A Fusion Protein Based Pneumococcal Vaccine

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BY

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Author's declaration

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

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Abstract

The Gram-positive bacterium *Streptococcus pneumoniae* (pneumococcus) is a major human pathogen, causing Otitis media, pneumonia, bacteraemia and meningitis worldwide. There are over 90 different serotypes of pneumococcus and current pneumococcal vaccines are somewhat limited in their protection against pneumococcal diseases. Currently available vaccines are based on the capsular polysaccharide. The 23-valent polysaccharide (PSP) vaccine can protect most of adults and children older than five, but it cannot protect children under age 2, immunodeficiency patients and cannot protect the elderly very well. The polysaccharide/ protein conjugated vaccines which are complementary to the 23-valent PS vaccine have been introduced. The PSP conjugate vaccine is highly immunogenic and protects children under the age of 2 and immunodeficiency patients against invasive pneumococcal diseases, but its efficacy is threatened by strain replacement and serotype switching since it only protects against the limited number of serotypes contained within the vaccine. There is therefore a need to develop improved vaccines against pneumococcal diseases.

Pneumolysin (PLY), a cholesterol dependent cytolysin and an important virulence factor of pneumococcus, can act as a powerful mucosal adjuvant to induce both systemic and mucosal immunity to proteins genetically fused to PLY. Which regions of PLY are required for the novel adjuvant activity is not currently known. The model antigen eGFP was fused to domain 1-3 of PLY (D123PLY) and domain 4 of PLY (D4PLY) by ligation-dependent DNA recombinant technology during my master project. Balb/c mice were intranasally immunised with purified eGFPD4PLY or eGFPD123PLY fusion protein and immune response were then monitored by enzyme linked immunosorbent assay (ELISA). The ELISA data shows truncated PLY lost the adjuvant property and this adjuvant property was restored in the presence of free PLY or Δ6PLY when antigen was fused with truncated PLY.

The aim of the current work is to determine whether a fusion protein based pneumococcal vaccine can be developed to provide protection against three pneumococcal strains in animal models of colonisation and invasive diseases. For this purpose, pneumococcal virulence factors, PsaA, PspA, PspC and PhtD were

genetically fused to PLY or Δ6PLY separately by In-fusion cloning technology. Δ6PLY is a toxoid that lacks haemolytic activity but retains the immunogenic and adjuvant activity of PLY. MF1 mice were then vaccinated either intranasally or subcutanously by purified fusion protein antigens and immune responses were also monitored by ELISA; vaccinated mice were finally challenged intranasally by three strains of pneumococcus and monitored for colonisation. The immunity elicited by pneumococcal antigens fused to PLY/Δ6 PLY is protective in a colonisation model.

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Abbreviations

α	Anti
°C	Degree Celsius
μg	micro gram
μl	micro litre
μm	micrometer
μM	micro molar
ABC	ATP-binding cassette
AEC	Anion Exchange Chromatography
AOM	Acute Otitis Media
APC	Antigen Presenting Cell
APS	Ammonium Persulphate
B cell	B lymphocytes
BAB	Blood Agar Plate
BHI	Brain Heart Infusion
BMM	Bone Marrow Macrophage
BSA	Bovine serum albumin
CBP	Choline-Binding protein
CDC	Cholesterol-Dependent Cytolysin
cfu	clony forming unit
Clp	Caseinolytic protease
CSF	Cerebrospinal Fluid
CT	Cholera Toxin
CTB	Cholera Toxin B Fragment
DC	Dendritic Cell

DPBS	Dulbecco"s Phosphate Buffer Saline
DH5α *E.coli*	Library Efficiency® DH5α™ Chemically Competent *E.coli*
DNA	Deoxyribonucleic Acid
dNTP	deoxyribonucletide triphasphate
E. coli	Escherichia coli
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced Green Fluorescent Protein
eGFPD123PLY	eGFP fused with domain 1-3 of pneumolysin
eGFPD4PLY	eGFP fused with domain 4 of pneumolysin
ELISA	Enzyme Linked Immunosorbent Assay
EU	Endotoxin Units
FAE	Follicule-Associated Epithelium
g	Gram
g	Centrifugal Force
GFP	Green Fluorescent Protein
dH_2O	Distilling water
H_2O	Water
H_2O_2	Hydrogen Peroxide
His-Tag	Histidine Affinity Tag
hpi	hours post infection
HtrA	High temperature requirement A
Hyl	Hyaluronidase
HU	Haemolytic Units
IFN-γ	Interferon Gamma
Ig	Immunoglobin
IL	Interleukin

ILY	Intermedialysin
i.n.	Intranasal
i.p.	Intraperitoneal
IPTG	Isoproyl-β-D-Thiogalactopyranoside
Kb	Kilobase
kDa	Kilodalton
L	Litre
LLO	Listeriolysin O
LB	Luria Broth
LPS	Lipopolysaccharide
LPXTG	L(lysine)P(proline)X(any amino acid)T(Tyrosine)G(glysine)
Lyt	Autolysin
M	Molar
M cell	Microfold cell
mg	Milligram(s)
ml	Millilitre
mM	Millimolar
MW	Molecular Weight
NAC	Nickel Affinity Chromatography
Nan	Neuraminidase
NO	Nitric Oxide
OD	Optical Density
PavA	Pneumococcal adhesion and virulence A
PBS	Phosphate Buffer Saline
PBS-T	Phosphate Buffer Saline Tween
PclA	Pneumococcal collagen-like protein A
PCR	Polymerase Chain Reaction

PcsB	Protein required for cell wall separation of group B streptococcus
PdB	Pneumolysin carrying W433F substitution
PfbA	Plasmin and fibronectin-binding protein A
PFO	Perfringolysin O
PG	Peptidoglycan
Pht	Pneumococcal Histidine Triad Protein
Pia	Pneumococcal Iron Acquisition Protein
Piu	Pneumococcal Iron Uptake Protein
PLY	Pneumolysin
PpmA	Pneumococcal proteinase maturation protein A
PppA	Pneumococcal protective protein A
PPV	Pneumococcal Polysaccharide Vaccine
PrtA	Pneumococcal serine protease A
PotD	Substrate-binding protein of a polyamine ABC transporter
Psa	Pneumococcal surface adhesion protein
Psp	Pneumococcal surface protein
PS	Polysaccharide
PV	Polysaccharide Vaccine
rpm	Revolutions per minute
ROS	Reactive Oxygen Species
RrgA/B/C	Pneumococcal pilus 1 subunit A/B/C
S.pneumoniae	Streptococcus pneumoniae
SDM	Site-Directed Mutagenesis
SDS-PAGE	Sodium Dodecyl Sulphate-Polyarylamide Gel Electrophoresis
SrlA	Streptococcal lipoprotein rotamase A
SrtA	Sortase A

SEM	Standard Error of the Mean
SLO	Streptolysin O
ST	Sequence Type
StkP	Serine/threonine protein kinase
T cell	T lymphocytes
TEM	Transmission Electron Microscopy
TIGR	the Institute for Genomic Research
TLD	Traditional Ligation Dependent
TLR	Toll-like Receptor
TNF	Tumour Necrosis Factor
TT	Tetanus Toxin
TTCF	Tetanus Toxin C fragment
V	Volts
WT	wild type
Xen10	Bioluminescent *S. pneumoniae* strain serotype 3 A66.1
Xen35	Bioluminescent *S. pneumoniae* strain serotype 4 TIGR4
ZmpB	Zinc metalloprotease

Chapter 1 Introduction

1.1 Streptococcus pneumoniae

The Gram-positive bacterium S. pneumoniae (the pneumococcus) is a major pathogen causing community -acquired disease worldwide. The facultative anaerobe was probably first recognised by Edwin Klebs in infected sputum and lung tissue in 1875, and then isolated by Sternberg and Pasteur simultaneously in 1880 (Austrian 1999). The organism was initially named as *Diplococcus pneumoniae* and reclassified as S. pneumoniae in 1974 (Austrian 1999). At present, pneumococcus contains 93 serotypes depending on the immunochemistry of their capsular polysaccharides (PS)(Henrichsen 1995; Park *et al*. 2007). However, not all serotypes cause diseases. There are differences in serotype distribution. For example, serotype 1 was more likely to cause diseases in healthy adults whereas with invasive pneumococcal diseases (IPD) from serotype 11A are associated with adult patients with underlying diseases such as cancer and HIV (Sjostrom *et al*. 2006). The Quellung (swelling) reaction, used for determination of serotypes, was developed by Neufeld in 1902 using antisera raised against homologous capsular PS. The classification of pneumococcal serotypes was originally by chronological order of discovery (Dochez & Gillespie 1913), however, cross-reactivity between different serotypes result the reclassification of pneumococcal serotypes by the Danish nomenclature. The Danish system can reflect both the unique and the cross reactivity of 93 PS serotypes by placing them into 46 serogroups. For example, the serotypes 1 and 2 remain the same, but serotypes 6 and 26 that have similar capsules by American nomenclature are now named serotypes 6A and 6B (belonging to serogroup 6) under the Danish system (Smart *et al*. 1989). However, the Quellung reaction has several disadvantages. Some isolates have been mistyped due to the cross-reactivity of anti-sera, and it is unable to determine serotypes for which no anti-sera are available, unencapsulated strains or strains have reduced expression of capsular genes. These problems lead to development of

molecular typing techniques such as Multi Locus Sequence Typing (MLST), which are more accurate and easier to perform (Pai *et al*. 2005; Batt *et al*. 2005).

MLST involves sequencing fragments of seven house-keeping genes (*aroE, gdh, gki, recP, spi, xpt* and *ddl*) in pneumococcus, and each allele is assigned a number depending on its sequence in the MLST website (http://spneumoniae.mlst.net/). This digital barcode‟ is known as the sequence type (ST). MLST can enable different laboratories to use the same standard. Different STs can exist within the same serotype and it has also revealed capsule switching(Jefferies *et al*. 2004; Beall *et al*. 2006; Jacobs *et al*. 2009) which make it a powerful tool for monitoring genetic variation in pneumococcal disease on a global scale. MLST data can be used to investigate evolutionary relationships among bacteria. MLST provides good discriminatory power to differentiate isolates. Microarray analysis of clinical isolates has revealed that gene variations still exist in isolates having the same serotype and MLST (Silva *et al*. 2006; Dagerhamn *et al*. 2008). However, it is too expensive and labour intensive to use microarray as a routine method of identification. Supplementing sequencing data of essential virulence genes in addition to MLST has been used in certain reference laboratories to improve the accuracy of the identification (Hanage *et al*. 2005). Genomic sequencing with recent high-throughput next generation sequencing technology such as Illumina (Solexa) sequencing and SOLiD sequencing has provided a more powerful and accurate method for identification of clinical isolates. However, the cost for next generation sequencing is still too high to use routinely. Promisingly, the cost for the third generation sequencing (Single molecule real time sequencing) will be less than $1,000 per human genome, which is much cheaper than that of next generation sequencing ($20,000 per human genome)(Hayden 2009). The cost of sequencing the pneumococcal genome is about $200 with Illumina sequencing, and that cost with the third generation sequencing will be likely to be less than $10. Of course, quicker and better software for analysing these sequencing data need to be developed.

1.2 Carriage of S. pneumoniae

The pneumococcus commonly colonises asymptomatically the mucosal surface of the upper respiratory tract of humans at least once in their lifetime (Bogaert *et al*. 2004a). Pneumococcus is a normal member of the microflora in the human nasopharynx. Common bacteria living in the human nasopharynx include *Haemophilus influenzae, Moraxella catarrhalis, Staphylococcus aureus, Neisseria meningititis* and other streptococcal spp (Bogaert *et al*. 2004b). When pneumococcus adheres to nasopharyngeal epithelial cells, normal healthy people can clear the infection with their own immunity, but bacteria may move to middle ear, eyes, sinuses or lungs and cause diseases in susceptible people (Mitchell 2006) (Figure 1). Because nasopharyngeal colonisation is an essential step for surviving, pneumococcus has evolved different ways to protect itself. For example, pneumococci are able to produce H_2O_2 (Pericone *et al*. 2000) and bacteriocins (Dawid *et al*. 2009) to inhibit or kill the other microflora in the nasopharynx. Pneumococcus is highly transformable and picks up genes from the other bacterial species in nasopharynx. For example, pneumococcus acquired genes that encode low affinity penicillin-binding proteins from *Streptococcus mitis* through interspecies DNA recombination events (Dowson *et al*. 1994; Tomasz 1999a). Pneumococcus becomes more competent for the acquisition of foreign DNA during the antibiotic stress (Prudhomme *et al*. 2006). Viral infection such as adenovirus and influenza virus in the respiratory tract can enhance colonisation of pneumococcus (Wadowsky *et al*. 1995; Tong *et al*. 2000).

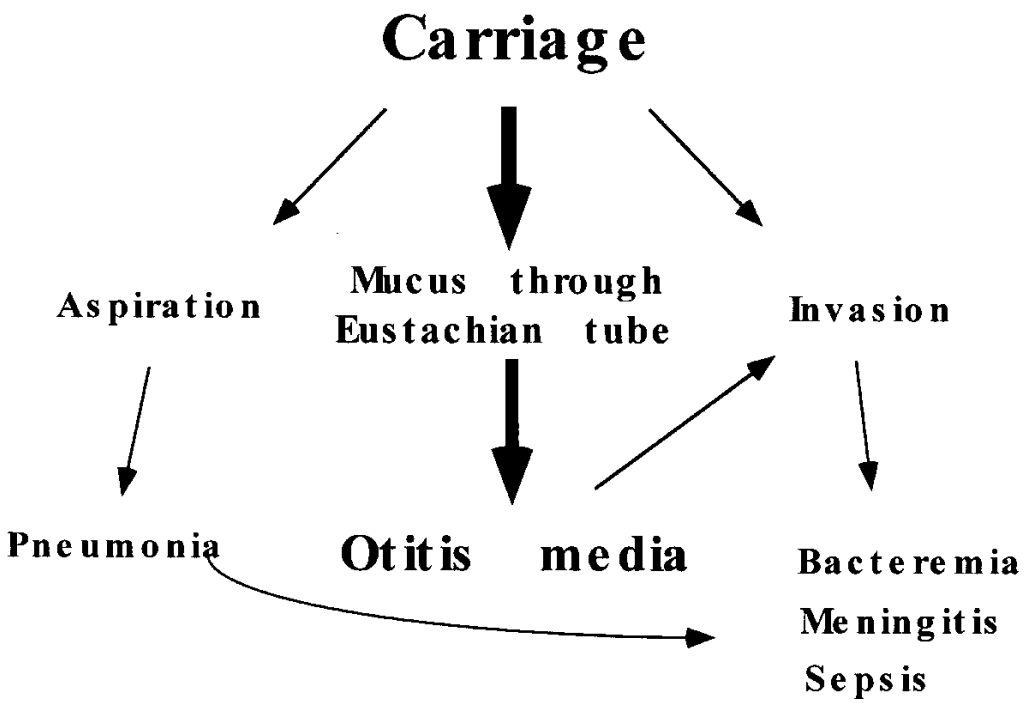


Figure 1-1 Correlation between pneumococcal carriage and diseases. Successfully colonising in the human nasopharynx is a key step toward causing diseases for pneumococcus.

Pneumococcus is spread through direct contact with the secretions of a colonised person. Pneumococcal carriage prevalence strongly depends on age. In developing counties, new born babies are quickly colonised by pneumococcus, the carriage reaches 90-100% when babies are several months old, declines to about 10% when children are older than ten years and remains at this level throughout adulthood (Gratten *et al.* 1986; Lloyd-Evans *et al.* 1996; Hill *et al.* 2006; Granat *et al.* 2007). In developed countries except certain groups such as aboriginal children in western Australia (Watson *et al.* 2006), children are not colonized by pneumococcus until several months old, the carriage reaches about 40-50% after two year old, goes down to 10% or less around the age of five and remains at this level during adulthood (Bogaert *et al.* 2001; Syrjanen *et al.* 2001; Hussain *et al.* 2005). Pneumococcal carriage in the elderly is normally about 5% (Flamaing *et al.* 2010). However, there are a number of factors including overcrowding (day care centres, homeless shelters and prisons), smoking, antibiotic use, heavy alcohol consumption and ethnicity (Kurtti *et al.* 1997; Obaro 2000; Bogaert *et al.* 2004a; Greenberg *et al.* 2006) that can increase frequency of pneumococcal carriage and therefore cause diseases.

1.3 Diseases caused by S. pneumoniae

The pneumococcus can cause both non-invasive diseases, such as acute otitis media (AOM), sinusitis and bronchitis, and more severe diseases, such as pneumonia, meningitis and bacteraemia, occasionally causing eye infection. Pneumococcus mainly infects children under the age of five, the elderly and immunodeficient patients (Johnston, Jr. 1991; Amdahl *et al*. 1995). Pneumococcal infection has high morbidity and mortality in both developing and developed countries (O'Brien *et al*. 2009).There were 14·5 million cases of pneumococcal diseases excluding upper respiratory diseases such as AOM, sinusitis and bronchitis worldwide in children under age five in 2000 (O'Brien *et al*. 2009) (Figure 1-2). The top ten countries with the highest numbers and proportions of pneumococcal diseases were all in Asia and Africa (India 27%, China 12%, Nigeria 5%, Pakistan 5%, Bangladesh 4%, Indonesia 3%, Ethiopia 3%, Congo 3%, Kenya 2%, and the Philippines 2%) (O'Brien *et al*. 2009).

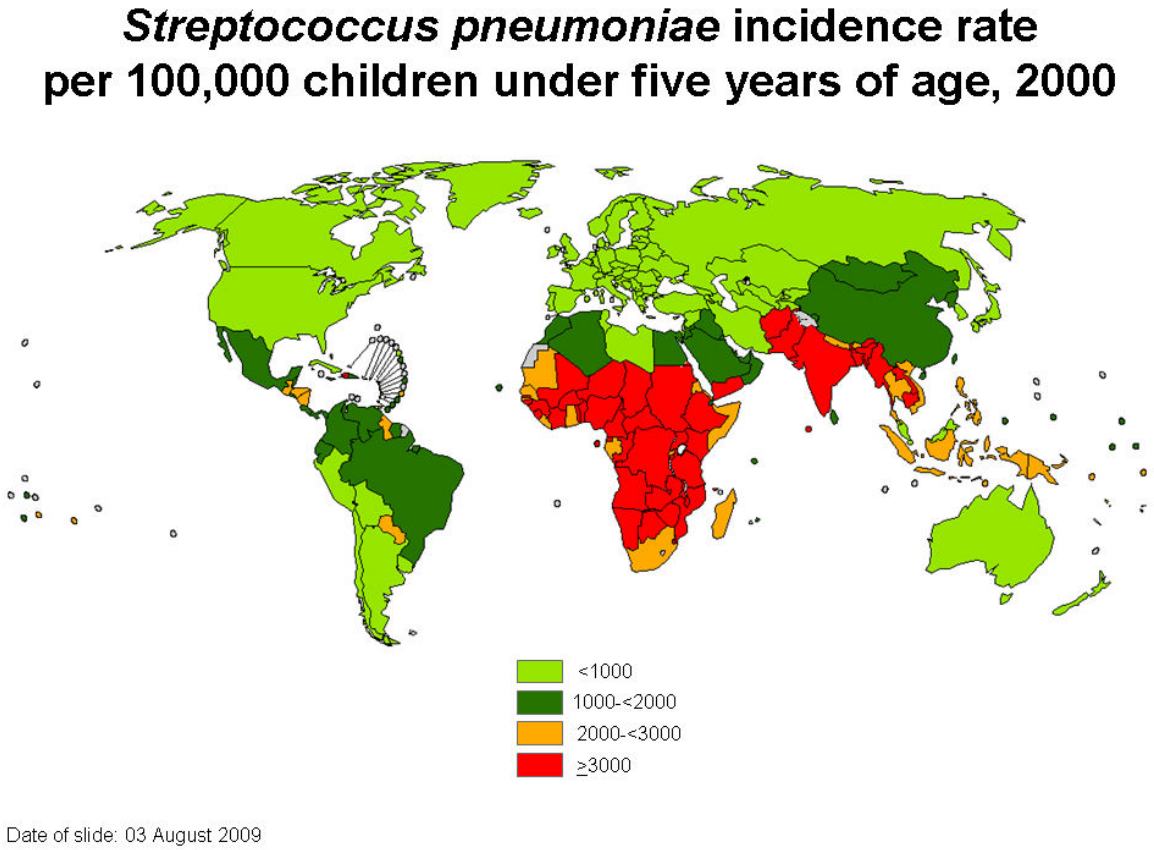


Figure 1-2 Incidence of invasive pneumococcal disease worldwide.
The rates of morbidity in developing countries are very high; particularly Africa has the highest morbidity rates. Picture is from WHO website
http://www.who.int/nuvi/pneumococcus/en/

Over 1 million children younger than 5 years old die from pneumoccocal infection per year, most of them in developing countries (Denny & Loda 1986) (Figure 1-3). The WHO estimated every minute at least one child dies from pneumococcal diseases. In recent years, antibiotic resistance has become a worldwide problem and further exacerbated the conditions since it limits the choices of antimicrobial agents (Tomasz 1999b). Further more, the antimicrobial agents have to be used in the early stages of infection for high-risk groups such as children, elderly and immunodeficiency patients. Vaccines offer the most effective protection against pneumococcal disease, therefore prevention of pneumococcal diseases by vaccination has become of great interest.

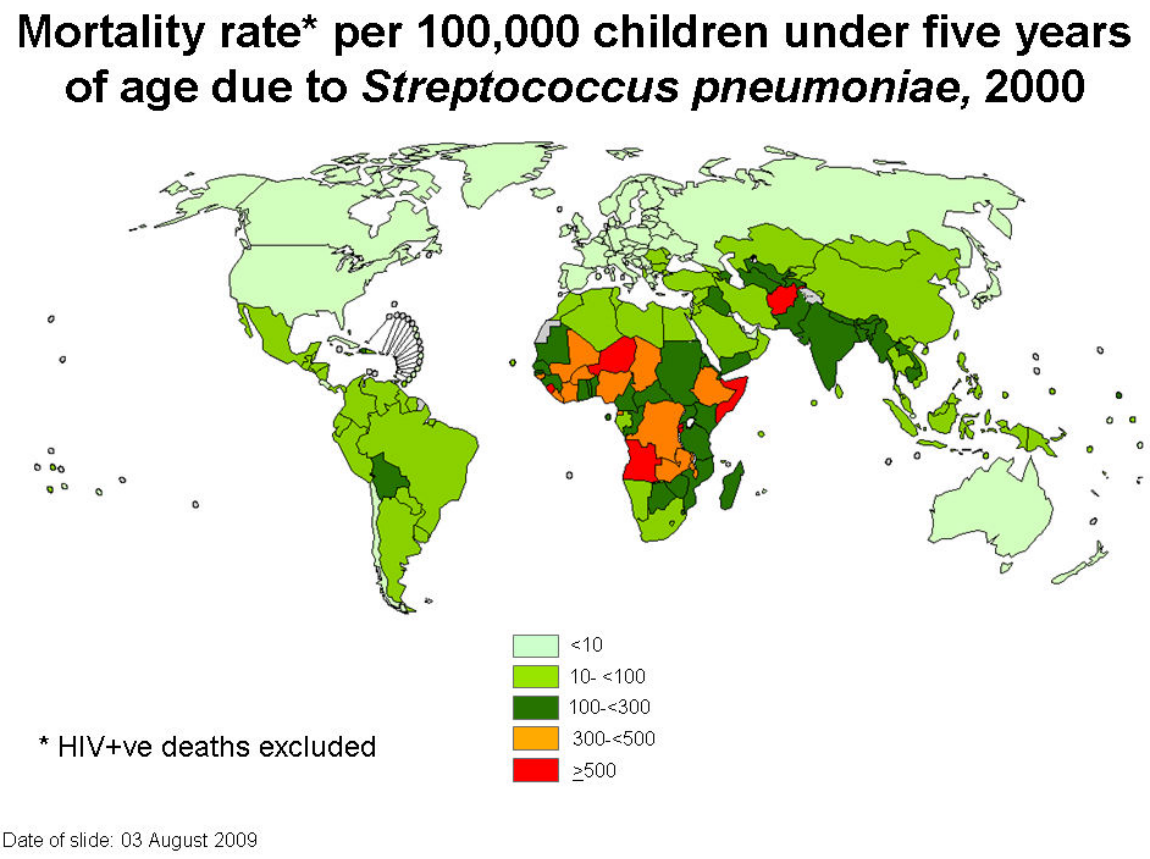


Figure 1-3 Mortality rates in children under age five because of pneumococcal diseases. The rate of mortality in developing countries are very high, particularly Africa has the highest mortality rates. Picture is from WHO website http://www.who.int/nuvi/pneumococcus/en/

1.3.1 Pneumococcal pneumonia and bacteraemia

Pneumonia is characterised by fluid in the lungs, which prevents oxygen reaching the bloodstream through the alveoli. Pneumonia is the most common cause of

death among all pneumococcal diseases (95.6% of total pneumococcal deaths) around the world (O'Brien *et al*. 2009). There were 13·8 million cases of incidence of pneumococcal pneumonia in children younger than 5 years worldwide in 2000 (O'Brien *et al*. 2009). According to the WHO, pneumonia kills more children than any other infectious disease including AIDS and malaria. Pneumococcus 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 developed countries, pneumococcal pneumonia is the most common community-acquired bacterial pneumonia with 100,000 cases/population and a 10-20% mortality rate, and this may reach above 50% in high-risk groups (Johnston, Jr. 1991; Amdahl *et al*. 1995; Rodriguez *et al*. 2009).

Bacteraemia is characterised by the presence of bacteria in the bloodstream. The WHO estimated the rate for bacteraemia to be 15–19 per 100 000 in Europe. There is a high mortality rate associated with pneumococcal bacteraemia and it is often associated with pneumonia. Antibiotics, particularly penicillin, is usually effective in the treatment of pneumococcus associated pneumonia and bacteraemia.

1.3.2 Pneumococcal meningitis

Meningitis is characterised by inflammation of the meninges (fluid filled membranes that surround the brain and spinal cord). There were 103,000 pneumococcal meningitis cases worldwide in 2000 (O'Brien *et al*. 2009). Pneumococcal meningitis is the most severe form of meningitis as 40-75% of pneumococcal meningitis patient in developing countries either die or become disabled (Goetghebuer *et al*. 2000). The mechanism by which colonising pneumococci reach the central nerve system (CNS) and cause meningitis remains unclear. It was originally thought that pneumococcus must enter the bloodstream and then pass the blood brain barrier before reaching the central nervous system (CNS). However, it has been demonstrated that pneumococcus can invade the CNS directly from the nasal cavity by axonal transport via olfactory nerves in a murine model (van Ginkel *et al*. 2003). Antibiotics are effective to treat pneumococcal meningitis.

1.3.3 Acute Otitis media

AOM is also called middle ear infection. AOM is rarely fatal and is one of the most common infectious diseases in children. The WHO has estimated that pneumococcus is responsible for 7 million cases of AOM per year. AOM is most prevalent in children under 2 years old. Approximately 60% children will experience at least one AOM episode by one year of age (Fireman *et al*. 2003) and this reaches 91% before their second birthday (Paradise *et al*. 1997). Up to 50% of all AOM is caused by pneumococcus (Prellner *et al*. 1999). This movement is likely to be easier than entering into the lungs or into the bloodstream.

1.4 Main virulence factors of S. pneumoniae

Pneumococcus produces many virulence factors such as capsular polysaccharide, pili, cell surface-associated proteins and toxins. Many putative virulence factors have been identified through genomic sequencing studies and signature-tagged mutagenesis (Polissi *et al*. 1998; Lau *et al*. 2001; Hava & Camilli 2002). The more characterised virulence factors and their roles in pathogenesis are summarised in Table 1-1 below.

Table 1-1 Pneumococcal virulence factors and their functions.

Virulence factor	Location	Function
Capsular PS	Outer Surface	Anti-phagocytotic Masks cell surface antigens from host immune system (Hammerschmidt *et al*. 2005)
PavA	Surface	Binds to fibronectin (Pracht *et al*. 2005)
Pili	Surface	Initial adhesion to host cells and important for ability to invade host(Barocchi *et al*. 2006) Backbone of pilus provided by RrgB and assembled by sortase 1, with RrgA and C acting as accessory proteins (Manzano *et al*. 2008)
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)
PspA	Surface	Binds pneumococcal 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)
PspC (CbpA)	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)
PhtA PhtB PhtD PhtE	Surface	Deletion of all Pht proteins is required to abolish virulence, relative to wild type, due to functional redundancy and it is required for inhibition of complement through binding of Factor H (Ogunniyi *et al.*, 2009) Pht proteins contain histidine triad motifs capable of binding zinc, which is scarce in the nasopharynx and binding of zinc, rather than other divalent cations, causes a change in conformation that changes the pattern of trypsin digested PhtD (Horsham, 2009) Pht proteins provide protection against sepsis (Adamou *et al.*, 2001)
Nan A NanB NanC		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).
PLY	Cytoplasm	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 1-3 for detailed list of roles of PLY)

LytA LytB LytC	Cell Wall	LytA releases other virulent factors e.g PLY 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)
ClpC ClpP	Cytoplasm	Involved in autolysis in some strains and pneumococcal growth in the lungs and bloodstream (Ibrahim et al. 2005) Involved in virulence, thermotolerance and resistance to oxidative stress (Ibrahim et al. 2005)
Hyl	Surface	Breaks down hyaluronic acid in mammalian connective tissue possibly to promote pneumococcal dissemination (Paton et al. 1993)
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)
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)
SlrA	Surface	Lipoprotein involved in colonisation of the nasopharynx and protects pneumococci from phagocytosis (Hermans et al. 2006)
ZmpB	Surface	Induces inflammation in respiratory tract (Blue et al. 2003)
PiaA	Surface	Lipoprotein component of pneumococcal iron ABC transporter and is required for full virulence in mouse model. 100% conserved and absent from oral streptococci (Whalan *et al.*, 2006
PiuA	Surface	Lipoprotein component of pneumococcal iron ABC transporter and is required for full virulence in mouse model. Highly conserved (0.3% variation) conserved but also present in other oral streptococci (Whalan *et al.*, 2006) Highly protective but via opsonophagocytosis, rather than inhibition of iron transport
PpmA	Surface	PpmA deficient cells are phagocytosed in a strain dependent manner and are less capable of persisting in the nasopharynx of mice (Cron *et al.*, 2009)
PclA	Surface	Found by analysis of sequenced genomes in selected strains as part of two open reading frames. The other coded for a transcriptional regulator. Analysis of clinical isolates found in 39% of the strains examined. Deletion mutants were unattenuated in a pneumonia model but were defective in adherence *in vitro* (Paterson *et al.*, 2008)
PfbA	Surface	Identified due to LPXTG. Mutants attenuated in adhesion. Caused morphological changes in epithelial cell lines that did not occur with wild type cells. Anti-PfbA antibodies inhibited growth in whole blood culture (Yamaguchi *et al.*, 2008)

PcsB	Surface	Down regulates expression of PcsB created a cell separation defect, causing the cells to form chains with excess but ordered cell wall synthesis at every cell equator and septum (Barendt *et al.*, 2009) Severe depletion led to rapid cessation of growth accompanied by the appearance of aberrantly shaped cells with unusual regions of cell wall synthesis (Ng *et al.*, 2004) High temperature and osmolarity leads to an increase in expression (Mills *et al.*, 2007)
StkP	Cell wall	Highly conserved protein that contributes to penicillin sensitivity independently from genes encoding penicillin-binding proteins (Dias *et al.*, 2009; Osaki *et al.*, 2009)

1.4.1 Capsule

The capsular polysaccharide (PS) is the most important virulence factor for pneumococcus. Capsular PS prevents entrapment in the nasal mucus and enables the bacteria to evade and resist oposonphagocytosis (Jonsson *et al*. 1985). The majority of strains and clinical isolates of pneumococcus have polysaccharide capsule. As described earlier, 93 different capsular PS have been found so far in pneumococcus, and most of capsular serotypes can cause diseases (Kalin 1998; Bratcher *et al*. 2009). Non-capsulated pneumococci also exist naturally, and have been recovered from the nasopharynx of health individual, immunocompromised or conjunctivitis patients (Muller-Graf *et al*. 1999; Martin *et al*. 2003; Crum *et al*. 2004).

The colony morphology changes from transparent to opaque are because of thickening of the capsule. Opaque colonies are associated with invasive disease and usually recovered from the blood while transparent colonies are associated with colonisation. Recently it has been shown that those two different populations of pneumococci also exist during nasal colonisation, transparent colonies loosely attached to the nasal surface and opaque colonies closely attached to the nasal mucosa. Transparent colonies are gradually removed by nasal washing, whereas opaque colonies are maintained and isolated from homogenised nasal tissues (Briles *et al*. 2005; Dawid *et al*. 2009). These morphologies are reversible and aid in transition from colonisation to invasive disease (Hammerschmidt *et al*. 2005).

There are two different ways to synthesise capsular PS dependent on the polymerases used, the sythase-dependent synthesis and Wzy-dependent

synthesis (block type). Bentley and its colleagues recently sequenced the capsule loci from 90 serotypes known at that time (Bentley *et al*. 2006). The sequence analysis show only type 37 and type 3 exhibit sequences consistent with the sythase-dependent synthesis mechanism, and the rest of the 90 serotypes use the Wzy-dependent synthesis. The Wzy-dependent synthesis is similar to the production of peptidoglycan and used in most streptococci as well as many other Gram-positive and negative bacteria (Yother 1999).

1.4.2 Cell wall components

As described earlier, pneumococcus is Gram-positive bacterium. The cell wall mainly contains peptidoglycan (PG). Penicillin inhibits PG synthesis through binding to the PG synthesizing enzymes (penicillin-binding proteins), which are responsible for cross-linking cell wall. Teichoic acid is attached to PG directly and lipoteichoic acid is anchored to the plasma cell membrane. Both teichoic and lipoteichoic acid contain phosphorylcholine (PC) which serves as an adhesin and is the place for binding of choline-binding proteins (Yother & White 1994). Cell wall is also the place for attaching of many other cell surface proteins. Mutation in licD2 gene which is involved in synthesis of PC resulted in significantly impaired virulence of the pneumococcus (Zhang *et al*. 1999). Cell wall components can reproduce many of the symptoms of pneumonia, Otitis media and meningitis (Tuomanen *et al*. 1987; Giebink *et al*. 1988; Riesenfeld-Orn *et al*. 1989; Hoffmann *et al*. 2007).

1.4.3 Pneumococcal cell surface proteins

Four main groups of surface-exposed proteins have been described in the pneumococcus: lipoproteins, choline-binding proteins, LPXTG-anchored proteins and family of non-typical surface proteins. Figure 1-4 illustrates the main protein virulence factors of pneumococcus.

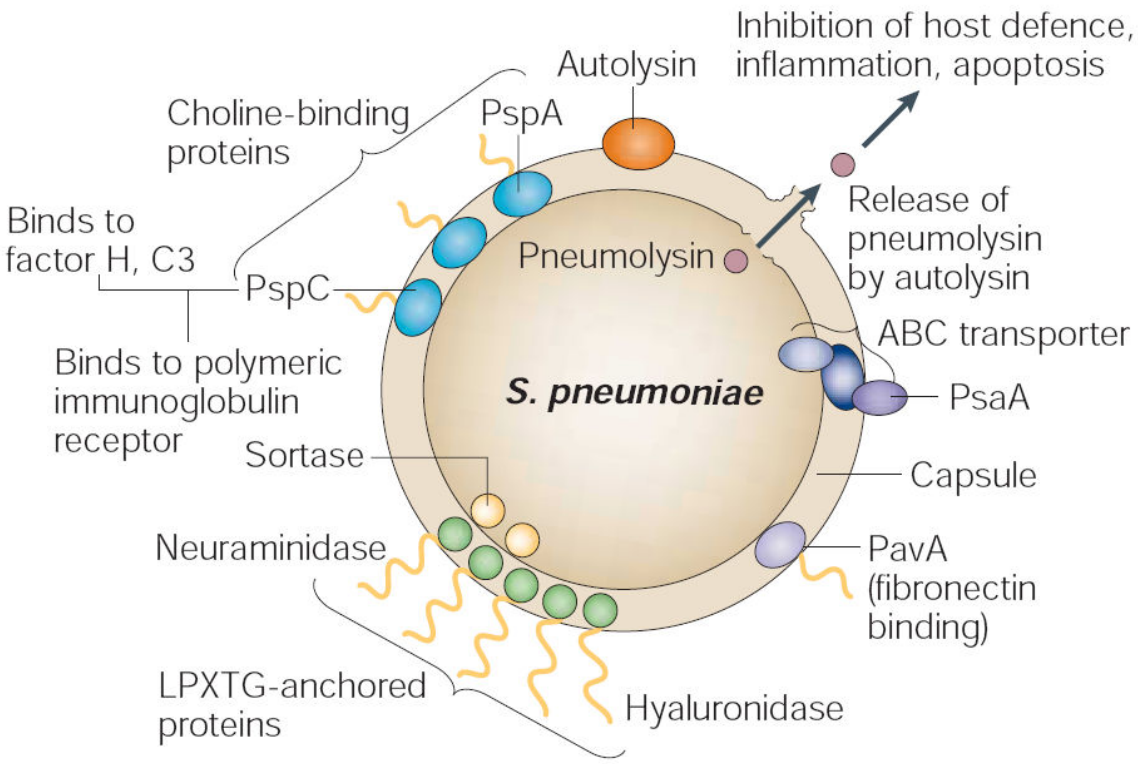


Figure 1-4 The main protein virulence factors of pneumococcus (Mitchell 2003).

1.4.3.1 Lipoproteins

Surface exposed lipoproteins are involved in transport of metal ions, sugars and other small molecules. Around 42-45 cell surface lipoproteins have been identified in pneumococcus (Bergmann & Hammerschmidt 2006). Lipoproteins have very diverse functions. Many of them are the substrate binding component of the ATP-binding cassette (ABC) metal transporters, which include pneumococcal surface adhesin A (PsaA), pneumococcal iron uptake protein A (PiuA) and pneumococcal iron acquisition protein A (PiaA)(Jomaa *et al*. 2005; Whalan *et al*. 2005; Whalan *et al*. 2006). Streptococcal lipoprotein rotamase A (SlrA) is important in colonisation of the host and AOM (Hermans *et al*. 2006; Stol *et al*. 2009) and putative proteinase maturation protein A (PpmA) is involved in pneumococcal pneumonia (Cron *et al*. 2009).

The size of PsaA is about 37 kDa. It is highly conserved among strains and produce by all pneumococci. PsaA belongs to a member of the family of metal binding lipoproteins and is the substrate binding component of the ABC transporter for manganese (Dintilhac *et al*. 1997; Lawrence *et al*. 1998). PsaA

shows similar sequence to the other adhesins from streptococci (Sampson *et al*. 1994), and was demonstrated to bind to E-cadherin of human nasopharyngeal epithelial cells (Anderton *et al*. 2007). PsaA pneumococcal mutant has reduced binding ability to human A549 cells (Berry & Paton 1996), anti-PsaA antibody reduces the binding of pneumococcus to human nasopharyngeal epithelial cell (Romero-Steiner *et al*. 2003). PsaA is important for virulence as PsaA knockout strains are avirulent in murine models of colonisation and invasive diseases (Berry & Paton 1996; Marra *et al*. 2002).

1.4.3.2 Choline-binding proteins

Choline-binding proteins (CBP) possess carboxy terminal repeat regions that non-covalently anchor to the phosphorylcholine of the cell wall (Yother & White 1994). Choline binding proteins have three major structural domains. The N-terminus has a sequence consistent with a coiled-coil α helix. In the centre of the molecule is a proline-rich region of ~ 60–80 amino acids. The C-terminal end of the molecule contains 9 or 10 repeats of 20 amino acids each. These repeats form a phosphocholine binding site and bind to the choline group of teichoic acid through ionic interaction. Four to five repeats are usually sufficient to mediate binding to choline. Pneumococci possess 13 to 16 different choline-binding proteins including pneumococcal surface protein A (PspA), pneumococcal surface protein C (PspC) and the autolysins (Bergmann & Hammerschmidt 2006). The non-CBP domain located at the N terminus in most cases is responsible for the functional activity of each CBP.

The size of PspA is from 60 to 100 kDa, its structure and antigenicity are variable among different strains of pneumococcus (Crain *et al*. 1990; Waltman *et al*. 1990). PspA has been divided into three different families and subclassed to six different clades based on the amino acid sequences of α-helical region at its N-terminus (Hollingshead *et al*. 2000). The “clade-defining region” is located 100 amino acids upstream of the proline-rich domain. Clades 1 and 2 belong to Family 1; family 2 includes clades 3, 4, and 5; and clade 6 is family 3. There is only 54% sequence identity in the α-helical region of family 1 and 2 PspAs, and the similarities of other regions are between 84 to 100%. PspA antisera are not able to distinguish individual PspA clades within each family (Briles *et al*. 2000b). Families 1 and 2 account for 94-99% of isolates in the USA, Colombia,

Brazil and Argentina (Vela Coral *et al*. 2001; Brandileone *et al*. 2004; Mollerach *et al*. 2004).

A PspA deficient mutant has significantly reduced virulence in *vivo* (Tu *et al*. 1999). PspA inhibits the complement pathway mediated by factor B (Tu *et al*. 1999), and blocks to iron transport lactoferrin bactericidal activity through direct binding (Hammerschmidt *et al*. 1999; Hakansson *et al*. 2001; Shaper *et al*. 2004). King et al recently showed PspA also contributes in secondary infection with pneumococcus after influenza virus infection (King *et al*. 2009). Human $CD4^+$ T cell related responses to PspA following natural exposure have been reported (Baril *et al*. 2006). Purified PspA stimulates release of cytokine IL-6 and chemokines including CXCL8, CCL2, CCL4 and CCL5 in human monocytes via activation of the extracellular signal-regulated kinase (ERK), c-Jun N-terminal kinase (JNK), p38 mitogen activated protein kinase (MAPK) and nuclear factor (NF)-kappaB signalling pathways (Cao *et al*. 2010a).

PspC, a polymorphic protein, has the molecular weight between 69 kDa and 120 kDa. Different allelic forms of PspC have been called by different names including choline-binding protein A (CbpA) (Rosenow *et al*. 1997), S. pneumoniae secretory IgA binding protein (SpsA) (Hammerschmidt *et al*. 1997) and C3-binding protein A (PbcA) (Cheng *et al*. 2000). They are divided into different groups based on the highly variable N-terminus region (Iannelli *et al*. 2002). PspA and PspC are paralogous, and have a very similar domain structure and have similarity in much of their sequences (Brooks-Walter *et al*. 1999). PspC is divided into choline-binding and peptidoglycan-linked families depending on the cell wall anchor mechanisms. The choline binding family is named CbpA. PspC members in this family are further divided into two clades, A and B, basing on the presence or absence of a PspA-like region upstream of the proline-rich region. Approximately 4% of PspC in tested strains belong to clade A and 96% clade B (Brooks-Walter *et al*. 1999). The peptidoglycan-linked family contains a sortase-dependent domain with LPXTG motif near the C-terminus of PspC. PspC is only found in 75% of strains of pneumococcus and antibody elicited by PspC is cross-reactive with PspA (Brooks-Walter *et al*. 1999). Furthermore, the *pspC* gene is replaced by the *hic* at the locus of *pspC* in those strains that do not contain *pspC*. HiC, a factor H-binding inhibitor of complement (Hic)(Janulczyk *et al*. 2000), is highly similar to PspC in the N-terminal half (Iannelli *et al*. 2002).

However, Hic lacks a choline binding domain and has a sortase-dependent domain with LPXTG motif. PspC has several different functions. PspC is an important adhesin for colonisation. A PspC deficient pneumococcus has a reduced binding ability to nasopharynx of infant rats and mice (Rosenow *et al*. 1997; Balachandran *et al*. 2002). PspC has an important role for translocation of pneumococci across human nasopharyngeal epithelial cells through bind to the human polymeric immunoglobulin receptor pIgR and transition from nasopharynx to lower respiratory tract (Orihuela *et al*. 2004). PspC also binds to the secretory component of IgA (Hammerschmidt *et al*. 1997; Hammerschmidt *et al*. 2000). PspC is a essential virulence factor because a PspC deficient strain was unable to infect or multiply in the lung in a mouse pneumonia model (Balachandran *et al*. 2002). PspC is involved in protecting the pneumococcus through inhibition of complement pathway via direct binding to factor H (Dave *et al*. 2004a; Dave *et al*. 2004b; Quin *et al*. 2005). PspC binds to short consensus repeats 5 to 13 of factor H, and Hic binds to 8 to 11 (Duthy *et al*. 2002). Binding of factor H by PspC also increases adherence to umbilical vein endothelial, pharyngeal epithelial and lung epithelial human cells *in vitro,* and enhances invasion of mouse lungs *in vivo* (Quin *et al*. 2007). PspA and PspC work synergically to protect pneumococci against complement-dependent clearance during invasive infection (Li *et al*. 2007). PspC increases release of proinflammatory molecules from human alveolar epithelial cells and the expression of intercellular adhesion molecule 1 (CD54) by A549 cells (Murdoch et al. 2002). Purified PspA or PspC can induce production of IL-8 in human neutrophils individually, and human neutrophils treated with mixture of PspA and PspC release much more IL-8 than those treated with PspA or PspC alone (Cao *et al*. 2010b). Allele variations in PspC structure between different strains alter PspC-dependent effects. Kerr et al showed deleting PspC in strains of serotypes 2, 3, and 19F did not significantly change the virulence of pneumococcus in the murine pneumonia model. In contrast, deleting PspC in serotype 4 strain TIGR4 significantly increases host survival times in both the pneumonia and bacteraemia models (Kerr *et al*. 2006). It was reported that PspC binds to the complement inhibitor C4b-binding protein in a allele-dependent fashion (Dieudonne-Vatran *et al*. 2009). It was shown that strains background and capsular serotype influence the effects of PspC on complement-mediated immunity to pneumococci(Yuste *et al*. 2010).

1.4.3.3 LPXTG proteins

LPXTG-proteins are anchored to peptidoglycan in the cell wall through sortases that recognise the amino acid sequence LPXTG (X can be any amino acid) in the C-terminal anchoring motif (Schneewind *et al.* 1993). Different sortases are involved in processing and anchoring the different surface-exposed proteins, sortase A (SrtA) responds for attaching most LPXTG-proteins when more sortase genes exist (Hava & Camilli 2002). SrtA is highly conserved and exists in all pneumococci, and SrtB, C, D were found in only 17% of clinical isolates (Paterson & Mitchell 2006). It has been shown that the SrtA deficient mutant has reduced adhesion to human pharyngeal cells due to changed location of Srt A dependent surface proteins (Kharat & Tomasz 2003). SrtA has also been reported to have roles in colonisation, pneumonia and bacteraemia in animal models (Chen *et al.* 2005; Paterson & Mitchell 2006). There are about 20 LPXTG proteins in pneumococcus including Neuraminidase A (NanA), hyaluronidase (Hyl) and IgA1 protease. However, IgA1 protease has N-terminal LPXTG (Bender & Weiser 2006), which is in the C-terminal of other LPXTG proteins.

NanA and Hyl are the two best-characterised LPXTG proteins. Hyl degrades hyaluronic acid which a key component in mammalian connective tissue and extracellular matrix thereby promoting invasion of epithelial layers (Feldman *et al.* 2007). There are three neuraminidases in pneumococcus, NanA, NanB and NanC, which are found in 100%, 96% and 51% of 442 clinical isolates in a screen study respectively (Pettigrew *et al.* 2006). Neuraminidases cleave the terminal sialic acid from polysaccharides, glycolipids, glycoproteins and oligosaccharides on the cell surface, and expose receptors to pneumococcus for adherence and colonisation (Mitchell 2000). Only NanA is secreted from pneumococcus and has LPXTG sequence, which suggests they may have different functions *in vivo*. NanB was found to work at a very low pH optimum (Berry *et al.* 1996), which suggested NanB may be active in host lysosome. NanA and NanB are both important for pneumococcal colonisation in the respiratory tract and survival in the bloodstream (Manco *et al.* 2006). NanA is important to promote pneumococcal adherence to and invasion of human brain in an *in vitro* infection model. Lectin like domain of NanA is responsible for invasion of blood brain barrier, activation of chemokine expression and recruitment of neutrophils during infection (Uchiyama *et al.* 2009; Banerjee *et al.* 2010).

1.4.3.4 Family of non-typical surface proteins (FNTSP)

FNTSP contains the pneumococcal histidine triad (Pht) protein family, pneumococcal adherence and virulence factor A (PavA), endolase and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) (Bergmann & Hammerschmidt 2006). The Pht protein family that is a newly recognised group of surface proteins each contains 4 or 5 copies of the histidine triad motif (HXXHXH) and comprises four members: PhtA, PhtB, PhtD and PhtE (Adamou *et al*. 2001). PhtA was one of five protective proteins found a genomic-wide screen for potential vaccine antigens (Wizemann *et al*. 2001). The protein sequence is highly conserved among individual Pht proteins in pneumococcus, and the serum against individual Pht protein cross-react (Hamel *et al*. 2004). Pht proteins have been shown to be zinc and manganese scavengers (Rioux *et al*. 2011), especially for zinc. The crystal structure of PhtA-166-220 has been shown to contain a novel zinc binding motif (Riboldi-Tunnicliffe *et al*. 2005). Pht proteins have been shown to be highly regulated by Zn^{2+} -dependent repressor AdcR. High concentration of Zn^{2+} up-regulated expression of PhtD proteins (Ogunniyi *et al*. 2009; Loisel *et al*. 2011; Shafeeq *et al*. 2011). Binding of zinc to histidine triad motifs of PhtD results in a change in conformation, which was detected through trypsin digestion, but this change was not observed when other divalent cations were added (Horsham M, unpublished data). It is controversial Pht protein is required for inhibition of complement through binding of Factor H. Ognniyi and his colleagues show that Pht proteins inhibit the complement pathway through the recruitment of complement factor H (Ogunniyi *et al*. 2009). In a later study, Melin and colleagues showed deletion of all four Pht proteins resulted in increased C3 deposition only on the serotype 4 but not on the other strains, and Pht proteins did not bind factor H. (Melin et al. 2010). Data from our lab always showed PhtD did not bind factor H through Western blotting (Horsham M unpublished data). Deletion of all Pht proteins is required to abolish virulence due to functional redundancy (Ogunniyi et al. 2009).

1.4.4 Pneumolysin

Pneumolysin (PLY), one of the most important virulent factors of pneumococcus, has many roles in the pathogenesis of disease (Mitchell & Andrew 1997). PLY is produced in almost all strains of pneumococcus (Kanclerski & Mollby 1987).

There are serotypes that express non-haemolytic forms of PLY (Kirkham et al. 2006a). PLY is a multifunctional toxin and is a member of the cholesterol-dependent cytolysins (CDCs) (Table-2). PLY is a soluble 53 kDa monomer, and forms oligomeric pores containing 30 to 50 PLY monomers after it binds to cholesterol-containing cell membrane, and lyses cells by osmotic force (Mithchell 2006). The CDCs are found in at least seven genera of Gram-positive bacteria. The CDCs form pores in cholesterol containing membranes and are very cytotoxic to all mammalian cells (Billington et al. 2000; Palmer 2001; Gilbert 2002). Table 1-2 is a list of all identified CDCs and the bacteria which produce them. Release of PLY is thought to be dependent on autolysin A to degrade the cell wall as PLY has no secretion signal sequence, which is different from all other CDCs. However, it has been suggested that LytA may not be required for PLY release (Balachandran et al. 2001). Recently , Price and Camilli reported PLY localised to the cell wall compartment(Price & Camilli 2009).

Table 1-2 Bacteria that produce cholesterol dependent cytolysins (CDCs).

Species	Name of CDC
Arcanobacterium pyogenes	Pyolysin
Bacillus anthracis	Anthrolysin O
B. cereus	Cereolysin
B. thuringiensis	Thuringiolysin O
B. sphaericus	Sphaericolysin
Brevibacillus laterosporous	Laterosporolysin
Clostridium perfringens	Perfringolysin O
C. bifermentas	Bifermentolysin
C. botulinum	Botulinolysin
C. chauvoei	Chauveolysin
C. histolyticum	Histolyticolysin
C. septicum	Septicolysin
C. sordellii	Sordellilysin
C. tetan	Tetanolysin
C. novyi	Novyilisin
Gardnerella vaginalis	Vaginolysin
Lactobacillus iners	Inerolysin
Listeria ivanovii	Ivanolysin O
L. monocytogenes	Listeriolysin O
L. seeligeri	Seeligeriolysin O
Paenibacillus alvei	Alveolysin
Streptococcus canis	Streptolysin O
S. dysgalactiae (ssp. equisimilis)	Streptolysin O
S. intermedius	Intermedilysin
S. pneumoniae	Pneumolysin
S. pyogenes	Streptolysin O
S. suis	Suilysin
S. mitis	Lectinolysin

Data from a review of the cholesterol-dependent cytolysins (Rosado *et al.* 2008) except lectinolysin (Farrand *et al.* 2008) and inerolysin (Rampersaud *et al.* 2011) which are identified and characterised recently.

1.4.4.1 Structure of PLY

The sequence of PLY is largely conserved among pneumococcal strains; there is not much difference with the primary structure (Mitchell 2006). How PLY binds cells and forms oligomeric pores is still not clear. The structure of PLY has not been solved yet. However, the structure of perfringolysin O (PFO) provides a model of the conformational transition into the pore form of model for PLY (Rossjohn et al. 1997). PFO which is one of the virulence factors of *Clostridium perfringens* has almost the same molecular mass as PLY. There is 48% sequence identity and 67% sequence similarity between PLY and PFO. Current

understanding of PLY structure and molecular mechanism are guided by a model built on the basis of the crystal structure of PFO. There are two possible ways for pore formation by the CDC family. Studies from streptolysin O suggest that oligomerisation starts to form the pore after monomers bind and insert into the membrane (Palmer 2001). In contrast, studies from PFO suggest that before the entire oligomer inserts to form the final pore, a prepore forms after monomers bind to the membrane (Hotze *et al*. 2001; Hotze *et al*. 2002). However, streptolysin O also could form a prepore (Heuck *et al*. 2003). More recently work with PLY suggests PLY forms a prepore before insertion of the oligomer takes place (Tilley *et al*. 2005). PLY contains four domains (Figure 1-5): domain 1 (residues 6-21, 58-147, 198-243, 319-342), domain 2 (residues 22-57, 343-359), domain 3 (residues 148-197, 244-318) and domain 4 (residues 360-471). Domain 1 plays a scaffold role that the other domains can reorganize during pore forming. Domain 4 is responsible for initially binding to cholesterol containing cell membranes by inserting a tryptophan rich loop into the upper layer of a lipid bilayer. As more and more toxin molecules bind to the membrane, these monomers start to assemble a pre-pore due to interaction of domain 1 and 3 among the molecules. Domain 2 begins to bend and dissociate with domain 3, and two sets of three α-helices in domain 3 begin to refold into two long β hairpins. Finally, the β hairpins insert into the lipid bilayer and form pores.

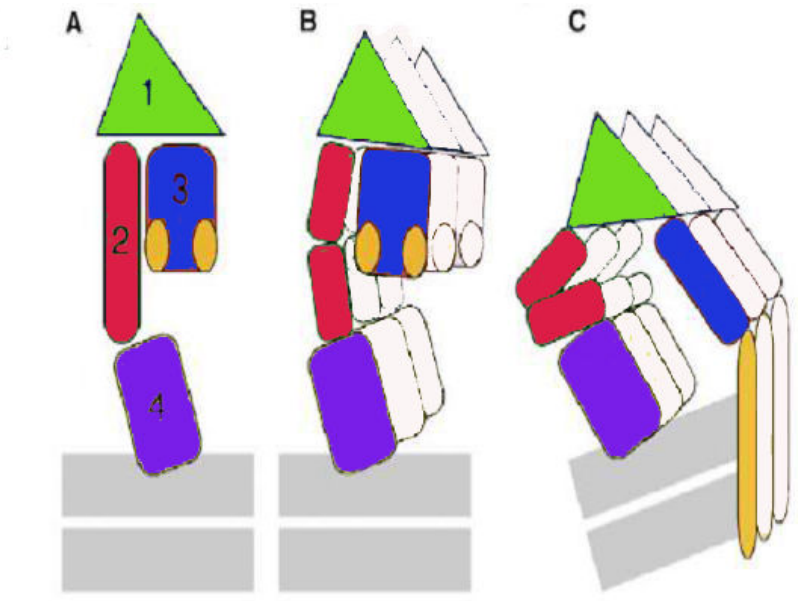


Figure 1-5 Pore formation by Pneumolysin.
The structural rearrangements accompanying the pore formation (A) monomer, (B) prepore, (C) membrane inserted pore.

1.4.4.2 Biologic effects of PLY

PLY has been detected in the cerebrospinal fluid (CSF) of meningitis patients (Spreer *et al*. 2003), middle ear fluid from Otitis media cases (Virolainen *et al*. 1994), lung sputum of pneumonia patients (Wheeler *et al*. 1999) and lung tissue from mice with experimental pneumonia (Canvin *et al*. 1995).

The biological effects of PLY have been studied using several systems. Study the interaction of the purified protein with isolated cells, with organ cultures and with whole animals. The role of PLY in host and pathogen interaction was also studied with the above systems using pneumococcal PLY proficient and knockout strains. PLY has at least two important activities in causing biological effect in host tissue and cells; forming pores in the cell membrane and the ability to activate the complement pathway (Mitchell 2006). Isogenic PLY mutant pneumococci with reduction in either cytotoxic activity or complement activation by PLY had significantly decreased virulence. PLY has different biological effects in different cells and tissues (Table 1-3) which are dependent on concentration of PLY and sensitivity of those cells to PLY (Hirst *et al*. 2002).

The toxin interferes with human immunity and inflammatory responses. Sub-lytic concentrations of PLY are able to trigger inflammation.

The role of PLY in nasopharyngeal colonisation is dependent on strain or serotype of pneumococcus. Nasopharyngeal epithelial cells have also been shown to act as a early sensitive detector through osmotic stress via activation of P38 MAPK in response to PLY (Ratner *et al*. 2006). Toll-like receptor (TLR) is one of most important pattern-recognition receptors, and myeloid differentiation factor 88 (MyD88) is an adaptor for TLRs. Recently, PLY has been shown to activate TLR4 via MyD88 and promote interleukin-8 (IL-8) production through calcium flux and activation of MAPK in nasopharyngeal epithelial cells (Dogan *et al*. 2010). In the absence of TLR2, clearance of pneumococcal nasopharyngeal colonization is delayed (van Rossum *et al*. 2005; Akira *et al*. 2006). Mice carrying a defined mutation in TLR4 showed diminished responses to PLY, and were more susceptible to nasopharyngeal colonization with pneumococcus (Malley et al. 2003). CD4+ T cells are required for mediating antibody-independent acquired immunity in responding to clearance of pneumococcal colonisation in

nasopharynx (Malley et al. 2005). The migration and activation of CD4+T cells are partially induced by PLY (Kadioglu et al. 2004). Zhang et al showed PLY strongly induced CD4+ T cell proliferation during nasopharyngeal colonisation of pneumococcus in children (Zhang et al. 2007). IL-8 is the main chemokine that promotes recruitment of neutrophils in nasopharynx (Yoon et al. 2010). It has been shown that neutrophils moved and took up pneumococcus by phagocytosis in the lumen of the nasopharynx between 1 and 3 days after pneumococcus colonised in nasopharynx of mice (Nelson *et al*. 2007). PLY deficient mutant pneumococcus did not recruit neutrophils and stayed much longer period in a murine colonisation mode (van Rossum *et al*. 2005). The neutrophils also contribute to the processing of bacterial antigen and delivery of those products to the mice nasal-associated lymphoid tissue (MALT) (Matthias et al. 2008). However, this early influx of neutrophils does not correlate with the decline in the density of pneumococci during colonisation, which takes up to a few weeks after primary challenge, indicating that neutrophils may not be the only phagocyte responding for clearance (McCool & Weiser 2004). The decline in pneumococcal load also requires the migration of monocytes/macrophages into the lumen of the upper airway in a TLR2-, IL-17A-, and CD4+ T cell–dependent manner (Zhang et al. 2009). It has been recently reported that the pore forming ability of PLY is required for peptidoglycan activated Nod2-dependent expression of CCL2 on recruitment of monocyte/macrophages into the lumen of the upper airway (Davis et al. 2011; Nakamura et al. 2011). PLY has previously shown to aid Nod1 sensing through delivery of peptidoglycan fragments moved into the cytoplasm of epithelial cells in culture (Ratner et al. 2007).

During spread of pneumococcus to the lung through the respiratory tract, PLY can slow down ciliary beats in the nasopharynx and bronchus (Steinfort *et al*. 1989; Feldman *et al*. 1990). Mice intranasally challenge with PLY deficient mutants show significantly lower numbers of pneumococcus in the nasopharynx, trachea and lung., increased bacterial clearance from the lung and prolonged survival (Kadioglu *et al*. 2002; Orihuela *et al*. 2004). PLY and PLY proficient pneumococcus induce significantly less proinflammatory cytokine expression at the early stages of infection than later stages of infection in epithelial cells (Yoo *et al*. 2010).

Additional functions have been suggested for PLY using PLY mutants. Pneumococcus expressing non-haemolytic PLY is more virulent than PLY deficient mutant (Alexander *et al*. 1998). A truncated PLY mutant is still able to induce interferon-γ and nitric oxide (NO) in lymphocytes from mice spleen cells *in vitro* (Baba *et al*. 2002). However, non-haemolytic PLY does exist naturally. A non-haemolytic PLY (ST306) was detected in the clone of serotype 1, which is the most prevalent pneumococcus detected in IPD (Mitchell 2006). Richard M. Harvey and his colleagues recently showed the pneumococcal D39 mutant strain which PLY is replaced with the low haemolytic activity PLY allele (ST3018) from serotype 1 strain 4496 has obtained an advantage to grow in the early stages of bacteraemia compared to the wild type D39 in a murine model (Harvey *et al*. 2011).

PLY is a crucial virulence factor in pneumonia (Canvin *et al*. 1995; Rubins *et al*. 1996; Alexander *et al*. 1998; Jounblat *et al*. 2003). PLY is also responsible for human lung mast cell mediated killing of pneumococcus; there is no response to PLY deficient pneumococcus (Cruse *et al*. 2010). PLY causes apoptosis in neutrophils (Zysk *et al*. 2000) and macrophages (Marriott *et al*. 2004), which helps bacterial clearance. Neutrophils action is extremely important in clearance of pneumococcus in pneumonia since neutrophils are the major cell for phagocytosing pneumococcus (Kolling *et al*. 2001). Functions of leukocytes are directly inhibited by PLY. Alveolar macrophages and DCs are important in phagocytosis of bacteria and coordinating the immune response during infections (Underhill & Ozinsky 2002). Resident alveolar macrophage are reduced in response to PLY (Maus *et al*. 2004). PLY also inhibits reactive oxidative species (ROS) production and impairs degranulation in monocytes (Nandoskar *et al*. 1986). PLY inhibits activation, maturation and cytokine production in human dendritic cells (DCs) (Littmann *et al*. 2009). The apoptotic effect of PLY in macrophage depended on activation of TLR4 (Srivastava *et al*. 2005).

Many proinflammatory and physiological mediators are induced by PLY in different cells and tissues (Table 1-3). PLY promotes production of tumour necrosis factor-α (TNF- α) and IL-1β in human alveolar and bronchial epithelial cells *in vitro* (Yoo *et al*. 2010). PLY activates phospholipase A in bovine pulmonary epithelial cells *in vitro* (Rubins *et al*. 1994). IL-8, superoxide, elastase and several physiological effectors were induced by PLY in neutrophils *in vitro*

(Cockeran *et al*. 2001a; Cockeran *et al*. 2002). PLY increased NO levels, which in turn contribute to upregulation of other cytokines such as TNF-β, IL- β and IL-6 in macrophages (Braun *et al*. 1999; Shoma *et al*. 2008). IL-6 and TNF-α production were induced by PLY in macrophages through activation of TLR4 via MyD88 (Malley *et al*. 2003). The direct interaction between PLY and TLR 4 has been suggested by ELISA (Malley *et al*. 2003). PLY stimulates ROS in human neutrophils (Martner *et al*. 2008). PLY induces NO and interferon-γ production in lymphocytes from mouse spleen (Baba *et al*. 2002). TNF-β and IL-1β are two key cytokines to the early host response to pneumococcus in the lung (Rubins & Pomeroy 1997). TNF-β and IL-1β can activate nuclear factor kappa-light-chain-enhancer of activated B cells (NF- $_{K}$B) (Mizgerd 2008). NF- $_{K}$B is essential for cytokine expression, neutrophil recruitment and bacterial clearance in pneumonia (Calbo & Garau 2010). IL-6 can delay apoptosis and increase cytotoxic function including ROS in neutrophils (Biffl *et al*. 1996; Rijneveld *et al*. 2002). McNeela *et al* recently showed that PLY induces TNF- α and IL17 A production *in vitro* and *in vivo,* and promotes IL-1ß production through activation of NOD-like receptor 3 (NLRP3) inflammasome independent of TLR4 in murine DC, and also demonstrates the NLRP3 is needed for protection against pneumococcus *in vivo* (McNeela *et al*. 2010). Witzenrath and colleagues also recently showed that IL-1ß induction by PLY is through activation of the NLRP3 inflammasome in murine bone marrow macrophages (BMMs) and non-haemolytic PLY (ST306) from serotype 1 pneumococcus does not stimulate production of IL-1ß in human peripheral blood monocytes (Witzenrath *et al*. 2011b). Activation of NLRP3 Inflammasome results in caspase-1 activation. Activated caspase-1 then converts IL-1ß and IL-18 precursor (pro-IL- ß and pro-IL-8) into mature cytokines. However, Littman et al showed expression of haemolytic PLY inhibits human DC maturation, induction of proinflammatory cytokines and activation of Inflammasome (Littmann *et al*. 2009). Human DCs produces significantly higher levels of cytokines than murine bone marrow-derived DCs (BMDCs) in response to PLY proficient pneumococcus, and murine BMDCs internalise much lower number of pneumococcus than human DCs (Littmann *et al*. 2009).

