increased levels (Bergeron *et al.*, 1998). The Greiss reaction can be used to determine nitric oxide concentration (Bernatowicz *et al.*, 1995).

Evaluation of the immune cell types present or recruited to the site of infection over time is important to determine the extent of inflammation and the potential source of increased TNF-α observed during infection with wild-type bacteria (Kerr *et al.*, 2002). A variety of cell types can produce TNF-α, including macrophages, neutrophils, lymphocytes and mast cells (Tracey and Cerami, 1994). Some of these cell types, such as macrophages and mast cells, will be present in the lung tissue. Mast cells in particular are thought to be important in the early release of TNF-α, having pre-formed TNF-α (Gordon *et al.*, 1990 and Kerr *et al.*, 2002). Other cells, such as neutrophils, will be recruited to the site of infection, with TNF-α itself serving to attract and activate neutrophils. These cells are important for their role in pneumococcal killing, although overactivity could contribute to tissue pathology. Thus a large influx of inflammatory cells would exacerbate the inflammation and result in further tissue damage. Immunohistochemistry is currently being performed on lung samples from several time points following infection with D39 and the *Δ739* mutant to characterise the immune cells present (by A. Kadioglu, Leicester). Production of TNF-α by different cells, such as alveolar macrophages and lung cells could also be determined using tissue culture assays.

The nature of the host response to pneumococcal infection is highly complex and is likely to involve many components too numerous to study in individual experiments such as those described above or used as part of this thesis. A more comprehensive way of studying the response to infection would be to use microarray analysis. Mouse arrays comprising 3000 immune response genes are available and could be used to examine gene profiles in tissue from infected mice, or from specific cells stimulated *in vitro* with bacterial strains. This work could

provide important clues to the differences in virulence of mutants compared with the isogenic parental strains.

With respect to the role of ZmpB in generating increased levels of TNF-α, there are several possible explanations. The protein itself could function as a chemoattractant to recruit immune cells capable of producing TNF-α to the site of infection. Other possible functions that would result in increased TNF-α, could depend on the putative protease activity of ZmpB (discussed later), whereby host proteins such as cytokines and their receptors or matrix metalloproteases (MMPs) could be targets for proteolysis. ZmpB may also act to cleave pneumococcal proteins, either within the cell or on the cell surface and serve to activate or inhibit the function of such proteins.

Cytokines and their receptors

A number of pathogens produce proteases capable of degrading cytokines and are thus thought to be important virulence factors. *P. aeruginosa* produces two distinct proteases both capable of degrading cytokines. The elastase enzyme has been shown to degrade IL-2 and TNF-α (Parmely *et al.*, 1990), and the alkaline protease degrades IFN-γ (Horvat *et al.*, 1988). A zinc metalloprotease from *Legionella pneumophila* can also cleave IL-2 and TNF-α (Hell *et al.*, 1993; Mintz *et al.*, 1993) and has been shown to have structural and functional homologies with the *P. aeruginosa* elastase (Black *et al.*, 1990). In contrast, there are examples of bacterial proteases that are able to activate cytokines via cleavage of the precursor form into its active form (Kapur *et al.*, 1993).

Similarly, bacterial proteases have been found that can cleave cytokine receptors. This can result in the cleaved receptor acting as a competitive inhibitor for the cytokine or the receptor may bind to cells that do not usually express it, thus rendering the cells responsive to the cytokine. Proteases from a range of organisms, including *Staphylocuccus aureus*, *P. aeruginosa* and *Serratia marcescens*, have been shown to cause the release of IL-6 receptor (IL-6R) from human monocytes and macrophages (Vollmer *et al.*, 1996). The IL-6R released by the *S. marcescens* protease was found to be biologically active and able to render hepatoma cells, which do not usually express IL-6R, responsive to IL-6 (Vollmer *et al.*, 1996).

There are several ways in which *S. pneumoniae* ZmpB could induce high levels of TNF- α based on cleavage of cytokines and their receptors. For instance, it could cleave membrane bound pro-TNF- α to its mature, more active form and thus mimic the activities of the natural host TNF-α converting enzyme, which is itself, a zinc metalloprotease. To test this hypothesis, macrophages or mast cells could be incubated with live and heat killed wild type and mutant bacteria. Following incubation, target cells would be subject to flow cytometry to detect the presence of surface bound TNF-α. ZmpB could also cleave cytokines or mediators involved in regulation the inflammatory response, rendering them inactive. IL-10 would be an example of such a candidate, although levels of this cytokine were not found to be significantly different following infection with mutant bacteria compared to wild-type infection.

Matrix metalloproteases

ZmpB could also mimic the activity of matrix metalloproteinases (MMPs) or activate MMPs themselves. MMPs are a family of zinc metalloprotease that are able to degrade virtually every component of the extracellular matrix (ECM) (Leppert *et al.*, 2001). They are generally secreted as inactive precursors and activated to their mature form by extracellular cleavage. Many cell types, including neutrophils and macrophages, produce MMPs. Other than ECM components, MMPs can cleave cytokine precursors, including TNF- α, and their receptors, and pro forms of MMPs themselves (Leppert *et al.*, 2001). Cytokines, including TNF- α, can stimulate activation of MMPs. This can result in a massive loop of amplification where MMPs and TNF- α can cause activation of each other and themselves. Unsurprisingly, MMPs have potential for massive tissue degradation and are thought to play a role in the pathogenesis of several disease states (Nakamura *et al.*, 1998; Leppert *et al.*, 2001; Warner *et al.*, 2001). Indeed an increase in MMP9 and TNF- α in the CSF of experimental pneumococcal meningitis has been shown (Leib *et al.*, 2000).

Proteolysis of ECM is a key event in the progression of inflammatory processes and may contribute to infection by allowing spread of pathogens. Several bacterial pathogens, including *P. aeruginosa* and *Vibrio cholerae*, have been found to have proteases able to activate different MMPs (Okamoto *et al.*, 1997). Such enzymes are likely to be important virulence factors. It is feasible that *S. pneumoniae* ZmpB could also function as an MMP-activating enzyme within the

lung environment and thus facilitating the activation of TNF- α indirectly. For example, ZmpB could mimic the activity of MMP-12, which is the predominant metalloelastase of macrophages in the lungs. MMP-12 has been linked with damage to lung tissue, recruitment of neutrophils into the lungs and ability to shed biologically active TNF-α (Warner *et al.*, 2001). MMP-12 activity is up-regulated in cigarette smokers, a condition which also predisposes to pneumococcal infection (Hautamaki *et al.*, 1997; Ahmer *et al.*, 1999). Although specific MMP inhibitors are generally not available, MMP deficient mice (including MMP-12 -/-) have been developed (Warner *et al.*, 2001) and would be a useful model in which to study pneumococcal infection in general. Furthermore, this model could be used with the *Δ739* mutant and its isogenic parental strain. Proteolytic destruction of ECM components by ZmpB could be one way of inducing an inflammatory response that would result in increased TNF- α levels.

