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Construction and Immunological Characterisation of a Non-Toxic form of Pneumolysin for use in Pneumococcal Vaccines

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By

Lea-Ann Stirling Kirkham B.Sc.

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Division of Infection and Immunity
Institute of Biomedical and Life Sciences
Biomedical Research Centre
Glasgow University
120 University Place
Glasgow G12 8TA

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Abstract

There are 90 different serotypes of *Streptococcus pneumoniae* and current pneumococcal vaccines are somewhat limited in their protection against invasive pneumococcal disease. The adult pneumococcal vaccine is composed of capsule polysaccharide from 23 of the most common disease causing serotypes. Infants are the major 'at risk' group from pneumococcal disease; however, polysaccharide-based vaccines are not protective in this age group. This has led to the development of a paediatric conjugate vaccine, which is composed of polysaccharide from seven of the most prevalent disease causing serotypes individually conjugated to a carrier protein. Although the conjugate vaccine is highly efficacious, it only elicits protection against disease caused by the seven homologous pneumococcal serotypes. Serotype specific immunisation is only a short-term solution to combating pneumococcal disease. Problems with serotype replacement have already arisen within five years since licensure of the paediatric vaccine, with non-vaccine serotypes replacing the eliminated vaccine serotypes. A solution to this is the development of pneumococcal vaccines containing a species wide pneumococcal protein to elicit non-serotype specific protection.

Pneumolysin, the pore-forming toxin produced by *S. pneumoniae*, may have an application in future pneumococcal vaccines as it is produced by all invasive isolates, is a major virulence factor and has been demonstrated to confer non-serotype specific protection against pneumococcal disease. The toxicity of pneumolysin is problematic in terms of vaccine use and existing pneumolysin mutants possess residual cytotoxicity. By mutating a region involved in protein oligomerisation, pneumolysin pore formation was abolished. This resulted in a non-toxic form of pneumolysin that retained the immunogenicity of wild type pneumolysin without the associated effects such as hypothermia, inflammatory cytokine production and vascular leakage following intranasal administration to mice.

Vaccination of mice with this pneumolysin toxoid elicited high titres of neutralising antibody, which significantly protected animals from pneumococcal infection.

Conjugation of the pneumolysin toxoid to capsule polysaccharide from serotype 4 *S. pneumoniae* elicited full protection against infection from the homologous serotype. This indicated that the pneumolysin toxoid was as effective as the carrier protein used in the current pneumococcal conjugate vaccine. Active vaccination with free pneumolysin toxoid significantly increased survival times in mice following challenge with a non-vaccine serotype. This research implies that combining conjugated and free pneumolysin toxoid may be more efficacious than current vaccines in protection against pneumococcal disease.

The importance of the pore-forming property of pneumolysin in pathogenesis of pneumococcal disease was investigated by construction of a pneumococcal strain carrying the same mutation used to construct the pneumolysin toxoid for vaccination. Pore formation was found not to be important for pathogenesis during murine pneumococcal pneumonia. This was further supported by the identification of serotype 1 strains from clinical disease that expressed non pore-forming pneumolysin yet were isolated from patients with invasive pneumococcal disease.

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Abbreviations

α-	Anti
°C	Degrees Celsius
Δ	Deletion
-/-	Deficient
µl	micro litre
µm	micrometer
µM	micro molar
A	Absorbance
Ab	Antibody
AEC	Anion Exchange Chromatography
AOM	Acute Otitis Media
Alum	Aluminium phosphate
APS	Ammonium persulphate
BAB	Blood Agar Base
BALF	Bronchoalveolar Lavage Fluid
BBB	Blood Brain Barrier
BHI	Brain Heart Infusion
BSA	Bovine Serum Albumin
C	Complement
Cbp	Choline Binding Protein
CDC	Cholesterol-dependent Cytolysin
CFU	Colony Forming Unit
Clp	Caseinolytic protease
CNS	Central Nervous System
CPS	Capsule Polysaccharide
CSF	Cerebrospinal Fluid

CSP	Competence Stimulating Peptide
CV	Column Volumes
D39	*Streptococcus pneumoniae* serotype 2 strain D39 (NCTC number: 7466)
DNA	Deoxyribonucleic acid
dNTP	deoxyribonucleotide triphosphate
E. coli	*Escherichia coli*
eGFP	Enhanced Green Fluorescent Protein
ELISA	Enzyme Linked Immunosorbent Assay
EMEM	Eagle's Minimum Essential Media
EU	Endotoxin Units
FBS	Foetal Bovine Serum
g	Gram
g	centrifugal force
gDNA	Genomic DNA
h	Hour
H_2O_2	Hydrogen peroxide
HIC	Hydrophobic Interaction Chromatography
His-Tag	Histidine Affinity tag
HRP	Horseradish peroxidase
HU	Haemolytic Units
Hyl	Hyaluronidase
IFN-γ	Interferon gamma
Ig	Immunoglobulin
IL	Interleukin
ILY	Intermedilysin
i.n.	Intranasal
i.p.	Intraperitoneal
IPTG	Isopropyl-β-D-Thiogalactopyranoside

Kb	Kilobase
KC	Cytokine-induced neutrophil chemoattractant
kDa	Kilodalton
L	Litre
LAL	Limulus Amebocyte Lysate
LB	Luria Broth
LPS	Lipopolysaccharide
LytA	Autolysin A
M	Molar
mAb	Monoclonal Antibody
MAD	Median Absolute Deviation
mg	Milligram(s)
min	Minute(s)
ml	Millilitre
MLST	Multi-Locus Sequence Typing
mM	Millimolar
MTT	3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrazoliumbromide
MW	Molecular weight
n	Group size
Nan	Neuraminidase
NK	Natural Killer
nm	Nanometre
NO	Nitric Oxide
OD	Optical Density
PAFr	Platelet activating factor receptor
PBS	Phosphate Buffered Saline
PB-S	Phosphate Buffer minus NaCl
PCR	Polymerase Chain Reaction

PCV	Pneumococcal Conjugate Vaccine
PdB	Pneumolysin carrying W433F substitution
PdT	Pneumolysin carrying triple mutations: D385N, C428G, W433F
PFO	Perfringolysin O
Pht	Pneumococcal histidine triad protein
Pia	Pneumococcal iron acquisition protein
Piu	Pneumococcal iron uptake protein
Ply	Pneumolysin
PPV	Pneumococcal Polysaccharide Vaccine
PS	Polysaccharide
Psa	Pneumococcal surface adhesion protein
psi	Pounds per square inch
Psp	Pneumococcal surface protein
rpm	Revolutions per minute
RT	Room Temperature (~20°C)
S. pneumoniae	*Streptococcus pneumoniae*
sdm	Site-directed mutagenesis
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
sec	Second(s)
SLO	Streptolysin O
SMPRL	Scottish Meningococcal and Pneumococcal Reference Laboratory
Spp.	Species
ST	Sequence Type
Tc	Core body temperature
TEM	Transmission Electron Microscopy
TIGR	The Institute for Genomic Research
TIGR4	Serotype 4 *S. pneumoniae* genome sequenced by TIGR

	(ATCC number: BAA-334)
TLR	Toll-like Receptor
TMB	3, 3′, 5, 5′-tetramethylbenzidine
TMH	Transmembrane Helices
TNF	Tumour Necrosis Factor
U	Units
V	Volts
v/v	Volume/volume
wk	Week
WT	Wild type

Introduction

1.1. *Streptococcus pneumoniae*

Streptococcus pneumoniae (the pneumococcus) is a Gram-positive facultative anaerobe that was discovered simultaneously by Pasteur in France and Sternberg in America in 1880. It has since been found to be the predominant cause of fatal infections such as bacterial pneumonia and meningitis. Pneumococci can be divided into 90 serotypes depending upon the immunochemistry of their capsular polysaccharide (Henrichsen 1995). The classification of pneumococcal serotypes was originally by chronological order of discovery (Dochez et al. 1913), however, many of the capsule polysaccharides are cross-reactive and this has led to the reclassification of serotypes using the Danish system of nomenclature. Where serotypes 1 and 2 remain the same but serotypes that have similar capsules, for example serotypes 6 and 26 by American nomenclature, are now termed serotypes 6A and 6B (belonging to serogroup 6) (Smart 1987). The Quellung (literally 'swelling') reaction developed by Neufeld in 1902 allows the determination of serotypes by their reactivity with antibodies raised against homologous capsule polysaccharide. This technique is still employed in reference laboratories today. However, cross reactivity hinders the quelling reaction resulting in some isolates being mistyped or nonserotypeable (due to the lack of capsule expression). Molecular serotyping techniques are currently under investigation in attempt to develop serotyping techniques that are more accurate in determining serotype rather than serogroup and are less expensive and easier to perform (Batt et al. 2005; Pai et al. 2005a). Multiplex PCR has recently been shown to provide an accurate high through-put approach to the serotyping of pneumococci (Pai et al. 2006).

With the advent of Multi Locus Sequence Typing (MLST), pneumococci can now be genetically categorised (Enright et al. 1998). MLST involves the sequencing of seven housekeeping genes (*aroE, gdh, gki, recP, spi, xpt and ddl*) where each allele is assigned a number depending upon its sequence using the MLST website (http://www.mlst.net). This results in a seven digit 'barcode' for each isolate known as the sequence type (ST). MLST

is a powerful tool in pneumococcal characterisation, providing an electronic form of molecular typing that allows standardisation between laboratories. It has become evident that different STs exist within one serotype and that polysaccharide capsules can switch (Coffey et al. 1998; Coffey et al. 1999; Jefferies et al. 2004), making MLST a more accurate tool for the genetic surveillance of pneumococcal disease on a global scale. More in depth analysis can be achieved using microarray to compare whole genomes. This allows assessment of gene content in individual isolates, however this technique provides an overwhelming amount of data and remains too expensive and labour intensive for routine clinical use. Microarray analysis of pneumococcal isolates has indicated that there are genetic differences within an individual sequence type (Silva et al, manuscript submitted), highlighting that variation occurs within a group considered identical by MLST. Supplementary sequencing of essential virulence genes in addition to the seven housekeeping genes may make MLST more powerful in reference laboratories (Hanage et al. 2005) until microarray technology becomes more accessible.

1.2. Carriage of *S. pneumoniae*

S. pneumoniae colonises the upper respiratory tract of most of the human population at least one time in their lives. Nasopharyngeal colonisation is an evolving and transient process with different species and serotypes present at different times. This niche is a highly competitive environment with *Haemophilus influenzae, Moraxella cattarrhalis, Neisseria meningitiis, Staphylococcus aureus* and other *Streptococcal spp.* all colonisers of the nasopharynx (Bogaert et al. 2004). *S. pneumoniae* has been shown to produce H_2O_2 which inhibits or kills other microflora in the nasopharynx giving the pneumococcus a competitive advantage for colonising this environment (Pericone et al. 2000). The nasopharynx is the source of transmission from host to host and also where *S. pneumoniae*, which is highly transformable, is thought to pick up genes from other bacterial species. An example of this is the pneumococcal acquisition of genes that encode low affinity

penicillin-binding proteins from other species such as *Streptococcus mitis* (Dowson et al. 1994) to confer penicillin resistance.

There are a number of factors that are associated with an increased frequency of pneumococcal carriage and therefore disease including overcrowding (as found in day care centres, large families in cramped housing, homeless shelters and prisons), smoking, antibiotic use and ethnicity (DeMaria et al. 1980; Kurtti et al. 1997; Obaro 2000; Bogaert et al. 2004). The most common serotypes isolated from nasopharyngeal swabbing of European children are 6A, 6B, 9V, 14, 18C, 19F and 23F; and the range of carried serotypes declines with age (Bogaert et al. 2004). These serotypes are also some of the most common disease causing serotypes in Europe, indicating that carriage preludes disease.

1.3. Diseases caused by *S. pneumoniae*

Although usually asymptomatically carried, *S. pneumoniae* can cause non-invasive diseases such as otitis media, sinusitis and bronchitis and the more severe and often fatal invasive diseases: pneumonia, meningitis and bacteraemia. In 2003, The World Health Organisation (WHO) estimated that at least one child dies from pneumococcal disease every minute (http://www.who.int/vaccines/en/pneumococcus.shtml). Host–pathogen interactions during pneumococcal pneumonia, bacteraemia, meningitis and otitis media are discussed in sections 1.7 to 1.9.

Although there is an overlap in pneumococcal strains that cause disease and those associated with carriage, some serotypes are more likely to be recovered from invasive disease (serotypes 1, 4, 14 and 18C) and others are more commonly isolated from nasopharyngeal swabbing of healthy individuals (serotype 3 ST180, 6B, 19F and 23F) (Brueggemann et al. 2003a). Invasive pneumococcal disease (IPD) has been shown to

originate from a serotype with which the host was already colonised (Lloyd-Evans et al. 1996), though in some cases this colonisation period may be brief, e.g. with serotype 1 (Brueggemann et al. 2003b). The factors involved in a carried strain becoming invasive are not fully understood. There is increasing evidence that viral infections are the predisposing factor in converting pneumococcal carriage to invasive disease (McCullers et al. 2002; Ishizuka et al. 2003; van der Sluijs et al. 2006). However, the host-pathogen interaction is complex and mechanisms by which internalised pneumococci escape endocytosis-mediated killing is not clear (Tuomanen et al. 1997). It is known that the inflammatory cytokines IL-1 and TNF-α, produced during viral infections, up-regulate expression of receptors on host cells, which facilitates pneumococcal binding. One identified receptor is the platelet-activating-factor receptor (PAFr) on epithelial and endothelial cells that pneumococcal cell surface phosphorylcholine binds to (Cundell et al. 1995; Tuomanen 1999). This interaction is thought to result in the internalisation of the bacteria, permitting the translocation through the respiratory epithelium and vascular endothelium (Cundell et al. 1995; McCullers et al. 2002).

1.4. Serotype 1 pneumococci

Serotype 1 *S. pneumoniae*, initially classified in 1913 (Dochez et al. 1913), remain one of the most prevalent invasive serotypes with reports of increases in serotype 1 IPD in Scotland (McChlery et al. 2005), Sweden (Henriques Normark et al. 2001) and Denmark (Konradsen et al. 2002) and a high prevalence of serotype 1 disease throughout Europe, South America, Africa and Asia (Scott et al. 1996; Hausdorff et al. 2000a; Hausdorff et al. 2000b). Interestingly, serotype 1 disease has been reported to be decreasing in North America (Feikin et al. 2002) [ranked 17th (Brueggemann et al. 2003b)] and Egypt (Wasfy et al. 2005). In developing countries, serotype 1 is associated with meningitis (Koek et al. 2005; Leimkugel et al. 2005) whereas in westernised countries serotype 1 is associated with pneumonia, complicated pneumonia, pulmonary empyema, peritonitis and salpingitis

(McFarlane et al. 1979; Westh et al. 1990; Sirotnak et al. 1996; Tan et al. 2002; Eltringham et al. 2003; Sjostrom et al. 2006). Serotype 1 and 7F have recently been identified as 'primary pathogens' in that these serotypes are most commonly isolated from IPD patients with no underlying disease, whereas other serotypes, such as 11A, were only found to cause disease in immunocompromised patients and have been termed 'opportunistic pathogens' (Sjostrom et al. 2006). The primary pathogens, although predominant in the study group and rarely found in carriage, were less likely to cause death compared with the opportunistic serotypes that are usually more carriage associated. Along with serotypes 5 and 7, serotype 1 is associated with higher ratio of hospitalisation versus ambulatory care compared with pneumococcal infections from other serotypes (Alpern et al. 2001). Serotype 1 is one of the few serotypes linked with non-hospital outbreaks of pneumococcal disease, such outbreaks have occurred in over crowded institutions and/or where alcoholism is a problem (DeMaria et al. 1980; Mercat et al. 1991; Gratten et al. 1993; Dagan et al. 2000).

Nasopharyngeal swabbing of healthy patients rarely results in the isolation of serotype 1 pneumococci, because of this and a global association with IPD, serotype 1 is said to have a high attack rate (Brueggemann et al. 2003b). It may be that serotype 1 pneumococci have a different niche, i.e. adenoid tissue, which is not swabbed during collection of carriage isolates. Indeed, *Escherichia coli* O157:H7 has recently been shown to have a highly specific niche in cattle (Naylor et al. 2003). It may be that carriage of serotype 1 is short lived as it remains sensitive to antibiotics (Brueggemann et al. 2003b) unlike strains associated with carriage that are thought to be under antibiotic pressure and in an environment which allows transfer of antibiotic resistance genes. During outbreaks of serotype 1 disease, the carriage rate increases, indicating that brief nasopharyngeal colonisation may occur with serotype 1 pneumococci.

1.5. Virulence Factors of *S. pneumoniae*

S. pneumoniae produces an array of virulence factors involved in pathogenesis including capsule polysaccharide, cell surface-associated proteins and toxins. Signature-tagged mutagenesis and full genomic sequencing studies have resulted in the identification of many putative virulence factors (Polissi et al. 1998; Lau et al. 2001; Tettelin et al. 2001; Hava et al. 2002). The more characterised pneumococcal virulence factors and their roles in disease are listed in Table 1.1.

Table 1.1. Pneumococcal virulence factors

Virulence factor	Location	Function
Autolysin A (LytA) Autolysin B (LytB) Autolysin C (LytC)	Cell wall	Releases other virulence factors, e.g. Ply, by breaking down cell wall LytA knockout strain is less virulent (Berry et al. 1989a) Involved in transition of pneumococci from nasopharynx to lower respiratory tract (Orihuela et al. 2004) Required for pneumococcal survival and replication in the lungs (Orihuela et al. 2004) LytB and C are involved in nasopharyngeal colonisation (Gosink et al. 2000)
Capsule polysaccharide (CPS)	Outer surface	Anti-phagocytotic Masks cell surface antigens from host immune system Expression levels are altered during disease (Hammerschmidt et al. 2005)
Cell Wall (including teichoic acids, phosphorylcholine, peptidoglycan and lipoteichoic acids)	Beneath capsule	Inflammatory: activates complement cascade and cytokine production Provides a surface for anchoring surface-exposed proteins
Choline binding protein A (CbpA, PspC, SpsA)	Attached to cell wall	Involved in immune cell recruitment (Murdoch et al. 2002) Has a major role in adhesion of pneumococci to nasopharyngeal cells (Rosenow et al. 1997) and translocation of pneumococci across human nasopharyngeal epithelial cells and for transition from nasopharynx to lower respiratory tract (Zhang et al. 2000; Orihuela et al. 2004)

		Binds human complement factor H to avoid attack and opsonophagocytosis (Quin et al. 2005) Inhibits sIgA Ab binding to pneumococci
Cbp D Cbp E (or Pce) CbpG		CbpD, E and G are involved in nasopharyngeal colonisation (Gosink et al. 2000) CbpD is involved in triggering the release of Ply from non-competent pneumococci (Guiral et al. 2005) CbpE and G are involved in adherence to human cells (Gosink et al. 2000) CbpE removes phosphorylcholine from the cell wall (de las Rivas et al. 2001) CbpG has a role in sepsis (Gosink et al. 2000)
Caseinolytic protease C (ClpC)	Cytoplasm	Involved in autolysis in some strains and pneumococcal growth in the lungs and bloodstream (Ibrahim et al. 2005)
ClpP		Involved in virulence, thermotolerance and resistance to oxidative stress (Ibrahim et al. 2005)
Hyaluronidase (Hyl)	Surface (secreted)	Breaks down hyaluronic acid in mammalian connective tissue possibly to promote pneumococcal dissemination (Paton et al. 1993)
High temperature requirement A (HtrA)	Cytoplasm	Essential for virulence in pneumonia and bacteraemia, involved in resisting oxidative stress and high temperatures (Ibrahim et al. 2004)
Hydrogen peroxide (H_2O_2)	Produced during aerobic growth	Inhibits/kills other bacterial species competing to colonise the nasopharynx (Pericone et al. 2000)
IgA1 protease	Cytoplasm	Cleaves human IgA1 (Poulsen et al. 1996; Wani et al. 1996), possibly counteracts mucosal defences (Polissi et al. 1998) enabling pneumococci to persist on mucosal surfaces (Weiser et al. 2003)
Neuraminidase (Nan A)	Surface exposed (Camara et al. 1994)	Cleaves N-acetyl neuraminic acid from lipoproteins, glycolipids and oligosaccharides on mammalian cells and breaks down mucin on mucosal surface thereby exposing receptors on cell surface allowing pneumococci to bind to host cells (Tong et al. 2000) Thought to be involved in spread of pneumococci from the nasopharynx to the lung (Orihuela et al. 2003) Important in colonisation and AOM (Tong et al. 2000) but not meningitis (Winter et al. 1997).
Pili	Surface (extends beyond	Initial adhesion to host cells and important for ability to invade host (Barocchi et al. 2006)

	CPS)	
Pneumococcal surface adhesin A (PsaA)	Surface	Manganese ABC transporter required for competence and virulence (Dintilhac et al. 1997) Binds to host cells, protects pneumococci from oxidative damage (Tseng et al. 2002)
Pneumococcal surface protein A (PspA)	Surface	Binds pneumococci to host cells and inhibits complement activation on pneumococcal cell surface (Tu et al. 1999; Ren et al. 2004) Binds to lactoferrin (protein involved in iron acquisition) at respiratory mucosal sites (Hammerschmidt et al. 1999) May be involved in nasopharyngeal colonisation (LeMessurier et al. 2006)
Pneumolysin (Ply)	Cytoplasm	Potent toxin Facilitates host tissue invasion and spread of disease (Orihuela et al. 2004) Interferes with phagocyte function (Houldsworth et al. 1994) Slows ciliary beating (Feldman et al. 1990) Disrupts integrity of human respiratory epithelium (Feldman et al. 1990) Required for pneumococcal survival and replication in the lungs (Orihuela et al. 2004) (see Table 1.2 for detailed list of roles of Ply)
Pyruvate oxidase (encoded by *spxB*)	Cytoplasm	An enzyme responsible for hydrogen peroxide production. It decarboxylates pyruvate to give acetyl phosphate + H_2O_2 + CO_2 Involved in adhesion of pneumococci to host cells, *spxB* knockout strains are less able to colonise and are less virulent (Spellerberg et al. 1996; Orihuela et al. 2004) Required for pneumococcal survival and replication in the lungs (Orihuela et al. 2004)
Streptococcal lipoprotein rotamase A (SlrA)	Surface	Lipoprotein involved in colonisation of the nasopharynx and protects pneumococci from phagocytosis (Hermans et al. 2006)
Zinc metalloprotease (ZmpB)	Surface	Induces inflammation in respiratory tract (Blue et al. 2003)

1.5.1. Capsule

The capsule polysaccharide of *S. pneumoniae* is a major virulence factor that enables the micro organism to resist phagocytosis (Jonsson et al. 1985). Unencapsulated strains are generally avirulent with rare cases of unencapsulated strains being recovered from

immunocompromised hosts or from conjunctivitis patients (Muller-Graf et al. 1999; Ishizuka et al. 2003; Martin et al. 2003). Changes in colony phenotype from transparent to opaque are due to thickening of the capsule. The transparent phenotype is adapted to colonisation of the nasopharynx with a thin capsule layer that allows cell wall phosphorylcholine to bind to the platelet activating factor receptor (PAFr) on respiratory epithelial cells. This permits colonisation of the host. Opaque colonies are usually recovered from the bloodstream and are unable to adhere to PAFr. These phenotypes are reversible and thought to aid the progression from carriage to invasive disease (Tuomanen 1999; Hammerschmidt et al. 2005). Although 90 capsule polysaccharide types have been identified (Henrichsen 1995), less than 20% of serotypes are the major cause of disease (Hausdorff et al. 2000b).

The capsule has long been thought of as the first point of contact with host cells. However, pneumococci have recently been shown to possess pili that protrude the capsule polysaccharide and are responsible for the initial adhesion to host cells (Barocchi et al. 2006). Using mutant strains (rlrA$^-$) that did not have pili, it was shown that pili also facilitate the ability of the pathogen to cause invasive disease in mice.

1.5.2. Cell wall and surface-exposed proteins

The pneumococcal cell wall is important in the attachment of the organism to host respiratory cells and is comprised of an array of surface-associated virulence factors. The pneumococcal cell wall is composed of peptidoglycan, teichoic and lipoteichoic acids. Phosphorylcholine, which is a component of the teichoic and lipoteichoic acids, acts as an adhesin for the platelet-activating factor receptor (PAFr) of host cells (Cundell et al. 1995).

There are three major groups of pneumococcal surface-exposed proteins: choline-binding proteins, lipoproteins and LPXTG-anchored proteins. Based on genome data, different

pneumococcal strains have been shown to vary in the proportion of these surface proteins (Bergmann et al. 2006). Choline-binding proteins are non-covalently anchored to the phosphorylcholine of the cell wall by carboxy terminal repeats. Pneumococci produce 13 to 16 different choline-binding proteins including pneumococcal surface protein A (PspA), choline binding protein A (CbpA, also termed pneumococcal surface protein C), and the autolysins (LytA, *N*-acetyl-muramoyl-L-alanine amidase; LytB, *β-N*-acetylglucosaminidase and LytC, β-N-acetylmuramidase [lysozyme]) (Bergmann et al. 2006).

Pneumococcal surface exposed lipoproteins are involved in substrate transport and include pneumococcal surface adhesin A (PsaA), the substrate-binding protein of a manganese transport system (Dintilhac et al. 1997), pneumococcal iron acquisition protein A (PiaA) and pneumococcal iron uptake protein A (PiuA) (Brown et al. 2001), Streptococcal lipoprotein rotamase A (SlrA) involved in colonisation of the host (Hermans et al. 2006) and putative proteinase maturation protein A (PpmA) involved in pneumococcal pneumonia (Overweg et al. 2000).

LPXTG anchored proteins are covalently attached to peptidoglycan in the cell wall by sortases that recognise the LPXTG motif of such proteins. Different sortases are involved in processing the different surface-exposed proteins (Hava et al. 2002; Paterson et al. 2006b). Hyaluronidase (Hyl) and neuraminidase A (NanA) are two well-characterised LPXTG-anchored proteins. Neuraminidases cleave the terminal sialic acid from glycolipids, glycoproteins and oligosaccharides on the cells of the host tissue and fluids providing a means of invasion for the pneumococci (Tong et al. 2000). Hyl hydrolyses hyaluronic acid, which is found in mammalian connective tissue, permitting pneumococcal invasion of the host tissues (Paton et al. 1993).

Protective immunity from pneumococcal infection has been demonstrated with a number of these pneumococcal cell surface exposed proteins, this is discussed in section 1.14 and potential vaccine candidates are given in Table 1.5.

1.5.3. Pneumolysin

Pneumolysin (Ply) is a pore-forming cytoplasmic toxin that belongs to the family of cholesterol-dependent cytolysins (see section 1.12). Ply is a multifunctional toxin produced by all disease causing isolates (Kalin et al. 1987) and is an important pneumococcal virulence factor with many roles in the pathogenesis of disease (Mitchell et al. 1997). There are two major functions associated with Ply: cytolysis by forming pores in membranes (Alouf 2000) and the ability to activate complement in the absence of specific antibody (Paton et al. 1984; Mitchell et al. 1991). Due to Ply's ability to form pores in cholesterol containing membranes, it is cytotoxic to all eukaryotic cells tested to date including pulmonary epithelial and endothelial cells (Steinfort et al. 1989; Feldman et al. 1990; Rubins et al. 1992; Rubins et al. 1993; Rayner et al. 1995), cerebral epithelial cells (Zysk et al. 2001) and ciliated ependyma (Mohammed et al. 1999). Ply has been detected in the cerebrospinal fluid (CSF) of meningitis patients (Spreer et al. 2003b), middle ear fluid from otitis media cases (Virolainen et al. 1994), lung sputum from pneumonia patients (Wheeler et al. 1999) and lung tissue from mice with experimental pneumonia (Canvin et al. 1995). When Ply is administered intranasally to mice there is an immediate response with damage to lung integrity that results in vascular leakage (Maus et al. 2004). Ply is thought to have an essential role in pneumococcal pneumonia (See section 1.7.1) and has been shown to induce inflammatory responses in the host lung similar to that caused by *S. pneumoniae* (Feldman et al. 1991). Interestingly, although Ply has distinct roles in the pathogenesis of pneumococcal disease, a study of virulence gene expression during pneumococcal infection of mice found *ply* expression to be constantly up regulated in all niches from the nasopharynx to the blood. The authors suggest that it is

the controlled timing of Ply release that is important for pneumococcal dissemination rather than *ply* expression (LeMessurier et al. 2006). At sub-lytic concentrations, which are more likely to be found during pneumococcal infection, Ply has a plethora of roles in pathogenesis that are detailed in Table 1.2.

A recent microarray study compared cDNA from human monocytes following treatment with either WT *S. pneumoniae* or a Ply negative strain to assess Ply-dependent gene expression (Rogers et al. 2003). The authors found that of the 4133 genes assessed, 116 were up regulated and 26 were down regulated in response to Ply. Up-regulated genes included those involved in synthesis of lysozyme, an antimicrobial enzyme that induces autolysis of pneumococci and enhances the phagocytic activity of neutrophils (Cottagnoud et al. 1993; Ibrahim et al. 2001). Mannose-binding lectin genes were also up regulated in monocytes incubated with the Ply positive strain, this molecule is involved in phagocytosis, cytokine production and complement activation and the authors imply that it could also be a core mediator in the inflammatory response to *S. pneumoniae*. Chemokines involved in inflammation, chemotaxis and cell adhesion were also up regulated in response to Ply, confirming existing data. Such experiments provide a valuable insight into genetic responses to Ply and offer the possibility of investigating whole host responses through the use of host based microarray chips, an area that will probably expand in the near future.

Table 1.2 details information derived from investigation of the effect of Ply *in vitro* and usually with only one cell type. Of course, this is not the case *in vivo* with cell to cell signalling and it may be that during infection some of the cells listed in Table 1.2 are never exposed to Ply. Studies on the *in vivo* effects of Ply, either by treatment of animals with purified toxin or by comparisons of animals treated with wild type (WT) *S. pneumoniae* and a Ply negative derivative, therefore reveal more about the role of Ply during infection and are discussed in the following sections.

Table 1.2. Effects of sub-lytic levels of recombinant Ply on cells

Ply Target	Effect	Potential role in pathogenesis
Alveolar and bronchial epithelial cells	Disrupts cells (Rubins et al. 1993) and inhibits cilial beat (Steinfort et al. 1989; Feldman et al. 1990; Adamou et al. 1998)	Facilitates pneumococcal growth and dissemination in the lungs
Pulmonary endothelial cells	Disrupts integrity (Rubins et al. 1992) Activates phospholipase A (Rubins et al. 1994)	Breaches endothelial barrier possibly facilitating dissemination from lung to bloodstream (Rubins et al. 1992) May contribute to tissue damage and inflammation
Microglial and neuronal cells	Induces apoptosis (Braun et al. 2002)	Possible role during meningitis
Neutrophils	Inhibits respiratory burst and migration (Paton et al. 1983a; Nandoskar et al. 1986) Induces necrosis (Zysk et al. 2000) and calcium influx (Cockeran et al. 2001b)	Decreases ability of neutrophils to migrate towards and phagocytose pneumococci Damages neutrophils
Fc fragment of antibody	Activates classical complement cascade in the absence of specific anti-Ply antibody by binding to Fc (Paton et al. 1984; Mitchell et al. 1991).	Reduces serum opsonic activity (Mitchell et al. 1997) Complement attack results in inflammation of host tissue (Mitchell et al. 1997), which facilitates dissemination of pathogen
Monocytes	Stimulates TNF-α and IL-1β release (Houldsworth et al. 1994) Inhibits respiratory burst and phagocytosis (Nandoskar et al. 1986) Up regulation of ICAM-1 (Thornton et al. 2005)	May facilitate pneumococcal adherence by up regulating binding receptors on host cells Prevents pneumococcal clearance Facilitates transmediated endocytosis of neutrophils for pneumococcal clearance, the benefit to pneumococci is not clear
Dendritic cells (DC)	Induces DC apoptosis (Colino et al. 2003)	DCs are critical for primary immune response to pathogens therefore this permits evasion of immune system
Spleen cells	Stimulates IFN-γ and Nitric Oxide release (Baba et al. 2002),	Alteration in adaptive immunity?
B cells	Inhibits antibody production (Ferrante et al. 1984)	But can be used as a vaccine? Is adjuvant important?
Lymphocytes	Blocks proliferation of cells in response to mitogens (Ferrante et al. 1984)	Prevents T-cell proliferation, thereby altering adaptive immunity

1.6. Role of Ply in pneumococcal carriage

Ply has been to shown to facilitate adherence of *S. pneumoniae* to human epithelial cells but it was not essential for successful colonisation of the host (Rubins et al. 1998). Another study suggested that Ply was essential for colonisation of the nasopharynx in serotype 2 but not serotype 3 *S. pneumoniae* (Kadioglu et al. 2002). A recent study has suggested that Ply elicits inflammation during carriage, which actually promotes clearance of pneumococci (van Rossum et al. 2005). The authors suggest that the reason a virulence factor would aid clearance is that the induced inflammation facilitates the spread of pneumococci, therefore sacrifice of some of the population may be required to allow the rest to disseminate and cause infection in susceptible hosts.

Ply has recently been shown to interact with Toll-Like Receptor (TLR) 4 and is thought to be essential in preventing pneumococcal colonisation from progressing to invasive disease (Malley et al. 2003). This interaction is suggested to result in tolerated carriage of the pneumococcus, however, what is not clear is what happens when hosts get IPD. Mice with defective TLR-4 (C3/HeJ) were shown to be more susceptible to fatal pneumococcal infection than WT mice (C3/HeOuJ), highlighting an essential role for TLR-4 in host innate immunity against pneumococcal infection.

To assess whether the cytolytic property of Ply was essential for TLR-4 activation, peritoneal macrophages from C3/HeJ (TLR4 -/- mice) and C3/HeOuJ mice were treated with either WT Ply or PdT (a mutant form of Ply that carries 3 amino acid substitutions D385NC428GW433F that abrogates the haemolytic activity and complement-activating properties of the toxin, discussed in section 1.15.1). PdT was still found to interact with TLR-4 to produce the inflammatory cytokine TNF-α, as C3/HeOuJ mice elicited a TNF-response to PdT (and Ply) whereas the C3/HeJ strain did not. Therefore the presence of Ply, but not its haemolytic or complement activating activities is important for activation of

TLR-4 dependent inflammatory responses. This is in contrast to another study that claimed strains expressing Ply promoted clearance of the pneumococci and this was in a TLR-4 independent manner (van Rossum et al. 2005). These studies differ in that recombinantly purified Ply was used in one and a Ply knockout strain was used in the other. Further investigation into the interaction between Ply and TLR-4 suggests that apoptosis is involved (Srivastava et al. 2005). Ply induced apoptosis of macrophages was shown to be mediated by TLR-4 and that administration of an inhibitor of apoptosis into the nasopharynx of mice significantly increased their susceptibility to IPD (Srivastava et al. 2005). From this work the authors suggest that Ply induced apoptosis and interaction with TLR-4 in the nasopharynx is an innate immune response against pneumococcal disease.

1.7. Pneumococcal pneumonia and bacteraemia

Pneumonia is characterised by fluid in the lungs, which hinders oxygen from reaching the bloodstream through the alveoli. According to WHO (www.who.int/vaccines/en/pneumococcus.shtml), more children die each year from pneumonia than any other infectious disease including AIDS and Malaria. *S. pneumoniae* is the leading cause of pneumonia and is thought to be responsible for the deaths of 1 million children every year, mostly in developing countries. In industrialised countries, *S. pneumoniae* is also the most common cause of community acquired pneumonia with 100,000 cases/population and a 10-20% mortality rate, though this is greater in high-risk groups such as infants, the elderly and HIV patients (Johnston 1991; Amdahl et al. 1995). Recently differences in serotype distribution have been identified, with IPD from serotype 11A exclusively associated with adult patients with underlying diseases such as cancer and HIV whereas serotype 1 was more likely to cause disease in otherwise healthy adults (Sjostrom et al. 2006).

Bacteraemia is characterised by the presence of bacteria in the bloodstream and is a condition that progresses rapidly. There is a high mortality rate associated with pneumococcal bacteraemia and it is often associated with pneumonia, after the pneumococci enter the bloodstream. Antibiotics (in particular penicillin and clarithromycin) are usually effective in the treatment of pneumonia and bacteraemia though with the rise of antibiotic resistant strains, this may not be the case for much longer. Vaccines offer the most effective protection against pneumococcal disease (see section 1.13).

1.7.1. Role of Ply in pneumococcal pneumonia and bacteraemia

Ply knockout strains of *S. pneumoniae* are less virulent than WT strains during pneumococcal pneumonia (Berry et al. 1989b) and bacteraemia (Berry et al. 1989b; Berry et al. 1992), indicating an important role of Ply in the pathogenesis of these diseases. It is thought that Ply significantly contributes to stimulation of the host inflammatory response during pneumonia, as mice infected with Ply negative *S. pneumoniae* have a delayed and less intense cell influx into the lungs (Kadioglu et al. 2000). Ply has been shown to be particularly important during the early stages of pneumococcal pneumonia and bacteraemia (Benton et al. 1995; Canvin et al. 1995). The inflammation caused by the release of Ply is thought to facilitate pneumococcal dissemination throughout the host (van Rossum et al. 2005). It is thought that Ply prevents the initiation of host immunity for the first few hours, possibly diverting the immune system, to allow the pneumococci to establish within the host.

One of the major host responses to pneumococcal pneumonia is the recruitment of neutrophils from the vascular space to the alveolar airways by cytokines such as TNF-α,

IL-1 and IL-8 (or KC in mice). Neutrophils are the major immune cells responsible for clearance of the pneumococci from the lung (Jones et al. 2005) and neutrophil recruitment during pneumococcal pneumonia has been shown to be Ply dependent (Jounblat et al. 2003; Moreland et al. 2005), demonstrated in Figure 1.1. Neutrophil recruitment from the circulation has also been identified in the nasopharynx in response to pneumococcal colonisation (van Rossum et al. 2005).

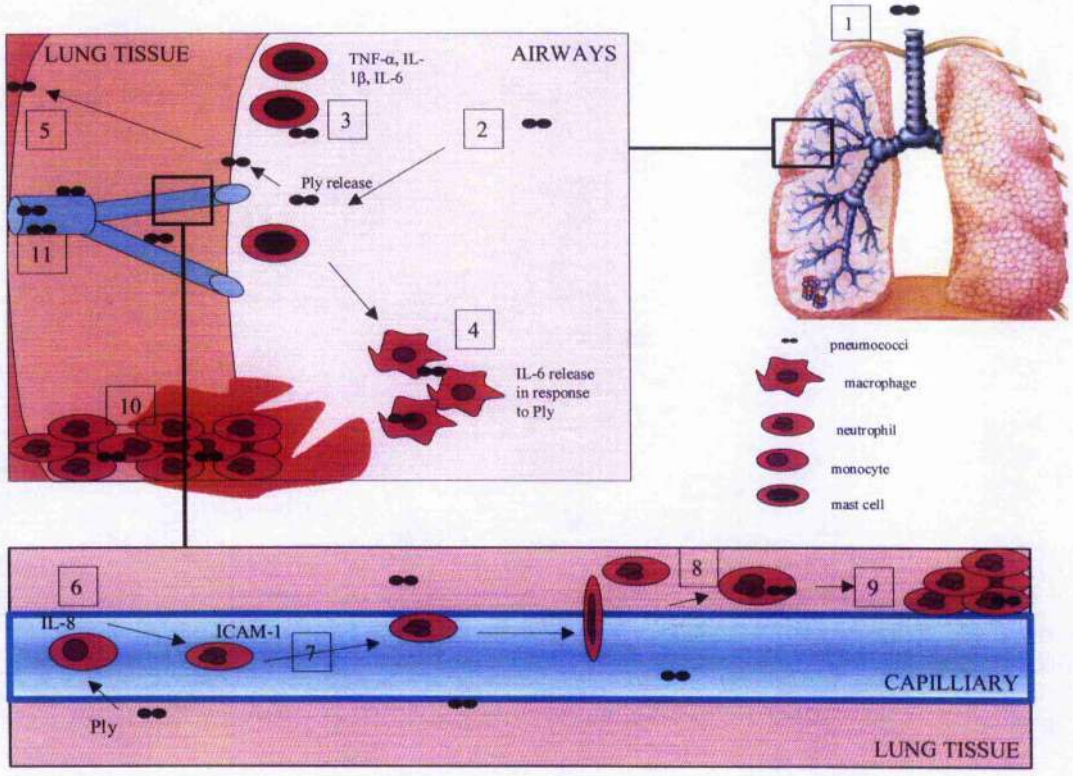
Recruitment of leukocytes (neutrophils, monocytes, eosinophils, lymphocytes and basophils) to sites of infection involves cell adhesion molecules, in particular ICAM-1 (intracellular cell adhesion molecule-1). Expression of ICAM-1 on the leukocyte cell surface adheres the leukocytes to the vascular endothelium enabling the leukocytes to migrate to sites of inflammation (Figure 1.1). Ply has been shown to up regulate the expression of ICAM-1 in human monocytes using both recombinant Ply and comparing serotype 3 (WU2) and serotype 2 (D39) *S. pneumoniae* with isogenic *ply* knockout mutants (Thornton et al. 2005). However, another research group found that both WT and a Ply negative mutant of D39 *S. pneumoniae* up regulated ICAM-1 in endothelial cells, indicating that at least for endothelial cells ICAM-1 expression is not Ply dependent (Moreland et al. 2005).

The regulation of cell adhesion molecules is controlled by inflammatory cytokines such as TNF-α and IL-8 (See section 1.10 for cytokines involved in pneumococcal infection). It is possible that the induction of cytokine release (e.g. IL-8 and the murine homolog KC) from monocytes and neutrophils by Ply (Cockeran et al. 2002; Rijneveld et al. 2002b; Thornton et al. 2005; Kirkham et al. 2006a), results in the up regulation of ICAM-1 to recruit leukocytes to the site of infection. In contrast to other studies, IL-8 production by endothelial cells was not found to be Ply dependent (Moreland et al. 2005). The variation in results may be due to the different cell types investigated.

Alveolar macrophages are the predominant cell type in bronchoalveolar space of healthy animals and there is a rapid recruitment of macrophages during inflammation (Reynolds 1987). These cells are involved in the immune response to pneumococcal pneumonia by phagocytosing invading bacteria and also inducing pulmonary inflammatory responses by secreting inflammatory mediators such as TNF-α and IL-6 that recruit other cells types, induce phagocytosis and antigen presentation (Reynolds 1987; Cavaillon 1994).

Previously, amino acid substitutions have been introduced into Ply in order to investigate whether it is the cytolytic (haemolytic) activity or complement activating ability of the toxin that contributes to pneumococcal virulence (Berry et al. 1995). These mutants are detailed in section 1.15.1. The cytolytic activity of Ply was demonstrated to be more important for virulence during bacteraemia rather than complement activating activity. The complement system is essential to innate immunity by protecting hosts from bacterial infections (Walport 2001). Humans with complement deficiencies are more susceptible to pneumococcal infection (Alper et al. 1970; Alper et al. 2003). During pneumococcal pneumonia, the complement protein C3 has been shown to be important for host survival during the initial stages of infection (Kerr et al. 2005b). By constructing a single amino acid substitution (D385N), the complement activating ability of Ply is abrogated (Mitchell et al. 1991). Cytolytic activity was shown to be important during early bacterial growth at 3h and 6h and for acute lung injury, whereas, the complement activating function was important for bacteraemia at 24h post infection (Rubins et al. 1996). The complement-activating factor of Ply has been shown to affect T cell accumulation and the cytolytic activity influences neutrophil recruitment into lung tissue during pneumonia (Jounblat et al. 2003).

Figure 1. 1. Interaction of Ply and the host immune system during pneumococcal pneumonia



1: Pneumococci enter the lung airways via the upper respiratory tract. Phosphorylcholine of the pneumococcal cell wall binds to PAF receptors on the respiratory tract epithelial cells to facilitate pneumococcal invasion into the lungs (Cundell et al. 1995). 2: Upon entering the bronchoalveolar airspace, pneumococci invade the lung epithelium, releasing the major virulence factor Ply. 3: Mast cells are in abundance at the lung/airway interface (Abraham et al. 1997) and may form part of the first defence against pneumococcal infection. They release prestored cytokines such as TNF-α to recruit immune cells to the site of infection inducing production of more proinflammatory cytokines. 4: Alveolar macrophages residing in the bronchoalveolar space are also some of the first immune cells to attack pneumococci. They phagocytose pneumococci and release inflammatory cytokines, which recruit more immune cells. 5: Pneumococci that successfully evade such immune responses then disseminate throughout the host. Ply destroys lung epithelial cells allowing pneumococcal invasion into the lung tissue. 6: Leukocytes in the blood release IL-8 in response to Ply (Cockeran et al. 2002). Monocytes do not need to interact directly with the bacteria, instead soluble inflammatory mediators such as those released by mast cells and macrophages can up regulate cytokine release from monocytes. IL-8 is a neutrophil

chemoattractant that directs the recruitment of neutrophils to the site of infection. 7: Ply up regulates ICAM-1 expression on the cell surface of leukocytes (Thornton et al. 2005), probably indirectly by Ply inducing IL-8 production. 8: Up regulation of ICAM-1 facilitates transmediated endocytosis of neutrophils from the blood to the site of infection, e.g. the lung tissue in the case of pneumococcal pneumonia. The complement system is important in the innate immune defence against pneumococcal invasion (Paterson et al. 2006a), including pneumonia (Kerr et al. 2005b). Ply has been shown to activate the complement cascade (Paton et al. 1984) and the inflammation induced by complement activation is thought to aid the spread of pneumococci. Complement products C3a and later C5a, in addition to IL-8, are neutrophil chemoattractants. Neutrophils phagocytose the pneumococci via C3b complement based opsonophagocytosis, resulting in the release of more cytokines, which induces further neutrophil (and macrophage) recruitment to the site of infection and therefore further phagocytosis of pneumococci (9). Ply release from pneumococci has been shown to induce nitric oxide (NO) production *in vitro* from host cells (Braun et al. 1999), this makes macrophages that have undergone phagocytosis more susceptible to apoptosis, resulting in clearance of engulfed pneumococci (Marriott et al. 2004). High levels of Ply release may increase the survival of invading pneumococci by inhibiting neutrophil and monocyte respiratory burst (Paton et al. 1983a). The mass influx of neutrophils into the lung and high levels of NO are, at least in part, responsible for acute inflammation in the lung during pneumococcal pneumonia (10). Acute inflammation results in oedema and release of serum proteins, which can act as a nutrient source and mode of transport for the pneumococci, thereby facilitating their spread throughout the host. If pneumococci are successful in evading the immune responses in the lung they can enter the bloodstream and cause bacteraemia (11). (Schematic of lungs from the oxford illustrated science encyclopaedia, www.oup.co.uk). This figure has been composed from *in vivo* experiments and *in vitro* analysis permitting hypothesis of the roles of cells during pneumococcal disease.

1.8. Pneumococcal meningitis

Meningitis is characterised by inflammation of the meninges (fluid filled membranes that surround the brain and spinal chord) and can be caused by viral or bacterial pathogens. *S. pneumoniae* causes the most severe form of meningitis with 40-75% of cases of pneumococcal meningitis in developing countries resulting in death or disability (www.who.int/vaccines/en/pneumococcus.shtml). There are often devastating sequelae associated with meningitis with high possibility of mental retardation, learning disabilities, focal neurological difficulties and hearing loss in patients that survive the infection (Bohr et al. 1984; Bohr et al. 1985).

The mechanism by which colonising pneumococci then cause meningitis remains unclear. It was originally thought that pathogens must gain access to the blood prior to invading the central nervous system (CNS), however, a recent report indicates that *S. pneumoniae* can enter the CNS directly via the olfactory neurones (van Ginkel et al. 2003). Providing there is no intervention by the immune system, circulating pneumococci can breach the Blood Brain Barrier (BBB) and colonise the CSF. Once in the CSF there is a massive inflammatory response by the host with intense leukocyte recruitment that often results in brain injury (Hirst et al. 2004a).

1.8.1. Role of Ply in pneumococcal meningitis

Although Ply is a potent neurotoxin, a decade ago Ply was not considered to be essential for the pneumococcus to cause meningitis (Friedland et al. 1995). It was shown that although recombinant Ply caused inflammation in rabbits when injected intracisternally a Ply negative strain of *S. pneumoniae* caused similar levels of inflammation to the wild type strain. However, further research has changed this view. Wellmer et al showed that intracerebral infection of mice with a *ply* negative strain of *S. pneumoniae* was less virulent

than the parent strain with reduced bacterial loads in the blood (Wellmer et al. 2002). However the cerebellar bacterial titres and meningeal damage were similar following infection whether Ply was present or not. Zysk et al have indicated that Ply is important during meningitis and that it may aid in penetration of the BBB to allow the pneumococci access to the CSF (Zysk et al. 2001). This was demonstrated *in vitro* with brain microvascular endothelial cells using both purified Ply and Ply expressing pneumococci.

Ply knockout strains have been used in a guinea pig model of pneumococcal meningitis to demonstrate that hearing loss, as a result of cochlear damage, is Ply dependent (Winter et al. 1997). Purified Ply has also been demonstrated to be toxic to ependymal cells (the cells that separate CSF from neuronal tissue) (Mohammed et al. 1999; Hirst et al. 2000) and to mediate apoptosis of human microglial cells (Braun et al. 2002). Braun et al have also shown that *S. pneumoniae* deficient in Ply and H_2O_2 production was unable to cause apoptosis of brain cells in comparison with the wild type parent strain (Braun et al. 2002). There are concerns with β-lactam treatment of pneumococcal meningitis patients in that it results in the release of Ply from the lysed pneumococci into the CSF and this may add to neurological damage (Spreer et al. 2003a).

1.9. Acute Otitis Media

Acute otitis media (AOM) is a middle ear infection that is highly prevalent in children, with an estimated 7 million cases of AOM in America each year (www.who.int/vaccines/en/pneumococcus.shtml) and even higher incidence rates in Australian aboriginals with 25-50% of children suffering from AOM at least once in their lives (Morris et al. 2005). Up to 50% of all AOM is caused by *S. pneumoniae* (Prellner et al. 1999), which is thought to migrate along the Eustachian tube from the nasopharynx. Such high levels of infection impose a significant burden on healthcare systems and there can be problems with integration of children into school that have suffered hearing loss as a result

of pneumococcal AOM. Serogroups 3, 19 and 23 may be more associated with an ability to cause otitis media than other types (Hausdorff et al. 2000a), however, other studies have failed to identify AOM associated serotypes or clonal groups (Hanage et al. 2004).

1.9.1. Role of Ply in AOM

The role of Ply in AOM is not known and it is thought that teichoic acids on the cell wall are more involved in inducing inflammation than the toxin. The ability of a Ply negative serotype 3 pneumococci to cause AOM in chinchillas was not significantly attenuated compared with WT serotype 3 (Sato et al. 1996). Anti-Ply antibodies have however been identified in the middle ear fluid of patients with AOM (Virolainen et al. 1995) and direct instillation of Ply into the cochlea of guinea pigs damages the inner and outer hair cells, suggesting that Ply is responsible for clinical outcomes such as deafness following AOM and meningitis (Comis et al. 1993). Purified streptolysin O (SLO), the cholesterol-dependent toxin produced by *Streptococcus pyogenes* (see section 1.12) that is related to Ply, has been shown to cause permeabilisation of the round window membrane of guinea pigs (Engel et al. 1998). The authors suggest that SLO is the cause of hearing loss in patients with otitis media caused by *S. pyogenes*.

1.10. Cytokines involved in pneumococcal infection

The host response to pneumococcal infection involves the mediation of phagocytes as the first line of the innate immune defence. During pneumococcal pneumonia, resident alveolar macrophages and, a few hours later, recruited neutrophils are the two major cell types involved in fighting infection (Figure 1.1). Recruitment of inflammatory cells to the site of infection is orchestrated by soluble proteins known as cytokines and chemokines (chemotactic cytokines) produced by the host immune system. Cytokines often function synergistically to control immune responses and they have different functions depending

on the cells involved, the stage at which they are produced and the combination of mediators involved (Borish et al. 2003). Cytokine production is a feature of both adaptive and innate immunity. During adaptive immunity, antigen-presenting cells stimulate cytokine production during the processing and presentation of antigen to T cells. The innate immune system uses pattern recognition receptors, such as Toll-like receptors (TLR), on cell surfaces that stimulate cytokine production upon recognition of an antigen (Borish et al. 2003).

Tumour Necrosis Factor (TNF)-α, Interleukin (IL)-1, IL-6, IL-8, IL-10, IL-12, Interferon (IFN)-γ (though this does not play a protective role in pneumococcal pneumonia as it does in pneumonia caused by other bacterial species), Macrophage Inflammatory Protein (MIP)-2 and murine cytokine-induced neutrophil chemoattractant KC (Ziegler-Heitbrock et al. 1992; Bergeron et al. 1998; Ebong et al. 1999; Madsen et al. 2000; Rijneveld et al. 2002a; Ling et al. 2003; Zwijnenburg et al. 2003; Albiger et al. 2005; Jones et al. 2005) have been identified as being involved in the complex host response to pneumococcal infection, though their exact roles remain undetermined. The balance of the host cytokine response to infection is crucial to the outcome of pneumococcal disease, as discussed below for TNF-α.

1.10.1. TNF-α

Mononuclear phagocytes, neutrophils, mast cells, activated lymphocytes, natural killer (NK) cells and endothelial cells produce TNF-α. This cytokine activates neutrophils, controlling their adherence, chemotaxis, degranulation and respiratory burst. A TNF-α response to pneumococcal infection has been shown to be essential for survival as CBA mice, that do not produce as much TNF-α in response to pneumococcal pneumonia as BALB/c mice, succumb to disease in contrast with BALB/c mice that survive infection

(Kerr et al. 2002). Although TNF-α is essential in a successful immune response to antigens, it also has detrimental effects on the host including vascular leakage and it is the main mediator involved in toxic shock and sepsis (Borish et al. 2003).

1.10.2. IL-6

The greatest source of IL-6 is from mononuclear phagocytic cells but T and B cells, endothelial cells, fibroblasts and hepatocytes also produce IL-6. IL-6 has pro and anti-inflammatory properties however during pneumococcal infection it is thought to play an anti-inflammatory role (Wang et al. 2000) by inhibiting TNF-α and IL-1β production. Serum IL-6 and TNF-α levels are elevated in mice with pneumococcal sepsis (Albiger et al. 2005). IL-6 has also recently been shown to induce the production of hepcidin from the liver (Nemeth et al. 2004), which sequesters free iron in the blood making it unavailable to pathogens thereby inhibiting their growth in the host. IL-6 production and hepcidin production have recently been linked during pneumococcal sepsis (Albiger et al. 2005).

1.10.3. KC

Produced by many cell types, KC (and the human homolog, IL-8) is involved in inducing neutrophil recruitment to sites of infection and degranulation (Cockeran et al. 2002; Borish et al. 2003). KC is a potent murine chemoattractant for neutrophils and is found in high levels in the lungs of mice with experimental pneumococcal pneumonia (Albiger et al. 2005). KC has also been shown to induce leukocyte recruitment into the CSF during pneumococcal meningitis (Zwijnenburg et al. 2003). The complexity of leukocyte recruitment during pneumococcal infection is an area currently under investigation, however, it does involve Ply (Kadioglu et al. 2000; Thornton et al. 2005), see section 1.7.1 and Figure 1.1.

1.10.4. Role of Ply in cytokine induction

The stimulation of cytokine production during pneumococcal infection has been indicated to be at least partly due to the release of Ply (Benton et al. 1998; Kerr et al. 2005a). IL-6, KC and MIP-2 levels are high in mice following treatment with purified Ply and cytokine production tends to be localised to the site of instillation of the toxin (Rijneveld et al. 2002b). The stimulation of cytokines may be a result of Ply's pore forming activity as mice treated with Ply carrying a point mutation that reduces haemolytic activity had lower cytokine responses than WT treated mice (Rijneveld et al. 2002b). In tissue culture experiments, IL-1β, TNF-α (Houldsworth et al. 1994) and IFN-γ (Baba et al. 2002) release has been observed in response to treatment with Ply. However, when Ply was administered intranasally to mice these cytokines were not detected in the BALF, lung tissue homogenate or serum within 24h post treatment (Rijneveld et al. 2002b; Kirkham et al. 2006a).

1.11. Cytokines involved in thermoregulation

Inducers of febrile responses in humans such as bacterial or viral infections often induce a hypothermic response in rodents (Roberts 1979), for example pneumococcal infection elicits a hypothermic response in mice (Kerr et al. 2002) but humans usually develop a febrile response. This may be due to the inability of rodents to maintain their core body temperature as they have a large surface area in comparison to body weight. Ambient temperature, host genetic background and age have been shown to be important in determining whether rodents develop a hypothermic or febrile response to LPS treatment (Derijk et al. 1994; Romanovsky et al. 1998). Hypothermia or fever during infection is a survival mechanism that can decease mortality (Romanovsky et al. 1998). This has been demonstrated with pneumococcal pneumonia in two strains of mice where the strain that exhibited a hypothermic response survived the infection whereas the strain that did not

elicit such a response succumbed to infection (Kerr et al. 2002). Hypothermia has been shown to attenuate lung injury by reducing the number and function of circulating neutrophils (Lim et al. 2003) and this was the case during pneumococcal pneumonia where animals that exhibited a hypothermic response had heightened TNF-α responses and different recruitment patterns of immune cells into the lungs (Kerr et al. 2002).

The main cytokines involved in the regulation and induction of hypothermia or fever are TNF-α, IFN-γ, IL-1 β, IL-6 and IL-10 (Leon 2004). TNF-α is involved in the initiation of hypothermia whereas IL-6 is involved in thermoregulation and anorexia during sepsis (Leon et al. 1998; Remick et al. 2005). Whether IL-6 is involved in hyperthermic or hypothermic responses during infection is unclear as there are many factors that can influence thermogenesis. One major influence is the ambient room temperature as was shown in an experimental sepsis model where mice maintained in a 30°C room developed fever but mice maintained at 22°C became hypothermic (Remick et al. 2005). In both cases the mice had elevated IL-6 levels during sepsis. In a model of experimental sepsis in ambient conditions, IL-6 -/- mice did not develop hypothermia but mortality was not altered compared with WT mice that did exhibit a hypothermic response. IL-6 is therefore involved in thermoregulation during infection, however other factors determine whether there is a hypothermic or hyperthermic response and also the outcome of disease. There are indications that IL-6 is the main inducer of hypothermia during bacterial infection (Leon 2004). IL-10 is an anti-inflammatory cytokine that is responsible for switching off inflammatory responses. IL-10 has been shown to modulate TNF-α production and therefore control hypothermia (Leon 2004).

Mild hypothermia modifies cytokine production from immune cells, favouring the production of pro-inflammatory cytokines. This has been shown with isolated human monocytes that are treated with LPS and incubated under hypothermic or normothermic

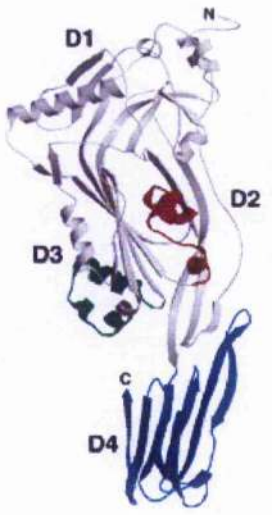
conditions for 48h. Mild hypothermia was found to result in an increase in IL-1β, IL-6, IL-12 and TNF-α production from monocytes treated with LPS (Matsui et al. 2006). The clinical relevance of this remains unclear, as does the actual function of these inflammatory cytokines during hypothermia. It is not known whether during infection, changes in body temperature result in the induction of pro-inflammatory cytokines or whether the production of pro-inflammatory cytokines induces the change in body temperature. In Dantzer's review 'How do cytokines say hello to the brain?' (Dantzer 1994), he points out that it is inadequate to think of the symptoms of infection and inflammation to merely be mediated by cytokines acting on the brain. Instead the interaction of cytokine activated immune cells should be taken into consideration and the neural pathways involved that then activate peripheral sensory nerves. It is generally thought that cytokines do not cross the blood brain barrier but that they interact with target cells at the circumventricular organs, which release signalling molecules such as prostaglandins that can diffuse throughout the brain. Indeed, Ply has been shown to stimulate prostaglandin E_2 (PGE_2) production from human neutrophils (Cockeran et al. 2001a) and during pneumococcal pneumonia there is an abundance of PGE_2 produced in the lungs (N'Guessan P et al. 2006). How such molecules control thermoregulation and behaviour is not yet clear.

1.12. The Cholesterol-Dependent Cytolysins

The cholesterol dependent cytolysin (CDC) family comprises of toxins from at least seven genera of Gram-positive bacteria. The toxins form large pores in cholesterol containing membranes making these toxins cytotoxic to all mammalian cells (Palmer 2001; Gilbert 2002). Identified CDCs and the species that produce them are listed in Table 1.3. Ply is different from all other CDCs as it has no secretion signal sequence and relies upon autolysin A to degrade the cell wall to allow its release into the surrounding environment. However, it has been indicated that LytA may not always be required for Ply release (Balachandran et al. 2001).

The molecular basis of pore formation has been the focus of several studies. Following membrane binding via cholesterol in a perpendicular manner (Ramachandran et al. 2005), the CDC monomers oligomerise into ring-shaped structures of 30-50 monomers and insert into the host cell membrane to create pores (Jedrzejas 2001; Palmer 2001; Gilbert 2002; Giddings et al. 2003). The molecular structures of Perfringolysin O (PFO) (Rossjohn et al. 1997) and Intermedilysin (ILY) (Polekhina et al. 2005) have been resolved. Most Ply structure/function studies use a homology model based on the PFO structure (Figure 1.2) (Gilbert et al. 1999b). CDCs are composed of 4 domains and it is domain 4 that is involved in host cell recognition and binding (see section 1.12.1).

Figure 1.2. PFO model with domains and transmembrane helices highlighted



Structural model of PFO with transmembrane helices (TMH) highlighted in red (TMH1) and green (TMH2) (Ramachandran et al. 2002). Domains are labelled D1 to 4 and the host cell-binding domain is highlighted in blue.

1.12.1. Cholesterol as the binding receptor of CDCs

CDCs bind to eukaryotic cell membranes as well as synthesised cholesterol-containing liposomes. A generally conserved undecapeptide region of domain 4 (ECTGLAWEWWR) has been shown to be involved in host cell binding (Jacobs et al. 1999) and is widely thought to form the receptor for cholesterol recognition on eukaryotic cells (Alouf 2000; Tweten et al. 2001). There is some variation at this region within the CDCs (Billington et al. 2000), with ILY, the human specific CDC possessing the most diverse sequence (GATGLAWEPW_R). This variation has been shown to be responsible for the human specificity of ILY (Nagamune et al. 2004). The undecapeptide region forms a loop that is thought to act as a dagger for insertion into lipid bilayers. It has been suggested that cholesterol is involved in insertion of oligomers into the membrane rather

than cell binding (Giddings et al. 2003). Recombinant forms of domain 4 of Ply or Streptolysin O (SLO) can block binding and subsequent pore formation of the native toxin to the host cell membrane (Baba et al. 2001; Weis et al. 2001). This demonstrates that domain 4 controls host cell binding, however, domain 4 does not self-associate to create pores, it is domain 3 that is instrumental in oligomerisation and pore formation. Intermedilysin is an exception in the CDC family in that it is human specific and the cytolytic action of this toxin is not blocked with pre-incubation with cholesterol (Nagamune et al. 2004). In 2004, the complement control protein CD59 was identified as the binding receptor for intermedilysin (Giddings et al. 2004).

Table 1.3. Bacteria that produce cholesterol dependent cytolysins (CDCs)

Species	CDC produced
Arcanobacterium pyogenes	Pyolysin
Bacillus anthracis	Anthrolysin O
B. cereus	Cereolysin
B. thuringiensis	Thuringiolysin O
Brevibacillus laterosporous	Laterosporolysin
Clostridium perfringens	Perfringolysin O
C. bifermentas	Bifermentolysin
C. botulinum	Botulinolysin
C. chauvoei	Chauveolysin
C. histolyticum	Histolyticolysin
C. septicum	Septicolysin
C. tetani	Tetanolysin
C. novyi	Novyilisin
Listeria ivanovii	Ivanolysin O
L. monocytogenes	Listeriolysin O
L. seeligeri	Seeligeriolysin O
Paenibacillus alvei	Alveolysin
Streptococcus canis	Streptolysin O
S. equisimilis	Streptolysin O
S. intermedius	Intermedilysin
S. pneumoniae	Pneumolysin
S. pyogenes	Streptolysin O
S. suis	Suilysin

Data from a CDC review (Billington et al. 2000), with the exception of Anthrolysin O produced by *Bacillus anthracis* which was identified and characterised in 2003 (Shannon et al. 2003).

1.12.2. CDC oligomerisation and pore formation

Cysteine labelling of amino acids throughout PFO was used to reveal which parts of the molecule are in hydrophilic or hydrophobic environments during pore formation (Shepard et al. 1998). The cysteine in each mutant was derivatised with NBD, (N'dimethyl N [iodeacyl]-N'- [7 nitrobenz-2-oxa-1, 3-diazolyl] ethylenediamine). When the derivatised cysteine is in a hydrophilic environment, the fluorescence is quenched but upon moving into the hydrophobic lipid bilayer, the fluorescence intensity increases. From this work, three α-helices in domain 3 were shown to move from the hydrophilic environment into the hydrophobic membrane during pore formation; suggesting that the α-helices form an

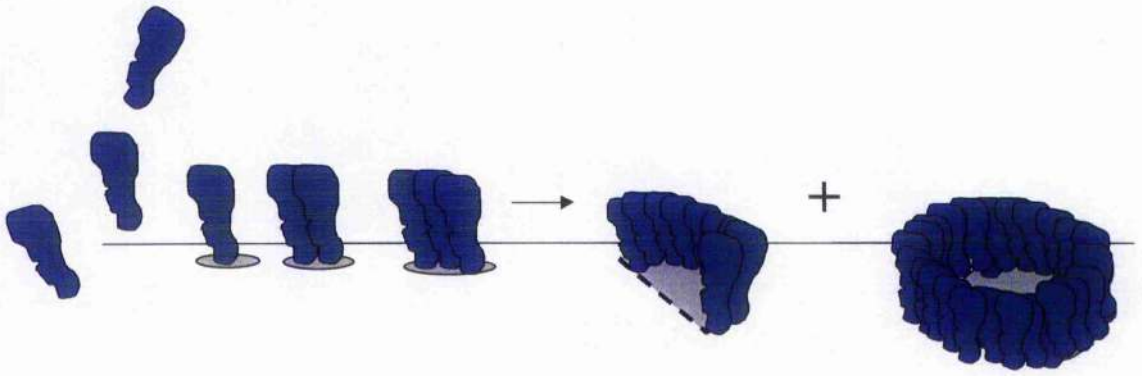
anti-parallel amphipathic β-hairpin during oligomerisation. A year later another region in domain 3 was identified to behave in a similar manner (Shatursky et al. 1999). This led to the theory that 2 hairpin loops (Figure 1.2) from each domain 3 monomer combine within the oligomers to form a β-barrel pore within host cell membranes. In 2000, Tweten and colleagues strengthened this data by showing that domain 3 of PFO inserts into the host cell membrane following binding to initiate pore formation (Heuck et al. 2000).