PLY also plays important roles when the pneumococcus moves from lung to blood stream (Berry *et al*. 1989b; Rubins *et al*. 1995; Berry *et al*. 1999; Kadioglu *et al*. 2002; Orihuela *et al*. 2004). The bronchial and lung epithelia are physical

barriers against bacteria (Hippenstiel *et al*. 2006). PLY plays a important role when pneumococci penetrate from alveoli into the interstitium and disseminate into the bloodstream as it induces separation of epithelial tight junctions and changes alveolar permeability by inducing inflammation (Steinfort *et al*. 1989; Duane *et al*. 1993; Rubins *et al*. 1993; Rubins *et al*. 1995). When PLY was directly applied to the lungs of rats, it caused inflammation, which is similar inflammation that of pneumococcal pneumonia (Feldman *et al*. 1991). Pneumococcal infection is reduced by human anti-PLY antibodies and therefore increases survival of animals (Musher *et al*. 2001). PLY activates the complement pathway (Paton *et al*. 1984) through binding to the Fc portion of human immunoglobulin (Mitchell *et al*. 1991). PLY proficient and deficient mutants of pneumococcus were cleared much slower from the lung of complement deficient mice $C5^-$ than wild type mice, especially with PLY proficient pneumococcus; the number of PLY deficient pneumococci increased in lung of $C5^-$ mice and were totally cleared from wild type mice (Rubins *et al*. 1995). Activation of the complement pathway is likely to be associated with T-cell recruitment as well as it directly contributing to clearance of pneumococcus (Jounblat *et al*. 2003). The impaired T-cell function in the early stages of infection may be associated with the impaired clearance of pneumococcus in $C5^-$ mice after infection (Kadioglu *et al*. 2004). It is also worth noting that Alexander et al. found that mice immunised with PLY were protected from nine of ten pneumococcal strain (Alexander *et al*. 1998).

It has been shown that a PLY knockout strain of pneumococcus has reduced virulence in a murine model of meningitis (Wellmer *et al*. 2002). PLY damages the ependymal cilia and induces apoptosis in brain (Hirst *et al*. 2000a; Hirst *et al*. 2000b; Braun *et al*. 2002). Mitochondrial damage and activation of P38 MAPK via calcium influx are responsible for PLY induced apoptosis in neuron cell (Stringaris *et al*. 2002; Braun *et al*. 2007). PLY causes stereocilia disorganization and apoptosis in cells of a rat model (Beurg *et al*. 2005). Interestingly, PLY-triggered apoptosis preferentially targeted inner hair cells. The calcium influx and the mitochondrial apoptotic pathway are also responsible for PLY-induced cochlear hair cell death. Sub-lytic concentrations of PLY remodel actin in host cells through activating GTPase, and induce extensive stabilisation and bundling via activating Src-kinase (Iliev *et al*. 2007; Iliev *et al*. 2009), which may facilitate

pneumococcal penetration of the endothelial cell barrier. PLY is important in eye infections. Johnson *et al.*, showed that a PLY knockout strain of pneumococcus has greatly reduced virulence in a rabbit ocular infection model (Johnson *et al*. 1990).

PLY may have an adverse effect in bacteraemia. A large number of bacteria were detected in the blood without obvious symptoms in mice infected with PLY deficient pneumococcus and chronic bacteraemia was developed (Benton *et al*. 1995; Kadioglu *et al*. 2000). 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).

Overall, on the one hand, PLY is a key virulence factor of pneumococcus to facilitate invasion of host tissues; on the other hand, PLY triggers production of key pro-inflammation mediators such as TNF-α, IL-1ß, IL-6 and IL-8 for activation of immune responses.

Table 1-3 Selected biological effects of PLY on different cells and tissues.

Cell types	Effects
Nasopharyngeal epithelial cells	Increases production of IL1- ß, IL-12, SDF-1, IL10, CXCL1-3 and IL-8 (Dogan *et al*. 2010)
Alveolar and bronchial epithelial cells	Disrupts cells (Rubins *et al*. 1993) and slows down cilial beat (Steinfort *et al*. 1989; Feldman *et al*. 1990)
Alveolar epithelial cells	Release of cytokine IL-8 through p38 MAPK activation (Ratner *et al*. 2006), also release IL-1ß and TNF-α (Yoo *et al*. 2010)
Pulmonary endothelial cells	Disrupts integrity (Rubins *et al*. 1992) Activates phospholipase A (Rubins *et al*. 1994)
Microglial and neuronal cells	Induces apoptosis (Stringaris *et al*. 2002; Braun *et al*. 2002) remodelling actin via RhoA and Rac1 activation (Iliev *et al*. 2007) Stabilises microtubule via Src-kinase (Iliev *et al*. 2009)
Neutrophils	Inhibits respiratory burst and migration (Paton & Ferrante 1983; Nandoskar *et al*. 1986) Induces necrosis and apoptosis (Zysk *et al*. 2000) Induces calcium influx (Cockeran *et al*. 2001b) Potentiates production of prostaglandin E(2) and leukotriene B(4) (Cockeran *et al*. 2001a) Induces reactive oxygen species (ROS)
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 & McDaniel 2005)
Mast cell	Stimulates release of antimicrobial peptides (Cruse *et al*. 2010)
Dendritic cells (DC)	Induces apoptosis (Colino & Snapper 2003) Inhibits DC maturation (Littmann *et al*. 2009) Release CCL2 and CCL5 (Bernatoniene *et al*. 2008) Increase release of cytokines including IL-12, IL-13, IL-6, IL-1α IL-ß and IFN-γ (McNeela *et al*. 2010)
Macrophage	Induces apoptosis (Srivastava *et al*. 2005) Increases production of TNF and IL-6 depends on MyD88 (Malley *et al*. 2003) Increases release of IL-1α, IL-1ß,IL-18 and IL-8 (Shoma *et al*. 2008; Witzenrath *et al*. 2011a)
Spleen cells	Stimulates IFN-γ and Nitric Oxide release (Baba *et al*. 2002)
B cells	Inhibits antibody production (Paton *et al*. 1984)
Lymphocytes	Blocks proliferation of cells in response to mitogens (Ferrante *et al*. 1984), and migrate T cells (Kadioglu *et al*. 2004)
Brains	Slows down ependymal cillia (Mohammed *et al*. 1999)
Lung	Pulmonary inflammation (Feldman *et al*. 1991; Rijneveld *et al*. 2002)
Middle ear	Cochlear damage (Comis *et al*. 1993; Skinner *et al*. 2004; Beurg *et al*. 2005)

1.5 Pneumococcal vaccine

Sir Almroth E. Wright developed the first pioneering pneumococcal vaccine using killed whole organisms, which was utilised to immunise the native workers of gold and diamond mines in South Africa in 1911 (Wright *et al*. 1914). However, the application failed because it only covered one or two serotypes. In 1913, Dochez and Avery successfully cured 10 of 11 pneumococcal pneumonia patients with pneumococcal anti-serum raised in animals (Dochez & Gillespie 1913). However, these therapies were too expensive and dangerous due to serum sickness. After Felton and Baily isolated pneumococcal capsular PS for the first time in 1926 (Felton & Baily 1926), the first successful pneumococcal vaccine developed with pneumococcal capsular PS aborted an outbreak of pneumococcal pneumonia in Worcester In 1931 (Smillie *et al*. 1938). The serum therapy and vaccine were later abandoned because of the application of antibiotic therapy. Emergence of multidrug-resistant pneumococci worldwide resulted in production of a 14-valent pneumococcal PS vaccine in 1977 (Merck Sharp and Dohme). The development of this first licensed pneumococcus vaccine was largely through Robert Austrian's efforts. The 14-valent pneumococcal PS vaccine was then developed to a 23-valent PS vaccine (PV23) in 1983. The licensed 23-valent PS vaccine developed by Merck Sharp and Dohme is named Pneumovax 23™, which uses 23-selected capsular PS from the 93 known serotypes. Pneumo 23™ is another licensed 23-valent PS vaccine marketed by Sanofi Pasteur.

Table 1-4 Big events in history of pneumococcal vaccines.

Year	Events
1911	killed whole cell pneumococcal vaccine failed pneumonia in South African miners
1913	Successfully cured pneumococcal pneumonia patient using serum therapy
1931	First pneumococcal PS vaccine
1977	Introduction of 14-valent polysaccharide vaccine (PPSV14) in 1977
1983	PPSV14 was upgraded to 23-valent PPSV (Pneumovax 23™ Merck) (Pneumo 23™ Sanofipasteur)
2000	Licensed 7-valent pneumococcal conjugated vaccine (Prevnar™, Wyeth, which was purchased by Pfizer in 2009)
2001	Re-emergence of research on whole-cell vaccine (Malley et al. 2001)
2009	Licensed 10-valent pneumococcal conjugated vaccine (Synflorix, GlaxoSmithKline)
2010	Licensed 13-valent pneumococcal conjugated vaccine (Prevnar 13 Pfizer)
Possible Future	Introduction of pneumococcal protein based or whole cell based vaccines

1.5.1 Current vaccine

1.5.1.1 The 23-valent pneumococcal polysaccharide vaccine (PPSV23)

PPSV23 contains 23 serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F and 33F. These serotypes account for 90% of invasive pneumococcal diseases (IPV) in developed countries. PPSV23 is immunogenic against 23 invasive pneumococcal serotypes and reduce about 36-52% invasive pneumococcal diseases (IPD) (Christenson *et al*. 2001). Following a single intramuscular injection, capular PSs specific IgM is seen in 60-80% of adults and children over the age of 2. Vaccination of children between 2 and 5 years of age saw protection against IPD caused by vaccine serotypes of 62% (Fiore *et al*. 1999). Older adults and people with chronic disease have enhanced risk of PID. PPSV23 is recommended for elderly people over 65 years old, solid organ transplant recipients and patient with immune conditions such as asplenia and sickle cell disease with the exception of HIV infection patients (Gebo *et al*. 1996; Fiore *et al*. 1999; Pebody *et al*. 2005). A vaccine trail of PPSV23 in Uganda

of HIV infected patients showedthat the vaccine was ineffective at preventing IPD and in terms of efficacy against pneumonia in HIV patients, PPSV23 was considered detrimental (French *et al*. 2000). Vaccinating old people in Scotland in winter 2003 resulted in apparent one third decrease in IPD (Mooney *et al*. 2008), and PPSV23 was effective in younger Alaska Native adults but not among people older 55 years (Singleton *et al*. 2007a). PPSV23 reduces the risk of bacteraemia in older adults (Shapiro *et al*. 1991; Butler *et al*. 1993; Jackson *et al*. 2003), but there is little or no evidence that the PPSV23 vaccine protects against pneumococcal pneumonia in adults (Carvalho *et al*. 2003; Huss *et al*. 2009). The PPSV23 has no effect on carriage of pneumococcus. There is also evidence that vaccination with PPSV23 results in protective immunity of shorter duration in younger adults (Simell *et al*. 2008) and that boosting with PPSV23 depletes memory B-cells and may induce long-lived T regulator suppressor cells (Musher *et al*. 2008).

Overall, PPSV23 has a very limited effect on the morbidity and mortality in some groups due to its poor immunogenicity in these groups, such as the very elderly, children under age two and immunodeficiency patients (Butler *et al*. 1999). It does not cover all pneumococcal serotypes and is not effective against acute Otitis media (AOM) (Wadwa & Feigin 1999). Children under two are unable to mount an appropriate immune response to a pure polysaccharide vaccine due to deficiency in maturation of distinct B lymphocyte subpopulations and imbalanced T cell regulatory influences (Barrett 1985). PPSV23 can only induce a B cell response that is $CD4^+$ T-cell independent (Harding *et al*. 1991; Mond *et al*. 1995) and therefore is limited in elicitation of germinal centre reaction, Ig class switching, somatic hypermutation and memory (Harding *et al*. 1991; Toellner *et al*. 2002). In addition, several capsular PSs including serotypes resistant to penicillin have very poor immunogenicity (Poland 1999).

1.5.1.2 Pneumococcal conjugate vaccine

Due to the above disadvantages associated with capsular PS vaccine, selected PSs have been conjugated with a protein carrier including tetanus toxoid or diphtheria toxoid to form the pneumococcal conjugate vaccine (PCV) with improved efficacy (Dagan *et al*. 1998). A 7-valent PS conjugate vaccine (PCV7, Prevnar) marketed by Wyeth which was purchased by Pfizer in 2009 was first

licensed in 2000 in USA and in 2001 in Europe. Purified capsular PS from serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F were chemically conjugated a non-toxic mutant diphtheria toxin CRM_{197} in PCV7. Recently, PCV7 is expanded to PCV13 vaccine after six more purified pneumococcal capsule including 1, 3, 5, 6A, 7F and 19A were added into PCV7 vaccine (PCV13) using CRM_{197} as a carrier protein. The PCV13 is named Prevnar 13, marked by Pfizer, was introduced in 2010. A 10-valent pneumococcal conjugate vaccine (PCV10) using H. *infuenzae* protein D as a carrier, named Synflorix, marked by GlaxoSmithKline, was introduced in 2009. PCV10 contains purified pneumococcal capsule PS 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F, and 23F. PCV is processed as a protein antigen rather than the polysaccharide. The conjugation of protein to polysaccharide results in a T-cell dependent response (Avery & Goebel 1931). PCV elicits protective, high level of anti-PS IgG (a shift from IgM and IgG2 to IgG1) response with good immunologic memory in all age group including children under age two (Rubinstein *et al*. 1998; Ahmad & Chapnick 1999).

PCV7 is highly effective in preventing PID in young children (Black *et al*. 2000; Mahon *et al*. 2006). A dramatic decrease was observed in IPD from 97 cases/100,000 to 24 cases/100,000 in 2005 and disease caused by serotypes in the vaccine fell from 80 cases/100,000 to 4.6 after PCV7 was introduced to the childhood schedule in 2000 In USA, (Mahon *et al*. 2006). A significant decrease in bacterial rhino sinusitis was also observed (Benninger 2008). Protection against IPD and clinical pneumonia in HIV-infected infants was also observed (Bliss *et al*. 2008). People also benefit through the herd immunity. Immunisation of children with PCV7 has been shown to result in over a 40% decrease in PID in people over 65 old years in USA (McBean *et al*. 2005).

PCV7 is less effective against radiological pneumonia (20-37% efficacy) than against PID (77-83% efficacy) (Cutts *et al*. 2005). PCV7 has limited effect on AOM. The PCV are able to reduce nasopharyngeal colonisation with vaccine serotypes of the pneumococcus (Mbelle *et al*. 1999; Eskola *et al*. 2001; Dagan *et al*. 2002; Singleton *et al*. 2007b), but strain replacement and serotype switching have already been observed (Veenhoven *et al*. 2003; Singleton *et al*. 2007b). A study about efficacy of PCV7 vaccine against AOM in Finland showed it has a 51% reduction rate in preventing vaccine-type AOM infection and only reduces 6% of all AOM episodes (Eskola et al. 2001). Another study of the efficacy of PCV

against AOM in Holland showed PCV7 has no effect on reduction of AOM episodes, serotype replacement occurred when nasopharyngeal carriage of serotypes included in PCV7 was greatly reduced (Veenhoven *et al*. 2003). From 2004, a 96% decrease in vaccine serotypes was matched by a 141% increase in non-vaccine serotypes in Alaskan Native children in the presence of high levels of PCV7 coverage. 28.3% of the increase in non-vaccine related serotypes was due to 19A (Singleton *et al*. 2007b). 40% of AOM isolates in a study in New York, during 2004-2006 expressed the 19A capsule. Eight different molecular sequence types (STs) expressed the 19A capsule; most of the strains were multi-drug resistant. In the case of ST-2722 (Legacy strain), it was resistant to all Food and Drug Administration (FDA)-approved AOM antimicrobial drugs (Pichichero & Casey 2007; Xu *et al*. 2009). However, a clinical trial of a 11-valent PCV pneumococcal conjugate vaccine (PCV11) in Czech Republic showed a 33.6% reduction in AOM episodes, which suggests that using *Haemophilus influenzae* protein D as a carrier protein in PCV vaccine can protect from AOM caused both by pneumococcus and H. *infuenzae* (Prymula *et al*. 2006).

In the USA, Prevnar costs $284.16 per course (4 doses) in state funded programmes. This price is far too high to be used in developing counties, with the result that the PCV7 only be used in most developed countries (Figure 1-6). The GAVI Alliance (the Global Alliance for Vaccines and Immunisation) is an organisation that coordinates private and public spending to create global access to vaccines. In 2006, The GAVI Alliance obtained funding available to 2015 to introduce PCVs into 72 countries with the lowest gross per capita income (<$1000 per year). To complement the financial support of the GAVI Alliance, a new mechanism called the Advance Market Commitment (AMC) was established in 2008. This is a binding contract from developed nations that guarantees vaccine makers a viable market for next-generation PCV and ensures a stable and cost effective supply for low-income countries (www.gavialliance.org). In order to effectively and substantially reduce childhood mortality in the GAVI Alliance -eligible countries, the GAVI currently provide the PCV7 at $3.50 in those countries (Sinha *et al*. 2007).

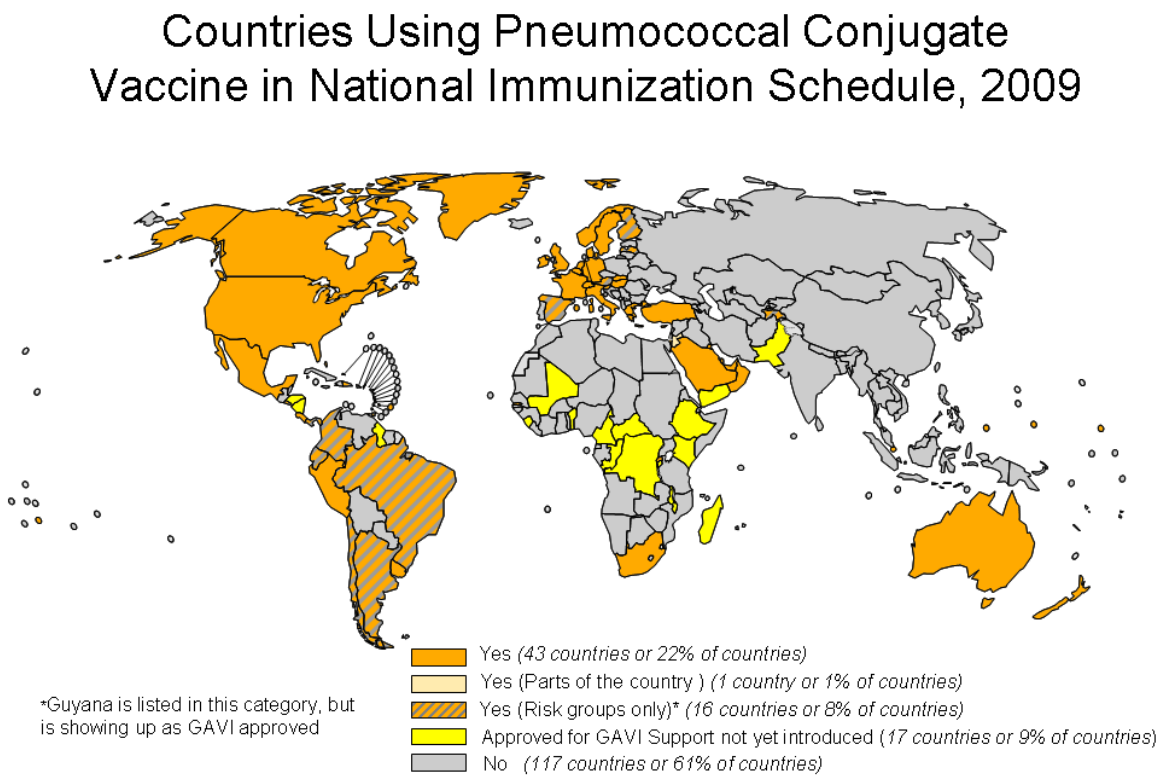


Figure 1-6 Countries using Pneumococcal conjugated vaccine in national immunisation schedule worldwide.
Picture is WHO website at http://www.who.int/nuvi/pneumococcus/en/

Overall, PCV7 is protective against invasive disease in young children, and induces long-term protection compared to PPSV23, which only induces relatively short-term protection. The PCV7 only covers certain vaccine serotype, is still serotype specific and does not include some serotypes highly prevalent in Africa, Asia and Oceania (Wuorimaa & Kayhty 2002). Recently, new licensed PCV13 has included 6 more purified pneumococcal capsular PS than PCV7. Further more, strain placement and serotype switching can cause long-term vaccine failure. Conjugating more different types of PS to carrier protein is unlikely to be a long term strategy as it will increase the cost, and it does not solve the issue of serotype replacement in the long term. The number of PSs linked to proteins is limited, and antigen competition and carrier protein epitope suppression, can impair the antibody response to PSs (Dagan *et al.* 1998). Finally, the PS conjugated vaccines are too expensive for developing countries. Therefore, it is important to develop new vaccines that can cope with these disadvantages. Some new strategies have been proposed such as the application of serotype independent protein based vaccines and whole cell vaccine.

1.5.2 Mucosal vaccines

As colonisation of the nasopharynx is the first step of pneumococcal infection and asymptomatic colonisation in the upper respiratory tract is common, especially in children, a mucosal vaccine which elicits strong local mucosal immunity to eliminate nasopharyngeal colonisation and interfering with invasion of the pneumococcus is potentially a good way to protect against pneumococcus (Zhang & Finn 2004). However, mucosal infections such as AOM, sinusitis and bronchitis are only partly prevented by licensed vaccines. The PCVs are able to reduce nasopharyngeal colonisation by vaccine serotypes of the pneumococcus (Mbelle et al. 1999; Dagan et al. 2002). But s mentioned earlier, PCVs can not protect diseases caused by serotypes not included within the vaccine. PCVs are less effective against pneumonia than against invasive diseases (Cutts *et al*. 2005; Qazi 2007). This observation might be as a result of difficulty either in the diagnosis of non-bacteraemic pneumococcal pneumonia or in distinguishing this diagnosis from other infective causes of pneumonia. Mucosal vaccination has shown promising results in protection against pneumococcal lung infection (Hanniffy *et al*. 2007; Cao *et al*. 2008; Medina *et al*. 2008a; Ferreira *et al*. 2009; Lu *et al*. 2009).

A mucosal vaccine also has several advantages since it is administered intranasally, orally or sublingually. This would avoid pain, and accidents associated with needles and transmission of infection, and save budget on medical equipment and staff training (Zhang & Finn 2004). Though the oral polio live vaccine is one of most successful immunisation strategies in the history of vaccination, oral vaccines are still very difficult to develop due to an arrays of barriers in the digestive tract including protease, bile salts, microvilli, enterointestinal junctions, and a large number of different bacteria on a thick layer of glycocalyx with digestive enzymes and carbohydrates (Nagler-Anderson 2001). Therefore, more study has focused on the development of nasal vaccines against respiratory pathogens including pneumococcus since the nasal mucosa is much easier to be accessible for administration of vaccine. There is evidence from murine model showing that oral immunisation of PspA fusion proteins delivered by attenuated *Salmonella* enterica serovar Typhimurium improved

protection against pneumococcus (Kang *et al*. 2002; Xin *et al*. 2009; Li *et al*. 2009).

A mucosal vaccine may also elicit good systemic immunity, i.e. oral polio vaccine, which would further strengthen the immune system for protection against the pathogen. All nasal pneumococcal vaccines studied so far elicit both good mucosal and systemic immunity (Baumann 2008). Studies have suggested that oral or nasal vaccines retain the same effectiveness as systemic application(Wu *et al*. 1997a; Seo *et al*. 2002). Nevertheless, there are number of studies which have shown the mucosal immunisation can elicit protection against pneumococcal colonisation and diseases. However, a potential problem with mucosal vaccines is that cross-reactivity. It can reduce or even eradicate of some species of the natural microflora on the surface of gut and respiratory tract. These species may protect humans from diseases caused by pathogens other than pneumococcus.

1.5.2.1 Mucosal associated lymphoid tissue (MALT) and immune response

MALTs are secondary lymphoid organs and found in various sites of the body, such as the respiratory tract, digestive tract, thyroid, breast, salivary glands, eye, and skin (Lavelle 2005). In rodents, nasal-associated lymphoid tissue (NALT) including a pair of lymphoid tissue locations at the floor of the nasal cavity and are lined by ciliated respiratory epithelium. Under the respiratory follicule-associated epithelium(FAE) is populated with immune cells including B cells, T cells, dendritic cells (DCs), Macrophages and other phagocytes (Heritage et al. 1997). NALT is also found in middle concha of the nasopharynx in children under age 2 in addition to adenoids (Debertin *et al*. 2003). The nasopharyngeal tonsils (adenoids) are part of Waldeyer's ring are major components of NALT in the human nose (Fujimura 2000).

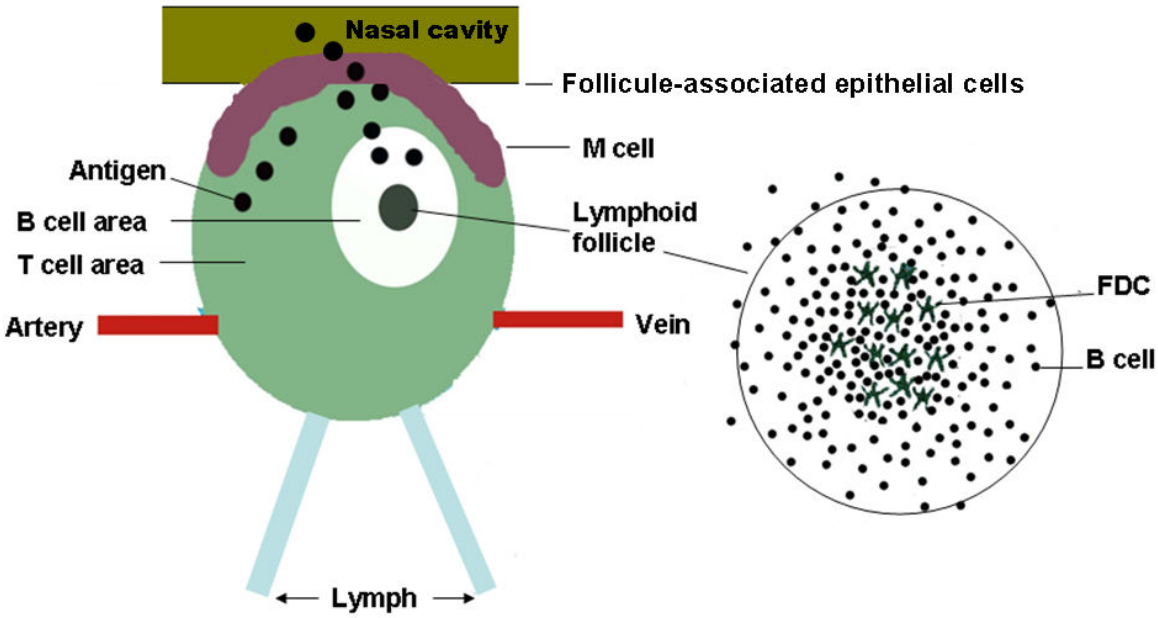


Figure 1-7 Nasal-associated lymphoid tissue (NALT).

In Figure 1-7, the FAE of the NALT are intercalated by microfold (M) cell, which does not have cilia and are selectively transporting antigens from the lumen of the nasal cavity into the NALT through endosomes. M cells only selectively transport antigens that can bind to molecules on the surface of the M Cells. M cells in the NALT are rich with TLR4 in their luminal location (Tyrer *et al*. 2006). Lymphoid follicles contain many B cells which are loosely embedded with follicular DCs. In germinal centres of lymphoid follicles, B cell proliferation takes place, and affinity maturation and B cell isotype switching occur. The lymphoid follicle is surrounded by a B cell zone which only contains B cells. The T cell zone containing multiple T cells is adjacent to the B cell areas. The transported antigens will either be removed by lymph to the lymph nodes, or picked up by follicular dendritic cells. DCs have been suggested to send dendrites across tight junctions between FAE and directly to sample antigens in the nasal lumen (Kamekura *et al*. 2008). FDCs then present the antigens to the B cells and activate them. Activated B cells start to divide and become very fragile. They will die by apoptosis unless they receive an appropriate second signal. T cells activated by other antigen presenting cells in the T cell area will move to lymphoid follicles and interact with B cells to provide a second signal. Activated B cells then either become plasma cells or undergo somatic hypermutation (Sompayrac 2003). Intranasal vaccination can induce humoral and cellular

immune responses in local sites and at distant mucosal sites such as the respiratory tract and cervico-vaginal mucosal tissue (Kiyono & Fukuyama 2004).

1.5.3 Pneumococcal whole cells vaccine

Incidence of pneumococcal diseases declined sharply before pneumococcal capsular specific antibodies are naturally elicited in infants, which suggests that antibodies to non-capsular antigens and another antibody independent protection involved in protection against pneumococcal diseases (Lipsitch *et al*. 2005). Antibodies to certain noncapsular antigens also arise early in life in response to colonization with pneumococci in mouse model, but those antibodies are not required for protection against pneumococcal colonization (Trzcinski *et al*. 2005). It was found this antibody independent protection requires functional CD4+ TH17 cells and is mediated by IL-17A through recruitment and activation of the neutrophils (Malley *et al*. 2005; Lu *et al*. 2008). Block of IL-17A significantly reduced immunity against pneumococcus in nasopharynx when purified cell wall PS was intranasally delivered, cell wall PS can elicit a similar immunity to PWCV (Malley *et al*. 2006).

The pneumococcal whole cell vaccine (PWCV) is not a novel idea. There were numerous studies about WCV back as early as 1891 (Klemperer & Klemperper 1891). The PWCV has a couple of advantages. PWCV should be very cheap and easy to manufacture as it uses killed cultured pneumococcus cells. PWCV is more immunogenic at the mucosal surface that soluble antigens, and less likely to be tolerogenic because it co-displays antigens that activate TLRs (Blander & Medzhitov 2006). PWCV is much more potent in preventing colonisation than purified cell wall PS (Malley *et al*. 2006). WCV can be freeze-dried and stored without refrigeration. PWCV can save costs associated with sterile needles and syringes since it can be delivered to non sterile sites such as the nasopharynx. PWCV elicits immunity through many different conserved pneumococcal antigens and should protect against all strains. Finally, PWCV has been to shown to reduce nasopharyngeal colonisation, middle ear-infection and pneumonia in animal studies (Malley *et al*. 2001; Malley *et al*. 2004; Roche *et al*. 2007; Lu *et al*. 2010b). An autolysin deleted unencapsulated PWCV (strain RM200, Rx1E PdT └lytA) substituted with TLR4-activating pneumolysin toxoid was produced with good manufacturing practice. The unencapsulated PWCV was administered parenterally with aluminium hydroxide (alum) and shown to offer protection

against colonisation, pneumonia and sepsis in mice challenged with pneumococcus (Lu *et al*. 2010a). Roche et al showed that mice immunised with live attenuated pneumococcal strains generated both mucosal and systemic protection against several pneumococcal strains (Roche *et al*. 2007).

Live *Salmonella typhimurium* mutants have also been used as a carrier for pneumococcal proteins. Live immunization may offer both mucosal immunity and systemic immunity to the antigens of interest (Curtiss, III *et al*. 2010). Mice vaccinated with avirulent *S. typhimurium* expressing PspA or PsaA have been shown to be protected against pneumococcal intraperitoneal or intranasal challenge, respectively (Wang *et al*. 2010a; Wang *et al*. 2010b). Mice orally immunised with a pneumococcal multiantigen DNA vaccine (containing *PsaA and PspA*) using attenuated S. *typhimurium* as a carrier had enhanced protection against colonisation by pneumococcus compared to mice intramuscularly vaccinated with PsaA and PLY together (Zhang *et al*. 2010).

1.5.4 Pneumococcal protein vaccine

Pneumococcal proteins have been studied extensively as potential vaccine candidates. A protein-based vaccine is expected to be immunogenic in all age groups including infants and the elderly. Broad and serotype independent protection might be elicited if highly conserved protein or protein epitopes or a combination of several proteins are included in the vaccine. It is relatively cheap and simple to produce protein vaccines by recombinant DNA technology. There are a number of papers showing that antibodies against pneumococcal proteins have been detected in human serum and mucosal secretions including children, adults and pregnant women, particularly in young children (Rapola *et al*. 2000; Vainio *et al*. 2005; Holmlund *et al*. 2006; Holmlund *et al*. 2007; Holmlund *et al*. 2009; Kaur *et al*. 2011; Lebon *et al*. 2011). Many pneumococcal proteins including PsaA, PspA, PspC, PLY, Pht protein family (PhtA, PhtB, PhtD and PhtE), hyaluronidase (hyl), autolysin, neuraminidase, ATP-dependent caseinolytic protease (ClpP), , putative lipoate-protein ligase (Lpl), pneumococcal protective protein A (PppA), caseinolytic protease (ClpP), serine/threonine protein kinase (StkP), and serine protease A (PrtA), have been suggested as potential vaccine

candidates (Table 1-1). PsaA, PspA, PspC, pht proteins and PLY are the main potential candidates.

Table 1-5 Protective efficacy in animal models of selected pneumococcal proteins.

Protein family	Antigen	Evidence of protection in animal models	
		Invasive disease	Colonisation
Toxins	Pneumolysin (Alexander *et al*. 1994)	+	-
Choline-binding proteins	PspA (Wu *et al*. 1997b; Briles *et al*. 2000c)	+	+
	PspC (CbpA) (Balachandran *et al*. 2002)	+	+
	PcpA (Glover *et al*. 2008)	+	
	LytA (Lock *et al*. 1992)	+	NR
	LytB/C (Wizemann *et al*. 2001)	+	NR
Sortase and sortase-dependent proteins	SrtA (Gianfaldoni *et al*. 2009)	+	-
	NanA (Lock *et al*. 1988; Long *et al*. 2004)	+	+
	RrgA/B/C (Gianfaldoni *et al*. 2007; Moschioni *et al*. 2010)	+	NR
	PrtA (Wizemann *et al*. 2001)	+	NR
ABC (ATP-binding cassette) transporter proteins	PsaA (Talkington *et al*. 1996; Whaley *et al*. 2010)	+	+
	PiaA and PiuA (Brown *et al*. 2001; Jomaa *et al*. 2006)	+	NR
	PotD (Shah & Swiatlo 2006; Shah *et al*. 2009)	+	+
	SP 2108 (Giefing *et al*. 2008; Moffitt *et al*. 2011)	-	+
	SP 0148 (Moffitt & Malley 2011)	NR	+
Histidine triad proteins	PhtA/B/D/E (Godfroid *et al*. 2011)	+	+
Enzymatic proteins	StkP (Giefing *et al*. 2008)	+	NR
	ClpP (Cao *et al*. 2008; Cao *et al*. 2009)	+	+
Other	PppA (Green *et al*. 2005; Medina *et al*. 2008b)	+	+
	PcsB (Giefing *et al*. 2008)	+	NR

SP 0148: TIGR4 locus name for substrate-binding lipoprotein of a methionine ABC transporter; NR: Not reported.

PsaA

The sequence of PsaA is highly conserved in pneumococcus. PsaA protects mice against pneumococcal carriage when they were immunised intranasally (Briles *et al*. 2000a). Mice subcutaneously vaccinated with PsaA using either Freund's or

titerMax adjuvants are protected against intravenous challenge with pneumococcus (Talkington *et al*. 1996). Immunisation studies with PsaA have shown significant protection against colonization but less protection against invasive disease in mice (Ogunniyi *et al*. 2000; Briles *et al*. 2000a). Co-delivery of PCV7 and PsaA can reduce nasopharyngeal colonisation by pneumococcal serotype 19A, whose capsular PS not included in PCV7, in mouse model (Whaley *et al*. 2010). It was shown that oral vaccination of mice with PsaA encapsulated in microalginate microspheres induced significant protection against colonisation, pneumonia and septicaemia when co-delivered with cholera toxin (CT) or the cholera toxin B subunit (CTB) as the adjuvant (Seo *et al*. 2002). Mice intraperitoneally co-vaccinated with DNA vaccine plasmid expressing the PsaA and purified PsaA generated humoral and cellular immune responses against the pneumococcus in mice (Miyaji *et al*. 2002). Holmlund and co-workers demonstrated detectable elevation of anti-PsaA IgG in the serum of 7 weeks old Filipino infants and that concentrations were higher in infants carrying pneumococcus (Holmlund *et al*. 2006).

PspA and PspC

As mentioned previously, the sequence and antigenicity of PspA and PspC are variable among different strains of pneumococcus (Crain *et al*. 1990; Waltman *et al*. 1990; Iannelli *et al*. 2002). Immunisation studies with PspA in mice showed significant protection against invasive disease but reduced efficacy against mucosal disease and nasopharyngeal carriage (Wu *et al*. 1997a; Ogunniyi *et al*. 2000; Arulanandam *et al*. 2001). Intranasal immunisation of mice with PspA using CTB as adjuvant is protective against nasopharyngeal colonisation, and prevents pneumococcal diseases (Wu *et al*. 1997a). Mice intranasally immunised with PspA clade 5 without an adjuvant induces PspA specific antibodies and protects mice against invasive challenge with pneumococcal strains ATCC 6303 (Ferreira *et al*. 2009). Sera from humans vaccinated with PspA significantly protected mice against intravenous challenge with pneumococcus producing heterologous PspA (Briles *et al*. 2000c). Mice intraperitoneally immunised with a DNA vaccine plasmid expressing the truncated PspA can protect against intraperitoneal challenge with the virulent pneumococcus (Miyaji *et al*. 2003). Vaccination with PspA was cross-protective against pneumococcal carriage with strains bearing different PspAs (Wu *et al*. 1997a). These studies mean that although PspA is

structurally variable, the protection elicited by two or three PspA variants should be enough to induce protection against most clinical strains (Briles *et al*. 2000b). PspA has been shown to interfere with complement deposition on pneumococcus. It has also been shown that vaccine-elicited rabbit and human antibodies to PspA can increase the deposition of C3 on several strains of pneumococcus (Ochs *et al*. 2008). However, development of new vaccine from PspA has slowed down because it was shown that PspA has the similarity to short sequences of human cardiac myosin throughout the molecule (Holmlund *et al*. 2006).

Intranasal immunisation with PspC using CTB as an adjuvant protected mice against intranasal challenge with pneumococcus (Balachandran *et al*. 2002). Mice intraperitoneally immunized with PspC were significantly better protected than mice immunized with PLY toxoid alone against intraperitoneal challenge with virulent pneumococcus (Ogunniyi *et al*. 2001). Both PspA and PspC are choline binding proteins, and have conserved proline rich regions at their C-termini. Vaccination with the proline-rich regions of PspA and PspC using alum as an adjuvant confers antibody mediated protection against pneumococcal bacteraemia in mice (Daniels *et al*. 2010). Mice intranasally immunised with PspC without an adjuvant did not generate PspC specific antibodies and failed to protect mice against invasive challenge with pneumococcal strains ATCC 6303 (Ferreira *et al*. 2009). However, intranasal immunisation with a live *Lactobacillus casei* expressing PspC generates anti-PspC IgG, and protects against the pneumococcus in both murine colonisation and invasive disease model (Ferreira *et al*. 2009; de Lucia *et al*. 2011).

Pht proteins family

All proteins of the Pht family have been shown to offer high levels of protection against pneumococcal infection with different strains and serotypes. PhtD appears to be the best antigen in Pht family (Zhang *et al*. 2001; Wizemann *et al*. 2001; Adamou *et al*. 2001; Hamel *et al*. 2004; Ogunniyi *et al*. 2009; Godfroid *et al*. 2011). Mice immunised with Pht proteins generate strong immunity against nasopharyngeal colonisation, pneumonia and sepsis (Zhang *et al*. 2001; Adamou *et al*. 2001; Hamel *et al*. 2004). Immunization with PhtD provides protection against fatal injection of pneumococcus in 80% of studies (Adamou *et al*. 2001).

Good protection was obtained when mice were was vaccinated with PhtD and injected with anti-polysaccharide antibodies specific for serotypes 1 and 3 (DeNoel *et al*. 2011a). A recent study showed PhtD and formaldehyde treated (dPLY) protects against pneumococcus in a rhesus macaque (Macaca mulatta) model of pneumonia using an a AS02-adjuvant system (DeNoel *et al*. 2011b). The chance of survival correlated with anti-PhtD and anti-dPLY antibody levels, which suggests the protection is at least partially because of PhtD and PLY specific antibodies (DeNoel *et al*. 2011b). Delivery of natural anti-PhtD human antibodies to mice significantly protected against lethal intranasal pneumococcal challenge.

PLY

Purified human antibody to PLY can protect mice against virulent pneumococcal strains (Musher *et al*. 2001). Immunization of mice with combinations of PLY and other pneumococcal proteins elicits protection against challenge with the pneumococcus (Ogunniyi *et al*. 2000; Ogunniyi *et al*. 2001). PLY has been used as carrier in PS conjugate vaccines (Kuo *et al*. 1995; Lee *et al*. 2001), and immunization of mice with PS PLY toxoid conjugate vaccine resulted in rapid clearance of the pneumococcus from the blood and provided cross-protection against challenge with heterologous serotypes of virulent pneumococcus (Lee *et al*. 2001), which means that PLY works not only as a conjugate carrier but also itself induces additional immunity for broad protection against pneumococcal infection out with protection elicited against PS. This suggests a PS conjugate vaccine using this pneumococcal protein as the carrier could provide a broader protective immunity than that using non-pneumococcal proteins. The PS conjugate vaccines induce mucosal immunity in addition to systemic immunity. However, PLY is too toxic to be used as vaccine directly. PLY toxoid, W433FPLY (Paton *et al*. 1991), which shows 1% haemolytic activity when compared to the WT-PLY has been widely used in animal immunisation studies and induced the significant degrees of protection against nasopharyngeal colonisation, pneumonia and sepsis (Alexander *et al*. 1994; Ogunniyi *et al*. 2001). ΔA146R147PLY (Δ6PLY), another PLY mutant, has been shown to be completely non-toxic at mg/ml concentrations (Kirkham *et al*. 2006c). It is able to bind to the cell surface but is unable to oligomerise and form pores that would damage

the host cells. Δ6PLY has recently been shown still be capable of generating an immune response when applied on the mucosal surface (Douce *et al*. 2010).

Other proteins

Previous immunization studies in a mouse intranasal challenged model using recombinant neuraminidase suggests that PLY is more valuable than the NanA as a vaccine candidate (Lock *et al*. 1988). However, Tong and colleagues showed immunization with NanA could protect chinchillas against nasopharyngeal colonisation with pneumococcus (Tong *et al*. 2005). Immunisation of mice with autolysin was shown to be effective against a virulent wild type pneumococcus (Berry *et al*. 1989a). Hyaluronidase (Hyl) is a surface bound enzyme that degrades hyaluronic acid. Hyl is involved in causing meningitis (Kostyukova *et al*. 1995). Vaccination with hyl can reduce the ability of pneumococcus to cross the blood/brain barrier (Our unpublished data). (Gianfaldoni *et al*. 2007; Moschioni *et al*. 2010)

Combined protein vaccines

Immunization with a combination of pneumococcal proteins elicited enhanced protection compared to immunization with individual proteins (Ogunniyi *et al*. 2000; Briles *et al*. 2000a; Ogunniyi *et al*. 2001; Briles *et al*. 2003; Ogunniyi *et al*. 2007). Intranasal vaccination with a mixture of PsaA and PspA can prevent nasopharyngeal carriage with pneumococcus, and the protection induced by the mixture of PsaA and PspA was significantly better than that induced by PsaA or PspA alone (Briles *et al*. 2000a). Mice vaccinated with PLY and PspA have improved protection against pneumococcus compared to active vaccination with PLY alone (Briles *et al*. 2003). Immunization with a mixture of PsaA and PspA elicited the best protection against colonisation, and PsaA elicited better protection than did PspA against colonisation (Briles *et al*. 2000a). Mice vaccinated with a mixture of a PLY toxoid (W433F, D385N and C428G), PsaA and PspA using CT as an adjuvant confers protection against pneumococcal colonisation in a CD4+ TH17 cell-dependent, antibody-independent fashion (Basset *et al*. 2007). In one study, immunisation with a combination of Lpl, ClpP and ΔA146 PLY has been shown to confer protection against pneumococcal infection with a mixture of four serotypes, and the combination vaccine was

reported to have the same efficacy as current vaccines PCV7 and PPSV23 (Wu *et al*. 2010). In another interesting study (Ogunniyi *et al*. 2007), the protective efficacy of immunization of mice with PdB (a pneumolysin toxoid), PspA, PspC (CbpA), PhtB, and PhtE were evaluated in an invasive-disease model. The combination of PdB, PspA, and PspC offers the best protection, which is better than protection in mice vaccination with one antigen or combination of any two antigens. However, protection in mice vaccinated with combination of PdB, PspA and PhtB is better than mice vaccinated with single antigen, but worse than combination of PdB and PspA. Different pneumococcal proteins play distinct roles in different stage of pneumococcal infection. Clearly, a synergistic effect was achieved when multiple proteins were delivered.

Fusion protein vaccine

Mice intranasally vaccinated with recombinant PspA fused to FlaB, a *Vibrio vulnificus* flagellin protein, have great cross-serotype protective immunity against pneumococcal infection (Nguyen *et al*. 2011). FlaB, a ligand of TLR5, has strong mucosal adjuvant activity and induces significantly higher levels of TT-specific mucosal and systemic IgA, and systemic IgG responses when administered with tetanus toxin (TT) (Lee *et al*. 2006). Mice intraperitoneally immunised with recombinant PsaA fused to B lymphocyte stimulator (BLyS) produce dramatically higher levels of PsaA specific antibodies in serum than mice immunised with non fused proteins (Gor *et al*. 2011).

1.5.5 The advantages of PLY as an protective antigen and adjuvant

PLY is a good candidate for developing a new pneumococcal vaccine. Firstly, PLY is produced by almost all clinical isolates and its primary structure varies little; secondly, PLY elicits T cell dependent immune responses and induces circulatory IgG and secretary IgA antibodies (Simell *et al*. 2001); third, PLY toxoid has the potential to be a effective carrier protein in PS conjugate vaccines; fourthly, even if the vaccine from PLY toxoid can not prevent diseases causes by all serotypes, vaccine elicited immunity to PLY may result the less severe disease. Finally, we have recently shown that PLY and its mutant Δ6PLY could act as

powerful mucosal adjuvant to carried protein antigens when they are genetically fused to the N – terminal of PLY/Δ6PLY to induce both systematic and mucosal immunity (Douce *et al*. 2010). At least one adjuvant was used in all animal experiments for testing pneumococcal protein antigens in the literature. These adjuvants are alum, Freund's solution, CT, CTB, titerMax, TT and tetanus toxin C fragment (TTCF) (Talkington *et al*. 1996; Seo *et al*. 2002). Only Alum and monophsopholipid A are licensed for use in humans as adjuvants (Ulanova *et al*. 2001; Casella & Mitchell 2008). Bacterial toxins or their derivatives such as labile toxin, TT, TTCF and CT or CTB have been widely used as adjuvants, but none of them need to be fused to the protein antigens. PLY has no adjuvant properties when only mixed with protein antigens, and needs to be fused to protein to be a potent adjuvant.

We initially used the model antigen enhanced green fluorescent protein (eGFP) to study the adjuvant properties of PLY. Intranasal challenge of mice with very low amounts of eGFPPLY or eGFPΔ6PLY (4 pmol) separately resulted in a rapid production of a significantly large amount of anti-eGFP IgG, and high levels of anti-eGFP IgA present both on the mucosal surface of the nasopharynx and in the pulmonary tracts of mice (Douce *et al*. 2010). No response was elicited when mice were administered intranasally with a mixture of unlinked proteins.

1.5.6 Cells which may play a role in the adjuvant properties of PLY

1.5.6.1 Antigen presenting cells

Antigens presenting cells (APCs) are cells that can simultaneously provide peptide/MHC II complex and co-stimulatory molecules that are essential for helper T cell (T_H cell) activation. Three types of APCs have been identified so far: dendritic cells (DCs), macrophages and B cells. DCs were firstly found just below the skin and called “Langerhans” cells, which are starfish-shaped and were discovered by Paul Langerhans in 1886 (Jolles 2002). The most important APCs are DCs as DCs are the most effective in activating naïve T_H cells, especially at the early stage of an infection. The activated DCs migrate into local lymph nodes to activate naïve T_H cells after receiving the right signal, for

example, TNF from activated residential macrophages. Activated macrophages do not travel to local lymph node. It is thought that their roles are to keep DCs and T_H cells activated when the latter migrate to the area of infection to help. B cells can not be APCs at the beginning of an infection as they are naïve cells and need to be activated by T_H cells first. However, after being activated, B cells play an important role in antigen presentation. B cells generally only present a antigen that is specifically recognised by its own B cell receptor, which is different to the other antigen-presenting cells such as DCs and macrophages which take up and display antigens non-specifically. After undergoing class switching and somatic hypermutation, activated B cells can then choose either to become plasma cells or memory B cells (Sompayrac 2003). Plasma cells are factories for producing antibodies.

1.5.6.2 T helper cells

In order to elicit antigen specific responses to purified proteins, T_H cells require to be activated through at least two different signals by antigen presenting cells (APCs) (Sompayrac 2003). Signal 1 is the cognate signal and delivered to T_H cells by peptide/MHC class II complexes on the surface of APCs (Lafferty *et al.* 1983). Signal 2 is generated in T cells by co-stimulatory molecules or cytokines released by APCs (Lafferty *et al.* 1983; Mueller *et al.* 1989). Signal 1 alone is usually not enough to activate T cells. In the absence of an effective signal 2, T_H cells become anergic (hyporesponsive) and can no longer be activated even if both signals are delivered later (Mueller *et al.* 1989). This is why generation of specific immune responses to a protein normally needs an adjuvant to induce production of co-stimulatory molecules or cytokines and to provide the second signal to T_H cells (Khoruts *et al.* 1998). T_H cells can only recognise peptide antigens but not the other antigens such as PS and cell wall fragments presented by APCs. However, it has been suggested that the peptide/MCH II signal during viral infection may activate T_H cells without second signals if the magnitude and intensity of the signal sent by APCs is sufficiently high (Bachmann *et al.* 1998). The activated T_H cell can then help activate a naïve B cell that specifically displays the same complex of foreign antigen on the class II MHC protein in a secondary lymphoid organ.

1.6 Aims of this project

The first aim of my project is to determine which regions of PLY are required for the novel adjuvant activity. The second aim of my project is to determine whether a fusion protein based pneumococcal vaccine can be developed to provide protection against three pneumococcal strains in animal models of colonisation.

Chapter 2 Materials and Methods

2.1 Bacterial strains, storage and growth conditions

S. pneumoniae strains were grown from a single colony in Brain Heart Infusion broth (BHI, Oxoid, UK) at 37°C without shaking to mid-log phase (OD600nm = 0.6) and stored in 1ml aliquots at -80°C with Protect beads (Technical Service Consultants, Lancashire, UK) or in 15% glycerol (Sigma-Aldrich, Dorset, UK). Strain purity and optochin sensitivity were checked by streaking the culture on BAB plates (Blood Agar Base; Oxoid, UK) supplemented with 5% horse blood (E & O Laboratories, Bonnybridge, UK). E. coli strains were grown overnight from a single colony with the appropriate antibiotic in Luria broth (LB: Appendix I) at 37°C with shaking at 220rpm. 1ml aliquots were made and then stored at -80°C in 10% glycerol.

2.2 Construction of plasmids

2.2.1 PsaA, PspA, PspC, PhtD and PLY nucleotide sequence

PsaA (SP_1650), PspA (SP_0117), PspC (SP_2190), PhtD (SP_1003) and PLY (SP_1923) gene sequences were extracted from the Centre Microbial Resource (CMR) database (http://cmr.jcvi.org/tigr-scripts/CMR/CmrHomePage.cgi) hosted by the J. Craig Venter Institute (JCVI-http://www.Jcvi.org/)

The amino acid sequence of PspA is not conserved across all strains of the pneumococcus (Appendix II). Based on amino acid sequence PspA can be divided into 6 clades and 3 families. Families 1 and 2 account for 94-99% of isolates in the USA, Colombia, Brazil and Argentina (Vela Coral *et al*. 2001; Brandileone *et al*. 2004; Mollerach *et al*. 2004). PspA from strain TIGR4 which is a family 2 protein is used in this study. It has previously been reported that systemic vaccination with full length family 2 PspA protects mice from intravenous challenge with strains of pneumococci bearing either family 1 or family 2 proteins (McDaniel *et al*. 1998). Colonization and virulence studies with 3 strains

which represent both family 1 (A66 and D39) and family 2 (TIGR4) PspA types are carried out in this study.

PspC also shows substantial sequence variation between strains (Appendix III). However, vaccination with the protein of the major clade (clade B) gives good protection in terms of survival rate and survival time of animals (reviewed in (Tai 2006).

2.2.2 Preparation of pneumococcal genomic DNA

10ml of mid-log phase grown pneumococci were centrifuged at 5000g for 20 min at 4°C to pellet cells. The cell pellet was resuspended and lysed with lysis buffer (10mM Tris, 100mM EDTA, 0.5% SDS in dH_2O), and the genomic (g) DNA was extracted using Qiagen mini columns (Qiagen, UK) following the manufacturer's instructions.

2.2.3 Plasmids

Plasmid pET33b (+) (Novagen, UK), containing a kanamycin resistance gene, was used. pET33b-D4PLY and pET33bD123PLY were constructed with ligation dependent cloning; pET33bPsaA, pET33bPspA, pET33bPspC and pET33bPhtD, pET33bPsaAPLY, pET33bPspAPLY, pET33bPspCPLY and pET33bPhtDPLY were constructed by in-fusion cloning (Clontech, France); pET33bΔ6PLY, pET33bPsaAΔ6PLY, pET33bPspAΔ6PLY, pET33bPspCΔ6PLY and pET33bPhtDΔ6PLY were constructed through Site-Directed Mutagenesis (Stratagene, UK).

2.2.4 Ligation dependent cloning

pET33bD4PLY, pET33bD123PLY and pET33beGFPPLY contain the gene sequences of domain 4 of PLY (D4PLY), domain 123 of PLY (D123PLY) and eGFPPLY respectively. pET33beGFPPLY and pET33bPLY separately were constructed by Mr Graeme J. M. Cowan.

2.2.4.1 Generation of DNA segment

50μl of pET33bD4PLY, pET33bD123PLY and pET33beGFPPLY were prepared separately from Library Efficiency® DH5α™ chemically competent (DH5α) *E.coli* (Invitrogen, Paisley, UK) using QIAprep Spin Miniprep Kit (Qiagen, West Sussex, UK) following the manufacturer's instructions. DNA quality was measured by agarose gel electrophoresis, and DNA quantity and quality was also measured by Nanodrop 1000 spectrophotometer (Thermo Scientific, UK). The eGFP coding sequence was amplified from pET33beGFPPLY by Polymerase Chain Reaction (PCR) using Taq polymerase (Promega, UK) and primers 20G and 20H (Table 2-3).

2.2.4.2 Enzyme digestion and ligation

The purified plasmids were then digested with 2μl each of *Nhe*I and *BamH*I restriction enzymes (New England Biolab, UK) at 37°C for 2 hours. The cut plasmids were treated with 1μl of calf intestinal alkaline phosphatase (Promega) at 37°C for 30mins and cleaned up using gel purification kits (Qiagen, UK). The PCR products from pET33b-eGFPPLY were cleaned using gel purification kit (Qiagen), digested using 2μl each of restriction enzyme *Nhe*I and *Bg*III (New England Biolab, UK) at 37°C for 2 hours and purified again using the gel purification kit. 100 ng of cleaned cut PCR products were ligated with 100 ng of cut plasmids using 10 units of T4 DNA ligase at room temperature for 4 hours.

2.2.5 In-fusion cloning

The in-fusion cloning system is ligation independent. It utilises the unique properties of the 3′–5′ exonuclease activity of poxvirus DNA polymerase. When linear double stranded DNAs with homologous ends (15- 18 base pairs) were incubated in the presence of Mg^{2+} and low concentrations of dNTP, the poxvirus DNA polymerase progressively removes nucleotides from the 3′ end using its 3′–5′ proofreading activity. This exposes complementary regions on DNAs that can then spontaneously anneal through base pairing and the nicks are repaired inside *E. coli* after transformation.

2.2.5.1 Design of overlap primers

In-Fusion can join up to 8 DNA fragments together as long as they share 15 ~ 18 base pairs (bps) of identical DNA segments at each end. Therefore, the PCR primers comprise two regions: vector specific and gene specific, and are between 35-55 bps long. The vector specific region is at least 15bps, which is identical to the end of the linearised vector; the gene specific region is between 20-30 bps. In addition, restriction sites, linkers, translation initiation sites or epitope tags can be added into the primer sequences. All primers are designed on Vector NTI™ Advance 10 software (Invitrogen, UK).

2.2.5.2 Generation of DNA segment

100ng of pET33b(+) was transferred into Library Efficiency® DH5α™ chemically competent *E.coli* (Invitrogen, UK), and 5μg of pET33bPLY and 5μg of pET33b(+) were prepared from 5ml overnight cultures using QIAprep Spin Miniprep Kit (Qiagen, UK) following the manufacturer's instructions. DNA quality was measured by agarose gel electrophoresis. The *PsaA, PspA, PspC, PhtD and PLY* genes were amplified from genomic DNA of the serotype 4 strain of TIGR4 *S. pneumoniae* by Polymerase Chain Reaction (PCR) using *pfuUltra* II fusion Hot Start (*pfuUltra* II) DNA polymerase (Stratagene, UK) and primers from Table 2-2 and Table 2-3. PspA is cloned from nucleotide 96 - 957(E32-L319). *PspC* is cloned from nucleotide 102 – 1479 (V34-Y493). *PhtD* is cloned from nucleotide 61 - 2574 (S21-I838). The use of high-fidelity polymerase can dramatically reduce errors and reaction time during PCR. The purified pET33b (+) plasmid was digested with 20 units each of *BamH*I and *HindIII* restriction enzymes (New England Biolab, UK) sequentially at 37^{o}C for 3 hours. The restriction enzyme-digested plasmid and all the PCR products were cleaned up using gel purification kits (Qiagen). DNA quality was measured by agarose gel electrophoresis, and DNA quantity and quality was also measured by Nanodrop 1000 spectrophotometer (Thermo Scientific, UK).

2.2.5.3 In-fusion reaction

As mentioned above, the In-Fusion Enzyme fuses PCR-generated DNA sequences and linearised vectors efficiently and precisely by recognizing a 15 ~

18 bps overlap at their ends. This 15 ~ 18 bps overlap can be engineered by designing custom primers for amplification of the desired sequences. 100-150ng of restriction enzyme-digested, gel-purified pET33b(+) plasmid were mixed at a molar ratio of 1 vector to 2 of each PCR DNA product in a total volume of 10 µl plus water in one tube of In-Fusion Dry-Down reaction mix (Clontech, France). The reaction was incubated for 15 min at 37°C, following 15 mins at 37°C, the reaction was then transferred to ice, and 40 µl Tris EDTA (TE) was added.

2.2.6 Site-Directed Mutagenesis

Stratagene's QuikChange® II XL Site-Directed Mutagenesis Kit was used. The method has been used successfully in making point mutation, replacing amino acid and deleting or inserting single or multiple amino acids. In order to introduce Δ6 mutation, 50-100ng of plasmids were amplified with the primers 23B and 23C (Table 2-3) by PCR (Table 2-1) using pfuUltra II DNA polymerase (Stratagene, UK). The mutagenic primers are complementary to each other and to opposite strands of the amplifying plasmid. The PCR product then was incubated with 1µl of restriction enzyme DpnI at 37°C for 2 hours in order to digest parental methylated plasmid. The nicked mutagenic plasmid was repaired inside XL-10 Gold chemically ultracompetent (XL10-Gold) *E.coli* cells after transformation.

Table 2-1 Site-directed mutagenesis PCR.

Reagent	Concentration and Volume µl
Forwards primer	15pmol, 15µl
Reverse primer	15pmol, 15µl
Template	1µl
DNTPs Mix	10mM, 10µl
DNase free d H_2O	Added to final volume of 50µl
10 × buffer	5µl
pfuUltra II	1µl

2.2.7 Transformation and DNA sequencing

1μl of ligation mix, diluted in-fusion reaction mixture or *DpnI* digested mutagenesis PCR product was transformed into XL10 Gold *E. coli* following the manufacture's instructions (Stratagene, UK). Bacteria were grown overnight on the Luria-Bertani (LB) agar (Appendix I) plate containing 50μg/ml kanamycin at 37°C. Colony PCR was performed in order to confirm the transformation using primers 7F and 7G (Table 2-3) flanking to the gene inserted. The PCR confirmed colonies were grown in 10ml LB containing 50μg/ml kanamycin overnight at 37°C with shaking at 220rpm. Bacterial stock with 10% glycerol was made and 1ml aliquots were then stored at -80°C. Plasmids were purified from culture (Qiagen, UK), checked by restriction enzyme digestion and finally sent for DNA sequence. All plasmids were sequenced in The Sequencing Service, University of Dundee using primers shown on Table 2-2 and Table 2-3. Data for DNA sequence were analysed by Vector NTI™ Advance 10 software (Invitrogen, UK). After the DNA sequences were proven to be correct, All Plasmids were electro-transformed into BL21 (DE3) *E.coli* (Stratagene, UK) by electroporation using BioRad Gene Pulser (BioRad, UK) following the manufacture's instructions. The colony PCR was performed before protein expression.

Table 2-2 Primers used for sequencing and constructing expression vectors.

Construct Name	Constructing Primer	Sequencing Primer
PET33beGFPD4PLY	20G and 20H	33Z, 34A, 7F
PET33beGFPD123PLY	20G and 20H	33Z, 34A, 7F
PET33bPsaA	65Y, 65Z	7F, 7G
PET33bPsaAPLY	65Y, 66A, 65W, 65X	4T, 7F, 7G, 65O
PET33bPsaAΔ6PLY	23B, 23C	4T, 7F, 7G, 65O
PET33bPspA	66B, 66C	7F, 7G
PET33bPspAPLY	66B, 66D, 65W, 65X	4T, 7F, 7G, 65P
PET33bPspAΔ6PLY	23B, 23C	4T, 7F, 7G, 65P
PET33bPspC	66E, 66F	7F, 7G
PET33bPspCPLY	66E, 66G, 65W, 65X	4T, 4U, 7F, 7G, 65T, 65U
PET33bPspCΔ6PLY	23B, 23C	4T, 4U, 7F, 7G, 65T, 65U
PET33bPhtD	66H, 66I	7F, 7G, 64O, 64P, 64Q
PET33bPhtDPLY	66H, 66J, 65W, 65X	4T, 4U, 7F, 7G, 64O, 64P, 64Q, 65V
PET33bPhtDΔ6PLY	23B, 23C	4T, 4U, 7F, 7G, 64O, 64P, 64Q, 65V

Table 2-3 Sequencing primers.

Primer Ref	Primer Name	Sequence (5' to 3')
7F	T7 old Fwd	TAATACGACTCACTATAGGG
7G	T7 old Rev	GCTAGTTATTGCTCAGCGGTG
20G	GFP pET33b Fwd	GTC AGG CTA GCA TGA GTA AAG GAG AAG AAC
20H	GFPpET33b Rev	CCA CGC AGA TCT TTG TAT AGT TCA TCC
23B	Delta6 forwards	GGTCAATAATGTCCCAATGCAGTATGAAAAAATAACGGCTC
23C	Delta6 reverse	GAGCCGTTATTTTTTCATACTGCATTGGGACATTATTGACC
33Z	eGFPseq1	TTGCACTACTGGAAAACTAC
34A	eGFPseq2	CATGGCAGACAAACAAAAAGAA
64O	PhtD Internal1 FWD	GTTCTAGTTATAATGCAAATCC
64P	PhtD Internal2 FWD	GTCGACAAGTTGATTTTGAGGC
64Q	PhtD Internal3 FWD	CCAAACGAACGTCCGCATTCAG
64R	PhtD Int-Ext FWD	GCAGAAGTAGATAGTCTCTTGG
65O	PsaA sequence for	CAAACATCCCAATCTACGCTC
65P	PspA sequence for	TGCTACTGCTCAACATCAAG
65S	PspC sequence rev	TCTTCAACCTTCTTCTCAGC
65U	PspC sequence rev2	ATTCTTCTTCTGATCTACGAGC
65T	PspC sequence for 2	TGAACAACCACAACCAGCG
65V	phtD sequence for	CGGCGACCATTACCATTAC
65W	PLY for new	GGTGGTGGTGGTTCCGCAAATAAAGCAGTAAATGACTT
65X	PLY rev new	GAGTGCGGCCGCAAGCTCTAGTCATTTTCTACCTTATCC
65Y	PsaA for Single	CAAATGGGTCGGGATCCGGCTAGCGGAAAAAAAGATACAACTTCTG
65Z	PsaA rev single	GAGTGCGGCCGCAAGCTTTATTTTGCCAATCCTTCAGCAATC
66A	PsaA rev double	GCGGAACCACCACCACCTTTTGCCAATCCTTCAGCAATCTTG
66B	PspA for single	GCAAATGGGTCGGGATCCGGAAGAATCTCCACAAGTTGTCG
66C	PspA rev single	GAGTGCGGCCGCAAGCTTATTCAGGATCAGCCCCTCCAAG
66D	PspA rev double	GCGGAACCACCACCACCTTCAGGATCAGCCCCTCCAAG
66E	PspC for single	GCAAATGGGTCGGGATCCGGTGGTTCATGCGACAGAGAAC
66F	PspC rev single	GAGTGCGGCCGCAAGCTTAATATTCTTCTTCTGATCTACGAGC
66G	PspC rev double	GCGGAACCACCACCACCATATTCTTCTTCTGATCTACGAGC
66H	PhtD for single	GCAAATGGGTCGGGATCCGTCCTATGAACTTGGTCGTCACC
66I	PhtD rev single	GAGTGCGGCCGCAAGCTTATATAGGAGCCGGTTGACTTTC
66J	PhtD rev double	GCGGAACCACCACCACCTATAGGAGCCGGTTGACTTTC

2.3 Protein expression and purification

2.3.1 Protein expression

The BL21 (DE3) *E.coli* containing different plasmids were grown separately in 2 litres of LB containing 50μg/ml kanamycin with shaking at 220rpm at 30°C. 1mM Isopropyl-β-D-thio-galactopyranoside (IPTG) was added to induce expression of the recombinant proteins when the OD_{600} of cell cultures reached 1. The bacterial cells were grown for a further 6 hours in LB at 30°C with shaking at 220rpm before harvesting by centrifugation at 4°C for 30 mins. Cell pellets were resuspended in 50 ml of pre-chilled phosphate buffered saline (PBS) (Oxoid, UK) with 10mg of the proteinase inhibitor Benzamidine and 300 units of DNase I (Sigma-Aldrich, Dorset, UK) per litre of cell pellets. Bacterial pellet suspension was sonicated 5 times for 30 seconds at 100% by cell sonicator (Constant systems Ltd, Warwick, UK) with a minute break between each time. The lysates were centrifuged at 18000g for 30 minutes at 4°C to remove cell debris. The supernatant was filtered by syringe using 0.22μm filter (Sartorius, Hannover, Germany).