8.6.2 Growth in the blood

The attenuated ability of the *Δ739* mutant to grow in the blood of mice indicates an important role for ZmpB within this environment. Phagocytosis is an important clearance mechanism for systemic pneumococcal infection and increased phagocytosis of mutant bacteria would reduce their survival in blood. Although this process is mediated by complement, and data presented in this work indicates that ZmpB does not significantly interfere with complement function, phagocytosis may also depend on factors other than complement opsonisation. Anticapsular antibodies can promote phagocytosis, although a humoral response to pneumococcal infection is thought to take at least 11 days to develop (Dallaire *et al.*, 2001) so would not be expected to play a role in the acute model of infection used within this work. In *Streptococcus pyogenes*, fibrinogen binding to the surface of bacteria cells has been shown to influence phagocytosis (Hortsmann *et al.*, 1992; Pandiripally *et al.*, 2002), suggesting that other host components may also influence phagocytosis in pneumococcal infection. Classical *in vitro* phagocyte killing assays could be used to determine if the *Δ739* mutant is more susceptible to phagocytosis, and the activity of phagocytic cells from both the blood and lungs could be compared (Jansen *et al.*, 2001). These assays have been described previously (chapter 6, section 6.2).

Alternatively, isolated whole human and / or murine blood could be used to determine if this medium supports the growth of the *Δ739* mutant. This could help determine if a component within the blood, or the lack of certain nutrients in the blood is preventing the growth of mutant

strains, or if this growth defect is due to a host component not present in isolated blood. Comparison of the bacterial strains in nutrient deplete broth could help identify if wild-type D39 bacteria are able to grow more efficiently in such conditions compared to the *Δ739* mutant strain.

8.6.3 Adhesion

Pneumococcal colonisation and adhesion to host tissues is a crucial step in initiation of the disease process and is thus a potential target of both prophylactic and therapeutic treatment. Pneumococci have been proposed to adhere to a range of cell types within the human respiratory system, including both resting and activated cells (Anderson, *et al.*, 1983; Gerard *et al.*, 1995; Cundell and Tuomanen, 1997). It has also been proposed that inflammatory cytokines induced by bacterial factors may up-regulate expression of host proteins that can subsequently be utilised by the pneumococcus for adhesion (Sollid *et al.*, 1987; Kelly *et al.*, 1994; Kadioglu *et al.*, 2002). It could thus be hypothesised that increased levels of TNF- α induced by wild type D39 may promote adhesion by the up-regulation of host factors used for pneumococcal attachment. ZmpB has previously been proposed to play a role in the colonisation of rat and human nasopharyngeal tissue, although the mutant strain used within these studies was later shown to be a non-pneumococcal *Streptococcus* species (Novak *et al.*, 2000; Bergé *et al.*, 2001). Data presented in this thesis indicate that the *Δ739* mutant was not impaired in its ability to colonise murine airways or lung tissue. Thus, data from the work in this thesis does not support a link between inflammation and increased adhesion, so it is necessary to study this phenomenon in further detail. Several differences (other than contaminating bacterial strains) could account for the conflicting data in the ability of *ΔzmpB* mutants to adhere. Novak and co-workers used a mutant in a serotype 4 pneumococcus and human cell lines or rat nasopharyngeal tissue to examine adhesion, whereas our mutants, in a type 2 background, were analysed for their ability to colonise murine airway and lung tissue *in vivo*. The *Δ739* mutant could thus be constructed in a type 4 pneumococcal strain and used to compare adhesion with its isogenic parental strain. Such a mutant would also be useful for characterisation in the murine infection model used for the ZmpB analysis. As the sequence of ZmpB between serotypes is so variable, this work could determine if ZmpB has similar functions in different serotypes.

8.6.4 Immungenicity and potential as a vaccine candidate

During this work, antibodies recognising the central region of ZmpB were produced following vaccination of mice with part of the ZmpB protein. These antibodies (and the purified ZmpB protein) can be used in a variety of ways to further characterise ZmpB. For example, the Western blot assay developed within this work could be used to determine if the antibody raised against ZmpB from a serotype 2 strain can recognise ZmpB in other serotypes. This is important based on the described sequence variation in ZmpB between serotypes. Similarily, pure ZmpB protein could be used to determine if antibodies to ZmpB are present during infection, both in murine sera following experimental infection and in convalescent sera from human infection. This would indicate if ZmpB is naturally immunogenic and produced during pneumococcal infection. Furthermore, vaccination studies with the ZmpB protein could be performed to determine the protection conferred by ZmpB against subsequent pneumococcal challenge. Anti-ZmpB antibodies could also be used to determine if passive protection could be achieved. Again, this work could be extended to include the protection elicited against different serotypes or strains of *S. pneumoniae*. All such studies would be important if ZmpB was to be considered as a potential vaccine candidate.

8.6.5 Cellular localisation

It is not known if the pneumococcal ZmpB protein is present on the cell surface or secreted into the surrounding medium. Sequence analysis of ZmpB reveals a LPXTG motif located at the N-terminal (http://www.tigr.org/). In other Gram-positive bacteria, this motif serves to anchor proteins to the cell surface, through the action of a sortase enzyme (Pallen *et al.*, 2001). This sortase cleaves between the threonine and the glycine residues and transpeptidates the protein to the pentaglycine cross-links of the cell wall (Mazmanian *et al.*, 1999). Such evidence indicates that ZmpB is exported to the pneumococcal cell surface. The LPXTG motif, however, is generally found at the C-terminal region of Gram positive proteins, so its relevance as a cell surface anchor for pneumococcal ZmpB is unclear. The LPXTG residues in ZmpB in D39 have been selectively deleted using Janus mutagenesis (G. Paterson, our laboratory). This technique is based on the Janus cassette constructed by Sung and co-workers (Sung *et al.*, 2001). Using two transformations, this cassette can be used to insert stop codons or DNA of arbitary sequence at any chosen site in the pneumococcal genome. The technique is extremely versatile, allowing

complete inactivation of genes, or more subtle mutations such as amino acid changes, or epitope tagging. The resulting mutations do not possess any antibiotic-selection markers that may interfere with further investigation. This also allows for the construction of multiple mutations in different genes without the need for various antibiotic selection markers. The technique allows for the transfer of large regions of DNA between different strains. Furthermore, polar effects on downstream genes are avoided. To determine cellular localisation, the Janus mutant and its isogenic parental strain could be analysed in conjunction with a sortase null mutant that has also been constructed (G. Paterson, our laboratory) using cell fractionation and subsequent detection of ZmpB with the anti-ZmpB antibodies in the different fractions. The *Δ739* mutant could be used as a negative control in such experiments.

