Screening of *Streptococcus pneumoniae* ABC transporters for their role in virulence and investigation of their lipoprotein components as vaccine candidates

Shilpa Basavanna

University College London

Thesis for doctoral degree (PhD), 2011

Declaration

I, Shilpa Basavanna confirm that the work presented in this thesis is my own. Where information has been derived from other sources, I confirm that this has been indicated in the thesis.

Abstract

Streptococcus pneumoniae causes life-threatening invasive diseases in children and older adults. Although effective at reducing the incidence of disease, the two currently available vaccines against *S. pneumoniae* have significant limitations that a vaccine based on protein antigens may overcome. For this thesis I have investigated the role of *S. pneumoniae* ABC transporters during infection, and have assessed as potential vaccine candidates the lipoprotein components of two ABC transporters.

Eleven ABC transporters were chosen for investigation of their role during infection, and disruption mutant strains were successfully constructed for 9 of these. Two mutant strains disrupting the Sp0148-52 and Sp0749-53 ABC transporters, which BLAST searches suggest have methionine and branched chain amino acids (BCAAs) as substrates respectively, were markedly attenuated in systemic and pulmonary mice models of virulence. Western blotting and PCR confirmed that the lipoprotein components of these ABC transporters, Sp0149 and Sp0749, are present in all the *S. pneumoniae* strains investigated and are membrane-localised. Radioactive and fluorescence ligand binding experiments showed the Sp0749 lipoprotein specifically bound to BCAAs, confirming Sp0749-53 encodes a BCAA ABC transporter.

Vaccination of mice with His_6-Sp0149 and His_6-Sp0749 induced specific IgG which was able to increase complement activity against and phagocytosis of *S. pneumoniae*. Intranasal immunisation of mice with His_6-Sp0749 and His_6-PiaA, affected the immune response to subsequent intranasal challenge with *S. pneumoniae* and increased lung inflammation upon subsequent *S. pneumoniae* intranasal challenge. However, both intraperitoneal and intranasal immunisation of mice with His_6-Sp0149 and His_6-Sp0749 resulted in only minor degrees of protection against *S. pneumoniae* septicaemia and pneumonia respectively. These results demonstrate that the *S.*

pneumoniae BCAA ABC transporter is required for the full virulence, and its lipoprotein component can elicit immune responses against invasive *S. pneumoniae* that although weakly protective could contribute towards a multivalent vaccine.

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Abbreviations

AOM	Acute otitis media
ATP	Adenosine triphosphate
ABC	ATP binding casette
A549	Human alveolar epithelial cell line
ahpD	Alkyl hydroxyl peroxidase D
AMP	Adenosine monophosphate
ARTEMIS	Primer designing software
AliA	Oligopeptide A
AmiACDEF	Oligopeptide ACDEF uptake ABC transporter
AdcABC	Zinc ABC uptake ABC transporter
AIB	amino isobutyric acid
B220	B cell surface marker
Balb/c	Inbred mouse strain
BALF	Bronchoalveolar lavage fluid
BgaA	β-galactosidase
BMEC	human brain microvascular endothelial cells
Blp	Bacteriocin-like peptide
BSA	Bovine serum albumin
BCA	Bicinchonic acid
BALF	Bronchoalveolar lavage fluid
BSA-T	Bovine serum albumin-Tween
BLAST	Basic Local Alignment Search Tool
*Bam*HI	Restriction enzyme
Bp	Base pairs

BCAA	Branched chain amino acids
CAP	Community acquired pneumonia
CNS	Central nervous system
CFU	Colony forming units
CSF	Cerebrospinal fluid
CRM197	7-valent vaccine conjugated to non-toxic mutant diphtheria toxoid
CbpA	Choline binding protein A
CBA/N	Inbred mouse strain
CBP	Choline binding proteins
COS-7	Cell line derived from the kidney cells of African green monkey
C3	Complement component 3
C3b	Component formed by cleavage of C3
iC3b	Inactive product of C3b
C1q	Multivalent complement complex
CRASP1	Complement regulator acquiring surface protein
CRP	C-reactive protein
CtsR	Negative regulator of heat shock response
Clp	Heat shock porteins
CCR	Carbon catabolite repressor
CcpA	Catabolite control protein A
CSP	Competence stimulating peptide
CO_2	Carbon-di-oxide
$CaCl_2$	Calcium chloride

CSP1	Competence stimulating peptide 1
cDNA	complementary DNA
CL	Cell lysates
CD1	Outbred mice
CI	Competetive index
CT	Cholera toxin
CD4	Cluster of differentiation 4
CD8	Cluster of differentiation 8
CD45RB	Cluster of differentiation 45 isoform
CD80	Cluster of differentiation 80
ComABCDE	Competence ABCDE
Cat gene	Chloramphenicol resistance gene
C	Complementation
Cden medium	Chemically defined medium
CDM	Chemically defined medium
^{14}C	Radiocarbon / radioactive isotope of carbon
Co^{2+}	Cobalt
DNA	Deoxyribonucleic acid
D562	Detroit cell line
DOLOP	Database of bacterial lipoproteins
dNTPs	Deoxynucleoside triphosphates
DTT	Dithiothreitol
DOC	Deoxycholic acid / deoxycholate
dNP	dinitrophenol
DMSO	Dimethyl sulfoxide

Δ	Delta symbol represents deletion mutation
ECM	Extracellular matrix
Eno	Enolase
End A	Endonuclease A
EDTA	Ethylenediaminetetraacetic acid
EB buffer	Elution buffer
erm gene	erythromycin gene
ECL	Enhanced chemiluminescence
ELISA	Enzyme linked immunosorbent assay
Fab fragment	Antigen binding fragment
Fc fragment	Crystallizable fragment
FimA	Fimbrial adhesion A
Fbp54	Fibronectin binding protein 54
FeoABC	*Salmonella typhimurium* iron uptake ABC transporter
FT	Flow through
FITC	Fluorescien isothiocyanate
FAMSE	5,6-carboxyfluorescein-succinidyl ester
FACS	Flourescence-activated cell sorting analysis
Fe^{3+}	Ferric iron
F primer	Forward primer
For	Forward primer
GlcNAc	*N*-acetylglucosamine
GalNAc	*N*-acetylgalactosamine
GAPDH	Glyceraldehyde-3-phosphate dehydrogenase
G+C	Guanine+Cytosine

G+LLP	Gram positive lipoprotein recognition pattern
Gln	Glutamime
GF/F	Glass microfibre filters
HIV	Human immunodeficiency virus
hpIgR	Human polymeric immunoglobin receptor
Hic	Factor H binding protein
Hep cell-line	Human laryngeal epidermoid carcinoma cell line
HUVEC	Human umbilical vein endothelial cells
HK	Histidine kinase
H_2O_2	Hydrogen peroxide
HRP	Hrse raddish peroxidase
HBSS	Hanks buffered salt solution
HBSS+ Ca+Mg	Hanks buffered salt solution + calcium + magnesium
HL60	Human promyelocytic leukemia cells
IL	Interleukin
IPD	Invasive pneumococcal disease
IgG	Immunoglobulin G
IgA	Immunoglobulin A
IgA1	Immunoglobulin A1
IL1 β	Interleukin 1 beta
IPTG	Isopropyl ß thiogalactosidase
IP	Intraperitoneal
IN	Intranasal
I-A-I-E	Subregions of major histocompatibility complex II (MHC II)
IQR	Interquartile range

IDM	Insertional duplication mutagenesis
Kb	Kilobases
KDa	Kilodalton
LytA	Autolysin
LTA	Lipotechoic acid
LraI	Lipoprotein receptor-associated antigen I
Lgt	Prolipoprotein diacylglyceryl transferase
Lsp	Prolipoprotein signal peptidase
Lnt	Apolipoprotein N-acyltransferase
LB	Luria Bertani
LIV	Leucine Isoleucine valine
MIP	Macrophage inflammatory protein
MBL	Mannose binding lectin
MSD	Membrane spanning domain
Msm	Multiple sugar binding ABC transporter
MtsABC	Iron uptake ABC transporter
mM	milli moles
µM	micro moles
$MgSO_4$	Magnesium sulfate
mA	milli Ampere
µCi	micro Curie
µL	micro litre
mL	milli litre
MOI	Multiplicity of infection
mRNA	messenger ribonucleic acid

mg ml^{-1}	milligrams per millilitre
MLST	Multi-Locus Sequencing Typing
MHC	Major Histocompatibility Complex
Nan A,B,C	Neuraminidase A, B, C
NETs	Neutrophil extracellular traps
NFAT	Nuclear factor of activated T cells
NO	Nitric oxide
nM	nano moles
nM	nano meters
NAES	Sodium acetate + EDTA + SDS
Ni-NTA	Nickel tagged-Nitrilotriacetic acid
NaH_2PO_4	Sodium phosphate
NCBI	National Center for Biotechnology Information
NaCl	Sodium chloride
N-terminal	Amino terminal of protein sequence
OMPC	Meningococcal outer membrane compex
O-variant	Opaque variant
OMP	Outer membrane proteins
OD	Optical density
OEP	Overlap extension PCR
OP	Opsonophagocytosis
Ori	Origin of replication
ORF	Open reading frame
Ply	Pneumolysin
PPV	Pneumococcal polysaccharide vaccine