2.3.2 Protein purification

2.3.2.1 Nickel affinity chromatography (NAC)

Expression vector pET33b is designed to leave six histidines (His tag) at either C-terminus or N-terminus of the proteins. Because the C-terminus of PLY is essential for cell binding, we leave the His tag at the N-terminus of the fusion proteins. All recombinant proteins can be purified by immobilised metal affinity chromatography since the His tag can interact tightly with metal. The bacterial cell supernatants were passed through a Nickel-charged NTA (Nitrilotriacetic acid resin) His-Trap FF crude column (GE healthcare, UK) by an AKTA purifier (GE Healthcare, UK). The tagged proteins stayed on the column, and the other proteins and bacterial components were eluted. His-tagged proteins were then eluted with a 0-500mM continuous gradient of imidazole in PBS. 25 fractions with increasing concentration of imidazole were collected. Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) was used to select the right

fraction which only contains one right size protein; the other fractions with contaminants were discarded.

2.3.2.2 Anion exchange chromatography (AEC)

Purified recombinant proteins were further purified by AEC column Hitrap Capto Q (GE healthcare, UK) on the AKTA purifier in order to exclude DNA, lipopolysaccharides (LPS) and contaminating proteins. The recombinant proteins were eluted with a gradient of a 0-1M continuous gradient of NaCl in 20mM Tris HCl buffer (PH = 8) while DNA, LPS and the other contaminants remained bound to the column.

2.3.2.3 Size Exclusion Chromatography (Gel filtration)

PspC was further purified on the AKTA purifier by gel filtration using S200 column (GE healthcare, UK).

2.3.2.4 Dialysis

Dialysis tubing with 8 kDa molecular weigh cut off (Medicell International Ltd, London, UK) was boiled in 10mM sodium bicarbonate solution with 1mM EDTA for 10 mins, washed with sterile PBS and stored at 4^{o}C with 20% ethanol. The proteins were dialysed three times against a greater than 50-fold volume of 1 × PBS at 4^{o}C over 36 hours. Amicon ultra centrifugal filters (Millipore, Watford, UK) were used to concentrate the proteins; Amicon ultra centrifugal filters were also used to remove the small amount of contaminating proteins. All proteins were filtered by syringe using 0.22μm filter (Sartorius, UK) before being aliquoted and stored at -80^{o}C.

2.4 Analysis of purified proteins

2.4.1 Protein concentration

Absorbance (A) of a protein sample depends on the molar concentration (c), light path length in centimetres (L), and molar extinction coefficient (ε) of protein at the specified wavelength. Purified samples have a low A260 (DNA

concentration) and a low A320 (protein aggregates). Only the amino acid tryptophan (W), tyrosine (Y) and to a lesser extent cysteine (C) contribute significantly to protein absorbance at 280nm. The amount of protein can be determined by measuring the absorbance (A) by spectrophotometer at 280nm. The molar protein concentration is equal to the absorbance at 280nm divided by the molar extinction coefficient (ε) of proteins.

$$\varepsilon = (nW \times 5500) + (nY \times 1490) + (nC \times 125)$$

$$A_{\lambda} = \varepsilon c L$$

$$c = A_{280nm} / \varepsilon L \ (= A_{280nm} / \varepsilon \text{ when } L = 1 \text{ cM})$$

2.4.2 SDS-PAGE and Western Blotting

Proteins were analysed using SDS-PAGE and Western blotting for presence of other contaminating proteins or degradation of the proteins. Western blots were also used to check if sera from mice immunised with different fusion proteins recognise native proteins in pneumococci. 10ml of mid-log phase grown pneumococci was centrifuged at 5000g for 20 min at 4°C to pellet cells. The cell pellet was resuspended in 1 ml PBS and sonicated 5 times for 15 seconds at 100% by cell sonicator (Constant systems Ltd, Warwick, UK) with a minute break between each time. 2-10μl of samples were mixed with 4 × loading buffer and boiled for 10 min at 70°C before loading on NuPAGE® Novex Bis-Tris 4-12% gels (Invitrogen, UK). Protein Seeblue®plus2 Pre-stained Standard marker (invitrogen, UK) was used for indicating sizes of proteins. All gels were run at 150 Volts for 60 min, then either stained or transferred to Hybond-C nitrocellulose membrane (Amersham Biosciences, Buckinghamshire, UK) and blotted at 30 Volts for 60 min using XCell™ Blot Module (Invitrogen, UK). The membranes were blocked in 3% skimmed milk (in Tris-NaCl pH 7.4) overnight at 4°C with shaking. Membranes were then incubated with first antibody (1:2000 dilutions in 3% skimmed milk) for two hours at 37°C with shaking. Membranes were then washed four times with Tris-Nacl (pH 7.4) buffer and incubated with horseradish hydrogen peroxidise-linked second antibody (1:2000 dilution in 3% skimmed milk; Amersham Biosciences) for two hours at 37°C with shaking.

Membranes were washed four times and finally developed with 4-Chloro-1-naphthol reaction solution (Sigma-Aldrich, UK) or chemiluminescent HRP substrate (Immobilon western system, Millipore, Watford, UK).

2.4.3 Lipopolysaccharide (LPS) levels in purified recombinant proteins

E.coli is a Gram-negative bacterium, LPS is a component of the cell wall of Gram-negative bacteria. LPS is highly immunogenic, high levels of LPS can affect mouse immunisation studies. An *in vitro* kinetic, quantitative chromogenic, limulus amebocyte lysate (LAL) endosafetm –pts cartridge (Charles River endosafe, USA) was used to determine the level of contaminating Gram-negative LPS in all protein samples. The LAL test is the most sensitive and specific assay available to measure LPS. The assay was run following manufacturer's instructions and with the help of Dr. Andrew Smith. In this assay, LPS triggers activation of a cascading series of serine proteases in LAL. The last activated enzyme in this series cleaves the colourless synthetic substrate to release yellow colour p-nitroaniline, which can be measured by a spectrophotometer at 385-410 nm.

2.4.4 Haemolytic assay

Round- and flat-bottomed 96-well plates (R-plate and F-plate; Nunc™, Denmark) were both used for this haemolytic assay. 1ml of horse blood was centrifuged for 1 min at 13k rpm in an Eppendorf tube. The supernatant was removed and the pellet was resuspended in same volume of PBS. Above process was repeated 5 times in order to move lysed cell and antibodies completely. 2% horse blood was prepared by adding 400μl washed horse blood to 20 ml PBS. 100μl of PBS was added into required wells of an R-plate by multi-channel pipette.100μl of PBS (negative control) and samples were then added into the wells in row 1, and 100μl of solution was transferred into row 2 from row 1. The tips were changed and 100μl of solution was transferred into row 3 from row 2. Above step was repeated until 100μl was discarded from the bottom row of the plate. 100μl of 2% horse blood was added into each well. The plate was covered by a lid and incubated at 37^{o}C for 30 minutes. The R-plate was centrifuged at 1000rpm for 1

minute and 100μl of supernatant from each well was transferred into a fresh F-plate by multi-channel pipette. The absorbance was measured by the spectrophotometer (96 well plate reader) at 540nM.

2.4.5 Formaldehyde treatment

Purified recombinant PsaAPLY fusion protein was incubated in PBS containing 25mg ml^{-1} lysine at 37°C for 48h with 4 different concentrations of formaldehyde separately; 0.03% (normally used to stabilize proteins for vaccine production), 0.15% (light treatment, LFT), 1.25% (medium treatment, MFT) and 5% formaldehyde (heavy treatment, HFT). The treated proteins were then tested by the haemolytic assay described previously. PsaAPLY was also incubated for 48h at 37°C in PBS containing 25mg ml^{-1} lysine separately. All the treated proteins then were dialysed 3 times with PBS overnight 24 hours at 4°C, and finally centrifuged at 4000 x *g* for 20 min. No precipitation was observed.

2.4.6 Characterisation of purified eGFP tagged proteins by fluorescence microscopy

Horse blood was prepared following the method in haemolytic assay. Lysed horse erythrocytes were also prepared with sterile water. 1ml of horse blood was centrifuged for 1 min at 13k rpm in an Eppendorf tube. The supernatant was removed and the pellet was resuspended in same volume of water. The cell suspension was centrifuged and the supernatant was removed again. The same process was repeated 3 times. 10 μl of horse blood and 1μl of proteins were added into 500 μl Eppendorf tubes, and incubated at 37°C for 15 minutes. 1 μl of each sample was put on a microscope slide and covered with a cover glass. The specimens were observed under the microscope with ultraviolet (UV) light.

2.4.7 Characterisation of purified eGFP tagged recombinant proteins by fluorescence-activated cell sorter (FACS)

In order to confirm the findng from microscopy, flow cytometry was performed using FACS. 4% horse blood cells were prepared by adding 800μl washed horse blood cells into 20ml PBS. All proteins were diluted to 14nM, 140nM and 1400nM

concentration. 500 μl of 4% horse blood and 500μl of protein sample were added into the Eppendorf tubes and results 7nM, 70nM and 700nM concentration. The mixture was then incubated at 37°C for 15 minutes, transferred into FACS tubes and measured by FACS. The result is analysed by WINMDI.

2.5 Immunisation study of purified recombinant proteins in mice

In vivo experiments were performed in accordance with the UK Animals 1986 (Scientific Procedures Act). All Balb/c and MF1 mice were purchased from Harlan Olac (UK). Mice were given at least one week to acclimatise prior to being placed on procedure. Food and water *ad libitum* were provided and mice were left at a constant room temperature of 20-22 °C with a 12 hours light/dark cycle. All animal work procedures were performed under personal licence and Home Office project approval. The programme of work was approved by the Ethics Committee of the University of Glasgow.

2.5.1 Determination of which region of PLY is required for the mucosal adjuvant activity

Six to eight week-old female Balb/c mice were intranasally (as described in section 2.5.2.1) immunised 3 times with antigens on days 1, 27 and 54 in the groups shown in Table 2-4. The experiment was based on giving 0.2μg of wild type (WT) PLY toxin and ten times equimolar concentration of D123PLY and D4PLY, and the final vaccine dose is 20 μl in PBS. Group 1 was immunised with 0.2μg of PLY and 0.1μg of eGFP, which ensure the ratio of PLY and eGFP was identical to that present in an equivalent dose of the eGFPPLY protein in group 2; group 2 was immunised with the equimolar concentration of eGFPPLY,; group 3 was immunised with ten times equimolar concentration of D4PLY and eGFP; group 4 was immunised with ten times equimolar concentration of eGFPD4PLY and eGFP; group 5 was immunised with ten times equimolar concentration of D123PLY and eGFP; group 6 was immunised with ten times equimolar concentration of eGFPD123PLY and eGFP; group 7 was immunised with ten times equimolar concentration of eGFPD4PLY and 0.2μg of PLY; group 8 was immunised with ten times equimolar concentration of eGFPD123PLY and 0.2μg of

PLY. Group 9 was immunised with ten times equimolar concentration of eGFPD4PLY and of Δ6PLY; group 10 was immunised with ten times equimolar concentration of eGFPD123PLY and 0.2μg of Δ6PLY. Group 11 was immunised with ten times equimolar concentration of eGFPD4PLY and D123PLY; group 12 was immunised with ten times equimolar concentration of eGFPD123PLY and D4PLY. Mouse sera from the day before each vaccination were collected from tails. Anti-eGFP IgG and IgA, anti-PLY IgG and IgA responses within individual serum samples were measured by enzyme linked immunoasorbent assay (ELISA).

Table 2-4 Mice immunisation study of purified recombinant proteins (eGFP as model antigen).

Group	Antigens used	Number of mice
1	Mixture of eGFP and PLY	6
2	eGFPPLY alone	5
3	Mixture of eGFP and D4PLY	5
4	eGFPD4PLY alone	5
5	Mixture of eGFP and D123PLY	5
6	eGFPD123PLY alone	5
7	Mixture of eGFPD4PLY and PLY	5
8	Mixture of eGFPD123PLY and PLY	5
9	Mixture of eGFPD4PLY and Δ6PLY	5
10	Mixture of eGFPD123PLY and Δ6PLY	5
11	Mixture of eGFPD4PLY and D123PLY	5
12	Mixture of eGFPD123PLY and D4PLY	5

2.5.2 A protein vaccine based on adjuvant activity of PLY

This project is funded by a charity named PATH (Program for Appropriate Technology in Health), so mice vaccinated intranasally and subcutaneously with PsaA fusion proteins were designated PATH 1 and 2 respectively; mice vaccinated intranasally and subcutaneously with PspA fusion proteins were designated PATH 3 and 4 respectively; mice vaccinated intranasally and subcutaneously with PspC fusion proteins were designated PATH 5 and 6 respectively; mice vaccinated intranasally and subcutaneously with PhtD fusions were designated PATH 7 and 8 respectively; mice vaccinated intranasally and

subcutaneously with the mixture of fusion protein antigens were designated PATH 9 and PATH 10 respectively. PATH 11 was to examine the impact of intranasal vaccination with the mixture of fusion proteins antigens on survival following invasive diseases.

2.5.2.1 Mucosal and systemic vaccination

Eight to twelve week-old outbred female MF1 mice (Harlan Olac, UK) were mucosally or systemically immunised 3 times with purified proteins in day 0, 14 & 28 in the groups shown in table 2-6. For mucosal immunization, 20μl 4pmol proteins in 20μl PBS (10μl per nostril) were intranasally instilled into the nares using a pipette. For systemic immunization, 4pmol proteins in 100μl PBS were subcutanously injected into the loose skin on the neck. For both delivery routes, mice were lightly anaesthetised with 3.5% isofluorane/1.5% oxygen (1.5 litre/min) (Astra-Zeneca, Macclesfield, UK) until the limb movement reflex had been lost. 35 animals were vaccinated by each route. Two weeks after the final vaccination (day 42), 100 μl blood were taken from mice 1-30 of each group by tail bleed, and mice 31-35 of each group were sacrificed. Samples were taken from the blood (cardiac puncture) and from the mucosal surfaces (nasopharynx and lung wash). Antigen specific IgG and IgA in blood and mucosal washes were measured by enzyme linked immunoasorbent assay (ELISA).

The remaining 30 immunised mice were challenged at day 56 (one month after final vaccination) using pneumococcal strain serotype 2 D39, bioluminescent pneumococcal strain serotype 3 A66.1 (Xen10) and bioluminescent pneumococcal strain serotype 4 TIGR4 (Xen35) separately via the respiratory tract. Colonisation model was used. All three strains were passaged through mice and stored as standard inocula with known viable counts. Virulence of three strains was confirmed in standard titration experiments in animals. Those mice were monitored for colonisation to determine the effect of vaccination. In order to monitor interaction between the pneumococcus and the mouse, standard bacteriology and *in vivo* imaging via IVIS spectrum system were used, *in vivo* imaging system allowed us to monitor growth and distribution of bacteria within the live mice in real time.

2.5.2.2 Preparation of mouse passaged standard inocula

Single colonies of *S. pneumoniae* TIGR4 (serotype 4), A66.1 Xen10 (serotype 3), TIGR4 Xen35 (serotype 4), and D39 (serotype 2) were grown up to mid-log phase (OD_{600nm} = 0.6) in BHI as described in section 2.1. 1ml aliquots were stored in 10% glycerol at -80ºC for at least 16 h then viable counts were assessed and $5x10^6$ cfu/200µl injected intraperitoneally (i.p.) into an MF1 mouse as previously described (Alexander et al. 1994). At 6 hpi the animal was sacrificed by terminal exsanguinations via cardiac puncture under general anaesthesia and blood was incubated overnight in 20 ml BHI at 37ºC and plated onto BAB plates as a sterility check. A 1:50 dilution of the overnight culture was inoculated into pre-warmed BHI with 15% fetal calf serum (FCS) and statically grown at 37ºC to mid-log phase (OD_{600nm} = 0.6). These standard inocula were then frozen at -80ºC for at least 16 h before viable counts were performed. Cultures were checked for purity and optiochin sensitivity strain as described in section 2.1. prior to freezing. The colony forming units (cfu)/ml in each culture were calculated.

2.5.2.3 Viable counts from standard inocula

24 h post freezing, at least three aliquots for each strain were defrosted rapidly in a 37ºC water bath for 2 min. The aliquots were then centrifuged at RT for 5 min at 13000g for 5 min. The supernatant was discarded and the cell pellet resuspended in 1ml PBS. 10-fold series of dilutions were made in a round-bottomed 96 well plate using sterile Dulbecco's PBS (DPBS; Sigma-Aldrich). 3 x 20µl of each dilution was spotted onto BAB plates divided into eight sectors and allowed to dry. Plates were incubated anaerobically overnight in a candle jar at 37ºC. The dilution sector where there were 10-70 colonies/20µl was then counted for calculating the cfu/ml for each strain. Before each challenge, the standard inocula were defrosted and prepared in the same way as described above and diluted in sterile DPBS to the desired dose. Viable counts of the inocula were checked before and after challenge to ensure that the bacteria remained viable during challenge and the correct dose was given.

2.5.2.4 IVIS Imaging

Mice were imaged for a maximum of 5 min at a number of time points post-infection using an IVIS Spectrum CCD camera (Xenogen Corporation). Images that were saturated were then retaken using modified settings. Total photon emission from selected and defined areas within the image of each mouse was quantified using Living Image 4.0 software package (Xenogen Corporation). The photon signal from the thorax was quantified from the ventral image of each mouse. The photon signal from the nasopharynx was quantified from the dorsal image of each mouse.

2.5.2.5 Colonisation model

2 x 10^7 cfu bacteria in 10µl were inoculated into the nares of the isofluorane anaesthetized animal (the small volume ensures colonization rather than infection of the lower respiratory tract). Distribution of the organisms was monitored at daily intervals using the *in vivo* imaging system for 3 days. Blood samples were collected at each day from tails. Mice were sacrificed after 3 days and nasopharyngeal washes were obtained using previously described method. Bacteria in the nasopharyngeal washes were quantified by viable counting. The level of colonization for the three selected pneumococcal strains was compared at the selected time point between fusion protein vaccinated groups and those given free protein.

2.5.3 Measuring antibody response to fusion proteins by ELISA

Flat-bottomed 96-well plates (Nunc™ Fisher scientific) were used for ELISA. Plate wells were coated with 100µl of 1µg/ml purified recombinant proteins separately in PBS overnight at 4°C. The plates were washed three times in PBS-T (PBS plus 0.05% Tween 20) and dried thoroughly. The plates were blocked with 50µl/well of 1% BSA in PBS (Sigma-Aldrich) and incubated for 1 hour at 37°C. The plates were washed 3 times in PBS-T and dried thoroughly. The plates were then incubated at 37°C with 100µl/well of serial dilution of the serum in duplicate (started at 1:50) in PBS for 2 hours. The plates were washed 3 times in PBS-T and dried thoroughly. The plates were incubated for 1 hour at 37°C with 100µl/well of a 1:1000 dilution of goat anti-mouse anti-IgG (γ-chain specific) or

IgA (α-chain specific) Biotin antibody (Sigma-Adrich, UK) in washing buffer. The plates were finally incubated for half an hour at 37°C with 100μl/well of a 1:2000 dilution of ExtrAvidin®−Peroxidase (Sigma-Adrich, UK) in washing buffer. In the negative control, 100μl/well of fresh coating buffer was added; in the positive control, a mouse serum that has been tested active was added. The plates were washed 3 times in PBS-T and dried thoroughly. 50μl of developing solution (Fast developing tablets, sigma-Aldrich, UK) were added into each well. The plates were incubated for 5 min at 37°C and the reaction was stopped with 50μl/well of 3M Hydrochloric acid. The absorbance was read by spectrophotometer (96 well plate reader) at 490nm and titres were calculated as the reciprocal of the highest serum dilution, which gave an absorbance of 0.3 above the background.

2.6 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 and Antibody titres 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. For groups of three or more, the data was compared using non-parametric Kruskal-Wallis with Dunn's post-test, which allows comparison of individual columns with control data. In all experiments, $P<0.05$ is considered significantly (*), $P<0.01$ is considered highly significant (**) and $P<0.001$ is considered extremely significant (***). P values are reported in the figure legends.

Chapter 3 Construction of expression vector, protein purification and in vitro characterisation of purified proteins

PLY and Δ6PLY can dramatically increase the antibody response to protein antigens genetically fused to themselves (Douce *et al*. 2010). In this chapter, the model antigen eGFP was fused to D123PLY and D4PLY. PsaA, PspA, PspC and PLY were also fused to PLY or Δ6PLY. Purified eGFP tagged proteins were then used to determine which domains of PLY are important for the novel adjuvant activity in Chapter 4. Purified PLY or Δ6PLY fused proteins were then used to develop a protein based pneumococcal vaccine in Chapter 5.

3.1 Construction of expression vectors

pET33bD4PLY, pET33bD4ePLY, pET33b123PLY and pET33beGFPPLY were constructed by Mr Graeme J.M.Cowan. A total of 14 plasmids were constructed in this work. pET33beGFPD4PLY and pET33beGFPD123PLY were constructed with ligation dependent cloning. pET33bPsaA, pET33bPsPA, pET33bPspC, pET33bphtD, pET33bPsaAPLY, pET33bPsaAPspAPLY, pET33bPspCPLY and pET33bphtDPLY were constructed by in-fusion cloning technology; pET33bPsaAΔ6PLY, pET33bPspAΔ6PLY, pET33bPspCΔ6PLY and pET33bPhtDΔ6PLY were constructed through Site-Directed Mutagenesis using pET33bPsaAPLY, pET33bPsaAPspAPLY, pET33bPspCPLY and pET33bphtDPLY as templates. The sequencing data from University of Dundee were analysed on Vector NTI™ Advance 10 software (Invitrogen, Paisley, UK) and showed all constructed expression plasmids have the right sequences.

3.2 Protein expression and purification

18 recombinant proteins with low levels of contaminating LPS (Table1-1) were purified successfully during this project. A two-step method of nickel affinity

chromatography (NAC) and anion exchange chromatography (AEC) was used for all proteins; the third step, size exclusion chromatography (gel filtration) was only used for purification of PspC.

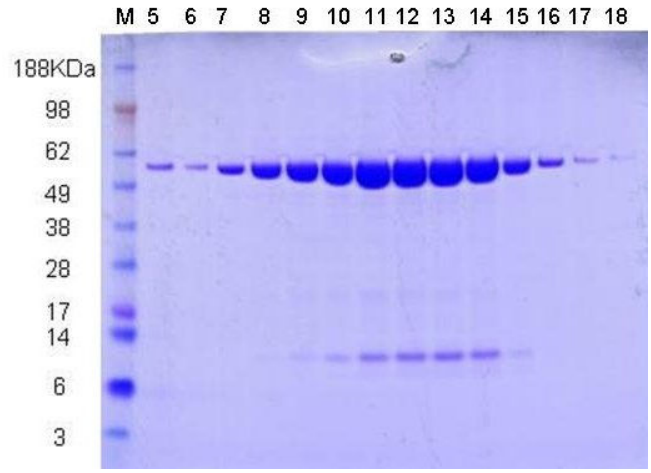


Figure 3-1 SDS-PAGE gel of PLY following NAC purification.
5µl of samples were loaded in from lane 5 to 18. 20 fractions were collected during NAC purification of PLY. Lane M is protein molecular weight marker (Invitrogen, UK). Fractions 5-18 contain a large amount of recombinant PLY, whose molecular weight is 57kDa.

PLY was firstly purified by NAC, and recombinant PLY with His_6-tag has a molecular weight of 57kDa and SDS PAGE confirmed this in comparison to molecular weight markers (Figure 3-1). The purified PLY was dialysed with Tris buffer and further purified with AEC purification (Figure 3-2 and Figure 3-3). During AEC purification, PLY bound to the negatively charged column, and was washed completely, then was eluted with a gradient of 0-0.5 M NaCl. The column was finally cleaned with 1M NaOH and 3M NaCl and stored at 20% ethanol. There are three peaks in the UV absorbance wave (blue line), the biggest peak is PLY, and the small peak before the biggest peak indicates contaminating proteins. The PLY collected after NEC was dialysed three times at 4^{o}C, 7 hours each time with 2 litres pre-chilled PBS buffer. In Figure 3-3, SDS-PAGE gel shows the PLY is over 99% pure after AEC purification.

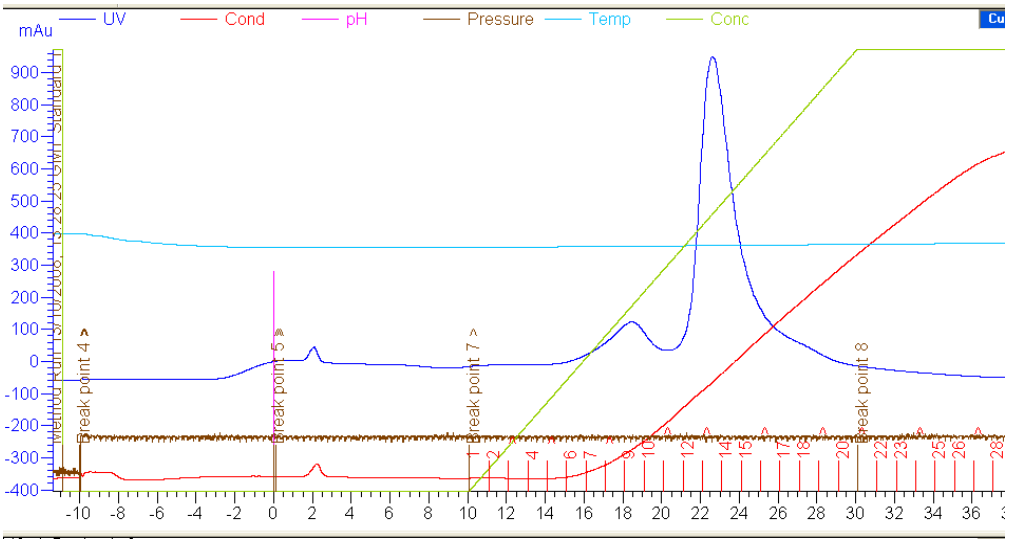


Figure 3-2 AEC purification of PLY.
Anion exchange Chromatography of PLY. The blue line (UV) is A280nm protein reading, which measures protein content. The yellow line represents NaCl concentration; the brown line presents the pressure in the column. Fraction 12-23 was kept for dialysis with PBS.

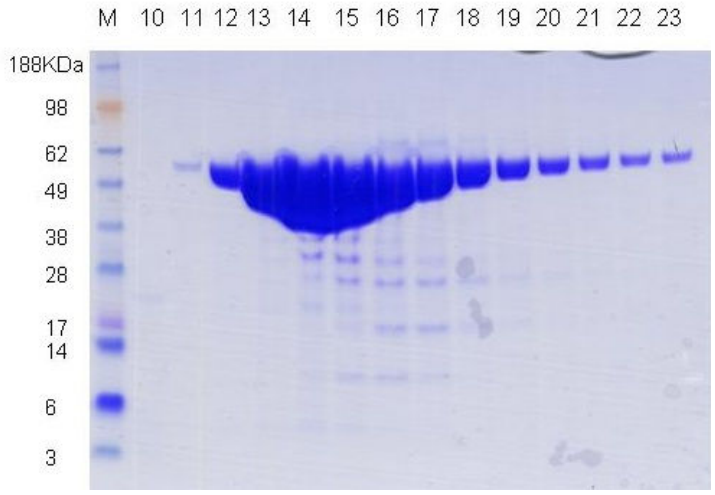


Figure 3-3 SDS-PAGE gel of PLY following AEC purification.
10µl of samples were loaded in each lane. Lane M is protein molecular weight marker. Lanes 10-23 are fractions eluted from the AEC column.

The purification of eGFPD123PLY (Figure 3-4), eGFPD4PLY (Figure 3-5), eGFPPLY (Figure 3-6), D123PLY (Figure 3-7), D4PLY (Figure 3-8), PsaA (Figure 3-9), PspA (Figure 3-10), PspC (Figure 3-11), PhtD (Figure 3-12), PsaAPLY (Figure 3-13), PsaAΔ6PLY (Figure 3-13), PspAPLY(Figure 3-14), PspAΔ6PLY (Figure 3-14), PspCPLY (Figure 3-16), PspCΔ6PLY (Figure 3-16), PhtDPLY (Figure 3-17) and PhtDΔ6PLY (Figure 3-17) are the same as that of PLY except PspC was further purified with gel filtration column. In each case, analysis by gel electrophoresis revealed a single protein of the expected size. All purified proteins are also over 95% pure.

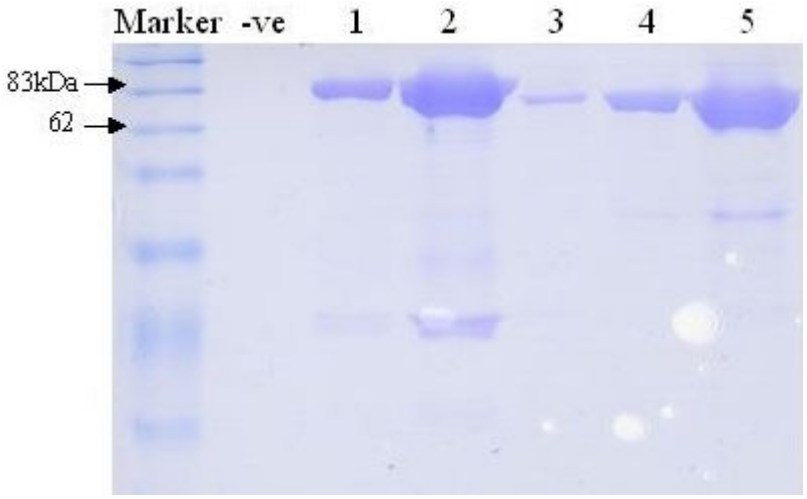


Figure 3-4 SDS-PAGE gel of eGFPD123PLY after NAC purification.
1µl of eGFPPLY protein was loaded in Lane 1, 8µl of eGFPPLY was loaded in Lane 2, 1µl of eGFPD123PLY protein was loaded in Lane 3, 3µl was loaded in Lane 4, and 8µl was loaded in Lane 5.

Recombinant eGFPD123PLY has a molecular weight of 70 kDa and SDS-PAGE confirmed this in comparison to molecular weight markers (Figure 3-4).

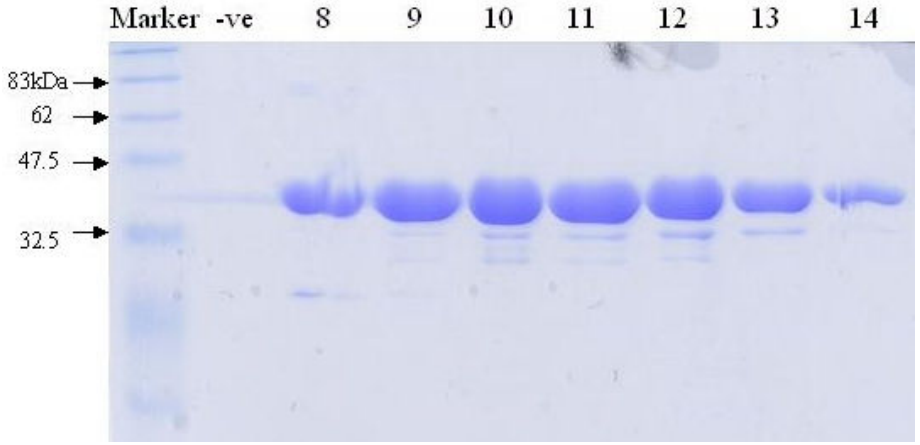


Figure 3-5 SDS-PAGE gel of eGFPD4PLY after NAC purification.
The different lane numbers represent different fractions, 8µl of samples were loaded in each lane; MW of recombinant eGFPD4ePLY is 44kDa. 20 fractions were collected during NAC purification of eGFPD4ePLY. Fraction 9-16 was kept for further purification. The eGFPD4ePLY was further purified as eGFPD123PLY.

Recombinant eGFPD4PLY has a molecular weight of 44 kDa and SDS-PAGE confirmed this in comparison to molecular weight markers (Figure 3-5).

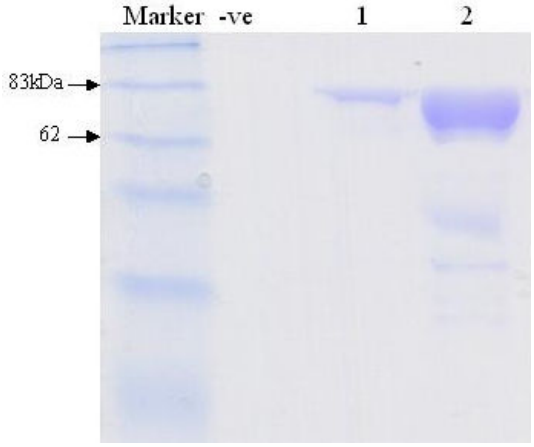


Figure 3-6 SDS-PAGE gel of eGFPPLY after AEC purification.
The samples were loaded in 10% SDS-PAGE gel, 1µl of protein was loaded in Lane 1, and 8µl was loaded in Lane 2. The gel was stained with coomassie brilliant blue solution. Negative control is loading buffer, MW of recombinant eGFPPLY is 83kDa. EGFPPLY was purified as the same as eGFPD123PLY.

Recombinant eGFPPLY has molecular weights of 83 kDa and SDS PAGE confirmed this in comparison to molecular weight markers (Figure 3-6).

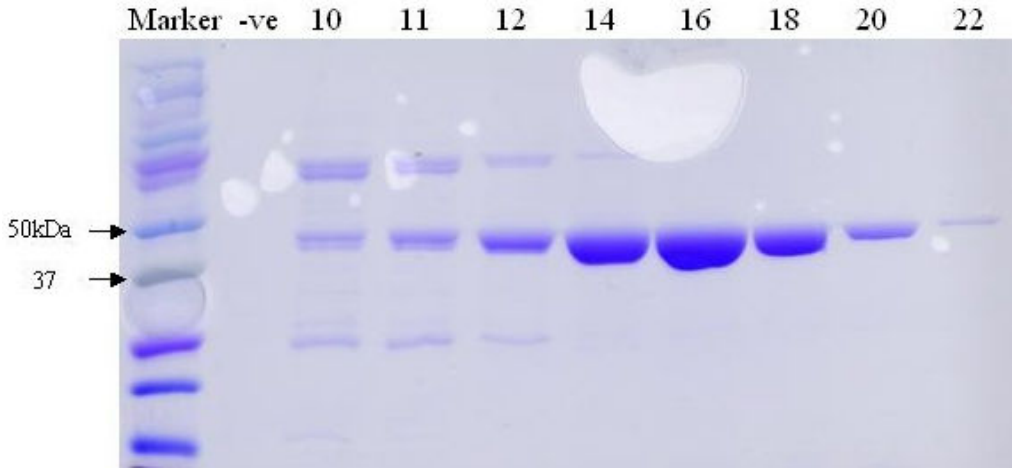


Figure 3-7 SDS-PAGE gel of D123PLY after AEC purification.
The different lane number represent different fractions, 8µl of samples were loaded in each lane; MW of recombinant D123PLY is 44 kDa. 25 fractions were collected during NAC purification of 123PLY. Fractions 16-22 were kept for further purification. The D123PLY was further purified as eGFPD123PLY

Recombinant D123PLY has a molecular weight of 44 kDa and SDS-PAGE confirmed this in comparison to molecular weight markers (Figure 3-7).

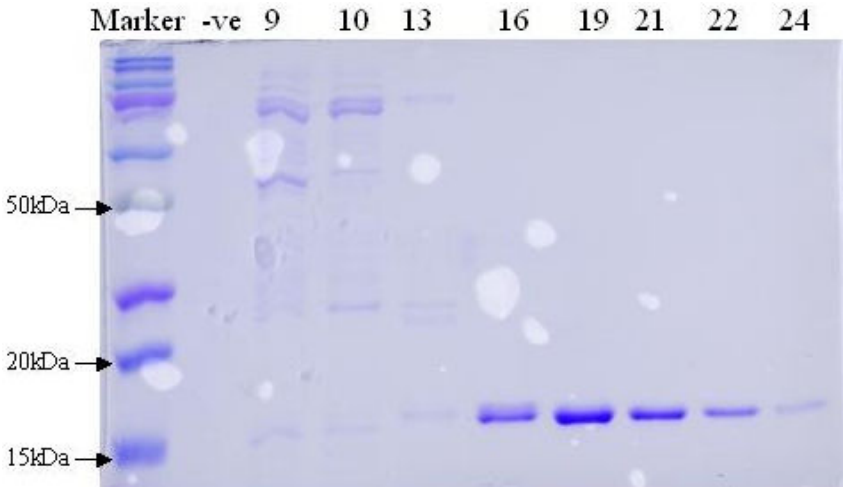


Figure 3-8 SDS-PAGE gel of D4PLY after AEC purification.
The different lane number represent different fractions, 8µl of samples were loaded in each lane; MW of recombinant D4ePLY is 18 kDa. 25 fractions were collected during NAC purification of D4PLY. Fractions 16-25 were kept for further purification.

Recombinant D4PLY has a molecular weight of 18 kDa and SDS PAGE confirmed this in comparison to molecular weight markers (Figure 3-8).

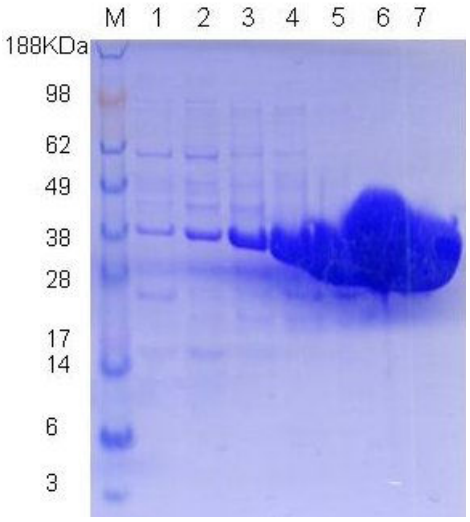


Figure 3-9 SDS-PAGE gel of PsaA after AEC purification.
10µl of samples were loaded in each lane. Lane M is protein molecular weight marker. Lanes 1-7 are fractions eluted from the AEC column. Fractions 5-7 were collected and dialysed against PBS.

Recombinant PsaA has a molecular weight of 37 kDa and SDS-PAGE confirmed this in comparison to molecular weight markers (Figure 3-9).

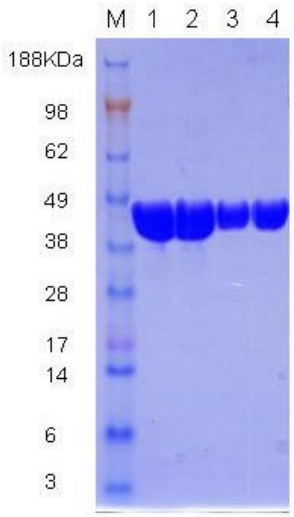


Figure 3-10 SDS-PAGE gel of PspA after AEC purification.
The different lane numbers represent different fractions, 7µl of samples were loaded in lane 1 and 2, 3 µl of samples were loaded in lane 3 and 4. Lane M is protein molecular weight marker. The recombinant PspA ran as 47 kDa in SDS-PAGE gel, even MW of recombinant PspA is predicted to be 38kDa.

In Figure 3-10, the estimated size of purified truncated PspA was approximately 47kDa on SDS-PAGE, which is larger than the predicted size of 38 kDa. However, anomalous migration of PspA was reported previously (Yother *et al*. 1992).

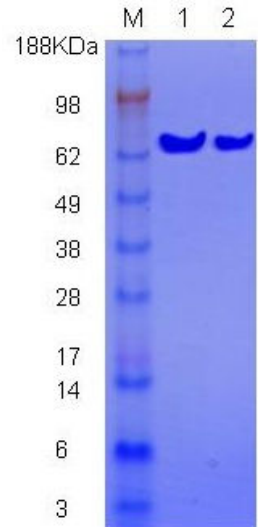


Figure 3-11 SDS-PAGE gel of PspC after gel filtration purification.
8µl of samples were loaded in each lane; recombinant PspC ran as 75 kDa, even MW of recombinant PspC is predicted to be 56kDa. Lane M is protein molecular weight marker.

The mobility of purified truncated PspC has a molecular weight of 75 kDa via SDS-PAGE although the molecular weight of recombinant PspC is predicted to be 56 kDa (Figure 3-11). Anomalous migration of PspC has been reported previously (Rosenow *et al*. 1997; Ogunniyi *et al*. 2001).

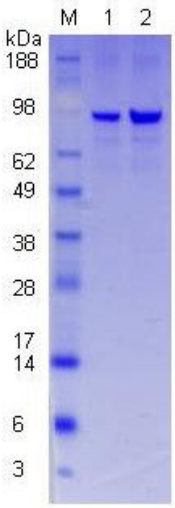


Figure 3-12 SDS-PAGE gel of PhtD after AEC purification.
3μl of sample was loaded in lane 1 & 2. PhtD is predicted to be 92 kDa. Lane M is protein molecular weight marker.

PhtD has a molecular weight of 92 kDa and SDS-PAGE confirmed this in comparison to molecular weight marker (Figure 3-12).

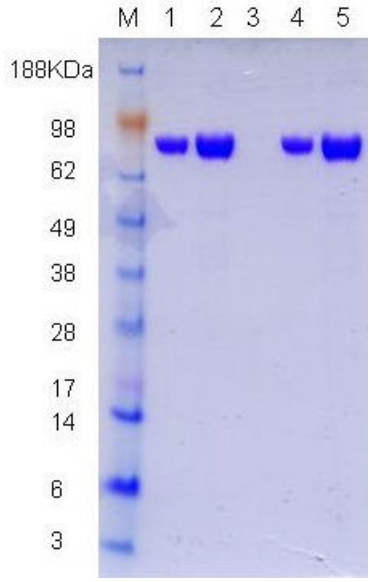


Figure 3-13 SDS-PAGE gel of PsaAPLY and PsaAΔ6PLY after AEC purification.
3μl of sample was loaded in lane 1 & 4. 6μl of sample was loaded in lane 2 & 5. Lane M is protein molecular weight marker. Lanes 1 & 2 were PsaAPLY and lanes 4 & 5 were PsaAΔ6PLY. Both PsaAPLY and PsaAΔ6PLY are predicted to be 89.7 kDa.

PsaAPLY and PsaAΔ6PLY have molecular weights of 89.7 kDa and SDS PAGE confirmed this in comparison to molecular weight markers (Figure 3-13).

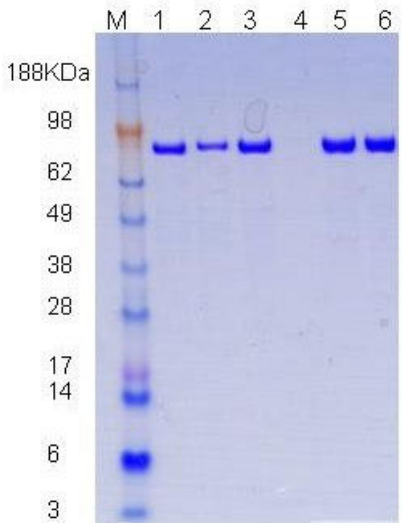


Figure 3-14 SDS-PAGE gel of PspAPLY and PspAΔ6PLY after AEC.
6μl of sample was loaded in each lane. Lane M is protein molecular weight marker. Lane 1-3 are PspAPLY, Lane 5 and 6 are PspAΔ6PLY. Both PspAPLY and PspAΔ6PLY are predicted to be 91 KDa.

PspAPLY and PspAΔ6PLY have molecular weights of 91 KDa and SDS-PAGE confirmed this in comparison to molecular weight markers (Figure 3-14).

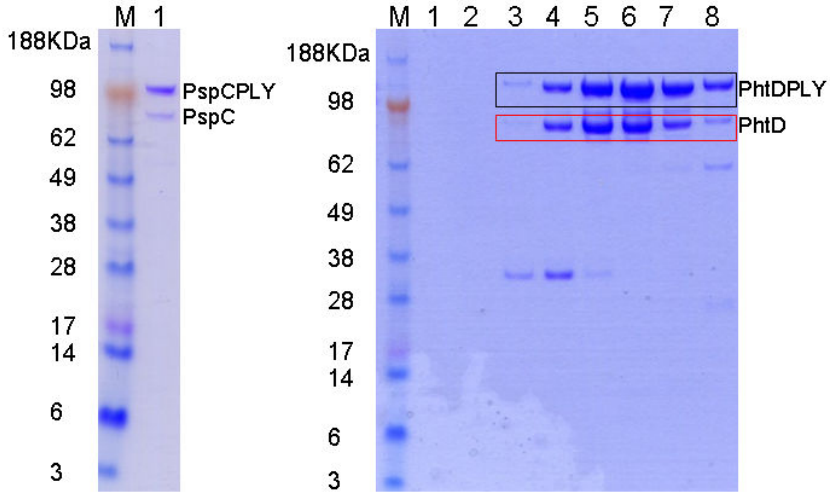


Figure 3-15 PspCPLY and PhtDPLY are cleaved during purification when GGGGS peptide linker were used.
10μl of sample was loaded each Lane. Lane M is protein molecular weight marker. PspCPLY has molecular weights of 109 kDa. PhtDPLY has molecular weights of 149 kDa.

At the beginning, PspCPLY and PhtDPLY failed to be purified as these two fusion proteins were cleaved to PspC/PLY or PhtD/PLY due to a linker problem (Figure 3-15). PAPAPAPAP peptide linker was used instead.

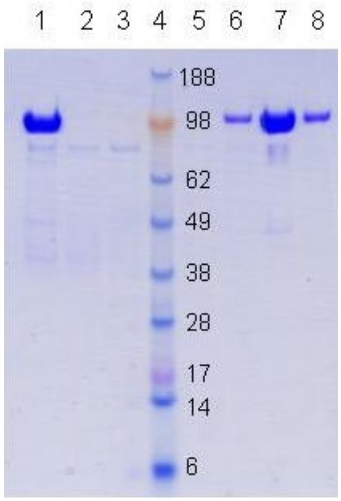


Figure 3-16 SDS-PAGE of PspCPLY and PspC∆6PLY following AEC purification.
4µl of sample was loaded in lane 1 & 7.Lane M is protein molecular weight marker. 1µl of sample was loaded in lane 6 & 8. Lanes 1 & 6 were PspCPLY and lanes 7 & 8 were PspCΔ6PLY.

After PAPAPAPAP peptide linker was introduced, PspCPLY and PspCΔ6PLY were successfully purified. PspCPLY and PspCΔ6PLY have molecular weights of 109 kDa and SDS-PAGE confirmed this in comparison to molecular weight markers (Figure 3-16).

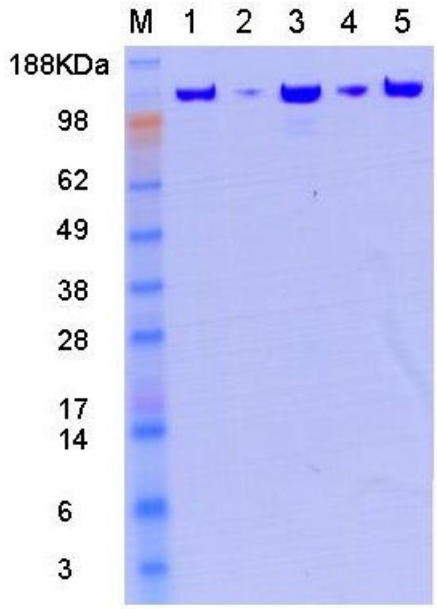


Figure 3-17 PhtDPLY and PhtDPLY after dialysis.
Lane M is protein molecular weight marker. Lane 1-3 are purified PhtDPLY. Lane 4 and 5 are purified PhtD∆6PLY. Both PhtDPLY and PhtD∆6PLY are predicted to be 149 KDa.

After PAPAPAPAP peptide linker was introduced, PhtDPLY and PhtDΔ6PLY were successfully purified. PhtDPLY and PhtDΔ6PLY have molecular weights of 149 kDa and SDS-PAGE confirmed this in comparison to molecular weight markers (Figure 3-17).

3.3 Concentration and LPS level of purified recombinant proteins

Protein concentration (Table 1-1) was determined using an absorbance at 280nm. Purified proteins have a low A260nm (DNA concentration) and a low A320nm (protein aggregates). The levels of LPS contamination (Table 3-1) were low (less than 5 International Unit (IU)/dose) and were considered to be insufficient to stimulate the immune system non-specifically (Douce *et al*. 2010).

Table 3-1 Properties of recombinant proteins used in future experiments.

Protein designation	Size (kDa)	Concentration ((μg/ml)	LPS level (IU/μg)	Haemolytic activity (HU/pmol)	Yield (mg/L)
PLY	57	520	0.103	22700	25
D123PLY	44	536	0.047	0	25
D4PLY	18	441	0.065-0.068	0	10
eGFPPLY	83	513	0.35-0.37	22700	40
eGFPD123PLY	70	450	0.334-0.373	0	50
eGFPD4PLY	44	434	0.067	0	25
PsaA	37	438	0.618	0	40
PsaAPLY	90	493	0.87	22650	7.5
PsaAΔ6PLY	90	467	0.78	0	8
PspA	38	1000	0.75	0	37.5
PspAPLY	91	700	1.1	22570	3.5
PspAΔ6PLY	91	1111	0.985	0	3
PspC	56	1700	0.234	0	5
PspCPLY	109	850	0.8	22690	2.5
PspCΔ6PLY	109	680	0.33	0	2.85
PhtD	92	2000	1.05	0	2
PhtDPLY	149	560	1.2	22534	3
PhtDΔ6PLY	149	650	1.3	0	2.5

3.4 Western Blotting

The purified recombinant proteins were further analyzed by Western Blotting. Figure 3-18 shows purified eGFPPLY, eGFPD123PLY, eGFPD4PLY, PLY, D123PLY and D4PLY were recognised by rabbit anti-PLY anti serum. Sera from mice intranasally immunised with purified Δ6PLY fusion protein were used to evaluate expression of native proteins in pneumococcal strains D39, Xen35 and Xen10 by Western blotting (Figure 3-19 to Figure 3-22). D39, Xen35 and Xen10 are three pneumococcal strains that were used to challenge vaccinated mice in later experiments. These sera were also used to evaluate purified recombinant proteins by Western Blotting. The levels of antigen specific IgG and IgA were also measured by ELISA and will be discussed in Chapter 5. Sera from mice immunised with purified recombinant proteins Δ6PLY fusion proteins recognised both purified recombinant proteins and native proteins in D39, Xen35 and Xen10 (Figure 3-19 to Figure 3-22).

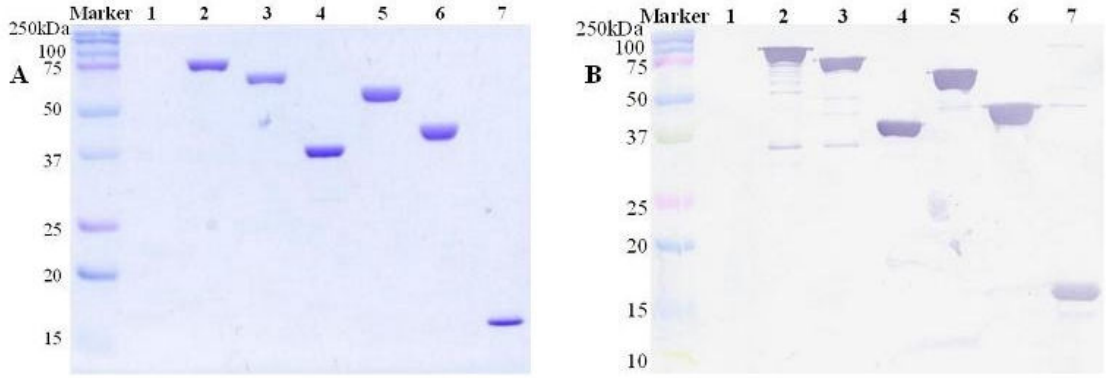


Figure 3-18 (A) SDS-PAGE and (B) Western Blotting analysis of the purified recombinant proteins.
1 µg of each sample was loaded on gel and the gel was stained with Coomasie brilliant blue solution. Rabbit anti-PLY antibody was used for Western Blotting. The specificity of this antibody has been previously tested with a pneumococcal cell lysate lack of PLY. Lane1, negative control; lane 2, eGFP-PLY is 83 kDa; lane 3, eGFP-D123PLY is 70 kDa; lane 4, eGFP-D4PLY is 42 kDa; lane 5, PLY is 57 kDa; lane 6, D123PLY is 44 kDa; lane 7, D4PLY is 18kDa.

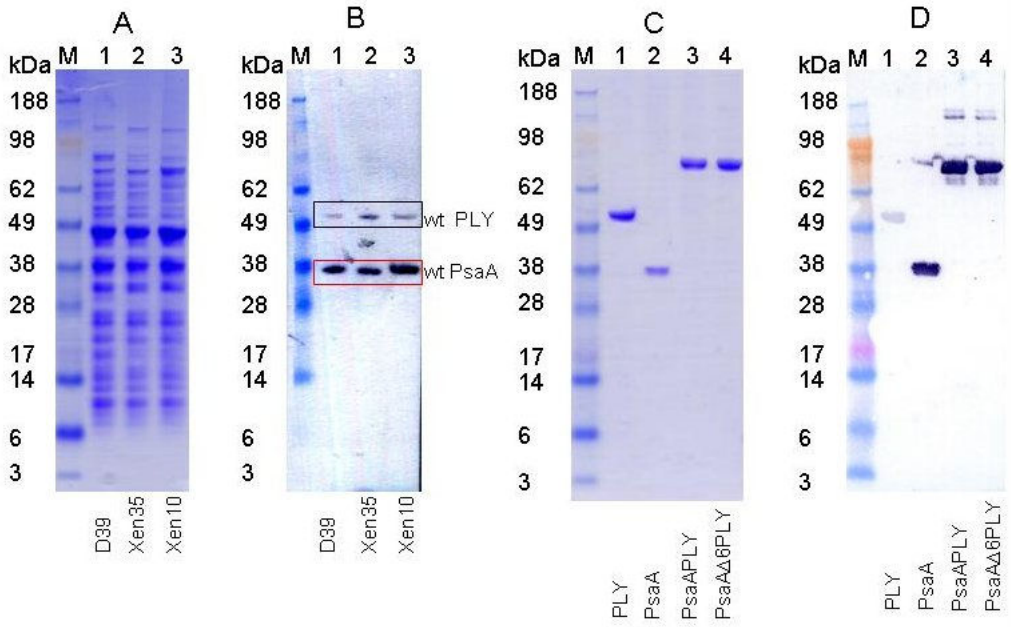


Figure 3-19 Sera from mice intranasally immunised with PsaAΔ6PLY recognise both recombinant and native proteins.
M is protein molecular weight marker. Lane 1, 2 and 3 contain 4 µl of pneumococcal whole cell lysate D39, Xen35 and Xen10 in A and B. A is SDS-PAGE gel stained with coomassie blue. B is western blot developed with anti-PsaAΔ6PLY mice sera, which recognised both wt PLY is 53 kDa (Black Square) and wt PsaA is 35 kDa (Red Square). Lane 1, 2, 3 and 4 contain 1µg of recombinant PLY (57 kDa), PsaA (37 kDa), PsaAPLY (90 kDa) and PsaAΔ6PLY (90 kDa) in C and D. C is SDS-PAGE gel stained with coomassie blue. D is western blot developed with anti-PsaAΔ6PLY mice sera.

In Figure 3-19, the mouse sera rose with PsaAΔ6PLY recognised recombinant PLY, PsaA, PsaAPLY and PsaAΔ6PLY; wt PLY and wt PsaA were nicely detected in all three strains with these sera. PsaAPLY and PsaAΔ6PLY were also recognised by rabbit polyclonal anti-PLY antibody (Figure 3-24B).

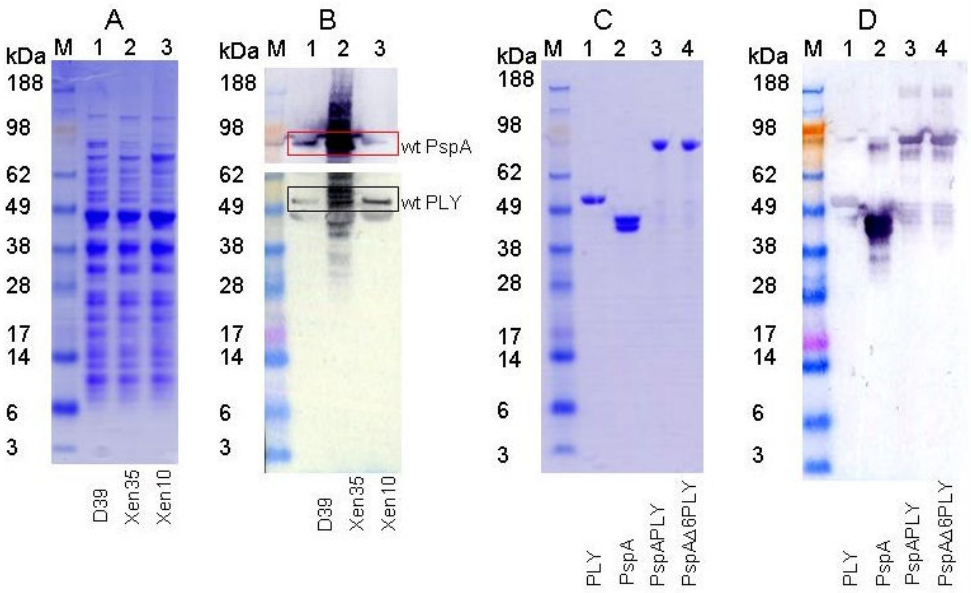


Figure 3-20 Sera from mice intranasally immunised with PspAΔ6PLY recognise both recombinant and native proteins.
M is protein molecular weight marker. Lane 1, 2 and 3 contain 4 µl of pneumococcal whole cell lysate D39, Xen35 and Xen10 in A and B. A is SDS-PAGE gel stained with coomassie blue. B is western blot developed with anti-PspAΔ6PLY mice sera, which recognised both wt PLY (53 kDa, Black Square) and wt PspA (69 ~ 82.8 kDa, Red Square). Lane 1, 2, 3 and 4 contain 1µg of recombinant PLY (57 kDa), PspA (predicted to be 38 kDa, but ran as 47 kDa), PspAPLY (91 kDa) and PspAΔ6PLY (91 kDa) in C and D. C is SDS-PAGE gel stained with coomassie blue. D is western blot with anti-PspAΔ6PLY mice sera. The same blot was cut to two parts and developed in different time.

In Figure 3-20, the sera raised against with PspAΔ6PLY recognised recombinant PLY, PspA, PspAPLY and PspAΔ6PLY; wt PLY and wt PspA were detected in all three pneumococcal strains.

In Figure 3-21, the sera from mice immunised with PspCΔ6PLY recognised recombinant PLY, PspC, PspCPLY and PspCΔ6PLY; wt PLY and wt PspC were detected in all three strains.

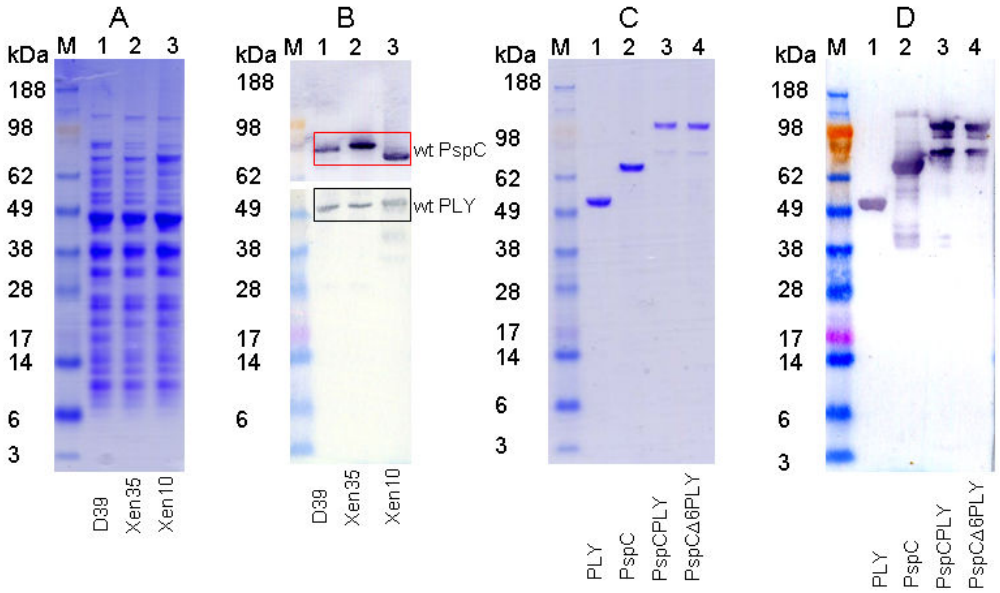


Figure 3-21 Sera from mice intranasally immunised with PspCΔ6PLY recognise both recombinant and native proteins. M is protein molecular weight marker.
Lane 1, 2 and 3 contain 4 µl of pneumococcal crude cell lysate D39, Xen35 and Xen10 separately in A and B. A is SDS-PAGE gel stained with coomassie blue. B is western blot probed with anti-PspCΔ6PLY mice sera, which recognised both wt PLY (53 kDa, Black Square) and wt PspC (78 ~ 79 kDa, Red Square). Lane 1, 2, 3 and 4 contain 1µg of recombinant PLY (56 kDa), PspC (predicted to be 56 kDa, but ran as 75 kDa), PspCPLY (109 kDa) and PspAΔ6PLY (109 kDa) in C and D. C is SDS-PAGE gel stained with coomassie blue. D is western blot with anti-PspCΔ6PLY mice sera. The same blot was cut to two parts and developed in different time.

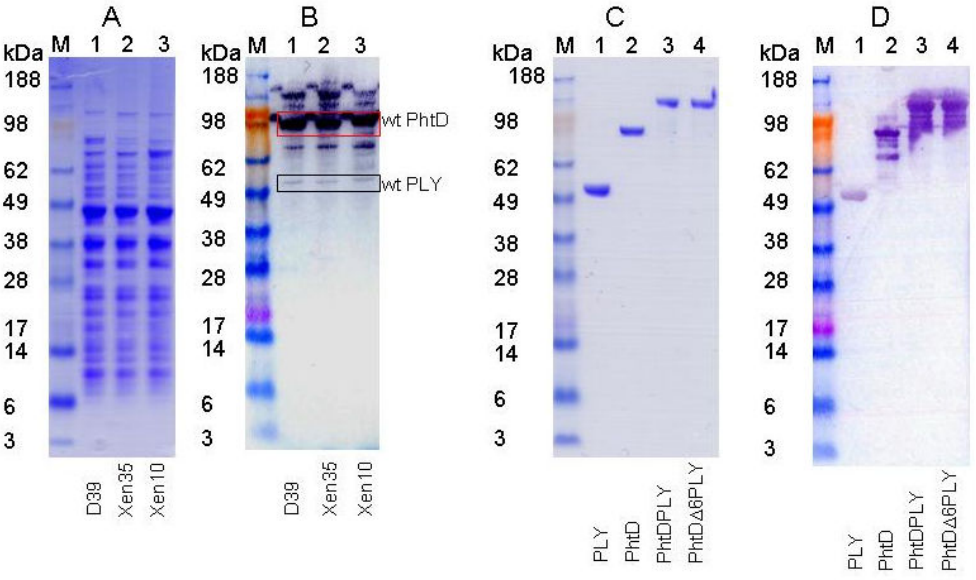
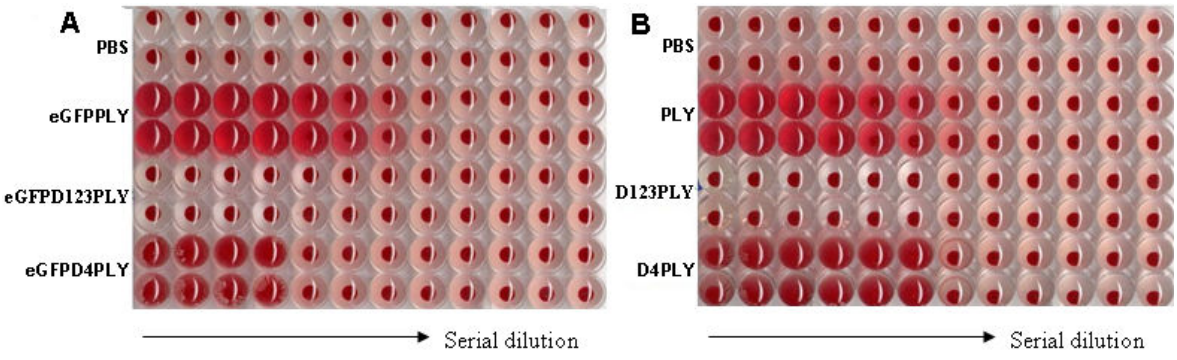


Figure 3-22 Sera from mice intranasally immunised with PhtDΔ6PLY recognise both recombinant and native proteins.
M is protein molecular weight marker. Lane 1, 2 and 3 contain 4 µl of pneumococcal crude cell lysate D39, Xen35 and Xen10 separately in A and B. A is SDS-PAGE gel stained with coomassie blue. B is western blot probed with anti-PhtDΔ6PLY mice sera, which detected both wt PLY (53 kDa, Black Square) and wt Pht proteins including PhtA (91 kDa, Red Square), PhtB (92 kDa, Red Square), PhtD (93.5 kDa, Red Square) and PhtE (116 kDa). In C and D, Lane 1, 2, 3 and 4 contain 1µg of recombinant PLY (56 kDa), PhtD (92 kDa), PhtDPLY (149 kDa) and PspAΔ6PLY (109 kDa). C is SDS-PAGE gel stained with coomassie blue. D is western blot probed with anti-PhtDΔ6PLY mice sera.

