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Genetic analysis of virulence of *Streptococcus pneumoniae*

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B.Sc.

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Author's Declaration

This thesis embodies the original work of the author unless otherwise stated.

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Abstract

Streptococcus pneumoniae (the pneumococcus) is known to be a genetically diverse species, and this important pathogen is amongst the most significant causes of bacterial disease in humans (such as pneumonia, bacteraemia, meningitis and otitis media). The infection process exposes the pneumococcus to numerous stress conditions, including temperature shift between the upper respiratory tract and deeper tissues, pH changes, exposure to reactive oxygen generated by host phagocytes, and nutritional deprivation. In order to survive, bacteria must have the ability to sense and respond to their environment in the host. A central role in this environment response is played by the two-component systems (TCS). The *S. pneumoniae* genome sequence contains 13 putative complete TCS and one orphan response regulator.

The project described in this thesis is aimed at the investigation of the regulation of expression of pneumococcal genes by TCS06 and TCS09 including those which are important to the virulence of this pathogenic bacteria. Furthermore, the putative virulence factor *dlt* operon regulated or potentially regulated by CiaR/H system was studied to investigate its contribution to the role of CiaR/H virulence. In addition, comparative genomic hybridization (CGH) was performed to investigate the genetic diversity in a collection of clinical isolates including several capsule serotype 14 and evaluate the diversity in virulence genes of the bacteria.

TCS06 was found to be important for the ability of the pneumococcus to invade the lungs and blood in a murine model of disease but it does not affect the overall outcome of pneumococcal disease. The phenotype associated with deficiency of *rr06* shows that the TCS06 is important for the bacteria when grown at higher temperatures. The transcriptional profile of a pneumococcal mutant lacking the response regulator of TCS06 found by microarray analysis allowed us to determine which transcriptional changes were occurring. The TCS09 was found to be attenuated in TIGR4 strain after intranasal infection in a murine model of infection. Microarray comparison of the transcriptional profiles of the wild-type strains with the *rr09* mutants showed that TCS09 appeared to (directly or indirectly) regulate different genes in D39 and TIGR4 strains. The *dlt* operon was found to be essential for bacterial growth at higher temperatures. Furthermore, this operon was shown to be involved in the acid tolerance response and sensitivity to antimicrobial peptides. However, no attenuation was found in murine model of disease using TIGR4 strain lacking the *dltA* gene. CGH of thirteen pneumococcal isolates showed that reference

strain TIGR4 contains twenty-five regions of diversity not shared in at least one of the strains tested, and three of these regions were identified for the first time in this study. In this study we provide a clear demonstration of genetic differences between strains of the same capsule serotype and ST. Furthermore, we show that clonal strains with the same serotype and ST behave differently in an animal model.

Keywords

Streptococcus pneumoniae; two-component systems; virulence factors; comparative genome hybridization; regions of diversity; microarrays.

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Abbreviations

APS	Ammonium persulphate
ATP	Adenosine triphosphate
BAB	Blood agar base
BHI	Brain heart infusion
BLAST	Basic local alignment search tool
bp	Base pair
BSA	Bovine serum albumin
BμG@S	Bacterial Microarray Group at St George's Hospital
CbpA	Choline biding protein A (same as PspC)
cDNA	Complementary DNA
CFU	Colony forming unit
CGH	Comparative genome hybridization
CSP	Competence stimulating peptide
Ct	Cycle threshold
Cy	Cyanine dye
DFI	Differential fluorescence induction
dH2O	Distilled water
DMSO	Dimethyl sulfoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphate
DTT	Dithiothritol
EDTA	Ethylenediaminetetraacetic acid
ermAM	Erythromycin resistance
g	Gram
h	Hours
H_2O_2	Hydrogen peroxide
HK	Histidine kinase
HtrA	High temperature requirement A
IgA	Immunoglobulin A
Kb	Kilobase pair
KDa	Kilo Dalton
L	Litre
LB	Luria Bertani
LytA	Autolysin A

MIC	Minimal inhibitory concentration
mg	Microgram
min	Minutes
ml	Milliliter
MLST	Multi locus sequence type
mm	Millimeter
mM	Millimoles
mRNA	Messenger ribonucleic acid
MW	Molecular weight
NanA/B/C	Neuraminidade A/B/C
OD600nm	Optical density at 600 nanometer
ORF	Open reading frame
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
pg	Picogram
Ply	Pneumolysin
PsaA	Pneumococcal surface antigen A
PspA	Pneumococcal surface protein A
PspC	Pneumococcal surface protein C (same as CbpA)
PTS	Phosphotransferase systems
qRT-PCR	Quantitative real-time reverse transcription polymerase chain reaction
RD	Region of diversity
RNA	Ribonucleic acid
rpm	Rounds per minute
RR	Response regulator
RT-PCR	Reverse transcription polymerase chain reaction
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SEM	Standard error of the mean
ST	Sequence type
STM	Signature-tagged mutagenesis
TCS	Two-component systems
TIGR	The institute for genomic research
U	Units
UV	Ultraviolet
V	Volts

x g	Centrifugal force
vol/vol	Volume per volume
w/vol	Weight per volume
ΔΔCT	Comparative Cycle value method
µg	Microgram
µl	Microlitre
µM	Micromoles
%	Percentage
°C	Degrees Celsius

Chapter 1

Introduction

1.1 Pneumococcus: general description

Infectious disease is the main cause of mortality in the world even with development of antibacterial agents over the last half century. Bacterial strains have developed, during this time, resistance to our best efforts to destroy them, and they have begun to compromise the treatment of infectious disease particularly in the hospital setting (Barrett *et al.*, 1998).

S. pneumoniae (the pneumococcus) is an important human pathogen, which was isolated for the first time over 124 years ago, in 1881 simultaneously by Pasteur in France and Sternberg in the USA (Pasteur, 1881; Sternberg, 1881). Pneumococcal diseases, especially pneumonia, were the most common causes of death before the "era of antibiotics". Nowadays the pneumococcus remains a major cause of morbidity and mortality in undeveloped and developed parts of the world, and resistance to common antibiotics is widespread (Hava *et al.*, 2003a). The pneumococcus has been the object of many investigations during the past century that resulted in important scientific discoveries (Watson *et al.*, 1993), including the transformation of live strains to a new serotype from DNA of dead pneumococci (Avery *et al.*, 1944), the therapeutic efficacy of penicillin in the 1940s (Abraham *et al.*, 1992), the elucidation of the role of the bacterial capsule in resistance to phagocytosis by cells of the host's immune systems (Issaef, 1893), and the ability of bacterial polysaccharides to induce protective antibodies (Baker, 1990; Felton *et al.*, 1955). However, with all this intensive investigation, many questions about the mechanisms of pathogenesis remain without answer.

S. pneumoniae is a component of the normal flora of the nasopharynges of approximately 50% of all adults, where it coexists with microflora in non-pathogenic state (Gray *et al.*, 1980). The pneumococcus is a Gram positive coccus and a member of the lactic acid bacteria, so named for their primary metabolic by product. The lactic acid bacteria include the *lactococci*, a group important in food and dairy industries, and the genera *Enterococcus* and *Streptococcus*. The bacteria belonging to the genus *Streptococcus* live in association with animal hosts, as either pathogenic or commensal organisms. Human pathogens include the beta-hemolytic species, such as *Streptococcus pyogenes* (Lancefield group A) and *Streptococcus agalactiae* (group B), as well as the human cariogenic species *Streptococcus mutans*. A number of commensal species of streptococci can occasionally cause opportunistic infections (Hoskins *et al.*, 2001).

The organism is an oval spherical coccus of 0.5-1.25 μm in diameter, occurring in pairs or short chains (Figure 1.1) and is usually surrounded by a polysaccharide capsule. Two

phenotypes of the pneumococcus have been identified, opaque and transparent, named after their colony appearance on transparent medium. This variation appears in all strains and there is spontaneous variation between colony phenotypes known as phase variation (Weiser, 1998).

On the basis of difference in capsular structure, pneumococci can be divided into about 40 serogroups and more that 90 serotypes (Henrichsen, 1995). The distribution of serotypes isolated from adults differs substantially from those isolated from children. Geographic and age-related differences in the incidence of certain *S. pneumoniae* serogroups have led to the proposal that, from an epidemiologic standpoint, each should be considered as a separate pathogen (Scott *et al.*, 1996). Two nomenclature systems have been used to classify the serotypes, the American nomenclature, introduced in 1944 by Eddy *et al.*, and reviewed by Henrichsen, 1999 (Henrichsen, 1999), where the serogroups and serotypes are nominated in the order of their discovery, and the Danish nomenclature, that classifies serotypes according to structural and antigenic characteristics, first published by Kaufmann *et al.* in 1940 and reviewed by Lund & Henrichsen 1978, Henrichsen 1999 (Henrichsen, 1999; Lund and Henrichsen, 1978), but has later been extended (Henrichsen, 1995). At present the Danish nomenclature is more widely accepted and is used throughout this study.

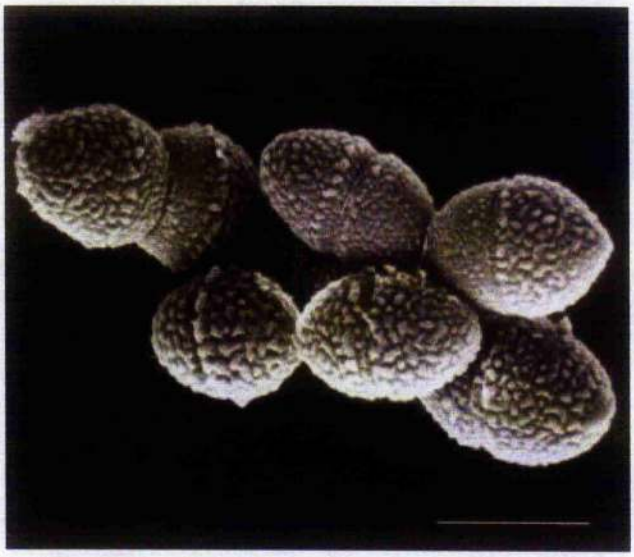


Figure 1.1- Morphology of *S. pneumoniae*.

Electron micrographs of *S. pneumoniae* ME19 (serotype 19F). Bar indicates 1 μm. Adapted from (Kobayashi *et al.*, 2005).

Some serotypes are more associated with invasive disease than others. Penicillin-resistance and multiple resistance are restricted world-wide to a few serogroups. These dominate also in the intermediate strains, but usually a much wider range of serogroups is represented in a smaller proportion (McGee *et al.*, 2001). Serogroups 6, 9, 14, 19 and 23 are most often associated with serious infection in children, these are also often associated with antibiotic resistance and carriage (Butler, 1997; Dagan *et al.*, 1994; McGee *et al.*, 2001; Scott *et al.*, 1996).

In recent years, there has been an increase in the understanding of interactions between the pneumococcus and its host, both in terms of how virulence factors of the organism contribute to pathogenesis and how the host's response to infection can be harmful as well as protective (Tuomanen and Masure, 1997). Despite the growing significance of such infections, little is known about the factors that govern the physiological responses of this organism, or the genetic repertoire that the pneumococcus employs to create disease. In spite of the advent of antibiotics, mortality from pneumococcal disease has remained unacceptably high (Gillespie, 1989).

1.2 Identification of pneumococcus

The pneumococcus is an aerobic or facultative anaerobic organism, and in laboratory cultures requires multiple nutritional factors, carbon dioxide and an ideal pH of 7.2-7.4. Methods of identification of *S. pneumoniae* include: Gram stain morphology; colony morphology and hemolysis on sheep blood agar; pyrrolidonyl arylamidase reactivity; optochin susceptibility; solubility in deoxycholate (bile); carbohydrate utilization; reaction with specific antisera (Quellung reaction); miniaturized manual systems such as the API 20 Strep system, the automated Gram Positive Identification Card and DNA probes (Kellogg *et al.*, 2001).

When grown on solid media the pneumococcus forms flat round colonies with depressed centers and when containing blood partial α-hemolysis of surrounding erythrocytes is detected. Sensitivity to optochin (ethylhydrocupreine, a derivative of quinine) is the most important identification criterion for pneumococcus (Lund and Henrichsen, 1978). A disc contain ethylhydrocupreine is placed on the surface of the plate inoculated with pneumococci and a zone of inhibition appears around the optochin disk after overnight incubation at 37°C in 5% CO2. This test is the most frequently used method to identify pneumococci in clinical laboratories (Kaijalainen *et al.*, 2002). In the bile solubility test, addition of bile salts, such as sodium deoxycholate, to a broth culture of pneumococci

results in prompt dissolution of the bacteria due to the activation of peptidoglycan degrading autolysin enzyme (Hawn and Beebe, 1965; Murray, 1979). The capsular swelling reaction (Quellung reaction) is an old-time method, but is still an useful method to identify pneumococcus isolates rapidly and with high certainty. The procedure is to mix loopfuls of bacteria in suspension, and equal volumes of methylene blue and antiserum to pneumococcal capsule on the surface of a glass slide and examine under a light microscope. If positive, a large capsule will surround the organism and agglutination of bacteria will be form. The capsular appearance is probably due both to capsular swelling and greater refraction (Roberts, 1979).

More recently, new possibilities for the identification of pneumococcus were introduced by the development of novel gene amplification methods for detection of pneumococci, such as identification of the virulence genes for pneumolysin (Kearns *et al.*, 2000; Salo *et al.*, 1999) and surface protein A (Scott *et al.*, 2003) by polymerase chain reaction. However, the pneumolysin gene proved to be unspecific for *S. pneumoniae*. The presence of the pneumolysin in other streptococci, in particular *Streptococcus mitis*, suggests that the applicability of pneumolysin in confirmation of the identification of *S. pneumoniae* is inappropriate (Neeleman *et al.*, 2004). Furthermore, DNA hybridization methods have also been used for the identification of pneumococcus (Denys and Carey, 1992; Fenoll *et al.*, 1990; Pozzi *et al.*, 1989). Multilocus sequence typing (MLST) is now widely used for determining the relatedness of pneumococcal strains, through which allelic profile of the housekeeping genes are directly assessed by nucleotide sequencing (Enright and Spratt, 1998; Jefferies *et al.*, 2003; Sa-Leao *et al.*, 2001).

1.3 Pneumococcal infections

S. pneumoniae is a common bacterial agent in the upper respiratory tract of healthy children and adults (Gray *et al.*, 1980) and most infections do not occur after prolonged carriage but follow the acquisition of new serotypes. Mortality due to pneumococcal infections is high, especially in developing countries and particularly among the young, elderly and immunocompromised (Feldman and Klugman, 1997). World-wide, pneumococcal infections have been estimated to cause 1.2 million pneumonia deaths per year, nearly 40% of all pneumonia deaths in children fewer than 5 years. The pneumococcus also causes 70, 000 deaths from meningitis and a similar number of deaths from sepsis and others infections in young children in developing countries each year (Mulholland, 1997). In industrialized countries, the pneumococcus is the major cause of

pneumonia in old age. The impact of pneumococcal disease is also significant in terms of morbidity (Obaro and Adegbola, 2002).

From the perspective of pathogen fitness, successful infections are marked by colonization, multiplication and ultimately transmission to a new host (Hava *et al.*, 2003b). The process of pathogenesis of the pneumococcal disease involves the translocation of the pneumococcus from the nasopharynx to other places, including the lung, blood and central nervous system. However these processes are poorly understood (Obaro *et al.*, 1996). The symptoms of all pneumococcal diseases are primarily due to the ability of the bacteria to promote an intense inflammatory response, either locally or systemically. In immunocompromised people, the elderly and young children, *S. pneumoniae* bacteria that initially colonize the nasopharynx may spread to distal sites, such as the middle ear, lower respiratory tract, or bloodstream (Hava *et al.*, 2003a) and cause several infections as sinusitis, otitis media (Mitchell, 2000; Tuomanen, 2000) pneumonia, arthritis, pericarditis, peritonitis (Musher, 1992; Novak *et al.*, 1998; Tuomanen *et al.*, 1995), and severe invasive infections such as meningitis and septicemia (Cundell *et al.*, 1995a; Sande and Tauber, 1999).

Pneumococci have three distinguishable colonial morphologies, opaque, semi-transparent and transparent (Weiser *et al.*, 1994). These distinct phenotypes have different abilities to colonize the nasopharynx, while the transparent phenotype has an enhanced capacity to adhere and colonize the nasopharynx, the more phagocytosis-resistant opaque phenotype predominates in blood (Obaro and Adegbola, 2002). Differences in colony morphology correlate with differences in virulence. Transparent variants demonstrate an increased ability to adhere to human lung epithelial cells and are selected for during nasopharyngeal colonization in an infant-rat model but are unable to induce sepsis (Cundell *et al.*, 1995c). This may relate to the enhanced binding of transparent pneumococci to epithelial cells and their glycoconjugate receptors when compared to opaque pneumococci (Cundell *et al.*, 1995b). While, the opaque variant is more virulent in an animal model of systemic infection following intraperitoneal inoculation of adult mice (Kim and Weiser, 1998). The higher virulence of the opaque variant in bacteraemia model may correlate with decreased opsonophagocytic killing of opaque pneumococci (Kim *et al.*, 1999). Phase variation appears to play a role in the adaptation of pneumococcus to changes in the receptors presented on activated host cells (Cundell *et al.*, 1995c). Strains with more adhesive capacity cause localized infections and the less adhesive bacterium causes invasive disease such as bacteraemia and meningitis. Translocation of the organism either by aspiration or penetration of the mucosa results in bacteraemia or sepsis, or both, and seeding in different

body systems may cause recognized disease syndromes such as meningitis and arthritis (Obaro and Adegbola, 2002).

1.3.1 Pneumonia

Pneumococcal pneumonia accounts for about one-sixth to two-thirds of all cases of community-acquired pneumonia (Mufson, 1999). The infection and case fatality rates of pneumococcal pneumonia are highest in elderly patients (Feldman, 2001; Sullivan *et al.*, 1972). Pneumonia causes about three million deaths in young children, nearly all of which are in developing countries. The Pneumococcus is the most important bacterial cause of pneumonia in young children and so is likely to be responsible for a high proportion of these deaths (Greenwood, 1999).

In most cases pneumococcal pneumonia results from the aspiration of pneumococci from the nasopharynx (Boulnois, 1992). The nasopharynx is protected by several specific and nonspecific defence mechanisms (Busse, 1991). Failure of these defences may facilitate access of pneumococci to the bronchi and the lungs (Boulnois, 1992; Musher, 1992). Viral upper respiratory tract infections may play a particularly important role, facilitating pneumococcal invasion by compromising the nonspecific defence mechanisms of the lung and causing epithelial cell damage. *In vitro* and *in vivo* adherence assays demonstrated that the influenza virus neuraminidase potentiates the pneumococcal adherence and the development of pneumonia (Hakansson *et al.*, 1996; McCullers and Bartmess, 2003; Peltola *et al.*, 2005). On the other hand, the pneumococcal neuraminidases, described in more detail in section 1.4.6.2, seems to be important in promoting adhesion of the bacterium to host epithelial cells (Giebink, 1999; Tong *et al.*, 1999). However pneumococcus itself can contribute to its spread to the lungs by causing impairment of the ciliary activity and pneumolysin-dependent disruption of the epithelial tight junctions (Rayner *et al.*, 1995). The characteristic symptoms of pneumococcal pneumonia are cough and sputum production, which reflect the proliferation of bacteria and the inflammatory response in the alveoli, and fever, which results from the release of cytokines and other pyrogenic substances both locally and systemically (Musher, 1992).

Bacteria that colonize the lungs may gain access to the bloodstream. Bloodstream infections are a common complication of bacterial pneumonia; bacteria invade the alveolar spaces and cause enough tissue damage to disrupt the barriers between alveoli and blood vessels. Blood cultures are positive in 15-30% of cases of pneumococcal pneumonia,

depending upon the population under study and, to a lesser extent, the serotype of pneumococcus (Musher, 1992).

1.3.2 Otitis media

The pneumococcus is the leading cause of otitis media wich is extremely common disease during childhood (Eskola and Kilpi, 2000). Nasopharyngeal carriage of middle ear pathogens is considered to be the prerequisite for otitis media (Syrjanen *et al.*, 2005). The carriage of these pathogens is common particularly in young children, which may be one explanation for the high incidence of otitis media among young individuals (Stenfors and Raisanen, 1990). To gain access to the middle ear, pneumococci must progress up the Eustachian tube from the nasopharynx. Once in the ear, bacterial replication leads to an influx of neutrophilis driven by release of IL-6, IL-1 and TNF (Carlsen *et al.*, 1992). It is the most common reason for the prescription of antibiotics to children. Bacterial lysis induced by antibiotics releases large amounts of bacterial cell wall fragments and pneumolysin, characterizing this infection by profound inflammation (Carlsen *et al.*, 1992; Ripley-Petzoldt *et al.*, 1988; Tuomanen *et al.*, 1985a; Tuomanen *et al.*, 1987a). Animal models have shown that cell wall fragments are sufficient to induce the entire inflammatory cascade of otitis media (Bhatt *et al.*, 1991; Bhatt *et al.*, 1993; Bhatt *et al.*, 1995; Carlsen *et al.*, 1992; Ripley-Petzoldt *et al.*, 1988).

Most cases of otitis media are preceded by a viral upper respiratory tract infection. The viral infection predisposes the child to the development of otitis media by causing Eustachian tube dysfunction and enhancing nasopharyngeal carriage of the middle ear pathogens (Bluestone, 1996; Faden *et al.*, 1991; Sanyal *et al.*, 1980). The most evident way to prevent viral or bacterial otitis is immunization. The only respiratory viral vaccine currently available is the influenza virus vaccine. Its use has been associated with a reduction of otitis media episodes (Stenfors and Raisanen, 1990). The ability of the bacterial vaccines to prevent otitis media has not been promising. For example, immunization of infants with a 7-valent pneumococcal conjugate vaccine (Prevnar®, Wyeth®) has been shown to be only modestly beneficial against otitis media (Black *et al.*, 2000; Eskola *et al.*, 2001; Kilpi *et al.*, 2003). An increase in otitis media caused by non-vaccine serotypes has been detected.

1.3.3 Meningitis

Meningitis is an inflammation of the meninges, the membranes that cover the brain and spinal cord protecting them from harmful substances in blood. The main causes of meningitis are *Neisseria meningitidis*, *S. pneumoniae* and *Haemophilus influenzae*. Incidence of disease due to *H. influenzae* has been reduced since the introduction of a conjugate vaccine. Bacterial meningitis remains a major problem with 10% of survivors suffering from neurological sequelae such as neurological deficits, neuropsychological impairment and most commonly, hearing loss (Arditi *et al.*, 1998; Grimwood *et al.*, 1995; van de Beek *et al.*, 2002; Woolley *et al.*, 1999). Meningitis is the most severe form of pneumococcal disease. Meningitis caused by *S. pneumoniae* is rapidly fatal, if it is not treated early and effectively, and it is associated with a higher mortality than meningitis caused by other common meningeal pathogens (Baraff *et al.*, 1993; Goetghebuer *et al.*, 2000). The morbidity and mortality of pneumococcal meningitis in children of industrialized countries are approximately 30% and 10%, respectively (Arditi *et al.*, 1998; Kornelisse *et al.*, 1995). In developing countries the statistics are dramatically higher: about 50% of children with pneumococcal meningitis die while in hospital (Goetghebuer *et al.*, 2000; Muhe and Klugman, 1999). The pneumococcal serogroups most often isolated from cerebrospinal fluid (CSF) include groups 6, 10 and 23 at all ages (Hausdorff *et al.*, 2000).

Pneumococcal meningitis usually occurs in relatively few individuals as a result of seeding of the meninges during high-grade bacteraemia or a head trauma (Musher, 1992). In order to cause meningitis, bacteria need to cross the blood-brain barrier. It has been suggested, in an *in vitro* blood-brain barrier model, that pneumococci cause rounding and detachment of brain microvascular endothelial cells and decrease the transendothelial electrical resistance, indicating an opening of tight junctions (Zysk *et al.*, 2001). Intra-cerebral inoculation of pneumococci also causes an opening of tight junctions between brain endothelial cells (Quagliarello *et al.*, 1986).

The actual mechanisms and route used by pneumococci to migrate to the meninges are not clear. It is widely believed that pneumococcal meningitis is acquired via colonization of the nasopharynx, followed by bacteraemia and invasion of the central nervous system (CNS) (Ring *et al.*, 1998). However the spread to the CNS can occur in the absence of blood infection (Marra *et al.*, 2002a). It has been suggested that local inflammation caused by pneumococci breaches the blood-brain barrier and admits entry of bacteria and phagocytes to this fragile area. The inflammatory reaction, rather than the pathogen itself,

is largely responsible for the damage that results from bacterial meningitis (Pfister and Scheld, 1997). Several animal models have been developed to study the course and treatment of meningitis. The two most widely used are the infant rat model and the adult rabbit model (Moxon *et al.*, 1974; Tauber and Zwahlen, 1994). Neither replicates the human course of the disease, but they have been very valuable research tools nonetheless. In the infant rat model, infection is initiated either via intranasal instillation or intraperitoneal injection, resulting in bacteraemia and entry of the bacteria into the CNS (Rodriguez *et al.*, 1991; Tauber and Zwahlen, 1994). Some reports indicated that the blood-brain barrier damage is caused by pneumolysin produced by *S. pneumoniae* (Braun *et al.*, 2002; Hirst *et al.*, 2004; Zysk *et al.*, 2001). The adult rabbit model relies on direct injection of bacteria into the CSF (Tauber and Zwahlen, 1994). The ease with which multiple CSF samples may be drawn is a major benefit of this model; however, its main drawback is the nonphysiological route of infection. Other models involve intracerebral injection of rats for examining the efficacy of therapies to clear the infection (Strake *et al.*, 1996).

1.3.4 Septicaemia

Septicaemia is a systemic disease in which microorganisms multiply in the blood or are continuously seeded into the bloodstream. Pneumococcal septicemia occurs frequently as a complication of pneumococcal pneumonia. Septicemia may occur also as a primary bacteraemia (bacteria present in bloodstream) in the absence of a clinically evident focus of infection (Balakrishnan *et al.*, 2000).

Epithelial damage caused by previous viral upper respiratory tract infections can increase the opportunity of pneumococci to reach the bloodstream. Previous reports suggest that *S. pneumoniae* is responsible for 1-11% of neonatal sepsis cases (Dawson *et al.*, 1999; Gladstone *et al.*, 1990; Greenberg *et al.*, 1997; Kaplan *et al.*, 1993) and the incidence of pneumococcal bacteraemia is high in infants up to two years of age (Jacobs *et al.*, 1979; Kaplan *et al.*, 1998). The incidence is low among teenagers and young adults, increases in patients of middle age, and reaches a high level among population over 65 years (Breiman *et al.*, 1990). Pneumococcal septicaemia in children seems to be associated with low risk of death, while increasing age, an extra pulmonary site of infection, the presence of chronic disease, or infection with certain serotypes (particularly type 3) contribute to a higher risk of death (Mufson *et al.*, 1974).

1.4 Pneumococcus virulence factors

The production of disease due to bacterial infection requires temporal and coordinated expression of a series of genes that allow the prospective pathogen to adapt to the hostile environment in the host. The expression of these genes contributes to the virulence of these pathogens, and such genes encode products frequently termed "virulence factors." Such products could include enzymes required to metabolize complex proteins and glycoproteins found in connective tissues or blood, bacterial toxins, cell surface proteins that mediate bacterial attachment, cell surface carbohydrates and proteins that protect a bacterium, and hydrolytic enzymes that may contribute to the pathogenicity of the bacterium (Barrett and Hoch, 1998). From the standpoint of the bacterium, virulence factors contribute to the ability of the microorganism to survive and grow at the site of infection and to its pathogenicity, and thus, in an ecological sense, virulence factors contribute to how well a microorganism propagates in a mammalian host (Barrett and Hoch, 1998).

S. pneumoniae is a member of the commensal microflora present on the mucosal surfaces in a non-pathogenic state, but can also cause a range of serious infections. To cause disease, the pneumococcus must be able to adhere to a tissue surface, and compete with the normal flora present on that surface. Normally the bacteria are found attached to the mucous membranes of the nasopharynx, but cause disease when aspirated into the lower respiratory tract (Mitchell, 2003).

The pneumococci interact with the host and its tissues through the polysaccharide capsule and a variety of usually surface-exposed protein molecules. These interactions are essential for the full pathogenicity of these bacteria and are likely involved in the disease causing processes (Jedrzejas *et al.*, 2002). In the past, the polysaccharide capsule was considered the primary virulence factor of *S. pneumoniae* because nonencapsulated bacteria are almost completely harmless compared with the isogenic encapsulated strain. Recent studies, however, have suggested that certain pneumococcal proteins significantly contribute to pathogenesis of the bacteria. In many cases, these proteins are involved in direct interactions with host tissues or in concealing the bacterial surface from the host defense mechanisms (Jedrzejas, 2001). The main pneumococcal virulence factors are illustrated in Figure 1.2 and listed in Table 1.1.

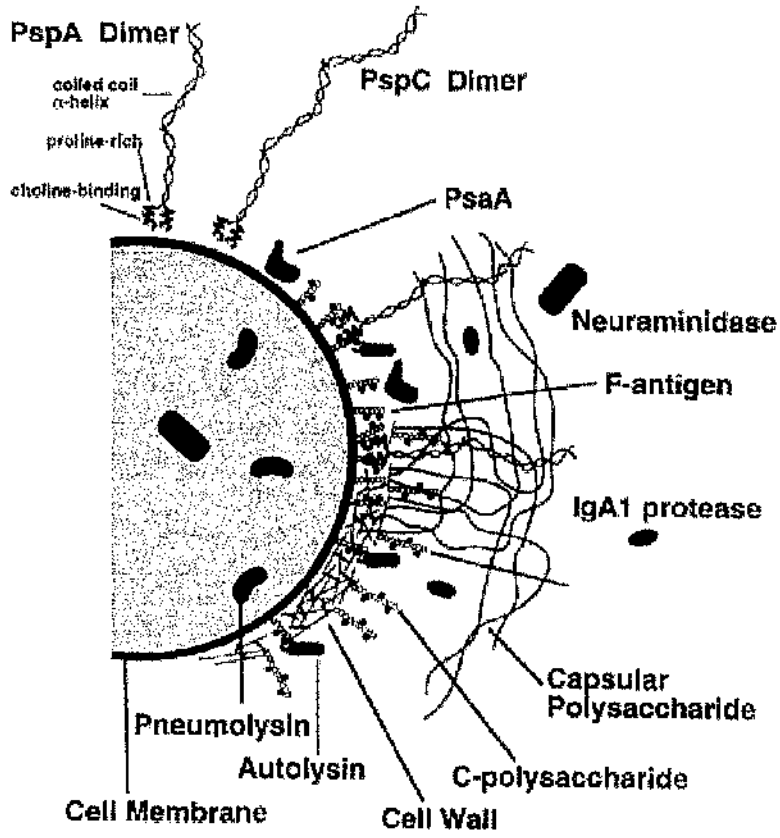


Figure 1.2- Schematic structure of the surface of S. *pneumoniae*.

Hypothetical representation of the pneumococcal surface showing several noncapsular antigens. C-polysaccharide (teichoic acid) attached to the cell wall is thought to be similar in structure to F-antigen (lipoteichoic acid), except that the latter contains lipids allowing it to insert in the cell membrane. Pneumolysin is depicted in the cytoplasm of the cell shown here, since its release is dependent on the autolytic activity of autolysin. Neuraminidase has been depicted both in the cytoplasm and beyond the capsule, since it is thought to be secreted by pneumococci. For PspA, an effort has been made to draw its extension from the surface to scale with respect to the thickness of the cell wall and capsule. It has been hypothesized that the lysines of the PspA α-helix interact with the capsular polysaccharides to stabillze the coverage of the surface by the capsule. PspC, as PspA, is attached to the surface through the choline of both lipoteichoic and teichoic acids. Since the location of PsaA with respect to other cell surface structures is unknown, its depiction here is completely hypothetical. Adapted from (Briles *et al.*, 1998).

Table 1.1- Summary of the main virulence factors of *S. pneumoniae*.

Virulence factor	Role in virulence	Reference
Capsule	Lacks intrinsic ability to activate the alternate complement pathway.	(Fine, 1975; Giebink *et al.*, 1977)
	Resistance to phagocytosis.	(Braconier and Odeberg, 1982; Matthay *et al.*, 1981)
	No or low immunogenicity of some serotypes.	(Kamboj *et al.*, 2003; van Dam *et al.*, 1990)
	Colonization	(Magee and Yother, 2001)
Cell wall and cell wall polysaccharide	Inflammatory effects:	
	Activation of the alternative complement pathway, resulting in anaphylatoxin production.	(Winkelstein and Tomasz, 1977, 1978)
	Enhancement of vascular permeability, mast cell degranulation, PMN activation.	(Johnston, 1991)
	IL-1 production increased, cytopathic for endothelium.	(Geelen *et al.*, 1993; Riesenfeld-Orn *et al.*, 1989)
	Mediator of attachment to endothelial cells.	(Geelen *et al.*, 1993)
Pneumolysin	Cytotoxic effect	(Feldman *et al.*, 1990; Rubins *et al.*, 1993)
	Cytolytic activity	(Cockeran *et al.*, 2002a)
	Overexpression of ICAM-1 in monocytes.	(Thornton and McDaniel, 2005)
	Increased concentrations of (Il.)-6.	(Rijneveld *et al.*, 2002)
	Nitric oxide production from macrophages.	(Braun *et al.*, 1999)
Pneumococcal surface protein A	Inhibits complement deposition.	(Ren *et al.*, 2003a; Ren *et al.*, 2004; Tu *et al.*, 1999)
	Binding of lactoferrin.	(Shaper *et al.*, 2004)
Pneumococcal surface protein C	Binds the secretory component of SIgA.	(Dave *et al.*, 2004; Hammerschmidt *et al.*, 1997; Zhang *et al.*, 2000)
	Binds human factor H.	(Dave *et al.*, 2001)
	Inhibits complement activation.	(Duthy *et al.*, 2002; Smith and Hostetter, 2000)
Autolysin	Generating inflammatory cell wall degradation products.	(Mitchell *et al.*, 1997)
	Releasing the pneumococcal virulence factors.	
Pneumococcal surface antigen A	Putative adhesin.	(Berry and Paton, 1996)
	Resistance to oxidative stress	(Tseng *et al.*, 2002)
Hyaluronidase	Degrades hyaluronan and certain chondroitin sulfates.	(Boulnois, 1992; Menzel and Farr, 1998)
Neuraminidase	Cleaves terminal sialic acid from cell surface glycans.	(Shakhnovich *et al.*, 2002; Tong *et al.*, 2001)
IgA1 Protease	Facilitate adherence by modification of the IgA1 antibody.	(Weiser *et al.*, 2003)

1.4.1 Capsule

For many years the capsule has been recognized as the major virulence factor of *S. pneumoniae* based on its capacity to confer resistance to complement-mediated opsonophagocytosis (Austrian, 1981; Bruyn *et al.*, 1992; Watson *et al.*, 1995; Wood and Smith, 1949). Experimental proof for this was provided by the difference in 50% lethal dose between encapsulated and unencapsulated strains. The encapsulated pneumococcus strains have been found to be at least 10^5 times more virulent in mice than the strains lacking the capsule (Avery and Dubos, 1931; Watson *et al.*, 1995). The capsule consists of high-molecular weight polymers made up of units of repeating oligosaccharides, which can contain 2 to 8 monosaccharides (AlonsoDeVelasco *et al.*, 1995). More than 90 polysaccharide capsular types have been described on the surface of the pneumococcus (Swiatlo and Ware, 2003), synthesis of the serotype-specific capsular polysaccharide is encoded by distinct clusters of up to 20 tightly linked genes transcribed as single operons (Paton and Morona, 2000).

The major mechanism by which the capsule promotes pneumococcal virulence is the protection of the bacteria against phagocytosis by resident pulmonary macrophages or recruited polymorphonuclear neutrophils (Tuomanen *et al.*, 1985b; Tuomanen *et al.*, 1987b). The capsule forms a physical barrier that separates bound, fixed complement components from complement receptors on host phagocytes (Brown *et al.*, 1982; Winkelstein, 1984). It may also function in the electrostatic repulsion of phagocytes from bacteria (Kasper, 1986). The association between capsular type and disease is well documented. Pneumococcal virulence and invasiveness depend on both the composition and quantity of the capsule produced. The thickness of the capsule may influence the degree of exposure of other important pneumococcal surface structures, such as the adhesins that are required during this initial colonization phase (Morona *et al.*, 2004).

Antibodies to the capsular components are highly protective (Austrian *et al.*, 1976; MacLeod *et al.*, 1945), and the global impact of pneumococcal disease has led to the development of polyvalent polysaccharide and polysaccharide -protein conjugate vaccine formulations (Ogunniyi *et al.*, 2002).

1.4.2 Cell wall and cell wall polysaccharide

The pneumococcal wall, located just under the capsular polysaccharide, consists of two major components, a peptidoglycan polymer, common to all bacterial cells, and teichoic

acid, a polysaccharide covalently linked to peptidoglycan. While teichoic acid is found in many Gram positive cell walls, pneumococcal teichoic acid is unique in that it contains phosphorylcholine (Mosser and Tomasz, 1970; Sorensen and Henrichsen, 1987; Tomasz, 1967). This phosphorus-containing teichoic acid is designated as a cell wall polysaccharide (C-polysaccharide) and functions as a recognition site for activation of the alternative complement pathway (Hummell *et al.*, 1981; Winkelstein and Tomasz, 1977, 1978) as well as for the of C-reactive proteins (Mold *et al.*, 1981), certain myeloma proteins (Szu *et al.*, 1983; Winkelstein and Tomasz, 1978), and pneumococcal autolysin (Giudicelli and Tomasz, 1984; Mosser and Tomasz, 1970). Cell wall polysaccharide is covalently linked to the peptidoglycan layer of the cell wall (Tomasz, 1981) and is uniformly distributed in both sides (Skov Sorensen *et al.*, 1988).

Since C-polysaccharide is common to all pneumococci (Sorensen and Henrichsen, 1987), it has been suggested that antibodies against this antigen might confer species-specific protection against pneumococcal infections (Gray *et al.*, 1983; Szu *et al.*, 1986). In addition to the cell-wall linked C-polysaccharide, pneumococci possess another common polysaccharide antigen called lipoteichoic acid (Briles and Tomasz, 1973). This antigen inhibits the function of LytA, the major enzyme responsible for the degradation of the peptidoglycan backbone of pneumococcus, which leads to cell lysis. During the stationary phase of growth pneumococcus cells release the lipoteichoic acid enabling the unrestrained autolytic activity of LytA and the destruction of the cell wall (Horne and Tomasz, 1985). The activity of the autolysin releases the components of the pneumococcus cell wall as fragments. The cell wall fragments induce release of proinflammatory cytokines from mononuclear macrophages (TNF-α, IL-1 and IL-6) (Tuomanen *et al.*, 1987b). Typical pneumococcal diseases such as otitis media, meningitis, and pneumonia can be mimicked in animals that have received injection of purified cell wall or its degradation products (Carlsen *et al.*, 1992; Tuomanen *et al.*, 1985a; Tuomanen *et al.*, 1987b). Also, purified cell wall is a powerful stimulus for the production of IL-1 by human monocytes (Riesenfeld-Orn *et al.*, 1989). Cell wall also was shown to be involved in the attachment of unencapsulated pneumococcus to human endothelial cells and to have cytopathic effects on these cells (Geelen *et al.*, 1993). Anti-cell wall polysaccharide or anti- phosphorylcholine antibodies have been demonstrated to protect animals against pneumococcal challenge (Briles *et al.*, 1981; Briles *et al.*, 1989; Briles *et al.*, 1992), however the protective activity of anti- phosphorylcholine antibodies is substantially weaker than that of anti-capsular polysaccharides antibodies (Briles *et al.*, 1981; Briles *et al.*, 1989).

1.4.3 Pneumolysin

Pneumolysin is an intracellular protein that belongs to the family of cholesterol-dependent cytolysin (CDC) (Boulnois, 1992). It is a 53 kDa protein produced by all clinical isolates of the pathogen (Paton *et al.*, 1983; Paton *et al.*, 1986; Paton *et al.*, 1993). It is a cytoplasmic enzyme and is released upon lysis of the pneumococcus under the action of the cell-bound autolysin (LytA) and has been shown to function in pathogenesis in several animal models of disease (Alcantara *et al.*, 1999; Benton *et al.*, 1995; Berry *et al.*, 1989b; Canvin *et al.*, 1995; Comis *et al.*, 1993). However, few pneumococcal strains express extracellular pneumolysin prior to stationary phase (Benton *et al.*, 1997). This finding raised the possibility that pneumolysin might be able to be released by a nonlytic mechanism. A mutant lacking autolysin in strain WU2 (type 3) showed the same pattern of pneumolysin as the wild-type strain demonstrating that pneumolysin is not dependent upon autolytic activity but it might occur via active secretion of the pneumolysin protein by an unidentified mechanism (Balachandran *et al.*, 2001).

Pneumolysin belongs to the family of thiol-activated cytolysins, which are produced by several Gram-positive bacteria and inactivated by cholesterol (Johnson *et al.*, 1980; Paton, 1996). The thiol-activated toxins presumably act by binding to the target cell membrane via interaction with cholesterol, which results in the insertion of the monomeric toxin into the lipid bilayer. Subsequently, the monomers oligomerize and form transmembrane pores, which cause cell lysis. (Boulnois *et al.*, 1991b). In addition to its cytolytic properties, pneumolysin has several effects at lower concentrations: it has a cytotoxic effect on ciliated bronchial epithelial cells, slows ciliary beating in organ culture, disrupts tight junctions and the integrity of the bronchial epithelial monolayer (Rayner *et al.*, 1995; Steinfort *et al.*, 1989), has a role in evasion of the immune system as decreases the bactericidal activity and migration of neutrophils (Paton and Ferrante, 1983), inhibits lymphocyte proliferation and antibodies synthesis (Ferrante *et al.*, 1984) and interferes with the complement pathway (Paton *et al.*, 1984). This toxin also stimulates the production of inflammatory mediators, such as TNF-α, IL-1β (Houldsworth *et al.*, 1994), nitric oxide (Braun *et al.*, 1999), IL-8 (Cockeran *et al.*, 2002b), and prostaglandins and leukotrienes (Cockeran *et al.*, 2001), activates phospholipases in endothelial cells (Rubins *et al.*, 1994) and is toxic to pulmonary endothelial and epithelial cells (Rubins *et al.*, 1992; Rubins *et al.*, 1993).

Although native pneumolysin has strong toxic effects, several derivatives which are nontoxic but retain the immunogenicity and protective activity of the native protein have

been engineered (Paton *et al.*, 1991). These constructs therefore seem to meet many criteria for inclusion in a pneumococcal conjugate vaccine (Alexander *et al.*, 1994; Kirkham *et al.*, 2006; Paton *et al.*, 1993).

1.4.4 Surface proteins

1.4.4.1 Choline-binding proteins

Pneumococci display an unusual surface molecule, phosphocholine, on the cell wall teichoic acid and the membrane bound lipoteichoic acid (Tomasz *et al.*, 1975). Studies have shown that choline-biding proteins (CBPs) noncovalently bind to the choline of both lipoteichoic and teichoic acids via its C-terminal end, consisting of the repeat region, also called the choline binding region (Cundell *et al.*, 1995b; McDaniel *et al.*, 1991; Yother and Briles, 1992; Yother and White, 1994). The pneumococcal surface protein (PspA) is a protein present on the surface of all clinically important pneumococcal serotypes and is required for full virulence (Crain *et al.*, 1990; McDaniel *et al.*, 1987). Other CBPs are present on the surface of this pathogen such as the autolysin (LytA) (Garcia *et al.*, 1986b) and pneumococcal surface protein C (PspC) (Brooks-Walter *et al.*, 1999). LytA is an amidase functioning in the separation of daughter cells during cell division (Ronda *et al.*, 1987) and is required for cell lysis (Tomasz *et al.*, 1971), whereas PspC appears to be the first known protein adhesin on the pneumococcal surface (Rosenow *et al.*, 1997). The pneumococcal family of virulence factors, CBPs, will be described below in more detail.

1.4.4.1.1 Pneumococcal surface protein A

PspA is a surface protein with variable molecular size ranging from 67 to 99 kDa in different pneumococcal strains (Waltman *et al.*, 1990). It is expressed by all pneumococci and is important for virulence (Crain *et al.*, 1990; McDaniel *et al.*, 1987). Bacteria unable to produce PspA are less virulent in models of systemic disease because they are more easily cleared from the bloodstream (McDaniel *et al.*, 1987). PspA is immunogenic and elicits protective antibody response in mice (McDaniel *et al.*, 1994).

Based on sequence analyses, the protein has four distinct domains: an amino-terminal, highly charged α-helical coiled-coil structure (288 amino acids in strain Rx1); a proline-rich domain (83 amino acids); a stretch of 10 highly conserved repeats of 20 amino acids comprising the choline-binding component and a slightly hydrophobic tail of 17 amino acids at the carboxy-terminus (Yother and Briles, 1992). The choline-binding component

of PspA is homologous with other pneumococcal CBPs, and is responsible for the attachment of PspA to the pneumococcal surface (Yother *et al.*, 1992). This orientation results in the amino-terminal α-helical domain of the molecule being exposed on the surface and thus available to interact with the human host (Gray, 1996). PspA is a serologically highly variable molecule and on the basis on the relatedness of the nucleotide and amino acid sequences, the different PspA molecules are grouped into two major allelic types (families), and further subdivided intro clades (Hollingshead *et al.*, 2000).

The importance of PspA in virulence has been well established in murine infection models with pneumococcal mutants that no longer express cell surface PspA (Briles *et al.*, 1988; McDaniel *et al.*, 1987). Although the mechanism of action of PspA is not fully understood it appears to be in protection against the host complement system (Cundell *et al.*, 1995b; Yother and White, 1994). The interference of PspA with complement activation it suggests facilitates pneumococcal survival and host invasion. PspA blocks the C3 convertase of the alternative complement pathway and/or accelerates its dissociation. This leads to an inhibition of the downstream events of the complement pathway, which would result in opsonization of pneumococcus and chemotaxis (Tu *et al.*, 1999). In addition PspA functions as a specific receptor for lactoferrin, the amino-terminal part being responsible for lactoferrin binding (Hammerschmidt *et al.*, 1999). Lactoferrin is an iron-sequestering glycoprotein, which predominates in mucosal secretions. It has been suggested that by binding lactoferrin pneumococci are able to interfere with the host's immune functions (Hakansson *et al.*, 2001).

PspA is considered as a pneumococcal protein vaccine candidate. This protein has been shown to bring protective immunity against pneumococcal infection in mice (Briles *et al.*, 1996; McDaniel *et al.*, 1991; Talkington *et al.*, 1991; Tart *et al.*, 1996). Both oral and intranasal immunization of mice with PspA elicited protective immunity against pneumococcal carriage and systemic infection (Wu *et al.*, 1997; Yamamoto *et al.*, 1997). Intranasal immunization of mice with a mixture of PsaA and PspA proved to be highly protective against pneumococcal carriage (Briles *et al.*, 2000), the mixture of these two proteins elicited better protection than either alone (Simell *et al.*, 2001).

1.4.4.1.2 Pneumococcal surface protein C

PspC is also known as CbpA (Rosenow *et al.*, 1997), SpsA (Hammerschmidt *et al.*, 1997), PbcA (Cheng *et al.*, 2000) and Hic (Janulczyk *et al.*, 2000) based on its ability to bind choline, secretory component of immunoglobulin A (IgA), complement component C3 and

factor H, respectively. This pneumococcal surface protein belongs to the family of pneumococcal choline-binding proteins that bind to the phosphocholine (Rosenow *et al.*, 1997) present in the teichoic acid and the lipoteichoic acid of the cell membrane and the cell wall (Tomasz, 1967). This protein specifically bind to secretory component of human secretory immunoglobulin A (Hammerschmidt *et al.*, 1997), human factor H (Dave *et al.*, 2001), and complement component C3 (Janulczyk *et al.*, 2000; Smith and Hostetter, 2000). PspC has been shown to interact with the human polymeric immunoglobulin receptor (pIgR), which is expressed by cells in the respiratory epithelium, thereby facilitating invasion of the mucosa (Zhang *et al.*, 2000).

PspC is a 110-kDa protein with eight choline-biding repeats similar to PspA. The consensus sequences of these domains of PspC and PspA are from 90 to 95% identical (Brooks-Walter *et al.*, 1999; McDaniel *et al.*, 1998). It consists of an N-terminal α-helical domain followed by a proline-rich domain. The C-terminal half of PspC contains the choline-binding domain. A variant of PspC, Hic, contains the LPXTG motif that anchors the protein covalently to the bacterial surface (Janulczyk *et al.*, 2000). Analysis of the deduced amino acid sequence of different PspC variants has revealed 11 major groups of PspC proteins (PspC1-11). Single proteins within a group display only minor variations in the amino acid sequence (Iannelli *et al.*, 2002).

PspC plays an important role in pneumococcal pathogenesis by functioning as an adhesin (Rosenow *et al.*, 1997) and was the first known protein adhesin identified on the pneumococcal surface (Weiser *et al.*, 1996). The importance of PspC in adherence and colonization of *S. pneumoniae* to epithelial cells of nasal passages and lungs in mice is well established (Balachandran *et al.*, 2002; Rosenow *et al.*, 1997). In the absence of PspC, pneumococci fail to enter and cross an *in vitro* blood-brain barrier, indicating a critical role for this protein in invasion (Ring *et al.*, 1998). Pneumococcal strains with mutations in PspC are also unable to colonize the mucosal surface or infect the lungs. The adherence properties of PspC may be due to its ability to bind glycoconjugates such as sialic acid and lactotetraoses, as well as C3 on activated epithelial cells of the host (Rosenow *et al.*, 1997; Smith and Hostetter, 2000). Zhang and co-workers by PspC-affinity chromatography had shown that the pneumococcus PspC biddings to human pIgR, and consequent expression of pIgR in human nasopharyngeal cells facilitates the pneumococcal adherence and invasion. Furthermore, PspC deletion or antibodies against human pIgR or PspC abolished the adherence and invasion. These results suggest that the PspC-pIgR interaction mediates the translocation of the pneumococcus across the mucosal barrier by transcytosis through epithelial cells (Zhang *et al.*, 2000).

1.4.4.1.3 Autolysin

LytA is a 36-kDa N-acetylmuramoyl-L-alanine amidase (Holtje and Tomasz, 1976; Mosser and Tomasz, 1970) which is located in the cell envelope (Diaz *et al.*, 1989). LytA is the major enzyme responsible for the pneumococcal cell wall turnover. It is a cell wall-associated protein that belongs to the family of pneumococcal CBPs. LytA is responsible for the degradation of the peptidoglycan backbone of the pneumococcus, which leads to cell lysis (Garcia-Bustos and Tomasz, 1987; Giudicelli and Tomasz, 1984). Therefore, the activity of LytA allows the release of intracellular toxins, such as the pneumolysin, and highly inflammatory cell wall fragments. The enzyme is activated under conditions in which biosynthesis stops, such as nutrient starvation, the end of logarithmic phase of growth or penicillin treatment (Mitchell, 2000; Tuomanen and Tomasz, 1990).

Autolysin-deficient mutants of *S. pneumoniae*, resulting from chemical mutagenesis, have been described (Garcia *et al.*, 1986a; Lopez *et al.*, 1986; Sanchez-Puelles *et al.*, 1986). All of these failed to undergo autolysis during the stationary phase of growth and were resistant to the lytic consequences of treatment with penicillin or deoxycholate. The mutants grew normally, except for the tendency to form short chains of cells rather than discrete diplococci suggesting that autolysin might play a role in daughter-cell separation. The normal phenotype is reestablished when the mutants are transformed with a recombinant plasmid carrying the wild-type gene (Ronda *et al.*, 1987). Previous studies using autolysin-negative mutants showed a marked reduction in virulence for mice; intranasal and intraperitoneal median lethal dose were 10^2-10^5 fold greater, respectively, than those of wild-type.These mutants did not spontaneously autolyse and did not release pneumolysin into the culture medium, even after the addition of sodium deoxycholate, suggesting a role for autolysin and possibly for the inflammation that follows autolysis in pneumococcal virulence and pathogenesis (Berry *et al.*, 1989a; Berry *et al.*, 1992; Canvin *et al.*, 1995). However, recent studies have shown that the extracellular release of pneumolysin was not dependent on autolysin action. The WU2 strain that has an autolysin defective mutation showed the same pattern of pneumolysin release as the wild-type strain. Furthermore, although the autolysin-deficient strain in BALB/cByJ mice showed similar effects on virulence to the pneumolysin mutant, using highly susceptible CBA/N mice, the loss of autolysin had no effect in infection in opposition of pneumolysin mutants. This suggest that autolysin and pneumolysin have different effects on virulence and that autolysin is not a required for the effect on virulence by pneumolysin (Balachandran *et al.*, 2001)

Immunization of mice with LytA confers limited protection against intranasal challenge with wild-type pneumococci (Berry *et al.*, 1989a). Subsequent studies demonstrated that immunity to autolysin and pneumolysin provide similar degrees of protection against intraperitoneal challenge (Berry *et al.*, 1992; Canvin *et al.*, 1995). Immunity to both factors did not result in greater protection than immunity to either one alone (Lock *et al.*, 1992). Furthermore, autolysin was shown to be triggered by human lysozyme, a defence factor released upon infection and inflammation (Bruyn *et al.*, 1992).

1.4.5 Cytoplasmic lipid bilayer attached macromolecules:

1.4.5.1 Pneumococcal surface antigen A

Pneumococcal surface adhesin A (PsaA) is a 37-kDa surface lipoprotein essential for pneumococcal virulence (Tharpe and Russell, 1996). Although PsaA was assigned as being a pneumococcal adhesin (Berry and Paton, 1996) due to the sequence analyses of the *psaA* gene revealing a strong degree of homology with the streptococcal putative lipoprotein adhesins AdcA from *S. pneumoniae* (Dintilhac and Claverys, 1997), ScaA from *Streptococcus gordonii* (Kolenbrander *et al.*, 1998), SsaB from *Streptococcus sanguis* (Ganeshkumar *et al.*, 1991) and FimA from *Streptococcus parasanguis* (Burnette-Curley *et al.*, 1995), and by the fact the PsaA mutants of *S. pneumoniae* exhibited a reduced ability to adhere to A549 pneumocytes (Berry and Paton, 1996), studies on the genomic sequence comparison (Berry and Paton, 1996) and structure of PsaA (Lawrence *et al.*, 1998) revealed that it is a component of an ATP-binding cassette-type (ABC-type) permease membrane transport system and by the fact PsaA is not exposed on the surface of the bacterial cell (Johnston *et al.*, 2004) demonstrate that the function as an adhesin is not consistent. However, some data related with inhibition of pneumococcal adherence to human nasopharyngeal epithelial cells by anti-PsaA Antibodies, continue supporting the argument that PsaA is an adhesin (Romero-Steiner *et al.*, 2003). This permease membrane transport system is composed of the products of three genes, *psaB* (ATP-binding protein), *psaC* (integral membrane protein), and *psaA* (solute- binding lipoprotein), which are organized in an operon with a gene encoding *PsaD*, a thiol peroxidase (Novak *et al.*, 1998).

It is supposed that PsaA is responsible for the uptake of Mn^{2+} and probably Zn^{2+} into the bacterium (Dintilhac *et al.*, 1997). Mn^{2+} is known to be a cofactor of streptococcal pyruvate kinase and lactate dehydrogenase. These enzymes are critical for glycolysis and homolactic fermentation, respectively (Crow and Pritchard, 1977). In *S. pneumoniae*, Mn^{2+}

has been shown to be required for the activity of CpsB, a tyrosine phosphatase involved in the regulation of capsule production (Bender and Yother, 2001; Morona *et al.*, 2002). Pneumococcal *pspA* mutants were shown to be more sensitive to the products of superoxide dismutase activity, while Mn^{2+} supplementation provided more protection against killing by H_2O_2 (Tseng *et al.*, 2002). In some streptococcal species, lectin-mediated adherence requires Mn^{2+} (Drake *et al.*, 1988; Lu *et al.*, 1992).

PsaA is considered a pneumococcal protein vaccine candidate. Initially, immunization of mice with PsaA was observed to increase protection against invasive pneumococcal disease (De *et al.*, 1999; Ogunniyi *et al.*, 2000; Talkington *et al.*, 1996). Protection against carriage was more effective when combinations of PsaA with PspA was administered to a greater degree than immunization with PsaA alone (Briles *et al.*, 2000). Despite the protective effects against carriage, immunization with PsaA consistently failed to protect against systemic infection (Ogunniyi *et al.*, 2000). Mutations in the psa operon result in an almost complete attenuation of virulence for all tested models of animal infection, including respiratory tract, systemic, intraperitoneal chamber, and otitis media models (Berry *et al.*, 1996; Marra *et al.*, 2002b). These mutations cause a requirement for added manganese for growth (Dintilhac *et al.*, 1997). The pneumococcus produces a significant amount of H_2O_2 during growth, mainly due to the presence of pyruvate oxidase (Spellerberg *et al.*, 1996). The production of H_2O_2 is believed to be important in the virulence of pneumococci and as a mechanism to eliminate competition by other pathogens (Duane *et al.*, 1993; Pericone *et al.*, 2000). The virulence attenuation may be a result of an inability to regulate oxidative stress and intracellular redox homeostasis on the part of *psaA* mutant strains (Tseng *et al.*, 2002).

1.4.6 LPXTG-anchored proteins

A group of pneumococcal surface proteins are covalently anchored to the cell wall through a carboxy-terminal motif-LPXTG. These proteins include hyaluronidase, neuraminidase and IgA1 protease.

1.4.6.1 Hyaluronidase

Hyaluronate lyase (Hyaluronidase) (Hynes *et al.*, 2000) is another major surface protein of *S. pneumoniae* with potentially antigenically variable properties that might be essential for full pneumococcal virulence (Boulnois *et al.*, 1991a). Hyaluronidase primarily degrades hyaluronan (Laurent and Fraser, 1992), the predominant polysaccharide component of

animal and human connective tissues and the nervous system, into unsaturated disaccharide units as the end products (Li *et al.*, 2000), and certain chondroitin sulfates (Pritchard *et al.*, 1994). This activity facilitates the host tissue invasion by the pneumococci and causes an increased level of tissue permeability, which plays an essential role in wound infections, pneumonia and in other sepsis (Ponnuraj and Jedrzejas, 2000). Because of this reason hyaluronate lyase is also know as a "spreading factor" (Akhtar and Bhakuni, 2003). Strains with higher hyaluronidase activity could breach the blood-brain barrier and disseminate more effectively (Kostyukova *et al.*, 1995).

In fresh *S. pneumoniae* cultures, most of the hyaluronidase activity is cell associated (Li *et al.*, 2000), which is consistent with the presence of the Gram positive cell surface anchorage domain (LPXTG) near its C terminus. The protein sequence of four bacterial hyaluronate lyases from *S. pneumoniae* (Berry *et al.*, 1994), *S. agalactiae* (Lin *et al.*, 1994), *Staphylococcus aureus* (Farrell *et al.*, 1995), and *Propionibacterium acnes* (Steiner *et al.*, 1997) have been reported so far. Their sequence homologies range from 28 to 68% suggesting their structural, functional, and evolutionary similarities (Akhtar and Bhakuni, 2003).

Deletion of the hyaluronidase gene does not affect virulence in a mouse model of infection. However, hyaluronidase deletion mutants in pneumolysin-negative backgrounds were significantly less virulent than derivatives with single mutations. These may represent another alternative for a pneumococcal vaccine or drug target, especially when combined with pneumolysin (Berry and Paton, 2000).

1.4.6.2 Neuraminidase

Neuraminidase cleaves terminal sialic acid residues from a wide variety of glycolipids, glycoproteins, and oligosaccharides on cell surfaces or in body fluids, and such activity has the potential to cause great damage to the host. Neuraminidase might also unmask potential cell surface receptors for putative pneumococcal adhesins (Krivan *et al.*, 1988).

Pneumococcal neuraminidase, like hyaluronidase, belongs to another family of pneumococcal surface proteins that are anchored to the pneumococcal cell wall by covalent linkage to peptidoglycan through a carboxy-terminal motif LPXTG (Mitchell, 2003). Pneumococci have at least two enzymes with neuraminidase activity, NanA and NanB (Berry *et al.*, 1996; Camara *et al.*, 1991; Camara *et al.*, 1994). NanB, unlike NanA, does not contain a LPXTG motif in their C-terminal. In some strains, there is also a NanB

homolog, NanC. NanA has a molecular mass of ~108 kDa (Camara *et al.*, 1994) and NanB has a mass of 74.5 kDa (Berry *et al.*, 1996). They possess very little amino acid homology. It has been suggested that neuraminidase activity promotes colonization by exposing host cell receptors otherwise covered by sialic acid (Tong *et al.*, 2001). Both enzymes seem to have a propensity for degradation to smaller fragments during *in vitro* growth and protein purification, and some of these fragments preserve neuraminidase activity. For NanA, active fragments as small as 85 kDa were isolated (Lock *et al.*, 1988). NanA has been located on the surface of pneumococci through antibody studies (Camara *et al.*, 1994). The activity of NanB is approximately 100 times lower than that of NanA (Berry *et al.*, 1996; Lock *et al.*, 1988). Both proteins are exported proteins with typical signal peptides, but NanB lacks the typical surface anchorage domain (LPXTG) present in NanA, which probably reflects the covalent binding of NanA to peptidoglycan structures of *S. pneumoniae* (Camara *et al.*, 1994; Schneewind *et al.*, 1995).

The exact role of pneumococcal neuraminidase in pathogenesis has not been clearly established, however, it has been proposed that neuraminidase could enhance colonization by decreasing viscosity of mucus or by exposing cell surface receptors for *S. pneumoniae* (Linder *et al.*, 1992; Linder *et al.*, 1994; Rosenfeld *et al.*, 1992). Histochemical studies of organs from mice dying after intraperitoneal administration of partially purified pneumococcal neuraminidase have indicated marked decreases in the sialic acid contents of the kidneys and liver compared with those of controls (Kelly and Greiff, 1970). It has also been shown that both coma and bacteraemia occur significantly more often among patients with pneumococcal meningitis when the concentration of N-acetylneuraminic acid in the cerebrospinal fluid is elevated (O'Toole *et al.*, 1971). *S. pneumoniae* neuraminidase has been implicated as a virulence factor in the pathogenesis of pneumococcal otitis media. Disruption of *nanA* diminishes the ability of pneumococci to colonize and persist in nasopharynx and middle ear in the chinchilla model (Tong *et al.*, 2000). Furthermore, immunization with neuraminidase resulted in a significant reduction in nasopharyngeal colonization as well as in the incidence of otitis media with effusion (Long *et al.*, 2004; Tong *et al.*, 2005).

1.4.6.3 Other LPXTG-anchored proteins

In addition, other LPXTG-anchored proteins, such as IgA1 protease, cell wall-associated protease PrtA and β-galactosidase (BgaA), are present on the surface of pneumococci.

IgA is the most abundant class of Igs on mucosal surfaces and its contribution to host defence results from its ability to block colonization-initiating adhesive interactions of microorganisms with host tissues (Russell *et al.*, 1999). *S. pneumoniae*, *H. influenzae* (Mulks *et al.*, 1980), *N. meningitides* (Vidarsson *et al.*, 2005) and oral streptococci (Kilian and Holmgren, 1981) produce a variety of enzymes called IgA1 proteases that specifically cleave the hinge region of IgA1 into Fab and Fc fragments (Collin and Olsen, 2001; Male, 1979; Plaut, 1983). These proteases may be important virulence factors because they are produced *in vivo* (Blake *et al.*, 1979; Insel *et al.*, 1982). This Zn-metalloproteinase with 1927 amino acids is characterized by the absence of C-terminal motif-LPXTG and is associated with the bacterial cell surface via an N-terminal motif-LPXTG (Poulsen *et al.*, 1996), and is highly homologous to the IgA1 protease from *Streptococcus sanguis* (Gilbert *et al.*, 1991). Wani and co-workers identified, cloned and characterized the IgA1 in *S. pneumoniae* (Wani *et al.*, 1996). IgA1 protease was identified as a virulence factor in both pneumonia and septicemia models by the STM screen (Polissi *et al.*, 1998). Further functions of IgA1 protease include impairment of host defences at the mucosal surfaces and support of colonization of the nasopharynx (Kilian *et al.*, 1996; Weiser *et al.*, 2003).

Protease activity of two distinct serine type proteases has been demonstrated in pneumococcal culture supernatant (Courtney, 1991). One of these two proteins, PrtA, was identified by computational analysis of the pneumococcal genome to be a surface located protein (Wizemann *et al.*, 2001). The protease PrtA has been identified as a serine protease belonging to the family of subtilisin-like proteases, also called subtilases. PrtA was identified as a virulence factor in an animal model of infection after intraperitoneal challenge. Survival of mice infected with the PrtA-deficient mutant strain was significantly longer than the survival during infection with the wild-type strain (Bethe *et al.*, 2001). This protease was identified by an immunological screening of an expression library of the pneumococcal genome using human convalescent-phase serum (Zulty and Barcak, 1995). Two parts of this protein, including the N-terminal and the C-terminal third, were demonstrated to be protective in mice (Wizemann *et al.*, 2001). The part of the protective N-terminal third is highly conserved in clinical pneumococcal isolates (Bethe *et al.*, 2001) and may display a promising candidate for vaccine development to protect humans from invasive pneumococcal infection.

The *bgaA* gene of *S. pneumoniae* encodes a putative 2235-amino-acid protein with the two amino acid motifs characteristic of the glycosyl hydrolase family of proteins. The LPXTG-anchored surface proteins described previously have been described as virulence factors, and it is conceivable that β-galactosidase is a virulence factor as well (Zahner and

Hakenbeck, 2000). The presence of antibodies against β-galactosidase in convalescent-phase serum from a patient with a history of pneumococcal infection is in agreement with this hypothesis (Zysk *et al.*, 2000).

1.5 Pneumococcal antibiotic resistance

The increase of antibiotic resistance in bacteria has accompanied the therapeutic use of antimicrobial agents, with a steady erosion of antimicrobial activity against Gram positive cocci (Moellering, 1998). Since the 1980s the development and dissemination of strains of *S. pneumoniae* with resistance to penicillin has become an important problem in the treatment of infections caused by this bacterium (Munoz *et al.*, 1991). There is a pandemic of resistance among clinical isolates of *S. pneumoniae* (Dagan *et al.*, 2001; Felmingham and Gruneberg, 2000), and pneumococcal disease is among the leading causes of infective mortality in children and the elderly (Ball *et al.*, 2002; Breiman *et al.*, 1994). Because 80% of antibiotic consumption is in the treatment of respiratory tract infections (Huovinen and Cars, 1998), this can explain the emergence of resistance in *S. pneumoniae* (Arason *et al.*, 1996; Perez-Trallero *et al.*, 2001) by selective pressure (Doern, 2001).

Over the past two decades, antibiotic use has led to increased resistance to the so-called "antibiotic group markers", specifically penicillin, erythromycin and ciprofloxacin for β-lactams, macrolides and quinolones respectively (Prieto *et al.*, 2002). The problem of resistance is heightened by the difficulty in choice among commonly used groups of antibiotics, since resistance to erythromycin is more prevalent among penicillin-resistant strains (Perez-Trallero *et al.*, 2001), and resistance to ciprofloxacin is more prevalent among penicillin-non-susceptible and erythromycin-resistant strains. (Garcia-Rey *et al.*, 2000; Perez-Trallero *et al.*, 2001).

Mathematical models predict that rates of resistance decay more slowly than they emerge (Austin *et al.*, 1999). A pharmacological strategy in the use of antibiotics including further widespread interventions to reduce global antibiotic consumption, protection of the effectiveness of antibiotic classes to which little resistance has emerged, and active surveillance of the serotypes and antimicrobial susceptibilities of nasopharyngeal isolates following the introduction of the pneumococcal conjugate vaccines appear to offer the most promise for limiting the spread of drug resistance among the pneumococci (Nuermberger and Bishai, 2004).

1.6 Pneumococcal vaccines

It has been many years, since the beginning of the 20 century (early as 1911), that pneumococcal vaccines were used (Wright *et al.*, 1914). In the1930s, the immunogenicity of purified capsular polysaccharides was demonstrated and the first pneumococcal capsular polysaccharide vaccine was developed. However, after the World War II the interest in these vaccines declined due to the demonstration of the therapeutic efficacy of antibiotics and the pneumococcal vaccine was withdrawn from the market (Austrian, 1985). Even with the use of antibiotics, the mortality rate of systemic pneumococcal diseases remained high (Gillespie, 1989), and the emergence of the first penicillin-resistant pneumococcus in the mid-1960s (Hansman and Bullen, 1967) induced renewed efforts to develop improved pneumococcal vaccines.

At the moment there are two main vaccines available for the prevention of pneumococcal disease, the polysaccharide and conjugate vaccines. The polysaccharide vaccine contains per dose 25 μg of purified capsular polysaccharide from each of the 23 serotypes of *S. pneumoniae* (Fedson and Musher, 2004). The effectiveness of the polysaccharide vaccine is difficult to know with accuracy, but overall efficacy in preventing pneumococcal bacteraemia is probably 50-70% (Butler *et al.*, 1993; Fedson, 1999; Fine *et al.*, 1994; Mangtani *et al.*, 2003; Melegaro and Edmunds, 2004). This vaccine is immunogenic and protective in most adults and in children over 5 years of age but fails in certain high risk groups, and children under 2 years old (O'Brien *et al.*, 1996). Furthermore, polysaccharide vaccines do not induce immunological memory, which is required for subsequent booster responses (Girard *et al.*, 2005) and current evidence suggests that the polysaccharide vaccine is not effective in protecting against non-bacteraemic pneumococcal pneumonia (Jackson *et al.*, 2003).

In order to develop vaccines for infant use, pneumococcal capsular polysaccharide of the epidemiologically most important pneumococcal serotypes have been covalently coupled with various carrier proteins. The conjugate vaccines have been shown to be safe and effective in children under 2 years (Dagan *et al.*, 1996; Kayhty *et al.*, 1995; Mbelle *et al.*, 1999; Puumalainen *et al.*, 2002; Shinefield *et al.*, 1999). These vaccines induce high concentrations of serum antibodies (Eskola *et al.*, 2001; Rennels *et al.*, 1998) and reduce nasopharyngeal carriage of vaccine serotypes (Dagan *et al.*, 1996; Dagan *et al.*, 2002; Obaro *et al.*, 1996). The seven-valent pneumococcal conjugate vaccine Pnc-CRM7 has been shown to be highly efficacious in preventing vaccine-serotype invasive disease in

young children and to be modestly beneficial against acute otitis media (Black *et al.*, 2000).

The problem of the conjugate vaccines is that only a limited number of serotypes may be included in the conjugated formulation due to logistic difficulties in the manufacturing process, and the attendant high cost (Shinefield *et al.*, 1999; Zimmerman, 2001).

The disadvantages of capsular polysaccharide and conjugate vaccines have stimulated an interest in alternative pneumococcal vaccination strategies. A promising complementary or alternative approach for prevention of pneumococcal infections is to develop vaccines directed against an antigenic moiety common to all pneumococcal serotypes. The use of pneumococcal proteins has been reported as a potential vaccine candidate. The proteins pneumolysin, PspA, PspC, and the pneumococcal adhesin A (PsaA) are currently the leading vaccine candidates (Briles, 2004; Paton, 1998).

1.7 Two-component signal transduction

All living cells must sense changes in their environment and respond appropriately (Appleby *et al.*, 1996). In their environment bacteria are continually bombarded by a large number of chemicals, some of which may serve as potential sources of carbon, nitrogen, and energy, while others may be harmful for their metabolic and regulatory processes (Barrett and Hoch, 1998). It is now clear that bacterial virulence is an adaptive genetic response requiring the induction of genes coding for virulence factors. This response implies that the infectious agent must be able to sense when it is in position to invade (Barrett *et al.*, 1998). Microorganisms must modulate the expression of specific genes in response to a large number of environmental signals and process much of this information using two-component signal transduction or two-component systems (TCS) (Barrett and Hoch, 1998; Hoch, 2000; Perego and Hoch, 1996; Stock *et al.*, 1989), also known as histidine-aspartate phosphorelay systems (Throup *et al.*, 2000).

Such systems also are found in plants and some lower eukaryotes (fungi, protozoa) and archaea (Barrett *et al.*, 1998; Chang and Stewart, 1998; Koretke *et al.*, 2000). Studies indicate that two-component elements are involved in plant hormone, stress, and light signaling (Hwang *et al.*, 2002). A number of TCS in some prokaryotic and eukaryotic organisms and their regulatory functions are shown in Table 1.2.

Table 1.2- Summary of two-component systems in some prokaryotic and eukaryotic organisms.

Organism	TCS	Characteristics Functions	References
Halobacterium salinarum	ChA/Y	Chemotaxis, phototaxis.	(Rudolph and Oesterhelt, 1995)
Bacillus subtilis	YycG/YycF	Cell wall homeostasis regulation.	(Szurmant *et al.*, 2005)
Escherichia coli	UhpB/UhpA	Hexose phosphateuptake.	(Verhamme *et al.*, 2002)
	BasS/BasR	Iron response.	(Hagiwara *et al.*, 2004)
	BarA/UvrY	Oxidative stress response.	(Pernestig *et al.*, 2003)
	CheA/CheY,B	Chemotaxis.	(Stock and Surette, 1996)
Helicobacter pylori	HP166/HP165	Host colonization.	(Panthel *et al.*, 2003)
Staphylococcus aureus	ArlS/ArlR	Regulation of drug efflux components.	(Fournier *et al.*, 2000)
	SrrB/SrrA	Response to oxygen modifies levels.	(Pragman *et al.*, 2004)
Listeria monocytogenes	LisK/R	Response to heat, acid, and penicillin stress.	(Kallipolitis and Ingmer, 2001; Stack *et al.*, 2005)
	CesK/R	Response to ethanol, resistance to antibiotics.	(Kallipolitis *et al.*, 2003)
Mycobacterium tuberculosis	DevS/DevR	Response to hypoxia.	(Saini *et al.*, 2004)
Salmonella typhimurium	PhoQ/PhoP	Acid tolerance response.	(Bearson *et al.*, 1998)
Streptococcus gordonii	BfrB/ BfrA	Biofilm formation.	(Zhang *et al.*, 2004)
Rhizobium meliloti	FixL/FixJ	Nitrogen fixation.	(Miyatake *et al.*, 1999; Monson *et al.*, 1995; Weinstein *et al.*, 1992)
	ExoS/ChvI	Regulation of succinoglycan production.	(Cheng and Walker, 1998)
Saccharomyces cerevisiae	Sln1p-Ssk1p	Response to oxidative stress.	(Singh, 2000)
	Chk1p	Quorum Sensing regulation.	(Kruppa *et al.*, 2004)
Arabidopsis thaliana	AtHK1	Putative osmosensor	(Urao *et al.*, 1999)
	AtRR1	Cytokinin signalling	(Osakabe *et al.*, 2002)

These systems have been shown to regulate a wide variety of cellular responses, including osmoregulation, competence, photosynthesis, expression of adhesions, chemotaxis, sporulation, antibiotic production and pathogenicity in a number of different bacteria (Appleby *et al.*, 1996; Hoch and Silhavy, 1995). Perhaps more importantly, evidence is

now accumulating that links phosphorelay signalling with the cell cycle and bacterial viability. This mechanism of genetic control had been adopted to regulate vital functions in the cell (Fabret and Hoch, 1998; Quon *et al.*, 1996).

TCS consist of a sensor histidine kinase (HK), often located in the cytoplasmic membrane, and a cytoplasmic response regulator (RR) (Figure 1.3) (Stock *et al.*, 1995). The mode of molecular communication between the sensor kinase and cognate response regulator is principally based on histidine-to-aspartate (His-Asp) phosphotransfer (Stock *et al.*, 1995). Upon receipt of a specific stimulus, the kinase domain of the sensor kinase protein is activated, resulting in the autophosphorylation of a conserved histidine residue. Once phosphorylated, the histidine kinase donates the high-energy phosphate group to a cognate response regulator. Phosphorylation of a conserved aspartate residue that lies within the receiver domain of the regulator is thought to lead to structural changes in the protein, allowing the response regulator to mediate changes in gene expression or protein function (Egger *et al.*, 1997). At this point, it is important to realize that response regulators are subject to regulation from a variety of sources and the phosphorylated (active) state of these proteins may be subject to dephosphorylation reactions that return it to an inactive state (Barrett and Hoch, 1998).

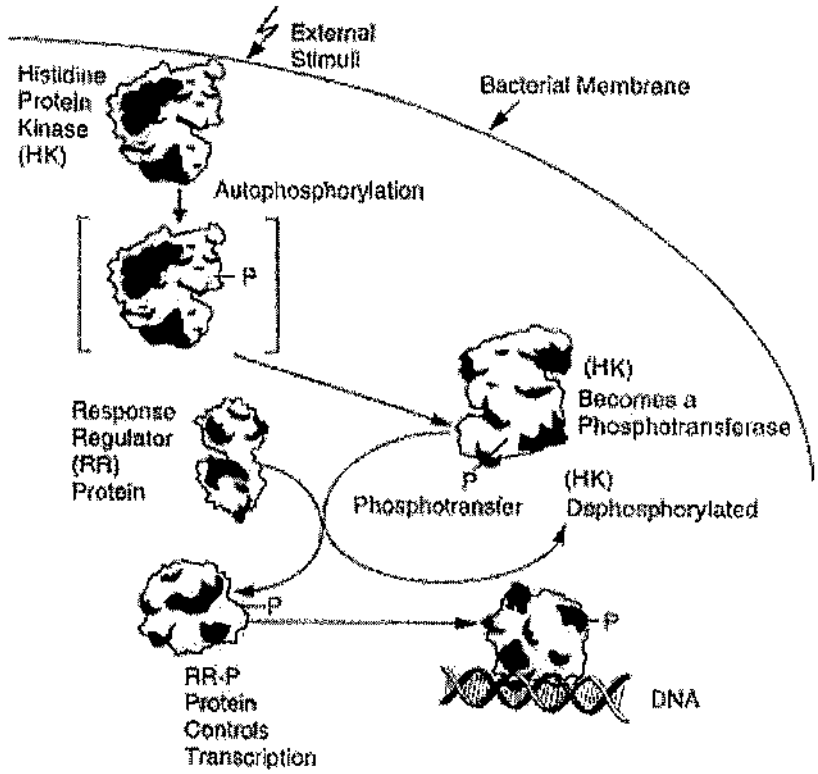


Figure 1.3- Two-component signal transduction mechanisms.

A Typical TCS consists of membrane sensor histidine kinase (HK) and a cytoplasmic response regulator (RR). The HK interacts directly with a signal ligand or with a receptor that binds to the signal ligand and induces an autophosphorylation. Subsequently, this phospho-group is transferred to the response regulator, which can then fulfil its regulatory function. Adapted from (Barrett *et al.*, 1998).

1.7.1 Sensor histidine kinase

In several respects, HKs are similar to the well-defined family of receptor Tyr kinases (Stock *et al.*, 1991): HKs operate as dimers and autophosphorylate; they are associated with the cytoplasmic membrane, usually via one or two membrane-spanning sequences; and they typically contain extracellular sensory input modules fused to the protein kinase catalytic module (Bourret *et al.*, 1991). This arrangement makes it easy to envision environmental stimuli impinging on the HK in a manner that regulates its kinase activity (Chang and Stewart, 1998).

There are also operational features that distinguish HKs from other protein kinases. First, HKs do not catalyze direct transfer of a phosphate from ATP to their "substrate" RR; rather, each HK must first autophosphorylate, and then the phosphoryl group from HK-P is passed to the RR. A second difference is that the site of HK autophosphorylation is a His residue, and the site of RR phosphorylation is an Asp residue (Bourret *et al.*, 1991).

Several hundred HKs (some well characterized, some surmised based on sequence analysis) have been found in bacteria, and amino acid sequence comparisons have identified a common 250-amino acid "transmitter module" in each of these. This module is thought to encompass the autokinase active site and, in most cases, the hisphosphorylation site (Chang and Stewart, 1998; Parkinson and Kofoid, 1992; Stock *et al.*, 1995).

Some HKs also have phosphatase activities, i.e. they can catalyze dephosphorylation of their cognate RRs (Igo *et al.*, 1989; Makino *et al.*, 1989). This dephosphorylation appears to involve a mechanism that is distinct from simple reversal of the HK-RR phosphotransfer reaction (Hsing and Silhavy, 1997).

1.7.2 Response regulator

The sensor HK regulates the activity of a cytoplasmic RR by directing its phosphorylation as depicted in Figure 1.3. Analysis of the amino acid sequences of known and suspected RRs has established two general themes: (i) RRs have an approximately 110-amino acid domain referred to as a "receiver module" that contains the Asp phosphorylation site; and (ii) most RRs are two-domain proteins in which the receiver module is fused to a second domain having some kind of output or effector activity (Parkinson and Kofoid, 1992). In many cases, the output domain is a DNA-binding module whereby the RR functions as a transcription factor, and Asp phosphorylation serves to control its ability to either bind its target DNA sequence or interact with other components of the transcription machinery (Hakenbeck and Stock, 1996). However, in *B. subtilis* the response regulator CheY does not interact with DNA, but interacts with the flagellar motor switch complex to induce counterclockwise rotation of the flagella, resulting in smooth swimming behavior (Bischoff *et al.*, 1993).

1.7.3 Control points

Different TCS appear to control RR phosphorylation levels via somewhat distinct mechanisms. For example, in response to a stimulus some systems alter RR-phosphorylation levels by controlling the rate of HK autophosphorylation (Borkovich and Simon, 1990), whereas in other systems it is the phosphatase activity of the HK or an additional component that is regulated in response to a stimulus (Atkinson *et al.*, 1994; Perego and Hoch, 1996). This diversity underscores the impressive flexibility of two-component circuitry; it can be modified to operate in a variety of different contexts using different aspects of the basic protein structures of receiver and transmitter modules and the

basic biochemistry of the phosphorylation/ dephosphorylation chemistry (Chang and Stewart, 1998).

1.7.4 Virulence regulation by two-component systems

It is now clear that bacterial pathogens are very adept at resolving host-environment-pathogen interactions by evolving virulence factors and regulation systems that allow them to survive in many hostile environments. TCS are the only common regulatory elements shared by a wide range of virulence systems, raising the possibility that a broad-spectrum inhibitor of such elements may suppress virulence in a variety of microorganisms (Barrett and Hoch, 1998; Novick *et al.*, 1995).

TCS are recognized for their role in the regulation of many of the virulence factors and other important genes required for bacteria to survive in the foreign host (Hecht *et al.*, 1995). One of the more interesting aspects of TCS control is the role of these systems in resistance to certain antibacterial agents (Barrett and Hoch, 1998).

Certain reviews (Bliska *et al.*, 1993; Dziejman and Mekalanos, 1995; Galan, 1994; Salmond *et al.*, 1995; Schneewind *et al.*, 1993; Stock *et al.*, 1995) have described the importance to bacteria of networks of intracellular signaling in the bacterium in response to its environment. The interruption of these signals may lead to the interruption of virulence and/or a decrease in the levels of bacterial virulence factors. Such targets may offer the opportunity for a totally new class of antibacterial agents (Highlander and Weinstock, 1992).

Four features in particular make the two-component family attractive as a potential target for antimicrobials:

Significant homology is shared among kinase and response regulator proteins of different genera of bacteria, particularly in those amino acid residues located near active sites (Parkinson and Kofoid, 1992);

Pathogenic bacteria use TCS transduction to regulate expression of essential virulence factors that are required for survival inside the host (Dziejman and Mekalanos, 1995);

Bacteria contain many TCS, and some of them are essential for viability (Hecht *et al.*, 1995; Quon *et al.*, 1996);

Signal transduction in mammals occurs by a different mechanism (Barrett *et al.*, 1998).

In pathogenic bacteria, the TCS often regulate the expression of virulence factors that are required for survival inside the host. TCSs are not found in higher eukaryotic organisms, making this an important new target that could lead to a novel class of antibacterial drugs. Compounds that inhibit TCSs could be expected to block important bacterial signaling pathways that may lead to bacterial cell death (Hlasta *et al.*, 1998).

1.7.5 Two-component systems in *Streptococcus pneumoniae*

Many important functions of bacteria, such as expression of adhesins, competence, osmoregulation, and chemotaxis, are regulated by TCS (Hoch and Silhavy, 1995).

Only four TCS had been identified previously to genome sequencing in *S. pneumoniae* (Guenzi *et al.*, 1994; Novak *et al.*, 1999a; Pestova *et al.*, 1996). Subsequently, genomic analysis of the pneumococcus revealed the full repertoire of the TCS. Screening for pneumococcal TCS identified thirteen hk:rr pairs and one unpaired response regulator (Table 1.3) (Lange *et al.*, 1999; Throup *et al.*, 2000). Although the number of TCS found in *S. pneumoniae* is less than in either *Bacillus subtilis* (34/35 *hk/rr* genes) or *Escherichia coli* (29/34 *hk/rr* genes), their presence suggests that His-Asp phosphorelay signal transduction is an important mechanism for regulating gene expression even in highly adapted pathogens such as *S. pneumoniae*. Previous contradictory reports about the role of these systems in virulence were described (Lange *et al.*, 1999; Throup *et al.*, 2000). While analysis of *rr* mutants in pneumococcal serotypes 3 and 22 by Lange and co-workers (Lange *et al.*, 1999) yielded no attenuation in interperitoneal infection of mice compared with wild-type, Throup and co-workers (Throup *et al.*, 2000) demonstrated an important role in pneumococcal virulence for most tested TCS in a mouse pneumonia model using serotype 3 strain 0100993. These opposing results show the complexity of the pneumococcal TCS in virulence and likely reflect experimental differences in bacterial and mouse strains and site of infections. Subsequent analysis confirmed the importance of bacterial strain and infection type in the TCS studies.

Table 1.3- Gene organization of the TCS, G+C content and their contribution to virulence. Adapted from (Lange *et al.*, 1999).