PCV	Pneumococcal conjugate vaccine
Pnc D	PCV with diphtheria toxoid as protein carrier
Pnc T	PCV with tetanus toxoid as protein carrier
PspA	Pneumococcal surface protein A
PspC	Pneumococcal surface protein C
PsaA	Pneumococcal surface antigen A
PiaA	Pneumococcal iron acquisition A
PiuA	Pneumococcal iron utilisation A
PpmA	Putative proteinase maturation protein
PrtA	Cell-wall associated precursor protein
PavA	Pneumococcal fibronectin binding protein
PC	Phosphorylcholine
PAFr	Platelet activating factor receptor
PAF	Platelet activating factor
Pce	Phosphorylcholine esterase
pIgR	polymeric immunoglobulin receptor
PPI	Pneumococcal pathogenicity island
PLG	Plasminogen binding proteins
PA	Plasminogen activators
PMNL	Polymorphonuclear leukocytes
Por1A	Porin 1 A
PAMPs	Pathogen associated molecular patterns
PcsB	Protein required for cell division and separation
PotABCD	Polyamine transporter ABCD
PstS	Phosphate specific transporter

PCR	Polymerase chain reaction
PBS	Phosphate buffered saline
PitADBC	Pneumococcal iron transporter ADBC
PO_4 buffer	Phosphate buffer
p-value	Statistical significance
%	Percentage
Rlr islet	RofA like-regulator
Rrg ABC	RlrA regulated gene ABC
ROI	Reactive oxygen intermediates
RNI	Reactive nitrogen intermediates
Reg M,R	Orthologues of Ccp
RR	Response regulator
Rpm	Rotations per minute
RT-PCR	Reverse transcriptase-polymerase chain reaction
RPMI	Roswell Park Memorial Institute
RBC	Red blood cells
R primer	Reverse primer
Rev	Reverse primer
16S rRNA	16 subunit ribosomal ribonucleic acid
ST	Serotype
SrtH	β-*N*-acetylglucosaminidase
ScaA	Streptococcal coaggregation adherence A
SsaB	Streptococcus sanguis adhesion B
SpsA	*S. pneumoniae* secretory IgA binding protein
SIgA	Secretory IgA

SlrA	Streptococcal lipoprotein rotamase
Srt	Sortase
SK-MES-1	Lung squamous cell carcinoma cell line
Spx	Puruvate oxidase
STM	Signature tagged mutagenesis
SBP	Substrate binding protein
SRP	Signal recognition particle
Sec	Sec translocase
Sit ABCD	Salmonella iron transporter
SloABC	*S. mutans* LraI operon
SDS-PAGE	Sodium dodecyl sulfate –polyacrylamide gel electrophoresis
s	seconds
*Sal*I	Restriction enzyme
*Sac*I	Restriction enzyme
TIGR	The Institute of Genomic Research
TNF-α	Tumor necrosis factor alpha
TI	T-cell independent immune response
TA	Teichoic acid
T-variant	Transparant variant
TLR-4	Toll-like receptor-4
TCSTS	Two-component signal transduction system
TCS	Two-component system
THY	Todd-Hewit yeast extract
TBS-T	Tris buffered saline-tween
Tris-HCL	Tris-hydrochloric acid

TSA	Tris sodium chloride azide
TA	Thymine Adenine
Th1, 2 response	T-helper 1, 2 response
USA	United States of America
UK	United Kingdom
UCL	University College London
WHO	World Health Organisatin
W1, 2	Washes 1, 2
*Xba*I	Restriction enzyme
YidC	*E. coli* secretory protein
Ybt	Yersinobactin
Zn^{2+}	Zinc

Acknowledgements

Thank you is an inadequate word to express my gratitude towards Dr. Brown. I consider myself fortunate to have a supervisor like Dr. Brown who has been extremely supportive ever since I started working with him. His invaluable guidance, encouragement and patience has made a lot of difference in the preparation of this thesis. I also convey my thanks to Professor Geoff Laurent, Graduate tutor Dr. Rachel Chambers and my secondary supervisor Dr. Helen Baxenadale for their inputs and constructive criticism. My thesis preparation would not have been fun and enjoyable experience without Jose, Suneeta and Catherine who have given me both technical and emotional support which I will always remember and appreciate. I would also like to express my sincere thanks to Dr. Arthur Hosie and Alex Webb, KCL and Dr. Gavin Thomas, University of York who helped and guided me with radioactive and spectroscopy assays. I also thank Mr. Steve Bottoms for performing histological analysis, and Sylwia Wilkosz who gladly agreed to correct some of my thesis sections inspite of her busy work. I would also like to thank all the CRR members who made the experience memorable. Thanks to UCLH charities and British Lung Foundation for funding this project.

Importantly, I would like to thank my husband, Madhu and lovely daughter, Apthi who have been an inspiration and my parents, brother and in-laws who have always been very supportive.

Dedicated to my mother Gnanamba

Chapter 1
Introduction to *Streptococcus pneumoniae*

1. 1 GENERAL

Streptococcus pneumoniae are Gram-positive, encapsulated cocci, facultative anaerobic and α-haemolytic bacteria usually arranged in short chains. *S. pneumoniae* is a human pathogen which inhabits the upper respiratory tract of healthy individuals and commonly causes diseases such as pneumonia, septicaemia, acute otitis media and meningitis. It can rarely cause other infections such as sinusitis, arthritis, pericarditis and peritonitis. Infants, elderly people and patients with predisposing medical conditions such as chronic lung disease, alcohol abuse, malignant disease, immunosuppressive therapy, diabetes, splenectomy and renal dialysis are particularly susceptible to *S. pneumoniae* infections (McKenzie *et al.*, 2000). Based on the polysaccharide capsule, more than 90 serotypes of *S. pneumoniae* have been identified.

S. pneumoniae belongs to the *Streptococcus mitis-Streptococcus oralis* group (Smit group) of viridans group streptococci, also known as the oral streptococcal group which includes *S. mitis, S. oralis, S. cristatus, S. infantis, S. peroris*. To differentiate *S. pneumoniae* from other viridans group streptococci, four phenotype tests are routinely performed, which are colony morphology, optochin sensitivity and bile solubility tests and agglutination with antipolysaccharide capsular antibodies. However, some *S. pneumoniae* strains resistant to optochin and bile insoluble (bile insoluble-atypical pneumococci) have been reported (Whatmore *et al.*, 2000), and concerns have also been raised on the sensitivity and specificity of the commercially

available agglutination test kits containing capsular antibodies to known *S. pneumoniae* serotypes as *S. pneumoniae* cells lacking capsule (atypical pneumococci) may not react and cross-reaction has been observed with other *S. mitis* group bacteria (Arbique *et al.*, 2004). The availability of complete genome sequence of the capsular serotype 4 strain (TIGR4) of *S. pneumoniae* and virulence studies performed in animal models has allowed conserved virulence genes to be used as an alternative way to identify *S. pneumoniae*. Amplification of virulence genes of *S. pneumoniae* such as autolysin gene (*lytA*), pneumolysin gene (*ply*), pencillin binding protein genes and specific regions of 16s rRNA gene have all been used to identify *S. pneumoniae* from other viridans group streptococci (Arbique *et al.*, 2004), although contradictory results suggest that the *lytA*, and *ply* genes can be occasionally present in both *S. mitis* and *S. oralis* (Kawamura *et al.*, 1999; Muller-Graf *et al.*, 1999; Whatmore *et al.*, 2000). Despite the inaccuracies of each of the above techniques, optochin sensitivity test and bile solubility tests remain the routine methods for identification of *S. pneumoniae*.

