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Stress response proteins in *Streptococcus pneumoniae*

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A thesis submitted to the University of Glasgow for the degree of PhD

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Yasser Musa Ibrahim

Abstract

Streptococcus pneumoniae (the pneumococcus) is a bacterial pathogen that continues to be associated with considerable morbidity and mortality worldwide. 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 species generated by host phagocytes, and nutritional deprivation.

This thesis aimed at the investigation of the role of the stress response in the virulence process. The functions of caseinolytic protease specificity subunit, ClpC and the protease subunit, ClpP were investigated as an example of ATP-dependent proteases. In addition, the role of the high temperature requirement A protein, HtrA, which is an example of the ATP-independent proteases, was also studied. Mutants lacking these proteins were constructed in different genetic backgrounds and their phenotypes were compared to the wild types both *in vitro* and *in vivo*.

ClpC was found to contribute to autolysis of the pneumococcus in a strain-dependent manner. ClpC was required for the release of autolysin A and pneumolysin in serotype 2 *S. pneumoniae* strain D39. *In vivo*, ClpC was required for the growth of the pneumococcus in the lungs and blood in a murine model of disease but it does not affect the overall outcome of pneumococcal disease. This thesis also reports the requirement of ClpP for the growth at elevated temperature and virulence of serotype 4 strain, TIGR4 and confirms its contribution to the thermotolerance, oxidative stress resistance and virulence of D39. Data also revealed that HtrA is a key virulence factor of *S. pneumoniae*. The virulence of Δ*htrA* mutants were completely abolished (type 2) or significantly reduced (type 4) in mouse pneumonia and bacteraemia models. HtrA was involved in the ability of the pneumococcus to grow at higher temperatures, to resist oxidative stress and to undergo genetic transformation. Furthermore, the regulation of virulence by the two-component system CiaR/H was found to be mediated through HtrA. Further analysis of Δ*htrA* mutants by proteomics and microarray is also shown in this thesis.

Acknowledgements

Praise be to ALLAH, without His willing and guidance this thesis would not have been possible. I also implore Him to render all my work for His sake

This thesis is dedicated to the memory of my beloved mother. She spared no effort in supporting and encouraging me during her life as well as being my everlasting inspiration even after her death

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Abbreviations

ABC	ATP-binding cassette
ail	Adhesion-invasion locus
ami	Aminopterin resistance operon
Amp	Ampicillin
ANOVA	Analysis of variance
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
CbpA	Choline-binding protein A
cDNA	Complementary deoxyribonucleic acid
CFU	Colony forming unit
CI	Competitive index
CIRCE	Controlling inverted repeat of chaperone expression
Clp	Caseinolytic protease
Cm-R	Chloramphenicol resistance
CRF	Central research facility
CSP	Competence stimulating peptides
CtsR	Class three gene repressor
Cup	Competence up
D39	*S. pneumoniae* strain D39
D39(LA)	Laboratory-adapted strain of D39
D39(MA)	Mouse-adapted strain of D39
DFI	Differential fluorescence induction
dH_2O	Distilled water
DMSO	Dimethyl sulphoxide
DNA	Deoxyribonucleic acid
dNTPs	Deoxynucleoside triphosphate
DOC	Deoxycholate
DTT	Dithiothritol
EDTA	Ethylenediamine tetra acetic acid

ELISA	Enzyme-linked immunosorbent assay
Em-R	Erythromycin resistance
Ery	Erythromycin
FCS/FBS	Foetal calf serum/Foetal bovine serum
g.DNA	Genomic DNA
GFP	Green fluorescent protein
h	Hours
HA	Hyaluronic acid
HK	Histidine kinase
HSPs	Heat shock proteins
HtrA	High temperature requirement A
HU	Haemolytic unit
I.N.	Intranasal
I.P.	Intraperitoneal
I.V.	Intravenous
IEF	Isoelectric focusing
IgA	Immunoglobulin A
IL-6	Interleukin-6
IPG	Immobilized pH gradient
IPTG	Isopropyl-beta-D-thiogalactopyranoside
Kan	Kanamycin
kb	Kilobase pair
kDa	Kilo Dalton
LB	Luria-Bertani broth
LytA	Autolysin A
MAD	Median absolute deviation
MALDI-TOF	Matrix-assisted laser desorption ionization–time of flight
MIC	Minimum inhibitory concentration
min	Minutes
ml	millilitre
MLST	Multilocus sequence typing
mM	Millimoles
MS	Mass spectrometry
MUAN	2'-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid
***mur*M/N**	Muropeptides M/N genes
NanA/B	Neuraminidase A/B

NBD	Nucleotide-binding domain
OD_{600nm}	Optical density at 600 nanometer
ORF	Open reading frame
PAF	Platelet activating factor
PBPs	Penicillin-binding proteins
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEP	Phosphoenolpyruvate
pg	Picogram
***p*I**	Isoelectric point
Ply	Pneumolysin
PS	Polysaccharides
PsaA	Pneumococcal surface antigen A
PspA	Pneumococcal surface protein A
PTS	Phosphotransferase systems
RBS	Ribosome binding site
RNA	Ribonucleic acid
RR	Response regulator
RTI	Respiratory tract infection
RT-PCR	Reverse transcription polymerase chain reaction
SBA	Sheep blood agar
SDS	Sodium dodecyl sulphate
SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
sec	Seconds
SEM	Standard error of the mean
SodA	Superoxide dismutase
Spec	Spectinomycin
STM	Signature-tagged mutagenesis
TCS	Two-component system
TCSTS	Two-component signal-transducing systems
TIGR	The institute for genomic research
TIGR4	*S. pneumoniae* strain TIGR4
TNF-α	Tumour necrosis factor alpha
U	Units
UV	Ultraviolet
V	Volts

vol/vol	Volume per volume
w/vol	Weight per volume
WT	Wild type
%	Percent
°C	Degrees Celsius
µg	Microgram
µl	microlitre
µM	Micromoles
2D-PAGE	Two-dimensional polyacrylamide gel electrophoresis
3D	Three dimensional

Chapter 1

INTRODUCTION

1.1 History of the pneumococcus

The pneumococcus, *Streptococcus pneumoniae*, has been one of the most extensively studied microorganisms since its first isolation in 1881. It was the object of many investigations that led to important scientific discoveries (Watson *et al.*, 1993). Recently, Rosenow has summarized the findings of the pneumococcal research between 1881 and 1917. These are shown in table 1.1. In 1928, Griffith observed that when heat-killed encapsulated pneumococci and live strains constitutively lacking any capsule were concomitantly injected into mice, the nonencapsulated could be converted into encapsulated pneumococci with the same capsular type as the heat-killed strain (Watson *et al.*, 1993).

In 1933, DNA was discovered to be the basis of heritable changes in living organisms, when it was found that DNA from a killed pneumococcus could transform a living strain to a new serotype (Avery *et al.*, 1944). Soon after the commercial production of penicillin, it was recognized that penicillin resistance could similarly be transformed by DNA from penicillin-resistant killed pneumococcus to live susceptible strains (Hotchkiss, 1951). In 1975, *Streptococcus pneumoniae* phages were first isolated from throat swabs of healthy children (McDonnell *et al.*, 1975). These phages have represented a useful tool for expanding the knowledge of genetic interchange mechanisms in the pneumococcus as well as to gain information on the relationship between host and parasite. In recent years, several pneumococcal bacteriophages from different sources have been isolated and characterized. Lysins encoded by pneumococcal bacteriophages have been characterized such as lysozyme from Cp-9 and the amidase from the temperature bacteriophage HB-3. The structural analysis of these enzymes provided the basis to establish the hypothesis on the modular evolution of the pneumococcal cell wall lytic enzymes. The analysis of choline-binding proteins encoded by bacteriophages has also been of great relevance to determine the molecular basis of choline recognition (Garcia *et al.*, 1990, Romero *et al.*, 1990, Diaz *et al.*, 1992).

Table 1.1- Pneumococcus: time line of early discoveries.

Year	Finding(s)/advance(s)	Investigator(s)
1881	First demonstration, microscopically, of pneumococci in blood of rabbits that died after injection of saliva from a child who died of rabies (in study by Pasteur) and after injection of normal saliva (in study by Sternberg)	L. Pasteur (Paris), Sternberg (New York)
1883	Observation, on the basis of Gram stain, of encapsulated diplococci in alveolar exudates of all but a few of 50 cases of pneumonia; cultivation of pneumococci on Koch's coagulated blood serum	C. Friedlander (Germany)
1884	Isolation of pneumococcus from all cases of "fibrinous pneumonia," then fulfillment of Koch's postulates	A. Fraenkel (Germany)
1886	Separation of pneumonia into lobar, lobular, and hypostatic types; identification of pneumococcus with lobar types	A. Weichselbaum (Austria)
1891	Initial underpinnings of anti-pneumococcic-serum therapy: serum (postcrisis) from patients with pneumonia protected rabbits injected with pneumococci, whereas similar serum (4-6 ml, subcutaneous) given to patients with pneumonia led, in all of them, to decrease in temperature in 6-12 h (in 2 of these patients, it returned to normal, where it remained)	G. Klemperer and F. Klemperer (Germany)
1902	Capsular swelling (quelling) reaction produced by exposure to homologous antiserum	F. Neufeld (Germany)
1910	Observation that pneumococci differ in immunologic properties, by use, in animals, of antiserum obtained by use of strains from patients with pneumonia: serum against 1 strain (designated "typical") protected mice against majority of strains (all other strains were designated "atypical")	F. Neufeld and L. Haendel (Germany)
1913	Subdivision of pneumococci into 3 distinct groups (I-III) (>60% strains) and a fourth, heterogeneous group (IV), on the basis of homologous-serum agglutination reactions and mouse-protection tests	L. Gillespie and A. Dochez (New York)
1917	Demonstration of immunologic differentiation of pneumococci into types based on specific soluble substances comprising capsules	A. Dochez and O. Avery (New York)
1917	Establishment of basis for serum therapy, by demonstration that, to be effective, antiserum must be type specific and given in adequate amounts and that, to be most effective, must be administered early	O. Avery, H. Chickering, H. Cole, and A. Dochez (New York)

Adapted from (Rosenow, 2004)

Because of its importance as a human pathogen, *S. pneumoniae* has been studied intensively for many decades. In 1977, a pneumococcal vaccine based on the capsular polysaccharide surrounding the pneumococcal cells was licensed in South Africa (Austrian, 1999).

It was recently that the advent of whole-genome sequencing of microbes, including microbial pathogens, has revolutionized the methods by which these organisms are studied and has heightened expectations regarding the ability to predict potential targets for antimicrobial agents and vaccines (Nowak, 1995, Baltz *et al.*, 1998) and *S. pneumoniae* is not an exception to this approach.

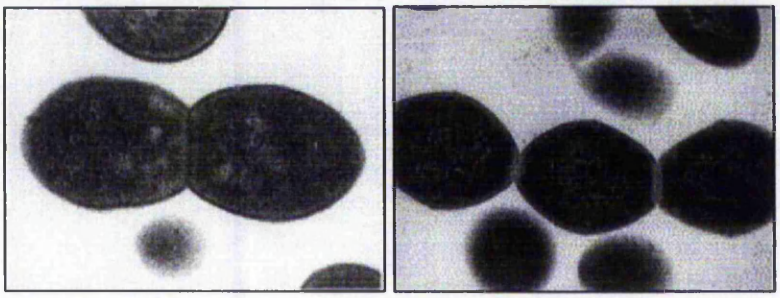
1.2 Microbiology of the organism

1.2.1 Colonial morphology

Colonies of the pneumococcus on blood agar appear smooth, glistening, and flat or dome-shaped with a diameter of 0.5-1.5 cm. Encapsulated organisms form mucoid colonies on agar. After 48 hours of incubation, the centre of the colony collapses due to autolysis, producing a doughnut or umbilicated form. If incubation exceeds 72 hours, the remainder of the organism spontaneously lyses and only a depression in the agar remains. Colonies of the pneumococcus produce greenish discolouration on blood agar (α-haemolysis) (Roberts, 1979, Murray *et al.*, 1998).

1.2.2 Direct morphology

The pneumococcus is a Gram-positive coccus, which may be spherical or oval. Typically, it is lancet-shaped and occurs in pairs (diplococcus). The cells are 0.5-1.2 μm in diameter. It may also occur singly or in short chains (figure 1.1). Chain formation is more likely to occur in broth culture. Older cultures decolourize readily and appear Gram-negative. Pneumococci are non-motile and do not form spores (Roberts, 1979, Murray *et al.*, 1998).



(A) (B)

Figure 1.1- Morphology of *S. pneumoniae*

Electron micrographs of pneumococcal strain Rx1. (A), Growth in pairs and (B), chain formation, > 100 cells (only part of it is shown). Magnification × 50,000. Adapted from (Yother *et al.*, 1998).

1.2.3 Taxonomy and classification

Although the pneumococcus has for many years been referred to as either *Streptococcus pneumoniae* or *Diplococcus pneumoniae*, the organism has been officially classified in the genus Streptococcus. The similarities between the pneumococcus and other streptococci include the following characteristics: (1) Pneumococci grow in short chains in broth cultures. (2) Streptococci, including the pneumococcus, are lactic acid bacteria, which ferment glucose to yield lactic acid. (3) Both pneumococci and streptococci contain group-specific carbohydrate and M protein. However, the pneumococcal M protein is not anti-phagocytic. (4) Antibiotic resistance and other genetic markers have been transferred from one species to another. (5) Nucleic acid homology; the GC content of DNA in 15 streptococci species, including the pneumococcus, has ranged from 33-42 % (Roberts, 1979).

Skerman and colleagues (Skerman *et al.*, 1980) describe the taxonomy of *Streptococcus pneumoniae* as follows: Bacteria (eubacteria), Firmicutes (Gram-positive bacteria), Bacillus/Clostridium group (low G+C Gram-positive bacteria), Streptococcaceae, Streptococcus. In addition, the capsular polysaccharide of the pneumococcus has been used for serological classification of strains. More than 90 serotypes are currently recognized (Murray *et al.*, 1998).

1.2.4 Identification

Methods that may be used for the laboratory identification of *S. pneumoniae* include; Gram stain morphology, colony morphology and haemolysis on sheep blood agar, pyrrolidonyl

arylamidase reactivity, optochin susceptibility, solubility in deoxycholate (bile), carbohydrate utilization, latex agglutination, Quellung reaction, and DNA probes based on the detection of specific ribosomal RNA sequences that are unique to *S. pneumoniae* (Kellogg *et al.*, 2001).

For the optochin test, a disk containing ethylhydrocupreine HCl is placed on the surface of freshly streaked SBA plate and a zone of inhibition is measured after overnight incubation at 37°C in 5 % CO_2. An isolate with a zone of 14 mm or greater is considered susceptible (Chandler *et al.*, 2000). Isolates of *S. pneumoniae* are rapidly lysed when the autolysins are activated after exposure to bile. The organism can be identified presumptively by placing a drop of bile on an isolated colony. Most colonies of *S. pneumoniae* are solubilized within a few minutes. This test can also be performed by adding bile to a broth culture, which causes the organisms to be rapidly lysed and the broth to clear (Murray *et al.*, 1998). For the DNA probe test, 1-2 freshly propagated colonies are lysed and hybridized to a DNA probe, then read on luminometer. The measured relative light units (RLU) are scored as positive when it is more than 50,000 (Chandler *et al.*, 2000). The Quellung reaction, or capsular precipitin reaction, provides rapid identification of pneumococci in various clinical specimens. The procedure is to mix loopfuls of bacteria in suspension, antiserum to pneumococcal capsule, and methylene blue on the surface of a glass slide and examine under oil immersion. If positive, the organism will be surrounded by a large capsule. The capsular appearance is probably due both to capsular swelling and greater refraction (Roberts, 1979).

Chandler and co-workers (Chandler *et al.*, 2000) suggested that α-haemolytic *Streptococcus pneumoniae* from an appropriate source (respiratory system, blood, cerebrospinal fluid) should be tested for optochin susceptibility. If the colony morphology is consistent with *S. pneumoniae*, the bile solubility or latex test could be used. The DNA probe could be used to evaluate any isolates giving questionable results by any of the other methods. Other methods currently used for the identification of the pneumococcus include the colony blot assay developed by Bogaert and co-workers (Bogaert *et al.*, 2004b), which allows the detection of multiple serotypes of the pneumococcus, i.e. mixed colonization within clinical specimens. Multilocus sequence typing (MLST) is now widely used for determining the relatedness of pneumococcal strains, through which alleles of several housekeeping genes are directly assessed by nucleotide sequencing, each unique allele combination determining a sequence type of a strain (Platonov *et al.*, 2000, Sa-Leao *et al.*, 2001, Jefferies *et al.*, 2003).

1.2.5 Nutrition and growth

Members of the genus *Streptococcus* are facultatively anaerobic. They are unique among bacteria able to grow anaerobically in that they do not synthesize porphyrins, cytochromes or catalase, and are not capable of forming ATP via an electron transport system. They generate energy through fermentation of carbohydrates. The pneumococcus is an obligate parasite with nutritional requirements resembling those of the host. It is classified among the lactic acid bacteria, facultative anaerobes that derive energy primarily from fermentation of carbohydrates to lactic acid. The ability to ferment inulin differentiates pneumococci from most α-haemolytic streptococci. Because pneumococci lack catalase, the H_2O_2 formed during growth is thought to cause loss of viability and it is not entirely understood how the pneumococcus can survive oxidative stress caused by its own production of H_2O_2. Recently, Pericone *et al* have shown that pyruvate oxidase (SpxB), the enzyme responsible for endogenous H_2O_2 production, was required for survival of *S. pneumoniae* during exposure to high levels of exogenously added H_2O_2 suggesting that SpxB is among the factors that contribute to hydrogen peroxide resistance of the pneumococcus (Pericone *et al.*, 2003).

An uncommon growth requirement of the pneumococcus is choline, which is incorporated into cell wall teichoic acid. Choline plays a crucial role in the structure and functions of the cell wall, for substitution of ethanolamine for choline in a synthetic medium gives rise to several abnormalities: (1) the cells fail to divide normally and form long chains; (2) they are resistant to autolysis even when grown in the presence of penicillin; (3) they lose their ability to undergo transformation; (4) they do not adsorb phages (Austrian, 1990).

1.3 Pattern of Pneumococcal Infection in Man

Streptococcus pneumoniae is carried asymptomatically in the upper respiratory tract by many healthy individuals and most infections do not occur after prolonged carriage but follow the acquisition of recently acquired serotypes. This suggests that the immune status of the host at the moment of colonization, as well as the virulence of the particular strain, determines whether pneumococci will remain confined to the nasopharynx or become invasive (Gray *et al.*, 1980, Johnston, 1991).

During the establishment of pneumococcal disease, pneumococci undergo spontaneous phase variation marked by switching from opaque to transparent colony morphotypes.

Although the nature of the molecular switch is unknown, transparent strains are adapted for nasopharyngeal adherence and display less capsule, more surface choline and more of the adhesin, choline binding protein A (CbpA). Opaque strains show improved survival in the bloodstream and bear more capsule, less choline and more of the protective antigen, pneuomococcal surface protein A (PspA) (Kim and Weiser, 1998).

The first step in the development of pneumococcal infection in man is the colonization of the nasopharynx by *S. pneumoniae*. From this site, *S. pneumoniae* can gain access to the lung or eustachian tube. If the bacteria enter the eustachian tube and begin growing there, they trigger an inflammatory response that causes the pain and fever associated with the middle ear infection (otitis media). Evidence indicates that otitis media occurs only in the presence of eustachian tube dysfunction, which is caused principally by respiratory viruses (adenovirus, influenza) (Giebink, 1999). If the bacteria are inhaled into the lung, they survive by evading phagocytosis by the alveolar macrophages and cause a local inflammatory response that results in a breakdown of the gas exchange mechanism, and the patient becomes cyanotic. The classical form of pneumococcal pneumonia is rapid onset of symptoms, high temperature, shaking chills, a productive cough, and blood in the sputum. In 15-30 % of patients with pneumonia, the bacteria enter the bloodstream, where lysis of bacteria releases cell wall components that trigger cytokine release, resulting in fever and shock. Meningitis occurs when bacteria that have gained access to the bloodstream infect the meninges, a set of membranes that covers the brain and spinal column, protecting them from harmful substances in blood (blood-brain barrier). Normally only glucose and electrolytes can cross the blood-brain barrier, but local inflammation caused by the bacteria breaches the blood-brain barrier and admits bacteria and phagocytes to this fragile area. Damage to the brain can lead to hearing loss, blindness, learning disabilities, paralysis, and death. Pneumococcal meningitis is characterized by fever, irritability, and drowsiness in early stages and seizures and coma in later stages (Salyers and Whitt, 1994, Giebink, 1999).

1.4 Virulence Factors

Pneumococci elaborate a variety of factors that contribute to virulence. These factors include the capsule, surface proteins like PspA, PspC and PsaA, proteins like pneumolysin (Ply), and others. Recent advances in the field of bacterial pathogenesis and the availability of genome sequence of the organism have allowed a large-scale identification of various genes involved in the pathogenicity of such life-threatening bacteria (Polissi *et al.*, 1998,

Paton and Giammarinaro, 2001, Hava and Camilli, 2002, Marra *et al.*, 2002a). Figure 1.2 shows some of the possible virulence factors of *S. pneumoniae* (Briles *et al.*, 1998).

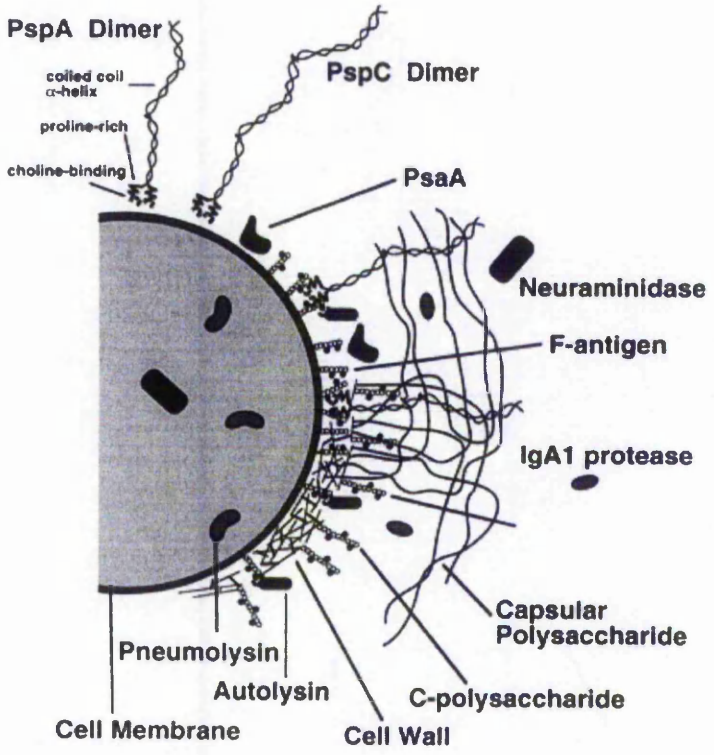


Figure 1.2- Hypothetical representation of the pneumococcal surface showing possible virulence factors.

Adapted from (Briles *et al.*, 1998).

1.4.1 The Capsule

The capsule surrounding the pneumococcal cell forms the outermost layer of the majority of fresh clinical isolates of the pneumococcus. A total of 90 distinct types of capsule are currently known (Henrichsen, 1995). It consists of high-molecular weight polymers made up of units of repeating oligosaccharides, which can contain between two and eight monosaccharides (VanDam *et al.*, 1990). 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 same encapsulated strain and the loss of the capsule is accompanied by a 100,000-fold reduction of virulence (Avery and Dubos, 1931). Capsular types were originally differentiated by the Quellung reaction in which addition of type specific antiserum causes agglutination and microscopically visible swelling of the capsule. Capsular type is classified by one of two systems: the American system, which assigns numbers in the chronological order of their discovery, and the Danish system, which is based on antigenic similarities. The lowest numbered serotypes

such as 1 and 2 are classified identically by both systems. The Danish serotype nomenclature is now generally employed (VanDam *et al.*, 1990). Some pneumococcal capsular serotypes are listed in table 1.2.

Table 1.2- The capsule loci for which DNA sequence is available.

Capsule locus	Reference
1	(Munoz *et al.*, 1997)
2	(Iannelli *et al.*, 1999)
3	(Arrecubieta *et al.*, 1995, Dillard *et al.*, 1995)
4	(Tettelin *et al.*, 2001)
14	(Kolkman *et al.*, 1997)
19A	(Morona *et al.*, 1999b)
19B	(Morona *et al.*, 1999b)
19C	(Morona *et al.*, 1999b)
19F	(Morona *et al.*, 1997)
23F	(Morona *et al.*, 1999a)
33F	(Llull *et al.*, 1998)

Adapted from (Mitchell and Kerr, 2002).

The various capsular polysaccharides in purified form are completely non-toxic and cannot themselves account for death from pneumococcal infection. Mizrachi Nebenzahl and co-workers have shown that the virulence of the pneumococcus can be determined independently of the capsule (Mizrachi Nebenzahl *et al.*, 2004). However, the role of the capsule in pathogenicity is quite apparent in that it provides resistance to phagocytosis and thus promotes the escape of pneumococci from the host immune defence. It was found that nonencapsulated pneumococci are readily phagocytized when added to a suspension of leukocytes in normal serum, whereas mucoid capsulated organisms are resistant to phagocytosis and multiply rapidly (Wood and Smith, 1949). More recently, a reduction of virulence was achieved by transposon mutagenesis of one gene apparently essential for pneumococcal type 3 capsular biosynthesis (Watson and Musher, 1990). It was also shown that the capsule plays an important role in the nasopharyngeal colonization. The requirement for the capsule in colonization may reflect its ability to prevent clearance of the organism by innate defences. Alternatively, or in addition, the capsule itself may be an adhesin (Magee and Yother, 2001) promoting adhesion of the pneumococcus to human cells. Moreover, Fernebro *et al* have demonstrated that capsular polysaccharides negatively influence the lytic process and contribute to antibiotic tolerance in clinical isolates of pneumococci (Fernebro *et al.*, 2004).

1.4.2 Pneumolysin

Pneumolysin (Ply) is a 53-kDa protein produced by all clinical isolates of *S. pneumoniae* (Paton *et al.*, 1983, Paton *et al.*, 1993). It is an intracellular protein that belongs to the family of thiol-activated toxins (Boulnois, 1992), which is widespread among Gram-positive pathogens. Unlike the thiol-activated toxin produced by other members of the family, pneumolysin lacks an N-terminal export signal sequence for transport to the extracellular environment (Walker *et al.*, 1987). Although pneumolysin is not secreted by pneumococci, it can be released upon lysis of pneumococci under the influence of the major lytic enzyme autolysin (LytA). The virulence properties of Ply are therefore directly dependent on the action of autolysin. However, the extracellular release of pneumolysin from the pneumococcal strain WU2 (type 3) was found to be independent of autolysin action. A mutant lacking autolysin showed the same pattern of pneumolysin release as the wild-type strain (Balachandran *et al.*, 2001).

The role of pneumolysin in pathogenesis has been established in several ways. It was found that pneumolysin-negative mutants are less virulent than their parental strains and the immunization with pneumolysin prolongs the survival of mice after challenge with pneumococci of different serotypes (Paton *et al.*, 1983, Berry *et al.*, 1989b, Alexander *et al.*, 1994). Pneumolysin has at least two biological activities that contribute to virulence in animal models; lytic activity and ability to activate complement (Mitchell, 2000). Ply action during pneumococcal infection disrupts the alveolar-capillary boundary, which produces an alveolar flooding providing nutrients for bacterial growth and facilitates penetration through the epithelium into the bloodstream (Rubins and Janoff, 1998). Pneumolysin stimulates the production of inflammatory cytokines like tumor necrosis factor alpha and interlukin-1β by human monocytes and inhibits the beating of cilia on human respiratory epithelial cells (Houldsworth *et al.*, 1994). The ability of these cells to clear mucus from the lower respiratory tract is reduced and as a result, facilitates the spread of pneumococcal infection. Ply was also found to be responsible for hearing loss in experimental pneumococcal meningitis by inducing specific and devastating cochlear damage (Winter *et al.*, 1997).

1.4.3 Surface proteins

The pneumococcal cell displays a range of surface proteins that may play a role in pathogenesis. These proteins have a wide range of functions, including adherence to host tissues, binding to specific immune system components, protein processing, nutrient

acquisition, and uptake of DNA from the environment (Overweg *et al.*, 2000b). Proteins that are attached to the cell surface can be divided into choline binding proteins (CBPs), LPXTG-anchored proteins, and lipoproteins. Examples of these proteins are discussed below.

1.4.3.1 Choline binding proteins

CBPs are proteins that are linked to the pneumococcal cell surface by non-covalent interactions with cell wall phosphorylcholine. This group includes LytA, PspA, CbpA and others.

1.4.3.1.1 Autolysins

Autolysins are members of a widely distributed group of enzymes that degrade the peptidoglycan backbone of bacterial organisms. These enzymes are located in the cell envelope and are postulated to play a role in a variety of physiological cell functions associated with cell wall growth, its turnover, and cell separation in microorganisms (Tomasz, 1984). An example of one such enzyme is the major autolytic enzyme N-acetylmuramoyl-L-alanine amidase (LytA) of *Streptococcus pneumoniae* (Lopez *et al.*, 1992). Other autolysins such as LytB have also been identified (Garcia *et al.*, 1999). The pneumococcal LytA is thought to be bound to choline moieties of lipotechoic acid, which in turn is anchored to the cell membrane. In this form, autolysin is inactive but when cell wall biosynthesis ceases, either because of nutrient starvation or treatment with antibiotics, this association is disrupted and the enzyme is then able to cleave the covalent bond between the glycan chain and the peptide side chain of the choline-containing cell wall, thereby bringing about cellular autolysis (Briese and Hakenbeck, 1985).

The contribution of autolysin to pneumococcal virulence has been studied using autolysin-negative mutants of pneumococci constructed by insertion-duplication mutagenesis (Berry *et al.*, 1992). It was found that these mutants have attenuated virulence in animal models compared to that of wild-type pneumococci. This suggests a role for autolysin and possibly for the inflammation that follows autolysis in pneumococcal virulence and pathogenesis (Canvin *et al.*, 1995). It has been suggested that autolysin plays its role in pathogenesis in two ways (1) by generating inflammatory cell wall degradation products and (2) by releasing the pneumococcal cytoplasmic contents, including virulence factors such as pneumolysin (Mitchell *et al.*, 1997).

Tuomanen *et al* (Tuomanen *et al.*, 1985) demonstrated that both peptidoglycan and teichoic acid components of the pneumococcal cell wall were potent mediators of meningeal inflammation in a rabbit model. They proposed that treatment with β-lactam antibiotics, which induce autolysin-mediated release of the above components, might further contribute to inflammatory tissue injury and possibly mortality. Other studies on LytA also have shown that this amidase induces a protective response in mice to streptococci when inoculated in the lungs (Berry *et al.*, 1994, Canvin *et al.*, 1995). The degree of protection in mice immunized with autolysin and pneumolysin were shown to be similar, and no additional protection was observed in animals immunized with both proteins. These data are consistent with the idea that the anti-autolysin antibodies exert their effects primarily by preventing the release of pneumolysin. On the other hand, autolysin played a major effect in middle ear infection in a chinchilla otitis media model, where the role of Ply was more limited (Sato *et al.*, 1996).

Tomasz and co-workers have reported that LytA mutant strains showed the same degree of virulence in mice as did the isogenic LytA+ parent (Tomasz *et al.*, 1988), a finding that contradicts other reports (Berry *et al.*, 1989a, Canvin *et al.*, 1995). Despite this controversy regarding the role of autolysin in pathogenesis, its protective property for mice challenged with pneumococci makes it a potential component of novel anti-pneumococcal vaccines (Lock *et al.*, 1992).

1.4.3.1.2 Pneumococcal surface protein A

Pneumococcal surface protein A (PspA) is a surface protein with variable molecular size ranging from 67 to 99 kDa. Based on sequence analysis, the protein has four distinct domains: an N-terminal highly charged α-helical region, a proline-rich domain, a stretch of 10 highly conserved 20-amino-acid repeats, and a tail of 17 slightly hydrophobic residues at the C-terminus. The N-terminal portion of this choline-binding protein has been predicted to have a coiled-coil structure reminiscent of the M protein of group A streptococci and this might be expected to protrude through the capsule and contains the epitopes recognized by protective monoclonal antibodies while the C-terminal portion is involved in attachment of the protein to the cell (Watman *et al.*, 1990, Yother and Briles, 1992).

Although the precise function of PspA is uncertain, it has been shown to inhibit complement activation and is proposed to exert a function in virulence by recruitment of the alternative complement pathway, thereby reducing the effectiveness of complement receptor-mediated pathways of clearance (Tu *et al.*, 1999). In addition, PspA functions as a

lactoferrin-binding protein and is suggested to be involved in iron uptake, thus contributing to pneumococcal growth under iron-limited conditions in the human host (Hammerschmidt *et al.*, 1999). It was found that immunization with rough pneumococci protected mice against challenge with virulence organisms, but no protection was conferred by immunization with rough pneumococci in which the PspA gene had been inactivated by insertion-duplication mutagenesis. Moreover, PspA-deficient type 2 pneumococci were more readily cleared from the blood of mice during the first hour after intravenous challenge than were otherwise isogenic wild-type organisms (McDaniel *et al.*, 1987).

1.4.3.1.3 Choline binding protein A

Another example of CBPs is the choline binding protein CbpA, also referred to as PspC and SpsA (Hammerschmidt *et al.*, 1997, Brooks-Walter *et al.*, 1999) and was identified as a major pneumococcal adhesin (Rosenow *et al.*, 1997). It is a 110-kDa protein with eight choline-binding repeats that are nearly identical to PspA, with shared amino acid sequence in the α-helix, proline-rich, and choline-binding regions (Brooks-Walter *et al.*, 1999). In the absence of CbpA, pneumococci fail to enter and traverse an *in vitro* blood-brain barrier, indicating a critical role for this protein in invasion (Ring *et al.*, 1998). If the gene for CbpA is disrupted by insertion-duplication mutagenesis the mutant pneumococci have a reduced ability to bind to cytokine-activated type II pneumocytes and endothelial cells *in vitro* suggesting that this protein plays a role in adherence. Moreover, the mutant organism is less able to colonize the nasopharyx of infant rats (Rosenow *et al.*, 1997). Immunization with CbpA of strain D39 (PspC/D39) elicits antibodies that cross-react with PspA/D39 and are able to protect against pneumococcal infection. CbpA interacts with the immune system in a variety of ways. CbpA binds specifically to the secretory component of immunoglobulin A (Hammerschmidt *et al.*, 1997). PspC may also regulate the complement system by either adhering to glycoconjugates, sialic acid, and lactotetraoses on the surface of activated human epithelial cells or binding to the C3 component of complement system (Rosenow *et al.*, 1997, Smith and Hostetter, 2000). More recently, PspC was found to bind human complement factor H (Dave *et al.*, 2001).

1.4.3.2 LPXTG-anchored proteins

LPXTG-anchred proteins are attached to the pneumococcal cell surface by the covalent interaction of the conserved amino acid sequence LPXTG with the cell wall. Examples of this group are neuraminidase, hyaluronidase and IgA1 protease.

1.4.3.2.1 Neuraminidase

Neuraminidase is another potential *Streptococcus pneumoniae* virulence factors. The enzyme cleaves terminal sialic acid from cell surface glycans such as mucin, glycolipids, glycoproteins, and oligosaccharides, 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). At least two studies on fresh, clinical isolates of *S. pneumoniae* showed that all strains examined (104 in all) had neuraminidase activity (Kelly *et al.*, 1967, O'Toole *et al.*, 1971). Moreover, *S. pneumoniae* neuraminidase has been detected in 78 % of culture-positive middle ear effusions from patients with acute otitis media and in 96 % of *S. pneumoniae* positive middle ear effusions from patients with chronic otitis media (Diven *et al.*, 1988). 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). Coma and bacteraemia occur significantly more often in patients with pneumococcal meningitis when the concentration of N-acetylneuramic acid in the cerebrospinal fluid is elevated (O'Toole *et al.*, 1971).

There appear to be at least two forms of the pneumococcal neuraminidase enzymes, NanA and NanB. The structural genes for both have been cloned and sequenced (Camara *et al.*, 1994, Berry *et al.*, 1996). The benefits to the pneumococcus of production of two distinct neuraminidases are unclear. Apart from their difference in size, the two enzymes have widely different pH optima, which implies that these enzymes may assist exploitation of distinct environmental niches (Berry *et al.*, 1996).

The role of neuraminidase in streptococcal pathogenesis has been inferred from immunization studies showing that the purified protein partially protected mice against pneumococcal infection (Lock *et al.*, 1988a). Although the precise role for neuraminidase in the pathogenesis of *S. pneumoniae* has not been established, 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, Rosenfeld *et al.*, 1992, Linder *et al.*, 1994). It has been demonstrated that the treatment of chinchilla tracheas with neuraminidase *in vitro* increases *S. pneumoniae* adherence and reverses the inhibitory effects of lacto-N-neotetraose (LNnT), suggesting that neuraminidase treatment results in an increase in the number of available receptors of *S. pneumoniae* (Tong *et al.*, 1999). Like pneumolysin, the purified neuraminidase was toxic for mice, and immunization with the protein partially protected mice from challenge with virulent *S.*

pneumoniae (Lock *et al.*, 1988a). This protection, however, was not as great as that achieved by immunization with pneumolysin, and no additive protection could be obtained when mice were immunized with both proteins. In addition, Tong and colleagues indicated that the disruption of *nanA* gene diminishes the ability of *S. pneumoniae* to colonize and persist in the chinchilla nasopharynx and middle ear (Tong *et al.*, 2000). In a study by Long and co-workers, immunization of chinchillas with recombinant neuraminidase resulted in a significant reduction in nasopharyngeal colonization as well as in the incidence of otitis media with effusion (Long *et al.*, 2004). Recently, King *et al* demonstrated that NanA has no contribution to adherence of *S. pneumoniae* to epithelial cells or colonization in an animal model. However, they observed NanA-dependent desialylation of human airway components that bind to the organism and may mediate bacterial clearance (King *et al.*, 2004).

1.4.3.2.2 Hyaluronidase

Hyaluronidase is an enzyme produced by a variety of wound and mucosal pathogens, including the pneumococcus (Humphery, 1944). The enzyme degrades hyaluronic acid, which is found associated with mammalian connective tissues and extracellular matrix. Thus hyaluronidase might play a role in pneumococcal pathogenesis by allowing greater microbial access to host tissues for colonization. It may also take part in the migration of the organism between tissues, for example translocation from the lung to the vascular system. As well as playing a role in tissue integrity, hyaluronic acid also plays a role in the generation of the inflammatory response. Production of hyaluronidase by the pneumococcus could also affect the inflammatory response in the lung (Mitchell, 2000).

Increased tissue permeability caused by the action of hyaluronidase on the extracellular matrix appears to play a role in wound infections, pneumonia, and other sepses such as bacteraemia and meningitis. In *S. pneumoniae* cultures, the enzyme is found in both the culture and the cell-associated fractions. This may suggest at least part of the enzyme is released by the pathogen to surrounding host tissues during infection to facilitate the bacterial invasion (Berry *et al.*, 1994). Kostyukova and colleagues have confirmed the hypothesis that hyaluronidase enzyme plays an important role in bacterial dissemination and breaching of the blood brain barrier by pneumococci. They have reported that high hyaluronidase activity is the most important factor contributing to the development of pneumococcal meningitis (Kostyukova *et al.*, 1995).

1.4.3.2.3 IgA1 Protease

Secretory IgA protects mucous membranes from attack by pathogenic microorganisms. It acts by neutralizing toxins, enzymes, and viruses, agglutinating bacteria, and preventing bacterial adhesion to mucous membranes by blocking receptors and, by virtue of its hydrophobic nature, causing repelling interactions with the mucosal epithelium (Liljemark *et al.*, 1979, Kilian and Russell, 1994). However, a few pathogenic bacteria such as *Streptococcus pneumoniae*, *Haemophilus influenzae*, and *Neisseria gonorrhoeae* produce a variety of enzymes called IgA1 proteases. They are so called because they cleave only human IgA1 and not the IgA2 isotype. These enzymes may be important virulence factors because they are produced *in vivo* (Blake *et al.*, 1979, Insel *et al.*, 1982). A survey of 114 isolates of *S. pneumoniae* revealed that at least 94 % demonstrated the ability to hydrolyze human IgA1 (Lomholt, 1995). The protease in *S. pneumoniae* demonstrates marked antigenic variation, since at least 17 antigenic forms have been identified (Lomholt, 1995).

The *S. pneumoniae* gene encoding the IgA1 enzyme has been cloned before, but instability prevented any further characterization (Pratt and Boulnois, 1987). More recently, Wani *et al* have succeeded to identify, clone and characterize the pneumococcal IgA1 protease (Wani *et al.*, 1996) but the role of the enzyme in the virulence of the pneumococcus has not been investigated. Access to the cloned gene will permit construction of defined IgA1 protease-negative mutant and will enable the assessment of the impact of such enzyme on the pathogenicity of the pneumococcus. Weiser and colleagues have reported a role of IgA1 protease in the antibody-enhanced pneumococcal adherence (Weiser *et al.*, 2003). The authors of this study provided evidence that this protease enables the pneumococci to subvert the antigen specificity of the humoral immune response to facilitate adhesive interactions and persistence on the mucosal surface.

1.4.3.3 Lipoproteins

Lipoproteins are attached to the cell surface via palmitic acid and the motif LXXC in the N-terminus of the protein serves as the point of lipidation. Pneumococcal surface adhesin A (PsaA) is an example of these lipoproteins. Pneumococci possess an ATP-binding cassette (ABC) transporter for Mn^{2+}, the Psa transporter (Dintilhac *et al.*, 1997, Novak *et al.*, 1998). This transporter 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). PsaA protein was originally identified as being located on the pneumococcal cell surface and acts as an adhesin and virulence factor (Berry and Paton, 1996). There is a

recent report, however, showing that PsaA is not exposed on the surface of the bacterial cell, ruling out its possible function as a direct adhesin (Johnston *et al.*, 2004). The likely function of PsaA is the transport of Mn^{2+} and Zn^{2+} into the cytoplasm of the bacteria (Berry and Paton, 1996, Dintilhac *et al.*, 1997). Intranasal immunization of mice with PsaA was observed to reduce the bacterial load in nasopharyngeal carriage (Briles *et al.*, 2000a, De *et al.*, 2000). PsaA negative mutants of pneumococci were avirulent in a mouse model. 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 (Marra *et al.*, 2002b).

1.5 Competence for genetic transformation

Transformation is one of the simplest modes of genetic exchange and involves uptake of exogenous native donor DNA and subsequent homology-dependent integration into the recipient chromosome. *Streptococcus pneumoniae* (the pneumococcus) is naturally competent for genetic transformation. Indeed, genetic transformation was originally discovered in *S. pneumoniae* (Griffith, 1928). Genetic transformation is likely to play a significant role in the lifestyle of this bacterium by favouring genetic plasticity. Transformation with naked DNA allows intraspecies and interspecies gene transfer, which helps this human pathogen to adapt to its host and bypass host defences. Such exchanges involve homologous recombination and so, they did not result in the creation of novel sequences but simply in a redistribution of previously existing genes. Homologous recombination then leads to the production of mosaic genes, as exemplified in the case of the genes for penicillin binding proteins (*pbp*) of *S. pneumoniae* that encode altered penicillin-binding proteins with decreased affinity for β-lactam antibiotics (Hakenbeck *et al.*, 1999).

1.5.1 Induction of competence

Competence, the state of cells able to take up DNA, develops suddenly in response to a cell-cell signal at some point during exponential growth phase and reaches a maximum about 20 min after its induction (induction phase). It then disappears abruptly (shutoff phase) and remains off for about 40 to 60 min during a period in which cells are refractory to the signal (Tomasz and Hotchkiss, 1964, Chen and Morrison, 1987, Morrison, 1997). In *S. pneumoniae* and related streptococcal species from the mitis phylogenetic group, the competent state is not a constitutive property but is induced by a peptide pheromone

through a quorum-sensing mechanism. The quorum-sensing signal responsible for competence induction is a heptadecapeptide, named CSP (competence-stimulating peptide) (Havarstein *et al.*, 1995), which derives from a precursor (ComC) by cleavage and transport into the medium by an ATP-binding cassette (ABC) transporter, ComAB (Hui *et al.*, 1995). The competence is triggered when CSP reaches an extracellular concentration of 1-10 ng/ml, which corresponds to a population density of about 10^7 cells/ml (Havarstein *et al.*, 1995). The signalling cascade, which leads to the induction of competence state, begins with the interaction of CSP with its histidine kinase receptor (ComD), which responds by transferring a phosphate group to its cognate response regulator (ComE). After phosphorylation, the response regulator activates transcription of several genes containing a ComE-binding site, such as *comAB* and *comCDE* operons (Pestova *et al.*, 1996), establishing a positive feedback loop ensuring an abrupt rise in CSP levels, making all cells in a culture competent simultaneously. Recently, the mechanism through which this quorum-sensing circuit (*comCDE* and *comAB*) evokes the expression of the genes for the machinery of genetic transformation started to be revealed. ComE (the quorum-sensing transducer) might act as a transcription factor to induce both the competence machinery genes and those of the CSP circuit (Alloing *et al.*, 1998). ComE was also reported to induce the expression of ComX, which acts as an alternative sigma factor to enable core polymerase to transcribe competence-specific genes whose up-regulation is required for competence (Lee and Morrison, 1999, Luo *et al.*, 2003). How competence is shut off after induction is unknown, although it has been proposed that ComE has dual functions, activation at low doses of the CSP stimulus and repression at high doses for the regulation of *comCDE*, which could account for the successive induction and suppression of competence in response to increasing levels of CSP (Alloing *et al.*, 1998).

Lee and colleague (Lee and Morrison, 1999) identified ComX as a competence-specific transcription modulator, and showed that it (1) is induced through ComE by the competence-stimulating peptide, (2) is required for the expression of competence-specific operons which contain a cin-box but not of those of the quorum-sensing operons or of *comX* itself, and (3) is present in two copies in the Rx derivative strain studied. Lee and colleague also reported that the quorum-sensing system alone causes neither competence shutoff nor the CSP refractory period, and proposed that a ComX-induced gene may be responsible for these post-competence phenomena. Luo and co-workers (Luo and Morrison, 2003) have tested the hypothesis that ComX acts as a transient alternative sigma factor and found that ComX polymerase produced transcripts for the competence-specific genes *ssbB*, *cinA*, *cglA*, *celA* and *dalA* and was inhibited by anti-ComX antibody. This report concluded that ComX is unstable and acts as a competence-specific sigma factor

(Luo and Morrison, 2003). More recently, Luo *et al* identified ComW as a new positive factor involved in competence regulation (Luo *et al.*, 2004). The authors of this study reported that co-expression of ComW and ComX complemented the ComE deficiency. The model of genetic transformation in *S. pneumoniae* proposed by Luo *et al* (Luo *et al.*, 2004) is shown in Figure 1.3.

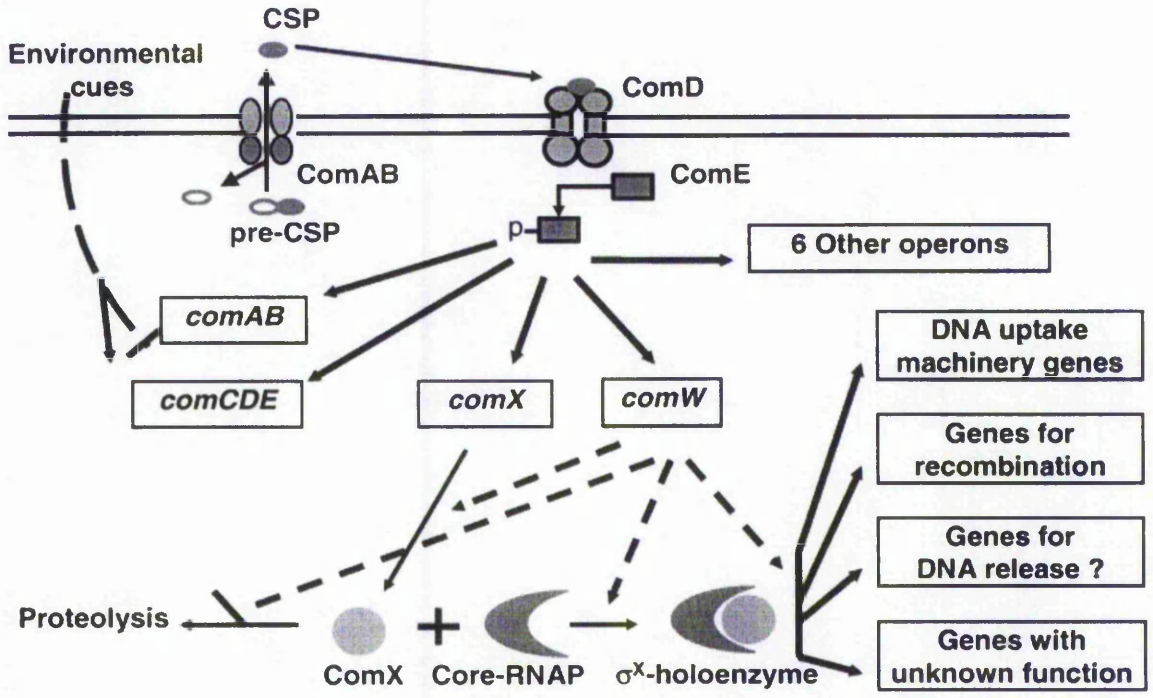


Figure 1.3- Model for the genetic transformation in *Streptococcus pneumoniae*.

The quorum-sensing system that accumulates CSP and an active form of ComE induces the expression of *comX*. ComX acts as an alternative sigma factor to enable core polymerase to transcribe competence-specific genes, whose up-regulation is required for competence. ComW, a putative ComE-dependent gene, promotes the translation of ComX and the expression of ComX-dependent genes, and may function at multiple sites in the regulation pathway. Solid arrows indicate processing (thin lines) or activation (thick lines) steps that are supported by previous and current results. Dashed lines suggest hypothetical links, T-bars indicate negative regulation and the double line represents the cell membrane. Adapted from (Luo *et al.*, 2004).

1.5.2 Uptake of DNA

During the period of competence, double-stranded DNA encountered by pneumococcal cells is bound to the cell surface and one strand of the DNA is degraded into short oligonucleotides in the medium while part of the other strand is imported inside the cell (Mejean and Claverys, 1988). The nuclease responsible for degradation in *S. pneumoniae* is the product of the *endA* gene (Lacks *et al.*, 1975, Puyet *et al.*, 1990). EndA is constitutively expressed membrane-located protein (Lacks and Neuberger, 1975) and is required for transport but not for binding of donor DNA to the cell surface (Lacks *et al.*,

1975). The imported single strand of DNA is protected from nuclease activity by a single-stranded DNA binding protein (SSB) (Morrison and Mannarelli, 1979) and is finally incorporated into the chromosome by homologous recombination involving the general recombination machinery, including RecA (Mortier-Barriere *et al.*, 1998).

Several competence-specific operons which are probably involved with the DNA uptake process and recombination were identified, such as *cilA*, *cilB* (Karudapuram *et al.*, 1995), *cilC*, *cilD*, *cilE*, and *coi* (Campbell *et al.*, 1998, Pestova and Morrison, 1998, Luo *et al.*, 2003). These operons and additional competence-related operons, such as *cinA-recA* and *cfl* (Londono-Vallejo and Dubnau, 1993, Lee *et al.*, 1999, Luo *et al.*, 2003), all contain an unusual perfectly conserved consensus sequence, TACGAATA (cin-box), at position 10 from the transcription start and a T-rich region at 25 (Campbell *et al.*, 1998).

1.5.3 Competence and virulence

Competence and virulence are highly regulated, and there is increasing evidence linking the two phenomena together. For example, the histidine kinase, ComD, the target for CSP is required for virulence (Lau *et al.*, 2001). Transcription of the *comCDE* operon is negatively controlled by the two-component system CiaR/H (Echenique *et al.*, 2000), which is also involved in virulence (Throup *et al.*, 2000). In addition, the virulence factor LytA, a choline binding protein showing autolytic activity (Berry *et al.*, 1989a), belongs to a late competence operon (Mortier-Barriere *et al.*, 1998). Clearly, virulence expression depends on events involved in the competence-signalling pathway. Furthermore, the ABC transporters encoded by *psa* (and *adc*), involved in the uptake of Mn^{2+} (and possibly Zn^{2+}), are important for growth and competence (Dintilhac *et al.*, 1997), and the *psaA* product is an essential virulence factor (Berry and Paton, 1996). Mutations of LicD2 and of Nox lead to the alteration of both competence and virulence expression (Auzat *et al.*, 1999, Zhang *et al.*, 1999, Lau *et al.*, 2001, Yu *et al.*, 2001).

Using differential fluorescence induction (DFI) technology, Marra and colleagues have reported that competence genes were up-regulated during infection (Marra *et al.*, 2002a). Recently, the two-component signalling system, MicAB, which contributes to repression of competence when oxygen is limited, was also found to be involved in the adaptive response of the pneumococcus to changes in oxygen level during the course of infection. A mutation in the histidine kinase, *micB* attenuates the pneumococcal strain for virulence when given intranasally (Kadioglu *et al.*, 2003). Chapuy-Regaud *et al* have investigated the role of the global regulator, RegR in competence development and virulence of the

pneumococcus by mutational analysis. RegR was found in this study to be involved in the adaptive response of *S. pneumoniae* through its control of competence and virulence (Chapuy-Regaud *et al.*, 2003). Furthermore, the role of the serine/threonine kinase StkP in competence signalling and in experimental virulence was investigated by Echenique *et al* (Echenique *et al.*, 2004). Data presented in this study demonstrated that StkP belongs to the signalling network involved in competence triggering *in vitro* and lung infection and bloodstream invasion *in vivo*. In competence, functional StkP is required for activation of *comCDE* upstream of the autoregulated ring orchestrated by the competence-stimulating peptide. This was the first description of positive regulation of *comCDE* transcription in balance with its repression by CiaR/H (Echenique *et al.*, 2004).

1.6 Adherence and Invasion

Adherence to and invasion of eukaryotic cells are the main strategies used by pathogenic bacteria for colonization, evasion of immune defences, survival, and causing disease in the mammalian hosts. It is becoming apparent that microorganisms, using their surface proteins, interact with host cell receptor molecules and regulate intercellular signalling pathways to induce their own adherence, colonization, and internalization (Pancholi, 2000). The ability of *Streptococcus pneumoniae* to adhere to specific sites on host tissues has been investigated by using various cell culture models, such as human nasopharyngeal epithelial cells and alveolar type II epithelial cells, as well as human vascular endothelial cells (Andersson *et al.*, 1983, Geelan *et al.*, 1993, Cundell and Tuomanen, 1994). It is widely believed that *S. pneumoniae* adherence to nasopharyngeal epithelium is a prerequisite for induction of otitis media, pneumonia, and other pneumococcal diseases. Initially, *S. pneumoniae* is believed to target an anatomical niche, such as the nasopharynx, in the host by binding to any of several surface glycoconjugates, which exist on normal, resting respiratory epithelial or endothelial cells. Pneumococcal adherence in the nasopharynx is presumably mediated by *N*-acetyl-D-glucosamine β1-3 galactose (GlcNAcβ1-3Gal) glycoconjugate receptors (Andersson *et al.*, 1983). The second phase of adherence, which is believed to lead to invasion and disease, is induced by cytokine activation, and results in the *de novo* expression of new sugar specificities on the cytokine-activated cells which results in a concomitant increase in pneumococcal adherence (Tong *et al.*, 1999). Activation of human epithelial and endothelial cells by interleukin, IL–1α and tumor necrosis factor, TNF–α produced in inflamed sites has been shown to induce the adherence of *S. pneumoniae* to platelet activating factor receptor, PAF-R (Cundell *et al.*, 1995a). This has been confirmed by Ishizuka *et al* who have demonstrated that *S.*

pneumoniae adheres to cultured human tracheal epithelial cells via binding to PAF-R (Ishizuka *et al.*, 2001). The latter group also suggested that rhinovirus infection stimulates *S. pneumoniae* adhesion to airway epithelial cells via increases in PAF-R and that increased adherence of *S. pneumoniae* may be one of the reasons that pneumonia develops after rhinovirus infection (Ishizuka *et al.*, 2003).

Adherence by *S. pneumoniae* is a complex process and adhesins or ligands that potentially contribute to the regulation of adherence are actively being investigated. These include peptide permeases, choline-binding proteins, opaque-transparent phenotypic variants, and the release of bacterial cell wall components which are responsible for epithelial and endothelial cell activation (Rosenow *et al.*, 1997, Tuomanen, 1997). A chinchilla trachea organ culture model was used by Tong and colleagues to evaluate adherence of *S. pneumoniae* otitis media strains *in vitro*. The strains were showed to adhere within 30 minutes of exposure to chinchilla tracheal epithelial tissues and neuraminidase was found to enhance the adherence of *S. pneumoniae* to upper respiratory tract epithelium (Tong *et al.*, 1999).

In contrast to colonization, invasion appears to be a more stringently selected capability. Pneumococci can traverse the respiratory epithelium *in vivo* and *in vitro*, but the route taken from the respiratory epithelium to the bloodstream may be via lymphatics or direct invasion of endothelial cells. Invasion is considered a multistage process initiated by adherence. A key step in the transition from adherence to invasion is the activation of host cells. Activated cells synthesize glycoconjugates bearing sialic acid and lacto-N-tetraoses. These determinants and others are recognized by pneumococci, such as platelet activating factor (PAF) receptor, secretory IgA, and the third component of complement. A critical adhesive ligand of the pneumococcus that binds all of these targets is CbpA (Cundell *et al.*, 1995a, Hammerschmidt *et al.*, 1997, Gosink and Tuomanen, 2000). Phase variation of pneumococci also determines the ability of the bacteria to invade the host cells. Ring *et al* have investigated the ability of pneumococci to invade and transmigrate through monolayers of rat and human brain microvascular endothelial cells (BMEC). Results of this study indicated that phase variation to the transparent phenotype increased invasion as much as 6-fold. Invasion of transparent pneumococci required choline in the pneumococcal cell wall and was partially inhibited by PAF receptor on the BMEC. Most of the opaque variants were killed in that assay. This study suggested that interaction of pneumococci with the PAF receptor results in sorting so as to transcytose bacteria across the cell while non-PAF receptor entry shunts bacteria for exit and re-entry on the apical surface in a novel recycling pathway (Ring *et al.*, 1998).

Most studies on pneumococcal epithelial cell interactions and on invasion have been done *in vitro* with cultured cell lines. However, Kadioglu and colleagues (Kadioglu *et al.*, 2001) introduced the first report of pneumococcal invasion and cellular internalization of broncho-epithelial cells *in vivo* by making pneumococci expressing green fluorescent protein (GFP) and used it to trace pneumococcal adherence and invasion. Since broncho-epithelial cells are the first host cells to be encountered and penetrated by the pneumococcus in the lower respiratory system, they would appear to play a vital role in subsequent pneumococcal invasiveness and lung pathogenesis. It was demonstrated that pneumococcal invasion into host cells not only involves cell surface contact and penetration, but also bacterial trans-cellular migration throughout epithelial cells. *S. pneumoniae* was shown to cause the separation of tight junctions in an organ culture of human adenoid ciliated and unciliated epithelium (Rayner *et al.*, 1995). However, Kadioglu and colleagues did not find pneumococci within cell junctions *in vivo*, indicating differences between the *in vivo* and the *in vitro* culture systems. The key to this discrepancy may lie with epithelial cell receptors expressed only under inflammatory *in vivo* conditions or to other factors not present within *in vitro* systems (Kadioglu *et al.*, 2001).

1.7 Antibiotic Resistance

Management of pneumococcal infections used to be relatively straightforward, and penicillin generally was the antibiotic of choice. However, the emergence of penicillin-resistant and multi-resistant strains during the last 20 years has posed serious problems in the treatment of pneumococcal diseases. The first reports on resistance to penicillin were made in the 1930s in Australia and New Guinea (Meis and Neeleman, 1996). It took until 1977 before highly resistant pneumococci were reported in South Africa (MIC >1.0 mg/l). In addition, these strains were also resistant to other penicillins and cephalosporins (Appelbaum *et al.*, 1977). It has been reported that penicillin kills pneumococci through two distinct mechanisms; one triggers an autolytic enzyme, amidase, and the other, which is enzyme-independent, involves the *cid* gene (Moreillon *et al.*, 1990). The mechanism of resistance of pneumococci to penicillin is due to alterations of the penicillin binding proteins (PBPs) that have reduced their affinity for penicillin and other related β-lactams. *S. pneumoniae* possesses five high molecular weight penicillin binding proteins PBP1a, PBP1b, PBP2a, PBP2b, PBP2x, and the low molecular weight D, D-carboxypeptidase PBP3. These PBPs together with β-lactamases are members of a superfamily of penicilloyl serine transferases that interact with β-lactam antibiotics via the formation of covalent

serine ester-linked penicilloyl complexes (Ghuysen and Dive, 1991, Krauss and Hackenbeck, 1997, Hakenbeck *et al.*, 1998). These alterations in PBPs are believed to be due to the ability of *S. pneumoniae* to acquire DNA by transformation of small segments of DNA from organisms sharing the same ecological niche. Molecular techniques have identified genetic mosaicism among the PBPs of resistant pneumococcal isolates. This mosaicism suggests that genes for these proteins were imported from some heterogeneous non-pneumococcal source (such as the mitis group) rather than evolving in an individual organism (Dowson *et al.*, 1994).

Higher mortality has been found among patients infected with *S. pneumoniae* strains resistant to penicillin than among patients infected with susceptible strains (Choi and Lee, 1998) and few studies have investigated the relationship between the overuse of penicillin and the colonization and/or infection risk with penicillin-resistant pneumococci. Other risk factors appear to be adolescent age group, previous hospitalization history, attending a nursery and living in areas from which resistant strains are frequently isolated (Klugman, 1996). Filipe and Tomasz (Filipe and Tomasz, 2000) have identified two genes, *murM* and *murN*, that encode enzymes involved with the biosynthesis of branched muropeptides. The inactivation of these genes restores penicillin susceptibility to β-lactam-resistant pneumococci. These genes therefore appear to be essential for pneumococci to make the branched cell wall associated with penicillin resistance. It is conceivable therefore, that antibiotics able to inhibit the action of these enzymes may be used in conjunction with penicillin or amoxicillin to prevent the expression of penicillin resistance.

Concurrent with this growing penicillin resistance problem is an associated decrease in susceptibility among *S. pneumoniae* to a range of other traditional empiric antimicrobials such as macrolides, trimethoprim-sulfamethoxazole, tetracyclines, and to a lesser extent chloramphenicol (Pallares *et al.*, 1987). In 1998, 24 % of all invasive pneumococcal isolates captured by a multistate, population-based surveillance network were resistant to penicillin, and 14% of all isolates were resistant to three or more drug classes (Whitney *et al.*, 2000). In addition, recent studies have reported cases of vancomycin-tolerant pneumococcal strains, although vancomycin is an antibiotic that had not previously been shown to have any pneumococcal resistance (Novak *et al.*, 1999, McCullers *et al.*, 2000). Cefditoren is a novel cephalosporin that is unusual in that it has greater antimicrobial activity against the pneumococcus than does penicillin. In an analysis of 312 pneumococcal strains, Johnson and co-workers showed that all these strains were inhibited by a cefditoren MIC of 1 μg/ml or less. Only 14 % of these strains were fully penicillin

resistant (Johnson *et al.*, 2000). These data clearly suggest that more studies on the susceptibility of pneumococcus to cefditoren are required.

The CDC National Center for Infectious Diseases has published that seven pneumococcal serotypes, namely (6A, 6B, 9V, 14, 19A, 19F, and 23F) account for most drug resistant pneumococci. Until 2000, *S. pneumoniae* infections caused 60,000 cases of invasive disease each year and up to 40 % of these were caused by pneumococci non-susceptible to at least one drug. These figures have decreased substantially following the introduction of the pneumococcal conjugate vaccine for children. In the year 2002, there were 37,000 cases of invasive pneumococcal disease. Of these, 34 % were caused by pneumococci non-susceptible to at least one drug and 17 % were due to a strain non-susceptible to 3 or more drugs (information from CDC National Center for Infectious Diseases at http://www.cdc.gov/).

1.8 Pneumococcal vaccines

It has long been recognized that the best management of most infectious diseases is prevention. Moreover, the possibility that antibiotics might one day be ineffective in curing at least some cases of pneumococcal pneumonia and other pneumococcal infections has increased the importance of having an effective vaccine. Soon after the pneumococcal capsule was identified as a major immunogen in 1930s, subsequent vaccine development focused on purified polysaccharides (PS). Pneumococci are now known to possess one of at least 90 different PS types, although pneumococci of less than 30 types cause the vast majority of human disease. It has become apparent that immunization with pneumococcal polysaccharide was effective in preventing bacteraemic pneumococcal pneumonia, and two hexavalent polysaccharide vaccines were commercially introduced. However, in the 1940s antibiotics were becoming widely available and the perceived impact of pneumococcal infections had greatly diminished. The two vaccines were withdrawn from the market for lack of demand (Austrian, 1981a, b). Despite the use of antibiotics, morbidity and mortality from invasive pneumococcal infections remained high. Renewed interest in pneumococcal vaccines led to further clinical trials of polysaccharide vaccines. Based on two large trials in South Africa and New Guinea, a 14-valent polysaccharide vaccine was licensed in the United States in 1977 and the formulation was expanded to include 23 PS types in 1983 (Salyers and Whitt, 1994, Briles *et al.*, 2000c).