TCS	Alternative name [1]	Gene organisation [2]	G+C content	Demonstrated role in virulence	References for role in virulence
TCS01	480		~41%	Yes	(Hava and Camilli, 2002; Throup *et al.*, 2000)
TCS02	Vic, MicAB, YycFG, 492		~40%	Yes	(Kadioglu *et al.*, 2003; Wagner *et al.*, 2002)
TCS03	474		~45%	No	
TCS04	PnpR/S, 481		~41%	Yes	(McCluskey *et al.*, 2004; Throup *et al.*, 2000)
TCS05	CiaR/H, 494		~38%	Yes	(Ibrahim *et al.*, 2004b; Marra *et al.*, 2002a; Throup *et al.*, 2000)
TCS06	478		~35%	Yes	(Throup *et al.*, 2000)
TCS07	539		~41%	Yes	(Hava and Camilli, 2002; Throup *et al.*, 2000)
TCS08	484		~42%	Yes	(Throup *et al.*, 2000)
TCS09	488		~41%	Yes	(Blue and Mitchell, 2003; Hava and Camilli, 2002; Lau *et al.*, 2001; Throup *et al.*, 2000)
TCS10	VncR/S, 491		~44%	No	
TCS011	479		~44%	No	
TCS12	ComD/E, 498		~31%	Yes	(Bartilson *et al.*, 2001; Hava and Camilli, 2002)
TCS013	BlpR/H, 486		~40%	Yes	(Throup *et al.*, 2000)
Orphan RR	RitR, 489		~42%	Yes	(Throup *et al.*, 2000; Ulijasz *et al.*, 2004)

[1] sp numbers annotations as published in TIGR4 genome (http://www.tigr.org).
[2] Black arrows histidine kinase; gray arrows response regulator.

The functions of most of the TCS in *S. pneumoniae* are largely unknown. Some of the TCS have been examined for functionality including TCS02, 04, 05, 09, 12, 13 and the orphan RR 14.

1.7.5.1 TCS02

TCS02, also known as Vic (Wagner *et al.*, 2002), MicAB (Echenique and Trombe, 2001), YycFG (Mohedano *et al.*, 2005) and 492 (Throup *et al.*, 2000) is the only TCS essential for pneumococcal viability, while the response regulator and histidine kinase shows homology

to the essential YycFG TCS in *B. subtilis* and *S. Aureus* (Fabret and Hoch, 1998; Lange *et al.*, 1999; Throup *et al.*, 2000). However, in pneumococci only *rr*02 appears to be essential, while both *rr* and *hk* are essential in *B. subtilis* and *S. Aureus*. Presumably, this shows that *rr*02 is phosphorylation-independent or is phosphorylated by other donor. The receiver domain of the response regulator of the TCS02 has recently been crystallized and its structure characterized (Bent *et al.*, 2003, 2004; Riboldi-Tunnicliffe *et al.*, 2004). The role of the TCS02 remains uncertain. However, recent reports indicate that TCS02 modulates expression of fatty acid biosynthesis genes and determines the fatty acid chain lengths in membrane lipids (Mohedano *et al.*, 2005). Also, TCS02 whose HK carries a PAS domain used as a signal sensor domain, is involved in competence repression under oxygen limitation (Echenique and Trombe, 2001). Furthermore, microarray analysis of *rr*02 of a deletion mutant suggest that this TCS regulated positively the transcription of a set of genes encoding important surface proteins, including PspA (Ng *et al.*, 2005).

Overexpression and deletions of the TCS02 components, including the downstream gene *vicX* caused attenuated virulence in interperitoneal infections (Wagner *et al.*, 2002). Furthermore, mutation of *hk*02 in two different strains (serotypes 2 and 6B) decreased virulence in a pneumonia model (Kadioglu *et al.*, 2003). However no virulence attenuation was found by Throup and co-workers in *hk*02 knock-out in 0100993 strain (Throup *et al.*, 2000). These last results may be related to the use of different experimental methods and strains.

1.7.5.2 TCS04

TCS04, also know as PnpR/S was first identified by Novak and co-workers (Novak *et al.*, 1999a). Throup and co-workers referred to TCS04 as 481 and identified it as a pneumococcal virulence factor (Throup *et al.*, 2000). The TCS04 is similar to PhoP/R of *B. subtilis* (Hulett, 1996), and presents considerable similarity to PhoP/Q systems of different bacteria such as *E. coli* (Kato *et al.*, 1999) *Mycobacterium tuberculosis* (Perez *et al.*, 2001), *Salmonella typhimurium* (Miller *et al.*, 1989), and *N. meningitides* (Johnson *et al.*, 2001). The role of TCS04 in pneumococcal virulence remains unclear. No attenuation was found in pneumococcal infection in serotype 22 and serotype 3 strains using a systemic model of infection (Lange *et al.*, 1999). On the other hand, Throup and co-workers reported a high level of attenuation of a serotype 3 strain (0100993) (Throup *et al.*, 2000), while McCluskey and co-workers using a comparison of *rr04* mutants in three strains (TIGR4, D39 and 0100993), in a similar pneumonia model, only observed attenuation in the TIGR4 strain (McCluskey *et al.*, 2004), demonstrating a serotype-specific variation of virulence of

rr04 mutants of *S. pneumoniae*. The discrepancy between the attenuation in the 0100993 *rr04* mutant strain in both studies may be caused by the use of different infection models, and by the complexity of studying of these systems. Microarray analysis of the transcriptome profile of these mutants demonstrated a considerable variation of the genes regulated by *rr04* (McCluskey *et al.*, 2004). Of interest, the three genes which encode a manganese transport system (Dintilhac *et al.*, 1997) were down regulated in TIGR4 *rr04* mutant but not in D39 and 0100993 mutants. This *psa* operon, consisting of *psaB*, *psaC* and *psaA* is known to contribute to pneumococcal virulence and resistance to oxidative stress (McAllister *et al.*, 2004). The down-regulation of the *psa* operon in TIGR4 *rr04* mutant may contribute to the specific attenuation on this strain.

1.7.5.3 TCS05

The TCS05 (CiaR/H) was the first pneumococcal TCS identified (Guenzi *et al.*, 1994). CiaR/H, appears to operate upstream of the ComDE pathway and has been shown to modulate competence expression. Pleotropic effects caused by *cia* mutations in the pneumococcus include sensitivity to cefotaxime, ability to form protoplasts, susceptibility to lysis by deoxycholate (Giammarinaro *et al.*, 1999). Other phenotypes in *cia* mutants have since been described, such as growth defects associated with the tendency for early lysis (Giammarinaro *et al.*, 1999; Hakenbeck *et al.*, 1999a; Lange *et al.*, 1999). In addition, the CiaR/H system is required to protect cells from the stress during differentiation to competence (Dagkessamanskaia *et al.*, 2004). *ciaH* mutants are also deficient in transformation deficiency (Echenique *et al.*, 2000; Giammarinaro *et al.*, 1999; Hakenbeck *et al.*, 1999a). The role of the CiaR/H system in virulence using knockout mutants has been demonstrated including the contribution to colonization of the mouse lung (Throup *et al.*, 2000) and the nasopharynx of infant rats (Sebert *et al.*, 2002), and involvement in systemic infection in mice (Marra *et al.*, 2002a). Furthermore, mutation in *ciaR* reduces virulence in both pneumonia and bacteraemia models of infection (Ibrahim *et al.*, 2004b).

Studies of the CiaR/H regulon identified the high-temperature requirement A gene (*htrA*), as being down-regulated in *ciaR/H* mutants (Ibrahim *et al.*, 2004b; Mascher *et al.*, 2003; Sebert *et al.*, 2002). Ibrahim and co-workers demonstrated that *htrA* is the key component in the contribution of ciaR/H to virulence (Ibrahim *et al.*, 2004b). However, several other known and putative virulence factors were regulated or potentially regulated by *ciaR/H* system than may contribute to the role of *ciaR/H* in virulence, including the *dlt*, and *pit2* operons (Mascher *et al.*, 2003; Sebert *et al.*, 2002).

1.7.5.4 TCS09

TCS09, also described as 488 (Throup *et al.*, 2000) was identified as having a significant contribution to pneumococcal virulence. In *in vivo* studies, an *rr*09 mutant in strain D39 was essentially avirulent in pneumonia and bacteraemia models. In contrast to wild-type, all *rr*09 infected mice survived infection with bacteria being rapidly cleared (Blue and Mitchell, 2003). However, the same mutation in 0100993 strain did not show the same pattern of attenuation (Blue and Mitchell, 2003; Throup *et al.*, 2000). These results demonstrate that the role of TCS09 in virulence appears to be dependent on the genetic background and the infection model used. In strain 0100993, RR09 was suggested to be involved in the dissemination from the lung to the systemic circulation, since the attenuation in a pneumonia model seems to relate to the reduction of 0100993 *rr*09 mutant in blood in contrast with no significant reduction of bacterial count in the lung. Furthermore no attenuation was observed in bacteraemia model of infection, showing the 0100993 *rr*09 mutant was able to survive in blood as the wild-type. (Blue and Mitchell, 2003).

TCS09 has the potential to contribute significantly to pneumococcal virulence but this contribution varies between pneumococcal strains and infection sites. So far, no gene targets of RR09 have been identified that could account for the observed *in vivo* phenotypes of *rr09* mutants, although preliminary studies suggested involvement of TCS09 in nutrient perception (Blue and Mitchell, 2003; Lange *et al.*, 1999).

1.7.5.5 TCS12

The pneumococcus is naturally competent for genetic transformation. Genetic transformation is likely to play a significant role in the lifestyle of this bacterium by favouring genetic diversity (Claverys and Havarstein, 2002). TCS12 (ComD/E) was described as being responsible for induction of competence for DNA transformation (Cheng *et al.*, 1997; Havarstein *et al.*, 1996; Pestova *et al.*, 1996). The quorum-sensing signal responsible for competence induction is a heptadecapeptide, named CSP (competence-stimulating peptide) (Havarstein *et al.*, 1995), which derives from its precursor ComC by cleavage and transport into the medium by an ATP-biding cassette (ABC) transporter, ComAB (Hui *et al.*, 1995). *comC* is the first gene of an operon, *com*, consisting of two other downstream elements, *comD/E* system. Null mutants with defects in either *comC* or *comD* were transformation deficient and failed to respond to exogenous CSP (Cheng *et al.*, 1997). The association between the competence and virulence in the

pneumococcus was shown in models of both pneumonia and bacteraemia using a *comD* mutant in D39 strain (Bartilson *et al.*, 2001). Additionally, *comD* was demonstrated to contribute to pneumonia and bacteraemia models in competitive infections with wild-type (Lau *et al.*, 2001). Furthermore, *comD* was identified as a virulence factor in pneumonia by the TIGR4 signature-tagged mutagenesis screen (STM) (Hava and Camilli, 2002). The link between the competence and virulence is not fully understood. However, 18 of the 124 up-regulated genes induced by CSP observed by Peterson and co-workers were identified in the TIGR4 STM screen as virulence factors, thereby providing a mechanistic explanation for the role of competence in virulence (Hava and Camilli, 2002). These up-regulated virulence factors included the autolysin *lytA*, *htrA*, a stress response protein and a choline-binding protein gene (*cbpD*).

The relationship between the TCS and competence is not restricted to the TCS12. Expression of *comC* is part of a complex regulatory network involving the ComD/E system for induction and the TCS02 and the CiaR/H system, which inhibits expression of the *comCDE* operon (Echenique *et al.*, 2000; Echenique and Trombe, 2001).

1.7.5.6 TCS13

TCS13 was identified by Throup and co-workers as contributing to pneumococcal virulence (Throup *et al.*, 2000). *rr*13 mutation in 0100993 strain was found to be significantly attenuated in their respiratory tract infection model. The TCS13 subsequently named BlpR/H for bacteriocin-like peptide controls a regulon including genes encoding Blps (de Saizieu *et al.*, 2000). The BlpR/H system is similar to the competence system ComD/E, with the peptide BlpC signalling via BlpR/H to up-regulate target genes including *blpC* itself. Microarray analysis revealed than 16 genes are significantly up-regulated when stimulated by BlpC. The relationship with virulence of BlpR/H system is shown in TIGR4 STM screen were *blpA*, apparently involved in BlpC export, was identified as a virulence factor (Hava and Camilli, 2002). Bacteriocins are commonly defined as compounds produced by bacteria that selectively inhibit or kill closely related species. Although poorly characterised, the pneumococcal bacteriocins activity presumably provides a growth advantage in microbial competition (Mindich, 1966). However, lungs are usually sterile and thus no microbial competitors for *S. pneumoniae* should be present, raising the question of the biological role of a bacteriocin regulon in the lung. So, it is proposed *blp* bacteriocins may be acting via a cytotoxic affect on host cells (de Saizieu *et al.*, 2000).

1.7.5.7 Orphan response regulator

Unlike the other pneumococcal TCS the rr489, designated RitR (Ulijasz *et al.*, 2004), is not adjacent to a kinase gene like the other thirteen TCS, and this response regulator is not known to be phosphorylated by any of the thirteen known histidine kinases. However, inactivation of *ritR* by antibiotic cassette mutagenesis in 0100993 strain demonstrates a significant reduction in pulmonary bacterial counts in a murine pneumonia model (Throup *et al.*, 2000). The importance of *ritR* in virulence was confirmed by Ulijasz and co-workers (Ulijasz *et al.*, 2004). In a cyclophosphamide-treated mouse lung infection model and thigh infection model (Andes and Craig, 2002), the inactivation of *ritR* reduced the ability of the pneumococcus to proliferate in the mouse lung but did not reduce its ability to infect the thigh. Microarray analysis revealed that RitR was associated with the transcription of genes involved in iron uptake and the oxidative stress response. In DNA footprinting experiments, RitR was shown to bind directly to three sites in the promoter region of the *piu* operon, thereby providing a direct link between RitR and iron uptake regulation (Ulijasz *et al.*, 2004). The mechanism for attenuation of virulence appears to be due, at least in part, to repression by RitR of the *piu* iron uptake system by negative regulation. While iron is essential for the growth of most bacteria it can also be deleterious through the Fenton reaction which catalyses the synthesis of reactive oxygen intermediates from H_2O_2 (Andrews *et al.*, 2003; Imlay, 2003). The way that RitR is activated in the absence of a cognate HK and the mechanisms by which the pneumococcus senses iron are unknown. Even though RitR contains the conserved aspartate residue which is phosphorylated in other response regulators, various phosphate donors did not alter RitR binding to the *piu* operon as expected in phosphorylation-dependent regulation (Ulijasz *et al.*, 2004).

1.7.5.8 Other TCS

The remaining seven TCS are poorly characterised. No contribution to virulence of pneumococcus has been reported for the TCS03, 10 and 11. TCS10 (VncR/S) has been suggested to play a key role in the regulation of cell death and autolysis in response to vancomycin challenge (Novak *et al.*, 1999b), although using distinct mutations in *vncS*, (Robertson *et al.*, 2002) demonstrated that the loss of VncS function alone does not result in tolerance to vancomycin challenge or to other autolysis-inducing antibiotics *in vitro* and *in vivo*. TCS06 is reported to be involved in regulation of pneumococcal virulence factor *cbpA*, also known as *pspC* (Standish *et al.*, 2005).

1.8 Regulation of *dlt* operon by TCS

The wall of the Gram positive bacterium constitutes a multifaceted fabric that is essential for survival, shape, and integrity. Macromolecular assemblies of cross-linked peptidoglycan (murein), polyanionic teichoic acids (TAs), and surface proteins function within this envelope. TAs are composed of wall teichoic acid (WTA) and lipoteichoic acid (LTA). WTA is covalently linked to the peptidoglycan, whereas LTA is a macroamphiphile with its glycolipid anchored in the membrane and its poly(glycerophosphate) (Gro-P) chain extending into the wall. Protonated D-alanyl ester residues, one of the principal substituents of TAs in many low G+C Gram positive bacteria, are covalently linked to these chains and provide counter ions for determining the net anionic charge of the TA. Together with peptidoglycan, WTA and LTA make up a polyanionic network or matrix that provides functions relating to the elasticity, porosity, and tensile strength (Neuhaus and Baddiley, 2003).

The D-alanylation of LTA allows Gram positive bacteria to modulate their surface charge, regulate ligand binding and control the electromechanical properties of the cell wall. Genetic studies of the biosynthesis of LTA in various Gram positive bacteria have shown that the incorporation of D-Ala residues requires the activity of four gene products (DltA-D) (Figure 1.4), which are encoded by the *dlt* operon (Abachin *et al.*, 2002; Poyart *et al.*, 2001; Poyart *et al.*, 2003).

Although numerous *dlt* operons from Gram positive bacteria have been characterized, little is known about the regulation of their expression. In *S. agalactiae*, (group B streptococcus) a two-component transduction system was identified that was encoded by *dltR* and *dltS* located upstream of the *dlt* operon that directs incorporation of D-alanine residues into lipoteichoic acid (Poyart *et al.*, 2001), suggesting that DltR/DltS functions to control expression of the *dlt* operon, thereby regulating the level of D-alanine esters in *S. agalactiae* lipoteichoic acid. *S. pneumoniae* contains phosphorylcholine esters instead of D-alanyl esters in both LTA and WTA (Fischer, 1997). However, the *dlt* operon is activated in the end of exponential phase when bacteria grown in Todd-Hewitt broth (THB) (Mascher *et al.*, 2003) and pneumococci enhanced expression of the *dlt* operon during epithelial cell contact (ECC) (Orihuela *et al.*, 2004), indicating that addition of D-alanine to teichoic acids in pneumococci can occur. DNA binding assays for CiaR demonstrate that CiaR binding at the region immediately located upstream of the *dlt* operon and R6 *ciaR* defective strain mutant shown down-regulation of *dlt* genes compared

with wild-type, suggest that the CiaR/H system potentially regulated directly or indirectly this operon (Mascher *et al.*, 2003).

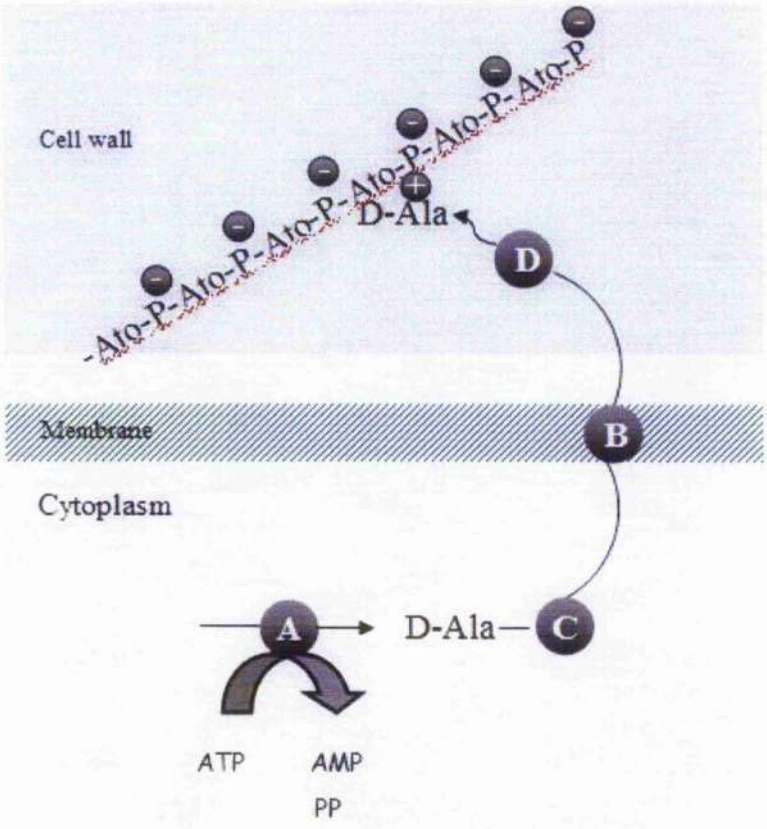


Figure 1.4- Schematic representation of the putative pathway of D-alanine transfer into teichoic acids.

A teichoic acid molecule is depicted as a chain of alternating alditol (Ato) and phosphate (P) residues. D-Alanine (D-Ala) is activated in the cytoplasm by DltA (A) via ATP hydrolysis and the release of pyrophosphate and is coupled to the phosphopantetheine prosthetic group of the D-alanine carrier protein DltC (C). The hydrophobic protein DltB (B) is likely to be involved in the transfer of D-alanine across the cytoplasmic membrane, and DltD (D), which bears a putative N-terminal signal peptide, is assumed to catalyze the esterification of teichoic acid alditol groups with D-alanine resulting in the introduction of positive charges into the otherwise negatively charged teichoic acids. Adapted from (Peschel *et al.*, 1999).

1.9 Genetic variability of *S. pneumoniae*

S. pneumoniae as the classical transformable organism is certainly prone to genetic variation, and the mosaic genes in antibiotic-resistant strains are a paradigm for intra- and interspecies gene transfer events (Hakenbeck *et al.*, 1999b). The pneumococcus can be divided into more than 90 serotypes based on capsular diversity but ~15 of these serotypes are the cause of the majority of invasive disease. Due to the clinical importance of this organism and the lack of a good correlation of the capsular serotypes to epidemiological spread of the species (Coffey *et al.*, 1998) much effort has been put into characterizing the population structure at the molecular level. The pneumococci are a naturally transformable species and the population structure is characterized by frequent horizontal gene transfers and recombination-mediated gene plasticity (Enright *et al.*, 1998). Multi-locus sequence typing (MLST) of housekeeping genes has been suggested as a solution to interrogating the population structure of pneumococci and has shown that individual serotypes may consist of a number of genetically diverse clonal complexes or ST types (Brueggemann *et al.*, 2003; Gertz *et al.*, 2003). Importantly, a recent study has shown that certain ST types are more commonly associated with disease than others (Brueggemann *et al.*, 2003).

The draft genome sequence of strain G54, a type 19F clinical isolate (Dopazo *et al.*, 2001), and the complete genome sequences of strain TIGR4, a type 4 virulent isolate (Tettelin *et al.*, 2001), and the avirulent laboratory strain R6 (Hoskins *et al.*, 2001) were published recently. Several studies have already taken advantage of DNA Microarrays to aid in the understanding of complex regulatory pathways in *S. pneumoniae*. One of the first high-density microarray studies in bacteria was done using pneumococcal DNA arrays (de Saizieu *et al.*, 1998). The use of an oligonucleotide Affymetrix array was employed to examine genetic diversity within a group of 20 *S. pneumoniae* isolates (Hakenbeck *et al.*, 2001). These isolates represented major antibiotic resistant clones isolated from throughout Europe, United States, South Africa and Papua New Guinea. PCR-based microarrays have also been used to compare the genomic composition of a smaller number of pneumococcal strains (Bruckner *et al.*, 2004; Hakenbeck *et al.*, 2001; Tettelin and Hollingshead, 2004). Comparison of complete *S. pneumoniae* genomes between TIGR4, R6 and G54 strains has shown that approximately 1802 genes (~80%) were shared by all of these three genomes (Tettelin and Hollingshead, 2004). Comparative genome hybridization using DNA microarrays allows the identification of regions of diversity. Analysis of genome hybridization of R6 and parental encapsulated strain D39 shown nine regions in TIGR4 that did not hybridize with the other two strains. Comparative genome hybridization of 13

additional strains, including clones that carry multiple drug resistance, show 13 regions of diversity involving multiple genes absent or divergent compared with TIGR4 (Tettelin and Hollingshead, 2004). Similar results were observed in a previous study of the R6 strain (Hakenbeck *et al.*, 2001). Also, in this comparative genome study 13 extra regions of diversity were identified for 17 additional strains belonging to 12 clonal groups. On the other hand, six clusters of genes were identified in R6 that were not present in TIGR4 (Hakenbeck *et al.*, 2001). These studies confirmed the genetic diversity that was previously thought to exist in populations of these naturally transformable bacteria.

1.10 Microarrays

The first report about DNA microarrays appeared in 1995 (Schena *et al.*, 1995) and since than microarray applications have been reported in many organisms including plants (Schena, 1996), yeast (DeRisi *et al.*, 1997; Lashkari *et al.*, 1997; Shalon *et al.*, 1996), filamentous fungus (Allen *et al.*, 2003), virus (Li *et al.*, 2001; Striebel *et al.*, 2004), protozoa (Bozdech *et al.*, 2003) and humans (DeRisi *et al.*, 1996; Schena *et al.*, 1996). DNA microarray technology has become an important research tool for biotechnology and microbiology (Polen and Wendisch, 2004). This technology is the result of automation and miniaturization of differential genes and permits a parallel analysis of gene expression and DNA homology for thousands of genes in a single experiment. Over the past several years, this unique technology has been used to explore hundreds of transcriptional patterns and genome differences for a variety of microbial species. Applications of microarrays extend beyond the boundaries of basic biology into diagnostics, environmental monitoring, pharmacology, toxicology and biotechnology (Majtan *et al.*, 2004).

1.11 Applications of DNA microarrays to bacterial systems

The two common applications of DNA microarray technology in microbiology are the exploration of genome-wide transcriptional profiles and the measurement of the similarities or differences in genetic contents among different microbes. However, other applications have been reported such as determination of virulence factors of microbial pathogens (Chizhikov *et al.*, 2001), host responses to microbial infection (McCaffrey *et al.*, 2004; Ren *et al.*, 2003b), gene profiling in response to drugs (Kato-Maeda *et al.*, 2001), analysis of microbial evolution and epidemiology (Leonard *et al.*, 2003; Saunders *et al.*, 2004), diagnostic applications (Bae *et al.*, 2005; Korczak *et al.*, 2005; Yu *et al.*, 2004) and predicting biochemical pathways (Doran *et al.*, 2003; Phue *et al.*, 2005). DNA microarray

technology is being used to study many bacterial species ranging from standard laboratory strains and pathogens to environmental isolates (Table 1.4).

Table 1.4- Use of DNA microarray technology in the study of different bacterial species.

Bacteria species	Application study	Reference
E. coli	Genome-wide transcriptional profiles.	(Oh and Liao, 2000; Wendisch *et al.*, 2001; Winterberg *et al.*, 2005)
B. subtilis	Genome-wide transcriptional profiles.	(Caldwell *et al.*, 2001; Guedon *et al.*, 2003; Stanley *et al.*, 2003)
M. tuberculosis	Genome-wide transcriptional profiles; gene profiling and drugs.	(Butcher, 2004; Gryadunov *et al.*, 2005; Stewart *et al.*, 2002)
H. pylori	Comparative genomics; genome-wide transcriptional profiles.	(Bjorkholm *et al.*, 2001; Thompson *et al.*, 2003; Wen *et al.*, 2003)
N. meningitidis	Comparative genomics; genome-wide transcriptional profiles.	(Grifantini *et al.*, 2003; Grifantini *et al.*, 2004; Swiderek *et al.*, 2005)
Pseudomonas aeruginosa	Comparative genomics; genome-wide transcriptional profiles;	(Ernst *et al.*, 2003; Wagner *et al.*, 2003; Whiteley *et al.*, 2001)
S. pneumoniae	Comparative genomics; genome-wide transcriptional profiles.	(Hakenbeck *et al.*, 2001; McCluskey *et al.*, 2004; Mohedano *et al.*, 2005; Sung and Morrison, 2005)

Two major types of DNA microarray have been developed, one is the oligonucleotide-based array and the other is the PCR product-based array (Ye *et al.*, 2001). The DNA microarray is a powerful tool that allows the development of our investigation of genome-wide functions.

1.11.1 Transcriptome

Measuring thousands of transcript levels in parallel is one of the most widespread applications of DNA microarray technology. Microarrays allow the production of a gene expression profile for a particular organism grown under different environmental conditions (Majtan *et al.*, 2004). DNA microarrays can also detect the presence of an mRNA transcript, and can estimate its abundance relative to other mRNA species within the same sample (Majtan *et al.*, 2004). Furthermore, DNA microarrays can be used to study regulatory systems controlling gene expression such as sigma factors, global transcriptional regulators, and TCS (Dharmadi and Gonzalez, 2004). DNA microarray has been used to investigate the regulation of virulence genes by the TCS in *S. pneumoniae* (McCluskey *et al.*, 2004; Sebert *et al.*, 2002).

1.11.2 Comparative genomics and genomotyping

Genomic hybridization of a whole genome array detects the presence or absence of similar DNA regions in other microorganisms. It is an effective way to conduct a comparative genomic study in the absence of complete genome sequences. Strain comparison by hybridizing genomic DNA to microarrays (genomotyping) is a more realistic approach than the whole-genome sequencing of dozens of strains (Majtan *et al.*, 2004). DNA microarrays can facilitate a better understanding of the genetic differences between closely related organisms, providing useful information for the identification of virulence factors, exploration of molecular phylogeny, improvement of diagnostics and development of vaccines (Dhiman *et al.*, 2001). DNA microarray technology is also an excellent way to identify changes in the genetic contents of the same strain after long-term adaptation or strain optimization (Riehle *et al.*, 2001). Regions of diversity in *S. pneumoniae* were found in studies of strain comparison using DNA microarrays (Bruckner *et al.*, 2004; Hakenbeck *et al.*, 2001; Tettelin and Hollingshead, 2004).

1.11.3 Determination of virulence factors

Many genes associated with virulence are regulated by specific conditions. Virulence gene expression ca be monitored by growing the pathogens in the appropriate *in vivo* models (cell cultures or animals) and, after recovering the bacteria for RNA preparation, compared with the expression of the genes under in *vitro* conditions (Grandi, 2001). Microarray analysis of pneumococcal gene expression during invasive disease showed dramatic changes in a variety of genes (Orihuela *et al.*, 2004).

The second approach relates to genome comparison studies, by identification of candidate virulence genes in different strains. Also microarray technology has been used to identify the presence of specific markers in bacterial genomes associated with pathogenesis (Majtan *et al.*, 2004).

1.12 Aims of this project

The aims of this project were focused on the study of:

1. Pneumococcal two-component systems. To analyze the transcriptional profiles and identify the genes regulated by RR06 and RR09 in different strains: TIGR4, D39, R6, and 0100993. To examine how mutation of these genes affects the virulence of the bacterium. To determine the role of putative virulence factor *dlt* operon in the phenotype of the *ciaR/H* mutants;

2. Analysis of genetic variation within *S. pneumoniae*. Use of comparative genome hybridizations using DNA microarray of a selected group of 10 pneumococcal strains that represent 6 major multi-locus sequence types found in the UK, and evaluation of the importance of the genetic variation in virulence of the bacteria. To analyze the differences in the gene regulation by the TCSs in strains TIGR4, R6, D39 and 0100993.

Chapter 2

Material and Methods

2.1 Bacterial strains and growth conditions

S. pneumoniae strains used for this study are described in Table 2.1. The bacteria were grown on blood agar Nº 2 (Oxoid, Basingstoke, UK) supplemented with 5% (vol/vol) defibrinated horse blood (E&O Laboratories, Bonnybridge, United Kingdom) overnight at 37 °C in the presence or absence of oxygen (candle extinction jar) or in Brain Heart Infusion (Oxoid, Basingstoke, UK) at 37 °C. *E. coli* strains were grown in Luria-Bertani broth at 37 °C overnight with agitation at 200 rpm or on Luria Bertani (LB) agar plates. When necessary appropriate antibiotics were added to the growth media cultures: *S. pneumoniae*, 1μg/ml of Erythromycin and 100 μg/ml of spectinomycin; *E. coli* 200 μg/ml of spectinomycin and 100 μg/ml of ampicillin.

2.2 Glycerol stock and culture check

One individual colony of blood agar culture was inoculated in to 20 ml of Brain Heart Infusion (BHI) and grown at 37 °C overnight. 100 μl of overnight culture was inoculated in to 10 ml of BHI, and grown at 37 °C until mid-log phase (OD_{600nm} ~0.6). Glycerol stocks were prepared by adding sterile glycerol, to the mid-log phase BHI cultures, to a final concentration of 20% (vol/vol) and 1 ml aliquots of the cultures were frozen immediately at -80 °C in 2 ml cryotubes. All pneumococcal cultures were checked by plating out and examining morphology, production of α-haemolysis on blood agar plates and sensitivity to diffusion of optochin disk (5μg of ethylhydrocupreine). Serotypes were confirmed by the Quellung reaction using specific antisera (Statens Seruminstitut, Copenhagen, Denmark) against capsular polysaccharide.

Table 2.1- List of strains used in this study.

Strain	Details	Reference
TIGR4	Serotype 4, clinical isolate	(Aaberge *et al.*, 1995)
D39	Serotype 2, NCTC 7466	(Avery *et al.*, 1944)
R6	Subclone of R36A, derivative of D39	(Smith and Guild, 1979)
0100993	Serotype 3, clinical isolate	(Throup *et al.*, 2000)
TIGR4 Δ*rr06*	TIGR4 with replacement of *rr06* with ermAM cassette	This work
D39 Δ*rr06*	D39 with replacement of *rr06* with ermAM cassette	This work
R6 Δ*rr06*	R6 with replacement of *rr06* with ermAM cassette	This work
0100993 Δ*rr06*	0100993 with replacement of *rr06* with ermAM cassette	(Throup *et al.*, 2000)
TIGR4 Δ*dltA*	TIGR4 with replacement of *dltA* with spec cassette	This work
TIGR4 Δ*rr09*	TIGR4 with replacement of *rr09* with ermAM cassette	This work
D39 Δ*rr09*	D39 with replacement of *rr09* with ermAM cassette	(Blue and Mitchell, 2003)
0100993 Δ*rr09*	0100993 with replacement of *rr09* with ermAM cassette	(Throup *et al.*, 2000)
D39 Δ*ciaR*	D39 with replacement of *ciaR* with spec cassette	(Ibrahim *et al.*, 2004b)
D39 Δ*htrA*	D39 with replacement of *htrA* with spec cassette	(Ibrahim *et al.*, 2004b)
TIGR4 Δ*pspC*	TIGR4 with replacement of *pspC* with ermAM cassette	(Tuomanen, 1999)
D39 Δ*pspC*	D39 with replacement of *pspC* with ermAM cassette	(Tuomanen, 1999)
P11	Serotype 14, ST9	(Jefferies *et al.*, 2004)
N16	Serotype 14, ST9	(Jefferies *et al.*, 2004)
P33	Serotype 14, ST9	(Jefferies *et al.*, 2004)
10	Serotype 14, ST124	(Jefferies *et al.*, 2004)
48	Serotype 14, ST124	(Jefferies *et al.*, 2004)
50	Serotype 14, ST124	(Jefferies *et al.*, 2004)
P49	Serotype 3, ST180	(Jefferies *et al.*, 2004)
PMEN7	Serotype 19A, ST75	(Smith and Klugman, 1997)
PMEN13	Serotype, 19A ST41	(Smith and Klugman, 1997)
PMEN23	Serotype 6A, ST37	(Richter *et al.*, 2002)

2.3 Primers and plasmids

The oligonucleotide primers used in this study are listed in Table 2.2. The primers were designed with Vector NTI 9.1.0 (Invitrogen Corporation) and provided by Sigma-Genosys. Concentrations of 100 µM were prepared as main stock and 20 µM as work stock in nuclease free water (Ambion®). The primers used in PCR for the genomic microarrays confirmation were designed and provided by Bacterial Microarray Group at St. George's Hospital (BµG@S). The primers used in Real-Time quantitative PCR (Table 2.3) were designed in Vector NTI 9.1.0 or by Sigma-Genosys, and were designed to give a product length between 50 to 200 bp, and similar Tm to minimize difference in the amplification efficiency. Specific conditions were kept to ensure quality of the primers to avoid primer dimers, hairpin loops formation. Also a BLAST of the primer sequence, using BLAST tool of TIGR web site (http://tigrblast.tigr.org/cmr-blast), against the whole genome of pneumococcus TIGR4 and R6 strains was performed to avoid cross-reaction with non-specific sequences. PCR-Script™ plasmid (Stratagene) was used for cloning the *dltA* gene and pCR® 4-TOPO® plasmid (Invitrogen™) was used for cloning the *spr0062* gene (annotated as *sp0063* in TIGR4 strain sequence).

Table 2.2- List of primers used in this study.

Primer	Sequence	Used for
TCS06-For	CTTTAGTGGCAAGTTTGGCTG	Amplification *rr06* fragment with erythromycin cassette
TCS06-Rev	TTGCATTTTACTAGTCACTTC	Amplification *rr06* fragment with erythromycin cassette
TCS09-For	CCTGTCATTGATGCCAGCAAATATCC	Amplification *rr09* fragment with erythromycin cassette
TCS09-Rev	CCACAAGAGCTGACTCCGAACCGG	Amplification *rr09* fragment with erythromycin cassette
dltA Amp For	GTGTCAAATAAACCAATAGC	Amplification of *dltA*
dltA Amp Rev	TCTCTTATTCACCTCGTT	Amplification of *dltA*
dltA Amp inv1	GGCGCGCCGTCAATGACTGCAGC	Knockout of *dltA*
dltA Amp inv2	GGCGCGCCCGATATTACCAAGGC	Knockout of *dltA*
dltA For	GCTCAGGTTCAAGAAGCCTTT	*dltA* internal fragment amplification
dltA Rev	TGATTCCAGGCATTTTCTCAC	*dltA* internal fragment amplification
ErmAM-For	CATGAACAAAAATATAAAATATTCTC	Amplification of erythromycin cassette
ErmAM-Rev	CTCATAGAATTATTTCCTCCCG	Amplification of erythromycin cassette
Spec up	GGCGCGCCATCGATTTTCGTTCGTGAATA	Amplification of spectinomycin cassette
Spec dn	GGCGCGCTATGCAAGGGTTTATTGTT	Amplification of spectinomycin cassette
gyrA For	TACGCCATGAGTGTTATCGTAGC	RT-PCR of *gyrA*
gyrA Rev	ACTATCTCCATCCATGGAACC	RT-PCR of *gyrA*
010-up4-*cbpA*	ATGTTTGCATCAAAAAGCGAAAGAAAAGTACA TTATTCAATTCG	RT-PCR of *cbpA*
cbpA-SKH2	CATACCGTTTTCTTGTTTCCAGCC	RT-PCR of *cbpA*
spr0062 P-1	TCTATGATTGGTATTTCTATCGTAGG	Knockout *spr0062*
spr0062 P-2	GGCGCGCCTGAGGTAAGATCATGTAAAGGTAA CC	Knockout *spr0062*
spr0062 P-3	TCTTACCTCAGGCGCGCCACTGCCTTTATCTTC TGGTTGCTTGG	Knockout *spr0062*
spr0062 P-4	CAAATTTAGCAGTAAATTCTTCTGGG	Knockout *spr0062*

Table 2.3- List of primers for quantitative Real-Time PCR used in this study.

Gene	Sense primer	Anti-sense primer	Product length (bp)
gyrA	GCGCGAGCTCTTCCTGATGT	TATGGGGTTTGTCTGGGGTC	100
ddl	AGTTCCTTATGTGGCTATCGTTG	CGGAGTTCTTCTTGGTTTTCAG	150
sp0060	ACGGTAGAGACTTATGTTGCTTG GA	AGATAAATGGAGACGGACGC	145
sp0061	TGGTTGTAGACGACGAAGTTG	CGAGCCACGATAAAGAGACG	160
sp0062	CTACCTTCTTCGCTCACCGT	GTACAAAGGCACCACCAAAA	150
sp0063	GGGATCTTCCTTTGGATTGC	AAGACACCAAGTACAGATGC	152
sp0064	ATGGTCGCTTCTGTGAGGAG	GTGTAAATGTTGTCTTGTGGGC	71
sp0065	TCTATGACGAACGCAAATGG	GAGCAAAAGACACAAGGACAGT	111
sp0066	TGGCTTGACTCTCTACACAG	CATAGGCACCAGTTTCTTCC	101
PspA[1)]	GGTAGCACTAGCGAAGAAAG	ACTTGATGTTGAGCAGTAGC	108
PspA[2)]	TATCTTAGGGGCTGGTTTTGT	CGCTGCATCATAGTCTTTCTCA	103
sp0090	GTGGTTGCCTTCAAAGACTAC	TCCGACCCATTTACTATTTATGAT AC	75
sp0091	GCAACCATCACGCTCTTAAC	GCCATACGACCTGTTTCTTTC	197
sp0092	GGTCTTGTTTACGGTCCAG	TGTTACCAGTGTTCCATCC	121
sp2141	AGTCTGACCAAGCATCTATCTC	TGTAAGCCAAATCTTCGTAAGC	168
sp2142	CAGTCTGGGTGGCTCTATC	GTGCGACCGTGTATTCTTC	101
sp2143	CTACATTGACGAGGGCAAAC	CGGACATTGGCTTCACTGG	84
sp2144	CGACAGCCCAACTCAGAC	CCAGATCCAGCCGTTAAGG	191
sp0461	CCATCGCAACAGGCTACC	TGTGACCCAATCCATACTTCC	185
sp0462	AACCAGTCCAGCGATAGG	CTTCTGTCAAGGTGTATGTCC	185
sp0463	ATACACCTGTGAACCACCAAG	CATTCTATCGCTCCAGTTTGC	104
sp0464	GTATCTTCTTTGTTATGGCTCTG	ATCATCATAGGAATACGAATCAT C	185
sp0466	GGTGTCTCGCTTGTATTATCG	TGTCAGCCTCATCCAACG	86
sp0467	GTGTCTCGTTATTATTATCGTAT TG	CCTCAAGTTCTGCCTTATCC	91
sp0468	TCTCGCCTACAATCAACGC	ATAATCTGCTCCCAAATAAACCG	169
sp0304	AGTTAAGACAAAGGACACAGG	ATACGAATACCATGAGCAAGC	93
sp0305	GTCTTCATCACTTATGGCACAG	GCACTAATAGCATCAACTACAAT ATC	81
sp0306	TGATTTCAGAAAGATACGAGTG ATG	TGAGACATAAACTCCTTTATTCG C	82
sp0310	ATGTGTGTTAAACATAATTGGG	CCATTGCTACAAGAAGACC	139

Table 2.3- Continuation.

Gene	Sense primer	Anti-sense primer	Product length (bp)
sp2173	TCTTTGGCTCTAGTGAATGGC	ATAAGGACGGTAGGAACGATTG	86
sp2174	GAAGTTATTGAAATTATTGATGAGTTG	CCATACTATCCAAGACACCTG	94
sp2175	GGAATCCGTAGCCCTATCAAC	GGAACCAGAAGGACAGACTC	97
sp2176	GCTATCCTGTCTATAATGTTTTG	GCAGCCAAACTATCCGAATC	76
sp2190[1)]	TTAACGAGTTGAACAACATTAAGAAC	CATCAGTATCTGTAGTTGGCTTTC	83
spr1995[2)]	GCGACAGAGAACGAGGGAAGTA	GTTTAAGGCGACATTTTGGG	162
sp2240	AATGGCGCCAGCAAAGTAGA	GCAATATCAGAGAAAGTGTCAGC	89
htrA	TCCCTATCAACCCCGAAAAG	CGATAGCGTCTCTCTCCTGC	147
ciaR	GACATGGATTTGAACTGGGAGCGGA	TGAACGTTTGAGAAGGGCCTGAA	95
ciaH	CGTGTGATTTTGATGGAGACC	GACAAAATCCAGAAACTAGCCATC	142

[1)] TIGR4 sequence annotation
[2)] R6 sequence annotation

2.4 Preparation of pneumococcal chromosomal DNA

S. pneumoniae chromosomal DNA was prepared by a phenol extraction method (Saito and Miura, 1963). 10^6 CFU/ml from a glycerol stock was inoculated in to 20 ml of BHI, and incubated at 37 °C until mid-log phase (OD_{600nm} ~0.6). The bacterial cells were harvested by centrifugation at 5,000 x *g* at room temperature for 15 min (Sigma laboratory centrifuge 4K15). In the case of serotypes 3 strains the centrifugation had to be increased to 12,000 x *g* for 20 min. The pellet was resuspended in 1 ml of extraction buffer (10 mM Tris pH 8.0, 100 mM EDTA pH 8.0, 0.5% SDS) and incubated for 1 h at 37 °C. Proteinase K (Sigma-Aldrich) was added to a final concentration of 100 µg/ml and the mixture was further incubated for 3 h at 50°C. RNase (Sigma-Aldrich) was added to a final concentration of 20 µg/ml, and the mixture was incubated at 37°C for 30 min. An equal volume of phenol/chloroform/isoamylalcohol (25:24:1 vol/vol, Fisher Scientific UK, Ltd.) was then added and the mixture was well mixed by inverting the tube several times. Following centrifugation at 18,000 x *g* (Eppendorf centrifuge 5417C, Germany) for 5 min, the aqueous layer was carefully removed to a new 1.5 ml tube and DNA was precipitated by adding 0.2 volume of 10 M ammonium acetate (Sigma-Aldrich) and 1 volume of 100%

ethanol (Fisher Scientific UK, Ltd). The mixture was then centrifuge at 18,000 x g for 15 min and the DNA pellet was left to air dry for 10-15min and them resuspended in TE buffer (Qiagen) pH 7.4 and store at -20 °C.

The DNA used in microarray experiments was isolated from Genomic-tip 100/G (Qiagen) columns and with DNA buffer set (Qiagen) to avoid any phenol contamination. The strains were grown as described above and the extraction was made as detailed in the manufacturer's instructions. The DNA was precipitated by adding 0.5 volume of isopropyl alcohol (Fisher Scientific UK, Ltd) and the mixture was then centrifuge at 5,000 x *g* at 4°C for 15 min. The pellet was washed with cool 70% ethanol and then centrifuge at 5,000 x *g* at 4°C for 10 min. The pellet was left to air dry for 10-15min and resuspended in TE buffer (Qiagen) pH 7.4 by incubation overnight at room temperature and stored at -20 °C.

2.5 Analysis and measurement of DNA

DNA sample concentrations were measured with NanoDrop® ND-1000 UV-Vis Spectrophotometer (NanoDrop® Technologies, USA). The sample concentration is given in ng/ul calculated using the Beer's Law based on absorbance at 260 nm against a blank of TE buffer. The ratio of absorbance at 260 and 280 nm is used to assess the purity of DNA. A ratio of ~1.8 is generally accepted as "pure" for DNA. Also the DNA was analysed by running 1% (w/vol) agarose gel (Gibco BRL Life Technologies, UK) containing 0.5 µg/ml ethidium bromide (Sigma-Aldrich) in TAE buffer. The DNA gel was visualized under UV transilluminator (Spectroline, TVC-312A) and images were captured using an UVP GelDoc system (UVP Laboratories).

2.6 DNA techniques and *E. coli* transformation

2.6.1 Polymerase chain reaction

Polymerase chain reaction (PCR) was processed in reactions of 50 µl consisting of: ~ 500 ng of genomic DNA, 5 µl of thermophilic DNA polymerase Buffer (pH 9.0), 2.5 mM magnesium, 0.5 mM each dNTP, 0.8 µM each primer (table 2.2.) and 2.5 U of Taq polymerase (all purchased from Invitrogen™). The reaction volume was made up to 50 µl with nuclease free H_2O. The PCR conditions were: 95°C for 5 min followed by 30-35 cycles of 95 °C for 30 s, 50-65 °C for 30 seconds and 72°C for 30-120 s. A final extension of 72°C for 10 min was performed. The PCR products were analysed in 1-2% agarose gel

simultaneously with 100 bp or 1 kb ladder (Promega) to confirm the molecular weight of dsDNA bands.

2.6.2 Cloning and transformation of *E. coli* with plasmid constructs

E. coli strain Top10 electrocompetent cells were used for transformation, carrying the plasmid constructs to use in later transformation of pneumococcus strains. The whole gene to be mutated was amplified from the pneumococcal chromosomal DNA, and PCR products were run in 1% agarose gels. DNA bands were excised from the gel and purified using the Qiaquick Gel Extraction Kit (Qiagen) according to the manufacturer's protocol. PCR-Script™ Amp SK (+) plasmid (Statagene appendix A2) and pCR® 4-TOPO® (Invitrogen™) were used for cloning according to the manufacturer's recommendations. The orientation of genes was confirmed by digestion with restriction endonuclease enzymes. The plasmid construct was used to transform the *E. coli* strain Top10 electrocompetent cells and amplified. 100 µl aliquots of competent cells were left in ice and 5 µl of plasmid DNA was added. The cells were incubated on ice for 20 min. The mixture was then transferred to pre-chilled 1 mm gap electroporation cuvettes (Molecular BioProducts) and electroporated using Gene PulserTM (Bio-Rad) with the following settings: voltage of 1.5 kV; capacitance of 25 µF and resistance of 200 Ohm. The usual time constant with these settings was ~ 4.6. The cuvettes were placed back on ice and 200 µl of SOC medium (Invitrogen™) was added and transferred to 1.5 ml microcentrifuge tubes and incubated at 37°C for 1 hour. The *E. coli* cells were plated onto LB agar plates with the appropriate antibiotic and with IPTG and X-gal to facilitate the identification of transformed cells by the blue/white selection. Cells containing the vector with the insert form white colonies while the cells that do not contain the vector with the insert form blue colonies. Individual white colonies were picked up and inoculated in 10 ml of LB broth and incubated at 37°C overnight.

2.6.3 Plasmid preparation and purification

The transformed *E. coli* cells were collected, after the overnight incubation, by centrifugation at 5,000 x *g* at room temperature for 5 min. The plasmids were isolated from the cells using the Qiaprep Spin Miniprep Kit (Qiagen) according to the manufacturer's instructions. Plasmid DNA concentration and quality was confirmed as described in section 2.5.

2.6.4 DNA sequencing

The plasmid carrying the insert was sequenced to confirm the correct inserts. The samples were sequenced in MBSU (Molecular Biology Support Unit, University of Glasgow, UK) using the appropriate primers with concentration at 3.2 μM. The sequences were performed with MegaBACE™ 1000 DNA sequencer (Amersham Biosciences) and a protocol based on the dideoxy method (Sanger *et al.*, 1977). Sequence alignment was confirmed with AlignX® (Invitrogen Corporation).

2.7 Construction of pneumococcal mutants

The *S. pneumoniae* mutants were constructed to study the role of TCSs 06 and 09 in expression of genes and virulence of the bacteria. The *dltA* gene, supposedly regulated by the TCS *ciaR/H,* was mutated to study the importance of the *dlt* operon in the phenotype of the *ciaH* mutant. The gene *spr0062* (annotated as *sp0063* in TIGR4 strain sequence) also was mutated to study its importance in the phenotype of *rr09* mutant.

For transformation, the whole of the gene to be mutated was amplified from the pneumococcal chromosomal DNA (strain TIGR4 and D39) using forward and reverse specific primers for each gene (Table 2.2). The genes were cloned in PCR-Script™ Amp SK(+) plasmid (Stratagene) or pCR® 4-TOPO® (Invitrogen™). Inverse internal primers (Table 2.2) were designed to amplify the 5' and 3' ends of the gene together with plasmids and to create *AscI* sites in the resulting PCR product. This PCR product was self-ligated to produce a plasmid carrying the interrupted gene. The plasmids were purified as described in section 2.6.3 and sequenced, using primers T3 and T7 specific for PCR-Script™ and for pCR® 4-TOPO® plasmids to confirm the deletion of the segment. An *AscI*-generated antibiotic cassette (spectinomycin) (Ibrahim *et al.*, 2004a) was ligated into plasmid after digestion with *AscI*. The fragment containing the gene to be modified with antibiotic resistance cassette was amplified by PCR and used to transform the pneumococcus. These strategies were used for making *dltA* and *spr0062* null mutants in TIGR4 and D39 respectively.

2.7.1 Transformation of *S. pneumoniae* strains

Transformation of *S. pneumoniae* strains TIGR4 (serotype 4), D39 and R6 (both serotype 2) was done using a modification method of Lacks and Hotchkiss (Lacks and Hotchkiss, 1960). Competence-stimulating proteins (Havarstein *et al.*, 1995) (CSP-1 for D39 and R6

and CSP-2 for TIGR4) were used for inducing competence in these different serotypes (Pozzi *et al.*, 1996). For preparation of competent cells, 10 ml of CAT medium (Porter and Guild, 1976) (see appendix A1) supplemented with 20% glucose and 0.5M K_2HPO_4 (CAT/GP medium) were inoculated with 200 µl of glycerol stock of TIGR4, D39 or R6 strains, and grown a 37°C until they reached OD_{600nm} 0.3-0.4. The cells were harvested by centrifugation at 5,000 x *g* for 10 min at 4°C (Sigma laboratory centrifuge 4K15). The cells were resuspended in CAT/GP medium containing 20% glycerol and were frozen at -80°C or used immediately for transformation.

For pneumococcus transformation, 900 µl of CAT/GP medium supplemented with 4% BSA and 0.1M $CaCl_2$ (CTM medium) and 100 ng of CSP1 or CSP2 was added to 100 µl of cells. The 1 ml competent cells were divided into 200 µl aliquots, different concentrations of transforming DNA (0.5-2 µg) were then added as well positive control DNA (pVA 383 erythromycin resistant plasmid) and a no DNA control. The cells were incubated 37°C for 10 min followed by 30°C for 20 min. 500 µl of CTM was added and the cells were incubated 37°C for 2 hours. Transformed cells were selected on blood agar plates with appropriate antibiotic selection.

2.7.2 Moving mutation from existing mutants

Some of the mutant strains used in this study were made by PCR amplification of the mutated genes from existing mutants of different serotypes.

2.7.2.1 *rr06* mutants

The original *S. pneumoniae* 0100993 *rr06* mutant was generated previously (Throup *et al.*, 2000) by allelic replacement with a constitutively expressed erythromycin resistance cassette. PCR amplification of the mutated region using the primers TCS06-F and TCS06-R (Table 2.2) was performed and the amplicon transformed into *S. pneumoniae* D39 (serotype 2) and TIGR4 (serotype 4) strains using the method described in section 2.7.1.

2.7.2.2 *rr09* mutants.

As the *rr06* mutant, the 0100993 *rr09* mutant was generated previously (Throup *et al.*, 2000). The mutation was moved by PCR amplification of the fragment containing the mutated *rr09* gene and carrying the erythromycin resistance cassette using the primers TCS09-F and TCS09-R (Table 2.2) and using this PCR product to transformed the *S.*

pneumoniae D39 (Blue and Mitchell, 2003) and TIGR4 (serotype 4) strains as described in section 2.7.1.

2.7.3 Confirmation of mutants

The successful replacement of the wild-type gene with the antibiotic resistance cassette in *S. pneumoniae* TIGR4, D39 and R6 was confirmed by PCR. Genomic DNA was prepared by phenol treatment as described in section 2.4 from cultures grown on blood agar supplemented with appropriate antibiotic. PCR of fragment containing the mutated gene and respective antibiotic resistance cassette was performed and the size of this fragment was compared in a 1% agarose gel. Homologous recombination of mutated gene resulted in an increase/decrease of the PCR product size for mutants when compared with wild-type. To confirm that the mutations occurred at the correct position in chromosome, PCR reactions with primers specific to genomic regions flanking the insertion region and antibiotic resistance cassettes gene primers were performed. Also, RNA of mutant strains and wild-type were prepared, as described in section 2.12 and reverse transcriptase PCRs of target genes were performed to ensure no transcription of mutant gene in transformed strains.

2.8 Phenotype study *in vitro*

2.8.1 Bacterial growth

2.8.1.1 Growth curve

For growth phenotype comparison, aliquots of glycerol stocks of pneumococcus strains were quickly thawed at 37°C water bath and 1x 10^6 CFU/ml were inoculated in 20 ml of BHI and incubated at 37°C or 40°C (preheated BHI) for 12h. At intervals of 1 hour 1 ml aliquot were withdrawn for each strain sample and the OD_{600nm} were measured in spectrophotometer (Unicam UV2, UV/Vis) against BHI as a blank.

2.8.1.2 Viable count

To enumerate the number of colony forming units (CFU) of pneumococcal strains stored as glycerol stocks, cells were pelleted by centrifugation at 13,000 x g (Eppendorf centrifuge 5417C, Germany) for 3 min and resuspended in equal volume of phosphate

buffered saline (PBS). The viable count was performed with serial 10-fold dilutions in PBS in 96-well plates (Nunclon® surface, Nalge International™). 3 x 20 µl of each dilution were plated on blood agar plates (in triplicate) and incubated at 37°C overnight. The colonies were counted in dilutions that presented a number of 30-200 colonies and the number of CFU/ml was calculated by the average of the number of colonies x 50 x dilution factor.

In a growth phenotype comparison, at intervals of 2 hours aliquots of 20 µl were withdrawn from BHI cultures and viable count was made as described above.

2.8.2 Aerobic and anaerobic growth

For growth phenotype comparison in aerobic and anaerobic conditions, 10 µl of glycerol bacterial stocks were dropped in the middle of blood plates and uniformly spread over the plate and incubated at 37 and 40°C in an anaerobic environment (CO_2 candle jar) or an aerobic environment (without CO_2 candle jar) overnight.

2.8.3 Oxidative stress

The sensitivity to H_2O_2 was tested by exposing aliquots of 1 ml of cultures grown to OD_{600nm} 0.3 to 40 mM of H_2O_2 (Sigma-Aldrich) for 5, 10 and 15 minutes at room temperature. A bacterial viable count, as described in section 2.8.1.2 was made before and after the exposure to H_2O_2.

2.8.4 Adherence assay

The adherence assays were performed as described previously by Kharat and Tomasz (Kharat and Tomasz, 2003). Human pharyngeal cell line Detroit 562 cells (Pancholi and Fischetti, 1997) were inoculated into 24-well tissue culture plates (Corning Incorporated, Costar®). The plates were maintained at 37°C in 5% CO_2-95% air with RPMI 1640 medium (Schuster, 2002) without phenol red (Gibco BRL, Life Technologies, UK) but supplied with 1 mM sodium pyruvate and 10% fetal bovine serum. Glycerol bacterial stocks were quickly thawed at 37°C and cells were harvested by centrifugation before resuspension in RPMI 1640 without phenol red supplemented with 1% fetal bovine serum to give a suspension of 10^7 CFU/ml. Monolayers of Detroit 562 cells in 24 well plates were washed with 2 × 1 ml PBS and incubated with 1 ml of bacterial suspension for 2 h. After incubation at 37°C in 5% CO_2, the bacterial suspension was decanted from each well and

the Detroit 562 cells gently washed with 3 × 1 ml PBS to remove non-adherent bacteria. Human pharyngeal cells were detached by treatment with 200 µl of 0.25% trypsin-0.1% /EDTA prepared in PBS and lysed by addition of 800 µl ice cold 0.025% Trition-X-100 in PBS. The number of bacteria adherent to and/or internalised by the pharyngeal cells were quantified by viable counts of the Detroit cell lysates, as described in section 2.8.1.2. Wild-type and mutant strains grow comparably in media without Detroit 562 cells.

2.8.5 Lowest pH for growth initiation

To study the pH tolerance of TIGR4 *dltA* mutant in comparison to wild-type, 1x 10^6 CFU/ml were inoculated in 20 ml of BHI adjusted to pH 5.0, 5.5, 6.0, 6.5, 7.0, 7.5 and incubated at 37°C for 12 hours. The assay was done in triplicate and each hour, an aliquot of 1 ml was taken to measure the OD_{600nm} in a spectrophotometer and the lowest pH at which the bacteria survived was notated (Diaz-Torres and Russell, 2001).

2.8.6 Antimicrobial peptides

It is very important to use polypropylene and not polystyrene microtitre plates, since cationic peptides bind polystyrene. The antibacterial peptide activities were assayed in TIGR4 wild-type and *dltA* mutant strains, using the minimal inhibitory concentration (MIC). The MIC for cationic anitimicrobial peptides were determined using a broth microdilution assay modified from the method of Amsterdam (Amsterdam, 1996). Mueller Hinton agar plates (MHA) (Oxoid, Basingstoke, UK) were inoculated with pneumococcus strains from glycerol stock, and grown overnight at 37°C. 10 ml of Mueller Hinton Broth (MHB) (Oxoid, Basingstoke, UK) were inoculated with individual colonies of overnight culture and grown at 37°C until OD_{600nm} 0.3-0.6. The bacterial cultures were diluted in MHB to give 1x 10^6 CFU/ml. Serial dilutions of Nisin, Gramidicin D, Indolicidin, Magainin II, Cecropin B and Defensin HNP1-2 (All from Sigma-Aldrich) were made in polypropylene 96-wells plates (Corning Incorporated, Costar®). The test peptides were dissolved in MilliQ water to obtain a concentration 1 mg/ml. An equal volume of 0.02% acetic acid, 0.4% BSA (Sigma-Aldrich) were added to give a concentration 500 µg/ml. Serial doubling dilutions in 0.01% acetic acid, 0.2% BSA were performed to get serial dilutions at 10 times the required test concentrations. A control with 0.01% acetic acid, 0.2% BSA without peptide was made as a blank medium. 100 µl of bacterial suspension were added in each well of new polypropylene 96-well plates, except in blank medium that contained 100 µl of MHB for sterility control, and 11 µl of 10x test peptide dilutions. The microtiter plates were incubated at 37°C with slow agitation, to prevent cell precipitation,

and measured after 8 hours of incubation at 630 nm in MRX revelation microplate reader (Dynex Technologies, Denkendorf, Germany). The MIC was taken as the lowest concentration of antimicrobial peptide that reduced growth more than 50%.

2.8.7 Morphology of mutants

To observe morphological changes in the capsule of TIGR4 wild-type, *rr06* and *dltA* mutants, a quellung reaction was done. The bacteria were grown in BHI at 37°C until medium log phase, and a loop of each culture was placed onto a slide, air dried for ~15 min and mixed with 10 µl of methylene blue and 10 µl of antiserum type 4 capsule (Statens Seruminstitut, Copenhagen, Denmark). A glass cover slip was placed over the slide and incubated at 37°C for 1 hour in humid box. The slides were observed using an optical microscope with magnification 400 and 1,000 x. The quellung reaction was used also to confirm the capsule type of the different strains.

2.9 Phenotype Study *in vivo*

The animal work was done in collaboration with Dr. Alison Kerr and Dr. Gavin Paterson. The studies were carried out under appropriate licensing from the Home Office, UK, and obeyed local regulations of the University of Glasgow.

2.9.1 Mice and inocula preparation

Female outbred MF1 mice (Harlan Olac, Bicester, United Kingdom) aged 9 to 13 weeks and weighing 30 to 35 g were used as a standard model of pneumococcal pneumonia and bacteraemia. All mice were kept in appropriate cages and provided with sterile pelleted food (B&K Universal, North Humberside, United Kingdom) and water ad libitum.

All the strains analysed *in vivo* were prepared by passaging through the mice as described previously (Alexander *et al.*, 1994). *S. pneumoniae* from a glycerol stock of each strain were collected by centrifugation at 18,000 x *g* (Eppendorf centrifuge 5417C, Germany) for 3 min. Pellets were resuspended in 900 µl of sterile PBS and diluted to give approximately 1x 10^6 CFU/ml. Mice were injected with 200 µl of bacterial suspension into the peritoneal cavity using 1-ml insulin syringe (Micro-fine, 12.7 mm, Becton Dickinson). 24-48 hours following injection, mice were sacrificed by cervical dislocation and the chest cavity opened. Blood was collected from the right ventricle of the heart using 23-gauge needle. 10 ml BHI (+/- antibiotic) were inoculated with four to five colonies of *S. pneumoniae* taken

from a fresh culture plate of mouse-passaged pneumococci and incubated overnight at 37°C. Bacteria were harvested by centrifugation at 5,000 x *g* at room temperature for 15 min and resuspended in 1 ml of BHI. 100 µl of this suspension was used to inoculate 10 ml of BHI containing 20% (vol/vol) heat-inactivated FBS (Gibco BRL, Life Tecnologies, UK) and incubated at 37°C until OD_{600nm} of 0.6-0.7 (~ 4 to 5 h). The cultures were aliquoted and stored at -80°C. Viable cell counts were determined in triplicate on blood agar plates. Pneumococci could be stored for at least 3 months at -80°C with no significant loss of viability. When required, the suspension was thawed rapidly at 37°C, and bacteria were harvested by centrifugation before resuspension in sterile PBS.

2.9.2 Intranasal infection

For intranasal challenge, mice were lightly anaesthetized with 2.5% (vol/vol) halothane (Zeneca Pharmaceuticals, Adlerley Edge, UK) over oxygen (1.5 L/min) using a calibrated vaporizer, and 1 x 10^6 CFU resuspended in sterile PBS was administered into the nares of mice held vertically (Alexander *et al.*, 1994) in a total volume of 50 µl using a Proline® pipette (Biohit). At predetermined time points, a small volume of blood was removed from a tail vein by using a 1-ml insulin syringe (Micro-fine, 12.7 mm; Becton Dickinson), and bacteraemia was determined by viable count as described in section 2.8.1.2. To determine the bacterial load in the lung tissue, mice were challenged intranasally as described above. Subsequently, mice were sacrificed at predetermined time points by cervical dislocation. Lungs were removed, weighed, and homogenized in 5 ml of sterile PBS by using an electric tissue homogenizer. Viable counts of bacterial suspensions were determined.

2.9.3 Intravenous infection

For intravenous infection, mice were placed in a ventilated, heated Perspex box for 5 min in order to expose veins and were restrained in appropriate apparatus. 1.0x 10^6 CFU, resuspended in sterile PBS, was administrated directly into a tail vein. At predetermined time points, a small volume of blood was removed from a tail vein by using a 1-ml insulin syringe (Micro-fine; 12.7 mm; Becton Dickinson), and bacteraemia was determined by viable enumeration as described in section 2.8.1.2. Symptoms were monitored for 168 h post-infection, and mice were culled prior to reaching, or upon reaching, a moribund state.

2.9.4 Intraperitoneal infection

For intraperitoneal infection, mice were each given 1.0×10^6 CFU, resuspended in sterile PBS, administered intraperitoneally. Survival times was monitored for two weeks and a small volume of blood was removed from a tail vein, 24h post-infection, by using a 1-ml insulin syringe (Micro-fine, 12.7 mm; Becton Dickinson) to determined bacteraemia by viable-cell enumeration as described in section 2.8.1.2.

2.9.5 Mice survival and disease symptoms

In order to determine experimental end points, mice were monitored frequently for symptoms of infection and were culled prior to reaching, or upon reaching, a moribund state. When animals showed signs of lethargy or upon becoming moribund, mice were humanely euthanized, the time was recorded and used as a measure of "mortality" for analysis of survival data (Toth, 1997). Mice that survived the course of infection (7 days, unless stated otherwise) were assigned an arbitrary survival time of 168 h for statistical analysis.

2.9.6 Bacteriological investigation

At predetermined times following infection mice were sacrificed by cervical dislocation ensuring intact trachea and the skin and muscles surrounding the trachea were exposed and the thoracic cavity opened. For bronchoalveolar lavage, the trachea was clamped, and 2 ml of sterile PBS was passed through the nasopharynx via a 16-gauge nonpyrogenic Angiocath (F. Baker Scientific, Runcorn, UK). Bronchoalveolar lavage and lung tissue sampling were carried out as previously described. Lavaged lungs were homogenized in 5 ml of sterile PBS with a glass handheld tissue homogenizer (Jencons, Leighton Buzzard, UK). Viable bacteria in lung samples were counted as described in section 2.8.1.2. To measure the levels of bacteraemia, blood samples were collected from the right ventricle of the heart after the chest cavity was opened. Following intravenous infection, blood samples were taken from peripheral veins.

2.9.7 Statistical analysis

Survival times were analysed using Mann-Whitney U test. Bacteriology results are expressed as geometric mean ± standard errors of the mean (SEM). Comparison of

bacterial loads in the time course experiment was performed using Student's *t* tests. In all analyses, $p < 0.05$ was considered statistically significant.

2.10 Protein analysis

2.10.1 Protein preparation

Bacterial strains were grown in 20 ml of BHI at 37°C until the mid-log phase (OD_{600nm} 0.6). The cells were collected by centrifugation at 5,000 x *g* 4°C for 15 min and the pellet resuspended in 1 ml of PBS. In case of choline binding proteins the pellets were resuspended in PBS containing 2% choline chloride (Sigma- Sigma-Aldrich) and incubated at 4°C for 15 min. The cells were disrupted by sonication for 4 times at 30 seconds each with 13 mm probe (Sonicator Vibra Cell, Sonics & Materials Inc.) using a power output of 36W, keeping the tubes with samples on ice during sonication process. A centrifugation at 13,000 x *g* for 10 min was performed to remove cell debris in case of non-membrane associated proteins.

2.10.2 Protein quantification

The total proteins were quantified by Bradford assay (Bradford, 1976). Standards were prepared with BSA in MilliQ water at concentrations between 62.5-1500 µg/ml. The protein quantification was preformed in 96-well plate (Nunclon® surface, Nalge International™). 10 µl of each standard were added to the first column of the microlitre plate (in duplicate) and 10 µl of each protein sample, or PBS into triplicate wells. 200 µl of Bradford reagent were added to each well, and incubated on room temperature for 10 min. Bubbles were avoided to the maximum, and the absorbance at 570 nm was read in microplate reader (Dynex Technologies, Denkendorf, Germany) and analysed with Dynex revelation 4.22 sostware (Dynex Technologies, Denkendorf, Germany). A standard curve was created and the concentration for each protein sample was calculated automatically.

2.10.3 Western immunoblot

To prepare a western immunoblot, sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to separate the proteins under study according to a standard protocol (Sambrook 1989). Mini gels were prepared in electrophoresis apparatus (Bio-Rad) with 10% separating gel (see appendix A1) and 4% stacking gel (see appendix

A1). Pre-cast gels (Bio-Rad) also were used in this study. Equal amounts of total proteins from each strain in loading buffer (see appendixA1) were heating at 95°C for 5 min and applied in the gels along with protein ladders (SeeBlue® Plus2 Pr-Staimed Standard, Invitrogen ™) to determine the size of protein bands. Gels were run at 120 V for ~90-100 min using a Biorad power pack 300 (Bio-Rad). When the bromophenol blue dye of the loading buffer reached the bottom of the gels, the electrophoresis was stopped, the gels were removed and placed into transfer buffer, cooled to 4°C, (see appendix A1) for 30 min with agitation to equilibrate. Hybond-C nitrocellulose membrane (Amersham Biosciences) and filter paper were cut to the size of the gels and soaked together with fibre pads in transfer buffer. The blotting apparatus (Bio-Rad) was assembled with grey side of holder, fibre pad, filter paper, gel, nitrocellulose membrane, filter paper and fibre pad, and ice pack were placed in the tank and the proteins were blotted at 100 V for 60 min. After proteins transfer, the membranes were blocked with 3% skimmed milk in Tris-NaCl (see appendix A1) overnight at 4°C with shaking. The membranes were transferred to fresh blocking buffer containing 1:500 or 1:1000 of the primary antibody and incubated at 37°C for 3 hours in a shaker. The membranes were washed 4 times, 5 minutes each with Tris-NaCl and then transferred to 1:1000 of secondary antibody (anti-mouse or anti-rabbit, peroxidase-liked whole antibody, Amersham Biosciences) in Tris-NaCl buffer. Membranes were washed as described before and then transferred to the developer solution (see appendix A1) in dark for until the bands developed. The membranes were washed with double distilled water to stop the previous reaction. The membranes with the blots were scanned.

2.11 Haemolytic assay of crude lysates

Pneumolysin activity of D39 *rr09* was compared with wild-type using a haemolytic assay, based on the ability of pneumolysin to cause lysis of red blood cells. The bacteria were grown in 20 ml of BHI at 37°C until the mid-log phase (OD_{600nm} 0.6). The total protein samples were prepared as described in section 2.10.1. The haemolytic assay was performed in a 96-well plate (Nunclon® surface, Nalge International™) in duplicate. 50 µl of PBS was added to each well, and 50 µl of total protein sample or PBS (negative control) added to the first column). Two-fold dilutions were made across the plate, and 50 µl of 2% defibrinated sheep red blood cells (E&O Laboratories, Bonnybridge, UK) was added to each well. The plate was closed and incubated at 37°C for 30 min followed by 30 min at room temperature. Results were analysed by visual examination comparing with negative control, where the red cells formed a pellet while in positive wells the activity of

pneumolysin caused the lysis of red cells preventing the pellet formation. The end point (50% lysis) was taken as the well in which half of the pellet is observed.

2.12 Pneumococcus RNA extraction

Bacteria grown in 10 ml of BHI to mid-log phase (OD_{600nm} of 0.6) at 37°C were harvested by centrifugation at 5,000 x *g* for 5 min at room temperature (Sigma laboratory centrifuge 4K15). The bacterial pellet was snap-frozen in liquid nitrogen to preserve RNA as much as possible. RNA isolation was performed using Qiagen RNeasy-Midi Kit (Qiagen) with the following modifications. Bacteria were resuspended in the presence of 200 µl of lysozyme (15mg/ml) prepared in TE buffer (10mM Tris-Cl pH 8.0, 1 mM EDTA pH 8.0) and vortexed vigorously for approximately 30 seconds. Suspensions were incubated at room temperature for 10-15 min, mixed vigorously, for 10 seconds every 2 min in a vortex mixer. After incubation in lysis buffer, 700 µl of RLT buffer (Qiagen) containing 10% (vol/vol) β-mercaptoethanol (Sigma-Aldrich) were added and mixed vigorously, for 10 seconds in vortex mixer. The mixture was transferred to 2 ml centrifuge tube containing ~50 mg of acid-washed glass beads, 100 µm (Sigma-Aldrich) and was submitted a mechanical disruption using a Hybaid Ribolyser (Hybaid^{TM}) at speed 4, 3 times for 20 seconds to facilitate the disruption of the cells. A brief centrifuge at 14,000 x *g* for 10 seconds was performed to remove the glass beads and the supernatant was transferred to a 15 ml centrifuge tube. 3.2 ml of RLT buffer were added to the supernatant, mixed vigorously for 10 seconds and 4 ml of 70% ethanol (prepared with Nuclease-free water) were added and mixed gently. The samples were applied twice (4 ml each) to RNeasy columns, with centrifugation at 5,000 x *g* at room temperature between each loading. The wash steps were subsequently processed according to the manufacturer's protocol and the RNA eluted by adding 150 µl of RNase-free water, leaving 1-3 min in room temperature and centrifuged at 5,000 x *g* at room temperature for 3 min. A second elution with the first elute was performed to increase the final RNA concentration. An on-column DNase digestion step was performed, using an RNase-free DNase set (Qiagen) according to the manufacturer's instructions.

2.12.1 RNA analysis

The total RNA concentration was measured using the NanoDrop® ND-1000 spectrophotometer (NanoDrop® Technologies, USA). The sample concentration is given in ng/ul calculate using the Beer's Law based on absorbance at 260 nm against a blank of

RNase-free water.. The ratio of absorbance at 260 and 280 nm is used to assess the purity of RNA. A ratio of ~2.0 is generally accepted as "pure" for RNA.

The integrity of total RNA samples were analysed using the RNA LabChip® kit with the Agilent 2100 bioanalyzer (Aligent Technologies, UK).1 µl of total RNA samples, with a maximum concentration of 200 ng/µl were applied to a RNA 6000 Nano LabChip. For concentrations below 10 ng/µl 1 µl of RNA, a RNA 6000 Pico LabChip was used. The system automatically calculates the ratio of ribosomal bands in total RNA samples, shows the percentage of ribosomal impurities in RNA samples and provides enhanced concentration estimation and comparison of samples and it performs automated integrity control of total and messenger RNA (Wang *et al.*, 2003) (Figures 3.21-3.23).

2.13 Reverse transcriptase PCR

cDNA was synthesized from RNA of pneumococcus strains, obtained as described in section 2.12, with ThermoScript™ RT-PCR system (Invitrogen™). To 2-5 µg of total RNA were added 50 ng of random primer (Invitrogen™) or 10 µM of reverse specific prime, 2 µl of 10 mM dNTP mix (Invitrogen™) was added and the volume brought to 12 µl with DEPC-treated water. The RNA and primer mixture were denatured by incubating at 65°C for 5 min and then placed on ice. Master mix containing 4 µl of 5 x cDNA synthesis buffer, previously vortexed, 1 µl of 0.1 DTT, 1 of µl RNaseOUT™ (40 U/µl), 1 µl of DEPC-treated water and 1 µl of ThermoScript™ RT (15U/µl) were added to the reaction tubes and transferred to a thermal cycler (DNA Engine Dyad® Peltier Thermal Cycler, MJ Research) and incubated at 25°C for 10 min then at 50-65°C for 50 min. In case of specific primers the reaction was performed at 50-65°C for 50 min. The reactions were terminated by incubating at 85°C for 5 min. A reaction without ThermoScript™ RT was performed as negative control. 1 µl of Rnase H was then added to each reaction and reactions were incubated at 37°C for 20 min to remove any residual RNA. The cDNA reactions were stored at -20°C or used immediately as a DNA template in PCR reactions as described in section 2.6.1.

2.14 Microarray analysis

In this project two different types of pneumococcal genome microarray slides have been used. One array used for transcriptome analysis was designed at the Pathogen Functional Genomics Resource Centre at TIGR (http://pfgrc.tigr.org) v.1 and v.2, and the second type

of slides used in transcriptome and genomic comparison was designed at the Bacterial Microarray Group at St George's Hospital BμG@S (http://www.bugs.sghms.ac.uk). The slides were stored in plastic slide boxes at room temperature in a desiccator containing a bottom layer of anhydrous calcium sulphate. The slides were kept away from dust and were handled with extreme care to prevent scratching or rubbing that may damage the printed DNA.

2.14.1 TIGR arrays

For analyses of the genes regulated by *rr06* and *rr09*, using TIGR slides for microarrays, two independent cultures of bacteria were grown in 10 ml of BHI on different days, to mid-log phase and the RNA extracted as described above. The method for using TIGR array describes the production of labelled DNA from microbial RNA with aminoallyl-labeled nucleotides via first strand cDNA synthesis with aminoallyl-dUTP followed by coupling of the aminoallyl groups to either Cyanine-3 or Cyanine-5 (Cy3/Cy5) fluorescent molecules.

2.14.1.1 Array design

The full genome array of *S. pneumoniae* microarray v.1 consists of amplicons representing segments of 2,131 open reading frames from *S. pneumoniae* reference strain TIGR4 (Tettelin *et al.*, 2001) spotted in quadruplicate on glass slides. Also, the array contains an additional 118 open reading frames from strains R6 (37) (Hoskins *et al.*, 2001) and G54 (81). Version 2 slides have an additional 164 and 399 open reading frames from strains R6 and G54 respectively.

2.14.1.2 Aminoallyl-labelled cDNA synthesis

The reactions were prepared in 0.2 ml PCR tubes. For each sample (control and test) 10 μg of total RNA was mixed with 2 μl of 3 mg/ml random hexamers (Invitrogen™), 1 ul of RNaseOUT™ (40 U/μl) and the volume was brought to 18.5 μl with nuclease free water. After gently mixing, the mixture was incubated at 70°C for 10 min in a DNA Engine Dyad® Peltier Thermal Cycler (MJ Research) to prevent any RNA loop formation. The mixture was cooled to 4°C on ice and a brief centrifugation (Eppendorf® MiniSpin centrifuge, Germany) was done to bring down any condensation. The remaining steps were done at room temperature. A master mix containing 6 μl of 5 x First Strand buffer (Invitrogen™), 3 μl of 0.1 M DTT (Invitrogen™), 1.2 μl of 12.5 mM dNTP/aa-UTP

labelling mix (12.5 mM dATP, 12.5 mM dCTP, 12.5 mM dGTP, 7.5 dTTP all from Invitrogen™ and 10 mM aa-dUTP Ambion®), and 2 µl of 200U/µl SuperScript II or III RT (Invitrogen™) was added to a final volume of 30.7 µl. After gently mixing the reaction was incubated overnight (~16 hours) at 42°C in a thermal cycler. The first strand synthesis reaction was stopped with addition of 10 µl of 0.5 M EDTA (Ambion®) and 10 µl of 1M NaOH to hydrolyze the RNA in the cDNA/RNA mixture, and the mixture was incubated at 65°C for 15 min in a thermal cycler. 25 µl of 1 M Tris, pH 7.0 (Ambion®) was added to the mixture to neutralise the pH.

NucleoSpin® Extract II Kit (Macherey-Nagel) was used to remove unincorporated aa-dUTP and free amines. The wash and elution buffer from the kit was substituted by phosphate buffers to avoid contamination with free amines that compete with the aa-dUTP in the Cy-dye coupling reaction.400 µl (5 x reaction volume) of buffer NT2 (Macherey-Nagel) was added to the cDNA reaction, mixed, transferred to NucleoSpin column, centrifuged at 14,000 x *g* for 1 min (Eppendorf® MiniSpin centrifuge, Germany) and the collection tubes were emptied. The columns were washed with 750 µl of phosphate wash buffer (see appendix A1), and centrifuged at 14,000 x *g* for 1 min. The collection tubes were emptied and an additional centrifugation at 14,000 x *g* for 1 min was performed to remove residual ethanol. The columns were transferred to a 1.5 ml centrifuge tube and the cDNA was eluted two times with 30 µl of phosphate elution buffer (see appendix A1) with incubation at room temperature for ~1 min and following centrifugation at 14,000 x *g* for 1 min. The final eluate (60 µl) was transferred to open 0.2 ml PCR tubes and dried down in speed vac (Savant DNA 110 Speed Vac, Global Medical Instruments, USA) at medium temperature for ~1 hour.

2.14.1.3 Cy3/Cy5 indirectly labelling of cDNA

To prevent photobleaching of the Cy-dyes, all reactions tubes were wrapped with foil and protected from light as much as possible. The dry aminoallyl-labelled cDNA samples was resuspend in 4.5 µl of freshly 0.1 M sodium carbonate buffer pH 9.3 (see appendix A1) by pipetting up and down for ~4 min. One vial of each Cy3 and Cy5 (Cy™ reactive dye, Amersham Biosciences) was prepared by resuspending in 15 µl of DMSO. 4.5 µl of the appropriate Cy dye, Cy3 or Cy5, was added and mixed to the aminoallyl-labelled cDNA sample and incubated for 2 hours in room temperature in the dark. After coupling had finished, 35 µl of 100 mM NaOAc pH 5.2 (see appendix A1) was added to the mixture to stop the incorporation reaction. The free Cy3 or Cy5 were removed by using the NucleoSpin® Extract II Kit (Macherey-Nagel). The cDNA reaction was mixed with 250 µl

(5 x reaction volume) of NT2 buffer (Macherey-Nagel), transferred to a NucleoSpin column, centrifuged at 14,000 x *g* for 1 min (Eppendorf® MiniSpin centrifuge, Germany) and the collection tubes were emptied. The columns were washed and dried as above and the columns were transferred to a 1.5 ml centrifuge tube. The dye-labelled cDNA was eluted two times with 50 µl of phosphate elution buffer with incubation at room temperature for ~1 min and a following centrifugation at 14,000 x *g* for 1min.

2.14.1.4 Analysis of labelling reaction

For each sample the concentration of cDNA and the total picomoles of Cy3 or Cy5 dye incorporated were calculated using the NanoDrop® ND-1000 UV-Vis Spectrophotometer, Microarray application (NanoDrop® Technologies, USA). The total picomoles of cDNA samples were calculated through of following formula:

$$\text{pmol nucleotides} = \frac{\text{cDNA (ng)} * \text{volume (µl)} * 1000 \text{ pg/ng}}{324.5 \text{ pg/pmol}^{1)}}$$

[1)] 324.5 pg/pmol represent the average molecular weight of dNTP.

The incorporation ratio of cDNA to dye was calculated through of following formula:

$$\text{nucleotides/dye incorporated} = \frac{\text{pmol cDNA}}{\text{pmol Cy dye}}$$

Dye incorporation per sample above 200 pmol and a nucleotides/dye incorporation ratio less than 50 were considered optimal for hybridisations (Hegde *et al.*, 2000).

The measurement of these parameters permit calculation of the efficiency of the labelling reaction and eliminate potentially flawed samples to use in hybridization probes for gene expression.

2.14.1.5 Pre-hybridization

It is extremely important that slides be perfectly clean to avoid background problems and prevent any scratching between the slides in a Coplin jar. 60 ml pre-hybridization buffer (for a maximum of 5 slides) was prepared (15 ml of 20x SSC (Ambiom®), 600 µl of 10%

SDS (Ambiom®), 0.6 gm of BSA fraction V powder (Sigma-Aldrich), MilliQ water to a volume of 60 ml. The pre-hybridization buffer was filtered with a 0.22 µm filter (Fisher Scientific) transferred to a clean Coplin jar and preheated at 42°C for at least 30 min. The printed slides were placed with their labels up and their printed side down in a Copling jar containing preheated prehybridization buffer and incubated at 42°C for ~2 hours. The slides were removed from the Coplin jar and by carefully gripping them by the label using forceps and placed in the slide holder of glass staining dish filled with MilliQ water filtered with 0.22 µm filter (Millipore). The slides were washed in rotary shaker (Edmund Bühler KM-2 shaker) and the water was changed every 2 min until ~2 L of water had been used. The slides were then washed with isopropyl alcohol (Fisher Scientific) for 2 min on the rotary shaker. The slides were put in a clean slide plastic box lined with paper towels and centrifuged at 450 x *g* for 10 min (Eppendorf Centrifuge 5804, Germany) for drying. The slides were used immediately to ensure optimal hybridization efficiency.

2.14.1.6 Hybridization of cDNA probes to array

After analysis of the labelling reaction the two differentially labelled probes (Cy3 and Cy5) were mixed together (final volume of 200 µl) and were transferred to 0.2 ml PCR tubes and dried down in a speed vac (Savant DNA 110 Speed Vac, Global Medical Instruments, USA) at medium temperature for ~2 hours. 1 ml of hybridization buffer (50% formamide (Sigma-Aldrich), 5 x SSC (Ambion®), 0.1% SDS (Ambion®), 300 µg Salmon Sperm DNA (Ambion®) was prepared and filtered with surface-free cellulose acetate syringe filter 0.45 µm (Nalgene™). The labelled probes were resuspended in 30 µl of filter hybridization buffer, by finger flicking for about 1 min and heating at 95°C for 5 min following by an additional mix by finger flicking for about 1 min and heated again at 95°C for 5 min. 100 µl of unused hybridization buffer were added to the well of the hybridization chamber (HybChamber, GeneMachines), to prevent the drying of the hybridization solution under the cover slip. The entire labelled probe mixture was applied onto the printed area of the slides preventing any air bubbles. A glass cover slip 24mm x 60mm, cleaned with compressed air was placed over the slide printed area and air bubbles were avoided. The hybridization chamber was well-sealed, wrapped in aluminium foil and incubated 42 °C for 18 hours in GeneChip® Hybridization oven 640 (Affymetrix).

2.14.1.7 Post-hybridization

After hybridization, the hybridization chamber was opened and the slides carefully removed, to avoid cover slip disturbance. The slide was submerged in 55°C preheated low

stringency buffer (50 ml of 20x SSC, 5 ml of 10% SDS, 445 ml of MilliQ water) and the cover slip was removed with smooth up and down movements and the slides were washed vigorously for an additional 1 min. The slides were transferred to a holder in a glass staining dish filled with new preheated low stringency buffer and an additional washing for 5 min was performed in a rotary shaker, followed by a 5 min wash using medium stringency (2.5 ml of 20x SSC, 5 ml of 10% SDS, 492.5 ml of MilliQ water) buffer and then to a final 5 min wash in higher stringency buffer (2.5 ml of 20x SSC, 997.5 ml of MilliQ water). The slides were dried by centrifugation as mentioned in section 2.14.1.5, and kept in dark until ready for scanning.