Cryo-Electron Microscopy has been used with Ply to reveal that domain 2 of the CDCs is situated on the outside of the oligomers and domain 3 and 4 face inwards (Gilbert et al. 1999b), this is also the case in PFO (Dang et al. 2005a). Domain 4 also appears to rotate during oligomerisation (Gilbert et al. 1999b). The mechanism by which CDC monomers oligomerise and insert into the membrane to form pores is a subject of intense research with two main theories as to how pores are formed.

1.12.2.1. Simultaneous binding and insertion theory

Weis and Palmer suggested that CDC monomers independently insert into the lipid bilayer, 'skating' around the membrane surface and colliding with other monomers to associate and form multimers in a unidirectional or bi-directional manner (Weis et al. 2001). A schematic is shown in Figure 1.3. Pores are then thought to form when oligomerisation is stopped by a limiting factor, possibly toxin concentration or oligomer size. This stage may be controlled by a conserved tryptophan in domain 4 of CDCs as Korchev and co-workers showed that a W433F substitution made in Ply increased the functional size of pores but reduced their frequency (Korchev et al. 1998). This theory allows for an explanation of arcs that are observed by EM on membranes treated with CDC toxins. Arcs may be incomplete pores, however, it is difficult to imagine that a free edge of lipid is viable.

Figure 1.3. Simultaneous binding, insertion and pore formation theory



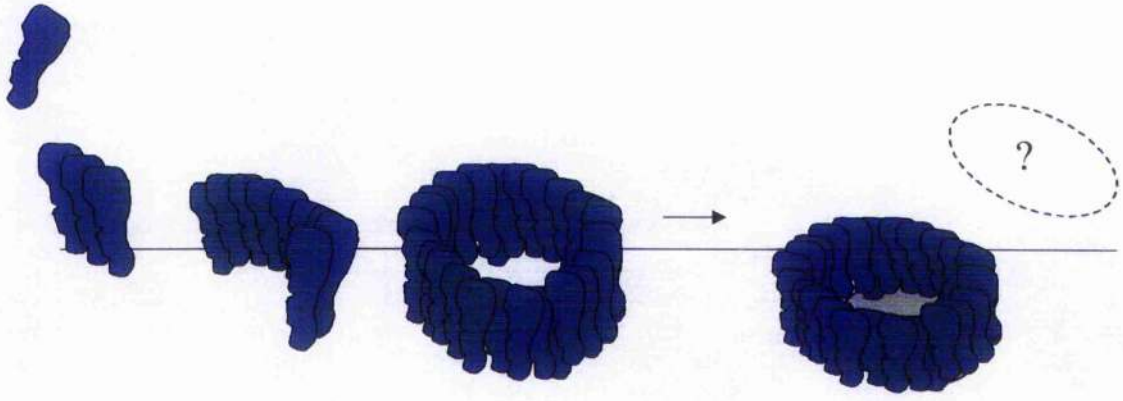
Monomers of CDC toxin (in blue) bind to and insert into lipid membranes either independently or as dimmers and trimers. As the oligomer grows so does the hole in the membrane. This process may stop to form arcs or continue to form pores. The dashed line indicates the 'free edge' of lipid.

1.12.2.2. The 'prepore' theory

It has recently been proposed that CDCs oligomerise on the surface of membranes to form 'a prepore complex' prior to insertion of the pore into the lipid bilayer (Shepard et al. 2000), see Figure 1.4. This group have continued to strengthen this theory with intricate investigation of the interaction of the domains of PFO and the host cell membrane (Hotze et al. 2001b; Hotze et al. 2002; Heuck et al. 2003; Czajkowsky et al. 2004; Dang et al. 2005a; Ramachandran et al. 2005). Atomic force microscopy has been used to demonstrate that the prepore collapses upon membrane insertion (Czajkowsky et al. 2004) with a reduction in height between a mutant form of PFO that remains in the prepore form (Hotze et al. 2002) and the inserted PFO pore. This theory has been further strengthened by the use of Cryo-EM with Ply to directly observe the transition from prepore to pore (Tilley et al. 2005). The prepore to pore theory can also explain data such as the ability of Ply to independently form oligomers in solution (Gilbert et al, 1998). However, insertion of such a large entity (the prepore) into the cell membrane would require a large amount of

energy. Ramachandran proposed that the energy for this insertion comes from the unfolding of two transmembrane helices in domain 3 upon oligomerisation which act as coiled springs (Ramachandran et al. 2005). Arcs are not accounted for in this theory and although they are evident in the figures of these papers (Czajkowsky et al. 2004; Dang et al. 2005a), their existence is not addressed. Also, the fate of the lipid membrane following pore insertion has not been investigated (Waltz 2005).

Figure 1.4. Prepore theory of CDC pore formation



CDC monomers (blue) bind to the lipid membrane surface but do not insert. The oligomer builds up to form a complete prepore which then inserts into the membrane as one entity. Note the change in height of the prepore as it 'collapses' upon insertion into the membrane (Czajkowsky et al. 2004). What happens to the eliminated piece of membrane, represented with the dashed line, is not known (Waltz 2005).

The prepore to pore theory seems to be more accepted with further research (Gilbert et al. 1999a; Tilley et al. 2005). Using FRET (Fluorescence Resonance Energy Transfer), Hotze and colleagues suggested that insertion of the prepore into the membrane is an all or nothing process and that monomers can not insert their transmembrane loops independently (Hotze et al. 2001a; Hotze et al. 2002). This has been developed further

with evidence that the TMHs are poised high above the membrane in the prepore and then insert to form the pore (Ramachandran et al. 2005).

It is possible that what actually occurs is a combination of the two theories, as eluded to by Gilbert (Gilbert 2005) or it may be that different CDCs have different mechanisms of pore formation. Indeed, the two theories are based on studies with different CDCs with Tweten's group using PFO and Palmer focusing on SLO as representative CDCs. Although related, there may be differences in their interaction. Indeed PFO was shown to exist in solution as an anti-parallel dimer whereas Ply exists as a monomer (Solovyova et al. 2004) and ILY has a unique human–specific binding receptor though it shares considerable structural homology with PFO (Giddings et al. 2004; Polekhina et al. 2005). Studies, such as those reviewed here for the CDCs, which allow an understanding of the mechanisms of action of virulence factors permits the rational design of vaccine candidates such as genetically modified toxoids.

1.13. Pneumococcal vaccines

Protection against invasive disease was generally accepted to be antibody mediated, with high circulating anti-pneumococcal antibodies (in particular anti-capsule antibodies) in patients that survive pneumococcal infection. However, recent work has disputed this and shown that protection against pneumococcal disease can be independent of antibodies but requires functional CD4+ T cells (Lipsitch et al. 2005; Malley et al. 2005). This research was instigated from the observation that the sharp decline in pneumococcal disease in infants occurs before the natural acquisition of anti-capsular antibodies (Lipsitch et al. 2005). This finding may have major implications for future pneumococcal vaccine design, although the research focuses on CD4+ T cells providing protection from carriage rather than protection against invasive disease (Malley et al. 2005). Currently, the basis of all

licensed pneumococcal vaccines is aimed at raising protective antibodies to capsule polysaccharide.

Advances in the development of pneumococcal vaccines are shown in Table 1.4. The first pneumococcal vaccines were composed of killed pneumococci, but with the advent of antibiotics in the 1940s, such vaccines were thought to no longer be required. With the development of antibiotic resistant pneumococci, which was first observed in Australia and Papua New Guinea in the 1960s and by the 1970s multi-drug resistant strains were responsible for hospital-acquired pneumococcal outbreaks in South Africa (Tomasz 1997), new pneumococcal vaccines were introduced using purified capsule polysaccharide from disease causing serotypes (section 1.13.1). The newest generation of pneumococcal vaccines involve conjugation of capsule polysaccharides to a carrier protein to improve their immunogenicity, especially in infants (section 1.13.2). Although the new polysaccharide protein conjugate vaccine is highly efficacious against disease caused by the vaccine serotypes, protection is not afforded against non-vaccine serotypes. New approaches to pneumococcal vaccine design are currently being investigated with the possible inclusion of conserved pneumococcal proteins to confer species wide protection (section 1.14). Investigation into the use of killed unencapsulated pneumococci as a vaccine has also re-emerged, which has been reported to prevent pneumococcal colonisation and IPD in mice and rats (Hvalbye et al. 1999; Malley et al. 2001).

1.13.1. Pneumococcal polysaccharide vaccines

In 1977, the first pneumococcal vaccine based on purified capsule polysaccharide (CPS) was introduced and was composed of CPS from the top 14 disease causing serotypes. In the 1980s the valency of the pneumococcal polysaccharide vaccine (PPV) was increased to 23 with the amount of polysaccharide for each serotype being reduced from 50μg to 25μg (French 2003). The 23-valent PPV is still administered to adults >65 years of age and to

patients with immune conditions such as asplenia, sickle cell disease and HIV infection where there is an increased risk of IPD (Gebo et al. 1996; Fiore et al. 1999; Pebody et al. 2005). However, the efficacy of PPV in these patients requires further investigation as immunisation trails of 23-valent PPV in Uganda of HIV infected patients proved that the vaccine was ineffective at preventing IPD and in terms of efficacy against pneumonia in HIV patients, 23-PPV was considered detrimental (French et al. 2000). Although PPV protects healthy immunocompetent adults (<65yr) from IPD, protection of the elderly is disputable (Ortqvist et al. 1998) and nonexistent for children <2yr. CPS from serotype 1 is included in the 23-valent PPV vaccine yet it is poorly immunogenic in all age groups (Scott et al. 1996).

Vaccination with free polysaccharide induces production of anti-CPS antibodies in a T-cell independent manner (Weintraub 2003). The injected CPS stimulates the clonal expansion of B-cells by binding and cross-linking sIg on the B-cell. This results in the maturation of B-cells into antibody producing plasma cells. However, these cells have a short life span (a few days) and no immune memory is created. The B-cell response is improved by opsonins e.g. C3 cleavage products which bind to the CPS and act as ligands for CD21 (a complement receptor). Children have poor CD21 expression levels and this may be a reason why PPV is poorly immunogenic in children (Weintraub 2003). As memory B-cells are not produced with PPV, boosting does not increase immunity. The antibody levels produced to free CPS are not long lasting and revaccination is recommended depending on the age/condition of patients. As PPV does not confer protection to infants, the major age group at greatest risk to pneumococcal disease, an alternative to polysaccharide vaccination has been developed (section 1.13.2).

Table 1.4. Development of pneumococcal vaccines

Year	Advances in pneumococcal vaccines
1914	Whole killed pneumococci prevented pneumonia in South African miners*
1930s	Antibodies developed in response to injection of CPS *
1940s	Prevention of pneumonia by immunisation with particular serotypes*
1970s	Protection from CPS proven, leading to the introduction of 14-valent PPV in 1977*
1980s	PPV was modified to include CPS from 23 serotypes*
2000	Licensure of Prevnar in the USA (7-valent PCV)
2001	Licensure of Prevenar in Europe
2001	Re-emergence of investigation into a whole-cell based vaccine (Malley et al. 2001)
2006	Proposed introduction of Prevenar into the UK immunisation schedule
2007	Proposed introduction of a 10-valent PCV (Streptorix, GlaxoSmithKline)
Future	Plans to develop a 13-valent PCV (Wyeth)
Future	Predicted incorporation of pneumococcal proteins into the next generation pneumococcal vaccines (see section 1.14)

* from review in 2003 (French 2003)

1.13.2. Pneumococcal conjugate vaccines

Conjugation of CPS to various proteins has been shown to improve the immunogenicity of CPS and also elicits a T-cell dependent immune response in infants. The first conjugate vaccine to be used in routine childhood vaccination was for protection against *Haemophilus influenzae* type b (Hib b), the major cause of invasive *H. influenzae* disease out of 6 serotypes (Kelly et al. 2004). The protein conjugated to CPS from *H. influenzae* type b varies depending upon the company that produces the vaccine, however, vaccination with these conjugates has successfully resulted in a 90-100% reduction in invasive disease from serotype b *H. influenzae* (Peltola 2000; Kelly et al. 2004). The problem with *S. pneumoniae* is the large number of serotypes and due to the complexity, and therefore expense of the conjugation process, only a small number of polysaccharides can be included in each PCV vaccine. In 2000, Wyeth Vaccines launched Prevnar, a heptavalent PCV using the diphtheria toxoid CRM_{197} as the protein component to confer an increased immune response to the capsule polysaccharides in infants (Black et al. 2000; Pelton et al. 2003). Serotype coverage by Prevnar is limited and varies globally with pneumococcal serotype prevalence (Hausdorff et al. 2000b; Spratt et al. 2000) as only seven out of a possible ninety (Henrichsen 1995) pneumococcal serotypes are included (serotypes 4, 6B, 9V, 14, 18C, 19F and 23F). In 2000, when Prevnar was first introduced the included serotypes covered almost 90% of IPD causing serotypes in North America and Canada but <60% of the predominant serotypes in Asia where serotypes 1 and 5 are the predominant cause of IPD (Hausdorff et al. 2000b).

Prevnar is currently the most effective paediatric vaccine available for protection against pneumococcal disease. However, the extent of protection against pneumococcal pneumonia (in comparison with protection observed against pneumococcal meningitis, bacteraemia and otitis media) is difficult to determine due to the difficulties in diagnosing pneumonia with radiographs (Black et al. 2000; Black et al. 2002; Obaro 2002). In the

Kaiser trial Black and co-workers found that overall, Prevnar was effective in reducing pneumococcal pneumonia in children <5 years of age and that globally this vaccine could offer considerable protection to this age group (Black et al. 2002). Prevnar has also been found to be immunogenic in HIV infected adults, regardless of previous exposure to 23-PPV (Miiro et al. 2005) but this vaccine is not as efficacious in elderly populations (Briles 2004).

Vaccination within a population not only protects those that are given the vaccine but can protect non-vaccinated individuals by reducing carriage rates and transmission of disease. This is known as herd immunity and is becoming apparent for pneumococcal disease in the United States since the implementation of childhood vaccination with Prevnar. Since 2000, there has been a dramatic decrease in IPD in non-vaccinated adults from the seven vaccine serotypes. By 2004, a 53% decrease in the 18-39 year group and a 26% decrease in the 40-64 year group of vaccine type IPD has been observed (Whitney 2005). In >65yr olds in America, there has been a decrease in IPD from vaccine types by 75% since the introduction of Prevnar (thought to be due to herd immunity from vaccinated grandchildren), however this coincides with a significant increase of 14% for IPD from non vaccine types (Lexau et al. 2005). There was also a 50% reduction in vaccine type IPD in children out with the vaccination range (<2months and >5-17years) (Whitney 2005).

In order to improve global protection with polysaccharide conjugate vaccines, separate vaccines composed of the most invasive serotypes in each region would be required. The problem with vaccinating against a subset of serotypes is that the serotypes that are circulating in the population change. This has been observed in America since the introduction of Prevnar, which has been so successful in terms of herd immunity, that now coverage of IPD from the seven vaccine serotypes in the United States is currently only

20% (Whitney 2005). This leads to the largest problem with vaccinating against a subpopulation of serotypes, which is vaccine evasion by the pathogen. Termed 'replacement disease' or 'serotype replacement', many studies have monitored serotype distribution and have observed the replacement of vaccine serotypes with non-vaccine serotypes either in carriage or IPD in vaccinated children and in adults as a result of contact with immunised children (Whitney 2005). Carriage studies in children in the United States over four years since Prevnar was introduced have revealed a 98% decrease in vaccine serotypes and a 79% decrease in vaccine-related serotypes but this has coincided with a 148% increase in carriage of serotype 19A (and an increase in 19A disease) and a 45% increase in carriage of other non vaccine types (Pai et al. 2005b; Whitney 2005). Serotype 19A is also becoming increasingly antibiotic resistant and more frequently switching capsule with vaccine type strains (Pai et al. 2005b). In a study of pre and post childhood immunisation with Prevnar, there was found to be an overall reduction in IPD by 19% in adult HIV patients since introduction of the vaccine and a 62% decrease in IPD from the seven vaccine types. However, a 44% increase in IPD from non vaccine serotypes in HIV patients was also observed over this time period (Flannery et al. 2006). Earlier vaccine trials of children vaccinated with Prevnar in Finland revealed a 57% decrease of AOM caused by the seven vaccine serotypes, however, this coincided with a 34% increase in AOM caused by non-vaccine serotypes (Kipli et al. 2000). Such promoted selection for non-vaccine serotypes is a major shortcoming of current pneumococcal vaccines, with an increase in serotype replacement reported by most countries where PCVs have been evaluated or introduced (Obaro et al. 1996; Kipli et al. 2000; McEllistrem M. C. 2003; Frazao et al. 2005); reviewed by Bernatoniene and Finn (Bernatoniene et al. 2005).

Capsule polysaccharide from serotype 1 pneumococci is not included in Prevnar and with the rise in non-vaccine serotypes this situation needs to be closely monitored. Trials with a 9-valent and 11-valent vaccine containing serotype 1 CPS have shown increased protection

against IPD causing serotypes (Klugman et al. 2003; Nurkka et al. 2004), and serotype 1 CPS will be included in the 10-valent PCV soon to be licensed by GlaxoSmithKline (PneumoADIP, www.pneumoadip.org). Although this will significantly increase protection against pneumococcal disease, it will also increase the cost of what is already a vaccine that is too expensive for use in developing countries. CPS from all 90 pneumococcal serotypes cannot be included in one vaccine (Klein 1995), and even within the PCV some CPSs are less immunogenic than others (Kamboj et al. 2003). Therefore research is focusing on alternatives to serotype specific vaccination such as the use of species-common immunogenic pneumococcal proteins in future vaccines (Alexander et al. 1994; Michon et al. 1998; Briles et al. 2001; Ogunniyi et al. 2001).

1.14. Potential candidates for the next generation of pneumococcal vaccines

The use of conserved pneumococcal proteins for vaccine production should confer broad serotype-independent protection against pneumococcal disease in all age groups. Recombinant protein vaccines are cheaper than the prohibitively expensive conjugate vaccines ($50/dose), making protein-based vaccines globally accessible. The main vaccine candidates are pneumococcal virulence factors, in particular surface proteins, as antibodies raised against them are likely to protect against pneumococcal infection. Pneumococcal surface proteins are of particular interest, as antibodies against them should promote opsonophagocytosis of the pneumococci. However, surface-exposed proteins are subjected to more selective pressure and tend to be variable between serotypes. Cytoplasmic proteins are more conserved as they are protected from such environmental pressure. Advances in genomics have opened up research of potential vaccine candidates with lists of putative virulence factors being produced (Tettelin et al. 2001; Wizemann et al. 2001). Potential vaccine candidates are shown in Table 1.5 with their homogeneity throughout the 90 pneumococcal serotypes given where known and also the protection

elicited by each protein. Some proteins protect against colonisation such as PsaA (Briles et al. 2000a; Briles et al. 2000b) and others protect against bacteraemia and/or pneumonia e.g. CbpA and Ply (Paton et al. 1983b; Ogunniyi et al. 2001). Protection is also dependent upon the route of administration for example NanA protects animals against carriage when administered intranasally (Tong et al. 2005) but it is not as protective when administered intraperitonealy (Lock et al. 1988). Whether these proteins eliminate carriage or invasive disease needs to be thoroughly investigated prior to inclusion in vaccines and also the outcome of eliminating carriage needs to be assessed.

The proteins that seem to have the most potential are PsaA and Ply due to their sequence conservation throughout the serotypes and that they are highly immunogenic in animal models (Paton et al. 1983b; Talkington et al. 1996; Briles et al. 2000a; Ogunniyi et al. 2001). PspA was a promising vaccine candidate as it is highly immunogenic and can confer protection against carriage and IPD (Briles et al. 2003), however, when PspA entered Phase I of clinical trials it was found to share 27% sequence similarity with human cardiac myosin (Maleckar 2004); further investigation has ceased.

Table 1.5. Pneumococcal proteins that are potential vaccine candidates

Protein	Sequence conservation	Protection studies (reference)
CbpA (PspC, SpsA)	Only present in 75% of serotypes, highly variable N-terminus (Berry et al. 2000)	Protects mice against bacteraemia and carriage (Brooks-Walter et al. 1999; Ogunniyi et al. 2001)
ClpP	-	Protects against sepsis (Kwon et al. 2004)
LytA	Present in all strains (Neeleman et al. 2004) but sequence conservation not determined	Partially protects mice against infection when used alone (Berry et al. 1989a) or as a carrier protein to 9V CPS (Lee et al. 2001b)
Nan A	Highly diverse (King et al. 2005)	Protects against pneumonia and sepsis though not as well as Ply (Lock et al. 1988) and protects chinchillas against nasopharyngeal colonisation (Tong et al. 2005)
Pht proteins (Histidine triad)	Conserved	Protect mice against lethal sepsis (Adamou et al. 2001) PhpA protects mice against pneumonia (Zhang et al. 2001)
PiaA & PiuA	Highly conserved (Whalan et al. 2006)	Protects against bacteraemia (Brown et al. 2001)
Ply	Originally thought to be highly conserved (Mitchell et al. 1990), but further analysis has revealed variation between and also within serotypes (Kirkham et al. 2006b) (Jefferies et al, manuscript in preparation)	Humans with α-Ply IgG have reduced risk of bacteraemia Human α-Ply IgG passively protects mice from challenge with type 1 and 4 pneumococci (Musher et al. 2001) Protects against pneumonia (Paton et al. 1983b) and bacteraemia (Ogunniyi et al. 2001) An effective carrier protein when conjugated to CPS (Alexander et al. 1994; Lee et al. 2001b)
PpmA	Limited variation (Overweg et al. 2000)	Hyper-immune anti-PpmA serum is effective in phagocytosis of pneumococci (Overweg et al. 2000) Immunogenic in humans (Vainio et al. 2006)
PppA	Conserved	Reduces colonisation (Green et al. 2005)
PsaA	Variable (Berry et al. 1996)	PsaA protects mice against pneumococcal colonisation (Briles et al. 2000a) and sepsis (Talkington et al. 1996)
PspA	Serologically variable (2 families, 6 clades)	Anti-PspA antibodies protect against carriage and invasive disease in humans (Palaniappan et al. 2005) Mucosal immunisation protects mice against carriage (Wu et al. 1997) Confers protection against AOM (White et al. 1999) and pneumonia (Briles et al. 2003)

CbpA, choline binding protein A; ClpP, caseinolytic protease P; Lyt A, Autolysin A; NanA, neuraminidase A; Pht, pneumococcal histidine triad protein; PiaA, pneumococcal iron acquisition protein A; Piu A, pneumococcal iron uptake protein A; Ply, pneumolysin; PpmA, putative proteinase maturation protein A; PppA, pneumococcal protective protein A; PsaA, pneumococcal surface adhesin A; PspA, pneumococcal surface protein A; -, sequence conservation between pneumococcal serotypes has not been determined.

1.14.1. Combinations of pneumococcal proteins may be more efficacious

It may be that a combination of pneumococcal proteins would provide the best protection against pneumococcal disease. This area requires further investigation though some groups are beginning to focus on this (in particular the research groups of David Briles in Alabama and James Paton in Adelaide), see Table 1.6 for a list of vaccination studies with combinations of pneumococcal proteins. There is also developing interest on mucosal presentation of pneumococcal proteins to the host to elicit protection against carriage or pneumonia. Mucosal immunity against pneumococcal pneumonia has been demonstrated by vaccination with *Lactococcus lactis* ghosts displaying combinations of pneumococcal proteins (van Selm et al. 2005). Another area of research is the use of DNA based vaccines where DNA encoding the protein antigen is directly injected into the host to raise a protective immune response. There have been a few studies using DNA encoding pneumococcal antigens such as Ply, type 4 polysaccharide, PspA and PsaA (Lee et al. 2001a; Lesinski et al. 2001; Miyaji et al. 2001; Miyaji et al. 2002; Miyaji et al. 2003). Generally the major problems with DNA vaccination is that a large amount of DNA is required to elicit a good immune response and although promising cell-mediated protection was observed in animal vaccination models, this was not the case in human trials of DNA

vaccination where antibody mediated immune responses were poor if raised at all (Dr. Gill R. Douce, personal communication, 2006).

Table 1.6. Combinations of pneumococcal proteins that increase protection

Protein combination	Reports of additive protection against pneumococci
Ply (PdB) + PspA	Improves protection against bacteraemia (Ogunniyi et al. 2000) and pneumonia (Briles et al. 2003)
Ply (PdB) + PsaA	Protects against bacteraemia better than PdB alone (Ogunniyi et al. 2000) Significantly improves protection against pneumonia (Briles et al. 2003)
PspA + PsaA	Elicits additive protection against bacteraemia (Ogunniyi et al. 2000), pneumonia (Briles et al. 2003) and carriage* (Briles et al. 2000a; Briles et al. 2001)
Ply (PdB) + CbpA	Protects against bacteraemia better than PdB alone (Ogunniyi et al. 2001)
Suggested Ply + Hyl	Double knockout of these genes attenuates pneumococci (Berry et al. 2000)

Ply, pneumolysin; PdB, Ply with reduced cytolytic activity; PspA, pneumococcal surface protein A; PsaA, pneumococcal surface adhesin A; CbpA, choline binding protein A, * when administered mucosally; Hyl, hyaluronidase.

1.15. Potential use of Ply as a vaccine candidate

Pneumolysin, is of particular interest as a vaccine candidate as it is produced by all strains of *S. pneumoniae* (Kalin et al. 1987) and is protective in animal models of vaccination (Paton et al. 1983b; Alexander et al. 1994; Kuo et al. 1995; Michon et al. 1998). Ply could be used alone (Paton et al. 1983b; Alexander et al. 1994) or as a carrier protein to the polysaccharides in current vaccine preparations (Paton et al. 1991; Kuo et al. 1995; Michon et al. 1998), conferring increased protection against pneumococcal disease, including pneumonia that is not well protected by the current conjugate vaccine.

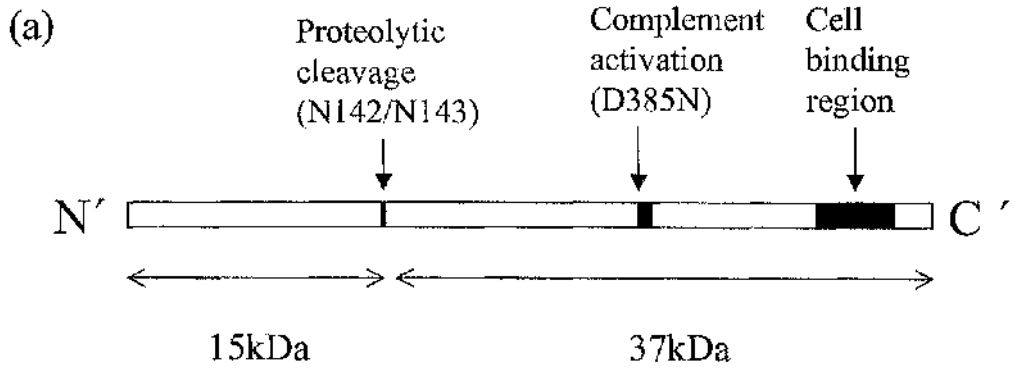
Generally, the amino acid sequence of pneumolysin was thought to be highly conserved throughout all pneumococcal serotypes with little variance over time and geographic distance (Mitchell et al. 1990). However, in 1996 Ply from serotypes 7 and 8 was reported to possess a threonine to isoleucine substitution at amino acid position 172 that reduces the specific activity of the toxin (Lock et al. 1996). Ten years later, Ply from serotype 1 clinical isolates has also been found to be variable (Kirkham et al. 2006b), see chapter 7.

1.15.1. The PdB pneumolysin toxoid and other existing Ply mutants

Immunogenic Ply mutants (Paton et al. 1991; Alexander et al. 1994) with reduced cytotoxicity have previously been constructed (Boulnois et al. 1990; Baba et al. 2001), however, these mutants retain the ability to form pores in host cell membranes. One Ply mutant extensively researched and commonly referred to as the PdB toxoid has a tryptophan to phenylalanine substitution at amino acid position 433 in domain 4 (W433F). This mutant retains 0.1 to 1% haemolytic activity compared with WT Ply (Paton 1996; Korchev et al. 1998). Another Ply mutant with negligible activity was reported (Michon et al. 1998), yet the location of the mutation was not identified and protection against pneumococcal disease was not proven. It is important to note that residual haemolytic activity of Ply is adequate for full virulence of the pneumococcus as demonstrated by the chromosomal replacement of WT Ply with Ply carrying a mutation that reduces the specific activity of the toxin to 0.1% of WT Ply (Berry et al. 1995). PdT is a triple point mutation D385N, C428G, W433F derived from PdB that is devoid of lytic and complement activating activities (Berry et al. 1995). However, this mutant is highly unstable with degradation observed in Western blots with a monoclonal antibody (PLY5) that recognises the C-terminal end of the protein (Prof. Tim J. Mitchell, personal communication, 2005). It may be that the complement activating properties of Ply are of an actual benefit in terms of use as a vaccine candidate as complement activation may promote a protective immune response.

In 1994, a series of Ply mutations were constructed by random mutagenesis and resulted in the first N-terminal mutations that affected haemolytic activity of the toxin (Hill et al. 1994). Substitution of histidine at amino acid position 156 with tyrosine (H156Y) resulted in a 98% reduction in haemolytic activity. In 1996, monoclonal antibodies (mAbs) were raised against various regions of Ply and used to probe the whole toxin and a 'proteinase K nicked' form (de los Toyos et al. 1996). Proteinase K cuts Ply into a 37kDa and 15kDa fragment and sequencing of the 37kDa fragment revealed that the N' terminus began NVPAR (amino acids 143-147, Figure 1.5). This N143 region in Ply has been shown to be highly antigenic by epitope scanning and is recognised by both human sera and rabbit hyper-immune sera (Salo et al, 1993). Some of the mAbs were found to neutralise the haemolytic activity of Ply (de los Toyos et al. 1996). All mAbs probed against Ply recognised both whole Ply and the 37kDa fragment, except mAb Ply 4, which recognised whole Ply but not the fragments, indicating that the epitope for this antibody was within the nicked region. Pre-incubation of Ply with mAb Ply 5 and Ply 8 was found to block binding of the toxin to the host cell membrane whereas pre-incubation with mAb Ply 4 prevented pore formation on liposome membranes.

Figure 1.5. Proteinase K cleaves pneumolysin into two fragments



(b)

(a) When pneumolysin is treated with proteinase K it is cleaved into two fragments (15kDa and 37 kDa) and the 37kDa fragment begins with amino acid N143 (de los Toyos et al. 1996). (b) the site of proteolytic cleavage (N142/N143) is highlighted in red and is in domain 1 at the linker sequence to domain 3.

This implied that the site blocked by mAb Ply 4 (thought to include the N143 region) is responsible for interaction of Ply with other Ply monomers to form pores. Indeed, this region has recently been confirmed to be involved in CDC oligomerisation (Ramachandran et al. 2004), discussed in section 1.12. Fine epitope mapping revealed that the epitope for mAb Ply 4 was actually located further downstream of Ply than previously suggested (Suarez-Alvarez et al. 2003). The Ply 4 epitope was found to be conformation dependent and only recognised fragments of Ply where domain 1 and 3 were together (as occurs in the structural model). From this work, the epitope for mAb Ply 4 was shown to encompass a core structure between amino acids E151 and Y247 (Suarez-Alvarez et al. 2003). This region is highlighted in red in Figure 1.6 and demonstrates that this epitope spans domains 1 and 3. The epitope on Ply for mAb Ply 8 was determined to include amino acids 450 to 458 at the C' terminus, which are known to be involved in host cell binding (see section 1.12). More recent work has shown that these monoclonal antibodies can significantly increase survival times of mice from pneumococcal pneumonia (Garcia-Suarez et al. 2004). Intravenous treatment with combinations of these monoclonal antibodies resulted in an even greater increase in survival times following pneumococcal challenge, suggesting a synergistic effect from the antibodies blocking different actions of Ply such as oligomerisation and host cell binding.

Prior to the epitope mapping in 2003 (Suarez-Alvarez et al. 2003), modifications around the N143 region in Ply were hypothesised to result in a non-oligomerising form of the toxin by altering the Ply-Ply interaction site. A N142N143 deletion and N143D substitution within Ply were previously constructed in our laboratory as initial steps to understanding this region and its role in oligomerisation, however both mutants behaved identically to native Ply in terms of haemolysis and pore formation (Search, 2002).

Figure 1.6. Proposed epitope for mAb Ply 4 that blocks oligomerisation

The epitope in Ply for monoclonal antibody Ply 4 was conformation dependent and fine epitope mapping identified the epitope to encompass amino acids E151 to Y247, highlighted in red (Suarez-Alvarez et al. 2003), that spans domains 1 and 3.

1.16. Aims of this project

The aim of this project was to construct mutations at the site of Ply thought to be involved in monomer-monomer recognition, thereby preventing oligomerisation and pore formation of the toxin. As the project progressed there was more information available as to the regions of Ply that may be involved in oligomerisation (Suarez-Alvarez et al. 2003; Ramachandran et al. 2004). Construction of a non-oligomerising Ply mutant was in view of creating a non-toxic form of Ply for use in the next generation of pneumococcal vaccines. Purification of the mutants would be investigated to determine the most efficient techniques in LPS removal, protein yield and purity. Upon production of a non-oligomerising form of Ply, toxicity of the mutants would be determined using existing assays and with the development of a more sensitive *in vitro* assay. Non-toxic mutants would then be assessed *in vivo* and their immunological properties compared with WT Ply.

Materials and Methods

2.1. Bacterial strains, storage and growth conditions

S. pneumoniae strains were grown from a single colony in BHI (Brain Heart Infusion broth: Oxoid) at 37°C without shaking to mid-log phase (OD_{600nm} 0.6) and stored in 1ml aliquots at -80°C with Protect beads (Technical Service Consultants, Lancashire, UK) or in 10% glycerol (Sigma-Aldrich, Dorset, UK). Strain purity and optochin sensitivity was checked by streaking the culture on BAB plates (Blood Agar Base; Oxoid) supplemented with 5% horse blood (E & O Laboratories, Bonnybridge, UK) prior to freezing. *E. coli* strains were grown overnight from a single colony with the appropriate antibiotic in LB (Luria broth; Sigma-Aldrich) at 37°C with shaking at 200rpm. 1ml aliquots were then stored at -80°C in 10% glycerol.

2.2. Preparation of pneumococcal genomic DNA and *E. coli* plasmid DNA

10ml of mid-log phase grown pneumococci were centrifuged at 3000g for 20 min at 4°C to pellet cells. The culture media was discarded and the cell pellet lysed with lysis buffer (10mM Tris, 100mM EDTA, 0.5% SDS in dH_2O) to extract the genomic (g) DNA using Qiagen midi columns (Qiagen, West Sussex, UK) and following the manufacturers instructions. Plasmids carrying mutations made by site-directed mutagenesis were purified from 5ml overnight cultures of *E. coli* using a plasmid miniprep kit (Qiagen) and following the manufacturers instructions. DNA quantity and quality was monitored by agarose gel electrophoresis.

2.3. Pneumolysin PCR and DNA sequencing

Pneumococcal *ply* genes (1.4Kb) were amplified by PCR from gDNA preparations using Taq polymerase (Promega, Southampton, UK) and primers 27R and 27S (Table 2.1) to

give an amplicon size of 2406bp. PCR products were cleaned using PCR purification columns (Qiagen) and then sent for DNA sequencing (DBS Genomics, Durham, UK) using primers 27R, 4T, 4V, 4W, 9Y and 27T (Table 2.1). Sequence data for each *ply* gene was assembled, aligned and translated using Vector NTI™ software (Invitrogen, Paisley, UK). Plasmid DNA, containing the *ply* gene, was sent for sequencing with the same primers. The *ply* gene was amplified by PCR from gDNA of the 00-3645 strain using primers 9Y and 9Z that encode restriction enzyme sites (Table 2.1, enzyme sites underlined) for subsequent digests to give an amplicon size of 1418bp. The 00-3645 Ply PCR product was cut with *Bam*HI and *Sac*I (Promega) and ligated into *Bam*HI/*Sac*I digested pET33b (Merck Biosciences) to give pETply00-3645. This plasmid was transformed into *E. coli* XL-1 cells (Stratagene, Amsterdam, Zuidoost, Netherlands) and the sequence of the insert confirmed. Work with 00-3645 was in collaboration with Dr. Johanna Jefferies and Ms Yu Jing.

Table 2.1. Primers used for *ply* PCR and DNA sequencing

LAB reference	Sequence 5′-3′
4V	CAATACAGAAGTGAAGGCGG
4T	GTTGATCGTGCTCCGATGAC
4W	GATCATCAAGGTAAGGAAGTC
27R	CTTGGCTACGATATTGGC
27S	TACTTAGTCCAACCACGG
27T	ATAAGTCATCGGAGCACG
9Y	CGGGATCCGGCAAATAAAGCAGTAAATGACTTT
9Z	GACGGAGCTCGACTAGTCATTTTCTACCTTATC
15C	GGAGGTAGAAGATGGCAAATAAAGC
15D	CTAGTCATTTTCTACCTTATCCTCTACC

2.4. Multi-locus Sequence Typing (MLST)

MLST was carried out at the Scottish Meningococcal and Pneumococcal Reference Laboratory using previously described primers (Enright et al. 1998) and a semi-automated method (Jefferies et al. 2004). MLST involves DNA sequencing of seven housekeeping genes (*aroE, gdh, gki, recP, spi, xpt and ddl*) from the gDNA of pneumococcal isolates (Enright et al. 1998). Using the MLST website (http://www.mlst.net), each allele is assigned a number depending upon its sequence and this results in a seven digit 'barcode' for each isolate. This barcode is then used to determine the sequence type (ST) of the isolate, allowing isolates with new allelic numbers to become a new ST and isolates with barcodes already in the database are assigned the same ST number. This allows the global comparison and surveillance of pneumococcal strains at a genetic level rather than by serotype.

2.5. Preparation of pneumococcal cell extract for analysis of Ply

Single colonies of each isolate were selected from blood agar base plates (Oxoid) supplemented with 5% horse blood (E & O laboratories) and grown to mid log phase in 15ml or 50ml BHI. 10ml of each 15ml culture and 40ml of each 50ml culture were centrifuged at 3000g for 20min at 4°C and the cell pellet stored overnight at –20°C. The cell pellets were resuspended in 1.5ml 1 × PBS (Phosphate Buffered Saline: Oxoid, Basingstoke, UK) and sonicated on ice using a high intensity ultrasonic processor (Jencons Scientific Ltd, Bedfordshire, UK) with 3 × 10 sec and 10 sec in between at an amplitude of 10%. Cell lysates were centrifuged in eppendorfs at 20,000g for 30min at 4°C. The soluble fraction (cell extract) was transferred to a separate tube and the insoluble fraction (pellet) washed in 1 × PBS and resuspended in 1.5ml PBS. The total protein content of the cell extract and pellet was determined using Bradford's assay (Bradford 1976), detailed in

section 2.7.1. Cell extracts were diluted to 300μg/ml total protein (or 2.35mg/ml total protein for the concentrated samples) to give a baseline from which Ply expression levels and haemolytic activity were measured.

2.6. Expression and purification of recombinant Ply

2.6.1. Protein expression

The template plasmid was the high expression vector pKK233-2 (Clontech Laboratories Inc, Palo Alto, CA) in which *ply* from D39 *S. pneumoniae* was previously inserted (Paton et al. 1991). All Ply mutants were made in pKK233-2, unless otherwise stated. Wild type (WT) Ply and all Ply mutants were expressed in *E. coli* XL-1 cells (Stratagene) in either 1L HySoy J media (Sigma-Aldrich, manufacture discontinued in 2004) or Terrific Broth (TB, see appendix for recipe). Each litre was inoculated with 5ml of overnight culture plus 100μg/ml of ampicillin and filter sterilised glucose to give 1% glucose/L in the case of Hysoy J media or phosphate buffer to TB. Flasks were shaken at 37°C, 200rpm until cultures reached an OD_{600nm} 1.5 then induced with 1mM IPTG (Sigma-Aldrich). Flasks were shaken for a further 3 hours at 37°C. Cells were harvested by centrifugation for 15min at 4000g at 4°C using a 4K15 centrifuge. Pelleted cells were frozen overnight at -20°C then resuspended in 20ml 1 × PBS per litre of harvested cell culture. To disrupt cells and release Ply, cells were passed through the One-shot Cell Disrupter (Constant systems Ltd, Warwick, UK) at pressure of 12,000 psi. Cell lysates were centrifuged at 20,000g for 30 min at 4°C to remove cell debris.

2.6.2. Purification of Ply and derivatives

2.6.2.1. Hydrophobic interaction chromatography

Different columns were used to purify WT Ply and the mutants to avoid cross contamination. Cell lysate containing Ply was diluted in chilled 3M NaCl to give a final salinity of 1.5M NaCl. This was performed in 10ml aliquots and prepared immediately before purification. The sample was then filtered using 0.2μM syringe filters (Sartorious, Hannover, Germany). The matrix used for Hydrophobic Interaction Chromatography (HIC) was POROS 20 PE packed into a 100mm column with 4.66mm diameter (Applied Biosystems Ltd, Warrington, UK). The BioCAD 700E workstation (Applied Biosystems Ltd) was used for both HIC and anion exchange chromatography (AEC) purification. All buffers were made in Millipore distilled water and filtered and degassed prior to use (see Appendix for buffer recipes). The column was flushed with dialysis buffer (PB-S, appendix I) to prepare the system, removing ethanol from column storage and then equilibrated with 1.5M NaCl. 5ml of sample was loaded onto the column that was then washed with a NaCl gradient ranging from 1.5M NaCl to 0M over 7 column volumes, collecting wash off from the column in 2ml aliquots. Protein is eluted from the column in 0.2M – 0.1M NaCl. For WT Ply this was in fractions 6-9. Eluted fractions were run on 10% SDS-PAGE and stained with coomassie blue using standard protocols (Laemmli 1970).

2.6.2.2. Dialysis of HIC purified protein for further purification

Fractions containing >98% pure Ply were pooled and dialysed overnight to remove any traces of salt. Dialysis tubing with a molecular weight cut off point of 14,000Da (Medicell International Ltd, London, UK) was previously boiled in 2% sodium bicarbonate solution with 1mM EDTA for 10min, thoroughly washed in distilled water and stored in 20%

ethanol at 4°C. Prior to use, a section of dialysis tubing was cut and washed in distilled water to remove ethanol traces, one end was sealed with two dialysis clips and the HIC purified protein transferred into the tubing and sealed with two clips. The pooled protein fractions were dialysed overnight in 1L phosphate buffer (without NaCl), this was then changed a further two times prior to 2-3 fold concentration of the sample using Amicon ultra-15 centrifugal filter tubes (Millipore, Watford, UK). Samples were centrifuged in the filter tubes at 1000g at 4°C and the concentrated samples were pooled together.

2.6.2.3. Anion Exchange Chromatography

The concentrated sample from overnight dialysis was further purified by AEC using Poros® HQ20 Micron media and the BioCAD® 700E workstation (Applied Biosystems Ltd, Warrington, UK). A NaCl gradient from 0M to 1M NaCl was introduced once the protein was bound to the column and washed. The protein eluted in 0.1M NaCl whilst contaminants, DNA and LPS, remained bound to the column until higher salt concentrations were passed through the column. Ply eluted in fraction 14 (in the 24th column volume for both WT and mutant Ply) and this fraction from each run was pooled to give purified protein, which was stored in small aliquots at -20°C. Samples were not repeatedly freeze/thawed.

2.6.2.4. Construction, expression and purification of eGFP tagged proteins

Mr. Graeme Cowan constructed, expressed and purified eGFP tagged versions of WT Ply and Δ6 Ply in the following manner: the coding sequence of Ply was amplified by PCR using primers 9Y and 9Z (Table 2.1). The PCR product was ligated into *Bam*HI/*Sac*I (Promega) digested pET33b (Merck Biosciences, Nottingham, UK) to produce pET33bPly and transformed into TOP10 *E. coli* (Invitrogen). The GFP coding sequence was amplified

from pNF320 (Freitag et al. 1999) by PCR using primers GFPpet33bfwd and GFPpet33brev (Table 2.3). The PCR product was cut with *Nhe*I and *Bgl*II (Promega), ligated into *Nhe*I/*Bam*HI digested pET33bPly and transformed into TOP10 *E. coli*. Mutations F64L and S65T (Cormack et al. 1996) were introduced into GFP by site-directed mutagenesis as described in section 2.8 using primers EGFP-fwd and EGFP-rev (Table 2.3) to give enhanced(e)GFP. Δ6eGFP-Ply was created by site directed mutagenesis of pET33bEGFPPly using the Δ6 fwd and Δ6 rev primers (Table 2.3). Sequences were confirmed and the plasmids were transformed into BL21 (DE3) *E. coli* (Stratagene).

Cell extracts of recombinant eGFP WT Ply and eGFP Δ6 Ply were expressed and disrupted as described for WT Ply in section 2.6. The pET33b vector was used to clone a six-histidine tag to the N′ terminus of eGFP-Ply. The histidine tag allows purification of proteins using immobilised metal affinity chromatography (IMAC). The principle of IMAC is based on the interaction between histidine and nickel. Cell extract is passed through a Ni-NTA (Nickel-Nitrilotriacetic acid resin) column (charged with immobilised nickel cations) and the histidine tagged protein is retained on the column and non-specific proteins are eluted. An imidazole gradient, from 0 - 300mM (Search 2002), is then introduced to compete with histidine in binding to the nickel charged column resulting in the elution of the histidine-tagged protein (pneumolysin elutes between 150-200mM imidazole). Fractions containing purified toxins were dialysed three times at 4°C against a greater than 50-fold volume of 1 × PBS. The disadvantages of IMAC are that the sample is eluted in imidazole, which must be removed by dialysis prior to in vivo studies, and that the incorporation of a histidine tag may affect the structure or function of the protein.

2.7. Analysis of purified proteins (and pneumococcal lysates)

2.7.1. Determination of protein concentration by Bradford's reaction

Total protein content of samples was determined using a standard Bradford's assay (Bradford 1976). This assay is based on the principle of an absorbance shift from A465 to A595 when protein binds to acidic Brilliant Blue G-250. The amount of absorbance shift is directly proportional to the amount of protein present. A standard curve was prepared from serial dilutions of 2mg/ml BSA (Bovine Serum Albumin; Sigma) from 1.5mg/ml to 0mg/ml, diluted in PBS. 10µl of standards and samples were transferred in duplicate to a flat bottom 96-well plate and 200µl Bradford's reagent was added (Bio-rad, Hertfordshire, UK). Absorbance was read at 570nm and the protein concentration of the sample was determined using the standard curve. For pneumococcal cell lysates, all samples were diluted to the same total protein level prior to further analysis.

2.7.2. Haemolytic Assay

The haemolytic activity of purified protein (or pneumococcal cell extract with standardised protein content) was assessed by a haemolysis assay (Walker et al. 1987) using a 2% (vol/vol) sheep erythrocyte suspension (E & O laboratories) or human erythrocyte suspension (Blood Transfusion Services, Scotland) in 1 × PBS. Two fold dilutions of samples were prepared in duplicate in U-bottom 96-well plates with 1×PBS. For purified protein 50µl of sample and 50µl PBS were placed in the first well, whereas for pneumococcal lysates 100µl of lysate was used neat in the first well. Following dilution, 50µl of 2% erythrocyte suspension was added to each well and the plates were incubated at 37°C for 30min. Plates were then left for 30min at RT to allow red blood cells to settle. The endpoint is the well where there is 50% lysis of erythrocytes, leaving 50% pelleted

erythrocytes. The reciprocal of the dilution at which the endpoint is reached is used to give Haemolytic Units (HU) per ml of sample, e.g. the first well has 50µl of sample and 50µl PBS, which is a two-fold dilution, then 50µl is removed for subsequent dilutions and 50µl of erythrocyte suspension is added resulting in a four-fold dilution but this is only in a total volume of 100µl and is therefore multiplied by 10 to give the dilution per ml, which is 1/40 for the first well. This means that well 2 represents a 1/80 dilution and well 3 a 1/160 dilution, therefore, if the endpoint was well 3, there would be 160 HU per ml of sample, as shown in Table 2.2.

Table 2.2. Haemolytic titre endpoints to give HU/ml

Well No.	Titre (HU/ml)	Well No.	Titre (HU/ml)	Well No.	Titre (HU/ml)
1	40	7	2560	13	1.6×10^5
2	80	8	5120	14	3.3×10^5
3	160	9	1.0×10^4	15	6.6×10^5
4	320	10	2.0×10^4	16	1.3×10^6
5	640	11	4.1×10^4	17	2.6×10^6
6	1280	12	8.2×10^4	18	5.2×10^6

In order to measure the percentage of haemolysis for a quantitative haemolytic assay, 100µl of supernatant was carefully transferred from each well into a flat bottom plate and the amount of haemoglobin released into the supernatant measured with an absorbance of 540nm. The percentage haemolytic activity was calculated using 0.04% ammonia to give 100% lysis and plotted against toxin concentration.

2.7.3. SDS-PAGE and Western Blotting

SDS-PAGE was used to assess the purity of fractions at each stage of purification. Unless otherwise stated 10% gels were used throughout. Samples were diluted two fold in sample buffer and boiled for 5min prior to loading on gels. Kaleidoscope marker (Bio-Rad) was used for all SDS-PAGE intended for Western blotting. Gels were run for 80min at 100 Volts and either stained or transferred to Hybond-C nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK) and blotted for 90 minutes at 80 Volts. For detection of Ply expression, Western blots were blocked overnight at RT in 3% skimmed milk in Tris-NaCl pH 7.4 with shaking and then incubated at 37°C with shaking for 2h in 3% skimmed milk with 1:2000 polyclonal rabbit anti-Ply serum (Mitchell et al. 1989). Membranes were then washed × 4 in Tris-NaCl pH 7.4 and incubated for 1h with 1:1000 HRP-linked anti-rabbit IgG (Amersham Biosciences) in 3% skimmed milk, washed × 4 and developed in developing solution. The reaction was stopped with distilled water. For all recipes see Appendix I.

2.7.4. Analysis of Lipopolysaccharide (LPS) contamination in purified proteins

2.7.4.1. Silver staining for LPS

The presence of LPS in the samples post purification was assessed using a slightly modified method from Tsai and Frasch (Tsai et al. 1982) which is explained below, recipes for all solutions are given in Appendix I. Prior to running protein samples on SDS-PAGE, samples were incubated with 100µg of Proteinase K (Sigma)/ml of sample for 1h at 37°C in order to digest the protein and leave only LPS in the sample. An equal volume of 5 × SDS sample buffer (e.g. 10µl sample + 10µl buffer) was added to each sample and boiled for 5min. The samples were then loaded on to a 15% SDS-PAGE gel and run until the

smallest marker reached 2/3 down the gel (LPS runs at 6kDa). The LPS in the gel was then fixed by shaking overnight in Fixing solution. The gel was then placed into 0.7% periodic acid in Fixing solution for 5 min to oxidise the LPS. The gel was then washed by shaking for 15 min × 3 in 500ml distilled water. Following washing, the gel was immersed in staining solution and shaken vigorously for 10min while covered, as the stain is light sensitive. The gel was then washed again × 3 and then placed in the formaldehyde developer for 2-5min until a band was visible on the gel for the positive LPS sample (kindly provided by Mr. Gordon Cheung). The reaction was stopped before there was any background staining by placing the gel in 10% acetic acid for 30 sec. The gels were washed and stored in water.

2.7.4.2. Quantification of LPS using the Limulus Amebocyte Lysate assay

Lipopolysaccharide (LPS) content of purified protein was determined using the Limulus Amebocyte Lysate (LAL) Kinetic-QCL® Kit (Cambrex, Nottingham, UK), which was run according to manufacturer's instructions and with the help of Dr. Rosie Smith. The LAL assay is a standardised protocol approved by the Food and Drug Administration for the measurement of endotoxin levels in pharmaceuticals and biological products. In 1964, endotoxin was discovered to react with a protein in the circulating amebocytes of horseshoe crabs (*Limulus polyphemus*). This protein was identified as coagulase and purified for exploitation of its enzymatic reaction with Gram negative endotoxin (Young et al. 1972). Samples are mixed with a LAL substrate reagent (Ac-Ile-Glu-Ala-Arg-pNA) and the time taken for the conversion of this colourless substrate to p-nitroaniline, which is yellow in colour, is measured photometrically. The reaction time is inversely proportional to the amount of endotoxin present in the samples and a standard curve using known amounts of endotoxin from *E. coli* 055:B5 is used to determine endotoxin levels in samples.

All test tubes, plates, pipette tips and water were endotoxin free (Cambrex). LPS from *E. coli* 055:B5 (Cambrex) was reconstituted in endotoxin free water and vortexed for 10min to give 50EU (Endotoxin Units)/ml. Four 10-fold dilutions of this were prepared to give a standard curve ranging from 50EU/ml to 0.005EU/ml, with thorough vortexing in between dilutions. Samples were diluted by 1/100 and 1/10,000. Samples and standards were added in quadruplicate to a flat bottom 96-well plate. Two wells for each sample were then spiked with a known amount of endotoxin. Incorporation of a known amount of endotoxin into samples allows for investigation of whether or not the sample inhibits or enhances the enzymatic reaction, which is automatically calculated by the Kinetic-QCL software. The blank was endotoxin free water, which was the last sample to be added. The plate was then incubated in the plate reader at 37°C for 10min. The supplied lysate was reconstituted with the appropriate volume of water and 100µl added to each well. The plate was then read at an absorbance of 405nm over 90min. The time taken for the reaction to reach saturation was recorded and samples within the range were selected for reading from the standard curve to give the amount of EU/ml of sample.

2.8. Construction of Ply mutants by site-directed mutagenesis

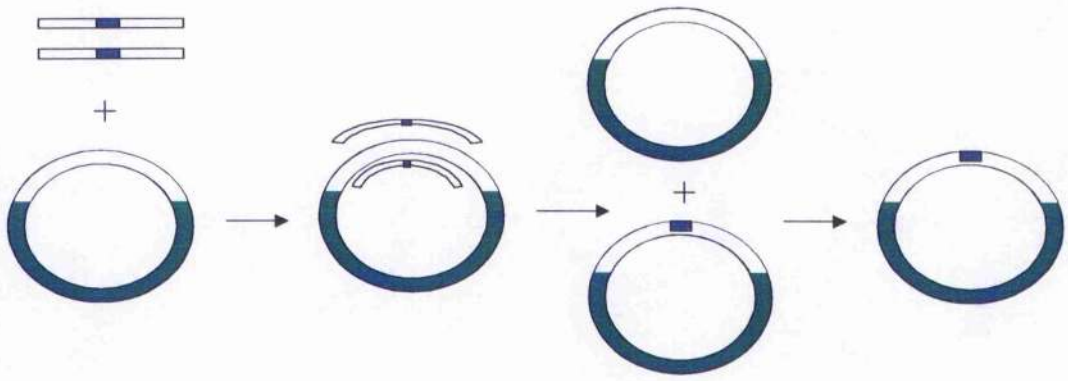
Eight double amino acid deletions and a single A146 deletion were created in the *ply* gene using a Quikchange® site directed mutagenesis kit (Stratagene). The template plasmid for all *ply* manipulation unless otherwise stated was the high expression vector pKK233-2 (Clontech Laboratories Inc) in which *ply* was previously inserted (Paton et al. 1991). Mr. Graeme J. M. Cowan cloned Perfringolysin O into the pKK233-2 vector (Solovyova et al. 2004). Primers (Sigma-Genosys Ltd, Haverhill, UK) designed to delete or substitute the relevant amino acids are shown in Table 2.3. The principles of site-directed mutagenesis are shown in Figure 2.1. Complementary forward and reverse primers (25-40 bases) were designed against the region of toxin to be mutated with the desired mutation made in the

middle of each primer (Primer sequences in Table 2.3). PCR reactions were set up in 50µl dH_2O with 5µl 10×reaction buffer, 125ng of each primer, 1-3µl purified plasmid DNA (~10ng) carrying the *ply* gene, 2mM dNTPs, and 1µl *PfuTurbo*® polymerase (2.5U), which is a high fidelity enzyme that reads the whole plasmid on each cycle, resulting in the creation of plasmids carrying the desired mutation. PCR conditions varied depending upon the mutation required (12 cycles for point mutations and 18 cycles for deletions or insertions) and the size of the template plasmid.

PCR conditions:	95°C for 30sec
12 or 18 cycles of	95°C for 30sec
	55°C for 1min
	68°C for 2min/Kb of plasmid
Final extension of	68°C for 10min

An enzyme digest with 10U *Dpn*I/50µl sample is used after PCR in order to digest the methylated parental DNA (the parental plasmid DNA must be isolated from a *dam*+ *E. coli* strain to enable the isolation of the daughter DNA through digestion of methylated parental DNA. According to the manufacturers guidelines, DNA from almost all strains of *E. coli* is methylated with the exception of the JM110 and SCS110 series. In this work, plasmids were isolated from XL-1 and XL-10 *E. coli*). The unmethylated PCR product, which is not digested, can then be transformed into *E. coli* cells such as XL-1 blue supercompetent cells for protein expression and purification (see Figure 2.1. for schematic of s.d.m. process).

Figure 2.1. Principal of site-directed mutagenesis



(a) (b) (c) (d)

Site-directed mutagenesis. (a) Shows parental plasmid DNA with insert of the gene that will be mutated e.g. *ply* in white. The primers are the bars above, which are complementary to each other and have the desired mutation in the middle of the primer (shown in blue); this can be base pair substitutions, deletions or insertions. (b) The parental plasmid and the primers are mixed and used in a PCR reaction with PFU Turbo polymerase, dNTP's and reaction buffer, supplied in kit from Stratagene. The DNA polymerase replicates the whole of the plasmid with the new primers annealed to this. This results in the production daughter plasmid DNA containing the desired mutation. However, the parental plasmid DNA remains (c). A *Dpn*I digest is introduced to digest the methylated parental DNA, leaving the daughter plasmid DNA (d) that can then be transformed into *E. coli* for protein expression and purification.

Table 2.3. Primers used for site-directed mutagenesis

Primer	Lab. Ref.	Sequence 5′-3′	Amino acid change
Δ1 fwd	22P	CGATTTGTTGGCTAAGCAAGATTATGGTCAGG	ΔW134H135
Δ1 rev	22Q	CCTGACCATAATCTTGCTTAGCCAACAAATCG	
Δ2 fwd	22R	GTTGGCTAAGTGGCATTATGGTCAGGTCAATAATGTC CC	ΔQ136D137
Δ2 rev	22S	GGGACATTATTGACCTGACCATAATGCCACTTAGCCA AC	
Δ3 fwd	22T	GGCTAAGTGGCATCAAGATCAGGTCAATAATGTCCC	ΔY138G139
Δ3 rev	22U	GGGACATTATTGACCTGATCTTGATGCCACTTAGCC	
Δ4 fwd	22V	GGCATCAAGATTATGGTAATAATGTCCCAGCTAG	ΔQ140V141
Δ4 rev	22W	CTAGCTGGGACATTATTACCATAATCTTGATGCC	
Δ5 fwd	22Z	GGTCAGGTCAATAATGCTAGAATGCAGTATG	ΔV144P145
Δ5 rev	23A	CATACTGCATTCTAGCATTATTGACCTGACC	
Δ6 fwd	23B	GGTCAATAATGTCCCAATGCAGTATGAAAAAATAAC GGCTC	ΔA146R147
Δ6 rev	23C	GAGCCGTTATTTTTTCATACTGCATTGGGACATTATT GACC	
Δ7 fwd	23D	GGTCAATAATGTCCCAGCTAGATATGAAAAAATAAC GGCTC	ΔM148Q149
Δ7 rev	23E	GAGCCGTTATTTTTTCATATCTAGCTGGGACATTATT GACC	

Δ8 fwd	23F	GTCCCAGCTAGAATGCAGAAAATAACGGCTCACAGC	ΔY150E151
Δ8 rev	23G	GCTGTGAGCCGTTATTTTCTGCATTCTAGCTGGGAC	
Δ9 fwd	27V	GCTAGAATGCAGTATGAAACGGCTCACAGCATGG	ΔK152I153
Δ9 rev	27W	CCATGCTGTGAGCCGTTTCATACTGCATTCTAGC	
Δ10 fwd	27X	GCAGTATGAAAAAATACACAGCATGGAACAACTCAA GGTC	ΔT154A155
Δ10 rev	27Y	GACCTTGAGTTGTTCCATGCTGTGTATTTTTTCATACT GC	
Δ11 fwd	27Z	GCAGTATGAAAAAATAACGGCTATGGAACAACTCAA GGTC	ΔH156S157
Δ11 rev	28A	GACCTTGAGTTGTTCCATAGCCGTTATTTTTTCATACT GC	
Δ12 fwd	28B	CGGCTCACAGCCAACTCAAGGTCAAGTTTGG	ΔM158E159
Δ12 rev	28C	CCAAACTTGACCTTGAGTTGGCTGTGAGCCG	
Δ16 fwd	28D	GGAACAACTCAAGGTCAAGTTTGACTTTGAAAAGAC AGGG	ΔG166S167
Δ16 rev	28E	CCCTGTCTTTTCAAAGTCAAACTTGACCTTGAGTTGT TCC	
ΔA146 fwd	33I	GGTCAGGTCAATAATGTCCCAAGAATGCAGTATGAA AAAATAAC	ΔA146
ΔA146 rev	33J	GAGCCGTTATCATACTGCATTCTTGGGACATTATTGA CCTGACC	
Δ6 PFO fwd	31Y	CTACACATACTTTACCAACTCAATATTCAGAATCTAT GG	ΔA191R192
Δ6 PFO rev	31Z	CCATAGATTCTGAATATTGAGTTGGTAAAGTATGTGT AG	

GFPpET 33b fwd	20G	GTCAGGCTAGCATGAGTAAAGGAGAAGAAC	
GFPpET 33b rev	20I	CCACGCAGATCTTTGTATAGTTCATCC	
EGFPpl y fwd	24W	CACTTGTCACTACTCTGACTTATGGTGTTCAATGC	Introduces F64L and S65T in GFP
EGFPpl y rev	24X	GCATTGAACACCATAAGTCAGAGTAGTGACAAGTG	

2.9. Janus mutagenesis

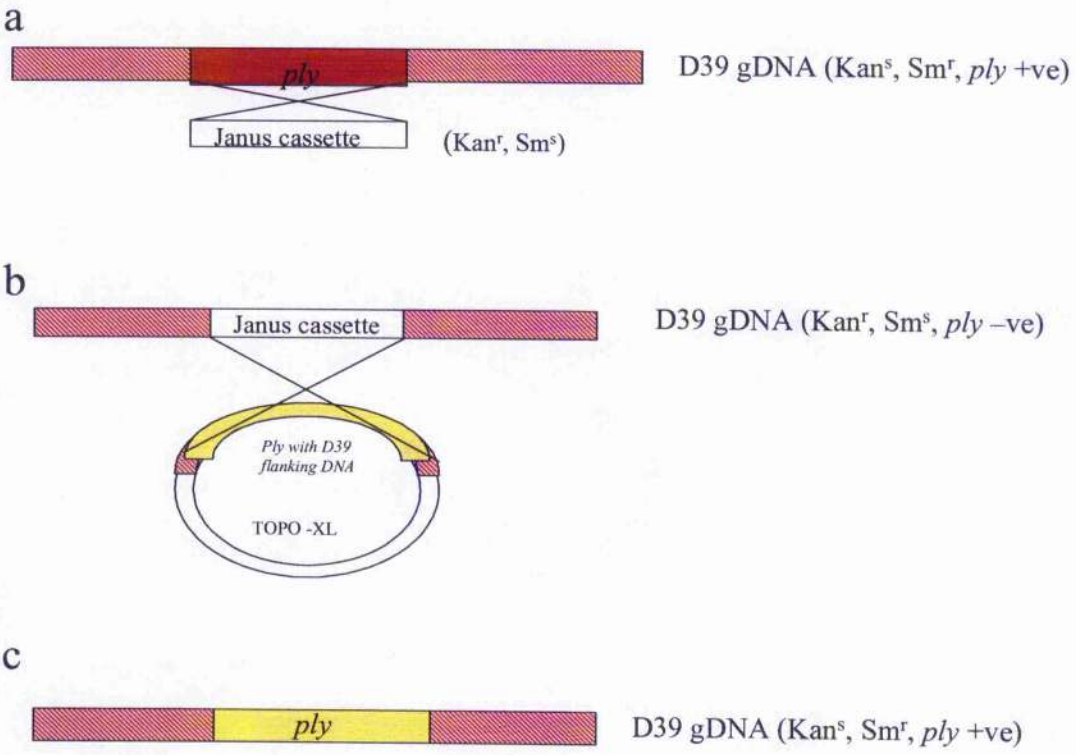
In order to assess the effect of the double deletion of A146 and R147 in Ply within a pneumococcal genetic background, the mutation was constructed in the chromosomal DNA of serotype 2 D39 *S. pneumoniae* using Janus mutagenesis (Sung et al. 2001), see schematic in Figure 2.2. The advantage of Janus mutagenesis is that it allows the introduction of mutations with non-selectable phenotypes into the pneumococcal genome.

The Janus cassette carries a kanamycin resistance marker and an *rpsL+* marker that confers streptomycin susceptibility, which is dominant over the *rpsL-* resistant allele of the wild type strain. This cassette is inserted into a desired site in the pneumococcal genome using homologous recombination (Figure 2.2.a). The resulting strain is kanamycin resistant and streptomycin susceptible compared with the parent strain that is kanamycin sensitive and streptomycin resistant. This allows the selection of strains carrying the Janus cassette. Homologous recombination is used again to insert a DNA sequence of choice (e.g. the *ply* gene carrying desired mutations, Figure 2.2.b) in place of the Janus cassette. This results in the loss of kanamycin resistance and streptomycin susceptibility and is used to select for transformants carrying the desired mutation (Figure 2.2.c).