The use of a 23-valent vaccine containing capsular polysaccharides from pneumococci commonly causing disease has had limited effect in reducing the morbidity and mortality

associated with this organism especially in the elderly and young children (Shapiro *et al.*, 1991, Butler *et al.*, 1998). One frequently postulated reason for the incomplete protection afforded by the polysaccharide vaccine is the poor immunogenicity of PS in elderly adults. Certain PS types in the 23-valent vaccine, notably 6B, 9V, 19F, and 23F, induce relatively weak antibody responses that diminish to prevaccination levels in a short time (Sankilampi *et al.*, 1997). Young children also respond poorly to polysaccharide antigens and are particularly susceptible to infections with encapsulated bacteria. The reasons for this immune response are complex and not fully known, but it is clear that children less than 2 years of age do not have a fully developed capacity to respond to many T-cell-independent polysaccharide antigens (Bijkers *et al.*, 1996).

The poor immunogenicity of polysaccharides in the very young and elderly has spurred the development of pneumococcal vaccine that involves T-cell responses. Covalent attachment of polysaccharide antigens to protein (conjugate vaccine) should improve the efficacy of the vaccine by making it more immunogenic in young children than polysaccharide vaccine. The proteins in the conjugate vaccine cause a switch in the immune response to polysaccharide from T-cell-independent to T-cell-dependent. This results in an increase in the antibody response and the generation of memory T-lymphocytes (Butler, 1997). Vaccine manufacturers have prepared many conjugate vaccines such as pncOMPC vaccine, which contains polysaccharides from seven serotypes conjugated to the meningococcal outer membrane complex, the pncCRM vaccine which contains either oligosaccharides or polysaccharides coupled to a non-toxic mutant of diphtheria toxin CRM197, the pncT vaccine that contains eight PSs coupled to tetanus toxoid, and pncD vaccine which contains the same PSs coupled to diphtheria toxoid (Hogerman *et al.*, 1995, Ahman *et al.*, 1996, Anderson *et al.*, 1996). Some pneumococcal conjugate vaccines and their manufacturers are shown in table 1.3.

Table 1.3- Some pneumococcal polysaccharide-protein conjugate vaccines currently or previously used or approaching licensure.

Saccharid(s)	Protein(s)	Manufacturer(s)	Trade name
Serotypes 4, 9V, 14, 19F and 23F polysaccharides; 6B saccharide	Mutant non-toxic diphtheria toxin CRM197	Wyeth	Prevenar®
As above plus types 1 and 5 polysaccharides	Mutant non-toxic diphtheria toxin CRM197	Wyeth	Not-licensed
1, 4, 5, 7F, 9V, 19F and 23F polysaccharides; types 3, 14 18C and 6B polysaccharides	Tetanus toxoid; Diphtheria toxoid	Aventis Pasteur	Not-licensed
1, 3, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F polysaccharides	Non-typeable *H. influenzae* outer membrane protein	Glaxo SmithKline	Not-licensed

Adapted from (Finn, 2004).

Black and co-workers have carried out a clinical efficacy trial of pneumococcal conjugate vaccine in infants. Seven-valent vaccine conjugated to CRM197, or meningococcal type C conjugate vaccine (Placebo) were given to 37, 868 children in northern California at 2, 4, and 6 months of age, and a booster was given at 12-15 months of age. The vaccine efficacy was 97.4 % against invasive disease caused by the vaccine serotypes (Black *et al.*, 2000). On the other hand, evidence recently reported from a clinical trial of pneumococcal conjugate vaccine suggests that vaccinated children have higher rates of otitis media from non-vaccine serotypes than do unvaccinated children (Eskola *et al.*, 2001). Conjugate vaccines have also been tested in elderly adults and found to be safe and immunogenic (Powers *et al.*, 1996, Shelly *et al.*, 1997). However, the pneumococcal serotypes responsible for invasive infections in adults are more diverse than those in children, and the formulation of conjugate vaccines to cover this larger variety of serotypes may prove to be a developmental and financial hurdle that may be difficult to overcome.

A possible solution to the vaccine problem was suggested by the discovery that a pneumococcal surface protein, PspA, elicits a protective antibody response that is effective against more than one capsular serotype (Salyers and Whitt, 1994). Moreover, because it is a protein, it would be an effective vaccine for high-risk groups as infants and the elderly. Other pneumococcal virulence factors have been considered as promising vaccine candidates or as carrier proteins in pneumococcal conjugate vaccine, these include

neuraminidase, autolysin, pneumolysin, and pneumococcal surface adhesin A (PsaA) (Lock *et al.*, 1988a, Sampson *et al.*, 1994). PspA, PsaA and pneumolysin are currently the leading vaccine candidates (Briles *et al.*, 2000b). However, none of the proteins are considered to elicit species-wide pneumococcal protection so far. This can be explained by the occurrence of allelic variation within most individual proteins (Briles *et al.*, 1998, Iannelli *et al.*, 2002). Antibodies raised against a single protein may not recognize allelic variants. Therefore, immunological interference using multiple variants of a single protein or using multiple proteins will limit immunological escape by the pneumococcus. Therefore, a combination of proteins should be considered in future protein vaccine strategies. Current strategies used for producing pneumococcal vaccines are reviewed in (Bogaert *et al.*, 2004a).

1.9 Signal transduction in bacteria

1.9.1 An overview

Bacteria must modulate the expression of specific genes in response to changes in environmental conditions. This adaptive response is mediated in part by two-component regulatory systems, also known as histidine-aspartate phosphorelay systems. One component of the system acts as an environmental sensor, often located in the cytoplasmic membrane, that transmits a signal to the second component, a cytoplasmic response regulator, which then mediates changes in gene expression (Stock *et al.*, 1995). The mode of molecular communication between a "sensor kinase" and its cognate "response regulator" is principally based on histidine-to-aspartate (His-Asp) phosphotransfer. According to the model that has been proposed for the transduction of environmental signals by these two-component systems, the sensor protein, commonly a histidine kinase (HK), responds to an environmental stimulus by autophosphorylation on a histidine residue. The phosphoryl group is then transferred to an aspartate residue in the response regulator (RR), which generates a cascade of events leading to transcription factors whose affinity for specific DNA sequence is modulated by phosphorylation (Stock *et al.*, 1995). By these means, bacterial two-component pathways control a dazzling array of functions including cell division, virulence, antibiotic resistance, metabolite fixation and utilization, response to environmental stress and sporulation. A number of two-component systems in some prokaryotic organisms and their regulatory functions are shown in table 1.4. In addition, the existence of a cross talk between the sensor of one system and the regulator of another system has been observed (Verhamme *et al.*, 2002). However, the mechanisms by

which different two-component systems are integrated into a coordinated cellular response is not well understood.

Table 1.4- Two-component systems in some prokaryotic organisms.

Organism	HK/RR	Regulatory function	Reference
Escherichia coli	CheA/CheY,B	Chemotaxis.	(Hess *et al.*, 1988)
	EnvZ/OmpR	Adaptation to external osmotic changes by regulating the levels of the outer membrane porin proteins.	(Egger *et al.*, 1997, Yoshida *et al.*, 2002)
	ArcB/ArcA	Aerobic/anaerobic regulation of respiration/fermentation.	(Lynch and Lin, 1996)
	BasS/BasR	Responses to iron.	(Hagiwara *et al.*, 2004)
Bacillus subtilis	VanS/VanR	Vancomycin tolerance.	(Evers and Courvalin, 1996)
	KinA/Spo0F	Regulation of sporulation.	(Wang *et al.*, 2001)
	ComP/ComA	Regulation of competence.	(Weinrauch *et al.*, 1990)
	DegS/DegU	Regulation of exprotein production, catabolism, competence development and motility.	(Ogura *et al.*, 2001)
Staphylococcus aureus	SrhS/SrhR	Regulation of energy transduction in response to changes in oxygen availability.	(Throup *et al.*, 2001)
	YycF/YycG	Regulation of bacterial cell wall.	(Martin *et al.*, 1999)
Campylobacter jejuni	RacR-RacS	Temperature-dependent growth and colonization.	(Bras *et al.*, 1999)
Vibrio fischeri	LuxI/LuxR	Quorum sensing and induction of bioluminescent genes.	(Salmond *et al.*, 1995)
Listeria monocytogenes	LisR/LisK	Response to ethanol, pH and H_2O_2 stresses, Cephalosporin tolerance.	(Kallipolitis and Ingmer, 2001, Cotter *et al.*, 2002)
Caulobacter crescentus	Cck/CtrA	Coordination of cell cycle progression and polar morphogenesis.	(Jacobs *et al.*, 1999, Wheeler and Shapiro, 1999)
Rhodobacter sphaeroides	PrrA/PrrB	Expression of photosynthetic and Calvin cycle CO_2 fixing operons.	(Emmerich *et al.*, 2000)
Streptomyces coelicolor	AbsA1/AbsA2	Antibiotic synthesis.	(Brian *et al.*, 1996, Anderson *et al.*, 2001)
Rhizobium meliloti	FixL/FixJ	Nitrogen fixation.	(Weinstein *et al.*, 1992)

The basic biochemical events of two-component signal transduction were first established in *Escherichia coli* (Ninfa and Magasanik, 1986) for the NR system, a regulatory system

that controls gene expression in response to nitrogen-source availability. At about the same time, Nixon and co-workers (Nixon *et al.*, 1986) recognized amino acid sequence similarities between the components of the NR system and components of numerous other bacterial sensory systems that had not been characterized at a biochemical level. Such similarities raised the exciting possibility that these other systems operated via a signalling mechanism analogous to that utilized by the NR system. Subsequent work has supported this idea, and the list of two-component systems has expanded to include hundreds of distinct systems and numerous review articles on the topic have been published (Bourret *et al.*, 1991, Parkinson and Kofoid, 1992, Hoch and Silhavy, 1995, Chang and Stewart, 1998, Stock *et al.*, 2000, Wick and Egli, 2004).

1.9.1.1 Domain structure and phosphorylation pathway

Histidine kinases are generally composed of a highly variable transmembrane-spanning amino-terminal sensor kinase and a more conserved carboxyl-terminus composed of the transmitter and kinase domains. Within the carboxyl-terminal domain are four, or occasionally five, recognizable amino acid motifs or boxes, which are named after the single-letter amino acid code for the most conserved residues, the H, N, D, F and G boxes. The H box centres on a strictly conserved histidine residue, the site of autophosphorylation (Stock *et al.*, 1989). The amino-terminal receiver domain of response regulators typically contains a stretch of four hydrophobic residues followed by the D1 and D2 boxes, each of which contains a conserved aspartate residue, and an additional K box, which contains an invariant lysine residue. The conserved aspartate, which lies within the D2 box, is considered to be the site of phosphorylation (Volz, 1993). Comparison of different response regulators reveals a highly conserved region, called the receiver domain, within the N-terminal half (approximately 125 amino acids). The C-terminal part corresponds to the effector or output domain. In most cases this domain has DNA binding properties, and many response regulators have been shown to function as transcriptional regulators.

A prototypical two-component pathway (figure 1.5) serves to demonstrate the basic phosphotransfer mechanism that forms the core of both simple and more complicated systems. Stimuli, detected by a sensor domain of the histidine kinase, regulate HK activities. The HK catalyzes ATP-dependent autophosphorylation of a specific His residue within the HK dimerization domain. The RR then catalyzes transfer of the phosphoryl group from the phosphoHis to one of its own Asp residues. Phosphorylation of the conserved regulatory domain of the RR activates an effector domain that elicits the specific output response (West and Stock, 2001).

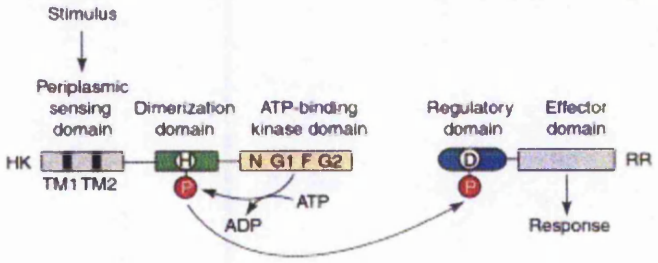


Figure 1.4- Two-component mechanism.

A typical two-component phosphotransfer system consists of a dimeric transmembrane sensor histidine kinase (HK) and a cytoplasmic response regulator (RR). A monomer of a representative HK is shown with transmembrane segments indicated by TM1 and TM2. Conserved sequence motifs N, G1, F and G2, are located in the ATP-binding domain. HKs catalyze ATP-dependent autophosphorylation of a specific conserved His residue (H). The activities of HKs are modulated by environmental signals. The phosphoryl group (P) is then transferred to a specific Asp residue (D) located within the conserved regulatory domain of an RR. Phosphorylation of the RR typically activates an associated (or downstream) effector domain, which ultimately elicits a specific cellular response. Adapted from (West and Stock, 2001).

1.9.1.2 Role of TCS in virulence

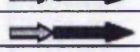
Pathogenic bacteria coordinate an intricate network of virulence factors, whose expression must be precisely controlled to maximize the chance of establishing a successful infection. Two-component systems are recognized for their role in the regulation of genes important for different stages of the infection process. For example, elevated temperature and low pH might be early signals heralding entry of an enteric pathogen into the host. Chemotactic signals affecting motility might then enable the pathogen to reach its desired local site. Once there, the pathogen must express new genes to compete with normal host flora for essential and limited nutrients (Hoch and Silhavy, 1995). All of these steps and many others are coordinated in part by TCS. The *Staphylococcus aureus* Agr is a very well characterized TCS and acts as a global regulator capable of inducing and repressing different groups of virulence-associated genes depending on the site of infection (Lyon *et al.*, 2000, Bronner *et al.*, 2004). Moreover, Zahrt and co-workers (Zahrt *et al.*, 2003) presented evidence demonstrating that sensor kinase MprB and response regulator MprA of *Mycobacterium tuberculosis* function as an intact signal-transducing pair both *in vitro* and *in vivo*. Sensor kinase MprB can be autophosphorylated, can donate phosphate to MprA, and can act as a phospho-MprA phosphatase *in vitro*. Mutagenesis of residues His^{249} in MprB and Asp^{48} in MprA abolished the ability of these proteins to be phosphorylated *in vitro*. Introduction of these alleles into *Mycobacterium bovis* BCG attenuated virulence in macrophages *in vivo* (Zahrt *et al.*, 2003). The role of two-component systems in virulence has been reported in many other pathogenic bacteria,

including *Helicobacter pylori* (Panthel *et al.*, 2003), *Haemophilus influenzae* (De Souza-Hart *et al.*, 2003), *Brucella abortus* (Guzman-Verri *et al.*, 2002), *Enterococcus faecalis* (Teng *et al.*, 2002), *Campylobacter jejuni* (Mackichan *et al.*, 2004) and many others. The integral role of two-component systems in virulence and antibiotic sensitivity, and the existence of essential two-component systems in several pathogenic bacteria, suggest that these systems may be novel targets for antimicrobial intervention (Stephenson and Hoch, 2002).

1.9.2 Pneumococcal two-component systems

Two separate genomic-based approaches have identified the two-component signal transduction systems in *S. pneumoniae* (Lange *et al.*, 1999, Throup *et al.*, 2000). A total of 14 open reading frames (ORFs) were identified as putative response regulators, 13 of which were adjacent to genes encoding probable histidine kinases (Throup *et al.*, 2000). Analysis of protein sequences of the 13 TCS identified by Lange and co-workers (Lange *et al.*, 1999) revealed that two of them have been described before, *ciaR/H* and *comDE*, and two are homologous to the *yycFG* (Fabret and Hoch, 1998) and the *phoRP* (Hulett, 1996) genes of *Bacillus subtilis*, these are the pneumococcal TCS02 and TCS04, respectively. All the 13 TCS identified by Lange and co-workers (Lange *et al.*, 1999) and their possible functions are shown in table 1.5.

Table 1.5- The pneumococcal two-component systems.

TCS	Acc. No.	Gene Organisation	G+C-Content	Possible regulatory function
TCS01	AJ06391		~41%	
TCS02	AJ06392		~40%	redox/energy sensing
TCS03	AJ06393		~45%	
TCS04	AJ06394		~41%	phosphate sensing
CiaH/R	AJ05926		~38%	competence and penicillin susceptibility
TCS06	AJ06395		~35%	
TCS07	AJ06396		~41%	
TCS08	AJ06397		~42%	
TCS09	AJ06398		~41%	nutrient perception
TCS10	AJ06399		~44%	
TCS11	AJ06400		~44%	
ComD/E	AJ05925		~31%	quorum sensing/competence
TCS13	AJ06401		~40%	pheromone/peptide sensing

All response regulators (short arrows, light gray) and histidine kinases (long arrows, dark gray) are arranged in pairs (column 3). Adapted from (Lange *et al.*, 1999).

Best described among these systems is ComDE, which activates the genetic competence system in response to competence-stimulating peptide (Pestova *et al.*, 1996, Cheng *et al.*, 1997). A second system, CiaR/H, appears to operate upstream of the ComDE pathway and has been shown to modulate competence expression but is also associated with other characteristics such as resistance to the β-lactam antibiotic cefotaxime (Guenzi *et al.*, 1994, Echenique *et al.*, 2000). Other pneumococcal TCS have been linked to a coordinated cell-death pathway (Novak *et al.*, 1999, Novak *et al.*, 2000b) and to the regulation of a bacteriocin-like peptide locus (de Saizieu *et al.*, 2000).

The response regulator of the TCS02 has recently been crystallized (Riboldi-Tunnicliffe *et al.*, 2004b). This system has been shown to be essential for bacterial growth as knockout mutations in these genes lead to non-viable bacterial strains, thus making it a prime target for new antibacterial agent design (Lange *et al.*, 1999). It has also been shown to regulate the transcription of ComCDE under oxygen limiting conditions and therefore involved in competence repression (Echenique *et al.*, 2000). Recently, the essentiality of TCS02 (referred to as VicRK) was shown to be due to its positive regulation of the expression of PcsB, a protein potentially involved in cell wall hydrolysis and the regulation of cell wall biosynthesis. The *pcsB* gene was identified as a target of the TCS02 (Ng *et al.*, 2003). Constitutive expression of $pcsB^{+}$ suppressed the essential requirement for VicRK and

allowed the isolation of VicR-null mutants (Ng *et al.*, 2003). Reduced expression of PcsB resulted in formation of long chains of cells suggesting a role for PcsB as a murein hydrolase that balances the extent of cell wall synthesis in *Streptococcus pneumoniae* R6 strain (Ng *et al.*, 2004). Physiological defects that resulted from depletion of PcsB strongly resembled those caused by depletion of TCS02, an observation that supports the hypothesis that PcsB is positively regulated by the TCS02 (Ng *et al.*, 2004). Furthermore, TCS04 has been linked to the ATP-binding transporter Psa, which has been associated with virulence (Marra *et al.*, 2002b) and resistance to oxidative stress and reduced adhesion to tissue cultured cells (Berry and Paton, 1996). The *psa* operon was found to be down-regulated in a rr04 mutant of TIGR4 which could contribute to the reduced virulence of that mutant (McCluskey *et al.*, 2004).

Signal transduction systems are also important for the *in vivo* adaptation and pathogenesis of *S. pneumoniae*. The capability of null mutants in these systems to cause disease in animals has been previously examined in two studies of virulence. In a murine model of pneumonia developing after intranasal inoculation in which all of the TCS except ComDE were tested, deletions in eight of the systems led to a dramatic attenuation of growth in the mouse (Throup *et al.*, 2000). In contrast, a second study testing a collection of single-crossover mutants in a different genetic background showed no evidence of decreased virulence for any of the strains in a murine model of intraperitoneal infection in which the requirements of adaptation to the mucosal surface of the airway were circumvented (Lange *et al.*, 1999). TCS04 and TCS09 have been reported to play a role in the virulence of *S. pneumoniae* and have been found to be strain-dependent (Blue and Mitchell, 2003, McCluskey *et al.*, 2004). The CiaR/H system has previously been reported to play a role in mouse lung colonization (Throup *et al.*, 2000) and colonization of the nasopharynx of infant rats (Sebert *et al.*, 2002) and has also been identified as playing a role in a mouse model of systemic disease (Marra *et al.*, 2002a). In this thesis, the relationship between HtrA, a stress response protein of the serine protease family, and the CiaR/H system has been investigated (see chapter 5).

1.10 Bacterial stress responses

Exposure to many different, constantly changing stresses is the normal lifestyle of bacteria. Stress can be defined as exposure to any environmental situation that results in damage of cellular components in the absence of a cellular response. Given the genome expression data available for certain bacteria, stress may be defined as a situation that stimulates the

expression of genes known to respond to a specific environmental condition. Bacterial cells respond to alterations in their environment by activating small or large groups of genes under the control of a common regulatory protein (Hengge-Aronis, 2000). In their attempts to eliminate pathogenic bacteria, host organisms present stressful conditions for bacteria, including environments limited in essential nutrients such as iron, suboptimal physical conditions such as acidic pH, and attacks of the immune system such as oxidative bursts. Pathogenic bacteria have to display rapid responses to these hostile environmental conditions including regulation of metabolic factors and exoprotein production (Charpentier *et al.*, 2000). The intriguing mechanisms by which pathogenic bacteria evade or cope with these stresses are just beginning to be revealed.

Heat shock or stress proteins (HSPs) are among the most highly conserved and abundant proteins found in nature. They are produced by all cells in response to various physiological and non-physiological stimuli (Lindquist, 1986). These proteins are being extensively studied and are classified into many families. The HSP100/Clp family is one example of such proteins with a great diversity of function, such as increased tolerance to high temperatures, promotion of proteolysis of specific cellular substrates and regulation of transcription. HSP100 proteins are classified on the basis of the presence of either one or two ATP-binding domains, on the length of the spacer region separating the two conserved nucleotide-binding regions and on the occurrence of specific signature sequences. They are divided into two major classes with eight subfamilies within these classes. Members of the first class contain two nucleotide binding domains (NBDs) flanked by amino-terminal, middle (or spacer) and carboxy-terminal regions. On the other hand, members of the second class are shorter in length, containing only a single NBD, which more closely resembles the second NBD of class I, and the carboxy-terminal region (Schirmer *et al.*, 1996).

In *Escherichia coli*, a Gram-negative bacterium, members of the Clp family of ATPases associate with the ClpP proteolytic subunit to form ATP-dependent proteases that degrade certain fusion proteins as well as several phage replication proteins (Gottesman, 1996, Gottesman *et al.*, 1998). Data suggest that the Clp ATPases are not only specificity factors that help to present various protein substrates to the ClpP subunit, but they also act as molecular chaperones independently of ClpP. For example, ClpA acts as a chaperone for the activation of P1 replication protein RepA (Wickner *et al.*, 1994, Wawrzynow *et al.*, 1996). Regulation of the *E. coli* heat shock response has been studied extensively. Transcription of *E. coli* heat shock regulon, consisting of twenty genes, requires the σ^{32} subunit of RNA polymerase as well as the minor sigma factor σ^{E}. Whereas σ^{32} regulon

plays a major role in coping with cytoplasmic protein damage, the σ^E regulon functions primarily to protect cells against extracytoplasmic or extreme heat stress (Yura *et al.*, 2000). The cellular concentration of σ^{32} is very low at 30°C, but rises rapidly at 42°C because of increases in σ^{32} synthesis, stability, and activity (Bukau, 1993).

Gram-positive bacteria use very different and diverse regulatory strategies depending on the specific heat shock genes and specific bacteria. For instance, the heat shock response in the soil bacterium *Bacillus subtilis* involves at least three different classes of heat-inducible genes distinguished by their regulatory mechanisms (Hecker *et al.*, 1996). Class I genes encode classical chaperones such as DnaK, GroES, and GroEL. Their expression involves a σ^A-dependent promoter and the highly conserved CIRCE (Controlling Inverted Repeat of Chaperone Expression) operator sequence, which is the binding site for the HrcA repressor (Yuan and Wong, 1995, Schulz and Schumann, 1996, Mogk *et al.*, 1997). Class II genes are the largest class; their expression is induced by heat shock but also by general stress conditions such as exposure to salt or ethanol, or starvation for glucose, phosphate or oxygen (Benson and Haldenwang, 1993). Expression of these genes requires σ^B factor whose synthesis and activity are increased under stress conditions. Class III genes (CtsR regulon) were defined as those devoid of CIRCE operator sequence and whose induction by heat shock and general stress conditions is σ^B-independent. These genes encode the ClpC and ClpX ATPases as well as the proteolytic subunit ClpP (Msadek *et al.*, 1994, Gerth *et al.*, 1996, Msadek *et al.*, 1998). ClpP and ClpC have been shown to play essential roles within the stationary phase regulatory network of *Bacillus subtilis*. ClpP is required for competence development, motility, degradative enzyme synthesis, growth at high temperature and sporulation (Msadek *et al.*, 1998). ClpC acts as a molecular switch to inhibit the activity of ComK, which is a transcriptional activator of competence genes (Msadek *et al.*, 1994, Turgay *et al.*, 1997). The expression of ClpP and ClpC is negatively controlled by the product of the first gene of the *clpC* operon, referred to as *ctsR* (Derre *et al.*, 1999b). This control by CtsR appears to be a highly conserved heat shock regulatory mechanism in low G+C Gram-positive bacteria.

Clp genes play important roles in the virulence and *in vivo* survival of several pathogens. For instance, ClpC was found to be required for intracellular growth, survival, and virulence of *Listeria monocytogenes*. The ClpC-deficient mutant became highly susceptible to various stresses, such as elevated temperature and osmolarity. Growth of the mutant was restricted within macrophages. However, The ClpC ATPase was not involved in phagocytosis, as bacterial uptake was similar in both wild type and mutant bacteria

(Rouquette *et al.*, 1998). In another study, transcription of *clpE* gene of *Listeria monocytogenes* required for prolonged survival at elevated temperature, was found to increase in the absence of *clpC* (Nair *et al.*, 1999). This was the first example implicating regulation of one clp by another. While ClpE expression was controlled by ClpC, ClpC expression appeared to be unaffected by the presence or absence of ClpE. In contrast to a *clpC* mutant, which was susceptible to salt stress, growth of the *clpE* mutant was unaffected. ClpP has been shown to modulate transcription of the adhesion-invasion locus (*ail*) in *Yersinia enterocolitica* (Pederson *et al.*, 1997) and patients with leprosy or tuberculosis were found to have antibodies specifically directed against mycobacterial ClpC (Misra *et al.*, 1996). Thus, elucidating the regulatory pathways controlling *clp* gene expression is likely to be important for understanding the virulence of pathogenic bacteria including the pneumococcus.

S. pneumoniae is carried in the nasopharynx where it experiences changes in temperature, pH, and ethanol concentration. This hostile environment may act as a strong stress to the pneumococcus and may influence gene expression of heat shock proteins (HSPs). A change in the environmental niche of the host, such as penetration of pneumococci from the nasopharynx into the bloodstream, can provoke remarkable morphological changes as well as changes in gene expression. For instance, it has been demonstrated that pneumococci in the nasopharynx are predominantly of the transparent colony phenotype and tend to express less capsule and more choline-binding protein A (CbpA) than those in the bloodstream. On the other hand, pneumococci in the bloodstream are predominantly of the opaque colony morphology and tend to produce more capsule and less CbpA than those in the nasopharynx (Kim and Weiser, 1998, Tuomanen, 1999). Furthermore, *S. pneumoniae* may encounter heat stress after penetration from the nasal mucosa (30 to 34°C) into the blood and/or meninges (37°C) during the pathogenic process (Lindemann *et al.*, 2002). Such changes in temperature may serve as a key trigger for a rapid, transient increase in the synthesis of the highly conserved heat shock proteins (HSPs).

It was found that after a thermal shock, *S. pneumoniae* response appeared as induction of three major HSPs of approximate molecular mass of 80, 72, 62 kDa and the shutdown of other proteins (Hamel *et al.*, 1997). HSP72 is present under normal growth conditions and the absolute amounts of it do not significantly increase after heat shock. Although this contrasts with what has been reported for most *E. coli* HSPs, this behaviour was also found to occur in other organisms (Engel *et al.*, 1990). Investigation of protein antigens involved in the immune response to *S. pneumoniae* led to the observation that HSP62 and HSP72 are targets of the immune response (Hamel *et al.*, 1997). Other pneumococcal stress

proteins including HSPs, GroEL, ClpL and alcohol dehydrogenase families were also identified (Choi *et al.*, 1999). However, despite their biological importance, little is known about the role of molecular chaperones/heat shock proteins in the response of *S. pneumoniae* to environmental stresses.

In response to heat stress, *S. pneumoniae* was also found to produce ClpC ATPase, which belongs to the HSP100 family of heat shock proteins. Members of this family have a dual function: they manifest chaperone activity on their own and are also involved in targeting disposable or damaged proteins for degradation by a protease moiety such as ClpP (Price, 2000). ClpC could therefore serve to sort damaged proteins, restoring the less impaired and presenting the terminally damaged for destruction by ClpP protease (Gottesman *et al.*, 1997). Charpentier and colleagues (Charpentier *et al.*, 2000) reported that insertion duplication mutagenesis of the *clpC* gene in *S. pneumoniae* revealed a pleiotropic phenotype of improved survival at high temperature, chain formation, penicillin and vancomycin tolerance, and adhesion and transformation deficiency. In this study, when the growth rates of the parent strain and the ClpC-deficient mutant were compared at temperatures ranging from 37°C to 46°C, the wild type did not grow well at temperatures higher than 40°C, whereas the mutant grew well at temperatures up to 44°C. Other studies suggest that the ClpC protein plays no role in these processes (Chastanet *et al.*, 2001, Robertson *et al.*, 2002). The function of ClpP-mediated proteolysis in the stress response and virulence of many Gram-negative and Gram-positive bacteria has been studied extensively. ClpP was found to be essential for virulence of *Salmonella enterica* serovar Typhimurium when examined in both a mouse model and in isolated macrophages (Webb *et al.*, 1999, Yamamoto *et al.*, 2001b). In *L. monocytogenes*, disruption of the *clpP* gene reduced haemolytic activity due to the major virulence factor, listeriolycin O that helps the bacteria to escape from the phagosomes of macrophages (Gaillot *et al.*, 2000). ClpP-negative mutants of *B. subtilis* and *Lactococcus lactis* were unable to survive heat shock and other stress conditions (Gerth *et al.*, 1998, Frees and Ingmer, 1999). Furthermore, it was also shown that ClpP was essential for the growth of *Staphylococcus aureus* in the presence of oxidative stress or at low temperature. The virulence of a *ΔclpP* mutant of *S. aureus* was severely attenuated when investigated in a murine abscess model (Frees *et al.*, 2003). Recent reports have shown the requirement of ClpP protease for thermotolerance, development of competence, and virulence of *S. pneumoniae* (Chastanet *et al.*, 2001, Robertson *et al.*, 2002, Kwon *et al.*, 2003). A part of this thesis was concerned with the investigation into the role of ClpC and ClpP, as an example of the ATP-dependent proteases, in the stress response and virulence of the pneumococcus.

This thesis has also tried to shed some light on HtrA as an example of the ATP-independent proteases. Homologues of the high temperature requirement A protein, HtrA have been described in a wide range of organisms including bacteria, yeast, plants, and humans (Zumbrunn and Trueb, 1996, Ponting, 1997, Gray *et al.*, 2000). Some bacteria have more than one paralogue of HtrA (Noone *et al.*, 2001). In all cases, the evidence points to a major role for these proteases in helping organisms to survive environmental stresses such as elevated temperature, oxidative and osmotic stresses. HtrA is known to be involved in the virulence of many Gram-negative bacteria such as *Salmonella enterica* serovar Typhimurium (Baumler *et al.*, 1994), *Brucella abortus* (Elzer *et al.*, 1996), and *Yersinia enterocolitica* (Li *et al.*, 1996). This protease is also required for full virulence of the Gram-positive bacterium *Streptococcus pyogenes* (Jones *et al.*, 2001). A HtrA homologue has also been identified in *Streptococcus pneumoniae* (Gasc *et al.*, 1998) and is regulated by the CiaR/H two-component system (Sebert *et al.*, 2002, Mascher *et al.*, 2003). HtrA was identified as a virulence factor of the pneumococcus in a signature tagged mutagenesis screen (Hava and Camilli, 2002).

The aim of this work was to answer the following questions:

- What proteins are involved in the stress response of the pneumococcus?
- Do these proteins affect the regulation of other proteins or metabolic factors?
- Are these proteins necessary for virulence of *S. pneumoniae*?
- Are mutations in such stress genes likely to affect growth pattern or virulence? and in what way?

Chapter 2

MATERIALS AND METHODS

2.1 Bacterial strains and growth conditions

Streptococcus pneumoniae strains used in this study are listed in table 2.1. Bacteria were grown on blood agar base number 2 (Oxoid, Basingstoke, United Kingdom) supplemented with 5% (vol/vol) defibrinated horse blood (E&O Laboratories, Bonnybridge, United Kingdom) (BAB) and in Brain Heart Infusion (BHI). *Escherichia coli* strains DH5α and XL-Gold ultracompetent cells (Stratagene) were grown in Luria- Bertani broth or Luria-Bertani (LB) agar plates. Where appropriate, antibiotics were added to the growth media at the following concentrations: ampicillin at 50 μg/ml, erythromycin at 1.0 mg/ml for *E. coli* or 1.0 μg/ml for *S. pneumoniae* and spectinomycin at 200 μg/ml for *E. coli* or 100 μg/ml for *S. pneumoniae*. Agar plates or broth inoculated with bacteria were incubated at 37 °C unless otherwise stated.

2.2 Primers and plasmids

The oligonucleotide primers used in this study are listed in table 2.2. PCR-Script™ plasmid (Stratagene) was used for cloning according to the manufacturers' recommendations. pDL278 plasmid (LeBlanc *et al.*, 1992) was used as a template to amplify the *Asc*I-generated spectinomycin resistance cassette (used as antibiotic marker in some mutants) using primer pair spec up and spec dn (table 2.2). pAL2 plasmid (Beard *et al.*, 2002) was used for expression of HtrA in *S. pneumoniae*.

2.3 Preparation of glycerol stocks

All pneumococcal strains used in this study were confirmed by colony morphology, production of α-haemolysis on blood agar plates, Gram stain and sensitivity to 5 μg of ethylhydrocupreine (optochin). Serotypes were confirmed by the Quellung reaction using specific antisera against capsular polysaccharide. Glycerol stocks of wild type and mutant strains were prepared by growing a single colony of the culture in BHI to mid-log phase (OD_{600nm} ~ 0.6-0.7). Sterile glycerol was then added to a final concentration of 20 % (vol/vol) and 1-ml aliquots of the cultures were frozen at – 80 °C in cryotubes.

Table 2.1- List of pneumococcal strains used in this study.

Strain	Relevant features	Reference or source
D39 or D39(MA)	Serotype 2; NCTC 7466	(Avery *et al.*, 1944)
D39 (LA)	Serotype 2, avirulent strain	Laboratory stock
TIGR4	Serotype 4, clinical isolate	(Aaberge *et al.*, 1995)
R6	Subclone of R36A, derivative of D39	(Smith and Guild, 1979)
R800	R6 derivative	(Lefevre *et al.*, 1979)
0100993	Serotype 3, clinical isolate	(Throup *et al.*, 2000)
D39 (MA) *ΔclpC*	D39 (MA) with replacement of *clpC* with Ery cassette	This work
D39 (LA) *ΔclpC*	D39 (LA) with replacement of *clpC* with Ery cassette	This work
R6 *ΔclpC*	R6 with replacement of *clpC* with Ery cassette	This work
R800 *ΔclpC*	R800 with replacement of *clpC* with Ery cassette	This work
TIGR4 *ΔclpC*	TIGR4 with replacement of *clpC* with Ery cassette	This work
SP 2000	*ΔclpP* mutant with Kan cassette	(Chastanet *et al.*, 2001)
D39 *ΔclpP*	D39 (MA) with replacement of *clpP* with Kan cassette	This work
TIGR4 *ΔclpP*	TIGR4 with replacement of *clpP* with Kan cassette	This work
D39 *ΔhtrA*	D39 with replacement of *htrA* with spec cassette	This work
TIGR4 *ΔhtrA*	TIGR4 with replacement of *htrA* with spec cassette	This work
D39*ΔhtrA/phtrA*$^+$	D39*ΔhtrA* complemented with pAL2-HtrA	This work
cia spc 136b	R6 mariner mutant of *ciaR*	(Martin *et al.*, 2000)
D39*ΔciaR*	D39 with mariner insertion in *ciaR*	This work
0100993*ΔciaR*	0100993 with replacement of *ciaR* with Ery cassette	(Throup *et al.*, 2000)
D39/pAL2YI	D39 with the empty pAL2YI vector	This work
D39/*phtrA*$^+$	D39 with pAL2-HtrA plasmid	This work
D39*ΔciaR/phtrA*$^+$	D39*ΔciaR* complemented with pAL2-HtrA	This work

Table 2.2- List of oligonucleotide primers used in this study.

Primer	Sequence (5'→ 3')	Used for
ClpC and ClpP work		
ClpC a1	ATGAACTATTCAAAAGCATTG	*clpC* amplification
ClpC a2	TGCAATATCAAATTTTAACTGG	*clpC* amplification
Clp inv1	GGCGCGCCTATCATGCAAAATCGCATAG	Deletion of *clpC*
Clp inv2	GGCGCGCCAACTGAAAAAAGCTTATAGACC	Deletion of *clpC*
Ery 3	GGCGCGCCTAACTATCGTCTTGAGTCCAACCC	Amplification of erythromycin cassette
Ery 5	GGCGCGCCGTTCATATTTATCAGAGCTCGTGC	Amplification of erythromycin cassette
MCP1.for	CATACAAGAGGTAGATAGAAAAGG	Confirmation of chromosomal insertion of interrupted *clpC*
MCP2.rev	TCAAACCAGTGTTTTGAGCAACCT	Confirmation of chromosomal insertion of interrupted *clpC*
ClpP for	ATGATTCCTGTAGTTATTGAAC	*clpP* amplification
ClpP rev	GTTCAATGAATTGTTGGCCATA	*clpP* amplification
AC94	TGACCATGGTTCCAGCTGCTAAAGTTGGC	*clpP* deletion (Chastanet *et al.*, 2001)
AC97	GCTACCATGGCAAGCGCCACAAACGATAG	*clpP* deletion (Chastanet *et al.*, 2001)
HtrA and CiaR work		
HtrA for	ATGAAACATCTAAAAACATT	Amplification of *htrA*
HtrA rev	AGATTCTAAATCACCTGAAC	Amplification of *htrA*
HtrA inv1	GGCGCGCCTACGCTATTTTGTCTGTT	Deletion of *htrA*
HtrA inv2	GGCGCGCCTTGGGAATCCAGATGGTTAAT	Deletion of *htrA*
Spec up	GGCGCGCCATCGATTTTCGTTCGTGAATA	Amplification of spectinomycin cassette
Spec dn	GGCGCGCCTATGCAAGGGTTTATTGTT	Amplification of spectinomycin cassette
htrA C1	AGTTATAATTAAGCTAGTCAG	Confirmation of chromosomal insertion of interrupted *htrA*
htrA C2	TGATTCTACACTAACACATCT	Confirmation of chromosomal insertion of interrupted *htrA*
HtrA1	CTGAATTCATAGGAGGACTATGAAACATC	Cloning of *htrA* into pAL2 plasmid
HtrA2	CTGAATTCAGATTCTAAATCACCTGAAC	Cloning of *htrA* into pAL2 plasmid
pAL2y1	CGAAAATTTGTTTGCTTTTTAATGG	*htrA* orientation in pAL2
pAL2y2	GGATACATTTCTATTGAGACTGGAT	*htrA* orientation in pAL2
MP144	CGAACCAAGCTGGAATATCT	PCR of *ciaR/H.* Upstream of *ciaR* (Martin *et al.*, 2000)
MP145	AACCATGTCAGGATCGTAGT	PCR of *ciaR/H.* Downstream of *ciaH* (Martin *et al.*, 2000)

2.4 Preparation of genomic DNA

Pneumococcal genomic DNA was prepared according to a published protocol (Blue and Mitchell, 2003). A single bacterial colony grown on blood agar plate was picked up and used to inoculate 10 ml of BHI and incubated overnight at 37 °C. Bacterial cells were collected by centrifugation at 5,000 ×g at room temperature for 15 min (Sigma laboratory centrifuge 4K15), resuspended in 1-ml extraction buffer (10 mM Tris pH 8.0, 100 mM EDTA pH 8.0, 20 μg/ml RNAseA, 0.5 % SDS w/vol) and incubated at 37 °C for 1h. Proteinase K (Sigma-Aldrich, UK) was added to a final concentration of 100 μg/ml and the mixture was further incubated for 3 hours at 50°C. An equal volume of phenol/ chloroform/isoamylalcohol (25:24:1 vol/vol, BDH & Fisher Scientific UK. Ltd.) was then added and the mixture was gently mixed. Following centrifugation at 13,000 ×g for 3 min (Eppendorf centrifuge 5417C, Germany), the aqueous layer was removed and DNA was precipitated by adding 0.2 volume of 10 M ammonium acetate (Sigma-Aldrich, UK) and 5 volumes of 100 % ethanol. The mixture was then centrifuged at 13,000 ×g for 30 min and DNA pellet was left to air dry for 30 - 60 minutes. DNA was resuspended in TE buffer (Qiagen) pH 7.4 and stored at – 20 °C.

2.5 Analysis of DNA

DNA samples were analyzed by measuring the OD_{260nm} and OD_{280nm} (Unicam UV2, UV/Vis spectrophotometer) where an OD_{260nm} of 1.0 corresponds to 50 μg/ml of DNA and OD_{260nm}/ OD_{280nm} is 1.8 for pure DNA. DNA was also analyzed by running on a 1% agarose gel (Gibco BRL Life Technologies, UK) containing 0.5 μg/ml ethidium bromide (Sigma-Aldrich, UK) in TAE buffer. DNA was visualized using an UV transilluminator (Spectroline, TVC-312A) and images were taken using a UVP GelDoc system (UVP Laboratories).

2.6 DNA techniques and transformation

2.6.1 Polymerase chain reaction

Genomic DNA was prepared as described above (section 2.4) and specific primers for different genes are listed in table 2.2. PCR reactions (100 μl) were set up as follows; 5 μl (~500 ng) genomic DNA, 1 μM of each primer (Sigma-Genosys, UK), 10 μl of thermopol

buffer, 2 µl of 100 mM $MgSO_4$, 2 units of Vent® polymerase (all purchased from New England Biolabs, UK) and 50 µM of dNTPs (Promega). The reaction volume was made up to 100 µl using dH_2O. The PCR reactions were heated to 95 °C for 5 min and run through 35 cycles of (95 °C for 30s, 55 °C for 30s, 72 °C for 1 min) and then heated to 72 °C for 5 min. Annealing and extension temperatures were variable and were optimized using a gradient program (Hybaid Omnigene PCR machine). PCR products were visualized as mentioned above (section 2.5).

2.6.2 DNA purification and cloning

DNA samples to be purified were run in DNA loading buffer (appendix, A3) in 1% agarose gels as described previously (section 2.5). 1 kb plus ladder (Promega) was also run on the gels to confirm the molecular weight of DNA fragments. DNA bands were excised from the gel and purified by using Qiaquick Gel Extraction Kit (Qiagen) according to the manufacturer's instructions. PCR-Script™ Amp SK (+) plasmid (Stratagene, see appendix, A1) was used for cloning according to the manufacturer's recommendations. Genes were cloned at the *SrfI* site as blunt-ended products and the proper orientation of genes was confirmed by restriction digestion with the appropriate endonuclease enzymes.

2.6.3 Transformation of E. coli with plasmid constructs

Escherichia coli strains DH5α and XL-Gold ultracompetent cells (Stratagene) were used for propagation of plasmid constructs following the manufacturer's instructions. The cells were plated onto LB agar plates with the appropriate antibiotic and also with IPTG and X-gal to facilitate the identification of transformed cells by blue/white selection. Cells containing vector with no insert form blue colonies while those containing vector carrying the insert form white colonies. Single white colonies were picked up and used to inoculate LB broth with the appropriate antibiotic and incubated overnight at 37 °C. In some experiments, Top10 electrocompetent cells (Invitrogen) were used for transformation as follows; 100-µl aliquots of competent cells were thawed on ice and 1-5 µl of plasmid DNA was added. The tubes were incubated on ice for 20 min. The mixture was then transferred to pre-chilled 1-mm-gap electroporation cuvettes (Molecular BioProducts) and the samples were electroporated using Gene Pulser™ (BIORAD) with the following settings; voltage: 1.5 V, capacitor: 25 µF, resistor: 200 Ohm. A typical time constant with these settings was ~ 4.6. After electroporation, the cuvettes were placed back on ice, 200 µl of SOC or LB medium was added and the contents transferred to microcentrifuge eppendorf tubes and

incubated at 37 °C for an hour. Different volumes of the cells were then plated onto LB agar with the appropriate antibiotic and incubated overnight at 37 °C.

2.6.4 Plasmid preparation and purification

Following overnight incubation, transformed cells were harvested by centrifugation at 5,000 ×g at room temperature for 5 min (Sigma laboratory centrifuge 4K15). Plasmids were isolated from bacterial pellets using the commercially available Qiaprep Spin Miniprep Kit (Qiagen). Plasmid DNA concentration and purity was determined as described in section 2.5.

2.6.5 DNA sequencing

To confirm that plasmids carry the correct inserts, Plasmid DNA samples were sent to the MBSU (Molecular Biology Support Unit, University of Glasgow, UK) for sequencing using the appropriate primers. Primers were used at 3.2 μM concentration for sequencing reactions. The MBSU sequence facility used ABI377 and MegaBACE1000 DNA sequencers (Amersham Bioscience) and a protocol based on the dideoxy method developed by Sanger *et al* (Sanger *et al.*, 1977). Sequence homology searches were performed using BLAST (Altschul and Lipman, 1990) and the Institute for Genomic Research (TIGR, CMR, http://www.tigr.org/) database.

2.7 Construction of pneumococcal mutants

2.7.1 Strategy for introducing mutation

Isogenic mutants of *S. pneumoniae* were constructed to study the role played by different stress response proteins in the virulence of this organism. The strategy used for making mutants is outlined in figure 2.1. The whole gene to be mutated was amplified from the pneumococcal chromosomal DNA (strain D39 unless otherwise mentioned) using forward and reverse primers specific for each gene (table 2.2) and cloned in PCR-Script™ Amp SK (+) plasmid (Stratagene). Internal primers, (table 2.2) were designed to amplify the 5' and 3'ends of the gene together with the PCR-Script™ and to create *Asc*I sites in the resulting PCR product. This PCR product was self-ligated to produce a plasmid carrying the interrupted gene. The resulting plasmid was purified as described in section 2.6.4 and sequenced in-house (MBSU) as described above, using primers T3 and T7 specific for

PCR-Script™ plasmid to confirm the knockout of the gene. To facilitate the selection of transformants, an *Asc*I-generated antibiotic resistance cassette (either erythromycin or spectinomycin) was ligated into this plasmid after digestion with *Asc*I. The modified version of the gene was amplified with PCR and used to transform *S. pneumoniae.* This method was used for making ClpC- and HtrA-null mutants in a serotype 2 background (strain D39).

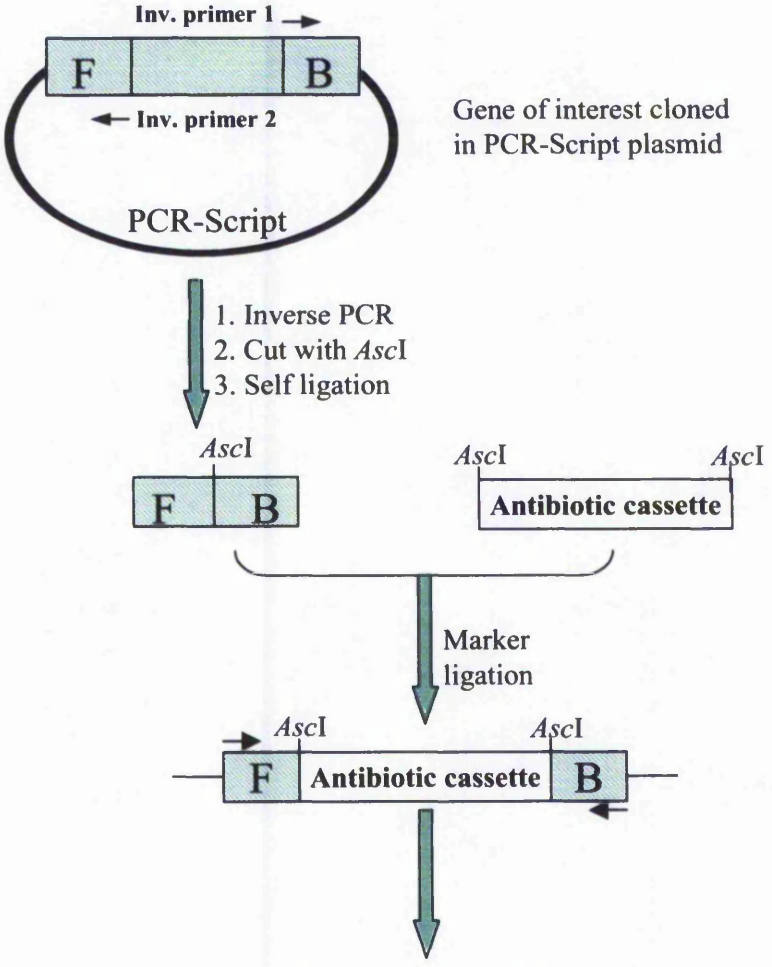


Figure 2.1- Strategy for making pneumococcal mutants

2.7.2 Transformation of S. pneumoniae

Transformation of *S. pneumoniae* strains D39 (serotype 2) and TIGR4 (serotype 4) was done by using a modification of an existing method (Lacks and Hotchkiss, 1960). Competence stimulating proteins (CSP-1 for D39 and CSP-2 for TIGR4) were used for inducing competence in these different serotypes (Pozzi *et al.*, 1996). Competent cells of

D39 or TIGR4 were prepared as follows; 200 µl of glycerol stock of cells (previously frozen down at OD_{600nm} ~ 0.6) were used to inoculate 10 ml of CAT medium (Porter and Guild, 1976) (see appendix, A3) supplemented with 20 % glucose and 0.5M K_2HPO_4 (CAT/GP medium) and incubated at 37 °C until they reached an OD_{600nm} of 0.3-0.4. Cells were then harvested by centrifugation at 5,000 × g for 10 min at 4 °C (Sigma laboratory centrifuge 4K15) and cell pellets were resuspended in CAT/GP medium containing 20% glycerol and frozen down at – 80 °C as 100-µl aliquots. Aliquots of competent cells were thawed and diluted 1/10 in CAT/GP medium supplemented with 4% bovine serum albumin and 0.1M $CaCl_2$ (CTM medium), and 100 ng of CSP/ml was added. Different amounts of transforming DNA (0.5-2 µg) were then added and the cells were incubated at 37 °C for 10 min and then at 30 °C for 20 min. Transformed cells were selected on BAB plates with appropriate antibiotic selection.

2.7.3 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.

2.7.3.1 ClpP mutants

The Δ*clpP* mutants of TIGR4 and the mouse-adapted strain of D39 (D39 MA, table 2.1) were constructed using a Δ*clpP* mutant strain, SP2000 (Chastanet *et al.*, 2001) kindly provided by Jean-Pierre Claverys (Toulouse, France). The mutation was moved by PCR amplification of a 1.6-kb fragment corresponding to the mutated *clpP* gene carrying a kanamycin resistance cassette using primer pair AC94 and AC97 (table 2.2) and using the PCR product to transform D39 and TIGR4. Transformed cells were selected on agar plates containing 250 µg/ml kanamycin.

2.7.3.2 CiaR mutants

The *ΔciaR* mutant strain cia spc 136b (Martin *et al.*, 2000) kindly provided by Jean-Pierre Claverys (Toulouse, France) was used to move the *ΔciaR* mutation into D39 by PCR amplification of a 6-kb fragment corresponding to the *ΔciaR* mutation using primer pair MP144 and MP145 (Martin *et al.*, 2000) (table 2.2) and using it directly to transform D39 wild type to create D39*ΔciaR*. Transformed cells were selected on agar plates containing 100 µg/ml spectinomycin.

2.7.4 Confirmation of mutants

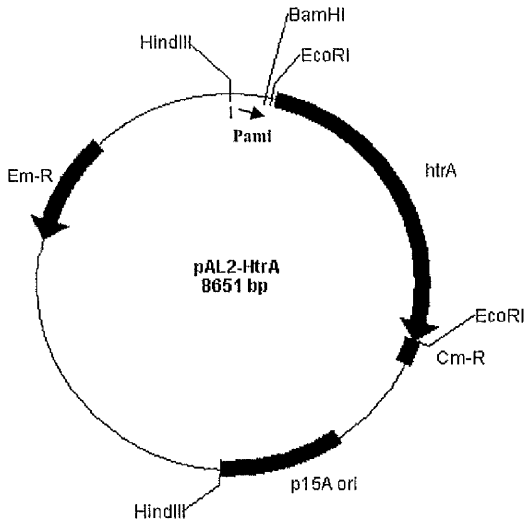
To confirm the mutations, genomic DNA was prepared from the transformed cells grown on blood agar plates supplemented with the appropriate antibiotic as described previously (section 2.4) and PCR was used to compare the mutated genes to those of the wild types. Homologous recombination of mutated DNA into the pneumococcus chromosome resulted in an increase/decrease in PCR product size for mutants when compared to the wild type copy of the gene. To ensure that the mutations occurred at the correct position of the chromosome, mutations were also confirmed by sequencing. Primers flanking the target genes were designed to amplify the genes with flanking DNA in the wild types and mutants and the PCR products were sent to the MBSU for sequencing as described in section 2.6.5. In the correct mutants, the sequence showed the 5' end of the gene followed by the antibiotic resistance marker and the 3' end of the gene whereas, sequence of the wild type copy showed the full length of the gene.

To confirm that the observed phenotype of D39 Δ*clpC* mutant strain was due to the disruption of *clpC* and not mutations elsewhere in the chromosome the *clpC* mutation was repaired. A 2,578-bp PCR product was amplified from wild-type D39 genomic DNA using primer pair MCP1 for and MCP2 rev (table 2.2). This fragment contained 30 bases upstream of the start codon (*ctsR* gene) and an additional 115 bases downstream of the stop codon for *clpC*. This PCR product was purified using the Qiagen gel purification kit and used directly to transform D39 Δ*clpC* strain by homologous recombination. Transformation of *S. pneumoniae* D39 Δ*clpC* strain was carried out as described in section 2.7.2. The transformation reactions were screened for transformants by plating onto selective plates (with 1 μg/ml erythromycin) and non-selective plates (without antibiotic). Colonies that lost erythromycin resistance were selected and checked by PCR and nucleotide sequencing for the presence of a complete copy of *clpC*. The effect of HtrA mutation was confirmed by complementation of HtrA from a plasmid (see below).

2.8 Construction of the plasmid expressing *htrA* gene in the pneumococcus

htrA was expressed in pAL2 (appendix, A2) plasmid (Beard *et al.*, 2002) from the constitutive promoter of *S. pneumoniae* aminopterin resistance operon (*ami*) (Alloing *et al.*, 1990). Primer pair htrA1 and htrA2 (table 2.2) was used to amplify *htrA* with *Eco*RI sites and a Gram-positive Ribosome Binding Site (RBS). This fragment was ligated to the

*Eco*RI-digested pAL2 carrying the *ami* promoter to create pAL2-HtrA plasmid. Proper orientation of *htrA* gene in pAL2-HtrA plasmid was confirmed by PCR using primer pair pAL2y1 and pAL2y2 (table 2.2) and the *htrA* gene was sequenced using the forward primer pAL2y1 and the reverse primer htrA2 for further confirmation. A schematic representation of pAL2-HtrA is shown in figure 2.2.



*Bam*HI -35 -10 *Eco*RI RBS

5'-ggatccgaaaatttgt**ttgctt**ttaatggataatgtga**tataat**-n14-gaattc-ac**AGGAGG**actATG-3'

Figure 2.2- A schematic representation of pAL2-HtrA plasmid.

pAL2-HtrA plasmid used for expression of *htrA* gene in *Streptococcus pneumoniae*. It was modified from pAL2 plasmid (Beard *et al.*, 2002) as explained in the text of this section. Arrows indicate the location and orientation of open reading frames. The nucleotide sequence shows the region between the *ami* promoter and the start codon of *htrA*. The Gram-positive ribosome binding site is shown in bold and uppercase, the *htrA* start codon is shown in uppercase, and restriction sites are underlined. Em-R, erythromycin resistance gene, Cm-R, chloramphenicol resistance gene.

2.9 Complementation with HtrA

The pAL2-HtrA plasmid described above, which carries an erythromycin resistant cassette, was used to transform D39*ΔhtrA* and D39*ΔciaR* mutants to generate the complemented strains D39*ΔhtrA/phtrA*$^+$ and D39*ΔciaR/phtrA*$^+$ in which HtrA is expressed from the plasmid. To test whether the effect of pAL2-HtrA plasmid was solely due to *htrA* insertion,

the *Eco*RI-digested pAL2 fragment containing the *ami* promoter was self ligated to create the empty plasmid designated pAL2YI. This plasmid was used to transform D39 wild type to generate the D39/pAL2YI strain (table 2.1). Strains complemented with HtrA were compared to the knockout strains with a number of assays to confirm that the phenotypes of these knockout strains are solely due to HtrA.

2.10 *In vitro* assays

2.10.1 Determination of bacterial growth

Growth phenotypes of the strains at normal and elevated temperatures were determined by measuring optical densities at 600 nm (OD_{600nm}) and the viable counts as described below

2.10.1.1 Growth curves

Aliquots of bacterial standard inocula (glycerol stocks) were quickly thawed at 37 °C in a water bath and the same number of viable cells (10^6 CFU/ml) of each strain was used to inoculate BHI prewarmed at 37 °C or 40 °C or otherwise stated. At 1h-intervals, samples were withdrawn to measure the OD_{600nm} (Unicam UV2, UV/Vis spectrophotometer) with the use of BHI as a blank.

2.10.1.2 Viable count

To enumerate the number of colony forming units (CFU) of bacteria in frozen stocks, an aliquot of glycerol stock was thawed and 900 µl was removed and centrifuged at 13,000 ×g (Eppendorf centrifuge 5417C, Germany) for 3 min to collect cells. The cell pellet was resuspended in 900 µl of sterile phosphate buffered saline (PBS). Serial 10-fold dilutions in PBS (up to 10^6) were made in a 96-well round-bottom plate (Nunclon® surface, Nalge Nunc International™). 6 × 20 µl of each dilution were plated onto BAB plates that were divided into six segments at the base and the plates were incubated at 37 °C overnight. Next morning, the number of bacterial colonies in the plates was counted (a number of 50-200 colonies/spot was considered) and the number of CFU/ml was calculated from the formula (Average number of colonies × 50 × dilution factor).

For growth phenotype comparison, equal numbers of viable cells (10^6 CFU/ml) of each strain was used to inoculate BHI prewarmed to the desired temperatures and samples were withdrawn at 1h-intervals to determine the viable count as described above.

2.10.2 Stress experiments

2.10.2.1 Penicillin-induced loss of viability

The penicillin-induced cell lysis of mutant strains and their wild types was studied according to the method of Charpentier and co-workers (Charpentier *et al.*, 2000). 10 ml of cultures grown in BHI to OD_{600nm} ~ 0.3 were exposed to 10 times the minimal inhibitory concentration (MIC) of benzylpenicillin (0.1 μg/ml) and the effect on cell viability was recorded by plating samples of penicillin-treated cultures taken at hourly intervals on BAB as described in section 2.10.1.2.

2.10.2.2 Deoxycholate-induced lysis

Bacterial strains were grown in BHI at 37 °C until they reached mid-log phase of growth (OD_{600nm} ~ 0.6-0.7). 10 % (w/vol) of deoxycholate (DOC; Sigma-Aldrich, UK) prepared in PBS was added to 5-ml aliquots of each culture to a final concentration of 0.05 %. Viable count was determined before the addition of DOC and at different time points following treatment with DOC.

2.10.2.3 Sensitivity to hydrogen peroxide

The sensitivity of strains to H_2O_2 was tested by using the method of Johnson and co-workers (Johnson *et al.*, 1993) with a slight modification. Aliquots of cultures grown to OD_{600nm} ~ 0.3 were exposed to 40 mM of H_2O_2 for 5,10 and 15 minutes at room temperature and the viable cells were counted by plating onto blood agar plates before and after the exposure to H_2O_2 and the result was expressed as percentage survival.

2.10.2.4 Sensitivity to paraquat

The effect of the redox compound methyl viologen (paraquat) was studied. A total of 10^4 cells were exposed to 60 mM paraquat (Sigma-Aldrich, UK) over a period of two hours. At various time intervals, samples were taken and the number of viable cells was determined by plating onto BAB (Tseng *et al.*, 2002).

2.10.2.5 PH tolerance

To study the role of stress proteins in pH tolerance, equal numbers of cells of mutant strains and wild types were used to inoculate BHI adjusted at pH 4, 5, 6, 7, 8, and 9. The growth was recorded after overnight incubation at 37 °C (Diaz-Torres and Russell, 2001).

2.10.3 Analysis of protein

2.10.3.1 Protein preparation

Bacterial strains were grown in BHI to the desired optical densities at the appropriate temperatures. Cell pellets from 20-ml cultures were collected by centrifugation at 5,000 × g for 15 min and were resuspended in 1 ml of phosphate buffered saline (PBS). Cell suspensions were sonicated 4 times of 30s each with a 13-mm probe (Sonicator, Vibra cell, Sonics & Materials Inc.) using a power output of 36W. The tubes containing the samples were kept in crushed ice during sonication. The cell debris was removed by centrifugation at 13,000 × g for 10 min.

2.10.3.2 Protein quantitation

Concentrations of total proteins were determined by Bradford assay with the use of bovine serum albumin (BSA) as a standard (Bradford, 1976). Different concentrations of BSA standard, protein samples or PBS (in triplicates) were mixed with Bradford reagent (Sigma-Aldrich, UK) and the absorbances at 280nm (A_{280}) were measured using a Unicam UV2, UV/Vis spectrophotometer. A standard curve was created by plotting the average readings of standards against their concentrations. The concentration of protein in each sample was calculated from this standard curve.

2.10.3.3 SDS-PAGE

Sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) was used to visualize proteins according to a standard protocol (Sambrook *et al.*, 1989). After assembling the minigel apparatus (BIO-RAD), a 10-12 % separating gel (appendix, A3) was poured, overlayed with dH_2O and allowed to set. Stacking gel (appendix, A3) was then poured on top of separating gel, a 10-well comb was inserted and the gel was left to set. While the gel was allowed to set, protein samples in loading buffer (appendix, A3) were heated to 95 °C for 5 min. The combs were then removed and protein samples were

loaded along with a standard protein ladder (Kaleidoscope Prestained Standards, BIO-RAD) to determine protein band size. Gels were run at 150 V for approximately 90 min using a Biorad power pack 300. When protein separation was completed, as indicated by migration of the bromophenol blue dye in the loading buffer towards the bottom of the gels, the gels were carefully removed from the glass plates and stained with Coomassie stain (appendix, A3) for 3-4 hours with shaking. Gels were then destained using destain solution (appendix, A3) until protein bands were clearly visible.

2.10.3.4 Western immunoblot

For western blot analysis, equal amounts of total proteins from each strain were separated on SDS-PAGE as described above and gels were equilibrated in the transfer buffer (appendix, A3) for 30 min shaking. Hybond-C nitrocellulose membrane optimized for protein transfer (Amersham) and filter paper were cut to the size of the gel and soaked along with fibre pads in transfer buffer. The BIO-RAD blotting apparatus was assembled as follows; grey side of holder, fibre pad, filter paper, gel, membrane, filter paper, filter pad. An ice pack and stirrer bar were placed in the tank and the protein bands were blotted at 100 V for 80 min. The membranes were blocked by transferring them into 3 % skimmed milk in Tris-NaCl (appendix, A3) for 1h or overnight. Membranes were then transferred into 3 % skimmed milk in Tris-NaCl with 1:1000 dilution of the primary antibody and shaken at 37 °C for 2-3 hours before washing four times with Tris-NaCl for 5 min each. They were then transferred to 1:1000 dilution of the secondary antibody (anti-mouse or anti-rabbit horseradish peroxidase-linked whole antibody, Amersham) in the same solution and shaken at 37 °C for 1 h. Membranes were washed as before and transferred to the developer solution (appendix, A3) in the dark for 15 min or until bands developed. Blots were air dried and scanned.