1.2 CLINICAL CONSIDERATIONS

1.2.1 Nasopharyngeal carriage

S. pneumoniae is a commensal residing in the upper respiratory tract of healthy children and adults. Transmission of the bacteria between people is through airborne droplets of respiratory secretions (Obaro and Adegbola, 2002). The first step in the nasopharyngeal colonisation of *S. pneumoniae* is adherence of the bacteria on to the epithelial surface with the help of various surface proteins (McCullers and Tuomanen, 2001). Transition from asymptomatic nasopharyngeal carriage to invasive disease is

thought to depend on the balance between the host's defence mechanisms and the ability of the bacteria to cause the disease.

The normal flora of the nasopharynx includes various haemolytic streptococci, *S. pneumoniae, Haemophilus influenzae, Neisseria meningitidis, Staphylococcus aureus* and *Moraxella cattarrhalis* (Bogaert *et al.*, 2004). Despite the genetic dissimilarities between *S. pneumoniae, H. influenzae and N. meningitidis*, they share some common features in their mode of pathogenesis, such as asymptomatic nasopharyngeal carriage with the capability of invading the lungs, blood stream, ear, brain and a tendency to target the elderly and the very young (McCullers and Tuomanen, 2001). Competition between the pathogenic bacteria during the colonisation in the respiratory tract has also been reported, where hydrogen peroxide produced by *S. pneumoniae* is bactericidal to other bacteria residing in the respiratory tract. Phosphorylcholine, a cell wall component produced by *S. pneumoniae* and *H. influenzae* mediates the adherence to the receptor of platelet activating factor leading to competition between the two bacteria. Since phosphorylcholine is antigenic, antibodies raised against one species of bacteria can cross-react and aid in the clearance of various bacterial species also expressing phosphorylcholine (Pericone *et al.*, 2000). Bogaert *et al* and Regev-Yochay *et al* have shown an age effect on the dominant pathogen colonising the nasopharynx. *S. aureus* colonizes the nasopharynx of 50% of children by the age of 10 years compared to 10% in their early years, whilst *S. pneumoniae* colonisaton was highest in infants (43%). Bogaert *et al* performed a cohort study on age-dependent carriage in children and adults aged 1-19 years and found that the peak of *S. pneumoniae* colonisation was 55% at the age of 3 years, declining to 8% after the age of 10 years. Other factors of relevance in *S. pneumoniae* colonisaton include socioeconomic background and environmental factors such as the

number of siblings, larger family size, income, smoking, children attending day care centre and previous respiratory viral infections (Vives *et al.*, 1997) (Bogaert *et al.*, 2004). The incidence of nasopharyngeal carriage in children in developing countries such as Gambia (Lloyd-Evans *et al.*, 1996), Pakistan (Mastro *et al.*, 1993), Papua New Guinea (Gratten *et al.*, 1989), Zambia (Frederiksen and Henrichsen, 1988), Australian aborinal infants (Watson *et al.*, 2006) and the Philippines (Lankinen *et al.*, 1994) is higher than in developed countries.

1.2.2 Diseases caused by *S. pneumoniae*

S. pneumoniae causes less severe mucosal infections such as sinusitis and acute otitis media and also causes more severe diseases such as pneumonia, septicaemia, meningitis. Transition from an asymptomatic carrier state to disease causing state occurs when the bacteria are aspirated from the nasopharynx into the lungs causing pneumonia or when the bacteria penetrate the nasopharyngeal mucosa and invade the blood stream to cause septicaemia and invasion across the blood-brain barrier leading to meningitis (Figure 1.1). *S. pneumoniae* also invades the blood from the lungs as a consequence of pneumonia. Risk of invasive disease after pneumococcal colonisation is higher in certain ethnic groups such as African American, Native American and Alaskan native population.

(a) Acute otitis media

Acute otitis media (AOM) is a common infection of the middle ear and is associated with the upper respiratory tract infections in up to 90% children. Nasopharyngeal carriage is the main risk factor for bacterial AOM. *S. pneumoniae* serotypes isolated from the nasopharynx and the middle ear during AOM are usually the same (Syrjanen

et al., 2005), and the rate of nasopharyngeal carriage is higher in children with respiratory infections and AOM (Syrjanen *et al.*, 2001). A Finnish cohort study performed in children found *S. pneumoniae* in 26% of patients with AOM, with most of the other cases due to *M. cattarrhalis* (23%) and *H. influenzae* (23%) (Kilpi *et al.*, 2001). Synergy between *S. pneumoniae* and the influenza viruses has been reported (Syrjanen *et al.*, 2005) (McCullers, 2006) in which a previous viral infection of the respiratory tract helps the establishment of bacterial superinfection. Influenza virus induces epithelial damage, and impairs the ability of respiratory epithelium to clear pathogens. Furthermore, the neuraminidase activity of the virus cleaves the terminal sialic acid from the cell surface aiding *S. pneumoniae* adherence (McCullers, 2006).

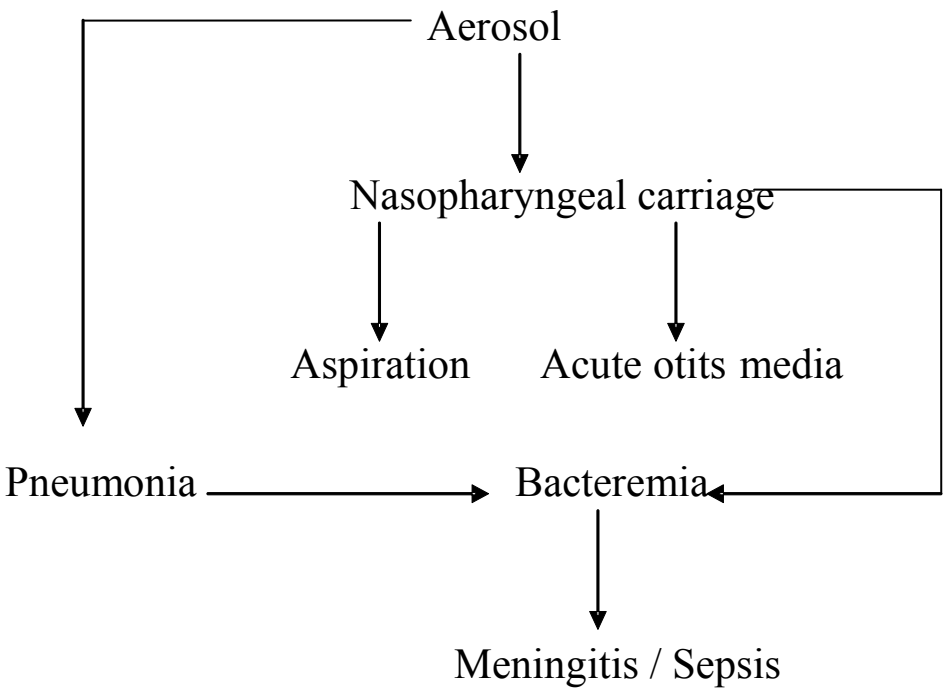


Figure 1.1: Diseases caused by *S. pneumoniae*

(b) Septicaemia

Septicaemia is a systemic infection during which the bacteria invade in to the blood and multiply, and may seed other sites to cause focal disease. Septicaemia caused by *S. pneumoniae* usually occurs secondary to pneumonia, but *S. pneumoniae* is also capable of establishing bacteremia in the absence of evidence of infection, presumably due to direct invasion from the nasophayrnx, a phenomena observed in 15% of cases of bacteremia in children (Gillespie and Balakrishnan, 2000). The incidence of bacteremia caused by *S. pneumoniae* is higher than bacteremia caused by other bacterial pathogens such as *H. influenzae, N. meningitidis* and *S. aureus* in children ≤ 1 year, the incidence decreasing with age (Schutzman *et al.*, 1991; Eskola *et al.*, 1992; Brent *et al.*, 2006). The incidence of bacteremia is also high in people over 65 years of age, and in adults with predisposing medical conditions (Breiman *et al.*, 1990). Other factors contributing to the high incidence of bacteremia are ethnicity, living conditions such as crowding, socioeconomic conditions such as family size, active / passive smoking and recent antibiotic use and age. The incidence of invasive *S. pneumoniae* infection in certain ethnic groups such as Alaskan natives were 4 times higher than non-natives and are associated with lack of breast feeding, attendance of day care centre and smoking tobacco (Gessner *et al.*, 1995; Davidson *et al.*, 1994).