2.9.1. Construction of Janus intermediates

The streptomycin resistance gene was amplified from R6 CP1200 *S. pneumoniae* (Sung et al. 2001) and transformed into D39 *S. pneumoniae* (Paterson 2003) to give a streptomycin resistant D39 *S. pneumoniae* (SmR1) for Janus mutagenesis. The Janus cassette was amplified from strain R1036 (Sung et al. 2001) and inserted into the genome of the streptomycin resistant D39 at the *ply* gene by Dr. Gavin Paterson using homologous recombination. This resulted in an intermediate strain of D39 *S. pneumoniae*, JP PCR B, which is kanamycin resistant and streptomycin sensitive (and pneumolysin negative). Dr. Gavin K. Paterson also amplified the *ply* gene from D39 *S. pneumoniae* with flanking D39 DNA (2.345 kb) and cloned it into the 3.5 kb TOPO-XL vector (Invitrogen). This provided the donor DNA for homologous recombination into the JP PCR B strain.

Figure 2.2. Janus mutagenesis



The principle of Janus mutagenesis is demonstrated with the replacement of the wild type pneumolysin gene (*ply*) shown in red (a) with a mutant form of *ply* (c). The Janus cassette, carrying kanamycin resistance and streptomycin susceptibility is inserted into the desired site on the chromosome of a streptomycin resistant strain of D39 *S. pneumoniae* by homologous recombination (a). A plasmid carrying the DNA that is to take place of the original gene is used for a further homologous recombination step to insert the desired DNA (yellow) in place of the Janus cassette (b). This results in a strain of D39 that carries the new chromosomal DNA without selection markers (c).

2.9.2. Construction of Δ6 Ply in D39 Ply

The TOPO-XL vector containing D39 WT *ply* and flanking DNA was purified from TOP-10 *E. coli* cells (Invitrogen) with a miniprep kit (Qiagen) to provide the parental DNA in which to construct the Δ6 mutation (ΔA146ΔR147) by site-directed mutagenesis using the original Δ6 Ply primers (23B and 23C, Table 2.3) and as described in section 2.8. This mutation was checked by DNA sequencing and provided the donor DNA for transformation into D39 *S. pneumoniae* gDNA. The parental plasmid with WT *ply* was also used to donate DNA to give a positive control that had been through the same transformation process as the Δ6 Ply mutant.

2.9.3. Transformation of mutants into the D39 chromosome by Janus mutagenesis

10ml BHI, supplemented with 1mM $CaCl_2$, was inoculated with 300µl of JP PCR B from a glycerol stock and grown statically at 37°C to an OD_{600} 0.1. 1ml aliquots of the cell culture were taken, one aliquot per sample, e.g. 1: negative control (no DNA), 2: positive control with streptomycin resistant WT gDNA, 3: 30µl miniprep plasmid DNA of Δ6 Ply in TOPO-XL, 4: 30µl miniprep plasmid DNA of WT *ply* in TOPO-XL vector. CSP-1 (Complement Stimulating Peptide; synthesised by Sigma-Aldrich) was added to each aliquot at 100ng/ml to promote uptake of external DNA. The cultures were incubated in a 37°C water bath for 15 min and DNA was added to the appropriate tubes and the samples were further incubated for 75 min. The transformations were plated out onto BAB plates supplemented with 5% horse blood and 300µg/ml streptomycin. Plates were incubated for 9h at 37°C under anaerobic conditions using GasPak™ Pouches (Becton Dickenson, Oxford, UK). This short anaerobic incubation time minimises the chance of streptomycin resistant revertants occurring that have lost the Janus cassette.

2.9.4. Selection of Janus mutants

100 single colonies for each transformation were picked and stabbed onto two blood agar plates, one with streptomycin (300μg/ml) and another with kanamycin (150μg/ml). Plates were incubated overnight at 37°C in a candle jar. Stabbed colonies that were streptomycin resistant and kanamycin sensitive were selected from the BAB plates and grown up at 37°C overnight in 20ml BHI with 300μg/ml streptomycin. Overnight cultures of these transformants were streaked onto BAB plates to check for purity and optochin sensitivity with optochin discs (Mast Group Ltd., Merseyside, UK). The overnight cultures were used to seed 20ml BHI containing 300μg/ml streptomycin, which was grown to mid-log phase then 10ml was frozen as 1ml aliquots in 20% glycerol. 10ml of mid-log phase culture was used to prepare gDNA (see section 2.2) for PCR analysis to check for presence of the *ply* gene using primers 15C and 15D (Table 2.1), which anneal to the 5' and 3' ends of the *ply* gene. Positive *ply* tranformants were further characterised for Ply expression, Ply activity and virulence using techniques already described in this chapter.

2.10. *In vitro* assays with Ply and derivatives

2.10.1. L929 fibroblast cytotoxicity assay

L929 murine fibroblasts (ECACC no.85011425, Wiltshire, UK) were cultured in RPMI 1640 media supplemented with 10% Foetal Bovine Serum, 1% of 5U/ml penicillin G sodium/5μg/ml Streptomycin sulphate and 2mM L-glutamine (Invitrogen, Paisley, UK). Cells were quantified using a haemocytometer, diluted in media to give 3×10^5 cells/ml and transferred as 200μl aliquots to a 96-well plate and incubated for 24h at 37°C, 5% CO_2. Serial dilutions of purified WT Ply and derivatives were prepared in RPMI 1640 media

from a stock concentration of 0.03mg/ml and added to the L929 fibroblasts in triplicate. Cell viability upon 24h incubation with Ply was assessed using 50µl/well of 1µg/ml of MTT (3-[4,5-Dimethylthiazol-2-yl]-2,5-diphenyltetrzolium bromide) (Sigma-Aldrich). Absorbance was read at 540nm.

2.10.2. Mast cell degranulation assay

RBL-2H3 rat mast cells (ATCC number CRL-2256 Lot. 2454194) were cultured in Eagle's Minimal Essential Media (EMEM; ATCC) supplemented with 15% heat inactivated FBS. Degranulation of mast cells was measured by the release of β-hexosaminidase (Stassen et al. 2003). 200µl of 5×10^4 cells/ml were transferred into each well of a 96-well Costar® plate (Sigma-Aldrich) to give 1×10^4 cells/well and incubated for 2 hours at 37°C, 5% CO_2 to allow cells to adhere. Cells were stimulated with 50µl of toxin in varying concentrations for 1.5h. (Heat-treated samples were heated to 65°C for 10min prior to incubation with mast cells.) The plate was then centrifuged at 200g for 3 min and the supernatant transferred into a new 96-well plate. Remaining cells were lysed with 180µl of 0.5% Triton X-100 (BDH biosciences, Dorset, UK) in EMEM. 2 × 20µl of lysate and 2 × 20µl supernatant was transferred into a fresh plate. 50µl of substrate solution (1.3mg p-nitrophenyl-N-acetyl-β-D-glucosamine [pNAG]/ml citrate buffer; Sigma-Aldrich) was added to each well and incubated for 90mins at 37 °C. The reaction was stopped with 150µl 0.2M Glycine NaOH, pH 10.7 and hydrolysis of the substrate was read at 405nm with an MRX plate reader. Recipes for buffers are given in Appendix I. Mast cell degranulation was expressed as the percentage of β-hexosaminidase released into the supernatant. The amount of β-hexosaminidase in the supernatant and lysate were added to give the total amount of β-hexosaminidase present. Mean percentage degranulation was plotted against toxin concentration.

2.10.3. Cell binding by Immunoblotting

Pneumococcal cell extracts or purified Ply and derivatives were incubated with a 2% horse erythrocyte suspension, washed × 6 with distilled water to remove unbound toxin and lyse the cells. The samples were then prepared by boiling with sample buffer and were then run on 10% SDS-PAGE for Western blotting with anti-Ply antibody (see section 2.7.3). This allowed analysis of Ply's ability to bind to erythrocytes as previously described (Owen et al. 1994).

2.10.4. Cell binding by Fluorescence Microscopy

Erythrocyte ghosts were generated from 0.1ml human blood by repeated washing with distilled water. The erythrocyte ghosts were then incubated with 50µg eGFP WT Ply or 50µg eGFP Δ6 Ply in 1ml 1 × PBS for 30 min at 37°C. The ghost membranes were pelleted, washed × 3 in PBS and were visualised by fluorescence microscopy by Mr. Graeme Cowan using a Zeiss Axioscop 20.

2.10.5. Binding inhibition assays

Dilutions of Δ6 Ply (10, 5, 2.5, 1.25 and 0µg/ml) were prepared in 1×PBS and transferred to round-bottomed 96-well plates, allocating two rows for each dilution of Δ6 Ply (50µl/well). For pre incubation of Δ6 Ply with erythrocytes, 50µl of a 2% sheep erythrocyte suspension (prepared as described for the haemolytic assay in section 2.7.2) was added to each well and the plate was incubated for 30min at 37°C. Serial dilutions of purified WT Ply were prepared in a separate plate and transferred to the plate (50µl/well) containing Δ6 Ply and the erythrocytes and incubated for a further 30min at 37°C. For competitive binding analysis, Δ6 Ply and WT Ply were combined and then incubated with

the erythrocyte suspension. The absorbance at 630nm of each supernatant was then read to measure the turbidity of the sample and plotted against the amount of WT Ply added to each sample. The A540nm was also measured to quantitate haemoglobin release and was found to produce similar results.

2.10.6. Cross-linking of oligomers

25µl of 0.5mg/ml WT Ply and Δ6 Ply were incubated with 25µl erythrocyte membranes (prepared from a 2% erythrocyte suspension) at 37°C for 30 min. The membranes were then pelleted and washed × 6 in PBS to remove unbound toxin. 5µl of 25mM DSS (Disuccinimidyl suberate; Pierce Biotechnology, Rockford, IL) dissolved in dimethyl sulfoxide (DMSO; Sigma-Aldrich) was added to the membrane bound toxin and the solution incubated at room temperature (RT) for 30 min in order to cross-link any toxin that has bound to the membranes. The reaction was quenched with 2.5µl 1M glycine for 15 min then boiled with SDS-PAGE sample buffer, run on 5% SDS-PAGE with high molecular weight markers (Bio-Rad, Hertfordshire, UK) and Western blotted with anti-Ply antibodies as described in section 2.7.3.

2.10.7. Transmission Electron Microscopy

200µl of 2% (vol/vol) horse erythrocyte solution was incubated with an equal volume of 0.2mg/ml Ply or filtered pneumococcal cell extract at 37°C for 30min then centrifuged at maximum speed using a bench top centrifuge to pellet the membranes. The cells were lysed and membranes were washed 5 times with distilled water to remove unbound toxin. The membranes were then resuspended in 50µl dH_2O. 5µl of sample was placed onto glow discharged carbon coated grids and negatively stained with NanoVan® (Nanoprobes, Yaphank, NY) according to manufacturer's instructions. Grids were viewed at ×25000 magnification using an LEO 912 Energy Filter Transmission Electron Microscope.

2.10.8. Measurement of Ply by ELISA

A Ply ELISA developed by Cima–Cabal et al (Cima-Cabal et al. 2001) was used to quantify Ply expression levels from pneumococcal cell lysate with the following modifications: Wells were coated with 100µl of 2.5µg/ml capture antibody, Ply 7 (de los Toyos et al. 1996) in coating buffer (see Appendix I). Plates were blocked with 10% foetal calf serum (Invitrogen) in PBS; washes were with 0.05% Tween 20 (Sigma-Aldrich) in 1 × PBS and dilutions were made in assay buffer (blocking buffer + 0.05% Tween 20). Pneumococcal cell extracts were diluted to 1:500 for the 300µg/ml total protein samples (or 1:2500 for the 2.35mg/ml total protein samples) and added in duplicate at 100µl/well. Purified Ply, prepared as described previously (Mitchell et al. 1989), was used to provide a standard curve with a range from 2000pg/ml to 31.25pg/ml. 100µl/well polyclonal α-Ply antibody (Mitchell et al. 1989) was then added at a 1:2000 dilution in assay buffer and plates were shaken for 30min at 37°C. Following 4 washes, 100µl/well of biotinylated α-rabbit IgG (Amersham Biosciences) was added at 1:500 and shaken for 30min at 37°C. Plates were washed × 4 and incubated with a 1:2000 dilution of streptavidin HRP (KPL, Gaithersburg, Maryland) and developed with 3, 3′, 5, 5′-tetramethylbenzidine (TMB) substrate (KPL) according to the manufacturers instructions.

2.11. *In-vivo* analysis of non-toxic Ply

All *in vivo* experiments were carried out in accordance with the UK Animals (Scientific Procedures Act) 1986. Mice had food and water *ad libitum* and were kept at a constant room temperature of 20-22°C and with a 12h light/dark cycle. All animal handling was by Dr. Alison R. Kerr and Dr. Gill R. Douce due to licensing restraints. However, all experimental design, sample processing, data acquisition and interpretation was by the author, Lea-Ann S. Kirkham.

2.11.1. Inflammatory responses to i.n. instillation of Ply

Eight week-old female MF1 mice (Harlan, Bichester, UK) were lightly anaesthetised with 2% halothane/1.5% oxygen (1.5 litre/min) (Astra-Zeneca, Macclesfield, UK). Purified WT Ply was administered intranasally (i.n.) at 1μg, 0.25μg, or 0.1μg/dose and ΔA146 Ply at 1μg/dose in 50μl of sterile saline (0.09% NaCl, Baxter International Inc, Deerfield, IL) with a saline only control group (n=6). Mice were monitored to a 24h end-point. TNF-α, IL-6 and IFN-γ cytokine levels were measured in the serum, BALF and lung tissue by ELISA using commercial matched anti-cytokine antibody pairs (BD Biosciences Pharmigen, San Diego, CA). Total protein levels in the BALF were measured using the standard Bradford's assay described in section 2.7.1.

2.11.2. Retrieval of BALF, serum, lung and brain tissue for cytokine analysis

Methods used for BALF and lung retrieval and processing were similar to those described before (van der Poll et al. 1996). To carry out lavage of the lungs, mice were culled by cervical dislocation and the skin and muscles above the trachea were separated. The trachea was clamped with forceps (Fisher Scientific, Loughborough, UK) and a 16-gauge angiocath was inserted into the trachea to open the airways. Lavage was carried out with 2×1ml volumes of PBS with a recovery volume of ~1.5ml, which was transferred to a sterile cryotube and immersed in liquid nitrogen. Following lavage, lungs and brains were removed and wrapped in aluminium foil then immediately immersed in liquid nitrogen. Blood was taken from the heart using a 23-gauge needle and transferred to an eppendorf to clot for 1h at RT or overnight at 4°C. The blood was then centrifuged at 1400g using a bench top centrifuge for 3 min. The supernatant (serum) was then transferred to a fresh tube. All samples were stored at -80°C prior to analysis.

2.11.3. Processing of BALF, serum, lung and brain tissue for cytokine analysis

Whole lungs and brains were weighed then homogenised 50 times in 5ml 1×PBS using handheld glass tissue homogeniser (Jencons, Leighton Buzzard, UK). Homogenates were transferred to 10ml falcon tubes and centrifuged at 1600g for 30mins at 4°C. Cell free supernatant was then transferred to a fresh 10ml tube and centrifuged at 5000g for 30mins at 4°C and then filter sterilised using 0.2μM syringe filters (Sartorious) into 5ml bijous, which could then be stored at -80°C. Immediately prior to analysis BALF was defrosted and centrifuged for 3min at maximum speed using a bench top centrifuge to remove cell debris.

2.11.4. Analysis of core body temperature of mice in response to Ply treatment

Six week-old female BALB/c mice were surgically implanted with telemetry chips as previously described (Kerr et al. 2002) in order to record core body temperature (Tc). Briefly, telemetry chips were sterilised in Cidex (Johnston & Johnston Medical Ltd, Skipton, UK) for 10min and then washed in sterile water. Mice were lightly anaesthetised with 2% v/v halothane over oxygen (1.5 litre/min) and the abdomen of the animals was shaved. Mice were then placed on a sterile drape over a heat mat and deeply anaesthetised with 3-5% v/v halothane over oxygen (1.5 litre/min). An incision was made in the abdomen with a sterile scalpel then sterile scissors were used to open the peritoneum. The telemetry chip was placed in the peritoneum, the inner wound fastened by suture and the outer wound fastened with surgical clips (Becton Dickinson, Oxford, UK). Mice were monitored for two weeks to ensure complete recovery, and then they were lightly anaesthetised and treated intranasally (i.n.) with purified WT Ply or ΔA146 Ply at 1μg/dose in 50μl of saline with a saline only control group (n=12-22). Immediately after treatment,

mice were placed in cages on individual receiver boxes to allow recording of Tc. Mice were monitored for 24h and Tc readings collected every minute. Data was collected using Vital View® software (Mini Mitter, Oregon) and transferred to Excel for analysis, where the data was reduced from one minute intervals to 30 minute intervals.

2.11.5. Sampling from telemetry experiments for subsequent cytokine analysis

Bronchoalveolar lavage (BALF), serum, brain and lung tissue samples were recovered at 2h, 6h and 24h from 5-6 mice from the WT Ply and ΔA146 Ply treated groups. Samples were retrieved and processed as previously described in section 2.11.2 and 2.11.3 and stored at –80°C prior to analysis.

2.11.6. Multiplex cytokine analysis of samples from telemetry experiments

Commercial cytokine ELISA antibody pairs for IL-6, TNF-α and IFN-γ (BD Biosciences Pharmigen, San Diego, CA) were used for initial analysis of individual cytokines with the appropriate buffers and at the concentrations given in Appendix I. Multiplex bead immunoassay kits (Biosource, Nivelles, Belgium) were used in conjunction with the Luminex™100 system (Bio-Rad) to measure inflammatory mediators produced in response to Ply treatment. Analytes can be combined from different sets to give a custom set of analytes for sample analysis. The technique is based on ELISA but unique antibody coated beads are used rather than the fixed base of 96-well plates. The Luminex system uses colour-coded bead sets with different fluorescent intensities which permits >100 colour codes. This allows the simultaneous measurement of a range of analytes in the same sample. The protocol was run according to manufacturers instructions. Briefly, 96-well plates with a filter base were filled with 200µl wash buffer and aspirated using a vacuum

manifold. Beads coated with antibody for all of the analytes of interest were mixed together and 25µl of mixture added to each well. 200µl wash solution was then added and the beads were washed by aspiration. 50µl incubation buffer was then added to each well and standards (comprising of all the analytes to be measured) were added in duplicate to the first two columns of the plate. Samples were diluted two-fold in assay buffer by adding 50µl assay buffer to sample wells then 50µl sample. The plates were protected from light with aluminium foil and shaken at RT for 2h at 500rpm. Wells were washed twice with wash solution and a mixture of biotinylated detection antibody for each analyte was added at 100µl/well and incubated at RT for 1h. The wells were washed twice and 100µl streptavidin-RPE was added at the relevant concentration for a 30min incubation. Plates were washed ×3 then 100µl wash buffer added to each well. Plates were either shaken for 2-3 min and read immediately with the xMap™ system (Biosource) or stored at 4°C overnight and read the following day by Dr. J. Alastair Gracie after a 2-3 min shake. Precision fluidics aligns the beads into single file to pass through two lasers, one that excites the tagged detection antibodies and another that excites the bead. This allows reactions to be measured in real time using fluorescent intensity to identify and quantify the reaction.

2.12. Mouse infection studies

2.12.1. Preparation of mouse passaged standard inocula

Single colonies of serotype 1 pneumococci from clinical isolates (ST227, strains 01-2696 and 00-3645; ST306, strain 01-1956) and the Janus mutants (WT D39 and Δ6 D39 *S. pneumoniae*, see section 2.9) were selected and grown up to mid-log phase in BHI as described in section 2.1. 1ml aliquots were stored in 20% FCS at -80°C for at least 16h then viable counts were assessed and 5×10^5 CFU/dose injected i.p. into an MF1 mouse as previously described (Alexander et al. 1994). At 24h the animal was sacrificed by cervical dislocation and blood taken by cardiac puncture and plated out onto BAB plates and incubated overnight at 37°C. A single colony was then selected and statically grown in 15ml BHI at 37°C to an OD_{600nm} 0.6. Frozen stocks of mouse passaged serotype 4 TIGR4 *S. pneumoniae* (ATCC: BAA-334, ST205), virulent serotype 1 *S. pneumoniae* (ST615) and serotype 2 D39 *S. pneumoniae* (ST128) were also streaked out for single colonies and grown to mid-log phase at which point 20% FCS (Invitrogen) was then added to each culture and 1ml aliquots were frozen at -80°C in cryotubes. Cultures were checked for purity and optochin sensitivity by streaking a loopful culture onto BAB plates prior to freezing. The Colony-Forming Units (CFU)/ml in each culture was calculated as described below.

2.12.2. Viable counts from standard inocula

24h post freezing, a vial for each strain was thawed in a 37°C water bath for 2 min. 900µl was then transferred to an eppendorf and centrifuged at RT for 3 min at 6600g using a bench top centrifuge. The supernatant was discarded and the cells resuspended in 900µl sterile PBS (Sigma-Aldrich). 1/10 dilutions of the sample were made in a 96-well U-bottomed plate using sterile PBS to give dilutions ranging from 10^{-1} to 10^{-6}. 3 × 20µl of

each dilution was spotted onto BAB plates divided into 6 sectors, 2 plates per sample, and allowed to dry. Plates were incubated anaerobically overnight in a candle jar at 37°C. The dilution sector where there were 10-70 colonies/20µl was then counted in order to calculate the CFU/ml for each strain.

Immediately before challenge, the standard inocula were thawed and prepared in the same way as described above and diluted in sterile PBS to the desired dose. Viable counts of the inocula were assessed prior and post challenge to check that the bacteria remained viable during challenge.

2.13. Challenge of mice

2.13.1. Intranasal (i.n.) challenge of mice

Eight to ten week old outbred female MF1 mice, or C3 deficient mice [C3-/- (Wessels et al. 1995), bred in house] for challenge with the serotype 1 clinical isolates (Chapter 7), were lightly anaesthetised as described in section 2.11.1 and challenged with the relevant dose and strain of *S. pneumoniae* in 50µl of sterile saline. 25µl was administered to each nare.

2.13.2. Intraperitoneal (i.p.) challenge of mice

Eight to ten week old female MF1 or BALB/c mice (Harlan) were scruffed at the neck and challenged i.p. by injection of the relevant dose of *S. pneumoniae* in 100µl sterile PBS. Mice were older (16-18 weeks) when challenged post vaccination due to the length of the active vaccination protocol.

2.13.3. Viable counts from blood, lung tissue and BALF

Following challenge with *S. pneumoniae*, mice were bled from the lateral tail vein or by cardiac puncture to assess bacteraemia and lungs and BALF were retrieved as described in section 2.11.2, however, lungs and BALF were not frozen in liquid nitrogen but were analysed immediately. Lungs were homogenised as described in preparation of lung tissue for cytokine analysis but the tissue was not centrifuged and the filtered. Whole blood and BALF were plated immediately. Samples were diluted in sterile PBS and plated out in the same way as described in section 2.12.2, with the exception of lung tissue, which was used neat in the first well and diluted thereafter. The CFU/ml of sample is plotted as a log value with a detection limit of 1.92.

2.13.4. Assessment of survival of mice post challenge with *S. pneumoniae* or Ply

Mice were monitored at frequent time intervals post treatment and were pain scored on a regular basis. Pain score ranged from normal, hunched stance and/or stary coat to lethargy where a pain score of ++ lethargic resulted in the humane sacrifice of the animal by cervical dislocation. If an animal was found dead, which was not often, the survival time was calculated as the intermediate time between when the animal was last pain scored and the time it was found dead to give an estimate of when the animal reached the endpoint of ++ lethargic.

2.14. Vaccination

2.14.1. Active Vaccination with WT Ply and ΔA146 Ply

Six-week-old female BALB/c mice (Harlan) were caged into group sizes of 8. Mice were bled from the lateral tail vein a day prior to immunisation to provide a baseline for

antibody titres (day 0 bleed). Serum was processed as described in section 2.11.2 and stored at -20°C. The mice were then immunised sub cutaneously with a 100µl dose of either 20µg WT Ply plus 100µg Alum (Wyeth, Pearl River, NY), 20µg ΔA146 Ply plus 100µg Alum, sterile NaCl plus 100µg Alum or NaCl only (0.9% NaCl, Baxter International Ltd). Bleeds were taken for antibody analysis a day before each boost, of which there were 3 at approximately 17 days apart. The animals were left for a month before a final bleed to check antibody titres prior to challenge. The mice were then challenged i.p. with 2×10^2 CFU/100µl dose TIGR4 *S. pneumoniae* and monitored for survival where the endpoint was when mice were deemed lethargic. 18h post challenge, blood was taken from the lateral tail vein of each mouse to assess the levels of bacteraemia in CFU/ml as described in 2.13.3.

2.14.2. Active vaccination with Δ6 Ply-type 4 polysaccharide conjugate

The protocol was the same as for active vaccination with free proteins but with the following modifications. Group sizes were increased to 10 to allow the groups to later be divided into two groups of five for i.p. challenge with either 10^2 CFU serotype 1 or TIGR4 *S. pneumoniae*. Δ6 Ply was used instead of ΔA146 Ply, as this was the protein that was conjugated to type 4 capsule polysaccharide (CPS) by Dr. Maya Koster, Wyeth Vaccines. The method used for conjugation of Ply to type 4 polysaccharide was reductive amination where oxidised polysaccharide was combined with protein at a 2:1 ratio and incubated with sodium cyanoborohydride for 4-6h at 25°C then 48h at 37°C and then incubated with sodium borohydride solution for 24h at 4°C to cap the reaction. The conjugates were filtered through a 100Kda MW cut off filter and assessed for carbohydrate and protein content. The dose of free protein used for immunisation was 0.64µg rather than 20µg in section 2.14.1 as this was determined by the amount of Δ6 Ply conjugated to type 4 capsule

polysaccharide (0.87mg/ml Δ6 Ply: 1.36mg/ml type 4 polysaccharide). The amount of Alum was increased from 100μg/dose in the free protein active vaccination to 200μg/dose to match the amount of Alum in Prevnar. BALB/c mice were given a sub cutaneous 200μl dose of either 1μg type 4 CPS plus 200μg Alum, 1μg type 4 CPS plus free 0.64μg Δ6 Ply plus 200μg Alum, 1μg type 4 CPS conjugated to 0.64μg Δ6 Ply plus 200μg Alum, 0.64μg Δ6 Ply plus 200μg Alum, 200μg Alum, PBS only, Prevnar containing 0.8μg type 4 CPS and 200μg Alum per dose or Prevnar plus 0.64μg Δ6 Ply.

2.14.3. α-Ply IgG ELISA of serum following active vaccination

Serum from each day before vaccination or boosting was collected from the lateral tail vein and analysed for anti-Ply IgG by ELISA (de los Toyos et al. 1996) using 5μg/ml Ply to coat the plates overnight and HRP-labelled anti-mouse IgG as the detection antibody (Amersham). Five fold dilutions of serum were prepared from a starting dilution of 1/50. The negative control on each plate was coating buffer with no antigen and then positive sera was added, this gave a background level, which was subtracted from each reading. Absorbance was read at 450nm and titres were taken at a cut off of OD0.3.

2.15. Statistical Analysis

For a statistical test to be chosen, the data was first assessed for normal distribution using GraphPad® Instat (GraphPad® Software Inc., San Diego, CA), where data that is normally distributed (parametric) fits a parabolic curve and the curve is centred at the mean. Bacterial load experiments fit normal distribution (Blue 2002), allowing the mean of the data to be compared between two groups using an unpaired t-test for parametric data, which compares one variable between two groups. Bacterial loads were expressed as the

mean ± SEM (standard error of the mean) and were statistically analysed with an unpaired t-test.

Inflammatory mediator levels, total protein levels in samples and survival times did not fit normal distribution curves and were therefore assessed with non-parametric analysis using a two-tailed Mann-Whitney U test (GraphPad® Software Inc.). This is a conservative test, also known as the rank sum test, which does not assume normal distribution and compares the median of two groups (this test cannot be used to compare more than two groups). The median is more robust than the mean as it is less sensitive to outliers. Two-tailed analysis, in comparison with one-tailed analysis, does not assume that the median of one group is expected to be higher than the other group prior to commencing the experiment. Inflammatory mediator levels were expressed as the mean ± SEM in graphs and tables but statistical analysis was with the median. Total protein levels in the BALF were expressed as the median ± MAD (Median Absolute Deviation) and survival times are shown for individual animals with the median survival times indicated. $P<0.05$ is considered significant (*) and $P<0.01$ considered very significant (**).

Chapter 3

Results

Construction, purification and *in vitro* characterisation of pneumolysin mutants

3: Purification and *in vitro* characterisation of pneumolysin mutants

Summary

The region surrounding amino acid residue N143 in Ply has been implicated in playing a role in oligomerisation of the protein (de los Toyos et al. 1996). Initial attempts to understand the importance of the N143 region in Ply were made by Dr. Jennifer Search who previously created a double amino acid deletion (ΔN142N143) and a substitution (N143D) in Ply. However the mutants behaved in a similar manner to WT Ply (Search 2002). In this study, eight double amino acid deletions were made surrounding the N142N143 site (four on either side). Of the eight double amino acid deletions constructed, the four upstream of N143 resulted in expression of a non-haemolytic form of Ply (Δ5-8, Table 3.1.). Mutants were purified for further characterisation.

Methods of Ply purification were also investigated and further developed. Previously recombinant Ply was purified using hydrophobic interaction chromatography (HIC) (Search 2002). This technique is capable of powerful resolution of native proteins from cell extracts, using high salt concentrations in the column media for adsorption of the protein and low salt concentrations for desorption. The column media is phenyl ether, which has no charge. However, HIC is not specific to Ply and should be used in combination with other chromatography steps as the resulting protein may not be completely pure. The major problem with purifying proteins from Gram-negative bacteria is the high levels of contaminating cell wall LPS, which is a potent inflammatory mediator. Inclusion of an additional purification step, anion exchange chromatography (AEC), was introduced in attempt to remove contaminating proteins and LPS in the HIC purified sample. AEC separates molecules based on their charge

and is complementary to HIC. The column media involves the use of a highly positively charged media that retains the highly negatively charged LPS and DNA longer than Ply, allowing separation of Ply from contaminants by the introduction of a salt gradient from 0M to 2M.

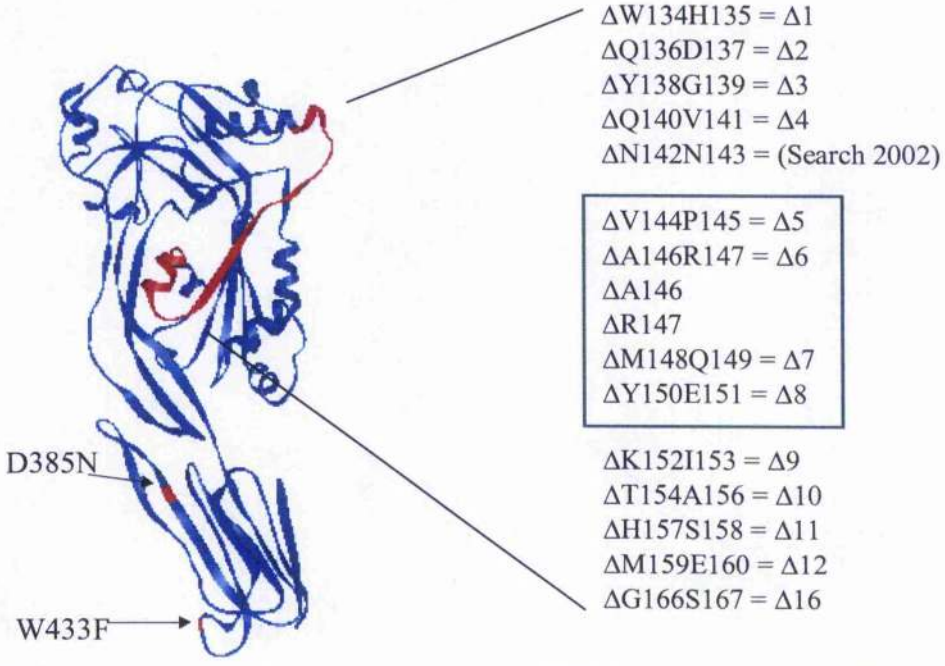
Cytotoxicity of the mutants to nucleated cell lines in addition to erythrocytes was assessed and compared with WT Ply and the existing PdB toxoid. A mast cell assay was developed to investigate a sensitive method to measure residual toxicity of Ply mutants. Mast cells are abundant in the alveoli and nasal mucous membranes and are therefore in prime position to detect inhaled antigens. They form the first line of defence as part of the innate immune system and can proliferate at the site of inflammation unlike neutrophils and macrophages (Abraham et al. 1997). They are highly sensitive to antigens and have been shown to degranulate during the early host response to pneumococcal pneumonia (Kerr et al. 2002). Two mutants, ΔA146 Ply and ΔA146R147 were further characterised in terms of their interaction with cells in culture, with assessment of binding, oligomerisation and pore formation prior to *in vivo* analysis.

Results

3.1. Construction of Ply mutants by site directed mutagenesis

Thirteen double amino acid deletions and two single amino acid deletions were constructed within the Ply protein by site-directed mutagenesis (Figure 3.1). Cell extracts of four double deletions and the two single deletions were found to have no detectable haemolytic activity (ΔV144P145, ΔA146R147, ΔM148Q149, ΔY150E151, ΔA146, ΔR147) highlighted in the green box in Figure 3.1. A list of all the mutations made in this work (and by others) is given in Table 3.1 with their haemolytic activity in comparison with WT Ply.

Figure 3.1. Site of mutations made within Ply



The location of the amino acid mutations are highlighted in red on the Ply homology model based on PFO (Rossjohn et al. 1997). Structural changes in relation to these mutations have not been accounted for by modelling. All mutations are at the domain 1-3 interface and D385N and W433F are also highlighted. The mutants in the green box have no haemolytic activity.

Table 3.1. Mutations made in Ply (and other CDCs) and haemolytic activity of cell lysates

Ply mutation[a]	Reference	% haemolytic activity compared with WT Ply
ΔW134H135 (Δ1)	This work	100
ΔQ136D137 (Δ2)	This work	100
ΔY138G139 (Δ3)	This work	100
ΔQ140V141 (Δ4)	This work	100
ΔV144P145 (Δ5)	This work	0
ΔN142N143	(Search 2002)	100
N143D	(Search 2002)	100
ΔA146R147 (Δ6)	This work	0
ΔM148Q149 (Δ7)	This work	0
ΔY150E151 (Δ8)	This work	0
ΔK152I153 (Δ9)	This work	37
ΔT154A155 (Δ10)	This work	77
ΔM158E159 (Δ12)	This work	42
ΔG166S167 (Δ16)	This work	83
ΔA146	This work	0
ΔR147	This work	0
Y150A	This work	57
W433F	(Mitchell 1992)	1
D385N	(Mitchell 1992)	100
D385NW433F	This work	1
Δ6D385N	This work	0
Δ6W433F	This work	0
Δ6D385NW433F	This work	0
ΔA191R192 PFO[b]	This work	0
ΔA204R205 ILY[b]	G. Cowan	0
ΔA190R191 ALO[b]	G. Cowan	0

[a] all mutations were constructed using the pKK233-2 vector and transformed into *E. coli* XL-1 blue supercompetent cells (ALO and ILY were cloned into pET-33b for His-tagged purification)

[b] equivalent Δ6 mutation made in CDCs Perfringolysin O, Intermedilysin and Anthrolysin O

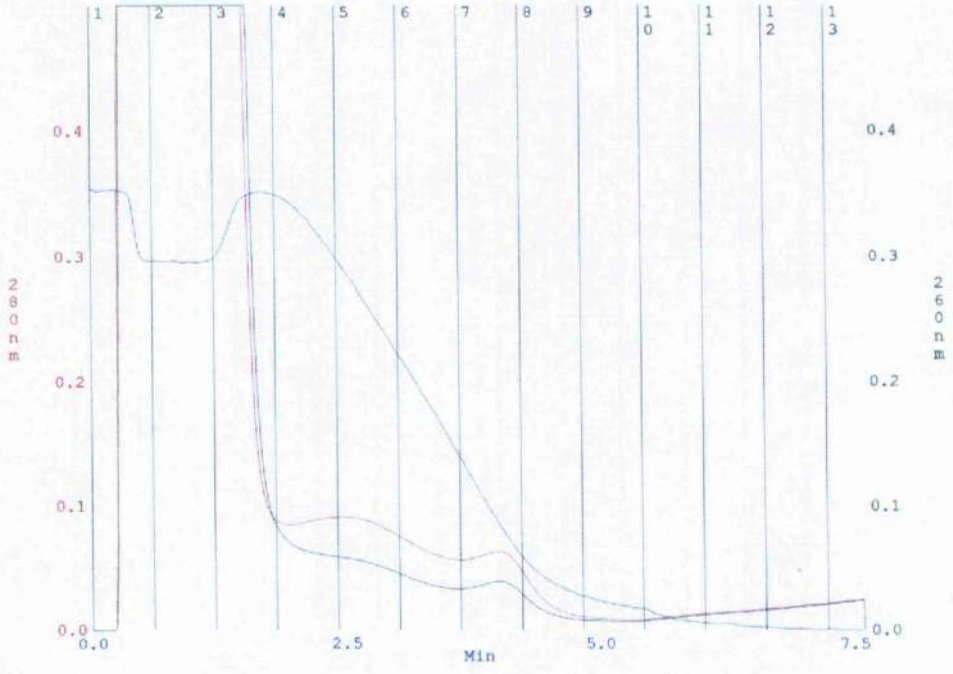
bold type denotes mutants that were purified for further characterisation

3.2. Purification of Ply and derivatives

All proteins were purified using a two-step method of hydrophobic interaction chromatography (HIC) and anion exchange chromatography (AEC). This improved protein purity and yield, which was 15-20mg/L culture. In general, the mutants were easier to purify than WT Ply and could be concentrated to at least 3mg/ml without the aggregation that often occurs during WT Ply purification. The isoelectric point (pI) of Ply is 5.7, making it slightly negatively charged at neutral pH whereas PFO has no charge at PH7, with a pI of 7.1. As a result of this, PFO did not bind to the AEC column but the DNA and contaminating proteins did, allowing PFO to be collected in the wash through. The inclusion of an anion exchange step removed residual contaminating proteins and also reduced endotoxin levels 100 fold (section 3.4).

Figure 3.2.1. shows a representative HIC purification of WT Ply. Fractions 1-4 are the non-specific proteins, which are washed through the column, and the peak over fractions 5-8 is Ply, as shown in SDS-PAGE gels of the HIC fractions (Figure 3.2.3).

Figure 3.2.1. HIC purification of WT Ply

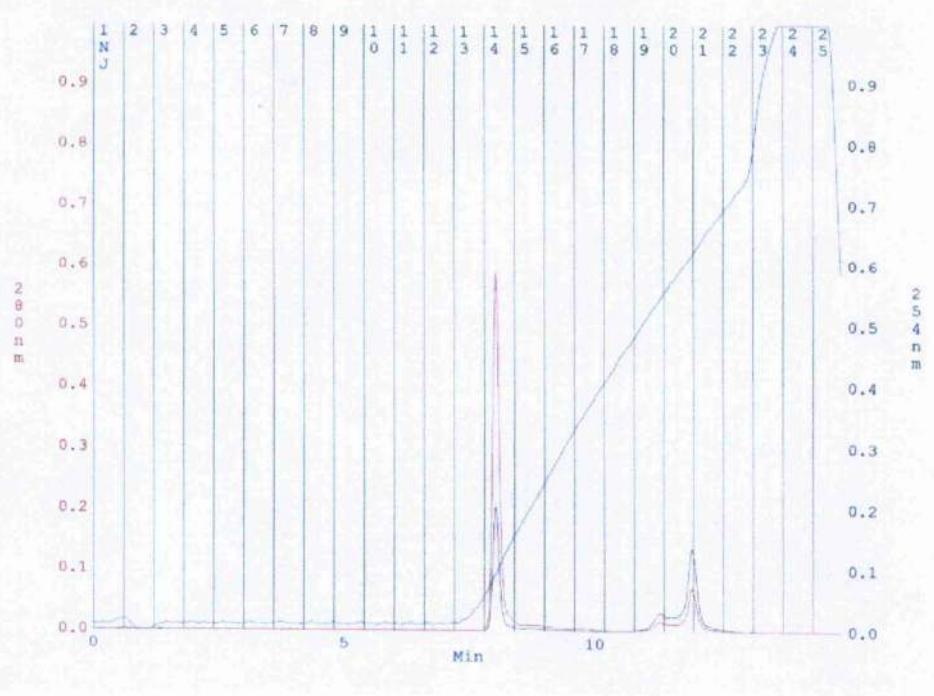


Hydrophobic Interaction Chromatography of WT Ply. The vertical numbered lines represent fractions that were collected in separate test tubes. The red line is the A280nm reading, which measures protein content. The green line is the A260nm reading, which measures DNA levels. The blue line represents conductivity (M) using NaCl.

Fractions 5-8 were pooled from each run and dialysed overnight at 4°C in pre-chilled dialysis buffer (see Appendix I) to remove NaCl. The following day, the buffer was replaced with fresh dialysis buffer and left for a further 4h then the HIC purified Ply was concentrated at 4°C using amicon centrifugation filters at 2080g (30kDa MW cut off). The concentrated sample was then further purified using AEC, Figure 3.2.2. Ply binds to the negatively charged column, allowing thorough washing and is eluted with 0.1M NaCl, in fraction 14 (run on SDS-PAGE, Figure 3.2.3). The DNA remains tightly

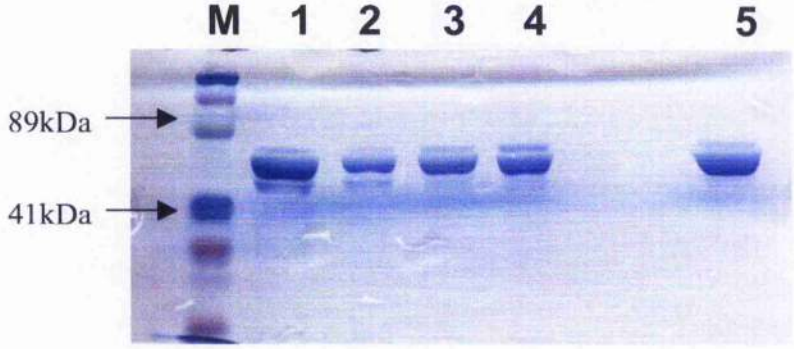
bound to the column and is only eluted when 1.5M NaCl is reached. This further purification step also removed significant amounts of LPS as shown by LPS silver staining of proteins before and after AEC (Figure 3.4.1 and Table 3.3).

Figure 3.2.2. AEC purification of WT Ply



Anion Exchange Chromatography of WT Ply. Green vertical numbered lines represent fractions. Red line is A280nm protein reading, green line is the A254nm DNA reading. The blue line represents NaCl concentration (M). Fraction 14 was pooled from each run.

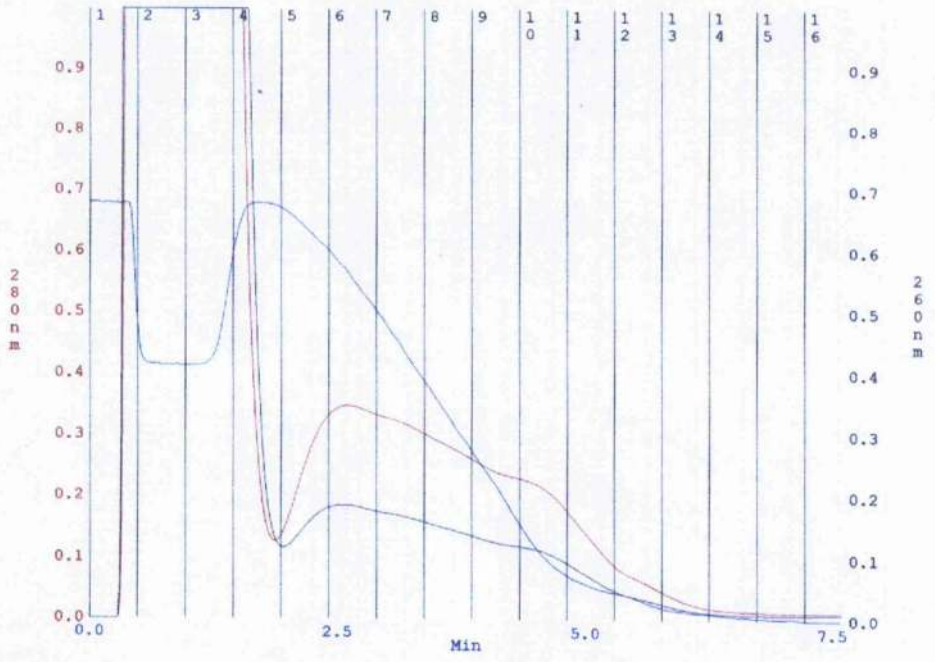
Figure 3.2.3. Coomassie stained SDS-PAGE of WT Ply purification



M, kaleidoscope marker; 1-4, HIC WT Ply fractions 5-8; 5, AEC WT Ply fraction 14 (4.8μg).

When Ply is over-expressed in some strains of *E. coli* two bands are observed in SDS-PAGE and Western blotting. This is thought to occur when there is occasional read through of the stop codon for the *ply* gene. When *sup*A$^+$ *E. coli* strains are used the stop codon is always read and a single band of expressed Ply is observed on gels (T. J. Mitchell, personal communication, 2003). A single band of Ply is observed in Western blots of Ply expressed by *S. pneumoniae* (See blots in Chapter 7).

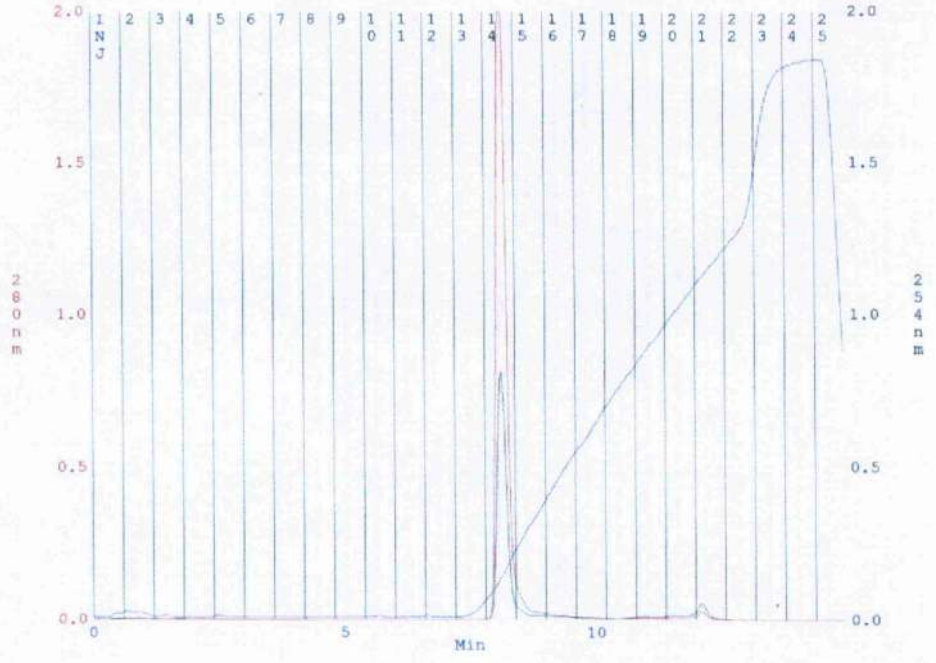
Figure 3.2.4. HIC purification of ΔA146 Ply



Hydrophobic Interaction Chromatography of ΔA146 Ply. Green vertical numbered lines represent fractions. Red line is A280nm protein reading; green line is the A260nm DNA reading. The blue line represents NaCl concentration (M).

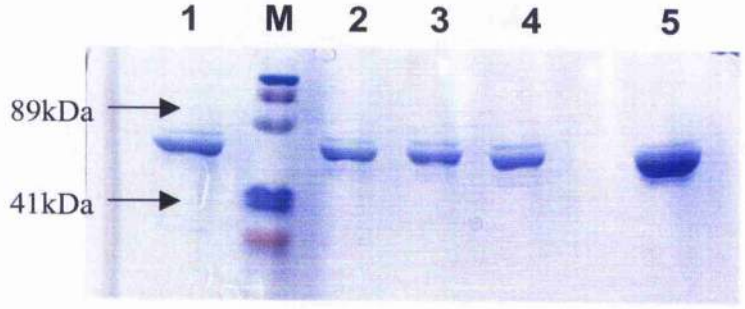
Fractions 6-9 from HIC purification of ΔA146 Ply were pooled from each run (Figure 3.2.4), dialysed overnight and concentrated as for WT Ply. As there was more protein for HIC, the AEC yield was also greater as shown in Figure 3.2.5. where fraction 14 was collected. Protein purity is shown by SDS-PAGE, Figure 3.2.6.

Figure 3.2.5. AEC of ΔA146 Ply



Anion Exchange Chromatography of ΔA146 Ply. Green vertical numbered lines represent fractions. Red line is A280nm protein reading; green line is the A254nm DNA reading. The blue line represents NaCl concentration (M). Fraction 14 was pooled from each run.

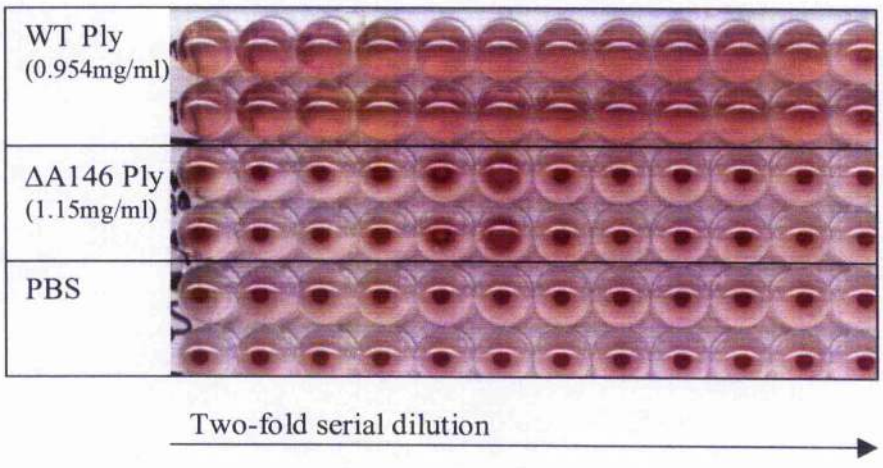
Figure 3.2.6. Coomassie stained SDS-PAGE of ΔA146 Ply HIC and AEC fractions



1, HIC ΔA146 Ply fraction 6; M, kaleidoscope marker; 2-4, HIC ΔA146 Ply fraction 7-9; 5, AEC ΔA146 Ply faction 14 (5.8μg).

Following purification, the samples were assessed in a haemolytic assay (Figure 3.2.7) to determine their specific activity. Two fold serial dilutions of the purified toxin were incubated with a 2% erythrocyte suspension and the endpoint was taken as the well with 50% lysis and 50% pelleted erythrocytes (as shown in the last wells of the WT Ply sample in Figure 3.2.7). The reciprocal titre of the endpoint dilution is used to give Haemolytic Units (HU) per ml of sample (explained in section 2.7.2). Figure 3.2.7 shows that the purified WT Ply has 8.2×10^4 HU/ml (giving a specific activity of 8.4×10^4 HU/mg) whereas the purified ΔA146 Ply mutant was not haemolytic. The aggregation of erythrocytes by ΔA146 Ply was observed in haemolytic assays and appeared to be concentration dependent. This effect is not observed with WT Ply in these assays due to the lysis of the erythrocytes however, fluorescence microscopy of GFP tagged WT Ply revealed that this aggregation is not mutant specific.

Figure 3.2.7. Haemolytic assay of purified WT Ply and ΔA146 Ply



3.3. Determination of protein concentration and specific activity of purified protein

The concentration of purified protein was determined using an absorbance scan at 280nm. Purified samples were to have a low A260nm (DNA concentration) and a low A320nm (protein aggregates). The absorbance at 280nm was then divided by the extinction coefficient (E) of Ply = 1.3 (Morgan et al. 1993). Protein concentrations were confirmed with a Bradford's assay.

The concentration of purified protein is then divided by the HU/ml to give the specific activity of Ply in HU per mg of protein. The concentration and specific activities of purified proteins used in this study are given in Table 3.2.

Table 3.2. Concentration and specific activity of purified proteins

Protein	Concentration (mg/ml)	Specific Activity (HU/mg)
WT Ply	0.522	6×10^5
ΔA146 Ply	1.31	0
Δ7 Ply[+]	5.63	0
Δ8 Ply[+]	3.88	0
W433F	0.33	5×10^4
Δ6D385N	3.71	0
Δ6W433F	1.26	0
Δ6D385NW433F	3.37	0
PFO[*]	0.338	2×10^5
Δ6 PFO	0.2	0

[*] The isoelectric point of PFO is 7.1, this means that at a neutral pH of 7, PFO has no charge. The PH of buffers could have been changed to 6.5 give PFO a +ve charge for cation purification. Instead, the column was made more hydrophobic by purifying PFO at 2M NaCl rather than 1M that is used for Ply purification. [+] purified by Wyeth.

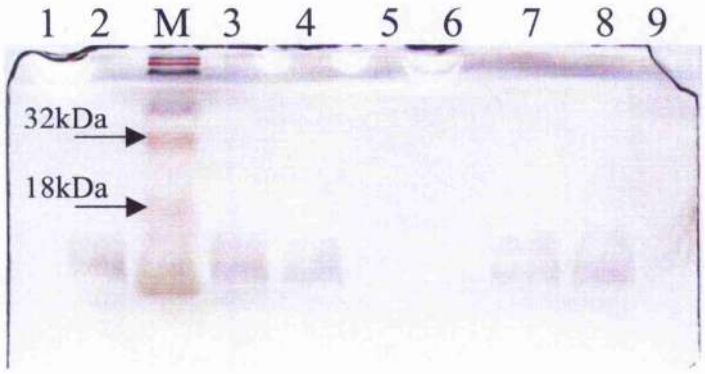
3.4. Measurement of LPS levels in purified proteins

As the recombinant proteins are purified from Gram-negative *E. coli*, the samples can be contaminated with LPS from the cell wall. LPS is a potent inflammatory agent and there are limitations of LPS levels in vaccine preparations. Because of this, LPS levels were assessed in purified proteins and mechanisms to remove LPS were investigated.

3.4.1. Assessment of LPS in HIC and AEC purified samples using silver staining

From previous work in the laboratory, LPS levels in HIC purified samples were known to be high (Table 3.3). Posidyne syringe filters were assessed in their ability to remove LPS from Ply but this was not successful (Figure 3.4.1). An additional AEC chromatography step was introduced and this resulted in considerable removal of contaminating LPS (Figure 3.4.1. and Table 3.3). Samples were incubated with Proteinase K to digest all proteins so that only LPS (6kDa) was stained on an SDS-PAGE gel. The only sample that had no detectable levels of LPS was the HIC and AEC purified Ply (Figure 3.4.1).

Figure 3.4.1. Silver stained SDS-PAGE gel of LPS in WT Ply samples



1, HIC and AEC WT Ply; 2, WT Ply cell lysate; M, kaleidoscope marker; 3, filtered WT Ply cell lysate; 4, posidyne filtered WT Ply cell lysate; 5-6, blank; 7-9, HIC purified fractions 6-8.

3.4.2 Quantification of LPS contamination of purified proteins using the LAL assay

The LAL (Limulus Amebocyte Lysate) assay was used to quantify LPS in the purified proteins before and after AEC. A 100 to 300-fold decrease in LPS levels was observed following AEC (Table 3.3). All proteins used for further investigation were HIC and AEC purified to ensure the lowest LPS levels possible.

Table 3.3. LPS levels in protein samples following different types of purification

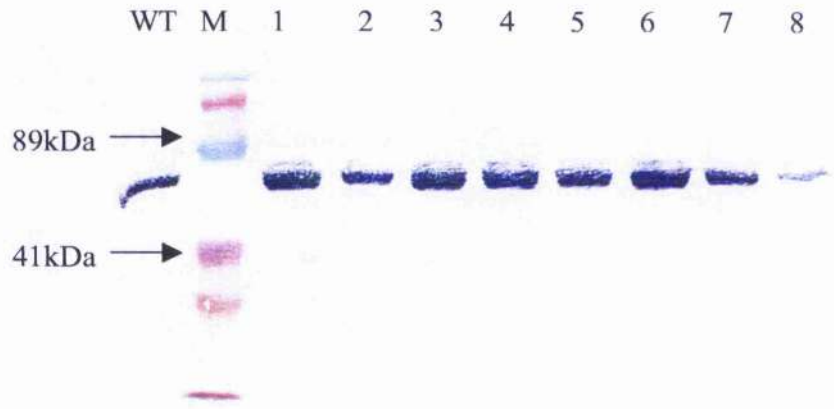
Sample	Reference	Purification*	EU/mg	pgLPS/μg toxin
WT Ply	(Search 2002)	MC	6,800	680
WT Ply	(Search 2002)	HIC	20,000	2000
ΔA146 Ply	This work	HIC	14,607	1460
WT Ply	This work	**HIC + AEC**	**641**	**64**
ΔA146 Ply	This work	**HIC + AEC**	**125**	**12.5**

* HIC, Hydrophobic Interaction Chromatography; AEC, Anion Exchange Chromatography; MC, Metal Chelate purification. Bold type denotes batch of WT Ply and ΔA146 Ply used for all *in vivo* analysis.

3.5. Expression of mutated forms of Ply

All mutants were detected in Western blots with both rabbit polyclonal anti-Ply antibody (raised in our laboratory, blot not shown) and mouse monoclonal antibody Ply4 (kindly provided by Juan de los Toyos, Oviedo, Spain), Figure 3.5. Expression levels were similar, though possibly less was produced by Δ8 Ply (Δ8 Ply was also found to be the least soluble in expression studies by Wyeth; Dr. Deborah Dilts, 2005, personal communication).

Figure 3.5. Western blot of Ply double amino acid deletions detected by monoclonal antibody Ply 4



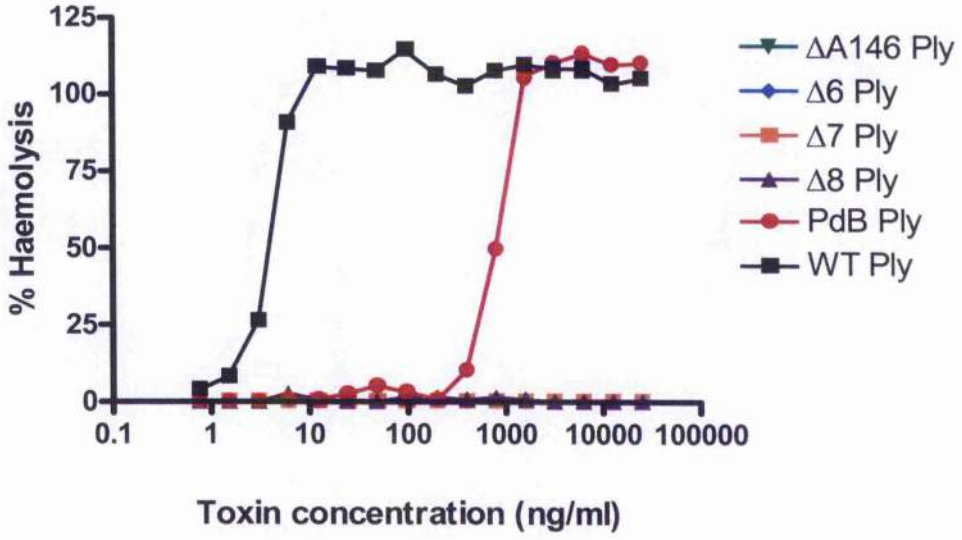
WT, wild type Ply; M, Kaleidoscope marker; 1, ΔW134H135 Ply (Δ1); 2, ΔQ136D137 Ply (Δ2); 3, ΔY138G139 Ply (Δ3); 4, ΔQ140V141 Ply (Δ4); 5, ΔV144P145 Ply (Δ5); 6, ΔA146R147 Ply (Δ6); 7, ΔM148Q149 Ply (Δ7); 8, ΔY150E151 Ply (Δ8).

Δ6 Ply (ΔA146R147) and ΔA146 Ply were purified as described. Δ7 Ply and Δ8 Ply were purified by Wyeth. These mutants were further characterised and compared with WT Ply and the PdB mutant.

3.6. Haemolytic activity of Ply mutants

Purified Δ6 (A146R147) Ply, Δ7, Δ8 and ΔA146 Ply were found to be non-haemolytic to human erythrocytes at concentrations 10,000 times greater than lytic concentrations of WT Ply (Fig. 3.6). PdB (the W433F Ply mutant) was 100 fold less haemolytic than WT Ply but was still lytic at 1μg/ml. ΔA146 Ply was concentrated to 3mg/ml and was still not lytic to human erythrocytes. Δ6, Δ7, Δ8 Ply and ΔA146 Ply also had no haemolytic activity on horse or sheep erythrocytes.

Figure 3.6. Haemolytic activity of Ply and derivatives



The haemolytic activity of pneumolysin mutants Δ6, Δ7, Δ8 Ply, ΔA146 Ply and PdB were compared with WT pneumolysin in a quantitative haemolytic assay. Following incubations of known concentrations of toxin with a human erythrocyte solution, the supernatants were collected and the haemoglobin content read at 540nm.

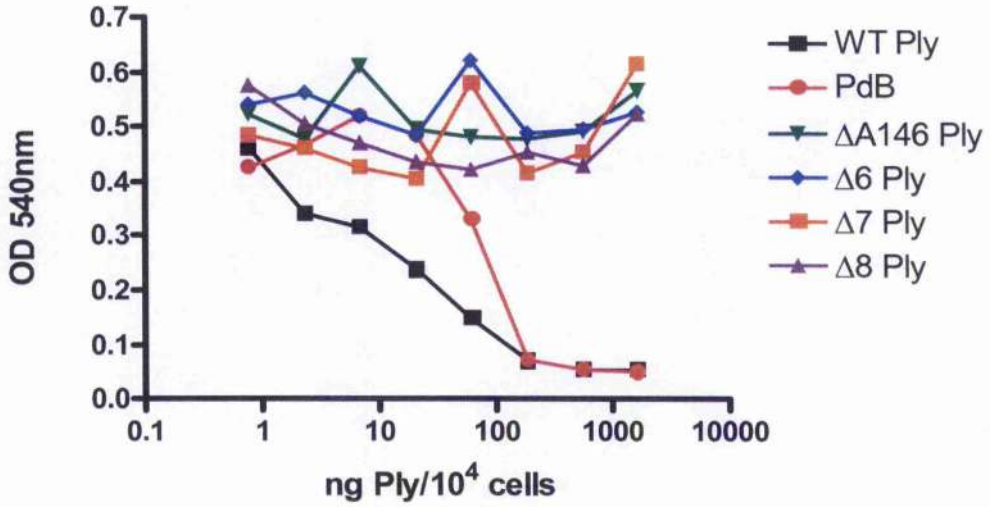
3.7. Cytotoxicity of Ply mutants to nucleated cell lines

As the Ply mutants were not found to lyse erythrocytes they were then tested for cytotoxicity to nucleated cell lines. In order to investigate this, murine fibroblasts (Figure 3.7.1), rat mast cells (Figure 3.7.2) and human monocytes (data not shown) were treated with Ply and non-haemolytic derivatives and assessed for cell damage.

3.7.1. L929 murine fibroblast cytotoxicity assay

L929 murine fibroblasts were used to assess the toxicity of Δ6, Δ7, Δ8 Ply and ΔA146 Ply to nucleated cells compared with WT Ply and PdB (W433F Ply). An MTT dye was used where mitochondria in live cells convert the yellow dye to a purple precipitate, thereby giving a high absorbance at 540nm compared with dead cells where the dye remains yellow. At concentrations of 100μg/ml (1.7μg Ply/10^4 cells), Δ6, Δ7, Δ8 Ply and ΔA146 Ply were not toxic to fibroblasts, whereas 725 fold less WT Ply (2.3ng Ply/10^4 cells) and 26 fold less PdB (63ng Ply/10^4 cells) were cytotoxic to the fibroblasts (Figure 3.7.1).

Figure 3.7.1. Cytotoxicity of Ply and derivatives to L929 murine fibroblasts



The cytotoxicity of WT Ply and the Ply mutants to nucleated cells was assessed using L929 murine fibroblasts. An MTT stain was used to assess viability of the cells following incubation with the toxins. High OD540nm indicates that the cells are alive as the mitochondria can convert MTT into a purple product. A low OD540nm indicated that the cells are dead and the MTT remains yellow.

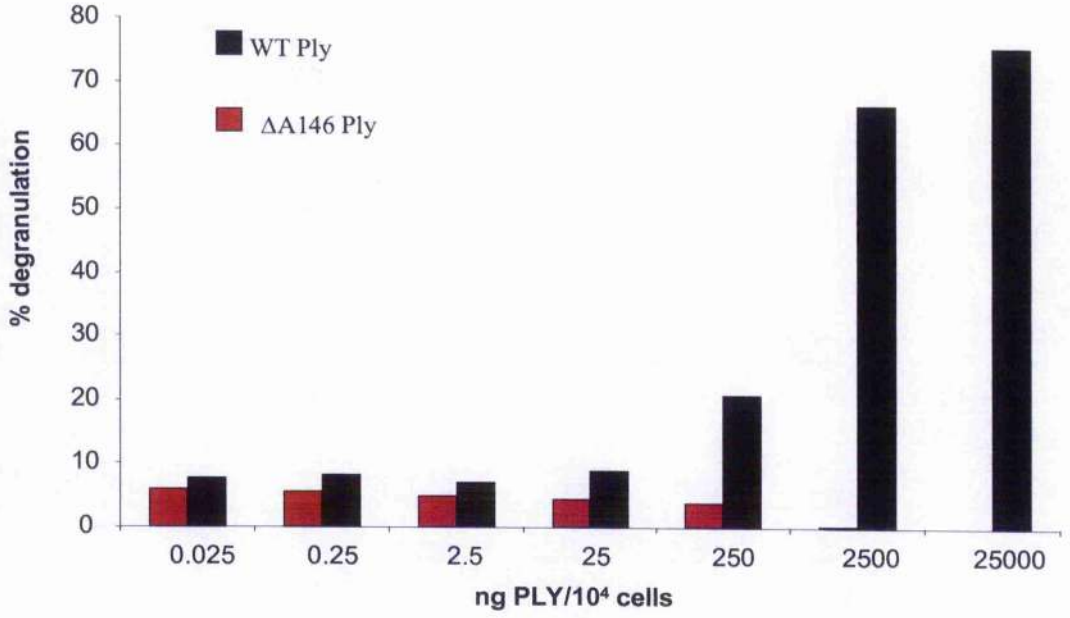
The PdB mutant was ~30 times less cytotoxic to fibroblasts than WT Ply. At 185ng, both WT Ply and PdB were completely toxic to 10^4 fibroblasts. The sensitivity of the fibroblast cytotoxicity assay (Figure 3.7.1) was similar to the haemolytic assay (Figure 3.6), though erythrocytes were not accurately quantified, with ng levels of WT Ply detected. This assay provides a sensitive measurement of toxicity to nucleated cells and revealed that the mutants made in the Δ6 region of the protein had no residual toxicity.

