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STUDIES OF *STREPTOCOCCUS PNEUMONIAE* COLONISATION: ROLE OF CAPSULE AND INDUCTION OF PROTECTIVE IMMUNITY

A thesis submitted for the degree of Doctor of Philosophy

by

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May, 2010

Declaration

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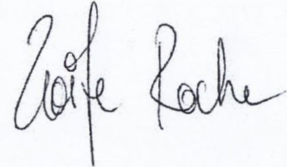

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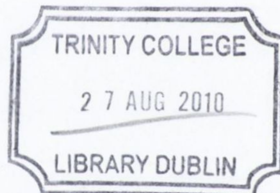
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Summary

Streptococcus pneumoniae (the pneumococcus) is a major human pathogen responsible for an estimated 1.6 million deaths each year, most of which are of young children in the developing world. Current pneumococcal vaccines target the polysaccharide capsule and despite their success in reducing invasive pneumococcal disease, there are limitations associated with their use and efficacy. These limitations, coupled with an increase in the prevalence of antibiotic resistance, have spurred research to find alternative vaccines.

S. pneumoniae asymptomatically colonises the human nasopharynx, yet this colonisation is a pre-requisite for invasive disease. Therefore, the study of nasopharyngeal colonisation is critical to understand the biology and pathogenesis of this opportunistic pathogen. The pneumococcus is enveloped by a thick layer of polysaccharide capsule, the serotype determining factor, of which there are over 90 currently identified. This capsule is also the major pneumococcal virulence factor which functions to mask underlying structures from host defences (anti-phagocytic), yet the importance of the capsule during nasal colonisation remained unknown. This thesis describes a novel role for the capsule during colonisation and also demonstrates that strains lacking this capsule act as potent live-attenuated vaccines.

Using a murine model of colonisation it was shown that unencapsulated strains remain capable of nasal colonisation, albeit at reduced duration and density compared to wild-type strains. This deficit in colonisation was not due to enhanced opsonophagocytic clearance. Immunohistochemistry of tissue sections from murine nasopharynx during colonisation showed that encapsulated strains reside along the surface of the epithelium following escape from lumenal mucus at the initial stage of colonisation. Conversely, unencapsulated strains remain agglutinated in this mucus and cannot efficiently transit to the site of stable colonisation. This suggests that the role of capsule in colonisation may be to aid in escape from lumenal mucus and subsequent mucociliary clearance. The inhibitory effect of encapsulation on mucus binding may be mediated by electrostatic repulsion, since both mucus and the majority of pneumococcal capsule types are negatively charged. Accordingly, removal of negatively charged sialic acid residues on mucus diminished the anti-adhesive effect of the capsule.

The observation that unencapsulated strains, which are attenuated in their ability to cause disease, can colonise the nasopharynx for at least seven days, led to the investigation of these strains as live-attenuated vaccines (LAV). Colonisation by these LAV strains, albeit limited, induced an immune response sufficient for both mucosal and systemic protection from challenge with a high dose of the virulent parent isolate. Immunisation also protected against a distantly related isolate (cross-protection). LAV immunisation elicited robust serum IgG and mucosal IgA responses and the humoral response is required for systemic protection as demonstrated using knock-out mice.

Western blot analysis of the humoral response induced by LAV identified three prominent cross-reactive antigens, PspA, PpmA and PsaA. These immunogenic antigens have all previously been suggested as candidates for a protein component vaccine. However, despite the robust response generated against PspA, and the amino-acid sequence similarity of PpmA and PsaA among clinical isolates, they are not necessary for mucosal cross-protection. This work highlights the need for a multi-component vaccine for efficient and broad protection, and confirms the effect of LAV protection against colonisation.

List of publications arising from research embodied in this thesis

Roche AM, Nelson AL, Gould JM, Ratner AJ, Weiser JN. (2007). Capsule enhances pneumococcal colonization by limiting mucus-mediated clearance. *Infect Immun.* **75**:83–90.

Roche AM, King SJ, Weiser JN. (2007). Live attenuated *Streptococcus pneumoniae* strains induce serotype-independent mucosal and systemic protection in mice. *Infect Immun* **75**:2469–2475.

Roche AM, Weiser JN. (2010). Identification of the targets of cross-reactive protein antigens induced by *Streptococcus pneumoniae* colonization. *Infect Immun.* **78**:2231–2239.

Other publications on *Streptococcus pneumoniae* during postgraduate registration

Dawid S, **Roche AM**, Weiser JN. (2007). The *blp* bacteriocins of *Streptococcus pneumoniae* mediate intraspecies competition both in vitro and in vivo. *Infect Immun.* **75**:443–451.

Matthias KA, **Roche AM**, Standish AJ, Shchepetov M, Weiser JN. (2008). Neutrophil-toxin interactions promote antigen delivery and mucosal clearance of *Streptococcus pneumoniae. J Immunol.* **180**:6246–6254.

Oral Presentations

Identification of the targets of cross-reactive antibodies induced by *Streptococcus pneumoniae* colonization. *Abstracts of the 7th International Symposium on Pneumococci and Pneumococcal Disease*, Tel Aviv, Israel. 14–18 March 2010, Abstract no. 36, p. 24.

Poster presentations

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Key to Abbreviations

2D	Two dimensional
aa	Amino acid
AOM	Acute otitis media
APC	Antigen presenting cell
bp	Base pair
BCIP	5-Bromo-4-Chloro-3′-Indolyphosphate-*p*-Toluidine
BSA	Bovine serum albumin
C4BP	C4b-binding protein
CBP	Choline-binding protein
CFU	Colony forming units
ChoP	Phosphorylcholine
CoVF	Cobra venom factor
Cps	Capsule
CRP	C-reactive protein
CSF	Cerebrospinal fluid
CSP	Competence stimulating peptide
d	Day
DAB	Diaminobenzidine tetrahydrochloride
DAPI	4′,6-diamidino-2-phenylindole
dH_2O	Distilled water
dNTP	Deoxynucleoside triphosphate
ECM	Extracellular matrix
ELISA	Enzyme-linked immunosorbent assay
Erm	Erythromycin
FITC	Fluorescein isothiocyanate
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
GMT	Geometric mean titre
h	Hour
H&E	Haematoxylin and eosin

HFCS	Hank's buffer supplemented with 5% foetal calf serum
Hic	Factor H binding inhibitor of complement
HIV	Human immunodeficiency virus
HPLC	High performance liquid chromatography
HRP	Horseradish peroxidise
Ig	Immunoglobulin
IM	Intramuscular
IN	Intranasal
IP	Intraperitoneal
IPD	Invasive pneumococcal disease
kb	Kilobase pair
kDa	Kilodalton
LAV	Live attenuated vaccine
LRR	Leucine rich repeats
LTA	Lipoteichoic acid
MDP	Muramyl dipeptide
MFI	Mean fluorescence intensity
Min	Minute
MLST	Multi-locus sequence typing
MMP	Matrix metalloproteinases
MSCRAMM	Microbial surface components recognising adhesive matrix molecules
NanA	Neuraminidase A
NBF	Neutral buffered formalin
NBT	Nitro blue tetrazolium
NHS	Normal human serum
NMS	Normal mouse serum
nt	Nucleotide
O	Opaque
OD	Optical density

Key to Abbreviations

PAF	Platelet activating factor
PAFr	Platelet activating factor receptor
PavA	Pneumococcal adherence and virulence factor A
PBR	Protein blocking reagent
PBS	Phosphate buffered saline
PBT	PBS with Triton X-100
PcpA	Pneumococcal choline-binding protein A
PCR	Polymerase chain reaction
PcsB	Protein required for cell wall separation of group B *Streptococcus*
PCV	Polysaccharide conjugate vaccine
PdB	Pneumolysin toxoid
PE	Phycoerythrin
Pht	Pneumococcal histidine triad
pIgR	Polymeric immunoglobulin receptor
PLG	Plasminogen
Ply	Pneumolysin
PPIase	Peptidyl-prolyl isomerase
PpmA	Putative proteinase maturation protein A
PPSV	Pneumococcal polysaccharide vaccine
PsaA	Pneumococcal surface adhesion A
PspA	Pneumococcal surface protein A
PspC	Pneumococcal surface protein C
PsrP	Pneumococcal serine-rich repeat protein
PVDF	Polyvinylidene fluoride
Rpm	Revolutions per minute
RT	Room temperature
s	seconds
SC	Subcutaneous
SCD	Sickle cell disease

Key to Abbreviations

SDS-PAGE	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
SlrA	Streptococcal lipoprotein rotamase A
StkP	Serine/threonine protein kinase
T	Transparent
TA	Teichoic acid
TD	Thymus-dependent
TS	Tryptic soy
U	Units
URI	Upper respiratory infection
WBC	White blood cells
WCV	Whole cell vaccine

This thesis is dedicated to my parents and my fiancé.

Chapter 1

Introduction

1.1 History of pneumococcal disease and treatment

Streptococcus pneumoniae (the pneumococcus) was first isolated in 1880 independently by both George Miller Sternberg and Louis Pasteur following inoculation of rabbits with their own saliva (Austrian, 2004). The rabbits quickly succumbed to septicaemia and investigation of their blood yielded large numbers of micrococci. The morphological features characteristic of the pneumococcus were observed and documented, the diplococcus form and the thick capsule (Sternberg, 1882). However, it was not until 1886 that the pneumococcus got its name and was associated with disease. Hans Christian Gram, who designed a staining protocol to better visualise microbes in tissue sections, noticed that bacteria appear to stain differentially with his method. Through the work of Gram, Carl Friedlander and Albert Fraenkel the pneumococcus was shown to cause lobar pneumonia, if the lung tissue was previously damaged. Likewise, it was also shown to be associated with pulmonary disease following injury. *S. pneumoniae* was also isolated from many other tissues and secretions in the body in the 1880's, confirming its association with a variety of diseases. However, it was noted that primary infections involved the respiratory tract (pneumonia, otitis media), but more invasive infections (meningitis, bacteraemia) were caused by haematogenous spread (López, 2006).

The human immune response to pneumococcus was also the subject of research. X-linked agammaglobulinaemia was first described in 1952, a rare genetic disorder with patients unable to generate mature B-cells (Conley, 1992). This disorder is commonly associated with increased pneumococcal infections. Other genetic conditions that are defective in antibody production, polymorphonuclear leukocytes or complement are also associated with increased incidences of pneumococcal infections, demonstrating their roles in protection from the pneumococcus (Janoff & Rubins, 1997). It is largely through work by Felix and Georg Klemperer that the importance of antibodies in protecting against the pneumococcus was realised (Klemperer & Klemperer, 1891). Immunisation with serum from rabbits previously inoculated with dead pneumococci conferred passive protection against virulent challenge. Elie Metchnikoff was the first to observe the clumping of pneumococci in the presence of rabbit serum in 1891 (Metchnikoff, 1891). This led to the discovery of multiple types of pneumococci, as some reacted with rabbit serum, while others did not. This variation in types was attributed to a carbohydrate substance in 1917

(Dochez & Avery, 1917). It was soon afterwards concluded that antibody to this capsular polysaccharide was responsible for immunity to pneumococcal infection. This led to the era of serum therapy to treat pneumococcal disease, by passively immunising patients with large doses of anti-capsular antibodies. Horse serum was the primary source of these antibodies due to the large volumes that could be obtained. However, the success of this serum therapy was minimal (Heffron, 1939), and coupled with the complication and expense of the therapy, this was soon surpassed by antibiotics to control pneumococci.

Optochin was the first drug realised to have inhibitory effects on the pneumococcus, yet it was not safe for human use (Morgenroth & Levy, 1911). Novel antimicrobials such as sulphanilamide, followed quickly by penicillin, became the most successful method of treating pneumococcal infection. However, *S. pneumoniae* is an adaptable foe and it did not take long for antibiotic-resistant strains to be identified, with resistance involving several complex mechanisms. Despite the introduction of antibiotics, pneumococcal infections remained a big problem, as resistant strains became more prevalent. This is one of the key reasons that a pneumococcal vaccine was developed. Vaccination began with whole cell vaccines in the early 1900's but quickly moved to polysaccharide-based vaccines in the 1940's. These vaccines have led to the significant decrease in pneumococcal disease and carriage.

The pneumococcus also played an important role in many scientific breakthroughs. Most noteworthy was through the study of how the pneumococcus obtained new capsule types from dead bacteria, in a process known as transformation. This led to the discovery that DNA is the basic unit of genetic material (Avery *et al.*, 1944; Lederberg, 1994).

1.2 The biology of streptococci

The genus *Streptococcus* comprises facultative anaerobic Gram-positive cocci that belong to the phylum Firmicutes and the lactic acid bacteria (Ryan *et al.*, 2004; Patterson, 1996). These bacteria are ovoid to spherical in shape and divide along a central plane leading to the characteristic growth in pairs or chains. They are catalase- and oxidase-negative and ferment glucose to lactic acid. Streptococcal species are usually classified based on their haemolytic activity on sheep blood agar. There are three groups: alpha-, beta- and gamma-haemolytic streptococci. Alpha-haemolysis is the greening or partial destruction of red

blood cells, and is produced primarily by the production of hydrogen peroxide by the streptococci (Barnard & Stinson, 1996). This leaves a green halo surrounding the colonies. *Streptococcus pneumoniae* and the viridans group fall into this category, with *S. pneumoniae* distinguished between other alpha-haemolytic species by optochin sensitivity, inulin fermentation, and bile solubility. The viridans group is mostly comprised of commensal oral streptococci. Beta-haemolysis is the complete rupture of red blood cells, leading to a zone of clearing surrounding the colonies. Beta-haemolytic streptococci are further divided by the expression of Lancefield antigens, which are grouped according to specific carbohydrate structures on the cell wall. Group A and B streptococci are both important pathogens that are aetiological agents of streptococcal pharyngitis ("strep throat") and neonatal meningitis, respectively (Facklam, 2002).

1.2.1 Streptococcus pneumoniae *biology and morphology*

Streptococcus pneumoniae has two prominent morphological characteristics – growth in chains or pairs (diplococcus) and the thick polysaccharide capsule that surrounds the bacterium which is recognised by the Quellung reaction (Neufeld, 1902). The polysaccharide capsule is the serotype-determining factor of the bacterium with over 91 serotypes identified to date. Beneath the capsule, the cell wall is predominantly composed of teichoic acid (TA) and peptidoglycan, which are found in roughly equal proportions (on a weight for weight basis) (Mosser & Tomasz, 1970). The pneumococcal peptidoglycan consists of layers of a glycan backbone connected via the *N*-acetylmuramic acid residue to the L-alanine of the stem peptide. TA (also known as C-polysaccharide), a membrane polysaccharide, is covalently attached by a phosphodiester bond to part of the *N*-acetylmuramyl residues of peptidoglycan (Ghuysen & Hackenbeck, 1994). Lipoteichoic acid (LTA) (also known as F-antigen) is identical in repeat and chain structures to TA apart from the lipid tail which anchors LTA in the plasma membrane. This is a unique trait to this bacterium as other bacteria have distinct TA and LTA structures (Fischer, 1997). Choline is a requirement for growth of *S. pneumoniae* as it is needed for TA biosynthesis. It is incorporated onto both TA and LTA, and these choline residues anchor a family of pneumococcal proteins known as choline-binding proteins (CBP) which non-covalently bind choline (Fischer, 2000). These CBP's have a range of functions including cell lysis and evasion of the host immune response (see **Section 1.5.2**).

Another key feature of *S. pneumoniae* is its natural competence. This competence is initiated by a competence-stimulating peptide (CSP) by a quorum-sensing mechanism. This ability to take up foreign DNA allows the pneumococcus to adapt to the nasopharyngeal niche it occupies (Claverys & Håvarstein, 2002; Johnsborg & Håvarstein, 2009).

1.3 Pneumococcal disease

S. pneumoniae is an important pathogen, killing around 1.6 million annually worldwide, most of whom are children in the developing world. The pneumococcus asymptomatically colonises the human nasopharynx with colonisation rates highest among children. This opportunistic pathogen is capable of causing disease if it progresses from the nasopharynx to normally sterile sites in the body, causing a range of diseases such as respiratory tract infections (otitis media, pneumonia) and systemic infections (meningitis, bacteraemia) (Durbin, 2004; Cartwright, 2002; Lynch & Zhanel, 2009b). Although colonisation represents the commensal state between host and bacteria, it is also the main risk factor for pneumococcal disease and is considered a pre-requisite for disease (Bogaert *et al.*, 2004a; Gray *et al.*, 1980; Faden *et al.*, 1997). The disease burden is highest in the youngest and oldest populations. Antibiotics (β-lactams and cephalosporins) are currently used to treat patients with pneumococcal infections. However, resistance to β-lactam drugs has been increasing and, therefore, there is an increasing need for prevention rather than treatment of pneumococcal disease (Weiser, 2010).

1.3.1 Otitis media and sinusitis

Otitis media is inflammation of the middle ear, which can be caused by viral or bacterial infection (Morris & Leach, 2009; Vergison, 2008). It is very common early in childhood, partly because the Eustachian tube is underdeveloped at this stage, with many children suffering from 2 to 3 episodes a year on average (Tonnaer *et al.*, 2006; Tan, 2002a; Tan, 2002b). While it is most often associated with viral upper respiratory infection (URI), the most common bacterial causes are *S. pneumoniae*, followed by *Haemophilus influenzae* and *Moraxella catarrhalis* (Vergison, 2008; Marchisio *et al.*, 2007). If a few displaced bacteria get into the Eustachian tube, normal clearance mechanisms, such as ciliary action, lead to swift elimination. However, in circumstances in which the opening of the

Eustachian tube is obstructed, such as viral infection or allergy, the bacteria may proliferate. Acute infection of the middle ear (AOM) leads to a build up of pressure and, if the issue is not resolved, rupture of the tympanic membrane (Morris & Leach, 2009). The traditional method of treatment of pneumococcal otitis media with β-lactam antibiotics is threatened by the spread of resistance. Indiscriminate treatment of children following inconclusive diagnosis may have promoted overuse of antibiotics and resistance.

The introduction of the heptavalent pneumococcal capsular polysaccharide conjugate vaccine (PCV7), which contains 5 of the most common serotypes that cause acute otitis media (6B, 9V, 14, 19F, and 23F) (Eskola *et al.*, 2001), has led to the decrease in AOM caused by *S. pneumoniae* by 34%, and reduced the overall AOM by 6–8% (Eskola *et al.*, 2001; Black *et al.*, 2000). Recent studies have also shown that PCV7-vaccinated children have an increased proportion of *H. influenzae* and *M. catharralis* in middle ear fluid (Eskola *et al.*, 2001).

Biofilms are highly organised three-dimensional aggregates of bacteria that are encased in an extracellular matrix consisting mainly of exopolysaccharide, along with DNA and proteins, in close association with a surface (Moscoso *et al.*, 2009; Tonnaer *et al.*, 2006; Murphy *et al.*, 2009). It is estimated that 60% of all human bacterial infections are the result of populations in biofilms (Hall-Stoodley *et al.*, 2004). Biofims were first suggested to play a role in otitis media due to the difficulty of antibiotic treatment of otitis media, and also the recurrent nature of the infection (Costerton, 2001). This infection mimics persistent lung infection in cystic fibrosis, which is attributed to the presence of a *Pseudomonas aeruginosa* biofilm. Evidence to support this hypothesis was later found, as pneumococcal biofilms have been detected in the middle ear of patients suffering from chronic and recurrent otitis media, and well as experimental otitis media in chinchillas (Hall-Stoodley *et al.*, 2006; Reid *et al.*, 2009). These populations are refractory to antibiotics and thus might explain why some patients improve while on antibiotics but relapse after completion of therapy leading to a chronic infection (McCullers *et al.*, 2007). Bacteria can be protected by this community organisation, and are up to 1000 times more resistant to antibiotics than phenotypically distinct planktonic bacteria (Slinger *et al.*, 2006; Kaji *et al.*, 2008; Starner *et al.*, 2008). Biofilms can also protect bacteria from host attack (Costerton *et al.*, 1999), and allow a reservoir for dissemeniation. Therefore, biofilms create an environment which enhances infection of the middle ear. Additionally, the

presence of viral variants also contributes to biofilm formation, with 70% of animals developing OM in the presence of influenza virus (Costerton *et al.*, 1999).

Sinusitis occurs when the drainage of the sinus is obstructed, via the osteomeatal complex, by prior infection. This allows the collection of fluid in the paranasal sinus cavity which supports bacterial growth and subsequent acute infection. Like otitis media, sinusitis is most commonly associated with *S. pneumoniae* and *H. influenzae* (Marchisio *et al.*, 2007).

1.3.2 Pneumonia

Pneumonia is an infection of the lungs which leads to inflammation, and is the leading cause of death in children worldwide (Bryce *et al.*, 2005). There are two types of pneumonia: bronchial pneumonia, where the infection is patchy around the large airways, and lobar pneumonia, where the infection is concentrated in one or more lobes. Pneumonia can be caused by microbes (bacteria, viruses, parasites or fungi) or lung injury. However, bacterial pneumonia is generally the most common and severe form. While *S. pneumoniae* can be the aetiological agent of both bronchial and lobar pneumonia, it is more commonly associated with lobar pneumonia, as 80% of cases are caused by the pneumococcus (van der Poll & Opal, 2009; Madeddu *et al.*, 2009).

If bacteria that are inhaled into the lungs are not cleared efficiently, they proliferate in the alveolar spaces and are carried along the alveolar septa. In these sites they activate complement, generate cytokine production and up-regulate receptors on vascular endothelial surfaces, which lead to filling of these spaces with exudate and white blood cells (WBC). This allows for the diagnosis of pneumonia, as X-rays will show non-lucent areas due to fluid build-up.

Patients often have underlying conditions or compromising cofactors that affect their ability to fight the infection, such as antecedent viral infection or smoking. Major risk factors for pneumococcal pneumonia include human immunodeficiency virus (HIV) infection, sickle cell disease (SCD), haemolytic uraemic syndrome, dialysis and, most importantly, influenza A infection (Dowell *et al.*, 2003; Feldman & Anderson, 2009; Hord *et al.*, 2002; Tokars *et al.*, 2002). Childhood pneumonia has declined by 39% (65% reduction in pneumococcal pneumonia) following the introduction of PCV7 vaccination in

the United States (Grijalva *et al.*, 2007). However, the pneumococcal polysaccharide vaccine appears to be less effective at preventing pneumonia in adults (Johnstone, 2009; Cicutto, 2009).

1.3.3 Meningitis

Meningitis is a disease caused by inflammation of the protective covering of the brain and spinal cord (the meninges). Meningitis has many causes, most commonly bacterial and viral, with bacterial meningitis more severe than viral (Kim, 2010; Andersen, 2007). Pneumococcal meningitis is much less frequent than pneumococcal pneumonia, yet it is much more severe (O'Brien *et al.*, 2009). Globally, there are 17 cases of pneumococcal meningitis per 100,000 children under the age of five years (incidence rate), with incidence rates as low as 6 in Europe and as high as 38 in Africa. Fatality occurs in up to 59% of cases of pneumococcal meningitis with brain and/or cochlear damage occurring in up to 50% of the survivors (O'Brien *et al.*, 2009; Bedford *et al.*, 2001). Treatment is entirely dependent on antibiotics, and requires rapid high doses of antibiotics intravenously (Chaudhuri *et al.*, 2008). Without treatment mortality approaches 100% and therefore rapid treatment in cases of meningitis is essential (Chang *et al.*, 2004). The pneumococcal vaccine has greatly reduced childhood pneumococcal meningitis in the USA, which means that adults are now the most likely age group to suffer from meningitis (Riordan, 2010).

1.3.4 Bacteraemia

Bacteraemia is the presence of bacteria in the blood (Myers & Gervaix, 2007; Perez *et al.*, 2004). There are two types of bacteraemia, namely, primary and secondary. Primary, or occult, bacteraemia is when bacteraemia occurs without any sign or focus of infection. In the case of the pneumococcus this usually happens by direct invasion through the nasal mucosa where it colonises. Secondary infection is when the pneumococcus gets into the bloodstream at the site of infection, e.g., such as in the lungs. In adults, most cases of pneumococcal bacteraemia are secondary. In cases of presumed primary bacteraemia where treatment is delayed, a focus of infection, such as meningitis, may appear. Bacteraemia can lead to septicaemic infection around the body if not controlled properly.

1.3.5 Conjunctivitis

Conjunctivitis is inflammation of the conjunctiva, the clear membrane that covers the sclera of the eye and lines the inner surface of the eyelids (Friedlaender, 1995). The mucous layer over the ocular surfaces normally protects the eye from invading pathogens, yet there have been a number of outbreaks of conjunctivitis caused by unencapsulated pneumococci in the recent years (Buck *et al.*, 2006; Crum *et al.*, 2004; Martin *et al.*, 2003). Unencapsulated pneumococci rarely cause other diseases as the polysaccharide capsule is anti-phagocytic and protects the bacterium from recognition and opsonisation. However, unencapsulated pneumococci adhere better to epithelial surfaces, including conjunctival epithelial cells. This increased adherence may, for instance, allow pneumococcal neuraminidase to breakdown the mucin protecting the eye (Williamson *et al.*, 2008).

1.4 Antibiotic resistance

Treatment of pneumococcal disease is often empirical and is primarily with β-lactams, or anti-pneumococcal fluoroquinolones (Segreti *et al.*, 2005). Like many important pathogens, *S. pneumoniae* is rapidly evolving mechanisms of resistance against the antibiotics that are used to treat pneumococcal infections (Jacobs, 2004; Croucher *et al.*, 2009). β-lactam resistance was first reported in *S. pneumoniae* in the 1960's (Hansman & Andrews, 1967), and rapidly spread worldwide (Lynch & Zhanel, 2005). The remarkable increase in antibiotic resistance globally is due to a few international clones (Gherardi *et al.*, 2007). A study in the US in the early 1990's showed that 16.4% of hospital isolates were resistant to at least one class of antibiotics used to treat the pneumococcus, with 85% of resistant isolates of the types included in the PCV7 vaccine (Breiman *et al.*, 1994). The introduction of the PCV7 vaccine has led to the decrease in carriage of penicillin-resistant pneumococci (Dagan, 2009a), and hence disease caused by these strains. More recently, a worldwide study demonstrated that 50% of isolates were resistant to at least one antibiotic class with resistance particularly high against β-lactams and macrolides (Felmingham *et al.*, 2007).

Resistance to β-lactams occurs by restructuring the targets of the drugs, the penicillin-binding proteins, to reduce the binding affinity of the antibiotics (Dowson *et al.*, 1989). There are two major mechanisms of resistance against macrolides – methylation of bacterial ribosomal RNA drug targets via enzymes encoded by the *ermA* and *ermB* genes

(Leclercq & Courvalin, 1991) and drug efflux mediated by the *mefA/E* gene products (Varaldo *et al.*, 2009). Resistance mediated by the *ermB* gene product is the most common in Europe, and is associated with high-level resistance, while efflux-mediated resistance predominates in North America, and is associated with low-level resistance (Felmingham *et al.*, 2007). It is also becoming increasingly common to find strains with both resistance mechanisms.

Despite concerns about the escalation of antibiotic resistance in pneumococci, there has also been some controversy as to whether this leads to increases in mortality caused by pneumococcal disease (Lynch & Zhanel, 2009a). The relationship between drug resistance and treatment failures has not been established convincingly (Pallares *et al.*, 1987; Pallares *et al.*, 1995; Yu *et al.*, 2003). Some studies have shown no independent effect of penicillin resistance on outcome of disease (Pallares *et al.*, 1987; Raz *et al.*, 1997; Aspa *et al.*, 2004), yet others have shown higher mortality rates in patients infected with strains resistant to cefotaxime or penicillin (Feikin *et al.*, 2000; Turett *et al.*, 1999). However, it may be difficult to fully dissect out the effect of antibiotic resistance on the outcome of pneumococcal disease due to many confounding factors, such as host variability (age, health) and pneumococcal virulence factor variations.

1.5 Virulence factors

Streptococcus pneumoniae has many factors that aid in persistence, immune evasion and disease progression (Jedrzejas, 2004; Mitchell & Mitchell, 2010; Preston & Dockrell, 2008; Weiser, 2010; Kadioglu *et al.*, 2008). **Table 1.1** lists the pneumococcal virulence factors known to date, and this section will discuss selected virulence factors in more detail. **Fig. 1.1** illustrates many of these on the surface of the pneumococcus. These include proteins which directly interact with host tissues as well as structures designed to block/hide binding of immune components. The contribution of these virulence factors appears to vary among different disease states and in different animal models.

Table 1.1

Pneumococcal virulence factors

Name	Function	% strains	Reference
Capsule (cps)	Modulates interaction with environment, Masking, anti-phagocytic	100	(Avery & Dubos, 1931; Watson & Musher, 1990) (Jonsson *et al.*, 1985)
CbpD	Unknown		(Gosink *et al.*, 2000)
CbpE	Unknown		(Gosink *et al.*, 2000)
CbpG	Putative serine protease		(Gosink *et al.*, 2000)
Enolase	Binds plasminogen		(Bergmann *et al.*, 2005; Eberhard *et al.*, 1999)
GAPDH	Binds plasminogen		(Bergmann *et al.*, 2004; Eberhard *et al.*, 1999)
Hyaluronate lyase	Breaks down connective tissue	99	(Berry *et al.*, 1994; Berry & Paton, 2000)
LytA	Cell wall hydrolysis		(Berry *et al.*, 1989a)
LytB	Cell wall hydrolysis		(Gosink *et al.*, 2000)
LytC	Cell wall hydrolysis		(Gosink *et al.*, 2000)
Neuraminidase	Cleaves off sialic acid from glycoproteins and glcolipids	100	(Tong *et al.*, 2000; Trappetti *et al.*, 2009)
PavA	Binds fibronectin		(Holmes *et al.*, 2001; Pracht *et al.*, 2005)
PcpA	Protein-protein interactions		(Sanchez-Beato *et al.*, 1998)
PhtA	Zinc uptake, Binds Factor H		(Ogunniyi *et al.*, 2009)
PhtB	Zinc uptake		(Ogunniyi *et al.*, 2009)
PhtD	Zinc uptake		(Ogunniyi *et al.*, 2009)
PhtE	Zinc uptake		(Ogunniyi *et al.*, 2009)
Pia	Iron uptake		(Jomaa *et al.*, 2005; Brown *et al.*, 2001)
Pili	Attachment to cells Inflammation	30	(Barocchi *et al.*, 2006a)
Piu	Iron uptake		(Jomaa *et al.*, 2005; Brown *et al.*,

			2001)
Pneumolysin	Pore-formation	100	(Berry *et al.*, 1989b)
PpmA	Peptidyl prolyl isomerases		(Cron *et al.*, 2009)
PrtA	Serine protease	100	(Bethe *et al.*, 2001)
PsaA	Transport manganese		(Talkington *et al.*, 1996; Berry & Paton, 1996; Marra *et al.*, 2002)
PspA	Protects against complement and lactoferrin	100	(McDaniel *et al.*, 1987; Ren *et al.*, 2003; Hammerschmidt *et al.*, 1999)
PspC	Protects against complement, adhesion		(Zhang *et al.*, 2000; Dave *et al.*, 2001)
SlrA	Peptidyl prolyl isomerases		(Hermans *et al.*, 2006)
Teichoic acid/ Lipoteichoic acid	Structure, inflammation	100	(Tillett & Francis, 1930; Goebel & Adams, 1943; Keller *et al.*, 1992)

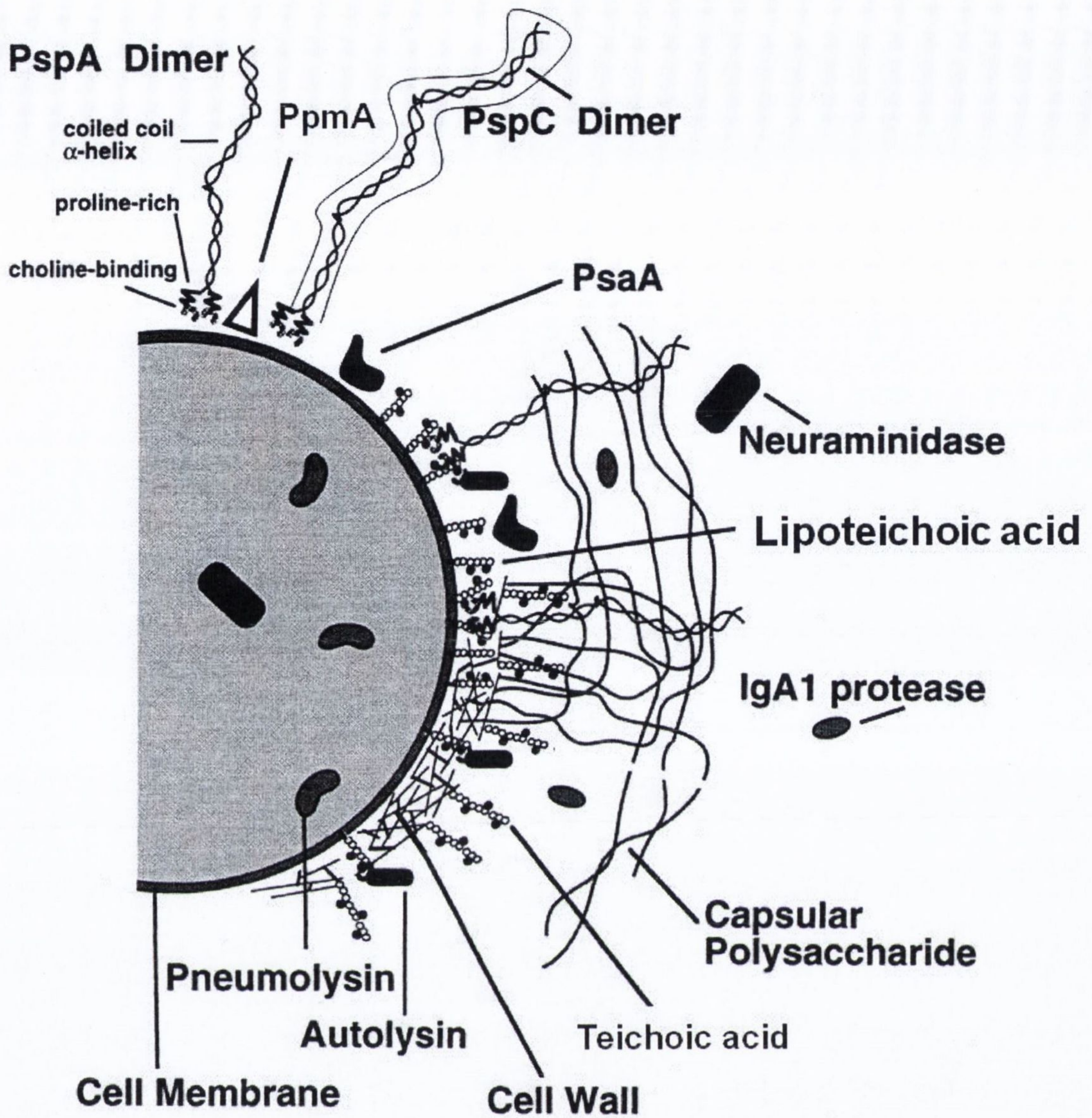


Fig. 1.1 Schematic diagram of the pneumococcal surface.

Major surface components, along with a number of important virulence factors, are shown. These include Ply, PspA, PspC and IgA1 protease. Adapted from Briles *et al.* (1998).

1.5.1 Capsule

Ninety-one pneumococcal polysaccharide capsule types have been identified to date (McEllistrem, 2009; López, 2006). Although the capsule types vary in sugar composition and linkages (Kamerling, 2000), they all have the same main function, i.e., to shield the bacterium from recognition by the host clearance mechanisms. The first study to demonstrate the virulence potential of the capsule used an enzymatic process to remove the capsule of a virulent type 3 strain, resulting in subsequent avirulence in mice (Avery & Dubos, 1931). This virulence has since been attributed to the capsule's ability to affect the quantity of complement component C3b deposited on the surface of the bacterium by obscuring underlying structures. Also, the capsule limits phagocytes from accessing the bound C3b, which marks the cell for opsonophagocytosis (Abeyta *et al.*, 2003). Antibodies against capsule can overcome this inhibitory effect of the capsule, by acting as an opsonin and activating the classical complement cascade. This polysaccharide structure is highly immunogenic in adults, and generates high titres of type-specific antibodies which can promote killing of the bacterium. However, if the host has never been exposed to a certain type before, this individual is more likely to be susceptible to persistence by and disease caused by this pneumococcal type.

Individual capsule types vary in their ability to evade these immune mechanisms. A recent study investigating why the current conjugate vaccine showed poor protection against 19F serotypes but good protection against 6B serotypes, both of which are included in the vaccine, demonstrated that the 19F capsular isolates require more anti-capsular antibody than the 6B isolates for complement deposition and opsonophagocytosis (Melin *et al.*, 2009).

Regulation of capsule production is important for the survival of the pneumococcus. Phase variation leads to two distinct phenotypes, opaque and transparent strains, which have increased and decreased amounts of capsular polysaccharide per cell, respectively, differing by up to 5.6 fold (Kim & Weiser, 1998; Weiser *et al.*, 1994; Kim *et al.*, 1999) (**Fig. 1.2**). Reduced amounts of capsule are important for exposing adhesins required for efficient colonisation of the nasopharynx (Weiser *et al.*, 1994), while more capsule helps

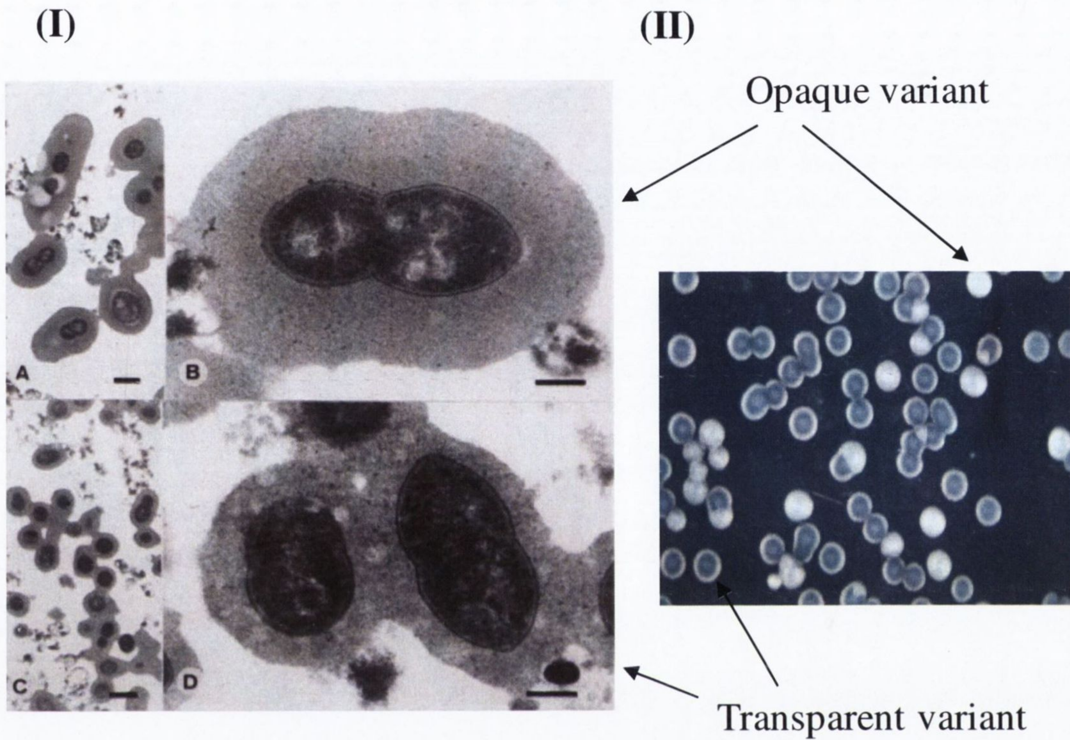


Fig. 1.2 Pneumococcal phase variation.

Streptococcus pneumoniae phase varies between an opaque phenotype and a transparent phenotype. This is characterised by the expression of more or less capsule, respectively. (I) Immunoelectron microscopy of pneumococcal capsules showing an increased zone of capsular material in opaque (A and B) compared to transparent (C and D) variants of a type 6B pneumococcal strain. Bar, 1 μm (A and C) and 0.3 μm (B and D). Kim *et al.* (1999). (II) Opaque and transparent colonies on transparent media (tryptic soy agar) visualised with oblique, transmitted light, and 60× magnification. Adapted from Weiser *et al.* (1994).

to evade opsonophagocytic killing in invasive disease (Kim & Weiser, 1998). Therefore, the type and amount of capsule produced affects the virulence potential of each strain by resisting immune mechanisms.

The majority of capsule types are negatively charged due to the presence of uronic acid, phosphate and/or pyruvate in their structure, with the few remaining types having a net neutral charge (Kamerling, 2000). This charge is important for another potential virulence function of the capsule. Most antimicrobial peptides are cationic and work by binding to the anionic surface of the bacterium. It has recently been demonstrated that the negative charge of capsule is responsible for antimicrobial peptides binding to the capsule, thus preventing them from binding to the surface of the bacterium, where they can damage the cell (Llobet *et al.*, 2008).

1.5.2 Choline-binding proteins (CBPs)

Pneumococci, and closely related oral streptococci, are unique among choline-containing bacteria as they express surface proteins which specifically and non-covalently bind choline as a cell wall attachment mechanism (Hakenbeck *et al.*, 2009; Hammerschmidt, 2009; Bergmann & Hammerschmidt, 2006). Genome sequencing identified 15 genes encoding proteins with similar choline-binding domains in the type 4 TIGR4 isolate (Tettelin *et al.*, 2001). However, the number of CBP's varies by strain (Hoskins *et al.*, 2001). CBPs have distinct functions, many of which are unknown, but they all share a 20 aa choline-binding repeat domain on the C-terminus (the choline-binding domain lies on the N-terminus of the LytB and LytC murein hydrolases (Lopez *et al.*, 2000)) for attachment to choline moieties on TA and LTA. They have a modular organisation and generally consist of a leader peptide, for secretion across the membrane, and a biologically active N-terminal region along with the choline-binding domain. Many of these surface-exposed structures have been well characterised as virulence factors.

1.5.2.1 LytA

The LytA protein was the first pneumococcal protein identified to interact with choline (Lopez *et al.*, 1997). It does not appear to be bound to choline in the cell wall, but requires binding to choline for full enzymatic activity (Garcia *et al.*, 1994). In the stationary phase of growth, when nutrients become depleted, *S. pneumoniae* undergoes autolysis through

the activation of this cell-wall-bound *N*-acetylmuramoyl-L-alanine amidase which breaks down the peptidoglycan layer. This is responsible for the phenotypic indentation in the centre of colonies. Autolysis-deficient strains have been shown to have reduced virulence in animal models (Berry & Paton, 2000).

The role of autolysis in virulence is unclear. However, there are many hypotheses for why this occurs. First, it has been suggested that autolysis occurs to release pneumolysin, Ply, the intracellular pore-forming toxin (Mitchell *et al.*, 1997). However, recent studies have suggested that autolysis is not required for the release of Ply (Balachandran *et al.*, 2001). Ply has been shown to interfere with host defence mechanisms (see **Section 1.5.3**). Secondly, autolysis releases cell wall degradation products which augment inflammation (Tuomanen, 1999). This inflammation has been suggested to contribute to the pathogenesis of the disease. Additionally, fragments from autolysed bacteria are unable to stimulate phagocyte-activating cytokines to the same extent as whole cells, and this reduces phagocyte-mediated elimination of live pneumococci in the blood (Martner *et al.*, 2009). Finally, autolysis provides a source of DNA for uptake by other strains to promote fitness.

1.5.2.2 PspA

Pneumococcal surface protein A (PspA) was the first surface-exposed protein identified that attaches to the cell wall via choline. PspA is a highly diverse protein as seen by immunological studies and sequencing of various pneumococcal strains, and is expressed by all pneumococci (Crain *et al.*, 1990; Waltman *et al.*, 1990; McDaniel *et al.*, 1998). The *pspA* gene has a mosaic structure, and encodes a protein with a highly variable N-terminus separated from the choline-binding C-terminus by a proline-rich region (McDaniel *et al.*, 1992) (**Fig. 1.3**). It is classified into three families and six clades by regions of sequence similarities, yet >98% of strains express PspA from families 1 and 2 (Hollingshead *et al.*, 2000). PspA is predicted to be arranged in long anti-parallel coils where it is thought that the negative charge of the distal tip of the coil forces the protein to stick out beyond the negative polysaccharide capsule (Daniels *et al.*, 2006; Jedrzejas, 2006).