2.10.4 Haemolytic assay

Pneumolysin activity of the mutant strains was compared to that of the wild type by the haemolytic assay, which is based on the ability of pneumolysin to cause lysis of red blood cells. 50-µl aliquots of PBS were added to the first-column wells of two round-bottomed 96-well plates (Nunclon® surface, Nalge Nunc International™) and 50 µl of total protein samples prepared as described in section 2.10.3.1 or PBS control were added and two-fold dilutions were made across the two plates. 50 µl of 2 % defibrinated sheep red blood cells (E&O Laboratories, Bonnybridge, UK) was added to each well and the plates were

incubated at 37 °C for 30 min and then left at room temperature until blood cells in negative wells had settled down. Results were read by visual examination of wells where in negative wells, blood cells form compact pellets whereas in positive wells pneumolysin caused the lysis of cells and therefore prevented pellet formation. The end point was taken as the well in which the pellet is half the size of the control wells. The haemolytic units were calculated from the number of dilutions required to cause 50 % cell lysis. For instance, in the first well, pneumolysin is diluted 1:2 in PBS and further diluted 1:2 when blood is added i.e. diluted by a factor of 4. If the first well is the endpoint, so there are 4 haemolytic units (HU) in 100 µl i.e. 40 HU/ml.

2.10.5 Competence assay

Competent cells of D39 wild type and *ΔhtrA* and *ΔhtrA/htrA*$^+$ mutants were made as described in section 2.7.2 and stored as 100-µl aliquots with 20% glycerol at – 80 °C. To compare the ability of the *ΔhtrA* and *ΔhtrA/htrA*$^+$ mutants to take up external DNA to that of the wild type D39, competent cells were thawed, diluted 1:10 in CTM medium described before (section 2.7.2) and 100 ng/ml of competence stimulating peptide (CSP) was added. 0.3 µg of pneumococcal genomic DNA conferring kanamycin resistance (from a Δ*clpP* mutant containing kanamycin resistance cassette (Chastanet *et al.*, 2001) was then added and the cells were incubated at 37 °C for 10 min, then at 30 °C for 20 min. Transformed cells were incubated on BAB without antibiotic selection for 2 hours at 37°C, then 250 µg/ml of kanamycin was added and incubation continued overnight. The efficiency of transformation was expressed as percentage of transformants to the total number of cells used.

2.10.6 Neuraminidase activity measurement

Neuraminidase activity in different cell fractions (Soluble, insoluble and supernatant) was assayed fluorometrically by mixing 10 µl of enzyme sample (total protein extracts), known concentrations of purified neuraminidase (positive control) or PBS (negative control) in triplicates with 10 µl of 0.35 % (w/vol) of the fluorogenic substrate 2'-(4-methyl-umbelliferyl)-α-D-*N*-acetylneuraminic acid (MUAN) (Sigma) in a 96-well plate as previously described (Lock *et al.*, 1988b). The reaction mixture was incubated for 10 min at 37 °C, and the reaction was stopped by the addition of 180 µl of 50 mM sodium carbonate buffer, pH 9.6. Fluorescence resulting from the release of 4-methylumbelliferone

from MUAN was detected using a Perkin-Elmer LS2B fluorimeter at an excitation wavelength of 366 nm and an emission wavelength of 446 nm.

2.10.7 Hyaluronidase activity measurement

The hyaluronidase activity was detected using a protocol based on two published plate methods (Smith and Willett, 1968, Richman and Baer, 1980) with the use of hyaluronic acid (HA) as a standard. Hyaluronic acid (Sigma-Aldrich) was dissolved in water at 8 mg/ml concentration and 0.8 % agarose (Roche Diagnostics, Germany) was dissolved in 0.3 M sodium phosphate buffer pH 7.0. 1 ml of HA was mixed with 9 ml of agarose heated to 55 °C and the mixture was poured into Petri dishes. After solidification, 3-mm wells were punched and emptied. 100 µl of samples or hyaluronidase control (Sigma-Aldrich) was then added to the wells and the plates incubated at 37 °C overnight (17-24 hours). Wells were then emptied and the surface medium flooded with 10 % (w/vol) of cetylpyridinium chloride made up in dH_2O and plates left on the bench for 30 min. A clear zone around the well suggested substrate degradation and the cloudy background resulted from undigested HA precipitation by cetylpyridinium chloride. The clear zones were measured in different cell fractions of the wild type and mutant strains and the activity of hyaluronidase compared.

2.11 *In vivo* assays

All animal work was carried out after receiving proper training and under appropriate project and personal licenses from the Home Office, UK. Local regulations of the University of Glasgow were also abided by.

2.11.1 Mice

Female outbred MF1 mice (25-30 g) were purchased from Harlan Olac, Bicester, UK. They were used when they were 9 weeks old as a standard model of pneumococcal pneumonia and bacteraemia. They were housed at the Central Research Facility (CRF), University of Glasgow, UK. All mice were kept in appropriate cages and provided with sterile pelleted food (B&K Universal, North Humberside, England) and water throughout the experiments.

2.11.2 Preparation of standard inocula

Bacterial strains were passaged through mice to prepare standard inocula before studying their *in vivo* phenotypes. Cells from 900 µl of glycerol stock of each strain were resuspended by centrifugation at 13,000 ×g (Eppendorf centrifuge 5417C, Germany) for 3 min. Pellets were resuspended in 900 µl of sterile PBS and diluted to give approximately 1×10^6 CFU/ml. Mice were injected with 200 µl of bacterial suspension (2×10^5 CFU) into the peritoneal cavity using a 1-ml insulin syringe (Microfine, 12.7 mm, Becton Dickinson). 24 hours (or 48 h for some mutants) following injection, mice were sacrificed by cervical dislocation and the chest cavity opened. Blood was collected from the right ventricle of the heart using a 23-gauge needle. 10 ml of BHI (+/- antibiotic) was inoculated with 50 µl of collected blood and incubated overnight (10-12 h) at 37 °C. Bacteria were collected by centrifugation at 5,000 ×g at room temperature for 15 min and resuspended in 1 ml of BHI. 100 µl of this suspension was used to inoculate 20 ml of BHI containing 20 % (vol/vol) heat-inactivated FBS (Gibco BRL, Life Technologies, UK) and incubated at 37 °C until mid-log phase of growth (OD_{600nm} 0.6-0.7). Glycerol was added to a final concentration of 20 % (vol/vol) and cultures kept as 1-ml aliquots at – 80 °C. Viable count of stocks was determined as described before (section 2.10.1.2) at least 24 hours following freezing.

2.11.3 Intranasal infection

Bacterial suspensions were prepared from standard inocula to give 2×10^7 CFU/ml. Mice were lightly anaesthetized with 1.5 % (vol/vol) halothane (Zeneca Pharmaceuticals, Macclesfield, UK) over oxygen (1.5 L/min) using a calibrated vaporizer. Anaesthesia was confirmed when mice failed to produce a reflex reaction when gently pinched. The infectious dose (1×10^6 CFU/mouse) was administered in 50 µl of bacterial suspension to the nostrils of mice held vertically (Kadioglu *et al.*, 2000) using a Proline® pipette (Biohit) and mice were laid on their backs until they recovered. Viable counts of bacterial suspensions were determined before and immediately after infection as described in section 2.10.1.2.

2.11.4 Intravenous infection

Bacterial suspensions were prepared from standard inocula to give 2×10^6 CFU/ml. Mice were placed in a ventilated, heated Perspex box for 5 min in order to expose veins and were restrained in appropriate apparatus. 100 µl of bacterial suspension was administered

directly into the bloodstream via the lateral vein using a 1-ml insulin syringe (Microfine, 12.7 mm, Becton Dickinson). Blood was taken from a separate vein immediately following injection to ensure successful infection (Kerr *et al.*, 2002) and bacteria enumerated as described in section 2.10.1.2.

2.11.5 Mice survival and pain scoring

Following infections, mice were observed and the development of symptoms was recorded over a period of eight days unless stated otherwise. Symptoms progressed from normal to severe with mice showing piloerection (starry coat), hunched posture and lethargy. Severe infection eventually resulted in mice becoming moribund, indicated by their inability to move when encouraged to do so. When showing signs of lethargy or upon becoming moribund, mice were humanely euthanized and were considered to reach the endpoint of the experiment (Toth, 1997). Mice that survived the course of infection were assigned an arbitrary survival time of 192 h for statistical analysis.

2.11.6 Bacteriological investigation

2.11.6.1 Bacterial loads in lung airways

At pre-chosen time points after intranasal infection, groups of mice were sacrificed by cervical dislocation ensuring intact trachea and the skin and muscles surrounding the trachea were exposed and the thoracic cavity opened. The trachea was clamped using Spencer-Wells forceps (Fisher Scientific Ltd, UK) to prevent backflow of fluid up the trachea. For bronchoalveolar lavage, a 16-gauge nonpyrogenic angiocath (F. Baker Scientific, Runcorn, United Kingdom) was inserted into the trachea and the lungs were lavaged twice with a total volume of 2 ml of sterile PBS. Viable bacteria in the lung airways were counted by plating out serial 10-fold dilutions on BAB (Kerr *et al.*, 2002) as described in section 2.10.1.2.

2.11.6.2 Bacterial loads in lung tissue

Following intranasal infection, lungs were lavaged as mentioned in the previous section. Lavaged lungs were removed into 5 ml of sterile PBS and were homogenized with a glass handheld tissue homogenizer (Jencons, Leighton Buzzard, United Kingdom). Viable bacteria in lung homogenate samples were counted by plating out serial 10-fold dilutions on BAB (Kerr *et al.*, 2002) as described in section 2.10.1.2.

2.11.6.3 Levels of bacteraemia

At pre-determined time points following intranasal infection, groups of mice were sacrificed by cervical dislocation and 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. Mice were restrained as described in section 2.11.4 and a maximum of 100 µl of blood samples was removed from the lateral tail veins and stored on ice. Enumeration of bacteria in blood samples collected by either method was carried out as described in section 2.10.1.2.

2.11.7 Nasopharyngeal colonization assay

Bacteria colonizing the nasopharynx were collected for counting by doing nasopharyngeal lavage early (6h-post-infection) and late in infection (48h-post-infection). Groups of MF1 mice were intranasally infected with 1×10^6 CFU/mouse as described in section 2.11.3. At the indicated time points, mice were sacrificed and the nasopharynx was washed by injecting 1-ml of sterile PBS into the trachea and collecting the fluid coming from the nares. The nasopharyngeal washes were then plated onto BAB with 20 µg/ml of neomycin to limit the growth of other bacteria.

2.11.8 Histological analysis

Mice were infected intranasally as described in section 2.11.3. At time points following infection animals were sacrificed and their trachea exposed. The trachea was clamped as described in section 2.11.6.1 and inflated with 1ml of formal saline (appendix, A3) using a catheter. Using a second set of forceps, the trachea was clamped just below catheter to keep the lung inflated. The first clamp and catheter were removed and the trachea was clamped just above the heart and lung using the first clamp. The second clamp was then removed and trachea was carefully cut above the first clamp to free the heart and lung and then pulled up. The heart/lung was placed into formal saline and covered with a piece of tissue paper to keep the inflated lungs submerged in formal saline. After 24 hours, the lungs were taken out and the heart carefully dissected away and discarded. The lungs were placed in new formal saline until ready for further processing. Following fixation, the lungs were embedded in paraffin and blocked by utilizing standard histological protocols. Lung blocks were sectioned at 5 µm prior to staining with hematoxylin and eosin (BDH Laboratory supplies, Poole, United Kingdom) (Kerr *et al.*, 2002). Lung tissue sections

were examined by microscopy and the number of areas at the pleural interface that were swollen with recruited inflammatory cells (pleurisy) as well as the number of perivascular alterations were scored in individual mice (Kerr *et al.*, 2002).

2.11.9 Measurement of immune modulators

At pre-determined time points following intranasal infection, as described in section 2.11.3, mice were sacrificed and the thoracic cavity opened up to expose the lungs. Bronchoalveolar lavage was done as described in section 2.11.6.1, but the fluid was snap-frozen by immersion in liquid nitrogen. Lungs were then removed, wrapped in aluminium foil, and snap-frozen in liquid nitrogen. Samples were then stored at - 80 °C until further processing. Upon thawing, the whole lungs were homogenized as described in section 2.11.6.2. Homogenates were centrifuged at 1,600 × g for 30 min at 4 °C (Sigma laboratory centrifuge 4K15). The supernatants were then centrifuged at 5,000 × g at 4 °C, filter-sterilized using 0.2 μM filter (Gelman Sciences, Northampton, United kingdom), and stored at – 80 °C. Upon thawing, lavage fluids were centrifuged at 17,900 × g for 3 min.

2.11.9.1 Measurement of IL-6

IL-6 was measured by Enzyme-linked immunosorbent assay (ELISA) by utilizing commercially available antibody pairs. Flat-bottomed 96-well maxisorb™ plates (Life Technologies) were coated with 50 μl per well of a 2 μg/ml preparation of purified anti-mouse mAb (Pharmingen Cat. No. 554400, Becton Dickinson) in coating buffer (appendix, A3), sealed with cling film to prevent evaporation and incubated at 4 °C overnight. Wells were then emptied and unbound areas blocked by the addition of 200 μl of blocking buffer (appendix, A3) and incubation at room temperature for 2 hours. Blocking buffer was removed from all wells and the plates were washed three times with PBS/0.05% tween 20 (Polyoxyethylenesorbitan monolaurate) (wash buffer). The recombinant murine IL-6 standards (Pharmingen Cat. No. 19251V) were prepared in blocking buffer/tween (appendix, A3) in the range of 1000 pg/ml to buffer only. Lavage fluids and lung homogenates prepared as described above were diluted 1:2 and 1:5 respectively in blocking buffer/tween prior to the addition of 100 μl of standard or sample in duplicates. Plates were then incubated at room temperature for 2 h or for 16 h at 4 °C. Plates were washed four times with wash buffer, 100 μl of 1 μg/ml solution of biotinylated anti-mouse mAb to IL-6 (Pharmingen Cat. No. 18082D, Becton Dickinson) was added and the plates were incubated for an hour at room temperature. Following four times of washing, 100 μl

of 1:200 dilution of streptavidin-HRP (Genzyme Cat. No. 80-3546-03) was added and incubated for 30 min at room temperature. After five washes with wash buffer, plates were developed with 100 µl of ELISA substrate (appendix, A3). Plates were incubated in the dark at room temperature for 30 min and the reaction stopped by addition of 100 µl of 1M sulphuric acid. The A_{450} was determined by reading the plates on a Dynatech MRX ELISA plate reader with correction set at A_{570}.

2.11.9.2 Measurement of TNF-α

TNF-α levels were measured by using TNF-α ELISA kit, OptEIA™ mouse TNF-α set (Pharmingen cat. no. 555268). Plastic flat-bottomed 96-well maxisorb™ plates (Life Technologies) were coated overnight at 4 °C with anti-mouse TNF-α capture antibody, diluted 1:350 in coating buffer (appendix, A3). Wells were washed three times with wash buffer (appendix, A3) and blocked with 200 µl of assay diluent (appendix, A3) for an hour at room temperature. TNF-α standards were prepared in assay diluent using two fold dilutions to concentrations ranging between 15.6 to 1000 pg/ml and assay diluent used as zero standard. Wells were washed three times, 100 µl of samples or standards was added and plates were sealed and incubated at room temperature for 2 hours. Wells were washed five times and 100 µl of working detector (biotinylated anti-mouse TNF-α detection antibody 1:350) was added and plates were sealed and incubated for an hour at room temperature. Wells were washed seven times, 100 µl of substrate solution (appendix, A3) was added to each well and the plates incubated in the dark for 30 min. reactions were stopped by adding 50 µl of 1 M phosphoric acid and absorbance (A_{450}) read using Dynatech MRX ELISA reader with correction set at A_{570}.

2.12 Proteomic analysis

I performed the proteomic analysis of D39 Δ*htrA* mutant in collaboration with Dr. Fran Mulholland at the Institute of Food Research (IFR), Norwich, UK.

2.12.1 Preparation of protein extracts

S. pneumoniae cultures were grown in BHI (100 ml) to an OD_{600nm} of 0.4 then harvested by centrifugation at 6,700 × g (Sigma laboratory centrifuge 4K15) for 15 min at room temperature and washed twice with cold PBS buffer, pH 8.0 (10 mM Na_2HPO_4, 1 mM KH_2PO_4, 140 mM NaCl, 3 mM KCl). The pellets were kept at –80 °C. Frozen pellets from

the 100-ml cultures were defrosted and resuspended in 4.67 ml Solution 1 (10 mM SDS, 200 mM DTT, 28 mM Tris base, 20 mM Tris-HCl) and 330 µl Solution 2 (24 mM Tris-base, 0.47 M Tris-HCl, 50 mM $MgCl_2$, 10 mg/ml RNAse, 10 mg/ml DNAse in 5 mM $CaCl_2$). Total extracts were prepared by passing cells through a French pressure cell, One Shot Benchtop Cell Disrupter (Constant Systems Ltd, Warwick, UK) at 12,000 lb/in^2 four times keeping samples on ice between cycles. Cell debris was removed by centrifugation at 13,200 × g for 15 min. the supernatant was then aliquoted and stored at –80 °C. Protein concentrations in the extract were measured by using Plus One Protein Quant kit (Amersham Biosciences).

2.12.2 *Two-dimensional electrophoresis*

2.12.2.1 First dimension

Bacterial lysates containing 50 µg of proteins prepared by the method described in the previous section were analysed in individual gels. The proteins were separated in the first dimension using pHaser system (Genomic solutions, Ann Arbor, Michigan, USA) and immobilized pH gradient gel strips (IPG) pH 4-7 (Amersham Pharmacia, Uppsala, Sweden) as recommended by the manufacturers with minor modifications. Proteins were mixed with lysis buffer (7 M urea, 2 M thiourea, 4 % CHAPS, 40 mM Tris-base) to a final volume of 50 µl, and 350 µl of rehydration buffer (7 M urea, 2 M thiourea, 2 % CHAPS, 2 % IPG buffer pH 4-7 (Amersham Pharmacia), 18.2 mM DTT, bromophenol blue). Gel strips were rehydrated with the total 400 µl overnight at 21 °C. Electrode wicks were put under the ends of the strips to allow better isoelectric focusing (IEF) and the strips were covered with dry strip cover fluid (Amersham Pharmacia) and the gel strips were run for 24 hours. The gel strips were rinsed with MilliQ water and equilibrated in filtered equilibration buffer (56 % vol/vol Tris acetate equilibration buffer pH 7.0 (Genomic solutions), 5 % SDS, 6 M Urea, 30 % glycerol) containing 52 mM DTT for 30 min and after that in filtered equilibration buffer containing 1.35 M iodoacetamide for another 30 min.

2.12.2.2 Second dimension

The proteins were separated in the second dimension on 10 % duracryl gels. Gels were cast according to the protocol (Investigator^{TM} 2-D Electrophoresis System, operating and maintenance manual), except that glass plates were sealed with silicon (low modulus silicon sealant, ARBO, Adshead Ratcliff & Company Ltd, Derbyshire, England). The

silicon was allowed to dry for at least one day. Gels were kept at 4 °C for one week to one month before use. Second dimension gels were run according to the protocol (Investigator™ 2-D Electrophoresis System, operating and maintenance manual) using cathode buffer (top running buffer; 0.2 M Tris, 0.2 M Tricine, 0.4 % SDS) and anode buffer (bottom running buffer; 25 mM Tris/acetate) and the Investigator system of Genomic solutions. Second dimension gels were taken out of the cold room and their wells rinsed with top running buffer several times using squeeze wash bottle and the wells were left full with top running buffer. A ring gasket with a silicone half moon gasket was put at each end round each gel cassette. First dimension gel strips were then placed in the well of the gel cassette and pushed gently to ease air bubbles away making sure all strips were inserted in the same orientation. Four gels were placed into each gel tank and the tank was sealed. The upper tank was filled with top buffer until the gasket rings were fully covered and the upper tank was placed into the lower tank unit and filled with top buffer until the gels were completely covered taking care not to disturb the strips resting on top of the second dimension gel. The gels were run for about four hours.

2.12.3 Staining, imaging and analysis

After separation, gels were stained with Sybro Ruby (Molecular Probes) according to the manufacturer's recommendations. The gels were taken out of the tank and the glass plates were opened. Whilst still on the plates, the sides of the gels where no protein should be located were cut off. Gels were carefully lifted off the plates and placed into boxes containing 400 ml of Sybro Ruby Fix solution (40 % methanol and 10 % acetic acid in MilliQ water) and gels were left for at least one hour on a shaker. The fix solution was discarded and 330 ml of Sybro Ruby stain was added in the dark, boxes were put into black plastic bags to avoid light and gels left for at least 90 min on a shaker. The Sybro Ruby was removed and 400 ml of the destain solution (10 % methanol and 6 % acetic acid in MilliQ water) was added and the boxes were covered again with black bags and left on the shaker for about one hour. The proXPRESS Proteomic Imaging System (Perkin Elmer, UK) was used for imaging. Gel images were analyzed using the ProteomeWeaver software.

2.12.4 Mass spectrometric analysis

Protein spots of interest were picked up for analysis using ProPick Spot Picker (Genomic Solutions). Protein spots were washed three times for 20 min in 100 µl solution A (freshly

prepared 80% (vol/vol) 50 mM ammonium bicarbonate, 20% (vol/vol) acetonitrile). The gel plugs were washed in 100 µl acetonitrile for 10-15 min and air dried for 10 min. For trypsin digestion, 5 µl of sequencing grade porcine modified trypsin (lyophilized, Promega) containing 50 ng of trypsin in 10 mM ammonium bicarbonate, was added and the plugs incubated at 37 °C for 2-4 hours. Trypsin was inactivated by adding 5 % formic acid and the plugs were incubated at 37 °C for 20 min, flash frozen and stored at –80 °C.

For matrix-assisted laser desorption ionization–time of flight mass spectrometry (MALDI-TOF-MS) analysis, the spots were analyzed by the John Innes Centre Protein Sequencing Facility. Peptide peak lists were searched against all bacteria databases at (http://www.matrixscience.com/).

2.13 Microarray analysis

Extra care was taken while working with RNA to avoid RNA degradation by RNases. Powder-free gloves were worn at all times to prevent contamination by RNases present on the skin and to avoid contamination of the array with dust particles. Before preparing or working with RNA, bench area, pipettes, glassware and gloves were treated with RNaseZap® RNase Decontamination Solution (Ambion Ltd, UK) to remove any RNase contamination. Filter tips (Greiner Bio-One Ltd, Gloucestershire, UK) were used for pipetting all liquids during this work.

2.13.1 Extraction of RNA

Bacterial strains were grown in BHI at the desired temperatures until they reached the mid-logarithmic phase of growth (OD_{600nm} ~ 0.6). Cells from 10-ml cultures were collected by centrifugation at 5000 ×g for 5 min at room temperature (Sigma laboratory centrifuge 4K15) and snap-frozen in liquid nitrogen to preserve RNA as quickly as possible. RNeasy® Midi Kit (Qiagen) was used to prepare RNA according to the protocol of the manufacturer with slight modification. All reagents were provided with the kit unless mentioned otherwise. Bacterial cell pellets were resuspended in 200 µl of lysis buffer, 15 mg/ml of lysozyme (Sigma-Aldrich, UK) in TE buffer (10 mM Tris pH 8.0; 1 mM EDTA pH 8.0) and vortexed for 30 seconds. Suspensions were then left at room temperature for 10 min with vortexing every 2 min. 700 µl of RLT buffer containing β-mercaptoethanol (10 µl of β-mercaptoethanol per 1-ml RLT buffer, freshly prepared prior to use) was added and mixed by vortexing. This mixture was transferred to cryotubes containing acid-washed

glass beads (106 μm, Sigam-Aldrich, UK) and bacterial cells disrupted using a HYBAID RiboLyser™ Cell Disrupter (Hybaid Ltd, Middlesex, UK) four times of 20s each. The tubes were then centrifuged for 30s at 13,000 ×g to remove the glass beads and the supernatants were transferred to 15-ml tubes. 3.2 ml of RLT buffer containing β-mercaptoethanol was added and tubes vortexed. 4 ml of 70% ethanol (prepared using RNase-free water) was added, mixed and applied to the columns and the columns were centrifuged at 5000 ×g for 5 min at room temperature. The flow-through was discarded and the columns washed by adding 4 ml of RW1 buffer and centrifuged at 5000 ×g for 5 min, then by adding 2.5 ml of RPE buffer and centrifuged at 5000 ×g for 2 min and a final wash by adding 2.5 ml of RPE buffer and centrifuged at 5000 ×g for 5 min before elution. RNeasy columns were transferred to new 15-ml tubes and RNA was eluted by adding 150 μl of RNase-free water, let stand for 1 min and centrifuged at 5000 ×g for 3 min and then for a second time using the first eluate to increase the final concentration of RNA.

2.13.2 Analysis of RNA

Total RNA concentrations were determined using a NanoDrop® ND-1000 UV/Vis spectrophotometer (NanoDrop® Technologies, USA). The RNA samples were also checked for integrity and concentration using the RNA 6000 Nano assay with the Agilent 2100 Bioanalyzer (Agilent Technologies, UK) according to the protocol of the manufacturer.

2.13.3 Array design and storage

The pneumococcal genome microarray slides were designed at the Pathogen Functional Genomics Resource Centre at TIGR (http://www.tigr.org/). They consist of PCR products representing segments of 2131 ORFs from *S. pneumoniae* strain TIGR4 in addition to 118 unique ORFs from strains R6 (37 ORFs) and G54 (81 ORFs), which are not present in the TIGR genome. They also contain 10 control spots printed at various locations. The slides were printed on 96 grids with each grid containing 7 columns and 14 rows. Slides were stored in a plastic slide box at room temperature in a dessicator containing a bottom layer of anhydrous calcium sulphate. Slides were kept away from dust and were handled with extreme care to prevent scratching or rubbing that may damage the printed DNA.

2.13.4 Aminoallyl-labelled cDNA synthesis

10 μg of RNA was mixed with 2 μl of 3 mg/ml random hexamer (Invitrogen) and the volume was brought to 18.5 μl using DEPC water (Ambion). The mixture was incubated at 70 °C for 10 min, cooled on ice for 30s and centrifuged briefly to bring down any condensation. Incubation was continued at room temperature. RNA/primer mix was mixed with 6 μl 5× first strand buffer, 3 μl of 0.1 M DTT, 1.2 μl of 12.5 mM dNTP/aa-UTP labelling mix (Ambion), and 2 μl of 200 U/μl of SuperScript II RT (Invitrogen), to a final volume of 30.7 μl. The mixture was incubated at 42 °C overnight (~ 16 hours). The first strand synthesis reaction was stopped by adding 10 μl of 0.5 M EDTA (Ambion) and 10 μl of 1 M NaOH to hydrolyze RNA in the cDNA/RNA mixture. The mixture was incubated at 65 °C for 15 min and 25 μl of 1 M Tris, pH 7.0 (Ambion) was added to neutralize pH. The unincorporated aa-dUTP and free amines were removed using a modification of the Qiagen protocol for their PCR purification kit. The cDNA reaction was mixed with 400 μl of PB buffer (Qiagen) and transferred to a Qiaquick column, centrifuged at 13,000 ×g for 1 min and the collection tubes were emptied. The columns were washed with 750 μl of phosphate wash buffer (5 mM KPO_4 pH 8.0 and 80 % ethanol in MilliQ water) and centrifuged at 13,000 ×g for 1 min. The collection tubes were emptied and the columns were centrifuged for an additional 1 min. The cDNA was eluted twice with 30 μl-each of phosphate elution buffer (4 mM KPO_4 pH 8.0 in MilliQ water). The final elution volume (60 μl) was transferred to open tubes and dried down in a speed vac (Savant DNA 110 Speed Vac, Global Medical Instruments, USA) for 1 hour.

2.13.5 Cy3/Cy5 labelling of cDNA

All reaction tubes were wrapped in foil and kept sequestered from light as much as possible to prevent photobleaching of the Cy-dyes. The aminoallyl-labelled cDNA was resuspended in 4.5 μl of 0.1 M of freshly prepared sodium carbonate (Fisher) buffer pH 9.3 (0.53 gm of Na_2CO_3 in 50-ml MilliQ water, lower pH to 9.3 using HCl) by pipetting up and down for several minutes. 4.5 μl of the appropriate Cy-dye, Cy3 or Cy5 (Amersham Biosciences) was added and the mixture incubated for 2 hours at room temperature in the dark. After coupling was complete, 35 μl of 100 mM sodium acetate pH 5.2 (1 ml of 3 M NaOAc pH 5.5, Ambion, in 29 ml of MilliQ water) was added and the free dye was removed using the Qiagen purification protocol. The cDNA reaction was mixed with 250 μl of PB buffer (Qiagen), transferred to Qiaquick columns and centrifuged at 13,000 ×g for

1 min. The columns were washed with 750 µl of PE buffer (Qiagen) and centrifuged as above and for an additional 1 min. The dye-labelled cDNA was eluted twice with 50 µl each of EB buffer (Qiagen). The incorporation of the Cy-dye into the cDNA was measured in 1-µl sample using the NanoDrop® ND-1000 UV/Vis spectrophotometer (NanoDrop® Technologies, USA). The final elution volume (100 µl) was transferred to open tubes and dried down in a speed vac (Savant DNA 110 Speed Vac, Global Medical Instruments, USA) for 2 hours.

2.13.6 Hybridization of cDNA probes to array

2.13.6.1 Pre-hybridization

It is extremely important that slides be perfectly clean to prevent background staining. The printed slides were placed with their labels up and their printed side down in a Coplin jar containing 60 ml of prehybridization buffer (15 ml of 20× SSC (Ambion); 600 µl of 10 % SDS (Ambion); 0.6 gm of BSA fraction V powder (Sigma); 44.4 ml of MilliQ water) preheated at 42 °C and incubated for at least an hour at 42 °C. The slides were removed from the Coplin jar by carefully gripping them by the label using forceps and placed in the slide holder of a glass-staining dish filled with MilliQ water. The entire staining dish apparatus was placed on top of a rotor shaker (Edmund Bühler KM-2 shaker) and shaken for 2 min. The water was changed every 2 min until ~ 2 L of wash water was used. The slides were then washed with isopropyl alcohol (Fisher Scientific) for 2 min on the rotary shaker. The slides were put into a clean centrifuge with a flat plate-holder lined with paper towel and centrifuged at 700 rpm for at least 10 min for drying.

2.13.6.2 Hybridization

The slides were used immediately following prehybridization to ensure optimal hybridization efficiency. The 1× hybridization buffer (500 µl of formamide (Sigma), 250 µl of 20× SSC; 10 µl of 10 % SDS, 75 µl of 10 mg/ml sheared salmon sperm DNA (Ambion) and 165 µl MilliQ water) was prepared and filtered using 0.22 µM filter (Fisher Scientific) just prior to use. The labelled probes prepared as described in section 2.13.5 were resuspended in 30 µl of hybridization buffer by finger flicking for about 1 min and the mixture was heated to 95 °C for 5 min. The suspension was mixed by finger flicking for an additional minute and heated again at 95 °C for another 5 min. The microarray slides were placed between the two plastic teeth in the bottom half of a hybridization chamber

(Corning) and the entire labelled probe mixture was applied onto the printed area of the slides keeping air bubbles to a minimum. A glass cover slip 24mm × 60mm was dusted with compressed air and placed over the slide printed area and any large bubbles were worked towards the edge by gently pressing the cover slip surface. 20 µl of unused hybridization buffer were added to the small wells at each end of the hybridization chamber. The chamber was then sealed with the metal edge bars, wrapped in aluminium foil and incubated at 42 °C for 16-20 hours (GeneChip® Hybridization oven 640).

2.13.6.3 Post-hybridization

The low stringency buffer (100 ml of 20× SSC, 10 ml of 10 % SDS, 890 ml of MilliQ water) was prepared and preheated to 55 °C. The slides were removed from the chamber and submerged in a Pyrex glass dish (Fisher Scientific) filled with the low stringency buffer. The slides were shaked to loosen the cover slip until it slid free of the slide surface and the slides were washed vigorously for an additional 1 min. The buffer was changed and slides washed for another 5 min followed by 5-min wash using medium stringency buffer (5 ml of 20× SSC, 10 ml of 10 % SDS, 985 ml of MilliQ water) and then with final 5-min wash with high stringency buffer (5 ml of 20× SSC, 995 ml of MilliQ water). The slides were dried by centrifugation as mentioned before (section 2.13.6.1) and kept in dark until ready for scanning.

2.13.7 Scanning array and data processing

For each strain, two or three independent RNA preparations were analyzed using the microarrays. Hybridized slides were scanned using a ScanArray™ Express microarray scanner (Packard Bioscience, Biochip Technologies) according to the manufacturer's instructions and the median pixel intensity values for each element on the array were quantified using Quantarray™ (Packard Bioscience). The data was further analyzed using GeneSpring™ 6.0 (Silicon Genetics). The 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 ratio change threshold of at least 2 standard deviations compared to the median ratio for each strain.

2.13.8 RT-PCR

RNA was extracted from cultures grown in BHI to OD_{600nm} of 0.6 using an RNeasy midi kit (Qiagen) as described in section 2.13.1. The synthesis of cDNA was carried out using ThermoScript™ RT-PCR system (Invitrogen). The reverse transcription reactions were prepared by combining the following in 0.5-ml eppendorf tubes; 50 ng of random hexamers, 5 μg of RNA, 2 μl of 10 mM dNTP mix. The reaction volumes were adjusted to 12 μl using DEPC-treated water and tubes incubated at 65 °C for 5 min to denature RNA and primers. The 5× cDNA synthesis buffer was vortexed briefly just prior to use. The following were then added to the reaction mixtures; 4 μl of 5× cDNA synthesis buffer, 1 μl of 0.1 M DTT, 1 μl of RNaseOUT™ (40U/μl), 1 μl of DEPC-treated water, and 1 μl of ThermoScript™ RT. A reaction without ThermoScript™ RT was used as a negative control. The reactions were then transferred to a thermal cycler and incubated at 25 °C for 10 min followed by 30 min at 50 °C. The reverse transcription reactions were terminated by incubating them at 85 °C for 5 min, 1 μl of RNase H was then added and tubes incubated at 37 °C for 20 min to get ride of any residual RNA and the cDNA synthesis reactions were stored at –20 °C. 5 μl of each cDNA reaction was used as a DNA template in PCR reactions, which were carried out using Vent® polymerase (Promega) as described in section 2.6.1 and the results analyzed by agarose gel electrophoresis.

2.14 Statistical analysis

Statistical analyses were carried out using StatView 4.1 (Abacus Concept). Survival times and comparisons of cytokine levels were analyzed by using nonparametric Mann-Whitney U analysis. Cytokine levels were expressed as the median ± the median absolute deviation (MAD) (Kerr *et al.*, 2002). Bacteriology results were expressed as geometric mean ± standard error of the mean (SEM). Bacteriologic assay results below the detection limits of the viable count assays (log 1.92 units per ml of blood and log 0.92 unit per ml of lung lavage fluid or lung homogenate samples) were ascribed values just below the detection limits (log 1.91 and 0.91, respectively). Comparisons of bacterial loads in the time course bacteriology experiments were performed using an unpaired *t*-test. Comparisons of bacterial loads at different time points within a group were carried out using the analysis of variance (ANOVA) with the Bonferroni post hoc test. The inter-group variation was controlled for by ensuring no statistical differences in any parameter between wild type and mutant-infected mice immediately following infection. In all analyses, a *p*-value less than 0.05 was considered statistically significant.

Chapter 3

ROLE OF CLPCP PROTEASE IN THE PNEUMOCOCCAL STRESS RESPONSE, RESULTS

Upon exposure to stress conditions, particularly heat shock, bacteria respond by mediating a cascade of events leading to the synthesis of a unique group of proteins called heat shock proteins (HSPs). The production of these proteins represents a protective cellular response to cope with the stress-induced damage of proteins. HSPs are highly conserved in both prokaryotes and eukaryotes (Lindquist, 1986, Craig *et al.*, 1993, Hendrick and Hartl, 1993). Many HSPs are molecular chaperones or ATP-dependent proteases and play major roles in protein folding, repair, and degradation under normal and stress conditions (Gottesman, 1999, Wickner *et al.*, 1999).

The ATP-dependent caseinolytic protease, ClpCP consists of an ATPase specificity factor, ClpC, which is a member of HSP100/clp family (Schirmer *et al.*, 1996) and a proteolytic subunit, ClpP, which by itself represents a unique family of serine proteases (Maurizi *et al.*, 1990). Both *clpC* and *clpP* genes are members of class III group of heat shock genes and their expression is negatively controlled by the class three stress gene repressor, CtsR (Derre *et al.*, 1999b). The presence of ClpC, ClpP and other Clp proteases in bacterial cells is fundamental for stress survival as mutations in some *clp* genes results in organisms unable to grow in the presence of stress conditions (Squires and Squires, 1992, Kruger *et al.*, 1994, Gerth *et al.*, 1998, Spiess *et al.*, 1999, Chastanet *et al.*, 2001). The ClpC protein, also identified as MecB (Msadek *et al.*, 1994) in *Bacillus subtilis*, was reported to be essential for growth at high temperature, cell division, sporulation and also to act as a negative regulator of ComK synthesis preventing late competence gene expression (Msadek *et al.*, 1994, Kruger *et al.*, 1997, Turgay *et al.*, 1997, Nanamiya *et al.*, 1998). ClpC was implicated in the virulence of *Listeria monocytogenes* by promoting early bacterial escape from the phagosomal compartment of macrophages (Rouquette *et al.*, 1996, Rouquette *et al.*, 1998). It was also involved in the invasion of hepatocytes *in vivo* during infection by modulating the invasion virulence factors (Nair *et al.*, 2000b). The role of ClpP-mediated proteolysis in the stress tolerance and virulence of Gram-negative and Gram-positive bacteria has been the subject of many recent studies. In *Salmonella enterica* serovar Typhimurium, ClpP was found to be necessary for virulence when examined in both a mouse model and in isolated macrophages (Webb *et al.*, 1999, Yamamoto *et al.*, 2001b). ClpP-null mutants of *B. subtilis* and *Lactococcus lactis* were impaired for survival during heat shock and other stress conditions (Gerth *et al.*, 1998, Frees and Ingmer, 1999). In *L. monocytogenes*, disruption of the *clpP* gene reduced the haemolytic activity due to the major virulence factor, listeriolysin O that helps the bacteria to escape from the phagosomes of macrophages (Gaillot *et al.*, 2000). It was also reported that ClpP was important for the growth of *Staphylococcus aureus* in the presence of oxidative stress or at

low temperature. The virulence of a *ΔclpP* mutant of *S. aureus* was severely attenuated when investigated in a murine abscess model (Frees *et al.*, 2003).

During the course of this study, a number of reports about the role of pneumococcal ClpC have been published but the contribution of ClpC to the autolysis and expression of competence genes and virulence factors is still debated (Charpentier *et al.*, 2000, Chastanet *et al.*, 2001, Robertson *et al.*, 2002). ClpC has been reported to play a role in thermal tolerance, control of autolysis and chain formation in the pneumococcus (Charpentier *et al.*, 2000) while other studies suggest that the protein plays no role in these processes (Chastanet *et al.*, 2001, Robertson *et al.*, 2002). Charpentier and co-workers (Charpentier *et al.*, 2000) also reported that ClpC plays a major role in processes related to the virulence of the organism including adherence to human cells and production of known virulence factors such as pneumolysin, LytA, CbpA and other choline-binding proteins. This study suggests that ClpC may play a major role in the virulence of the pneumococcus. A signature-tagged mutagenesis screen identified ClpC as a virulence factor in the pneumococcus (Polissi *et al.*, 1998). However, it was showed that ClpC null mutants of the pneumococcus are not greatly affected in their virulence (Robertson *et al.*, 2002). Recent studies have agreed on the requirement of ClpP for thermotolerance, development of competence, and virulence of *Streptococcus pneumoniae* (Chastanet *et al.*, 2001, Robertson *et al.*, 2002, Kwon *et al.*, 2003).

The study presented here was undertaken to define the role played by ClpC and ClpP in the stress response and virulence of *S. pneumoniae*. Isogenic mutants of pneumococcal strains lacking either ClpC or ClpP genes were constructed and analyzed *in vitro* and *in vivo*.

3.1 Characterization of ClpC and ClpP proteins

3.1.1 ClpC

Proteins of the Clp ATPase family are highly conserved and ubiquitous in prokaryotes and higher organisms and are classified into two classes based on the presence of either one or two ATP binding domains as well as on the occurrence of specific signature sequences (Gottesman *et al.*, 1990a, Schirmer *et al.*, 1996). ClpC belongs to class 1 Clp ATPase proteins, members of which contain N-terminal and C-terminal domains, two highly conserved ATP binding domains and a variably sized middle domain (Ingmer *et al.*, 1999). Analysis of the complete genome sequence of *Streptococcus pneumoniae* strain TIGR4

(The Institute for Genomic Research at http://www.tigr.org/) revealed a single copy of the gene for ClpC. The 2.433 kb gene encodes for an 810-amino-acid protein with a molecular weight of 90.2 kDa and a pI of 5.82. The pneumococcal ClpC protein shares sequence similarity with ClpC/MecB of *B. subtilis* (47.2% identity) and the ClpC ATPases of *Lactococcus lactis* (53.4% identity). The amino acid alignment with other prokaryotic ClpC ATPases showed that the structural organization of the pneumococcal ClpC is typical for proteins of this family. ClpC contains the two nucleotide-binding regions ATP-binding 1 (amino acids 206-436) and ATP-binding 2 (amino acids 474-667), harbouring the characteristic ATPase A and B boxes. These two domains are separated by a spacer region of 37 amino acids (amino acids 437-473) and enclosed between a leader sequence of 205 amino acids at the N-terminus and a trailer sequence of 143 amino acids at the C-terminus. Figure 3.1 shows the amino acid alignment for the pneumococcal ClpC with those of *B. subtilis* and *L. lactis*.

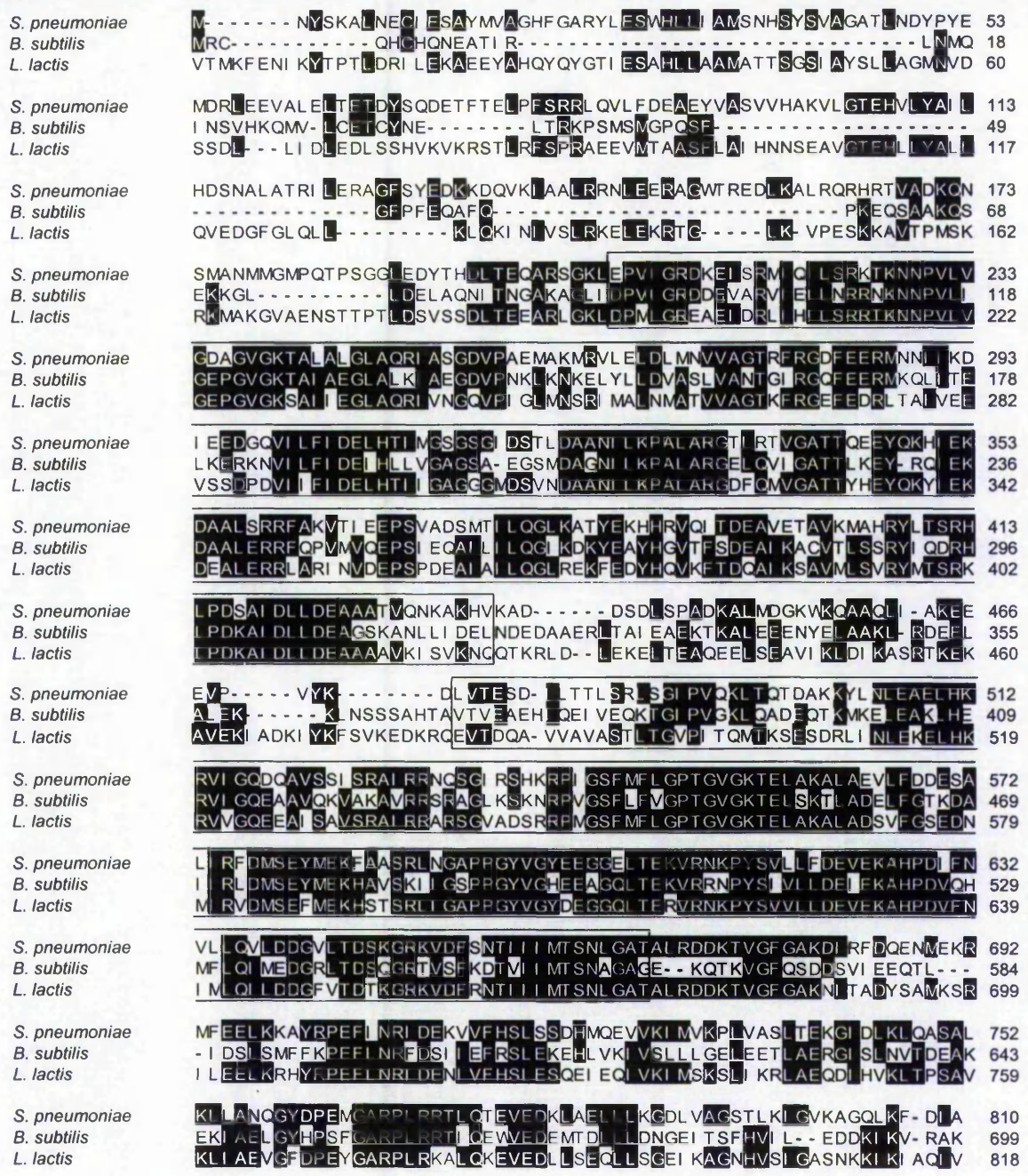


Figure 3.1- Multiple sequence alignment of ClpC protein from some Gram-positive bacteria.

Alignment of the *S. pneumoniae* ClpC amino acid sequence with those of *B. subtilis* and *L. lactis*. Amino acid sequences were aligned using *ClustalV Method* (MegAlign, DNASTAR Inc.). Numbers indicate positions in the amino acid sequence. Identical residues are shaded. The characteristic nucleotide-binding domains, ATP-1 (amino acids 206-436) and ATP-2 (amino acids 474-667), are boxed. The 37-amino-acid spacer region separates the two ATP binding domains (amino acids 437-473).

3.1.2 ClpP

The *S. pneumoniae* genome has recently been shown to contain only a single copy of *clpP* (Hoskins *et al.*, 2001). The 588 bp gene encodes for a 196-amino-acid protein with a molecular weight of 21.3 kDa and a pI of 4.49. The pneumococcal ClpP protein shares

amino acid identity with that of *Bacillus subtilis* and *Lactococcus lactis*, 85.1 % and 58.9 % respectively. The high degree of identity over the entire length of the aligned sequences (figure 3.2) indicates that *S. pneumoniae* ClpP is a member of the unique family of serine proteases (Maurizi *et al.*, 1990). The serine-96 residue, the histidine-121 residue and the aspartate-172 residue, which constitute the catalytic triad of the serine protease, are conserved (figure 3.2).

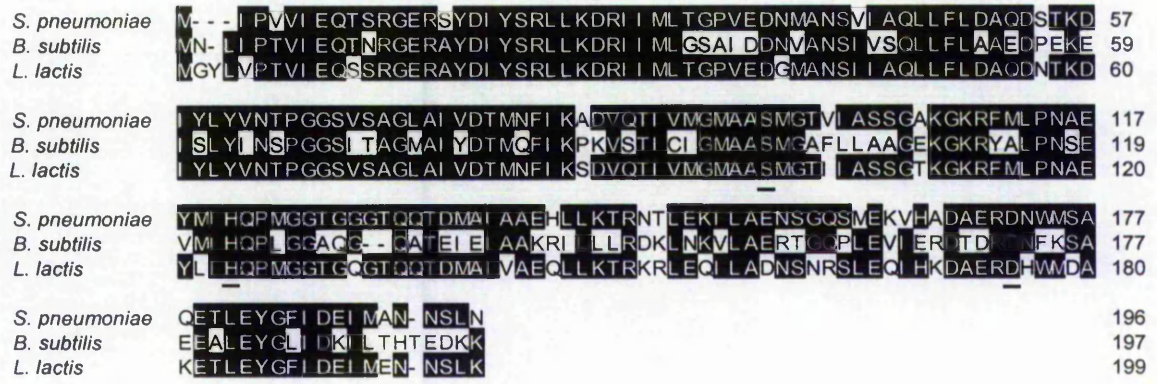


Figure 3.2- Multiple sequence alignment of ClpP protein from some Gram-positive bacteria.

Alignment of the *S. pneumoniae* ClpP amino acid sequence with those of *B. subtilis* and *L. lactis*. Amino acid sequences were aligned using *ClustalV Method* (MegAlign, DNASTAR Inc.). Identical residues are shaded. The conserved catalytic Ser^{96}, His^{121}, and Asp^{172} residues (*S. pneumoniae* numbering) are underlined.

3.2 Construction of mutants

3.2.1 ClpC mutation

The *clpC* gene of *S. pneumoniae* strains D39, R6, R800 and TIGR4 was disrupted by the introduction of an erythromycin resistance cassette into the gene. The whole *clpC* gene was amplified from the chromosomal DNA of a laboratory-adapted strain of D39, D39(LA), using primers ClpC a1 and ClpC a2 (table 2.2) and cloned into PCR-Script™ cloning vector (Stratagene). Internal primers, Clp inv1 and Clp inv2 (table 2.2) were designed to carry out inverse PCR for removing the middle region of *clpC* creating *Asc*I restriction sites in the resulting PCR product. *Asc*I-generated erythromycin cassette, using Ery3 and Ery5 primers (table 2.2), was ligated to the inverse PCR product to be used as a selection marker.

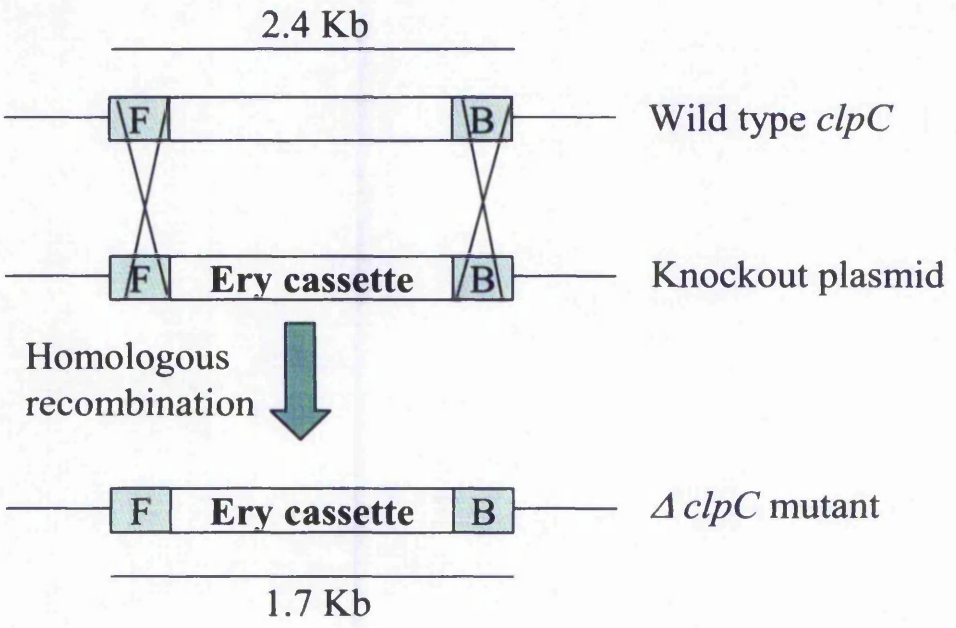


Figure 3.3- Introducing *clpC* mutation in *S. pneumoniae*.

3.2.1.1 Confirmation of inverse PCR product

The product of the inverse PCR described above was 3.6 kb. This PCR product was gel purified using a Qiagen gel purification kit, self ligated and transformed into *E. coli* cells. The transformed cells were selected by plating onto LB agar with 50 μg/ml of ampicillin and the plasmid was recovered from cultures grown overnight on LB broth with ampicillin using a Qiagen miniprep kit. The knockout of *clpC* was then confirmed by restriction digestion and PCR. Digesting the resulting plasmid with *Eco*RI and *Asc*I resulted in a 300-bp band and a 3.3 kb band (figure 3.4 B) confirming the diagrammatic representation shown in Figure 3.4. When primer pair clpC a1 and clpC a2 was used, the PCR product was about 700 bp and when primer pair T3 and T7 used, the product was about 750 bp (figure 3.4 C). The PCR products again confirm the orientation shown in the upper panel of figure 3.4.

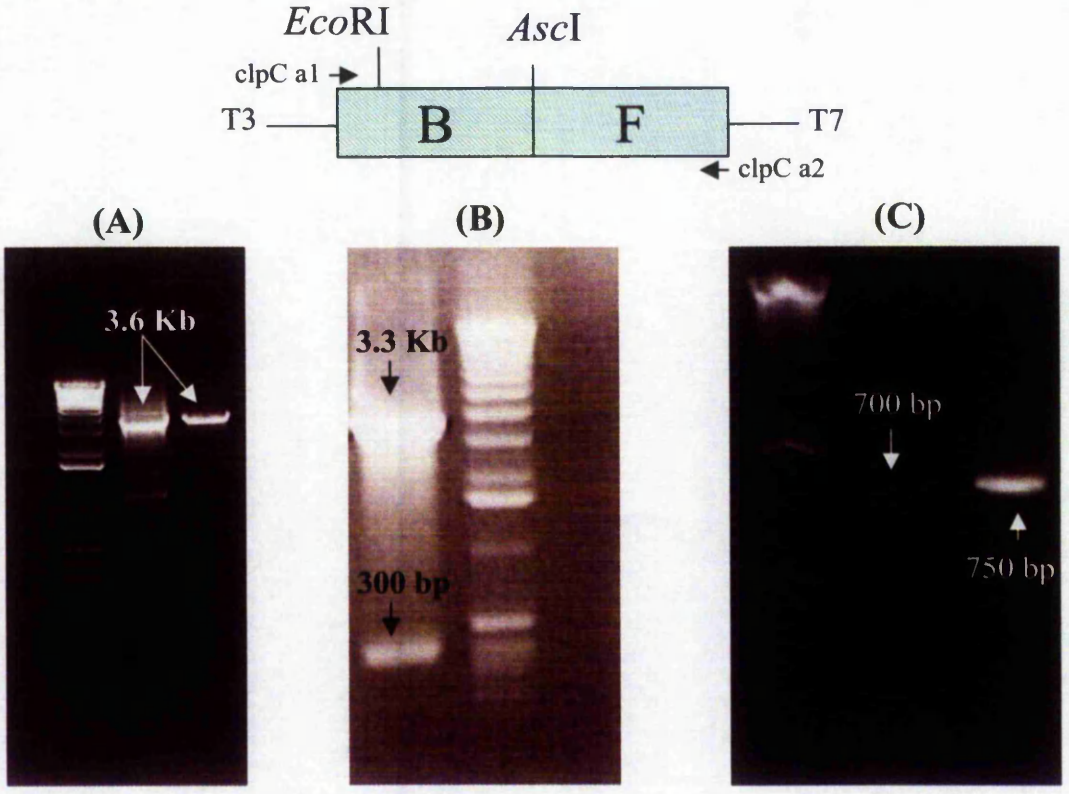


Figure 3.4- Inverse PCR and confirmation of product.

Agarose gel electrophoresis of the inverse PCR product and its purification in (A) lanes 2 and 3 respectively, confirmation of self-ligated inverse PCR product by restriction digestion with *Asc*I and *Eco*RI in (B) and confirmation of self-ligated inverse PCR product by PCR using primer pairs, (T3 and T7) and (clpC a1 and clpC a2) in (C). Band sizes as indicated on the gel pictures. The DNA marker used is the 1 kb plus ladder.

3.2.1.2 Marker ligation

To facilitate the selection of transformants, an *Asc*I-generated erythromycin cassette was ligated to the plasmid described above after being digested with *Asc*I and the new plasmid was cloned into *E. coli* cells. The transformed cells were selected by plating onto LB agar with 1 mg/ml of erythromycin and the plasmid was recovered from cultures grown overnight on LB broth with erythromycin using a Qiagen miniprep kit. The ligation of the erythromycin cassette was confirmed by PCR and restriction digestion. Using the primer pair clpC a1 and clpC a2 resulted in 1.7-kb DNA frgment (figure 3.5 A) and digestion of the plasmid with *Asc*I released the 1 kb fragment corresponding to the erythromycin gene (figure 3.5 B).

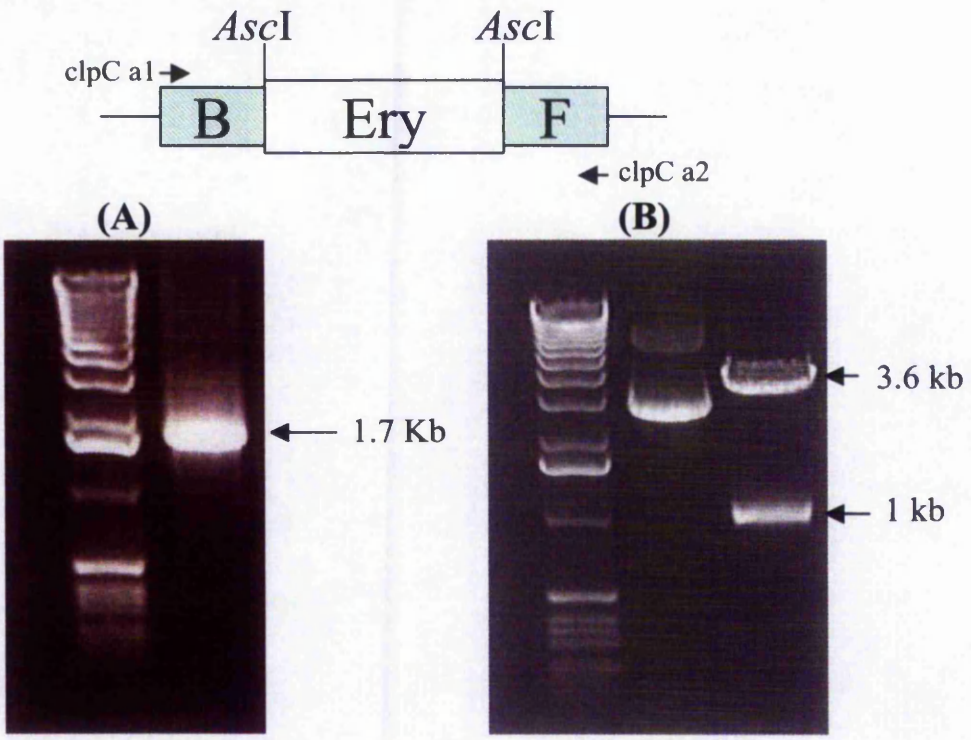


Figure 3.5- Confirmation of marker ligation in the knockout plasmid.

Agarose gel electrophoresis of PCR product using primer pair clpC a1 and clpC a2 (1.7 kb) in (A) and digestion of the knockout plasmid with *Asc*I in (B). In (B), lane 2, uncut plasmid. Lane 3, *Asc*I-cut plasmid. The DNA marker used is the 1 kb plus ladder.

3.2.1.3 Transformation of pneumococcal strains

The modified version of *clpC* contains the erythromycin cassette in the middle of about 350 bases of the original gene copy at each end of the gene, which was used for homologous recombination. This construct was amplified out of the plasmid using primers ClpC a1 and ClpC a2 primers and used to transform a laboratory-adapted strain of D39, termed D39(LA) and mutants were selected by growth on 1 μg/ml erythromycin. Mutation was confirmed by using diagnostic primers MCP1 for and MCP2 rev (table 2.2) which amplify a 2.6 kb fragment in the D39(LA) wild type and a 1.9 kb fragment in the D39(LA) Δ*clpC* mutant (figure 3.6 A). It was also confirmed by nucleotide sequencing and the growth of the pneumococcus on erythromycin. This mutation was moved into strains R6 and R800 to test whether the phenotype resulting from *clpC* deletion in these strains is consistent with phenotypes reported by other groups (Charpentier *et al.*, 2000, Chastanet *et al.*, 2001, Robertson *et al.*, 2002). The *clpC* mutation was also moved into TIGR4 and a mouse-adapted strain of D39, termed D39(MA) to be used in the *in vivo* studies. Confirmation of *clpC* mutation in these strains is shown in figure 3.6 B.

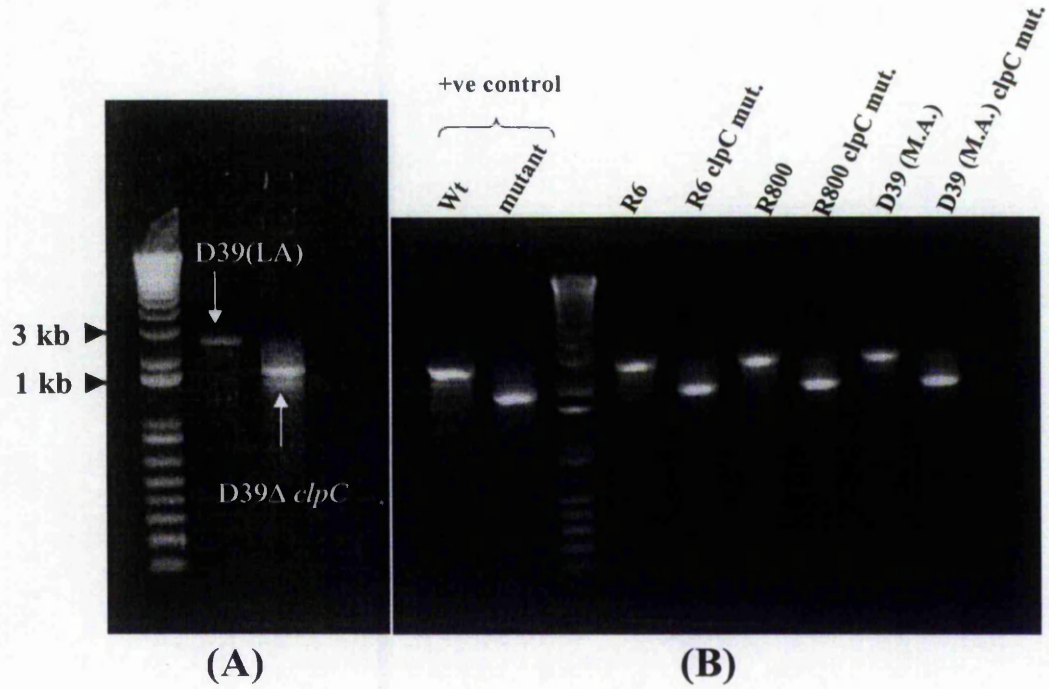


Figure 3.6- Confirmation of ClpC mutation in some pneumococcal strains.

Agarose gel electrophoresis of PCR products using primer pair MCP1 for and MCP2 rev for confirmation of *clpC* mutation in D39(LA) strain in (A) and in other strains in (B). PCR products using this primer pair were 2.6 kb fragment in the wild type and 1.9 kb fragment in the mutant. The DNA marker used is the 1 kb plus ladder.

3.2.2 ClpP mutation

A *clpP* mutant strain, SP2000 (Chastanet *et al.*, 2001) was generously provided by Jean-Pierre Claverys (CNRS-Université Paul Sabatier, Toulouse, France) as a ligation between ~500 bp fragments upstream and downstream from *clpP* gene and a kanamycin resistance (Km^r) gene. This mutant was used to construct Δ*clpP* mutants of TIGR4 and the mouse-adapted strain of D39, D39(MA), by PCR amplification of a 1.6 kb fragment corresponding to *clpP* mutation carrying kanamycin resistance cassette using primer pair AC94 and AC97 (table 2.2). This PCR product was used to transform D39 and TIGR4. Transformed cells were selected on 250 μg/ml kanamycin. The introduction of mutation in TIGR4 and D39 was confirmed by PCR using *clpP* specific primers, clpP for and clpP rev (table 2.2) and primer pair AC94 and AC97 (Chastanet *et al.*, 2001). Using primer pair clpP for and clpP rev gave a 600 bp fragment in the wild type and about a 1.4 kb fragment in the mutants. The product of PCR when using primers AC94 and AC97 was about 1.4 kb in the case of the wild type, while in case of the mutants resulted in a 1.6 kb fragment (figure 3.7).

