# CXC Chemokine Responses of Respiratory Epithelial Cells to *Streptococcus pneumoniae*


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# Abstract

*Streptococcus pneumoniae* (the pneumococcus) remains a major cause of morbidity and mortality worldwide, particularly in young children and the elderly. It is responsible for a spectrum of diseases ranging from otitis media, to potentially fatal conditions such as pneumonia and meningitis, and is estimated to cost health services billions of dollars each year. The interaction of *S. pneumoniae* with the host generally begins in the nasopharynx, and invasive disease is almost invariably preceded by nasopharyngeal colonisation. In some circumstances, *S. pneumoniae* may translocate from the nasopharynx to the lungs where pneumonia can develop, and inflammation is believed to play a role in this process. The presence of pneumococci in the lungs also triggers an inflammatory response, which is important for clearance of the bacteria. However, a prolonged inflammatory response leads to tissue damage, and is linked with a poor prognosis of disease. It has been shown that respiratory epithelial cells are able to play an active part in the response to respiratory pathogens by releasing chemokines that are responsible for neutrophil recruitment, and it has recently been shown that infection of type II pneumocytes with *S. pneumoniae* leads to the release of interleukin (IL)-8. In order to determine the role of specific pneumococcal factors in eliciting a CXC chemokine response from type II pneumocytes (A549) and nasopharyngeal cells (Detroit-562), monolayers of these cells were infected with wild type (WT) *S. pneumoniae* D39, or mutants deficient in choline binding protein A (CbpA), pneumococcal surface protein A (PspA), or pneumolysin (Ply), and the CXC chemokine mRNA response was measured by real-time RT-PCR. Release of IL-8 was also measured by ELISA. In response to WT D39, both A549 and Detroit-562 cells showed a significant increase in CXC chemokine mRNA, and IL-8 protein. This response was increased 2-fold when a CbpA-negative (ΔCbpA) mutant was used to infect cells, suggesting that CbpA may have an

inhibitory effect on the CXC chemokine response of these cells. Further investigation demonstrated that this activity is dependent on the N-terminal region of CbpA and that all three N-terminal domains are required for this effect, as deletion of any one of these domains had the same effect on the CXC chemokine response as removing CbpA altogether.

Infection with a PspA-negative mutant (ΔPspA) led to a 2-fold decrease in the CXC chemokine response of A549 cells, compared to infection with WT D39 at 2 h, but no difference was seen in the response of Detroit-562 cells to this mutant compared to WT D39. Thus, PspA appears to have the ability to stimulate an early CXC chemokine release from A549 cells. Deletion of the first of 2 regions of the N-terminal α-helical domain of PspA reduced the ability of *S. pneumoniae* to elicit a chemokine response to the same degree as removing PspA altogether, indicating that it is this region that is responsible for the chemokine inducing ability of PspA.

Ply appeared to have no effect on the CXC chemokine response of A549 cells with no obvious difference seen in the response of these cells to ΔPly compared to WT D39. However, infection of Detroit-562 cells with ΔPly led to a 2-fold decrease in IL-8 mRNA and protein release compared to WT D39. Using D39 strains producing mutant forms of Ply with reduced cytotoxicity and/or complement activating abilities, the role of the cytotoxic activity of Ply was demonstrated to be important in generation of a chemokine response from both cell lines. Infection of A549 or Detroit-562 cells with mutants producing Ply with only 0.02% or 0.1% haemolytic activity led to a 2-fold decrease in IL-8 release compared to that elicited by WT D39. The complement activating ability of Ply also appeared to be important in the generation of a CXC chemokine response from A549 cells. Cells infected with a mutant that produced Ply with no complement activating ability released significantly less IL-8 than cells infected with WT D39. This activity of Ply did not appear to have an effect on the CXC chemokine release of Detroit-562 cells. Thus all three virulence factors investigated had some role in the ability of *S. pneumoniae* to generate a CXC chemokine response from

respiratory epithelial cells, although their roles and the cell lines that were affected differed.

# Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the text.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying.

Rikki Marie Ann Graham

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# Abbreviations used in this Thesis

Abbreviations acceptable to the American Society for Microbiology are used without definition in this thesis. Additional abbreviations are defined when first used in the text, and are listed below.

| | |
|---|---|
| $A_{450, 570, 600}$ | Absorbance at 450, 570 or 600 nm, respectively |
| aa | Amino acid |
| Ag | Antigen |
| ALF | Apolactoferrin |
| Amp | Ampicillin |
| AP | Alkaline phosphatase |
| BA | Blood agar |
| BAL | Broncho-alveolar lavage |
| BSA | Bovine serum albumin |
| C3 | Complement component 3 |
| C3b | Complement component 3b |
| C5 | Complement component 5 |
| CbpA | Choline binding protein A |
| CBPs | Choline-binding proteins |
| CFU | Colony forming units |
| ChoP | Phosphorylcholine |
| Cml | Chloramphenicol |
| CRP | C-reactive protein |
| CSP-1 | Competence stimulating peptide 1 |
| CXC | Cysteine (C)-X-C motif |
| C-terminus | Carboxy terminus |
| DIG | Digoxigenin |
| DMEM | Dulbecco's modified essential medium |
| DMSO | Dimethylsulfoxide |
| DOC | Sodium deoxycholate |
| EDTA | Ethylene diamine tetraacetic acid |
| ELISA | Enzyme linked immunosorbent assay |
| ELR | Glutamine (E)-leucine (L)-arginine (R) motif |
| EMEM | Eagle's modified essential medium |
| ENA-78 | Epithelial derived neutrophil activating peptide-78 |
| ERK | Extra-cellular signal-regulated kinase |
| *erm* | Erythromycin resistance gene on pVA891 |
| Ery | Erythromycin |
| EryBA | Blood agar with erythromycin |
| EU | Endotoxin units |
| FB | Factor B |
| FCS | Foetal calf (bovine) serum |
| fH | Factor H |
| *g* | Gravity units |
| GAPDH | Glyceraldehyde-3-phosphate dehydrogenase |
| GCP-2 | Granulocyte chemotactic protein-2 |
| GRO | Growth related oncogene |

| | |
|---|---|
| h | Hour(s) |
| Hib | *Haemophilus influenza* type b |
| HIV | Human immunodeficiency virus |
| HK | Heat-killed |
| HU | Haemolytic units |
| i.n. | Intranasal |
| i.p. | Intraperitoneal |
| IFNγ | Interferon-γ |
| Ig | Immunoglobulin |
| IL | Interleukin |
| IPTG | Isopropyl-β-D-thiogalactoside |
| Kan | Kanamycin |
| LAL | Limulus amoebocyte lysate |
| LB | Luria Bertani broth |
| $LD_{50}$ | 50% lethal dose |
| Lf | Lactoferrin |
| LPS | Lipopolysaccharide |
| LTA | Lipoteichoic acid |
| LytA | Autolysin A |
| mAb | Monoclonal antibody |
| MAPK | Mitogen activated protein kinase |
| MGSA | Melanoma growth stimulatory activity |
| MIP-2 | Macrophage inflammatory protein 2 |
| NanA | Neuraminidase A |
| NFκB | Nuclear factor kappa B |
| Ni-NTA | Nickel nitrilotriacetic acid |
| N-terminus | Amino terminus |
| ORF | Open reading frame |
| PAF | Platelet activating factor |
| PBP | Penicillin binding protein |
| PBS | Phosphate buffered saline |
| PCR | Polymerase chain reaction |
| PdB | Pneumolysoid |
| PFO | Perfringolysin O |
| pIgR | Polymeric immunoglobulin receptor |
| PMN | Polymorphonuclear leukocyte |
| PS | Polysaccharide |
| PsaA | Pneumococcal surface antigen A |
| PspA | Pneumococcal surface protein A |
| R | Resistant |
| RBC | Red blood cell |
| RT | Room temperature |
| RT-PCR | Reverse-transcription polymerase chain reaction |
| S | Sensitive |
| SC | Secretory component |
| SCR | Short consensus repeat |
| SDS | Sodium dodecyl sulphate |
| SDS-PAGE | Sodium dodecyl sulphate polyacrylamide gel electrophoresis |
| sIgA | Secretory immunoglobulin A |
| SLO | Streptolysin O |
| SN | Supernatant |
| STM | Signature-tagged mutagenesis |

| | |
|---|---|
| TA | Teichoic acid |
| TEMED | N,N,N'N'-tetramethyl-ethylene-diamine |
| THY | Todd-Hewitt broth supplemented with yeast extract |
| TLR | Toll-like receptor |
| TNFα | Tumour necrosis factor-alpha |
| TTBS | Tween-Tris buffered saline |
| VEGF | Vascular endothelial growth factor |
| WCL | Whole cell lysate |
| WT | Wild type |

# Chapter One – General Introduction

## 1.1 Historical background

*Streptococcus pneumoniae* (the pneumococcus) was independently isolated in 1880 by two microbiologists, Louis Pasteur in France who named it *Microbe septicemique du salive*, and George M. Sternberg in the U.S., who named it *Micrococcus pasteuri* (Watson *et al.*, 1993). The discovery was made when rabbits that had received subcutaneous injection of human saliva as a negative control developed systemic disease. Examination of blood from the diseased rabbits showed "an immense number of micrococci, usually joined in pairs and having a diameter of 0.5 μ." (Sternberg, 1881). In describing the appearance of the organism, Pasteur noted, "Each of these little particles is surrounded at a certain focus with a sort of aureole which corresponds perhaps to a material substance" (Austrian, 1981a), a reference to what is now known to be the polysaccharide capsule that surrounds the bacteria. Neither of the saliva samples injected into the rabbits came from individuals suffering from pneumococcal disease, but rather from carriers, thus the link between this organism and pneumonia was not made for several years after its discovery. It was the German scientist Frankel who ultimately made this link in the early 1880s, and by the late 1880s it was known also to be the cause of meningitis and otitis media (Watson *et al.*, 1993). In 1920 the organism was renamed *Diplococcus pneumoniae*, a name that referred to the pairs of cocci found in samples from the lungs of pneumonia patients. Sternberg objected to this name because he noted that the organism also formed longer chains in culture media, and thus was

in fact Streptococcus, but it was not until 1974, many years after his death, that the name *Streptococcus pneumoniae* was adopted (Austrian, 1981b).

*S. pneumoniae* played a central role in the development of the Gram stain, an assay still commonly used today in diagnosis and research. Christian Gram developed the stain bearing his name in 1884 when experimenting with techniques for visualising bacteria in pathological specimens (Austrian, 1981a; Gram, 1884; Watson *et al.,* 1993). After exposing lung tissue sections from patients with lobar pneumonia to the reagents used in Gram staining he found that the sections contained large numbers of paired cocci, which retained this stain. The observation that certain bacteria in other specimens failed to retain the violet stain led to the classification of all bacteria as being either Gram-positive or Gram-negative (Watson *et al.,* 1993), a classification that is still in use today.

Studies into capsular transformation of *S. pneumoniae* led to one of the most important discoveries in biomedical science: that DNA is the carrier of genetic information. Griffith (1928) found that when mice were injected with heat-killed encapsulated pneumococci together with a live unencapsulated strain the bacteria isolated from the blood had become encapsulated and were serologically identical to the heat-killed cells in the inoculum. DNA was found to be the genetic carrier responsible for this capsular transformation by Avery, MacLeod and McCarty who published their seminal paper in 1944 (Avery *et al.,* 1944), and this was confirmed by experiments in which the addition of DNase resulted in a "marked loss of biological activity" of the transforming principle (McCarty and Avery, 1946). McCarty also stated in this paper "It remains one of the challenging problems for future research to determine what sort of configurational or structural differences can be demonstrated between desoxyribonucleates of separate specificities."

## 1.2 The pneumococcal surface

The pneumococcus has three distinct surface layers: the plasma membrane, the cell wall, and the capsule (Figure 1.1) (Briles *et al.,* 1998). The capsule is the thickest layer, largely obscuring the inner structures. Although being completely non-toxic, it has antiphagocytic properties that aid in evasion of the host immune system (Briles *et al.,* 1998) making it an essential virulence determinant, against which most vaccines to date have been targeted. Both the cell wall and the capsule contain polysaccharide (PS), but whilst the cell wall PS (also known as teichoic acid (TA) or C-polysaccharide) is common to all pneumococci, the capsular PS is structurally variable. It is on the basis of these differences in capsular PS that pneumococci are divided into 90 serotypes (AlonsoDeVelasco *et al.,* 1995; Henrichsen, 1995). The large number of pneumococcal serotypes has made development of an effective vaccine difficult, because antibodies against one serotype are generally not protective against pneumococci of different serotypes. This has led to development of new strategies for pneumococcal vaccination, which do not rely solely on the capsular PS as an antigen. Capsular PS is not the only factor important for virulence, and there are a number of other molecules that have been shown to influence the pathogenesis of pneumococcal disease (Figure 1.1). One component of the cell surface that is of major importance is the phosphorylcholine (ChoP) residues found on cell wall TA and lipoteichoic acid (LTA). ChoP is important for many cellular functions of *S. pneumoniae* such as transformability, separation of daughter cells following cell division, and the activity of autolysins (Fischer, 2000). In addition, it also serves as an anchor for the various choline-binding proteins displayed on the surface of the cell, these have a variety of functions ranging from nutrient scavenging to adherence, and several are believed to have a role in pneumococcal virulence. The choline binding protein (CBP) family includes pneumococcal surface protein A (PspA) and choline-binding protein A (CbpA), which will be discussed in more detail in Section 1.6.

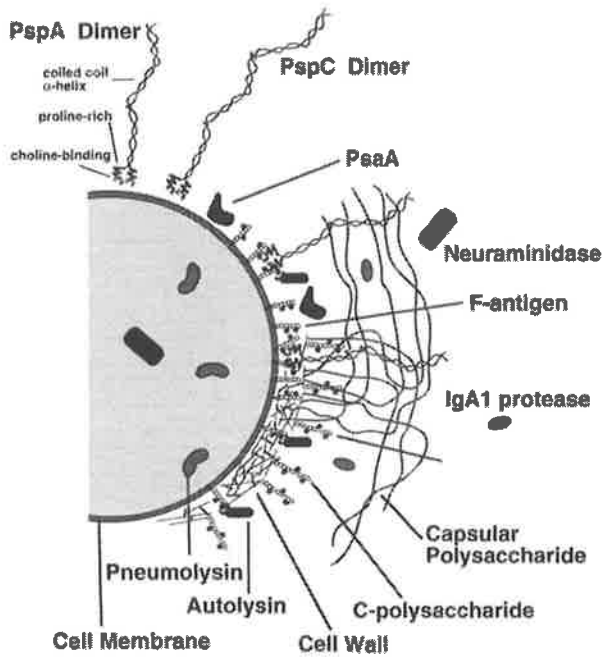


**Figure 1.1 Schematic diagram of the pneumococcal surface.**
Major surface components, as well as some important virulence factors are shown, including the capsular polysaccharide, the cell-wall teichoic acid (C-polysaccharide) containing phosphorylcholine residues, surface proteins PspA and PspC (CbpA), and Pneumolysin. Reproduced from Briles *et al*. (1998).

### 1.2.1 Phase variation

*S. pneumoniae* has been shown to undergo spontaneous phase variation between opaque and transparent colony phenotypes (Cundell *et al.,* 1995b), and this is associated with differences in the expression of various surface molecules. This change in colony morphology is associated with differences in the ability of the variants to interact with the host at different stages of the pathogenic process. Organisms with the opaque phenotype display an upregulation of capsular PS, and a decrease in the expression of cell wall TA and CbpA when compared to transparent variants (Overweg *et al.,* 2000). An increase in capsular PS expression increases the antiphagocytic capacity of the pneumococcus, and accordingly, opaque variants show increased virulence in a sepsis model due to increased survival in the blood (Kim and Weiser, 1998). In contrast, transparent variants have increased levels of cell wall TA (and thus ChoP and its associated proteins), and decreased capsular PS, and are more efficient at colonising the nasopharynx and adhering to cytokine activated lung epithelial cells (Weiser *et al.,* 1996; Overweg *et al.,* 2000). This adherence occurs via an interaction between ChoP and platelet-activating factor (PAF) receptors, which are upregulated on activated cells (Cundell *et al.,* 1995a). Pneumococci have also been shown to have an increased ability to undergo transcytosis across brain microvascular endothelial cells by utilising the same receptor (PAF receptor), at least in an *in vitro* model (Ring *et al.,* 1998). The ability of the phenotypic variants to interact with the host in distinct ways and at various host sites reflects the relative importance of different surface molecules in the pathogenic process of pneumococcal infection. It also illustrates the importance of the capsule, cell wall TA and ChoP, as well as its associated proteins, in the interaction of the pneumococcus with the host.

## 1.3 Streptococcus pneumoniae as a pathogen

*Streptococcus pneumoniae* is a major human pathogen, estimated to be responsible for approximately 1 million deaths in the under 5 age group each year (WHO

[http://www.who.int/mediacentre/factsheets/fs178/en], 2004). It is the leading cause of serious invasive conditions such as pneumonia, and bacteremia, and in countries where vaccination has led to a decrease in the incidence of meningitis caused by *Haemophilus influenzae* and *Neisseria meningitidis* infections, *S. pneumoniae* is the leading cause of this diseases in children (Bogaert *et al.,* 2004). It is also a very common cause of less severe conditions such as sinusitis and otitis media, which although not usually associated with high mortality, are nevertheless a major drain on the public health system of developed countries.

### 1.3.1 Nasopharyngeal colonisation and progression to disease

Carriage of *S. pneumoniae* in the nasopharynx is not uncommon in the community, and depending on factors such as age, geographic location and socio-economic status, the rate of carriage in the population can range from 5 - 95% (Crook *et al.,* 2004). Carriage does not always lead to invasive disease, but it can lead to infection of others, which is proportional to the frequency and intimacy of contact between people. In fact, acquisition of pneumococci usually occurs via carriers rather than from infected individuals (Briles *et al.,* 2000a). Although invasive disease is almost invariably preceded by colonisation, it generally occurs soon after acquisition of a new strain rather than after prolonged carriage (Boulnois, 1992). In most cases carriage is asymptomatic, and after a period of weeks the pneumococcus is cleared by the host immune system. In healthy adults, colonisation frequently leads to production of serotype-specific antibodies, which contributes to elimination of the organism. It may also result in protection from future infection with the same pneumococcal serotype (Catterall, 1999). However, under certain conditions, the bacteria translocate from the nasopharynx to other sites in the body, leading to disease. Otitis media may occur when infection with a respiratory virus causes blockage of the Eustacian tube, resulting in decreased pressure in the middle ear cavity and movement of pneumococci from the nasopharynx to this site. Alternatively, damage to the respiratory epithelium, or upregulation of host cell receptors induced by inflammation, may enhance penetration of pneumococci into lung tissue

resulting in pneumonia (Sollid *et al.,* 1987; Phillips *et al.,* 1990; Briles *et al.,* 1996b; Blanch *et al.,* 1999; Zhang *et al.,* 2000). From the lungs, the pneumococcus may move into the bloodstream causing bacteremia and sepsis if the numbers are large enough. Recently, the use of pneumococci expressing green fluorescent protein has made visualisation of invasion possible (Kadioglu *et al.,* 2001). Pneumococci were initially found on the mucosal surface of the respiratory tract, and at later stages, they could be visualised in different layers of the lung tissues. The bacteria were found inside epithelial cells, and not in-between the junctions of these cells, indicating that invasion can occur via trans-cellular migration. Trans-cellular migration of pneumococci has also been demonstrated *in vitro* with the nasopharyngeal epithelial cell line Detroit-562. R6x *S. pneumoniae* were able to migrate across the epithelial cells without compromising the integrity of the cell monolayer (Zhang *et al.,* 2000). Once in the blood, in some cases pneumococci can reach the brain and cause meningitis, which is fatal in up to 30% of patients (Briles *et al.,* 1996a).

## 1.4 Pneumococcal Vaccines

The first vaccine against pneumococcal disease was a killed whole cell vaccine developed in the early 1900s, but studies into the effectiveness of this vaccine were inconclusive (Paton, 2004). Since then three major strategies for development of an effective vaccine have been investigated: polysaccharide vaccines, polysaccharide-protein conjugate vaccines, and common protein vaccines (Mulholland, 1999). The ideal pneumococcal vaccine would provide broad range protection, and protect not only against invasive disease, but also would reduce or eliminate nasopharyngeal carriage, thus reducing spread of pneumococci within the community.

### 1.4.1 Polysaccharide Vaccines

Until recently, all pneumococcal vaccines were composed of capsular PS. A tetra-valent PS vaccine, and two hexavalent vaccines were developed in the 1940s and successfully

trialed in military recruits, but the introduction of antibiotics led to a reduced interest in the use of a pneumococcal vaccine and these were taken off the market (Paton 2004). In 1977, a 14-valent polysaccharide vaccine that gave protection against the 14 major disease-causing serotypes became available in the U.S. Later a 23-valent PS vaccine became available and this is still in use today (AlonsoDeVelasco *et al.,* 1995), this provides protection against the 23 serotypes that cause about 90% of invasive pneumococcal infections in the U.S or Europe (Paton, 2004). However, due to the high degree of variability in the capsular structure between the different serotypes, antibodies against one capsular type do not provide protection against pneumococci of unrelated capsular types. Thus, although the 23-valent vaccine provides protection against the 23 most prevalent serotypes involved in human disease (Briles *et al.,* 1998), it does not offer protection against any of the other 67 serotypes of *S. pneumoniae* apart from a handful of cross-reacting types, and due to variation in serotype prevalence in different geographic regions, coverage can be as low as 60% in some countries (Paton, 2004). Another problem with PS vaccines is that although they elicit long lasting antibody responses in healthy adults, polysaccharides are poorly immunogenic in those groups at highest risk of pneumococcal infection, (eg. asplenic patients, HIV patients, the elderly, and children under 2 years of age). They do not elicit immunological memory, because they are thymus independent antigens (T-independent Ags) (Briles *et al.,* 1998), and responses in children are particularly poor towards the very serotypes that most commonly cause invasive paediatric disease (Douglas *et al.,* 1983).

### 1.4.2 Polysaccharide-Protein Conjugate Vaccines

The success of the *Haemophilus influenzae* type b (Hib) polysaccharide-protein conjugate vaccine in controlling meningitis caused by Hib in children encouraged the development of a similar conjugate vaccine for *S. pneumoniae* (Briles *et al.,* 2001). These vaccines consist of capsular PS that has been conjugated to a protein carrier such as tetanus or diphtheria toxoids, which gives it the properties of a thymus-dependent antigen (T-dependent

Ags) (Briles *et al.,* 1998). The conjugates are more immunogenic in infants than PS alone, elicit immunological memory, and studies have shown that such antigens are able to elicit protection against carriage in children (Dagan *et al.,* 1996; Obaro *et al.,* 1996). The Hib conjugate vaccine requires only a single PS conjugate type, but because there are 90 different serotypes of pneumococcus, a pneumococcal conjugate vaccine would need to contain as many PS conjugate types as possible in order to give broad protection (Briles *et al.,* 1998; Briles *et al.,* 2001). The conjugate vaccine currently licensed for use in Australia is 7-valent, but others under development contain up to 11 capsular PS types (the serotype coverage has been reduced from 23 due to the high cost of producing protein-PS conjugates). The conjugate vaccine is highly protective against invasive disease caused by the serotypes included, but like the PS vaccines, they will not offer protection against non-included, unrelated serotypes. Moreover, vaccination with the conjugate vaccine leads to an increase in carriage of non-vaccine serotypes (Dagan *et al.,* 1996; Obaro *et al.,* 1996). Carriage of one strain of *S. pneumoniae* may interfere with colonisation of the host by a second serotype to which there is exposure. Thus, reduction in carriage of vaccine serotypes as a result of vaccination may remove this interference, providing a mechanism by which serotype distribution of carriage and invasive disease could be altered in a population (Obaro *et al.,* 1996; Lipsitch, 1997). There has also been concern expressed about the use of PS vaccines in infants, because of the possibility of producing antibodies that may cross-react with polysialyated glycoproteins found in the human embryonal brain and newborn rat kidney, heart, and muscle. These glycoproteins are not found in the corresponding adult tissues, and may be the reason that infants exhibit poor immunogenicity to PS (Briles *et al.,* 1998). However, it remains to be seen whether these concerns are founded. It is also unfortunate that due to the need for multiple PS serotypes to be included in the conjugate vaccine, the cost will be very high, which will probably preclude use in developing countries, where pneumococcal disease kills so many children each year (Briles *et al.,* 2001).

### 1.4.3 Common Protein Vaccines

The problems associated with the PS and conjugate vaccines have led to investigations into the possibility of using pneumococcal proteins as vaccine antigens. A protein based vaccine would have several advantages over PS and conjugate vaccines: 1) proteins are T-dependent antigens, therefore making them more immunogenic than PS in infants; 2) due to the cross reactive nature of the proteins used, there is potential for greater, perhaps universal, serotype coverage; 3) because capsular PS is not used as an antigen, the problem of serotype specificity is overcome, and use of the vaccine will not lead to an increase in carriage of non-vaccine serotypes; and 4) they will probably be less expensive than conjugate vaccines, therefore allowing widespread use in developing countries (Briles *et al.,* 2000b; Briles *et al.,* 2001). There are several well-characterised protein antigens that are expressed on the surface or released by the pneumococcus, that are known to elicit protective antibodies. These proteins include pneumolysin (Ply) (Paton and Ferrante, 1983) or toxoided derivatives thereof such as PdB (Alexander *et al.,* 1994), pneumococcal surface protein A (PspA) (Briles *et al.,* 1998), and choline binding protein A (CbpA) (Rosenow *et al.,* 1997).

## 1.5 Antimicrobial resistance

Penicillin has been the mainstay of treatment for pneumococcal infection for more than 50 years. However in the U.S., penicillin resistance has increased from 4-5% of all isolates in the 1980s to 30-40% in 1997 (Barry, 1999; Tomasz, 1999). In some parts of the world where antibiotic use is poorly regulated, resistance rates may be as high as 70% (Marton *et al.,* 1991). The mechanism of penicillin resistance involves alteration of Penicillin Binding Proteins (PBPs) in the pneumococcal cell wall, lowering their affinity for penicillin (Di Guilmi *et al.,* 1999; Hakenbeck *et al.,* 1999). This prevents penicillin from binding and interfering with the final steps of peptidoglycan biosynthesis (Di Guilmi *et al.,* 1999). It is thought that penicillin resistance is acquired via homologous recombination of PBP genes

between a penicillin resistant and penicillin susceptible pneumococcus (Tomasz, 1999). The rise in penicillin resistance has led to increased use of other antimicrobial drugs, but many pneumococci are now developing resistance to these as well, and are therefore multiply resistant. In some parts of the world, up to 89% of penicillin resistant isolates are also resistant to other antimicrobials (Tarasi *et al.,* 1997). Molecular fingerprinting techniques have shown diversity amongst resistant strains from around the world, but has also shown that many multiply resistant strains belong to recognisable clonal groups such as one which was initially isolated in Spain, known as the Spanish serotype 23F clone (Coffey *et al.,* 1999; Tomasz, 1999). Isolates of the Spanish clone have been recovered in many countries throughout Europe, South East Asia, the U.S., and South Africa (Coffey *et al.,* 1999). Pneumococci are particularly prone to capsular switching events involving recombinational exchanges at the capsular polysaccharide biosynthesis locus (*cps*), and this has led to the appearance of serotype variants of this highly penicillin resistant clone (Coffey *et al.,* 1999; Tomasz, 1999). The rising prevalence of penicillin resistant, and multiply resistant clones of pneumococci worldwide has led to renewed interest in developing an effective pneumococcal vaccine, which would prevent initial infection, reducing the need to use antibiotics, and thus the selection for further resistant strains.

## 1.6 Pneumococcal virulence factors

The pneumococcal capsule is the major virulence factor of this organism, and as described above, it is essential for virulence. However, there are several other factors that contribute to the virulence of *S. pneumoniae*. There are a number of proteins present on the pneumococcal surface. These include sortase-dependent proteins such as IgA protease, and neuraminidase A (NanA), lipoproteins such as pneumococcal surface antigen A (PsaA), and CBPs such as autolysin A (LytA), and choline binding proteins A, C, D, E, F, G, I, and J (CbpA-J), that bind to ChoP residues on the pneumococcal surface. These proteins have a

range of functions involved in all aspects of pneumococcal infection, including cleavage of human immunoglobulin (Ig) A1 (IgA protease), $Mn^{2+}$ transport (PsaA), and autolysis (LytA) (Gosink *et al.,* 2000). Large-scale analyses of pneumococcal virulence factors using signature-tagged mutagenesis (STM), have identified many genes that play a role in virulence (Polissi *et al.,* 1998; Lau *et al.,* 2001; Hava and Camilli, 2002). Microarray analysis of global gene expression has also shown that a number of genes are upregulated *in vivo* compared to *in vitro* suggesting that they may play a role in virulence (Orihuela *et al.,* 2004b). Although several of the genes identified by these studies were previously identified virulence factors, other factors not previously thought to be involved in virulence were also identified. These include housekeeping genes involved in metabolism, nutrient uptake, and DNA mutation repair. Thus, the definition of a virulence factor has had to be broadened to include factors that not only have a direct role in pathogenesis of disease but also those that help the pneumococcus to survive in the host. Three pneumococcal virulence factors that have been studied in considerable detail are the CBPs Pneumococcal surface protein A (PspA), and Choline binding protein A (CbpA), and the toxin Pneumolysin (Ply), and these are described in more detail below.

### 1.6.1 Choline Binding Protein A (CbpA)

CbpA is a multifunctional surface protein bound non-covalently to ChoP residues of the cell wall TA. It was initially isolated based on its choline binding properties by Rosenow *et al.,* (1997). Other groups independently isolated the same molecule based on other properties and named it accordingly. Thus, it is also known as *Streptococcus pneumoniae* secretory IgA binding protein (SpsA) (Hammerschmidt *et al.,* 1997), and pneumococcal surface protein C (PspC) (Brooks-Walter *et al.,* 1999). In some strains of *S. pneumoniae* that lack CbpA, the gene for factor H-binding inhibitor of complement (Hic) is present in the same locus as *cbpA* (Janulczyk *et al.,* 2000). Hic has an N-terminus with a high degree of sequence homology with CbpA, and is thought to have similar functions. However, Hic does not

contain a choline-binding domain and is instead anchored to the pneumococcal surface by an LPXTG motif (Janulczyk *et al.,* 2000). Iannelli *et al.,* (2002) divided the CbpA proteins of 43 strains of *S. pneumoniae* into 11 groups based on DNA sequence. The CbpA molecule of the well studied type 2 *S. pneumoniae* strain D39 belongs to group 3 and is comprised of an N-terminal domain, a proline rich region and a choline binding domain responsible for binding of the molecule to ChoP residues on the surface of the pneumococcus (Brooks-Walter *et al.,* 1999; Iannelli *et al.,* 2002) (Figure 1.2). It shows a high degree of sequence similarity to PspA. Based on this similarity it is predicted to be an elongated molecule with an N-terminal region that is α-helical in structure (Jedrzejas, 2001; Iannelli *et al.,* 2002; Luo *et al.,* 2004). This N-terminal region contains 2 short repeat regions, both of which contain a hexapeptide motif that is important in binding to sIgA (Iannelli *et al.,* 2002; Luo *et al.,* 2004). The solution structure of the second of these domains (CbpA-R2) has been determined and been shown to be composed of 3 α-helices which adopt a raft like structure, through antiparallel helix-helix interactions (Luo *et al.,* 2004) (Figure 1.3). CD spectral analysis has suggested that the first small repeat region (CbpA-R1) and the hypervariable region are also α-helical in nature, but are separately folded, suggesting that the N-terminus of CbpA is comprised of 3 independent α-helical domains (Luo *et al.,* 2004).

CbpA appears to have differing importance in the virulence of *S. pneumoniae* depending on the site of infection. The role of CbpA in colonisation of the nasopharynx is well established both *in vitro* and *in vivo*. Microarray analysis has demonstrated an upregulation of *cbpA* expression in tissue culture medium with nasopharyngeal epithelial cells compared to expression in tissue culture medium alone (Orihuela *et al.,* 2004b), and CbpA has been shown to contribute to adherence to Detroit-562 cells via interaction with the polymeric immunoglobulin receptor (pIgR) (Zhang *et al.,* 2000). *In vivo*, CbpA-negative mutants demonstrated a decreased ability to adhere to the rat nasopharynx, and to translocate from this site to the lungs when compared to the wild-type (Rosenow *et al.,* 1997; Orihuela *et*

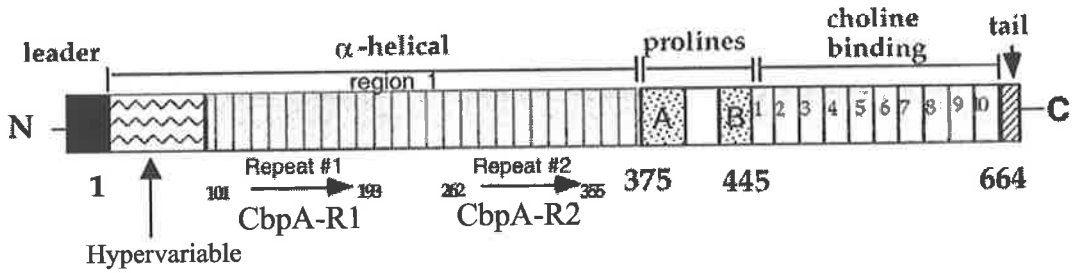


**Figure 1.2. Schematic representation of CbpA** showing the distinct domains of the molecule. Numbers represent amino acid residues. Modified from Brooks-Walter *et al.* (1999).

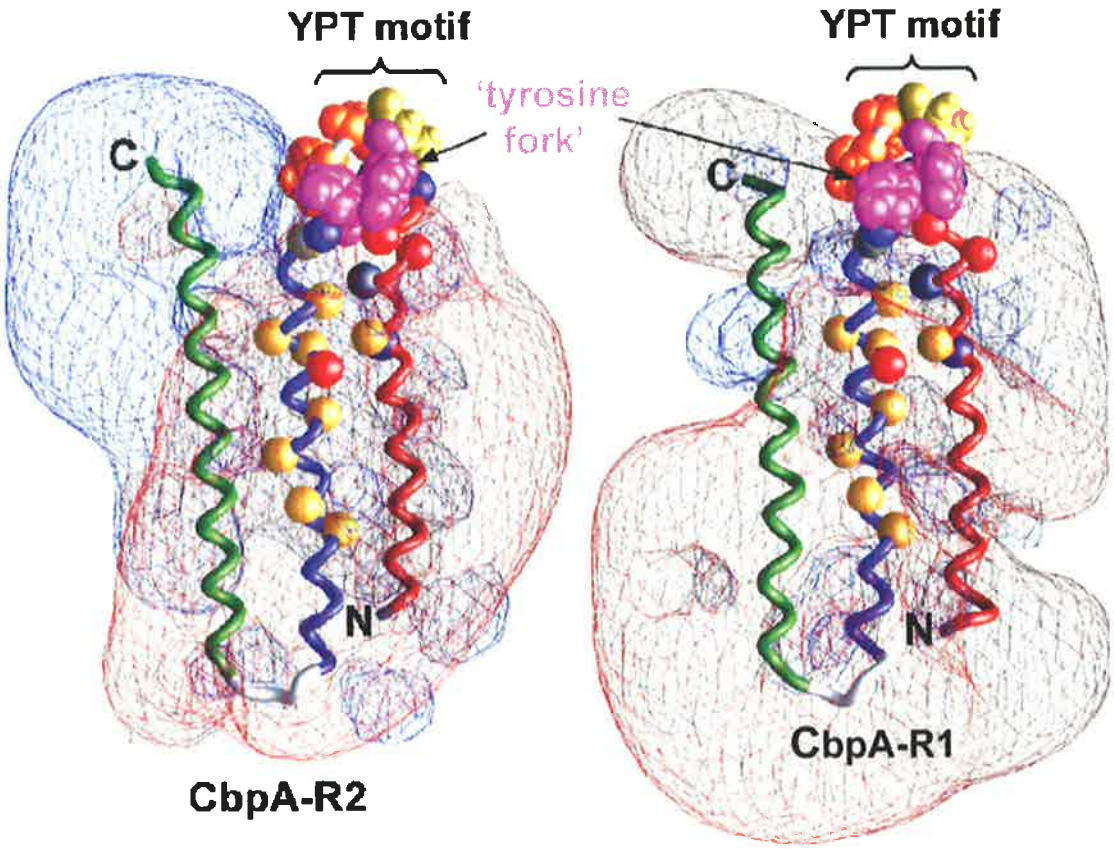


**Figure 1.3. Small repeat domains of CbpA.** Contour maps of electrostatic potentials (±1kt; red, negative potential; blue, positive potential) for CbpA-R2 (left) and homology model of CbpA-R1 (right). The 3 α-helices of each domain are coloured as follows:Helix 1 red, helix 2 blue, and helix 3 green. The YPT motifs involved in sIgA binding are also illustrated. Tyr 358 and 363 (magenta; labeled 'tyrosine fork'), Pro 359 (yellow) and Thr 360 and 362 (orange). Adapted from Luo *et al.*, (2004). The positions of these domains are indicated in Figure 1.2.

*al.,* 2004a), further reinforcing the importance of this molecule in colonisation. However, the role of CbpA in systemic disease remains unclear. A recent study suggested it was involved in sepsis, with a CbpA-negative strain showing a 19-fold reduction in virulence after intravenous injection compared to WT D39 (Iannelli *et al.,* 2004), but studies in this laboratory (Berry and Paton 2000), and by Orihuela *et al.,* (2004a) using the same *S. pneumoniae* strain suggest that it is of little importance in systemic disease, with no difference in virulence seen between CbpA-negative strains and WT D39. There was also no increase in *cbpA* expression by pneumococci in blood compared with *in vitro* expression as measured by either quantitiative RT-PCR (Ogunniyi *et al.,* 2002), or microarray analysis (Orihuela *et al.,* 2004b). Notwithstanding these findings, the fact that CbpA binds the serum component factor H (fH) (Dave *et al.,* 2001; Duthy *et al.,* 2002; de Cordoba *et al.,* 2004) may indicate some role in systemic virulence.

#### 1.6.1.1 CbpA as an adhesin

Rosenow *et al.,* (1997) demonstrated that a CbpA-negative strain had a decreased ability to bind to cytokine activated human lung epithelial cells and endothelial cells when compared to the parent strain, and subsequent studies demonstrated the importance of this molecule in adherence to and invasion of nasopharyngeal epithelial cells (Zhang *et al.,* 2000; Elm *et al.,* 2004a). Adherence of pneumococci to lung epithelial cells (A549) has previously been shown to be mediated by interaction of TA with the platelet activating factor (PAF) receptor (Cundell and Tuomanen, 1994; Cundell *et al.,* 1995a). However, CbpA has also been shown to bind the complement component C3, displayed on activated cells (Cheng *et al.,* 2000; Smith and Hostetter, 2000), and this could be another mechanism of attachment to these cells by pneumococci. In the nasopharynx the receptor for CbpA is believed to be the pIgR (Zhang *et al.,* 2000). Binding of pneumococci to nasopharyngeal epithelial cells (Detroit-562) *in vitro* is inhibited by antibodies against pIgR, but not by antibodies against the PAF receptor (Zhang *et al.,* 2000). pIgR is responsible for translocation of polymeric

immunoglobulins such as IgA across mucosal epithelial cells from the basolateral to the apical surface where they are released (Mostov, 1994). It is composed of an N-terminal ligand binding domain, a single membrane-spanning region and a short cytoplasmic tail. The extracellular N-terminal region is further divided into 5 Ig-like domains, with domain 1 (D1) being the initial pIgA-binding domain (Bakos *et al.,* 1994). Upon release of IgA at the apical surface, the ligand binding domain is cleaved from the pIgR, and remains bound to IgA as the secretory component (SC), protecting it from proteolysis; this IgA-SC complex is known as sIgA. CbpA can bind sIgA, but it is unable to bind serum IgA, which lacks SC (Hammerschmidt *et al.,* 1997), and CbpA can bind free SC with the same affinity as sIgA (Hammerschmidt *et al.,* 1997; Zhang *et al.,* 2000). The CbpA binding sites on pIgR have been mapped to domains D3 and D4, with deletion of either of these diminishing binding to CbpA (Elm *et al.,* 2004a). A recombinant fragment of pIgR containing only D3 and D4 also binds CbpA as well as complete pIgR (Lu *et al.,* 2003). On CbpA, a hexapeptide motif that is present once in CbpA from certain type 1 pneumococci, and twice in most other types including D39 has been shown to be essential for binding to pIgR (Elm *et al.,* 2004a; Elm *et al.,* 2004b; Luo *et al.,* 2004). This motif is present once in each of the small repeat regions of the D39 CbpA N-terminus (Figure 1.3). Mutation of one of these motifs reduced binding of CbpA to pIgR, and mutation of both completely abolished binding (Luo *et al.,* 2004). Latex beads coated in CbpA attached to, and were take up by Detroit-562 cells which express high levels of pIgR (Zhang *et al.,* 2000; Brock *et al.,* 2002), and binding of CbpA to pIgR can drive the transcytotic pathway from the apical to basolateral surface (Zhang *et al.,* 2000; Kaetzel 2001; Elm *et al.,* 2004a) (Figure 1.4). Thus, binding of pneumococci to pIgR via CbpA could represent a mechanism by which the bacteria are able to cross the epithelium and initiate invasive disease.

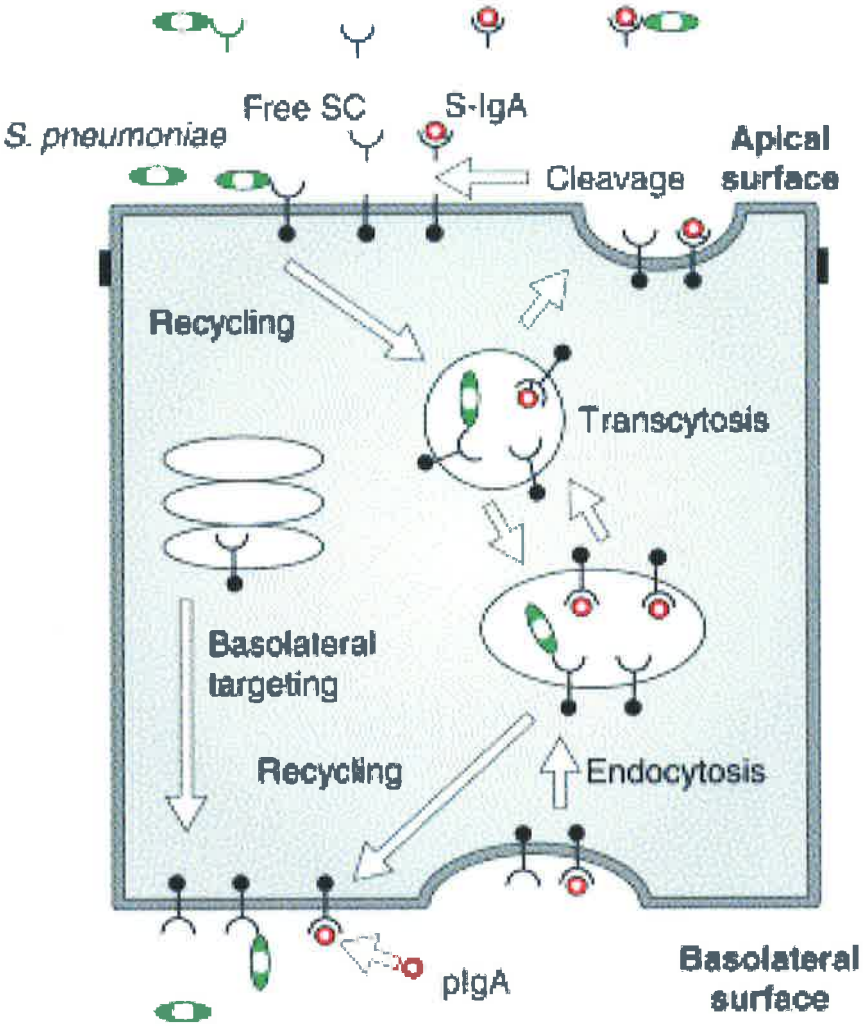


**Figure 1.4. Invasion of mucosal epithelial cells by *S.pneumoniae*.**
*S. pneumoniae* (green), binds to uncleaved pIgR on the apical surface, and is then are internalised by the cell and translocated to the basolateral surface. Reproduced from Kaetzel (2001).

### 1.6.1.2 Binding of Factor H

CbpA has also been shown to bind factor H (fH), a serum protein involved in regulating the alternate complement pathway (Dave *et al.,* 2001; de Cordoba *et al.,* 2004). Factor H protects host cells from C3b-mediated complement attack by inhibiting binding of factor B to C3b, acting as a cofactor for factor I-mediated cleavage of C3b, and accelerating the decay of C3bBb (Jarva *et al.,* 2003). The structure of fH comprises 20 repetitive domains known as short consensus repeats (SCRs), and distinct SCRs are responsible for the different functions of the molecule. Those involved in binding to C3b are localised to SCR1-5, SCR6-10 or 12-14, and SCR19-20 (Duthy *et al.,* 2002). Binding to CbpA has been shown by different groups to be either at SCR 8-15 (Duthy *et al.,* 2002), or SCR6-10 (Dave *et al.,* 2004), but the exact location is yet to be determined. Nevertheless, binding of CbpA is known to be via a site distinct from that of C3b binding, since the ability of fH to bind CbpA was unaffected by a 50-fold molar excess of C3b (Duthy *et al.,* 2002), and fH is still fully functional when CbpA is bound (Jarva *et al.,* 2002; Dave *et al.,* 2004). Thus it is likely that once bound to CbpA on the pneumococcal surface, fH is then able to bind C3b preventing amplification of C3b deposition on the surface of the pneumococcus, and subsequent opsonophagocytosis. The region of CbpA to which fH binds has not been definitively mapped, but fragments of CbpA in which the proline rich region are absent show a diminished ability to bind fH (Duthy *et al.,* 2002). Thus, this region may itself contain the binding site, or may be important in presenting the N-terminal α-helix in the correct conformation for binding. It is known that the binding site for fH is distinct to that for sIgA, which as mentioned above, binds via hexapeptide motifs in the small repeat regions of the N-terminal α-helix. Binding of fH to CbpA was not affected by sIgA, and fragments of CbpA that were unable to bind sIgA were still able to bind fH (Dave *et al.,* 2004), showing that there are 2 distinct binding domains for these molecules on CbpA.

#### 1.6.1.3 Chemokine release in response to CbpA

It has been reported that CbpA contributes to the release of IL-8 from respiratory epithelial cells in response to *S. pneumoniae*. This was based on the observation that culture supernatants from exponentially growing pneumococci elicited release of IL-8 from type II pneumocytes, and this response was significantly reduced when supernatant from a CbpA-negative strain was used (Madsen *et al.,* 2000; Murdoch *et al.,* 2002). These cells also reportedly exhibited increased IL-8 release when treated with purified recombinant CbpA, to levels that were comparable to those elicited by IL-1α, a potent cell activator (Murdoch *et al.,* 2002). However, the ability of CbpA to stimulate chemokine release from host cells is still contentious, as a recent study by Peppoloni *et al.,* (2005) has suggested that CbpA may actually suppress the release of chemokines by brain microglial cells, with CbpA-negative pneumococci eliciting higher levels of macrophage inflammatory protein (MIP)-2 than their wild type counterparts (Peppoloni *et al.,* 2005). Thus CbpA may have the ability to both upregulate and downregulate the inflammatory response from host cells depending on the cell type.