2.14.2 BμG@S arrays

For analyses of the genomic comparison between TIGR4 reference strain and test strains using the BμG@S microarrays slides, bacteria were grown in 20 ml BHI to mid-log phase and the DNA extracted as described above. These arrays have also been used to analyse the genes regulated by the *rr06*. The method using for BμG@S arrays describe the production of labelled DNA by a direct labelling reaction with Cyanine-3 or Cyanine-5 (Cy3/Cy5) fluorescent molecules.

2.14.2.1 Array design

Microarrays were constructed by robotic spotting of PCR amplicons onto poly L-lysine coated glass microscope slides (MicroGrid II. BioRobotics, UK) (Hinds *et al.*, 2000a). Amplicons were designed to represent each of the annotated ORFs present in *S. pneumoniae* strains TIGR4 (Tettelin *et al.*, 2001) and R6 (Hoskins *et al.*, 2001) to create a composite, inclusive array for these two strains. The design process essentially designed multiple amplicons using Primer3 for all TIGR4 ORFs plus strain-specific ORFS only present in R6, as determined by automated analysis of BLAST comparisons (Hinds *et al.*, 2000b). A single amplicon was selected to represent each ORF based on its lack of similarity to other ORFs on the array using BLAST analysis to ensure minimal cross-hybridisation.

2.14.2.2 Cy3/Cy5 directly labelling of genomic DNA and cDNA

A direct labelling method was used to label cDNA and genomic DNA. Reverse transcription of RNA was done using the method described previously (Stewart *et al.*, 2002). To prevent photobleaching of the Cy-dyes, all reactions tubes were wrapped with

foil and kept sequestered from light as much as possible. 2 µg of total RNA (control and test strains) was mixed with 2 µl of 3 mg/ml random hexamers (Invitrogen™) and the volume was brought to 11µl with nuclease free water. After gentle mixing the mixture was incubated at 70°C for 10 min in DNA Engine Dyad® Peltier Thermal Cycler (MJ Research) to prevent any RNA loop formation. The mixture was cooled to 4°C in ice and a briefly centrifuged (Eppendorf® MiniSpin centrifuge, Germany) to bring down any condensation. A master mix containing 5 µl of 5 x First Strand buffer (Invitrogen™), 2.5 µl of 0.1 M DTT (Invitrogen™), 2.3 µl of dNTP mix (5mM dA/G/TTP, 2mM dCTP, Invitrogen™), and 2 µl of 200U/µl SuperScript II RT (Invitrogen™) was added. 1.7 µl of Cy3 or Cy5 was added and after gently mixed the reaction was incubated at 25°C for 10 min followed 42°C for 90 min in Thermal Cycler.

For Cy3/Cy5 labelled DNA, 3.5 µg of genomic DNA (control and test strains) was mixed with 1 µl of 3 mg/ml random hexamers (Invitrogen™) and the volume was brought to 41.5 µl with nuclease free water. After gentle mixing the mixture was incubated at 95°C for 5 min in DNA. The mixture was cooled to 4°C on ice and a brief centrifugation was done to bring down any condensation. A master mix containing 5 µl of 10 x React 2 buffer (Invitrogen™), 1 µl of dNTP mix (5mM dA/G/TTP, 2mM dCTP, Invitrogen™), and 1 µl of 5U/µl Large Fragment DNA Polymerase I (Klenow) (Invitrogen™) was added. 1.5 µl of Cy3 or Cy5 (Amershan Biosciences) was added and after gently mixed the reaction was incubated at 37°C for 90 min in Thermal Cycler.

2.14.2.3 Pre-hybridization

50 ml pre-hybridization buffer (for a maximum of 5 slides) was prepared (8.75 ml of 20x SSC (Ambiom®), 500 µl of 10% SDS (Ambiom®), 5 ml of 100 mg/ml BSA (Sigma-Aldrich), MilliQ water to 50 ml). The pre-hybridization buffer was filtered with a 0.22 µm filter (Fisher Scientific) transferred to a clean Coplin jar and preheated at 65°C for at least 90 min. The printed slides were placed with their labels up and their printed side down in a Copling jar containing prehcated prehybridization buffer and incubated at 65°C for at least 20 min. The slides were removed from the Coplin jar and by carefully gripping them by the label using forceps and placed in the slide holder of glass staining dish filled with MilliQ water filtered with a 0.22 µm filter (Millipore). The slides were washed for 1 min and then washed with isopropyl alcohol (Fisher Scientific) for 1 mim. The slides were put in a clean slide plastic box lined with paper towels paper and centrifuged at 900 x *g* for 5 min (Eppendorf Centrifuge 5804, Germany) for drying. The slides were used immediately to ensure optimal hybridization efficiency.

2.14.2.4 Hybridization of template probes to array

MinElute PCR Purification kit (Qiagen) was used to purify the labelled cDNA or DNA. The Cy3 and Cy5 labelled sample were combined in a single 1.5 ml centrifuge tube and 500 µl Bufffer PB (Qiagen) was added and mixed. The samples were transferred to MinElute columns and centrifuged at 14,000 x *g* for 1 min (Eppendorf® MiniSpin centrifuge, Germany). The columns were washed with 500 µl of PE buffer (Qiagen), and centrifuged at 14,000 x *g* for 1 min. The collection tubes were emptied and a second wash with 250 µl of PE buffer was performed. An additional centrifugation at 14,000 x *g* for 1 min was performed to remove residual ethanol. The columns were transferred to a 1.5 ml centrifuge tube and the labelled cDNA or DNA was eluted in 10.5 µl of nuclease-free water with incubation at room temperature for ~1 min and a following centrifugation at 14,000 x *g* for 1 min.

The purified cDNA or DNA mixture was adjusted to 16 ml in with 3.2 µl of filtered (0.2 µm filter) 20x SSC (Ambion ®) and 2.3 µl of filtered (0.2 µm filter) 2% SDS (Ambion®). The hybridization solution was heated to 95°C for 2 min and cooled at room temperature. The slides were carefully placed in hybridization chamber (ArrayIt®) and the entire probe mixture was applied onto the printed area of the slide preventing any air bubbles. A glass cover slip 22mm x 22mm was dusted with compressed air and placed over the slide printer area and air bubbles formation was avoided. 15 µl of MilliQ water was added to the small wells at each add of the hybridization chamber. The hybridization chamber was well-sealed and incubated in 65°C preheated water bath for 18 hours in dark.

2.14.2.5 Post-hybridization

After hybridization, the hybridization chamber was opened and the slides carefully removed, to avoid cover slip disturbance. The slide was submerged in 65°C preheated wash A buffer (20 ml of 20x SSC, 2 ml of 10% SDS, 378 ml of MilliQ water) in a holder in a glass staining dish and the cover slip was removed with smooth up and down movements and the slides were washed vigorously for 2 min. The slides was transferred to a new holder in a glass staining dish filled containing the wash B buffer (1.2 ml of 20x SSC and 398.8 ml of MilliQ water) and washed vigorously for 4 min. The slides were dried by centrifugation as mentioned in section 2.14.2.3, and kept in dark until ready for scanning.

2.14.3 Scanning array and quantification

Hybridized slides were scanned using a ScanArray ™ Express microarray scanner (Packard Biosciences Biochip Technologies, Perkin Elmer). The two laser channels were switched on 15 min before the scanning of the slides. When ready, the slides were submitted to an easy scan with low resolution to confirm the hybridization of the labelled probe and to normalize the 2 channels (Cy3 and Cy5). In TIGR arrays the *gyrA* and *ddl* house keeping gene spots were used to normalize the Cy3 and Cy5 channels, while in the BμG@S arrays the rRNA serial dilutions spots were used for normalization. After normalization the slides were finally scanned with a resolution of 10 μm. The dual channel array images Cy3 and Cy5 spot intensities were saved as TIF image files prior to quantification analysis. The median pixel intensities values for each element of the array were quantified with the QuantArray (Packard BioScience, Perkin Elmer) or with BlueFuse for Microarray 3.1 (BlueGnome Ltd.).

2.14.4 Microarray analysis

2.14.4.1 Transcriptone

For each strain, two independent RNA preparation and 2 or 4 microarrays slides were used to analyse the genes regulated by *rr06* and *rr09*. The data was analysed with GeneSpring 7.0 (Silicon Genetics). LOWESS intensity-dependent normalization was used to perform per-spot and per-array normalization, and the cross-gene error model was based on the replicate measurements for each strain. Statistically significant differences were defined as those with a *t*-test *P*-value of less than 0.05 and a ratio change threshold of at least 2 standard deviations compared to the median ratio for each strain.

2.14.4.2 Genomic Array

Our experimental design for whole genome scanning was to perform comparative hybridizations between a Cy5 labelled genomic DNA of the reference strain (TIGR4) in competition with Cy3 labelled genomic DNA of the strain being tested (Table 2.1). One-dye swap experiment was accomplished to minimize the differences in intensities and background generated by each fluorochrome. The data were further analysed using GeneSpring 7.0 (Silicon Genetics). LOWESS intensity-dependent normalization was used to perform per-spot and per-array normalization, and the cross-gene error model was based on the replicate measurements for each strain. A cut-off of 2 for the normalised intensity

ratio was applied to discriminate the presence and absence of the gene in test strain compared with reference strain TIGR4. On the basis of PCR validations, the data was reanalysed using a cut-off for the mean ratio between TIGR4 and test strain>1.5 for the absence of a gene, unless the same gene its present in an intensity greater that 1500 in both channels (TIGR4 and test strain) and in this case we decided that the gene was present. For intensities of less than 600 for both channels the result was recorded as unclear. Since the genes with a ratio 1.4 gave an ambiguous result in PCR validations we decided to validate the data by doing a PCR for all the genes with this ratio. In Figure 2.1 a flowdiagram summarises the genomic array experiment and analysis.

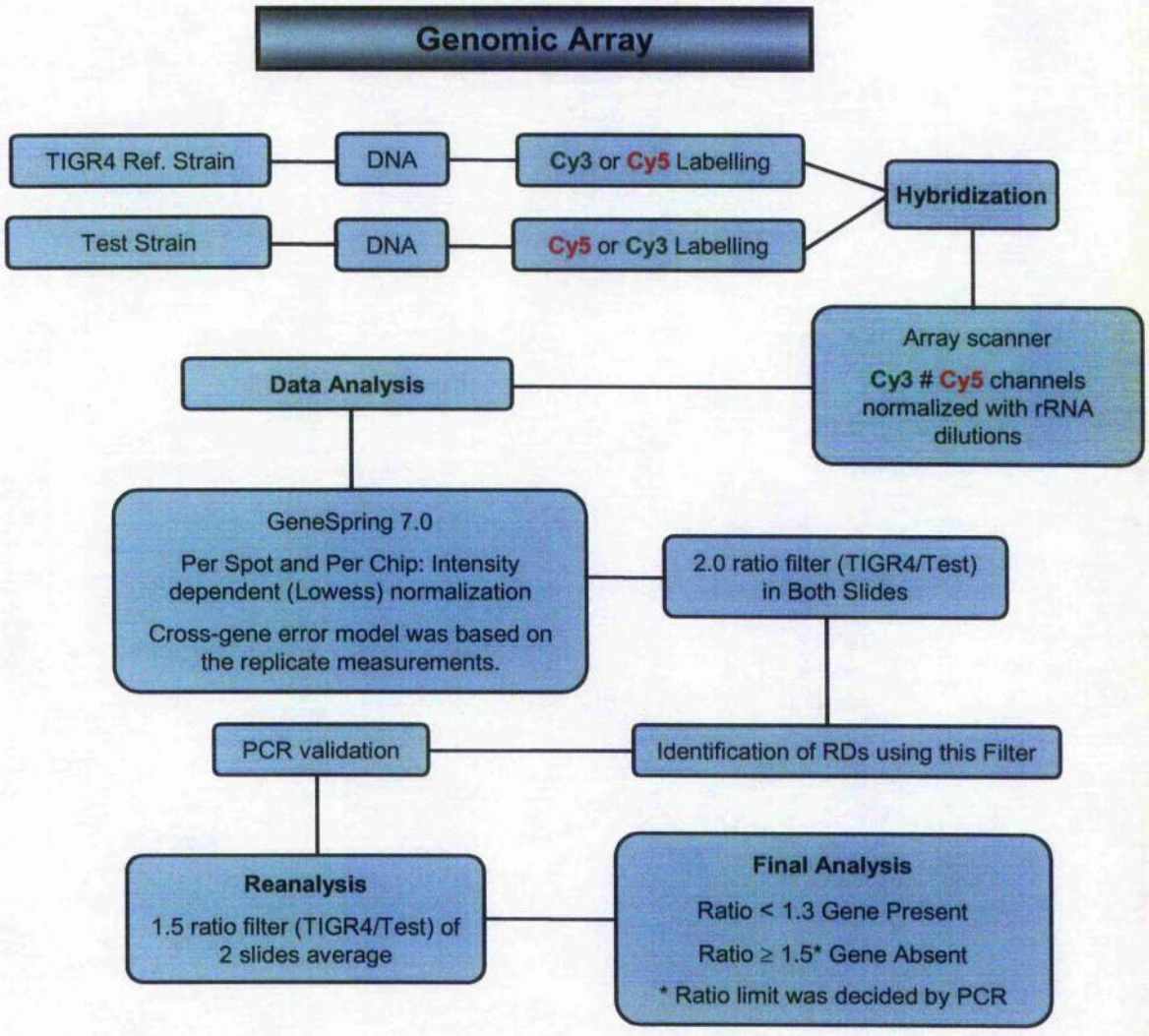


Figure 2.1- Summary of genomic array comparison experiment between TIGR4 reference strain and test strain.

Genomic DNA of reference strain TIGR4 and test strain was labelled with Cy3 or Cy5 and hybridized in DNA microarray slides. The slides were scanned and the data was further analysed using GeneSpring 7.0. A cut-off of 2 for the normalised intensity ratio was applied to discriminate the presence and absence of the gene, and the RDs identified. A final analysis was done on the basis of the PCR validations and a cut-off of 1.5 was used to determinate the presence and absence of the gene.

2.15 Real-Time quantitative PCR

2.15.1 cDNA synthesis

For each sample two micrograms of total RNA was added to 30.7 µl containing 6 µl cDNA synthesis buffer, 10 mM DTT, 40 U RNaseOUT, 0.5 mM dNTP/aa-UTP mix, 6 µg random hexamer, 400 U SuperScript III (Invitrogen™) and the reverse transcription reaction was performed overnight at 42°C. Reverse transcription was terminated by incubating for 15 min at 70 °C and template RNA removed by incubating at 37°C for 20 min in presence of 2 U RNase H.

2.15.2 Analysis of expression of the genes by qRT-PCR

The analysis of gene expression by qRT-PCR, were performed using the SYBR Green method. The SYBR Green is a dye that intercalates into double-stranded DNA and produces a fluorescent signal. The intensity of the signal is proportional to the amount of dsDNA present in the reaction. Therefore, at each step of the PCR reaction, the signal intensity increases as the amount of product increases, proving a reliable method to quantify PCR reactions in real time.

qRT-PCR were performed in quadruplicate in DNA Engine Opticon 2 (MJ research®, Bio-Rad) and carried out in white 96-well plates (MJ research®, Bio-Rad). 24 µl of master mix containing 12.5 µl of SYBR Green PCR master mix (DyNAmo™), 0.3 µl M of each specific oligonucleotide primer (Sigma-Genosys), and 9.5 µl of nuclease-free water were added with multistep electronic pipette in the 96-well plates. 1 µl of cDNA or of serial dilutions of the reverse transcriptase PCR product of the reference or target gene was added in each well and closed with transparent plastic caps. The 96-well plate was carefully agitated in vortex to mix the reaction and a brief centrifuged at 2,250 x *g* for 20 seconds was performed to keep away air bubbles. The 96-well plates with 25 µl of reaction were placed in DNA Engine Opticon 2 and the real time PCR reaction conditions were as follows: 15 min at 95°C for enzyme activation and 40 cycles of 30 seconds at 95°C, 30 seconds at 60°C and 30 seconds at 72°C. Fluorescence due to the binding of the SYBR green fluorochrome to double-stranded DNA was measured once per cycle. In the final step a melting curve analysis, 70-95°C, was performed to confirm the presence of the specific product and development of primers dimers. The analysis was made with MJOpticon Monitor™ 3.1 (MJ research®, Bio-Rad). The increment in fluorescence versus

reaction cycle was plotted, the baseline was subtracted using the global minimum method and the threshold cycle value (CT) was obtained by positioning of the threshold on the data graph above to the baseline on the beginning of the exponential phase of the curve. The reaction efficiency was calculated on the basis of the slope of the plot between the Ct values for the dilutions against concentration. The comparative Ct method ($\Delta\Delta C_T$) (Livak and Schmittgen, 2001) was used to measure the relative mRNA levels of the target gene between the wild-type and the mutant test strains and normalized against the housekeeping gene *gyrA* to control the cDNA loaded into the reaction. A second housekeeping gene, *ddl* was used in all reactions to double check the normalization between the control gene and the test gene.

Chapter 3

Two-component signal transduction system 06

Two-component systems (TCS) play a central role in bacterial survival by regulating various cellular processes such as osmoregulation, genetic competence, and chemotaxis in response to environmental changes (Appleby *et al.*, 1996; Szurmant and Ordal, 2004). The *S. pneumoniae* genome sequence contains 13 putative TCSs, along with one orphan RR (Lange *et al.*, 1999; Throup *et al.*, 2000). The pneumococcal TCS HK/RR06 also referred as 478HK/RR (Throup *et al.*, 2000) consists of a 51 kDa membrane-associated sensory protein called a histidine kinase (HK06), and 25 kDa cognate cytosolic DNA-binding response regulator protein, that presumably acts as a transcriptional regulator (RR06). The HK/RR06 system has been shown to regulate the pneumococcal virulence factor PspC (also known as CbpA) (Standish *et al.*, 2005). This study was completed during the course of the project presented here. The importance of PspC in pneumococcal pathogenesis is well described (Balachandran *et al.*, 2002; Ring *et al.*, 1998; Rosenow *et al.*, 1997). The genomic organisation shows that *rr06* has an upstream position relative to the virulence factor *pspC* (Figure 3.1).

The study presented here investigates the role of RR06 in the virulence of *S. pneumoniae* and the genes regulated by this response regulator in TIGR4, D39, R6 and 0100993 strains that may contribute to the *in vitro* and *in vivo* phenotype in *rr06* mutants (Δ*rr06*). These studies also investigated the involvement of the RR06 in the regulation of the pneumococcal virulence factor PspC.

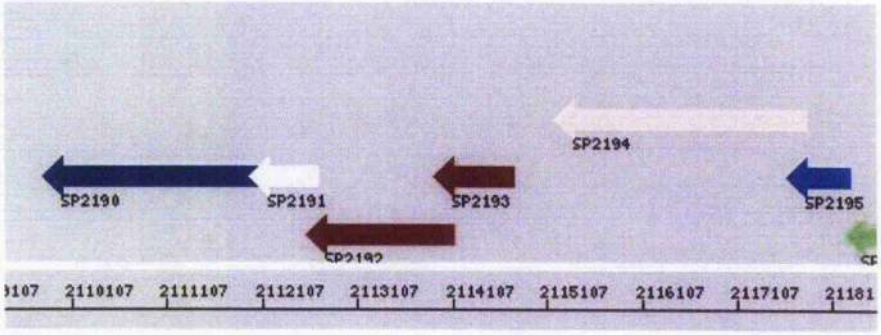


Figure 3.1- Genomic organization of TCS *hk/rr06*.

The TCS *hk/rr06* locus encodes a response regulator (*sp2193*) and a sensor histidine kinase (*sp2192*) and is located upstream of the gene encoding the virulence factor *pspC* (*sp2190*). The gene names use the TIGR4 gene annotation (http://www.tigr.org).

3.1 Contribution of RR06 to pneumococcal virulence

3.1.1 Construction of pneumococcal mutants

The *rr06* gene of pneumococcal strains TIGR4, D39 and R6 were disrupted by introduction of an erythromycin resistance cassette into the gene. The fragment containing the mutation was amplified by PCR from chromosomal DNA of the strain 0100993 (Throup *et al.*, 2000) using the primers TCS06-For and TCS06-Rev (Table 2.2). Following purification the fragments were used to transform the *S. pneumoniae* strains and were integrated by homologous recombination (Figure 3.2) and the final mutants were selected by growth on erythromycin.

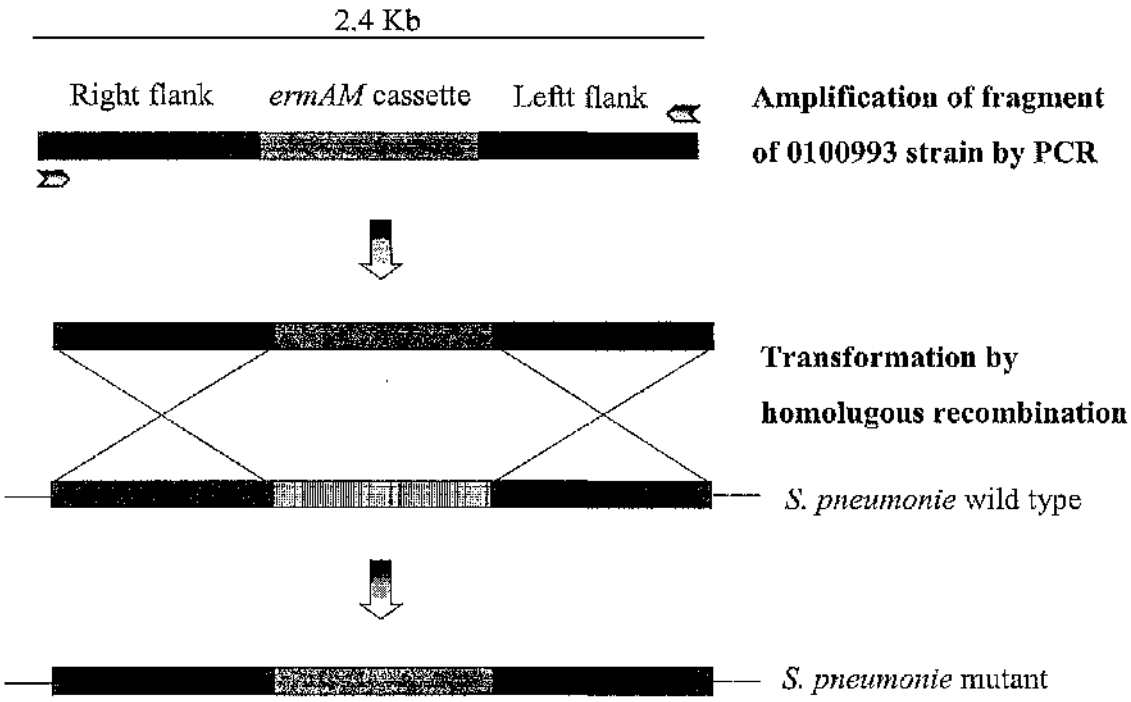


Figure 3.2- Construction of *S. pneumoniae rr06* mutants.

The fragment containing the mutant *rr06* gene disrupted with erythromycin resistance cassette was amplified by PCR from strain 0100993. Following purification the fragments were used to transform *S. pneumoniae* strains and the final mutants were selected by grown on erythromycin.

3.1.2 Confirmation of mutation

In order to construct the RR06 mutation in selected strains of the pneumococcus, the original strain constructed by Throup and co-workers (Throup *et al.*, 2000) was used as a source of the 2.4 kb fragment containing the *rr06* gene disrupted with the erythromycin resistance cassette. This fragment was amplified as a single fragment of 2.4 kb from their strain and used to transform the pneumococcal strains. The successful replacement of the wild-type *rr06* gene with the erythromycin resistance cassette in *S. pneumoniae* TIGR4, D39 and R6, was confirmed by PCR using the primers TCS06-For and TCS-Rev (Table 2.2) in chromosomal DNA of the mutants and wild-type strains and the size of the PCR product was confirmed by agarose gel electrophoresis (Figure 3.3). The size of the PCR product expected in wild-type strain was 2.073 kb while in the mutants strain 2.428 kb: a difference of 0.355 kb (difference between the erythromycin resistance cassette and *rr06* gene).

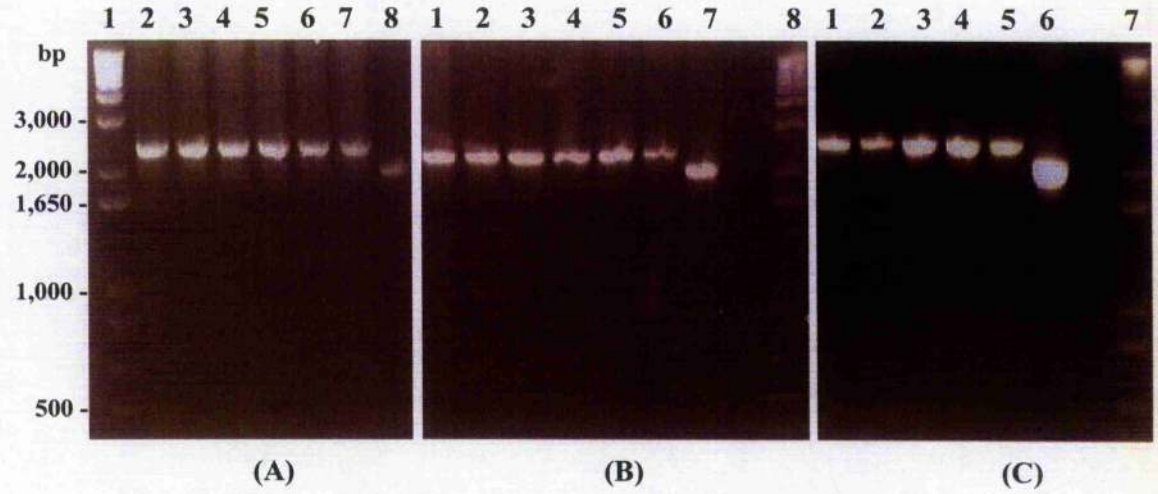


Figure 3.3 Confirmation of mutation in *rr06* mutants of *S. pneumoniae* strains.

PCR products using primer pair TCS06-For and TCS06-Rev running in 1% of agarose gel. Confirmation of R6 transformation, lane 2-7 putative *rr06* mutants, lane 8 wild-type (A); Confirmation of D39 transformation, lane 1-6 putative *rr06* mutants, lane 7 wild-type (B); Confirmation of TIGR4 transformation, lane 1-5 putative *rr06* mutants, lane 6 wild-type (C). The DNA ladder marker used was the 1 kb plus ladder (Invitrogen™).

Reactions with erythromycin cassette specific primers (ErmAM-For and ErmAM-Rev) and primers specific to genomic regions lying outside the insertion region were carried out to confirm correct insertion of the erythromycin resistance cassette into the *rr06* gene of mutants (Figure 3.4). As expected, no PCR amplicons were obtained for any of the wild-type strains (Figure 3.4 (A) and (B), lanes 2, 4, 7, and 9) while an amplicon of approximately 1.9 kb was obtained from TIGR4, 0100993, D39 and R6 Δ*rr06* mutants using primers TCS06-For and ErmAM-Rev (Figure 3.4 (A) and (B), lanes 3 and 5) and approximately 1.4 kb using primers ErmAM-For and TCS06-Rev (Figure 3.4 (A) and (B), lanes 8 and 10). These results confirmed successful allelic replacement of the *rr06* gene by the erythromycin resistance cassette in all four mutants.

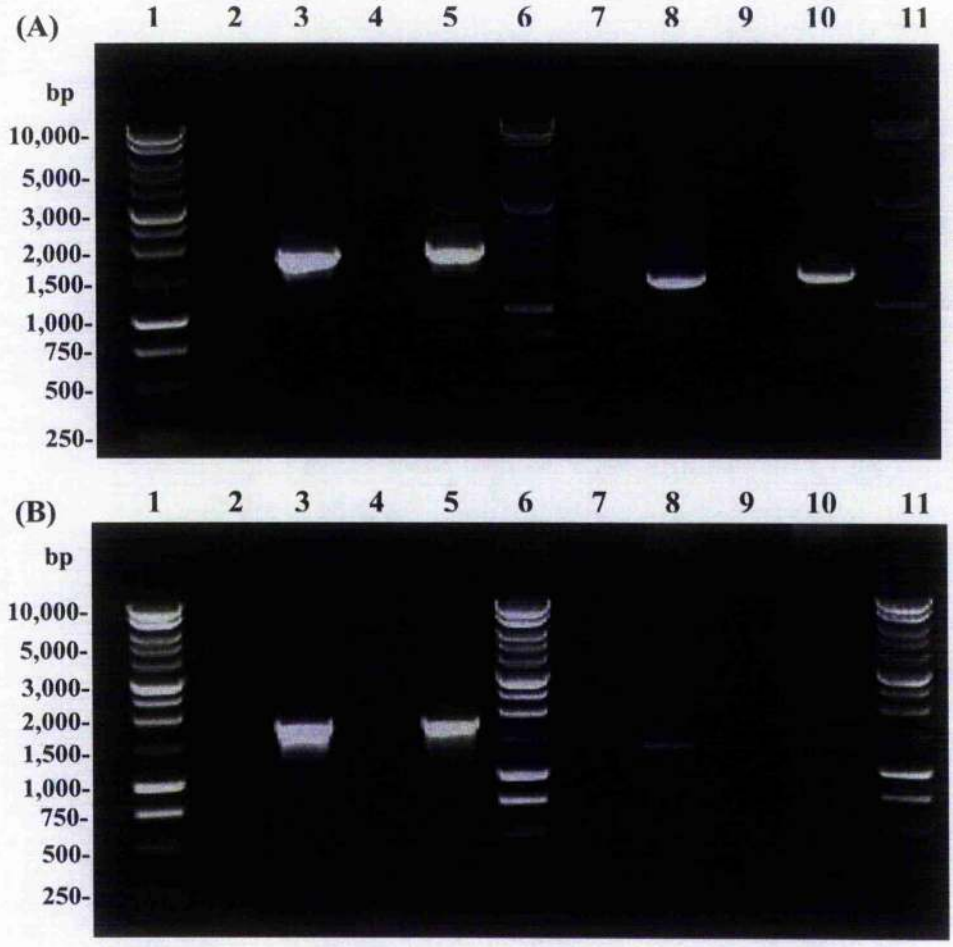


Figure 3.4- Allelic replacement confirmation of *rr06* with a constitutive erythromycin resistance cassette.

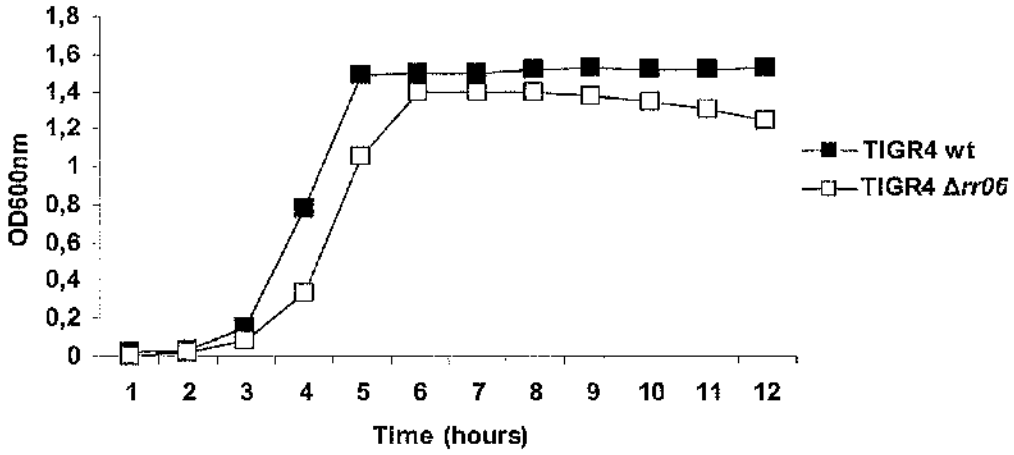
Correct replacement of *rr06* by erythromycin resistance cassette, in TIGR4, D39 and R6 was confirmed using primers TCS06-For and ErmAM-Rev (lanes 2-5 A, B) and primers ErmAM-For and TCS06-Rev (lanes 7-10 A, B). Genomic DNA from the following strains were used as templates: TIGR4 wild-type (lanes 2 and 7), TIGR4 *rr06* mutant (lanes 3 and 8), 0100993 wild-type (lanes 4 and 9) and 0100993 *rr06* mutant (lanes 5 and 10) (A); D39 wild-type (lanes 2 and 7), D39 *rr06* mutant (lanes 3 and 8), R6 wild-type (lanes 4 and 9) and R6 *rr06* mutant (lanes 5 and 10). The DNA ladder marker used was the 1 kb plus ladder (Promega).

3.2 *In vitro* analysis

3.2.1 Growth measurements of *rr06* mutants by optical density

The effect of the *rr06* mutation on the growth of *S. pneumoniae* at different temperatures was studied. Cultures of the *rr06* mutant and the wild-type strains were grown in BHI broth at 37 and 40°C. 1x 10^6 CFU/ml of each strain was used to inoculate 20 ml of BHI and incubated at the indicated temperatures. At 1-hour intervals, samples were withdrawn to measure the optical density at OD_{600nm}. When the growth of *rr06* mutants of *S. pneumoniae* strains TIGR4, D39, R6 and 0100993 was compared with the respective wild-type strains, no significant difference was detected at 37°C (Figures 3.5-3.8). A slight difference in growth of the TIGR4, D39 wild-types and respective Δ*rr06* mutant was reproducibly observed. However when the bacteria were grown at 40°C a great reduction was observed in TIGR4 Δ*rr06* when compared with wild-type (Figure 3.5). In the D39 mutant a small reduction in growth was observed in 40°C, this observation was similar to that at 37°C and the difference was in growth rate rather than total growth, both mutant and wild-type strains reaching the same stationary phase. In serotype 3 (0100993) strain the result remained unclear because the wild-type bacteria had a little growth at 40°C (Figure 3.8). However a longer growth for 0100993 strains should be done to investigate any difference in growth progress between the wild-type and Δ*rr06* mutant.

37°C



40°C

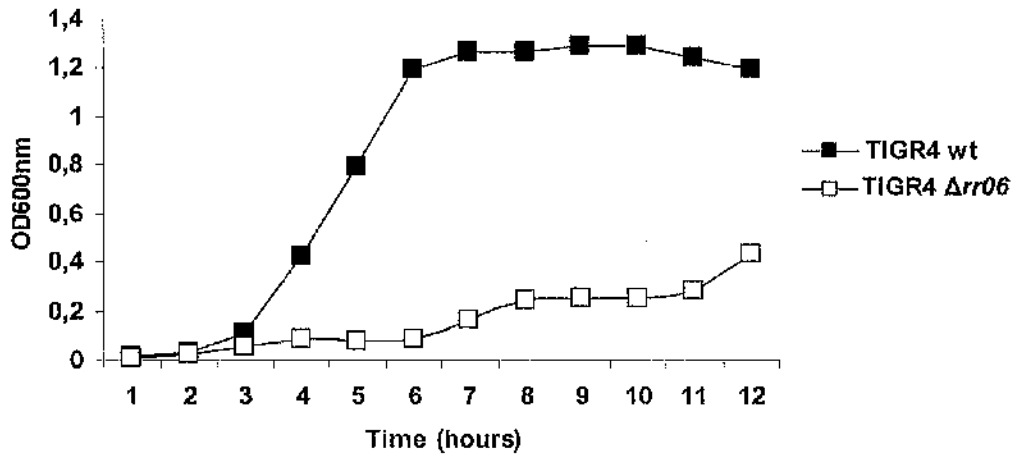
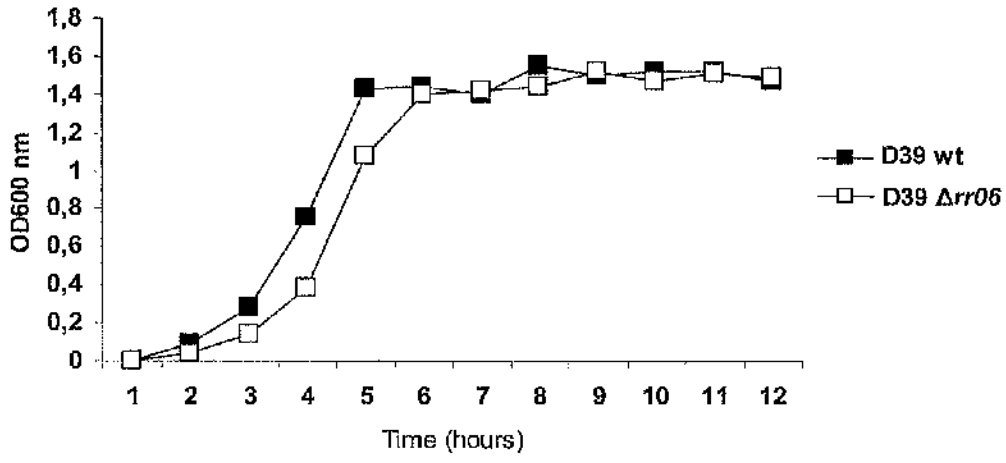


Figure 3.5- Comparison growth curves of TIGR4 wild-type and Δ*rr06* strains at 37 and 40°C.

10^6 CFU/ml of each strain was inoculated in 20 ml of prewarmed BHI and incubated at the indicated temperatures. 1 ml of samples was taken at 60 min-intervals to measure the optical density at 600nm. Each point on the graph represents the mean of 2-3 independent experiments.

37°C



40°C

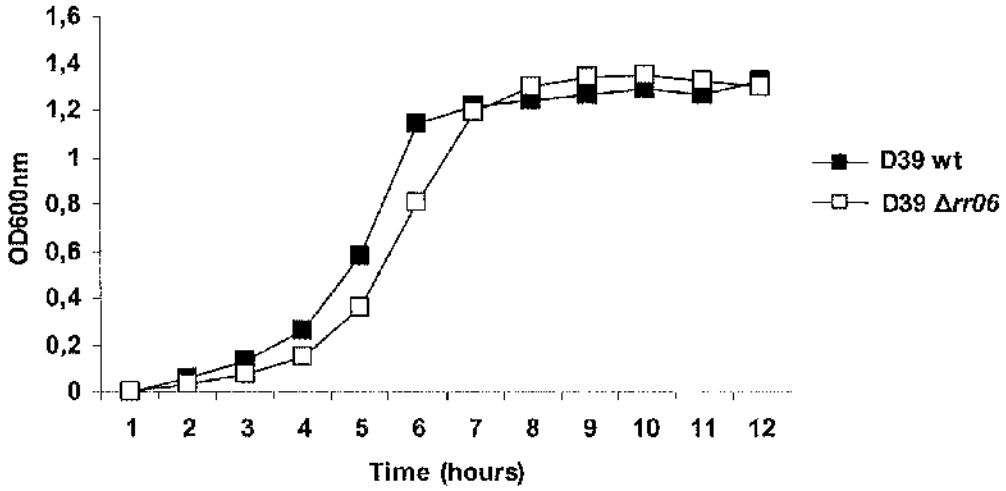


Figure 3.6- Comparison growth curve of D39 wild-type and Δ*rr06* strains at 37 and 40°C.

10^6 CFU/ml of each strain was inoculated in 20 ml of prewarmed BHI and incubated at the indicated temperatures. 1 ml of samples was taken at 60 min-intervals to measure the optical density at 600nm. Each point on the graph represents the mean of 2-3 independent experiments.

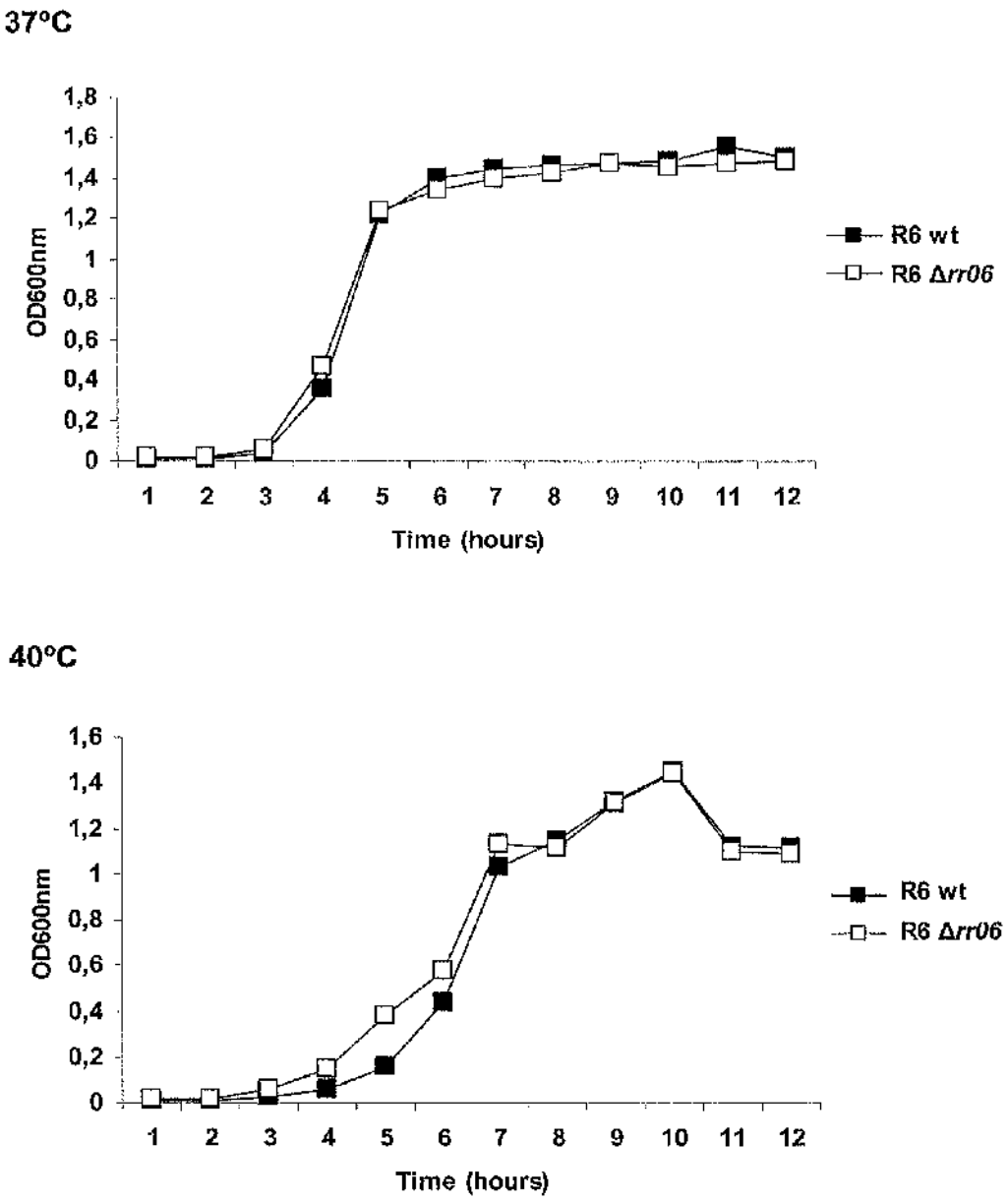


Figure 3.7- Comparison growth curve of R6 wild-type and Δ*rr06* strains at 37 and 40°C.

106 CFU/ml of each strain was inoculated in 20 ml of prewarmed BHI and incubated at the indicated temperatures. 1 ml of samples was taken at 60 min-intervals to measure the optical density at 600nm. Each point on the graph represents the mean of 2-3 independent experiments.

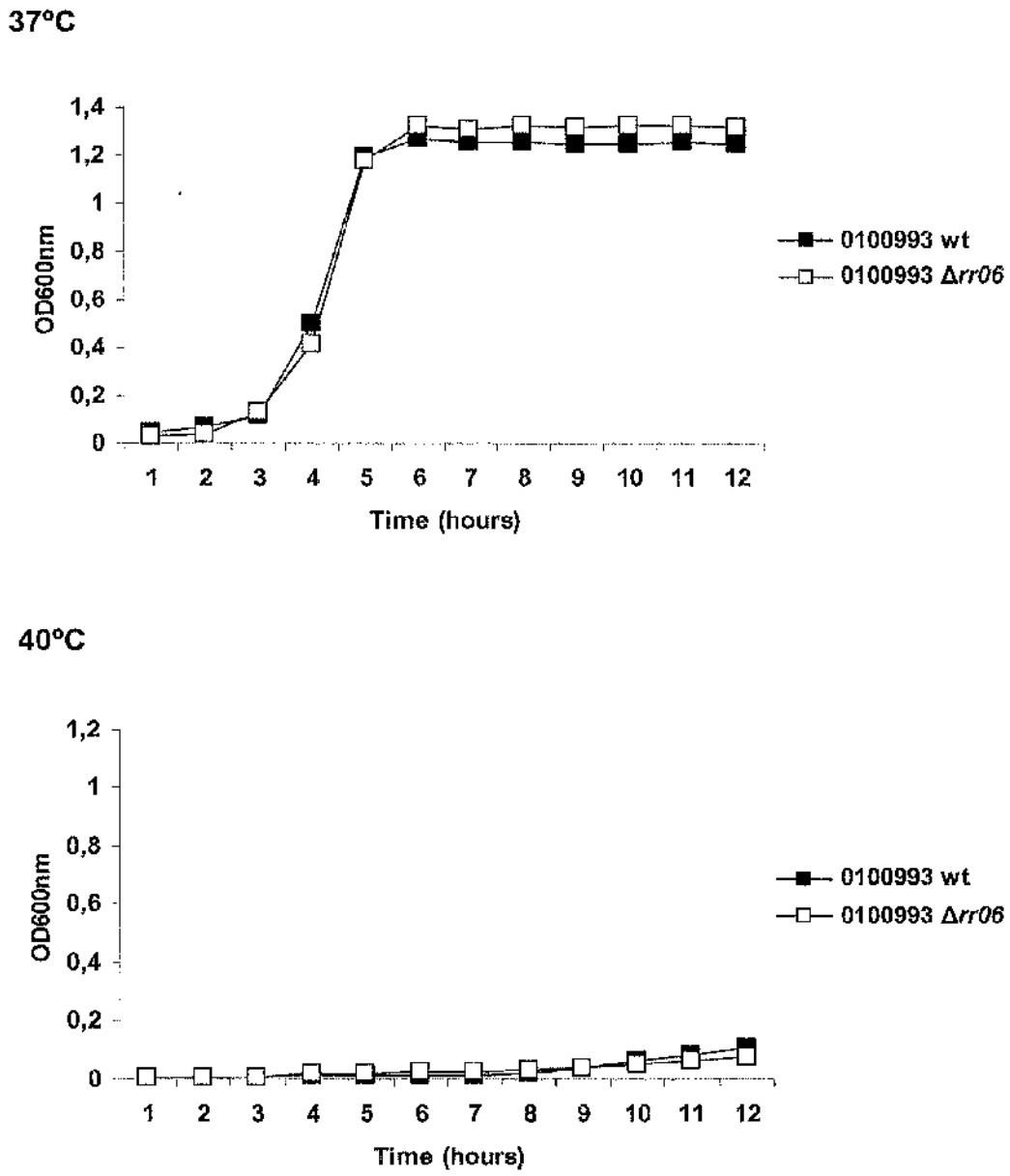


Figure 3.8- Comparison growth curve of 0100993 wild-type and Δ*rr06* strains at 37 and 40°C.

10^8 CFU/ml of each strain was inoculated in 20 ml of prewarmed BHI and incubated at the indicated temperatures. 1 ml of samples was taken at 60 min-intervals to measure the optical density at 600nm. Each point on the graph represents the mean of 2-3 independent experiments.

3.2.2 Growth measurements of *rr06* mutants by viable counting

To confirm the growth trend as measured by optical density measurements and the beginning of autolysis, viable count of TIGR4, D39, R6 and 0100993 wild-types were compared to their Δ*rr06* mutants each 2-hour intervals until 12 hours of growth in BHI broth. No significant differences are observed at both temperatures in D39, R6 and 0100993 strains (Figures 3.10-3.12), while in the TIGR4 background the results reflected the optical density measurements (Figure 3.9). The autolysis in TIGR4 Δ*rr06* strain at

37°C occurs later than wild-type, due to the lower growth rate and at 40°C the mutant maintained the cell viability until 12 hours due the low growth rate when compared with the wild-type. The effect of *rr06* deletion in growth of *S. pneumoniae* is strain dependent. Mutation affected the growth rate of the TIGR4 strain at 40°C but not of two serotype 2 strains.

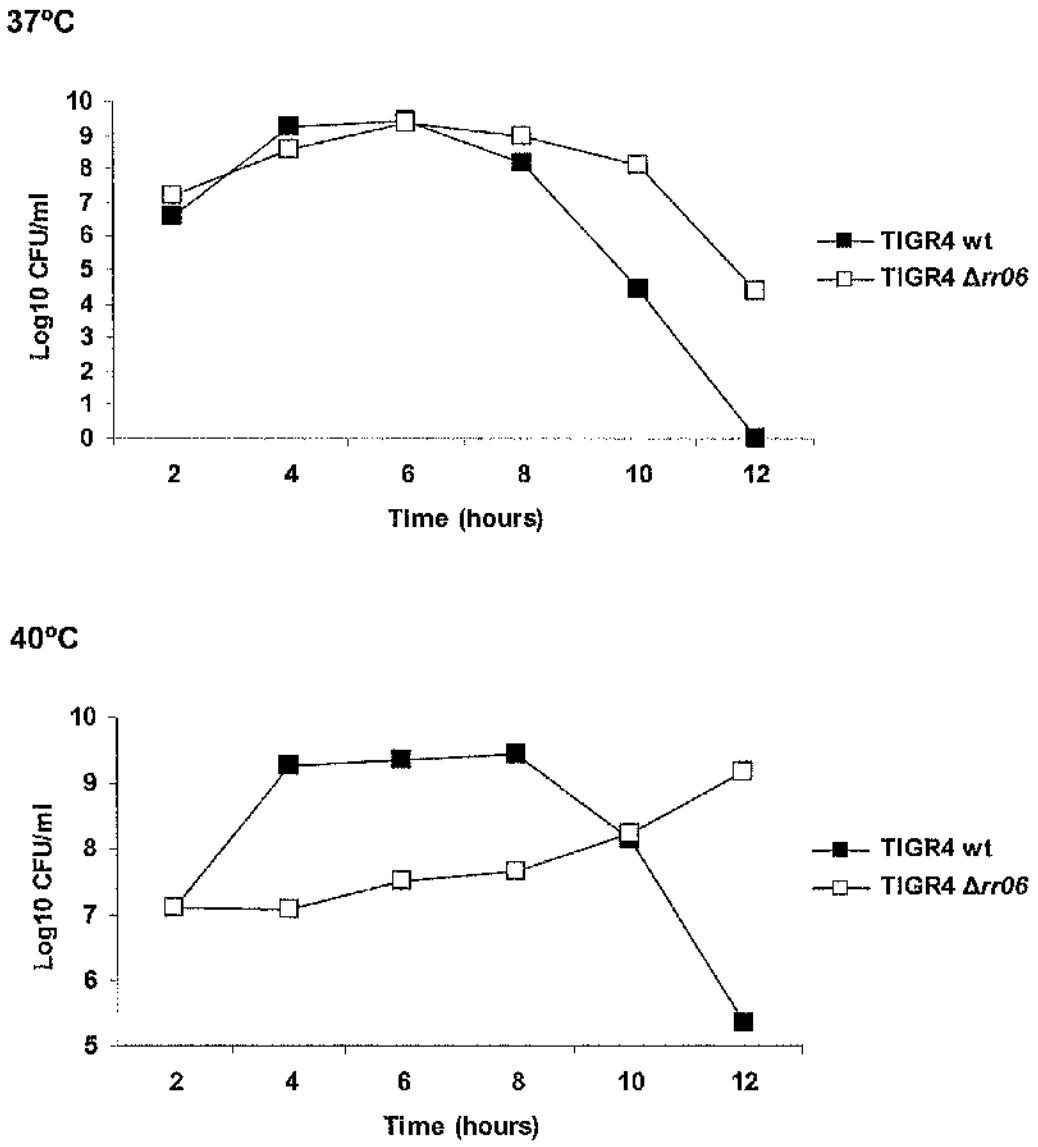


Figure 3.9- Comparison of the *in vitro* growth of TiGR4 wild-type and Δ*rr06* strains at 37 and 40°C.

10^6 CFU/ml of each strain was inoculated in 20 ml of prewarmed BHI and incubated at the indicated temperatures. Samples were taken at 2 hours-intervals to measure the viable bacterial count on blood plates. Each point on the graph represents the mean of 2-3 independent experiments.

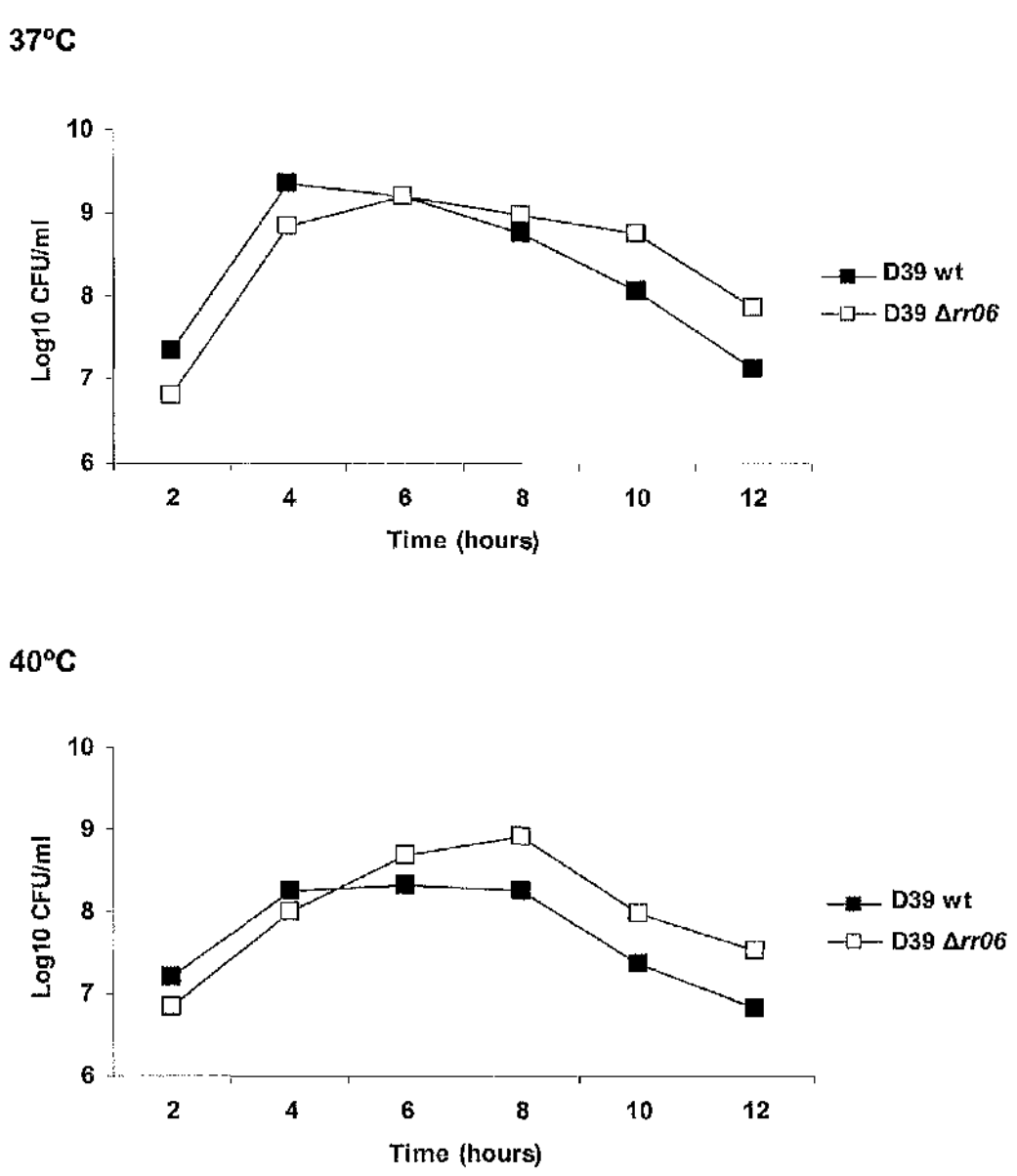


Figure 3.10- Comparison of the *in vitro* growth of D39 wild-type and Δ*rr06* strains at 37 and 40°C.

10^6 CFU/ml of each strain was inoculated in 20 ml of prewarmed BHI and incubated at the indicated temperatures. Samples were taken at 2 hours-intervals to measured the viable bacterial count on blood plates. Each point on the graph represents the mean of 2-3 independent experiments.

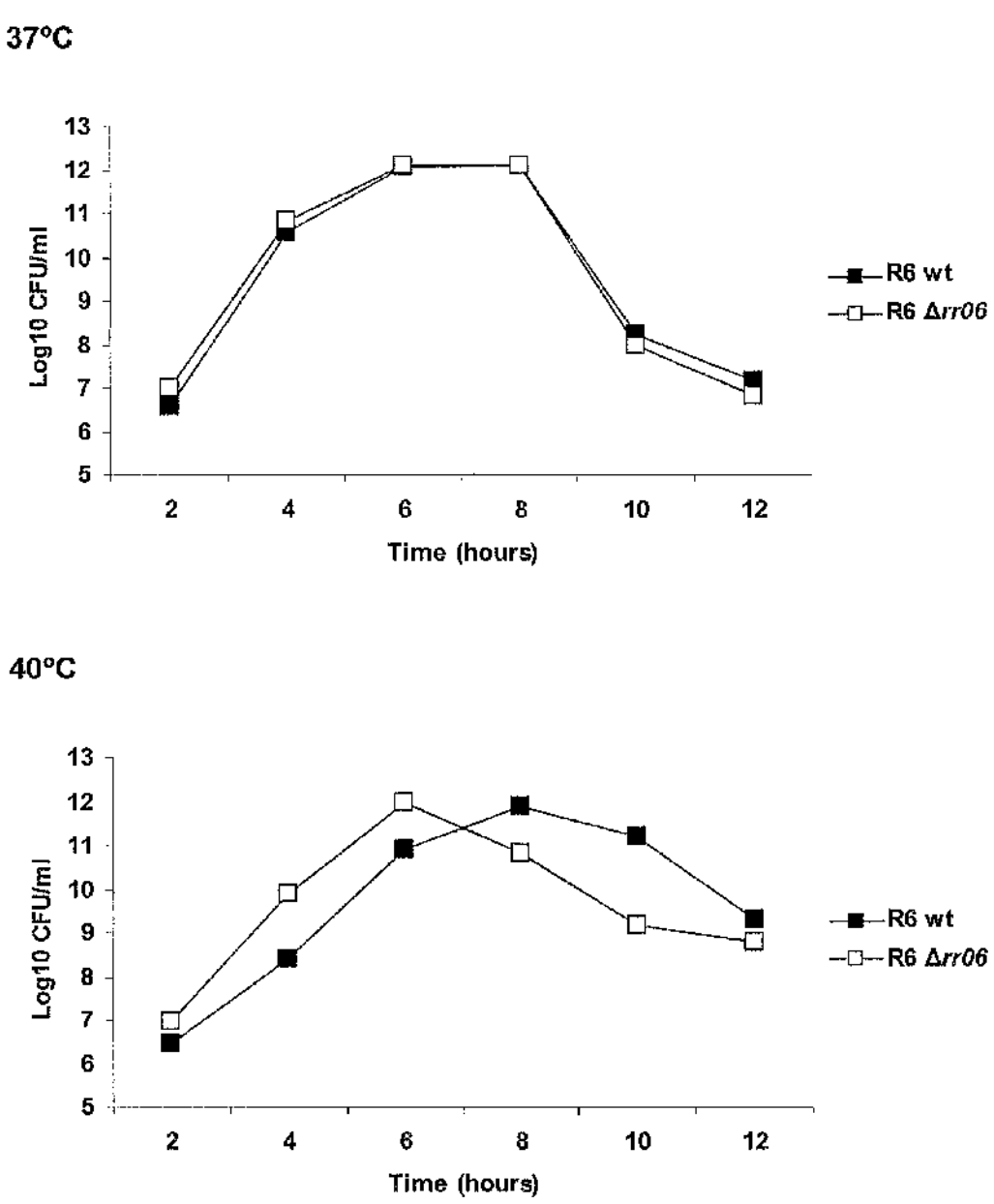


Figure 3.11- Comparison of the *in vitro* growth of R6 wild-type and Δ*rr06* strains at 37 and 40°C.

10^6 CFU/ml of each strain was inoculated in 20 ml of prewarmed BHI and incubated at the indicated temperatures. Samples were taken at 2 hours-intervals to measured the viable bacterial count on blood plates. Each point on the graph represents the mean of 2-3 independent experiments.

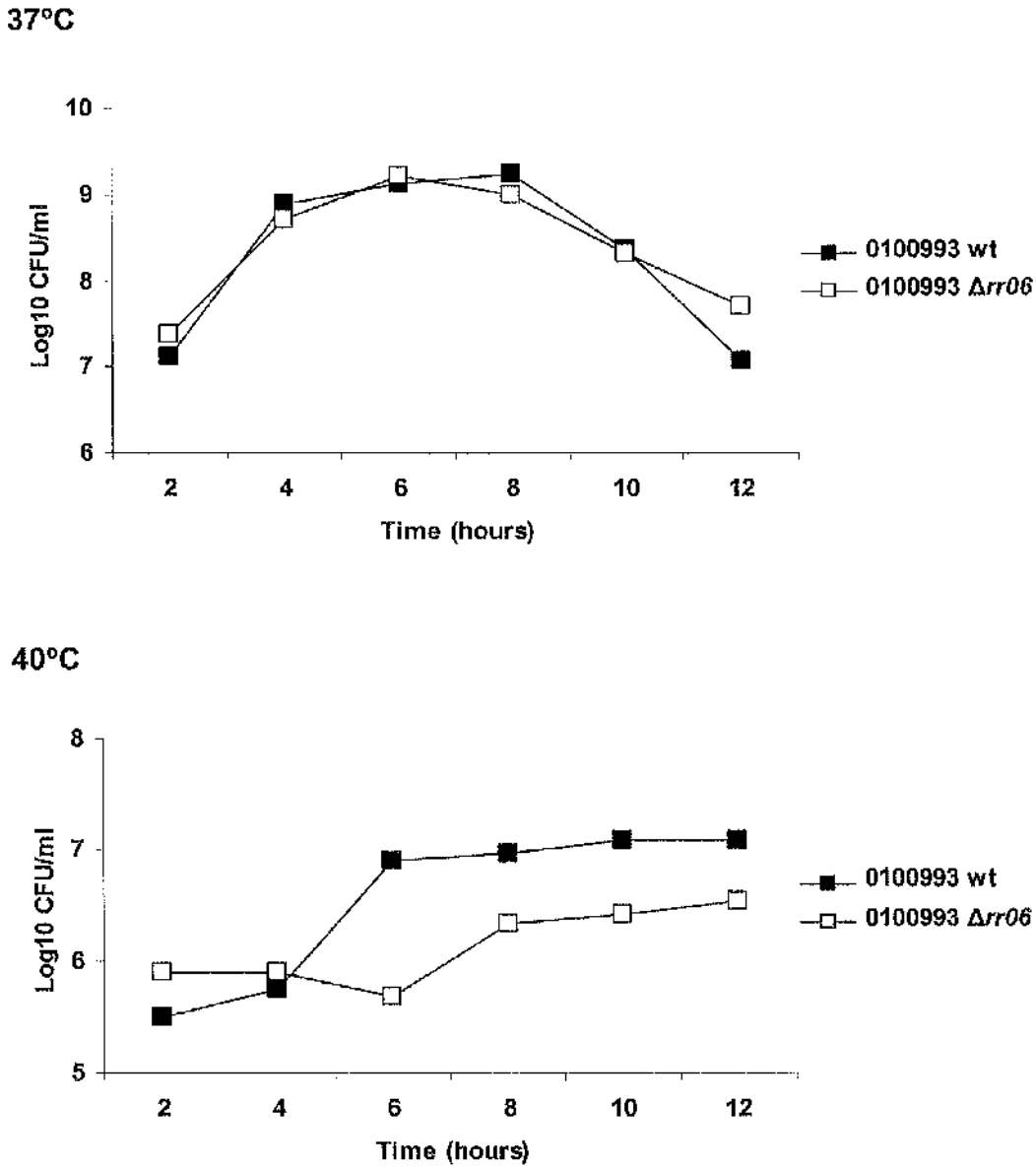


Figure 3.12- Comparison of the *in vitro* growth of 0100993 wild-type and Δ*rr06* strains at 37 and 40°C.

10^6 CFU/ml of each strain was inoculated in 20 ml of prewarmed BHI and incubated at the indicated temperatures. Samples were taken at 2 hours-intervals to measured the viable bacterial count on blood plates. Each point on the graph represents the mean of 2-3 independent experiments.

3.2.3 Growth in anaerobic and aerobic conditions

To study the possible influence of oxygenation on growth of *rr06* mutants at high temperature, the *S. pneumoniae* TIGR4 wild-type and *rr06* mutant strains were inoculated on blood plates and incubated in CO_2 candle jars or in aerobic conditions at 37 and 40°C. No difference was observed between the bacteria grown in anaerobic and aerobic

conditions (Figure 3.13 and Figure 3.14). The *rr06* mutation seems to have had a huge impact on the phenotype when grown at 40°C in presence or absence of O_2 Figure 3.14.

A summary of the influence of temperature on the growth of *S. pneumoniae rr06* mutants and wild-type strains are listed in Table 3.1.

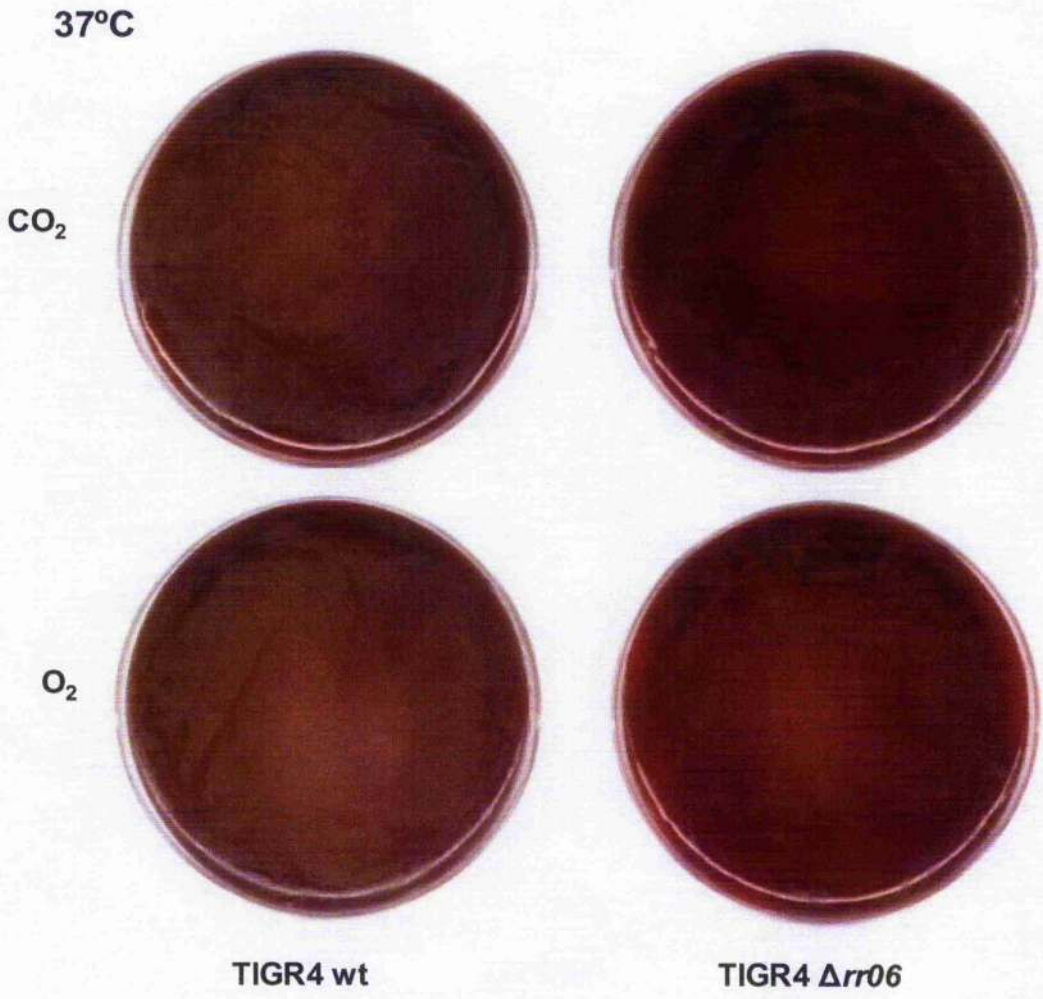


Figure 3.13- Growth of TIGR4 wild-type and Δ*rr06* strains at 37°C.

The pneumococcal strains were grown on blood plates at 37°C in anaerobic conditions (CO_2 candle jar) or in aerobic conditions during overnight.

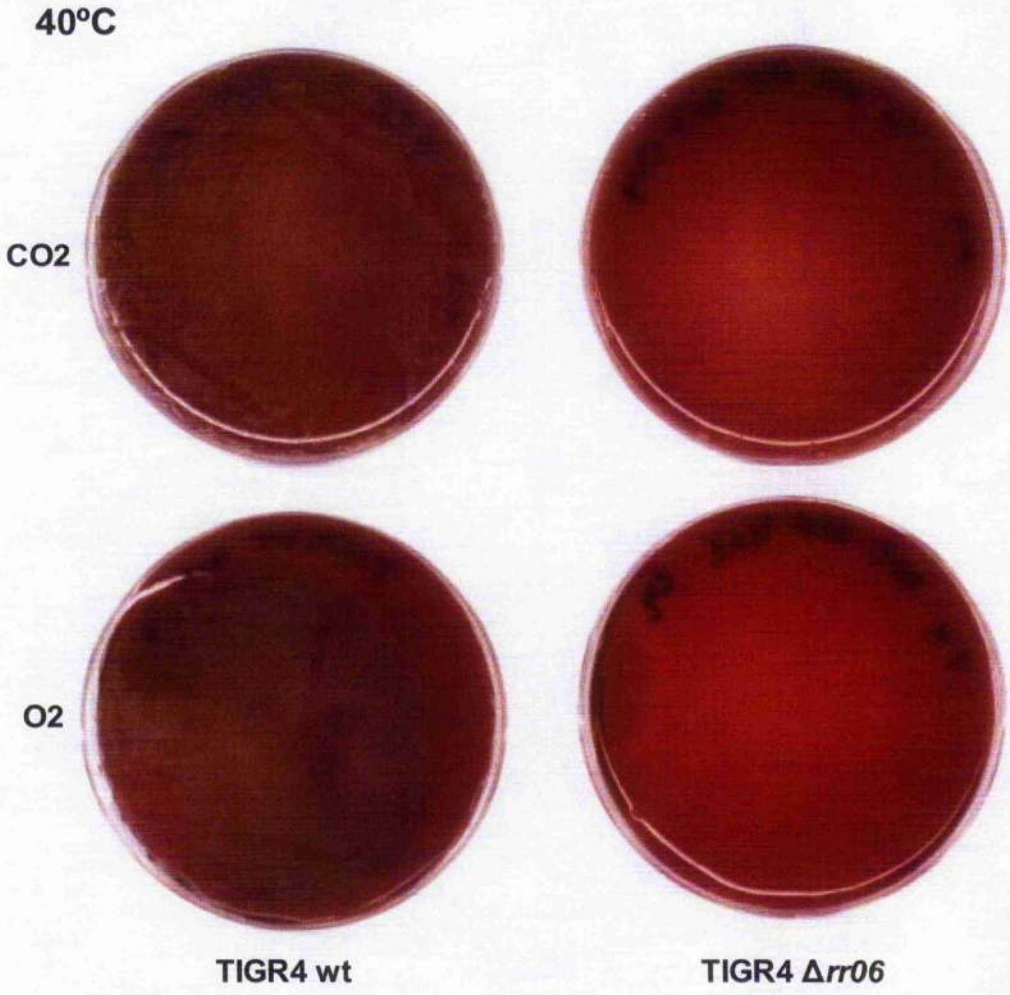


Figure 3.14- Grown of TIGR4 wild-type and Δ*rr06* strains at 40°C

The pneumococcal strains were grown on blood plates at 40°C in anaerobic conditions (CO_2 candle jar) or in aerobic conditions during overnight.

Table 3.1- Summary of growth of *S. pneumoniae rr06* mutants and wild-type strains in broth and blood plates.

Strains	Broth		Plates			
	37°C	40°C	37°C/O_2	37°C/CO_2	40°C/O_2	40°C/CO_2
TIGR4 wt	++++	+++	++++	++++	+++	+++
TIGR4 Δ*rr06*	+++	+	++++	++++	-	-
D39 wt	++++	+++	N/A	N/A	N/A	N/A
D39 Δ*rr06*	+++	+++	N/A	N/A	N/A	N/A
R6 wt	++++	+++	N/A	N/A	N/A	N/A
R6 Δ*rr06*	++++	+++	N/A	N/A	N/A	N/A
0100993 wt	++++	+	N/A	N/A	N/A	N/A
0100993 Δ*rr06*	++++	+	N/A	N/A	N/A	N/A

(++++) Normal growth.
(+++) Slow growth.
(+) Poor growth.
(-) No grow.
(N/A) Data not available.

3.2.4 Resistance of mutants to oxidative stress

To study the role of RR06 in oxidative stress tolerance, the sensitivity of TIGR4 Δ*rr06* to peroxide was compared to the TIGR4 wild-type using the hydrogen peroxide survival test (Tseng *et al.*, 2002). The bacteria were grown to OD_{600nm} 0.3 in BHI broth and subjected to 40 mM of H_2O_2 to 5, 10 and 15 min and the survival rate was calculated from viable count on blood plates against a control for both strains without H_2O_2. TIGR4 Δ*rr06* seems more sensitive to peroxide than wild-type (Figure 3.15), but no statistically significant difference was observed in the 3 times of exposure to H_2O_2, due to the large statistical variation observed.

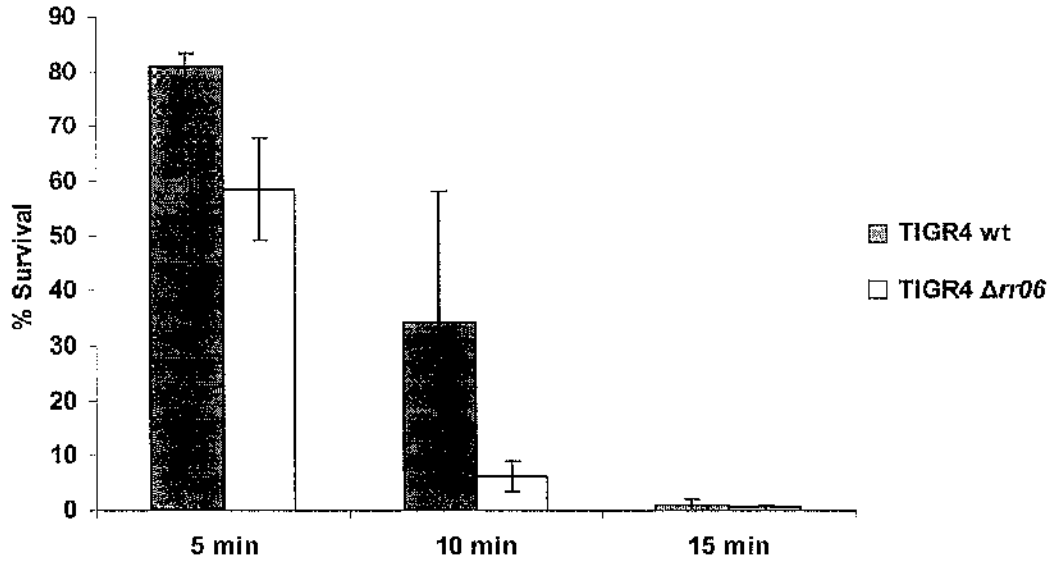


Figure 3.15- H_2O_2 survival test of *S. pneumoniae* TIGR4 wild-type and Δ*rr06*.

Bacteria were grown in BHI to OD_{600nm} 0.3 and 40 mM of H_2O_2 was added to 1 ml aliquot of the culture. Cells were incubated at room temperature for 5, 10 and 15 min with or without H_2O_2 and viable counts carried out in blood pates. Values are expressed as the survival percentage and represent the mean (SEM) of three independent experiments.

3.2.5 Adherence assay

The virulence factor PspC is know to function as an adhesin, playing an important role in adherence and colonization of *S. pneumoniae* to epithelial cells (Rosenow *et al.*, 1997). As the HK/RR06 system has been shown to regulate the PspC, the effect of the *rr06* deletion on adherence of *S. pneumoniae* was studied using the human pharyngeal cell line Detroit 562 cells. No significant difference was observed in adherence capacity of TIGR4 Δ*rr06* when compared with wild-type Figure 3.16.

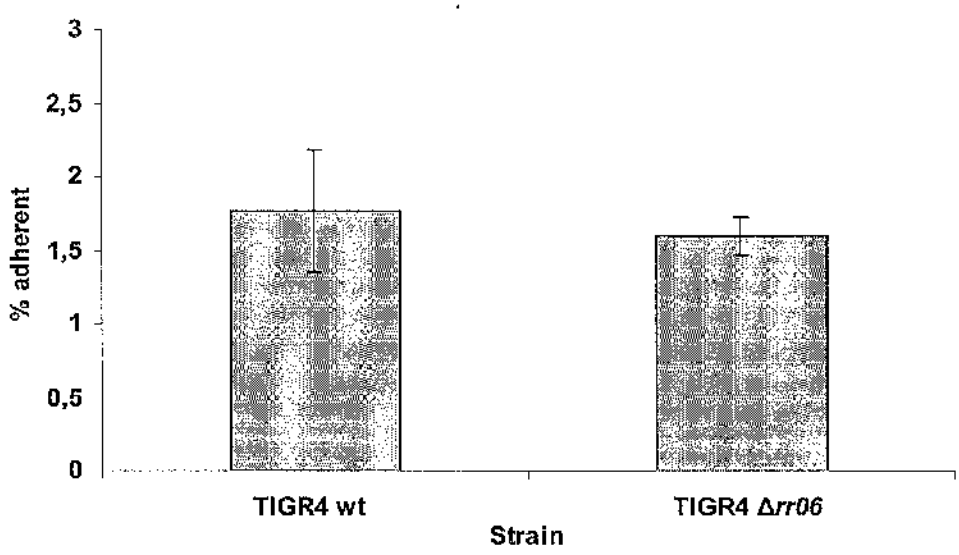


Figure 3.16- Adherence of TIGR4 wild-type and Δ*rr06* mutant strains to human nasopharyngeal cells.

Bacteria were added to a confluent monolayer of washed Detroit 562 cells and the fraction remaining associated with the cells determined as described in 2.8.4. Data expressed as the percentage of cells adherent/ internalised from the initial inoculum. No significant difference was found between the two groups Data represent mean ± SEM, pooled from two experiments (each n = 4, n = 8 in total). This experiment was done in collaboration with Dr. Gavin Paterson.

3.3 *In vivo* analysis

The effect of RR06 on the growth of the bacteria at 40°C in TIGR4 strain and the possibility that it regulates the virulence factor PspC in *S. pneumoniae* suggests that RR06 may play an important role in the virulence of the *S. pneumoniae*. Murine models of pneumonia and bacteraemia were used to study the presumable contribution of RR06 to the pathogenesis of pneumococcal disease.

3.3.1 Intranasal infection

TIGR4 Δ*rr06* and Δ*pspC* were analysed for their abilities to cause disease in a pneumonia model of infection. Mice were infected with 1 x 10^6 CFU intranasally and the development of symptoms was monitored over a period of 2 weeks. No differences were observed in TIGR4 Δ*rr06* compared with wild-type. In both strains all mice succumbed to the infection at the same rate and had similar survival times. On the other hand, 80% of the mice survived to infection with Δ*pspC*, presenting a significantly longer survival times than TIGR4 wild-type (Figure 3.17).

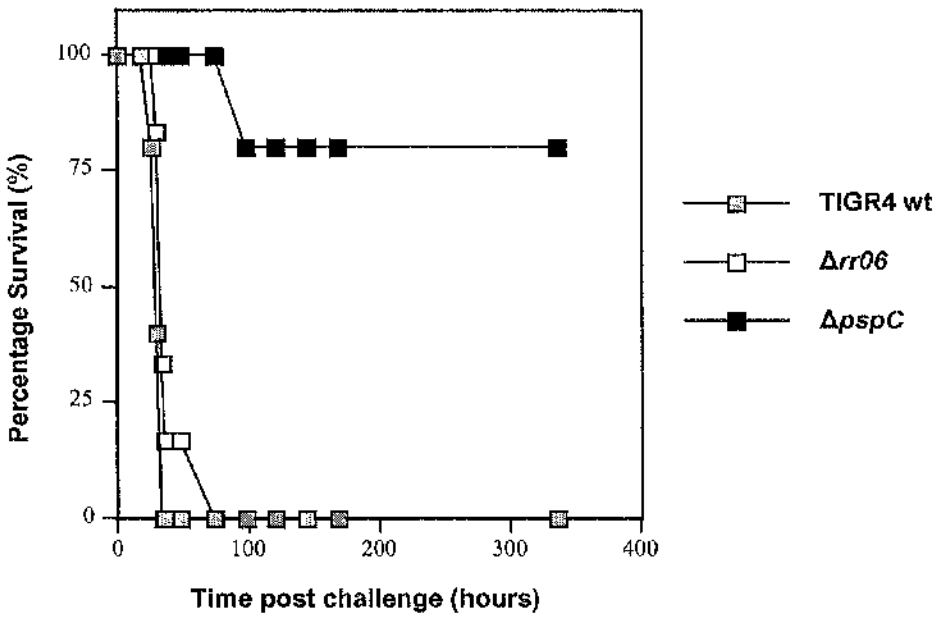


Figure 3.17- Survival of mice following intranasal infection with TIGR4 wild-type, Δ*rr06* and Δ*pspC* strains.

Mice were infected intranasally with 10^6 CFU/mouse. In survival data, no significant difference was observed between Δ*rr06* (median 33h) and TIGR4 (median 30h). Δ*pspC* had significantly longer survival times than TIGR4 ($P<0.01$), presenting a median survival time 336 hours (ie they survived challenge). 4/5 mice infected with Δ*pspC* did survive, the other 1 died at 97.5h. This experiment was done in collaboration with Dr. Alison Kerr.

3.3.2 Intravenous Infections

To study the importance of RR06 in a bacteraemia model, TIGR4 wild-type, Δ*rr06* and Δ*pspC* mutants were injected directly into the bloodstream by tail vein. Mice were given 1.0 x 10^6 CFU/mouse and survival times was determined. There were no significant differences between the TIGR4 Δ*rr06* and its parent strain to cause death in mice. All animals in both strains succumbed after 336 hours post-infection, presenting TIGR4 Δ*rr06* a median of 56 hours of survival against 33.5 hours in wild-type. As in the pneumonia model, a statistically significant difference was observed in TIGR4 Δ*pspC* compared with wild-type strain. 60% of the MF1 mice had survived following 336 hours of intravenous infection (Figure 3.18).

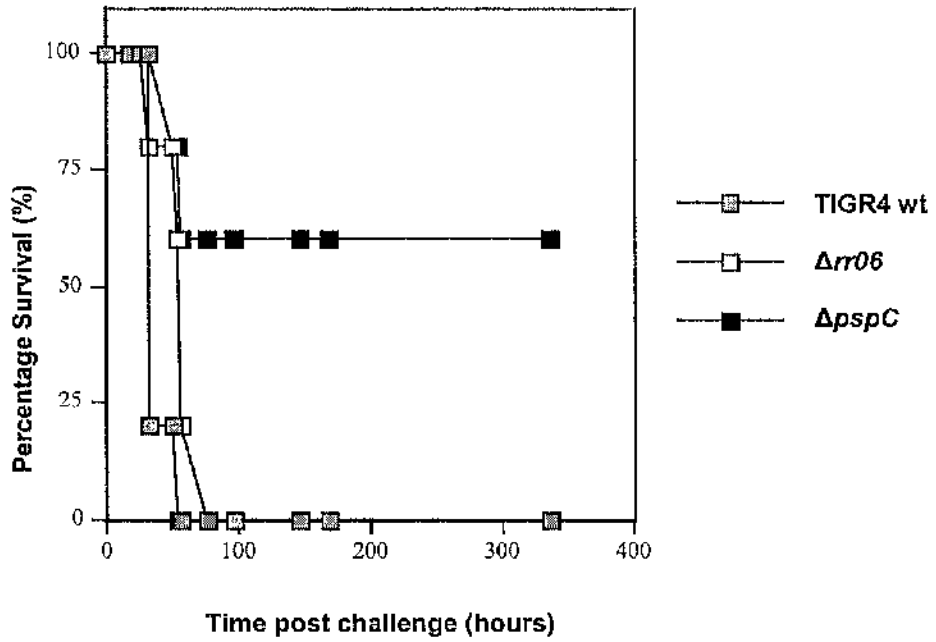


Figure 3.18- Survival of mice following intravenous infection with TIGR4 wild-type, Δ*rr06* and Δ*pspC* strains.

Mice were infected intravenously with 10^6 CFU/mouse. In survival data, no significant difference was observed between Δ*rr06* (median 56h) and TIGR4 wild-type (median 33.5h). Δ*pspC* had significantly longer survival times than TIGR4 ($P<0.05$), with a median survival time 336 hours (ie they survived challenge). 3/5 mice infected with Δ*pspC* did survive, the other 2 died at 49h and 56h. This experiment was done in collaboration with Dr. Alison Kerr.

3.3.3 Bacteriological investigation

The bacterial loads at 24h post-intranasal infection were studied in TIGR4 wild-type and in the mutants Δ*rr06* and Δ*pspC*. Slight differences from the results in survival times were observed. The levels of bacteria in nasopharynx were similar in Δ*rr06* compared with wild-type, however a statistically significant decrease in bacterial levels were observed in lung airways and lung tissue in *rr06* mutants. *pspC* mutants presented significant decrease in bacterial loads in all cases (Figure 3.19). In the bacteraemia model, 24h following intravenous infections (Figure 3.20) a decrease in bacterial counts was observed in TIGR4 Δ*rr06* and Δ*pspC* compared with wild-type however the lower levels of bacteria in Δ*rr06* was not statically significant. On the other hand, 24h after intranasal infection a significant decreased was observed in both mutants compared with wild-type.