The Janus mutagenesis technique could also be used to mutate the HEXXH-E region thought to be essential for the activity of zinc metalloproteases. Analysis of resulting mutants using the same experiments described in this thesis for *Δ739* would help determine if the putative protease activity of ZmpB contributes to its role in virulence. This could also help provide evidence that ZmpB does indeed have protease activity.

8.6.6 Other functions

A BLAST search (http://www.ncbi.nlm.nih.gov:80/BLAST/) revealed that the ZmpB proteins of the TIGR4 and R6 strains both had highest homology with IgA1 proteases (*iga*) from *S. pneumoniae* and other bacteria, mainly Streptococcal species. These proteases function to cleave IgA1 and are thus thought to function in immune evasion (Plaut, 1983). Unlike the homology with Hic/PspC, the homology to IgA1 proteases was present across the whole protein sequence. Table 8.2 shows the region of homology of the TIGR4 ZmpB protein with other proteins. The homology with Streptococcal IgA1 proteases is intriguing as this group of proteins have other features in common with ZmpB. It has been demonstrated that heterogeneity is present within the IgA1 proteases, even within the same species (Poulsen *et al.*, 1996). The IgA1 proteases of Streptococcal species also have the HEXXH-E motif characteristic of zinc metalloproteases and have a LPXTG motif unusually located at the N-terminus region of the protein. It is thought that the heterogeniety between the *iga* genes is the result of horizontal transfer and recombination within and between species (Poulsen *et al.*, 1998). Isolates of *S. pneumoniae* vary in their IgA1 protease activity and this has been attributed to the polymorphism within this species (Reinholdt

and Kilian, 1997). Although annotated as IgA1 proteases, these proteins may have other functions. Indeed in two, independent STM screens set up to identify pneumococcal virulence factors in different pneumococcal serotypes, disruptions in *iga* genes were found to be attenuated in virulence (Polissi *et al.*, 1998; Hava and Camilli, 2002). As murine models were used for both screens, and bacterial IgA proteases specifically cleave human but not mouse IgA (Plaut, 1983), this data suggested that these proteins have other functions (Hava and Camilli, 2002). One of the STM screens demonstrated reduced virulence of a pneumococcal *iga* mutant in models of pneumonia and septicaemia (Polissi *et al.*, 1998). As IgA1 acts predominantly at the mucosal surface, reduced virulence of the *iga* mutant in a septicaemia model is further evidence that this gene has roles in virulence other than IgA1 cleavage. Furthermore, the IgA1 protease of *N. gonorrhoeae* has been shown to cleave LAMP1, a lysosome integral membrane protein (Hauck and Meyer, 1997), indicating that other proteins are indeed the target of IgA1 proteolytic activity in bacterial pathogens.

Repeat structures showing variation in number and sequence have been identified in many Streptococcal *iga* genes. Indeed, the *zmpB* gene of TIGR4 was identified as containing a stretch of iterative DNA (CAAAACAAAACAAAA) (Novak *et al.*, 2000; Tettelin *et al.*, 2001). This repeat sequence could not be found in the R6 or G54 *zmpB* nucleotide sequences however. Repeated structures are found in many surface exposed and secreted proteins of Streptococci and may allow for phase variation and hence immune evasion (Kehoe, 1994).

Novak and colleagues tested ZmpB for IgA1 and IgA2 proteolytic activity but could not detect any (Novak *et al.*, 2000). While it would be interesting to perform further IgA1 / IgA2 proteolysis assays to determine if ZmpB does possess this activity, this would not explain the attenuation in virulence for *Δ739* in our murine model of infection as pneumococcal IgA1 proteases are specific for human IgA only (Plaut, 1983). Furthermore, one study deleted the gene encoding the IgA1 protease a type 2 pneumococcus (R6) and found that the resulting mutant had a complete loss of human IgA1 proteolytic activity (Wani *et al.*, 1996). This indicates that ZmpB may not cleave human IgA1, although the assay conditions used for the above study may not have been optimal for detecting IgA1 protease activity present in other genes.

Protein description	Identity	Positives	ZmpB region of alignment
ZmpB, *Streptococcus pneumoniae*, strain R6	98 %	99 %	Across first 300 amino acids
	40 %	58 %	Across remainder of protein
ZmpB, *Streptococcus pneumoniae*, strain G54 (type 19F)	99 %	99 %	Across first 300 amino acids
	41 %	59 %	Across remainder of protein
IgA1 protease, *Streptococcus pneumoniae*, strain TIGR4	28 %	46 %	Across whole protein
IgA1 protease, *Streptococcus pneumoniae*, strain R6	26 %	45 %	Across whole protein
IgA1 protease, *Streptococcus sanguinis*	26 %	44 %	Across whole protein
IgA1 protease, *Streptococcus mitis*	25 %	44 %	Across whole protein
Factor H-binding region, PspC, *Streptococcus pneumoniae*, strain A66 (type 3)	35 %	47 %	Residues 154-396

Table 8.2 Proteins showing homology to ZmpB (TIGR4)

A BLAST search was performed to determine proteins showing homology to *S. pneumoniae* ZmpB (from the TIGR 4 strain). The table shows a selection of the proteins showing highest homology, together with the region over which homology was present.

The *Δ739* mutant described in this thesis is currently being analysed in a *C. elegans* model of infection to determine if it is attenuated compared to D39 wild-type in this model (W. Jansen, CX Utrecht, the Netherlands). As discussed in chapter 4, the use of such models are a cheaper and simpler alternative to murine models and may enhance our understanding of the infection process.

8.7 Regulation of ZmpB expression

RT-PCR data presented in this work suggests that TCS09 does not prevent the expression of ZmpB, at least under the conditions used. This would not be unusual, as close proximity of a gene to a TCS does not necessarily imply its regulation by that system. This observation, does

not however, preclude the possibility that TCS09 is involved in regulating *zmpB* as the RT-PCR analysis used is not quantitative. It could be possible that TCS09 may up- or down-regulate *zmpB* expression and this could be assessed using a quantitative technique such as real-time RT-PCR. Although it was hoped that *zmpB* regulation by TCS09 could be confirmed by microarray analysis using the *Δrr09* mutant discussed in chapter 6, the ZmpB probe on the array was designed based on the TIGR4 sequence and shows limited homology to the type 2 R6 sequence. Thus to overcome this problem a *rr09* mutant will be constructed in the type 4 TIGR4 strain and re-analysed for ZmpB expression.