In Figure 3-22, the sera from mice immunised with PhtDΔ6PLY recognised recombinant PLY, PhtD, PhtDPLY and PhtDΔ6PLY; wt PLY and wt Pht proteins including PhtA, PhtB, PhtD and PhtE were detected in all three strains.

3.5 Haemolytic assay

The purified proteins were analysed with a haemolytic assay to determine their toxicity (Figure 3-23). The haemolytic units for the purified proteins are detailed in Table 3-1. There was no statistically significant difference between the haemolytic activity of native PLY and its fusion proteins. EGFPPLY, PsaAPLY, PspAPLY, PspCPLY and PhtDPLY are as haemolytic as PLY. 1nM of recombinant PLY and PLY fusion proteins were sufficient to completely lyse 2% horse blood cell. These results mean fusing a protein to the N-terminus of PLY does not affect haemolytic activity. D123PLY, D4PLY, eGFPD123PLY, eGFPD4PLY, PsaA, PspA, PspC, PhtD and all Δ6PLY fusion proteins are non-haemolytic as expected, even at 3000 nM concentrations, which is 1000 times higher than the concentration of PLY and PLY fusion proteins. Interestingly, the high concentration of D4PLY and eGFPD4PLY caused horse erythrocytes to form aggregates in PBS solution (Figure 3-23A and B); the supernatant in the cells treated with high concentrations of D123PLY and eGFPD123PLY is clearer than that its control (PBS) (Figure 3-23A and B).



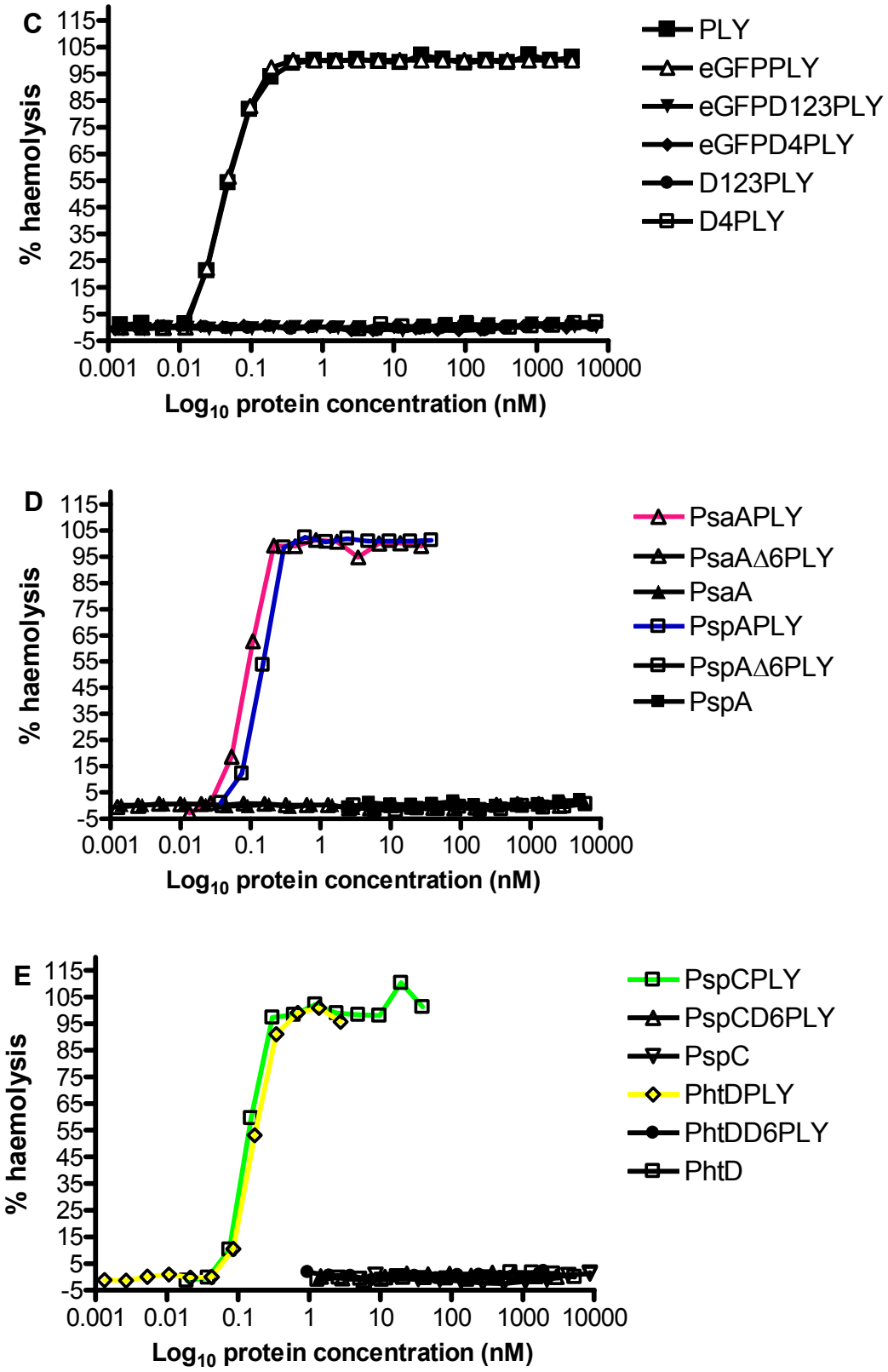


Figure 3-23 Haemolytic activity of purified proteins.
2% horse blood erythrocyte were treated with decreasing concentrations of purified recombinant proteins in PBS and % of haemolysis was determined by measurement of the resultant supernatant at OD_{540} after incubated for 30 min at 37°C. In A and B, the concentrations of proteins are decrease from left to right.

3.6 Formaldehyde treatment

Formaldehyde treatment has been used widely in vaccine preparation to inactivate the toxicities of bacterial toxins, and in some cases, to increase the immunogenicity (Lagergard et al. 2007). Formaldehyde treatment should be also considered for PLY, which may inactivate its toxicity but keep its immunogenicity. A recent study showed PhtD and formaldehyde treated (dPLY) protects against pneumococcus in a rhesus macaque (Macaca mulatta) model of pneumonia using a AS02-adjuvant system (DeNoel *et al*. 2011b). This type of treatment results in the chemical cross linking of those domains of PLY that interact with the cell and can reduce its capacity to lyse cells, but at very low concentrations, formaldehyde treatment could also stabilize the protein and help in storage and transport.

PsaAPLY at 0.2mg/ml was treated with 0.03%, 0.15%, 1.25% and 5% of formaldehyde separately. 25mg ml^{-1} lysine was also added to all samples. The haemolytic activities were totally abolished with formaldehyde treatment even with 0.03 % formaldehyde (Figure 3-25). High molecular weight products were observed with all concentrations in SDS-PAGE gel (Figure 3-24A). Those products were formed due to chemical cross-linking. PsaAPLY and formaldehyde treated PsaAPLY were detected by rabbit raised polyclonal antibody except PsaAPLY treated with 5% of formaldehyde (Figure 3-24B), although the detection became weaker with higher concentration of formaldehyde treatment. High molecular weight products were not detected by anti-PLY antibody (Figure 3-24B). In order to assess its ability to stimulate immune responses and the ability of this formulation to induce protection against colonization and disease, the PsaAPLY treated with1.25% formaldehyde was used in a mouse immunisation study.

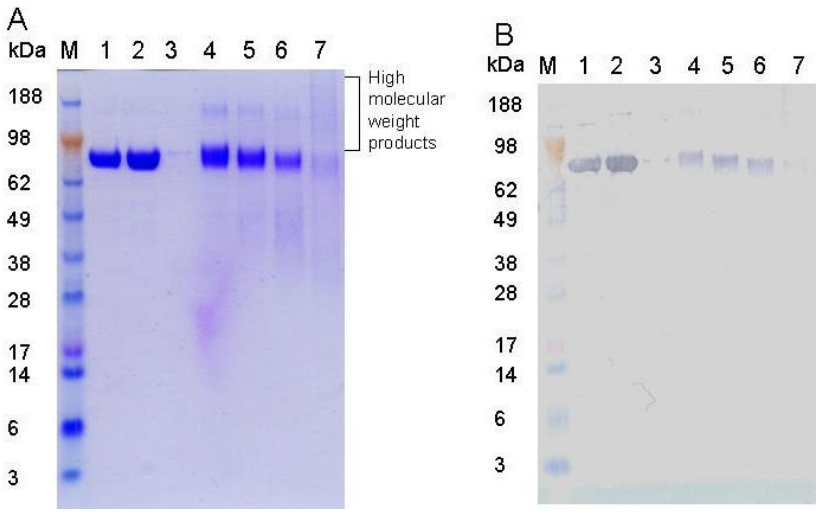


Figure 3-24 SDS-PAGE (A) and Western Blotting (B) analysis of the purified PsaAPLY and formaldehyde treated PsaAPLY.
2 µg of each sample was loaded on SDS-PAGE gels. A is SDS-PAGE gel stained with coomassie blue. B is Western Blots probed with rabbit polyclonal anti-PLY antibody. M is molecular weight marker. Lane 1: untreated PsaA∆6PLY; lane 2: untreated PsaAPLY; lanes 4: PsaAPLY treated with 0.03% (0.012M) formaldehyde; lanes 5: PsaAPLY treated with 0.25% (0.1M) formaldehyde; lanes 6: PsaAPLY treated with 1.25% (0.5M) formaldehyde; lanes 7: PsaAPLY treated with 5% (2M) formaldehyde.

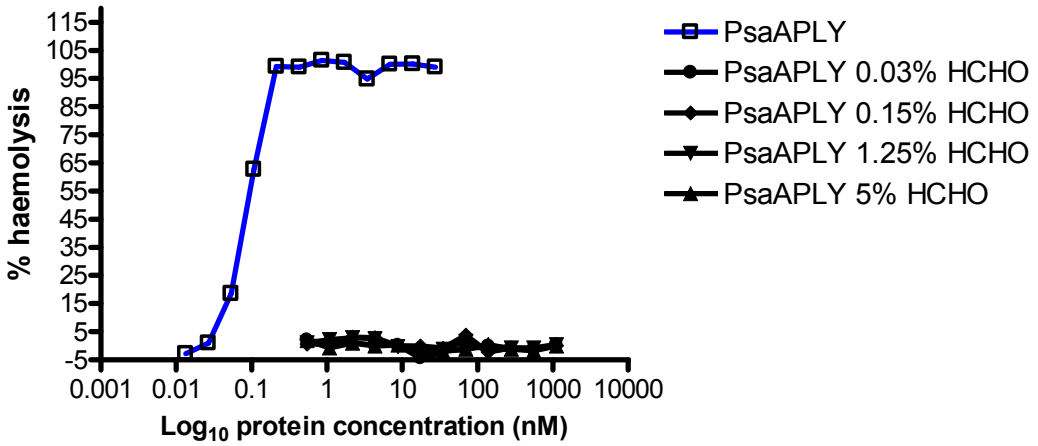


Figure 3-25 Haemolytic activity of formaldehyde (HCHO) treated PsaAPLY.
2% horse blood erythrocyte were treated with decreasing concentrations of formaldehyde treated PsaAPLY in PBS and % of haemolysis was determined by measurement of the resultant supernatant at OD_{540} after incubated for 30 min at 37°C.

3.7 Cell binding assessed by Fluorescence Microscopy

Florescence microscopy images showed eGFPPLY (Figure 3-26C) and eGFPD4PLY (Figure 3-26A) but not eGFPD123PLY (Figure 3-26B) bind to horse erythrocytes, and eGFPPLY lysed horse erythrocytes but eGFPD4PLY did not. Furthermore, eGFPD4PLY bound horse erythrocytes to form a large aggregate of cells. These results are consistent to our findings in the haemolytic assay. Interestingly,

eGFPPLY also aggregated lysed horse blood cells. Previously work in our lab by Dr Graeme J. M. Cowan showed eGFPΔ6PLY can also bind to and aggregate horse blood cells (Figure 3-26D).

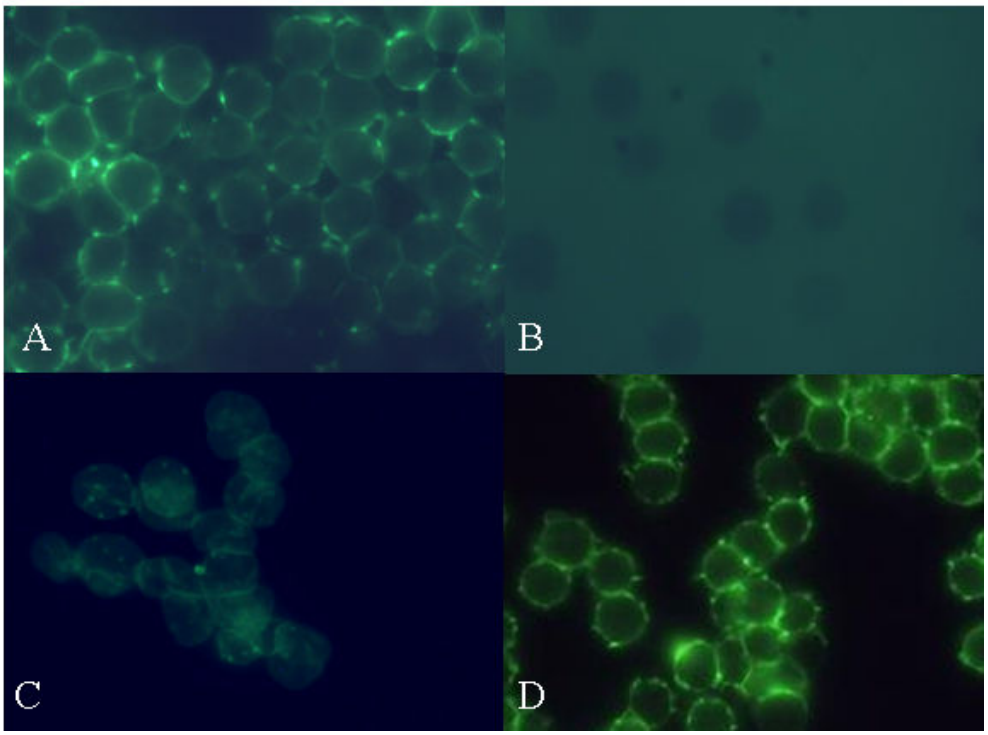


Figure 3-26 Fluorescence microscopy of horse erythrocytes treated with purified eGFP tagged recombinant proteins.
(A) eGFPD4PLY bound to horse erythrocytes and caused aggregation. (B) eGFPD123PLY was unable to bind to horse erythrocytes. (C) eGFPPLY bound to erythrocyte and lysed cells. (D) eGFPΔ6PLY bound to erythrocyte. Viewed with × 100 objective. Image D is from Dr Graeme J. M. Cowan.

3.8 Characterisation of purified eGFP tagged proteins by FACS

Flow cytometry analysis was used to confirm the findings with the florescence microscope (Figure 3-27). eGFPD4PLY binds to horse erythrocytes, and eGFPD123PLY does not bind to horse erythrocytes. More fluorescent events were detected with increase of concentration of eGFPD4PLY (7nM, 70nM and 700nM), but not with eGFP and eGFPD123PLY; the horse erythrocytes were lysed at 7nM eGFPPLY since 1nM eGFPPLY is enough to lyse 2% horse erythrocyte.

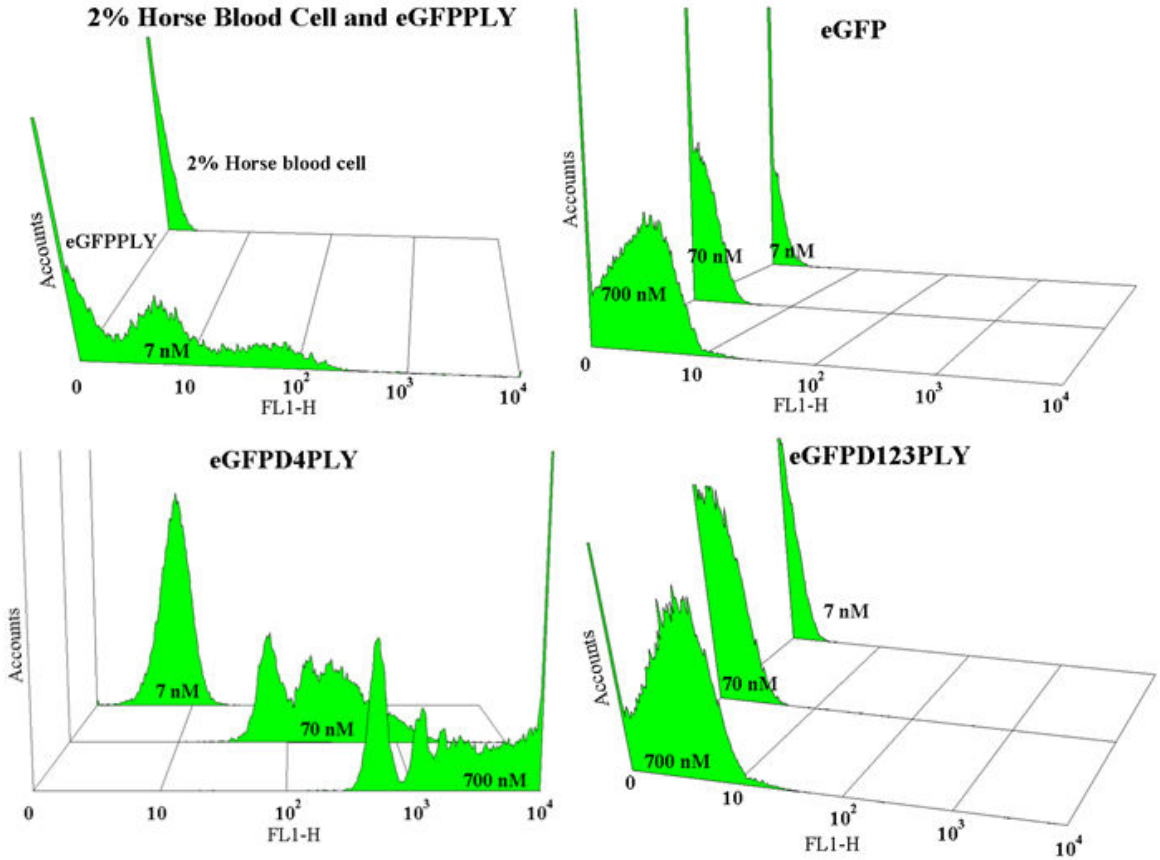


Figure 3-27 Flow cytometry profiles of horse blood cells treated with eGFP, eGFPPLY, eGFPD123PLY and eGFPD4PLY.
2% horse blood cells were incubated with 7 nM, 70 nM and 700 nM of eGFP, eGFPD123PLY or eGFPD4PLY respectively at 37°C for 15 mins. Only 7 nM eGFPPLY was used since this concentration is enough to interrupt horse blood cell. The treated cells were then measured by FACS.

3.9 Discussion

Gene fusion technologies are important tools for both basic biochemical research and biopharmaceuticals development. Recombinant fusion proteins are routinely constructed to increase the expression of soluble proteins and to facilitate protein purification. Other engineering approaches that link two proteins or protein domains by a peptide linker include immunoassays (e.g., using chimeras between antibody fragments and proteins), selection and production of antibodies, and engineering of multi-functional enzymes. Here we fused PsaA, PspA, PspC and PhtD to PLY and its mutant Δ6PLY in order to increase their immunogenicity.

It can be laboured intensive and difficult to make fusion proteins with the traditional ligation dependent (TLD) method, even with overlap PCR techniques and the Gateway cloning system. DNAs need to be cloned into the entry vector

first, and then exchanged into the destination vector in Gateway cloning. We constructed pET33beGFPD4PLY and pET33beGFPD123PLY plasmids using TLD method. It requires cloning of D4PLY and D123PLY DNA encoding sequence into pET33b+ plasmid first, and insertion of the eGFP gene into the same vector later. With TLD techniques, the fusion protein often contains undesired amino acids either encoded by restriction sites engineered to give a joining site where two DNAs can be ligated or encoded by the sequence between two different restriction sites when using four restriction sites to clone the two DNAs. The undesired amino acids can interfere in structure, reduce expression, or be antigenic.

In-fusion cloning can join multiple fragments of DNA together in one reaction (Zhu *et al*. 2007), therefore facilitating the design of seamless fusion protein constructs. It is not restriction site limited can clone any insert into any vector and is suitable for high throughput cloning. It also facilitates introduction of different peptide linkers to separate different moieties of fusion proteins. In this work, we successfully constructed 8 DNA vectors including pET33bPsaA, pET33bPspA, pET33bPspC, pET33bPhtD, pET33bPsaAPLY, pET33bPsaAPspAPLY, pET33bPspCPLY and pET33bphtDPLY by In-fusion. PET33bPsaAΔ6PLY, pET33bPspAΔ6PLY, pET33bPspCΔ6PLY and pET33bPhtDΔ6PLY were constructed through Site-Directed Mutagenesis to introduce the Δ6 mutation within the existing constructs.

Recombinant PsaA, PspA, PspC, PhtD and PLY were expressed and purified. The peptide linkers with shortest length were used in our study. All fusion proteins were initially connected by glycine rich flexible (FL) linkers containing 5 amino acid residues (GGGGS) since it is the most commonly used linker to separate different moieties of fusion proteins. PsaAPLY, PsaAΔ6PLY, PspAPLY and PspAΔ6PLY were highly expressed and purified successfully (Figure 3-13 and Figure 3-14). Unfortunately, PspCPLY and PhtDPLY were expressed well but were difficult to purify as these two fusion proteins were cleaved to PspC/PLY or PhtD/PLY (Figure 3-16).

There are two potential solutions for solving the stability problem, either increase the length of FL linkers from 5 amino acid residues (GGGGS) to 10 amino acid residues (GGGGSGGGGS), even to 15 or 20 amino acids long, or use

different peptide linkers. FL linker can actually fold back and bring two different moieties into direct contact each other (Wriggers *et al*. 2005). This is the possible reason why PspCPLY and PhtDPLY with FL linker were not stable. Because proline polymers can provide a predictable end-to-end distance over a length of more than 100 residues (Schimmel & Flory 1967), the rigid linker (RL) containing nine amino acids residue (PAPAPAPAPAP) was used (Zhao *et al*. 2008). PspCPLY, PsaCΔ6PLY, PhtDPLY and PhtDΔ6PLY were highly expressed and purified successfully later after RL were introduced (Figure 3-16 and Figure 3-17).

SDS is an ionic detergent that binds to the vast majority of proteins at a constant ratio of 1.4 gm SDS/gm protein. Proteins contain an abundance of hydrophobic residues—such as the transmembrane (TM) sequences of membrane—have helical regions do not bind at this ratio and this is one reason why some proteins migrate anomalously on SDS-PAGE gel (Rath *et al*. 2009). My purified PspA and PspC only contain N-terminal coiled-coil α-helix and part of proline rich region. This is why both purified PspA and PspC run anomalously on SDS-PAGE gel.

eGFPPLY, PsaAPLY, PspAPLY, PspCPLY and PhtDPLY are as haemolytic as PLY, which means fusing other antigens to the N terminus appears not to affect haemolytic activity. Furthermore, this also means FL and RL linkers are suitable for those fusion proteins. D123PLY, D4PLY, PsaA, PspA, PspC, PhtD, eGFPD123PLY, eGFPD4PLY, PsaAΔ6PLY, PspA Δ6PLY, PspC Δ6PLY and PhtDΔ6PLY have no haemolytic activity at all. Another reason to perform haemolytic assay with those non-haemolytic proteins is to confirm that there is no contamination with haemolytic proteins during the purification. Our results from fluorescence microscopy and FACS suggested the domain 4 of PLY is essential for PLY binding to cell membrane, and responsible for aggregation of cells.

In our haemolytic assay, the phenomenon that D4PLY caused horse erythrocytes to form aggregates in PBS solution has also been found with PLY and Δ6PLY. Fluorescence microscopy images further showed this phenomenon with D4PLY and PLY. The reasons why D4PLY causes horse erythrocytes to aggregate still remains unclear, further investigations are being conducted.

A second membrane binding site, rather than the D4PLY cholesterol binding site has been suggested. High concentrations of PLY426 or PLY437 (the C-terminal 51 or 40 amino acid were removed from the full length of PLY) were shown to induce γ-interferon and NO in mouse spleen cells (Baba, Kawamura et al. 2002). The results suggested this site was independent of cholesterol binding and pore forming ability of PLY. The clearance property of D123PLY in the haemolytic assay has been found with the other PLY mutants (unpublished data). The second cell membrane binding site different to the cholesterol binding site in domain 4 of PLY may exist in domain 123 of PLY. Dubail et al. showed Domain 1-3 of listeriolysin (D123LLO) alone expressed and secreted from a LLO deleted *Listeria monocytogenes* mutant can still bind to epithelial cells without Domain 4 of LLO (D4LLO) in human epithelial cells (Dubail *et al*. 2001). However, we can not detect this interaction between D123PLY and horse blood cells with fluorescent microscopy and FACS. The His tag in the purified D123PLY may block this interaction, and horse blood cells possibly do not have the right receptors for the D123PLY. The next step is to make tag free D123PLY and also try with different cell lines. More interestingly, D123LLO and D4LLO expressed in the LLO deleted *Listeria monocytogenes* mutant reassembled into a heamolytically active form (Dubail *et al*. 2001). It was also reported that K1 (D123PLY) and K2 (D4PLY) fragments, two products of protease K treated PLY, remained closely associated as "nicked" PLY; the nicked PLY is haemolytic and forms similar pores to non protease K treated PLY on the surface of liposomes under electron microscopy (Morgan *et al*. 1997).

Our haemolytic assay may not be sensitive enough to detect low levels of cytolysis with truncated form of PLY and Δ6PLY. Flow cytometry using Fluorescent DNA dyes labelling can be a potential method to detect low level of permeabilisation of different cell lines.

Sera from mice intranasally immunised with purified recombinant Δ6PLY fusion proteins recognised both purified recombinant and native protein partners in D39, Xen35 and Xen10 as shown by Western blot (Figure 3-19 to Figure 3-22). The serum against individual pht proteins is cross-reacts (Hamel *et al*. 2004). In this study, anti-PhtD serum is indeed cross-reactive against wild type PhtA, B and E from all three strains. D39, Xen35 and Xen10 are the three pneumococcal

strains will be used to challenge vaccinated mice in later experiments. The levels of antigen specific IgG and IgA were also measured by ELISA and will be discussed in Chapter 5.

Formaldehyde treatment was also used for inactivating PLY as it has been widely applied in vaccine preparations to inactivate the toxicities of bacterial toxins, and in some case, to increase the immunogenicity (Lagergard et al. 2007). Formaldehyde treated PsaAPLY had no haemolytic activities at all even with 0.03 % formaldehyde (Figure 3-25). High molecular weight products were observed with all concentrations in SDS-PAGE gel (Figure 3-24A).

Chapter 4 Serology of mice immunised with eGFPPLY, eGFPD4PLY and eGFPD123PLY

The previous work in our lab showed that PLY and Δ6PLY are potent mucosal adjuvants (Douce *et al*. 2010). Both PLY and Δ6PLY are capable of dramatically increasing antigen specific IgG and IgA to protein antigens fused to PLY and Δ6PLY. Intranasal challenge of mice with very low amounts of eGFPPLY or eGFPΔ6PLY (4 pmol) separately resulted a fast production of a significantly large amount of anti-eGFP IgG, and high levels of anti-eGFP IgA present in both the mucosal surface of nasopharynx and pulmonary tracts of mice (Douce *et al*. 2010). No response was elicited when mice were inoculated intranasally with a mixture of unlinked proteins. In order to understand the underlying mechanism of adjuvant properties of PLY, I further investigation of which domains of PLY are required for the novel adjuvant activity using purified D123PLY, D4PLY, and PLY, eGFPD123PLY, eGFPD4PLY and eGFPPLY in Chapter 2 were performed.

4.1 Immunising mice with purified recombinant proteins

65 Balb/c mice (Harlan Olac, UK) were divided into 12 groups and were intranasally immunised with appropriate antigens on days 1, 27 and 54 (Table 4-1). Bleeds were taken one day before each immunisation; the last bleed was taken two weeks after the third boost (day 54). One mouse died in group 2 and three mice died in group 7 on the day after first immunisation.

Table 4-1 Mice immunisation study of purified recombinant proteins.

Group	Antigens used	Number of mice
1	Mixture of eGFP and PLY	6
2	eGFPPLY alone	6
3	Mixture of eGFP and D4PLY	5
4	eGFPD4PLY alone	5
5	Mixture of eGFP and D123PLY	5
6	eGFPD123PLY alone	5
7	Mixture of eGFPD4PLY and PLY	8
8	Mixture of eGFPD123PLY and PLY	5
9	Mixture of eGFPD4PLY and Δ6PLY	5
10	Mixture of eGFPD123PLY and Δ6PLY	5
11	Mixture of eGFPD4PLY and D123PLY	5
12	Mixture of eGFPD123PLY and D4PLY	5

4.2 Immune response in mice immunised with purified proteins.

Systemic IgG and mucosal IgA responses within individual serum samples were measured by enzyme linked immunoasorbent assay (ELISA).

4.2.1 Systemic response

4.2.1.1 eGFP specific IgG response

No eGFP Specific IgG antibody was detected in the pre-immune bleed of all the groups (Figure 4-1). Mice immunised i.n. with very low level of eGFPPLY (4 pmol) rapidly produced statistically significant levels of IgG antibody to eGFP after the first immunisation (day 26), and the level of anti-eGFP IgG increased significantly after each boost (A). No anti-eGFP IgG was detected in the group administered a mixture of eGFP and PLY (Figure 4-2). The level of anti-eGFP IgG in mice immunised with eGFPPLY is significantly higher than those in other groups except compared to either eGFPD4PLY mixed with PLY or a mixture of eGFPD123PLY and PLY (Figure 4-1 and Figure 4-2). These results confirmed our previous finding that PLY can efficiently deliver fused antigens to the mucosal

surface of the respiratory tract, resulting in the rapid production of IgG antibody to the conjugated antigen in the blood. They also demonstrated that D123PLY or D4PLY can deliver antigen that elicits an immune response in the presence of free PLY or Δ6PLY.

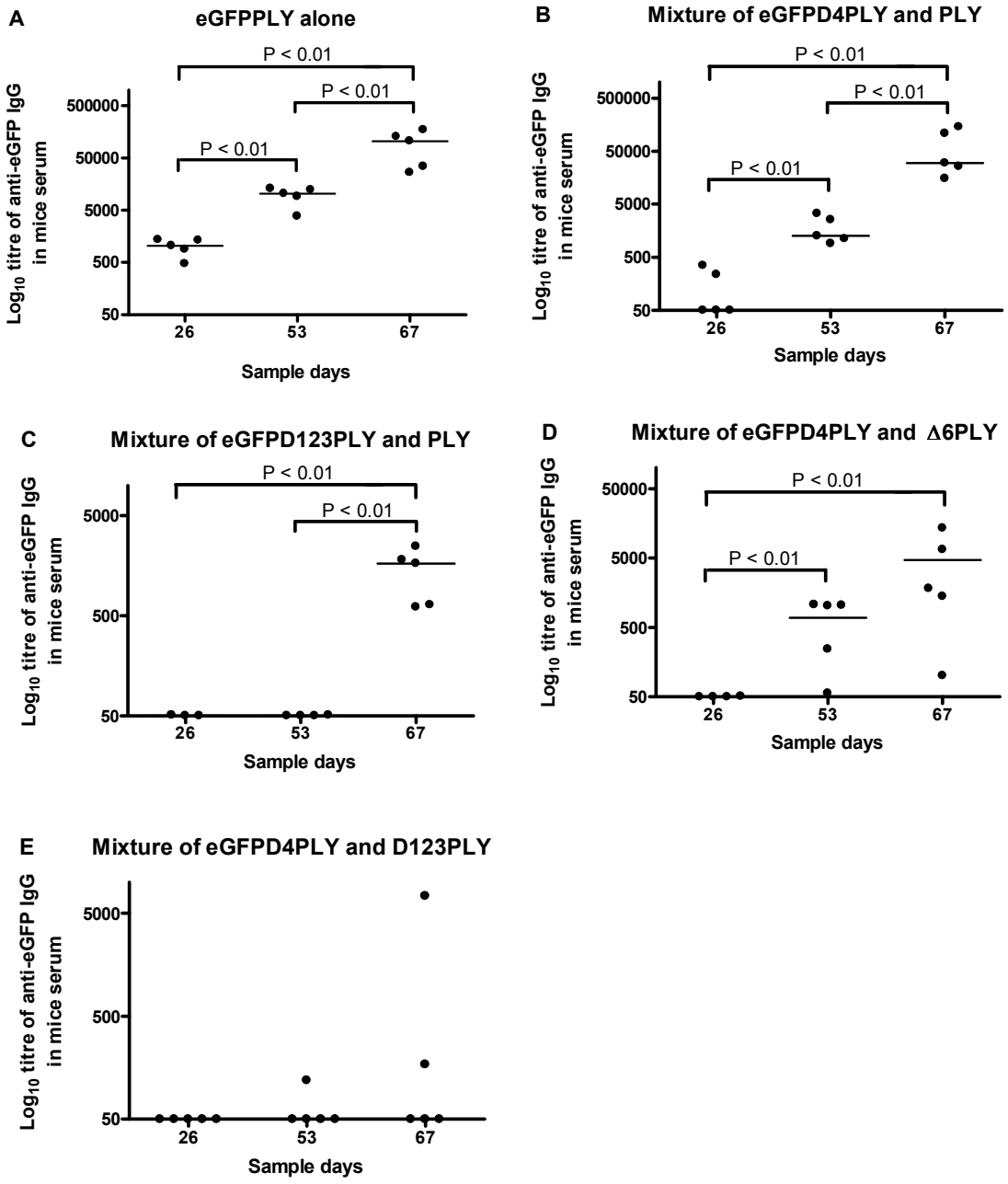
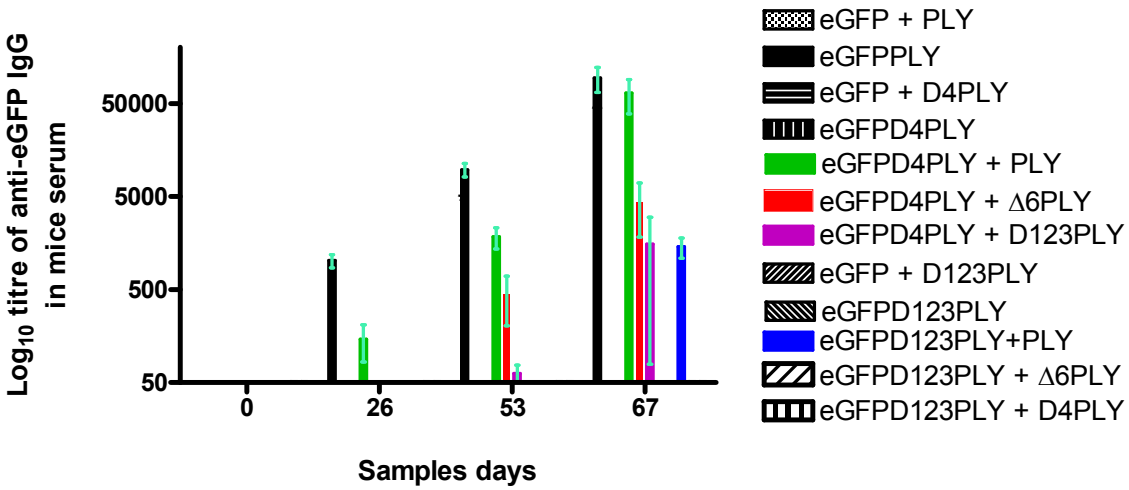


Figure 4-1 eGFP specific IgG response generated in sera of mice immunised i.n. with three doses of different combination of antigens.
(A) Mice immunised with eGFPPLY alone. (B) Mice immunised with mixture of eGFPD4PLY and PLY. (C) Mice immunised with mixture of eGFPD123PLY and PLY. (D) Mice immunised with mixture of eGFPD4PLY and Δ6PLY. (E) Mice immunised with mixture of eGFPD123PLY and D4PLY. (F) Time course of changing response. In A, B, C, D and E, each dot represents an individual mouse and each line presents the median response of total mice. The detection limit of anti-eGFP IgG titre is 50. Groups were compared using GraphPad Prizm 4.0 (California, USA) by Mann-Whitney test.



Groups	P value	Summary
eGFPPLY vs eGFP + PLY	P < 0.01	**
eGFPPLY vs eGFP + D4PLY	P < 0.01	**
eGFPPLY vs eGFPD4PLY	P < 0.01	**
eGFPPLY vs eGFPD4PLY + PLY	P > 0.05	Ns
eGFPPLY vs eGFPD4PLY + Δ6PLY	P < 0.05	*
eGFPPLY vs eGFPD4PLY + D123PLY	P < 0.05	*
eGFPPLY vs eGFP +D123PLY	P < 0.01	**
eGFPPLY vs eGFPD123PLY	P < 0.01	**
eGFPPLY vs eGFPD123PLY + PLY	P < 0.05	*
eGFPPLY vs eGFPD123PLY + Δ6PLY	P < 0.01	**
eGFPPLY vs eGFPD123PLY + D4PLY	P < 0.01	**
eGFPD4PLY + PLY vs eGFP + D4PLY	P < 0.01	**
eGFPD4PLY + PLY vs eGFPD4PLY	P < 0.01	**
eGFPD4PLY + PLY vs eGFPD4PLY + Δ6PLY	P < 0.01	**
eGFPD4PLY + PLY vs eGFPD4PLY + D123PLY	P > 0.05	Ns
eGFPD4PLY + PLY vs eGFPD123PLY + PLY	P < 0.05	*
eGFPD123PLY + PLY vs eGFP + D123PLY	P < 0.01	**
eGFPD123PLY + PLY vs eGFPD123PLY	P < 0.01	**
eGFPD123PLY + PLY vs eGFPD123PLY + D4PLY	P < 0.01	**
eGFPD123PLY + PLY vs eGFPD123PLY + Δ6PLY	P < 0.01	**
eGFPD4PLY + Δ6PLY vs eGFP + D4PLY	P < 0.01	**
eGFPD4PLY + Δ6PLY vs eGFPD4PLY	P < 0.01	**
eGFPD4PLY + Δ6PLY vs eGFPD123PLY + Δ6PLY	P < 0.01	**

Figure 4-2 Time course of changing response for eGFP specific IgG.
The graph shows median titres ± the interquartile range from the median from five mice before each challenge. Each bar represents the median titre of total mice per group and each error bar presents the interquartile range from the medianThe detection limit of anti-eGFP IgG titre is 50. The table shows statistical comparison of anti-eGFP IgG response between different groups. NS = not significant, * P<0.05 was significant and ** P<0.01 was very significant. Groups were compared using GraphPad Prizm 4.0 (California, USA) by Mann-Whitney test.

No anti-eGFP IgG antibody was detected in mice immunised with either eGFP mixed with D4PLY or with combination of eGFP and D123PLY (Figure 4-2). Interestingly, no anti-eGFP IgG antibody was produced in mice immunised with either eGFPD4PLY or eGFPD123PLY separately, but high level of anti-eGFP IgG response was detected in mice vaccinated with mixture of eGFPD4PLY and PLY (Figure 4-1C and Figure 4-2). The final titre of anti-eGFP IgG in mice vaccinated with a mixture of eGFPD4PLY and PLY is equivalent to that in eGFPPLY vaccinated mice. The significant level of anti-eGFP IgG response was also found in mice vaccinated with mixture of eGFPD123PLY and PLY after two boosts (Figure 4-1D and Figure 4-2). More interestingly, low but significant level of anti-eGFP IgG was produced in mice immunised with mixture of eGFPD4PLY and Δ6PLY after two boosts (Figure 4-1D and Figure 4-2), and no anti-eGFP IgG was found in mice vaccinated with mixture of eGFPD123PLY and Δ6PLY (Figure 4-2). No significant level of anti-eGFP IgG response was observed mice immunised with mixture of eGFPD4PLY and D123PLY or mixture of eGFPD123PLY and D4PLY (Figure 4-1E and Figure 4-2). Together these results mean intranasal administration of eGFP tagged truncated forms of PLY are unable to induce anti-eGFP IgG responses in mice unless intact PLY or Δ6PLY were also administered.

4.2.1.2 PLY Specific IgG response

The response to carrier protein pneumolysin was different to carried protein antigens (Figure 4-4). No PLY specific IgG response was observed after a single dose of the toxin. The level of anti-PLY IgG increased significantly after each boost. Detectable level of anti-PLY IgG was found in mice vaccinated with PLY, eGFPPLY, mixture of eGFP and PLY, mixture of eGFPD4PLY and PLY or mixture of eGFPD123PLY and PLY respectively after first boost. A statistically significantly higher level of anti-PLY IgG response was produced in the above groups after two doses of the antigens were given. Low but significant PLY specific IgG responses were also detected in mice immunised with mixture of eGFPD4PLY and Δ6PLY or mixture of eGFPD123PLY and Δ6PLY. No PLY specific IgG (Figure 4-4) was detected in mice vaccinated with eGFPD4PLY, eGFPD123PLY, mixture of eGFP and D4PLY, mixture of eGFP and D123PLY, mixture of eGFPD4PLY + D123PLY or mixture of eGFPD123PLY + D4PLY respectively. These results mean intranasal administration of PLY or Δ6PLY is

able to elicit systemic PLY specific IgG response in the blood of mice, but this does not happen with truncated form of PLY.

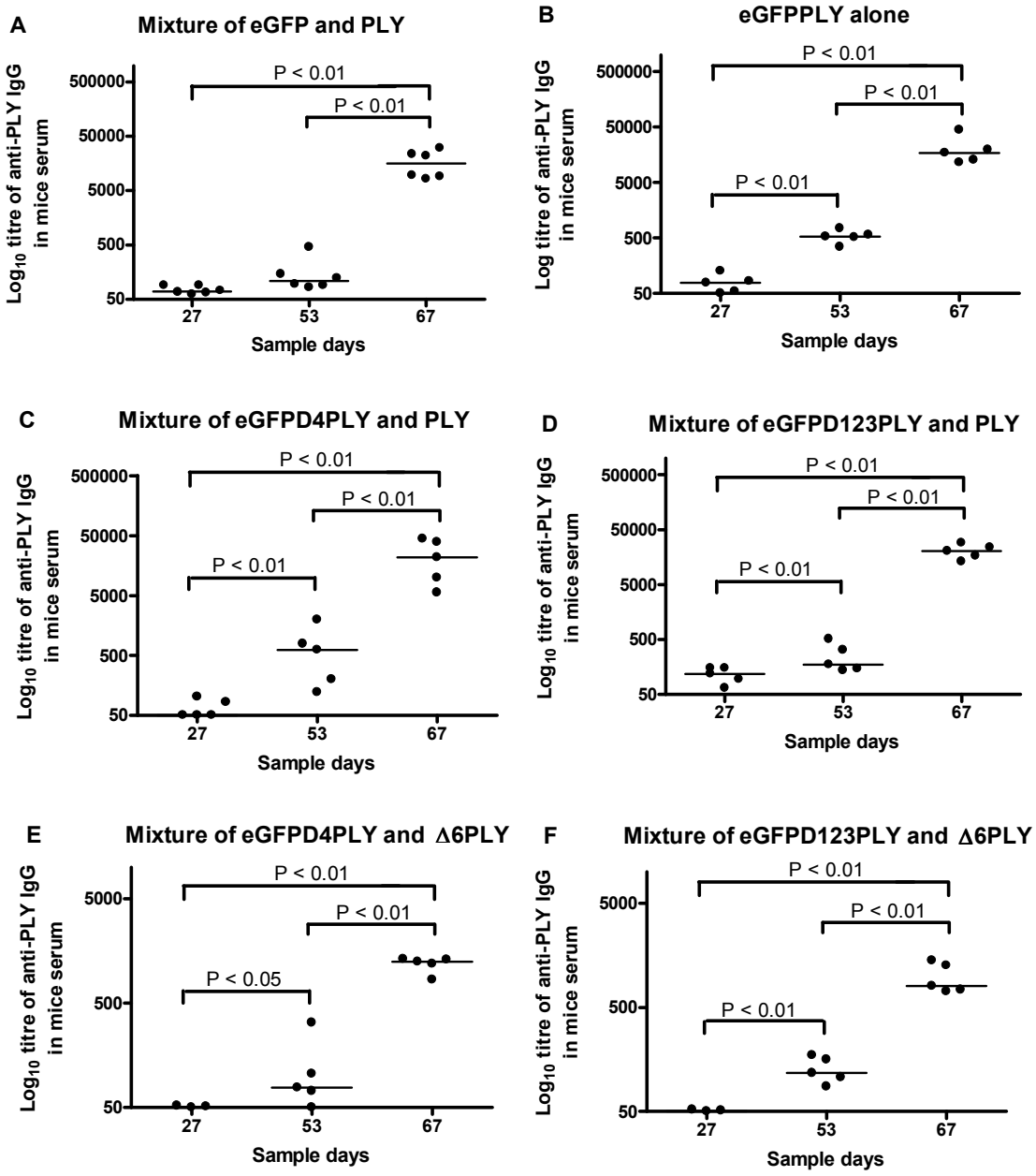
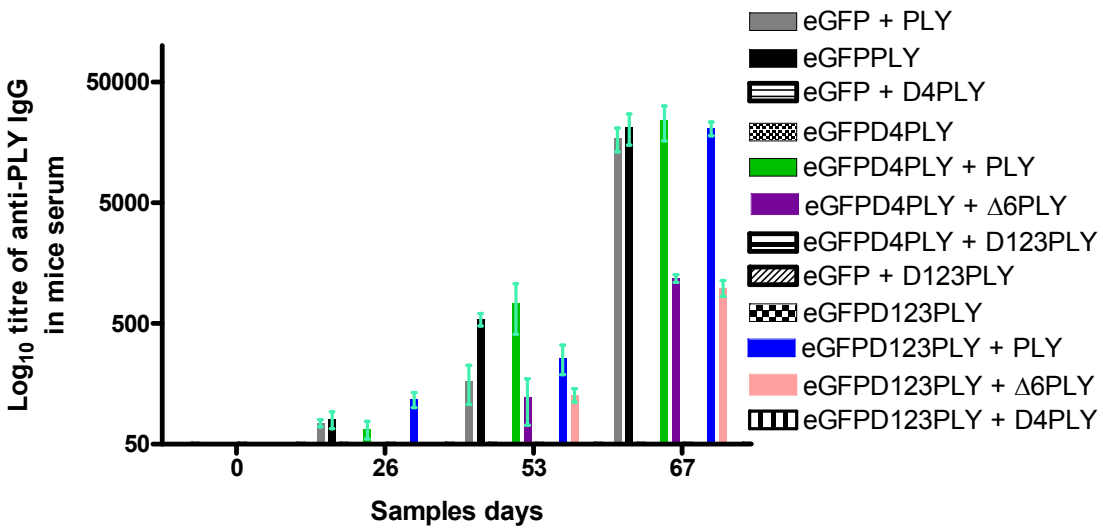


Figure 4-3 PLY specific IgG response generated in sera of mice immunised i.n. with three doses of different combination of antigens.
(A) Mice immunised with eGFP and PLY alone. (B) Mice immunised with eGFPPLY alone. (C) Mice immunised with mixture of eGFPD4PLY and PLY. (D) Mice immunised with mixture of eGFPD123PLY and PLY. (E) Mice immunised with mixture of eGFPD4PLY and Δ6PLY. (F) Mice immunised with mixture of eGFPD123PLY and Δ6PLY. (G) Time course of changing response. In A, B, C, D, E and F, each dot represents an individual mouse and each line presents the median response of total mice. The detection limit of anti-eGFP IgG titre is 50. Groups were compared using GraphPad Prizm 4.0 (California, USA) by Mann-Whitney test.



Groups	P value	Summary
eGFPPLY vs eGFP + PLY	P >0.05	Ns
eGFPPLY vs eGFP + D4PLY	P < 0.01	**
eGFPPLY vs eGFPD4PLY	P < 0.01	**
eGFPPLY vs eGFPD4PLY + PLY	P > 0.05	Ns
eGFPPLY vs eGFPD4PLY + Δ6PLY	P < 0.05	*
eGFPPLY vs eGFPD4PLY + D123PLY	P < 0.01	**
eGFPPLY vs eGFP +D123PLY	P < 0.01	**
eGFPPLY vs eGFPD123PLY	P < 0.01	**
eGFPPLY vs eGFPD123PLY + PLY	P > 0.05	Ns
eGFPPLY vs eGFPD123PLY + Δ6PLY	P < 0.05	*
eGFPPLY vs eGFPD123PLY + D4PLY	P < 0.01	**
eGFPD4PLY + PLY vs eGFP + D4PLY	P < 0.01	**
eGFPD4PLY + PLY vs eGFPD4PLY	P < 0.05	*
eGFPD4PLY + PLY vs eGFPD4PLY + Δ6PLY	P < 0.05	*
eGFPD4PLY + PLY vs eGFPD4PLY + D123PLY	P > 0.05	Ns
eGFPD4PLY + PLY vs eGFPD123PLY + PLY	P > 0.05	Ns
eGFPD123PLY + PLY vs eGFP + D123PLY	P < 0.05	*
eGFPD123PLY + PLY vs eGFPD123PLY	P < 0.05	*
eGFPD123PLY + PLY vs eGFPD123PLY + D4PLY	P < 0.05	*
eGFPD123PLY + PLY vs eGFPD123PLY + Δ6PLY	P < 0.05	*
eGFPD4PLY + Δ6PLY vs eGFP + D4PLY	P < 0.05	*
eGFPD4PLY + Δ6PLY vs eGFPD4PLY	P < 0.05	*
eGFPD4PLY + Δ6PLY vs eGFPD123PLY + Δ6PLY	P > 0.05	Ns

Figure 4-4 Time course of changing response for PLY specific IgG.
The graph shows median titres ± the interquartile range from the median from five mice before each challenge. Each bar represents the median titre of total mice per group and each error bar presents the interquartile range from the median. The detection limit of anti-eGFP IgG titre is 50. The table shows statistical comparison of anti-PLY IgG response between different groups. NS = not significant, * P<0.05 was significant, ** P<0.01 was very significant and *** P<0.001 was extremely significant. Groups were compared using GraphPad Prizm 4.0 (California, USA) by Mann-Whitney test.

4.2.2 Mucosal response

In addition to systemic responses, mucosal responses were also observed (Figure 4-5 and Figure 4-6). The level of eGFP specific IgA in all nasal and lung wash of mice immunised i.n. with eGFPPLY or a mixture of eGFPD4PLY and PLY were significantly higher than with the other combinations of antigens, which did not elicit any anti-eGFP IgA. Anti-eGFP IgA was also detectable but not significant in nasal and lung wash of mice vaccinated i.n.with combination of eGFPD123PLY and PLY. The anti-eGFP IgA was not found in intestine and vigina (data not shown). These results suggest that PLY is able to efficiently deliver fused antigens to the mucosal surface of the respiratory tract, resulting in the rapid production of IgA antibody to the conjugated antigen at the mucosal surface. Again, these also demonstrated that D123PLY or D4PLY can deliver antigen that elicits an immune response in the presence of PLY or Δ6PLY.

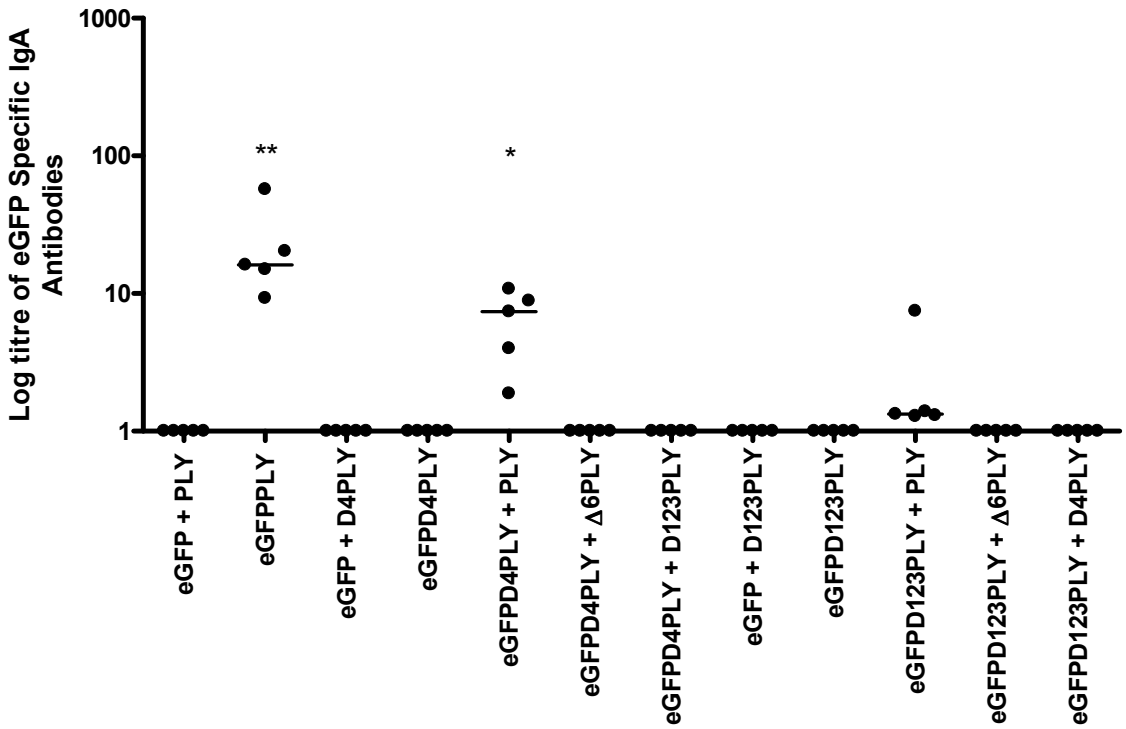


Figure 4-5 Mucosal eGFP specific IgA responses generated by three i.n. doses of antigen in nasal lavage samples.
Each dot represents individual animals and indicates the level of variation in the immune response observed. Lines represent median antibody titres. Animals immunised with eGFPPLY (P < 0.01) or eGFPD4PLY + PLY (P < 0.05) generated specific responses that were significantly higher than animals given the other combinations except eGFPD123PLY + PLY (P > 0.05). Groups were compared using GraphPad Prizm 4.0 (California, USA) by Kruskal-Wallis with Dunn's post test. * P<0.05 was significant and ** P<0.01 was highly significant.

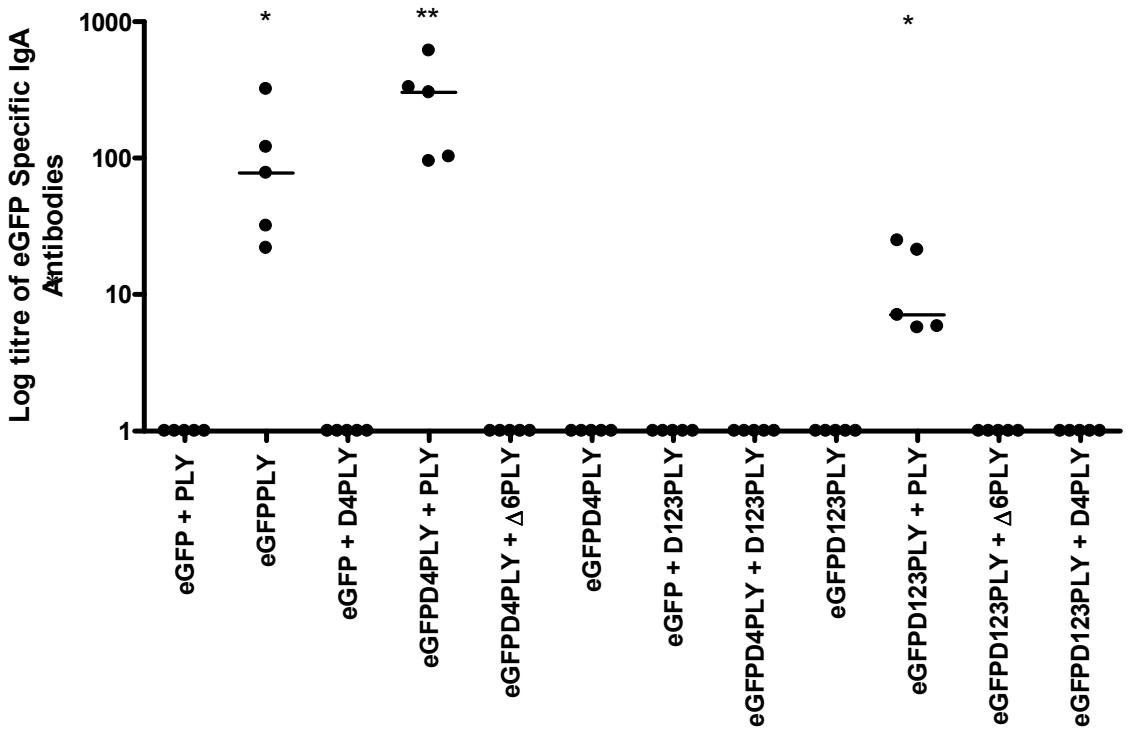


Figure 4-6 Mucosal eGFP specific IgA responses generated by three i.n. doses of antigen in pulmonary lavage samples.
The symbols represent individual animals and indicate the level of variation in the immune response observed. Lines represent median antibody titres. Animals immunised with eGFPPLY ($p < 0.05$) or eGFPD4PLY + PLY ($p < 0.01$) generated specific responses that were significantly higher than animals given the other combinations except eGFPD123PLY + PLY ($p > 0.05$). Groups were compared using GraphPad Prizm 4.0 (California, USA) by Kruskal-Wallis with Dunn's post test. * $P<0.05$ was significant and ** $P<0.01$ was highly significant.

4.3 Discussion

Mice challenged i.n. with very low levels of eGFPPLY (0.3g) rapidly produce of a high level of IgG antibody to eGFP, but i.n. administration of a mixture of eGFP and PLY does not induce the above response. This response was further amplified after additional doses of eGFPPLY were applied and induced significant amount of antigen specific IgA responses to eGFP in the nose and lung. The anti-eGFP IgA was not found in intestine and vigina (data not shown). These results showed PLY can act as a good mucosal adjuvant to eGFP when eGFP and PLY are conjugated together, and unconjugated PLY can not work as an adjuvant to the free antigen. These finding was recently published (Douce *et al*. 2010). High levels of anti-PLY IgG were detected in the groups immunised with PLY and eGFPPLY, but not found in groups immunised with truncated forms of PLY (Figure 4-3), which means PLY remains highly immunogenic after been

fused to another protein, and truncated PLY including D123PLY and D4PLY are not immunogenic.

The previous work in our group also suggests ∆6PLY, a mutant which has lost haemolytic property totally but remains immunogenic without PLY associated toxic effects (Kirkham *et al*. 2006b), can still act as a adjuvant in eGFP∆6PLY fusion protein during immunisation (Douce et al., 2010). Since the adjuvant property of native PLY is better than ∆6PLY, so the adjuvant property of the proteins is: PLY> ∆6PLY > truncated PLY (D123PLY and D4PLY).

Intranasal administration of eGFP conjugated to truncated forms of PLY is unable to induce anti-eGFP IgG response in mice unless free PLY or Δ6PLY was also administered. eGFPD123PLY or eGFPD4PLY alone was not able to provide a second signal to stimulate T helper cell response during antigen-presenting cell and T helper cell interaction. This second signal can not be provided through adding truncated forms of PLY when a mixture of eGFPD123PLY and D4PLY, and a mixture of eGFPD4PLY and D123PLY were delivered i.n. to mice. However, the second signal maybe is provided by PLY or Δ6PLY when a mixture of eGFPD123PLY and PLY, a mixture of eGFPD4PLY and PLY or a mixture of eGFPD4PLY and Δ6PLY were delivered i.n. to mice.

Initial experiments used the model antigen (eGFP) but we believe that this strategy of vaccination could be applied to any protein antigen such as viral, bacterial and parasitic antigens. For the next step, the eGFP protein needs to be replaced with pneumococcal proteins. One or more fusion proteins are expected to elicit serotype independent protection, which would provide a broader protection against pneumococcus. To confirm the relevance of this approach, four pneumococcal proteins PsaA, PspA, PspC and PhtD were fused to PLY and ΔPLY separately. The mice were immunised with these fusion proteins and challenged with three different strains of pneumococci. The results are presented and discussed in Chapter 5 and 6. The adjuvant properties of PLY also exist in other members of cholesterol dependent cytolysin family (McInally C unpublished data). This technology could be also used to help raise antibodies to poorly immunogenic protein antigens.

Chapter 5 Serology of mice immunised with pneumococcal proteins fused to PLY or ∆6PLY

It has previously been demonstrated that PLY and Δ6PLY are potent mucosal adjuvants with the model antigen eGFP when delivered intranasally (Douce *et al*. 2010). However, the next step is to test whether this technology when applied to other antigens and other delivery routes can generate similar responses. In this chapter, PsaAPLY, PsaAΔ6PLY, PspAPLY, PspAΔ6PLY, PspCPLY, PspCΔ6PLY, PhtDPLY and PhtDΔ6PLY were tested using both intranasal and subcutaneous delivery routes. My PhD is funded by PATH (Programme for Appropriate Technology in Health). Experiments PATH 1 - 8 each consist of delivery of a single fusion protein antigen intranasally and subcutaneously, respectively. PATH 9 and 10 consist of delivery of a mixture of fusion protein antigens intranasally and subcutaneously. No antigen specific IgG or anti-PLY IgG were detected in the mice immunised with PBS and in the bleed before immunisation.

5.1 Systemic IgG and mucosal IgA responses in mice vaccinated with PsaA fusion proteins (Path 1 and 2)

As shown in Figure 5-1, both intranasal and subcutaneous administration of three doses of the conjugate protein PsaAPLY and PsaAΔ6PLY resulted in very rapid production of a high levels of IgG antibodies to PsaA. All animals responded strongly to applied i.n. or s.c. PsaAPLY and PsaA∆6PLY. Responses to with PsaAΔ6PLY were approximately 10 fold lower than PsaAPLY when immunised i.n. Mice responded more strongly to subcutaneously administered PsaAΔ6PLY than when it was applied intranasally. There is no significant difference in responses in between PsaAPLY and PsaA∆6PLY when applied s.c. Because Δ6PLY has been shown to be completely non-toxic, it may be possible to boost the immune response by increasing the dose of protein in future vaccinations since we only used 4 pmol PsaAΔ6PLY in each dose. There was also a significant immune response to the formaldehyde treated PsaAPLY injected subcutaneously without

using an adjuvant, and that was not seen at all when it was delivered intranasally. A few mice responded to the PsaA delivered both i.n. and s.c.