(c) Pneumonia

S. pneumoniae is the commonest cause of community acquired pneumonia (CAP) (Lim, 2004). Translocation of the pneumococci from the nasopharynx into lower respiratory tract (lungs) is thought to occur through microaspiration (Obaro and Adegbola, 2002). *S. pneumoniae* is particularly likely to be established in the lungs in conditions where the host's structural barriers are altered such as damage to epithelial

lining and ciliary function following respiratory viral infection and smoking. The incidence of pneumonia is increased in metabolic and nutritional abnormalities such as diabetes mellitus and vitamin A deficiency (Obaro and Adegbola, 2002) and in patients with impaired immunity such as HIV infection, patients on immunosuppressive therapy and congenital immunoglobulin and complement deficiences (Bogaert *et al.*, 2004). Respiratory tract infections with influenza virus and / or *S. pneumoniae* are a serious worldwide health problem and together are the 6th leading cause of death worldwide (McCullers and Tuomanen, 2001).

(d) Meningitis

Meningitis is usually caused by nasopharyngeal colonisation with *S. pneumoniae* followed by asymptomatic bacteraemia and invasion of the central nervous system (CNS). *S. pneumoniae* meningitis has a 20% mortality and is particularly associated with moderate to severe neurological damage which is observed in 50% of the survivors (Arditi *et al.*, 1998) (Muhe and Klugman, 1999). Invasion of the CNS occurs by crossing the blood-brain barrier but the mechanism used by *S. pneumoniae* to cause meningitis is not properly understood. In the CSF, *S. pneumoniae* can multiply and reach cell densities of 10^9 cfu ml^{-1}, which induces a strong inflammatory reaction in response to cell wall components after autolysis of the bacteria. Proinflammatory cytokines (TNF-α, IL-1 and IL-6) and chemokines [IL-8, macrophage inflammatory protein (MIP-1 and MIP-2)] are known to be raised, which leads to the recruitment of macrophages and granulocytes for the clearance of bacteria. The neurological damage partially results as a consequence of the severity of the inflammatory reaction to *S. pneumoniae* (Meli *et al.*, 2002).

1.3 VACCINES AGAINST *S. PNEUMONIAE*

After *S. pneumoniae* was isolated and cultured by Sternberg and Pasteur simultaneously in 1881, pursuit for an effective vaccine against *S. pneumoniae* infections has continued until today. Although a whole-cell pneumococcal vaccine clinical trial performed in 1911 was unsuccessful, groundwork for the role of antibodies against *S. pneumoniae* capsular polysaccharide was laid which initiated the development of the polyvalent pneumococcal capsular polysaccharide vaccine (Reinert, 2004). At present, the pneumococcal capsular polysaccharide vaccines (PPV) and pneumococcal conjugate vaccine (PCV) have their own advantages and disadvantages. Protein based vaccines may offer some advantages over the PPV and are also currently under investigation.

(a) S. pneumoniae serotype distribution

As the existing vaccines protect against specific *S. pneumoniae* serotypes, the distribution of disease causing serotypes will dictate the potential efficacy of the vaccine at reducing *S. pneumoniae* disease. There are more than 90 serotypes of *S. pneumoniae* and the serotype distribution varies with geographic area and age group. In the USA, serotypes 4, 6, 9, 14, 18, 19, 23 causes 80-90% of invasive diseases, and in Europe the same serotypes cause 61-81% invasive diseases in children. In India, serotypes 14, 8, 19F, 7 and 11 are known to cause systemic and ophthalmic infections (Kar *et al.*, 2006) and serotypes 1, 6, 19, 5, 23 and 7 are prevalent in south India, with serotype 1 being the main cause of meningitis and pneumonia (Kanungo and Rajalakshmi, 2001). In Taiwan, serotypes 14, 23F, 6B, 19F, and 3 were found to be prevalent in 1999-2004**.** The reasons for the variation in serotype prevalence with

geographic location are unclear, but could include socio-economic status and differences in the age distribution in the study population (Hausdorff, 2002).

Table 1.1 *S. pneumoniae* serotype distribution causing invasive pneumococcal diseases (IPD) in various parts of the world (Hausdorff, 2002, Sleeman *et al*, 2001)

Geographical distribution of IPD ST	*S. pneumoniae* ST causing IPD
Developing countries	1, 5, 7, 14, 19, 23
Latin America, W. Europe	1, 3, 4, 6, 7, 9, 14, 15, 18, 19, 23
United Kingdom.	1, 3, 4, 6, 7, 8, 14, 18, 19
Australia, Canada, U.S.A.	4, 6, 9, 12, 14, 18, 19, 23

(b) S. pneumoniae capsular polysaccharide vaccines

The pneumococcal capsular polysaccharide vaccine is a polyvalent vaccine based on the formulation of various capsular polysaccharide antigens. In 1977, a 14-valent capsular PPV was licensed first and was followed by a 23-valent vaccine in 1983 which is still in current use (French, 2003). The capsular polysaccharide antigens included are from 23 serotypes of *S. pneumoniae* that commonly cause invasive disease and provides a broad spectrum of protection (up to 90% of invasive strains) even though the epidemiology of the distribution of serotypes differs according to the geographical area (Jefferson and Demicheli, 2002). In the U.S.A, PPV are recommended for elderly people aged 65 and over, and in children above 2 years or adults with the predispositions to *S. pneumoniae* infections. Although the PPV is effective in healthy adults, it is not recommended to children under the age of 2 years as it only induces a T-cell independent immune response (TI) which is ineffective in

infants. The antibody concentration and protection induced by PPV wanes with time and this vaccine does not effectively induce protection in the elderly (French, 2003). Furthermore PPV induces protection mainly against septicaemia and meningitis and its efficacy at preventing *S. pneumoniae* pneumonia is poor (Huss *et al.*, 2009; Moberley *et al.*, 2008).

(c) S. pneumoniae conjugate vaccines

To improve the efficacy of PPV, epidemiologically important serotypes of *S. pnemoniae* are covalently coupled to protein carriers such as diphtheria toxoid (PncD conjugate vaccine), tetanus toxoid (PncT), and meningococcal outer membrane complex (OMPC). Covalent coupling of the polysaccharide antigens with the carrier proteins converts the immune response to T-cell dependent and increases the immunogenicity.

The first PCV licensed in the U.S in 2000 was 7-valent vaccine conjugated to non-toxic mutant diphtheria toxoid (CRM197), commercially known as Prevenar® (Wyeth-Ayerst laboratories, Philadelphia, USA) and later introduced in Europe in 2001. The serotypes present in the 7-valent PCV are 4, 6B, 9V, 14, 18C, 19F and 23F which cause 51-82% of invasive infections in children and 58% of those causing AOM. Two more PCVs, a 9-valent PCV including the additional serotypes 1 and 5 and an 11-valent vaccine including the additional serotypes 3 and 7V are in clinical trials. Although the addition of 4 more serotypes covers 73-92% of invasive infections, increasing the vaccine valency within the limits of the quantity of protein carrier and also maintaining the immunogenicity is technically difficult.

High antibody responses are observed in children to conjugate vaccines compared to no response to the unconjugated polysaccharide antigens (Feikin *et al.*,

2004). In addition to children, other risk group such as elderly and immunocompromised patients (bone marrow transplant patients, HIV-infected people) who have shown suboptimal immunogenicity to polysaccharide vaccines have exhibited good antibody response to the conjugate vaccine (Reinert, 2004). Clinical trials have shown that conjugated vaccines are very effective at preventing invasive infections due to *S. pneumoniae*, and unlike the unconjugated vaccine also prevent pneumonia and otitis media in children (WHO report, 2007, 2008). In addition, the incidence of *S. pneumoniae* infections caused by vaccine serotypes in adults is reduced presumably due to herd immunity effects (WHO report, 2008).

The main disadvantages with conjugate vaccines are the limitation of the number of capsular serotypes that could be used in vaccine formulation and high costs of its production. As more than 90 capsular serotypes are present and only a limited number of capsular serotypes are used in the conjugate vaccine, serotype replacement may occur, where the 'vaccine serotype' may be replaced by the 'non-vaccine serotype' (Singleton *et al.*, 2007) (Munoz-Almagro *et al.*, 2008). Similarly, the conjugate vaccine formulation may need to be varied based on the geographic area as the prevalence of serotypes in different countries may vary. Although 9 and 11 valent conjugate vaccines offer better protection against *S. pneumoniae* invasive diseases, nevertheless, as the number of serotypes increases, the cost of preparation increases. Due to this reason, there are major difficulties in introducing a conjugated vaccine to developing countries (Ortqvist, 2001).