### 1.6.2 Pneumococcal Surface Protein A (PspA)

PspA is another member of the pneumococcal choline binding protein family that is expressed by all clinically important pneumococcal serotypes (Crain *et al.,* 1990). It consists of four distinct structural domains: 1) the N-terminus consisting of a coiled-coil α-helical structure; 2) a highly flexible, tether-like proline rich region; 3) a repeat region responsible for choline binding; and 4) a hydrophobic tail at the C terminus, (Figure 1.5) (Brooks-Walter *et al.,* 1999; Jedrzejas *et al.,* 2001). The N-terminal domain of the PspA molecule is highly variable between different serotypes of pneumococci (Crain *et al.,* 1990). Even so, it is highly cross-reactive, and immunisation with a PspA molecule of one type produces antibodies that are cross-reactive with all PspA types tested (Briles *et al.,* 2001), and provides protection against pneumococci with serologically distinct PspA types (Briles *et al.,* 1998).

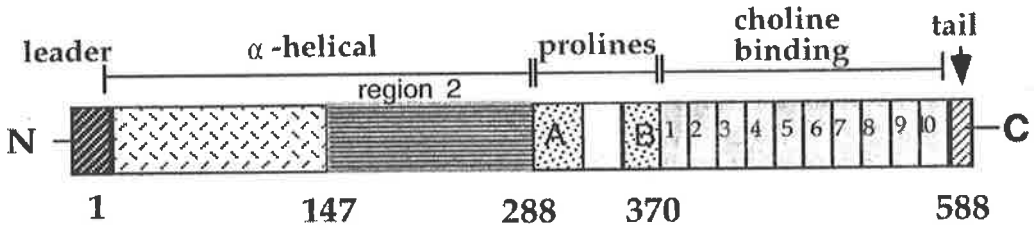


**Figure 1.5. Schematic representation of PspA** showing the distinct domains of the molecule. Numbers represent amino acid residues. Modified from Brooks-Walter *et al.* (1999).

The structure of the N-terminus of PspA has been analysed using a variety of biophysical methods (Jedrzejas *et al.,* 2001). The molecule was found to be highly α-helical, and to be arranged in monomeric coiled-coils, similar in shape to an elongated rod (Figure 1.6). It is highly charged and polar in nature, with the electropositive region interacting with the electronegative capsule, and the electronegative tip pointing away from the bacterial cell wall. Most protective epitopes of PspA have been mapped to this tip, and specifically the regions containing amino acids 1-115, and 192-260 are particularly protective (Yother *et al.,* 1992; McDaniel *et al.,* 1994; Briles *et al.,* 1996a; Tart *et al.,* 1996; Roche *et al.,* 2003). This region of PspA is reported to be responsible for the functional properties of the molecule, such as complement inhibition, and binding of human lactoferrin (Lf) (Hakansson *et al.,* 2001; Ren *et al.,* 2004a).

The importance of PspA in virulence of *S. pneumoniae* was first demonstrated by McDaniel *et al.,* (1987). Insertional inactivation of the gene for PspA led to 10-fold greater clearance of pneumococci from the blood (McDaniel *et al.,* 1987). A later study showed that challenge of mice with a PspA-negative mutant resulted in significant increases in both the median survival time and the survival rate compared to the parent strain in both systemic and intranasal models of infection (Briles *et al.,* 1988; Berry and Paton, 2000; Balachandran *et al.,* 2002). In an *in vivo* model, infection with a PspA-negative strain led to a significant decrease in serum C3, indicative of complement activation, but infection with a PspA positive strain resulted in diminished activation of complement and no significant consumption of serum C3 (Tu *et al.,* 1999), suggesting that PspA is able to interfere with complement activation. In order to elucidate the stage of complement activation at which PspA exerts its effect, mice deficient in complement components C3, Factor B (FB), or C5, as well as wild type mice, were challenged with PspA positive or PspA-negative strains of pneumococci. The PspA-negative mutant was cleared rapidly from wild type mice, but was unable to be cleared from $C3^-$ or $FB^-$ mice, causing bacteremia as intense as that caused by PspA positive pneumococci.

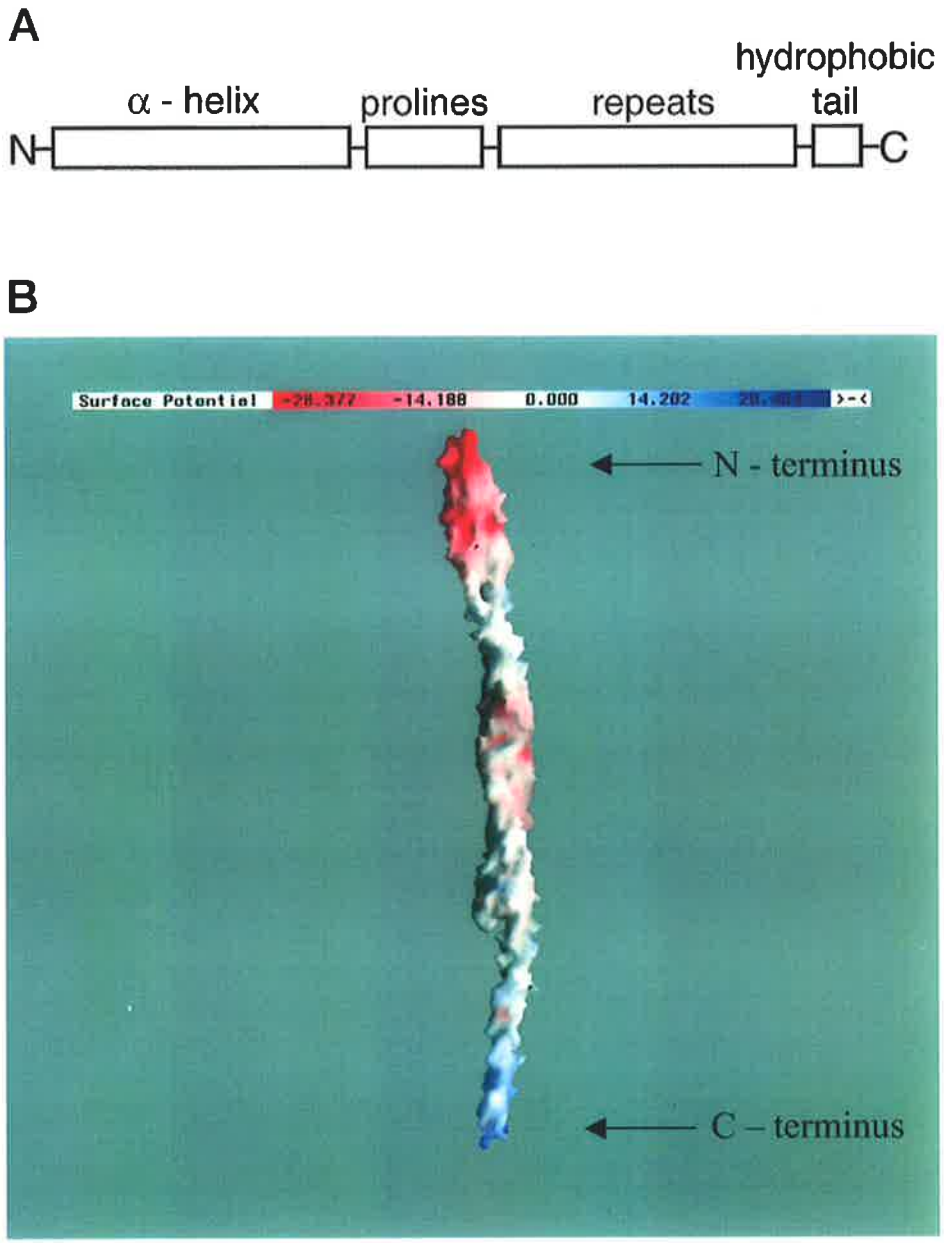


**Figure 1.6. Pneumococcal surface protein A (PspA).**
**A.** Schematic representation of the PspA molecule showing the distinct domains. **B.** Model of PspA displaying the elongated rod shape of the molecule. Blue areas are electropositive, red areas are electronegative. Adapted from Jedrzejas *et al.*, (2001).

$C5^-$ mice showed no increase in virulence for the PspA-negative strain (Tu *et al.,* 1999). These results indicate that the inhibition of complement activation elicited by PspA is targeted at factor B-mediated activation of C3 to C3b. It has also been observed that deposition of C3 on PspA-negative pneumococci is markedly increased compared to PspA positive strains, and antibodies against PspA increase C3 deposition on WT pneumococci (Ren *et al.,* 2004a; Ren *et al.,* 2004b; Yuste *et al.,* 2005). Thus, PspA prevents deposition of the opsonin C3b onto the bacterial surface, enabling the pneumococcus to avoid complement-mediated opsonophagocytosis. This makes PspA an important virulence factor of *S. pneumoniae*, facilitating evasion of host defences and allowing it to persist in host tissues and cause disease. The exact mechanism by which PspA interacts with the complement factors to prevent deposition of C3b on the pneumococcal surface, and the specific regions of PspA responsible for this activity remain unknown, although the N-terminal α-helical region is believed to be important. PspA has also been shown to bind human Lf, an iron-sequestering glycoprotein present in mucosal secretions (Hammerschmidt *et al.,* 1999; Hakansson *et al.,* 2001). Characterisation of the interaction between Lf and PspA by the use of truncated PspA fragments has shown that Lf binds in region 2 of the α-helical domain of PspA (aa 167-288) (Hakansson *et al.,* 2001). Binding of Lf by PspA has been shown to protect *S. pneumoniae* from killing by the iron-depleted form of Lf, apolactoferrin (ALF), which is bactericidal against pneumococci (Shaper *et al.,* 2004). PspA-negative *S. pneumoniae* were killed more efficiently by ALF than the WT, and antibodies to PspA enhanced ALF killing of WT *S. pneumoniae* (Shaper *et al.,* 2004). In addition, free PspA not bound to the pneumococcal surface was also able to bind to ALF and prevent it killing pneumococci (Shaper *et al.,* 2004). Thus, binding of Lf by PspA may contribute to colonisation by preventing killing of pneumococci at mucosal surfaces.

### 1.6.3 Pneumolysin

Pneumolysin (Ply) is a 53 kDa protein toxin expressed by all clinically relevant strains of *S. pneumoniae*. It belongs to the group of cholesterol dependent cytolysins, a family that includes toxins from several Gram-positive species including perfringolysin O (PFO) from *Clostridium perfringens*, and streptolysin O (SLO) from *Streptococcus pyogenes*. Its primary mode of action is as a cytotoxin, binding to host cell membranes where it forms transmembrane pores and leads to cell lysis. However, it also has other roles in virulence; it can directly activate complement (Paton *et al.,* 1984), and at sublytic concentrations it can stimulate cells of the immune system to produce cytokines (Houldsworth *et al.,* 1994; Baba *et al.,* 2002).

#### 1.6.3.1 Ply in pathogenesis

The role of Ply in pathogenesis of pneumococcal disease has been investigated in a number of *in vitro* and *in vivo* models (Paton 1996). It inhibits ciliary beating of human respiratory epithelium, preventing clearance of pneumococci from the respiratory tract, and allowing penetration of the bacteria to the lower respiratory tract, where presence of pneumococci can lead to pneumonia (Boulnois *et al.,* 1991). Berry *et al.,* (1989) established the role of Ply in pneumococcal virulence by inactivation of the *ply* gene in *S. pneumoniae* D39. This led to an increase in $LD_{50}$, and survival time after challenge of mice when compared with the otherwise isogenic parent strain. In addition, although the rate of growth *in vitro* was identical between the Ply-negative mutant and the parent strain, there was a clear reduction in net multiplication of the Ply-negative mutant in the blood of infected mice (Berry *et al.,* 1989). Subsequent studies have confirmed and expanded upon these findings. Rubins *et al.,* (1995) demonstrated that Ply-negative pneumococci had a reduced ability to multiply within the lung and a decrease in their ability to injure the alveolar-capillary barrier, when compared to the wild type. It was also shown that Ply-negative mutants were less able to penetrate the lung interstitium from the alveoli, and invade the bloodstream compared to the

wild type (Rubins *et al.,* 1995). If Ply-negative pneumococci are injected intravenously, they are unable to cause the rapid sepsis characteristic of wild type strains, but instead cause chronic bacteremia, persisting in the blood for up to 7 days post-infection. Mortality was reduced by 50% compared to mice infected with the wild type, which caused death within 24-28 h post-infection (Benton *et al.,* 1995). After intranasal infection, levels of both WT and Ply-negative D39 in the blood were identical until 12-16 h post infection. After this time however, numbers of the WT increased rapidly reaching a peak at 24-48 h post infection, at which time mortality was high with the majority of mice succumbing to infection (Benton *et al.,* 1995; Kadioglu *et al.,* 2000). Numbers of Ply-negative in the blood did not increase past 28 h, and at this time reached a plateau of $10^6$-$10^7$ CFU/ml; these levels could be maintained for up to 7 days with no signs of illness (Benton *et al.,* 1995; Kadioglu *et al.,* 2000) indicating that Ply is a major contributing factor to the sequelae associated with sepsis. A similar occurrence was seen in the lungs, with numbers of Ply-negative pneumococci increasing more slowly than the WT, but reaching the same levels by 48 h post infection. Once again, although mice infected with the WT bacteria were dying by 48 h, those infected with Ply-negative showed little or no signs of illness (Alexander *et al.,* 1998; Kadioglu *et al.,* 2000). Recently the use of real-time bioluminescent imaging has allowed the role of Ply in disease to be examined further. Ply deletion mutants were unable to colonise the nasopharynx, translocate to the lungs or survive in the blood, demonstrating the many functions of the molecule (Orihuela *et al.,* 2004a). The collective results of these studies illustrate the important role that Ply plays in the development of pneumococcal pneumonia, enabling penetration into the lower respiratory tract and intra-alveolar multiplication of pneumococci. Ply also facilitates entry of pneumococci into the bloodstream from the alveoli, a crucial step in the development of systemic pneumococcal disease, and once pneumococci have entered the blood stream, Ply facilitates exponential growth leading to sepsis by interfering with the host immune response (Benton *et al.,* 1995). Once in the blood, Ply may help *S. pneumoniae*

to gain entry to the CSF, contributing to meningitis (Zysk *et al.,* 2001). Ply has been shown to damage ciliated ependymal cells and inhibit ciliary beat frequency of these cells (Mohammed *et al.,* 1999; Hirst *et al.,* 2000; Hirst *et al.,* 2003), and a Ply-deficient mutant was significantly less virulent than WT *S. pneumoniae* in a mouse model of meningitis (Wellmer *et al.,* 2002). Thus, Ply is a multi-functional pneumococcal virulence factor and appears to play a role in pathogenesis at multiple host sites.

### 1.6.3.2 Effects of Ply at a cellular level

The role of Ply in virulence of *S. pneumoniae* can be explained to some degree by the effects it has on the host at a cellular level. It has been shown to damage both monocytes (Nandoskar *et al.,* 1986), and epithelial cells (Rubins *et al.,* 1993) in a dose dependant manner, causing membrane lesions 100-200 nm in diameter, and loss of pseudopodia in monocytes (Hirst *et al.,* 2002); these effects are likely to be due to the cytotoxic activity of Ply. Ply-induced damage to epithelial cells was accompanied by separation of the tight junctions of the cells leading to increased alveolar permeability (Rubins *et al.,* 1993; Rayner *et al.,* 1995). This contributes to the influx of fluid and leukocytes associated with pneumococcal pneumonia. Ply induced damage to respiratory epithelia was shown to be dependent upon Ply itself, rather than the recruitment of leukocytes. Treatment of mouse lungs with Ply increased vascular permeability and recruitment of leukocytes to this site, while treatment with its non-cytolytic derivative PdB did not. Mice that were pre-treated with anti-CD18 to block leukocyte recruitment, or had undergone depletion of neutrophils, still showed an increase in lung injury after treatment with Ply demonstrating that this injury was due to the cytotoxic effects of Ply and not to leukocyte influx (Maus *et al.,* 2004). Ply has also been shown to interfere with ciliary beating of both respiratory epithelial cells and ependymal cells of the brain. In organ culture experiments, the ciliary beating frequency of respiratory epithelial cells was significantly reduced in response to Ply-positive *S. pneumoniae* when compared to Ply-negative mutants (Rayner *et al.,* 1995). This activity of

Ply presumably allows the pneumococcus to persist in the respiratory tract by interfering with the important ciliary clearance mechanism.

### 1.6.3.3 Ply and cytokine release

Ply has the ability to stimulate cytokine release from host cells and thus influence the inflammatory response generated towards the pneumococcus. Intranasal inoculation of mice with Ply is associated with a dose dependent increase in neutrophil numbers in broncho-alveolar lavage (BAL) and in levels of interleukin (IL)-6, MIP-2, and growth related oncogene α (GRO-α) (Rijneveld *et al.,* 2002). At sublytic concentrations, it also has the ability to increase the pro-inflammatory activity of neutrophils, and increase IL-8 release from these cells (Cockeran *et al.,* 2001; Cockeran *et al.,* 2002). Monocytes and macrophages also respond to Ply by increasing levels of pro-inflammatory cytokines such as tumour necrosis factor (TNF)α and IL-1β (Houldsworth *et al.,* 1994; Malley *et al.,* 2003), and monocytes produce significantly less IL-8 mRNA and protein *in vitro* in response to Ply-negative *S. pneumoniae* compared to the WT (Rogers *et al.,* 2003). Recently is has been demonstrated that the macrophage response to Ply is dependent on an interaction with Toll-like receptor (TLR) 4, and macrophages from mice with a mutation in TLR4 show no response to Ply (Malley *et al.,* 2003). The role of the cytotoxic activity of Ply in inducing cytokine responses from host cells remains controversial with studies showing conflicting results. In the absence of pore-forming activity Ply was able to induce the production of interferon (IFN) γ from spleen cells, but this induction was eliminated when cells were treated with cytotoxic Ply (Baba *et al.,* 2002). However, it was also shown that Ply mutants with reduced cytotoxicity were less potent in inducing an influx of neutrophils into the lungs, and levels of MIP-2 in the lungs of mice infected with this mutant were reduced compared to the WT (Rijneveld *et al.,* 2002). In addition, while Ply treatment of respiratory epithelial cells infected with *H. influenzae* increased IL-8 release in a similar fashion as whole *S. pneumoniae*, the non-cytotoxic derivative PdB did not have this effect (Ratner *et al.,* 2005). Thus, the role of the

cytotoxic activity of Ply in cytokine induction remains debatable, and this process is likely to involve the interaction of many factors.

### 1.6.3.4 Pneumolysin structure and function

The structure of Ply has been modelled on the structure of PFO, a molecule with which it shares 48% sequence identity, and 60% sequence similarity (Rossjohn *et al.,* 1998). The model of Ply depicts a rod-shaped molecule consisting of 4 domains (Figure 1.7).

Domain 1 is thought to be involved in oligomerisation of the molecule and is structurally associated with domain 3 (Baba *et al.,* 2001). Domain 2 acts as a junction between domain 1 and domain 4, and domain 4 is thought to be the functional domain, involved in cholesterol binding, membrane insertion, and complement activation (Mitchell *et al.,* 1991; Kelly and Jedrzejas, 2000). Studies using truncated forms of Ply have shown that only truncates containing domain 4 could bind to erythrocyte membranes; those containing domains 1-3 only were not able to bind or cause haemolysis, while purified domain 4 was able to completely inhibit haemolysis caused by full-length Ply in a dose dependent manner (Baba *et al.,* 2001). This domain contains the longest stretch of sequence identity between Ply and other members of the cholesterol dependent cytolysin family, an undecapeptide (ECTGLAWEWWR) containing the so-called Trp-rich loop (Rossjohn *et al.,* 1998). It is proposed that cholesterol binds close to this motif causing a conformational change in which hydrophobic residues are revealed forming a "hydrophobic dagger" that inserts into the cell membrane (Rossjohn *et al.,* 1998; Kelly and Jedrzejas, 2000). Trp433 is part of a hydrophobic pocket that is made up of the side chains of Tyr376, Cys428, Arg426, and Gln374. It is thought that when cholesterol binds, it displaces Trp433 from this pocket causing it to flip out, forming the hydrophobic dagger (Rossjohn *et al.,* 1998). Trp433 is important in maintaining the conformation of the Trp-rich loop, and in membrane insertion overall, as when it is substituted with Phe, Ply loses 99.9% of its haemolytic activity, presumably due to a reduced ability of the molecule to insert into the cell membrane.

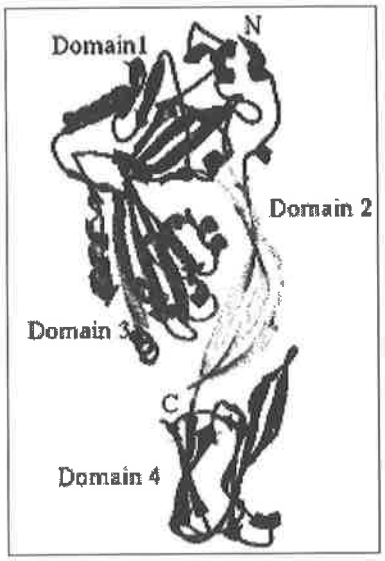


**Figure 1.7. Ribbon structure of pneumolysin.**
Model of pneumolysin structure based on homology to perfringolysin from *C. perfringens*, as predicted by Rossjohn *et al.*, 1998. Different domains of the molecule are indicated and shaded.

However, the residual cytotoxic activity of the Trp433→Phe mutant is still enough to cause disease, as there was no significant reduction in virulence of a D39 derivative expressing this mutant toxin in mice, when compared to WT D39 using either an intranasal or intraperitoneal infection model (Berry *et al.,* 1995; Alexander *et al.,* 1998), suggesting that 0.1% haemolytic activity may still be enough for Ply to exert a significant deleterious effect. Another domain 4 residue believed to play an important role in the cytolytic function of Ply is His367. Substitution of this residue with Arg reduces cytolytic activity of the molecule to 0.02% of that of WT Ply, and although it can bind cell membranes, this molecule was unable to oligomerise and form membrane pores (Boulnois *et al.,* 1991). D39 expressing the His367→Arg Ply derivative showed significantly decreased virulence in mice, when challenged by both the intranasal and intraperitoneal routes (Berry *et al.,* 1995; Alexander *et al.,* 1998).

Pneumolysin was originally labelled a "thiol-activated" toxin, based on the observation that it was only active in the reduced state, and that its activity was abolished by sulfhydryl blocking agents. This idea was further strengthened by the fact that the entire molecule contains only one cysteine residue, present at position 428 and located in the conserved Trp-rich undecapeptide. Thus it was thought that the thiol group of this residue played an essential role in the activity of Ply. However, mutagenesis studies have demonstrated that this thiol group is not essential for function. Substitution of Cys428 with Ala did not affect the cytotoxicity of the molecule, and the activity of this mutant was completely inhibited by cholesterol (Saunders *et al.,* 1989). Nevertheless, the Cys428 residue itself may be important in the function of Ply, because when substituted with Ser or Gly activity was significantly reduced (Saunders *et al.,* 1989). When substitution of this residue with Gly is combined with point mutation of Trp433→Phe, the haemolytic activity of Ply is reduced to 0.0001% of the WT, and is also reduced compared to mutation of Trp433 only. In both intranasal and intraperitoneal models of infection, the virulence of this mutant was

significantly reduced when compared to the WT and to the Trp433→Phe mutant (Berry *et al.*, 1995; Alexander *et al.*, 1998).

The ability of Ply to activate complement via the classical pathway (Paton *et al.*, 1984) was subsequently shown to be due to a region of the molecule with similarity to a domain of human C-reactive protein (CRP) (Mitchell *et al.*, 1991). Studies in which residues in this region were altered using site-directed mutagenesis demonstrated that Asp385 is important for this function. When this residue was changed to Asn, complement-activating ability was abolished, probably due to a reduction in binding of the Fc portion of IgG (Mitchell *et al.*, 1991). The significance of complement activation in the function of Ply as a virulence factor has been demonstrated in *in vivo* studies. Cirrhotic rats in which complement is depleted clear Ply-negative and D39 Asp385→Asn mutants significantly faster than WT (Alcantara *et al.*, 2001), presumably because Ply activates the small amount of remaining complement factors in the fluid phase, away from the pneumococcal surface. After intranasal infection of normal mice, growth of the Ply Asp385→Asn mutant in the lungs and the blood was slower than the wild type, with the same growth kinetics observed as for the $Ply^-$ strain in the first 24 h. After this time growth was similar to that of the wild type, demonstrating that the requirement of complement activation wanes as the infection progresses (Alexander *et al.*, 1998). In an intranasal model, mice infected with D39 expressing the Ply Asp385→Asn mutant survived significantly longer than those infected with the wild type; survival rates were 30% and 0% respectively (Alexander *et al.*, 1998). In this model, a significant difference was also seen in the numbers of pneumococci that were present in the blood. This is consistent with the proposed function of Ply in the blood to activate complement away from the pneumococcus, thus interfering with clearance. Numbers of the Asp385→Asn mutant increased more slowly in the blood in the first 24 h post infection when compared to the WT, and showed a rate of growth in the blood that was similar to that of a Ply-negative mutant

(Alexander *et al.,* 1998). However an intraperitoneal model of infection showed that the D39 Asp385→Asn mutant was as virulent as the wild type (Berry *et al.,* 1995).

## 1.7 Pathology of pneumococcal disease

### 1.7.1 Histology

The development of pneumococcal disease, and in particular pneumonia, involves dramatic changes in morphology and histology of the lungs, eventually leading to complete disruption of lung architecture and death of the animal. Several studies have sought to understand the events leading to the development of disease, and the host factors involved. Intranasal infection of mice with doses of *S. pneumoniae* ranging from $5 \times 10^4$ to $1 \times 10^7$ CFU led to the same general trends in progression of disease although the kinetics of events in the mice infected with $5 \times 10^4$ CFU were slightly delayed. At approximately 12 h post infection, there was a decline in numbers of bacteria in the lungs, as neutrophils recruited to this site by pro-inflammatory cytokines attempted to clear the infection (Bergeron *et al.,* 1998; Kadioglu *et al.,* 2000). Presence of pneumococci in the lungs caused localised oedema and inflammation of bronchioles plus a significant increase in lung weight at 24 h post infection (Bergeron *et al.,* 1998; Kadioglu *et al.,* 2000). At this time, all mice were beginning to show signs of illness, and tissue injury became visible in the lungs, with disruption of alveolar integrity and an influx of leukocytes (Bergeron *et al.,* 1998; Kadioglu *et al.,* 2000; Kerr *et al.,* 2002). By 48 h post infection, up to 75% of the lung was affected by inflammation (Bergeron *et al.,* 1998; Kadioglu *et al.,* 2000; Kerr *et al.,* 2002). There was infiltration of neutrophils and erythrocytes into interstitial tissues, and inflammatory exudate into the alveolar spaces; in some lung sections the alveoli were barely distinguishable due to extensive damage (Kadioglu *et al.,* 2000; Dallaire *et al.,* 2001). The entire lung was encompassed by inflammation by 72 h post infection with the higher doses, with widespread tissue damage and an increase in lung weight due to oedema and cellular influx (Figure 1.8) (Bergeron *et al.,* 1998; Kadioglu *et al.,*

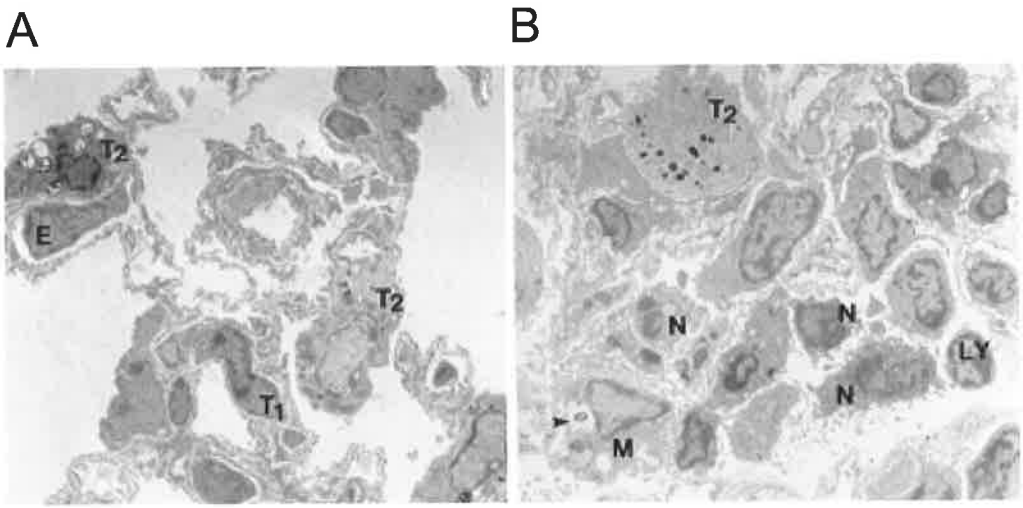


**Figure 1.8. Lung architecture of mice infected with S. pneumoniae.** Electron micrograph of normal mouse lungs (A), and lungs of a mouse infected with $10^7$ CFU *S. pneumoniae* (B), and sacrificed 72 h post infection. Macrophages (M), lymphocytes (LY), and neutrophils (N) can be seen in the lungs of infected mice, as well as disruption of the lung structure. E, endothelial cell; T1, type I pneumocyte; T2 type II pneumocyte. Magnification x 5000. Adapted from Bergeron *et al.* (1998).

2000). Alveoli were completely indistinguishable due to extensive tissue damage, and lung integrity was severely disrupted. At this point most mice had succumbed to infection and bacteria could be detected in the blood of all surviving mice (Bergeron *et al.,* 1998; Kadioglu *et al.,* 2000; Kerr *et al.,* 2002).

### 1.7.2 Roles of cytokines in inflammation

Progression of pneumococcal disease, influx of inflammatory cells and changes in lung histology are mediated largely by cytokines, and this cytokine response plays an important role in the ability of the host to overcome pneumococcal infection. The increased susceptibility of alcoholics to pneumococcal pneumonia is thought to be largely due to an inability to effectively recruit neutrophils to the site of infection. Studies in mice have shown that this is due to suppression of MIP-2 release by ethanol (Boe *et al.,* 2001; Boe *et al.,* 2003). Such mice are more susceptible to pneumococcal infection, demonstrating the importance of chemokine induced neutrophil recruitment in overcoming pneumonia caused by *S. pneumoniae.* The chemokine profile of pneumococcal infection has been studied in mice using intranasal infection models. After infection, bacteria move quickly to the lungs where they can be detected almost immediately after infection (Dallaire *et al.,* 2001). An early increase in MIP-2 is seen in the lung tissue and BAL, and this is accompanied by an influx of neutrophils to the site of infection (Dallaire *et al.,* 2001). In mice that went on to recover, there was an early, sharp MIP-2 and IL-1β response that helped to overcome infection. This response was short lived with levels of chemokines in the lungs of these mice returning to normal levels by 48 h post infection. Mice that died had a delayed response that became prolonged due to an inability to overcome the numbers of pneumococci, with levels of chemokines remaining elevated in the lungs and blood of these mice until death (Dallaire *et al.,* 2001). This prolonged inflammatory response and the accompanying presence of neutrophils probably leads to an increase in damage to the lungs of the host and it is likely that this contributes to mortality.

Thus, it appears that the early response of the host is crucial in determining the outcome of pneumococcal infection. Experiments in which mice were injected with pro-inflammatory compounds (LPS, recombinant TNF (rTNF), or heat killed *S. pneumoniae*) concurrently with the challenge dose supported this. The additional inflammatory response these compounds generated early in infection helped mice to overcome the infection, and significantly increased survival (Dallaire *et al.,* 2001).

In the studies described above, an obvious role for chemokines in response to pneumococcal infection is seen. However in an *in vivo* model it is difficult to isolate the source of these chemokines, because the lung is a complex environment with many cell types present. The use of *in vitro* models allows the response of individual cell types to be investigated so that the specific contributions that particular cells make to inflammation can be better understood. While leukocytes such as neutrophils and alveolar macrophages contribute greatly to the release of pro-inflammatory cytokines in the lungs in response to infection, respiratory epithelial cells are also able to contribute to this response. Infection with *Mycoplasma pneumoniae*, or *Chlamydia pneumoniae* led to an increase in release of the pro-inflammatory cytokines TNFα, IL-8 and IL-1β from type II pneumocytes (A549) (Yang *et al.,* 2002; Yang *et al.,* 2003), and infection with *H. influenzae* and *S. pneumoniae*, both singly and in combination, led to an increase in IL-8 release from Detroit-562 and A549 cells (Ratner *et al.,* 2005). Thus epithelial cells, which are the first line of defence against invading pathogens, are able to contribute directly to the inflammatory response by releasing chemokines that may recruit leukocytes to the site of infection thereby facilitating clearance of the bacteria.

## 1.8 Chemokines

The chemokines are a family of small molecular weight (8-12 kDa) cytokines that have a number of important biological functions, including control of leukocyte chemotaxis

(Rollins, 1997). The structure of chemokines is highly conserved with up to 70% sequence identity existing at the amino acid level. All have the same basic structure consisting of a disorganised N-terminus anchored to the rest of the molecule by disulphide bonds involving the cysteine residues that are the major feature of this family. Most chemokines contain at least four cysteine residues, with disulphide bonds forming between the first and third and the second and fourth. The chemokine superfamily is further divided into subfamilies based on the relative positions of these residues (Luster, 1998; Gangur *et al.,* 2002). At present 4 families of chemokines exist, the largest of these are the α and β families in which the first two cysteine residues are separated by one amino acid (CXC), or are directly adjacent to each other (CC).

### 1.8.1 CXC Chemokines

The family of CXC chemokines can itself be further subdivided into two groups based on the presence or absence of the tripeptide Glutamate, Leucine, Arginine (ELR) adjacent to the CXC motif (Gale and McColl, 1999). This tripeptide plays an important role in the target cell specificity of the chemokine, with ELR containing chemokines having chemotactic activity for neutrophils and those not containing this motif attracting lymphocytes (Luster, 1998). IL-8, MIP-2α (also known as GRO-β), MIP-2β (also known as GRO-γ), melanoma growth stimulatory activity (MGSA) (also known as GRO-α), epithelial derived neutrophil activating peptide-78 (ENA-78), and granulocyte chemotactic protein-2 (GCP-2) are all members of the ELR containing group of CXC chemokines, and thus have neutrophil chemotactic activity (Rollins, 1997). Of these, IL-8 has been studied most closely. It is released by a range of cells including epithelial cells, and besides neutrophil chemotaxis has a variety of functions including basophil activation and angiogenesis (Baggiolini *et al.,* 1994). MIP-2α, MIP-2β, and MGSA were initially isolated in transformed cells, and shown to be

important for growth regulation, hence their alternative designation as "growth related oncogene" (GRO) (Rollins, 1997). These chemokines are also reported to have a role in angiogenesis (Baggliolini *et al.,* 1997). ENA-78 and GCP-2 were also first isolated in tumor cells lines, and although they are chemotactic for neutrophils, their activity is approximately 10-fold lower than that of IL-8 (Baggiolini *et al.,* 1994; Rollins, 1997). Neutrophil recruitment is an important step in the host response to pneumococcal infection, thus it is likely that these chemokines also play an important role in pneumococcal pathogenesis.

### 1.8.2 Bacterial induction of CXC chemokines

Pattern recognition receptors such as toll like receptors (TLRs) present on host cells are part of the innate immune response enabling rapid recognition of and response to invading bacteria (Sabroe *et al.*, 2002). Binding of bacterial products to TLRs triggers a cascade of intracellular signalling events that ultimately leads to activation of the transcription factor nuclear factor kappa B (NFκB). NFκB binds the promoter region of specific genes including the genes for CXC chemokines such as IL-8, and activates transcription of these genes (Akira and Takeda, 2004). Expression of IL-8 can also be regulated at the post-transcriptional level by the p38 and extra-cellular signal-regulated kinase (ERK) mitogen activated protein kinase (MAPK) pathways, possibly by increasing IL-8 mRNA stability (Hoffmann *et al.*, 2002, Jijon *et al.*, 2002).

## 1.9 Aims of the work in this thesis

As described above, the development of pneumococcal disease is a complex process involving interactions between multiple host and pneumococcal factors. The inflammatory response is known to play a key role in the outcome of disease, and the recruitment of neutrophils to the site of infection is believed to be an important part of this response. CXC chemokines are important mediators of inflammation, responsible for the recruitment of

neutrophils to the site of infection thereby facilitating phagocytosis. There are some indications that *S. pneumoniae* may modulate the release of CXC chemokines from epithelial cells during infection, but the specific pneumococcal factors responsible for this activity remain to be determined. Characterisation of pneumococcal factors involved in the release of CXC chemokines from respiratory epithelial cells will allow a better understanding of the interaction between *S. pneumoniae* and the respiratory epithelium, and an improved knowledge of the early steps in pathogenesis of pneumococcal disease.

Accordingly, the specific aims of the work described in this thesis are:

1. To develop a procedure for the detection and quantitation of CXC chemokine mRNA in respiratory epithelial cell lines (A549 and Detroit-562) in response to infection with *S. pneumoniae*.
2. To determine the CXC chemokine response of A549 and Detroit-562 cells *in vitro* to *S. pneumoniae* D39.
3. To elucidate the role of the pneumococcal virulence factors CbpA, PspA and Ply in the CXC chemokine response of these cells towards *S. pneumoniae*.
4. To identify the specific domains of these proteins that are responsible for their modulating effects on CXC chemokine responses.

# Chapter Two– Materials and Methods

## 2.1 Chemicals and Reagents

Most chemicals used were AnalaR grade and were purchased from Ajax Chemicals (NSW, Australia). Tris was purchased from Progen Industries (QLD, Australia). Maleic acid, and Sodium dodecyl sulphate were purchased from Sigma Chemical Company (St. Louis, MO., USA). Acrylamide, ammonium persulphate and N,N,N'N'-tetramethyl-ethylene-diamine (TEMED) were purchased from Bio-Rad Laboratories (Hercules, CA, USA). The deoxyribonucleoside triphosphates (dNTPs), Isopropyl-β-D-thiogalactoside (IPTG), and herring sperm DNA were purchased from Roche Diagnostics (Mannheim, Germany). Sodium Deoxycholate (DOC) was purchased from BDH Biochemicals (Poole, England). All enzymes were purchased from Roche Diagnostics.

### 2.1.1 Antibiotics

Ampicillin (Amp) was purchased from CSL (Victoria, Australia); Kanamycin sulphate (Kan), Erythromycin (Ery), and Chloramphenicol (Cml) were purchased from Roche.

## 2.2 Bacterial Growth Media

*E. coli* strains were grown in Luria-Bertani broth (LB) (10 g/l tryptone-peptone, 5 g/l yeast extract, 5 g/l NaCl), or Terrific broth (24 g/l yeast extract, 12 g/l tryptone, 0.4% (v/v)

glycerol, 0.17 M $KH_2PO_4$, 0.72M $K_2HPO_4$), or on LB agar plates (LB with 15 g agar). Where appropriate, Amp, Kan, or Cml were added to the growth medium at concentrations of 50, 50, and 25 μg/ml respectively. *E. coli* strains were preserved in LB in the presence of 30% glycerol at -80°C.

*S. pneumoniae* were routinely grown in Todd Hewitt broth with yeast (THY) (36.4 g/l Todd Hewitt (Oxoid), 10 g/l yeast extract), or on blood agar plates (39 g/l Columbia base agar (Oxoid), 5% (v/v) defibrinated horse blood). Where appropriate, Ery was added to the growth medium at a concentration of 0.2 μg/ml.

For storage, pneumococci were grown in serum broth (10% (v/v) donor horse serum in nutrient broth (10 g/l of peptone (Oxoid), 10 g/l of Lab Lemco (Oxoid) and 5 g/l of NaCl)), then stored at -80°C.

## 2.3 Eukaryotic cell line growth and maintenance

Eukaryotic cells were routinely grown and maintained in 75 $cm^2$ vented tissue culture flasks (Falcon). Detroit-562 (pharyngeal epithelial) cells (ATCC CCL-138) were grown in Eagle's modified essential medium (EMEM) supplemented with 10% (v/v) foetal calf serum (FCS) (Gibco BRL-Life Technologies, Grand Island, NY, USA), 1mM sodium pyruvate (Trace), 100 IU/ml penicillin, and 100 μg/ml streptomycin (Gibco Life technologies), at 37°C in the presence of 5% $CO_2$. A549 cells (type II pneumocytes) (ATCC CCL-185) were grown in Dulbecco's modified essential medium (DMEM) (Trace biosciences), supplemented with 5% (v/v) FCS, 100 IU/ml penicillin, and 100 μg/ml streptomycin, at 37°C. Upon reaching confluence, cell monolayers were detached by the addition of a solution of 0.25% (w/v) trypsin and 0.2% (w/v) EDTA in phosphate buffered saline (PBS) (0.137 M NaCl, 2.7 mM KCl, 8.1 mM $Na_2HPO4$, 1.5 mM $KH_2PO_4$, pH 7.4) and flasks were reseeded at a ratio of 1:15 (cell suspension: media). Cells were stored in the appropriate growth medium supplemented

with 20% (v/v) FCS and 10% (v/v) dimethylsufoxide (DMSO) at -80°C or -275°C for long-term storage.

## 2.4 Trypan Blue exclusion assay for cell viability

Confluent monolayers of eukaryotic cells were assayed for viability after infection with *S. pneumoniae* by exposure to 0.4% (w/v) Trypan blue dye in cell culture medium for 5 min after which the dye was removed and 1 ml cell culture media was added to cells. Cell viability was assessed by ability to exclude the dye using microscopy at 400 × magnification.