3.7.2. Release of β-hexosaminidase from RBL-2H3 mast cells in response to Ply treatment

Mast cells are highly sensitive and degranulate in response to antigens, releasing a range of molecules into the surrounding environment including β-hexosaminidase (Abraham et al. 1997). As mast cells are found in environments more relevant to the route of pneumococcal infection, such as mucosal lining and the lung, they were used in attempts to develop a highly sensitive assay to measure any residual activity that the ΔA146 Ply mutant may possess. 10^4 rat RBL-2H3 mast cells were incubated with serial dilutions of either WT Ply or ΔA146 Ply. Release of β-hexosaminidase was measured by the colourimetric conversion of pNAG substrate (see section 2.10.2). Degranulation of mast cells increased with increasing WT Ply concentrations but not with ΔA146 Ply treatment (Figure 3.7.2).

Heat-treated samples were also assessed to ensure the response was due to the protein and not LPS. The heat-treated samples did not elicit β-hexosaminidase release (data not shown) and also the highest concentration of ΔA146 Ply and therefore LPS did not result in degranulation. The lowest WT Ply concentration to result in degranulation of 10^4 mast cells was >250ng. This, although a more relevant cell line in terms of pneumococcal infection was not as sensitive as the fibroblast cytotoxicity assay (Figure 3.7.1) where concentrations <10ng WT Ply were cytotoxic to 10^4 L929 fibroblasts. This is discussed at the end of the chapter.

Figure 3.7.2. Mast cell degranulation in response to Ply or ΔA146 Ply treatment



Mast cell degranulation was assessed by measuring the release of β-hexosaminidase in response to treatment with either WT Ply or the non-haemolytic mutant ΔA146 Ply. WT Ply caused degranulation of RBL-2H3 mast cells in a concentration-dependent manner. This did not occur with ΔA146 Ply.

3.8. Analysis of cell binding, oligomerisation and pore forming ability of non-toxic Ply

As the mutants were not cytotoxic, their ability to form pores must be disrupted, though the stage at which this occurs i.e. host cell binding, oligomerisation or pore insertion was not known. The non-toxic mutants were analysed alongside WT Ply and PdB.

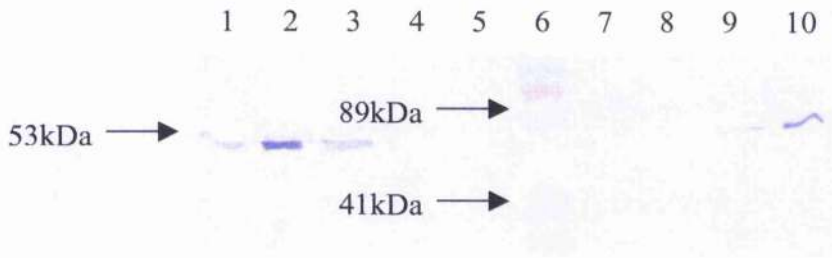
3.8.1. Binding of Ply and derivatives to erythrocyte membranes

The mutations surrounding the Δ6 region are not in domain 4, the binding domain, and therefore the binding ability of the mutants would probably not be altered. This was investigated using a modified binding assay (Owen et al. 1994) and fluorescence microscopy of GFP-tagged WT Ply and Δ6 Ply. The ability of Δ6 Ply to protect erythrocytes from WT Ply lysis was also investigated.

3.8.1.1. Binding assessed by Western blotting

10μg/ml of toxin was incubated with equal volumes of a 2% sheep erythrocyte suspension for 30 min at 37°C. Following incubation, the membranes were washed to remove unbound toxin and the sample run on SDS-PAGE. Immunoblotting with polyclonal α-Ply antibody was then used to detect any toxin that has bound to the erythrocyte membrane. Lanes 2 and 10 of Figure 3.8.1 show that Δ6 Ply and WT Ply bind to erythrocyte membranes. After the first wash of the membranes, unbound toxin was still present in the wash (Figure 3.8.1, lanes 3 and 9), demonstrating that further washes of the membranes were required to remove unbound toxin (Figure 3.8.1, lanes 4, 5, 7 and 8).

Figure 3.8.1. Western blot of Ply and Δ6 Ply bound to erythrocyte membranes



1, 10ug/ml WT Ply control; 2, washed erythrocyte membranes incubated with Δ6 Ply; 3, 1st wash of membranes incubated with Δ6 Ply; 4, 2nd wash of membranes incubated with Δ6 Ply; 5, 3rd wash of membranes incubated with Δ6 Ply; 6, kaleidoscope marker; 7, 3rd wash of membranes incubated with WT Ply; 8, 2nd wash of membranes incubated with Δ6 Ply; 9, 1st wash of membranes incubated with Δ6 Ply; 10, washed erythrocyte membranes incubated with WT Ply.

Further Ply derivatives were assessed for their binding ability. Figure 3.8.2 shows that the triple mutant Δ6D385NW433F, Δ6, Δ7 and Δ8 Ply bind to erythrocyte membranes. Although the W433F mutation is in the binding domain, binding does not seem to be altered. This technique is not quantitative but a FACS based method using GFP tagged versions of the proteins would provide such results.

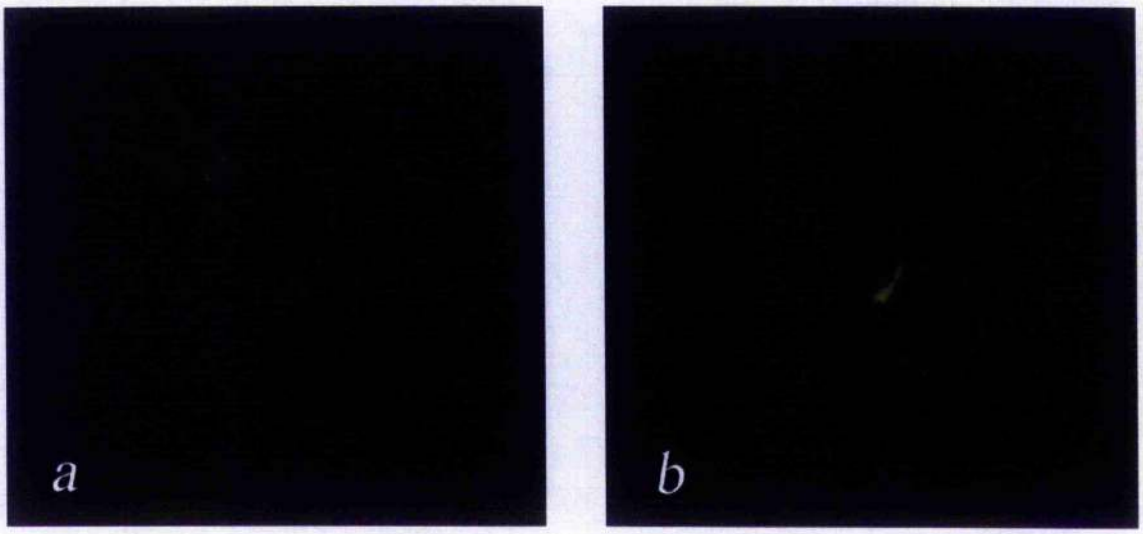
Figure 3.8.2. Western blot of Δ6, Δ7, Δ8, Δ6D385NW433F and W433F Ply bound to erythrocyte membranes

M, Precision^{+} kaleidoscope marker; 1, WT Ply only; 2, Δ6 Ply + erythrocytes; 3, Δ7 + erythrocytes, 4, Δ8 + erythrocytes; 5, blank; 6, Δ6D385NW433F Ply + erythrocytes; 7, W433F Ply + erythrocytes; 8, blank; 9, WT Ply + erythrocytes. All samples were thoroughly washed to remove unbound protein prior to running on the gel.

3.8.1.2. Fluorescence Microscopy of eGFP tagged Ply and Δ6 Ply.

The ability of Δ6 Ply to bind to human erythrocyte membranes was confirmed by visualising GFP tagged forms of Δ6 Ply and WT Ply (constructed by Mr. Graeme J. M. Cowan) by fluorescence microscopy (Figure 3.8.3). Erythrocyte ghosts treated with eGFP alone were not visualised under fluorescence, confirming that it is Ply that binds to the cell membrane.

Figure 3.8.3. Fluorescence microscopy of erythrocyte ghosts treated with eGFP-tagged Ply



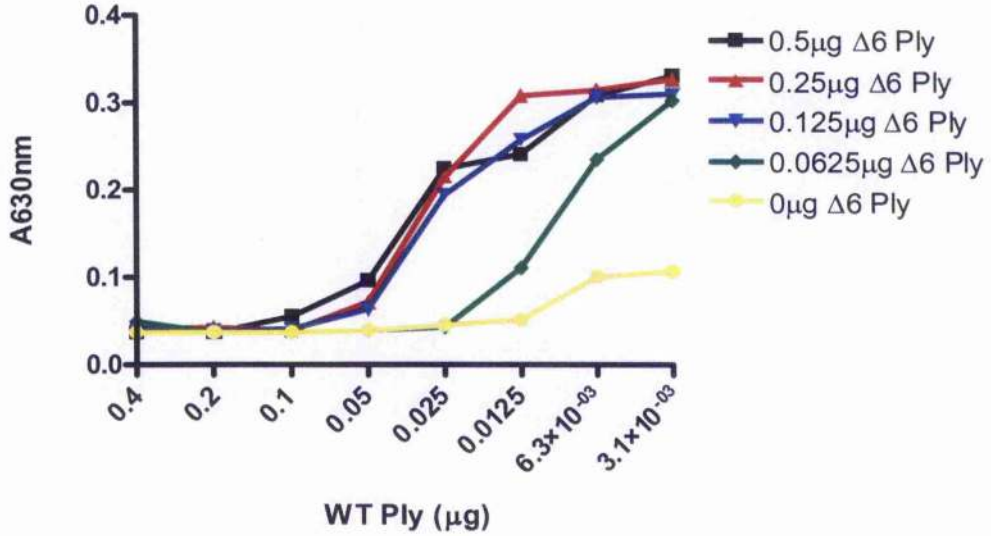
Fluorescence microscopy of (a) eGFP-tagged WT Ply bound to erythrocyte ghosts and (b) eGFP-tagged Δ6 Ply bound to erythrocyte ghosts. Human erythrocyte ghosts were treated with 0.05mg/ml eGFP-tagged toxin. Viewed with × 100 objective. Images courtesy of Mr. Graeme J. M. Cowan.

3.8.1.3. Inhibition of WT Ply lysis of erythrocytes by prior treatment with Δ6 Ply

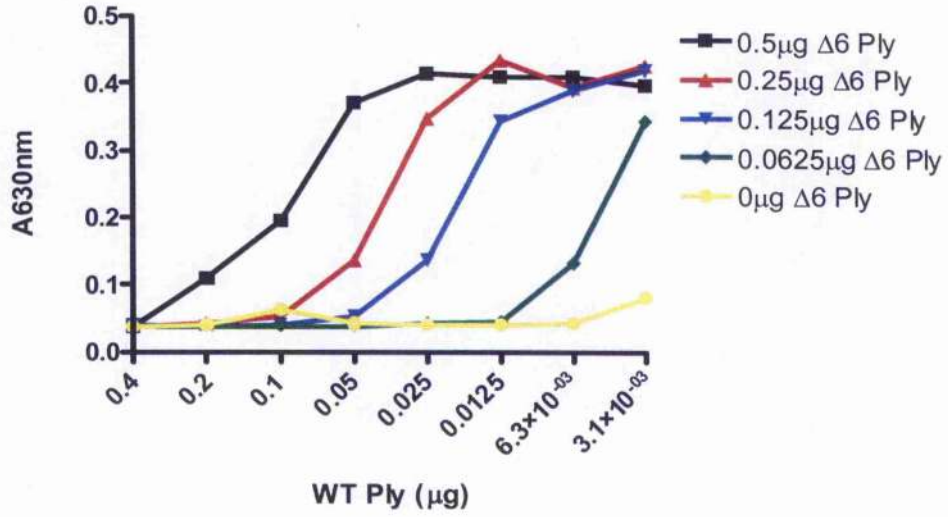
The ability of Δ6 Ply to protect erythrocytes from WT lysis was assessed by treating sheep erythrocytes with various concentrations of Δ6 Ply and WT Ply either by incubating both proteins together with the erythrocytes to investigate competition for binding (Figure 3.8.4.a) or by pre-incubating the erythrocytes with Δ6 Ply and then treating with WT Ply (Figure 3.8.4.b).

Figure 3.8.4. Inhibition of haemolysis from WT Ply by binding of Δ6 Ply

(a)



(b)



Inhibition of sheep erythrocyte lysis from WT Ply by competitive treatment with Δ6 Ply (a) or by incubation of erythrocytes with Δ6 Ply prior to WT Ply treatment (b).

Figure 3.8.4.a. shows that erythrocytes are protected from WT Ply lysis by treatment with Δ6 Ply. Both proteins were added at the same time thereby suggesting that there is competition for binding. 0.1μg WT Ply was lytic to the erythrocytes irrespective of the amount of Δ6 Ply added. However, at lower concentrations of WT Ply, Δ6 Ply prevented erythrocyte lysis in a concentration dependent manner. 0.0125μg of WT Ply was lytic to erythrocytes that were not treated with Δ6 Ply, however, concentrations ≥0.25μg of Δ6 Ply protected erythrocytes from complete lysis with this concentration of WT Ply. Protection from lysis increased when erythrocytes were pre-incubated with Δ6 Ply for 30min and then treated with WT Ply, Figure 3.8.4.b. Erythrocytes were protected from otherwise lytic concentrations of WT Ply with pre-incubation with ≥0.125μg of Δ6 Ply. Erythrocytes were completely protected from WT Ply lysis when pre-incubated with 10 times as much Δ6 Ply, for example, 0.5μg Δ6 Ply prevented erythrocyte lysis from 0.05μg WT Ply and 0.25μg Δ6 Ply protected erythrocytes from lysis with 0.025μg WT Ply (Figure 3.8.4.b).

3.8.2. Investigation of oligomerising and pore forming abilities of non-toxic Ply

As the binding step in pore formation was not altered by construction of non-toxic mutants, the next steps to investigate were oligomerisation and pore formation. As the region mutated is thought to be involved in monomer-monomer recognition, oligomerisation may be affected. Oligomerisation was investigated by cross linking membrane bound toxin with DSS (Disuccinimidyl suberate), which cross-links the amines on lysines and is lipophilic and membrane permeable, making it useful for cross-linking oligomers that are associated with the host cell membrane. Transmission

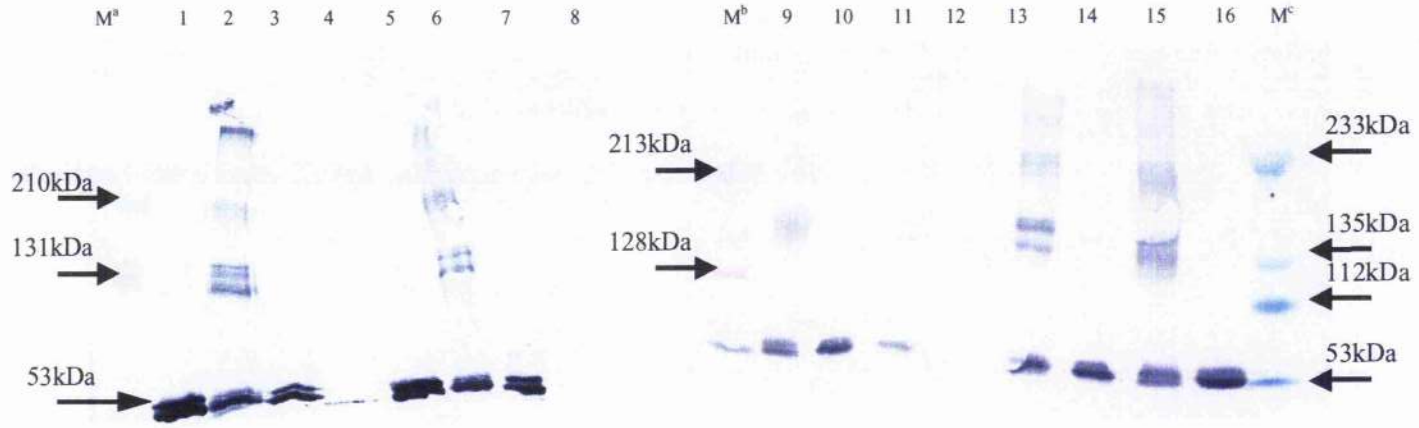
electron microscopy was used to assess the ability of a non-toxic mutant to form pores in host cell membranes.

3.8.2.1. Cross-linking of toxin bound to erythrocyte membrane

Erythrocyte ghosts were incubated with either WT Ply or Δ6 Ply for 30min at 37°C and washed to remove unbound toxin as for the binding assays. 2mM DSS was then added to cross-link any membrane bound toxin prior to running on 5% SDS-PAGE, which was then Western blotted with anti-Ply serum (Figure 3.8.5).

Both WT Ply and Δ6 Ply were found to form multimeric structures when cross-linked with DSS (lanes 2, 6, 9, 11, 13 and 15 of Figure 3.8.5). When the proteins were incubated with erythrocyte membranes prior to DSS cross-linking, the higher molecular weight bands are more defined (lanes 2, 6, 11 and 13) with the approximate size of trimers and tetramers (~160kDa and ~212kDa respectively). This data suggests that Δ6 Ply can form multimeric structures in solution and may not bind to the membrane as a monomer.

Figure 3.8.5. Western blot of cross-linked membrane bound toxin using anti-Ply antibody



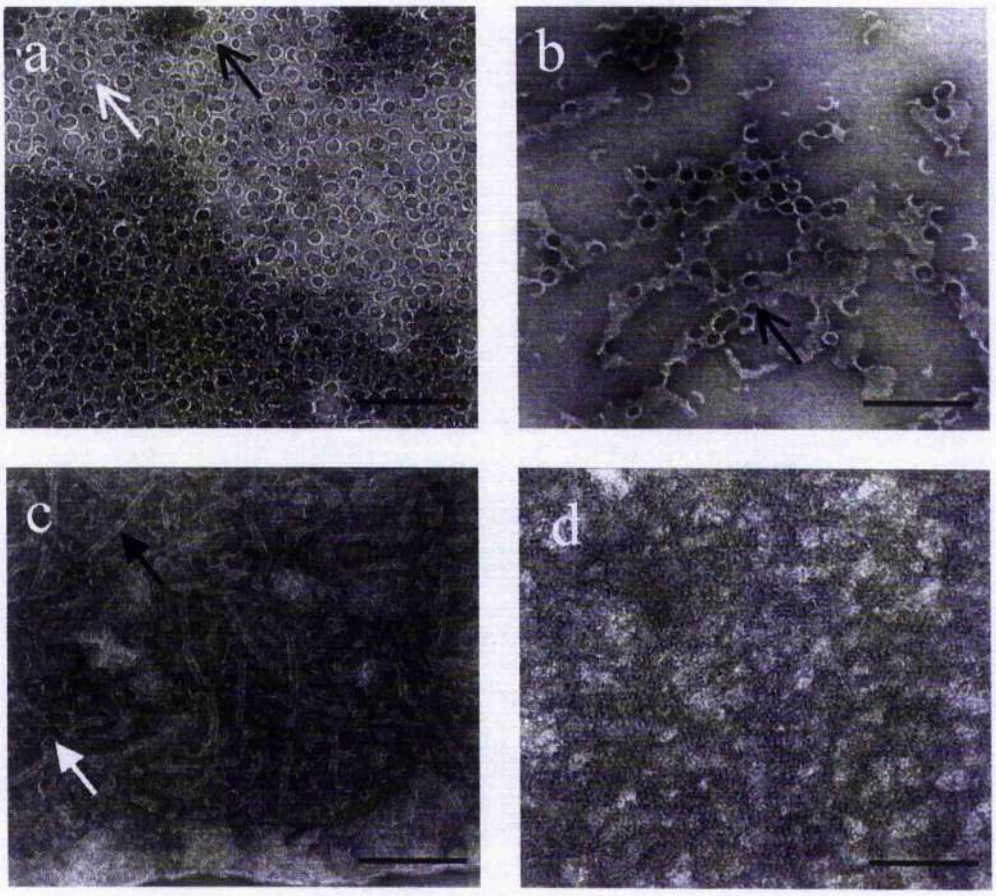
Treatment	M[a]	1	2	3	4	5	6	7	8	M[b]	9	10	11	12	13	14	15	16	M[c]
WT Ply		•	•	•							•	•	•						
Δ6 Ply						•	•	•							•	•	•	•	
DSS			•		•		•				•		•	•	•		•		
cells			•	•	•		•	•	•			•	•	•	•	•			

M[a], high molecular weight kaleidoscope marker; M[b], precision plus high molecular weight kaleidoscope marker; M[c], high molecular weight marker; 1, WT Ply ; 2 and 11, DSS treated WT Ply incubated with erythrocytes; 3 and 10, WT Ply incubated with erythrocytes; 4 and 12, DSS and erythrocytes; 5 and 16, Δ6 Ply ; 6 and 13, DSS treated Δ6 Ply incubated with erythrocytes; 7 and 14, Δ6 Ply incubated with erythrocytes; 8, erythrocytes; 9, WT Ply and DSS; 15, Δ6 Ply and DSS.

3.8.2.2. Investigation of pore formation using Transmission Electron Microscopy

Pores were readily visualised by Transmission Electron Microscopy of negatively stained erythrocyte membranes treated with WT Ply (Figure 3.8.6.a) and PdB (Figure 3.8.6.b) but not on membranes treated with ΔA146 Ply (Figure 3.8.6.c). Instead, arcs and rod-like structures were observed on ΔA146 Ply treated membranes that were not present on untreated membranes (Fig. 3.8.6.d).

Figure 3.8.6. Transmission Electron Microscopy of erythrocyte membrane treated with Ply and derivatives



Electron micrograph of negatively stained horse erythrocyte membranes treated with (a) 0.2mg/ml WT Ply; (b) 0.2mg/ml PdB; (c) 0.2mg/ml ΔA146 Ply; (d) PBS. Black horizontal scale bar represents 200nm, magnification × 25000. White arrows highlight arc structures, the black arrows point to pores (>) or rod structures (►).

Discussion

The combination of HIC and AEC resulted in highly purified Ply with reduced levels of contaminating LPS. For this work and all future research in our laboratory this double purification method has been adopted and in the case of His-tagged proteins the AEC step is included after metal chelate chromatography.

The Ply mutants (Δ5 to Δ8) were recognised by mAb Ply4 (Figure 3.5). Recently, pre-treatment with mAb Ply 4 has been shown to passively protect mice from otherwise lethal doses of WT Ply treatment and *S. pneumoniae* infection (Garcia-Suarez et al. 2004). This may be a result of mAb Ply4 blocking Ply's ability to oligomerise *in vivo*, a property that has already been demonstrated *in vitro* (de los Toyos et al. 1996). It is important to note that mAb Ply4's recognition of these mutants shows that an essential epitope has been retained. This epitope has previously been shown to be highly antigenic by epitope scanning and is recognised by both human convalescent sera and rabbit hyper-immune sera (Saio et al. 1993).

In vitro characterisation of the double amino acid deletion ΔA146R147 (Δ6 Ply) and the single amino acid deletion ΔA146 Ply revealed that they were not cytotoxic to erythrocytes or nucleated cells unlike native Ply or the PdB mutant. Erythrocytes were shown to be protected from WT Ply lysis by pre-incubation with Δ6 Ply (Figure 3.8.4.b) and this has been previously shown with pre-incubation of membranes with domain 4 of Ply (Baba et al. 2001). Such protection of cells is likely to be a result of the saturation of the membrane binding sites by the non-lytic mutant thereby preventing the native toxin from binding to and damaging the host cell membrane.

The ability of the mutants to activate the complement cascade was assessed using an ELISA that measures production of C3a by C3 cleavage (data not shown). Although Δ6 Ply and ΔA146 Ply activated complement to approximately the same levels as WT Ply, there was a lot of variation in the assay and time did not permit optimisation.

Mast cells are prominent beneath the epithelial layer of skin and mucosal linings and are some of the first cells to encounter and attack antigens (Abraham et al. 1997) making a mast cell based assay relevant to pneumococcal infection. The involvement of mast cells during pneumococcal colonisation or disease is not well documented. A recent study has shown that *S. pneumoniae* stimulates degranulation of RBL-2H3 mast cells without the release of prestored cytokines (Barbuti et al. 2006). However, in this paper the pneumococci are added to culture media containing penicillin, which would lyse the bacteria and result in the release of a range of inflammatory mediators including Ply. This would indeed represent the clinical situation where a patient with IPD was treated with a β-lactam antibiotic but does not prove that the pneumococcus stimulates mast cell degranulation in the absence of antibiotics. SLO, the CDC produced by *S. pyogenes*, has also been shown to cause degranulation of mast cells (Stassen et al. 2003), but this is the first report that purified WT Ply does this. It may be that only when Ply is released by *S. pneumoniae*, from the action of LytA or β-lactams, are mast cells degranulated *in vivo*. The ΔA146 Ply mutant did not cause mast cell degranulation, indicating that it is the haemolytic activity of Ply that is responsible for mast cell degranulation.

The mast cell assay was not as sensitive as the cytotoxicity assay using fibroblasts, which was able to detect cytotoxicity of WT Ply at 2ng WT Ply/10^4 L929 fibroblasts compared with a minimum cytotoxic level of 250ng WT Ply/10^4 RBL-2H3 mast cells. Different cell types have previously been shown to vary in their sensitivity to Ply, with human U937 monocytes more resistant to the lytic action of Ply compared with human lung epithelial

cell lines A549 and L132 (Hirst et al. 2002). Differentiated cell lines have also been shown to become either more or less sensitive to treatment with toxins Ply and SLO (Tanigawa et al. 1996; Hirst et al. 2002). Such differences in sensitivity to toxin treatment have been attributed to differences in the structure and dynamics of the cell membrane. In the work presented here we can not make such conclusions as the different cell types were investigated in different assays with one measuring viability of cells in response to Ply and the other a biochemical measure of sensitivity to Ply.

We have shown that although Δ6 Ply binds to erythrocyte membranes (Figure 3.8.1 and 3.8.3) and forms multimeric structures (Figure 3.8.5), it does not disrupt cell membranes (as shown by the haemolytic assay in Figure 3.6 and the viability assays in Figures 3.7.1 and 3.7.2). According to the Ply structural model based on perfringolysin O (PFO) (Rossjohn et al. 1997), the CDC produced by *Clostridium perfringens,* the Δ6 Ply mutation is at the domain 1 and 3 interface (Figure 3.1). This region is packed in an awkward manner, possibly to provide energy for the movement of domain 3 during membrane insertion (Tweten et al. 2001). Domain 3 of PFO has two transmembrane β-hairpins (TMH1 and TMH2) that unfold upon oligomerisation to allow insertion of the oligomer into the lipid bilayer (Shepard et al. 1998). A Y181A substitution in PFO prevents insertion into the membrane and arrests the oligomer in a 'prepore' state on the membrane surface (Hotze et al. 2002). This mutation is immediately upstream of TMH1 and is in the same conserved region as Δ6 in Ply. If Δ6 Ply behaved in the same manner, we would expect to detect prepore structures on the membrane surface. Although ΔA146 Ply appears to self-associate to some extent, oligomeric rings (prepores) have not been observed. Instead, arcs (also evident in the WT Ply treated sample and discussed in section 1.12) and long chains were observed by electron microscopy of ΔA146 Ply treated erythrocytes (Figure 3.8.6.c). As arcs are formed on membranes treated with ΔA146 Ply (Figure 3.8.6.c), the 'oligomerisation preceding pore formation' theory fits this observation, as

ΔA146 Ply does not lyse cells. However, the arcs observed in WT Ply treated membranes appear to be inserted into the membrane (Figure 3.8.6.a). Arcs also appear to insert in a recent study with PFO but this is not addressed by the author (Dang et al. 2005b). The long chains formed by ΔA146 Ply treatment of erythrocytes have not been described before. Helical structures have been shown to form with high concentrations of Ply, both in solution and on membranes (Gilbert et al. 1998; Gilbert et al. 1999b), however the diameter of these structures is similar to the 30nm ring structures and not <10nm as observed by electron microscopy (Figure 3.8.6.c). A possible explanation for these long chains is that the self-recognition site has been altered on the ΔA146 Ply monomers, not so much that association is inhibited but enough to prevent the regulation that must occur in native Ply to give the uniform oligomer ring shape and size of ~50 monomers (Morgan et al. 1995). Figure 3.9 shows possible Δ6 Ply monomer interactions to give the long chains based on previously published dimensions of the monomer and pore (Andrew et al. 1997). This could be further investigated using streptavidin labelling and EM (Dang et al. 2005a) to investigate the orientation of domains.

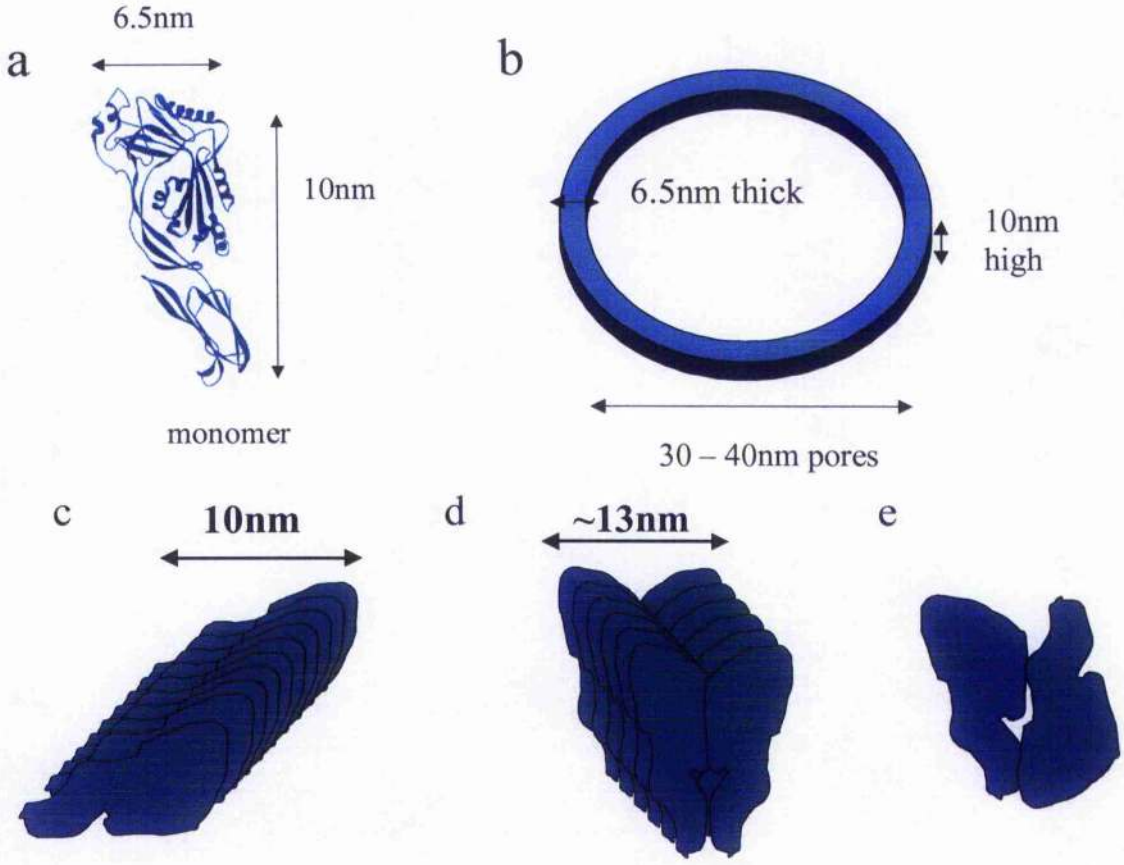
Recently, specific sites on domain 3 of PFO have been identified as being involved in monomer-monomer interaction (Ramachandran et al. 2004). TMH1 and TMH2 are linked together by 5 β-strands, β_1 to β_5. In the monomeric PFO form, β_1 is exposed whereas β_4 is shielded from protein interaction by β_5. When the PFO monomer binds to the cell membrane, a conformational change occurs that exposes β_4 to allow association with the β_1 strand of another PFO monomer. The residue on β_1 that is proposed to recognise β_4 is T179, immediately downstream of A177 and R178, the residues homologous to those deleted in Δ6 Ply (A146 R147). Deletion of A177 R178 in PFO and A204 R205 in ILY (constructed by Mr. Graeme J. M. Cowan) gives the same non-lytic phenotype as Δ6 Ply. This region is highly conserved throughout the CDCs (Table 3.4). By combining the experimental evidence with ΔA146 Ply and detailed analysis of PFO, deleting the residues

within the VPARMQYE region probably modifies a self-recognition site of domain 3 on the β_1 strand that abolishes the ability of the toxin to form oligomeric rings and insert into the host cell membrane.

Table 3.4. Homology of Δ6 region in some of the sequenced CDCs

Cholesterol Dependent Cytolysin	**Consensus sequence to Ply**
Pneumolysin	VPARMQYE
Perfringolysin O	LPARTQYSE
Septicolysin	LPARTQYSE
Streptolysin O	LPARTQYTE
Intermedilysin	VPARMQYE
Alveolysin	LPARLQYAE
Anthrolysin	LPARTQYTE
Cereolysin	LPARTQYTE
Novylisin	LPARTQYSE

Figure 3.9. Possible interaction of ΔA146 Ply molecules on erythrocyte membranes



The dimensions of the Ply monomer (a) and WT Ply pores (b) have been previously calculated (Andrew et al. 1997). From the electron microscopy images (Figure 3.8.6), the width of the ΔA146 Ply rods are about 10-12nm and this ties in with the height of WT Ply pores, so it is possible that ΔA146 Ply multimerises in the manner shown in (c), possibly due to hydrophobic interactions of domain 3 with the membrane. ΔA146 Ply does bind to membranes, presumably via domain 4, this could also occur in the manner shown in (d) where domain 4 still binds to the membrane in a perpendicular manner and self association occurs between the hydrophobic domain 3 to give rods with a width of ~13nm. Or there may be a random association of hydrophobic regions of Ply binding to monomers already bound to cholesterol on the membrane (e).

Chapter 4

Results

In vivo responses to intranasal treatment with non-toxic pneumolysin

4: *In vivo* responses to i.n. treatment with non-toxic pneumolysin

Summary

Δ6 Ply and ΔA146 Ply were demonstrated to be non-toxic to erythrocytes and nucleated cell lines in chapter 3. If either of these Ply toxoids are to be considered for inclusion in the next generation of pneumococcal vaccines then they must be demonstrated to be non-toxic *in vivo*. Both outbred and inbred mice were treated intranasally (i.n.) with mutant Ply and the host responses were compared with mice given WT Ply. I.n. administration of Ply is more sensitive than other routes of administration and it also is a more realistic way in which a host with pneumonia may be exposed to Ply. Gross symptoms, inflammatory cytokine responses, core body temperature and lung integrity in response to Ply treatment were assessed to investigate whether the non-pore forming Ply caused any detrimental effects to the host.

4.1. Gross symptoms following Ply treatment

BALB/c mice treated i.n. with 1μg ΔA146 Ply recovered from anaesthesia quicker than mice receiving any dose of WT Ply from 0.1μg to 1μg. In the initial hours post treatment the behaviour of ΔA146 Ply treated mice was similar to that of the saline control group but WT Ply treated mice exhibited piloerection, laboured breathing and a hunched stance over a 6h period, recovering within 24h. When a dose of 1μg WT Ply was administered i.n. to five MF1 mice, three reached a moribund state within 6h whereas i.n. administration of 10μg of Δ6 Ply had no adverse effects on MF1 mice; higher doses were not investigated.

4.2. Inflammatory responses of MF1 mice to i.n. administration of Ply

Anaesthetised female MF1 mice were administered i.n. with 1μg of either WT Ply or ΔA146 Ply in 50μl of sterile saline. Less than 50% of the WT Ply treated group survived this treatment (2/5), therefore the dose of WT Ply was reduced to 0.25μg (n=6). 24h post treatment, the lungs were washed with saline to give bronchoalveolar lavage fluid (BALF) and the lungs were removed and homogenised to give lung tissue. Both BALF and lung tissue were assessed for the presence of the inflammatory cytokines IL-6, TNF-α and IFN-γ by ELISA.

4.2.1. Cytokines in BALF and lung tissue of MF1 mice, 24h post Ply treatment

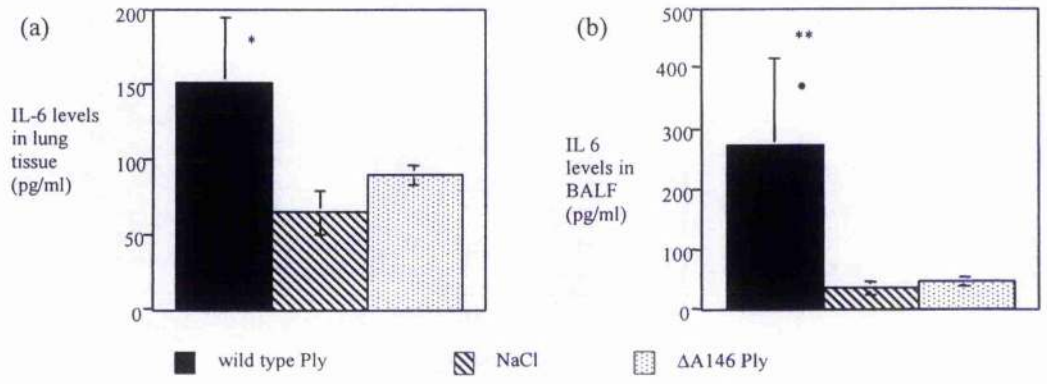
IL-6 levels were measured in the lung tissue and BALF of MF1 mice as a marker of Ply induced inflammation to the host, 24h post-treatment. IL-6 levels in the lung (and survival) in response to lower WT Ply doses of 0.1μg and 0.25μg were investigated (Table 4.1). All mice survived treatment with both 0.1μg and 0.25μg WT Ply. A dose of 0.25μg WT Ply was then administered to a larger group size (n=6) and IL-6 levels in the lungs at 24h post treatment were compared with lungs from mice treated with 1μg ΔA146 Ply, Figure 4.1.

Table 4.1. Average IL-6 levels in lung 24h post i.n. treatment with WT Ply

WT Ply dose (sample size)	Average IL-6 in lung tissue (pg/ml)	Average IL-6 in BALF (pg/ml)
0.1μg (3)	132	244
0.25μg (3)	174	308
1.0μg (2)	295	4278

An increase in IL-6 was observed in the lung tissue of MF1 mice treated with 0.25μg WT Ply ($P<0.05$) compared with the saline control however, there were no significant levels of IL-6 in the lung tissue of ΔA146 Ply treated mice compared with the saline control group (Figure 4.1.a). A 10-fold increase in IL-6 was found in the BALF of the WT Ply treated group (Figure 4.1.b) compared with ΔA146 Ply ($P<0.01$) and saline treatment ($P<0.01$). The median IL-6 level in the BALF from the WT Ply treated group was 272 pg/ml (range of 90 - 651 pg/ml) whereas the median background IL-6 level in saline treated mice was 33 pg/ml with no significant increase in mice treated with 1μg ΔA146 Ply (44 pg/ml). TNFα and IFNγ levels in the lung tissue and BALF samples were also investigated, however, the levels were low in all treatment groups at 24h.

Figure 4.1. IL-6 levels in the lungs of MF1 mice 24h post i.n. treatment with 0.25μg WT Ply or 1μg ΔA146 Ply

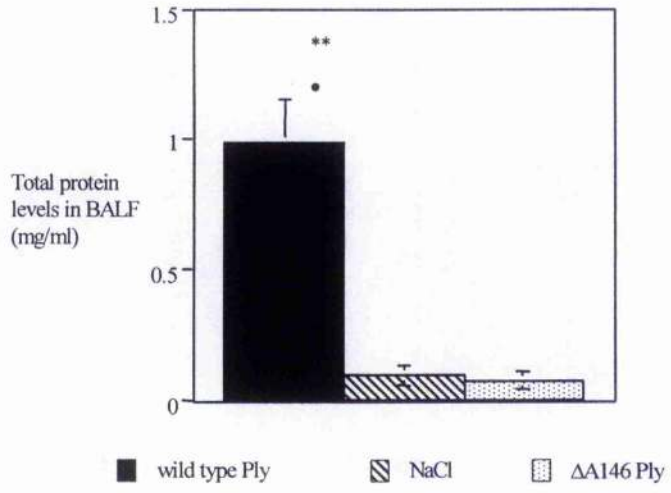


Median (± MAD) IL-6 levels in lung tissue (a) and BALF (b) at 24h post treatment with either 0.25μg WT Ply (black bar), 1μg ΔA146 Ply (spotted bar) or saline (striped bar). *P<0.05 when compared with the saline control, **P<0.01 when compared with ΔA146 Ply, •P<0.01 when compared with the saline control; n=6.

4.3. Assessment of lung integrity following i.n. Ply administration to MF1 mice

When the lung/airway interface is damaged, host serum proteins (i.e. albumin) flow into the airways. MF1 mice treated i.n. with 0.25μg WT Ply were found to have 10 times the level of total protein in their BALF, 1mg/ml total protein, compared with MF1 mice treated with 4 times as much ΔA146 Ply, 0.1mg/ml total protein (P<0.01, n = 6). Total protein levels in BALF from ΔA146 Ply treated mice were similar to healthy lavage samples, Figure 4.2.

Figure 4.2. Total protein levels in BALF from MF1 mice at 24h post i.n. treatment with 0.25μg WT Ply or 1μg ΔA146 Ply



Significantly greater levels of protein were found in the airways of mice treated with 0.25μg WT Ply (black bar) in comparison with mice treated with 1μg ΔA146 Ply (**P < 0.01, spotted bar) and the saline treated group (*P <0.01, striped bar). Values are expressed as the median ± MAD, n = 6.

4.4. Analysis of core body temperature in response to Ply treatment

The behaviour of mice following WT Ply treatment has been described earlier in this chapter and is characterised with a hunched stance and piloerection, with the animal feeling cold to the touch. This was thought to be evidence of a hypothermic response to the toxin. BALB/c mice were used for further investigation with Ply treatment as this allowed the core body temperature (Tc) in response to Ply to be correlated with the previously published observation of Tc in response to *S. pneumoniae* infection (Kerr et al. 2002). BALB/c mice can also tolerate higher doses of WT Ply compared with MF1

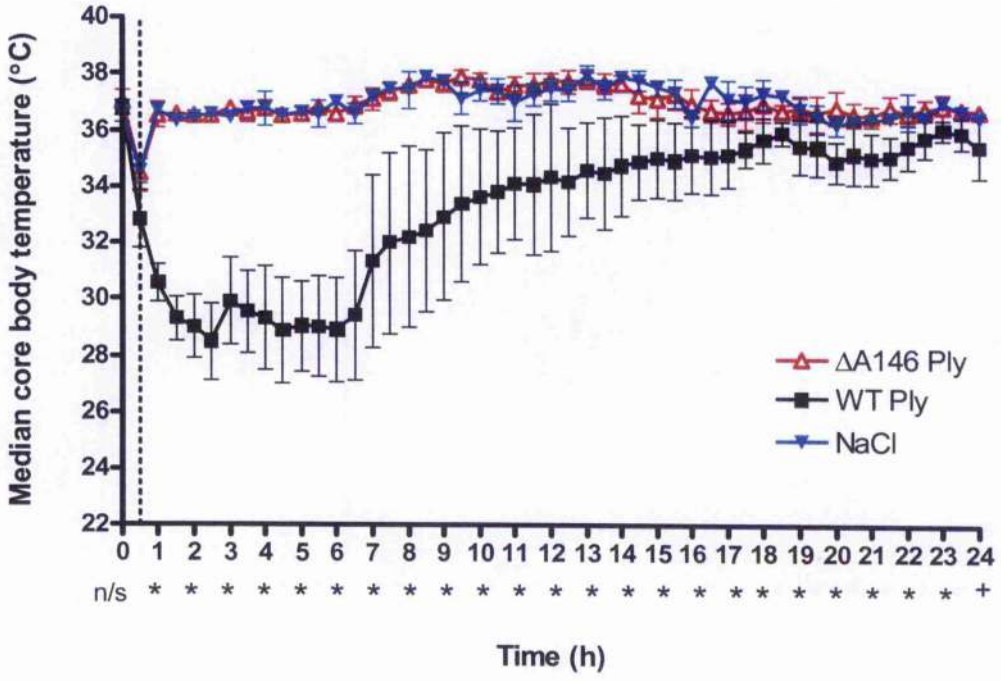
mice as demonstrated in section 4.1, this allowed direct comparison between i.n. treatment with 1μg of WT Ply and 1μg of non-haemolytic mutant protein. Female BALB/c mice were implanted with telemetry chips that record the Tc of mice via radio waves from the chip to a transmitter box upon which an individually caged animal is placed. Mice were monitored for two weeks following chip implantation and then 24h prior to toxin treatment were placed in individual cages to record their Tc. Data was acquired electronically, at chosen intervals. Immediately after i.n. treatment with either 1μg WT Ply (n=22), 1μg ΔA146 Ply (n=22) or NaCl (n=5), the mice were placed on the transmitters for 24h of data acquisition (Figure 4.3). At 2h, 6h and 24h, five mice (six for the 24h group) from the WT Ply and ΔA146 Ply treatment groups were sacrificed and sampled for analysis of inflammatory mediators in the lung, brain, BALF and serum. Total protein levels were measured in the BALF of WT Ply and ΔA146 Ply treated groups at each time point.

4.4.1. Treatment of BALB/c mice with ΔA146 Ply does not result in a hypothermic response in comparison with WT Ply treatment

BALB/c mice treated with 1μg ΔA146 Ply did not exhibit the hypothermic response observed in mice treated with 1μg WT Ply (Figure 4.3). The Tc of all treated groups dropped initially by approximately 1°C for the ΔA146 Ply and NaCl treated groups whereas the Tc of the WT Ply treated group significantly dropped by 2°C ($P<0.01$). Following this initial drop in Tc, the ΔA146 Ply and NaCl treated groups recovered to their pre-treatment Tc whereas the median Tc of the WT Ply treated mice continued to decrease to 27°C within 6h post treatment. From treatment to the 24h endpoint, the Tc of the WT Ply treated group was statistically significantly lower than the ΔA146 Ply

and NaCl treated groups. After 6h, the Tc of WT Ply treated mice increased to 35°C, however, the pre-treatment median Tc of 37°C was not obtained within 24h.

Figure 4.3. Core body temperature of BALB/c mice in response to Ply treatment



Median (± MAD) core body temperatures (Tc) of BALB/c mice over 24h following i.n. administration of either 1μg WT Ply, 1μg ΔA146 Ply (n=22 at 0h, 17 at 2h, 12 at 6h and 6 at 24h), or NaCl (n=5). The dashed vertical line represents the time of treatment. From 1h to 23h, * $P<0.01$and at 24h $^{+}P<0.05$, where the Tc of the WT Ply treated group is significantly lower than the NaCl and ΔA146 Ply groups.

4.5. Analysis of inflammatory mediator production in response to Ply treatment

In attempt to identify mediators involved in the hypothermic response induced by WT Ply or any responses to ΔA146 Ply treatment, the Luminex system was adopted to allow simultaneous measurement of multiple cytokines and chemokines in each sample at each time point. BALF, serum, lungs and brains were recovered at 2h, 6h and 24h post Ply treatment from telemetry chip implanted BALB/c mice. The samples were processed and analysed for the following analytes: IL-1β, IL-5, IL-6, IL-10, IL-12, IFN-γ, TNF-α, GM-CSF and KC.

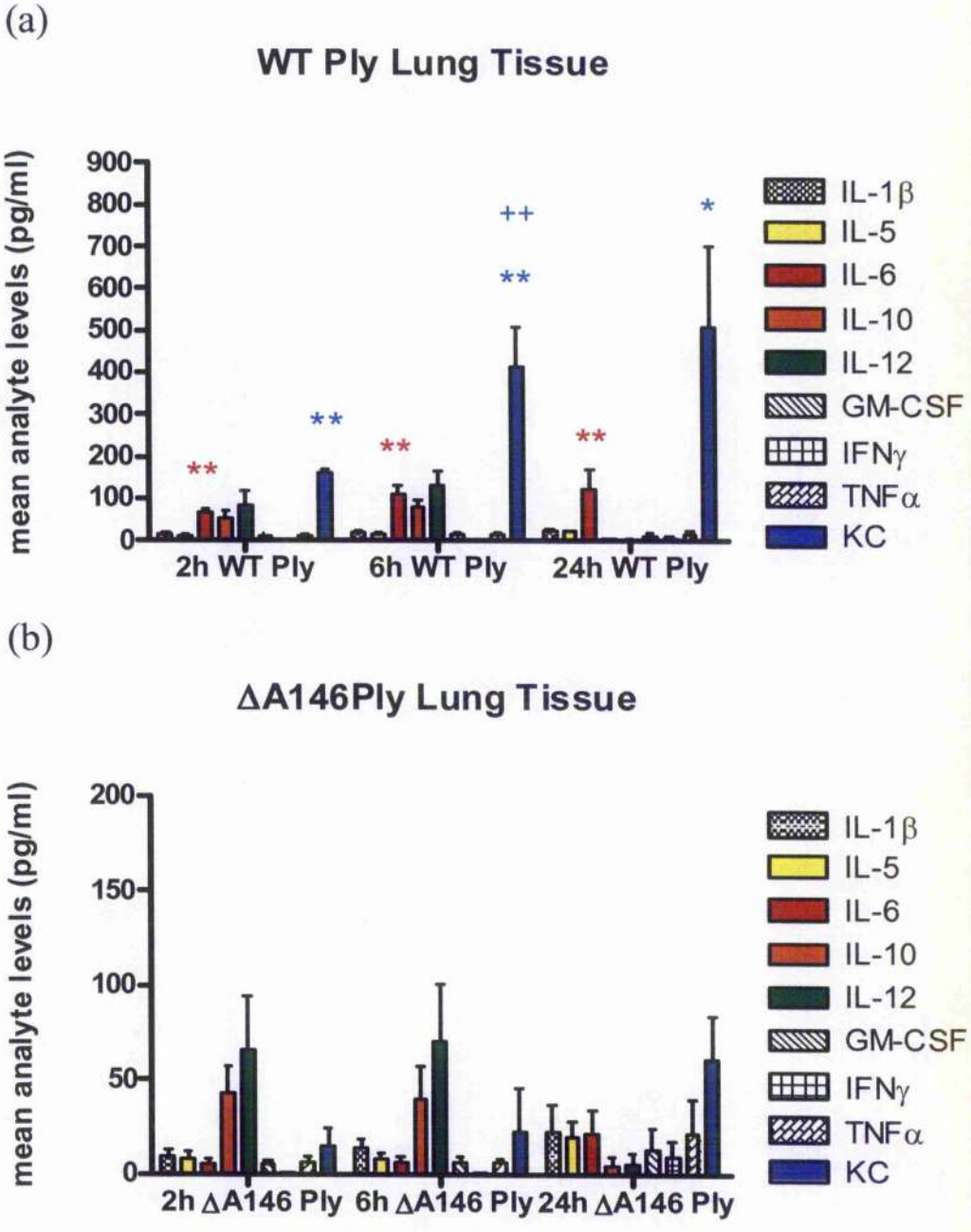
4.5.1. IL-6 and KC levels were significantly increased in the lungs of WT Ply treated BALB/c mice compared with ΔA146 Ply treatment

IL-6 and KC levels were significantly higher in lung tissue from WT Ply treated mice compared with ΔA146 Ply treatment at all time points (Figure 4.4). By 24h the WT lung tissue had 8-fold more KC and 5-fold more IL-6 than lung tissue from the ΔA146 Ply treated group. IL-6 levels in the lung tissue from WT Ply treated mice increased over time from 63pg/ml at 2h to 120pg/ml at 24h (Figure 4.4.a). KC levels in lung tissue from the WT Ply treated group increased over 24h from 159pg/ml at 2h to 507pg/ml at 24h, with a significant increase from 2h to 6h ($^{++}P<0.01$, Figure 4.4.a). There were no significant increases in any of the other cytokines measured in the lung tissue of WT Ply treated mice compared with ΔA146 Ply treatment.

In the BALF, IL-6 levels were significantly higher in the WT Ply treated group at all time points ($P<0.01$) compared with the ΔA146 Ply treated group (Figure 4.5). The level of IL-6 in WT Ply BALF increased over time from a mean of 63pg/ml at 2h to 590pg/ml by 24h post treatment. IL-6 levels were higher in the BALF than the lung tissue whereas KC levels were higher in the lung tissue. The levels of KC were significantly greater in the BALF of WT Ply treated mice at 24h compared with ΔA146 Ply treatment ($P<0.05$). IL-5 levels were also significantly increased by 5-fold in the BALF of WT Ply treated mice at 24h (52 pg/ml; $P<0.05$) when compared with ΔA146 Ply treatment (10 pg/ml), however the IL-5 levels were still low in comparison with IL-6 and KC levels.

Low levels of IL-10 and IL-12 were present in the lung tissue (Figure 4.4) and BALF (Figure 4.5) of both groups at 2h and 6h post treatment. IL-10 and IL-12 were not present in the lung tissue or BALF 24h post treatment. There were no significant differences in analyte levels in serum and brain tissue from WT Ply treated mice compared with ΔA146 Ply treated mice. However, IL-10 and IL-12 levels were high in the serum of both treatment groups at 2h and 6h but not at 24h (Figure 4.6.a and 4.6.c). These cytokines were also present at low levels in the brain tissue of both treatment groups at 2h and 6h (Figure 4.6.b and 4.6.d). KC was also present in the serum of both treatment groups at 24h (Figure 4.6.a and 4.6.c), but not in the brain tissue. KC levels in the lung tissue (Figure 4.4b) and BALF (Figure 4.5b) of ΔA146 Ply treated mice were below 50pg/ml at all time points. TNFα, IFNγ and IL-1β were not found at any time point in any of the samples.

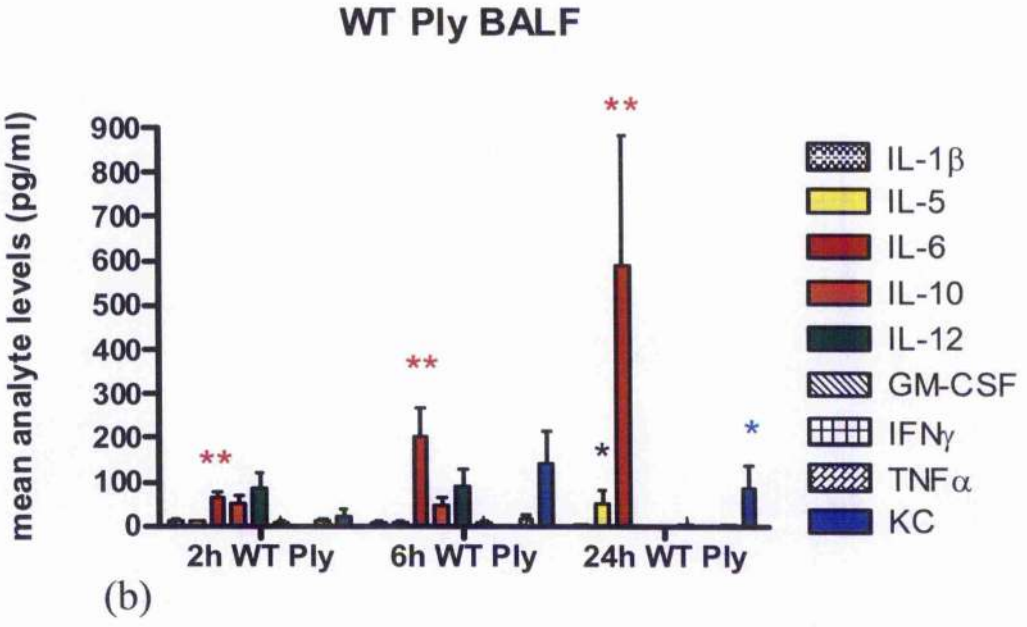
Figure 4.4. Analyte levels in lung tissue at 2h, 6h and 24h post Ply treatment



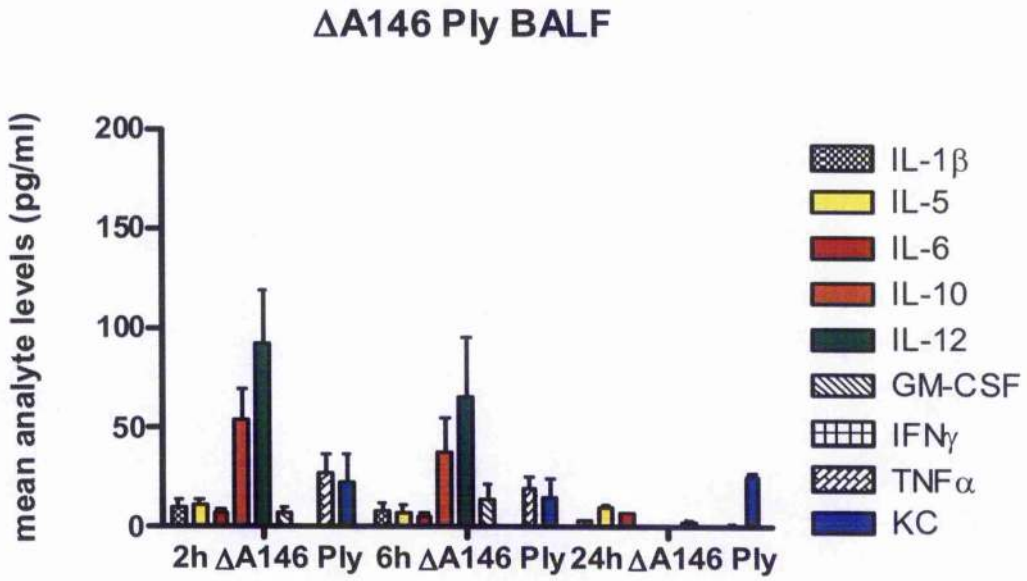
Mean (± SEM) levels of IL-1β, IL-5, IL-6, IL-10, IL-12, GM-CSF, IFN-γ, TNF-α and KC in the lung tissue of BALB/c mice at 2h, 6h and 24h post i.n. treatment with either 1μg WT Ply (a) or 1μg ΔA146 Ply (b). $^{*}P < 0.05$, $^{**}P < 0.01$ when compared with the ΔA146 Ply treated group; $^{++}P < 0.01$ when compared with 2h WT Ply KC level (asterisk colour corresponds with analyte); n=5/6. Statistical analysis was with the median.

Figure 4.5. Analyte levels in BALF at 2h, 6h and 24h post Ply treatment

(a)

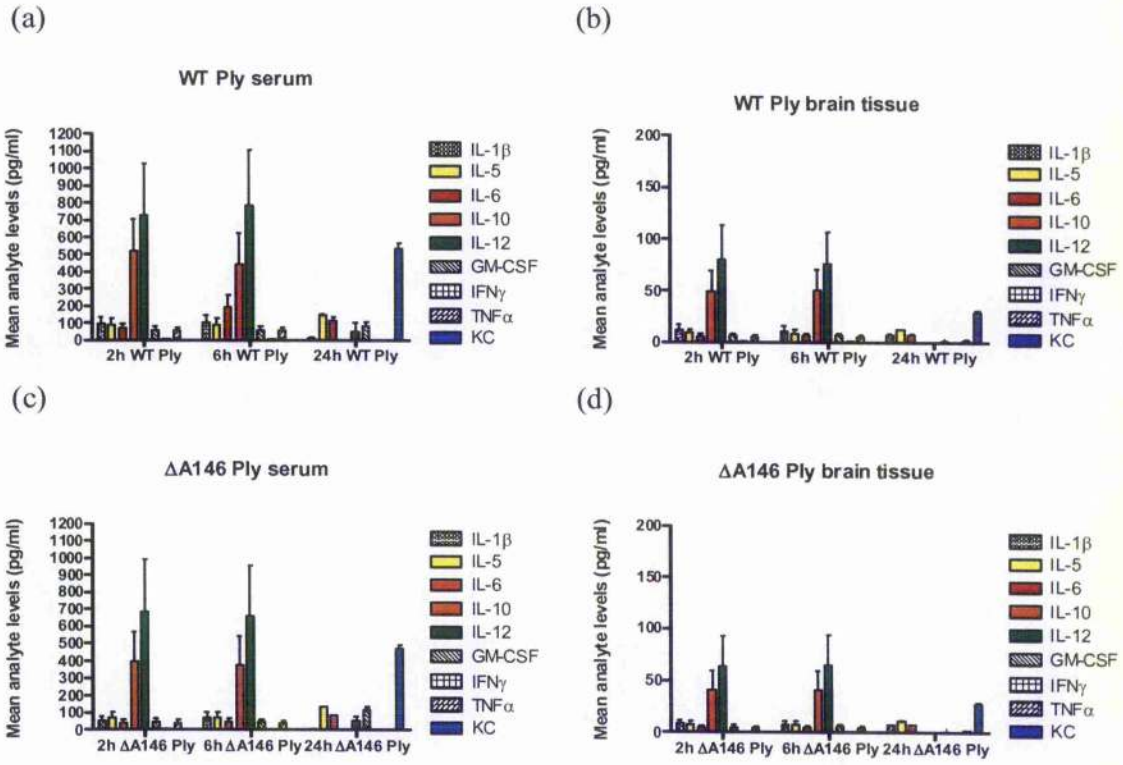


(b)



Mean (± SEM) levels of IL-1β, IL-5, IL-6, IL-10, IL-12, GM-CSF, IFN-γ, TNF-α and KC in the BALF of BALB/c mice at 2h, 6h and 24h post i.n. treatment with either 1μg WT Ply (a) or 1μg ΔA146 Ply (b). *P <0.05, **P<0.01 when compared with the ΔA146 Ply treated group (asterisk colour corresponds with analyte, except IL-5 where asterisk is black rather than yellow); n=5/6.

Figure 4.6. Analyte levels in serum and brain tissue at 2h, 6h and 24h post Ply treatment

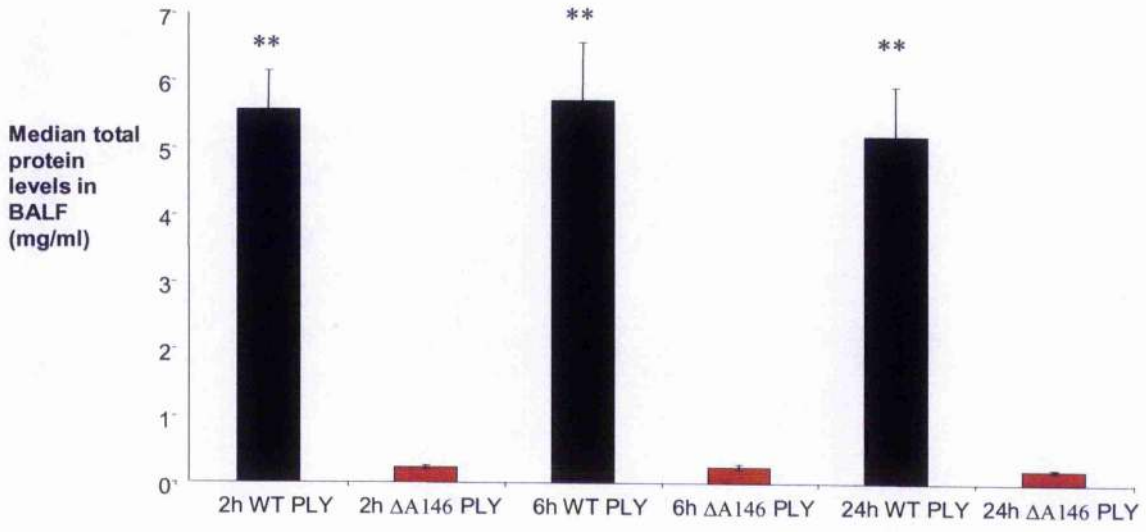


Mean (± SEM) levels of IL-1β, IL-5, IL-6, IL-10, IL-12, GM-CSF, IFN-γ, TNF-α and KC in the serum and brain tissue of BALB/c mice at 2h, 6h and 24h post i.n. treatment with either 1μg WT Ply (a, serum; b, brain tissue) or 1μg ΔA146 Ply (c, serum; d, brain tissue); n=5/6.

4.6. Total protein in BALF of BALB/c mice at 2h, 6h and 24h post Ply treatment

The total protein levels in BALF were significantly higher in the 1μg WT Ply i.n. treated group compared with 1μg ΔA146 Ply at all time points (**P<0.01, Figure 4.7). Total protein levels in the BALF of ΔA146 Ply treated mice were comparable to background protein levels in healthy lavage samples. Within 2h post WT Ply treatment the total protein in the lung airways significantly increased to levels 25-fold greater than the ΔA146 Ply group. This level was maintained over 24h. The damage to lung integrity by WT Ply corresponds with the IL-6 and KC profile in the lung tissue and BALF, as at 2h, 6h and 24h total protein levels, IL-6 and KC are significantly higher than the ΔA146 Ply treated group.

Figure 4.7. Total protein levels in BALF at 2h, 6h and 24h post i.n. Ply treatment



Total protein levels in BALF at 2h, 6h and 24h post treatment with either 1μg WT Ply (black bars) or 1μg ΔA146 Ply (red bars). Values are expressed as the median ± MAD (n=5/6, **P<0.01 when compared with ΔA146 Ply group).