(d) S. pneumoniae protein vaccines and lipoproteins as vaccines

The limitations posed by the polysaccharide and conjugate vaccines have stimulated research for an alternative *S. pneumoniae* vaccine. Targeting conserved antigens that

are important for survival of *S. pneumoniae* in the host could be a promising vaccination strategy and could be used as an alternative to the polysaccharide and conjugate vaccines. Protein based vaccines are known to induce a thymus-dependent immune response in young children contributing to immunological memory. Hence the development of a protein based vaccine can provide protection against most of the *S. pneumoniae* serotypes, thereby overcoming limitations of serotype-specific protection as observed in capsular polysaccharide vaccines and serotype replacement phenomenon observed in conjugate vaccines. Furthermore, protein based vaccines can be produced by relatively inexpensive recombinant DNA technology.

Several *S. pneumoniae* protein antigens are being investigated and their efficacies as vaccines in animal models are currently under evaluation. Most of the *S. pneumoniae* proteins evaluated in animal models are cell surface proteins, although proteins that are secreted or cytoplasmic have also been invesitgated. Potential protein vaccine candidates that have shown protection in animal models are the choline binding proteins PspA and PspC (also called CbpA), the lipoproteins PsaA, PiaA and PiuA (Brown *et al.*, 2001; Garmory and Titball, 2004; Jomaa *et al.*, 2006), inactivated toxin Ply (Paton, 1998), PpmA, a putative proteinase maturation protein which is important during the secretion of proteins (Overweg *et al.*, 2000), PrtA, a cell-wall associated precursor protein (Bethe *et al.*, 2001) and PavA, a fibronectin binding protein (Swiatlo and Ware, 2003). As it is evident that these proteins contribute at different stages during pathogenesis, a combination of protein antigens rather than a single antigen may provide a superior degree of protection against nasopharyngeal carriage and invasive *S. pneumoniae* diseases (Ogunniyi *et al.*, 2000), (Briles *et al.*, 2000; Brown *et al.*, 2001; Jomaa *et al.*, 2006).

(e) Live, killed and DNA vaccines

Roche *et al* have used *S. pneumoniae* deletion mutant strains lacking capsular polysaccharide, pneumolysin and PspA as live attenuated vaccines. The live attenuated vaccine was able to colonise the upper respiratory tract, demonstrating significant increased levels of serum and mucosal antibody titres, and a single intranasal administration of the live attenuated vaccine without adjuvant was sufficient to induce both systemic and mucosal protection against *S. pneumoniae* challenge (Roche *et al.*, 2007). Oliveria *et al* have expressed PsaA antigen in certain species of lactic acid bacteria such as *Lactobacillus casei, L. plantarum, and L. helveticus*. Following the intranasal inoculation of these lactic acid bacteria expressing PsaA in mice, increased levels of specific IgG and IgA antibodies were obtained. Also the intranasal immunisation with these recombinant lactic acid bacteria reduced the nasopharyngeal colonisation upon *S. pneumoniae* challenge (Oliveira *et al.*, 2006). Similar experiments performed by Campos *et al* in *L. casei* expressing PspA as mucosal vaccine in mice induced specific anti-PspA antibodies, deposition of complement on the surface of *S. pneumonaie* and led to increased survival of immunized mice after a systemic challenge with *S. pneumoniae* (Campos *et al.*, 2008). Hvalbye *et al* demonstrated that the intranasal immunisation of mice with heat inactivated *S. pneumoniae* (serotype 4) induced specific anti-capsular polysaccharide antibodies in serum and mucosal secretions. Following intraperitoneal *S. pneumoniae* serotype 4 challenge, the intranasally immunised mice were protected against both systemic and pulmonary infection (Hvalbye *et al.*, 1999). Lesinski *et al* have synthesised the oligodeoxynucleotides encoding the peptide mimic of the serotype 4 capsular polysaccharide of *S. pneumoniae*, which was then ligated into an expression vector and used for the immunisation in Balb/c mice. Epidermal immunisation of this

DNA vaccine in Balb/c mice was able to elicit antibodies specific to serotype 4 capsular polysaccharide of *S.pneumoniae* (Lesinski *et al.*, 2001). Ferreira *et al* have shown that the DNA vaccine vectors expressing the N-terminal region of PspA confer systemic protection against *S. pneumoniae* in the intraperitoneally immunised mice, and that the level of protection using PspA as a DNA vaccine was similar to that of protection observed with PspA as protein vaccine (Ferreira *et al.*, 2006). Moore *et al* have demonstrated protection against serotype 4 *S. pneumoniae* in the CBA/N mice immunised with PspA DNA and PspA protein using prime / boost strategy and have demonstrated enhanced antibody response using this method (Moore *et al.*, 2006). Table 1.2 summarises the different approaches of experimental immunisation and the efficacy as vaccine candidates are evaluated based on the survival rate after *S. pneumoniae* challenge.

Identifying novel conserved protein antigens and investigating the best protein combinations and / or alternate vaccines such as DNA, live and killed vacines may provide an alternative method to PPV and PCV vaccines that are effective at preventing *S. pneumoniae* nasopharyngeal carriage and invasive infection. However, there is lack of consensus of best method to compare the efficiency of the vaccine candidates due to variations in mouse strain, route of infection, bacterial strain, and adjuvant used.

1.4 VIRULENCE FACTORS INVOLVED IN THE PATHOGENESIS OF *S. PNEUMONIAE* INFECTIONS

As discussed above, *S. pneumoniae* inhabit the nasopharynx as a commensal and spread to different hosts as an aerosol or by mucosal contact (Hava *et al.*, 2003). During invasive disease, various virulence factors of *S. pneumoniae* contribute to the

Table 1.2: Different approaches of immunisation against *S. pneumoniae*

Vaccine candidates	Route of immunisation / Whether successful	*In vivo* models	References
Protein			
Pneumolysin	IN (Yes), IP	Mic	Briles *et al*. 2003, 2000, Ogunniyi *et*
Pneumococcal surface	IV (Yes), IP	Mic	Seo *et al*. 2002, Gor *et al*. 2002, Talkington *et*
Pneumococcal surface	IN (Yes), IV (Yes),	Mic	Briles *et al*. 2000, 2003, Arulanandam *et*
Pneumococcal surface	IN (No), IV (Yes),	Mic	Ogunniyi *et al*. 2001, Balachandran *et*
Pneumococcal histidine triad	SC	Mic	Adamou *et al*. 2001, Hamel et al. 2004, Zang *et*
Neuraminidase A	IP (No), IN	Chinchilla,	Long *et al*. 2004, Lock *et al*.
Autolysin A (lyt	IN (No), IP	Mic	Berry *et al*. 1989, Lock *et al*.
Pneumococcal iron	IN (Yes), IP	Mic	Brown *et al*. 2001, Jomaa *et al*.
Pneumococcal iron	IN (Yes), IP	Mic	Brown *et al*. 2001, Jomaa *et al*.
Novel surface proteins (Sp46, Sp91, Sp128,	SC	Mic	Wizemann *et al*.

Vaccine	Route of immunisation / Whether successful	*In vivo*	Referenc
DNA vaccines + protein			
psp A DNA + Psp A	IM	Mic	Moore *et al*.
Live attenuated *S. pneumoniae*			
Mutant of *cps*, *psp A*	IN	Mic	Roche *et al*.
psa A, *psp A* gene expressed	IN	Mic	Oliveira *et al*. 2006, Campos *et*
Killed *S. pneumoniae*			
Heat inactivated serotype 4	IN	Mic	Hvalbye *et al*.

IP: Intraperitoneal, IM: Intramuscular, IN: Intranasal, IV: Intravenous, SC: Subcutaneous, cps: capsular polysaccharide. Partly

establishment of the bacteria in different parts of the human body such as ear, lungs, blood and brain. Stages important for the development of invasive *S pneumoniae* disease can be divided into the following categories:

(1) adhesion to nasopharyngeal epithelium

(2) invasion of underlying tissue and the blood.