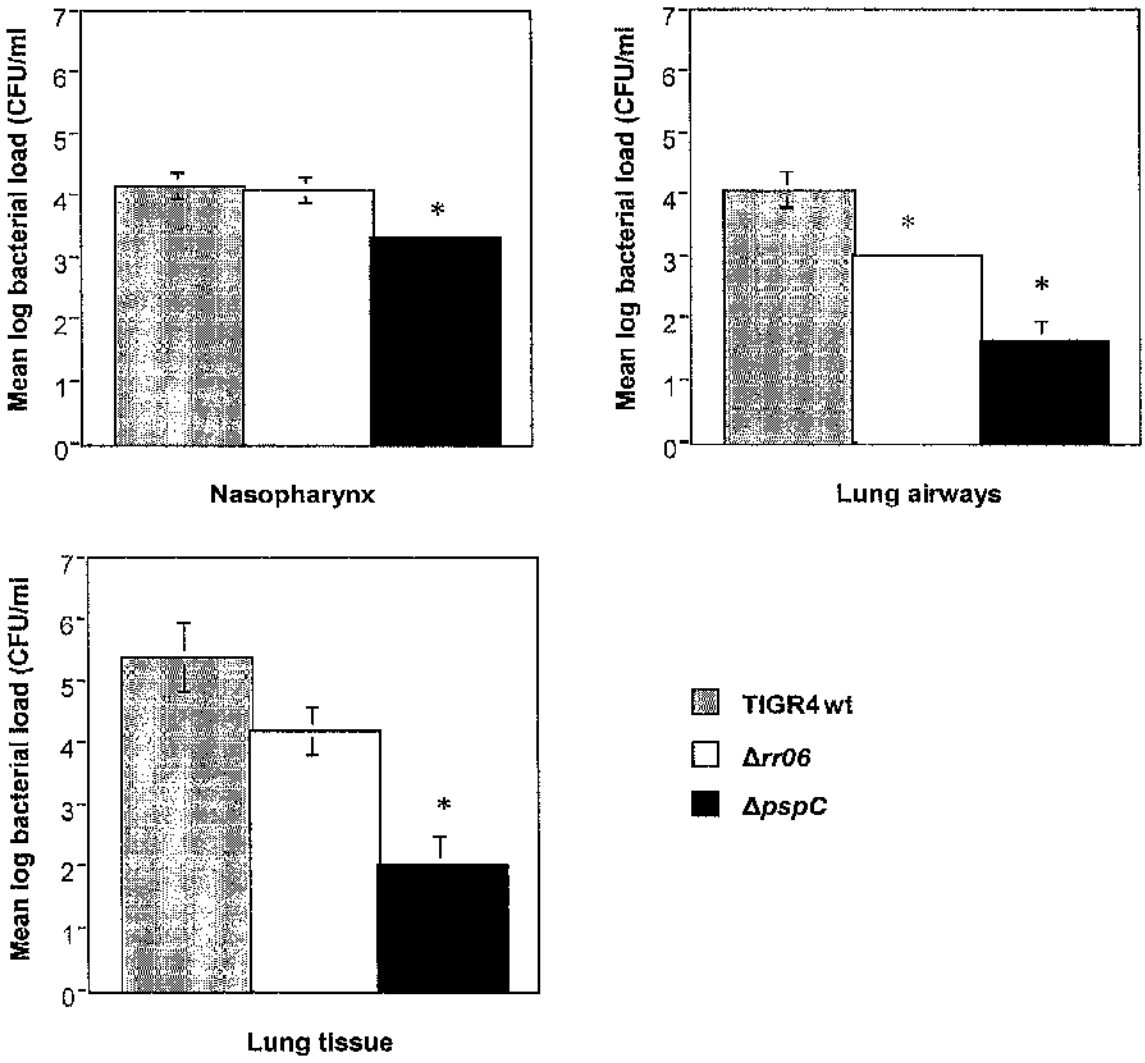


Figure 3.19- Bacterial loads of TIGR4 wiid-type, Δ*rr06* and Δ*pspC* after intranasai infection.

Effect of RR06 and PspC in the TIGR4 background on nasopharyngeal, lung airway and lung tissue bacterial load in mice. Bacterial counts were performed on blood plates after 24h post-intranasal infection. n=5 mice/group, * P<0.01 signlficantly different from TIGR4 results. This experiment was done in collaboration with Dr. Alison Kerr.

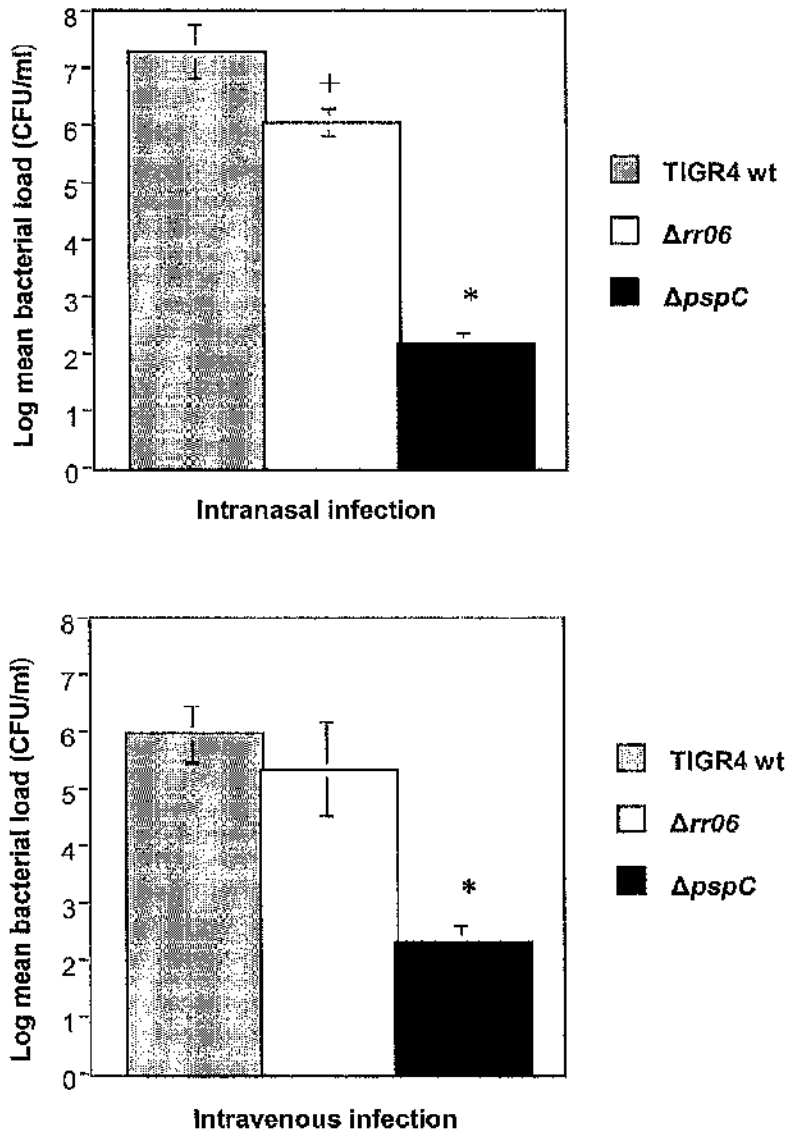


Figure 3.20- Bacterial loads in blood of TIGR4 wild-type, Δ*rr06* and Δ*pspC* after intranasal and intravenous infection.

Effect of RR06 and PspC in the TIGR4 background on bacteraemia in mice. Bacterial counts were performed on blood plates after 24h post-intranasal or intravenous infection. n=5 mice/group, * P<0.01 and + P<0.05 significantly different from TIGR4 results. This experiment was done in collaboration with Dr. Alison Kerr.

The RR06 seems to play an important role in the capacity of *S. pneumoniae* TIGR4 to invade the lungs and blood. Furthermore, the mutation in *rr06* gene affected the ability of the bacteria grow at higher temperatures, however this phenotype seems to be strain-dependent. To investigate the genes regulated by the RR06 that can be involved in the Δ*rr06* mutants phenotypes described above, a microarray analysis was performed between the wild-types strain TIGR4, R6 and 0100993 and the *rr06* deficient mutants.

3.4 Microarray analysis

Microarray analysis was performed to compare the transcriptome of *S. pneumoniae* wild-type strain TIGR4 and the isogenic mutant Δ*rr06*. This study was intended to analyse the genes regulated by the RR06 in strain TIGR4 and also, identify putative genes that can be involved *in vitro* and *in vivo* phenotype of the Δ*rr06* strain. During this analysis, two different amplicon-based microarrays were used: TIGR arrays v.1, designed to represent 2131 ORFs from *S. pneumoniae* TIGR4 in addition to 118 ORFs from R6 (37) and G54 (81); BμG@S arrays, designed to represent each of the annotated ORFs present in *S. pneumoniae* strains TIGR4 and R6. The use of two distinct amplicon-based microarrays in this study is due to the availability of theses arrays in our laboratory, and also to compare the reproducibility between both arrays and methods. For each array type comparison, an independent RNA preparation was used. For each RNA preparation four array slides were used and a dye swap was performed to minimize the variation between the Cy3 and Cy5 incorporation and intensities, i.e., in two slides cDNA from TIGR4 wild-type was labelled with Cy3 and cDNA fromTIGR4 Δ*rr06* with Cy5, while in the other two slides the cDNA from TIGR4 wild-type was labelled with Cy5 and cDNA from TIGR4 Δ*rr06* with Cy3. Furthermore, due the differences of *in vitro* phenotype in TIGR4 Δ*rr06* compared with the D39, R6 and 0100993 Δ*rr06* strains, preliminary microarray analysis was performed to study the transcriptional profiles in one of the serotype 2 (R6) and 0100993 wild-type strains and their isogenic mutant Δ*rr06*.

3.4.1 RNA analysis

The RNA samples were prepared from TIGR4 wild-type and Δ*rr06* strains grown in 10 ml of BHI to mid-log phase (OD_{600nm} of 0.6) at 37°C. Total RNA was isolated using Qiagen RNeasy-Midi Kit and the concentration calculated using the NanoDrop® ND-1000 spectrophotometer. Concentrations of total RNA between 1.0 -2.0 μg/μl were obtained for the TIGR4 Δ*rr06* and its parent strains. Because it is essential to use high-quality messenger RNA in the context of gene expression analysis via microarray analysis, the integrity and purification of total RNA sample were tested using the Agilent Bioanalyser. The system calculates the ratio of ribosomal bands in total RNA samples and shows the percentage of ribosomal impurities in RNA samples (Wang *et al.*, 2003). Figure 3.21 and Figure 3.22 illustrate the quality of total RNA from the samples used in TIGR and BμG@S arrays respectively, indicating the purity of the RNA (no genomic DNA contamination) as well no degradation of the RNA samples. RNase degradation of total RNA is easily

detected by a shift in the RNA size distribution towards smaller fragments and a decrease in fluorescence signal of ribosomal peaks, while genomic DNA contamination is characterized by larger molecular weight peak that is well separated from rRNA peaks (Figure 3.23).

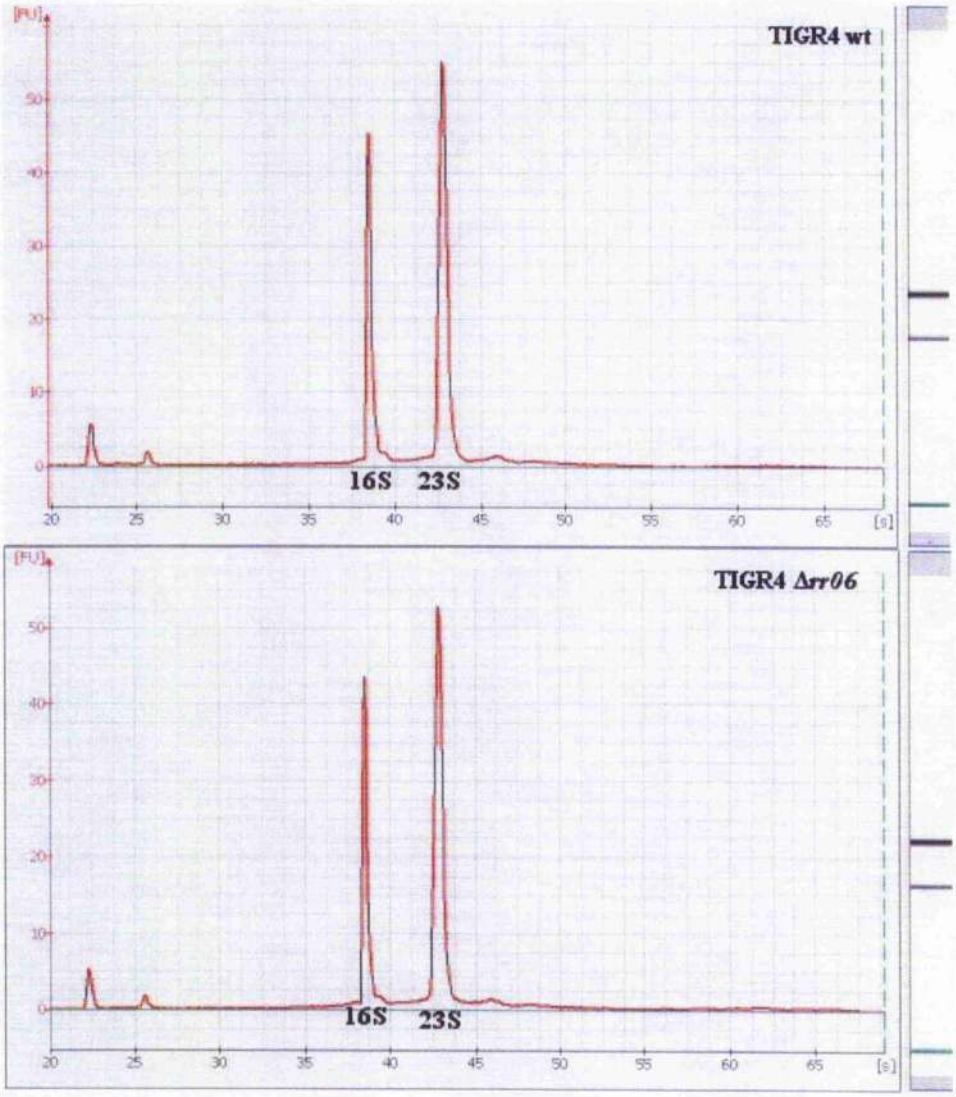


Figure 3.21- Analysis of total RNA samples from TIGR4 wild-type and Δ*rr06* strains used in TIGR microarrays.

Integrity and purity of total RNA prepared from 10 ml of BHI cultures grown to mid-log phase (OD_{600nm} of 0.6) at 37°C using Qiagen RNeasy-Midi Kit and checked with Agilent 2100 Bioanalyser.

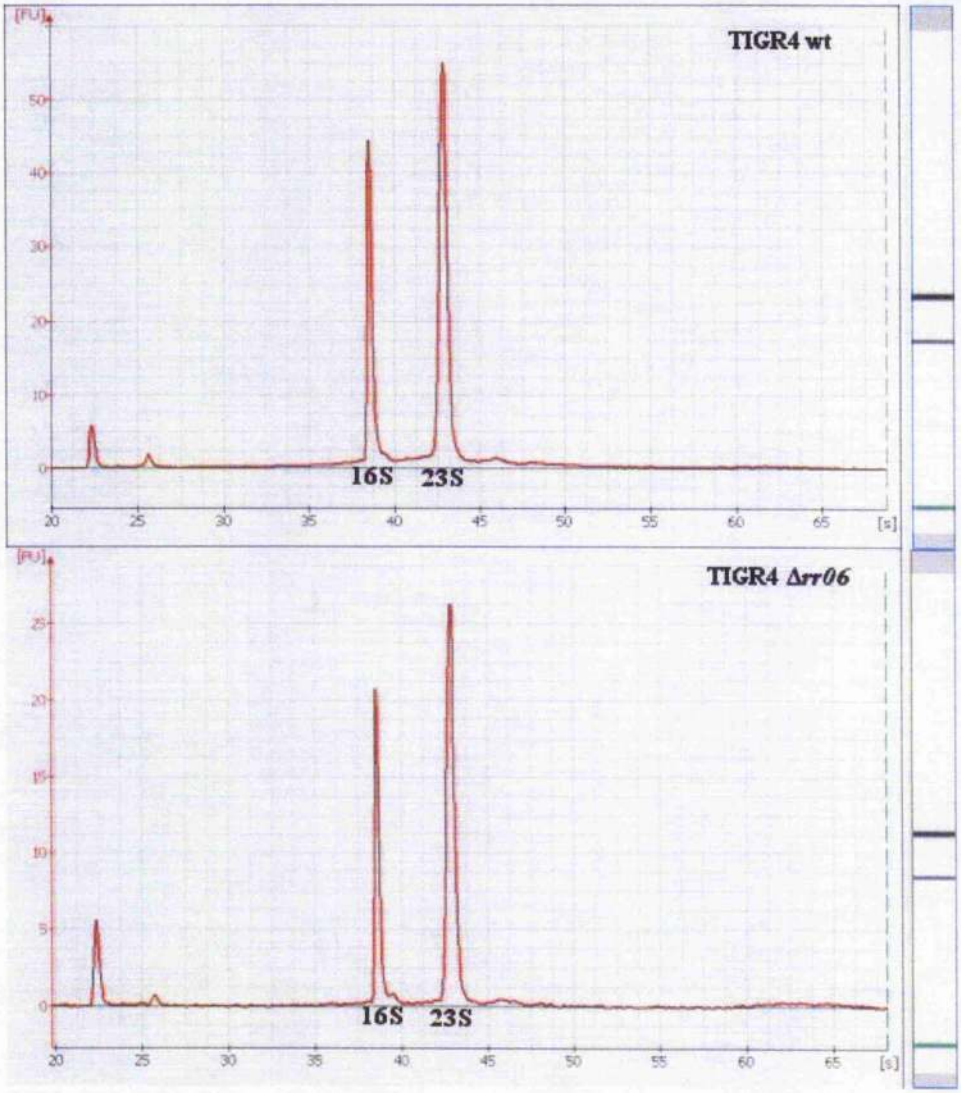


Figure 3.22- Analysis of total RNA samples from TIGR4 wild-type and Δ*rr06* strains used in BμG@S arrays.

Integrity and purity of total RNA prepared from 10 ml of BHI cultures grown to mid-log phase (OD_{600nm} of 0.6) at 37°C using Qiagen RNeasy-Midi Kit and checked with Agilent 2100 Bioanalyser.

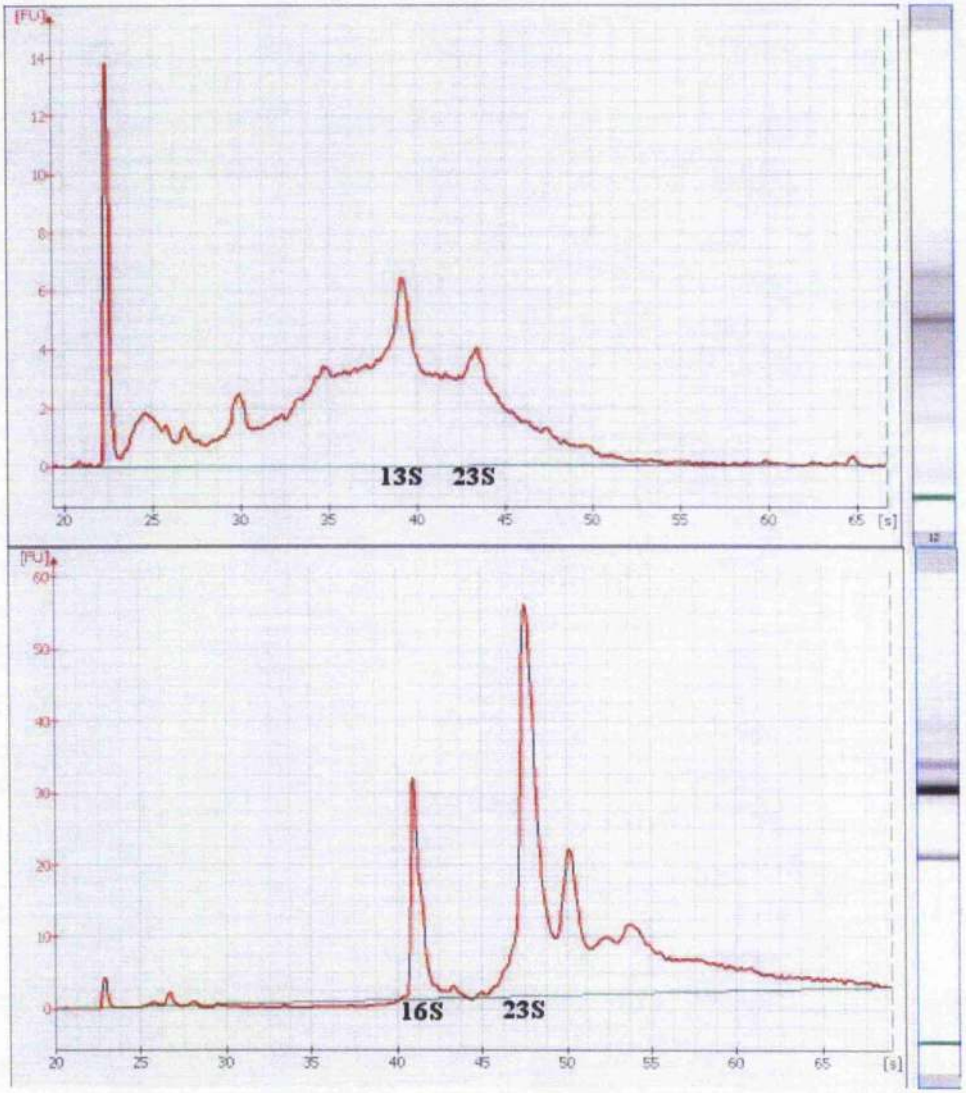


Figure 3.23- Different types of low quality of RNA for microarray sample preparation.

Agilent 2100 bioanalyzer electropherograms of RNA. Degraded total RNA, the baseline fluorescence is elevated for both 16S and 23S making the rRNA peaks appear to be riding on top of the baseline (A); Contaminated total RNA with high molecular weight genomic DNA (B).

3.4.2 TIGR arrays

The transcriptional profiles of *S. pneumoniae* TIGR wild-type and Δ*rr06* strains were performed using TIGR microarray slides using an indirect labelling method. 10 μg of total RNA prepared from each strain was used in reverse transcriptase reactions to generate cDNA, which was labelled with Cy3 and Cy5 fluorochromes. Four slide replicates were used and a dye swap was performed.

Following the hybridization at 42°C overnight the slides were scanned using ScanArray ™ Express microarray scanner and the signal intensities for each spot of both sample were measured using the Quantarray™. The microarray data is shown as a ratio of signal intensities for each spot getting a measure for fold changes in gene expression. Nevertheless, ratios computed from raw data typically have a skewed frequency distribution. For this reason a manual normalization for the two dyes during the scanning was performed as well the LOWESS intensity-dependent normalization in Genespring™ software prior to analysis. The purpose of normalization is to adjust the effects in the variation of microarray technology such as the fluorescence intensities of the two colour channels (Cy3 and Cy5), differences between the RNA samples or the printed probes, efficiency of dye incorporation, experimental variability in probe coupling, scanner sensitivity and signal amplification (Smyth and Speed, 2003; Yang *et al.*, 2002).

After the normalization, the data from the four arrays slides were analysed using Genespring™. Statistically significant differences in expression between the wild-type and Δ*rr06* strains were defined as those with a Student's *t* test *p*-value < 0.05. Since this *p*-value is purely a statistical measure of differential gene expression an additional ratio-change threshold of at least 2 standard deviations over the median ratio for each strain was applied.

3.4.2.1 TIGR microarray data

Microarray comparisons of the transcription profiles of the TIGR4 Δ*rr06* and the respective wild-type were used to identify the genes controlled by RR06. The scatter plot in Figure 3.24 showed the relationship of the mean ratio of hybridization intensities between the TIGR4 wild-type (control strain), and the Δ*rr06* mutant (test strain). This graphical representation shows an overview of the transcription profiles, characterized with a range of colours for the intensities of expression. The genes up-regulated in TIGR4 Δ*rr06* appear with red colour, down-regulated genes with blue colour and the genes

without significant variation, between the wild-type and the mutant strain, appear with yellow spots.

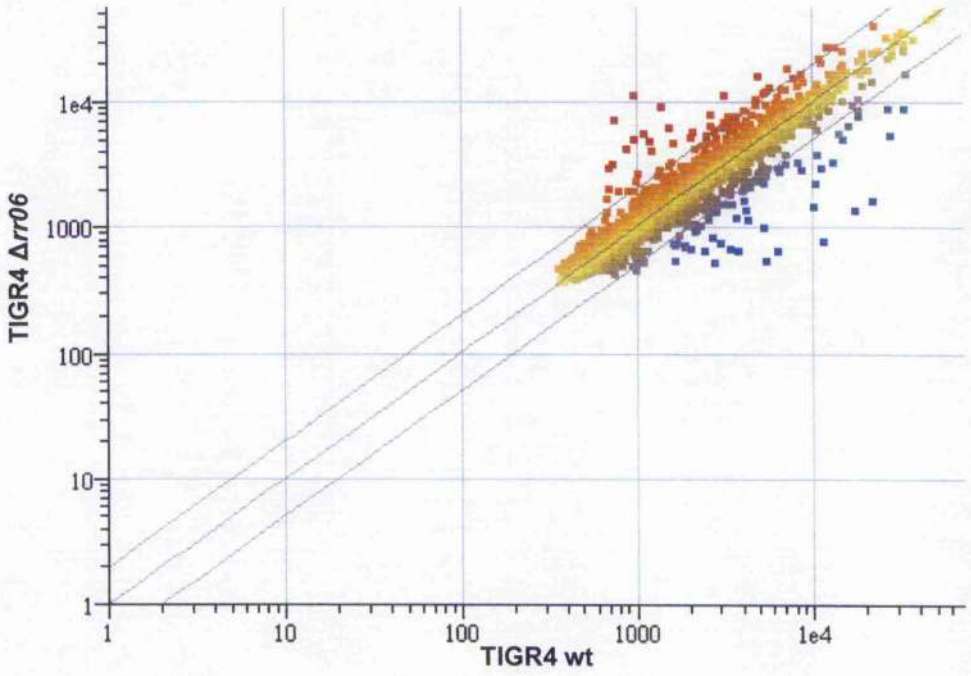


Figure 3.24- Scatter plot comparing the gene expression between TIGR4 Δ*rr06* and TIGR4 wild-type in TIGR arrays slides.

cDNA labelled with Cy3 and Cy5 were hybridized in TIGR arrays slides and analysed in Genespring™. The scatter plot showed the gene expression intensities between the TIGR4 wild-type (control strain) and TIGR4 Δ*rr06* (test strain). Blue spots (bellow the diagonal lines) mean the genes down- regulated in TIGR4 Δ*rr06* and the red spots (above the diagonal lines) the genes up-regulated. The yellow spots (inside the diagonal lines) describe the genes without significant variation between theTIGR4 Δ*rr06* and the wild-type strains.

Whereas most of the pneumococcal genes remained unaltered, showing comparable expression levels between the wild-type and the Δ*rr06* strains. Approximately 3% of the genes were shown to have significant differences in expression levels between the wild-type and Δ*rr06* mutants. Eighteen genes were found to be up-regulated in TIGR4 Δ*rr06* and fifty three genes down regulated. The up and down-regulated genes were selected if they demonstrated a two-fold difference in signal intensity between TIGR4 wild-type and Δ*rr06* mutant and if this intensity was found to be statistically significant ($P \leq 0.05$). The altered genes confirmed by hybridization of TIGR DNA microarray slides are listed in Table 3.2 (up-regulated genes) and Table 3.3 (down-regulated genes).

The up-regulated genes found in comparison of the expression profile of the TIGR4 Δ*rr06* with wild-type, of particular interest were the genes that encodes the *rlrA* pathogenicity islet that include the cell wall surface proteins (*sp0462-sp0464*) and two sortases (*sp0466* and *sp0467*) (Paterson and Mitchell, 2005) (Figure 3.25). A recent study has shown that these genes regulate the formation of pneumococcal adhesin pili-like appendage, contributing to adherence, virulence and host inflammatory response (Barocchi *et al.*, 2006). These up-regulated genes do not include the gene *sp0465* that encodes a small ORF and consequently a low signal intensity is obtained in the microarray hybridization.

Table 3.2- Up-regulated genes in TIGR4 Δ*rr06* compared with its parental strain using TIGR arrays.

Genes[1)]	Gene symbol	Mean intensity ratio[2)]	*p*-value[3)]	Annotation[4)]
sp0220		2.1	1.45E-02	Ribosomal protein
sp0232		2.5	3.55E-03	Translation initiation factor
sp0234		2.1	4.57E-04	Ribosomal protein
sp0415		3.2	8.01E-04	Enoyl-Coa-hydratase/isomerase family
sp0462	*rrgA*	8.8	1.37E-05	Cell wall surface anchor family protein
sp0463	*rrgB*	8.9	2.69E-04	Cell wall surface anchor family protein
sp0464	*rrgC*	11.2	2.52E-05	Cell wall surface anchor family protein
sp0466	*srtB*	3.2	6.65E-04	Sortase putative
sp0467	*srtC*	2.2	1.95E-02	Sortase putative
sp0501		3.8	3.54E-05	Transcriptional regulator, Merr family
sp0502		3.4	1.41E-04	Glutamine synthetase, type I
sp0742		3.9	5.52E-05	Conserved hypothetical protein
sp0783		4.7	2.55E-04	Conserved hypothetical protein
sp0875		4.5	2.72E-04	Lactose phosphotransferase system repressor
sp0876		4.8	1.50E-03	1-Phosphofructokinase, putative
sp0877		4.9	6.46E-05	PTS System, Fructose specific II ABC components
sp0959		2.7	3.52E-04	Translation initiation factor IF
sp1586		2.9	1.64E-03	ATP-Dependent RNA helicase, putative

[1)] Gene designation of microarray ORFs in agreement with TIGR (http://www.tigr.org).
[2)] Ratios intensities of TIGR4 wild-type/Δ*rr06* determined in microarray experiments. The value represents the mean intensities for the four replicate experiments. Genes up-regulated with ≥ two-fold expression changes between the wild-type and mutant were selected.
[3)] *P*- value represents the mean *P*-value calculated from individual *t*-tests of intensity changes between the wild-type and mutant. Genes with *P*-value ≤0.05 were selected.
[4)] Annotations as published in TIGR4 genome (http://www.tigr.org).

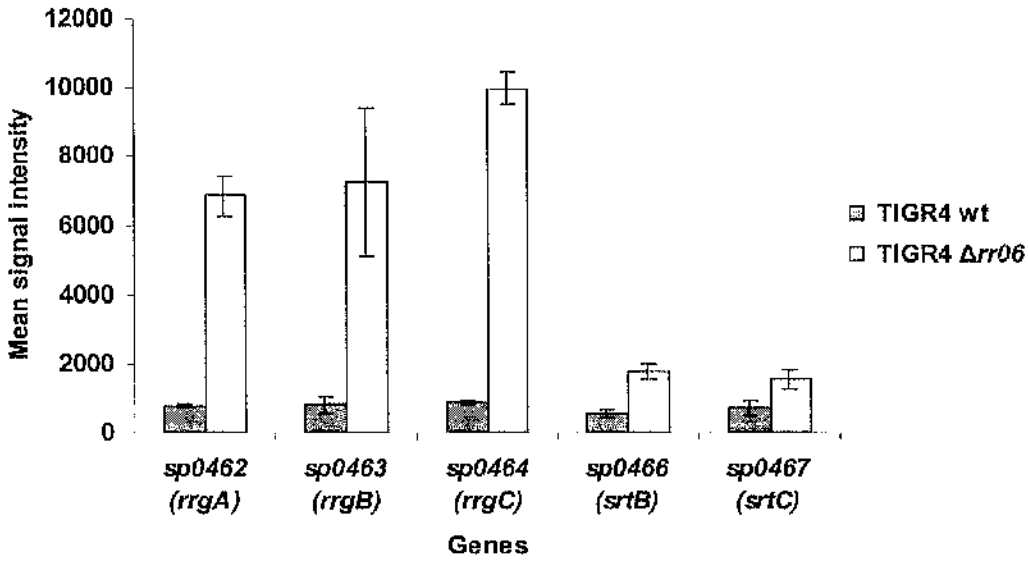


Figure 3.25- Genes up-regulated in TIGR4 Δ*rr06* associated with expression of *rlrA* pathogenicity islet.

Expression of *rlrA* pathogenicity islet up-regulated in TIGR4 Δ*rr06* compared with wild-type strain. Values are determined by microarray hybridization and are given as the geometric mean fluorescence intensity values from four replicate hybridizations. Error bars represent ± SEM.

As expected the gene *rr06* was down-regulated. However, the mean ratio was not particularly high due to the individual low intensity spots in TIGR4 wild-type. The remaining signal fluorescence in Δ*rr06* was similar to the background signal. Nevertheless RT-PCR and qRT-PCR analysis showed high levels of expression of *rr06* in TIGR4 wild-type (section 3.6.1.2). Of interest were the down-regulated genes associate with: (i) stress response (Figure 3.26); (ii) surface wall metabolism proteins (Figure 3.27) with relevance of *dlt* operon (*sp2173*, *sp2175*, *sp2176*) that promotes the D-alanylation of lipoteichoic acid (LTA) in Gram positive bacteria (Abachin *et al.*, 2002; Poyart *et al.*, 2001; Poyart *et al.*, 2003); (iii) energy metabolism (Figure 3.28); (iv) protein degradation (Figure 3.29), with one gene encoding a stress response and known virulence factor, *htrA* (Ibrahim *et al.*, 2004a, b; Sebert *et al.*, 2002). Furthermore the transcriptional levels in TIGR4 Δ*rr06* showed down-regulation of another pneumococcal TCS, *sp0386* (*hk03*) *sp0387* (*rr03*). Regarding the regulation of the virulence factor PspC, no significant differences were observed in expression levels in TIGR4 Δ*rr06* compared to its parental strain. The microarray analysis also showed that the expression of the hypothetical protein (*sp2191*), which is located immediately downstream from TCS06, was found to be unaltered. Analysis of the data demonstrates an expression ratio for *sp2191* of 1.0. This finding confirms that insertion of the erythromycin cassette into *rr06* does not have a polar effect on downstream genes.

Table 3.3- Down-regulated genes in TIGR4 Δ*rr06* compared with its parental strain using TIGR arrays.

Genes[1)]	Gene symbol	Mean intensity ratio[2)]	*p*-value[3)]	Annotation[4)]
sp0064		0.5	2.44E-03	PTS system, IIA component
sp0097		0.2	1.18E-05	Conserved domain protein
sp0098		0.3	1.15E-02	Hypothetical protein
sp0099		0.4	3.03E-03	Hypothetical protein
sp0100		0.4	2.30E-04	Conserved hypothetical protein
sp0107		0.3	1.19E-03	LYSM domain protein
sp0285		0.5	3.56E-03	Alcohol dehydrogenase, ZINC-containing
sp0338		0.3	2.03E-02	ATP-dependent CLP protease, ATP-binding subunit
sp0386	*hk03*	0.4	1.57E-03	Sensor histidine kinase, putative
sp0387	*rr03*	0.4	6.71E-04	DNA-binding response regulator
sp0459	*pfl*	0.4	3.40E-03	Formate acetyltransferase
sp0515	*hrcA*	0.2	4.51E-04	Heat-inducible transcription repressor HRCA
sp0516	*grpE*	0.2	7.83E-03	Heat shock protein GRPE
sp0517	*dnaK*	0.1	1.10E-04	DnaK protein
sp0519	*dnaJ*	0.1	2.91E-04	DnaJ PROTEIN
sp0641		0.4	2.51E-03	Serine protease, subtilase family
sp0647		0.4	2.07E-03	PTS system, IIC component, putative
sp0715	*lctO*	0.4	1.61E-03	Lactate oxidase
sp0726		0.4	9.68E-03	Phosphomethylpyrimidine Kinase
sp0820	*clpE*	0.3	5.83E-04	ATP-dependent CLP protease, ATP-binding subunit CLPE
sp1027		0.3	9.93E-04	Conserved hypothetical protein
sp1190		0.4	2.14E-03	Tagatose 1,6-diphosphate aldolase
sp1191		0.5	1.44E-04	Tagatose-6-phosphate kinase
sp1340		0.3	5.22E-04	Hypothetical protein
sp1465		0.4	6.90E-03	Hypothetical protein
sp1466		0.4	3.80E-04	Hemolysin
sp1686		0.3	2.23E-03	Oxidoreductase, GFO/IDH/MOCA Family
sp1687	*NanB*	0.2	2.15E-03	Neuraminidase B
sp1689		0.3	4.85E-04	Transporter, permease protein
sp1793		0.1	1.71E-04	Hypothetical protein
sp1852	*galT*	0.2	2.97E-04	Galactose-1-phosphate uridylyltransferase
sp1860	*proWX*	0.4	1.39E-04	Choline transporter
sp1861	*proV*	0.4	4.57E-04	Choline transporter
sp1862		0.4	2.19E-04	Hypothetical protein
sp1895	*msmG*	0.3	2.34E-03	Sugar ABC transporter, permease protein
sp1897	*msmE*	0.2	7.57E-04	Sugar ABC transporter, sugar-binding protein
sp1906	*groEL*	0.3	5.07E-05	Chaperonin
sp2026		0.2	3.52E-05	Alcohol dehydrogenase, iron-containing
sp2106		0.3	2.18E-03	Glycogen phosphorylase family protein
sp2107	*malQ*	0.4	2.41E-04	4-Alpha-glucanotransferase
sp2148		0.06	4.37E-06	Arginine deiminase
sp2150	*argF*	0.1	4.87E-05	Ornithine carbamoyltransferase
sp2151		0.08	2.14E-05	Carbamate kinase
sp2152		0.07	1.66E-04	Conserved hypothetical protein
sp2153		0.3	7.82E-04	Peptidase, M20/M25/M40 family
sp2173	*dltD*	0.5	3.46E-03	DltD protein
sp2175	*dltB*	0.4	2.12E-04	DltB protein
sp2176	*dltA*	0.5	6.20E-04	D-Alanine-activating enzyme
sp2193	*rr06*	0.5	2.39E-03	DNA-binding response regulator
sp2232		0.3	1.78E-04	Conserved hypothetical protein
sp2233		0.2	1.18E-04	Hypothetical protein
sp2239	*htrA*	0.4	7.31E-03	Serine protease
sp2240		0.3	1.13E-03	SPSPOJ protein

[1)] Gene designation of microarray ORFs in agreement with TIGR (http://www.tigr.org).
[2)] Ratios intensities of TIGR4 wild-type/Δ*rr06* determined in microarray experiments. The value represents the mean intensities for the four replicate experiments. Genes up-regulated with ≥ two-fold expression changes between the wild-type and mutant were selected.
[3)] *P*- value represents the mean *P*-value calculated from individual *t*-tests of intensity changes between the wild-type and mutant. Genes with *P*-value ≤0.05 were selected.
[4)] Annotations as published in TIGR4 genome (http://www.tigr.org).

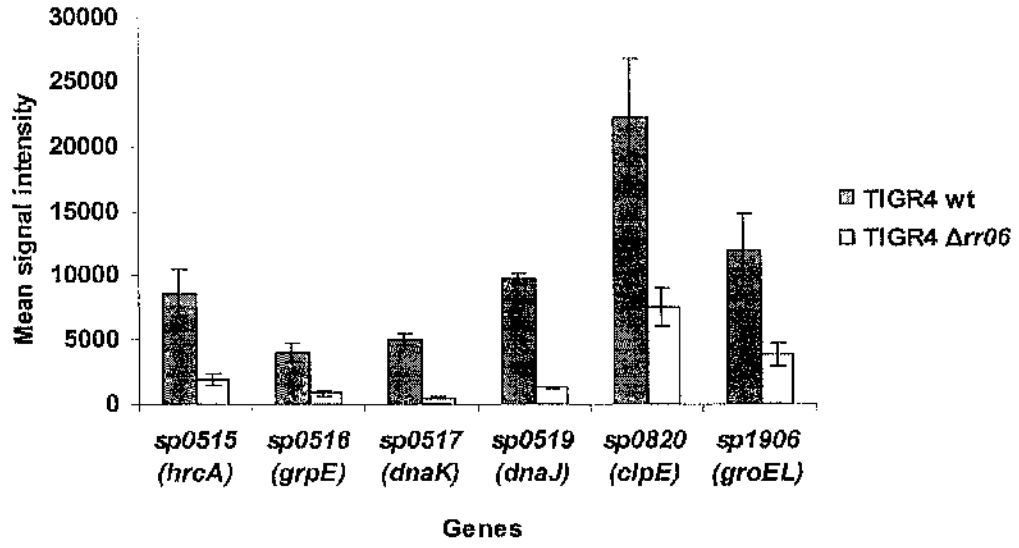


Figure 3.26- Genes down-regulated in TIGR4 Δ*rr06* associated with stress response.

Expression of heat shock associate proteins down-regulated in TIGR4 Δ*rr06* compared with wild-type strain. Values are determined by microarray hybridization and are given as the geometric mean fluorescence intensity values from four replicate hybridizations. Error bars represent ± SEM.

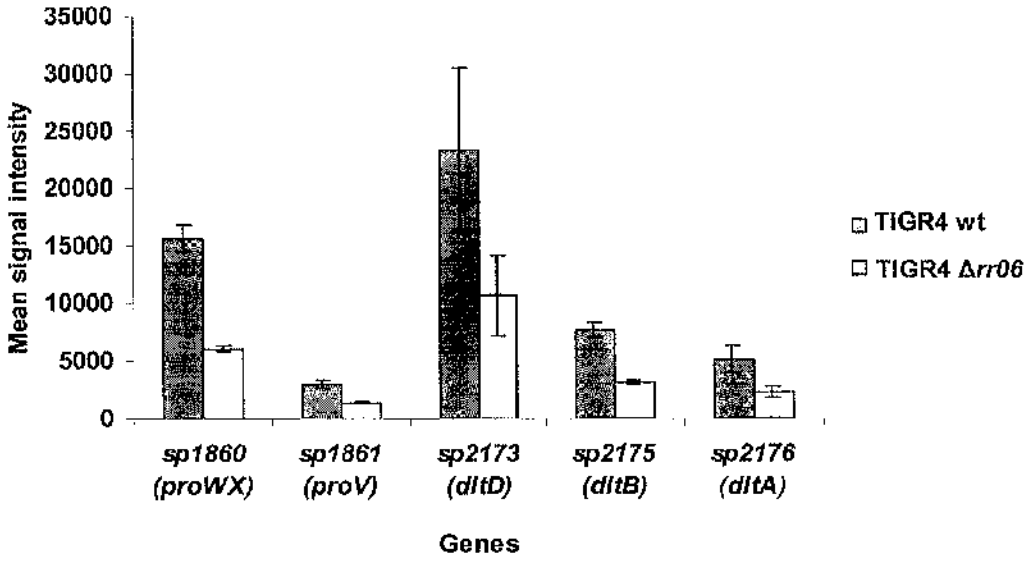


Figure 3.27- Genes down-regulated in TIGR4 Δ*rr06* associated with surface wall metabolism.

Expression of surface wall metabolism associate proteins down-regulated in TIGR4 Δ*rr06* compared with wild-type strain. Values are determined by microarray hybridization and are given as the geometric mean fluorescence intensity values from four replicate hybridizations. Error bars represent ± SEM.

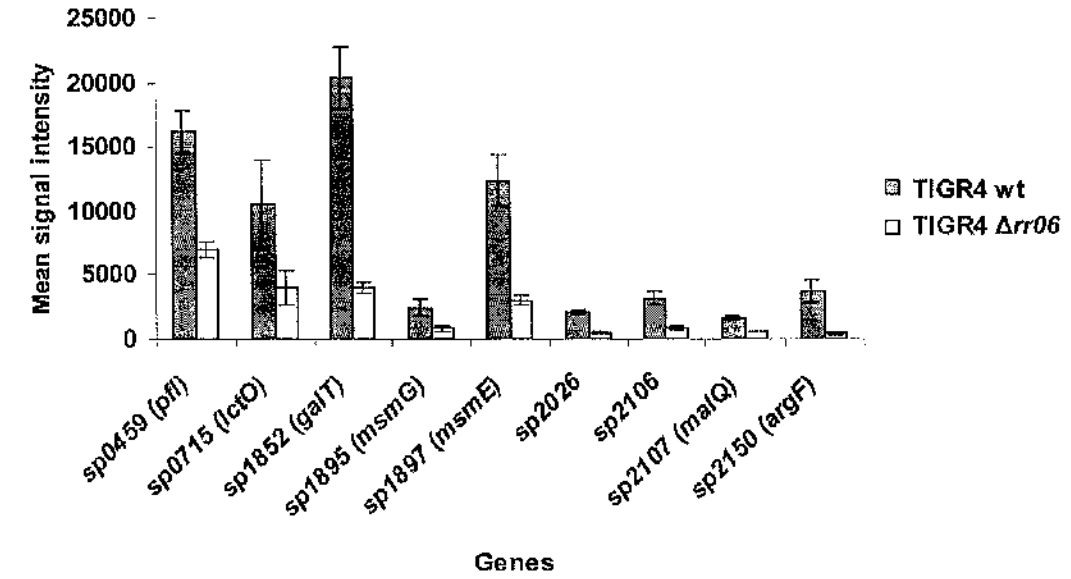


Figure 3.28- Genes down-regulated in TIGR4 Δ*rr06* associated with energy metabolism.

Expression of energy metabolism associate proteins down-regulated in TIGR4 Δ*rr06* compared with wild-type strain. Values are determined by microarray hybridization and are given as the geometric mean fluorescence intensity values from four replicate hybridizations. Error bars represent ± SEM.

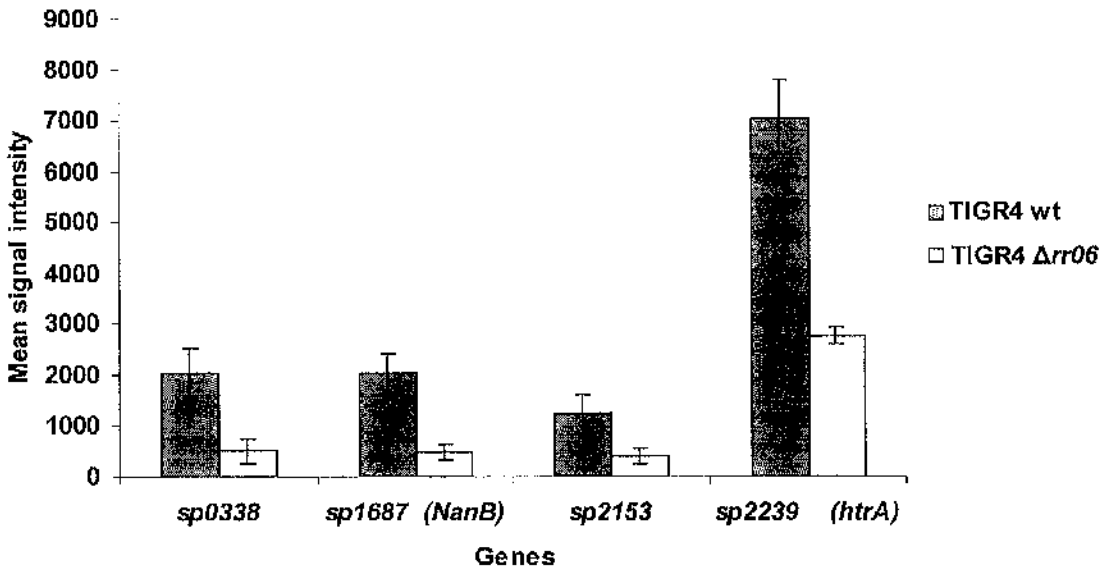


Figure 3.29- Genes down-regulated in TIGR4 Δ*rr06* associated with degradation of protein, peptides and glycopeptides.

Expression of genes associates with degradation of proteins, peptides, and glycopeptides down-regulated in TIGR4 Δ*rr06* compared with wild-type strain. Values are determined by microarray hybridization and are given as the geometric mean fluorescence intensity values from four replicate hybridizations. Error bars represent ± SEM.

3.4.3 BμG@S arrays

The transcriptional profiles of *S. pneumoniae* TIGR wild-type and Δ*rr06* strains was also performed using BμG@S microarrays slides. cDNA prepared from 2 μg of total RNA by reverse transcriptase reactions was labelled with cy3 and cy5 fluorochromes. As in TIGR arrays study, four BμG@S arrays slides were used in this analysis as well a dye swap.

3.4.3.1 BμG@S microarray data

Microarray comparisons of the transcription profiles of the TIGR4 Δ*rr06* and their respective wild-type was used to identify the genes controlled by RR06. The correlation of the mean ratio of hybridisation intensities between the TIGR4 wild-type (control strain), and the Δ*rr06* mutant (test strain) are listed Figure 3.30. The scatter plot shows an overview of the transcription profiles, characterized with a range of colours for the intensities of expression.

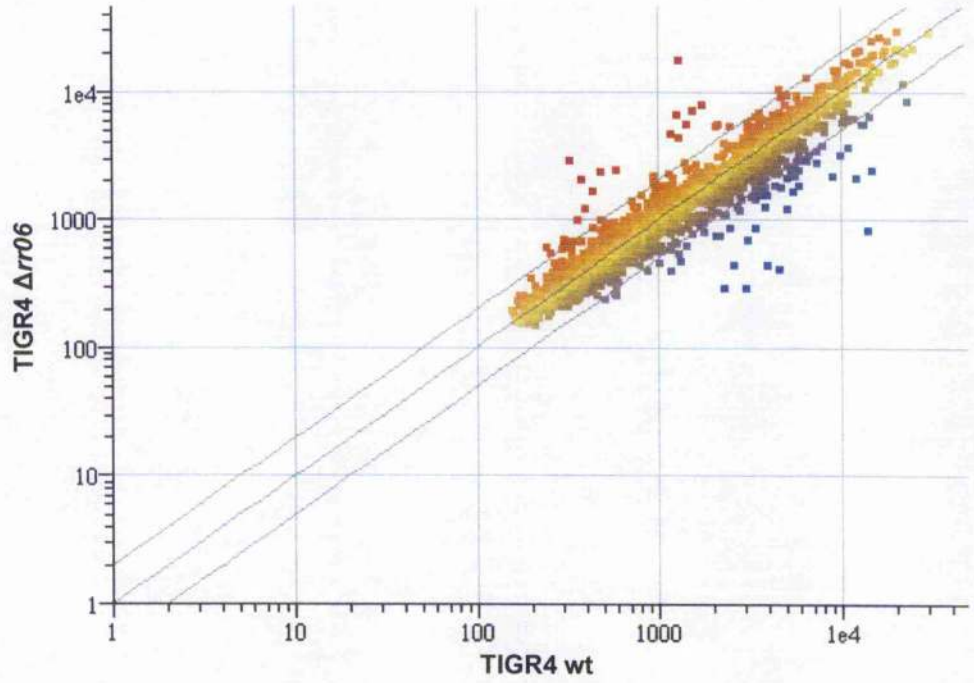


Figure 3.30- Scatter plot comparing the gene expression between TIGR4 Δ*rr06* and TIGR4 wild-type in BμG@S arrays slides.

cDNA labelled with Cy3 and Cy5 were hybridized in BμG@S arrays slides and analysed in Genespring™. The scatter plot showed the gene expression intensities between the TIGR4 wild-type (control strain) and TIGR4 Δ*rr06* (test strain). Blue spots (bellow the diagonal lines) mean the genes down- regulated in TIGR4 Δ*rr06* and the red spots (above the diagonal lines) the genes up-regulated. The yellow spots (inside the diagonal lines) describe the genes without significant variation between theTIGR4 Δ*rr06* and the wild-type strains.

As in the previous analysis, using TIGR arrays slides, most of the pneumococcal genes remained unaltered. However, about 2% of the genes were shown to have significant differences in expression levels between the wild-type and Δ*rr06* mutant using the BμG@S arrays slides. Fifteen genes were found to be up-regulated in TIGR4 Δ*rr06*.and twenty nine genes down-regulated. The up and down-regulated genes were selected for the spots with a two-fold difference in signal intensity between TIGR4 wild-type and Δ*rr06* mutant and statistically significant ($P \leq 0.05$). All genes with altered expression are listed in Table 3.4 (up-regulated genes) and Table 3.5 (down-regulated genes).

Table 3.4- Up-regulated genes in TIGR4 Δ*rr06* compared with its parental strain using BμG@S arrays.

Genes[1)]	Gene symbol	Mean intensity ratio[2)]	p-value[3)]	Annotation[4)]
sp0461	*rlrA*	3.7	3.41E-02	Transcriptional regulator, putative
sp0462	*rrgA*	4.9	1.75E-02	Cell wall surface anchor family protein
sp0463	*rrgB*	13.1	1.13E-02	Cell wall surface anchor family protein
sp0464	*rrgC*	7.6	2.13E-02	Cell wall surface anchor family protein
sp0645		2.2	2.00E-02	PTS system IIA component, putative
sp0742		4.6	3.67E-02	Conserved hypothetical protein
sp0783		2.9	4.87E-02	Conserved hypothetical protein
sp1898	*aga*	2.6	3.89E-02	Alpha-galactosidase
sp2000	*rr11*	3.5	3.15E-02	DNA-binding response regulator
sp2001	*hk11*	3.9	3.16E-02	Sensor histidine kinase, putative
sp2002		4.9	1.36E-02	Conserved hypothetical protein
sp2003		5.1	7.95E-03	ABC transporter, ATP-binding protein
sp2004		4.5	3.83E-02	Hypothetical protein
sp2005		4.0	1.73E-02	Hypothetical protein
sp2197		2.3	3.55E-02	ABC transporter, substrate-binding protein, putative

[1)] Gene designation of microarray ORFs in agreement with TIGR (http://www.tigr.org).
[2)] Ratios intensities of TIGR4 wild-type/Δ*rr06* determined in microarray experiments. The value represents the mean intensities for the four replicate experiments. Genes up-regulated with ≥ two-fold expression changes between the wild-type and mutant were selected.
[3)] *P*- value represents the mean *P*-value calculated from individual *t*-tests of intensity changes between the wild-type and mutant. Genes with *P*-value ≤0.05 were selected.
[4)] Annotations as published in TIGR4 genome (http://www.tigr.org).

The up-regulated genes found in the comparison of the expression profile of the TIGR4 Δ*rr06* with wild-type of interest included the transcriptional regulator *rlrA*, identified by signature-tagged mutagenesis (STM) (Hava and Camilli, 2002) and three cell wall surface proteins (*sp0462-0464*) (Figure 3.31). Furthermore the transcriptional levels in TIGR4 Δ*rr06* showed another up-regulation pneumococcal TCS, response regulator *sp2000* (*rr11*) and sensor histidine kinase *sp2001* (*hk11*).

The transcriptome analysis showed an appreciable agreement between the two-microarray slides used in this study. Some of the divergence results between the slides TIGR and BμG@S apparently due to the different methods used for each slide (indirectly and directly labelling) and due to the differences of the probes printed in the slides.

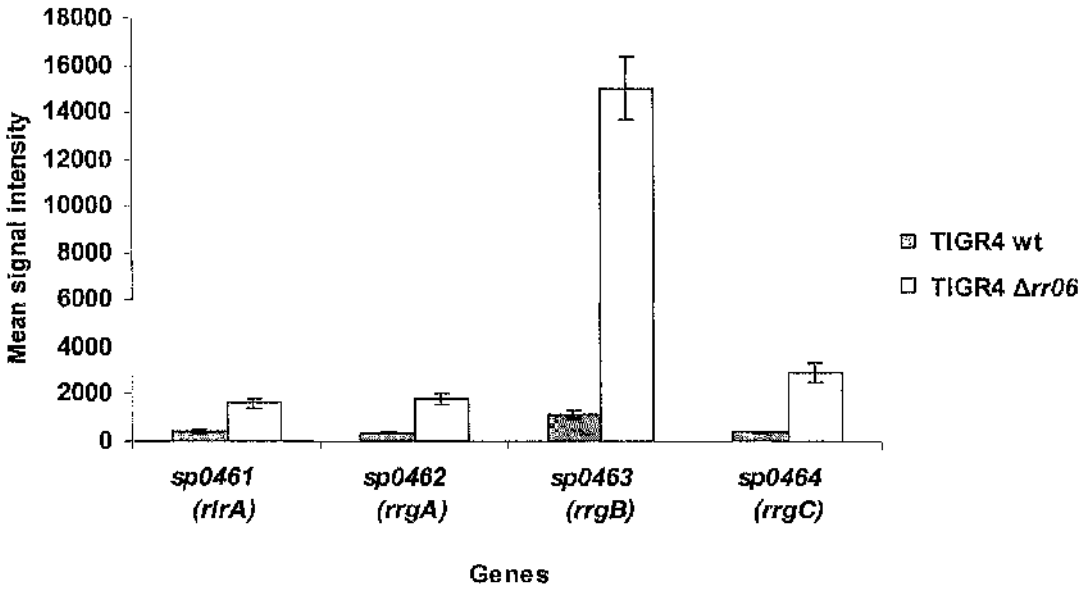


Figure 3.31- Genes up-regulated in TIGR4 Δ*rr06* associated with expression of *rlrA* pathogenicity islet using BμG@S arrays slides.

Expression of *rlrA* pathogenicity islet up-regulated in TIGR4 Δ*rr06* compared with wild-type strain. Values are determined by microarray hybridization and are given as the geometric mean fluorescence intensity values from four replicate hybridizations. Error bars represent ± SEM.

As in TIGR microarray analysis, the *rr06* was down-regulated using BμG@S arrays slides. Of interest were the down-regulation of the response regulator *ciaR* (*sp0798*) and the CiaR/H-regulated genes *htrA* (*sp2239*) and *sp2240* (Ibrahim *et al.*, 2004b; Sebert *et al.*, 2002). In this microarray analysis the sensor histidine kinase *ciaH* had an expression ratio more that two-fold down-regulated in Δ*rr06* mutant, however the difference was not statistical significant. Together with *rr06* a set of additional sixteen genes were found down-regulated in both analyses, using the TIGR and BμG@S microarrays slides (Figure 3.32).

Table 3.5- Down-regulated genes in TIGR4 Δ*rr06* compared with its parental strain using BμG@S arrays

Genes[1]	Gene symbol	Mean intensity ratio[2]	p-value[3]	Annotation[4]
sp0107		0.21	3.04E-02	LysM domain protein
sp0338		0.09	1.62E-02	ATP-dependent Clp protease, ATP-binding subunit, putative
sp0516	*grpE*	0.44	3.02E-02	heat shock protein GrpE
sp0517	*DnaK*	0.18	1.34E-02	DnaK protein
sp0519	*DnaJ*	0.26	2.79E-02	DnaJ protein
sp0702	*pyrE*	0.46	4.08E-02	Orotate phosphoribosyltransferase PyrE
sp0789		0.34	3.46E-02	Conserved hypothetical protein
sp0798	*ciaR*	0.44	3.66E-02	DNA-binding response regulator CiaR
sp0981		0.43	5.66E-03	Protease maturation protein, putative
sp1275	*carB*	0.43	2.11E-02	Carbamoyl-phosphate synthase, large subunit
sp1438		0.34	2.80E-02	ABC transporter, ATP-binding protein
sp1465		0.34	1.35E-02	Hypothetical protein
sp1466		0.38	1.22E-02	Hemolysin
sp1722		0.32	1.69E-02	PTS system IIABC components
sp1793		0.07	1.67E-02	Hypothetical protein
sp1794		0.13	1.30E-02	Hypothetical protein
sp1826		0.35	1.84E-02	ABC transporter, substrate-binding protein
sp1953		0.35	4.47E-02	Toxin secretion ABC transporter, ATP-binding/permease protein
sp1975		0.40	3.74E-02	SpoIIIJ family protein
sp2063		0.24	1.86E-02	LysM domain protein, authentic frameshift
sp2106		0.16	3.21E-03	Glycogen phosphorylase family protein
sp2107	*malQ*	0.22	2.31E-02	4-alpha-glucanotransferase
sp2175	*dltB*	0.45	3.36E-02	DltB protein
sp2176	dltA	0.45	2.04E-02	D-alanine-activating enzyme
sp2193	*Rr06*	0.22	4.07E-02	DNA-binding response regulator
sp2232		0.10	1.41E-02	Conserved hypothetical protein
sp2233		0.15	2.64E-02	Hypothetical protein
sp2239	*htrA*	0.38	1.16E-02	Serine protease
sp2240		0.33	3.85E-02	SpspoJ protein

[1] Gene designation of microarray ORFs in agreement with TIGR (http://www.tigr.org).
[2] Ratios intensities of TIGR4 wild-type/Δ*rr06* determined in microarray experiments. The value represents the mean intensities for the four replicate experiments. Genes up-regulated with ≥ two-fold expression changes between the wild-type and mutant were selected.
[3] *P*- value represents the mean *P*-value calculated from individual *t*-tests of intensity changes between the wild-type and mutant. Genes with *P*-value ≤0.05 were selected.
[4] Annotations as published in TIGR4 genome (http://www.tigr.org).

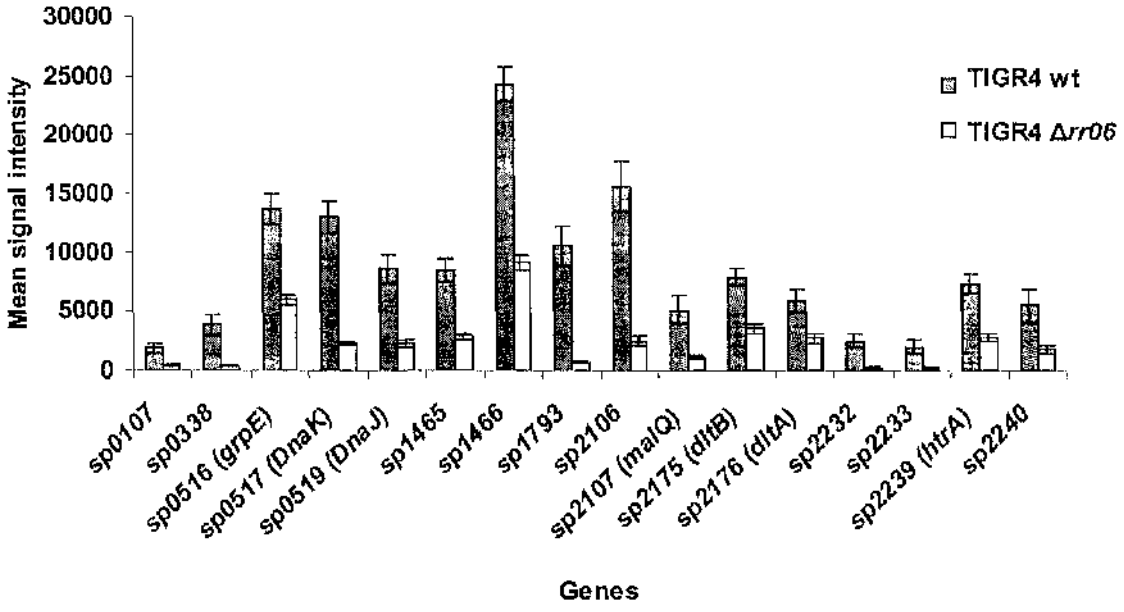


Figure 3.32- Down-regulated genes in TIGR4 Δ*rr06* using BμG@S microarrays slides common to TIGR microarray analysis.

Expression of down-regulated genes in TIGR4 Δ*rr06* compared with wild-type strain. Values are determined by microarray hybridization and are given as the geometric mean fluorescence intensity values from four replicate hybridizations. Error bars represent ± SEM.

3.4.4 Transcriptional profiles of Δ*rr06* mutants.

Transcriptome study by microarray analysis was performed to compare the R6 and 0100993 Δ*rr06* mutants with respective wild-type, using BμG@S microarray slides. Two slide replicates were used and a dye swap was performed to minimize the variation between the Cy3 and Cy5 incorporation and intensities. In the R6 strain, no significant differences of gene expression were observed in array analysis, except for the *rr06* gene. On the other hand, a little difference in expression of Δ*rr06*, compared with the wild-type, was observed in 0100993 strain (Figure 3.33). In 0100993 strain, nine genes were found to be up-regulated in Δ*rr06* mutant (Table 3.6) however, no genes were found to be significantly down-regulated. The *rlrA* islet that encodes the pneumococcal pilus (Barocchi *et al.*, 2006) was not found to be regulated by TCS06 in 0100993 strain. However, analysis of genetic variation (chapter 6) showed that the pneumococcal *rlrA* islet is absent of the genome in 0100993 strain. The transcriptional profiles of R6, 0100993 and TIGR4 indicated a strain-specific role of RR06 in gene regulation.

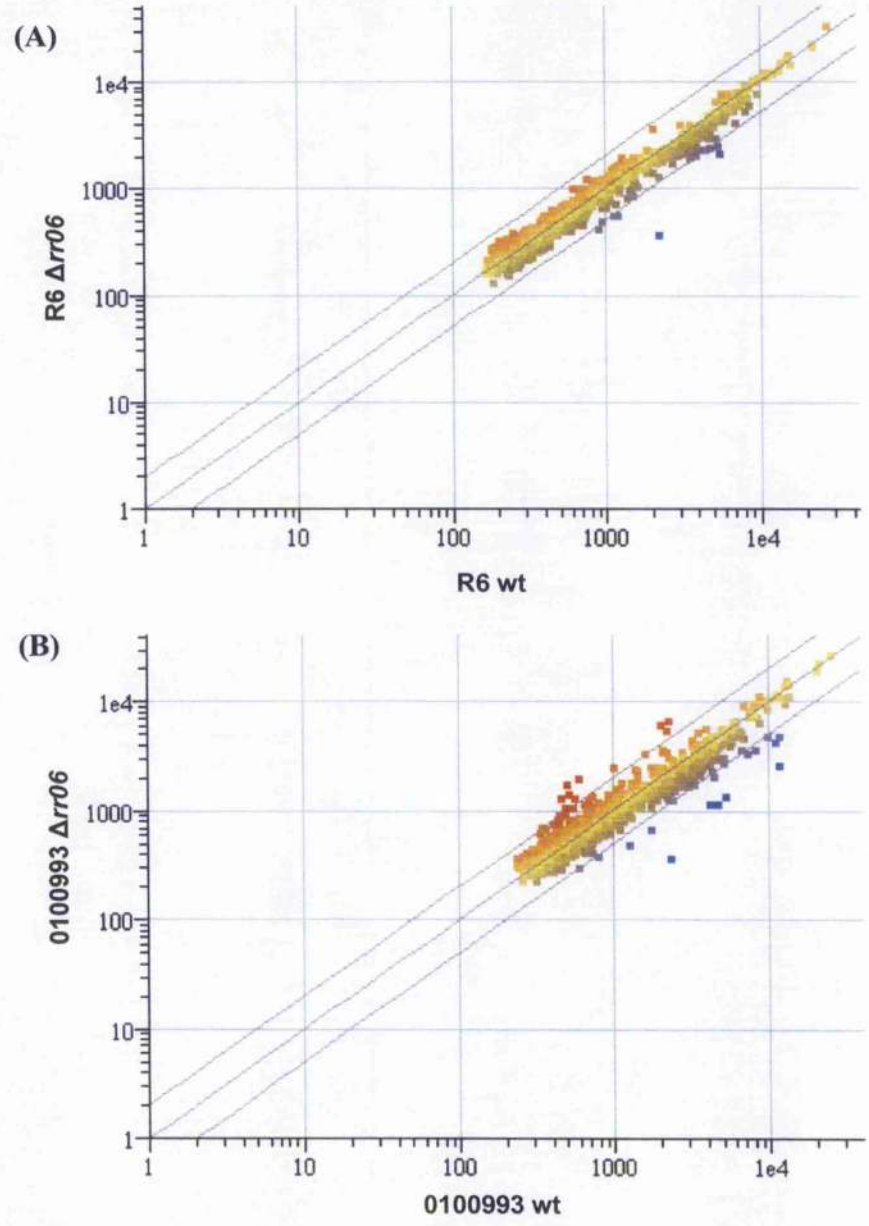


Figure 3.33- Scatter plot comparing the gene expression between R6 and 0100993 Δ*rr06* and their parental wild-type.

cDNA labelled with Cy3 and Cy5 were hybridized in BμG@S arrays slides and analysed in Genespring™. The scatter plots showed the gene expression intensities between the R6 wild-type (control strain) and R6 Δ*rr06* (test strain) (A), and between the 0100993 wild-type (control strain) and 0100993 Δ*rr06* (test strain) (B). Blue spots (bellow the diagonal lines) mean the genes down-regulated in TIGR4 Δ*rr06* and the red spots (above the diagonal lines) the genes up-regulated. The yellow spots (inside the diagonal lines) describe the genes without significant variation between the Δ*rr06* mutants and the wild-type strains.

Table 3.6- Up-regulated genes in 0100993 Δ*rr06* compared with its parental strain using BμG@S arrays.

Genes[1)]	Gene symbol	Mean intensity ratio[2)]	p-value[3)]	Annotation[4)]
sp0717	*thiM*	2.8	1.130E-02	Hydroxyethylthiazole kinase
sp0720		2.4	1.760E-02	ABC transporter. ATP-binding protein
sp0722	*tenA*	2.3	9.505E-03	Transcriptional activator TenA
sp0867		2.9	6.325E-03	ABC transporter. ATP-binding protein
sp0869		3.4	4.105E-03	Aminotransferase. class-V
sp0870		2.8	4.805E-03	NifU family protein
sp2196		2.4	1.561E-02	ABC transporter. ATP-binding protein
sp2197		2.9	4.165E-03	ABC transporter. substrate-binding protein. putative
sp2199		2.2	1.339E-02	Conserved hypothetical protein

[1)] Gene designation of microarray ORFs in agreement with TIGR (http://www.tigr.org).
[2)] Ratios intensities of 0100993 wild-type/Δ*rr06* determined in microarray experiments. The value represents the mean intensities for the four replicate experiments. Genes up-regulated with ≥ two-fold expression changes between the wild-type and mutant were selected.
[3)] *P*- value represents the mean *P*-value calculated from individual *t*-tests of intensity changes between the wild-type and mutant. Genes with *P*-value ≤0.05 were selected.
[4)] Annotations as published in TIGR4 genome (http://www.tigr.org).

The contribution of the TCS06 in gene regulation in *S. pneumoniae* appears to be strain-dependent. To confirm that the strain-specific gene regulation by RR06 is dependent of their genomic background, absence or presence of the genes, a comparative genomic hybridization (CGH) study was performed using R6, D39 and 0100993 strains in comparison with reference strain TIGR4 (see chapter 6). Comparisons of the altered gene regulation of the *rr06* mutants and the absence or presence of these genes are illustrated in the Table 3.7. The majority of the genes altered in expression profile in microarray analysis of the TIGR4 Δ*rr06* strain are found to be present in the genome background of R6 and 0100993 strains. Interestingly the *rlrA* pathogenicity islet, up-regulated only in TIGR4 Δ*rr06* was found to be absent in the genome of R6 and 0100993 strains.

Table 3.7- Genes regulated by RR06 and presence or absence of the gene by CGH in different strains.

	Transcriptional profile			CGH		
Genes[1)]	TIGR4	R6	0100993	TIGR4	R6	0100993
sp0064	+	-	-	+	+	+
sp0097	+	-	-	+	+	+
sp0098	+	-	-	+	+	+
sp0099	+	-	-	+	+	+
sp0100	+	-	-	+	+	+
sp0107	+	-	-	+	+	+
sp0220	+	-	-	+	+	+
sp0232	+	-	-	+	+	+
sp0234	+	-	-	+	+	+
sp0285	+	-	-	+	+	+
sp0338	+	-	-	+	+	+
sp0386	+	-	-	+	+	+
sp0387	+	-	-	+	+	+
sp0415	+	-	-	+	+	+
sp0459	+	-	-	+	+	+
sp0461	+	-	-	+	+	+
sp0462	+	-	-	+	-	-
sp0463	+	-	-	+	-	-
sp0464	+	-	-	+	-	-
sp0466	+	-	-	+	-	-
sp0467	+	-	-	+	-	-
sp0501	+	-	-	+	+	+
sp0502	+	-	-	+	+	+
sp0515	+	-	-	+	+	+
sp0516	+	-	-	+	+	+
sp0517	+	-	-	+	+	+
sp0519	+	-	-	+	+	+
sp0641	+	-	-	+	+	-
sp0645	+	-	-	+	+	+
sp0647	+	-	-	+	+	+
sp0702	+	-	-	+	+	+
sp0715	+	-	-	+	+	+
sp0717	-	-	+	+	+	+
sp0720	-	-	+	-	+	+
sp0722	-	-	+	+	+	+
sp0726	+	-	-	+	+	+
sp0742	+	-	-	+	+	+
sp0789	+	-	-	+	+	+
sp0783	+	-	-	+	+	+
sp0798	+	-	-	+	+	+
sp0820	+	-	-	+	+	+
sp0869	-	-	+	+	+	+
sp0870	-	-	+	+	+	+
sp0867	-	-	+	+	+	+
sp0875	+	-	-	+	+	+
sp0876	+	-	-	+	+	+
sp0877	+	-	-	+	+	+
sp0959	+	-	-	+	+	+
sp0981	+	-	-	+	+	+
sp1027	+	-	-	+	+	+
sp1190	+	-	-	+	+	+
sp1191	+	-	-	+	+	+
sp1275	+	-	-	+	+	+
sp1340	+	-	-	+	+	+
sp1438	+	-	-	+	+	+
sp1465	+	-	-	+	+	+
sp1466	+	-	-	+	+	+
sp1586	+	-	-	+	+	+
sp1686	+	-	-	+	+	+
sp1687	+	-	-	+	+	+
sp1689	+	-	-	+	+	+

Table 3.7- Continuation.

Genes[1)]	Transcriptional profile			CGH		
	TIGR4	R6	0100993	TIGR4	R6	0100993
sp1722	+	-	-	+	+	-
sp1793	+	-	-	+	-	+
sp1794	+	-	-	+	-	+
sp1826	+	-	-	+	+	+
sp1852	+	-	-	+	+	+
sp1860	+	-	-	+	+	+
sp1861	+	-	-	+	+	+
sp1862	+	-	-	+	+	+
sp1895	+	-	-	+	+	+
sp1897	+	-	-	+	+	+
sp1898	+	-	-	+	+	+
sp1906	+	-	-	+	+	+
sp1953	+	-	-	+	+	-
sp1975	+	-	-	+	+	+
sp2000	+	-	-	+	+	+
sp2001	+	-	-	+	+	+
sp2002	+	-	-	+	+	+
sp2003	+	-	-	+	-	+
sp2004	+	-	-	+	+	-
sp2005	+	-	-	+	-	+
sp2026	+	-	-	+	+	+
sp2063	+	-	-	+	+	+
sp2106	+	-	-	+	+	+
sp2107	+	-	-	+	+	+
sp2148	+	-	-	+	+	+
sp2150	+	-	-	+	+	+
sp2151	+	-	-	+	+	+
sp2152	+	-	-	+	+	+
sp2153	+	-	-	+	+	+
sp2173	+	-	-	+	+	+
sp2175	+	-	-	+	+	+
sp2176	+	-	-	+	+	+
sp2193	+	-	-	+	+	+
sp2197	-	-	+	+	+	+
sp2196	-	-	+	+	+	+
sp2199	-	-	+	+	+	+
sp2232	+	-	-	+	+	+
sp2233	+	-	-	+	+	+
sp2239	+	-	-	+	+	+
sp2240	+	-	-	+	+	+

[1)] Gene designation of microarray ORFs in agreement with TIGR (http://www.tigr.org).
(+) Altered gene expression (transcriptional profile) or gene present (CGH).
(-) Unaltered gene expression (transcriptional profile) or gene absent (CGH).

3.5 Validation of microarray data

To validate the microarray data, relative transcript levels were determined by quantitative real-time PCR on a selection of genes up and down-regulated in Δ*rr06* compared to parental strain.

3.5.1 Quantitative Real-Time PCR

To confirm the microarray data, 2 μg of the same RNA prepared from either TIGR4 wild-type and Δ*rr06*, used in microarray assays, was used in the reverse transcription reaction to synthesize cDNA.

3.5.1.1 Reaction efficiency

To calculate the efficiency of qRT-PCR for each pair primer reaction, serial 10-fold dilutions between 1000-0.001 pg (Figure 3.34) of reverse transcriptase PCR product of control gene and target genes were made. 1 μl of each dilution were applied in qRT-PCR and the efficiency was calculated on basis of the slope of the plot between the cycle threshold (Ct) values for the dilutions against concentration (Figure 3.35).

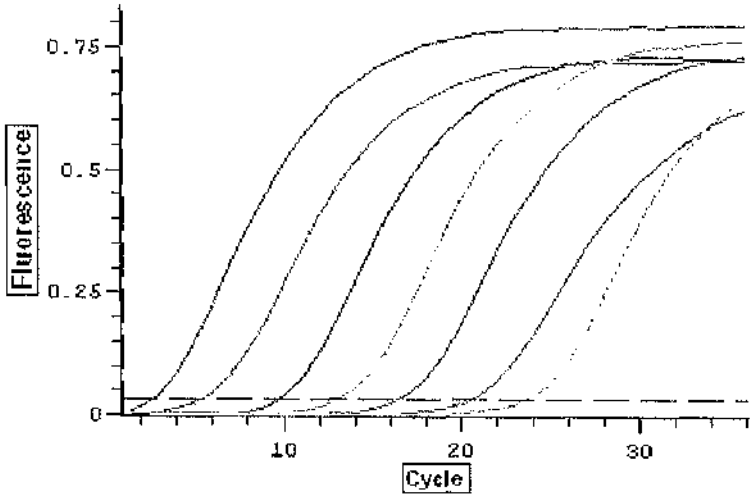


Figure 3.34- qRT-PCR data graph of 10-fold serial dilutions.

Plot between the Ct values and fluorescence signal of 10-fold serial dilutions (1000-0.001 pg) of reverse transcriptase PCR product of control or target gene.

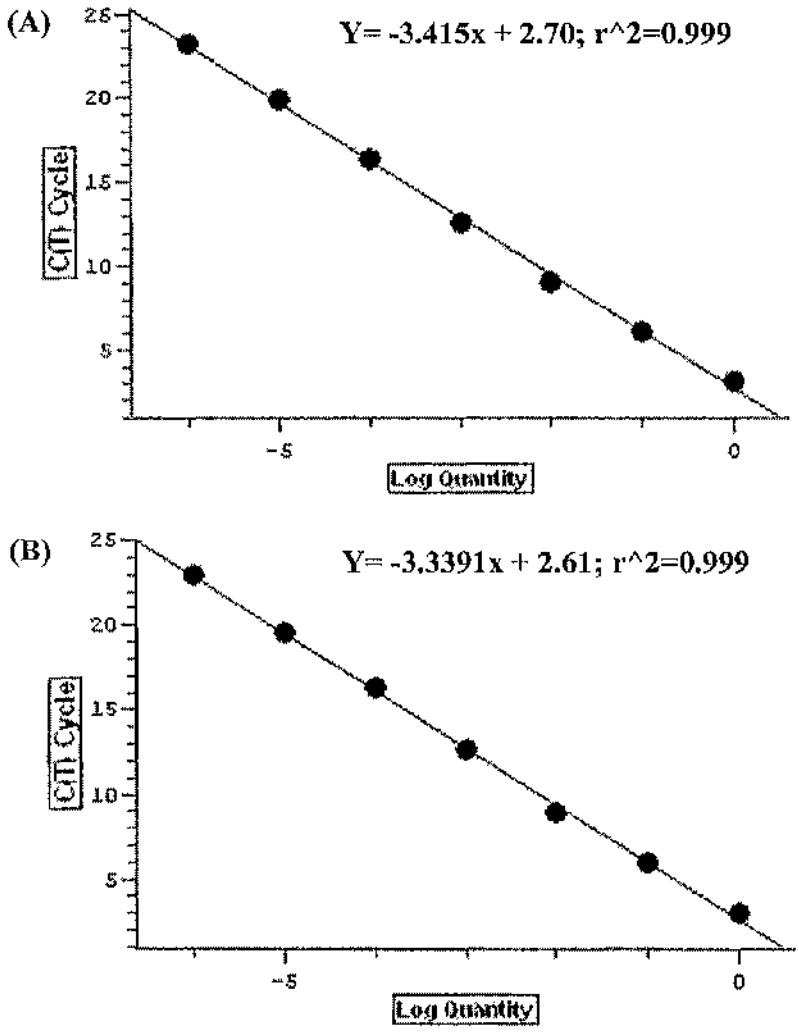


Figure 3.35- Efficiency of qRT-PCR reaction of control and target genes.

Efficiency was calculated on basis in the slope of the plot between the Ct values for the 10-fold dilutions against concentration. Efficiency for pair primer of control gene *gyrA* = 96% (A); Efficiency for pair primer of target gene *ddl* = 99% (B). The efficiency of the reaction was calculated by the formula: $E=10^{(-1/slope)}-1$.

3.5.1.2 Melting curve analysis

After the qRT-PCR reactions, a melting curve analysis between 70-95°C was performed to confirm the presence of the specific product and ensure no-development of primers dimers. A typical plot of the dissociation curve is shown in Figure 3.36. The melting curves are displayed as first negative derivative of the fluorescence versus the temperature (-dI/dT). Thus, a peak can be seen as the melting temperature (Figure 3.37) (Wilkening and Bader, 2004).

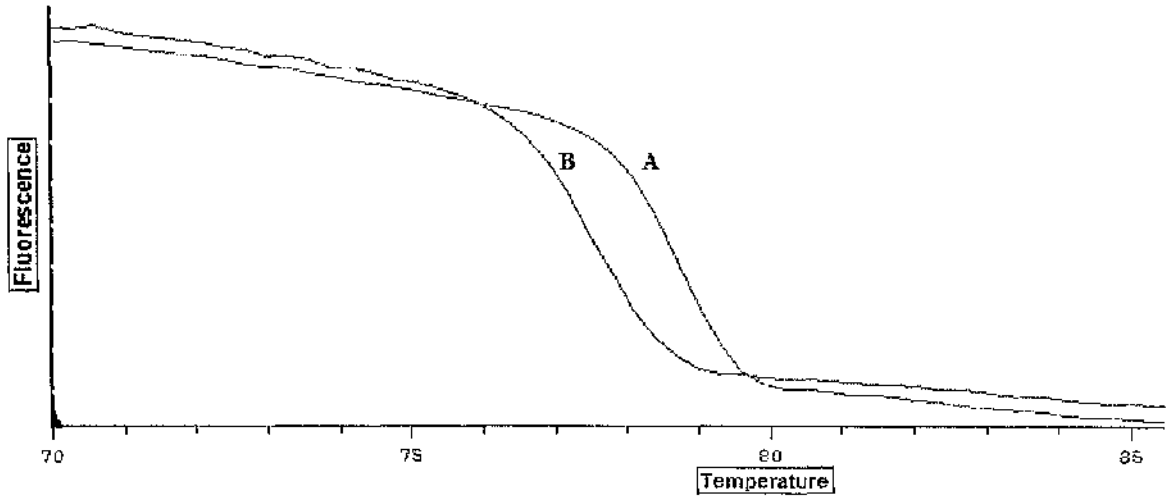


Figure 3.36- Melting curve analysis of qRT-PCR reaction.

LightCycler melting curves analysis from the amplification of control gene *gyrA* (B) and target gene *ddl* (A). The Tm of the amplicon starts at the point of inflection of the melting curve profile The qRT-PCR reactions were made in quadruplicate using SYBR Green method.

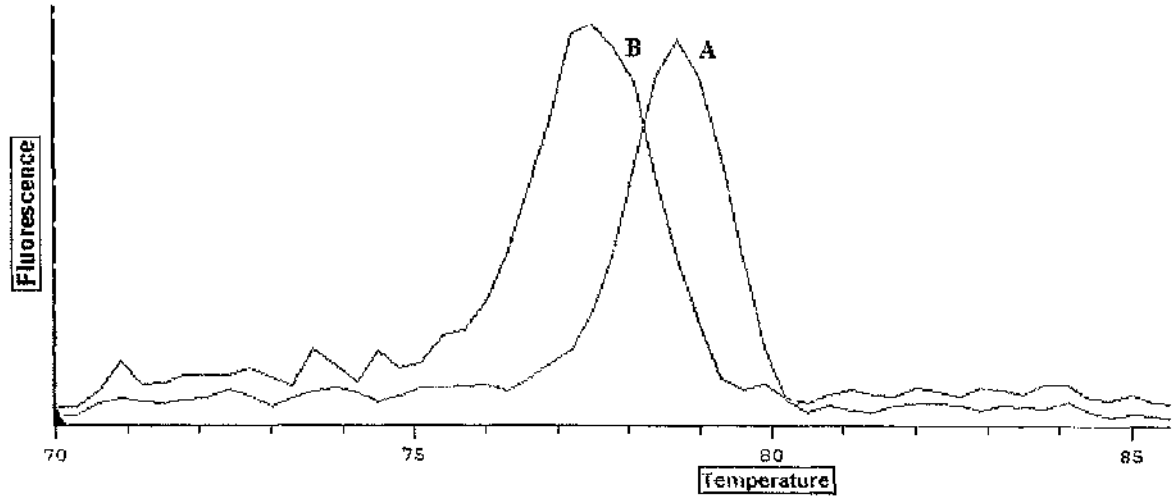


Figure 3.37- Derivate melting curve analysis of qRT-PCR reaction.

LightCycler melting curves analysis from the amplification of control gene *gyrA* (B) and target gene *ddl* (A). The melting curves are displayed as first negative derivative of the fluorescence versus the temperature (-dl/dT). Thus the maximun -dl/dT value for a curve corresponds to the melting temperature of the product. The qRT-PCR reactions were made in quadruplicate using SYBR Green method.