Discussion

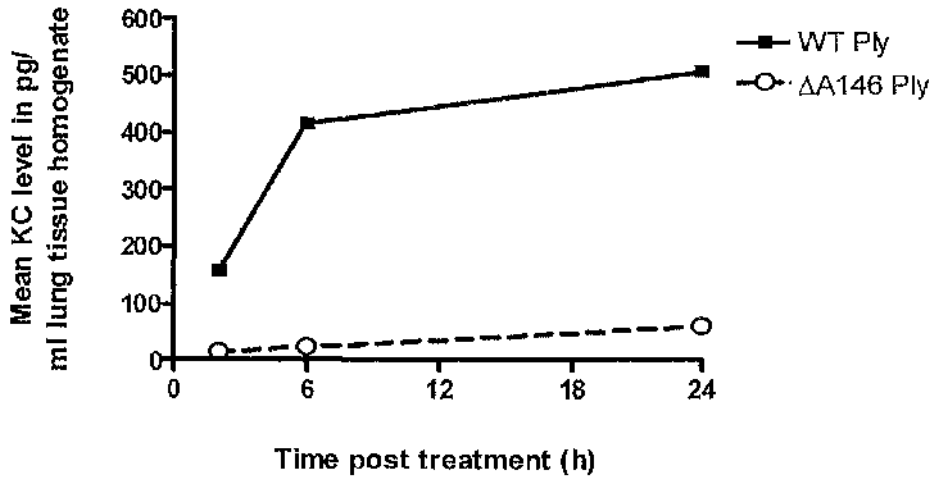
Treatment of inbred and outbred strains of mice with ΔA146 Ply does not induce the inflammatory effects that are associated with WT Ply treatment. Mice treated with WT Ply had severely damaged lungs in comparison with lungs retrieved from mice treated with ΔA146 Ply. The large amount of total protein observed in the airways of WT Ply treated mice is a result of Ply's disruption of tight junctions at the capillary/airway barrier (Rubins et al. 1995) allowing an influx of host serum proteins to flood the lung airways. This vascular leakage has recently been shown to be entirely due to the cytolytic action of Ply and is independent of recruited leukocytes (Maus et al. 2004), though cytokines produced in response to Ply may intensify lung damage.

Although the nine analytes investigated were not an exhaustive list, they were chosen as the analytes with known or implicated roles in inflammation or hypothermia (Houldsworth et al. 1994; Bergeron et al. 1998; Leon et al. 1998; Ebong et al. 1999; Fillion et al. 2001; Baba et al. 2002; Rijneveld et al. 2002a; Rijneveld et al. 2002b; Search 2002; Borish et al. 2003; Strieter et al. 2003; Leon 2004; Jones et al. 2005). When BALB/c mice were treated i.n. with WT Ply there was a localised inflammatory response within 24h following treatment, characterised by significant increases in IL-6 and KC levels in lung tissue and BALF. This response was not observed in samples from mice treated with ΔA146 Ply. Localised production of IL-6 and KC in response to WT Ply and pneumococcal infection has previously been reported and is likely to be from alveolar macrophages and recruited neutrophils (Fillion et al. 2001; Rijneveld et al. 2002b; Jones et al. 2005). WT Ply treated mice had higher KC levels in their lung tissue compared with the BALF, which only had significant levels of KC at 24h. This is possibly due to the high numbers of neutrophils recruited into the lung tissue. KC levels may increase in the BALF following vascular leakage and/or neutrophil recruitment caused by WT Ply.

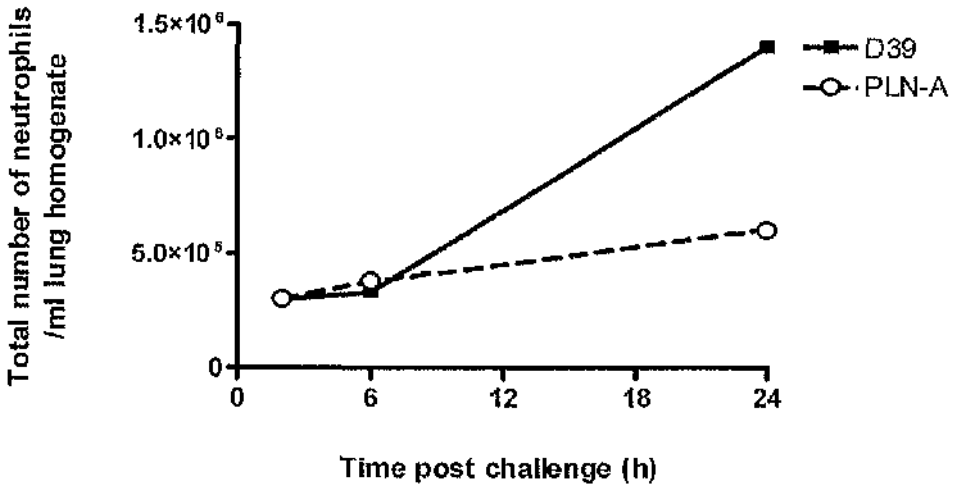
KC and C5a are the main neutrophil recruiters, however, mice deficient in the C5a receptor did not differ from WT mice in neutrophil counts in the lung during pneumococcal pneumonia (Kerr et al. 2005b). This indicates that KC in mice, or IL-8 from human neutrophils (Cockeran et al. 2002), is responsible for the mass influx of neutrophils into the lungs during pneumococcal pneumonia. The timing of the increase in KC levels in the lung following WT pneumolysin treatment mirrors with the profile of neutrophil recruitment into the lung over 24h post i.n. infection with D39 *S. pneumoniae* (Figure 4.8). By 6h post WT Ply treatment the KC level has almost reached the level observed at 24h (Figure 4.8.a) and it is from 6h post infection that the number of neutrophils in the lung tissue substantially increases (Figure 4.8.b). Comparing the data that shows neutrophil recruitment during pneumococcal infection is due to Ply (Kadioglu et al. 2000) with the KC levels in response to WT Ply (Figure 4.4.a) suggests that neutrophil recruitment is driven by Ply's induction of KC. It is important to note that this is not a direct comparison as Ply negative *S. pneumoniae* is being compared with purified Ply and in different mouse strains. The induction of KC by purified Ply is, however, due to the cytolytic property of Ply, as treatment of mice with ΔA146 Ply did not induce KC production in the lung tissue.

Figure 4.8. Comparison of lung KC levels following Ply treatment with neutrophil counts in lung tissue following pneumococcal pneumonia

(a)



(b)



(a) Mean KC level in lung tissue homogenate (pg/ml) of BALB/c mice over 24h post i.n. treatment with 1μg of purified WT Ply (filled squares) or 1μg of purified ΔA146 Ply (open circles) (n=5/6), data plotted from results in Figure 4.4. (b) Total number of neutrophils in the lung tissue of MF1 mice (n=5) post i.n. challenge with 10^6 CFU of D39 *S. pneumoniae* (filled squares) or the *ply* knockout strain of D39, PLN-A (open circles), data adapted from Kadioglu et al, 2000 with an approximation of neutrophil counts (Kadioglu et al. 2000).

IL-6 levels were greater in the BALF of WT treated mice compared with the lung tissue (in contrast to KC, which was greater in the lung tissue). This is probably due to the alveolar macrophages residing in the BALF, which instantly release IL-6 and recruit other IL-6 releasing cells, explaining the continual increase in IL-6 production. The interaction between KC and IL-6, if any, has not been identified though both are involved in pneumococcal pneumonia (Albiger et al. 2005) and are produced in response to WT Ply treatment (Rijneveld et al. 2002b). IL-6 profiles in CD-1 mice post pneumococcal infection were similar in timing to this work but the levels were 10-fold greater in the lung tissue rather than the BALF (Bergeron et al. 1998), this is the opposite of WT Ply treatment in BALB/c mice where the IL-6 levels were greater in the BALF (Figure 4.5) compared with lung tissue (Figure 4.4). This difference is probably due to the timing and location of Ply release, as Ply treatment and release of Ply during infection may occur in different locations within the host, thereby involving different cell types and inflammatory responses and interaction with other components. For example, CbpA can stimulate IL-8 production from human alveolar epithelial cells (Murdoch et al. 2002).

IL-6 has been shown to inhibit neutrophil recruitment in the lungs by blocking the expression of TNF-α and other inflammatory mediators involved in this process (Mizgerd 2002). It may be that induction of IL-6 by Ply limits neutrophil influx in the alveolar space thereby assisting pneumococcal growth in the lungs. This can also be taken from the perspective of the host in that IL-6 production as an anti-inflammatory cytokine may protect the host tissue from the damage caused by the mass influx of neutrophils. Hypothermia, which involves IL-6 (Leon et al. 1998), has also been shown to reduce the pulmonary sequestion of neutrophils to attenuate lung injury (Lim et al. 2003). The cytolytic activity of Ply may be important in the release of iron from erythrocytes to promote pneumococcal growth in the host, this is in counterbalance with hosts production

of IL-6 in response to Ply which has recently been shown to result in hypoferraemia to inhibit proliferation of pathogens (Nemeth et al. 2004).

IL-5 levels were increased in the BALF of WT Ply treated mice compared with mice treated with ΔA146 Ply (Figure 4.5). IL-5 plays a key role in eosinophil-based inflammation as it stimulates eosinophil production and prolongs their survival by blocking their apoptosis (Borish et al. 2003). Interestingly increases in albumin levels in the BALF (as observed with WT Ply treatment, Figure 4.7) have been shown to correlate with an increase in eosinophils in the BALF (Lampinen et al. 2004), which are the main IL-5 producing cells. In addition to eosinophils, mast cells and NK cells are known to express IL-5. Although the IL-5 levels are low in comparison with IL-6 and KC, it is important to note that low levels of cytokines can have high levels of biological activity.

TNF-α, IFN-γ and IL-1β were not found in any of the samples despite previous research showing that *in vitro* treatment of cells with Ply induces IFN-γ (Braun et al. 1999; Baba et al. 2002), IL-1β and TNF-α release (Houldsworth et al. 1994). However, the findings in this work correlate with other studies showing no TNF-α or IFN-γ in the lungs of mice at 6h post WT Ply treatment (Rijneveld et al. 2002b). This highlights that although tissue culture experiments can indicate inflammatory mediators involved in pathogenesis, host cytokine responses are complex and involve the interaction of an array of immune cells. *In vivo* investigation therefore provides a more realistic indication of the cytokines induced following Ply treatment.

Direct i.n. instillation of saline to mice did not elevate cytokine levels in the lung (Section 4.2) compared with WT Ply. Therefore, due to home office regulations that do not permit repetition of such experiments, a saline group was not assessed for cytokine analysis in the telemetry experiment. Instead, this experiment was a direct comparison to investigate

whether i.n. treatment of mice with ΔA146 Ply elicited the same inflammatory response as WT Ply, which it clearly does not. All inflammatory mediators implicated in Ply's role in infection were not produced in mice treated with the non-cytolytic form of Ply.

The initial drop in core body temperature (Tc) observed in all groups (Figure 4.3) has been reported to be an effect of anaesthesia due to the high ratio of surface area to body weight, which makes mice particularly susceptible to hypothermia (Flecknell 1996; Leon et al. 1998). Hypothermic responses to bacteria and endotoxins have previously been suggested to be beneficial to the host (Kerr et al. 2002; Lim et al. 2003; Leon 2004). BALB/c mice have a profound early hypothermic response to *S. pneumoniae* infection but are able to clear the infection, whereas CBA/Ca mice do not have the early hypothermic response and subsequently succumb to pneumococcal infection (Kerr et al. 2002). The dramatic drop in Tc in response to WT Ply alone has not been reported before. It is possible that the 12-hour hypothermia observed with pneumococcal infection (Kerr et al. 2002) may occur when the bacteria have established within the host and are lysing, resulting in the release of Ply. The immediate hypothermic response observed with purified WT Ply may be due to the direct intranasal administration of the toxin but this may also be a result of the mice being unable to maintain their Tc following treatment with the anaesthetic. Further experiments recording the Tc of BALB/c mice infected with the Ply negative strain of *S. pneumoniae*, PLN-A, would provide information on whether the hypothermic response found with D39 *S. pneumoniae* infection is directly due to Ply and IL-6 production.

Although the mechanisms of thermoregulation are not fully understood, cytokines are known to be involved in regulation/induction of hypothermia (Leon 2004). IL-6 may play a role in murine hypothermia following exposure to Ply and ultimately *S. pneumoniae* infection. IL-6 has been shown to act as pyrogen, inducing an increase in Tc (Gruol et al. 1997; Leon et al. 1998), it may be that the IL-6 increase observed over 24h post Ply

treatment counteracts the early hypothermic response to restore the Tc of the hypothermic animals. However, IL-6 production is also known to induce hypothermia (Leon et al. 1998) and it may be that IL-6 is a contributing factor to the induction of hypothermia as high IL-6 levels in the BALF occur within the 2-6h that hypothermia is observed. However, the IL-6 level continues to rise in the BALF within 24h post treatment with Ply (Figure 4.5). Treatment of IL-6 -/- mice with WT Ply would permit the investigation into whether the hypothermic response involves IL-6.

Measurable levels of IL-10 were present in the samples in particular in the serum at 2h and 6h post treatment. IL-10 is an anti-inflammatory cytokine that switches off the hosts inflammatory response by modulating TNF-α and IL-6 production. However, IL-10 was also found in the serum from mice treated with ΔA146 Ply and these mice did not have a hypothermic response or IL-6 production that would need to be controlled by IL-10.

Ultimately, what is important in the context of this work is that treatment of mice with ΔA146 Ply did not result in the prolonged hypothermia or inflammatory cytokines induced by WT Ply treatment, further supporting the safety of this toxoid for use as a vaccine component.

Chapter 5

Results

Vaccination with pneumolysin and derivatives

5. Vaccination with Ply and derivatives.

Summary

Current pneumococcal vaccines are somewhat limited as they only protect against serotypes for which the capsule polysaccharide (CPS) is a component of the vaccine. It is thought that the inclusion of a common pneumococcal protein in the next generation of vaccines may confer protection against disease caused by all serotypes of *S. pneumoniae*. Healthy humans were recently shown to have higher anti-Ply titres in their saliva and serum compared with *S. pneumoniae* infected patients, suggesting that anti-Ply antibodies may protect humans from pneumococcal disease (Huo et al. 2004). Passive immunisation of mice with purified anti-Ply IgG from humans with non-bacteraemic pneumococcal pneumonia was shown to protect mice from challenge with two different pneumococcal serotypes (Musher et al. 2001). Ply makes a good vaccine candidate as it is produced by all invasive strains of *S. pneumoniae* (Kanclerski et al. 1987), it is relatively conserved (see chapter 7) and has been shown to be immunogenic in mice as a free protein (Paton et al. 1983b; Alexander et al. 1994; Ogunniyi et al. 2001) or as a carrier protein to the polysaccharides in current vaccine preparations (Paton et al. 1991; Lee et al. 1994; Kuo et al. 1995; Michon et al. 1998; Lee et al. 2001b), conferring increased protection against pneumococcal disease. All previous studies either use chemically inactivated WT Ply or genetically produced mutants with residual haemolytic activity. The cytotoxicity of Ply and the PdB derivative has been demonstrated in chapters 3 and 4. This chapter compares the immunogenicity of non-toxic ΔA146 Ply with WT Ply and also investigates the protection elicited by conjugating Δ6 Ply to CPS from serotype 4 *S. pneumoniae*.

Results

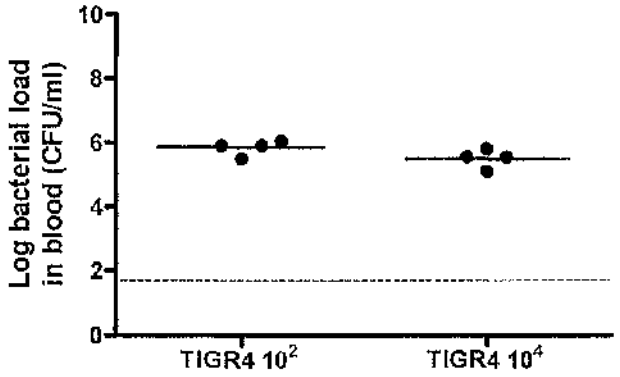
5.1. Determining an infectious i.p. dose of *S. pneumoniae*

In order to ensure that the challenge dose of *S. pneumoniae* to vaccinated mice would be lethal for the control groups, infectious doses were determined in unvaccinated mice. This allowed any increase in protection afforded by vaccination to be determined.

5.1.1. Determining an infectious i.p. dose of TIGR 4 *S. pneumoniae*

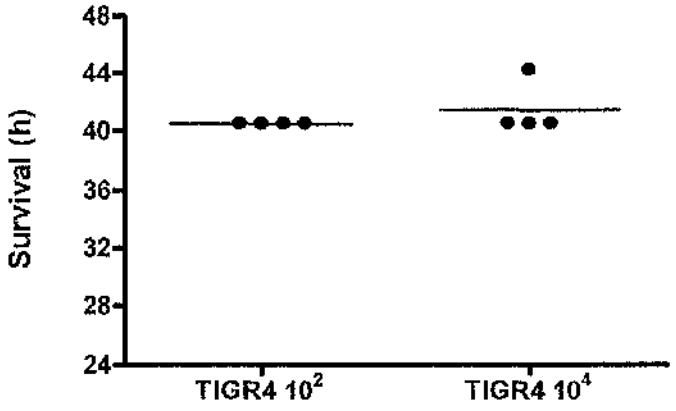
BALB/c mice were given either 10^2 or 10^4 CFU TIGR4 *S. pneumoniae* intraperitonealy (i.p.) in a 100µl dose (n=4). Doses were checked immediately before and after challenge to ensure that the dose was correct and the pneumococci remained viable. 18h post infection, blood was taken from the lateral tail vein and assessed for bacteraemia. Both groups had similar mean bacterial loads, 5.9×10^5 CFU/ml for the 10^2 CFU challenged group and 5.5×10^5 CFU/ml for the 10^4 CFU challenged group (Figure 5.1.1). The survival times of the groups did not differ significantly, in fact, the lower dose group became lethargic quicker than the higher dose, but group sizes were small and it was only one outlier that increased the survival time of the high dose group (Figure 5.1.2.).

Figure 5.1.1. Bacteraemia in BALB/c mice at 18h following i.p. infection with either 10^2 or 10^4 CFU TIGR4 *S. pneumoniae*



Log bacterial load in the blood of BALB/c mice 18h post i.p. infection with either 10^2 CFU or 10^4 CFU TIGR4 *S. pneumoniae*. Horizontal bar represents the mean; each dot represents an individual animal. Dashed horizontal line represents the detection limit.

Figure 5.1.2. Survival of BALB/c mice following i.p. infection with either 10^2 or 10^4 CFU TIGR4 *S. pneumoniae*



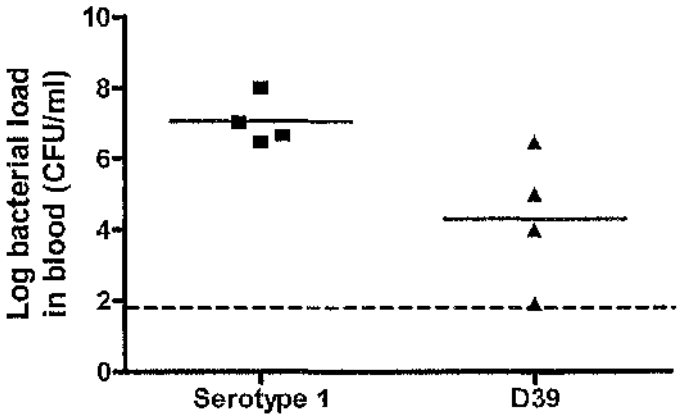
Survival (h) of BALB/c mice post i.p. challenge with a either 10^2 CFU or 10^4 CFU TIGR4 *S. pneumoniae* (n=4). Horizontal bar represents the mean; each dot represents an individual animal.

The lower dose of 10^2 CFU TIGR4 *S. pneumoniae* was chosen for challenging the actively vaccinated mice. For a second vaccination experiment, a serotype for which capsule polysaccharide was not a vaccine component was required for challenge.

5.1.2. Determining an infectious i.p. dose of D39 *S. pneumoniae* (serotype 2)

D39 *S. pneumoniae*, the common serotype 2 laboratory strain, was initially chosen as a non-vaccine serotype, however this was not suitable, as the 24h viable counts in the blood of MF1 mice were so variable compared with a virulent serotype 1 strain ST615 (Figure 5.1.3).

Figure 5.1.3. 24h bacteraemia in blood from MF1 mice following challenge with serotype 1 or D39 *S. pneumoniae*

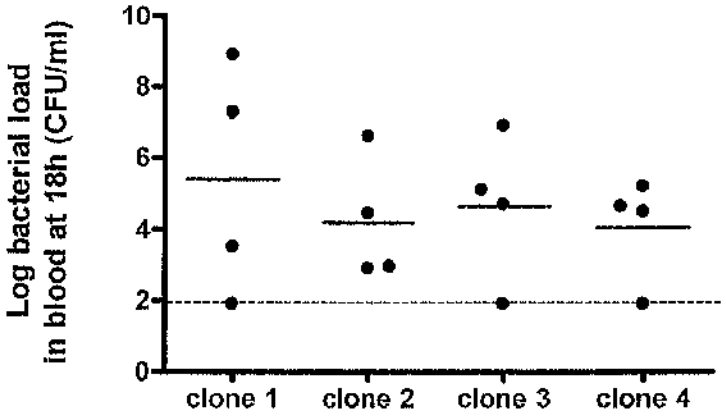


Log bacterial load in blood of MF1 mice 24h post i.p. challenge with either 10^5 CFU serotype 1 *S. pneumoniae* or 10^5 CFU D39 *S. pneumoniae* (n=4). Horizontal bar represents the mean. Dashed horizontal line represents the detection limit.

The reason for the variability of D39 *S. pneumoniae* was unknown. To investigate whether this variability was due to the standard inoculum, the strain was mouse passaged by infecting MF1 mice with 10^5 CFU D39 (n=4) and taking a terminal bleed from the first animal to succumb to infection. The blood was spread onto BAB plates supplemented with 5% horse blood for single colonies and four single colonies were selected for preparation of inoculum (clones 1-4). Each clone was assessed for ability to cause infection in BALB/c mice. Even in an inbred strain of mouse, challenged with freshly mouse-passaged clones, the bacterial loads remained highly variable, with 3 animals for 3 different clones having no detectable bacteraemia and other mice with bacterial loads of 10^7 CFU/ml and greater, Figure 5.1.4 (n=4).

All of the mice challenged with 10^5 CFU serotype 1 *S. pneumoniae* had similar levels of bacteraemia at 24h post infection in comparison with mice infected with the same dose of D39 *S. pneumoniae* (Figure 5.1.3). Serotype 1 was therefore chosen as the non-vaccine serotype for challenge of vaccinated animals, however the minimum infectious dose was still to be determined.

Figure 5.1.4. Bacteraemia in BALB/c mice at 18h post challenge with mouse virulent clones of D39 *S. pneumoniae*

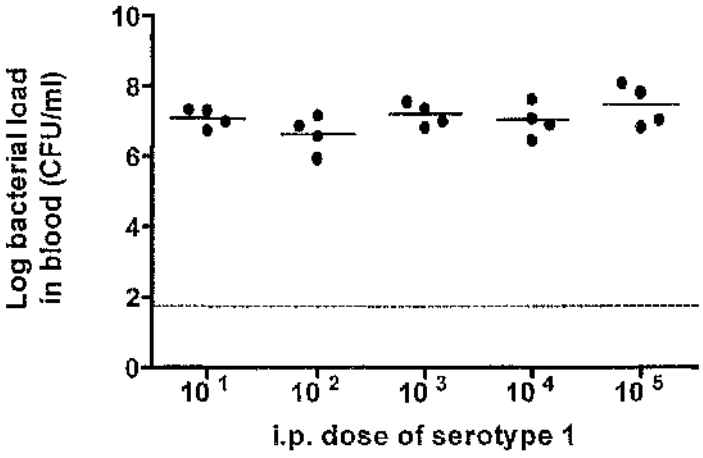


Bacterial loads in blood of BALB/c mice, 18h post i.p. challenge with 10^5 CFU D39 *S. pneumoniae* (n = 4). Horizontal bar represents the mean; each dot represents an individual animal. Dashed horizontal line represents the detection limit. Clones 1-4 are single clones of mouse virulent D39 *S. pneumoniae*.

5.1.3. Determining the minimum infectious dose of serotype 1 *S. pneumoniae*

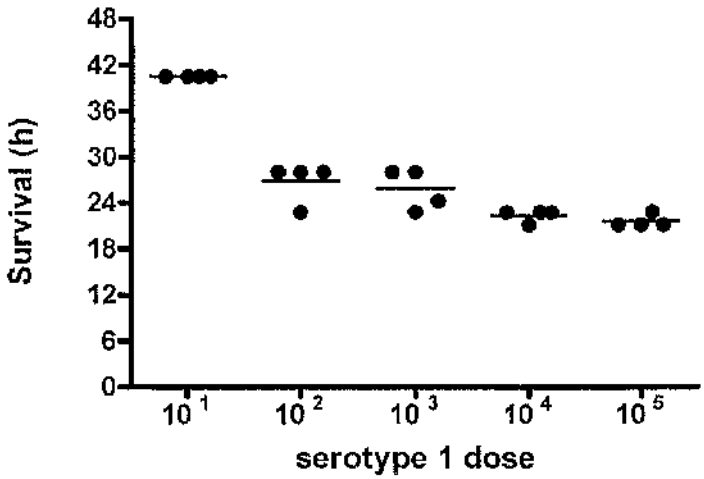
The minimum infectious i.p. dose of serotype 1 was determined by administering varying doses to BALB/c mice (n=4) and taking an 18h sample bleed for viable counts (Figure 5.1.5) and monitoring survival times (Figure 5.1.6). Bacterial loads in the blood at 18h post challenge were between 10^6 and 10^7 CFU/ml for all groups irrespective of the challenge dose (Figure 5.1.5). There was little variation in survival times within each group and between groups administered with different doses, except the group given 10^1 CFU that survived almost 20h longer than the 10^2 CFU dose (Figure 5.1.6). From these experiments, a dose of 10^2 CFU serotype 1 *S. pneumoniae* was chosen for challenge of vaccinated mice.

Figure 5.1.5. 18h bacteraemia following infection of BALB/c mice with a dose range of serotype 1 *S. pneumoniae*



Bacterial loads in blood of BALB/c mice 18h post i.p. infection with different doses of serotype 1 *S. pneumoniae* (n=4). Horizontal bar represents the mean; each dot represents an individual animal. Dashed horizontal line represents the detection limit.

Figure 5.1.6. Survival of BALB/c mice following i.p. challenge with serotype 1 *S. pneumoniae*

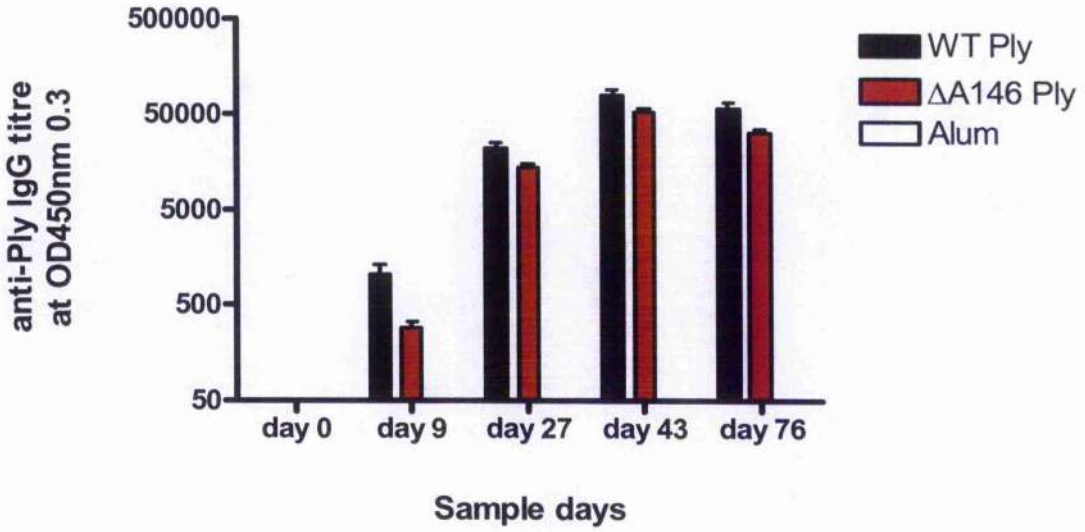


Survival (h) of BALB/c mice post i.p. challenge with 10^1 – 10^5 CFU serotype 1 *S. pneumoniae* (n=4). Horizontal bar represents the median survival time; each dot represents an individual animal.

5.2. Active vaccination with purified Ply and derivatives

BALB/c mice were subcutaneously administered 20μg of WT Ply or ΔA146 Ply plus 100μg Alum, 100μg Alum or PBS (n=8). They were boosted three times on days 10, 28 and 44. Sample bleeds were taken before immunisation (day 0) and each boost (days 9, 27, 43) and one month after the last boost (day 76). Anti-Ply IgG antibody levels were measured in each sample bleed (Figure 5.2.1.). After the first immunisation (day 9), there were detectable levels of anti-Ply IgG in the groups immunised with WT Ply or ΔA146 Ply. By day 27 after one boost, anti-Ply IgG titres in sera from groups vaccinated with ΔA146 Ply plus Alum and WT Ply plus Alum were high and plateaued after two boosts (day 43). There were no anti-Ply IgG antibodies found in serum from the control mice that were vaccinated with Alum only (Figure 5.2.1.) or PBS (data not shown).

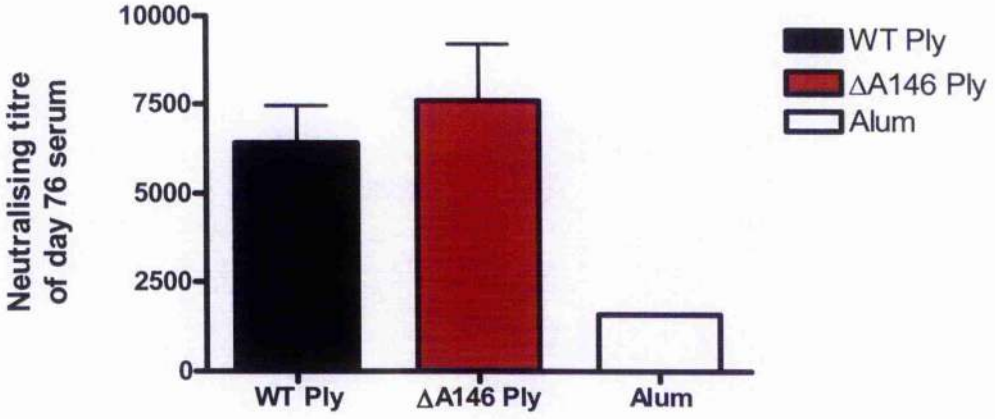
Figure 5.2.1. Anti-Ply IgG titre in serum during active vaccination with Ply



Mean anti-Ply IgG titre at OD450nm 0.3 (± SEM) in BALB/c mice before vaccination (day 0), the day before each boost (days 9, 27 and 43) and one month after the last boost (day 76). The detection limit was a titre of 50, n=8.

The anti-Ply IgG antibodies from day 76 serum of the WT Ply plus Alum and ΔA146 Ply plus Alum group were also found to neutralise Ply's haemolytic activity (3HU/well) to a neutralising titre of 3200-12800 in comparison with background levels of 800-1600 for the Alum only group (Figure 5.2.2.).

Figure 5.2.2. Neutralising titre of day 76 serum from active vaccination with Ply

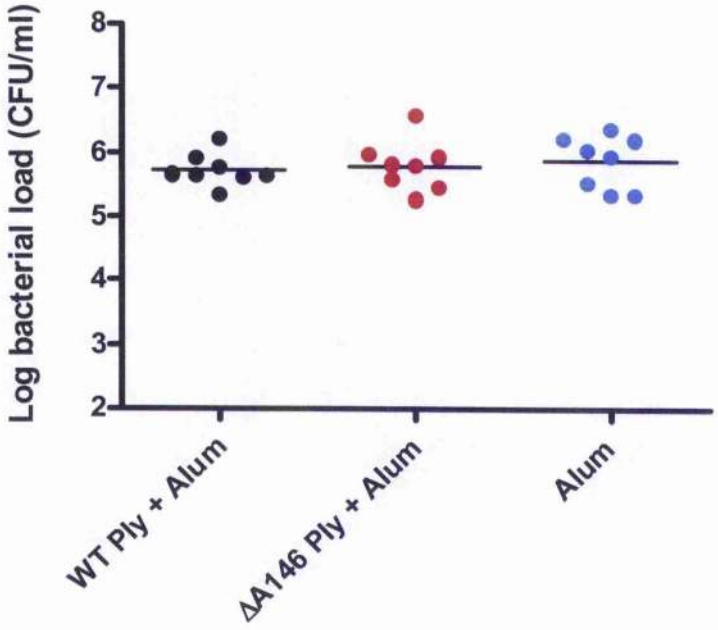


Mean neutralising titre (±SEM) of day 76 serum from BALB/c mice following vaccination with either WT Ply plus Alum, ΔA146 Ply plus Alum or Alum alone (n=8). The titre is calculated as the reciprocal of the dilution where the antibodies no longer block the haemolytic action of 60HU/ml of WT Ply.

Following vaccination, mice were challenged i.p. with 10^2 CFU TIGR4 *S. pneumoniae*. 18h following challenge, the bacterial loads in the blood of all vaccinated groups were at 10^5 CFU/ml (range of 10^5 – 10^6 CFU/ml), with no significant difference between Ply vaccinated groups and control groups (Figure 5.2.3). However, mice immunised with WT Ply plus Alum or ΔA146 Ply plus Alum survived the infection significantly longer than the Alum control group, $P<0.05$ (Figure 5.2.4.) and the PBS only group. There was no

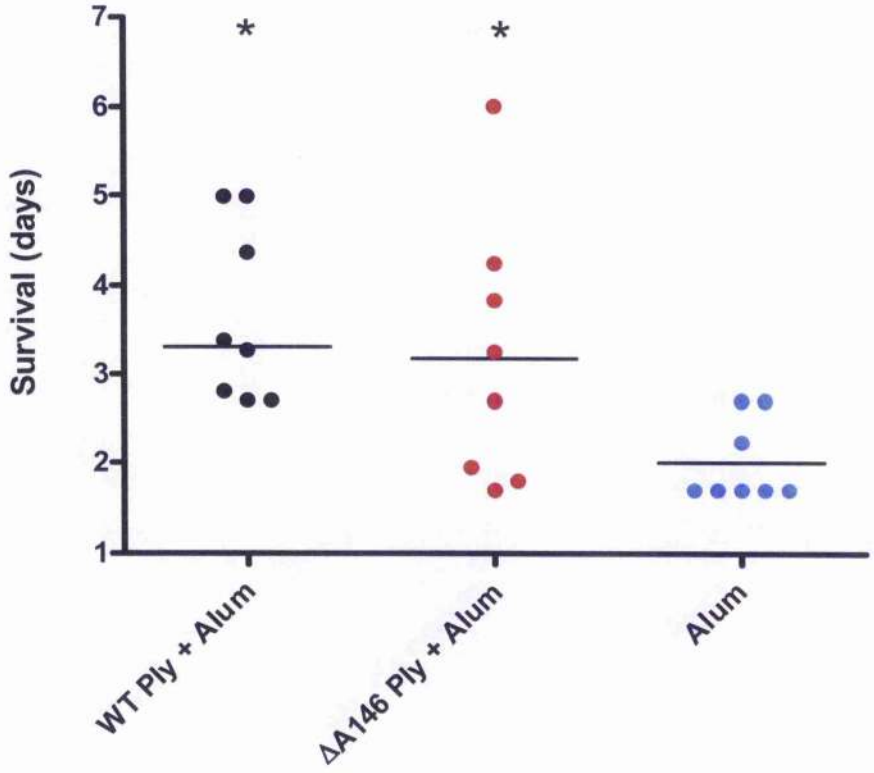
significant difference between the protection afforded by ΔA146 Ply compared with WT Ply. One mouse from the ΔA146 Ply plus Alum group, which had a blood bacterial load of 2×10^5 CFU/ml TIGR4 *S. pneumoniae* at 18h post infection, cleared the infection within 6 days and survived (Figure 5.2.4).

Figure 5.2.3. Bacteraemia at 18h in vaccinated mice following challenge with 10^2 CFU TIGR4 *S. pneumoniae*



Bacterial load in blood from vaccinated BALB/c mice at 18h post i.p. challenge with 10^2 CFU TIGR4 *S. pneumoniae*. Horizontal bar represents the mean log bacterial load; each dot represents an individual animal (n=8).

Figure 5.2.4. Survival of vaccinated mice following challenge with 10^2 CFU TIGR4 *S. pneumoniae*



Survival (days) of vaccinated BALB/c mice following challenge with 10^2 CFU TIGR4 *S. pneumoniae* (n=8). Each dot represents an individual animal and the horizontal bar is the median survival time (*P<0.05 when compared with Alum alone group). There is no difference in the survival times between WT Ply and ΔA146 Ply protection; both are equally protective.

5.3. Conjugate vaccination

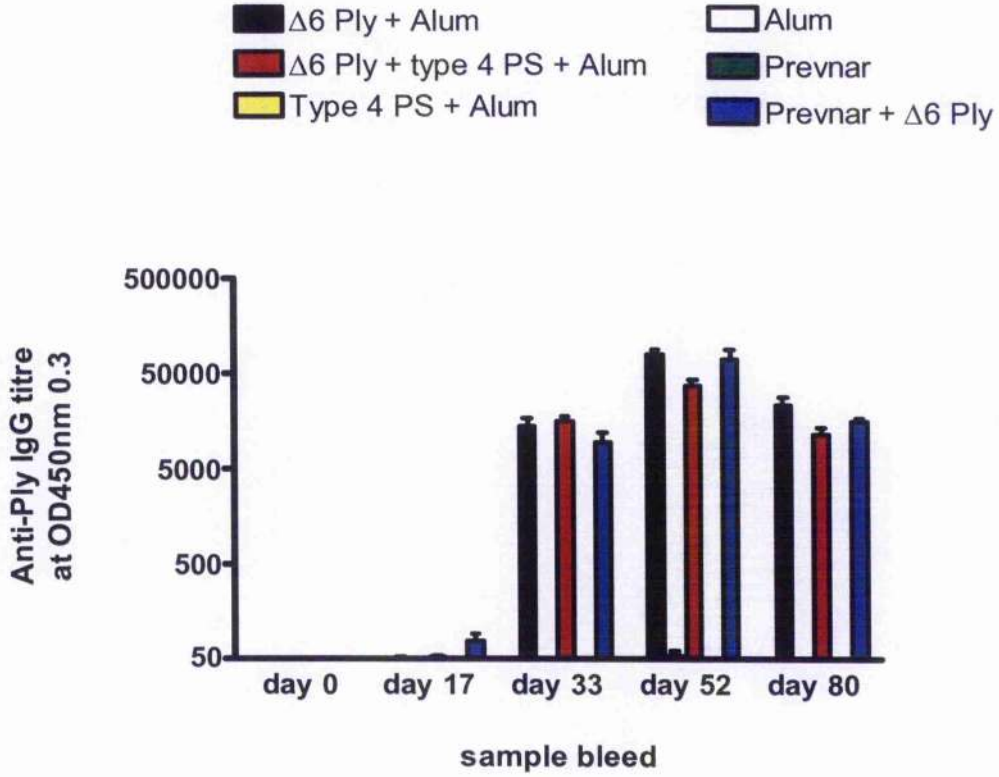
Δ6 Ply was covalently conjugated to CPS from serotype 4 *S. pneumoniae* to give Δ6 Ply-type4 CPS (this was carried out at Wyeth Vaccines by Dr. Maya Koster). This allowed for the comparison of Δ6 Ply with CRM_{197} (the conjugate protein in Prevnar) in ability to act as a carrier protein to improve protection against infection from disease caused by a vaccine serotype: serotype 4 TIGR4 *S. pneumoniae*. The reason for using a pneumococcal protein as the carrier protein is that it may confer additional protection against disease caused by non-vaccine serotypes. BALB/c mice were vaccinated with Δ6 Ply-type4 CPS and then challenged with either TIGR4 *S. pneumoniae* or serotype 1 *S. pneumoniae* and assessed for bacteraemia and survival in comparison with mice vaccinated with free Δ6 Ply plus Alum, free type 4 CPS plus Alum and Alum alone. A Prevnar vaccinated group and a Prevnar plus Δ6 Ply group were also included to assess whether adding free Ply to the existing vaccine could confer protection against infection with a non-vaccine serotype.

5.3.1. Active vaccination with Δ6Ply-type4 CPS

BALB/c mice were vaccinated subcutaneously with 200μl of either Δ6 Ply-type4 CPS (containing 1μg CPS and 0.64μg Δ6 Ply) plus 200μg Alum, 1μg type 4 CPS plus 200μg Alum, 0.64μg Δ6 Ply plus 200μg Alum, Prevnar (containing 0.8μg type 4 CPS and 200μg Alum), Prevnar plus 0.64μg free Δ6 Ply, 200μg Alum, or PBS alone (n=10). All preparations were standardised to contain comparable amounts of CPS, protein and Alum. Note that the dose of protein (0.64μg) is low in comparison with the 20μg dose of ΔA146 Ply administered in the previous vaccination experiment but this was determined by the dose of CPS. Also the dose of adjuvant was increased from 100μg to 200μg, as this was the amount of Alum in Prevnar. Blood was taken before vaccination and before each of the three boosts on day 17, 33 and 52. A final bleed was taken 4 weeks after the last boost

on day 80 and one week prior to challenge. Anti-Ply IgG levels were measured in the serum from each bleed (Figure 5.3.1). The anti-Ply IgG titres were high and plateaued in the groups vaccinated with either free Δ6 Ply or Δ6Ply conjugated to type 4 CPS after the first boost on day 18 (day 33 bleed). These titres are comparable to the anti-Ply IgG titres in BALB/c mice vaccinated with 20μg free WT Ply or ΔA146 Ply, however, when more free protein was administered there was an earlier anti-Ply IgG response (Figure 5.2.1).

Figure 5.3.1. Anti-Ply IgG titres in serum post vaccination with Ply conjugate



Mean anti-Ply IgG titre at OD450nm 0.3 (± SEM) in BALB/c mice before vaccination (day 0), the day before each boost (days 17, 33 and 52) and one month after the last boost (day 80). The detection limit was a titre of 50, n=10. Δ6 Ply + type4PS + Alum is the conjugate vaccine.

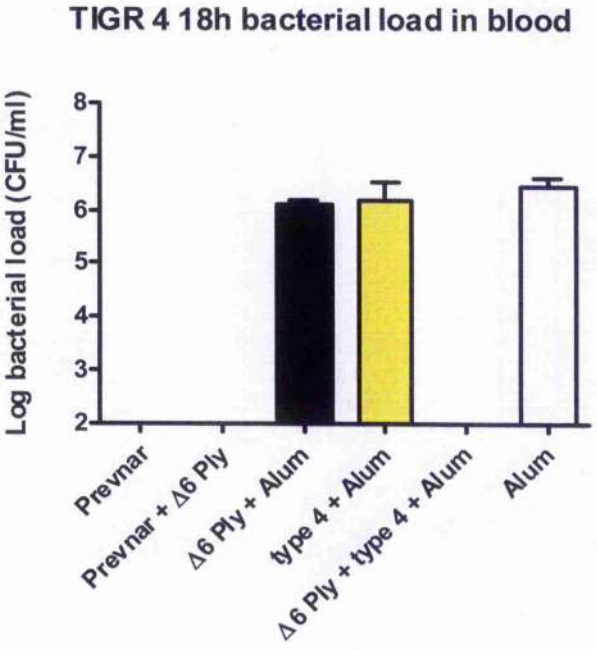
Each group of 10 vaccinated mice was divided to give 5 to be challenged i.p. with 10^2 TIGR4 *S. pneumoniae* and 5 with 10^2 serotype 1 *S. pneumoniae*. Blood samples were

taken at 18h post infection and assessed for bacteraemia (Figure 5.3.2). Mice vaccinated with Prevnar, Prevnar plus Δ6 Ply and Δ6 Ply conjugated to type 4 CPS were completely protected from infection with TIGR4 *S. pneumoniae*, Figure 5.3.2.a. Whereas mice given free type 4 CPS plus Alum, free Δ6 Ply plus Alum or Alum only were bacteraemic within 18h post infection. The mean bacterial loads in blood from the Δ6 Ply plus Alum and type 4 CPS plus Alum vaccinated animals were lower (1.2×10^6 and 1.6×10^6 CFU/ml respectively) than the Alum only control group (2.8×10^6 CFU/ml); however this difference was not statistically significant.

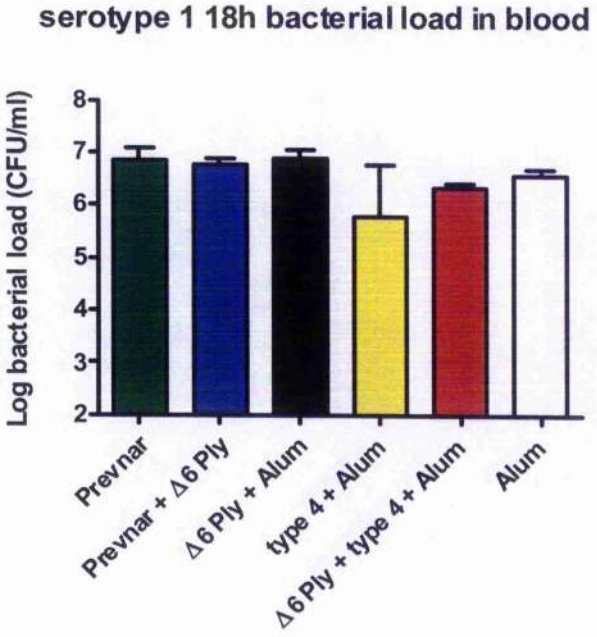
All mice challenged with serotype 1 *S. pneumoniae* were bacteraemic within 18h post infection (Figure 5.3.2.b) with no significant differences in bacterial loads between groups. Three mice in separate groups did not survive through the vaccination protocol and died of natural causes, this resulted in a group size of 4 for three of the groups challenged with serotype 1 *S. pneumoniae*.

Figure 5.3.2. Bacteraemia in conjugate vaccinated animals at 18h post challenge with TIGR4 (a) or serotype 1 (b) *S. pneumoniae*

(a)



(b)



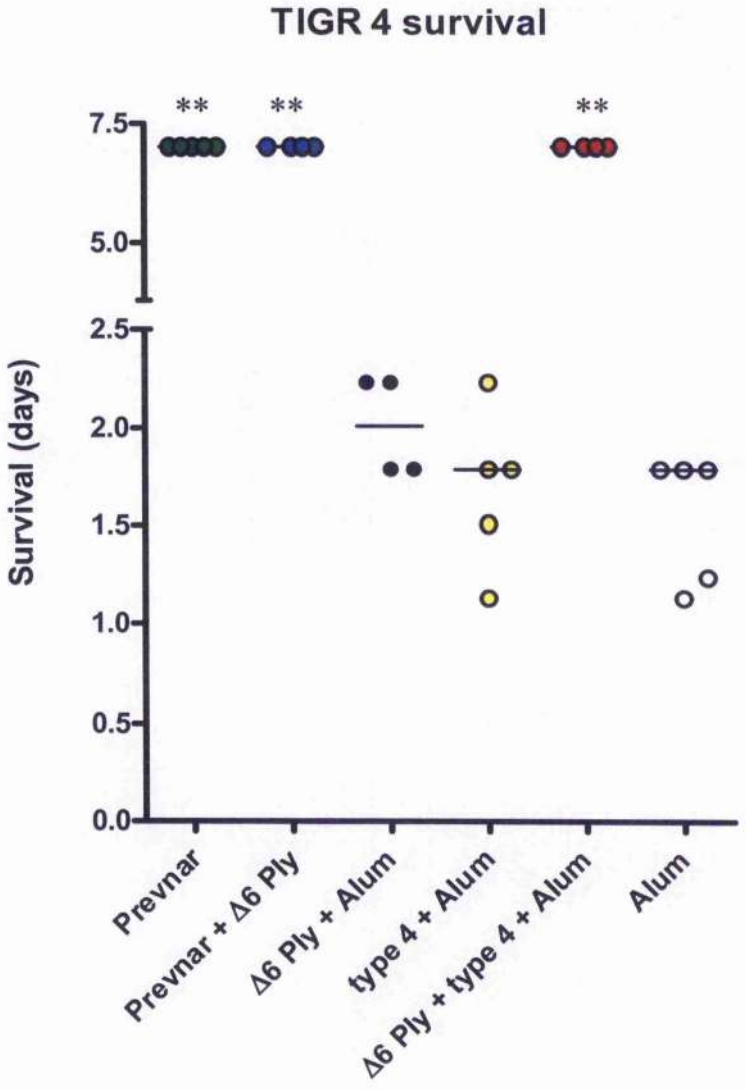
Mean log bacterial loads (± SEM) in blood of BALB/c mice 18h post i.p. challenge with 10^2 CFU of either (a) TIGR4 *S. pneumoniae* or (b) serotype 1 *S. pneumoniae* (n=4/5). Δ6 Ply + type4PS + Alum is the conjugate vaccine.

Survival following infection was monitored and mice were culled when they became lethargic. Mice were considered to have completely survived infection if they survived >7 days. The groups vaccinated with Prevnar, Prevnar plus Δ6 Ply and Δ6 Ply conjugated to type 4 CPS survived infection with TIGR4 *S. pneumoniae* (Figure 5.3.3.a). There was no significant difference in survival of mice given free type 4 CPS plus Alum or Δ6 Ply plus Alum compared with the Alum only control group and all mice succumbed to infection within 54h.

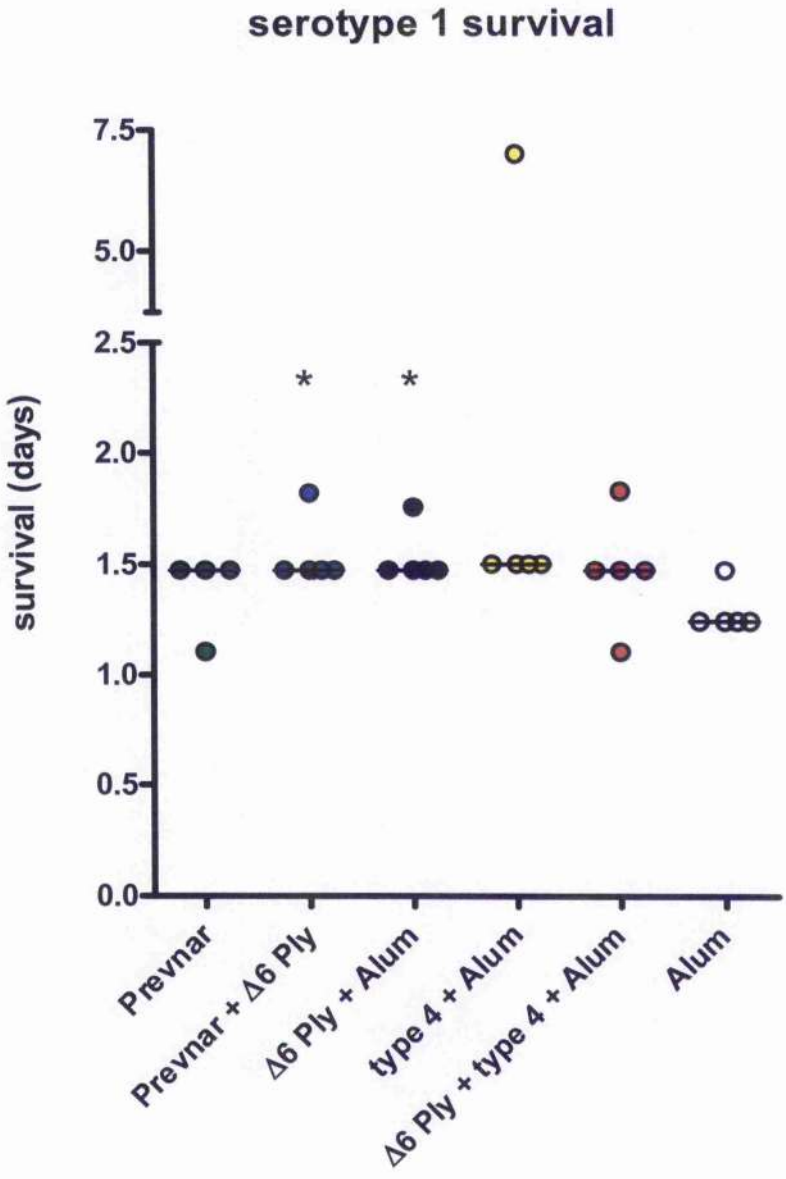
When mice were challenged with serotype 1 *S. pneumoniae* no groups were completely protected from infection, apart from one mouse in the type 4 CPS only group. This mouse did not have bacteraemia at 18h and may have not received the whole challenge dose; it was therefore not included in statistical analysis. Groups immunised with free Δ6 Ply (Prevnar plus Δ6 Ply group and the Δ6 Ply plus Alum group) survived infection significantly longer than the Alum only group ($P<0.05$), Figure 5.3.3.b.

Figure 5.3.3. Survival of conjugate vaccinated mice following i.p. challenge with either TIGR4 or serotype 1 *S. pneumoniae*.

(a)



(b)



Survival (days) of vaccinated BALB/c mice following challenge i.p. with 10^2 CFU (a) TIGR4 *S. pneumoniae* (b) serotype 1 *S. pneumoniae*. Each dot represents an individual animal and the horizontal bar is the median survival time. Mice that were alive at 7 days post challenge were considered to have survived infection. **P<0.01, *P<0.05 when compared with Alum alone group; n=4/5.

Discussion

The reason for variation in the ability of *S. pneumoniae* D39 to cause invasive disease was not determined. Even when a single colony of mouse virulent D39 was used as the standard inoculum for i.p. challenge of inbred mice, there was still variation in bacterial loads from mice with no detectable bacteraemia to mice with 8×10^8 CFU/ml (Figure 5.1.4). The variation was not due to the strain of mouse as both MF1 and BALB/c mice were assessed (Figures 5.1.3 and 5.1.4 respectively). Variation has previously been observed when D39 is used for challenge experiments (Ogunniyi et al. 2001; Blue 2002). The genome of D39 is almost complete and it has been found to possess hypervariable regions that are different in clones from the same strain (T. J. Mitchell, personal communication, 2006). This variable region may be related to the variation observed in virulence and requires further investigation. For the purposes of this study we decided not to use D39 as it was too variable. We therefore selected a serotype 1 strain for challenge experiments.

A similar immunogenic response to ΔA146 Ply, Δ6 Ply and WT Ply demonstrates that antibodies are still produced against these mutants. The ability of the raised antibodies to block the lytic action of Ply indicates that they are fully functional and recognise an active region of the toxin. Mice vaccinated with ΔA146 Ply plus Alum or WT Ply plus Alum were protected from infection with TIGR4 *S. pneumoniae* significantly longer than animals that were given Alum alone (Figure 5.2.4). One mouse from the ΔA146 Ply plus Alum group, had a blood bacterial load of 2×10^5 CFU/ml TIGR4 *S. pneumoniae* at 18h post infection but cleared the infection within 6 days. It may be that the anti-Ply antibodies can inhibit infection to allow anti-capsule opsonising antibodies to develop and clear the infection. To assess this, anti-type 4 CPS IgM could be measured in serum from the mouse that cleared the infection. Highly neutralising anti-Ply antibodies are important in 'mopping up' Ply release by *S. pneumoniae*, thereby preventing inflammation and

subsequent tissue invasion. Such antibodies have recently been shown to protect ciliated ependymal cells from the toxic effects of Ply release following penicillin lysis of pneumococci (Hirst et al. 2004b).

Having shown that ΔA146 Ply protects against bacteraemia, the next step is to investigate protection against pneumonia by challenging vaccinated mice intranasally rather than by the intraperitoneal route. As ultimately a vaccine that protects against pneumonia would add to the existing vaccines where such protection is disputable in adults and low in children (Black et al. 2002; Parijs et al. 2004). Prior to investigation into the variability of bacteraemia using D39 as the challenge strain, vaccinated MF1 mice were challenged intranasally with D39. Mice immunised with Δ6 Ply plus Alum survived infection considerably longer than the Alum group (5.9 and 3.8 days respectively, n=10, data not shown), however this was not statistically significant and was probably due to the variability with D39 infection. This experiment should be repeated using TIGR4 for the challenge. Different routes of immunisation should also be investigated, e.g. mucosal immunisation to investigate whether the non-toxic Ply mutants confer mucosal protection that may protect against carriage or the transition from carriage to pneumonia.

In the conjugate vaccination, mice immunised with 30 times less free protein were only slightly protected from TIGR4 infection though this was not significant when compared with the Alum only group. The reason for this may be the lower dose of Ply administered in the second vaccination, however, this is unlikely as the anti-Ply IgG titres were similar in both groups (Figures 5.2.1 and 5.3.1) irrespective of the dose of Ply administered. What is more likely is that the increase in the amount of Alum in the conjugate vaccination experiment from 100μg/dose to 200μg/dose, possibly resulted in Alum conferring some non-specific protection as the median survival time following i.p. challenge with 10^2 CFU TIGR4 increased from 40.5h with 100μg Alum (Figure 5.2.4) to 42.9h with 200μg Alum

(Figure 5.3.3a). For this data, the increase in survival time for the Alum groups from 40.5h to 42.9 was not significant. Larger group sizes are required to thoroughly investigate whether increasing the dose of Alum significantly increases survival times of mice. However, bacterial loads were significantly reduced in the Δ6 Ply group when compared with the PBS alone group ($P<0.05$, data not shown) though not with the Alum group following TIGR4 challenge (Figure 5.3.2a). Also following serotype 1 challenge, there were significantly lower counts in the Alum group compared with the PBS group ($P<0.05$) further supporting the evidence that the high dose of Alum provides a slight increase in protection that may mask any subtle increases in protection afforded by Ply or CPS. The choice of adjuvant is important as demonstrated by the fact that a combination of MPL (monophosphoryl lipid A) and Alum increased the immunogenicity of PdB compared with the use of Alum alone (Ogunniyi et al. 2001).

Mice vaccinated with Prevnar, Prevnar plus Δ6 Ply and Δ6 Ply-type 4 CPS were completely protected from infection with TIGR4 *S. pneumoniae* (Figure 5.3.3.a). This suggests that Δ6 Ply is as effective a carrier protein as CRM_{197} in preventing infection from a vaccine serotype, though comparison with CRM_{197} in a titration experiment is required to prove this. The use of Ply as a carrier protein has already been demonstrated with WT Ply and PdB (Paton et al. 1991; Lee et al. 2001b). Protection was not conferred against TIGR 4 *S. pneumoniae* when mice were vaccinated with free Δ6 Ply. If a larger dose of free Δ6 Ply was administered in the conjugate vaccination experiment then an increase in survival times would be expected (e.g. 20μg as in Figure 5.2.4, which significantly increased survival times of BALB/c mice challenged with TIGR4 *S. pneumoniae*). Therefore, Δ6 Ply must be conjugated to CPS to confer complete protection against infection with a vaccine serotype, acting as a carrier protein like CRM_{197} in Prevnar.

As free polysaccharide is the basis of 23-PPV vaccines, we hypothesised that mice vaccinated with free type 4 CPS would be protected from TIGR4 challenge (at least partially). However, protection against TIGR4 *S. pneumoniae* challenge was not conferred when mice were vaccinated with free type 4 CPS. Immunisation with free type 4 CPS has previously been shown to fail to elicit an antibody response in adult NIH outbred mice and inbred BALB/c mice (Peeters et al. 1991). This may be a result of the low dose administered or that for some reason protective antibodies are not produced in these mice.

The only groups significantly protected from serotype 1 infection were those vaccinated with free Δ6 Ply (Prevnar plus Δ6 Ply and Δ6 Ply plus Alum; Figure 5.3.3.b). As the Δ6 Ply-type 4 CPS group was not significantly protected from serotype 1 infection, conjugated Ply may not confer additive protection against non-vaccine serotypes. This suggests that free protein significantly increases survival time against infection with a non-vaccine serotype but conjugated Ply is required for full protection against infection from vaccine serotypes. Perhaps the conjugation chemistry affects the nature of the Ply antigen; indeed the effect of reductive amination on the activity of pneumolysin is not well-defined and requires further investigation. It has been suggested that epitopes can be altered during reductive amination (Lee 2002). WT Ply conjugated to CPS was not available to investigate whether it loses haemolytic activity upon conjugation to CPS. If the haemolytic activity is destroyed by conjugation then it could be argued that there is no requirement for a non-toxic form of Ply. However, there is the potential of incomplete conjugation, resulting in free toxin. In terms of safety for clinical trials, a non-toxic form of Ply is preferable for vaccine design. This is the first report that Ply provides different levels of protection depending on whether it is administered as free protein or conjugated to CPS and signifies an area that requires further investigation with larger group sizes.

As mice immunised with Δ6 Ply-type 4 CPS were completely protected from TIGR4 *S. pneumoniae* infection but not those given free type 4 CPS, it can be assumed that the anti-type 4 CPS antibody response is improved when Δ6 Ply is conjugated to type 4 CPS. Anti-type 4 CPS antibodies will be analysed by ELISA. Previously anti-Ply and anti-CPS titres following immunisation with free PdB and free 19F CPS were compared with titres following immunisation with PdB conjugated to 19F CPS (Mitchell 1992). The levels of anti-Ply IgG elicited by the conjugate formulation were significantly lower than levels elicited by free PdB. In this present conjugate vaccination study the anti-Ply IgG titres were comparable irrespective of whether Ply was administered as a free protein or not (Figures 5.2.1 and 5.3.1).

It would be worthwhile vaccinating mice with Δ6 Ply-type 4 CPS plus free Δ6Ply to investigate whether this confers protection against serotype 1 infection. It may mean that free Ply should be incorporated into new vaccines to give broader protection and conjugated Ply can be used to confer vaccine serotype protection in place of CRM_{197}. If free Ply was to be used in vaccines, then Δ6 Ply or ΔA146 Ply would be safer alternatives to WT Ply or PdB Ply and more likely to be licensed.

Chapter 6

Results

Chromosomal replacement of *ply* in D39 *S. pneumoniae* with Δ6 *ply*

6: Chromosomal replacement of Ply in D39 *S. pneumoniae* with Δ6 Ply

Summary

Previously, Ply mutants have been used to investigate whether it is the cytolytic activity or complement activating ability of the toxin that contributes to pneumococcal virulence (Berry et al. 1995). This work indicated that with an i.p. model of infection, it was the cytotoxic activity of Ply that was important for virulence rather than the complement activating ability. However the mutants that were used, including the W433F (PdB) mutant, retained some haemolytic activity. As the Δ6 Ply mutation results in the complete abrogation of pore-forming activity and therefore lytic activity (as shown in chapter 3) the importance of pore-forming activity for pneumococcal virulence could be investigated. *ply* carrying the Δ6 mutation was used to replace the WT *ply* gene in D39 *S. pneumoniae* using Janus mutagenesis (Sung et al. 2001), which utilises the natural transformability of *S. pneumoniae* to allow the insertion of synthetic DNA into a desired site on the chromosomal DNA by negative selection. The resulting mutant strain, Δ6 D39, was then assessed for virulence in a murine model of pneumococcal pneumonia. This work was carried out prior to the vaccination studies in chapter 5 when D39 was the common laboratory reference strain in which most mutations were constructed, including the reagents for Janus mutagenesis with *ply*. The Δ6 Ply mutation should now be made in another serotype such as TIGR4 to give more accurate *in vivo* results.

6.1. Construction of Δ6 Ply in D39 *S. pneumoniae* using Janus mutagenesis

Dr. Gavin K. Paterson cloned the Janus cassette, which encodes a kanamycin resistance marker and an *rpsL+* marker, in place of the *ply* gene in streptomycin resistant D39 *S. pneumoniae* (also constructed by Gavin Paterson using the marker kindly supplied by Jean

Pierre Claverys, Toulouse, France). The *rpsL+* marker confers streptomycin sensitivity that dominates over the *rpsL-* allele of the host strain that gives spontaneous streptomycin resistance. This gives an intermediate strain, JP PCR B, which is streptomycin sensitive and kanamycin resistant. Successful transformation of the synthetic gene in place of the Janus cassette results in transformants that are streptomycin resistant and kanamycin sensitive. This allows for easy selection of true tranformants by plating on to streptomycin plates then selecting colonies for double stabbing on to streptomycin and kanamycin plates and choosing colonies that only grow on the streptomycin plates.

Dr. Gavin K. Paterson cloned the *ply* gene from D39 *S. pneumoniae* with flanking D39 DNA (2.345Kb) into the 3.5 Kb TOPO-XL vector to provide the donor DNA for transformation into D39 gDNA. This plasmid was used to create the Δ6 *ply* mutation (ΔA146ΔR147) by site-directed mutagenesis. Purified plasmid DNA for the Δ6 *ply* mutant and WT *ply* was then transformed into the JP PCR B strain. The WT donor DNA was included to restore the WT *ply* gene and give a control that had been through the same process as the Δ6 *ply* mutant. The transformation reaction was spread on to streptomycin plates for single colonies. 5/100 WT *ply* D39 transformants (WT_{15}, WT_{24}, WT_{47}, WT_{60}, WT_{64}) and 4/100 Δ6 *ply* D39 transformants ($\Delta6_8$, $\Delta6_{41}$, $\Delta6_{48}$, $\Delta6_{76}$) were streptomycin resistant and kanamycin sensitive. They were all optochin sensitive and pure cultures. Genomic DNA was prepared from cultures of each transformant and presence of the *ply* gene was checked by PCR (Figure 6.1).

6.1.2. *ply* PCR of Janus transformants

Primers 15C and 15D (Table 2.1) anneal to the 5' and 3' end of the *ply* gene respectively. PCR with these primers only gives a positive result for gDNA that contains *ply* (1.4Kb). The original D39 strain was shown to possess the *ply* gene (Figure 6.1. lane 2), whereas the

intermediate JP PCR B strain was *ply* negative (Figure 6.1. lane 3) as the Janus cassette is in place of *ply*. 4/5 WT *ply* transformants and 3/4 Δ6 *ply* tranformants were positive for the *ply* gene (Figure 6.1: lanes 4, 6, 7, and 12 for WT *ply* and lanes 8, 9,and 10 for Δ6 *ply*), indicating that these strains were successfully transformed with the donor DNA. This was confirmed by DNA sequencing.