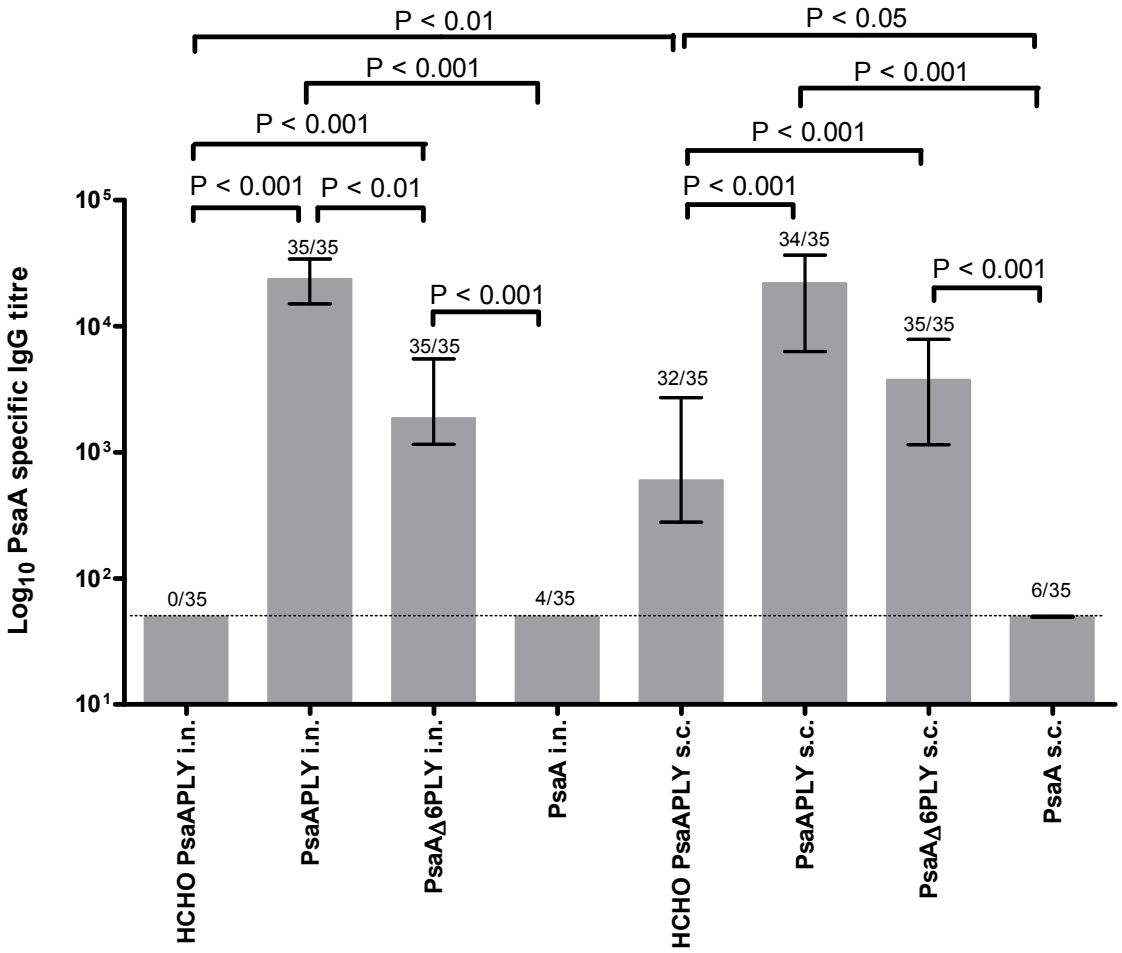


Figure 5-1 PsaA IgG specific detected in sera of mice either intranasally (i.n.) or subcutanously (s.c.) vaccinated with three doses of purified proteins.
The graph shows median titres ± the interquartile range from the median from 35 mice. The number of responding individuals in a group is shown above each bar. The dotted horizontal line represents the detection limit of the assay. Groups were compared using GraphPad Prizm 4.0 (California, USA) by Kruskal-Wallis with Dunn's post test. P<0.05 was significant, P<0.01 was highly significant and P<0.001 was extremely significant. HCHO means formaldehyde treated.

The level of PsaA specific IgA in all nasal and lung washes of mice immunised i.n. with PsaAPLY were significantly higher than with the other combinations of antigens (Figure 5-2). Detectable levels of PsaA specific IgA were found in both the nasal (Figure 5-2A) and pulmonary tract (Figure 5-2B) of mice immunised i.n. with PsaAΔ6PLY. No significant IgA was detected in mice immunised s.c. with any of the antigens.

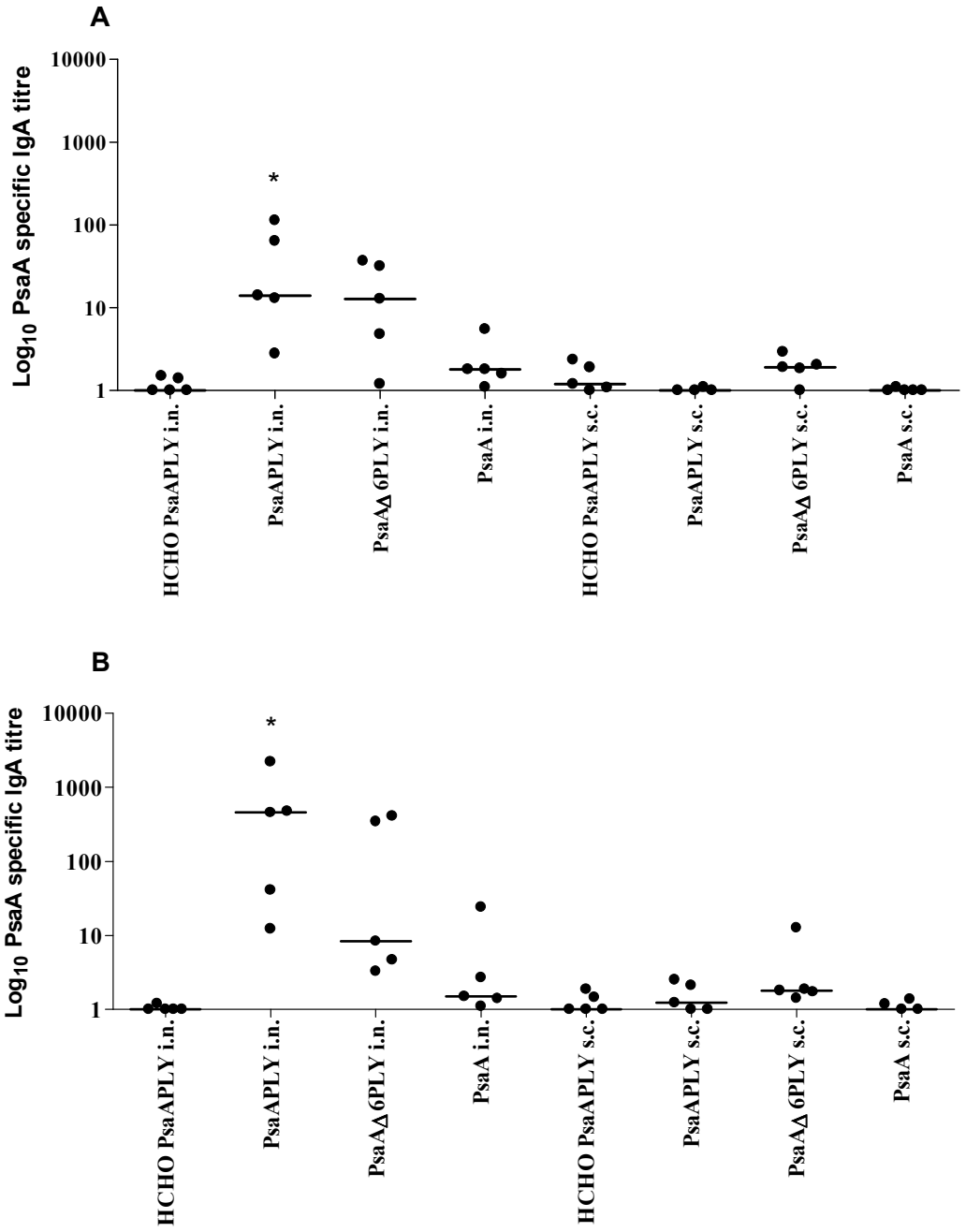


Figure 5-2 Mucosal PsaA specific IgA responses generated by three i.n. or s.b. doses of antigen in nasal (A) and pulmonary (lung) lavage samples (B).
The symbols represent individual animals and indicate the level of variation in the immune response observed. Bars represent median antibody titres. Mice immunised i.n. with PsaAPLY generated specific responses that were significant higher than mice given the other antigens. Groups were compared using GraphPad Prizm 4.0 (California, USA) by Mann-Whitney non-parametric test. * P<0.05 was significant and ** P<0.01 was highly significant.

5.2 Systemic IgG and mucosal IgA responses in mice vaccinated with PspA fusion proteins (Path 3 and 4)

As shown in Figure 5-3, a statistically significant ($p < 0.001$) level of IgG antibodies to PspA were detected in mice immunised i.n. and s.c. with three doses of the conjugate protein PspAPLY or PspAΔ6PLY individually. Responses with PspAPLY were approximately 20 fold higher than those with PspAΔ6PLY, and about 17% of mice (6/35) did not respond to PspAΔ6PLY when it was delivered intranasally. Mice responded more strongly to subcutaneous (97% 34/35) administration of PspAΔ6PLY compared to when it was applied intranasally (83% 29/35) on both titres and numbers. High dose of PspAΔ6PLY should be considered to be used in future vaccinations to solve this problem. There was no significant difference between responses to PspAPLY and PspAΔ6PLY when applied s.c. When the proteins were administered s.c., mice responded much more strongly to PspAΔ6PLY both in titres and numbers than when it was applied i.n., but surprisingly mice responded more weakly to PspAPLY than when it was administered i.n. A few mice responded to s.c. delivery of PspA.

Significant levels of PspA specific IgA were detected in all animals immunised i.n. with PspAPLY and those responses were found in both the nasal (nasal lavage) and pulmonary tract (pulmonary lavage); a detectable level of PspA specific IgA were observed in 3 out of five mice immunised i.n. with PspAΔ6PLY (Figure 5-4). No PspA specific IgA was detected in mice given any of the antigens s.c..

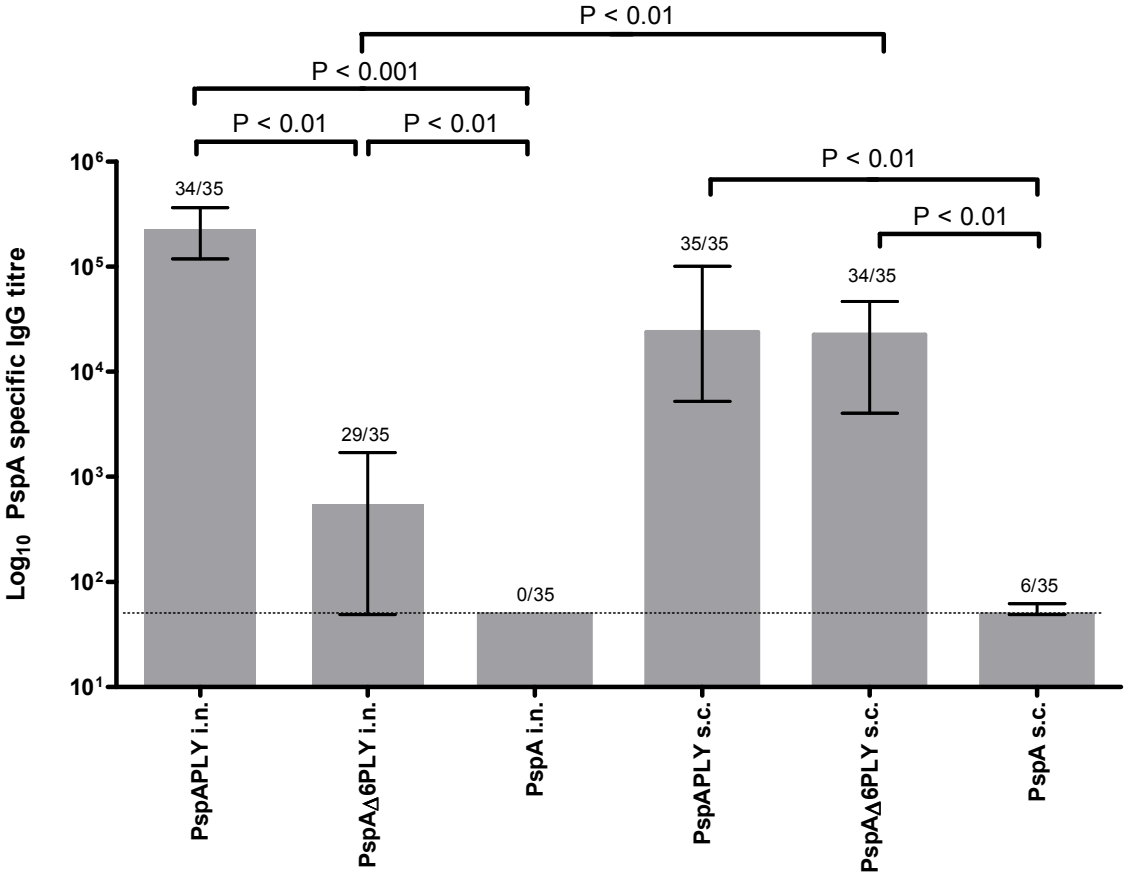


Figure 5-3 PspA specific IgG detected in sera of mice either intranasally (i.n.) or subcutanously (s.c.) vaccinated with three doses of purified proteins.
Bars represent median titres ± the interquartile range from the median from 35 mice. The number of responding individuals in a group is shown above each bar. The dotted horizontal line represents the detection limit of the assay. Groups were compared using GraphPad Prizm 4.0 (California, USA) by Kruskal-Wallis with Dunn's post test. P<0.05 was significant, P<0.01 was highly significant and P<0.001 was extremely significant.

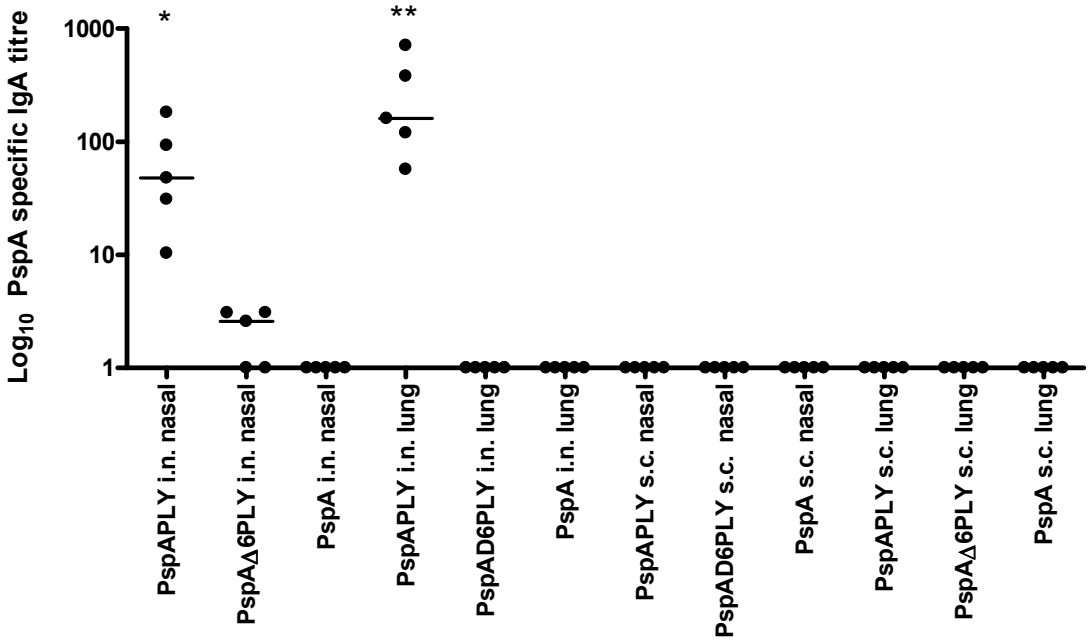


Figure 5-4 Mucosal PspA specific IgA responses generated by three i.n. or s.b. doses of antigen in nasal and pulmonary (lung) lavage samples.
The symbols represent individual animals and indicate the level of variation in the immune response observed. Bars represent median antibody titres. Mice immunised i.n. with PspAPLY generated specific responses that were significant higher than mice given the other antigens. Groups were compared using GraphPad Prizm 4.0 (California, USA) by Mann-Whitney non-parametric test. * P<0.05 was significant and ** P<0.01 was highly significant.

5.3 Systemic IgG and mucosal IgA in mice vaccinated with PspC fusion proteins (Path 5 and 6)

A similar pattern was observed in PspC fusion proteins vaccination (Figure 5-5). Following three doses of the protein, significant levels of IgG antibodies to PspC were observed in the blood of animals vaccinated with PspCPLY and PspCΔ6PLY, but were absent from those given PspC alone (Figure 5-5). Responses with PspCΔ6PLY were approximately 10 fold lower than that with PspCPLY in both i.n. and s.c delivery routes, and about 23% of mice did not respond to PspCΔ6PLY with i.n. delivery route. Again, high doses of the Δ6PLY version of the fusion protein need to be used in future vaccinations. When those proteins were administered subcutaneously, more mice (34/35) responded to PspCΔ6PLY than when it was applied i.n. (27/35) (Figure 5-5).

PspC specific IgA was only detected in both the nasal (nasal lavage) and pulmonary tract (pulmonary lavage) of all mice immunised i.n. with PspCPLY, but not with PspCΔ6PLY (Figure 5-6). Low level of PspC specific IgA was also detected in 2 out of five mice vaccinated s.c. with PspCPLY.

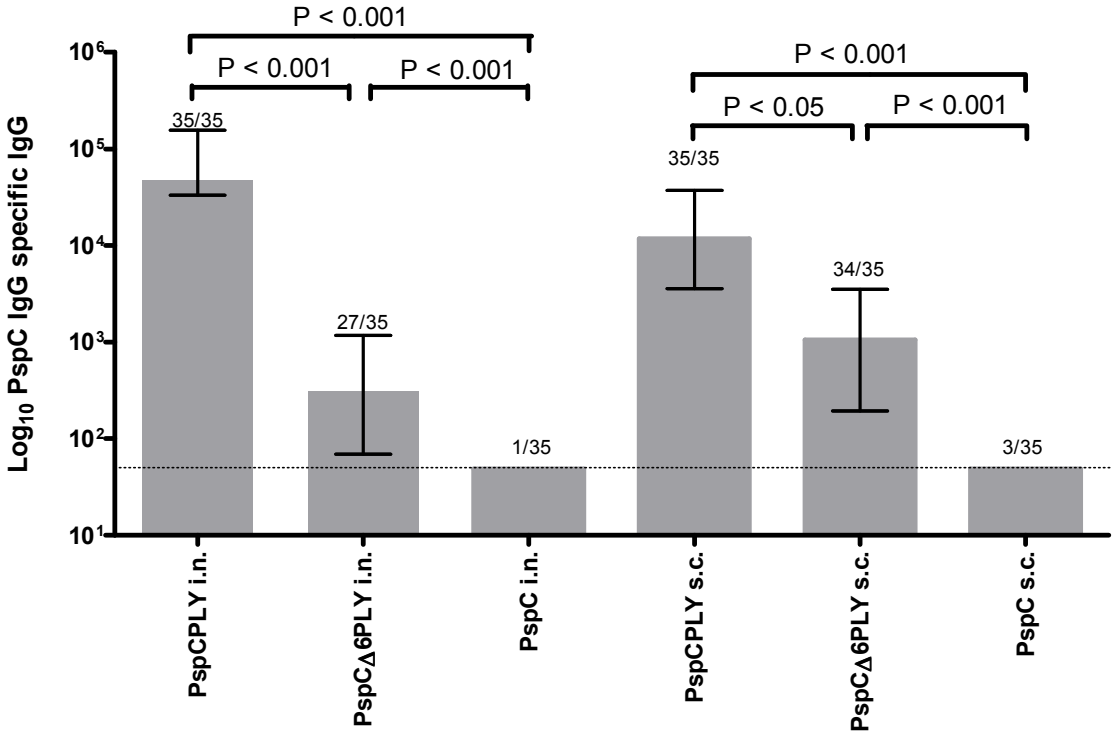


Figure 5-5 PspC specific IgG detected in sera of mice intranasally (i.n.) or subcutanously (s.c.) vaccinated with three doses of purified proteins.
Bars represent median titres ± the interquartile range from the median from 35 mice. The number of responding individuals in a group is shown above each bar. The dotted horizontal line represents the detection limit of the assay. Groups were compared using GraphPad Prizm 4.0 (California, USA) by Kruskal-Wallis with Dunn's post test.

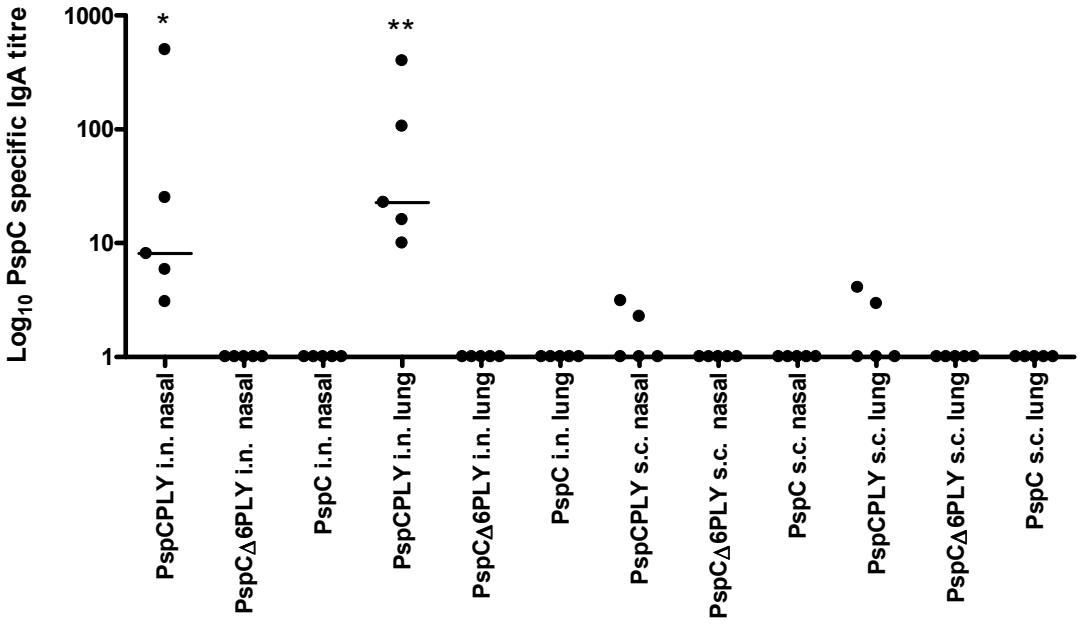


Figure 5-6 Mucosal PspC specific IgA responses generated by three i.n. or s.b. doses of antigen in nasal and pulmonary (lung) lavage samples.
The symbols represent individual animals and indicate the level of variation in the immune response observed. Bars represent median antibody titres. Mice immunised i.n. with PspCPLY generated specific responses that were significant higher than mice given the other antigens. Groups were compared using GraphPad Prizm 4.0 (California, USA) by Mann-Whitney non-parametric test. * P<0.05 was significant and ** P<0.01 was highly significant.

5.4 TLR4 is not required for adjuvant activity of PLY

Several studies have indicated TLR4 may interact with PLY directly (Malley *et al*. 2003; Dessing *et al*. 2009). In our experiment (Figure 5-7), there is no significant difference in PspC specific IgG response between Balb/c wild type and TLR4 knockout mice when PspCPLY was delivered i.n., which suggests TLR4 is not required for adjuvant properties of PLY in this model.

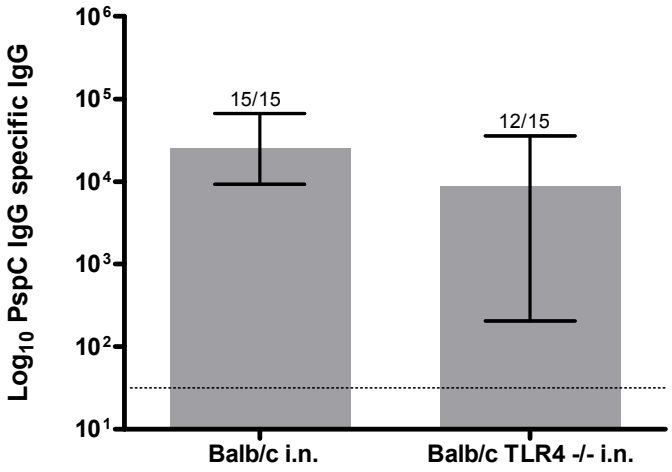


Figure 5-7 Anti-PspC IgG detected in sera of Balb/c and Balb/c TLR4 -/- mice intranasally (i.n.) vaccinated with three doses of PspCPLY.
Bars represent median titres ± the interquartile range from the median. The number of responding individuals in a group is shown above each bar. The dotted horizontal line represents the detection limit of the assay. Groups were compared using GraphPad Prizm 4.0 (California, USA) by Mann-Whitney test.

5.5 Systemic IgG in mice vaccinated with PhtD fusion proteins (Path 7 and 8)

Almost all animals responded strongly to PhtDPLY and PhtDΔ6PLY applied i.n. or s.c.. High level of IgG antibodies to PhtD were detected in mice immunised i.n. or s.c. with three doses of the fusion proteins PhtDPLY and PhtDΔ6PLY individually. Responses with PhtDΔ6PLY were approximately 5 fold lower than PhtDPLY when immunised i.n.. A few mice responded to intranasal delivery of PhtD alone and IgG responses in these animals were not particularly high titre. Mice responded more strongly to s.c. administered PhtDΔ6PLY than when it was applied i.n. The response in PhtDPLY and PhtDΔ6PLY are almost equivalent when they were applied s.c. Interestingly, a significant immune response was also detected in mice injected subcutaneously with PhtD alone, which means PhtD

itself is immunogenic when applied s.c. It is also worth noting that the IgG response in mice vaccinated s.c. with PhtD alone is significantly lower than that with PhtDPLY and PhtDΔ6PLY, which suggests that fusing PhtD to PLY and Δ6PLY can still further boost the immune response in those animals.

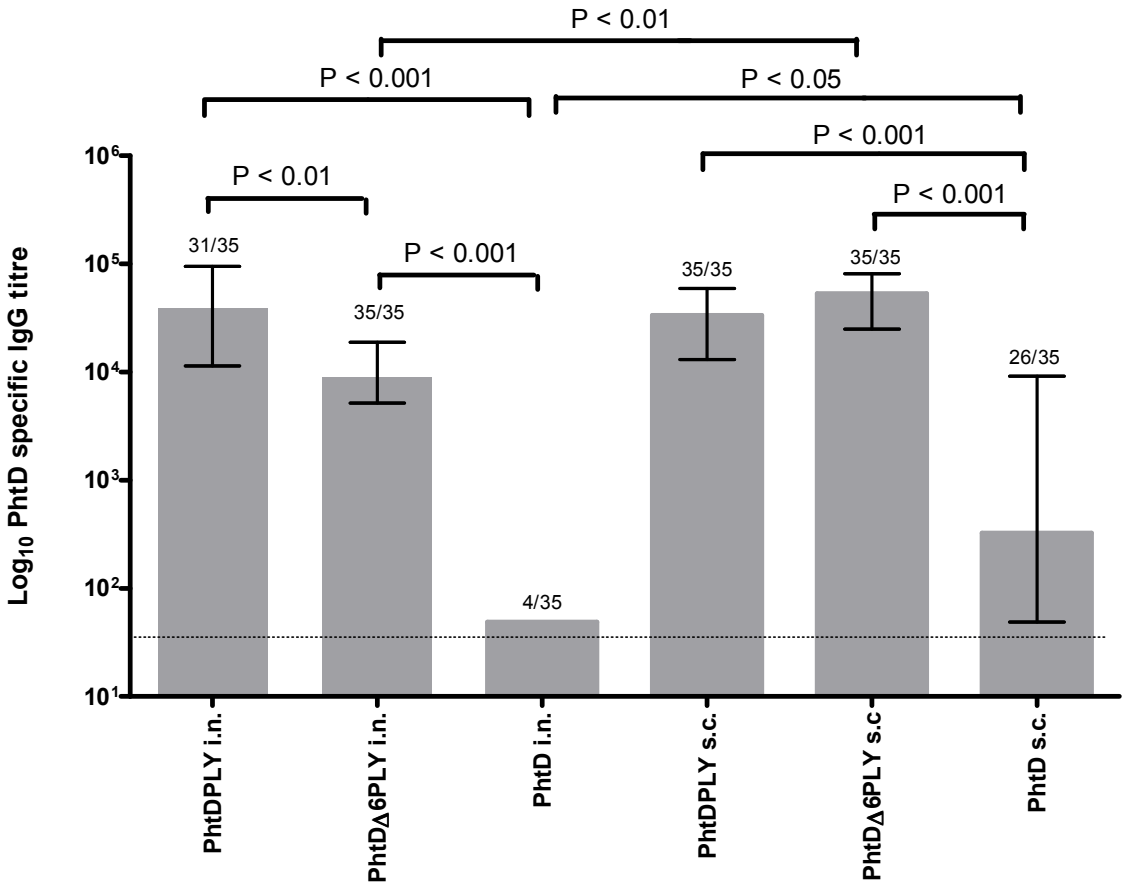


Figure 5-8 PhtD specific IgG titre detected in sera of mice intranasally (i.n.) or subcutanously (s.c.) vaccinated with three doses of purified proteins.
Bars represent median titres ± the interquartile range from the median from 35 mice. The number of responding individuals in a group is shown above each bar. The dotted horizontal line represents the detection limit of the assay. Groups were compared using GraphPad Prizm 4.0 (California, USA) by Kruskal-Wallis with Dunn's post test. P<0.05 was significant, P<0.01 was highly significant and P<0.001 was extremely significant.

5.6 PLY specific IgG response

We previously showed that intranasal administration of eGFPPLY can generate better anti-PLY IgG response than that of eGFPΔ6PLY (Douce *et al*. 2010). Similar results were observed in mice vaccinated with PspCPLY or PspCΔ6PLY. In Figure 5-9, anti-PLY IgG responses were detected in animal vaccinated i.n. with PLY, PspCPLY, PspCΔ6PLY and PhtDΔ6PLY. However, surprisingly PhtDPLY elicited very poor PLY specific IgG response both in titres and numbers (10\35). The anti-PLY IgG responses in PhtDPLY is significant lower than in mice

vaccinated with PLY, eGFPPLY and PspCPLY separately, which generated almost equivalent level of anti-PLY IgG antibodies. These results suggest the anti-PLY IgG response is clearly affected by carried antigens when mice are immunised with PLY fusion proteins. In addition, subcutaneous delivery of PLY, PLY or Δ6PLY fusion protein is able to elicit anti-PLY IgG response (data not shown).

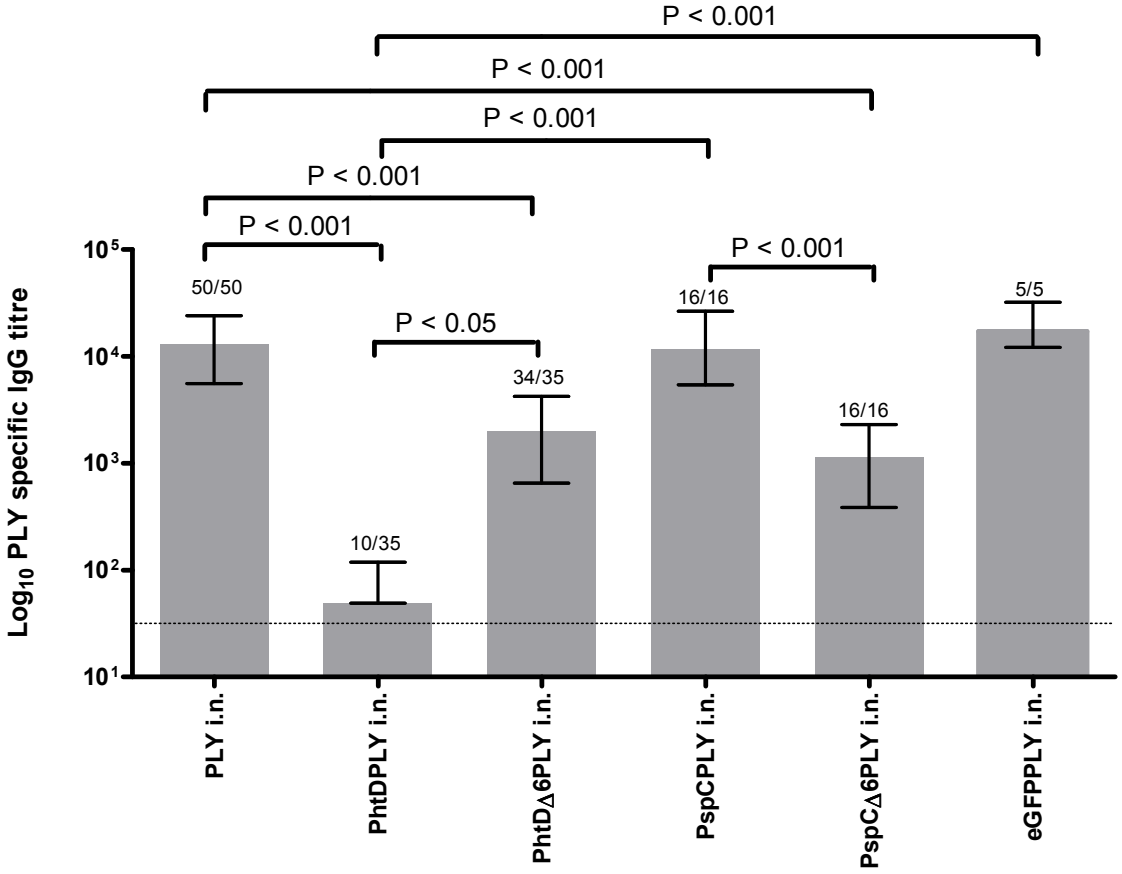


Figure 5-9 Anti-PLY IgG antibodies detected in sera of mice intranasally (i.n.) vaccinated with three doses of purified proteins.
Bars represent median titres ± the interquartile range from the median. The number of responding individuals in a group is shown above each bar. The dotted horizontal line represents the detection limit of the assay. Groups were compared using GraphPad Prizm 4.0 (California, USA) by Kruskal-Wallis with Dunn's post test. P<0.05 was significant, P<0.01 was highly significant and P<0.001 was extremely significant.

5.7 IgG responses in mice immunised with mixtures of fusion proteins

Higher concentrations of protein were used in order to boost antibody response since ∆6PLY has no cytotoxicity (Kirkham *et al*. 2006c).

5.7.1 Systemic IgG response to intranasal delivery of 8 µg mixed ∆6PLY fusion proteins

As shown in Figure 5-10, in the ∆6PLY fusion mixture group, anti-PsaA (120/120), anti-PspA (120/120), anti-PhtD (120/120) and anti-PLY IgG (117/120) were detected in almost all 120 animals. Antigen specific titres were significantly higher in the ∆6PLY fusion mixture group than in those that received the single antigen mixture except anti-PhtD IgG, which was equivalent in both groups. Anti-PLY IgG in animals that received PLY alone was significantly higher than that in the ∆6PLY fusion mixture group, which received 10 fold more ∆6PLY per dose.

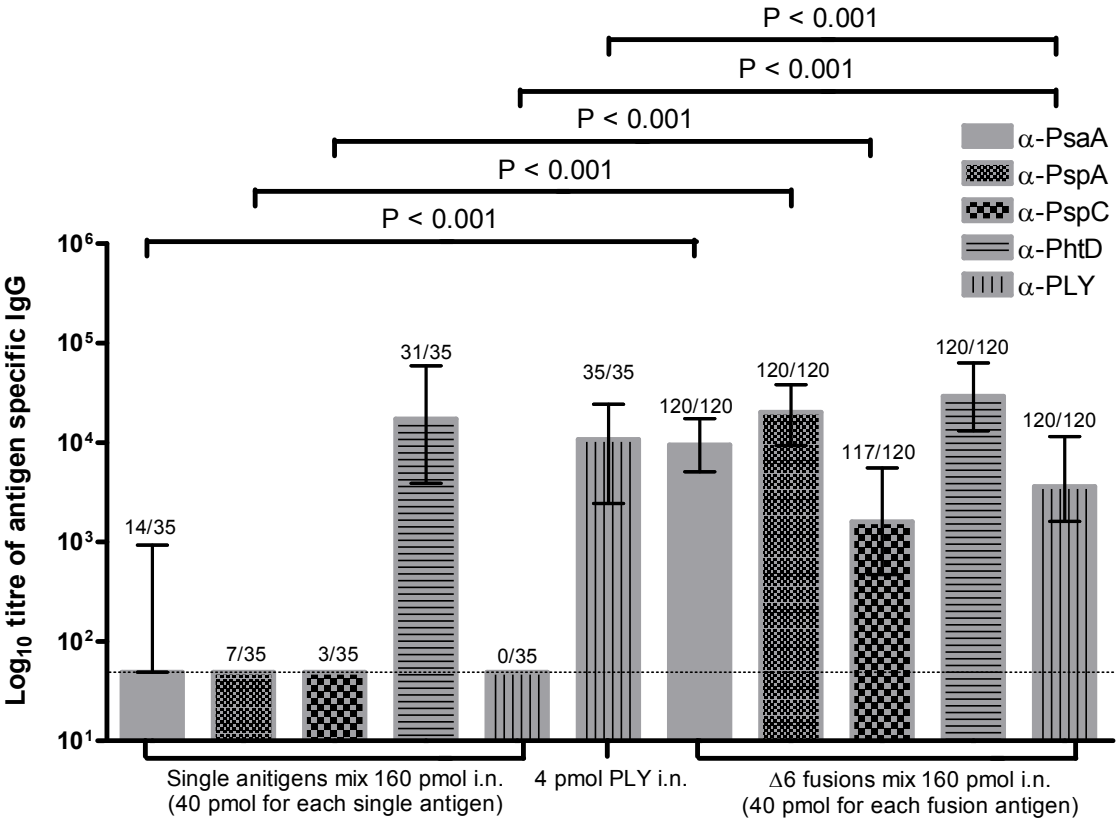


Figure 5-10 Antigen specific IgG antibodies detected in sera of animals vaccinated intranasally with a mixture of single antigens, PLY alone or with a mixture of Δ6PLY fusions. Bars represent median titres ± the interquartile range from the median. The number of responding individuals in a group is shown above each bar. The dotted horizontal line represents the detection limit of the assay. Groups were compared by Mann-Whitney non-parametric t-test using GraphPad Prizm 4.0 (California, USA). There was a significant difference in anti-PsaA, anti-PspA, anti-PspC and anti-PLY serum IgG (P <0.001) in those mice that received the mixture of Δ6PLY fusions compared with those that received a mixture of single antigens. No other statistically significant differences were seen.

Significantly more antigen specific IgG was generated in response to the Δ6PLY fusion mixture group in comparison to the equivalent group in PATH 1, 3, 5 & 7 (Figure 5-11), which is consistent to the results in the trial experiment (Figure 5-10). The percentage of mice responding to PspA (from 83% to 100%) and PspC (from 77% to 100%) in the mixture group was significantly increased in comparison to when each antigen was given alone. This is possibly due more protein being used rather than synergic effect among the antigens since the Δ6PLY fusion mixture group received 10 fold more protein than the group who received individual antigen (PATH 1, 3, 5 and 7).

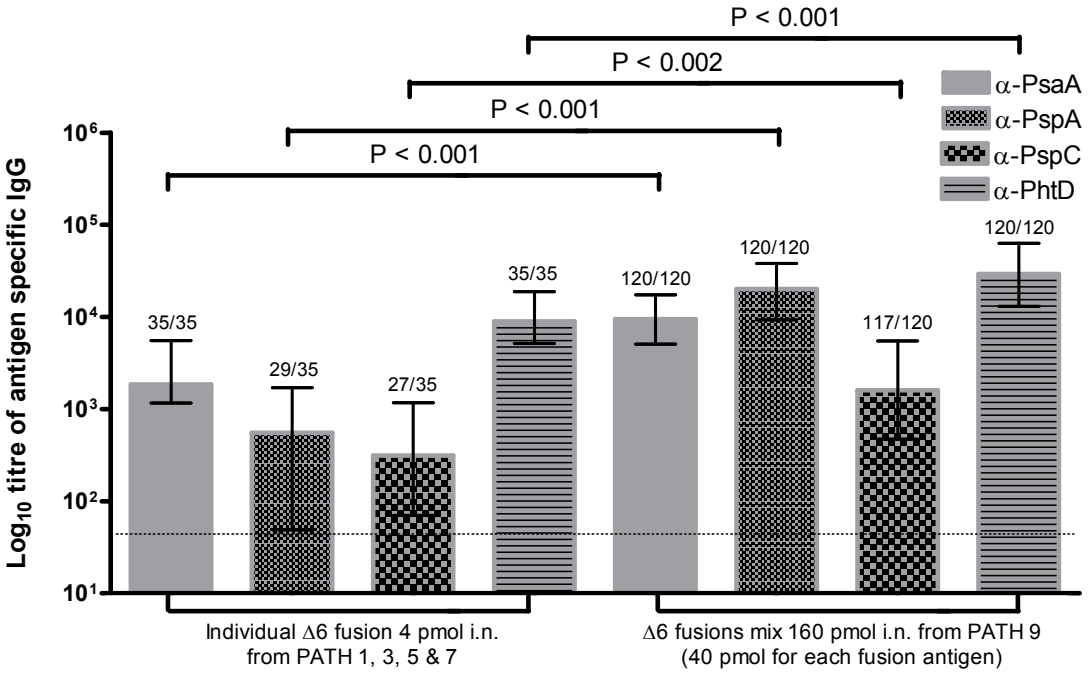


Figure 5-11 Antigen specific IgG antibodies detected in sera of animals vaccinated intranasally with 4 pmol individual Δ6PLY fusion protein (from PATH 1, 3, 5 & 7) or with a mixture of Δ6PLY fusion proteins (160 pmol in total, 40 pmol each from PATH 9). Bars represent median titres ± the interquartile range from the median. The number of responding individuals in a group is shown above each bar. The dotted horizontal line represents the detection limit of the assay. Groups were compared by Mann-Whitney non-parametric t-test using GraphPad Prizm 4.0 (California, USA). There was a significant difference in anti-PsaA, anti-PspA and anti-PhtD serum IgG (P <0.001) and anti-PspC (P 0.002) in those mice that received the mixture of Δ6PLY fusions compared with those that received individual Δ6PLY fusions.

As shown in Figure 5-12, in the single antigen mixture control group, 14/35 recognised PsaA, 7/35 recognised PspA, 3/35 recognised PspC, 31/35 recognised PhtD but none recognised PLY. This is in comparison to PATH 1, 3, 5 & 7, where 3/35 recognised PsaA, 1/35 recognised PspA, 1/35 recognised PspC, 4/35 recognised PhtD and 2/35 recognised PLY. The number of mice responding to individual antigens in the mixture group was slightly increased in comparison to when each antigen was given alone. In addition, titres were fractionally elevated except that the number of mice responding to PhtD was substantially increased in comparison to PATH 7 and the titres were significantly higher (P <0.001) as seen in Figure 5-12 below. It is possible that this is because 10 times more antigens are delivered. Interestingly, PhtD becomes much more immunogenic when a high concentration is used.

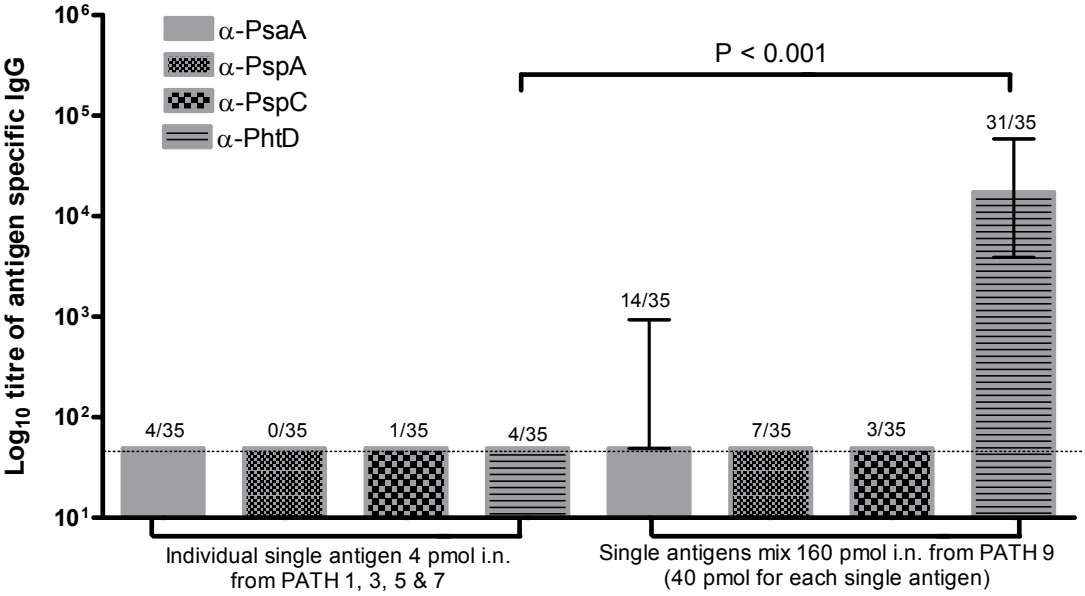


Figure 5-12 Antigen specific IgG antibodies detected in sera of animals vaccinated intranasally with single antigens (data from PATH 1, 3, 5 & 7) or with a mixture of single antigens (data from PATH 9).
Bars represent median titres ± the interquartile range from the median. The number of responding individuals in a group is shown above each bar. The dotted horizontal line represents the detection limit of the assay. Groups were compared by Mann-Whitney non-parametric t-test using GraphPad Prizm 4.0 (California, USA). There was a significant difference in anti-PhtD IgG (P <0.001) in those mice that received the mixture of antigens compared with those that received each antigen individually. No other statistically significant differences were seen.

The above data suggested administration of 160 pmol of mixture of Δ6PLY fusion proteins (40 pmol for each fusion protein) is well tolerated and induced good antigen specific IgG titres in mice. It is likely that other vaccine candidates could easily be added to the fusion protein mix.

5.8 Discussion

All fusion proteins tested, following delivery by both intranasal and subcutaneous routes, were capable of generating statistically significant levels of systemic antibodies to the protein (PsaA, PspA, PspC and PhtD) fused to either PLY or Δ6PLY in mice. The serology data of PsaAPLY and PsaAΔPLY were published recently (Douce *et al*. 2010). Formaldehyde treated PsaAPLY induces anti-PsaA IgG responses when delivered s.c. but not when delivered i.n.. The formaldehyde treated PsaAPLY possibly behaves as micro-depot of antigen that is slowly released from the site of injection into draining lymph nodes. i.n. or s.c. administration of Individual antigens such as PsaA, PspA and PspC alone to

mice were not capable of generating antigen specific antibodies. PhtD was capable of inducing antigen specific antibodies in the absence of an adjuvant.

Intranasal delivery route induces good antigen specific mucosal IgA in both nasal and pulmonary tracts in response to the fusion proteins. Subcutaneous delivery route induces very poor mucosal response in both nasal and pulmonary tracts in response to the fusion proteins. In fact, only very low levels of antigen specific mucosal IgA were detected in 2 out of 5 mice immunised s.c. with PspCPLY and no antigen specific mucosal IgA was induced when the other fusion proteins were applied s.c.. PLY fusion proteins elicited significant levels of IgA in nasal and pulmonary tracts. Δ6PLY fusion proteins did not elicit significant level of IgA except PsaAΔ6PLY, which elicited detectable level of PsaA specific IgA in both nasal and pulmonary tracts (Figure 5-2).

We selected a combination of antigens for intranasal delivery as a mixture in PATH 9 & 10 empirically determined to be 40 pmol of each antigen administered as a mixture (160 pmol in total) in three doses, with an equimolar mix of single antigens and a 4 pmol PLY group as a control. Delivery of a mixture of ∆6PLY fusions intranasally is capable of generating significant titres of antigen specific IgG in mice sera (Figure 5-10).

A greater number of individuals in the mixed single antigens group responded to individual antigens, and this response is particularly strong in response to PhtD (Figure 5-11 and Figure 5-12). The anti-PhtD IgG response is so strong that there is not a significant difference between the single antigen mixture and the ∆6PLY fusion mixture. As mentioned earlier, PhtD was capable of inducing antigen specific antibodies when delivered s.c. in the absence of an adjuvant. More interestingly, PhtD inhibited production of anti-PLY IgG in mice intranasally immunised with PhtDPLY through unknown mechanisms, but this did not occur in mice immunised with PhtDΔ6PLY. Both PLY and PhtD have been shown to interfere with complement pathways (Paton et al. 1984; Mitchell et al. 1991; Ogunniyi et al. 2009). The data for PhtD indicate that PhtD has additional important functions during pneumococcal infection, especially in the colonisation stage.

Overall, antigen specific responses are significantly stronger in the Δ6PLY fusion mixture group, when compared with the single antigen mixture as well as the results from PATH 1, 3, 5 & 7, where 2μg (40 pmol) of each Δ6PLY fusion was given individually. There is some variation in final titres between the antigens in the Δ6PLY fusion mixture group, but this may be related to the properties of each individual protein antigen. There is a robust response to the Δ6PLY adjuvant in the Δ6PLY fusion mixture group, although it is lower than the response to the fully toxic form and lower than all the other antigens in the Δ6PLY fusion mixture. We have demonstrated producing antibody responses to 5 pneumococcal proteins through adjuvant properties of PLY and Δ6PLY. In next chapter, the protective efficiency of these antigens in vaccination challenge models is described.

Chapter 6 ∆6PLY fusion proteins confer protection against colonisation by pneumococcus

The data presented in chapter 5 show that mice either vaccinated intranasally (i.n.) or subcutanously (s.c.) with PLY or Δ6PLY fusion proteins generate good antigen specific antibody responses. This chapter explores whether these immune responses elicited by PLY or Δ6PLY fusion proteins can confer protection against three strains of pneumococcus in a murine model of colonisation. Vaccinated mice were challenged i.n. with 2 x 10^7 cfu Xen10, D39 or Xen35 respectively. The nasopharynx of each mouse was washed and the number of bacteria present enumerated after 72 hours post infection (hpi).

6.1 Mice vaccinated i.n. or s.c. with PsaA fusion proteins and colonised with pneumococcus

After 72hpi, no significant difference on bacterial load was detected in nasal wash of mice vaccinated i.n. or s.c. with PsaA, PsaAPLY, PsaAΔ6PLY or formaldehyde treated (HCHO) PsaAPLY respectively (Figure 6-1). Intranasal or subcutaneous delivery of PsaAPLY, PsaAΔ6PLY or formaldehyde treated PsaAPLY alone were not protective against colonisation with pneumococcus Xen10, D39 and Xen35.

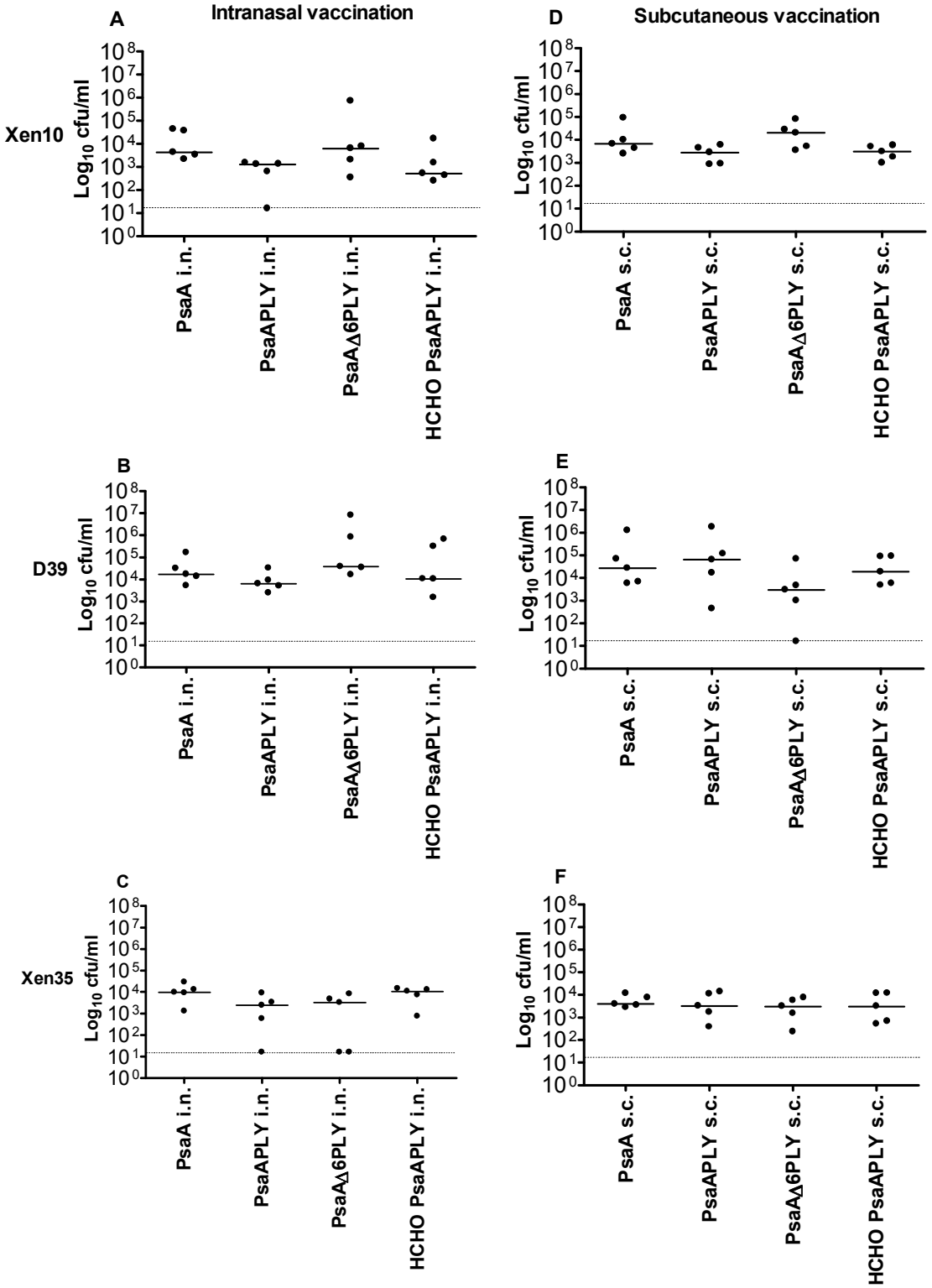

A
Intranasal vaccination
D
Subcutaneous vaccination
Xen10
B
D39
E
C
Xen35
F
Log10 cfu/ml
PsaA i.n.
PsaAPLY i.n.
PsaAΔ6PLY i.n.
HCHO PsaAPLY i.n.
PsaA s.c.
PsaAPLY s.c.
PsaAΔ6PLY s.c.
HCHO PsaAPLY s.c.

Figure 6-1 PsaA fusion proteins are not protective against nasopharyngeal colonisation with pneumococcus.
Bacterial count in nasal washes at 72 hours post infection in animals vaccinated intranasally (i.n.) or subcutanously (s.c.) with PsaA, PsaAPLY, formaldehyde treated PsaAPLY (HCHO PsaAPLY) or PsaA∆6PLY respectively, and colonised with Xenogen 10 A66.1 (Xen10), D39 or Xenogen 35 TIGR4 (Xen35) respectively. (A) Vaccinated i.n. and colonised with Xen10. No statistical difference between groups. (B) Vaccinated i.n. and colonised with D39. No statistical difference between groups. (C) Vaccinated i.n. and colonised with Xen35. No statistical difference between groups. (D) Vaccinated s.c. and colonised with Xen10. No statistical difference between groups. (E) Vaccinated s.c. and colonised with D39. No statistical difference between groups. (F) Vaccinated s.c. and colonised with Xen35. No statistical difference between groups. Five mice were used for each group. Each spot represents a single individual. Horizontal lines represent the mean for each group. The dotted line represents the limit of detection for the assay. All groups were compared by Kruskal-Wallis with Dunn's post test using GraphPad Prizm 4.0 (California, USA).

6.2 Mice vaccinated i.n. or s.c. with PspA fusion proteins and colonised with pneumococcus

In mice vaccinated i.n. with PspA, PLY, PspAPLY or PspA∆6PLY respectively, bacterial load was significantly lower in the nasal wash of mice immunised i.n. with PspA∆6PLY than those with PspA after these mice were infected i.n. with Xen10 or D39 for 72 hours (Figure 6-2A and B). After 72 hours post infection (hpi) with Xen35, no significant difference was observed in the nasal lavage between any of the groups, although there did appear to be a trend towards lower counts in PspA∆6PLY compared to that of PspA (Figure 6-2C). In mice vaccinated i.n. with PspA∆6PLY, no bacteria were recovered in nasal lavage in 3 out of 5 mice challenged i.n. with Xen10 or D39, and no bacteria were recovered in nasal lavage of 2 out of 5 mice challenged i.n. with Xen35. These results suggested intranasal delivery of PspA∆6PLY protected mice against Xen10 and D39, and potentially against Xen35, and intranasal delivery of PspAPLY did not protect mice against pneumococcus.

In mice vaccinated s.c. with PspA, PLY, PspAPLY or PspA∆6PLY respectively, no significant differences in the bacterial load were observed between any of the groups infected i.n. with Xen10 or D39 (Figure 6-2D and E). After 72 hours post infection (hpi) with Xen35, bacterial load was significantly lower in the nasal wash of mice s.c immunised with PspA∆6PLY than those with PspA (Figure 6-2F). These results suggested subcutaneous delivery of PspA∆6PLY only protected mice against Xen35, and subcutaneous delivery of PspAPLY did not protect mice against any of three pneumococcal strains used in this murine colonisation study.

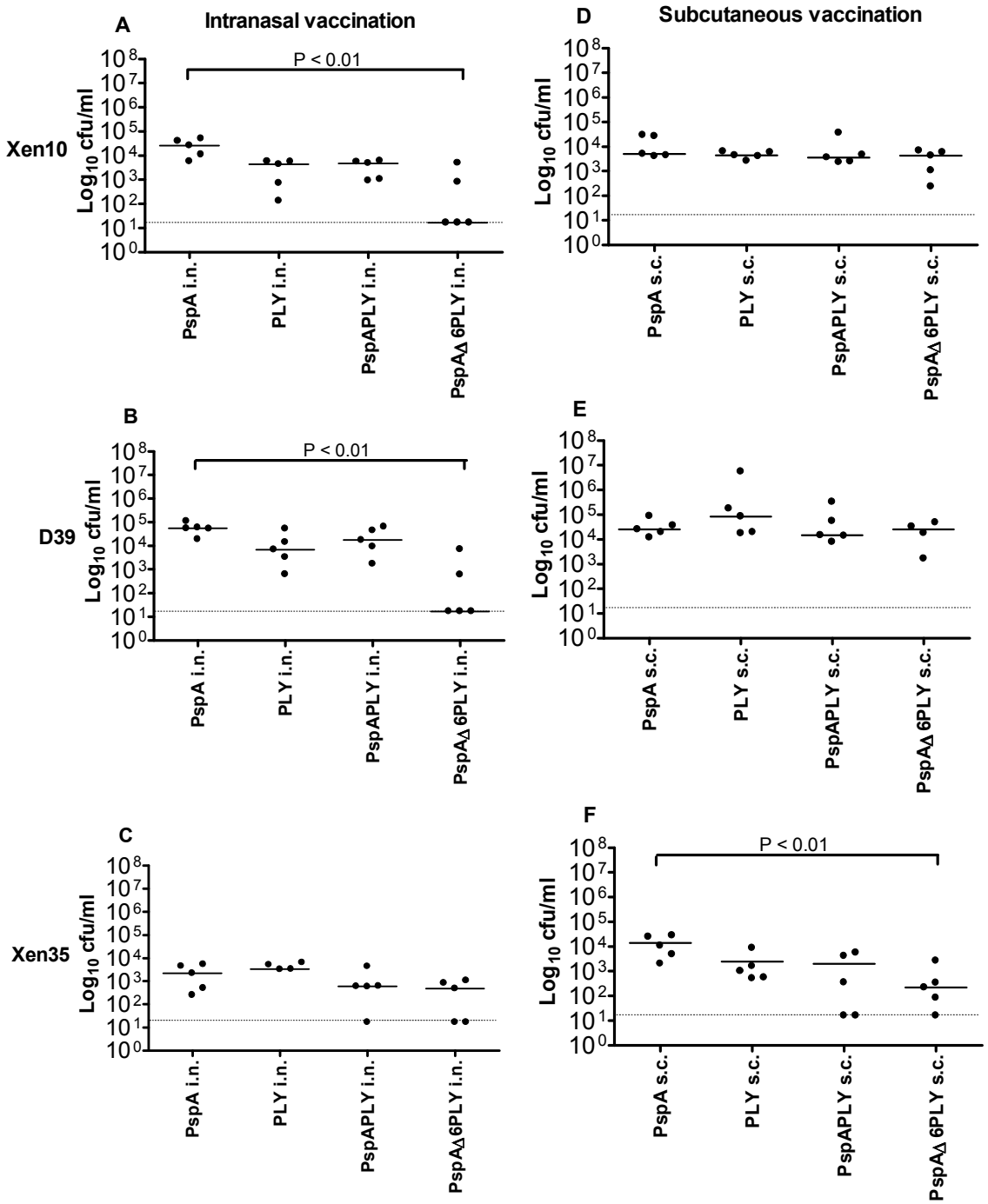

A
Intranasal vaccination
Xen10
P < 0.01
Log10 cfu/ml
PspA i.n.
PLY i.n.
PspAPLY i.n.
PspAΔ6PLY i.n.
D
Subcutaneous vaccination
PspA s.c.
PLY s.c.
PspAPLY s.c.
PspAΔ6PLY s.c.
B
D39
E
C
Xen35
F

Figure 6-2 PspA fusion proteins protects against pneumococcal colonisation. Bacterial count in nasal washes at 72 hps in animals vaccinated intranasally or subcutanously with PspA, PLY, PspAPLY or PspA∆6PLY respectively, and colonised with Xen10 A66.1, D39 or Xen35 TIGR4 respectively. (A) Vaccinated i.n. and colonised with Xen10. There was a significant difference (P < 0.01) between the PspA and PspA∆6PLY vaccinated mice. (B) Vaccinated i.n. and colonised with D39. There was a significant difference (P < 0.01) between the PspA and PspA∆6PLY vaccinated mice. (C) Vaccinated i.n. and colonised with Xen35. No statistical difference between groups. (D) Vaccinated s.c. and colonised with Xen10. No statistical difference between groups. (E) Vaccinated s.c. and colonised with D39. No statistical difference between groups. (F) Vaccinated i.n. and colonised with Xen35. There was a significant difference (P < 0.01) between the PspA and PspA∆6PLY vaccinated mice. Five mice were used for each group. Each spot represents a single individual. Horizontal lines represent the median for each group. The dotted line represents the limit of detection for the assay. All groups were compared by Kruskal-Wallis with Dunn's post test using GraphPad Prizm 4.0 (California, USA).

6.3 Mice vaccinated i.n. or s.c. with PspC fusion proteins and challenged with pneumococcus

No significant difference on the bacterial load was detected in the nasal lavage between any of the groups vaccinated i.n. with PspC, PLY, PspCPLY or PspCΔ6PLY respectively (Figure 6-3A, B and C). These results suggested intranasal delivery of PspCPLY or PspCΔ6PLY alone did not protect mice against colonisation with pneumococcus Xen10, D39 and Xen35. However, very low bacterial counts were detected in three out of five PspC∆6PLY vaccinated mice compared to that of PspA after 72 hpi with D39 or Xen35. The trend with D39 and Xen35 colonization was towards protection but it was not statistically significant (Figure 6-3B and C).

In mice vaccinated s.c. with PspC, PLY, PspCPLY or PspCΔ6PLY respectively, no significant differences in the bacterial load were observed between any of the groups infected i.n. with Xen10 or D39 (Figure 6-3D and E). After 72 hpi with Xen35, bacterial load was significantly lower in the nasal wash of mice s.c immunised with PspAPLY than that with PspA, as well as between PspAΔ6PLY and PspA (Figure 6-3F). These results suggested subcutaneous delivery of PspCPLY or PspCΔ6PLY only protected mice against Xen35.

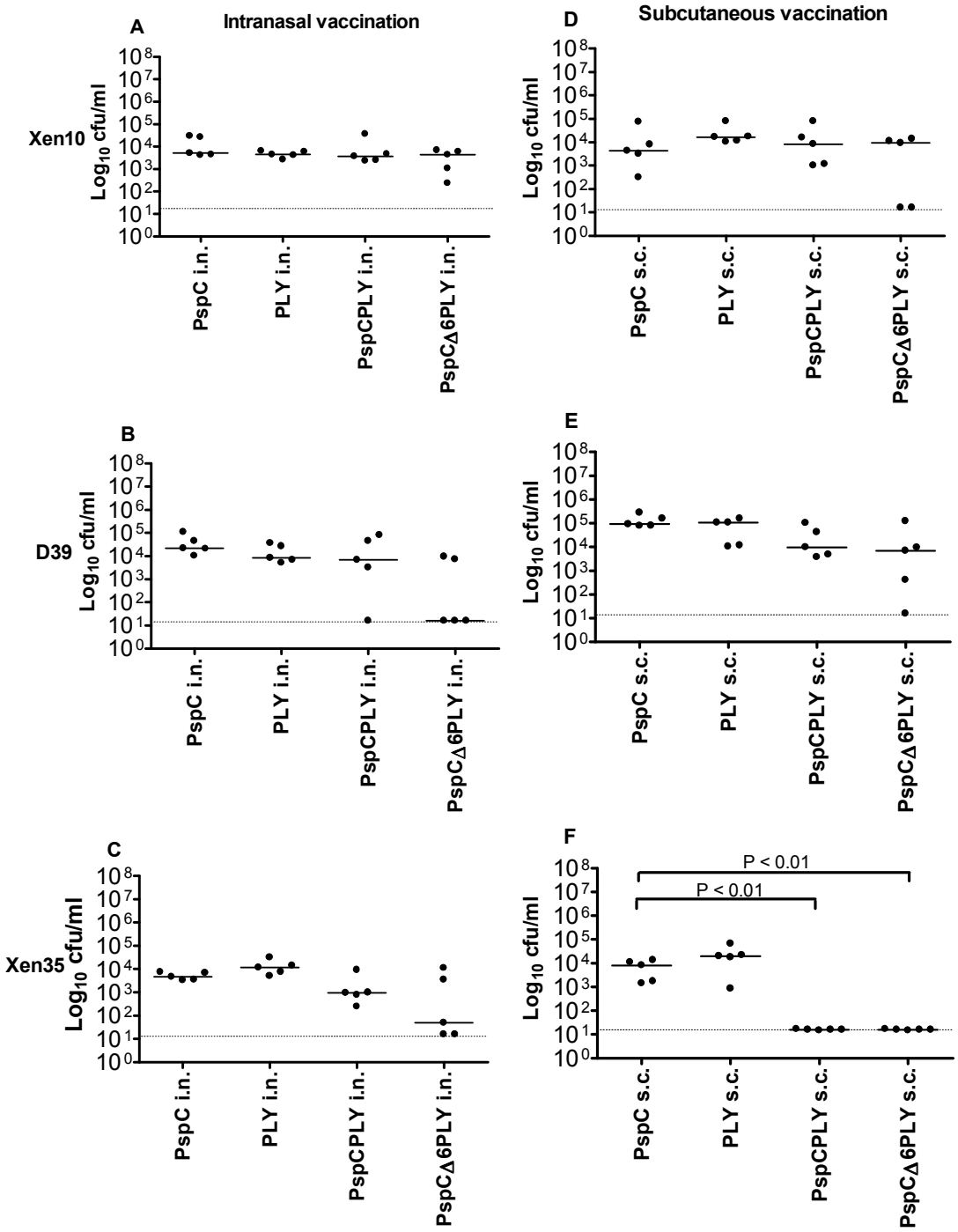


Figure 6-3 PspC fusion proteins reduces pneumococcal carriage in nasopharynx with Xen35.
Bacterial count in nasal washes at 72 hps in animals vaccinated intranasally or subcutanously with PspC, PLY, PspCPLY or PspCΔ6PLY respectively, and colonised with Xen10 A66.1, D39 or Xen35 TIGR4 respectively. (A) Vaccinated i.n. and colonised with Xen10. No statistical difference between groups. (B) Vaccinated i.n. and colonised with D39. No statistical difference between groups. (C) Vaccinated i.n. and colonised with Xen35. No statistical difference between groups. (D) Vaccinated s.c. and colonised with Xen10. No statistical difference between groups. (E) Vaccinated s.c. and colonised with D39. No statistical difference between groups. (F) Vaccinated s.c. and colonised with Xen35. There is a significant difference (P < 0.01) were detected between the PspC and PspCPLY, as well as PspC and PspCΔ6PLY (P < 0.01) vaccinated mice. Five mice were used for each group. Each spot represents a single individual. Horizontal lines represent the median for each group.