## 2.5 Bacterial strains and cloning vectors

The strains and plasmids used in this study are indicated in Table 2.1

**Table 2.1** Strains and plasmids used in this study

| ***S. pneumoniae* strain** | **Description** | **Reference** |
|---|---|---|
| D39 | Capsular serotype 2 (NCTC 7466) | Avery *et al.*, (1944) |
| CbpA⁻ D39 | $Ery^R$, CbpA mutant of D39 | Berry and Paton, (2000) |
| ΔCbpA D39 | *cbpA* deletion mutant of D39 | This study |
| CbpAΔHyp D39 | Hypervariable region of *cbpA* (nt 424-720) deletion mutant of D39 | This study |
| CbpAΔSR1 D39 | Small repeat region 1 of *cbpA* (nt 721-1206) deletion mutant of D39 | This study |
| CbpAΔSR2 D39 | Small repeat region 2 of *cbpA* (nt 1207-1548) deletion mutant of D39 | This study |
| CbpAΔPro D39 | Proline rich region of cbpA (nt 1549-1761) deletion mutant of D39 | This study |
| PspA⁻ D39 | $Ery^R$, PspA mutant of D39 | McDaniel *et al.*, (1987) |
| ΔPspA D39 | *pspA* deletion mutant of D39 | This study |
| PspAΔh1 D39 | α-helical region 1 of *pspA* (nt 210-657) deletion mutant of D39 | This study |
| PspAΔh2 D39 | α-helical region 2 of *pspA* (nt 658-1083) deletion mutant of D39 | This study |
| PspAΔhelix D39 | α-helical region of *pspA* (nt 210-1083) deletion mutant of D39 | This study |
| PspAΔpro D39 | Proline rich region of *pspA* (nt 1084-1329) deletion mutant of D39 | This study |
| ΔPly D39 | *ply* deletion mutant of D39 | Berry *et al.* (1999) |
| 367 | Ply mutant of D39 with point mutation of residue 367 from His to Arg | Berry *et al.*, (1995) |
| 385 | Ply mutant of D39 with point mutation of residue 385 from Asp to Asn | Berry *et al.*, (1995) |

| | | |
|---|---|---|
| 433 | Ply mutant of D39 with point mutation of residue 433 from Trp to Phe | Berry *et al.*, (1995) |
| Triple | Ply mutant of D39 with point mutations of residues 433 from Trp to Phe, 428 from Cys to Gly, and 385 from Asp to Asn | Berry *et al.*, (1995) |
| | | |
| ***E. coli* strain** | **Description** | **Source/Reference** |
| DH5α | *E. coli* K-12 derivative: $F^-$, *deoR, supE44 Δ(lacZYA-argF) U169 [ϕ80 dlacΔ(lacZ M15)] hsdR17recA1 endA1 gyrA96 thi-1 relA1,* $\lambda^-$ | Laboratory collection |
| BL21(DE3) *lpx*$M^-$ | $Cml^R$ | Cognet *et al.*, (2003) |
| M15 | *E. coli* K-12 derivative: $NaI^S$, $Str^S$, $Rif^S$, $F^-$, $th^-$, $lac^-$, $ara^+$, $gal^+$, $mtl^-$, $recA^+$, $uvr^+$, $lon^+$ | Qiagen |
| JM109 | *E. coli* K-12 derivative: F', *traD36* $proA^+$ $B^+$ $lacI^q$ *Δ(lacZ)M15/ Δ (lac-proAB) glnV44 e14*$^-$ *gyrA96 recA1 relA1 endA1 thi-1 hsdR17* | Laboratory collection |
| | | |
| **Plasmids** | **Characteristics** | **Source/Reference** |
| pGEM®-T Easy | PCR product cloning vector (T-tailed; $Amp^R$) | Promega |
| pQE30 | $His_6$-tag expression vector, $Amp^R$ | Qiagen |
| pVA891 | $Cml^R$, $Ery^R$ | Macrina *et al.*, (1983) |
| pJCP191 | Ply expression vector (*ply* cloned into pUC18), $Amp^R$ | Laboratory collection |

## 2.6 Oligodeoxynucleotides

All oligodeoxynucleotides used in this study were purchased from Sigma Genosys (Sigma-Aldrich), and are listed in Table 2.2

**Table 2.2** Oligonucleotides used in this study. Lower case letters represent oligonucleotide tails.

| Name | Sequence 5`→ 3` | (Accession No.)/Location |
|---|---|---|
| CbpAprom4 | ATG AAG TTA TAA TCC CAA ATG GAA GC | R6 genome (NC_003098) complementary nt 1989739-1989765 |
| CbpAR2 | TCT CTA GCG CTA TAA ATC CGG C | D39 *cbpA* (AF068646) complementary nt 2551-2572 |
| IDPspAa | ACA AGT CTA GCC AGC GTC GCT | D39 *pspA* (M74122) nt 151-171 |
| IDPspAb | TAT CTG ATA CTT TGA ACC ATT GGC | D39 *pspA* (M74122) complementary nt 1874-1897 |
| PspAF | CAA GTC TAG CCA GCG TCG | D39 *pspA* (M74122) nt 152-169 |
| PspApromF | AAGCTTATGATATAG | D39 *pspA* (M74122) nt 1-15 |
| PspAR2 | CCT GTA GCC ATA GCA CCA TTG C | D39 *pspA* (M74122) complementary nt 1433-1454 |
| RMAG1 | aat tta atg ggc TTA CTT ATT CAT CTA AAT TTA CCT CTT TT | R6 genome (NC_003098) complementary nt 128339-128367 |
| RMAG2 | tag atg aat aag TAA GCC GAT TAA ATT AAA GCA TGT TAA | R6 genome (NC_003098) nt 130213-130239 |
| RMAG3 | TTA GAA CGG CTT AAA ATC AGA TAT GA | R6 genome (NC_003098) nt 126448-126473 |
| RMAG4 | CGC CAC CTA GAA CAC TCT TCG | R6 genome (NC_003098) complementary nt 131668-131687 |
| RMAG5 | ata aac atg ttt GTA AAC TAA ACC TAA TAT AAC TAG TTA | R6 genome (NC_003098) complementary nt 1987525-1987552 |
| RMAG6 | tta ggt tta gtt TaC AAA CAT GTT TAT TTC CTT CTA TAT | R6 genome (NC_003098) nt 1989641-1989667 |
| RMAG7 | GCT GCA CCG ATA GAC AGA CGC | R6 genome (NC_003098) nt 1985081-1985101 |
| RMAG8 | TCC TTG ACC ATA TCT GCT CAC C | R6 genome (NC_003098) complementary nt 1991426-1991447 |
| RMAG12 | tga aga agt cgc tGA AGG AGT GAT TAC ATG AAC AA | pVA891 *ery* nt 5103-5125 |
| RMAG13 | tgg ctc ttc agc CTC ATA GAA TTA TTT CCT CCC G | pVA891 *ery* nt 4363-4384 |
| RMAG14 | att cac tcc ttc AGC GAC TTC TTC AGC ATC CAC | D39 *pspA* (M74122) complementary nt 718-738 |
| RMAG15 | taa ttc tat gag GCT GAA GAG CCA TCG CAA CCA | D39 *pspA* (M74122) nt 1075-1095 |
| PspA Helix1 1 | ttt agc ttc ttc TGC TCT TAC AAC AGT AGG CTG | D39 *pspA* (M74122) complementary nt 199-209 |
| PspA Helix1 2 | gtt gta aga gca GAA GAA GCT AAA GCA AAA TTA GAA | D39 *pspA* (M74122) nt 658-678 |
| PspA Helix2 1 | tgg ttt ttc tgg TAG TTT TTT AGT AAG TTC TGG TGC | D39 *pspA* (M74122) complementary nt 634-657 |
| PspA Helix2 2 | act aaa aaa cta CCA GAA AAA CCA GCT CCA GCT | D39 *pspA* (M74122) nt 1084-1104 |
| PspA Pro 1 | ttt cca gcc tgt CTC ATT AAC TGC TTT CTT AAG GTC | D39 *pspA* (M74122) complementary nt 1063-1083 |

| | | |
|---|---|---|
| PspA Pro 2 | gca gtt aat gag ACA GGC TGG AAA CAA GAA AAC G | D39 *pspA* (M74122) nt 1330-1350 |
| PspA helix 1 | tgg ttt ttc tgg TGC TCT TAC AAC AGT AGG CTG | D39 *pspA* (M74122) complementary nt 199-209 |
| PspA helix 2 | gtt gta aga gca CCA GAA AAA CCA GCT CCA GCT | D39 *pspA* (M74122) nt 1084-1104 |
| CbpA hyp1 | ctt ttc tcc tgg CGC ATG AAC CAC ACT TCC CAT | D39 *cbpA* (AF068646) complementary nt 403-423 |
| CbpA hyp2 | gtg gtt cat gcg CCA GGA GAA AAG GTA GCA GAA | D39 *cbpA* (AF068646) nt 721-741 |
| CbpA sm rep1 1 | ctt ttt tcc tga TTT CAA TGT ATC TTT TTT AAA CTT CTC | D39 *cbpA* (AF068646) complementary nt 694-720 |
| CbpA sm rep1 2 | gat aca ttg aaa TCA GGA AAA AAG GTA GCA GAA GCT | D39 *cbpA* (AF068646) nt 1207-1230 |
| CbpA sm rep2 1 | ttg ttc agc tgg TTT CAG GGA TGA GCT TGG AAG | D39 *cbpA* (AF068646) complementary nt 1186-1206 |
| CbpA sm rep2 2 | tca tcc ctg aaa CCA GCT GAA CAA CCA CAA CCA | D39 *cbpA* (AF068646) nt 1549-1569 |
| CbpA Pro 1 | ttg ttt cca gcc TTT TTC TTT AAC TTT ATC TTC TTC TG | D39 *cbpA* (AF068646) complementary nt 1523-1548 |
| CbpA Pro 2 | gtt aaa gaa aaa GGC TGG AAA CAA GAA AAC GGT | D39 *cbpA* (AF068646) nt 1762-1782 |
| IL-8 Fwd | GAA GGA ACC ATT CTC ACT GTG TGT A | IL-8 mRNA (M28130) nt 75-99 |
| IL-8 Rev | TTA TGA ATT CTC AGC CCT CTT CAA AAA C | IL-8 mRNA (M28130) complementary nt 402-375 |
| ENA-78 Fwd | GAA CCC GCG ACC GCT CGC | ENA-78 mRNA (XM_003507) nt 62-79 |
| ENA-78 Rev | AGA AAA GGG GCT TCT GGA TCA A | ENA-78 mRNA (XM_003507) complementary nt 393-372 |
| GCP-2 Fwd | CTC CAC CCA GCT CAG GAA CC | GCP-2 mRNA (XM_003502) nt 14-33 |
| GCP-2 Rev | GAA AAG GGG CTT CCG GGT CCA | GCP-2 mRNA (XM_003502) complementary nt 351-331 |
| MGSA Fwd | AGC CAC ACT CAA GAA TGG GCG | MSGA mRNA (XM_003504) nt 304-324 |
| MGSA Rev | TGG CAT GTT GCA GGC TCC TC | MSGA mRNA (XM_003504) complementary nt 758-777 |
| MIP-2α Fwd | ATT TGT TAA TAT TTC TTC GTG ATG ACA TAT CA | MIP-2α mRNA (X53799) nt 709-740 |
| MIP-2α Rev | TCG AAA CCT CTC TGC TCT AAC AC | MIP-2α mRNA (X53799) complementary nt 1,010-1,032 |
| MIP-2β Fwd | AGA ACA TCC AAA GTG TGA ATG TAA GG | MIP-2β mRNA (X53800) nt 198-223 |
| MIP-2β Rev | TCC TTT CCA GCT GTC CCT AGA A | MIP-2β mRNA (X53800) complementary nt 458-479 |
| GAPDH Fwd | TCC TTG GAG GCC ATG TGG GCC AT | GAPDH mRNA (XM_033258) nt 206-228 |
| GAPDH Rev | TGA TGA CAT CAA GAA GGT GGT GAA G | GAPDH mRNA (XM_033258) complementary nt 445-421 |

## 2.7 Protein purification

### 2.7.1 Expression of recombinant proteins

The N-terminal fragments of CbpA and PspA from the type 2 *S. pneumoniae* D39 were expressed as $His_6$-fusion proteins using the vector pQE31 (Qiagen, Hilden, Germany), in the *E. coli* K12 expression strain M15 (Qiagen), or *E. coli* BL21 (DE3) *lpxM*$^-$. Expression strains were grown in 100 ml Luria-Bertani (LB) or Terrific broth overnight at 37°C with agitation in the presence of 50 mg/ml Amp. This starter culture was then diluted 1/10 into 2 × 500 ml LB or Terrific broth with 50 mg/ml Amp, and incubated at 37°C with agitation until an Absorbance at 600nm ($A_{600}$) of 0.5 had been reached. High-level expression of $His_6$-tagged proteins was induced by the addition of 2 mM IPTG, and incubation at 37°C for 3 h with agitation. The culture was then centrifuged at 4°C for 8 min at 11,440 × *g* (Beckman J2-MI). The pellet was resuspended in 200 ml of lysis buffer (50 mM sodium-phosphate, 20 mM imidazole, 2 M NaCl, pH 8.0), and the cells were lysed using a French pressure cell (SLM Instruments) operated at 12,000 psi. The lysate was then centrifuged at 120,000 × *g* for 1 h at 4°C, to remove cellular debris.

### 2.7.2 Purification of CbpA and PspA by Ni-NTA chromatography

Recombinant $His_6$-tagged CbpA and PspA proteins were purified by Nickel-nitrilotriacetic acid (Ni-NTA) affinity chromatography (Qiagen). A 2 ml Ni-NTA bed was equilibrated with 20 ml lysis buffer. Lysate supernatant was treated with 20 mM β-mercaptoethanol, 1% (v/v) Triton-X100, DNase and RNase for 10 min on ice, and then was loaded onto the column at a rate of 15 ml/h. After the entire supernatant had been loaded, the column was washed with 20 ml wash buffer (10 mM sodium-phosphate, 1 M NaCl, 0.5%(v/v) Triton-X100, pH 6.0). Bound $His_6$-tagged protein was eluted from the column with a 0-500 mM imidazole gradient in elution buffer (10 mM sodium-phosphate, pH 6.0). Fractions were collected and immediately placed on ice. 10 µl from each of the fractions was subjected to analysis by

Sodium dodecyl sulphate-polyacrylamide gel electrophoresis (SDS-PAGE), as described in Section 2.8.1, to confirm the presence and purity of the desired protein. Fractions containing the protein were concentrated in 10 mM sodium-phosphate (pH 7.0), and stored in 50% glycerol at –20°C. Protein concentration was determined by the method of Bradford (Bradford, 1976) using bovine serum albumin (BSA) as a standard.

### 2.7.3 Purification of Ply by column chromatography

Pneumolysin was expressed in *E.coli* JM109 carrying pJCP191, and purified by a modification of the method of Paton *et al.*, (1991). The expression strain was grown in 50 ml Terrific broth with 200 μg/ml Amp overnight at 37°C with agitation. This culture was then diluted 1/4 into 200 ml Terrific broth with 200 mg/ml Amp and grown for 4 h at 37°C with agitation. This culture was added to 2.5 l Terrific broth with 200 μg/ml Amp and grown overnight at 37°C with agitation. Cultures were centrifuged at 4°C for 10 min at 11,440 × *g*. Pellets were resuspended in 10 mM sodium phosphate (pH 7.0) and lysed using a french pressure cell operated at 12,000 psi. The lysate was then centrifuged at 120,000 × *g* for 1 h at 4°C, to remove cellular debris. Supernatant was subjected to ion-exchange chromatography on DEAE-sepharose, eluted with a 10 mM – 250 mM sodium phosphate (pH 7.0) gradient. Fractions were collected and analysed for the presence of Ply by haemolysis assay (Section 2.8.4). Fractions containing Ply were pooled and concentrated in 50 mM sodium phosphate (pH 7.0). Concentrated fractions were then subjected to gel permeation chromatography on sephacryl S200 and eluted with the same buffer. Fractions were analysed for the presence of Ply by haemolysis assay, and SDS-PAGE, and peak fractions were pooled, concentrated in 10 mM sodium phosphate (pH 7.0), and stored at –20°C in 50% (v/v) glycerol.

## 2.8 Protein analysis

### 2.8.1 SDS-PAGE

SDS-PAGE was performed using the method described by Laemmli (1970). Proteins were stained by gentle agitation for 1 h at 65°C in 0.1% (w/v) Coomasie Brilliant Blue R250, dissolved in 10% (v/v) glacial acetic acid and 25% (v/v) methanol. Destaining was performed by agitation with several changes of a solution of 10% (v/v) acetic acid and 10% (v/v) isopropanol.

### 2.8.2 Preparation of Whole Cell Lysates (WCL)

Pellets of 10 ml 4 h pneumococcal cultures, or overnight *E. coli* cultures were resuspended in 500 µl 2 × LUG (5% (v/v) β-mercaptoethanol, 62.5 mM Tris-HCl, 2% (w/v) SDS, 10% (v/v) glycerol, 0.05% (w/v) bromophenol blue, pH 6.8). Samples were boiled for 10 min, centrifuged at 15,000 × *g* for 5 min, and stored at -20°C in 50 µl aliquots.

### 2.8.3 Western blot analysis

Proteins were separated by SDS-PAGE, and electroblotted onto nitrocellulose (Pall Life Sciences, MI., USA) at 300 mA for 1 h as described by Towbin *et al.* (1979). After transfer, the membrane was blocked in 5% (w/v) skim milk powder (Diploma) in TTBS (20 mM Tris-HCl, 154 mM NaCl, 0.5% (v/v) Tween-20, pH 7.4) for 20 min, with gentle agitation. The blocking solution was removed and the membrane probed with various polyclonal antisera at a dilution of 1/3000 in TTBS overnight at room temperature with gentle agitation. The filters were washed with TTBS 3 times for 10 min, before the addition of blotting grade goat anti-mouse IgG-Alkaline Phosphate conjugate (BioRad Laboratories, Hercules, CA., USA) at a dilution of 1/15 000 in TTBS, and incubated for 1 h at room temperature with gentle agitation. The membrane was washed 4 times for 5 min with TTBS, and then equilibrated in 15 ml DIG 3 (100 mM Tris-HCl, 100 mM NaCl, 50 mM $MgCl_2$, pH 9.5) for 2 min before addition of 45

µl DIG 4 (75 mg/ml nitroblue tetrazolium salt in 70% dimethylformamide), and 35 µl DIG 5 (50 mg/ml 5-bromo-4-chloro-3-indolylphosphate toluidinium salt in dimethylformamide). After the desired colour reaction had taken place, it was stopped by the addition of TE (10 mM Tris, 1 mM EDTA, pH 8.0). The membrane was then rinsed in water and dried.

### 2.8.4 Haemolysis assay

To assess the total haemolytic activity of cultures containing approximately $5 \times 10^7$ CFU *S. pneumoniae* D39, 10 ml cultures were grown in THY to an $A_{610}$ of 0.22, and pelleted at 2500 × *g* for 10 min. The pellet was resusupended in 1 ml of PBS with 0.1% (w/v) sodium deoxycholate (DOC), incubated at 37°C for 10 min to induce autolysis, and centrifuged for 3 min at 10,000 × *g*. Lysates were serially diluted 2-fold in PBS in 96-well plates (Sarstedt, South Australia); 100 µl of each dilution was added to an equal volume of a 3% washed human red blood cell (RBC) suspension in PBS. Trays were incubated at 37°C for 30 min, and then centrifuged at 2500 × *g* for 10 min. 50 µl of the supernatant was taken from each well and added to a new 96-well tray. The Absorbance of the wells was read at 570 nm, and the results graphed as average absorbance against dilution. The haemolytic activity was determined as the reciprocal of the dilution at which 50% of the RBCs had lysed. Haemolytic activity of purified Ply was determined using a similar method except that 1 µl of Ply, or dilutions thereof, was added to the RBCs instead of culture lysate.

### 2.8.5 LPS Removal from protein preparations with Polymyxin-B

Preparations of recombinant pneumococcal proteins were treated with Polymyxin-B in an attempt to remove contaminating LPS. 2 ml of a 50% slurry of Detoxi-Gel™ endotoxin removing gel (Pierce chemical company, IL USA) was packed into a column and allowed to settle for 30 min creating a 1 ml bed. The column was initially washed with 5 column volumes of 1% (w/v) sodium deoxycholate, followed by 5 column volumes of pyrogen free

PBS, using a peristaltic pump at a rate of 15 ml per h. 2.8 mg of protein in a total volume of 1 ml was added to the column followed by 200 µl pyrogen free PBS. Once the protein had entered the gel bed, the pump was switched off for at least 1 h to increase binding efficiency. The sample was eluted with pyrogen free PBS and 300 µl fractions were collected. Fractions were subjected to SDS-PAGE to locate the protein, and protein concentration was determined using the method of Bradford. Samples were then analysed for the presence of LPS at the Institute for Medical and Veterinary Science (IMVS) (Frome Rd Adelaide, Australia) using the Limulus Amoebocyte Lysate (LAL) assay.

## 2.9 DNA isolation and manipulation

### 2.9.1 Agarose gel electrophoresis

DNA was electrophoresed through horizontal agarose gels (0.8-2% (w/v) agarose dissolved in TBE buffer [44.5 mM Tris, 44.5 mM boric acid, 1.25 mM EDTA, pH 8.4]) immersed in TBE buffer containing ethidium bromide (100 µg in 2.5 l) at 140-180 V. Prior to loading DNA samples, a one tenth volume of tracker dye was added (15% (w/v) Ficoll, 0.1% (w/v) bromophenol blue, 100 ng/ml RNase A). DNA bands were visualised by short wave UV transillumination and images were captured by a Tracktel video imaging system attached to a Mitsubishi thermal printer. Approximate sizes of visualised fragments were calculated by comparison of their mobility with that of DNA size markers derived from *Eco*RI digested *Bacillus subtilis* bacteriophage SPP1 DNA (fragment sizes: 8.56, 7.43, 6.11, 4.90, 3.64, 2.80, 1.95, 1.88, 1.52, 1.41, 1.16, 0.99, 0.71, 0.49, 0.36, 0.08 kilobase pairs), or *Hpa*II digested pUC19 DNA (fragment sizes: 501, 404, 331, 242, 190, 147, 111, 110, 67, 34, 26 base pairs).

### 2.9.2 Pneumococcal chromosomal DNA isolation

Pneumococcal chromosomal DNA was isolated using the Wizard Genomic DNA purification kit (Promega Life Sciences, WI USA), according to the manufacturer's instructions, with the

exception that 0.1% (w/v) DOC was added to the pellet during the initial lysis step when resuspending in 200 µl 50 mM EDTA.

### 2.9.3 Plasmid miniprep

A 10 ml overnight culture of *E. coli* in LB was pelleted by centrifugation at 2880 × *g* for 10 min in a bench top centrifuge (Sigma). Plasmid DNA was isolated using the UltraClean Mini Plasmid Prep kit (Mo Bio Laboratories, CA USA) according to the manufacturer's instructions.

### 2.9.4 Restriction endonuclease digestion of DNA

Restriction endonuclease digestions of DNA were carried out in a volume of 20 µl. Each reaction contained approximately 1 µg DNA, and 1-2 units of the restriction enzyme(s), in the buffers recommended and supplied by the enzyme's manufacturer (Roche Diagnostics, Mannheim, Germany). Reactions took place at the manufacturer's suggested temperature overnight. Restriction digests were analysed using agarose gel electrophoresis.

### 2.9.5 DNA ligation

Ligation reactions were performed in a 2-step process. The first step took place in a final volume of 15 µl of 1 × ligation buffer (20 mM Tris-HCl, 10 mM MgCl2, 10 mM dithioerythritol, 0.6 mM ATP, pH 7.6). Each reaction contained ~3 units of T4 DNA ligase (Roche), and approximately 500 ng each of the vector and insert DNA. This mixture was incubated at 37°C for 1 h. Step 2 involved addition of a further ~2 units of T4 ligase, and increasing the reaction volume to 40 µl with 1 × ligation buffer. The ligation proceeded at room temperature overnight.

### 2.9.6 Polymerase Chain Reaction (PCR)

Standard PCR reactions were carried out on a thermal cycler (Hybaid), in a final volume of 50 µl, using Taq DNA polymerase according to the manufacturer's instructions (Roche diagnostics Pty Ltd).

High fidelity PCR reactions were carried out on a thermal cycler (Hybaid), in a final volume of 50 µl, using the Expand™ Long Template PCR System (Roche). Each reaction contained approximately 100 ng of the DNA template, 1-2 units of the Expand™ DNA polymerase, 4 µM of each oligonucleotide primer, 200 µM of each dNTP in 1 × Expand PCR buffer 3. Reaction conditions comprised 30 cycles as follows: Denaturation at 94°C for 1 minute; annealing at 60°C for 1 minute; and extension at 68°C for various times depending on the length of the product (approximately 1 minute for each kb of product).

Overlap extension PCR was carried out on a thermal cycler (Hybaid), in a final volume of 50 µl using the Expand™ Long Template PCR System (Roche). Each reaction contained approximately 100 ng of the DNA templates, 1-2 units of the Expand™ DNA polymerase, 4 µM of each oligonucleotide primer, 200 µM of each dNTP in 1 × Expand PCR buffer 3. The reaction conditions were as follows: Denaturation at 94°C for 2 min, followed by 2 cycles of denaturation at 92°C for 30 sec, annealing at 40°C for 1 min, and extension at 68°C for 7 min, 30 sec, and then 33 cycles of denaturation at 92°C for 30 sec, annealing at 55°C for 1 min, extension at 68°C for 7 min, 30 sec, with a final extension at 68°C for 8 min.

### 2.9.7 Purification of PCR product

PCR products were purified using the UltraClean PCR Clean-up DNA Purification Kit (Mo Bio Laboratories, Solana Beach, CA, USA) according to the manufacturer's instructions.

### 2.9.8 Cloning of PCR product

For cloning, PCR products were directly ligated into pGEM®-T Easy as described in the manufacturer's specifications (Promega). Briefly, the ligation mixture consisted of the purified PCR product (Section 2.9.7), 50 ng pGEM®-T Easy vector DNA, 3 U T4 DNA ligase and 1 × ligation buffer, in a total volume of 10–15 µl. The reaction mixture was incubated overnight at 4°C. Ligation mixtures were then transformed into *E. coli* DH5α as described in Section 2.11

### 2.9.9 DNA sequencing and analysis

DNA sequencing reactions were carried out using the method described in the PRISM™ Dye Primer Cycle Sequencing Ready Reaction Kit manual (Applied Biosystems, Foster City, CA., USA). Each reaction contained double stranded DNA template (50-200 ng), 4 µl Big Dye terminator mix, and 3.2 pmol of a single primer, made up to a final reaction volume of 20 µl with sterile water. Sequencing reactions were performed on a thermal cycler (Hybaid) under the following conditions: heat denaturation at 95°C for 30 sec, primer annealing and extension at 60°C for 4 min for 25 cycles. When the reaction was completed, the dye terminator labelled-DNA was precipitated with 80 µl of 75% (v/v) isopropanol and 20 µg of glycogen (Roche). The mixture was left at RT for 2 h, after which the precipitate was pelleted by centrifugation at 14,900 × *g* for 30 min. The pellet was subsequently washed with 250 µl of 70% (v/v) ethanol and centrifuged for a further 5 min at 14,900 × *g* at 4°C. The DNA pellets were then dried at 65°C for 10 min. Sequencing was performed by the Molecular Pathology Unit at the IMVS, Adelaide, S.A., Australia using an Applied Biosystems 3700 DNA sequencing analyser. DNA sequence data were analysed using the DNAMAN program (Lynnon Biosoft®, Vaudreuil, Quebec, Canada), and BLAST searches were used to identify homologies with sequence data from the National Center for Biotechnology Information (Bethesda, MD, USA [www.ncbi.nlm.nih.gov/blast]) (Altschul *et al.*, 1997).

## 2.10 RNA isolation and manipulation

### 2.10.1 RNA extraction from eukaryotic cells

Whole cellular RNA was extracted using TRIZOL® reagent according to the manufacturer's instructions (Invitrogen Life Technologies). Briefly, 1 ml TRIZOL® reagent was added directly to cell monolayers and the cells suspended by passing through a pipette several times. The homogenised samples were incubated for 5 min to permit the complete dissociation of nucleoprotein complexes, before the addition of 200 µl chloroform. The mixture was agitated for 15 sec then centrifuged at 12,000 × *g* for 15 min. After centrifugation the aqueous phase containing the RNA was transferred to a clean reaction tube and RNA was precipitated by the addition of 500 µl isopropanol and incubation at RT for 10 min. RNA was pelleted by centrifugation at 12,000 × *g* for 10 min and the pellet washed with 1 ml 75% ethanol for 5 min at 7500 × *g*. The pellet was then air dried for 10 min and resuspended in 45 µl RNase free water (Promega) at 58°C for 10 min to facilitate resuspension. RNA was reprecipitated by the addition of 1/10 volume sodium acetate (pH 4.8) and 2 volumes absolute ethanol and incubation at –80°C overnight. The following day, RNA was pelleted by centrifugation at 12,000 × *g* for 30 min at 4°C, washed in 75% (v/v) ethanol, and resuspended in nuclease-free water. RNasin® Ribonuclease Inhibitor was added to RNA, and contaminating DNA was digested with RQ1 RNase-free DNase, followed by DNase stop solution, according to the manufacturer's instructions (Promega).

### 2.10.2 Reverse transcription-PCR (RT-PCR)

RT-PCR was performed using the One-step Access RT-PCR system (Promega) according to the manufacturer's instructions. Briefly, each reaction was performed in a final volume of 25 µl, containing 1 × AMV/*Tfl* reaction buffer, 0.2 mM dNTPs, 20 nmol of each oligonucleotide, 1 mM $MgSO_4$, 2.5 U AMV reverse transcriptase, 2.5 U *Tfl* DNA polymerase, and 1 ng to 1

μg RNA. Reactions were performed on a Hybaid PCR Sprint thermal cycler and included the following steps: 45 min of reverse transcription at 48°C, followed by 2 min denaturation at 94°C and 30 cycles of amplification (denaturation at 94°C for 30 sec, annealing at 56–60°C for 30 sec and extension at 72°C for 45 sec), unless indicated otherwise. The absence of DNA contamination in all RNA preparations was confirmed by RT-PCR analysis using primers specific for the gene encoding the house-keeping enzyme glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Table 2.2). The gene encoding GAPDH contains an intron such that mRNA template directs amplification of a 239-bp product, whereas chromosomal DNA template directs amplification of a 341-bp product. The absence of DNA contamination was determined by electrophoresis of 10 μl of each PCR product on a 2% (w/v) agarose gel.

### 2.10.3 Real-time reverse transcription-PCR

Oligonucleotide primer pairs used are specified in Table 2.2. RT-PCR was performed using the one-step access RT-PCR system (Promega) according to the manufacturer's instructions. Each reaction was performed in a final volume of 20 μl, essentially as described in Section 2.10.2, but also containing a 1/20,000 dilution of SYBR® Green I nucleic acid stain. The quantitative RT-PCR was performed on a Rotorgene RG-2000 cycler (Corbett Research, Mortlake, N.S.W., Australia) and included the following steps: 45 min of reverse transcription at 48°C, followed by 2 min denaturation at 94°C and 40 cycles of amplification (denaturation at 94°C for 30 sec, annealing at 56–60°C for 30 sec and extension at 72°C for 45 sec), unless indicated otherwise. Each RNA sample was assayed in triplicate using primers specific for the various chemokine mRNAs, or mRNA for GAPDH, which was used as an internal control.

Results were calculated using the comparative cycle threshold ($2^{\Delta\Delta Ct}$) method (User Bulletin no.2 [http://docs.appliedbiosystems.com/pebiodocs/04303859.pdf]; Applied

Biosystems), in which the amount of target mRNA is normalized to a reference (0 h control) relative to an internal control (GAPDH mRNA). Results are expressed as relative changes in chemokine mRNA levels compared to 0 h control levels. Standard deviations (SD) were initially determined as the $\sqrt{((\text{SD sample})^2 + (\text{SD GAPDH})^2)}$, and this was then applied to the formulas: $SD+ = 2^{\Delta\Delta CT-SD} - 2^{\Delta\Delta CT}$ and $SD- = 2^{\Delta\Delta Ct} - 2^{\Delta\Delta CT+SD}$.

## 2.11 Transformation of *E. coli*

### 2.11.1 Preparation of competent cells

*E. coli* cells were grown at 37°C to an $A_{600}$ of 0.4, then centrifuged for 10 min at 2880 × *g* at 4°C. The pellet was then resuspended in 0.1 M $MgCl_2$, and placed on ice for 10 min before pelleting at 2880 × *g* for 10 min at 4°C. The pellet was then resuspended in a one fifth volume of 0.1 M $CaCl_2$, and placed on ice for 90 min. Competent cells were stored at -80°C in 100 µl aliquots.

### 2.11.2 Transformation of *E. coli*

Transformation of *E. coli* involved adding ~1 µg DNA directly to competent cells, and leaving on ice for 30 min. Cells were then heat-shocked at 42°C for 2 min, and placed on ice for a further 30 min. 1 ml LB was then added and after shaking for 60 min at 37°C, the culture was pelleted, resuspended in 200 µl LB, and plated on LB agar plates with the appropriate antibiotic selection.

## 2.12 Transformation of *S. pneumoniae*

### 2.12.1 Preparation of competent cells

Pneumococcal cells were grown in c-CAT media (10 g/l Casamino acids [Difco Bacto], 5 g/l Tryptone [Difco Bacto] 5 g/l NaCl, 10 g/l Yeast Extract, 4%(v/v) 0.4 M $K_2HPO_4$, 0.002%

(w/v) glucose, 15 mg glutamine) to an $A_{600}$ of 0.25-0.3, then diluted to an $A_{600}$ of 0.01 in 10 ml CTM media (c-CAT with 0.2% (w/v) BSA, 1% (v/v) 0.1 M NaOH) and grown to an $A_{600}$ of 0.1. The cells were then pelleted in a bench centrifuge (Heraeus biofuge 13 centrifuge, 10 min, 13 000 rpm), resuspended in 812 µl CTM-pH 7.8 and 188 µl 80% glycerol, and stored at –80°C in 100 µl aliquots.

### 2.12.2 Transformation of pneumococci

500 µl of CTM-pH 7.8 and 30 ng competence stimulating peptide-1 (CSP-1) (Chiron-tech) were added to an aliquot of competent cells. The cells were then incubated at 37°C for 5 min before the addition of 1–100 ng of DNA, and incubated at 32°C for 30 mins and then at 37°C for 2-4 h. After incubation, cells were plated onto BA plates containing 0.2 µg/ml Ery (EryBA), and incubated overnight at 37°C in the presence of 5% $CO_2$.

### 2.12.3 Back transformation of pneumococci

500 µl of CTM-pH 7.8 and 30 ng CSP-1 were added to an aliquot of competent cells. The cells were then incubated at 37°C for 20 min before addition of 1–100 ng DNA and incubated at 32°C for 30 min then at 37°C for 2 h. The total volume of cells was split between 2 tubes and 5 µl Ery (0.01 mg/ml) was added to each tube before incubation at 37°C for 30 min. 50 µl Amp (25 mg/ml) was then added and each tube incubated for 4 h at 37°C. Cells were then pelleted by centrifugation and washed 2 times in CTM before finally resuspending in 1 ml CTM. 100 µl of a 1/1000 dilution was then plated onto BA plates and incubated overnight at 37°C in the presence of 5% $CO_2$. Colonies were replica plated onto BA and EryBA plates to identify $Ery^S$ back-transformants.

## 2.13 IL-8 Enzyme Linked Immunosorbent Assay (ELISA)

Levels of IL-8 protein in cell culture supernatant were measured using a commercial sandwich enzyme linked immunosorbent assay (ELISA) kit (R&D Systems Inc.). Each well of a 96 well tray (Maxisorp Nunc-immuno plates, Nunc, Roskilde, Denmark) was coated with 200 ng of the capture antibody (monoclonal anti-human IL-8 antibody) diluted in PBS RT overnight. Plates were washed 3 times with ELISA wash buffer (0.05% (v/v) Tween 20 in PBS pH 7.4) using an ELx50 automatic strip washer (Bio-Tek instruments Inc., Winooski, VA, USA.) (all wash steps were performed in this manner). Wells were then blocked by addition of 300 µl blocking buffer (1% (w/v) BSA, 5% (w/v) sucrose, and 0.05% (w/v) $NaN_3$ in PBS) and incubated at RT for 1 h before washing 3 times. 100 µl of culture supernatant or IL-8 standard (R&D Systems) diluted in ELISA diluent (0.1% (w/v) BSA, 0.05% (v/v) Tween-20 in Tris buffered saline (TBS) [20 mM Trizma base, 150 mM NaCl pH 7.3]) was added to the wells, the plate was gently tapped for 1 min to ensure thorough mixing of the reagents and incubated at RT for 2 h before washing 3 times. 100 µl of 20 ng/ml biotinylated goat anti-human IL-8 polyclonal detection antibody diluted in ELISA diluent was added to each well and the plate incubated for 2 h at RT before washing a further 3 times. To each well, 100 µl of a 1/10,000 dilution of Horseradish peroxidase-conjugated streptavidin diluted in TBS + 0.1% (w/v) BSA was added, the plate was incubated at RT for 20 min, and then washed 4 times. 100 µl substrate solution (1 tablet each of the o-phenylenediamine, and dihydrochloride tablet set (Sigma) in 20 ml water) was added to each well and incubated in the dark for 30 min at RT. The reaction was stopped by the addition of 50 µl stop solution (1 M $H_2SO_4$) and gently tapping the plate to ensure thorough mixing. The colour reaction was measured by determining the $A_{450}$ using correction at $A_{570}$ on an ELISA plate reader (Dynatec MR5000).

## 2.14 Infection of epithelial cell monolayers with *S. pneumoniae* D39

For chemokine assays, A549 cells or Detroit-562 cells were seeded in 6-well tissue culture trays and allowed to attach overnight. Cells were used at 90–100% confluence. Cells were washed twice with PBS, 1 ml of the appropriate culture medium (without antibiotics or FCS) was added to each well, and cells were left to rest in air at 37°C for A549, or at 37°C in 95% air/5% $CO_2$ for Detroit-562 for at least 2 h. *S. pneumoniae* from overnight plates was inoculated into 10 ml THY medium and grown to an $A_{600}$ of 0.15. *S. pneumoniae* cultures were then pelleted at 2500 × *g* for 10 min in a benchtop centrifuge, and the pellets resuspended in 2 ml of the appropriate cell culture medium without FCS or antibiotics to remove residual THY before pelleting again. Pellets were then resuspended in 1.5 ml of cell culture medium and 100 µl of this suspension (approximately $5 \times 10^7$ CFU) was used to infect cell culture monolayers. 2 and 4 h control cells received 100 µl cell culture medium in place of *S. pneumoniae* culture, with the 0 h control receiving nothing. Cell monolayers were then incubated in air at 37°C for A549 cells, or 37°C in 95% air/5% $CO_2$ for Detroit-562 cells for 2 or 4 h, at which time the supernatant was collected and stored at –20°C for analysis by IL-8 ELISA (Section 2.13), and the monolayer was lysed in 1 ml TRIzol® Reagent for RNA extraction (Section 2.10.1). Samples were also collected at 0 h to determine baseline chemokine expression in A549 or Detroit-562 cells before stimulation with *S. pneumoniae*.

# Chapter Three - Characterisation of Respiratory Epithelial Cell CXC Chemokine Responses to *Streptococcus pneumoniae*

## 3.1 Introduction

There is growing evidence that aspects of the immune response contribute to the high mortality rate seen with pneumococcal pneumonia. In mice, death from pneumococcal infection is associated with a severe inflammatory response involving the influx of leukocytes, in particular neutrophils, to the site of infection (Kadioglu *et al.*, 2000; Dallaire *et al.*, 2001). The presence of the pneumococcus and the resulting inflammatory response causes damage to the host epithelium, breaking down the tight junctions between cells and enabling the pneumococcus to infiltrate the host. The extensive tissue damage resulting from this inflammatory response ultimately proves detrimental to the host and is responsible for the mortality seen in pneumococcal pneumonia (Kadioglu *et al.*, 2000). CXC chemokines are important mediators of inflammation, responsible for recruiting neutrophils to the site of infection. Prolonged increases in levels of the chemokines MCP-1, MIP-1α, GROα, and MIP-2 in the lungs of mice have been linked to a detrimental outcome of infection (Dallaire *et al.*, 2001), with mice that showed sustained increases in levels of these chemokines later dying of infection, while mice in which levels of these chemokines quickly returned to normal after a sharp increase, generally went on to recover from infection. In human infection with *S.*

*pneumoniae*, increases in TNF-α, IL-1β, IL-6, and IL-10 levels are typically seen in the blood upon admission to hospital (Bruunsgaard *et al.*, 1999). Elderly patients generally show higher levels of these molecules compared to younger patients, and these levels remain elevated for a longer period of time. This prolonged expression of pro- and anti- inflammatory cytokines is indicative of a long-lasting inflammatory response, possibly due to an inability to control the infection in the early stages, and may be associated with the increased morbidity that is associated with pneumococcal infections in the elderly.

It is now understood that epithelial cells are able to control the inflammatory response by secreting a variety of cytokines (Simon and Paine, 1995; Yang *et al.*, 2002; Yang *et al.*, 2003). Type II pneumocytes, and nasopharyngeal epithelial cells have been shown to express a number of cytokines important in the recruitment of inflammatory cells and modulation of their activity. These epithelial cells can produce CXC chemokines that may be important in pneumococcal infection, such as MIP-2α, MIP-2β, and IL-8, and their release could be elicited by specific pneumococcal factors.

To date, the majority of studies into the inflammatory response generated towards pneumococcal infection have been performed using animal models, and while these have proven useful in providing information on the effect of this inflammation on pathogenesis and the outcome of pneumococcal infection, the insight that they provide into the situation that occurs in humans is limited, because although there are some murine analogues of human chemokines, mice are not the natural host of *S. pneumoniae* and thus may respond differently to infection. This study utilises human epithelial cell lines as an *in vitro* model to examine the chemokine response that might occur in humans upon infection with *S. pneumoniae*. Another question that remains unanswered regarding pneumococcal infection is whether there is a difference in the chemokine response elicited by the pneumococcus in the nasopharynx when compared to the response that occurs in the lungs. It is possible that the differences in the behaviour of the pneumococcus in these two host niches, (asymptomatic colonisation vs.

invasive disease), may be a consequence of differences in the way the different cell types react to the presence of the pneumococcus. Both of these host sites are complex environments with multiple cell types present, many of which probably contribute to the inflammatory response to pneumococcal infection. However, it has been shown that epithelial cells are able to contribute to the inflammatory response by releasing chemokines (Simon and Paine, 1995), and there may be differences in the way that the epithelial cells of these two host sites react to pneumococcal infection. The use of two different cell lines, A549 (type II pneumocytes) and Detroit-562 (nasopharyngeal epithelial cells), will enable differences in the CXC chemokine response generated by epithelial cells at these two sites to be examined. Unlike epithelial tissues which are polarised, these cell-lines are unpolarised. Nevertheless, they are an established *in vitro* model for epithelial cells, and have been used previously to measure chemokine responses to infection (Yang *et al.,* 2002; Yang *et al.,* 2003).

In this chapter, procedures for the detection and quantitation of chemokine mRNA and IL-8 protein production by A549 and Detroit-562 cells in response to infection with live *S. pneumoniae* were developed. These were used to quantitate the relative levels of IL-8, ENA-78, GCP-2, MGSA, MIP-2α and MIP-2β mRNA in cells infected with *S. pneumoniae* D39, relative to uninfected control cells, using the mRNA for the housekeeping gene GAPDH as an internal standard. IL-8 protein production by *S. pneumoniae*-infected cells was also assessed to confirm that an increase in IL-8 mRNA results in an increase in secretion of the chemokine itself.

## 3.2 Results

### 3.2.1 Determination of optimal dose of *S. pneumoniae* infection

To determine the maximum dose of live *S. pneumoniae* that could be administered to cells without damaging or killing them, confluent monolayers of A549, and Detroit-562 cells

were incubated with *S. pneumoniae* D39 at doses ranging from $1 \times 10^7$ to $1 \times 10^8$ CFU per well (a ratio of approximately 10 to 100 bacteria per cell), and cell morphology was examined at 1, 2, 3, and 4 h at 400 × magnification. Results obtained are summarised in Table 3.1, and the maximum dose compatible with cell survival was found to be between $4 \times 10^7$ and $6 \times 10^7$ CFU per well.

Viability at 2 and 4 h was also assessed by Trypan blue exclusion as described in Section 2.4. After exposure to $5 \times 10^7$ CFU *S. pneumoniae* D39 for 4 h, cell viability was still >95%, confirming that this dose is not lethal during this time frame. Thus in all future experiments, cells were incubated with approximately $5 \times 10^7$ CFU *S. pneumoniae.*

| *S. pneumoniae* D39 CFU | Time (hours) | | | |
|---|---|---|---|---|
| | 1 | 2 | 3 | 4 |
| Media (control) | +++ | +++ | +++ | +++ |
| $1 \times 10^7$ | +++ | +++ | +++ | +++ |
| $4 \times 10^7$ | +++ | +++ | +++ | +++ |
| $6 \times 10^7$ | +++ | +++ | ++ | + |
| $8 \times 10^7$ | +++ | ++ | + | - |
| $1 \times 10^8$ | +++ | ++ | +/- | - |

**Table 3.1 Integrity of cell monolayers after incubation with *S. pneumoniae.*** Cell monolayers were incubated with indicated numbers of *S. pneumoniae* for 1 – 4 h and monolayer integrity scored, such that an intact monolayer scored +++, and complete disruption of the monolayer scored –.

The growth of *S. pneumoniae* D39 in wells under these conditions was also assessed by plating out serial dilutions at each time point and determining the viable count. It was found that numbers of viable bacteria (CFU) remained constant throughout the experiment (data not shown).

The viability of Detroit-562 cells under the same conditions was assessed and it was found that incubation with $5 \times 10^7$ CFU for up to 4 h did not affect viability of these cells (data not shown). Accordingly, this dose was used for all experiments with Detroit-562 cells.

### 3.2.2 Determination of the optimal timepoints of infection

In order to determine the optimal timepoints at which to collect cell culture supernatant and extract cellular RNA for analysis, A549 monolayers were incubated with $5 \times 10^7$ CFU *S. pneumoniae* D39, or DMEM (control cells) for 1, 2, 3, 4, or 6 h, at which time cellular RNA was extracted and analysed for the presence of IL-8 mRNA by real time RT-PCR, as described in Sections 2.10.1, and 2.10.3. Culture supernatant was also collected and analysed for IL-8 protein by ELISA as described in Section 2.13. RNA extracts and supernatant from uninfected cells at 0 h were also analysed for IL-8 mRNA and IL-8 protein to determine basal expression levels in A549 cells.

The greatest increases in IL-8 mRNA expression were observed at 2 and 4 h (Figure 3.1 A). Levels of IL-8 protein in the supernatant were too low to detect at 1, 2, and 3 h, while similar levels were seen at 4 and 6 h (Figure 3.1 B). Thus, in all future experiments RNA was collected from infected and control cells at 2 and 4 h. Supernatant was also collected from cells at 4 h and analysed by ELISA.

Experiments with Detroit-562 cells showed that these timepoints were also optimal for this cell line (data not shown), and so in all experiments with these cells RNA was collected at 2 and 4 h, and cell culture supernatants were collected at 4 h.

### 3.2.3 CXC chemokine mRNA response of A549 cells to *S. pneumoniae* D39

Initial experiments involved examining the induction of a range of CXC chemokines in A549 cells by infection with the serotype 2 *S. pneumoniae* strain D39. Confluent monolayers of A549 cells were incubated with approximately $5 \times 10^7$ CFU of *S. pneumoniae* for 2 or 4 h after which total cellular RNA was extracted as described in Section 2.10.1 and analysed for the presence of mRNA specific for IL-8, ENA-78, GCP-2, MGSA, MIP-2α, and MIP-2β by real-time RT-PCR, as described in Section 2.10.3 (Figure 3.2). Increases of approximately 5-fold in mRNA levels for IL-8, MIP-2α, and MIP-2β were seen in response to

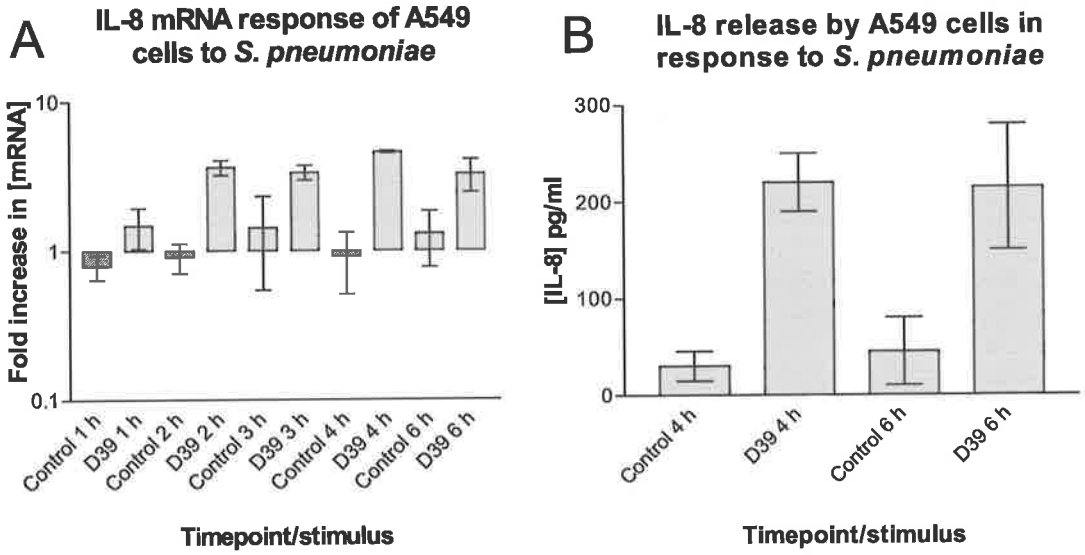


**Figure 3.1. IL-8 mRNA and protein expression after incubation with *S. pneumoniae* D39.** A549 cell monolayers were incubated with 5 × $10^7$ CFU *S. pneumoniae* D39 for the times indicated before extraction of cellular RNA and analysis for IL-8 mRNA expression by real-time RT-PCR (A), and IL-8 secretion by ELISA of cell culture supernatant (B). Data shown are mean ± SE from 2 experiments.

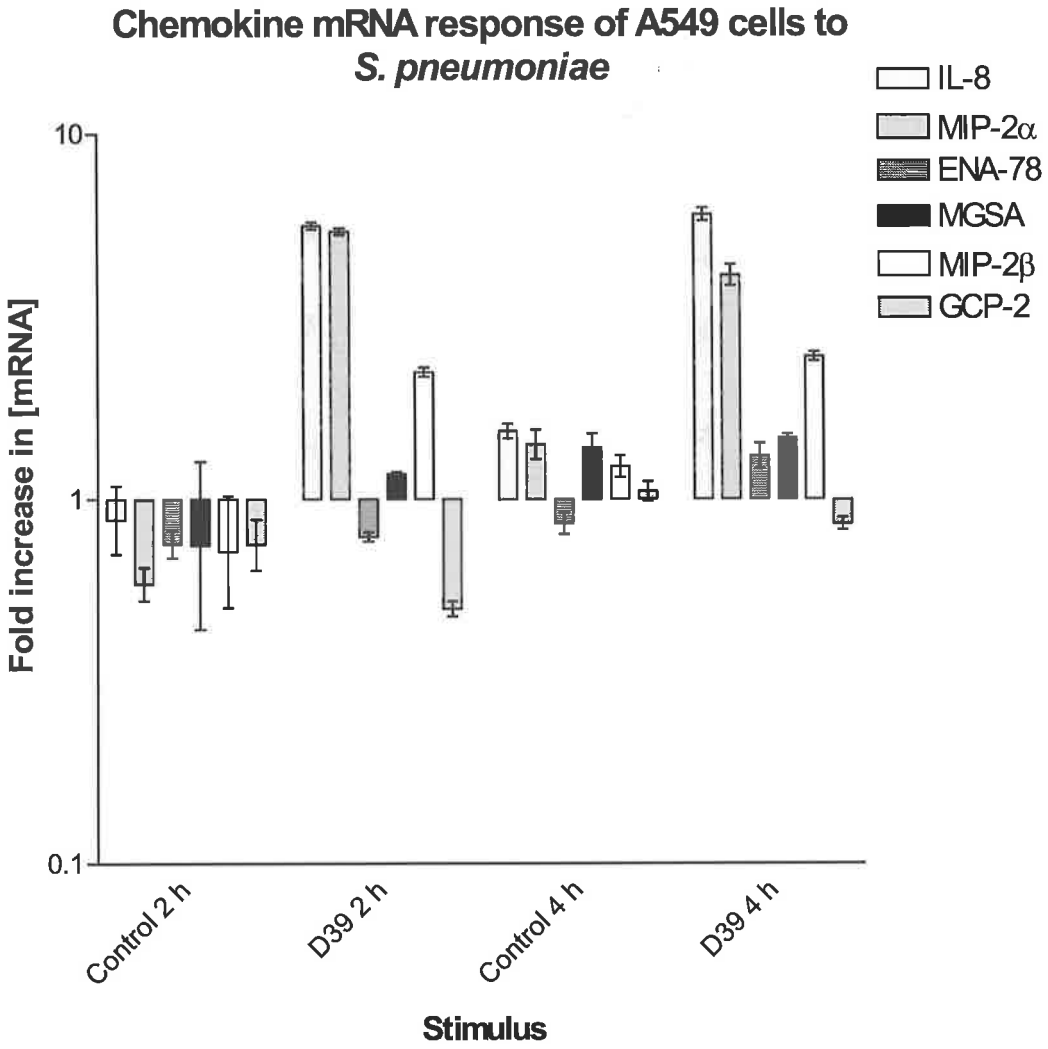


**Figure 3.2. CXC chemokine mRNA response of type II pneumocytes after infection with *S. pneumoniae* D39.** Confluent monolayers of A549 cells were infected with $5 \times 10^7$ CFU *S. pneumoniae* D39 for 2 or 4 h at which times cellular RNA was extracted and analysed by real-time RT-PCR using oligonucleotides specific for IL-8, MIP-2α, ENA-78, MGSA, MIP-2β, and GCP-2. GAPDH mRNA was used as an internal control, and results are expressed as fold increase in mRNA at 2 or 4 h relative to an uninfected 0 h control.