Appendix

A1. *S. pneumoniae* strains

Strain	Description
D39	Obtained from NCTC (7466), Central Public Health Laboratory, London. Encapsulated virulent isolate, related to the non-encapsulated R6 strain. Serotype 2. MLST type 128
ΔpAS1	Mutation in *rr08*. Created by insertion duplication mutagenesis (in this study).
Δrr08	Mutation in *rr08*. Created by allelic replacement (in this study).
ΔpRPL7	Mutant in *hk08*. Created by insertion duplication mutagenesis (in this study).
ΔrRPL50	Mutation in gene immediately upstream of TCS08, *sp0082*. Created by insertion duplication mutagenesis (in this study)..
Δrr09	Mutation in *rr*09. Created by allelic replacement (in this study)..
Δhk09	Mutation in *hk*09. Created by allelic replacement (in this study)..
Δ738	Mutation in *zmpB*. Created by Mariner mutagenesis (in this study).. Resistant to spectinomycin.
Δ739	Mutation in *zmpB*. Created by Mariner mutagenesis (in this study).. Resistant to spectinomycin. Mutation results in a more truncated version of *Δzmp738*.
0100993	Encapsulated, clinical isolate, serotype 3.Obtained from M.K.R. Burnham, GlaxoSmithKline (NCIMB no. 40794) MLST type 595
Δ484hk	Mutation in *hk08* gene, created by allelic replacement. Obtained from M.K.R. Burnham, GlaxoSmithKline.
Δ484rr	Mutation in *rr08* gene, created by allelic replacement. Obtained from M.K.R. Burnham, GlaxoSmithKline.
Δ488	Mutation in *rr09* gene, created by allelic replacement. Obtained from M.K.R. Burnham, GlaxoSmithKline.
TIGR4	Encapsulated, clinical isolate, serotype 4. Used for the TIGR genome sequencing project (Tettelin *et al*, 2000). Other names for this strain includeJNR.7/87, KNR.7/87 and N4. MLST type 205
R6	Unencapsulated, avirulent derivative of strain D39. MLST type 128. Obtained from J-P. Claverys, Toulouse, France

Table A1 *S. pneumoniae* strains
Parental strains are illustrated in bold. Mutant strains are illustrated in italics. Mutant strains all have cassettes encoding erythromycin resistance, unless stated otherwise.

A2: Hoffmann-La Roche Constructs and vector for TCS08 insertion-duplication mutants

Construct	Function
pRPL7	To knock out *hk08* gene
pAS1RR08	To knock out *rr08* gene
pRPL50	To knock out *sp0082*surface protein gene

Table A2 Plasmid constructs and associated mutations

The table lists the constructs received from Hoffmann-La Roche which, when transformed into growing cultures of *S. pneumoniae*, create the mutations described upon homologous recombination into the target gene. All constructs are based on the 7.4 Kb pAS1 vector, see below (Mortier-Barriére *et al.*, 1998) and produce an erythromycin-resistant phenotype when integrated into the pneumococcal genome. Failure to integrate into the genome will result in loss of the plasmid. The pRPL50 vector includes a tag that will target any translated protein sequence for degradation (Keiler *et al.*, 1996 and Molnos *et al.*, 2000).

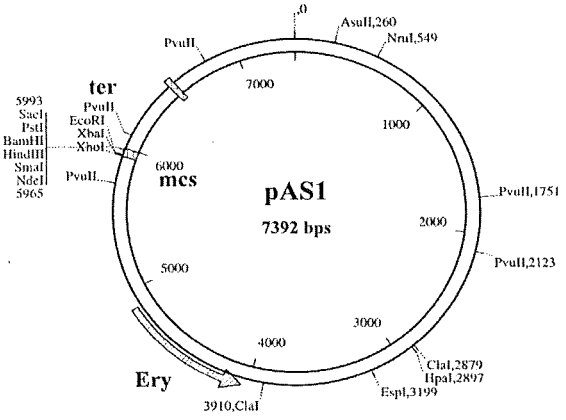


Figure A2; pAS1 suicide vector used to construct the TCS08 insertion-duplication mutants

The pAS1 plasmid is a suicide vector unable to replicate in *S. pneumoniae* (Mortier-Barriére *et al.*, 1998). A region homologous to the gene targeted for mutagenesis was inserted into the multi-cloning site (mcs) and the resulting construct was used to transform strain D39. Integration into the target gene occurs by homologous recombination. Transformants are selected by their resistance to erythromycin, encoded within the pAS1 vector. Failure to integrate into the chromosome results in loss of the plasmid from the host cell. For the *ΔpRPL50* mutant, the coding sequence of a tmRNA molecule was inserted into the pAS1 vector to target any translated protein for degradation.

A3 Primers used

General primers		
Name	**Sequence (5'-3')**	**Description**
PlyF	CGGGATCCGGCAAATAAAGCAGT AAATGACTTT	Forward primer for pneumolysin gene
PlyR	GACGGAGCTCGACTAGTCATTTTC TACCTTATC	Reverse primer for pneumolysin gene
T3	ATTAACCCTCACTAAAGGGA	Forward primer for sequencing PCR products in pCR®4-TOPO®
T7	TAATACGACTCACTATAGGG	Reverse primer for sequencing PCR products in pCR®4-TOPO® and pET33b
16SF	GATAACTATTGGAAACGATAGCT AATACCG	Forward primer for 16S RNA gene. Control for RT-PCR.
16SR	GCTAATGCGTTAGCTACGGCACT AAACCC	Reverse primer for 16S RNA gene. Control for RT-PCR.