(3) evasion of the host's immune response

(4) induction of inflammation and direct tissue damage

(5) replication and growth *in vivo*

(6) co-ordinated expression of genes responsible for *S. pneumoniae* growth and survival at different stages of infection

Nasopharyngeal epithelial adhesion is the prerequisite for *S. pneumoniae* colonisation which may progress to invasion of the underlying tissues and further dissemination of the bacteria into the blood. While stages 3, 5 and 6 are essential for nasopharyngeal colonisation of *S. pneumoniae*, invasive infection also leads to stage 4, the induction of inflammation. These stages of infection do not necessarily occur in a sequential manner; for example, although invasion usually follows adhesion, growth *in vivo* and coordinated expression of genes in reaction to environmental changes will occur continuously.

Various *S. pneumoniae* virulence factors contribute to each of these stages and their involvement in pathogenesis is discussed in the following section (Table 1.3).

1.4.1 Adhesion

Adherence of *S. pneumoniae* to the mucosa of the nasopharynx is the first step to either remain as a commensal or to progress to cause an invasive disease. *S. pneumoniae* engages cell surface proteins, enzymes and other surface molecules to

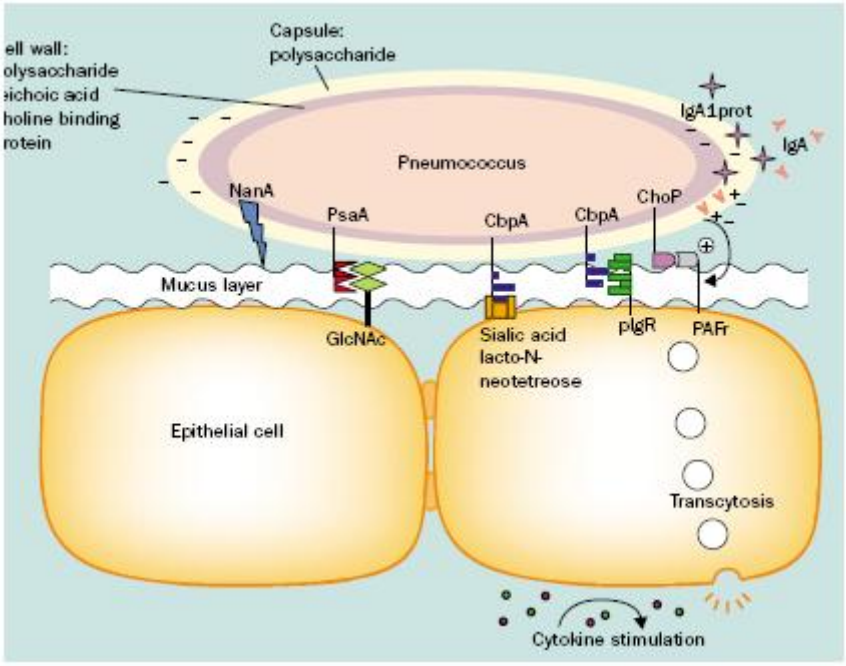


Fig 1.2: Schematic diagram of various virulence factors involved in *S. pneumoniae* adherence to host cells. Adapted from Bogaert *et al.*, 2004.

successfully adhere to the host's tissues. These virulence factors not only initiate the adherence but also promote the localised persistence / colonisation of *S. pneumoniae*, which would otherwise be cleared from the nasopharynx by the host's physiological fluids such as mucus and saliva. *S. pneumoniae* virulence factors often exhibit specificity for the host's cell surface receptors and mediate adhesion (Fig 1.2). Some of the identified *S. pneumoniae* virulence factors known to contribute to *S. pneumoniae* adherence including the capsule, IgA1 protease, phosphorylcholine, neuraminidases, exoglycosidases, pneumococcal surface adhesin, choline binding protein A, pneumococcal adhesion and virulence factor A, streptococcal lipoprotein rotamase and the newly identified streptococcal pili and are discussed below.

(a) Capsule

The capsule of *S. pneumoniae* is the outermost covering comprised of polysaccharide. A possible role of the *S. pneumoniae* polysaccharide capsule in the nasopharyngeal colonisation has been recently identified by Nelson *et al* using both *in vitro* and *in vivo* methods. Histological observations of the nasal tissue of mice following intranasal inoculation of the *S. pneumoniae* wild-type and mutant unencapsulated strains demonstrated that the encapsulated *S. pneumoniae* were able to remain on the mucosal surface for as long as 2 weeks, suggesting the establishment of a stable nasopharyngeal colonisation. Unlike the wild-type strain, the unencapsulated strain of *S. pneumoniae* was agglutinated in the luminal mucus, and was unable to transit to the mucosal surface suggesting a role of capsule in avoiding mucus-mediated clearance of *S. pneumoniae* from the nasopharynx (Nelson *et al.*, 2007). A probable mechanism for the evasion of the mucosal entrapment of encapsulated *S. pneumoniae* is thought to be the electrostatic repulsion created due to similar negative charge imparted by sialic acid of the mucus and the *S. pneumoniae* capsular polysaccharide. This electrostatic repulsion created between mucus and the *S. pneumoniae* capsule may hinder mucociliary clearance of *S. pneumoniae* in the nasal passage and thereby contribute to the early stages of *S. pneumoniae* colonisation (Nelson *et al.*, 2007).

(b) IgA1 protease

IgA1 proteases are enzymes produced by *S. pneumoniae* and other bacteria residing in the upper respiratory tract. These enzymes cleave the IgA1 immunoglobulin which comprises of over 90% of IgA antibody in the respiratory secretions. IgA1 proteases produced by *S. pneumoniae* are cell wall associated (Weiser, 2006), and specifically cleave the proline-threonine or proline-serine peptide bonds at the hinge region of

human IgA1, thereby separating the Fab fragments (antigen-binding region) from the Fc fragment (responsible for secondary effector functions) of the IgA1 antibody. It has been suggested that following binding of the host's IgA1 antibodies to *S. pneumoniae* capsular polysaccharide, secreted IgA1 protease cleaves the Fc region of the IgA1 antibody, and this facilitates *S. pneumoniae* adhesion to host cells. The authors have shown that the incubation of encapsulated, IgA1 protease producing *S. pneumoniae* strain with human IgA1 antibody increased the adherence of *S. pneumoniae* to the Detroit cell line (a human pharyngeal epithelial cell line), where as *S. pneumoniae* mutant strains unable to produce IgA1 protease failed to adhere to the Detroit cell line upon treatment with IgA1 antibody. The suggested mechanism behind IgA1 protease mediated *S. pneumoniae* adherence is that the ionic interactions between IgA1-Fab and the *S. pneumoniae* polysaccharide capsule strips the *S. pneumoniae* polysaccharide capsule from the bacteria, thereby unmasking the cell wall phosphorylcholine (PC) of *S. pneumoniae*. The cell wall PC of *S. pneumoniae* can then interact with the platelet activating factor receptor (PAFr) present on the host epithelial cells for the adherence (Weiser, 2006). However, the specificity of *S. pneumoniae* IgA1 protease to human IgA1 antibodies prevents effective experiments in mouse models of infection to support the above *in vitro* data (Weiser *et al.*, 2003).

(c) Phosphorylcholine (PC)

The cell wall of *S. pneumoniae* plays a very important role in the adhesion of *S. pneumoniae* to the host tissues. The cell wall of *S. pneumoniae* is composed of peptidoglycan, teichoic acid (TA) and lipoteichoic acid (LTA). Although LTA is chemically identical to TA, LTA is attached to the cell membrane by a lipid moiety. Both LTA and TA contain phosphorylcholine (PC), which plays a major role in *S.*

pneumoniae adhesion and also anchors an important class of *S. pneumoniae* surface proteins called choline binding proteins (CBPs). The role of PC in the adherence of *S. pneumoniae* during asymptomatic colonisation is unknown but *in vitro* and *in vivo* experiments have demonstrated an important role of PC in the adherence of *S. pneumoniae* during the conversion from an asymptomatic colonisation to the onset of an invasive disease. Studies have demonstrated that PC of *S. pneumoniae* has increased affinity for a host cell surface receptor, PAFr which is present only on the activated cells, therefore enhancing *S. pneumoniae* adherence in the presence of inflammation (Prescott *et al.*, 2000). PAFr is a G-protein coupled receptor for the platelet activating factor (PAF), a glycerophospholipid which is produced predominantly by platelets as well as epithelial cells, endothelial cells, neutrophils and macrophages. *In vitro* studies have demonstrated PC mediated adherence of *S. pneumoniae* to human endothelial, epithelial and PAFr-transfected COS-7 cells (Cundell *et al.*, 1995) and human tracheal epithelial cells *in vitro* ((Ishizuka *et al.*, 2001). Furthermore, Cundell and colleagues have demonstrated the interaction of *S. pneumoniae* PC with the PAFr in an *in vivo* rabbit model of pneumonia. The authors have observed that following administration of exogenous IL-1 during the intranasal challenge of *S. pneumoniae* in rabbits, there was an increased recovery of *S. pneumoniae* from the bronchoalveolar lavage than from control rabbits, while administration of a PAFr antagonist reduced the colonisation and progression to pneumonia by >90% (Cundell *et al.*, 1995). Further evidence for the importance of PC has been provided by the studies of the cell wall hydrolase, phosphorylcholine esterase, Pce (CbpE) which modulates the amount of PC on the *S. pneumoniae* cell wall. Inactivation of the gene encoding Pce causes an altered *S. pneumoniae* colony morphology and decreased colonisation of the nasopharynx in rats (Vollmer and