3.5.1.3 Relative quantification method ($\Delta\Delta C_T$)

To validate our microarray data, relative transcript levels were determined by quantitative real-time PCR on a selection of genes. An interesting target altered in expression profile in microarray analysis of the TIGR4 Δ*rr06* compared with the wild-type, was the *rlrA* pathogenicity islet (Hava *et al.*, 2003a), up-regulated in TIGR4 Δ*rr06*, composed of a

putative transcriptional regulator *rlrA* (*sp0461*), three cell wall-anchored surface proteins (*sp0462-0464*) and three sortase enzymes (*sp0466-0468*). The up-regulation of the *rlrA* pathogenicity islet in TIGR4 Δ*rr06* was checked by quadruplicate reactions by qRT-PCR using cDNA of TIGR4 wild-type and Δ*rr06* strains, from two independent RNA preparations, and tested using the SYBR Green method. The qRT-PCR analysis of the *rlrA* pathogenicity islet by relative quantification method ($\Delta\Delta C_T$) (Livak and Schmittgen, 2001) showed strong up-regulation of this locus in Δ*rr06* strain and a positive correlation with the expression ratio obtained by microarray analysis Figure 3.38. Other interesting loci identified in microarray analysis was the *dlt* operon, down-regulated in TIGR Δ*rr06* strain. qRT-PCR analysis showed agreement with microarray analysis in two genes of this operon, *dltA* (*sp2176*) and *dltD* (*sp2173*). The remaining genes, *dltB* (*sp2175*) and *dltC* (*sp2174*) were not observed to have a significant decrease (Figure 3.39).

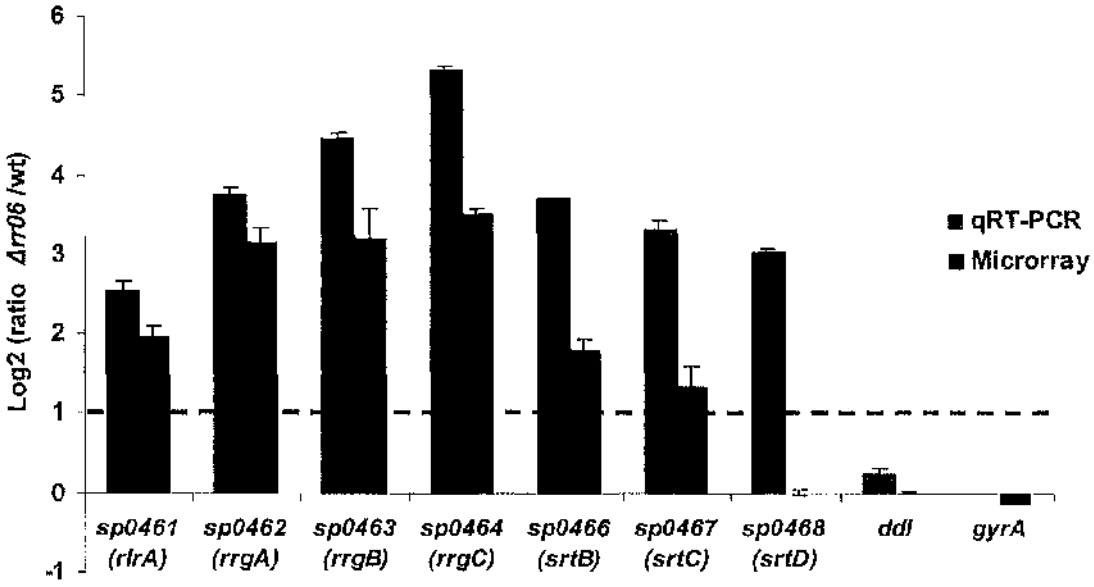


Figure 3.38- Expression ratio of *rlrA* pathogenicity islet in TIGR4 Δ*rr06* compared with its parental strain.

The expression ratio of *rlrA* pathogenicity islet in TIGR4 Δ*rr06* compared with wild-type strain was determined by microarray and qRT-PCR. The dashed line indicates the two-fold change microarray cut-off value for differential expression. No microarray data was obtained for *sp0468*. Expression of two control house keeping genes D-alanine-D-alanine ligase (*ddl*) and DNA gyrase subunit A (*gyrA*) are shown.

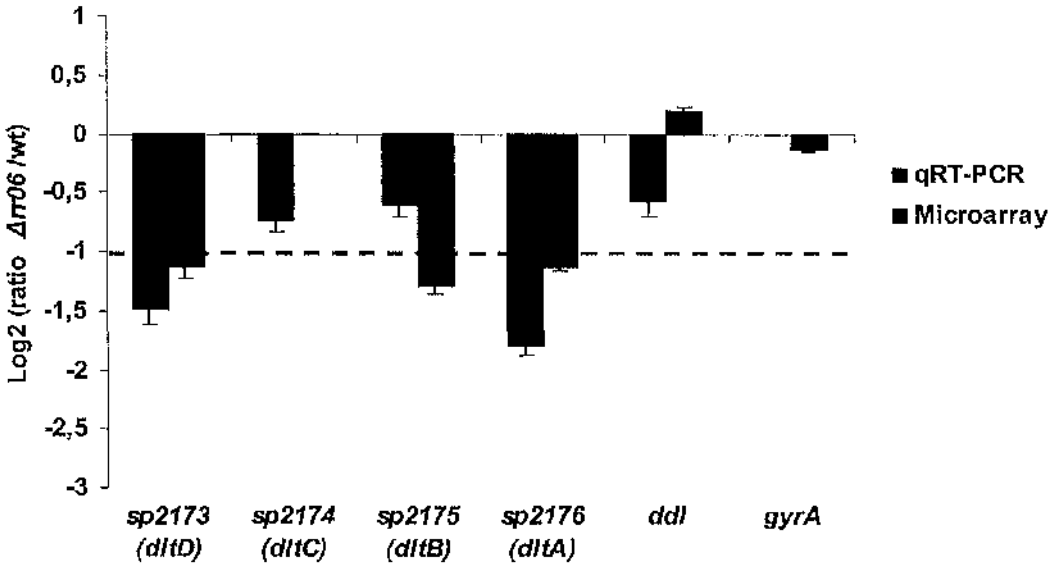


Figure 3.39- Expression ratio of *dlt* operon in TIGR4 Δ*rr06* compared with its parental strain.

The expression ratio *dlt* operon in TIGR4 Δ*rr06* compared with wild-type strain was determined by microarray and qRT-PCR. The dashed line indicates the two-fold change microarray cut-off value for differential expression. No microarray data was obtained for *sp2174*. Expression of two control house keeping genes *ddl* and *gyrA* are shown.

3.6 Does RR06 regulate the virulence factor PspC?

The pneumococcal surface protein C (PspC), also know as CbpA, plays an important role in the pneumococcus pathogenesis by functioning as an adhesin (Rosenow *et al.*, 1997). It also binds soluble host factors such as the secretory component, the third component of complement (C3) and complement factor H (Cheng *et al.*, 2000; Dave *et al.*, 2001; Hammerschmidt *et al.*, 1997; Janulczyk *et al.*, 2000). During the time of work on this project it was suggested that RR06 regulates the virulence factor *pspC*. Genomic organisation shows that *rr06* has an upstream position to the virulence factor *pspC*. Furthermore, *S. pneumoniae* Δ*rr06* mutants strains have *in vivo* a similar phenotype that the Δ*pspC*. Evidence of this regulation have recently been published. Electrophoresis mobility shift assays shows that the RR06 binds to a region upstream of the *pspC* gene and acts as a transcriptional activator for the *pspC* promoter (Standish *et al.*, 2005). These findings show than RR06 can presumably contribute to the regulation of *pspC*. To confirm if RR06 regulates the expression of this virulence factor, microarrays, RT-PCR or qRT-PCR and western immunoblot analysis were done in TIGR4, D39, R6 and 0100993 strains.

3.6.1 Expression of *pspC* on *S. pneumoniae* Δ*rr06* mutants

3.6.1.1 Microarray analysis

To verify if the virulence factor PspC is regulated by the TCS06, the expression levels of *pspC* gene were analysed using microarray technology. The transcriptional levels of *pspC* were compared in *S. pneumoniae* wild-type strains TIGR4, D39, R6 and 0100993 and their isogenic mutant Δ*rr06* growing at BHI up to mid-log phase (OD_{600nm} of 0.6) at 37°C. During this analysis, different amplicon-based microarrays were used, TIGR and BμG@S arrays. Due to the variability in *pspC* (Brooks-Walter *et al.*, 1999), both amplicon-based microarrays include two-*pspC* sequence probes using TIGR4 annotation (*sp2190*) and R6 annotation (*spr1995*).

No differences were observed in expression levels of *pspC* in TIGR4 Δ*rr06* compared to the parental strain (Figure 3.40). The mean signal of expression in the four replicate microarray hybridization slides in both TIGR and BμG@S microarrays slides were similar in the two strains. The regulation analysis of *pspC* by the Δ*rr06* was also studied in the serotype 2 R6 strain and in 0100993 strain (serotype 3). No significant difference was observed in expression of the virulence factor *pspC* in both Δ*rr06* strains, presenting in Δ*rr06* strain a small increase in transcription relative to the parental wild-type in BμG@S arrays analysis (Figure 3.40).

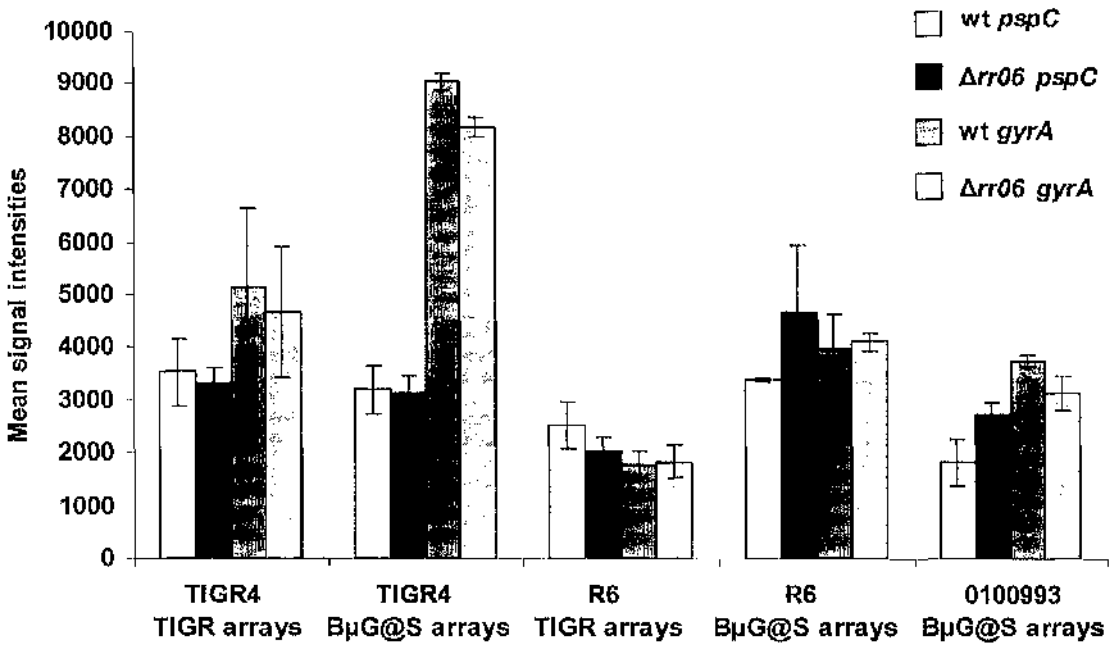


Figure 3.40- Microarray expression analysis of *pspC* in Δ*rr06* mutants.

The expression of *pspC* of Δ*rr06* mutants compared with parental wild-types strains were studies using TIGR and BμG@S microarrays. Values are determined by microarray hybridization and are given as the geometric mean fluorescence intensity values from four replicate hybridizations of each type of array slide in TIGR4 strain and two replicate hybridizations in R6 and 0100993 strains. Error bars represent ± SEM. The expression of control house keeping gene *gyrA* is shown.

Standish and co-workers, observed that the mutation of *rr06* in D39 background affects the transcription of *pspC*. Real-Time PCR analysis demonstrated 3-fold down-regulation compared with the wild-type parent (Standish *et al.*, 2005). However, the R6 unencapsulated laboratory strain used in this study is derived from the D39 strain. For this reason in the present study, a microarray analysis was performed using the exact strains D39 wild-type and their isogenic *rr06* mutant, kindly supplied by Standish and co-workers. The expression of *pspC* was reduced in Δ*rr06* strain compared with wild-type (Figure 3.41). However, this reduction was not statistically significant. Expression of the *pspC* gene in the D39 Δ*rr06* mutant was only down-regulated 1.6 fold compared with the wild-type parent.

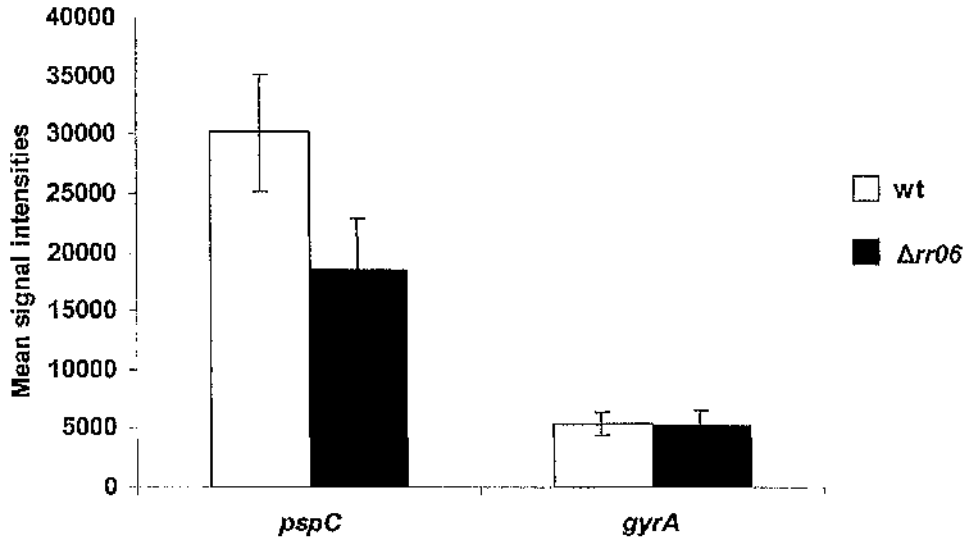


Figure 3.41- Microarray expression analysis of *pspC* in D39 Δ*rr06* mutant.

The expression of *pspC* in D39 Δ*rr06* compared with wild-type strain was study using TIGR microarrays. Values are determined by microarray hybridization and are given as the geometric mean fluorescence intensity values from two replicate hybridizations. Error bars represent ± SEM. The expression of control house keeping gene *gyrA* is shown.

3.6.1.2 RT-PCR and qRT-PCR analysis

The microarray data of *pspC* expression on R6 Δ*rr06* compared with the isogenic wild-type strain was confirmed by RT-PCR, while the expression on TIGR4 strain was confirmed by real-time PCR (qRT-PCR).

In the *S. pneumoniae* R6 strain, cDNA was synthesized from 2 μg of RNA, prepared from 10 ml of BHI cultures, using ThermoScript™ RT-PCR system (Invitrogen™). The RT-PCR reactions samples were preformed using the pair primers 010-up4-*cbpA* and *cbpA*-SKH2 (Table 2.2). No significant differences were observed in expression of *pspC* between the Δ*rr06* mutant and parental wild-type (Figure 3.42).

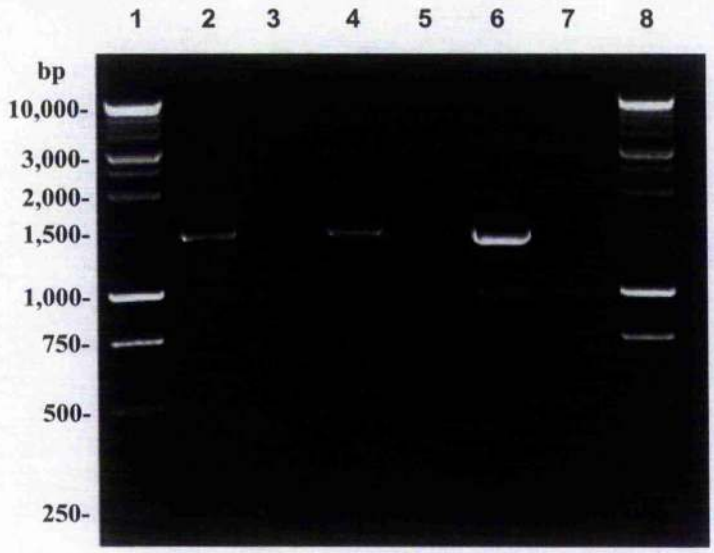


Figure 3.42- RT-PCR of *pspC* on *S. pneumoniae* R6 strain.

cDNA was obtained from 2 μg of RNA using the ThermoScript™ RT-PCR system (Invitrogen™). The RT-PCR reactions were prepared using the pair primers up4-*cbpA* and *cbpA*-SKH2 and run in 1.5% agarose gel. Lane 2 R6 wild-type, lane 3 R6 wild-type negative control, lane 4 R6 Δ*rr06*, lane 5 R6 Δ*rr06* negative control, lane 6 R6 genomic DNA, lane 7 R6 genomic DNA negative control. The DNA ladder marker used was the 1 kb plus ladder (Promega).

Because of the limitations of RT-PCR to compare the levels of expression between control and test samples, the expression of the *pspC* in TIGR4 Δ*rr06* compared with parental wild-type was confirmed by q-RT-PCR. 2 μg of RNA, prepared from 10 ml of BHI, were reverse-transcribed using SuperScript II RT (Invitrogen™). 1 μl of each cDNA sample was used to quantify the expression levels of *pspC* using the Sybr green method. No significant differences were observed in expression of the *pspC* gene in TIGR4 Δ*rr06* compared with wild-type. The qRT-PCR data confirm the previous microarray analysis, showing no direct regulation by RR06 in expression of the virulence gene *pspC*, remaining at the ratio of the expression similar to the control genes *gyrA* and *dll* (Figure 3.43).

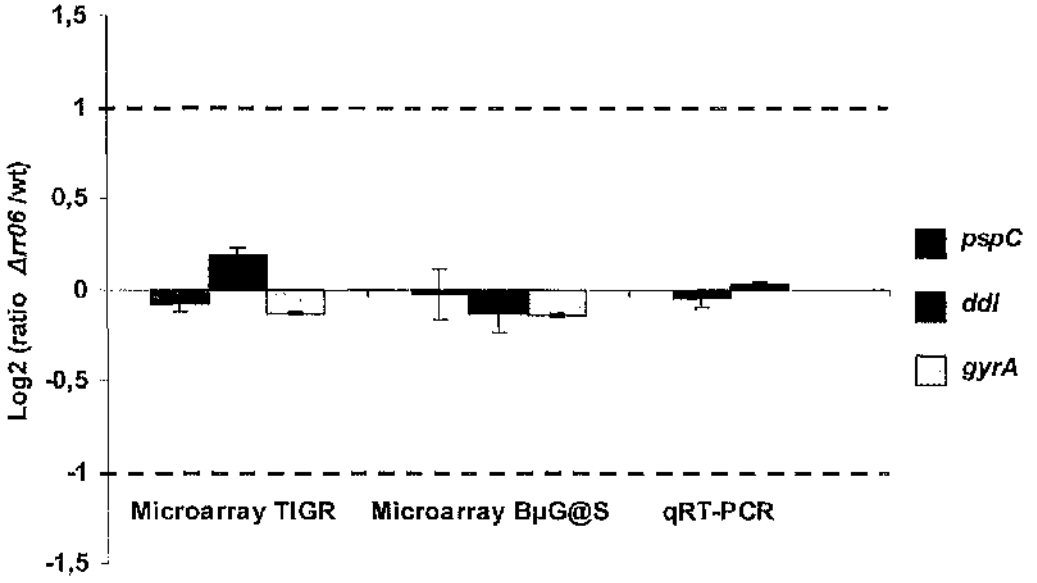


Figure 3.43- Expression ratio of *pspC* in TIGR4 Δ*rr06* compared with its parental strain.

The expression ratio of *pspC* in TIGR4 Δ*rr06* compared with wild-type strain was determined by microarray and qRT-PCR. The dashed line indicates the two-fold change microarray cut-off value for differential expression. The comparative $\Delta\Delta C_T$ method was used for qRT-PCR analysis and the test genes were normalized against the control gene *gyrA*. Expression of two control house keeping genes *ddl* and *gyrA* are shown.

3.6.1.3 Western immunoblot analysis

Western immuboblot was used to examine the levels of virulence factor PspC expressed by *S. pneumoniae* TIGR4, D39 and R6 strains. Expression of the PspC protein was studied in pneumococcal cultures growing in BHI until late-log phase (OD_{600nm} 1.0) (Figure 3.44). The expression of the protein was shown to be similar in all Δ*rr06* strains compared with wild-type. The western immunoblot analysis confirmed the previous microarrays, RT-PCR and qRT-PCR analysis using cultures in mid-log phase (OD_{600nm} 0.6), showing no regulation of PspC by RR06 in the previous conditions.

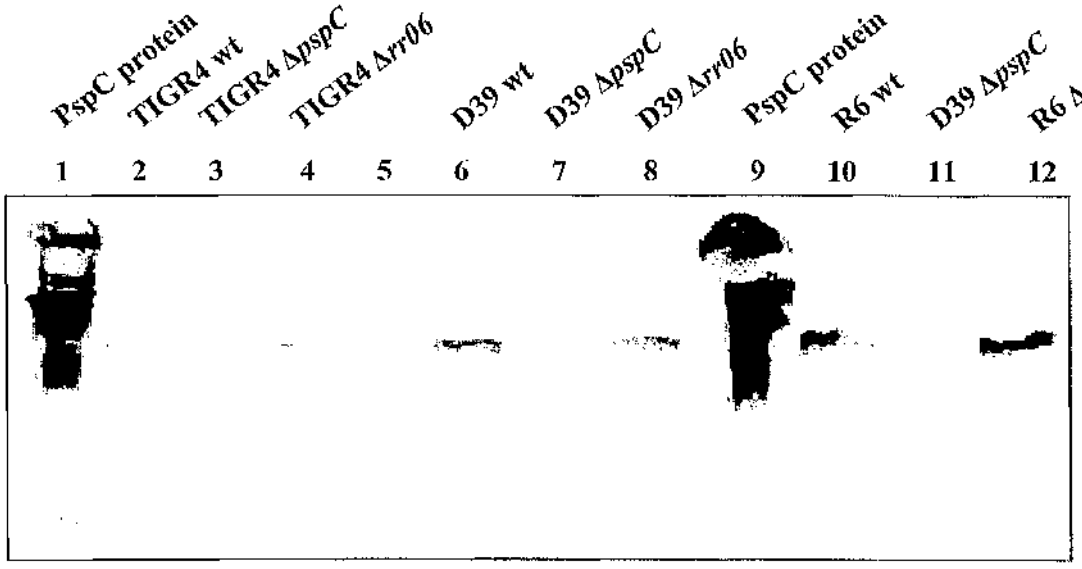


Figure 3.44- Western immunobloot analysis of PspC in different pneumococcal strains.

Expression of PspC in TIGR4, D39 and R6 wild-types and Δ*rr06* mutants strains. 15 μg of total protein for each sample were applied in 10% SDS-polyacrylamide gel following by western immunoblot using anti-PspC and anti-rabbit serum. Lane 1 and 9 PspC protein, Lanes 2, 6 and 10 TIGR4, D39 and R6 wild-type strains, Lane 3 TIGR4 Δ*pspC* mutant strain, lanes 7 and 11 D39 Δ*pspC* mutant strain, Lanes 4, 8 and 12 TIGR4, D39 and R6 Δ*rr06* mutant strains. The proteins marker used (lane 5) was the Precision Plus Protein standards (Bio-Rad). The Western Immunobloot analysis of PspC was done in two independent experiments.

Standish and co-workers had shown that the TCS06 regulated the virulence factor *pspC* (Standish *et al.*, 2005). However, our microarray analysis did not show significant changes in *pspC* mRNA in TIGR4, R6, 0100993 and D39 Δ*rr06* strain compared with parental wild-types. This result correlates with RT-PCR and qRT-PCR analysis, and also with Western immunoblotting analysis data, indicating that RR06 do not regulate, at least in these conditions, the *pspC*.

Chapter 3 discussion

Bacteria live in a constantly changing environment in which nutrients, temperature, osmolarity, acidity, humidity, and many other conditions are subject to abrupt and unexpected changes. In order to survive, bacteria must have the ability to sense and respond to their environment. A recognised key mechanism through which bacteria perceive and respond to their environment is played by the two-component systems (TCS) also referred to as the two-component signal transduction systems. These systems typically consist of two modular proteins; a sensor histidine kinase (HK) that auto-phosphorylates at a histidine residue in response to environmental stimuli creating a high-energy phosphoryl group and a cognate response regulator (RR) to which an activated phosphate is transferred and that then mediates a downstream response, often acting as a DNA-biding protein to cause changes in gene expression (Parkinson, 1993; Stock *et al.*, 2000).

Molecular advances in the form of genome sequencing, signature-tagged mutagenesis, differential fluorescence induction and microarray analysis have yielded considerable progress in the study of these systems in *S. pneumoniae*.

TCS06 system and virulence

When *S. pneumoniae* causes disease it needs to adapt in a wide range of sites, including the lung, middle ear, sinuses, blood, and meninges. The continued existence of the pneumococcus to its environments in the host requires adaptive response that involves several ways of regulating gene expression including the TCS. Thirteen HK/RR pairs with an additional orphan unpaired RR were identified in the pneumococcal genome (Lange *et al.*, 1999; Throup *et al.*, 2000). The TCS06, also know as system 478, is relatively poorly characterised, however this system has been shown to contribute to virulence. Deletion in the histidine kinase and response regulator genes, *hk/rr06*, in serotype 2 strain conferred attenuation in a mouse respiratory tract infection (RTI) model of infection (Throup *et al.*, 2000). A Recent study, using strain D39 (serotype 2) has shown the importance of the TCS06 for the ability of the pneumococcus to survive and proliferate an *in vivo* mouse model and to adhere to epithelial cells (Standish *et al.*, 2005). Furthermore, Standish and co-workers observed that TCSO6 regulates an important virulence factor *cbpA*, also know as *pspC* (Standish *et al.*, 2005).

In the present study, we investigated the importance of RR06 in the ability of the pneumococcus to colonize the nasopharynx and translocate to the lungs and blood in TIGR4 strain. After intranasal infection, the RR06 was not shown to have an important role in the capacity of pneumococci to colonize the nasopharynx however, RR06 seems to

be important for the ability of the pneumococcus to invade the lungs and blood. A large reduction in growth in the lung was observed by Throup and co-workers in strain 0100993, who detected a reduction of approximately 3.5 logs in growth in the lung due to disruption of the *hk/rr06* system (Throup *et al.*, 2000). In the experiments reported here, the same mutation used in TIGR4 strain, reduced significantly the amount of the bacteria in the lungs airways and in the blood after intranasal challenge. In the *in vivo* studies reported by Standish and co-workers, the results observed were unclear. The *hk06* mutant of strain D39 was much reduced in lung tissue and in the blood 96 hours after intranasal infection while, no reduction is observed at early times. However, unexpectedly the numbers of *rr06* mutants in the lungs and in the blood increased significantly when compared with wild-type (Standish *et al.*, 2005). This observation contradicts what was reported by Throup and co-workers where, in preliminary experiments for strain 0100993, the inactivation of histidine kinase genes alone resulted in substantially less attenuation than that obtained with strains harbouring the corresponding response regulator deletion (Throup *et al.*, 2000). These results perhaps reflect differences in pneumococcal strains. It was recently shown that the contribution of two-component system to virulence could be strain-dependent (Blue and Mitchell, 2003). Furthermore, these different observations can be due to the method of generating mutants, or even the type of mice used.

Colonization of the nasopharynx is an important step in pneumococcal pathogenesis. Using the MF1 mouse model to study the importance of the TCS06 in the pneumococcus nasopharynx colonization, no significant reductions were observed in TIGR4 *rr06* mutant. A similar effect was observed in a D39 background using both *hk06* and *rr06* mutations. However in this study, the number of bacteria found after 48, 96 hours of infection increased significantly in D39 *rr06* mutant when compared with wild-type (Standish *et al.*, 2005). Therefore this two-component system seems to be important for colonization and growth of the bacteria in the lungs, and also for invasion of the bloodstream but not in colonization of the nasopharynx.

Effect of RR06 on *in vitro* growth

Photogenic bacteria as well as other living organisms have evolved adaptational regulatory networks to maintain cell viability under stressful environmental conditions and to ensure persistence and re-growth in host tissues. When entering the host from the environment, a microbial pathogen is confronted by several changes, including nutrient limitation, changes in temperature, humidity, and osmolarity. Furthermore, the bacteria are exposed to natural host resistance mechanisms such as phagocytosis by specialized phagocytes (Kaufmann,

1998) and confronted by reactive oxygen and nitrogen intermediates. The TCS have been shown to regulate gene expression in response to a large changes in the environment (Barrett and Hoch, 1998; Hoch, 2000; Perego and Hoch, 1996; Stock *et al.*, 1989).

In this study, we investigated the importance of TCS06 for *S. pneumoniae* to grow at higher temperatures and to resist to oxidative stress. We had shown that RR06 plays a role in resistance to elevated temperatures. The TIGR4 Δ*rr06* mutant has a slow growth at 40°C and presents a slight decrease in the growth rate to 37°C, where the mutant reaches a final optical density three times less than wild-type. Furthermore, the influence of TCS06 on growth or survival of the pneumococcus at higher temperature was more evident when the bacterium was grown on blood agar. The TIGR4 Δ*rr06* did not grow at 40°C in either aerobic or anaerobic conditions. However, the effect of RR06 on temperature sensitivity appeared to be strain-dependent. In serotype 2 strains, R6 and D39 no significant changes in growth were observed between the Δ*rr06* mutant and the wild-types, while in 0100993 background, both strains did not grow at 40°C. Some reports have shown the importance of the TCS in different bacteria to mediate stress responses, for example adaptation to growth and survival in higher temperature environments (Fabret and Hoch, 1998; Morel-Deville *et al.*, 1998). In *S. pneumoniae* the CiaR/H system was been reported to regulated many genes in response to stress environment, included the high-temperature requirement A gene (*htrA*) implicated in the success of the bacteria grows at higher temperatures (Ibrahim *et al.*, 2004b).

Hydrogen peroxide is an example of reactive oxygen species that can cause damage to DNA and other cell component. The sensitivity to hydrogen peroxide of TIGR4 Δ*rr06* was increased after 5-10 min exposure to 40 mM of hydrogen peroxide (Figure 3.15). However, the sensitivity to oxidative stress of the *rr06* mutant was not statistically significant due to the great differences between the three independent replicates used in this study. The effect of hydrogen peroxide treatment on TIGR4 Δ*rr06* remains unclear due to the higher variability of this methodology observed in our and other laboratories, making a statistical validation for this analysis difficult.

Gene regulation by TCS06

TCS probably plays an essential role in regulating genes necessary for successfully colonization and infection by human pathogens, such as *S. pneumoniae* (Hava and Camilli, 2002; Kadioglu *et al.*, 2003; Lau *et al.*, 2001; Throup *et al.*, 2000). Several studies have shown the regulation of key genes known to contribute to pneumococcal virulence. TCS04

has been shown to regulate the *psa* operon that encodes a manganese ABC transporter system in TIGR4 strain (McCluskey *et al.*, 2004). Investigation of the CiaR/H system regulon identified the major virulence factor *htrA*, as being down-regulated in *ciaR/H* mutants (Ibrahim *et al.*, 2004b; Mascher *et al.*, 2003; Sebert *et al.*, 2002). Recently, microarray analysis has shown that TCS02 regulated positively the transcription of a set of genes encoding important surface proteins, including the PspA virulence factor (Ng *et al.*, 2005).

The expression of important pneumococcal virulence factors has been shown to be upregulated *in vivo*. However, such studies are limited by focusing on only a small number of genes (Ogunniyi *et al.*, 2002; Orihuela *et al.*, 2000; Orihuela *et al.*, 2001; Quin *et al.*, 2005). A large-scale identification of *S. pneumoniae* virulence determinants has been attempted, such as the studies of STM screens (Hava and Camilli, 2002; Lau *et al.*, 2001; Polissi *et al.*, 1998), or by development of differential fluorescence induction (DFI) (Marra *et al.*, 2002a). Furthermore, more recently a full examination of pneumococcal transcription *in vivo* during bacteraemia, meningitis, and epithelial cell contact models was done through the use of whole-genome microarray (Orihuela *et al.*, 2004).

Genetic regulation by TCS reveals an important challenge to understand the role of these systems during infection. Unfortunately, analysis of *in vivo* bacterial gene expression is beset by the technical difficulties of recovering sufficient quantities of pure and intact bacterial RNA from infected host tissues. For these reasons, the genetic regulation by TCS06 report here was made by whole-genome microarray analysis using RNA extracted *in vitro* standard conditions. The microarray is a powerful tool for the analysis of transcriptional changes in gene expression. Recently, several pneumococcal microarray studies have been used to find the genes directly or indirectly regulated by the TCS, TCS02 (Ng *et al.*, 2005), TCS04 (McCluskey *et al.*, 2004), CiaR/H (Sebert *et al.*, 2002), and TC13 (de Saizieu *et al.*, 2000).

In this study, we investigated the genes regulated by the RR06 in TIGR4 strain, using two whole-genome microarrays from independent sources, and two independent RNA samples. Due to time limitation, microarray analysis from strains R6 and 0100993 were preformed from only one RNA sample. Moreover, some of the transcriptional changes were confirmed by quantitative Real-Time PCR (qRT-PCR). The transcriptional changes occurring in TIGR4 Δ*rr06* compared with wild-type varied between 3% in TIGR arrays and 2% in BμG@S arrays slides. The transcriptome analysis showed an appreciable agreement between the two-microarray slides. However, the divergences in transcriptome

profile of the two distinct amplicon-based microarrays probably reflect differences in the method or/and due to the differences of the probes printed in the slides. In TIGR4 strain, the set of RR06-regulated genes was found to be enriched for genes predicted to encode proteins associated with stress response, surface wall metabolism, energy metabolism, and degradation of protein, peptides and glycopeptides. Of the genes down-regulated in *rr06* mutant, the *clpE* have being reported to play a role in the thermo-tolerance of *S. pneumoniae* (Chastanet *et al.*, 2001). *dnaK*, *dnaJ*, *grpE*, and *groEL* are known to play a role in the protection of proteins against stress (Choi *et al.*, 1999; Frydman, 2001; Linke *et al.*, 2003). Another down-regulated gene, *hrcA*, is known to regulate stress response genes in bacteria (Narberhaus, 1999). Furthermore, a major virulence factor, *htrA* was also found as being down-regulated in Δ*rr06*. This serine protease was described to being involved in the ability of the pneumococcus to grow at high temperatures (Ibrahim *et al.*, 2004a). These findings may be the reason of the role of RR06 on growth of the pneumococcus at higher temperatures. Others genes of interest which transcriptional levels decreased significantly in TIGR4 Δ*rr06* strain were: *dlt* operon (discussed in more detail in chapter 4); and *nanB*. The *dlt* operon encodes four gene products DltA, DltB, DltC and DltD that promote the incorporation of D-alanine residues into the lipoteichoic acids LTAs in Gram positive bacteria (Abachin *et al.*, 2002; Poyart *et al.*, 2001; Poyart *et al.*, 2003). The *nanB* is one of at least two known pneumococcal enzymes with neuraminidase activity (Berry *et al.*, 1996; Camara *et al.*, 1991; Camara *et al.*, 1994).

Some of these genes, found in this study, directly or indirectly controlled by TCS06, such as the *dlt* operon and *htrA*, may be the component key in the contribution of TCS06 to virulence. Some reports have demonstrated that the incorporation of D-Ala residues into the LTA is important for the virulence of two major Gram-positive pathogens *L. monocytogenes* and *S. aureus* (Abachin *et al.*, 2002; Collins *et al.*, 2002; Peschel *et al.*, 1999) while, *htrA* is known to be involved in the virulence of many Gram-negative bacteria such as *S. typhimurium* (Baumler *et al.*, 1994), *Brucella abortus* (Elzer *et al.*, 1996), *Yersinia enterocolitica* (Li *et al.*, 1996), and *Klebsiella pneumoniae* (Cortes *et al.*, 2002), and is required for full virulence of the Gram-positive bacterium *S. pyogenes* (Jones *et al.*, 2001). The exact role of *htrA* in virulence of *S. pneumoniae* is unclear but well-established (Hava and Camilli, 2002; Ibrahim *et al.*, 2004a). However, *htrA* and the *dlt* operon were reported as being regulated or potentially regulated by the CiaR/H system (Mascher *et al.*, 2003; Sebert *et al.*, 2002). The down-regulation of these genes in *ciaR/H* and *rr06* mutants demonstrates that TCS06 may indirectly control de expression of *htrA* and the *dlt* operon.

The *rlrA* pathogenicity islet in TIGR4, showed a strong up-regulation in the *rr06* mutant, suggesting an indirect regulation by TCS06. RlrA has been shown to positively regulate the expression of the seven genes on the pathogenicity islet. Of the six genes, three have homology to the LPXTG family of cell wall-anchored surface proteins (*rrgA*, *rrgB*, *rrgC*) and three encode putative sortase (*srtB*, *srtC*, and *srtD*) (Hava *et al.*, 2003a). In addition to *rlrA*, *srtD* was also identified as an essential virulence gene through an STM screen and was confirmed to be essential to the survival of *S. pneumoniae* during lung infection. RlrA was also found to be essential for colonization of the nasopharynx but not bacteraemia while, StrD is dispensable in both of these models (Hava and Camilli, 2002). A recent study had shown evidence that *rlrA* pathogenicity islet encoded pilus-like structures on the surface of *S. pneumoniae*. This structure seems to influence the pneumococcal adherence and the development of pneumonia and bacteraemia in mice and also stimulate the host inflammatory response (Barocchi *et al.*, 2006).

Furthermore, we investigated the transcriptional changes in pneumococcal mutants lacking the response regulator of TCS06 (Δ*rr06*) by microarray analysis of two additional strains, R6 and 0100993. A differential gene expression pattern between the TIGR4 Δ*rr06* and R6 and 0100993 strain was observed. While, a significant expression change, up or down-regulated, of a large number of genes was observed in the TIGR4 strain, in strain 0100993 only 9 genes were identified to be up-regulated in 0100993 Δ*rr06* strain. Curiously, no changes in gene expression were observed in R6 Δ*rr06* when compared with wild-type. The genetic diversity between and within different strains is likely to have a significant impact on the repertoire of genes regulated by TCS06. In line with this, mutants of TCS04 (McCluskey *et al.*, 2004) and TCS09 (Blue and Mitchell, 2003) were recently found to confer strain-dependent phenotypes in a mouse infection model, caused probably by differential transcription regulation. These findings come in agreement with observed on effect of RR06 *in vitro* growth, where only the *rr06* mutation in TIGR4 strain presents a dramatic impact on growth at 40°C.

S. pneumoniae undergoes spontaneous phase variation between a transparent phenotype and an opaque phenotype (Kim and Weiser, 1998); the transparent phenotype has an enhanced capacity to adhere and colonize the nasopharynx, whereas the more phagocytosis-resistant opaque phenotype predominates in blood. An increased capacity to adhere by the transparent phenotype corresponds to higher levels of PspC, phosphorylcholine, teichoic acid, and autolysin (LytA) than do opaque variants (Kim and Weiser, 1998; Rosenow *et al.*, 1997; Weiser *et al.*, 1994). In contrast, the opaque phenotype produces more capsular polysaccharide and PspA is expressed in greater

amounts in the opaque variant (Kim and Weiser, 1998). Because these numbers of proteins are reported to be differentially expressed in opaque and transparent phase pneumococci (Rosenow *et al.*, 1997), is important to confirm if all strains present the same phenotypic variation before the transcriptome analysis. However, due to the difficulty in distinguishing with accuracy between opaque and transparent colonies, a final confirmation of the phase variation of all strains was done by the analysis of the levels of these proteins, where no differences of PspA, PspC, LytA and capsular polysaccharide were observed.

Regulation of virulence factor PspC by TCS06

PspC, also known as CbpA and SpsA, is considered a major virulence factor and protective antigen, and belongs to the family of choline-binding proteins that are present on the surface of *S. pneumoniae*. These proteins noncovalently bind to the phosphorylcholine on the cell wall teichoic acid and the membrane-bound lipoteichoic acid (McDaniel *et al.*, 1991). Several functions are attributed to PspC, including binding to the secretory component of human immunoglobulin A and to complement factors C3 and H (Cheng *et al.*, 2000; Dave *et al.*, 2001; Hammerschmidt *et al.*, 1997; Hammerschmidt *et al.*, 2000; Janulczyk *et al.*, 2000). Binding to factor H is a defence strategy used by certain microorganisms for protection against complement attack and opsonophagocytosis. The importance of PspC in adherence and colonization of *S. pneumoniae* to epithelial cells of nasal passages and lungs in mice is well established (Balachandran *et al.*, 2002; Rosenow *et al.*, 1997). It has been shown that PspC-deficient mutants have a reduced ability to colonize the nasopharynx and infect the lungs (Balachandran *et al.*, 2002). This study had as purpose to investigate the involvement of the RR06 in the regulation of the pneumococcal virulence factor PspC. The genomic organisation shows that RR06 has an upstream position to a virulence factor *pspC*, and a number of prokaryotic TCS regulate adjacent genes on the chromosome, including the TCS13 from the pneumococcus (de Saizieu *et al.*, 2000). Also, the RR06 knockout has an *in vivo* phenotype similar to that of the *pspC*-deficient mutants. Furthermore, electrophoresis mobility shift assays (previously shown in our laboratory) demonstrates that the RR06 binds to a region upstream of the *pspC* gene. These findings suggesting that maybe the RR06 can regulate the *pspC* gene.

Standish and co-workers (Standish *et al.*, 2005) reported that TCS06 regulated directly the major virulence factor PspC. Western immunoblotting and qRT-PCR analysis shown down-regulation in *rr06* deletion mutants relative to wild-type *S. pneumoniae* D39. However, unexpectedly up-regulation of *pspC* was observed in D39 *hk06* mutant, suggesting that RR06 can be activated independently of HK06. Nevertheless, this finding

does not explain why the *pspC* is up-regulate ~5 fold relative to D39 wild-type. The results shown by our investigation demonstrated that RR06 does not regulate the virulence factor *pspC*, at least in the conditions presents in our study. Microarray analysis of two independent RNA samples, and using two different whole-genome microarray did not shown any significant change in expression of *pspC* in four different Δ*rr06* mutants strains, TIGR4, D39, R6 and 0100993 compared with wild-type. The qRT-PCR analysis, for TIGR4 strains, confirmed the previous microarray study. Furthermore, western immunoblotting was implemented to study the level of PspC produced by these different strain. Again, the blots indicated no significant changes of the levels of PspC in Δ*rr06* mutants when compared with parental wild-type. A similar result was observed by Standish and co-workers (Standish *et al.*, 2005) for the strain TIGR4 Δ*rr06* mutant, where no differences in *pspC* expression were found for TIGR4 *rr06* mutant by both Western immunoblotting and qRT-PCR.

These inconsistent observations between our studies and Standish and co-workers (Standish *et al.*, 2005) about the regulation of the expression of PspC in D39 strain, reflects perhaps differences in methodologies of quantification of *pspC* expression, the use of different broth cultures (Todd Hewitt broth with 1% yeast extract instead of BHI used in this study) or by the fact the RNA was collected at different optical densities of growth of bacteria. However, the D39 strains (wild-type and *rr06* mutant) using by Standish and co-workers (Standish *et al.*, 2005) were kindly sent to our laboratory, and microarray analysis demonstrated again no significant changes in expression of *pspC*. Nevertheless, PspC mRNA has recently been shown to upregulated a massive 870-fold *in vivo* compared to growth *in vitro* (Quin *et al.*, 2005). This finding demonstrates that *in vivo S. pneumoniae* needs to adapt to a different environment, and some regulatory network is activated to increase the expression of PspC. Here, *in vivo* environment the TCS06 maybe has a key role in regulation of the *pspC*. To prove this theory, future work needs to be done as compared the transcript levels of PspC between the *rr06* mutans and wild-type from bacterial RNA recovered from *in vivo* conditions.

Summary

Data presented in this chapter confirm that TCS06 is important for the ability of the pneumococcus to invade the lungs and blood but not for colonization of the nasopharynx. The *in vitro* phenotype associated with deficiency of *rr06* was shown that the TCS06 is important for the bacteria grown at higher temperatures and maybe to survive at oxidative stress. The transcriptional profile of the pneumococcal mutant lacking the response

regulator of TCS06 (Δ*rr06*), as determined by microarray analysis, showed differences in expression levels of some genes in TIGR4 background when compared with its wild-type: about 1% of the genes were upregulated and 2-3% were down-regulated in the *rr06* mutant. On the other hand a different transcription profile was obtained for R6 and 0100993 *rr06* deficient mutants, revealing that the gene expression controlled by the TCS06 is strain dependent. It has also been shown by microarray analysis, qRT-PCR and Western immunoblotting that TCS06 does not regulate the major virulence factor *pspC* in the *in vitro* conditions used in this study. This contrasts with the findings of Standish and co-workers (Standish *et al.*, 2005), who showed that *pspC* was differentially expressed relative to D39 background. In conclusion, our results, *in vitro* and *in vivo*, suggest that TCS06 regulates genes that are important for the virulence of the pneumococcus and in the ability to cause disease.

Chapter 4

Role of *dlt* operon in pneumococcal virulence

4.1 *Characterization of dlt* operon

The cell wall of Gram-positive bacteria contains two types of anionic polymers: the wall teichoic acids (WTA), which are covalently linked to the peptidoglycan; and the lipoteichoic acids (LTAs), which are polyphosphoglycerol substituted with a D-Ala ester or a glycosyl residue and are anchored in the membrane by their glycolipid moiety (Fischer, 1988; Fischer *et al.*, 1990). Incorporation of D-alanine residues into the LTAs necessitates the activity of four gene products (DltA, DltB, DltC and DltD) (Figure 4.1) that are encoded by the *dlt* operon (Poyart *et al.*, 2001). DltA is a cytoplasmic D-alanine-D-alanyl carrier protein ligase that catalyses the D-alanylation of the D-alanyl carrier protein DltC; DltB is a transmembrane protein thought to be involved in the efflux of activated D-alanine to the site of acylation; and DltD is thought to be a membrane associated protein that may have multifunctional activities (hydrolysis of mischarged DltC, facilitation of D-alanine ligation to DltC and D-alanylation of LTAs) (Debabov *et al.*, 2000).

Inactivation of any genes within this operon results in the complete absence of D-ala ester in the LTA and these D-Ala-deficient LTA mutants were found to exhibit a variety of phenotypic changes that could be attributed to the resulting charge modification of their cell surface. In *S. aureus, S. agalactiae* and *Staphylococcus xylosus*, inactivation of *dlt* operon results in increased sensitivity of these bacteria to defensins, protegrins, tachyplesins, magainin II, and other cationic peptides (Peschel *et al.*, 1999; Poyart *et al.*, 2003). Insertional mutagenesis of *dltA* in *S. gordonii* resulted in a loss of intrageneric coaggregation and in the formation of pleomorphs (Clemans *et al.*, 1999). Defects in D - alanyl- LTA synthesis could also result in acid sensitivity, as shown in the case of *S. mutans* (Boyd *et al.*, 2000). Some reports have demonstrated that the incorporation of D-Ala residues into the LTA is important for the virulence of two major Gram-positive pathogens *L. monocytogenes* and *S. aureus* (Abachin *et al.*, 2002; Collins *et al.*, 2002; Peschel *et al.*, 1999).

Although the pneumococcus contains phosphorylcholine esters instead of D-alanyl esters in both LTA and WTA (Fischer, 1997) the *dlt* operon was identified in the genomic sequences of *S. pneumoniae* R6 (Hoskins *et al.*, 2001) and TIGR4 (Tettelin *et al.*, 2001). In addition, the *dlt* operon is expressed only in certain media and activated in certain points of growth (Mascher *et al.*, 2003), indicating that the *dlt* operon maybe play an important role in virulence of the bacteria.

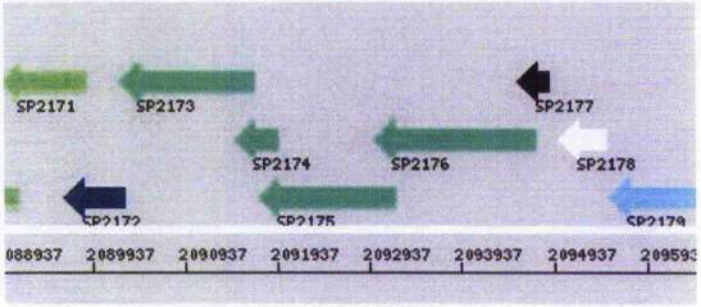


Figure 4.1- Genomic organization of *dlt* operon.

The *dlt* operon is composed by the genes *dltA* (*sp2176*), *dltB* (*sp2175*), *dltC* (*sp2174*) and *dltD* (*sp2173*).

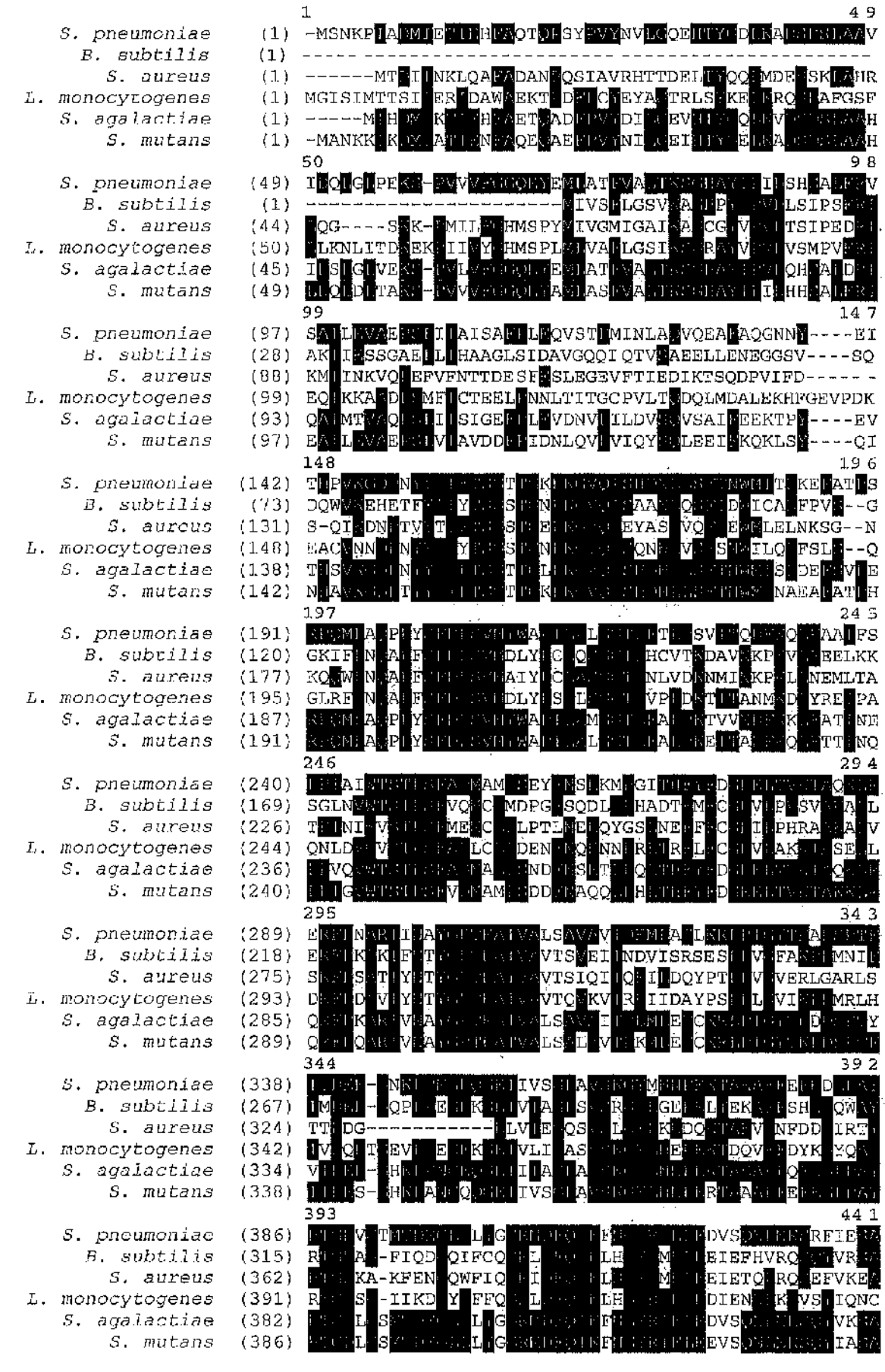
In the present study we have demonstrated by microarray and qRT-PCR analysis that the *dlt* operon is down-regulated in TIGR4 RR06 defective mutant. On the other hand, Mascher and co-workers had shown that CiaR binding directly in *dlt* promoter region and potentially regulated the *dlt* operon (Mascher *et al.*, 2003). In this study we investigated the role of the *dltA* gene in virulence of *S. pneumoniae* TIGR4 strain to determine if the DltA is responsible for the *rr06* defective mutant phenotype.

4.2 DltA

DltA is an enzyme responsible for activation of the D-Alanine residues in the cytoplasm via ATP hydrolysis and the release of pyrophosphate and is coupled to the phosphopantetheine prosthetic group of the D-alanine carrier protein DltC. The enzyme is a member of a large protein family that both activates and transfers amino or fatty acids via a 4'-phosphopantetheine prosthetic group of a carrier protein or coenzyme A CoA (Kleinkauf and Von Dohren, 1996). DltA protein not only activates D-alanine but also ligates the activated ester to the 4'-phosphopantetheine prosthetic group of the carrier protein. Therefore, the activating enzyme is now designated D-alanine:DltA ligase (AMP forming) (Neuhaus and Baddiley, 2003).

Analysis of complete genome of *S. pneumoniae* strain TIGR4 in The Institute for Genomic Research (TIGR) at www.tigr.org revealed a single copy of the gene for *dltA*. The 1551 kb gene encodes for an 516 amino-acid protein with a molecular weight of 57 kDa and a pI of 4.6. In Figure 4.2 are represented a multiple sequence alignment of DltA of *S. pneumoniae* with other Gram-positive bacteria. The pneumococcal DltA protein shares sequence similarity with DltA of *B. subtilis* (79% identity), *S. aureus* (36% identity), *L. monocytogenes* (44% identity), *S. agalactiae* and *S. mutans* (79% identity). There is one

motif, SGXTGXPKG, common to each strain that may represent the phosphate-binding loops (Saraste *et al.*, 1990). The enzymes also have two additional regions whose sequences are highly conserved with nonribosomal peptide synthetases (adenylation domain) (Heaton and Neuhaus, 1992). The two common motifs at the DltA proteins, in these six bacteria species, are GRXDFQIKXXGYRXE and PXKX$_9$TXNGKIDXKXX.



S. pneumoniae (1)
B. subtilis (1)
S. aureus (1)
L. monocytogenes (1)
S. agalactiae (1)
S. mutans (1)

S. pneumoniae (49)
B. subtilis (1)
S. aureus (44)
L. monocytogenes (50)
S. agalactiae (45)
S. mutans (49)

S. pneumoniae (97)
B. subtilis (28)
S. aureus (88)
L. monocytogenes (99)
S. agalactiae (93)
S. mutans (97)

S. pneumoniae (142)
B. subtilis (73)
S. aureus (131)
L. monocytogenes (148)
S. agalactiae (138)
S. mutans (142)

S. pneumoniae (191)
B. subtilis (120)
S. aureus (177)
L. monocytogenes (195)
S. agalactiae (187)
S. mutans (191)

S. pneumoniae (240)
B. subtilis (169)
S. aureus (226)
L. monocytogenes (244)
S. agalactiae (236)
S. mutans (240)

S. pneumoniae (289)
B. subtilis (218)
S. aureus (275)
L. monocytogenes (293)
S. agalactiae (285)
S. mutans (289)

S. pneumoniae (338)
B. subtilis (267)
S. aureus (324)
L. monocytogenes (342)
S. agalactiae (334)
S. mutans (338)

S. pneumoniae (386)
B. subtilis (315)
S. aureus (362)
L. monocytogenes (391)
S. agalactiae (382)
S. mutans (386)

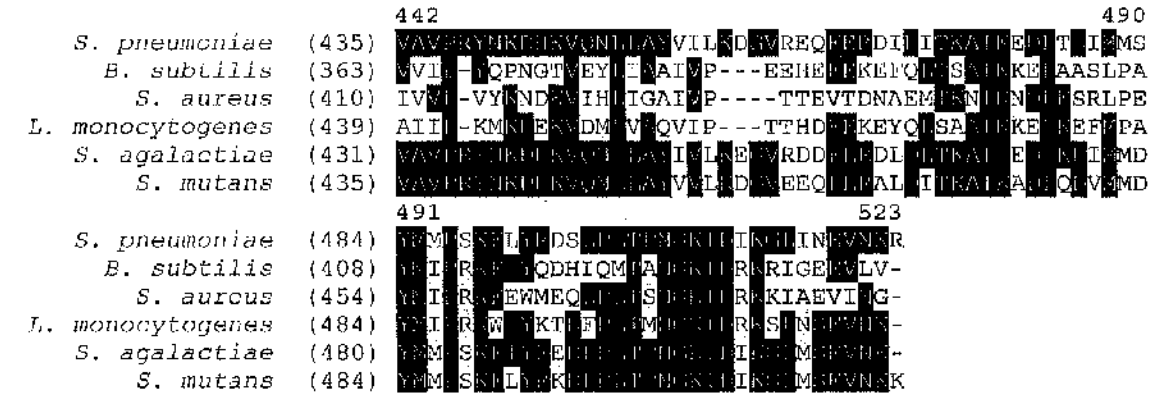


Figure 4.2- Multiple sequence alignment of DltA protein from some Gram-positive bacteria.

Alignment of the *S. pneumoniae* TIGR4 amino acid sequence with those of *B. subtilis* 168 strain, *S. aureus* N315 strain, *L. monocytogenes* EGD-e strain, *S. agalactiae* NEM316 strain and *S. mutans* UA159 strain. All sequences were obtained from TIGR (www.tigr.org). Genes were aligned using Align X, a component of the Vector NTI 9.1.0 (Invitrogen Corporation).

4.2.1 Construction of *dltA* mutant

The *dltA* gene of *S. pneumoniae* TIGR4 strain was disrupted by the introduction of spectinomycin resistance cassette into the gene. The whole *dltA* gene was amplified from the genomic DNA of pneumococcal TIGR4 strain using primers *dltA* Amp For and *dltA* Amp Rev (Table 2.2) and cloned into PCR-Script™ cloning vector (Stratagene). The primers *dltA* Amp inv1 and *dltA* Amp inv2 (Table 2.2) were used to removed the middle region of *dltA* gene creating AscI restriction site as a result of inverse PCR. Spectinomycin resistance cassette with AscI restriction sites was ligated to the inverse PCR product to use as selection marker.

4.2.1.1 Confirmation of inverse PCR product

The inverse PCR product was gel purified using Qiagen gel purification kit, self ligated and transformed into *E. coli* Top10 electrocompetent cells (Invitrogen™). The transformed cells were plated onto LB agar with 50 µg/ml of ampicillin and the plasmid isolated from overnight cultures on LB broth with ampicillin using Qiagen plasmid DNA isolation. The inverse PCR product was 3.3kb (Figure 4.3 A) and the deletion of the *dltA* gene was then confirmed by restriction digestion (Figure 4.3 B). Digestion of the plasmid with *AscI* and *EcoRI* resulted in a 200 bp and 3.1 kb bands.

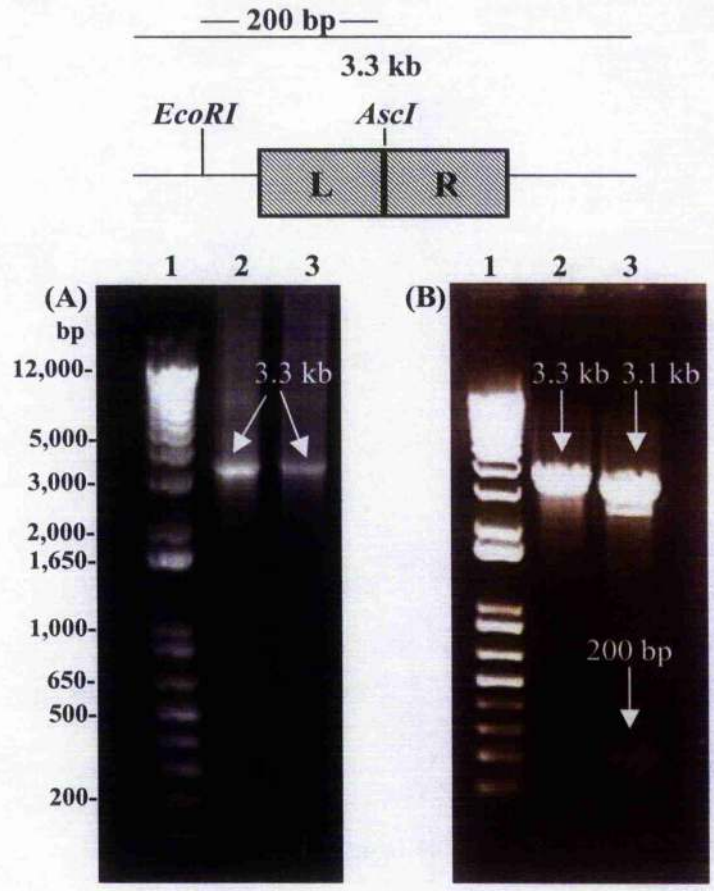


Figure 4.3- Inverse PCR and confirmation of product.

Inverse PCR product run in 1% agarose gel (lane 2 and 3) (A); confirmation of self-ligated inverse PCR product by restriction digestion with Asc I (lane 2) and *AscI* plus *EcoRI* (lane 3) (B). The DNA marker used is the 1 kb plus ladder (Invitrogen™).

4.2.1.2 Marker ligation

The spectinomycin resistance cassette was used as selectable marker to facilitate the selection of transformants. An AscI-generated spectinomycin resistance cassette was then ligated to the inverse PCR described above and cloned into *E. coli* Top10 electrocompetent cells (Invitrogen™). The transformed cells were plated onto LB agar with 200 μg/ml of spectinomycin and the plasmid isolated from overnight cultures on LB broth with spectinomycin using Qiagen plasmid DNA isolation. The ligation of the cassette was confirmed by restriction digestion. Digestion of the plasmid with *EcoRI* resulted in 4.4 kb band, with *AscI* in 3.3 and 1.1 kb bands and the digestion with *EcoRI* and *AscI* together resulted three bands with 3.1, 1.1 and 0.2 kb Figure 4.4.

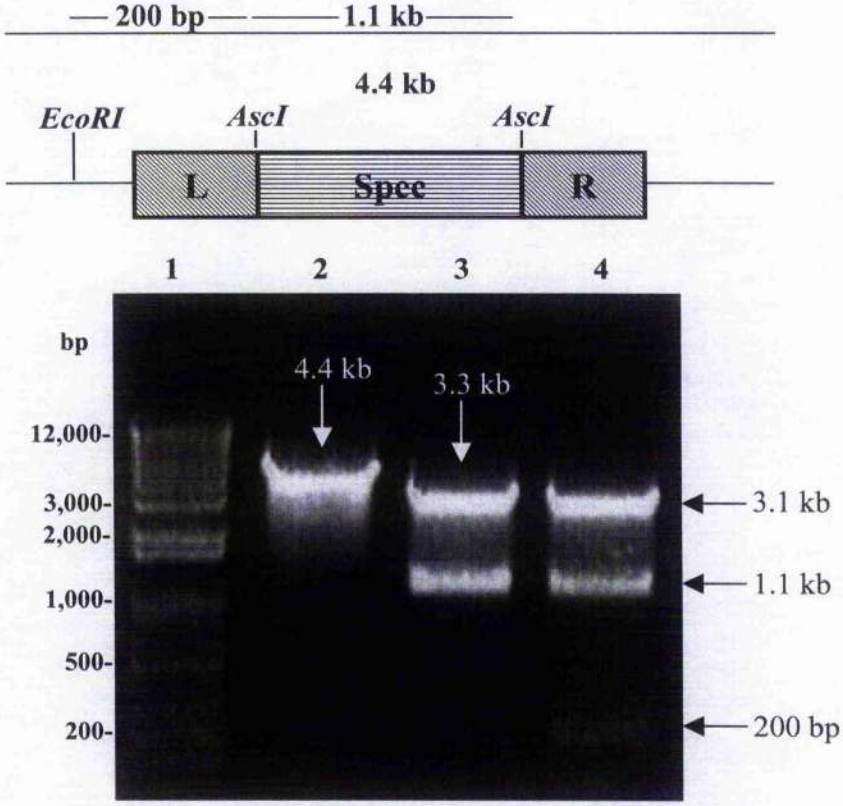


Figure 4.4- Confirmation of marker ligation in the plasmid with *dltA* mutation.

Digestion of knockout plasmid with spectinomycin resistance cassette running in 1% agarose gel. Lane 2 digestion with *EcoRI*, lane 3 digestion with *AscI* and lane 4 disgestion with *EcoRI* plus *AscI*. The DNA marker used is the 1 kb plus ladder (Invitrogen™).

4.2.1.3 Transformation of *S. pneumoniae* TIGR4 strain

The modified fragment containing the deletion of *dltA* gene with spectinomycin resistance cassette was amplified by PCR out of the plasmid using the primer pair *dltA* Amp For and *dltA* Amp Rev (Table 2.2). Following purification, the fragments were used to transform the *S. pneumoniae* TIGR4 strain by homologous recombination (Figure 4.5). The mutants were selected by growing on blood plates with 100 μg/ml of spectinomycin. The successful replacement of *dltA* gene with the spectinomycin resistance cassette in *S. pneumoniae* TIGR4, was confirmed by PCR (Figure 4.6). In one reaction the amplification of *dltA* gene was done using the primers *dltA* Amp For and *dltA* Amp Rev (Table 2.2) which amplified a 1.6 kb fragment in the TIGR4 wild-type and 1.5 kb in TIGR4 Δ*dltA* strain, a second reaction using the internal primers *dltA* For and *dltA* Rev (Table 2.2) for amplification of the region modified which amplified a 430 bp fragment in wild-type strain of *dltA* gene, and a third reaction, using the primers Spec up and Spec dn (Table 2.2) to amplify the spectinomycin resistance cassette in TIGR4 Δ*dltA* strain. The mutation was also confirmed by nucleotide sequencing.

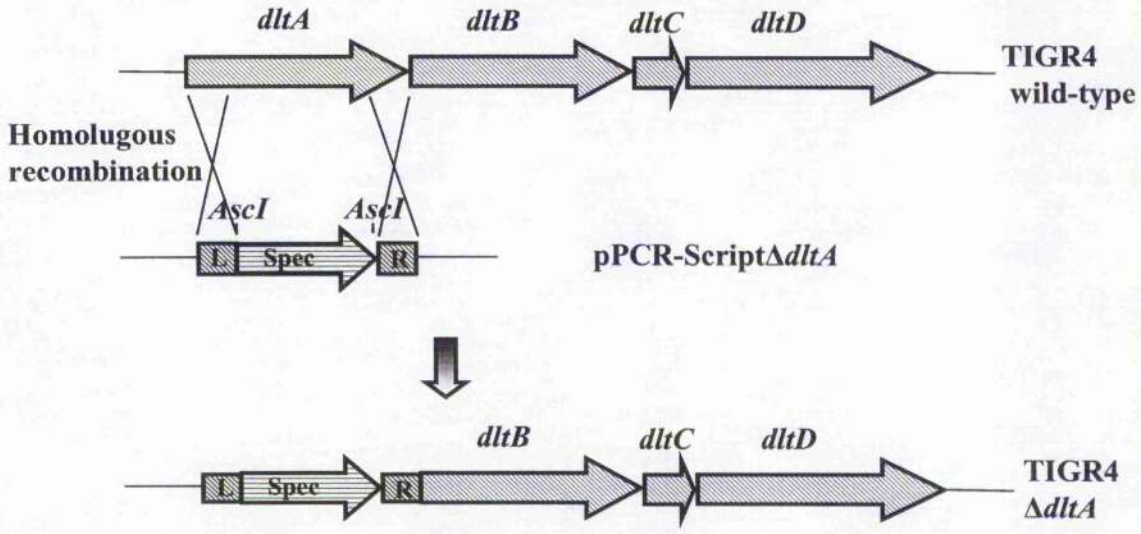


Figure 4.5- Organization of *dlt* genes in *S. pneumoniae* and disruption by spectinomycin resistance gene insertion.

The *dlt* operon of *S. pneumoniae* was disrupted by replacing the *dltA* gene with the spectinomycin resistance gene spec as shown. The spec gene and PCR fragments of *dltA* gene were cloned to produce the integration vector pPCR-Script*ΔdltA* and used to transform *S. pneumoniae* TIGR4 strain.

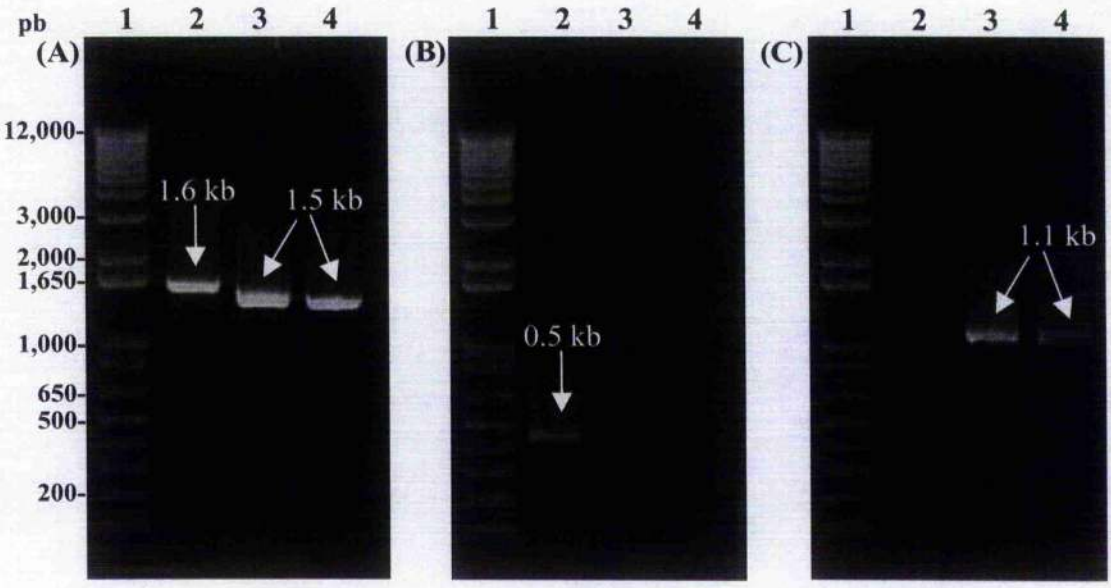


Figure 4.6- Confirmation of *dltA* deletion in *S. pneumoniae* TIGR4 strain.

PCR reactions for confirmation of *dltA* knockout running in 1% agarose gel. Amplification of *dltA* gene lane 2 TIGR4 wild-type and lanes 3 and 4 TIGR4 Δ*dltA* (A); amplification of internal segment of *dltA* gene, lane 2 TIGR4 wild-type and lanes 3 and 4 TIGR4 Δ*dltA* (B); amplification of spectinomycin cassette, lane 2 TIGR4 wild-type and lanes 3 and 4 TIGR4 Δ*dltA* (C). The DNA marker used is the 1 kb plus ladder (Invitrogen™).

4.3 *In vitro* analysis

4.3.1 Growth measurements of *dltA* mutants by optical density

The effect of the *dltA* mutation on the growth of *S. pneumoniae* at different temperatures was studied. Cultures of the Δ*dltA* mutants and the wild-types grown in BHI broth at 37 °C were monitored. 1x 10^6 CFU/ml of each strain was used to inoculate BHI and incubated at the indicated temperatures. At 1-hour intervals, samples were withdrawn to measure the optical density at OD_{600nm}. The growth of Δ*dltA* at 37°C was reduced compared to that of the wild-type. This difference was in growth rate and in total growth measured by optical density. The TIGR4 Δ*dltA* in comparison to the wild-type undergoes autolysis immediately after reaching stationary phase (Figure 4.7). At 40°C the mutant did not grow, revealing this high temperature has a great effect in TIGR4 Δ*dltA* strain (Figure 4.9).

4.3.2 Growth measurements of *dltA* mutants by viable counting

To confirm the growth trend in optical measurement and the beginning of autolysis, viable count of TIGR4 wild-type were compared to their respective Δ*dltA* mutants at 2-hour intervals up to 12 hours during growth in BHI broth. No significant differences were observed at 37°C, both strains tended to undergo autolysis after the 8 hours, suggesting that the mutation does not interfere in the growth of the bacteria (Figure 4.8). However, in microscopy analysis (section 4.3.3) we observed a variation in morphology between the wild-type and Δ*dltA* mutant as the emergence of long chains of bacteria cells in the mutant. Because the viable count is measured as colony forming unit (CFU) the formation of these long chains makes a direct comparison with the growth curve difficult. At 40°C the results reflected the optical density measurements. A significant reduction of the cell viability was observed in the TIGR4 Δ*dltA* strain, indicating that the mutant does not survive at higher temperatures (Figure 4.10).

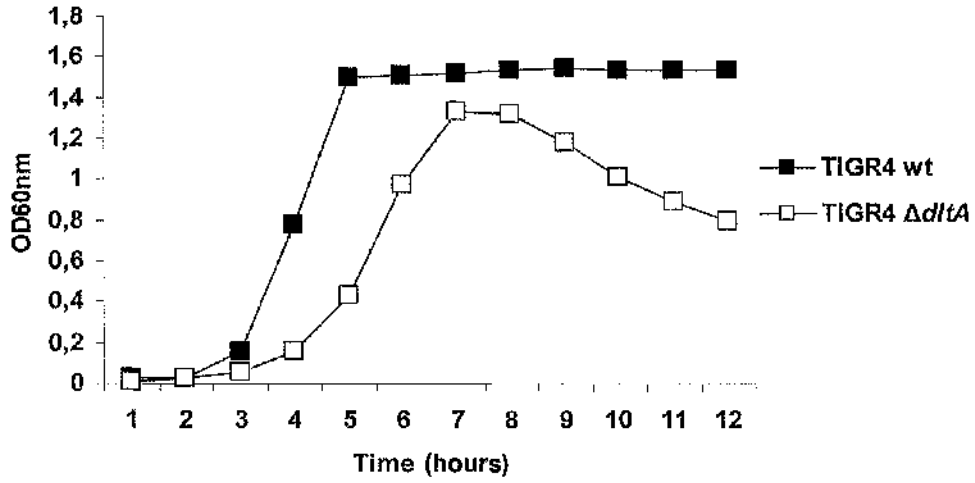


Figure 4.7- Comparison growth curves of TIGR4 wild-type and Δ*dltA* strains at 37 °C.

10^6 CFU/ml of each strain were inoculated in 20 ml of prewarmed BHI and incubated at 37 °C. 1 ml of sample were taken at 60 min-intervals to measured the optical density at 600nm.

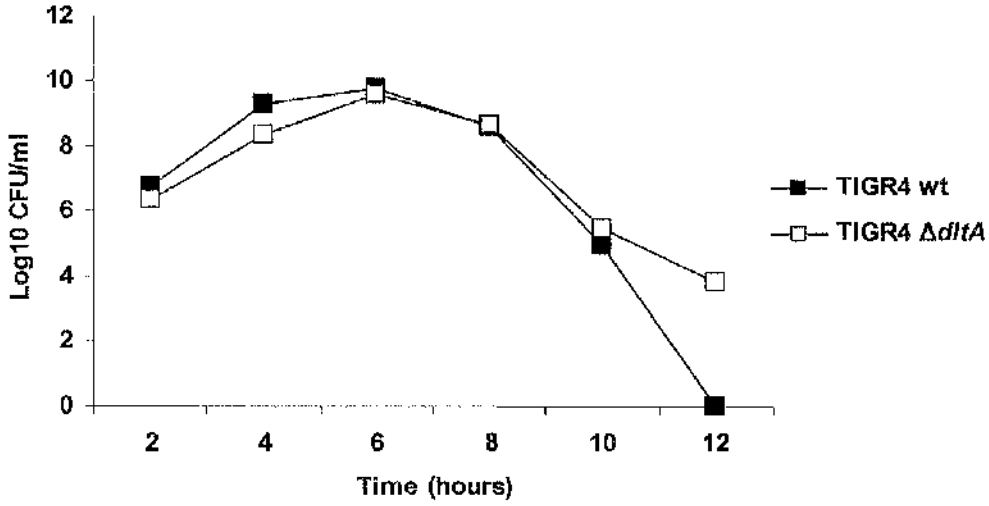


Figure 4.8- Comparison of the *in vitro* growth of TIGR4 wild-type and Δ*dltA* strains at 37°C.

10^6 CFU/ml of each strain were inoculated in 20 ml of prewarmed BHI and incubated 37°C. Samples were taken at 2 hours-intervals to measured the viable bacterial count on blood plates.

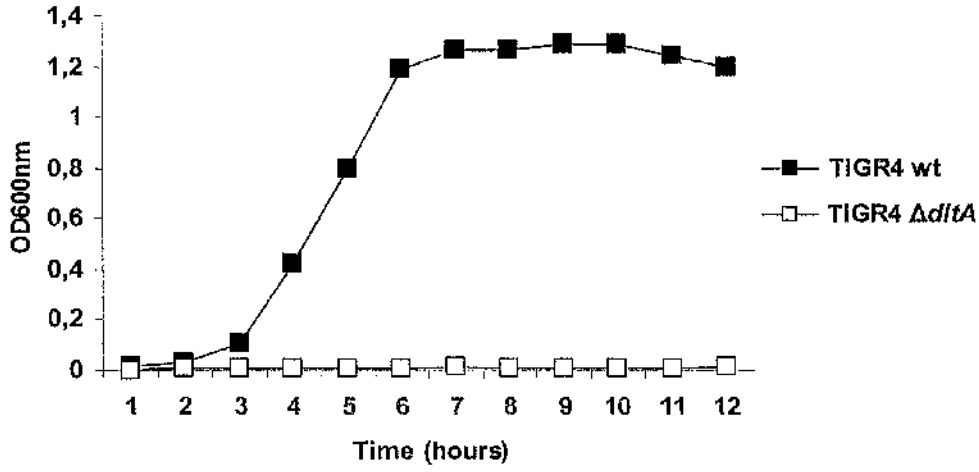


Figure 4.9- Comparison growth curves of TIGR4 wild-type and Δ*dltA* strains at 40 °C.

10^6 CFU/ml of each strain were inoculated in 20 ml of prewarmed BHI and incubated at 40 °C. 1 ml of sample were taken at 60 min-intervals to measured the optical density at 600nm.

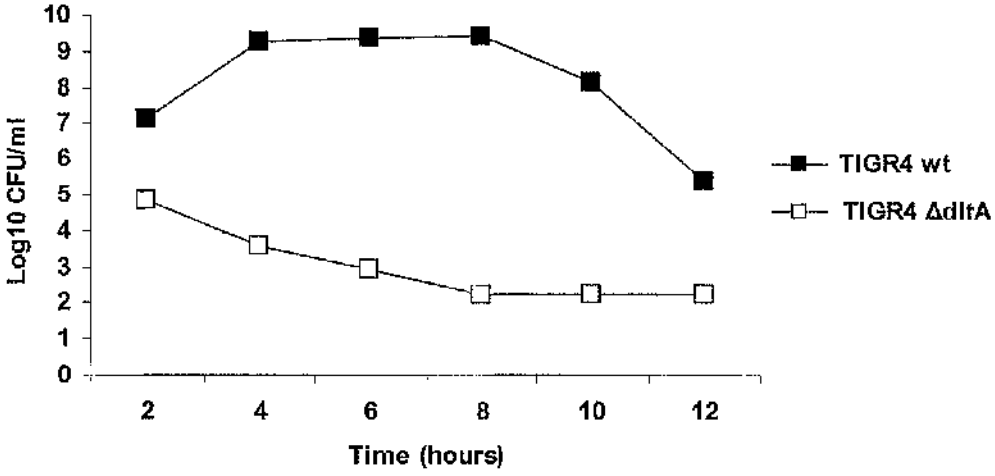


Figure 4.10- Comparison of the *in vitro* growth of TIGR4 wild-type and Δ*dltA* strains at 40°C.

10^6 CFU/ml of each strain were inoculated in 20 ml of prewarmed BHI and incubated 40°C. Samples were taken at 2 hours-intervals to measured the viable bacterial count on blood plates.

4.3.3 Effect of *dltA* deletion in morphology

The difference observed in optical density after stationary phase between the TIGR4 wild-type and Δ*dltA* strains, apparently not related with autolysis, suggests that differences in morphology of the cell may have interfered with optical density measurements. Therefore, the TIGR4 wild-type and the Δ*dltA* mutant were examined by light microscopy in early-stationary phase BHI broth and overnight blood plate cultures. The Quellung reaction also

was used to observe differences in strains layer surface (Figure 4.11). This reaction creates a swelling of the capsule surrounding a bacterium as a result of interaction with anticapsular antibody, consequently the capsule becomes more refractile and conspicuous. No changes were observed in surface of Δ*dltA* mutants compared with wild-type, however there was visible emergence of long chains of bacterial cells in Δ*dltA* mutant in both types of cultures while no chains more that 6 organisms were observed in the wild-type cultures.

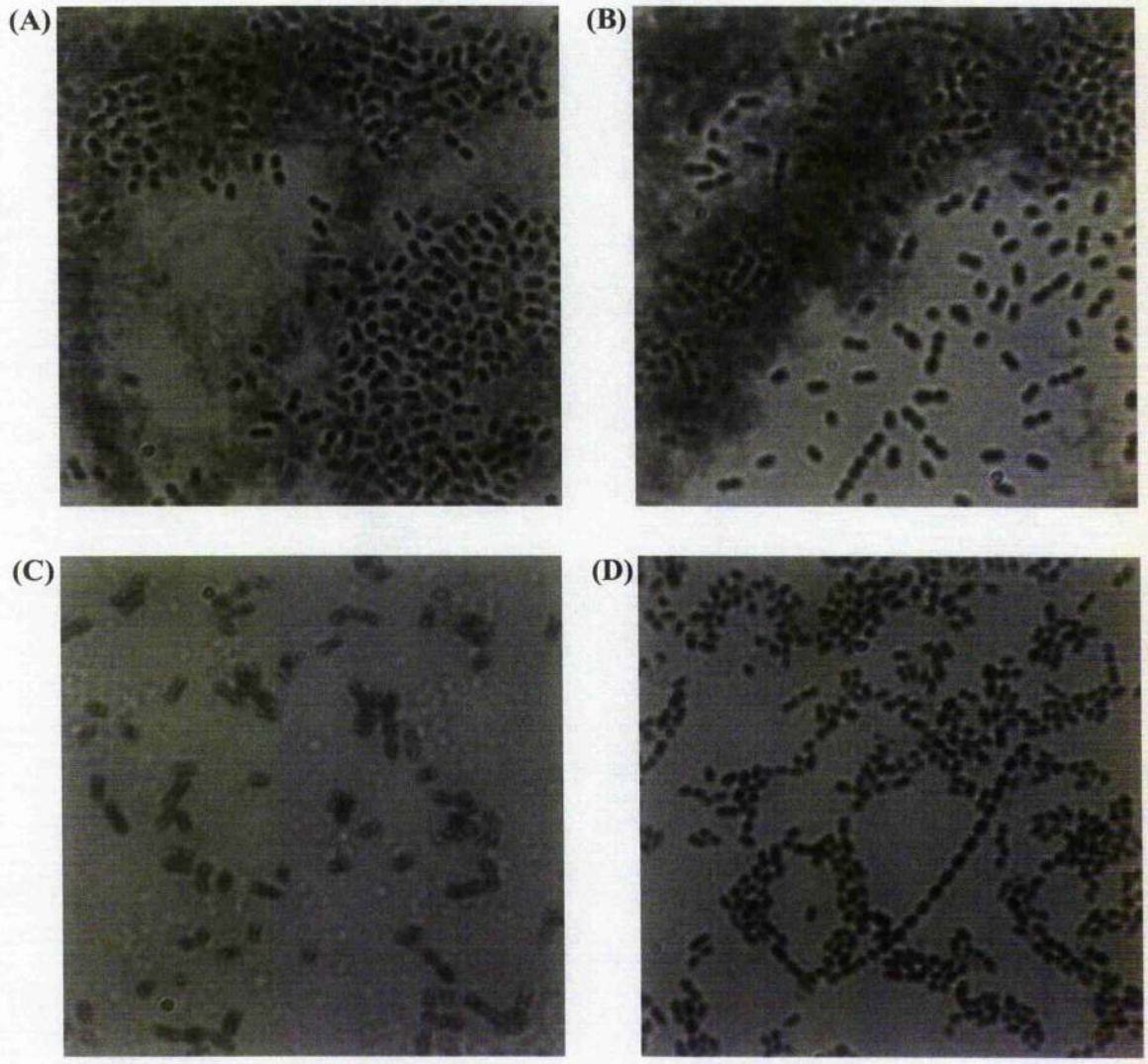


Figure 4.11- Effect of *dltA* deletion on the morphology of the *S. pneumoniae*.

TIGR4 Δ*dltA* and wild-type strains were grown to early-stationary phase on BHI or on blood plates and morphology observed in light microscopic. TIGR4 wild-type grown in BHI culture and examined through quellung reaction with antiserum type 4 (A); TIGR4 Δ*dltA* grown in BHI culture and examined through quellung reaction with antiserum type 4 (B); TIGR4 wild-type grown on blood plates (C); TIGR4 Δ*dltA* grown on blood plates (D). Magnification x1000.

4.3.4 Effect of pH on growth initiation

The effect of the *dltA* deletion on the ability to grow at different pHs were studied. *In vitro* growth of TIGR4 wild-type and their Δ*dltA* mutant was performed in BHI at 37°C in an pH range between 6.0-7.5. The TIGR4 Δ*dltA* strain was less acid tolerant than the wild-type strain. The data in Figure 4.12 showed that the mutant was unable to initiate growth with pH of 6.0. In cultures at pH6.5 the growth is highly reduced in Δ*dltA* mutant, initiating the process of growing after 8 hours of incubation and with lower growth rate. At pH below 6.0 both strains did not grown.