Figure 6.1. *ply* PCR of genomic DNA from *S. pneumoniae* transformants

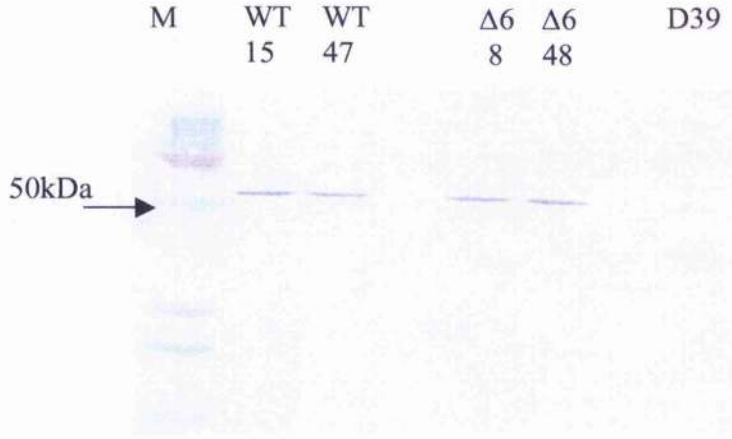
M, 1kb^{+} marker; lane 1, no DNA; lane 2, D39 *S. pneumoniae* streptomycin resistant strain; lane 3, JP PCR B *S. pneumoniae* intermediate strain with Janus cassette in chromosomal position of *ply*; lanes 4-7 and 12, WT *ply* transformants; lanes 8-11, Δ6 *ply* transformants.

6.2. Expression of Ply from *S. pneumoniae* Janus mutants

Two successful *S. pneumoniae* Janus mutants for WT *ply* (WT_{15} and WT_{47}, lanes 4 and 6 in Figure 6.1) and Δ6 *ply* ($\Delta6_8$ and $\Delta6_{48}$, lanes 8 and 10 in Figure 6.1) were grown up to mid log phase, harvested and lysed for analysis of Ply expression (Figure 6.2) and haemolytic activity (Figure 6.3). There was no difference in growth rates between transformants. The total amount of protein in each cell lysate was determined by

Bradford's assay then standardised to 200μg/ml prior to analysis. Both WT D39 and Δ6 D39 Janus transformants were positive for Ply expression (Figure 6.2).

Figure 6.2. Western blot of cell lysate from Janus transformants

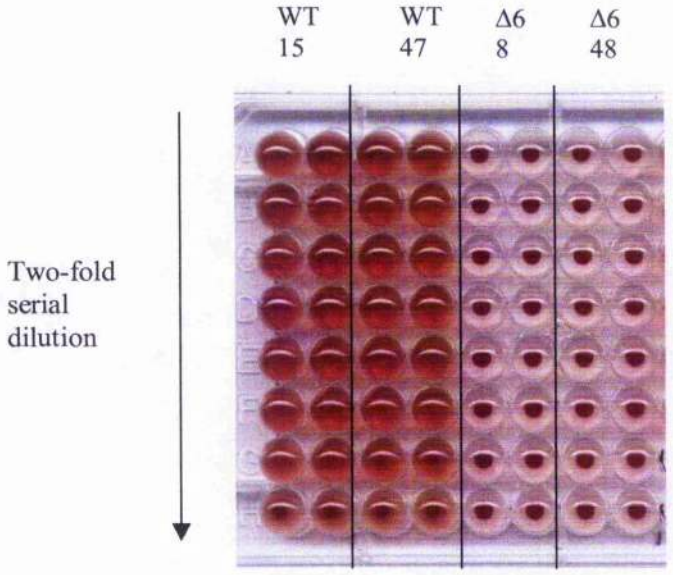


M, Precision^{+} kaleidoscope marker; lane 1, WT_{15} lysate; lane 2, WT_{47} lysate; lane 3, blank; lane 4, $\Delta6_{8}$ lysate; lane 5, $\Delta6_{48}$ lysate; lane 6, blank; lane 7, D39 *S. pneumoniae* lysate.

6.3. Haemolytic activity of Δ6 D39 *S. pneumoniae*

The haemolytic activity of cell lysate from two WT D39 transformants and two Δ6 D39 transformants was assessed. Ply expressed by Δ6 D39 was not haemolytic, whereas WT D39 produced haemolytic Ply (Figure 6.3.).

Figure 6.3. Haemolytic assay of cell lysate from restored WT D39 and Δ6 D39 Janus transformants.



Haemolytic assay of pneumococcal cell lysate from two WT D39 *S. pneumoniae* strains (WT_{15} and WT_{47}) and two strains carrying the Δ6 Ply mutation ($\Delta6_8$ and $\Delta6_{48}$).

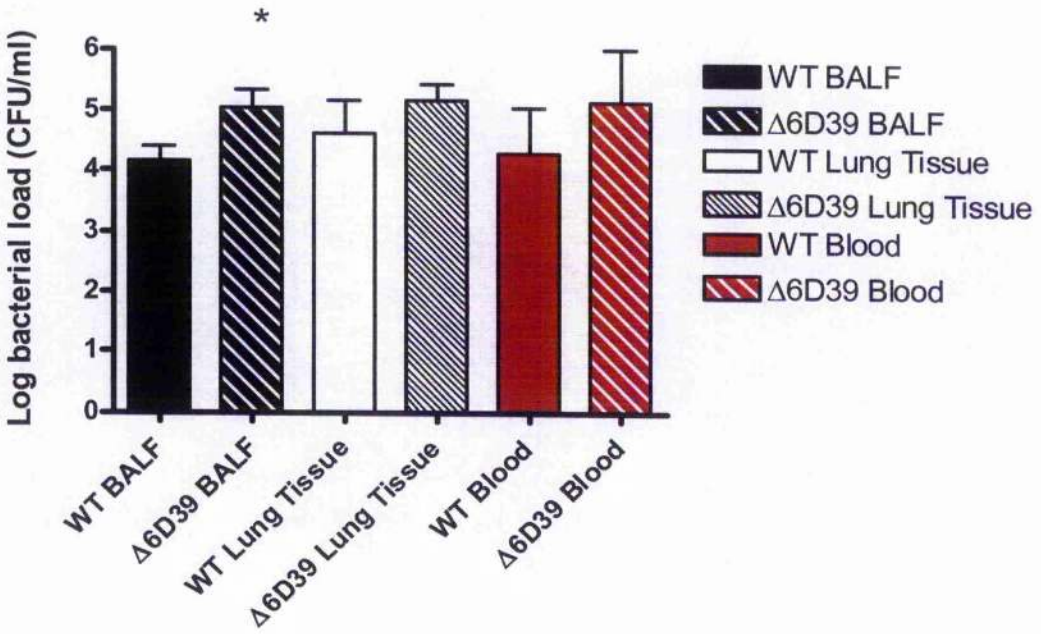
6.4. Virulence of Δ6 D39 *S. pneumoniae*

In order to assess the importance of pore-forming activity in Ply for *in vivo* pneumococcal infection, MF1 mice were infected i.n. with either 10^6 CFU WT D39 *S. pneumoniae* (WT_{47}) or Δ6 D39 *S. pneumoniae* ($\Delta6_{48}$; n=20). At 24h post infection, 10 from each group were sacrificed for analysis of bacterial loads in the lungs (pneumonia) and blood (bacteraemia), Figure 6.4.1. The remaining animals were monitored for survival over a two-week period, Figure 6.4.2. (n=10).

6.4.1. Bacteriology at 24h post i.n. infection with Δ6 D39 *S. pneumoniae*

Bacterial loads were determined from BALF, lung tissue and blood samples 24h post i.n. infection with WT D39 or Δ6 D39 (Figure 6.4.1.). The bacterial load in the BALF was significantly higher for the Δ6 D39 infected group compared with the WT D39 group ($P<0.05$).

Figure 6.4.1. Bacterial load (CFU/ml) in BALF, lungs and blood at 24h post i.n. challenge with either WT D39 *S. pneumoniae* or Δ6 D39 *S. pneumoniae*.

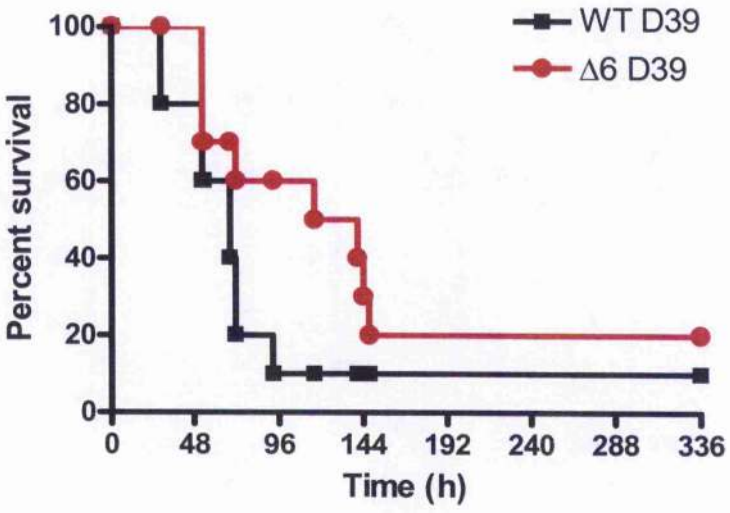


Mean log bacterial load (± SEM) in BALF, lung tissue and blood of MF1 mice at 24h post i.n. challenge with 10^6 CFU WT D39 *S. pneumoniae* or Δ6 D39 *S. pneumoniae*. *$P<0.05$ when bacterial loads in BALF of Δ6 D39 infected mice is compared with bacterial loads in BALF from the WT D39 group; n=10.

6.4.2. Survival of MF1 mice following intranasal infection with Δ6 D39 *S. pneumoniae*

There was no significant difference in survival times of mice infected with Δ6 D39 *S. pneumoniae* compared with mice infected with WT D39 *S. pneumoniae* (Figure 6.4.2, P=0.1203 at 336h). However, the Δ6 D39 infected group took longer to succumb to infection with 50% survival (5/10 mice) at 116 hours compared with 10% survival (1/10 mice) in the WT group (this was not quite significant, P=0.0874 at 116h).

Figure 6.4.2. Survival of MF1 mice following i.n. challenge with *S. pneumoniae*



Survival (h) of MF1 mice following i.n. challenge with 10^6 CFU of either WT D39 *S. pneumoniae* (black squares) or Δ6 D39 *S. pneumoniae* (red circles, n=10).

Discussion

Cloning Δ6 *ply* into the chromosomal DNA of D39 *S. pneumoniae*, shows that *in situ*, the Δ6 mutation still results in expression of a non-haemolytic form of Ply. However, it appears that for pneumonia, the pneumococcus does not require haemolytically active Ply to be fully virulent. This mutant may prove a useful tool for investigating other functions of Ply during pneumococcal infection such as TLR-4 activation (Malley et al. 2003) and binding to epithelial cells.

As there are significantly higher numbers of Δ6 D39 *S. pneumoniae* in the BALF at 24h compared with WT D39 *S. pneumoniae* (Figure 6.4.1.), it is possible that the mutant strain is not cleared from the site of infection as effectively as WT D39 *S. pneumoniae*. Improved clearance of D39 *S. pneumoniae* from the BALF has previously been shown to be a result of the host's inflammatory response to the cytotoxicity of WT Ply (van Rossum et al. 2005). When pneumococci attach to the respiratory mucosa, an array of inflammatory mediators are produced (Bergeron et al. 1998) in response to the numerous virulence factors of the pneumococcus. This results in an influx of leukocytes to the site of infection and WT Ply has been associated in stimulating this immune response (Kadioglu et al. 2000). Comparative analysis of the inflammatory responses following infection with WT D39 and Δ6 D39 *S. pneumoniae* may reveal mediators induced by the pore forming activity of Ply, this would probably include IL-6 and KC that was shown in chapter 4 to be produced by the murine model in response to WT Ply treatment but not the non-pore forming mutants.

Ply has been directly proven to facilitate bacterial invasion of epithelial cells and treatment with toxin alone recreates symptoms of pneumococcal infection in the lung (Feldman et al. 1990; Feldman et al. 1991). Ply negative strains are less virulent (Berry et al. 1989b),

however, there was no difference in bacterial loads in the lung tissue following infection with Δ6 D39 compared with WT D39 and the bacterial loads were actually increased in the BALF after Δ6 D39 infection (Figure 6.4.1). This indicates that there is some other function of Ply that does not involve pore-forming activity that is important for pneumococcal pneumonia.

It is important to address the problem in comparing host immune responses to the attenuated PLN-A knockout with wild type infection. Ply is obviously important for establishment of pneumococcal infection as the knockout strain does not grow well within the host but when immune responses such as cytokine induction are compared in hosts with WT infection and PLN-A this may just be due to the lack of bacteria and not directly to the omission of Ply. In light of this, point mutations that allow a known activity of Ply to be investigated are more favourable. Differences tend to not be noticed in growth in the host but subtle changes in infection are observed. Previously, Ply mutants have been used to investigate whether it is the cytolytic activity or complement activating ability of the toxin that contributes to pneumococcal virulence (Berry et al. 1995). This work indicated that it was the cytotoxic activity of Ply that was important for virulence in bacteraemia rather than the complement activating ability. However, in our intranasal model of infection, a strain of D39 with non-cytotoxic Ply is still capable of causing disease with no significant difference in survival times or lower bacterial loads compared with WT D39. It may be the route of infection, involving different cell types, that gives variable results or the fact that the Ply mutant used in the previous study had another function altered in addition to pore-forming ability. Cytolytic activity has been shown to be important during early bacterial growth at 3h and 6h and for acute lung injury, whereas, the complement activating function was important for bacteraemia at 24h post infection (Rubins et al. 1996). It is possible that at earlier time points post challenge, Δ6 D39 may be less able to establish in the host as a result of its dysfunctional Ply, but this was not apparent by 24h.

Growth of a Ply mutant with 0.02% haemolytic activity of WT Ply (H367R) (Alexander et al. 1998) was found to be inhibited in the lung during the early stages of pneumococcal pneumonia (Jounblat et al. 2003). Indicating that if earlier time points of infection were investigated with Δ6 D39 there may be a difference in bacterial loads. The involvement of Ply in pneumococcal infection is complex and it is unlikely that the function of Ply is limited to these two activities.

Janus mutagenesis provides an elegant system for insertion of mutations into chromosomal DNA. Previous *ply* mutants of *S. pneumoniae* in which the whole *ply* gene is disrupted by insertion-duplication mutagenesis are more intrusive and the downstream effects are not known. From this work, the serotype 1 data in chapter 7 and the fact that strains with *ply* mutations remain more virulent than *ply* knockout strains, it appears that there is an additional function of Ply other than complement activation and cytolytic activity that is required for full virulence.

Chapter 7

Results

Naturally occurring Ply mutants in serotype 1 clinical isolates

7. Naturally occurring Ply mutants in serotype 1 clinical isolates

Summary

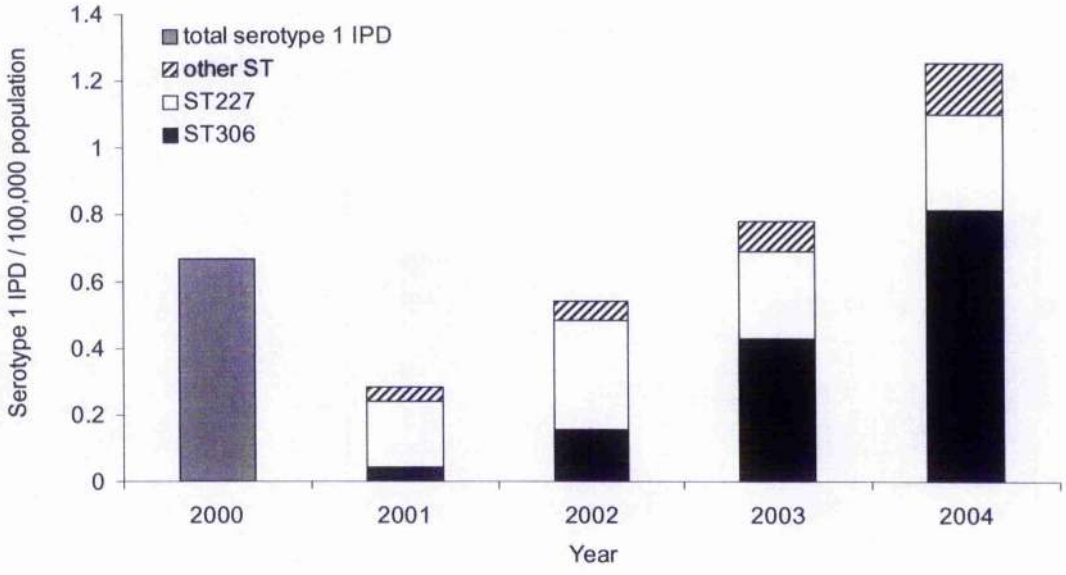
Recently, there has been an increase in invasive pneumococcal disease (IPD) caused by serotype 1 *S. pneumoniae* throughout Europe. Serotype 1 IPD is associated with bacteraemia and pneumonia in Europe and North America, especially in neonates, and is ranked among the top five most prevalent pneumococcal serotypes in at least 10 countries. The currently licensed paediatric pneumococcal vaccine does not afford protection to serotype 1.

As part of an on-going study of pneumococcal virulence genes (Jefferies, Kirkham et al, manuscript in preparation), a number of clinical isolates with mutations in their *ply* gene were identified. The mutations were predominantly in the *ply* gene of serotypes 1, 7 and 8. The serotype 1 isolates had additional mutations in the *ply* gene to those previously described for serotype 7 and 8 (Lock et al. 1996) and were chosen for further investigation due to their high attack rate and recent reports of an increase in serotype 1 disease (see section 1.4). From the initial 252 Scottish clinical pneumococcal isolates (Jefferies et al. 2004) (chosen to represent all serotypes received by the Scottish Meningococcus and Pneumococcus Reference Laboratory, SMPRL), four were serotype 1 and of these, two had mutations within the *ply* gene. Further analysis of an additional 28 serotype 1 isolates revealed that more than half had mutations within the *ply* gene, which resulted in the abrogation of the haemolytic activity of the toxin. Multi Locus Sequence Typing (MLST; carried out at SMPRL) of the serotype 1 isolates revealed a correlation between mutations in the *ply* gene and sequence type (Kirkham et al. 2006b).

7.1. Incidence of serotype 1 IPD in Scotland

The incidence rate of serotype 1 IPD in Scotland has increased from 0.67 cases/100,000 population in 2000 to 1.25 cases/100,000 population in 2004 (Figure 7.1, calculated from an overall incidence of IPD in Scotland of 12/100,000 with a population of 5 million). It is important to note that surveillance in Scotland improved in 2001 resulting in 90-95% of all cases in Scotland being reported to SMPRL. MLST data was available from 2001 onwards, allowing the incidence of each ST within serotype 1 IPD to be calculated. The incidence of ST306 increased from 0.04/100,000 population in 2001 to 0.813/100,000 population in 2004 whereas the incidence of ST227 remained fairly unchanged with 0.196 cases/100,000 population in 2001 and 0.286 cases/100,000 population in 2004 (Figure 7.1).

Figure 7.1. Incidence of serotype 1 IPD in Scotland from 2000 to 2004



Incidence of serotype 1 caused IPD in Scotland from 2000 to 2004. MLST data for 2001 to 2004 allows sequence type distribution within serotype 1 isolates to be recorded. Black bar represents ST306, white bar represents ST227 and diagonal striped bar represents all other sequence types. Grey bar represents total number of isolates. It is important to note that surveillance of IPD in Scotland improved from 2001 resulting in 90-95% of all cases being reported to SMPRL.

In 2003 there were 41 cases of serotype 1 IPD in Scotland (McChlery et al. 2005), resulting in an incidence rate of 0.76/100,000 population. MLST of the 2003 samples revealed that 23 of these isolates were ST306, 13 were ST227 and there were 5 others: ST217 (1), ST1310 (2), ST1247 (1) and ST1239 (1). This study sampled all isolates from the first 9 months of 2003, of which there were 28 cases. Twelve of the 28 were ST227 and 16 were ST306. From this group size of 28, 25 were available for further study. This gave a final sample group of 29 isolates (25 from 2003 and 4 from 2000-

2001), 16 were ST306, and 13 were ST227 (Table 7.1). ST306, allelic profile 12 8 13 5 16 4 20, is a double locus variant of ST227, allelic profile 12 5 13 5 17 4 20, sharing 5 of the 7 housekeeping genes assessed by MLST.

7.2. Sequencing of the Ply gene from clinical pneumococcal isolates

The *ply* genes from the 29 serotype 1 pneumococci were sequenced by DBS genomics and analysed by Dr. Johanna M. C. Jefferies alongside the Ply sequence from the serotype 2 laboratory strain D39 (Walker et al. 1987). At the DNA level, five different alleles of the *ply* gene were present in this collection, on translation this resulted in two protein alleles. DNA sequences have been deposited at NCBI under accession numbers DQ251177, DQ251178, DQ251179, DQ251180, DQ251181 by Dr. Johanna M. C. Jefferies. The two Ply protein alleles correlated with the ST of the serotype 1 pneumococci in that all isolates of ST306 (n=16) contained mutations at six amino acid positions (Y150H, T172I, K224R, A265S, ΔV270 and ΔK271) when compared with the D39 Ply sequence (Figure 7.2.1). This protein allele is referred to as Ply306. Twelve of the 13 isolates of ST227 differed from the D39 sequence at only one residue, D380N. This protein allele is referred to as Ply227 (Figure 7.2.1) and has the same sequence as Ply from TIGR4 *S. pneumoniae* (Tettelin et al. 2001). One ST227, strain 00-3645, was found to have a 24 base pair insertion in the *ply* gene, which was a direct repeat of the preceding sequence and resulted in an eight amino acid insertion in the protein (NVRNLLKG, at residues 417-424). Structural modelling of this insert by Dr. Alan Riboldi-Tunnicliffe was found to result in the extension of a loop in domain 4, highlighted in red in Figure 7.2.2.

Figure 7.2.1. Alignment of Ply 306 and 227 protein alleles with D39 *S. pneumoniae*

```
          1                                                   50
PlyD39    MANKAVNDFI LAMNYDKKKL LTHQGESIEN RFIKEGNQLP DEFVVIERKK
Ply227    MANKAVNDFI LAMNYDKKKL LTHQGESIEN RFIKEGNQLP DEFVVIERKK
Ply306    MANKAVNDFI LAMNYDKKKL LTHQGESIEN RFIKEGNQLP DEFVVIERKK

          51                                                  100
PlyD39    RSLSTNTSDI SVTATNDSRL YPGALLVVDE TLLENNPTLL AVDRAPMTYS
Ply227    RSLSTNTSDI SVTATNDSRL YPGALLVVDE TLLENNPTLL AVDRAPMTYS
Ply306    RSLSTNTSDI SVTATNDSRL YPGALLVVDE TLLENNPTLL AVDRAPMTYS

          101                                                 150
PlyD39    IDLPGLASSD SFLQVEDPSN SSVRGAVNDL LAKWHQDYGQ VNNVPARMQY
Ply227    IDLPGLASSD SFLQVEDPSN SSVRGAVNDL LAKWHQDYGQ VNNVPARMQY
Ply306    IDLPGLASSD SFLQVEDPSN SSVRGAVNDL LAKWHQDYGQ VNNVPARMQH

          151                                                 200
PlyD39    EKITAHSMEQ LKVKFGSDFE KTGNSLDIDF NSVHSGEKQI QIVNFKQIYY
Ply227    EKITAHSMEQ LKVKFGSDFE KTGNSLDIDF NSVHSGEKQI QIVNFKQIYY
Ply306    EKITAHSMEQ LKVKFGSDFE KTGNSLDIDF NSVHSGEKQI QIVNFKQIYY

          201                                                 250
PlyD39    TVSVDAVKNP GDVFQDTVTV EDLKQRGISA ERPLVYISSV AYGRQVYLKL
Ply227    TVSVDAVKNP GDVFQDTVTV EDLKQRGISA ERPLVYISSV AYGRQVYLKL
Ply306    TVSVDAVKNP GDVFQDTVTV EDLKQRGISA ERPLVYISSV AYGRQVYLKL

          251                                                 300
PlyD39    ETTSKSDEVE AAFEALIKGV KVAPQTEWKQ ILDNTEVKAV ILGGDPSSGA
Ply227    ETTSKSDEVE AAFEALIKGV KVAPQTEWKQ ILDNTEVKAV ILGGDPSSGA
Ply306    ETTSKSDEVE AAFESLIKGV KVAPQTEWKQ ILDNTEVKAV ILGGDPSSGA

          301                                                 350
PlyD39    RVVTGKVDMV EDLIQEGSRF TADHPGLPIS YTTSFLRDNV VATFQNSTDY
Ply227    RVVTGKVDMV EDLIQEGSRF TADHPGLPIS YTTSFLRDNV VATFQNSTDY
Ply306    RVVTGKVDMV EDLIQEGSRF TADHPGLPIS YTTSFLRDNV VATFQNSTDY

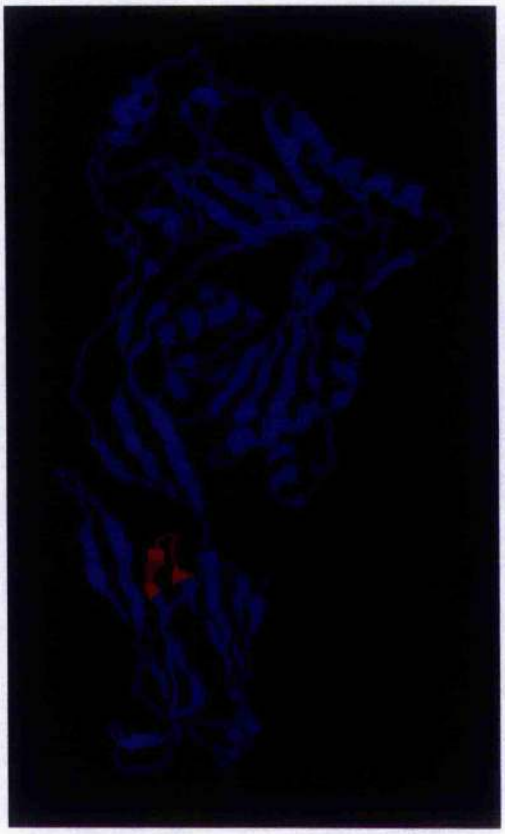
          351                                                 400
PlyD39    VETKVTAYRN GDLLLDHSGA YVAQYYITWD ELSYDHQGKE VLTPKAWDRN
Ply227    VETKVTAYRN GDLLLDHSGA YVAQYYITWN ELSYDHQGKE VLTPKAWDRN
Ply306    VETKVTAYRN GDLLLDHSGA YVAQYYITWD ELSYDHQGKE VLTPKAWDRN

          401                                                 450
PlyD39    GQDLTAHFTT SIPLKGNVRN LSVKIRECTG LAWEWWRTVY EKTDLPLVRK
Ply227    GQDLTAHFTT SIPLKGNVRN LSVKIRECTG LAWEWWRTVY EKTDLPLVRK
Ply306    GQDLTAHFTT SIPLKGNVRN LSVKIRECTG LAWEWWRTVY EKTDLPLVRK

          451                 470
PlyD39    RTISIWGTTL YPQVEDKVEN D
Ply227    RTISIWGTTL YPQVEDKVEN D
Ply306    RTISIWGTTL YPQVEDKVEN D
```

Alignments of the amino acid sequence for ST227 and ST306 pneumolysin compared with the D39 pneumolysin sequence. The mutations are highlighted in grey. ST227 pneumolysin differs from D39 pneumolysin by one amino acid substitution (D380N). ST306 pneumolysin differs from D39 pneumolysin by 4 amino acid substitutions (Y150H, T172I, K224R, A265S) and two amino acid deletions (V270K271).

Figure 7.2.2. Structure of 00-3645 Ply based on PFO homology model



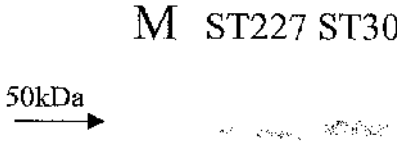
Structure of Ply produced by strain 00-3645 with the eight amino acid repeat highlighted in red. This results in the extension of a loop in domain 4.

7.3. Expression levels and haemolytic activity of Ply in serotype 1 clinical isolates

Western blots were run with the cell extract from each sample and all, except 00-3645, were positive for Ply expression. Ply expressed by ST306 samples were found to run slightly higher on 12% SDS-PAGE than ST227 Ply despite possessing two amino acid deletions (demonstrated in Figure 7.3.1). Haemolytic assays were carried out on lysates from the 29 serotype 1 pneumococcal isolates and D39 *S. pneumoniae* (Table 7.1). 55% (16/29) of the serotype 1 isolates produced Ply that did not lyse human erythrocytes, all

of which were ST306. Lysates prepared from ST227 serotype 1 pneumococci and the positive control D39, were haemolytic. Analysis of Ply expression levels by ELISA revealed that all ST306 non-haemolytic lysates contained titres comparable to, and in some cases higher, than lysates expressing haemolytically active Ply (Table 7.1). The specific activity of Ply was calculated from the haemolytic assay (Haemolytic Units; HU/ml) and expression levels (mg/ml) to give the specific activity in HU/mg of toxin. The specific activity of Ply produced by D39 and ST227 isolates was similar, however Ply expressed by ST306 strains had no specific activity (Table 7.1). Ply expression by strain 00-3645 was not detectable by ELISA.

Figure 7.3.1. Western blot of Ply from an ST227 and ST306 strain

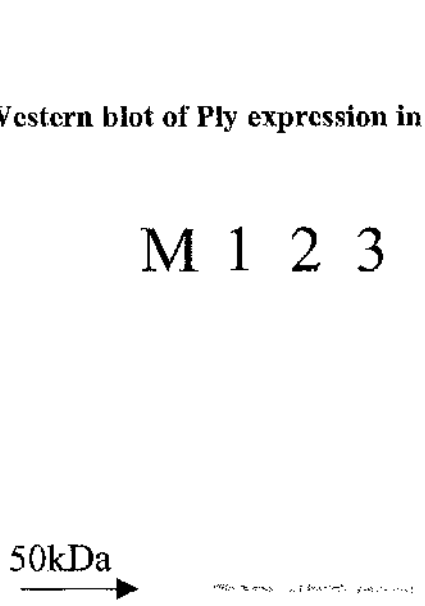


M, Precision^{+} kaleidoscope marker; ST227, cell lysate from concentrated strain 01-2696; ST306, cell lysate from concentrated strain 01-1956. Blot is from samples run on 12% SDS-PAGE using polyclonal anti-Ply serum from rabbit.

To further investigate whether the Ply306 allele produced non-haemolytic Ply or Ply with reduced haemolytic activity, preparations were made of a representative ST306 (01-1956) and ST227 (01-2696) strain to give cell extract with 8-10 fold more Ply. Cell extract from strain 01-1956 (ST306) contained 6.4μg/ml Ply and strain 01-2696 (ST227) contained 5.8μg/ml Ply as determined by ELISA, however, the ST306 Ply

remained non-haemolytic. Ply from 00-3645 was still not detected by ELISA in a concentrated sample, however a faint band was detected by Western blotting with anti-Ply antibody (Figure 7.3.2). The insoluble fraction from 00-3645 lysis was negative for the presence of Ply by Western blotting and ELISA. Although ELISA did not detect expression of Ply by strain 00-3645, the cell extract from this strain was found to have residual haemolytic activity in the first well of the haemolytic assay.

Figure 7.3.2. Western blot of Ply expression in concentrated samples



M, Precision kaleidoscope marker; 1, ST227 cell lysate (strain 01-2696), 2, ST306 cell lysate (01-1956); 3, same as 1; 4, 00-3645 cell lysate. All cell lysates were standardised to 2.35mg/ml total protein prior to analysis. Blot is from samples run on 10% SDS-PAGE using polyclonal anti-Ply serum from rabbit.

Yu Jing cloned 00-3645 *ply* into pET-33b for recombinant expression of 00-3645 Ply. This was achieved, but the 00-3645 Ply was almost exclusively found in the insoluble cell fraction. The r00-3645Ply that was soluble possessed some haemolytic activity but at levels ~100 times less that of recombinant WT Ply. Expressed r00-3645 Ply proved difficult to purify, despite the incorporation of a His-tag.

Table 7.1. Sequence type, haemolytic activity and Ply expression levels of serotype 1 *S. pneumoniae* isolates

Sample ID	Patient age (years)	Diagnosis[c]	Sequence Type (ST)	Haemolytic Activity (+/-)[a]	Ply (ng/ml)[b]	Specific Activity (HU/mg)[b]
D39 (reference)	-		128	+	989	3.2×10^5
00-3645	40		227	-	0	-
01-1956	5	bacteraemia	306	-	1203	0
03-1769	58		306	-	1663	0
03-1828	63		306	-	2214	0
03-1986	65		306	-	1654	0
03-2412	84	pyrexia	306	-	1608	0
03-2473	38	pyrexia	306	-	1454	0
03-2672	25	pneumonia	306	-	1976	0
03-2759	66	pneumonia	306	-	1695	0
03-3425	22	pyrexia	306	-	1516	0
03-3431	12	pyrexia	306	-	1701	0
03-3485	56	pyrexia	306	-	982	0
03-3582	36		306	-	1651	0
03-3788	35	pneumonia	306	-	1245	0
03-3828	32	pneumonia	306	-	1326	0
03-3921	64	pneumonia	306	-	1355	0
03-1063	18		306	-	2621	0
01-2696	33		227	+	1155	4.2×10^5
03-2135	38	pneumonia	227	+	1520	4.2×10^5
03-2219	76		227	+	1401	4.6×10^5
03-2234	46		227	+	1230	5.2×10^5
03-2406	75	septicaemia	227	+	1447	4.4×10^5
03-2920	42		227	+	1606	2.0×10^5
03-2809	9	pyrexia	227	+	1431	2.2×10^5
03-3629	67	pyrexia	227	+	1063	1.5×10^5
03-3920	28	pneumonia	227	+	1329	4.8×10^5
01-2526	6		227	+	670	7.2×10^5
03-1510	14	pneumonia	227	+	1518	4.2×10^5
03-3856	20		227	+	901	7.1×10^5

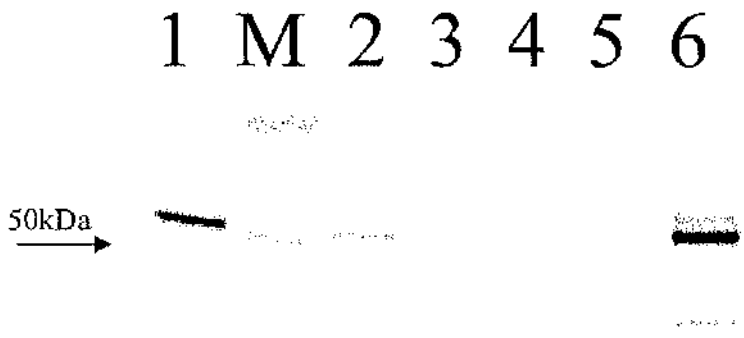
[a] Haemolytic activity assessed with human erythrocytes; [b] cell lysates diluted in 1xPBS to 300µg/ml total protein prior to analysis;

[c] All isolates were from blood cultures and therefore caused bacteraemia

7.4. Analysis of binding and pore forming ability of isolates expressing non-haemolytic Ply

Erythrocyte membranes were treated with cell extract from an ST306 and ST227 isolate and from strain 00-3645. Following washing to remove unbound toxin, samples were run on SDS-PAGE and Western blotted using anti-pneumolysin antibody. Ply from both ST306 and ST227 bound to erythrocytes (Figure 7.4.1), however, ST306 Ply did not bind as well. 00-3645 Ply was not observed to bind at all, though expression levels were so low that detection of binding would be minimal.

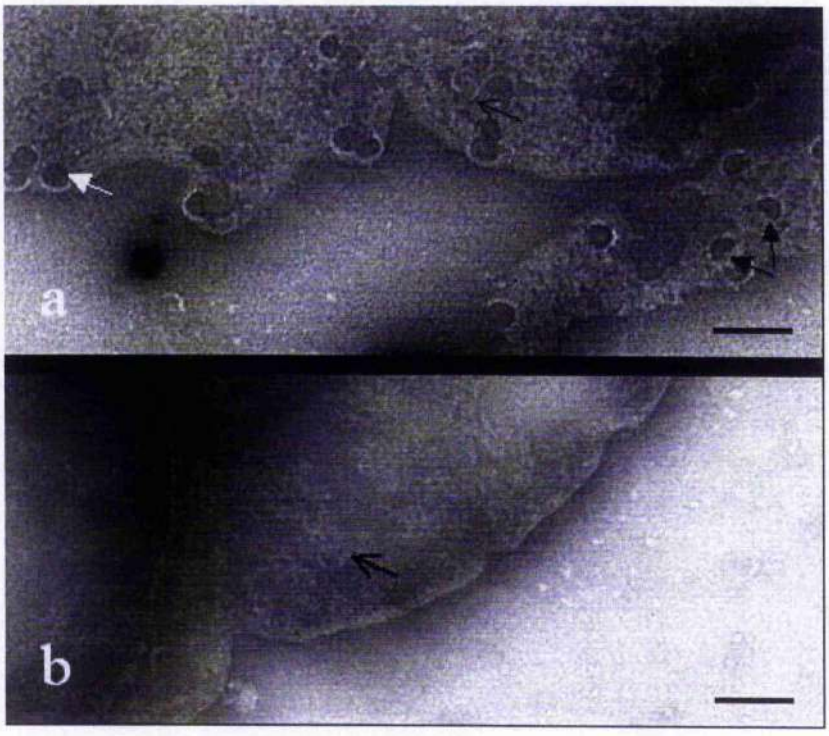
Figure 7.4.1. Analysis of binding of serotype 1 Ply to erythrocyte membranes



1, 10μg/ml recombinant WT Ply; M, Precision™ kaleidoscope marker; 2, washed erythrocyte membranes incubated with cell extract from ST227 strain 01-2696 (5.8μg/ml Ply); 3, washed erythrocyte membranes incubated with cell extract from ST306 strain 01-1956 (6.4μg/ml Ply); 4, washed erythrocyte membranes incubated with 00-3645 cell extract; 5, blank; 6, washed erythrocyte membranes incubated with recombinant WT Ply.

Analysis of pore formation by Transmission Electron Microscopy revealed that Ply expressed by ST306 was unable to form pores on erythrocyte membranes (Figure 7.4.2.b) in comparison with ST227 Ply (Figure 7.4.2.a). Arcs were observed on membranes treated with cell extract from both STs but the Ply 306 toxin did not assemble to form functional pores in the host cell membrane. PBS treated membrane was included as a negative control and was similar in appearance to ST306 treated membrane without the presence of arc structures.

Figure 7.4.2. TEM of erythrocyte membrane treated with serotype 1 Ply



Transmission Electron Microscopy of negatively stained erythrocyte membrane treated with filtered cell extract from an ST227 isolate (a) or ST306 isolate (b). Viewed at ×25000 magnification, black bar represents 100nm. White arrow indicates a pore, filled black arrows show arcs that appear to be inserted into the membrane and fine black arrows indicate arcs of toxin that do not appear to be inserted into the membrane.

7.5. Virulence of ST306, ST227 and strain 00-3645

MF1 mice were challenged i.p. with 5×10^7 CFU 00-3645 *S. pneumoniae* but this did not result in infection. In order to establish infection, complement deficient C3-/- mice were given the same dose but still did not succumb to infection. The mice did not display any clinical signs of illness following challenge. Thus 00-3645 was deemed to be avirulent in mice. In contrast, i.p. injection of 5×10^7 CFU 01-1956 (ST306) or 01-2696 (ST227) resulted in bacteraemia within 24h in both MF1 and C3-/- mice. I.n. inoculation of MF1 mice with 5×10^7 CFU 01-1956 or 01-2696 resulted in transient signs of infection in all animals. These signs ranged from a hunched stance to lethargy but all animals survived the challenge. At 24h post challenge 3/5 mice inoculated with either 01-2696 or 01-1956 were bacteraemic. There was no difference in the levels of bacteraemia caused by 01-2696 or 01-1956.

Discussion

Serotype 1 has remained one of the most prevalent IPD causing serotypes and has recently been ranked amongst the top five IPD causing serotypes in England, Germany, Spain, Quebec, Chile and South Africa (Brueggemann et al. 2003b). Data collected in Scotland from 1999-2001 ranked serotype 1 as the 10th most prevalent serotype, causing 3.5% of IPD (Kyaw et al. 2003). When data from 2002 was included, serotype 1 ranked 9th causing 3.8% of IPD in Scotland (Denham et al. 2005) and by 2003, serotype 1 ranked as the 4th most prevalent serotype, causing 6.7% of IPD (McChlery et al. 2005). MLST data reveals that this increase in serotype 1 disease in Scotland, beginning in 2002, is from the expansion of ST306 (Figure 7.1). This has previously occurred in Sweden where serotype 1 caused IPD increased from 1% in 1992 to 10% in 1997 and this was shown to be solely due to the emergence of ST306 (Henriques Normark et al. 2001).

ST227 and ST306 are from the same lineage and clonal group (Brueggemann et al. 2003b) and are almost exclusive to serotype 1 pneumococci, although an ST227 has been identified with 19F capsule polysaccharide (Jefferies et al. 2004). ST306 IPD is predominant in continental Europe whereas ST227 is the most prevalent serotype 1 ST in England, North America and Canada (Brueggemann et al. 2003b). The reasons for this distribution are unclear and larger sample groups would be required for such analysis. Brueggemann and Spratt (Brueggemann et al. 2003b) explained the differences in ST distribution between England and the rest of Europe to be a result of the low carriage rate of serotype 1, which thereby reduces the probability of transfer of clones between populations in comparison with more carriage-associated serotypes. The difference in ST distribution between England and Scotland is surprising, with ST306 only present in the Scottish samples. As isolates in the two studies are from different time periods, it may be that ST306 is now also found in England; however, there are no ST306 isolates from

England in the MLST database. We are possibly witnessing the clonal expansion of serotype 1 with the emergence of ST306 in Scotland.

All of the ST306 isolates were shown to have mutations in their *ply* gene that resulted in the expression of non-haemolytic Ply, yet these clinical isolates were from patients with IPD. Despite serotype 1 pneumococci being traditionally highly virulent in mice (Kostyukova et al. 1995), the strains in this study were found to cause mild disease in MF1 mice. Strain 00-3645, expressing low levels of Ply, was avirulent in both WT and immune compromised C3-/- mice. Intraperitoneal injection of strains 01-2696 and 01-1956, producing WT and non-haemolytic Ply respectively, caused terminal septicaemia. These strains were less virulent intranasally, causing only transient infection. There was no difference observed in the survival times or 24h bacteraemia of mice infected with either ST227 (strain 01-2696) or ST306 (strain 01-1956). Thus, the haemolytic activity of Ply does not have an effect on the virulence of these serotype 1 isolates in our model. This correlates with the fact that all of the serotypes analysed caused IPD in patients irrespective of Ply phenotype.

Ply has long been known as an important virulence factor of the pneumococcus but a number of studies with a *ply* knockout mutant of *S. pneumoniae* have revealed that the toxin is not essential for pneumococcal induced inflammation during meningitis (Friedland et al. 1995; Winter et al. 1997; Wellmer et al. 2002). D39 *S. pneumoniae* carrying the *ply* gene was found to cause acute sepsis in mice following intravenous challenge compared with chronic bacteraemia if the *ply* gene was absent (Benton et al. 1995). Studies with defined point mutations in the *ply* gene showed that the haemolytic activity of Ply was important during sepsis (Berry et al. 1995). The haemolytic property of Ply has also been shown to be involved in the initial stages of pneumonia including invasion of the lung tissue and neutrophil recruitment, however haemolytic activity is not important in

facilitating pneumococcal growth in the alveoli (Rubins et al. 1995; Rubins et al. 1996). As serotype 1 disease is associated with pneumonia rather than meningitis in Europe and the United States (Hausdorff et al. 2000a; Ispahani et al. 2004; Sjostrom et al. 2006) (and Table 7.1) it may be that haemolytically active Ply is not essential for the pathogenesis of certain serotype 1 sequence types, such as ST306. This may mean that there will be less selective pressure to conserve the nucleotide sequences that encode the haemolytic activity of Ply and may allow the mutations we have identified to arise. Recently, an increase in pneumococcal meningitis in Ghana has been attributed to serotype 1 clonal complex ST217 (Leimkugel et al. 2005), which the authors propose may be better equipped to cause meningitis. We have sequenced the *ply* gene from an ST217 strain from the Ghana meningitis outbreak and found it carried the Ply 227 allele. This may indicate that haemolytically active Ply is required for pneumococci to cause meningitis but not pneumonia. This highlights the need for closer investigation of not only serotype specific virulence factors but the possibility of variation within serotypes and underlines the importance of MLST surveillance.

As ST306 causes IPD, haemolytic activity appears to not be essential for virulence. This is further supported by the *in vivo* results in chapter 6, where mice challenged i.n. with a strain of D39 *S. pneumoniae* carrying non-haemolytic pneumolysin had similar bacterial loads in the lungs and blood at 24h (Figure 6.4.1) and similar survival times compared with mice challenged with the wild type strain (Figure 6.4.2). Indeed, the bacterial load in the BALF of mice challenged with D39 carrying non-haemolytic Ply was significantly greater than the pneumococcal counts in BALF of mice challenged with WT D39 (Figure 6.4.1). Strains carrying Ply lacking haemolytic activity and complement binding activity have been demonstrated to be more virulent than strains with the *ply* gene knocked out, thereby indicating that Ply has an additional function yet to be determined (Benton et al. 1997).

The majority of the mutations observed in Ply306 are those previously implicated in the reduction of haemolytic activity for certain serotype 7 and serotype 8 strains (Lock et al. 1996). These mutations have not, until now, been associated with serotype 1 strains. Further analysis of these serotype 7 and 8 strains revealed that substitution of threonine with isoleucine at residue 172 was responsible for reducing the haemolytic activity of the pneumococcal lysates from 10^6 HU/mg Ply to 10^4 HU/mg Ply and was also attributable for anomalously slow migration of the toxin on SDS PAGE gels. From more recent studies, the T172I mutation probably affects insertion of domain 3 of the toxin into the host cell membrane (Ramachandran et al. 2002; Ramachandran et al. 2004). Ply 306 also ran anomalously on SDS-PAGE (Figure 7.3.1) but in contrast with the serotype 7 and 8 isolates, there was no haemolytic activity observed even when the samples were concentrated. The abrogation of haemolytic activity of Ply306 is probably due to the combination of the T172I substitution with a further mutation, Y150H, not observed in the serotype 7 or 8 strains. The Y150H mutation in Ply is comparable with the Y181A mutation made in the related toxin perfringolysin O (PFO) (Hotze et al. 2001a). PFO Y181A is unable to form pores in cell membranes thus reducing haemolytic activity to less than 1% activity of WT PFO. Mutation of Y150 in Ply to a histidine residue would probably have a similar effect, indeed, substitution of this residue in Ply to alanine reduces the haemolytic activity to 0.2% of WT Ply (Chapter 3, Table 3.1) and deletion of this residue and the adjacent glutamic acid (ΔY150E151; Δ8) in Ply results in a non-haemolytic form of Ply (Chapter 3).

The reduced binding ability of Ply306 and therefore inability to form pores is likely to be a result of the combination of mutations in the *ply* gene. Functional pores were not observed on membranes treated with Ply306, however arc structures were observed on the surface of membrane treated with Ply306 and Ply227 (Figure 7.4.2). Arcs remain a subject of contention within cholesterol dependent cytolysin research (Tweten et al. 2001) and there

is debate as to whether arcs can insert into the membrane and cause membrane damage. Here, Ply306 was not found to be cytolytic and thus may provide evidence that arcs are non-functional structures formed by the toxin. Although reduced binding was observed, there were no mutations in the binding domain (domain 4) of Ply306. This has been shown before with mutations in domain 3 of PFO that altered the binding ability of PFO (Heuck et al. 2000).

The eight amino acid insertion present in Ply from strain 00-3645 occurs just upstream of the highly conserved undecapeptide region involved in membrane binding (Jacobs et al. 1999). A single amino acid substitution in this region reduces the haemolytic activity of Ply by 100 fold (Korchev et al. 1998). The residual haemolytic activity of r00-3645Ply indicates that it can bind to eukaryotic cells and form pores but not as effectively as the native toxin. This residual activity may mean that the eight amino acid repeat, which results in the extension of the loop involved in host cell binding, does not completely disrupt folding and therefore functioning of the toxin. Due to the low levels of expression, pore formation was not investigated with the 00-3645 strain.

The fact that Ply may be less conserved than previously assumed should not affect its use as the protein component of next-generation conjugate vaccines. Antibodies raised against WT Ply still recognised the Ply expressed by all serotype 1 pneumococci in Western blots and ELISA. Although mAb PLY7 (de los Toyos et al. 1996) recognised the serotype 1 Ply alleles, it has since been found to not detect some variants of Ply expressed by other serotypes (Dr. Johanna Jefferies, personal communication, 2005). This is important in terms of using Ply ELISAs in clinical assays. Also, the use of *ply* PCR as a clinical diagnostic tool may not be advisable in light of such variation. Some laboratories have already found the *ply* PCR can be negative for pneumococci or positive for other species

such as *S. mitis* (Neeleman et al. 2004) and have therefore changed to using *lyt*A PCR to distinguish pneumococci.

The Ply306 allele may be of some benefit to the pneumococci, possibly prolonging carriage in the nasopharynx compared with other serotype 1 STs. Indeed in Sweden, ST306 was found in the nasopharynx of 2 healthy siblings when ST306 was at its most prevalent (Sandgren et al. 2004), and was suggested to either be adapting to colonisation or possibly the short carriage time of this ST had been captured. Recently, ST306 has been found to cause less severe IPD compared with serotype 1 ST227 (Sjostrom et al. 2006) and serotype 1 ST228 (Sandgren et al. 2005). The increase in ST306 observed in Scotland and Sweden may be due to an increased ability to colonise the host, however there is insufficient MLST data of serotype 1 isolates from carriage and sequences of the *ply* gene of the Swedish isolates were not investigated. We may be witnessing the evolution of serotype 1 strains to becoming less invasive and more carriage associated. This highlights the requirement for global MLST surveillance of both carriage and IPD associated pneumococci.

Chapter 8

Final Discussion

Final Discussion

A non pore-forming form of pneumolysin was successfully constructed and shown to retain the immunogenicity of WT Ply without the associated toxic effects. Abrogation of pore formation also enabled the investigation of this role in pneumococcal pneumonia. Previous Ply mutants with reduced cytolytic activity have been used to try to understand this role in pathogenesis (Berry et al. 1995), however, mutants with abrogated pore-forming activity have not been investigated before. We have shown that the pore forming activity of Ply is not essential for *S. pneumoniae* to cause pneumonia. As the other identified activity, complement activation, is also not essential for IPD and mutant pneumococcal strains with residual cytolytic activity and abolished complement activating ability are still more virulent than the Ply knockout strain (Benton et al. 1997), Ply possibly has additional activities yet to be determined.

Our work has also shown that pore forming activity is not essential for serotype 1 *S. pneumoniae* to cause IPD as clinical isolates were found to contain Ply alleles with mutations that rendered Ply non-haemolytic. We have shown that Ply is not as conserved as once thought with 5 DNA alleles and 2 protein alleles for Ply from 29 clinical isolates of serotype 1 pneumococci. We also found that the non-haemolytic Ply allele was intrinsically associated with ST306. Similar levels of Ply expression were found in all isolates; however, the non-haemolytic form expressed by ST306 had reduced binding and therefore pore-forming activity. It is interesting to note that although the mutations affected binding, they were not in the binding domain of the toxin. This is in contrast to the non pore-forming Δ6 Ply and ΔA146 Ply mutants, which were able to bind to cells as well as WT Ply but did not form pores. Previous work has shown that mutations in domain 3 of PFO have an affect on domain 4's ability to bind to the host cell (Heuck et al. 2000) and this probably occurs with Ply expressed by ST306.

By analysis of MLST data from SMPRL we have also identified the possible clonal expansion of ST306 as being responsible for the recent increase in serotype 1 disease in Scotland. This has also occurred in Sweden with a 10 fold increase in serotype 1 IPD over five years that was attributable to a rise in ST306 (Henriques Normark et al. 2001). It would be of interest to sequence the *ply* genes from the Swedish isolates. ST217 has recently been associated with meningitis in Ghana (Leimkugel et al. 2005), sequencing of the ST217 *ply* allele revealed that it was WT *ply* (the same as ST227 and that carried by TIGR4 *S. pneumoniae*). It may be that haemolytically active Ply is required by serotype 1 pneumococci to cause meningitis. This will be further investigated.

Further analysis of 252 isolates from SMPRL has found 12 different Ply protein alleles, with 2 protein alleles resulting in the loss of haemolytic activity (Jefferies et al, manuscript in preparation). This, combined with our serotype 1 data (Kirkham et al. 2006b), highlights the importance of surveillance of virulence genes in addition to the housekeeping genes monitored by MLST. The reasons for the increase in ST306 may be due to a favourable trait that carrying non-lytic Ply permits, such as increased tolerance of the host to allow carriage, yet the serotype 1 isolates were all from invasive disease. Another possibility is that the ST306 strain is retained in the lungs and is unable to disseminate throughout the lung tissue to enter the bloodstream due to the inability of Ply 306 to lyse host cells. This could result in an increase in the bacterial load of ST306 in the lungs, as was observed with the Δ6D39 mutant in chapter 6, possibly providing a source of spread for serotype 1 pneumococci by aerosol from coughing of patients. It would be interesting to investigate whether ST306 is only associated with pneumonia and is unlikely to go on to cause bacteraemia and this would suggest that cytotoxic Ply is required for transmission of the bacteria from the lungs into the bloodstream as has been shown in mice using bioluminescent imaging (Orihuela et al. 2004). Interestingly serotype 1 is associated with meningitis in Africa (Leimkugel et al. 2005) and pneumonia in Europe or

North America. Large scale analysis of sequence type and virulence genes of carried and invasive isolates may help to understand this association, the role of Ply and the high attack rates of serotype 1 pneumococci.

The variability of Ply may not be so important in terms of its use as a vaccine candidate as the major epitopes remain conserved and all expressed Ply was recognised by polyclonal and monoclonal anti-Ply antibodies. However, thought must be taken in using *ply* as a diagnostic tool (Toikka et al. 1999; Greiner et al. 2001; Saukkoriipi et al. 2002; Cima-Cabal et al. 2003) as some primers used in PCR of clinical samples may not detect mutant forms of *ply*. Also, Ply was found to be expressed by *Streptococcus mitis* and this was detected by polyclonal anti-Ply antibody but not by the monoclonal antibody (PLY7) used in the Ply ELISA (our unpublished data). Recently *ply* sequencing in addition to MLST has been suggested to be used to group difficult to type or non-typeable pneumococci (Hanage et al. 2005); the variability of *ply* should be taken into consideration in these circumstances.

WT Ply treatment of mice was found to result in hypothermia and high levels of IL-6 and KC in the lungs of mice. These cytokines may play a role in the host response to Ply treatment, though their role was not determined in this study and they were not found systemically. Studies have shown that very small quantities of circulating cytokines are required to exert an effect on the brain and therefore thermogenesis (Dantzer 1994). It may be that WT Ply damage of the blood brain barrier (Zysk et al. 2001) permits direct influx of cytokines to the brain, giving the immediate hypothermic response observed. To investigate whether IL-6 and KC are involved in the hypothermic response to Ply, telemetry chip implanted mice could be treated with anti-IL6 or anti-KC antibodies then treated with WT Ply and placed on the telemetry system to monitor their Tc in comparison with mice treated with WT Ply only. If the hypothermic response is blocked by anti-

cytokine antibody then it is likely that those cytokines are involved in the host response to Ply treatment and ultimately pneumococcal infection, which could also be investigated in the same manner. This could be confirmed with knockout host strains such as IL-6-/- mice and investigating their response to Ply.

It is likely that a combination of pneumococcal proteins will be used in the next generation of pneumococcal vaccines and as Ply confers protection against pneumonia and bacteraemia (Alexander et al. 1994), it will probably be included. An additional factor to investigate is whether conjugated and/or free proteins will be used, as we have demonstrated that Ply may function differently depending upon presentation to the host. WT Ply and Δ6 Ply have been demonstrated to be as effective as CRM_{197} in functioning as a carrier protein to boost the immunogenicity of capsule polysaccharides. An advantage of using Ply as a carrier protein instead of CRM_{197} is that it reduces the risk of carrier suppression that has been demonstrated with tetanus toxoid (TT) (Dagan et al. 1998). When infants were given multiple vaccinations using TT as the carrier protein, their antibody levels were significantly lower than when formulations with different carrier proteins were administered. As CRM_{197} is also in the licensed and highly successful Hib vaccine, there are concerns that carrier suppression may occur with CRM_{197} and result in reducing the efficacy of this vaccine and Prevnar. If a pneumococcal protein such as Ply, which we have demonstrated to be as effective as CRM_{197}, is used as the carrier protein in the next generation of pneumococcal vaccines then this phenomenon could be avoided.

In addition to functioning as a carrier protein, immunisation with free Ply, ΔA146 Ply or Δ6 Ply was shown to increase survival times following challenge with two pneumococcal serotypes (type 1 and 4). Such protection may inhibit pneumococcal growth to allow the development of anti-capsule antibodies to clear the infection. An increase in protection against a non-vaccine serotype by mixing free Δ6 Ply with Prevnar revealed that the simple

inclusion of Δ6 Ply with the existing vaccine conferred additional protection. If free Ply was to be included in the next generation of pneumococcal vaccines then a toxoided version such as ΔΔ146 Ply or Δ6 Ply provides a safe alternative to the WT toxin.