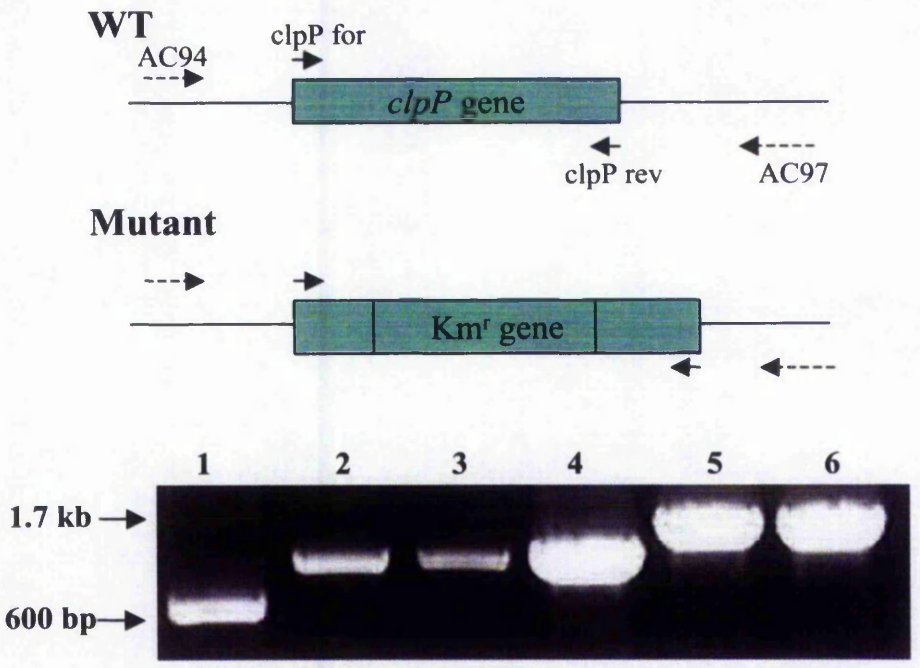


Figure 3.7- Confirmation of *clpP* mutation in TIGR4 and D39.

Agarose gel electrophoresis of PCR products for confirmation of *clpP* mutation in TIGR4 and D39 strains. Lanes 1-3, using primer pair clpP for and clpP rev, Lane 1, D39 wild type *clpP* (600bp). Lane 2, D39Δ*clpP* mutant (~1.4 kb). Lane 3, TIGR4Δ*clpP* mutant (~1.4 kb). Lanes 4-6 using primer pair AC94 and AC97. Lane 4, D39 wild type (~1.4 kb). Lane 5, D39Δ*clpP* mutant (~1.6 kb). Lane 6, TIGR4Δ*clpP* mutant (~1.6 kb). In the diagram, solid arrows represent clpP for and clpP rev and dashed arrows represent AC94 and AC97.

3.3 *In vitro* analysis of mutants

3.3.1 Growth measurements of ΔclpC mutants

3.3.1.1 By optical density

ClpC has been shown to play an essential role in growth at high temperatures, presumably due to its role in the degradation of misfolded and damaged proteins generated by heat stress. The ability of Δ*clpC* mutants to grow at different temperatures was studied. 1×10^6 CFU/ml of each strain was used to inoculate BHI prewarmed at 37 and 40°C and incubated at the indicated temperatures. At 30-min intervals, samples were withdrawn to measure the optical density at 600 nm (OD_{600nm}). The growth of D39(LA) Δ*clpC* mutant was reduced compared to that of the wild type at 37 °C as measured by optical density. This difference was in growth rate rather than total growth as both mutant and wild type strains reached the same stationary phase OD_{600nm} (figure 3.8). The slower growth of the mutant was not

observed at higher temperature (40 °C), as the growth of both wild type and mutant organisms was very similar (figure 3.8). In addition, the Δ*clpC* mutant organism did not undergo autolysis after entering the stationary phase of growth both at 37 and 40 °C compared to the wild type (figure 3.8).

3.3.1.2 By viable counting

To confirm the lack of autolysis in the Δ*clpC* mutant, the viable counts of all wild type strains, laboratory-adapted D39(LA), mouse-adapted D39(MA), R6, R800 and TIGR4 were compared to their Δ*clpC* mutants over time. There was no defect in growth at 40 °C suggesting that ClpC is not involved in the heat stress tolerance of the pneumococcus. However, both at 37 and 40 °C, the Δ*clpC* mutants of both the laboratory-adapted strain D39(LA) and the mouse-adapted strain D39(MA) maintained cell viability after reaching the stationary phase of growth compared to their parent strains which tended to undergo autolysis rapidly when a certain cell density had been reached (figure 3.9). This observation of the maintenance of viability was not recorded in the Δ*clpC* mutants of R6, R800 and TIGR4 strains (data not shown).

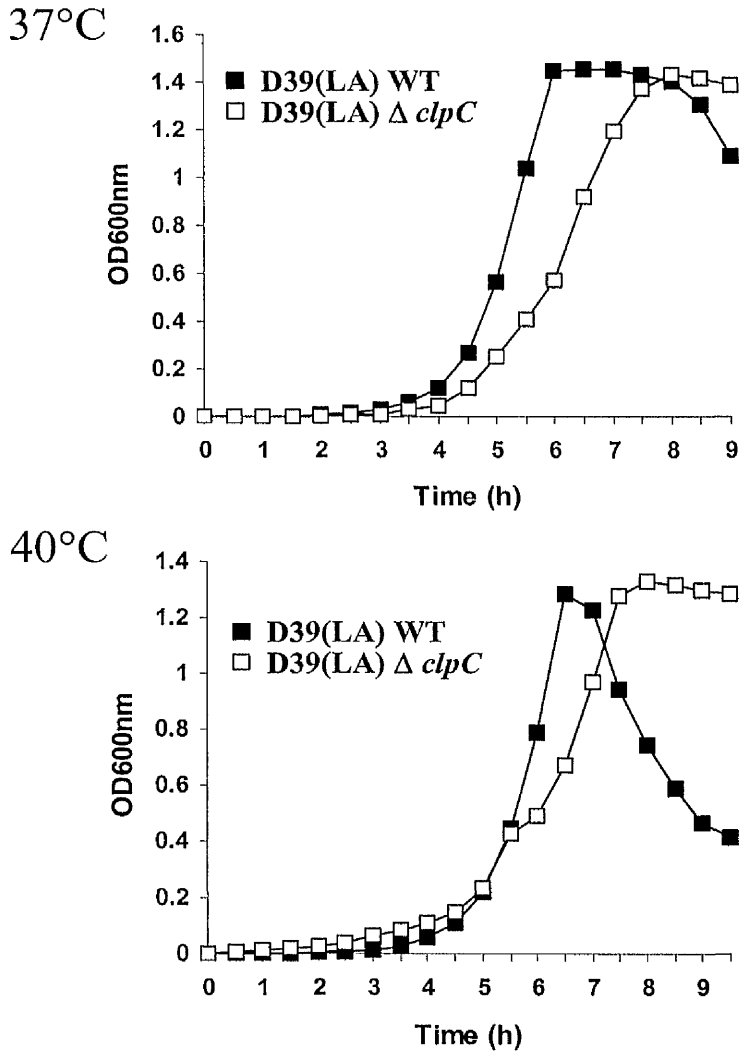


Figure 3.8- Representative growth curves of D39(LA) and its Δ*clpC* mutant at 37 and 40 °C.

10^6 CFU/ml of each strain was used to inoculate BHI prewarmed at the indicated temperatures and samples were taken at 30 min-intervals to measure the optical density at 600 nm (OD_{600nm}).

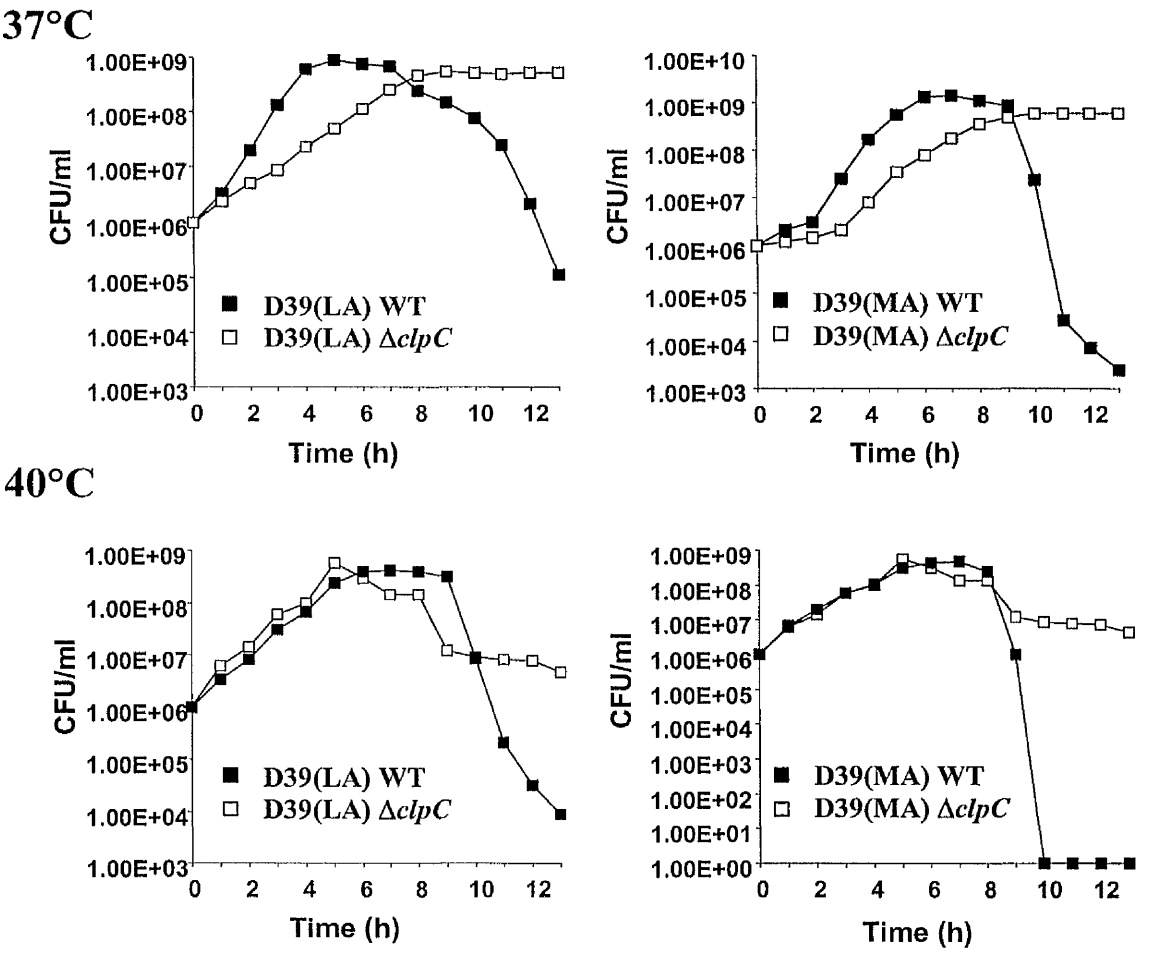


Figure 3.9- Representative graphs of *in vitro* growth of D39(LA) and D39(MA) wild types and their Δ*clpC* mutants at 37 °C and 40 °C.

10^6 CFU/ml of each strain was used to inoculate BHI prewarmed at the indicated temperatures and samples were taken at 1h-intervals to measure the viable bacterial count on BAB plates. The growth of Δ*clpC* mutants was similar to that of the wild types at both temperatures. However, the mutant strains maintain viability in the stationary phase of growth i.e. the autolysis rate of the Δ*clpC* mutants was very much reduced compared to that of the wild types.

3.3.2 Effect of clpC deletion on morphology

Cell morphology of cultures grown at different optical densities was examined by light microscopy of the Gram-stained samples. The Δ*clpC* mutants of both D39 strains grew in long chains of bacterial cells rather than the typical diplococcus form of *S. pneumoniae* (figure 3.10). No chains longer than 10 organisms were observed in the wild type cultures while at least one chain of this length or greater was seen in 12 randomly selected fields in the mutant cultures as shown in figure 3.10. Appearance of these chains suggests an impairment in cell separation, which is believed to be induced by the autolytic action of autolysin A (LytA) (Briese and Hakenbeck, 1985, Novak *et al.*, 1999).

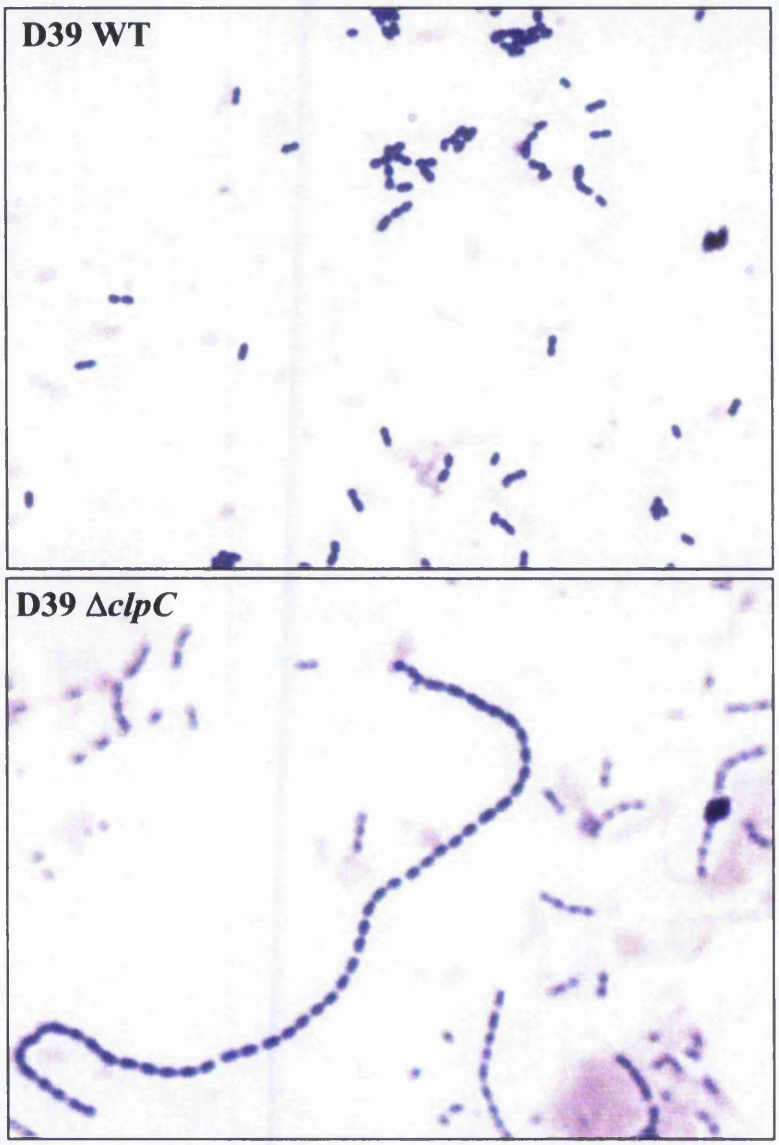


Figure 3.10- Effect of *clpC* deletion on the morphology of the pneumococcus.

The mutant strains of both D39(LA) and D39(MA) grow in long chains of bacterial cells compared to the characteristic diplococcus form of *Streptococcus pneumoniae*. Samples of the same optical density were withdrawn, Gram stained and examined by light microscopy. Magnification ×1000.

3.3.3 Penicillin-induced loss of viability

To further investigate the phenomenon of less autolysis in the Δ*clpC* mutant, the penicillin-induced autolysis of Δ*clpC* mutants and their wild types was studied (figure 3.11). This assay was carried out by exposing cultures grown to an OD_{600nm} of ~ 0.3 to 0.1 μg/ml (10× the MIC) of benzylpenicillin at 37 °C and the viable bacteria were counted by plating samples of the cultures onto BAB plates at 1-hour intervals over a period of six hours. The

Δ*clpC* mutant of D39(LA) strain showed a small decline in viable count over a six-hour period of exposure to penicillin while its parent strain lost viability over time and no viable cells were recorded after six hours of exposure to the antibiotic. The Δ*clpC* mutant of the D39(MA) strain also maintained viability in the presence of penicillin but, surprisingly, its parent strain showed no penicillin-induced autolysis (figure 3.11). The TIGR4 wild type and TIGR4 Δ*clpC* mutant were also penicillin resistant (data not shown). Similar to their parent strains, R6 Δ*clpC* and R800 Δ*clpC* mutants underwent autolysis when treated with penicillin (data not shown).

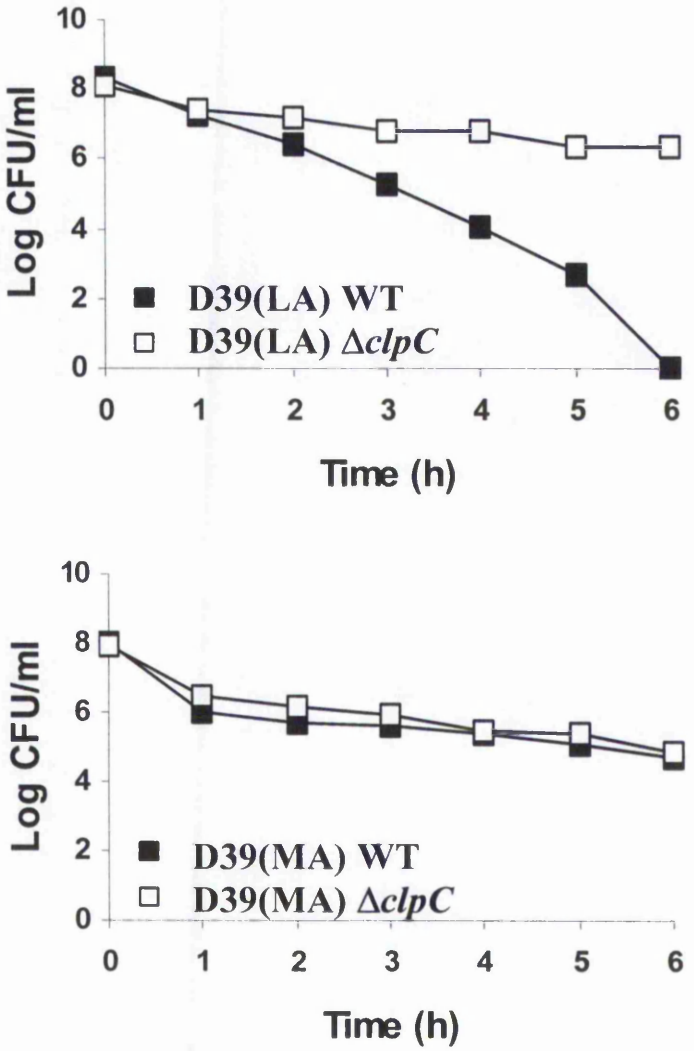


Figure 3.11- Penicillin-induced loss of viability assay for *clpC* mutants and wild types.

Penicillin-induced autolysis of Δ*clpC* mutants compared to their wild types. Cultures grown to OD ~ 0.3 were exposed to 0.1 μg/ml of benzylpenicillin and the viable bacteria were counted on BAB plates at 1h-intervals over a six-hour period.

3.3.4 Role of ClpC in the release of LytA and Ply

It is believed that the toxin pneumolysin is released upon lysis of pneumococcal cells by the influence of autolysin A (Tomasz, 1984). The decreased rate of autolysis in the Δ*clpC* mutant encouraged the investigation of the effect of the *clpC* deletion on LytA and Ply release. Proteins in the cellular fraction and those in the culture supernatant of the mouse-adapted strain D39(MA), which was chosen for the *in vivo* analysis, and its Δ*clpC* mutant grown in BHI for 11 hours at 37 °C were separated, transferred to nitrocellulose membranes and reacted with antisera to LytA and Ply. LytA was not detected in the supernatant of Δ*clpC* mutant but found in that of the wild type and in the cellular fraction of both strains. Ply was detected in the wild type and the mutant both in the cellular fraction and the supernatant. However, the amount of the toxin was less in the supernatant of the Δ*clpC* mutant (figure 3.12). This again suggests a role of ClpC in the autolysis of some strains and serotypes of *S. pneumoniae*.

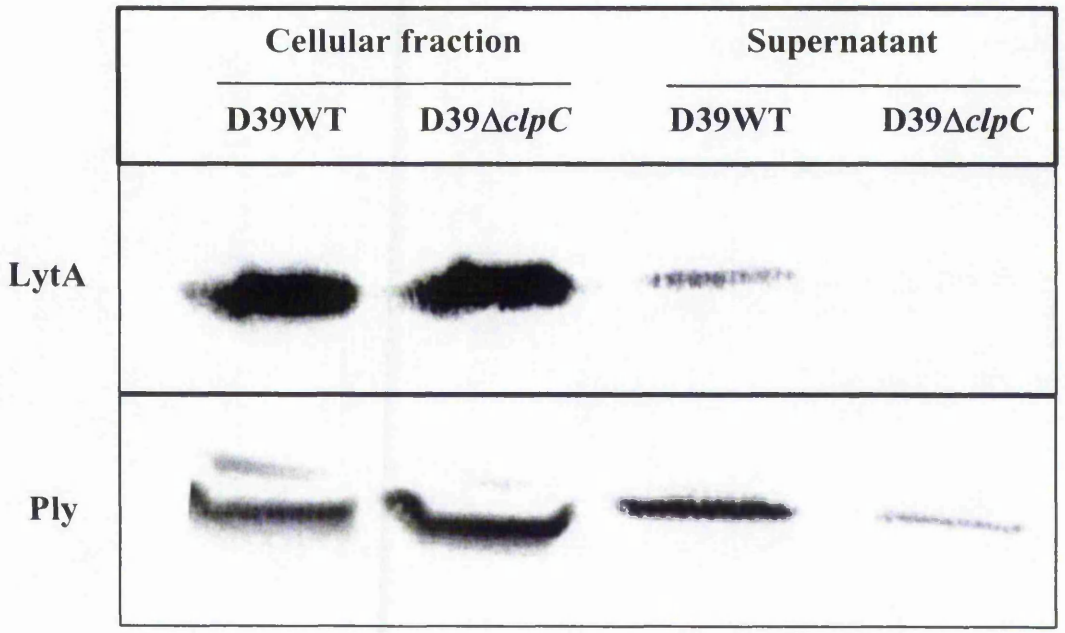


Figure 3.12- Western blot analysis of LytA and Ply in the D39(MA) WT and its Δ*clpC* mutant.

Total proteins in the cellular fraction and in the culture supernatant were separated by SDS-PAGE, transferred to nitrocellulose membranes and reacted with antisera specific to lytA and Ply. The amount of LytA and Ply produced was not affected by *clpC* deletion but the amount of Ply released to the supernatant upon cell lysis was much less in the mutant compared to that of the parent strain. In addition, no LytA was detected in the supernatant of D39 Δ*clpC* mutant.

3.3.5 Growth phenotype of ΔclpP mutants

The requirement of the pneumococcal ClpP for resistance to heat stress was reported before in the R6 and D39 strains (Chastanet *et al.*, 2001, Robertson *et al.*, 2002). To address the question of whether the ClpP protein is required for stress tolerance, the Δ*clpP* mutants generated in this study were assessed for altered phenotypes with respect to high and low temperatures. The role of ClpP in D39 and TIGR4 strains was investigated by growing equal numbers of viable cells of the wild types and their Δ*clpP* mutants in BHI at 30, 37 and 40 °C and recording the optical density at 600nm at time intervals (figure 3.13). The growth of both mutants at 37 °C was very much similar to that of their wild types. However, the Δ*clpP* mutants of both strains were unable to grow at 40 °C. The D39 Δ*clpP* mutant was less able to grow at 30 °C than its D39 parent strain while the Δ*clpP* mutant of TIGR4 and its wild type strain were identical in their growth at this lower temperature (figure 3.13). The reduced growth rate of D39 Δ*clpP* mutant at 30 °C has been reported previously (Robertson *et al.*, 2002).

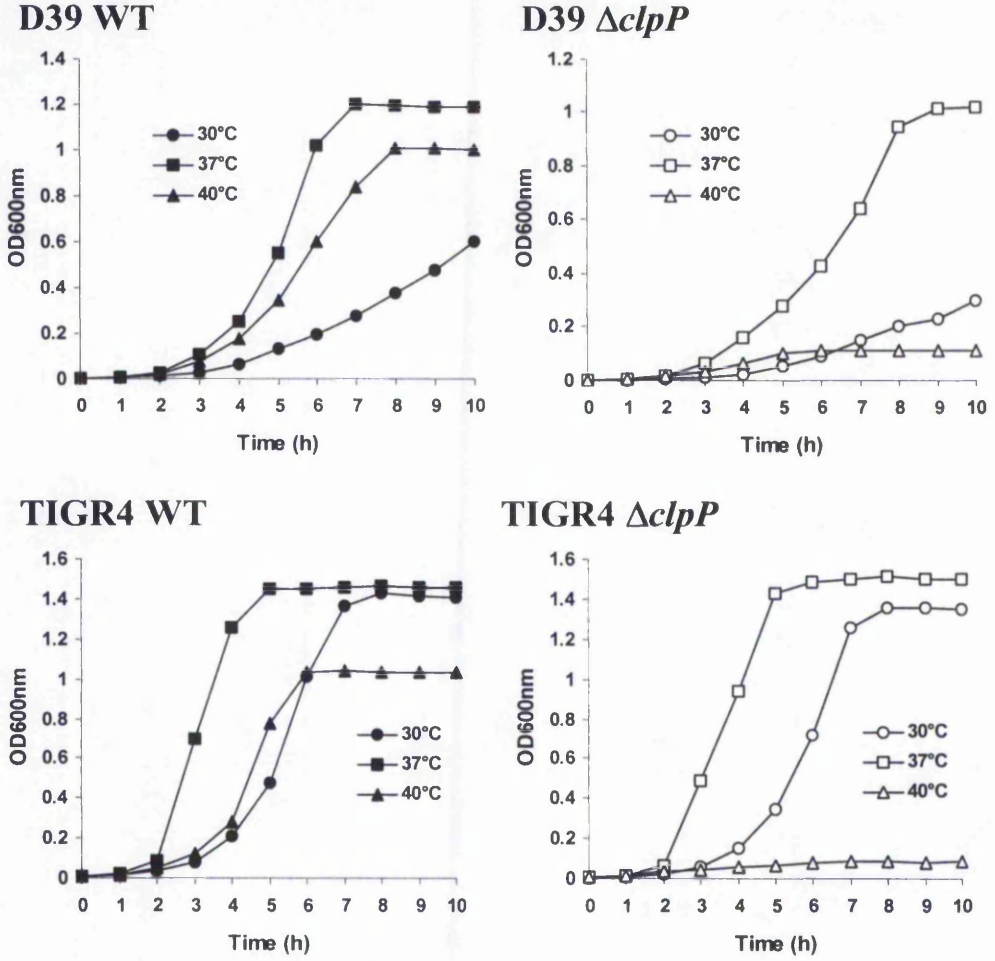


Figure 3.13- Representative growth curves of D39 and TIGR4 parent strains and their Δ*clpP* mutants at different temperatures.

10^6 CFU/ml of each strain was used to inoculate BHI prewarmed at the indicated temperatures and samples were taken at 1h-intervals to measure the OD_{600nm}. The Δ*clpP* mutants of both strains were impaired for growth at 40 °C and the growth of D39 Δ*clpP* mutant was less than that of the D39 wild type at 30 °C.

3.3.6 Response of mutants to oxidative stress

The proteolytic activity of ClpP is required by bacterial cells to prevent the accumulation of misfolded or damaged proteins resulting from different stress conditions (Frees and Ingmer, 1999, Kruger *et al.*, 2000). To study the role of ClpC and ClpP in the oxidative stress tolerance, the sensitivity of Δ*clpC* and Δ*clpP* mutants to hydrogen peroxide were compared to their parent strain D39(MA). As reported before (Robertson *et al.*, 2002), the Δ*clpP* mutant was more sensitive to peroxide than the wild type (figure 3.14). On the other hand, the response of Δ*clpC* mutant to peroxide was very much similar to that of the wild type (figure 3.14) suggesting that ClpC does not play a role in pneumococcal resistance to oxidative stress. The contribution of ClpC to pH tolerance was also investigated. No

differences were recorded between D39 WT or Δ*clpC* mutant in growth at different pHs suggesting that ClpC has no influence on the acidic tolerance of the pneumococcus (data not shown).

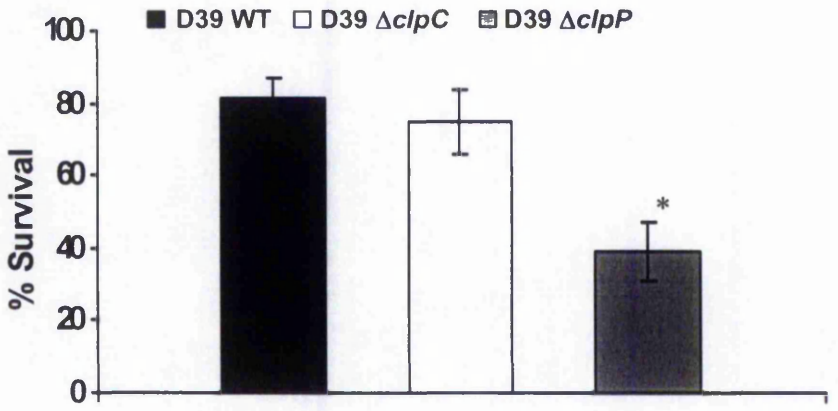


Figure 3.14- Peroxide sensitivity assay for D39 wild type and its Δ*clpC* and Δ*clpP* mutants.

40 mM of H_2O_2 were added to 1-ml aliquots of culture grown to OD_{600nm} of ~ 0.3 and left at room temperature for 15 min. Viable counts were performed on BAB plates before and after the addition of peroxide and the % survivals were calculated. Values expressed are the mean (SEM) of three independent experiments. *, p< 0.05 lower survival for Δ*clpP* mutant than wild-type.

3.4 *In vivo* analysis of mutants

3.4.1 ClpC mutants

The effect of ClpC on the rate of autolysis and thereby the release of pneumolysin suggested that ClpC might play a role in the virulence of the pneumococcus. Murine models of pneumonia and bacteraemia were used to investigate the contribution, if any, of ClpC to the pathogenesis of pneumococcal disease.

3.4.1.1 Intranasal infection

Groups of MF1 mice were infected intranasally as described in the Materials and Methods section. After administering the infectious dose (10^6 CFU/mouse), mice were observed and the development of symptoms was recorded over a period of two weeks. All mice infected with D39Δ*clpC* mutant succumbed to the infection at the same rate and had similar survival times to those infected with the D39 wild type (figure 3.15).

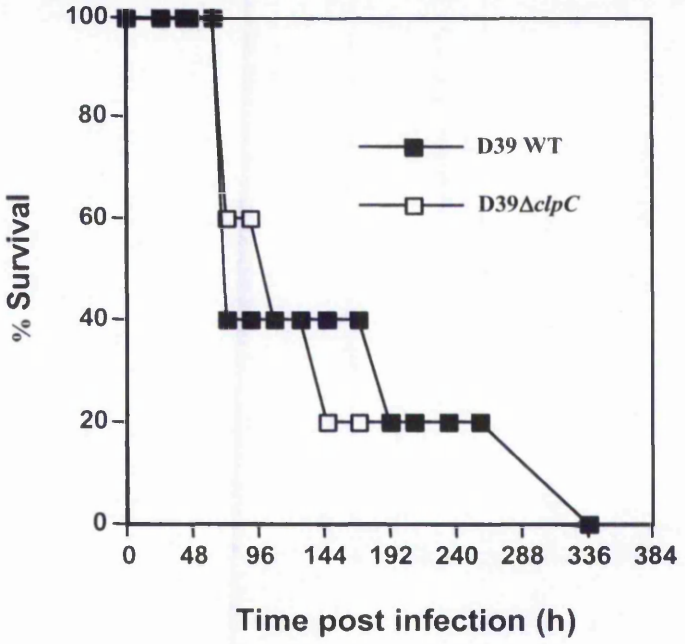


Figure 3.15- Survival of MF1 mice after intranasal infection with D39 WT or its *clpC* mutant.

Mice infected intranasally with 10^6 CFU/mouse of D39 or D39 Δ*clpC* (n=5). The results are given as the percentage of mice survival over time.

3.4.1.2 Bacteriological investigation

The bacterial loads of the D39Δ*clpC* mutant compared to the D39 wild type in the lung airways, lung tissue and blood after intranasal infection was studied at 0, 6, 12, 18, 24, and 48 h following challenge. Both the wild type and mutant strains were cleared from the lung airways (figure 3.16A). However, the bacterial counts of D39Δ*clpC* mutant in the lung tissue were significantly lower than those of the D39 wild type 12, 18, 24, and 48 hours post-infection (figure 3.16B). In the bloodstream, the wild type organism grew to levels of about 10^6 CFU/ml while the D39Δ*clpC* mutant caused a transient bacteraemia in mice 24 hours post-challenge and then started to clear after 48 h (figure 3.16C) where the blood concentration of Δ*clpC* mutant was significantly lower than that of the wild type.

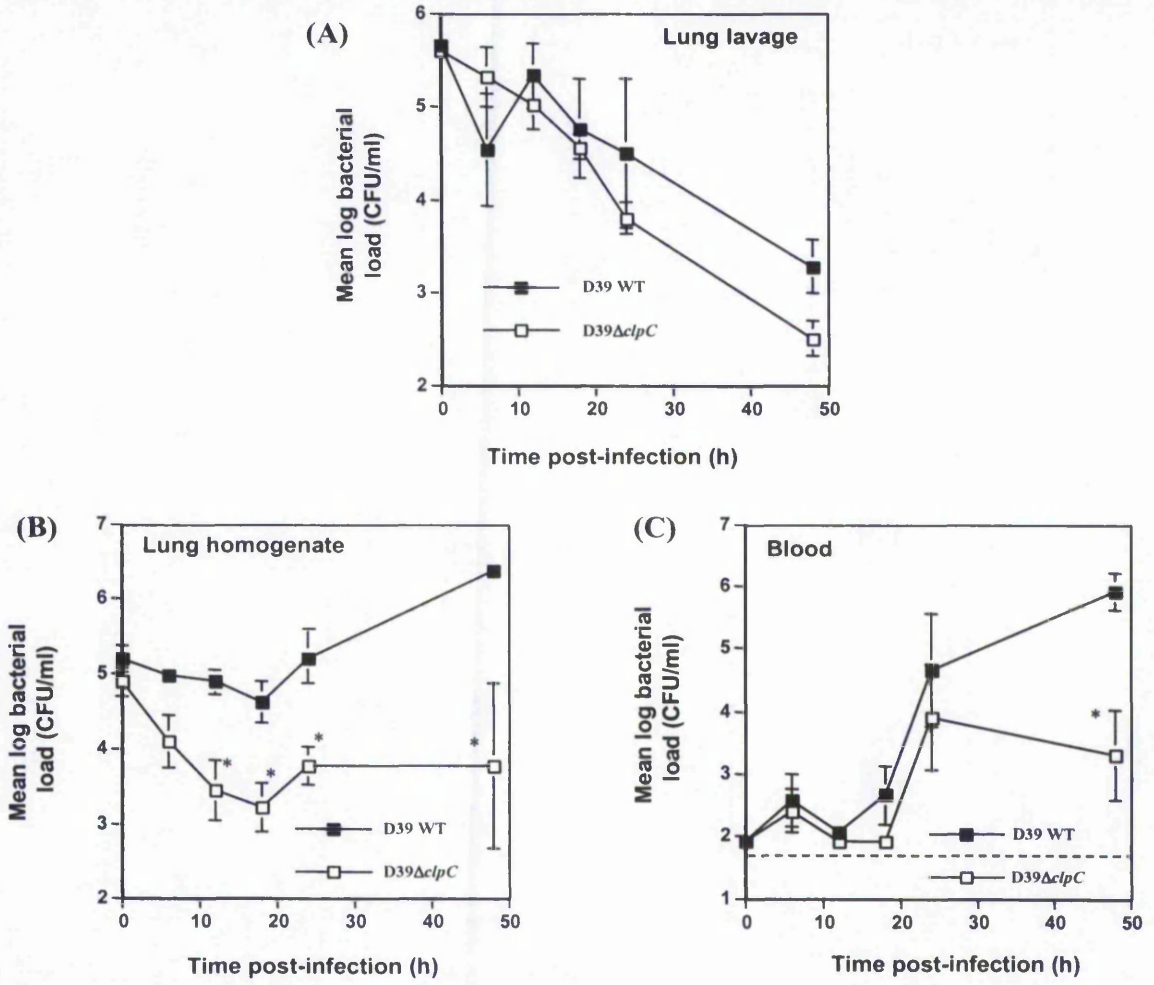


Figure 3.16- Bacterial loads of D39 WT or D39Δ*clpC* after intranasal infection.

Effect of ClpC on the virulence of the pneumococcus. (A) Bacterial counts in the lung airways. (B) Bacterial counts in the lung tissue. (C) Bacterial counts in blood. Bacterial counts were performed on BAB plates at different time points after intranasal infection with 10^6 CFU/mouse of each strain (n=5). Dashed line represents limit of detection of the assay. *, p <0.05 lower bacterial loads for D39Δ*clpC* mutant than for D39 wild type.

3.4.1.3 Bacterial loads in sequential blood sampling

Because the data in figure 3.16C are derived from groups of animals sacrificed at each time point, it is not possible to determine the extent of transient bacteraemia in individual animals. To measure the levels of transient bacteraemia caused by the D39Δ*clpC* mutant organisms, the number of bacteria in blood of individual mice was followed at different time points after intranasal infection by counting bacteria in blood samples taken by sequential tail bleeding (figure 3.17). Again, the mutant organisms grew to levels of about 10^4 CFU/ml 24 h post-challenge and then cleared after 48 h while the wild type organisms grew dramatically (figure 3.17). Results from the experiment in figure 3.16C are not significantly different from those in figure 3.17.

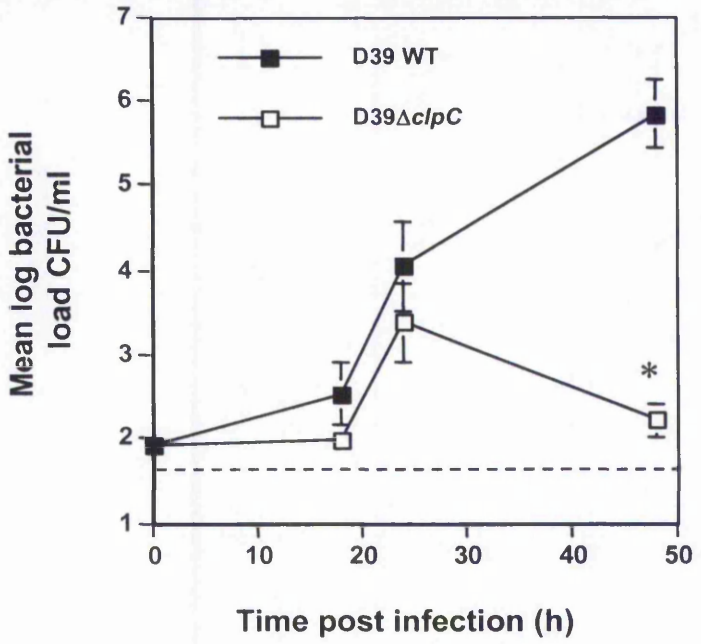


Figure 3.17- Bacterial count in blood taken by tail bleeding after intranasal infection.

Bacterial counts were performed on BAB plates at different time points after intranasal infection with 10^6 CFU/mouse of each strain (n=5). Dashed line represents limit of detection of the assay. *, p <0.05 lower bacterial loads for D39Δ*clpC* mutant than for D39 wild type.

3.4.1.4 Intravenous infection

To determine whether the ClpC role was specific for the lung, wild type and mutant organisms were injected directly into the bloodstream to by-pass the respiratory tract. MF1 mice were given 1×10^5 CFU/mouse and both survival of mice and bacterial loads in blood were determined. There were no statistically significant differences between the D39 Δ*clpC* mutant and its parent strain in the ability to cause death in mice when given intravenously. All mice in both groups succumbed to the infection by 34 h post challenge. The median survival time for D39-infected mice was 24 hours and that for D39Δ*clpC*-infected ones was 31 hours.

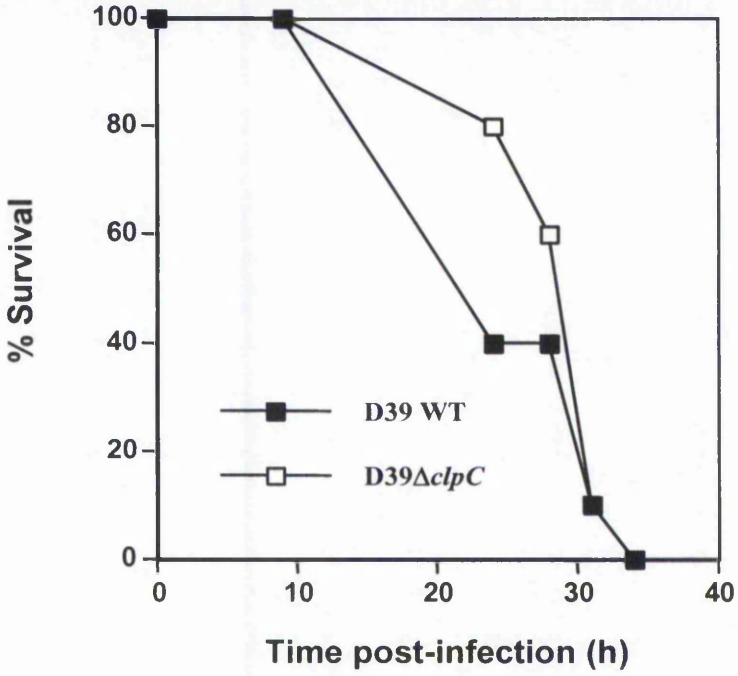


Figure 3.18- Survival of MF1 mice after intravenous infection with D39 WT or its Δ*clpC* mutant.

Mice infected intravenously via lateral tail veins with 10^5 CFU/mouse of D39 or D39 Δ*clpC* (n=5). The results are given as the percentage of mice survival over time.

The bacterial loads of both strains in blood were also followed over time. Blood samples were taken by sequential tail bleeding at 0, 12, 24 and 31 hours after intravenous infection and the bacterial counts were determined by plating serial 10-fold dilutions onto BAB plates. Both the wild type and mutant organisms grew to a level of more than 10^6 CFU/ml by 12 hours post-challenge and grew dramatically thereafter causing the death of 60% of the D39-infected mice and 20% of D39Δ*clpC*-infected mice by 24 hours. However, 80% of mice in both groups died by 31 hours and no mice in either group survived the challenge more than 34 hours post-infection. The log CFU/ml of both strains at these time points is given in table 3.1. There were no statistically significant differences at 0 h and 12 h time points and the death of mice 24 h and 31 h post-challenge did not allow any more statistical analysis.

Table 3.1- Bacterial counts in blood after intravenous infection with D39 WT or D39Δ*clpC* mutant.

Mouse no.	Bacterial loads at indicated time (log_{10} CFU/ml)							
	0h		12h		24h		31h	
	D39	Δ*clpC*	D39	Δ*clpC*	D39	Δ*clpC*	D39	Δ*clpC*
1	5.06	4.97	6.49	6.28	9.04	--	--	--
2	4.99	4.95	7.11	6.34	--	7.77	--	--
3	4.86	5.08	7.25	6.18	--	7.86	--	--
4	5.08	5.00	7.51	6.48	--	8.25	--	--
5	4.72	4.83	6.34	6.28	7.49	6.86	9.63	9.39

Mice were bled 0 h, 12 h, 24 h and 31 h after intravenous infection and the viable count was determined by plating on BAB plates. --, Dead mouse.

3.4.1.5 Histological analysis

Tissue histology of lungs infected with wild type and those infected with D39Δ*clpC* mutant organisms was examined in order to determine the host response to both strains. Mice were sacrificed 48 h after intranasal challenge and their lungs removed, fixed, sectioned and stained. Sections were examined for the influx of inflammatory cells into the lungs by light microscopy and the number of pleurisy, areas at the pleural interface that were swollen and congested with recruited inflammatory cells and also the number of perivascular alterations were scored. The results are shown in table 3.2. Statistical analysis of these scores revealed no significant differences between lungs infected with the wild type organism and those infected with the mutant strain suggesting that the host response to D39 Δ*clpC* mutant was not altered and that the mutant organisms caused the same effect on lungs of infected mice as that caused by the wild type.

Table 3.2- Histological scores of lungs from mice infected with wild type D39 or Δ*clpC* mutant organisms.

Mouse no.	D39 WT-infected lungs			D39 Δ*clpC*-infected lungs		
	Pleurisy	Perivascular alterations	Total	Pleurisy	Perivascular alterations	Total
1	10	12	**22**	2	6	**8**
2	0	25	**25**	6	22	**28**
3	2	1	**3**	2	10	**12**
4	0	0	**0**	1	1	**2**
5	4	42	**46**	5	0	**5**

3.4.1.6 Analysis of TIGR4Δ*clpC* mutant

To examine the role of ClpC in the virulence of other pneumococcal strains, a Δ*clpC* mutant of the serotype 4 strain TIGR4 was constructed and compared to its parent strain for the ability to cause disease in mice. Groups of MF1 mice (five each) were infected intranasally with 1×10^5 CFU/mouse of either the wild type or the mutant organisms and the symptoms of pneumococcal disease were followed over a period of four days. Mice, which reached the moribund state, were humanely sacrificed and their survival times recorded. Mice infected with the TIGR4 wild type strain succumbed to the infection slightly faster than those infected with the mutant strain. Statistical analysis revealed that the median survival time of TIGR4 wild type-infected mice was 48 hours and that of the mutant-infected mice was 72 hours, however, this difference was not significant. The results are represented in figure 3.19.

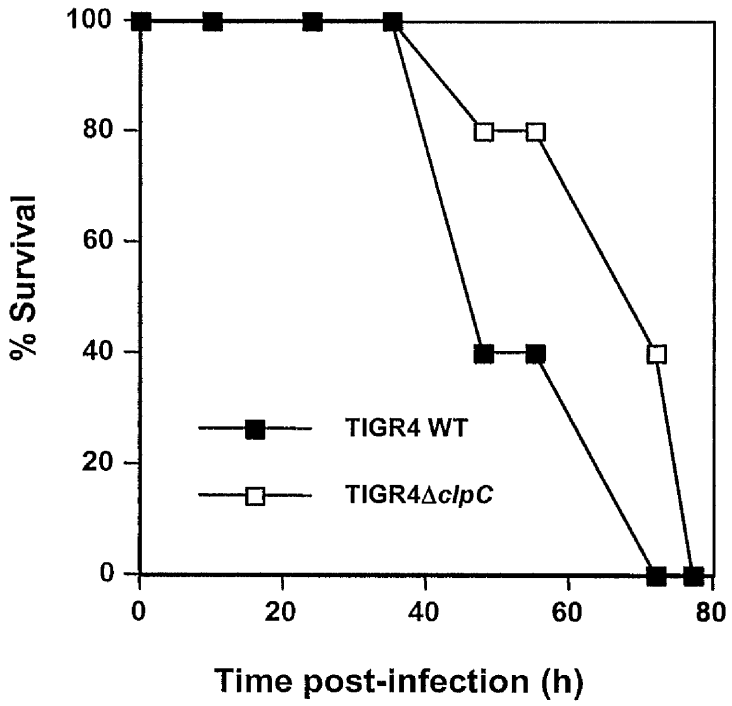


Figure 3.19- Survival of MF1 mice after intranasal infection with TIGR4 WT or its Δ*clpC* mutant.

Mice infected intranasally with 10^5 CFU/mouse of TIGR4 WT or TIGR4 Δ*clpC* (n=5). The results are given as the percentage of mice survival over time.

3.4.2 ClpP mutants

ClpP negative mutants of the serotype 2 strain D39 were reported to be attenuated in a murine intratracheal (Robertson *et al.*, 2002) and intraperitoneal (Kwon *et al.*, 2003)

models of infection. In this study, the virulence of Δ*clpP* mutants of D39 and TIGR4 was tested in our pneumonia model. None of the mice in the D39-infected group survived (median survival time: 51 h) while D39 Δ*clpP* infected mice did not show any symptoms of illness (median survival time: 336 h) and survived until the end of the experiment (figure 3.20A). The virulence of TIGR4 Δ*clpP* mutant was significantly reduced (median survival time: 54 h) compared to that of the TIGR4 wild type (median survival time: 28 h) although no TIGR4 Δ*clpP*-infected mice survived the challenge (figure 3.20B).

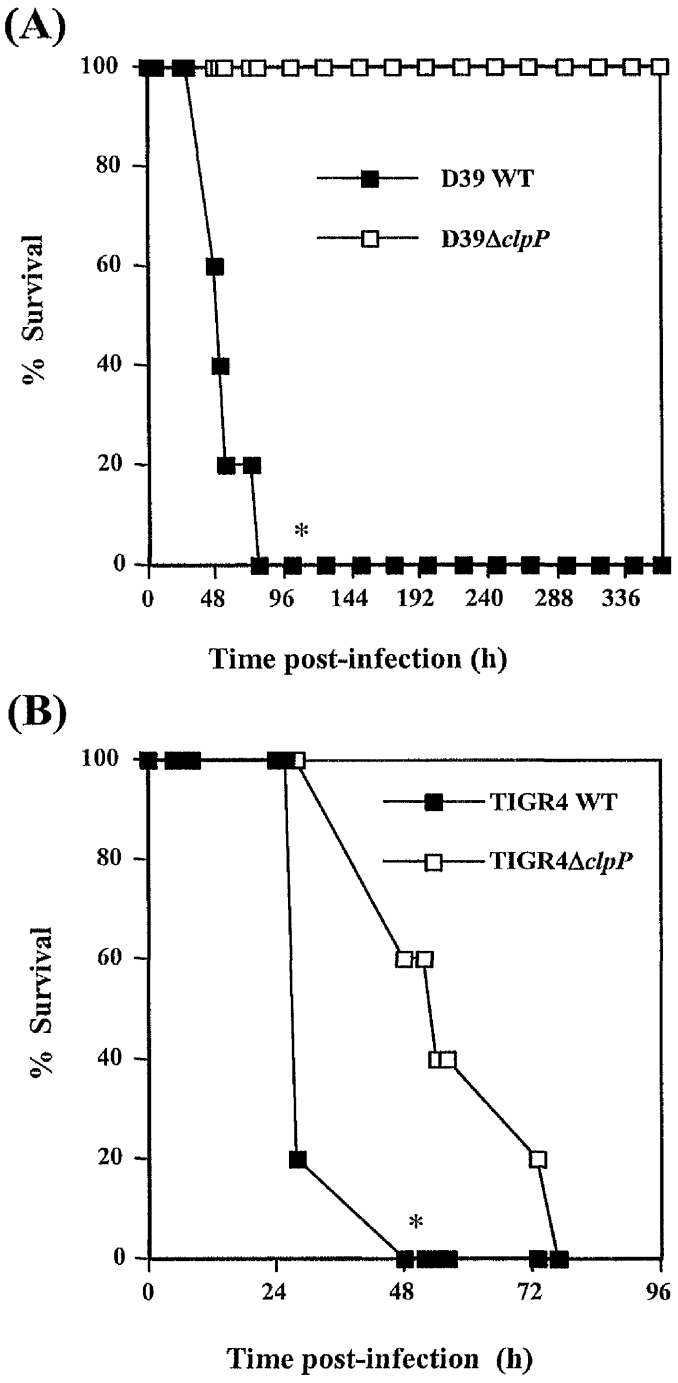


Figure 3.20- Effect of *clpP* deletion on the virulence of pneumococcal D39 and TIGR4 strains.

Survival of MF1 mice infected intranasally with 10^6 CFU/mouse (n=5) of D39 or D39 Δ*clpP* in (A) and TIGR4 or TIGR4 Δ*clpP* mutant in (B). The D39 Δ*clpP* mutant was completely avirulent while the TIGR4 Δ*clpP* mutant was less virulent than the TIGR4 WT. *, $p<0.05$ shorter survival times for D39 and TIGR4 wild types than for Δ*clpP* mutants.

In addition, the number of bacteria in the lung airways, lung tissue and blood of mice infected with either D39 wild type or the D39Δ*clpP* mutant was investigated at 24h and 48h post-infection with 1×10^5 CFU/mouse. The bacterial number in the lung tissue and

blood of the D39Δ*clpP* mutant-infected mice was significantly less than that in mice infected with D39 wild type 24 h and 48 h post-challenge (figure 3.21). There was no significant difference in the number of bacteria in the lung lavage at either time points (figure 3.21).

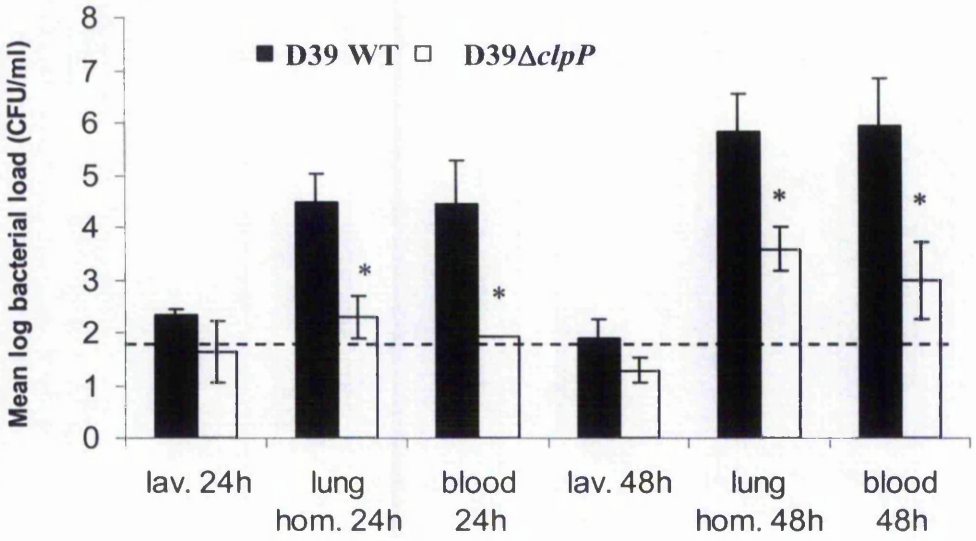


Figure 3.21- Bacterial loads of D39 wild type and its Δ*clpP* mutant.

Viable counts of D39 wild type and its Δ*clpP* mutant in the lung lavage, lung tissue homogenate, and blood 24 h and 48 h after intranasal infection with 10^6 CFU/mouse of each strain (n=5). Dashed line represents the limit of detection of the assay for blood samples. *, $p<0.05$ lower bacterial loads for Δ*clpP* mutant than for D39 parent strain.

3.5 Confirmation of the role of ClpC

Recently, complementation of the *clpP* mutation has been reported by Robertson and co-workers (Robertson *et al.*, 2002). This report stated that the growth of the Δ*clpP*/*clpP*$^+$ merodiploids was indistinguishable from that of the R6 and D39 parent strains on different media at 30, 37, and 40 °C and, therefore, concluded that the loss of *clpP* function alone is responsible for all of the observed growth defects at reduced and elevated temperatures. Because ClpP data in the study presented in this chapter agree with the previous reports, only confirmation of the ClpC role has been sought.

In order to confirm that the construction of the *clpC* mutation did not have a polar effect on expression of downstream genes, the expression levels of these genes were examined using

microarrays. Genes downstream from *clpC* include the response regulator and the histidine kinase genes of the two-component system number six (SP2193 and SP2192, respectively), a conserved hypothetical protein (SP2191) and the choline binding protein A gene (SP2190). Organization of the *clpC* gene as published on the TIGR website (http://www.tigr.org/) is shown in figure 3.22.

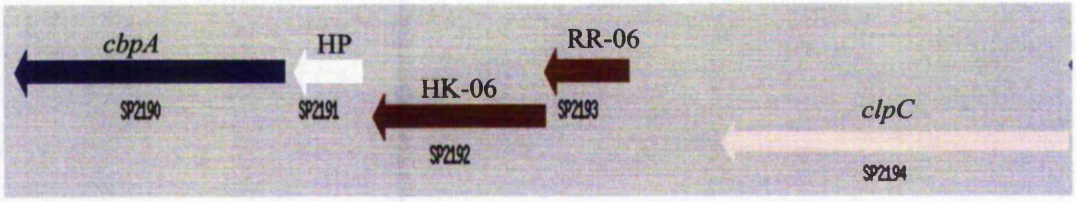


Figure 3.22- Organization of the *clpC* gene locus.

Adapted from TIGR website at (http://www.tigr.org/). RR-06, Response regulator of the two-component system number 06. HK-06, Histidine kinase of the two-component system number 06. HP, hypothetical protein. CbpA, Choline binding protein A.

The *clpC* mutation was also reverted by transforming the D39Δ*clpC* mutant with a wild type copy of *clpC* and screening the transformation reaction for colonies that lost the erythromycin resistance. The revertant strain was then analyzed both *in vitro* and *in vivo*.

3.5.1 Microarray analysis

The pneumococcal genome microarray slides (2nd version) designed at the Pathogen Functional Genomics Resource Centre at TIGR (http://www.pfgrc.tigr.org/) were used in this analysis. The full genome array consists of amplicons representing segments of 2131 ORFs from *S. pneumoniae* reference strain TIGR4 spotted in quadruplicate on glass slides. Also, the array contains an additional 563 ORFs from strains R6 (164) and G54 (399).

3.5.1.1 RNA preparation, analysis and hybridization

The bacterial RNA was extracted from cultures grown in BHI at 37 °C to an OD_{600nm} of 0.6 using RNeasy® Midi (Qiagen) columns according to the manufacturer's protocol. Preparation of RNA under these conditions yielded 2.3 µg/µl of wild type RNA and 1.8 µg/µl of the mutant RNA. The concentrations of RNA were determined using NanoDrop® ND-1000 UV/Vis spectrophotometer (NanoDrop® Technologies, USA). Because it is essential for the microarray analysis to have a high quality RNA, the RNA samples were also tested for integrity and concentration using the agilent technology. Figure 3.23 shows

the results of this analysis, which clearly indicates the purity, and integrity of RNA samples.

The RNA was reverse transcribed and labelled with Cy3 or Cy5 probes (Amersham Pharmacia) by using the aminoallyl labelling method. Denatured probes were then hybridized to the glass slides and the hybridized slides were scanned using a laser scanner. The microarray data was analyzed to get the global gene expression levels.

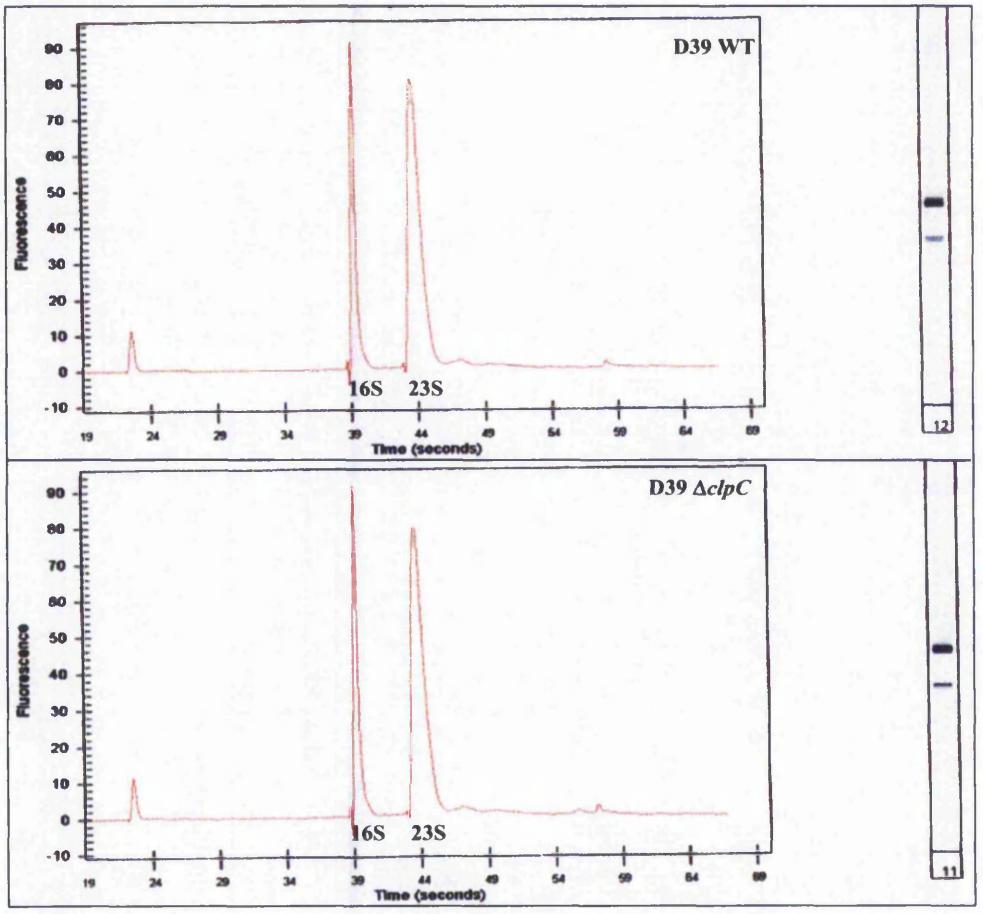


Figure 3.23- Analysis of RNA samples prepared from D39 WT and D39 Δ*clpC* mutant.

The RNA was prepared from cultures grown in BHI at 37 °C to OD600 ~ 0.6 using Qiagen RNeasy® Midi Kit. RNA samples were checked for integrity and concentration using RNA 6000 Nano assay with the Agilent 2100 Bioanalyzer.

Since the labelling efficiencies of Cy3 and Cy5 can be different, as reported previously (Ye *et al.*, 2001), a dye-swapping experiment was performed to eliminate any artificial effect in labelling, as well as to provide an independent means to verify the results for the first chip. The data from the dye-swapping experiments in this study agreed very well, indicating that the two dyes labelled consistently. Statistically significant differences were defined as

those with a *t*-test *P*-value of less than 0.0001 and a ratio change threshold of at least 2 standard deviations compared to the median ratio for each strain.

3.5.1.2 Array results

The scatter plot in figure 3.24 represents the relationship between the average spot intensities of the wild-type control cells (X-axis) and D39Δ*clpC* mutant cells (Y-axis). This simple procedure provided a general idea of the data and confirmed that most of the gene spots fell along the diagonal lines and were similarly labelled. The spots that were not on the diagonal lines were candidates for genes with changes in their expression levels.

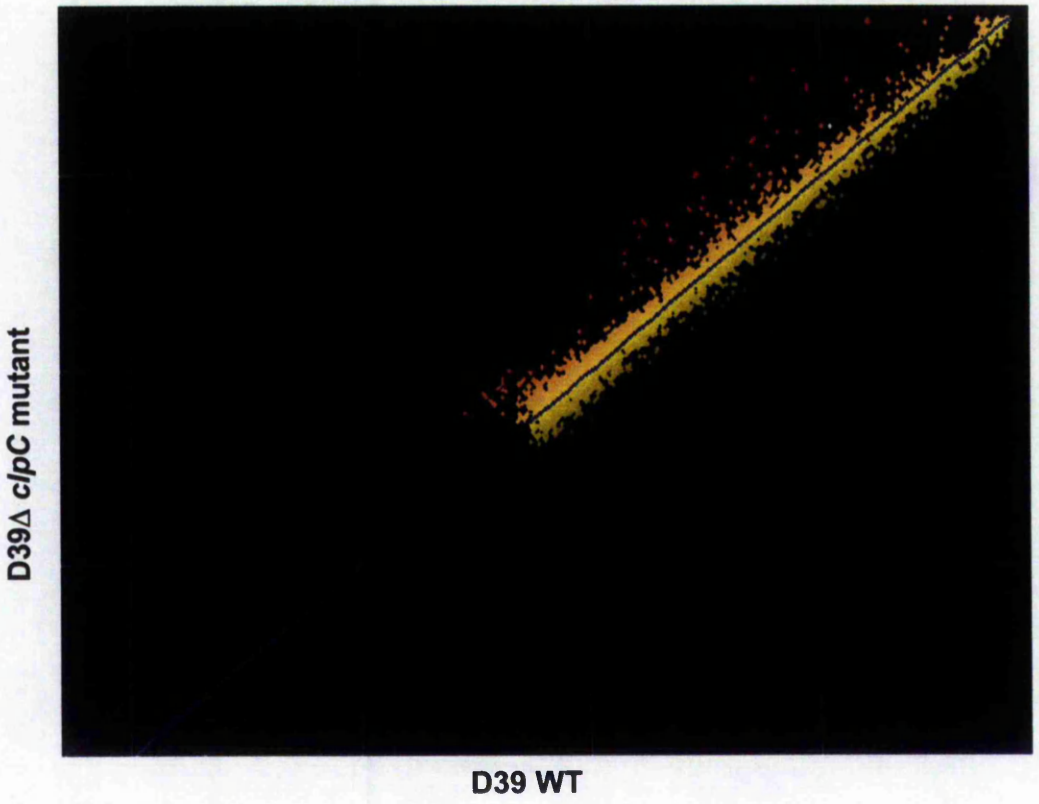


Figure 3.24- Representative scatter plot comparing gene expression in the D39Δ*clpC* mutant to that of the wild type D39.

Labelled cDNA samples were hybridized to the probes on the array and scanned. Data were analyzed using the Genespring™ software. The plot illustrates the spot intensities of all genes detected for the wild type on the X-axis and the mutant on the Y-axis. Yellow spots (within the diagonal lines) indicate similar expression level, red spots (above the diagonal lines) indicate up-regulated genes and blue spots (below the diagonal lines) indicate down-regulated genes in the mutant strain compared to wild type strain.