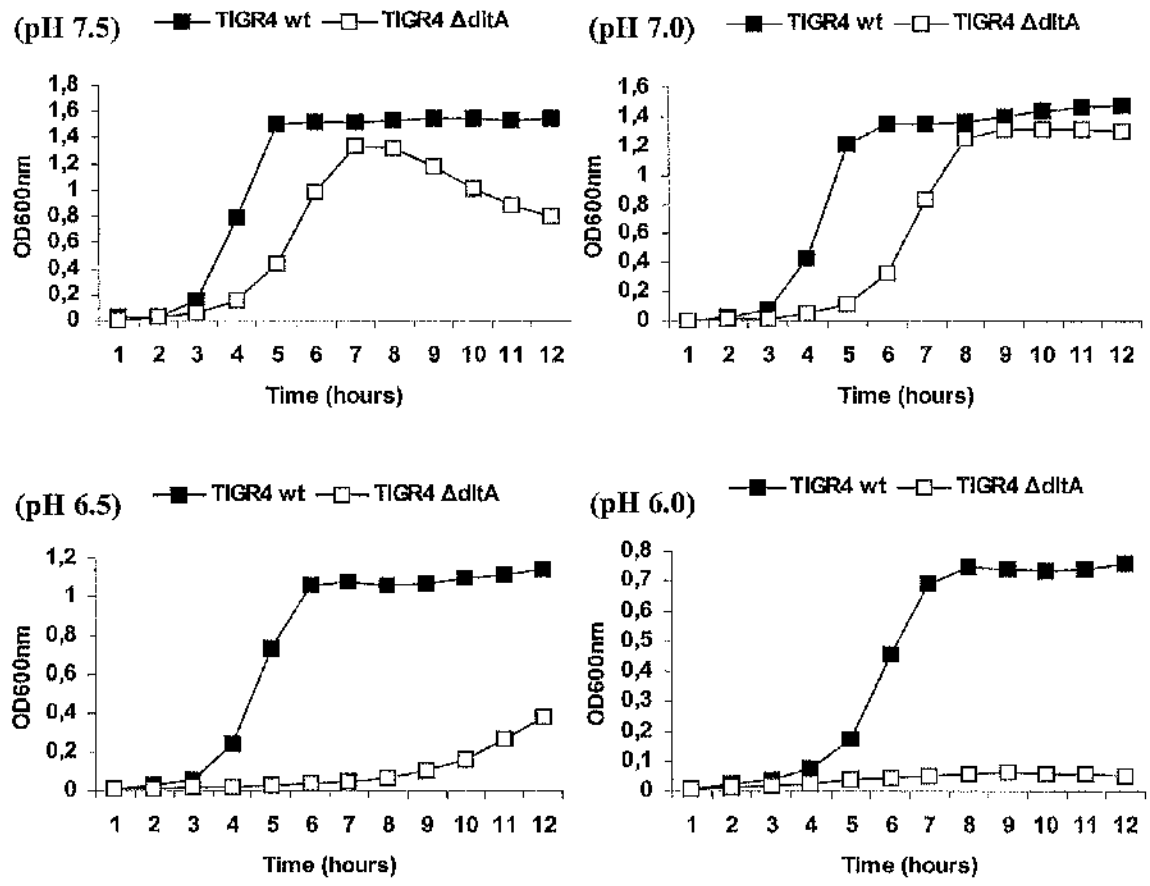


Figure 4.12- Comparison of acid sensitive *in vitro* growth of TIGR4 wild-type and Δ*dltA* strains.

10^6 CFU/ml of TIGR4 wild-type and their Δ*dltA* mutant was inoculated in 20 ml of BHI at different values of pH, at 37°C. Optical density was measured at 600nm in intervals of 1 hour.

4.3.5 Effect of antimicrobial peptides

The minimal inhibitory concentrations (MIC) of antimicrobial peptides, that cause membrane-damage, were determined for the *S. pneumoniae* wild-type and Δ*dltA* mutant. The MIC for cationic anitimicrobial peptides were determined using a broth microdilution assay modified from the method of Amsterdam (Amsterdam, 1996), and the was taken as the lowest concentration of antimicrobial peptide that reduced growth more than 50%.

The mutant was more sensitive to nisin and Magainin II, positive net charge peptides, than the wild-type. The inhibitory concentration of these antimicrobial peptides was 8 and 2-fold higher in wild-type than in Δ*dltA* strain respectively. No difference was observed using gramicidin D, peptide with a neutral net charge (Table 4.1). The increased sensitivity of *dlt* mutants seems related to cationic peptides, since no considerable change were observed in the inhibitory concentrations of the neutral peptide gramicidin D, however no changes were observed in other cationic peptides, as indolicidin, cecropin, defensin HNP1-2 and colistin, indicating that cationic properties are not sufficient for activity of a peptide against the TIGR4 Δ*dltA* mutant.

Table 4.1- Activity of antimicrobial peptides against TIGR4 wild-type and Δ*dltA* mutant.

		Minimal inhibitory concentration against (μ g/ml)	
Antimicrobial peptide	**Net charge**	**TIGR4 wt**	**Δ*dltA***
Nisin	+3	12.5	1.56
Gramicidin D	0	25	25
Indolicidin	+4	1,56	1,56
Magainin II	+4	12,5	6,25
Cecropin B	+5	> 100	> 100
Defensin HNP1-2	+2/+3	> 50	>50
Colistin	+5	> 200	> 200

4.4 *In vivo* analysis

4.4.1 Intranasal infection

DltA plays an import role in virulence of some Gram-positive bacteria. Significant attenuation in virulence of *dltA* mutants was observed in *S. agalactiae* (Poyart *et al.*, 2003), in *S. aureus* (Kristian *et al.*, 2003), and *L. monocytogenes* (Abachin *et al.*, 2002). In this study the role of DltA in virulence of *S. pneumoniae* TIGR4 strain was studied. In the pneumonia model, mice were infected with 1 x 10^6 CFU intranasally with TIGR4 wild-type and Δ*dltA* strains and the development of symptoms was monitored. No statistical significant difference was observed in survival of mice between wild-type and their isogenic mutant Δ*dltA* (Figure 4.13). However, mice infected with TIGR4 Δ*dltA* mutant did not show symptoms of disease, suggesting than the mutation in *S. pneumoniae* TIGR4 induces less inflammation during infection.

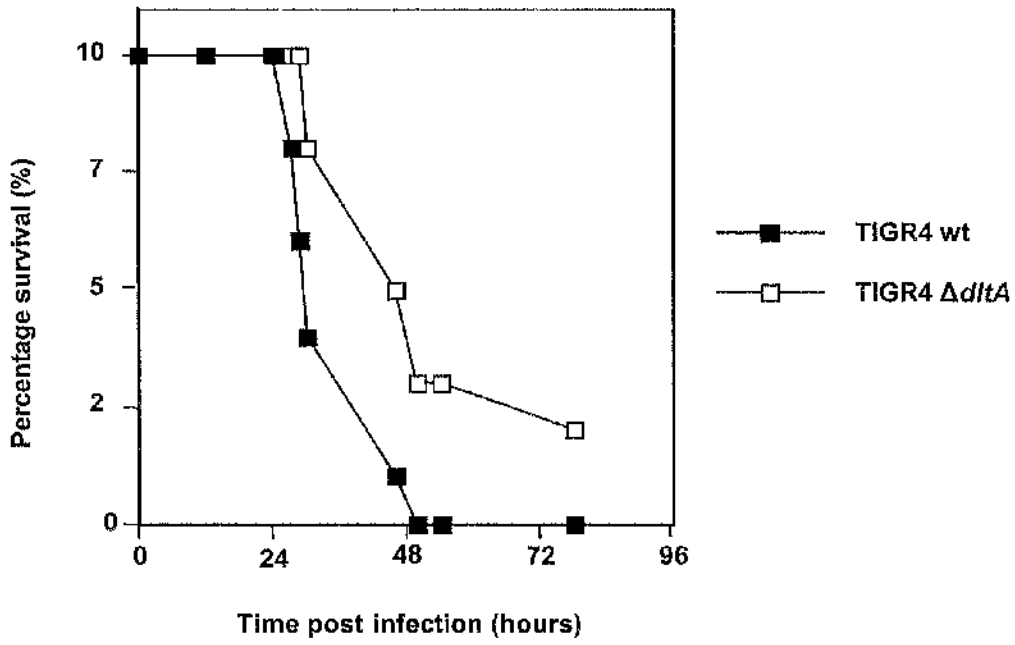


Figure 4.13- Survival of mice following intranasal infection with TIGR4 wild-type and Δ*dltA* strains.

Numbers of 5 mice were infected intranasally with 10^6 CFU/mouse. In survival data, no significant difference was observed between Δ*dltA* and TIGR4. Done in collaboration with Dr. Alison Kerr.

4.4.2 Bacteriological investigation

The bacterial loads of TIGR4 Δ*dltA* mutant compared to the wild-type in the lung airways, lung tissue and blood were studied following 24h post-intranasal infection. No difference in bacterial counts was observed in bronchio-alveolar lavage fluid in both strains. Slight differences in the bacterial counts recovered from homogenized lungs were observed. The number of bacteria recovered from the blood was also reduced in the Δ*dltA*-infected mice however in both, the reduction did not reveal statistically significant differences (Figure 4.14).

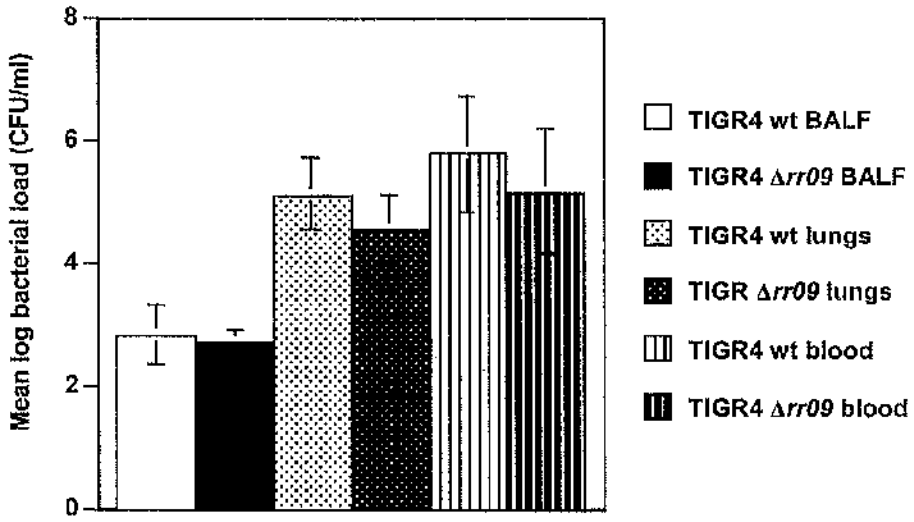


Figure 4.14- Bacterial loads of TIGR4 wild-type and Δ*dltA* strains after intranasal infection.

Bacterial loads in bronchio-alveolar lavage fluid (BALF), homogenized lungs (lungs), and blood after 24 hours upon intranasal infection with 10^6 CFU. Done in collaboration with Dr. Alison Kerr.

Chapter 4 discussion

The cell wall of the Gram-positive bacterium is characterized by macromolecular assemblies, of cross-linked peptidoglycan, polyanionic teichoic acids, and surface proteins that function within this envelope. It is essential for survival, shape, and integrity (Baddiley, 1972, 1989; Weidel and Pelzer, 1964). Teichoic acids include the wall teichoic acids (WTA), which are covalently linked to the peptidoglycan; and the lipoteichoic acids (LTAs), which are polyphosphoglycerol substituted with a D-alanyl (D-Ala) ester or a glycosyl residue and anchored in the membrane by a terminal glycolipid moiety (Fischer, 1988; Fischer *et al.*, 1990). The highly charged teichoic acids are essential for viability of Gram-positive bacteria and seem to be involved in the control of cell shape, autolytic enzymes, and magnesium ion concentration within the cell envelope (Bierbaum and Sahl, 1987; Pooley and Karamata, 1994). The D-alanylation of LTA allows Gram-positive bacteria to modulate their surface charge, regulate ligand binding and control the electromechanical properties of the cell wall. Genetic studies of the biosynthesis of LTA in various Gram-positive bacteria demonstrated that the incorporation of D-Ala residues requires the activity of four gene products (DltA, DltB, DltC and DltD), whose products are highly homologous to those encoded by the *dlt* operons of various Gram-positive bacteria characterized so far (Abachin *et al.*, 2002; Boyd *et al.*, 2000; Clemans *et al.*, 1999; Debabov *et al.*, 2000; Neuhaus *et al.*, 1996; Perego *et al.*, 1995; Peschel *et al.*, 1999; Poyart *et al.*, 2001).

dlt operon and virulence

The *dlt* operon plays an import role in virulence of some Gram-positive bacteria. Significant attenuation in virulence of *dltA* mutants was observed in *S. agalactiae* in several animal experiment models, showing that the incorporation of D-Ala residues into the LTA is important for the virulence of this bacterium (Poyart *et al.*, 2003). The importance of incorporation of D-Ala residues into the LTAs in virulence also was observed in *L. monocytogenes*, where a *dltA* mutant was eliminated more rapidly from the blood, liver and spleen than the wild-type strain (Abachin *et al.*, 2002). Furthermore, Kristian and co-workers (Kristian *et al.*, 2003) showed the importance of D-alanylation of teichoic acids for survival of *S. aureus in vivo* in a mouse tissue cage infection model.

Very little is known about the characterization and the role of *dlt* operon in *S. pneumoniae*. Although no D-alanylation of teichoic acids has been observed in *S. pneumoniae* (Fischer, 1997), it is curious that the both genome sequences of *S. pneumoniae* R6 (Hoskins *et al.*, 2001) and TIGR4 (Tettelin *et al.*, 2001) contain the *dlt* operon that, in other Gram-positive bacteria encodes enzymes involved in teichoic acid modification. Furthermore, the *dlt*

operon is expressed in certain media and activated in certain points of growth (Mascher *et al.*, 2003). Here, we showed by microarray and qRT-PCR analysis that the *dlt* operon is activated in BHI broth during mid-log phase. The regulation of the level of transcription of the *dlt* operon was also observed to be growth-phase dependent in many others Gram-positive bacteria. In *L. monocytogenes* and *B. subtilis* the *dlt* operon is activated during late-log phase of growth (Abachin *et al.*, 2002; Perego *et al.*, 1995). In *S. mutans* the maximal expression of the *dlt* genes occurs during the mid-log phase of growth when the medium contains carbohydrates internalized via the phosphoenolpyruvate phosphotransferase system (PTS) whereas it is constitutively expressed during all stages of growth in medium containing non-PTS sugars (Spatafora *et al.*, 1999) while, in *S. agalactiae,* the *dlt* operon is maximal activated during the exponential phase of growth and is regulated by a two-component regulatory system when the amount of D-Ala incorporated into the LTAs decreases (Poyart *et al.*, 2001). Furthermore, Orihuela an co-workers (Oribuela *et al.*, 2004) observed enhanced expression of the *dlt* operon during epithelial cell contact, indicating enhanced addition of and D-alanine to teichoic acids. These findings indicates that is possible that the failure to detect D-alanylation of teichoic acids by Fischer (Fischer, 1997) is related to analyzing cells grown under conditions where the *dlt* operon is not active, and presumably the *dlt* operon can play an important role in virulence of *S. pneumoniae.*

Poyart and co-workers discovered two regulatory genes, *dltR* and *dltS*, upstream of the *dlt* operon in *S. agalactiae* (Poyart *et al.*, 2001). These genes encode putative regulatory and sensor proteins of a two-component regulatory system. This TCS modulates expression of the operon and would appear to sense an environmental or external signal related to the absence of D-alanyl esters in LTA. In *S. pneumoniae* R6 strain, Mascher an co-workers had identified the *dlt* operon as a putative CiaR target region (Mascher *et al.*, 2003), on the other hand, in this present study we had shown that the *dlt* operon is down-regulated in TIGR4 strain carrying a deletion of the response regulator of the TCS06. These findings suggest that both TCS potentially regulated directly or indirectly the *dlt* operon. In the present study, we investigated the role of the DltA in the phenotype of the TIGR4 Δ*rr06* mutant.

The role of DltA on virulence of *S. pneumoniae* in TIGR4 strain, using the pneumonia model of infection was studied. Previous studies identified the *dltA* and *dltB* genes as virulence factors of the pneumococcus in an STM screen (Hava and Camilli, 2002). Here, we shown that mice infected with Δ*dltA* mutant survived longer than the mice infect with wild-type. However, the survival rates between both strains was not statistically

significant. Here, we have shown that RR06 seems important for the ability of bacteria translocate to the lungs and blood. However, *in vivo* bacteriological investigation using the TIGR4 Δ*dltA* mutant strain does not show statically significant reduction of the bacteria in the lungs and blood. These preliminaries experiments indicate that *dlt* operon is not particularly important for the virulence of *S. pneumoniae* using a small number of animals. The use of a large number of mice per experiment would possibly show a significant difference in attenuation of *dltA* mutant.

Effect of *dlt* operon on *in vitro* growth

The *dltA* genes of *S. pneumoniae*, *B. subtilis*, *S. aureus*, *L. monocytogenes*, *S. agalactiae* and *S. mutans* are similar in sequence and organization. Studies in *L. casei* have demonstrated a role of DltA as a D-alanine-D-alanyl carrier protein ligase, which activates D-alanine by hydrolysis of ATP and transfers it to the phosphopantetheine cofactor of a specific D-alanine carrier protein, which is encoded by *dltC* (Debabov *et al.*, 1996; Heaton and Neuhaus, 1992). In this study, we investigated the importance of *dltA* on the growth of *S. pneumoniae* TIGR4 strain at standard and higher temperatures, the sensitivity to antimicrobial peptides and to acid tolerance. Furthermore, the effect of a *dltA* deletion in morphology of the bacteria was observed.

The growth rate of TIGR4 Δ*dltA* mutant compared with wild-type was reduced at 37ºC and the mutant underwent autolysis immediately after reaching the stationary phase. However, in viable counting measurement no differences were observed after stationary phase between both strains. However, due to the differences in morphology namely, the emergence of long chains of bacteria of cells in the TIGR4 Δ*dltA*, it is difficult to compare directly the growth rate with the viable counting measurement. Furthermore, the mutation revealed has a great effect in growth at higher temperatures, the TIGR4 Δ*dltA* did not grow at 40ºC, a similar effect in growth was found in TIGR4 Δ*rr06* mutant.

The Δ*dltA* mutant, derived from the wild-type strain TIGR4 displayed an increased susceptibility to antimicrobial peptides nisin and magainin II (positive net charge peptides), while no difference was observed using gramicidin D, a neutral net charge, indicating an increased sensitivity of TIGR4 Δ*dltA* to cationic peptides. Susceptibility of other Gram-positive *dlt* mutans to antimicrobial peptides is clear related with positive net charge (Peschel *et al.*, 1999; Poyart *et al.*, 2003). However, in the present study, no changes in susceptibility of Δ*dltA* mutant was observed in positive peptides indolicidin, cecropin B, colistin and the human defensins HNP1-2, not allowing confirmation that the increased

sensitivity is directly related to altered electrostatic interaction of the peptides with the mutant cells.

The study of the acid-sensitive phenotype of TIGR4 Δ*dltA* was based on the importance of D-alanylation of LTAs in the growth and physiology of Gram-positive bacteria (Archibald *et al.*, 1973; Boyd *et al.*, 2000; Fischer *et al.*, 1981; Koch *et al.*, 1982; Perego *et al.*, 1995; Wecke *et al.*, 1996). The link between the theoretical D-alanylation of teichoic acids in *S. pneumoniae* and acid sensitivity was done by inactivation of *dltA* of the *dlt* operon in strain TIGR4. The mutant was shown to be acid-sensitive displaying a defective acid tolerance response, possibly due to an increased permeability protons compared to wild-type. The TIGR4 Δ*dltA* was unable to initiate growth below pH 6.5 and presented a dramatic reduction in growth at pH 7.0-6.5 compared with wild-type. Unexpectedly, transcriptional analysis of the short and long-term acid tolerance response (pH 6.0) did not show any significant changes in all four genes of *dlt* operon (Martin-Galiano *et al.*, 2005). These findings again, can be related by the fact *dlt* operon is expressed only in certain media cultures and activated in certain points of growth.

Inactivation of genes in the *dlt* operon in various Gram-positive bacteria shows an array of phenotypic changes in morphology of bacteria. Inactivation of the *dltA* gene of *S. gordonii* (Clemans *et al.*, 1999) and *dltC* gene in *S. mutans* (Boyd *et al.*, 2000) resulted in a mutant with multiple septation sites and exhibited a smooth and unstructured surface with a thickened, cap-like cell wall, while, in *S. agalactiae dltA* mutants formed visible clumps and a significant number of the cells possessed an aberrant morphology (Poyart *et al.*, 2001; Poyart *et al.*, 2003). In this study, no differences in morphology was observed in TIGR4 Δ*dltA* mutants compared with wild-type, however, by optical microscopy long chains of bacterial cells were observed in the Δ*dltA* mutant that were not apparent in the wild-type.

Summary

The *dlt* operon of Gram-positive bacteria comprises four genes (*dltA*, *dltB*, *dltC*, and *dltD*) that catalyze the incorporation of D-alanine residues into the LTAs. In this work, we demonstrated that DltA is important for growth of *S. pneumoniae* and is essential when the bacterium is subjected to higher temperatures. The *DltA* mutant was shown to be defective in mounting an effective acid tolerance response and sensitive to antimicrobial peptides nisin and magainin II. The mutation seems to have influence in morphology of *S. pneumoniae*, resulting in the occurrence of long chains of bacterial cells in Δ*dltA*. The

DltA seems not to be responsible for the TIGR4 Δ*rr06* mutant *in vivo* phenotype, however like the Δ*rr06* mutant the TIGR4 Δ*dltA* mutant had a reduction in the growth rate at 37°C compared with wild-type, and the mutation has a large effect when the bacterium was cultured at 40°C.

Chapter 5

Two-component signal transduction system 09

5.1 Contribution of RR09 in pneumococcal virulence

The pneumococcal genome contains 13 putative complete TCS and one orphan response regulator (Lange *et al.*, 1999). Eight of the pneumococcal TCS have been shown to be important for virulence (Paterson *et al.*, 2006; Throup *et al.*, 2000). Recent studies in a murine model of infection have demonstrated that TCS09 of *S. pneumoniae* is virulence-associated. However the contribution of this TCS to the virulence in the pneumococcus is strain dependent. While, in a D39 background an *rr06* mutant is avirulent via intraperitoneal, intranasal, and intravenous routes of infection, the mutation of the response regulator in a 0100993 background results in a strain that is fully virulent intraperitoneally and intravenously (Blue and Mitchell, 2003). The genomic organisation of pneumococcal TCS HK/RR09 also referred as 488 HK/RR (Throup *et al.*, 2000) is shown in Figure 5.1. The *sp0662* gene (TIGR4 annotation) encodes a 63 kDa membrane-associated sensory protein called a histidine kinase (HK09), and the *sp0661* (TIGR4 annotation) encodes a 28 kDa cognate cytosolic DNA-binding response regulator protein, which acts as a transcriptional regulator (RR09). Amino acid sequence homology suggests that the sensory domain is related to the extra-cellular part of McpA and McpB of *B. subtilis* (Lange *et al.*, 1999). These proteins are believed to be involved in the control of chemotaxis through sensing of environmental nutrient concentrations The extra-cellular stimulus of TCS09, however, is as yet unknown.

The present study evaluated the role of RR09 in the virulence of *S. pneumoniae* TIGR4 strain using a murine model of infection. This study investigated the transcriptional changes in pneumococcal mutants lacking the response regulator of TCS09 (Δ*rr09*) by microarray and qRT-PCR analysis of three strains, D39, TIGR4 and 0100993, that may contribute to the *in vivo* phenotype. Furthermore, the gene *sp0063*, encoding a putative IIC component of an PTS system, was investigated in the pneumococcus D39 strain to evaluate the role in phenotype of Δ*rr09* mutant. This transmembrane protein together with the functional domains IIA, IIB catalyzes simultaneous the translocation and phosphorylation of a particular carbohydrate (Kotrba *et al.*, 2001).

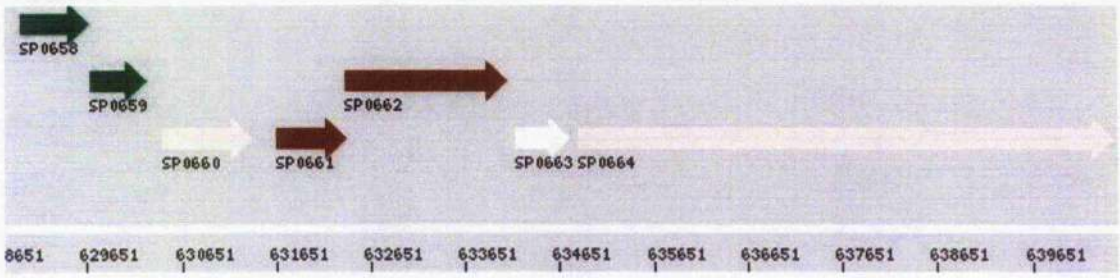


Figure 5.1- Genomic organization of TCS *hk/rr09*.

The TCS *hk/rr09* genes is composed by a response regulator (*sp2193*) and a sensor histidine kinase (*sp2192*). The genes names use the TIGR4 gene annotation (http://www.tigr.org).

5.1.1 Constructions of pneumococcal mutants

The *rr09* gene of pneumococcal strains TIGR4 and D39 were disrupted by introduction of an erythromycin resistance cassette into the gene. The fragment containing the mutation was amplified by PCR from chromosomal DNA of the strain 0100993 (Throup *et al.*, 2000) using the primers TCS09-For and TC09S-Rev (Table 2.2). Following purification the fragments were used to transform the *S. pneumoniae* strains by homologous recombination (Figure 3.2) and the final mutants were selected by grown on erythromycin.

5.1.2 Confirmation of mutation

The allelic replacement of *rr09* with a constitutive erythromycin resistance cassette was originally carried out in strain 0100993 by Throup *et al.* (Throup *et al.*, 2000). The erythromycin resistance cassette, ~1 kb, was inserted in the target gene (*rr09*) ~0.73 kb. This was amplified by PCR and used to transform the pneumococcal strains. The successful replacement of the wild-type *rr09* gene with the erythromycin resistance cassette in *S. pneumoniae* TIGR4 and D39 was confirmed by PCR. Reactions with erythromycin cassette specific primers (ErmAM-For and ErmAM-Rev) and primers specific to genomic regions lying outside the insertion region (TCS09-For and TCS09-Rev) (Table 2.2) were carried out to confirm correct insertion of the erythromycin resistance cassette into the *rr09* gene of both mutants (Figure 5.2). As expected, no PCR amplicons were obtained for any of the wild-type strains, lanes 2, 4, 8, and 10) while an amplicon of approximately 1.5 kb was obtained from TIGR4 and D39 Δ*rr09* mutants using primers TCS09-For and ErmAM-Rev, lanes 3 and 5 and approximately 1.5 kb using primers ErmAM-For and TCS06-Rev lanes 9 and 11). These results confirmed successful allelic replacement of the *rr09* gene by the erythromycin resistance cassette in the two mutants. The mutation also was confirmed by sequencing.

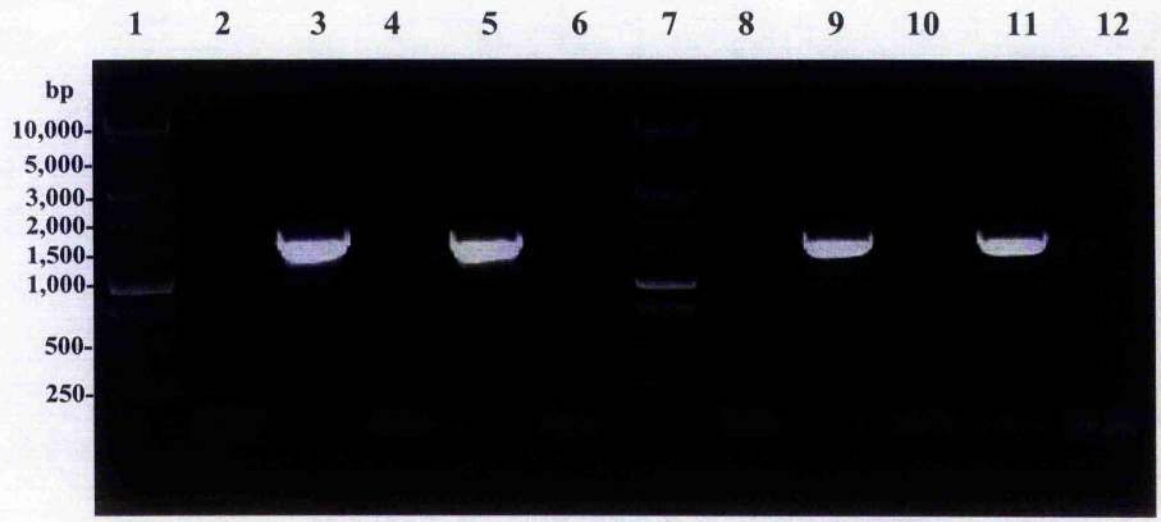


Figure 5.2- Allelic replacement confirmation of *rr09* with a constitutive erythromycin resistance cassette.

Correct replacement of *rr09* by erythromycin resistance cassette, in TIGR4 and D39 was confirmed using primers TCS06-For and ErmAM-Rev (lanes 2-6) and primers ErmAM-For and TCS06-Rev (lanes 8-12). Genomic DNA from the following strains were used as templates: TIGR4 wild-type (lanes 2 and 8), TIGR4 Δ*rr09* mutant (lanes 3 and 9), D39 wild-type (lanes 4 and 10), D39 *rr09* mutant (lanes 5 and 11). Negative controls are shown in lanes 6 and 12 The DNA ladder marker used was the 1 kb plus ladder (Promega).

5.2 *In vivo* analysis

5.2.1 Intranasal infection

Previous studies indicated a strain-specific role for TCS09 in pneumococcal virulence (Blue and Mitchell, 2003). To extend these studies, *rr09* was deleted in *S. pneumoniae* strain TIGR4 by allelic replacement, and the resulting TIGR Δ*rr09* mutant was compared to its wild-type in a murine pneumonia model. Mice were infected with 1 x 10^6 CFU intranasally with TIGR4 wild-type and Δ*rr09* strains and the development of symptoms was monitored. Mice infected with TIGR4 Δ*rr09* were found to have significantly longer survival times than mice infected with the parental strain (Figure 5.3). The mice infected with wild-type did not survive more than 30 hours after inoculation while, 80% of the mice infect with Δ*rr09* succumbed after 48 hours and the remained after 96 hours.

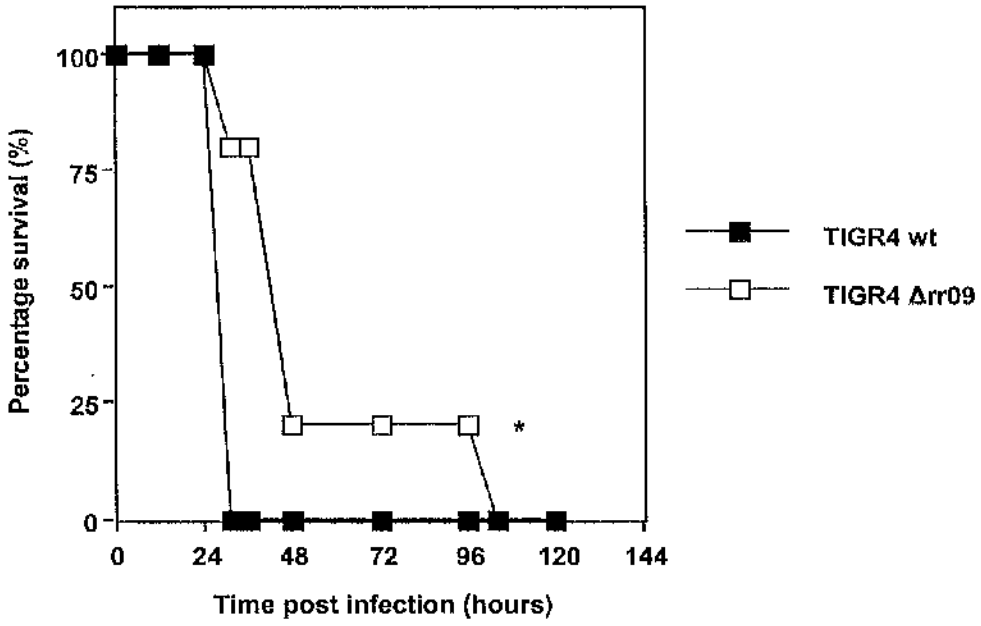


Figure 5.3- Survival of mice following intranasal infection with TIGR4 wild-type and Δ*rr09* strains.

Numbers of 5 mice were infected intranasally with 10^6 CFU/mouse. * $P < 0.01$ for longer survival of TIGR4 Δ*rr09* compared to TIGR4 wild-type. Done in collaboration with Dr. Alison Kerr.

5.2.2 Bacteriological investigation

The bacteria loads of TIGR4 Δ*rr09* mutant were compared to the wild-type in the lung airways, lung tissue and blood 24h post-intranasal infection. A significant reduction in bacterial load was observed in bronchio-alveolar lavage fluid compared to the wild-type strain ($p < 0.01$), while a similar reduction was observed for bacteria recovered from homogenized lungs (Figure 5.4). The number of bacteria recovered from the blood was also significantly reduced in the Δ*rr09*-infected mice ($p < 0.01$; Figure 5.4).

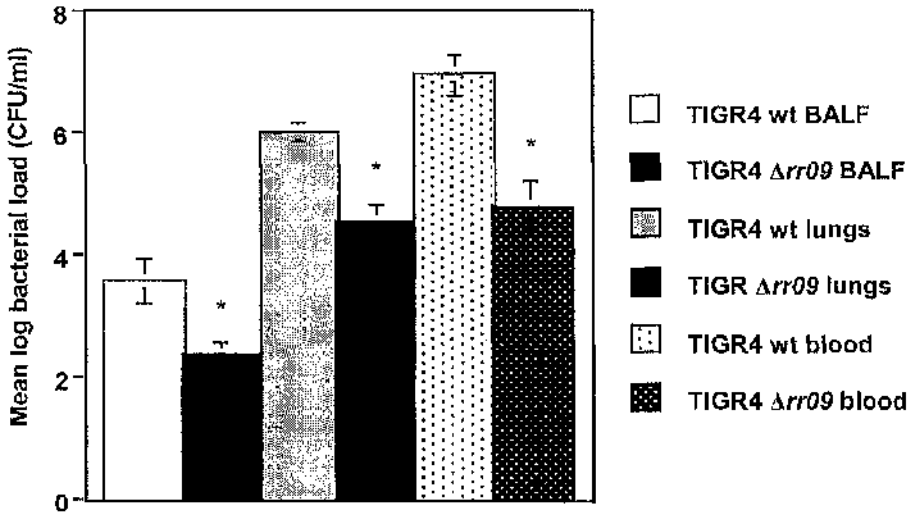


Figure 5.4- Bacterial loads of TIGR4 wild-type and Δ*rr09* strains after intranasal infection.

Bacterial loads in bronchio-alveolar lavage fluid (BALF), homogenized lungs (lungs), and blood after 24 hours upon intranasal infection with 10^6 CFU. Done in collaboration with Dr. Alison Kerr.

5.3 Microarray analysis

To identify genes controlled by RR09, microarray analysis was performed to compare the transcriptome of *S. pneumoniae* wild-type strains TIGR4 and D39 and their isogenic mutant. Furthermore, the microarray analyses allowed identification of putative genes regulated by RR09 and possibly involved in the strain dependent virulence.

The second version of the pneumococcal genome microarray slides designed by TIGR (http://www.tigr.org) was used in this analysis. The full genome array consists of amplicons representing 2131 ORFs from *S. pneumoniae* TIGR4 strain in addition to 563 ORFs from R6 (164) and G54 (399) strains. For each strain, two independent RNA preparations and a total of four array slide replicates with dyes swapped were used.

5.3.1 RNA analysis

The RNA samples were prepared from TIGR4 and D39 wild-type strains and their isogenic Δ*rr09* mutants, grown in 10 ml of BHI to mid-log phase (OD_{600nm} of 0.6) at 37°C. Total RNA was isolated using Qiagen RNeasy-Midi Kit and the concentration calculated using the NanoDrop® ND-1000 spectrophotometer. Concentrations of total RNA between 1.0-2.0 μg/μl were obtained for the TIGR4 Δ*rr09* and its parent strains. The integrity and purification of total RNA sample were tested using the Agilent Bioanalizer. Figure 5.5 and Figure 5.6 illustrate the quality of total RNA from the samples used in microarrays and qRT-PCR analysis.

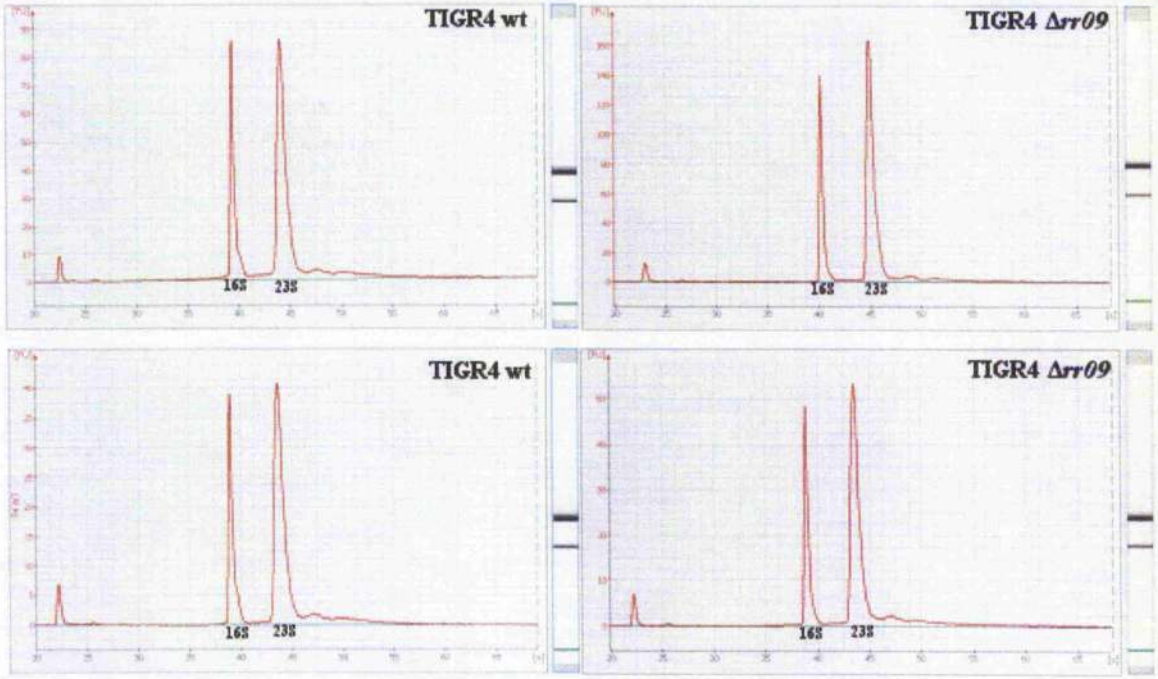


Figure 5.5- Analysis of total RNA samples from TIGR4 wild-type and Δ*rr09* strains used in TIGR microarrays.

Integrity and purity of two independent total RNA samples prepared from 10 ml of BHI cultures grown to mid-log phase (OD_{600nm} of 0.6) at 37°C using Qiagen RNeasy-Midi Kit and checked with Agilent 2100 Bioanalyser.

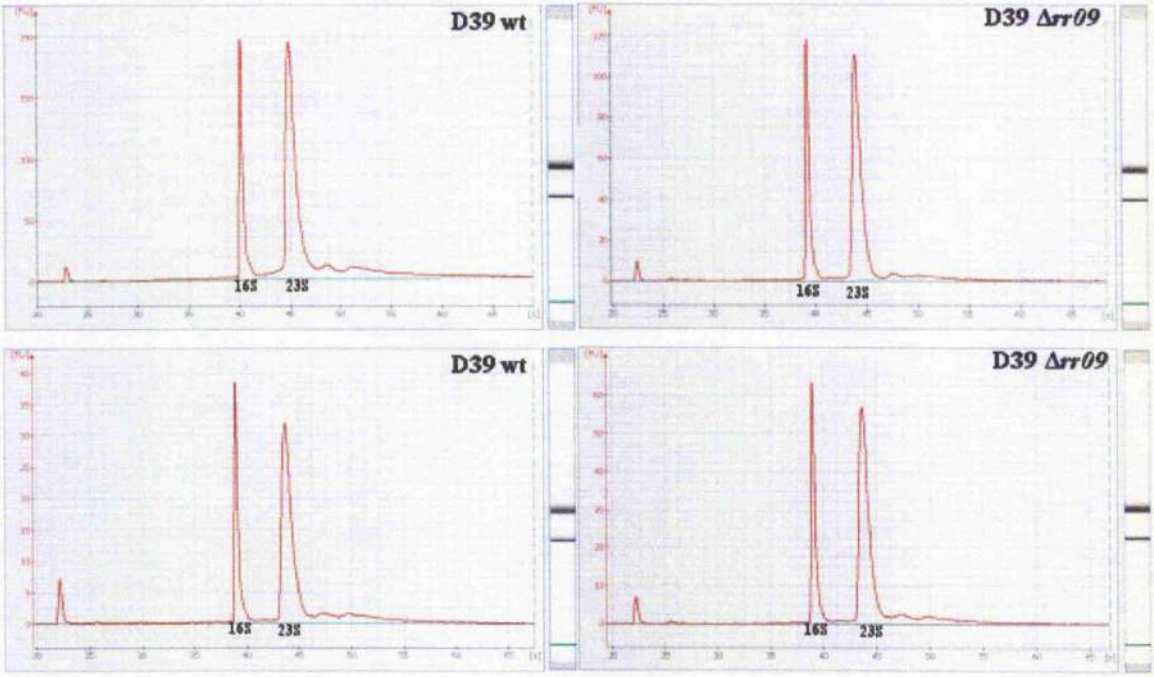


Figure 5.6- Analysis of total RNA samples from D39 wild-type and Δ*rr09* strains used in TIGR microarrays.

Integrity and purity of two independent total RNA samples prepared from 10 ml of BHI cultures grown to mid-log phase (OD_{600nm} of 0.6) at 37°C using Qiagen RNeasy-Midi Kit and checked with Agilent 2100 Bioanalyser.

5.3.2 Comparison of TIGR4 and D39

Microarray comparisons of the transcription profiles of the TIGR4 and D39 Δ*rr09* and their respective wild-type was used to identify the genes controlled by RR09. The relationships of the mean ratio of hybridizations intensities between the wild-types (control), and the Δ*rr09* mutant strains (test) are shown in scatter plots (Figure 5.7).

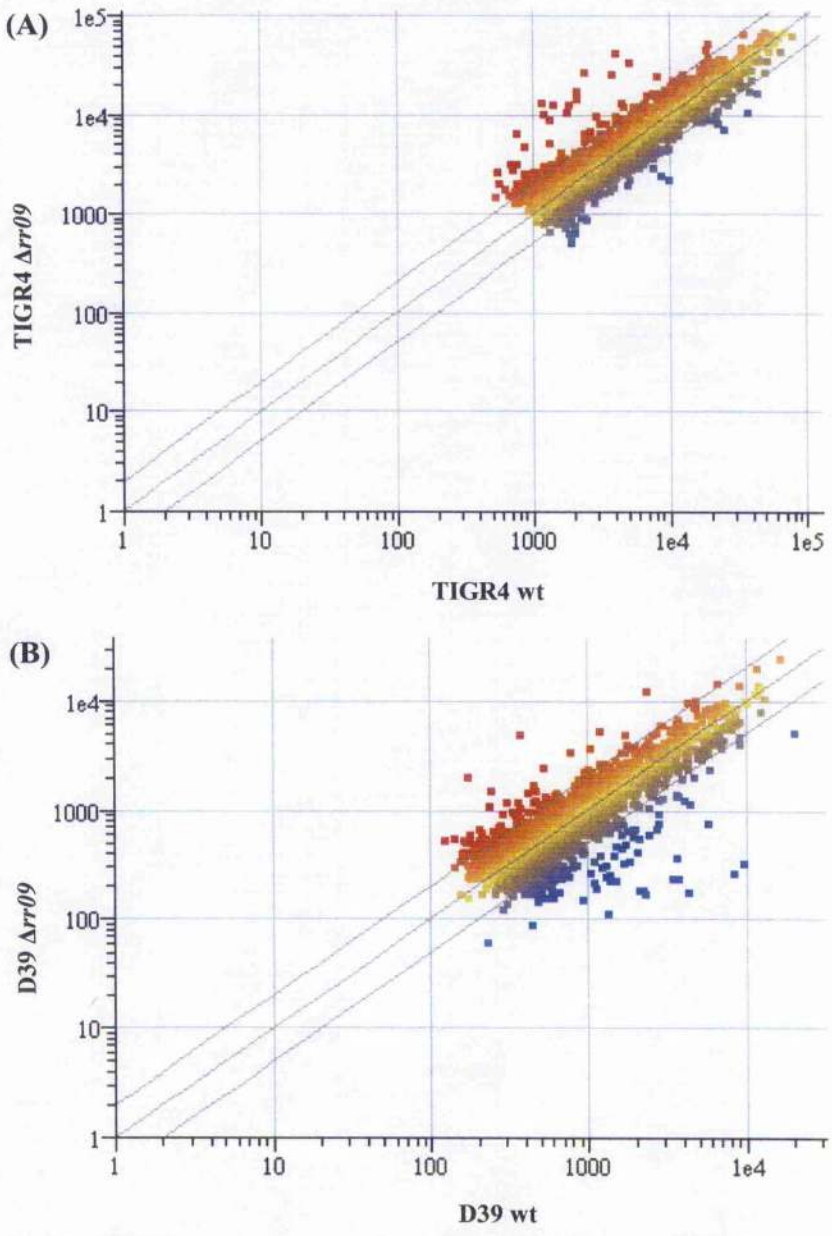


Figure 5.7- Scatter plot comparing the gene expression between Δ*rr09* and wild-type strains.

cDNA labelled with Cy3 and Cy5 were hybridized in TIGR arrays slides and analysed in Genespring™. The scatter plot shows the gene expression intensities between the wild-type (control strain) and Δ*rr09* (test strain). Hybridization intensities in TIGR4 strains (A), and D39 strains (B). Blue spots (bellow the diagonal lines) indicate the genes are down-regulated and the red spots (above the diagonal lines) indicates the genes up-regulated in Δ*rr09* compared to wild-type. The yellow spots (inside the diagonal lines) describe the genes without significant variation between theTIGR4 Δ*rr09* and the wild-type.

Whereas most of the pneumococcal genes remained unaltered, showing comparable expression levels between the wild-type and the Δ*rr09* strains, approximately 3.7% of the genes were shown to have significant differences in expression levels between the wild-types and Δ*rr09* mutants. Twenty-three genes were found to be up-regulated in TIGR4 Δ*rr09* and fifteen genes down regulated, while in D39 Δ*rr09* ten genes were found to be up-regulated and thirty-six genes down regulated. The up and down-regulated genes were selected if they demonstrated a two-fold difference in signal intensity between wild-types and Δ*rr09* mutants and if this intensity was found to be statistically significant ($P \leq 0.05$).

Little overlap was observed in the set of genes controlled by RR09 in D39 and TIGR, with a few notable exceptions. Two genes, encoding a putative lactose phosphotransferase system repressor (*sp0875*) and a 1-phosphofructokinase (*sp0876*) showed an increased transcription level in both D39 and TIGR4 Δ*rr09*. Furthermore, the gene encoding the virulence factor PspA (*sp0117*, TIGR annotation; *spr0121* R6 annotation) was found to be down-regulated in both Δ*rr09* mutants.

5.3.2.1 Strain-specific regulation by RR09: TIGR4

The altered genes from the DNA microarray experiments, regulated by RR09 in TIGR4 strain, are listed in Table 5.1 (up-regulated genes) and Table 5.2 (down-regulated genes). 38 genes showed a significant difference in expression, twenty-three genes were up-regulated in TIGR4 Δ*rr09*, such as the genes located on the *rlrA* pathogenicity islet (Figure 5.8). This 12-kb islet, which is not present on the D39 genome (Tettelin *et al.*, 2001), codes for the transcriptional activator RlrA, three surface proteins, and three putative sortases and is required for colonization and lung infection, but dispensable for systemic infection (Hava *et al.*, 2003a). The *rlrA* pathogenicity islet was recently reported to encode a pilus-like structure on the cell surface of the pneumococcus, influencing the virulence and host inflammatory responses (Barocchi *et al.*, 2006). Increased expression of a cluster of genes predicted to be involved in purine metabolism (*sp0044-sp0056*) was also observed (Figure 5.9).

Table 5.1- Up-regulated genes in TIGR4 Δ*rr09* compared with its parental strain.

Genes[1]	Gene symbol	Mean intensity ratio[2]	*p*-value[3]	Annotation[4]
sp0044	*purC*	4.0	2.31E-04	Phosphoribosylaminoimidazole-succinocarboxamide synthase
sp0045		4.4	1.01E-04	Phosphoribosylformylglycinamidine synthase, putative
sp0046	*purF*	10.7	1.30E-04	Amidophosphoribosyltransferase
sp0047	*purM*	4.4	2.21E-04	Phosphoribosylformylglycinamide cyclo-ligase
sp0048	*purN*	5.6	1.44E-04	Phosphoribosylglycinamide formyltransferase
sp0050	*purH*	6.0	5.00E-05	Phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase
sp0051	*purD*	7.4	1.07E-05	Phosphoribosylamine--glycine ligase
sp0053	*purE*	5.9	2.03E-05	Phosphoribosylaminoimidazole carboxylase, catalytic subunit
sp0054	*purK*	9.6	2.81E-05	Phosphoribosylaminoimidazole carboxylase, atpase subunit
sp0056	*purB*	2.9	3.59E-05	Adenylosuccinate lyase
sp0287		6.1	7.24E-05	Xanthine/uracil permease family protein
sp0461	*rhrA*	2.5	3.81E-02	Transcriptional regulator, putative
sp0462	*rrgA*	4.9	3.16E-04	Cell wall surface anchor family protein
sp0463	*rrgB*	4.0	2.29E-04	Cell wall surface anchor family protein
sp0464	*rrgC*	5.0	3.70E-04	Cell wall surface anchor family protein
sp0466	*srtB*	2.8	4.60E-04	Sortase, putative
sp0845		2.1	1.95E-04	Lipoprotein
sp0875	*lacL*	2.6	8.12E-05	Lactose phosphotransferase system repressor
sp0876		2.1	2.61E-04	1-phosphofructokinase, putative
sp1229	*fhs*	2.0	6.23E-04	Formate--tetrahydrofolate ligase
sp1249		2.5	5.77E-04	Conserved hypothetical protein
sp1587		5.4	3.33E-05	Oxalate:formate antiporter

[1] Gene designation of microarray ORFs in agreement with TIGR (http://www.tigr.org).
[2] Ratios Intensities of TIGR4 wild-type/Δ*rr09* determined in microarray experiments. The value represents the mean intensities for the four replicate experiments. Genes up-regulated with ≥ two-fold expression changes between the wild-type and mutant were selected.
[3] *P*-value represents the mean *P*-value calculated from individual *t*-tests of intensity changes between the wild-type and mutant. Genes with *P*-value ≤0.05 were selected.
[4] Annotations as published in TIGR4 genome (http://www.tigr.org).

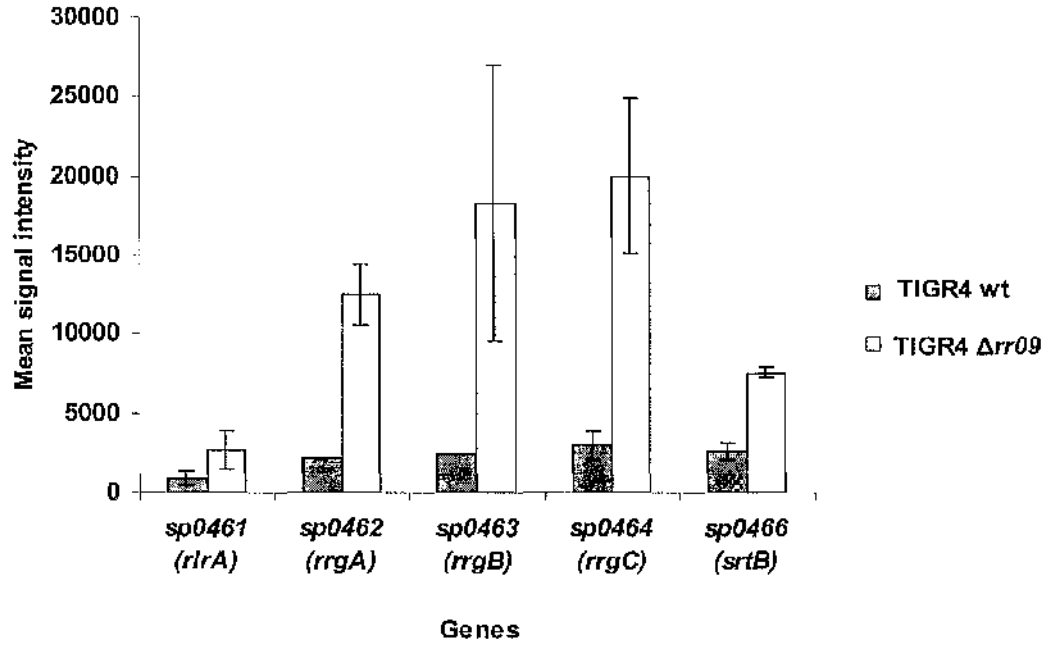


Figure 5.8- Genes located on the *rlrA* pathogenicity islet up-regulated in TIGR4 Δ*rr09*.

Expression of *rlrA* pathogenicity islet associate genes up-regulated in TIGR4 Δ*rr09* compared with wild-type strain. Values are determined by microarray hybridization of two independent RNA samples and are given as the geometric mean fluorescence intensity values from four replicate hybridizations. Error bars represent ± SEM.

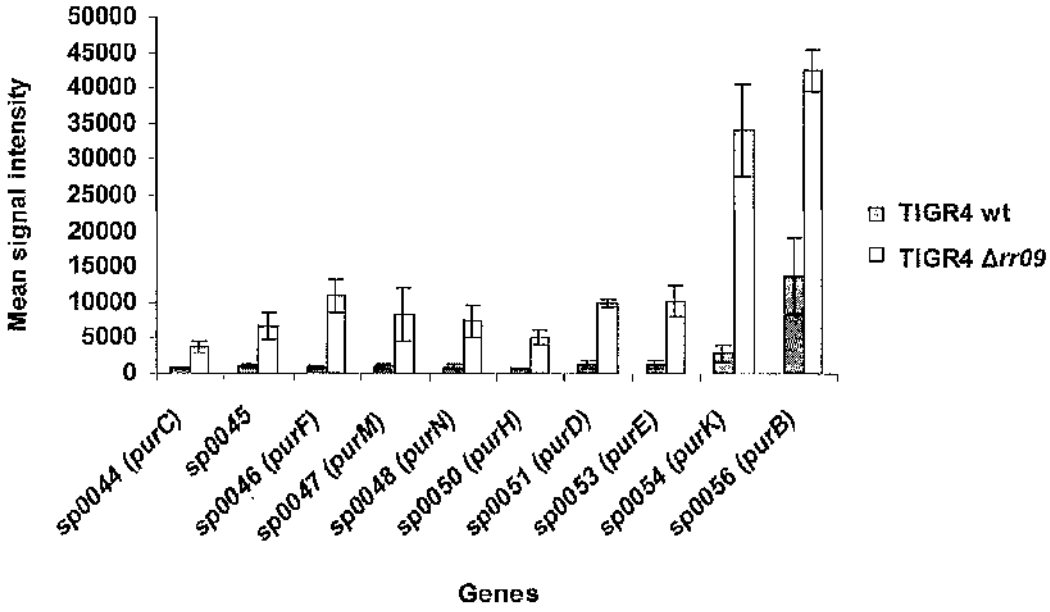


Figure 5.9- Genes up-regulated in TIGR4 Δ*rr09* associated with purine metabolism.

Expression of purines metabolism associate proteins up-regulated in TIGR4 Δ*rr09* compared with wild-type strain. Values are determined by microarray hybridization of two independent RNA samples and are given as the geometric mean fluorescence intensity values from four replicate hybridizations. Error bars represent ± SEM.

As expected the *rr09* gene was down-regulated, the signal fluorescence in Δ*rr09* remained similar to the background signal. Of interest was the *sp0117* down-regulated in Δ*rr09* mutant, than encodes the virulence factor PspA. This pneumococcal virulence factor interferes with the complement system and functions as a specific receptor for lactoferrin (Hammerschmidt *et al.*, 1999; Tu *et al.*, 1999). A recent study showed that *pspA* is also regulated by the VicRK two-component system (TCS02) (Ng *et al.*, 2005). Other interesting genes were associate with a sugar ABC transporter (*sp1895-sp1997*) (Figure 5.10) as well as various stress-response genes (*hrcA*, *grpE*, *dnaK*, and *dnaJ*) (Figure 5.11).

Table 5.2- Down-regulated genes in TIGR4 Δ*rr09* compared with its parental strain.

Genes[1]	Gene symbol	Mean intensity ratio[2]	*p*-value[3]	Annotation[4]
sp0117	*pspA*	0.40	5.26E-04	Pneumococcal surface protein A
sp0338		0.37	1.31E-02	ATP-dependent CLP protease, ATP-binding subunit, putative
sp0424	*fabZ*	0.46	1.50E-03	Hydroxymyristoyl- dehydratase
sp0515	*hrcA*	0.41	4.41E-04	Heat-inducible transcription repressor HRCA
sp0516	*grpE*	0.32	8.72E-04	Heat shock protein GRPE
sp0517	*dnaK*	0.40	1.21E-03	DnaK protein
sp0519	*dnaJ*	0.49	5.98E-04	DnaJ protein
sp0661	*rr09*	0.5	5.41E-03	DNA-binding response regulator
sp0647		0.46	4.36E-03	PTS system, IIC component, putative
sp0648	*bgaA*	0.29	4.48E-04	Beta-galactosidase
sp0724		0.47	1.12E-04	Hydroxyethylthiazole kinase, putative
sp1804		0.49	1.55E-02	General stress protein
sp1883		0.37	1.46E-02	Dextran glucosidase DEXS, putative
sp1884		0.27	2.07E-03	PTS system, IIABC components
sp1895	*msmG*	0.41	2.30E-03	Sugar ABC transporter, permease protein
sp1896	*msmF*	0.45	1.48E-03	Sugar ABC transporter, permease
sp1897	*msmE*	0.37	1.35E-03	Sugar ABC transporter, sugar-binding protein

[1] Gene designation of microarray ORFs in agreement with TIGR (http://www.tigr.org).
[2] Ratios intensities of TIGR4 wild-type/Δ*rr09* determined in microarray experiments. The value represents the mean intensities for the four replicate experiments. Genes up-regulated with ≥ two-fold expression changes between the wild-type and mutant were selected.
[3] *P*-value represents the mean *P*-value calculated from individual *t*-tests of intensity changes between the wild-type and mutant. Genes with *P*-value ≤0.05 were selected.
[4] Annotations as published in TIGR4 genome (http://www.tigr.org).

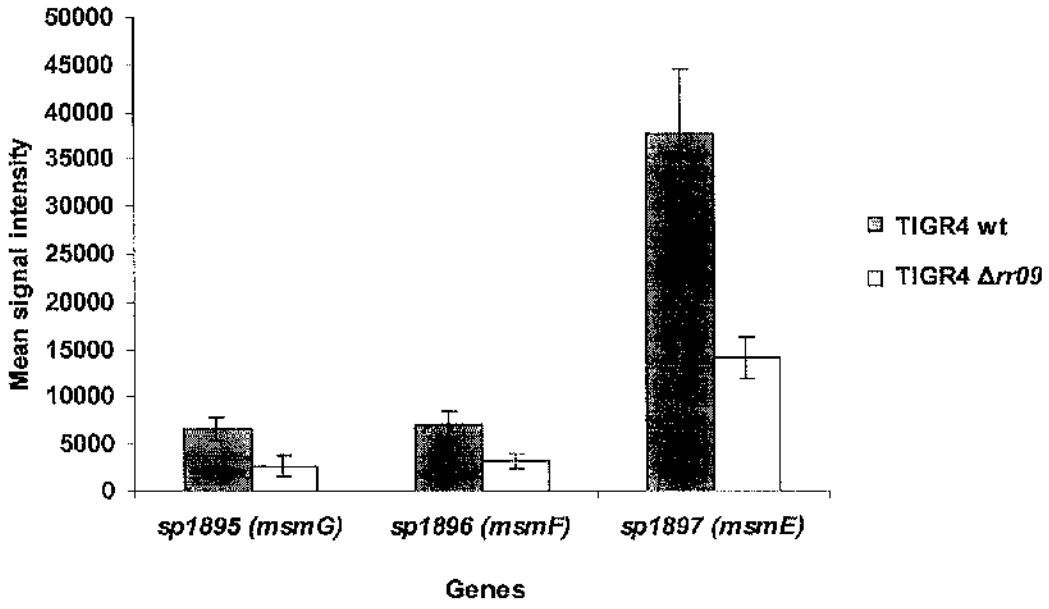


Figure 5.10- Genes down-regulated in TIGR4 Δ*rr09* associated with sugar ABC transporter.

Expression of sugar ABC transporter proteins down-regulated in TIGR4 Δ*rr09* compared with wild-type strain. Values are determined by microarray hybridization of two independent RNA samples and are given as the geometric mean fluorescence intensity values from four replicate hybridizations. Error bars represent ± SEM.

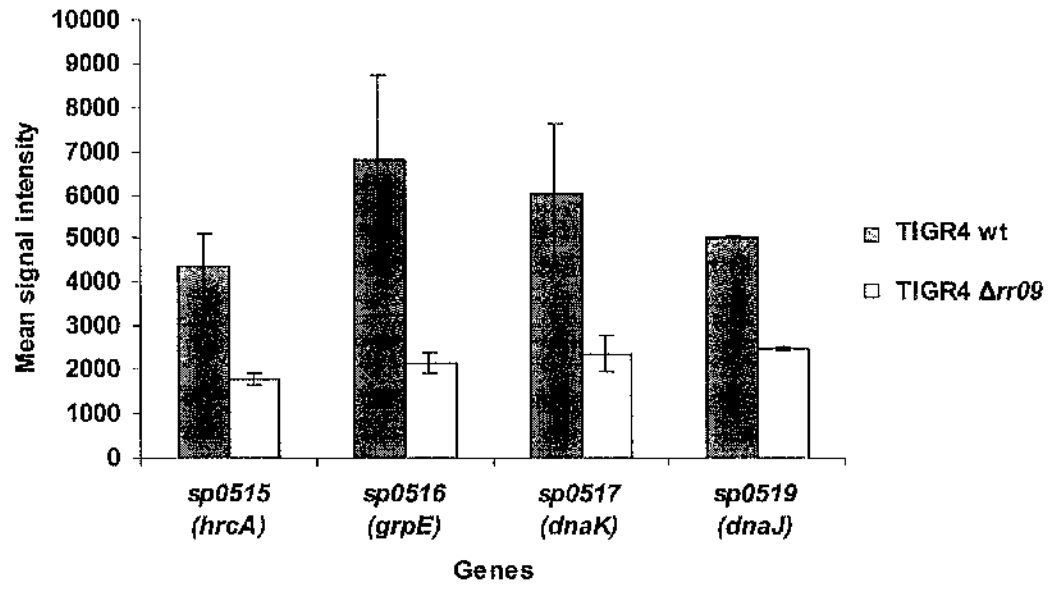


Figure 5.11- Genes down-regulated in TIGR4 Δ*rr09* associated with stress-response.

Expression of stress-response associated proteins down-regulated in TIGR4 Δ*rr09* compared with wild-type strain. Values are determined by microarray hybridization of two independent RNA samples and are given as the geometric mean fluorescence intensity values from four replicate hybridizations. Error bars represent ± SEM.

5.3.2.2 Strain-specific regulation by RR09: D39

The altered genes from the DNA microarrays experiments, regulated by RR09 in D39 strain, are listed in Table 5.3 (up-regulated genes) and Table 5.4 (down-regulated genes). A total of 48 genes appeared to be differentially expressed between the D39 wild-type and Δ*rr09* mutant strains. Of these, 10 genes were up-regulated in the D39 Δ*rr09*, included *glnAR*, encoding glutamate synthetase and its repressor, genes encoding a putative lactose phosphotransferase system repressor (*sp0875*), a 1-phosphofructokinase (*sp0876*) and a fructose-specific phosphotransferase system (PTS IIABC) (*sp0877*). Furthermore, the expression of a gene cluster containing the pneumolysin gene (*sp1923*) as well as several hypothetical ORFs (*sp1922-sp1926*) was found to be up-regulated in D39 Δ*rr09* (Figure 5.12).

Table 5.3- Up-regulated genes in D39 Δ*rr09* compared with its parental strain.

Genes[1]	Gene symbol	Mean intensity ratio[2]	*p*-value[3]	Annotation[4]
sp0501	*glnR*	2.8	6.21E-03	Transcriptional regulator
sp0502	*glnA*	3.0	6.64E-04	Glutamine synthetase, type I
sp0875	*lacR*	15.1	6.71E-05	Lactose phosphotransferase system repressor
sp0876		13.4	2.28E-05	1-phosphofructokinase, putative
sp0877		8.3	6.97E-04	PTS system, fructose specific IIABC components
sp1922		3.4	8.15E-05	Conserved hypothetical protein
sp1923	*ply*	4.6	2.28E-03	Pneumolysin
sp1924		2.9	4.49E-04	Hypothetical protein
sp1925		3.1	1.36E-02	Hypothetical protein
sp1926		2.9	1.75E-04	Hypothetical protein

[1] Gene designation of microarray ORFs in agreement with TIGR (http://www.tigr.org).
[2] Ratios intensities of D39 wild-type/Δ*rr09* determined in microarray experiments. The value represents the mean Intensities for the four replicate experiments. Genes up-regulated with ≥ two-fold expression changes between the wild-type and mutant were selected.
[3] *P*-value represents the mean *P*-value calculated from individual *t*-tests of intensity changes between the wild-type and mutant. Genes with *P*-value ≤0.05 were selected.
[4] Annotations as published in TIGR4 genome (http://www.tigr.org).

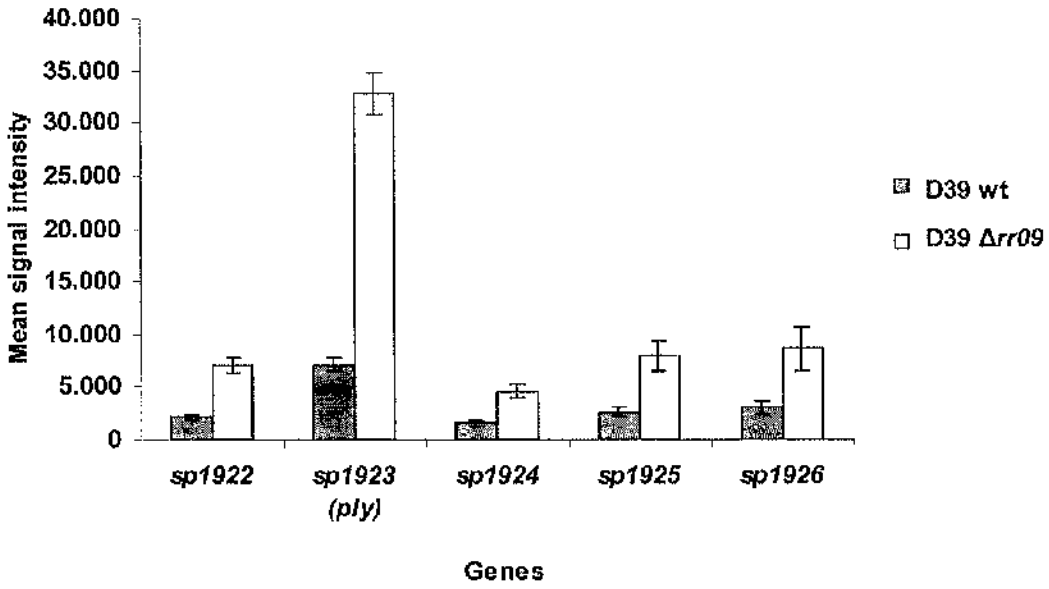


Figure 5.12- Gene cluster up-regulated in D39 Δ*rr09*.

Expression of a gene cluster containing the pneumolysin gene (*sp1923*) up-regulated in D39 Δ*rr09* compared with wild-type strain. Values are determined by microarray hybridization of two independent RNA samples and are given as the geometric mean fluorescence intensity values from four replicate hybridizations. Error bars represent ± SEM.

A total of 38 genes, including *rr09*, showed significantly decreased transcriptional levels in D39 Δ*rr09*, indicating that they are directly or indirectly activated by RR09. These included two putative operons, *sp0060-sp0066* (Figure 5.13) and *sp0303-sp0310* (Figure 5.14). These clusters include several genes encoding putative PTSs. These PTSs are involved in metabolism of sugars such as mannose, and glucose. Other putative operons, *sp2141-sp2144* (Figure 5.15) predicted to be involved in N-glycan degradation, and *sp0090-sp0092* (Figure 5.16) encoding an ABC transporter were down-regulated in Δ*rr09* mutant. Furthermore, two genes encoding known or proposed virulence factors, PspA, and HtrA (Ibrahim *et al.*, 2004a; Sebert *et al.*, 2002), were down-regulated.

Table 5.4- Down-regulated genes in D39 Δ*rr09* compared with its parental strain.

Genes[1]	Gene symbol	Mean intensity ratio[2]	*p*-value[3]	Annotation[4]
sp0057	*strH*	0.16	3.97E-04	Beta-N-acetylhexosaminidase
sp0060	*bga*	0.03	6.00E-05	Beta-galactosidase
sp0061		0.07	3.43E-04	PTS system, IIB component
sp0062		0.03	3.30E-05	PTS system, IIC component
sp0063		0.03	3.75E-05	PTS system, IID component
sp0064		0.04	8.85E-05	PTS system, IIA component
sp0065	*agaS*	0.19	1.83E-02	Sugar isomerase domain protein AgaS
sp0066	*galM*	0.12	6.80E-04	Aldose 1-epimerase
sp0090		0.25	6.69E-03	ABC transppermease protein
sp0091		0.31	1.58E-03	ABC transporter, permease protein
sp0092		0.24	4.46E-02	ABC transporter, substrate-binding protein
sp0285		0.36	6.18E-03	Alcohol dehydrogenase, zinc-containing
sp0303	*bglA*	0.04	5.17E-03	6-phospho-beta-glucosidase
sp0305		0.09	3.59E-03	PTS system, IIB component
sp0306		0.22	9.44E-03	Transcriptional regulator putative
sp0307		0.30	4.97E-03	PTS system, IIA component
sp0308		0.21	5.40E-03	PTS system, IIA component
sp0310		0.15	1.64E-04	PTS system, IIC component
sp0368		0.22	6.46E-04	Cell Wall Surface Anchor family protein, authentic frameshift
sp0386		0.42	4.39E-03	Sensor histidine Kinase, Putative
sp0498		0.19	1.06E-04	Endo-beta-N-acetylglucosaminidase, putative
sp0577		0.27	3.85E-03	PTS system, beta-glucosides-specific IIABC components
sp0661	*rr09*	0.43	9.21E-03	DNA-binding response regulator
sp1027		0.26	4.13E-03	Conserved hypothetical protein
sp1695		0.22	2.13E-03	Acetyl xylan esterase, putative
sp1802		0.18	6.58E-04	Hypothetical protein
sp2026		0.24	2.66E-03	Alcohol dehydrogenase, iron-containing
sp2055		0.34	2.13E-04	Alcohol dehydrogenase, zinc-containing
sp2056	*nagA*	0.34	7.82E-04	N-acetylglucosamine-6-phosphate deacetylase 1
sp2107	*malQ*	0.27	2.74E-03	4-alpha-Glucanotransferase
sp2141		0.08	7.01E-04	Glycosyl hydrolase-related protein
sp2142		0.08	2.14E-04	ROK family protein
sp2143		0.16	1.57E-02	Conserved hypothetical protein
sp2144		0.09	1.46E-04	Conserved hypothetical protein
sp2146		0.20	2.71E-03	Conserved hypothetical protein
sp2239	*htrA*	0.21	1.51E-02	Serine protease
sp2240		0.21	5.45E-03	spspoJ protein
spr0121	*pspA*	0.22	8.66E-04	R6 surface protein PspA precursor

[1] Gene designation of microarray ORFs in agreement with TIGR (http://www.tigr.org).
[2] Ratios intensities of D39 wild-type/Δ*rr09* determined in microarray experiments. The value represents the mean intensities for the four replicate experiments. Genes up-regulated with ≥ two-fold expression changes between the wild-type and mutant were selected.
[3] *P*-value represents the mean *P*-value calculated from individual *t*-tests of intensity changes between the wild-type and mutant. Genes with *P*-value ≤0.05 were selected.
[4] Annotations as published in TIGR4 genome (http://www.tigr.org).

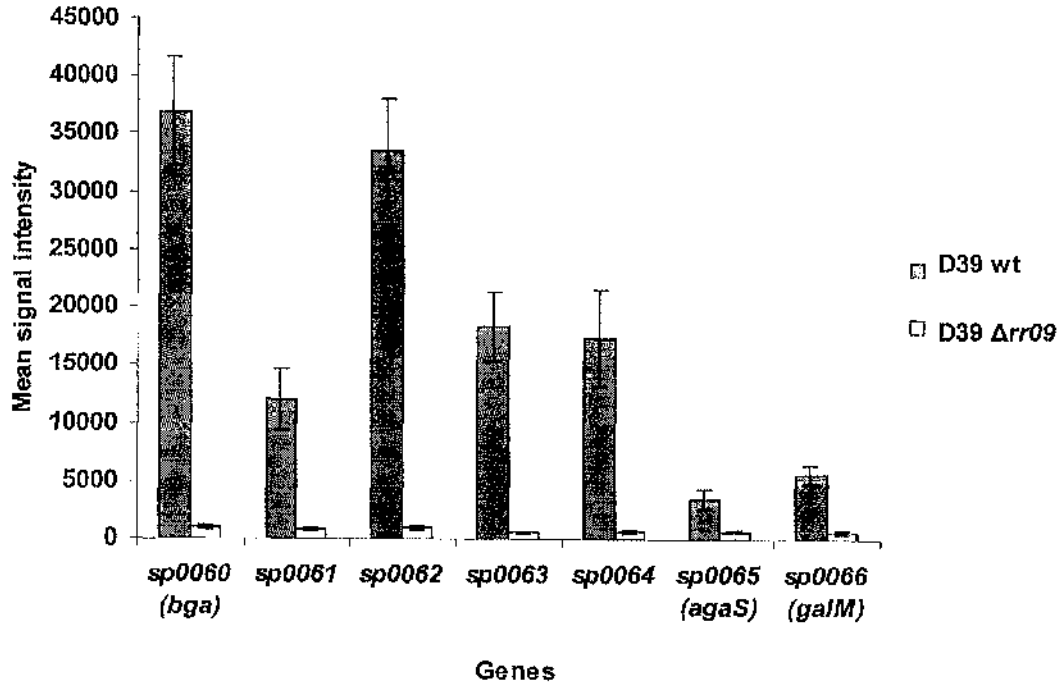


Figure 5.13- Mannose-specific PTS IIABCD system down-regulated in D39 Δ*rr09*.

Expression of Mannose-specific PTS IIABC system down-regulated in D39 Δ*rr09* compared with wild-type strain. Values are determined by microarray hybridization of two independent RNA samples and are given as the geometric mean fluorescence intensity values from four replicate hybridizations. Error bars represent ± SEM.

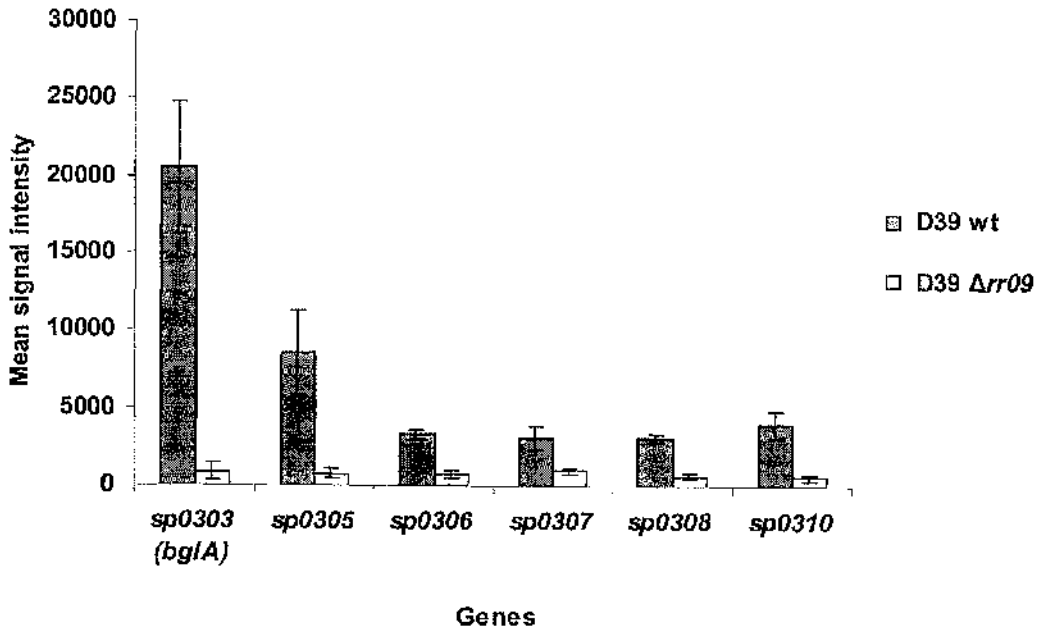


Figure 5.14- Glucose-specific PTS IIABC system down-regulated in D39 Δ*rr09*.

Expression of Mannose-specific PTS IIABC system down-regulated in D39 Δ*rr09* compared with wild-type strain. Values are determined by microarray hybridization of two independent RNA samples and are given as the geometric mean fluorescence intensity values from four replicate hybridizations. Error bars represent ± SEM.

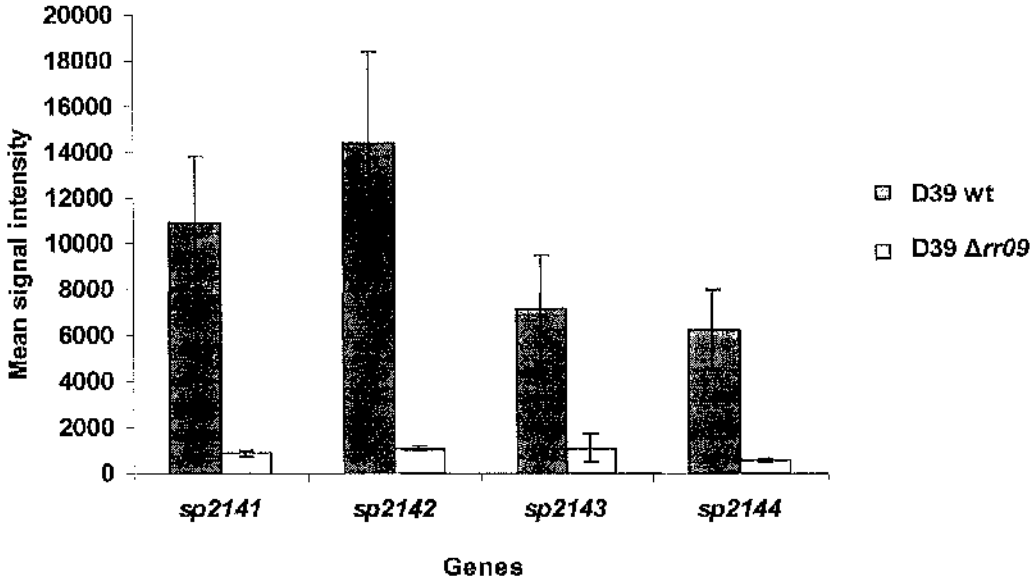


Figure 5.15- Putative operon involve in N-glycan degradation down-regulated in D39 Δ*rr09*.

Expression of putative operon *sp2141-sp2144* down-regulated in D39 Δ*rr09* compared with wild-type strain. Values are determined by microarray hybridization of two independent RNA samples and are given as the geometric mean fluorescence intensity values from four replicate hybridizations. Error bars represent ± SEM.

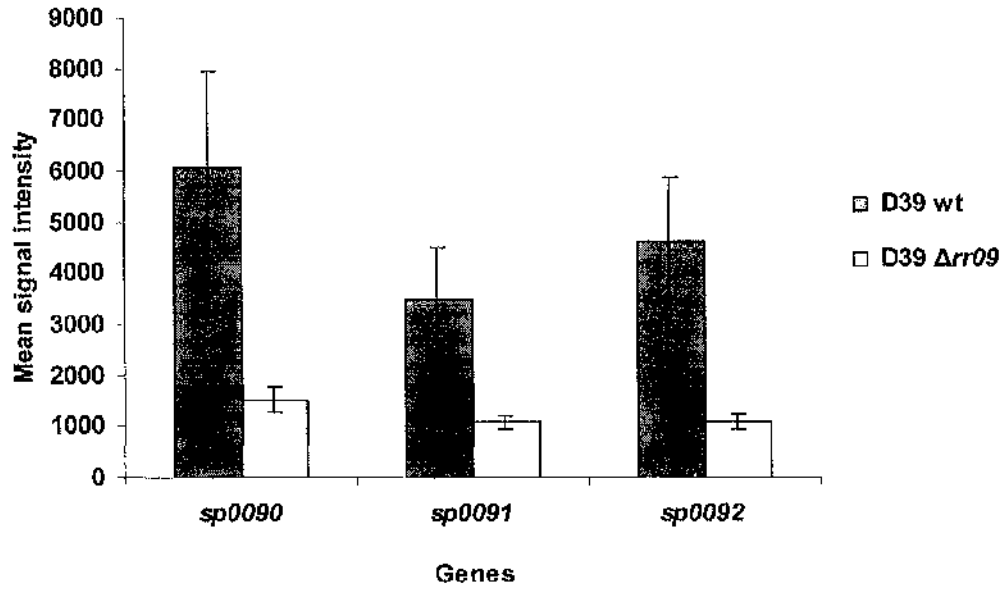


Figure 5.16- ABC transporter down-regulated in D39 Δ*rr09*.

Expression of ABC transporter down-regulated in D39 Δ*rr09* compared with wild-type strain. Values are determined by microarray hybridization of two independent RNA samples and are given as the geometric mean fluorescence intensity values from four replicate hybridizations. Error bars represent ± SEM.

As for TCS06, the gene regulation of TCS09 appears to be strain-dependent. A comparative genomic hybridization (CGH) (see chapter 6) was done to verify the presence or absence of these genes regulated by *rr09* in one strain background but not in the other. Once more, the majority of the genes altered in expression profile in microarray analysis of the TIGR4 or Δ*rr09* strains is present in the genome background of D39 strain. The *rlrA* pathogenicity islet, up-regulated in TIGR4 Δ*rr09*, but not in D39 Δ*rr09* strain, is absent in D39 genome.

Table 5.5- Genes regulated by RR09 and presence or absence of the gene by CGH in different strains.

	Transcriptional profile		CGH	
Genes[1)]	TIGR4	D39	TIGR4	D39
sp0044	+	-	+	+
sp0045	+	-	+	+
sp0046	+	-	+	+
sp0047	+	-	+	+
sp0048	+	-	+	+
sp0050	+	-	+	-
sp0051	+	-	+	+
sp0053	+	-	+	+
sp0054	+	-	+	+
sp0056	+	-	+	+
sp0057	-	+	+	+
sp0060	-	+	+	+
sp0061	-	+	+	+
sp0062	-	+	+	+
sp0063	-	+	+	+
sp0064	-	+	+	+
sp0065	-	+	+	+
sp0066	-	+	+	+
sp0090	-	+	+	+
sp0091	-	+	+	+
sp0092	-	+	+	+
sp0117	+	+	+	+
sp0285	-	+	+	+
sp0287	+	-	+	+
sp0303	-	+	+	+
sp0305	-	+	+	+
sp0306	-	+	+	+
sp0307	-	+	+	+
sp0308	-	+	+	+
sp0310	-	+	+	+
sp0338	+	-	+	+
sp0368	-	+	+	+
sp0386	-	+	+	+
sp0424	+	-	+	+
sp0461	+	-	+	-
sp0462	+	-	+	-
sp0463	+	-	+	-
sp0464	+	-	+	-
sp0466	+	-	+	-
sp0498	-	+	+	+
sp0501	-	+	+	+
sp0502	-	+	+	+
sp0515	+	-	+	+
sp0516	+	-	+	+
sp0517	+	-	+	+
sp0519	+	-	+	+

Table 5.5- Continuation.

Genes[1)]	Transcriptional profile		CGH	
	TIGR4	D39	TIGR4	D39
sp0577	-	+	+	+
sp0647	+	-	+	+
sp0661	+	+	+	+
sp0648	+	-	+	+
sp0724	+	-	+	+
sp0845	+	-	+	+
sp0875	+	+	+	+
sp0876	+	+	+	+
sp0877	-	+	+	+
sp1027	-	+	+	+
sp1229	+	-	+	+
sp1249	+	-	+	+
sp1587	+	-	+	+
sp1695	-	+	+	+
sp1802	-	+	+	+
sp1804	+	-	+	+
sp1883	+	-	+	+
sp1884	+	-	+	+
sp1895	+	-	+	+
sp1896	+	-	+	+
sp1897	+	-	+	+
sp1922	-	+	+	+
sp1923	-	+	+	+
sp1924	-	+	+	+
sp1925	-	+	+	+
sp1926	-	+	+	+
sp2026	-	+	+	+
sp2055	-	+	+	+
sp2056	-	+	+	+
sp2107	-	+	+	+
sp2141	-	+	+	+
sp2142	-	+	+	+
sp2143	-	+	+	+
sp2144	-	+	+	+
sp2146	-	+	+	+
sp2239	-	+	+	+
sp2240	-	+	+	+

[1)] Gene designation of microarray ORFs in agreement with TIGR (http://www.tigr.org).
(+) Altered gene expression (transcriptional profile) or gene present (CGH).
(-) Unaltered gene expression (transcriptional profile) or gene absent (CGH).