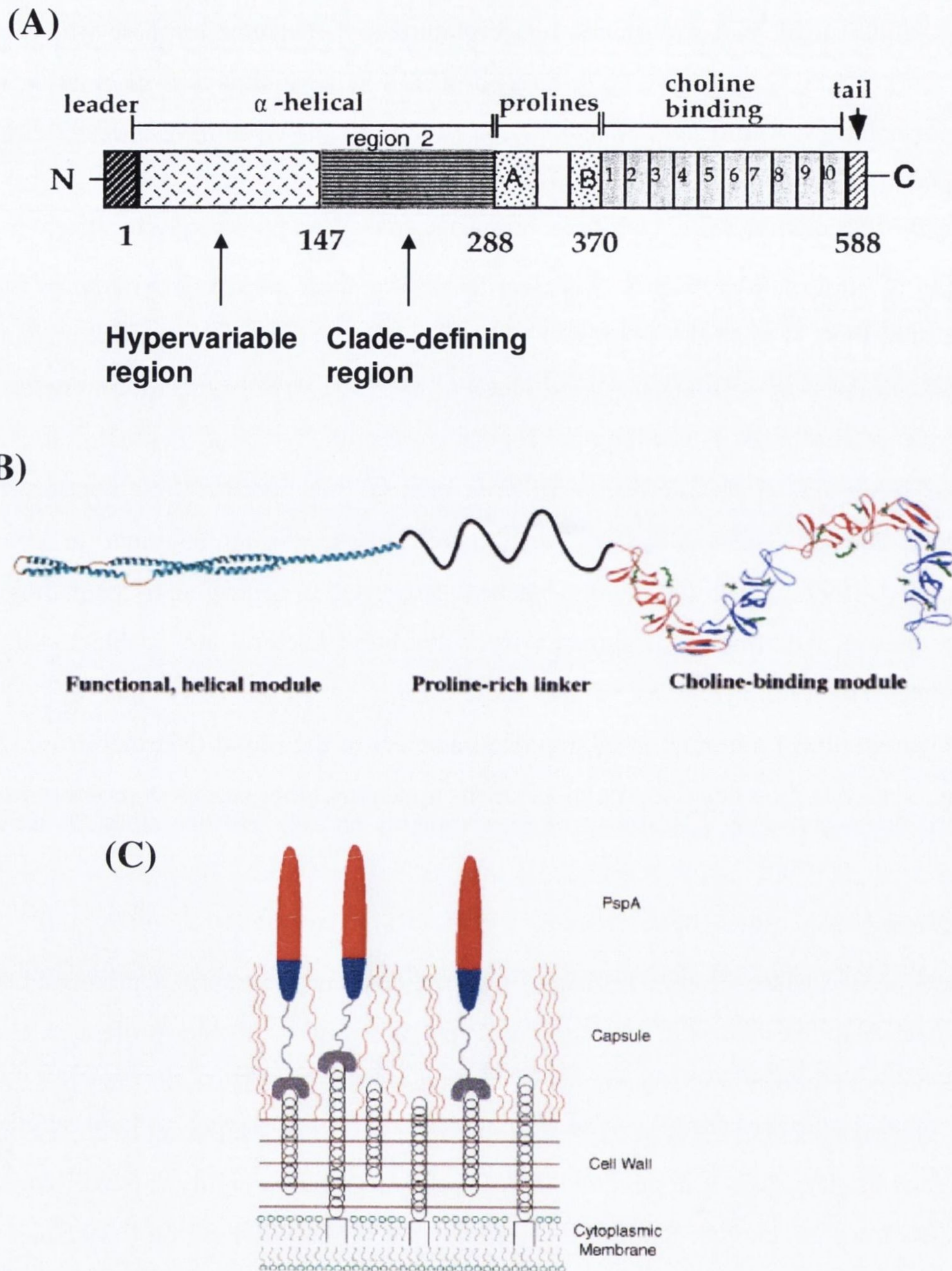


Fig. 1.3 Schematic diagram of the structure of pneumococcal surface protein A (PspA).

(A) Domains of PspA showing modular arrangement. Numbers represent amino acid residues. Adapted from Brooks-Walter *et al.* (1999). (B) Schematic diagram of a domain structure of full-length PspA. The first 288 amino acids are in the charged/helical coiled-coil conformation followed by the flexible peptide linker of the proline-rich region (aa 289–370), the CBD repeats (aa 371–571), and the hydrophobic 17 aa at the extreme C-terminal part. (C) Proposed view of PspA on the surface of *S. pneumoniae.* Interactions between the negative charge of many capsule types and the negatively charged parts of PspA are thought to result in PspA pointing its negatively charged end in the direction away from bacterial cells. (B-C) Adapted from Jedrzejas (2006).

The importance of PspA in virulence has been demonstrated by the rapid clearance of Δ*pspA* strains from the blood compared to the wild-type strain (Tu *et al.*, 1999). This has been attributed to the ability of PspA to interfere with C3 deposition and complement activation (Jarva *et al.*, 2003; Tu *et al.*, 1999; Ren *et al.*, 2004b). This complement activity is important for opsonophagocytosis, which is thought to be essential to eliminate pneumococci from the circulation (Brown *et al.*, 1983; Neeleman *et al.*, 1999). This interference appears to occur primarily through the classical pathway. However, the exact mechanism has yet to be defined.

Another virulence function of PspA involves binding human lactoferrin. Lactoferrin is an important component of innate immunity at mucosal surfaces, where it is found in abundance, and is therefore more important for controlling colonisation than invasive disease (Jenssen & Hancock, 2009; Shaper *et al.*, 2004; Lizzi *et al.*, 2009; Actor *et al.*, 2009). It has both bacteristatic and bactericidal activity against incoming pathogens. The bacteristatic activity is due in part to its iron-chelating ability, which can deplete iron and restrict bacterial growth (Otto *et al.*, 1992). The bactericidal activity is mediated by the N-terminal peptide lactoferricin, which is thought to attach to and destabilise negatively charged bacterial membranes (Zasloff, 2002). PspA has recently been shown to bind to lactoferrin at this lactoferricin domain, thus preventing the peptide from binding to and damaging the cell (Senkovich *et al.*, 2007). Therefore, PspA has virulence activities which contribute to the two phases of pneumococcal pathogenesis, i.e., invasive disease and mucosal colonisation. PspA is also immunogenic in humans. Antibodies to PspA have been shown to negatively correlate with susceptibility to carriage (McCool *et al.*, 2002), demonstrating the importance of this virulence factor in humans. However, the variability of this protein limits the cross-reactivity of anti-PspA antibodies (Miyaji *et al.*, 2002).

1.5.2.3 PspC

Pneumococcal surface protein C (PspC), also known as CbpA and SpsA, is a paralogue of PspA (Brooks-Walter *et al.*, 1999). PspC is regulated by the two-component signal transduction system RR06/HK06 (Standish *et al.*, 2005). It has a highly variable alpha-helical N-terminus, a similar choline-binding domain to PspA and a proline-rich region separating the N- and C-terminal portions. The N-terminal portion is important for the virulence function of PspC and mediates binding to secretory component, C3, Factor H

and C4BP (Hammerschmidt *et al.*, 2007; Quin *et al.*, 2007; Dieudonné-Vatran *et al.*, 2009; Jarva *et al.*, 2003; Hammerschmidt *et al.*, 2000a).

There are two main virulence functions of PspC. First is the role of PspC in colonisation. PspC is upregulated during colonisation (LeMessurier *et al.*, 2006), when the transparent phenotype is predominant, and binds in a human-specific manner to the secretory component of the polymeric immunoglobulin receptor (pIgR). This allows for adherence for colonisation, as well as transcytosis across the epithelial barrier (Zhang *et al.*, 2000). The importance of this protein in colonisation has been demonstrated by reduced carriage of PspC mutants in infant rats and pIgR-deficient mice (Rosenow *et al.*, 1997; Zhang *et al.*, 2000).

The second virulence function of PspC involves its interaction with complement components and is important for invasive disease (Jarva *et al.*, 2003; Yuste *et al.*, 2010). PspC has been shown to bind C3, as well as human factor H and C4b-binding protein (C4BP), which regulate the alternative and classical pathways, respectively (Dieudonné-Vatran *et al.*, 2009; Dave *et al.*, 2001; Neeleman *et al.*, 1999; Cheng *et al.*, 2000; Lu *et al.*, 2008a). Binding of these regulatory proteins factor H and C4BP in the blood can prevent complement attack by inhibition of C3b, thus preventing complement-mediated phagocytosis. Binding of PspC to factor H has also been shown to increase adherence to human cells and enhance lung invasion (Quin *et al.*, 2007). In models of lung infection and bacteraemia, PspC mutants were reduced in virulence compared to isogenic wild-type strains (Balachandran *et al.*, 2002). PspC is also immunogenic in humans with significantly increased titres of anti-PspC generated following colonisation (McCool *et al.*, 2003).

1.5.2.4 PcpA

Another CBP associated with virulence is the pneumococcal choline-binding protein A (PcpA). This is a large protein expressed in the exponential phase of growth and contains several leucine-rich repeats (LRR) in the N-terminal domain (Sanchez-Beato *et al.*, 1998). PcpA is differentially regulated in the presence of manganese and zinc by the transcriptional regulator PsaR (Johnston *et al.*, 2006; Kloosterman *et al.*, 2008). Manganese represses PcpA expression. Therefore PcpA has been shown to be important in

areas of low manganese, such as the lungs, where PcpA mutants colonise the lungs at a lower level compared to wild-type strains (Johnston *et al.*, 2006; Hava & Camilli, 2002). The mechanism of virulence is unknown. However, LRR domains have been proposed to be involved in protein-protein and protein-lipid interactions, and therefore PcpA may be an adhesin (Sanchez-Beato *et al.*, 1998). In contrast, the nasopharynx contains high levels of manganese and PcpA should be repressed in this location. Accordingly, PcpA was shown to have no effect on murine nasal colonisation (Johnston *et al.*, 2006).

1.5.3 Pneumolysin

Pneumolysin (Ply) is a 53 kD protein produced by virtually all clinical isolates of *S. pneumoniae*, is highly conserved among strains and is expressed during the late log phase of growth of the pneumococcus (Benton *et al.*, 1997). It is one of two haemolysins produced by *S. pneumoniae* (Canvin *et al.*, 1997), and is a cholesterol-dependent cytolysin, formerly known as thiol-activated cytolysins, which is part of a family of pore-forming toxins expressed by a range of Gram-positive organisms (Marriott *et al.*, 2008; Palmer, 2001; Alouf, 2000). These toxins bind to eukaryotic cells with cholesterol-rich membranes. It is primarily a cytoplasmic protein with no known signal sequence or anchoring motif. Autolysis of the cell leads to the release of Ply, but is not required, as Ply is also released by autolysis-deficient strains and localises to the cell wall by an unknown mechanism (Balachandran *et al.*, 2001; Price & Camilli, 2009).

Ply is a known virulence factor as mutants are attenuated in intranasal and systemic infection (Berry *et al.*, 1989b; Hirst *et al.*, 2004; Kadioglu *et al.*, 2000; Braun *et al.*, 2002; Wellmer *et al.*, 2002). There are two main functions associated with Ply virulence. First is the ability of Ply to bind to cholesterol on host cells and form large pores by oligomerisation of up to 50 monomers, thereby lysing the cell (Morgan *et al.*, 1995). This pore-forming function is required for full virulence in an intraperitoneal (IP) infection model (Berry *et al.*, 1999).

The second virulence function of Ply is its interaction with the complement system. Ply can activate the classical pathway without toxin-specific antibody by binding directly to C1q and indirectly by binding to the Fc portion of immunoglobulins (Ig). The ability to bind C1q is thought to be due to amino acid sequence similarity with the acute phase

protein C-reactive protein (CRP) (Mitchell *et al.*, 1991). This interaction with complement components has been shown to lead to reduced complement deposition and increased virulence (Yuste *et al.*, 2005), by quenching complement away from the pneumococcal surface. However, in some models of infection, where the pore-forming and complement-binding activities of Ply were disrupted, there was still increased virulence compared to the Δ*ply* strain (Berry *et al.*, 1999). This suggests that there are additional biological virulence functions of pneumolysin. Indeed, Ply has been associated with many other effects on host cells and tissues. Ply may also reduce non-specific host defence systems as it slows ciliary beating and disrupts the tight junctions of human respiratory epithelium (Rayner *et al.*, 1995; Steinfort *et al.*, 1989).

Ply also affects the production of proinflammatory mediators, such as TNF-α, IL-1β and IL-6, can stimulate nitric oxide release from macrophages and interacts with PMNs to increase bactericidal products (Rijneveld *et al.*, 2001; Houldsworth *et al.*, 1994; Braun *et al.*, 1999; Jones *et al.*, 2005). These effects may be due to interaction with Toll-like receptor 4 (TLR4). Ply does not induce an inflammatory response in macrophages from TLR4-deficient mice, and these TLR4-deficient mice are more susceptible to pneumococcal sepsis and colonisation, which is dependent on the expression of Ply (Malley *et al.*, 2003). This is not due to the cytotoxic or complement-activating ability of Ply, and may be by direct interaction with TLR4 (Srivastava *et al.*, 2005; Malley *et al.*, 2003). IL-17, a cytokine produced by Th17 T-cells, is also induced by Ply (Malley *et al.*, 2006). Interestingly, both of these host immune components, TLR4 and IL-17, are required for protection from pneumococcal colonisation, and the effects are dependent on Ply (Malley *et al.*, 2006; Malley *et al.*, 2003). IL-17 enhances neutrophil production and migration to sites of infection (Kolls & Linden, 2004) and, therefore, Ply has been shown to interfere with the ability of neutrophils to migrate towards and to kill, the pneumococci (Paton & Ferrante, 1983).

1.5.4 IgA Protease

IgA is the most abundant Ig isotype present in the nasal mucosa of which 90% is in the form of IgA1. Therefore, many pathogens at this site make an IgA1 protease to inactivate this major component of mucosal humoral immunity (Mistry & Stockley, 2006). All *S.*

pneumoniae isolates possess a zinc metalloproteinase, encoded by the *iga* gene, that specifically cleaves the hinge region of human IgA1 (Kilian *et al.*, 1979; Poulsen *et al.*, 1996). It is anchored non-covalently to a fragment linked to the peptidoglycan by sortase enzymes recognising the LPNTG motif on the amino-terminus (Bender & Weiser, 2006). The cleavage of the Fc_{α} region prevents recognition by phagocytes and leaves the underlying antigen obscured by bound Fab_{α} fragments, which is an important immune evasion mechanism. The bound cleaved IgA also enhances binding of the pneumococcus to human epithelial cells by neutralising the negative charge of the capsule and exposing choline on the bacterial surface to bind to the PAF receptor (Mahalingam & Lidbury, 2003; Weiser *et al.*, 2003). Pneumococcal IgA1 protease mutants are attenuated in pneumonia models (Hava & Camilli, 2002; Chiavolini *et al.*, 2003). Since pneumococcal IgA1 protease cannot cleave murine IgA, this attenuation must result from other functions of this protein. Pneumococcal IgA protease is also immunogenic and elicits antibodies early in life (Adrian *et al.*, 2004).

1.5.5 Other Proteases

The pneumococcus also produces other zinc metalloproteinases ZmpB, ZmpC and ZmpD (Camilli *et al.*, 2006). ZmpB is involved in virulence, mediated by induction of an inflammatory response, as Δ*zmpB* mutants are attenuated in an IP model and induce lower levels of TNFα (Blue *et al.*, 2003). ZmpC cleaves human matrix metalloproteinase 9, activating the human protease which cleaves extracellular matrix gelatine and collagen (Oggioni *et al.*, 2003). This has been shown to be important for virulence in a murine pneumonia model.

ClpE is one of five ATP-dependent caseinolytic proteases produced by *S. pneumoniae* (Zhang *et al.*, 2009a). It is required for pneumococcal growth at high temperatures (Chastanet *et al.*, 2001), and has also been demonstrated as a virulence factor in a murine IP infection model (Zhang *et al.*, 2009a). This has been attributed to a reduction in adhesion to host cells by ClpE dependent modulation of virulence factors.

1.5.6 NanA

Neuraminidase A (NanA) is an exoglycosidase that cleaves terminal sialic acid on host cells and proteins (Roggentin *et al.*, 1993). Virtually all pneumococcal strains express NanA (King *et al.*, 2005), and some also produce another neuraminidase, NanB (Paton *et al.*, 1993), and a putative neuraminidase NanC (Pettigrew *et al.*, 2006). NanA is anchored to the cell wall by an LPXTG sortase motif, whereas NanB and NanC are secreted (Navarre & Schneewind, 1999). However, a frameshift mutation in the TIGR4 genome sequence strain leads to secretion of NanA (Pericone *et al.*, 2002; King *et al.*, 2005).

One function of NanA has been attributed to nutrient acquisition, by cleaving monosaccharides from mucin as well as other glycoconjugates (Burnaugh *et al.*, 2008; Yesilkaya *et al.*, 2008). This decreases the viscosity of mucin which may inhibit mucin-mediated clearance. NanA has also been attributed a role in the niche competitiveness of pneumococci. Some respiratory pathogens attach sialic acid to their surfaces to mimic host cells and evade complement-mediated phagocytosis. NanA has been shown to cleave terminal sialic acid from its competitors *Haemophilus influenzae* and *Neisseria meningitidis*, exposing them to complement and thereby giving the pneumococcus an advantage in this niche (Shakhnovich *et al.*, 2002).

NanA has also been shown to be important for mucosal colonisation in both mice and rats (Manco *et al.*, 2006; Orihuela *et al.*, 2004; Tong *et al.*, 2000). This is due at least in part to the cleavage of sialic acid on pneumococcal host targets, such as secretory component, lactoferrin and IgA2, which may help persistence in the respiratory tract (King *et al.*, 2004). Also, NanA has been suggested to enhance adherence during colonisation, by changing the carbohydrate moieties in the tracheal epithelium and exposing receptors to pneumococci (Tong *et al.*, 2002). Therefore, immunisation with NanA protein leads to protection against nasopharyngeal colonisation and experimental OM (Long *et al.*, 2004). Furthermore, NanA has also been associated with biofilm formation (Parker *et al.*, 2009; Trappetti *et al.*, 2009). It has been suggested that sialic acid is a signalling molecule that induces NanA expression leading to enhanced adherence and survival within the biofilm environment (Trappetti *et al.*, 2009). This has been demonstrated to enhance pneumococcal colonisation and lung invasion.

A NanA mutant was not attenuated in an IP model of infection (Berry & Paton, 2000). Accordingly, expression of the *nanA* gene was found to be higher in the nasopharynx than in the blood or lungs (LeMessurier *et al.*, 2006). However, surface-anchored NanA has also recently been shown to contribute to meningitis as NanA is necessary and sufficient to promote pneumococcal adherence to, and invasion of, human brain microvascular endothelial cells, which depends on the N-terminal lectin-like domain (Uchiyama *et al.*, 2009).

The *nanA* gene has a high level of sequence diversity between strains, up to 14.8% at the amino acid level (King *et al.*, 2005). The diversity occurs in regions not involved in enzymatic function. This diversity suggests NanA may be an important target of the humoral immune response. Indeed, antibodies against NanA are elicited early in life, but antibody levels are not associated with a reduced risk of subsequent pneumococcal carriage or AOM (Simell *et al.*, 2006).

1.5.7 Other exoglycosidases

S. pneumoniae also produces other exoglycosidases such as BgaA and StrH, and an O-glycosidase (King *et al.*, 2006; Marion *et al.*, 2009). BgaA and StrH have been shown to act along with NanA to sequentially remove sialic acid, galactose and *N*-acetylglucosamine and expose mannose on human glycoproteins, allowing the pneumococcus to bind to the airway. Therefore, a triple *nanA*, *bgaA* and *strH* mutant displayed reduced adherence to epithelial cells in vitro (King *et al.*, 2006). The recently identified O-glycosidase has also been demonstrated to be involved in adherence to epithelial cells, and furthermore, plays a role in colonisation (Marion *et al.*, 2009).

1.5.8 PpmA

Putative proteinase maturation protein A (PpmA) is a lipoprotein regulated by the response regulator CiaR (Halfmann *et al.*, 2007). It shows amino acid sequence similarity to the parvulin family of peptidyl-prolyl *cis/trans* isomerases (PPIase) which are involved in protein folding, yet no detectable PPIase activity has been found to date (Hermans *et al.*, 2006; Overweg *et al.*, 2000a). PpmA sequence is conserved among pneumococci

(Overweg *et al.*, 2000a; Overweg *et al.*, 2000b), but expression is reduced in opaque phenotypic variants. It is unclear whether this surface-associated protein is exposed beyond the capsule as there are data supporting and opposing this possibility (Overweg *et al.*, 2000a; Gor *et al.*, 2005). However, antibodies against PpmA are found early in life and are increased in children with pneumococci colonising the nasopharynx but not the middle ear and decreased when pneumococci are found in the middle ear, suggesting that these antibodies are protective against middle ear colonisation (Vainio *et al.*, 2006). PpmA mutants have reduced virulence in a murine pneumonia model (Overweg *et al.*, 2000a), are attenuated in nasopharyngeal colonisation and are more efficiently phagocytosed in a strain-specific manner (Cron *et al.*, 2009).

1.5.9 SlrA

Streptococccal lipoprotein rotamase A (SlrA) is another peptidyl-prolyl isomerise produced by the pneumococcus (Hermans *et al.*, 2006). It is a functional, cyclophilin-type PPIase and contributes to pneumococcal nasopharyngeal colonisation, but not invasive infection. This occurs most likely by modulating the biological function of important virulence proteins. SlrA is also immunogenic, and antibodies are produced against SlrA early in life, yet the antibody titres do not correlate with reduced pneumococcal carriage or infection (Adrian *et al.*, 2004)

1.5.10 PsaA

Pneumococcal surface adhesion A (PsaA) is another surface-associated lipoprotein involved in virulence (Rajam *et al.*, 2008a). All pneumococci express the 37-kD PsaA with strong amino acid sequence identity across strains (Sampson *et al.*, 1997; Morrison *et al.*, 2000). One main function of PsaA is to transport Mn^{2+} into the cell as part of an ABC-type transport protein complex (Dintilhac *et al.*, 1997). The N-terminus of PsaA is directly attached to the lipid of the cytoplasmic membrane, yet similarly to PpmA, there is some controversy about the surface exposure of PsaA (Gor *et al.*, 2005; Johnston *et al.*, 2004; Jedrzejas, 2001; Russell *et al.*, 1990). Due to its size it is not expected to extend beyond the cell wall (Lawrence *et al.*, 1998). However, antibodies against PsaA increase in adults

and children in response to pneumococcal carriage and disease, suggesting surface exposure (Rapola *et al.*, 2001a; Rapola *et al.*, 2003; Simell *et al.*, 2001).

PsaA has been shown to be an adhesin which binds to human nasopharyngeal epithelial cells (Romero-Steiner *et al.*, 2003; Berry & Paton, 1996) which also suggests that it may be surface-exposed under some conditions. The receptor for PsaA binding has recently been described as E-cadherin (Anderton *et al.*, 2007), and binding to this tight junction protein has been shown to increase internalisation of pneumococci across the epithelial layer (Rajam *et al.*, 2008b). Therefore, PsaA mutants have reduced colonisation *in vivo* (McAllister *et al.*, 2004; Berry & Paton, 1996). Mutants lacking PsaA are also less virulent than wild-type pneumococci in an IP infection model, and this reduced virulence has been attributed to inefficient Mn^{2+} uptake and to reduced adherence, as well as hypersensitivity to oxidative stress (Berry & Paton, 1996; McAllister *et al.*, 2004; Tseng *et al.*, 2002). Further evidence of the contribution of PsaA to virulence is that transcription of the *psaA* gene is high in an IP infection model (Ogunniyi *et al.*, 2002).

1.5.11 Pili

Pili are hair-like structures which extend beyond the cell surface and aid in host-cell attachment (Scott & Zahner, 2006). Gram-positive pili, which are distinct from Gram-negative pili, are formed by covalent linkage of subunit proteins in a sortase-dependent manner (Scott & Zahner, 2006). Pili were only recently observed on the surface of the pneumococcus, and two types have been identified to date (LeMieux *et al.*, 2006; Barocchi *et al.*, 2006b; Bagnoli *et al.*, 2008). The first pneumococcal pilus consists of three subunits. RrgA anchors the pilin to the cell surface, while RrgB decorates the length of the pilus structure and RrgC is the tip subunit. The genes encoding pili are found on the pneumococcal *rlrA* pathogenicity island, flanked by insertion sequences characteristic of mobile genetic elements, along with three sortase enzymes (Barocchi *et al.*, 2006b). The second pilus is located on pilus islet 2, with PitB as the backbone subunit (Bagnoli *et al.*, 2008).

The first pneumococcal pilus has been associated with virulence. Strains expressing pili outcompete wild-type strains in murine models of colonisation, pneumonia and bacteraemia (Barocchi *et al.*, 2006a; Hava & Camilli, 2002). However, pili are not

associated with increased virulence in clinical isolates (Basset *et al.*, 2007). Piliated strains also elicit an enhanced IL-6 host response, demonstrating that pneumococcal pili can also stimulate inflammatory responses (Barocchi *et al.*, 2006b). Furthermore, immunisation with pilus subunits can protect against invasive disease (Gianfaldoni *et al.*, 2007).

Interestingly, few pneumococcal strains have genes encoding pili (Basset *et al.*, 2007; Aguiar *et al.*, 2008; Moschioni *et al.*, 2008; Moschioni *et al.*, 2009), with up to 30% of clinical isolates expressing the first pilus, while only 7% express the second pilus. Strains can express both pilus types, yet the second pilus is not present in the absence of the first pilus (Moschioni *et al.*, 2009). This infrequency among strains suggests the role of pili in virulence is redundant.

1.5.12 Other virulence factors

In this age of genomics and sequence mining some groups are using genomic library screens to identify novel virulence factors, often with the goal of uncovering unique vaccine targets (Shen *et al.*, 2006; Hava & Camilli, 2002). These studies have identified many novel putative virulence factors in both clinical isolates and reference sequenced strains (TIGR4), with variation in expression among strains, demonstrating the plasticity of the pneumococcal genome. One such study looking at tissue-specific virulence factors, using signature-tagged mutagenesis to screen 6149 *mariner*-transposon insertion strains, identified 387 attenuated mutants in a murine pneumonia model (Hava & Camilli, 2002). The virulence potential of the pneumococcal pilus was first demonstrated in this study. Other novel factors identified include a subtilisin-like serine protease, which has amino acid sequence similarity to another known pneumococcal virulence factor, and a platelet binding protein. These proteins, and the other identified factors, have yet to be fully characterised.

1.6 Adherence

The first step in colonisation requires attachment to the epithelial surface via host receptors and matrix proteins (Hammerschmidt, 2006; Nobbs *et al.*, 2009). This allows for stable colonisation of, and translocation across, the mucosal barrier. This can lead to subcellular

dissemination within the host and invasive infection, which is enhanced by adherence to host immune components to prevent clearance. This requires adhesins and receptors on the bacterial surface. **Table 1.2** lists some of the known pneumococcal adherence factors and MSCRAMMS (microbial surface components recognising adhesive matrix molecules). **Fig. 1.4** shows a schematic representation of how these pneumococcal adherence factors play a role in colonisation and invasive disease. Some of these adherence factors will be discussed in this section in greater detail.

Table 1.2. Pneumococcal adherence factors

(Adapted from Hammerschmidt (2006))

Adherence Molecule	**Receptor(s)/Target protein(s)**	**Target cell/Host niche**	**References**
Enolase	Plasmin(ogen)	Mucosal surface, serum	(Bergmann *et al.*, 2001; Bergmann *et al.*, 2003)
GAPDH	Plasmin(ogen)	Mucosal surface, serum	(Bergmann *et al.*, 2004)
Hic	Factor H	Serum	(Janulczyk *et al.*, 2000; Jarva *et al.*, 2004; Jarva *et al.*, 2002)
PavA	Fibronectin	Mucosal surface	(Holmes *et al.*, 2001; Pracht *et al.*, 2005)
ChoP	PAFr	Stimulated cells	(Cundell *et al.*, 1995a)
Pneumolysin	TLR4	Antigen presenting cells	(Malley *et al.*, 2003)
PsaA	E-Cadherin	Mammalian cells	(Anderton *et al.*, 2007)
PspA	(Apo-)lactoferrin	Respiratory tract	(Hammerschmidt *et al.*, 1999; Shaper *et al.*, 2004)
	Inhibitor of C3b deposition		(Ren *et al.*, 2003; Ren *et al.*, 2004b; Ren *et al.*, 2004a; Tu *et al.*, 1999)
PspC	Polymeric Ig receptor	Respiratory tract cells	(Zhang *et al.*, 2000; Luo *et al.*, 2005; Elm *et al.*, 2004; Lu *et al.*, 2003; Rosenow *et al.*, 1997)
	Secretory IgA and secretory component	Respiratory tract	(Hammerschmidt *et al.*, 1997; Hammerschmidt *et al.*, 2000b)
	Factor H	Serum	(Dave *et al.*, 2001; Dave *et al.*, 2004; Duthy *et al.*, 2002)
	Complement protein C3	Serum	(Smith & Hostetter, 2000)

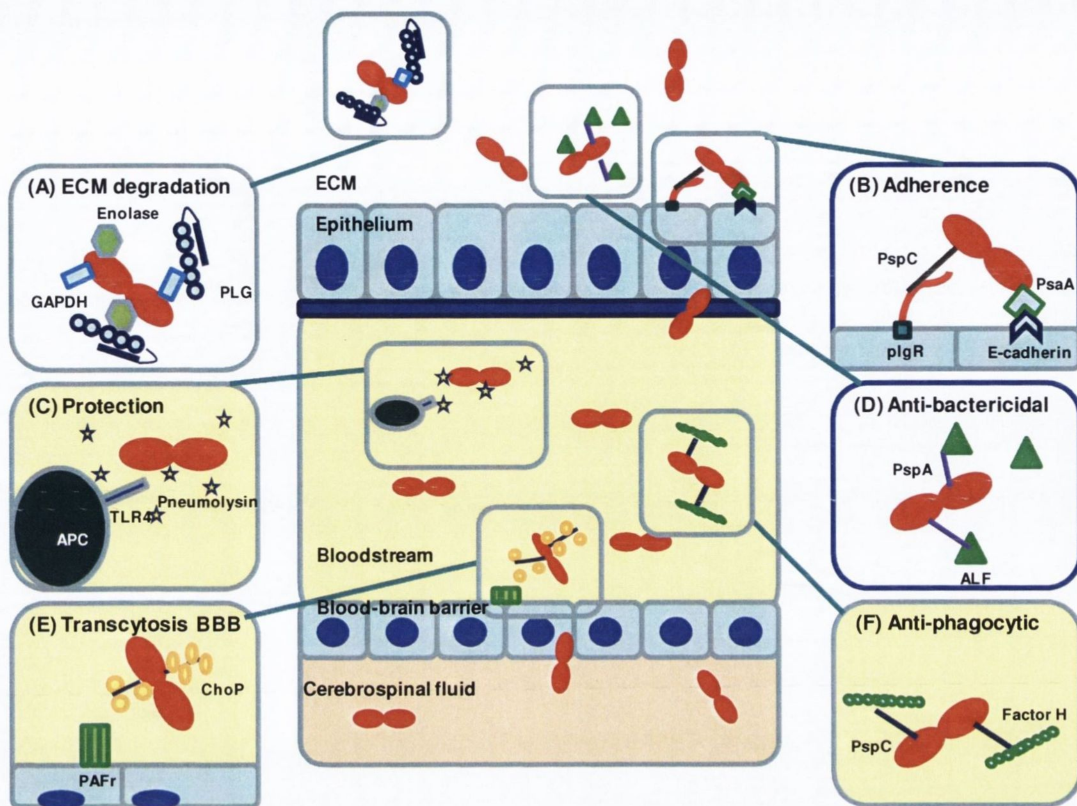


Fig. 1.4 Schematic diagram of pneumococcal adherence factors and their role in colonisation and invasive disease.

(A) GAPDH and enolase promote binding of plasminogen (PLG) which can degrade the extracellular matrix (ECM). (B) Pneumococcal surface proteins PsaA and PspC can bind to E-cadherin and pIgR, respectively, to promote adherence to the epithelium. (C) Pneumolysin can interact with TLR4 on APCs and may promote protection against innate immune responses. (D) PspA can bind to apolactoferrin (ALF), preventing killing by the bactericidal peptide. (E) ChoP binds to PAFr which can enhance adherence to, and transcytosis across, the blood brain barrier. (F) PspC can bind to the complement regulatory protein Factor H, preventing complement-mediated clearance. Adapted from Hammerschmidt (2006).

1.6.1 Pili

Pili, which extend beyond the surface of the bacteria, are known for their role in adherence to host cells (Mandlik *et al.*, 2008; Scott & Zahner, 2006). Both pneumococcal pili have been associated with adherence (Barocchi *et al.*, 2006b; Bagnoli *et al.*, 2008). RrgA, one of the three pilin MSCRAMM subunits from the first pneumococcal pilus, has been demonstrated to be the adhesive subunit (Nelson *et al.*, 2007; Izoré *et al.*). Strains lacking this subunit, which still form a pilus composed of the two remaining subunits, are significantly less adherent to human epithelial cells *in vitro*, and also have reduced colonisation in a murine model (Nelson *et al.*, 2007).

1.6.2 PavA

Pneumococcal adherence and virulence factor A (PavA) is a surface-exposed protein, despite the lack of leader peptide and membrane-anchoring sequence (Holmes *et al.*, 2001). PavA binds to immobilised fibronectin, an extracellular matrix protein found in various body fluids and tissues (Joh *et al.*, 1999; Holmes *et al.*, 2001). Binding to fibronectin represents a critical first step in the infection process. However, inactivation of the *pavA* gene only reduces binding of the pneumococcus to fibronectin by 50%, suggesting that other fibronectin-binding proteins are present on the cell surface which may contribute to binding (Holmes *et al.*, 2001). PavA also binds to epithelial and endothelial cells in a fibronectin-independent manner (Holmes *et al.*, 2001; Pracht *et al.*, 2005). Since adherence is not affected by preincubation with anti-PavA sera or exogenous PavA protein, it is thought that PavA may modulate other surface proteins rather than acting directly as an adhesin (Pracht *et al.*, 2005). PavA is also essential for full virulence in murine septicaemia and meningitis models (Pracht *et al.*, 2005).

Recently, another fibronectin-binding protein was identified in the pneumcoccus. Plasminogen- and fibronectin-binding protein B (PfbB), containing an LPXTG anchoring motif, was found to directly mediate bacterial adhesion to human epithelial cells via binding to both fibronectin and plasminogen (Papasergi *et al.*, 2010). This was demonstrated by binding fluorescent beads coated with PfbB to epithelial cells.

1.6.3 Plasminogen binding proteins

Plasmin is an extracellular matrix (ECM) protein present in the blood that breaks down many plasma proteins, in particular fibrin clots (Levi & van der Poll, 2010). Binding to plasmin(ogen) has also been described for the pneumococus. Two proteins associated with this binding are the glycolytic enzymes α-enolase and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Bergmann *et al.*, 2001; Bergmann *et al.*, 2004). However, neither of these proteins contains classic anchoring domains or secretion signals, and the mechanism of how they are secreted or anchored remains unknown. Pneumococci acquire host-derived proteolytic activity by binding plasmin(ogen) on their surface. Surface-bound plasmin activity has been associated with invasion and dissemination of infections (Lähteenmäki *et al.*, 2005). Indeed, enolase-deficient mutants were found to be attenuated in an intranasal infection model, due to decreased plasmin-mediated degradation of host ECM and transmigration through fibrin matrices (Bergmann *et al.*, 2005).

1.6.4 Choline-binding proteins

Many choline-binding proteins have been associated with adherence to host cells, leading to enhanced immune evasion and colonisation (Bergmann & Hammerschmidt, 2006; Hakenbeck *et al.*, 2009). PspA inhibits activation and deposition of human complement C3 through an unknown mechanism (Ren *et al.*, 2003; Ren *et al.*, 2004b). PspA also binds to lactoferrin, thus preventing killing by the bactericidal effects of this protein (Shaper *et al.*, 2004).

PspC is a multifunctional choline-binding protein with many known host ligands. PspC can bind to human secretory component on polymeric immunoglobulin receptors (Hammerschmidt *et al.*, 1997; Hammerschmidt *et al.*, 2000a). Binding to this receptor leads to adherence and transcytosis across mucosal respiratory epithelial cells (Zhang *et al.*, 2000; Brock *et al.*, 2002). Binding to free secretory component, along with secretory IgA which also contains the secretory component, may also allow the pneumococcus to evade phagocytosis by masking surface structures (Elm *et al.*, 2004; Hammerschmidt *et al.*, 1997). PspC can also bind to complement component C3 and factor H, which help the pneumococcus evade host recognition and clearance (Smith & Hostetter, 2000; Dave *et al.*, 2001).

1.6.5 Hic

The PspC-like protein Hic (factor H binding inhibitor of complement) also binds to factor H (Janulczyk *et al.*, 2000). The N-terminal sequence is similar to the N-terminus of PspC, yet Hic differs from PspC most noticeably through the mechanism of anchoring to the cell wall. Hic is bound to the cell wall by an LPXTG motif rather than a CBD (Janulczyk *et al.*, 2000). Binding of Hic to factor H has also been demonstrated to improve the survival of pneumococci in a systemic mouse model (Iannelli *et al.*, 2004).

1.6.6 ChoP

The pneumococcus can adhere to activated host cells by binding to platelet-activating factor receptor (PAFr) via phosphorylcholine (ChoP) (Cundell *et al.*, 1995a). ChoP decorates the bacterial surface, and its binding to PAFr enables the pneumococcus to adhere to epithelial cells, as well as to trancytose across the blood-brain barrier (Ring *et al.*, 1998). The choline-binding protein Pce (or CbpE) is a phosphorylcholine esterase which regulates the expression of ChoP on the surface to enhance colonisation and infection (Vollmer & Tomasz, 2001). Functional inactivation of Pce leads to changes in colony morphology, reduced colonisation, decreased adherence to epithelial cells and decreased survival (de las Rivas *et al.*, 2001; Vollmer & Tomasz, 2001). CRP also binds to ChoP to tag the bacteria for phagocytosis (Kaplan & Volankis, 1974). Therefore, regulation of the expression of ChoP is essential to maintain a balance between survival and pathogenesis.

1.6.7 PsrP

PsrP (pneumococcal serine-rich repeat protein) is a surface protein that is a member of the serine-rich repeat protein family (Shivshankar *et al.*, 2009). This family of proteins is known to comprise adhesins, with roles in biofilm formation, colonisation and invasive disease (Froeliger & Fives-Taylor, 2001; Bensing *et al.*, 2004b; Bensing *et al.*, 2004a). PsrP is also the largest bacterial protein identified (Rose *et al.*, 2008; Shivshankar *et al.*, 2009). PsrP is important for binding of the pneumococcus to lung epithelium via binding to keratin 10 in a lectin-independent manner, yet does not bind to keratin 10 on nasopharyngeal epithelium (Shivshankar *et al.*, 2009). Therefore, PsrP is required for

persistence in the lungs but not for colonisation or sepsis (Rose *et al.*, 2008). Furthermore, active immunisation with recombinant PsrP, or passive immunisation with antibodies against PsrP, leads to decreased bacteria in the blood and lungs and increased survival of mice (Shivshankar *et al.*, 2009; Rose *et al.*, 2008).

1.6.8 PsaA

PsaA was first suggested to be an adhesin based on amino acid sequence similarity to known adhesins in other *Streptococcus* species (Sampson *et al.*, 1994; Rajam *et al.*, 2008a). This was later confirmed, as strains lacking PsaA had reduced ability to bind to type II pneumocytes (Berry & Paton, 1996). Recently the receptor for PsaA has been characterised as E-cadherin, a tight junction protein that is important for cell adhesion (Anderton *et al.*, 2007). PsaA-deficient mutants also have reduced ability to colonise the nasopharynx (Berry & Paton, 1996; McAllister *et al.*, 2004), possibly due to inefficient adherence during colonisation.

1.7 Colonisation

S. pneumoniae resides on the mucosal surface of the nasopharynx (colonisation) in a largely commensal state with the host (Bogaert *et al.*, 2004a). Many other microorganisms share a similar niche, such as *Haemophilus influenzae*, *Neisseria meningitidis*, *Moraxella catarrhalis* and *Staphylococcus aureus* (Margolis *et al.*, 2010; Murphy & Parameswaran, 2009; de Vries *et al.*, 2009; Erwin & Smith, 2007; Yazdankhah & Caugant, 2004; van Belkum *et al.*, 2009). Nasopharyngeal colonisation is a dynamic process with fluctuations in the microorganisms colonising this niche partially mediated by competition between species and serotypes (Lysenko *et al.*, 2005; Pericone *et al.*, 2000; Dawid *et al.*, 2007).

Until recently, humans were thought to be the only natural host for the pneumococcus (Austrian, 1982). However, there is increasing evidence that pets and laboratory animals can also be colonised and infected with the pneumococcus (van der Linden *et al.*, 2009; Benson & Sweeney, 1984; Chanter, 1994). Isolates found in many animal species may be identical to human clones, and transmitted by close contact to humans. Yet isolates found in guinea pigs and horses appear to be specialised clones for these animals (van der Linden *et al.*, 2009; Whatmore *et al.*, 1999).

Nasopharyngeal colonisation is asymptomatic but is considered a prerequisite to disease (Bogaert *et al.*, 2004a; Gray *et al.*, 1980; Faden *et al.*, 1997). Disease will not occur without preceding nasopharyngeal carriage by the homologous strain (Faden *et al.*, 1997; Gray *et al.*, 1980). Also the risk of progression from asymptomatic colonisation to disease is greatest soon after the exposure and acquisition of a new isolate into the nasopharynx, but disease may also occur months after acquisition (Gray *et al.*, 1980; Austrian *et al.*, 1977). Serotype 1 strains are an anomaly, as they are rarely isolated from healthy carriers, yet are frequently associated with outbreaks of invasive disease (Hausdorff *et al.*, 2005; Mehiri-Zghal *et al.*, 2010). It has been proposed that rarely carried strains, such as serotype 1 strains, are poor colonisers because of a short duration of colonisation and/or a low density (Hausdorff *et al.*, 2005). However, the introduction of the conjugate vaccine has lead to increased prevalence of these strains among carriers (Nunes *et al.*, 2008).

Stable and asymptomatic colonisation requires adherence to non-inflamed epithelial cells. However, the transition from asymptomatic colonisation to invasion requires the local generation of inflammatory factors, such as TNF and IL-1, as seen with viral infections, which change the type and number of receptors on target epithelial and endothelial cells, and allow internalisation of pneumococci to occur (Cundell *et al.*, 1995a).

Colonisation of the nasopharynx is also the source of horizontal transfer of this pathogen, and crowding in day-care centres, hospitals and prisons increases this spread (Hoti *et al.*, 2009; Hoge *et al.*, 1994; Principi *et al.*, 1999; Mandigers *et al.*, 1994). Children are heavily colonised with the pneumococcus, followed by an age-related decline in colonisation associated with the maturation of the immune system. One study established that colonisation rates peak at 55% at the age of three years with a steady decline to stable rates of less than 10% after 10 years (Bogaert *et al.*, 2003). Other studies have mirrored these results (Mühlemann *et al.*, 2003; Daw *et al.*, 1997). Children as young as one-day-old can acquire one or more strains of *S. pneumoniae*, with up to five coexisting pneumococcal serotypes found in the upper respiratory tract of healthy children in a classic study (Gundel, 1933) (Tonnaer *et al.*, 2006). Serotypes 19F, 6B, 14, 23F and 6A are most common in young children in Europe and the USA, with serotype distributions varying slightly by area around the world (Bogaert *et al.*, 2001; Yeh *et al.*, 2003). Clearance can occur weeks to years after acquisition. However, the duration of carriage decreases with

successive acquisition of new strains (Gray *et al.*, 1980). The mucosal immune response has a key role in the regulation of nasopharyngeal pathogens, with a robust response necessary for clearance. In contrast, a poor response leads to persistence in this niche. This is seen in HIV and sickle cell disease patients who have increased risk for pneumococcal disease but not colonisation, as their primary mucosal barriers and immune responses are still intact (Polack *et al.*, 2000; Overturf, 2000).

The reported rates of bacterial acquisition and colonisation depend on age, geographical region, crowding, genetic background and other socioeconomic conditions (Principi *et al.*, 1999). There are also many pneumococcal factors associated with colonisation. These include adhesins (PsaA, choline), antiphagocytic factors (PspA, PspC,) and masking structures (capsule). These are discussed elsewhere in this chapter. *S. pneumoniae* undergoes phase variation between transparent and opaque phenotypes. Transparent strains have decreased amounts of capsule and increased expression of cell-surface proteins and carbohydrate-containing cell wall structures (Kim *et al.*, 1999) and thus adhere to epithelial cells better. Accordingly such strains are selected for during colonisation (Weiser *et al.*, 1996). The mechanism of phase variation is unknown, yet this variation allows for the survival of the pneumococcus in very different environments. Therefore, colonisation is a complicated and dynamic process with many contributing factors.

1.8 Animal models of *Streptococcus pneumoniae*

In order to better understand the pathogenesis of the pneumococcus, it is necessary to use appropriate animal models to mimic the infection in a controlled manner (Chiavolini *et al.*, 2008). There are many important considerations when choosing an animal model, including the type of animal and pneumococcal strain used. Some animal species are available as inbred and/or outbred varieties. Inbred animals have the advantage of uniform and consistent host responses, while outbred animals have more heterozygosity and therefore better mimic the variability in human responses. The bacterial strain used is also important, as the capsule type and genetic background can significantly affect the outcome of infection (Kelly *et al.*, 1994; Sandgren *et al.*, 2005). Many groups choose to study a limited set of strains, such as D39, WU2 and TIGR4 (types 2, 3 and 4 respectively), in order to compare results between studies.

Rodents, rabbits, chinchillas and guinea pigs have all been used to study pneumococcal disease (Chiavolini *et al.*, 2008). However, mice are increasingly the most popular choice due to the low cost, ability to use large numbers in a study and ease of manipulation. There are murine models of pneumonia, sepsis, meningitis, otitis media and colonisation which are readily used (Chiavolini *et al.*, 2008; Medina, 2010; Malley & Weiser, 2008). One such murine colonisation model, with IN inoculation without anaesthesia, has been validated by a human experimental colonisation study which demonstrated similar duration and density of colonisation between species (McCool *et al.*, 2002). There are also many models tailored for the study of protein or polysaccharide vaccines (Tai, 2006; González-Fernández *et al.*, 2008).

Even within species there are many choices. Many mouse species have been used in the study of pneumococcal disease, with the most common being C57BL/6, BALB/C, DBA and CBA mice (Chiavolini *et al.*, 2008). C57BL/6 mice have the advantage of the availability of many immunocompromised models, such as those lacking antibodies, T-cells or specific cytokines (Kitamura *et al.*, 1991; Grusby *et al.*, 1991; Dalton *et al.*, 1993). This allows the dissection of the host components involved in the response to pneumococcal infection. However, there are differences between mice which can sometimes lead to altered responses to pneumococcal infection (Ripoll *et al.*, 2010). C57BL/6 and BALB/C mice are known to have differences in the timing and vigour of the inflammatory response. C57BL/6 mice have a tendency towards a Th1 response, whereas BALB/C mice have a tendency towards a Th2 respone (Lohoff *et al.*, 1998). However, this does not always affect the outcome of pneumococcal infection (Gabr *et al.*, 2001).