Analysis of microarray data revealed that the expression of genes downstream from *clpC* was not affected by the disruption of *clpC* gene (table 3.3). Analysis of the levels of RNA

in the region showed that the only transcript level significantly altered in this region is that of ClpC.

Table 3.3- Levels of expression of *clpC* and downstream genes in the D39Δ*clpC* mutant compared to the wild type as determined by microarray.

Gene[a]	Spot Intensity ratio[b]	*p*-value[c]	Annotation[d]
SP2194	0.25	4.7E-05	ATP-dependent Clp protease, ATP-binding subunit
SP2193	1.23	0.254	DNA-binding response regulator
SP2192	1.55	0.003	Sensor histidine kinase
SP2191	1.25	0.033	Conserved hypothetical protein
SP2190	1.20	0.269	Choline binding protein A

***a*, Gene designation of microarray ORFs which match the TIGR designations (http://www.tigr.org). *b*, Mutant/wild type intensity ratios as determined in microarray experiments. *c*, *p*-value represents the mean *p*-value calculated from individual *t*-tests of intensity changes between the wild type and mutant. A *p*-value of less than 0.0001 is considered significant for up- or down-regulated genes. *d*, Annotations as published in TIGR4 genome (http://www.tigr.org).**

3.5.1.3 Regulation of gene expression by ClpC

Besides confirming that *clpC* mutation was done in a proper way with no downstream polar effects, the array results also identified some up- or down-regulated genes as a result of ClpC loss of function. Analysis of data revealed the down regulation of an operon of three genes (SP0875-77) in addition to the absence of ClpC transcripts in the mutant. The down-regulated genes are given in table 3.4.

Table 3.4- Genes down-regulated as a result of *clpC* deletion.

Gene	Spot Intensity ratio	*p*-value	Annotation
SP0875	0.07	6.7E-6	Lactose phosphotransferase system repressor
SP0876	0.09	6.8E-5	1-phosphofructokinase, putative
SP0877	0.14	2.8E-5	PTS system, fructose specific IIABC components

As expected, the *clpC* gene was highly down regulated in the D39Δ*clpC* mutant strain. In fact, examination of the individual spot intensities revealed that the fluorescence signals of *clpC* spots in case of D39Δ*clpC* mutant were identical to the background signals suggesting the absence of ClpC transcripts in the mutant RNA. The microarray analysis also revealed the up regulation of twenty other genes. These genes are shown in table 3.5.

Table 3.5- Genes up-regulated as a result of *clpC* deletion.

Gene[a]	Spot Intensity ratio[b]	*p*-value[c]	Annotation[d]
SP0285	2.2	4.1E-6	Alcohol dehydrogenase, zinc containing
SP0368	3.2	5.0E-4	Cell wall surface anchor family protein
SP0577	6.1	5.1E-5	PTS system, beta-glucosidase-specific IIABC components
SP0578	3.7	2.6E-5	6-phospho-beta-glucosidase
SP0648	2.3	1.1E-3	Beta-galactosidase
SP0706	2.2	1.8E-3	Hypothetical protein
SP1185	3.1	1.5E-4	PTS system, lactose-specific IIBC component
SP1190	2.8	1.9E-4	Tagatose 1,6-diphosphate aldolase
SP1191	2.2	3.7E-4	Tagatose-6-phosphate kinase
SP1471	3.2	1.2E-3	Oxidoreductase, putative
SP1472	3.4	2.3E-4	Oxidoreductase, putative
SP1802	3.4	5.9E-5	Hypothetical protein
SP1803	2.8	6.5E-4	Conserved hypothetical protein
SP2026	2.2	6.3E-5	Alcohol dehydrogenase, iron-containing
SP0057	2.1	1.2E-3	Beta-N-acetylhexosaminidase
SP0061	3.3	5.6E-4	PTS system, IIB component
SP0063	4.0	3.3E-7	PTS system, IID component
SP0064	4.2	1.5E-5	PTS system, IIA component
SP0066	2.4	4.7E-4	Aldose 1-epimerase
SP0092	2.3	5.6E-4	ABC transporter, substrate-binding protein

***a*, Gene designation of microarray ORFs which match the TIGR designations (http://www.tigr.org). *b*, Mutant/wild type intensity ratios as determined in microarray experiments. *c*, *p*-value represents the mean *p*-value calculated from individual *t*-tests of intensity changes between the wild type and mutant. A *p*-value of less than 0.0001 is considered significant for up- or down-regulated genes. *d*, Annotations as published in TIGR4 genome (http://www.tigr.org/).**

3.5.2 *In vitro* analysis of the revertant strain

To confirm that the phenotype observed for D39Δ*clpC* mutant strain is due to the mutation in *clpC* gene and not due to other mutations introduced elsewhere into the chromosome, the *clpC* mutation was repaired by transformation with the wild type gene. Following transformation of D39Δ*clpC* with the wild type *clpC* gene, several pneumococcal colonies that had lost the erythromycin resistance phenotype were recovered. Analysis using PCR and nucleotide sequencing revealed the existence of wild type *clpC* allele in the revertant strain. The *in vitro* growth of the revertant strain, D39 *clpC*$^{+}$, compared to that of the wild type and the D39Δ*clpC* mutant strains in BHI media at 37 °C was studied to confirm that the decreased autolysis phenotype of D39Δ*clpC* mutant is due to the disruption of *clpC*. The growth of the revertant strain was identical to that of the wild type as measured both by optical density at 600nm (OD_{600nm}) and by counting the viable bacteria. The revertant

strain grew at the same rate as the wild type to about 10^9 CFU/ml after 7 hours and then underwent autolysis after reaching the stationary phase of growth to reach a viable count of less than 10^4 CFU/ml after 12 hours in a way that is identical to that of the wild type (figure 3.25). On the other hand, the D39Δ*clpC* mutant strain also grew to 10^9 CFU/ml but did not undergo autolysis. The viable count of the mutant remained at 10^9 CFU/ml after 12 hours. These data suggest that the observed phenotype of the D39Δ*clpC* mutant, decreased rate of autolysis, is solely due to the loss of function of ClpC and not due to any mutations elsewhere in the chromosome.

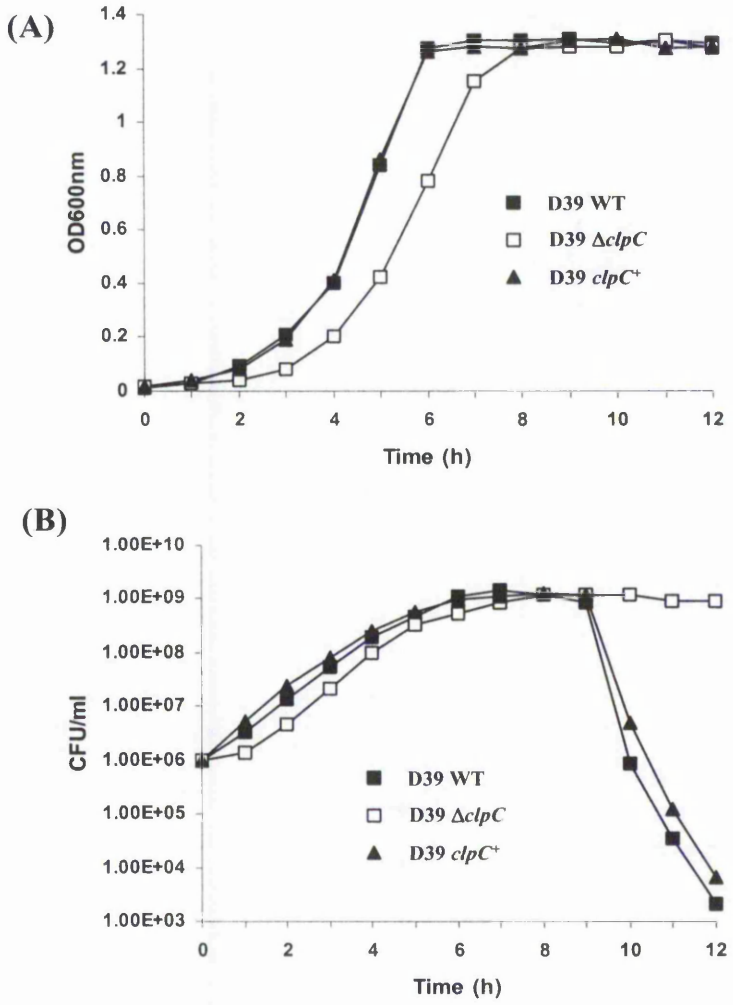


Figure 3.25- Growth of the *clpC* revertant strain, D39 *clpC*$^+$, compared to the wild type D39 and the D39Δ*clpC* mutant.

Representative graphs of *in vitro* growth of D39 WT, D39 Δ*clpC* mutant and D39 *clpC*$^+$ at 37 °C. 10^6 CFU/ml of each strain was used to inoculate BHI prewarmed at the indicated temperature and samples were taken at 1h-intervals to measure the optical density at 600nm (A) and the viable bacterial count (B). The rate of growth and autolysis of D39 *clpC*$^+$ strain was almost identical to that of the wild type.

3.5.3 In vivo analysis of the revertant strain

The *in vivo* phenotype of the revertant strain was also studied to confirm that the transient bacteraemia caused by D39Δ*clpC* mutant in mice is entirely due to *clpC* disruption. The bacterial loads of the revertant strain in tail bleed were very similar to that of the wild type (table 3.6).

Table 3.6- Bacterial counts in blood samples taken by tail bleeding after intranasal infection.

Mouse no.	log_{10} bacterial loads (CFU/ml) at indicated time (h) post-infection											
	D39 WT				D39Δ*clpC*				D39 *clpC*+			
	0	18	24	48	0	18	24	48	0	18	24	48
1	NF	2.20	4.57	7.70*	NF	NF	NF	2.96	NF	2.39	4.56	7.28*
2	NF	2.21	2.87	4.72	NF	2.76	4.98	3.02	NF	NF	NF	6.43
3	NF	4.11	5.79	7.86	NF	2.40	3.87	NF	NF	4.88	6.32	8.23*
4	NF	3.22	5.16	6.43	NF	2.22	4.99	2.52	NF	4.90	6.41	7.59*
5	NF	3.70	5.40	6.62	NF	NF	3.77	NF	NF	3.57	4.96	6.36

Values given are log_{10} CFU/ml. NF, None found. *, Mouse sacrificed 44 h post-infection due to reaching moribund state.

Finally to test for the presence of suppressor mutations that compensate for the lack of ClpC, organisms recovered from the blood of animals infected with D39Δ*clpC* and showing signs of disease were used to re-challenge a further group of animals. The phenotype observed using serial tail bleeding was very similar to the original D39Δ*clpC* (table 3.7). The mutant organisms grew in blood to a viable count of about 10^4 CFU/ml by 48 hours after intranasal infection and then the count declined to about 10^3 CFU/ml by 72 hours. These data clearly indicate that the virulence profile of the D39Δ*clpC* is not affected by serial passage. The results of the gene repair and the serial passage suggest that the phenotype observed is due to the lack of ClpC and not other mutational effects.

Table 3.7- Bacterial count in blood taken by tail bleeding from a second infection of mice with D39Δ*clpC* mutant.

Mouse	Log_{10} bacterial loads at indicated time post-infection				
no.	0h	18h	24h	48h	72h
1	NF	NF	1.92	4.44	3.60
2	NF	NF	NF	4.28	--
3	NF	NF	NF	3.41	4.20
4	NF	NF	2.22	4.92	4.05
5	NF	1.92	NF	3.37	3.65
6	NF	NF	NF	2.52	NF

Mice were infected intranasally with 10^6 CFU/mouse (n=5) and the number of bacteria in sequential tail bleeds was determined. Values given are log_{10} CFU/ml. NF, None found. --, None detected (dead mouse)

Chapter 3 Discussion

Heat shock proteins and stress response

Pathogenic 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, some of which are highly stressful. These include nutrient limitation, changes in temperature, humidity, and osmolarity. Moreover, the pathogen is exposed to natural host resistance mechanisms such as phagocytosis by specialized phagocytes (Kaufmann, 1998). Once engulfed by phagocytes, the pathogen is confronted with reactive oxygen and nitrogen intermediates. To protect itself against the host, the pathogen activates various evasion mechanisms including the synthesis of heat shock proteins (HSPs). The importance of HSPs for pathogen survival in this stressful environment was illustrated by experiments with a mutant of the intracellular pathogen *Salmonella enterica* serovar Typhimurium, which overexpresses HSPs (Buchmeier and Heffron, 1990). This mutant was shown to be resistant to a variety of oxidizing agents and heat. On the contrary, mutants of *S. typhimurium* with specific HSP gene defects are highly susceptible to killing by activated macrophages and also express decreased virulence *in vivo* (Fields *et al.*, 1986, Johnson *et al.*, 1991).

Heat shock proteins (HSPs) were identified as proteins whose synthesis was enhanced by stresses such as an increase in temperature. HSPs can be classified into Hsp100, Hsp70, Hsp60 and small Hsp families according to their molecular weight and are universally present in prokaryotes and eukaryotes (Craig *et al.*, 1993, Hendrick and Hartl, 1993). Reversing polypeptide unfolding and preventing protein aggregation are major functions of HSPs, especially under stress (Craig *et al.*, 1993, Becker and Craig, 1994, Hartl, 1996). In non-stressed cells HSPs are present in low concentrations while in stressed cells they accumulate at high levels. In *Escherichia coli*, for example, the hsp60 homolog GroEL, which was first described by Hendrix *et al* (Hendrix, 1979), represents 1 to 2 % of the total protein content under normal conditions. Under stress conditions, however, its concentration is increased four- to fivefold (Shinnick, 1991). Although HSPs do not accumulate to such high levels in eukaryotes, their concentrations are also increased after heat shock (Welch, 1990, Shinnick, 1991).

While the importance of HSPs for survival in the host holds true for a diversity of intracellular pathogens, HSP induction seems to be less appropriate for some other microbes, including *Listeria monocytogenes*. The ability of *L. monocytogenes* to survive in macrophages in the absence of increased HSP synthesis could be explained by the potential

of this pathogen to evade the stressful endosomal environment at an early phase after phagocytosis and therefore can grow in macrophages without the support of proteins induced by environmental stresses (Hanawa *et al.*, 1995). Thus, the impact of HSPs on microbial survival in the host varies in different infections.

One of the mechanisms that bacteria have developed to survive in unfavourable conditions is the induction of targeted intracellular proteolysis performed by energy-dependent proteases such as Clp ATP-dependent proteases (Parsell and Lindquist, 1993, Gottesman, 1996, Fedhila *et al.*, 2002). The Clp proteins constitute a family of stress proteins, which are highly conserved and universal. Members of this family have been shown to exist in many prokaryotes such as *Escherichia coli* (Squires *et al.*, 1991), *Streptococcus mutans* (Shiroza and Kuramitsu, 1988) and *Streptococcus pneumoniae* (Pearce *et al.*, 1993) and in several eukaryotes, including *Saccharomyces cerevisiae* (Parsell *et al.*, 1991) and *Trypanosoma brucei* (Gottesman *et al.*, 1990b).

HSPs as molecular chaperones or proteases

The folding of many newly synthesized proteins in the cell depends on a set of conserved proteins known as molecular chaperones. These prevent the formation of misfolded protein structures, both under normal conditions and when cells are exposed to stresses such as high temperature. Although many chaperones are classified as stress proteins, they also carry out essential functions under normal physiological conditions. Some HSPs temporarily stabilize unfolded or partially folded proteins and thus promote the generation of the correct tertiary structure (Becker and Craig, 1994, Hartl, 1996). For those "hopeless case proteins" that are impossible to be refolded, the chaperone molecules may act as regulators of proteolysis by presenting these proteins for degradation by the protease subunit. These proteases are integral parts of the cell's protein quality control system which is responsible for clearing the cell of non-functional proteins (Wickner *et al.*, 1999, Dougan *et al.*, 2002). Most of these proteases also perform important regulatory functions by controlling the availability of transcriptional regulators, enzymes and other proteins via conditional degradation (Jenal and Hengge-Aronis, 2003). Proteolysis by Clp requires a serine type peptidase ClpP subunit and a regulatory ATPase subunit (Schirmer *et al.*, 1996). The proteolytic subunits, ClpP, perform the actual hydrolysis of substrates. However, their active sites are buried within the cavity of the so-called proteolytic core formed by 14 ClpP subunits. Therefore, hexamers of ATPase subunits (ClpA, ClpC or ClpX), which are members of the Clp/Hsp100 superfamily (Schirmer *et al.*, 1996) and associate with the core, are required in order to recognize, unfold and ultimately

translocate substrate proteins into it (Hlavacek and Vachova, 2002). *Streptococcus pneumoniae* contains putative orthologues of four ATPase specificity factors (ClpC, ClpE, ClpL and ClpX) (Hoskins *et al.*, 2001, Tettelin *et al.*, 2001) and a single protease subunit ClpP.

Regulation of Clp ATPases

The cellular concentration of Clp has to be adapted in response to environmental conditions resulting in the accumulation of non-functional, i.e. truncated, misfolded or aggregated, proteins. The principal regulatory phenomena are well studied in enterobacteria and low G+C Gram-positive bacteria. In *Escherichia coli,* RNA polymerase (RNAP) containing the alternative sigma factor σ^{32} is responsible for heat-triggered transcriptional activation of *clpX, clpP* and other stress genes (Arsene *et al.*, 2000), whereas the transcriptional repressor CtsR controls expression of the *clpC, clpE* and *clpP* genes in *Bacillus subtilis* (Kruger and Hecker, 1998, Derre *et al.*, 1999a). The activity of σ^{32} as well as of CtsR is at least partially controlled at the level of protein stability. σ^{32} is normally directed by the DnaKJ-GrpE chaperone machinery into a degradation pathway involving several ATP-dependent proteases. However, σ^{32} stability increases rapidly during heat stress because the amounts of DnaKJ-GrpE available for σ^{32} sequestration become limited as the chaperone machinery is now required for disaggregating and refolding non-native proteins. In contrast, CtsR stability is apparently modulated via heat-triggered post-translational modification by a putative protein kinase, McsB, and subsequent ClpCP-dependent degradation (Kruger *et al.*, 2001). In *Bacillus subtilis* and *Listeria monocytogenes*, CtsR negatively controls expression of the class III heat shock genes *clpP*, *clpE* and of the *clpC* operon (which encompasses *ctsR* and is thus autoregulated) by binding specifically to a direct heptanucleotide repeat in their promoter regions (Derre *et al.*, 1999b, Nair *et al.*, 2000a)

As *S. pneumoniae* lacks the sigma factor σ^{32} of *E. coli*, concentration of Clp is likely to be regulated by alternative mechanisms. Sequence analysis of the genome database of *S. pneumoniae* released by The Institute for Genomic Research (TIGR) revealed that *clpC* in *S. pneumoniae* belongs to a locus organized differently from that in *B. subtilis* and *L. monocytogenes*. Immediately upstream of *clpC*, an open reading frame encoding a protein with 43% amino acid sequence identity with CtsR of *B. subtilis* was found (Derre *et al.*, 1999b). Analysis of the pneumococcal amino acid sequence of CtsR revealed that the protein contains a predicted helix-turn-helix DNA-binding motif as noted previously for

CtsR in *B. subtilis* (Derre *et al.*, 1999b). The translational termination codon of *ctsR* is located four bases downstream of the translational start codon of *clpC*. A consensus CtsR recognition sequence (AGTCAAACAAGGTCAAG) was identified 502 bp upstream of the *clpC* translational start codon within the promoter region of *clpC*, at the beginning of the coding sequence of *ctsR*. Upstream of *clpC*, three genes potentially encoding members of an ABC transporter were identified (Charpentier *et al.*, 2000). Six hundred and forty-two base pairs downstream of the *clpC* gene, a putative two-component system was found. Reverse transcriptase polymerase chain reaction (RT-PCR) analysis revealed that this two-component system is transcribed from its own promoter (Charpentier *et al.*, 2000).

Analysis *in vitro*

As the role of ClpC in the autolysis and virulence of *S. pneumoniae* is controversial (Polissi *et al.*, 1998, Charpentier *et al.*, 2000, Chastanet *et al.*, 2001, Robertson *et al.*, 2002), the role of ClpC in different pneumococcal strains was studied here. In addition, the contribution of ClpP to the stress response and virulence of the pneumococcus was also investigated. Two versions of the pneumococcal strain D39 have been used in this analysis, one of which is mouse virulent (termed mouse-adapted; MA) and the other is avirulent in mice and termed laboratory-adapted (LA). The basis for the difference in the virulence of these two isolates is unknown but appears not to be related to phase variation or capsule expression as judged by visual analysis of colonies. These strains differed not only in their virulence for mice but also their resistance to penicillin-induced lysis. The mouse-adapted strain was resistant to penicillin-induced autolysis whereas the laboratory-adapted strain was not. As penicillin-induced autolysis is believed to involve the action of the major autolysin (LytA), these strains may differ in their basal activity of LytA. The nucleotide sequence of the LytA gene has been determined and is not mutated when compared to the D39 sequence present in the database. Total levels of LytA, as judged by Western blotting are also similar in D39(MA) compared to D39(LA). The difference may be related to activation of the LytA enzyme or by some other aspect of differences in the cell wall on the strains. Whether the difference in penicillin-induced lysis in these strains is reflected in their virulence is not clear at the present time. ClpC and ClpP seem to play different roles in different strains and serotypes (see below).

Role of ClpC in autolysis

The Δ*clpC* mutants of all strains studied were able to grow at high temperature in a manner similar to the parent strains. However, the Δ*clpC* mutants of type 2 strain D39 did not

undergo autolysis after stationary phase of growth, grew in long chains, and were resistant to penicillin-induced lysis suggesting an impairment in the function of LytA. In the case of the mouse-adapted strain of D39, the parent strain was also resistant to penicillin-induced lysis. Charpentier and co-workers reported that a Δ*clpC* mutant of strain R6 (a derivative of D39) showed increased tolerance to high temperatures, formed long chains and failed to undergo lysis after penicillin treatment (Charpentier *et al.*, 2000). Contrary to this report, Chastanet and colleagues reported that a Δ*clpC* mutant of strain R348 grew as diplococcal cells and did not form chains and was indistinguishable from the wild type in response to penicillin or DOC-induced autolysis and also in the growth at different temperatures (Chastanet *et al.*, 2001). A third paper by Robertson *et al* reported that ClpC was not involved in the growth at elevated temperatures and also did not affect autolysis in strains R6 and D39 (Robertson *et al.*, 2002). The dramatic phenotype of the Δ*clpC* mutant observed by Charpentier and co-workers (Charpentier *et al.*, 2000) may be due to a strain not being a pneumococcus. The ZmpB phenotype reported by the same group (Novak *et al.*, 2000a) has been found by another group to be due to a strain related to *Streptococcus viridans* (Berge *et al.*, 2001). Data presented in this chapter suggest that ClpC is not involved in thermal tolerance but can play a role in autolysis, cell-separation and resistance to penicillin-induced lysis. However, the findings with the two variants of D39 also show that this phenotype can vary according to the history of the strain. This presumably reflects accumulation of other mutations that affect the phenotype of ClpC mutants. Differences also occur between strains as shown by the effect of the ClpC mutation in the TIGR4 strain reported here.

In a Western blot analysis, the quantity of released pneumolysin was less in the Δ*clpC* mutant than in the wild type and no release of LytA in the culture supernatant of the mutant was detected. Autolysin is activated during the stationary phase or upon penicillin treatment to cause lysis of bacteria and the release of cytoplasmic contents including pneumolysin and other virulence factors (Ronda *et al.*, 1987, Mitchell *et al.*, 1997). Thus it appears that ClpC is involved in the control of this autolytic process and in the release of pneumococcal virulence factors. However, it should be noted that the extracellular release of pneumolysin from the pneumococcal strain WU2 (type 3) can also be independent of the action of LytA (Balachandran *et al.*, 2001). The lack of autolysis was not observed for strains R6, R800 and TIGR4 suggesting that the contribution of ClpC to the autolysis of the pneumococcus is strain-dependent.

ClpC was also implicated in the ability of bacteria to respond to stresses other than heat shock. A *clpC* insertional mutant of *Bacillus subtilis* had an impaired tolerance for salt stress (Kruger *et al.*, 1994). In addition, Scharf *et al* reported that in *Bacillus subtilis*, ClpC and TrxA are induced at least partially in a σ^B-dependent mechanism and raise the capacity of the bacteria to recover proteins damaged by oxidative stress (Scharf *et al.*, 1998). The contribution of ClpC to the pH tolerance and oxidative stress resistance of the pneumococcus was tested here. ClpC was found to have no effect on the pneumococcal acid tolerance or resistance to peroxide. The lack of apparent stress sensitivity of Δ*clpC* mutants to heat and other stresses may be explained by compensation for ClpC deficiency by other chaperones.

In vitro phenotype of *clpP* mutants

The ClpP proteolysis is indispensable for survival under conditions of stress. ClpP of *E. coli* removes abnormal proteins that accumulate during stress conditions and controls the half-life of regulatory proteins including the stationary phase sigma factor σ^S (Zhou and Gottesman, 1998). ClpP-null mutants of *Bacillus subtilis* and *Lactococcus lactis* were unable to survive during heat shock and other stress conditions (Gerth *et al.*, 1998, Frees and Ingmer, 1999). It was also reported that ClpP was necessary for the ability of *Staphylococcus aureus* to resist oxidative stress and to grow at low temperature (Frees *et al.*, 2003). Furthermore, ClpP-mediated proteolysis was also essential for many other processes such as, natural genetic competence and sporulation in *B. subtilis* (Msadek *et al.*, 1998), biofilm development in *Pseudomonas fluorescens* (O'Toole and Kolter, 1998) and viability and cell cycle progression in *Caulobacter crescentus* (Jenal and Fuchs, 1998).

Similar to the role of ClpP in other bacteria, this study reports that ClpP is necessary for the growth of both type 2 and type 4 strains of the pneumococcus at elevated temperatures. This study also found that ClpP is required for the growth of D39 at lower temperature (30°C) but not for TIGR4. The defect in growth at 30°C for a ClpP mutant of D39 has been reported previously (Robertson *et al.*, 2002). The *in vitro* growth data in this chapter also indicate that D39 and TIGR4 wild type strains are different in their growth at lower temperatures. The D39 grew at 30 °C to an OD_{600nm} of 0.6 after 10 hours of inoculation while the OD_{600nm} of TIGR4 at the same time and temperature was 1.4 (Figure 3.13). The requirement of ClpP for the survival of TIGR4 under heat shock has not been reported before but again highlights strain differences in the role of these molecules. Data presented in this study also confirm the involvement of ClpP in the resistance of *S. pneumoniae* strain

D39 to oxidative stress, which was reported by Robertson and co-workers (Robertson *et al.*, 2002).

Analysis *In vivo*

ClpC was identified as a virulence factor in a signature-tagged mutagenesis screen (Polissi *et al.*, 1998). It was suggested to play a role in the virulence of *S. pneumoniae* by affecting expression of choline-binding proteins (Cbps) and other virulence factors such as pneumolysin (Charpentier *et al.*, 2000). Data shown in this chapter suggest that ClpC infuences Ply release by affecting autolysis (Figure 3.12). The virulence of D39Δ*clpC* was also reported to be marginally attenuated in an intratracheal model of infection (Robertson *et al.*, 2002). In the pneumonia and bacteraemia models used for this study, ClpC did not affect the overall outcome of pneumococcal disease judged by survival times. However, the growth in the lungs and blood after intranasal infection was dependent on ClpC. The number of Δ*clpC* mutants recovered from lungs declined from approximately 10^5 to less than 10^4 whereas the wild type increased to more than 10^6. Appearance of bacteria in the bloodstream following intranasal challenge was similar for wild type and Δ*clpC* in that bacteria appeared in the bloodstream at a similar time and grew to about 10^4 per ml of blood by 24 h (significantly higher counts than time 0 h for both groups). Therefore, although Δ*clpC* is less able to grow at 37 °C *in vitro*, it is able to grow normally for the first 24 h in mouse blood. However, the wild type bacteria then continued to grow to levels of approximately 10^6 per ml by 48 h whereas numbers of the mutant reduced to levels that were no longer significantly different to counts at time 0 h. The effect of disruption of the *clpC* gene on bacteriology is therefore dramatic, with very low numbers of the mutant present in the lung and blood at 48 h when animals begin to show signs of disease. This suggests that the lethal event is triggered early in the infection or that low numbers of bacteria are able to cause the effect. In fact, analysis of serial tail bleed from the same animal (table 3.6) shows that the mouse can actually clear transient bacteraemia to levels below detection but still go on to die from the infection suggesting the former is more likely. It also remains a possibility that the levels of bacteraemia in *ΔclpC* infected mice increases again between 48 h and the time of death. The event triggered by the pneumococcus that results in death is still not known.

ClpP-controlled proteolysis is also essential for disease progression and virulence of bacterial pathogens, favouring survival in the host organism or modulating the activity of virulence factors. The stress-induced ClpP serine protease contributes to virulence in

Salmonella enterica serovar Typhimurium (Webb *et al.*, 1999) and modulates adhesion invasion locus (*ail*) gene expression in *Yersinia enterocolitica* (Pederson *et al.*, 1997). In *Listeria monocytogenes*, ClpP is essential for intracellular parasitism and virulence (Gaillot *et al.*, 2000). Kwon and co-workers reported that ClpP also plays an essential role in the virulence of *S. pneumoniae* (Kwon *et al.*, 2003). In the present study, the D39Δ*clpP* mutant was completely avirulent in a pneumonia model of infection while the TIGR4Δ*clpP* mutant was less virulent than its TIGR4 wild type (mirroring the less pronounced effect of the Δ*clpP* mutation on *in vitro* growth, figure 3.13). A Δ*clpP* mutant of the strain D39 was reported to be strongly attenuated for virulence in murine lung and sepsis infection models (Robertson *et al.*, 2002). Despite the agreement on the contribution of ClpP to the virulence of the pneumococcus, it is not completely understood whether this contribution is due to its proteolytic effect on misfolded or stress-damaged proteins or due to regulation of other virulence factors. Recently, Kwon and co-workers reported that expression of virulence genes such as pneumolysin and pneumococcal surface antigen A is modulated by ClpP protease (Kwon *et al.*, 2003). The same group further investigated the underlying mechanism of virulence attenuation by the Δ*clpP* mutant and reported that the mRNAs of pneumolysin (*ply*) and a serotype 2 capsule locus (*cpsA*) were regulated post-transcriptionally by ClpP and that ClpP is translocated into the cell wall after heat shock (Kwon *et al.*, 2004). In addition, ClpP was found to be required for colonization of the nasopharynx and survival in the lungs of mice after intranasal infection (Kwon *et al.*, 2004).

Global transcriptional profile of Δ*clpC* mutant

To address the genetic basis for the Δ*clpC* phenotype, analysis of global transcription of the D39Δ*clpC* mutant compared to the D39 wild type was performed. As an internal control of this method, *clpC* transcript was not detected from the D39Δ*clpC* compared to the parent strain. Furthermore, the level of transcription from the *clpC*-downstream genes was the same in D39Δ*clpC* and its parent strain D39, indicating the lack of polarity on the surrounding genes.

The global transcription analysis showed that only an operon of three genes (table 3.4) was repressed by *clpC* deletion. This operon contains the gene for LacR (13.4-fold down regulation), which belongs to a group of DNA-binding repressors including GalR and LacI, and most of the members of this group contain a helix-turn-helix structure at their N-terminal regions (Weickert and Adhya, 1992). It also contains genes homologous to the *L.*

monocytogenes FruA and FruB, which are involved in fructose metabolism (11.4- and 6.9-fold down regulation, respectively). The most notable among the up-regulated genes were some of those genes involved in sugar transport and metabolism including PTS systems and the beta-glucosidase, *bglA* (table 3.5) in addition to a cell wall surface anchor family protein and some hypothetical proteins of unknown function. The phospho- transferase systems (PTS) are multicomponent complexes involved in transport and concomitant phosphorylation of various sugars in bacteria. The phosphorylation cascade takes place from phosphoenolpyruvate (PEP) to the sugar via two cytoplasmic proteins (EI and HPr) commonly used by all the PTS, and a carbohydrate specific enzyme II (EII). The latter is composed of at least three domains, two cytoplasmic IIA and IIB, and one membrane bound domain IIC, (Postma *et al.*, 1993).

The significance of up-regulation of these genes involved in sugar metabolism in response to *clpC* mutation is not clear at present. In addition, as ClpC probably regulates protein folding or directs damaged proteins for degradation by the proteolytic subunit, it is likely that microarray is not the best approach to study the effect of *clpC* deletion.

Summary

The ATP-dependent caseinolytic proteases (Clp) are fundamental for stress tolerance and virulence in many pathogenic bacteria. The role of ClpC in the autolysis and virulence of *S. pneumoniae* is controversial. In this study, the role of ClpC was tested in a number of *S. pneumoniae* strains and the contribution of ClpC to autolysis was found to be strain-dependent. ClpC is required for the release of autolysin A and pneumolysin in serotype 2 *S. pneumoniae* strain D39. *In vivo*, ClpC is required for the growth of the pneumococcus in the lungs and blood in a murine model of disease but it does not affect the overall outcome of pneumococcal disease. Repair of *clpC* mutation and expression of downstream genes, determined by microarray analysis, revealed the lack of downstream polar effect of the mutation and confirmed that the phenotype observed is solely due to lack of ClpC. This study also reports the requirement of ClpP for growth at elevated temperature and virulence of serotype 4 strain TIGR4 and confirms its contribution to the thermotolerance, oxidative stress resistance and virulence of D39.

The difference in virulence phenotype between Δ*clpC* and Δ*clpP* shows that other ATPase specificity factors interact with ClpP or that ClpP has roles independent of the activity of these factors. Investigation into the role of other ATPase specificity factors in the virulence process is therefore required.

Chapter 4

ROLE OF HtrA IN THE PNEUMOCOCCAL STRESS RESPONSE, RESULTS

Bacteria use multiple regulatory pathways to respond to stresses that affect cytoplasmic physiological processes. The synthesis of a variety of heat shock proteins (HSPs) consisting of cytoplasmic chaperones and proteases is induced in response to elevated temperature, oxidative stress and other stressful stimuli. The function of HSPs is to facilitate survival by mediating refolding or degradation of misfolded or stress-damaged proteins.

The High Temperature Requirement A (HtrA), also known as DegP or DO Protease (Seol *et al.*, 1991), is a heat shock-induced serine protease that was first described in *Escherichia coli* to be involved in the degradation of periplasmic misfolded proteins (Strauch *et al.*, 1989). This ATP-independent protease has both general molecular chaperone and proteolytic activities and switches from chaperone to protease in a temperature-dependent manner (Spiess *et al.*, 1999), the protease activity being most apparent at elevated temperature.

This chapter summarizes the investigation into the role of HtrA protein in the pneumococcal stress response. HtrA-null mutants were constructed and their *in vitro* and *in vivo* phenotypes compared to the wild types.

4.1 Characterization of HtrA

Analysis of the complete genome sequence of *Streptococcus pneumoniae* strain TIGR4 (The Institute for Genomic Research at http://www.tigr.org/) revealed a single copy of the gene for HtrA. The 1179 bp gene encodes for a 393-amino-acid protein with molecular weight of 42 kDa. The protein possesses an amino-terminal hydrophobic region, a trypsin like catalytic domain (residues 116-277) with conserved His^{122}, Asp^{152} and Ser^{234} residues and a PDZ domain at the carboxyl-terminal (residues 289-382). Purified HtrA shows ATP-independent endopeptidase activity against β-casein that can be inhibited by diisopropyl fluorophosphate, a specific inhibitor of serine proteases, confirming that HtrA is a serine protease (Lipinska *et al.*, 1990). The pneumococcal HtrA is homologous to HtrA of many Gram-positive bacteria. The amino acid sequence alignment shown in figure 4.1 highlights this homology.

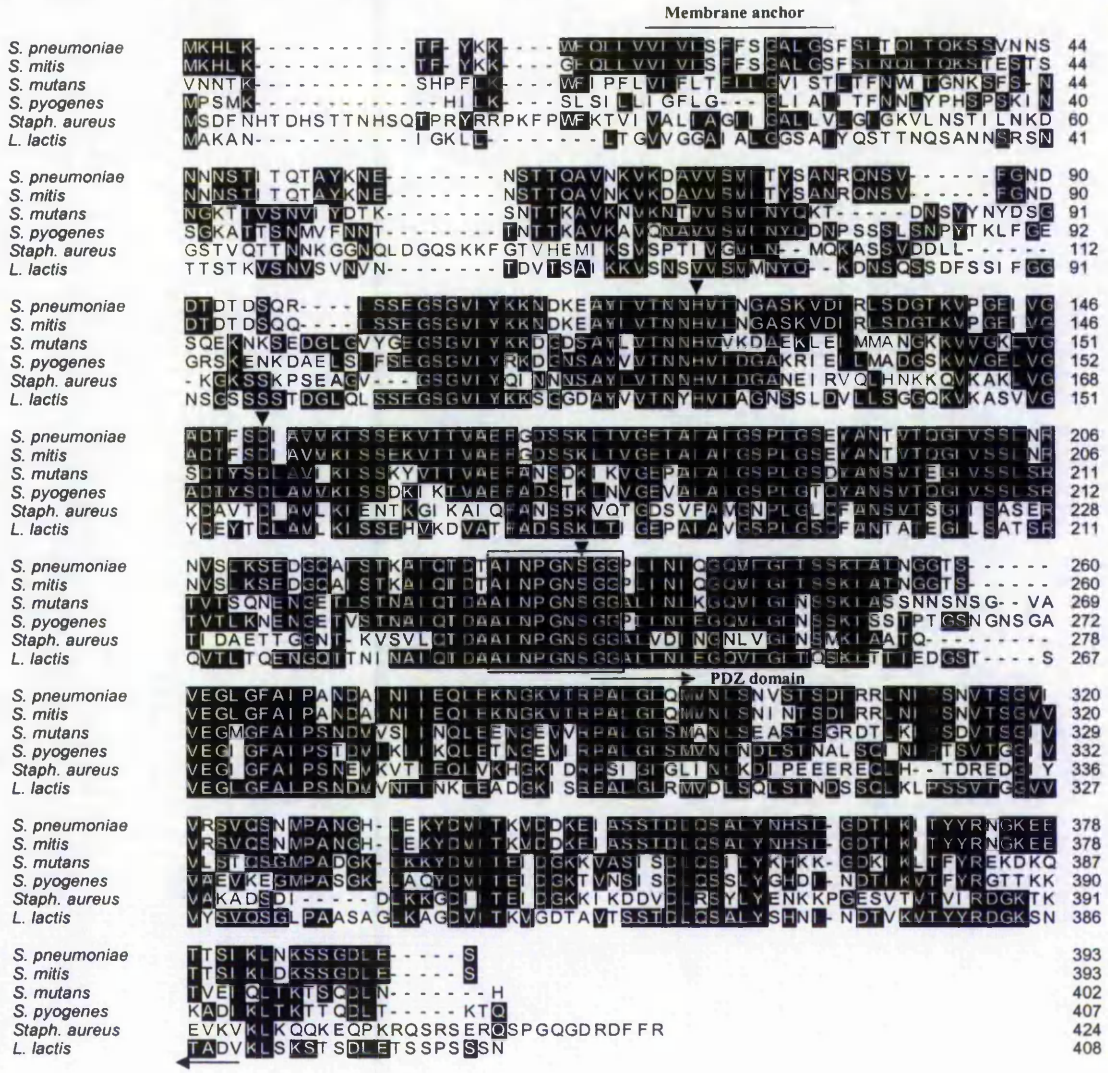


Figure 4.1- Multiple sequence alignment of HtrA protein from some Gram-positive bacteria.

Amino acid sequences were aligned using *ClustalV Method* (MegAlign, DNASTAR Inc.). Conserved residues are highlighted. The conserved sequence around the active site is boxed. Arrowheads point at the catalytic triad of His[122], Asp[152] and Ser[234] (*S. pneumoniae* numbering). The membrane anchor region of *S. pneumoniae* HtrA lies between valine 17 and serine 29. The PDZ domain is the region between the two arrows.

4.2 Construction of Δ*htrA* mutants

The *htrA* gene of *S. pneumoniae* strains D39 and TIGR4 was disrupted by the introduction of a spectinomycin resistance cassette into the gene. A knockout plasmid was constructed as discussed below and used to mutate the pneumococcal *htrA* gene using homologous recombination. Figure 4.2 summarizes this process.

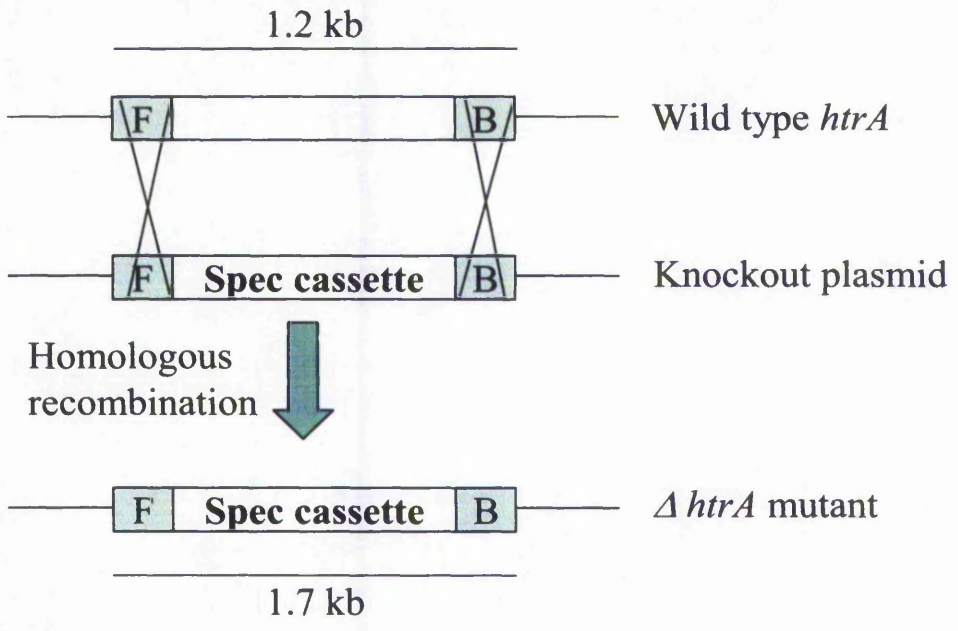


Figure 4.2- Construction of *htrA* mutant

4.2.1 Plasmid construction and confirmation

A 1.2 kb fragment representing the whole *htrA* gene was cloned in PCR-Script™ Amp cloning vector (3 kb). The inverse PCR resulted in ~ 3.5 kb DNA fragment with *Asc*I restriction sites, which represent the 5' and 3' ends of *htrA* together with PCR-Script. The *Asc*I-cut inverse PCR product was ligated to a spectinomycin resistance cassette (1.2 kb) to facilitate the selection of transformed cells and the resulting plasmid was used to transform *E. coli* competent cells (see Materials and Methods). This plasmid was confirmed by restriction digestion and PCR.

4.2.1.1 Confirmation by restriction digestion

The construction of the plasmid was confirmed by digestion with *Asc*I endonuclease. The 10-µl reaction included 5 µl of the plasmid, 3.6 µl of dH_2O, 1 µl of buffer 4 and 0.4 µl of *Asc*I (New England Biolabs, UK). The reaction was incubated at 37 °C in a water bath for 2 hours and then run in loading buffer on a 1 % agarose gel to visualize DNA bands. Figure 4.3 shows the result of this restriction digestion.

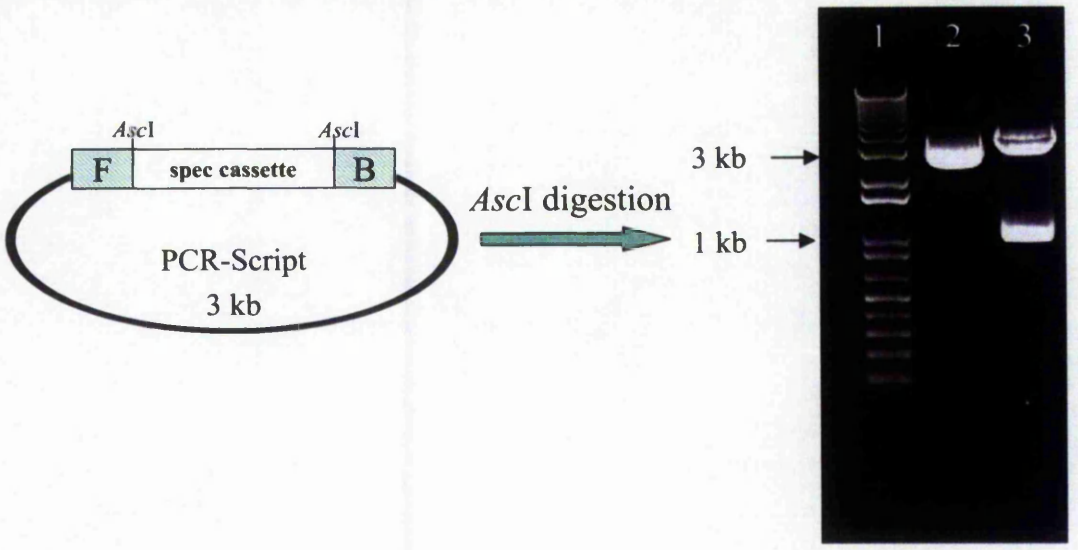


Figure 4.3- Agarose gel electrophoresis of *Asc*I-digestion of the plasmid used for making Δ*htrA* mutant.

Lane 1, 1 kb plus ladder; lane 2, uncut plasmid; lane 3, *Asc*I-cut plasmid showing 1.2 kb fragment representing the spectinomycin cassette and ~3.5 fragment representing PCR-Script and the 5' and 3' ends of *htrA*.

4.2.1.2 Confirmation by PCR

The plasmid was also confirmed using different combinations of *htrA* forward and reverse primers (HtrA for and HtrA rev, table 2.2) and spectinomycin forward and reverse primers (Spec up and Spec dn, table 2.2) as shown in figure 4.4. When HtrA for and HtrA rev primers were used, a 1.7 kb fragment was amplified instead of 1.2 kb corresponding to *htrA* gene. The spectinomycin resistance cassette (1.2 kb) was amplified when primer pair Spec up and Spec dn was used. PCR reactions using HtrA for and Spec dn or HtrA rev and Spec up combinations were negative. However, those reactions using HtrA for and Spec up or HtrA rev and Spec dn resulted in 1.45 kb products. These PCR products confirmed the construction of the plasmid shown in figure 4.4.

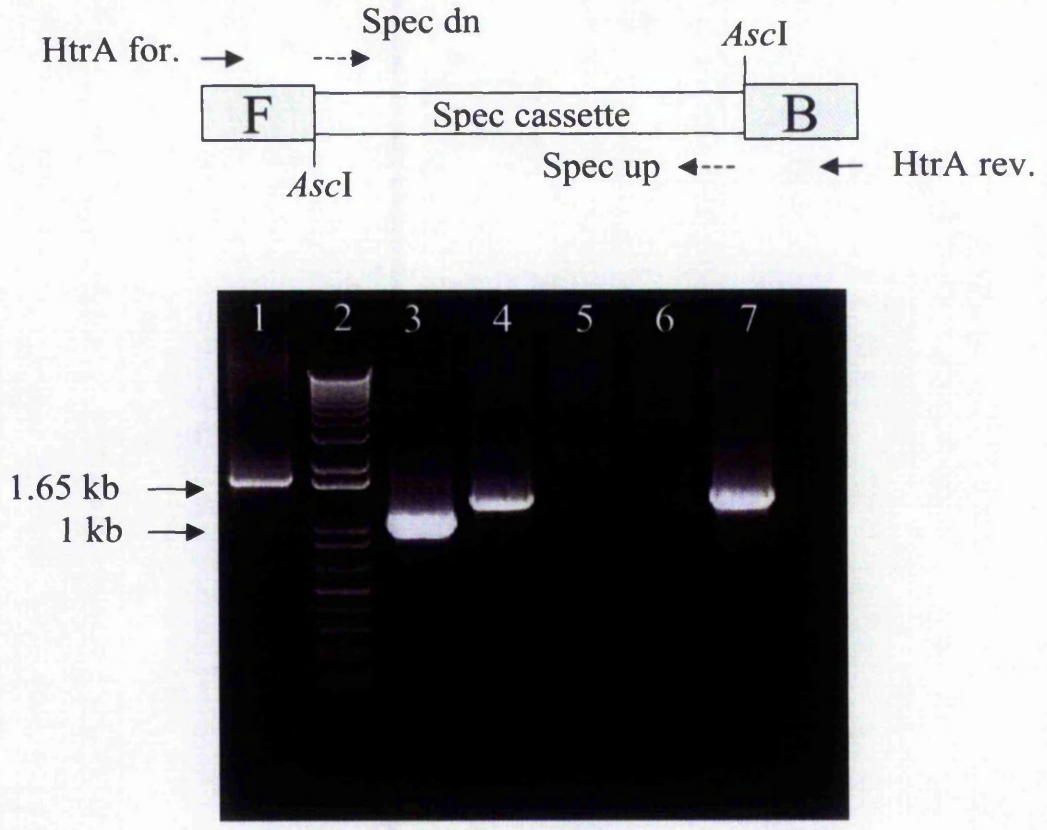


Figure 4.4- Agarose gel electrophoresis of PCR reactions carried out for confirmation of the plasmid used for making Δ*htrA* mutant.

Lane 1, 1.7 kb product using primer pair HtrA for and HtrA rev; lane 2, 1 kb plus ladder; lane 3, 1.2 kb product using primer pair Spec up and Spec dn; lane 4, 1.45 product using primer pair HtrA for and Spec up; lane 5, negative reaction using primer pair HtrA for and Spec dn; lane 6, negative reaction using primer pair HtrA rev and Spec up; lane 7, 1.45 product using primer pair HtrA rev and Spec dn.

4.2.2 Transformation of S. pneumoniae

The mutated *htrA* gene was amplified by PCR with primer pair HtrA for and HtrA rev (1.7 kb) and used to transform *S. pneumoniae* strain D39 (serotype 2) as mentioned in the Materials and Methods. Genomic DNA was prepared from the transformed cells and the mutation was confirmed by using diagnostic primers, htrA C1 and htrA C2 (table 2.2) flanking *htrA* to amplify a PCR fragment of 1.7 kb from the chromosomal DNA of the transformed cells grown on spectinomycin compared to the 1.2 kb fragment of the wild type copy of *htrA* (Figure 4.5). This PCR fragment was sequenced to further confirm that the mutation occurred in the right position of the pneumococcal chromosome. This mutation was later moved to the TIGR4 strain (serotype 4) to investigate the role of HtrA in the virulence of the pneumococcus in different backgrounds.

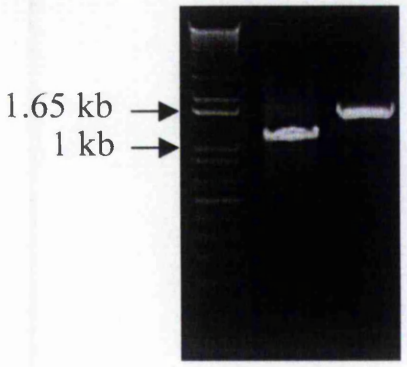


Figure 4.5- Confirmation of *htrA* mutation in strain D39.

Primer pair htrA C1 and htrA C2 flanking *htrA* gene was used to amplify a 1.7 kb band from the D39 Δ*htrA* mutant (Lane 3) compared to the 1.2 kb fragment representing the wild type copy of *htrA* (Lane 2). The 1 kb plus DNA ladder is shown in lane 1.

4.3 *In vitro* analysis of Δ*htrA* mutant

The *in vitro* phenotypes of Δ*htrA* mutants were compared to the wild type strains in a number of assays to investigate the role of HtrA in pneumococcal biology and stress response. The pneumococcal strain D39 was used in these assays unless stated otherwise.

4.3.1 Growth phenotype

The growth of D39 Δ*htrA* mutants at normal and elevated temperatures was compared to D39 wild type; 1×10^6 CFU/ml from glycerol stocks of known viable counts of wild type and mutant strains was used to inoculate BHI prewarmed at 37 °C and 40 °C and the cultures incubated in water baths adjusted at these temperatures. At 1-hour intervals, samples of the cultures were withdrawn under aseptic conditions and the OD_{600nm} and viable counts were determined.

The D39Δ*htrA* mutant was found to grow at a similar rate as the wild type at 37 °C. However, the mutant grew more slowly at 40 °C compared to growth of D39 wild type. The effect of *htrA* deletion was to slow growth rather than prevent it and the mutant organisms still reached the same final optical density (figure 4.6 A). Moreover, the D39Δ*htrA* mutant had a decreased rate of autolysis compared to its parent strain at 40 °C as indicated by the viable count at stationary and decline phases of growth (figure 4.6 B).

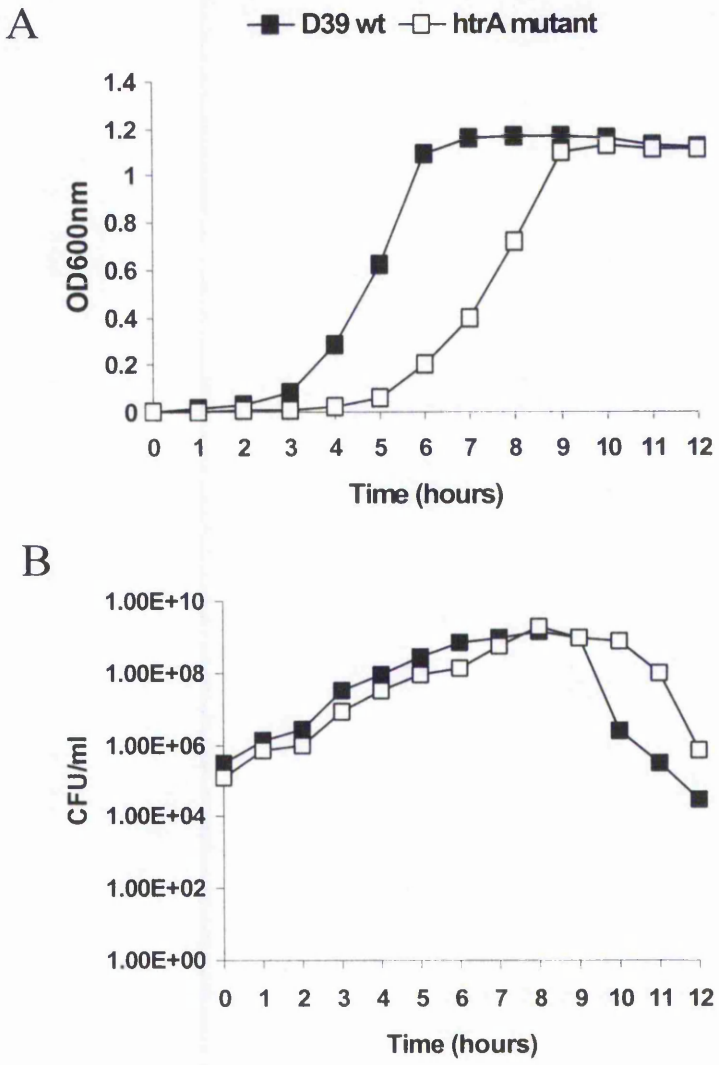


Figure 4.6- Growth phenotype of Δ*htrA* mutant compared to D39 wild type at 40 °C.

Representative graphs of three independent experiments showing growth of D39Δ*htrA* mutant and D39 wild type measured by OD_{600nm} in (A) and by viable counts in (B). BHI prewarmed at 40 °C was inoculated with either bacterial strain to 10^6 CFU/ml. Samples were taken at 1h-intervals to measure the OD_{600nm} and viable counts on BAB.

4.3.2 Hydrogen peroxide sensitivity

Sensitivity to hydrogen peroxide, which represents oxidative stress, was studied for the D39Δ*htrA* mutant and its D39 wild type. 1-ml aliquots of bacterial cultures grown to OD_{600nm} ~ 0.3 were exposed to 40 mM of H_2O_2 for 5, 10, and 15 min at room temperature and the viable counts measured before and after exposure to peroxide. Results were taken as percentage of cells survived per total cells. The D39Δ*htrA* was more sensitive to H_2O_2 than D39 wild type. The percentage survival of the mutant was significantly less than that of the wild type at all indicated time points (figure 4.7).

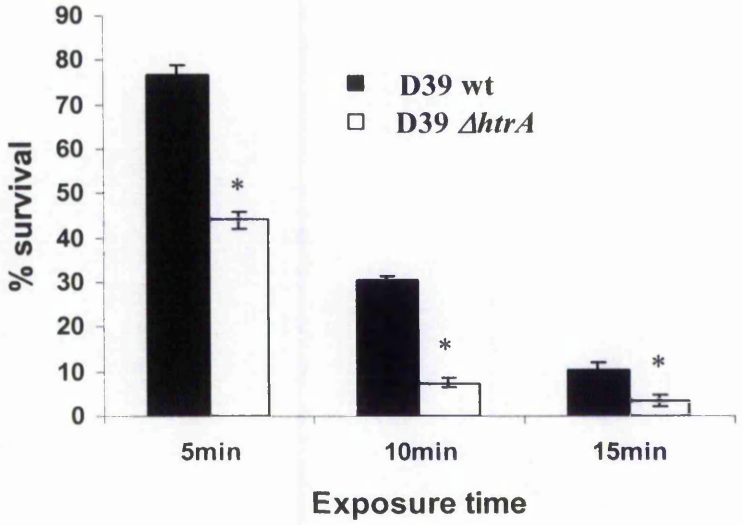


Figure 4.7- Hydrogen peroxide sensitivity assay for D39 wild type and D39Δ*htrA* mutant.

Hydrogen peroxide (final concentration 40 mM) was added to 1-ml aliquots of culture grown to OD_{600nm} ~ 0.3. Viable counts were performed on BAB plates before and after the addition of peroxide and the % survivals were calculated. Values expressed are the mean (SEM) of three independent experiments. *, $p <$ 0.05 lower survival for Δ*htrA* mutant than wild-type.

4.3.3 Transformation efficiency

The ability of the strains to take up exogenous DNA was also compared. Pneumococcal genomic DNA conferring kanamycin resistance (from a Δ*clpP* mutant containing kanamycin resistance cassette) (Chastanet *et al.*, 2001) was used as the transforming DNA. 0.3 μg of this DNA was used to transform competent cells of D39 wild type and Δ*htrA* and *ΔhtrA/phtrA*$^+$ mutants as described in the Materials and Methods. The efficiency of transformation was expressed as the percentage of transformed cells to the total number of cells used. The disruption of the *htrA* gene caused a decrease in transformation efficiency of approximately 32-fold. This reduction could be reversed by complementation with a plasmid containing the *htrA* gene, pAL2-HtrA plasmid (table 4.1).

Table 4.1- Transformability of Δ*htrA* mutant compared to D39 wild type.

Experiment number	% transformants		
	D39 wild type	D39Δ*htrA*	D39Δ*htrA*/*phtrA*$^+$
1	0.825	0.024	0.522
2	0.394	0.017	0.321
3	0.408	0.011	0.517
Mean ± SEM	**0.542 ± 0.141**	**0.017 ± 0.004**	**0.453 ± 0.066**

D39Δ*htrA* mutant had a lower transformation efficiency than D39 wild type (*P* <0.05). This decrease in transformation efficiency was reverted in the complemented mutant D39Δ*htrA*/*htrA*$^+$ by expression of HtrA using the pAL2-HtrA plasmid. The percentage transformants was indicated as (kanamycin-resistant colonies per total viable cells used) ×100.

4.3.4 Other in vitro analyses

The D39Δ*htrA* mutant was also analyzed by a number of other assays. The sensitivity to Paraquat, which is taken up by bacterial cells and generates superoxide within the cytoplasm (Hassett *et al.*, 1987), and the effect of *htrA* deletion on the ability to grow at different pHs were also studied. There was no difference in the effect of paraquat and no significant differences between wild type and mutant strains in the growth at different pHs (data not shown).

The amount of the toxin pneumolysin and the major autolytic enzyme LytA expressed at 37 °C and 40 °C were studied by Western blotting. Total protein samples from cultures grown to mid-logarithmic phase were separated on 12 % SDS-PAGE, electroblotted onto nitrocellulose membranes and reacted with specific antisera against pneumolysin and autolysin A by standard methods (Sambrook *et al.*, 1989). There was no significant difference between the wild type and mutant strains in the amount of pneumolysin or LytA (data not shown).

The distribution of three known surface proteins of the pneumococcus was also examined. No difference in the levels of CbpA, hyaluronidase or neuraminidase associated with the different cell fractions could be found as determined by Western blotting. In addition, there were no differences in the distribution of enzymatic activity of hyaluronidase or neuraminidase between the mutant and wild-type organisms (data not shown).

4.4 *In vivo* analysis of Δ*htrA* mutants

The effect of *htrA* deletion on the virulence of the pneumococcus was studied in two different serotypes, serotype 2 (strain D39) and serotype 4 (strain TIGR4) using a number of *in vivo* assays.

4.4.1 Intranasal infection

Groups of MF1 mice (5 mice each) were infected intranasally with either type 2 or type 4 wild types or their Δ*htrA* mutants. The effect of HtrA was examined in two genetic backgrounds as it was recently shown that genetic background could have a major influence on the role of virulence factors (Blue and Mitchell, 2003). After administering the infectious dose (10^6 CFU/mouse in case of D39 and either 10^5 or 10^6 CFU/mouse in case of TIGR4), mice were observed and the development of symptoms was recorded over a period of eight days. Mice infected with D39 wild type started to show symptoms of illness 24 h post-infection and all mice were moribund by 72 h. However, none of the D39Δ*htrA* infected mice showed any signs of illness throughout the experiment (Figure 4.8 A). In the case of the TIGR4 strain, which is more virulent than D39, all mice infected with a dose of 10^5 CFU of TIGR4Δ*htrA* survived the experiment, while 80 % of those infected with the wild type TIGR4 became moribund (Figure 4.8 B). When using a dose of 10^6 CFU/mouse, mice infected with wild type TIGR4 became moribund very rapidly (all were sacrificed by 29 h) while the first moribund case of TIGR4Δ*htrA* infected mice was not recorded until 120 h post-infection and 60% of the animals were normal at the endpoint of the experiment, eight days. (Figure 4.8 C).

4.4.2 Bacteriological investigation

The behavior of D39Δ*htrA* mutant compared to the D39 wild type in the lung airways, lung tissue and blood after intranasal infection was studied at 0, 6, 12, 18, 24, and 48 h after infection. In the lung airways, both D39 and D39Δ*htrA* mutant were cleared over time. However, the D39Δ*htrA* mutant was cleared more rapidly. The number of D39Δ*htrA* recovered was significantly lower than that of D39 at 6 and 12 hours post-infection ($p<0.05$) (Figure 4.9 A). In the lung tissue however, while D39 grew very well, D39Δ*htrA* was unable to survive (Figure 4.9 B). Furthermore, D39 appeared in blood 12 h post-infection and grew dramatically, whereas, D39Δ*htrA* mutant was never detected in blood (Fig 4.9 C).

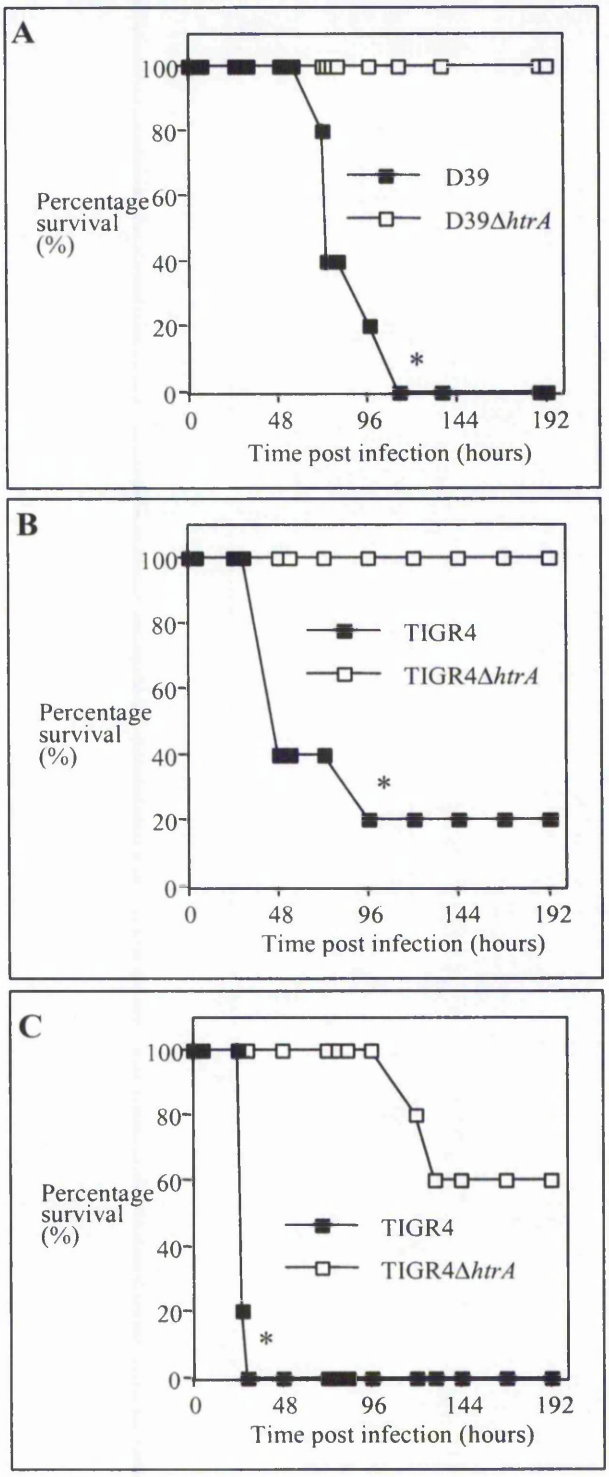


Figure 4.8- Survival of MF1 mice infected intranasally with D39 and TIGR4 wild types or their Δ*htrA* mutants.