The dotted line represents the limit of detection for the assay. All groups were compared by Kruskal-Wallis with Dunn's post test using GraphPad Prizm 4.0 (California, USA).

6.4 Mice vaccinated i.n. or s.c. with PhtD fusion proteins and challenged with pneumococcus

In mice vaccinated i.n. with PhtD, PLY, PhtDPLY or PhtDΔ6PLY respectively, bacterial load was significantly lower in the nasal wash of PhtDΔ6PLY immunised mice than PhtD vaccinated mice after 72 hpi with Xen10, D39 or Xen35 (Figure 6-4 A,B and C). After 72 hours post infection (hpi) with Xen35, a significant difference was observed in the nasal lavage between PhtDPLY and PhtD vaccinated mice, and there was no significant difference in mice infected i.n. with Xen10 or D39; These results suggested intranasal delivery of PhtDΔ6PLY alone protected mice against Xen10, D39 and Xen35, and intranasal delivery of PhtDPLY alone only protected mice against Xen35.

In mice vaccinated s.c. with PhtD, PLY, PhtDPLY or PhtDΔ6PLY respectively, no significant differences on the bacterial loads were detected between any of the groups infected i.n. with Xen10 , D39 or Xen35(Figure 6-4D, E and F). These results suggested subcutaneous delivery of PhtDPLY or PhtDΔ6PLY alone did not protect mice against any one of three pneumococcal strains.

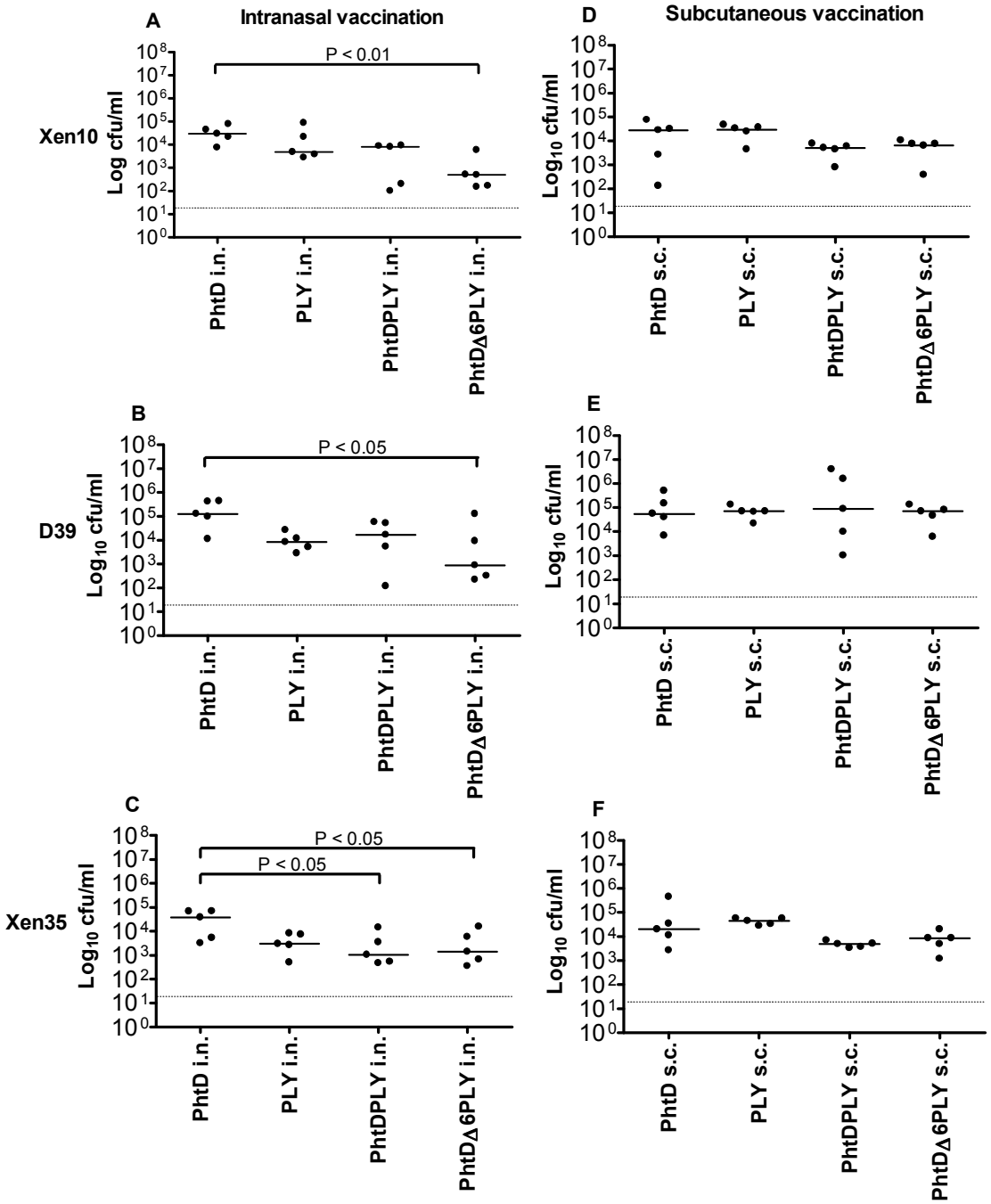


Figure 6-4 PhtD fusion proteins reduced nasopharyngeal colonisation with pneumococcus. Bacterial count in nasal washes at 72 hpi in animals vaccinated intranasally or subcutanously with PhtD, PLY, PhtDPLY or PhtDΔ6PLY respectively, and colonised with Xen10, D39 or Xen35 respectively. (A) Vaccinated i.n. and colonised with Xen10. There was a significant difference (P < 0.01) between the PhtD and PhtDΔ6PLY vaccinated mice. (B) Vaccinated i.n. and colonised with D39. There was a significant difference (P < 0.05) between the PhtD and PhtDΔ6PLY vaccinated mice. (C) Vaccinated i.n. and colonised with Xen35. No statistical difference between groups. There was a significant difference between the PhtD and PhtDPLY (P < 0.05), as well as the PhD and PhtDΔ6PLY (P < 0.05 vaccinated mice. (D) Vaccinated s.c. and colonised with Xen10. No statistical difference between groups. (E) Vaccinated s.c. and colonised with D39. No statistical difference between groups. (F) Vaccinated s.c. and colonised with Xen35. No statistical difference between groups. Five mice were used for each group. Each spot represents a single individual. Horizontal lines represent the median for each group. The dotted line represents the limit of detection for the assay. All groups were compared by Kruskal-Wallis with Dunn's post test using GraphPad Prizm 4.0 (California, USA).

6.5 Mice vaccinated i.n. or s.c. with a mixture of ∆6PLY fusion proteins and challenged with pneumococcus

After encouraging protection results were obtained with delivery of individual Δ6PLY fusion proteins, protection efficacy of mixed Δ6PLY fusion proteins were tested. In previous chapter, a 10 times mixture of PsaAΔ6PLY, PspAΔ6PLY, PspCΔ6PLY and PhtDΔ6PLY (10 x Δ6PLY fusions mix, 40 pmol each, 160 pmol in total) was selected to use in the animal protection study as it gave better antigen specific responses than 1 time mixture of PsaAΔ6PLY, PspAΔ6PLY, PspCΔ6PLY and PhtDΔ6PLY (Δ6PLY fusions mix, 4 pmol each, 16 pmol in total). A significantly lower bacterial load was observed in the nasal lavage of mice inoculated i.n. with 10 times Δ6PLY fusion proteins mix than mice vaccinated i.n. with either 1 time single antigen alone or 10 times single antigens mix, but this was not observed with s.c. vaccinated mice. Mice vaccinated i.n. with Δ6PLY fusion proteins mix were protected against Xen10, D39 or Xen35 (Figure 6-5A, B and C), but s.c. delivery of Δ6PLY fusion proteins mix was not protective against pneumococcal colonisation.

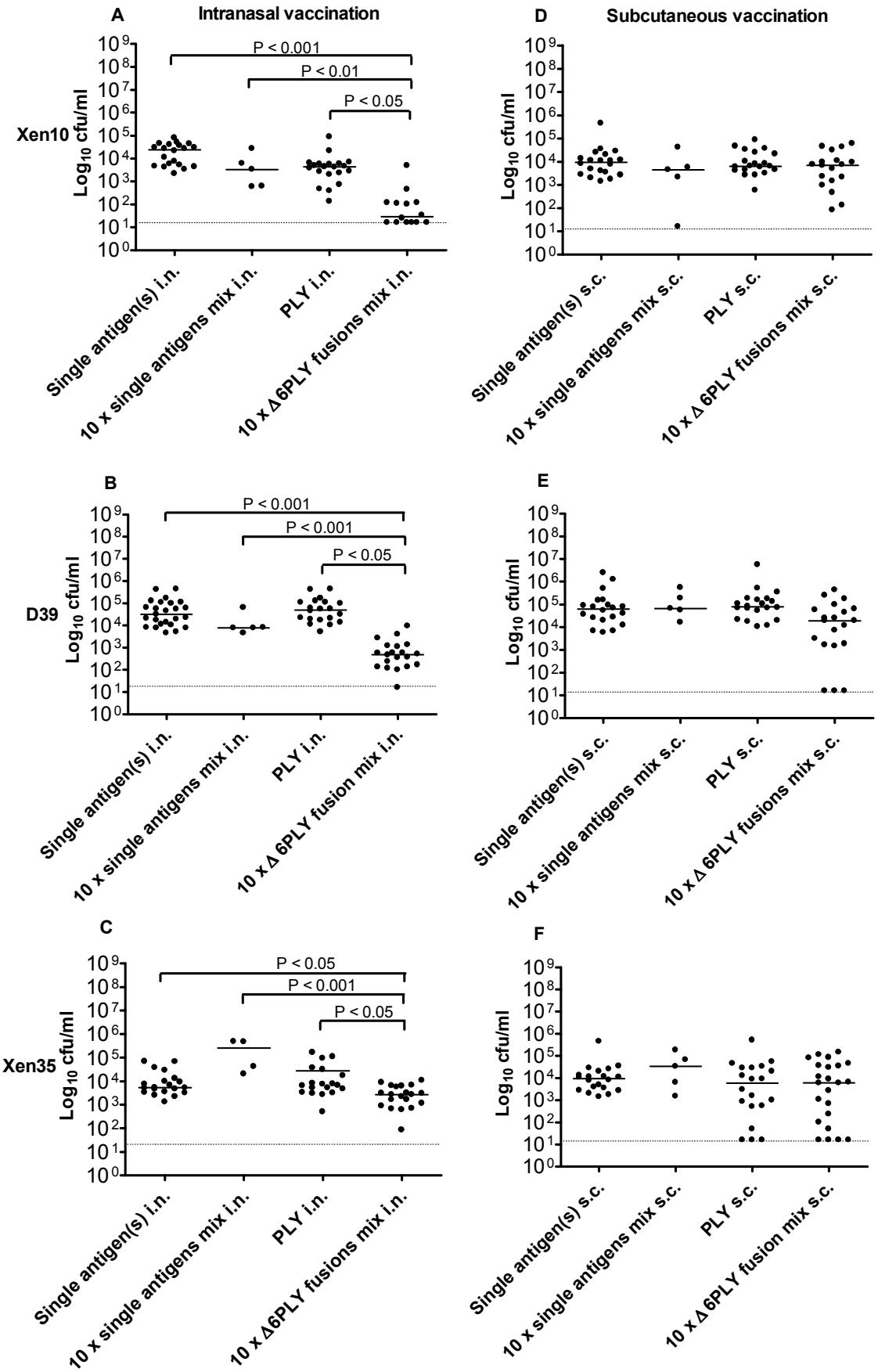

A
Intranasal vaccination
D
Subcutaneous vaccination
Xen10
Log10 cfu/ml
P < 0.001
P < 0.01
P < 0.05
Single antigen(s) i.n.
10 x single antigens mix i.n.
PLY i.n.
10 x Δ 6PLY fusions mix i.n.
Single antigen(s) s.c.
10 x single antigens mix s.c.
PLY s.c.
10 x Δ 6PLY fusions mix s.c.
B
E
D39
P < 0.001
P < 0.001
P < 0.05
10 x Δ 6PLY fusion mix i.n.
10 x Δ 6PLY fusion mix s.c.
C
F
Xen35
P < 0.05
P < 0.001
P < 0.05
10 x Δ6PLY fusions mix i.n.

Figure 6-5 mixture of Δ6 fusion proteins protects mice against pneumococcal colonisation. Bacterial count in nasal washes at 72 hpi in animals vaccinated intranasally with Single antigen(s) (combined results from mice vaccinated with PsaA, PspA, PspC and PhtD individually), 10 times mixture of single antigens (10 x single antigens mix) including PsaA, PspA, PspC and PhtD, PLY, 10 times mixture of Δ6PLY fusion proteins (10 x △6PLY fusions mix) including PsaA△6PLY, PspA△6PLY, PspC△6PLY and PhtD△6PLY, and colonised with Xen10, D39 or Xen35 respectively. (A) Vaccinated i.n. and colonised with Xen10. There was a significant difference (P < 0.01) between the PhtD and PhtD△6PLY vaccinated mice. There was a statistically significant difference between single antigens and 10xMix Δ6PLY fusions (P <0.001), as well as between PLY and 10Xmixed Δ6PLY fusion proteins (P <0.01). (B) Vaccinated i.n. and colonised with D39. There was a significant difference (P < 0.05) between the PhtD and PhtD△6PLY vaccinated mice. There was a statistically significant difference between single antigens and 10xMix Δ6PLY fusions (P <0.001), as well as between PLY and 10XMix Δ6PLY fusions (P <0.001). (C) Vaccinated i.n. and colonised with Xen35. No statistical difference between groups. There was a statistically significant difference between single antigens and 10xMix Δ6PLY fusions (P <0.05), as well as between PLY and 10xMix Δ6PLY fusions (P <0.01). (D) Vaccinated s.c. and colonised with Xen10. No statistical difference between groups. (E) Vaccinated s.c. and colonised with D39. No statistical difference between groups. (F) Vaccinated s.c. and colonised with Xen35. No statistical difference between groups. 20 mice were used for each group. Each spot represents a single individual. Horizontal lines represent the median for each group. The dotted line represents the limit of detection for the assay. All groups were compared by Kruskal-Wallis with Dunn's post test using GraphPad Prizm 4.0 (California, USA).

Table 6-1 Summary of protection levels against colonisation with the different antigens via single antigen control.

Antigen	Strain / Model Of Vaccination	Xenogen 10 A66.1	D39	Xenogen 35 TIGR4
PsaAPLY	i.n.	-	-	-
PsaAΔ6PLY	i.n.	-	-	-
Formaldehyde treated PsaAPLY	i.n.	-	-	-
PsaAPLY	s.c.	-	-	-
PsaAΔ6PLY	s.c.	-	-	-
Formaldehyde treated PsaAPLY	s.c.	-	-	-
PspAPLY	i.n.	-	-	-
PspAΔ6PLY	i.n.	+ P < 0.05	++ P < 0.01	T
PspAPLY	s.c.	-	-	-
PspAΔ6PLY	s.c.	-	-	+ P < 0.05
PspCPLY	i.n.	-	-	-
PspCΔ6PLY	i.n.	-	T	T
PspCPLY	s.c.	-	-	++ P < 0.01
PspCΔ6PLY	s.c.	-	-	++ P < 0.01
PhtDPLY	i.n.	-	-	+ P < 0.05
PhtDΔ6PLY	i.n.	++ P < 0.01	+ P < 0.05	+ P < 0.05
PhtDPLY	s.c.	-	-	-
PhtDΔ6PLY	s.c.	-	-	-
Δ6PLY fusion protein mixture	i.n.	+++ P < 0.001	+++ P < 0.001	+ P < 0.05
Δ6PLY fusion protein mixture	s.c.	-	-	-

P < 0.05 significant (+), P < 0.01 very significant (++), P < 0.001 extremely significant (+++), not significant (-), trends towards significant (T).

Overall, Δ6PLY fusion protein applied i.n. alone was more protective than i.n. delivery of PLY fusion protein alone against pneumococcus, particularly in against Xen35 (Table 6-1). In our system, PspA or PhtD are much more protective than PsaA or PspC after being fused to Δ6PLY. PsaA failed to protect against any of three pneumococcal stains after being fused to PLY or Δ6PLY using either i.n. or s.c delivery route. PspC only protected against Xen35 after being fused to PLY

or Δ6PLY and applied s.c. Whereas PspA and especially PhtD protect against colonisation. The PhtD managed to protect against all 3 strains. Xen35 is the most protected strain among the three challenging strains.

6.6 Discussion

A number of studies have shown PsaA, PspA, PspC or PhtD applied i.n. alone with an adjuvant protected animals against pneumococcal carriage (Wu et al. 1997a; Briles et al. 2000a; Balachandran et al. 2002; Godfroid et al. 2011). PLY was also shown to facilitate the clearance of pneumococcal colonisation in the nasopharynx, although there are also some studies suggesting PLY increases or has no role in pneumococcal carriage (Kadioglu et al. 2002; van Rossum et al. 2005). Increased CD4+ T cell proliferative responses to PLY was shown to be associated with clearance of nasopharyngeal colonisation of pneumococcus in children (Zhang et al. 2007). Immunization with combination of above pneumococcal proteins elicited better protection than immunization with individual protein alone (Ogunniyi et al. 2000; Briles et al. 2000a; Ogunniyi et al. 2001; Briles et al. 2003; Ogunniyi et al. 2007).

I.n. delivery of PspAΔ6PLY or PhtDΔ6PLY alone was protective against all three pneumococcal strains, but not PspAPLY or PhtDPLY alone. The anti-PspA IgG response in mice immunised with PspAPLY is better than that with PspAΔ6PLY, which indicates antibody independent protection may be involved. Intranasal delivery of a mixture of PsaAΔ6PLY, PspAΔ6PLY, PspCΔ6PLY and PhtDΔ6PLY conferred better protection against nasopharyngeal colonisation with pneumococcus than administration of PspAΔ6PLY or PhtDΔ6PLY alone. Subcutaneous vaccination with PspAΔ6PLY, PspCΔ6PLY or PhtDΔ6PLY alone protected mice against nasopharyngeal carriage of pneumococcal strain Xen35 (TIGR4), but subcutaneous immunising mixture of PsaAΔ6PLY, PspAΔ6PLY, PspCΔ6PLY or PhtDΔ6PLY was unable to protect against the same strain of pneumococcus. Clearly, a synergistic was achieved when multiple proteins were delivered. It has been shown that mice vaccinated with a mixture of a PLY toxoid (W433F, D385N, and C428G), PsaA and PspA using CT as an adjuvant confers protection against pneumococcal colonisation in a CD4+ TH17 cell-dependent, antibody-independent fashion (Basset et al. 2007). Intranasal

delivery of PspAΔ6PLY alone, PhtDΔ6PLY alone or mixture of Δ6PLY fusion proteins may induce the similar antibody-independent protection in our system. However, the antibody-dependent protection may also play a role in protection against colonisation with pneumococcus in the nasopharynx.

Overall, neither systemic nor mucosal antibody responses to the different fusion proteins, as described in chapter 5, were reliable predictors of the efficacy of the fusions as vaccines against nasal colonisation. Antibodies to certain noncapsular antigens also arise early in life in response to colonization with pneumococci in mouse model, but those antibodies are not required for protection against pneumococcal colonization (Trzcinski *et al*. 2005). Higher concentration (higher than sub-lytic level) of PLY inhibits immune responses through direct effects on immune cells such as macrophage, monocytes and DCs (Nandoskar *et al*. 1986; Maus *et al*. 2004; Littmann *et al*. 2009). PLY fusion proteins may inhibit proliferation of CD4+ T cell and impair antibody independent protection. It is not well recognised that T regulator cells (Tregs) and TH17 are two T cell subsets with opposing actions and interplay in regulation of inflammation and autoimmunity (Mai *et al*. 2010). Zhang et al recently showed adenoidal CD4+ $CD25^{hi}$ Tregs has a potent inhibitory effect on CD4+ TH17 cells in the persistence of pneumococcal carriage in children (Zhang *et al*. 2011). This regulatory role of $CD25^{hi}$ Tregs on CD4+ TH17 cell was also showed in tonsil of adults and elderly (Pido-Lopez *et al*. 2011). Protease treatment reduced the inhibitory effect on Whole cell antigen-induced CD4+ T cell proliferation, suggesting the pneumococcal proteins may contribute to the accumulation of adenoidal Tregs (Zhang *et al*. 2011). Anti-pneumococcal PLY Tregs were detected in tonsil and likely contributing to the suppression of PLY anti-pnumococcal CD4+ cell responses; PLY Anti-pneumococcal CD4+ T cell responses are greater in mucosal lymphoid tissue than in the blood (Pido-Lopez *et al*. 2011). Δ6PLY fusions may lose this inhibitory effect of PLY but remain the capability to stimulate TH17 response. The adjuvant property of Δ6PLY may further amplify the IL-17 stimulation.

It was found the antibody independent protection requires functional CD4+ TH17 cells and is mediated by IL-17A through recruitment and activation of the neutrophils (Malley *et al*. 2005; Lu *et al*. 2008). Block of IL-17A significantly reduced immunity against pneumococcus in nasopharynx when purified cell wall

PS was intranasally delivered, cell wall PS can elicit a similar immunity to PWCV (Malley *et al*. 2006). Next step, IL-17 Receptor Knockout (RAKO) Mice will be used to assess if IL-17A is important for this Δ6PLY fusion proteins stimulated protection against pneumococcal colonisation in nasopharynx. Δ6PLY fusion proteins and their individual partners including PsaA, PspA, PspC, PhtD and Δ6PLY will be also checked if those proteins can induce IL-17 production. It will be also interesting to check PLY specific TH17 cells and Tregs exist in both NALT and blood of mice immunised i.n. with PLY fusion proteins. We can also hypothesise that PLY specific TH17 cells may exist but Tregs may be absent in NALT of mice immunised i.n. with Δ6PLY fusion proteins.

Chapter 7 Final discussion: a hypothesis

Mice immunised i.n. with PLY alone generated a good anti-PLY IgG response, which is not surprising as PLY can activate and promote release cytokines such as TNF, IL-1ß and IL-18 in APCs including macrophage and DCs, and activated APCs in turn activated the naïve $CD4^+$ T cell (Figure 7-1). Because eGFPPLY is as haemolytic as PLY, intranasally applied eGFPPLY should stimulate macrophages or DCs to produce the same cytokines as PLY. Intranasal delivery of eGFPPLY results in good antibody responses to both eGFP and PLY, but intranasal administration of a mixture of eGFP and PLY to mice only results in good anti-PLY IgG response. When eGFPPLY was applied i.n. to mice, eGFPPLY may have bound quickly to cells in the nasopharynx such as nasal epithelium, M cells and residential macrophages and DCs, and activated them as PLY is a pore forming toxin and has high affinity for cholesterol containing cell membranes. After receiving TNF from activated macrophages or themselves, activated DCs quickly move into local lymph nodes and activates naïve $CD4^+$ T cells, which then activated B cells displaying eGFPPLY epitopes. However, when a mixture of eGFP and PLY were applied intranasally to mice, PLY quickly bound to residential macrophages and DCs, and activated them, but eGFP was slowly taken up by other APCs due to the lack of a delivery system. Activated DCs displaying PLY epitopes then successfully primed T cells, but the few DCs displaying eGFP epitopes failed to activate naïve $CD4^+$ T cells as those DCs could not provide a second signal. Basically, there was a time difference in uptake of eGFP and PLY by different DCs due to high binding ability of PLY to cholesterol rich cell membranes. DCs which took up PLY moved into local lymph nodes and interacted with T cells first. The above hypotheses are needed to be carefully proven by experiments.

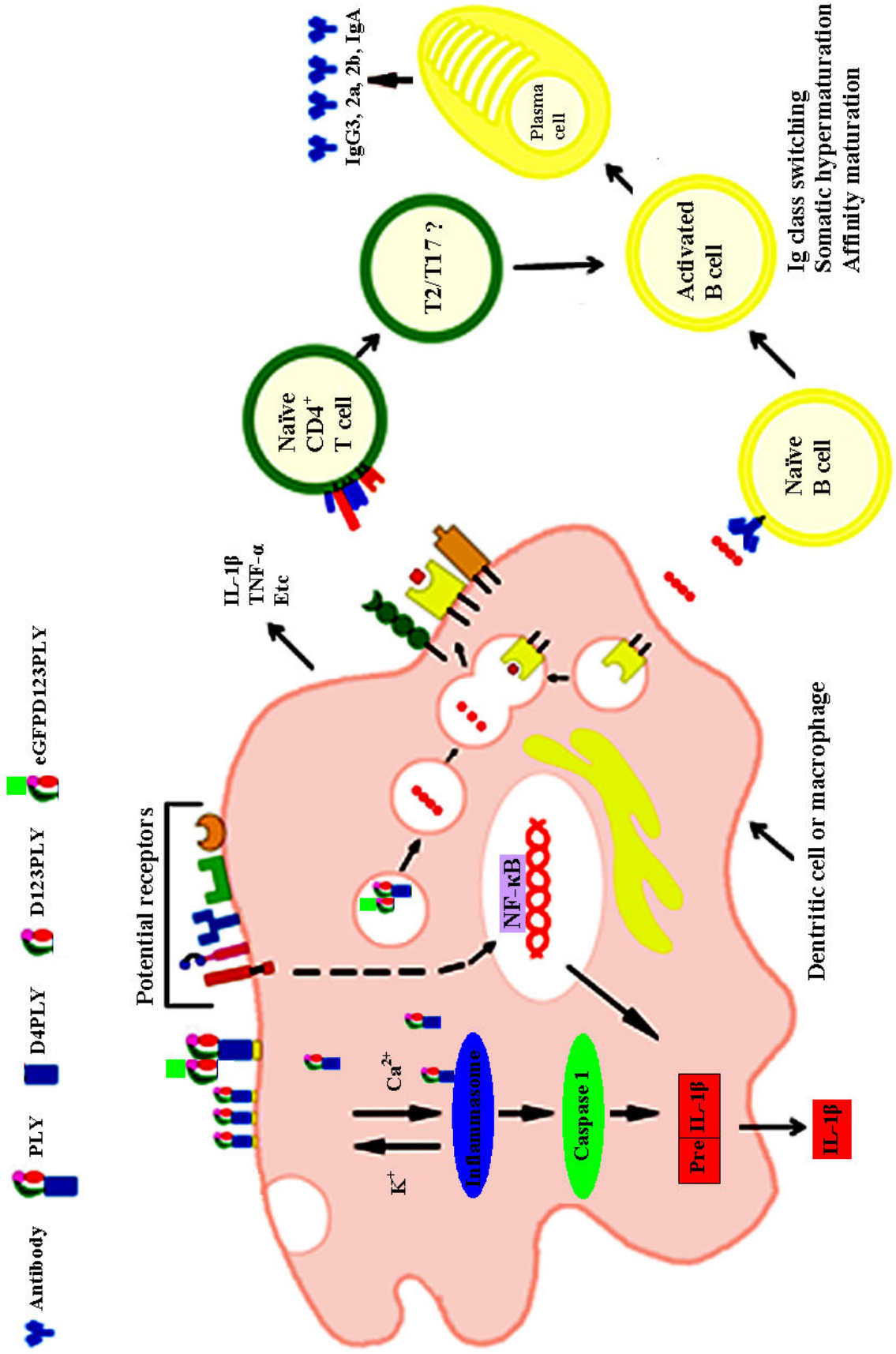


Figure 7-1 Immune responses to PLY and its derivatives.

eGFP + D4PLY, eGFP + D123PLY, eGFPD4PLY + D123PLY, eGFPD123PLY + D4PLY, eGFPD4PLY alone and eGFPD123PLY alone failed to stimulate mice to generate specific antibody responses, because DCs that have taken up those proteins could not provide a second signal besides the cognate signal during activation of T_H cells. Purified D123PLY and D4PLY may interact with each other on the surface of cell membrane. D123LLO and D4LLO expressed in the LLO deleted *Listeria monocytogenes* mutant reassembled into a heamolytically active form (Dubail *et al*. 2001). It was also reported that K1 (D123PLY) and K2 (D4PLY) fragments, two products of protease K treated PLY, remained closely associated as "nicked" PLY; nicked PLY is haemolytic and forms similar pores to non protease K treated PLY on the surface of liposomes under electron microscopy; a mixture of purified K1 and K2 fragments was found to be non-haemolytic suggesting that re-forming of a active form did not occur (Morgan *et al*. 1997). The microscopy and FACS data suggest D4PLY can deliver eGFP, and potentially other antigens, to the surface of cell membrane when those antigens are fused to D4PLY.

The level of anti-eGFP IgG, anti-PLY and anti-eGFP IgA in mice immunised i.n. with a mixture of eGFPD4PLY and PLY is equivalent to those in mice intranasally immunised with eGFPPLY. When a mixture eGFPD4PLY and PLY were applied intranasally together, both eGFPD4PLY and PLY were able to quickly bind to the surface of DCs at the same time. The Δ6PLY is not as effective as PLY as an adjuvant, with the result that the level of anti-eGFP IgG and anti-PLY response in mice vaccinated i.n. with a mixture of eGFPD4PLY and Δ6PLY is much lower than those in mice vaccinated i.n. with a mixture of eGFPLYD4PLY and PLY.

Detectable levels of anti-GFP and anti-PLY IgG were observed in mice immunised i.n. with a mixture of eGFPD123PLY and PLY, and the anti-eGFP IgG level is much lower than that in mice intranasally immunised with eGFPPLY or a mixture of eGFPD4PLY and PLY respectively. The hypothesis is that eGFPD123PLY may bind to cells through its interaction with PLY (Figure 7-1). Purified PLY can form dimer or trimers in solutions. PLY forms multimer rings on the surface of cholesterol rich membranes of mammalian cells. Previous work during my Master's study with NMR suggested purified D4PLY and D123PLY were folded properly and maintain the 3 D structure correctly (MA Master thesis). eGFPD123PLY and PLY may form some hetero-dimers or –trimers, which

facilitate eGFPD123PLY interactions with DCs. A small amount of eGFPD123PLY would be taken up by DCs with PLY at the same time through this weaker interaction between PLY and eGFPD123PLY. D123PLY may bind to the cell membrane itself. Dubail et al. showed Domain 1-3 of listeriolysin (D123LLO) alone expressed and secreted from a LLO deleted *Listeria monocytogenes* mutant can still bind to human epithelial cells without Domain 4 of LLO (D4LLO) (Dubail et al. 2001). However, we can not detect this interaction between D123PLY and horse blood cell with fluorescent microscopy and FACS. The His tag in the purified D123PLY may block this interaction, and horse blood cells possibly do not have the right receptors for D123PLY.

No significant anti-eGFP IgG was detected in mice intranasally immunised with eGFPD123PLY and Δ6PLY. eGFPD123PLY should not interact with ∆6PLY as Δ6PLY was created through deletion of 146A and 147R in the pore forming site. Pores were readily visualised by Transmission Electron Microscopy of negatively stained erythrocyte membranes treated with WT PLY, but arcs and rod-like structures were observed on ∆6PLY treated membranes (Kirkham et al. 2006c).

When eGFPD4PLY and PLY bind to cholesterol rich membrane of DCs, eGFPD4PLY may also possibly interact with the PLY, forming transient hetero multimers rings on the cell membrane. In order to elicit anti-eGFP responses in the host, eGFPD4PLY does not require to be applied simultaneously with PLY or Δ6PLY. eGFPD4PLY and D123PLY, eGFPD123PLY and D4PLY may form dimer or trimers, but no second signal can be provided during APC and T cell interaction.

All fusion proteins stimulated good antigen specific IgG when applied to mice individually, but only PLY fusion proteins stimulated good antigen specific IgA responses. The results from my project demonstrated that it is essential for PLY and Δ6PLY to be genetically fused to its carried antigen, binding of the PLY and Δ6PLY to the membrane is required for adjuvanticity and the Δ6PLY is a good adjuvant but is not effective as PLY; the full length of PLY or Δ6PLY is needed for the adjuvant activity as truncated forms of PLY, D123PLY and D4PLY, have no adjuvant activity; D123PLY and D4PLY can deliver antigens to elicit an immune response in the presence of PLY or Δ6PLY. The reduction of the adjuvant property maybe due to reduced release of cytokines as Δ6PLY is not cytotoxic due to lose of pore-forming ability (Kirkham et al. 2006c). So far, no cytokines or

pro-inflammatory mediators have been detected in mammalian cells treated with Δ6PLY. Exposure of neutrophils to very low concentration of PLY (8 ng/ml) resulted in influx of Ca (2+) and significant release of matrix metalloproteinase-9 (MMP-9) and generation of eukotriene B (4) (LTB (4)). However, treatment of the cells with Δ6PLY at concentrations of up to 1000 ng/ml had only trivial effects on Ca(2+) influx and no effects on either release of MMP-9 or LTB(4) production (Cockeran *et al.* 2011). Our unpublished data shown Δ6PLY can not induce IL-ß production in mouse bone marrow derived macrophage. However, the fact of Δ6PLY is a good adjuvant suggests that Δ6PLY can provide a second signal during activation of naïve CD4+ T cells through unknown pathways besides the cognate signal. Both PLY and Δ6PLY induce CD4+ T cell responses in the child's nasopharynx (Pope et al., unpublished data). Δ6PLY may possibly activate complement pathways as the essential amino acids for this still exist in Δ6PLY (Mitchell et al. 1991).

Intranasal delivery of PspAΔ6PLY protected mice against nasopharyngeal colonisation with pneumococcus in mice, and both i.n. and s.c. vaccination with PspAPLY did not confer protection. The level of anti-PspA IgG response in mice immunised with PspAPLY is much higher than that with PspAΔ6PLY, which indicates antibody independent protection may be involved. Intranasal delivery of a mixture of PsaAΔ6PLY, PspAΔ6PLY, PspCΔ6PLY and PhtDΔ6PLY conferred better protection against nasopharyngeal colonisation with pneumococcus than using PspAΔ6PLY or PhtDΔ6PLY alone. Clearly, a synergistic effect was achieved when multiple antigens were delivered. It was reported before that immunization with PspA can cross-protect against carriage strains bearing different PspAs (Wu et al. 1997b). The antibodies against individual pht protein are cross-reactive (Hamel et al. 2004). In this study, Anti-PhtD serum is possibly cross-reactive against wild type PhtA, B and E besides PhtD from all three strains. However, use of more antigens does not definitely mean better protection. For example, Ogunniyi et al showed that protection in mice vaccinated with combination of PdB, PspA and PhtB is worse than a combination of PdB and PspA (Ogunniyi et al. 2007). Subcutaneous vaccination with PspAΔ6PLY, PspCΔ6PLY or PhtDΔ6PLY alone protected mice against nasopharyngeal carriage of pneumococcal strain Xen35 (TIGR4), but subcutaneous immunisation with a mixture of PsaAΔ6PLY, PspAΔ6PLY,

PspCΔ6PLY or PhtDΔ6PLY was unable to protect against the same strain of pneumococcus. Intranasal delivery of a mixture of PspAΔ6PLY and PhtDΔ6PLY could possibly achieve similar or even better levels of protection compared to all four Δ6PLY fusion proteins.

It has been that shown mice vaccinated with a mixture of a PLY toxoid (W433F, D385N, and C428G), PsaA and PspA using CT as an adjuvant confers protection against pneumococcal colonisation in a CD4+ TH17 cell-dependent, antibody-independent fashion (Basset et al. 2007). PspAΔ6PLY, PhtDΔ6PLY or a mixture of Δ6PLY fusion proteins may induce the similar antibody-independent protection in our system. It was found this antibody independent protection is mediated by IL-17A through recruitment and activation of the neutrophils (Malley *et al*. 2005; Lu *et al*. 2008). Block of IL-17A significantly reduced immunity against pneumococcus in nasopharynx when purified cell wall PS was intranasally delivered, cell wall PS can elicit a similar immunity to PWCV (Malley *et al*. 2006). However, the antibody-dependent protection can not be totally ruled out for playing a role against nasopharyngeal carriage of pneumococcus.

Overall, neither systemic nor mucosal antibody responses to the different fusion proteins were reliable predictors of the efficacy of the fusons as vaccines against nasal colonisation. PLY fusion proteins may inhibit proliferation of CD4+ T cell and impair antibody independent protection. Higher concentration (higher than sub-lytic level) of PLY inhibits immune responses through direct effects on immune cells such as macrophage, monocytes and DCs (Nandoskar *et al*. 1986; Maus *et al*. 2004; Littmann *et al*. 2009). Zhang et al recently showed adenoidal CD4+ $CD25^{hi}$ Tregs has a potent inhibitory effect on CD4+ TH17 cells in the persistence of pneumococcal carriage in children (Zhang *et al*. 2011). This regulatory role of $CD25^{hi}$ Tregs on CD4+ TH17 cell was also showed in tonsil of adults and elderly (Pido-Lopez *et al*. 2011). Protease treatment reduced the inhibitory effect on Whole cell antigen-induced CD4+ T cell proliferation, suggesting the pneumococcal proteins may contribute to the accumulation of adenoidal Tregs (Zhang *et al*. 2011). Anti-pneumococcal PLY Tregs were detected in tonsil and likely contributing to the suppression of PLY anti-pneumococcal CD4+ cell responses; PLY Anti-pneumococcal CD4+ T cell responses are greater in mucosal lymphoid tissue than in the blood (Pido-Lopez *et al*. 2011). Δ6PLY fusions may lose this inhibitory effect of PLY on TH17 cells

through Tregs but remain the capability to stimulate proliferation of TH17 cells. The adjuvant property of Δ6PLY may further promote IL-17 release from TH17 cells. Proliferation on AMNC from children in response to Δ6PLY was significantly higher than to PLY, and greater IL17A and IL-22 responses are detected in PBMC incubated PsaA, PspA and PspC than in AMNC which may be due an increased regulation of T-cell responses (Pope et al., unpublished data), as it have recently shown there are more Tregs in adenoids than in blood (Zhang *et al.* 2011). It will be interested to assess if the adjuvant effect of Δ6PLY promotes IL17A release in mouse MALT when Δ6PLY fusion protein is delivered i.n. alone or as a mixture.

PsaA, PspA and PspC are not immunogenic and PhtD is immunogenic. There was a high level of anti-PhtD IgG detected in mice immunised s.c. with PhtD alone. Intranasal delivery of PhtD alone did not induce anti-PhtD IgG responses in mice. A significantly higher level of anti-PhtD IgG response was detected in mice immunised i.n. with PsaA, PspA, PspC and PhtD together in comparison to when PhtD was given i.n. alone. 10 times more antigen was used when those antigens were delivered together in comparison to delivered alone. More interestingly, PhtD inhibited production of anti-PLY IgG in mice intranasally immunised with PhtDPLY through unknown mechanisms, but this did not occur in mice immunised with PhtDΔ6PLY. Both PLY and PhtD have been shown to interfere with complement pathways (Paton et al. 1984; Mitchell et al. 1991; Ogunniyi et al. 2009). The data for PhtD indicate that PhtD has additional important functions during pneumococcal infection, especially in the colonisation stage. These unexpected results need to be further carefully investigated.

In addition, we are also aware of the need to determine whether these toxins are able to interfere with the CNS via the olfactory nerve. Similar studies with LT and CT have shown that nasal application can result in potential toxicity to the CNS via binding of the toxin to bind olfactory lobes via GM1 gangliosides. Whilst it is possible for this to occur, PLY is more readily manipulated genetically than LT and CT holotoxins and therefore provides opportunities to alter the protein to maximise the adjuvant activity whilst limiting the potential for CNS involvement.

The ability of these fusion protein antigens to elicit protection against nasopharyngeal carriage in mice raises the possibility that mucosal immunity to these antigens might confer protection against carriage in humans.

Appendix I Buffers and recipes

A. Media recipes

LB broth
10g Tryptone
10g NaCl
5g Yeast Extract
In 1L of dH2O, autoclave.

LB Agar
1 Litre of LB Broth as above plus 10g Bacteriological Agar
Autoclave, store at RT.
Melt in microwave, cool immediately or keep at 60-65^{o}C in water bath, pour plate

Terrific broth (TB)
12g bacto-tryptone
24g bacto-yeast extract
4ml glycerol
Make up to 0.9L with dH_2O and autoclave

In a separate flask dissolve in 100ml dH_2O:
2.31g KH_2PO_4 monobasic
12.54g K_2HPO_4 dibasic (for trihydrate 16.45 g)

B. Nickel affinity chromatography (NAC) buffer

10 × PBS
80g NaCl
2g KCl
14.4 Na_2HPO_4
2.4 KH_2PO_4
In 1 litre dH_2O

250mM EDTA PH 7.5-8
93.6g EDTA in 1 litre dH_2O

250mM imidazole
17.02g imidazole in 1L PBS

100mM $NiSO_4$
26.28g $NiSO_4$ in 1L dH_2O

3M guanidine HCl
286.59g guanidine HCl in 1L dH_2O

C. SDS PAGE gel

RUNNING GEL (two gels)	5%	12%	10%	7.5%
dH_2O	2.35ml	3.35	4.05	4.85
1.5M Tris PH 8.8	2.5ml	2.5	2.5	2.5
10% SDS	0.1ml	0.1	0.1	0.1
30% polyacrylamide	5.0ml	4	3.3	2.5
10% ammonium persulphate	0.05ml	0.05	0.05	0.05
TEMED	0.005ml	0.005l	0.005	0.005

STACKING GEL (two gels)

dH_2O	3.21ml
0.5M Tris pH6.8	1.25ml
10% SDS	0.05ml
30% polyacrylamide	0.488ml
10% ammonium persulphate	0.025ml
TEMED	0.005ml

5 × Sample Buffer
0.6ml 0.5M Tris pH6.8
5.0ml 50% glycerol
2.0ml 10% SDS
1.0ml 1% bromophenol blue
0.9ml dH_2O

10 × Running Buffer
30g Tris
144g Glycine
10g SDS
Make up to 1L with dH_2O

D.Western Blotting

Transfer Buffer
25mM Tris Base (3.03g)
192mM Glycine (14.4g)
20% Methanol (200ml)
Make up to 1L with dH_2O
(chilled to 4°C)

Tris NaCl PH 7.4
Tris base 1.2g
NaCl 8.7g
make up to 1L with dH_2O
conc.HCl 800µl

Developer (make just before use)
Dissolve 30mg 4-chloro-1-napthol in 10ml Methanol
Add to 40ml Tris NaCl pH7.4
Add 30µl H_2O_2 (30% w/v)

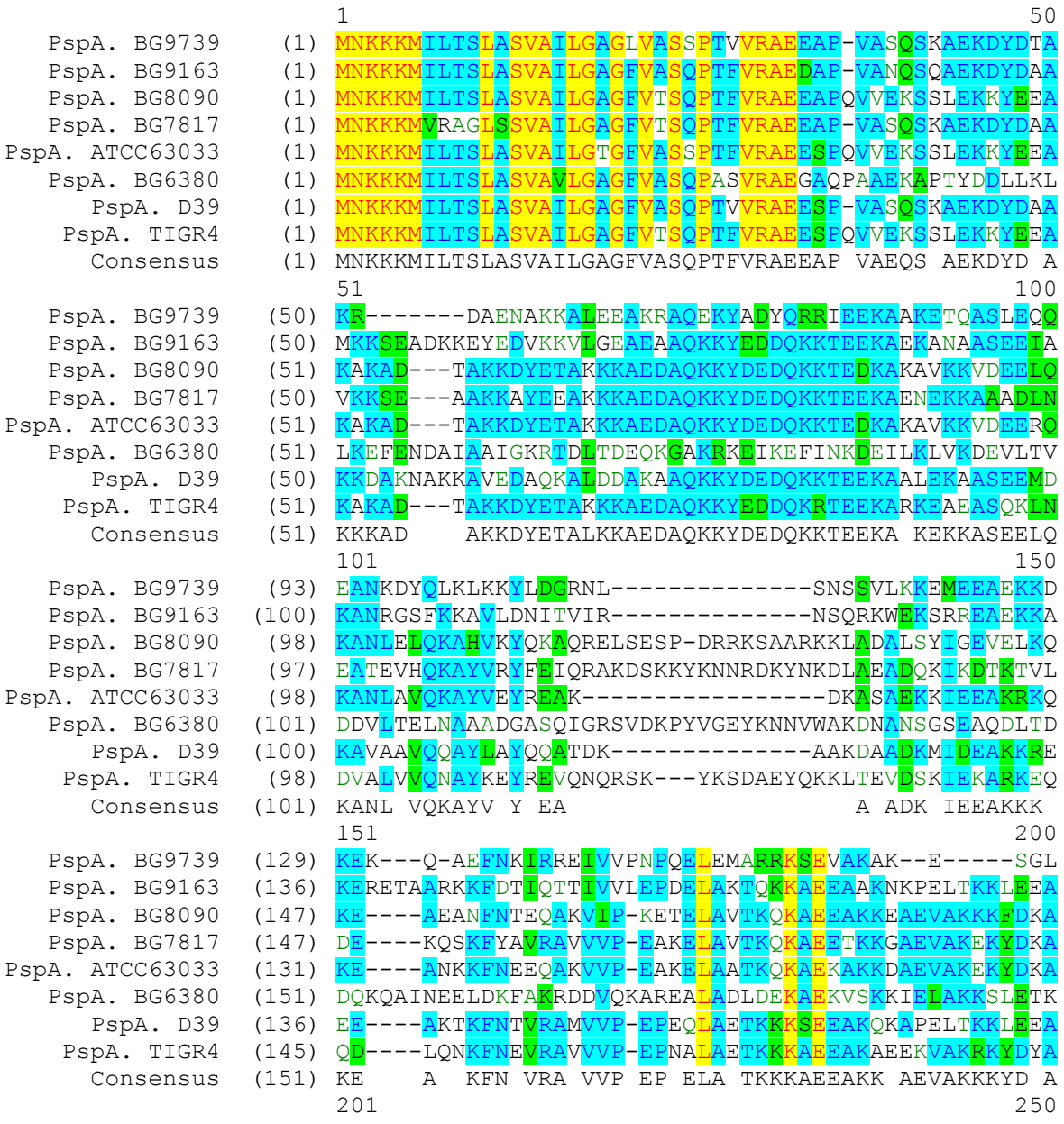
E.ELISA

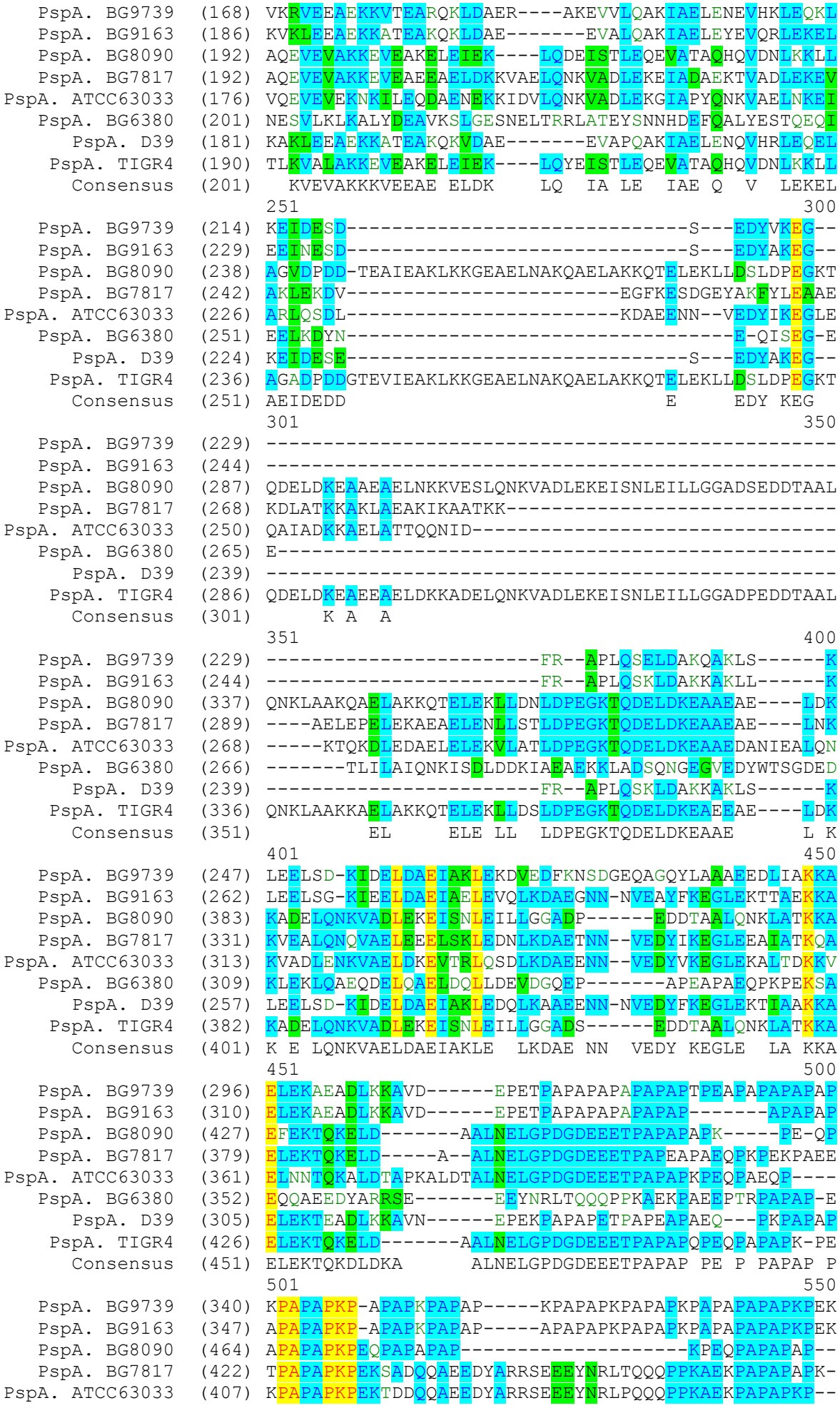
Developing solution (make just before use)
Dissolve one Fast™ o-phenylenediamine dihydrochroide tablet and buffer table in 20ml dH_2O

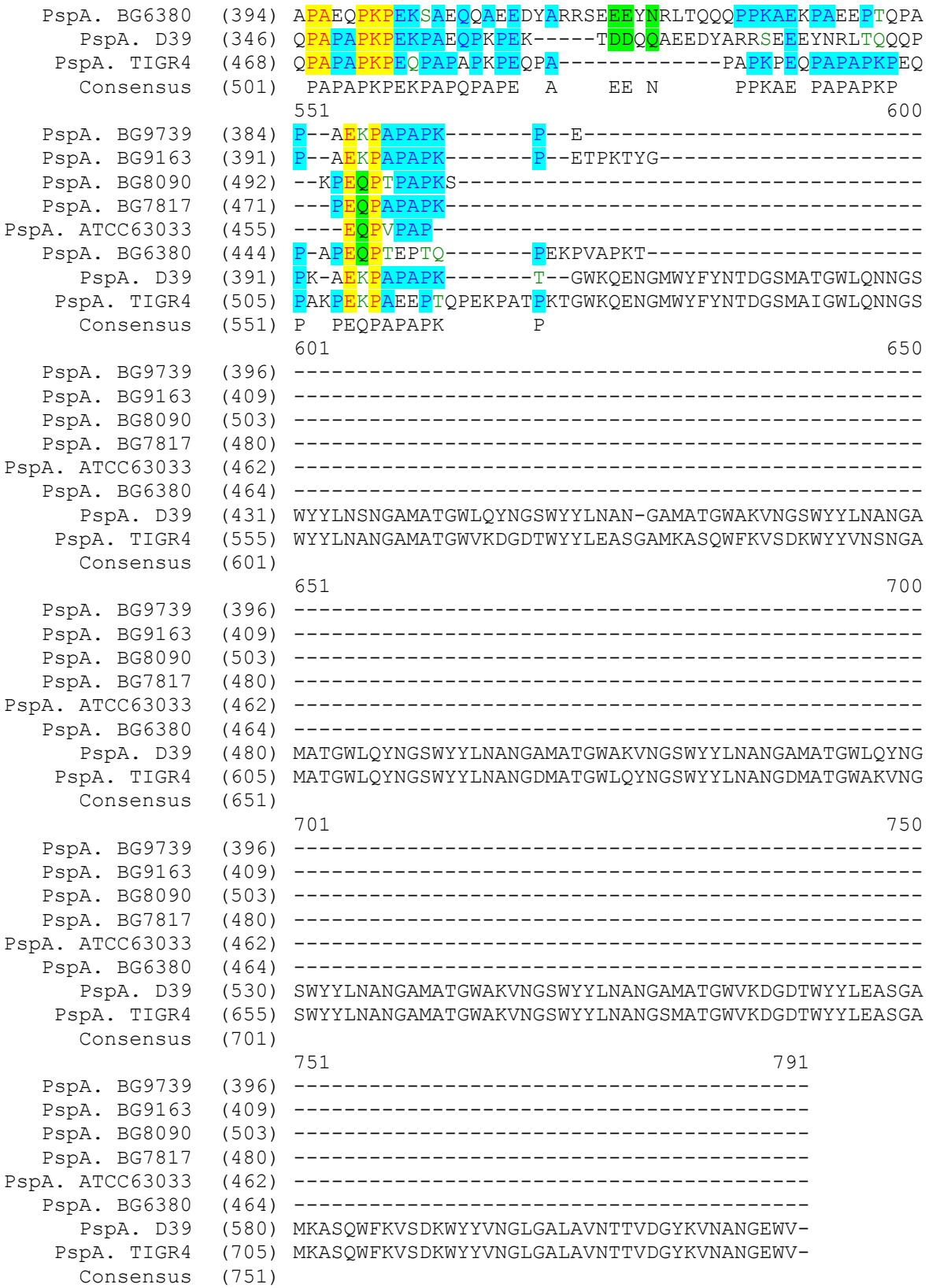
Unless otherwise stated, all reagents and chemicals are from Sigma-Adlrich, Dorset, UK.

Appendix II Alignment of PspA from different strains of pneumococcus

Strain name	Family	Clade	Capsule type	Accession no.
BG9739	1	1	4	AF071804
BG9163	1	2	6B	AF071815
BG8090	2	3	19	AF071817
BG7817	2	4	12	AF071826
ATCC63033	2	5	Not known	AF071821
BG6380	3	6	37	AF071823
D39	2	4	2	ABJ54172
TIGR4	2	3	4	AAK74303

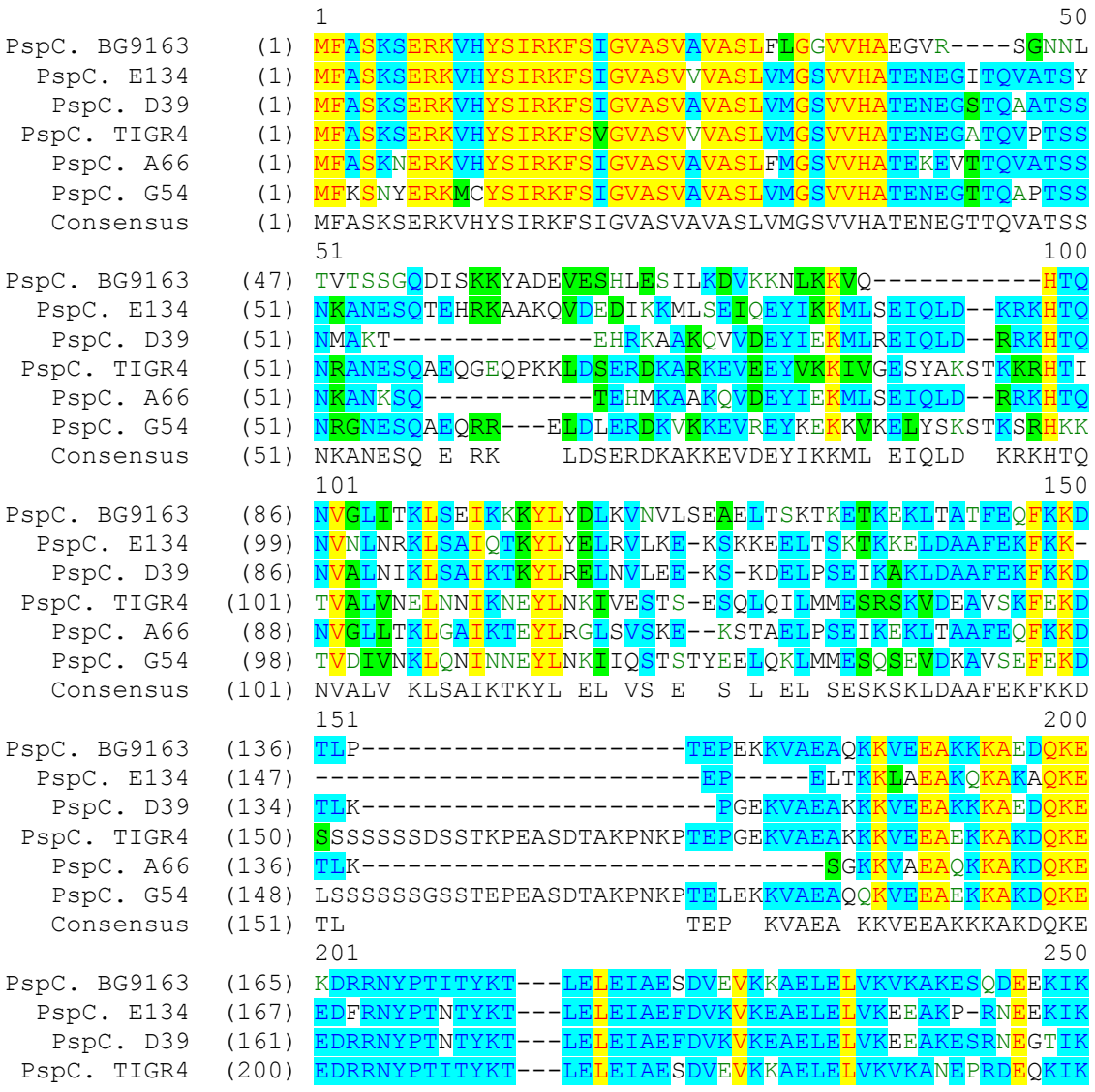



PspA. BG9739 (168) VKRVEEAEKKVTEARQKLDAER----AKEVVLQAKIAELENEVHKLEQKL
PspA. BG9163 (186) KVKLEEAEKKATEAKQKLDAE-------EVALQAKIAELEYEVQRLEKEL
PspA. BG8090 (192) AQEVEVAKKEVEAKELEIEK----LQDEISTLEQEVATAQHQVDNLKKLL
PspA. BG7817 (192) AQEVEVAKKEVEAEEAELDKKVAELQNKVADLEKEIADAEKTVADLEKEV
PspA. ATCC63033 (176) VQEVEVEKNKILEQDAENEKKIDVLQNKVADLEKGIAPYQNKVAELNKEI
PspA. BG6380 (201) NESVLKLKALYDEAVKSLGESNELTRRLATEYSNNHDEFQALYESTQEQI
PspA. D39 (181) KAKLEEAEKKATEAKQKVDAE-------EVAPQAKIAELENQVHRLEQEL
PspA. TIGR4 (190) TLKVALAKKEVEAKELEIEK----LQYEISTLEQEVATAQHQVDNLKKLL
Consensus (201) KVEVAKKKVEEAE ELDK LQ IA LE IAE Q V LEKEL
251 300
PspA. BG9739 (214) KEIDESD-------------------------------S---EDYVKEG--
PspA. BG9163 (229) EEINESD-------------------------------S---EDYAKEG--
PspA. BG8090 (238) AGVDPDD-TEAIEAKLKKGEAELNAKQAELAKKQTELEKLLDSLDPEGKT
PspA. BG7817 (242) AKLEKDV-----------------------EGFKESDGEYAKFYLEAAE
PspA. ATCC63033 (226) ARLQSDL-----------------------KDAEENN--VEDYIKEGLE
PspA. BG6380 (251) EELKDYN-----------------------------------E-QISEG-E
PspA. D39 (224) KEIDESE-------------------------------S---EDYAKEG--
PspA. TIGR4 (236) AGADPDDGTEVIEAKLKKGEAELNAKQAELAKKQTELEKLLDSLDPEGKT
Consensus (251) AEIDEDD E EDY KEG
301 350
PspA. BG9739 (229) --
PspA. BG9163 (244) --
PspA. BG8090 (287) QDELDKEAAEAELNKKVESLQNKVADLEKEISNLEILLGGADSEDDTAAL
PspA. BG7817 (268) KDLATKKAKLAEAKIKAATKK----------------------------
PspA. ATCC63033 (250) QAIADKKAELATTQQNID--------------------------------
PspA. BG6380 (265) E---
PspA. D39 (239) --
PspA. TIGR4 (286) QDELDKEAEEAELDKKADELQNKVADLEKEISNLEILLGGADPEDDTAAL
Consensus (301) K A A
351 400
PspA. BG9739 (229) ------------------------FR--APLQSELDAKQAKLS------K
PspA. BG9163 (244) ------------------------FR--APLQSKLDAKKAKLL------K
PspA. BG8090 (337) QNKLAAKQAELAKKQTELEKLLDNLDPEGKTQDELDKEAAEAE----LDK
PspA. BG7817 (289) ----AELEPELEKAEAELENLLSTLDPEGKTQDELDKEAAEAE----LNK
PspA. ATCC63033 (268) -----KTQKDLEDAELELEKVLATLDPEGKTQDELDKEAAEDANIEALQN
PspA. BG6380 (266) -------TLILAIQNKISDLDDKIAEAEKKLADSQNGEGVEDYWTSGDED
PspA. D39 (239) ------------------------FR--APLQSKLDAKKAKLS------K
PspA. TIGR4 (336) QNKLAAKKAELAKKQTELEKLLDSLDPEGKTQDELDKEAEEAE----LDK
Consensus (351) EL ELE LL LDPEGKTQDELDKEAAE L K
401 450
PspA. BG9739 (247) LEELSD-KIDELDAEIAKLEKDVEDFKNSDGEQAGQYLAAAEEDLIAKKA
PspA. BG9163 (262) LEELSG-KIEELDAEIAELEVQLKDAEGNN-NVEAYFKEGLEKTTAEKKA
PspA. BG8090 (383) KADELQNKVADLEKEISNLEILLGGADP------EDDTAALQNKLATKKA
PspA. BG7817 (331) KVEALQNQVAELEEELSKLEDNLKDAETNN--VEDYIKEGLEEAIATKQA
PspA. ATCC63033 (313) KVADLENKVAELDKEVTRLQSDLKDAEENN--VEDYVKEGLEKALTDKKV
PspA. BG6380 (309) KLEKLQAEQDELQAELDQLLDEVDGQEP-------APEAPAEQPKPEKSA
PspA. D39 (257) LEELSD-KIDELDAEIAKLEDQLKAAEENN-NVEDYFKEGLEKTIAAKKA
PspA. TIGR4 (382) KADELQNKVADLEKEISNLEILLGGADS------EDDTAALQNKLATKKA
Consensus (401) K E LQNKVAELDAEIAKLE LKDAE NN VEDY KEGLE LA KKA
451 500
PspA. BG9739 (296) ELEKAEADLKKAVD------EPETPAPAPAPAPAPAPTPEAPAPAPAPAP
PspA. BG9163 (310) ELEKAEADLKKAVD------EPETPAPAPAPAPAP-------APAPAP
PspA. BG8090 (427) EFEKTQKELD-------AALNELGPDGDEEETPAPAPAPK-----PE-QP
PspA. BG7817 (379) ELEKTQKELD-----A--ALNELGPDGDEEETPAPEAPAEQPKPEKPAEE
PspA. ATCC63033 (361) ELNNTQKALDTAPKALDTALNELGPDGDEEETPAPAPKPEQPAEQP----
PspA. BG6380 (352) EQQAEEDYARRSE-------EEYNRLTQQQPPKAEKPAEEPTRPAPAP-E
PspA. D39 (305) ELEKTEADLKKAVN------EPEKPAPAPETPAPEAPAEQ---PKPAPAP
PspA. TIGR4 (426) ELEKTQKELD-------AALNELGPDGDEEETPAPAPQPEQPAPAPK-PE
Consensus (451) ELEKTQKDLDKA ALNELGPDGDEEETPAPAP PE P PAPAP P
501 550
PspA. BG9739 (340) KPAPAPKP-APAPKPAPAP-----KPAPAPKPAPAPKPAPAPAPKPEK
PspA. BG9163 (347) APAPAPKP-APAPKPAPAP-----APAPAPKPAPAPKPAPAPAPKPEK
PspA. BG8090 (464) APAPAPKPEQPAPAPAP--------------------KPEQPAPAPAP--
PspA. BG7817 (422) TPAPAPKPEKSADQQAEEDYARRSEEEYNRLTQQQPPKAEKPAPAPAPK-
PspA. ATCC63033 (407) KPAPAPKPEKTDDQQAEEDYARRSEEEYNRLPQQQPPKAEKPAPAPKP--