1.9 Host response to *S. pneumoniae*

1.9.1 Host response to carriage

Nasopharyngeal colonisation of *S. pneumoniae* is the initial interaction with the host (Tuomanen, 1997; Käyhty *et al.*, 2006; Bogaert *et al.*, 2004a). This interaction lasts days to weeks, as host cells recognise and mount an immune response against the pathogen to prevent progression to disease. This colonisation leads to a robust influx of neutrophils

(van Rossum *et al.*, 2005), which associate with the bacteria in the nasal spaces (Matthias *et al.*, 2008). However, this early neutrophil influx is insufficient to control colonisation, as neutropenic mice have a similar density of colonising bacteria as wild-type mice at day 3 (Matthias *et al.*, 2008). Despite their limited role in the initial stages of colonisation, neutrophils may affect the immune response and contribute to the eventual clearance of pneumococci.

Colonisation also leads to an increase in type-specific antibodies, particularly IgA. It is unclear whether this antibody alone is sufficient to protect humans and animals from colonisation (Dagan *et al.*, 1996; Malley *et al.*, 1998). Also, vaccination with the capsular polysaccharide conjugate vaccine leads to an increase in type-specific IgG, which leads to decreased colonisation with included types (Huang *et al.*, 2009). However, studies investigating the natural mechanism of protection against carriage suggest that $CD4^+$ T-cells, but not antibodies, are required for naturally acquired mucosal protection in both naïve and previously exposed mice (van Rossum *et al.*, 2005; Malley *et al.*, 2005). Furthermore, the $CD4^+$ T-cell response in naïve mice is dependent on the pattern recognition receptor TLR2 and, via IL-17a, leads to recruitment of monocytes/macrophages which are necessary to clear the colonising bacteria (Zhang *et al.*, 2009b). This same study demonstrated that neutrophils may be key effectors in the clearance of colonisation in mice previously exposed to pneumococci.

1.9.2 Pathogenesis of pneumonia

Pneumococci are thought to gain entry into the normally sterile lower respiratory tract by being aerosolised from their site of colonisation in the nasopharynx into the alveolar spaces, bypassing the ciliated epithelium which they cannot bind to efficiently (Tuomanen, 1986; Novak & Tuomanen, 1999; van der Poll & Opal, 2009; McCullers & Tuomanen, 2001). Pneumococci localise to the alveolar epithelial cells via attachment to salivated cell surface glycoconjugates. It has been suggested that neuraminidases then cleave terminal sialic acid to enhance closer adherence (Paton *et al.*, 1997). Once localised to the airway, pneumococci release Ply and H_2O_2, which damage the epithelial cells.

An intense pulmonary inflammation is also initiated by bacterial cell-wall fragments (Tuomanen *et al.*, 1987b). Chemokine production from epithelium and C5a generated from

the activation of the complement system lead to recruitment of leukocytes to the site of infection. Mac-1, PAF receptor and Galectin-3 are important for leukocyte migration. This migration of leukocytes into the lesion is known as 'grey hepatisation' due to the change in colour of the lungs. The leukocytes trap the bacteria against the alveolar wall in a process known as 'surface phagocytosis'. Opsonins are crucial to the recognition and killing of bacteria by leukocytes in pneumonia. Both CRP, which binds to bacteria-associated choline, and complement play a critical role in tagging bacteria for phagocytosis (Mold *et al.*, 2002; Alexander *et al.*, 1998). The importance of complement-mediated phagocytosis is highlighted by patients deficient in complement components, who have increased susceptibility to recurrent pneumococcal infections (Alper *et al.*, 1970; Sampson *et al.*, 1982). Neutrophil-mediated killing of bacteria, by either phagocytosis or secretion of antimicrobial products, may be successful for controlling a low number of bacteria, but the presence of inadequate leukocytes often makes the inflammation worse. For example, release of neutrophil defensins enhances adherence of respiratory pathogens, which counteracts the benefit of their antimicrobial activity (Gorter *et al.*, 2000).

Key to the survival of the host is the production of TNF-α and IL-1 from leukocytes and epithelium (Takashima *et al.*, 1997). The resolution of the disease is followed by the replacement of neutrophils with macrophages to remove the debris (Robertson *et al.*, 1928). Therefore, the balance of the inflammatory response to control bacterial growth and yet prevent the release of inflammatory debris by extensive bacterial lysis is required for recovery. This may mean the difference between life and death in the progression of the disease (Dallaire *et al.*, 2001).

1.9.3 Pathogenesis of meningitis

Not all immune responses are beneficial for the host. Occasionally they are detrimental. In the case of pneumococcal meningitis, the neurological sequelae resulting from this invasive disease result from the overwhelming inflammatory response to bacterial products rather than the bacteria themselves (Tuomanen *et al.*, 1985; Brandt, 2010). This has been confirmed by treatment with anti-inflammatory agents, such as dexamethasone, or the blocking of adhesins required for leukocyte migration, which decreases the sequelae in mice and humans if given early in the progression of meningitis (de Gans & van de Beek, 2002; Täuber *et al.*, 1985; Tuomanen *et al.*, 1989). Despite the crucial role of pneumolysin

in pneumococcal pneumonia, the effect is more limited in meningitis (Friedland *et al.*, 1995). Rather, bacterial cell wall components are linked to this inflammation as they lead to a transient increase in meningeal inflammation (Tuomanen *et al.*, 1987a). The peptidoglycan constituent muramyl dipeptide (MDP), which is sensed via NOD2 receptors (Girardin *et al.*, 2003), is at least partially responsible for this vigorous inflammation, as studies confirm that these fragments are highly active in inducing inflammation in rabbits with experimental meningitis (Cottagnoud *et al.*, 2003). Sensing of these bacterial fragments in the cerebrospinal fluid (CSF) leads to production of TNF-α and other cytokines, which trigger a cascade of inflammatory mediators, such as matrix metalloproteinases (MMP), produced by neutrophils. These mediators contribute to the pathophysiology that can ultimately lead to hearing impairment, brain damage and/or death (Leib *et al.*, 2001; Leppert *et al.*, 2000).

1.10 Polysaccharide vaccines

The rise in antimicrobial resistance emphasises the importance of vaccine prophylaxis against pneumococcal disease (Bogaert *et al.*, 2004b; Bernatoniene & Finn, 2005; Poolman, 2004). The capsular polysaccharide is the major virulence factor of *S. pneumoniae* and when given as a purified component elicits capsule-specific antibodies which can afford protection (Austrian, 1976; Dagan *et al.*, 1996; Malley *et al.*, 1998) by tagging bacteria for uptake by phagocytic cells. Therefore, the capsule has been the focus of much of the vaccine design against *S. pneumoniae*. There are currently three licensed pneumococcal vaccines, all of which target the polysaccharide capsule, but are split into pneumococcal polysaccharide vaccines (PPSV) and polysaccharide conjugate vaccines (PCV).

1.10.1 PPSV

PPSVs were the first vaccines licensed against *S. pneumoniae*. The original PPSV was licensed in 1977 as a 14-valent vaccine. However, the current PPSVs, Pneumovax™23 manufactured by Merck and Co., and Pnu-Immune™23 manufactured by Lederle Laboratories, consist of highly purified polysaccharide capsules from 23 serotypes.

Pneumovax™ was first licensed in the USA in 1983 and is recommended for adults over 65 and children over the age of two years with certain chronic conditions who have problems clearing infections. It is immunogenic in adults and children (Borgono *et al.*, 1978), but because the immune response to the vaccine is T-cell independent, it is not immunogenic in children ≤ 2 years of age whose immune system is immature. Early studies demonstrated the vaccine's ability to protect against pneumococcal pneumonia and bacteraemia with vaccine efficacy between 76–79% and 82–92%, respectively (Austrian *et al.*, 1976; Smit *et al.*, 1977). However, recent studies have questioned the efficacy of the vaccine for preventing pneumonia in adults (Cabre, 2009).

1.10.2 PCV

Since the pneumococcal disease burden is particularly high in young children and the PPSV vaccine is not immunogenic in this age group, an alternative vaccine was needed. PCV7, or Prevnar™, was the first pneumococcal conjugate vaccine licensed for use in young children (Hsu *et al.*, 2003; Oosterhuis-Kafeja *et al.*, 2007; Jacobson & Poland, 2002; Murray & Jackson, 2002). Differing from PPSV, the protein conjugation of the capsular polysaccharides in the PCV7 vaccine ensures that this vaccine elicits a T-cell-dependent immune response, with antibody titres boosted by subsequent immunisation. It was licensed in 2000 in the US, and 2001 in Europe and has been incorporated into the national immunisation programmes in many countries around the world. Manufactured by Wyeth Pharmaceuticals, it consists of capsular polysaccharide from seven serotypes conjugated individually to CRM197, a non-toxic variant of the diphtheria toxin obtained by nitrosoguanidine mutagenesis of toxigenic corynephage β (Uchida *et al.*, 1971). The seven capsular types included in the vaccine (4, 6B, 9V, 14, 18C, 19F, 23F) are responsible for 80% of invasive pneumococcal disease (IPD) in the USA and 37–100% in various regions of Europe.

Since the introduction of this vaccine the incidence of IPD, and the frequency of antimicrobial-resistant pneumococci, have decreased (Whitney *et al.*, 2003; Isaacman *et al.*, 2009; Bettinger *et al.*, 2010). However, vaccine coverage has also declined, as types not included in the vaccine are becoming more prevalent due to serotype replacement. The incidence of IPD in adults has also been reduced following the introduction of PCV7

vaccination. This has been attributed to reduced colonisation by vaccine types in vaccinated children (Ghaffar *et al.*, 2004), which led to a decline in adult colonisation by decreased exposure and transmission as a result of herd immunity (Millar *et al.*, 2008; Hammitt *et al.*, 2006). Studies have shown the efficacy of the PCV7 vaccine in reducing pneumonia incidence and hospital admissions by at least 32% (Grijalva *et al.*, 2007; Black *et al.*, 2002) but the prevention of otitis media episodes is more limited, with efficacy rates from 6–7% in various studies (Black *et al.*, 2000; Eskola *et al.*, 2001).

To achieve greater vaccine coverage GlaxoSmithKline produced a 10-valent PCV, Synflorix™, which includes serotypes 1, 5 and 7F in addition to the PCV7 types (Croxtall & Keating, 2009). The conjugation protein in this vaccine is Protein D from non-typeable *H. influenzae*, and as a result the vaccine has been shown to protect against otitis media caused by both *S. pneumoniae* and *H. influenzae* (Prymula *et al.*, 2006). Synflorix™ is licensed in Canada, Australia and recently Europe, and affords vaccine coverage against up to 90% of IPD serotypes in parts of Europe (PneumoADP, 2008). It is predicted to prevent approximately 60–80% of IPD in young children worldwide and to provide greater protection than Prevnar™ (Hausdorff *et al.*, 2009). There is also a 13-valent PCV in the pipeline, Prevnar™13, which incorporates an additional 3 serotypes, including serotype 19A, which is increasing in prevalence around the world and is frequently associated with antibiotic resistance (Dagan, 2009a; Kaplan *et al.*, 2010).

Despite the success of these conjugate vaccines, there are also many limitations with their use, in addition to serotype replacement (Hsieh *et al.*, 2008). Firstly, the level of protection from mucosal infections is limited. Furthermore, the distribution of serotypes responsible for IPD varies by location, requiring that vaccines be tailored to each geographical region to ensure the greatest level of protection. This geographical specificity, coupled with the complexity of the vaccine, as each PS-protein conjugate is prepared separately, contributes to the prohibitive cost for those in most need in the developing world.

1.11 Protein component vaccines

Experience with pneumococcal vaccines has demonstrated that vaccination based on serotype-specific targets leads to a reduction in the disease burden due to vaccine types.

Importantly, this also leads to serotype replacement of pneumococci with types not included in the vaccine. To address this issue and to create a vaccine with broad coverage that will not lead to serotype replacement, pneumococcal vaccine research is currently focused on protein component vaccines (Bogaert *et al.*, 2004b).

Pneumococcal antigens that are common to all or most serotypes have received much interest as vaccine targets for their potential to induce broad protection (Briles *et al.*, 1998). Proteins in particular have received a lot of attention as they have many advantages over polysaccharides. Firstly, they induce T-cell-dependent immune responses, including immunological memory, in young children, the target population (Rijkers *et al.*, 1993). Also, there are large scale expression systems available for inexpensive manufacturing of protein vaccines, making it more accessible for people in the most need in developing countries. Some of the successful protein candidates are discussed below.

1.11.1 Ply

Ply was the first protein suggested as a vaccine candidate. Due to the toxicity of this protein it is necessary to inactivate the cytolytic activity by amino-acid changes, thereby reducing the possible adverse effects, for use of this protein in vaccine studies. Antigenicity is highly conserved among clinical isolates suggesting broad protective capabilities (Cima-Cabal *et al.*, 1999). Antibodies against Ply block the cytotoxic/cytolytic and complement activation activities *in vitro*. Studies using purified pneumolysin toxoid (Pdb) have demonstrated that it can protect against pneumonia and bacteraemia, and slow the progression of disease (Tai, 2006). Since Ply is predominantly contained within the cell, antibodies induced by immunisation are presumed to protect by neutralising the biological properties when it is released from the cell, thereby inhibiting infection, rather than stimulating opsonophagocytic clearance. This may be why Ply has not been successful in reducing carriage (Briles *et al.*, 2000a). The pneumolysin toxoid has also been demonstrated to be an effective protein carrier for polysaccharide vaccines (Michon *et al.*, 1998).

1.11.2 PsaA

One protein antigen which has been shown to be successful in reducing carriage is PsaA. Immunisation with purified PsaA, in combination with strong adjuvants, reduces

pneumococcal colonisation without affecting the microflora in the nasopharynx (Pimenta *et al.*, 2006). This is further evidence that perhaps PsaA is surface-exposed under certain conditions or in the transparent variant. Additionally, children with high titres of naturally acquired mucosal and serum anti-PsaA antibodies are less likely to have carriage that progresses to AOM. Therefore, it is likely that purified PsaA will protect against AOM also (Rapola *et al.*, 2001b).

1.11.3 PspA

PspA is the most well studied and characterised pneumococcal protein vaccine candidate (Briles *et al.*, 1997; Ferreira *et al.*, 2009). It was first identified as immunogenic by immunoscreening a λ-phage pneumococcal library with monoclonal antibodies elicited against heat-inactivated unencapsulated pneumococci (McDaniel *et al.*, 1984). PspA has proved successful in protecting against both invasive disease and colonisation in many mouse models (Swiatlo *et al.*, 2003; Oma *et al.*, 2009; Briles *et al.*, 2003), and has also been investigated as a possible protein carrier for a 5-valent capsular polysaccharide conjugate vaccine (Meng *et al.*, 2009). Surface-exposed PspA is highly variable and, although there is thought to be a higher degree of protection elicited within the same protein family, protection has been seen between PspA families (McDaniel *et al.*, 1991; Briles *et al.*, 2000b; McDaniel *et al.*, 1998). To enhance the cross protection of this protein, a fusion protein of PspA's from the two major families, including clades 3 and 4 from family 2, has been suggested (Hollingshead *et al.*, 2000; Darrieux *et al.*, 2007). The success of PspA vaccine studies led to a phase 1 clinical trial, where young adults were immunised with purified PspA in combination with the mucosal adjuvant alum. This immunisation induced cross-reactive antibodies that were able to passively protect mice against challenge from three virulent serotypes (Briles *et al.*, 2000b). The success of this trial highlights the potential for common surface proteins in protecting against IPD.

1.11.4 PhtB

Another successful protein vaccine candidate belongs to the family of histidine triad proteins (Pht) of the pneumococcus. This is a family of four proteins with an unusual histidine triad motif HXXHXH which is repeated 4–5 times in the sequence. Their role in virulence has recently been demonstrated, with attenuation only seen when all Pht proteins are removed. They act through inhibition of complement via recruitment of factor H

(Ogunniyi *et al.*, 2009). Surface-exposed PhtB has been studied in greatest detail, but all protein types have induced protection as demonstrated by increased survival rates. PhtB protects from fatal challenge by multiple strains and has also led to reductions in the numbers of bacteria in the nasopharynx (Adamou *et al.*, 2001; Hamel *et al.*, 2004).

1.11.5 Others

Recently there was a study taking an unbiased look for novel vaccine targets, using genomic antigenic fingerprinting of antibodies from patients who had been exposed to *S. pneumoniae* (Giefing *et al.*, 2008). This study identified two novel vaccine candidates, a protein required for cell wall separation of group B *Streptococcus* (PcsB) and serine/threonine protein kinase (StkP), which were highly conserved among strains and showed significant cross protection in murine models of sepsis and pneumonia.

Additionally, intranasal immunisation with cell wall polysaccharide, with adjuvant, induces serotype-independent immunity to pneumococci, which is mediated through IL-17A expressing $CD4^+$ T-cells (Malley *et al.*, 2006; Lu *et al.*, 2009b).

1.11.6 Combinations

To ensure the greatest level of protection that is broadly cross reactive, a number of studies have looked at combinations of antigens. Some of these combinations prove more successful than the individual antigens alone in eliciting protection. One such combination, Pdb, PspA and PspC, has been demonstrated to elicit enhanced protection against sepsis and pneumonia (Ogunniyi *et al.*, 2006), while a combination of PspA and PsaA has enhanced protection against colonisation (Briles *et al.*, 2000a). Additionally, combining cell wall polysaccharide with a fusion protein consisting of pneumolysin toxoid and PsaA confers protection against both colonisation and invasive disease (Lu *et al.*, 2009a). This confirms the need for a multi-component vaccine for the greatest efficacy against pneumococcal disease.

1.12 Killed whole-cell vaccines

Another novel vaccine approach against *S. pneumoniae* is killed whole-cell vaccines (WCV). Intranasal immunisation with the heat-inactivated whole-cell TIGR4 strain has

been demonstrated to induce protection against invasive disease, even in the absence of adjuvant (Hvalbye *et al.*, 1999). Ethanol-killed WCV's have been described in much detail. Malley et al. (2001) developed a WCV made from ethanol-inactivated unencapsulated cells from serotype 2 background. These cells which have been genetically modified to expose noncapsular antigens by the removal of the capsule-encoding genes, have increased density of growth due to defective LytA, and contain a non-toxic Ply (Pdb). Studies have demonstrated that intranasal immunisation with this WCV can induce protection against nasopharyngeal and middle-ear colonisation, as well as invasive disease, from at least two heterologous challenge strains, when administered with cholera toxin adjuvant (Malley *et al.*, 2003; Malley *et al.*, 2004). This immunisation was shown to elicit protective systemic antibodies, yet these antibodies are not required for protection against colonisation, since μMT mice, which do not make specific antibodies, are mucosally protected following immunisation (Malley *et al.*, 2005). However, $CD4^+$ T-cells are required for this protection, via the secretion of IL-17A, which attracts neutrophils and leads to increased killing (Lu *et al.*, 2008b).

1.13 Route of immunisation

The route of immunisation is a key factor in protection by vaccines (Belyakov & Ahlers, 2009; Chadwick *et al.*, 2009). Current pneumococcal vaccines are administered intramuscularly (IM) or subcutaneously (SC). Many studies of protein component vaccines use IM or SC immunisation protocols to address efficacy against invasive disease, and intranasal (IN) immunisation to look at reduction in colonisation. However, it is difficult to compare the route of immunisation as it depends on the route of challenge, and the endpoint of the study.

The focus of many other novel vaccine studies is mucosal delivery, to enhance the immune response at the location of initial host-pathogen interaction. Preventing this initial step, colonisation, would thus prevent invasive disease. Mucosal immune responses are most efficiently induced by administering stimulants onto the mucosal surface, whereas injected vaccines are generally poor inducers of mucosal immunity and, therefore, less effective at protecting against mucosal infections (Levine, 2000). However, assessing the efficacy of mucosal immunisation is difficult compared to that of injected vaccines due to complications in capturing and measuring the antibody and T-cell responses at these sites.

Moreover, the dose of mucosal vaccine that actually enters the body cannot be accurately measured. Nevertheless, there are successful mucosal vaccines available worldwide, including a nasal vaccine against influenza virus (Belshe *et al.*, 1998), suggesting an efficacy for mucosal vaccines against the pneumococcus.

Another important consideration in mucosal immunisation is the use of adjuvants. Mucosal immunisation is enhanced by adjuvants, yet few mucosal adjuvants are available for clinical use at this time, apart from Alum (aluminium hydroxide). For example, studies on killed WCV, which are administered IN and protect against invasive disease and colonisation, use cholera toxin as the adjuvant. However, this potent mucosal adjuvant is not approved for human use. Much research is focused on identifying and characterising novel adjuvants for mucosal immunisation, with the goal to elicit B-cell-mediated immunity by enhanced mucosal vaccination (Lore & Karlsson Hedestam, 2009; Gosselin *et al.*, 2009; O'Hagan & De Gregorio, 2009; Reed *et al.*, 2009).

Aims of the current study

This thesis set out to address two important topics. The first aim was to investigate the role of the pneumococcal capsule during nasal colonisation. It is widely known that the polysaccharide capsule is anti-phagocytic in invasive disease, yet the role of capsule during colonisation had not been addressed, despite colonisation being the most common outcome of this host-pathogen interaction. The capsule is also known to limit adhesion to epithelial cells, which is a necessary step for successful colonisation, by masking surface-located adhesins, yet this pathogen has retained capsule expression. Therefore, there must be a beneficial role of this capsule for the pathogen during colonisation. This study used a murine model of colonisation, as well as immuno-staining of tissue sections to address this aim.

The second aim of this thesis was to construct and evaluate a live-attenuated vaccine against *S. pneumoniae*. Current pneumococcal vaccines target the polysaccharide capsule, and have many limitations associated with their use and efficacy. The most noteworthy limitation is serotype replacement, with non-vaccine types increasing in prevalence due to the reduction in the prevalence of vaccine types. The limitations of current vaccines, along with an increase in frequency of antibiotic-resistant pneumococci, have motivated research on novel vaccines that target conserved antigens, and which are more broadly protective. Live-attenuated vaccines have the potential to be broadly protective, due to the presence of multiple conserved antigens, but have received little attention for bacterial pathogens. Therefore, this study set out to investigate live-attenuated *S. pneumoniae* vaccines in mucosal and systemic protection, and to identify the host and bacterial factors involved, using a murine model of colonisation.

Chapter 2

Polysaccharide capsule enhances pneumococcal colonisation by limiting mucus-mediated clearance

2.1 Introduction

A capsule is a prominent feature of many pathogens, particularly those causing invasive infection. For example, the most common aetiological agents of bacterial meningitis, *Streptococcus pneumoniae, Haemophilus influenzae* type b, and *Neisseria meningitidis,* are all encapsulated, a requirement for the sustained bacteraemia needed to breach the blood-brain barrier. The importance of capsule to their pathogenicity results from its inhibition of opsonophagocytosis. Unencapsulated mutants rarely cause invasive infection and are highly attenuated in models of infection due to more efficient opsonophagocytic clearance (Brown *et al.*, 1983; Watson & Musher, 1990).

Many encapsulated pathogens, including each of these species noted above, exist primarily in a commensal relationship with its human host, where they reside on the mucosal surface of the nasopharynx. This suggests that the main selective pressure on such organisms takes place during carriage. Any advantage a capsule confers to organisms residing on mucosal surfaces, where complement and phagocytes may be less abundant, has not been established. It is thought that the ability of a microbe to persist in this niche requires adherence to host cells and tissues. However, *in vitro* studies consistently show an anti-adhesive effect of the capsule, suggesting a potential biological disadvantage to capsule expression that must be balanced by its contribution to survival during colonisation (Hammerschmidt *et al.*, 2005; Ring *et al.*, 1998; Stephens *et al.*, 1993; St. Geme III & Falkow, 1991).

The pneumococcal capsule is diverse in nature, with many serotypes which vary in the composition and linkages of its component sugars. Additionally, most serotypes exhibit phase variation between opaque and transparent phenotypes, by varying the amount of polysaccharide capsule/cell. Similar to the capsules of many other species, the only common structural feature among this large array of polysaccharides is that none has a net positive charge. In fact, structures of more than half of the known capsule types have been determined and all but four types are negatively charged due to the presence of acidic sugars, pyruvate or phosphate, with the remainder being neutral. Despite the diversity of capsular structures, shared physical characteristics are thought to contribute to a conserved function in protecting the underlying bacterial surface structures from the deposition of antibody and complement. This chapter investigates the role of capsule during colonisation

by *S. pneumoniae*.

2.2 Materials and methods

2.2.1 Bacterial strains and culture conditions

S. pneumoniae strains were routinely grown on tryptic soy (TS) agar plates supplemented with 5000 Units (U) of catalase per plate, or on blood agar plates (TS supplemented with 5% sheep blood – Becton, Dickinson and Company, Franklin Lakes, New Jersey), at 37°C in 95% air/ 5% CO_2 for approximately 20 h. Alternatively, *S. pneumoniae* was grown in TS broth, static, in a 37°C water bath.

Strains used *in vivo* were selected because of their ability to efficiently colonise the murine nasopharynx and included the TIGR4 (type 4 clinical isolate, genome sequence strain), 6A (a mouse virulent, type 6A clinical isolate), 23F (a type 23F capsule-expressing *S. pneumoniae* isolate from experimental human colonisation studies) and D39 (a type 2 clinical isolate) strains (Avery *et al.*, 1944; Kim & Weiser, 1998; McCool *et al.*, 2002; Tettelin *et al.*, 2001) (**Table 2.1**).

2.2.2 Construction of unencapsulated mutants

The *cps* operon was deleted from spontaneously streptomycin-resistant mutants (200 µg/ml) of the TIGR4, 6A and D39 strains using the bicistronic positively and negatively selectable Janus cassette (Sung *et al.*, 2001). Otherwise isogenic strains, in the TIGR4 background, were made to express various capsule types by replacing the Janus cassette with *cps*6A, *cps*7F, *cps*14 or *cps*23F capsule operons, as previously described (Trzcinski *et al.*, 2003). These were kind gifts from Marc Lipsitch at Harvard School of Public Health.

Encapsulation was confirmed by positive Quellung reactions with type-specific antisera (Staten Seruminstitut, Copenhagen, Denmark). This was performed by mixing 5 µl of pneumococcal culture, grown to OD_{620} = 0.3, with 5 µl of typing serum, followed by microscopic examination for capsular swelling to confirm the production of type-specific capsule.

To differentiate between opaque and transparent colony phenotypes, pneumococci were grown on TS plates for approximately 20 h, and colony phenotype was determined using oblique, transmitted light (Weiser *et al.*, 1994).

All strains were passaged intranasally in mice prior to growth for preparation of frozen stocks.

Table 2.1 List of pneumococcal strains used in this study.

Strain	Serotype	Source/Reference
TIGR4	4	(Tettelin *et al.*, 2001)
TIGR4Δ*cps*	4	This study
TIGR4Δ*cps*Δ*pspA*	4	This study
TIGR4Δ*pspA*	4	This study
TIGR4Δ*ppmA*	4	This study
TIGR4Δ*cps*Δ*ppmA*	4	This study
TIGR4Δ*psaA*	4	This study
TIGR4Δ*pspA*Δ*ppmA*	4	This study
TIGR4Δ*cps*Δ*pspA*Δ*ppmA*	4	This study
TIGR4Δ*cps*:4-O	4	(Trzcinski *et al.*, 2003)
TIGR4Δ*cps*:4-T	4	(Trzcinski *et al.*, 2003)
TIGR4Δ*cps*:6A-O	4	(Trzcinski *et al.*, 2003)
TIGR4Δ*cps*:6A-T	4	(Trzcinski *et al.*, 2003)
TIGR4Δ*cps*:7F-O	4	(Trzcinski *et al.*, 2003)
TIGR4Δ*cps*:7F-T	4	(Trzcinski *et al.*, 2003)
TIGR4Δ*cps*:14	4	(Trzcinski *et al.*, 2003)
TIGR4Δ*cps*:23F-O	4	(Trzcinski *et al.*, 2003)
TIGR4Δ*cps*:23-T	4	(Trzcinski *et al.*, 2003)
6A	6A	(Kim & Weiser, 1998)
6AΔ*cps*	6A	This study
6AΔ*ply*	6A	This study
6AΔ*cps*Δ*ply*	6A	This study
6AΔ*pspA*	6A	This study
6AΔ*ply*Δ*pspA*	6A	This study
6AΔ*ppmA*	6A	This study
6AΔ*psaA*	6A	This study
6AΔ*cps*Δ*psaA*	6A	This study
D39	2	(Avery *et al.*, 1944)
D39Δ*cps*	2	(Sung *et al.*, 2001)
23F	23F	(McCool *et al.*, 2002)
23FΔ*pspA*	23F	This study
6B	6B	Clinical isolate
6BΔpspA	6B	This study
14	14	Clinical isolate
18c	18c	Clinical isolate
19F	19F	Clinical isolate

2.2.3 Mouse model of nasopharyngeal colonisation

Six-week-old, female C57BL/6J (wild type) or B6.129-S2-Igh-6^{tm1Cgn}/J (μMT) mice (Jackson Laboratories, Bar Harbor, Maine) were housed in accordance with Institutional Animal Care and Use Committee protocols. μMT mice are in the C57BL6/J background and contain a targeted mutation in the heavy chain locus of IgM and do not produce mature B-cells or antibody (Kitamura *et al.*, 1991). BALB/c mice were obtained from Taconic (Germantown, New York). Mice were colonised using a previously described model of nasopharyngeal colonisation with *S. pneumoniae* (**Fig. 2.1**) (McCool & Weiser, 2004). Groups of at least five mice per condition were inoculated intranasally (IN), without anaesthesia, with 10 μl containing 1–5 × 10^7 CFU of phosphate buffered saline (PBS)-washed, mid-log phase *S. pneumoniae* applied to a single naris. At the time indicated, the animal was killed by asphyxiation, the trachea was exposed and cannulated, and 200 μl of PBS was instilled. Lavage fluid was collected from the nares for determination of viable counts of bacteria. Serial dilutions were plated in 10 μl spots on selective medium containing neomycin (5 μg/ml for the TIGR4 strain, 20 μg/ml for other strains) to inhibit the growth of contaminants. Of the neat nasal lavage 50 μl was also spread onto a single TS plate. The lower limit of detection for bacteria in lavage culture was 20 CFU/ml.

2.2.4 Neutrophil and complement depletion

A rat anti-mouse IgG2b directed against Ly-6G on the surface of murine myeloid (and limited subpopulations of lymphoid) lineage cells, mAb RB6-8C5, was purified from ascites of nude mice given the RB6-8C5 hybridoma (Bryder *et al.*, 2004; Hestdal *et al.*, 1991). To deplete neutrophils, 150 μg of mAb/animal was administered by IP injection 24 h prior to intranasal challenge with bacteria. This dose had previously been shown to result in peripheral blood neutropenia (<10% of pretreatment levels) for up to 5 days (Jensen *et al.*, 1993). Controls were given the equivalent IP dose of total rat IgG (Sigma Chemical Co., St. Louis, Missouri). Hypo-complementaemia was induced by IP injection of 25 μg/animal of cobra venom factor (CoVF, Quidel, San Diego, California) in PBS, 18 h prior to bacterial challenge. This procedure had been previously shown to reduce levels of immunodetectable C3 to <3% of normal levels and result in a period of hypocomplementaemia of at least 48 h (Szalai *et al.*, 2000).

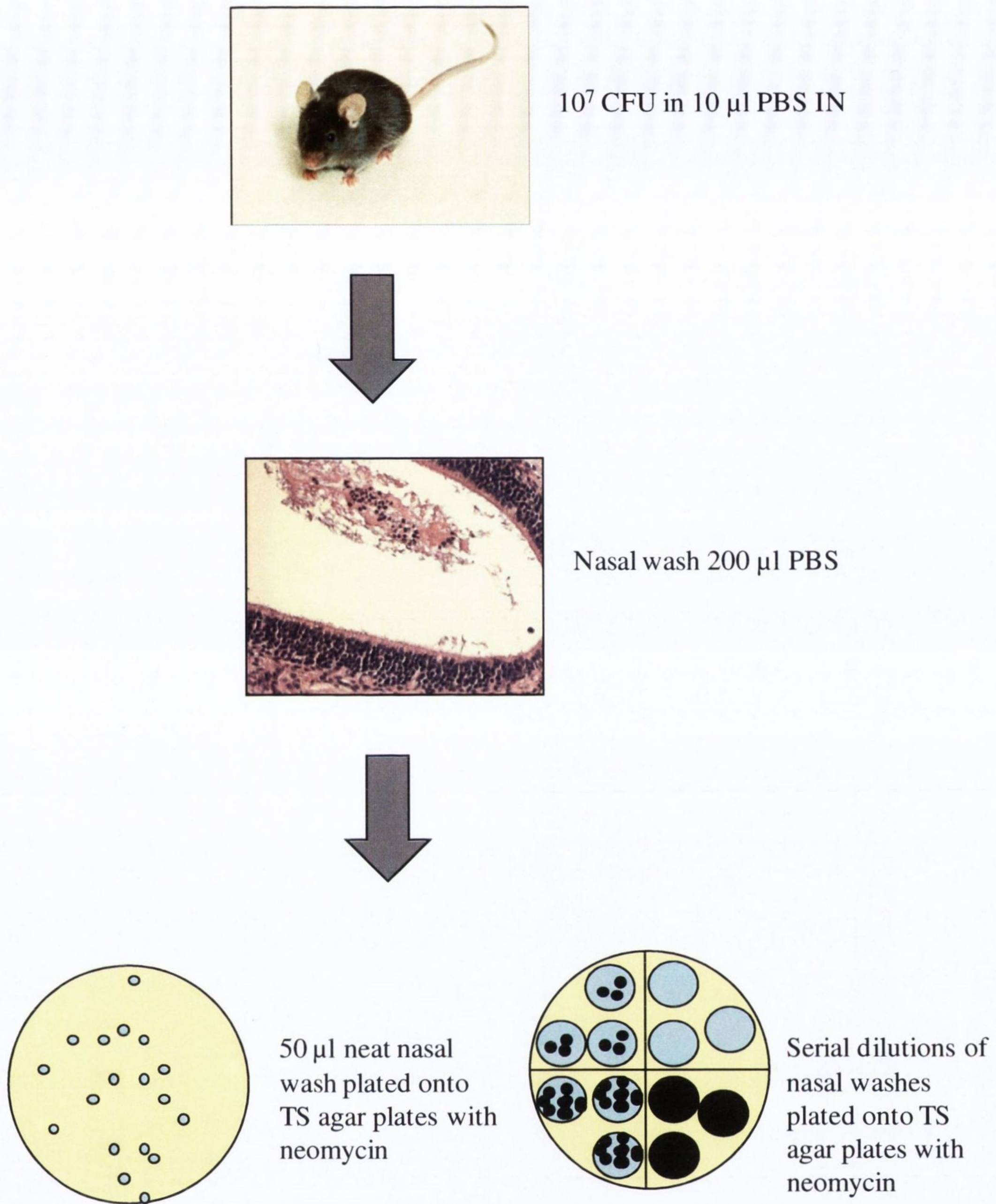


Fig. 2.1 Murine model of colonisation.

A mouse is inoculated with 10^7 CFU IN without anaesthetic in 10 µl of PBS. After the time indicated, the mouse is killed, trachea dissected, and 200 µl of PBS is flushed through the upper respiratory tract. Nasal lavage is collected from the nares. Of the neat nasal wash, 50 µl is spread onto a TS plate with neomycin to detect low numbers of colonising bacteria. Serial dilutions of 10 µl are also spotted onto TS plates with neomycin to detect large numbers of colonising bacteria.

2.2.5 Histology and immunofluorescence

At the time indicated post-inoculation, the animal was killed, decapitated, and the head was fixed for 24 h in 4% (v/v) paraformaldehyde in PBS, followed by another 24 h in fresh fixative. The head was then decalcified by serial incubations in 0.12 M EDTA (pH 7.0) over one month before freezing in Tissue-Tek O.C.T. embedding medium (Miles, Elkhart, Indiana) in a Tissue-Tek Cryomold. Sections, 5 μm thick, were cut and stored at –80°C. Frozen embedded tissue sections were stained with haematoxylin and eosin (H&E) following a 10-min fixation step in 10% (v/v) neutral buffered formalin (NBF). This was performed as follows. Slides were stained in haematoxylin for 2 min, dipped quickly in 0.5% hydrochloric acid/ 70% ethanol/ 29.5% dH_2O, immersed in 0.2% $NaHCO_3$ for 4 min, then quickly immersed in Eosin for 1–2 s. Sections were washed with gently running water after each step. Sections were then dehydrated, by serial incubation in alcohol of increasing concentration, cleared in xylene for 5 min, and mounted in Cytoseal™ (Richard-Allan Scientific, Kalamazoo, MI).

2.2.5.1 Immunohistochemistry

For immunohistochemistry, sections were post-fixed in 1:1 methanol:acetone at –20°C for 10 min followed by washing in dH_2O. Endogenous peroxidase was blocked by incubation in 2.25% H_2O_2 in dH_2O for 15 min. Sections were blocked with avidin and biotin, 15 min each, followed by a further 10-min incubation in protein blocking reagent (PBR, Coulter/Immunotech, Miami, FL) to prevent non-specific binding. Sections were then incubated in three steps: typing serum (Statens Serum Institute) at 4°C overnight, 1:500 v/v in PBT (1 × PBS, 0.1% BSA, 0.2% Triton X-100), biotinylated goat anti-rabbit IgG, 1:200 v/v in PBT, for 30 min, avidin-horse radish peroxidase (HRP) ABC reagent for 30 min (Vector Laboratories, Burlingame, CA), and the signal was developed using diaminobenzidine tetrahydrochloride (DAB) kit (Vector Laboratories). Sections were washed with PBS after each incubation step.

After DAB staining the slides were fixed in 10% NBF for 3 min followed by a 3-min wash in dH_2O. Sections were exposed to 3% (v/v) acetic acid in dH_2O (pH 2.5) for 3 min, before incubating with 1% (w/v) Alcian Blue in 3% acetic acid (pH 2.5) for 30 min. After washing in water for 10 min, sections were counterstained with 0.1% Nuclear Fast Red in dH_2O for 40 s then dehydrated in absolute alcohol, cleared in xylene, and mounted in

Cytoseal™. Bright-field imaging was performed on a Nikon E600 Eclipse microscope equipped with a liquid crystal (Micro-color RGB MS-C, CRI Inc, Boston, MA) and a high resolution CCD digital camera (CoolSnap CF, Roper Scientific, Tucson, AZ) with Nomarski optics.

2.2.5.2 Immunofluorescence

For immunofluorescent staining, tissue was post-fixed with 1:1 acetone:methanol as above, followed by blocking with PBR before addition of primary antibody, diluted 1:500 in PBT. Pneumococci were stained with typing sera as above. Neutrophils were stained using rat anti-mouse neutrophil IgG (allotypic marker - MCA771GA) (AbD Serotec, Raleigh, North Carolina) at 1:100 dilution in PBT overnight at 4°C. Signal was detected with Cy3- or Cy2-conjugated species-specific secondary antibodies (Jackson ImmunoResearch, West Grove, PA) diluted 1:400 in PBT and incubated for 2 h at room temperature (RT). After washing with PBS followed by dH_2O, sections were counterstained with DAPI (4',6-diamidino-2-phenylindole) (Molecular Probes, Invitrogen, Carlsbad, CA) diluted 1:10,000 in dH_2O. All image analysis was carried out using IPLab™ imaging software (Scanalytics, Fairfax, VA).

2.2.6 Human upper airway secretion binding assays (Completed by Dr. Jane Gould)

Mucus from human upper airway secretions was used in a modified solid-phase mucin-binding assay (Gould & Weiser, 2001; Ryan *et al.*, 2001). Filtered human nasal secretions were diluted 1:1 in sterile PBS, sonicated until homogeneous, and applied to a 96-well Polysorp plate (Thermo Fisher Scientific) by centrifugation at 250 × g for 3 min at RT. Following overnight incubation at 37°C, the unbound secretions were removed by washing with PBS and treated for an additional 3 h in sodium citrate buffer (50 mM, pH 6.0) with or without recombinant neuraminidase derived from *Clostridium perfringens* (25 U/ml, New England Biolabs, Beverly, MA). Bacterial cultures grown to mid-log phase (OD_{620} = 0.4) were diluted in PBS to a density of 10^5 CFU/ml and applied by centrifugation at 250 × g for 3 min at RT. Adherence of pneumococci to bound upper respiratory tract mucus was determined by incubation at 4°C for 60 min with gentle agitation. Supernatant fluids, and the first wash with PBS, were removed for colony counts. Bacteria in the supernatant fluid and first wash were considered to have not adhered and were compared to the inoculum to calculate the percentage of adherent bacteria. There was no significant adherence in the

absence of fixed nasal secretions (PBS alone control).

2.2.7 Statistical analysis

Colonisation density was expressed as the log_{10} CFU/ml for calculation of means ± standard error of the mean. Statistical comparisons of colonisation among groups were made by the non-parametric test indicated (GraphPad Prism 4, Graphpad Software Inc., La Jolla, CA).

2.3 Results

2.3.1 Unencapsulated mutants colonise the nasal spaces

The contribution of the capsule during colonisation was assessed by comparing encapsulated isolates with their isogenic unencapsulated mutants in a murine model of colonisation following intranasal inoculation. The TIGR4Δ*cps* mutant (type 4) consistently colonised C57BL/6J mice but at a density 10 to 100-fold less compared to its TIGR4 parent strain, as assessed by quantitative culture of upper airway lavages at 2 d post-inoculation (**Fig. 2.2**). A similar contribution of encapsulation to colonisation was also demonstrated by comparison of isolates of other types (2 and 6A) with and without *cps*.

To confirm that the decrease in fitness for colonisation was due to the loss of the capsule, the deletion of the *cps* locus in the TIGR4 strain was corrected by insertion of the *cps* locus derived from the TIGR4 strain or isolates of heterologous pneumococcal types. Phenotypic variants with opaque (O) or transparent (T) colony phenotypes were considered separately. Correction of capsule expression was sufficient to restore the density of colonisation to wild-type levels for T but not O variants (**Fig. 2.3**). However, in these constructs of the same genetic background, the contribution of encapsulation depended on the capsule type with the greatest effect for negatively charged types, 4, 6A and 23F, and a lesser effect for types with a neutral charge, 7F and 14.

To determine when encapsulation affects colonisation, the TIGR4 wild-type strain and its TIGR4Δ*cps* mutant were compared over the period during which pneumococci could be recovered from upper airway lavages. The TIGR4Δ*cps* mutant was able to persist for up to 7 d post-inoculation (**Fig. 2.4**). However, there was a marked decline in colonisation density between 30 min and 20 h post-inoculation for the TIGR4Δ*cps* mutant that was not observed for wild-type TIGR4 strain, which displayed a more gradual decrease over 14 d post-inoculation. After this initial decline for the TIGR4Δ*cps* mutant, the rate of decrease of colonising bacteria was similar, regardless of the expression of capsule.

Together these results demonstrate that the capsule is not necessary for, but may enhance, colonisation, particularly during initial events in the host. The extent of the contribution of capsule is dependent on its composition and amount.

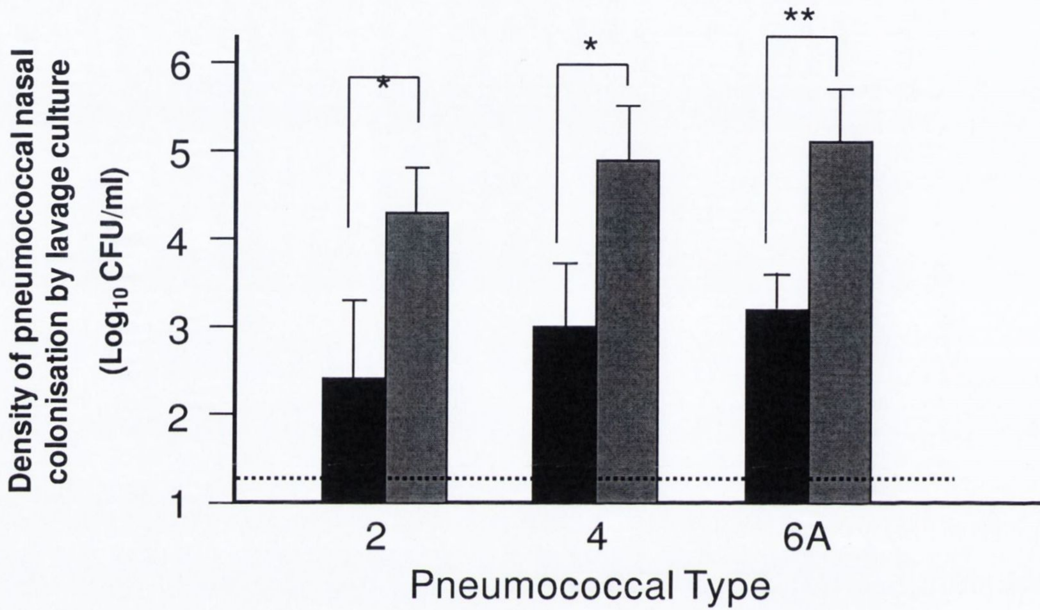


Fig. 2.2 Effect of pneumococcal capsule on the density of nasal colonisation.

Two days following IN challenge with 10^7 CFU of an isolate of the type indicated (gray bars) or its unencapsulated mutant (black bars), lacking the entire cps locus, C57BL/6J mice were killed for quantitative culture of upper respiratory tract lavage fluid. Values represent the mean colonisation density of 5–10 mice/group ± SEM. Statistical difference compared to encapsulated parent strain was determined by the Mann-Whitney test. $^*P<0.05$, $^{**}P<0.01$.