D39 after 2 h when compared to control cells. At 4 h post-infection, there was a 5-fold increase in IL-8 mRNA, a 4-fold increase in MIP-2α mRNA, and a 2-fold increase in MIP-2β mRNA compared to uninfected control cells. No obvious increases were seen in mRNA for ENA-78, GCP-1, or MGSA at either 2 or 4 h post-infection (Figure 3.2). These were pilot experiments so no statistical analysis was performed.

### 3.2.4 Secretion of IL-8 by type II pneumocytes in response to infection with *S. pneumoniae*

To confirm that an increase in IL-8 mRNA also resulted in an increase in IL-8 protein production by the cell, cell culture supernatant was collected at 4 h post infection and analysed by ELISA for IL-8 concentration as described in Section 2.13. It was found that the concentration of IL-8 in culture supernatant was increased approximately 6-fold in cells exposed to *S. pneumoniae* D39 when compared to control cells that were exposed to cell culture media alone ($P < 0.05$) (Figure 3.3).

### 3.2.5 CXC chemokine response of Detroit-562 cells to infection with *S. pneumoniae* D39

The nasopharyngeal epithelial cell line Detroit-562 was employed to model the chemokine response elicited by *S. pneumoniae* in the nasopharynx, and to determine whether there are any differences in the responses relative to the lung cells. Confluent monolayers of Detroit-562 cells were incubated with $5 \times 10^7$ CFU *S. pneumoniae* D39, or MEM in the case of control cells. Total cellular RNA was extracted at 2 and 4 h and analysed by real-time RT-PCR for the presence of mRNA for IL-8, ENA-78, GCP-2, MGSA, MIP-2α, and MIP-2β, as described in Sections 2.10.1, and 2.10.3. RNA was also extracted from uninfected cells at 0 h to determine baseline levels of mRNA for these chemokines in this cell line. After infection with *S. pneumoniae* D39, IL-8 mRNA was increased approximately 8-fold at 2 h, and approximately 60-fold at 4 h, compared to uninfected control cells. MIP-2α mRNA was

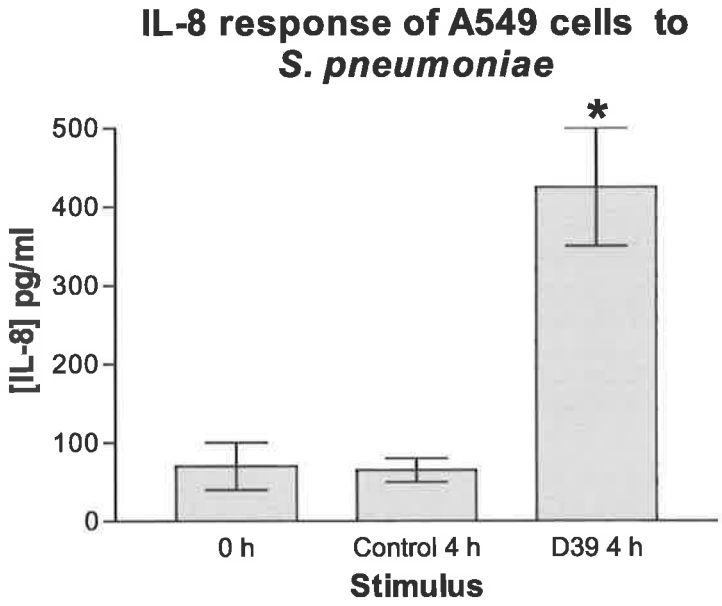


**Figure 3.3. IL-8 secretion by type II pneumocytes after infection with *S. pneumoniae*.** A549 monolayers were incubated with $5 \times 10^7$ CFU *S. pneumoniae* D39 for 4 h before collection of culture supernatant and analysis for IL-8 protein by ELISA. The experiment was performed in triplicate and mean ± SE shown. Data were analysed for statistical significance by one-way ANOVA with post-hoc Bonferroni test. * $P < 0.05$, compared to control cells.

increased approximately 8-fold and 70-fold compared to uninfected control cells at 2 and 4 h respectively. MIP-2β mRNA was increased approximately 4 and 29-fold at 2 and 4 h respectively, and MGSA mRNA levels were increased approximately 3 and 10-fold after incubation with *S. pneumoniae* D39 for 2 and 4 h respectively compared to uninfected control cells. Levels of mRNA for ENA-78 and GCP-2 were unaffected by infection with *S. pneumoniae* D39, with no considerable difference seen between infected cells and control cells (Figure 3.4). These were pilot experiments, so no statistical analyses were performed.

### 3.2.6 Secretion of IL-8 protein by nasopharyngeal epithelial cells in response to infection with *S. pneumoniae* D39

To confirm that an increase in chemokine mRNA was accompanied by an increase in chemokine production, cell culture supernatant was collected after infection with $5 \times 10^7$ CFU *S. pneumoniae* D39 for 4 h, and analysed for the presence of IL-8 by ELISA. Levels of IL-8 protein secreted by nasopharyngeal epithelial cells after exposure to *S. pneumoniae* were increased approximately 5-fold when compared to uninfected control cells ($P < 0.05$) (Figure 3.5).

## 3.3 Discussion

In this chapter, assays were developed to investigate the CXC chemokine responses of respiratory epithelial cells to infection with *S. pneumoniae* using type II pneumocyte (A549) and nasopharyngeal (Detroit-562) cell lines.

Initial experiments indicated that both type II pneumocytes and nasopharyngeal epithelial cells increase CXC chemokine production in response to infection with the type 2 *S. pneumoniae* strain D39. Cell viability studies showed that cells were not damaged by infection with *S. pneumoniae* at the doses and incubation times used in this study. Thus the chemokine response seen reflects an inflammatory response generated towards the presence of the bacteria. Type II pneumocytes showed a moderate (5-fold) increase in mRNA levels

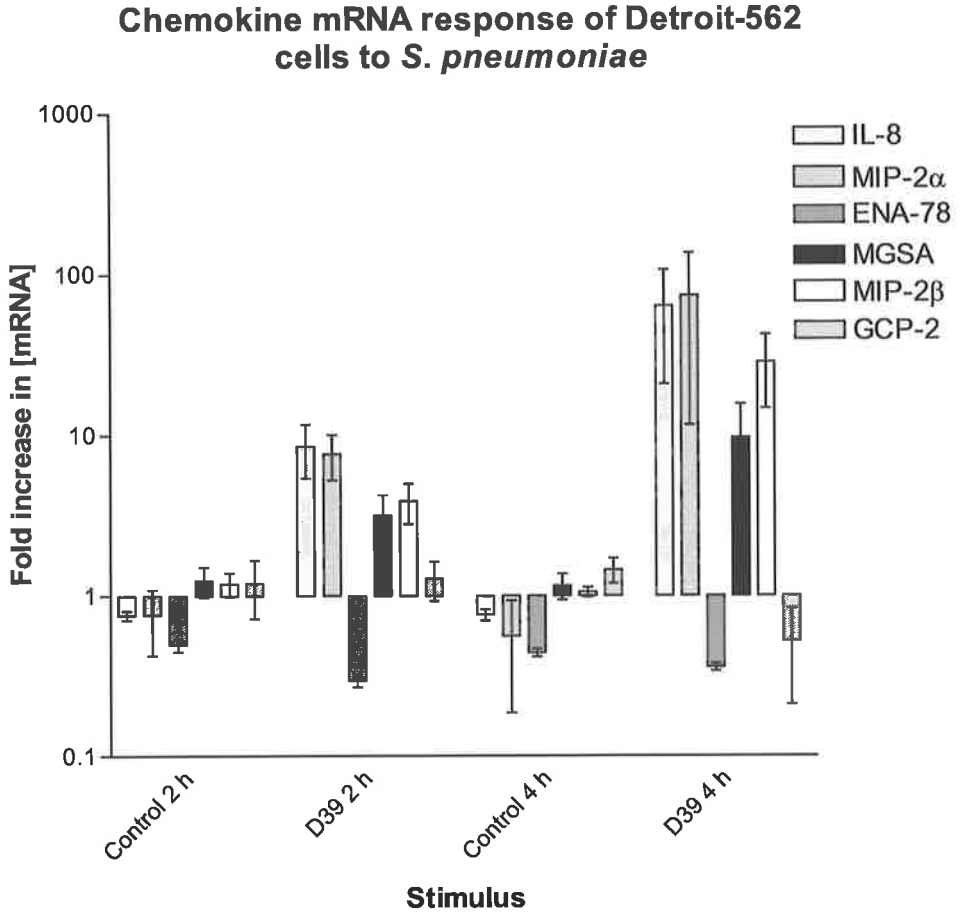


**Figure 3.4. CXC chemokine response of nasopharyngeal epithelial cells after infection with *S. pneumoniae* D39.** Confluent monolayers of Detroit-562 cells were infected with $5 \times 10^7$ CFU *S. pneumoniae* D39 for 2 or 4 h at which time cellular RNA was extracted and analysed by real-time RT-PCR using oligonucleotides specific for IL-8, MIP-2α, MIP-2β, MGSA, GCP-2, and ENA-78. GAPDH mRNA was used as an internal control, and results are expressed as fold increase in mRNA at 2 or 4 h relative to an uninfected 0 h control.

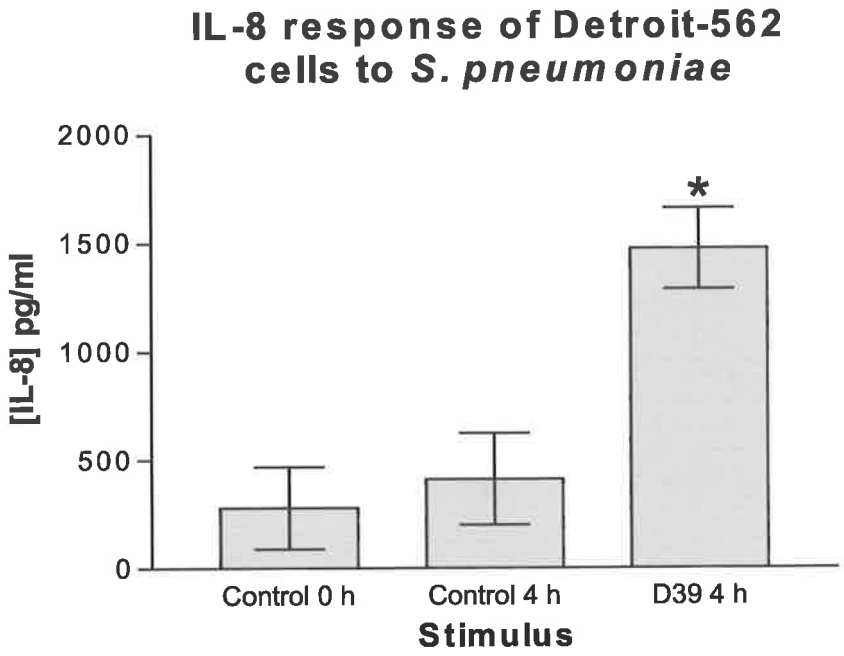


**Figure 3.5. IL-8 secretion by nasopharyngeal epithelial cells after infection with *S. pneumoniae*.** Detroit-562 monolayers were incubated with $5 \times 10^7$ CFU *S. pneumoniae* D39 for 4 h before collection of culture supernatant and analysis for IL-8 protein by ELISA. The experiment was performed in triplicate and mean ± SE shown. Data were analysed for statistical significance by one-way ANOVA and post-hoc Bonferroni test. * $P < 0.05$.

for IL-8, MIP-2α, and MIP-2β at both 2 and 4 h post infection, when compared to control cells, but no increase was seen in mRNA levels of ENA-78, MGSA, or GCP-2 (Figure 3.2). IL-8 protein release into the cell culture supernatant was also significantly increased compared to control cells (Figure 3.3). This suggests that the CXC chemokines IL-8, MIP-2α, and MIP-2β may be part of the lung cell response to pneumococcal infection, and are likely to play a part in recruiting neutrophils to this site of infection. While ENA-78, MGSA, and GCP-2 appeared to play little or no role in the response of epithelial cells to pneumococcal infection of the lungs, this does not rule out the possibility that they are released by other cell types present in the lungs, and therefore still play a role in response to pneumococcal infection at this site. Nasopharyngeal epithelial cells showed a response that was similar to A549 cells upon infection with *S. pneumoniae*. As was seen for A549 cells, Detroit-562 cells showed an increase in mRNA for IL-8, MIP-2α, and MIP-2β, but also showed an increase in mRNA for MGSA (Figure 3.4). Thus MGSA may have a role in the response of nasopharyngeal epithelial cells, but not type II pneumocytes to *S. pneumoniae* infection. An increase in mRNA levels for IL-8 correlated with an increase in the secretion of IL-8 by this cell line (Figure 3.5), demonstrating that at least in the case of IL-8, an increase in cellular chemokine mRNA correlates with an increase in secretion of the chemokine itself.

Overall, the response of Detroit-562 cells to infection with *S. pneumoniae* appeared to be much greater than that of A549 cells. Levels of IL-8 released by these cells were approximately 3 times greater than those released by A549 cells. However, these cells also had a higher basal expression level of IL-8, so the relative increase in expression upon infection with *S. pneumoniae* was similar between the 2 cell lines.

These experiments have shown that both type II pneumocytes and nasopharyngeal epithelial cells respond to infection with live *S. pneumoniae* by upregulating CXC chemokine expression, in particular that of IL-8, MIP-2α, and MIP-2β. Some caution needs to be exercised when interpreting the results obtained above, as A549 and Detroit-562 are

transformed, unpolarised epithelial cell lines. Nevertheless, results are consistent with previous *in vivo* studies that show that there is an increase in pro-inflammatory mediators in the lungs of mice following infection with *S. pneumoniae* (Bergeron *et al.,* 1998; Dallaire *et al.,* 2001; Kerr *et al.,* 2002). The specific pneumococcal factors responsible for this response remain to be elucidated, and investigation of these factors is the subject of the next chapter.

# Chapter Four – Role of Pneumococcal Proteins in Respiratory Epithelial Cell CXC Chemokine Responses

## 4.1 Introduction

In Chapter Three, assays were developed to measure the chemokine response of respiratory epithelial cells to infection with *S. pneumoniae*. In particular, mRNA levels of CXC chemokines were measured using real-time RT-PCR, and levels of secreted IL-8 were measured by ELISA. Infection with wild-type *S. pneumoniae* elicited a chemokine response from both nasopharyngeal epithelial cells and type II pneumocytes, but the specific pneumococcal factors responsible for this response remain to be elucidated. Pneumococcal proteins, and in particular the surface proteins CbpA and PspA, and the toxin Ply have an established role in virulence, as described in Chapter One (Section 1.6). CbpA has most commonly been described as an adhesin, promoting adherence to host cells by interacting with the polymeric immunoglobulin receptor (pIgR) (Cundell *et al.*, 1995a), but it also has other functions such as binding factor H (fH), (Dave *et al.*, 2001). Ply has been reported to have a number of effects on host cells including direct cytotoxicity and activation of complement, and at sublytic concentrations it is reported to slow ciliary beating, and inhibit the actions of leukocytes, (Hirst *et al.*, 2004). The first major role of PspA to be discovered

was its ability to inhibit complement activation (Tu *et al.*, 1999), but it has also been shown to bind human lactoferrin (Lf) (Hammerschmidt *et al.*, 1999).

There are also indications that these proteins may have the ability to stimulate chemokine release from host cells. CbpA has previously been reported to stimulate IL-8 release from type II pneumocytes (Madsen *et al.*, 2000; Murdoch *et al.*, 2002), and Ply has been shown to cause an inflammatory response in the lungs when administered to mice (Rijneveld *et al.*, 2002), although the actual role of epithelial cells in this response remains to be elucidated. PspA has not previously been reported to have a role in chemokine induction, but its importance as a virulence factor, and its structural similarity to CbpA make it worthy of investigation. In this chapter the role of these proteins in generating epithelial cell CXC chemokine responses to *S. pneumoniae* was investigated, using the assays established in Chapter Three, with mutants deficient in production of these proteins, as well as purified recombinant CbpA, PspA and Ply.

Because the general trend of the chemokine mRNA response to *S. pneumoniae* was similar for all the chemokines that were upregulated, it was decided to use IL-8 and MIP-2α mRNA as an indicator of the overall CXC chemokine mRNA response.

## 4.2 Mutant studies

To examine the role that the pneumococcal surface proteins CbpA and PspA may have in the generation of a CXC chemokine response by host epithelial cells, mutants created by insertion-duplication mutagenesis of the genes for these proteins were utilised in the chemokine induction assays developed in Chapter Three ($CbpA^-$ and $PspA^-$, Table 2.1). Insertion-duplication mutagenesis is a simple and rapid method of creating mutants in specific genes. It involves cloning an internal fragment of the gene of interest into a suicide vector. Transformation of this vector into the recipient WT strain leads to a single crossover event between the insert on the vector and the homologous region on the gene. This results in

duplication of the cloned fragment and integration of the vector into the gene, disrupting the open reading frame (ORF) such that a functional, full-length product is no longer produced (Figure 4.1). The possible role that Ply may play in the host chemokine response was investigated using a mutant in which the *ply* gene had been deleted in frame (ΔPly; Table 2.1) (Berry *et al.*, 1995).

To examine the potential role that the pneumococcal surface proteins CbpA and PspA play in the chemokine response to *S. pneumoniae*, A549 or Detroit-562 cells were infected with approximately $5 \times 10^7$ CFU *S. pneumoniae* D39 or CbpA$^-$ or PspA$^-$ mutants. Cellular RNA was extracted at 2 or 4 h and analysed for the presence of chemokine-specific mRNA by real-time RT-PCR, as described in Section 2.10.3. Cell culture SN was collected at 4 h and analysed for IL-8 by ELISA, as described in Section 2.13.

### 4.2.1 Chemokine mRNA response of Type II pneumocytes to *S. pneumoniae* D39 CbpA$^-$ and PspA$^-$ insertion-duplication mutants

After incubation with *S. pneumoniae*, A549 cells showed a significant increase in chemokine mRNA when compared to uninfected control cells (IL-8 mRNA: $P < 0.001$ for all strains; MIP-2α mRNA: $P < 0.05$ for WT D39, $P < 0.01$ for CbpA$^-$ and PspA$^-$). After 2 h exposure to *S. pneumoniae*, no significant difference was seen in the IL-8 mRNA response elicited by either the CbpA$^-$ or PspA$^-$ mutants when compared to WT D39. However, after 4 h IL-8 responses elicited by CbpA$^-$ and PspA$^-$ were approximately 2-fold greater than those elicited by WT D39 ($P < 0.05$, and $P < 0.01$ respectively) (Figure 4.2).

The MIP-2α mRNA response to CbpA$^-$ at 2 h was approximately 2-fold higher than that to WT D39 ($P < 0.05$). This difference was also observed at 4 h, ($P < 0.05$). At 2 h, there was no significant difference in the MIP-2α mRNA response to PspA$^-$ when compared to WT D39, but at 4 h, the response to PspA$^-$ was approximately 2-fold higher than that to WT D39 ($P < 0.01$) (Figure 4.2).

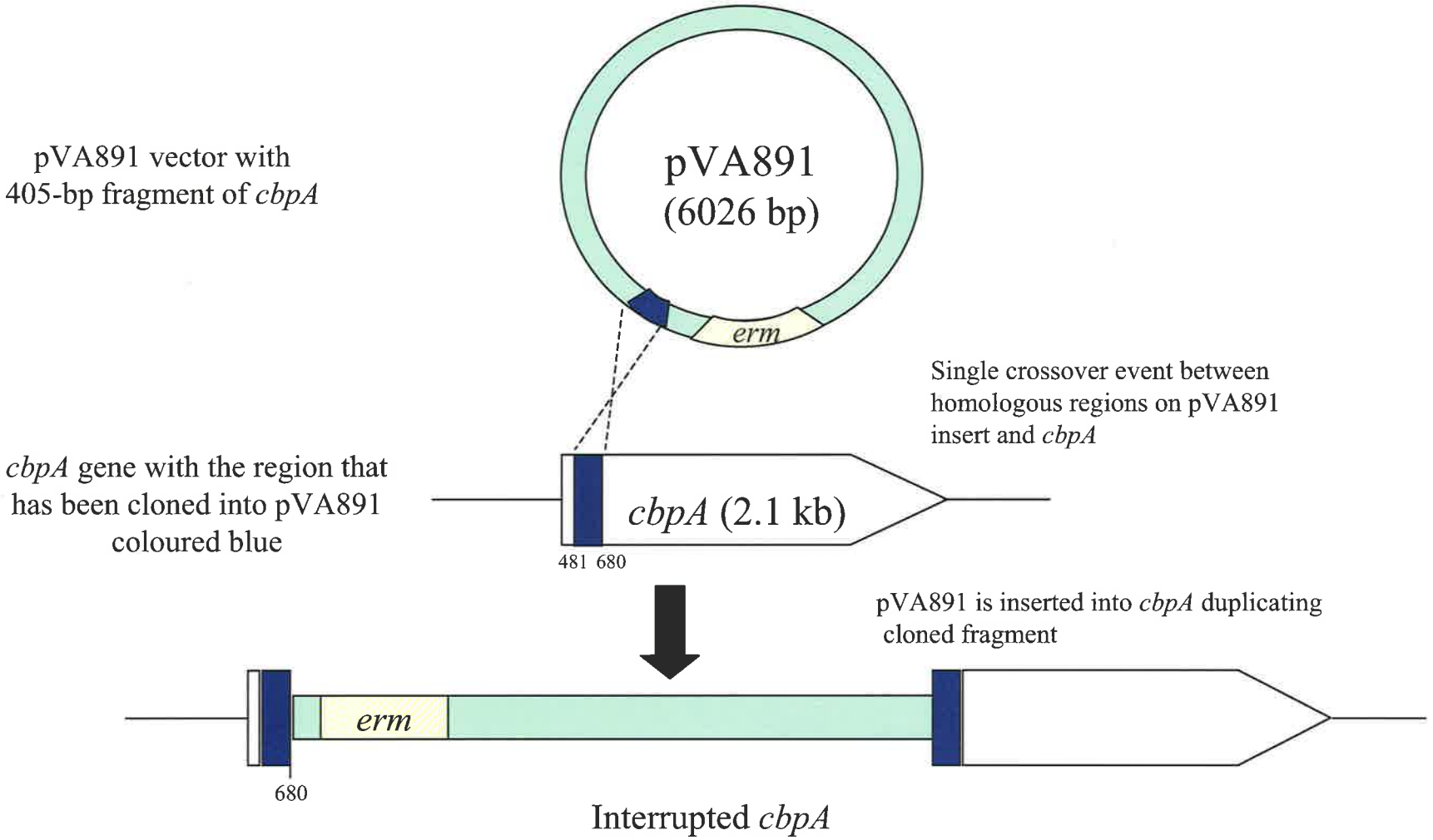


**Figure 4.1 Insertion duplication mutagenesis of *cbpA*.** A 200-bp internal fragment of the *cbpA* gene (nt 481-680) (dark blue) was amplified by PCR and cloned into the vector pVA891. When this vector is transformed into *S. pneumoniae*, a single cross over event occurs between the *cbpA* fragment on the vector and the homologous region in the chromosomal *cbpA* gene. This results in integration of the vector into the chromosome and thus interruption of *cbpA*.

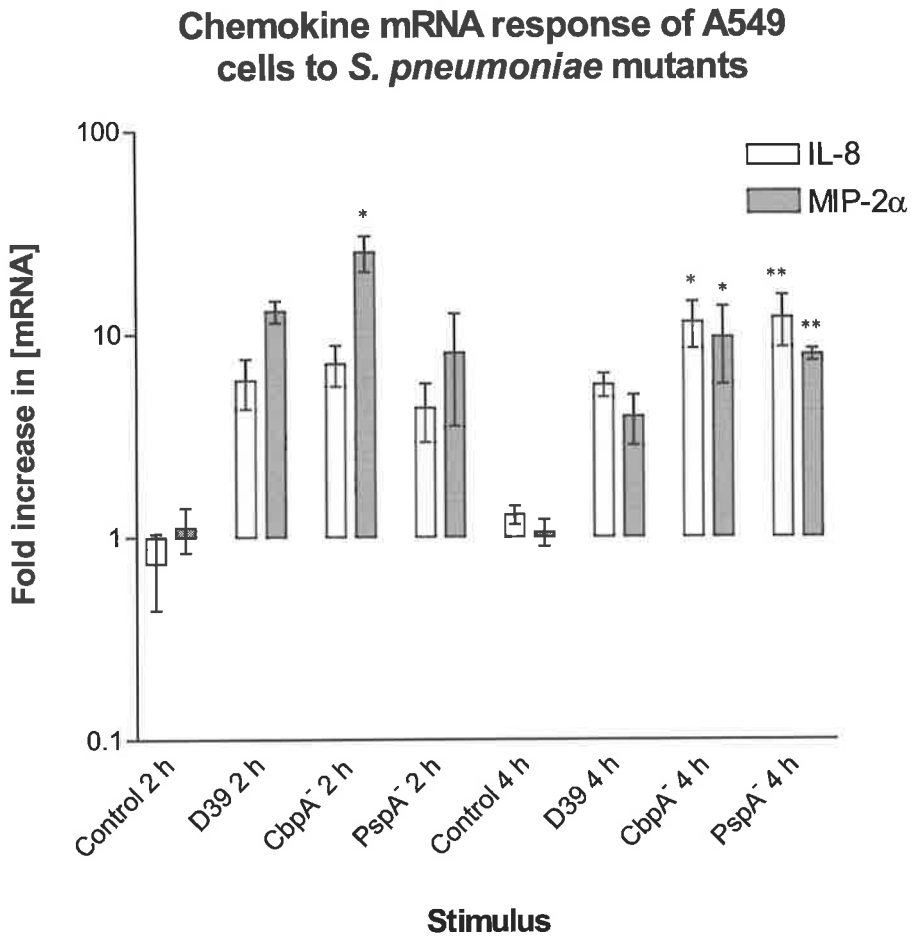


**Figure 4.2. CXC chemokine mRNA response of type II pneumocytes to WT D39, CbpA$^{-}$ and PspA$^{-}$.** Confluent monolayers of A549 cells were incubated with approximately $5 \times 10^7$ CFU WT *S. pneumoniae* D39, CbpA$^{-}$ or PspA$^{-}$ for 2 or 4 h before extraction of cellular RNA and analysis for chemokine specific mRNA by real-time RT-PCR. Experiments were performed in quadruplicate, and results are expressed as fold increase of chemokine mRNA relative to a 0 h control (mean ± SE). Data were analysed for statistical significance by One-way ANOVA with a post-hoc Bonferroni test. ** $P < 0.01$; * $P < 0.05$, compared to WT D39 at the same timepoint.

### 4.2.2 Chemokine mRNA response of nasopharyngeal epithelial (Detroit-562) cells to *S. pneumoniae* D39, CbpA⁻ and PspA⁻

After incubation of Detroit-562 cells with the various *S. pneumoniae* strains for 2 h, levels of mRNA for IL-8 and MIP-2α were increased significantly when compared to uninfected control cells ($P < 0.001$ for all); this increase was also apparent at 4 h ($P < 0.001$ for all) (Figure 4.3). When infected with the equivalent dose of PspA⁻ *S. pneumoniae* D39, nasopharyngeal epithelial cells exhibited a similar response at 2 h to that seen against WT D39. However after 4 h, the mRNA response to the PspA⁻ mutant was significantly higher for both IL-8 and MIP-2α mRNA when compared to WT D39. IL-8 mRNA was increased approximately 3 fold, while MIP-2α mRNA was increased approximately 9-fold compared to WT D39 (Figure 4.3) ($P < 0.05$, and $P < 0.01$, respectively). Infection with the CbpA⁻ mutant elicited an approximately 3-fold increase in IL-8 and MIP-2α mRNA when compared to WT D39 at 2 h ($P < 0.01$, and $P < 0.05$ respectively), and at 4 h IL-8 mRNA was increased approximately 3-fold, while MIP-2α mRNA was increased approximately 5-fold ($P < 0.05$ for both) (Figure 4.3).

### 4.2.3 Release of IL-8 by respiratory epithelial cells in response to WT D39, CbpA⁻ and PspA⁻

In order to confirm results obtained by real-time RT-PCR, cell culture SN from A549 and Detroit-562 cells infected with $5 \times 10^7$ CFU *S. pneumoniae* D39, CbpA⁻ or PspA⁻ were collected and assayed for IL-8 by ELISA as described in Section 2.13. Both cell types showed a significant increase in IL-8 levels in response to any of the *S. pneumoniae* strains when compared to uninfected control cells. A549 cells showed an approximate 2-fold increase in IL-8 in response to both CbpA⁻ ($P < 0.05$) and PspA⁻ ($P < 0.001$) compared with WT D39 (Figure 4.4 A). Detroit-562 cells also showed an increase of approximately 4-fold in IL-8 in response to CbpA⁻ and PspA⁻ compared with WT D39 ($P < 0.001$ for both) (Figure 4.4 B). These data are consistent with the results obtained for IL-8 by real-time RT-PCR.

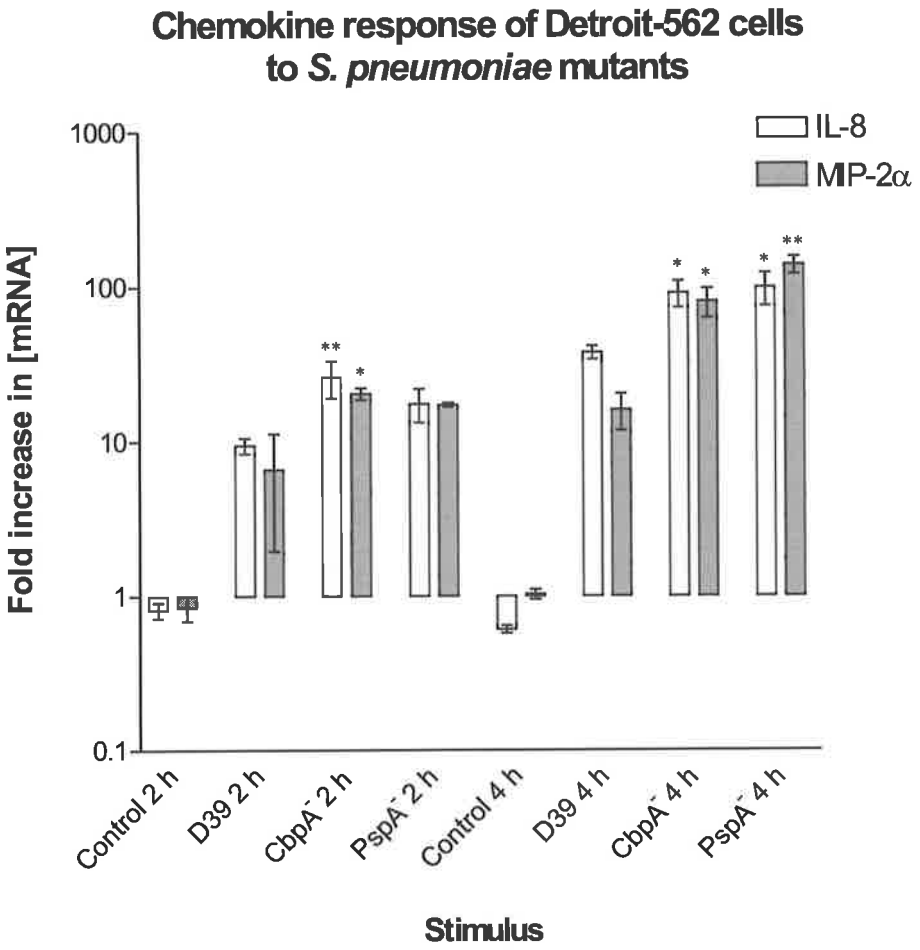


**Figure 4.3. CXC chemokine mRNA response of nasopharyngeal epithelial cells to WT D39, CbpA$^-$ and PspA$^-$.** Confluent monolayers of Detroit-562 cells were incubated with approximately 5 × $10^7$ CFU WT *S. pneumoniae* D39, CbpA$^-$ or PspA$^-$ for 2 or 4 h before extraction of cellular RNA and analysis for chemokine specific mRNA by real-time RT-PCR. Experiments were performed in quadruplicate, and results are expressed as fold increase of chemokine mRNA relative to a 0 h control (mean ± SE). Data were analysed for statistical significance by One-way ANOVA with a post-hoc Bonferroni test. ** $P < 0.01$; * $P < 0.05$, compared to WT D39 at the same timepoint.

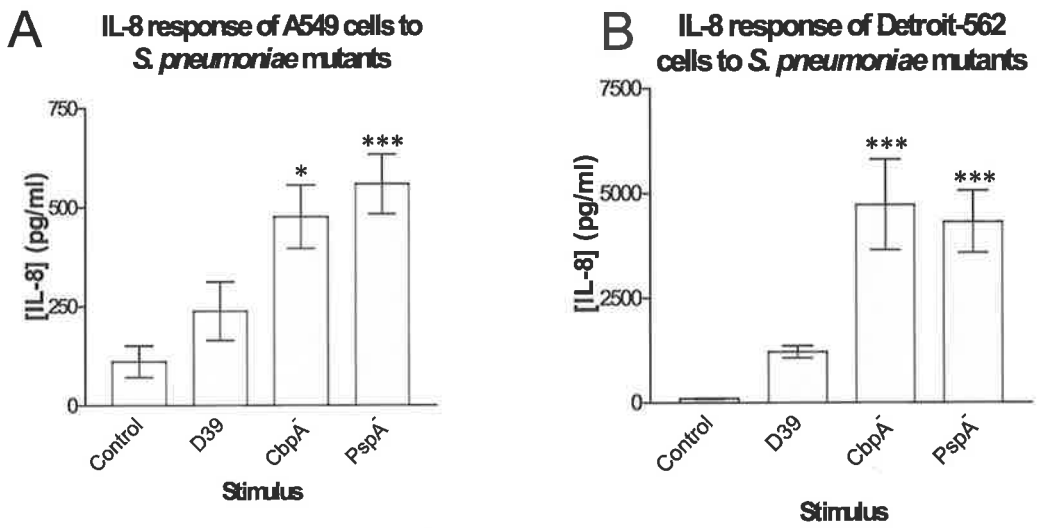


**Figure 4.4. IL-8 response of respiratory epithelial cells to WT D39, CbpA$^-$ and PspA$^-$.** Cell culture supernatants from A549 (A) and Detroit-562 (B) cells infected with $5 \times 10^7$ CFU WT D39, CbpA$^-$ or PspA$^-$ were analysed for the presence of IL-8 by ELISA. Data are mean ± SE from 3 independent experiments. Results were analysed for statistical significance by One-way ANOVA with a post-hoc Bonferroni test. *** $P < 0.001$; * $P < 0.05$, relative to D39.

Thus, after infection with CbpA$^-$ and PspA$^-$, a significant increase was seen in the CXC chemokine mRNA response of both A549 and Detroit-562 cells compared to that elicited by WT D39, and this was consistent with the increases seen in release of IL-8 protein.

### 4.2.4 Production of deletion mutants

The results obtained with the insertion-duplication mutants CbpA$^-$ and PspA$^-$ were surprising, since that which had previously been published suggested that mutants in these proteins would show a decrease in chemokine production, rather than the increase that was observed. However, it should be noted that due to the nature of the mutagenesis procedure (as shown in Figure 4.1), the CbpA$^-$ and PspA$^-$ insertion-duplication mutants might still produce residual fragments of the proteins. When the CbpA$^-$ mutant was constructed, the vector pVA891 was inserted approximately 680-bp into the *cbpA* ORF, thus there is the potential for a truncated, approximately 20 kDa form of CbpA to be produced. This fragment would consist of the N-terminal region of the molecule which is the region implicated to function in host interaction. Another important point is that this N-terminal fragment would not contain the choline-binding domain, and so it would not be expected to be anchored to the cell wall of the pneumococcus, but rather it would be released into the culture SN. A soluble CbpA fragment could potentially interact better with the epithelial cells than cell-bound CbpA, and hence elicit a greater response. Likewise, the PspA mutant, which was constructed in a similar way, also has the potential to produce a truncated N-terminal fragment of the molecule. This would also not contain the choline-binding domain and thus might be secreted by the pneumococcus into the SN, potentially allowing it to interact with the epithelial cells more easily than surface bound PspA. Sequencing of the interrupted *pspA* locus of PspA$^-$ showed that the insertion occurs around 260 nt into the ORF (data not shown). Thus it was possible that the mutant was producing the leader sequence and the first portion of the α-helical region. Since it is proposed that the α-helical N-terminus is the biologically active region of the molecule, this could be responsible for eliciting a chemokine response from type

II pneumocytes. Western blot analysis of SN from cultures of CbpA$^-$ and PspA$^-$ failed to show the presence of truncated fragments of these proteins, but their presence cannot be ruled out entirely (data not shown). To distinguish whether the increase in chemokine response that was seen in these cells was due to the absence of full-length versions of these proteins or to residual expression and possible secretion of truncated forms of these molecules, deletion mutants in which the entire gene of interest was deleted in-frame were constructed.

### 4.2.4.1 Construction of in-frame deletion mutants

Mutants in which the genes encoding CbpA or PspA were deleted in frame were constructed using a strategy analogous to that utilised by Berry *et al.*, (1999) to construct the mutant in which the region encoding aa 55-437 of Ply was deleted in-frame (ΔPly).

To construct the CbpA deletion mutant, overlap extension PCR was used to create a linear DNA fragment comprising the 5` and 3` flanking regions of *cbpA*, but with the *cbpA* ORF itself deleted. This product was then back-transformed into the CbpA$^-$ mutant used in Section 4.2.1 (Table 2.1), where it recombined with the *cbpA* gene deleting it from the chromosome. The *S. pneumoniae* R6 genome (Genbank accession number NC_003098) was used to design oligonucleotides that bound 1.8 kb upstream and 2.4 kb downstream of *cbpA*, (RMAG7 and RMAG8) (Table 2.2). Oligonucleotides were also designed to bind at the beginning and end of the *cbpA* gene, incorporating the start codon and the following 2 codons (RMAG6), and the stop codon with the preceding codon (RMAG5). Products of the PCR reactions using RMAG5 + RMAG8 and RMAG6 + RMAG7 contained overlapping, complementary sequences at one end as a result of extensions incorporated into the oligonucleotides, such that when both products were combined and used as a template in a PCR reaction using the oligonucleotides RMAG7 and RMAG8, the overlapping sequences annealed bringing the 2 products together. Thus, the flanking regions of *cbpA* were amplified together, with nt 10-2100 of the *cbpA* ORF deleted (Figure 4.5). This product was transformed into competent CbpA$^-$ cells as described in Section 2.12.3, and a homologous

recombination event between the PCR product and the regions flanking *cbpA* on the chromosome resulted in deletion of *cbpA* from the chromosome (Figure 4.5). Transformants were selected by loss of Ery resistance by replicate patching on BA and EryBA plates, and deletion of *cbpA* was confirmed by PCR (Figure 4.6), sequence analysis (data not shown), and Western blotting (Figure 4.7). The confirmed *cbpA* deletion mutant was designated ΔCbpA.

For reasons that remain unclear, repeated attempts to construct a *pspA* deletion mutant by transformation of the insertion-duplication mutant PspA$^-$ were unsuccessful. Deletion of the *pspA* gene in *S. pneumoniae* D39 was therefore achieved using a two-step process. The first step involved interrupting *pspA* with a 762-bp Ery resistance cassette by allelic replacement, thus creating an Ery$^R$ recipient strain (*pspA*::*erm*). The second step utilised overlap extension PCR to create a PCR product containing the upstream and downstream regions of *pspA* but with *pspA* itself deleted. This product was then back-transformed into the *erm* insertion mutant as described in Section 2.12.3, resulting in a recombination event in which the complete *pspA* ORF was replaced by the deletion product and thus the gene was deleted from the chromosome.

To create the *pspA*::*erm* insertion mutant, the Ery resistance gene (*erm*) from the vector pVA891 (Macrina *et al.*, 1983) was amplified using the oligonucleotides RMAG12 and RMAG13 (Table 2.2). These oligonucleotides were designed to contain tails that overlap with complementary tails on other oligonucleotides designed to bind within the *pspA* gene (RMAG14 and RMAG15, Table 2.2), (designed using the D39 *pspA* sequence [Genbank accession number M74122]). The regions of *pspA* between nucleotides 151 and 738, and 1075 and 1897 were amplified using the oligonucleotides IDPspAa and RMAG14, and RMAG15 and IDPspAb (Table 2.2). The tails on the oligonucleotides resulted in the presence of extensions on the *erm* gene PCR product and on the amplified *pspA* regions that overlapped such that when the products were combined, the three fragments could anneal. The entire fragment could then be amplified by PCR with oligonucleotides IDPspAa and

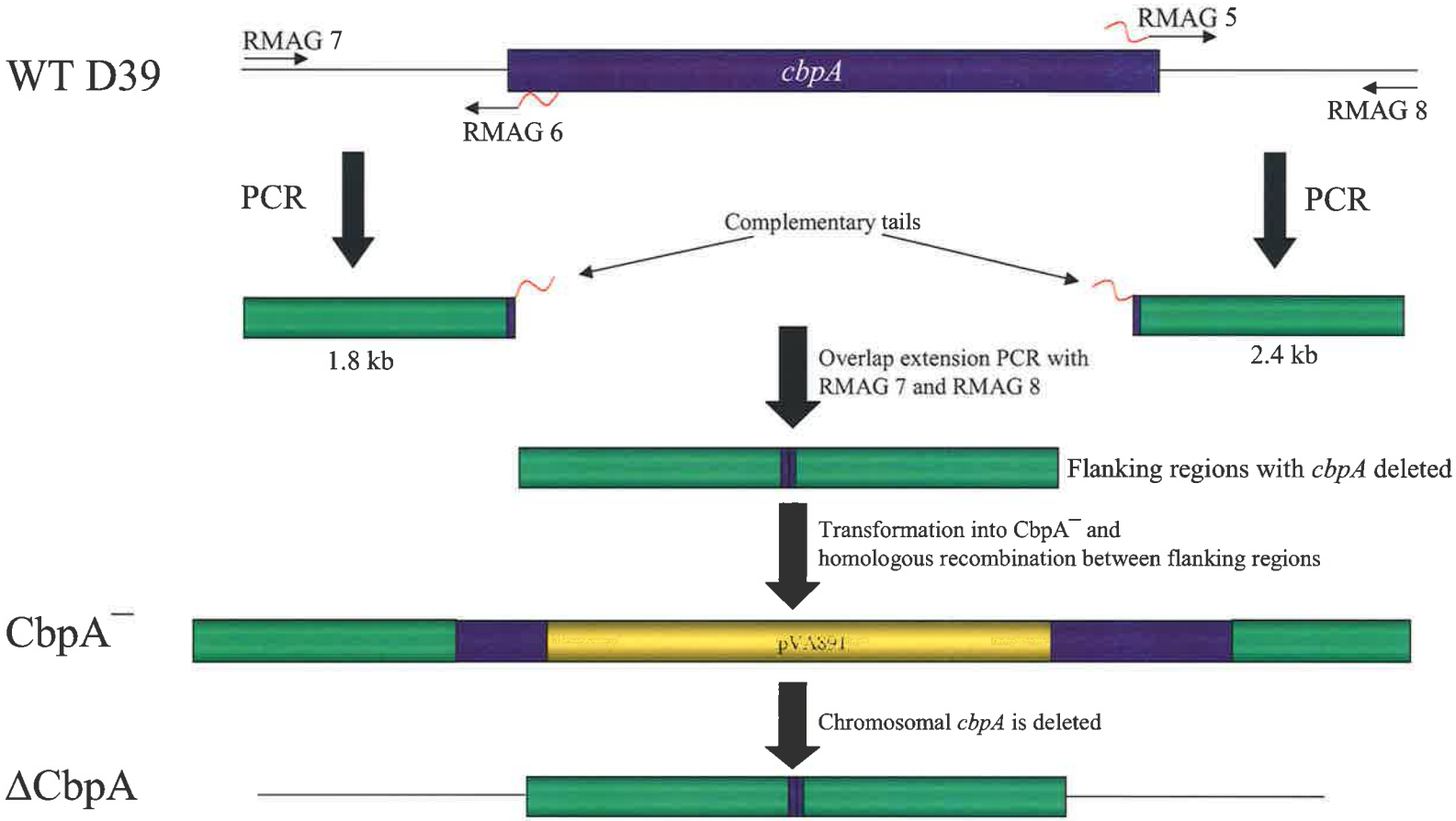


**Figure 4.5. Deletion of *cbpA* from the pneumococcal chromosome.** CbpA$^-$ *S. pneumoniae* were transformed with a PCR product created by overlap extension such that the flanking regions of *cbpA* are amplified without *cbpA* itself. This results in homologous recombination of flanking regions of *cbpA* on the PCR product with those on the chromosome, such that the gene is deleted from the chromosome.

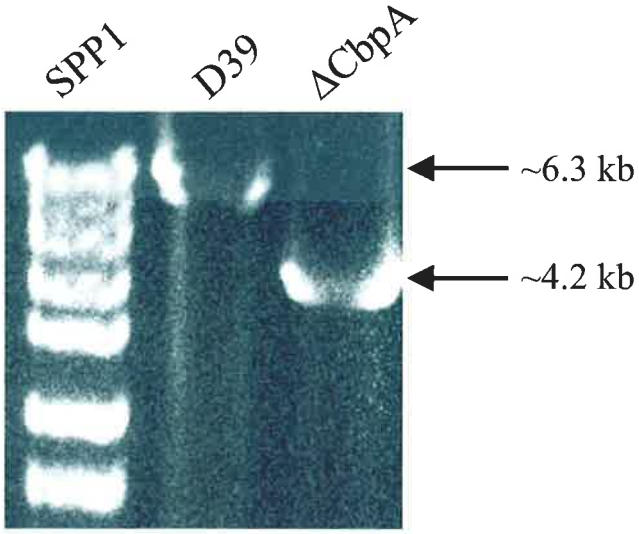


**Figure 4.6. PCR analysis of ΔCbpA.** Colonies of WT *S. pneumoniae* D39 and ΔCbpA were subjected to direct PCR analysis using the oligonucleotides RMAG7 and RMAG8 (Table 2.2). Products were electrophoresed on a 1% agarose gel and stained with ethidium bromide (as described in Section 2.9.1). Sizes of products were estimated according to mobilities relative to the SPP1 marker and are indicated. WT D39 gave a product with an approximate size of 6.3 kb, and ΔCbpA gave a product with an approximate size of 4.2 kb consistent with deletion of *cbpA* (2.1 kb).