Tomasz, 2001). Pce has also been shown to be important for the virulence of some *S. pneumoniae* strains, such as a serotype 3 strain, possibly by increasing the choline residues on the cell wall thereby increasing the affinity to PAFr and hence invasion (Hammerschmidt, 2006).

(d) Neuraminidases (NanA, NanB, NanC)

Neuraminidase enzymes are another group of virulence factors produced by *S. pneumoniae*. These enzymes cause damage to the host tissue by cleaving the terminal sialic acid of glycans, mucins and glycoproteins present on the cell surface, which is thought to expose receptors that facilitate *S. pneumoniae* adherence and invasion of the host tissues. To date three neuraminidases have been identified, NanA and NanB with molecular weight of 108 and 75 kDa respectively (Camara *et al.*, 1994) and NanC which is a homologue of NanB, with as yet unknown function. Both NanA and NanB are produced by all *S. pneumoniae* strains, however NanC, a homologue of NanB, is produced by only some strains of *S. pneumoniae* (Pettigrew *et al.*, 2006). NanA and NanB exhibit very little homology at the amino acid level and the activity of NanA is much higher than NanB (Jedrzejas, 2001). Both enzymes are exported proteins, however NanA has a C-terminal LPXTGX motif suggesting that it is covalently anchored to peptidoglycan. It is unclear why *S. pneumoniae* produces two neuraminidases, but it has been suggested that they may function at different stages during adhesion or invasion and this is supported by the differences in their molecular weights and optimum pH (maximum activity of NanA at pH 5.0, and of NanB is at pH 7.0) (Berry *et al.*, 1996; Jedrzejas, 2001). Both NanA and NanB have been shown to be important for colonisation, pneumonia and sepsis (Manco *et al.*, 2006).

(e) Exogylcosidases

Glycosylated human cell surfaces aid in functions such as cell-cell interactions and binding and transport of positively charged molecules. Glycosylation of human cell surface involves the deposition of sugar residues on the inner mannose residues with later elongation by linkage of *N*-acetylglucosamine (GlcNAc), then galactose residues which are further linked to sialic acid residues (King *et al.*, 2006). *S. pneumoniae* recognises and cleaves the glycosylated surface of the host's nasopharyngeal epithelium to aid its adherence. *S. pneumoniae* has been shown to produce cell-surface associated enzymes, known as exoglycosidases. These include NanA (Camara *et al.*, 1991), β-galactosidase (BgaA) (Zahner and Hakenbeck, 2000) and β-*N*-acetylglucosaminidase (StrH) (King *et al.*, 2006). NanA contributes to the adhesion of *S. pneumoniae* by cleaving the terminal sialic acid present on the host epithelial cells as discussed in an earlier section. BgaA, another *S. pneumoniae* exoglycosidase, specifically cleaves the terminal β(1-4) galactose linked to GlcNAc, and StrH cleaves the terminal β 1-linked *N*-acetylglucosamine residues on the host cell surfaces. Studies have shown that NanA, BgaA and StrH cleave the terminal sialic acid, terminal galactose and terminal *N*-acetylglucosamine in a sequential manner to expose the mannose residues necessary for the adherence of *S. pneumoniae* (King *et al.*, 2006). Although *in vivo* studies of colonisation in infant rats demonstrated no attenuation of virulence following the intranasal inoculation of the *S. pneumoniae* triple mutant strain of *nanA, bgaA* and *strH*, *in vitro* this triple mutant showed significant reduction in adherence to the Detroit cell line when compared to the wild-type *S. pneumoniae* (King *et al.*, 2006). These results suggest that although NanA, BgaA and StrH contribute to *S. pneumoniae* adherence *in vitro* the role *in vivo* of these exoglycosidases remains unclear.

(f) Pneumococcal surface adhesion (PsaA)

PsaA is a 37-kDa surface lipoprotein component of a *S. pneumoniae* manganese uptake ATP binding cassette transporter (ABC transporter) (Berry and Paton, 1996). *psaA*$^-$ mutants have reduced degree of adhesion to type II pneumocytes (A549 cell line) (Berry and Paton, 1996) and the Detroit cell line (D562) (Romero-Steiner *et al.*, 2003) suggesting that PsaA is important for the nasopharyngeal colonisation. PsaA belongs to a family of surface associated proteins called lipoprotein receptor-associated antigen I (Lra I) and the PsaA homologues such as FimA of *Streptococcus. parasanguis* (Burnette-Curley *et al.*, 1995), ScaA of *Streptococcus gordonii* (Kolenbrander *et al.*, 1998) SsaB of *Streptococcus sanguis* (Ganeshkumar *et al.*, 1993) are identified to function as adhesins and some also transport manganese (Kolenbrander *et al.*, 1998). It is unclear why a cation ABC transporter affects adhesion, and it may be that the secondary effects of loss of manganese uptake cause the reduced adhesion of *psaA*$^-$ strains to the epithelial cells. However, a recent study by Anderton *et al*. has demonstrated that the PsaA protein of *S. pneumoniae* specifically binds to the transmembrane glycoprotein, E-cadherin, in D562 monolayers (Anderton *et al.*, 2007). So perhaps PsaA in common with many virulence factors has dual functions during infection, acting as both a direct adhesin and for manganese uptake.

(g) Pneumococcal surface protein C / Choline binding proteinA (PspC / CbpA)

CbpA is a cell surface expressed CBP. CbpA, also known as PspC, SpsA (*S. pneumoniae* secretory IgA binding protein) and Hic (Factor H binding protein) has multiple functions in adhesion during carriage and lung infection (Balachandran *et al.*, 2002). CbpA is known to bind to the human polymeric Ig receptors (hpIgR) produced

by human nasopharyngeal cell lines *in vitro* (Zhang *et al.*, 2000). pIgR is synthesised and exported to the apical surface of epithelial cells. Once at the cell surface, the secretory component of pIgR is released which then binds to IgA, forming secretory IgA (SIgA) (Phalipon and Corthesy, 2003). It has been suggested that the presence of excess free secretory component of IgG and secretory IgA (SIgA) in the mucosal cavity may lead to the binding of CbpA to SIgA or IgG, thereby aiding the adhesion of *S. pneumoniae* to the nasopharynx (Elm *et al.*, 2004; Hammerschmidt *et al.*, 1997). This has been supported by *in vivo* studies, where *S. pneumoniae* mutant strains deficient in CbpA have reduced colonisation of infant rats nasopharynx (Rosenow *et al.*, 1997). In addition, *S. pneumoniae* nasopharyngeal colonisation is reduced in pIgR knockout mice (Zhang *et al.*, 2000; Hammerschmidt, 2006).

In addition to the pIgR binding, *S. pneumoniae* CbpA also exhibits binding to the complement components C3 (Smith and Hostetter, 2000) and factor H (fH, a glycoprotein and also functions as fluid phase regulator of host's complement components). Surface bound fH on *S. pneumoniae* has been shown to prevent the complement mediated opsonophagocytosis of *S. pneumoniae* (Dave *et al.*, 2001) and will be discussed in evasion of immunity section. A recent study has demonstrated that host cell surface fH bound to CbpA thereby mediating the adherence of *S. pneumoniae* to the nasopharyngeal cells (D562 cell line), lung epithelial cells (A549 cell line) and human brain derived endothelial cells *in vitro* (Hammerschmidt *et al.*, 2007). Furthermore, microarray analysis of gene expression demonstrated an upregulation of CbpA by *S. pneumoniae* attached to the nasopharyngeal epithelial cells (Detroit cells) *in vitro*, but not by the *S. pneumoniae* isolated from the blood and CSF of mice suggesting CbpA is important for the interaction of *S. pneumonaie* with epithelium (Orihuela *et al.*, 2004).