Primers for TCS08 work		
Name	**Sequence (5'-3')**	**Description**
AP70	GACCATGATTACGCCAAG	Forward primer to isolate 3' end of *ΔpAS1*, *ΔpRPL7* and *ΔpRPL50*. Specific for inserted plasmid (pAS1) DNA
RPL22	GTCACGACGTTGTAAAAC	Reverse primer to isolate 3' end of *ΔpAS1*, *ΔpRPL7* and *ΔpRPL50*. Specific for inserted plasmid (pAS1) DNA
URR8F	TGACGACGAGGTAGAAAT	Forward primer to isolate 5' end of *ΔpAS1*. Specific for chromosomal DNA
RR08R1	TGTCCTCTCGGTTTCTCT	Reverse primer to isolate 3' end of *ΔpAS1*. Specific for chromosomal DNA
HK8PFW	ACTCAAGGAGCATGCCAA	Forward primer to isolate 5' end of *ΔpRPL7*. Specific for chromosomal DNA
HK8PR	GGGTAAAGGTACTTCCTAG	Reverse primer to isolate 3' end of *ΔpRPL7*. Specific for chromosomal DNA
RPL102	**	Forward primer to isolate 5' end of *ΔpRPL50*. Specific for chromosomal DNA
RPL109	GTGTCCCATCCACATCAGC	Reverse primer to isolate 3' end of *ΔpRPL50*. Specific for chromosomal DNA

RR08F	GATGGATAGTGGGCTGAC	Forward primer for rr08 gene to confirm absence in *ΔpAS1* mutant.
RR08R	AATCCCAGCACCGACAAG	Reverse primer for rr08 gene to confirm absence in *ΔpAS1* mutant.
HK08F	ATAGAGAAACCGAGAGGA	Forward primer for hk08 gene to confirm absence in *ΔpRPL7* mutant.
HK08R	TCTACCATGAATAGCTGG	Reverse primer for hk08 gene to confirm absence in *ΔpRPL7* mutant.
Sp0644F	AGACTCTAAGGAGAATCC	Forward primer for sp0082 gene to confirm absence in *ΔpRPL50* mutant.
Sp0644R	CCTGATTGCTGATTGAGT	Reverse primer for sp0082 gene to confirm absence in *ΔpRPL50* mutant.
484P1	ACGATGGCAGGTGAAAACA	Forward primer to isolate *rr08* gene for type 3 and allelic replacement (Throup *et al*, 2000)
484P2	GCGACAACTGGGCAATCATCAA	Reverse primer to isolate *rr08* gene for type 3 and allelic replacement (Throup *et al*, 2000)

Primers for TCS09 work		
Name	**Sequence (5'-3')**	**Description**
488P1	GCGGAGCCGAGTAGGAGATTCTC ACC	Forward primer to isolate *rr09* gene for type 3 and allelic replacement (Throup *et al*, 2000)
488P4	TGTAGAAATGACCTGACCAG	Reverse primer to isolate *rr09* gene for type 3 and allelic replacement (Throup *et al*, 2000)
RR09F	GCAGCCTACGATATGGAA	Forward primer for RT-PCR of *rr09* gene, region A
RR09R	CCTCAATATCCTGACGAG	Reverse primer for RT-PCR of *rr09* gene, region A
MsrAF	CAGTTAGCCAAGGAGGAA	Forward primer within *msrA* gene
MsrAR	TGACATGGACGGTTTCTG	Reverse primer within *msrA* gene
ZmpR	ACAGATGCTTCTGTCCCT	Reverse primer within *zmpB* gene

Primers for ZmpB work		
Name	**Sequence (5'-3')**	**Description**
ZmpUp	AGTCTTACCCAAGCTGGTTCC	Forward primer for isolation of *zmpB* gene (Bergé *et al*, 2001)
ZmpDo	CCCCCAAAGTCCTAAAATCA	Reverse primer for isolation of *zmpB* gene (Bergé *et al*, 2001)
MP128	TACTAGCGACGCCATCTATGTG	Primer specific for Mariner cassette (Bergé *et al*, 2001)
ZmpIntF1	GTAGTTCCGGATGACACA	Forward primer for RT-PCR of *zmpB* gene, region 1
ZmpIntR1	TTGAGGTGCTTGGAGAAC	Reverse primer for RT-PCR of *zmpB* gene, region 1
ZmpIntF2	GAGCGATGGCGAAGATTT	Forward primer for RT-PCR of *zmpB* gene, region 2
ZmpIntR2	CACCGTATTCACCATTGG	Reverse primer for RT-PCR of *zmpB* gene, region 2
ForBamZmp	GGTCATGGATCCGGCTAATCCA ATGGGTGAG	Forward primer for expression of ZmpB middle region. Contains BamHI restriction site
RevSacZmp	GGTCATGAGCTCCTAATCCTCT GTAGTCGTCTC	Reverse primer for expression of ZmpB middle region. Contains SacI restriction site and a stop codon
ZmpseqgF	TGGAGATAAAGGCTGGTG	Forward primer for internal sequencing of expressed ZmpB middle region.
ZmpseqgR	GCTACTGGAGAAAGTACC	Reverse primer for internal sequencing of expressed ZmpB middle region.
ForBamZmp2	GGTCATGGATCCGGAGACGAC TACAGAGGAT	Forward primer for expression of ZmpB C-terminal region. Contains BamHI restriction site
RevSacZmp2	GGTCATGAGCTCCTATGCTTGT GGTGCAGT	Reverse primer for expression of ZmpB C-terminal region. Contains SacI restriction site

Table A3 Primers used
** RPL102 primer supplied by Hoffman-La-Roche. Sequence not available.

A4 Common solutions and buffers

Extraction Buffer (for genomic DNA preparation)

10 mM Tris pH 8.0 (Sigma-Aldrich, UK), 100 mM EDTA pH 8.0 (Fisher Scientific UK Ltd.), 0.5 % SDS (w/v)(Fisher Scientific Ltd.UK).

TE (Tris-EDTA) Buffer pH7.4

10 mM Tris-Cl (pH 7.4, Sigma-Aldrich , U.K.)

1 mM EDTA (pH 8.0, Fisher Scientific U.K. Ltd.)

PBS, pH 7.4

One tablet (phosphate buffered saline tablets, Dulbecco A, Oxoid Ltd. Basingstoke) was dissolved in 100 ml dH_2O and autoclaved.

DNA Gel Loading Buffer (6x)

0.25 % Bromophenol Blue (Sigma-Aldrich)

0.25 % Xylene cyanole FF

40 % (w/v) sucrose in dH_2O

Protein Gel Sample Buffer (5x)

60 mM Tris-HCl (pH 6.8)

25 % glycerol

2 % SDS

14.4 mM 2-mercaptoethanol

0.1 % bromophenol blue

Protein Gel Running Buffer (1x)

25 mM Tris

192 mM glycine

0.1 % SDS

1 % Agarose

1 g agarose (GibcoBRL)

100 ml TAE buffer

1 Kb Ladder

10 µl DNA gel loading buffer (6x)

50 µl DNA ladder (GibcoBRL)

1 x SSC

0.15 M NaCl

0.015 M sodium citrate

Coating buffer for ELISA

11.8 g Na_2HPO_4

16.1 g NaH_2PO_4

Made up to 1 L with dH_2O

Wash buffer for ELISA

PBS + 0.05 % Tween-20 [polyoxyethylene sorbitol monolaurate] (Sigma-Aldrich, UK)

Assay diluent for ELISA

PBS + 10 % heat inactivated FCS (Gibco BRL, Life Technologies)