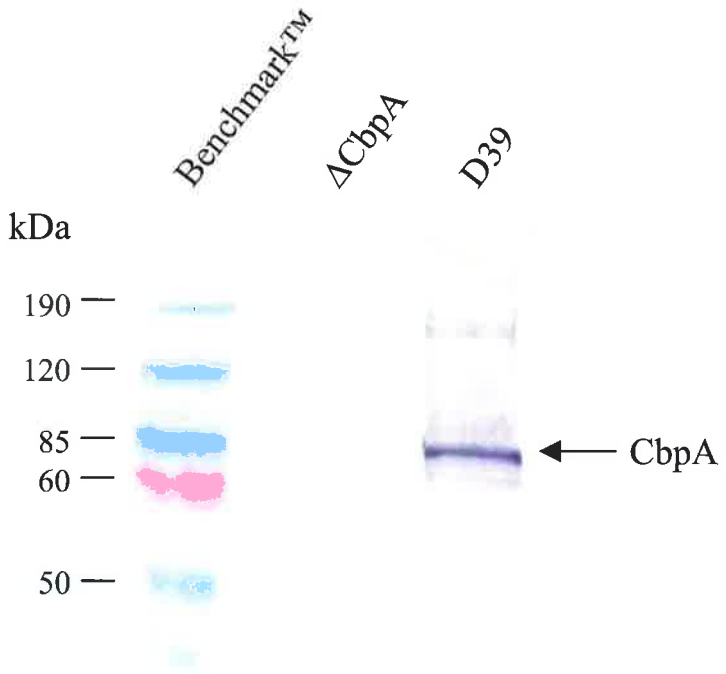


**Figure 4.7. Western blot analysis of ΔCbpA.** Lysates of WT D39 and ΔCbpA were separated by SDS-PAGE, electroblotted onto nitrocellulose and reacted with mouse polyclonal antiserum specific for CbpA (as described in Section 2.8). The position of CbpA is indicated, as are the mobilities of the pre-stained protein size marker species. A band of the appropriate size for CbpA was seen in the lysate of WT D39, but no reactivity with anti-CbpA was seen for the ΔCbpA lysate. The observed smear is characteristic of the reactivity of polyclonal mouse serum with CbpA in D39 lysates.

IDPspAb. The resulting product comprised the *pspA* regions 151-738 and 1075-1897 flanking *erm* as shown in Figure 4.8. This product was transformed into competent *S. pneumoniae* D39 cells as described in Section 2.12.2, and homologous recombination between the *pspA* regions on the PCR product and the corresponding regions of *pspA* on the chromosome resulted in *erm* being inserted into *pspA* on the chromosome, thus interrupting the gene. Transformants were selected by plating on EryBA, and confirmed by PCR (Figure 4.9), sequence analysis (data not shown), and Western blotting with PspA-specific antiserum (Figure 4.10). The resulting *erm* insertion mutant was designated *pspA*::*erm*.

The next step of the process involved deleting the entire *pspA* gene. This was achieved using the oligonucleotides RMAG1 and RMAG3 (Table 2.2), designed using the *S. pneumoniae* R6 genome sequence to amplify a 1.9 kb fragment immediately upstream of *pspA*, incorporating the start codon, and the first three codons of the *pspA* ORF. RMAG2 and RMAG4 (Table 2.2) were also used to amplify a 1.3 kb fragment immediately downstream of *pspA*, and incorporating the *pspA* stop codon. Products of both reactions contained overlapping, complementary sequences at one end as a result of extensions incorporated into the oligonucleotides, such that when both products were used as a template in a PCR reaction using the oligonucleotides RMAG3 and RMAG4, the overlapping sequences annealed and the resulting 3.2 kb product comprised the two flanking regions of *pspA* with nt 13-1857 of the *pspA* ORF deleted (Figure 4.11). When this product was transformed into *pspA*::*erm* as described in Section 2.12.3, a homologous recombination event occurred between the regions flanking *pspA*, resulting in chromosomal *pspA* being replaced by the PCR product, thus deleting *pspA* from the chromosome (Figure 4.11). Successful transformants were screened for loss of Ery resistance by replicate patching onto BA and EryBA plates after Ery/Amp enrichment (as described in Section 2.12.3), and mutants were confirmed by PCR (Figure 4.12), sequence analysis (data not shown), and Western blotting (Figure 4.13). The confirmed *pspA* deletion mutant was designated ΔPspA.

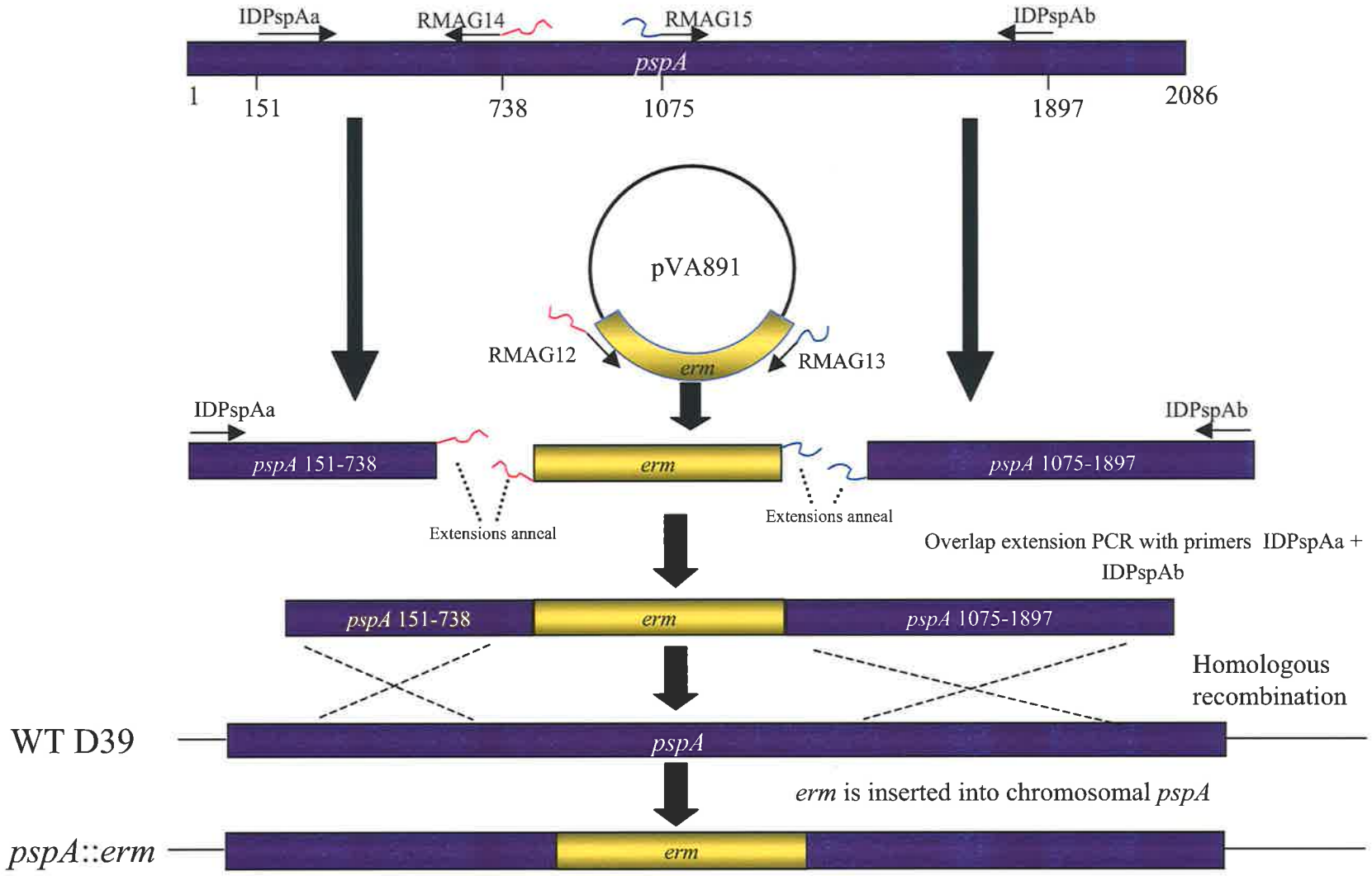


**Figure 4.8. Creation of *erm* insertion mutant of *pspA*.** A PCR product containing the *erm* gene from pVA891 flanked by regions of *pspA* is created by overlap extension PCR. Transformation of this PCR product into competent *S. pneumoniae* D39 results in recombination between homologous regions on the PCR product with *pspA* on the chromosome and thus insertion of *erm* into *pspA*.

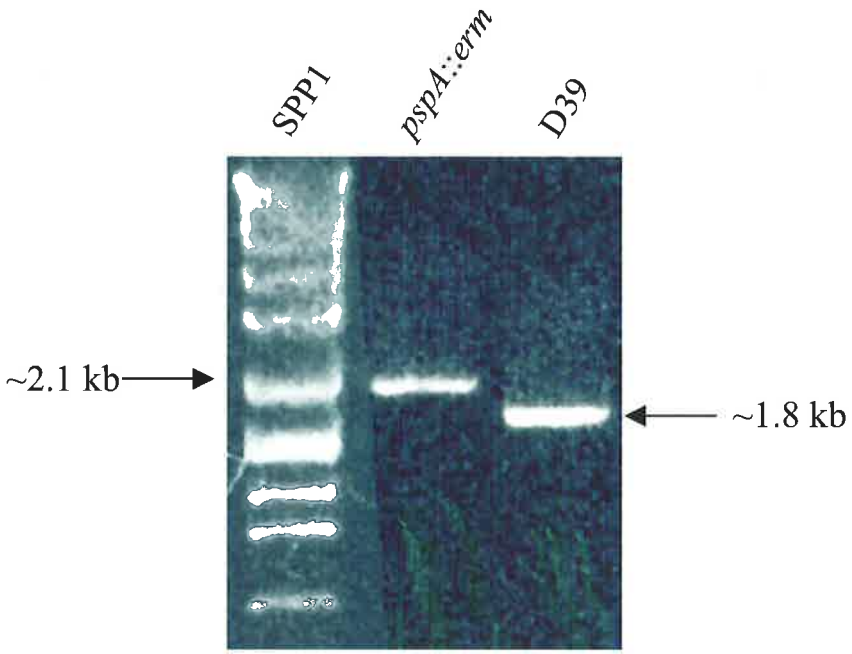


**Figure 4.9. PCR analysis of *pspA*::*erm*.** Colonies of WT *S. pneumoniae* D39 and *pspA*::*erm* were subjected to direct PCR analysis using the oligonucleotides IDPspAa and IDPspAb (Table 2.2). Products were electrophoresed on a 1% agarose gel and stained with ethidium bromide (as described in Section 2.9.1). Sizes of products were estimated according to mobilities relative to the SPP1 marker and are indicated. The PCR product used to create *pspA*::*erm* was 2.1 kb in size, consistent with the 2.1 kb band evident for *pspA*::*erm*, while WT D39 gave a product of approximately 1.8 kb, (the expected size).

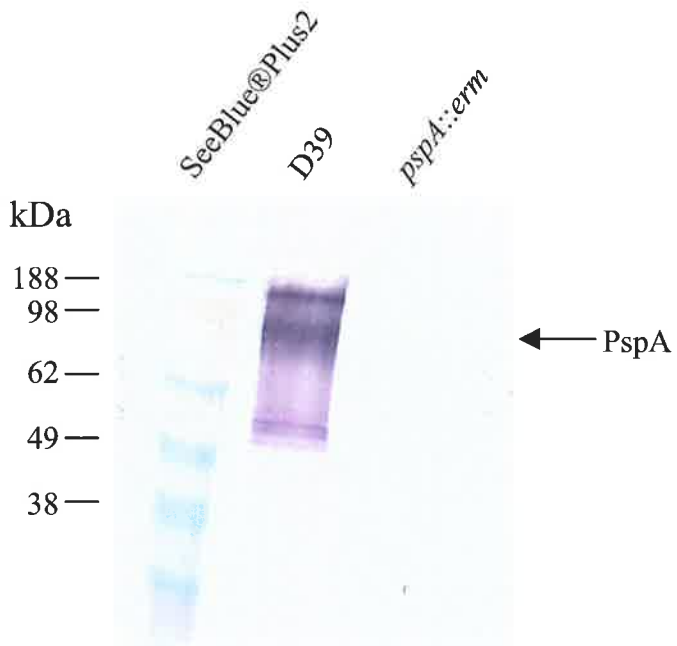


**Figure 4.10. Western blot analysis of *pspA*::*erm*.** Lysates of WT D39 and *pspA*::*erm* were separated by SDS-PAGE, electroblotted onto nitrocellulose and reacted with mouse polyclonal antiserum specific for PspA (as described in Section 2.8). The position of PspA is indicated, as are the mobilities of the pre-stained protein size marker species. A band of the appropriate size for PspA was seen in the lysate of WT D39, but no reactivity with anti-PspA was seen for the *pspA*::*erm* lysate. The observed smear is characteristic of the reactivity of polyclonal mouse serum with PspA in D39 lysates.

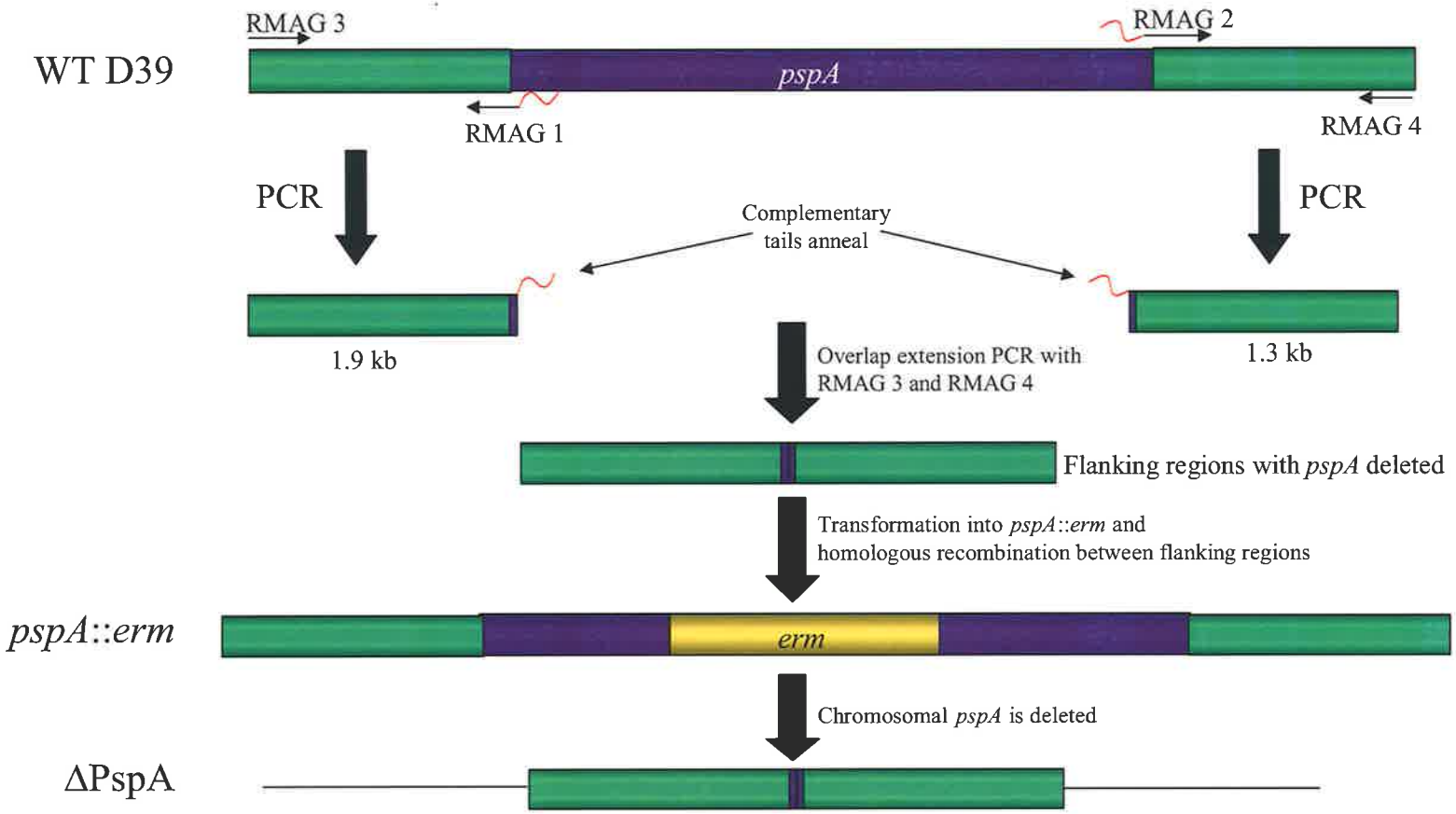


**Figure 4.11. Deletion of *pspA* from the pneumococcal chromosome.** Transformation of *pspA*::*erm* with a PCR product created by overlap extension such that the flanking regions of *pspA* are amplified without *pspA* itself, results in homologous recombination between flanking regions of *pspA* on the PCR product with those on the chromosome such that the gene is deleted from the chromosome.

### 4.2.5 Chemokine response of A549 and Detroit-562 cells to infection with D39, ΔCbpA, ΔPspA, and ΔPly

In order to assess whether the chemokine response seen towards the insertion-duplication mutants was due to expression of truncated forms of the proteins or the absence of the protein of interest, A549 or Detroit-562 cell monolayers were incubated with $5 \times 10^7$ CFU WT *S. pneumoniae* D39, ΔCbpA, ΔPspA, or ΔPly. At 2 and 4 h total cellular RNA was extracted and analysed for the presence of IL-8 and MIP-2α mRNA by real-time RT-PCR using specific oligonucleotides, as described in Section 2.10.3. Cell culture SN was collected at 4 h and analysed for IL-8 by ELISA, as described in Section 2.13.

All A549 cells incubated with *S. pneumoniae* showed a significant increase in both IL-8 and MIP-2α mRNA when compared to uninfected control cells that were incubated with media alone ($P < 0.001$). After 2 h, A549 cells showed an approximately 2-fold increase in IL-8 mRNA when infected with ΔCbpA compared to WT D39 ($P < 0.01$). However, infection with ΔPspA for 2 h led to IL-8 and MIP-2α mRNA levels in cells that were approximately 2-fold lower compared to those infected with WT D39 ($P < 0.01$ for both) (Figure 4.14). This finding is different to the results seen with $PspA^-$ (see Section 4.2.1) in which no difference was seen for IL-8 or MIP-2α mRNA at 2 h. No significant difference in chemokine mRNA levels were observed in the A549 cells exposed to ΔPly for 2 h when compared to those exposed to WT D39 (Figure 4.14).

Levels of both IL-8 and MIP-2α mRNA were increased approximately 2-fold and 4-fold respectively in A549 cells incubated with ΔCbpA for 4 h when compared to those incubated with WT D39 ($P < 0.01$, and $P < 0.001$ respectively) (Figure 4.14), in agreement with previous results (see Section 4.2.1). Levels of both IL-8 and MIP-2α mRNA were also raised approximately 2-fold in A549 cells infected with ΔPly in comparison to WT D39 ($P < 0.05$, and $P < 0.01$ respectively). However, there was no significant difference in chemokine

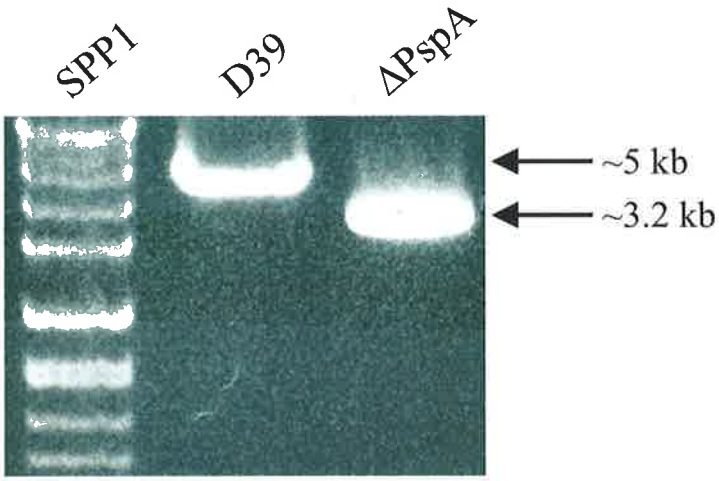


**Figure 4.12. PCR analysis of ΔPspA.** Colonies of WT *S. pneumoniae* D39 and ΔPspA were subjected to direct PCR analysis using the oligonucleotides RMAG3 and RMAG4 (Table 2.2). Products were electrophoresed on a 1% agarose gel and stained with ethidium bromide (as described in Section 2.9.1). Sizes of products were estimated according to mobilities relative to the SPP1 marker and are indicated. WT D39 gave a product with an approximate size of 5 kb, and ΔPspA gave a band with an approximate size of 3.2 kb consistent with deletion of *pspA* (1.8 kb).

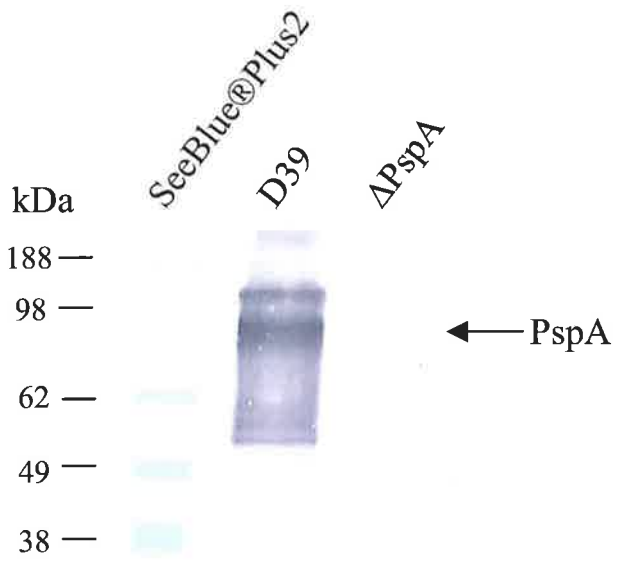


**Figure 4.13. Western blot analysis of ΔPspA.** Lysates of WT D39 and ΔPspA were separated by SDS-PAGE, electroblotted onto nitrocellulose and reacted with mouse polyclonal antiserum specific for PspA (as described in Section 2.8). The position of PspA is indicated, as are the mobilities of the pre-stained protein size marker species. A band of the appropriate size for PspA was seen in the lysate of WT D39, but no reactivity with anti-PspA was seen for the ΔPspA lysate. The observed smear is characteristic of the reactivity of polyclonal mouse serum with PspA in D39 lysates.

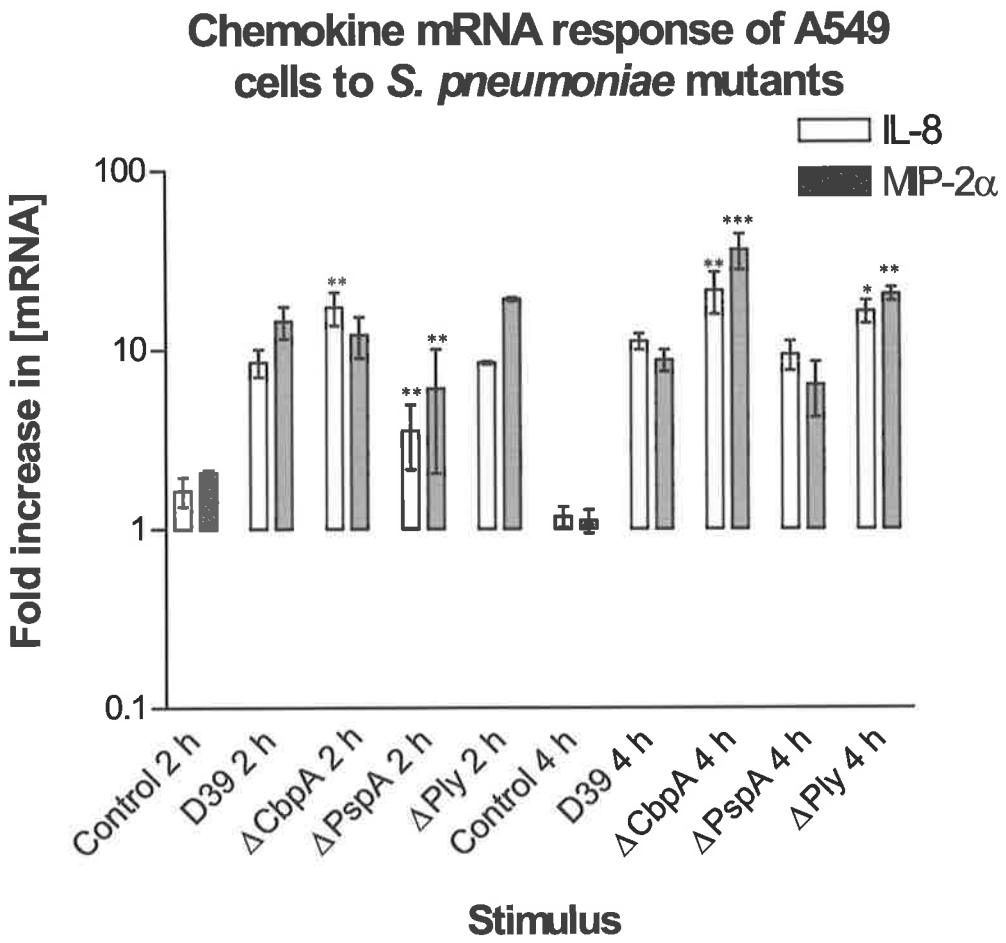


**Figure 4.14. CXC chemokine mRNA response of type II pneumocytes to *S. pneumoniae* D39, ΔCbpA, ΔPspA, and ΔPly.** Confluent monolayers of A549 cells were incubated with approximately $5 \times 10^7$ CFU WT *S. pneumoniae* D39, ΔCbpA, ΔPspA, or ΔPly for 2 or 4 h before extraction of cellular RNA and analysis for chemokine specific mRNA by real-time RT-PCR. Results are expressed as fold increase of chemokine mRNA relative to a 0 h control. Experiments were performed in quadruplicate and average results analysed for statistical significance by One-way ANOVA with a post-hoc Bonferroni test. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$, in comparison to WT D39 at the same timepoint.

mRNA levels in A549 cells exposed to ΔPspA for 4 h compared to those exposed to WT D39 (Figure 4.14), again in contrast with results obtained using PspA$^-$ (see Section 4.2.1).

All Detroit-562 cells incubated with *S. pneumoniae* showed a significant increase in both IL-8 and MIP-2α mRNA when compared to uninfected control cells that were incubated with media alone ($P < 0.001$). In these cells, the only mutant to elicit a significantly different response to that of WT D39 at 2 h was ΔPly, which showed an approximately 2-fold decrease in IL-8 mRNA ($P < 0.05$) (Figure 4.15). However, after 4 h incubation, levels of chemokine mRNA elicited by this mutant were similar to those elicited by WT D39, and were significantly higher than levels seen at 2 h (IL-8: $P < 0.001$, MIP-2α $P < 0.01$). Detroit-562 cells exposed to ΔCbpA for 4 h showed an increase in IL-8 mRNA of approximately 2-fold and an increase in MIP-2α mRNA levels of approximately 3-fold when compared to WT D39 ($P < 0.05$, and $P < 0.01$ respectively). ΔPspA and ΔPly did not elicit significantly different chemokine mRNA responses from nasopharyngeal epithelial cells after 4 h incubation compared to WT D39 (Figure 4.15).

#### 4.2.5.1 IL-8 release by respiratory epithelial cells in response to *S. pneumoniae* deletion mutants

In order to confirm the results obtained by real-time RT-PCR for chemokine mRNA induction in cells infected with the various deletion mutants, cell culture SN collected from the same experiments presented above were assayed for IL-8 by ELISA, as described in Section 2.13. Both A549 and Detroit-562 cells showed a significant increase in IL-8 when exposed to all strains of *S. pneumoniae* when compared to uninfected control cells ($P < 0.001$ for all). IL-8 release by A549 cells corresponded with IL-8 mRNA levels measured by real-time RT-PCR, with an approximately 2-fold increase observed in IL-8 production by cells exposed to ΔCbpA when compared to WT D39 ($P < 0.01$). A549 cells exposed to ΔPspA also released approximately 1.4-fold less IL-8 than those infected with WT D39 ($P < 0.05$). There was no significant difference in IL-8 secretion in A549 cells infected with ΔPly

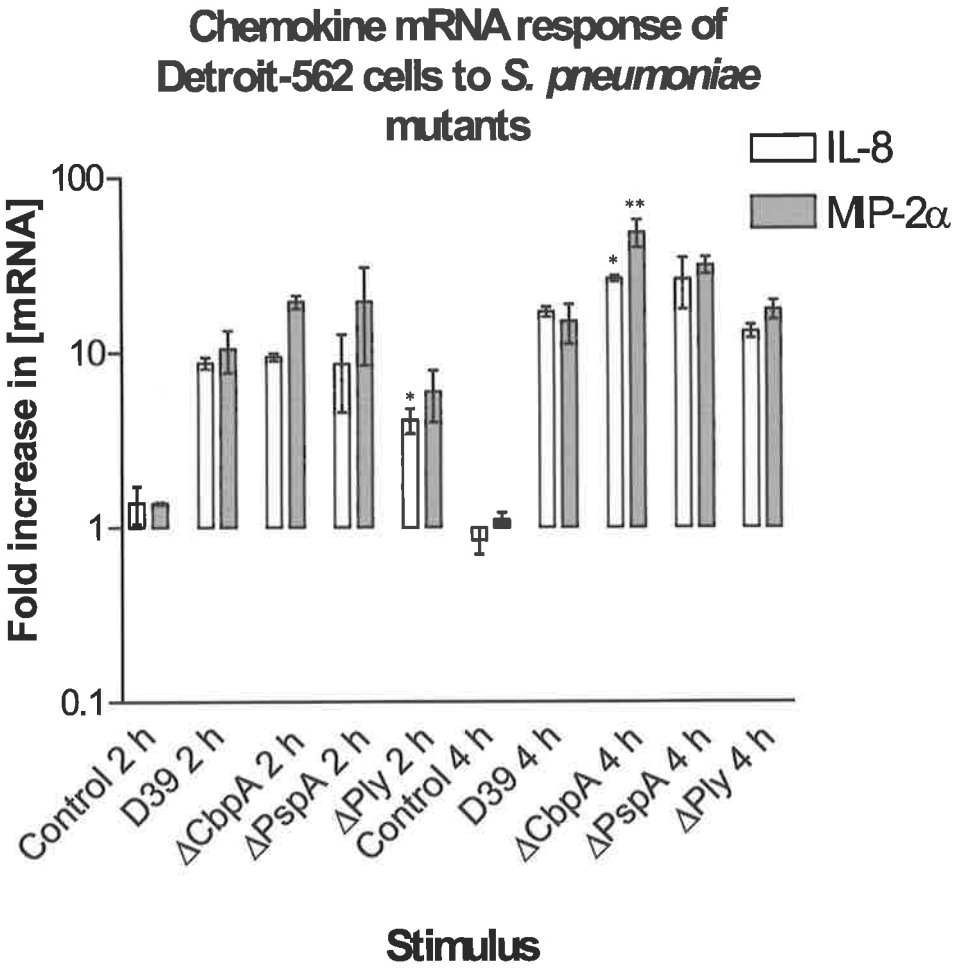


**Figure 4.15. CXC chemokine mRNA response of nasopharyngeal epithelial cells to *S. pneumoniae* D39, ΔCbpA, ΔPspA, and ΔPly.** Confluent monolayers of Detroit-562 cells were incubated with approximately $5 \times 10^7$ CFU WT *S. pneumoniae* D39, ΔCbpA, ΔPspA, or ΔPly for 2 or 4 h before extraction of cellular RNA and analysis for chemokine specific mRNA by real-time RT-PCR. Results are expressed as fold increase of chemokine mRNA relative to a 0 h control. Experiments were performed in quadruplicate and analysed for statistical significance by One-way ANOVA with a post-hoc Bonferroni test. ** $P < 0.01$; * $P < 0.05$, in comparison to WT D39 at the same timepoint.

compared to the WT (Figure 4.16 A). Release of IL-8 by Detroit-562 cells also followed a similar trend to that seen for IL-8 mRNA, with ΔCbpA eliciting levels that were approximately 2-fold higher than WT D39 ($P < 0.01$). ΔPspA induced a response from these cells that was not statistically different to that for WT D39, while cells incubated with ΔPly showed an approximately 1.5-fold decrease in IL-8 production compared to those incubated with WT D39 ($P < 0.05$) (Figure 4.16 B).

Thus, for both cell types and for all mutants, differences in IL-8 protein levels in culture SN were largely consistent with differences in cellular [mRNA].

The results of the studies using the deletion mutants were not entirely consistent with those presented in Sections 4.2.1, 4.2.2, and 4.2.3, which employed insertion-duplication mutants. ΔCbpA and $CbpA^-$ showed a similar upregulation of chemokine mRNA and IL-8 from A549 and Detroit-562 cells relative to WT D39, apparently confirming that CbpA may be directly inhibiting the IL-8 response from these cells. However, the responses to ΔPspA were not the same as those to $PspA^-$, with ΔPspA eliciting a significantly reduced IL-8 protein response, and a significantly reduced IL-8 and MIP-2α mRNA response from A549 cells at 2 h, and having no effect on Detroit-562 cells when compared to WT D39. ΔPly had different effects on the two cell lines, eliciting a significant increase in chemokine mRNA but not IL-8 protein from A549 cells at 4 h, but a significant decrease in IL-8 mRNA response at 2 h, and reduced IL-8 secretion in Detroit-562 cells compared to cells incubated with WT D39.

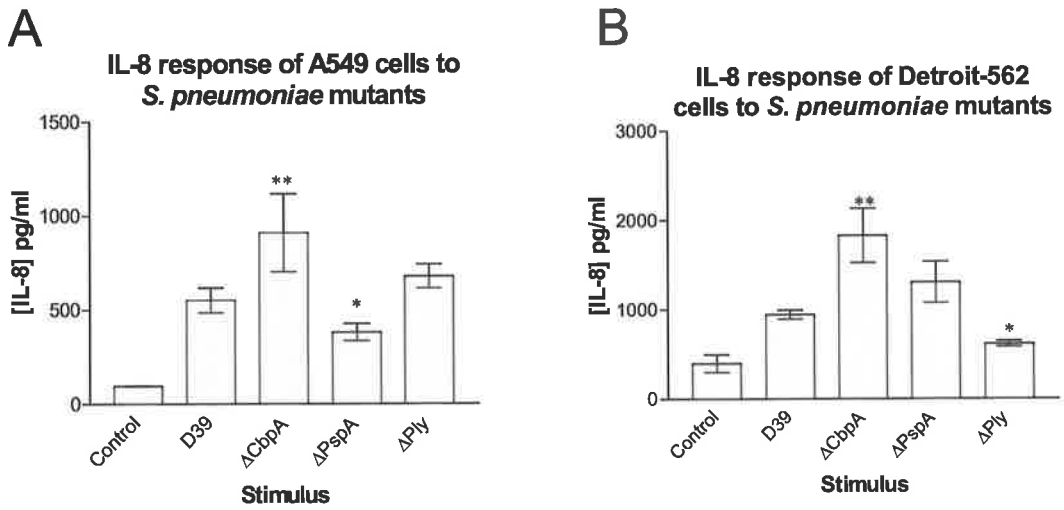


**Figure 4.16. IL-8 response of respiratory epithelial cells to WT D39, ΔCbpA , ΔPspA, or ΔPly.** Cell culture supernatants from A549 (A) and Detroit-562 (B) cells infected with $5 \times 10^7$ CFU WT D39, ΔCbpA, ΔPspA, or ΔPly were analysed for the presence of IL-8 by ELISA. Data shown are the mean ± SE from 3 independent experiments. Results were analysed for statistical significance by One-way ANOVA with a post-hoc Bonferroni test. ** $P < 0.01$; * $P < 0.05$, in comparison to D39.

## 4.3 Protein studies

### 4.3.1 Chemokine responses to pneumococcal proteins purified from recombinant *E. coli*

The N-terminal portion of the mature CbpA polypeptide (amino acids 1-445) containing the α-helical and proline rich regions, but lacking the signal peptide and the choline binding domain was expressed as a $His_6$ fusion protein in the *Escherichia coli* K-12 expression strain M15, using the vector pQE31 containing ampicillin resistance (Table 2.1). The exclusion of the choline-binding domain of CbpA in the recombinant protein was essential, because the presence of this region makes it impossible to purify. $His_6$-CbpA was purified from the expression construct by Ni-NTA affinity chromatography, as described in Section 2.7.2. Fractions collected from the Ni-NTA column were electrophoresed on an SDS-PAGE gel and stained with Coomasie brilliant blue to assess the purity of $His_6$-CbpA. The purified protein in the fractions had an apparent size of 75 kDa, consistent with the known mobility of the $His_6$-CbpA fusion protein previously established in this laboratory (Ogunniyi *et al.*, 2001). The concentration of CbpA was established by Bradford protein assay as described in Section 2.7.2. CbpA was judged to be >95% pure by SDS-PAGE and Coomasie brilliant blue staining. The 43 kDa N-terminal portion of the PspA molecule containing the α-helical and proline rich regions, but again lacking the choline binding domain, was purified by a method identical to that used to purify CbpA, as described in Section 2.7.2. PspA and CbpA antigens used for chemokine induction assays were judged to be >95% pure by SDS-PAGE and Coomasie blue staining (Figure 4.17).

In order to clarify the results obtained with the pneumococcal mutants concerning the potential roles of the pneumococcal surface proteins PspA, and CbpA in the generation of chemokine responses, respiratory epithelial cell monolayers were incubated with 10 μg/ml of recombinant PspA or CbpA (purified as described in Section 2.7.2) for 2 or 4 h. Cell culture SN was collected and cellular RNA extracted and assayed for the presence of IL-8 protein or

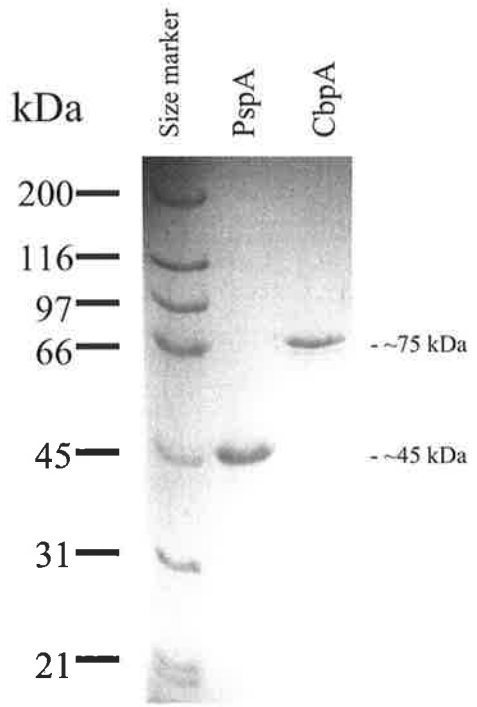


**Figure 4.17. SDS-PAGE analysis of PspA and CbpA.** Purified recombinant PspA and CbpA to be used in chemokine induction assays were subjected to SDS-PAGE analysis and stained with Coomasie brilliant blue (as described in Section 2.8.1). The mobilities of the size markers and the proteins are indicated in kDa.

chemokine mRNA as described in Sections 2.10.3 and 2.13. This concentration was chosen after initial pilot experiments that showed that the response to 1 μg/ml or 5 μg/ml was too low to measure (Results not shown). Moderate increases were seen in the levels of IL-8 and MIP-2α mRNA in response to CbpA and PspA but these did not reach statistical significance (Figure 4.18).

Use of CbpA and PspA purified from recombinant *E. coli* in chemokine induction assays is potentially complicated by LPS contamination of the protein preparations. Because A549 and Detroit-562 cells are sensitive to LPS, its presence in the preparations of CbpA and PspA used in the chemokine induction assays could evoke a response that is not due to the proteins themselves. To confirm that the increases seen in chemokine mRNA levels were due to the pneumococcal proteins and not to contaminating LPS, preparations were heat-inactivated (HI) by boiling for 30 min to denature any protein present. Upon incubation of A549 cells with HI CbpA, IL-8 mRNA levels were increased significantly when compared to uninfected control cells ($P < 0.01$), but were not significantly different compared to those elicited by the native protein. Levels of IL-8 and MIP-2α mRNA in Detroit-562 cells did not change significantly (Figure 4.18). PspA did not elicit a significantly increased response from either cell line, and there was no significant difference in the responses elicited by native or HI PspA (Figure 4.18). To determine whether this difference in response to the purified pneumococcal proteins was also evident at the protein level, culture SN collected from A549 or Detroit-562 cells incubated with or without 10 μg/ml native or HI CbpA or PspA was analysed for IL-8 by ELISA as described in Section 2.13. Results from the ELISA were consistent with the real-time RT-PCR results. IL-8 levels in A549 cells treated with native CbpA were 35-fold higher than control cells ($P < 0.001$), and levels of IL-8 released in response to HI CbpA were 68-fold higher than control cells ($P < 0.001$), and 2-fold higher than levels released in response to native CbpA ($P < 0.001$) (Figure 4.19). In Detroit-562 cells, both native and HI CbpA elicited an approximately 17-fold increase in IL-8 compared

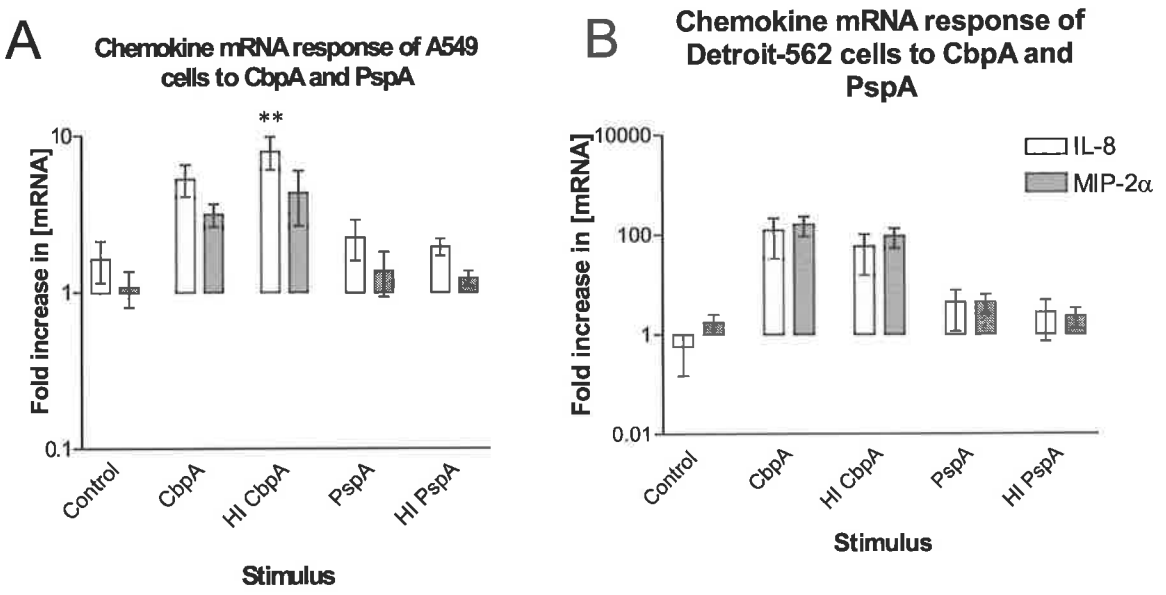


**Figure 4.18. CXC chemokine mRNA response of A549 and Detroit-562 cells to pneumococcal proteins.** Confluent monolayers of A549 (A) or Detroit-562 (B) cells were incubated with or without 10 μg/ml native or HI CbpA or PspA for 4 h before extraction of RNA and analysis for chemokine mRNA by real-time RT-PCR using specific oligonucleotides. Data shown are mean ± SE from 3 independent experiments. Data were analysed for statistical significance by One-way ANOVA with post hoc Bonferroni test. ** $P < 0.01$, compared to control cells.

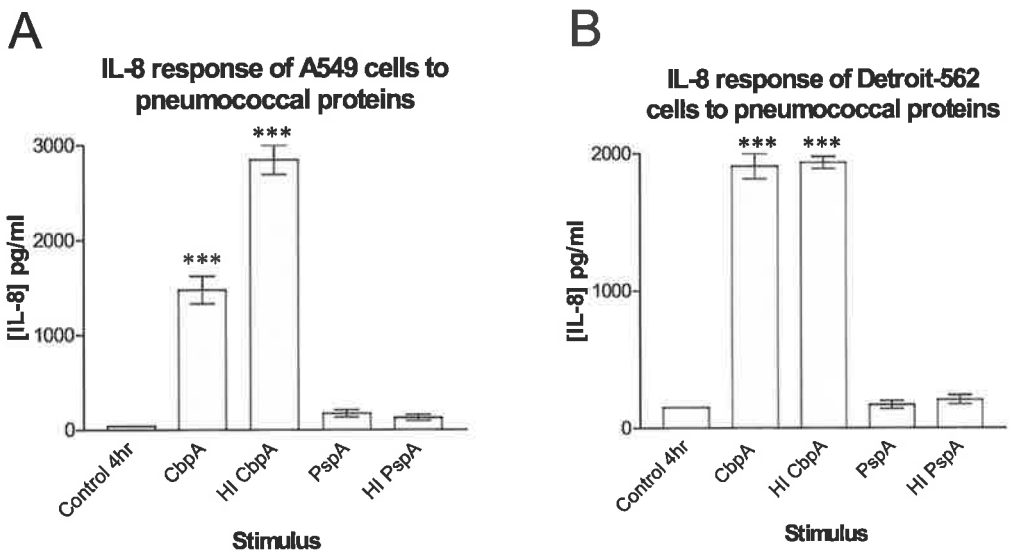


**Figure 4.19. IL-8 response of respiratory epithelial cells to pneumococcal proteins**. Confluent monolayers of A549 or Detroit-562 cells were incubated with 10 μg/ml native or HI CbpA or PspA, for 4 h before cellular supernatant was collected and assayed for IL-8 by ELISA. Data shown are mean ± SE from 3 independent experiments. Data were analysed for statistical significance by One-way ANOVA with post hoc Bonferroni test. *** $P < 0.001$, compared to control cells.

to control cells ($P < 0.001$ for both) (Figure 4.19). Overall, the responses to CbpA preparations were much greater than those to PspA preparations, which did not elicit a significant increase in IL-8 from either cell type compared to control cells (Figure 4.19).

The fact that heat denaturation did not reduce chemokine responses to the purified proteins suggested that the preparations contained significant levels of lipopolysaccharide (LPS). Samples were analysed for LPS contamination using the Limulus amoebocyte lysate (LAL) assay at the IMVS (Frome Rd, Adelaide) and found to contain > 80 endotoxin units (EU)/ml, which is enough to stimulate chemokine production from these cells. This LPS appeared to be stimulating the cells such that any effect that the pneumococcal proteins had on chemokine production was being masked by the response to LPS itself. Thus, it was important to remove contaminating LPS if the role of these proteins in chemokine production was to be determined. Protein preparations were treated with polymyxin-B as described in Section 2.8.5 in an attempt to absorb LPS. After treatment with polymyxin-B, LPS levels in the protein preparations were reduced to approximately 50 EU/ml (Results not shown), but were still sufficiently high that cells treated with both HI preparations, elicited responses that were greater than those to non-HI preparations (Figure 4.20).