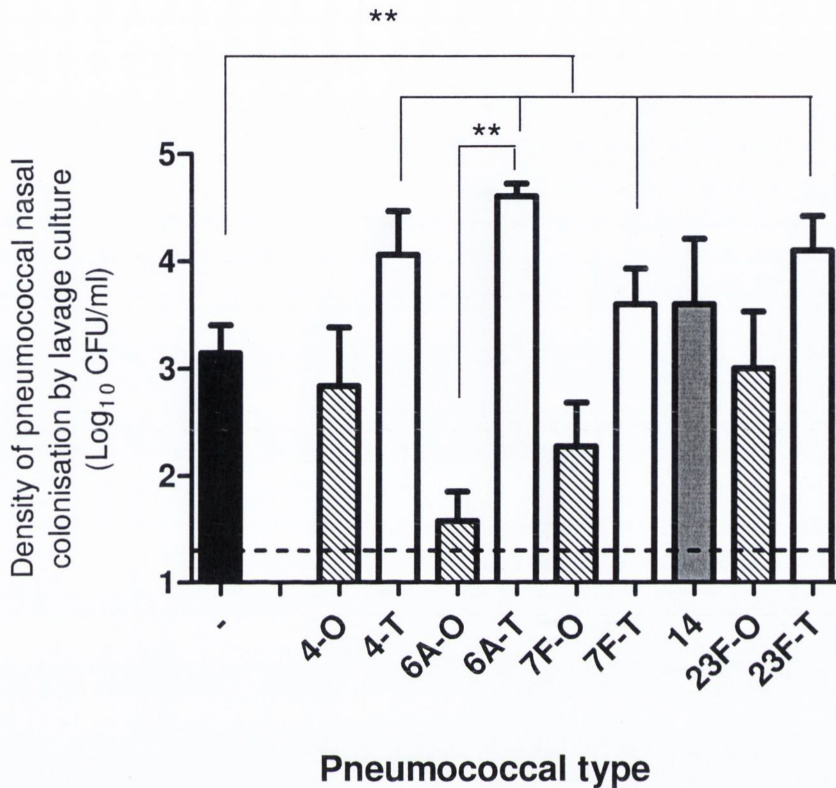


Fig. 2.3 Capsule type and amount determines colonisation density.

Effect of restoration of encapsulation on different serotypes. The TIGR4Δ*cps* mutant (black bar) or transformants with the cps locus of the type indicated, that corrected the loss of capsule expression, were compared for their abilities to colonise C57BL/6J mice. Following IN challenge with 10^7 CFU of the isolate type indicated, colonisation density was determined at 2 d post-inoculation. Acquisition of the type indicated was confirmed by Quellung reactions. For encapsulated transformants of types 4, 6A, 7F, and 23F, opaque (O – hatched bars) and transparent (T – white bars) variants were tested separately. For the type 14 transformant (gray bar), only one phenotype was observed. Dashed line represents the limit of detection. Values represent the mean colonisation density of 5–10 mice/group ± SEM. ** P< 0.0032. The TIGR4Δ*cps* mutant was compared to transparent encapsulated transformants using Kruskal-Wallis test with Dunn's post test for multiple comparisons. The 6A-O and 6A-T variants were compared using the Mann-Whitney test.

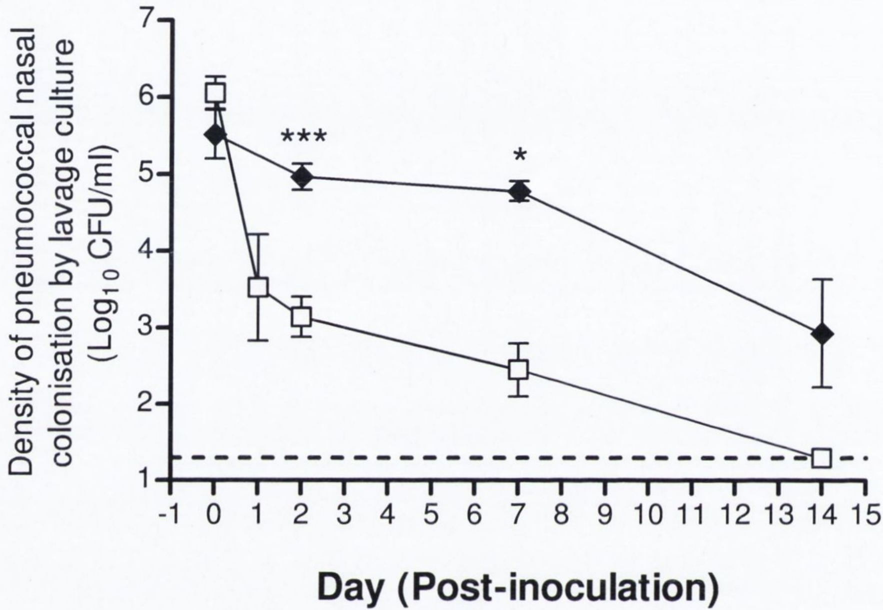


Fig. 2.4 Effect of pneumococcal capsule on the duration of nasal colonisation.

Following IN challenge of C57BL/6J mice with 10^7 CFU of the TIGR4 wild-type strain (closed diamonds) or the TIGR4Δ*cps* mutant (open squares), the density of colonisation was assessed by quantitative culture of upper respiratory tract lavage fluid on the day post-inoculation indicated. The dashed line indicates the limit of detection. Values represent the mean of 5–10 mice/strain at each time point ± SEM. Statistical difference compared to encapsulated parent strain was determined by the Mann-Whitney test. *P=0.0128, ***P=0.0005.

2.3.2 The capsule does not impact on opsonophagocytic clearance during colonisation

Histological examination of colonised nasal tissues of BALB/c mice confirmed that colonising pneumococci induce a neutrophil influx into lateral nasal spaces by 1 d with a maximal response by 3 d post-inoculation (**Fig. 2.5**), as had been previously seen (van Rossum *et al.*, 2005). Similar results were seen with C57BL/6J mice. Immunofluorescent staining of frozen tissue to detect bacteria, demonstrated that these dense clusters of neutrophils have engulfed pneumococci, but that not all pneumococci become associated with neutrophils (**Fig. 2.6**).

To test whether neutrophil-mediated clearance accounted for the lower density of colonisation by unencapsulated pneumococci, mice were treated with monoclonal antibody RB6-8C5 to deplete neutrophils prior to intranasal challenge (Hestdal *et al.*, 1991). If opsonophagocytic clearance was important during colonisation, this treatment would be expected to enhance colonisation by more readily opsonised unencapsulated pneumococci. However, there was no significant effect of mAb RB6-8C5 treatment compared to controls on the density of the TIGR4Δ*cps* mutant at 2 d post-inoculation (**Fig. 2.7**). Similarly, no effect on the colonisation density of the TIGR4Δ*cps* mutant was observed following complement depletion by systemic administration of cobra venom factor prior to nasal challenge. In addition, genetically modified congenic mice (μMT) that fail to generate specific antibody did not demonstrate significantly enhanced colonisation by the TIGR4Δ*cps* mutant when compared to parental mice.

These findings indicated that neutrophil-, complement- or antibody-mediated clearance mechanisms do not contribute substantially to the lower density of colonisation by unencapsulated mutants and left in question the role of capsule during colonisation.

2.3.3 Effect of capsule on the dynamics of colonisation

To define the role of the capsule in pneumococcal colonisation, the events during the initial 2 d period post-inoculation, during which the majority of the deficit in colonisation by unencapsulated mutants occurs, were visualised in tissue sections. For the TIGR4 wild-type strain, bacteria were initially (30 min) confined to the lumen of nasal spaces, where

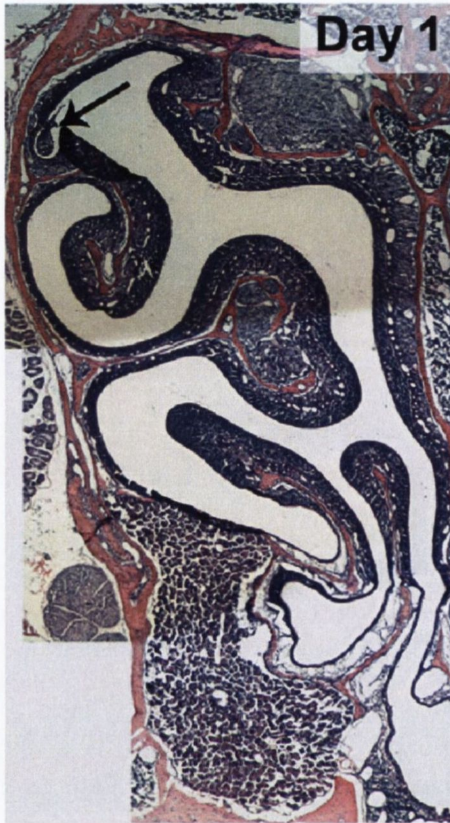


Fig. 2.5 Neutrophil influx during *S. pneumoniae* colonisation.

BALB/c mice were given an IN dose of 10^7 CFU of the 23F strain, and sacrificed at the time indicated. Frozen embedded tissue, from one side of the nasal septum, was stained with H&E. Arrows indicate neutrophil-rich clusters in the lumen of lateral nasal spaces. Composite of images taken at 40×.

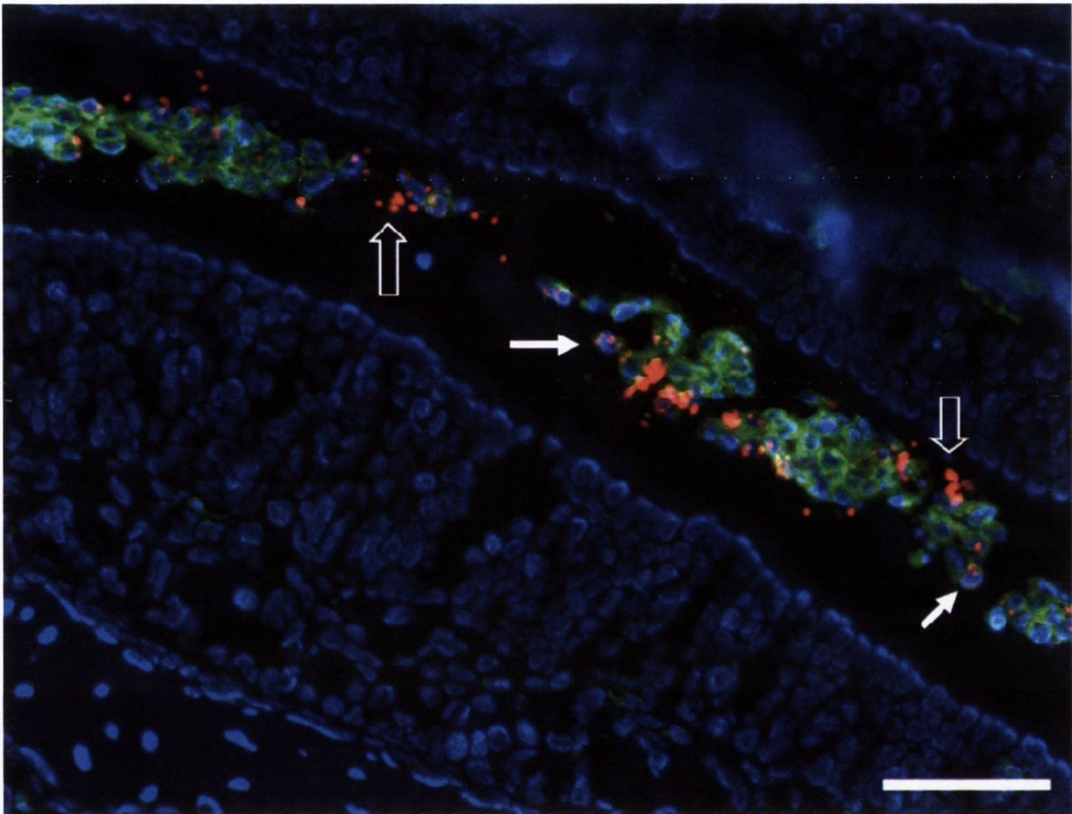


Fig. 2.6 *S. pneumoniae* associates with neutrophils in the nasopharynx.

C57BL/6J mice were given an IN dose of 10^7 CFU of the TIGR4 strain, and killed at 1 d post-inoculation. Frozen embedded tissue was stained with pneumococcal type-specific antiserum, detected by Cy3 secondary antibody (red), with anti-neutrophil marker detected by Cy2 secondary antibodies (green), and with DAPI (blue). Pneumococci associated (closed arrows), and not associated (open arrows), with neutrophils are indicated. 50 µm scale bar.

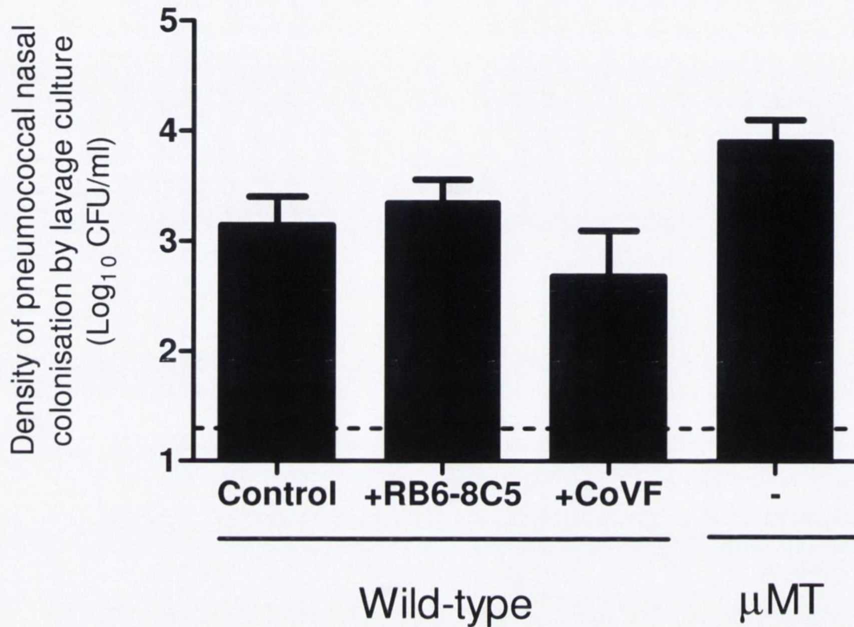


Fig. 2.7 Effect of neutrophils, complement and functional antibody on colonisation by an unencapsulated mutant.

C57BL/6J mice were pre-treated with mAb RB6-8C5 to deplete neutrophils (or with rat IgG as control), or cobra venom factor (CoVF) to deplete complement (or with vehicle control) prior to IN challenge with 10^7 CFU of the TIGR4Δ*cps* mutant. The density of colonisation was assessed by quantitative culture of the upper respiratory tract lavage fluid on 2 d post-inoculation. Colonisation of congenic μMT mice was determined in parallel experiments. Values represent the mean of 5–18 mice/strain at each time point ± SEM. Statistics were determined by Kruskal-Wallis test with Dunn's post test for multiple comparisons. P>0.05.

they associate with amorphous, acellular material (**Fig. 2.8A**). This lumenal material is mucus, based on its staining with Alcian Blue that identifies acidic mucopolysaccharides (**Fig. 2.8C**). By 1 d post-inoculation, these encapsulated pneumococci had transited to the mucosal surfaces where they were found along the glycocalyx, a circa 0.3 μm thick meshwork of mucopolysaccharides and glycoproteins that project from the plasma membrane of the epithelial cell (**Fig. 2.8B, 2.8D**). At later time points up to 14 d, pneumococci remained in the glycocalyx indicating that this was the site of stable colonisation (**Fig. 2.9**).

The TIGR4Δ*cps* mutant was also seen initially in the lumenal mucus (30 min), but unlike the encapsulated parent, was heavily agglutinated in mucus (**Fig. 2.10A v. 2.10B**). Similar results were noted when the 6A strain and its 6AΔ*cps* mutant were compared at 30 min post-inoculation (**Fig. 2.10C v. 2.10D**). When rare unencapsulated mutants of TIGR4 were seen later at 20 h post-inoculation, these were still confined to lumenal mucus rather than along the epithelial surface suggesting an inability to transit to the epithelial surface as occurred for the encapsulated parent (**Fig. 2.10E v. 2.10F**).

2.3.4 Effect of the capsule on interaction with mucus

Consequently, the role of capsule in allowing for escape from mucus was examined. This was addressed by investigating binding of wild-type and unencapsulated strains to mucus in human nasal secretions bound to a 96-well plate, and calculating the percentage of the inoculum that was bound after 30-min incubation at 4°C. When the TIGR4 and 6A wild-type strains were compared to their unencapsulated mutants for their relative abilities to adhere to immobilised human airway mucus *in vitro*, the encapsulated strains showed significantly reduced mucus binding in the solid-phase assay (**Fig. 2.11**). The ability of these encapsulated strains, but not their unencapsulated mutants, to evade binding to mucus was reduced by removing negatively charged sialic acid residues on mucus by pre-treatment of nasal secretions with neuraminidase (**Fig. 2.11**).

These results suggested that the higher amounts of capsule/cell on O variants may increase their fitness during early events of colonisation when escape from lumenal mucus is required. This was tested *in vivo* using an inoculum consisting of equal proportions of O and T variants of the same isolate and calculating the ratio of phase variants after

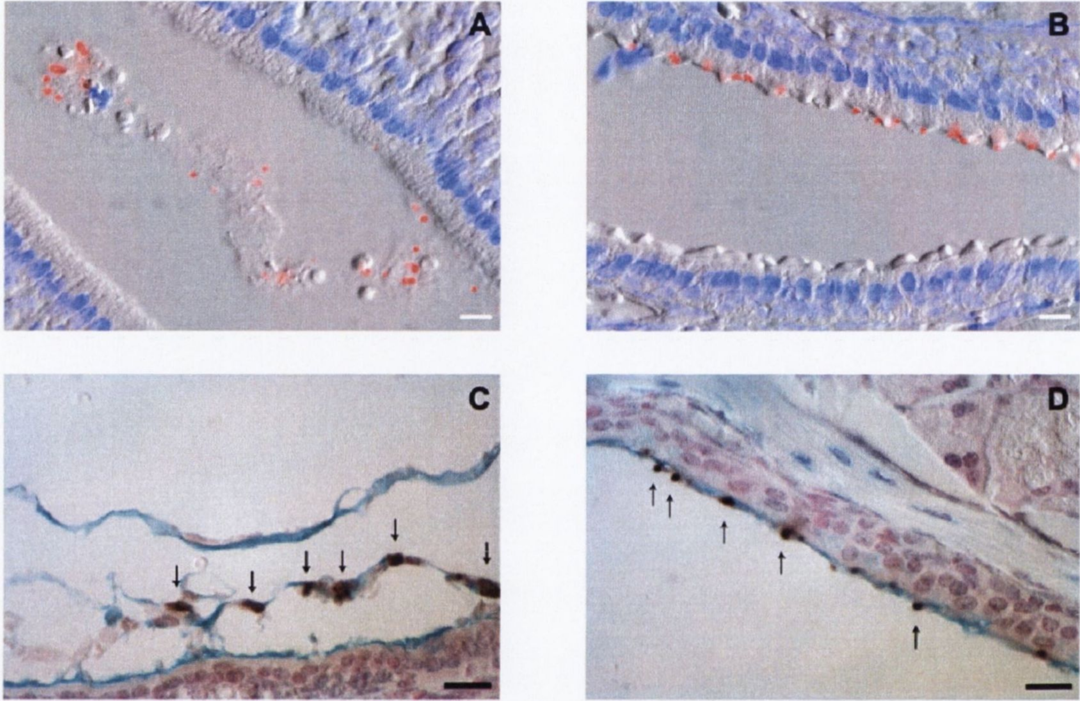


Fig. 2.8 Early events in colonisation, showing transition from mucus to the epithelial surface for encapsulated pneumococcal strain TIGR4.

Frozen nasal tissue from C57BL/6J mice colonised for 30 min (A) or 2 d (B) and stained with *S. pneumoniae* type-specific serum, detected with Cy3 secondary antibody (red), and with DAPI (blue) superimposed with Normarski bright-field optics to show the epithelial border. Frozen nasal tissue from C57BL/6J mice colonised 30 min (C) or 2 d (D) stained with pneumococcal type-specific serum and detected with HRP-conjugated secondary antibody and DAB substrate (brown), Alcian Blue (pH 2.5) (blue) and Nuclear Fast Red (red). Arrows indicate bacteria. 10 μm scale bar.

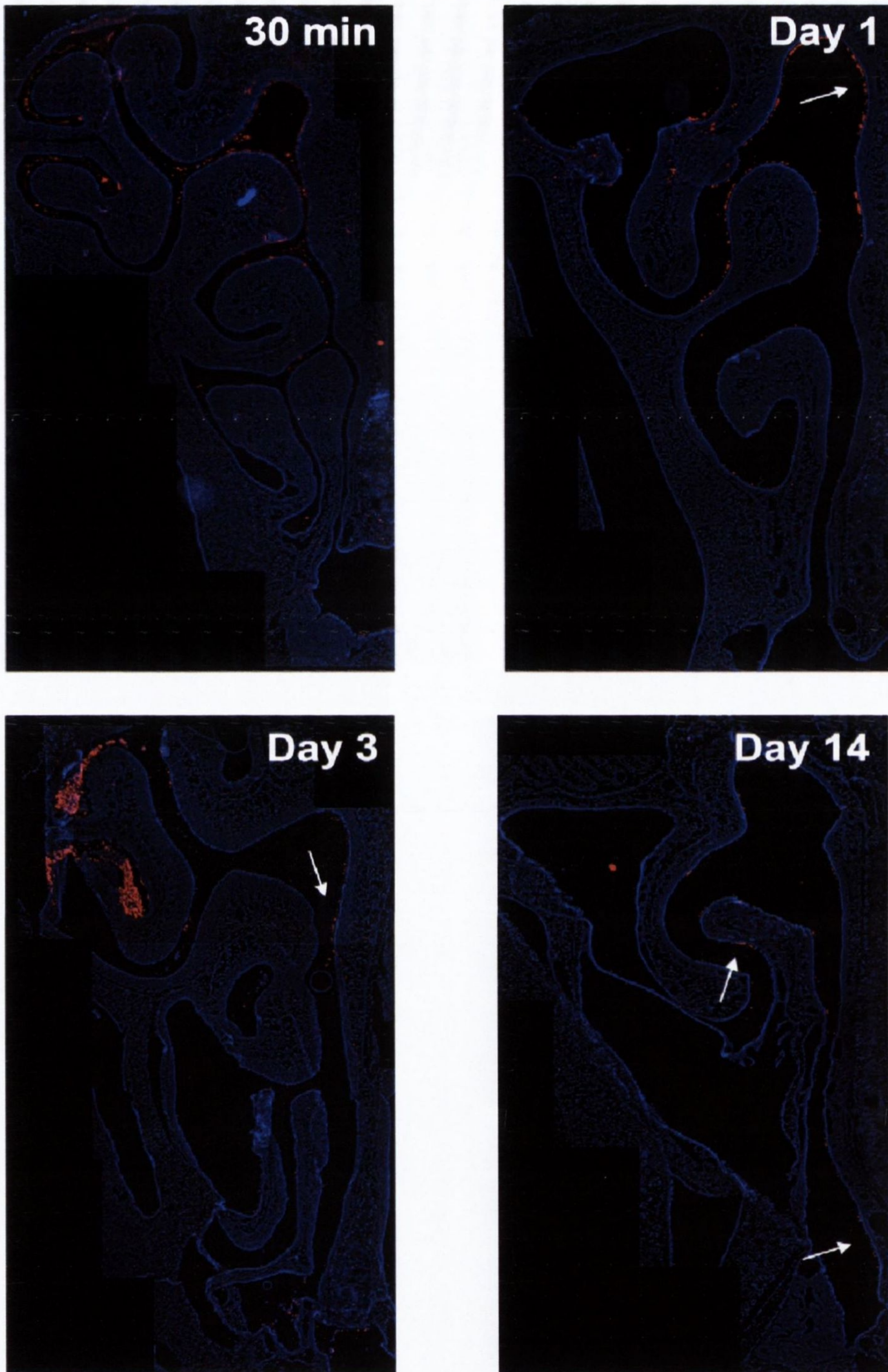


Fig. 2.9 Distribution of pneumococci over the time course of stable carriage.

BALB/c mice were given an IN dose of 10^7 CFU of the 23F strain, and killed at the time indicated. Frozen embedded tissue, from one side of the nasal septum, was stained with pneumococcal typing serum followed by Cy3 secondary antibody (red) and DAPI (blue). Arrows indicate the site of pneumococcal colonisation along the nasal epithelium. Composite of images taken at 40×. Similar colonisation dynamics were seen in C57BL/6J mice colonised with the TIGR4 strain.

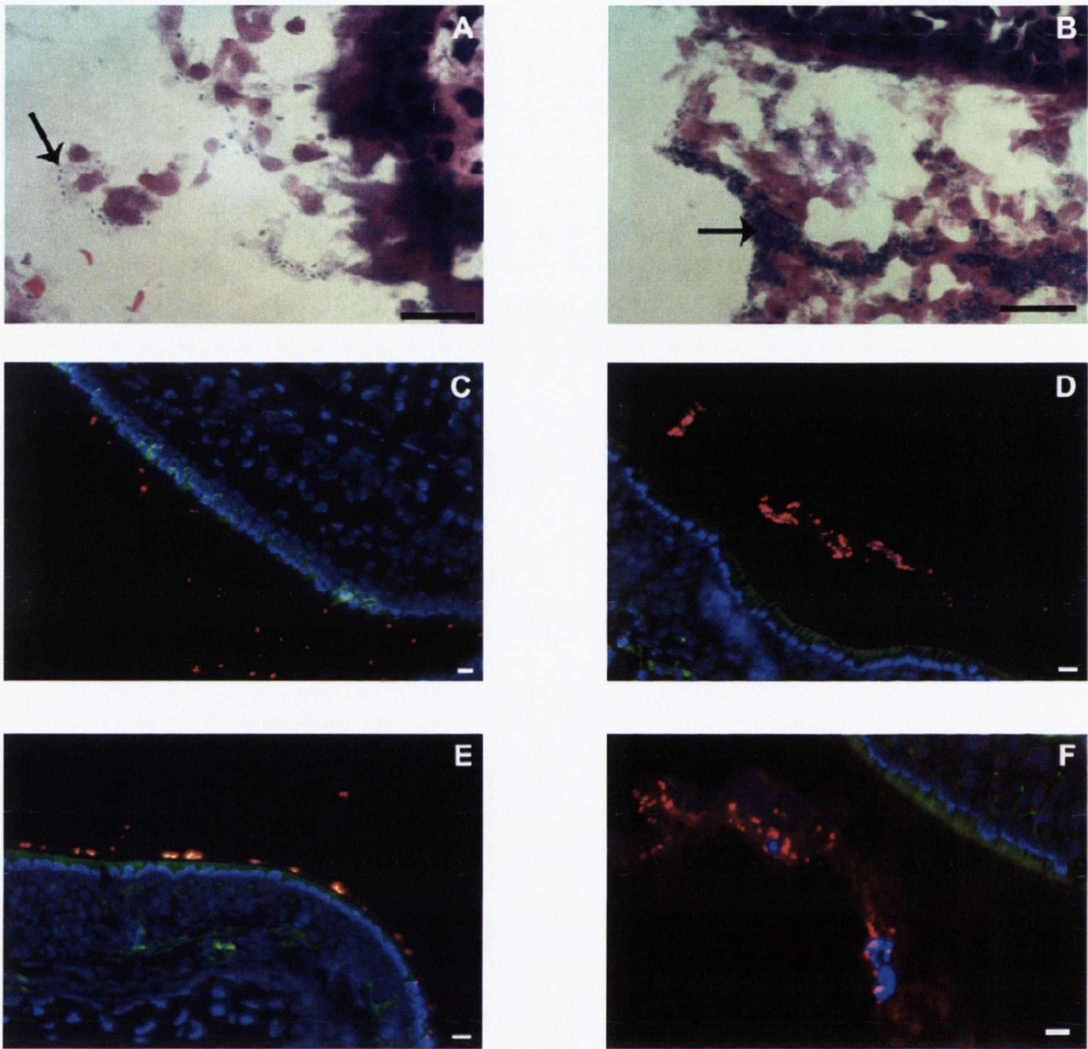


Fig. 2.10 Unencapsulated pneumococci remain trapped within lumenal mucus.

A-B. Haematoxylin and eosin (H&E) stained frozen nasal tissue from C57BL/6J mice 30 min post-inoculation with (A) the wild-type TIGR4 strain or (B) the TIGR4Δ*cps* mutant. Only the unencapsulated mutant is heavily agglutinated in mucoid material in the lumen. Pneumococci are marked by arrows. **C-F**. Frozen nasal tissue from C57BL/6J mice comparing the 6A strain (C) and its 6AΔ*cps* mutant (D) or the TIGR4 strain (E) and its TIGR4Δ*cps* mutant (F) at 30 min (C, D) or 20 h post-inoculation (E, F) using staining with pneumococcal type-specific sera detected with Cy3 secondary antibody (red) and DAPI (blue). Tissue autofluorescence (green) reveals the epithelial border. 10 μm scale bar.

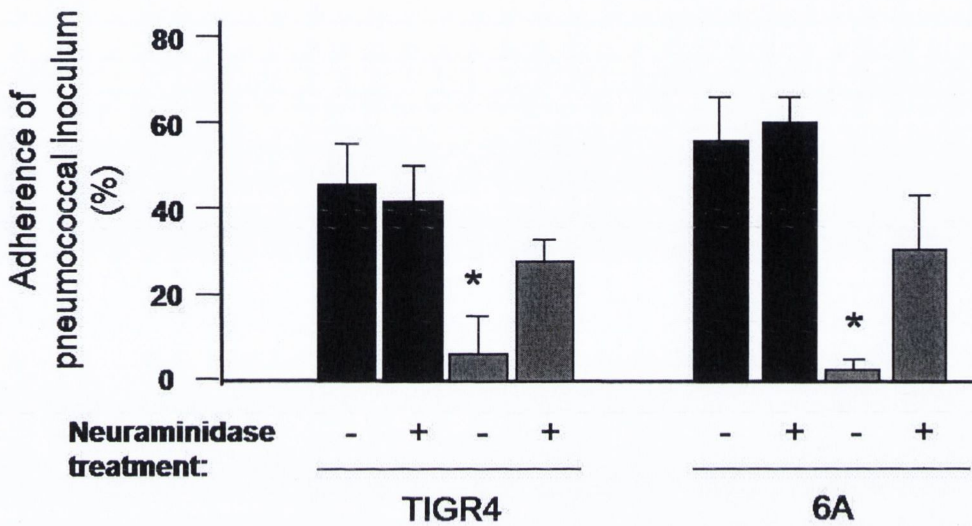


Fig. 2.11 Capsule inhibits mucus association.

The binding of unencapsulated (black bars) compared to encapsulated (grey bars) strain TIGR4 or strain 6A to immobilised mucus from human nasal secretions was compared. Where indicated, the nasal secretions were pretreated with neuraminidase. Adherent bacteria were quantified by determining the proportion of the inoculum removed by 30 min incubation at 4°C (non-adherent bacteria). Means of three independent experiments in duplicate ± SEM are shown. Statistics compared to other experimental conditions for the same strain were determined by Kruskal-Wallis test with Dunn's post test for multiple comparisons. * $P < 0.03$.

colonisation at various early time points. For isolates of two pneumococcal types, 6A and 23F, there was a strong selective advantage for O variants during initial colonisation (<3 h), corresponding to the period when pneumococci are still predominately within the lumen of the nasal spaces (**Fig. 2.12**). These findings suggest that capsule acts to inhibit the association with lumenal mucus that promotes clearance in the early phases of colonisation.

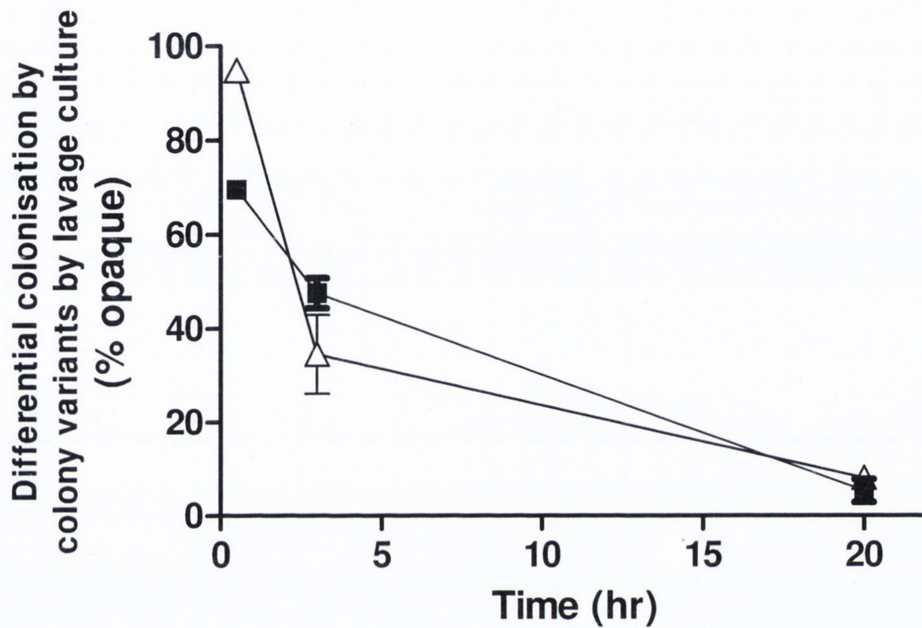


Fig. 2.12 Opaque phenotypes dominate at the initial stage in colonisation.

C57BL/6J mice were inoculated IN with 10^7 CFU of a 1:1 ratio of O and T variants of the 6A strain (solid squares) or the 23F strain (open triangles). At the time indicated post-inoculation, mice were killed and colonisation density was determined. Values are the percent of opaque variants colonising at the time indicated ± SEM. $n \geq 3$. Colony phenotype in nasal lavages was determined by analysis under oblique transmitted light.

2.4 Discussion

This study examined the role of capsule in the biology of the mucosal pathogen *S. pneumoniae*. The commensal state of the organism was the focus of this study, since colonisation is the first step in all pneumococcal disease and by far the most common outcome of the bacterial–host interaction (Bogaert *et al.*, 2004a). A murine model of nasal colonisation was used, as this model embodies many of the key features of experimental and natural colonisation of humans (McCool *et al.*, 2002). Findings in this chapter reveal that only encapsulated organisms are able to transit efficiently from their initial site in a host, the lumenal mucus, to the epithelial surface. The capacity of encapsulated, but not unencapsulated, pneumococci to escape from lumenal mucus would allow access to host-cell receptors on the epithelial surface of the nasal mucosa. Since only the bacteria closely approximated to these epithelial surfaces demonstrate stable colonisation, escape from the mucus appears to be an important step in persistence and may explain the contribution of encapsulation to this mucosal pathogen.

In considering the mechanism for these observations, it seems unlikely that the capsule facilitates the transition from the mucus to the epithelial surface by increasing the adhesive properties of the organism. Capsules are highly diverse in structure, and *in vitro* studies confirm that the capsule substantially inhibits, rather than promotes, bacterial adhesion to host cells (Hammerschmidt *et al.*, 2005; Adamou *et al.*, 1998). The possibility that capsule inhibits killing by antimicrobial substances can not be fully excluded. However, the marked effect of the capsule on the rate of decrease in the density of colonisation during the first hours following challenge, but not in subsequent days, is not supportive of such a hypothesis. Therefore, it is concluded that the events seen by histological examination during early colonisation are most consistent with the capsule enhancing escape of the organism from mucus and evasion of clearance by mucociliary flow, an important innate defense mechanism (Stannard & O'Callaghan, 2006; Schwab & Zenkel, 1998). A further implication is that the optimal strategy for successful colonisation requires a balance between sufficient capsule to escape mucus and excessive capsule inhibiting adherence to the epithelium.

Pneumococci appear to deal with the advantages and disadvantages of the capsule by spontaneously varying its expression between two phases. These findings provide an

explanation for O-variants, previously shown to be relatively deficient in adherence and sustained colonisation, which are more likely to survive in the bloodstream in animal models and humans because of increased amounts of capsule/cell (Cundell *et al.*, 1995b; Kim & Weiser, 1998; Weiser *et al.*, 2001). Increased encapsulation by the more virulent O-variants facilitates escape from lumenal mucus, ensuring a portion of the population will overcome this initial clearance mechanism. The present findings also offer an explanation for a prior report showing that a minimal amount of capsule is needed for efficient colonisation (Magee & Yother, 2001).

Why does the capsule allow for escape from mucus? Mucus, comprised largely of mucopolysaccharides that are highly negatively charged due to an abundance of sialic acid and other anionic residues, traps and removes particles such as bacteria (Cone, 2009; Fokkens & Scheeren, 2000; Rubin, 2002; Tabak, 1995). Microbes with surface characteristics that promote interaction with the mucus and subsequent removal by mucociliary flow may benefit from an altered surface charge that increases electrostatic repulsion. This hypothesis is supported by data showing that (i) restoration of capsule expression with negatively charged polysaccharides had the greatest impact on the density of colonising bacteria, and (ii) treatment of mucus with neuraminidase, to remove negatively charged sialic acid residues, increased the adherence of encapsulated bacteria. In the case of the pneumococcus, the negatively charged (or in a few instances uncharged) capsule may act to obscure positively charged surface features such as the quaternary amines on choline residues on its structurally conserved teichoic acids (Fischer *et al.*, 1993). In this regard, mucin glycoproteins have been previously shown to agglutinate unencapsulated oral streptococci in a manner dependent on their sialic acid content (Levine *et al.*, 1978).

The finding that pretreatment of human mucus with neuraminidase leads to increased adherence of two encapsulated strains to immobilised mucus from human nasal secretions, suggests that endogenous neuraminidase activity may enhance agglutination in lumenal mucus. *S. pneumoniae* expresses three exoglycosidases, including NanA, which sequentially remove sugars, including sialic acid, found on mucus and other human glycoconjugates that bind to the organism (Phalipon *et al.*, 2002; Weiser *et al.*, 2003). The current mucin-binding assay was performed with pneumococcal strains that express NanA. Why would the pneumococcus express a protein that would enhance clearance on the

mucosa? Perhaps the excess neuraminidase in the assay lead to an artificial effect on mucus adherence. Furthermore, pretreatment of mice with a sialidase, which is currently in clinical development for treatment of influenza virus, did not affect colonisation of the pneumococcus (Hedlund *et al.*, 2010). These data suggest that sialidase treatment of the upper airways does not lead to enhanced agglutination and, therefore, clearance of the pneumococcus. Different experimental systems have shown conflicting evidence about the role of pneumococcal NanA in initial colonisation (King *et al.*, 2006; Orihuela *et al.*, 2004; Tong *et al.*, 2000). However, cleavage of sialic acid residues by NanA also reduces the viscosity of mucus, which may aid in escape from entrapment in lumenal mucus (Scanlon *et al.*, 1989). Other surface characteristics of the pneumococcus may also serve to release it from entrapment in mucus. These include its IgA1-specific protease, which cleaves off the Fcα-containing fragment linked to the mucus through secretory component (King *et al.*, 2006).

Although capsules also act to inhibit opsonisation of underlying bacterial surface components, the present findings suggest that this may not be the primary function for which these structures have evolved on mucosal organisms. Despite an influx of neutrophils during colonisation, opsonophagocytosis-mediated clearance of unencapsulated mutants did not account for their lower density during colonisation. Therefore, pathogenicity may not have been selected for among these organisms, but may be a consequence of encapsulation that evolved primarily to serve a different purpose, namely, escape from mucociliary clearance during their commensal state.

Chapter 3

Live-attenuated *Streptococcus pneumoniae* vaccine elicits antibody-dependent cross-protection

3.1 Introduction

Streptococcus pneumoniae is a leading human pathogen causing a range of diseases that result in an estimated 1.6 million deaths per year worldwide (Levine *et al.*, 2006). The surface of the nasal mucosa is the major reservoir for *S. pneumoniae* and at this site they reside primarily in a commensal relationship in their human host. Carriage is extremely common with >50% of children acquiring at least one isolate during their first year. By three years of age carriage rates steadily decline until adulthood when rates plateau at 10–20%. The global decrease in carriage and disease rates that occurs after early childhood suggests that protective immunity may be induced by prior carriage events and develops in a serotype-independent manner (Lipsitch, 1999; Lipsitch *et al.*, 2005).

Increasing rates of resistance of *S. pneumoniae* to antibiotics highlight the priority of preventing pneumococcal disease (Jacobs, 2004; Lipsitch, 1999). There are currently three commercially available vaccines against *S. pneumoniae*, all of which are based on the polysaccharide capsule. Pneumovax©, a 23-valent polysaccharide vaccine, is effective in adults, but not in early childhood. The advent of the pneumococcal conjugate vaccine has led to the reduction of pneumococcal disease in children and adults (Whitney *et al.*, 2003; Tsai *et al.*, 2008), by direct vaccination and through herd immunity, respectively. Prevnar©, a 7-valent polysaccharide conjugate vaccine, is the most widely used conjugate vaccine. However, the conjugate vaccine has been shown to induce selective pressure and the gradual replacement of vaccine types colonising the nasopharynx with non-vaccine types (serotype replacement) (Toltzis & Jacobs, 2005). Moreover, its effectiveness against the most frequent manifestations of infection, mucosal infection (pneumonia and otitis), seems far more limited than for invasive disease (Eskola *et al.*, 2001; Musher *et al.*, 2006). In addition, the distribution of serotypes responsible for IPD varies worldwide. Therefore, vaccines need to be tailored to each geographical region to ensure the greatest level of protection. This geographical specificity, coupled with the complexity of the vaccine, contributes to the prohibitive cost for those in most need in the developing world. It is, therefore, desirable to find new and innovative strategies for vaccine development.

A main focus of current research is aimed at component vaccines comprised of protein antigens, with particular attention to virulence factors with antigenic determinants widely shared among strains. Among these are the pneumolysin toxoid, PspA, NanA,

pneumococcal histidine triad proteins A, B and D, and PsaA. Inclusion of a combination of two or more antigens will most likely be necessary to confer broad protection against the highly heterogeneous pneumococcal population (Ogunniyi *et al.*, 2006). Another strategy uses killed whole bacteria and is able to confer broad protection in mouse models after multiple intranasal doses administered with an adjuvant (Malley *et al.*, 2001).

An alternative approach is the development of live-attenuated vaccines. Live-attenuated vaccines have been highly successful for prevention of viral disease (O'Ryan & Linhares, 2009; Chen & Subbarao, 2009; Amanna & Slifka, 2009; Fiore *et al.*, 2009). However, they have received relatively little attention for bacterial disease, exceptions including the *Mycobacterium bovis* BCG vaccine against tuberculosis (Delogu & Fadda, 2009; Hoft, 2008; Young, 2003), the *Salmonella enterica* serovar Typhi Ty21a vaccine against typhoid (Fraser *et al.*, 2007; Wahdan *et al.*, 1982), and live-attenuated oral vaccines against *Vibrio cholera* (Chowdhury *et al.*, 2009; Ryan *et al.*, 2006). Here, a live-attenuated vaccine of *S. pneumoniae* is described that can elicit an immune response sufficient to reduce colonisation and protect against otherwise lethal challenge in a serotype-independent manner.

3.2 Methods

3.2.1 Bacterial strains and culture conditions

S. pneumoniae strains were grown as described in **Chapter 2, Section 2.2.1**.

Strains 6A and TIGR4 were compared by multi-locus sequence typing (MLST) (Maiden *et al.*, 1998), performed by Jason Stewart at Columbus Childrens Research Institute, as previously described (Enright & Spratt, 1998). The isolates represent two sequence types (6A ST460 and 4 ST1982) with different alleles at 7/7 loci analysed. Thus, the 6A and TIGR4 isolates represent different clonal complexes as defined by eBURST (Feil *et al.*, 2004).

3.2.2 Transformation of pneumococci

A pneumolysin-negative 6A mutant was used in this study (van Rossum *et al.*, 2005). This mutation was transferred to the other mutants 6AΔ*cps* and 6AΔ*pspA* and to the TIGR4 isolate, by transformation using cell lysates from mutant 6AΔ*ply*. Cell lysates were prepared as follows: 10 ml cultures were grown in TS to OD_{620} = 0.5, then pelleted, resuspended in 100 µl of lysis buffer (0.1% sodium deoxycholate, 0.01% SDS, 0.15M sodium citrate) and incubated at 37°C for 10 min. To the lysates 900 µl of SSC (0.15M NaCl, 0.015M sodium citrate) was then added, followed by incubation at 65°C for 15 min.

Transformation was then completed as follows. The host strain (6AΔ*cps*, 6AΔ*pspA* or TIGR4) was grown in broth to an OD_{620} = 0.15 in C+Y pH 6.8 medium (Lacks & Hotchkiss, 1960) (see **Appendix**). Then 50 µl of cells was added to 950 µl C+Y pH 8.0 medium, along with 2.5 µl of 10 µg/ml CSP-1 (amino acid sequence: MRLSKFFRDFILQRKK) and CSP-2 (EMRISRIILDFLFLRKK) (Hävarstein *et al.*, 1995), and 10 µl 100 mM $CaCl_2$. The cells were then incubated at 30°C for 10 min before addition of approximately 200 ng of DNA (100 µl cell lysate from the 6AΔ*ply* mutant), and then incubated at 30°C for 40 min followed by 37°C for 2 h. After incubation, cells were plated onto TS plates containing 1 µg erythromycin (Erm)/ml, and incubated for approximately 20 h at 37°C in 95% air/ 5% CO_2. This was followed by serial back transformation to ensure no other mutations were incorporated into Ply-deficient strains (Dawid *et al.*, 2007).

3.2.3 Confirmation of Ply-deficient strains using Western blotting

Loss of pneumolysin expression was confirmed by Western blotting using a mouse monoclonal antibody against pneumolysin (Novocastra, Newcastle upon Tyne, UK) (**Fig. 3.1A**).

Whole cell lysates were prepared as follows. Mutant and wild-type strains were grown to mid-log phase in TS broth. Of these, 1 ml aliquots were pelleted, washed and resuspended in 50 μl of Laemmli sample buffer (Laemmli, 1970) and boiled for 5 min. Samples of 12 μl were loaded onto 10% polyacrylamide gels (BioRad, Hercules, CA) prior to sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) at 100 V, using the Mini-Protean II system (BioRad). Proteins were transferred to PVDF membrane (Thermo Scientific, Waltham, MA) by the Trans-Blot SD semi-dry transfer system (BioRad) at 18 V for 0.6 h. Where indicated, membranes were stained with Ponceau S (0.1% (w/v) Ponceau S in 5% acetic acid) for 5 min, to confirm equal loading between samples by intensity of stain, and washed in water to remove dye prior to addition of antibodies.