Working detector for ELISA

Assay diluent + biotinylated detection antibody + avidin-horseradish peroxidase conjugate (1:350 dilution, unless stated otherwise)

TMB substrate solution for ELISA

Equal volumes of TMP Peroxidase Substrate (containing 3,3',5,5'-tetramethylbenzidine) and Peroxidase Solution B (containing H_2O_2 in citric acid buffer) (KPL Laboratories/Insight Biotechnologies)

Prepared within 15 min of use

Stop solution for ELISA

10 % phosphoric acid (H_3PO_4)

BHI contents

Calf brain infusion solids, beef heart infusion solids, protease peptone, glucose, NaCl, disodium phosphate

Growth media for tissue culture

(all reagents from Gibco BRL, Life Technologies)

RPMI 1640

10 % heat inactivated FCS

50 IU ml^{-1} penicillin

50 µg ml^{-1} streptomycin

Coomassie Blue

250 mg Coomassie Blue R250 (Sigma-Aldrich, UK.) was dissolved in 100 ml ethanol:dH_2O (1:1 v/v). 50 ml of acetic acid:dH_2O (1:1 v/v) added and the stain filtered through a Whatman No. 1 filter.

Destain for SDS-PAGE

500 ml dH_2O

400 ml ethanol

100 ml acetic acid

Transfer Buffer, pH 8.1-8.4 for Western blot

25 mM Tris (Sigma-Aldrich, U.K.)

192 mM glycine

20 % (v/v) methanol

Developing solution for Western blot

30 mg 4-chloro-1-napthol (Sigma-Aldrich, UK.) dissolved in 10 ml methanol.

50 ml Tris NaCl, pH 7.4

30 µL H_2O_2 (30 % w/w, Sigma-Aldrich)

Pre-hybridisation solution for microarrays

(in 50 ml dH_20)

8.75 ml 20 x SSC

250 µl 20 % SDS

5 ml BSA (100 mg ml^{-1})

Wash A for microarrays

(in 400 ml dH_20)

20 ml 20 x SSC

1 ml 20 % SDS

Wash B for microarrays

(in 400 ml d H_20)

1.2 ml 20 x SSC

A5 Useful Web Addresses

MLST database	http://fi-srvmlst1-ide.sm.med.ic.ac.uk/new/
BLAST	http://www.ncbi.nlm.nih.gov:80/BLAST/
PubMed	http://www.ncbi.nlm.nih.gov/entrez/query.fcgi
The Institute of Genomic Research	http://www.tigr.org/
Bacterial Microarray Group, St. Georges	http://www.sghms.ac.uk/depts/medmicro/bugs/
Sanger centre, Artemis Software	http://www.sanger.ac.uk/Software/Artemis

A6 ZmpB Alignments

Figures on following pages. Alignments were performed using the ClustalIV programme for multiple alignments (DNAStar) and dashes within alignments were introduced by this software programme.

Figure A6a Alignment of ZmpB from strains G54, R6 and TIGR4

Protein alignment of ZmpB from strains G54 (top sequence), R6 (middle sequence) and TIGR4 (lower sequence). The arrow represents the signal peptidase site and the boxed region shows the LPxTG motif. The thick, unbroken black line above the sequence and the black broken line represent the HETTH zinc binding motif with the conserved glutamate (E) residue 20 amino acids downstream, respectively. Identical residues between all 3 strains are shaded in red. Residues displaying similarity are not shown. The TIGR4 and R6 sequences have 40 % identity, whilst TIGR4 and G54 have 41 % identity across the whole protein (excluding the first 300 amino acids / homologous region). Identity between strains G54 and R6 is discussed below.

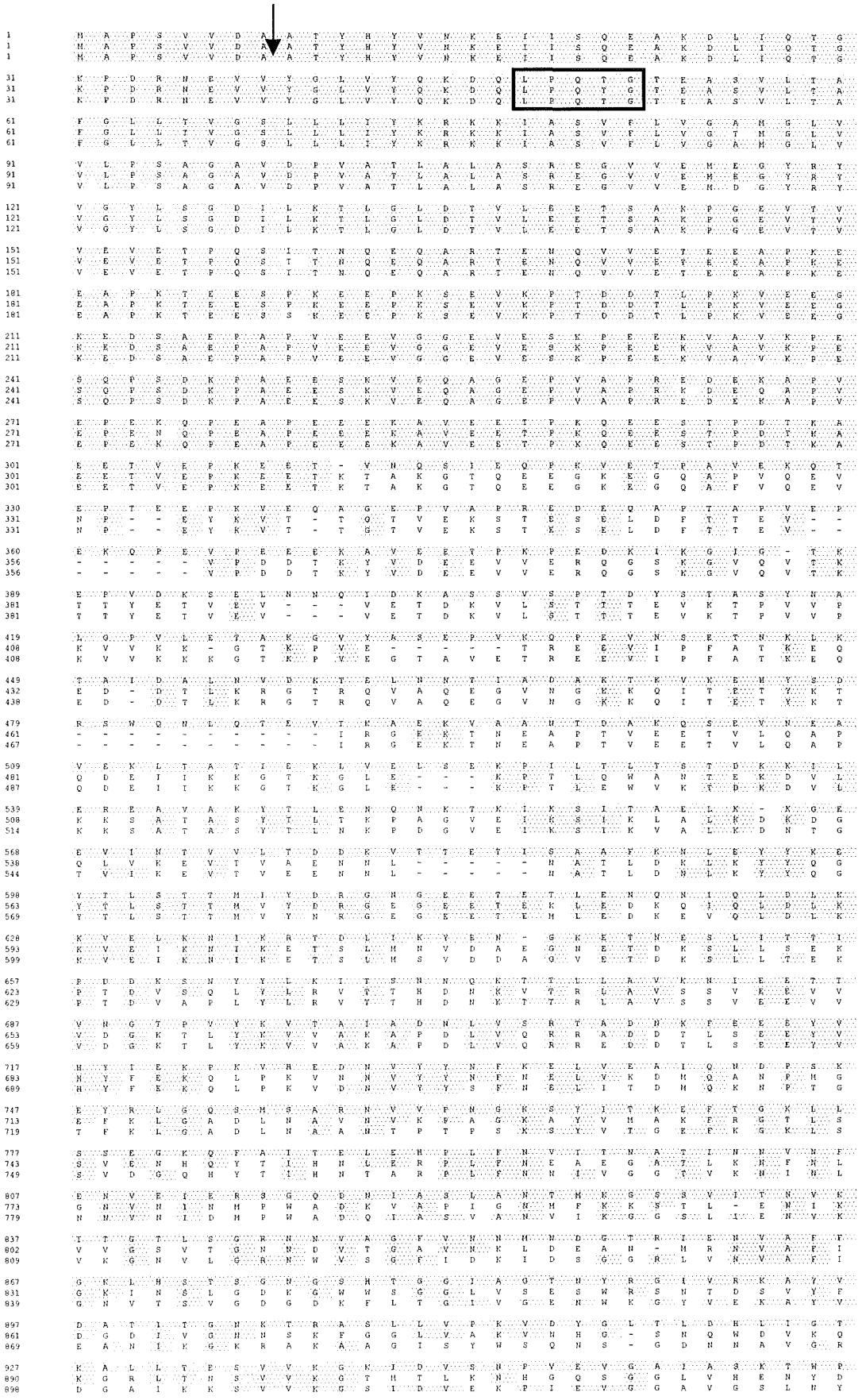
Figure A6b Alignment of ZmpB from strains G54 and R6