### 4.3.2 Expression of pneumococcal proteins in *E. coli* BL21(DE3) *lpxM*$^-$

The problem of LPS contamination could theoretically be overcome if the pneumococcal proteins were expressed in a strain of *E. coli* that produced a mutant form of LPS unable to stimulate epithelial cells. In 2003 Cognet *et al.* described a derivative of the *E. coli* expression strain BL21(DE3) that has a mutation in the *lpxM* gene which is responsible for myristoylating LPS. These mutants express a non-myristoylated form of LPS that is unable to stimulate LPS sensitive cells, as assessed by TNFα and IL-8 production from dendritic cells, and activation of TLR4 on these cells (Cognet *et al.*, 2003). This strain was kindly provided by Jean-Francois Gauchat, from The University of Montreal, Quebec,

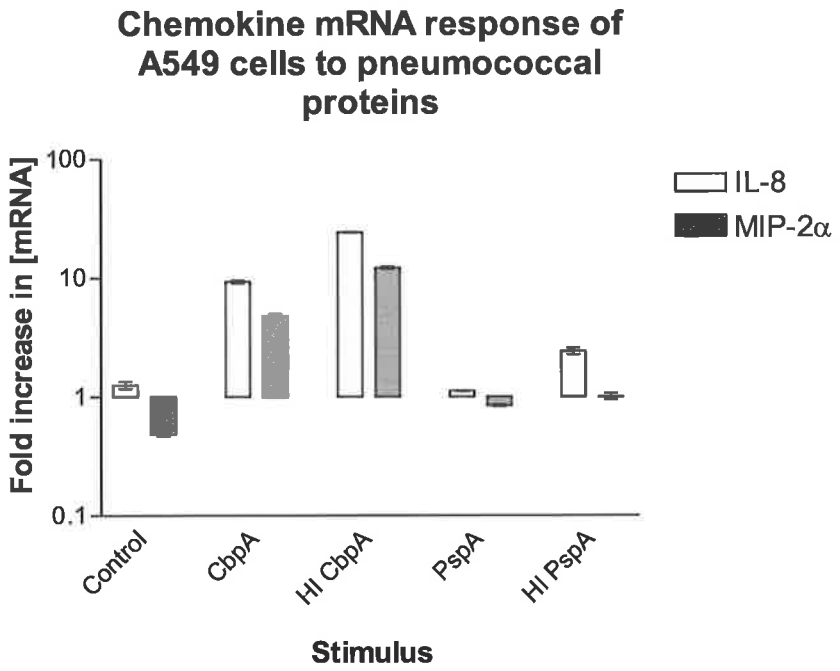


**Figure 4.20. Response of A549 cells to pneumococcal proteins after treatment with polymyxin B.** Confluent monolayers of A549 cells were incubated with 10 μg/ml CbpA or PspA that had been treated with Polymyxin B in an attempt to remove LPS from protein preparations, or HI preparations of the same. RNA was extracted at 4 h and analysed for chemokine specific mRNA by real-time RT-PCR. Data shown are mean ± SE of 3 independent experiments.

Canada. The pQE30-based expression constructs for the recombinant $His_6$ tagged CbpA and PspA proteins used in the above experiments were isolated from their original expression strain and transformed into competent BL21(DE3) *lpxM*$^-$ *E. coli* as described in Section 2.11. The recombinant proteins were then expressed in this strain and purified by Ni-NTA chomatography, as described in Sections 2.7.1 and 2.7.2. Both CbpA and PspA were judged to be pure by SDS-PAGE analysis (Figure 4.21).

### 4.3.3 IL-8 and MIP-2α mRNA response of respiratory epithelial cells to CbpA and PspA

To assess whether or not expression of CbpA and PspA in this mutant strain of *E. coli* overcame the problem of cell activation by contaminating LPS, and to elucidate the abilities of these proteins to elicit a chemokine response in these cell lines, A549 and Detroit-562 cells were incubated with 10 μg/ml native or HI preparations of these proteins for 4 h, at which time RNA was extracted and analysed for the presence of chemokine mRNA by real-time RT-PCR, as described in Section 2.10.3. After being incubated with CbpA for 4 h, A549 cells showed an approximately 7-fold increase in MIP-2α mRNA when compared to control cells ($P < 0.01$); IL-8 mRNA was also elevated approximately 3-fold, but this did not reach statistical significance (Figure 4.22).

However, when the preparation of CbpA was heat inactivated both the IL-8 and MIP-2α mRNA response was significantly increased when compared to control cells (approximately 9-fold, and 24 fold respectively) ($P < 0.001$ for both), and the increase in the IL-8 mRNA response by cells exposed to this preparation was approximately 3-fold when compared to the response elicited by native CbpA ($P < 0.01$) (Figure 4.22). Cells exposed to PspA exhibited an approximately 6-fold increase in both IL-8 and MIP-2α mRNA when compared to control cells ($P < 0.01$ for both), and this response was reduced in cells treated with HI protein. The IL-8 mRNA response elicited by HI PspA was approximately 3-fold less than to native PspA, and not significantly higher than that of control cells. The MIP-2α

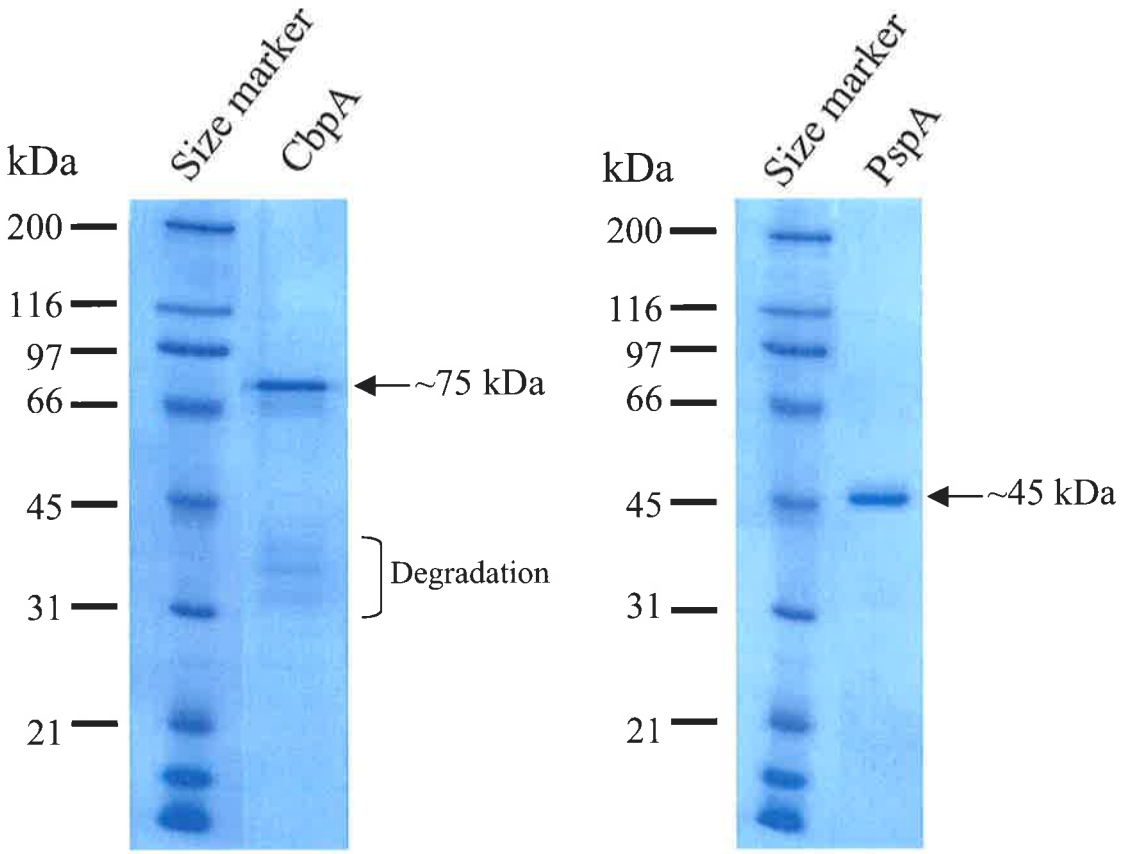


**Figure 4.21. SDS-PAGE analysis of PspA and CbpA.** Purified recombinant PspA and CbpA from *E. coli* BL21 (DE3) *lpxM*$^{-}$ to be used in chemokine induction assays were subjected to SDS-PAGE analysis and stained with Coomasie brilliant blue (as described in Section 2.8.1). The mobilities of the size marker and the proteins are indicated in kDa. A small amount of degradation was observed in CbpA as indicated.

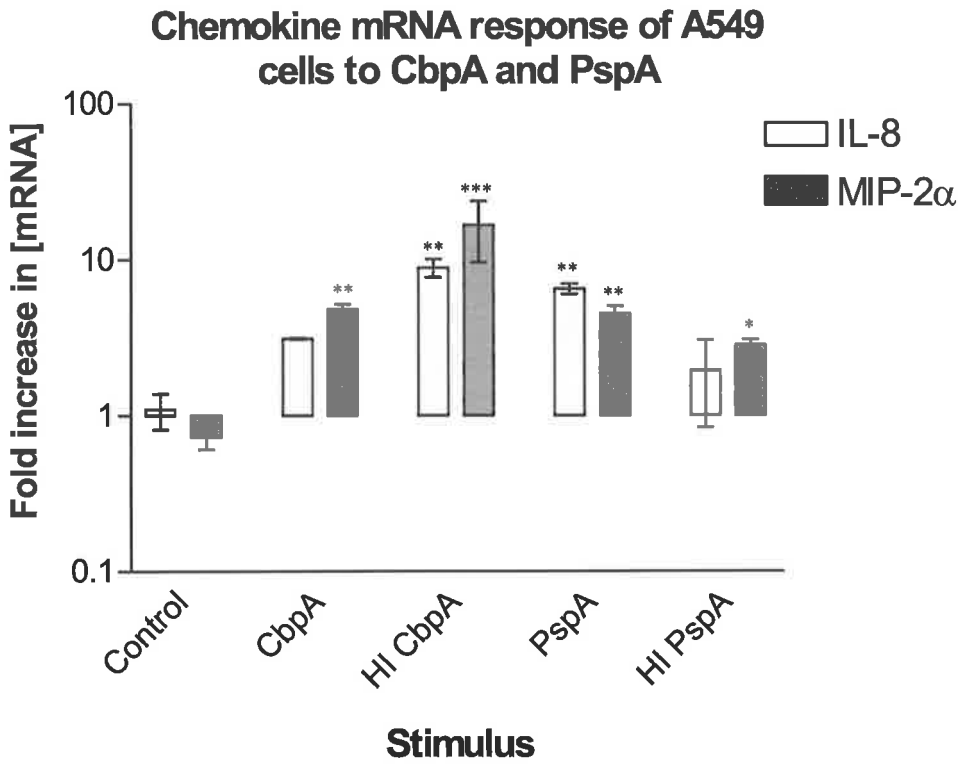


**Figure 4.22. CXC chemokine mRNA response of type II pneumocytes to CbpA and PspA.** Confluent monolayers of A549 cells were incubated with 10 μg/ml native or HI CbpA or PspA for 4 h before extraction of cellular RNA and analysis for chemokine mRNA by real-time RT-PCR. Data shown are mean ± SE of 3 independent experiments. Results were assessed for statistical significance by One-way ANOVA with post-hoc Bonferroni test. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$, when compared to uninfected control cells.

mRNA response to HI PspA was reduced approximately 1.5-fold compared to that for native PspA but was still approximately 4-fold higher than control cells ($P < 0.05$) (Figure 4.22).

Similar results were seen with nasopharyngeal epithelial cells; with both preparations of CbpA eliciting a significant increase in chemokine mRNA when compared to control cells. Native CbpA elicited an approximately 6-fold increase in IL-8 mRNA compared to control cells, and HI CbpA elicited an approximately 19-fold increase in IL-8 mRNA (CbpA, $P < 0.01$; HI CbpA, $P < 0.001$) (Figure 4.23). The HI preparation elicited an approximately 3-fold higher chemokine mRNA response than native CbpA ($P < 0.05$ for both IL-8 and MIP-2α). Native PspA did not elicit a significant increase in the chemokine mRNA response when compared to control cells (Figure 4.23), but HI PspA elicited a significantly increased MIP-2α mRNA response in Detroit-562 cells ($P < 0.05$). However, there was no significant difference in the response generated by these cells towards HI PspA when compared to native PspA (Figure 4.23).

### 4.3.4 Secretion of IL-8 by respiratory epithelial cells in response to CbpA and PspA

To determine whether any observed increase in chemokine mRNA was accompanied by an increase in IL-8 protein production by the cells, culture SN from the A549 and Detroit-562 monolayers incubated for 4 h with 10 μg/ml native or HI CbpA or PspA was assayed for IL-8 by ELISA as described in Section 2.13 (Figure 4.24).

In agreement with the increase seen in chemokine mRNA in Section 4.3.3, a significant (approximately 8-fold) increase in production of IL-8 protein was seen in A549 cells treated with CbpA compared to control cells ($P < 0.001$). This response was increased to 10-fold when CbpA was heat-inactivated ($P < 0.001$), although the difference between CbpA and HI CbpA did not reach statistical significance (Figure 4.24 A). PspA induced an approximately 7-fold increase in IL-8 response in these cells when compared to control cells ($P < 0.001$), and this response was reduced approximately 3-fold when PspA was heat-

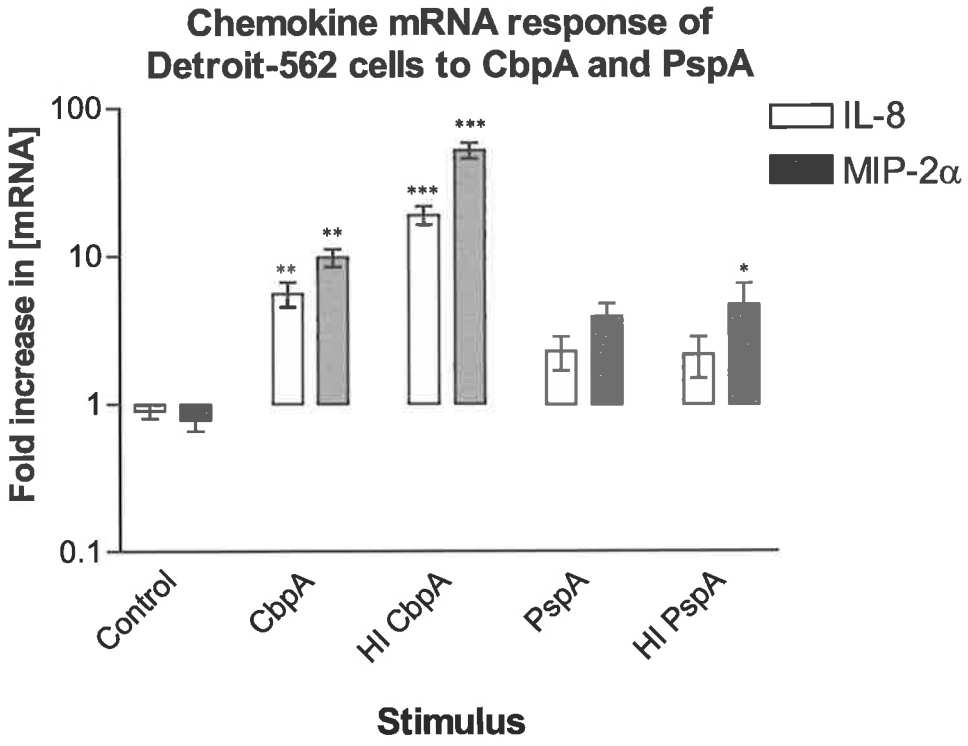


**Figure 4.23. CXC chemokine mRNA response of nasopharyngeal epithelial cells to CbpA and PspA.** Confluent monolayers of Detroit-562 cells were incubated with 10 μg/ml native or HI CbpA or PspA for 4 h before extraction of cellular RNA and analysis for chemokine mRNA by real-time RT-PCR. Data shown are mean ± SE from 3 independent experiments. Results were assessed for statistical significance by One-way ANOVA with post-hoc Bonferroni test. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$, when compared to control cells.

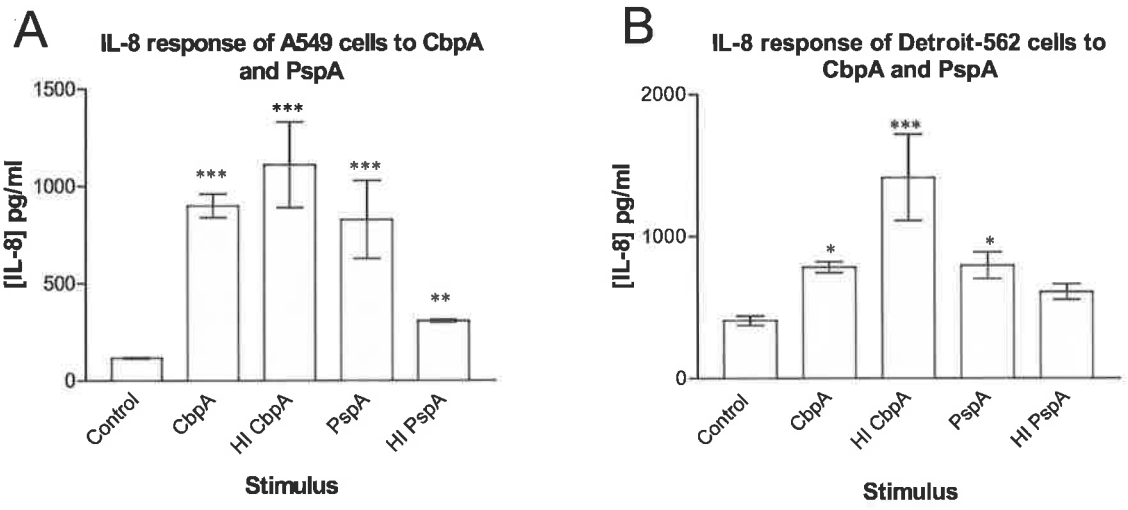


**Figure 4.24. IL-8 response of respiratory epithelial cells to CbpA and PspA.** Confluent monolayers of A549 (A) or Detroit-562 (B) cells were incubated with 10 μg/ml native or HI CbpA or PspA for 4 h before recovery of cell culture supernatant and analysis for IL-8 by ELISA. Data shown are mean ± SE from 3 independent experiments. Results were assessed for statistical significance by One-way ANOVA with post-hoc Bonferroni test. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$, when compared to control cells.

inactivated, although it was still increased approximately 2-fold compared to control cells ($P < 0.01$) (Figure 4.24 A).

Detroit-562 cells displayed an approximately 2-fold increase in IL-8 response to CbpA relative to control cells ($P < 0.05$), and an approximately 4-fold increase was seen in response to HI CbpA ($P < 0.001$), which was also 2-fold greater than the response to native CbpA ($P < 0.05$). The IL-8 response of these cells to PspA was approximately 2-fold higher than uninfected control cells ($P < 0.05$), and heat-inactivation of PspA reduced this response to levels not significantly different from the control cells (Figure 4.24 B).

### 4.3.5 Chemokine induction by type II pneumocytes and nasopharyngeal epithelial cells in response to Ply

To investigate the role of Ply in inducing a chemokine response from type II pneumocytes and nasopharyngeal epithelial cells, confluent monolayers of A549 or Detroit-562 cells were incubated with 300 haemolytic units (HU)/ml of native Ply purified for 4 h before collection of cellular RNA analysis for chemokine specific mRNA by real-time RT-PCR as described in Section 2.10.3. 300 HU/ml was chosen as the appropriate concentration of Ply to use, because haemolysis assays of D39 culture SN, (see Section 2.8.4), indicated that this is the approximate amount of Ply present in $5 \times 10^7$ CFU of WT D39. LPS contamination of Ply did not appear to be a problem, since upon heat-inactivation of the preparations, levels of stimulation of cells were the same as control levels (Results not shown). After incubation with Ply for 4 h, A549 cells showed no significant increase in either IL-8 or MIP-2α mRNA when compared to untreated control cells (Figure 4.25 A). Detroit-562 cells showed an approximately 3-fold increase in IL-8 mRNA when compared to control cells ($P < 0.05$), but no significant difference was seen in MIP-2α mRNA levels (Figure 4.25 B). Analysis of cell culture SN from these experiments for the presence of IL-8 was consistent with mRNA levels. No significant increase in IL-8 was observed in A549 cells

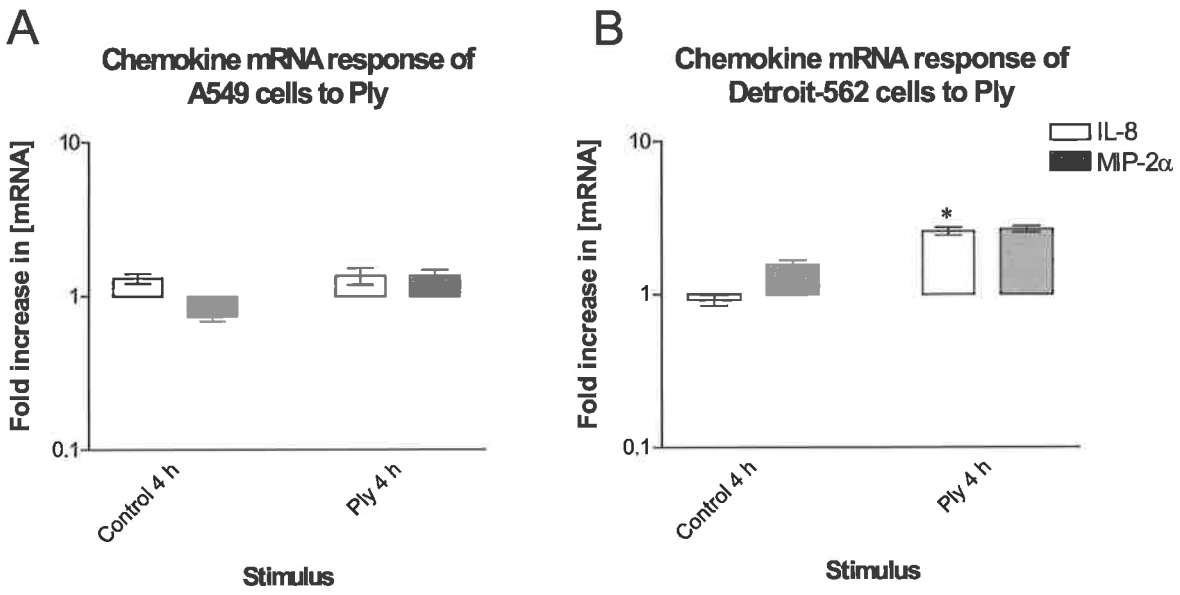


**Figure 4.25. CXC chemokine mRNA response of A549 and Detroit-562 cells to Ply.** Confluent monolayers of A549 (A) or Detroit-562 (B) cells were incubated with 300 HU of Ply for 4 h before extraction of cellular RNA and analysis for chemokine mRNA by real-time RT-PCR. Data shown are mean ± SE from 3 independent experiments. Results were assessed for statistical significance by One-way ANOVA with post-hoc Bonferroni test. * $P < 0.05$, when compared to uninfected control cells.

treated with Ply compared to control cells, and an approximately 3-fold increase in IL-8 levels was seen in Detroit-562 cells ($P < 0.05$) (Figure 4.26 A and B).

Overall, results obtained from experiments using purified CbpA, PspA, and Ply were largely consistent with the results seen using the various D39 deletion mutants. However, due to the inability to eliminate the possibility that contaminating factors in the protein preparations were affecting the responses, interpretation of these protein studies is difficult.

## 4.4 Discussion

In Chapter Three it was shown that infection of type II pneumocytes (A549), and nasopharyngeal epithelial cells (Detroit-562) with *S. pneumoniae* leads to the generation of a CXC chemokine response, as measured by chemokine mRNA and IL-8 protein. In this chapter, the contributions of the known pneumococcal virulence factors CbpA, PspA, and Ply to generation of these responses were examined. Each was found to play some role in modulation of the CXC chemokine response from these cells under the conditions used in this assay.

### 4.4.1 CbpA suppresses IL-8 and MIP-2α production from both A549 and Detroit-562 cells

The importance of CbpA in pneumococcal pathogenesis has been well established; it has been shown in several studies to contribute to nasopharyngeal colonisation (Rosenow *et al.*, 1997; Balachandran *et al.*, 2002; Orihuela *et al.*, 2004a), and may mediate invasion across epithelial cells by binding pIgR (Zhang *et al.*, 2000). CbpA has previously been reported to have a role in stimulating IL-8 release from type II pneumocytes (Madsen *et al.*, 2000; Murdoch *et al.*, 2002). However, the results presented in this study are not consistent with such a role, and suggest that CbpA actually suppresses the IL-8 response from both type II pneumocytes and nasopharyngeal epithelial cells. Initial results obtained using cells exposed

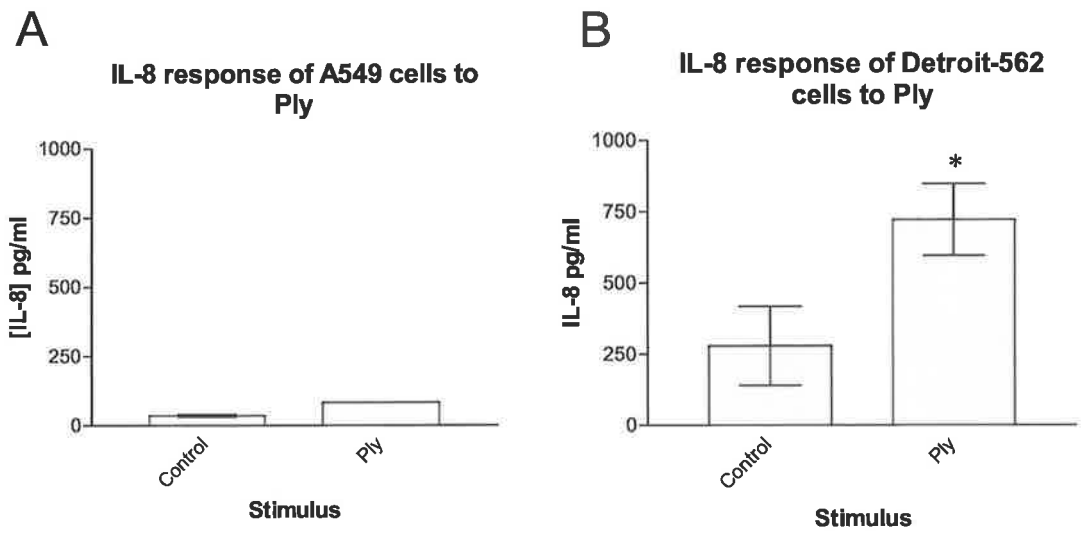


**Figure 4.26. IL-8 response of respiratory epithelial cells to Ply.** Confluent monolayers of A549 (A) or Detroit-562 (B) cells were incubated with 300 HU of Ply for 4 h before recovery of cell culture supernatant and analysis for IL-8 by ELISA. Data are mean ± SE from 3 independent experiments. Results were assessed for statistical significance by One-way ANOVA with post-hoc Bonferroni test. * $P < 0.05$, compared to control cells.

to the insertion-duplication mutant CbpA$^-$ showed a significant increase in CXC chemokine mRNA and IL-8 protein production in both A549 and Detroit-562 cells compared to cells incubated with WT D39 (Figures 4.2, 4.3 and 4.4). The incompatibility of these results with those previously published led to the hypothesis that the mutant may be releasing a truncated N-terminal fragment of CbpA, due to the mutagenesis procedure employed. Such a truncated CbpA fragment would probably not be anchored to the pneumococcal cell wall, and therefore could potentially interact better with cell monolayers, stimulating these cells to produce higher levels of IL-8 than those elicited by WT D39. To test this hypothesis, an in-frame deletion mutant was constructed in which the entire gene for CbpA was deleted from the D39 chromosome, and the ability of this mutant to elicit a chemokine response from A549 and Detroit-562 cells was assessed. Results obtained using ΔCbpA were consistent with those obtained using CbpA$^-$; both A549 and Detroit-562 cells generated an IL-8 and MIP-2α mRNA and IL-8 protein response to ΔCbpA that was approximately 2-fold higher than to WT D39 (Figures 4.14, 4.15 and 4.16). This suggests that the increase in CXC chemokine responses observed towards CbpA$^-$ was due to the absence of CbpA, and not to production of a soluble truncated form of the protein. The reasons for the contradiction between the results reported here and previously published results regarding the role of CbpA in CXC chemokine induction are unclear, but there are a number of differences in the experimental models used in these studies.

The first study to suggest a role for CbpA in stimulation of a chemokine response from respiratory epithelial cells was that of Madsen *et al.*, (2000). In this study, A549 monolayers were stimulated with $1 \times 10^8$ CFU of a heat-killed (HK) descendent of the *S. pneumoniae* Rx strain (CP1200), or SN from $1 \times 10^8$ CFU CP1200 grown in THY for 4 h, and IL-8 secretion from these cells was measured by ELISA. After 4 h, levels of IL-8 were increased 3-fold in response to the HK cells when compared to uninfected control cells, and 33-fold in response to the culture SN. Murdoch *et al.*, (2002), also measured the IL-8

response of A549 cells to $1 \times 10^8$ CFU HK *S. pneumoniae* of a range of strains, including D39, or SN from these bacteria. After 4 h, the IL-8 release by cells in response to HK *S. pneumoniae* was approximately 3-fold greater than that by uninfected control cells, and the response to culture SN was approximately 28-fold greater than control cells. There was no significant difference seen in the response to any of the different serotypes tested. The results presented in Chapter Three also showed a significant increase (approximately 6-fold) in the IL-8 response of A549 cells to *S. pneumoniae* after infection for 4 h when compared to control cells (Figure 3.3). However in this study, live *S. pneumoniae* were used to infect cells, not HK as were used in previous studies. The use of live *S. pneumoniae* presumably allows the bacteria to interact with the epithelial cells in a manner that better reflects the situation that occurs during natural infection.

When the bacterial culture SN from CP1200 that was used to stimulate cells was subjected to SDS-PAGE, a 90 kDa band was visualised, and this protein was found to have an amino acid sequence identical to that for CbpA, except for a reduction in the number of choline-binding repeats (Madsen *et al.*, 2000). This reduction in choline-binding repeats may explain why a substantial amount of the protein could be released into the SN and not remain anchored to the pneumococcal surface. Culture SN from D39 was also shown to contain CbpA at levels that were measurable by Western blot, but only after concentrating the samples (Murdoch *et al.*, 2002), which may suggest that CbpA is actually present in the SN of D39 at very low levels. Both of these studies claim that CbpA in the SN was responsible for the observed increase in IL-8 response of A549 cells.

Stimulation of A549 cells with SN from CbpA-negative D39 or CP1200 led to an IL-8 response that was approximately 50% of that generated towards SN from WT D39 or CP1200 (Madsen *et al.*, 2000; Murdoch *et al.*, 2002), suggesting a role for CbpA in the IL-8 response observed towards the WT. The differences observed in these studies compared to the results presented in this chapter may be related to differences in the experimental design and the

strains used. CbpA is not normally a secreted protein and is usually found anchored to the pneumococcal surface, so the use of *S. pneumoniae* culture SN to stimulate cells may not present CbpA to the cells in the same conformation, or context as when it is attached to the surface, and in the presence of other pneumococcal factors.

Studies presented in this chapter with live ΔCbpA, and CbpA$^-$ *S. pneumoniae* demonstrated an approximately 2-fold increase in the IL-8 response of A549 cells towards these mutants when compared to WT D39 (Figures 4.4 A and 4.16 A). A previous report demonstrated that stimulation of A549 cells with HK CbpA-negative CP1200 led to a nearly 2-fold reduction in IL-8 release compared to HK WT CP1200, or SN from WT CP1200 (Madsen *et al.*, 2000). Heat killing may denature some of the proteins produced by *S. pneumoniae*, and would also not allow the bacteria to interact with the A549 cell monolayer in a way that reflects natural infection. This, together with the fact that unlike D39, CP1200 is an unencapsulated strain may account for the differences seen between the results presented here and those reported by Madsen *et al.*, (2000).

Experiments with purified recombinant CbpA supported the results obtained with ΔCbpA. Exposure to a CbpA preparation contaminated with LPS led to an increase in CXC chemokine mRNA and IL-8 from type II pneumocytes, and this response was increased upon heat inactivation of CbpA (Figures 4.18 A and 4.19 A). This suggests that native CbpA was inhibiting the response of the epithelial cells to the LPS present in the preparation, such that when this property of CbpA was abolished by heat inactivation, the response to LPS was increased. Similar results were seen in response to CbpA purified from the *lpxM*$^-$ strain of *E. coli* with cells exposed to native CbpA showing a significantly reduced response when compared to cells exposed to HI CbpA ($P < 0.01$) (Figures 4.22, 4.23 and 4.24). These preparations should have been free from active LPS, but it is possible that there may have been other factors in the preparation that were stimulating the cells. The continued presence of heat stable stimulatory factors in protein preparations, and the problems this presented in

interpreting results meant that purified proteins were not used in future experiments to investigate the role of these proteins in CXC chemokine induction.

In previous studies, treatment of A549 cells with 10 μg/ml recombinant CbpA for 8 h led to an increase in IL-8 and MCP-1 mRNA. IL-8 protein production was also increased in response to 0.01 – 10 μg/ml CbpA in a dose dependent manner (Murdoch *et al.*, 2002). However, the CbpA used in these experiments is the same preparation as that used in the initial studies presented in Section 4.3 that was found to be contaminated with substantial levels of LPS. This could account for the response of A549 cells that was observed, and since no data were shown on the effects of heat-inactivation of the CbpA preparations used, it seems likely that the observed response is due to contaminating LPS and not to CbpA itself. When A549 cells were treated with CbpA purified based on its C3 binding properties, from CP1200, there was a dose response peaking at treatment with 7.2 pmoles (approximately 648 ng/ml) of CbpA. This concentration elicited a 16-fold increase in IL-8 compared to control cells (Madsen *et al.*, 2000). The same study also demonstrated that treatment with greater than 1 μg/ml CbpA reduced the IL-8 response of A549 to levels below that of control cells. This suggests that CbpA at concentrations above 1 μg/ml inhibits the release of IL-8 from A549 cells. These results are partially compatible with the results presented in this chapter. In this study, treatment of A549 cells with 10 μg/ml of CbpA that had been heat inactivated led to a greater increase in IL-8 release than did treatment with native CbpA, suggesting that the IL-8 response to other factors in the protein preparation was being suppressed by the presence of functional CbpA (Figures 4.19 A and 4.24 A).

A recent study by Peppoloni *et al.*, (2005), demonstrated that CbpA$^-$ *S. pneumoniae* induced higher levels of MIP-2 from microglial cells than WT *S. pneumoniae* (Peppoloni *et al.*, 2005), thus providing evidence that CbpA may have a inhibitory effect on CXC chemokine release by some host cell types. Overall, the results presented here suggest that contrary to previously published data, in this model CbpA appears to inhibit chemokine

production from respiratory epithelial cells. This may provide a mechanism by which *S. pneumoniae* is able to inhibit stimulation of the chemokine response, and thus prevent the subsequent neutrophil influx, which would contribute to clearance of the bacteria. Suppressing the early inflammatory response may also allow bacterial numbers to reach higher levels, causing a prolonged inflammatory response that is damaging to the epithelium, aiding invasion and spread of pneumococci to other host sites. However, the mechanism by which it does this and the actual region(s) of the molecule responsible for this activity remain unknown, the latter being the subject of investigation in Chapter Five.

### 4.4.2 PspA stimulates IL-8 and MIP-2α production from A549 cells but not Detroit-562 cells

PspA is thought to have the ability to inhibit complement activation, preventing deposition of C3 onto the pneumococcal surface and thus enabling the pneumococcus to avoid opsonophagocytosis (Tu *et al.*, 1999). This function makes PspA important for survival of *S. pneumoniae* in the blood, as indicated by studies in which PspA-negative mutants showed a significant reduction in systemic virulence compared to WT D39 (McDaniel *et al.*, 1987; Briles *et al.*, 1988; Berry and Paton, 2000; Balachandran *et al.*, 2002). The *pspA* gene has also been shown by RNA analysis to be upregulated in the blood of infected mice compared to *in vitro* levels (Ogunniyi *et al.*, 2002; Orihuela *et al.*, 2004b), further implicating its importance in this host niche. The importance of PspA for survival of *S. pneumoniae* in the nasopharynx is less well established. It has been demonstrated that PspA-negative mutants are able to colonise the nasopharynx as well as WT D39 (Orihuela *et al.*, 2004a), suggesting that it plays little role in virulence at this site. However, a PspA-negative mutant was significantly less virulent than WT D39 after intranasal infection of mice (Berry and Paton, 2000), and immunisation of mice with PspA has been shown to protect against nasopharyngeal carriage (Wu *et al.*, 1997; Briles *et al.*, 2000a). There are no previously published data on the effect of PspA on chemokine release by epithelial cells. In the present

study, a significantly higher IL-8 and MIP-2α mRNA, and IL-8 protein response was elicited from both A549 and Detroit-562 cells by the insertion-duplication mutant PspA$^-$ compared to WT D39 at 4 h. An in-frame deletion mutant of *pspA* (ΔPspA) was then constructed in D39 for the same reasons outlined above for CbpA. Interestingly, infection of A549 cells with ΔPspA led to a different response when compared to PspA$^-$. The CXC chemokine mRNA and IL-8 protein response of these cells changed from being increased 2-fold compared to WT D39 in response to PspA$^-$, to being decreased 2-fold compared to WT D39 in response to ΔPspA (Figures 4.2, 4.4 A, 4.14 and 4.16 A). This suggests that PspA has the ability to stimulate an early IL-8 and MIP-2α mRNA and IL-8 protein response from A549 cells. Although no fragment of PspA could be detected by Western blotting in culture SN from PspA$^-$, that does not completely discount its existence. Indeed, these results suggest that a fragment was present and able to stimulate cells infected with PspA$^-$, such that they increased CXC chemokine release compared to WT D39. Experiments with purified recombinant PspA showed a much lower chemokine response to this protein than to CbpA, but supported results obtained using the PspA mutants, with native PspA inducing significantly increased chemokine mRNA and IL-8 protein responses in type II pneumocytes, which were reduced upon heat-inactivation. These results suggest that PspA may have a direct role in eliciting a chemokine response from type II pneumocytes in this model. This has not been suggested by previous studies, with PspA mainly thought to be important in sepsis (Ogunniyi *et al.*, 2002; Orihuela *et al.*, 2004b), and prevention of pneumococcal clearance from the blood (Neeleman *et al.*, 1999; Tu *et al.*, 1999). Thus, this study may indicate a new role for PspA in the generation of a chemokine response from type II pneumocytes, at least in the model used here. Chemokine release from respiratory epithelial cells is important in recruiting neutrophils to the site of infection where clearance of the bacteria can occur (Bergeron *et al.*, 1998; Kadioglu *et al.*, 2000). The increase in chemokine release from A549 cells in response to PspA may represent a mechanism by which the host is able to recognise the presence of

pneumococci at this site and recruit neutrophils. *In vivo*, *S. pneumoniae* taken from the lungs of mice have been shown to decrease expression of *pspA* compared to expression *in vitro,* as measured by levels of mRNA (Le Messurier *et al.*, 2005). *S. pneumoniae* may decrease expression of PspA in the lungs to avoid stimulating a chemokine response from type II pneumocytes that would lead to neutrophil influx and promote clearance of the pneumococci.

Infection of Detroit-562 cells with PspA$^-$ led to a significant increase in chemokine mRNA and IL-8 protein 4 h post infection when compared to levels elicited by WT D39. However, this effect was eliminated upon infection of cells with ΔPspA, with no significant difference seen in the IL-8 or MIP-2α response of Detroit-562 cells to this mutant compared to WT D39 (Figures 4.3, 4.4 B, 4.15 and 4.16 B). The active fragment of PspA that was potentially produced by PspA$^-$ probably stimulated the Detroit-562 cells, because it was able to interact directly with the monolayer and was not attached to the pneumococcal surface. It is also possible that this fragment exists in a different conformation when not in the context of the rest of the molecule, and so may have a different effect on cells. These cells showed little response to purified PspA that comprised the entire N-terminal region (Figures 4.23 and 4.24 B), suggesting that in this cell line the N-terminal region of PspA has little or no role in stimulating a CXC chemokine response. PspA-negative mutants are able to colonise the nasopharynx of mice as well as WT *S. pneumoniae* (Balachandran *et al.* 2002; Orihuela *et al.*, 2004a). However, expression of *pspA* is upregulated in this *in vivo* niche compared to *in vitro* expression (Le Messurier *et al.*, 2005). This may provide an advantage to the pneumococcus because of the ability of PspA to bind Lf, which may help it to survive at mucosal surfaces by protecting it from killing by apolactoferrin (Hammerschmidt *et al.*, 1999; Hakansson *et al.*, 2001; Shaper *et al.*, 2004). The results presented here suggest that an increased expression of PspA at this host site might not induce an increase in CXC chemokine release from nasopharyngeal epithelial cells, and thus would not affect colonisation by the recruitment of neutrophils to clear the bacteria.

### 4.4.3 The role of Ply in the CXC chemokine response of respiratory epithelial cells

Ply is a multifunctional protein with a clearly demonstrated role in pneumococcal virulence. It has previously been shown to elicit a pro-inflammatory cytokine response from leukocytes (Houldsworth *et al.*, 1994), and Ply negative mutants induce much less inflammation than WT *S. pneumoniae in vivo* (Kadioglu *et al.*, 2000). However the ability of Ply to stimulate the release of CXC chemokines from epithelial cells has not previously been investigated. Type II pneumocytes demonstrated no difference in chemokine response to ΔPly compared to WT D39 at 2 h. At 4 h a significant increase was seen in the IL-8 mRNA response of these cells to ΔPly, but this was not observed at the protein level (Figures 4.15 and 4.16 A). Treatment of A549 cells with purified Ply also had no significant effect on release of IL-8 or MIP-2α mRNA or IL-8 protein (Figures 4.25 A and 4.26 A). Ply has previously been shown to be responsible for an increase in pro-inflammatory cytokines in the lungs, and recruitment of leukocytes to the site of infection (Bergeron *et al.*, 1998; Kadioglu *et al.*, 2000) but the results presented in this study suggest that type II pneumocytes are unlikely to be the source of chemokines involved in neutrophil recruitment to the lungs in response to Ply. Chemokines released by other cells present in the lungs, such as alveolar macrophages, may be responsible for recruiting neutrophils and other leukocytes to the site of infection (Houldsworth *et al.*, 1994). These results also suggest that although Ply has been shown to promote lung injury (Kadioglu *et al.*, 2000), and is reported to be important in translocation of pneumococci from the lungs to the blood (Orihuela *et al.*, 2004a), CXC chemokine release from type II pneumocytes does not appear to be mechanistically involved in these processes.

Infection of Detroit-562 cells with ΔPly led to a significant decrease in IL-8 mRNA and protein compared to cells infected with WT D39, and incubation of these cells with purified Ply led to a significant increase in IL-8 mRNA and protein compared to control cells

(Figures 4.15, 4.16 B, 4.25 B and 4.26 B). These results suggest that Ply is responsible for eliciting an IL-8 response from nasopharyngeal epithelial cells in this model. *In vivo*, Ply negative mutants are less capable of colonising the nasopharynx than their WT counterparts, and show a reduced capacity to translocate to the lungs from this site (Kadioglu *et al.*, 2002; Orihuela *et al.*, 2004a). It has also recently been shown that Ply interacts with TLR4 to stimulate the release of IL-6 and TNFα from macrophages, and levels of nasopharyngeal colonisation in TLR4 deficient mice were higher than in wild-type mice (Malley *et al.*, 2003). TLR4 deficient mice were also more susceptible to intranasal challenge with WT *S. pneumoniae* than WT mice, but this difference was not seen when the challenge strain was Ply-negative (Malley *et al.*, 2003). It is possible that Ply interacts with TLR4 on Detroit-562 cells to stimulate a chemokine response from these cells. However, any connection between the importance of Ply in nasopharyngeal colonisation and invasion *in vivo* and its suggested role in IL-8 induction from nasopharyngeal epithelial cells *in vitro* remains unclear, and further study is required before the relevance of the observed IL-8 response can be determined.

# Chapter Five – Investigation of the Specific Domains Responsible for CXC Chemokine Inhibition by CbpA

## 5.1 Introduction

As described in Chapter One, CbpA is considered an important virulence factor with reported roles in adherence to host cells (Cundell *et al.*, 1995a; Zhang *et al.*, 2000) and induction of an IL-8 response from respiratory epithelial cells (Madsen *et al.*, 2000; Murdoch *et al.*, 2002). The latter role was contradicted in Chapter Four, where it was shown that a ΔCbpA mutant exhibited an increase in IL-8 mRNA when compared to the response to WT D39. Purified CbpA also appeared to suppress production of IL-8 and MIP-2α mRNA and IL-8 protein when administered to respiratory epithelial cells, with HI CbpA eliciting a significantly higher response than native CbpA. What remains unknown are the specific regions of CbpA that are responsible for this effect, and these were investigated in this chapter. Although the crystal structure has not been determined, based on sequence data, and similarity to PspA, CbpA is predicted to be an elongated molecule with an N-terminal region that is α-helical in structure (Jedrzejas, 2001; Iannelli *et al.*, 2002; Luo *et al.*, 2004). CbpA has a number of C-terminal repeats that make up the choline-binding domain of the molecule. This region allows CbpA to bind to the surface of the pneumococcus via ChoP residues on cell wall TA and LTA. The N-terminus of the molecule can be divided further into 3 distinct domains (Iannelli *et al.*, 2002; Luo *et al.*, 2004). The first 100 – 150 aa of the N-terminus of

the mature protein show very little sequence similarity between strains, and this section is named the hypervariable region (Brooks-Walter *et al.*, 1999). Downstream of this hypervariable region there are 2 direct repeats, which are highly conserved between strains and are reported to play a role in binding of CbpA to sIgA (Elm *et al.*, 2004a; Elm *et al.*, 2004b; Luo *et al.*, 2004). Following these direct repeats is a proline-rich region that in D39 shows a high degree of similarity to the proline-rich region of PspA (Brooks-Walter *et al.*, 1999). Antibodies directed at this region have been shown to be protective against infection (Brooks-Walter *et al.*, 1999), and this region may be important in binding of CbpA to factor H (fH) (Duthy *et al.*, 2002). In this chapter, *S. pneumoniae* strains were constructed expressing CbpA derivatives in which each of these N-terminal domains and the proline rich region were individually deleted in-frame. These were then used in the chemokine induction assay described previously, in an attempt to identify the specific domain(s) of the molecule involved in the apparent inhibitory effect of CbpA on the chemokine response of respiratory epithelial cells to pneumococcal infection. Due to difficulties purifying CbpA free from LPS contamination, it was decided not to use recombinant truncated forms of CbpA to investigate the roles of these domains in the chemokine response, but rather to use CbpA domain deletion *S. pneumoniae* mutants.

## 5.2 Results

### 5.2.1 Investigation of regions of CbpA responsible for inhibition of the chemokine response from A549 and Detroit-562 cells

To elucidate the specific domain(s) of the CbpA molecule that may be involved in modulation of the chemokine response seen against *S. pneumoniae*, mutants were constructed that produce forms of CbpA in which specific domains had been deleted in-frame. Regions of *cbpA* encoding the hypervariable region (aa 1-100), the first of two small repeats (aa 101-260), the second of these repeats (aa 261-375), and the proline rich region (aa 376-445) were each individually deleted in-frame. In all mutants the leader sequence and choline-binding

domain were left unaltered to allow correct export of the protein and binding to the pneumococcal surface (Figure 5.1).

### 5.2.2 Construction of CbpA domain deletion mutants

In order to construct CbpA mutants in which specific domains were deleted, a similar strategy to that used to create ΔCbpA was utilised (Section 4.2.4.1). Using the D39 *cbpA* sequence (Genbank accession number AF068646), oligonucleotide pairs were designed to flank the regions to be deleted; each of these oligonucleotides incorporated tails complementary to the other (Table 2.2). The same method of overlap PCR utilised in construction of ΔCbpA was used to generate products in which specific regions of the gene corresponding to specific domains of the protein were deleted in frame such that translation of the rest of the molecule was unaffected (Figure 5.2). These products were transformed into competent D39 CbpA$^-$ cells as described in Section 4.2.4.1, and transformants selected by loss of Ery resistance by replicate plating onto BA and EryBA plates following Ery/Amp enrichment, as described in Section 2.12.3. Deletion of the correct region of the gene was confirmed by PCR (Figure 5.3), and sequence analysis (data not shown). For each mutant, expression of a truncated CbpA protein was confirmed by Western blot analysis using CbpA specific polyclonal antiserum (Figure 5.4). Confirmed mutants producing CbpA lacking the hypervariable region, the small repeat region 1, the small repeat region 2, or the proline-rich region were designated CbpAΔHyp, CbpAΔSR1, CbpAΔSR2, or CbpAΔPro, respectively.