Mice infected intranasally with 10^6 CFU/mouse (n=5) of D39 or D39 Δ*htrA* in (A), 10^5 or 10^6 CFU/mouse of TIGR4 or TIGR4Δ*htrA* mutant in (B) and (C) respectively. *, p <0.05 longer survival times for the Δ*htrA* compared to wild type.

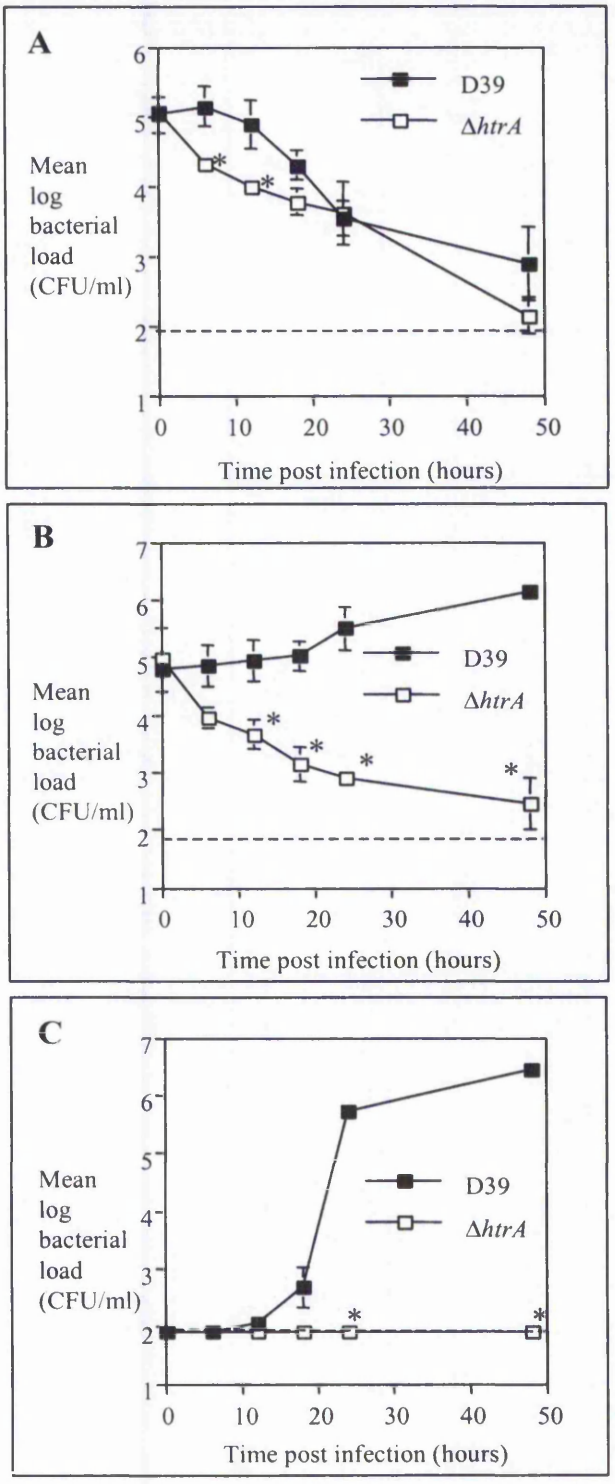


Figure 4.9- Bacterial loads in the lung and blood of MF1 mice after intranasal infection with D39 wild type or D39Δ*htrA* mutant.

Time course of mean (SEM) bacterial growth in the lung lavage (A), lung tissue (B), and blood (C) after intranasal infection with 10^6 CFU/mouse (n=5) of D39 wild type or its Δ*htrA* mutant. Broken line represents the limit of detection of the assay. *, p <0.05 lower bacterial loads for D39Δ*htrA* mutant than for D39.

4.4.3 Bacteraemia after intranasal infection (TIGR4)

The ability of TIGR4Δ*htrA* mutant to cause bacteraemia in mice after intranasal infection was also tested. Mice were bled 24 h and 48 h after challenge with 10^5 CFU/mouse of either TIGR4 wild type or TIGR4Δ*htrA* mutant and the number of pneumococci in blood was determined by plating on BAB. The number of TIGR4 wild type was between log 5-6.8 in 60 % of mice after 24 h of infection while no bacterial count was detected in the rest of the group. Mice with detectable bacterial count died and those with no count survived 48 h post-infection. In contrast the TIGR4Δ*htrA* mutant was not found in the blood of mice at any time point (table 4.2).

Table 4.2- Counts of bacteria in tail blood from MF1 mice after intranasal infection with 10^5 CFU/mouse of TIGR4 wild type or TIGR4Δ*htrA* mutant.

Mouse number	Log_{10} bacterial load (CFU/ml) at indicated time post-infection			
	24h		48h	
	TIGR4	TIGR4Δ*htrA*	TIGR4	TIGR4Δ*htrA*
1	6.041	ND	--	ND
2	6.791	ND	--	ND
3	ND	ND	ND	ND
4	5.097	ND	--	ND
5	ND	ND	ND	ND

Mice were bled 24h and 48h post-infection and the viable count was determined by plating on BAB plates. TIGR4Δ*htrA* was not detected in blood of all infected mice at either time points. $P < 0.05$ at 24 h post-infection. ND, none detected. --, Dead mouse.

4.4.4 Intravenous infection

To determine if the role played by HtrA was specific for the lung, organisms were injected directly into the bloodstream to by-pass the respiratory tract. MF1 mice were given 1×10^5 CFU/mouse and both survival of mice and bacterial loads in blood were determined. Wild type D39 grew in the blood and resulted in all injected animals becoming moribund while Δ*htrA* mutant organism caused no disease, all mice infected with Δ*htrA* mutant were quite healthy at the endpoint of the experiment (figure 4.10 A). The wild type strain established itself well and multiplied rapidly while the mutant strain was cleared from the blood (figure 4.10 B). HtrA therefore plays a critical role in the survival and growth of the pneumococcus in the vascular compartment.

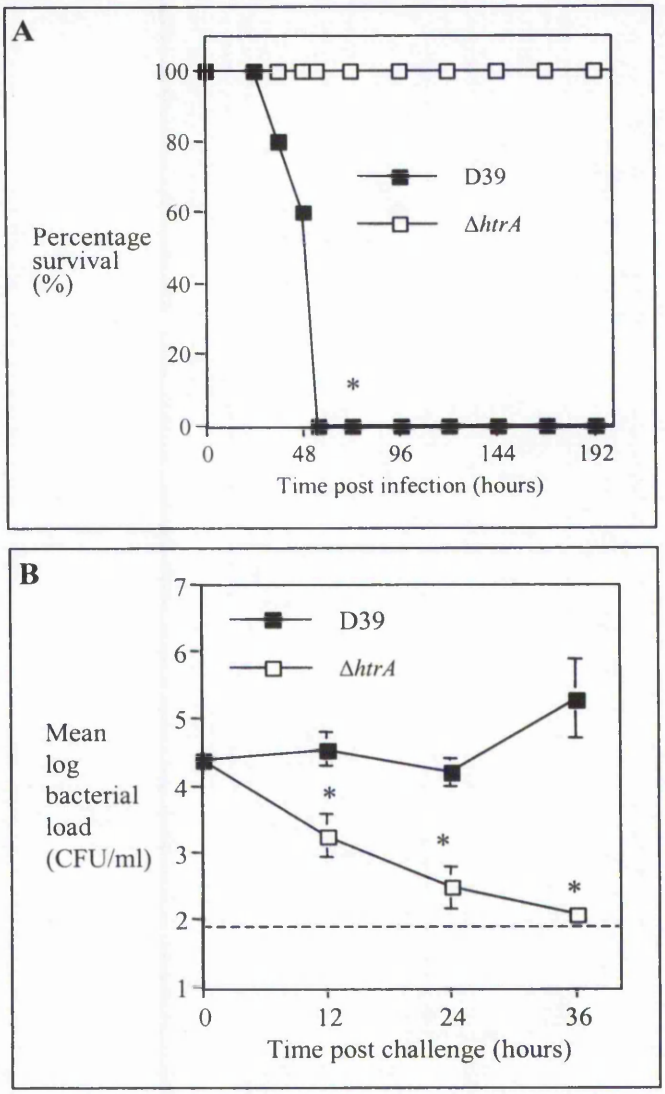


Figure 4.10- Survival of MF1 mice and bacterial load in blood after intravenous infection with D39 wild type or D39Δ*htrA* mutant.

Survival of MF1 mice (A) and time course of mean (SEM) bacterial growth in blood (B) after intravenous challenge with 10^5 CFU/mouse (n=5) of D39 wild type or its Δ*htrA* mutant. In A *, $p<0.05$ shorter survival times for D39 than for Δ*htrA* mutant and in B *, $p<0.05$ lower bacterial loads for Δ*htrA* than for D39 wild type. Dashed line represents the limit of detection of the assay.

4.4.5 Measurement of immune modulators

To study the inflammatory response to D39Δ*htrA* compared to that of D39 wild type, IL-6 and TNF-α levels in the lung lavage and in the lung tissue of MF1 mice after intranasal infection were measured (figure 4.11). D39 induced high levels of IL-6 at 24 h post-infection, which was reduced after 36 h in the lung lavage and increased by time in the lung tissue. No or very low IL-6 levels were detected both in the lung lavage and in the lung tissue in the case of D39Δ*htrA* mutant. D39 gave peak TNF-α levels in the lung lavage after 24 h which declined sharply while TNF-α concentrations were increased over

time in the lung tissue. In contrast, the D39Δ*htrA* mutant induced very low TNF-α in the lung lavage fluid, and levels in the lung tissue that were less than those induced by D39. Although these results are complicated by the fact that the lungs contain different numbers of organisms, it is clear that D39Δ*htrA* induces much less inflammation in the lung. The bacterial count of both organisms present in the lavage fluid at 24 h was very similar (Figure 4.9) but the mutant still did not induce IL-6 or TNF-α production.

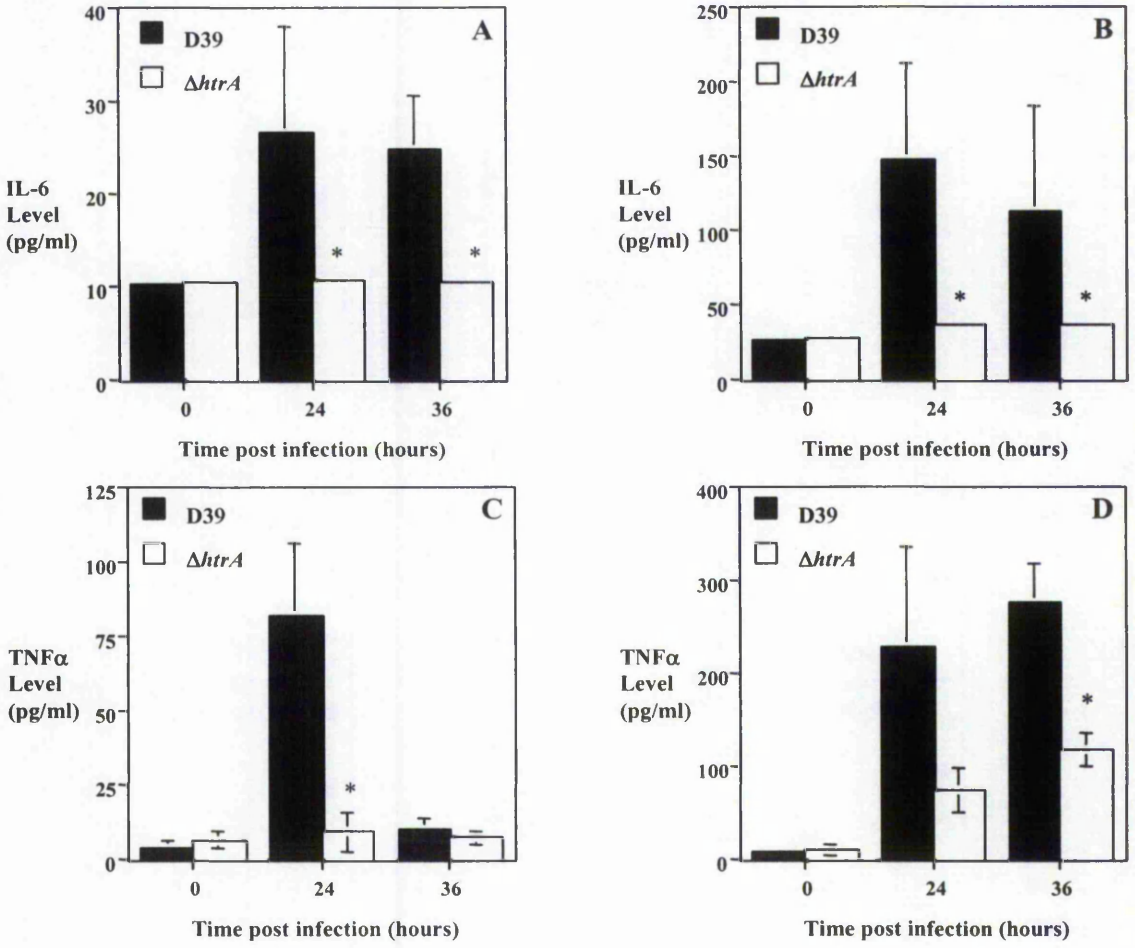


Figure 4.11- Levels of IL-6 and TNF-α in the lung lavage and lung tissue of MF1 mice after intranasal infection with D39 wild type or its Δ*htrA* mutant.

Median (MAD) levels of IL-6 and TNF-α induced in the lung lavage and in the lung tissue of MF1 mice (n=5) infected intranasally with 10^6 of D39 wild type or its Δ*htrA* mutant. (A) Levels of IL-6 in the lung lavage, (B) levels of IL-6 in the lung tissue, (C) levels of TNF-α in the lung lavage, and (D) levels of TNF-α in the lung tissue. *, p< 0.05 lower cytokine levels for Δ*htrA* mutant than for D39 wild type.

4.4.6 Histological analysis

Histological examination revealed that the influx of inflammatory cells into the lungs of mice infected with wild type bacteria was greater than in those infected with D39Δ*htrA*

mutant. At 48 h post infection lungs of mice infected with D39 contained more lesions, especially at the pleural interface (figure 4.12 A). These areas were swollen and congested with recruited cells (nuclear morphology indicates the majority of cells were neutrophils). In contrast, lungs from mice infected with D39Δ*htrA* had few inflammatory lesions (figure 4.12 B). Perivascular alterations were also scored. The perivascular areas of D39 wild type-infected mice had inflammatory cell recruitment, whereas some of lung sections of mutant-infected mice had no such cells (figure 4.12 C & D). This difference in perivascular alteration between the wild type and mutant organisms was not statistically significant however.

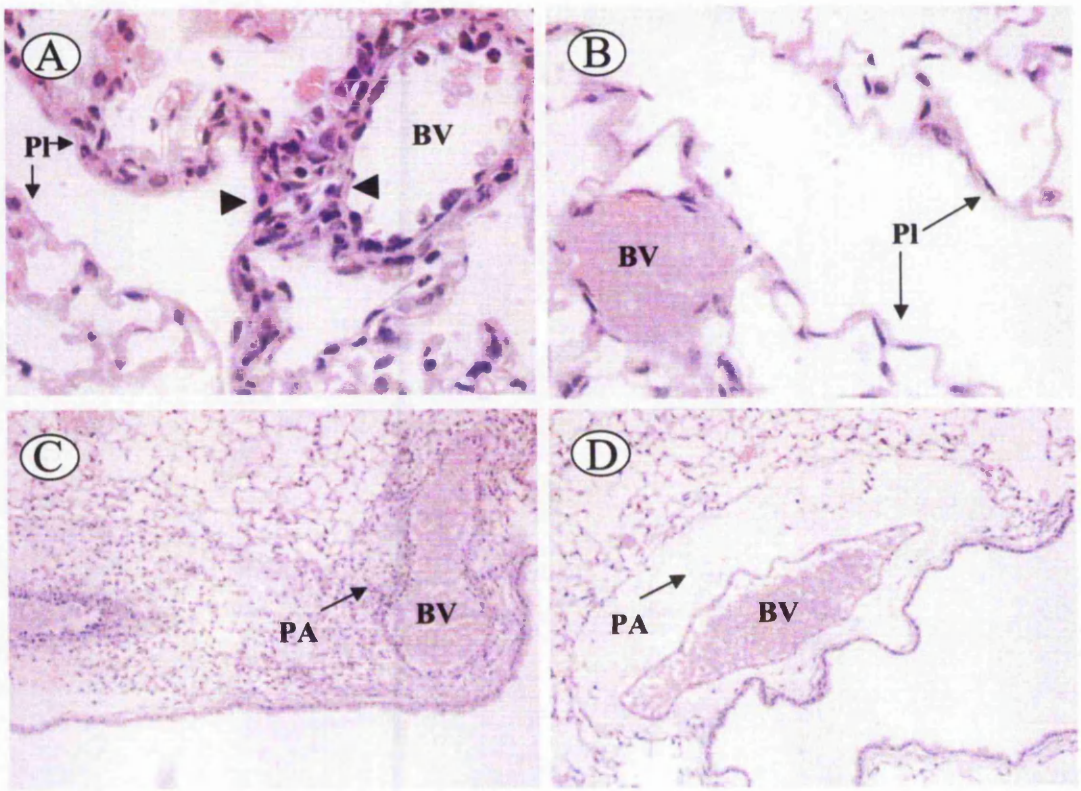


Figure 4.12- Lung tissue sections from mice infected intranasally with either D39 wild type or D39Δ*htrA* mutant.

Mice were sacrificed 48 h post-infection and their lungs removed, fixed, and stained with H+E. The sections show a blood vessel (BV) in close proximity to the pleural interface (PI). The pleural interface is swollen with inflammatory cells in the lungs infected with D39 shown in (A) but is narrow and healthy in the lungs infected with the D39Δ*htrA* mutant shown in (B). Inflammatory cells can also be seen marginating out of the blood vessel towards the pleural interface between the two large arrowheads in panel A. The perivascular areas of wild type-infected mice were noticed to have inflammatory cell recruitment (C) whereas in some mutant-infected mice, these perivascular alterations (PA) contained no cells (D). However, this difference in perivascular alterations was not statistically significant.

4.4.7 Nasopharyngeal colonization

The colonization of the nasopharynx by D39Δ*htrA* mutant compared to that of D39 wild type was also studied (figure 4.13). The number of bacteria recovered from the nasopharynx was about the same in both cases early (6 h post-challenge) and late in the infection (48 h post-challenge).

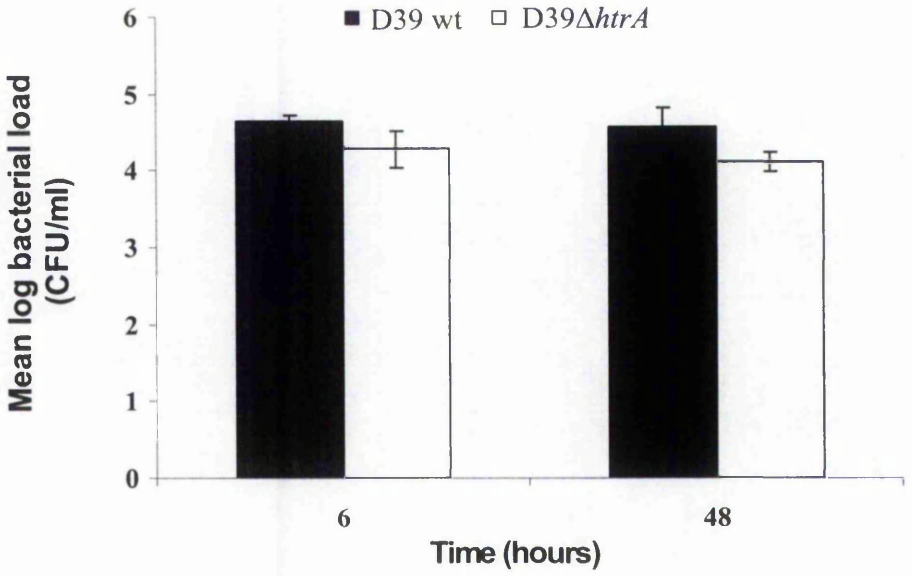


Figure 4.13- Nasopharyngeal colonization with either D39 wild type or D39Δ*htrA* mutant.

Groups of mice were infected with 10^6 CFU/mouse (n=5) of either D39 wild type or D39Δ*htrA* mutant. At the indicated time points, bacteria colonizing the nasopharynx were collected and counted on BAB plates. Data given are the mean ± SEM of bacterial counts in individual mice.

4.4.8 Effect of complementation with HtrA on virulence

To confirm that the attenuation of virulence in the D39Δ*htrA* mutant was entirely due to lack of HtrA, this mutant was complemented with the pAL2-HtrA plasmid, which expresses HtrA constitutively from the Gram-positive *ami* promoter. When the HtrA level was restored in the D39Δ*htrA* mutant, the strain was again fully virulent in a pneumonia model of infection (figure 4.14). The virulence phenotype of D39Δ*htrA* mutant is therefore confirmed to be solely due to deletion of *htrA*.

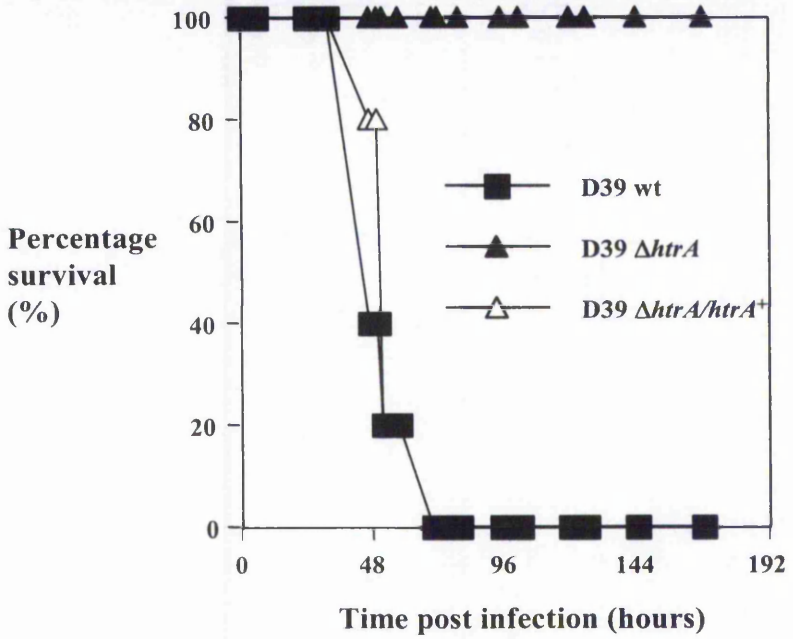


Figure 4.14- Effect of complementation with HtrA on virulence of D39Δ*htrA* mutant.

D39Δ*htrA* strain showed attenuation in virulence as judged by survival time of mice (n=5) after intranasal infection with 10^6 CFU/mouse. Complementation with HtrA (from plasmid pAL2-HtrA) reverted this strain to full virulence.

Chapter 4 Discussion

Members of the HtrA class of stress response proteins are serine proteases, which apparently function by degrading oxidatively or otherwise damaged proteins before they can accumulate to toxic levels in the cells. Homologues of HtrA have been described in a wide range of organisms including bacteria, yeast, plants, and humans (Zumbrunn and Trueb, 1996, Ponting, 1997, Gray *et al.*, 2000). Some bacteria have more than one paralogue of HtrA (Noone *et al.*, 2001). In all cases, the evidence points to a major role for these proteases in helping organisms to survive environmental stresses such as elevated temperature, oxidative and osmotic stresses. HtrA is of interest because HtrA-null mutants of several Gram-negative and Gram-positive bacteria have been shown to be attenuated in animal models of infection (Elzer *et al.*, 1996, Li *et al.*, 1996, Jones *et al.*, 2001, Cortes *et al.*, 2002) and it can also be used as vaccine (Pallen and Wren, 1997).

Identification of pneumococcal HtrA

An HtrA homologue has been identified in *Streptococcus pneumoniae* (Gasc *et al.*, 1998). Searching of the *S. pneumoniae* strain TIGR4 sequence database (at http://www.tigr.org/) revealed a gene for HtrA about 1.2 kb that encodes for a 42-kDa protein. The BLASTP search (Altschul *et al.*, 1990) was used to compare the amino acid sequence homology of pneumococcal HtrA to that of other serine proteases. The pneumococcal HtrA homologue revealed extensive amino acid sequence identity with *Streptococcus mitis* (95.7%), *Streptococcus mutans* (53.6%), *Lactococcus lactis* (55.2%), *Streptococcus pyogenes* (53.7%), *Bacillus subtilis* (42.2%), *Staphylococcus aureus* (36%), *Listeria monocytogenes* (46.3%), and *E. coli* (36.8%).

HtrA has an N-terminal protease domain and a single C-terminal PDZ domain in addition to the transmembrane domain located near the N-terminal by which HtrA is anchored to the membrane. The protease domain contains the conserved sequence GNSGGPL, which is similar to the consensus sequence GDSGGPK surrounding the active site serine residue of many trypsin-like serine proteases (Lipinska *et al.*, 1990). It also contains the catalytic triad of His, Ser, Asp residues. The PDZ domain of HtrA protease plays a critical role in substrate recognition by binding misfolded proteins. PDZ domains are identified as 80-100 residue repeats and named after the three eukaryotic proteins, PSD-95 (post-synaptic density), Dgl (Discs large), and ZO (zonula occludens) (Ponting, 1997). They generally mediate protein-protein interactions by specific binding to the last several residues at the C-terminal ends of target proteins (Doyle *et al.*, 1996, Harrison, 1996, Songyang *et al.*, 1997, Harris and Lim, 2001, Walsh *et al.*, 2003, Wilken *et al.*, 2004), and some protease PDZ domains appear to function in substrate recognition and tethering (Beebe *et al.*, 2000,

Spiers *et al.*, 2002). Other protease PDZ domains, however, seem to negatively regulate activity by blocking access to the active sites of the protease domain. For example deletion of the HtrA2 PDZ domain enhances degradation of a non-specific substrate (Clausen *et al.*, 2002, Krojer *et al.*, 2002, Li *et al.*, 2002).

Construction of mutants

To study the role of HtrA in *S. pneumoniae* stress response, Δ*htrA* mutants were constructed in two different backgrounds by insertion of a spectinomycin resistance cassette into the gene and inactivation of the wild type copy by double-crossover homologous recombination. Prior to studying the *in vitro* and *in vivo* phenotypes, mutant organisms were analyzed for their ability to grow in the presence of an antibiotic selection and also were confirmed to be *S. pneumoniae* using the relevant tests mentioned in section 2.3.

Sensitivity of *htrA* mutant to high temperature and peroxide

The HtrA proteases are essential for the removal of misfolded and aggregated proteins in the periplasm of *E. coli* (Strauch *et al.*, 1989) and many other Gram-negative bacteria and, therefore, promoting growth at elevated temperature, oxidative stress, or any other conditions that may result in protein damage. HtrA orthologs have been also identified in many Gram-positive bacteria (Poquet *et al.*, 2000, Jones *et al.*, 2001, Noone *et al.*, 2001, Sebert *et al.*, 2002). These bacteria lack the outer membrane of Gram-negative bacteria, and as a consequence, lack the periplasmic space. Thus, the folding of the secretory proteins is likely to occur at the membrane-cell wall interface following translocation across the cytoplasmic membrane (Tjalsma *et al.*, 2000, van Wely *et al.*, 2001). HtrA in Gram-positive bacteria is thought to be anchored to the membrane by a single transmembrane domain located near the N-terminous of the protein (Poquet *et al.*, 2000, Noone *et al.*, 2001).

The results in this chapter indicate that HtrA plays a role in resistance to elevated temperature and oxidative stress. However, the effect of a lack of HtrA at higher temperature was to slow growth rather than prevent it and mutant organisms still reached the same final optical density the wild type reached. HtrA appears to have specific functions in different microorganisms. For instance, while HtrA was found to be essential for bacterial survival at an elevated temperature in many bacteria (Seol *et al.*, 1991, Jones *et al.*, 2001, Cortes *et al.*, 2002, Foucaud-Scheunemann and Poquet, 2003, Wonderling *et*

al., 2004), the *Salmonella enterica* serovar Typhimurium Δ*htrA* mutants have been shown to be temperature insensitive (Johnson *et al.*, 1991). Furthermore, inactivation of the *ykdA/htrA* in *Bacillus subtilis* unexpectedly improves survival to heat shock and H_2O_2 exposure due to overexpression of the other genes (Noone *et al.*, 2001). There is also a variation of phenotypes associated with *htrA* deletions. Elzer *et al* reported that *Brucella abortus htrA* mutants were sensitive to both temperature and oxidative stress (Elzer *et al.*, 1996), but Tatum and colleagues observed that *B. abortus htrA* mutants were insensitive to both stimuli (Tatum *et al.*, 1994). The effect of HtrA on protection from oxidative stress was observed for hydrogen peroxide but not for parquat. Paraquat is taken up by bacterial cells and generates superoxide within the cytoplasm (Hassett *et al.*, 1987). This suggests that HtrA provides resistance to hydrogen peroxide but not to superoxide probably because superoxide is generated inside the cells while HtrA is anchored to the membrane. Skorko-Glonek and co-workers have reported a similar finding where the *E. coli* HtrA was found to participate in bacterial survival against ferrous ions and cumene hydroperoxide but not against H_2O_2 or paraquat (Skorko-Glonek *et al.*, 1999). Resistance to oxidative stress is known to be mediated in part through the Mn^{2+} ABC transport system encoded by the Psa operon (Tseng *et al.*, 2002). This operon is known to control the level of expression of superoxide dismutase and NADH oxidase (Tseng *et al.*, 2002) and is involved in virulence. Whether there is an interaction between HtrA and the Psa proteins remains to be determined.

Effect of *htrA* deletion on transformation

HtrA also plays a role in the transformation of the pneumococcus. Deletion of the gene for HtrA resulted in an approximate 32-fold decrease in transformation efficiency, which could be restored by expression of HtrA from a plasmid. Competence development is controlled in *S. pneumoniae* by the quorum-sensing signal, competence-stimulating peptide (CSP) (Havarstein *et al.*, 1995), which is derived from a ribosomally synthesized precursor, ComC (Morrison, 1997). CSP is sensed by the ComDE two-component system (Havarstein *et al.*, 1996, Pestova *et al.*, 1996). A second system, CiaR/H operates upstream of the ComDE pathway and has been shown to modulate competence (Guenzi *et al.*, 1994, Echenique *et al.*, 2000). The CiaR/H system also positively regulates HtrA, as Δ*ciaR/H* mutants are characterized by strongly decreased expression of HtrA (Sebert *et al.*, 2002, Mascher *et al.*, 2003). The possibility raised by Sebert and co-workers (Sebert *et al.*, 2002) that HtrA might play a role in the competence pathway is therefore confirmed. However, the presence of HtrA seems to be required for transformation, rather than for inhibition of

competence. It may be that HtrA is required for the correct folding of one of the protein components of the competence pathway.

The role of HtrA in pneumococcal pathogenesis

HtrA is known to be involved in the virulence of many Gram-negative bacteria such as *Salmonella enterica* serovar Typhimurium (Baumler *et al.*, 1994), *Brucella abortus* (Elzer *et al.*, 1996), *Yersinia enterocolitica* (Li *et al.*, 1996) and *Klebsiella pneumoniae* (Cortes *et al.*, 2002). This protease is required for full virulence of the Gram-positive bacterium *Streptococcus pyogenes* (Jones *et al.*, 2001). A *S. pneumoniae* strain lacking the *htrA* gene showed a decreased fitness in a competitive model of colonization (Sebert *et al.*, 2002). HtrA was identified as a virulence factor of the pneumococcus in a signature tagged mutagenesis screen (Hava and Camilli, 2002). The *in vivo* data presented in this chapter clearly indicate that HtrA-negative mutants of *S. pneumoniae* are dramatically reduced in virulence. The effect of a *htrA* deletion was tested in two strains of the pneumococcus. In D39, deletion of the gene completely attenuated the organism. All animals infected with the Δ*htrA* mutant survived the challenge whereas those infected with the wild type succumbed to the infection. In the case of TIGR4 strain, virulence was dramatically reduced, although using higher doses of the more virulent TIGR4 strain did result is some disease in the Δ*htrA* group. Analysis of the infection by measuring bacterial loads in different locations showed that the mutant organism could not survive in the lung and was cleared rapidly from the air spaces. The mutant never invaded the bloodstream from the lungs. The wild type organism grew dramatically to more than 10^6 CFU/ml in the lung tissue by 48 h post-infection while the number of mutant organisms was less than 10^3 CFU/ml at the same time point. Furthermore, wild type bacteria appeared in the bloodstream 18 h post challenge and multiply rapidly to more than 10^6 CFU/ml by 48 h while the mutant organism was not detected in blood at any of the time points studied. To investigate whether the role of HtrA was specific to the lungs, the organisms were injected directly into the bloodstream to bypass the respiratory tract. The wild type strain overcome the host defenses and was able to multiply in blood to a level of 10^5 CFU/ml by 36 h post-infection causing bacteraemia that led to the death of all animals infected. The mutant strain, on the other hand, was unable to survive in the blood, the bacterial load declined from more than 10^4 CFU/ml to levels just above the limit of detection of the assay (about 10^2 CFU/ml) and the mutant-infected mice were healthy at the endpoint of the challenge. The Δ*htrA* mutant strains are therefore potential live vaccine candidates. The *in vivo* phenotype of the D39Δ*htrA* mutant appears to be consistent with the proposed function of

bacterial HtrA proteases as component of a line of defense against oxidative damage caused by the host. However, an *htrA* mutant of strain of *Brucella abortus* was reported to be dispensable for virulence in a mouse model (Phillips and Roop, 2001). This *htrA* mutant was not attenuated in BALB/c mice through 4 weeks post-infection despite being more sensitive to oxidative killing and less resistant to killing by cultured murine neutrophils and macrophages *in vitro* than the virulent parent strain.

Colonization of the nasopharynx is an important step in pneumococcal pathogenesis. Using the MF1 mouse model in this study, no difference was observed between the wild type and mutant strains in the number of bacteria colonizing the nasopharynx early or late in the infection (6 h and 48 h post challenge respectively). Sebert and colleagues reported that an *htrA* mutant of *S. pneumoniae* 0100993 strain (type 3) showed decreased fitness in a competitive model of colonization using newborn Sprague-Dawley rats (Sebert *et al.*, 2002). This again may indicate that HtrA proteases have specific functions in different strains or backgrounds (e.g. the virulence of *htrA* mutants was slightly different between D39 and TIGR4 strains, see figure 4.8) or it could simply be attributed to the difference in animal models used. This could also be explained by the difference between the competitive infection assay used in the study by Sebert and co-workers (Sebert *et al.*, 2002) and the direct infection assay used here.

Altered inflammatory response to D39Δ*htrA* mutant

Pneumococcal infection is characterized by an intense inflammatory response that is coordinated mainly by cytokines. Tumor necrosis factor (TNF) is a pro-inflammatory cytokine that exerts powerful effects on the immune system including the release of other pro-inflammatory cytokines, activation of neutrophils, and the induction of adhesion molecules, which lead to a rapid attraction of inflammatory cells to the inflammatory site (Ming *et al.*, 1987, Neumann *et al.*, 1996). TNF has been implicated as a central mediator of the host response to bacterial infection, in which it may play a dual role. Systemic and excessive release of TNF into the circulation is harmful to the host, as documented by numerous laboratory studies in which anti-TNF strategies prevented death during otherwise rapidly fatal sepsis (Tracey *et al.*, 1987, Hinshaw *et al.*, 1990). However, local production of TNF, at the site of an infection, is important for adequate antibacterial defense. Following recruitment of phagocytic cells, TNF can promote antimicrobial activity by activating the respiratory burst of neutrophils (Dusi *et al.*, 1996) and by activating the capacity to degranuolate (Klebanoff *et al.*, 1986). Moreover, this importance has been demonstrated in murine models of pneumonia in which treatment with anti-TNF

impaired host defense against various respiratory pathogens, including *S. pneumoniae* (van der Poll *et al.*, 1997a, Rijneveld *et al.*, 2001). Reduced capacity of CBA/Ca mice to induce rapid TNF-α activity within lung airways following infection with *S. pneumoniae* was reported to be a factor in their elevated susceptibility to pneumococcal pneumonia (Kerr *et al.*, 2002). In light of its presumed detrimental role in the pathogenesis of overwhelming sepsis, anti-TNF has been evaluated in a fairly large number of controlled clinical trials of patients with sepsis. Although individual trials did not reveal a significant benefit for patients treated with anti-TNF, pooled data from trials that evaluated monoclonal antibodies directed against TNF demonstrated a statistically significant reduction in mortality (Marshall, 2000). In another study, the effect that anti-TNF therapeutically administered together with antibiotics has in mice with ongoing pneumococcal pneumonia was determined. Treatment with anti-TNF was associated with both an enhanced inflammatory response in lung tissue and unaltered or modestly reduced IL-6 level. It was also associated with a diminished effect of the antibiotic, ceftriaxone (Rijneveld *et al.*, 2003).

The cytokine interleukin 6 (IL-6) has been shown to have multiple biological activities against many cellular targets. It has been ascribed both pro- and anti-inflammatory effects. IL-6 can activate monocytes (Borish *et al.*, 1989) and can synergize with TNF to increase the respiratory burst of neutrophils *in vitro* (Mullen *et al.*, 1995). This synergy between IL-6 and TNF-α may represent an important mechanism by which IL-6 potentiates the effect of TNF-α *in vivo*. However, in pneumococcal pneumonia IL-$6^{-/-}$ mice have been shown to mount significantly higher levels of inflammatory responses in comparison to wild-type animals (van der Poll *et al.*, 1997b). IL-6 may play a significant role in host defense against bacterial infection (van der Poll *et al.*, 1997b) whereas previously it was considered merely a marker for the severity of the bacterial challenge.

In this study, the measurement of the cytokines IL-6 and TNF-α was used as an indicator of the overall pattern of inflammatory cytokine release after stimulation with either wild type or Δ*htrA* mutant strains. Analysis of the inflammatory response showed that a Δ*htrA* mutant induced markedly less inflammation than D39. These results are complicated by the differences in number of organisms present in the wild type and mutant infected lungs and the lack of inflammatory cytokine production induced by the mutant may simply be a reflection that the number of organisms present is below that needed to trigger the response. However, the number of organisms present in the lavage fluid at 24 h was very similar between wild type and mutant but the mutant still does not induce IL-6 or TNF-α

production. Histological examination of the tissues confirms an altered host response to the mutant. Lungs exposed to the mutant organism have much fewer lesions of inflammatory cells at the pleural interface. A possible use of pneumococcal *htrA* mutant as a live vaccine should therefore be considered. As both the *htrA* mutant and the wild type strains similarly produce pneumolysin (Ply) *in vitro* and Ply is known to induce the inflammatory response to the pneumococcus, it is likely that the effect of Ply in the *htrA* mutant is masked somehow *in vivo*. The possibility of pneumolysin being not produced or released by the mutant strain *in vivo* is yet to be investigated.

Changes in inflammatory response and tissue histology may reflect either a direct action of the serine protease on some unknown substrate or may reflect altered expression of other virulence factors. With this in mind, the expression of several known virulence factors of the pneumococcus was examined. No effect of the *htrA* mutation could be found on the amount of pneumolysin, autolysin, CbpA (PspC), hyaluronidase or neuraminidase made by the organism. Recent work on *L. lactis* showed that in an HtrA-null mutant several surface proteins failed to be properly processed (Poquet *et al.*, 2000). Therefore the distribution and activity of several pneumococcal surface proteins in Δ*htrA* mutant were examined. Cellular fractionation and Western blotting showed there was no difference in the distribution of CbpA, hyaluronidase and neuraminidase between the wild type and Δ*htrA* mutant. Moreover, there was no difference between wild type and Δ*htrA* mutant in the amount of neuraminidase and hyaluronidase enzymatic activity associated with the different cell fractions. Mutagenesis of either the gene for neuraminidase (*nanA*) or that for hyaluronidase (*hyl*) had no significant impact on virulence (Berry and Paton, 2000). Other studies have showed that these enzymes are involved in pneumococcal invasion (Berry *et al.*, 1994) and in colonization (Tong *et al.*, 2000). It is thought that hyaluronidase might contribute directly to invasion as it hydrolyzes hyaluronic acid, which is an important component of connective tissues. It has also been suggested that neuraminidase activity promotes colonization by exposing host cell receptors otherwise covered by sialic acid (Tong *et al.*, 2001). HtrA therefore does not appear to play a role in cell surface protein location and activity, at least for the proteins examined. The HtrA homologue in *S. pyogenes* was also shown not to affect cell surface protein expression (Jones *et al.*, 2001).

Recently, Lyon and co-workers reported that in-frame deletion of *htrA* of *Streptococcus pyogenes* did not result in attenuation in a murine model of subcutaneous infection (Lyon and Caparon, 2004) while the insertional mutagenesis of *htrA* of the same organism has been associated with a reduced capacity to cause disease in an animal model of systemic

infection (Jones *et al.*, 2001) raising the issue of the possibility of polar effect of mutation on the downstream genes (e.g. *spoJ*). To address this, the Δ*htrA* mutant was complemented with pAL2-HtrA plasmid, which constitutively expresses HtrA and the virulence of the complemented strain was tested in a pneumonia model of infection. When HtrA level was corrected, the strain was again fully virulent suggesting that the observed virulence phenotype of the mutant is solely due to absence of HtrA. In addition, microarray analysis of Δ*htrA* mutant also suggested that the expression of downstream genes was almost identical to those of the wild type confirming no polar effect of *htrA* mutation occurred (see chapter 6).

Summary

Data in this chapter demonstrate that HtrA is a major virulence factor of *S. pneumoniae*. Deletion of the gene for HtrA from strain D39 (serotype 2) completely abolished its virulence in mouse pneumonia and bacteraemia models, while the virulence of the TIGR4 (serotype 4) strain was dramatically reduced. HtrA-negative mutants induced much less inflammation in the lungs during pneumonia than the wild type. HtrA is involved in the ability of the pneumococcus to grow at higher temperatures, to resist oxidative stress and to undergo genetic transformation. The expression and cellular location of several known virulence factors of the pneumococcus were not affected by the lack of HtrA.

Chapter 5

RELATIONSHIP BETWEEN HtrA AND THE TWO-COMPONENT SYSTEM CiaR/H, RESULTS

Signal transduction across biological membranes is essential in enabling bacterial cells to identify and respond to environmental stimuli. Recent studies have demonstrated the role of bacterial two-component signal-transducing systems (TCSTS) in mediating adaptive responses to environmental signals (Parkinson, 1993, Stock *et al.*, 1995). The pneumococcal CiaR/H two-component system (figure 5.1) consists of a sensor histidine kinase, CiaH, anchored in the cell membrane and a cytoplasmic response regulator, CiaR which is a DNA-binding protein involved in the regulation of genes in response to environmental signals sensed by CiaH (Guenzi and Hakenbeck, 1995). This system was identified as one of thirteen TCSTS in two genomic screens (Lange *et al.*, 1999, Throup *et al.*, 2000). Pleotropic effects caused by *cia* mutations in the pneumococcus include sensitivity to cefotaxime, the ability to form protoplasts, susceptibility to lysis by deoxycholate (Giammarinaro *et al.*, 1999) and tendency to early lysis (Lange *et al.*, 1999). CiaH mutants also have a transformation deficiency (Giammarinaro *et al.*, 1999, Hakenbeck *et al.*, 1999, Echenique *et al.*, 2000). *In vivo*, the CiaR/H has been shown to contribute to colonization of the mouse lung (Throup *et al.*, 2000) and the nasopharynx of infant rats (Sebert *et al.*, 2002) and to be involved in systemic infection in mice (Marra *et al.*, 2002a). Previous studies have shown that the CiaR/H regulon contains many genes, including the High Temperature Requirement A gene and that the *htrA* gene is highly down-regulated in CiaR-null mutants (Sebert *et al.*, 2002, Mascher *et al.*, 2003). These studies demonstrated that the regulation of *htrA* is probably direct as CiaR was shown to bind to DNA upstream of the *htrA* gene.

Data in the previous chapter demonstrated that HtrA is a crucial virulence factor of the pneumococcus. As HtrA is down regulated in Δ*ciaR* mutants, the study presented in this chapter was intended to investigate to what degree the phenotype resulting from deletion of *ciaR* can be attributed to HtrA. The Δ*ciaR* mutant was complemented with HtrA that was constitutively expressed from a plasmid and the effect of this complementation on the *in vitro* phenotype and virulence was studied.

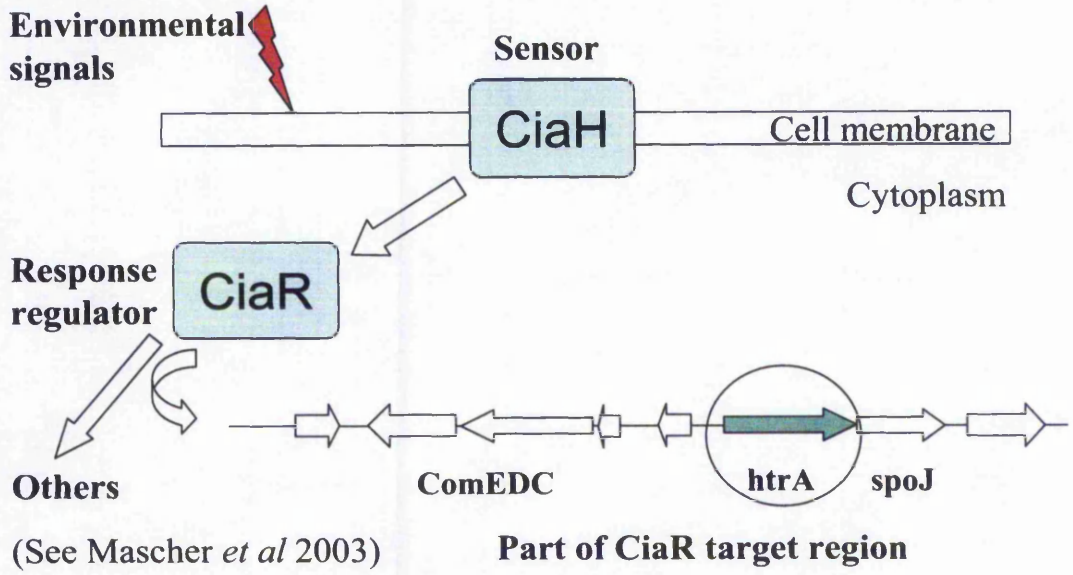


Figure 5.1- The CiaR/H two-component system.

The environmental signal is sensed by the membrane histidine kinase CiaH, which then transmits the signal to the cytoplasmic response regulator CiaR which in turn mediates the appropriate response to this signal.

5.1 Construction of plasmid pAL2-HtrA

Plasmid pAL2 (Beard *et al.*, 2002) was used for constitutive expression of *htrA* from the pneumococcal *ami* promoter (Alloing *et al.*, 1990). The pAL2 plasmid (~12.5 kb, see appendix) was kindly provided by Dr. V. Salisbury (University of the West of England). The gene for HtrA containing 5' and 3' *Eco*RI sites and a ribosome binding site (RBS) at the 5' end was amplified with PCR (Figure 5.2A) and the pAL2 plasmid was digested with *Eco*RI to release the lux operon (figure 5.2B). The *htrA* gene was then ligated to the *Eco*RI-digested pAL2 fragment, which carries the *ami* promoter to create plasmid pAL2-HtrA. The construction of pAL2-HtrA was confirmed by restriction digestion and the orientation of *htrA* in the plasmid was confirmed by PCR as described below. The *htrA* gene was also sequenced using the forward primer pAL2Y1 upstream from *htrA* and the reverse primer HtrA2 for further confirmation.

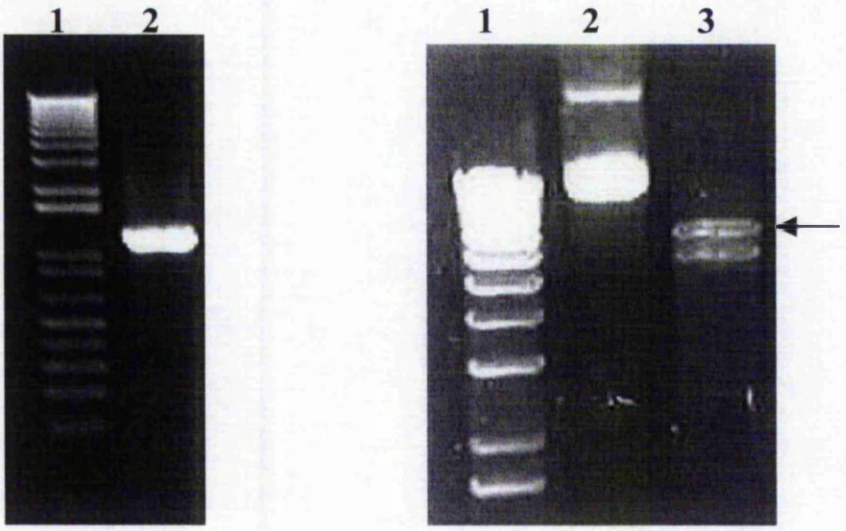


Figure 5.2- Construction of pAL2-HtrA plasmid used for expression of HtrA in *S. pneumoniae*.

Agarose gel electrophoresis of DNA products used for construction of the pAL2-HtrA plasmid. In A, 1 kb plus ladder is shown in lane 1 and an amplified fragment containing *htrA* gene is shown in lane 2 (1.2 kb). In B, digestion of pAL2 with *Eco*RI. Lane 1, 1 kb plus ladder; lane 2, uncut plasmid (12.5 kb); lane 3, *Eco*RI-cut pAL2. The upper band indicated by the arrow (~7 kb), which contains the *ami* promoter, was used for ligation.

5.1.1 Confirmation of pAL2-HtrA

After ligation of *htrA* to pAL2, the resulting plasmid was propagated in *E. coli* Top10 electrocompetent cells (Invitrogen) using electroporation and the plasmid was prepared from transformed cells by using a miniprep kit (Qiagen). The construction of plasmid pAL2-HtrA was confirmed by restriction digestion with *Eco*RI. The restriction digestion reactions were analyzed by running on 1 % agarose gels. From the six clones analyzed, three carried the *htrA* gene, clones 2, 3 and 5 as shown (figure 5.3) in lanes 3,4 and 6.

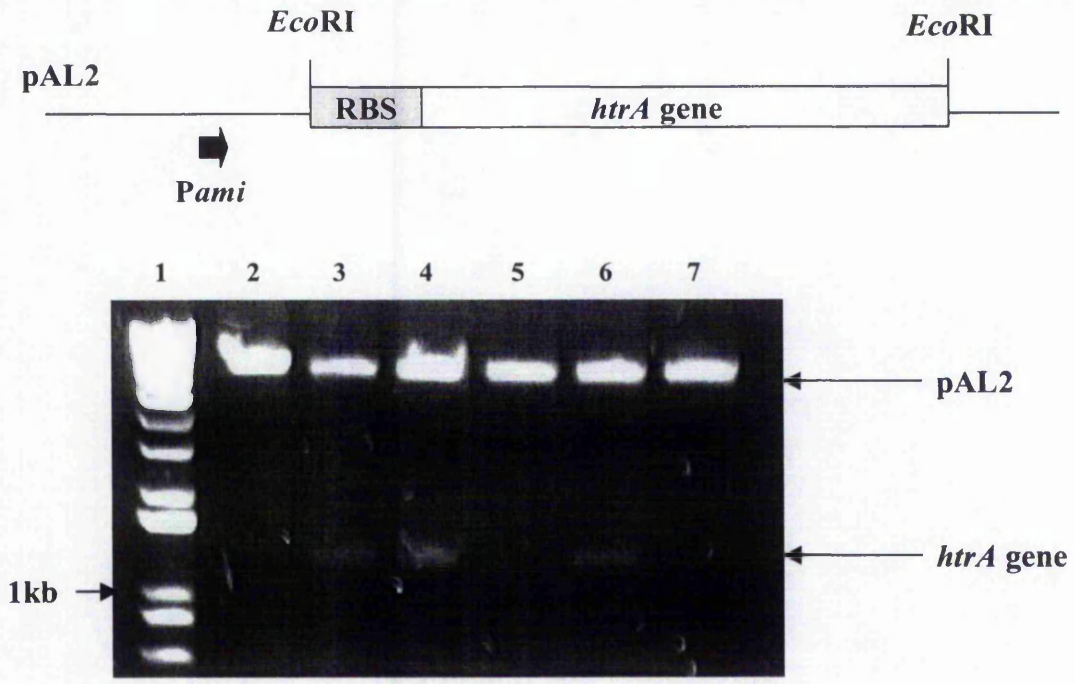


Figure 5.3- Confirmation of pAL2-HtrA plasmid by restriction digestion.

Agarose gel electrophoresis of *Eco*RI-digested pAL2-HtrA plasmids. Lane 1, 1 kb plus ladder; lanes 2-7 plasmids from different bacterial clones. Plasmids 2, 3, and 5 were positive (shown in lanes 3, 4 and 6). RBS, Ribosome Binding Site. P*ami*, the *ami* promoter.

5.1.2 Orientation of htrA in pAL2-HtrA plasmid

For expression of *htrA* from the *ami* promoter in pAL2-HtrA, the gene should be in the proper orientation with respect to the *ami* promoter. This was confirmed by PCR using primer pair pAL2Y1 and pAL2Y2 (table 2.2). The forward primer pAL2Y1 was designed to anneal upstream from *htrA* and downstream from the *ami* promoter. The sequence of the reverse primer pAL2Y2 was within the *htrA* gene. A PCR product of 600 bp would result from the *htrA* gene in the correct orientation whereas no product would be expected from the gene in the opposite orientation. The forward and reverse primers HtrA for and HtrA rev (table 2.2), which amplify the whole gene were also used to confirm the presence of the *htrA* gene. Analysis of PCR products using agarose gel electrophoresis revealed that plasmids from clones 2 and 3 were confirmed to carry *htrA* gene in the correct direction while the plasmid from clone 5 contained the gene in the opposite orientation (figure 5.4).

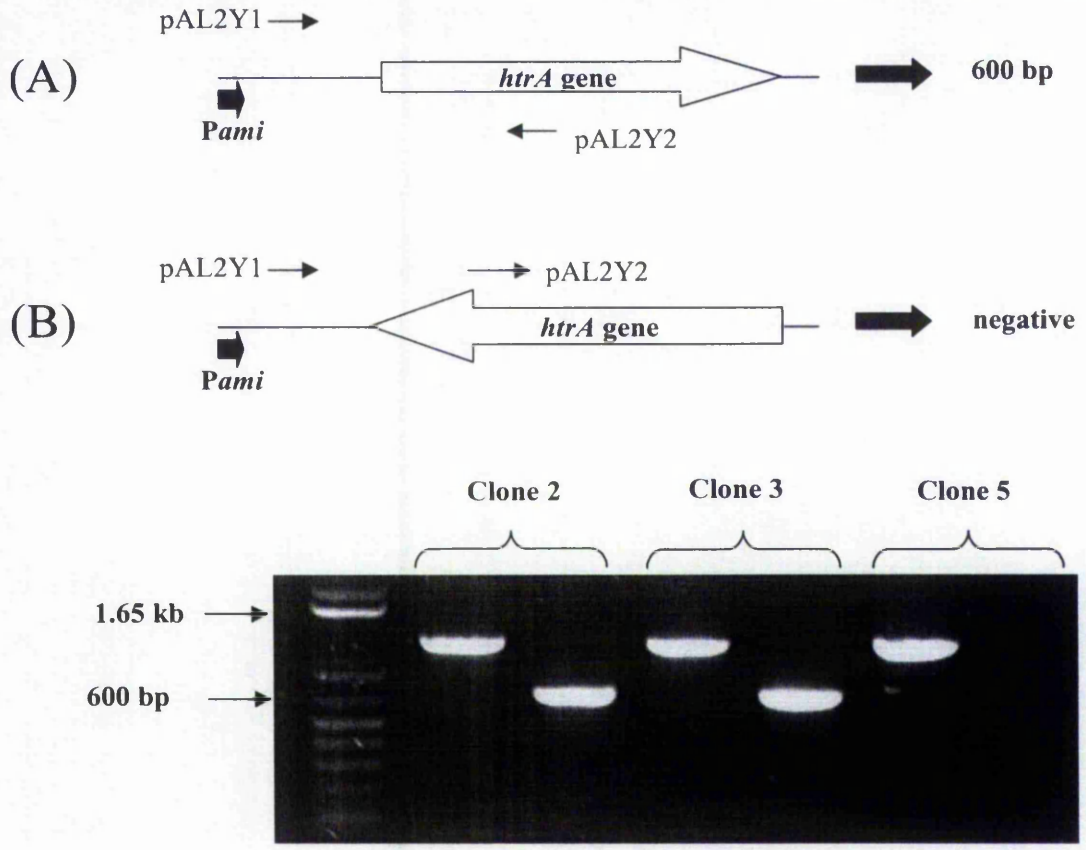


Figure 5.4- Determination of *htrA* gene orientation in pAL2-HtrA plasmid.

Agarose gel electrophoresis of PCR reactions carried out for determining the orientation of the *htrA* gene in pAL2-HtrA plasmids. Clones 2 and 3 gave 1.2-kb bands using primers HtrA for and HtrA rev and 600 kb fragments when using pAL2Y1 and pAL2Y2 primers. This confirms the orientation shown in A. Clone 5 gave a 1.2-kb band using the gene specific primers confirming the presence of *htrA*, but no product was found when using pAL2Y1 and pAL2Y2 primers, suggesting that *htrA* is found in the orientation shown in B. P*ami*, the *ami* promoter.

5.2 CiaR mutation and complementation with HtrA

The *ciaR* mutation in strain 0100993 (type 3) was made by allelic replacement with an erythromycin resistance marker and was kindly provided by Martin Burnham (Throup *et al.*, 2000). In order to perform the complementation experiments with the pAL2-HtrA plasmid (erythromycin selection) another antibiotic marker was needed to disrupt the *ciaR* gene. Therefore, the *ciaR* mutation with the spectinomycin resistance gene was moved into D39 (type 2) from the Δ*ciaR* mutant strain cia spc 136b (Martin *et al.*, 2000) kindly provided by Jean-Pierre Claverys (Toulouse, France). A single colony from cia spc 136b strain streaked on blood agar plates was picked and used to inoculate BHI. After overnight incubation at 37 °C, genomic DNA was prepared and used as a template in PCR reactions. Primer pair MP144 and MP145 (Martin *et al.*, 2000) (table 2.2) was used in PCR for

amplification of a 6 kb fragment corresponding to the Δ*ciaR* mutation carrying the spectinomycin resistance cassette. This PCR product was used to transform D39 to create the D39Δ*ciaR* mutant strain and transformed cells were selected on spectinomycin agar plates. The genomic DNA was then prepared from transformed cells and PCR was used for confirmation. Using primer pair MP144 and MP145 resulted in a 5 kb fragment from the wild type strain and a 6 kb fragment from Δ*ciaR* mutants (figure 5.5).

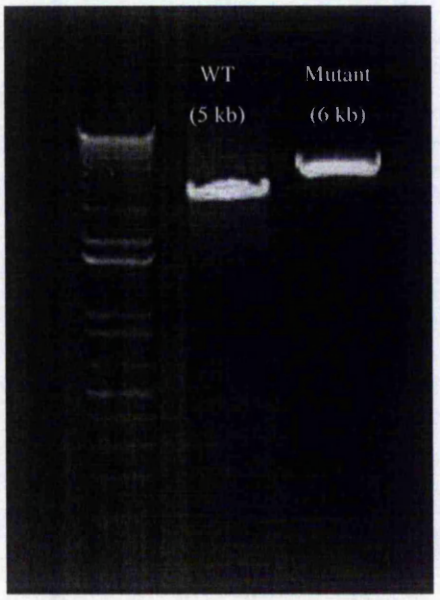


Figure 5.5- Confirmation of introduction of a *ciaR* mutation into D39.

Agarose gel electrophoresis of PCR reactions used for confirming the *ciaR* mutation. Lane 1, 1-kb plus ladder; lane 2, *ciaR* from wild type D39 (5 kb); lane 3, mutated *ciaR* amplified from transformed cells (6 kb).

The CiaR/H-null mutants are known to have a transformation deficiency and therefore difficulty in complementation of the Δ*ciaR* mutant with HtrA from pAL2-HtrA plasmid was expected. To overcome this problem, the pAL2-HtrA plasmid was used to transform wild type D39 strain first and then the *ciaR* mutation was introduced into the resulting strain to create the complemented strain D39Δ*ciaR/phtrA*$^{+}$ in which the HtrA level is corrected by constitutive expression from the pAL2-HtrA plasmid.

5.3 HtrA Western immunoblot analysis

As CiaR is involved in virulence and regulates *htrA*, which has been shown in the previous chapter to be a crucial virulence factor, the question of whether the CiaR phenotype was associated with the down-regulation of *htrA* was addressed. In order to test this, a plasmid was constructed to allow the constitutive expression of *htrA* in the pneumococcus. Plasmid

pAL2-HtrA (section 2.8, figure 2.2) allows the expression of HtrA from the pneumococcal *ami* promoter (Alloing *et al.*, 1990). Western blotting was used to examine the levels of HtrA expressed by pneumococcal strains. Expression of HtrA was shown to be higher at 40 °C than 37 °C in the wild type D39 but not in the D39Δ*ciaR* mutant (figure 5.6 A) confirming HtrA to be a heat shock protein of the pneumococcus and to be up-regulated by CiaR in response to heat shock. As expected, HtrA expression was abolished in D39*ΔhtrA*. Levels of expression of HtrA could be restored in the D39Δ*htrA* mutant to levels similar to wild type with the plasmid pAL2-HtrA. The level of HtrA was reduced but not abolished in Δ*ciaR* and introduction of pAL2-HtrA into the Δ*ciaR* strain resulted in expression of levels of HtrA similar to wild type (figure 5.6 B). Visual examination of these blots suggested that deletion of CiaR resulted in an approximate 4-fold reduction in production of HtrA. Expression of HtrA in the Δ*ciaR* strain from the plasmid returned the level of HtrA to that of the wild type. Expression of HtrA from the plasmid in the wild-type strain did not increase total levels of HtrA.

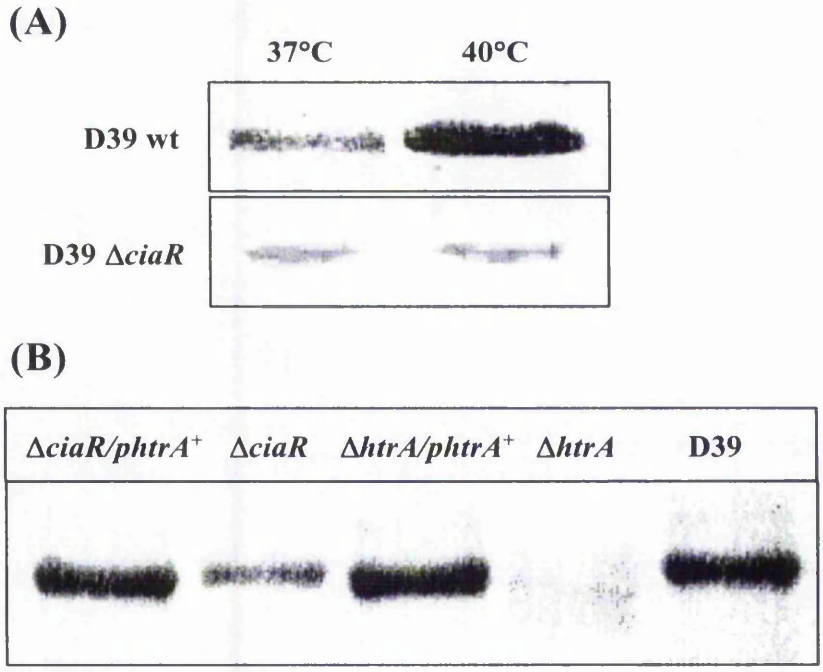


Figure 5.6- Western immunoblot analysis of HtrA levels in different pneumococcal strains.