Protein alignment of ZmpB from strains G54 (top sequence), R6 (lower sequence). Identical residues between the strains are shaded in red. Residues displaying similarity are not shown. The sequences show 67 % identity across the whole protein (excluding the first 300 amino acids / homologous region).

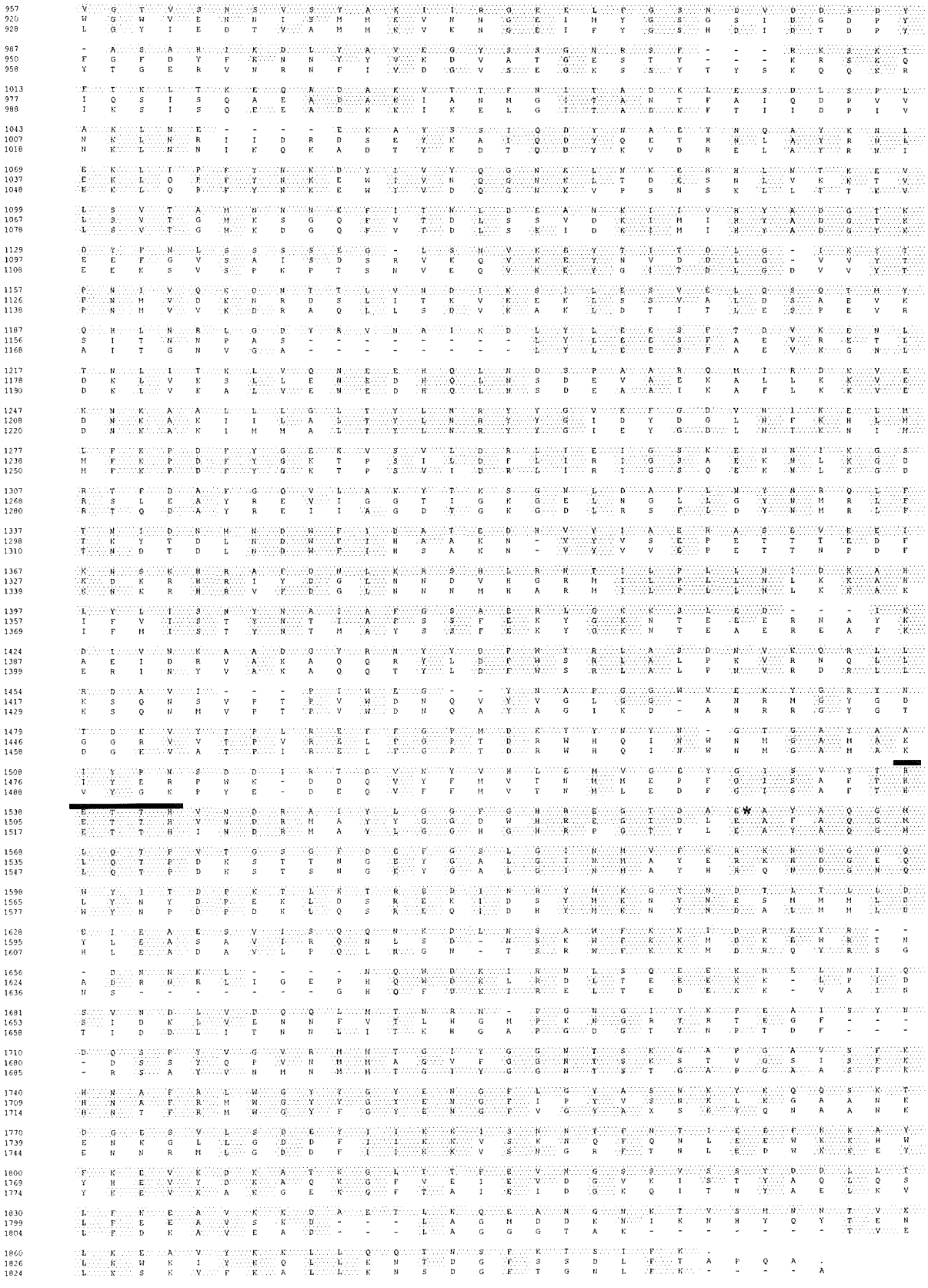
Figure A6c Alignment of ZmpB from G54, R6 and TIGR4 with Hic / Pspc from strain A66

Alignment of regions of ZmpB from different strains showing homology with N-terminal region of PspC of strain A66 (type 3). This region in strain A66 is thought to be involved in binding complement factor H, and has been designated Hic (factor H inhibitor of complement, Janulczk et al., 2000). The numbers to the left of the figure represent actual protein residues of PspC or ZmpB. Identical residues are shaded in red. Residues displaying similarity are not shown. All three ZmpB proteins display approximately 35 % identity and 47 % similarity to this region of PspC of A66.