Membranes were then blocked in PBS supplemented with 1% BSA prior to incubation with anti-pneumolysin antibody at 1:50 dilution in PBS (1% BSA) at RT overnight. Following three 5-min washes, bound antibody was detected by 2-h incubation with anti-mouse secondary antibody conjugated to alkaline phosphatase (Sigma), at 1:4000 dilution in PBS (1% BSA). Alkaline phosphatase signal was developed by incubation with 2.5 mg 5-bromo-4-chloro-3′-indolyphosphate-*p*-toluidine and 3.2 mg nitro-blue tetrazolium (Fisher Scientific, Pittsburgh, PA), and was stopped by addition of H_2O.

3.2.4 Construction of PspA-deficient strains

The 6AΔ*pspA* strain was constructed by amplifying a 1.3-kb fragment of the *pspA* gene from the 6A strain with primers LSM13 and SKH2 (Hollingshead *et al.*, 2000) (**Table 3.1**), using *Taq* polymerase to ensure 'A' overhangs.

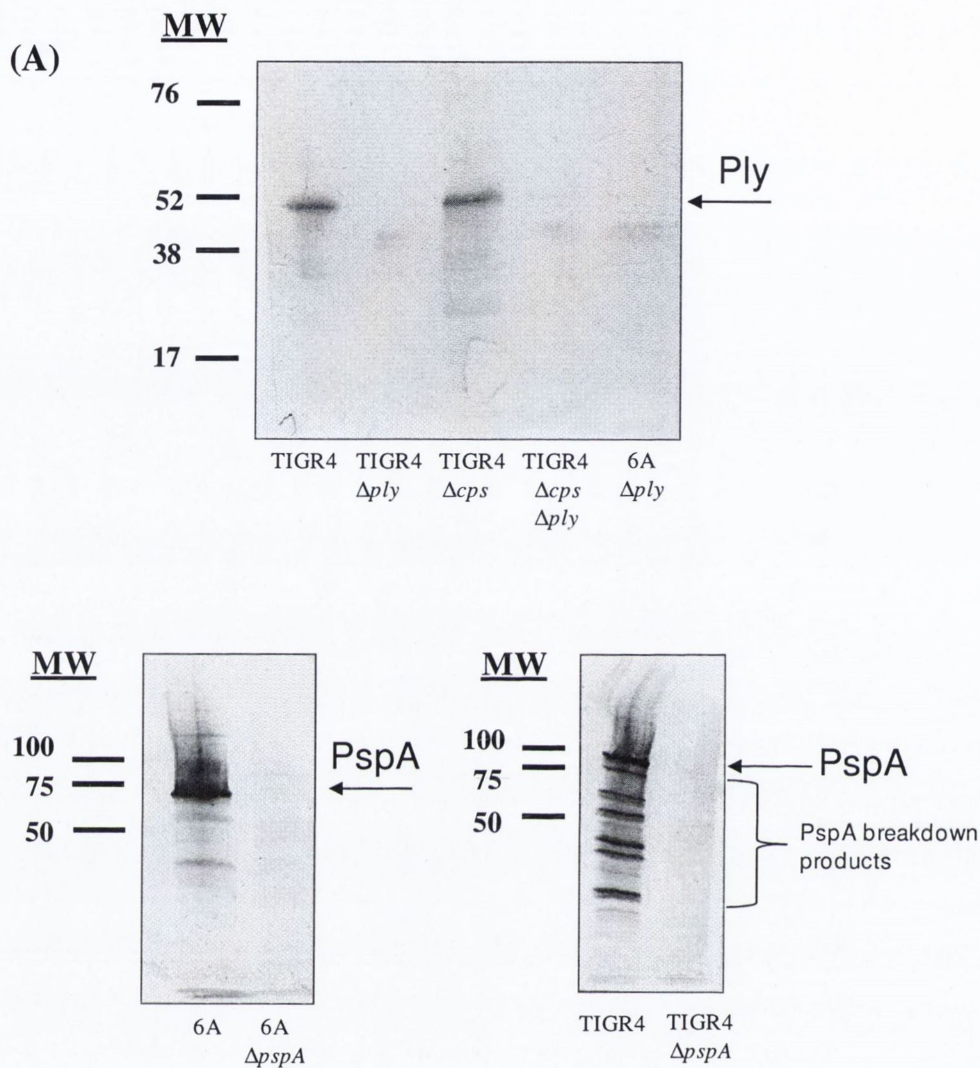


Fig. 3.1 Confirmation of Ply and PspA mutants by Western immunoblotting.

Whole cell lysates from strains listed above were separated on 10% SDS-PAGE gels, transferred to PVDF membranes, and probed with a monoclonal antibody against Ply (A) or polyclonal anti-clade 2 or 3 PspA, for strains 6A and TIGR4, respectively (B). MW, molecular weight. Size markers are in kDa.

Standard PCR reactions were carried out on a thermal cycler (Hybaid, Middlesex, England), in a final volume of 50 µl. Each reaction contained 2.5 mM $MgCl_2$, 200 µM (each) deoxynucleoside triphosphates (dNTPs), 50 pmol of each primer, and 1 U of Platinum *Taq* polymerase, according to the manufacturer's instructions (Invitrogen). Cycling consisted of 95°C for 1 min, 62°C for 1 min, and 72°C for 3 min, repeated 30 times. PCR products were purified from agarose gels using the QIAquick™ Gel Extraction Kit (Qiagen), according to the manufacturer's instructions.

The PCR product was cloned using the TOPO TA Cloning Kit (Invitrogen, Carlsbad, CA). The cloning vector in this kit, the TOPO PCR2.1 plasmid, contains 'T' overhangs for easy ligation of *Taq*-generated PCR products. The TOPO cloning reaction contained 1 µl TOPO vector, 3 µl PCR product, 1 µl salt solution and 1 µl dH_2O, and was incubated at RT for 5 min. After 30 min resting on ice, the resulting cloned plasmid was transformed into One Shot® Top10F´ chemically competent *E. coli* (Invitrogen), by adding 2 µl of cloning reaction to competent cells and heat shocking at 42°C for 30 s, followed by 2 min on ice. Then 250 µl SOC medium was added to the cells and they were incubated at 37 °C with shaking for 1.5 h before plating on selective kanamycin plates containing X-Gal and IPTG (Fisher Scientific) for blue/white screening. White colonies were picked and PCR was used to confirm insertion of the gene of interest, using primers LSM13 and SKH2.

A 390 bp deletion was made in this 1.3-kb fragment using inverse PCR, with primers pspa390del6AF and pspa390del6AR, with *Sal*I sites incorporated into the sites of the deletion (**Table 3.1**). High fidelity PCR was used to amplify this deletion product, and carried out on a thermal cycler (Hybaid), in a final volume of 50 µl, using Platinum *Pfx* DNA polymerase. Each reaction contained approximately 100 ng of the DNA template, 1.5 units of Platinum *Pfx* polymerase, 1 µM of each oligonucleotide primer, 0.3 mM of each dNTP in 1 × *Pfx* amplification buffer supplemented with 1 mM $MgSO_4$. Reaction conditions comprised 30 cycles as follows: Denaturation at 94°C for 1 min, annealing at 55°C for 1 min, and extension at 68°C for various times depending on the length of the product (approximately 1 min for each kb of product). The PCR product was then cut with *Sal*I.

Table 3.1 List of primers used in this study.

Primer name	Sequence (5′ → 3′)
LSM13	GCA AGC TTA TGA TAT AGA AAT TTG TAA C
SKH2	CCA CAT ACC GTT TTC TTG TTT CCA GCC
pspa390del6AF	ACG CGT CGA CGA TTC AGA AGA TTA TGC TA
pspa390del6AR	ACG CGT CGA CTC CTC TGT TGC CTT AGC TA
ErmF	TTA AGA AGC AGT GAT TAC ATG AAC
ErmR	CTC ATA GAA TTA TTT CCT CCC G
PpmAR6	AGA AAC ACA ATT ATC CCA TTT TTA TCG
PpmAF7	TCA TGG GAC TTA CAC ACG TTT GAT G
PpmAR7	CGC TGC TAT TAC GAC ACA GGC TGA A
PpmAF8	GTG GTG CCT ATC AAG CTC TTT ATG C
PpmAOER1	***CAC TCC TTC*** **GGC ACC TGC** CAA TAA TTT TTT C
PpmAOEF1	***GCA GGT GCC*** **GAA GGA GTG** ATT ACA TGA AC
PpmAOER2	***AAT CTC CAC C*****AT TAT TTC CTC CC**G TTA AAT AAT AG
PpmAOEF2	***GGA GGA AAT AAT*** **GGT GGA GAT T**CA AGC TCA AGC TC
PsaAFHind32	GCA AGC TTA TGA AAA AAT TAG GTA CAT TAC TCG
PsaAR	TTA TTT TGC CAA TCC TTC AGC AAT C
InvpsaAFmfe1	GCC AAT TGA TTG TAA CCA GCG AAG GAG CAT TC
InvpsaARmfe1	GCC AAT TGA AAC CAA GCA TTG CCA CCT GTT TC

- The following restriction sites used are underlined: *Sal*I: GTCGAC, *Hin*dIII: AAGCTT, *Mfe*I: CAATTG.
- Primers used in overlap extension PCR – the sequence of the region that anneals to the complementary sequence of the second primer in **bold**, and the region that cannot bind to DNA in the first PCR step is shown in *italics*.

A spectinomycin-resistance cassette (*aad9*, GenBank accession number U30830) (Smith *et al.*, 1995), cut out of the TOPO PCR2.1 plasmid with endogenous *Sal*I sites, was ligated into the *Sal*I sites on the plasmid containing the deleted *pspA* gene. Mutants 6AΔ*pspA* and 6AΔ*ply*Δ*pspA* were generated by transformation with the resulting plasmid DNA (10 μl), with selection for spectinomycin resistance (200 μg/ml). The loss of PspA was confirmed by sequencing and Western blotting, using antibodies against clade-specific PspA (gift from M.L.S de Oliveira, Instituto Butantan, São Paulo, Brazil) at a 1:1000 dilution (**Fig. 3.1B**). Primers are listed in **Table 3.1**.

3.2.5 Mouse model of nasopharyngeal colonisation

Six-week-old female C57BL/6J (wild type), B6.129-S2-Igh-6^{tm1Cgn}/J (μMT) (Jackson Laboratories, Bar Harbor, Maine), B6.129-H2-$Ab1^{tm1Gru}$N12 ($MHCII^{-/-}$) (Taconic, Germantown, New York) and $IgA^{-/-}$ (gift from Yimin Yu, University of Pennsylvania) mice were housed in accordance with IACUC protocols. μMT mice contain a targeted mutation in the heavy chain locus of IgM and do not produce mature B cells or antibody (Kitamura *et al.*, 1991). MHC-II deficient mice exhibit a depletion of $CD4^+$ T-cells through the disruption of the H2-Ab1 gene (Grusby *et al.*, 1991). $IgA^{-/-}$ mice contain a targeted deletion in the alpha-chain constant region and do not express IgA (Harriman *et al.*, 1999). Mice were colonised as described in **Chapter 2, Section 2.2.3**. Nasal lavage fluid was stored at –20°C for determination of antibody concentrations by ELISA.

3.2.6 **S. pneumoniae** *challenge*

Mice where challenged intranasally with 1–5 × 10^7 CFU of *S. pneumoniae* parent isolate at 5 wk post-immunisation with an attenuated mutant (**Fig. 3.2**). Data from **Chapter 2** (**Section 2.3.1**) demonstrated that these attenuated unencapsulated strains are cleared from the upper respiratory tract by 1 wk post-inoculation. Where two-dose immunisation is indicated, the second dose was given 2 wk after the first dose with challenge 5 wk after the second dose. Mice were observed for signs of sepsis over a 9-d period post-challenge. Animals showing signs of sepsis were euthanased and the spleens cultured to confirm the presence of pneumococci. After 9 d the remaining animals were euthanased and nasal washes were obtained for quantitative culture. The vaccine strain given up to 65 d earlier was never detected by selective plating of nasal washes, including in those obtained from

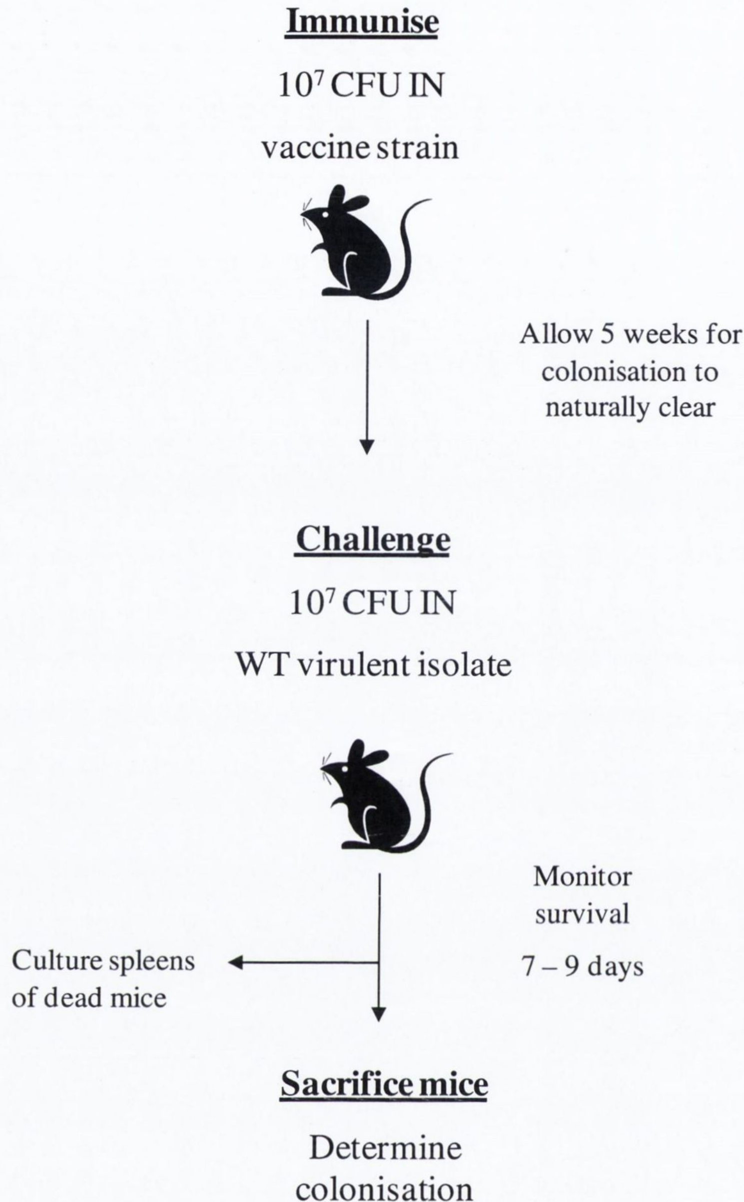


Fig. 3.2 Murine immunisation model.

Mice were immunised IN with 1–5 × 10^7 CFU of vaccine strain in 10 µl of PBS. 5 wk post-immunisation mice were challenged IN with 1–5 × 10^7 CFU of wild-type S. pneumoniae. Where two-dose immunisation is indicated, the second dose was given 2 wk after first dose with challenge 5 wk after the second dose. Mice were observed for signs of sepsis over a 7–9 d period post-challenge. Animals showing signs of sepsis were euthanased and the spleens cultured to confirm the presence of pneumococci. After 9 d the remaining animals were euthanased and nasal washes were obtained for quantitative culture. The vaccine strain given up to 65 d earlier was never detected by selective plating of nasal washes, including in those obtained from immunodeficient mice.

immunodeficient mice. Blood was also collected from cardiac punctures and the sera were stored at –20°C for ELISA.

3.2.7 Measurement of IgG and IgA levels by ELISA

Whole cell bacteria were used as the solid-phase antigen to determine the antibody titre to pneumococci. PBS-washed whole bacteria, diluted with coating buffer (0.015 M Na_2CO_3, 0.035 M $NaHCO_3$) to a final OD_{620} = 0.1, were fixed by overnight incubation at 4°C on Immulon 2HB 96-well plates (Thermo, Milford, MA). Plates were washed with PBS-Brij (0.05%). After blocking with 1% BSA (Sigma) for 1 h and washing, serum, or nasal washes, in 1% BSA were added to the plate in 10-fold serial dilutions, and incubated overnight at 4°C. For serum samples, antigen-specific antibodies were detected using goat anti-mouse IgG (heavy and light chains) conjugated to alkaline phosphatase (Sigma), diluted 1/4000, with incubation for 1.5 h. The ELISA was developed with *p*-nitrophenyl phosphate (Sigma), and the absorbance at 415 nm recorded after a standardised period of 1 h.

For nasal-wash samples a goat anti-mouse IgA was used, and developed as with serum samples. End-point titres were determined in triplicate by calculating the sample dilution at which the absorbance was equal to 0.1. Protein concentrations in the nasal-wash samples were determined by the BCA™ protein assay kit (Pierce, Rockford, IL) to correct for variation in the dilution in nasal washes and used to calculate the geometric mean titre (GMT) of antibody per mg of total protein.

3.2.8 Passive protection studies

100 µl of each serum sample from five mice that received either the 6AΔ*cps* LAV (immune sera) or the PBS control (naïve sera) was pooled. µMT mice were given an IP injection with 100 µl of pooled immune or naïve sera one day prior to intranasal challenge with 10^7 CFU/mouse of the virulent 6A strain. Mice were monitored for 9 d following challenge and euthanased when showing signs of sepsis.

3.2.9 Statistical analysis

Colonisation density was expressed as the $\log_{10}$ CFU/ml for calculation of means ± standard error of the mean. Statistics were carried out on ELISA data using the Mann-Whitney test. Statistical comparisons of survival and colonisation among groups were made by the non-parametric test indicated (GraphPad Prism 4).

3.3 Results

3.3.1 Evaluation of attenuation of, and of colonisation by, vaccine strains

To generate live-attenuated vaccine candidates, genes of each of the three major virulence determinants of *S. pneumoniae* (*cps*, *ply*, *pspA*) were independently interrupted in a type 6A isolate capable of inducing sepsis following intranasal challenge. To establish which strains were attenuated in the mouse model, the survival of C57BL/6J mice was followed after a high dose of these mutant strains (10^7 CFU/mouse) given intranasally. The 6AΔ*ply* mutant remained as virulent as the parent strain, while the 6AΔ*pspA* mutant displayed partial attenuation and the 6AΔ*cps* mutant was completely avirulent (**Fig. 3.3**). Because of the limited attenuation of the vaccine strains lacking the proteinaceous virulence determinants, a double mutant was constructed (6AΔ*ply*Δ*pspA*) which was found to be significantly, but still not completely attenuated.

Since the ability of live vaccine strains to induce protection would be enhanced by their persistence on the mucosal surface, the effect of attenuating mutations was determined by comparing quantitative colonisation of the upper respiratory tract. At 9 d post-inoculation the 6AΔ*ply*, 6AΔ*pspA* and 6AΔ*ply*Δ*pspA* mutants displayed similar colonisation density compared to the parent strain (**Fig. 3.4A**). In contrast, colonisation by the Δ*cps* mutants was not significantly above the limit of detection by 9 d. A similar result was seen with a Δ*cps* mutant of the TIGR4 strain. To further investigate the unencapsulated mutants, the density of colonisation was also determined at 2 d post-inoculation. The Δ*cps* mutants of both serotypes demonstrated colonisation albeit at a reduced density compared to the corresponding parent strains (**Fig. 3.4B**), confirming earlier results (**Chapter 2**, **Fig. 2.2**).

3.3.2 Protection against sepsis and colonisation induced by vaccine strains

The ability to protect against sepsis was assessed for the vaccine strains that showed the greatest attenuation (6AΔ*cps* and 6AΔ*ply*Δ*pspA*). Colonisation by live-attenuated vaccine strains was used to immunise mice, and 5 wk post-immunisation the previously colonised mice were challenged intranasally with a high dose of the 6A parent strain (10^7 CFU/mouse). Both the 6AΔ*cps* mutant and the double mutant (6AΔ*ply*Δ*pspA*) vaccine strains induced significant protection (**Fig. 3.5**).

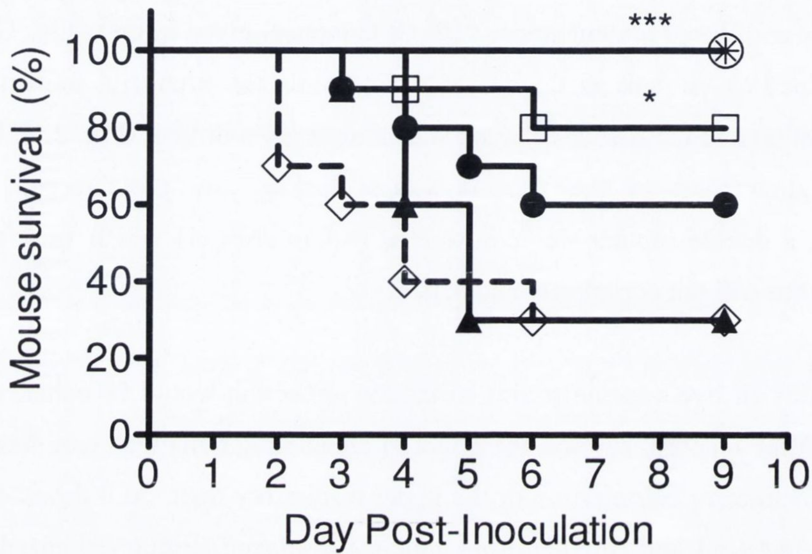


Fig. 3.3 Attenuation of *S. pneumoniae*.

Comparison of the level of attenuation of various *S. pneumoniae* type 6A live vaccines over a 9-d period post-inoculation of C57BL/6J mice with 10^7 CFU, as assessed by mouse survival. Data are based on a minimum of 10 animals in each group. Statistical difference, compared to the parent strain, was determined by the Kaplan-Meier log-rank test. ◊ = 6A,

▲ = 6AΔ*ply*, ● = 6AΔ*pspA*, □ = 6AΔ*ply*Δ*pspA*, ○ = 6AΔ*cps*, ∗ = PBS control.
* P=0.0155, *** P<0.0001.

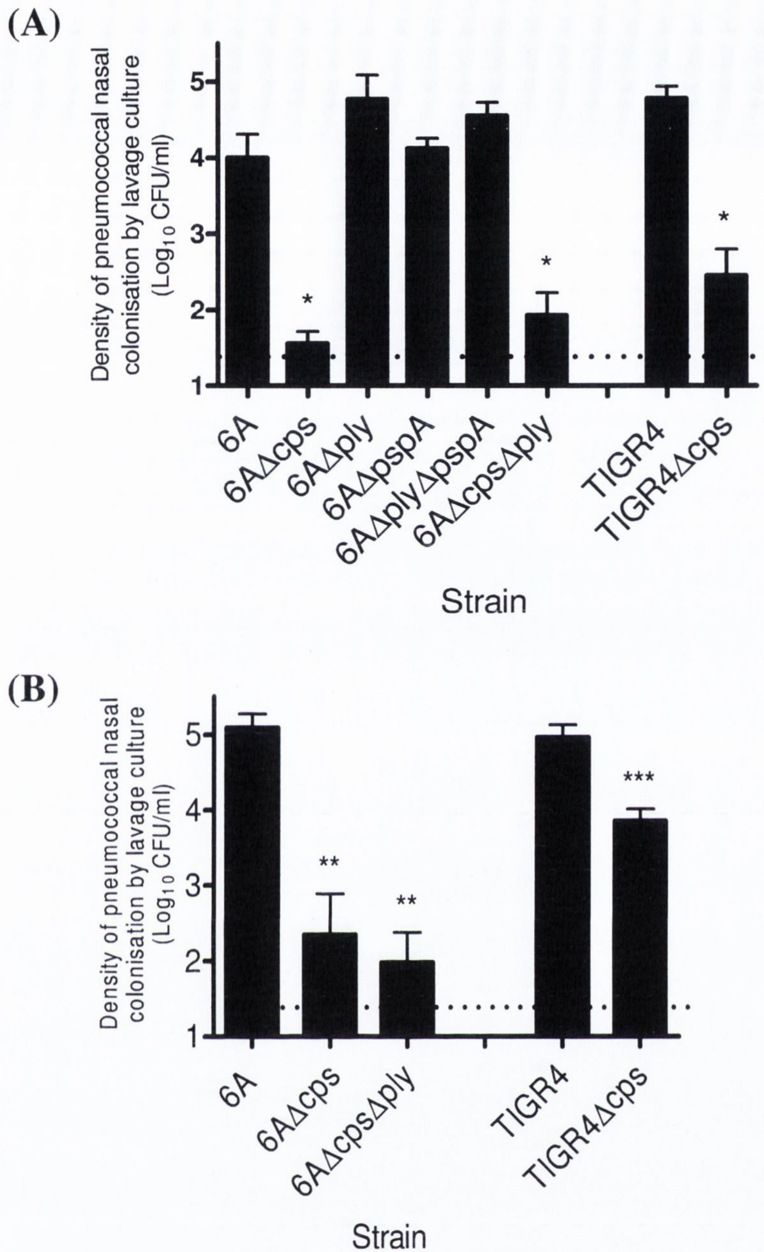


Fig. 3.4 Colonisation of attenuated *S. pneumoniae*.

Comparison of the ability of various live vaccine strains to colonise the C57BL/6J mouse nasopharynx at (A) 9 d and (B) 2 d post-inoculation IN with 10^7 CFU. Density of pneumococci in upper respiratory tract lavage fluid is shown by the mean $\log_{10}$ CFU/ml ± SEM. n = 4–9 mice per group per time point. Dashed line indicates limit of detection. Statistical difference, compared to the corresponding parent strain, was determined using Mann-Whitney test. *P<0.0286, **P=0.004, ***P=0.0008.

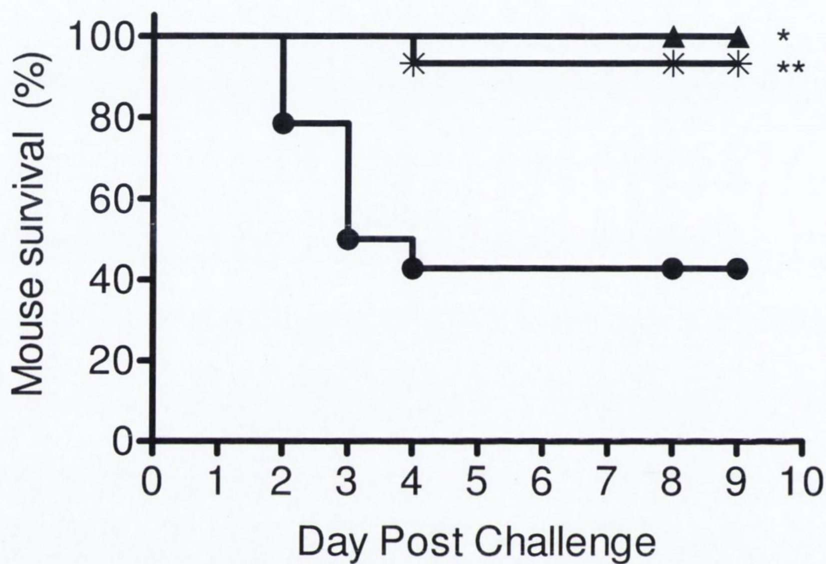


Fig. 3.5 Systemic protection induced by live-attenuated vaccine strains.

Survival rates of C57BL/6J mice immunised IN with 10^7 CFU of the 6AΔ*ply*Δ*pspA* double mutant (▲, n=8), the 6AΔ*cps* mutant (*, n=15), or vehicle only control (•, n=14), following IN challenge with 10^7 CFU of the wild-type 6A strain 5 wk post-immunisation. Statistical difference, compared to the vehicle control, was determined by the Kaplan-Meier log-rank test. * P=0.0109, ** P=0.0026.

Mucosal protection induced by prior colonisation by vaccine strains was also assessed. The density of colonisation of challenge strains was determined 9 d post-inoculation with the parent isolate. Following challenge with either the TIGR4 or 6A parent isolates, the density of colonisation was significantly reduced for mice that were immunised with the corresponding Δ*cps* vaccine strains (**Fig. 3.6**). Combinations of mutations in 6A vaccine strains, including Δ*ply*Δ*pspA* and Δ*cps*Δ*ply*, induced mucosal protection against the 6A parent isolate, but for the later mutant a significant effect was only seen after two doses of the live-attenuated vaccine. Together these data suggest that the attenuated vaccine strains can induce mucosal and systemic protection.

3.3.3 Cross-protection induced by vaccine strains

The Δ*cps* mutant was able to protect significantly from sepsis, and also showed the greatest mucosal protection. Therefore, the TIGR4Δ*cps* mutant was used to assess whether immunity induced by a strain of one serotype could protect against a strain of another serotype (cross-protection). Colonisation with the TIGR4Δ*cps* mutant was used to immunise mice before challenge with a high dose of the wild-type 6A strain (10^7 CFU/mouse). With a single dose of the TIGR4Δ*cps* vaccine strain mice had partial protection from sepsis. However, with two doses of this vaccine strain there was complete protection from sepsis with the 6A challenge (**Fig. 3.7**). Again, mucosal protection was investigated. A single dose of the TIGR4Δ*cps* mutant partially reduced colonisation by the wild-type 6A strain, with further reduction following a second vaccine dose (**Fig. 3.8**). The single dose 6A vaccine strains (Δ*cps* and Δ*ply*Δ*pspA*) were also used to investigate cross protection from colonisation with a high dose TIGR4 challenge. The TIGR4 strain causes sepsis at a low rate in the mouse model used, so mucosal not systemic protection was evaluated. Both the Δ*cps* and Δ*cps*Δ*pspA* vaccine strains were proficient at reducing colonisation by a distantly related isolate (**Fig. 3.8**). These results suggest that the live-attenuated strains are able to elicit protective immunity that is serotype independent.

3.3.4 Roles of humoral and cell-mediated immunity in protection induced by vaccine strains

The roles of humoral and cell-mediated immunity were investigated to determine the immunological basis of protection against the live-attenuated vaccine strains. To assess the

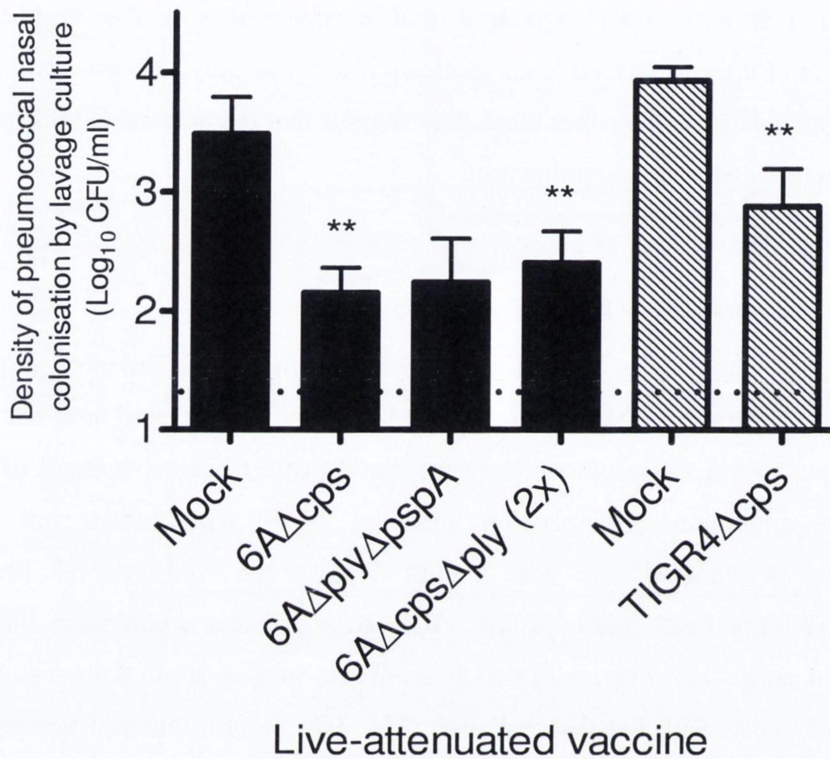


Fig. 3.6 Mucosal protection induced by live-attenuated vaccine strains.

Colonisation density at 9 d post-inoculation of C57BL/6J mice challenged with strain 6A (solid bars) or TIGR4 (hatched bars) after IN immunisation with 10^7 CFU of the indicated live vaccine. Challenge dose of 10^7 CFU was administered IN five weeks after the final immunisation. The 6AΔ*cps*Δ*ply* vaccine was delivered with 2 doses (2×) prior to challenge. Values represent the mean of 6–14 mice/group ± SEM. Dashed line indicates limit of detection. Colonisation density was analysed in surviving mice. Eight mice in mock immunised group, and one animal in 6AΔ*cps* group, which did not survive, were not included in the analysis. Statistical difference, compared to the corresponding mock immunised group, was determined using Mann-Whitney test. ** P<0.0083.

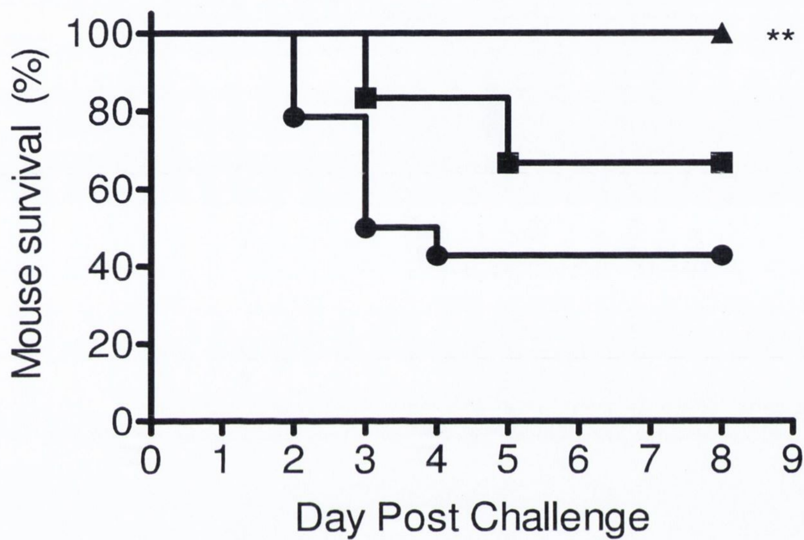


Fig. 3.7 Systemic cross-protection induced by live-attenuated vaccine strains.

Survival rates of C57BL/6J mice immunised IN with 10^7 CFU of the TIGR4Δ*cps* mutant as a single dose (■, n=12) versus two doses (▲, n=12), compared to vehicle only control (●, n=15), following IN challenge with 10^7 CFU of the wild-type 6A strain five weeks post-immunisation. Statistical difference, compared to the vehicle control, was determined by the Kaplan-Meier log-rank test. ** P=0.0021.

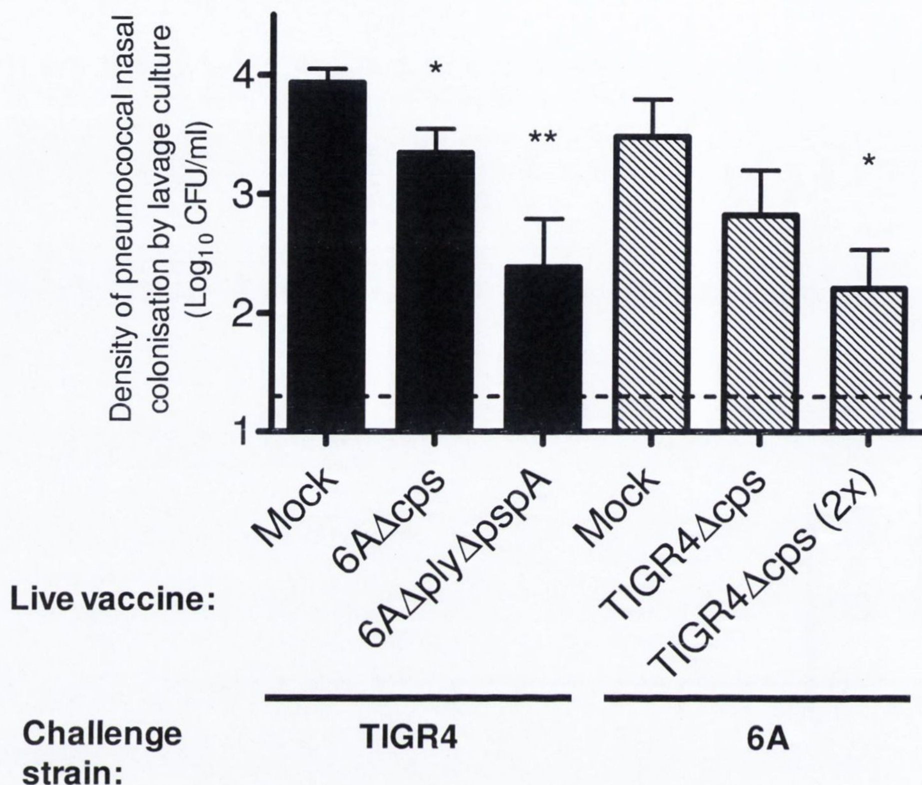


Fig. 3.8 Mucosal cross-protection induced by live-attenuated vaccine strains.

Colonisation density at 9 d post-inoculation of C57BL/6J mice challenged with 10^7 CFU of the parent TIGR4 (solid bars) or the parent 6A (hatched bars) strains after IN immunisation with 10^7 CFU of the indicated live vaccine of the other serotype. One dose of the vaccine strain was administered except where a second dose is indicated (2×). Values represent the mean of 6–12 mice/group ± SEM. Dashed line indicates limit of detection. Colonisation density was analysed in surviving mice. Eight mice in mock immunised group, and four animals in the TIGR4Δ*cps* single dose group, which did not survive, were not included in the analysis. Statistical difference, compared to the corresponding mock immunised group, was determined using Mann-Whitney test. * $P<0.0278$, ** $P=0.0028$.

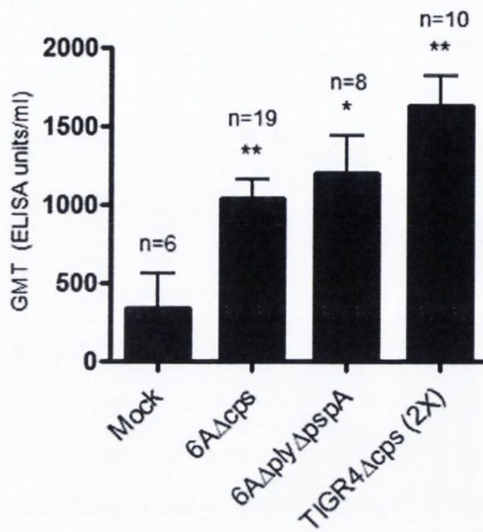
humoral immune response, the IgG titres to whole pneumococci were determined in sera for groups in which there was protection against either 6A or TIGR4 challenge (**Fig. 3.9A** and **B,** respectively). The levels of pneumococcus-specific IgG in sera were significantly increased after immunisation with vaccine strains (Δ*cps*, Δ*ply*Δ*pspA*). The levels of IgA were then quantified in nasal wash samples to determine the antibody titre on the mucosal surface. These values were corrected for variation in dilution of nasal wash samples by determination of total protein concentrations. The mice immunised with vaccine strains displayed significant increases in IgA titres (**Fig. 3.9C**).

Having demonstrated an increase in antibody titres due to immunisation, protection from sepsis was investigated for dependence on humoral or cell-mediated immunity. μMT mice (which are unable to generate specific antibody) and $MHCII^{-/-}$ mice (exhibiting a depletion of $CD4^+$ T-cells) were immunised with the 6AΔ*cps* vaccine strain to see if protection was sustained. Neither the μMT, nor the $MHCII^{-/-}$ mice, were protected against challenge with the parent 6A strain (**Fig. 3.10**). This suggested that the protection from sepsis was antibody dependent but also required $CD4^+$ T-cells.

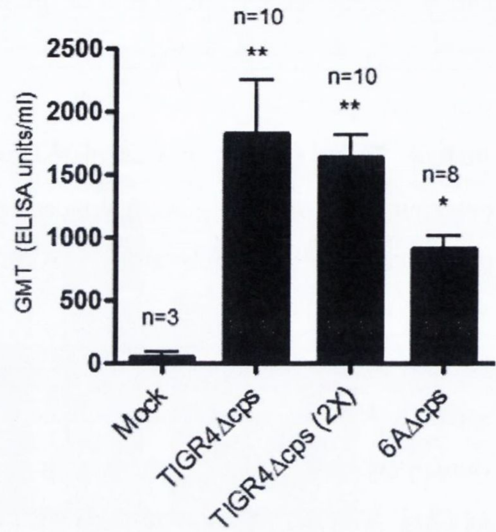
Whether or not IgA was mediating mucosal protection was also investigated since IgA is the most abundant Ig on the mucosal surface of the nasopharynx. A type 6A isolate (6A), that causes invasive infection in mice following intranasal challenge, was used to determine if IgA was important for protection. This was carried out by colonising $IgA^{-/-}$ mice with the 6AΔ*cps* mutant, as unencapsulated strains are unable to cause invasive infection, followed by challenge with virulent parental 6A and monitoring of mouse survival. Unlike μMT mice, which lack specific antibody, $IgA^{-/-}$ mice were protected during secondary challenge, suggesting that IgA is not required to prevent sepsis following intranasal challenge (**Fig. 3.10**).

Antibody dependence in mucosal protection was also investigated. The reduction in colonisation seen with the immunocompetent C57BL/6J mice following immunisation was not seen with the μMT nor the $MHCII^{-/-}$ mice (**Fig. 3.11**). The role of IgA was also investigated in mucosal protection, by monitoring the levels of colonisation at 8 d post-challenge. There was a wide range of colonisation density in previously colonised $IgA^{-/-}$ mice. However, 40% of these mice were still able to clear colonisation, suggesting that

(A)



(B)



(C)

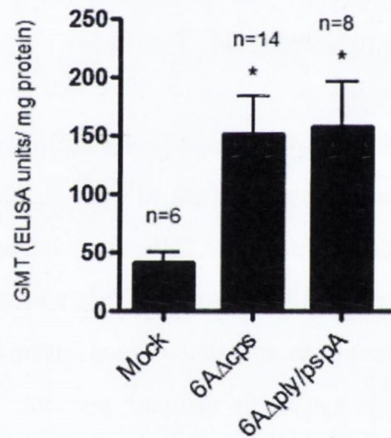


Fig. 3.9 ELISA titres of IgG and IgA following LAV immunisation.

Levels of IgG in serum against (A) strain 6A or (B) strain TIGR4 (whole bacteria), from C57BL/6J mice immunised IN with 10^7 CFU of the indicated live vaccine. Values shown are the geometric mean titres (GMT) ± SEM following one or two (2×) doses of the vaccine strain. GMT is calculated as the dilution at which the OD_{415} of the ELISA equals an arbitrary value of 0.1. (A) *P=0.0293, **P<0.01. (B) *P=0.0121, **P=0.007. (C) Levels of IgA against strain 6A (whole bacteria) in nasal wash samples from C57BL/6J mice immunised IN with 10^7 CFU of the indicated live vaccine. Values shown are GMT/mg total protein ± SEM. *P<0.02. Statistical difference, compared to mock immunised control group, was determined using Mann-Whitney test.

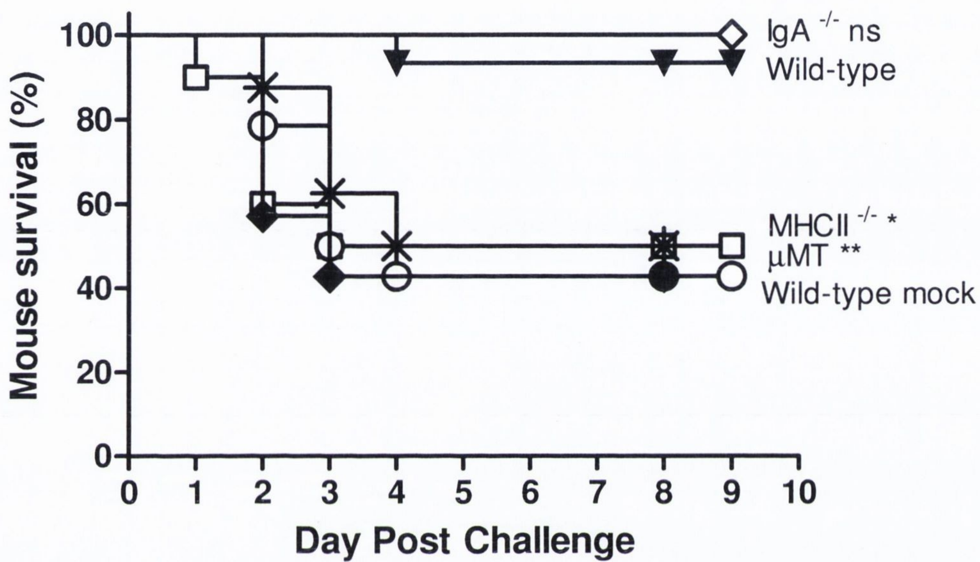


Fig. 3.10 Mechanism of systemic protection.

Survival rates of μMT mice (□, n=10), MHCII$^{-/-}$ mice (*, n=8), IgA$^{-/-}$ mice (◊, n=10) and wild-type C57BL/6J mice (▼, n=15) immunised IN with 10^7 CFU of the 6AΔ*cps* mutant, PBS mock immunised μMT (♦, n=7) or PBS mock immunised C57BL/6J mice (○, n=14) and challenged with 10^7 CFU of the 6A parent strain. Statistical difference, compared to immunised wild-type mice, was determined by Kaplan-Meier log-rank test. * *P*=0.0117, ** *P*=0.0096, ns, non-significant.