Appendices

Appendix I

Buffers and Recipes

Appendix I (buffers and recipes)

Unless otherwise stated, all reagents are from Sigma-Aldrich, Dorset, UK

A. Media

Terrific broth (TB)
12 g bacto-tryptone
24 g bacto-yeast extract
4 mL glycerol
in 900 ml dH_2O
Autoclave

In a separate flask dissolve in 90 mL H_2O:
2.31 g KH_2PO_4 monobasic
12.54 g K_2HPO_4 dibasic (for trihydrate 16.45 g)
Adjust volume to 100 mL with H_2O

Hysoy J
10g Hysoy J (now discontinued in the UK)
5g NaCl
5g yeast extract
5.7g Na_2HPO4
3.74g $NaH_2PO4.2H_2O$
in 1L dH_2O
autoclave
then add 20ml of filter sterilised 50% glucose

B. BioCAD buffers

1×PB-S(dialysis buffer)
KCl - 0.2g,
KH2PO4 - 0.24g,
Na2HPO4 - 1.44g

3M NaCl
175.32g NaCl
in 1L millipore dH_2O

20% Ethanol
200ml Ethanol A. R.
800ml millipore dH_2O

C. SDS-PAGE gel recipes

Stacking gel (two gels)

dH_2O	3.21ml
0.5M Tris pH 6.8	1.25ml
10% SDS	0.05ml
30% polyacrylamide	0.488ml
10% ammonium persulphate	0.025ml
TEMED	0.005ml

10% Resolving gel (two gels)

dH_2O	4.05ml
1.5M Tris pH8.8	2.5ml
10% SDS	0.1ml
30% polyacrylamide	3.3ml
10% ammonium persulphate	0.05ml
TEMED	0.005ml

(15% resolving gel: 2.35ml dH_2O and 5ml polyacrylamide)

10× Running buffer

SDS	10g
Glycine	144g
Tris base	30g

In 1L dH_2O

D. Western blot recipes

Transfer Buffer (4°C)

Tris Base	3.03g
Glycine	14.4g
Methanol	200ml

in 1L dH_2O

Tris NaCl pH7.4

Tris base	1.2g
NaCl	8.7g

in 1L dH_2O
conc. HCl 800µl

Developer
(prepare just before use)
Dissolve 30mg 4-chloro-1-napthol in 10ml methanol
Add 30µl H_2O_2 (30% w/v) to 40ml Tris NaCl pH7.4
Mix
Stop developing with water

E. LPS Silver Staining solutions

Fixing solution
Ethanol 400ml
Acetic acid 50ml
dH_2O 550ml

Periodic acid (0.7%)
1.4g periodic acid in 200ml fixing solution

Silver staining solution*
0.1M Sodium hydroxide 28ml
Ammonium hydroxide 2ml
while stirring solution,
add 5ml of 20% silver nitrate
(1g silver nitrate in 5ml dH_2O)

Silver stain developer
dH_2O 200ml
Citric Acid (10mg/ml)1ml
Formaldehyde (37%) 0.1ml
dH_2O 115ml

(*note a brown precipitate will form but disappears in seconds; ensure reagent does not dry out and is neutralised with HCl acid as it may become explosive when dry)

F. Mast cell degranulation solutions

Mast cell lysate
0.5% Triton X-100 (BDH)
in EMEM media (ATCC)

Substrate solution
1.3mg pNAG/ml citrate buffer, filter before use.
(pNAG = p-nitrophenyl-N-acetyl-β-D-glucosamine)

Citrate buffer
0.1M Sodium Citrate pH 4.5 made by mixing the following solutions:

Solution A
300ml 1M NaOH
31.5g Citric Acid (Fisher Scientific)
700ml distilled water
Solution B
0.1M HCl

Add 67.8ml solution A to 32.3ml solution B

Stop solution (store at 4°C)
0.2M Glycine NaOH pH 10.7, made up by mixing the following solutions:
Solution A
7.5g/l Glycine (Fisher Scientific)
5.8g/l NaCl (BDH)
1L distilled water
Solution B
0.2M NaOH

Add 52.8ml solution A to 47.2ml solution B

G. ELISA buffers
n.b. pH is important. Check each time before use.
Storage: 4 weeks at 4°C

Anti-Ply and Ply ELISA
coating buffer (pH 9.6)
1.59g Na_2CO_3
2.93g $NaHCO_3$
in 1L dH_2O

IFN-γ ELISA
coating buffer (pH9): 0.1M Na_2HPO_4 adjust to pH9 using 0.1M NaH_2PO_4
Coating Ab: 2μg/ml
Detection Ab: 0.5μg/ml
Standard: Top standard 2000pg/ml (two fold dilutions)

IL-6 ELISA
coating buffer (pH9): 0.1M Na_2HPO_4 adjust to pH9 using 0.1M NaH_2PO_4
Coating Ab: 2μg/ml
Detection Ab: 1μg/ml
Standard: Top standard 1000pg/ml (two-fold dilutions)

TNF-α ELISA
coating buffer (pH 9): same as above
Coating Ab: 4μg/ml
Detection Ab: 1μg/ml
Standard: Top standard 10,000pg/ml (two-fold dilutions)

Appendix II

Sequence Alignments

Appendix II

DNA and amino acid alignments of mutants made by site-directed mutagenesis

Deletion

Substitution
(A to G at base 791 is a silent mutation in *ply* to remove an internal Hind III site to allow subsequent cloning steps, our unpublished data)

Alignment of Δ6 Ply with D39 Ply

```
          201                                                       250
delta_6                                                ATGGCAAA TAAAGCAGTA
D39_PLY                                                ATGGCAAA TAAAGCAGTA

          251                                                       300
delta_6   AATGACTTTA TACTAGCTAT GAATTACCAT AAAAAGAAAC TCTTGACCCA
D39_PLY   AATGACTTTA TACTAGCTAT GAATTACGAT AAAAAGAAAC TCTTGACCCA

          301                                                       350
delta_6   TCAGGGAGAA AGTATTGAAA ATCGTTTCAT CAAAGAGGGT AATCAGCTAC
D39_PLY   TCAGGGAGAA AGTATTGAAA ATCGTTTCAT CAAAGAGGGT AATCAGCTAC

          351                                                       400
delta_6   CCGATGAGTT TGTTGTTATC GAAAGAAAGA AGCGGACCTT GTCGACAAAT
D39_PLY   CCGATGAGTT TGTTGTTATC GAAAGAAAGA AGCGGAGCTT GTCGACAAAT

          401                                                       450
delta_6   ACAAGTGATA TTTCTGTAAC AGCTACCAAC GACAGTCGCC TCTATCCTGG
D39_PLY   ACAAGTGATA TTTCTGTAAC AGCTACCAAC GACAGTCGCC TCTATCCTGG

          451                                                       500
delta_6   AGCACTTCTC GTAGTGGATG AGACCTTGTT AGAGAATAAT CCCACTCTTC
D39_PLY   AGCACTTCTC GTAGTGGATG AGACCTTGTT AGAGAATAAT CCCACTCTTC

          501                                                       550
delta_6   TTGCGGTTGA TCGTGCTCCG ATGACTTATA GTATTGATTT GCCTGGTTTG
D39_PLY   TTGCGGTTGA TCGTGCTCCG ATGACTTATA GTATTGATTT GCCTGGTTTG

          551                                                       600
delta_6   GCAAGTAGCG ATAGCTTTCT CCAAGTGGAA GACCCCAGCA ATTCAAGTGT
D39_PLY   GCAAGTAGCG ATAGCTTTCT CCAAGTGGAA GACCCCACCA ATTCAAGTGT

          601                                                       650
delta_6   TCGCGGAGCG GTAAACGATT TGTTGGCTAA GTGGCATCAA GATTATGGTC
D39_PLY   TCGCGGAGCG GTAAACGATT TGTTGGCTAA GTGGCATCAA GATTATGGTC

          651                                                       700
delta_6   AGGTCAATAA TGTCCCA[...]ATGCAGT ATGAAAAAAT AACGGCTCAC
D39_PLY   AGGTCAATAA TGTCCCAGCT AGAATGCAGT ATGAAAAAAT AACGGCTCAC
```

```
           701                                                 750
delta_6    AGCATGGAAC AACTCAAGGT CAAGTTTGGT TCTGACTTTG AAAAGACAGG
D39_PLY    AGCATGGAAC AACTCAAGGT CAAGTTTGGT TCTGACTTTG AAAAGACAGG

           751                                                 800
delta_6    GAATTCTCTT GATATTGATT TTAACTCTGT CCATTCAGGT GAAAAGCAGA
D39_PLY    GAATTCTCTT GATATTGATT TTAACTCTGT CCATTCAGGT GAAAAGCACA



           801                                                 850
delta_6    TTCACATTGT TAATTTTAAG CAGATTTATT ATACAGTCAG CGTAGACGCT
D39_PLY    TTCAGATTGT TAATTTTAAG CAGATTTATT ATACAGTCAG CGTAGACGCT

           851                                                 900
delta_6    GTTAAAAATC CAGGAGATGT GTTTCAAGAT ACTGTAACGG TAGAGGATTT
D39_PLY    GTTAAAAATC CAGGAGATGT GTTTCAAGAT ACTGTAACGG TAGAGGATTT

           901                                                 950
delta_6    AAAACAGAGA GGAATTTCTG CAGAGCGTCC TTTGGTCTAT ATTTCGAGTG
D39_PLY    AAAACAGAGA GGAATTTCTG CAGAGCGTCC TTTGGTCTAT ATTTCGAGTG

           951                                                1000
delta_6    TTGCTTATGG GCGCCAAGTC TATCTCAAGT TGGAAACCAC GAGTAAGAGT
D39_PLY    TTGCTTATGG GCGCCAAGTC TATCTCAAGT TGGAAACCAC GAGTAAGAGT

           1001                                               1050
delta_6    GATGAAGTAG AGGCTGCTTT TGAGGCTTTG ATAAAAGCAC TCAAGGTAGC
D39_PLY    GATGAAGTAG ACGCTGCTTT TGAAGCTTTG ATAAAAGGAG TCAAGGTAGC

           1051                                               1100
delta_6    TCCTCAGACA GAGTGGAAGC AGATTTTGGA CAATACAGAA GTGAAGGCGG
D39_PLY    TCCTCAGACA GAGTGGAAGC AGATTTTGGA CAATACAGAA GTGAAGGCGG

           1101                                               1150
delta_6    TTATTTTAGG GGGCGACCCA AGTTCGGGTG CCCGAGTTGT AACAGGCAAG
D39_PLY    TTATTTTAGG GGGCGACCCA ACTTCGGGTG CCCGAGTTGT AACAGGCAAG

           1151                                               1200
delta_6    GTGGATATGG TAGAGGACTT GATTCAAGAA GGCAGTCGCT TTACAGCACA
D39_PLY    GTCGATATGG TAGAGCACTT GATTCAAGAA GGCAGTCGCT TTACAGCAGA

           1201                                               1250
delta_6    TCATCCAGGC TTGCCGATTT CCTATACAAC TTCTTTTTTA CGTGACAATG
D39_PLY    TCATCCAGGC TTGCCGATTT CCTATACAAC TTCTTTTTTA CGTGACAATG

           1251                                               1300
delta_6    TAGTTGCGAC CTTTCAAAAC AGTACAGACT ATGTTGAGAC TAAGGTTACA
D39_PLY    TAGTTGCGAC CTTTCAAAAC AGTACAGACT ATGTTGAGAC TAACGTTACA

           1301                                               1350
delta_6    GCTTACAGAA ACGGAGATTT ACTGCTGGAT CATAGTGGTG CCTATGTTGC
D39_PLY    GCTTACAGAA ACGGAGATTT ACTGCTGGAT CATAGTGGTG CCTATGTTGC

           1351                                               1400
delta_6    CCAATATTAT ATTACTTGGG ATGAATTATC CTATGATCAT CAAGGTAAGG
D39_PLY    CCAATATTAT ATTACTTGGG ATCAATTATC CTATGATCAT CAAGGTAAGG

           1401                                               1450
delta_6    AAGTCTTGAC TCCTAAGGCT TGGGACAGAA ATGGGCACCA TTTGACGGCT
D39_PLY    AAGTCTTCAC TCCTAAGGCT TGGGACAGAA ATGGGCAGGA TTTGACGGCT

           1451                                               1500
delta_6    CACTTTACCA CTAGTATTCC TTTAAAAGGG AATGTTCGTA ATCTCTCTGT
D39_PLY    CACTTTACCA CTAGTATTCC TTTAAAAGGG AATGTTCGTA ATCTCTCTGT
```

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          1501                                                 1550
delta_6   CAAAATTAGA GAGTGTACCC GGCTTGCCTG GGAATGGTGG CGTACGGTTT
D39_PLY   CAAAATTAGA GAGTGTACCG GGCTTGCCTG GGAATGGTGG CGTACGGTTT

          1551                                                 1600
delta_6   ATGAAAAAAC CGATTTGCCA CTAGTGCGTA AGCGGACGAT TTCTATTTGG
D39_PLY   ATGAAAAAAC CGATTTGCCA CTAGTGCGTA AGCGGACGAT TTCTATTTGG

          1601                                                 1650
delta_6   GGAACAACTC TCCATCCTCA GGTAGAGGAT AAGCTAGAAA ATGACTAGGA
D39_PLY   GGAACAACTC TCCATCCTCA GGTAGAGGAT AAGGTAGAAA ATGACTAGGA

          1651
delta_6   GACGAGAATG CTTGCGACAA AAAGA
D39_PLY   GAGGAGAATG CTTGCGACAA AAAGA
```

Alignment of ΔA146 Ply with D39 Ply

```
                201                                                 250
delta_A146                                                          ATG
D39_PLY_DNA                                                         ATG

                251                                                 300
delta_A146      GCAAATAAAG CAGTAAATGA CTTTATACTA GCTATGAATT ACGATAAAAA
D39_PLY_DNA     GCAAATAAAG CAGTAAATGA CTTTATACTA GCTATGAATT ACGATAAAAA

                301                                                 350
delta_A146      GAAACTCTTG ACCCATCAGG GAGAAAGTAT TGAAAATCGT TTCATCAAAG
D39_PLY_DNA     GAAACTCTTG ACCCATCAGG GAGAAAGTAT TGAAAATCGT TTCATCAAAG

                351                                                 400
delta_A146      AGGGTAATCA GCTACCCGAT GAGTTTGTTG TTATCGAAAG AAAGAAGCGG
D39_PLY_DNA     AGGGTAATCA GCTACCCGAT GAGTTTGTTG TTATCGAAAG AAAGAAGCGG

                401                                                 450
delta_A146      AGCTTGTCGA CAAATACAAG TGATATTTCT GTAACAGCTA CCAACGACAG
D39_PLY_DNA     AGCTTGTCGA CAAATACAAG TGATATTTCT GTAACAGCTA CCAACGACAG

                451                                                 500
delta_A146      TCGCCTCTAT CCTGGAGCAC TTCTCGTAGT GGATGAGACC TTGTTAGAGA
D39_PLY_DNA     TCGCCTCTAT CCTGGAGCAC TTCTCGTAGT GGATGAGACC TTGTTAGAGA

                501                                                 550
delta_A146      ATAATCCCAC TCTTCTTGCG GTTGATCGTG CTCCGATGAC TTATAGTATT
D39_PLY_DNA     ATAATCCCAC TCTTCTTGCG GTTGATCGTG CTCCGATGAC TTATAGTATT

                551                                                 600
delta_A146      GATTTGCCTG GTTTGGCAAG TAGCGATAGC TTTCTCCAAG TGGAAGACCC
D39_PLY_DNA     GATTTGCCTG GTTTGGCAAG TAGCGATAGC TTTCTCCAAG TGGAAGACCC

                601                                                 650
delta_A146      CAGCAATTCA AGTGTTCGCG GAGCGGTAAA CGATTTGTTG GCTAAGTGGC
D39_PLY_DNA     CAGCAATTCA AGTGTTCGCG GAGCGGTAAA CGATTTGTTG GCTAAGTGGC

                651                                                 700
delta_A146      ATCAAGATTA TGGTCAGGTC AATAATGTCC CA[  ]AGAAT GCAGTATGAA
D39_PLY_DNA     ATCAAGATTA TGGTCAGGTC AATAATGTCC CAGCTAGAAT GCAGTATGAA

                701                                                 750
delta_A146      AAAATAACGG CTCACAGCAT GGAACAACTC AAGGTCAAGT TTGGTTCTGA
D39_PLY_DNA     AAAATAACGG CTCACAGCAT GGAACAACTC AAGGTCAAGT TTGGTTCTGA

                751                                                 800
delta_A146      CTTTCAAAAG ACAGGGAATT CTCTTGATAT TGATTTTAAC TCTGTCCATT
D39_PLY_DNA     CTTTCAAAAG ACAGGGAATT CTCTTGATAT TGATTTTAAC TCTGTCCATT

                801                                                 850
delta_A146      CAGGTGAAAA GCAGATTCAG ATTGTTAATT TTAAGCAGAT TTATTATACA
D39_PLY_DNA     CAGGTGAAAA GCAGATTCAG ATTGTTAATT TTAAGCAGAT TTATTATACA

                851                                                 900
delta_A146      GTCAGCGTAG ACGCTGTTAA AAATCCAGGA GATGTGTTTC AAGATACTGT
D39_PLY_DNA     GTCAGCGTAG ACGCTGTTAA AAATCCAGGA GATGTGTTTC AAGATACTGT

                901                                                 950
delta_A146      AACGGTAGAG GATTTAAAAC AGAGAGGAAT TTCTGCAGAG CGTCCTTTGG
D39_PLY_DNA     AACGGTAGAG GATTTAAAAC AGAGAGGAAT TTCTGCAGAG CGTCCTTTGG
```

```
                951                                                1000
delta_A146      TCTATATTTC GAGTGCTTGCT TATCGGCGCC AAGTCTATCT CAAGTTGCAA
D39_PLY_DNA     TCTATATTTC GAGTCTTGCT TATGGGCGCC AAGTCTATCT CAAGTTGGAA

               1001                                               1050
delta_A146      ACCACGAGTA AGAGTGATGA AGTAGAGGCT GCTTTTGAGG CTTTGATAAA
D39_PLY_DNA     ACCACGAGTA AGAGTGATGA AGTAGAGGCT GCTTTTGAAG CTTTGATAAA

               1051                                               1100
delta_A146      AGGAGTCAAG GTAGCTCCTC AGACAGAGTG GAAGCAGATT TTGGACAATA
D39_PLY_DNA     AGGAGTCAAG GTAGCTCCTC AGACAGAGTG GAAGCAGATT TTGGACAATA

               1101                                               1150
delta_A146      CAGAAGTGAA GGCGCTTATT TTAGGGGGCG ACCCAAGTTC GGGTGCCCGA
D39_PLY_DNA     CAGAAGTGAA GGCGGTTATT TTAGGGGGCG ACCCAAGTTC GGGTGCCCGA

               1151                                               1200
delta_A146      GTTGTAACAG GCAAGGTGGA TATGGTAGAG GACTTGATTC AAGAAGGCAG
D39_PLY_DNA     GTTGTAACAG GCAAGGTGGA TATGGTAGAG GACTTGATTC AAGAAGCCAC

               1201                                               1250
delta_A146      TCGCTTTACA GCAGATCATC CAGGCTTGCC GATTTCCTAT ACAACTTCTT
D39_PLY_DNA     TCGCTTTACA GCAGATCATC CAGGCTTGCC GATTTCCTAT ACAACTTCTT

               1251                                               1300
delta_A146      TTTTACGTGA CAATGTAGTT GCGACCTTCC AAAACAGTAC AGACTATGTT
D39_PLY_DNA     TTTTACGTGA CAATGTAGTT GCGACCTTCC AAAACAGTAC AGACTATGTT

               1301                                               1350
delta_A146      GAGACTAAGG TTACAGCTTA CAGAAACGGA GATTTACTGC TGGATCATAG
D39_PLY_DNA     GAGACTAAGG TTACAGCTTA CAGAAACGGA GATTTACTGC TGGATCATAG

               1351                                               1400
delta_A146      TGGTGCCTAT CTTGCCCAAT ATTATATTAC TTGGGATGAA TTATCCTATG
D39_PLY_DNA     TGGTGCCTAT GTTGCCCAAT ATTATATTAC TTGGGATGAA TTATCCTATG

               1401                                               1450
delta_A146      ATCATCAAGG TAAGGAAGTC TTGACTCCTA AGGCTTGGGA CAGAAATGGG
D39_PLY_DNA     ATCATCAAGG TAAGGAAGTC TTGACTCCTA AGGCTTGGGA CAGAAATGGG

               1451                                               1500
delta_A146      CAGGATTTGA CGGCTCACTT TACCACTAGT ATTCCTTTAA AAGGGAATGT
D39_PLY_DNA     CAGGATTTGA CGGCTCACTT TACCACTAGT ATTCCTTTAA AAGGGAATGT

               1501                                               1550
delta_A146      TCGTAATCTC TCTGTCAAAA TTAGAGAGTG TACCGGGCTT GCCTGGGAAT
D39_PLY_DNA     TCGTAATCTC TCTGTCAAAA TTAGAGAGTG TACCGGGCTT GCCTGGGAAT

               1551                                               1600
delta_A146      GGTGGCGTAC GGTTTATGAA AAAACCCATT TGCCACTAGT GCGTAAGCGG
D39_PLY_DNA     GGTGGCGTAC GGTTTATGAA AAAACCGATT TGCCACTAGT GCGTAAGCGG

               1601                                               1650
delta_A146      ACGATTTCTA TTTGGGGAAC AACTCTCTAT CCTCAGGTAG AGGATAAGGT
D39_PLY_DNA     ACGATTTCTA TTTGGGGAAC AACTCTCTAT CCTCAGGTAG AGGATAAGGT

               1651
delta_A146      AGAAAATGAC TAGGAGAGGA GAATGCTTGC GACAAAAAGA
D39_PLY_DNA     AGAAAATGAC TAGGAGAGGA GAATGCTTGC GACAAAAAGA
```

Amino acid sequence alignment of sequenced Δ6 W433F Ply with D39 Ply

```
           1                                                   50
 D39_Ply   MANKAVNDFI LAMNYDKKKL LTHQGESIEN RFIKEGNQLP DEFVVIERKK
d6_W433F   MANKAVNDFI LAMNYDKKKL LTHQGESIEN RFIKEGNQLP DEFVVIERKK

           51                                                  100
 D39_Ply   RSLSTNTSDI SVTATNDSRL YPGALLVVDE TLLENNPTLL AVDRAPMTYS
d6_W433F   RSLSTNTSDI SVTATNDSRL YPGALLVVDE TLLENNPTLL AVDRAPMTYS

           101                                                 150
 D39_Ply   IDLPGLASSD SFLQVEDPSN SSVRGAVNDL LAKWHQDYGQ VNNVPARMQY
d6_W433F   IDLPGLASSD SFLQVEDPSN SSVRGAVNDL LAKWHQDYGQ VNNVP[ ]MQY

           151                                                 200
 D39_Ply   EKITAHSMEQ LKVKFGSDFE KTGNSLDIDF NSVHSGEKQI QIVNFKQIYY
d6_W433F   EKITAHSMEQ LKVKFGSDFE KTGNSLDIDF NSVHSGEKQI QIVNFKQIYY

           201                                                 250
 D39_Ply   TVSVDAVKNP GDVFQDTVTV EDLKQRGISA ERPLVYISSV AYGRQVYLKL
d6_W433F   TVSVDAVKNP GDVFQDTVTV EDLKQRGISA ERPLVYISSV AYGRQVYLKL

           251                                                 300
 D39_Ply   ETTSKSDEVE AAFEALIKGV KVAPQTEWKQ ILDNTEVKAV ILGGDPSSGA
d6_W433F   ETTSKSDEVE AAFEALIKGV KVAPQTEWKQ ILDNTEVKAV ILGGDPSSGA

           301                                                 350
 D39_Ply   RVVTGKVDMV EDLIQEGSRF TADHPGLPIS YTTSFLRDNV VATFQNSTDY
d6_W433F   RVVTGKVDMV EDLIQEGSRF TADHPGLPIS YTTSFLRDNV VATFQNSTDY

           351                                                 400
 D39_Ply   VETKVTAYRN GDLLLDHSGA YVAQYYITWD ELSYDHQGKE VLTPKAWDRN
d6_W433F   VETKVTAYRN GDLLLDHSGA YVAQYYITWD ELSYDHQGKE VLTPKAWDRN

           401                                                 450
 D39_Ply   GQDLTAHFTT SIPLKGNVRN LSVKIRECTG LAWEWWRTVY EKTDLPLVRK
d6_W433F   GQDLTAHFTT SIPLKGNVRN LSVKIRECTG LAFEWWRTVY EKTDLPLVRK

           451                 471
 D39_Ply   RTISIWGTTL YPQVEDKVEN D
d6_W433F   RTISIWGTTL YPQVEDKVEN D
```

Amino acid sequence alignment of sequenced Δ6D385NW433F Ply with D39 Ply

```
                1                                                   50
d6_D385N_W433F  MANKAVNDFI LAMNYDKKKL YTHQGESIEN RFIKEGNQLP DEFVVIERKK
       D39_Ply  MANKAVNDFI LAMNYDKKKL YTHQGESIEN RFIKEGNQLP DEFVVIERKK

               51                                                  100
d6_D385N_W433F  RSLSTNTSDI SVTAINDSRL YPGALLVVDE TLLENNPTLL AVDRAPMTYS
       D39_Ply  RSLSTNTSDI SVTATNDSRL YPGALLVVDE TLLENNPTLL AVDRAPMTYS

              101                                                  150
d6_D385N_W433F  IDLPGLASSD SFLQVEDPSN SSVRGAVNDL LAKWHQDYGQ VNNVPARMQY
       D39_Ply  IDLPGLASSD SFLQVEDPSN SSVRGAVNDL LAKWHQDYGQ VNNVPARMQY

              151                                                  200
d6_D385N_W433F  EKITAHSMEQ LKVKFGSDFE KTGNSLDIDF NSVHSGEKQI QIVNFKQIYY
       D39_Ply  EKITAHSMEQ LKVKFGSDFE KTGNSLDIDF NSVHSGEKQI QIVNFKQIYY

              201                                                  250
d6_D385N_W433F  TVSVDAVKNP GDVFQDTVTV EDLKQRGISA ERPLVYISSV AYGRQVYLKL
       D39_Ply  TVSVDAVKNP GDVFQDTVTV EDLKQRGISA ERPLVYISSV AYGRQVYLKL

              251                                                  300
d6_D385N_W433F  ETTSKSDEVE AAFEALIKGV KVAPQTEWKQ ILDNTEVKAV ILGGDPSSGA
       D39_Ply  ETTSKSDEVE AAFEALIKGV KVAPQTEWKQ ILDNTEVKAV ILGGDPSSGA

              301                                                  350
d6_D385N_W433F  RVVTGKVDMV EDLIQEGSRF TADHPGLPIS YTTSFLRDNV VATFQNSTDY
       D39_Ply  RVVTGKVDMV EDLIQEGSRF TADHPGLPIS YTTSFLRDNV VATFQNSTDY

              351                                                  400
d6_D385N_W433F  VETKVTAYRN GDLLLDHSGA YVAQYYITWD ELSYNHQGKE VLTPKAWDRN
       D39_Ply  VETKVTAYRN GDLLLDHSGA YVAQYYITWD ELSYDHQGKE VLTPKAWDRN

              401                                                  450
d6_D385N_W433F  GQDLTAHFTT SIPLKGNVRN LSVKIRECTG LAFEWWRTVY EKTDLPLVRK
       D39_Ply  GQDLTAHFTT SIPLKGNVRN LSVKIRECTG LAFEWWRTVY EKTDLPLVRK

              451                 471
d6_D385N_W433F  RTISIWGTTL YPQVEDKVEN D
       D39_Ply  RTISIWGTTL YPQVEDKVEN D
```

Alignment of DNA sequence of Janus Δ6 D39 *ply S. pneumoniae* with D39 *ply*

```
                201                                                 250
d6D39_janus     .......ATG GCAAATAAAG CAGTAAATCA CTTTATACTA GCTATGAATT
     WT_PLY     .......ATG GCAAATAAAG CAGTAAATGA CTTTATACTA GCTATGAATT

                251                                                 300
d6D39_janus     ACGATAAAAA GAAACTCTTG ACCCATCAGG GAGAAAGTAT TGAAAATCGT
     WT_PLY     ACGATAAAAA GAAACTCTTG ACCCATCAGG GAGAAAGTAT TGAAAATCGT

                301                                                 350
d6D39_janus     TTCATCAAAG AGGGTAATCA GCTACCCGAT GAGTTTGTTG TTATCGAAAG
     WT_PLY     TTCATCAAAG AGGGTAATCA GCTACCCGAT GAGTTTGTTG TTATCGAAAG

                351                                                 400
d6D39_janus     AAAGAAGCGG AGCTTGTCGA CAAATACAAG TGATATTTCT GTAACAGCTA
     WT_PLY     AAAGAAGCGG AGCTTGTCGA CAAATACAAG TGATATTTCT GTAACAGCTA

                401                                                 450
d6D39_janus     CCAACGACAG TCGCCTCTAT CCTGGAGCAC TTCTCGTAGT GGATGAGACC
     WT_PLY     CCAACGACAG TCGCCTCTAT CCTGGAGCAC TTCTCGTAGT GGATGAGACC

                451                                                 500
d6D39_janus     TTGTTAGAGA ATAATCCCAC TCTTCTTGCG GTTGATCGTG CTCCGATGAC
     WT_PLY     TTGTTAGAGA ATAATCCCAC TCTTCTTGCG GTTGATCGTG CTCCGATGAC

                501                                                 550
d6D39_janus     TTATAGTATT GATTTGCCTG GTTTGGCAAG TACCGATAGC TTTCTCCAAG
     WT_PLY     TTATAGTATT GATTTGCCTG GTTTGGCAAG TAGCGATAGC TTTCTCCAAG

                551                                                 600
d6D39_janus     TGGAAGACCC CAGCAATTCA AGTGTTCGCG GAGCGGTAAA CGATTTGTTG
     WT_PLY     TGGAAGACCC CAGCAATTCA AGTGTTCGCG GAGCGGTAAA CGATTTGTTG

                601                                                 650
d6D39_janus     GCTAAGTGGC ATCAAGATTA TGGTCAGGTC AATAATGTCC CA[illegible]AT
     WT_PLY     GCTAAGTGGC ATCAAGATTA TGGTCAGGTC AATAATGTCC CAGCTAGAAT

                651                                                 700
d6D39_janus     GCAGTATGAA AAAATAACGG CTCACAGCAT GGAACAACTC AAGGTCAAGT
     WT_PLY     GCAGTATGAA AAAATAACGG CTCACAGCAT GGAACAACTC AAGGTCAAGT

                701                                                 750
d6D39_janus     TTGGTTCTGA CTTTGAAAAG ACAGGGAATT CTCTTGATAT TGATTTTAAC
     WT_PLY     TTGGTTCTGA CTTTGAAAAG ACAGGGAATT CTCTTGATAT TGATTTTAAC

                751                                                 800
d6D39_janus     TCTGTCCATT CAGGTGAAAA GCAGATTCAG ATTGTTAATT TTAAGCAGAT
     WT_PLY     TCTGTCCATT CAGGTGAAAA GCAGATTCAG ATTGTTAATT TTAAGCAGAT

                801                                                 850
d6D39_janus     TTATTATACA GTCAGCGTAG ACGCTGTTAA AAATCCAGGA GATGTGTTTC
     WT_PLY     TTATTATACA GTCAGCGTAG ACGCTGTTAA AAATCCAGGA GATGTGTTTC

                851                                                 900
d6D39_janus     AAGATACTGT AACGGTAGAG GATTTAAAAC AGAGAGGAAT TTCTGCAGAG
     WT_PLY     AAGATACTGT AACGGTAGAG GATTTAAAAC AGAGAGGAAT TTCTGCAGAG

                901                                                 950
d6D39_janus     CGTCCTTTGG TCTATATTTC GAGTGTTGCT TATGGGCGCC AAGTCTATCT
     WT_PLY     CGTCCTTTGG TCTATATTTC GAGTGTTGCT TATGGGCGCC AAGTCTATCT

                951                                                1000
d6D39_janus     CAAGTTGGAA ACCACGAGTA AGAGTGATGA AGTAGAGGCT GCTTTTGAAG
```

```
     WT_PLY  CAAGTTGGAA ACCACGAGTA AGAGTGATGA AGTAGAGGCT GCTTTTGAAG

                1001                                          1050
d6D39_janus  CTTTGATAAA AGGAGTCAAG GTAGCTCCTC AGACAGAGTG GAAGCAGATT
     WT_PLY  CTTTGATAAA AGGAGTCAAG GTAGCTCCTC AGACAGAGTG GAAGCAGATT

                1051                                          1100
d6D39_janus  TTGGACAATA CAGAAGTGAA GGCGGTTATT TTAGGGGGCG ACCCAAGTTC
     WT_PLY  TTGGACAATA CAGAAGTGAA GGCGGTTATT TTAGGGGGCG ACCCAAGTTC

                1101                                          1150
d6D39_janus  CGCTCCCCCA GTTGTAACAC GCAACCTGGA TATGGTAGAG GACTTGATTC
     WT_PLY  GGGTGCCCGA GTTGTAACAG GCAAGGTGGA TATGGTAGAG GACTTGATTC

                1151                                          1200
d6D39_janus  AAGAAGGCAG TCGCTTTACA GCAGATCATC CAGGCTTGCC GATTTCCTAT
     WT_PLY  AAGAAGGCAG TCGCTTTACA GCAGATCATC CAGGCTTGCC GATTTCCTAT

                1201                                          1250
d6D39_janus  ACAACTTCTT TTTTACGTGA CAATGTAGTT GCGACCTTTC AAAACAGTAC
     WT_PLY  ACAACTTCTT TTTTACGTGA CAATGTAGTT GCGACCTTTC AAAACAGTAC

                1251                                          1300
d6D39_janus  AGACTATGTT GAGACTAAGG TTACAGCTTA CAGAAACGGA GATTTACTGC
     WT_PLY  AGACTATGTT GAGACTAAGG TTACAGCTTA CAGAAACGGA GATTTACTGC

                1301                                          1350
d6D39_janus  TGGATCATAG TGGTGCCTAT GTTGCCCAAT ATTATATTAC TTGGGATGAA
     WT_PLY  TGGATCATAG TGGTGCCTAT GTTGCCCAAT ATTATATTAC TTGGGATGAA

                1351
d6D39_janus  TTATCCTATG
     WT_PLY  TTATCCTATG
```

Alignment of DNA sequence of Janus WT *ply S. pneumoniae* with D39 *ply*

```
                201                                                 250
janus_WT_Ply    .......ATG GCAAATAAAG CAGTAAATGA CTTTATACTA GCTATGAATT
D39_ply_DNA     .......ATG GCAAATAAAG CAGTAAATGA CTTTATACTA GCTATGAATT

                251                                                 300
janus_WT_Ply    ACGATAAAAA GAAACTCTTG ACCCATCAGG GAGAAAGTAT TGAAAATCGT
D39_ply_DNA     ACGATAAAAA GAAACTCTTG ACCCATCAGG GAGAAAGTAT TGAAAATCGT

                301                                                 350
janus_WT_Ply    TTCATCAAAG AGGGTAATCA GCTACCCGAT GAGTTTGTTG TTATCGAAAG
D39_ply_DNA     TTCATCAAAG AGGGTAATCA GCTACCCGAT GAGTTTGTTG TTATCGAAAG

                351                                                 400
janus_WT_Ply    AAAGAAGCGG AGCTTGTCGA CAAATACAAG TGATATTTCT GTAACAGCTA
D39_ply_DNA     AAAGAAGCGG AGCTTGTCGA CAAATACAAG TGATATTTCT GTAACAGCTA

                401                                                 450
janus_WT_Ply    CCAACGACAG TCGCCTCTAT CCTGGAGCAC TTCTCGTAGT GGATGAGACC
D39_ply_DNA     CCAACGACAG TCGCCTCTAT CCTGGAGCAC TTCTCGTAGT GGATGAGACC

                451                                                 500
janus_WT_Ply    TTGTTAGAGA ATAATCCCAC TCTTCTTGCG GTTGATCGTG CTCCGATGAC
D39_ply_DNA     TTGTTAGAGA ATAATCCCAC TCTTCTTGCG GTTGATCGTG CTCCGATGAC

                501                                                 550
janus_WT_Ply    TTATACTATT GATTTCCCTG CTTTGGCAAG TAGCGATAGC TTTCTCCAAG
D39_ply_DNA     TTATAGTATT GATTTGCCTG CTTTGGCAAG TAGCGATAGC TTTCTCCAAG

                551                                                 600
janus_WT_Ply    TGGAAGACCC CAGCAATTCA AGTGTTCGCG GAGCGGTAAA CGATTTGTTG
D39_ply_DNA     TGGAAGACCC CAGCAATTCA AGTGTTCGCG GAGCGGTAAA CGATTTGTTG

                601                                                 650
janus_WT_Ply    GCTAAGTGGC ATCAAGATTA TGGTCAGGTC AATAATGTCC CAGCTAGAAT
D39_ply_DNA     GCTAAGTGGC ATCAAGATTA TGGTCAGGTC AATAATGTCC CAGCTAGAAT

                651                                                 700
janus_WT_Ply    GCAGTATGAA AAAATAACGG CTCACAGCAT GGAACAACTC AAGGTCAAGT
D39_ply_DNA     GCAGTATGAA AAAATAACGG CTCACAGCAT GGAACAACTC AAGGTCAAGT

                701                                                 750
janus_WT_Ply    TTGGTTCTGA CTTTGAAAAG ACAGGGAATT CTCTTGATAT TGATTTTAAC
D39_ply_DNA     TTGGTTCTGA CTTTGAAAAG ACAGGGAATT CTCTTGATAT TGATTTTAAC

                751                                                 800
janus_WT_Ply    TCTGTCCATT CAGGTGAAAA GCAGATTCAG ATTGTTAATT TTAAGCAGAT
D39_ply_DNA     TCTGTCCATT CAGGTGAAAA GCAGATTCAG ATTGTTAATT TTAAGCAGAT

                801                                                 850
janus_WT_Ply    TTATTATACA GTCAGCGTAG ACGCTGTTAA AAATCCAGGA GATGTGTTTC
D39_ply_DNA     TTATTATACA GTCAGCGTAG ACGCTGTTAA AAATCCAGGA GATGTGTTTC

                851                                                 900
janus_WT_Ply    AAGATACTGT AACGGTACAG GATTTAAAAC ACAGACGAAT TTCTCCAGAG
D39_ply_DNA     AAGATACTGT AACGGTAGAG GATTTAAAAC AGAGAGGAAT TTCTGCAGAG

                901                                                 950
janus_WT_Ply    CCTCCTTTCG TCTATATTTC GAGTCTTCCT TATGGGCGCC AAGTCTATCT
D39_ply_DNA     CGTCCTTTGG TCTATATTTC GAGTGTTGCT TATGGGCGCC AAGTCTATCT