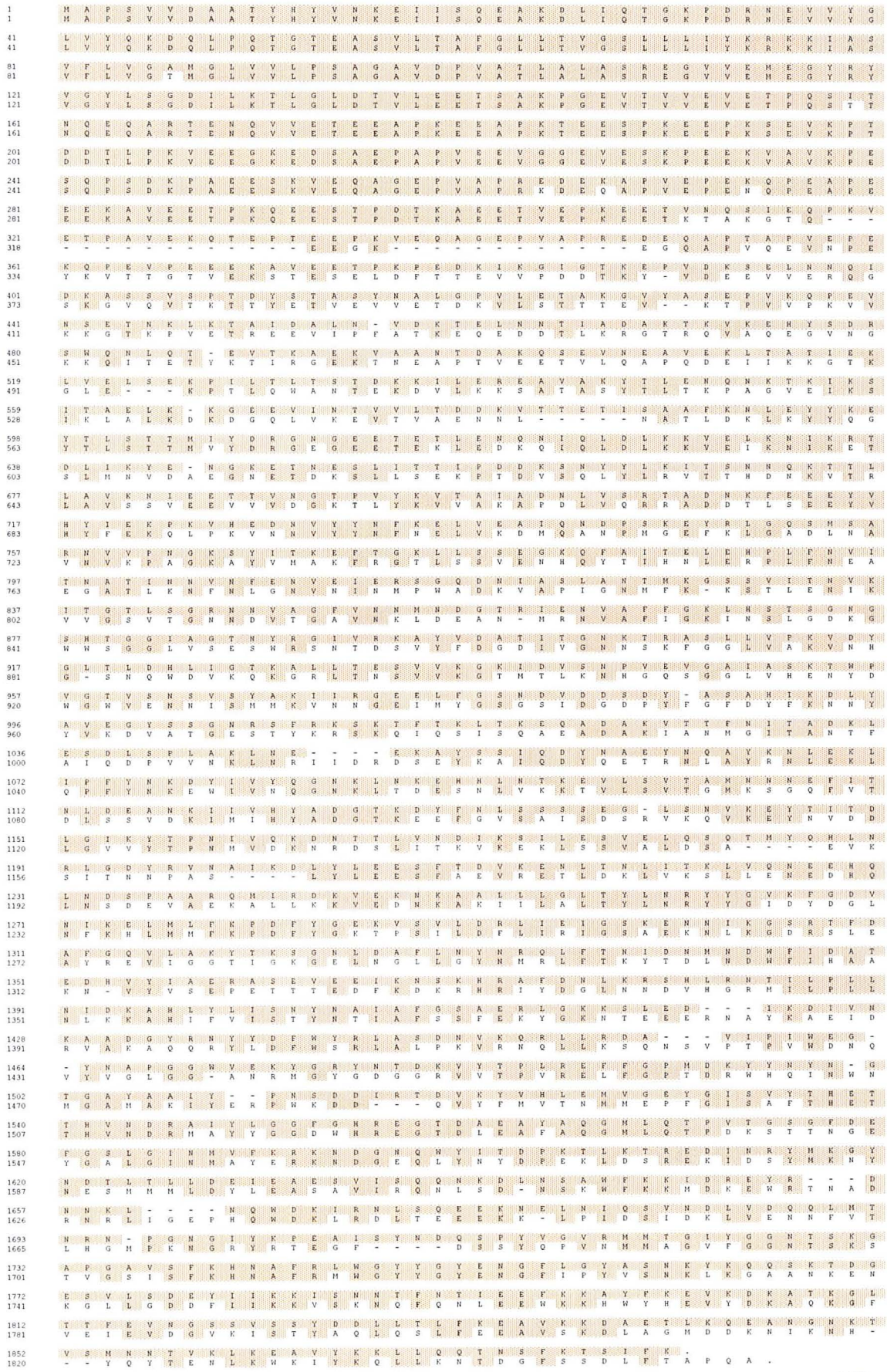
A6a Alignment of ZmpB from strains G54, R6 and TIGR4



A6a Alignment of ZmpB from strains G54, R6 and TIGR4 (continued)



A6b Alignment of ZmpB from strains G54 and R6



A6c Alignment of ZmpB from TIGR4 with Hic / Pspc from strain A66

```
268  - - - E T P Q P E T P K P E V K P E L E T P K P E V K P E P E T P K P E V K P E  PspC
151  V E V E T P Q S I T N Q E Q A R T E N Q V V E T E E A P K E E A P K T E E S S K  G54
151  V E V E T P Q S T T N Q E Q A R T E N Q V V E T E E A P K E E A P K T E E S P K  R6
151  V E V E T P Q S I T N Q E Q A R T E N Q V V E T E E A P K E E A P K T E E S P K  TIGR4

305  P E T P K P E V K P E L E T - P K P E - V K P E P E T P K P - - - - - - E V K P  PspC
191  - E E P K S E V K P T D D T L P K V E E G K E D S A E P A P V E E V G G E V E S  G54
191  - E E P K S E V K P T D D T L P K V E E G K E D S A E P A P V E E V G G E V E S  R6
191  - E E P K S E V K P T D D T L P K V E E G K E D S A E P A P V E E V G G E V E S  TIGR4

337  E P E T P K P E V K P E P E T P - K P E V K P E L E T P K P E V K P - - - E L E  PspC
230  K P E E - K V A V K P E S Q P S D K P A E E S K V E Q A G E P V A P R E D E K A  G54
230  K P E E - K V A V K P E S Q P S D K P A E E S K V E Q A G E P V A P R K D E Q A  R6
230  K P E E - K V A V K P E S Q P S D K P A E E S K V E Q A G E P V A P R E D E K A  TIGR4

373  T P K P E V K P E - P E T P K P - E V K P E L E T P K P E V K P E P E T P K P E  PspC
269  P V E P E K Q P E A P E E E K A V E E T P K Q E E S T P D T K A E - E T V E P K  G54
269  P V E P E N Q P E A P E E E K A V E E T P K Q E E S T P D T K A E - E T V E P K  R6
269  P V E P E K Q P E A P E E E K A V E E T P K Q E E S T P D T K A E - E T V E P K  TIGR4

411  V K P E L E T P K P E V K P E P E T P K P E V K P E - - L E T P K P E V K P E P  PspC
308  E E T K T A K G T Q E E G K E G Q A F V Q E V N P E Y K V T T G T V E K S T E S  G54
308  E E T K T A K G T Q E E G K E G Q A P V Q E V N P E Y K V T T G T V E K S T E S  R6
308  E E T - V N Q S I E Q P K V E T P A V E K Q T E P T - - - E E P K V E Q A G E P  TIGR4

449  E T P - K P E V K P E P E T P K P E V K P E P E T P K P - E V K P E L E T P K P  PspC
348  E L D F T T E V V P D D T K Y V D E E V V E R Q G S K G V Q V T K T T Y E T V E  G54
348  E L D F T T E V V P D D T K Y V D E E V V E R Q G S K G V Q V T K T T Y E T V E  R6
344  V A P R E D E Q A P T A P V E P E K Q P E V P E E E K A V E E T P K P E D K I K  TIGR4

487  E V K P E L E T P K P E V K P E P E T P K P E V K P E L E T P K P E V K P E L    PspC
388  V V E T D K V - - - - - L S T T T E V K T P V V P K V V K K K G T K P V E G      G54
388  V V E T D K V - - - - - L S T T T E V K T P V V P K V V K K - G T K P V E T R    R6
384  G I G T K E P V D K S E L N N Q I D K A S S V S P T D Y S T A S Y N A L G      TIGR4
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