### 5.2.3 Chemokine mRNA response of respiratory epithelial cells to CbpA domain mutants

To measure the chemokine mRNA response of respiratory epithelial cells to the CbpA domain mutants of *S. pneumoniae* and thus gain information on the region(s) of the molecule responsible for the apparent inhibitory effect of CbpA on chemokine induction, confluent monolayers of A549 or Detroit-562 cells were incubated with $5 \times 10^7$ CFU WT *S.*

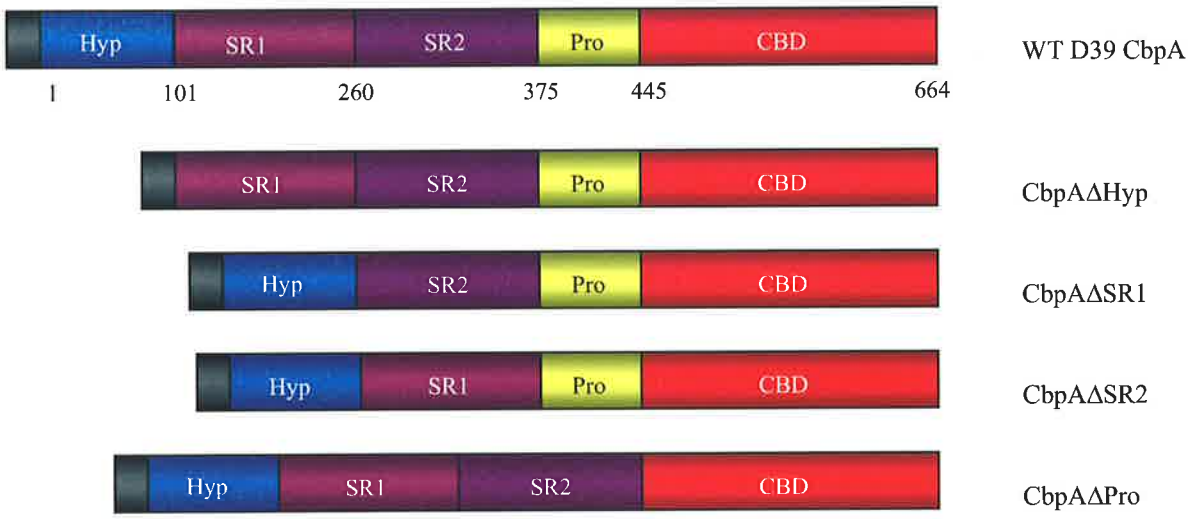


**Figure 5.1. CbpA domain deletion mutants**. Schematic representation of the CbpA molecule and the mutants with specific domain deletions. Black represents the leader sequence, blue represents the hypervariable region, plum represents the small repeat region 1, violet represents the small repeat region 2, yellow represents the proline rich region, and red represents the choline binding domain. Numbers denote amino acid residues.

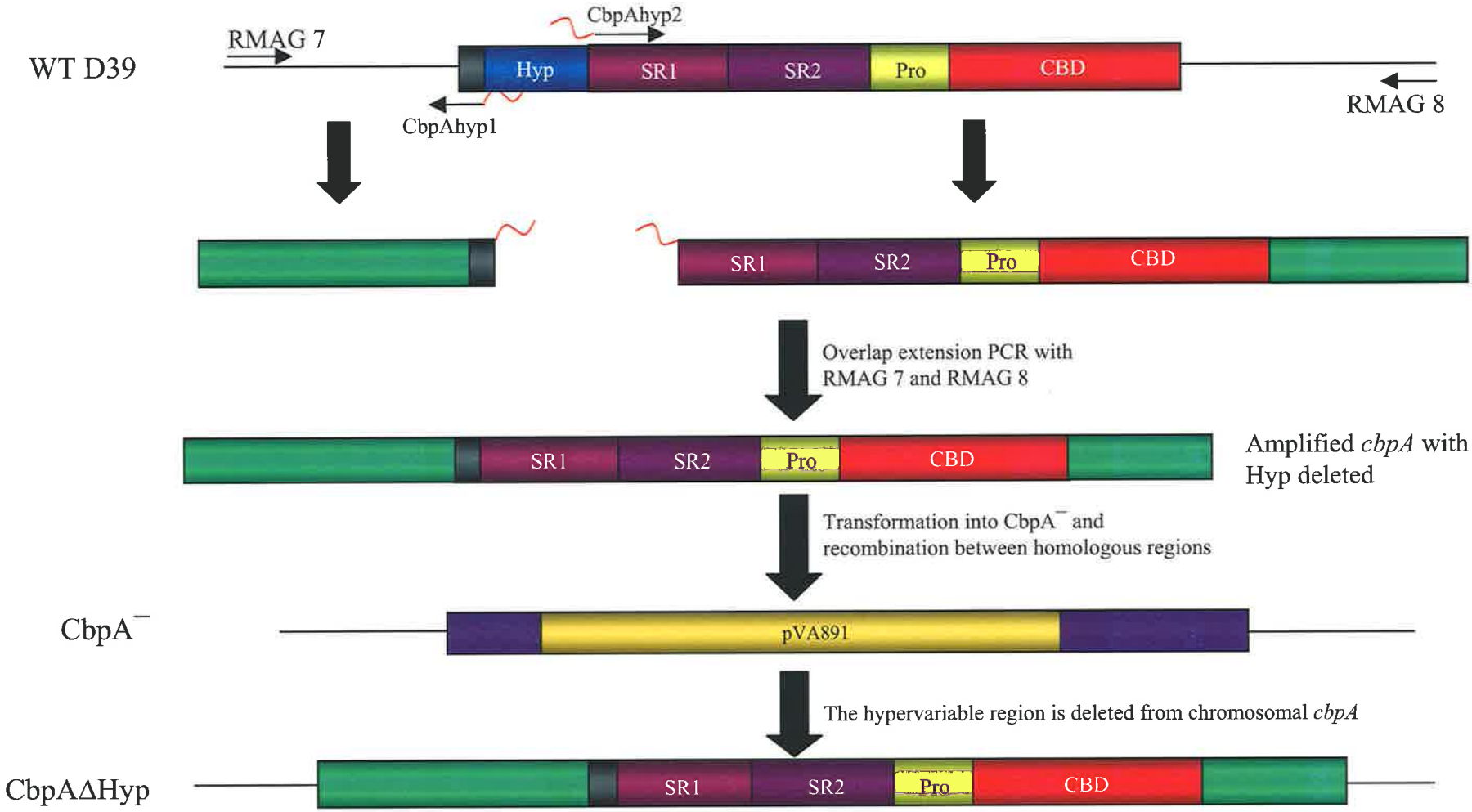


**Figure 5.2. Deletion of the hypervariable region of CbpA.** A PCR product created by overlap extension such that the sequences flanking the region encoding the hypervariable domain are amplified, without the region itself, was transformed into CbpA⁻. This results in homologous recombination between flanking regions of *cbpA* on the PCR product and those on the chromosome such that the region that encodes the hypervariable domain of CbpA is deleted from the chromosome. Other CbpA domain deletion mutants were constructed using an analogous method.

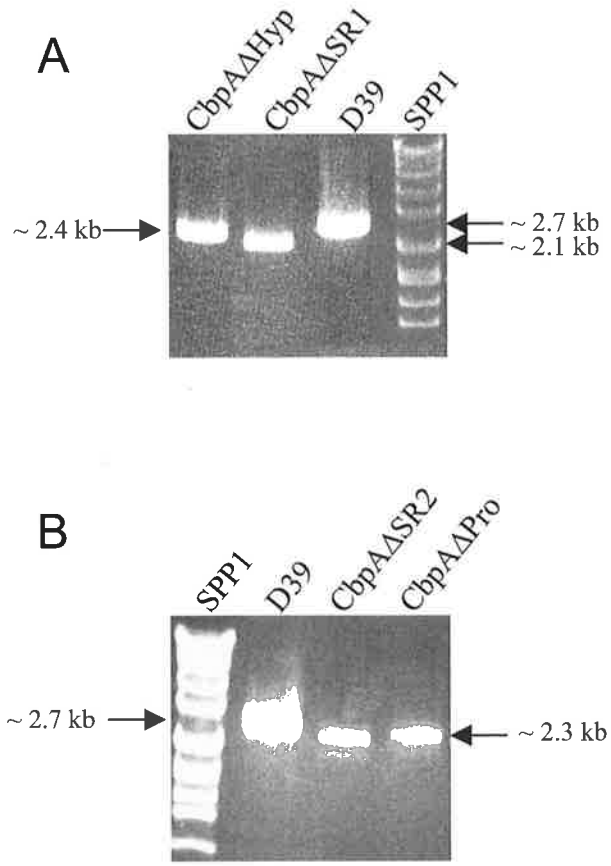


**Figure 5.3. PCR analysis of CbpA domain deletion mutants.** **A**. Colonies of *S. pneumoniae* WT D39, CbpAΔHyp, and CbpAΔSR1were subjected to direct PCR analysis using the oligonucleotides CbpAprom4 and CbpAR2 (Table 2.2). **B**. Colonies of *S. pneumoniae* WT D39, CbpAΔSR2, and CbpAΔPro were subjected to direct PCR analysis using the oligonucleotides CbpAprom4 and CbpAR2 (Table 2.2). Products were electrophoresed on a 1% agarose gel and stained with ethidium bromide as described in Section 2.9.1. Sizes of products were estimated according to mobilities relative to the SPP1 marker and are indicated.


**Figure 5.4. Western blot analysis of CbpA domain mutants.** Lysates of WT D39 and *cbpA* domain mutants were separated by SDS-PAGE, electroblotted onto nitrocellulose and reacted with mouse polyclonal antiserum specific for CbpA (as described in Section 2.8). A band of the appropriate size for CbpA (~95 kDa) was seen in the lysate of WT D39, while the sizes of bands seen in the lysates of the domain mutants that were consistent with deletion of the specific domains (~85 kDa in each case).

*pneumoniae* D39 or the various *cbpA* domain deletion mutants referred to above. Total cellular RNA was extracted at 2 and 4 h post-infection and analysed for the presence of IL-8 and MIP-2α mRNA by real-time RT-PCR using specific oligonucleotides (Section 2.10.3).

After 2 h, all A549 cells incubated with *S. pneumoniae* showed a significant increase in chemokine mRNA when compared to uninfected control cells ($P < 0.001$), but there was no significant difference in chemokine mRNA response between cells infected with WT D39 and those infected with the mutants (data not shown). After 4 h cells incubated with CbpAΔHyp showed an approximate 2-fold increase in IL-8 mRNA, and 5- fold increase in MIP-2α mRNA, when compared to cells infected with WT D39 (IL-8: $P < 0.05$, MIP-2α: $P < 0.01$) (Figure 5.5). Small increases in IL-8 mRNA levels were seen in cells infected with CbpAΔSR1, and CbpAΔSR2 when compared to cells incubated with WT D39 but these did not reach statistical significance. However, these cells showed approximate 6-fold, and 3-fold increases in MIP-2α mRNA when compared to those infected with WT D39 ($P < 0.001$, and $P < 0.05$ respectively). No significant increase in either IL-8 or MIP-2α mRNA was seen in cells infected with CbpAΔPro when compared to WT D39. Incubation with ΔCbpA led to an increase of approximately 2-fold in IL-8, and 4-fold in MIP-2α mRNA when compared to WT D39, as was seen previously in Chapter Four ($P < 0.05$, and $P < 0.01$ respectively) (Figure 5.5).

After 2 h incubation with *S. pneumoniae*, Detroit-562 cells showed a significant increase in chemokine mRNA when compared to uninfected control cells, but there was no significant difference in the chemokine mRNA response elicited by any of the mutants when compared to WT D39 (data not shown). However, after 4 h incubation levels of IL-8 mRNA were increased approximately 2-fold in cells exposed to CbpAΔHyp, CbpAΔSR1, or CbpAΔSR2, when compared to cells infected with WT D39 ($P < 0.01$, $P < 0.05$, and $P < 0.05$, respectively) (Figure 5.6). As was previously shown in Chapter Four, levels of IL-8 mRNA were also significantly increased (approximately 4-fold), in cells exposed to the mutant in

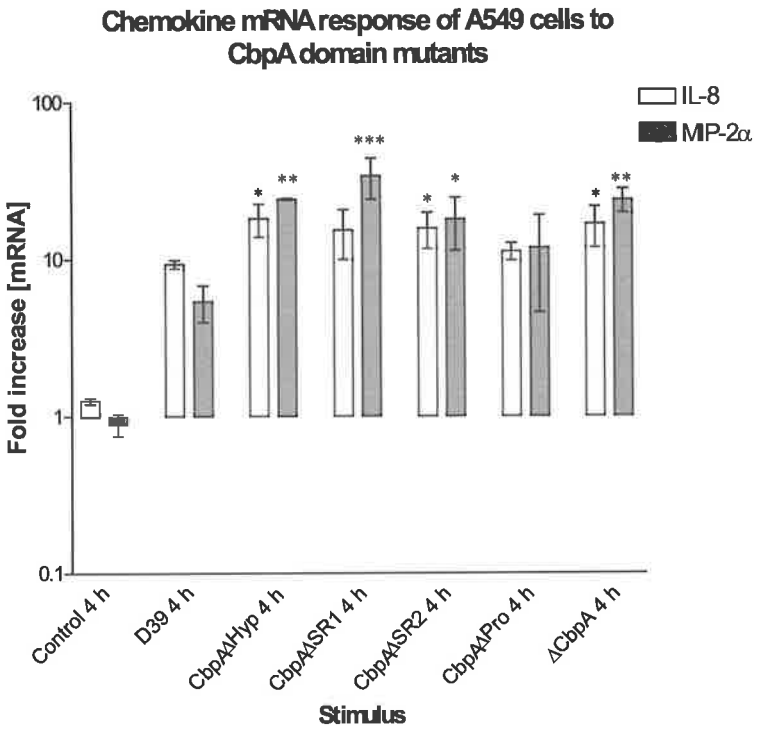


**Figure 5.5. CXC chemokine mRNA response of type II pneumocytes cells to CbpA domain mutants.** Confluent monolayers of A549 cells were incubated with $5 \times 10^7$ CFU *S. pneumoniae* D39 or otherwise isogenic mutants with in-frame deletions of regions encoding specific domains of CbpA for 4 h before extraction of cellular RNA and analysis for chemokine mRNA by real-time RT-PCR. Data are mean ± SE from 3 independent experiments. Results were analysed for statistical significance by One-way ANOVA with post-hoc Bonferroni test. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$, relative to WT D39.

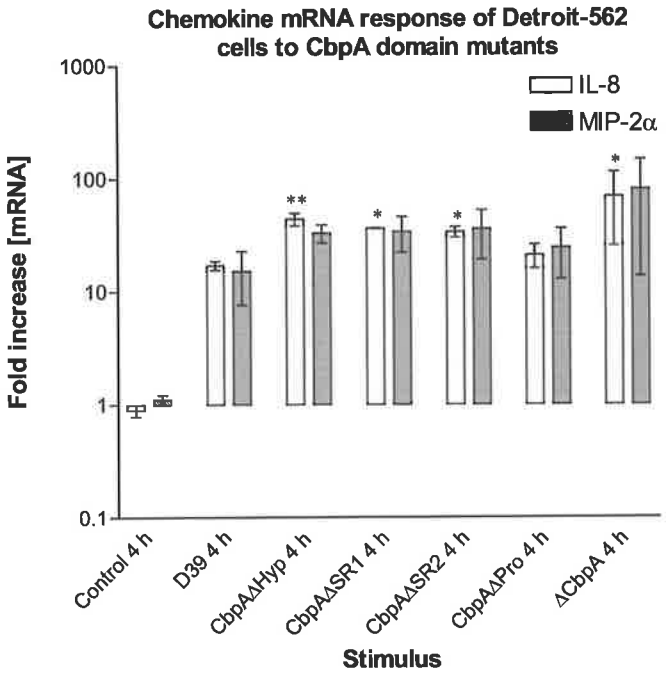


**Figure 5.6. CXC chemokine mRNA response of nasopharyngeal epithelial cells to CbpA domain mutants.** Confluent monolayers of Detroit-562 were incubated with $5 \times 10^7$ CFU *S. pneumoniae* D39 or otherwise isogenic mutants with in-frame deletions of regions encoding specific domains of CbpA for 4 h before collection of total cellular RNA and analysis for chemokine mRNA by real-time RT-PCR. Data are mean ± SE from 3 independent experiments. Data were analysed for statistical significance by One-way ANOVA with post-hoc Bonferroni test. ** $P < 0.01$; * $P < 0.05$, relative to WT D39.

which the entire *cbpA* gene had been deleted (ΔCbpA) ($P < 0.05$), and levels were comparable to those elicited by the above mentioned mutants. CbpAΔPro elicited similar levels of IL-8 mRNA as WT D39 (Figure 5.6). Differences in levels of MIP-2α mRNA followed the same trends as those seen for IL-8 mRNA, but these differences did not reach statistical significance.

### 5.2.4 IL-8 production by respiratory epithelial cells in response to CbpA domain mutants

Cell culture supernatants from the experiments described above were analysed for the presence of IL-8 protein by ELISA as described in Section 2.13. Differences in IL-8 production by the different CbpA domain mutants of *S. pneumoniae* were consistent with those observed at the mRNA level (Figure 5.7 A and B). For both A549 and Detroit-562 cells, infection with CbpAΔHyp, CbpAΔSR1, or CbpAΔSR2 elicited a significantly increased IL-8 response in comparison to WT D39, and gave a response similar to that elicited by ΔCbpA. The response elicited by CbpAΔHyp was approximately 1.7 fold greater than that elicited by WT D39 in both cell types ($P < 0.01$ and $P < 0.001$, for A549 and Detroit-562 cells, respectively). The response of A549 cells to CbpAΔSR1, and CbpAΔSR2 was approximately 2 fold greater than that to WT D39 ($P < 0.001$ for both) (Figure 5.7 A), and Detroit-562 cells showed an approximately 1.5 fold increase in IL-8 release in response to these mutants when compared to WT D39 ($P < 0.01$ and $P < 0.001$, for CbpAΔSR1 and CbpAΔSR2, respectively) (Figure 5.7 B). CbpAΔPro elicited an IL-8 response similar to that elicited by WT D39 in both cell types. The IL-8 response of both cell types to ΔCbpA was approximately 2 fold greater than that to WT D39, ($P < 0.01$ and $P < 0.001$, for A549 and Detroit-562 cells, respectively) (Figure 5.7 A and B).

Overall, these results suggest that the N-terminal domains all contribute to the inhibitory effect of CbpA on chemokine production by both A549 and Detroit-562 cells.

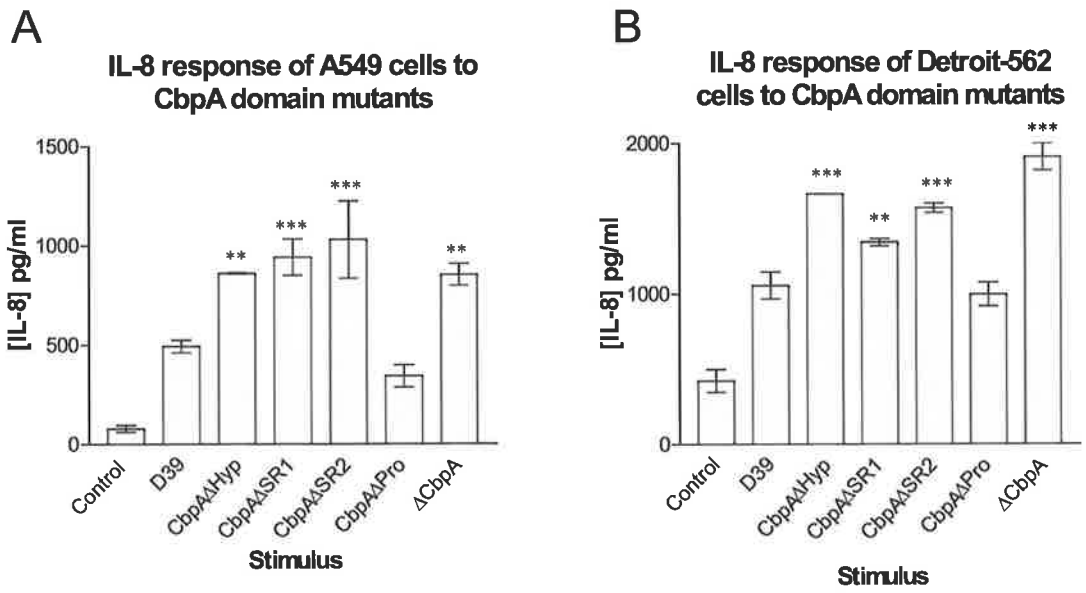


**Figure 5.7. IL-8 production by respiratory epithelial cells in response to CbpA domain mutants.** Confluent monolayers of A549 or Detroit-562 cells were incubated with WT *S. pneumoniae* D39 or CbpA domain mutants for 4 h before collection of cell culture supernatant and analysis for IL-8 by ELISA. Data are mean ± SE from 3 independent experiments. Data were analysed for statistical significance by One-way ANOVA with post hoc Bonferroni test. *** $P < 0.001$; ** $P < 0.01$, relative to WT D39.

Incubation of either cell type with CbpAΔHyp, CbpAΔSR1, or CbpAΔSR2 led to a significant increase in CXC chemokine mRNA and IL-8 protein when compared to the levels elicited by WT D39, and levels elicited by these mutants were similar to those generated in response to ΔCbpA.

## 5.3 Discussion

As described in Section 1.6.1, CbpA is comprised of 3 major regions, the C-terminal choline binding domain, a proline rich region, and an N-terminal α-helical region. The N-terminus is further divided into 2 small repeat regions, each of which is independently folded into an α-helical conformation (Luo *et al.*, 2004). Binding to pIgR has been shown to be dependent on these small repeat regions. Truncated forms of CbpA in which both of these domains are removed show a reduced affinity for pIgR, compared to WT CbpA molecules (Elm *et al.*, 2004a; Luo *et al.*, 2004). Another function of CbpA is fH binding, and the proline-rich region of the molecule appears to be important for this. Fragments of CbpA lacking the proline rich region bind significantly less fH than complete CbpA (Duthy *et al.*, 2002), but it is not clear if the proline rich region plays a direct role in binding fH or instead is necessary for presentation of the N-terminal regions in the correct conformation to allow binding to occur.

In this chapter, mutants were constructed in which the various CbpA domains were deleted in-frame in order to investigate the contribution of these regions to the ability of CbpA to suppress the CXC chemokine responses of A549 and Detroit-562 cells. Experiments with these mutants demonstrated a clear role for the N-terminal region of the molecule in the ability to suppress the IL-8 and MIP-2α response of these epithelial cells to *S. pneumoniae*. Mutants in which any of the N-terminal α-helical domains were deleted (CbpAΔHyp, CbpAΔSR1, and CbpAΔSR2) elicited an IL-8 response from nasopharyngeal epithelial cells that was significantly higher than that to WT D39, and that was similar to that elicited by

ΔCbpA (Figures 5.6 and 5.7 B). Type II pneumocytes showed an increase of at least 2-fold in IL-8 or MIP-2α mRNA or IL-8 protein in response to CbpAΔHyp, CbpAΔSR1, and CbpAΔSR2 compared to WT D39. Once again these mutants elicited a similar response to that elicited by ΔCbpA (Figures 5.5 and 5.7 A). In other words, removing any one of these N-terminal domains had the same effect on chemokine induction as removing CbpA completely, and this suggests that all three N-terminal regions are necessary for CbpA to exert its inhibitory effect on the CXC chemokine response of respiratory epithelial cells. It has been suggested that the hypervariable region and the 2 small repeat regions form independent α-helical structures (Luo *et al.*, 2004), but the way in which these are arranged to form the N-terminus of the molecule has not been determined. It is possible that the mechanism by which CbpA exerts its effect on chemokine responses is dependent upon presentation of the molecule in a specific conformation that relies on all three regions being present. Removal of any of the regions could disrupt this conformation leading to the observed diminished effect. It is unlikely that the observed differences in CXC chemokine induction are due to differences in adherence of the CbpA mutants to host cells, since adherence occurs via the N-terminal region of CbpA, and it is this region that appears to inhibit the CXC chemokine response. Purified CbpA is also capable of inhibiting CXC chemokine induction, further indicating that CbpA itself is responsible for the observed effects on the chemokine response.

It appears that the proline-rich region does not have any significant role in the chemokine suppressing activity of CbpA, since the mutant in which the proline-rich region is deleted (CbpAΔPro) was unaffected in its ability to inhibit chemokine production from either cell line, and elicited an IL-8 response that was similar to that elicited by WT D39 (Figures 5.7 A and B). In this mutant, the hypervariable region, and the 2 small repeat regions are present. Thus, it appears that unlike binding of fH, which requires the proline rich region to be present, the ability of CbpA to suppress CXC chemokine expression is dependent only on the first three domains.

The exact mechanism by which the N-terminal regions of CbpA exert their inhibitory effect on CXC chemokine expression is unknown. CbpA is able to bind fH and sIgA at the same time, without competitive interference, demonstrating that these molecules have different binding sites on CbpA (Dave *et al.*, 2004). However, it is not known if the chemokine suppressing action of CbpA is affected by binding of either of these molecules. The fact that the ability of both A549 and Detroit-562 cells to produce CXC chemokines is affected by CbpA suggests that the cognate receptor for CbpA is common to both cell types. The identity of this receptor remains unknown and should be the subject of further investigation, so that the interaction between CbpA and host cells, and the mechanism by which CbpA suppresses the chemokine response can be better understood.

# Chapter Six – Investigation of the Specific Domains Responsible for CXC Chemokine Induction by PspA

## 6.1 Introduction

Results from Chapter Four suggested that PspA has the ability to stimulate early chemokine release from type II pneumocytes (A549), as shown by a significant reduction in the CXC chemokine response to ΔPspA compared to WT D39 after 2 h of infection. As described in Chapter One, PspA consists of four distinct structural domains: 1) the N-terminus consisting of a coiled-coil α-helical structure, 2) a highly flexible, tether-like proline rich region, 3) a repeat region responsible for choline binding, and 4) a hydrophobic tail at the C terminus (Jedrzejas *et al.*, 2001). The structure of the N-terminus of PspA has recently been analysed using a variety of biophysical methods (Jedrzejas *et al.*, 2001). The N-terminal α-helical region (aa 1-288) is thought to be the active domain of the molecule responsible for the anti-complement functions of the protein (Jedrzejas *et al.*, 2001). PspA also binds to human lactoferrin (Lf), an iron-sequestering glycoprotein found predominantly in mucosal secretions, via the C-terminal half of this α-helical domain (Hammerschmidt *et al.*, 1999; Hakansson *et al.*, 2001). This domain has a structure that folds back on itself, and so is divided into 2 regions. In D39, region 1 of the mature PspA α-helix comprises aa 1-146, and region 2 is from aa 147-288. The proline rich region (aa 289-370) is believed to act as a flexible tether, connecting the α-helical region to the choline-binding domain of the molecule

(Jedrzejas *et al.*, 2001). In this chapter *S. pneumoniae* mutants were constructed with deletions of specific N-terminal regions of PspA. These were analysed in an attempt to determine which region(s) contribute to the generation of a chemokine response from A549 and Detroit-562 cells. Due to the low response elicited by purified recombinant PspA, and difficulties purifying protein free of LPS contamination, it was decided not to use recombinant truncated forms of PspA to investigate the roles of these domains in the chemokine response, but rather to use PspA domain deletion *S. pneumoniae* mutants.

## 6.2 Results

### 6.2.1 The role of specific PspA domains in eliciting a chemokine response from respiratory epithelial cells

In order to investigate the specific domain(s) of PspA responsible for eliciting a chemokine response from respiratory epithelial cells, a series of in-frame deletion mutants were constructed in which specific domains of the molecule were deleted. The 2 regions of the N-terminal α-helix (aa 1-146, and aa 147-288) were deleted individually and together, and the proline-rich (aa 289-370) region was also deleted (Figure 6.1). In all mutants, the leader sequence and choline-binding domain were left unaltered to allow correct export of the protein and binding to the pneumococcal surface.

### 6.2.2 Production of PspA domain deletion mutants

*S. pneumoniae* D39 derivatives in which specific regions of the *pspA* gene were deleted in-frame were constructed using a method analogous to that used to create the CbpA domain mutants (Section 5.2.2). The D39 *pspA* sequence (Genbank accession number M74122) was used to design the oligonucleotides used (Table 2.2). Overlap PCR was used to generate PCR products in which the specific regions of the gene were deleted as shown in Figure 6.2. These products were transformed into competent D39 *pspA*::*erm* cells that were constructed in Section 4.2.4.1, and transformants were selected by loss of Ery resistance by

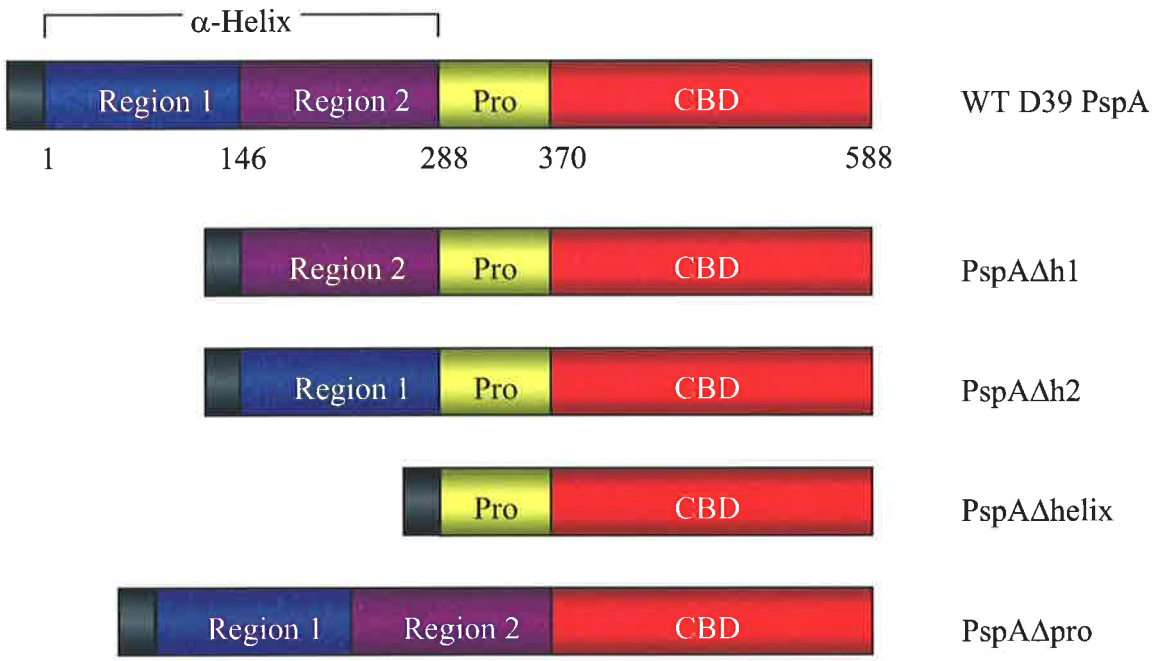


**Figure 6.1. PspA domain deletion mutants**. Schematic representation of the PspA molecule and the mutants with specific domain deletions. Black represents the leader sequence, blue represents region 1 of the α-helix region, violet represents region 2 of the α-helix, yellow represents the proline rich region (Pro), and red represents the choline binding domain (CBD). Numbers denote amino acid residues.

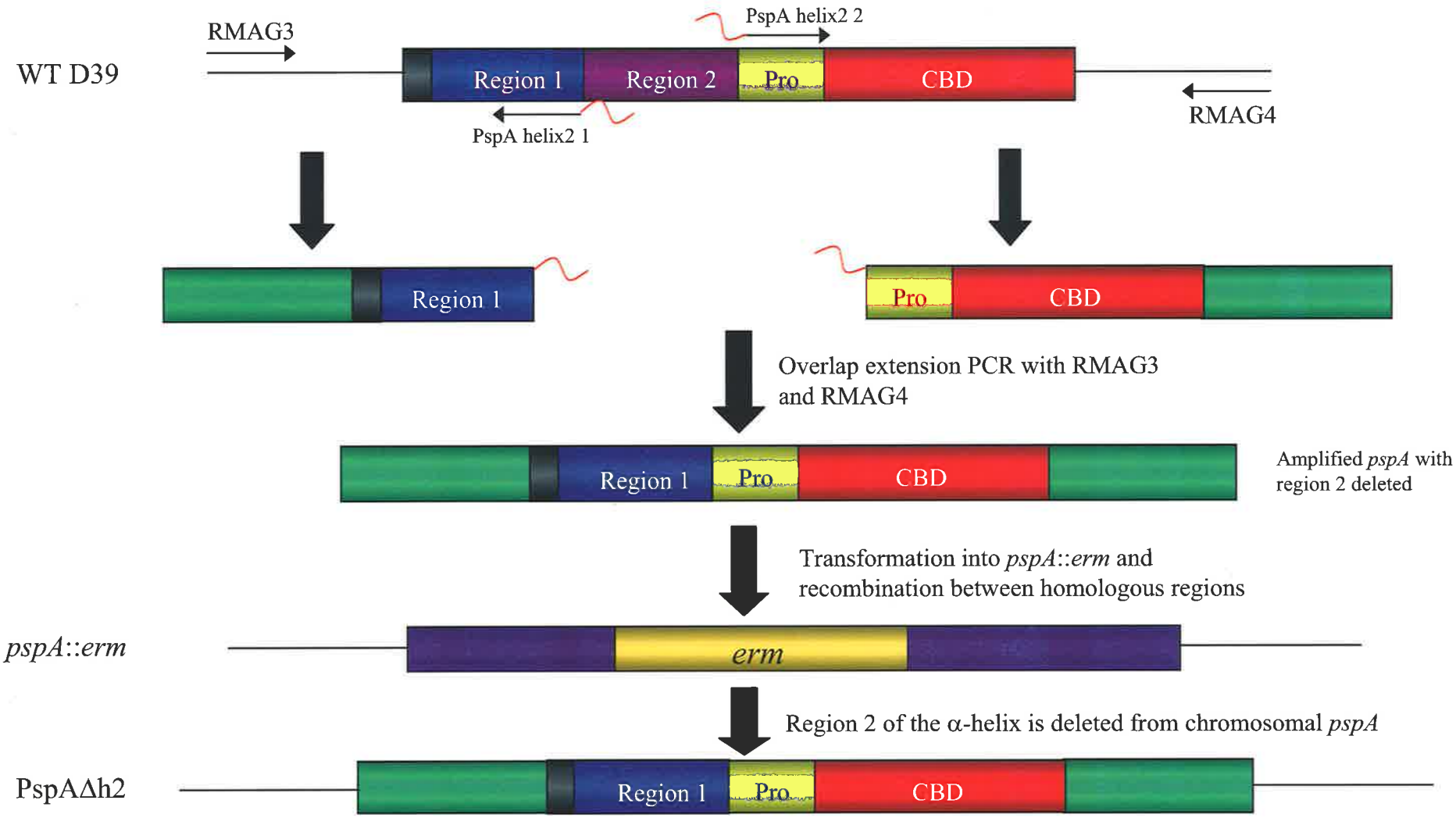


**Figure 6.2. Deletion of region 2 of the PspA N-terminal α-helix.** A PCR product created by overlap extension such that the sequences flanking the section encoding region 2 of the α-helix the are amplified, without the section itself, was transformed into *pspA*::*erm*. This results in homologous recombination between flanking regions of *pspA* on the PCR product and those on the chromosome such that the section that encodes region 2 of the α-helix of PspA is deleted from the chromosome. Other PspA domain deletion mutants were constructed using an analogous method.

replicate plating onto BA and EryBA plates following Ery/Amp enrichment, as described in Section 2.12.3. For each mutant, deletion of the selected region of the *pspA* gene was confirmed by PCR (Figure 6.3), and sequence analysis (data not shown). Production of truncated PspA protein of the expected size by the various mutants was confirmed by Western blot analysis with PspA-specific polyclonal mouse antiserum (Figure 6.4). Confirmed mutants producing PspA lacking region 1 of the α-helix, region 2 of the α-helix, the entire α-helix, and the proline-rich region were designated PspAΔh1, PspAΔh2, PspAΔhelix, and PspAΔpro, respectively.

### 6.2.3 Chemokine response of respiratory epithelial cells to PspA domain deletion mutants of *S. pneumoniae*

To investigate the possible role of specific PspA domains in the induction of a chemokine response in respiratory epithelial cells, confluent monolayers of A549 or Detroit-562 cells were infected with $5 \times 10^7$ CFU WT *S. pneumoniae* D39 or the various PspA domain deletion mutants, or the D39 mutant in which the entire *pspA* gene had been deleted in-frame (ΔPspA) (see Section 4.2.4.1). After incubation with *S. pneumoniae* for 2 or 4 h, total cellular RNA was extracted and analysed for the presence of chemokine specific mRNA by real-time RT-PCR with specific oligonucleotides as described in Section 2.10.3.

When incubated with WT D39, or PspA domain mutants for 2 h, all A549 cells showed a significant increase in chemokine mRNA when compared to uninfected control cells ($P < 0.001$), except for PspAΔPro which did not induce a statistically significant increase in MIP-2α mRNA. PspAΔh1, elicited an IL-8 mRNA response that was approximately 2-fold lower than that elicited by WT D39, ($P < 0.05$), and PspAΔhelix elicited an IL-8 mRNA response that was approximately 3-fold lower than WT-D39 ($P < 0.05$) (Figure 6.5). The IL-8 mRNA response to ΔPspA was approximately 2-fold lower than that to WT D39 ($P < 0.05$), consistent with results from the experiments described in Chapter Four (Figures 4.14 and 6.5).

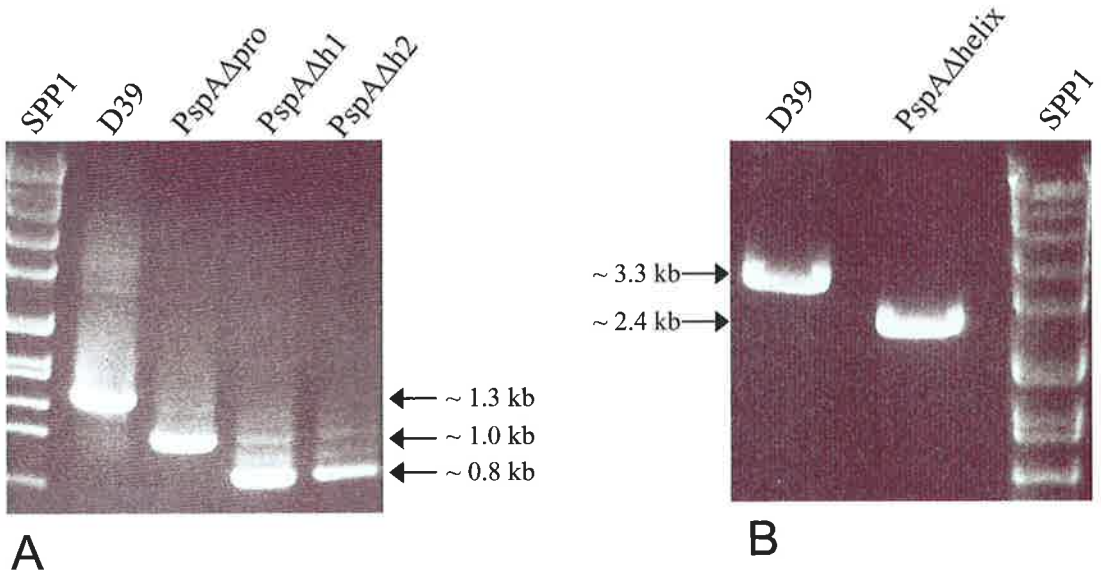


**Figure 6.3. PCR analysis of PspA domain deletion mutants.** **A**. *S. pneumoniae* colonies of WT D39 and PspAΔpro, PspAΔh1, and PspAΔh2 were subjected to direct PCR analysis using the oligonucleotides PspAF and PspAR2 (Table 2.2). **B**. *S. pneumoniae* colonies of WT D39 and PspAΔhelix were subjected to direct PCR analysis using the oligonucleotides PspApromF and RMAG4 (Table 2.2). Products were electrophoresed on a 1% agarose gel and stained with ethidium bromide as described in Section 2.9.1. Sizes of products were estimated according to mobilities relative to the SPP1 marker and are indicated.

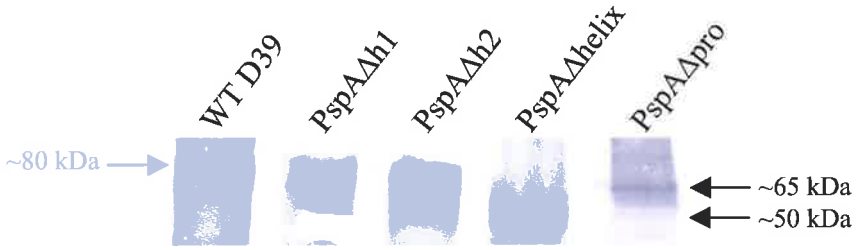


**Figure 6.4. Western blot analysis of PspA domain mutants.** Lysates of WT D39 and *pspA* domain mutants were separated by SDS-PAGE, electroblotted onto nitrocellulose and reacted with mouse polyclonal antiserum specific for PspA (as described in Section 2.8). A band of the appropriate size for PspA (~80 kDa) was seen in the lysate of WT D39, while the sizes of bands seen in the lysates of the domain mutants that were consistent with deletion of the specific domains (~65 kDa for PspAΔh1, PspAΔh2, and PspAΔpro, and ~50 kDa for PspAΔhelix).

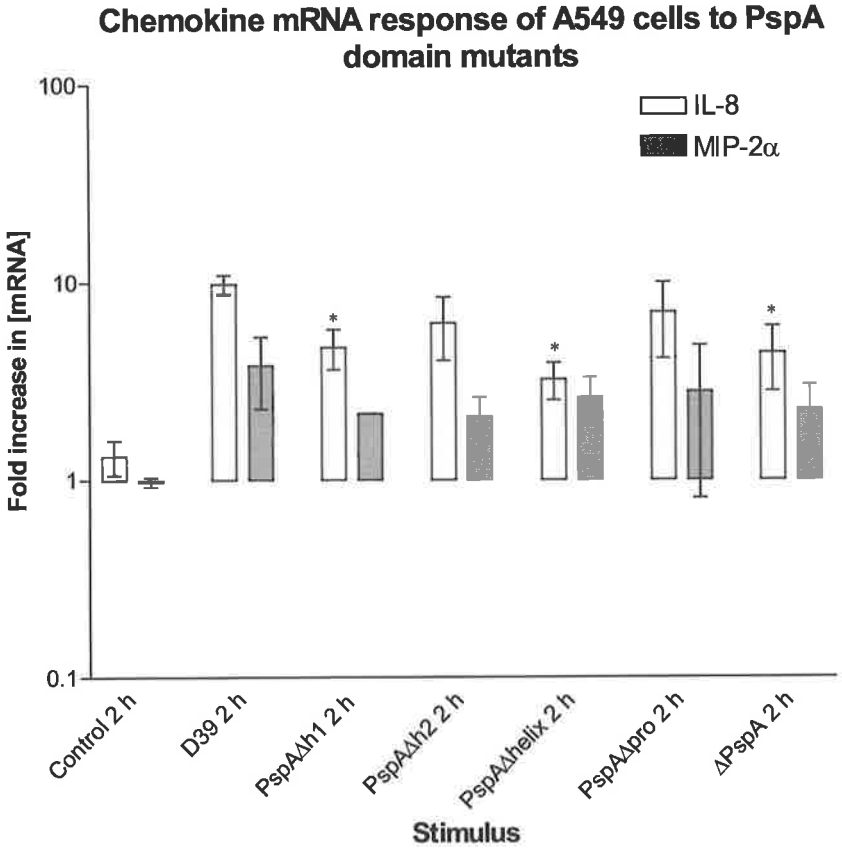


**Figure 6.5. CXC chemokine mRNA response of type II pneumocytes cells to PspA domain mutants.** Confluent monolayers of A549 cells were incubated with $5 \times 10^7$ CFU *S. pneumoniae* D39 or otherwise isogenic mutants with in-frame deletions of specific domains of PspA for 2 h before extraction of cellular RNA and analysis for chemokine mRNA by real-time RT-PCR with specific oligonucleotides. Data are mean ± SE from 3 independent experiments. Results were analysed for statistical significance by One-way ANOVA with post-hoc Bonferroni test. * $P < 0.05$.

There was no significant difference between the responses elicited by PspAΔpro and D39 at 2 h. There was also no significant difference in the responses generated by any of the mutants compared to WT D39 at 4 h (data not shown). This is also consistent with the results seen in Chapter Four, where ΔPspA did not show a significant difference in the IL-8 mRNA response elicited by these cells at 4 h when compared to WT D39, in spite of the significant decrease seen in response to this mutant at 2 h (Figure 4.14).

Although incubation with all strains of *S. pneumoniae* tested led to a significant increase in the chemokine mRNA response of Detroit-562 cells when compared to uninfected control cells ($P < 0.001$ for all), there was no significant difference in the responses elicited by the PspA domain mutants when compared to that elicited by WT D39 at either the 2 h (data not shown) or 4 h timepoint (Figure 6.6). This is consistent with the results seen in Chapter Four, where there was no significant difference observed in the response of Detroit-562 cells to ΔPspA when compared to WT D39 (Figure 4.15)

### 6.2.4 IL-8 release by type II pneumocytes and nasopharyngeal epithelial cells in response to PspA domain mutants

To confirm the results obtained by mRNA analysis, culture supernatant was collected from cells incubated with $5 \times 10^7$ CFU WT *S. pneumoniae* D39 or the various PspA mutants for 4 h, and analysed for IL-8 by ELISA, as described in Section 2.13. There was a significant increase in IL-8 production by all groups of cells exposed to *S. pneumoniae* when compared to uninfected control cells ($P < 0.05$). In both cell types, the IL-8 response to PspAΔh1 and PspAΔhelix was approximately 2-fold lower compared to that seen in response to WT D39 ($P < 0.01$ for both mutants in A549, and $P < 0.05$ for both mutants in Detroit-562). These responses were also reduced compared to the response elicited by PspAΔh2, although this did not reach statistical significance (Figure 6.7 A and B). PspAΔh2 did not elicit a significantly different response from either cell line compared to that elicited by WT D39. ΔPspA elicited an IL-8 response in A549 cells that was reduced approximately 2-fold

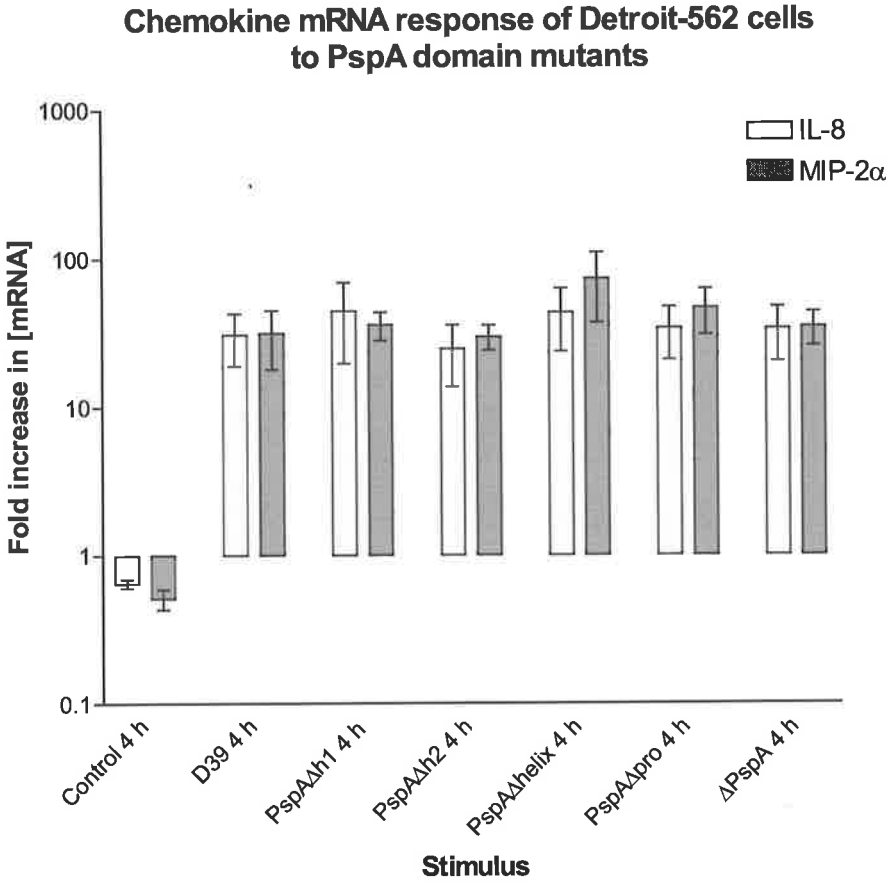


**Figure 6.6. CXC chemokine mRNA response of nasopharyngeal epithelial cells to PspA domain mutants.** Confluent monolayers of Detroit-562 cells were incubated with $5 \times 10^7$ CFU *S. pneumoniae* D39 or otherwise isogenic mutants with in-frame deletions of specific domains of PspA for 4 h before extraction of cellular RNA and analysis for chemokine mRNA by real-time RT-PCR with specific oligonucleotides. Data are mean ± SE from 3 independent experiments. Results were analysed for statistical significance by One-way ANOVA with post-hoc Bonferroni test.