5.4 Validation of microarray data

5.4.1 Quantitative Real-Time PCR

Selected genes from microarray experiments were validated by quantitative Real-Time PCR (qRT-PCR) analysis performed using the relative quantification method ($\Delta\Delta C_T$) (Livak and Schmittgen, 2001). The reactions were carried out in quadruplicate reactions using cDNA of different strains, from two independent RNA preparations and tested with SYBR green. The microarray and qRT-PCR analysis strongly correlated in TIGR4 and D39 strains. An interesting target altered in expression profile in microarray analysis of the

TIGR4 Δ*rr09* compared with the wild-type, was the *rlrA* pathogenicity islet (Hava *et al.*, 2003a), up-regulated in TIGR4 Δ*rr09*, comprising a putative transcriptional regulator *rlrA*, three cell wall-anchored surface proteins (*sp0462-0464*) and three sortase enzymes (*sp0466-0468*). The *rlrA* islet was recently shown to encode the pneumococcal pilus (Barocchi *et al.*, 2006). The qRT-PCR analysis of all genes of this locus confirmed the strong up-regulation in Δ*rr09* strain Figure 5.17. In this present project we also identified the *rlrA* pathogenicity islet to be up-regulated in TIGR4 Δ*rr06* mutant compared to the wild-type (Figure 3.38). Both, TCS06 and TCS09 seems regulate the *rlrA* pathogenicity islet.

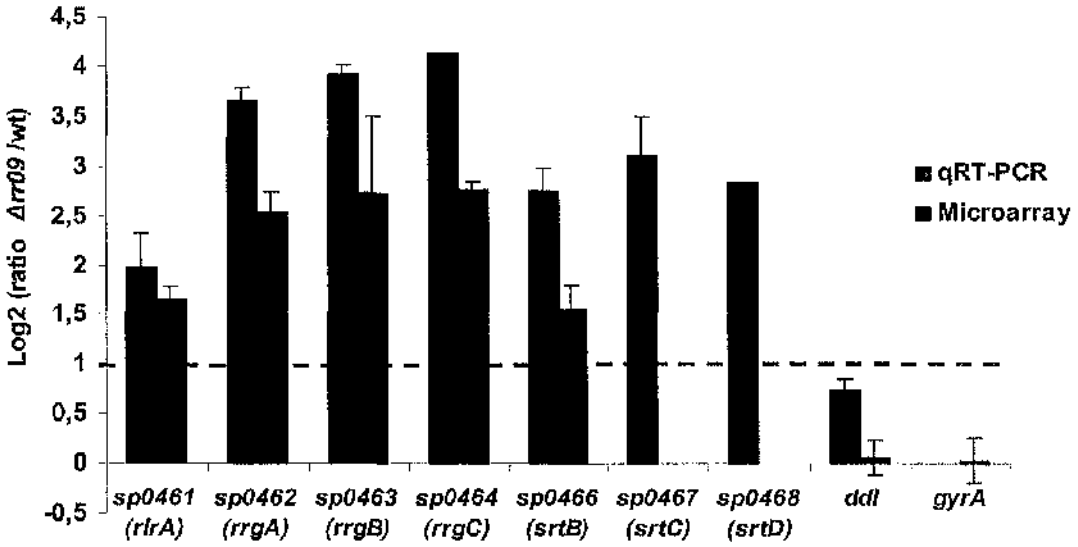


Figure 5.17- Expression ratio of *rlrA* pathogenicity islet in TIGR4 Δ*rr09* compared with its parental strain.

The expression ratio of *rlrA* pathogenicity islet in TIGR4 Δ*rr09* compared with wild-type strain was determined by microarray and qRT-PCR. The dashed line indicates the two-fold change microarray cut-off value for differential expression. No microarray data was obtained for *sp0467* and *sp0468* genes. Expression of two control house keeping genes *ddl* and *gyrA* are shown.

Of interest genes in qRT-PCR analysis for D39 strains were the putative operons *sp0060-sp0066* encoding a putative β-galactosidase, a mannose-specific PTS, a sugar isomerase, and an aldose 1-epimerase (Figure 5.19), and the *sp2141-sp2142* involved in N-glycan degradation (Figure 5.18). The putative operon *sp0060-sp0066* was identified to be down-regulated in strain D39 Δ*rr09* for the first time in preliminary microarray studies done in our laboratory using array slides designed and provided by BμG@S (Blue, 2002). In both putative operons the expression of all genes were highly reduced in D39 Δ*rr09* mutant, confirming the previous microarray analysis.

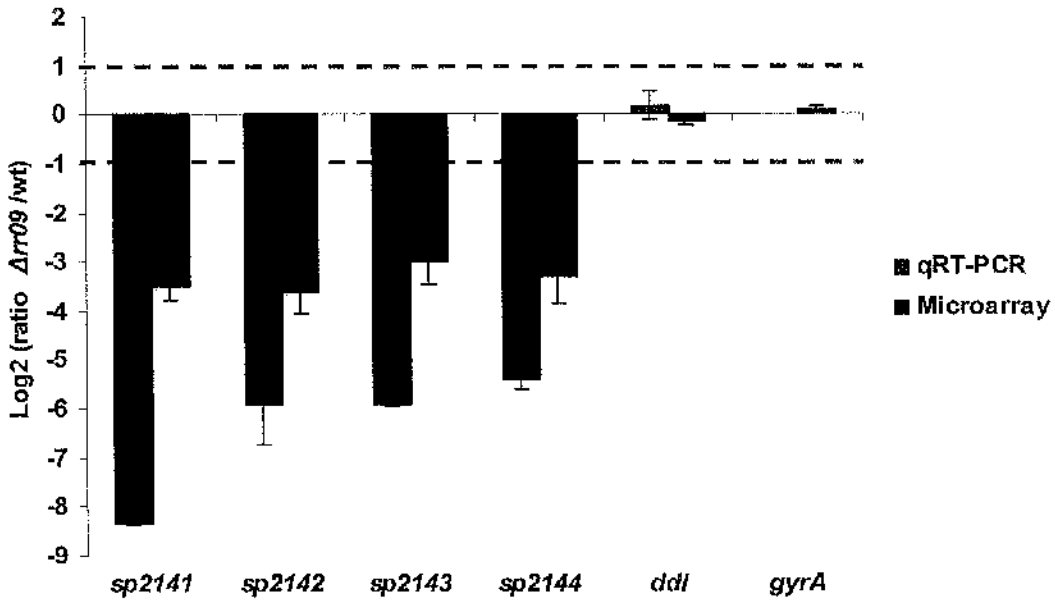


Figure 5.18- Expression ratio *sp2141-sp2142* in D39 Δ*rr09* compared to parental strain.

The expression ratio of putative operon *sp2141-sp2144* in D39 Δ*rr09* compared with wild-type strain was determined by microarray and qRT-PCR. The dashed line indicates the two-fold change microarray cut-off value for differential expression. Expression of two control house keeping genes *ddl* and *gyrA* are shown.

The contribution of a RR09 to the virulence of *S. pneumoniae* is strain dependent. The microarray analysis showed an expression reduction in the putative operon *sp0060-sp0066* as well as the gene that encodes the virulence factor HtrA only in the D39 Δ*rr09* strain. Furthermore, the virulence factor PspA, was found to be down-regulated in D39 as well in TIGR4 but not in 0100993 strain (Figure 5.20). Although the virulence factor *pspA* is down-regulated in both D39 and TIGR4 Δ*rr09* mutant strains, its contribution in virulence of the *rr09* mutants seems to be strain-dependent. The high attenuation in the virulence of the D39 Δ*rr09* mutant in a murine model of infection compared to TIGR4 can be explained by the fact that *pspA* is down-regulated approximately four times more in D39 than in TIGR4 Δ*rr09* mutant strains. Furthermore, the down-regulation of the virulence factor *htrA* and others genes in D39 Δ*rr09* may also contribute for this difference in virulence.

To confirm that the expression regulation of these genes by RR09 is strain dependent, a qRT-PCR analysis was done in all strains. The putative operon *sp0060-sp0066* appeared to be differentially regulated between D39 and the TIGR4 and 0100993 strains. While strong down-regulation of *sp0060-sp0066* was observed in D39Δ*rr09*, no significant RR09-dependent regulation was observed in either TIGR4 or 0100993 (Figure 5.19). The expression levels of theses genes are a clear example of strain-specific regulation by RR09. In addition the virulence factor *pspA* and the serine protease *htrA* as well the gene *sp2240*, which was found to be expressed on the same transcript as *htrA*, and the TCS CiaR/H, which were reported to regulate *htrA* (Ibrahim *et al.*, 2004b; Sebert *et al.*, 2002) was found to be differently expressed in the three strains (Figure 5.20). The expression of *pspA* was reduced in both TIGR4 and D39 Δ*rr09* mutants, while in 0100993 no significant different was found in Δ*rr09* strain compared it wild-type. On the other hand, the expression of *htrA* and *sp2240* was significantly down-regulated in D39 Δ*rr09* strain, not showing significant variation in expression in the other two strains. Both genes encoding the TCS CiaR/H also were down-regulated in D39 Δ*rr09* strain. The down-regulation of this TCS in the D39 Δ*rr09* mutant may be related to the observed reduction in expression of *htrA* in this mutant compared it parental strain (Ibrahim *et al.*, 2004b; Sebert *et al.*, 2002).

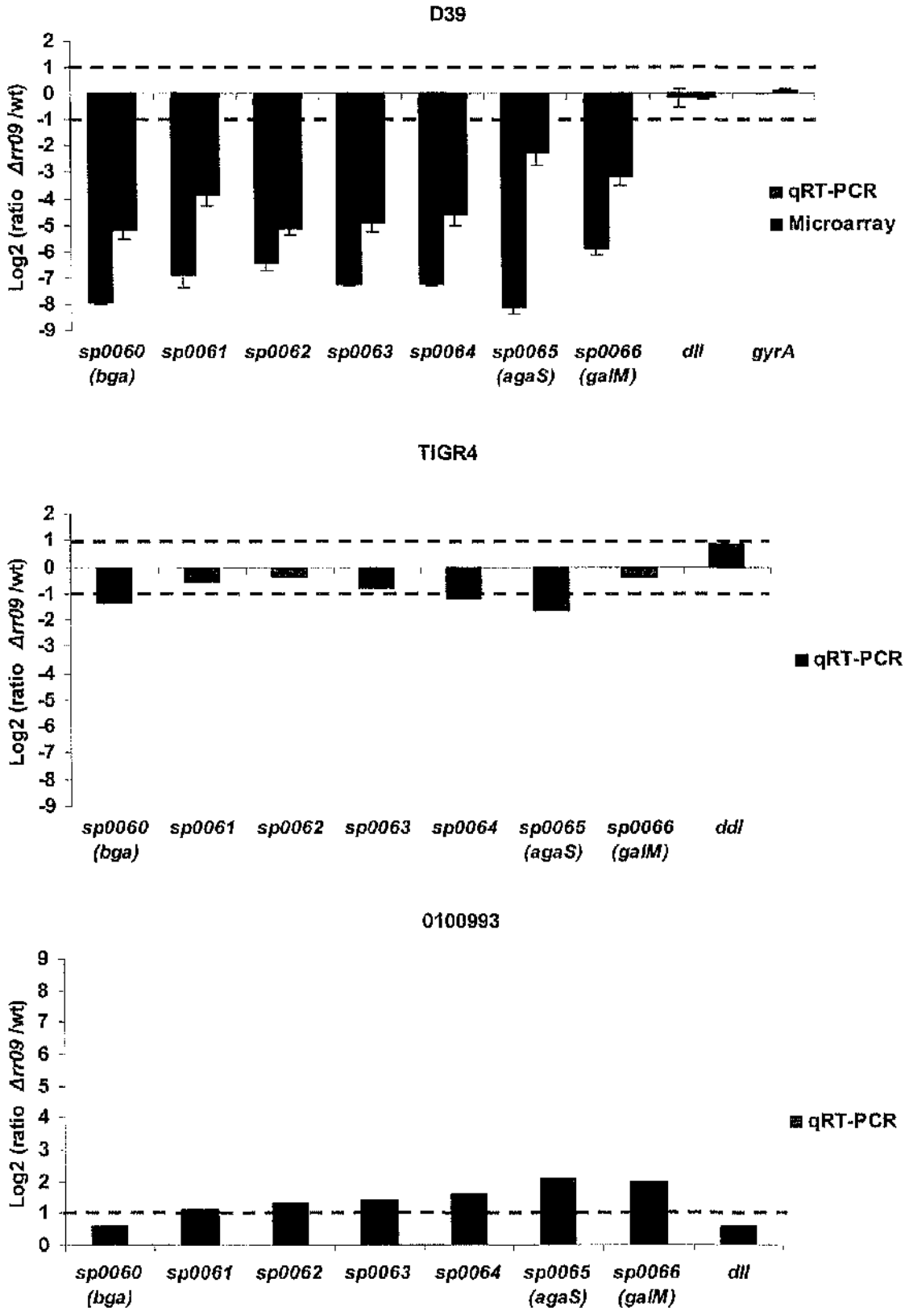


Figure 5.19- Expression ratios of *sp0060-sp0066* in D39, TIGR4 and 0100993.

The expression ratios of putative operon *sp0060-sp0066* in D39, TIGR4 and 0100993 Δ*rr09* mutants compared with wild-type was determined by microarray and qRT-PCR. The dashed line indicates the two-fold change microarray cut-off value for differential expression. No microarray data was obtained for the TIGR4 and 0100993 strains. Expression of two control house keeping genes *ddl* and *gyrA* are shown

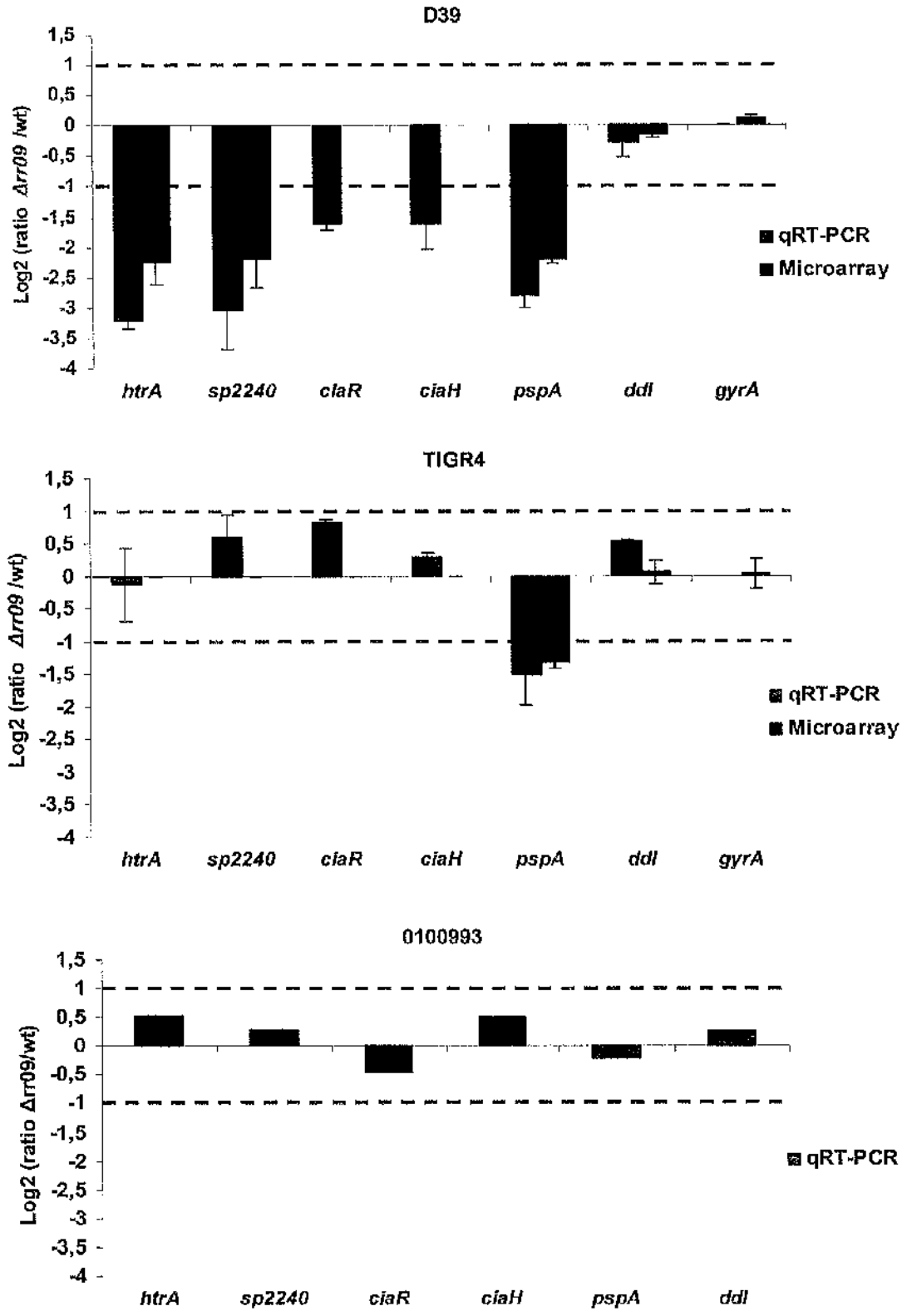


Figure 5.20- Expression ratios of virulence factors *htrA* and *pspA* in D39, TIGR4 and 0100993 strains.

The expression ratios of htrA and *pspA* in D39, TIGR4 and 0100993 Δ*rr09* mutants compared with wild-type was determined by microarray and qRT-PCR. The dashed line indicates the two-fold change microarray cut-off value for differential expression. No microarray data was obtained for 0100993 as well for the *ciaR/H* in the D39 and TIGR4 strains and *htrA*, *sp2240* in TIGR4Expression of two control house keeping genes *ddl* and *gyrA* are shown.

5.4.2 Western Immunoblot

Microarray and qRT-PCR analysis showed a significant down-regulation of the virulence factors *pspA* and *htrA* in D39 Δ*rr09*. The amount of protein of both virulence factors, expressed in wild-type and the D39 Δ*rr09* was checked by western immunoblot. A positive control, using primary antibody against the protein PspC was also used. The western immunoblot confirmed the microarray and qRT-PCR expression analysis for both proteins. Expression of protein PspA was shown to be higher in D39 wild-type than D39 Δ*rr09* (Figure 5.21A). No difference in expression of PspC was observed in both strains (Figure 5.21B), also this protein control did shown significant variation in previous microarray analysis. As with PspA, the expression of protein HtrA was shown to be higher in D39 wild-type than D39 Δ*rr09* (Figure 5.22). Furthermore, the levels of HtrA in *rr09* mutant demonstrated a similar expression of this protein with D39 Δ*ciaR* strain (Ibrahim *et al.*, 2004b).

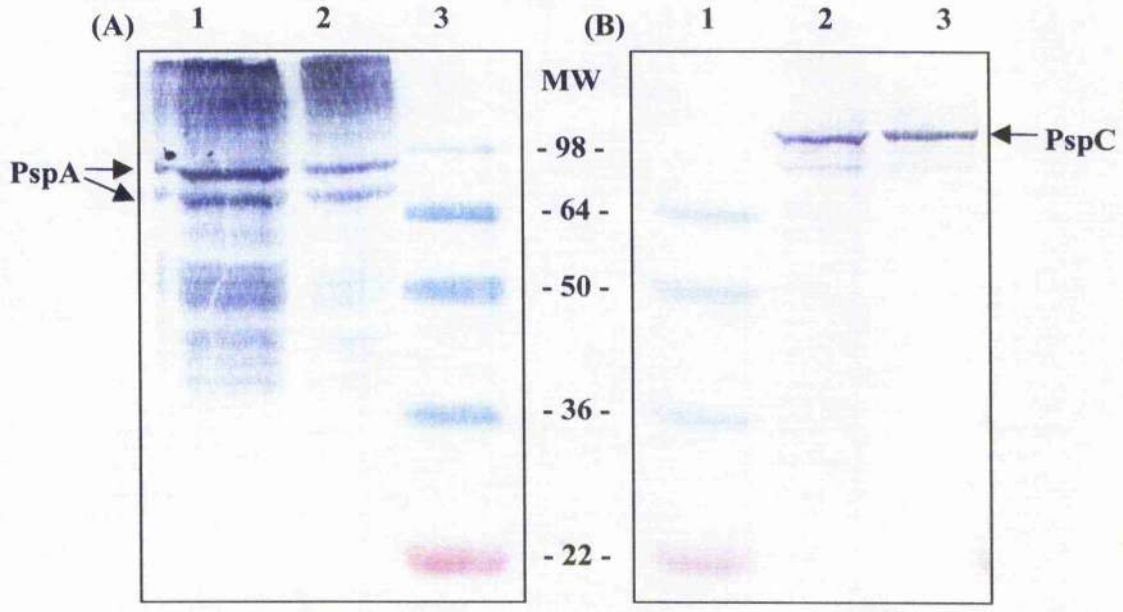


Figure 5.21- Western immunoblot analysis of PspA and PspC levels in D39 Δ*rr09* compared to wild-type.

15 μg of total protein was run in 10% SDS PAGE, the protein transferred to nitrocellulose membrane and the levels of PspA and PspC expression in D39 wild-type were compared with D39 Δ*rr09* mutant. Levels of PspA in strain D39 wild-type (lane 1) and D39 Δ*rr09* (lane2) (A). Levels of PspC in strain D39 wild-type (lane 1) and D39 Δ*rr09* (lane2) (B). The proteins marker used (lane 3) was the SeeBlue® Plus2 Pr-Staimed Standard (Invitrogen™).

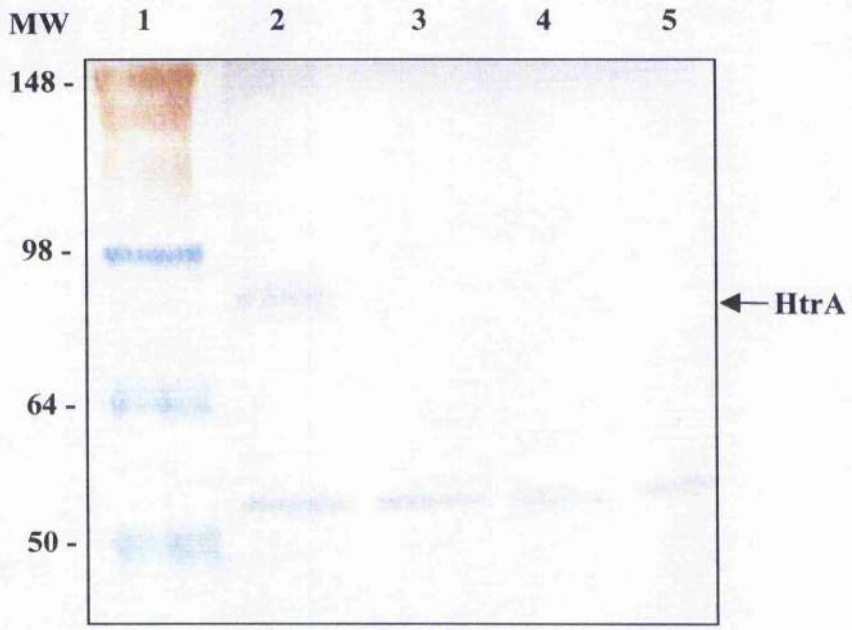


Figure 5.22- Western immunoblot analysis of HtrA levels in D39 Δ*rr09* compared to wild-type.

15 μg of total protein was run in 10% SDS PAGE, the protein transferred to nitrocellulose membrane and the levels of HtrA expression in D39 wild-type were compared with D39 Δ*rr09* mutant. D39 wild-type (lane 2), D39 Δ*rr09* (lane 3) D39 Δ*ciaR* (lane 4) and D39 Δ*htrA* (lane5). The proteins marker used (lane 1) was the SeeBlue® Plus2 Pr-Staimed Standard (Invitrogen™).

5.5 *In vitro* analysis

5.5.1 Haemolytic assay

Pneumolysin is the major cytoxin of the pneumococcus and plays an important role in the early pathogenesis of invasive pneumococcal pneumonia. Microarray analysis showed a significant increase in expression of pneumolysin in D39 Δ*rr09* compared with wild-type strain. To confirm the microarray results, the amounts of the toxin pneumolysin expressed by both strains was studied used a haemolytic assay using a method described previously (Johnson *et al.*, 1980). Interestingly the haemolytic activity did not show any difference between the wild-type and the Δ*rr09* mutant (Figure 5.23).

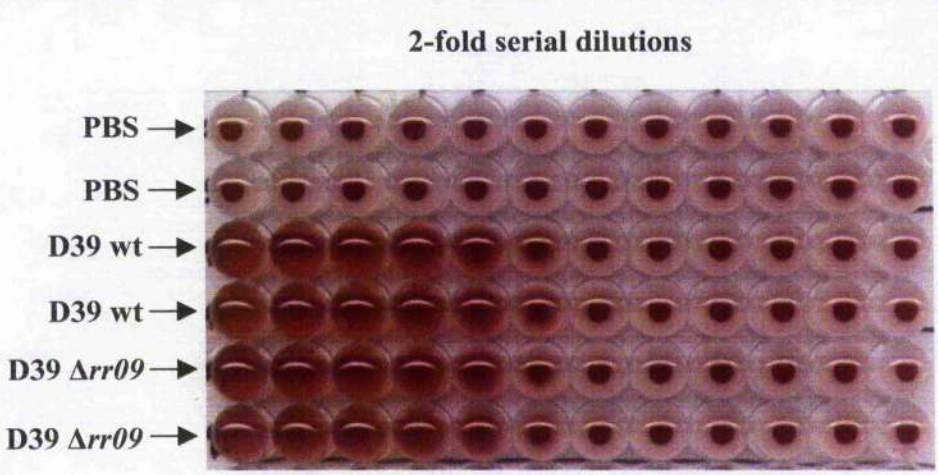


Figure 5.23- Crude haemolytic assay of pneumolysin toxin.

2-fold dilutions of crude lysate, grown to mid-log phase, of D39 wild-type and Δ*rr09* mutant were added to sheep RBC. The relative amount of pneumolysin was estimate by eye. Haemolysis activity was performed in duplicate and PBS was used as a negative control.

5.6 Role of gene *sp0063* in phenotype of RR09 mutation

The transcriptional profiles of *S. pneumoniae* wild-type strain D39 and the isogenic mutant Δ*rr09* showed significantly decreased transcriptional levels in *rr09* knockout mutant of mannose-specific PTS system IIABC (*sp0061-sp0064*). One of these genes, *sp0063* (IID component) was identified as a virulence factor in the signature-tagged mutagenesis screen of TIGR4 (Hava and Camilli, 2002). To verify the contribution of *sp0063* in the phenotype of D39 Δ*rr09* strain *in vivo* analysis, a mutation of the gene was made in D39 wild-type strain by the replacement of nucleotides 176-676 of *sp0063* (according to the R6 annotation) with a spectinomycin resistance cassette.

5.6.1 *In vivo* analysis

Previous studies demonstrated that the TCS09 is crucial in pneumococcal virulence of D39 strain (Blue and Mitchell, 2003). As demonstrated in this chapter, the *sp0063* is down regulated in D39 *rr06* mutant. This study was intended to investigate how much of the phenotype resulting from deletion of *rr09* is attributed to *sp0063*.

The effect of the D39 Δ*sp0063* mutant on virulence was compared to its wild-type and Δ*rr09* mutant (Figure 5.24). Mice were infected by interperitoneal injection with 10^6 CFU and the survival of animals monitored for 336 hours. The bacterial loads in the blood were studied following 24h post-interperitoneal infection. No significant difference was seen in survival rates between wild-type D39 and the *sp0063* mutant. All five wild-type infected

mice died while a single *sp0063* infected mouse survived. Likewise, blood counts at 24h post-infection were similar. In contrast the *rr09* mutant was essentially avirulent. In agreement with previous studies (Blue and Mitchell, 2003) all infected mice survived and blood counts at 24h were below the detection limit (~83 CFU/ml).

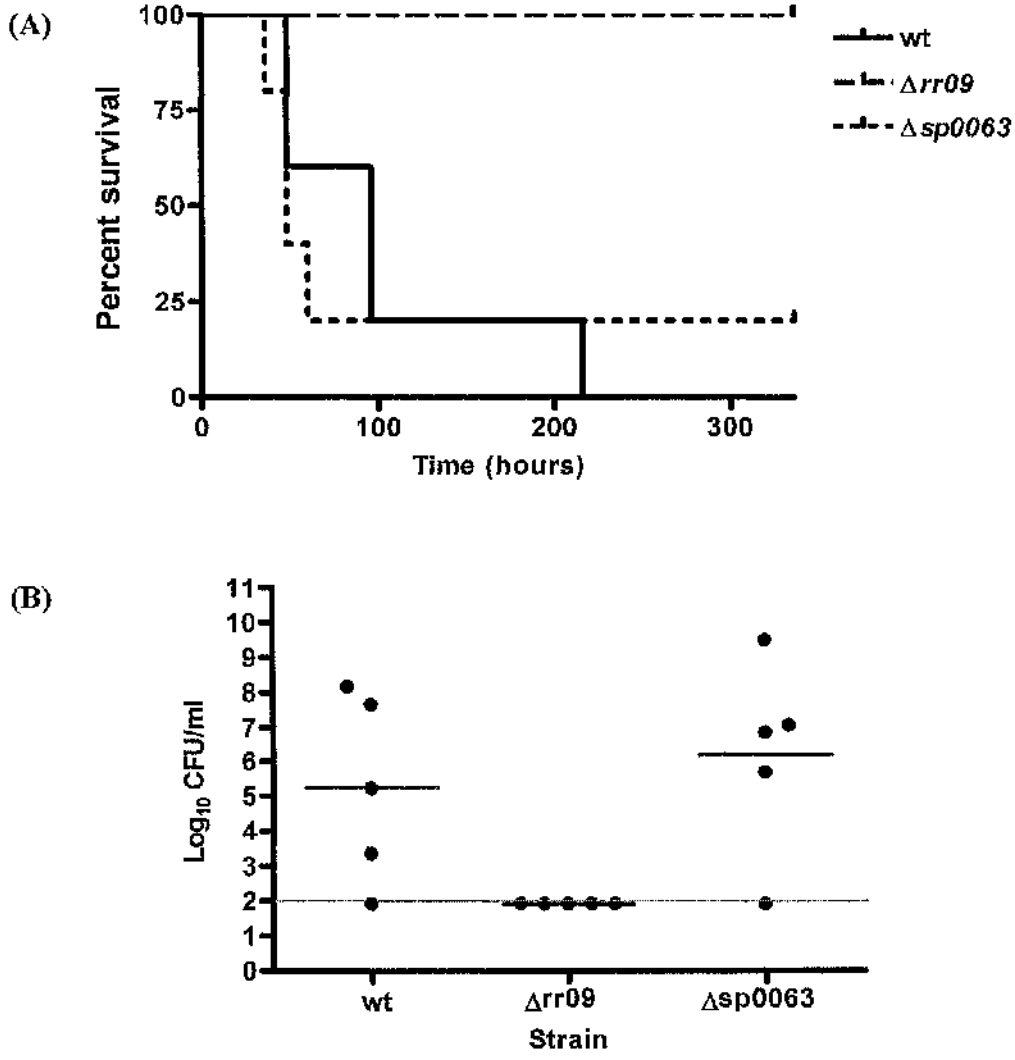


Figure 5.24- *In vivo* analysis of D39 Δ*sp0063* strain.

Effect of *sp0063* on virulence of strain D39. Survival times were monitored for 336 hours after interperitoneal infection with 10^6 CFU of wild-type, Δ*rr09* and Δ*sp0063* strains, n=5 (A). Bacterial blood counts measured at 24h post-infection via tail bleeds. Each point indicates the data from an individual mouse; the horizontal bar indicates the mean (n=5). Dashed line indicates detection limit. Mice with bacterial counts below the detection limit (log_{10} 1.92) were assigned a value of 1.90. Done in collaboration with (B). Dr. Gavin Paterson.

Chapter 5 discussion

TCS09 system and virulence

The pneumococcus needs to adapt and orchestrate its gene expression to persist in different environments. A central role in this environment response is played by TCS by regulating various cellular processes such as osmoregulation, genetic competence, and chemotaxis in response to environmental changes (Appleby *et al.*, 1996; Hoch and Silhavy, 1995). Relatively little is known about TCS09. Together with HK09, the RR09 forms the TCS09 and its gene names are *sp0661-sp0662* (TIGR4 sequence) or *spr0578-spr0579* (R6 sequence) (http://www.tigr.org). Amino acid sequence homology suggests that the sensory domain is related to the extra-cellular part of McpA and McpB of *Bacillus subtilis*. These two proteins are believed to be involved in the control of chemotaxis through sensing of environmental nutrient concentrations (Lange *et al.*, 1999). The extra-cellular stimulus of TCS09, however, is as yet unknown. *In vivo* studies in a murine model of infection suggest that TCS09 plays a role in virulence although this appeared to be dependent on the genetic background and the infection model used (Blue and Mitchell, 2003). In strain D39 (serotype 2) the lack of RR09 led to an avirulent phenotype upon intranasal, intravenous, and intraperitoneal infection, while in strain 0100993 (serotype 3) RR09 was suggested to be involved in the dissemination of the bacteria from the lung tissue into the bloodstream (Blue and Mitchell, 2003).

In the present study, we extended the investigation of the strain-specific role of TCS09 in pneumococcal virulence using TIGR4 (serotype 4) *rr09* mutant in a murine pneumonia model of infection. Similar to 0100993 Δ*rr09*, the TIGR4 Δ*rr09* mutant was found to be attenuated, with prolonged murine survival after intranasal infection with this mutant. Furthermore, significantly fewer bacteria were recovered from in bronchio-alveolar lavage fluid, lung tissue and from blood of mice infected with TIGR Δ*rr09* when compared to wild-type infected mice. In contrast to TIGR4 Δ*rr09*, mice intranasally infected with 0100993 Δ*rr09* had no reduction of recovered bacteria from the lungs when comparable with wild-type infected mice (Blue and Mitchell, 2003).

Gene regulation by TCS09

No gene targets of RR09 have yet been identified that could relate to the *in vivo* phenotypes observed in *rr09* mutants although preliminary studies suggested involvement of TCS09 in nutrient perception (Blue and Mitchell, 2003; Lange *et al.*, 1999). In this study, we investigated the strain-specific regulation by RR09 by comparing transcriptional profiles of mutants in two different genetic backgrounds, namely *S. pneumoniae* strains

D39 and TIGR4 using whole-genome microarray analysis and two independent RNA samples analysed *in vitro* during mid-log phase of each strains. Moreover, some of the transcriptional changes were confirmed by quantitative Real-Time PCR (qRT-PCR).

The transcriptional changes occurring in TIGR4 Δ*rr09* were ~1% of the genome for up-regulated and ~0.8% for down-regulated genes. An interesting pattern of regulation was observed for genes located on the *rlrA* pathogenicity islet, absent in strain D39, which is strongly up-regulated in the TIGR4 *rr09* mutant strain. Curiously, the same pattern was observed during the study of the transcriptional profile of the *rr06* mutant (chapter 3), indicating that, more that one TCS can be involved in regulation of a particular or cluster of genes. Hendriksen (Hendriksen, personal communication), observed that this islet is strongly down-regulated in early phases when the bacteria are grown in Todd Hewitt broth supplemented with 5 g/l Yeast extract (THY-broth). These findings suggest an indirect regulation of the *rlrA* pathogenicity islet by the TCS09. RlrA has been shown to positively regulate the expression of the seven genes on the pathogenicity islet. Of the six genes, three have homology to the LPXTG family of cell wall-anchored surface proteins (*rrgA*, *rrgB*, *rrgC*) and three encode putative sortases (*srtB*, *srtC*, and *srtD*) (Hava *et al.*, 2003a). A recent study showed that the *rlrA* pathogenicity islet encodes a pilus-like structure previously unknown to exist in the pneumococcus. The pneumococcal pilus seems to be essential for bacterial adhesion and subsequent ability to cause invasive disease as well as in stimulation of the host inflammatory response (Barocchi *et al.*, 2006). Repression of the pathogenicity islet by MgrA has been suggested (Hemsley *et al.*, 2003), but no differential expression of *mgrA* was observed in this study. The genes, *rlrA,* and *srtD* were found in the STM screen and have been described to be essential for efficient colonization (Hava and Camilli, 2002). Furthermore in this present study, the virulence factor *pspA* was observed to be down-regulated in the *rr09* mutant, suggesting that the regulation of these genes (*rlrA*, *srtD* and *pspA*) by RR09 may be the cause of the attenuation in a murine model of TIGR4 Δ*rr09*.

In total, 48 genes were found to be differentially expressed between wild-type and D39 Δ*rr09* mutant strains. 10 genes were found to be up-regulated in *rr09* mutant, and include pneumolysin, the major pneumococcal cytoxin. However, a haemolytic assay did not show differences between the wild-type and the Δ*rr09* mutant. This suggests that the difference in the pneumolysin expression does not reflect the amount of protein. Additional experiments need to be done, such as qRT-PCR and western immunoblot to determine at what level pneumolysin is up-regulated in D39 Δ*rr09*. Of genes differentially expressed, 38 were found to be down-regulated in the D39 *rr09* mutant. Some of these genes were

predicted to encode proteins involved in carbohydrate metabolism, in particular genes encoding enzyme II (EII) of a sugar-specific phosphotransferase systems (PTS). These membrane-associated proteins facilitate uptake of carbohydrates, such as mannose and fructose, by PTSs. The genes *sp0061-sp0064*, encoding a putative mannose-specific PTS, were strongly down-regulated in D39 Δ*rr09*, but not regulated in TIGR4 Δ*rr09* and 0100993 Δ*rr09*. Genes adjacent to this PTS are also involved in N-glycan degradation, with putative roles in hydrolysis of aminosugar side chains of glycan. Several other genes involved in this process were co-regulated with these genes in D39 Δ*rr09*. Another down-regulated putative PTS is encoded by the genes *sp0307-sp0310* and *sp0577* (glucose-specific PTS). As for the previous PTS, no changes in regulation were observed in TIGR4 Δ*rr09* strain. Although the exact function of TCS09 in *S. pneumoniae* remains unclear, the regulation of different PTS by the RR09 is in agreement with Lange and co-workers, that predicted a putative role in nutrient perception based on homology with chemotaxis-associated sensors found in *B. subtilis* (Lange *et al.*, 1999). Furthermore, genes encoding other components of these systems, namely HPr, and CcpA, (global regulator of carbon metabolism) (Kotrba *et al.*, 2001; Titgemeyer and Hillen, 2002), are involved in repression of the β-galactosidase in *S. pneumoniae* D39 strain (Giammarinaro and Paton, 2002). In contrast with the TIGR4 strain (Iyer *et al.*, 2005), this repression is not mediated by glucose. However, none of these genes appear to be altered or upregulated in our studies in D39 strain, indicating that CcpA is not responsible for the down-regulation of the putative mannose-specific PTS encoding by the *sp0061-sp0064* genes.

As well as the down-regulation of PTS, the differences observed between the avirulent phenotype of D39 Δ*rr09*, and TIGR4 and 0100993 in mouse model of infection, could be the result of the down-regulation of two virulence factors, *htrA* and *pspA* in D39 Δ*rr09*. The *htrA* gene, down-regulated only in D39 Δ*rr09*, is thought to be regulated by CiaR/H (Ibrahim *et al.*, 2004b; Sebert *et al.*, 2002; Sebert *et al.*, 2005). While, no significant change in expression of *ciaR/H* was detected by microarray analysis, qRT-PCR analysis had shown 3-fold decrease of *ciaR/H* expression in Δ*rr09*. This small change in CiaR/H system may explain the down-regulation of *htrA* in Δ*rr09*, confirming probably an indirect regulation of this virulence factor by the RR09. The down-regulation of HtrA in D39 Δ*rr09* was also confirmed by western immunoblotting, and this change could contribute to the D39 Δ*rr09 in vivo* phenotype, because virulence of D39 *htrA* mutants is completely abolished (Ibrahim *et al.*, 2004a, b). The *pspA* gene was also found to be down-regulated in TIGR4 Δ*rr09* but not in 0100993 Δ*rr09*. However, the change in transcriptional levels of *pspA* in D39 Δ*rr09* was approximately double that observed in TIGR4 Δ*rr09*. Furthermore, western immunoblotting was implemented to study the level of PspA

produced D39 Δ*rr09* against wild-type. As with the transcription analysis, the blot indicated significant changes of the level of PspA in *rr09* mutant. Microarray analysis has shown that TCS02 positively regulates the PspA (Ng *et al.*, 2005). Band shift and footprinting experiments using purified unphosphorylated RR02 and phosphorylated RR02 demonstrate that the RR02 biding to regions upstream of *pspA*, demonstrating a direct regulation of PspA by TCS02. As with *htrA*, these results suggest that TCS09 may indirectly regulated the virulence factor PspA.

The study of transcriptional profiles of D39 Δ*rr09* in early phases of growth sampled (Hendriksen, personal communication) demonstrated an increased of the expression of competence-related genes like *comAB*, *comDE*, *cinA* and the *cglABCD*-operon compared with wild-type. This regulation appears to be strain-specific, since no change of expression was observed in TIGR4 Δ*rr09*, and confined to early phases of growth of the pneumococcus, because no changes in this genes were observed in mid-log phase of growth in the present study. Upregulation of *comDE* leads to induction of competence, which in turn induces transcription of *comX*. Genes induced by competence stimulating peptide (CSP) were described by Peterson and co-workers as well three groups of responding genes: early, late and delayed genes (Peterson *et al.*, 2004). The early genes are believed to be directly regulated by ComE. The upregulation of the early CSP-induced genes found by Hendriksen study (Hendriksen, personal communication) may be the consequence of premature development of competence by the lack of RR09. On the other hand, some of the late genes were found to be upregulated (*cglABCD* and *cinA*) probably due to downstream regulation of competence development. The delayed CSP-induced gene *htrA* was shown here to be down-regulated at the OD_{600} of 0.6 in BHI-broth but upregulated at an OD_{600} of 0.2 in THY-broth (Hendriksen, personal communication).

Some genes were found to be controlled by RR09 in our microarray analysis, as well in the early phases of growth sampled report by Hendriksen (Hendriksen, personal communication), while others appeared to be regulated in a growth-phase-dependent manner. Although this differential expression could be the consequence of the different growth conditions used, BHI-broth for OD_{600} of 0.6 and THY-broth for OD_{600} of 0.1 and 0.2 (Hendriksen, personal communication) and/or the use of two different microarrays slides, the considerable overlap between the set of genes regulated at the different growth phases (e.g., *sp0060-sp0066*, *htrA*, *sp0647-sp0648*, and the *rlrA* pathogenicity island), makes this explanation less likely. Although expression of genes has been shown to differ substantially between different broths (Ogunniyi *et al.*, 2002), our results indicate that apparent putative targets can be identified with different experimental set-ups.

Role of gene *sp0063* in phenotype of RR09 mutation

Phosphotrasferase systems (PTS) are responsible for the transport of specific sugars into the cell. The PTS transporters consist of three subunits, IIA, IIB, and IIC. One exception is the mannose family transporters, which also contains the subunit, IID (Kotrba *et al.*, 2001). The genes *sp0061-sp0064*, encoding a putative mannose-specific PTS. This system was strongly down-regulated in D39 Δ*rr09*, but not regulated in TIGR4 Δ*rr09* and 0100993 Δ*rr09*. Lowered sugar uptake caused by the down-regulation of this locus could result in a reduced supply of sugar building blocks for the polysaccharide capsule, which in turn might explain the avirulent phenotype of D39 Δ*rr09* in a sepsis model of infection. In contrast, Δ*rr09* derivatives of TIGR4 and 0100993 showed a less severe *in vivo* phenotype, consistent with the unaltered expression of *sp0061-sp0064*. Furthermore, the *sp0063* was identified as a virulence factor in a STM screen (Hava and Camilli, 2002). To test the role of this putative mannose-specific PTS in the *rr09* mutant phenotype, the gene encoding the putative IIC component of this PTS (*sp0063*) in D39 was deleted by allelic replacement, and its experimental virulence was assessed. No significant difference was observed in survival rates of mice infected with wild-type D39 and the *sp0063* mutant. Likewise, blood counts at 24h post-infection were similar. In contrast, the D39 Δ*rr09* mutant was essentially avirulent. The gene product of *sp0063* alone does not significantly contribute to virulence in D39 in this model and alone does not explain the dramatic phenotype observed following deletion of *rr09* in D39. Moreover, our preliminary data indicate that the *rr09* and *sp0063* mutants are still able to ferment mannose (data not shown), which could be a consequence of the apparent redundancy of PTSs in the pneumococcal genome, complementing the lower expression of this particular PTS.

Summary

Recent murine studies have demonstrated that TCS09 of *S. pneumoniae* is virulence-associated, and moreover, is strain-specific (Blue and Mitchell, 2003). In the present study, we used a murine model of infection to assess the virulence of a TIGR4 *rr09*-mutant, and found that TIGR4 Δ*rr09* was attenuated after intranasal infection and mice infected with *rr09*-mutant had significantly longer survival times than the wild-type infected mice. Furthermore, we investigated the transcriptional changes in pneumococcal mutants lacking the response regulator of TCS09 (Δ*rr09*) by microarray analysis of two strains, D39 and TIGR4. The transcriptional pattern of D39 Δ*rr09* and TIGR4 Δ*rr09* displayed clear differences compared to their parental wild-type strains. Moreover, TCS09 appeared to (directly or indirectly) regulate different genes in D39 and TIGR4. Our microarray and

qRT-PCR analysis showed that in TIGR4 Δ*rr09* the *rlrA* pathogenicity islet is strongly up-regulated, while the virulence factor *pspA* is down-regulated. In D39 Δ*rr09* genes involved in sugar uptake (e.g. PTS systems) *sp0061-sp0064* were drastically down-regulated. Furthermore, we found that the putative operon *sp2141-sp2144*, and genes encoding a β-galactosidase, a putative sugar isomerase and putative aldose epimerase were strongly down-regulated in D39, and not regulated in TIGR4. qRT-PCR was extended to strain 0100993 in which these genes were not regulated by RR09 in strain 0100993. In addition, the down-regulation of pneumococcal virulence factors PspA and HtrA, found in previous analysis was confirmed by western immunoblot. The gene *sp0063*, found in STM screen (Hava and Camilli, 2002) was drastically down-regulated in D39 Δ*rr09*. D39 *sp0063* deficient mutant did not show significant attenuation in an animal model, excluding the possibility that the control of virulence by TCS09 is directly mediated via *sp0063*. In conclusion, our results indicate that TCS09 regulated several genes that are important for the virulence of the pneumococcus, and the regulation of pneumococcal genes by TCS09 is strain-specific.

Chapter 6

Analysis of genetic variation within *S. pneumoniae*

6.1 Comparative genomic hybridization

S. pneumoniae can be divided into more than 90 serotypes based on capsular diversity. The pneumococci are a naturally transformable species and the population structure is characterised by frequent horizontal gene transfers and recombination-mediated gene plasticity (Enright *et al.*, 1998). Genome comparison studies have provided a new basis to understand differences in virulence (presence or absence of regions containing virulence genes and antibiotics resistance determinants) as well to understand the gene transfer events by the analysis of transposable elements, insertion sequences, prophages and phage like elements. Two complete genome sequences for *S. pneumoniae* TIGR4 and R6 strains (Hoskins *et al.*, 2001; Tettelin *et al.*, 2001) and a draft genome sequence of strain G54, a type 19F clinical isolate (Dopazo *et al.*, 2001) were published. The genome sequence of serotype 23F strain as well as another 3 strains are currently being sequenced by Sanger institute (http://www.sanger.ac.uk/). Furthermore, an additional genome sequence of strain 670-6B is in progress by TIGR institute (http://www.tigr.org). One remarkable feature of the pneumococcal genomes is the presence of a high number of repeated sequences classified as insertion sequence, BOX and RUP elements.

Previous studies of transcriptome profile between *rr06* and *rr09* mutants and the respective wild-types showed that the gene regulation by TCS06 and TCS09 is strain-dependent. An example was found for the *rlrA* pathogenicity islet. This pathogenicity islet was shown to be up-regulated in both TIGR4 mutants but not in R6, D39 and 0100993 strains. Recent studies from our laboratory have also demonstrated strain-specific effects in gene regulation by the TCS04 (McCluskey *et al.*, 2004). The different transcriptional profiles observed for the *rr* mutants suggest that the TCS may have different gene regulation in the different strain backgrounds. To address the question of the different transcriptional profile occurring due to the variation in their respective genomes, i.e. as a result of absence of the genes found in the transcriptome analysis, a comparative genomic hybridization (CGH) study was performed using R6, D39 and 0100993 strains in comparison with reference strain TIGR4. With the exception of a small number of genes, only the genes that compose the *rlrA* pathogenicity islet, differentially expressed in TIGR4 *rr06* and *rr09* mutants but not in other strains, were found to be absent in R6, D39 and 0100993 strains. To characterize the genetic diversity of *S. pneumoniae* an additional group of 10 *S. pneumoniae* strains was selected to represent 6 major multi-locus sequence types found in the UK associated with disease or carriage and compared with reference strain TIGR4 by DNA microarray analysis.

6.2 Overall genomic diversity in *S. pneumoniae*

The genomic content of a collection of 13 *S. pneumoniae* strains was examined using PCR-based microarray chips designed to represent all genes of the 2 currently available complete pneumococcal genome sequences from TIGR4 (serotype 4) and R6 (unencapsulated laboratory strain derived from the type 2 strain D39). Our collection of isolates was chosen to represent diverse serotypes (x7) and multi-locus sequence types (x8). These include the reference strain TIGR4 and 3 other strains used in our laboratory, D39 (Avery *et al.*, 1944), R6 (Smith and Guild, 1979) and 0100993 (Throup *et al.*, 2000). The remaining isolates include six serotype 14, currently a prominent serotype among disease isolates in the UK and elsewhere (Brueggemann *et al.*, 2003; Denham and Clarke, 2005; Hanage *et al.*, 2005b; Kalin, 1998). Importantly, strains of the same serotype and multi-locus sequence type were included to investigate the levels of diversity among these apparently closely related strains. All strains together with relevant strain information are listed in Table 6.1. Genomic DNA from each isolate was tested against DNA from the reference strain, TIGR4 and the presence or absence of individual genes was predicted based on the relative hybridisation levels observed for each gene. Since the PCR products used in microarray analysis did not necessarily cover variable regions, the "absence" of the gene in this study means the gene is missing or is highly variable. The PCR-based microarray was designed to include all TIGR4 genes and additional genes present in the R6 genome sequence, this microarray comparison identified the absence of genes in the test isolate compared to TIGR4 and identify genes which were common to the test isolate and R6 but absent in TIGR4. In the analysis these genes appeared as insertions. This current study will focus on genes which were absent in the test isolates in comparison with reference strain TIGR4 and illustrate the R6 genes present in test strains but absent in TIGR4. A diagrammatic representation of these comparisons is also shown in Figure 6.1.

Table 6.1- *S. pneumoniae* strains used in genomic comparison study.

Strain	Specimen	Serotype /ST type	Year of isolation	Location	Reference
TIGR4	Blood	4/205	N/A	Norway	(Aaberge *et al.*, 1995; Tettelin *et al.*, 2001)
0100993	N/A	3/180	N/A	N/A	(Throup *et al.*, 2000)
R6	Laboratory strain derived from *in vitro* passage	2/128	1950s	N/A	(Ravin, 1959)
D39	N/A	2/128	Deposited NCTC 1948	N/A	(Avery *et al.*, 1979)
P11	Blood	14/9	2003	Aberdeen, Scotland	(Jefferies *et al.*, 2004)
N16	Blood	14/9	2000	Dundee, Scotland	(Jefferies *et al.*, 2004)
P33	Eye	14/9	2001	Dumfries, Scotland	(Jefferies *et al.*, 2004)
10	Blood	14/124	2000	Aberdeen, Scotland	(Jefferies *et al.*, 2004)
48	Blood	14/124	2000	Glasgow, Scotland	(Jefferies *et al.*, 2004)
50	Blood	14/124	2001	Glasgow, Scotland	(Jefferies *et al.*, 2004)
P49	Blood	3/180	2002	Glasgow, Scotland	(Jefferies *et al.*, 2004)
PMEN7	N/A	19A/75	<1997	South Africa	(Smith and Klugman, 1997)
PMEN13	N/A	19A/41	<1997	South Africa	(Smith and Klugman, 1997)
PMEN23	N/A	6A/37	1994-2000	North Carolina, USA	(Richter *et al.*, 2002)

(N/A) Data not available or not known.

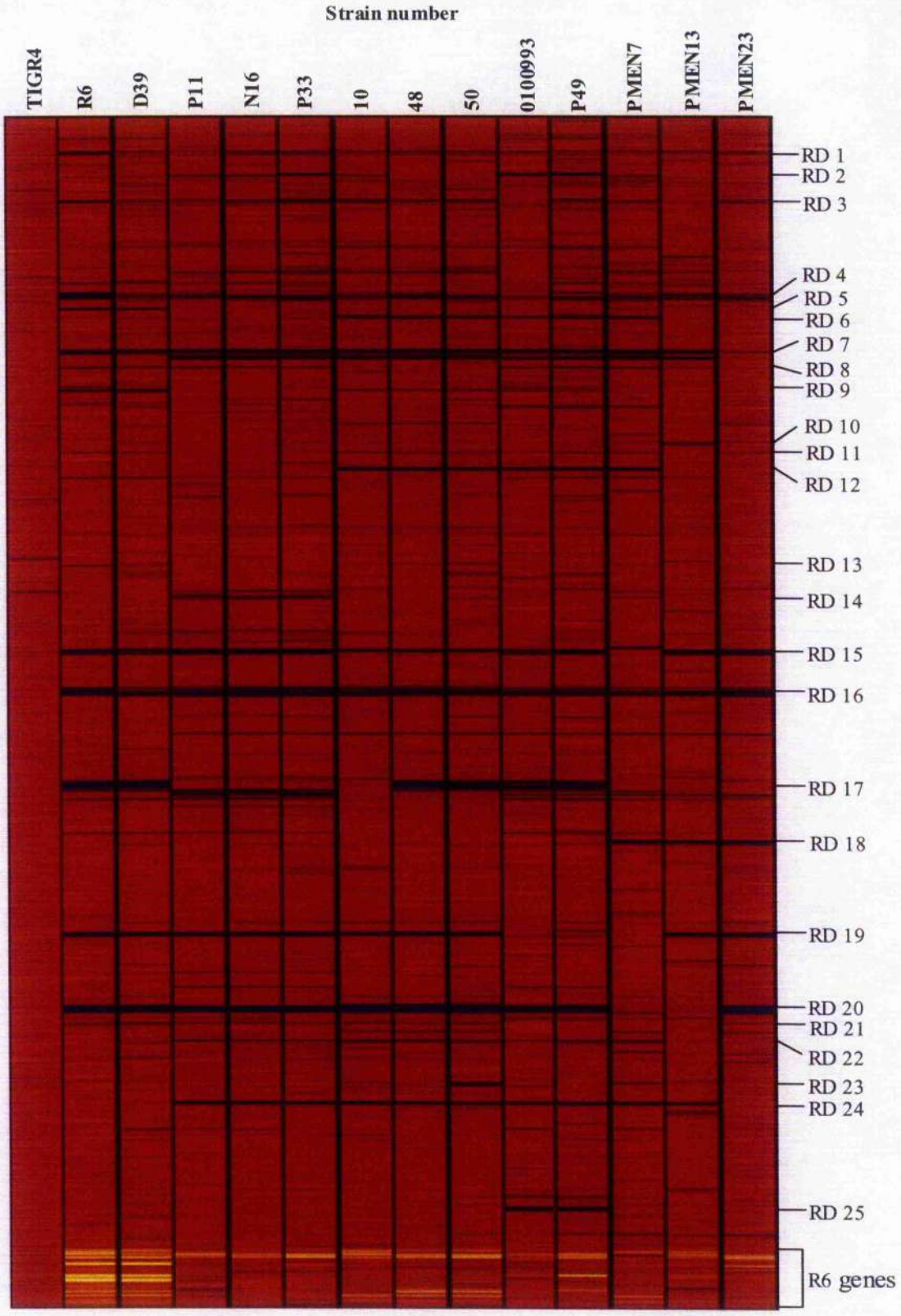


Figure 6.1- Genome wide distribution of ORFs among the *S. pneumoniae* strains studied.

The genes are arranged according to the annotated genome. Low hybridizations signals compared with reference strain TIGR4 are indicated by blue lines. The 25 regions of diversity (RD) that represent three or more contiguous genes not conserved in at least one of the strains are marked on the right side. The low hybridizations signals in TIGR4 compared with the test strains (R6 genes annotation) are indicated by yellow lines.

From the 2236 genes analysed (reference strain), 1903 genes were common to all strains, in contrast, 333 genes (~15%) were missing from at least one strain. Only 29 of the genes were missing in all strains. This noncore fraction of 15% is less than has been reported in similar studies with *Helicobacter pylori* (22%) (Salama *et al.*, 2000). With the exception of IgA1 protease gene (*iga*) (Adrian *et al.*, 2004), none of the genes implicated in virulence including those for hyaluronidase, neuraminidase A and B, autolysin, pneumolysin, *pspA*, and other surface antigens PsaA and PavA, are present in this core set (Paton *et al.*, 1997).

The thirteen-pneumococcal two-component systems and the orphan *rr*14 (*ritR*) were also found to be present in all strains examined. The numbers of deleted genes from each test isolate is given in Table 6.2 together with the percentage, which falls into the different functional categories described on the TIGR website (http://www.tigr.org). The most common category of absent genes in all strains was genes that encode hypothetical proteins or proteins with unknown functions. A substantial number of other genes could be categorised as encoding cell surface-associated proteins (including all genes classified as cell envelope, transport and binding or protein fate). Between 33% (strain D39) and 24.1% (strain N16) of the absent genes were of this type. Genes encoding proteins involved in cellular processes, energy metabolism, regulatory function, mobile and extrachromosomal element functions and signal transduction were also found to be absent in all strains tested.

Table 6.2- Different functional categories of deleted genes.

Strain	**R6**	**D39**	**P11**	**N16**	**P33**	**10**	**48**	**50**	**0100993**	**P49**	**PMEN7**	**PMEN13**	**PMEN23**
N° of deleted genes	172	154	145	148	129	126	148	163	179	177	127	112	116
Category[1)]	**% total in each category**												
Hypothetical proteins	32.2	26.6	34	37.8	32	34.2	33.1	34.3	29.1	26.6	31.5	28,7	32
Cellular processes	6.3	3.6	6.1	6.7	4.9	3.5	2.1	3.8	4.5	5.2	3.9	6.1	3.6
Unknown function	6.3	5	4.8	3.4	4.1	4.4	4.8	6.3	5.6	5.8	5.5	3.5	5.4
Transport and binding Protein	10.2	11.5	6.8	6	4.9	5.3	10.3	10.6	11.2	11	7.8	7	8.9
Energy metabolism	2.9	2.9	4.1	4	4.9	5.3	5.5	5.6	4.5	4.6	4.7	2.6	1.8
Cell envelope	13.7	16.5	13.6	13.4	16.4	21	14.5	12.5	14	11.6	14.1	16.5	19.6
Regulatory function	5.1	6.5	4.1	5.4	4.9	6.1	6.9	6.9	5.6	5.2	7.1	6.1	6.3
Mobile and extrachromosomal element functions	12	13	11.6	11.4	10.7	3.5	4.8	4.4	8.9	5.8	18.1	8.6	8
Protein fate	4	5	4.8	4.7	5.7	5.3	4.8	3.8	5	6.3	6.3	5.2	3.5
Signal transduction	2.3	2.9	4.8	4.7	5.7	7	5.5	5	5	4.6	3.1	7.4	3.5
Other	5.1	5.8	5.4	5.4	5.7	8.8	7.6	3.8	6.7	5.2	12.6	7.8	8

[1)] Category assigned using the gene information on http://www.tigr.org.

6.3 Examination of variability at a select locus

The *S. pneumoniae* choline binding proteins (CBPs) have been implicated in adhesion; 12 CBPs have been identified (Novak and Tuomanen, 1999). They are surface-exposed proteins associated with the pneumococcal choline-containing wall teichoic acid via C-terminal repeats. Four of the 10 *cbp* genes included on the microarray indicated sequence variation (encoded by *cpbJ*, *pspC*, *pcpA* and *cbpI*), in agreement with reported variability in *pspC* (Brooks-Walter *et al.*, 1999).

Because of the known heterogenous characteristics of the PspC protein we had included a PspC probe designed to the non-heterogeneous part of this protein (*spR6-1995*) in the initial design of the microarray. Analysis of the hybridisation patterns for this probe (data not shown), demonstrated the presence of the *pspC* gene only in the strains R6, TIGR4, D39, 0100993, PMEN 7, PMEN13 and PMEN 23. A PCR validation confirms the presence or absence of the *spR6-1995* in the different strains when we use the R6 *pspC* probe (Figure 6.2). The PCR analysis through the variable size or absence of PCR product demonstrates, even with the probe designed for the conserved region of *pspC*, variability in this part of *pspC* sequence between the different strains.

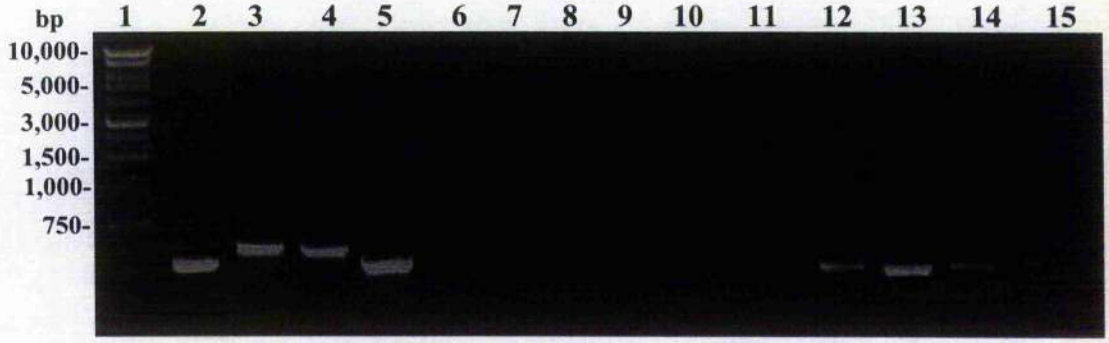


Figure 6.2- PCR of test strains and reference TIGR4 for the *pspC* gene of *spR6-1995* probe.

Validation of microarray analysis of *pspC* using non-heterogeneous probe *spR6-1995*. R6 (lane 2), TIGR4 (lane 3), 0100993 (lane 4), D39 (lane 5), P11 (lane 6), N16 (lane 7), 10 (lane 8), 50 (lane 9), P33 (lane 10) P49 (lane 11) PMEN7 (lane 12), PMEN13 (lane 13), PMEN23 (lane 14), 48 (lane 15). The DNA ladder marker used was the 1 kb plus ladder (Promega).

In addition to choline-biding proteins described previously as having a highly variable sequence in different strains, *S. pneumoniae* has others surface proteins that are anchored to the cell wall via a structure close to the C terminus consisting of an LPXTG sequence motif followed by a membrane spanning hydrophobic region and a charged sequence. Several of these cell surface proteins are highly variable when analyzed in different *S. pneumoniae* strains such as the PrtA protease and the IgA protease (associated with the bacterial cell surface via an N-terminal motif-LPXTG) (Bethe *et al.*, 2001; Lomholt, 1995; Poulsen *et al.*, 1996). The Table 6.3 shows the percentage of sequence similarity between these variable genes and known genome sequences of three different strains, TIGR4, R6 and G54. Thus the apparent deletion of these genomic regions in all strains is more likely due to the considerable heterogeneity between the TIGR4 *pspC*, *iga*, *zmpB*, *prtA* and *pcpA* and those of the test strains.

Table 6.3- Homology of selected variable genes of know genome sequences.

	TIGR4[3]	R6[3]	G54[3]
Genes		**% of homology[4]**	
pspC (sp2190)[1]	100	81.2	61.7
pspC (spr1995)[2]	81.2	100	58.1
iga	100	86.5	69
zmpB	100	61.4	63.7
prtA	100	97.2	96.4
pcpA	100	86.9	96.4

[1] TIGR4 sequence annotation
[2] R6 sequence annotation
[3] Genome sequences published in TIGR institute (http://www.tigr.org).
[4] Percentage of homology of select genes with sequence heterogeneity. Percentage was calculated on alignment program AlignX (Vector NTI 9.1.0, Invitrogen Corporation).

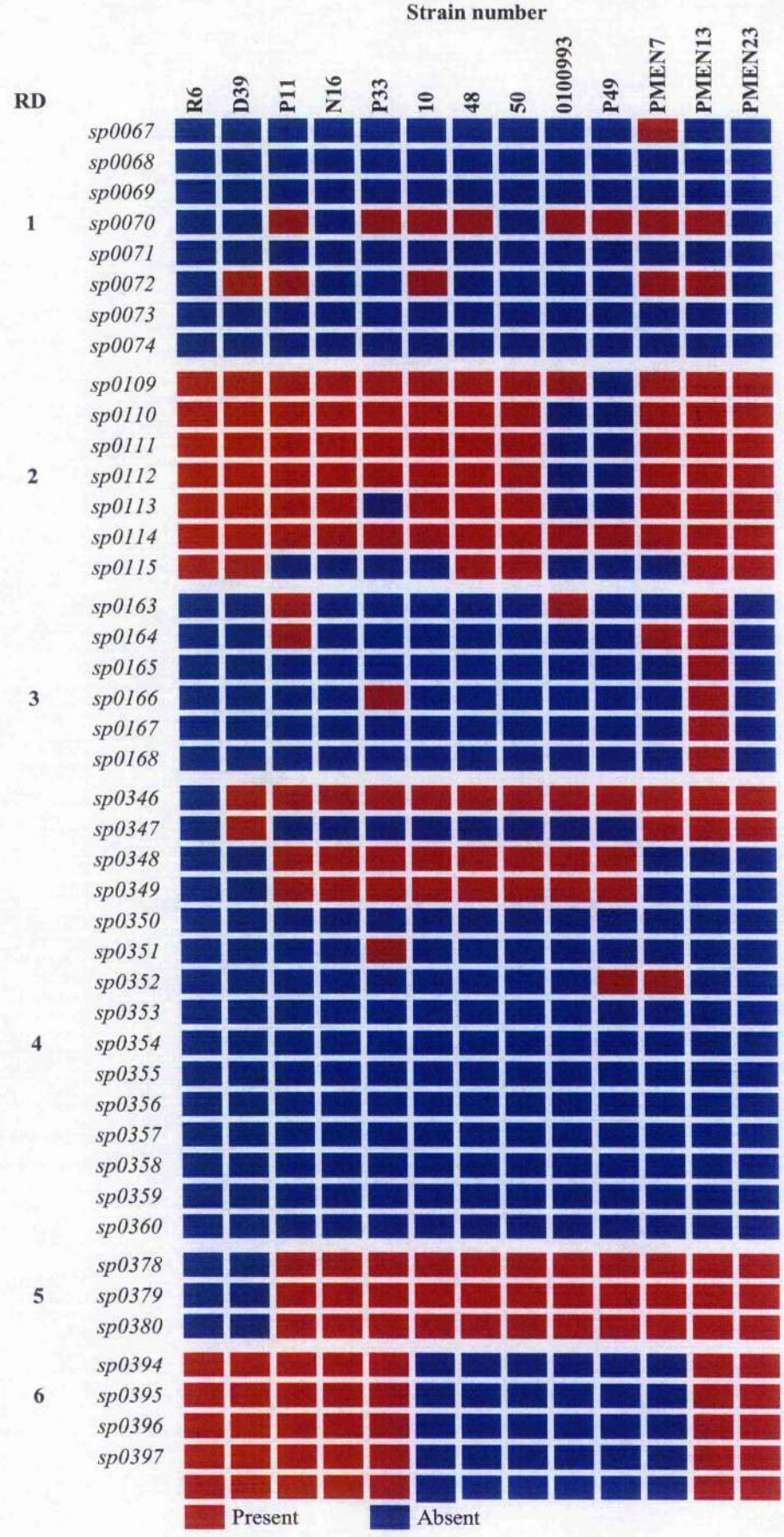
6.4 Genomic distribution and characteristics of chromosomal deletions

The comparative genome hybridization of the 13 strains compared to the reference strain showed at least twenty-five regions of diversity (RD), where three or more contiguous genes (according to the TIGR4 annotation) where not conserved in all strains (Figure 6.1 and Table 6.4). Previous studies in other organisms have referred these clusters as regions of difference (Broekhuijsen *et al.*, 2003; Fitzgerald *et al.*, 2001). Importantly, there was strong agreement between the array results and the sequenced R6 genome sequence. Of the 248 RD genes identified, the array agreed with the result expected based on the R6 genome sequence for 242 of these genes (97.5%). In addition the distribution of 200 genes from the other test strains was investigated by PCR. Of these 200, 189 (94.5%) showed agreement between the microarray and PCR. Together these data confirm the microarray analysis to be a good predictor of the presence/absence of genes/probe sequences. The RDs were numbered the pneumococcal regions RD1 to RD25 accordingly and ranging considerably in size from 1.7 kb to 36.9 kb. These twenty-five RDs represent approximately 242 kb corresponding to ~11% of the total genome of TIGR4. As will become evident, these regions are often not completely deleted and sometimes only a fraction of the genes are deleted in certain strains (Figure 6.3). Fourteen of these regions in TIGR4 do not hybridize with the serotype 2 strain R6, twelve of these nonconserved regions were identified by Bruckner and co-workers and the remaining two RDs were also identified but not as clusters (Bruckner *et al.*, 2004).

Table 6.4- Regions of diversity identified in 13 test strain.

Region of diversity	5' coordinate	3' coordinate	Size	Variable Sp[1)] n°
RD 1	72141	81204	9.0 kb	*sp0067-0074*
RD 2	110794	116973	6.2 kb	*sp0109-0115*
RD 3	159192	164810	5.6 kb	*sp0163-0168*
RD 4	320076	336443	16.4 kb	*sp0346-0360*
RD 5	356986	359377	3.3 kb	*sp0378-0380*
RD 6	372309	377694	5.4 kb	*sp0394-0397*
RD 7	436037	447414	12.6 kb	*sp0460-0468*
RD 8	451961	459021	7.1 kb	*sp0473-0478*
RD 9	511718	517349	5.6 kb	*sp0531-0544*
RD 10	611196	622142	11.0 kb	*sp0643-0648*
RD 11	634854	642899	8.0 kb	*sp0664-0666*
RD 12	663182	667616	4.4 kb	*sp0692-0700*
RD 13	837760	839434	1.7 kb	*sp0888-0891*
RD 14	897939	902629	4.6 kb	*sp0949-0954*
RD 15	988049	1000382	12.6 kb	*sp1050-1065*
RD 16	1064391	1073637	9.2 kb	*sp1129-1147*
RD 17	1239143	1272833	33.7 kb	*sp1315-1352*
RD 18	1352438	1364506	12.1 kb	*sp1433-1444*
RD 19	1512872	1523192	10.3 kb	*sp1612-1622*
RD 20	1656781	1692953	36.9 kb	*sp1756-1773*
RD 21	1709981	1715310	5.3 kb	*sp1793-1799*
RD 22	1740005	1741506	3.2 kb	*sp1828-1830*
RD 23	1824670	1827823	3.2 kb	*sp1911-1918*
RD 24	1850362	1859718	9.4 kb	*sp1948-1955*
RD 25	2077252	2082547	5.3 kb	*sp2159-2166*

[1)] TIGR4 annotation (http://www.tigr.org)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P11
N16
P33
10
48
50
0100993
P49
PMEN7
PMEN13
PMEN23
RD
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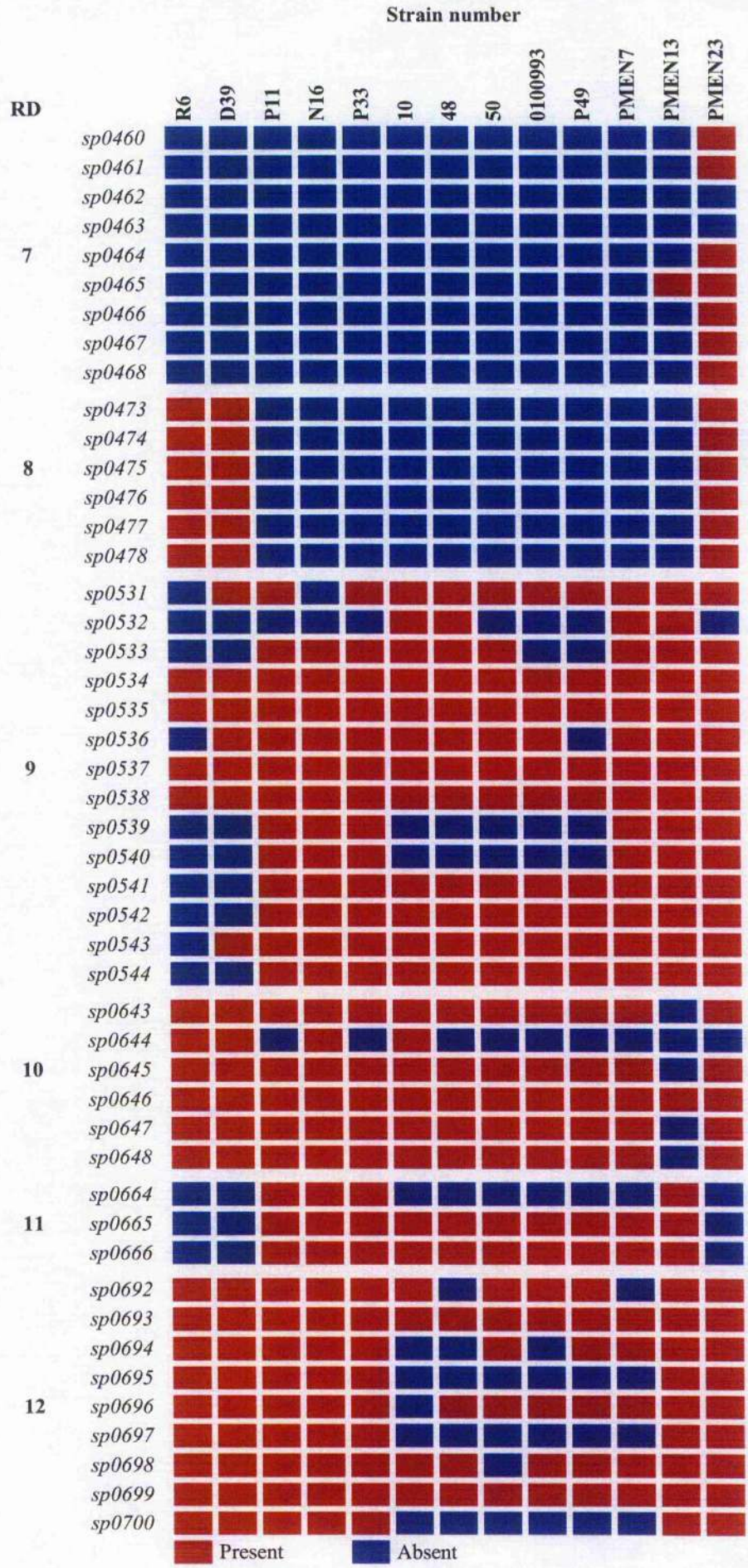
sp0068
sp0069
1
sp0070
sp0071
sp0072
sp0073
sp0074
sp0109
sp0110
sp0111
2
sp0112
sp0113
sp0114
sp0115
sp0163
sp0164
sp0165
3
sp0166
sp0167
sp0168
sp0346
sp0347
sp0348
sp0349
sp0350
sp0351
sp0352
sp0353
4
sp0354
sp0355
sp0356
sp0357
sp0358
sp0359
sp0360
sp0378
5
sp0379
sp0380
sp0394
6
sp0395
sp0396
sp0397
Present
Absent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11
sp0665
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12
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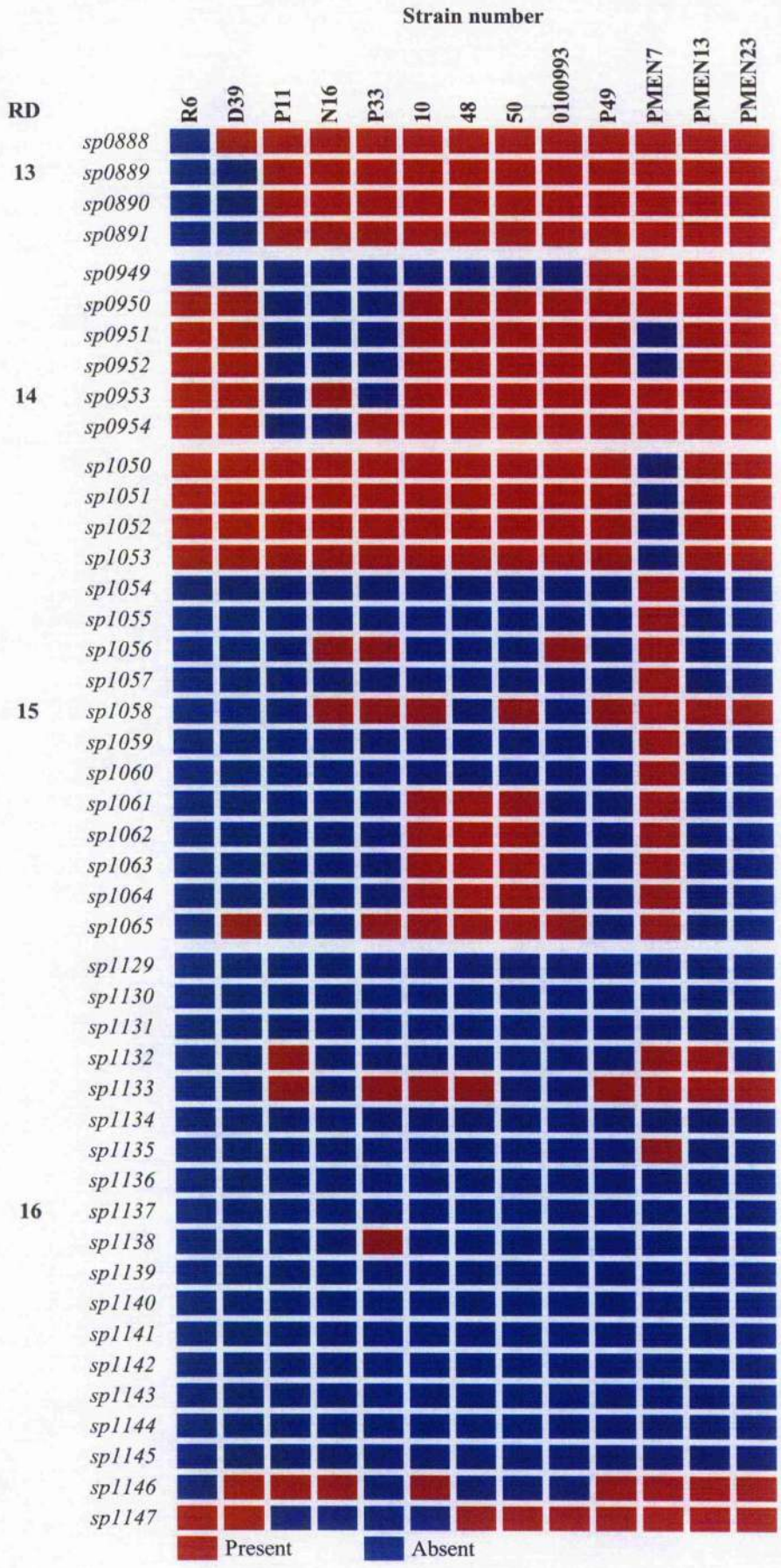
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P33
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0100993
P49
PMEN7
PMEN13
PMEN23
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15
16
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sp1136
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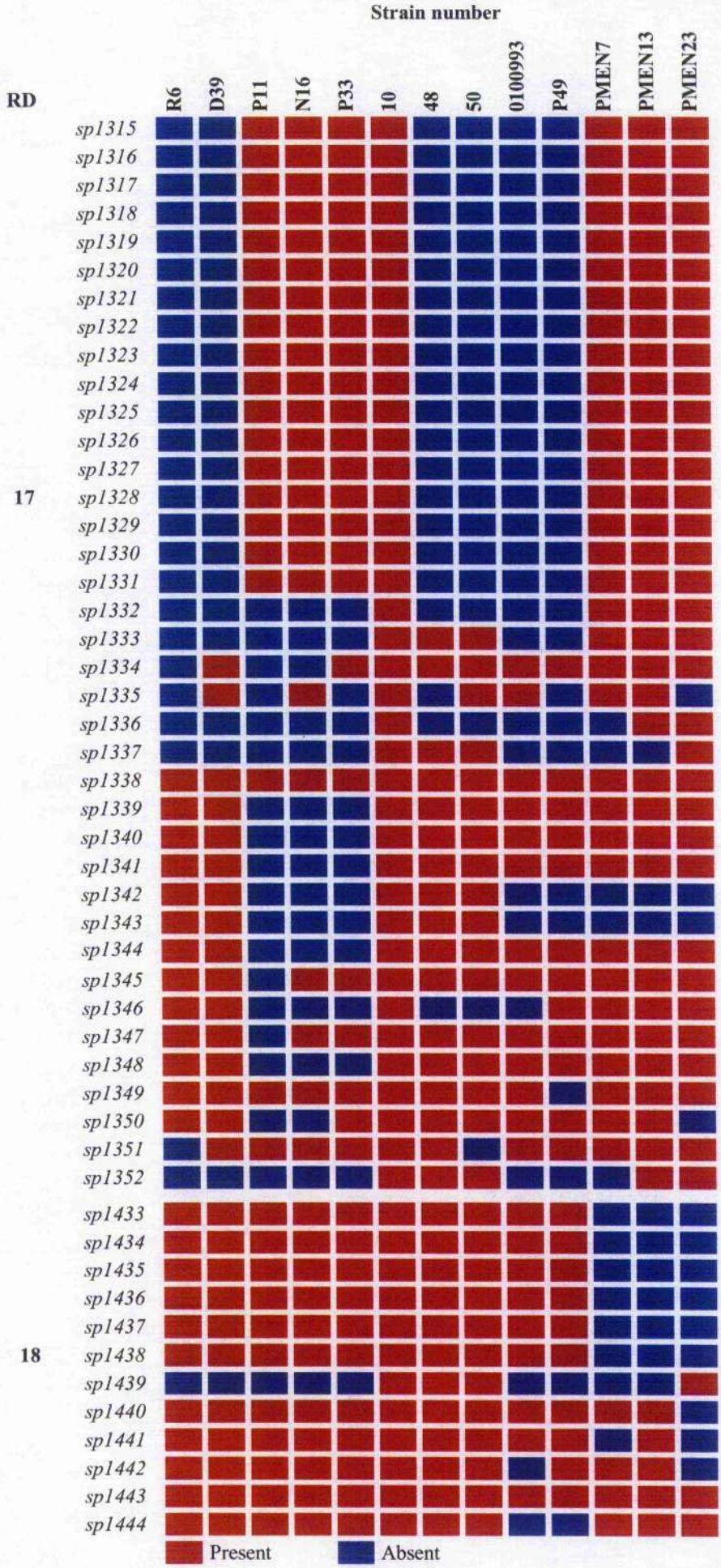
sp1145
sp1146
sp1147
Present
Absent


Strain number
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10
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P49
PMEN7
PMEN13
PMEN23
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sp1318
sp1319
sp1320
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sp1433
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sp1444
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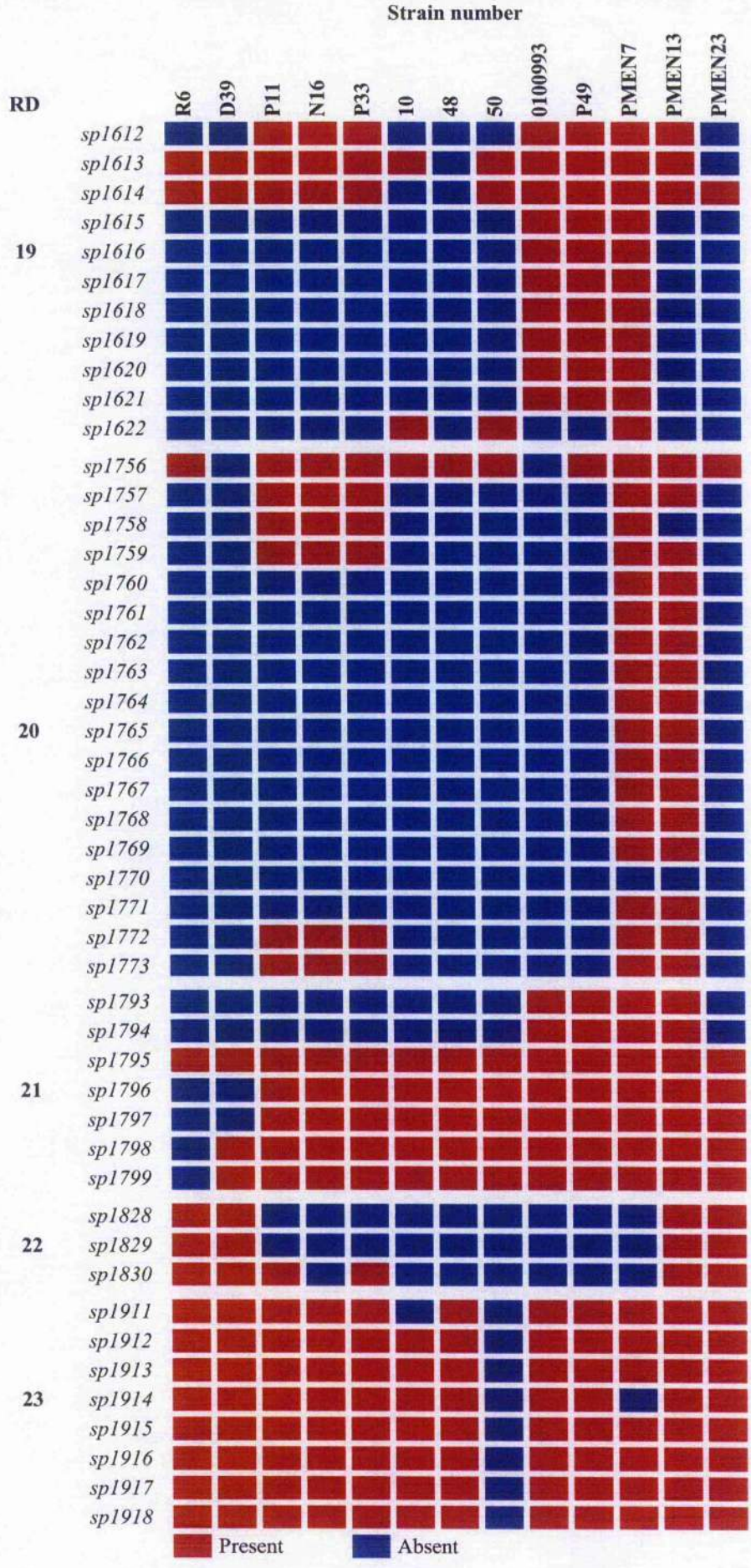
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P49
PMEN7
PMEN13
PMEN23
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sp1828
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sp1912
sp1913
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sp1915
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sp1918
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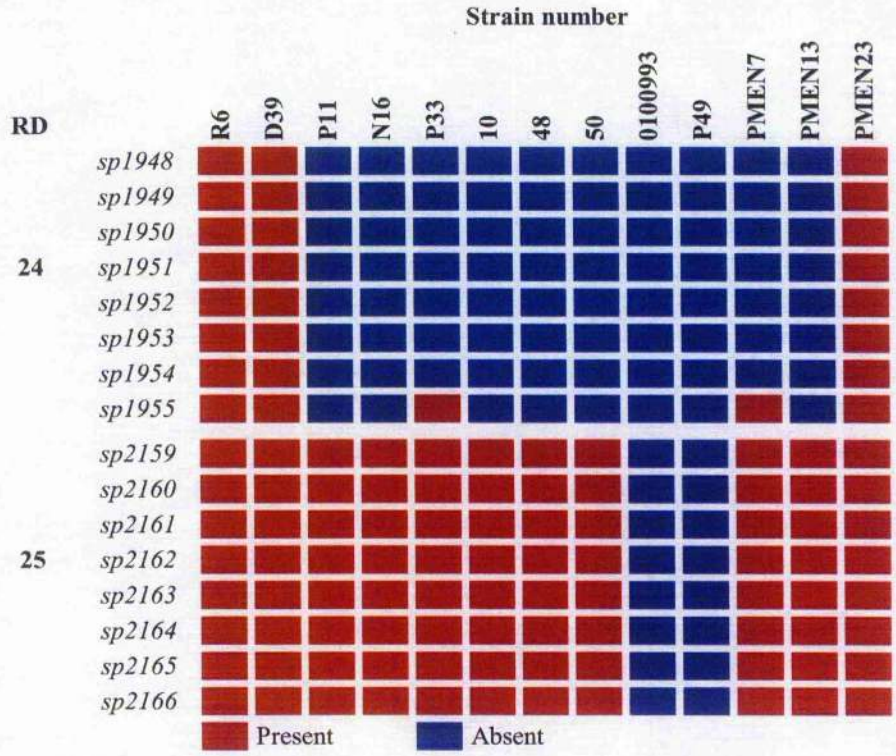


Figure 6.3- Regions of diversity.