                951                                                1000
janus_WT_Ply    CAAGTTCGAA ACCACGAGTA AGACTGATCA AGTACAGGCT GCTTTTGAAC
D39_ply_DNA     CAAGTTGGAA ACCACGAGTA AGAGTGATGA AGTACAGGCT GCTTTTGAAG
```

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                1001                                                1050
janus_WT_Ply    CTTTGATAAA AGGAGTCAAG GTAGCTCCTC AGACAGAGTG GAAGCAGATT
 D39_ply_DNA    CTTTGATAAA AGGAGTCAAG GTAGCTCCTC AGACAGAGTG GAAGCAGATT

                1051                                                1100
janus_WT_Ply    TTGGACAATA CAGAAGTGAA GGCGGTTATT TTAGGGGGCG ACCCAAGTTC
 D39_ply_DNA    TTGGACAATA CAGAAGTGAA GGCGGTTATT TTAGGGGGCG ACCCAAGTTC

                1101                                                1150
janus_WT_Ply    GGGTGCCCGA GTTGTAACAG GCAAGGTGGA TATGGTAGAG GACTTGATTC
 D39_ply_DNA    GGGTGCCCGA GTTGTAACAG GCAAGGTGGA TATGGTAGAG GACTTGATTC

                1151                                                1200
janus_WT_Ply    AAGAAGGCAG TCGCTTTACA GCAGATCATC CAGGCTTGCC GATTTCCTAT
 D39_ply_DNA    AAGAAGGCAG TCGCTTTACA GCAGATCATC CAGGCTTGCC GATTTCCTAT

                1201                                                1250
janus_WT_Ply    ACAACTTCTT TTTTACGTGA CAATGTAGTT GCGACCTTTC AAAACAGTAC
 D39_ply_DNA    ACAACTTCTT TTTTACGTGA CAATGTAGTT GCGACCTTTC AAAACAGTAC

                1251                                                1300
janus_WT_Ply    AGACTATGTT GAGACTAAGG TTACAGCTTA CAGAAACGGA GATTTACTGC
 D39_ply_DNA    AGACTATGTT GAGACTAAGG TTACAGCTTA CAGAAACGGA GATTTACTGC

                1301                                                1350
janus_WT_Ply    TGGATCATAG TGGTGCCTAT GTTGCCCAAT ATTATATTAC TTGGGATGAA
 D39_ply_DNA    TGGATCATAG TGGTGCCTAT GTTGCCCAAT ATTATATTAC TTGGGATGAA

                1351
janus_WT_Ply    TTATCCTATG
 D39_ply_DNA    TTATCCTATG
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Publications

L. A. S. Kirkham, A. R. Kerr, G. R. Douce, G. K. Paterson, D. A. Dilts, D. F. Liu and T. J. Mitchell (2006). "Construction and immunological characterisation of a novel non-toxic protective pneumolysin mutant for use in future pneumococcal vaccines." Infect Immun **74**(1): 586-93.

L. A. S. Kirkham, J. M. C. Jefferies, A. R. Kerr, Y. Jing, S. C. Clarke, A. Smith and T. J. Mitchell (2006). "Identification of invasive serotype 1 pneumococcal isolates that express nonhemolytic pneumolysin." J. Clin. Microbiol. **44**(1): 151-159.

A. R. Kerr, L. S Kirkham, A. Kadioglu, P. Andrew, P. Garside, H. Thompson and T.J. Mitchell (2005). Identification of a detrimental role for NK cells in pneumococcal pneumonia and sepsis in immunocompromised hosts. Microbes and Infection. 7(5-6): 845-852. 2005.

Conference contributions (presenting author is underlined)

L. S. Kirkham, G. R. Douce, D.A. Dilts, M. Koster and T. J. Mitchell. (2006, poster) Vaccination with non-toxic pneumolysin conjugated to capsule polysaccharide protects against invasive disease. International Symposium on Pneumococci and Pneumococcal Disease-5, Alice Springs, Australia.

D.A. Dilts, D.F. Lui, M. Douglas, L. Nūnez, L. S. Kirkham and T. J. Mitchell. (2006, poster) Solubility and carrier potential of three non-hemolytic pneumolysin proteins. International Symposium on Pneumococci and Pneumococcal Disease-5, Alice Springs, Australia.

L. S. Kirkham, A. R. Kerr, D. A. Dilts and T. J. Mitchell. (2005, poster) Development of a novel non-toxic pneumolysin mutant for use in future pneumococcal vaccines. Interscience Conference on Antimicrobial Agents and Chemotherapy, Washington D.C., USA.

L. S. Kirkham, J. M. C. Jefferies, A. R. Kerr, S. C. Clarke, A. Smith and T. J. Mitchell. (2005, poster) Identification of invasive serotype 1 isolates that express non-haemolytic pneumolysin. Europneumo, Braunschweig, Germany.

L. S. Kirkham, A. R. Kerr, G. J. M. Cowan and T. J. Mitchell. (2004, poster) Construction and characterisation of a non-toxic pneumolysin mutant. Pore-forming toxins meeting, Mainz, Germany.

L. S. Kirkham, A. R. Kerr, D.A. Dilts and T. J. Mitchell. (2004, poster) Creation and characterisation of a non-toxic pneumolysin mutant. International Symposium on Pneumococci and Pneumococcal Disease-4, Helsinki, Finland.

J. M.C. Jefferies, L. S. Kirkham, Y. Jing, A. Smith, S. C. Clarke and T. J. Mitchell. (2004, selected for oral presentation) Characterisation of a novel form of pneumolysin from a clinical pneumococcal isolate. Society of General Microbiology AGM, Bath, UK.

Patents

L. S. Kirkham and T.J. Mitchell. Mutant pneumolysin proteins. (May 2004, Patent number: GB0410220.8)

L. S. Kirkham, G. J. M. Cowan and T.J. Mitchell. Mutant Cholesterol-binding cytolysin proteins. (May 2004, Patent number: GB0410221.8)

References

Abraham, S. N. and Malaviya, R. (1997). "Mast cells in infection and immunity." Infect Immun **65**(9): 3501-3508.

Adamou, J. E., Heinrichs, J. H., Erwin, A. L., Walsh, W., Gayle, T., Dormitzer, M., Dagan, R., Brewah, Y. A., Barren, P., Lathigra, R., Langermann, S., Koenig, S. and Johnson, S. (2001). "Identification and characterization of a novel family of pneumococcal proteins that are protective against sepsis." Infect Immun **69**(2): 949-58.

Adamou, J. E., Wizemann, T. M., Barren, P. and Langermann, S. (1998). "Adherence of *Streptococcus pneumoniae* to human bronchial epithelial cells (BEAS-2B)." Infect Immun **66**(2): 820-2.

Albiger, B., Sandgren, A., Katsuragi, H., Meyer-Hoffert, U., Beiter, K., Wartha, F., Hornef, M., Normark, S. and Normark, B. H. (2005). "Myeloid differentiation factor 88-dependent signalling controls bacterial growth during colonization and systemic pneumococcal disease in mice." Cellular Immunology **7**(11): 1603-1615.

Alexander, J. E., Berry, A. M., Paton, J. C., Rubins, J. B., Andrew, P. W. and Mitchell, T. J. (1998). "Amino acid changes affecting the activity of pneumolysin alter the behaviour of pneumococci in pneumonia." Microb Pathog **24**(3): 167-74.

Alexander, J. E., Lock, R. A., Peeters, C. C., Poolman, J. T., Andrew, P. W., Mitchell, T. J., Hansman, D. and Paton, J. C. (1994). "Immunization of mice with pneumolysin toxoid confers a significant degree of protection against at least nine serotypes of *Streptococcus pneumoniae*." Infect Immun **62**(12): 5683-8.

Alouf, J. E. (2000). "Cholesterol-binding cytolytic protein toxins." Int J Med Microbiol **290**(4-5): 351-6.

Alper, C. A., Abramson, N., Johnston, R. B., Jr., Jandl, J. H. and Rosen, F. S. (1970). "Increased susceptibility to infection associated with abnormalities of complement-

mediated functions and of the third component of complement (C3)." N Engl J Med **282**(7): 350-4.

Alper, C. A., Xu, J., Cosmopoulos, K., Dolinski, B., Stein, R., Uko, G., Larsen, C. E., Dubey, D. P., Densen, P., Truedsson, L., Sturfelt, G. and Sjoholm, A. G. (2003). "Immunoglobulin deficiencies and susceptibility to infection among homozygotes and heterozygotes for C2 deficiency." J Clin Immunol **23**(4): 297-305.

Alpern, E. R., Alessandrini, E. A., McGowan, K. L., Bell, L. M. and Shaw, K. N. (2001). "Serotype prevalence of occult pneumococcal bacteremia." Pediatrics **108**(2): E23.

Amdahl, B. M., Rubins, J. B., Daley, C. L., Gilks, C. F., Hopewell, P. C. and Janoff, E. N. (1995). "Impaired natural immunity to pneumolysin during human immunodeficiency virus infection in the United States and Africa." Am J Respir Crit Care Med **152**(6 Pt 1): 2000-4.

Andrew, P. W., Mitchell, T. J. and Morgan, P. J. (1997). "Relationship of structure to function in pneumolysin." Microb Drug Resist **3**(1): 11-7.

Baba, H., Kawamura, I., Kohda, C., Nomura, T., Ito, Y., Kimoto, T., Watanabe, I., Ichiyama, S. and Mitsuyama, M. (2001). "Essential role of domain 4 of pneumolysin from *Streptococcus pneumoniae* in cytolytic activity as determined by truncated proteins." Biochemical and Biophysical Research Communications **281**(1): 37-44.

Baba, H., Kawamura, I., Kohda, C., Nomura, T., Ito, Y., Kimoto, T., Watanabe, I., Ichiyama, S. and Mitsuyama, M. (2002). "Induction of gamma interferon and nitric oxide by truncated pneumolysin that lacks pore-forming activity." Infect Immun **70**(1): 107-13.

Balachandran, P., Hollingshead, S. K., Paton, J. C. and Briles, D. E. (2001). "The autolytic enzyme LytA of *Streptococcus pneumoniae* is not responsible for releasing pneumolysin." J Bacteriol **183**(10): 3108-16.

Barbuti, G., Moschioni, M., Censini, S., Covacci, A., Montecucco, C. and Montemurro, P. (2006). "*Streptococcus pneumoniae* induces mast cell degranulation." Int J Med Microbiol.

Barocchi, M. A., Ries, J., Zogaj, X., Hemsley, C., Albiger, B., Kanth, A., Dahlberg, S., Fernebro, J., Moschioni, M., Masignani, V., Hultenby, K., Taddei, A. R., Beiter, K., Wartha, F., von Euler, A., Covacci, A., Holden, D. W., Normark, S., Rappuoli, R. and Henriques-Normark, B. (2006). "A pneumococcal pilus influences virulence and host inflammatory responses." Proc Natl Acad Sci U S A.

Batt, S. L., Charalambous, B. M., McHugh, T. D., Martin, S. and Gillespie, S. H. (2005). "Novel PCR-restriction fragment length polymorphism method for determining serotypes or serogroups of *Streptococcus pneumoniae* isolates." J. Clin. Microbiol. **43**(6): 2656-2661.

Benton, K. A., Everson, M. P. and Briles, D. E. (1995). "A pneumolysin-negative mutant of *Streptococcus pneumoniae* causes chronic bacteremia rather than acute sepsis in mice." Infect Immun **63**(2): 448-55.

Benton, K. A., Paton, J. C. and Briles, D. E. (1997). "The hemolytic and complement-activating properties of pneumolysin do not contribute individually to virulence in a pneumococcal bacteremia model." Microb Pathog **23**(4): 201-9.

Benton, K. A., VanCott, J. L. and Briles, D. E. (1998). "Role of tumor necrosis factor alpha in the host response of mice to bacteremia caused by pneumolysin-deficient *Streptococcus pneumoniae*." Infect Immun **66**(2): 839-42.

Bergeron, Y., Ouellet, N., Deslauriers, A. M., Simard, M., Olivier, M. and Bergeron, M. G. (1998). "Cytokine kinetics and other host factors in response to pneumococcal pulmonary infection in mice." Infect Immun **66**(3): 912-22.

Bergmann, S. and Hammerschmidt, S. (2006). "Versatility of pneumococcal surface proteins." Microbiology **152**(Pt 2): 295-303.

Bernatoniene, J. and Finn, A. (2005). "Advances in pneumococcal vaccines." Drugs **65**(2): 1-27.

Berry, A., Alexander, J., Mitchell, T., Andrew, P., Hansman, D. and Paton, J. (1995). "Effect of defined point mutations in the pneumolysin gene on the virulence of *Streptococcus pneumoniae*." Infect. Immun. **63**(5): 1969-1974.

Berry, A. M., Lock, R. A., Hansman, D. and Paton, J. C. (1989a). "Contribution of autolysin to virulence of *Streptococcus pneumoniae*." Infect Immun **57**(8): 2324-30.

Berry, A. M. and Paton, J. C. (1996). "Sequence heterogeneity of PsaA, a 37-kilodalton putative adhesin essential for virulence of *Streptococcus pneumoniae*." Infect Immun **64**(12): 5255-62.

Berry, A. M. and Paton, J. C. (2000). "Additive attenuation of virulence of *Streptococcus pneumoniae* by mutation of the genes encoding pneumolysin and other putative pneumococcal virulence proteins." Infect Immun **68**(1): 133-40.

Berry, A. M., Paton, J. C. and Hansman, D. (1992). "Effect of insertional inactivation of the genes encoding pneumolysin and autolysin on the virulence of *Streptococcus pneumoniae* type 3." Microb Pathog **12**(2): 87-93.

Berry, A. M., Yother, J., Briles, D. E., Hansman, D. and Paton, J. C. (1989b). "Reduced virulence of a defined pneumolysin-negative mutant of *Streptococcus pneumoniae*." Infect Immun **57**(7): 2037-42.

Billington, S. J., Jost, B. H. and Songer, J. G. (2000). "Thiol-activated cytolysins: structure, function and role in pathogenesis." FEMS Microbiol Lett **182**(2): 197-205.

Black, S., Shinefield, H., Fireman, B., Lewis, E., Ray, P., Hansen, J. R., Elvin, L., Ensor, K. M., Hackell, J., Siber, G., Malinoski, F., Madore, D., Chang, I., Kohberger, R., Watson, W., Austrian, R. and Edwards, K. (2000). "Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children.

Northern California Kaiser Permanente Vaccine Study Center Group." Pediatr Infect Dis J **19**(3): 187-95.

Black, S. B., Shinefield, H. R., Ling, S., Hansen, J., Fireman, B., Spring, D., Noyes, J., Lewis, E., Ray, P., Lee, J. and Hackell, J. (2002). "Effectiveness of heptavalent pneumococcal conjugate vaccine in children younger than five years of age for prevention of pneumonia." Pediatr Infect Dis J **21**(9): 810-5.

Blue, C. E. (2002). PhD thesis: Characterisation of the role of two two-component signal transduction systems and a putative zinc metalloprotease in the virulence of *Streptococcus pneumoniae*. Infection and Immunity. Glasgow, University of Glasgow.

Blue, C. E., Paterson, G. K., Kerr, A. R., M., B., Claverys, J. P. and Mitchell, T. J. (2003). "ZmpB, a novel virulence factor of *Streptococcus pneumoniae* that induces Tumor Necrosis Factor alpha production in the respiratory tract." Infect Immun **71**(9): 4925-4935.

Bogaert, D., de Groot, R. and Hermans, P. (2004). "*Streptococcus pneumoniae* colonisation: the key to pneumococcal disease." The Lancet Infectious Diseases **4**(3): 144-154.

Bohr, V., Paulson, O. B. and Rasmussen, N. (1984). "Pneumococcal meningitis. Late neurologic sequelae and features of prognostic impact." Arch Neurol **41**(10): 1045-9.

Bohr, V., Rasmussen, N., Hansen, B., Gade, A., Kjersem, H., Johnsen, N. and Paulson, O. (1985). "Pneumococcal meningitis: an evaluation of prognostic factors in 164 cases based on mortality and on a study of lasting sequelae." J Infect **10**(2): 143-57.

Borish, L. C. and Steinke, J. W. (2003). "Cytokines and chemokines." J Allergy Clin Immunol **111**(2): S460-S475.

Boulnois, G. J., Mitchell, T. J., Saunders, F. K., Mendez, F. J. and Andrew, P. W. (1990). Structure and function of pneumolysin, the thiol-activated toxin of *Streptococcus pneumoniae*. Bacterial Protein Toxins. R. Rapuoli, Stuttgart: pp. 43-51.

Bradford, M. M. (1976). "A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding." Anal Biochem **72**: 248 - 254.

Braun, J. S., Novak, R., Gao, G., Murray, P. J. and Shenep, J. L. (1999). "Pneumolysin, a protein toxin of *Streptococcus pneumoniae*, induces nitric oxide production from macrophages." Infect Immun **67**(8): 3750-6.

Braun, J. S., Sublett, J. E., Freyer, D., Mitchell, T. J., Cleveland, J. L., Tuomanen, E. I. and Weber, J. R. (2002). "Pneumococcal pneumolysin and H_2O_2 mediate brain cell apoptosis during meningitis." J Clin Invest **109**(1): 19-27.

Briles, D. E. (2004). "Protection of the elderly from pneumococcal pneumonia with a protein-based vaccine?" Mechanisms of Ageing and Development **125**(2): 129-131.

Briles, D. E., Ades, E., Paton, J. C., Sampson, J. S., Carlone, G. M., Huebner, R. C., Virolainen, A., Swiatlo, E. and Hollingshead, S. K. (2000a). "Intranasal immunization of mice with a mixture of the pneumococcal proteins PsaA and PspA is highly protective against nasopharyngeal carriage of *Streptococcus pneumoniae*." Infect Immun **68**(2): 796-800.

Briles, D. E., Hollingshead, S., Brooks-Walter, A., Nabors, G. S., Ferguson, L., Schilling, M., Gravenstein, S., Braun, P., King, J. and Swift, A. (2000b). "The potential to use PspA and other pneumococcal proteins to elicit protection against pneumococcal infection." Vaccine **18**(16): 1707-11.

Briles, D. E., Hollingshead, S., Paton, J. C., Ades, E., Novak, L., van Ginkel, F. and Benjamin, W. H. (2003). "Immunizations with pneumococcal surface protein A and pneumolysin are protective against pneumonia in a murine model of pulmonary infection with *Streptococcus pneumoniae*." J Infect Dis **188**: 339-348.

Briles, D. E., Hollingshead, S. K., Nabors, G. S., Paton, J. C. and Brooks-Walter, A. (2001). "The potential for using protein vaccines to protect against otitis media caused by *Streptococcus pneumoniae*." Vaccine **19 Suppl 1**: S87-95.

Brooks-Walter, A., Briles, D. E. and Hollingshead, S. K. (1999). "The pspC gene of *Streptococcus pneumoniae* encodes a polymorphic protein, PspC, which elicits cross-reactive antibodies to PspA and provides immunity to pneumococcal bacteremia." Infect Immun **67**(12): 6533-42.

Brown, J. S., Ogunniyi, A. D., Woodrow, M. C., Holden, D. W. and Paton, J. C. (2001). "Immunization with components of two iron uptake ABC transporters protects mice against systemic *Streptococcus pneumoniae* infection." Infect Immun **69**(11): 6702-6.

Brueggemann, A. B., Griffiths, D. T., Meats, E., Peto, T., Crook, D. W. and Spratt, B. G. (2003a). "Clonal relationships between invasive and carriage *Streptococcus pneumoniae* and serotype- and clone-specific differences in invasive disease potential." J Infect Dis **187**(9): 1424-32.

Brueggemann, A. B. and Spratt, B. G. (2003b). "Geographic distribution and clonal diversity of *Streptococcus pneumoniae* serotype 1 isolates." J. Clin. Microbiol. **41**(11): 4966-4970.

Camara, M., Boulnois, G. J., Andrew, P. W. and Mitchell, T. J. (1994). "A neuraminidase from *Streptococcus pneumoniae* has the features of a surface protein." Infect Immun **62**(9): 3688-95.

Canvin, J. R., Marvin, A. P., Sivakumaran, M., Paton, J. C., Boulnois, G. J., Andrew, P. W. and Mitchell, T. J. (1995). "The role of pneumolysin and autolysin in the pathology of pneumonia and septicemia in mice infected with a type 2 pneumococcus." J Infect Dis **172**(1): 119-23.

Cavaillon, J. M. (1994). "Cytokines and macrophages." Biomed Pharmacother **48**(10): 445-53.

Cima-Cabal, M. D., Mendez, F. J., Vazquez, F., Aranaz, C., Rodriguez-Alvarez, J., Garcia-Garcia, J. M., Fleites, A., Martinez Gonzalez-Rio, J., Molinos, L., de Miguel, D. and de los Toyos, J. R. (2003). "Immunodetection of pneumolysin in human urine by ELISA." J Microbiol Methods **54**(1): 47-55.

Cima-Cabal, M. D., Mendez, F. J., Vazquez, F., del Mar Garcia-Suarez, M. and de los Toyos, J. R. (2001). "A specific and ultrasensitive chemiluminescent sandwich ELISA test for the detection and quantitation of pneumolysin." J Immunoassay Immunochem **22**(2): 99-112.

Cockeran, R., Durandt, C., Feldman, C., Mitchell, T. J. and Anderson, R. (2002). "Pneumolysin activates the synthesis and release of interleukin-8 by human neutrophils *in vitro*." J Infect Dis **186**(4): 562-5.

Cockeran, R., Steel, H. C., Mitchell, T. J., Feldman, C. and Anderson, R. (2001a). "Pneumolysin potentiates production of prostaglandin E(2) and leukotriene B(4) by human neutrophils." Infect Immun **69**(5): 3494-6.

Cockeran, R., Theron, A. J., Steel, H. C., Matlola, N. M., Mitchell, T. J., Feldman, C. and Anderson, R. (2001b). "Proinflammatory interactions of pneumolysin with human neutrophils." J Infect Dis **183**(4): 604-11.

Coffey, T., Daniels, M., Enright, C. and Spratt, B. (1999). "Serotype 14 variants of the Spanish penicillin-resistant serotype 9V clone of *Streptococcus pneumoniae* arose by large recombinational replacements of the cpsA--pbp1a region." Microbiology **145**(8): 2023-2031.

Coffey, T. J., Enright, M. C., Daniels, M., Morona, J. K., Morona, R., Hryniewicz, W., Paton, J. C. and Spratt, B. G. (1998). "Recombinational exchanges at the capsular polysaccharide biosynthetic locus lead to frequent serotype changes among natural isolates of *Streptococcus pneumoniae*." Molecular Microbiology **27**(1): 73-83.

Colino, J. and Snapper, C. M. (2003). "Two distinct mechanisms for induction of dendritic cell apoptosis in response to intact Streptococcus pneumoniae." J Immunol **171**(5): 2354-65.

Comis, S. D., Osborne, M. P., Stephen, J., Tarlow, M. J., Hayward, T. L., Mitchell, T. J., Andrew, P. W. and Boulnois, G. J. (1993). "Cytotoxic effects on hair cells of guinea pig cochlea produced by pneumolysin, the thiol activated toxin of *Streptococcus pneumoniae*." Acta Otolaryngol **113**(2): 152-9.

Cormack, B. P., Valdivia, R. H. and Falkow, S. (1996). "FACS-optimized mutants of the green fluorescent protein (GFP)." Gene **173**(1 Spec No): 33-8.

Cottagnoud, P. and Tomasz, A. (1993). "Triggering of pneumococcal autolysis by lysozyme." J Infect Dis **167**(3): 684-90.

Cundell, D. R., Gerard, N. P., Gerard, C., Idanpaan-Heikkila, I. and Tuomanen, E. I. (1995). "*Streptococcus pneumoniae* anchor to activated human cells by the receptor for platelet-activating factor." Nature **377**(6548): 435-8.

Czajkowsky, D. M., Hotze, E. M., Shao, Z. and Tweten, R. K. (2004). "Vertical collapse of a cytolysin prepore moves its transmembrane beta-hairpins to the membrane." EMBO: 1-10.

Dagan, R., Eskola, J., Leclerc, C. and Leroy, O. (1998). "Reduced response to multiple vaccines sharing common protein epitopes that are administered simultaneously to infants." Infect. Immun. **66**(5): 2093-2098.

Dagan, R., Gradstein, S., Belmaker, I., Porat, N., Siton, Y., Weber, G., Janco, J. and Yagupsky, P. (2000). "An outbreak of *Streptococcus pneumoniae* serotype 1 in a closed community in southern Israel." Clin Infect Dis **30**(2): 319-21.

Dang, T. X., Hotze, E. M., Rouiller, I., Tweten, R. K. and Wilson-Kubalek, E. M. (2005a). "Prepore to pore transition of a cholesterol-dependent cytolysin visualized by electron microscopy." Journal of Structural Biology **150**(1): 100-108.

Dang, T. X., Milligan, R. A., Tweten, R. K. and Wilson-Kubalek, E. M. (2005b). "Helical crystallization on nickel-lipid nanotubes: Perfringolysin O as a model protein." J Struct Biol.

Dantzer, R. (1994). "How do cytokines say hello to the brain? Neural versus humoral mediation." Eur. Cytokine Netw. **5**(3): 271-3.

de las Rivas, B., Garcia, J. L., Lopez, R. and Garcia, P. (2001). "Molecular characterization of the pneumococcal teichoic acid phosphorylcholine esterase." Microb Drug Resist **7**(3): 213-22.

de los Toyos, J. R., Mendez, F. J., Aparicio, J. F., Vazquez, F., Del Mar Garcia Suarez, M., Fleites, A., Hardisson, C., Morgan, P. J., Andrew, P. W. and Mitchell, T. J. (1996). "Functional analysis of pneumolysin by use of monoclonal antibodies." Infect Immun **64**(2): 480-4.

DeMaria, A., Browne, K., Berk, S. L., Sherwood, E. J. and McCabe, W. R. (1980). "An outbreak of type 1 pneumococcal pneumonia in a men's shelter." J. American Medical Association **244**(13): 1446-1449.

Denham, B. C. and Clarke, S. C. (2005). "Serotype incidence and antibiotic susceptibility of *Streptococcus pneumoniae* causing invasive disease in Scotland, 1999-2002." J Med Microbiol **54**(Pt 4): 327-31.

Derijk, R. H., Van Kampen, M., Van Rooijen, N. and Berkenbosch, F. (1994). "Hypothermia to endotoxin involves reduced thermogenesis, macrophage-dependent mechanisms, and prostaglandins." Am J Physiol Regul Integr Comp Physiol **266**(1): R1-8.

Dintilhac, A., Alloing, G., Granadel, C. and Claverys, J. P. (1997). "Competence and virulence of *Streptococcus pneumoniae*: Adc and PsaA mutants exhibit a requirement for Zn and Mn resulting from inactivation of putative ABC metal permeases." Mol Microbiol **25**(4): 727-39.

Dochez, A. r. and Gillespie, L. J. (1913). "A biologic classification of pneumococci by means of immunity reactions." A.M.A. **6**: 727-732.

Dowson, C. G., Coffey, T. J. and Spratt, B. G. (1994). "Origin and molecular epidemiology of penicillin-binding-protein-mediated resistance to beta-lactam antibiotics." Trends Microbiol **2**(10): 361-6.

Ebong, S., Call, D., Nemzek, J., Bolgos, G., Newcomb, D. and Remick, D. (1999). "Immunopathologic alterations in murine models of sepsis of increasing severity." Infect Immun **67**(12): 6603-10.

Eltringham, G., Kearns, A., Freeman, R., Clark, J., Spencer, D., Eastham, K., Harwood, J. and Leeming, J. (2003). "Culture-negative childhood empyema is usually due to penicillin-sensitive *Streptococcus pneumoniae* capsular serotype 1." J. Clin. Microbiol. **41**(1): 521-522.

Engel, F., Blatz, R., Schliebs, R., Palmer, M. and Bhakdi, S. (1998). "Bacterial cytolysin perturbs round window membrane permeability barrier in vivo: possible cause of sensorineural hearing loss in acute otitis media." Infect Immun **66**(1): 343-6.

Enright, M. C. and Spratt, B. G. (1998). "A multilocus sequence typing scheme for *Streptococcus pneumoniae*: identification of clones associated with serious invasive disease." Microbiology **144 (Pt 11)**: 3049-60.

Feikin, D. R. and Klugman, K. P. (2002). "Historical changes in pneumococcal serogroup distribution: implications for the era of pneumococcal conjugate vaccines." Clin Infect Dis **35**(5): 547-55.

Feldman, C., Mitchell, T. J., Andrew, P. W., Boulnois, G. J., Read, R. C., Todd, H. C., Cole, P. J. and Wilson, R. (1990). "The effect of *Streptococcus pneumoniae* pneumolysin on human respiratory epithelium in vitro." Microb Pathog **9**(4): 275-84.

Feldman, C., Munro, N. C., Jeffery, P. K., Mitchell, T. J., Andrew, P. W., Boulnois, G. J., Guerreiro, D., Rohde, J. A., Todd, H. C., Cole, P. J. and et al. (1991). "Pneumolysin

induces the salient histologic features of pneumococcal infection in the rat lung in vivo." Am J Respir Cell Mol Biol **5**(5): 416-23.

Ferrante, A., Rowan-Kelly, B. and Paton, J. C. (1984). "Inhibition of *in vitro* human lymphocyte response by the pneumococcal toxin pneumolysin." Infect Immun **46**(2): 585-9.

Fillion, I., Ouellet, N., Simard, M., Bergeron, Y., Sato, S. and Bergeron, M. G. (2001). "Role of chemokines and formyl peptides in pneumococcal pneumonia-induced monocyte/macrophage recruitment." J Immunol **166**(12): 7353-7361.

Fiore, A. E., Levine, O. S., Elliott, J. A., Facklam, R. R. and Butler, J. C. (1999). "Effectiveness of pneumococcal polysaccharide vaccine for preschool-age children with chronic disease." Emerg Infect Dis **5**(6): 828-31.

Flannery, B., Heffernan, R. T., Harrison, L. H., Ray, S. M., Reingold, A. L., Hadler, J., Schaffner, W., Lynfield, R., Thomas, A. R., Li, J., Campsmith, M., Whitney, C. G. and Schuchat, A. (2006). "Changes in invasive pneumococcal disease among HIV-infected adults living in the era of childhood pneumococcal immunization." Ann Intern Med **144**(1): 1-9.

Flecknell, P. (1996). Laboratory animal anaesthesia., Academic Press. San Diego and London.

Frazao, N., Brito-Avo, A., Simas, C., Saldanha, J., Mato, R., Nunes, S., Sousa, N. G., Carrico, J. A., Almeida, J. S., Santos-sanches, I. and Lencastre, H. (2005). "Effect of the seven-valent conjugate pneumococcal vaccine on carriage and drug resistance of *Streptococcus pneumoniae* in healthy children attending day-care centers in Lisbon." Pediatr Infect Dis J. **24**(3): 243-252.

Freitag, N. E. and Jacobs, K. E. (1999). "Examination of *Listeria monocytogenes* intracellular gene expression by using the green fluorescent protein of *Aequorea victoria*." Infect. Immun. **67**(4): 1844-1852.

French, N. (2003). "Use of pneumococcal polysaccaride vaccines: no simple answers." J Infect **46**: 78-86.

French, N., Nakiyingi, J., Carpenter, L. M., Lugada, E., Watera, C., Moi, K., Moore, M., Antvelink, D., Mulder, D., Janoff, E. N., Whitworth, J. and Gilks, C. F. (2000). "23-valent pneumococcal polysaccharide vaccine in HIV-1-infected Ugandan adults: double-blind, randomised and placebo controlled trial." Lancet **355**(9221): 2106-11.

Friedland, I. R., Paris, M. M., Hickey, S., Shelton, S., Olsen, K., Paton, J. C. and McCracken, G. H. (1995). "The limited role of pneumolysin in the pathogenesis of pneumococcal meningitis." J Infect Dis **172**(3): 805-9.

Garcia-Suarez, M. d. M., Cima-Cabal, M. D., Florez, N., Garcia, P., Cernuda-Cernuda, R., Astudillo, A., Vazquez, F., De Los Toyos, J. R. and Mendez, F. J. (2004). "Protection against pneumococcal pneumonia in mice by monoclonal antibodies to pneumolysin." Infect. Immun. **72**(8): 4534-4540.

Gebo, K. A., Moore, R. D. and Keruly, J. C. (1996). "Risk factors for pneumococcal disease in human immunodeficiency virus-infected patients." J Infect Dis **173**: 857-62.

Giddings, K. S., Johnson, A. E. and Tweten, R. K. (2003). "Redefining cholesterol's role in the mechanism of the cholesterol-dependent cytolysins." Proc Natl Acad Sci U S A **100**(20): 11315-11320.

Giddings, K. S., Zhao, J., Sims, P. J. and Tweten, R. K. (2004). "Human CD59 is a receptor for the cholesterol-dependent cytolysin intermedilysin." Nat Struct and Mol Biol **11**(12): 1173-1178.

Gilbert, R. J. (2002). "Pore-forming toxins." Cell Mol Life Sci **59**(5): 832-44.

Gilbert, R. J., Heenan, R. K., Timmins, P. A., Gingles, N. A., Mitchell, T. J., Rowe, A. J., Rossjohn, J., Parker, M. W., Andrew, P. W. and Byron, O. (1999a). "Studies on the structure and mechanism of a bacterial protein toxin by analytical

ultracentrifugation and small-angle neutron scattering." J Mol Biol **293**(5): 1145-60.

Gilbert, R. J., Jimenez, J. L., Chen, S., Tickle, I. J., Rossjohn, J., Parker, M., Andrew, P. W. and Saibil, H. R. (1999b). "Two structural transitions in membrane pore formation by pneumolysin, the pore-forming toxin of *Streptococcus pneumoniae*." Cell **97**(5): 647-55.

Gilbert, R. J., Rossjohn, J., Parker, M. W., Tweten, R. K., Morgan, P. J., Mitchell, T. J., Errington, N., Rowe, A. J., Andrew, P. W. and Byron, O. (1998). "Self-interaction of pneumolysin, the pore-forming protein toxin of *Streptococcus pneumoniae*." J Mol Biol **284**(4): 1223-37.

Gilbert, R. J. C. (2005). "Inactivation and activity of cholesterol-dependent cytolysins: what structural studies tell us." Structure **13**(8): 1097-1106.

Gosink, K. K., Mann, E. R., Guglielmo, C., Tuomanen, E. I. and Masure, H. R. (2000). "Role of novel choline binding proteins in virulence of *Streptococcus pneumoniae*." Infect. Immun. **68**(10): 5690-5695.

Gratten, M., Morey, F., Dixon, J., Manning, K., Torzillo, P., Matters, R., Erlich, J., Hanna, J., Asche, V. and Riley, I. (1993). "An outbreak of serotype 1 *Streptococcus pneumoniae* infection in central Australia." Med J Aust **158**(5): 340-2.

Green, B. A., Zhang, Y., Masi, A. W., Barniak, V., Wetherell, M., Smith, R. P., Reddy, M. S. and Zhu, D. (2005). "PppA, a surface-exposed protein of *Streptococcus pneumoniae*, elicits cross-reactive antibodies that reduce colonization in a murine intranasal immunization and challenge model." Infect. Immun. **73**(2): 981-989.

Greiner, O., Day, P. J., Bosshard, P. P., Imeri, F., Altwegg, M. and Nadal, D. (2001). "Quantitative detection of *Streptococcus pneumoniae* in nasopharyngeal secretions by real-time PCR." J Clin Microbiol **39**(9): 3129-34.

Gruol, D. L. and Nelson, T. E. (1997). "Physiological and pathological roles of interleukin-6 in the central nervous system." Mol Neurobiol **15**(3): 307-39.

Guiral, S., Mitchell, T. J., Martin, B. and Claverys, J. P. (2005). "Competence-programmed predation of noncompetent cells in the human pathogen Streptococcus pneumoniae: genetic requirements." Proc Natl Acad Sci U S A **102**(24): 8710-5.

Hammerschmidt, S., Bethe, G., H. Remane, P. and Chhatwal, G. S. (1999). "Identification of pneumococcal surface protein A as a lactoferrin-binding protein of *Streptococcus pneumoniae*." Infect. Immun. **67**(4): 1683-1687.

Hammerschmidt, S., Wolff, S., Hocke, A., Rosseau, S., Muller, E. and Rohde, M. (2005). "Illustration of pneumococcal polysaccharide capsule during adherence and invasion of epithelial cells." Infect Immun **73**(8): 4653-67.

Hanage, W. P., Auranen, K., Syrjanen, R., Herva, E., Makela, P. H., Kilpi, T. and Spratt, B. G. (2004). "Ability of pneumococcal serotypes and clones to cause acute otitis media: implications for the prevention of otitis media by conjugate vaccines." Infect. Immun. **72**(1): 76-81.

Hanage, W. P., Kaijalainen, T., Herva, E., Saukkoriipi, A., Syrjanen, R. and Spratt, B. G. (2005). "Using multilocus sequence data to define the pneumococcus." J. Bacteriol. **187**(17): 6223-6230.

Hausdorff, W. P., Bryant, J., Kloek, C., Paradiso, P. R. and Siber, G. R. (2000a). "The contribution of specific pneumococcal serogroups to different disease manifestations: implications for conjugate vaccine formulation and use, part II." Clin Infect Dis **30**(1): 122-40.

Hausdorff, W. P., Bryant, J., Paradiso, P. R. and Siber, G. R. (2000b). "Which pneumococcal serogroups cause the most invasive disease: implications for conjugate vaccine formulation and use, part I." Clin Infect Dis **30**(1): 100-21.

Hava, D. L. and Camilli, A. (2002). "Large-scale identification of serotype 4 *Streptococcus pneumoniae* virulence factors." Mol Microbiol **45**(5): 1389-406.

Henrichsen, J. (1995). "Six newly recognized types of *Streptococcus pneumoniae*." J. Clin. Microbiol. **33**(10): 2759-2762.

Henriques Normark, B., Kalin, M., Ortqvist, A., Akerlund, T., Liljequist, B. O., Hedlund, J., Svenson, S. B., Zhou, J., Spratt, B. G., Normark, S. and Kallenius, G. (2001). "Dynamics of penicillin-susceptible clones in invasive pneumococcal disease." J Infect Dis **184**(7): 861-9.

Hermans, P. W. M., Adrian, P. V., Albert, C., Estevao, S., Hoogenboezem, T., Luijendijk, I. H. T., Kamphausen, T. and Hammerschmidt, S. (2006). "The streptococcal lipoprotein rotamase A (SlrA) is a functional peptidyl-prolyl isomerase involved in pneumococcal colonization." J. Biol. Chem. **281**(2): 968-976.

Heuck, A. P., Hotze, E. M., Tweten, R. K. and Johnson, A. E. (2000). "Mechanism of membrane insertion of a multimeric beta-barrel protein: perfringolysin O creates a pore using ordered and coupled conformational changes." Mol Cell **6**(5): 1233-42.

Heuck, A. P., Tweten, R. K. and Johnson, A. E. (2003). "Assembly and topography of the prepore complex in cholesterol-dependent cytolysins." J Biol Chem **278**(33): 31218-25.

Hill, J., Andrew, P. W. and Mitchell, T. J. (1994). "Amino acids in pneumolysin important for hemolytic activity identified by random mutagenesis." Infect Immun **62**(2): 757-8.

Hirst, R. A., Kadioglu, A., O'Callaghan, C. and Andrew, P. W. (2004a). "The role of pneumolysin in pneumococcal pneumonia and meningitis." Clin Exp Immunol **138**(2): 195-201.

Hirst, R. A., Mohammed, B. J., Mitchell, T. J., Andrew, P. W. and O'Callaghan, C. (2004b). "*Streptococcus pneumoniae*-induced inhibition of rat ependymal cilia is attenuated by antipneumolysin antibody." Infect. Immun. **72**(11): 6694-6698.

Hirst, R. A., Rutman, A., Sikand, K., Andrew, P. W., Mitchell, T. J. and O'Callaghan, C. (2000). "Effect of pneumolysin on rat brain ciliary function: comparison of brain slices with cultured ependymal cells." Pediatr Res **47**(3): 381-4.

Hirst, R. A., Yesilkaya, H., Clitheroe, E., Rutman, A., Dufty, N., Mitchell, T. J., O'Callaghan, C. and Andrew, P. W. (2002). "Sensitivities of human monocytes and epithelial cells to pneumolysin are different." Infect Immun **70**(2): 1017-22.

Hotze, E. M., Heuck, A. P., Czajkowsky, D. M., Shao, Z., Johnson, A. E. and Tweten, R. K. (2002). "Monomer-monomer interactions drive the prepore to pore conversion of a beta-barrel-forming cholesterol-dependent cytolysin." J Biol Chem **277**(13): 11597-605.

Hotze, E. M., Wilson-Kubalek, E. M., Rossjohn, J., Parker, M., Johnson, A. E. and Tweten, R. K. (2001a). "Arresting pore formation of a cholesterol-dependent cytolysin by disulphide trapping synchronizes the insertion of the transmembrane β–sheet from a prepore intermediate." J Biol Chem **276**(11): 8261-8266.

Hotze, E. M., Wilson-Kubalek, E. M., Rossjohn, J., Parker, M. W., Johnson, A. E. and Tweten, R. K. (2001b). "Arresting pore formation of a cholesterol-dependent cytolysin by disulfide trapping synchronizes the insertion of the transmembrane beta-sheet from a prepore intermediate." J Biol Chem **276**(11): 8261-8.

Houldsworth, S., Andrew, P. W. and Mitchell, T. J. (1994). "Pneumolysin stimulates production of tumor necrosis factor alpha and interleukin-1 beta by human mononuclear phagocytes." Infect Immun **62**(4): 1501-3.

Huo, Z., Spencer, O., Miles, J., Johnson, J., Holliman, R., Sheldon, J. and Riches, P. (2004). "Antibody response to pneumolysin and to pneumococcal capsular polysaccharide in healthy individuals and *Streptococcus pneumoniae* infected patients." Vaccine **22**(9-10): 1157-1161.

Hvalbye, B. K., Aaberge, I. S., Lovik, M. and Haneberg, B. (1999). "Intranasal immunization with heat-inactivated *Streptococcus pneumoniae* protects mice against systemic pneumococcal infection." Infect Immun **67**(9): 4320-5.

Ibrahim, H. R., Thomas, U. and Pellegrini, A. (2001). "A helix-loop-helix peptide at the upper lip of the active site cleft of lysozyme confers potent antimicrobial activity with membrane permeabilization action." J. Biol. Chem. **276**(47): 43767-43774.

Ibrahim, Y. M., Kerr, A. R., McCluskey, J. and Mitchell, T. J. (2004). "Role of HtrA in the virulence and competence of *Streptococcus pneumoniae*." Infect. Immun. **72**(6): 3584-3591.

Ibrahim, Y. M., Kerr, A. R., Silva, N. A. and Mitchell, T. J. (2005). "Contribution of the ATP-dependent protease ClpCP to the autolysis and virulence of *Streptococcus pneumoniae*." Infect. Immun. **73**(2): 730-740.

Ishizuka, S., Yamaya, M., Suzuki, T., Takahashi, H., Ida, S., Sasaki, T., Inoue, D., Sekizawa, K., Nishimura, H. and Sasaki, H. (2003). "Effects of rhinovirus infection on the adherence of *Streptococcus pneumoniae* to cultured human airway epithelial cells." J Infect Dis **188**: 1928-39.

Ispahani, P., Slack, R. C., Donald, F. E., Weston, V. C. and Rutter, N. (2004). "Twenty year surveillance of invasive pneumococcal disease in Nottingham: serogroups responsible and implications for immunisation." Arch Dis Child **89**(8): 757-62.

Jacobs, T., Cima-Cabal, M. D., Darji, A., Mendez, F. J., Vazquez, F., Jacobs, A. A., Shimada, Y., Ohno-Iwashita, Y., Weiss, S. and de los Toyos, J. R. (1999). "The conserved undecapeptide shared by thiol-activated cytolysins is involved in membrane binding." FEBS Lett **459**(3): 463-6.

Jedrzejas, M. J. (2001). "Pneumococcal virulence factors: structure and function." Microbiol Mol Biol Rev **65**(2): 187-207 ; first page, table of contents.

Jefferies, J. M. C., Smith, A., Clarke, S. C., Dowson, C. and Mitchell, T. J. (2004). "Genetic analysis of diverse disease-causing pneumococci indicates high levels of diversity within serotypes and capsuleswitching." J. Clin. Microbiol. **42**(12): 5681-5688.

Johnston, R. B., Jr. (1991). "Pathogenesis of pneumococcal pneumonia." Rev Infect Dis **13 Suppl 6**: S509-17.

Jones, M. R., Simms, B. T., Lupa, M. M., Kogan, M. S. and Mizgerd, J. P. (2005). "Lung NF-{kappa}B activation and neutrophil recruitment require IL-1 and TNF receptor signaling during pneumococcal pneumonia." J Immunol **175**(11): 7530-7535.

Jonsson, S., Musher, D. M., Chapman, A., Goree, A. and Lawrence, E. C. (1985). "Phagocytosis and killing of common bacterial pathogens of the lung by human alveolar macrophages." J Infect Dis **152**(1): 4-13.

Jounblat, R., Kadioglu, A., Mitchell, T. J. and Andrew, P. W. (2003). "Pneumococcal behavior and host responses during bronchopneumonia are affected differently by the cytolytic and complement-activating activities of pneumolysin." Infect Immun **71**(4): 1813-9.

Kadioglu, A., Gingles, N. A., Grattan, K., Kerr, A., Mitchell, T. J. and Andrew, P. W. (2000). "Host cellular immune response to pneumococcal lung infection in mice." Infect Immun **68**(2): 492-501.

Kadioglu, A., Taylor, S., Iannelli, F., Pozzi, G., Mitchell, T. J. and Andrew, P. W. (2002). "Upper and lower respiratory tract infection by *Streptococcus pneumoniae* is affected by pneumolysin deficiency and differences in capsule type." Infect Immun **70**(6): 2886-90.

Kalin, M., Kanclerski, K., Granstrom, M. and Mollby, R. (1987). "Diagnosis of pneumococcal pneumonia by enzyme-linked immunosorbent assay of antibodies to pneumococcal hemolysin (pneumolysin)." J Clin Microbiol **25**(2): 226-9.

Kamboj, K. K., Kirchner, L., Kimmel, R., Greenspan, N. S. and Schreiber, J. R. (2003). "Significant variation in serotype-specific immunogenicity of the seven-valent *Streptococcus pneumoniae* capsular polysaccharide-CRM_{197} conjugate vaccine occurs despite vigorous T cell help induced by the carrier protein." J Infect Dis **187**: 1629-38.

Kanclerski, K. and Mollby, R. (1987). "Production and purification of *Streptococcus pneumoniae* hemolysin (pneumolysin)." J Clin Microbiol **25**(2): 222-5.

Kelly, D. F., Moxon, E. R. and Pollard, A. J. (2004). "*Haemophilus influenzae* type b conjugate vaccines." Immunology **113**(2): 163-174.

Kerr, A. R., Irvine, J. J., Search, J. J., Gingles, N. A., Kadioglu, A., Andrew, P. W., McPheat, W. L., Booth, C. G. and Mitchell, T. J. (2002). "Role of inflammatory mediators in resistance and susceptibility to pneumococcal infection." Infect Immun **70**(3): 1547-57.

Kerr, A. R., Kirkham, L. A., Kadioglu, A., Andrew, P. W., Garside, P., Thompson, H. and Mitchell, T. J. (2005a). "Identification of a detrimental role for NK cells in pneumococcal pneumonia and sepsis in immunocompromised hosts." Microbes Infect 7(5-6): 845-52.

Kerr, A. R., Paterson, G. K., Riboldi-Tunnicliffe, A. and Mitchell, T. J. (2005b). "Innate immune defense against pneumococcal pneumonia requires pulmonary complement component C3." Infect. Immun. **73**(7): 4245-4252.

King, S. J., Whatmore, A. M. and Dowson, C. G. (2005). "NanA, a neuraminidase from *Streptococcus pneumoniae*, shows high levels of sequence diversity, at least in part through recombination with *Streptococcus oralis*." J. Bacteriol. **187**(15): 5376-5386.

Kipli, T., Jokinen, J., Herva, E., Palmu, A., Lockhart, S., Siber, G., Eskola, J. and Group, t. F. O. M. S. (2000). Effect of a heptavalent pneumococcal vaccine (PNCCRM) on pneumococcal acute otitis media (AOM) by serotype. Second International Symposium on Pneumococci and Pneumococcal Disease, Sun City, South Africa, March 19-23, abstract O20.

Kirkham, L. A., Kerr, A. R., Douce, G. R., Paterson, G. K., Dilts, D. A., Liu, D. F. and Mitchell, T. J. (2006a). "Construction and immunological characterization of a

novel nontoxic protective pneumolysin mutant for use in future pneumococcal vaccines." Infect Immun **74**(1): 586-93.

Kirkham, L.-A. S., Jefferies, J. M. C., Kerr, A. R., Jing, Y., Clarke, S. C., Smith, A. and Mitchell, T. J. (2006b). "Identification of invasive serotype 1 pneumococcal isolates that express nonhemolytic pneumolysin." J. Clin. Microbiol. **44**(1): 151-159.

Klein, D. L. (1995). "Pneumococcal conjugate vaccines: review and update." Microb Drug Resist **1**(1): 49-58.

Klugman, K. P., Madhi, S. A., Huebner, R. E., Kohberger, R., Mbelle, N., Pierce, N. and the Vaccine Trialists Group (2003). "A trial of a 9-valent pneumococcal conjugate vaccine in children with and those without HIV infection." N Engl J Med **349**(14): 1341-1348.

Koek, J., Njanpop-Lafourcade, B., Cade, S., Varon, E., Sangare, L., Valjevac, S., Vergnaud, G. and Pourcel, C. (2005). "Evaluation and selection of tandem repeat loci for *Streptococcus pneumoniae* MVLA strain typing." BMC Micro **5**(66).

Konradsen, H. B. and Kaltoft, M. S. (2002). "Invasive pneumococcal infections in Denmark from 1995 to 1999: epidemiology, serotypes, and resistance." Clin. Diagn. Lab. Immunol. **9**(2): 358-365.

Korchev, Y. E., Bashford, C. L., Pederzolli, C., Pasternak, C. A., Morgan, P. J., Andrew, P. W. and Mitchell, T. J. (1998). "A conserved tryptophan in pneumolysin is a determinant of the characteristics of channels formed by pneumolysin in cells and planar lipid bilayers." Biochem J **329 (Pt 3)**: 571-7.

Kostyukova, N. N., Volkova, M. O., Ivanova, V. V. and Kvetnaya, A. S. (1995). "A study of pathogenic factors of *Streptococcus pneumoniae* strains causing meningitis." FEMS Immunol Med Microbiol **10**(2): 133-7.

Kuo, J., Douglas, M., Ree, H. K. and Lindberg, A. A. (1995). "Characterization of a recombinant pneumolysin and its use as a protein carrier for pneumococcal type 18C conjugate vaccines." Infect Immun **63**(7): 2706-13.

Kurtti, P., Isoaho, R., von Hertzen, L., Keistinen, T., Kivela, S. L. and Leinonen, M. (1997). "Influence of age, gender and smoking on *Streptococcus pneumoniae, Haemophilus influenzae* and *Moraxella (Branhamella) catarrhalis* antibody titres in an elderly population." Scand J Infect Dis **29**(5): 485-9.

Kwon, H.-Y., Ogunniyi, A. D., Choi, M.-H., Pyo, S.-N., Rhee, D.-K. and Paton, J. C. (2004). "The ClpP protease of *Streptococcus pneumoniae* modulates virulence gene expression and protects against fatal pneumococcal challenge." Infect. Immun. **72**(10): 5646-5653.

Kyaw, M. H., Christie, P., Clarke, S. C., Mooney, J. D., Ahmed, S., Jones, I. G. and Campbell, H. (2003). "Invasive pneumococcal disease in Scotland, 1999-2001: use of record linkage to explore associations between patients and disease in relation to future vaccination policy." Clin Infect Dis **37**(10): 1283-91.

Laemmli, U. K. (1970). "Cleavage of structural proteins during the assembly of the head of bacteriophage T4." Nature **227**(15): 680-685.

Lampinen, M., Carlson, M., Hakansson, L. D. and Venge, P. (2004). "Cytokine-regulated accumulation of eosinophils in inflammatory disease." Allergy **59**(8): 793-805.

Lau, G. W., Haataja, S., Lonetto, M., Kensit, S. E., Marra, A., Bryant, A. P., McDevitt, D., Morrison, D. A. and Holden, D. W. (2001). "A functional genomic analysis of type 3 *Streptococcus pneumoniae* virulence." Molecular Microbiology **40**(3): 555-571.

Lee, C.-J. (2002). "Quality control of polyvalent pneumococcal polysaccharide-protein conjugate vaccine by nephelometry." Biologicals **30**(2): 97-103.

Lee, C. J. and Li, Z. M. (2001a). "Protective immunity and gene expression related to pneumococcal glycoconjugate." Adv Exp Med Biol **491**: 505-14.

Lee, C. J., Lock, R. A., Andrew, P. W., Mitchell, T. J., Hansman, D. and Paton, J. C. (1994). "Protection of infant mice from challenge with *Streptococcus pneumoniae* type 19F by immunization with a type 19F polysaccharide--pneumolysoid conjugate." Vaccine **12**(10): 875-8.

Lee, C. J., Wang, T. R. and Frasch, C. E. (2001b). "Immunogenicity in mice of pneumococcal glycoconjugate vaccines using pneumococcal protein carriers." Vaccine **19**(23-24): 3216-25.

Leimkugel, J., Adams Forgor, A., Gagneux, S., Pfluger, V., Flierl, C., Awine, E., Naegeli, M., Dangy, J. P., Smith, T., Hodgson, A. and Pluschke, G. (2005). "An outbreak of serotype 1 *Streptococcus pneumoniae* meningitis in northern Ghana with features that are characteristic of *Neisseria meningitidis* meningitis epidemics." J Infect Dis **192**(2): 192-9.

LeMessurier, K. S., Ogunniyi, A. D. and Paton, J. C. (2006). "Differential expression of key pneumococcal virulence genes *in vivo*." Microbiology **152**(2): 305-311.

Leon, L. R. (2004). "Hypothermia in systemic inflammation: role of cytokines." Front Biosci. **9**(1): 1877-88.

Leon, L. R., White, A. A. and Kluger, M. J. (1998). "Role of IL-6 and TNF in thermoregulation and survival during sepsis in mice." Am J Physiol Regul Integr Comp Physiol **275**(1): R269-277.

Lesinski, G. B., Smithson, S. L., Srivastava, N., Chen, D., Widera, G. and Westerink, M. A. J. (2001). "A DNA vaccine encoding a peptide mimic of *Streptococcus pneumoniae* serotype 4 capsular polysaccharide induces specific anti-carbohydrate antibodies in Balb/c mice." Vaccine **19**(13-14): 1717-1726.

Lexau, C. A., Lynfield, R., Danila, R., Pilishvili, T., Facklam, R., Farley, M. M., Harrison, L. H., Schaffner, W., Reingold, A., Bennett, N. M., Hadler, J., Cieslak, P. R. and Whitney, C. G. (2005). "Changing epidemiology of invasive pneumococcal disease

among older adults in the era of pediatric pneumococcal conjugate vaccine." Jama **294**(16): 2043-51.

Lim, C., Kim, M., Ahn, J., Kim, M., Kwon, Y., Lee, I., Koh, Y., Kim, D. S. and Kim, W. D. (2003). "Hypothermia protects against endotoxin-induced acute lung injury in rats." Intensive Care Med. **29**(3): 453-9.

Ling, E., Feldman, G., Dagan, R. and Mizrachi-Nebenzahl, Y. (2003). "Cytokine mRNA expression in pneumococcal carriage, pneumonia and sepsis in young mice." J Infect Dis **188**: 1752-6.

Lipsitch, M., Whitney, C. G., Zell, E., Kaijalainen, T., Dagan, R. and Malley, R. (2005). "Are anticapsular antibodies the primary mechanism of protection against invasive pneumococcal disease?" PLoS Med **2**(1): e15.

Lloyd-Evans, N., O'Dempsey, T. J., Baldeh, I., Secka, O., Demba, E., Todd, J. E., McArdle, T. F., Banya, W. S. and Greenwood, B. M. (1996). "Nasopharyngeal carriage of pneumococci in Gambian children and in their families." Pediatr Infect Dis J **15**(10): 866-71.

Lock, R. A., Paton, J. C. and Hansman, D. (1988). "Comparative efficacy of pneumococcal neuraminidase and pneumolysin as immunogens protective against *Streptococcus pneumoniae*." Microb Pathog **5**(6): 461-7.

Lock, R. A., Zhang, Q. Y., Berry, A. M. and Paton, J. C. (1996). "Sequence variation in the *Streptococcus pneumoniae* pneumolysin gene affecting haemolytic activity and electrophoretic mobility of the toxin." Microb Pathog **21**(2): 71-83.

Madsen, M., Lebenthal, Y., Cheng, Q., Smith, B. L. and Hostetter, M. K. (2000). "A pneumococcal protein that elicits interleukin-8 from pulmonary epithelial cells." J Infect Dis **181**(4): 1330-6.

Maleckar, J. (2004). Quadrivalent pneumococcal protein vaccines - clinical and safety investigations (Oral presentation PROT-02). ISPPD-4, Helskini, Finland.

Malley, R., Henneke, P., Morse, S. C., Cieslewicz, M. J., Lipsitch, M., Thompson, C. M., Kurt-Jones, E., Paton, J. C., Wessels, M. R. and Golenbock, D. T. (2003). "Recognition of pneumolysin by Toll-like receptor 4 confers resistance to pneumococcal infection." Proc Natl Acad Sci U S A **100**(4): 1966-71.

Malley, R., Lipsitch, M., Stack, A., Saladino, R., Fleisher, G., Pelton, S., Thompson, C., Briles, D. and Anderson, P. (2001). "Intranasal immunization with killed unencapsulated whole cells prevents colonization and invasive disease by capsulated pneumococci." Infect Immun **69**(8): 4870-3.

Malley, R., Trzcinski, K., Srivastava, A., Thompson, C. M., Anderson, P. W. and Lipsitch, M. (2005). "CD4+ T cells mediate antibody-independent acquired immunity to pneumococcal colonization." PNAS **102**(13): 4848-4853.

Marriott, H. M., Ali, F., Read, R. C., Mitchell, T. J., Whyte, M. K. B. and Dockrell, D. H. (2004). "Nitric oxide levels regulate macrophage commitment to apoptosis or necrosis during pneumococcal infection

10.1096/fj.03-1450fje." FASEB J.: 03-1450fje.

Martin, M., Turco, J. H., Zegans, M. E., Facklam, R. R., Sodha, S., Elliott, J. A., Pryor, J. H., Beall, B., Erdman, D. D., Baumgartner, Y. Y., Sanchez, P. A., Schwartzman, J. D., Montero, J., Schuchat, A. and Whitney, C. G. (2003). "An outbreak of conjunctivitis due to atypical *Streptococcus pneumoniae*." N Engl J Med **348**(12): 1112-21.

Matsui, T., Ishikawa, T., Takeuchi, H., Okabayashi, K. and Mackawa, T. (2006). "Mild hypothermia promotes pro-inflammatory cytokine production in monocytes." J Neurosurg Anesthesiol **18**(1): 32-6.

Maus, U. A., Srivastava, M., Paton, J. C., Mack, M., Everhart, M. B., Blackwell, T. S., Christman, J. W., Schlondorff, D., Seeger, W. and Lohmeyer, J. (2004). "Pneumolysin-induced lung injury is independent of leukocyte trafficking into the alveolar space." J Immunol **173**(2): 1307-1312.

McChlery, S. M., Scott, K. J. and Clarke, S. C. (2005). "Clonal analysis of invasive pneumococcal isolates in Scotland and coverage of serotypes by the licensed conjugate polysaccharide pneumococcal vaccine: possible implications for UK vaccine policy." Eur J Clin Microbiol Infect Dis **24**: 262-267.

McCullers, J. A. and Rehg, J. E. (2002). "Lethal synergism between influenza virus and *Streptococcus pneumoniae*: characterization of a mouse model and the role of platelet-activating factor receptor." J Infect Dis **186**(3): 341-50.

McEllistrem M. C., A. J., Mason E. O. and Wald E.R . (2003). "Epidemiology of acute otitis media caused by *Streptococcus pneumoniae* before and after licensure of the 7-valent pneumococcal protein conjugate vaccine." J Infect Dis **71**(12): 7149-7153.

McFarlane, A. C., Hamra, I.. K., Reiss-Levy, E. and Hansman, D. (1979). "Pneumococcal peritonitis in adolescent girls." Med J Aust **1**(3): 100-1.

Mercat, A., Nguyen, J. and Dautzenberg, B. (1991). "An outbreak of pneumococcal pneumonia in two men's shelters." Chest **99**(1): 147-51.

Michon, F., Fusco, P. C., Minetti, C. A. S. A., Laude-Sharp, M., Uitz, C., Huang, C.-H., D'Ambra, A. J., Moore, S., Remeta, D. P., Heron, I. and Blake, M. S. (1998). "Multivalent pneumococcal capsular polysaccharide conjugate vaccines employing genetically detoxified pneumolysin as a carrier protein." Vaccine **16**(18): 1732-1741.

Miiro, G., Kayhty, H., Watera, C., Tolmie, H., Whitworth, J. A. G., Gilks, C. F. and French, N. (2005). "Conjugate pneumococcal vaccine in HIV-infected Ugandans and the effect of past receipt of polysaccharide vaccine." J Infect Dis **192**(10): 1801-1805.

Mitchell, T. J. and Andrew, P. W. (1997). "Biological properties of pneumolysin." Microb Drug Resist **3**(1): 19-26.

Mitchell, T. J., Andrew, P. W., Saunders, F. K., Smith, A. N. and Boulnois, G. J. (1991). "Complement activation and antibody binding by pneumolysin via a region of the toxin homologous to a human acute-phase protein." Mol Microbiol **5**(8): 1883-8.

Mitchell, T. J., Andrew, Boulnois, G.J., Lee, C.J., Lock, R.A. and Paton, J.C. (1992). "Molecular studies of pneumolysin, the thiol-activated toxin of *Streptococcus pneumoniae* as an aid to vaccine design." Bacterial protein toxins **23**: 429-438.

Mitchell, T. J., Mendez, F., Paton, J. C., Andrew, P. W. and Boulnois, G. J. (1990). "Comparison of pneumolysin genes and proteins from *Streptococcus pneumoniae* types 1 and 2." Nucleic Acids Res **18**(13): 4010.

Mitchell, T. J., Walker, J. A., Saunders, F. K., Andrew, P. W. and Boulnois, G. J. (1989). "Expression of the pneumolysin gene in *Escherichia coli*: rapid purification and biological properties." Biochim Biophys Acta **1007**(1): 67-72.

Miyaji, E. N., Dias, W. O., Gamberini, M., Gebara, V. C. B. C., Schenkman, R. P. F., Wild, J., Riedl, P., Reimann, J., Schirmbeck, R. and Leite, L. C. C. (2001). "PsaA (pneumococcal surface adhesin A) and PspA (pneumococcal surface protein A) DNA vaccines induce humoral and cellular immune responses against *Streptococcus pneumoniae*." Vaccine **20**(5-6): 805-812.

Miyaji, E. N., Dias, W. O., Tanizaki, M. M. and Leite, L. C. C. (2003). "Protective efficacy of pspA (pneumococcal surface protein A)-based DNA vaccines: contribution of both humoral and cellular immune responses." FEMS Immunology and Medical Microbiology **37**(1): 53-57.

Miyaji, E. N., Ferreira, D. M., Lopes, A. P. Y., Brandileone, M. C. C., Dias, W. O. and Leite, L. C. C. (2002). "Analysis of serum cross-reactivity and cross-protection elicited by immunization with DNA vaccines against *Streptococcus pneumoniae* expressing PspA fragments from different clades." Infect. Immun. **70**(9): 5086-5090.

Mizgerd, J. P. (2002). "Molecular mechanisms of neutrophil recruitment elicited by bacteria in the lungs." seminars in Immunology **14**: 123-132.

Mohammed, B. J., Mitchell, T. J., Andrew, P. W., Hirst, R. A. and O'Callaghan, C. (1999). "The effect of the pneumococcal toxin, pneumolysin on brain ependymal cilia." Microb Pathog **27**(5): 303-9.

Moreland, J. G. and Bailey, G. (2005). "Neutrophil transendothelial migration in vitro to *Streptococcus pneumoniae* is pneumolysin-dependent." Am J Physiol Lung Cell Mol Physiol.

Morgan, P. J., Hyman, S. C., Rowe, A. J., Mitchell, T. J., Andrew, P. W. and Saibil, H. R. (1995). "Subunit organisation and symmetry of pore-forming, oligomeric pneumolysin." FEBS Lett **371**(1): 77-80.

Morgan, P. J., Varley, P. G., Rowe, A. J., Andrew, P. W. and Mitchell, T. J. (1993). "Characterization of the solution properties and conformation of pneumolysin, the membrane-damaging toxin of *Streptococcus pneumoniae*." Biochem J **296 (Pt 3)**: 671-4.

Morris, P., Leach, A., Silberberg, P., Mellon, G., Wilson, C., Hamilton, E. and Beissbarth, J. (2005). "Otitis media in young Aboriginal children from remote communities in Northern and Central Australia: a cross-sectional survey." BMC Pediatrics **5**(1): 27.

Muller-Graf, C. D., Whatmore, A. M., King, S. J., Trzcinski, K., Pickerill, A. P., Doherty, N., Paul, J., Griffiths, D., Crook, D. and Dowson, C. G. (1999). "Population biology of *Streptococcus pneumoniae* isolated from oropharyngeal carriage and invasive disease." Microbiology **145 (Pt 11)**: 3283-93.

Murdoch, C., Read, R. C., Zhang, Q. and Finn, A. (2002). "Choline-binding protein A of *Streptococcus pneumoniae* elicits chemokine production and expression of intercellular adhesion molecule 1 (CD54) by human alveolar epithelial cells." J Infect Dis **186**(9): 1253-60.

Musher, D. M., Phan, H. M. and Baughn, R. E. (2001). "Protection against bacteremic pneumococcal infection by antibody to pneumolysin." J Infect Dis **183**(5): 827-30.

Nagamune, H., Ohkura, K., Sukeno, A., Cowan, G., Mitchell, T. J., Ito, W., Ohnishi, O., Hattori, K., Yamato, M., Hirota, K., Miyake, Y., Maeda, T. and Kourai, H. (2004). "The human-specific action of intermedilysin, a homolog of streptolysin o, is dictated by domain 4 of the protein." Microbiol Immunol **48**(9): 677-92.

Nandoskar, M., Ferrante, A., Bates, E. J., Hurst, N. and Paton, J. C. (1986). "Inhibition of human monocyte respiratory burst, degranulation, phospholipid methylation and bactericidal activity by pneumolysin." Immunology **59**(4): 515-20.

Naylor, S. W., Low, J. C., Besser, T. E., Mahajan, A., Gunn, G. J., Pearce, M. C., McKendrick, I. J., Smith, D. G. and Gally, D. L. (2003). "Lymphoid follicle-dense mucosa at the terminal rectum is the principal site of colonization of enterohemorrhagic *Escherichia coli* O157:H7 in the bovine host." Infect Immun **71**(3): 1505-12.

Neeleman, C., Klaassen, C. H. W., Klomberg, D. M., de Valk, H. A. and Mouton, J. W. (2004). "Pneumolysin is a key factor in misidentification of macrolide-resistant *Streptococcus pneumoniae* and is a putative virulence factor of *S. mitis* and other *Streptococci*." J. Clin. Microbiol. **42**(9): 4355-4357.

Nemeth, E., Rivera, S., Gabayan, V., Keller, C., Taudorf, S., Pedersen, B. K. and Ganz, T. (2004). "IL-6 mediates hypoferremia of inflammation by inducing the synthesis of the iron regulatory hormone hepcidin." J. Clin. Invest. **113**(9): 1271-1276.

N'Guessan P, D., Hippenstiel, S., Etouem, M. O., Zahlten, J., Beermann, W., Lindner, D., Opitz, B., Witzenrath, M., Rosseau, S., Suttorp, N. and Schmeck, B. (2006). "*Streptococcus pneumoniae*-induced p38 MAPK- and NF-{kappa}B-dependent COX-2 expression in human lung epithelium." Am J Physiol Lung Cell Mol Physiol.

Nurkka, A., Joensuu, J., Henckaerts, I., Peeters, P., Poolman, J., Kilpi, T. and Kayhty, H. (2004). "Immunogenicity and safety of the eleven valent pneumococcal polysaccharide-protein D conjugate vaccine in infants." Pediatr Infect Dis J **23**(11): 1008-14.

Obaro, S. K. (2000). "Prospects for pneumococcal vaccination in African children." Acta Trop **75**(2): 141-53.

Obaro, S. K. (2002). "The new pneumococcal vaccine." Clin Microbial Infect **8**(10): 623-633.

Obaro, S. K., Adegbola, R. A. and Banya, W. A. (1996). "Carriage of pneumococci after pneumococcal vaccination." Lancet **348**: 271-2.

Ogunniyi, A. D., Folland, R. L., Briles, D. E., Hollingshead, S. K. and Paton, J. C. (2000). "Immunization of mice with combinations of pneumococcal virulence proteins elicits enhanced protection against challenge with *Streptococcus pneumoniae*." Infect Immun **68**(5): 3028-33.

Ogunniyi, A. D., Woodrow, M. C., Poolman, J. T. and Paton, J. C. (2001). "Protection against *Streptococcus pneumoniae* elicited by immunization with pneumolysin and CbpA." Infect Immun **69**(10): 5997-6003.

Orihuela, C. J., Gao, G., Francis, K. P., Yu, J. and Tuomanen, E. I. (2004). "Tissue-specific contributions of pneumococcal virulence factors to pathogenesis." J Infect Dis **190**(9): 1661-9.

Orihuela, C. J., Gao, G., McGee, M., Yu, J., Francis, K. P. and Tuomanen, E. (2003). "Organ-specific models of Streptococcus pneumoniae disease." Scand J Infect Dis **35**(9): 647-52.

Ortqvist, A., Hedlund, J. and Burman, L. A. (1998). "Randomised trial of 23-valent pneumococcal capsular polysaccharide vaccine in prevention of pneumonia in middle-aged and elderly people." Lancet **351**: 399-403.

Overweg, K., Kerr, A., Sluijter, M., Jackson, M. H., Mitchell, T. J., de Jong, A. P., de Groot, R. and Hermans, P. W. (2000). "The putative proteinase maturation protein A of *Streptococcus pneumoniae* is a conserved surface protein with potential to elicit protective immune responses." Infect Immun **68**(7): 4180-8.

Owen, R. H., Boulnois, G. J., Andrew, P. W. and Mitchell, T. J. (1994). "A role in cell-binding for the C-terminus of pneumolysin, the thiol-activated toxin of *Streptococcus pneumoniae*." FEMS Microbiol Lett **121**(2): 217-21.

Pai, R., Gertz, R. E. and Beall, B. (2006). "Sequential multiplex PCR approach for determining capsular serotypes of *Streptococcus pneumoniae* isolates." J. Clin. Microbiol. **44**(1): 124-131.

Pai, R., Limor, J. and Beall, B. (2005a). "Use of pyrosequencing to differentiate *Streptococcus pneumoniae* serotypes 6A and 6B." J. Clin. Microbiol. **43**(9): 4820-4822.

Pai, R., Moore, M. R., Pilishvili, T., Gertz, R. E., Whitney, C. G., Beall, B. and Team, A. B. C. S. (2005b). "Postvaccine genetic structure of *Streptococcus pneumoniae* serotype 19A from children in the United States." J Infect Dis **192**(11): 1988-1995.

Palaniappan, R., Singh, S., Singh, U. P., Sakthivel, S. K. K., Ades, E. W., Briles, D. E., Hollingshead, S. K., Paton, J. C., Sampson, J. S. and Lillard, J. W., Jr. (2005). "Differential PsaA-, PspA-, PspC-, and PdB-specific immune responses in a mouse model of pneumococcal carriage." Infect. Immun. **73**(2): 1006-1013.

Palmer, M. (2001). "The family of thiol-activated, cholesterol-binding cytolysins." Toxicon **39**(11): 1681-9.

Parijs, B. A.-V. and Malinoski, F. J. (2004). "Post-marketing effectiveness of Prevnar [pneumococcal 7-valent conjugate vaccine (diphtheria CRM197 protein)] and implications for adult immunization." Mechanisms of Ageing and Development **125**(2): 147-148.

Paterson, G. K. (2003). PhD thesis: Identification and characterisation of novel pneumococcal virulence factors. Infection and Immunity. Glasgow, Glasgow University.

Paterson, G. K. and Mitchell, T. J. (2006a). "Innate immunity and the pneumococcus." Microbiology **152**(2): 285-293.

Paterson, G. K. and Mitchell, T. J. (2006b). "The role of *Streptococcus pneumoniae* sortase A in colonisation and pathogenesis." Microbes and Infection **8**: 145-53.

Paton, J. C. (1996). "The contribution of pneumolysin to the pathogenicity of Streptococcus pneumoniae." Trends Microbiol **4**(3): 103-6.

Paton, J. C., Andrew, P. W., Boulnois, G. J. and Mitchell, T. J. (1993). "Molecular analysis of the pathogenicity of *Streptococcus pneumoniae*: the role of pneumococcal proteins." Annu Rev Microbiol **47**: 89-115.

Paton, J. C. and Ferrante, A. (1983a). "Inhibition of human polymorphonuclear leukocyte respiratory burst, bactericidal activity, and migration by pneumolysin." Infect Immun **41**(3): 1212-6.

Paton, J. C., Lock, R. A. and Hansman, D. J. (1983b). "Effect of immunization with pneumolysin on survival time of mice challenged with *Streptococcus pneumoniae*." Infect Immun **40**(2): 548-52.

Paton, J. C., Lock, R. A., Lee, C. J., Li, J. P., Berry, A. M., Mitchell, T. J., Andrew, P. W., Hansman, D. and Boulnois, G. J. (1991). "Purification and immunogenicity of genetically obtained pneumolysin toxoids and their conjugation to Streptococcus pneumoniae type 19F polysaccharide." Infect Immun **59**(7): 2297-304.

Paton, J. C., Rowan-Kelly, B. and Ferrante, A. (1984). "Activation of human complement by the pneumococcal toxin pneumolysin." Infect Immun **43**(3): 1085-7.

Pebody, R., Leino, T., Nohynek, H., Hellenbrand, W., Salmaso, S. and Ruutu, P. (2005). "Pneumococcal vaccination policy in Europe." Euro Surveill **10**(9).

Peeters, C. C., Tenbergen-Meekes, A. M., Evenberg, D. E., Poolman, J. T., Zegers, B. J. and Rijkers, G. T. (1991). "A comparative study of the immunogenicity of pneumococcal type 4 polysaccharide and oligosaccharide tetanus toxoid conjugates in adult mice." J Immunol **146**(12): 4308-14.

Peltola, H. (2000). "Worldwide *Haemophilus influenzae* type b disease at the beginning of the 21st Century: Global analysis of the disease burden 25 years after the use of the polysaccharide vaccine and a decade after the advent of conjugates." Clin. Microbiol. Rev. **13**(2): 302-317.

Pelton, S. I., Dagan, R., Gaines, B. M., Klugman, K. P., Laufer, D., O'Brien, K. and Schmitt, H. J. (2003). "Pneumococcal conjugate vaccines: proceedings from an interactive symposium at the 41st Interscience Conference on Antimicrobial Agents and Chemotherapy." Vaccine **21**(15): 1562-71.

Pericone, C. D., Overweg, K., Hermans, P. and Weiser, J. N. (2000). "Inhibitory and bactericidal effects of hydrogen peroxide production by *Streptococcus pneumoniae* on other inhabitants of the upper respiratory tract." Infect Immun **68**(7): 3990-3997.

Polekhina, G., Giddings, K. S., Tweten, R. K. and Parker, M. W. (2005). "Insights into the action of the superfamily of cholesterol-dependent cytolysins from studies of intermedilysin." PNAS **102**(3): 600-605.

Polissi, A., Pontiggia, A., Feger, G., Altieri, M., Mottl, H., Ferrari, L. and Simon, D. (1998). "Large-scale identification of virulence genes from *Streptococcus pneumoniae*." Infect Immun **66**(12): 5620-5629.

Poulsen, K., Reinholdt, J. and Kilian, M. (1996). "Characterization of the *Streptococcus pneumoniae* immunoglobulin A1 protease gene (IgA) and its translation product." Infect. Immun. **64**(10): 3957-3966.

Prellner, K., Hermansson, A., White, P., Melhus, A. and Briles, D. (1999). "Immunization and protection in pneumococcal otitis media studied in a rat model." Microb Drug Resist **5**(1): 73-82.

Quin, L. R., Carmicle, S., Dave, S., Pangburn, M. K., Evenhuis, J. P. and McDaniel, L. S. (2005). "*In vivo* binding of complement regulator Factor H by *Streptococcus pneumoniae*." J Infect Dis **192**(11): 1996-2003.

Ramachandran, R., Heuck, A. P., Tweten, R. K. and Johnson, A. E. (2002). "Structural insights into the membrane-anchoring mechanism of a cholesterol-dependent cytolysin." Nat Struct Biol **9**(11): 823-7.

Ramachandran, R., Tweten, R. K. and Johnson, A. E. (2004). "Membrane-dependent conformational changes initiate cholesterol-dependent cytolysin oligomerization and intersubunit -strand alignment." Nat Struct and Mol Biol **11**: 697-705.

Ramachandran, R., Tweten, R. K. and Johnson, A. E. (2005). "The domains of a cholesterol-dependent cytolysin undergo a major FRET-detected rearrangement during pore formation." Proc Natl Acad Sci U S A **102**(20): 7139-44.

Rayner, C., Jackson, A., Rutman, A., Dewar, A., Mitchell, T., Andrew, P., Cole, P. and Wilson, R. (1995). "Interaction of pneumolysin-sufficient and -deficient isogenic variants of *Streptococcus pneumoniae* with human respiratory mucosa." Infect. Immun. **63**(2): 442-447.

Remick, D. G., Bolgos, G., Copeland, S. and Siddiqui, J. (2005). "Role of Interleukin-6 in mortality from and physiologic response to sepsis." Infect. Immun. **73**(5): 2751-2757.

Ren, B., Szalai, A. J., Hollingshead, S. K. and Briles, D. E. (2004). "Effects of PspA and antibodies to PspA on activation and deposition of complement on the pneumococcal surface." Infect. Immun. **72**(1): 114-122.

Reynolds, H. Y. (1987). "Lung inflammation: normal host defense or a complication of some diseases?" Annual Review of Medicine **38**(1): 295-323.

Rijneveld, A. W., Lauw, F. N., Schultz, M. J., Florquin, S., Te Velde, A. A., Speelman, P., Van Deventer, S. J. and Van Der Poll, T. (2002a). "The role of interferon-gamma in murine pneumococcal pneumonia." J Infect Dis **185**(1): 91-7.

Rijneveld, A. W., van den Dobbelsteen, G. P., Florquin, S., Standiford, T. J., Speelman, P., van Alphen, L. and van der Poll, T. (2002b). "Roles of interleukin-6 and macrophage inflammatory protein-2 in pneumolysin-induced lung inflammation in mice." J Infect Dis **185**(1): 123-6.

Roberts, N. J. (1979). "Temperature and host defense." Microbiol Rev **43**(2): 241-259.

Rogers, P. D., Thornton, J., Barker, K. S., McDaniel, D. O., Sacks, G. S., Swiatlo, E. and McDaniel, L. S. (2003). "Pneumolysin-dependent and -independent gene expression identified by cDNA microarray analysis of THP-1 human mononuclear cells stimulated by *Streptococcus pneumoniae*." Infect Immun **71**(4): 2087-94.

Romanovsky, A. A. and Szekely, M. (1998). "Fever and hypothermia: two adaptive thermoregulatory responses to systemic inflammation." Medical Hypotheses **50**: 219-226.

Rosenow, C., Ryan, P., Weiser, J. N., Johnson, S., Fontan, P., Ortqvist, A. and Masure, H. R. (1997). "Contribution of novel choline-binding proteins to adherence, colonization and immunogenicity of *Streptococcus pneumoniae*." Mol Microbiol **25**(5): 819-29.

Rossjohn, J., Feil, S. C., McKinstry, W. J., Tweten, R. K. and Parker, M. W. (1997). "Structure of a cholesterol-binding, thiol-activated cytolysin and a model of its membrane form." Cell **89**(5): 685-92.

Rubins, J., Charboneau, D., Fasching, C., Berry, A., Paton, J., Alexander, J., Andrew, P., Mitchell, T. and Janoff, E. (1996). "Distinct roles for pneumolysin's cytotoxic and complement activities in the pathogenesis of pneumococcal pneumonia." Am. J. Respir. Crit. Care Med. **153**(4): 1339-1346.

Rubins, J. B., Charboneau, D., Paton, J. C., Mitchell, T. J., Andrew, P. W. and Janoff, E. N. (1995). "Dual function of pneumolysin in the early pathogenesis of murine pneumococcal pneumonia." J Clin Invest **95**(1): 142-50.

Rubins, J. B., Duane, P. G., Charboneau, D. and Janoff, E. N. (1992). "Toxicity of pneumolysin to pulmonary endothelial cells in vitro." Infect Immun **60**(5): 1740-6.

Rubins, J. B., Duane, P. G., Clawson, D., Charboneau, D., Young, J. and Niewoehner, D. E. (1993). "Toxicity of pneumolysin to pulmonary alveolar epithelial cells." Infect Immun **61**(4): 1352-8.

Rubins, J. B., Mitchell, T. J., Andrew, P. W. and Niewoehner, D. E. (1994). "Pneumolysin activates phospholipase A in pulmonary artery endothelial cells." Infect Immun **62**(9): 3829-36.

Rubins, J. B., Paddock, A. H., Charboneau, D., Berry, A. M., Paton, J. C. and Janoff, E. N. (1998). "Pneumolysin in pneumococcal adherence and colonization." Microb Pathog **25**(6): 337-42.

Salo, P., Narvanen, A. and Leinonen, M. (1993). "Mapping of immunoreactive sites of pneumococcal pneumolysin by use of synthetic peptides." Infect Immun **61**(7): 2822-6.

Sandgren, A., Albiger, B., Orihuela, C. J., Tuomanen, E., Normark, S. and Henriques-Normark, B. (2005). "Virulence in mice of pneumococcal clonal types with known invasive disease potential in humans." J Infect Dis **192**(5): 791-800.

Sandgren, A., Sjostrom, K., Olsson-Liljequist, B., Christensson, B., Samuelsson, A., Kronvall, G. and Henriques Normark, B. (2004). "Effect of clonal and serotype-specific properties on the invasive capacity of *Streptococcus pneumoniae*." J Infect Dis **189**(5): 785-96.

Sato, K., Quartey, M. K., Liebeler, C. L., Le, C. T. and Giebink, G. S. (1996). "Roles of autolysin and pneumolysin in middle ear inflammation caused by a type 3 *Streptococcus pneumoniae* strain in the chinchilla otitis media model." Infect Immun **64**(4): 1140-5.

Saukkoriipi, A., Palmu, A., Kilpi, T. and Leinonen, M. (2002). "Real-time quantitative PCR for the detection of *Streptococcus pneumoniae* in the middle ear fluid of children with acute otitis media." Mol Cell Probes **16**(5): 385-90.

Scott, J. A., Hall, A. J., Dagan, R., Dixon, J., Eykyn, S. J., Fenoll, A., Hortal, M., Jette, L. P., Jorgensen, J. H., Lamothe, F., Latore, C., Macfarlane, J. T., Shlaes, D. M., Smart, L. E. and Taunay, A. (1996). "Serogroup-specific epidemiology of *Streptococcus pneumoniae*:associations with age, sex and geography in 7000 episodes of invasive disease." Clin Infect Dis **22**: 973-81.

Search, J. J. (2002). PhD thesis: The role of pneumolysin in pro-inflammatory mediator production. Infection and Immunity. Glasgow, University of Glasgow: 269.

Shannon, J. G., Ross, C. L., Koehler, T. M. and Rest, R. F. (2003). "Characterization of anthrolysin O, the *Bacillus anthracis* cholesterol-dependent cytolysin." Infect Immun **71**(6): 3183-9.

Shatursky, O., Heuck, A. P., Shepard, L. A., Rossjohn, J., Parker, M. W., Johnson, A. E. and Tweten, R. K. (1999). "The mechanism of membrane insertion for a cholesterol-dependent cytolysin: a novel paradigm for pore-forming toxins." Cell **99**(3): 293-9.

Shepard, L. A., Heuck, A. P., Hamman, B. D., Rossjohn, J., Parker, M. W., Ryan, K. R., Johnson, A. E. and Tweten, R. K. (1998). "Identification of a membrane-spanning domain of the thiol-activated pore-forming toxin *Clostridium perfringens* perfringolysin O: an alpha-helical to beta-sheet transition identified by fluorescence spectroscopy." Biochemistry **37**(41): 14563-74.

Shepard, L. A., Shatursky, O., Johnson, A. E. and Tweten, R. K. (2000). "The mechanism of pore assembly for a cholesterol-dependent cytolysin: formation of a large prepore complex precedes the insertion of the transmembrane beta-hairpins." Biochemistry **39**(33): 10284-93.

Sirotnak, A. P., Eppes, S. C. and Klein, J. D. (1996). "Tuboovarian abscess and peritonitis caused by *Streptococcus pneumoniae* serotype 1 in young girls." Clin Infect Dis **22**(6): 993-6.

Sjostrom, K., Spindler, C., Ortqvist, A., Kalin, M., Sandgren, A., Kuhlmann-Berenzon, S. and Henriques-Normark, B. (2006). "Clonal and capsular types decide whether pneumococci will act as a primary or opportunistic pathogen." Clin Infect Dis **42**(4): 451-459.

Smart, L. E. (1987). The serology of pneumococcal Infection. A study of the laboratory diagnosis of pneumococcal infection and the distribution of pneumococcal type, University of Glasgow.

Solovyova, A. S., Nollmann, M., Mitchell, T. J. and Byron, O. (2004). "The solution structure and oligomerization behavior of two bacterial toxins: Pneumolysin and Perfringolysin O." Biophys. J. **87**(1): 540-552.

Spellerberg, B., Cundell, D. R., Sandros, J., Pearce, B. J., Idanpaan-Heikkila, I., Rosenow, C. and Masure, H. R. (1996). "Pyruvate oxidase, as a determinant of virulence in *Streptococcus pneumoniae*." Mol Microbiol **19**(4): 803-13.

Spratt, B. G. and Greenwood, B. M. (2000). "Prevention of pneumococcal disease by vaccination: does serotype replacement matter?" The Lancet **356**: 1210-1211.

Spreer, A., Kerstan, H., Bottcher, T., Gerber, J., Siemer, A., Zysk, G., Mitchell, T., Eiffert, H. and Nau, R. (2003a). "Reduced release of pneumolysin by *Streptococcus pneumoniae in vitro* and *in vivo* after treatment with nonbacteriolytic antibiotics in comparison to ceftriaxone." Antimicrob Agents Chemother **47**(8): 2649-2654.

Spreer, A., Kerstan, H., Bottcher, T., Gerber, J., Siemer, A., Zysk, G., Mitchell, T. J., Eiffert, H. and Nau, R. (2003b). "Reduced Release of Pneumolysin by Streptococcus pneumoniae In Vitro and In Vivo after Treatment with Nonbacteriolytic Antibiotics in Comparison to Ceftriaxone." Antimicrob. Agents Chemother. **47**(8): 2649-2654.

Srivastava, A., Henneke, P., Visintin, A., Morse, S. C., Martin, V., Watkins, C., Paton, J. C., Wessels, M. R., Golenbock, D. T. and Malley, R. (2005). "The apoptotic response to pneumolysin is toll-like receptor 4 dependent and protects against pneumococcal disease." Infect. Immun. **73**(10): 6479-6487.

Stassen, M., Muller, C., Richter, C., Neudorf, C., Hultner, L., Bhadki, S., Walev, I. and Schmitt, E. (2003). "The Streptococcal exotoxin Streptolysin O activates mast cells to produce tumor necrosis factor alpha by p38 mitogen-activated protein kinase and protein kinase C-dependent pathways." Infect Immun **71**(11): 6171-6177.

Steinfort, C., Wilson, R., Mitchell, T., Feldman, C., Rutman, A., Todd, H., Sykes, D., Walker, J., Saunders, K., Andrew, P. W. and et al. (1989). "Effect of *Streptococcus pneumoniae* on human respiratory epithelium in vitro." Infect Immun **57**(7): 2006-13.

Strieter, R. M., Belperio, J. A. and Keane, M. P. (2003). "Host innate defences in the lung: the role of cytokines." Curr Opin Infect Dis **16**: 193-198.

Suarez-Alvarez, B., Garcia-Suarez, M. d. M., Mendez, F. J. and de los Toyos, J. R. (2003). "Characterisation of mouse monoclonal antibodies for pneumolysin: fine epitope mapping and V gene usage." Immunology Letters **88**(3): 227-239.

Sung, C. K., Li, H., Claverys, J. P. and Morrison, D. A. (2001). "An rpsL cassette, janus, for gene replacement through negative selection in *Streptococcus pneumoniae*." Appl Environ Microbiol **67**(11): 5190-6.

Talkington, D. F., Brown, B. G., Tharpe, J. A., Koenig, A. and Russell, H. (1996). "Protection of mice against fatal pneumococcal challenge by immunization with pneumococcal surface adhesin A (PsaA)." Microb Pathog **21**(1): 17-22.

Tan, T. Q., Mason, E. O., Jr, Wald, E. R., Barson, W. J., Schutze, G. E., Bradley, J. S., Givner, L. B., Yogev, R., Kim, K. S. and Kaplan, S. L. (2002). "Clinical characteristics of children with complicated pneumonia caused by *Streptococcus pneumoniae*." Pediatrics **110**(1): 1-6.

Tanigawa, T., Suzuki, J., Ueta, T., Katsumoto, T. and Tanaka, Y. (1996). "Different sensitivity to streptolysin-O of cells in macrophage lineage." Microbiol Immunol **40**(1): 81-4.

Tettelin, H., Nelson, K. E., Paulsen, I. T., Eisen, J. A., Read, T. D., Peterson, S., Heidelberg, J., DeBoy, R. T., Haft, D. H., Dodson, R. J., Durkin, A. S., Gwinn, M., Kolonay, J. F., Nelson, W. C., Peterson, J. D., Umayam, L. A., White, O., Salzberg, S. L., Lewis, M. R., Radune, D., Holtzapple, E., Khouri, H., Wolf, A. M., Utterback, T. R., Hansen, C. L., McDonald, L. A., Feldblyum, T. V., Angiuoli, S., Dickinson, T., Hickey, E. K., Holt, I. E., Loftus, B. J., Yang, F., Smith, H. O., Venter, J. C., Dougherty, B. A., Morrison, D. A., Hollingshead, S. K. and Fraser, C. M. (2001). "Complete genome sequence of a virulent isolate of *Streptococcus pneumoniae*." Science **293**(5529): 498-506.

Thornton, J. and McDaniel, L. S. (2005). "THP-1 monocytes up-regulate intercellular adhesion molecule 1 in response to pneumolysin from *Streptococcus pneumoniae*." Infect. Immun. **73**(10): 6493-6498.

Tilley, S. J., Orlova, E. V., Gilbert, R., Andrew, P. and Saibil, H. R. (2005). "Structural basis of pore formation by the bacterial toxin pneumolysin." Cell **121**: 247-256.

Toikka, P., Nikkari, S., Ruuskanen, O., Leinonen, M. and Mertsola, J. (1999). "Pneumolysin PCR-based diagnosis of invasive pneumococcal infection in children." J Clin Microbiol **37**(3): 633-7.

Tomasz, A. (1997). "Antibiotic resistance in *Streptococcus pneumoniae*." Clin Infect Dis **24**(suppl1): S85-88.

Tong, H. H., Blue, L. E., James, M. A. and DeMaria, T. F. (2000). "Evaluation of the virulence of a *Streptococcus pneumoniae* neuraminidase-deficient mutant in nasopharyngeal colonization and development of otitis media in the chinchilla model." Infect Immun **68**(2): 921-4.

Tong, H. H., Li, D., Chen, S., Long, J. P. and Demaria, T. F. (2005). "Immunization with recombinant *Streptococcus pneumoniae* neuraminidase NanA protects chinchillas against nasopharyngeal colonization." Infect Immun **73**(11): 7775-8.

Tsai, C. M. and Frasch, C. E. (1982). "A sensitive silver stain for detecting lipopolysaccharides in polyacrylamide gels." Anal Biochem **119**: 115-9.

Tseng, H.-J., McEwan, A. G., Paton, J. C. and Jennings, M. P. (2002). "Virulence of *Streptococcus pneumoniae*: PsaA mutants are hypersensitive to oxidative stress." Infect. Immun. **70**(3): 1635-1639.

Tu, A.-H. T., Fulgham, R. L., McCrory, M. A., Briles, D. E. and Szalai, A. J. (1999). "Pneumococcal surface protein A inhibits complement activation by *Streptococcus pneumoniae*." Infect. Immun. **67**(9): 4720-4724.

Tuomanen, E. (1999). "Molecular and cellular biology of pneumococcal infection." Curr Opin Microbiol **2**(1): 35-9.

Tuomanen, E. I. and Masure, H. R. (1997). "Molecular and cellular biology of pneumococcal infection." Microb Drug Resist **3**(4): 297-308.

Tweten, R. K., Parker, M. W. and Johnson, A. E. (2001). "The cholesterol-dependent cytolysins." Curr Top Microbiol Immunol **257**: 15-33.

Vainio, A., Fagerlund, R., Melen, K., Lehtinen, M. J., Julkunen, I. and Virolainen, A. (2006). "Serum antibodies to putative proteinase maturation protein A in children with acute otitis media." Vaccine **24**: 1792-9.

van der Poll, T., Marchant, A., Keogh, C. V., Goldman, M. and Lowry, S. F. (1996). "Interleukin-10 impairs host defense in murine pneumococcal pneumonia." J Infect Dis **174**(5): 994-1000.

van der Sluijs, K. F., van Elden, L. J., Nijhuis, M., Schuurman, R., Florquin, S., Shimizu, T., Ishii, S., Jansen, H. M., Lutter, R. and van der Poll, T. (2006). "Involvement of the platelet activating factor receptor in host defense against *Streptococcus*

pneumoniae during postinfluenza pneumonia." Am J Physiol Lung Cell Mol Physiol **290**: L194-9.

van Ginkel, F. W., McGhee, J. R., Watt, J. M., Campos-Torres, A., Parish, L. A. and Briles, D. E. (2003). "Pneumococcal carriage results in ganglioside-mediated olfactory tissue infection." Proc Natl Acad Sci U S A **100**(24): 14363-7.

van Rossum, A. M., Lysenko, E. S. and Weiser, J. N. (2005). "Host and bacterial factors contributing to the clearance of colonization by *Streptococcus pneumoniae* in a murine model." Infect Immun **73**(11): 7718-26.

van Selm, S., Adrian, P. V., Estevao, S., van Roosmalen, M., Audouy, S., Neef, J., Nieuwenhuis, E. E. S., de Groot, R., Robillard, G. T., Hermans, P. W. M. and Leenhouts, K. (2005). *Lactococcus lactis* ghosts displaying pneumococcal protein antigens: a mucosal vaccine. 7th European meeting on the molecular biology of the pneumococcus, Braunschweig, Germany.

Virolainen, A., Jero, J., Kayhty, H., Karma, P., Leinonen, M. and Eskola, J. (1995). "Antibodies to pneumolysin and pneumococcal capsular polysaccharides in middle ear fluid of children with acute otitis media." Acta Otolaryngol **115**(6): 796-803.

Virolainen, A., Salo, P., Jero, J., Karma, P., Eskola, J. and Leinonen, M. (1994). "Comparison of PCR assay with bacterial culture for detecting *Streptococcus pneumoniae* in middle ear fluid of children with acute otitis media." J Clin Microbiol **32**(11): 2667-70.

Walker, J. A., Allen, R. L., Falmagne, P., Johnson, M. K. and Boulnois, G. J. (1987). "Molecular cloning, characterization, and complete nucleotide sequence of the gene for pneumolysin, the sulfhydryl-activated toxin of *Streptococcus pneumoniae*." Infect Immun **55**(5): 1184-9.

Walport, M. J. (2001). "Complement (first of two parts)." N Engl J Med **344**(14): 1058-1066.

Waltz, T. (2005). "How cholesterol-dependent cytolysins bite holes into membranes." Mol Cell **18**: 393-394.

Wang, E., Simard, M., Ouellet, N., Bergeron, Y., Beauchamp, D. and Bergeron, M. G. (2000). "Modulation of cytokines and chemokines, limited pulmonary vascular bed permeability, and prevention of septicemia and death with ceftriaxone and interleukin-10 in pneumococcal pneumonia." J Infect Dis **182**(4): 1255-9.

Wani, J., Gilbert, J., Plaut, A. and Weiser, J. (1996). "Identification, cloning, and sequencing of the immunoglobulin A1 protease gene of *Streptococcus pneumoniae*." Infect. Immun. **64**(10): 3967-3974.

Wasfy, M. O., Pimentel, G., Abdel-Maksoud, M., Russell, K. L., Barrozo, C. P., Klena, J. D., Earhart, K. and Hajjeh, R. (2005). "Antimicrobial susceptibility and serotype distribution of *Streptococcus pneumoniae* causing meningitis in Egypt, 1998-2003." J Antimicrob Chemother **55**(6): 958-64.

Weintraub, A. (2003). "Immunology of bacterial polysaccharide antigens." Carbohydrate Research **338**(23): 2539-2547.

Weis, S. and Palmer, M. (2001). "Streptolysin O: the C-terminal, tryptophan-rich domain carries functional sites for both membrane binding and self-interaction but not for stable oligomerization." Biochim Biophys Acta **1510**(1-2): 292-9.

Weiser, J. N., Bae, D., Fasching, C., Scamurra, R. W., Ratner, A. J. and Janoff, E. N. (2003). "Antibody-enhanced pneumococcal adherence requires IgA1 protease." Proc Natl Acad Sci U S A **100**(7): 4215-20.

Wellmer, A., Zysk, G., Gerber, J., Kunst, T., Von Mering, M., Bunkowski, S., Eiffert, H. and Nau, R. (2002). "Decreased virulence of a pneumolysin-deficient strain of *Streptococcus pneumoniae* in murine meningitis." Infect Immun **70**(11): 6504-8.

Wessels, M. R., Butko, P., Ma, M., Warren, H. B., Lage, A. L. and Carroll, M. C. (1995). "Studies of group B streptococcal infection in mice deficient in complement

component C3 or C4 demonstrate an essential role for complement in both innate and acquired immunity." Proc Natl Acad Sci U S A **92**(25): 11490-4.

Westh, H., Skibsted, L. and Korner, B. (1990). "*Streptococcus pneumoniae* infections of the female genital tract and in the newborn child." Rev Infect Dis **12**(3): 416-22.

Whalan, R. H., Funnell, S. G. P., Bowler, L. D., Hudson, M. J., Robinson, A. and Dowson, C. G. (2006). "Distribution and genetic diversity of the ABC transporter lipoproteins PiuA and PiaA within *Streptococcus pneumoniae* and related *Streptococci*." J. Bacteriol. **188**(3): 1031-1038.

Wheeler, J., Freeman, R., Steward, M., Henderson, K., Lee, M. J., Piggott, N. H., Eltringham, G. J. and Galloway, A. (1999). "Detection of pneumolysin in sputum." J Med Microbiol **48**(9): 863-6.

White, P., Hermansson, A., Svanborg, C., Briles, D. and Prellner, K. (1999). "Effects of active immunization with a pneumococcal surface protein (PspA) and of locally applied antibodies in experimental otitis media." ORL J Otorhinolaryngol Relat Spec **61**(4): 206-11.

Whitney, C. G. (2005). What have you 'herd' about immunity? Interscience Conference on Antimicrobials Agents and Chemotherapy, Washington, D.C.

Winter, A. J., Comis, S. D., Osborne, M. P., Tarlow, M. J., Stephen, J., Andrew, P. W., Hill, J. and Mitchell, T. J. (1997). "A role for pneumolysin but not neuraminidase in the hearing loss and cochlear damage induced by experimental pneumococcal meningitis in guinea pigs." Infect Immun **65**(11): 4411-8.

Wizemann, T. M., Heinrichs, J. H., Adamou, J. E., Erwin, A. L., Kunsch, C., Choi, G. H., Barash, S. C., Rosen, C. A., Masure, H. R., Tuomanen, E., Gayle, A., Brewah, Y. A., Walsh, W., Barren, P., Lathigra, R., Hanson, M., Langermann, S., Johnson, S. and Koenig, S. (2001). "Use of a whole genome approach to identify vaccine molecules affording protection against *Streptococcus pneumoniae* infection." Infect Immun **69**(3): 1593-8.

Wu, H. Y., Nahm, M. H., Guo, Y., Russell, M. W. and Briles, D. E. (1997). "Intranasal immunization of mice with PspA (pneumococcal surface protein A) can prevent intranasal carriage, pulmonary infection, and sepsis with *Streptococcus pneumoniae*." J Infect Dis **175**(4): 839-46.

Young, N., Levin, J. and Prendergast, R. A. (1972). "An invertebrate coagulation system activated by endotoxin: evidence for enzymatic mediation." J. Clin. Invest. **51**: 1790-1797.

Zhang, J. R., Mostov, K. E., Lamm, M. E., Nanno, M., Shimada, S., Ohwaki, M. and Tuomanen, E. (2000). "The polymorphic immunoglobulin receptor translocates pneumococci accross human nasopharyngeal epithelial cells." Cell **102**(6): 827-37.

Zhang, Y., Masi, A. W., Barniak, V., Mountzouros, K., Hostetter, M. K. and Green, B. A. (2001). "Recombinant PhpA protein, a unique histidine motif-containing protein from *Streptococcus pneumoniae*, protects mice against intranasal pneumococcal challenge." Infect. Immun. **69**(6): 3827-3836.

Ziegler-Heitbrock, H. W., Passlick, B., Kafferlein, E., Coulie, P. G. and Izbicki, J. R. (1992). "Protection against lethal pneumococcal septicemia in pigs is associated with decreased levels of interleukin-6 in blood." Infect Immun **60**(4): 1692-4.

Zwijnenburg, P. J., Polfliet, M. M., Florquin, S., van den Berg, T. K., Dijkstra, C. D., van Deventer, S. J., Roord, J. J., van der Poll, T. and van Furth, A. M. (2003). "CXC-chemokines KC and macrophage inflammatory protein-2 (MIP-2) synergistically induce leukocyte recruitment to the central nervous system in rats." Immunol Lett **85**(1): 1-4.

Zysk, G., Bejo, L., Schneider-Wald, B. K., Nau, R. and Heinz, H. (2000). "Induction of necrosis and apoptosis of neutrophil granulocytes by *Streptococcus pneumoniae*." Clin Exp Immunol **122**(1): 61-6.

Zysk, G., Schneider-Wald, B. K., Hwang, J. H., Bejo, L., Kim, K. S., Mitchell, T. J., Hakenbeck, R. and Heinz, H. P. (2001). "Pneumolysin is the main inducer of

cytotoxicity to brain microvascular endothelial cells caused by *Streptococcus pneumoniae*." Infect Immun **69**(2): 845-52.