In (A) expression of HtrA at 37 °C and 40 °C in wild type strain D39 and strain D39Δ*htrA*. In (B) levels of HtrA in strain D39Δ*htrA* and strain D39Δ*ciaR* and their complemented strains grown at 37 °C.

5.4 *In vitro* growth phenotype of Δ*ciaR* mutants.

HtrA has been shown to be involved in the ability of the type 2 pneumococci to grow at an elevated temperature and that the effect of a *htrA* deletion was to slow growth rather than prevent it. The D39Δ*htrA* mutant was also found to have decreased rate of autolysis compared to its parent strain at 40 °C as indicated by viable count at stationary and decline phases of growth (chapter 4, section 4.3.1). The growth phenotype of D39Δ*ciaR* mutants at normal and elevated temperatures was also studied. Similar to D39Δ*htrA*, the growth of D39Δ*ciaR* mutant was less than that of the wild type at 40 °C as judged by OD (figure 5.7 A). D39Δ*ciaR* showed a decreased rate of autolysis after reaching the stationary phase of growth at both 37 °C and 40 °C compared to the D39 wild type (figure 5.7 B). This growth phenotype of strain D39Δ*ciaR* could be restored by increasing HtrA levels in the complemented strain D39Δ*ciaR/phtrA*$^{+}$ (figure 5.7 A&B). Introduction of the pAL2YI vector had no effect on the growth of D39 at either 37 °C or 40 °C (data not shown). Also, introduction of the pAL2-HtrA plasmid into D39 wild type did not result in altered growth at these temperatures (data not shown).

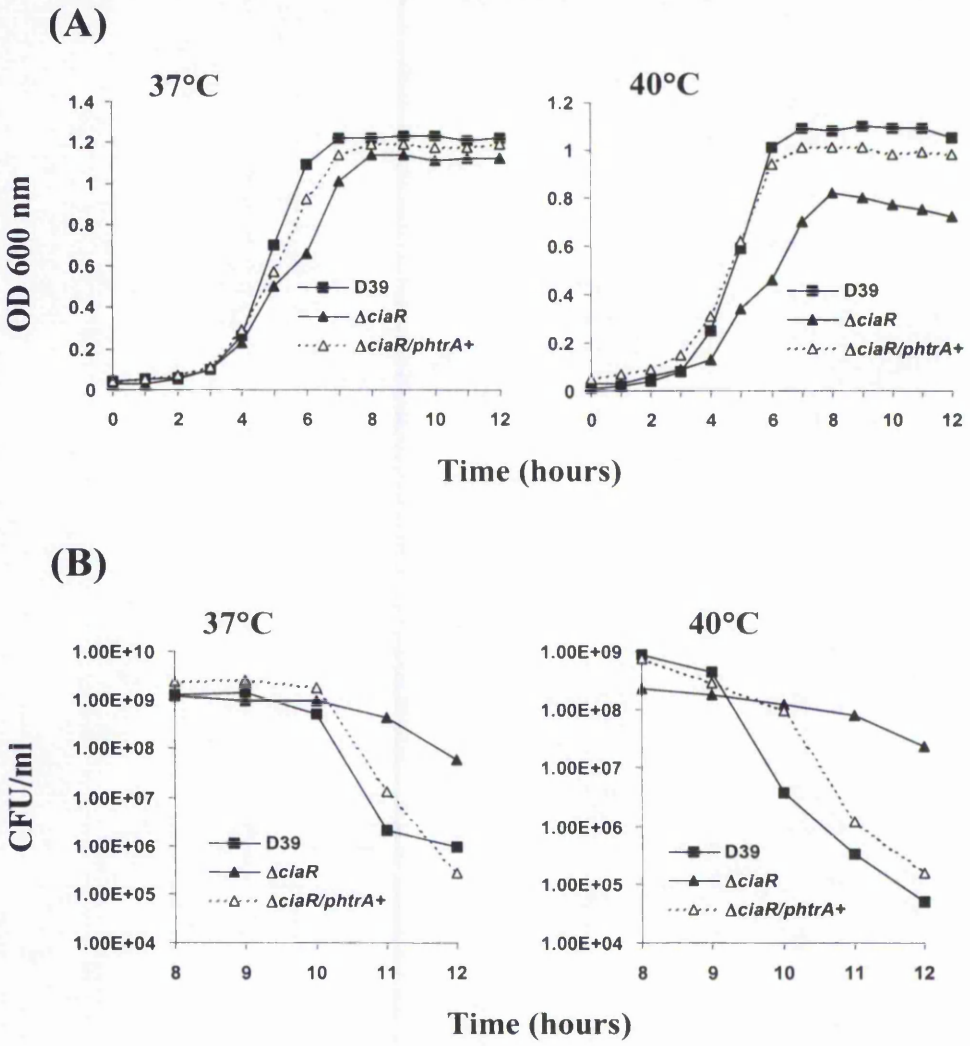


Figure 5.7- *In vitro* growth analysis of Δ*ciaR* mutant in type 2 background.

Growth curves of D39 WT, D39Δ*ciaR* and D39Δ*ciaR/phtrA*$^+$ at 37 °C and 40 °C represented as optical densities at 600nm in (A) and as viable counts after reaching the stationary phase of growth in (B). 10^6 CFU/ml of each strain was used to inoculate BHI prewarmed at the indicated temperatures and samples were withdrawn at 1-hour intervals to measure the OD_{600nm} and viable counts.

The effect of *ciaR* mutation in type 3 strain 0100993 was more dramatic at elevated temperature when compared to a type 2 strain. Growth of the 0100993Δ*ciaR* mutant was similar to the wild type and showed a similar rate of autolysis at 37 °C, whereas growth of the 0100993Δ*ciaR* mutant at 40 °C was prevented by the mutation (figure 5.8).

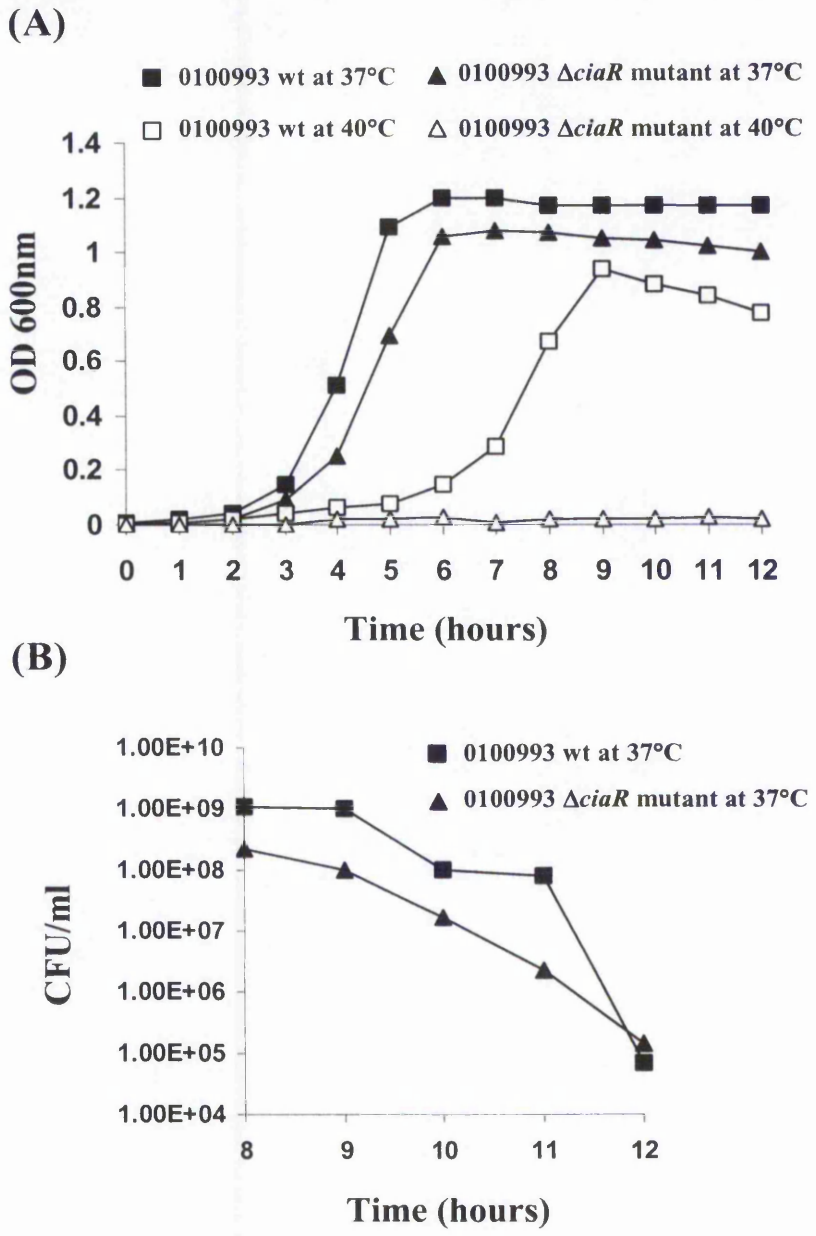


Figure 5.8- *In vitro* growth analysis of Δ*ciaR* mutant in type 3 background.

Growth curves of type 3 strain 0100993 wild type and its Δ*ciaR* mutant at 37 °C and 40 °C represented by OD_{600nm} in (A) and viable count in (B). The growth of the 0100993 Δ*ciaR* mutant was impaired at 40 °C and showed a rate of autolysis similar to that of the wild type at 37 °C.

5.5 Peroxide sensitivity of Δ*ciaR* mutant

HtrA has been shown in the previous chapter (section 4.3.2) to be involved in the ability of D39 to resist oxidative stress posed by hydrogen peroxide. To determine whether reduced expression of HtrA in D39Δ*ciaR* mutant resulted in sensitivity to oxidative stress, the D39Δ*ciaR* mutant strain was compared to the wild type and to the complemented strain

D39Δ*ciaR/phtrA*$^+$ for sensitivity to 40 mM of hydrogen peroxide. The D39Δ*ciaR* strain was significantly more sensitive to peroxide than the parent strain D39 after 10 and 15 min of exposure to hydrogen peroxide. There was no statistically significant difference 5 min after peroxide treatment probably because strain D39Δ*ciaR* still expresses some HtrA. By restoring HtrA to a level similar to the wild type in the complemented strain D39Δ*ciaR/phtrA*$^+$, the strain was again identical to the wild type in response to oxidative stress (figure 5.9). The sensitivity of strain D39Δ*ciaR* to oxidative stress is, therefore, similar to what was observed previously for strain D39Δ*htrA* and could be explained by the down-regulation of HtrA in the CiaR-null mutant.

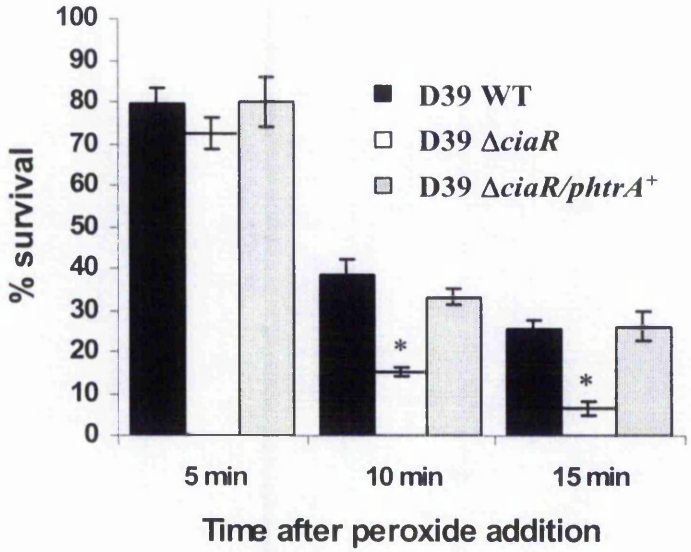


Figure 5.9- Hydrogen peroxide sensitivity of D39Δ*ciaR* mutant.

H_2O_2 sensitivity assay for D39 wild type, D39Δ*ciaR* and D39Δ*ciaR/phtrA*$^+$ strains. 40mM of H_2O_2 was added to 1-ml aliquots of culture grown to OD_{600nm} ~ 0.3. Viable counts were performed on BAB plates before and after the addition of peroxide and the % survival were calculated. Values expressed are the mean (SEM) of three independent experiments. *, $p < 0.05$ lower survival for D39Δ*ciaR* mutant than wild-type.

5.6 *In vivo* analysis of Δ*ciaR* mutant (type 3)

5.6.1 Intranasal challenge

The CiaR/H system has previously been reported to play a role in mouse lung colonization (Throup *et al.*, 2000) and colonization of the nasopharynx of infant rats (Sebert *et al.*, 2002) and has also been identified as playing a role in a mouse model of systemic disease (Marra *et al.*, 2002a). The role of CiaR in the virulence of the pneumococcus was

confirmed and further investigated in the MF1 model of infection. Initially, a Δ*ciaR* mutant on a serotype 3 background was compared with its wild type parent (strain 0100993). This is the same mutant as that used previously (Throup *et al.*, 2000, Sebert *et al.*, 2002) in the studies described above. All animals challenged intranasally with 10^7 CFU of the parent strain were moribund by 144 hours post infection. In contrast, the 0100993 Δ*ciaR* mutant only caused a moribund state in 25 % of the animals (figure 5.10 A). Bacteriological analysis showed that the 0100993 Δ*ciaR* strain grew to a viable count of approximately 10^3 CFU in the lung compared to 10^6 for the wild type organism. The level of bacteraemia caused by the 0100993 Δ*ciaR* strain was below the limit of detection of the assay (approximately 100 organisms per ml) (figure 5.10 B). CiaR was also confirmed to play a role in the virulence of strain D39 (see below).

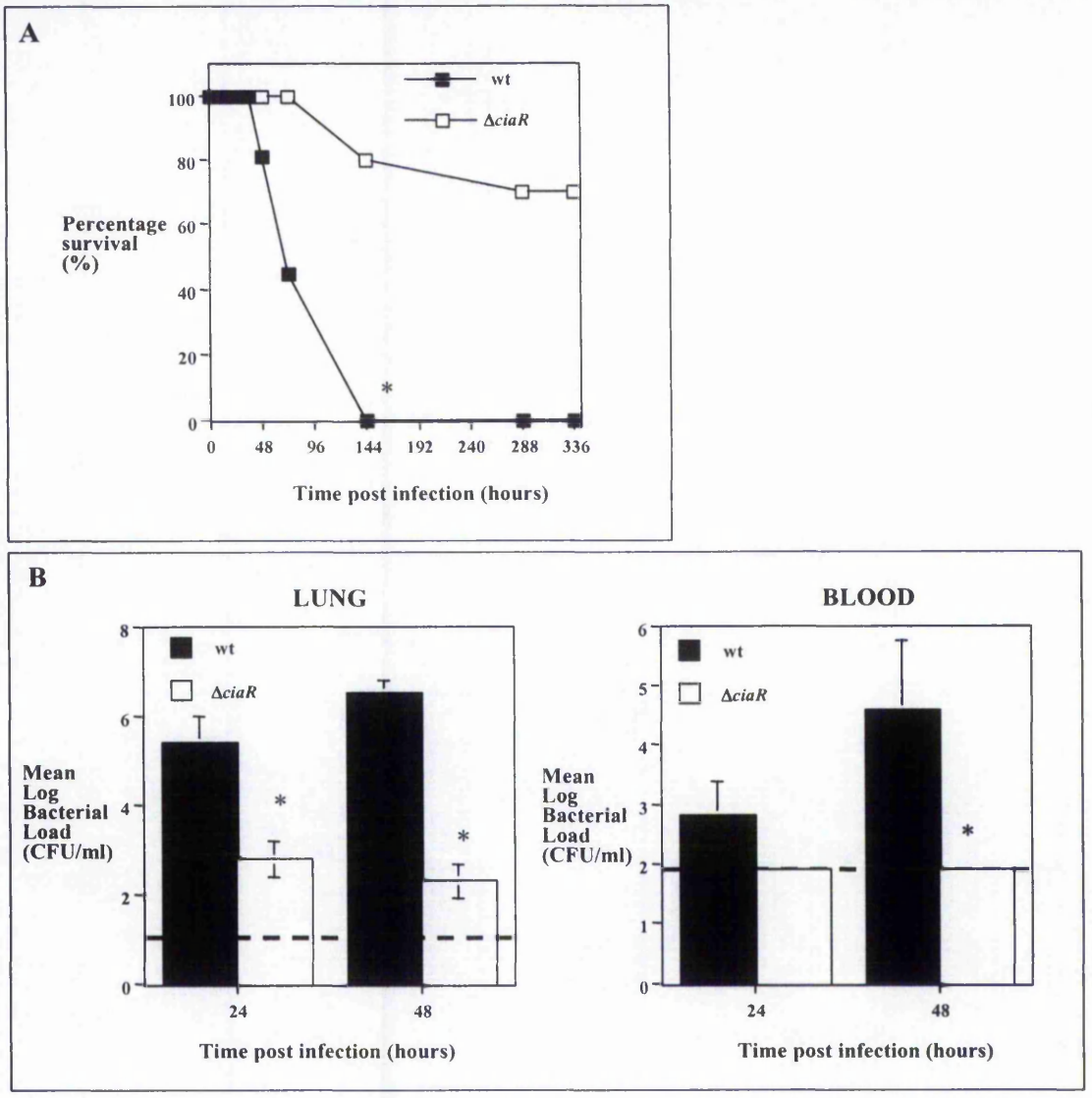


Figure 5.10- *In vivo* analysis of Δ*ciaR* mutant (type 3).

Effect of CiaR on virulence of strain 0100993. (A). Survival of animals given 10^7 CFU of wild-type or Δ*ciaR* mutant by the intranasal route (n=10). (B). Mean (SEM) bacteriology in lung and blood during infection, n=5. Broken line represents the limit of detection of the assay. In A *, $p<0.05$ shorter survival times for WT than for Δ*ciaR* mutant and in B *, $p<0.05$ lower bacterial loads for Δ*ciaR* than for WT.

5.6.2 Competitive analysis of virulence

Competitive analysis of virulence was examined using a slight modification of the method of Hava and Camilli (Hava and Camilli, 2002). Challenge doses of the wild type strain 0100993 and its Δ*ciaR* mutant were mixed in a 1:1 ratio and inoculated intranasally (2×10^6) or intraperitoneally (2×10^5) using two separate groups of mice. After 24 h and 48 h, bacteria were recovered from the lungs and blood. The number of wild type versus mutant bacteria recovered was determined by plating onto non-selective and selective (erythromycin) BAB plates and competitive indices calculated as the ratio of mutant to

wild type bacteria recovered from each animal. When the competitive index value is less than one, this means that the mutant is attenuated in the assay. Competition experiment results showed that CiaR plays a role in both the lung and systemic infection models (table 5.1).

Table 5.1- Competitive index for 0100993 WT vs 0100993 Δ*ciaR* mutant in intranasal and intraperitoneal infections.

Mouse no.	Competitive Index (CI)	
	Intranasal infection	Intraperitoneal infection
1	0.14	0.07
2	0.12	0.013
3	0.014	0.032
4	0.007	0.03
5	0.012	ND
Mean	**0.059**	**0.036**
SEM	**0.02**	**0.012**

The *in vivo* CI was calculated for each animal as the ratio of mutant to wild type divided by the input ratio of mutant to wild type. Intranasal and intraperitoneal infections were done in two separate groups of 5 mice. ND, none detected. SEM, standard error of the mean.

5.7 *In vivo* effect of complementation with HtrA (type 2)

As the growth phenotype and sensitivity to peroxide of D39Δ*ciaR* mutant could be reverted by complementation with HtrA, the virulence phenotype of the knockout and complemented strains was investigated in a mouse pneumonia model. For these studies all mutants used were in a D39 background (serotype 2) and a dose of 10^6 CFU was given intranasally. For D39 wild type all animals succumbed to the infection by 72 hours whereas no animals became sick when challenged with D39Δ*htrA* (figure 5.11). A Δ*ciaR* mutant of D39 showed attenuation in the model with 40 % of the animals becoming moribund during the experiment. When the pAL2-HtrA plasmid was introduced into the D39Δ*htrA* strain it reverted to full virulence. Moreover, when the level of HtrA expression was corrected in the D39Δ*ciaR* mutant, this also reverted to full virulence. HtrA therefore can complement the Δ*ciaR* mutant and restore virulence.

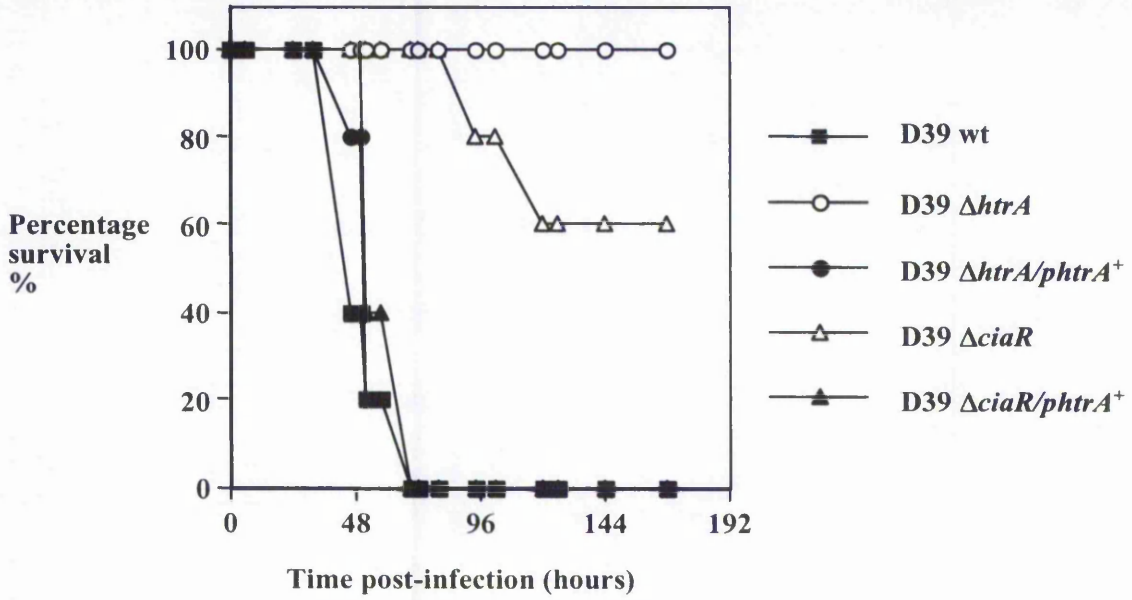


Figure 5.11- Effect of complementation with HtrA on the virulence of CiaR-null mutant.

Survival of MF1 mice after intranasal infection with 10^6 CFU/mouse (n=5). Strains D39Δ*htrA* and D39Δ*ciaR* show reduced virulence as judged by survival time. Complementation with HtrA (from plasmid pAL2-HtrA) reverts these strains to full virulence.

Chapter 5 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 monitor external conditions and alter their structure, morphology, and physiology accordingly. The sophisticated two-component signal transducing systems (TCSTS) have evolved in bacteria to elicit adaptive responses to environmental stimuli. These systems typically consist of two modular proteins; a sensor histidine kinase (HK) that auto-phosphorylates at a histidine residue in response to environmental stimulus 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-binding protein to cause changes in gene expression (Parkinson, 1993, Stock *et al.*, 2000).

CiaR/H system and virulence

Streptococcus pneumoniae causes disease in a wide range of sites, including the lung, middle ear, sinuses, blood, and meninges. Continued existence of the pneumococcus in these different environments requires adaptive responses that involve the utilization of TCSTS. Thirteen pairs of such two-component systems and an orphan response regulator have been identified in the pneumococcal genome (Lange *et al.*, 1999, Throup *et al.*, 2000). Two-component systems possibly play essential roles in regulating genes required for successful colonization and infection by human pathogens, such as *S. pneumoniae* (Lau *et al.*, 2001, Hava and Camilli, 2002, Hubbard *et al.*, 2003, Kadioglu *et al.*, 2003). The CiaR/H two-component system was the first to be identified (Guenzi *et al.*, 1994) and is known to be important in a number of aspects of pneumococcal biology. Mutations in the histidine protein kinase gene, *ciaH*, conferred resistance to β-lactam antibiotics suggesting that the system may control genes important in cell wall metabolism (Guenzi *et al.*, 1994). The system is also involved in the control of bacterial lysis (Giammarinaro *et al.*, 1999, Hakenbeck *et al.*, 1999, Lange *et al.*, 1999) and the regulation of genetic competence (Guenzi *et al.*, 1994, Echenique *et al.*, 2000, Martin *et al.*, 2000). Previous studies have shown that this system plays a role in colonization of the lungs of mice (Throup *et al.*, 2000) and the nasopharynx of infant rats (Sebert *et al.*, 2002). The same mutant of *S. pneumoniae* (kindly provided by Martin Burnham of SmithKlineBeecham; now GlaxoSmithKline) used in these two studies was also used here to show that CiaR/H also plays a major role in the causation of disease in the mouse. The Δ*ciaR* mutant of strain 0100993 was much reduced in its overall virulence (as judged by survival times) when given intranasally. The mutant organism grew to about 10^3 CFU/ml in lungs compared to 10^6 CFU/ml for the wild type organism (a reduction of 3 logs). A large reduction in growth

in the lung was also observed by Throup and co-workers (Throup *et al.*, 2000) who observed a reduction of approximately 5 logs in growth in the lung following disruption of the *ciaR/H* operon. Because the nasopharynx is the primary reservoir of the pneumococcus from which cases of pneumococcal disease arise, Sebert and colleagues have examined the ability of a Δ*ciaR/H* mutant to persist during nasopharyngeal carriage by using an infant rat model of colonization where carriage is maintained for up to several weeks without the development of either localized or invasive disease. Results of this study revealed that inoculation of wild type organisms resulted in maximum colonization levels of about 10^6 CFU/ml of nasal wash whereas the Δ*ciaR/H* mutant did not colonize the nasopharynx (Sebert *et al.*, 2002). In the experiments reported here, the mutant organism only invaded the bloodstream to very low levels (at the limit of detection of 100 organisms per ml). This two-component system therefore seems to be very important for colonization, growth in the lungs and also for invasion of the bloodstream.

Low-level bacteraemia following intranasal challenge could be caused either by poor invasion from the lungs or by poor growth in the blood. Competition experiments between wild type and mutant strains showed that the mutant was much less able to colonize the lungs and also less able to grow systemically. CiaR/H was also found to play a role in the virulence of the type 2 strain D39. It is important to investigate the role of these systems in more than one strain as it has recently been shown that the contribution of two-component systems to virulence can be strain-dependent (Blue and Mitchell, 2003). A role of CiaR/H in the systemic virulence of D39 was also shown by Marra and co-workers (Marra *et al.*, 2002a). The authors of this study used differential fluorescence induction (DFI) method to identify *S. pneumoniae* genes involved in pathogenesis and then studied the effect of deletion of these genes on virulence in both respiratory tract infection (RTI) model and a systemic model of infection. A CiaR/H-null mutant of strain D39 was attenuated in the systemic model but not in the RTI model, an observation that contradicts what was reported by Throup and co-workers for strain 0100993 (Throup *et al.*, 2000) concerning the role of CiaR/H in the RTI model. This perhaps reflects differences in pneumococcal strains, methods of generating mutants, or even mouse strains used. Here, CiaR/H was found to play a role in strain D39 using the pneumonia model of infection with 60 % of the animals surviving the challenge.

Level of HtrA expression in CiaR mutant

Western immunoblotting was implemented to study the level of HtrA produced by different strains. The blots indicated that D39Δ*ciaR* expressed approximately 4-fold less

HtrA protein than wild type D39. When HtrA was expressed from a plasmid (pAL2-HtrA) in the D39Δ*ciaR* strain, Western blotting showed the level of HtrA was restored to a wild-type level. When HtrA was expressed from the plasmid in the wild type strain, there was no increase in the level of HtrA protein. This finding is surprising and suggests that there is a regulatory mechanism for controlling maximal levels of HtrA and that this mechanism may not be dependent on CiaR/H. On the transcriptional level, Sebert and co-workers reported that the *htrA* gene was 27-fold down-regulated in CiaR-null mutant when they used microarray technology to identify transcriptional changes due to inactivation of nine TCSTS (Sebert *et al.*, 2002). They also found 37.2-fold down-regulation of *htrA* in the same mutant using real time quantitative RT-PCR. Mascher *et al* also reported that *htrA* was differentially down-regulated in Δ*ciaR* mutant (Mascher *et al.*, 2003). More recently, Dagkessamanskaia and co-workers reported that the ratios of *htrA* gene expression of competent to non-competent cells were 8.4/2.3 for wild type strain R800 and 1.4/1.0 for CiaR-null mutant and concluded that the extent of down-regulation for *htrA* is much higher when calculated for competent CiaR-null mutant cells than for non-competent cells (Dagkessamanskaia *et al.*, 2004). The results of HtrA Western blot shown in this chapter are therefore consistent with and further confirm these reports.

Effect of complementation with HtrA on *In vitro* growth

In this study, HtrA has been shown to behave as a typical heat shock protein with levels of the protein increasing after exposure of bacteria to a temperature of 40 °C. Interestingly, the increased expression of HtrA at high temperature was demonstrated to be dependent on the presence of CiaR/H. Based on these findings, the strain D39Δ*ciaR* was predicted to be defective in growth at 40 °C (due to decreased expression of HtrA). Analysis *in vitro* indicates that the growth of D39Δ*ciaR* mutant was less than the wild type at elevated temperature. The CiaR/H system is also involved in the control of bacterial lysis (Giammarinaro *et al.*, 1999, Hakenbeck *et al.*, 1999, Lange *et al.*, 1999, Mascher *et al.*, 2003) and in particular Lange and co-workers (Lange *et al.*, 1999) reported that a *ciaR* mutant of the strain R6 had an increased rate of autolysis when grown in Todd-Hewitt medium at 37 °C. Contrary to this report, the D39Δ*ciaR* mutant in this study showed decreased rate of autolysis as judged by viable count at stationary and decline phases of growth both at normal and elevated temperatures. This may reflect differences in the genetic content of R6 and D39, which are now known to differ at several loci (Tettelin *et al.*, 2001). Sebert and co-workers found that the *in vitro* doubling time (growth rates) of the Δ*ciaR/H* mutant strain was not significantly different from that of the wild type strain

0100993 and concluded that attenuation of the Δ*ciaR/H* mutant in nasal carriage could not be explained by a constitutive defect in growth (Sebert *et al.*, 2002). Autolysis is a characteristic mechanism that enables the pneumococcus to release and exchange its genetic material and also to release virulence factors during infection. The decreased rate of autolysis of the D39Δ*ciaR* mutant may therefore explain the attenuation in virulence of this mutant due to less virulence factors available at the site of infection. The *in vitro* growth phenotype of the *ciaR* mutant of serotype 3 strain was also studied. The effect of the *ciaR* deletion in this strain was more dramatic as the growth of this mutant was completely prevented at an elevated temperature but it showed a similar rate of autolysis to that of the parent strain at 37 °C. These findings highlight the differences of the effects of mutations in different strains. The growth phenotype of D39Δ*ciaR* was restored to wild type by introducing the pAL2-HtrA plasmid in the complemented strain suggesting that the D39Δ*ciaR* growth phenotype is due to the low level of HtrA expressed by this strain.

The strongly decreased expression of the *htrA* in the Δ*ciaR* mutant suggested that HtrA might participate in the Cup phenotype (for Competence up) of the Δ*ciaR* mutant (Sebert *et al.*, 2002) probably by degrading the secreted CSP at the surface of the cell or through some other functional connection. The observation that the D39Δ*htrA* mutant taken up external DNA less easily as shown in the previous chapter confirmed this possibility. Dagkessamanskaia *et al* found that *htrA* was depressed in non-competent *ciaR* cells (Dagkessamanskaia *et al.*, 2004); an observation that lends some weight to the proposal that HtrA is involved in competence for genetic transformation. However, authors of the same study reported that inactivation of *htrA* of the strain R800 by mariner mutagenesis did not up-regulate competence. They, therefore, concluded it is unlikely that the down-regulation of *htrA* in *ciaR* cells is responsible for the Cup phenotype of the *ciaR* mutant. With this controversy in mind, it can be postulated that HtrA seems to be required for transformation, rather than for inhibition of competence. Analysis of the growth curves of wild-type strain and its *ciaR* derivative in competence-permissive medium in the above-mentioned study (Dagkessamanskaia *et al.*, 2004) suggested a direct connection between competence and stationary phase autolysis. In the absence of CSP, the two strains exhibited very similar growth curves, whereas the addition of CSP had a dramatic effect on the *ciaR* mutant strain, competence induced stationary phase autolysis of this mutant. These data suggested that the CiaR/H system is required for *S. pneumoniae* to cope with physiological changes induced during differentiation to competence and for a normal exit from competence.

Sensitivity to oxidative stress

Hydrogen peroxide is an example of reactive oxygen species that can cause damage to DNA and other cell components. As it has been previously shown that deletion of HtrA is associated with increased sensitivity of the pneumococcus to oxidative stress, it was predicted that reduced levels of HtrA in strain D39Δ*ciaR* would also result in reduced resistance to oxidative stress. The sensitivity to hydrogen peroxide of D39Δ*ciaR* was increased although not to the same extent as found previously for D39Δ*htrA* mutant. After 5 min exposure to 40 mM hydrogen peroxide the survival of strain D39Δ*ciaR* was similar to that of the wild type (figure 5.9) whereas the survival of strain D39Δ*htrA* was significantly reduced at this time (figure 4.7 in the previous chapter). This probably reflects the expression of low levels of HtrA in strain D39Δ*ciaR*. Resistance of strain D39Δ*ciaR* to hydrogen peroxide could be reverted to wild type levels by complementation with HtrA, which again confirms that the D39Δ*ciaR* phenotype could be explained by down-regulation of HtrA in this mutant.

Complementation with HtrA restores virulence of CiaR mutant

As CiaR/H plays a role in virulence the next question is how does this system mediate pathogenesis of infection? The *cia* regulon has already been defined and includes genes important for the synthesis and modification of cell wall polymers, peptide pheromone and bacteriocin production and the *htrA-spoJ* region (Mascher *et al.*, 2003). Because it is already known that CiaR/H regulates the gene for HtrA (Sebert *et al.*, 2002, Mascher *et al.*, 2003) and that deletion of the *htrA* gene reduces the ability of the pneumococcus to colonize the nasopharynx (Sebert *et al.*, 2002) and attenuates a type 2 pneumococcal strain in both pneumonia and bacteraemia models of infection, attempts were made to define the contribution of HtrA to the overall CiaR/H phenotype.

Both CiaR and HtrA-null mutants have reduced virulence and the CiaR/H system is known to regulate HtrA expression. Therefore, whether the reduced virulence of a Δ*ciaR* mutant could be explained by a down-regulation of HtrA expression was investigated. In order to do this, a Δ*ciaR* version of D39 was made and confirmed to be less virulent than its parent strain. The reduction in lung colonization was of the order of several orders of magnitude, a finding similar to that of previous studies (Throup *et al.*, 2000). Restoring the level of HtrA to wild type levels in strain D39Δ*ciaR* produced a strain that was again fully virulent in mice. This shows that the virulence phenotype of D39Δ*ciaR* can be completely

explained by the reduced expression of HtrA. Interestingly D39 Δ*ciaR* still expresses low levels of HtrA and this may explain why it is more virulent than D39Δ*htrA*.

Summary

Data presented in this chapter confirm that the CiaR/H system is involved in controlling the levels of HtrA within the cell and have shown that up-regulation of HtrA in response to heat is dependent on the CiaR/H system. As the response regulator CiaR has been shown to bind physically to DNA in the region of HtrA, this regulation is proposed to be directly at the level of transcription (Mascher *et al.*, 2003). A number of the phenotypes associated with deficiency in CiaR/H has been shown to be due to alterations in levels of HtrA. Thus, the resistance of cells to autolysis, the increased sensitivity to oxidative stress and the decreased virulence of strain D39Δ*ciaR* can all be explained by alterations in levels of HtrA. It has also been shown that transformation efficiency is altered by HtrA. While the CiaR/H regulon is obviously very complex, many of the phenotypes observed in mutants of this regulon can be explained by changes in HtrA. The exact mechanism by which HtrA controls these processes is still unclear. Further analysis of *htrA* mutants is likely to provide clues as to how HtrA plays its role in the pneumococcal biology and stress response.

Chapter 6

PROTEOMIC AND MICROARRAY ANALYSES OF *htrA* MUTANTS, RESULTS

Determining protein expression and any changes in defined circumstances is a key to understanding cellular mechanisms. Integrated data sets from protein expression studies encompassing relative abundances, subcellular localization, profiling of isoforms and post-transcriptional modifications are very important to understanding physiological protein function (Steiner and Witzmann, 2000). Much of this information can be derived from two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) in combination with mass spectrometric identification. 2D-PAGE as a proteomic methodology is able to resolve and investigate the abundance of several thousands of proteins in a single sample. In addition, it can be used to compare quantities of proteins in related samples, such as those from altered environments or from mutant and wild type (Lilley *et al.*, 2002). Proteomics technology was used to examine the proteins synthesized by erythromycin-susceptible and erythromycin-resistant *S. pneumoniae* strains (Cash *et al.*, 1999). It was also used to compare the protein expression pattern of the transparent variant to that of the opaque variant of the pneumococcus (Overweg *et al.*, 2000a). Furthermore, among the attempts for new antimicrobial drug discovery, proteomics technologies should aid in the identification of novel drug targets and compounds with unique mechanisms of action other than those currently provided by the traditional antibiotics (Rachakonda and Cartee, 2004).

In fact, proteomics is now also yielding important findings across a wide range of applications concerning human health. Proteomic profiling is used as a diagnostic tool. For instance, proteomic approaches to the identification of novel biomarkers for cancer diagnosis and staging have relied on the identification of differentially expressed proteins between tumor cells and their normal counterparts based on the patterns of protein expression observed by two-dimensional gel electrophoresis (2D-PAGE) (Rodland, 2004). Proteomics has also been used for characterizing the modifications in protein expression during the development of lung diseases (Waldburg *et al.*, 2004), in mapping of human signalling pathways (Colland *et al.*, 2004) and in many other applications.

Another technology that is now widely used for the study of genome-wide expression profiles is the microarray. DNA microarrays provide a system for the simultaneous measurement of the expression level of thousands of genes in a single hybridization assay. Each array consists of a reproducible arrangement of thousands of DNAs (PCR products or oligonucleotides) attached to a solid support, usually glass. Fluorescently labelled cDNA prepared from messenger RNA is hybridized to complementary DNA on the array and then detected by laser scanning. Hybridization intensities for each DNA sequence on the array are determined using an automated process and converted to a quantitative read-out of relative gene expression levels. The data can then be further analyzed to identify

expression patterns and variation that correlate with cellular development, physiology and function (Harrington *et al.*, 2000). Several studies have already taken advantage of DNA microarrays to aid in the understanding of complex regulatory pathways in *S. pneumoniae* (de Saizieu *et al.*, 2000, Sebert *et al.*, 2002, Mascher *et al.*, 2003, McCluskey *et al.*, 2004). Microarrays were used to evaluate transcriptional changes associated with entrance of *S. pneumoniae* into a specialized state in which the bacterium becomes competent for DNA uptake, a process implicated in virulence (Lau *et al.*, 2001, Dagkessamanskaia *et al.*, 2004). It was also used to analyze responses to pneumococcal virulence factors (McDaniel *et al.*, 2004).

Transcriptome and proteome studies are now becoming more refined in their approach and are shedding light on the role of pathogen-specific mechanisms/structures in pathogenesis. In addition, studies of gene expression *in vivo* have shed new light on how the host influences the niche occupied by bacteria. Such approaches combined with modern functional genomics technologies represent a powerful tool in understanding host-bacteria interactions (Walduck *et al.*, 2004).

As HtrA was found to be a major virulence factor of the pneumococcus, it was selected for further analysis. The aim of the study presented in this chapter was to apply proteomics and microarray technologies to the analysis of the effect of *htrA* deletion in an attempt to explore the function of HtrA protein in the pneumococcal biology and stress response.

6.1 Proteomic analysis

The proteomic analysis was carried out at the Institute of Food Research (IFR), Norwich, UK. The proteome of the D39Δ*htrA* mutant was compared to that of the wild type D39 by separating equal amounts of protein samples by 2D-PAGE, picking up some of the protein spots showing differences and identifying them using MALDI-TOF-MS analysis.

6.1.1 Protein sample preparation

Protein samples from D39 wild type and the D39Δ*htrA* mutant were prepared from 100-ml cultures grown at 37 °C to OD_{600nm} ~ 0.4. Cell pellets were collected by centrifugation, washed with cold PBS buffer pH 8.0 and resuspended in solution 1 and solution 2 as described in the Materials and Methods (section 2.12.1). Total protein extracts were

prepared by passing cell suspensions through a French pressure cell and cell debris was removed by centrifugation. The supernatants were then aliquoted and stored at –80 °C.

The amount of protein in samples was determined using the PlusOne™ 2-D Quant Kit (Amersham Biosciences). This procedure has a precipitation step that removes interfering substances such as SDS and DTT present in samples. These substances are incompatible with common protein assays such as Bradford, Lowry and Biuret. This method was adopted to allow accurate quantification of the protein samples to be analyzed, as identical amounts of proteins to be loaded is necessary for proper interpretation of the 2D results. Protein samples were also visualized by running an SDS-PAGE. Figure 6.1 shows protein bands of both the D39 wild type and the D39Δ*htrA* mutant separated by SDS-PAGE. No differences (i.e. bands that are absent/present) between the wild type and mutant strains can be observed from this gel. Samples were then transported to the IFR in dry ice for analysis.

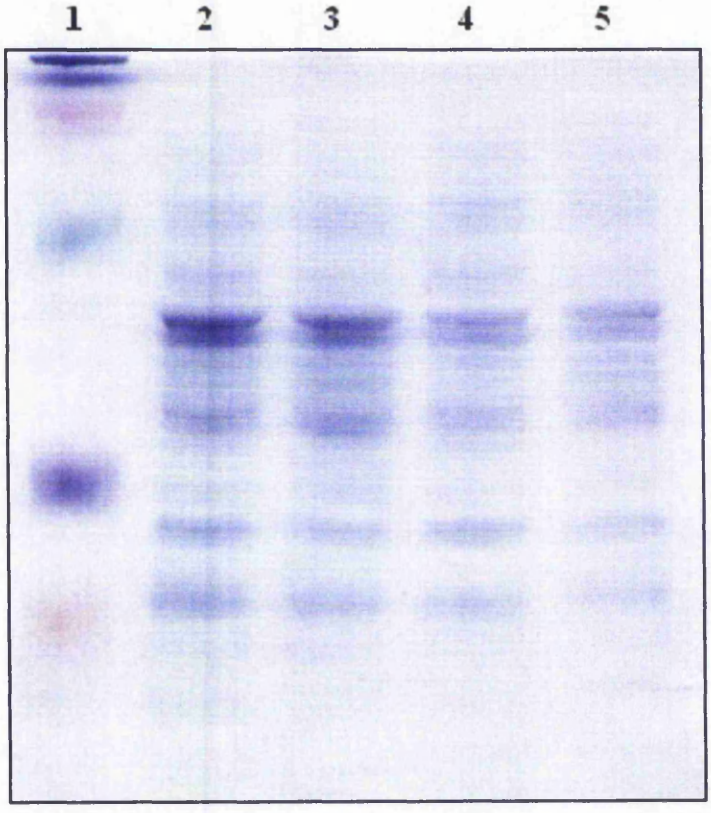


Figure 6.1- Representative SDS-PAGE analysis of protein samples of D39 wild type and D39Δ*htrA* used for proteomics study.

Lane 1, Kaleidoscope Prestained Standards ladder; lane 2, 15 μg of D39 wild type protein; lane 3, 15 μg of D39Δ*htrA* mutant protein; lane 4, 7.5 μg of D39 wild type protein; lane 5, 7.5 μg of D39Δ*htrA* mutant protein.

6.1.2 2D-gel electrophoresis

In the 2D-PAGE, proteins are separated first according to their isoelectric points (pI) and then on the basis of their molecular weights in the second dimension. Two different protein preparations of both D39 wild type and D39 Δ*htrA* mutant strain were analyzed in this assay. The first dimension electrophoresis was done by using 18-cm immobilized pH gradient gel (IPG) strips (pH 4-7; Amersham) and the pHaser system (Genomic Solutions). 50 µg of each protein sample was separated on individual IPG strips for 24 hours. After, the gel strips were equilibrated, the proteins were separated in the second dimension on 10 % duracryl gels. After separation, gels were stained with Sybro Ruby (Molecular Probes) according to the manufacturer's recommendations. Sybro Ruby is slightly less sensitive than silver but has a much better dynamic range and is mass spectrometry compatible. Images of the gels were taken after at least 1 hour of destaining using the proXPRESS Proteomic Imaging System (Perkin Elmer, UK). Representative 2D images of D39 wild type and D39 Δ*htrA* mutant are shown in figures 6.2 and 6.3, respectively.

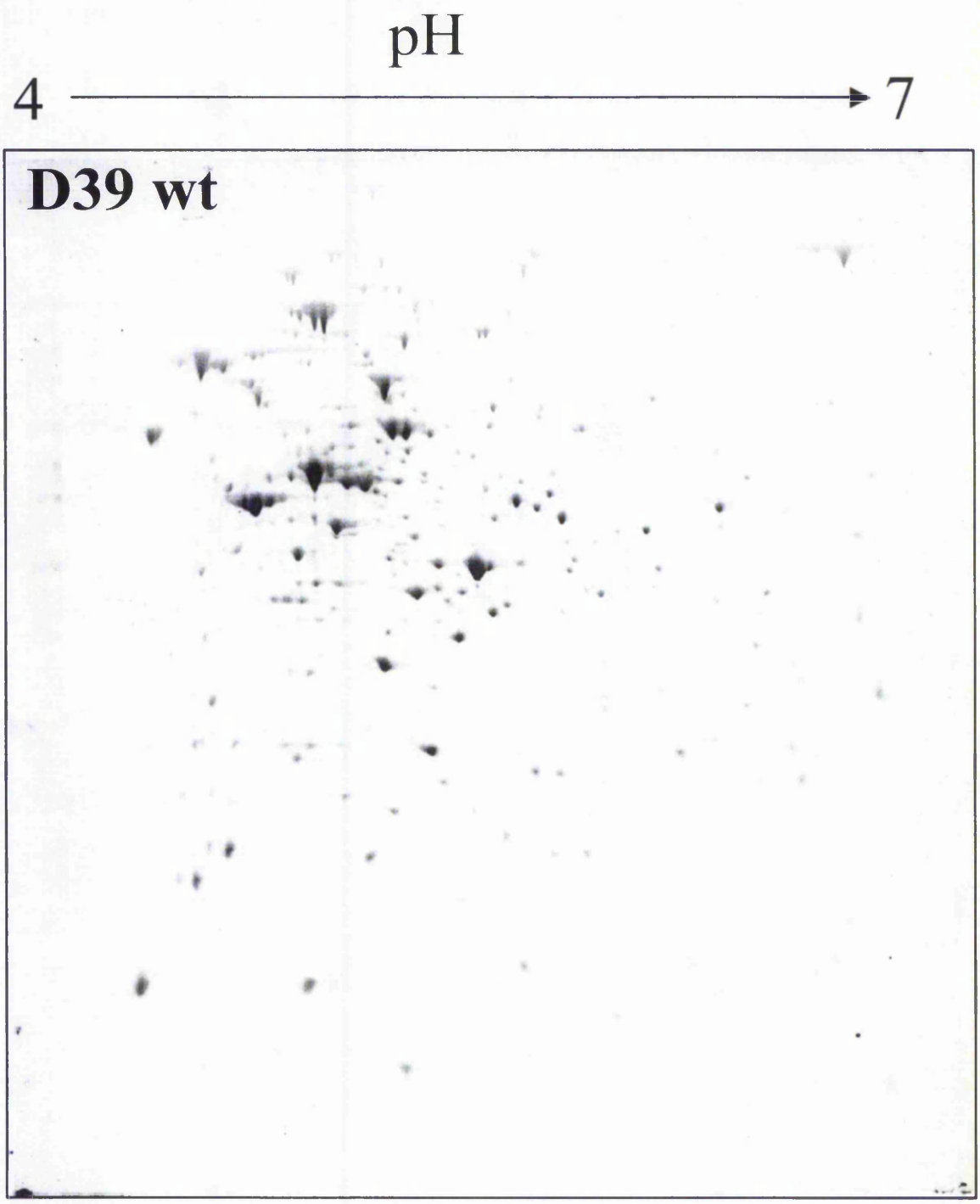


Figure 6.2- Representative 2D gel image of D39 wild type.

2D-PAGE analysis of total proteins of *S. pneumoniae* wild type strain grown at 37 °C. Extracted proteins were separated by isoelectric focusing in the pI range of 4 to 7 in the first dimension and 10% duracryl gel in the second dimension. Resolved proteins were visualized following staining with Sybro Ruby stain.

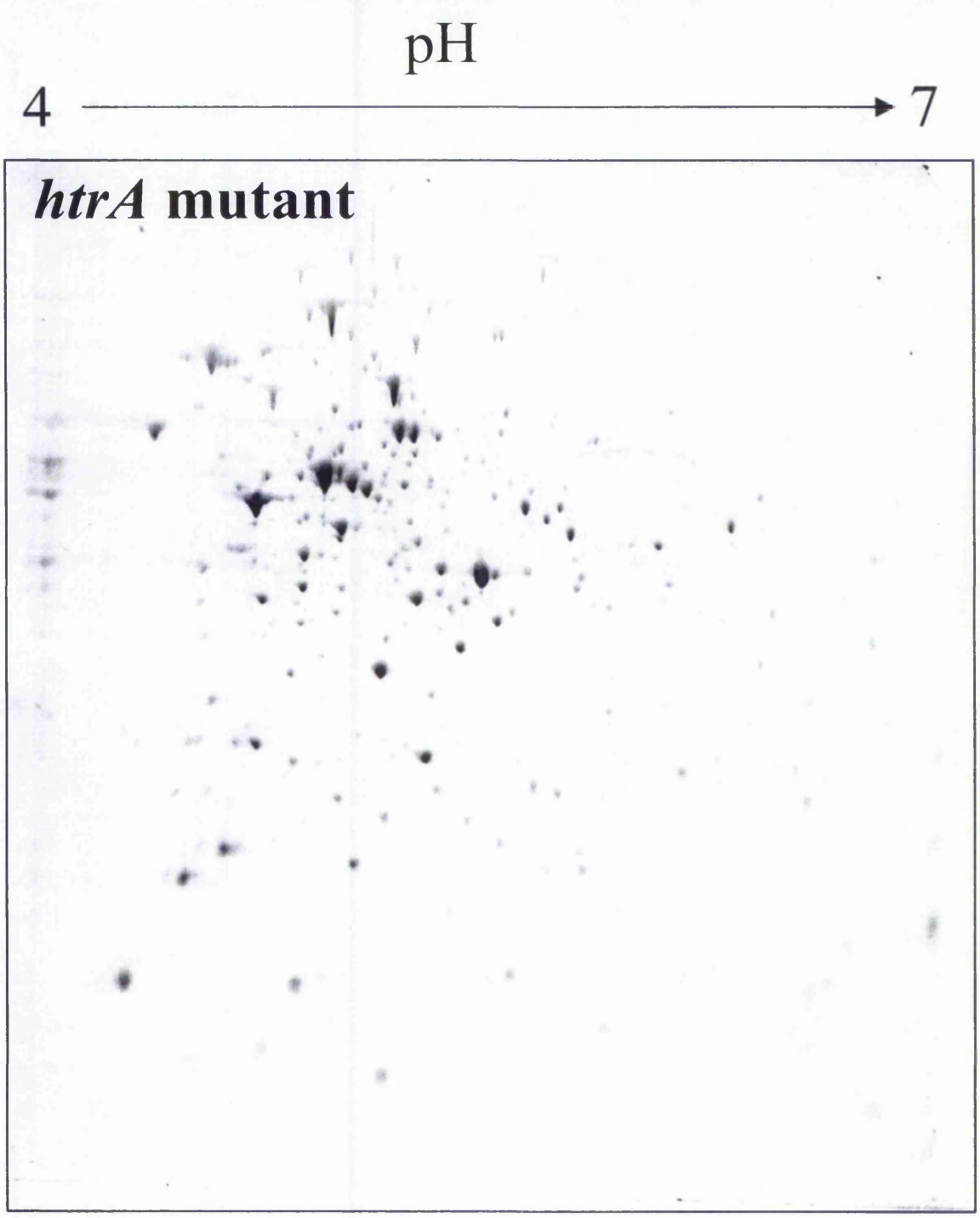


Figure 6.3- Representative 2D gel image of D39 Δ*htrA* mutant.

2D-PAGE analysis of total proteins of *S. pneumoniae* D39 Δ*htrA* mutant strain grown at 37 °C. Extracted proteins were separated by isoelectric focusing in the pI range of 4 to 7 in the first dimension and 10% duracryl gel in the second dimension. Resolved proteins were visualized following staining with Sybro Ruby stain.

6.1.3 Analysis of 2D images

The gel images of D39 wild type and D39Δ*htrA* mutant (four gels each) were analyzed using the ProteomeWeaver software (Definiens AG, Munich, Germany). Every gel image was compared to the rest of images to identify similar and different 2D samples. This software enables the determination of protein spot intensities and performs overlays of gel images. The 3D images of protein spots could also be compared using this analysis to identify real protein spots from any dye crystals or background. The results of this analysis showed many protein spots, which were unique to the proteome of D39Δ*htrA* mutant but not found in case of the D39 wild type. Figure 6.4 shows some examples of 3D images of proteins expressed in the D39Δ*htrA* mutant but not in the D39 wild type. The overlay analysis results also showed some protein spots whose intensities were different between the two groups. An example of these overlays is shown in figure 6.5. The protein spots showing differences between the two groups were picked up for identification analysis using mass spectrometry.

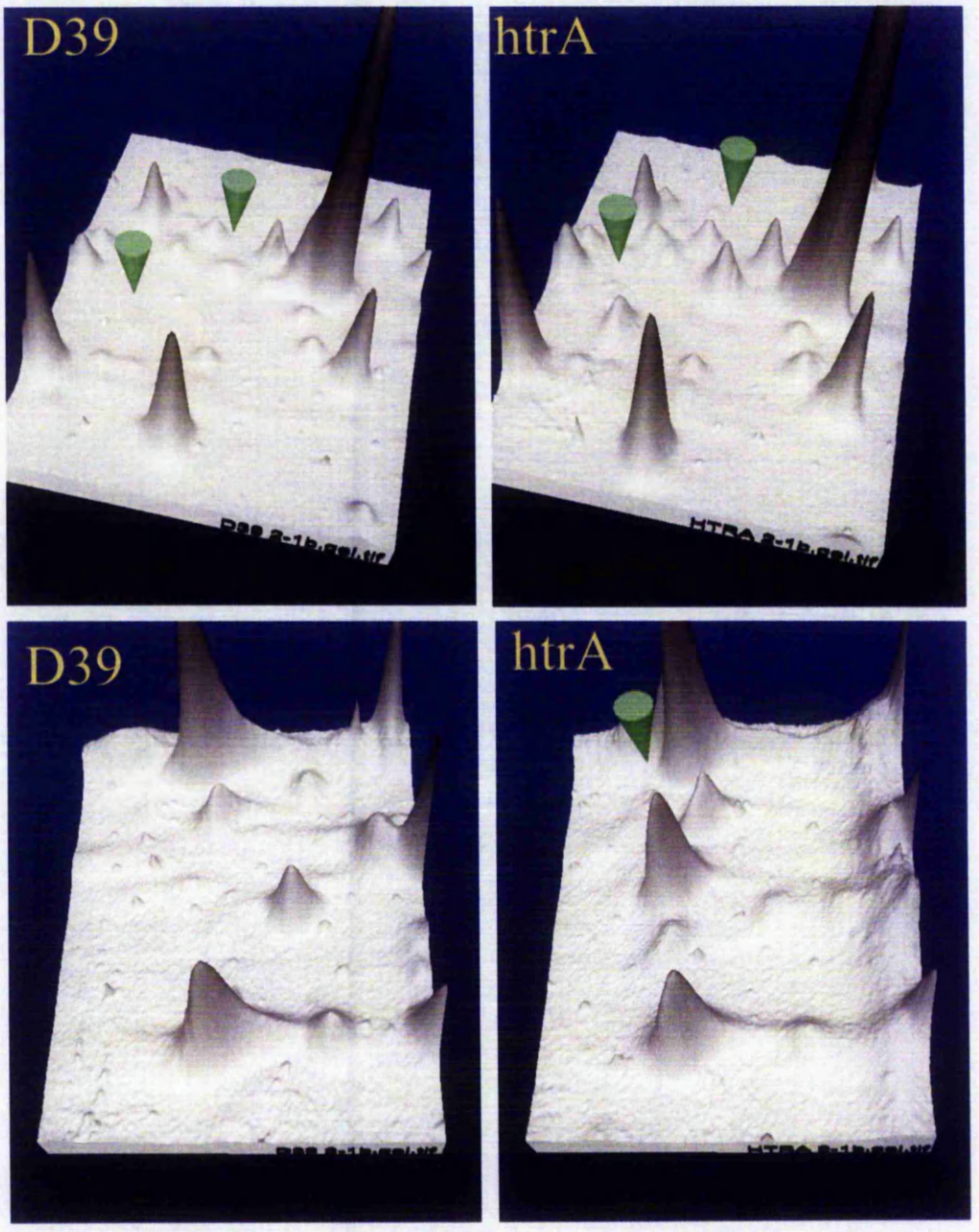


Figure 6.4- Example of 3D images of protein spots generated by the ProteomeWeaver.

The 3D image of protein spots in D39 wild type gel section was compared to that of the corresponding section of D39Δ*htrA* mutant. Spots pointed at by the green cones are unique in the D39Δ*htrA* mutant.

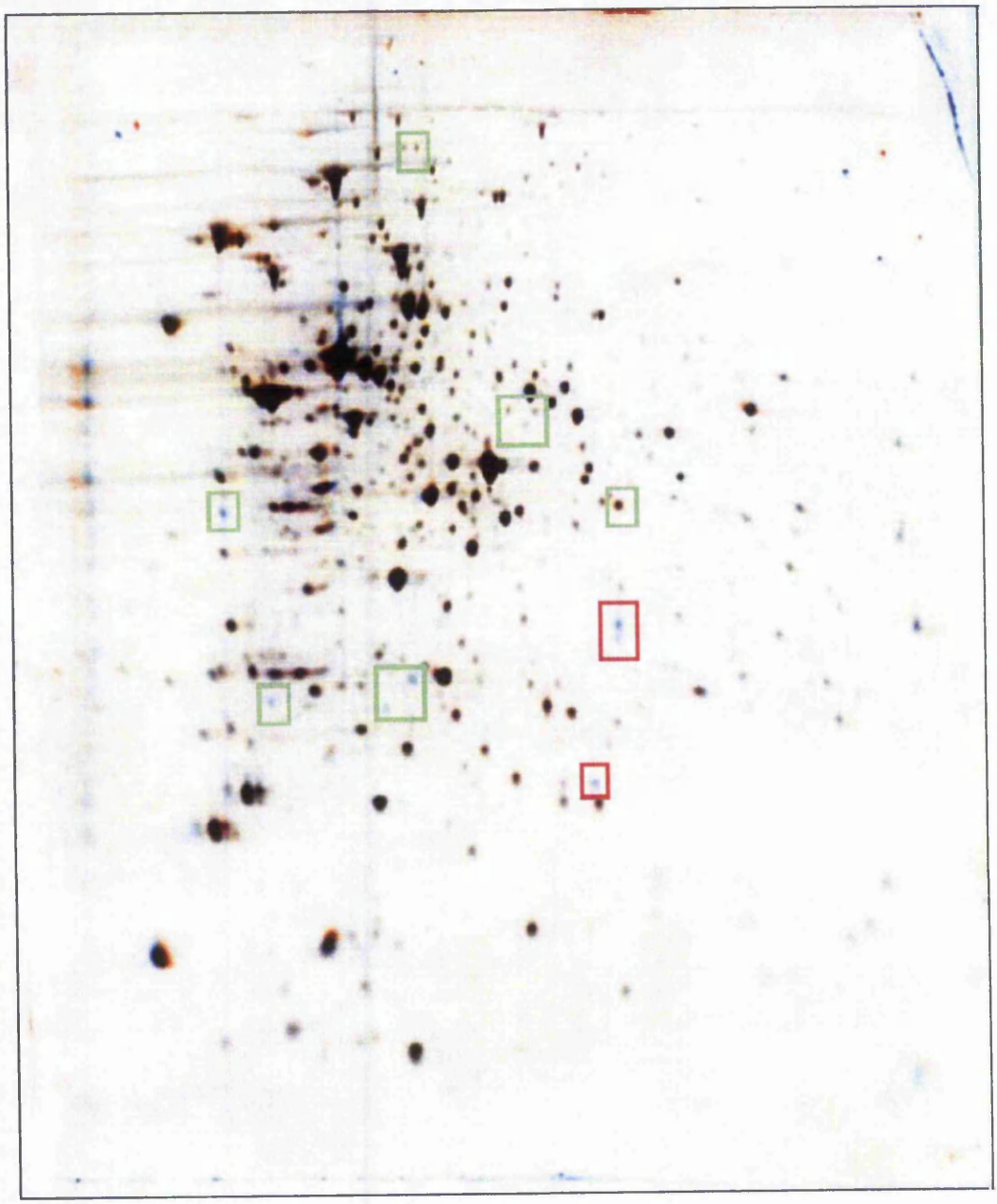


Figure 6.5- An example of 2D image overlays generated by the ProteomeWeaver.

The ProteomeWeaver was used to generate overlays of the 2D images of both D39 wild type and D39Δ*htrA* mutant to identify differences between their proteomes. Green boxes highlight spots showing consistent changes in protein spots between the D39 wild type and the D39Δ*htrA* mutant in both sample preparations 1 and 2. The red boxes show inconsistent changes between sample preparations 1 and 2.

6.1.4 Identification of protein spots

Current practices for the identification of proteins from 2D gels frequently involve interrogation of the genomic sequence data available for many species. Protein spots that

showed differences between the wild type and *htrA* mutant were excised for subsequent protein identification using ProPick Spot Picker (Genomic Solutions). These spots were digested with trypsin using in-gel protocol and analyzed using MALDI-TOF mass spectrometry to generate distinctive peptide mass fingerprints, which were compared to the annotated genomic database available at (http://www.matrixscience.com/) to allow the identification of unknown proteins. Because this analysis was carried out towards the end of the project, not all spots that showed differences were identified. These investigations provided identifications for some cellular proteins from *S. pneumoniae* that were differentially expressed due to *htrA* deletion. Among the identified proteins are those involved in cellular processes and stress response such as DnaK and superoxide dismutase (SodA), those involved in central and intermediary metabolism such as glyceraldehyde-3-phosphate dehydrogenase, lactate dehydrogenase, formate acetyltransferase and also transcription and translation proteins such as elongation factor Tu. Table 6.1 shows the identity of *S. pneumoniae* proteins up- or down-regulated, or showing no difference in expression due to loss of *htrA* and figures 6.6 and 6.7 show examples of gel sections of the identified proteins.

Table 6.1- Identity of *S. pneumoniae* proteins picked up from 2D gels.

Spot no.[a]	Gene symbol	Protein identity	No. of peptides matched[b]	Mean spot vol. ratio[c]
30	*pfl*	Formate acetyltransferase	7	0.81
65	*pfl*	Formate acetyltransferase	14	0.79
311	*pfl*	Formate acetyltransferase	16	0.89
2608	*pfl*	Formate acetyltransferase	12	1.00
275	*gap*	Glyceraldehyde-3-phosphate dehydrogenase	8	0.95
295	*eno*	Enolase	21	0.71
1374	*eno*	Enolase	12	1.27
1958	*eno*	Enolase	16	1.55
479	*ftsZ*	Cell division protein FtsZ	20	1.13
586	*phtA*	Hypothetical protein phtA	6	2.01
702		Spectinomycin adenyltransferase	9	9.46
1060		Spectinomycin adenyltransferase	10	N.A.
850	*rpsA*	30S Ribosomal protein S1	14	1.35
1033	*rpsA*	30S Ribosomal protein S1	12	1.08
1727	*rpsA*	30S Ribosomal protein S1	16	0.68
944		Type 2 capsule locus. Glucose-1-phosphate thimidylyl transferase (cps19aL)	26	1.18
966	*cysRS*	Cysteinyl-tRNA synthetase; cystein-tRNA ligase (CysRS)	23	0.56
986	*ldh*	Lactate dehydrogenase	14	0.76
1189		Nitroreductase	21	1.40
1249	*rpoA*	DNA-directed RNA polymerase	22	0.95
1721		Outer membrane protein A	19	1.01
1773	*pgm*	Phosphoglucomutase	20	0.97
1809	*accA*	Carboxyltransferase	14	1.23
1817		Hypothetical protein Spr0309	21	1.17
1853	*frr*	Ribosome recycling factor	17	1.03
1873	*sodA*	Superoxide dismutase (Fe)	9	3.06
2076	*tuf*	Elongation factor Tu	23	2.20
2335	*tuf*	Elongation factor Tu	18	0.48
2349	*tuf*	Elongation factor Tu	19	0.51
2456	*ppaC*	Pyrophosphate phosphohydrolase	20	0.95
2485	*dnaK*	DnaK	8	0.94
4608	*dnaK*	DnaK	13	1.67
2709		Aspartate-tRNA ligase	14	0.72

Analysis of the *htrA* mutant proteome compared to that of the wild type strain D39. Proteins were identified by MALDI-TOF MS *a*, Refers to the number of protein spot labelled in this analysis. *b*, Number of tryptic peptides observed in mass spectra. *c*, ratio of mean spot volume of mutant to wild type, values below one represent down-regulated proteins and those above one represent up-regulated proteins. N.A. none available.