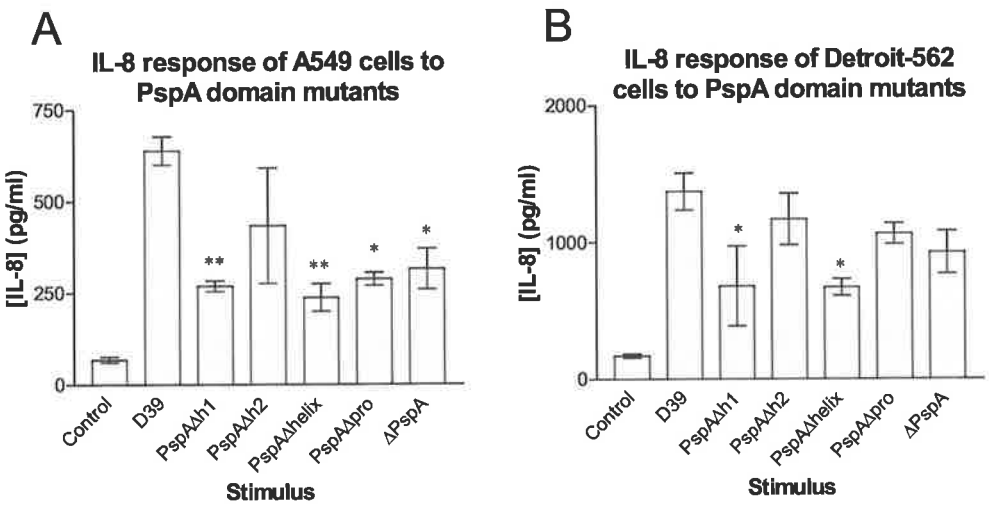


**Figure 6.7. IL-8 Response of Respiratory Epithelial Cells to PspA Domain Mutants.** Confluent monolayers of A549 (A) or Detroit-562 (B) cells were incubated with 5 × $10^7$ CFU WT *S. pneumoniae* D39 or PspA domain mutants for 4 h before collection of cell culture SN and analysis for IL-8 by ELISA. Data are mean ± SE from 3 independent experiments. Data were analysed for statistical significance by One-way ANOVA with post-hoc Bonferroni test. ** $P < 0.01$; * $P < 0.05$.

compared to WT D39 ($P < 0.05$), and was similar to that elicited by PspAΔh1 and PspAΔhelix (Figure 6.7 A). A549 cells also showed an approximately 2-fold decrease in IL-8 in response to PspAΔpro when compared to WT D39 (Figure 6.7 A). However, in Detroit-562 cells there was no significant difference seen in the response elicited by PspAΔpro or ΔPspA when compared to WT D39 (Figure 6.7 B).

Overall these results suggest a role for the first region of the N-terminal α-helical domain of PspA in generating an IL-8 response from A549 and possibly Detroit-562 cells. A549 cells showed a significant decrease in IL-8 mRNA and protein production in response to PspAΔh1, and PspAΔhelix when compared to WT D39, while Detroit-562 cells released significantly less IL-8 in response to these mutants when compared to WT D39 although this difference was not seen at the mRNA level in this cell line.

## 6.3 Discussion

As described above and in detail in Chapter One, the principal functional region of the PspA molecule in terms of host interaction is believed to be the N-terminus. Most protective epitopes are also located in this region. Protective antibodies are generally targeted towards the second region of the α-helix between aa residues 192 and 260, although some are found to bind to the first α-helical region between aa 1 and 115 (Yother *et al.*, 1992; McDaniel *et al.*, 1994; Briles *et al.*, 1996; Tart *et al.*, 1996; Roche *et al.*, 2003). It is likely that antibodies are directed against these regions because they are surface exposed and are therefore easily accessible to the immune system. However, it is also possible that they are protective because they target important PspA domains and neutralise functions such as complement inhibition, and binding of Lf. Binding of Lf by PspA has been shown to occur via region 2 of the α-helix, between aa residues 167-288 (Hakansson *et al.*, 2001), and a 10 aa fragment of PspA containing aa 269-278 was able to bind Lf and inhibit its function (Shaper *et al.*, 2004). In

addition, binding of the PspA α-helix by antibodies reduces its ability to prevent complement deposition on the pneumococcal surface (Ren *et al.*, 2004b), suggesting that these regions may be important for the complement inhibiting properties of PspA.

In Chapter Four, PspA was shown to stimulate the early CXC chemokine response of A549 cells to *S. pneumoniae* D39. In this chapter, the specific regions of the molecule responsible for this effect were investigated by constructing mutants in which the major N-terminal domains were deleted in frame (PspAΔh1, PspAΔh2, PspAΔhelix, and PspAΔpro). Results from this study indicate that as well as being important for complement inhibition and Lf binding, the α-helix of PspA is responsible for its ability to elicit a chemokine response from A549 cells, and that region 1 but not region 2 of the N-terminal α-helix is important for this function. A549 cells infected with PspAΔh1, and PspAΔhelix gave an IL-8 mRNA and protein response that was reduced approximately 2-fold compared to cells infected with WT D39, and the response elicited by these mutants was similar to that elicited by ΔPspA (Figures 6.5 and 6.7 A). In Chapter Four it was speculated that the increased chemokine response observed against $PspA^-$ was due to an active fragment released by the mutant. If present, this fragment would be comprised of the N-terminal part of region 1 of the α-helix, and its apparent ability to stimulate cells provides further evidence for the role of region 1 in chemokine stimulation. PspAΔh2 did not elicit a response that was significantly different to WT D39 at either the protein or mRNA level, suggesting that this region has little role in eliciting a chemokine response from these cells (Figures 6.5 and 6.7 A). PspAΔpro did not elicit a significantly different chemokine mRNA response from A549 cells compared to WT D39, but IL-8 protein release from cells infected with this mutant was reduced approximately 2-fold compared to cells infected with WT D39 (Figures 6.5 and 6.7 A). This inconsistency makes it difficult to comment on the likely role of this domain in chemokine release from these cells. Differences in the MIP-2α mRNA response of A549 cells to PspA domain mutants followed the same trend as was seen for the IL-8 mRNA response, but due to a high

degree of variation in mRNA levels between experiments these differences did not reach statistical significance (Figure 6.5). Thus it is possible that region 1 of the α-helix also plays a role in the MIP-2α mRNA response of these cells, but this cannot be confirmed by these experiments.

Chemokine induction from epithelial cells alerts the immune system to the presence of bacteria, and promotes recruitment of neutrophils to the site of infection, thus performing a function beneficial to the host. The role of the first but not the second region of the α-helix in chemokine induction from A549 cells may be related to the reason why the majority of protective epitopes in PspA are in the second region of the α-helix. Masking region 1 of the α-helix by the binding of pre-existing antibodies could prove detrimental to the host, by reducing the chemokine eliciting effect of the molecule, and thus preventing recruitment of neutrophils and subsequent clearance of the bacteria. Also, it appears that the regions important for chemokine induction and for Lf binding are different, so it is possible that these activities do not interfere with each other.

The results obtained with Detroit-562 cells were consistent with results seen in Chapter Four, where there was no significant difference in the CXC chemokine responses of cells infected with ΔPspA compared to WT D39 (Figures 6.6 and 6.7 B). At the mRNA level there was also no significant difference observed in the response of these cells to any of the PspA domain deletion mutants (Figure 6.6). However, there was a significant decrease in the amount of IL-8 released in response to PspAΔh1, and PspAΔpro when compared to WT D39 (Figure 6.7 B). The reasons for this discrepancy between the mRNA and protein results are unclear, as is the reason why PspAΔh1, and PspAΔhelix would elicit a significantly reduced IL-8 response from these cells while ΔPspA does not. Further experimentation is needed in order to clarify these results, and no specific conclusions can be drawn here.

Overall, PspA appears to have a role in stimulating IL-8 release from A549 cells, and according to the results presented here, region 1 of the N-terminal α-helix is largely

responsible for this activity. When the structure of PspA is considered, the fact that this region is likely to be surface exposed (beyond the capsule) and easily visible to the host puts it in a good position to interact with host cells. Narrowing down the position of the active site, and identifying the possible host cell receptors involved in this response are worthy subjects for further investigation.

# Chapter Seven – Investigation of the Specific Activities of Ply Responsible for Modulation of the CXC Chemokine Response

## 7.1 Introduction

It is well recognised that pneumolysin has an important role in virulence of *S. pneumoniae* via its cytotoxic and complement activating properties, as reviewed by Hirst *et al.,* (2004). However, the results of experiments with ΔPly described in Chapter Four suggest that it may have only a limited role in the induction of a CXC chemokine response from respiratory epithelial cells. Even so, since it is able to elicit chemokine release from other cell types, and this was shown to be influenced by cytotoxicity (Baba *et al.*, 2002; Rijneveld *et al.*, 2002; Ratner *et al.*, 2005), it was decided to investigate the role of pneumolysin cytotoxicity in CXC chemokine release from A549 and Detroit-562 cells.

There are a number of amino acid residues in pneumolysin that are known to be important in the function of the molecule, and mutation of these residues has varying degrees of effect on the function of Ply (as described in detail in Section 1.6.3.4). Individual mutations of Trp433, Cys428, or His367 all have an effect on cytotoxicity to varying degrees, while Asp385 is important for Ply's complement activating ability (Saunders *et al.*, 1989; Boulnois *et al.*, 1991; Mitchell *et al.*, 1991). Considering the importance of these residues in the overall function of Ply, it is reasonable to determine whether or not they affect the ability of Ply to elicit a CXC chemokine response from respiratory epithelial cells. In this chapter,

the relative contributions of these residues in the CXC chemokine response elicited by Ply was examined using mutants of *S. pneumoniae* D39 containing point mutations in the *ply* gene.

## 7.2 Results

To investigate the potential roles that the different activities of Ply may have on the CXC chemokine response of respiratory epithelial cells to *S. pneumoniae*, monolayers of A549 and Detroit-562 cells were incubated with $5 \times 10^7$ CFU of WT D39 or otherwise isogenic mutants with point mutations in *ply* (Table 2.1). In these mutants, specific residues known to be important in cytotoxicity and/or complement activation were changed by point mutation to eliminate or reduce these functions, as outlined in Table 7.1.

| Name | Mutation | Phenotype |
|---|---|---|
| 367 | His367→Arg | 0.02% Haemolytic activity of WT Ply |
| 385 | Asp385→Asn | Undetectable complement activating ability |
| 433 | Trp433→Phe | 0.1% Haemolytic activity of WT Ply |
| Triple | Trp433→Phe, Cys428→Gly, Asp385→Asn | 0.001% Haemolytic activity of WT Ply and undetectable complement activating ability |

**Table 7.1. D39 Ply point mutants used in this study (constructed by Berry *et al.* (1995)).**

### 7.2.1 CXC Chemokine mRNA response elicited by A549 and Detroit-562 cells towards *S. pneumoniae* Ply point mutants

After exposure to WT D39 and Ply mutants for 2 h, A549 cells showed a significant increase in IL-8 and MIP-2α mRNA when compared to uninfected control cells ($P < 0.001$). At this time-point, cells incubated with 367 or 433, both of which have mutations affecting cytotoxicity, generated an IL-8 mRNA response that was approximately 4-fold lower than that generated by WT D39 ($P < 0.001$ for both). Triple, which has highly attenuated cytotoxicity and complement activating ability generated a 4-fold lower response than WT

D39, and ΔPly elicited an approximately 2-fold lower response than WT D39 ($P < 0.001$, and $P < 0.01$ respectively). There was no significant difference in the response of these cells at 2 h to 385, which has reduced complement-activating ability but normal cytotoxicity, when compared to cells incubated with WT D39 (Figure 7.1). The MIP-2α mRNA response was similar to all strains at 2 h, except for the Triple mutant, which showed an approximately 4-fold decrease in MIP-2α mRNA in comparison to WT D39 ($P < 0.001$) (Figure 7.1). After incubation with *S. pneumoniae* for 4 h, an increase in IL-8 mRNA was seen for all A549 cells to which bacteria were added when compared to uninfected control cells ($P < 0.001$), and a significant increase in MIP-2α mRNA was seen for all mutants except 367 ($P < 0.001$ for all). There was an approximate 3-fold decrease in IL-8 mRNA produced by cells exposed to 367, and Triple when compared to WT D39 ($P < 0.001$ for both), but no significant difference in either IL-8 or MIP-2α mRNA was seen in the cells exposed to 433 or ΔPly at 4hr compared to WT D39 (Figure 7.1). At this timepoint there was also an approximately 2-fold decrease seen in the cells exposed to 385 when compared to WT D39 ($P < 0.05$). There was no significant decrease in the MIP-2α mRNA response towards any of the mutant strains in comparison to that elicited by WT D39 (Figure 7.1).

After 2 or 4 h exposure to all strains of *S. pneumoniae* tested, Detroit-562 cells showed a significant increase in IL-8 and MIP-2α mRNA when compared to uninfected control cells ($P < 0.001$). After 2 h, all cells treated with mutants showed an approximately 3-fold decrease in IL-8 mRNA when compared to WT D39, ($P < 0.01$ for all) with the exception of 385, which did show a decrease in mRNA levels but this did not reach statistical significance (Figure 7.2). After 4 h incubation, Detroit-562 cells incubated with 367, and 433 showed an approximately 3-fold decrease in IL-8 mRNA ($P < 0.01$, and $P < 0.01$ respectively), and infection with Triple led to an approximately 5-fold decrease in IL-8 mRNA ($P < 0.001$) (Figure 7.2). As was seen at 2 h, no significant difference was seen in Detroit-562 cells infected with the 385 mutant, and the ΔPly mutant showed no significant

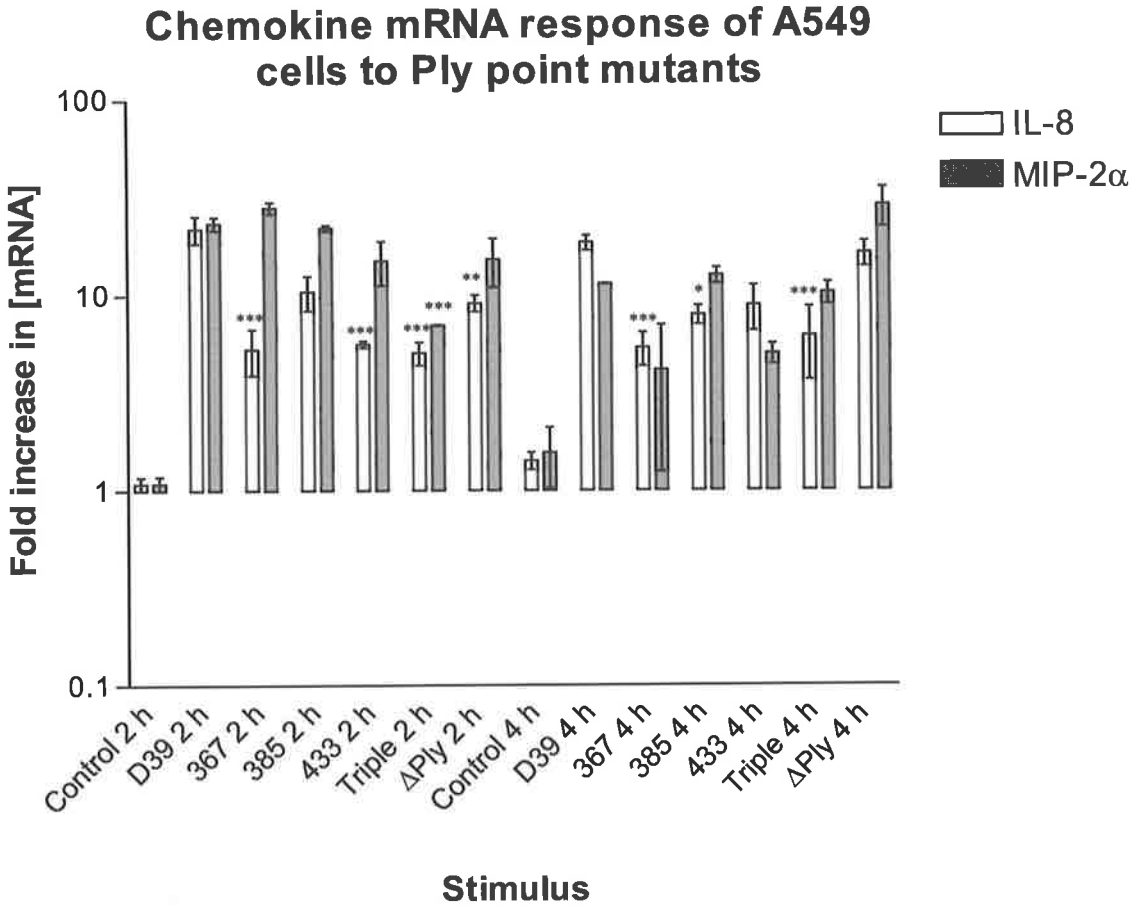


**Figure 7.1. CXC Chemokine response of type II pneumocytes to Ply point mutants.** Confluent monolayers of A549 cells were incubated with $5 \times 10^7$ CFU WT *S. pneumoniae* D39 or otherwise isogenic mutants with point mutations in the *ply* gene for 2 or 4 h before extraction of cellular RNA and analysis for IL-8 or MIP-2α-specific mRNA by real-time RT-PCR. Data are mean ± SE from 4 independent experiments. Results were analysed for statistical significance by One-way ANOVA with post-hoc Bonferroni test. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$, in comparison to WT D39 at the same time.

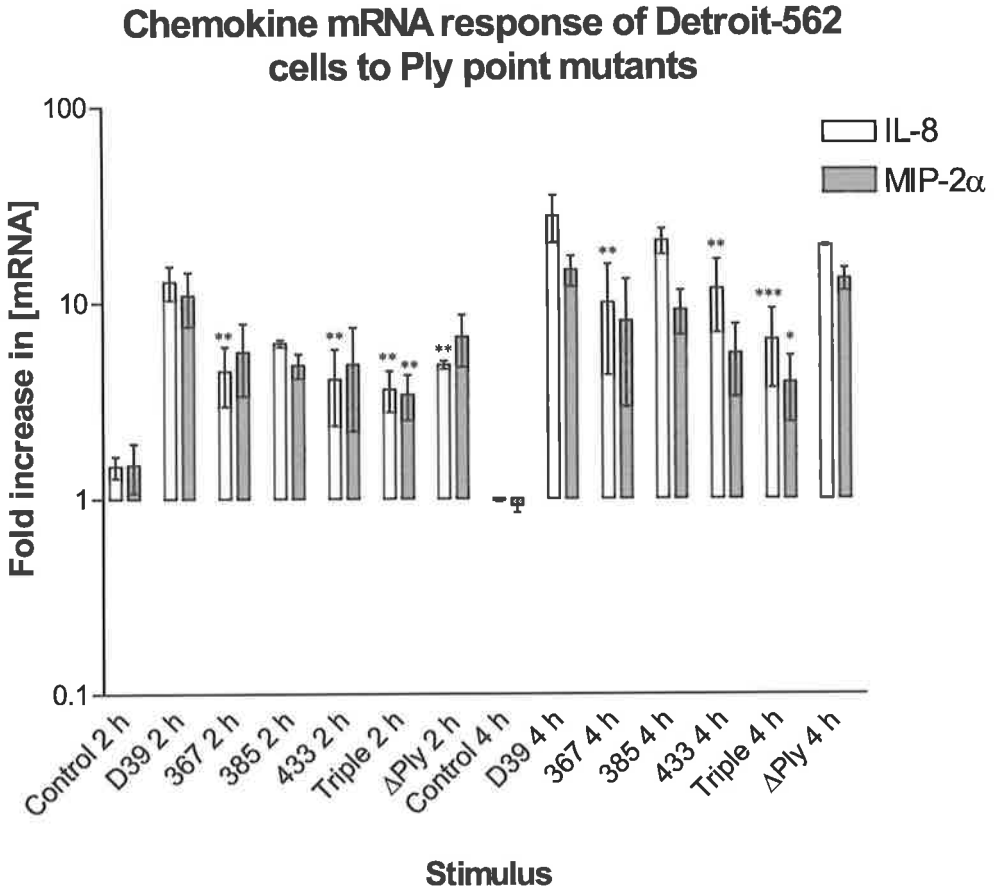


**Figure 7.2. CXC Chemokine response of nasopharyngeal epithelial cells to Ply point mutants.** Confluent monolayers of Detroit-562 cells were incubated with $5 \times 10^7$ CFU WT *S. pneumoniae* D39 or otherwise isogenic mutants with point mutations in the *ply* gene for 2 or 4 h before collection of cellular RNA and analysis for IL-8 or MIP-2α-specific mRNA by real-time RT-PCR. Data are mean ± SE from 4 independent experiments. Results were analysed for statistical significance by One-way ANOVA with post-hoc Bonferroni test. *** $P < 0.001$; ** $P < 0.01$; * $P < 0.05$, in comparison to WT D39 at the same time.

difference in induction of IL-8 mRNA levels in these cells when compared to WT D39 (Figure 7.2). At both 2 and 4 h, the only mutant to show a significant decrease in MIP-2α mRNA levels when compared to WT D39 was Triple, which elicited approximately 4-fold less mRNA at both timepoints ($P < 0.01$, and $P < 0.05$ for 2 and 4 h respectively), (Figure 7.2).

### 7.2.2 IL-8 secretion by A549 and Detroit-562 cells in response to D39 Ply point mutants

Culture supernatants from A549 and Detroit-562 cells exposed to *S. pneumoniae* D39 and isogenic *ply* point mutants for 4 h were assayed for IL-8 protein production by ELISA as described in Section 2.13. In A549 cells all *S. pneumoniae* strains caused a significant increase in IL-8 production when compared to uninfected control cells exposed to cell culture media alone ($P < 0.001$). The differences in the IL-8 response seen between WT D39 and the point mutants were largely consistent with the differences noted in mRNA expression. Levels of IL-8 produced by the cells in response to 367, 385, 433, and the Triple mutant were approximately 2-fold lower than levels produced in response to WT D39 ($P < 0.01$; $P < 0.05$; $P < 0.05$; and $P < 0.01$ respectively). Also in accordance with mRNA results, levels of IL-8 produced in response to ΔPly were not significantly different to those elicited by WT D39 (Figure 7.3 A).

In Detroit-562 cells all *S. pneumoniae* strains caused an increase in IL-8 secretion that was significant when compared to uninfected control cells ($P < 0.001$). When compared to the levels of IL-8 released in response to WT D39, 367, 433, and ΔPly all showed an approximately 2-fold decreased response ($P < 0.01$ for all), as did the Triple mutant ($P < 0.05$). As was noted for mRNA, the IL-8 response to 385 was not significantly different to that to WT D39 (Figure 7.3 B).

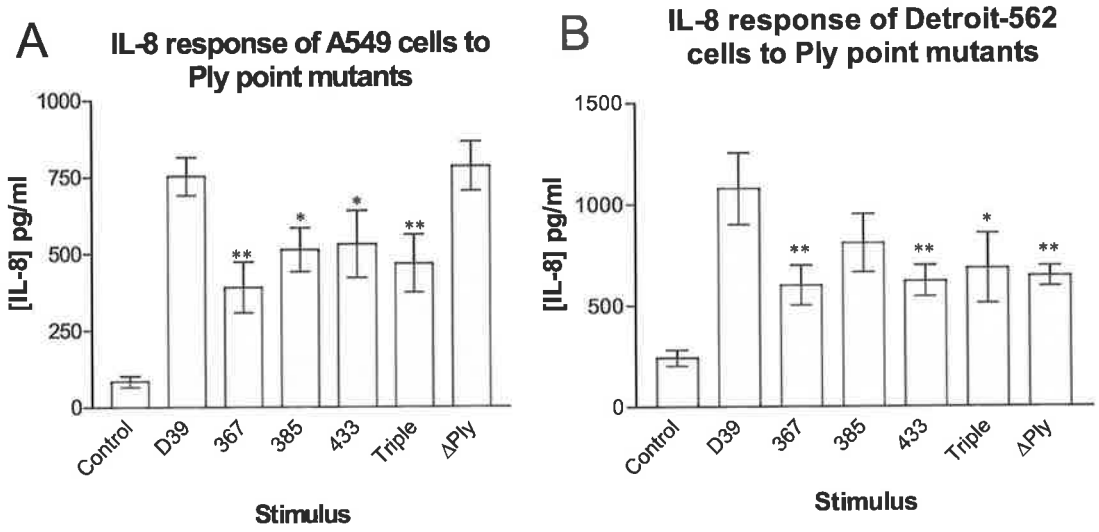


**Figure 7.3. IL-8 response of respiratory epithelial cells to *S. pneumoniae* Ply point mutants.** Cell culture supernatants from A549 (A) or Detroit-562 (B) cells infected with $5 \times 10^7$ CFU WT D39 or Ply point mutants were analysed for the presence of IL-8 by commercial ELISA. Data are mean ± SE from 4 independent experiments. Data were analysed for statistical significance by One-way ANOVA followed by a post-hoc Bonferroni test. ** $P < 0.01$; * $P < 0.05$ in comparison to D39.

## 7.3 Discussion

The most functionally important domain of Ply is believed to be domain 4, and it is in this region that the residues important for the cytotoxic and complement activating functions of Ply are located (Saunders *et al.*, 1989; Boulnois *et al.*, 1991; Mitchell *et al.*, 1991). The aa residues His367, and Trp433 are important in the cytolytic function of Ply, with mutants in which these residues are replaced showing only 0.02%, and 0.1% haemolytic activity respectively, when compared to the native toxin. Complement activation is dependent on the residue Asp385 and this activity is reduced in mutants in which this residue has been replaced (Saunders *et al.*, 1989; Boulnois *et al.*, 1991; Mitchell *et al.*, 1991).

Experiments with strains of *S. pneumoniae* carrying point mutations in the *ply* gene demonstrated that both the cytotoxic and complement-activating functions of the molecule play some role in the generation of an IL-8 response to pneumococcal infection in A549 cells, and cytotoxicity, but not complement activating activity, has a role in IL-8 induction in Detroit-562 cells. 367 elicited a significantly lower IL-8 response than WT D39 in both type II pneumocytes and nasopharyngeal epithelial cells, as shown at both the mRNA and protein levels (Figures 7.1, 7.2 and 7.3). This mutation is believed to decrease the ability of Ply to oligomerise in target cell membranes and Ply from this mutant has only 0.02% of the haemolytic activity of the wild type Ply.

In both type II pneumocytes and nasopharyngeal epithelial cells, 433 generated a significantly reduced IL-8 response at 2 and 4 h at both the mRNA and protein levels (Figures 7.1, 7.2 and 7.3). Although Ply from this mutant has a higher haemolytic activity than that from 367 (0.1% of that of WT Ply compared to 0.02% for 367), there was no significant difference in the chemokine responses elicited by these mutants. It has recently been demonstrated that the IL-8 response of A549 cells infected with *H. influenzae* was significantly increased when treated with purified Ply, but treatment with PdB, a toxoid form of Ply with the Trp433→Phe substitution, does not have this effect (Ratner *et al.*, 2005). This

is consistent with the role for the cytotoxic activity of Ply in the IL-8 response to infection with *S. pneumoniae* observed here.

The mutant in which complement activating activity was abolished (385) elicited a significant decrease in IL-8 mRNA and protein from type II pneumocytes at 4 h post infection, but no difference in response was seen in nasopharyngeal epithelial cells infected with this mutant when compared to WT D39. Thus, the complement activating function of Ply may play a role in CXC chemokine induction from type II pneumocytes, but the role it has in CXC chemokine induction from nasopharyngeal epithelial cells appears to be limited.

The Triple mutant, which has both the complement activating activity and cytotoxicity reduced, elicited a decreased chemokine mRNA and IL-8 response in both cell types when compared to WT D39, but this was not significantly less than that elicited by 433, 385, or 367, suggesting that there is no additive effect when both activities are reduced. However the CXC chemokine mRNA response to this mutant in Detroit-562 cells was significantly less than to 385, further suggesting that in these cells cytotoxicity but not complement activation affects the chemokine response. Thus, it appears that CXC chemokine induction attributable to Ply in nasopharyngeal epithelial cells is dependent mainly on cytotoxicity rather than complement activation.

The MIP-2α mRNA response was not significantly decreased in response to any of the mutants compared to WT D39, except for the Triple mutant at 2 h in A549 cells and 2 h and 4 h in Detroit-562 cells. However, the general trend observed in levels of mRNA for this chemokine were similar to those of IL-8 mRNA, and it is likely that results did not reach statistical significance because there was a high degree of variation in MIP-2α mRNA levels between experiments.

The complete absence of Ply in ΔPly leads to a significant decrease in IL-8 mRNA from both A549 and Detroit-562 cells at 2 h, but interestingly ΔPly showed no difference in IL-8 or MIP-2α mRNA levels produced by either cell type at 4 h when compared to WT D39.

However, at the protein level, although there was no difference seen in the IL-8 response of A549 cells to this mutant, Detroit-562 cells released significantly less IL-8 in response to ΔPly compared to WT D39, something that was also noted in Chapter Four (Figures 4.15 and 4.16 B). Although the reduction in IL-8 release from Detroit-562 cells in response to ΔPly was only observed at the protein level, it suggests that Ply does have a role in induction of this chemokine from nasopharyngeal epithelial cells. It is possible that WT levels of cytotoxicity lead to an increase in chemokine response compared to uninfected control cell. When cytotoxicity is reduced as in 367 and 433, this effect on the cells is also reduced, but even these low levels of cytotoxicity may mask the response of these cells to other pneumococcal factors that can influence the chemokine response. The use of whole, live *S. pneumoniae* to determine the role of specific factors in CXC chemokine induction is preferential to other methods because it better reflects natural infection. However, it can complicate the interpretation of results. As has been shown in Chapter Four, it appears that different pneumococcal factors have different effects on the chemokine responses of respiratory epithelial cells. Thus, CXC chemokine induction in respiratory epithelial cells infected with *S. pneumoniae* is a complex process that reflects composite responses to multiple pneumococcal factors. Removal of one pneumococcal factor such as Ply from the whole organism may upset this balance. It is possible that Ply has a role in modulating the chemokine response to other pneumococcal factors such as PspA and CbpA. Thus, when Ply is removed altogether as in ΔPly, its effect is abolished and instead of seeing the expected decrease in chemokine response by A549 cells, levels are the same as to WT D39, because the response to other pneumococcal factors such as PspA, which is able to stimulate chemokine release from A549 cells, is unmasked. However, there is no direct evidence to support this hypothesis, and further investigation is required to fully understand the reasons behind the responses observed towards the Ply mutants.

Previous studies on the role of Ply in cytokine release from host cells also have given

conflicting results regarding the effect that cytotoxicity has on this process. Production of IFNγ from spleen cells was shown to be reduced in response to cytotoxic Ply when compared to Ply with no pore-forming activity (Baba *et al.*, 2002). However, cytotoxicity was demonstrated to be important in the induction of MIP-2 from neutrophils in the lungs of mice (Rijneveld *et al.*, 2002), and as mentioned above, Ply but not PdB was able to increase IL-8 release from A549 cells infected with *H. influenzae* (Ratner *et al.*, 2005). Overall, the results of this study are confusing and it remains unclear the exact role that the different functions of Ply play in the CXC chemokine response of respiratory epithelial cells to pneumococcal infection. It appears that the cytotoxicity of Ply does play a role in increasing the chemokine response of A549 and Detroit-562 cells to *S. pneumoniae* infection, but it may also affect the cells in such a way that the response to other pneumococcal factors is modulated. More study is required in order to fully understand the way in which Ply interacts with epithelial cells to influence the CXC chemokine response to pneumococcal infection.

# Chapter Eight – Final Discussion

*S. pneumoniae* is a major cause of morbidity and mortality worldwide. It is generally carried asymptomatically in the nasopharynx, but in some circumstances it can translocate from this site to cause invasive diseases such as pneumonia, and meningitis. Inflammation is likely to play a role in this process by damaging host epithelium and increasing the presence of host cell receptors that enable pneumococci to transcytose across the epithelium (Sollid *et al.*, 1987; Phillips *et al.*, 1990; Briles *et al.*, 1996b; Blanch *et al.*, 1999; Zhang *et al.*, 2000). The presence of pneumococci in the lungs triggers an inflammatory response, which results in an influx of leukocytes such as neutrophils to this site. These are important for clearance of the bacteria, but a prolonged inflammatory response is detrimental to the host and is responsible for many of the symptoms associated with pneumonia such as lung oedema, and damage to tissues (Dallaire *et al.*, 2001). Epithelial cells play a direct role in the inflammatory response to respiratory pathogens by releasing pro-inflammatory cytokines, and in particular CXC chemokines that recruit neutrophils to the site of infection (Simon and Paine, 1995; Yang *et al.*, 2002; Yang *et al.*, 2003). Thus, it is also likely that epithelial cells play a direct role in the pathogenesis of pneumococcal infection by releasing CXC chemokines that recruit neutrophils to the site of infection, an important process in the development of disease.

In Chapter Three it was demonstrated that infection of A549 cells with *S. pneumoniae* D39 leads to a increase in mRNA for the CXC chemokines IL-8, MIP-2α, and MIP-2β, and

an increase in secretion of IL-8 when, compared to uninfected control cells (Sections 3.2.3 and 3.2.4). These results were consistent with previous *in vivo* studies that showed that levels of MIP-2 were increased in the lungs of mice infected with *S. pneumoniae* (Bergeron *et al.*, 1998; Dallaire *et al.*, 2001; Kerr *et al.*, 2002), and *in vitro* studies that showed an increase in IL-8 release by A549 cells in response to heat-killed *S. pneumoniae* (Madsen *et al.*, 2000; Murdoch *et al.*, 2002). Detroit-562 cells also responded to pneumococcal infection by increasing levels of CXC chemokine mRNA. Levels of IL-8, MIP-2α, MIP-2β, and MGSA mRNA, and IL-8 protein were increased compared to uninfected control cells (Sections 3.2.5 and 3.2.6). Therefore *in vitro*, *S. pneumoniae* infection leads to an increase in the CXC chemokine response of respiratory epithelial cells.

The pneumococcal virulence factors CbpA, PspA, and Ply each have a demonstrated role in pathogenesis of pneumococcal disease, as described in Sections 1.6.1, 1.6.2 and 1.6.3, respectively. The potential roles of these proteins in CXC chemokine release by respiratory epithelial cells was investigated in Chapter Four, and each was found to have some influence on the CXC chemokine response of these cells.

Infection of A549 monolayers with either CbpA$^-$ or ΔCbpA led to a 2-fold increase in levels of IL-8 and MIP-2α mRNA, and IL-8 protein compared with those elicited by WT D39 (Sections 4.2.1, 4.2.3 and 4.2.5). Detroit-562 cells showed an increase of at least 3-fold in IL-8 and MIP-2α mRNA after infection with CbpA$^-$ or ΔCbpA, compared to levels elicited by WT D39 (Sections 4.2.2 and 4.2.5). IL-8 release from these cells was also increased by infection with CbpA$^-$ or ΔCbpA, compared to WT D39 (Sections, 4.2.3 and 4.2.5.1). Thus, it appears that the response of these cells to *S. pneumoniae* is greater in the absence of CbpA, suggesting that CbpA suppresses the CXC chemokine response of respiratory epithelial cells, at least *in vitro*.

Investigation of the specific domains of CbpA involved in this activity by infecting cells with mutants in which individual regions of the molecule were deleted, indicated that the

three N-terminal domains (the hypervariable region, SR1 and SR2) were all required for CbpA to have this effect. Mutants expressing CbpA in which any one of these domains were deleted elicited a chemokine response from cells that was similar to that elicited by ΔCbpA (Sections 5.2.3 and 5.2.4). Thus removing any one of these regions had the same effect on CXC chemokine induction as removing CbpA altogether. The exact mechanism by which these domains exert their chemokine suppressing effect remains unknown, but it may be dependent upon presentation of CbpA in a specific conformation that requires all three regions to be present. The proline-rich region appeared to have no effect on the ability of CbpA to suppress CXC chemokine release from A549 or Detroit-562 cells, with mutants expressing CbpA in which this domain was deleted eliciting the same response from cells as WT D39 (Sections 5.2.3 and 5.2.4). CbpA was able to suppress the CXC chemokine response of both A549 and Detroit-562 cells, suggesting that the cognate receptor for this activity is common to both cell types.

The ability of CbpA to suppress CXC chemokine production from nasopharyngeal epithelial cells may help colonisation of this site by preventing the recruitment of neutrophils that would otherwise clear the bacteria. In the lungs, an early sharp response has been shown to be vital in overcoming pneumococcal infection (Dallaire *et al.*, 2001). Thus, CbpA-mediated attenuation of this early response may allow bacterial numbers in the lungs to reach a level that overwhelms the host immune system, preventing clearance and leading to a sustained inflammatory response that aids the entry of pneumococci into the blood.

In addition to having roles in complement inhibition and binding of Lf, PspA appears to be important in the stimulation of an early CXC chemokine response towards pneumococcal infection from A549 cells, but not Detroit-562 cells. Infection of A549 cells with ΔPspA led to a 2-fold decrease in levels of IL-8 and MIP-2α mRNA, and IL-8 protein when compared to levels elicited by WT D39 at 2 h (Section 4.2.5). No difference was seen

in the CXC-chemokine response elicited by ΔPspA from Detroit-562 cells compared to that elicited by WT D39 at either the mRNA or protein level (Section 4.2.5). These results suggest that PspA has the ability to stimulate an early CXC chemokine response from type II pneumocytes, and this may contribute to the early inflammatory response towards *S. pneumoniae* in the lungs. *S. pneumoniae* down regulates transcription of *pspA* when in the lungs relative to *in vitro* (Le Messurier *et al.*, 2005), and this may limit the chemokine response generated towards it by epithelial cells. It is possible that the ability of PspA to stimulate an early CXC chemokine response from type II pneumocytes is exploited by the host to detect the presence of *S. pneumoniae* and recruit neutrophils to the lungs where they can clear the bacteria. Mutants producing PspA molecules in which specific regions had been deleted were used to investigate the domain(s) of PspA involved in stimulating chemokine release from A549 cells. Experiments with these mutants showed that the first of 2 regions in the N-terminal α-helix of PspA was essential for the stimulatory activity of the molecule. Deletion of the entire α-helical region led to a 2-fold decrease in the IL-8 response of A549 cells. When only region 1 was deleted, the response elicited was also 2-fold less than that elicited by WT D39. However, when region 1 was present, and region 2 deleted, the response elicited was not significantly different to that elicited by WT D39 (Sections 6.2.3 and 6.2.4). Thus, region 1 but not region 2 of the α-helix is important in eliciting this IL-8 response. PspAΔh1 and PspAΔhelix elicited IL-8 responses that were similar to those elicited by ΔPspA (Sections 6.2.3 and 6.2.4). Thus, the effect on the IL-8 response of A549 cells of removing region 1 of the α-helix was the same as that of removing PspA altogether.

The inability of PspA to elicit a chemokine response from Detroit-562 cells suggests that PspA does not play a significant role in generating a CXC chemokine response from nasopharyngeal epithelial cells. PspA is reported to be important in protection of the pneumococcus from killing by ALF at mucosal sites such as the nasopharynx (Shaper *et al.*, 2004), and transcription of *pspA* is increased in *S. pneumoniae* isolated from the nasopharynx

of mice, relative to *in vitro* transcription (Le Messurier *et al.*, 2005). The results presented here suggest that increased expression of PspA would not lead to an increased inflammatory response, which might otherwise compromise survival of pneumococci in the nasopharyngeal niche.

Initial experiments with ΔPly suggested that Ply might have a role in eliciting a CXC chemokine response from Detroit-562 but not A549 cells (Section 4.2.5). Infection of Detroit-562 monolayers with this mutant led to a 2-fold decrease in IL-8 mRNA and protein compared to levels elicited by WT D39 (Figures 4.15 and 4.16 B). No significant decrease was seen in the response of A549 cells to ΔPly compared to WT D39 (Figures 4.14 and 4.16 A). The results obtained using Ply point mutants were confusing, with mutants in which Ply was completely absent showing no difference in chemokine induction from A549 cells compared to WT D39, but mutants producing Ply with reduced cytotoxicity or complement activating ability eliciting a significantly reduced CXC chemokine response compared to WT D39 (Figures 7.1 and 7.3 A). However, investigation of the roles of the specific activities of Ply in generation of a CXC chemokine response from Detroit-562 cells demonstrated that cytotoxicity of Ply influenced the IL-8 response. *S. pneumoniae* mutants that produced Ply with reduced cytotoxicity elicited a reduced CXC chemokine mRNA and IL-8 protein response from these cells (Sections 7.2.1 and 7.2.2). The complement activating function of Ply appeared to play no role in CXC chemokine induction from Detroit-562 cells with no difference observed in the response to this mutant compared to WT D39 (Figures 7.2 and 7.3 B).

Overall, it appears that nasopharyngeal epithelial cells respond to infection with live *S. pneumoniae* by upregulating CXC chemokines. CbpA may play a role in dampening this response, perhaps preventing neutrophil recruitment and subsequent clearance of the bacteria. It appeared that PspA did not have any effect on CXC chemokine responses from these cells.

The exact role of Ply at this site remains unclear, but it appears that it may have the ability to stimulate CXC chemokine production from these cells due to its direct cytotoxic activity. This activity may help to promote adherence by increasing the expression of pIgR on epithelial cells (Phillips *et al.*, 1990; Zhang *et al.*, 2000), and may promote invasion by damaging the epithelium.

Type II pneumocytes also appear to respond to pneumococcal infection by upregulating CXC chemokine production. PspA appears to contribute to this response by stimulating an early CXC chemokine response from these cells, a phenomenon that may be exploited by the host to recognise the presence of *S. pneumoniae* and promote clearance by recruiting neutrophils. CbpA appears to have a similar effect on type II pneumocytes as it does on nasopharyngeal epithelial cells, suppressing CXC chemokine release at 4 h. This may be to dampen the inflammatory response in the lungs, preventing clearance and leading to a prolonged response that damages the alveolar epithelium possibly enabling pneumococci to invade the blood.

It is important to note that there are many other cell types present in the nasopharynx and the lungs, and although PspA, CbpA, and Ply appear to have measurable effects on epithelial cells, they may have distinct effects on other cell types, which could influence the overall host response to pneumococcal infection. Moreover, none of the pneumococcal virulence factors investigated was solely responsible for chemokine induction, because none of the mutants tested reduced CXC chemokine induction from epithelial cells to control levels. It is also likely that additional pneumococcal factors are also involved in this activity.

Rather than relying on purified proteins to investigate the roles of CbpA, PspA, and Ply in generation of a CXC chemokine response from epithelial cells, this study mainly used intact, live pneumococci. This is preferential, because it reflects natural infection more accurately, and presents pneumococcal proteins in their natural context, and at physiologically relevant doses. However, this places some limitations on the assay conditions, particularly

duration of infection, meaning that the longer-term effects of pneumococcal infection on the CXC chemokine responses of these cells cannot be investigated. Even so, the very early responses have been shown to be vital in determining the outcome of pneumococcal infection, and so are worthy of investigation on their own. There is now evidence that there is substantial variation in the expression of key pneumococcal virulence factors *in vivo* compared to *in vitro*, and at different host sites (Le Messurier *et al.*, 2005; Orihuela *et al.*, 2004). Expression of PspA, and CbpA appeared to be increased in the nasopharynx, and decreased in the lungs of mice, compared to *in vitro* expression, and Ply expression was decreased in infected Detroit-562 cell culture, compared to expression in TC medium alone (Orihuela *et al.*, 2004). Given the influence of these proteins on CXC chemokine responses as described above, changes in expression will affect the response of epithelial cells at these different host sites to *S. pneumoniae* infection. Other pneumococcal factors involved in mediating the CXC chemokine response may also be expressed differently at distinct host sites, and so their influence on the CXC chemokine response will vary depending on the host site. Since the inflammatory response is a composite of all such factors, it is clearly a very complicated process, and defining the roles, and assessing the significance of contributions of individual pneumococcal factors remains a challenge.

This study provides a foundation that may lead to a better understanding of the way in which *S. pneumoniae* interacts with respiratory epithelial cells to stimulate a CXC chemokine response. It has characterised the involvement of three pneumococcal virulence factors (CbpA, PspA, and Ply) in this process, and the specific regions of these molecules responsible for the observed modulatory effects. A better understanding of the process by which *S. pneumoniae* triggers an inflammatory response in the host will provide further insights into the early steps in pathogenesis of pneumococcal disease.

## 8.1 Future directions

Clearly further research is needed to clarify the results presented here, and to fully understand the role of specific pneumococcal factors in eliciting an inflammatory response to infection with *S. pneumoniae*. Individually, CbpA, PspA and Ply all appear to influence the CXC chemokine response elicited by respiratory epithelial cells. Constructing *S. pneumoniae* mutants in which 2 or more of these proteins are deleted, and using these to infect cell monolayers, may indicate whether these proteins act in concert. It is also important to determine that differences in CXC chemokine release from epithelial cells *in vitro* correlate with differences in neutrophil recruitment *in vivo*. This could be achieved by measuring neutrophil numbers in the lungs and nasopharynx of mice infected with the mutants before and after infection, and comparing these to the numbers recruited in response to the wild-type strain. Identifying the host cell receptors to which CbpA, PspA, and Ply bind in order to exert their CXC chemokine modulating effects would also provide a greater understanding of the interaction of these molecules with epithelial cells. As mentioned above, it is also likely that there are other pneumococcal factors involved in eliciting a CXC chemokine response from respiratory epithelial cells, and identifying these could prove worthwhile. As mentioned previously, the nasopharynx and the lungs are complex environments with multiple cell types present, many of which probably contribute to the inflammatory response generated towards pneumococcal infection. Investigating the roles of pneumococcal factors in eliciting CXC chemokine responses from cells such as alveolar macrophages, lymphocytes, and endothelial cells could enable a broader understanding of the inflammatory response to *S. pneumoniae*, and the way in which it contributes to pathogenesis of pneumococcal disease.

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