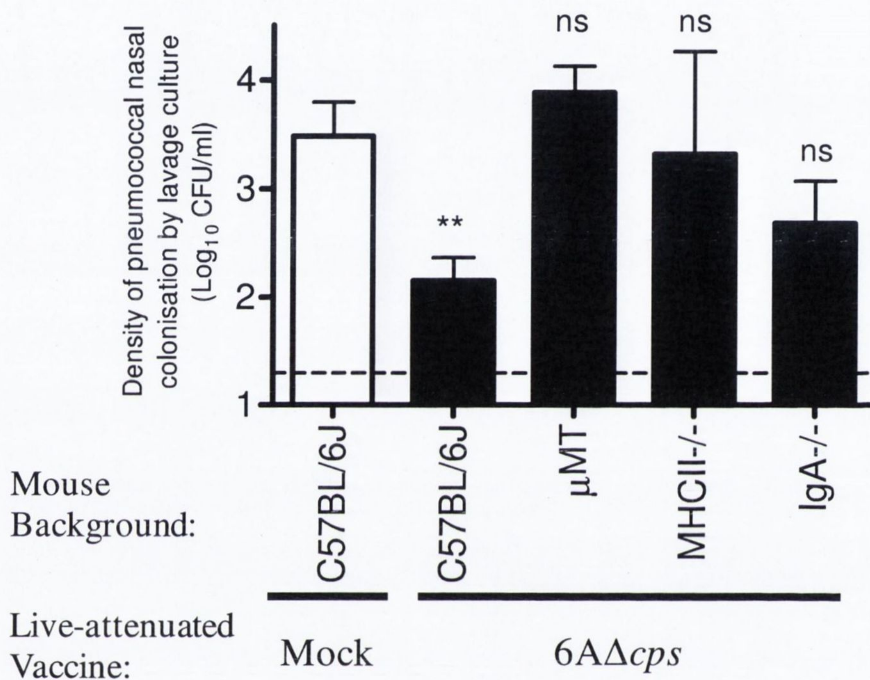


Fig. 3.11 Mechanism of mucosal protection.

Colonisation density at 9 d post-inoculation of C57BL/6J mice challenged IN with 10^7 CFU of the 6A parent strain after IN immunisation with 10^7 CFU of the 6AΔ*cps* mutant. The dotted line indicates the lower limit of detection. Values represent the means of 4–14 mice/group ± SEM. Statistical difference, compared to the mock immunised C57BL/6J mice, was determined by Mann-Whitney test. ** P=0.0078, ns, non-significant.

IgA was not required for mucosal protection (**Fig. 3.11**). This suggests that mucosal protection following immunisation is also antibody dependent and requires $CD4^+$ T-helper cells, probably to generate effective antibody.

3.3.5 Passive protection

To confirm that antibodies were required for systemic protection, μMT mice were injected IP with pooled immune or naïve sera and challenged with the virulent 6A strain. Mice that received the immune sera were partially protected from 6A challenge, compared to mice that received naïve sera (**Fig. 3.12**). However, this effect was minimal, possibly due to insufficient amounts of antibodies delivered.

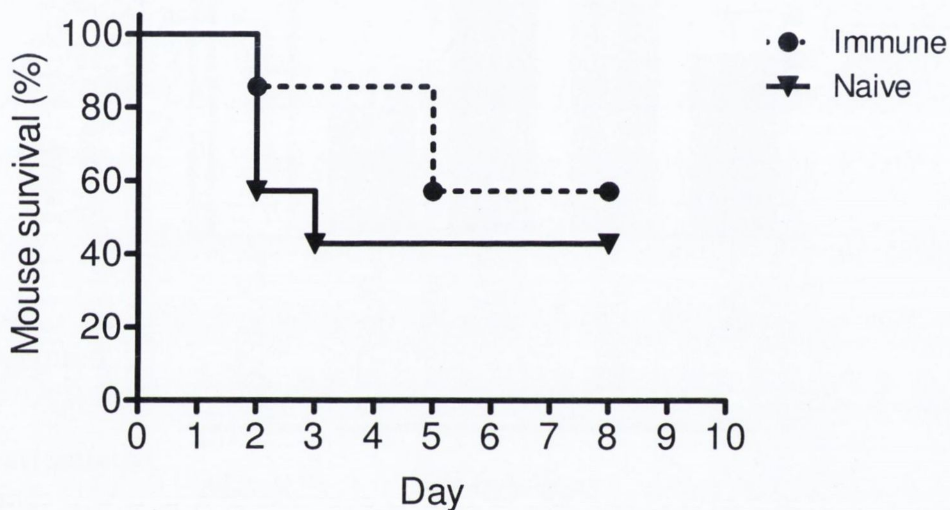


Fig. 3.12 Passive protection against systemic infection.

μMT mice were injected with 100 μl of pooled sera from C57BL/6J mice that received LAV (Immune, ●) or PBS control (Naïve, ▼) one day prior to IN challenge with 10^7 CFU of the virulent 6A strain. Mice were monitored for 9 d following challenge and euthanased when showing signs of sepsis. 100 μl of each serum sample from five mice was pooled in each group. Kaplan Meier curve with survival rates of mice. n = 7 mice per group.

3.4 Discussion

This study addressed whether protective immunity can be induced through colonisation with live-attenuated pneumococci. Observations that unencapsulated pneumococci can colonise the nasopharynx at a low density (**Chapter 2, Section 2.3.1**) led to questions about whether this limited colonisation would be sufficient to generate a protective response. The present approach to study live-attenuated vaccines also took advantage of previous studies that defined other pneumococcal virulence determinants (van Rossum *et al.*, 2005; Balachandran *et al.*, 2002) These virulence determinants were eliminated alone, and in combination, to generate mutants that are highly attenuated, but remain capable of colonising sufficiently to induce protective immunity. The response to live-attenuated strains could be useful in accelerating the immunity that occurs during early childhood, but could do so with safe mutants rather than potentially virulent isolates as occurs naturally. A further benefit of this approach is that it could provide more effective mucosal immunity than current strategies based on systemic immunisation and, furthermore, it could induce protection with a potentially inexpensive and easily administered vaccine. Beyond the potential advantages of this approach for children in the developing countries, live-attenuated vaccines could also be useful in boosting pre-existing natural immunity later in life.

These findings provide additional evidence that upper airway colonisation may be an immunising event. Prior studies have differed in showing whether murine model colonisation with natural isolates protects from subsequent intranasal challenge with the same isolate (McCool & Weiser, 2004; Malley *et al.*, 2001). The attenuated strains used in the present study may be cleared more efficiently compared to wild-type isolates and may, as a result, be more effective at inducing protective immune responses. Encapsulation, for example, may obscure the immune response to underlying surface antigens. In this regard, the Δ*cps* mutants tested showed limited and transient colonisation that, nevertheless, was sufficient to confer significant protection. Moreover, the current results demonstrate that colonisation by Δ*cps* mutants induces mucosal and systemic protection that does not depend on a response to capsular polysaccharide (serotype-independent protection). Serotype-independent protection against pneumococcal colonisation induced by prior exposure in a mouse model was demonstrated with constructs of different types in the same genetic background (Trzcinski *et al.*, 2005). This observation demonstrates that an

epitope or epitopes shared between two isolates, which are not closely related as assessed by MLST analysis, can also induce protective immunity. **Chapter 4** attempts to identify the non-capsular polysaccharide structure(s) inducing serotype-independent immunity. Based on the efficacy of mutants lacking PspA and Ply, these well-studied immunogenic proteins do not appear to be required for this effect, although in experimental human carriage studies pre-existing antibodies to PspA correlated with resistance to colonisation (McCool *et al.*, 2002). The immune response to common epitopes may underlie the broad, cross-serotype decrease in pneumococcal carriage and disease that occurs beyond the first years of life – an event that may be due to prior colonisation by a limited array of strains and types (Lipsitch *et al.*, 2005). The scope of cross-protection would need to be examined with a larger array of isolates to determine the number of live-attenuated vaccine strains required to stimulate broad immunity.

Of the live-attenuated vaccine strains tested, those lacking capsular polysaccharide and the combination of Ply and PspA were both significantly attenuated and able to induce protective immunity. However, the Δ*ply*Δ*pspA* vaccine strain was not fully attenuated in this model indicating that additional steps might be required before a strain with this combination of mutations could be used safely. In contrast, the Δ*cps* mutant was attenuated in an otherwise highly mouse virulent isolate. Although there have been occasional reports of infection caused by unencapsulated or non-typeable pneumococcal isolates, these are generally limited to superficial sites and unencapsulated mutants are completely avirulent in models of infection (Martin *et al.*, 2003; Mato *et al.*, 2005; Hanage *et al.*, 2006). An added margin of safety may be provided by inclusion of more than a single attenuating mutation in virulence determinants. A combination of Δ*cps* and Δ*ply* mutations was examined in this study, and shown to be as effective as the single Δ*cps* mutation in inducing mucosal protection when provided in a two-dose regimen. Such a step would also reduce the chances of reversion to a more virulent phenotype. Indeed, by incorporating mutation(s) in previously identified genes required for natural competence it should be possible to minimise this possibility (Lee *et al.*, 1999). The *comE* gene would be a good target, as this encodes the response regulator required for sensing the pheromone CSP required for DNA uptake. Disruption of the *comE* gene has been shown to abolish both the response to synthetic activator peptide and endogenous competence induction (Lee & Morrison, 1999).

Colonisation with live attenuated pneumococci induced increased levels of anti-pneumococcal serum IgG (and mucosal IgA). This serum IgG response could account for the observed protection from systemic infection and offers the possibility of long-acting immunity. The lack of protection seen following immunisation of μMT mice but not immunocompetent parental mice demonstrates the importance of antibody in mucosal and systemic protection. Passive protection did not fully protect mice against invasive infection, although sera from mice that received boosters of the LAV may increase the level of protection. The requirement for $CD4^+$ T-cells, as seen with $MHCII^{-/-}$ mice, could be because of the contribution of these cells to humoral immunity. Surprisingly, a similar requirement for humoral immunity was not observed in the clearance of colonisation in naïve C57BL/6J mice or in protection from colonisation induced by prior exposure to encapsulated pneumococci (McCool & Weiser, 2004; Trzcinski *et al.*, 2005). This difference in the requirement for antibody offers further evidence that the immune response to live-attenuated strains and wild-type isolates is distinct. Malley *et al.* (2001) have previously shown that intranasal vaccination with killed-whole pneumococci given in multiple doses with an adjuvant generates protection from colonisation in a serotype-independent manner. This effect was also shown to occur in mice that failed to produce specific antibody, suggesting a different protective immune response to live-attenuated and killed strains (Malley *et al.*, 2005). While both intranasal vaccine approaches are potentially less complex and more broadly acting than currently available products, the antibody-dependent effects demonstrated in this study included protection from systemic infection and did not require use of a pharmacological adjuvant.

Prior exposure to live-attenuated mutants, including the Δ*cps*, Δ*ply*Δ*pspA* and Δ*cps*Δ*ply* mutants, were shown here to decrease the density of colonisation by wild-type isolates when challenged intranasally 5 wk later. Since nasal colonisation is the reservoir for pneumococcal transmission, this effect on carriage suggests that a live-attenuated vaccine, like the conjugate vaccine, has the potential to induce herd immunity (Musher, 2006). In addition, because of the effect on carriage, previously described experimental human carriage studies offer a means of testing the safety and efficacy of immunisation using live-attenuated pneumococci in the natural host (McCool *et al.*, 2002).

Chapter 4

Bacterial factors involved in protection induced by live-attenuated *S. pneumoniae* colonisation

4.1 Introduction

Pneumococcal antigens that are common to all or most serotypes have received much interest as vaccine targets for their potential to induce broad protection. Some of these include surface proteins — choline-binding proteins (Briles *et al.*, 2000b; Brooks-Walter *et al.*, 1999), lipoproteins (Briles *et al.*, 2000a; Seo *et al.*, 2002), toxin (Alexander *et al.*, 1994), histidine triad proteins (Adamou *et al.*, 2001) and sortase-dependent surface proteins —, and cell wall structural components (Tanaka *et al.*, 2007; Malley *et al.*, 2006; Giefing *et al.*, 2008). These antigens given alone or in combination elicit both systemic and/or mucosal protection when administered by a variety of methods with adjuvants in animal models. Some of these protein antigens have been confirmed, by unbiased genomic approaches looking for antigens, to be recognised by antibodies from patients convalescing from pneumococcal diseases (Wizemann *et al.*, 2001; Giefing *et al.*, 2008). The success of studies involving these antigens highlights the potential for common surface proteins in protecting against IPD. An inexpensive broad-spectrum vaccine against a common antigen or antigens could also overcome the limitations of the conjugate vaccine.

Colonisation rates of *S. pneumoniae* decline significantly as age increases, suggesting that this early colonisation may be an immunising event (Granat *et al.*, 2009). However, the immune mechanism responsible for the decline in colonisation has yet to be fully defined. It is clear that reducing colonisation prevents pneumococcal disease. For example, experience with the PCV7 vaccine has demonstrated that decreased colonisation in vaccine recipients reduces transmission and leads to decreased disease in those who have not been vaccinated (Whitney *et al.*, 2003; Millar *et al.*, 2008). This suggests that the effectiveness of any novel vaccine that decreases carriage would be magnified in the community because of the contribution of herd immunity.

The results in **Chapter 3** demonstrated that carriage of live-attenuated *S. pneumoniae* can elicit antibody-dependent immunity and can also protect against a heterologous challenge strain. The focus of this chapter was to take an unbiased look at which pneumococcal antigen(s) induce broadly cross-reactive and cross-protective antibodies following nasal colonisation with LAV. In this study, LAV immunisation was used as a tool to identify

cross-reactive antigens by dissecting out the main targets of the humoral immune response using a mouse model of nasal colonisation.

4.2 Materials and Methods

4.2.1 Bacterial strains and culture conditions

S. pneumoniae strains were grown as described in **Chapter 2, Section 2.2.1**. Strains used in this study include strains from **Chapter 3, Section 3.2.1**, along with a 6B clinical isolate (**Chapter 2, Table 2.1**). The *pspA* gene from each strain was sequenced. The TIGR4 strain expresses PspA from family 2 (clade 3), whereas the 6A, 6B and 23F express PspAs from family 1 (clade 2, 1, and 1, respectively).

The Δ*cps* and Δ*pspA* mutants were constructed as described in **Chapter 2, Section 2.2.2** and **Chapter 3, Section 3.2.4**, respectively. The Δ*pspA* mutation was transferred to the TIGR4, 23F and 6B strains using sequential back-transformation with lysates of mutant 6AΔ*pspA*, selecting for spectinomycin resistance (200 µg/ml).

4.2.2 Construction of PpmA-deficient strains

The Δ*ppmA* mutant was constructed by insertion deletion, with an erythromycin cassette from shuttle vector pMU1328 (Achen *et al.*, 1986) replacing the majority of the *ppmA* gene. This mutant was constructed using overlap extension PCR (Ho *et al.*, 1989) [**Fig. 4.1,** adapted from (Choi & Schweizer, 2005)]. Primers used in overlap extension PCR are listed in **Chapter 3, Table 3.1**, with the sequence of the region that anneals to the complementary sequence of the second primer in bold, and the region that cannot bind to DNA in the first PCR step is shown in italics. In step one, primer pairs PpmAF7/PpmAOER1 and PpmAOEF2/PpmAR6 were used to amplify the two flanking regions of the *ppmA* gene from the TIGR4 strain, deleting most of the gene, and adding nucleotides complementary to the *erm* cassette. Primers PpmAOEF1 and PpmAOER2 were used to amplify the erythromycin cassette, adding nucleotides complementary to the *ppmA* gene flanking regions at the beginning and end of the sequences. All PCR reactions in step one were performed using high fidelity PCR, as described in **Chapter 3, Section 3.2.4**.

The resulting PCR products from step one were purified three times to ensure removal of primers, using QIAquick PCR Purification Kit (Qiagen, Valencia, CA). In step two, the

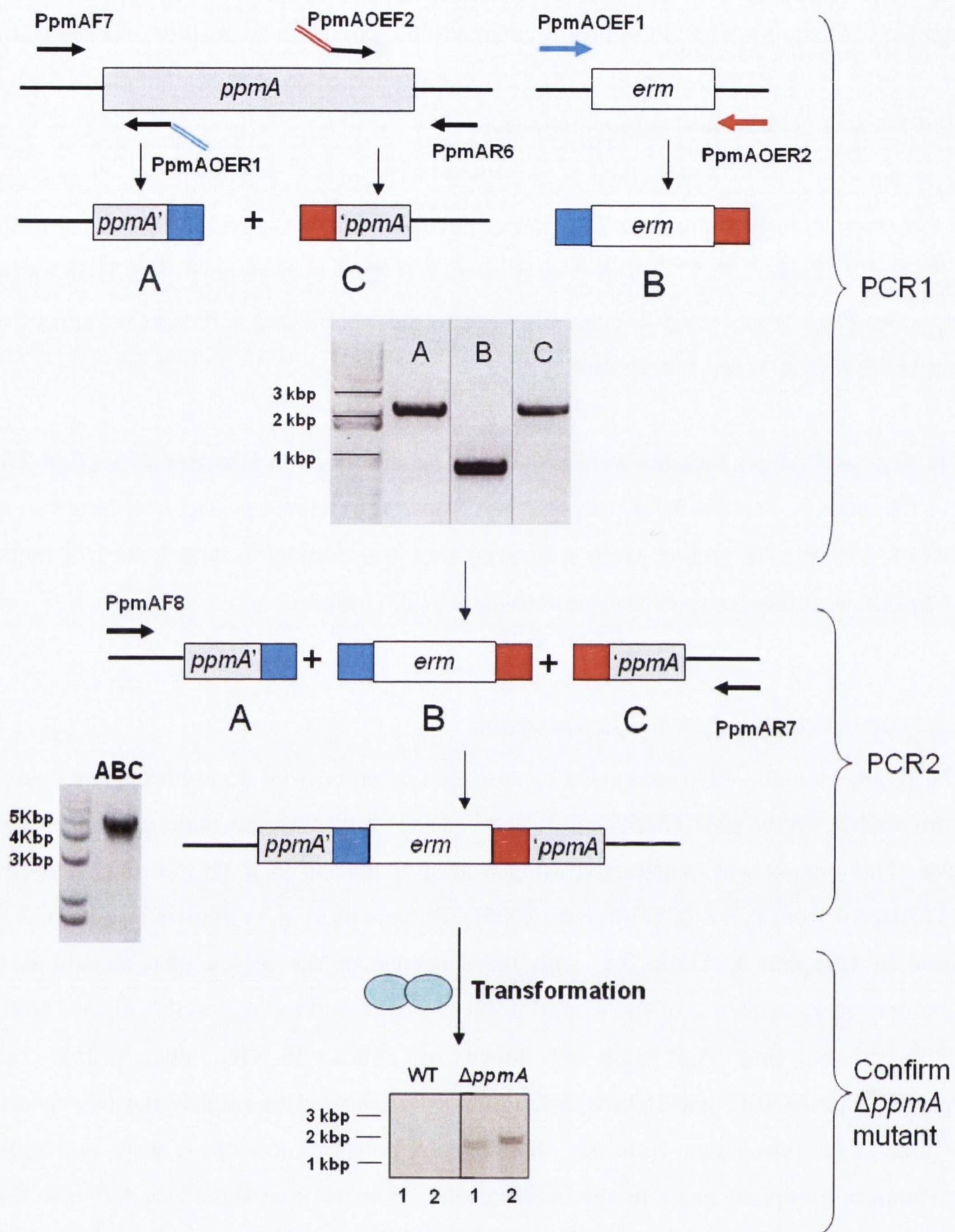


Fig. 4.1 Schematic diagram of overlap extension PCR construction of Δ*ppmA* mutant.

PCR 1 generates N- and C-terminal-encoding gene fragments (A + C respectively), removing the majority of the gene, and prepares the *erm* cassette with compatible ends for the *ppmA* gene fragments (B). PCR 2 splices these fragments together (ABC). The resulting mutant *ppmA* gene is transformed into *S. pneumoniae*, and confirmed by PCR with primers ErmF/PpmAR7 (1) and ErmR/PpmAF8 (2) (**Chapter 3,Table 3.1**), to confirm insertion of antibiotic cassette. Adapted from Choi & Schweiser (2005).

three fragments were added together as template for primers PpmAF8 and PpmAR7, and spliced together by overlap extension PCR.

Overlap extension PCR was carried out as described for high fidelity PCR (see **Chapter 3, Section 3.2.4**), using Platinum *Pfx* DNA polymerase (Invitrogen). The reaction conditions were as follows. Denaturation at 94℃ for 2 min, followed by 2 cycles of denaturation at 92℃ for 30 s, annealing at 40℃ for 1 min, and extension at 68℃ for 7 min, and then 33 cycles of denaturation at 92℃ for 30 s, annealing at 55℃ for 1 min, extension at 68℃ for 7 min, with a final extension at 68℃ for 8 min.

This PCR construct, with the *erm* cassette replacing most of the *ppmA* gene, was transformed into strains TIGR4, 6A and 23F with selection for erythromycin resistance (1 μg/ml), as described in **Chapter 3, Section 3.2.2**. The Δ*ppmA* mutations were confirmed by PCR using primer pairs PpmAF7/ErmR and PpmAR6/ErmF, to confirm insertion of the *erm* cassette within the *ppmA* gene (**Fig. 4.1**).

4.2.3 Construction of PsaA-deficient strains

The Δ*psaA* mutant was also constructed by insertion deletion, with an erythromycin cassette from shuttle vector pMU1328 replacing the majority of the *psaA* gene. Primers are listed in **Chapter 3, Table 3.1**. This mutant was constructed by amplifying the gene using primers PsaAFHind32 and PsaAR. This PCR product was cloned into the TOPO PCR2.1 plasmid and transformed into One Shot® Top10F′ chemically competent *E. coli* using the TOPO TA cloning kit (Invitrogen), as described in **Chapter 3, Section 3.2.4**. A 300-bp deletion was made in this gene using inverse PCR with primers InvpsaAFmef1 and InvpsaARmef1, and an erythromycin resistance cassette, cut out of TOPO PCR2.1 plasmid with *Eco*RI sites, was inserted into the plasmid cut with *Mfe*I. All PCR reactions were performed as high fidelity PCR, as described in **Chapter 3, Section 3.2.4**. *S. pneumoniae* Δ*psaA* mutants were generated by transformation with the plasmid DNA, with selection for erythromycin resistance (1 μg/ml). The Δ*psaA* mutations were confirmed by sequencing and Western blotting, using a mouse monoclonal antibody against PsaA (gift from Eddie Ades, CDC) at 1:1000 dilution (**Fig. 4.2**). The Western blotting was performed as described in **Chapter 3, Section 3.2.3**.

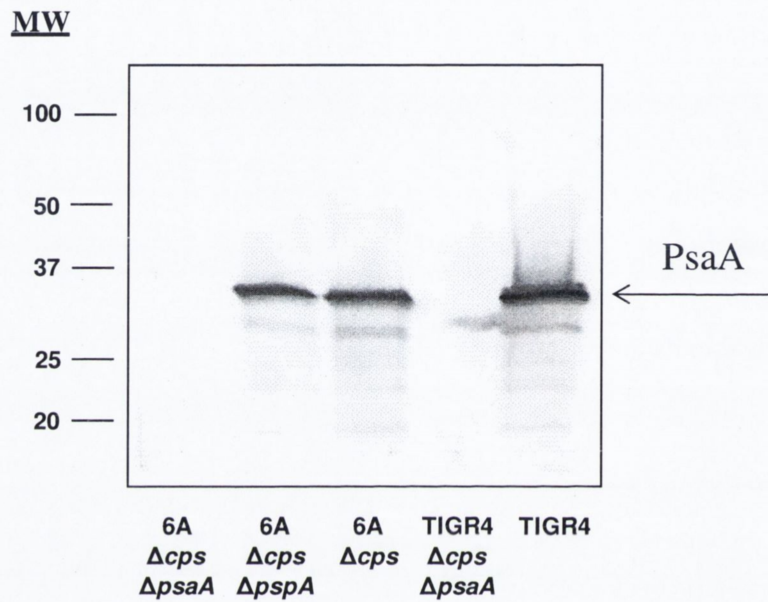


Fig. 4.2 Western blot confirmation of the Δ*psaA* mutants.

Whole cell lysates from strains listed above were separated on 10% SDS-PAGE gels, transferred to PVDF membranes, and probed with a mouse mAb against PsaA to confirm the absence of PsaA in mutants. MW, molecular weight. Size markers in kDa.

4.2.4 Mouse model of nasopharyngeal colonisation and **S. pneumoniae** *challenge*

The mouse model is described in **Chapter 3, Sections 3.2.2** and **3.2.3**. Briefly, C57BL/6J mice received two-dose intranasal immunisation with 10^7 CFU without adjuvant, with the second dose given 2 wk after the first dose. Sera were obtained 1 d prior to challenge by tail bleeds, and stored at –20°C. Mice where challenged intranasally with 1–5 × 10^7 CFU of *S. pneumoniae* strains 5 wk following the second immunisation. After 8–9 d the remaining animals were euthanased and nasal washes were obtained for quantitative culture. Blood was also collected by cardiac punctures and sera were stored at –20°C for flow cytometric analysis.

4.2.5 Fractionation of bacteria

Fractionation of whole-cell bacteria was performed by growing large cultures (500 ml) of bacteria to mid-log phase, washing, then resuspending in 20 ml fractionation buffer (30 mM Tris/150 mM NaCl). Cultures were then passed twice through a French press system (American Instrument Co., Silver Spring, MD) to lyse the bacteria. Unlysed bacteria were removed by centrifugation at low speed (7,500 × *g*). The lysed bacteria were separated into soluble and insoluble fractions by ultracentrifugation at 275,000 × *g*, with the soluble fraction remaining in the supernatant fluid. Insoluble fractions were washed twice with PBS prior to resuspension in 1 ml of PBS. The purity of fractions was assessed by Western blot, by loading equal amounts of protein in each lane, using antibodies against known insoluble components, PsaA (mAb-PsaA, 1:1000) and LTA (TEPC15, 1:1000 – Sigma), and were determined to be crude (**Fig. 4.3**). Samples were stored at –20°C for use in gel electrophoresis.

4.2.6 Proteinase K Treatment

Aliquots (1 ml) of mid-log phase bacteria, OD_{620} = 0.5, were pelleted, washed, and resuspended in either 50 µl of 0.5 mg proteinase K/ml in PBS, or PBS control. Samples were incubated at 37°C for 45 min, before addition of Laemmli buffer, boiling for 5 min and loading onto SDS-PAGE gels.

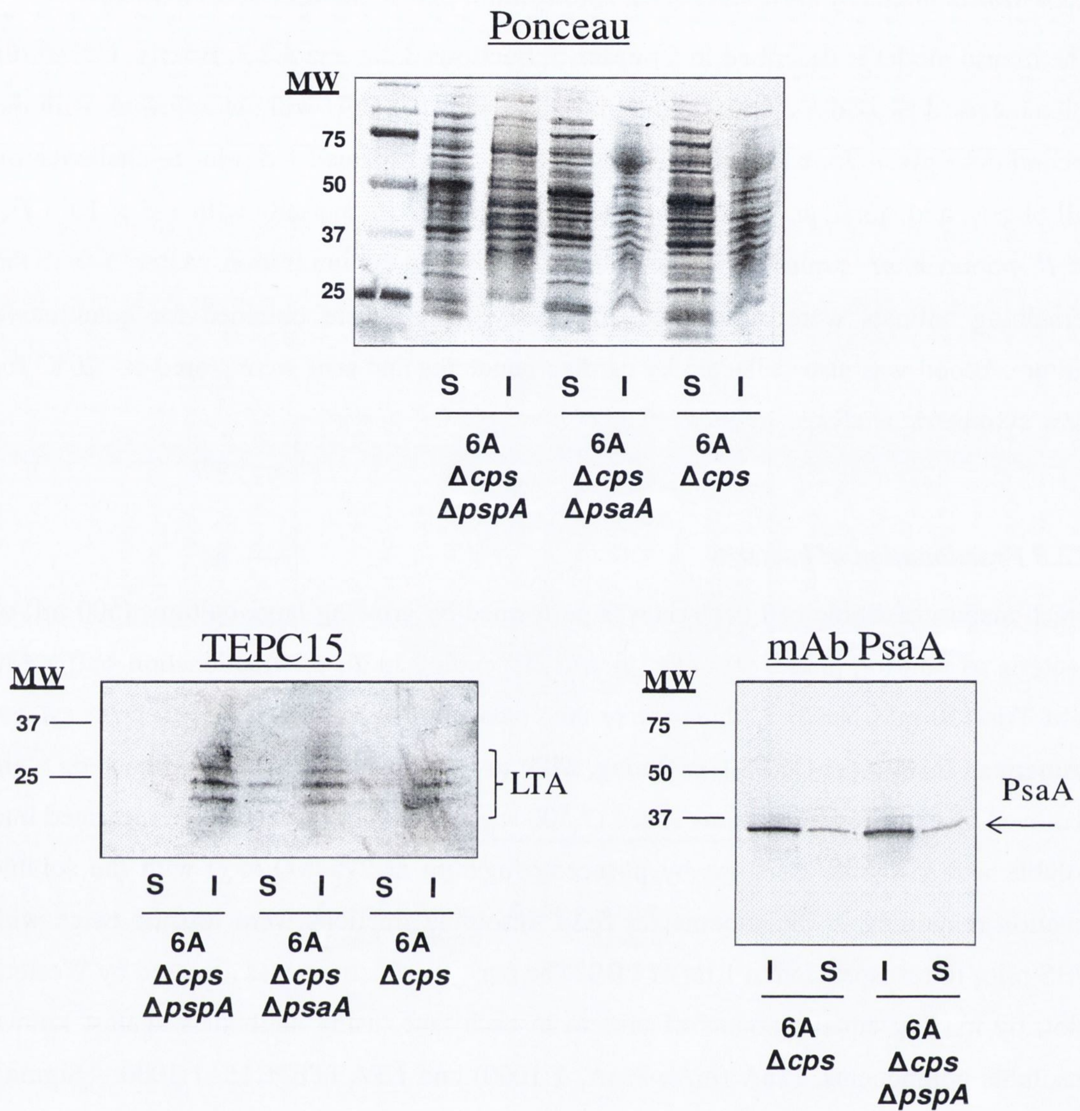


Fig. 4.3 Crude fractionation of bacteria.

Crude soluble (S) and insoluble (I) fractions were loaded on 10% SDS-PAGE gels. 1 μg protein/lane for PsaA Western blot, and 0.1 μg protein/lane for TEPC15 Western blot. Membranes were stained with Ponceau S prior to incubation with corresponding antibodies, to show equal loading between lanes. Fractions were demonstrated to be crude due to the presence of low levels of insoluble fraction contaminants in the soluble fraction. MW, molecular weight. Size markers in kDa.

4.2.7 Choline washing

Choline washing of insoluble fractions was performed by incubating 1 part protein fraction with 1 part choline chloride (Sigma) (4% in PBS) to a final concentration of 2% choline. Samples were rotated at 4°C for 1 h. CBPs were then separated from fractions by centrifugation, with the CBPs remaining in the supernatant fluid (Yother & White, 1994). Samples were stored at –20°C for use in gel electrophoresis.

4.2.8 Precipitation of proteins

Proteins present in the insoluble fractions were precipitated by chloroform-methanol extraction in order to purify samples and remove contaminating products such as detergents and lipids for clearer results in 2D-GE (Wessel & Flügge, 1984). This was completed as follows. To 100 µl of the insoluble fraction, 400 µl of methanol was added, and the mixture vortexed and centrifuged for 10 s at 9,000 × g. Next 100 µl of chloroform was added, vortexed and centrifuged for 10 s. Then 300 µl of water was added, and this mixture vortexed and centrifuged for 3 min at 9,000 × g at 4°C. The sample separated into two phases. The upper phase was aspirated off without disturbing the interface. Methanol (300 µl) was added to the lower phase, and the mixture vortexed and centrifuged at 14,000 × g for 10 min at 4°C. The protein sedimented as a pellet, and was resuspended in 125 µl of ReadyPrep Rehydration/Sample Buffer (BioRad).

4.2.9 Protein gel electrophoresis and staining

One dimensional SDS-PAGE was performed as described in **Chapter 3, Section 3.2.3**. Two dimensional SDS-PAGE involved separation of proteins by isoelectric point and by molecular weight, respectively.

Two dimensional SDS-PAGE was performed as follows: 7 mm ReadyStrips (pI 4.7–5.9) (BioRad) were rehydrated with precipitated sample at RT overnight. Isoelectric focusing was carried out in a Protean IEF cell (BioRad), with the following 3-step protocol: Step 1, rapid ramp to 250 V. Step 2, slow ramp to 4,000 V. Step 3, rapid ramp to 10,000 V-hr (volt-hours). All steps were completed at 20°C. After completion of isoelectric focusing, strips were then washed in Equilibration Buffers I and II (BioRad). The proteins were then separated in the second dimension using 10% polyacrylamide gels as described in

Chapter 3, Section 3.2.3. The proteins were stained in the gels using Coomassie brilliant blue R-250 (Fisher Scientific).

4.2.10 Western blot analysis

Protein mixtures were separated by one-dimensional and two-dimensional SDS-PAGE and transferred to PVDF membrane (Thermo Scientific) as described in **Chapter 3, Section 3.2.3**. Gels from two-dimensional SDS-PAGE were half transferred (18 V for 0.3 h, compared to 0.6 h for one-dimensional), and following transfer the remaining gel was stained using Coomassie brilliant blue R-250 to obtain a stained gel and Western blot membrane pair. Membranes were then blocked in PBS supplemented with 1% BSA prior to incubation with mouse serum (pooled from equal volume of serum from five mice colonised with either the TIGR4Δ*cps* or 6AΔ*cps* mutants or the 23F strain, or from naïve mice) at 1:1,000 dilution at RT overnight. Bound antibody was detected by anti-mouse secondary antibody conjugated to alkaline phosphatase (Sigma), and BCIP/ NBT (Fisher Scientific) development.

4.2.11 Mass spectroscopy

Spots identified by Western blot as cross-reactive were traced on the corresponding Coomassie stained gel, and proteins spots of interest were excised from the gel using a pipette tip. The protein within the gel plug was trypsin digested and injected onto a HPLC C18 column to separate the digested peptides. The separated peptides were sprayed into an LTQ ion-trap mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA). Mascot software (Mascot Software Technologies, Bloomington, IN) was used to search bacterial databases for sequence similarity. Cut-offs were assigned as a protein score of >70 with a unique peptide value of >2. This was completed by the Proteomics core at UPENN.

4.2.12 Measurement of serum antibody binding by flow cytometry

Aliqouts (100 μl) of mid-log-phase bacteria were pelleted and washed in Hanks buffer (Invitrogen) supplemented with 5% (v/v) foetal calf serum (HFCS) (Thermo Scientific). Cells were then incubated with antibody source (1% human sera, 10% pooled mouse serum or 1:500 TEPC15), diluted in HFCS, for 45 min at 37°C. Following washing in HFCS, the cells were incubated with FITC-conjugated secondary antibody against IgG

(Sigma) for 30 min on ice at a concentration of 1:100 in HFCS. After washing the cells were resuspended in 200 µl of 1% formaldehyde in dH_2O. Flow cytometric analysis was conducted on a FACSCalibur machine with CellQuest software (BD, Franklin Lakes, NJ). Bacteria were gated and 20,000 events were collected. The quantity of antibody bound to bacterial cells was calculated by measuring the geometric mean fluorescence intensity (MFI) of the strains incubated with antibody source minus the 'no antibody' control using FlowJo software (Tree Star, Ashland, OR).

4.2.13 Statistical analysis

Colonisation density was expressed as the $\log_{10}$ CFU/ml for calculation of means ± standard error of the mean. Statistical comparisons of survival and colonisation among groups were made by the non-parametric test indicated. Statistics were calculated using GraphPad Prism 4 (GraphPad).

4.3 Results

4.3.1 PspA, PpmA and PsaA identified as cross-reactive antigens

Since antibody, excluding IgA, is important for protection, the IgG responses from mice colonised with LAV strains were analysed to identify bacterial antigens inducing cross-reactive antibodies. To identify the targets of cross-reactive antibodies induced by carriage, mice were colonised with three strains (TIGR4Δ*cps*, 6AΔ*cps* or 23F) and the cross-reactive serum IgG response was analysed by Western blot. Whole-cell pneumococcal lysates were separated by SDS-PAGE and transferred to PVDF membranes that were then probed with sera from mice colonised with one of two heterologous strains to look at cross-reactive responses.

Pooled serum from naïve mice was probed against whole-cell lysates of the 6A and TIGR4 strains to confirm the absence of natural antibody against the pneumococcus (**Fig. 4.4A**). When the 6A lysate was probed with pooled serum from mice previously colonised with the TIGR4Δ*cps* mutant, three prominent areas of cross-reactive bands were seen (**Fig. 4.4B1**). A similar banding pattern was also seen with lysates from a type 2 strain (**Fig. 4.4B2**). Crude fractionation of the type 2 strain was performed by lysing cells with a French press system and separating the soluble and insoluble fractions by ultracentrifugation. Western blots of these fractions probed with pooled serum from mice previously colonised with the TIGR4Δ*cps* mutant indicated that the bands in area 2 were in the soluble or cytoplasmic fraction (**Fig. 4.4B2**). Pneumolysin is a well characterised pneumococcal virulence factor of approximately 50 kDa, which induces antibodies following natural infection (Rapola *et al.*, 2000; Simell *et al.*, 2001). However, analysis of two Δ*ply* mutants confirmed that the cross-reactive band in area 2 was not pneumolysin, which may be predominantly cytoplasmic (**Fig. 4.5**). This cytoplasmic antigen in area 2 was excluded from further analysis, since the focus of the present investigation was on cross-reactive surface antigens. The antigens in areas 1 and 3 were predominantly in the insoluble fraction and, therefore, likely to be surface exposed (**Fig. 4.4B2**). These antigens were also confirmed as proteins as prior treatment of lysates with proteinase K resulted in almost complete loss of cross-reactivity (**Fig. 4.6**). Therefore, the identification of these protein antigens was investigated further.

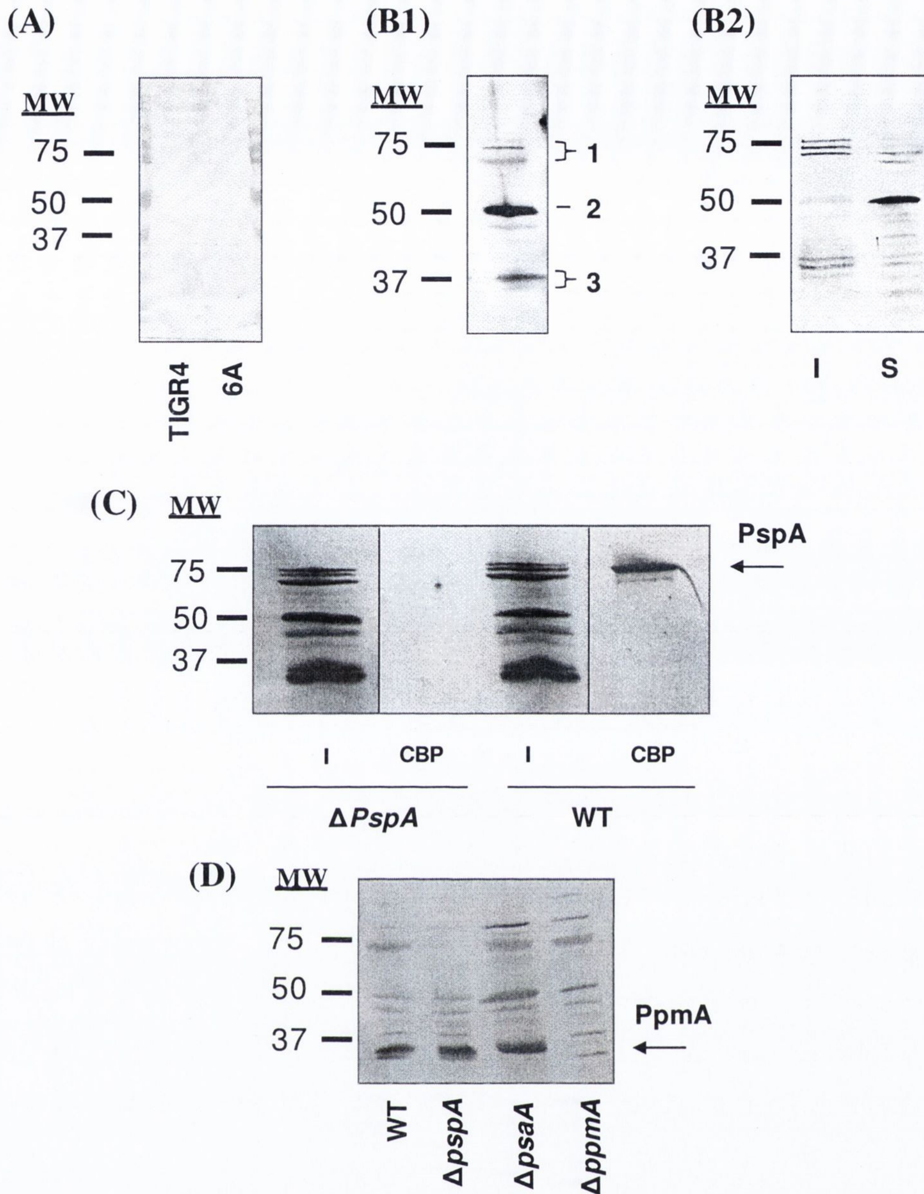


Fig. 4.4 Identification of cross-reactive antigens recognised by TIGR4Δ*cps*-induced IgG.

(A) Whole-cell lysates of strains TIGR4 and 6A were separated by SDS-PAGE and probed with pooled serum from naïve C57BL/6J mice. (B1) Whole-cell lysates of the 6A strain were separated by SDS-PAGE and probed with pooled serum from TIGR4Δ*cps*-colonised C57BL/6J mice. Three regions with cross-reactive bands were identified (1–3). (B2) Crude soluble and insoluble fractions from a type 2 strain were probed with pooled serum from TIGR4Δ*cps*-colonised mice to determine the subcellular location of the cross-reactive antigens (I, insoluble fraction; S, soluble fraction). (C) The insoluble fractions of the parent 6A strain and of its 6AΔ*pspA* mutant were washed with 2% choline prior to SDS-PAGE, to remove choline-binding proteins, and probed with pooled serum from TIGR4*Δcps*-colonised mice (I, insoluble fraction; CBP, choline-binding proteins removed in wash). (D) Whole-cell lysates of strain 6A and mutants probed with pooled serum from TIGR4*Δcps*-colonised mice. MW, molecular weight. Size markers in kDa.

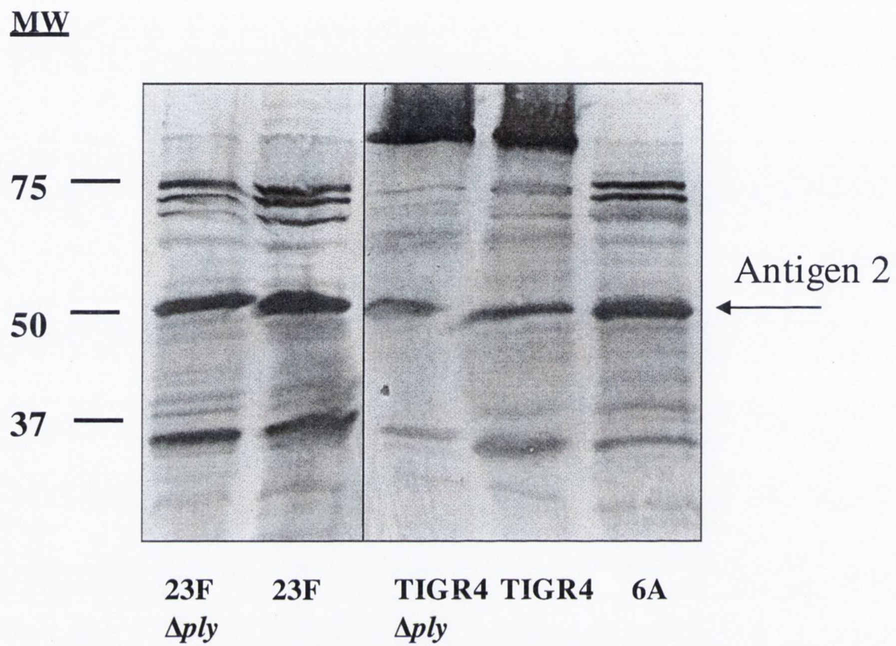
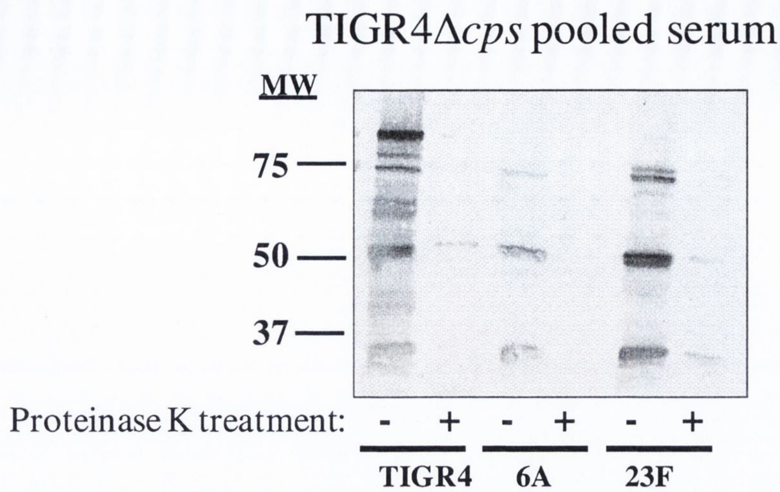


Fig. 4.5 Band in area 2 is not Pneumolysin.

Whole cell lysates of the strains indicated were separated by 10% SDS-PAGE gel and probed with pooled serum from C57BL/6J mice immunised with the TIGR4Δ*cps* mutant. The band in cross-reactive area 2 did not disappear in the absence of Ply, suggesting that Ply is not this band. MW, molecular weight. Size markers in kDa.