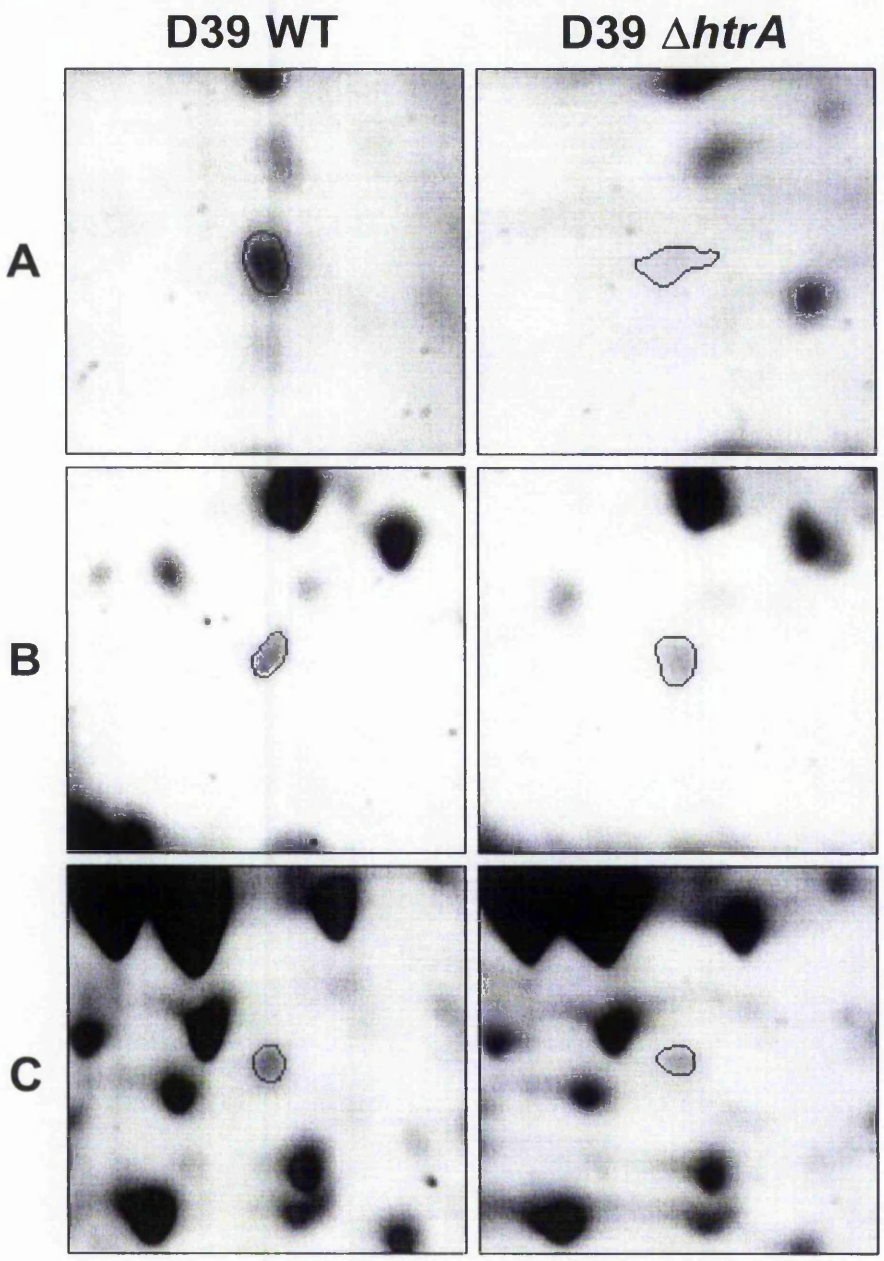


Figure 6.6- Representative *S. pneumoniae* proteins down regulated in *htrA* mutant.

2D sections from individual gels showing spots of proteins down regulated as a result of *htrA* deletion. Proteins from D39 wild type or *htrA* mutant grown at 37°C were separated by two dimensional gel electrophoresis and identified by MALDI-TOF MS. A, Elongation factor Tu. B, 30S Ribosomal protein S1. C, Cysteinyl-tRNA synthetase (CysRS).

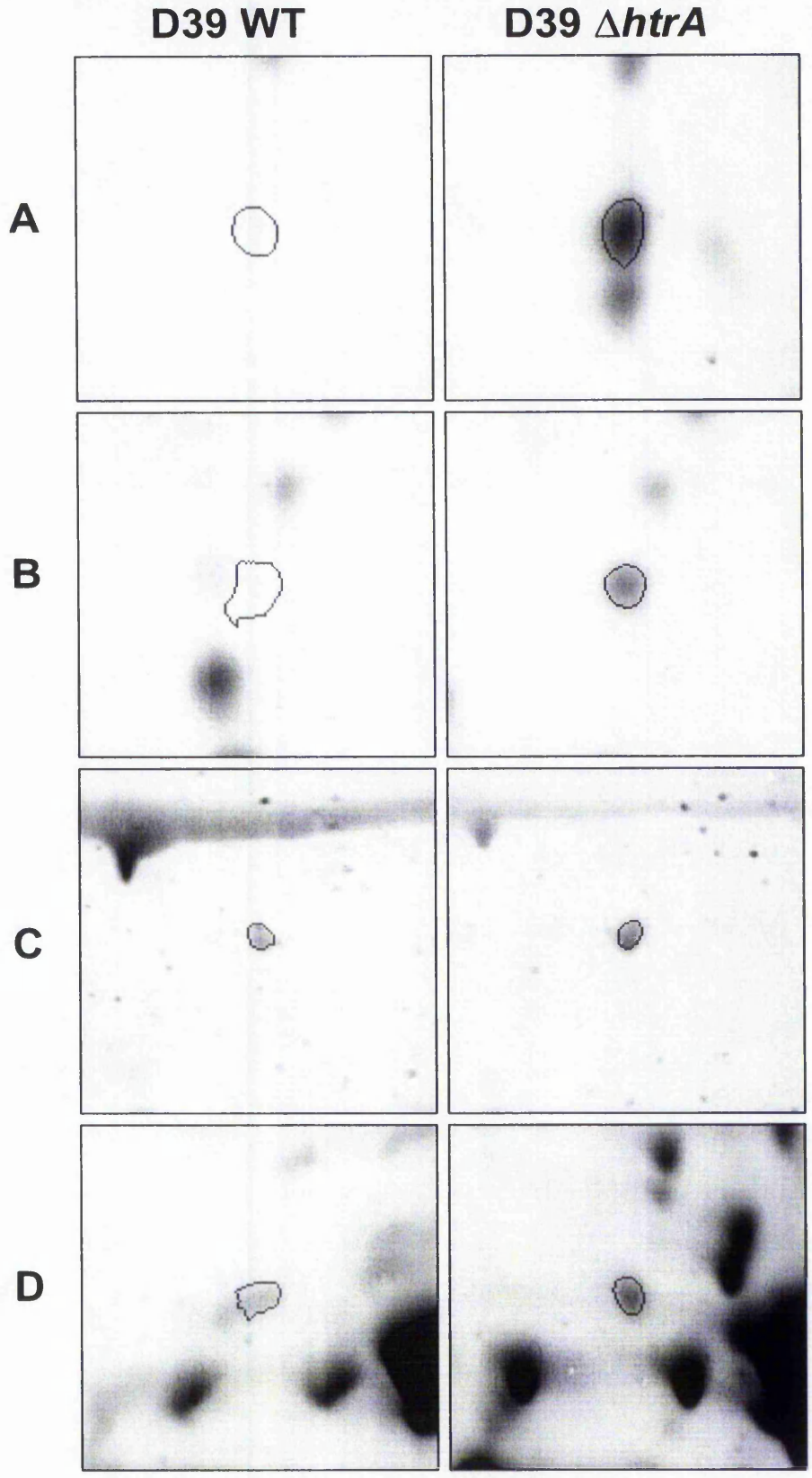


Figure 6.7- Representative *S. pneumoniae* proteins up regulated in *htrA* mutant.

2D sections from individual gels showing spots of proteins up regulated as a result of *htrA* deletion. Proteins from D39 wild type or *htrA* mutant grown at 37°C were separated by two-dimensional gel electrophoresis and identified by MALDI-TOF MS. A, Spectinomycin adenyltransferase. B, Superoxide dismutase (Fe). C, Hypothetical protein phtA. D, DnaK.

6.2 Microarray analysis

This analysis was intended to compare the transcriptome of Δ*htrA* mutants to that of the wild type strain in an attempt to understand the effect of *htrA* deletion on the global gene expression. The microarray was designed to represent 2131 ORFs from *S. pneumoniae* TIGR4 strain in addition to 118 unique ORFs from R6 (37) and G54 (81). Gene expression profiles of both strains were compared in microarray experiments using fluorescent probes, labelled with Cy3 and Cy5, consisting of the first strand cDNA made from randomly primed total cellular RNA (Eisen and Brown, 1999). The relative representation of a transcript between the two RNA pools was assayed by measuring the fluorescence intensities of the two dyes at a given target on the array. Each comparison with a given RNA pair was repeated twice with the dyes swapped and the experiment was repeated once.

6.2.1 Preparation and analysis of RNA

RNA samples were prepared from TIGR4 wild type or TIGR4Δ*htrA* mutant strains grown in BHI at 37 °C to mid-log phase using Qiagen RNeasy® Midi Kit (see section 2.13.1 in the Materials and Methods). Preparing RNA under these conditions yielded 1.9 μg/μl of wild type RNA and 2.1 μg/μl of the mutant RNA. The concentrations of RNA were determined using NanoDrop® ND-1000 UV/Vis spectrophotometer (NanoDrop® Technologies, USA). Because it is essential for the microarray analysis to have a high quality RNA, the RNA samples were also tested for integrity and concentration using the agilent technology. Figure 6.8 shows the results of this analysis, which clearly indicate the purity, and integrity of RNA samples.

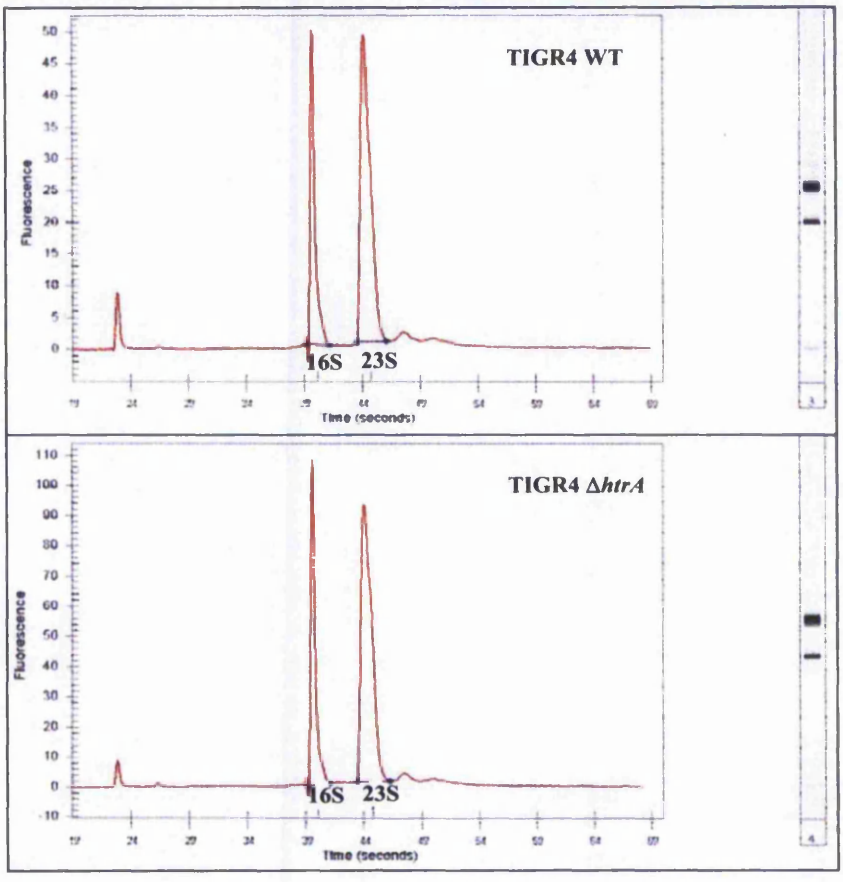


Figure 6.8- Analysis of RNA samples prepared from TIGR4 WT and TIGR4 Δ*htrA* mutant.

The RNA was prepared from cultures grown in BHI at 37 °C to OD_{600nm} ~ 0.6 using Qiagen RNeasy® Midi Kit. RNA samples were checked for integrity and concentration using RNA 6000 Nano assay with the Agilent 2100 Bioanalyzer.

6.2.2 RT-PCR for htrA

RT-PCR was used to confirm the absence of *htrA* transcript in the RNA sample prepared from Δ*htrA* mutant strain. RNA samples (2 μg each) prepared from TIGR4 wild type or TIGR4Δ*htrA* mutant strains grown at 37 °C were used to synthesize cDNA using random hexamers. The synthesis of cDNA was carried out using ThermoScript™ RT-PCR system as described in section 2.13.8. 5 μl of each cDNA reaction was used as a DNA template in PCR reactions, which were carried out using Vent® polymerase and the *htrA* specific primer pair HtrA for and HtrA rev (table 2.2 in the Materials and Methods). The PCR reactions were heated to 94 °C for 5 min and run through 30 cycles of (94 °C for 1 min, 52°C for 30s, 68 °C for 1 min). Samples of PCR reactions were withdrawn after 15 and 30 cycles and the products were visualized by agarose gel electrophoresis. Figure 6.9 shows the results of these PCR reactions. The gene for HtrA was amplified after 15 and 30 PCR cycles in case of TIGR4 wild type but not in TIGR4Δ*htrA* mutant.

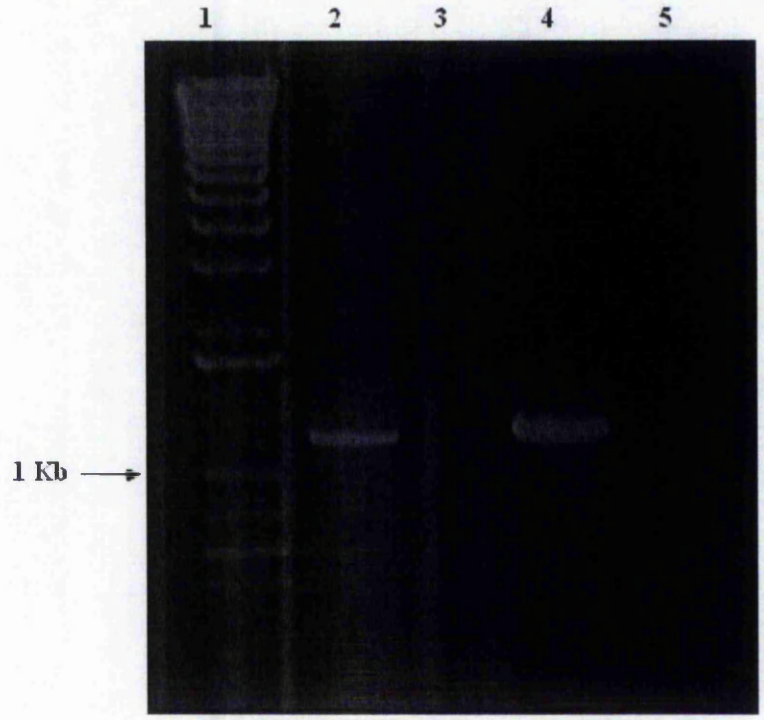


Figure 6.9- Agarose gel electrophoresis for the analysis of RT-PCR products for *htrA*.

5μl of cDNA samples prepared from RNA of both stains was used in PCR reactions to amplify *htrA* gene to examine the existence of a transcript for *htrA*. Lane 1, 1 kb plus ladder; lane 2, *htrA* (1.2 kb) from the wild type amplified after 15 cycles; lane 3, mutant after 15 cycles (negative); lane 4, *htrA* from the wild type after 30 PCR cycles; lane 5, mutant after 30 cycles (negative).

6.2.3 Microarray design and data analysis

Microarray analysis of large-scale gene expression patterns in the TIGR4 wild type and TIGR4Δ*htrA* was used in an attempt to identify the molecular basis for the virulence attenuation of Δ*htrA* mutants and to obtain a comprehensive view of the extent to which HtrA activity impacts pneumococcal gene expression. 10 μg of RNA prepared from either TIGR4 wild type strain or TIGR4Δ*htrA* mutant strain was used in the reverse transcription reactions to synthesize cDNA, which was labelled by the fluorescent dyes, Cy3 and Cy5 before hybridization to the array. The incorporation of Cy3 and Cy5 is known to be of different efficiencies. In order to overcome this problem, a dye swap was performed with the cDNA from the wild type labelled with Cy3 and that from the mutant labelled with Cy5 in one slide and the labelling was done in a reverse order in the second slide i.e. wild type with Cy5 and mutant with Cy3.

The labelled cDNA samples were hybridized to the probes spotted on the array slides and the hybridized slides were scanned using a ScanArray™ Express microarray scanner (Packard Bioscience, Biochip Technologies). The signal intensities for each spot of both

samples on the array were measured using Quantarray™ (Packard Bioscience). The calculated ratio of signal intensities for each spot delivers a measure for fold changes in gene expression. However, the corresponding fold changes might reflect experimental biases (e.g. dye bias) rather than changes in gene expression. For this reason, a normalization step was performed prior to data analysis. This step aimed to adjust the fluorescence intensities of the two colour channels, blue (Cy3) and red (Cy5) and to correct experimental factors such as differing labelling efficiencies, fluorescence quantum yields, background intensities, scanning sensitivity and signal amplification (Schuchhardt *et al.*, 2000, Holloway *et al.*, 2002, Yang *et al.*, 2002)

The array data from two independent experiments was imported to the Genespring™ software, which enables the determination of signal ratios, the identification of regulated genes and the performance of statistical analysis of results. In the Genespring™ analysis, a signal ratio of 1.0 indicates that the expression of particular genes is equal in both wild type and mutant, a ratio of more than 1.0 indicates increased expression in the mutant compared to the wild type and a ratio of less than 1.0 corresponds to lower expression in the mutant compared to the wild type. This software also allows spot intensities to be colour coordinated to help identify differential gene expression. Genes showing equal expression levels result in yellow spots, those showing up-regulation in the wild type appear blue and red spots represent those down-regulated genes.

6.2.4 Array results

The scatter plot in figure 6.10 shows the relationship between the average hybridization intensities of the wild-type control cells and *htrA* mutant cells. This simple procedure provided an overview of the data and indicated that most of the spots fell along the diagonal lines and were equally labelled. The spots that were not on the diagonal were candidates for genes with expression changes.

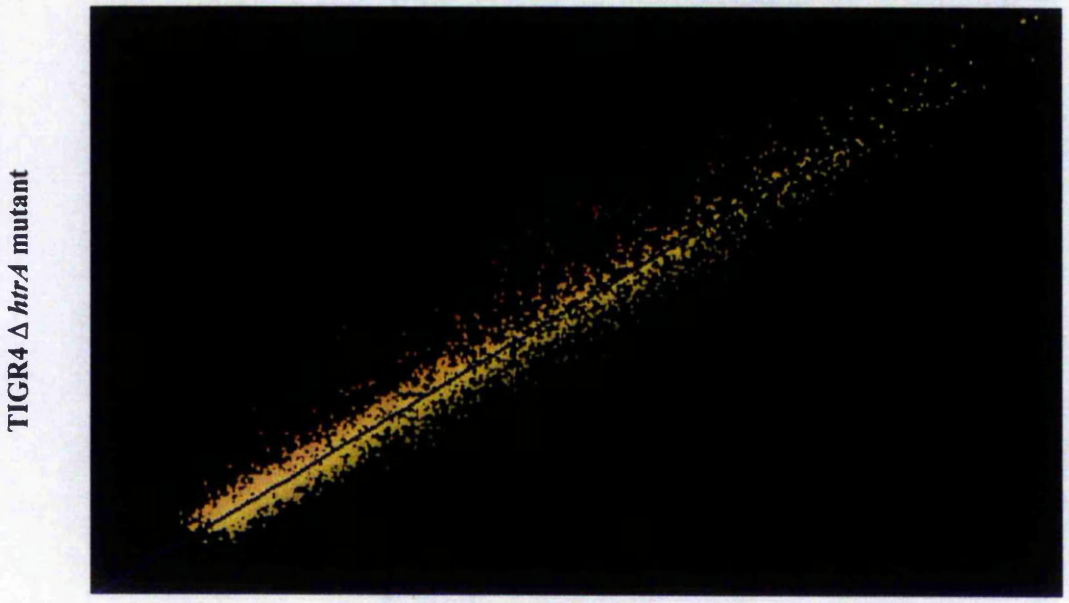


Figure 6.10- Representative scatter plot comparing gene expression in the TIGR4Δ*htrA* mutant to that of the wild type TIGR4.

Labelled cDNA samples were hybridized to the probes on the array and scanned. Data was analyzed using the Genespring™ software. The plot illustrates the spot intensities of all genes detected for the wild type on the X-axis and the mutant on the Y-axis. Yellow spots (within the diagonal lines) indicate similar expression level, red spots (above the diagonal lines) indicate up-regulated genes and blue spots (below the diagonal lines) indicate down-regulated genes in the mutant strain compared to wild type strain.

Whereas most of the pneumococcal genes remained unaltered, showing comparable expression levels between the TIGR4 wild type and TIGR4Δ*htrA* mutant, some genes were shown to have significant differences in expression levels between wild type and mutant organisms. Genes were selected if they demonstrated at least two-fold difference in signal intensity between the wild type and mutant and if this intensity was found to be statistically significant ($P \leq 0.05$) using *t*-test in the Genespring™ analysis. The microarray analysis revealed five down-regulated genes and twenty up-regulated genes. Lists of down- and up-regulated genes are shown in tables 6.2 and 6.3 respectively.

Table 6.2- TIGR4 down-regulated genes due to *htrA* deletion.

TIGR4 annotation	Gene symbol	Mean intensity ratio	*p*-value	Putative identification
SP0366	*aliA*	2.0	0.0012	Oligopeptide ABC transporter
SP0783		2.5	0.0011	Conserved hypothetical protein
SP1241		2.3	0.0000	Amino acid ABC transporter
SP2239	*htrA*	6.6	0.0002	Serine protease

As expected, the *htrA* gene was highly down regulated in the TIGR4Δ*htrA* mutant strain. In fact, examination of the individual spot intensities revealed that the fluorescence signals of *htrA* spots in case of Tigr4Δ*htrA* mutant were identical to the background signals. Of interest was the down-regulation of the oligopeptide ABC transporter *aliA*, which is an ATP-binding cassette transporter involved in nutrient uptake and in other processes such as colonization (Kerr *et al.*, 2004).

Table 6.3- TIGR4 up-regulated genes due to *htrA* deletion.

TIGR4 annotation	Gene symbol	Mean intensity ratio	*p*-value	Putative identification
SP0459	*pfl*	2.2	0.0006	Formate acetyltransferase
SP0517	*dnaK*	2.4	0.0009	DnaK protein
SP0519	*dnaJ*	3.1	0.0012	DnaJ protein
SP0875	*lacR*	2.1	0.0001	Lactose phosphotransferase system repressor
SP0876		2.6	0.0001	1-Phosphofructokinase, putative
SP0877		2.7	0.0004	PTS system, fructose specific II ABC components
SP1715		2.3	0.0001	ABC transporter, ATP-binding protein
SP1883	*dexS*	2.0	0.0005	Dextran glucosidase DexS, putative
SP1884		2.7	0.0001	PTS system, IIABC components
SP1906	*groEL*	2.7	0.0001	Chaperonin, 60 kDa
SP2030	*recP*	2.0	0.0003	Transketolase
SP2055		2.5	0.0001	Alcohol dehydrogenase, zinc-containing
SP2206	*yfiA*	2.0	0.0008	Ribosomal subunit interface protein
SP2216	*usp45*	2.2	0.0281	Secreted 45 kDa protein
SP0044	*purC*	2.9	0.0001	Phosphoribosylaminoimidazole-succinocarboxamide synthase
SP0045		3.1	0.0000	Phosphoribosylformylglycinamidine synthase, putative
SP0046	*purF*	2.1	0.0003	Amidophosphoribosyltransferase
SP0047	*purM*	2.1	0.0002	Phosphoribosylformylglycinamide cyclo-ligase
SP0050	*purH*	2.0	0.0000	Phosphoribosylaminoimidazolecarboxamide formyltransferase/IMP cyclohydrolase
SP0095		2.1	0.0019	Conserved hypothetical protein

This microarray analysis also confirmed no polar effect of *htrA* mutation occurred on the downstream gene *spoJ*. Analysis of data revealed *spoJ* average expression level of 2.6345 (normalized intensity) in the TIGR4 wild type and 3.1635 in the TIGR4Δ*htrA* mutant. This slight difference in the expression level of *spoJ* in both strains was not statistically significant. This observation rules out the possibility of the polar effect of the mutation highlighted by Lyon and co-workers (Lyon and Caparon, 2004) who observed that deletion

of *htrA* of *Streptococcus pyogenes* did not result in attenuation in a murine model of subcutaneous infection contradicting what Jones and colleagues have reported about the insertional mutagenesis of *htrA* of the same organism (Jones *et al.*, 2001).

Chapter 6 Discussion

HtrA protein contributes in a number of aspects to the biology and stress response of *Streptococcus pneumoniae*. This serine protease is implicated in the ability of the pneumococcus to grow at higher temperatures, to resist oxidative stress and to take up exogenous DNA in the process of transformation. Moreover, deletion of *htrA* resulted in organisms much attenuated in virulence using animal models of infection (see chapter 4). However, the exact mechanism by which HtrA controls these processes is still unclear. The study presented in this chapter was designed to explore this mechanism by employing the powerful tools of proteomics and microarray to investigate the effect of HtrA on the expression of other proteins.

Proteomics analysis

The resolving power of two-dimensional polyacylamide gel electrophoresis facilitates the monitoring of expression of thousands of proteins simultaneously. In this study, the methodologies of proteomics were employed to investigate the effect of *htrA* inactivation on the differential expression of proteins by comparing the proteome of the wild type D39 strain to that of D39Δ*htrA* mutant strain. Identification of proteins by MALDI-TOF enabled the confirmation that the proteomic experiment was carried out properly. The spectinomycin adenyltransferase, which was used as an antibiotic resistance marker in the mutant, was only identified in the four gels corresponding to D39Δ*htrA* mutant proteins but not in the wild type. However, the overall expression level of most of the identified proteins was found to be similar in the wild type and mutant organisms (table 6.1) due to differences between individual gels in the same group. Some of the identified proteins existed as isoforms, presumably due to deamination or phosphorylation or by modification with a group resulting in a shift of the pI toward the acidic range of the 2D map (Kilstrup *et al.*, 1997). Furthermore, due to time limitation, not all the protein spots showing differences were identified. This is why HtrA protein, for example, was not found among the identified proteins and also as expected from doing one experiment, no statistical analysis was properly carried out. Therefore, it should be noted that the results of this proteomic analysis are only preliminary and more experiments are needed to validate these data.

(i) Down-regulated proteins

The proteomic analysis presented here revealed that the most noticeably down-regulated proteins are those involved in the process of protein synthesis, namely 30S ribosomal protein S1, aminoacyl-tRNA synthetase (CysRS), and Elongation factor Tu. In bacteria,

two RNA–protein subunits consisting of a small 30S subunit and a large 50S subunit assemble around a mRNA template into a roughly spherical 70S particle that translates the nucleotide sequence into a polypeptide chain through repetitive, codon-dependent binding of aminoacylated tRNA. Translation of genetic information encoded by mRNA is the last step of the gene expression pathway. The essential components and principal steps of translation have been delineated through genetic, biochemical, and kinetic methods (Wilson *et al.*, 2002). The synthesis of peptide bonds is done by the ribosome, which is constructed using three rRNAs and more than fifty ribosomal proteins in bacteria. Translation involves additional factors that play important role in each step, as represented by initiation, peptide elongation, termination, and recycling of the ribosome. The process of polypeptide elongation has been most extensively characterized (Rodnina *et al.*, 2000). Gene expression is regulated at three steps, transcription, translation and post-translation. The critical regulation point in the translation process is initiation, during which the 30S ribosomal subunit binds to the Shine-Dalgarno (SD) sequence (Shine and Dalgarno, 1974) in the mRNA. Ribosomal protein S1 is the largest protein of eubacterial ribosomes. It is an RNA-binding protein involved in retention of mRNA during initiation of translation and, maybe, during elongation (Subramanian, 1983, Sorensen *et al.*, 1998).

Aminoacyl-tRNA synthetases play a critical role in protein biosynthesis, since they catalyze the accurate charging of a given set of tRNAs with the cognate amino acid. The insertion of cysteine into nascent peptides during protein synthesis is dependent on the interaction of cysteine codons with cysteinyl-tRNA (Cys-tRNA) in the ribosomal A site. The pneumococcal cysteinyl-tRNA synthetase (CysRS) was found in this analysis to be down regulated in the D39Δ*htrA* mutant. Disruption of the homologous gene *cysS* encoding CysRS of *B. subtilis* was found to be lethal for the cells, indicating that these genes code for essential and unique functions in these organisms (Gagnon *et al.*, 1994).

Elongation factor Tu (EF-Tu) (Krab and Parmeggiani, 1998) is a guanine nucleotide-binding protein responsible for the delivery of aminoacyl-tRNA (aa-tRNA) to the ribosome during the elongation phase of protein synthesis. EF-Tu belongs to a superfamily of regulatory GTP hydrolases (G proteins) that is implicated in the sorting and amplification of transmembrane signals and the direction of the synthesis and translocation of proteins (Sprang, 1997). A role of EF-Tu in attachment of lactobacilli to human cells was also reported. Granato and co-workers demonstrated an adhesin-like role of *Lactobacillus johnsonii* EF-Tu and showed that it is able to induce a pro-inflammatory response (Granato *et al.*, 2004). Work with *E. coli* elongation factors suggested that some of them possess chaperone properties in addition to their role in translation (Caldas *et al.*, 1998, Caldas *et*

al., 2000). Caldas *et al* have reported that the *E. coli* elongation factor EF-Tu increases the refolding of unfolded proteins in a manner similar to that of molecular chaperones, protects proteins against thermal denaturation, and forms complexes with unfolded proteins. This report proposed that, in addition to its role in translational elongation, EF-Tu protein might help in protein folding and renaturation in the cytoplasm (Caldas *et al.*, 1998). In the present study, proteomic analysis revealed the down-regulation of EF-Tu in the D39Δ*htrA* mutant. Giving the role of EF-Tu as a chaperone, it is sensible to postulate that the down-regulation of EF-Tu partly accounts for the accumulation of damaged proteins that results in the slower growth of Δ*htrA* mutant at elevated temperatures compared to the wild type.

(ii) Up-regulated proteins

SodA, PhtA, and DnaK were among the up-regulated proteins revealed by this proteomic analysis. Reactive oxygen intermediates, including superoxide anion ($\cdot O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($\cdot OH^-$), have many deleterious effects on living organisms, ranging from DNA damage to peroxidation of membrane lipids (Uchida *et al.*, 1965, Janssen *et al.*, 1993). Sources of reactive oxygen intermediates are abundant and include incomplete reduction of oxygen during respiration, exposure to radiation or to redox-active compounds, and the respiratory burst of phagocytes (Halliwell and Cutteridge, 1990). In the presented proteomic study, the oxidative stress protein superoxide dismutase (SOD) was identified among proteins up regulated as a result of *htrA* deletion. Superoxide dismutases (SODs) are metalloenzymes that catalyze the conversion of superoxide molecules to hydrogen peroxide and molecular oxygen and therefore form one of the cell's major defence mechanisms against oxidative stress (McCord and Fridovich, 1969). *Escherichia coli* contains two highly homologous SODs, a manganese- and an iron-containing enzyme (Mn-SOD and Fe-SOD, respectively). In contrast, a single Mn-SOD is present in *Bacillus subtilis*. In *E. coli*, the absence of SODs was found to be associated with an increased sensitivity to cadmium, nickel and cobalt ions. Mutants lacking either *sodA* or *sodB* exhibited metal resistance to levels comparable to that of the wild-type strain. On the other hand, in *B. subtilis*, the *sodA* mutation had no effect on cadmium and copper resistance (Geslin *et al.*, 2001). The *htrA* mutant studied here showed increased sensitivity to hydrogen peroxide but not to paraquat (see chapter 4) suggesting that SODs have no effect on the pneumococcal resistance to superoxide generated by paraquat.

Phagocytes undergo a sharp burst of oxygen consumption when engulfing bacteria. This oxygen is enzymatically reduced to toxic metabolites, which are essential to the bactericidal action of the cell. As well as being important in the detoxification of

superoxide radicals during aerobic metabolism, SOD has also been shown to be a virulence factor for several pathogenic bacteria. Pesci and co-workers (Pesci *et al.*, 1994) reported that a *sodB* insertion mutation in *Campylobacter jejuni* caused a significant reduction in the ability of the bacterium to invade an embryonic intestinal cell line. Inactivation of *sodA* in *Yersinia enterocolitica* resulted in a marked reduction in virulence of the organism in a mouse infection model after intravenous infection when compared to its parental strain (Roggenkamp *et al.*, 1997). More recently, it was reported that a superoxide dismutase mutant strain of *Neisseria meningitidis* was less virulent in a mouse intraperitoneal infection model than the wild-type strain (Wilks *et al.*, 1998). On the other hand, it should be noted that the role of superoxide dismutase is not the same in all microorganisms. A *sodA* deletion mutation of *Bordetella bronchiseptica* and *Bordetella pertussis* did not affect the virulence of these species in mice following intranasal infection (Graeff-Wohlleben *et al.*, 1997). SODs were reported to be a virulence factor in many other organisms, including *Candida albicans* (Hwang *et al.*, 2002), *Vibrio shiloi* (Banin *et al.*, 2003) and Leishmania (Ghosh *et al.*, 2003).

The pneumococcus is a facultative anaerobic bacterium that lacks catalase (Hardie, 1986). Thus, the absence of this enzyme suggests that superoxide dismutase may play a critical role in response to oxidative stress, affecting both the survival and, consequently, the virulence of the organism. Yesilkaya and co-workers (Yesilkaya *et al.*, 2000) reported that a *SodA* mutant of the virulent type 2 strain D39 had a lower growth rate than the wild type and exhibited susceptibility to the redox-active compound, Paraquat. *In vivo*, mice infected with the *SodA* mutant had a longer median survival rate than those infected with the wild type. The growth of the mutant in the lungs and its appearance in the bloodstream were also delayed (Yesilkaya *et al.*, 2000). The up-regulation of superoxide dismutase revealed by the proteomic analysis in this study therefore suggests the mutant pneumococcal cells may respond to the loss of HtrA function by increasing the amount of those proteins known to be involved in the resistance against oxidative stress to compensate for the absence of HtrA.

Among the up-regulated proteins is the pneumococcal histidine triad protein A (PhtA), also known as BVH-11 (Hamel *et al.*, 2004). This protein belongs to a family of analogous but separate proteins capable of eliciting protection against pneumococcal infections. The amino-acid sequences of these proteins contain three histidine triad motifs (HxxHxH). Within *S. pneumoniae*, there are four related pneumococcal histidine triad proteins (PhtA, PhtB, PhtD and PhtE). These proteins are approximately 800 amino acids in length and are highly conserved within this organism. They were selected as potential vaccine candidates

based upon their possession of hydrophobic leader sequences, which presumably target these proteins to the bacterial cell surface (Inouye *et al.*, 1982, Munoa *et al.*, 1991). This family of protein antigens has been reported to elicit protection against certain pneumococcal capsular types in a mouse model of systemic disease (Adamou *et al.*, 2001). Another report (Wizemann *et al.*, 2001) has shown that immunization using a fragment of PhtA (residues 18-230) protects mice from subsequent infection by several strains of *S. pneumoniae* (in contrast to other potential protein vaccines, which elicit a response but are strain-specific). The possible use of these proteins as vaccine components to control pneumococcal diseases has also been reported by Hamel and co-workers (Hamel *et al.*, 2004). Recently, residues 18-230 of the PhtA was expressed, purified and X-ray-characterized (Riboldi-Tunnicliffe *et al.*, 2004a) but its actual function is currently unknown. It is proposed that these proteins play a role in metal or nucleoside binding on the cell surface but no reports confirm that at present. The question of a possible role for this family of proteins in the virulence of the pneumococcus is also left unanswered. Adamou and co-workers have shown that sera from pneumococcal bacteremia patients recognize PhtA and PhtD, indicating that these proteins are exposed and recognized by the immune system during natural *S. pneumoniae* infection in humans (Adamou *et al.*, 2001). The up-regulation of proteins that are potential vaccine candidates such as PhtA in the *htrA* mutant reported here strengthens the idea of using these mutants as live vaccines as these proteins are exposed to the host immune system. This also signifies the role of proteomics and microarray approaches in providing information on the global changes in strains of particular interest.

As part of the major heat shock proteins (HSPs), DnaK promotes proper folding and translocation of proteins (Craig *et al.*, 1993, Hendrick and Hartl, 1993). Heat shock of the pneumococcus has been found to induce the synthesis of heat shock proteins. The major proteins induced by heat shock were 62, 72, and 84 kDa in size, identified subsequently as GroEL, DnaK, and ClpL, respectively (Choi *et al.*, 1999). The pneumococcal DnaK was cloned and characterized by Kim *et al* (Kim *et al.*, 1998). In *E. coli*, DnaK is part of a chaperone machine consisting of DnaK, DnaJ and nucleotide exchange factor, GrpE (Georgopoulos and Welch, 1993). The DnaK chaperone system functions mainly in the folding of nascent polypeptides and provides protection against aggregation during heat shock.

The major HSP, DnaK, is highly immunogenic in *S. pneumoniae* (Hamel *et al.*, 1997) and might be a good candidate as a vaccine (Kim *et al.*, 1998). The highly conserved and immunodominant antigenic nature of DnaK suggests that this protein may play some role

in defense against pneumococcal infection and pathogenesis. Since HSPs function as chaperones and promote renaturation of unfolded proteins (Hendrick and Hartl, 1993) and are induced during infection in a wide variety of bacterial pathogens (Buchmeier and Heffron, 1990, Dorman *et al.*, 1990, Fernandez *et al.*, 1996), survival *in vivo* could be enhanced by the stabilizing effect of HSPs on bacterial macromolecular complexes in hostile environments (Buchmeier and Heffron, 1990). Therefore, persistence of the HSPs upon return to normal conditions and induction of virulence proteins by heat shock might contribute to or enhance the virulence of the pneumococcus. The growth phenotype of *htrA* mutant at high temperatures, that the mutant cells eventually reached the same maximum OD as the wild type (see chapter 4), could therefore be explained by the up-regulation of DnaK, which helps refold damaged proteins resulting from heat stress.

Microarray analysis

Microarray is a powerful tool for the analysis of transcriptional changes in gene expression following different changes in the environment. Recently, pneumococcal microarrays have been used to measure transcriptional changes *in vitro* (Sebert *et al.*, 2002, McCluskey *et al.*, 2004) and also *in vivo*, during invasive disease (Orihuela *et al.*, 2004). A simple analysis has been chosen here, due to time limitation, to get an idea about alterations in gene expression as a result of *htrA* deletion. The transcriptome of both the TIGR4 wild type and its *htrA* mutant grown to mid-log phase at 37 °C were compared. The mutation was confirmed by the absence of HtrA transcripts. Lack of HtrA transcripts in the mutant was also confirmed by RT-PCR. Only three genes were down regulated, *aliA*, the gene for the amino acid ABC transporter (SP2141) and a hypothetical gene (SP0783) with unknown function. In addition, twenty more genes were up regulated (see below).

(i) Down-regulated genes

Streptococcus pneumoniae is an obligate parasite, which requires several amino acids for growth. Oligopeptide uptake mediated by the Ami ABC permease is therefore important for nutrition. AliA is an oligopeptide-binding lipoprotein, which is highly homologous to the characterized AmiA (Alloing *et al.*, 1994) and belongs to the ABC permease Ami-AliA/AliB (Alloing *et al.*, 1990). The Ami-AliA/AliB oligopeptide permease is involved in the uptake of nutrients from the environment and may also act as an environmental signal allowing the organism to adapt accordingly (Claverys *et al.*, 2000). Regarding the decrease in transformation efficiency of *htrA* mutants compared to wild types, microarray analysis did not reveal any down regulation in genes reported to be involved in competence, as

expected for the stage of growth from which RNA was extracted. One possible way to address this would be investigating differential gene expression in the mutant and wild type in the presence of CSP to induce genes involved in competence. It has been proposed that Ami-AliA/AliB may be involved in triggering competence for genetic transformation (Claverys *et al.*, 2000). Mutations in genes encoding components of the Ami oligopeptide transporter have been found to affect competence development (Alloing *et al.*, 1994, Pearce *et al.*, 1994). As the *htrA* mutant was less easy to take up external DNA than the wild type, it is likely that the down-regulation of *aliA* could account for this decrease in genetic competence. Peptide permeases also modulate pneumococcal adherence to epithelial and endothelial cells either by acting as adhesins or by modulating the expression of adhesins on the pneumococcal surface (Cundell *et al.*, 1995b). Recently, Kerr and co-workers have shown that although the Ami-AliA/AliB complex is not required for virulence during pneumococcal pneumonia, it does play a role in colonization of the nasopharynx (Kerr *et al.*, 2004). The number of *aliA* mutant organisms colonizing the nasopharynx of mice was significantly lower than that of the wild type organism 48 hours post challenge (Kerr *et al.*, 2004). Furthermore, in a microarray analysis of pneumococcal gene expression during invasive disease, designed to identify site-specific patterns of expression for virulence factors and others, the gene for AliA was identified among the genes whose expression was enhanced during epithelial cell contacts (ECC). The level of *aliA* was 3.9-fold up regulated (Orihuela *et al.*, 2004). Presumably, the genes whose expression is altered during invasive disease are those that are required by the bacteria for survival in the host (Hava *et al.*, 2003). Taken together, AliA is considered as a good candidate that could explain some of the phenotypes observed for the *htrA* mutant including competence and virulence and could be considered for future work investigating the role of HtrA.

(ii) Up-regulated genes

Analysis of microarray data also revealed the up-regulation of several genes. These include stress related genes encoding for DnaK and DnaJ that are involved in protein folding and stabilization in addition to the 60 kDa chaperonin GroEL. As discussed for the DnaK (see proteomic analysis above), the products of these genes play a significant role in the folding of nascent protein chains during normal growth conditions and in the refolding of proteins after thermal damage. Furthermore, the DnaK-DnaJ-GrpE chaperone complex participates in ATP-dependent proteolysis in the cell. It is proposed that these genes have been expressed in response to accumulation of misfolded or aggregated proteins, which resulted from lack of HtrA in the pneumococcal Δ*htrA* mutant. DnaK was up regulated in both

microarray and proteomic analyses. In addition, the gene for RecP was found to be up regulated. RecP belongs to a diverse transketolase family and is implicated in genetic recombination in the pneumococcus (Reizer *et al.*, 1993). RecP is involved only in the last step of DNA processing during transformation, i.e., recombination, because *recP* insertion and deletion mutants were not defective in binding, degradation, or uptake of donor DNA or in competence induction (Rhee and Morrison, 1988). Whether RecP is linked to the deficiency in DNA uptake observed for the Δ*htrA* mutant is yet to be investigated.

Genes involved in purine nucleotide biosynthesis pathway, SP0045, SP0046 (*purF*), SP0047 (*purM*), and SP0050 (*pur H*) were up regulated. These genes may be organized as a single transcription unit in the chromosome. They have been identified in the *in vivo* analysis of gene expression during invasive disease (Orihuela *et al.*, 2004). Their levels of expression were up regulated in the blood by, 6.1, 11.6, 8.4, and 10.3-fold, respectively (Orihuela *et al.*, 2004). Up regulation of these genes during bacteraemia indicates a role for the products of these genes in the pathogenicity of pneumococcal disease. Mutation in *purF* of *Mycobacterium smegmatis* resulted in impaired survival during oxygen-starved stationary phase (Keer *et al.*, 2001). Analysis of the genome of *S. pneumoniae* strain TIGR4, published at The Institute for Genomic Research at (http://www.tigr.org/), revealed an operon composed of a transcriptional regulator, *lacR* (SP0875), a phosphofructokinase (SP0876), and a fructose-specific ATP-binding cassette, ABC (SP0877). This operon was up-regulated in the Δ*htrA* mutant. Expression of genes required for nutrient acquisition is likely to be tightly regulated. Infection at different sites of the host may necessitate that bacteria can make use of a range of carbohydrates depending on the availability and streptococci are known to display metabolic flexibility (Neijssel *et al.*, 1997). Insertional inactivation of the genes encoding the ABC transporter substrate-binding protein and the fructosidase in the homologous operon of *Lactobacillus acidophilus* reduced the ability of the mutants to grow on fructo-oligosaccharides (Barrangou *et al.*, 2003).

Another upregulated gene is *yfiA*. The product of this gene is a ribosome-associated protein, YfiA, which is a cold shock protein in *E. coli*. Synthesis of Yfia and its subsequent binding to ribosomes were induced by a temperature decrease (Agafonov *et al.*, 2001). It blocks the binding of aminoacyl-tRNA to the ribosomal A site and therefore protein synthesis declines as the production of this protein is induced. This protein is also involved in sugar metabolism and mutational inactivation of the *yfiA* gene almost abolishes growth of *B. subtilis* in the minimal medium containing maltose (Yamamoto *et al.*, 2001a).

The microarray data discussed above was derived from two array chips that used the same RNA preparation taken from a single time point during *in vitro* growth. Thus, before drawing any conclusions, the array work needs to be repeated using different RNA preparations from bacteria grown to the same stage in identical growth conditions. Microarray analysis of gene expression *in vivo* during infection is now feasible (Orihuela *et al.*, 2004). It would be helpful, therefore, to study genes differentially expressed by *htrA* mutants at different infection sites. Studying the effect of an *htrA* deletion on gene expression in different strains is also required. This will, for example, confirm the proteomic data shown for D39 and also give an idea of the possible differences of the function of HtrA in different pneumococcal strains and serotypes. It should also be noted that altered expression of the genes discussed does not prove that HtrA directly regulates such genes. It is likely that some genes could be affected indirectly through other regulatory networks.

Summary

Studies presented here were designed to explore the mechanism by which HtrA exerts its functions in the biology and stress response of *S. pneumoniae*. These studies were done at the end of the project and are preliminary. The effect of *htrA* deletion on the expression of proteins was investigated using the powerful tools of proteomics and microarray. Analysis of *htrA* mutants by proteomics and microarray confirmed the vivacity of the mutation. The spectinomycin marker was only identified in the mutant proteins (proteomics) and no effect of the mutation was observed on the expression of *htrA*-downstream genes (microarray). Expression of proteins from a number of functional categories is modulated as a result of *htrA* loss of function. Some of these are stress response proteins while others are key components in central and intermediary metabolism. These approaches also revealed a number of targets for future gene inactivation or functional enzymology studies. These studies would further facilitate the assessment of their role in the stress response and any additional pleiotropic effects. However, the presented data are only in their infancy, they require validation by repeating some of the experiments and designing some more under different physiological conditions. Measuring differential gene expression after heat shock or oxidative stress would be examples of such experiments.

CONCLUDING REMARKS

Streptococcus pneumoniae is responsible for a large proportion of the bacterial disease involving the respiratory tract (acute otitis media, sinusitis, and pneumonia) as well as invasive infection (septicemia and meningitis) in humans. Colonization is the first step in the interaction between *S. pneumoniae* and its human host. Carriage of *S. pneumoniae* in the nasopharynx is common and typically asymptomatic, yet, in infants, the elderly, and persons with underlying medical conditions, the pneumococcus is a major cause of community-acquired pneumonia, bacteraemia, and meningitis. The limited efficacy of the current vaccine and the increasing emergence of antibiotic-resistant strains have urged the exploration as to how the pneumococcus cause such life-threatening diseases in an attempt to identify novel vaccine candidates and antimicrobial agents. Although the pneumococcus has been studied for over a century, an understanding of the mechanisms that underlie the course of disease remains fragmented. This work, therefore, was aimed to hopefully contribute to our understanding of such mechanisms.

Bacterial entry into the host organism involves significant environmental changes, which are expected to induce the regulation of heat shock genes. Indeed, some of the heat shock proteins (HSPs) are themselves virulence factors while others affect pathogenesis indirectly, by increasing bacterial resistance to host defences or regulating virulence genes. This thesis was concerned with the evaluation of the role of some proteins involved in the pneumococcual defence against different stress conditions. To study the contribution of these proteins to the stress response, this thesis 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 which one of these HSPs is deleted. The phenotypes of mutants were compared to the wild types *in vitro* as well as in murine models of pneumococcal disease.

Clp ATP-dependent proteases are involved in regulation by proteolysis in several bacteria and consist of a proteolytic subunit, ClpP, on which substrate specificity is conferred through association with ATPase subunits (ClpA, ClpC, and ClpX), which include members of the ubiquitous Hsp100 family. In this thesis, the role of ClpC and ClpP in the stress response of *S. pneumoniae* was investigated. ClpC has been identified previously as a virulence factor of the pneumococcus in a signature-tagged mutagenesis screen. In a previous report, ClpC was shown to play a role in thermal tolerance, control of autolysis and chain formation in the pneumococcus. Moreover, this report has also shown that ClpC plays a key role in processes related to virulence including adherence to human cells and production of known virulence factors such as pneumolysin, LytA, CbpA and other choline binding proteins. However, other reports did not confirm a role of ClpC in these processes.

Although this thesis did not resolve the controversy over the role of ClpC, it does report some interesting findings on the contribution of ClpC to different processes particularly autolysis and *in vivo* bacterial growth. Among all the Δ*clpC* mutants studied, Δ*clpC* mutants of the serotype 2 strain D39 showed a decreased autolysis rate after stationary phase of growth or under exposure to antibiotics. The mutants of both virulent and avirulent strains of D39 maintained viability after stationary phase both at optimal and elevated temperatures. Moreover, cell separation in these mutants was altered and the organisms grew in long chains of bacterial cells. This decrease in autolysis rate suggested impairment in the role of the major pneumococcal amidase responsible for cell separation and autolysis, LytA. Investigation by Western blotting of the amount of LytA produced and released by the mutant strains revealed that LytA was not released to the culture supernatant while it was detected, as expected, in the culture supernatant of the wild type. In addition, the quantity of released pneumolysin was less in the Δ*clpC* mutant than in the wild type. Thus it is likely that ClpC is involved in the control of autolytic process and in the release of pneumococcal virulence factors. The autolysis phenotype of D39Δ*clpC* mutants was not observed for Δ*clpC* mutants of the other strains tested suggesting a difference in ClpC function between different pneumococcal strains and serotypes. The contribution of ClpC to other stresses such as acid stress and oxidative stress was also studied. It appears that ClpC plays no role in the pneumococcal response to these stresses.

Analysis of Δ*clpC* mutants *in vivo* using pneumonia and bacteraemia models of disease revealed that ClpC has no effect on the overall outcome of pneumococcal disease. Mice infected with either the wild type or the mutant strains succumbed to the infection at similar rates. However, the growth of the pneumococcus in the lungs and blood following intranasal infection was dependent on ClpC. Numbers of Δ*clpC* mutant organisms recovered from lungs were significantly lower than that of the wild type at different time points after infection. Appearance of bacteria in the bloodstream following intranasal challenge was similar for wild type and Δ*clpC* mutant in that bacteria appeared in the bloodstream at a similar time and grew to a similar level by 24 hours. However, the wild type bacteria then continued to grow dramatically whereas numbers of the mutant were reduced to levels similar to that of time zero. Although the event triggered by the pneumococcus that results in death of mice in the Δ*clpC*-infected group is not known, the effect of *clpC* deletion on *in vivo* bacteriology seems to be dramatic.

This thesis confirms the contribution of ClpP to the stress response of *S. pneumoniae* reported previously for some strains and also reports a role of ClpP in other strains that has

not been reported before. ClpP is necessary for the growth of both serotype 2 (D39) and serotype 4 (TIGR4) strains of the pneumococcus at elevated temperatures (40 °C). ClpP is also required for the growth of D39 at a lower temperature (30 °C) but not for TIGR4. The requirement of ClpP for the survival of pneumococcal strains under heat shock highlights strain differences in the role of these molecules. Data presented in this thesis also confirm the involvement of ClpP in the resistance of *S. pneumoniae* strain D39 to oxidative stress. ClpP is also involved in the pneumococcal virulence. The virulence of the D39Δ*clpP* mutant was completely abolished in a pneumonia model of infection while it was reduced in TIGR4 Δ*clpP* mutant compared to its TIGR4 wild type when judged by survival of mice. Moreover, the bacterial counts of D39Δ*clpP* in the lungs and blood of infected mice were significantly lower than that of D39 wild type suggesting a role of ClpP in favouring survival of the pneumococcus in the host organism.

As an example of the ATP-independent proteases, the role of the high temperature requirement A protease, HtrA, was also investigated in different genetic backgrounds. The HtrA family of stress response proteins play fundamental roles in protecting bacteria against stresses posed by temperature, peroxide and others. This thesis reports that HtrA is a major virulence factor of the pneumococcus. Deletion of the gene for HtrA resulted in organisms that were more sensitive to heat and oxidative stresses. The effect of HtrA loss of function on the growth at elevated temperatures was different from that reported for other organisms in that it does not prevent growth completely but rather slows it. In addition, HtrA was found to provide resistance to hydrogen peroxide but not to superoxide. Production of reactive oxygen species (ROS) is considered an important feature of intracellular bactericidal mechanisms in phagocytes. To prevent oxidative damage, bacteria have evolved adaptive responses that result in the expression of numerous genes in response to the stresses exerted by the ROS. These adaptations include production of stress proteins such as HtrA. This thesis also reports that HtrA is involved in the ability of *S. pneumoniae* to take up exogenous DNA in the process of genetic transformation.

Mirroring the *in vitro* phenotypes, Δ*htrA* mutants were unable to cause disease in animal models of infection. The virulence of the type 2 mutant strain was completely abolished while that of the type 4 mutant was dramatically reduced. The Δ*htrA* mutants were cleared rapidly from the lung airways and lung tissues and failed to gain access to the bloodstream following intranasal infection. In addition, mutants were unable to cause disease when injected directly into blood. Thus the Δ*htrA* mutants neither caused pneumonia nor bacteraemia. Furthermore, the immune response to mutant organisms was altered

compared to that of the wild type as measured by levels of IL-6 and TNF-α as well as by tissue histology. The pneumococcal *htrA* mutant is therefore potentially suitable as a live vaccine and could also be used as a delivery system for several antigens.

Another interesting finding on the role of HtrA is its relation with the two-component system CiaR/H, which is involved in controlling the levels of HtrA within the cell. This thesis shows that up-regulation of HtrA in response to heat is dependent on the CiaR/H system. Moreover, most of the phenotypes observed for the CiaR-null mutant could be reverted by complementation with HtrA. Thus, the decreased autolysis, the increased sensitivity to peroxide and the attenuation in virulence of the D39Δ*ciaR* strain can all be explained by alterations in the levels of HtrA.

The interesting findings on HtrA encouraged further investigations using proteomics and microarray technologies to get an idea as to how HtrA is involved in these processes. New developments in the RNA analysis techniques now enable a comprehensive view on the bacterial physiology. Transcriptome analyses in combination with high resolution two-dimensional polyacrylamide gel electrophoresis (2D-PAGE) and mass spectrometry have been extensively applied for the description of general and specific stress and starvation responses of *Escherichia coli* and *Bacillus subtilis*. This thesis reports a number of genes/proteins that differentially expressed as a result of *htrA* deletion. However, more work is needed to validate these data.

To conclude, this thesis has shed some light on the role of some stress proteins in the pneumococcal biology and pathogenesis. It also showed that it is important to study the role of proteins and other molecules of interest in different strains and serotypes, as it could be misleading to draw a conclusion based on a study of a single serotype. As HtrA looks promising based on the findings in this thesis, future work could involve the production of recombinant HtrA. Access to the purified protein will enable the study of the properties of this enzyme as well as the assessment of its immunogenicity and a possible use of it in a vaccine formulation.

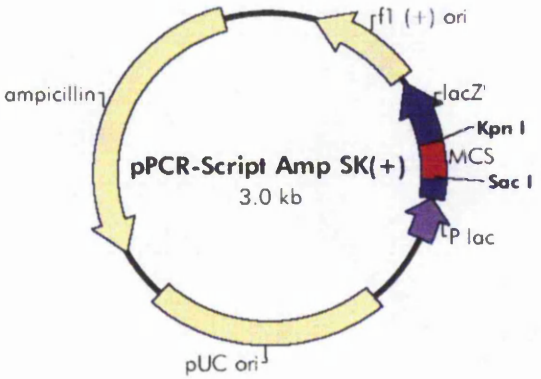
Future work

1. Site-directed mutagenesis could be used to mutate the active site of HtrA and the protease and chaperone activities of all mutant proteins and wild type HtrA compared *in vitro*.
2. Effect of these mutations on the virulence of the pneumococcus *in vivo* could be investigated to find out what activity (proteolytic or chaperoning) of HtrA is essential for virulence.
3. The protective effects of the purified proteins can be studied *in vivo* by vaccination. *htrA* mutants can also be tested as live vaccines.
4. Purified wild type HtrA can be crystallized and its structure determined.
5. Effect of HtrA on gene expression following exposure to hydrogen peroxide and other stress-inducing conditions could also be studied using microarrays.

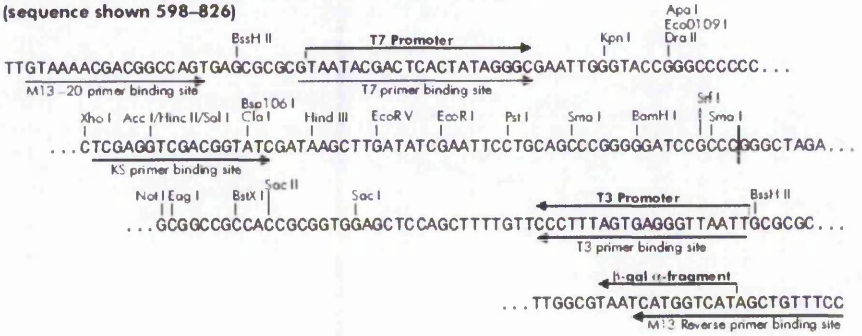
APPENDIX

A1. PCR-Script™ Plasmid

f1 (+) origin 135–441
β-galactosidase α-fragment 460–816
multiple cloning site 653–760
lac promoter 817–938
pUC origin 1158–1825
ampicillin resistance (*bla*) ORF 1978–2833

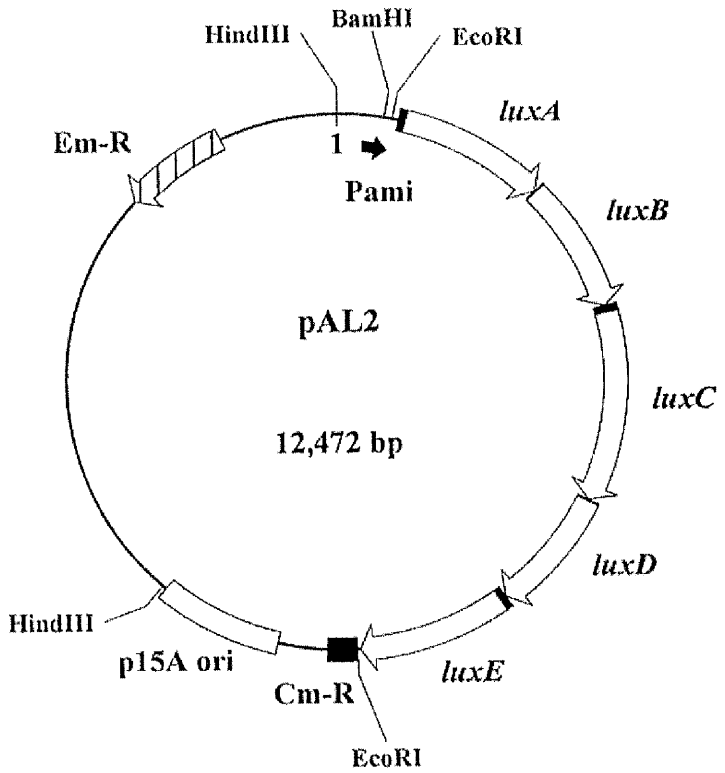


pPCR-Script Amp SK(+) Multiple Cloning Site Region
(sequence shown 598–826)



PCR-Script™ Amp cloning vector. adapted from Stratagene website at (http://www.stratagene.com).

A2. The pAL2 plasmid used for expression of HtrA



Schematic diagram showing the original pAL2 plasmid used for construction of pAL2-HtrA plasmid, which was used for expression of *htrA* gene in the pneumococcus. Arrows indicate the locations and orientation of open reading frames. Em-R, erythromycin resistance gene; Cm-R, chloramphenicol resistance gene. Adapted from (Beard *et al.*, 2002).

A3. Common 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) (Fischer Scientific, UK)

DNA loading buffer (6X)

0.25 % Bromophenol blue (Sigma-Aldrich, UK)

0.25 % Xylene cyanol FF

40 % (w/vol) Sucrose in dH_2O

Agarose (1 %)

1 g agarose (GibcoBRL)

100 ml of TAE buffer

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

Protein gel running buffer (1X)

25 mM Tris base (Sigma-Aldrich, UK)

192 mM glycine

0.1 % SDS

1L dH_2O

Protein separating gel (for two gels)

4.05 ml dH_2O

2.5 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 ammonium persulphate (APS)

5 μl of TEMED

Protein stacking gel (for two gels)

3.05 ml dH_2O

1.25 ml of 1.5 M Tris HCl, pH 6.8

50 μl of 10 % SDS

665 μl of 30 % acrylamide bis

25 μl of ammonium persulphate (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

Coomassie stain

Dissolve 250 mg of Coomassie blue R250 (Sigma-Aldrich, UK) in 100 ml ethanol: dH_2O (1:1 vol/vol). Add 50 ml of acetic acid: dH_2O (1:1 vol/vol). Filter through a whatman No. 1 filter paper

Destain solution for SDS-PAGE

40 % methanol

10 % acetic acid

50 % dH_2O

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 conc. HCl

Make to 1L with dH_2O

Developer (Western blot)

Dissolve 30 mg of 4-chloro-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 to use

PBS, pH 7.4

Dissolve one tablet of phosphate buffered saline (Dulbecco A, Oxoid Ltd, Basingstoke) in 100 ml of dH_2O and autoclave

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 bactocasitone or bacto-casmino acid

1 g of yeast extract

5 g of tryptone

5g of NaCl

1L of dH_2O

Formal saline

3.06 g NaH_2PO_4 anhydrous

6.70 g Na_2HPO_4 anhydrous

100 ml Formaldehyde

9 g NaCl, Made up to 1 litre with dH_2O

IL-6 ELISA coating buffer

0.1 M Na_2HPO_4, adjust pH to 9.0 with 0.1 M NaH_2PO_4

IL-6 ELISA blocking buffer

PBS with 10% FCS (vol/vol)

IL-6 ELISA standard diluent

PBS with 10% FCS (vol/vol) and 0.25% Tween-20 (Polyoxyethylene sorbitol monolaurate)

IL-6 ELISA substrate

Sufficient substrate for one plate is obtained by mixing 10 ml of solution A with 100 µl of solution B and adding 4 µl of H_2O_2

(A) 1.36 g Sodium acetate in 100 ml dH_2O, pH adjusted to 5.5 with citric acid

(B) 6 mg Tetramethylbenzidine (TMB) in 1 ml DMSO

TNF-α coating buffer

11.8 g Na_2HPO_4

16.1 g NaH_2PO_4

Dissolve in 1 L dH_2O, adjust pH to 6.5 (use in 7 days)

TNF-α wash buffer

25 μl of Tween-20

500 ml of PBS

TNF-α assay diluent

31.5 ml of PBS

3.5 ml of FBS

Use in 3 days, require 35 ml per plate.

TNF-α substrate solution

Equal volumes of tetramethylbenzidine (TMB) and hydrogen peroxide (solutions A and B of the Pharmingen OptEIA mouse TNF set, cat. 555268). Mix just prior to use. 10 ml per plate.

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