Presence and absence of the gene was determined by comparative genomic hybridization. Red positive, blue negative. Gene number and annotation taken from TIGR4 genome at http://www.tigr.org.

The first region RD1 is a 9 kb chromosomal region encoding 8 ORFs, *sp0067* to *sp0074*. Six of the eight ORFs encode hypothetical or proteins with unknown functions and the remaining 2 ORFs (*sp0069* and *sp0071*) encode the pneumococcal *cbpI* and the *zmpC* (zinc-metalloprotease) proteins. The *cbpI* is characterized by terminal repeats responsible for the attachment to the choline-containing teichoic acids of *S. pneumoniae* (Garcia *et al.*, 1998). The zinc-metalloprotease appears to have a role in invasion. Oggioni and co-workers demonstrate that human matrix metalloproteinase 9 (MMP-9) as the substrate the zinc metalloproteinase (ZmpC) located on the surface of *S. pneumoniae* (Oggioni *et al.*, 2003). The other 2 metalloprotease, ZmpB (*sp0664*) and the IgA (*sp1154*) are missing in some of the test strains (Figure 6.3 and Figure 6.4). The *zmpB* is absent in strains PMEN7, PMEN23, R6, D39, P49, 0100993 and the three strains of ST 124 group, and the *iga* is absent in PMEN13, PMEN23, R6, D39, P49, 0100993 and in the ST 9 strains group. IgA1 protease and ZmpB significantly contribute to the virulence of the pneumococcus, while the impact of ZmpC is less profound (Chiavolini *et al.*, 2003).

RD2 is a 6.2 kb chromosomal region encoding 7 ORFs (*sp0109* to *sp0115*). The *sp0109*, bacteriocin locus is missing only in strain P49. This region also encodes an ABC

transporter. The other 3 ORFs are hypothetical proteins and one disrupted reading frame. This RD is present in majority of test strains, but almost totally absent in 0100993 and P49.

The RD3 (5.6 kb) encoding 6 ORFs (*sp0163* to *sp0168*) and contains a putative macrolide efflux system. *sp0163* encodes the protein PlcR a transcriptional regulator. The other ORFs encode hypothetical proteins or unknown function. Analysis of the microarray data demonstrates the absence of this cluster in majority of the test strains, except in PMEN13. The regulatory protein is only present in 0100993, P11 and PMEN13.

RD4 is the capsule encoding region of the pneumococcal chromosome and not surprisingly was shown in this analysis to have genetic variation between the different strains tested. This RD was identified as 16.4 kb in length encoding 15 ORFs, most of which encode proteins involved in capsule biosynthesis. BLAST searches of the sequences from microarrays of the capsule genes of the *S. pneumoniae* capsular loci (http://www.sanger.ac.uk) for each different serotype strain, showed that the microarray analysis and BLAST searches for these ORFs coincide (Table 6.5). The *cpsB* (*sp0347*), *cpsC* (*sp0348*), or the *cpsD* (*sp0349*), genes that are more often conserved diverge in some strains. The microarray analysis did not show variation in the conserved gene *cspA* (*sp0346*) in any strain, except in unencapsulated strain R6. Variation in different capsule genes between different pneumococcal strains has been well documented (Claverys *et al.*, 2000; Garcia and Lopez, 1997).

Table 6.5- Homology of capsular genes in 13 *S. pneumoniae* test strains.

	R6	D39	P11	N16	P33	10	48	50	0100993	P49	PM7[1)]	PM13[2)]	PM23[3)]
Genes									% of homology[4)]				
sp0346	97	97	97	97	97	97	97	97	97	97	88	88	83
sp0347	97	97	83	83	83	83	83	83	97	97	88	95	98
sp0348	69	69	96	96	96	96	96	96	69	69	69	69	69
sp0349	77	77	97	97	97	97	97	97	77	77	75	75	76
sp0350	-	-	-	-	-	-	-	-	-	-	-	-	-
sp0351	-	-	-	-	-	-	-	-	-	-	-	-	-
sp0352	-	-	-	-	-	-	-	-	-	-	-	-	-
sp0353	-	-	-	-	-	-	-	-	-	-	-	-	-
sp0354	-	-	-	-	-	-	-	-	-	-	-	-	-
sp0355	-	-	-	-	-	-	-	-	-	-	-	-	-
sp0356	-	-	-	-	-	-	-	-	-	-	-	-	55
sp0357	57	57	-	-	-	-	-	-	57	57	71	71	-
sp0358	-	-	-	-	-	-	-	-	-	-	-	-	-
sp0359	-	-	-	-	-	-	-	-	-	-	-	-	-
sp0360	-	-	-	-	-	-	-	-	-	-	-	-	-

[1)] PMEN7
[2)] PMEN13
[3)] PMEN23
[4)] Percentage of homology of capsular genes shared between the sequences spotted in microarrays and the sequences of the different serotypes of *S. pneumoniae* (http://www.sanger.ac.uk).

RD5 contains only 3 genes (*sp0378-sp0380*). This small region (3.3 kb) is totally missing in both serotype 2 strains, and present in all remaining strains. The *sp0378* encodes the choline-biding protein CbpJ, while the other two genes encodes hypothetical proteins.

RD6 is a small cluster, 5.4 kb, composed of 4 ORFs (*sp0394-sp0397*) and contain a PTS system, a transcriptional regulator (*sp0395*) and an energy metabolism protein (*sp0397*). This cluster is totally absent in 0100993, 10, 48, 50, P49 and PMEN7 but present in other test strains.

The next region of variation, RD7 is particularly interesting and consists of 12.6 kb of DNA encoding 9 ORFs (*sp0460-sp0468*). This cluster encodes the *rlrA* pathogenicity islet and consists of seven genes of which *sp0462-sp0464* genes are predicted to encode LPXTG-containing microbial surface components recognizing adhesive matrix molecules that bind to components of the extracellular matrix of the host, and three sortase enzymes *sp0466-sp0468*, as well *sp0461* (*rlrA*) a positive regulator of these cluster. A recent study had shown evidence that *rlrA* pathogenicity islet encoded pilus-like structures on their surface, influencing pneumococcal adherence and the development of pneumonia and bacteraemia in mice and also host inflammatory response stimulation (Barocchi *et al.*, 2006). This region of cell wall surface protein genes is preceded by a transposase encoding

gene (*sp0460*). Our microarray studies demonstrated deletion of this complete region in all the strains except for PMEN13 and PMEN23. In PMEN13 the gene *sp0465* is present and in the PMEN23 only two of the three genes that encode the cell wall proteins are absent. A PCR for the PMEN23 was made and it confirmed the absence of these two cell wall proteins and the presence of the others ORFs.

RD8 is a 7.1 kb region encoding 6 ORFs (*sp0473-sp0478*). Included in this region are three genes (*sp0474*, *sp0476* and *sp0478*), which encode phosphotransferase systems, one specific for cellobiose transport and two for lactose transport. This cluster is present in R6, D39 and PMEN23, in other strains it is completely absent. The gene product of *sp0477* is 6-phospho-beta-galactosidase and was absent from all strains except for the two above. The other ORF encodes a hypothetical protein.

RD9 is a cluster of 5.6 kb (*sp0531-sp0544*) that contains 14 ORFs. This region encodes 5 bacteriocins (BlpI, BlpJ, BlpK, BlpM and BlpO) and all the five are missing only in R6 strain. Bruckner and co-workers also identified these bacteriocins absent in R6 (Bruckner *et al.*, 2004). These bacteriocins have been described in detail (de Saizieu *et al.*, 2000; Reichmann and Hakenbeck, 2000). The other ORFs encode other cellular processes proteins, hypothetical or unknown function proteins and two transposases.

The next region, RD10, has 11.0 kb and encodes 6 ORFs. Included in this region are 3 PTS systems. Two of these systems are missing in PMEN13, the other one (*sp0646*) it is present in all strains. This region was complete in R6, D39 and 10 and only the disrupted reading frame *sp0644*, except the three strains listed above, is missing in all strains. The last ORF encodes a beta-galactosidase and is absent only in PMEN13.

RD11 is an 8.0 kb region encoding 3 ORFs (*sp0664-sp0666*). All three genes are missing in PMEN23 and in the two serotypes 2 strains. On the other hand, this cluster is totally present in ST 9 strains and in the remaining strains only the first gene is absent. The *sp0664* encodes the zinc metalloprotease ZmpB (referred to in the description of RD1). The others ORF encodes hypothetical or unknown function proteins.

RD12 is a 4.4 kb region and encodes 9 ORFs (*sp0692-sp0700*). This cluster was complete in R6, D39, P11, N16, P33, PMEN13 and PMEN23. In all other strains examined the pattern of gene deletions was more complex. The *sp0697* gene encodes an ABC transporter and is missing in 0100993, 10, 48, 50, P49 and PMEN7. The other ORFs are hypothetical or proteins of unknown function.

RD13 was the small 1.7 kb region of diversity found in this analysis (*sp0888-sp0891*). This region is missing in serotype 2 strains. The *sp0888* gene is absent only in R6, while the other three genes are absent in both strains.

RD14 covers 4.6 kb and six genes in TIGR4 (*sp0949-sp0954*). This region is completely absent in P11 and in other ST9 strains only one gene is present. The *sp0949* encodes a transposase (IS1515) and is missing in R6, D39, P11, N16, P33, 10, 48, 50 and 0100993 strains. Although predicted to be inactive in TIGR4, due to a frameshift mutation introducing a premature stop codon, this element may be responsible for the unequal strain distribution of this region. *sp0950* encodes a predicted GNAT family acetyltransferase (GCN5-related N-acetyltransferase) as does *sp0953*, although they share only limited homology. *sp0951* encodes a conserved hypotetical protein, which contains a putative TfoX N-terminal domain. Identified in *H. influenza*, the TfoX/Sxy protein is essential for transformation in that species (Williams *et al.*, 1994; Zulty and Barcak, 1995). No data exists as yet for a role for *sp0951* in pneumococcal transformation and microarray analysis of the global response to competence stimulating peptide (CSP) in TIGR4 did not identify *sp0951* as being a CSP responsive gene (Peterson *et al.*, 2004). The *sp0952* is absent in PMEN7 and the ST9 strains and is annotated as encoding an alanine dehydrogenase carrying an authentic frameshift resulting in a premature stop codon. *sp0953* missing in P11 and N16 encodes a protein of unknown function. The last ORF of this cluster encodes a competence protein, CelA, one of the competence-specific genes involved in the natural transformation of *S. pneumoniae* (Luo and Morrison, 2003) and is absent in P11 and N16. None of the genes in this region were identified as virulence factors in the signature-tagged mutagenesis screen of TIGR4 and so their role in virulence is unclear as yet.

The next region of diversity, RD15, is 12.6 kb and encodes 16 ORFs (*sp1050-sp1065*). The first 3 OFRs are missing only in PMEN7 and encode hypothetical or proteins of unknown function. *sp1054* to *sp1056* encode transposon-associated proteins, and is absent in the majority of the strains except the PMEN7. *sp1057* encodes another protein PlcR a transcriptional regulator and the *sp1061* encodes a protein kinase. The protein PlcR is missing in all strains except in PMEN7 and the kinase is present in the ST 124 strain group and PMEN7. This cluster encodes an ABC transporter (*sp1062* and *sp1063*) and as the transposase protein (*sp*1064) is present in ST 124 strains group and PMEN7.

Most of the genes in RD16 are missing in all strains (Figure 6.3). RD16 is 9.2 kb in length and encodes 19 ORFs (*sp1129-sp1147*). The majority of the ORFs of this non-conserved region (*sp1132-sp1147*) encode hypothetical proteins. *sp1129* encodes the xerC4 integrase-

recombinase, involve in DNA metabolism (Reichmann and Hakenbeck, 2002) and *sp1130* and *sp1131* encode two transcriptional regulators that are absent in all the thirteen test strains.

The large RD17 is a 33.7 kb and contain 38 ORFs (*sp1315* to *sp1352*), the highest number found in this analysis. The first fraction of this region (*sp1315* to *sp1337*) was missing in its majority in the strains 0100993, P49, R6, D39 and two strains of the ST 124 group (48 and 50). The *sp1326* encode one of the three neuraminidases (NanC) and it was absent in the same strains mentioned above. The neuraminidases A and B as others pneumococcal proteins have been characterized as putative virulence factors (Wellmer *et al.*, 2002). The NanC encodes a polypeptide with the structural features of a neuraminidase exhibiting approximately 50% deduced amino acid sequence identity to NanB (Berry and Paton, 2000). Examination of *S. pneumoniae* R6 (http://www.tigr.org/) genome sequence indicates the presence only of the NanA and NanB and the absence of the NanC, in agreement with the microarray analysis. *sp1315* to *sp1322* encode proteins involved in sodium transport and release. 17 of the ORFs encode hypothetical proteins. *sp1341* to *sp1343* absent, in the strains ST 9 group appear to encode an ABC transporter involved in toxin secretion (Tettelin *et al.*, 2001). The *sp1336* encodes a protein involved in DNA metabolism and is missing in the majority of the strains. The presence or absence of these individual genes in the various strains is shown in Figure 6.3.

Another ABC transport system is encoded by the ORFs in RD18. This 12.1 kb region has 12 genes, 5 of which encode putative transposon proteins (*sp1439*, *sp1441-sp1444*), 2 encode hypothetical proteins (*sp1436-sp1437*), one encodes a transcriptional regulator (*sp1433*) and the remaining 4 ORFs encode the ABC transporter. All of the ABC transporter genes are missing in strain PMEN23 and three of then in PMEN7, PMEN13 as well the transcriptional regulator. This entire region is present in the three strains of ST 124 group, and in the R6, D39 and ST9 strain group only the *sp1439* (transposase) is absent.

RD19 is a 10.3 kb region (*sp1612-sp1622*) with 11 ORFs. This region encodes a PTS system with nitrogen metabolism (*sp1617* to *sp1621*), 3 transposon proteins (*sp1613*, *sp1614* and *sp1622*), 2 proteins involved in energy metabolism (*sp1615* and *sp1616*), and one hypothetical protein (*sp1612*). This RD is totally absent in strain 48, present in PMEN7 as the majority of the genes in the two serotype 3 strains. The PTS system is missing in all strains except in PMEN7, 0100993 and P49.

RD20, a region of 36.9 kb, is the largest region of diversity found in this genomic comparison, and encodes 18 ORFs (*sp1756-sp1773*). Most of the genes encode proteins with the LPXTG cell wall anchor motif which are important for the interaction with host cells and appear to be highly variable, similar to cell wall-associated choline-binding proteins (Bruckner *et al.*, 2004). The comparatively small number of ORFs for the size of this RD is due to the large size of one of the ORFs: *sp1772*. This ORF is 14.3 kb in length and encodes a large 4776-amino-acid serine-rich protein that is homologous to a glycoprotein in *S. gordonii* that has been shown to mediate platelet binding of this organism, and is therefore being considered to be important in the pathogenesis of infective endocarditis (Takamatsu *et al.*, 2004). Other genes in this locus encode accessory proteins that may play a role in export, expression and modification of the glycoprotein. *sp1759* and *sp1763* encode homologues of secretory proteins SecA and SecY (SecA2 and SecY2), respectively. SecA has a central and multifaceted role in pre-protein export. It can interact directly with the signal sequence of pre-secretory proteins (Akita *et al.*, 1990; Kimura *et al.*, 1991) and with the cytoplasmic chaperone SecB (Fekkes *et al.*, 1997; Hartl *et al.*, 1990). The organization of genes in the *secY2–A2* loci suggests that SecA2 and SecY2 are not essential for viability in *S. pneumoniae*, but may rather be required specifically for export of the serine-rich repeat proteins encoded upstream (Bensing and Sullam, 2002). The SecA-encoding gene was present in all strains except PMEN7, PMEN13 and the three ST 9 strains and the SecY-encoding gene is present only in PMEN7, PMEN13. A PCR for all genes of this region in strains PMEN7 and PMEN13 confirmed the microarray analysis for this locus. The presence or absence of the surface protein (*sp1772*) and the other ORFs can be seen clearly in Figure 6.3.

RD21 is a region with 5.3 kb (*sp1793* and *sp1799*). This region encode an ABC transporter (*sp1796* to *sp1798*), one gene (*sp1795*) involved in sucrose metabolism and a transcriptional regulator (*sp1799*), the two remaining genes encode hypothetical proteins. The majority of the ORFs (Figure 6.3) were missing in R6 and D39 strains. The strains 0100993, P49, PMEN7 and PMEN13 possessed all the genes found in TIGR4, the remaining strains had missing the 2 hypothetical proteins (*sp1793* and *sp1794*).

RD22 comprises 3 ORFs, *sp1828-sp1830* in a 3.2 kb region of the TIGR4 genome. All three genes were present in R6, D39, PMEN13 and PMEN23. In contrast, all were absent in N16, 10, 48, 50, 0100993, P49 and PMEN7. In the case of P11 and P33, only *sp1830* was found to be present. The genes are annotated, in numerical order as, UDP-glucose 4-epimerase (*galE*), galactose-1-phosphate uridylyltransferase (*galT*) and phosphate transport system regulatory protein (*phoU*). This latter gene was identified in the TIGR4 STM

screen and when the mutant was analysed further in competitive infections with wild-type it was attenuated in models of pneumonia, bacteraemia and nasal colonization (Hava and Camilli, 2002). The pneumococcus *galE* and *galT* genes revealed strong degree of homology with others bacteria, and these genes seem to be involved in galactose metabolism. Inactivation of *galE* and *galT* in *Lactococcus lactis* and *B. subtilis* respectively, results in the inability of bacteria grow on galactose (Krispin and Allmansberger, 1998; Vaughan *et al.*, 1998). Although these pneumococcal genes are uncharacterised, the STM data does show a potential for the selected distribution of these genes to influence the behaviour of strains.

The eight genes, *sp1911-sp1918* make up RD23 and cover a small region with 3.2 kb of the TIGR4 genome. This region is fully present in 12 strains. One other strain, PMEN7, lacks only a single gene, *sp1914*. However, in strain 50 this entire region is missing. This region is poorly characterised, with four of the genes annotated as encoding hypothetical proteins, the significance of the absence of this region in strain 50 is therefore unclear. A function for one of these hypothetical proteins, *sp1915*, is suggested by the presence of a LytTr DNA-binding domain found in various bacterial transcriptional factors. The remaining genes are annotated as a putative thioredoxin (*sp1911*) involved in energy metabolism, *cspC* (cold shock protein)-related protein with an authentic point mutation resulting in a premature stop codon (*sp1913*), PAP2 family protein (*sp1916*), a family of mainly phosphatase enzymes (PF-01569), and an ATP-binding protein (*sp1918*). None of the genes in this region were identified in the STM screen of TIGR4 (Hava and Camilli, 2002).

RD24 is a 9.4 kb region and contains 8 ORFs (*sp1948* to *sp1955*), which encode a bacteriocin formation protein (*sp1950*), a toxin secretion ABC transporter (*sp1953*), serine protease (*sp1954*) and five hypothetical proteins. The majority of the genes were missing in all strains, including the three proteins described above, except for PMEN23 and the two serotype 2 strains that possess all the genes (Figure 6.3).

The last region, RD25 is 5.3 kb with 8 ORFs (*sp2159* to *sp2166*). This region encodes a PTS system (*sp2161* to *sp2164*) and two genes involved in fucose metabolism (*sp2165* and *sp2166*). The remaining ORFs in this region encode hypothetical and unknown functions proteins. The two serotype 3 strains (1009933 and P49) were shown to be missing the 8 ORFs, the other strains were shown to possess the entire region found in TIGR4 (Figure 6.3).

The genome sequences of two strains, INV200 (ST 9, serotype 14) and OXC141 (ST 180, serotype 3) are almost complete (www.sanger.ac.uk). To validate the presence or absence of selected genes of ST9 (P11, N16 and P33) and ST180 strains (0100993 and P49) from four random RDs, the TIGR4 (reference strain) sequences of these genes were compared with known sequence of the INV200 and OXC141 strains. The majority of the BLAST searches for these genes showed a strong agreement with the microarray analysis. The differences between our analysis and the BLAST searches may be related to the diversity between strains of the same ST (see section 6.5).

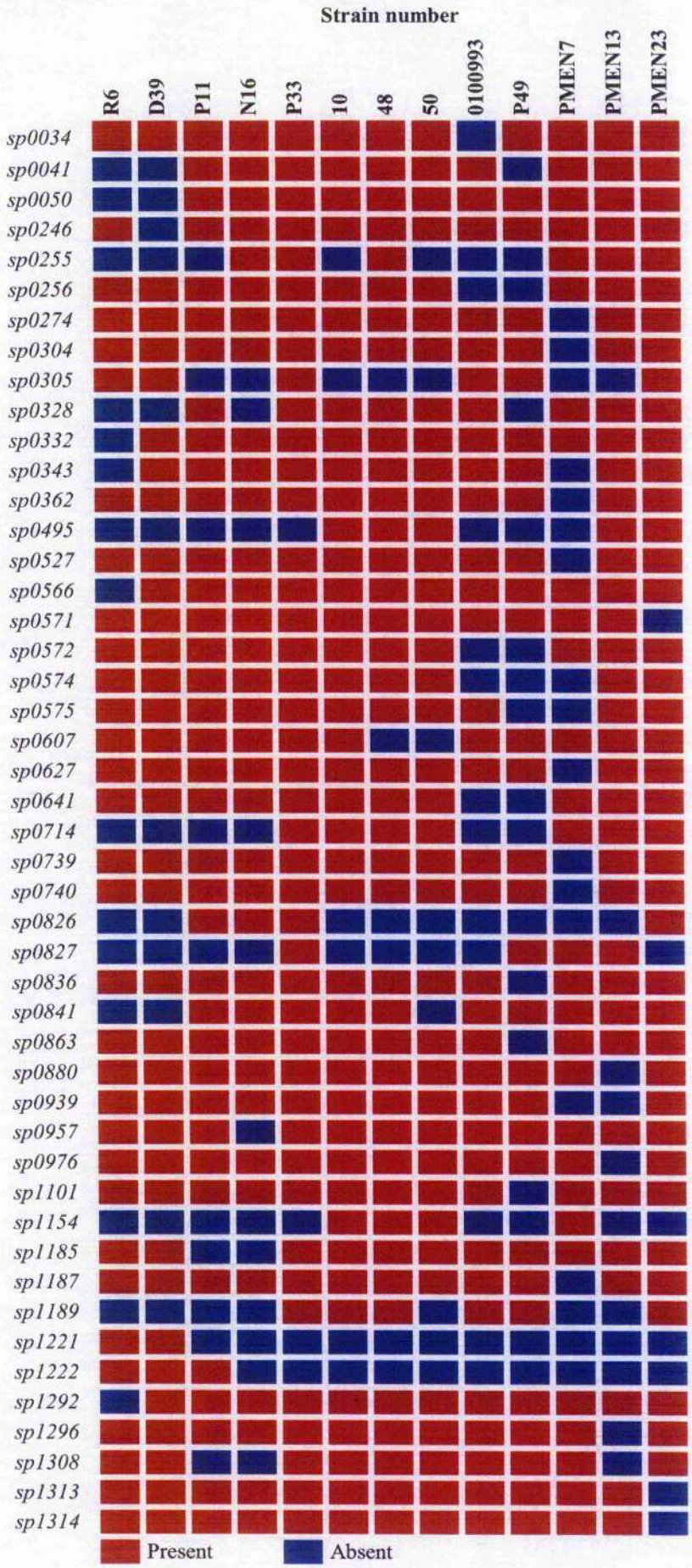
Table 6.6- Comparison between microarray analysis of ST9 and ST180 strains and known sequences of INV200 and OXC141 strains.

	RD	ST9 strains			INV200 BLAST[2)]	ST180		OXC141 BLAST[3)]
Gene[1)]		P11	N16	P33		0100993	P49	
sp0460	7	-	-	-	-	-	-	-
sp0461	7	-	-	-	-	-	-	-
sp0462	7	-	-	-	-	-	-	-
sp0463	7	-	-	-	-	-	-	-
sp0464	7	-	-	-	-	-	-	-
sp0465	7	-	-	-	-	-	-	-
sp0466	7	-	-	-	-	-	-	-
sp0467	7	-	-	-	-	-	-	-
sp0468	7	-	-	-	-	-	-	-
sp0949	14	-	-	-	-	-	-	-
sp0950	14	-	-	-	-	+	-	+
sp0951	14	-	-	-	-	+	+	+
sp0952	14	-	-	-	-	+	+	+
sp0953	14	-	+	-	+	+	+	+
sp0954	14	-	-	+	+	+	+	+
sp1315	17	+	+	+	+	-	-	-
sp1316	17	+	+	+	+	-	-	-
sp1317	17	+	+	+	+	-	-	-
sp1318	17	+	+	+	+	-	-	-
sp1319	17	+	+	+	+	-	-	-
sp1320	17	+	+	+	+	-	-	-
sp1321	17	+	+	+	+	-	-	-
sp1322	17	+	+	+	+	-	-	-
sp1911	23	+	+	+	+	+	+	+
sp1912	23	+	+	+	+	+	+	+
sp1913	23	+	+	+	+	+	+	+
sp1914	23	+	+	+	+	+	+	+
sp1915	23	+	+	+	-	-	+	+
sp1916	23	+	+	+	+	-	+	-
sp1917	23	+	+	+	+	+	+	-
sp1918	23	+	+	+	+	+	+	+

[1)] TIGR4 sequence annotation (http://www.tigr.org).
[2)] *S. pneumoniae* ST 9, serotype 14 (www.sanger.ac.uk).
[3)] *S. pneumoniae* ST 180, serotype 3 (www.sanger.ac.uk).
(+) Gene present.
(-) Gene absent.

Outside of the RDs we identified in total 85 genes that are of variable presence in one or more strains comparing with TIGR4 (Figure 6.4). Nevertheless some of these absent genes can have a diverse sequence in the different strains and appear absent in analysis, as we showed previously for the CBPs. Of these non-RD absent genes found in comparative genome hybridization thirteen were identified as virulence factors in the signature-tagged mutagenesis screen of TIGR4 (Hava and Camilli, 2002). Four of these genes encode cell surface proteins *prtA* (*sp0641*) *iga* (*sp1154*), *pcpA* (*sp2136*) and *pspC* (*sp2190*) described previously as having a highly variable sequence when analyzed in different *S. pneumoniae* strains. The remaining nine genes, encode a phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase (*sp0050*) and is missing in both serotype 2 strains, *sp0246,* missing only in D39, encodes a transcriptional regulator, *sp0274* encodes a DNA polymerase III, alpha subunit and is absent in PMEN7, *sp0571*, absent only in PMEN23 encodes a cell filamentation protein Fic-related protein, *sp1869* encodes an iron-compound ABC transporter and is missing in PMEN23 and the *sp1939* (*dinF*), missing in PMEN13, encodes a MATE efflux family protein DinF. While the genes *sp0332* and *sp1292* absent in R6, and *sp0939* absent in PMEN7 and PMEN13 encodes hypothetical and unknown function proteins.

The role of majority of these genes in *S. pneumoniae* genes remains unclear. The *sp0050* seems to be involved in purine biosynthetic pathways. The *sp1869* gene belongs to the *pia* operon, which encodes an ABC transporter involved in iron uptake. *In vitro* and *in vivo* phenotypes of strains containing mutation in this iron uptake ABC transporter demonstrate that the pia operon is probably the dominant *S. pneumoniae* iron transporter (Brown *et al.*, 2002). Of the iron transporter genes, the *piaA* (*sp1869*) mutant strains had the highest degree of attenuation in virulence in mouse models of nasal and systemic infection (Brown *et al.*, 2001). The *dinF* gene (*sp1939*), together with *cinA*, *recA*, and *lytA* constitutes the *cin* operon. Although the role of *dinF* remains unknown, it has been suggested that may play a role in competence and repairing DNA damage (Mortier-Barriere *et al.*, 1998).


Strain number
R6
D39
P11
N16
P33
10
48
50
0100993
P49
PMEN7
PMEN13
PMEN23
sp0034
sp0041
sp0050
sp0246
sp0255
sp0256
sp0274
sp0304
sp0305
sp0328
sp0332
sp0343
sp0362
sp0495
sp0527
sp0566
sp0571
sp0572
sp0574
sp0575
sp0607
sp0627
sp0641
sp0714
sp0739
sp0740
sp0826
sp0827
sp0836
sp0841
sp0863
sp0880
sp0939
sp0957
sp0976
sp1101
sp1154
sp1185
sp1187
sp1189
sp1221
sp1222
sp1292
sp1296
sp1308
sp1313
sp1314
Present
Absent

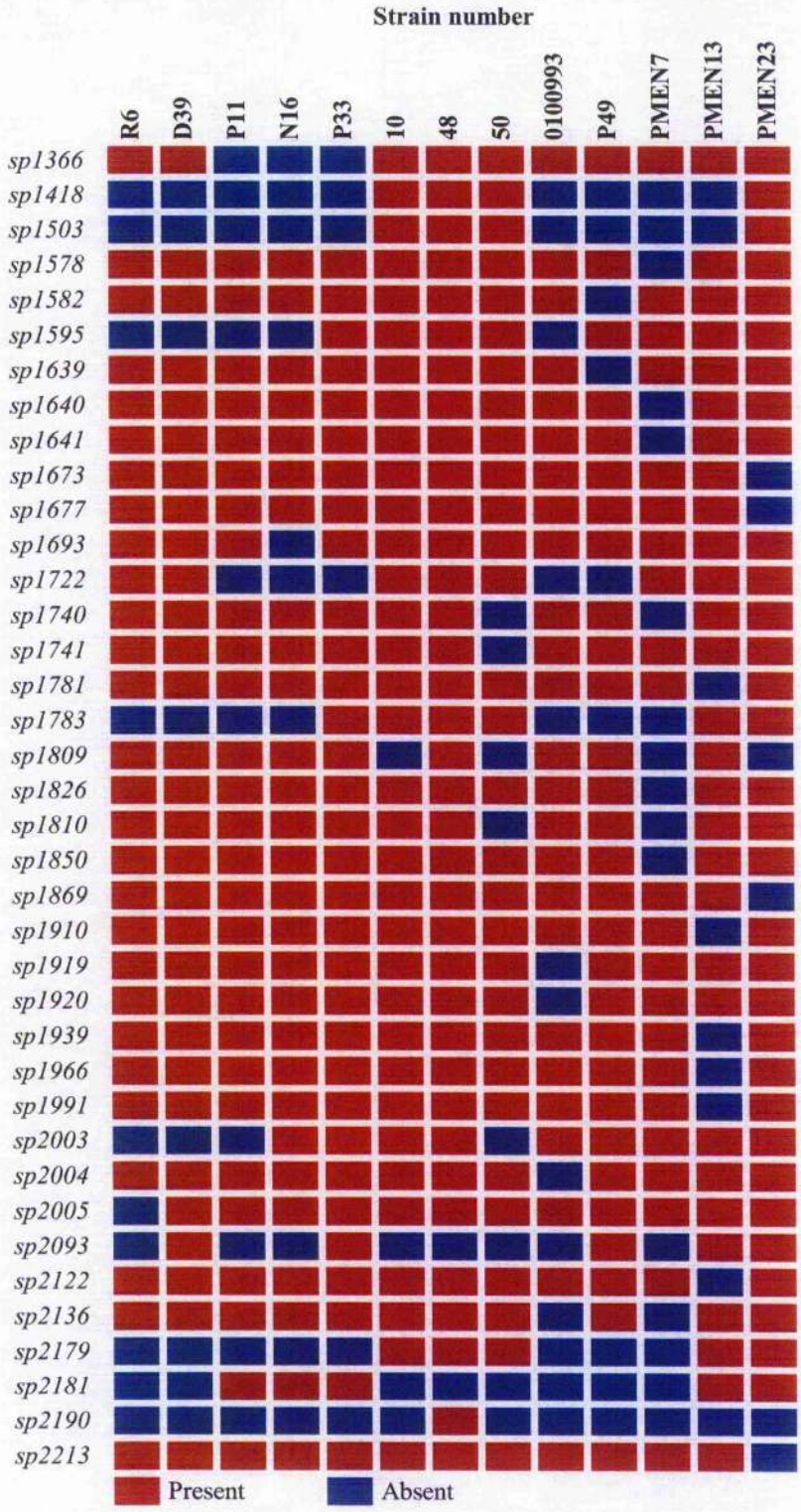


Figure 6.4- Isolated divergent genes.

Presence and absence of the gene was determined by comparative genomic hybridization. Red positive blue negative. Gene number and annotation taken from TIGR4 genome at http://www.tigr.org.

6.5 Diversity between *S. pneumoniae* strains

MLST is now widely used to type the pneumococcus and other pathogens providing high-resolution discrimination of a large number of clones (Enright and Spratt, 1998, 1999; Williams *et al.*, 1994). Strains of the same ST are assumed to be clonal and to have descended from a recent common ancestor. Strains of the same ST can be of different capsular serotypes showing that such strains are not necessarily identical despite being of the same ST. However, genetic differences in addition to the capsule locus have not been extensively characterised for strains of the same ST. Based on the genomic profile of each strain a cluster analysis was performed to define the relationship of the strains to each other using Genespring 7.0. A phylogenetic tree of these data is shown in Figure 6.5. In this comparison we included ST groups to represent different clonal groups of disease-associated, ST9 (P11, N16 and P33) and ST124 (10, 48 and 50), and also individual strains representing diverse serotypes and multi-locus sequence types. In phylogenetic tree it is evident that all the three ST9 and the three ST124 isolates are clustered. These results show that within these two clonal groups the strains are genetically very similar. Between these two ST types (serotype 14) there is more genetic diversity, forming two independent sub clusters. However, in this study the comparative genomic hybridization provides a first example of the phenomenon of differences between strains of the same ST extending to non-capsular genes. The Venn diagrams in Figure 6.6 and Figure 6.7 relates the number of common and singular absent genes between the strains for each these ST groups. In a total of 173 different genes in ST124 group 117 (~68%) are common in the three strains, and in ST9 group 120 (~74%) genes are common in total of 159 absent genes. The other ST types were clustering in agreement with the same capsular type, the two isolates serotypes 19A (PMEN7 and PMEN13) and ST3 (0100993 and P49) are clustered together. The strain R6 (unencapsulated strain derived from the type 2 strain D39) are clustered with D39 as expected. On the other hand, the ST strain 6A (PMEN23) formed an independent cluster.

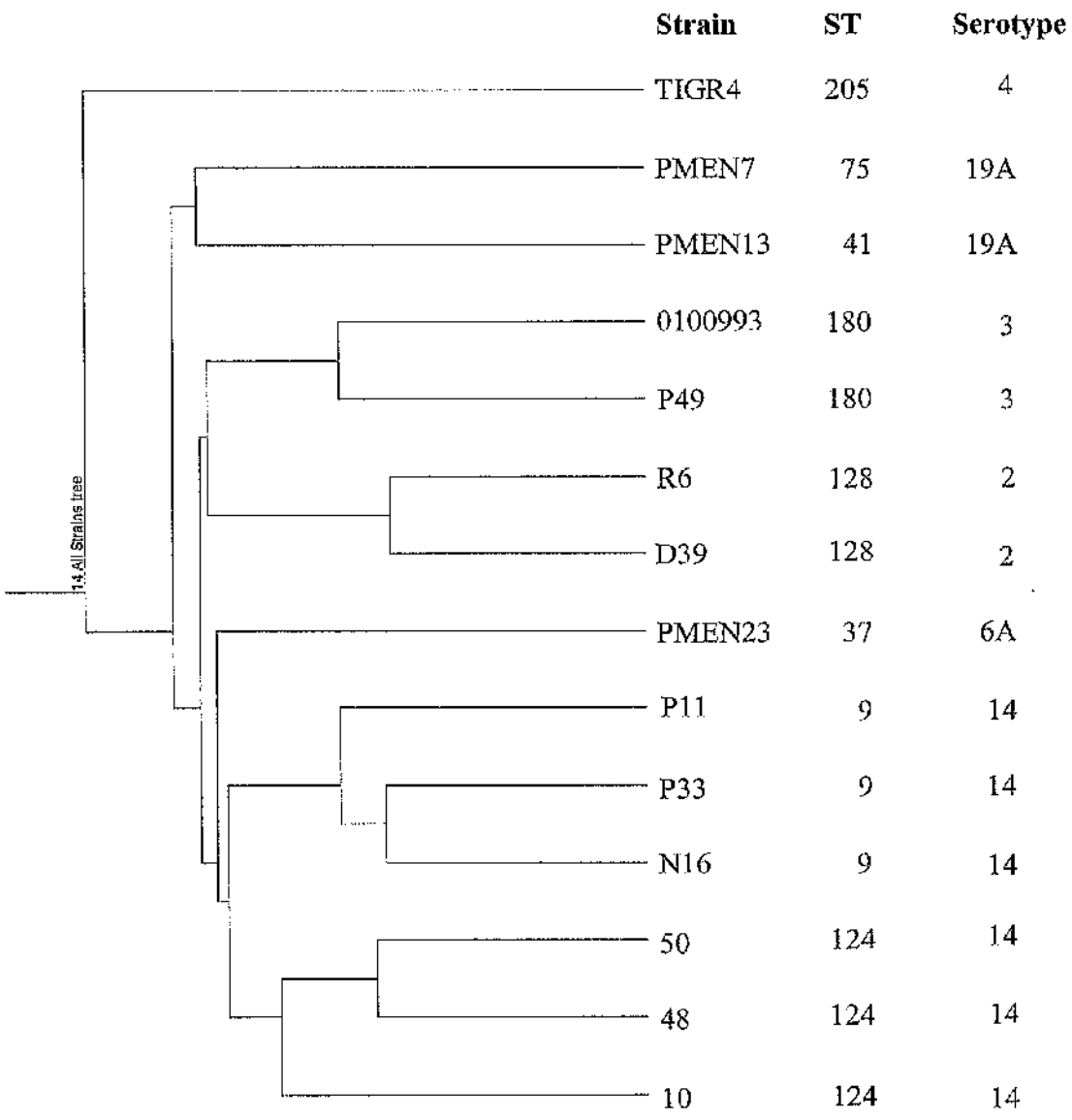


Figure 6.5- Comparative phylogenetic tree.

The phylogenetic tree was based on the genomic profile of each strain. Distances were calculated using the condition tree of Genepring 7.0.

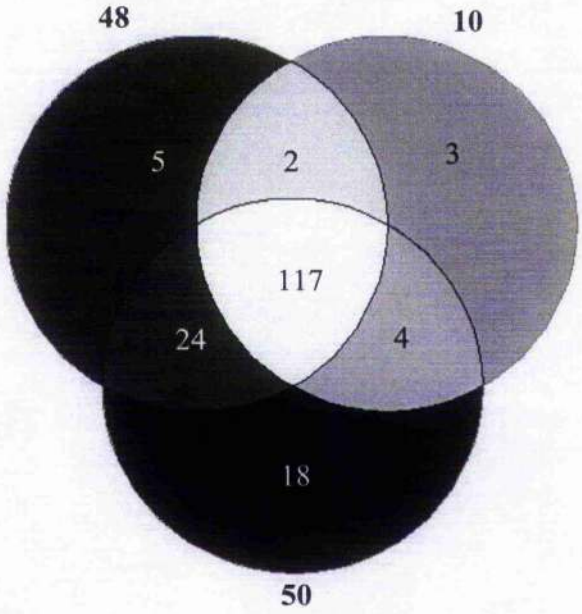


Figure 6.6- Genetic differences between pneumococcal ST124 strains.

Distribution of missing genes in strains 48, 10 and 50. Absence of the genes were determined by microarray analysis.

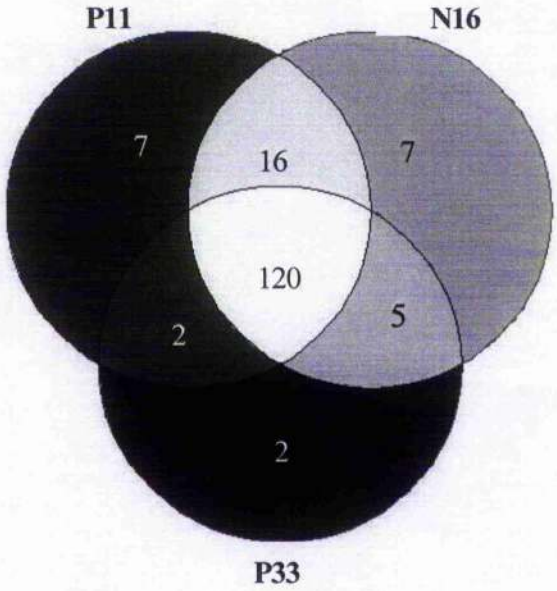


Figure 6.7- Genetic differences between pneumococcal ST9 strains.

Distribution of missing genes in strains P11, N16 and P33. Absence of the genes were determined by microarray analysis.

Relative to the distribution of the genes in RDs a diversity is shown in RD17 and 23 involving the three ST124 serotype 14 strains: 10, 48 and 50 (Figure 6.8). In the case of RD17, it is present in its entirely in strain 10 but the first half of this region is absent in strain 48 and 50. RD23, which is a new region of diversity, is absent in strain 50 but present in strains 10 and 48. Importantly, validation by PCR showed a strong agreement with the microarray results with $^{105}/_{108}$ (97%) genes agreeing between the two methods. The biological significance of these differences is uncertain however, both RD17 and 23 are poorly characterised. Within these regions, the TIGR4 STM screen identified two genes, *sp1321* and *sp1328*, with unequal strain distributions, as pneumococcal virulence factors (Hava and Camilli, 2002). There is therefore the potential for these genetic differences to effect phenotypic differences between these clonal strains. The distribution of the absence genes in ST9 strains was more uniform. Excluding singular genes, all of the missing RDs in ST9 group were common in the three strains.

(A)

		Strain number	Array 10	Array 48	Array 50	PCR 10	PCR 48	PCR 50
sp1315	v-type sodium ATP synthase, subunit D							
sp1316	v-type sodium ATP synthase, subunit B							
sp1317	v-type sodium ATP synthase, subunit A							
sp1318	v-type sodium ATP synthase, subunit G							
sp1319	v-type sodium ATP synthase, subunit C							
sp1320	v-type sodium ATP synthase, subunit E							
sp1321	v-type sodium ATP synthase, subunit K							
sp1322	v-type sodium ATP synthase, subunit I							
sp1323	Hypothetical protein							
sp1324	ROK family protein							
sp1325	Oxidoreductase, Gfo/Idh/MocA family							
sp1326	Neuraminidase, putative (nanC)							
sp1327	Conserved hypothetical protein							
sp1328	Sodium:solute symporter family protein							
sp1329	N-acetylneuraminate lyase							
sp1330	N-acetylmannosamine-6-P epimerase, putative							
sp1331	Phosphosugar-binding transcriptional regulator, putative							
sp1332	Conserved domain protein							
sp1333	Hypothetical protein							
sp1334	Conserved hypothetical protein							
sp1335	Hypothetical protein							
sp1336	Type II DNA modification methyltransferase spn5252IP							
sp1337	S1380-spn1, transposase							
sp1338	Hypothetical protein							
sp1339	Hypothetical protein							
sp1340	Hypothetical protein							
sp1341	ABC transporter, ATP-binding protein							
sp1342	Toxin secretion ABC transporter, ATP-binding/permease protein							
sp1343	Prolyl oligopeptidase family protein							
sp1344	Conserved hypothetical protein							
sp1345	Hypothetical protein							
sp1346	Membrane protein, putative							
sp1347	Hypothetical protein							
sp1348	Conserved hypothetical protein							
sp1349	Hypothetical protein							
sp1350	Conserved domain protein							
sp1351	Hypothetical protein							
sp1352	IS1380-spn1, transposase							

Present Absent

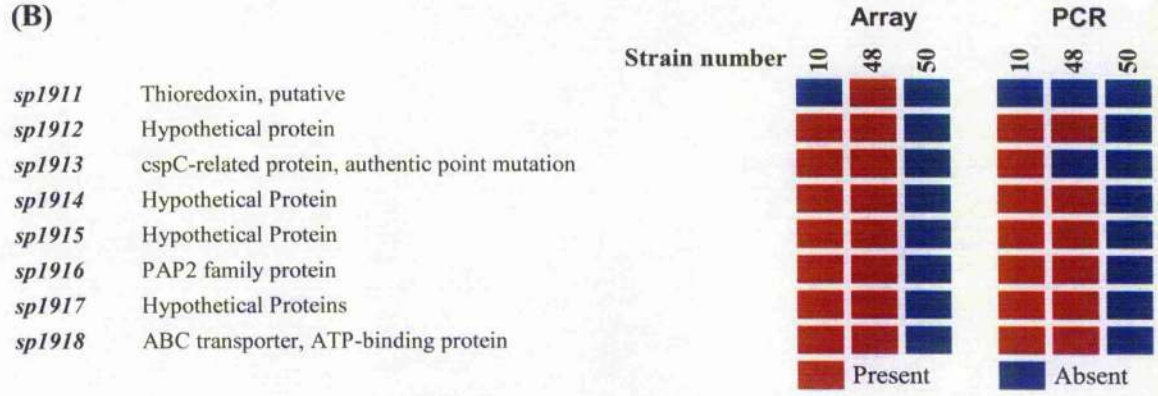


Figure 6.8- Genetic differences between pneumococcal strains of the same ST and serotype.

Strain distribution of RD17 (*sp1315-sp1352*) (A) and 23 (*sp1911-sp1918*) (B). Presence and absence of the gene was determined by microarray and PCR analysis. Red positive, blue negative. Gene number and annotation taken from TIGR4 genome at http://www.tigr.org.

6.5.1 Analysis of virulence of strains of the same ST and serotype

To test if the genetic differences between strains with the same ST and serotype could have biological significance the three strains: 10, 48 and 50 were tested for virulence in a mouse intraperitoneal infection model. Young (5 week old) female C57/Bl6 mice were infected by the intraperitoneal route with 10^7 CFU and survival and blood counts monitored. All mice survived the infection and none showed clinical signs (n=7-8). However, at 6 h post-infection a transient bacteraemia was noted that was cleared by 24 h. Comparison of the blood bacterial counts at 6hr. shows a significant difference between the strains, Figure 6.9. The blood counts for mice infected with strain 48 were significantly lower than those infected with strain 10 ($P<0.0001$) or strain 50 ($P=0.0441$). The mean bacterial count for strain 48 was approximately 24-fold lower than strain 10 and 5-fold lower than strain 50. In line with this, strain 48 had the lowest proportion of bacteraemic animals, Figure 6.9. Although there was a trend towards higher bacterial blood counts in mice infected with strain 10 compared to strain 50, the difference was not statistically significant ($P=0.0882$).

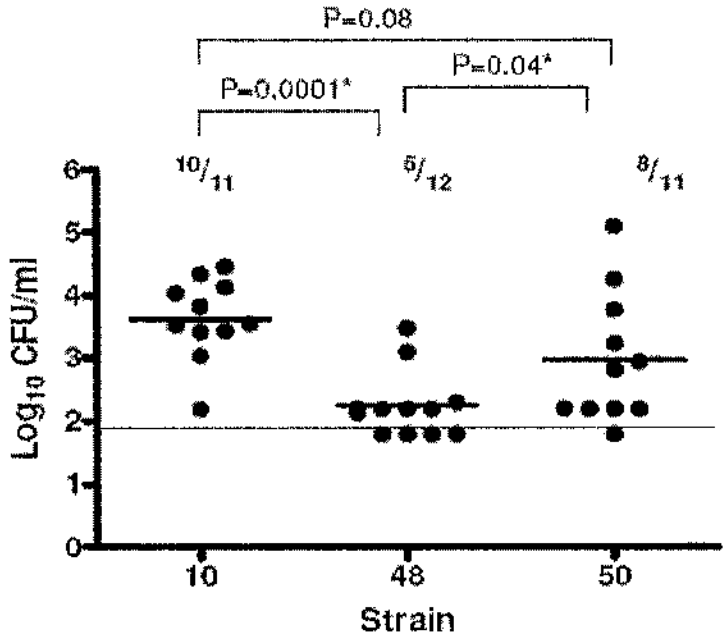


Figure 6.9- Blood bacterial counts 6 hours post-infection.

Three pneumococcal strains of the same ST and serotype (ST124 serotype 14) were injected by the intraperitoneal route into C57/Bl6 mice and the blood bacterial viable count taken at 6h post-infection. Each point indicates the data from an individual mouse; the horizontal bar indicates the mean (n=11-12). Dashed line indicates detection limit. Proportions indicate the number of mice that had bacteraemia above the detection limit (log 1.92 CFU/ml). *P* values (Student's *T* test) relate to bacterial counts with <0.05 considered significant (*). Done in collaboration with Dr. Gavin Paterson.

Chapter 6 discussion

Genomic diversity among multiple strains within a pathogenic bacterial species has been proposed to play a key role in virulence by the continual evolution of new strains via horizontal gene transfer (Ehrlich *et al.*, 2005; Kreth *et al.*, 2005; Lomholt, 1995; Post *et al.*, 2004; Shen *et al.*, 2005). Naturally transformable, pneumococcal genetic diversity and plasticity is evidenced by the presence of over 90 distinct capsular serotypes and the emergence of antibiotic resistance. Furthermore, comparative genomic studies using DNA microarrays analysis (Bruckner *et al.*, 2004; Hakenbeck *et al.*, 2001; Shen *et al.*, 2006; Tettelin and Hollingshead, 2004), sequencing of genomic libraries from eight pneumococcal clinical isolates (Shen *et al.*, 2006), genome sequences of *S. pneumoniae* strains TIGR4 (Tettelin *et al.*, 2001) and R6 (Hoskins *et al.*, 2001) and with information from the pneumococcal diversity project, in which multiple strains have been sequenced at multiple sites (Hollingshead and Briles, 2001), have established that within the pneumococcus there exists a substantial genetic heterogeneity as well as genomic plasticity. Indeed, pneumococcal genetic diversity and genetic exchange with related organisms makes it hard to clearly define the pneumococcus as a species (Arbique *et al.*, 2004; Hanage *et al.*, 2005a; Suzuki *et al.*, 2005). Along with this genetic diversity comes important phenotypic differences with regards to the propensity of strains and serotypes to cause disease. For example, approximately ~85% of disease is caused by only 20 different serotypes (Kalin, 1998). In addition, certain multilocus sequence types (ST) are more associated with disease than others (Brueggemann *et al.*, 2003). Although, capsular serotype is recognised as a crucial contributing factor in these differences (Brueggemann *et al.*, 2003), other, as yet uncharacterised genetic factors also contribute (Mizrachi Nebenzahl *et al.*, 2004; Sandgren *et al.*, 2004; Sandgren *et al.*, 2005). The advent of genome sequencing and microarray technology has allowed this genetic diversity to be probed more fully, offering the potential to better understand pneumococcal strain and serotype differences. In addition to helping to understand carriage and disease processes, such data may also contribute to antimicrobial and vaccine development through the identification of conserved targets found in all strains/serotypes. Furthermore, understanding the pneumococcal population structure may help predict and interpret its response to interventions such as antibiotics or vaccines, especially when these may be effective against only a subset of strains/serotypes.

Regions of genetic diversity among clinical isolates

Genome-to-genome comparisons through comparative genome hybridization (CGH) using DNA microarray reveal the footprints of genetic plasticity in the pneumococcal genomes.

One characteristic footprint is the presence of a number of regions of diversity that have different complements of genes in different isolates.

Of the twenty-five regions of diversity found in the 13 test strains compared to the reference strain TIGR4, by CGH, fourteen of these regions were found not to hybridize with the serotype 2 strain R6 representing approximately 155 kb corresponding to over 7% of the total genome. A previous study of CGH between these two fully sequenced pneumococcal strains also demonstrated these nonconserved regions in R6, however two of these RDs were not identified as cluster (Bruckner *et al.*, 2004). The ancestral strain D39 showed an almost identical differential pattern as the R6 strain when compared to the TIGR4 strain. The remaining eleven RDs, were identified as absent at least in one of the other pneumococcal isolate strains, and the majority of the genes belonging to these RDs are totally present in R6 strain. The limits of these RDs or clusters vary a little between the studies and likely reflect the use of different clinical strains or array probes. Three novel RDs identified in the present pneumococcal CGH analysis were not recognised in Bruckner and co-workers study (Bruckner *et al.*, 2004). One of the genes found in these novel RDs, *sp1830* (*phoU*), encoding a phosphate transport system regulator, was identified in the TIGR4 STM. Furthermore, attenuation in models of pneumonia, bacteraemia and nasal colonization was observed in competitive infections between *S. pneumoniae* wild-type and *phoU* mutants (Hava and Camilli, 2002). Moreover, Bruckner and co-workers identified five clusters that we have not observed (Bruckner *et al.*, 2004). Presumably, this is due to the use of different strains while the analysis of further strains would allow discovery of other RDs. These twenty-five RDs represent approximately 242 kb corresponding to ~11% of the total genome of TIGR4. As will become evident, these regions are often not completely deleted and sometimes only a fraction of the genes are deleted in certain strains.

Diversity between *S. pneumoniae* strains

In this CGH study, of the total of TIGR4 strain genes analysed 85% were common to all thirteen test strains, 15% were missing from at least one strain and 1.5% of the genes were missing in all test strains. With the exception of IgA1, none of the genes implicated in virulence, such as the pneumolysin, hyaluronidase, neuraminidase A and B, autolysin, *pspA* and other surface antigens PsaA and PavA were found to be missing in any strain. (Paton *et al.*, 1997). However, four of the 10 choline binding proteins genes incorporated in the microarray analysis, including the virulence factor *pspC*, are present in this set of missing genes, indicated sequence variation. This finding is in agreement with reported

variability in *pspC* (Brooks-Walter *et al.*, 1999). Interestingly, the thirteen- pneumococcal two-component systems and the orphan *rr*14 (Lange *et al.*, 1999; Throup *et al.*, 2000) were also found to be present in all strains examined, showing high levels of sequence conservation among of different strains. Many of the missing genes identified here and in similar array analysis are annotated as encoding hypothetical or conserved hypothetical proteins with little/no data available on their function(s). Furthermore, about 24.1-33% of missing genes encode a particular type of proteins, such as: transport and binding protein, cell envelope and protein fate. These findings may be related to the association of these proteins with the cell surface, and more directly relate with the environment and consequently subject to natural transformation.

This CGH study allowed us to demonstrate that: (i) within strains of the same serotype, great genetic diversity may still occur; (ii) individual strains of disease-associated ST9 and ST124 clustered tightly; (iii) and the ST180 strains (more associated with carriage) possessed greater genetic diversity and did not cluster with disease-associated groups. However, strains of the same ST and serotype have significant genetic differences. Although perhaps not a surprising finding, strains of the same ST can be of different capsular serotypes showing that such strains are not necessarily identical despite being of the same ST, this study was the first clear demonstration of genetic diversity, in addition to the capsule locus, for strains of the same ST. Microarray analysis may therefore be of utility in complementing current MLST and serotyping schemes by providing a higher resolution typing (Garaizar *et al.*, 2006).

Analysis of virulence of strains of the same ST and serotype

In the present study, we show differences in the virulence of strains 10, 48 and 50 in a mouse infection model. These strains have the same ST (124) and serotype (14). All mice survived the infection and none showed clinical signs. This observation can be due to the type of animal used, although the serotype 14 strains is associated with disease in humans, these strains are known to have low virulence in mice. However, comparison of the blood bacterial counts at 6 h shows a significant difference between the strains. The blood counts for mice infected with strain 48 were significantly lower than those infected with strain 10 or strain 50. The mean bacterial count for strain 48 was approximately 24-fold lower than strain 10 and 5-fold lower than strain 50. Therefore, these strains, despite being of the same serotype and ST show differences in virulence in this mouse model. Interestingly, a diversity is shown in CGH analysis in RD17 and 23 involving these three ST124 serotype 14 strains. The RD17 is entirely present in strain 10 but the first half of this region is

absent in strain 48 and 50, while RD23 is absent in strain 50 but present in strains 10 and 48. Furthermore, TIGR4 STM screen identified two genes, *sp1321* and *sp1328* (Hava and Camilli, 2002), with unequal strain distributions, as pneumococcal virulence factors. However, the biological significance of these differences is uncertain, since both RD17 and 23 are poorly characterised. A causal relationship between genotypic differences and phenotype remains however, to be confirmed empirically. A trend for differences in the behaviour of strains of the same ST and serotype during mouse infections was recently shown, but not examined further (Sandgren *et al.*, 2005). For example, two ST162 serotype 19F strains showed different propensities to cause pneumonia following intranasal infection. One strain caused pneumonia in 80% of infected C57BL/6 and BALB/c mice while the proportions for a second strain where 53% and 40% respectively.

Summary

The important human pathogen *S. pneumoniae* is known to be a genetically diverse species. In this present study we have used CGH microarray analysis to investigate this diversity in a collection of clinical isolates including several capsule serotype 14 pneumococci, a dominant serotype among disease isolates. The reference strain TIGR4 contains twenty-five regions with contiguous genes that are not shared in at least one of the strains tested. Three of these regions of diversity among pneumococcal isolates were identified for the first time in this study. Of the 2,236 TIGR4 genes analysed 85% genes were common to all strains, in contrast, 15% were missing from at least one strain and 1.3% were missing in all strains. Importantly, we provide a clear demonstration of genetic differences between strains of the same capsule serotype and ST. Thus, even though strains may appear identical based on current typing methods they may boast potentially important genetic and phenotypic differences. CGH may therefore, in certain circumstances, prove to be a valuable tool to supplement current typing methods. This will especially be valuable when particular virulence associated genes and genotypes are identified for example as done recently for otitis media (Pettigrew and Fennie, 2005). Finally, we show that these clonal strains with the same serotype and ST behave differently in an animal model. Strains of the same ST and serotype therefore have important genetic and phenotypic differences.

7 Concluding remarks

Streptococcus pneumoniae (the pneumococcus) is a causative agent of several important diseases involving the respiratory tract (pneumonia, sinusitis and otitis media) as well as invasive infection (meningitis and septicemia) and remains a major cause of morbidity and mortality in undeveloped and developed parts of the world. *S. pneumoniae* has become a worldwide problem which limits the choice of antimicrobial agents. The limited efficacy of the current vaccines and resistance to common antibiotics encourage continued interest in developing of new and alternative strategies to treat and prevent pneumococcal diseases. Although the pneumococcus has been studied for over a century, an understanding of the mechanisms that underlie the course of disease remains fragmented. In order to identify new points of intervention it is helpful to have an understanding of the pathogenesis of the infection and the bacterial factors that play a role in this process. The project aimed to contribute to our understanding of such mechanisms.

When the pneumococcus causes disease it needs to adapt to different environments such as the lower respiratory tract, the meninges and the blood. Bacteria have several ways of regulating gene expression in response to changes in the environment including the use of so-called two-component systems (TCS). These systems are recognised as a key mechanism through which bacteria perceive and respond to their environment. These systems have been shown to regulate a wide variety of cellular responses, including osmoregulation, competence, photosynthesis, expression of adhesions, chemotaxis, antibiotic production, and pathogenicity, in a number of different bacteria.

This project was concerned with the evaluation of the role of pneumococcal TCS06 and TCS09 in perception and response to their environment and understanding how these TCS contribute to pneumococcal virulence. Furthermore, the putative virulence factor *dlt* operon regulated or potentially regulated by the CiaR/H system was studied to investigate its contribution to the role of CiaR/H to virulence. To study the contribution of these TCS and *dlt* operon in virulence of bacteria, this project has made use of the available complete pneumococcal genome sequence and the current molecular biology techniques used for introducing mutations in individual genes to construct isogenic mutants in one of the RR of these TCS and in the first gene of the *dlt* operon (*dltA*). In addition, comparative genomic hybridization (CGH) was performed to investigate whether the apparent differential gene regulation in different *S. pneumoniae* strains by TCS06 and TSC09 was due to the absence of the genes identified to be differentially expressed. Furthermore, the CGH was also used to study the genetic diversity in a collection of clinical isolates and evaluate the importance of this diversity in virulence of the bacteria.

The functions of most of the TCS in *S. pneumoniae* are largely unknown. Some of the TCS have been examined for functionality for the sensing of phosphate, for development of competence for DNA transformation, penicillin susceptibility, maintenance of nasal carriage, regulation of genes that are involved in the biochemical makeup of the cell envelope, and maintenance of iron homeostasis. In this project the role of the TCS06 and TCS09 in the expression regulation and virulence of *S. pneumoniae* was investigated. TCS06 has been previously shown to be attenuated in animal models. Analysis of TIGR4 Δ*rr06* mutant *in vivo* using pneumonia and bacteraemia models of disease revealed that RR06 has no effect on the overall outcome of pneumococcal disease. Although the current study did not show clear evidence of the importance of TCS06 in our animal model of infection, the RR06 seems to be important for the ability of the pneumococcus to invade the lungs and blood, causing a significant reduction of the amount of the TIGR4 Δ*rr06* in the lungs airways and in the blood after intranasal challenge, compared with wild-type. Bacterial loads in the bloodstream 24 hours after intravenous challenge was similar for wild-type and Δ*rr06* mutant. Although the event triggered by the pneumococcus that results in death of mice in the Δ*rr06*-infected group mice is not known, the effect of *rr06* deletion on *in vivo* bacteriology seems to be significant.

This study confirms the contribution of RR06 in resistance to elevated temperatures. The TIGR4 Δ*rr06* mutant has a dramatic decrease in the growth rate at 40 °C, and this effect was more evident when the bacterium was grown on blood agar. However, the effect of RR06 on temperature sensitivity appeared to be strain-dependent. In serotype 2 strains, R6 and D39 no significant changes in growth were observed between the Δ*rr06* mutant and the wild-types, while in 0100993 background both strains did not grow at 40°C.

Transcriptome analysis between TIGR4 Δ*rr06* mutant and wild-type revealed an important change in the genetic regulation by this TCS. The set of RR06-regulated genes was found to be enriched for genes predicted to encode proteins associated with the stress response, and this could explain the effect of RR06 on growth of the pneumococcus at higher temperatures. The work described in this thesis has also shown that the genetic regulation by this TCS06 is strain-dependent. Although a significant expression change of a large number of genes was observed in TIGR4 strain, only a few genes was observed to be up-regulated in strain 0100993, and no changes were observed in R6. The genes regulated by TCS06 seem to have a significant impact in phenotype of the different genetic background Δ*rr06* mutants.

Previous studies have indicated that TCS06 regulated the virulence factor PspC. Here, we demonstrated by transcriptome and western immunoblot analysis in four different strains that TCS06 does not regulate this important virulence factor. Although the work described in this thesis did not resolve the controversy about the regulation of PspC, we show that *in vitro* regulation of *pspC* by TCS06 is not evident. However, we believed that during pneumococcal infection the regulation of PspC could occur, due to the massive increase in expression of this virulence factor *in vivo* compared to growth *in vitro*. To prove this theory, transcript levels of PspC between the *rr06* mutants and wild-type from bacterial RNA recovering after pneumococcal infection in animal models need to be done.

The role of TCS09 in pneumococcal virulence has been reported to be strain-specific. While D39 carrying a deletion of the response regulator RR09 was found to be avirulent in all murine models tested 0100993 Δ*rr09* was found to be attenuated upon intranasal infection only. Here, we extended these observations by analysis of a TIGR4 Δ*rr09* mutant in a murine pneumonia model of infection. Similar to 0100993 strain, the mutant was found to be attenuated, with prolonged murine survival after intranasal infection when compared with wild-type. A significant reduction was observed in the number of bacteria recovered from bronchio-alveolar lavage fluid, lungs, and the systemic circulation of mice infected with TIGR4 Δ*rr09*. To assess the genetic basis of these phenotypic differences between D39 and TIGR4 *rr09* mutants, a transcriptome analysis was performed to identify the complement of genes regulated by TCS09 *in vitro*.

This study allows us to identify several targets of RR09 in D39 and TIGR4 that could account for the phenotypes conferred by their respective mutants. While, in total 39 genes were found to be differentially expressed between wild-type and TIGR4 Δ*rr09*, in D39 the number of expression genes altered was 48. Furthermore, only 4 of these genes were common in both strains. In D39, the set of RR09-regulated genes was found to be enriched for genes predicted to encode proteins involved in carbohydrate metabolism. Pneumococci are likely to have different nutritional needs during the various stages of infection. Possibly, TCS09 plays a role in this process by regulating genes required for nutrient uptake in response to various conditions characteristic of different sites of the host. The signal that triggers TCS09 might be present at one site and not another, and particular strains might be more adept at responding to these signals, potentially explaining the phenotypical differences observed in animal studies.

One of these genes, *sp0063* encoding the IIC component of a putative mannose-specific PTS, was identified in the STM screen as virulence factor. However, the gene product of

sp0063 alone does not significantly contribute to virulence in D39 in our animal model of infection and alone does not explain the dramatic phenotype observed following deletion of *rr09* in D39.

Furthermore in this present study, the virulence factor *pspA* was observed, be down-regulated in both *rr09* mutants. The major virulence factor *htrA* was also found to be down-regulated in D39 Δ*rr09* mutant, suggesting that the regulation of these virulence factors can be related with the attenuation of *rr09* mutants in animal models of infection. However, previous studies have reported that *pspA* and *htrA* are directly regulated by the TCS02 and CiaR/H systems respectively. These suggest that the TCS09 maybe regulate these important virulence factors indirectly. However, both HK and RR of the TCS02 were not found to be altered or down-regulated in our studies, therefore is unclear the mode of regulation of the PspA by the TCS09. Our studies show a lower, but significant, down-regulation in CiaR and CiaH in D39 Δ*rr09* mutant. This finding can clarify the down-regulation of HtrA in our *rr09* mutant.

The gene regulation studies by microarray analysis of TCS, also shown a strong up-regulation of the *rlrA* pathogenicity islet in TIGR4 Δ*rr06* and Δ*rr09* strains. Both TCS seems to be indirectly related with the regulation of this pathogenicity islet, and therefore associated with the development of pilus-like structures on surface of *S. pneumoniae*.

Although, our transcriptome analysis in TCS06 and TCS09 allows us to understand the role of these TCS in virulence of *S. pneumoniae*, the exact role in pneumococcal virulence for most of these has still to be investigated. Additional studies will be required to obtain a complete understanding of how these TCS regulate the expression of the whole pneumococcal transcriptome in response to different environmental stimuli. Also, the signals to which the TCSs are sensitive need to be elucidated. Furthermore, predicted function of many of the novel targets identified in this study is still based on sequence homology only which makes it more difficult to identify their exact role in these regulatory systems.

The *dlt* operon of Gram-positive bacteria comprises four genes (*dltA*, *dltB*, *dltC*, and *dltD*) that catalyze the incorporation of D-alanine residues into the LTAs. Our previous studies of transcriptome analysis by TCS06 demonstrated that the *dlt* operon is regulated by the RR06. However, the mechanisms of control of expression of *dlt* operon by TCS06 are still unclear. Furthermore, this putative virulence factor has shown to be potentially regulated by the CiaR/H system.

In this work, we demonstrated that DltA is important for growth of *S. pneumoniae* and is essential when the bacterium is subjected to higher temperatures. Furthermore, this operon was shown to be defective in its acid tolerance response and sensitive to antimicrobial peptides nisin and magainin II. Mutations in *dltA* seem to have influence in morphology of *S. pneumoniae* occurring the presence of long chains of bacterial cells. Although previous studies identified the *dltA* and *dltB* as virulence factors of the pneumococcus in STM screen, no significant attenuation was found in murine model of disease using TIGR4 strain lacking the *dltA* gene. However, because in this work was used a single strain infection, attenuation in *dltA* mutant in our animal model of disease may be observed using a competitive model of infection, i.e. using the TIGR4 wild-type and Δ*dltA* strains simultaneously.

Although this preliminary study provides the first evidence of the importance of the *dlt* operon in *S. pneumoniae*, additional experiments need to be done to ensure the role of the *dlt* operon in D-alanylation of teichoic acids and consequent role in virulence of bacteria. Extending the mutation in other *S. pneumoniae* serotypes; using different model of infection; and increasing the number of mice in individual experiments. *In vitro* experiment; for example, investigating the maximal expression of the *dlt* genes in different types of media cultures and in the points of growth of the pneumococcus in prospective to optimize the effect of *dlt* operon in susceptibility of antimicrobial peptides.

The human pathogen *S. pneumoniae* is known to be a genetically diverse species. In this present study we have used CGH microarray analysis to investigate whether the differential gene regulation in different strains by TCS is related to the different genomic backgrounds. The *rlrA* pathogenicity islet is differential expressed in TIGR4 *rr06* and *rr09* mutants but not in R6, D39 and 0100993. However, this can be explained due to the absence of this pathogenicity islet in the genomic background of these later strains. Furthermore, the CGH analysis was also used to investigate the *S. pneumoniae* genome diversity in a collection of clinical isolates including several capsule serotype 14 pneumococci, a dominant serotype among disease isolates.

Although microarray analysis allows the whole genome to be interrogated easily there are several caveats to be acknowledged. Firstly, the true degree of population diversity is underestimated because test strain specific genes are not included. How many genes do the test strains carry that are absent in TIGR4? Indeed, sequencing of genomic libraries from eight pneumococcal clinical isolates revealed a number of putative ORFs distinct from TIGR4 with many also unrelated to known streptococcal sequences (Shen *et al.*, 2006).

Importantly, a major shortcoming of investigations like this is the inability to determine if the absence of an array signal represents absence of a particular gene or divergence in the probe sequence between strains. In addition, array analysis provides no details on gene location or number. For example, a gene may be present in multiple copies or in a difference location compared to other strains but this is overlooked in this analysis. Also, subtle but functionally significant differences will be missed such as promoter and coding sequence mutations that may alter the production and activity of gene products. Likewise the basis for genetic differences are unclear, i.e. is presence/absence due to acquisition by one strain and not another or loss from one strain and not another? Finally, many of the genes identified here and in similar array analysis are annotated as encoding hypothetical or conserved hypothetical proteins with little/no data available on their function(s). Furthermore, even those with annotations lack functional confirmation. The potential significance of the absence or presence of these genes is therefore hard to interpret until they have been characterised more fully.

However, acknowledging these drawbacks we have employed CGH to identify large genomic regions of diversity between pneumococcal strains. We confirm the previous identification of several variable regions and identify three addition regions that are not conserved among strains. We provide a clear demonstration of genetic differences between strains of the same serotype and ST. In addition we show differences in the virulence of these strains in a mouse infection model. Thus, although strains may appear identical based on current typing methods they may boast potentially important genetic and phenotypic differences. CGH may therefore be useful in providing higher resolution typing. This will be especially valuable when particular virulence associated genes and genotypes are identified for example as done recently for otitis media.

Future work:

1. Study in more detail the expression of the genes regulated by the TCS06 and TCS09, at different time points of *in vitro* growth.

2. Study the control of gene expression by TCS06 and TCS09 *in vivo* to complete understanding of the role of these systems during infection, and confirm or not the regulation of virulence factor PspC by the TCS06. Also, study the effect on the expression of *rr09* and other genes when the bacteria are subjected to broth cultures of different composition during *in vitro* growth.

3. Further evaluate temperature sensitivity of Δ*rr06* mutants.

4. Study the virulence of the *dltA* in different genetic background, and using competitive infections models.

5. Determine whether pneumococcal LTA or WTA is D-alanylated using techniques such as nuclear magnetic resonance and capillary electrophoresis mass spectroscopy.

6. Study the mechanisms of regulation: (i) Most response regulators act as transcriptional activators that recognize specific DNA binding sites in their target promoters. Electrophoretic gel mobility shift assay may allow us to ensure that some important genes identified here are directly or indirectly controlled by this TCS; (ii) the regulation mechanism of the TCS system presumes the activation of the response regulator by the histidine kinase. Investigate if the RR06 and the RR09 is phosphorylated by the respective HK06 and HK09 or by another kinase or sensitive mechanism.

7. Extend the CGH to other pneumococci in our collection of isolates, which can allow us to create a genetic distribution of the genes between disease and carriage-associated strains. Use of improve array based on new genome sequences.

Appendix

A1. Solutions and buffers

Genomic DNA extraction buffer

10 mM Tris, pH 8.0

100 mM EDTA (Fischer Scientific, UK), pH 8.0

0.5% SDS (w/vol) 8 Fischer Scientific, UK)

Agarose (1%)

1g agarose (GibcoBRL)

100 ml of TAE buffer

Dissolve and add 0.5 µg/ml ethidium bromide (Sigma-Aldrich, UK)

BHI medium contents, g/l (Oxoid)

Calf brain infusion solids (12.5), beef heart infusion solids (5.0), protease peptone (10), glucose (2.0), NaCl (5.0), disodium phosphate (2.5)

CAT medium

10 g of bacto-casamino acid

1 g of yeast extract

5g of tryptone

5 g of NaCl

Make to 1L with dH_2O

CAT/GP medium

100 ml CAT

1 ml glucose 20%

3 ml K_2HPO_4 0.5 M

CTM medium

100 ml CAT

2 ml glucose 20%

3 ml K_2HPO_4 0.5 M

4 ml BSA 4%

1 ml $CaCl_2$ 1%

PBS, pH 7.4

Dissolve one tablet of phosphate buffered saline (Dulbecco A, Oxoid Ltd, Basingstoke) in 100 ml of dH_20 and autoclave

DNA loading buffer (6X)

0.25% bromophenol blue (Sigma-Aldrich, UK)

0.25% xylene cyanol FF

40% (w/vol) sucrose in dH_2O

Protein gel running buffer (1x)

25 mM Tris base (Sigma-Aldrich, UK)

192 mM glyvine

0.1% SDS

Make to 1L with dH_2O

Protein separating gel (for two gels)

4.05 ml dH_20

2.05 ml of 1.5 M Tris-HCl, pH 8.8

100 µl of 10% SDS

3.30 ml of 30% acrylamide bis

50 µl of APS

5 ul of TEMED

Protein stacking gel (for two gels)

3.05 ml dH_2O

1.25 ml of 1.5 M Tris-HCl, pII 6.8

50 µl of 10% SDS

665 µl of 30% acrylamide bis

25 µl of APS

5 µl of TEMED

Protein gel loading (sample) buffer

60 mM Tris-HCl, pH 6.8

25% glycerol

2% SDS

14.4 mM 2-mercaptoethanol

0.1% bromophenol blue

Transfer buffer (western blot)

25 mM Tris base

192 mM glycine

20% vol/vol methanol

Tris NaCl, pH 7.4 (western blot)

1.2 g Tris base

8.7 g NaCl

800 µl of HCl 37%

Make to 1L with dH_2O

Blocking buffer (western blot)

3 g of skimmed milk

100 ml Tris NaCl, pH 7.4

Developer (western blot)

Dissolve 30 mg of 4-cloro-1-naphthol (Sigma-Aldrich, UK) in 10 ml methanol. Add to 50 ml Tris NaCl, pH 7.4. Add 30 µl of 30% (vol/vol) H_2O_2 just prior use

1 M KPO_4, pH 8.5

16.547 g K_2HPO_4

0.680 g KH_2PO_4

Make to 100 ml with MilliQ water

Phosphate wash buffer

0.25 ml of 1 M KPO_4, pH 8.5

7.625 ml of MilliQ water

Make to 50 ml with 95% ethanol (Sigma-Aldrich)

Phosphate elution buffer

0.2 ml of 1 M KPO_4, pH 8.5

Make to 50 ml with MilliQ water

100 mM NaOAc pH 5.2

1 ml 3M NaOAc, pH 5.2 (Ambiom®)

Make to 30 ml with MilliQ water

1 M NaoH

2 g NaOH pellets

Make to 50 ml with MilliQ water

0.1 M sodium carbonate buffer pH 9.3

0.53 g Na_2CO_3

50 ml MilliQ water

Add HCl 37% to lower pH to 9.3

Filter with 0.22 µm filter

Pre-hybridization solution

15 ml of 20x SSC (Ambiom®)

600 µl of 10% SDS (Ambiom®)

0.6 gm of BSA fraction V powder (Sigma-Aldrich)

Make to 60 ml with MilliQ water

Filter with 0.22 µm filter

Hybridization solution

500 µl of formamide (Sigma-Aldrich)

250 µl of 20x SSC (Ambiom®)

10 µl of 10% SDS (Ambiom®)

Salmon Sperm DNA (Ambion®)

Make to 1 ml with MilliQ water

Filter with 0.45 µm filter

Low stringency buffer

50 ml of 20x SSC (Ambiom®)

5 ml of 10% SDS (Ambiom®)

Make to 500 ml with MilliQ water

Filter with 0.22 µm filter

Medium stringency buffer

2.5 ml of 20x SSC (Ambiom®)

5 ml of 10% SDS (Ambiom®)

Make to 500 ml with MilliQ water

Filter with 0.22 µm filter

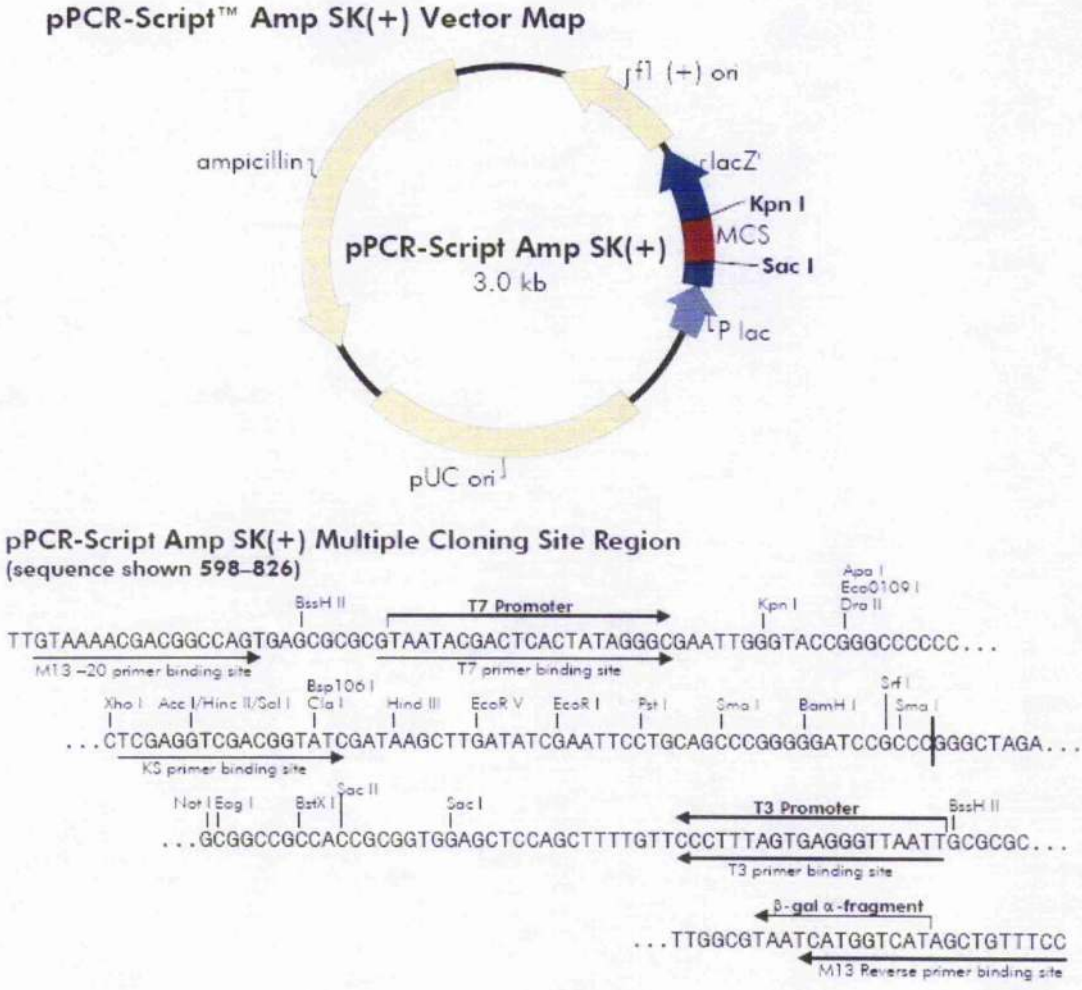
High stringency buffer

2.5 ml of 20x SSC (Ambiom®)

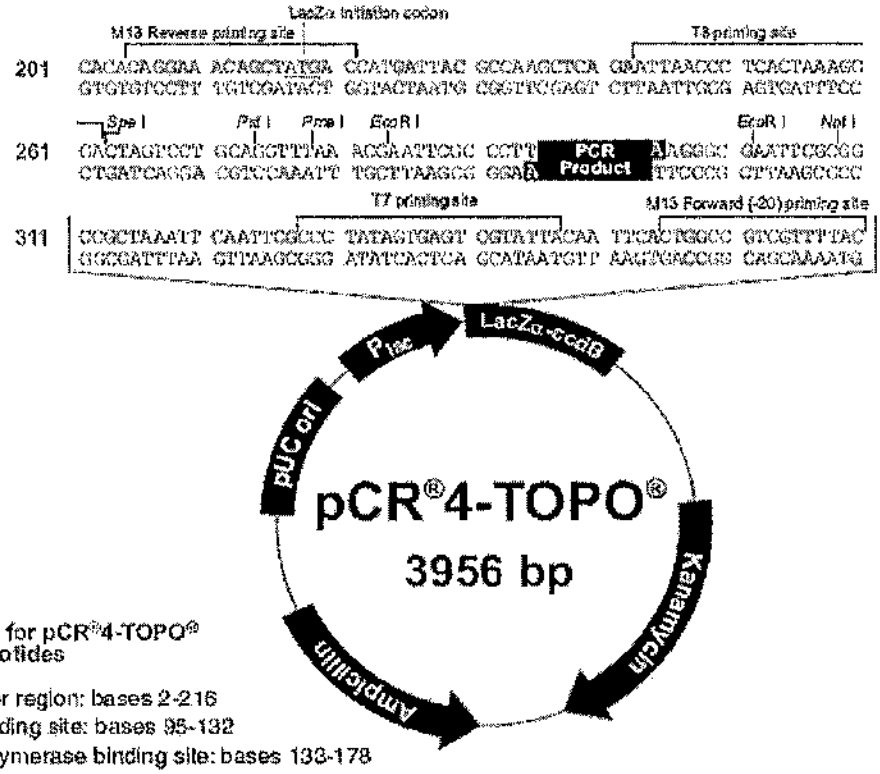
Make to 500 ml with MilliQ water

Filter with 0.22 μm filter

A2. PCR-Script™ plasmid



A3. pCR® 4-TOPO ® plasmid



Comments for pCR®4-TOPO®
3956 nucleotides

lac promoter region: bases 2-216
- CAP binding site: bases 95-132
- RNA polymerase binding site: bases 133-178
- Lac repressor binding site: bases 179-199

Start of transcription: base 179
M13 Reverse priming site: bases 205-221
LacZα-*ccdB* gene fusion: bases 217-810
- LacZα portion of fusion: bases 217-497
- *ccdB* portion of fusion: bases 508-810

T3 priming site: bases 243-262
TOPO® Cloning site: bases 294-295
T7 priming site: bases 328-347
M13 Forward (-20) priming site: bases 355-370
Kanamycin promoter: bases 1021-1070
Kanamycin resistance gene: bases 1159-1953
Ampicillin (*bla*) resistance gene: bases 2203-3063 (c)
Ampicillin (*bla*) promoter: bases 3064-3160 (c)
pUC origin: bases 3161-3834
(c) = complementary strand

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Ibrahim, Y.M., Kerr, A.R., Silva, N.A., and Mitchell, T.J. (2005) Contribution of the ATP-dependent protease ClpCP to the autolysis and virulence of *Streptococcus pneumoniae*. *Infect Immun* 73: 730-740.

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International congress abstracts

Silva N.A., McCluskey J. & Mitchell T.J Role of the two component signal transduction system 06 in *Streptococcus pneumoniae*. 4th International Symposium on Pneumococci and Pneumococcal Diseases. May 9-13, 2004 at Marina Congress Center, Helsinki, Finland.

Silva, N., McCluskey, Jefferies, J., Hinds, J., Paterson, G. and Mitchell, T. Genomic diversity of *Streptococcus pneumoniae*: Identification of common regions of diversity (RD) using microarray. Europneumo - 7th European Meeting on the Molecular Biology of the Pneumococcus. 2005 May 8-11, 2005 at Gesellschaft für Biotechnologische Forschung, Braunschweig, Germany.