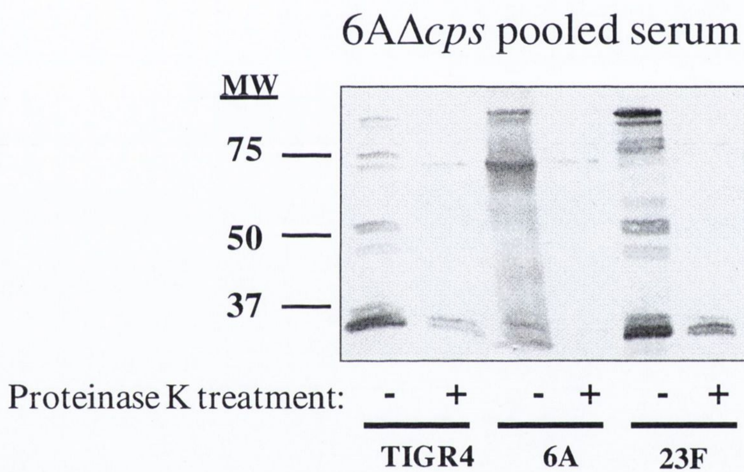


Fig. 4.6 Cross-reactive antigens confirmed as proteins.

Bacteria grown to mid-log phase was pelleted, washed, and incubated with either proteinase K (+) or PBS (–) for 1 h at 4°C. Resulting washed whole-cell lysates were probed with pooled serum from C57BL/6J mice immunised with either the TIGR4Δ*cps* or the 6AΔ*cps* mutant to confirm the loss of bands in the presence of proteinase K. MW, molecular weight. Size markers in kDa.

S. pneumoniae possesses a family of proteins on its surface that are non-covalently attached to choline residues on TA. To determine if pneumococcal CBPs were recognised by cross-reactive antibodies, insoluble fractions were washed with 2% choline to remove CBPs. The choline wash of strain 6A contained a single protein of ~75 kDa which reacted with pooled serum from mice colonised with the TIGR4Δ*cps* mutant (**Fig.4.4C**). Bands in area 1 were identified as PspA by the absence of this band in a 6AΔ*pspA* mutant.

2D electrophoresis and mass spectrometry were carried out to identify the predominant cross-reactive low molecular weight band (area 3) (**Fig. 4.7**). Two predominant low molecular weight spots were picked and peptide sequencing identified them both as a single protein, PpmA, which was confirmed by the loss of the band in area 3 in a 6AΔ*ppmA* mutant (**Fig. 4.4D**).

The serum IgM response was also analysed. Whole-cell lysates of the 6A strain were probed with pooled serum from TIGR4Δ*cps*-colonised mice, with anti-mouse IgM used as secondary antibody. The appearance of faint bands demonstrated that less IgM was induced compared to IgG. However, IgM also recognised PspA and PpmA, demonstrating that this response is similar to the serum IgG response (**Fig. 4.8**). The disappearance of an additional band of ~45kDa in the absence of PpmA may be due to effects of PpmA on folding of other proteins (Overweg *et al.*, 2000a).

When TIGR4 whole-cell lysates were probed with pooled serum from mice colonised with the 6AΔ*cps* mutant, a different pattern of cross-reactivity was noted, with only a single prominent band. This band contained a single protein which was identified by 2D electrophoresis and mass spectroscopy as PsaA (**Fig. 4.9**). This was confirmed by the loss of the band in a TIGR4Δ*psaA* mutant (**Fig. 4.10A**).

Mice colonised with a third isolate, 23F, induced cross-reactive antibodies that recognised PpmA in whole cell lysates of both strains TIGR4 and 6A. However, neither PspA nor PsaA were recognised (**Fig. 4.10B**). Therefore, PspA, PpmA and PsaA induce cross-reactive antibodies following murine colonisation. However, the cross-reactivity of each antigen varies depending on the colonising strain.

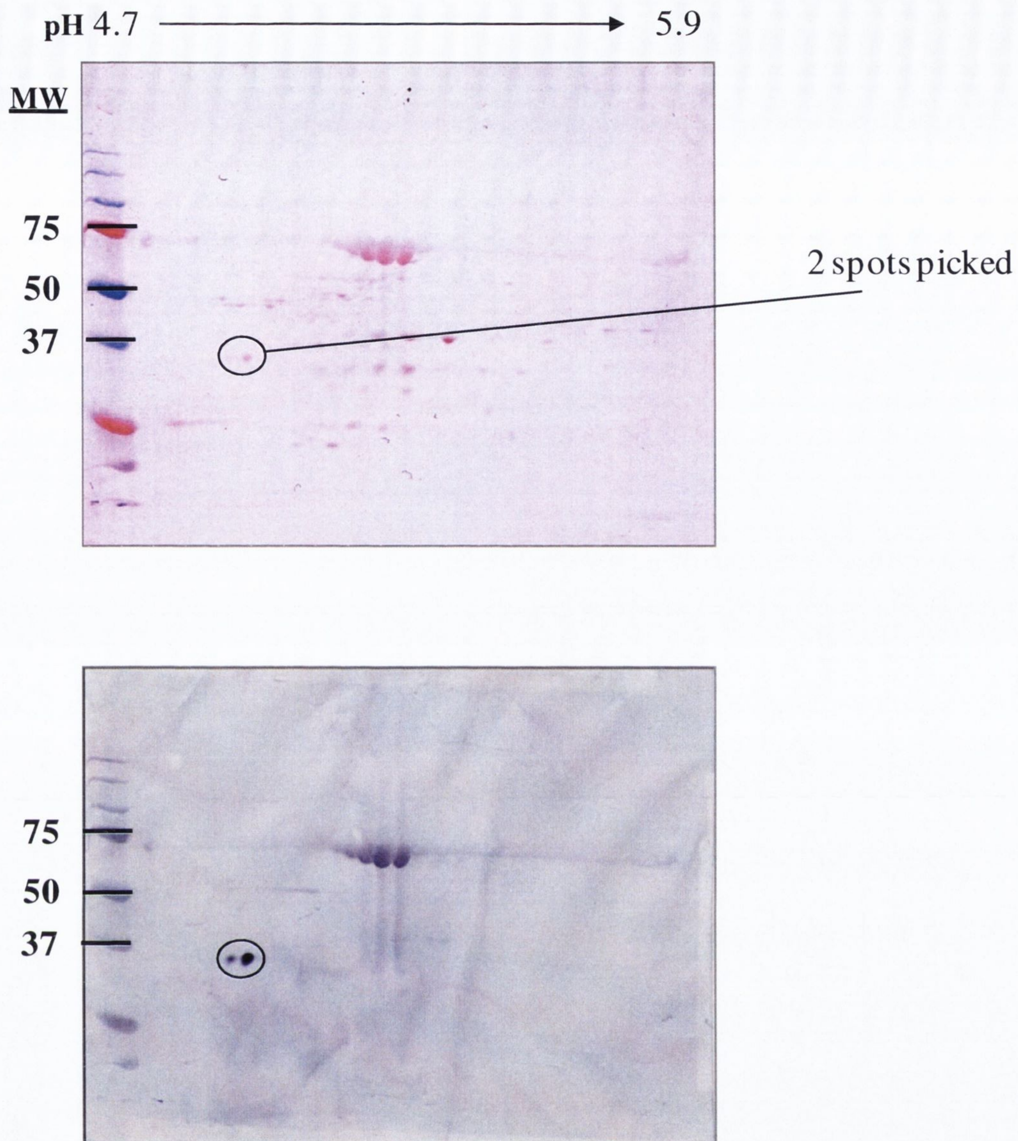


Fig. 4.7 PpmA identified as cross-reactive antigen.

The insoluble fraction of strain 6A was separated by 2D SDS-PAGE, and probed with pooled serum from C57BL/6J mice immunised with the TIGR4Δ*cps* mutant (bottom). The Ponceau S-stained membrane (top) illustrates the two spots that were picked for mass spectroscopy from the corresponding Coomassie stained gel. Both spots were identified as PpmA. MW, molecular weight. Size markers in kDa.

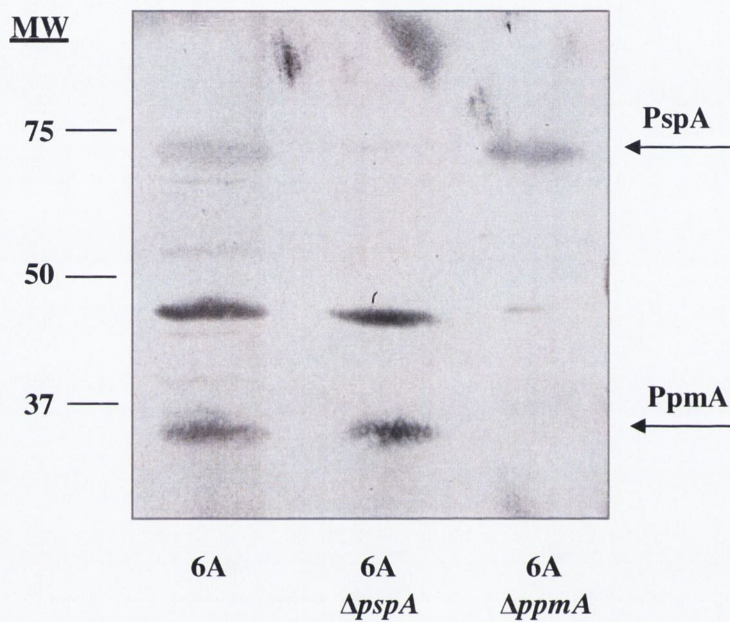


Fig. 4.8 Serum IgM response similar to IgG response in TIGR4Δ*cps*-immunised mice.

Whole-cell lysates were separated on a 10% SDS-PAGE gel, and probed with pooled serum from mice immunised with the TIGR4Δ*cps* mutant. Bound antibody was detected with anti-mouse IgM secondary antibody. The disappearance of bands in the absence of PspA and PpmA demonstrated that these antigens are the target of cross-reactive IgM as well as IgG. MW, molecular weight. Size markers in kDa.

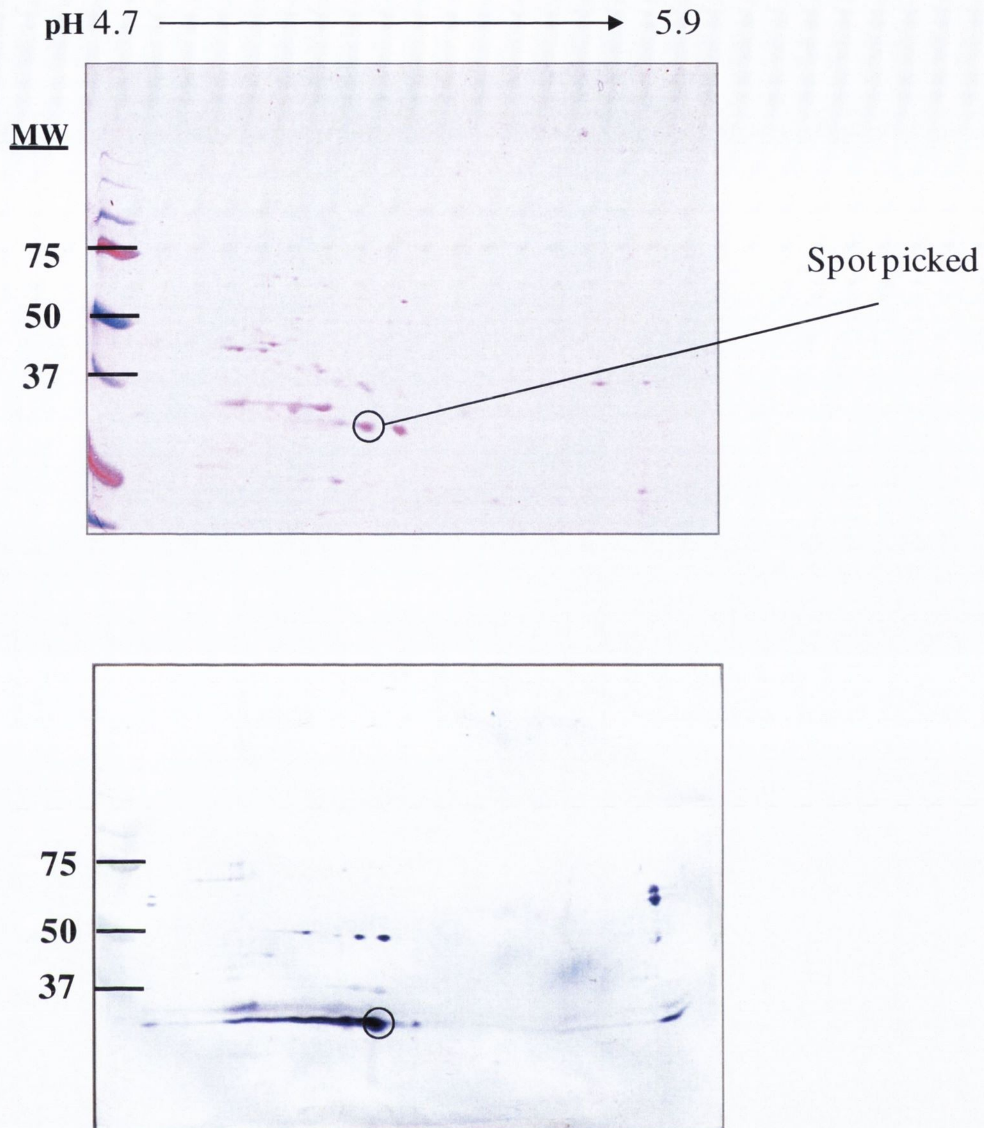


Fig. 4.9 PsaA identified as cross-reactive antigen.

The insoluble fraction of the TIGR4Δ*pspA* mutant was separated by 2D SDS-PAGE, and probed with pooled serum from mice immunised with the 6AΔ*cps* mutant (bottom). The Ponceau S-stained membrane (top) illustrates the spot that was picked for mass spectroscopy from the corresponding Coomassie stained gel. The spot was identified as PsaA. MW, molecular weight. Size markers in kDa.

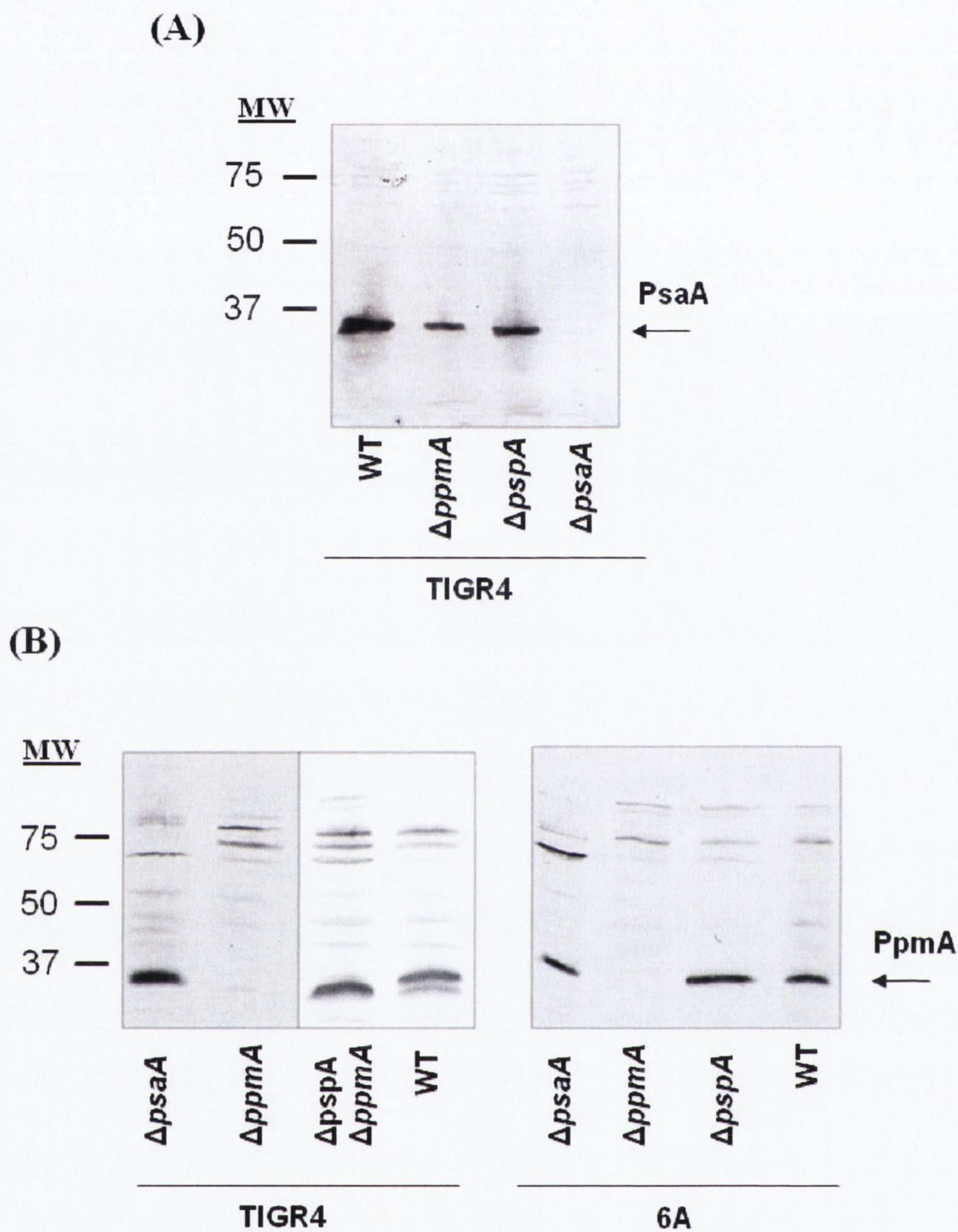


Fig. 4.10 Identification of cross-reactive antigens recognised by 6A- and 23F-induced IgG.

(A) Whole cell lysates from strain TIGR4 and its mutant derivatives probed with pooled serum from 6AΔ*cps*-colonised mice. (B) Whole cell lysates of both strains TIGR4 and 6A probed with pooled serum from 23F-colonised mice. MW, molecular weight. Size markers in kDa.

4.3.2 Carriage induces broadly cross-reactive PpmA and PsaA antibodies

PpmA and PsaA are both highly conserved among *S. pneumoniae* strains (Gor *et al.*, 2005). To determine the extent of cross-reactivity of induced antibodies, whole cell lysates from a range of diverse pneumococcal strains, including representatives of common serotypes, were separated by SDS-PAGE and probed with pooled serum from mice colonised with the TIGR4Δ*cps*, 6AΔ*cps* or 23F strains. A similar banding pattern of cross-reactivity was seen with pooled serum from TIGR4Δ*cps*-colonised mice. This serum recognised PpmA in 6/6 heterologous strains analysed (**Fig. 4.11A**).

Similarly, pooled serum from mice colonised with the 6AΔ*cps* mutant reacted with PsaA in 6/6 heterologous strains analysed (**Fig. 4.11B**). Pooled serum from mice colonised with the 23F mutant also reacted with PpmA in 6/6 heterologous strains analysed (**Fig. 4.11C**). Higher molecular weight cross-reactive bands of different sizes may be PspA, as this protein is highly variable in length between strains. With the pooled serum from TIGR4Δ*cps*-colonised mice the ~50 kDa cytoplasmic protein also appeared to be broadly cross-reactive in all strains tested. This confirmed that these antigens have the ability to generate broadly cross-reactive antibodies.

4.3.3 PspA is the dominant surface antigen recognised by immune sera

To determine the relative contribution of each antigen to the humoral immune response, flow cytometry was performed on wild-type strains and mutants lacking each of the three identified cross-reactive antigens (PspA, PpmA, and PsaA). Whole cell bacteria were incubated with sera pooled from five mice colonised with a heterologous strain to look at cross-reactive binding. The amount of cross-reactive surface antibody against each antigen was calculated as the difference in binding between wild type and mutants lacking the antigen.

IgG induced by colonisation of mutant TIGR4Δ*cps* recognised the wild-type 6A strain and this binding was greatly reduced in a 6AΔ*pspA* mutant (**Fig. 4.12A**). This suggests that PspA is responsible for the majority of the 6A cross-reactive surface antibody generated by TIGR4Δ*cps* colonisation. Loss of PpmA resulted in a minimal reduction in surface-bound antibody, suggesting that PpmA is the target for limited cross-reactive antibody to the

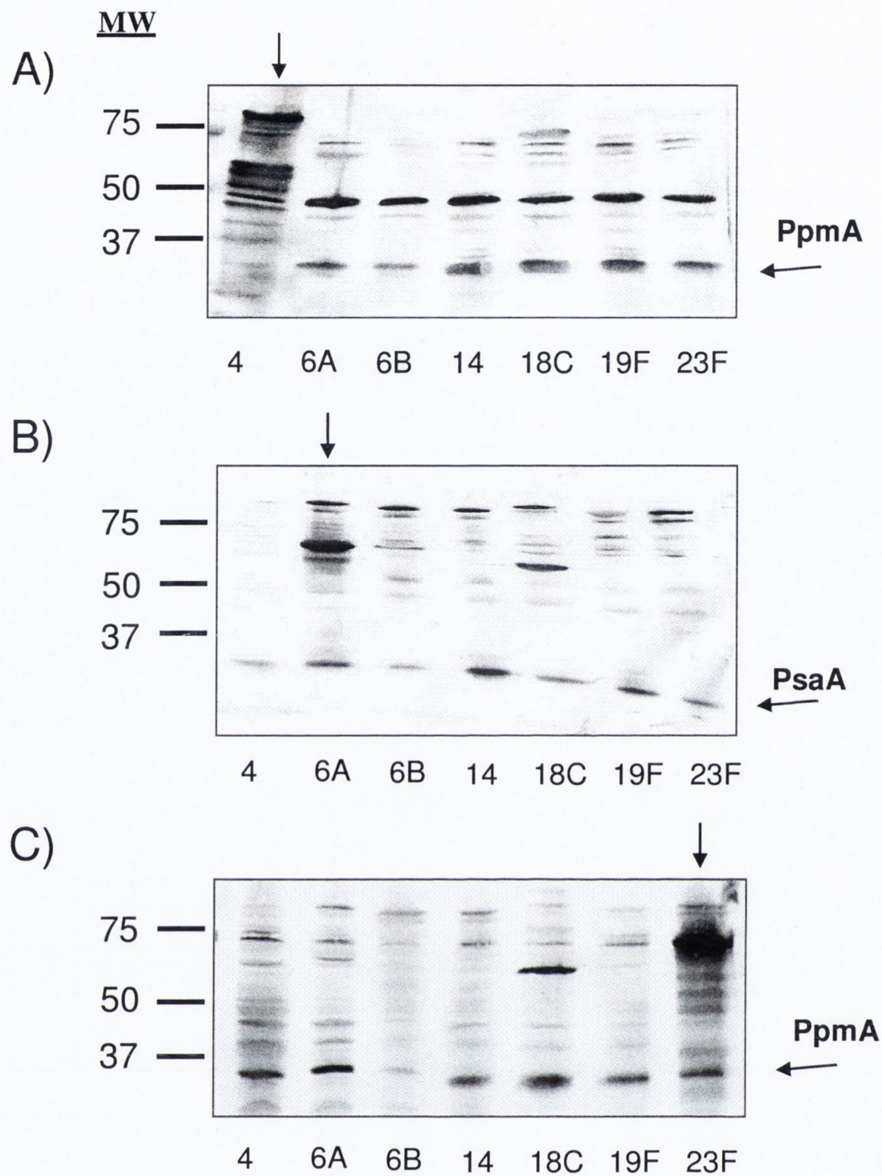


Fig. 4.11 Conservation of cross-reactive antigens.

Whole-cell lysates from seven strains, representing the common serotype indicated below, were separated by SDS-PAGE and probed with pooled serum from C57BL/6J mice colonised with strains (A) TIGR4Δ*cps*, (B) 6AΔ*cps* and (C) 23F. Location of PpmA and PsaA are indicated by arrows, and are based on controls using relevant mutants. MW, molecular weight. Size markers in kDa. Overhead arrows represent reactivity with the immunising strain.

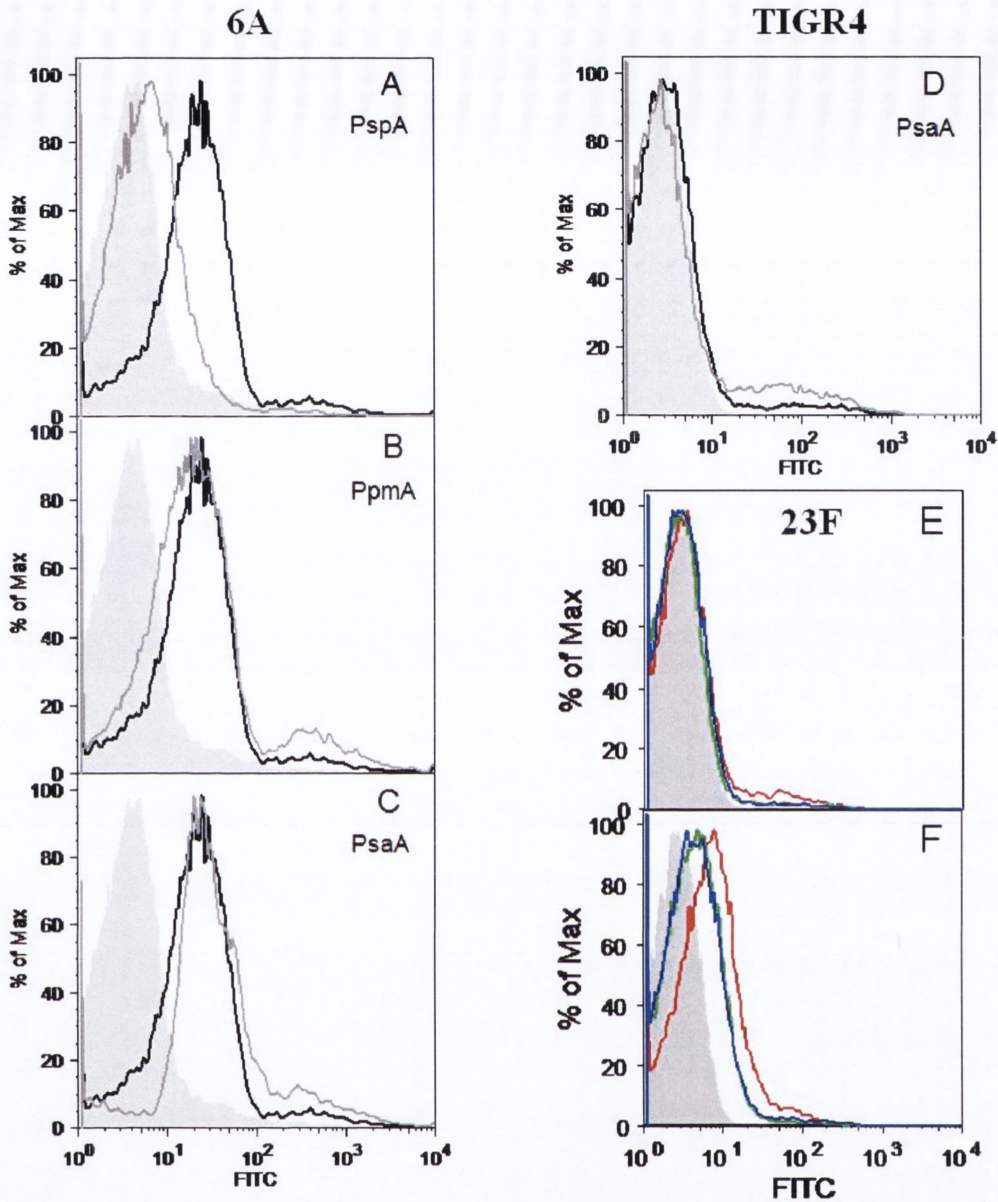


Fig. 4.12 Surface binding of cross-reactive antibody.

Representative histograms (from 2–3 independent experiments) of (A – C) serum IgG from TIGR4Δ*cps*-colonised C57BL/6J mice binding to 6A pneumococci and mutants. Black line represents binding to the wild-type 6A strain, and the grey line represents binding to mutants lacking (A) PspA, (B) PpmA or (C) PsaA. The shaded area represents binding of IgG from PBS mock-colonised mice. (D) Histogram of serum IgG from 6AΔ*cps*-colonised mice binding to TIGR4 pneumococci and its *psaA* mutant. The black line represents binding to wild-type TIGR4 and the grey line represents binding to a mutant lacking PsaA. (E & F) Histogram of serum IgG from 23F-colonised mice binding to strains TIGR4 (E) and 6A (F), as well as mutants in these backgrounds. Blue line = wild-type, green line = *ppmA* mutant, red line = *pspA* mutant.

bacterial surface (**Fig. 4.12B**). However, the loss of PsaA had no effect on surface bound antibody suggesting that PsaA does not contribute to cross-reactive surface antibody (**Fig. 4.12C**).

This was confirmed by the lack of surface-bound IgG when strain TIGR4 and its mutants were incubated with sera from 6AΔ*cps*-colonised mice (**Fig. 4.12D**), where PsaA was identified as the predominant cross-reactive antigen in Western blots. IgG induced by colonisation of strain 23F bound to the surface of strain 6A but not to the surface of strain TIGR4 (**Fig. 4.12F** and **4.12E**, respectively). However, no loss of binding was seen with the absence of PpmA or PspA, suggesting that neither of these antigens were responsible for this surface bound antibody. The increased binding of IgG of 23F-induced sera to mutant 6AΔ*pspA* will be discussed later (**Section 4.3.5**).

Therefore, PspA may induce a robust IgG response to the pneumococcal surface following colonisation, whereas the response to PpmA and PsaA is more limited. However, as suggested by Western blots, the cross-reactivity of each antigen varies depending on the colonising strain.

4.3.4 Normal human sera contains PpmA antibodies

Sera were taken from healthy adult volunteers and analysed to determine if prior exposure to *S. pneumoniae* induced antibodies against the identified cross-reactive antigens. Since there were many reactive bands in Western blots, flow cytometry was used to detect surface antibody to the three candidate antigens. The contribution of each antigen to the overall IgG response was measured by comparing binding to wild-type strains and looking for a loss in binding in mutants. For PpmA, in 11/18 cases (0/6 sera tested against strain TIGR4, 5/6 sera tested against strain 6A, and 6/6 sera tested against strain 23F), Δ*ppmA* mutants showed a significant decrease in human IgG binding compared to parental wild-type strain pairs in each serum sample, $p<0.05$ (**Fig. 4.13A**). This result was not due to differences in capsule expression between wild-type and *ppmA*-deficient strains, as assessed by flow cytometry comparing binding of monoclonal anti-capsular antibodies. However, as predicted based on the analysis of mouse sera, there was no difference in binding of human IgG in the absence of the *psaA* gene. These data suggests that most adult

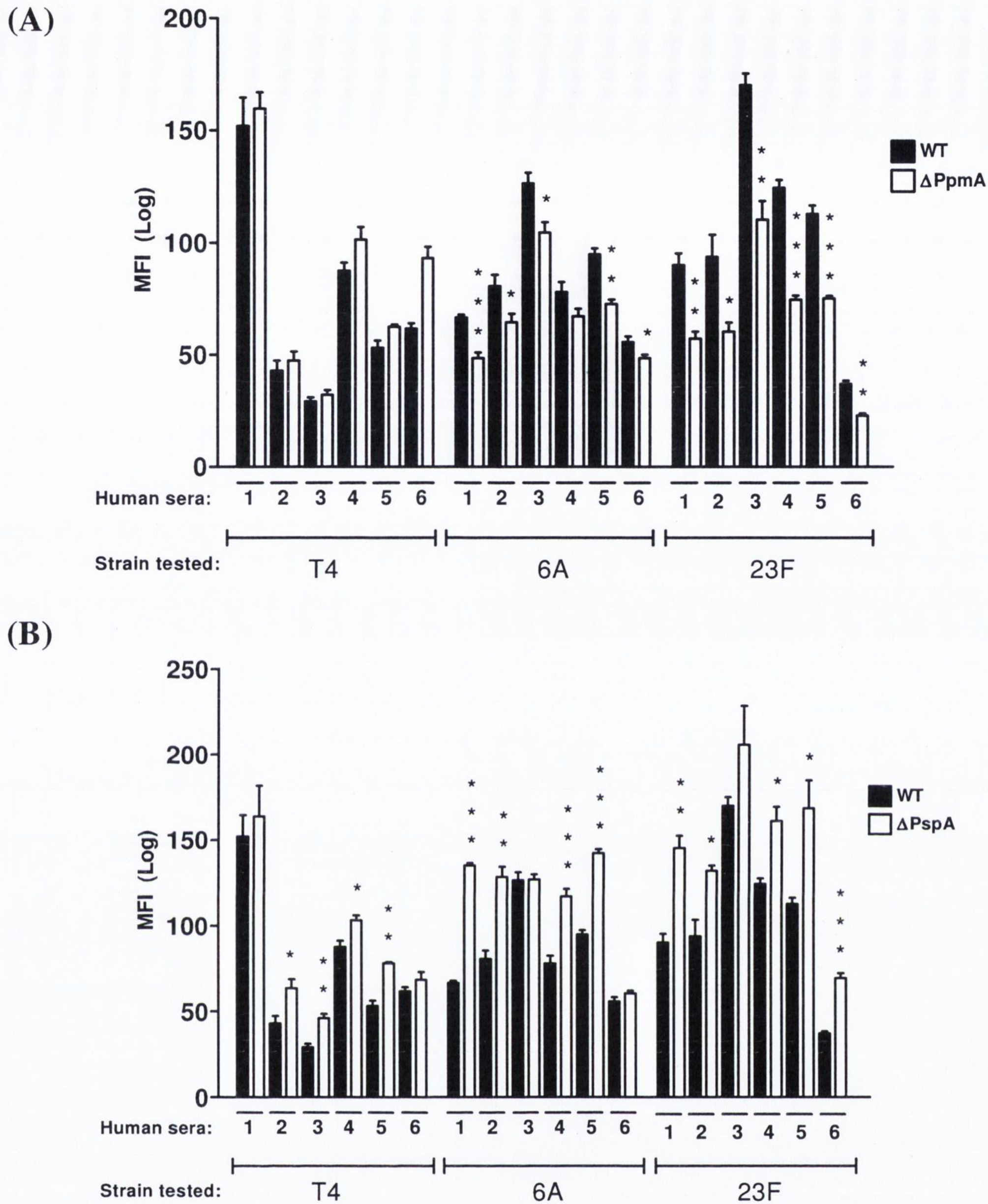


Fig. 4.13 Reactivity of normal human sera.

Whole cell bacteria, from three different strain backgrounds (strains TIGR4, 6A and 23F), were incubated with normal human sera from adult volunteers to compare IgG binding to wild-type strains and isogenic mutants lacking the *ppmA* (A) and *pspA* (B) genes. FITC-labelled bound IgG was detected by flow cytometry and 20,000 bacteria gated events were analysed. Each bar represents the mean fluorescence intensity (MFI) of binding of human IgG to the wild-type and mutant strains of the type indicated. MFI is the intensity of fluorescence which correlates to the quantity of fluorophores bound to the cell. Values are the mean of three independent experiments ± SEM. Statistics were determined by Students *t*-test. *P<0.049, **P<0.0065, ***P<0.0008.

human sera contains natural antibodies recognising surface-exposed epitopes of PpmA, with results varying depending on the target strain tested.

4.3.5 PspA inhibits antibody binding to underlying structures

While analysing human sera to determine if prior exposure to *S. pneumoniae* induced natural antibodies against cross-reactive antigens, it was noted that strains lacking PspA resulted in a significant increase in human IgG binding to whole cell bacteria by flow cytometry (**Fig. 4.12F**, **Fig. 4.13B**, and **Fig. 4.14A**). For PspA, in 13/18 cases (4/6 sera tested against strain TIGR4, 4/6 sera tested against strain 6A, and 5/6 sera tested against strain 23F), Δ*pspA* mutants showed a significant increase in human IgG binding compared to parental wild-type strains, $p<0.05$. Strains lacking PspA also had increased binding of human IgM (**Fig. 4.14B**).

Since PspA binds to phosphorylcholine (ChoP) residues on the surface of *S. pneumoniae*, and natural antibodies against ChoP are common in both mice and humans, whether or not PspA was inhibiting binding of antibodies against ChoP was investigated. Binding of TEPC-15, murine IgA antibodies directed against ChoP residues on TA, were increased in the absence of PspA (**Fig. 4.14A** and **4.14C**). Furthermore, binding of IgM, but not IgG, in BALB/c pooled normal mouse serum (naïve) was also increased in strains lacking PspA (**Fig. 4.15**). Pooled BALB/c mouse serum was used as these mice are known to have higher titres of natural antibodies against phosphorylcholine compared to C57BL/6J mice (Lieberman *et al.*, 1974). The increased binding of naïve mouse IgM but not IgG to the pneumococcus is not surprising, as natural antibody in mouse sera is mostly of the IgM isotype (Avrameas, 1991).

These data suggests that PspA may function to inhibit binding of natural antibodies against ChoP, with results varying depending on the strain background.

4.3.6 PspA, PpmA and PsaA are not required for cross-protection

The *in vivo* role of PspA and PpmA in cross-protection was investigated by colonising mice with mutants lacking these antigens in the TIGR4Δ*cps* background, and looking at mucosal cross-protection against strain 6A, since these two antigens were identified as cross-reactive using sera from TIGR4Δ*cps*-colonised mice. First, the effects of deletions of

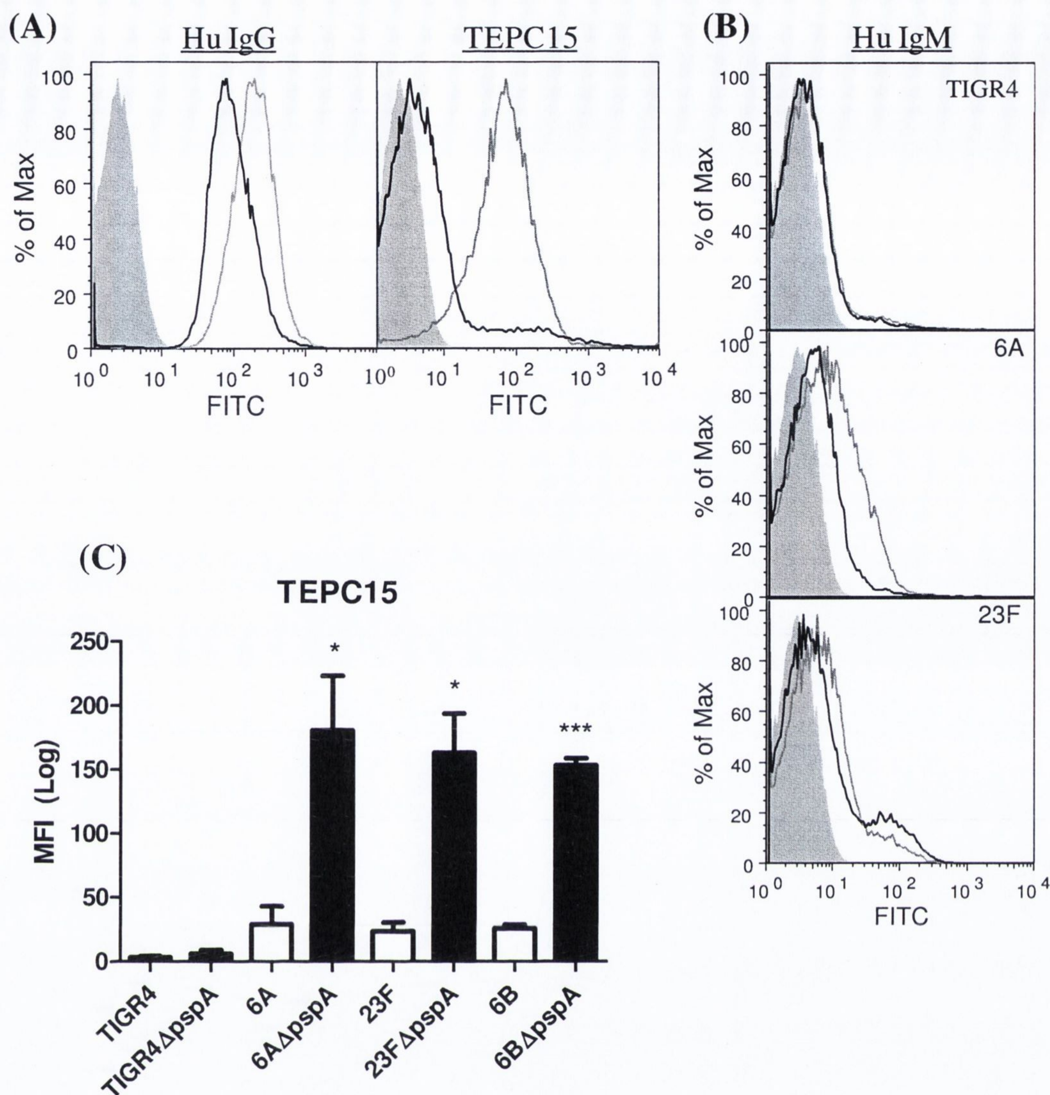


Fig. 4.14 PspA inhibits antibodies binding to the surface.

(A) Representative histograms of antibody bound to whole-cell bacteria by flow cytometry. Binding of TEPC-15 anti-ChoP IgA antibody, and binding of IgG in normal human sera to whole-cell wild-type and PspA-deficient pneumococci. Shaded line is the no antibody control, black line is wild-type 6A strain, and grey line is the mutant 6AΔ*pspA*. (B) Representative histograms of human IgM bound to whole-cell bacteria indicated. Shaded line is the no antibody control, black line is wild type, and grey line is Δ*pspA* mutant of the indicated strain. (C) Combined flow cytometry data demonstrating the significant increase in TEPC15 antibody binding to phosphorylcholine residues on TA in the absence of PspA. FITC-labelled bound antibody was detected by flow cytometry and 20,000 bacteria gated events were analysed. Each bar represents the log mean fluorescence intensity (MFI) of TEPC15 antibody binding to whole-cell pneumococci. MFI is the intensity of fluorescence, which correlates to the quantity of fluorophores bound to the cell. Values are the mean of 2–4 independent experiments ± SEM. Statistics were determined by Student's *t*-test. * $P<0.0472$, *** $P<0.0001$.

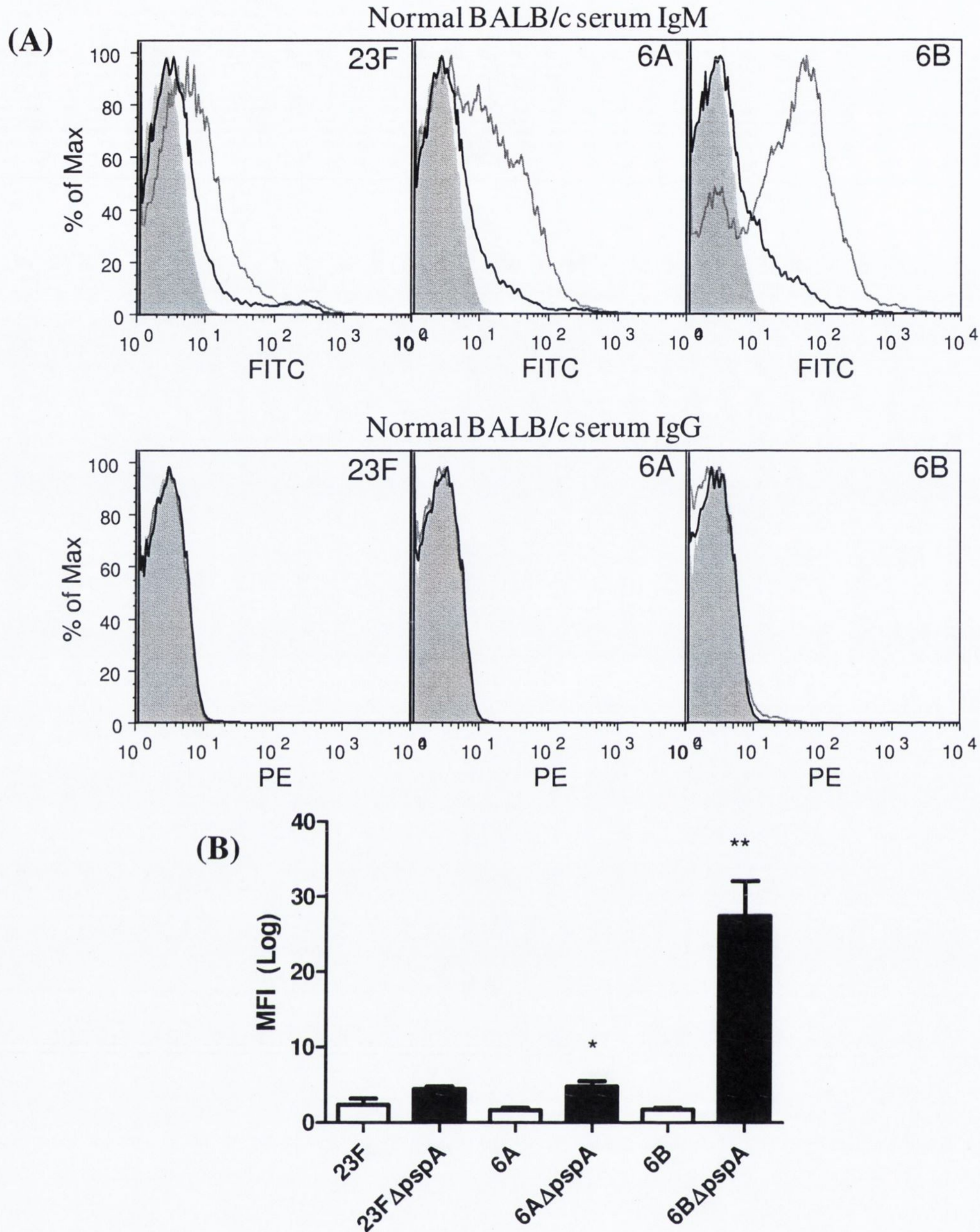


Fig. 4.15 Reactivity of normal mouse sera.