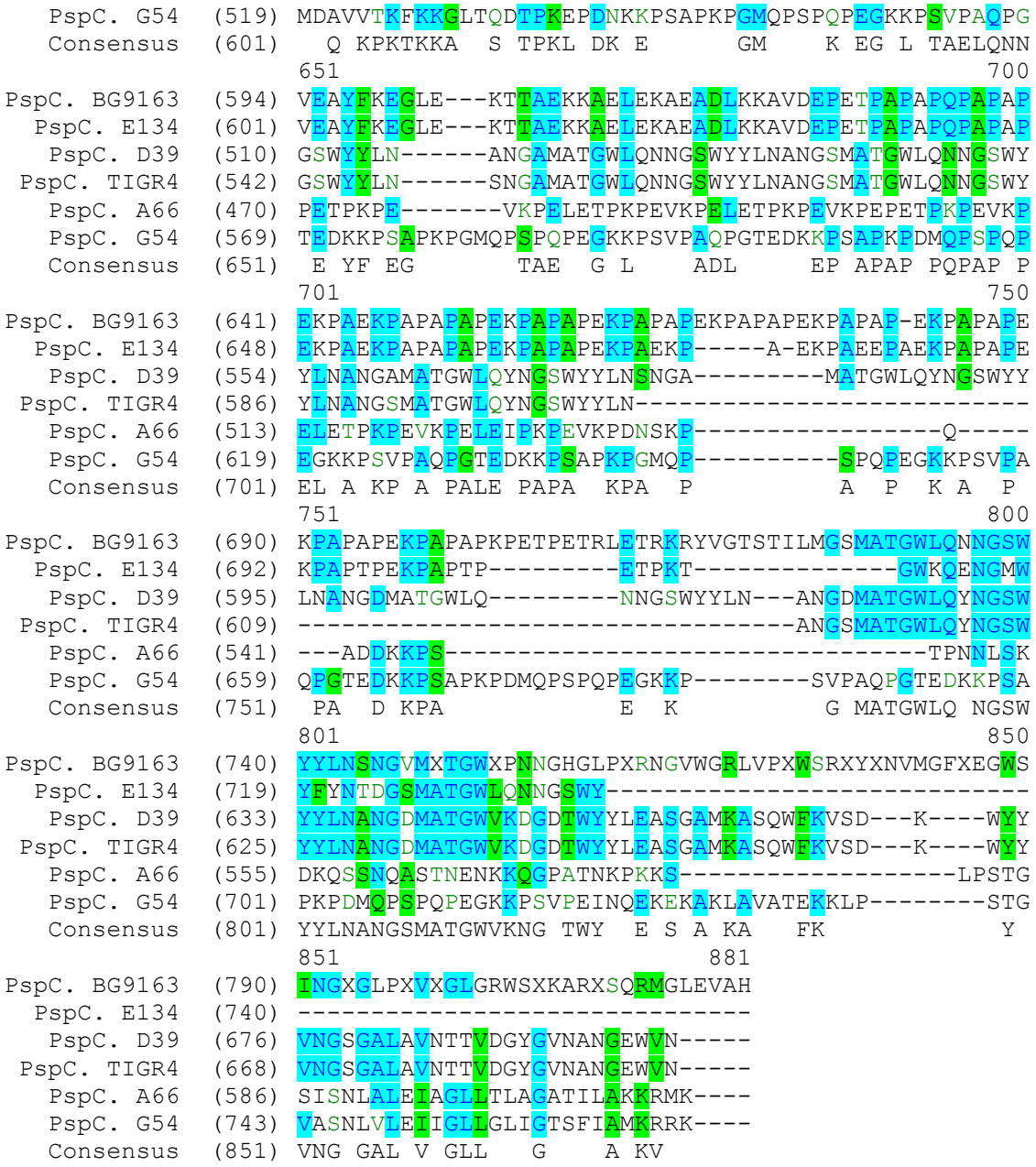

PspA. BG6380 (394) APAEQPKPEKSAEQQAEEDYARRSEEEYNRLTQQQPPKAEKPAEEPTQPA
PspA. D39 (346) QPAPAPKPEKPAEQPKPEK-----TDDQQAEEDYARRSEEEYNRLTQQQP
PspA. TIGR4 (468) QPAPAPKPEQPAPAPKPEQPA----------PAPKPEQPAPAPKPEQ
Consensus (501) PAPAPKPEKPAPQPAPE A EE N PPKAE PAPAPKP
551 600
PspA. BG9739 (384) P--AEKPAPAPK-------P--E----------
PspA. BG9163 (391) P--AEKPAPAPK-------P--ETPKTYG----------
PspA. BG8090 (492) --KPEQPTPAPKS----------
PspA. BG7817 (471) ---PEQPAPAPK----------
PspA. ATCC63033 (455) ----EQPVPAP----------
PspA. BG6380 (444) P-APEQPTEPTQ-------PEKPVAPKT----------
PspA. D39 (391) PK-AEKPAPAPK-------T--GWKQENGMWYFYNTDGSMATGWLQNNGS
PspA. TIGR4 (505) PAKPEKPAEEPTQPEKPATPKTGWKQENGMWYFYNTDGSMAIGWLQNNGS
Consensus (551) P PEQPAPAPK P
601 650
PspA. BG9739 (396) ----------
PspA. BG9163 (409) ----------
PspA. BG8090 (503) ----------
PspA. BG7817 (480) ----------
PspA. ATCC63033 (462) ----------
PspA. BG6380 (464) ----------
PspA. D39 (431) WYYLNSNGAMATGWLQYNGSWYYLNAN-GAMATGWAKVNGSWYYLNANGA
PspA. TIGR4 (555) WYYLNANGAMATGWVKDGDTWYYLEASGAMKASQWFKVSDKWYYVNSNGA
Consensus (601)
651 700
PspA. BG9739 (396) ----------
PspA. BG9163 (409) ----------
PspA. BG8090 (503) ----------
PspA. BG7817 (480) ----------
PspA. ATCC63033 (462) ----------
PspA. BG6380 (464) ----------
PspA. D39 (480) MATGWLQYNGSWYYLNANGAMATGWAKVNGSWYYLNANGAMATGWLQYNG
PspA. TIGR4 (605) MATGWLQYNGSWYYLNANGDMATGWLQYNGSWYYLNANGDMATGWAKVNG
Consensus (651)
701 750
PspA. BG9739 (396) ----------
PspA. BG9163 (409) ----------
PspA. BG8090 (503) ----------
PspA. BG7817 (480) ----------
PspA. ATCC63033 (462) ----------
PspA. BG6380 (464) ----------
PspA. D39 (530) SWYYLNANGAMATGWAKVNGSWYYLNANGAMATGWVKDGDTWYYLEASGA
PspA. TIGR4 (655) SWYYLNANGAMATGWAKVNGSWYYLNANGSMATGWVKDGDTWYYLEASGA
Consensus (701)
751 791
PspA. BG9739 (396) ----------
PspA. BG9163 (409) ----------
PspA. BG8090 (503) ----------
PspA. BG7817 (480) ----------
PspA. ATCC63033 (462) ----------
PspA. BG6380 (464) ----------
PspA. D39 (580) MKASQWFKVSDKWYYVNGLGALAVNTTVDGYKVNANGEWV-
PspA. TIGR4 (705) MKASQWFKVSDKWYYVNGLGALAVNTTVDGYKVNANGEWV-
Consensus (751)

Appendix III Alignment of PspC from different strains of pneumococcus

Strain name	Family	Clades	Capsule type	Accession no.
BG9163	choline-binding	A	4	AF068650
E134	choline-binding	A	6B	AF068647
D39	choline-binding	B	2	AF068646
TIGR4	choline-binding	B	4	AE007507
A66	peptidoglycan-linked		3	AF252857
G54	peptidoglycan-linked		19F	AF154034




PspC. A66 (156) AKQEIEALIVKHKGREIDLDRKKAKAAVTEHLKKLLNDIEKNLKKEQHTH
PspC. G54 (198) EDYRNYPTITYKT---LELEIAEFDVKVKEAELELVKVKAKESRDEKKIK
Consensus (201) EDRRNYPTITYKT LELEIAEFDVKVKEAELELVKVKAKESRDE KIK
251 300
PspC. BG9163 (212) QAEAEVESKQAEATRLKKIKTDR----EEAKRKADAKLKEAVEKN--VAT
PspC. E134 (213) QAKAKVESKKAEATRLEEIKTERKKAEEEAKRKAEESEKKAAEAKQKVDT
PspC. D39 (208) QAKEKVESKKAEATRLENIKTDRKKAEEEAKRKADAKLKEANVAT-----
PspC. TIGR4 (247) QAEAEVESKQAEATRLKKIKTDREEAEEEAKRRADAK----------
PspC. A66 (206) TVELIKNLKDIEKTYLHKLDESTQKAQLQKLIAESQS----------
PspC. G54 (245) QAEAEVESKQAEATRLKKIKTDRKKAEEEAKLKEAVEKNAATS-------
Consensus (251) QAEAEVESKQAEATRLKKIKTDRKKAEEEAKRKADAK K A
301 350
PspC. BG9163 (256) SEQDKPKRRAKRGVSGELATPDKKENDAKSSDSSVGEETLPSPSLNMANE
PspC. E134 (263) KEQGKPKRRAKRGVSGELATPDKKENDAKSSDSSVGEETLPSPSLNMANE
PspC. D39 (253) SDQGKPKGRAKRGVPGELATPDKKENDAKSSDSSVGEETLPSS-------
PspC. TIGR4 (284) -EQGKPKGRAKRGVPGELATPDKKENDAKSSDSSVGEETLPSP-------
PspC. A66 (243) -KLDEAFSKFKNGLS----SSS---NSGSSTKPETPQPETPKP-------
PspC. G54 (288) -EQGKPKRRVKRRALGEQATPDKKDYFEKDFRPAFNKNRQMVAIQESLNK
Consensus (301) EQGKPKRRAKRGVSGELATPDKKENDAKSSDSSVGEETLPSP N
351 400
PspC. BG9163 (306) SQTEHRKDVDEYIKKMLSEIQLDGRKHTPNVNLNIKLSAIKTKYLYELSV
PspC. E134 (313) SQTEHRKDVDEYIKKMLSEIQLDRRKHTQNVNLNIKLSAIKTKYLYELSV
PspC. D39 (296) --
PspC. TIGR4 (326) --
PspC. A66 (278) --
PspC. G54 (337) LDGETKTVPDG--
Consensus (351) E R D
401 450
PspC. BG9163 (356) LKENSKKEELTSKTKAELTAAFEQFKKDTLKPEKKVAEAEKKVEEAKKKA
PspC. E134 (363) LKENSKKEELTSKTKAELTAAFEQFKKDTLKPEKKVAEAEKKVEEAKKKA
PspC. D39 (296) ----------------------------SLKSGKKVAEAEKKVEEAEKKA
PspC. TIGR4 (326) ----------------------------SLKPEKKVAEAEKKVEEAKKKA
PspC. A66 (278) ----------------------------EVKPELETPKPEVKPEPETPKP
PspC. G54 (348) ----------------------------AKLTGEAGNAYNEVRDYAIKVVS
Consensus (401) SLKPEKKVAEAEKKVEEAKKKA
451 500
PspC. BG9163 (406) KDQKEEDRRNYPTNTYKTLELEIAESDVKVKEAELELVKEEANESRNEEK
PspC. E134 (413) KDQKEEDRRNYPTNTYKTLELEIAESDVKVKKAELELVKEEANESRNEEK
PspC. D39 (318) KDQKEEDRRNYPTNTYKTLDLEIAESDVKVKEAELELVKEEAKEPRDEEK
PspC. TIGR4 (348) EDQKEEDRRNYPTNTYKTLELEIAESDVEVKKAELELVKEEAKEPRNEEK
PspC. A66 (300) EVKPE------PETPKPEVKPELETPKPEVKPEPETPKPEVKPEPETPK-
PspC. G54 (371) ENKKLLSQTAVTMDELAMQLTKLNDAMSKLREAKAKLVPEVKPQPENPEH
Consensus (451) KDQKEEDRRNYPTNTYKTLELEIAESDVKVKEAELELVKEEA EPRNEEK
501 550
PspC. BG9163 (456) IKQAKEKVESKKAEATRLEKIKTDRKKAEEEAKRKAEESEKK---AAEAK
PspC. E134 (463) IKQAKEKVESKKAEATRLEKIKTDRKKAEEEAKRKAEESEKK---AAEAK
PspC. D39 (368) IKQAKAKVESKKAEATRLENIKTDRKKAEEEAKRKAAEEDKVKEKPAEQP
PspC. TIGR4 (398) VKQAKAEVESKKAEATRLEKIKTDRKKAEEEAKRKAAEEDKVKEKPAEQP
PspC. A66 (343) -PEVKPEPETPKPEVKPELETPKPEVKPELETPKPEVKPEPE--------
PspC. G54 (421) QRPTTPAPDTKPIPQPEGKKPSVPDINQEKEKAKLAVATYMS--KILDDI
Consensus (501) IKQAK KVESKKAEATRLEKIKTDRKKAEEEAKRKA ESEK K AE
551 600
PspC. BG9163 (503) QKVDAEEYALEAKIAELEYEVQRLEKELKEIDESDSEDYLKEG----LRA
PspC. E134 (510) QKVDAEEYALEAKIAELEYEVQRLEKELKEIDESDSEDYLKEG----LRA
PspC. D39 (418) QPAPATQPEK--PAPKPEKPAE-QPKAEKTDDQQAEEDYARRSEEEYNRL
PspC. TIGR4 (448) QPAPAPKAEKPAPAPKPENPAE-QPKAEKPADQQAEEDYARRSEEEYNRL
PspC. A66 (384) TPKPEVKPELETPKPEVKPEPE-TPKPEVKPELETPKPEVKPE------P
PspC. G54 (469) QKHHLQKEKHRQIVALIKELDEFKKQALSEIDNVNTKVEIENTVHKIFAD
Consensus (551) QP PA K ELEAPIPELE E E PKALKEIDQ SEDYLK S R
601 650
PspC. BG9163 (549) PLQSKLDTKKAKLSKLEELSDKIDE-----LDAEIAKLEVQLKDAEGNNN
PspC. E134 (556) PLQSKLDTKKAKLSKLEELSDKIDE-----LDAEIAKLEVQLKDAEGNNN
PspC. D39 (465) TQQQPPKTEKPAQPSTPKTGWKQEN-----GMWYFYNTDGSMATGWLQNN
PspC. TIGR4 (497) TQQQPPKTEKPAQPSTPKTGWKQEN-----GMWYFYNTDGSMATGWLQNN
PspC. A66 (427) E-TPKPEVKPELETPKPEVKPEPETP------KPEVKPEPETPKPEVKPE


PspC. G54 (519) MDAVVTKFKKGLTQDTPKEPDNKKPSAPKPGMQPSPQPEGKKPSVPAQPG
Consensus (601) Q KPKTKKA S TPKL DK E GM K EG L TAELQNN
651 700
PspC. BG9163 (594) VEAYFKEGLE---KTTAEKKAELEKAEADLKKAVDEPETPAPAPQPAPAP
PspC. E134 (601) VEAYFKEGLE---KTTAEKKAELEKAEADLKKAVDEPETPAPAPQPAPAP
PspC. D39 (510) GSWYYLN------ANGAMATGWLQNNGSWYYLNANGSMATGWLQNNGSWY
PspC. TIGR4 (542) GSWYYLN------SNGAMATGWLQNNGSWYYLNANGSMATGWLQNNGSWY
PspC. A66 (470) PETPKPE-------VKPELETPKPEVKPELETPKPEVKPEPETPKPEVKP
PspC. G54 (569) TEDKKPSAPKPGMQPSPQPEGKKPSVPAQPGTEDKKPSAPKPDMQPSPQP
Consensus (651) E YF EG TAE G L ADL EP APAP PQPAP P
701 750
PspC. BG9163 (641) EKPAEKPAPAPAPEKPAPAPEKPAPAPEKPAPAPEKPAPAP-EKPAPAPE
PspC. E134 (648) EKPAEKPAPAPAPEKPAPAPEKPAEKP-----A-EKPAEEPAEKPAPAPE
PspC. D39 (554) YLNANGAMATGWLQYNGSWYYLNSNGA---------MATGWLQYNGSWYY
PspC. TIGR4 (586) YLNANGSMATGWLQYNGSWYYLN--------------------------
PspC. A66 (513) ELETPKPEVKPELEIPKPEVKPDNSKP-----------------Q-----
PspC. G54 (619) EGKKPSVPAQPGTEDKKPSAPKPGMQP----------SPQPEGKKPSVPA
Consensus (701) EL A KP A PALE PAPA KPA P A P K A P
751 800
PspC. BG9163 (690) KPAPAPEKPAPAPKPETPETRLETRKRYVGTSTILMGSMATGWLQNNGSW
PspC. E134 (692) KPAPTPEKPAPTP---------ETPKT--------------GWKQENGMW
PspC. D39 (595) LNANGDMATGWLQ---------NNGSWYYLN---ANGDMATGWLQYNGSW
PspC. TIGR4 (609) ----------------------------------ANGSMATGWLQYNGSW
PspC. A66 (541) ---ADDKKPS--------------------------------TPNNLSK
PspC. G54 (659) QPGTEDKKPSAPKPDMQPSPQPEGKKP--------SVPAQPGTEDKKPSA
Consensus (751) PA D KPA E K G MATGWLQ NGSW
801 850
PspC. BG9163 (740) YYLNSNGVMXTGWXPNNGHGLPXRNGVWGRLVPXWSRXYXNVMGFXEGWS
PspC. E134 (719) YFYNTDGSMATGWLQNNGSWY----------------------------
PspC. D39 (633) YYLNANGDMATGWVKDGDTWYYLEASGAMKASQWFKVSD---K----WYY
PspC. TIGR4 (625) YYLNANGDMATGWVKDGDTWYYLEASGAMKASQWFKVSD---K----WYY
PspC. A66 (555) DKQSSNQASTNENKKQGPATNKPKKS--------------------LPSTG
PspC. G54 (701) PKPDMQPSPQPEGKKPSVPEINQEKEKAKLAVATEKKLP--------STG
Consensus (801) YYLNANGSMATGWVKNG TWY E S A KA FK Y
851 881
PspC. BG9163 (790) INGXGLPXVXGLGRWSXKARXSQRMGLEVAH
PspC. E134 (740) --------------------------------
PspC. D39 (676) VNGSGALAVNTTVDGYGVNANGEWVN-----
PspC. TIGR4 (668) VNGSGALAVNTTVDGYGVNANGEWVN-----
PspC. A66 (586) SISNLALEIAGLLTLAGATILAKKRMK----
PspC. G54 (743) VASNLVLEIIGLLGLIGTSFIAMKRRK----
Consensus (851) VNG GAL V GLL G A KV

Publications

Hupp S., Wippel C., Fortsch C., Ma J., Mitchell T J & Iliev A. 2011. Astrocytic tissue remodeling by the meningitis neurotoxin pneumolysin facilitates pathogen tissue. GLIA (Accepted)

Douce,G., Ross,K., Cowan,G., Ma,J. & Mitchell,T.J. 2010. Novel mucosal vaccines generated by genetic conjugation of heterologous proteins to pneumolysin (PLY) from Streptococcus pneumoniae. *Vaccine*, **28,** 3231-3237.

Förtsch,C., Hupp,S., Ma,J., Mitchell,T.J., Maier,E., Benz,R. & Iliev,A. 2011. The astrocytic shape changes by the cholesterol-dependent cytolysin pneumolysin in non-lytic conditions still require pore-forming capacity. *Toxins*, **3,** 43-62.

Lebon,A., Verkaik,N.J., Labout,J.A., de Vogel,C.P., Hooijkaas,H., Verbrugh,H.A., van Wamel,W.J., Jaddoe,V.W., Hofman,A., Hermans,P.W., Ma,J., Mitchell,T.J., Moll,H.A. & van Belkum,A. 2011. Natural antibodies against several pneumococcal virulence proteins in children during the pre-pneumococcal-vaccine era: the generation R study. *Infect.Immun.*, **79,** 1680-1687.

Wippel,C., Fortsch,C., Hupp,S., Maier,E., Benz,R., Ma,J., Mitchell,T.J. & Iliev,A.I. 2011. Extracellular calcium reduction strongly increases the lytic capacity of pneumolysin from streptococcus pneumoniae in brain tissue. *J Infect.Dis.*, **204,** 930-936.

Witzenrath,M., Pache,F., Lorenz,D., Koppe,U., Gutbier,B., Tabeling,C., Reppe,K., Meixenberger,K., Dorhoi,A., Ma,J., Holmes,A., Trendelenburg,G., Heimesaat,M.M., Bereswill,S., van der,L.M., Tschopp,J., Mitchell,T.J., Suttorp,N. & Opitz,B. 2011. The NLRP3 inflammasome is differentially activated by pneumolysin variants and contributes to host defense in pneumococcal pneumonia. *J Immunol.*, **187,** 434-440.

Conference contributions **(Presenting author is underlined)**

Ma JT, Ross KS, Ritchie R, Douce G, Mitchell TJ (2011, presentation). "A fusion protein based vaccine offers protection against pneumococcus in murine model of colonisation." Europneumo, Amsterdam, Holland.

Ma JT, Ross KS, Ritchie R, Douce G, Mitchell TJ (2010, Poster). "A fusion protein based mucosal pneumococcal vaccine" World Pneumonia Day: perspectives on pneumonia and pneumococcal disease. Glasgow, UK.

Ross KS, Ma JT, Ritchie R, Douce G, Mitchell TJ (2010, Poster). "Pneumolysin fusion proteins as novel vaccine candidates against experimental pneumococcal

diseases" World Pneumonia Day: perspectives on pneumonia and pneumococcal disease. Glasgow, UK.

Ma JT, Ross KS, Ritchie R, Douce G, Mitchell TJ (2010, Presentation). "Immunogenic and adjuvant prosperities of pneumolysin from Streptococcus pneumoniae." University of Glasgow Interdepartmental Conference, Glasgow, UK.

Ma JT, Ross KS, Ritchie R, Douce G, Mitchell TJ (2010, Poster). "A fusion protein based pneumococcal vaccine." SGM, Nottingham, UK.

Ross KS, Douce G, Ma JT, Wale R, Mitchell (2008, poster). "Novel adjuvant properties of pneumolysin, a toxin produced by *Streptococcus pneumoniae*." 6th International Symposium on Pneumococci & Pneumococcal Diseases, Reykjavik, Iceland.

Ma JT, Ross KS, Ritchie R, McInally C-A, Douce G, Mitchell TJ (2009, poster). "Adjuvant properties of cholesterol dependent cytolysins." Europneumo, Bern, Switzerland.

Ma JT, Ross KS, Ritchie R, Douce G, Mitchell TJ (2009, poster). "New generation of pneumococcal vaccine." University of Glasgow Internal presentations, Glasgow, UK.

List of References

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. & Johnson,S. 2001. Identification and characterization of a novel family of pneumococcal proteins that are protective against sepsis. *Infect.Immun.*, **69,** 949-958.

Ahmad,H.& Chapnick,E.K. 1999. Conjugated polysaccharide vaccines. *Infect.Dis.Clin.North Am.*, **13,** 113-33, vii.

Akira,S., Uematsu,S. & Takeuchi,O. 2006. Pathogen recognition and innate immunity. *Cell*, **124,** 783-801.

Alexander,J.E., Berry,A.M., Paton,J.C., Rubins,J.B., Andrew,P.W. & Mitchell,T.J. 1998. Amino acid changes affecting the activity of pneumolysin alter the behaviour of pneumococci in pneumonia. *Microb.Pathog.*, **24,** 167-174.

Alexander,J.E., Lock,R.A., Peeters,C.C., Poolman,J.T., Andrew,P.W., Mitchell,T.J., Hansman,D. & 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,** 5683-5688.

Amdahl,B.M., Rubins,J.B., Daley,C.L., Gilks,C.F., Hopewell,P.C. & 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,** 2000-2004.

Anderton,J.M., Rajam,G., Romero-Steiner,S., Summer,S., Kowalczyk,A.P., Carlone,G.M., Sampson,J.S. & Ades,E.W. 2007. E-cadherin is a receptor for the common protein pneumococcal surface adhesin A (PsaA) of Streptococcus pneumoniae. *Microb.Pathog.*, **42,** 225-236.

Arulanandam,B.P., Lynch,J.M., Briles,D.E., Hollingshead,S. & Metzger,D.W. 2001. Intranasal vaccination with pneumococcal surface protein A and interleukin-12 augments antibody-mediated opsonization and protective immunity against Streptococcus pneumoniae infection. *Infect.Immun.*, **69,** 6718-6724.

Austrian,R. 1999. The pneumococcus at the millennium: not down, not out. *J.Infect.Dis.*, **179 Suppl 2,** S338-S341.

Avery,O.T.& Goebel,F.W. 1931. Chemo-immunological studies on conjugated carbohydrate-proteins. V. The immunological specificity of an antigen prepared by combining the capsular polysacchride of type III pneumococcus with foreign protein. *J.Exp.Med*, **54,** 419-426.

Baba,H., Kawamura,I., Kohda,C., Nomura,T., Ito,Y., Kimoto,T., Watanabe,I., Ichiyama,S. & Mitsuyama,M. 2002. Induction of gamma interferon and nitric oxide by truncated pneumolysin that lacks pore-forming activity. *Infect.Immun.*, **70,** 107-113.

Bachmann,M.F., Zinkernagel,R.M. & Oxenius,A. 1998. Immune responses in the absence of costimulation: viruses know the trick. *J Immunol.*, **161,** 5791-5794.

Balachandran,P., Brooks-Walter,A., Virolainen-Julkunen,A., Hollingshead,S.K. & Briles,D.E. 2002. Role of pneumococcal surface protein C in nasopharyngeal carriage and pneumonia and its ability to elicit protection against carriage of Streptococcus pneumoniae. *Infect.Immun.*, **70,** 2526-2534.

Balachandran,P., Hollingshead,S.K., Paton,J.C. & Briles,D.E. 2001. The autolytic enzyme LytA of Streptococcus pneumoniae is not responsible for releasing pneumolysin. *J.Bacteriol.*, **183,** 3108-3116.

Banerjee,A., van Sorge,N.M., Sheen,T.R., Uchiyama,S., Mitchell,T.J. & Doran,K.S. 2010. Activation of brain endothelium by pneumococcal neuraminidase NanA promotes bacterial internalization. *Cell Microbiol.*.

Baril,L., Dietemann,J., Essevaz-Roulet,M., Beniguel,L., Coan,P., Briles,D.E., Guy,B. & Cozon,G. 2006. Pneumococcal surface protein A (PspA) is effective at eliciting T cell-mediated responses during invasive pneumococcal disease in adults. *Clin.Exp.Immunol.*, **145,** 277-286.

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. & Henriques-Normark,B. 2006. A pneumococcal pilus influences virulence and host inflammatory responses. *Proc.Natl.Acad.Sci.U.S.A*, **103,** 2857-2862.

Barrett,D.J. 1985. Human immune responses to polysaccharide antigens: an analysis of bacterial polysaccharide vaccines in infants. *Adv.Pediatr.*, **32,** 139-158.

Basset,A., Thompson,C.M., Hollingshead,S.K., Briles,D.E., Ades,E.W., Lipsitch,M. & Malley,R. 2007. Antibody-independent, CD4+ T-cell-dependent protection against pneumococcal colonization elicited by intranasal immunization with purified pneumococcal proteins. *Infect.Immun.*, **75,** 5460-5464.

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

Baumann,U. 2008. Mucosal vaccination against bacterial respiratory infections. *Expert Rev.Vaccines*, **7,** 1257-1276.

Beall,B., McEllistrem,M.C., Gertz,R.E., Jr., Wedel,S., Boxrud,D.J., Gonzalez,A.L., Medina,M.J., Pai,R., Thompson,T.A., Harrison,L.H., McGee,L. & Whitney,C.G. 2006. Pre- and postvaccination clonal compositions of invasive pneumococcal serotypes for isolates collected in the United States in 1999, 2001, and 2002. *J.Clin.Microbiol.*, **44,** 999-1017.

Bender,M.H.& Weiser,J.N. 2006. The atypical amino-terminal LPNTG-containing domain of the pneumococcal human IgA1-specific protease is required for proper enzyme localization and function. *Mol.Microbiol.*, **61,** 526-543.

Benninger,M.S. 2008. Acute bacterial rhinosinusitis and otitis media: changes in pathogenicity following widespread use of pneumococcal conjugate vaccine. *Otolaryngol.Head Neck Surg.*, **138,** 274-278.

Bentley,S.D., Aanensen,D.M., Mavroidi,A., Saunders,D., Rabbinowitsch,E., Collins,M., Donohoe,K., Harris,D., Murphy,L., Quail,M.A., Samuel,G., Skovsted,I.C., Kaltoft,M.S., Barrell,B., Reeves,P.R., Parkhill,J. & Spratt,B.G. 2006. Genetic analysis of the capsular biosynthetic locus from all 90 pneumococcal serotypes. *PLoS Genet.*, **2,** e31.

Benton,K.A., Everson,M.P. & Briles,D.E. 1995. A pneumolysin-negative mutant of Streptococcus pneumoniae causes chronic bacteremia rather than acute sepsis in mice. *Infect.Immun.*, **63,** 448-455.

Bergmann,S.& Hammerschmidt,S. 2006. Versatility of pneumococcal surface proteins. *Microbiology*, **152,** 295-303.

Bernatoniene,J., Zhang,Q., Dogan,S., Mitchell,T.J., Paton,J.C. & Finn,A. 2008. Induction of CC and CXC chemokines in human antigen-presenting dendritic cells by the pneumococcal proteins pneumolysin and CbpA, and the role played by toll-like receptor 4, NF-kappaB, and mitogen-activated protein kinases. *J.Infect.Dis.*, **198,** 1823-1833.

Berry,A.M., Lock,R.A., Hansman,D. & Paton,J.C. 1989a. Contribution of autolysin to virulence of Streptococcus pneumoniae. *Infect.Immun.*, **57,** 2324-2330.

Berry,A.M., Lock,R.A. & Paton,J.C. 1996. Cloning and characterization of nanB, a second Streptococcus pneumoniae neuraminidase gene, and purification of the NanB enzyme from recombinant Escherichia coli. *J.Bacteriol.*, **178,** 4854-4860.

Berry,A.M., Ogunniyi,A.D., Miller,D.C. & Paton,J.C. 1999. Comparative virulence of Streptococcus pneumoniae strains with insertion-duplication, point, and deletion mutations in the pneumolysin gene. *Infect.Immun.*, **67,** 981-985.

Berry,A.M.& Paton,J.C. 1996. Sequence heterogeneity of PsaA, a 37-kilodalton putative adhesin essential for virulence of Streptococcus pneumoniae. *Infect.Immun.*, **64,** 5255-5262.

Berry,A.M., Yother,J., Briles,D.E., Hansman,D. & Paton,J.C. 1989b. Reduced virulence of a defined pneumolysin-negative mutant of Streptococcus pneumoniae. *Infect.Immun.*, **57,** 2037-2042.

Beurg,M., Hafidi,A., Skinner,L., Cowan,G., Hondarrague,Y., Mitchell,T.J. & Dulon,D. 2005. The mechanism of pneumolysin-induced cochlear hair cell death in the rat. *J.Physiol*, **568,** 211-227.

Biffl,W.L., Moore,E.E., Moore,F.A. & Barnett,C.C., Jr. 1996. Interleukin-6 delays neutrophil apoptosis via a mechanism involving platelet-activating factor. *J Trauma*, **40,** 575-578.

Billington,S.J., Jost,B.H. & Songer,J.G. 2000. Thiol-activated cytolysins: structure, function and role in pathogenesis. *FEMS Microbiol.Lett.*, **182,** 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. & 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,** 187-195.

Blander,J.M.& Medzhitov,R. 2006. Toll-dependent selection of microbial antigens for presentation by dendritic cells. *Nature*, **440,** 808-812.

Bliss,S.J., O'Brien,K.L., Janoff,E.N., Cotton,M.F., Musoke,P., Coovadia,H. & Levine,O.S. 2008. The evidence for using conjugate vaccines to protect HIV-infected children against pneumococcal disease. *Lancet Infect.Dis.*, **8,** 67-80.

Bogaert,D., de Groot,R. & Hermans,P.W. 2004a. Streptococcus pneumoniae colonisation: the key to pneumococcal disease. *Lancet Infect.Dis.*, **4,** 144-154.

Bogaert,D., Engelen,M.N., Timmers-Reker,A.J., Elzenaar,K.P., Peerbooms,P.G., Coutinho,R.A., de Groot,R. & Hermans,P.W. 2001. Pneumococcal carriage in children in The Netherlands: a molecular epidemiological study. *J.Clin.Microbiol.*, **39,** 3316-3320.

Bogaert,D., van Belkum,A., Sluijter,M., Luijendijk,A., de Groot,R., Rumke,H.C., Verbrugh,H.A. & Hermans,P.W. 2004b. Colonisation by Streptococcus pneumoniae and Staphylococcus aureus in healthy children. *Lancet*, **363,** 1871-1872.

Brandileone,M.C., Andrade,A.L., Teles,E.M., Zanella,R.C., Yara,T.I., Di Fabio,J.L. & Hollingshead,S.K. 2004. Typing of pneumococcal surface protein A (PspA) in Streptococcus pneumoniae isolated during epidemiological surveillance in Brazil: towards novel pneumococcal protein vaccines. *Vaccine*, **22,** 3890-3896.

Bratcher,P.E., Park,I.H., Hollingshead,S.K. & Nahm,M.H. 2009. Production of a unique pneumococcal capsule serotype belonging to serogroup 6. *Microbiology*, **155,** 576-583.

Braun,J.S., Hoffmann,O., Schickhaus,M., Freyer,D., Dagand,E., Bermpohl,D., Mitchell,T.J., Bechmann,I. & Weber,J.R. 2007. Pneumolysin causes neuronal cell death through mitochondrial damage. *Infect.Immun.*, **75,** 4245-4254.

Braun,J.S., Novak,R., Gao,G., Murray,P.J. & Shenep,J.L. 1999. Pneumolysin, a protein toxin of Streptococcus pneumoniae, induces nitric oxide production from macrophages. *Infect.Immun.*, **67,** 3750-3756.

Braun,J.S., Sublett,J.E., Freyer,D., Mitchell,T.J., Cleveland,J.L., Tuomanen,E.I. & Weber,J.R. 2002. Pneumococcal pneumolysin and H(2)O(2) mediate brain cell apoptosis during meningitis. *J.Clin.Invest*, **109,** 19-27.

Briles,D.E., Ades,E., Paton,J.C., Sampson,J.S., Carlone,G.M., Huebner,R.C., Virolainen,A., Swiatlo,E. & 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,** 796-800.

Briles,D.E., Hollingshead,S., Brooks-Walter,A., Nabors,G.S., Ferguson,L., Schilling,M., Gravenstein,S., Braun,P., King,J. & Swift,A. 2000b. The potential to use PspA and other pneumococcal proteins to elicit protection against pneumococcal infection. *Vaccine*, **18,** 1707-1711.

Briles,D.E., Hollingshead,S.K., King,J., Swift,A., Braun,P.A., Park,M.K., Ferguson,L.M., Nahm,M.H. & Nabors,G.S. 2000c. Immunization of humans with recombinant pneumococcal surface protein A (rPspA) elicits antibodies that passively protect mice from fatal infection with Streptococcus pneumoniae bearing heterologous PspA. *J.Infect.Dis.*, **182,** 1694-1701.

Briles,D.E., Hollingshead,S.K., Paton,J.C., Ades,E.W., Novak,L., van Ginkel,F.W. & Benjamin,W.H., Jr. 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., Novak,L., Hotomi,M., van Ginkel,F.W. & King,J. 2005. Nasal colonization with Streptococcus pneumoniae includes subpopulations of surface and invasive pneumococci. *Infect.Immun.*, **73,** 6945-6951.

Brooks-Walter,A., Briles,D.E. & 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,** 6533-6542.

Brown,J.S., Ogunniyi,A.D., Woodrow,M.C., Holden,D.W. & Paton,J.C. 2001. Immunization with components of two iron uptake ABC transporters protects mice against systemic Streptococcus pneumoniae infection. *Infect.Immun.*, **69,** 6702-6706.

Butler,J.C., Breiman,R.F., Campbell,J.F., Lipman,H.B., Broome,C.V. & Facklam,R.R. 1993. Pneumococcal polysaccharide vaccine efficacy. An evaluation of current recommendations. *JAMA*, **270,** 1826-1831.

Butler,J.C., Shapiro,E.D. & Carlone,G.M. 1999. Pneumococcal vaccines: history, current status, and future directions. *Am.J.Med.*, **107,** 69S-76S.

Calbo,E.& Garau,J. 2010. Of mice and men: innate immunity in pneumococcal pneumonia. *Int.J.Antimicrob.Agents*, **35,** 107-113.

Canvin,J.R., Marvin,A.P., Sivakumaran,M., Paton,J.C., Boulnois,G.J., Andrew,P.W. & 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,** 119-123.

Cao,J., Chen,T., Gong,Y., Ying,B., Li,D., Xu,W., Zhang,X., Wang,L. & Yin,Y. 2010a. Molecular mechanisms of the secretion of cytokines and chemokines from human monocytes activated by pneumococcal surface protein A (PspA): Roles of mitogen-activated protein kinases and NF-kappaB. *Microb.Pathog.*, **48,** 220-229.

Cao,J., Chen,T., Li,D., Wong,C.K., Chen,D., Xu,W., Zhang,X., Lam,C.W. & Yin,Y. 2008. Mucosal immunization with purified ClpP could elicit protective

efficacy against pneumococcal pneumonia and sepsis in mice. *Microbes.Infect.*, **10,** 1536-1542.

Cao,J., Gong,Y., Yin,Y., Wang,L., Ying,B., Chen,T. & Zhang,X. 2010b. Pneumococcal proteins PspA and PspC induce CXCL8 production in human neutrophils: implications in pneumococcal infections. *Microbes.Infect.*.

Cao,J., Li,D., Gong,Y., Yin,N., Chen,T., Wong,C.K., Xu,W., Luo,J., Zhang,X., Lam,C.W. & Yin,Y. 2009. Caseinolytic protease: a protein vaccine which could elicit serotype-independent protection against invasive pneumococcal infection. *Clin.Exp.Immunol.*, **156,** 52-60.

Carvalho,M.G., Steigerwalt,A.G., Thompson,T., Jackson,D. & Facklam,R.R. 2003. Confirmation of nontypeable Streptococcus pneumoniae-like organisms isolated from outbreaks of epidemic conjunctivitis as Streptococcus pneumoniae. *J.Clin.Microbiol.*, **41,** 4415-4417.

Casella,C.R.& Mitchell,T.C. 2008. Putting endotoxin to work for us: monophosphoryl lipid A as a safe and effective vaccine adjuvant. *Cell Mol.Life Sci.*, **65,** 3231-3240.

Chen,S., Paterson,G.K., Tong,H.H., Mitchell,T.J. & DeMaria,T.F. 2005. Sortase A contributes to pneumococcal nasopharyngeal colonization in the chinchilla model. *FEMS Microbiol.Lett.*, **253,** 151-154.

Cheng,Q., Finkel,D. & Hostetter,M.K. 2000. Novel purification scheme and functions for a C3-binding protein from Streptococcus pneumoniae. *Biochemistry*, **39,** 5450-5457.

Christenson,B., Lundbergh,P., Hedlund,J. & Ortqvist,A. 2001. Effects of a large-scale intervention with influenza and 23-valent pneumococcal vaccines in adults aged 65 years or older: a prospective study. *Lancet*, **357,** 1008-1011.

Cockeran,R., Durandt,C., Feldman,C., Mitchell,T.J. & Anderson,R. 2002. Pneumolysin activates the synthesis and release of interleukin-8 by human neutrophils in vitro. *J.Infect.Dis.*, **186,** 562-565.

Cockeran,R., Steel,H.C., Mitchell,T.J., Feldman,C. & Anderson,R. 2001a. Pneumolysin potentiates production of prostaglandin E(2) and leukotriene B(4) by human neutrophils. *Infect.Immun.*, **69,** 3494-3496.

Cockeran,R., Steel,H.C., Theron,A.J., Mitchell,T.J., Feldman,C. & Anderson,R. 2011. Characterization of the interactions of the pneumolysoid, Delta6 PLY, with human neutrophils in vitro. *Vaccine*, **29,** 8780-8782.

Cockeran,R., Theron,A.J., Steel,H.C., Matlola,N.M., Mitchell,T.J., Feldman,C. & Anderson,R. 2001b. Proinflammatory interactions of pneumolysin with human neutrophils. *J.Infect.Dis.*, **183,** 604-611.

Colino,J.& Snapper,C.M. 2003. Two distinct mechanisms for induction of dendritic cell apoptosis in response to intact Streptococcus pneumoniae. *J.Immunol.*, **171,** 2354-2365.

Comis,S.D., Osborne,M.P., Stephen,J., Tarlow,M.J., Hayward,T.L., Mitchell,T.J., Andrew,P.W. & 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,** 152-159.

Crain,M.J., Waltman,W.D., Turner,J.S., Yother,J., Talkington,D.F., McDaniel,L.S., Gray,B.M. & Briles,D.E. 1990. Pneumococcal surface protein A (PspA) is serologically highly variable and is expressed by all clinically important capsular serotypes of Streptococcus pneumoniae. *Infect.Immun.*, **58,** 3293-3299.

Cron,L.E., Bootsma,H.J., Noske,N., Burghout,P., Hammerschmidt,S. & Hermans,P.W. 2009. Surface-associated lipoprotein PpmA of Streptococcus pneumoniae is involved in colonization in a strain-specific manner. *Microbiology*, **155,** 2401-2410.

Crum,N.F., Barrozo,C.P., Chapman,F.A., Ryan,M.A. & Russell,K.L. 2004. An outbreak of conjunctivitis due to a novel unencapsulated Streptococcus pneumoniae among military trainees. *Clin.Infect.Dis.*, **39,** 1148-1154.

Cruse,G., Fernandes,V.E., de Salort,J., Pankhania,D., Marinas,M.S., Brewin,H., Andrew,P.W., Bradding,P. & Kadioglu,A. 2010. Human lung mast cells mediate pneumococcal cell death in response to activation by pneumolysin. *J.Immunol.*, **184,** 7108-7115.

Curtiss,R., III, Xin,W., Li,Y., Kong,W., Wanda,S.Y., Gunn,B. & Wang,S. 2010. New technologies in using recombinant attenuated Salmonella vaccine vectors. *Crit Rev.Immunol.*, **30,** 255-270.

Cutts,F.T., Zaman,S.M., Enwere,G., Jaffar,S., Levine,O.S., Okoko,J.B., Oluwalana,C., Vaughan,A., Obaro,S.K., Leach,A., McAdam,K.P., Biney,E., Saaka,M., Onwuchekwa,U., Yallop,F., Pierce,N.F., Greenwood,B.M. & Adegbola,R.A. 2005. Efficacy of nine-valent pneumococcal conjugate vaccine against pneumonia and invasive pneumococcal disease in The Gambia: randomised, double-blind, placebo-controlled trial. *Lancet*, **365,** 1139-1146.

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

Dagan,R., Givon-Lavi,N., Zamir,O., Sikuler-Cohen,M., Guy,L., Janco,J., Yagupsky,P. & Fraser,D. 2002. Reduction of nasopharyngeal carriage of Streptococcus pneumoniae after administration of a 9-valent pneumococcal conjugate vaccine to toddlers attending day care centers. *J.Infect.Dis.*, **185,** 927-936.

Dagerhamn,J., Blomberg,C., Browall,S., Sjostrom,K., Morfeldt,E. & Henriques-Normark,B. 2008. Determination of accessory gene patterns predicts the same relatedness among strains of Streptococcus pneumoniae as sequencing of housekeeping genes does and represents a novel approach in molecular epidemiology. *J.Clin.Microbiol.*, **46,** 863-868.

Daniels,C.C., Coan,P., King,J., Hale,J., Benton,K.A., Briles,D.E. & Hollingshead,S.K. 2010. The proline-rich region of pneumococcal surface proteins A and C contains surface-accessible epitopes common to all

pneumococci and elicits antibody-mediated protection against sepsis. *Infect.Immun.*, **78,** 2163-2172.

Dave,S., Carmicle,S., Hammerschmidt,S., Pangburn,M.K. & McDaniel,L.S. 2004a. Dual roles of PspC, a surface protein of Streptococcus pneumoniae, in binding human secretory IgA and factor H. *J.Immunol.*, **173,** 471-477.

Dave,S., Pangburn,M.K., Pruitt,C. & McDaniel,L.S. 2004b. Interaction of human factor H with PspC of Streptococcus pneumoniae. *Indian J.Med.Res.*, **119 Suppl,** 66-73.

Davis,K.M., Nakamura,S. & Weiser,J.N. 2011. Nod2 sensing of lysozyme-digested peptidoglycan promotes macrophage recruitment and clearance of S. pneumoniae colonization in mice. *J Clin.Invest*, **121,** 3666-3676.

Dawid,S., Sebert,M.E. & Weiser,J.N. 2009. Bacteriocin activity of Streptococcus pneumoniae is controlled by the serine protease HtrA via posttranscriptional regulation. *J.Bacteriol.*, **191,** 1509-1518.

de Lucia,H.M., Ferreira,P.C., Ferreira,D.M., Miyaji,E.N., Ho,P.L. & Oliveira,M.L. 2011. Nasal immunization of mice with Lactobacillus casei expressing the pneumococcal surface protein C primes the immune system and decreases pneumococcal nasopharyngeal colonization in mice. *FEMS Immunol.Med Microbiol.*, **62,** 263-272.

Debertin,A.S., Tschernig,T., Tonjes,H., Kleemann,W.J., Troger,H.D. & Pabst,R. 2003. Nasal-associated lymphoid tissue (NALT): frequency and localization in young children. *Clin.Exp.Immunol.*, **134,** 503-507.

Denny,F.W.& Loda,F.A. 1986. Acute respiratory infections are the leading cause of death in children in developing countries. *Am.J.Trop.Med.Hyg.*, **35,** 1-2.

DeNoel,P., Godfroid,F., Hermand,P., Verlant,V. & Poolman,J. 2011a. Combined protective effects of anti-PhtD and anti-pneumococcal polysaccharides. *Vaccine*.

DeNoel,P., Philipp,M.T., Doyle,L., Martin,D., Carletti,G. & Poolman,J.T. 2011b. A protein-based pneumococcal vaccine protects rhesus macaques from pneumonia after experimental infection with Streptococcus pneumoniae. *Vaccine*.

Dessing,M.C., Hirst,R.A., de Vos,A.F. & van der,P.T. 2009. Role of Toll-like receptors 2 and 4 in pulmonary inflammation and injury induced by pneumolysin in mice. *PLoS One*, **4,** e7993.

Dieudonne-Vatran,A., Krentz,S., Blom,A.M., Meri,S., Henriques-Normark,B., Riesbeck,K. & Albiger,B. 2009. Clinical isolates of Streptococcus pneumoniae bind the complement inhibitor C4b-binding protein in a PspC allele-dependent fashion. *J.Immunol.*, **182,** 7865-7877.

Dintilhac,A., Alloing,G., Granadel,C. & 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,** 727-739.

Dochez,A.R.& Gillespie,L.J. 1913. A biological classification of pneumococci by means of immunity reactions. *JAMA*, **61,** 727-732.

Dogan,O., Gulmez,D. & Hascelik,G. 2010. Effect of new breakpoints proposed by Clinical and Laboratory Standards Institute in 2008 for evaluating penicillin resistance of Streptococcus pneumoniae in a Turkish University Hospital. *Microb.Drug Resist.*, **16,** 39-41.

Douce,G., Ross,K., Cowan,G., Ma,J. & Mitchell,T.J. 2010. Novel mucosal vaccines generated by genetic conjugation of heterologous proteins to pneumolysin (PLY) from Streptococcus pneumoniae. *Vaccine*, **28,** 3231-3237.

Dowson,C.G., Coffey,T.J. & Spratt,B.G. 1994. Origin and molecular epidemiology of penicillin-binding-protein-mediated resistance to beta-lactam antibiotics. *Trends Microbiol.*, **2,** 361-366.

Duane,P.G., Rubins,J.B., Weisel,H.R. & Janoff,E.N. 1993. Identification of hydrogen peroxide as a Streptococcus pneumoniae toxin for rat alveolar epithelial cells. *Infect.Immun.*, **61,** 4392-4397.

Dubail,I., Autret,N., Beretti,J.L., Kayal,S., Berche,P. & Charbit,A. 2001. Functional assembly of two membrane-binding domains in listeriolysin O, the cytolysin of Listeria monocytogenes. *Microbiology*, **147,** 2679-2688.

Duthy,T.G., Ormsby,R.J., Giannakis,E., Ogunniyi,A.D., Stroeher,U.H., Paton,J.C. & Gordon,D.L. 2002. The human complement regulator factor H binds pneumococcal surface protein PspC via short consensus repeats 13 to 15. *Infect.Immun.*, **70,** 5604-5611.

Eskola,J., Kilpi,T., Palmu,A., Jokinen,J., Haapakoski,J., Herva,E., Takala,A., Kayhty,H., Karma,P., Kohberger,R., Siber,G. & Makela,P.H. 2001. Efficacy of a pneumococcal conjugate vaccine against acute otitis media. *N.Engl.J.Med.*, **344,** 403-409.

Farrand,S., Hotze,E., Friese,P., Hollingshead,S.K., Smith,D.F., Cummings,R.D., Dale,G.L. & Tweten,R.K. 2008. Characterization of a streptococcal cholesterol-dependent cytolysin with a lewis y and b specific lectin domain. *Biochemistry*, **47,** 7097-7107.

Feldman,C., Cockeran,R., Jedrzejas,M.J., Mitchell,T.J. & Anderson,R. 2007. Hyaluronidase augments pneumolysin-mediated injury to human ciliated epithelium. *Int.J.Infect.Dis.*, **11,** 11-15.

Feldman,C., Mitchell,T.J., Andrew,P.W., Boulnois,G.J., Read,R.C., Todd,H.C., Cole,P.J. & Wilson,R. 1990. The effect of Streptococcus pneumoniae pneumolysin on human respiratory epithelium in vitro. *Microb.Pathog.*, **9,** 275-284.

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. & . 1991. Pneumolysin induces the salient histologic features of pneumococcal infection in the rat lung in vivo. *Am.J.Respir.Cell Mol.Biol.*, **5,** 416-423.

Felton,L.& Baily,G. 1926. Biological significance of the soluble specific substance of pneumococcus. *Journal of infectious dieseases*, **38,** 131-144.

Ferrante,A., Rowan-Kelly,B. & Paton,J.C. 1984. Inhibition of in vitro human lymphocyte response by the pneumococcal toxin pneumolysin. *Infect.Immun.*, **46,** 585-589.

Ferreira,D.M., Darrieux,M., Silva,D.A., Leite,L.C., Ferreira,J.M., Jr., Ho,P.L., Miyaji,E.N. & Oliveira,M.L. 2009. Characterization of protective mucosal and systemic immune responses elicited by pneumococcal surface protein PspA and PspC nasal vaccines against a respiratory pneumococcal challenge in mice. *Clin.Vaccine Immunol.*, **16,** 636-645.

Fiore,A.E., Levine,O.S., Elliott,J.A., Facklam,R.R. & Butler,J.C. 1999. Effectiveness of pneumococcal polysaccharide vaccine for preschool-age children with chronic disease. *Emerg.Infect.Dis.*, **5,** 828-831.

Fireman,B., Black,S.B., Shinefield,H.R., Lee,J., Lewis,E. & Ray,P. 2003. Impact of the pneumococcal conjugate vaccine on otitis media. *Pediatr.Infect.Dis.J.*, **22,** 10-16.

Flamaing,J., Peetermans,W.E., Vandeven,J. & Verhaegen,J. 2010. Pneumococcal colonization in older persons in a nonoutbreak setting. *J.Am.Geriatr.Soc.*, **58,** 396-398.

French,N., Nakiyingi,J., Carpenter,L.M., Lugada,E., Watera,C., Moi,K., Moore,M., Antvelink,D., Mulder,D., Janoff,E.N., Whitworth,J. & Gilks,C.F. 2000. 23-valent pneumococcal polysaccharide vaccine in HIV-1-infected Ugandan adults: double-blind, randomised and placebo controlled trial. *Lancet*, **355,** 2106-2111.

Fujimura,Y. 2000. Evidence of M cells as portals of entry for antigens in the nasopharyngeal lymphoid tissue of humans. *Virchows Arch.*, **436,** 560-566.

Gebo,K.A., Moore,R.D., Keruly,J.C. & Chaisson,R.E. 1996. Risk factors for pneumococcal disease in human immunodeficiency virus-infected patients. *J.Infect.Dis.*, **173,** 857-862.

Gianfaldoni,C., Censini,S., Hilleringmann,M., Moschioni,M., Facciotti,C., Pansegrau,W., Masignani,V., Covacci,A., Rappuoli,R., Barocchi,M.A. & Ruggiero,P. 2007. Streptococcus pneumoniae pilus subunits protect mice against lethal challenge. *Infect.Immun.*, **75,** 1059-1062.

Gianfaldoni,C., Maccari,S., Pancotto,L., Rossi,G., Hilleringmann,M., Pansegrau,W., Sinisi,A., Moschioni,M., Masignani,V., Rappuoli,R., Del Giudice,G. & Ruggiero,P. 2009. Sortase A confers protection against Streptococcus pneumoniae in mice. *Infect.Immun.*, **77,** 2957-2961.

Giebink,G.S., Ripley-Petzoldt,M.L., Juhn,S.K., Aeppli,D., Tomasz,A. & Tuomanen,E. 1988. Contribution of pneumococcal cell wall to experimental otitis media pathogenesis. *Ann.Otol.Rhinol.Laryngol.Suppl*, **132,** 28-30.

Giefing,C., Meinke,A.L., Hanner,M., Henics,T., Bui,M.D., Gelbmann,D., Lundberg,U., Senn,B.M., Schunn,M., Habel,A., Henriques-Normark,B.,

Ortqvist,A., Kalin,M., von Gabain,A. & Nagy,E. 2008. Discovery of a novel class of highly conserved vaccine antigens using genomic scale antigenic fingerprinting of pneumococcus with human antibodies. *J.Exp.Med.*, **205,** 117-131.

Gilbert,R.J. 2002. Pore-forming toxins. *Cell Mol.Life Sci.*, **59,** 832-844.

Glover,D.T., Hollingshead,S.K. & Briles,D.E. 2008. Streptococcus pneumoniae surface protein PcpA elicits protection against lung infection and fatal sepsis. *Infect.Immun.*, **76,** 2767-2776.

Godfroid,F., Hermand,P., Verlant,V., DeNoel,P. & Poolman,J.T. 2011. Preclinical evaluation of the Pht proteins as potential cross-protective pneumococcal vaccine antigens. *Infect.Immun.*, **79,** 238-245.

Goetghebuer,T., West,T.E., Wermenbol,V., Cadbury,A.L., Milligan,P., Lloyd-Evans,N., Adegbola,R.A., Mulholland,E.K., Greenwood,B.M. & Weber,M.W. 2000. Outcome of meningitis caused by Streptococcus pneumoniae and Haemophilus influenzae type b in children in The Gambia. *Trop.Med.Int.Health*, **5,** 207-213.

Gor,D.O., Ding,X., Li,Q., Sultana,D., Mambula,S.S., Bram,R.J. & Greenspan,N.S. 2011. Enhanced immunogenicity of pneumococcal surface adhesin A (PsaA) in mice via fusion to recombinant human B lymphocyte stimulator (BLyS). *Biol.Direct*, **6,** 9.

Granat,S.M., Mia,Z., Ollgren,J., Herva,E., Das,M., Piirainen,L., Auranen,K. & Makela,P.H. 2007. Longitudinal study on pneumococcal carriage during the first year of life in Bangladesh. *Pediatr.Infect.Dis.J.*, **26,** 319-324.

Gratten,M., Gratten,H., Poli,A., Carrad,E., Raymer,M. & Koki,G. 1986. Colonisation of Haemophilus influenzae and Streptococcus pneumoniae in the upper respiratory tract of neonates in Papua New Guinea: primary acquisition, duration of carriage, and relationship to carriage in mothers. *Biol.Neonate*, **50,** 114-120.

Green,B.A., Zhang,Y., Masi,A.W., Barniak,V., Wetherell,M., Smith,R.P., Reddy,M.S. & 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,** 981-989.

Greenberg,D., Givon-Lavi,N., Broides,A., Blancovich,I., Peled,N. & Dagan,R. 2006. The contribution of smoking and exposure to tobacco smoke to Streptococcus pneumoniae and Haemophilus influenzae carriage in children and their mothers. *Clin.Infect.Dis.*, **42,** 897-903.

Hakansson,A., Roche,H., Mirza,S., McDaniel,L.S., Brooks-Walter,A. & Briles,D.E. 2001. Characterization of binding of human lactoferrin to pneumococcal surface protein A. *Infect.Immun.*, **69,** 3372-3381.

Hamel,J., Charland,N., Pineau,I., Ouellet,C., Rioux,S., Martin,D. & Brodeur,B.R. 2004. Prevention of pneumococcal disease in mice immunized with conserved surface-accessible proteins. *Infect.Immun.*, **72,** 2659-2670.

Hammerschmidt,S., Bethe,G., Remane,P.H. & Chhatwal,G.S. 1999. Identification of pneumococcal surface protein A as a lactoferrin-binding protein of Streptococcus pneumoniae. *Infect.Immun.*, **67,** 1683-1687.

Hammerschmidt,S., Talay,S.R., Brandtzaeg,P. & Chhatwal,G.S. 1997. SpsA, a novel pneumococcal surface protein with specific binding to secretory immunoglobulin A and secretory component. *Mol.Microbiol.*, **25,** 1113-1124.

Hammerschmidt,S., Tillig,M.P., Wolff,S., Vaerman,J.P. & Chhatwal,G.S. 2000. Species-specific binding of human secretory component to SpsA protein of Streptococcus pneumoniae via a hexapeptide motif. *Mol.Microbiol.*, **36,** 726-736.

Hammerschmidt,S., Wolff,S., Hocke,A., Rosseau,S., Muller,E. & Rohde,M. 2005. Illustration of pneumococcal polysaccharide capsule during adherence and invasion of epithelial cells. *Infect.Immun.*, **73,** 4653-4667.

Hanage,W.P., Kaijalainen,T., Herva,E., Saukkoriipi,A., Syrjanen,R. & Spratt,B.G. 2005. Using multilocus sequence data to define the pneumococcus. *J.Bacteriol.*, **187,** 6223-6230.

Hanniffy,S.B., Carter,A.T., Hitchin,E. & Wells,J.M. 2007. Mucosal delivery of a pneumococcal vaccine using Lactococcus lactis affords protection against respiratory infection. *J.Infect.Dis.*, **195,** 185-193.

Harding,C.V., Roof,R.W., Allen,P.M. & Unanue,E.R. 1991. Effects of pH and polysaccharides on peptide binding to class II major histocompatibility complex molecules. *Proc.Natl.Acad.Sci.U.S.A*, **88,** 2740-2744.

Harvey,R.M., Ogunniyi,A.D., Chen,A.Y. & Paton,J.C. 2011. Pneumolysin with Low Hemolytic Activity Confers an Early Growth Advantage to Streptococcus pneumoniae in the Blood. *Infect.Immun.*.

Hava,D.L.& Camilli,A. 2002. Large-scale identification of serotype 4 Streptococcus pneumoniae virulence factors. *Mol.Microbiol.*, **45,** 1389-1406.

Hayden,E.C. 2009. Genome sequencing: the third generation. *Nature*, **457,** 768-769.

Henrichsen,J. 1995. Six newly recognized types of Streptococcus pneumoniae. *J.Clin.Microbiol.*, **33,** 2759-2762.

Heritage,P.L., Underdown,B.J., Arsenault,A.L., Snider,D.P. & McDermott,M.R. 1997. Comparison of murine nasal-associated lymphoid tissue and Peyer's patches. *Am J Respir.Crit Care Med,* **156,** 1256-1262.

Hermans,P.W., Adrian,P.V., Albert,C., Estevao,S., Hoogenboezem,T., Luijendijk,I.H., Kamphausen,T. & Hammerschmidt,S. 2006. The streptococcal lipoprotein rotamase A (SlrA) is a functional peptidyl-prolyl isomerase involved in pneumococcal colonization. *J.Biol.Chem.*, **281,** 968-976.

Heuck,A.P., Tweten,R.K. & Johnson,A.E. 2003. Assembly and topography of the prepore complex in cholesterol-dependent cytolysins. *J Biol.Chem.*, **278,** 31218-31225.

Hill,P.C., Akisanya,A., Sankareh,K., Cheung,Y.B., Saaka,M., Lahai,G., Greenwood,B.M. & Adegbola,R.A. 2006. Nasopharyngeal carriage of Streptococcus pneumoniae in Gambian villagers. *Clin.Infect.Dis.*, **43,** 673-679.

Hippenstiel,S., Opitz,B., Schmeck,B. & Suttorp,N. 2006. Lung epithelium as a sentinel and effector system in pneumonia--molecular mechanisms of pathogen recognition and signal transduction. *Respir.Res.*, **7,** 97.

Hirst,R.A., Rutman,A., Sikand,K., Andrew,P.W., Mitchell,T.J. & O'Callaghan,C. 2000a. Effect of pneumolysin on rat brain ciliary function: comparison of brain slices with cultured ependymal cells. *Pediatr.Res.*, **47,** 381-384.

Hirst,R.A., Sikand,K.S., Rutman,A., Mitchell,T.J., Andrew,P.W. & O'Callaghan,C. 2000b. Relative roles of pneumolysin and hydrogen peroxide from Streptococcus pneumoniae in inhibition of ependymal ciliary beat frequency. *Infect.Immun.*, **68,** 1557-1562.

Hirst,R.A., Yesilkaya,H., Clitheroe,E., Rutman,A., Dufty,N., Mitchell,T.J., O'Callaghan,C. & Andrew,P.W. 2002. Sensitivities of human monocytes and epithelial cells to pneumolysin are different. *Infect.Immun.*, **70,** 1017-1022.

Hoffmann,O., Braun,J.S., Becker,D., Halle,A., Freyer,D., Dagand,E., Lehnardt,S. & Weber,J.R. 2007. TLR2 mediates neuroinflammation and neuronal damage. *J.Immunol.*, **178,** 6476-6481.

Hollingshead,S.K., Becker,R. & Briles,D.E. 2000. Diversity of PspA: mosaic genes and evidence for past recombination in Streptococcus pneumoniae. *Infect.Immun.*, **68,** 5889-5900.

Holmlund,E., Quiambao,B., Ollgren,J., Jaakkola,T., Neyt,C., Poolman,J., Nohynek,H. & Kayhty,H. 2009. Antibodies to pneumococcal proteins PhtD, CbpA, and LytC in Filipino pregnant women and their infants in relation to pneumococcal carriage. *Clin.Vaccine Immunol.*, **16,** 916-923.

Holmlund,E., Quiambao,B., Ollgren,J., Nohynek,H. & Kayhty,H. 2006. Development of natural antibodies to pneumococcal surface protein A, pneumococcal surface adhesin A and pneumolysin in Filipino pregnant women and their infants in relation to pneumococcal carriage. *Vaccine*, **24,** 57-65.

Holmlund,E., Simell,B., Jaakkola,T., Lahdenkari,M., Hamel,J., Brodeur,B., Kilpi,T. & Kayhty,H. 2007. Serum antibodies to the pneumococcal surface proteins PhtB and PhtE in Finnish infants and adults. *Pediatr.Infect.Dis.J.*, **26,** 447-449.

Hotze,E.M., Heuck,A.P., Czajkowsky,D.M., Shao,Z., Johnson,A.E. & 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,** 11597-11605.

Hotze,E.M., Wilson-Kubalek,E.M., Rossjohn,J., Parker,M.W., Johnson,A.E. & Tweten,R.K. 2001. 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,** 8261-8268.

Houldsworth,S., Andrew,P.W. & Mitchell,T.J. 1994. Pneumolysin stimulates production of tumor necrosis factor alpha and interleukin-1 beta by human mononuclear phagocytes. *Infect.Immun.*, **62,** 1501-1503.

Huss,A., Scott,P., Stuck,A.E., Trotter,C. & Egger,M. 2009. Efficacy of pneumococcal vaccination in adults: a meta-analysis. *CMAJ.*, **180,** 48-58.

Hussain,M., Melegaro,A., Pebody,R.G., George,R., Edmunds,W.J., Talukdar,R., Martin,S.A., Efstratiou,A. & Miller,E. 2005. A longitudinal household study of Streptococcus pneumoniae nasopharyngeal carriage in a UK setting. *Epidemiol.Infect.*, **133,** 891-898.

Iannelli,F., Oggioni,M.R. & Pozzi,G. 2002. Allelic variation in the highly polymorphic locus pspC of Streptococcus pneumoniae. *Gene*, **284,** 63-71.