(A) Representative histograms of IgM and IgG in normal BALB/c mouse pooled serum binding to whole-cell wild-type strains and isogenic mutants lacking *pspA*. FITC-labelled bound IgM, and PE-labelled bound IgG, were detected by flow cytometry and 20,000 bacteria gated events were analysed. Shaded line is no antibody control, black line is wild-type strain and grey line is the Δ*pspA* mutant of the indicated strain. (B) Combined flow cytometry data of normal mouse IgM binding to the surface of the strains indicated. Each bar represents the mean fluorescence intensity (MFI) of binding. MFI is the intensity of fluorescence which correlates to the quantity of fluorophores bound to the cell. Values are the mean of three independent experiments ± SEM. Statistics were determined by Student's *t*-test. * P=0.0184, ** P=0.0052.

these antigens on colonisation were determined, since protection would be enhanced by persistence on the mucosal surface. The Δ*pspA* and Δ*ppmA* mutants colonised as well as the wild-type strains at 3 d (**Fig. 4.16A**), suggesting they would be effective at inducing protective immunity. Despite the robust immune response elicited against PspA and the broad cross-reactivity of PpmA, neither was necessary for cross-protection. Mice were still protected from strain 6A colonisation (**Fig. 4.18**) and sepsis (**Fig. 4.17**) in the absence of PspA. Additionally, there was still protection from colonization after immunisation with strain TIGR4 lacking both PspA and PpmA (**Fig. 4.18**).

Since PsaA was identified as cross-reactive with pooled serum from mice colonised with mutant 6AΔ*cps*, the role of PsaA was investigated by colonising mice with the 6AΔ*cps*Δ*psaA* mutant and looking at mucosal cross-protection against strain TIGR4. Again, the effect of deletions of the *psaA* gene on colonisation was determined. The Δ*psaA* mutants were highly attenuated in their ability to colonise at 3 d (**Fig. 4.16A**), suggesting they may not be effective at inducing protective immunity. Nevertheless, mice were colonised with the 6AΔ*cps* and 6AΔ*cps*Δ*psaA* mutants, and challenged with the TIGR4 strain. Analysis of the serum IgG from the immunised mice demonstrated that the 6AΔ*cps*Δ*psaA* mutant induced significantly less IgG against whole-cell 6A and TIGR4, compared to the 6AΔ*cps* mutant (**Fig. 4.16B**). The results indicated that PsaA was not a requirement for cross-protection as mice immunised with a Δ*psaA* mutant were still protected from colonisation with the TIGR4 strain (**Fig. 4.18**). This also demonstrated that despite poor colonisation and reduced antibody response, the 6AΔ*cps*Δ*psaA* mutant was able to induce sufficient protective immunity.

These data suggest that the three major cross-reactive antigens may contribute to, but are not necessary for, mucosal cross-protection.

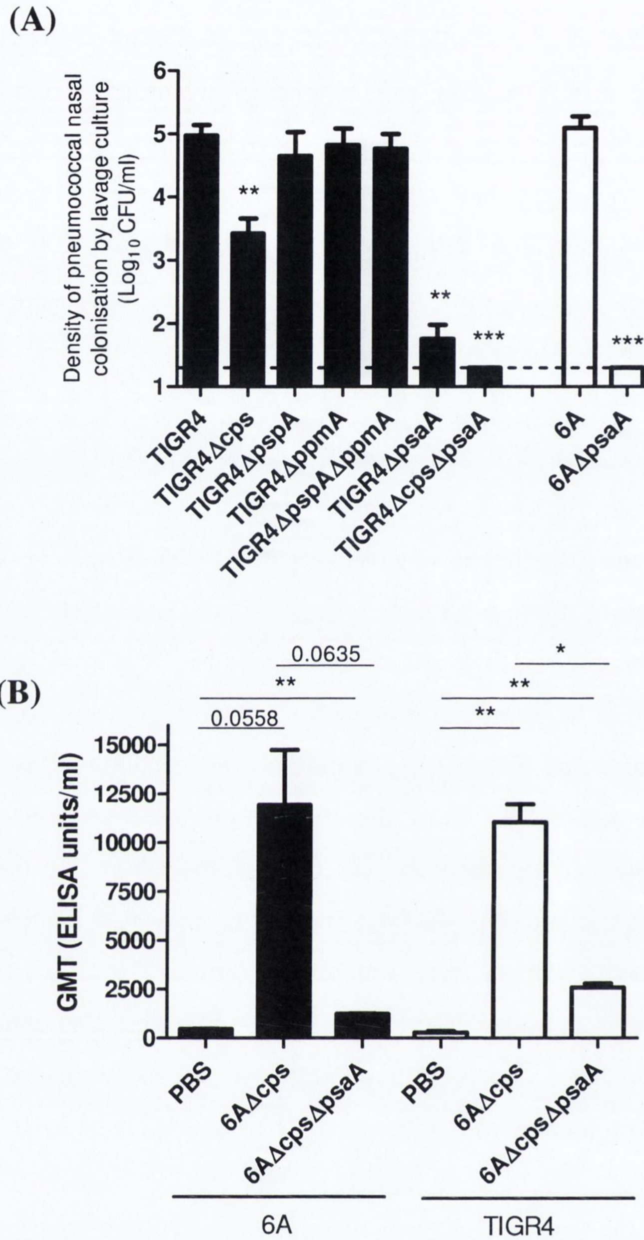


Fig. 4.16 Characterisation of strains lacking cross-reactive antigens.

(A) Colonisation data of strains lacking cross-reactive antigens at 3 d, following IN inoculation of C57BL/6J mice with 10^7 CFU of the strain indicated. Statistical difference, compared to wild-type, was determined by Mann-Whitney test. ** P<0.0014, *** P<0.0002. (B) IgG titres induced against whole-cell of strain 6A (black bars) and strain TIGR4 (white bars) following IN immunisation of C57BL/6J mice with 10^7 CFU of the indicated live-attenuated vaccine strain. GMT is calculated as the dilution at which the OD_{415} of the ELISA equals an arbitrary value of 0.1. Statistics were determined by Student's t-test. * P=0.0121, ** P<0.0085.

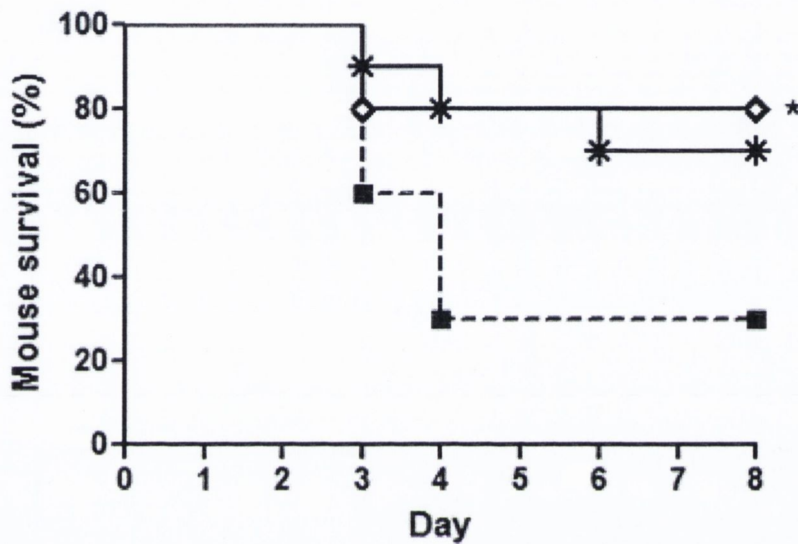


Fig. 4.17 ***In vivo*** **role of PspA in systemic cross-protection.**

Survival rates of immunised mice following virulent challenge. C57BL/6J mice were immunised by colonisation with two doses of 10^7 CFU of PBS (mock) (■), the TIGR4 strain (◊) or the TIGR4Δ*pspA* mutant (∗), prior to challenge 5 weeks later with 10^7 CFU of the virulent strain 6A. n = 10 mice per group. Statistical differences, compared to mock-immunised mice, was determined by Kaplan-Meier log-rank test. * P=0.0389.

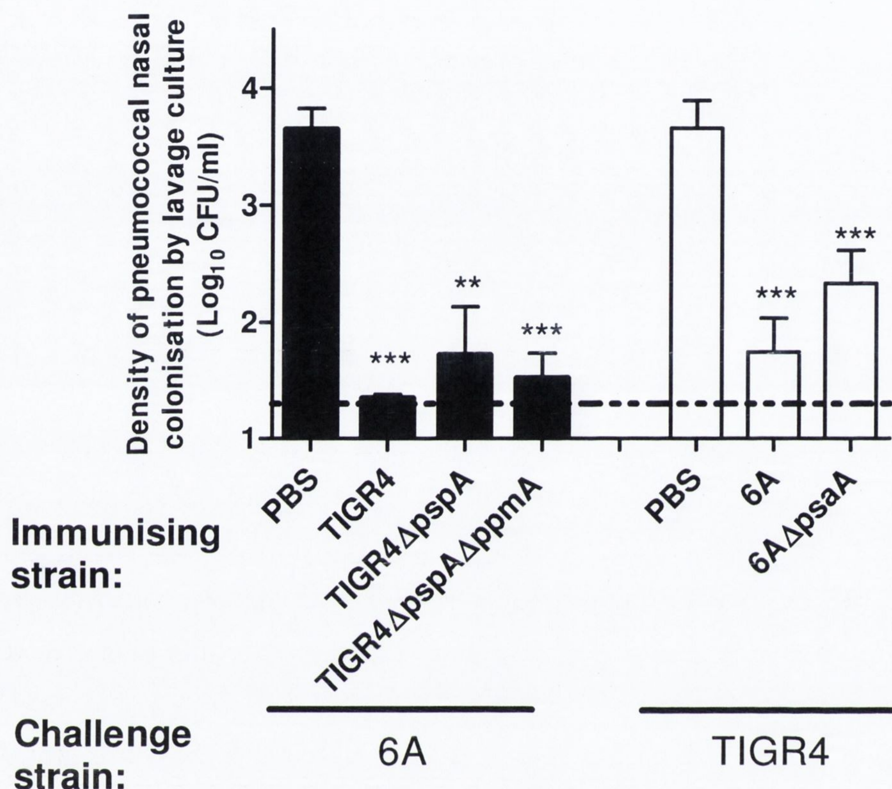


Fig. 4.18 ***In vivo*** **role of PspA, PpmA and PsaA in mucosal cross-protection.**

C57BL/6J mice were mock-immunised with PBS or by colonisation with two doses of 10^7 CFU of the TIGR4 strain or mutants lacking the *pspA* gene alone or in combination with the *ppmA* gene prior to challenge with 10^7 CFU IN of virulent strain 6A. Alternatively, mice were immunised by colonisation with strain 6A or a 6A mutant lacking the *psaA* gene prior to challenge with strain TIGR4. Colonisation density of challenge strain was assessed at 8 d post challenge. n > 8 mice per group. Results are displayed as means ± SEM. The dotted line indicates the lower limit of detection. Statistical differences comparing to PBS mock-immunised mice were determined by the Mann-Whitney test. ** P= 0.006, *** P< 0.0006.

4.4 Discussion

The goal of this study was to identify pneumococcal surface antigens eliciting a cross-reactive and cross-protective antibody response during colonisation. Experiments analysing antibody generated by murine colonisation by three different pneumococcal isolates revealed several prominent cross-reactive antigens. However, no single surface antigen was identified as a broad target of cross-reactive antibody among all strains tested. Therefore, attention was focused on the most prominent cross-reactive antigens recognised in Western blots. Three out of four prominent bands recognised by cross-reactive antibodies were found to be surface-associated proteins. Each of these three proteins, PspA, PpmA and PsaA, has been previously proposed as a vaccine candidate or shown to induce protective responses when given as a purified protein together with an adjuvant (Darrieux *et al.*, 2007; Briles *et al.*, 2000a; Overweg *et al.*, 2000a).

The present results provide confirmation of the potential of each of these antigens to induce a cross reactive immune response. However, following immunisation with mutants lacking the relevant cross-reactive antigen(s), there was no significant decrease in cross-protection as determined by colonisation density. This suggests unknown minor antigens, which generate weaker cross-reactive responses, may mediate cross-protection and were not identified in the present approach using Western analysis. Alternatively, there may be redundancy of cross-protective antigens which the current mouse model cannot account for. It is also possible that the serum IgG response is not representative of humoral protection at the mucosal surface, yet may be more important for preventing invasive infection. However, systemic immunisation with the PCV7 vaccine is known to induce IgG, which leaks into the nasal mucosa (Nurkka *et al.*, 2001) and protects against carriage.

In this regard, herein the investigations focused on IgG since data in **Chapter 3** supported a role of antibody other than IgA in mucosal and systemic protection. $CD4^+$ T-cells also played a role in protection induced by colonisation. The requirement for $CD4^+$ T-cells could be because of their contribution to humoral immunity. However, the possibility that these, or other, antigens may be important for $CD4^+$ T-cell-mediated, but not antibody-mediated cross-protection cannot be ruled out, as previously demonstrated (Malley *et al.*, 2005). Another possibily is that both arms of immunity are required, as a recent study demonstrated a role for both antibody- and cell-mediated immunity in protection induced

by prior colonisation of the pneumococcus (Richards *et al.*, 2009). A further consideration was that levels of antibody induced by carriage might be much lower than those generated in response to the PCV7 vaccine and, thus, might provide only limited cross-protection and require the combined effects of multiple antigens.

PspA was identified as one of the dominant targets of cross-reactive antibody induced by carriage. PspA, an abundant surface protein, is the most well-studied and characterised pneumococcal protein vaccine candidate. When given systemically or mucosally as a purified antigen it can protect against both invasive disease and colonisation in mouse models (Swiatlo *et al.*, 2003; Oma *et al.*, 2009; Briles *et al.*, 2003). PspA is also protective when expressed by recombinant attenuated *Salmonella* vaccines (Nayak *et al.*, 1998; Li *et al.*, 2009). PspA has a highly variable α-helical N-terminus with sequence similarities in other regions that allow for its classification into three families, and six clades. Although there is thought to be a higher degree of protection elicited within the same family, protection has been seen between families (McDaniel *et al.*, 1991; Briles *et al.*, 2000b; McDaniel *et al.*, 1998). In the present study, colonisation by strain TIGR4 elicited antibodies against PspA that cross-reacted with PspA from strain 6A. The PspA proteins expressed by these two isolates have been sequenced and belong to different families, confirming that carriage may induce cross-reactivity between families. However, sera from mice colonised with strain 6A did not show prominent cross-reactivity with PspA of strain TIGR4, suggesting that cross-reactivity between families is variable. Moreover, PspA was not found to be responsible for inducing high levels of cross-reactive antibody following colonisation by another isolate (23F) of the same PspA family as strain 6A.

Cross-reactive antibody to PspA in normal human serum, was not detected. However, the inhibitory effect of PspA on antibody binding may obscure the results. The presence of anti-PspA antibodies in human sera may not be accurately assessed using flow cytometry to compare total IgG binding to parental and Δ*pspA* strains. Indeed, a recent study confirmed that human sera contain anti-PspA antibodies, which negatively correlate with susceptibility to carriage (McCool *et al.*, 2002). Another group demonstrated that levels of anti-PspA IgG increase following colonisation in an outbred mouse model (Richards *et al.*, 2009). This increase in antibodies correlated with protection from colonisation and pneumonia induced by prior colonisation, confirming that PspA is a dominant target of the host response to colonisation.

The approach of the present study to identify cross-reactive antigens by Western analysis was limited to antibodies detecting denatured proteins, and would not identify conformational epitopes. However, the majority of sera induced by colonisation with mutant TIGR4Δ*cps* recognised PspA on the bacterial surface, suggesting that this approach accounted for the major targets of humoral immunity.

Another prominent target of cross-reactive IgG induced by colonisation is PpmA. PpmA is a highly conserved lipoprotein studied for its potential as a vaccine candidate, and is thought to be involved in the secretion and activation of cell-surface molecules (Overweg *et al.*, 2000a). Although the ability of PpmA to induce cross-protection has not been addressed in prior studies, antibodies generated against PpmA are cross-reactive against multiple strains of different serotypes as seen in the present study and others (Overweg *et al.*, 2000a). Serum antibodies against PpmA are detected early in life and correlate with carriage. Antibodies to PpmA are lower in children with *S. pneumoniae* found in the middle ear (Vainio *et al.*, 2006) than in colonised controls, suggesting that the immune response to PpmA may be protective. Herein, significant levels of anti-PpmA antibodies were detected in all adult normal human serum samples tested, although the level of surface-reactive anti-PpmA antibodies appeared to vary depending on the strain tested. This variation could be due to differential surface exposure of this antigen, or to other interfering components among strains. Whereas PspA has been confirmed to be surface-exposed on encapsulated *S. pneumoniae* (Daniels *et al.*, 2006), it is not as clear whether PpmA and PsaA are masked by the capsule (Overweg *et al.*, 2000a; Gor *et al.*, 2005). These data suggest that the surface accessibility of PpmA is strain-specific.

A third prominent target of cross-reactive IgG induced by colonisation is PsaA. PsaA is a highly conserved lipoprotein that is part of an ABC transporter involved in transport of manganese into the cell (Dintilhac *et al.*, 1997). PsaA is protective against carriage when given as a purified protein fused to cholera toxin (Pimenta *et al.*, 2006). Western blotting identified PsaA as the major cross-reactive antigen in sera from mice colonised with strain 6A. As reported in other studies, surface reactivity of antibody to PsaA following murine colonisation or in normal human serum was not detected (Gor *et al.*, 2005). PsaA is not required for mucosal cross-protection in the currently used model as mice are still protected in the absence of this antigen during immunisation. However, it is worth noting

that strains lacking PsaA colonise poorly compared to parental strains, and as a result, mice immunised with the 6AΔ*cps*Δ*psaA* mutant induced less total anti-6A specific antibody, as well as less anti-TIGR4 cross-reactive antibody (**Fig. 4.16B**). This may account for the partial protection following immunisation by mutant 6AΔ*cps*Δ*psaA* compared to the wild-type.

Many respiratory pathogens decorate their surface with ChoP, as this can aid in adherence to, and invasion of, host epithelial cells via the PAF receptor. However, ChoP is also a major target of the host immune response, via natural antibodies against ChoP and the acute phase reactant CRP (Mold *et al.*, 1981; Goldenberg *et al.*, 2004; Briles *et al.*, 1981). Therefore, many of these bacteria undergo phase variation of the expression of choline in order to hide this antigen when an immune response is mounted. However, the pneumococcus requires choline for TA biosynthesis and therefore cannot turn off expression of this structure, leaving it at a disadvantage compared to its competitors. This study demonstrated that strains lacking PspA exhibit increased binding of human IgG, murine IgM and TEPC-15 against ChoP. This has led to the hypothesis that *S. pneumoniae* binds proteins to ChoP on its surface as a novel mechanism of masking ChoP from host recognition. A significant effect is seen in the absence of PspA alone as this is the most abundant choline-binding protein expressed.

The pneumococcus has evolved many mechanisms to evade the host immune system and persist on the nasal mucosa and invasive sites. This includes evasion of humoral immunity, which is known to be crucial for the protection induced by current pneumococcal conjugate vaccines. For example, *S. pneumoniae* produces an IgA protease which cleaves and inactivates human IgA1, and also expresses PspC which binds to secretory component and prevents recognition of bound antibodies by phagocytes.

Opsonophagocytic killing is also presumed to be a crucial factor in controlling the pneumococcus. Therefore, opsonisation is also important. Accordingly, the pneumococcus has developed ways to prevent complement deposition via PspC, Ply and PspA. The mechanism of how PspA interferes with C3 deposition is still unclear, but may be through blocking of the binding of antibodies, and other opsonins, to underlying conserved structures (such as ChoP) which mediate this interference. Further research would be needed to confirm this hypothesis.

In summary, the vaccine potential of colonisation was analysed to understand how carriage may be an immunising event, with the potential benefit of identifying proteins that elicit a protective immune response as candidates for a component vaccine. Previous reports have demonstrated that these cross-reactive antigens are sufficient, yet this murine colonisation model shows they are not necessary for mucosal cross protection. These data suggest that a protein-based vaccine that targets carriage may have to include multiple components to generate broad mucosal protection against this highly heterogeneous pathogen. These findings also confirm the effect of live vaccination in protection from colonisation, with significant cross-protection achieved even in the absence of candidate vaccine targets. Broad protection is also an advantage of the killed whole cell vaccination approach (Malley *et al.*, 2001). These vaccination methods have the advantage of the exposure of multiple potential cross-protective antigens to the immune system, and may therefore overcome the limitations of narrow range component vaccines.

Chapter 5

General Discussion

5.1. Pneumococcal colonisation

Colonisation is the first step in pneumococcal pathogenesis and in the majority of cases results in asymptomatic carriage of the nasopharynx. Colonisation is eventually cleared, in days to months, without the knowledge of the unsuspecting host. Therefore, this host–pathogen interaction is an important element of *S. pneumoniae* pathogenesis to study and understand. This doctoral research project set out to determine factors involved in pneumococcal colonisation and also to study the effects of colonisation as a protective event.

One of the major virulence factors contributing to pneumococcal disease burden is the polysaccharide capsule which encases the bacteria (Preston & Dockrell, 2008). Many respiratory bacteria express polysaccharide capsules, which function as antiphagocytic shells in the event of host attack (Lindberg, 1999). However, like many opportunistic pathogens that colonise the respiratory tract, *S. pneumoniae* resides on the mucosal surface in a commensal state with the host where the anti-phagocytic role of the capsule may be minimal. Indeed, nasopharyngeal colonisation is also the mode of transmission of the pneumococcus, with invasive disease a dead end for the bacterium. Furthermore, capsular polysaccharide is the major target of the host immune response against the pneumococcus (Weiser, 2010), and capsule production expends a lot of energy (García & López, 1997). Therefore, if the pneumococcus spends most of its time colonising the nasopharynx, why/how would the main virulence factor of the pneumococcus be evolutionarily selected for if its main function was protection in an invasive setting? This suggested that there was another function that the capsule was selected for which enhanced colonisation, the carrier state.

Figure 5.1 illustrates the current model of pneumococcal colonisation, incorporating the results from this thesis. On arrival into the host nasopharynx the pneumococcus encounters several innate defence mechanisms. How the pneumococcus deals with these innate mechanisms is discussed in more detail elsewhere (see **Chapter 1**, **Sections 1.5** and **1.6**). These include, but are not limited to, antimicrobial peptides, antigen-presenting cells and mucus. Mucus serves to trap incoming foreign particles and remove them by the mechanical beating of the cilia on epithelial cells (Cone, 2009; Antunes & Cohen, 2007).

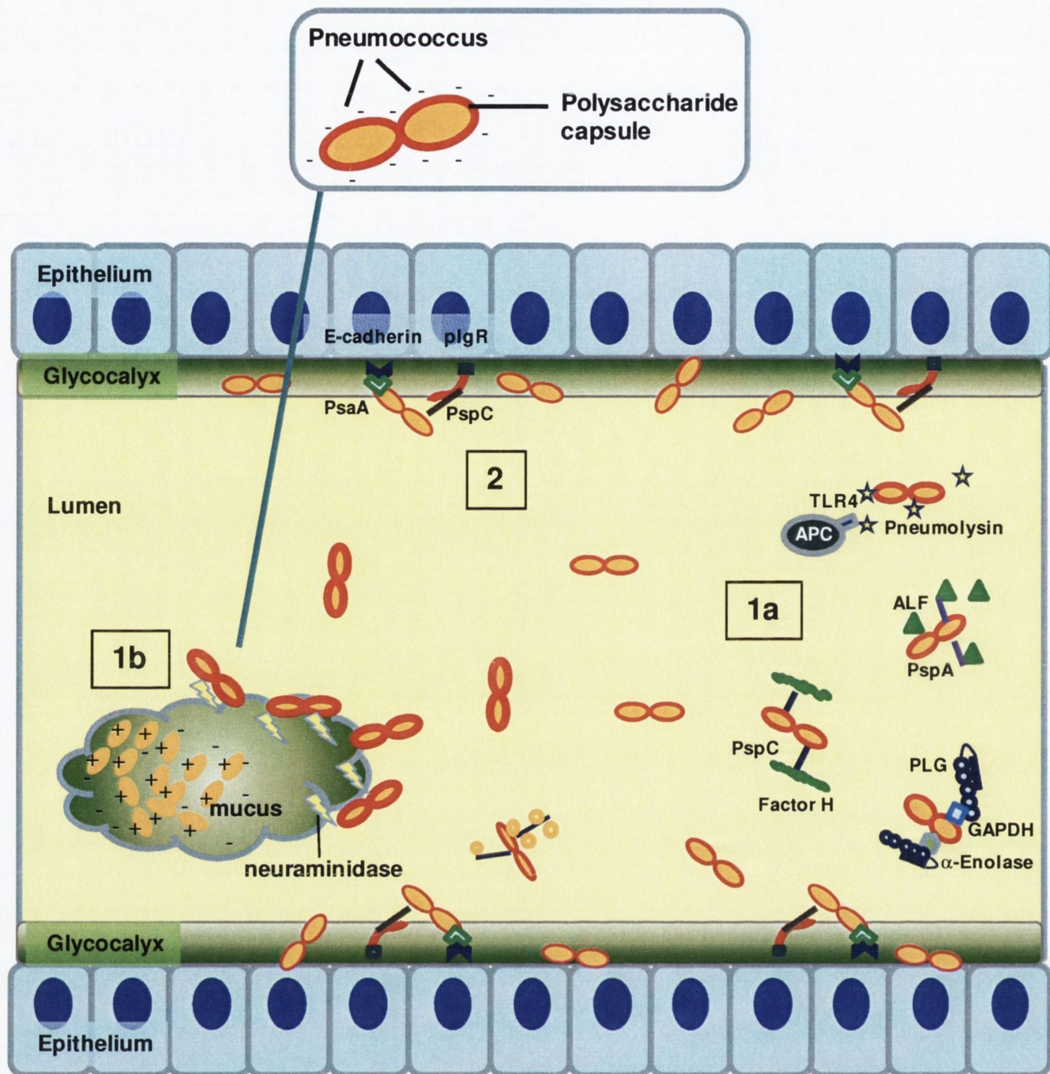


Fig. 5.1 Model of pneumococcal colonisation of the nasopharynx.

(1a) Pneumococci have to overcome many host immune responses to colonise the nasopharynx, such as antigen-presenting cells recognising Ply by TLR4, preventing killing by apolactoferrin (ALF) by binding to PspA, and evasion of complement-mediated killing by binding the regulatory protein Factor H to PspC. (1b) One of the first barriers to overcome for successful pneumococcal colonisation is entrapment in lumenal mucus. Unencapsulated strains (yellow – no red outline) remain agglutinated in mucus and are cleared by mucociliary clearance. Heavily encapsulated strains (thick red outline) escape lumenal mucus by electrostatic repulsion. Neuraminidase may also contribute to this escape by reducing the viscosity of the mucus. (2) Strains that escape mucus transit to the surface of the epithelium where they attach to host receptors. This stage of colonisation selects for transparent pneumcocci expressing less capsule (thin red outline). The pneumococci colonise this location for at least 14 days.

Immunostaining of tissue sections from early stages of colonisation demonstrated that bacteria must transit to the epithelial border for stable colonisation to occur. In order to reach the site of stable colonisation they must escape the lumenal mucus with which they are initially associated. Nasopharyngeal tissue sections from mice colonised with unencapsulated strains suggested an alternative role for capsule during colonisation, as strains lacking capsule are heavily agglutinated in lumenal mucus and fail to transit to the epithelial border. The mechanism is thought to rely on electrostatic repulsion due to the negative charge on both the capsule and the mucopolysaccharides of mucus. The present study demonstrates that this mucus entrapment selects for highly encapsulated strains which repel the mucus by charge allowing their escape from the mucopolysaccharides.

Following this escape, the next step is for the pneumococcus to transfer, and adhere, to the surface of the epithelium for stable colonisation to occur. Somewhere between escape from mucus and epithelial attachment, occurring after approximately 30 min to 20 h, selection shifts to less encapsulated strains. This is likely due to better exposure of underlying receptors for adherence. It still remains unclear if the pneumococcus is capable of changing between opaque and transparent phenotypic variants *in vivo*, or if one spontaneous phenotypic variant outcompetes the population to multiply and colonise successfully. This would be a very difficult, but important, question to answer. The present studies confirm a significant role for capsule in colonisation and also demonstrate the relative importance of opaque and transparent phenotypes in colonisation.

The pneumococcus is a multifactorial pathogen, with an arsenal of factors enabling evasion of host attack. Not only is the production of a plethora of virulence factors important but the pneumococcus has a physicochemical mechanism of immune evasion, namely, its negative surface charge. The current study highlighted this evasion strategy during mucus entrapment. Another example of this charge-mediated immune evasion in the setting of colonisation has recently been established. Llobet *et al.*(2008) demonstrated that the capsule mediates resistance to killing by antimicrobial peptides by acting as a bacterial decoy. Nasal secretions are home to a multitude of antimicrobial peptides, the majority of which are cationic. These cationic antimicrobial peptides bind to negatively charged capsules, thereby reducing the amount of these peptides reaching the bacterial surface where they can damage the cell. The latter study, along with the findings outlined in this thesis, implies that the surface charge of pneumococcal polysaccharide capsule

plays a vital role in successful colonisation in the nasopharynx. Something as simple as surface charge can have a tremendous effect on the outcome of the interaction between host and bacterium.

5.2. Adaptive immunity and live-attenuated vaccines

The present studies demonstrated that unencapsulated strains of the pneumococcus are capable of colonising the nasopharynx for up to 7 days, long enough for an adaptive immune response to be induced. This observation led to the question of whether colonisation by unencapsulated mutants could induce an immune response that could protect from subsequent challenge by a virulent strain. Murine studies demonstrated that colonisation by these attenuated unencapsulated strains can lead to both systemic and mucosal cross-protection, confirming that colonisation by live-attenuated vaccine (LAV) strains of *S. pneumoniae* can protect in a serotype-independent manner.

The mechanism of protection was investigated. It was noted that immunisation with LAV strains leads to a robust increase in anti-pneumococcal serum IgG and mucosal IgA. These antibodies are required for protection against colonisation, along with $CD4^{+}$ T-cells, as mice lacking either of these immune components were not protected following immunisation with LAV strains. These results suggest that there are thymus-dependent (TD) antigens on the bacterial surface that induce a protective immune response. This was an important result, as pneumococcal vaccines that induce T-cell-dependent immunity are required for successful vaccination of young children, who are at the greatest risk from pneumococcal disease. Also, TD antigens are more likely to induce immunological memory, thereby protecting the host long after vaccination.

Since humoral immunity is crucial for this colonisation-induced protection, the TD antigens mediating this protection were investigated by analysing serum IgG induced by colonisation. Western blotting and mutational analysis lead to the identification of three prominent cross-reactive antigens (PspA, PpmA and PsaA) induced by colonisation by at least one of three distinct strains. There was variation in the cross-reactivity of each strain, suggesting that there are differences in the expression of or surface exposure of these antigens between strains.

Two of these antigens, PpmA and PsaA, are highly conserved across strains. Such conservation of amino acid sequences suggests that these proteins may not be exposed to the immune response and therefore are not under immune pressure. However, flow cytometry demonstrated that PpmA may be exposed on some strains. In contrast, no surface exposure of PsaA was seen on any strain. Yet, studies demonstrate the potential for these antigens to induce a mucosal immune response (Pimenta *et al.*, 2006; McCool *et al.*, 2003; Simell *et al.*, 2001; Adrian *et al.*, 2004). However, unlike PpmA and PsaA, PspA is a highly variable surface-exposed immunogen expressed by all *S. pneumoniae* isolates. It is known to induce a strong humoral immune response which correlates with protection from subsequent carriage (McCool *et al.*, 2003), and it induced a robust serum IgG response in mice in the current study.

These proteins were identified as the dominant cross-reactive antigens recognised by sera induced by colonisation, yet they were not necessary for cross-protection. Initial studies on LAV strains confirmed that PspA was not needed to induce a protective response against colonisation, as immunisation with mutants lacking both PspA and Ply were protective. However, PspA given as a purified protein can be protective. Also, PspA is the most abundant protein on the surface of the pneumococcus and extends well beyond the capsule (Daniels *et al.*, 2006). Therefore, it is not surprising that it is a major target of the host response. However, there are many other immunogenic antigens exposed on strains lacking the capsule and it is likely that there is redundancy of these immunogenic antigens, allowing for protection without this dominant protein.

This study focused on the protective effects induced by surface-exposed antigens. This was a logical place to start as surface antigens are considered the best candidates for vaccines (Barocchi *et al.*, 2007). This rationale led us to disregard antigen 2 which elicited a robust humoral response on murine colonisation with the TIGR4 strain and, thus, may be a target for protective immunity. What is this antigen? And could it possibly be surface exposed, or exposed to the immune system during colonisation? This study established that it is not the pore-forming toxin pneumolysin, which is of a similar molecular weight and is found predominantly in the cytoplasmic fraction like antigen 2. Yet pneumolysin was recently demonstrated to be localised to the cell wall despite a lack of signal peptide or cell-wall-anchoring motif (Price & Camilli, 2009). There is also a number of other pneumococcal proteins that are exported and localised to the cell wall, yet do not contain motifs for

known export mechanisms. These are known as anchorless surface proteins. These include the α-enolase (Kolberg *et al.*, 2006; Bergmann *et al.*, 2001; Pancholi & Fischetti, 1998), PavA (Holmes *et al.*, 2001) and GAPDH (glyceraldehyde-3-phosphate dehydrogenase) (Bergmann *et al.*, 2004; Pancholi & Fischetti, 1992). Enolase is secreted by an unknown mechanism and reassociates to the surface via an unknown receptor (Bergmann *et al.*, 2001). PavA may also attach through this mechanism. It is possible that antigen 2 may be a novel anchorless surface protein, since the molecular weight of antigen 2 suggests that it may not be a known anchorless surface antigen. Therefore, antigen 2 may be important although it is primarily found in the cytoplasmic fraction. Important to note is that it is only conserved in mice colonised with the TIGR4 strain and, therefore, may not be a good candidate for inducing broad protection. Yet, it may be useful in a combination protein vaccine setting, and would warrant further investigation.

This thesis project adds much to the current knowledge of protective immunity against the pneumococcus. Firstly, it demonstrates the protective capacity of colonisation, which has been suggested by others, but never formally shown. This was also recently corroborated by another study (Richards *et al.*, 2009). This finding confirms that the events in early childhood play an important role in prevention of pneumococcal disease in later years. One wonders if the reduction of childhood colonisation due to routine vaccination with the conjugate vaccine will have a deleterious downstream effect on health in the increasingly aging population. It is unlikely that the vaccine-induced reduction of colonisation will lead to complete eradication of the pathogen, due to incomplete vaccination of the population. More probable, the pathogen will continue to be present and will be more deadly when it is finally acquired by a new host, whose immune system has not been properly primed for this pathogen due to the reduced transmission or through vaccination. However, this will occur in such a small percentage of people compared to the pre-pneumococcal vaccine era that the benefits of the vaccine outweigh the disadvantages.

Secondly, this research suggests the importance of antibody in natural protection against the pneumococcus. Previous studies have questioned the role of antibodies against the pneumococcus (van Rossum *et al.*, 2005; Malley *et al.*, 2005).These studies differed from the current study. One of these studies looked at the mechanism of clearance in naïve mice, which does not accurately represent the population (van Rossum *et al.*, 2005). Another study looked at the role of antibodies in previously colonised mice and demonstrated

antibody-independent protection against the same colonising type 6B strain (Malley *et al.*, 2005). The latter study differs from the current study as Malley *et al.* (2005) used encapsulated strains and this may better mimic the natural protection in the population. Yet further investigations would need to be completed to fully address this question, looking at protection induced by various encapsulated strains. It is unlikely, yet possible, that colonisation by strains with or without capsule would have a different mechanism of protection, particularly cross-protection against strains of different serotypes. Thus, although antibodies may play an important role in naturally acquired protection, the immune response may depend on the level and type of encapsulation of the protective colonising strain. Strains with a thicker, more complex capsule may prevent the exposure of the underlying protective protein antigens.

Finally, the results in this thesis project confirm that protein antigens are important in protection from colonisation. This has promising consequences for pneumococcal component vaccines. Many groups are trying to identify the best pneumococcal protein vaccine candidate, for example by genomic approaches (Barocchi *et al.*, 2007), that will induce broad protection and hopefully overcome some of the limitations of the current vaccines. However, it is proving difficult to find such an antigen, and therefore combinations of antigens will likely be required. The data in this thesis suggest that a combination of common antigens will be a promising new line of vaccine development against this pathogen.

The research embodied in this thesis proposes that adaptive immunity is more important than innate immunity in the prevention of pneumococcal colonisation and disease. One of the key innate components responsible for clearing foreign objects from the nasal passages, i.e., lumenal mucus and subsequent mucociliary clearance, is not effective at removing this encapsulated pathogen. Yet antibodies play a key role in the prevention of subsequent colonisation and disease. Contact with the pneumococcus is likely to occur numerous times in a lifetime, and thus the adaptive response will be crucial for controlling these interactions.

This thesis has presented strong evidence that a live attenuated vaccine would be highly successful in preventing not only invasive disease but also colonisation, and hence likely lead to herd immunity. However, a live attenuated vaccine is a difficult concept for the

pubic to accept, let alone the pharmaceutical manufacturers. The main concern would no doubt be the safety of the live strain. Herein ways are suggested to ensure that such a vaccine would remain attenuated (see **Chapter 3**, **Section 3.4**), namely, by removing the mechanism for transformation so that the vaccine strain cannot adapt and become virulent. One advantage of a live colonising vaccine strain would be its potential to transmit to other hosts, spreading the vaccine strain to unvaccinated individuals, thereby increasing the effectiveness of the vaccine. However, this may be unlikely since the vaccine strain poorly colonises its host and perhaps would not remain in the nasopharynx long enough for transmission. This potential for transmission would lead to concerns that the wrong population would acquire this vaccine, such as immunocompromised individuals. However, the present studies demonstrate that even in hosts lacking B- or T-cells the vaccine strain is completely attenuated, yet will not be effective in these populations. Such findings would need to be validated in humans. However, a live attenuated vaccine is a promising feasible answer to the current limitations of the polysaccharide vaccines.

One concern is that by targeting and eradicating nasopharyngeal colonisation would leave this niche open for replacement colonisation, and perhaps disease, caused by strains not targeted by live vaccination. Replacement of vaccine serotypes by non-vaccine serotypes has been described previously (Chibuk *et al.*, 2010; Dagan, 2009b). Yet live vaccination with unencapsulated strains could reveal many broadly reactive antigens and therefore could increase the number of strains targeted by immunisation. Eradicating pneumococcal colonisation has also raised concerns about the effect on co-colonising species (Bogaert *et al.*, 2004c). Recent large-scale population studies revealed an inverse relationship between nasopharyngeal *S. aureus* colonisation and nasopharyngeal colonisation with vaccine-type strains of *S. pneumoniae* (Bogaert *et al.*, 2004c; Regev-Yochay *et al.*, 2004). However, this negative correlation is not seen in HIV-infected individuals (McNally *et al.*, 2006), suggesting that the immune system, rather than direct bacterial interaction, may be mediating this apparent interference in upper respiratory tract flora. One group is investigating the potential role of antibodies directed against the pneumococcus in recognising specific staphylococcal surface antigens as a possible mechanism for this interference, thereby leading to clearance of *S. aureus* (R. Lijek, University of Pennsylvania, personal communication). Also, an augmentation of recurrent acute otitis media caused by *S. aureus* has been described following administration of the conjugate vaccine (Veenhoven *et al.*, 2003). Therefore, it is important to understand the effects of

reducing pneumococcal colonisation on the host microbiota to predict the outcome of future novel vaccines.

Despite not being necessary for cross-protection, analysis of human sera demonstrated the presence of anti-PpmA antibodies, by comparing binding of Ig in human sera to strains with or without PpmA. This further demonstrates that PpmA is immunogenic in the natural host. Interestingly, when analysing human sera for anti-PspA antibodies it was noted that strains lacking PspA on their surface bound more human IgG than wild-type strains. This suggests that PspA prevents binding of human antibodies to other surface moieties. Furthermore, the binding of antibodies against phosphorylcholine, and other epitopes on TA, was also increased in the absence of PspA. This suggests a possible novel mechanism whereby *S. pneumoniae* evades choline-dependent immune attack by decorating conserved choline antigens with variable proteins.

Other respiratory pathogens hide choline from the host immune response by phase variation of the expression of choline on the surface (Weiser *et al.*, 1997; Serino & Virji, 2000; Weiser *et al.*, 1998). In the case of *Haemophilus influenzae*, this is mediated by a translational switch within the *licA* gene, a putative choline kinase. Slip-strand repair of multiple tandem repeats can yield a gene with variable repeats which causes a sequence to jump into, or out of, frame. Once the gene is out of frame, the absence of a functioning enzyme prevents choline from being incorporated onto the LPS. However, as choline is a requirement for the growth of *S. pneumoniae*, the pneumococcus cannot turn off choline incorporation into TA, but rather just vary the amount incorporated (Fischer, 2000; Kim & Weiser, 1998). Concealing choline can prevent CRP-mediated opsonisation as well as recognition by naturally occurring anti-phosphorylcholine antibodies present in abundance in the host.

The antibody-blocking function of PspA may also explain the mechanism by which PspA interferes with complement activation, namely, by limiting antibody binding to the surface and hence leading to reduced complement activation by the classical pathway. The majority of studies investigating PspA and complement inhibition have used normal mouse sera (NMS) or normal human sera (NHS) as a complement source, both of which are known to contain natural antibodies recognising phosphorylcholine (Goldenberg *et al.*, 2004; Briles *et al.*, 1981). Also, it has been shown that PspA interferes predominantly with

the classical pathway, with the alternative pathway responding only after the classical has been initiated and acting to amplify the response (Ren *et al.*, 2004b). Therefore, antibodies are likely to play a role in the interference between PspA and complement.

In summary, the studies embodied in this thesis have unveiled a previously overlooked, yet key role of capsule in colonisation. Strains lacking this capsule are easily cleared by entrapment in mucus, yet provide a novel mechanism of immunisation. Such immunisation has the advantage of protecting not only against invasive disease, but also mucosal colonisation. Preventing colonisation would be expected to lead to herd immunity and protection against pneumococcal disease in unvaccinated individuals. Therefore, colonisation remains the key event to target in preventing pneumococcal disease in the population.

5.3 Future directions

Future studies could confirm that the role of capsule in colonisation is to aid in escape from mucus by electrostatic repulsion, and not by increased resistance to antimicrobial substances. One way this could be addressed is by an *in vitro* mucus killing assay, to see if unencapsulated pneumococci are killed more quickly and efficiently than encapsulated pneumococci due to presence of antimicrobial peptides. Also, studies could determine if electrostatic repulsion by mucus is a conserved function of the capsule during colonisation with other bacteria residing in the upper airways. *Haemophilus influenzae*, *Neisseria meningitidis*, and *Staphylococcus aureus* all have a requirement for a capsule during colonisation, and therefore, a role could be tested in similar studies with each bacterium.

This thesis demonstrated that LAV strains against *S. pneumoniae* act as potent vaccines. However, further studies are needed before these LAV strains can be considered for human use. Pre-clinical testing would require investigation of the extent of cross-reactivity of the vaccine strains. Also the induction of an anamnestic response to the vaccine strains, as well as the response in an infant mouse model should be established. Most importantly the safety of the vaccine strain would need to be confirmed, by generating a strain incapable of reverting to a virulent phenotype.

Future studies could also explore the cross-protective antigens of LAV strains. Since the major cross-reactive bands recognised by immune sera were not necessary for cross-protection, identification of the minor bands may be more informative. Also, the requirement for multiple antigens could be examined. Identification of cross-protective antigens may also indicate antigens for protein component vaccines, which may be successful for preventing nasal colonisation.

Future research could also focus on PspA-mediated inhibition of antibody binding, and the masking of ChoP antigens. To determine if the contribution of PspA to the inhibition of complement binding may be due to inhibition of anti-ChoP antibodies, sera from μMT mice, which lack specific antibody, could be used as a complement source. Also, studies could determine if CRP-mediated clearance is enhanced in strains lacking PspA. The downstream effect of inhibition of anti-ChoP antibody binding could be measured by *ex vivo* opsonophagocytic killing assays, as well as mouse models of infection, to determine if this leads to enhanced survival. Since natural antibody against ChoP is common, these studies would enhance understanding of how the pneumococcus evades the host immune response.

Appendix

C + Y MEDIA (pH 6.8 or 8.0)

1X Pre C	800 ml
Supplement	26 ml
Glutamine (1mg/ml)	20 ml
Adams III	20 ml
Pyruvate (2% w/v)	10 ml
KPO_4 (pH 6.8 or 8.0) (see below)	30 ml
Difco Yeast (5%)	20 ml

Filter sterilise and store at 4°C.

C+Y Medium components

4X Pre C (1L)

Sodium acetate (anhydrous)	4.84 g
Casamino acids (Difco)	20 g
L-Tryptophan	0.02 g
L-Cysteine HCl	0.2 g

Add H_2O to 1 litre

Adjust pH to 7.4–7.6

Autoclave and store at 4°C.

Adams I

Biotin (0.5 mg/ml)	0.06 ml
Nicotinic Acid	30 mg
Pyridoxine	35 mg
Calcium Pantothenate	120 mg (calcium salt of pantothenic acid)
Thiamine HCl	32 mg
Riboflavin	14 mg

Add H_2O to 200ml

Filter sterilise and store at 4°C in the dark.

Adams II

$FeSO_4.7H_2O$	50 mg
$CuSO_4$	50 mg
$ZnSO_4.7H_2O$	50 mg
$MgCl_2.4H_2O$	20 mg
HCl	1 ml

Add H_2O to 100 ml

Filter sterilise and store at 4°C in the dark.

Adams III

Adams I	64 ml
Adams II	16 ml
Asparagine	800 mg
Choline Chloride	80 mg
$CaCl_2$ (1%)	0.64 ml

Add H_2O to 400 ml

Filter sterilise and store at 4°C in the dark.

KPO_4 (pH 6.8 or pH 8.0)

KH_2PO_4 (1M)	255 ml
K_2HPO_4 (1M)	245 ml

pH to 6.8 or 8.0

Autoclave and store at 4°C.

3 in 1 salts

$MgCl_2.6H_2O$	100 g
$CaCl_2$ anhydrous	0.5 g
$MnSO_4$ (0.1 M)	0.2 ml

Add H_2O to 1 litre

Filter sterilise and store at 4°C.

Supplement

3 in 1 salts	60 ml

Glucose (20%)	120 ml
Sucrose (50%)	6 ml
Adenosine (2 mg/ml)	120 ml
Uridine (2 mg/ml)	120 ml

Filter sterilise and store at 4°C.

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