Iliev,A.I., Djannatian,J.R., Nau,R., Mitchell,T.J. & Wouters,F.S. 2007. Cholesterol-dependent actin remodeling via RhoA and Rac1 activation by the Streptococcus pneumoniae toxin pneumolysin. *Proc.Natl.Acad.Sci.U.S.A*, **104,** 2897-2902.

Iliev,A.I., Djannatian,J.R., Opazo,F., Gerber,J., Nau,R., Mitchell,T.J. & Wouters,F.S. 2009. Rapid microtubule bundling and stabilization by the Streptococcus pneumoniae neurotoxin pneumolysin in a cholesterol-dependent, non-lytic and Src-kinase dependent manner inhibits intracellular trafficking. *Mol.Microbiol.*, **71,** 461-477.

Jackson,L.A., Neuzil,K.M., Yu,O., Benson,P., Barlow,W.E., Adams,A.L., Hanson,C.A., Mahoney,L.D., Shay,D.K. & Thompson,W.W. 2003. Effectiveness of pneumococcal polysaccharide vaccine in older adults. *N.Engl.J.Med.*, **348,** 1747-1755.

Jacobs,M.R., Bajaksouzian,S., Bonomo,R.A., Good,C.E., Windau,A.R., Hujer,A.M., Massire,C., Melton,R., Blyn,L.B., Ecker,D.J. & Sampath,R. 2009. Occurrence, distribution, and origins of Streptococcus pneumoniae Serotype 6C, a recently recognized serotype. *J.Clin.Microbiol.*, **47,** 64-72.

Janulczyk,R., Iannelli,F., Sjoholm,A.G., Pozzi,G. & Bjorck,L. 2000. Hic, a novel surface protein of Streptococcus pneumoniae that interferes with complement function. *J.Biol.Chem.*, **275,** 37257-37263.

Jefferies,J.M., Smith,A., Clarke,S.C., Dowson,C. & Mitchell,T.J. 2004. Genetic analysis of diverse disease-causing pneumococci indicates high levels of diversity within serotypes and capsule switching. *J.Clin.Microbiol.*, **42,** 5681-5688.

Johnson,M.K., Hobden,J.A., Hagenah,M., O'Callaghan,R.J., Hill,J.M. & Chen,S. 1990. The role of pneumolysin in ocular infections with Streptococcus pneumoniae. *Curr.Eye Res.*, **9,** 1107-1114.

Johnston,R.B., Jr. 1991. Pathogenesis of pneumococcal pneumonia. *Rev.Infect.Dis.*, **13 Suppl 6,** S509-S517.

Jolles,S. 2002. Paul Langerhans. *J Clin.Pathol.*, **55,** 243.

Jomaa,M., Terry,S., Hale,C., Jones,C., Dougan,G. & Brown,J. 2006. Immunization with the iron uptake ABC transporter proteins PiaA and PiuA prevents respiratory infection with Streptococcus pneumoniae. *Vaccine*, **24,** 5133-5139.

Jomaa,M., Yuste,J., Paton,J.C., Jones,C., Dougan,G. & Brown,J.S. 2005. Antibodies to the iron uptake ABC transporter lipoproteins PiaA and PiuA promote opsonophagocytosis of Streptococcus pneumoniae. *Infect.Immun.*, **73,** 6852-6859.

Jonsson,S., Musher,D.M., Chapman,A., Goree,A. & Lawrence,E.C. 1985. Phagocytosis and killing of common bacterial pathogens of the lung by human alveolar macrophages. *J.Infect.Dis.*, **152,** 4-13.

Jounblat,R., Kadioglu,A., Mitchell,T.J. & 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,** 1813-1819.

Kadioglu,A., Coward,W., Colston,M.J., Hewitt,C.R. & Andrew,P.W. 2004. CD4-T-lymphocyte interactions with pneumolysin and pneumococci suggest a crucial protective role in the host response to pneumococcal infection. *Infect.Immun.*, **72,** 2689-2697.

Kadioglu,A., Gingles,N.A., Grattan,K., Kerr,A., Mitchell,T.J. & Andrew,P.W. 2000. Host cellular immune response to pneumococcal lung infection in mice. *Infect.Immun.*, **68,** 492-501.

Kadioglu,A., Taylor,S., Iannelli,F., Pozzi,G., Mitchell,T.J. & 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,** 2886-2890.

Kalin,M. 1998. Pneumococcal serotypes and their clinical relevance. *Thorax*, **53,** 159-162.

Kamekura,R., Kojima,T., Koizumi,J., Ogasawara,N., Kurose,M., Takano,K., Go,M., Himi ,T. & Sawada,N. 2008. Expression of thymic stromal lymphopoietin (TSLP) in allergic rhinitis: Induction of tight junction proteins in human nasal epithelial cells and dendritic cells by epithelial-derived TSLP. *Inflammation and Regeneration*, **8,** 160-165.

Kanclerski,K.& Mollby,R. 1987. Production and purification of Streptococcus pneumoniae hemolysin (pneumolysin). *J.Clin.Microbiol.*, **25,** 222-225.

Kang,H.Y., Srinivasan,J. & Curtiss,R., III 2002. Immune responses to recombinant pneumococcal PspA antigen delivered by live attenuated Salmonella enterica serovar typhimurium vaccine. *Infect.Immun.*, **70,** 1739-1749.

Kaur,R., Casey,J.R. & Pichichero,M.E. 2011. Serum Antibody Response to Five Streptococcus pneumoniae Proteins During Acute Otitis Media in Otitis-prone and Non-otitis-prone Children. *Pediatr.Infect.Dis.J.*.

Kerr,A.R., Paterson,G.K., McCluskey,J., Iannelli,F., Oggioni,M.R., Pozzi,G. & Mitchell,T.J. 2006. The contribution of PspC to pneumococcal virulence varies between strains and is accomplished by both complement evasion and complement-independent mechanisms. *Infect.Immun.*, **74,** 5319-5324.

Kharat,A.S.& Tomasz,A. 2003. Inactivation of the srtA gene affects localization of surface proteins and decreases adhesion of Streptococcus pneumoniae to human pharyngeal cells in vitro. *Infect.Immun.*, **71,** 2758-2765.

Khoruts,A., Mondino,A., Pape,K.A., Reiner,S.L. & Jenkins,M.K. 1998. A natural immunological adjuvant enhances T cell clonal expansion through a CD28-dependent, interleukin (IL)-2-independent mechanism. *J Exp.Med*, **187,** 225-236.

King,Q.O., Lei,B. & Harmsen,A.G. 2009. Pneumococcal surface protein A contributes to secondary Streptococcus pneumoniae infection after influenza virus infection. *J.Infect.Dis.*, **200,** 537-545.

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

Kirkham,L.A., Kerr,A.R., Douce,G.R., Paterson,G.K., Dilts,D.A., Liu,D.F. & Mitchell,T.J. 2006b. Construction and immunological characterization of a novel nontoxic protective pneumolysin mutant for use in future pneumococcal vaccines. *Infect.Immun.*, **74,** 586-593.

Kirkham,L.A., Kerr,A.R., Douce,G.R., Paterson,G.K., Dilts,D.A., Liu,D.F. & Mitchell,T.J. 2006c. Construction and immunological characterization of a novel nontoxic protective pneumolysin mutant for use in future pneumococcal vaccines. *Infect.Immun.*, **74,** 586-593.

Kiyono,H.& Fukuyama,S. 2004. *Nat.Rev.Immunol.*, **4,** 699-710.

Klemperer,G.& Klemperper,F. 1891. Wersuche uber immunisierung und Heilung bei der Pheumokokkeninfection. *Ber.Klin.Wochenscher.*, **28,** 830-835.

Kolling,U.K., Hansen,F., Braun,J., Rink,L., Katus,H.A. & Dalhoff,K. 2001. Leucocyte response and anti-inflammatory cytokines in community acquired pneumonia. *Thorax*, **56,** 121-125.

Kostyukova,N.N., Volkova,M.O., Ivanova,V.V. & Kvetnaya,A.S. 1995. A study of pathogenic factors of Streptococcus pneumoniae strains causing meningitis. *FEMS Immunol.Med.Microbiol.*, **10,** 133-137.

Kuo,J., Douglas,M., Ree,H.K. & 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,** 2706-2713.

Kurtti,P., Isoaho,R., von Hertzen,L., Keistinen,T., Kivela,S.L. & 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,** 485-489.

Lafferty,K.J., Prowse,S.J., Simeonovic,C.J. & Warren,H.S. 1983. Immunobiology of tissue transplantation: a return to the passenger leukocyte concept. *Annu.Rev.Immunol.*, **1,** 143-173.

Lagergard,T., Lundqvist,A., Wising,C., Gabrielsson,V. & Ahlman,K. 2007. Formaldehyde treatment increases the immunogenicity and decreases the toxicity of Haemophilus ducreyi cytolethal distending toxin. *Vaccine*, **25,** 3606-3614.

Lau,G.W., Haataja,S., Lonetto,M., Kensit,S.E., Marra,A., Bryant,A.P., McDevitt,D., Morrison,D.A. & Holden,D.W. 2001. A functional genomic analysis of type 3 Streptococcus pneumoniae virulence. *Mol.Microbiol.*, **40,** 555-571.

Lavelle,E.C. 2005. Generation of improved mucosal vaccines by induction of innate immunity. *Cell Mol.Life Sci.*, **62,** 2750-2770.

Lawrence,M.C., Pilling,P.A., Epa,V.C., Berry,A.M., Ogunniyi,A.D. & Paton,J.C. 1998. The crystal structure of pneumococcal surface antigen PsaA reveals a metal-binding site and a novel structure for a putative ABC-type binding protein. *Structure.*, **6,** 1553-1561.

Lebon,A., Verkaik,N.J., Labout,J.A., de Vogel,C.P., Hooijkaas,H., Verbrugh,H.A., van Wamel,W.J., Jaddoe,V.W., Hofman,A., Hermans,P.W., Ma,J., Mitchell,T.J., Moll,H.A. & van Belkum,A. 2011. Natural antibodies against several pneumococcal virulence proteins in children during the pre-pneumococcal-vaccine era: the generation R study. *Infect.Immun.*, **79,** 1680-1687.

Lee,C.J., Wang,T.R. & Frasch,C.E. 2001. Immunogenicity in mice of pneumococcal glycoconjugate vaccines using pneumococcal protein carriers. *Vaccine*, **19,** 3216-3225.

Lee,S.E., Kim,S.Y., Jeong,B.C., Kim,Y.R., Bae,S.J., Ahn,O.S., Lee,J.J., Song,H.C., Kim,J.M., Choy,H.E., Chung,S.S., Kweon,M.N. & Rhee,J.H. 2006. A bacterial flagellin, Vibrio vulnificus FlaB, has a strong mucosal adjuvant activity to induce protective immunity. *Infect.Immun.*, **74,** 694-702.

Li,J., Glover,D.T., Szalai,A.J., Hollingshead,S.K. & Briles,D.E. 2007. PspA and PspC minimize immune adherence and transfer of pneumococci from erythrocytes to macrophages through their effects on complement activation. *Infect.Immun.*, **75,** 5877-5885.

Li,Y., Wang,S., Scarpellini,G., Gunn,B., Xin,W., Wanda,S.Y., Roland,K.L. & Curtiss,R., III 2009. Evaluation of new generation Salmonella enterica serovar Typhimurium vaccines with regulated delayed attenuation to induce immune responses against PspA. *Proc.Natl.Acad.Sci.U.S.A*, **106,** 593-598.

Lipsitch,M., Whitney,C.G., Zell,E., Kaijalainen,T., Dagan,R. & Malley,R. 2005. Are anticapsular antibodies the primary mechanism of protection against invasive pneumococcal disease? *PLoS Med.*, **2,** e15.

Littmann,M., Albiger,B., Frentzen,A., Normark,S., Henriques-Normark,B. & Plant,L. 2009. Streptococcus pneumoniae evades human dendritic cell surveillance by pneumolysin expression. *EMBO Mol.Med.*, **1,** 211-222.

Lloyd-Evans,N., O'Dempsey,T.J., Baldeh,I., Secka,O., Demba,E., Todd,J.E., Mcardle,T.F., Banya,W.S. & Greenwood,B.M. 1996. Nasopharyngeal carriage of pneumococci in Gambian children and in their families. *Pediatr.Infect.Dis.J.*, **15,** 866-871.

Lock,R.A., Hansman,D. & Paton,J.C. 1992. Comparative efficacy of autolysin and pneumolysin as immunogens protecting mice against infection by Streptococcus pneumoniae. *Microb.Pathog.*, **12,** 137-143.

Lock,R.A., Paton,J.C. & Hansman,D. 1988. Comparative efficacy of pneumococcal neuraminidase and pneumolysin as immunogens protective against Streptococcus pneumoniae. *Microb.Pathog.*, **5,** 461-467.

Loisel,E., Chimalapati,S., Bougault,C., Imberty,A., Gallet,B., Di Guilmi,A.M., Brown,J., Vernet,T. & Durmort,C. 2011. Biochemical characterization of the histidine triad protein PhtD as a cell surface zinc-binding protein of pneumococcus. *Biochemistry*, **50,** 3551-3558.

Long,J.P., Tong,H.H. & DeMaria,T.F. 2004. Immunization with native or recombinant Streptococcus pneumoniae neuraminidase affords protection in the chinchilla otitis media model. *Infect.Immun.*, **72,** 4309-4313.

Lu,Y.J., Forte,S., Thompson,C.M., Anderson,P.W. & Malley,R. 2009. Protection against Pneumococcal colonization and fatal pneumonia by a trivalent conjugate of a fusion protein with the cell wall polysaccharide. *Infect.Immun.*, **77,** 2076-2083.

Lu,Y.J., Gross,J., Bogaert,D., Finn,A., Bagrade,L., Zhang,Q., Kolls,J.K., Srivastava,A., Lundgren,A., Forte,S., Thompson,C.M., Harney,K.F., Anderson,P.W., Lipsitch,M. & Malley,R. 2008. Interleukin-17A mediates acquired immunity to pneumococcal colonization. *PLoS Pathog.*, **4,** e1000159.

Lu,Y.J., Leite,L., Goncalves,V.M., Dias,W.O., Liberman,C., Fratelli,F., Alderson,M., Tate,A., Maisonneuve,J.F., Robertson,G., Graca,R., Sayeed,S., Thompson,C.M., Anderson,P. & Malley,R. 2010a. GMP-grade pneumococcal whole-cell vaccine injected subcutaneously protects mice from nasopharyngeal colonization and fatal aspiration-sepsis. *Vaccine*, **28,** 7468-7475.

Lu,Y.J., Yadav,P., Clements,J.D., Forte,S., Srivastava,A., Thompson,C.M., Seid,R., Look,J., Alderson,M., Tate,A., Maisonneuve,J.F., Robertson,G., Anderson,P.W. & Malley,R. 2010b. Options for inactivation, adjuvant, and route of topical administration of a killed, unencapsulated pneumococcal whole-cell vaccine. *Clin.Vaccine Immunol.*, **17,** 1005-1012.

Mahon,B.E., Hsu,K., Karumuri,S., Kaplan,S.L., Mason,E.O., Jr. & Pelton,S.I. 2006. Effectiveness of abbreviated and delayed 7-valent pneumococcal conjugate vaccine dosing regimens. *Vaccine*, **24,** 2514-2520.

Mai,J., Wang,H. & Yang,X.F. 2010. Th 17 cells interplay with Foxp3+ Tregs in regulation of inflammation and autoimmunity. *Front Biosci.*, **15,** 986-1006.

Malley,R., Henneke,P., Morse,S.C., Cieslewicz,M.J., Lipsitch,M., Thompson,C.M., Kurt-Jones,E., Paton,J.C., Wessels,M.R. & 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,** 1966-1971.

Malley,R., Lipsitch,M., Stack,A., Saladino,R., Fleisher,G., Pelton,S., Thompson,C., Briles,D. & Anderson,P. 2001. Intranasal immunization with killed unencapsulated whole cells prevents colonization and invasive disease by capsulated pneumococci. *Infect.Immun.*, **69,** 4870-4873.

Malley,R., Morse,S.C., Leite,L.C., Areas,A.P., Ho,P.L., Kubrusly,F.S., Almeida,I.C. & Anderson,P. 2004. Multiserotype protection of mice against pneumococcal colonization of the nasopharynx and middle ear by killed nonencapsulated cells given intranasally with a nontoxic adjuvant. *Infect.Immun.*, **72,** 4290-4292.

Malley,R., Srivastava,A., Lipsitch,M., Thompson,C.M., Watkins,C., Tzianabos,A. & Anderson,P.W. 2006. Antibody-independent, interleukin-17A-mediated, cross-serotype immunity to pneumococci in mice immunized intranasally with the cell wall polysaccharide. *Infect.Immun.*, **74,** 2187-2195.

Malley,R., Trzcinski,K., Srivastava,A., Thompson,C.M., Anderson,P.W. & Lipsitch,M. 2005. CD4+ T cells mediate antibody-independent acquired immunity to pneumococcal colonization. *Proc.Natl.Acad.Sci.U.S.A*, **102,** 4848-4853.

Manco,S., Hernon,F., Yesilkaya,H., Paton,J.C., Andrew,P.W. & Kadioglu,A. 2006. Pneumococcal neuraminidases A and B both have essential roles during infection of the respiratory tract and sepsis. *Infect.Immun.*, **74,** 4014-4020.

Manzano,C., Contreras-Martel,C., El Mortaji,L., Izore,T., Fenel,D., Vernet,T., Schoehn,G., Di Guilmi,A.M. & Dessen,A. 2008. Sortase-mediated pilus fiber biogenesis in Streptococcus pneumoniae. *Structure.*, **16,** 1838-1848.

Marra,A., Lawson,S., Asundi,J.S., Brigham,D. & Hromockyj,A.E. 2002. In vivo characterization of the psa genes from Streptococcus pneumoniae in multiple models of infection. *Microbiology*, **148,** 1483-1491.

Marriott,H.M., Ali,F., Read,R.C., Mitchell,T.J., Whyte,M.K. & Dockrell,D.H. 2004. Nitric oxide levels regulate macrophage commitment to apoptosis or necrosis during pneumococcal infection. *FASEB J.*, **18,** 1126-1128.

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. & Whitney,C.G. 2003. An outbreak of conjunctivitis due to atypical Streptococcus pneumoniae. *N.Engl.J.Med.*, **348,** 1112-1121.

Martner,A., Dahlgren,C., Paton,J.C. & Wold,A.E. 2008. Pneumolysin released during Streptococcus pneumoniae autolysis is a potent activator of intracellular oxygen radical production in neutrophils. *Infect.Immun.*, **76,** 4079-4087.

Matthias,K.A., Roche,A.M., Standish,A.J., Shchepetov,M. & Weiser,J.N. 2008. Neutrophil-toxin interactions promote antigen delivery and mucosal clearance of Streptococcus pneumoniae. *J.Immunol.*, **180,** 6246-6254.

Maus,U.A., Srivastava,M., Paton,J.C., Mack,M., Everhart,M.B., Blackwell,T.S., Christman,J.W., Schlondorff,D., Seeger,W. & Lohmeyer,J. 2004. Pneumolysin-induced lung injury is independent of leukocyte trafficking into the alveolar space. *J.Immunol.*, **173,** 1307-1312.

Mbelle,N., Huebner,R.E., Wasas,A.D., Kimura,A., Chang,I. & Klugman,K.P. 1999. Immunogenicity and impact on nasopharyngeal carriage of a nonavalent pneumococcal conjugate vaccine. *J.Infect.Dis.*, **180,** 1171-1176.

McBean,A.M., Park,Y.T., Caldwell,D. & Yu,X. 2005. Declining invasive pneumococcal disease in the U.S. elderly. *Vaccine*, **23,** 5641-5645.

McCool,T.L.& Weiser,J.N. 2004. Limited role of antibody in clearance of Streptococcus pneumoniae in a murine model of colonization. *Infect.Immun.*, **72,** 5807-5813.

McDaniel,L.S., McDaniel,D.O., Hollingshead,S.K. & Briles,D.E. 1998. Comparison of the PspA sequence from Streptococcus pneumoniae EF5668 to the previously identified PspA sequence from strain Rx1 and ability of PspA from EF5668 to elicit protection against pneumococci of different capsular types. *Infect.Immun.*, **66,** 4748-4754.

McNeela,E.A., Burke,A., Neill,D.R., Baxter,C., Fernandes,V.E., Ferreira,D., Smeaton,S., El Rachkidy,R., McLoughlin,R.M., Mori,A., Moran,B., Fitzgerald,K.A., Tschopp,J., Petrilli,V., Andrew,P.W., Kadioglu,A. & Lavelle,E.C. 2010. Pneumolysin Activates the NLRP3 Inflammasome and Promotes Proinflammatory Cytokines Independently of TLR4. *Plos Pathogens*, **6**.

Medina,M., Villena,J., Salva,S., Vintini,E., Langella,P. & Alvarez,S. 2008a. Nasal administration of Lactococcus lactis improves local and systemic immune responses against Streptococcus pneumoniae. *Microbiol.Immunol.*, **52,** 399-409.

Medina,M., Villena,J., Vintini,E., Hebert,E.M., Raya,R. & Alvarez,S. 2008b. Nasal immunization with Lactococcus lactis expressing the pneumococcal protective protein A induces protective immunity in mice. *Infect.Immun.*, **76,** 2696-2705.

Melin,M., Di Paolo,E., Tikkanen,L., Jarva,H., Neyt,C., Kayhty,H., Meri,S., Poolman,J. & Vakevainen,M. 2010. Interaction of pneumococcal histidine triad proteins with human complement. *Infect.Immun.*, **78,** 2089-2098.

Mitchell,T.J. 2000. Virulence factors and the pathogenesis of disease caused by Streptococcus pneumoniae. *Res.Microbiol.*, **151,** 413-419.

Mitchell,T.J. 2006. Streptococcus pneumoniae: infection, inflammation and disease. *Adv.Exp.Med.Biol.*, **582,** 111-124.

Mitchell,T.J.& Andrew,P.W. 1997. Biological properties of pneumolysin. *Microb.Drug Resist.*, **3,** 19-26.

Mitchell,T.J., Andrew,P.W., Saunders,F.K., Smith,A.N. & 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,** 1883-1888.

Miyaji,E.N., Dias,W.O., Tanizaki,M.M. & Leite,L.C. 2003. Protective efficacy of PspA (pneumococcal surface protein A)-based DNA vaccines: contribution of both humoral and cellular immune responses. *FEMS Immunol.Med.Microbiol.*, **37,** 53-57.

Miyaji,E.N., Ferreira,D.M., Lopes,A.P., Brandileone,M.C., Dias,W.O. & Leite,L.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,** 5086-5090.

Mizgerd,J.P. 2008. Acute lower respiratory tract infection. *N.Engl.J Med*, **358,** 716-727.

Moffitt,K.L., Gierahn,T.M., Lu,Y.J., Gouveia,P., Alderson,M., Flechtner,J.B., Higgins,D.E. & Malley,R. 2011. T(H)17-based vaccine design for prevention of Streptococcus pneumoniae colonization. *Cell Host Microbe*, **9,** 158-165.

Moffitt,K.L.& Malley,R. 2011. Next generation pneumococcal vaccines. *Curr.Opin.Immunol.*, **23,** 407-413.

Mohammed,B.J., Mitchell,T.J., Andrew,P.W., Hirst,R.A. & O'Callaghan,C. 1999. The effect of the pneumococcal toxin, pneumolysin on brain ependymal cilia. *Microb.Pathog.*, **27,** 303-309.

Mollerach,M., Regueira,M., Bonofiglio,L., Callejo,R., Pace,J., Di Fabio,J.L., Hollingshead,S. & Briles,D. 2004. Invasive Streptococcus pneumoniae isolates from Argentinian children: serotypes, families of pneumococcal surface protein A (PspA) and genetic diversity. *Epidemiol.Infect.*, **132,** 177-184.

Mond,J.J., Lees,A. & Snapper,C.M. 1995. T cell-independent antigens type 2. *Annu.Rev.Immunol.*, **13,** 655-692.

Mooney,J.D., Weir,A., McMenamin,J., Ritchie,L.D., Macfarlane,T.V., Simpson,C.R., Ahmed,S., Robertson,C. & Clarke,S.C. 2008. The impact and effectiveness of pneumococcal vaccination in Scotland for those aged 65 and over during winter 2003/2004. *BMC Infect.Dis.*, **8,** 53.

Morgan,P.J., Harrison,G., Freestone,P.P., Crane,D., Rowe,A.J., Mitchell,T.J., Andrew,P.W. & Gilbert,R.J. 1997. Structural and functional characterisation of two proteolytic fragments of the bacterial protein toxin, pneumolysin. *FEBS Lett.*, **412,** 563-567.

Moschioni,M., Emolo,C., Biagini,M., Maccari,S., Pansegrau,W., Donati,C., Hilleringmann,M., Ferlenghi,I., Ruggiero,P., Sinisi,A., Pizza,M., Norais,N., Barocchi,M.A. & Masignani,V. 2010. The two variants of Streptococcus pneumoniae pilus-1 RrgA adhesin retain the same function and elicit cross-protection in vivo. *Infect.Immun.*.

Mueller,D.L., Jenkins,M.K. & Schwartz,R.H. 1989. Clonal expansion versus functional clonal inactivation: a costimulatory signalling pathway determines the outcome of T cell antigen receptor occupancy. *Annu.Rev.Immunol.*, **7,** 445-480.

Muller-Graf,C.D., Whatmore,A.M., King,S.J., Trzcinski,K., Pickerill,A.P., Doherty,N., Paul,J., Griffiths,D., Crook,D. & Dowson,C.G. 1999. Population

biology of Streptococcus pneumoniae isolated from oropharyngeal carriage and invasive disease. *Microbiology*, **145 (Pt 11),** 3283-3293.

Murdoch,C., Read,R.C., Zhang,Q. & 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,** 1253-1260.

Musher,D.M., Phan,H.M. & Baughn,R.E. 2001. Protection against bacteremic pneumococcal infection by antibody to pneumolysin. *J.Infect.Dis.*, **183,** 827-830.

Musher,D.M., Rueda,A.M., Nahm,M.H., Graviss,E.A. & Rodriguez-Barradas,M.C. 2008. Initial and subsequent response to pneumococcal polysaccharide and protein-conjugate vaccines administered sequentially to adults who have recovered from pneumococcal pneumonia. *J.Infect.Dis.*, **198,** 1019-1027.

Nagler-Anderson,C. 2001. Man the barrier! Strategic defences in the intestinal mucosa. *Nat.Rev.Immunol.*, **1,** 59-67.

Nakamura,S., Davis,K.M. & Weiser,J.N. 2011. Synergistic stimulation of type I interferons during influenza virus coinfection promotes Streptococcus pneumoniae colonization in mice. *J Clin.Invest*, **121,** 3657-3665.

Nandoskar,M., Ferrante,A., Bates,E.J., Hurst,N. & Paton,J.C. 1986. Inhibition of human monocyte respiratory burst, degranulation, phospholipid methylation and bactericidal activity by pneumolysin. *Immunology*, **59,** 515-520.

Nelson,A.L., Roche,A.M., Gould,J.M., Chim,K., Ratner,A.J. & Weiser,J.N. 2007. Capsule enhances pneumococcal colonization by limiting mucus-mediated clearance. *Infect.Immun.*, **75,** 83-90.

Nguyen,C.T., Kim,S.Y., Kim,M.S., Lee,S.E. & Rhee,J.H. 2011. Intranasal immunization with recombinant PspA fused with a flagellin enhances cross-protective immunity against Streptococcus pneumoniae infection in mice. *Vaccine*, **29,** 5731-5739.

O'Brien,K.L., Wolfson,L.J., Watt,J.P., Henkle,E., Deloria-Knoll,M., McCall,N., Lee,E., Mulholland,K., Levine,O.S. & Cherian,T. 2009. Burden of disease caused by Streptococcus pneumoniae in children younger than 5 years: global estimates. *Lancet*, **374,** 893-902.

Obaro,S.K. 2000. Confronting the pneumococcus: a target shift or bullet change? *Vaccine*, **19,** 1211-1217.

Ochs,M.M., Bartlett,W., Briles,D.E., Hicks,B., Jurkuvenas,A., Lau,P., Ren,B. & Millar,A. 2008. Vaccine-induced human antibodies to PspA augment complement C3 deposition on Streptococcus pneumoniae. *Microb.Pathog.*, **44,** 204-214.

Ogunniyi,A.D., Folland,R.L., Briles,D.E., Hollingshead,S.K. & Paton,J.C. 2000. Immunization of mice with combinations of pneumococcal virulence proteins elicits enhanced protection against challenge with Streptococcus pneumoniae. *Infect.Immun.*, **68,** 3028-3033.

Ogunniyi,A.D., Grabowicz,M., Briles,D.E., Cook,J. & Paton,J.C. 2007. Development of a vaccine against invasive pneumococcal disease based on combinations of virulence proteins of Streptococcus pneumoniae. *Infect.Immun.*, **75,** 350-357.

Ogunniyi,A.D., Grabowicz,M., Mahdi,L.K., Cook,J., Gordon,D.L., Sadlon,T.A. & Paton,J.C. 2009. Pneumococcal histidine triad proteins are regulated by the Zn2+-dependent repressor AdcR and inhibit complement deposition through the recruitment of complement factor H. *FASEB J.*, **23,** 731-738.

Ogunniyi,A.D., Woodrow,M.C., Poolman,J.T. & Paton,J.C. 2001. Protection against Streptococcus pneumoniae elicited by immunization with pneumolysin and CbpA. *Infect.Immun.*, **69,** 5997-6003.

Orihuela,C.J., Gao,G., Francis,K.P., Yu,J. & Tuomanen,E.I. 2004. Tissue-specific contributions of pneumococcal virulence factors to pathogenesis. *J.Infect.Dis.*, **190,** 1661-1669.

Pai,R., Limor,J. & Beall,B. 2005. Use of pyrosequencing to differentiate Streptococcus pneumoniae serotypes 6A and 6B. *J.Clin.Microbiol.*, **43,** 4820-4822.

Palmer,M. 2001. The family of thiol-activated, cholesterol-binding cytolysins. *Toxicon*, **39,** 1681-1689.

Paradise, J. L., Rockette, H. E., Colborn, D. K., Bernard, B. S., Smith, C. G., Kurs-Lasky, M., and Janosky, J. E. Otitis media in 2253 Pittsburgh-area infants: prevalence and risk factors during the first two years of life. 99, 318-333. 24-5-1997. Pediatrcs .
Ref Type: Generic

Park,I.H., Pritchard,D.G., Cartee,R., Brandao,A., Brandileone,M.C. & Nahm,M.H. 2007. Discovery of a new capsular serotype (6C) within serogroup 6 of Streptococcus pneumoniae. *J.Clin.Microbiol.*, **45,** 1225-1233.

Paterson,G.K.& Mitchell,T.J. 2006. The role of Streptococcus pneumoniae sortase A in colonisation and pathogenesis. *Microbes.Infect.*, **8,** 145-153.

Paton,J.C.& Ferrante,A. 1983. Inhibition of human polymorphonuclear leukocyte respiratory burst, bactericidal activity, and migration by pneumolysin. *Infect.Immun.*, **41,** 1212-1216.

Paton,J.C., Lock,R.A., Lee,C.J., Li,J.P., Berry,A.M., Mitchell,T.J., Andrew,P.W., Hansman,D. & Boulnois,G.J. 1991. Purification and immunogenicity of genetically obtained pneumolysin toxoids and their conjugation to Streptococcus pneumoniae type 19F polysaccharide. *Infect.Immun.*, **59,** 2297-2304.

Paton,J.C., Rowan-Kelly,B. & Ferrante,A. 1984. Activation of human complement by the pneumococcal toxin pneumolysin. *Infect.Immun.*, **43,** 1085-1087.

Pebody,R.G., Leino,T., Nohynek,H., Hellenbrand,W., Salmaso,S. & Ruutu,P. 2005. Pneumococcal vaccination policy in Europe. *Euro.Surveill*, **10,** 174-178.

Pericone,C.D., Overweg,K., Hermans,P.W. & 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,** 3990-3997.

Pettigrew,M.M., Fennie,K.P., York,M.P., Daniels,J. & Ghaffar,F. 2006. Variation in the presence of neuraminidase genes among Streptococcus pneumoniae isolates with identical sequence types. *Infect.Immun.*, **74,** 3360-3365.

Pichichero,M.E.& Casey,J.R. 2007. Emergence of a multiresistant serotype 19A pneumococcal strain not included in the 7-valent conjugate vaccine as an otopathogen in children. *JAMA*, **298,** 1772-1778.

Pido-Lopez,J., Kwok,W.W., Mitchell,T.J., Heyderman,R.S. & Williams,N.A. 2011. Acquisition of pneumococci specific effector and regulatory Cd4+ T cells localising within human upper respiratory-tract mucosal lymphoid tissue. *PLoS Pathog.*, **7,** e1002396.

Poland,G.A. 1999. The burden of pneumococcal disease: the role of conjugate vaccines. *Vaccine*, **17,** 1674-1679.

Polissi,A., Pontiggia,A., Feger,G., Altieri,M., Mottl,H., Ferrari,L. & Simon,D. 1998. Large-scale identification of virulence genes from Streptococcus pneumoniae. *Infect.Immun.*, **66,** 5620-5629.

Pracht,D., Elm,C., Gerber,J., Bergmann,S., Rohde,M., Seiler,M., Kim,K.S., Jenkinson,H.F., Nau,R. & Hammerschmidt,S. 2005. PavA of Streptococcus pneumoniae modulates adherence, invasion, and meningeal inflammation. *Infect.Immun.*, **73,** 2680-2689.

Prellner,K., Hermansson,A., White,P., Melhus,A. & Briles,D. 1999. Immunization and protection in pneumococcal otitis media studied in a rat model. *Microb.Drug Resist.*, **5,** 73-82.

Price,K.E.& Camilli,A. 2009. Pneumolysin localizes to the cell wall of Streptococcus pneumoniae. *J.Bacteriol.*, **191,** 2163-2168.

Prudhomme,M., Attaiech,L., Sanchez,G., Martin,B. & Claverys,J.P. 2006. Antibiotic stress induces genetic transformability in the human pathogen Streptococcus pneumoniae. *Science*, **313,** 89-92.

Prymula,R., Peeters,P., Chrobok,V., Kriz,P., Novakova,E., Kaliskova,E., Kohl,I., Lommel,P., Poolman,J., Prieels,J.P. & Schuerman,L. 2006. Pneumococcal capsular polysaccharides conjugated to protein D for prevention of acute otitis media caused by both Streptococcus pneumoniae and non-typable Haemophilus influenzae: a randomised double-blind efficacy study. *Lancet*, **367,** 740-748.

Qazi,S. 2007. Global Action Plan for the Prevention and Control of Pneumonia (GAPP): Report of an Informal Consultation. *UNICEF/WHO*.

Quin,L.R., Carmicle,S., Dave,S., Pangburn,M.K., Evenhuis,J.P. & McDaniel,L.S. 2005. In vivo binding of complement regulator factor H by Streptococcus pneumoniae. *J.Infect.Dis.*, **192,** 1996-2003.

Quin,L.R., Onwubiko,C., Moore,Q.C., Mills,M.F., McDaniel,L.S. & Carmicle,S. 2007. Factor H binding to PspC of Streptococcus pneumoniae increases adherence to human cell lines in vitro and enhances invasion of mouse lungs in vivo. *Infect.Immun.*, **75,** 4082-4087.

Rampersaud,R., Planet,P.J., Randis,T.M., Kulkarni,R., Aguilar,J.L., Lehrer,R.I. & Ratner,A.J. 2011. Inerolysin, a cholesterol-dependent cytolysin produced by Lactobacillus iners. *J Bacteriol.*, **193,** 1034-1041.

Rapola,S., Jantti,V., Haikala,R., Syrjanen,R., Carlone,G.M., Sampson,J.S., Briles,D.E., Paton,J.C., Takala,A.K., Kilpi,T.M. & Kayhty,H. 2000. Natural development of antibodies to pneumococcal surface protein A, pneumococcal surface adhesin A, and pneumolysin in relation to pneumococcal carriage and acute otitis media. *J.Infect.Dis.*, **182,** 1146-1152.

Rath,A., Glibowicka,M., Nadeau,V.G., Chen,G. & Deber,C.M. 2009. Detergent binding explains anomalous SDS-PAGE migration of membrane proteins. *Proc.Natl.Acad.Sci.U.S.A*, **106,** 1760-1765.

Ratner,A.J., Aguilar,J.L., Shchepetov,M., Lysenko,E.S. & Weiser,J.N. 2007. Nod1 mediates cytoplasmic sensing of combinations of extracellular bacteria. *Cell Microbiol.*, **9,** 1343-1351.

Ratner,A.J., Hippe,K.R., Aguilar,J.L., Bender,M.H., Nelson,A.L. & Weiser,J.N. 2006. Epithelial cells are sensitive detectors of bacterial pore-forming toxins. *J.Biol.Chem.*, **281,** 12994-12998.

Riboldi-Tunnicliffe,A., Isaacs,N.W. & Mitchell,T.J. 2005. 1.2 Angstroms crystal structure of the S. pneumoniae PhtA histidine triad domain a novel zinc binding fold. *FEBS Lett.*, **579,** 5353-5360.

Riesenfeld-Orn,I., Wolpe,S., Garcia-Bustos,J.F., Hoffmann,M.K. & Tuomanen,E. 1989. Production of interleukin-1 but not tumor necrosis factor by human monocytes stimulated with pneumococcal cell surface components. *Infect.Immun.*, **57,** 1890-1893.

Rijneveld,A.W., van den Dobbelsteen,G.P., Florquin,S., Standiford,T.J., Speelman,P., van Alphen,L. & van der,P.T. 2002. Roles of interleukin-6 and macrophage inflammatory protein-2 in pneumolysin-induced lung inflammation in mice. *J.Infect.Dis.*, **185,** 123-126.

Rioux,S., Neyt,C., Di Paolo,E., Turpin,L., Charland,N., Labbe,S., Mortier,M.C., Mitchell,T.J., Feron,C., Martin,D. & Poolman,J.T. 2011. Transcriptional regulation, occurrence and putative role of the Pht family of Streptococcus pneumoniae. *Microbiology*, **157,** 336-348.

Roche,A.M., King,S.J. & Weiser,J.N. 2007. Live attenuated Streptococcus pneumoniae strains induce serotype-independent mucosal and systemic protection in mice. *Infect.Immun.*, **75,** 2469-2475.

Rodriguez,A., Lisboa,T., Blot,S., Martin-Loeches,I., Sole-Violan,J., De Mendoza,D. & Rello,J. 2009. Mortality in ICU patients with bacterial community-acquired pneumonia: when antibiotics are not enough. *Intensive Care Med.*, **35,** 430-438.

Romero-Steiner,S., Pilishvili,T., Sampson,J.S., Johnson,S.E., Stinson,A., Carlone,G.M. & Ades,E.W. 2003. Inhibition of pneumococcal adherence to human nasopharyngeal epithelial cells by anti-PsaA antibodies. *Clin.Diagn.Lab Immunol.*, **10,** 246-251.

Rosado,C.J., Kondos,S., Bull,T.E., Kuiper,M.J., Law,R.H., Buckle,A.M., Voskoboinik,I., Bird,P.I., Trapani,J.A., Whisstock,J.C. & Dunstone,M.A. 2008. The MACPF/CDC family of pore-forming toxins. *Cell Microbiol.*, **10,** 1765-1774.

Rosenow,C., Ryan,P., Weiser,J.N., Johnson,S., Fontan,P., Ortqvist,A. & Masure,H.R. 1997. Contribution of novel choline-binding proteins to adherence, colonization and immunogenicity of Streptococcus pneumoniae. *Mol.Microbiol.*, **25,** 819-829.

Rossjohn,J., Feil,S.C., McKinstry,W.J., Tweten,R.K. & Parker,M.W. 1997. Structure of a cholesterol-binding, thiol-activated cytolysin and a model of its membrane form. *Cell*, **89,** 685-692.

Rubins,J.B., Charboneau,D., Fasching,C., Berry,A.M., Paton,J.C., Alexander,J.E., Andrew,P.W., Mitchell,T.J. & Janoff,E.N. 1996. Distinct roles for pneumolysin's cytotoxic and complement activities in the pathogenesis of pneumococcal pneumonia. *Am.J.Respir.Crit Care Med.*, **153,** 1339-1346.

Rubins,J.B., Charboneau,D., Paton,J.C., Mitchell,T.J., Andrew,P.W. & Janoff,E.N. 1995. Dual function of pneumolysin in the early pathogenesis of murine pneumococcal pneumonia. *J.Clin.Invest*, **95,** 142-150.

Rubins,J.B., Duane,P.G., Charboneau,D. & Janoff,E.N. 1992. Toxicity of pneumolysin to pulmonary endothelial cells in vitro. *Infect.Immun.*, **60,** 1740-1746.

Rubins,J.B., Duane,P.G., Clawson,D., Charboneau,D., Young,J. & Niewoehner,D.E. 1993. Toxicity of pneumolysin to pulmonary alveolar epithelial cells. *Infect.Immun.*, **61,** 1352-1358.

Rubins,J.B., Mitchell,T.J., Andrew,P.W. & Niewoehner,D.E. 1994. Pneumolysin activates phospholipase A in pulmonary artery endothelial cells. *Infect.Immun.*, **62,** 3829-3836.

Rubins,J.B.& Pomeroy,C. 1997. Role of gamma interferon in the pathogenesis of bacteremic pneumococcal pneumonia. *Infect.Immun.*, **65,** 2975-2977.

Rubinstein,L.J., Garcia-Ojeda,P.A., Michon,F., Jennings,H.J. & Stein,K.E. 1998. Murine immune responses to Neisseria meningitidis group C capsular polysaccharide and a thymus-dependent toxoid conjugate vaccine. *Infect.Immun.*, **66,** 5450-5456.

Sampson,J.S., O'Connor,S.P., Stinson,A.R., Tharpe,J.A. & Russell,H. 1994. Cloning and nucleotide sequence analysis of psaA, the Streptococcus pneumoniae gene encoding a 37-kilodalton protein homologous to previously reported Streptococcus sp. adhesins. *Infect.Immun.*, **62,** 319-324.

Sato,K., Quartey,M.K., Liebeler,C.L., Le,C.T. & 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,** 1140-1145.

Schimmel,P.R.& Flory,P.J. 1967. Conformational energy and configurational statistics of poly-L-proline. *Proc.Natl.Acad.Sci.U.S.A*, **58,** 52-59.

Schneewind,O., Mihaylova-Petkov,D. & Model,P. 1993. Cell wall sorting signals in surface proteins of gram-positive bacteria. *EMBO J.*, **12,** 4803-4811.

Seo,J.Y., Seong,S.Y., Ahn,B.Y., Kwon,I.C., Chung,H. & Jeong,S.Y. 2002. Cross-protective immunity of mice induced by oral immunization with pneumococcal surface adhesin a encapsulated in microspheres. *Infect.Immun.*, **70,** 1143-1149.

Shafeeq,S., Kloosterman,T.G. & Kuipers,O.P. 2011. Transcriptional response of Streptococcus pneumoniae to Zn(2+) limitation and the repressor/activator function of AdcR. *Metallomics.*, **3,** 609-618.

Shah,P., Briles,D.E., King,J., Hale,Y. & Swiatlo,E. 2009. Mucosal immunization with polyamine transport protein D (PotD) protects mice against nasopharyngeal colonization with Streptococcus pneumoniae. *Exp.Biol.Med.(Maywood.)*, **234,** 403-409.

Shah,P.& Swiatlo,E. 2006. Immunization with polyamine transport protein PotD protects mice against systemic infection with Streptococcus pneumoniae. *Infect.Immun.*, **74,** 5888-5892.

Shaper,M., Hollingshead,S.K., Benjamin,W.H., Jr. & Briles,D.E. 2004. PspA protects Streptococcus pneumoniae from killing by apolactoferrin, and antibody to PspA enhances killing of pneumococci by apolactoferrin [corrected]. *Infect.Immun.*, **72,** 5031-5040.

Shapiro,E.D., Berg,A.T., Austrian,R., Schroeder,D., Parcells,V., Margolis,A., Adair,R.K. & Clemens,J.D. 1991. The protective efficacy of polyvalent pneumococcal polysaccharide vaccine. *N.Engl.J.Med.*, **325,** 1453-1460.

Shoma,S., Tsuchiya,K., Kawamura,I., Nomura,T., Hara,H., Uchiyama,R., Daim,S. & Mitsuyama,M. 2008. Critical involvement of pneumolysin in production of interleukin-1alpha and caspase-1-dependent cytokines in infection with Streptococcus pneumoniae in vitro: a novel function of pneumolysin in caspase-1 activation. *Infect.Immun.*, **76,** 1547-1557.

Silva,N.A., McCluskey,J., Jefferies,J.M., Hinds,J., Smith,A., Clarke,S.C., Mitchell,T.J. & Paterson,G.K. 2006. Genomic diversity between strains of the same serotype and multilocus sequence type among pneumococcal clinical isolates. *Infect.Immun.*, **74,** 3513-3518.

Simell,B., Korkeila,M., Pursiainen,H., Kilpi,T.M. & Kayhty,H. 2001. Pneumococcal carriage and otitis media induce salivary antibodies to pneumococcal surface adhesin a, pneumolysin, and pneumococcal surface protein a in children. *J.Infect.Dis.*, **183,** 887-896.

Simell,B., Lahdenkari,M., Reunanen,A., Kayhty,H. & Vakevainen,M. 2008. Effects of ageing and gender on naturally acquired antibodies to pneumococcal capsular

polysaccharides and virulence-associated proteins. *Clin.Vaccine Immunol.*, **15,** 1391-1397.

Singleton,R.J., Butler,J.C., Bulkow,L.R., Hurlburt,D., O'Brien,K.L., Doan,W., Parkinson,A.J. & Hennessy,T.W. 2007a. Invasive pneumococcal disease epidemiology and effectiveness of 23-valent pneumococcal polysaccharide vaccine in Alaska native adults. *Vaccine*, **25,** 2288-2295.

Singleton,R.J., Hennessy,T.W., Bulkow,L.R., Hammitt,L.L., Zulz,T., Hurlburt,D.A., Butler,J.C., Rudolph,K. & Parkinson,A. 2007b. Invasive pneumococcal disease caused by nonvaccine serotypes among alaska native children with high levels of 7-valent pneumococcal conjugate vaccine coverage. *JAMA*, **297,** 1784-1792.

Sinha,A., Levine,O., Knoll,M.D., Muhib,F. & Lieu,T.A. 2007. Cost-effectiveness of pneumococcal conjugate vaccination in the prevention of child mortality: an international economic analysis. *Lancet*, **369,** 389-396.

Sjostrom,K., Spindler,C., Ortqvist,A., Kalin,M., Sandgren,A., Kuhlmann-Berenzon,S. & Henriques-Normark,B. 2006. Clonal and capsular types decide whether pneumococci will act as a primary or opportunistic pathogen. *Clin.Infect.Dis.*, **42,** 451-459.

Skinner,L.J., Beurg,M., Mitchell,T.J., Darrouzet,V., Aran,J.M. & Dulon,D. 2004. Intracochlear perfusion of pneumolysin, a pneumococcal protein, rapidly abolishes auditory potentials in the Guinea pig cochlea. *Acta Otolaryngol.*, **124,** 1000-1007.

Smart,L.E., Dougall,A.J. & Girdwood,R.W. 1989. Pneumococcal serotyping. *J.Infect.*, **18,** 296-298.

Smillie,W., Warnock,G. & White,H. 1938. A study of a type I pneumococcus epidemical the state hospital in Worcester MA. *AM J Public Health*, **3(2),** 293-302.

Sompayrac,L. 2003. *How the Immune Sytem Works*.

Spreer,A., Kerstan,H., Bottcher,T., Gerber,J., Siemer,A., Zysk,G., Mitchell,T.J., Eiffert,H. & Nau,R. 2003. 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,** 2649-2654.

Srivastava,A., Henneke,P., Visintin,A., Morse,S.C., Martin,V., Watkins,C., Paton,J.C., Wessels,M.R., Golenbock,D.T. & Malley,R. 2005. The apoptotic response to pneumolysin is Toll-like receptor 4 dependent and protects against pneumococcal disease. *Infect.Immun.*, **73,** 6479-6487.

Steinfort,C., Wilson,R., Mitchell,T., Feldman,C., Rutman,A., Todd,H., Sykes,D., Walker,J., Saunders,K., Andrew,P.W. & . 1989. Effect of Streptococcus pneumoniae on human respiratory epithelium in vitro. *Infect.Immun.*, **57,** 2006-2013.

Stol,K., van Selm,S., van den,B.S., Bootsma,H.J., Blokx,W.A., Graamans,K., Tonnaer,E.L. & Hermans,P.W. 2009. Development of a non-invasive murine infection model for acute otitis media. *Microbiology*, **155,** 4135-4144.

Stringaris,A.K., Geisenhainer,J., Bergmann,F., Balshusemann,C., Lee,U., Zysk,G., Mitchell,T.J., Keller,B.U., Kuhnt,U., Gerber,J., Spreer,A., Bahr,M., Michel,U. & Nau,R. 2002. Neurotoxicity of pneumolysin, a major pneumococcal virulence factor, involves calcium influx and depends on activation of p38 mitogen-activated protein kinase. *Neurobiol.Dis.*, **11,** 355-368.

Syrjanen,R.K., Kilpi,T.M., Kaijalainen,T.H., Herva,E.E. & Takala,A.K. 2001. Nasopharyngeal carriage of Streptococcus pneumoniae in Finnish children younger than 2 years old. *J.Infect.Dis.*, **184,** 451-459.

Tai,S.S. 2006. Streptococcus pneumoniae protein vaccine candidates: properties, activities and animal studies. *Crit Rev.Microbiol.*, **32,** 139-153.

Talkington,D.F., Brown,B.G., Tharpe,J.A., Koenig,A. & Russell,H. 1996. Protection of mice against fatal pneumococcal challenge by immunization with pneumococcal surface adhesin A (PsaA). *Microb.Pathog.*, **21,** 17-22.

Thornton,J.& McDaniel,L.S. 2005. THP-1 monocytes up-regulate intercellular adhesion molecule 1 in response to pneumolysin from Streptococcus pneumoniae. *Infect.Immun.*, **73,** 6493-6498.

Tilley,S.J., Orlova,E.V., Gilbert,R.J., Andrew,P.W. & Saibil,H.R. 2005. Structural basis of pore formation by the bacterial toxin pneumolysin. *Cell*, **121,** 247-256.

Toellner,K.M., Jenkinson,W.E., Taylor,D.R., Khan,M., Sze,D.M., Sansom,D.M., Vinuesa,C.G. & MacLennan,I.C. 2002. Low-level hypermutation in T cell-independent germinal centers compared with high mutation rates associated with T cell-dependent germinal centers. *J Exp.Med.*, **195,** 383-389.

Tomasz,A. 1999a. New faces of an old pathogen: emergence and spread of multidrug-resistant Streptococcus pneumoniae. *Am.J.Med.*, **107,** 55S-62S.

Tomasz,A. 1999b. The challenge of multiresistant Streptococcus pneumoniae: international initiatives in day-care centers and the use of molecular epidemiologic techniques. *Clin.Microbiol.Infect.*, **5 Suppl 4,** S64-S68.

Tong,H.H., Fisher,L.M., Kosunick,G.M. & DeMaria,T.F. 2000. Effect of adenovirus type 1 and influenza A virus on Streptococcus pneumoniae nasopharyngeal colonization and otitis media in the chinchilla. *Ann.Otol.Rhinol.Laryngol.*, **109,** 1021-1027.

Tong,H.H., Li,D., Chen,S., Long,J.P. & DeMaria,T.F. 2005. Immunization with recombinant Streptococcus pneumoniae neuraminidase NanA protects chinchillas against nasopharyngeal colonization. *Infect.Immun.*, **73,** 7775-7778.

Trzcinski,K., Thompson,C., Malley,R. & Lipsitch,M. 2005. Antibodies to conserved pneumococcal antigens correlate with, but are not required for, protection against pneumococcal colonization induced by prior exposure in a mouse model. *Infect.Immun.*, **73,** 7043-7046.

Tseng,H.J., McEwan,A.G., Paton,J.C. & Jennings,M.P. 2002. Virulence of Streptococcus pneumoniae: PsaA mutants are hypersensitive to oxidative stress. *Infect.Immun.*, **70,** 1635-1639.

Tu,A.H., Fulgham,R.L., McCrory,M.A., Briles,D.E. & Szalai,A.J. 1999. Pneumococcal surface protein A inhibits complement activation by Streptococcus pneumoniae. *Infect.Immun.*, **67,** 4720-4724.

Tuomanen,E., Rich,R. & Zak,O. 1987. Induction of pulmonary inflammation by components of the pneumococcal cell surface. *Am.Rev.Respir.Dis.*, **135,** 869-874.

Tyrer,P., Foxwell,A.R., Cripps,A.W., Apicella,M.A. & Kyd,J.M. 2006. Microbial pattern recognition receptors mediate M-cell uptake of a gram-negative bacterium. *Infect.Immun.*, **74,** 625-631.

Uchiyama,S., Carlin,A.F., Khosravi,A., Weiman,S., Banerjee,A., Quach,D., Hightower,G., Mitchell,T.J., Doran,K.S. & Nizet,V. 2009. The surface-anchored NanA protein promotes pneumococcal brain endothelial cell invasion. *J.Exp.Med.*, **206,** 1845-1852.

Ulanova,M., Tarkowski,A., Hahn-Zoric,M. & Hanson,L.A. 2001. The Common vaccine adjuvant aluminum hydroxide up-regulates accessory properties of human monocytes via an interleukin-4-dependent mechanism. *Infect.Immun.*, **69,** 1151-1159.

Underhill,D.M.& Ozinsky,A. 2002. Phagocytosis of microbes: complexity in action. *Annu.Rev.Immunol.*, **20,** 825-852.

Vainio,A., Fagerlund,R., Melen,K., Lehtinen,M.J., Julkunen,I. & Virolainen,A. 2005. Serum antibodies to putative proteinase maturation protein A in children with acute otitis media. *Vaccine*.

van Ginkel,F.W., McGhee,J.R., Watt,J.M., Campos-Torres,A., Parish,L.A. & Briles,D.E. 2003. Pneumococcal carriage results in ganglioside-mediated olfactory tissue infection. *Proc.Natl.Acad.Sci.U.S.A*, **100,** 14363-14367.

van Rossum,A.M., Lysenko,E.S. & Weiser,J.N. 2005. Host and bacterial factors contributing to the clearance of colonization by Streptococcus pneumoniae in a murine model. *Infect.Immun.*, **73,** 7718-7726.

Veenhoven,R., Bogaert,D., Uiterwaal,C., Brouwer,C., Kiezebrink,H., Bruin,J., IJzerman,E., Hermans,P., de Groot,R., Zegers,B., Kuis,W., Rijkers,G., Schilder,A. & Sanders,E. 2003. Effect of conjugate pneumococcal vaccine followed by polysaccharide pneumococcal vaccine on recurrent acute otitis media: a randomised study. *Lancet*, **361,** 2189-2195.

Vela Coral,M.C., Fonseca,N., Castaneda,E., Di Fabio,J.L., Hollingshead,S.K. & Briles,D.E. 2001. Pneumococcal surface protein A of invasive Streptococcus pneumoniae isolates from Colombian children. *Emerg.Infect.Dis.*, **7,** 832-836.

Virolainen,A., Salo,P., Jero,J., Karma,P., Eskola,J. & 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,** 2667-2670.

Wadowsky,R.M., Mietzner,S.M., Skoner,D.P., Doyle,W.J. & Fireman,P. 1995. Effect of experimental influenza A virus infection on isolation of Streptococcus pneumoniae and other aerobic bacteria from the oropharynges of allergic and nonallergic adult subjects. *Infect.Immun.*, **63,** 1153-1157.

Wadwa,R.P.& Feigin,R.D. 1999. Pneumococcal vaccine: an update. *Pediatrics*, **103,** 1035-1037.

Waltman,W.D., McDaniel,L.S., Gray,B.M. & Briles,D.E. 1990. Variation in the molecular weight of PspA (pneumococcal surface protein A) among Streptococcus pneumoniae. *Microb.Pathog.*, **8,** 61-69.

Wang,S., Li,Y., Scarpellini,G., Kong,W., Shi,H., Baek,C.H., Gunn,B., Wanda,S.Y., Roland,K.L., Zhang,X., Senechal-Willis,P. & Curtiss,R., III 2010a. Salmonella vaccine vectors displaying delayed antigen synthesis in vivo to enhance immunogenicity. *Infect.Immun.*, **78,** 3969-3980.

Wang,S., Li,Y., Shi,H., Scarpellini,G., Torres-Escobar,A., Roland,K.L. & Curtiss,R., III 2010b. Immune responses to recombinant pneumococcal PsaA antigen delivered by a live attenuated Salmonella vaccine. *Infect.Immun.*, **78,** 3258-3271.

Watson,K., Carville,K., Bowman,J., Jacoby,P., Riley,T.V., Leach,A.J. & Lehmann,D. 2006. Upper respiratory tract bacterial carriage in Aboriginal and non-Aboriginal children in a semi-arid area of Western Australia. *Pediatr.Infect.Dis.J.*, **25,** 782-790.

Wellmer,A., Zysk,G., Gerber,J., Kunst,T., von Mering,M., Bunkowski,S., Eiffert,H. & Nau,R. 2002. Decreased virulence of a pneumolysin-deficient strain of Streptococcus pneumoniae in murine meningitis. *Infect.Immun.*, **70,** 6504-6508.

Whalan,R.H., Funnell,S.G., Bowler,L.D., Hudson,M.J., Robinson,A. & Dowson,C.G. 2005. PiuA and PiaA, iron uptake lipoproteins of Streptococcus pneumoniae, elicit serotype independent antibody responses following human pneumococcal septicaemia. *FEMS Immunol.Med.Microbiol.*, **43,** 73-80.

Whalan,R.H., Funnell,S.G., Bowler,L.D., Hudson,M.J., Robinson,A. & 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,** 1031-1038.

Whaley,M.J., Sampson,J.S., Johnson,S.E., Rajam,G., Stinson-Parks,A., Holder,P., Mauro,E., Romero-Steiner,S., Carlone,G.M. & Ades,E.W. 2010. Concomitant administration of recombinant PsaA and PCV7 reduces Streptococcus pneumoniae serotype 19A colonization in a murine model. *Vaccine*, **28,** 3071-3075.

Wheeler,J., Freeman,R., Steward,M., Henderson,K., Lee,M.J., Piggott,N.H., Eltringham,G.J. & Galloway,A. 1999. Detection of pneumolysin in sputum. *J.Med.Microbiol.*, **48,** 863-866.

Witzenrath,M., Pache,F., Lorenz,D., Koppe,U., Gutbier,B., Tabeling,C., Reppe,K., Meixenberger,K., Dorhoi,A., Ma,J., Holmes,A., Trendelenburg,G., Heimesaat,M.M., Bereswill,S., van der,L.M., Tschopp,J., Mitchell,T.J., Suttorp,N. & Opitz,B. 2011b. The NLRP3 Inflammasome Is Differentially Activated by Pneumolysin Variants and Contributes to Host Defense in Pneumococcal Pneumonia. *J Immunol*..

Witzenrath,M., Pache,F., Lorenz,D., Koppe,U., Gutbier,B., Tabeling,C., Reppe,K., Meixenberger,K., Dorhoi,A., Ma,J., Holmes,A., Trendelenburg,G., Heimesaat,M.M., Bereswill,S., van der,L.M., Tschopp,J., Mitchell,T.J., Suttorp,N. & Opitz,B. 2011a. The NLRP3 Inflammasome Is Differentially Activated by Pneumolysin Variants and Contributes to Host Defense in Pneumococcal Pneumonia. *J Immunol*..

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. & Koenig,S. 2001. Use of a whole genome approach to identify vaccine molecules affording protection against Streptococcus pneumoniae infection. *Infect.Immun.*, **69,** 1593-1598.

Wriggers,W., Chakravarty,S. & Jennings,P.A. 2005. Control of protein functional dynamics by peptide linkers. *Biopolymers*, **80,** 736-746.

Wright,A.E., Morgan,W.P., Colebrook,L. & Dodgson,R.W. 1914. Prophyatic inoculations against pneumococcus infections. *Lancet*, **1-10,** 87-95.

Wu,H.Y., Nahm,M.H., Guo,Y., Russell,M.W. & Briles,D.E. 1997a. 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,** 839-846.

Wu,H.Y., Virolainen,A., Mathews,B., King,J., Russell,M.W. & Briles,D.E. 1997b. Establishment of a Streptococcus pneumoniae nasopharyngeal colonization model in adult mice. *Microb.Pathog.*, **23,** 127-137.

Wu,K., Zhang,X., Shi,J., Li,N., Li,D., Luo,M., Cao,J., Yin,N., Wang,H., Xu,W., He,Y. & Yin,Y. 2010. Immunization with a combination of three pneumococcal proteins confers additive and broad protection against Streptococcus pneumoniae Infections in Mice. *Infect.Immun.*, **78,** 1276-1283.

Wuorimaa,T.& Kayhty,H. 2002. Current state of pneumococcal vaccines. *Scand.J.Immunol.*, **56,** 111-129.

Xin,W., Li,Y., Mo,H., Roland,K.L. & Curtiss,R., III 2009. PspA family fusion proteins delivered by attenuated Salmonella enterica serovar Typhimurium extend and enhance protection against Streptococcus pneumoniae. *Infect.Immun.*, **77,** 4518-4528.

Xu,Q., Pichichero,M.E., Casey,J.R. & Zeng,M. 2009. Novel type of Streptococcus pneumoniae causing multidrug-resistant acute otitis media in children. *Emerg.Infect.Dis.*, **15,** 547-551.

Yoo,I.H., Shin,H.S., Kim,Y.J., Kim,H.B., Jin,S. & Ha,U.H. 2010. Role of pneumococcal pneumolysin in the induction of an inflammatory response in human epithelial cells. *FEMS Immunol.Med.Microbiol.*.

Yoon,B.N., Choi,N.G., Lee,H.S., Cho,K.S. & Roh,H.J. 2010. Induction of interleukin-8 from nasal epithelial cells during bacterial infection: the role of IL-8 for neutrophil recruitment in chronic rhinosinusitis. *Mediators.Inflamm.*, **2010,** 813610.

Yother, J. Common themes in hte genetics of streptococcal capuslar polysaccharides, p.164-184 In J. B. Goldberg (ed). 1999. Genetics of bacterial polysaccharides., CRC Press, Boca Raton, FL.
Ref Type: Generic

Yother,J., Handsome,G.L. & Briles,D.E. 1992. Truncated forms of PspA that are secreted from Streptococcus pneumoniae and their use in functional studies and cloning of the pspA gene. *J.Bacteriol.*, **174,** 610-618.

Yother,J.& White,J.M. 1994. Novel surface attachment mechanism of the Streptococcus pneumoniae protein PspA. *J.Bacteriol.*, **176,** 2976-2985.

Yuste,J., Khandavilli,S., Ansari,N., Muttardi,K., Ismail,L., Hyams,C., Weiser,J., Mitchell,T. & Brown,J.S. 2010. The effects of PspC on complement-mediated immunity to Streptococcus pneumoniae vary with strain background and capsular serotype. *Infect.Immun.*, **78,** 283-292.

Zhang,J.R., Idanpaan-Heikkila,I., Fischer,W. & Tuomanen,E.I. 1999. Pneumococcal licD2 gene is involved in phosphorylcholine metabolism. *Mol.Microbiol.*, **31,** 1477-1488.

Zhang,Q., Bagrade,L., Bernatoniene,J., Clarke,E., Paton,J.C., Mitchell,T.J., Nunez,D.A. & Finn,A. 2007. Low CD4 T cell immunity to pneumolysin is associated with nasopharyngeal carriage of pneumococci in children. *J.Infect.Dis.*, **195,** 1194-1202.

Zhang,Q.& Finn,A. 2004. Mucosal immunology of vaccines against pathogenic nasopharyngeal bacteria. *J.Clin.Pathol.*, **57,** 1015-1021.

Zhang,Q., Leong,S.C., McNamara,P.S., Mubarak,A., Malley,R. & Finn,A. 2011. Characterisation of regulatory T cells in nasal associated lymphoid tissue in children: relationships with pneumococcal colonization. *PLoS Pathog.*, **7,** e1002175.

Zhang,Q., Ma,Q., Li,Q., Yao,W. & Wang,C. 2010. Enhanced protection against nasopharyngeal carriage of Streptococcus pneumoniae elicited by oral multiantigen DNA vaccines delivered in attenuated Salmonella typhimurium. *Mol.Biol.Rep.*.

Zhang,Y., Masi,A.W., Barniak,V., Mountzouros,K., Hostetter,M.K. & 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,** 3827-3836.

Zhang,Z., Clarke,T.B. & Weiser,J.N. 2009. Cellular effectors mediating Th17-dependent clearance of pneumococcal colonization in mice. *J.Clin.Invest*, **119,** 1899-1909.

Zhao,H.L., Yao,X.Q., Xue,C., Wang,Y., Xiong,X.H. & Liu,Z.M. 2008. Increasing the homogeneity, stability and activity of human serum albumin and interferon-alpha2b fusion protein by linker engineering. *Protein Expr.Purif.*, **61,** 73-77.

Zhu,B., Cai,G., Hall,E.O. & Freeman,G.J. 2007. In-fusion assembly: seamless engineering of multidomain fusion proteins, modular vectors, and mutations. *Biotechniques*, **43,** 354-359.

Zysk,G., Bejo,L., Schneider-Wald,B.K., Nau,R. & Heinz,H. 2000. Induction of necrosis and apoptosis of neutrophil granulocytes by Streptococcus pneumoniae. *Clin.Exp.Immunol.*, **122,** 61-66.