(h) Pneumococcal adhesion and virulence factor A (PavA)

Pneumococcal adhesion and virulence factor A (PavA) is an outer cell surface protein, although it lacks both the typical LPXTG anchorage motif of Gram positive cell wall associated proteins or choline binding domains typical of CBPs. PavA binds to immobilized fibronectin, a mammalian glycoprotein present in either soluble or less soluble forms. The soluble form of fibronectin is present in body fluids such as plasma, CSF and amniotic fluid, whereas the less soluble form is present in the extracellular matrix and basement membrane (van der *et al.*, 1995). Binding to fibronectin is observed for the oral bacterium, *S. gordonii,* which belongs to the mitis group of streptococci, and for other Gram positive bacteria such as *S. aureus* and *S. pyogenes*, and is thought to be important for adherence to epithelial cells. PavA of *S. pneumoniae* exhibits 67% amino acid identity to Fbp54, a fibronectin binding protein of *S. pyogenes* (Holmes *et al.*, 2001). *pavA*$^{-}$ strains of *S. pneumoniae* exhibited reduced adherence to A549 and Hep-2 epithelial cells *in vitro* suggesting that it also has a role for adhesion of *S. pneumoniae* in the nasopharynx (Pracht *et al.*, 2005).

(i) Streptococcal lipoprotein rotamase (SlrA)

SlrA is a *S. pneumoniae* lipoprotein and belongs to the family of peptidyl-prolyl isomerases (PPIases). Although the function of SlrA is not known, SlrA has been demonstrated to be important for the nasopharyngeal colonisation of *S. pneumoniae* in mice, as the mutant strains of SlrA exhibited increased clearance from the nasopharynx when compared to the wild-type *S. pneumoniae*. Moreover, *in vitro* adherence studies also demonstrated reduced adherence of the mutant strains of SlrA

to the Detroit cell line suggesting that SlrA may play an important role during adhesion of *S. pneumoniae* to the host cells (Hermans *et al.*, 2006).

(j) Pili

The presence of the pili and their role in *S. pneumoniae* adhesion has been recently identified (Barocchi *et al.*, 2006). *S. pneumoniae* pili are long appendages that extend beyond the polysaccharide capsule and *S. pneumoniae* capsular serotypes with better colonising and invasive potential such as serotype 4 and 19F have been identified to possess pili. The *S. pneumoniae* pilus is encoded by a pathogenicity island (PI), the *rlr* islet, which consist of DNA segments that differ in genetic content compared to the same location in strains that do not possess pili. PIs confer particular virulence traits on bacterial pathogens, and are often present in pathogenic but absent in non pathogenic strains of the same or related species (Hacker and Kaper, 2000). The *rlrA* pathogenicity islet is a cluster of 7 genes found in approximately 27% of the invasive *S. pneumoniae* isolates (Aguiar *et al.*, 2008) . Of the 7 genes, three of them (*rrgA, rrgB, rrgC*) encode proteins with LPXTG motifs suggesting they are surface proteins, and 3 encode for the sortase homologues *srtB, srtC* and *srtD* which catalyse the covalent attachment of *rrgA, rrgB, rrgC*, which contain LPXTG motifs, to the bacterial cell wall (Hava *et al.*, 2003). RrgA is an important pilus subunit and is responsible for increased adherence to the human respiratory epithelial cell line (A549 cell line) *in vitro* (Nelson *et al.*, 2007). *S. pneumoniae* mutant strains expressing no RrgA have a significant reduction in their adherence to the A549 cell line even though they still form pili, suggesting that the expression of RrgA on the pili is essential for adhesion. In support of the *in vitro* adherence studies, in a mouse model the *rrgA*$^{-}$ mutant strain was significantly reduced in its ability to colonise the nasopharynx

compared to the wild-type *S. pneumoniae*. In contrast, *rrgB*$^{-}$ and *rrgC*$^{-}$ mutant strains of *S. pneumoniae* did not exhibit any significant difference *in vitro* adherence studies or in nasopharyngeal colonisation of mice when compared to the wild-type *S. pneumoniae* (Nelson *et al.*, 2007).

(k) Phase variation

S. pneumoniae undergoes a spontaneous but reversible phenomenon known as phase variation. Three different phase variants have been identified, transparent (T), semitransparent and opaque phase variants (O) (Weiser *et al.*, 1994). T variants of *S. pneumoniae* are thought to be better colonisers of the nasopharynx due to the presence of high levels of TA and PC in the cell wall and higher expression of CbpA aiding adherence, whereas O variants are thought to be capable of escaping phagocytosis during blood stream infections or septicaemia because of the presence of more capsular polysaccharide (Cundell *et al.*, 1995). The frequency of spontaneous phase variation is known to be strain specific among *S. pneumoniae* serotypes, and is relatively low.

In vivo colonisation in the infant rat model demonstrated that the transparent variants of *S. pneumonaie* showed significantly higher nasopharyngeal colonisation than the opaque variants (Weiser *et al.*, 1994). The *in vivo* observations were supported by the *in vitro* adhesion studies where the T variants of *S. pneumoniae* exhibited increased adherence compared to opaque variants to the buccal epithelial, cytokine-activated A549 lung epithelia (which leads to increased expression of PAFr on the cell surface), and vascular endothelial cell lines. *In vitro* adhesion assays of *S. pneumoniae* to various carbohydrate sugars, which represent the glycoconjugate receptors such as Gal, GalNAc, GlcNAc on the resting epithelial cell surface,

demonstrated increased adherence of T variants to these immobilized glycoconjugates compared to the O variants of *S. pneumoniae* (Cundell *et al.*, 1995). These results suggest that phase variation of *S. pneumoniae* is an important phenomenon where the relative levels of capsule and PC expression are altered to enable better colonisation.

Overall, a large range of virulence factors have been identified that influence *S. pneumoniae* adherence to cell lines, some of which are important for nasopharyngeal colonisation. Why there is such variety in adhesins and variety in their corresponding host ligands is not at all clear, and whether each adhesin has a role at different stages of infection or whether there is broad overlap in their particular roles and therefore a great deal of redundancy requires further investigation.

1.4.2 Invasion of tissues

Following *S. pneumoniae* adhesion to the nasopharynx, invasion of the underlying tissues to cause diseases such as pneumonia and / or meningitis occurs through the interaction of *S. pneumoniae* virulence factors with the host and probably as a consequence of the host inflammatory response as well. *S. pneumoniae* is known to produce enzymes and harbour surface proteins that degrade the host's extracellular matrix (ECM) allowing access to deeper tissues and thus promoting invasion. Dissemination of *S. pneumoniae* into deeper tissues can occur by the paracellular route which involves breaking of tight junctions or by intracellular transmigration (transcytosis) of epithelial and endothelial cells. Intracellular transmigration requires the internalisation of *S. pneumoniae* by non-phagocytic host cells such as epithelial and endothelial cells and is thought to provide a survival advantage for bacteria which would otherwise be cleared by immune cells.

Some of the identified *S. pneumoniae* virulence factors known to contribute to tissue invasion include hyaluronidase, plasminogen binding proteins, PC, CbpA, PavA and pneumolysin are discussed below.

(a) Hyaluronidase

Hyaluronidase, an enzyme produced by *S. pnemoniae* degrades hyaluronic acid, a chemical component of the connective tissue. Degradation of the connective tissue helps in gaining access into the blood stream. It has been demonstrated that some *S. pneumoniae* strains with higher hyaluronidase acivity are capable of escaping into the blood brain barrier to cause meningitis (Kostyukova *et al.*, 1995). *S. pneumoniae* strains isolated from patients with meningitis and meningoencephalitis have exhibited an increased hyaluronidase activity than strains isolated from patients with otitis media (Volkova *et al.*, 1994; Gillespie and Balakrishnan, 2000)**.**

(b) Plasminogen (PLG) binding proteins

S. pneumoniae possesses housekeeping enzymes which are involved in various metabolic functions, but certain housekeeping enzymes also contribute to virulence. Two key housekeeping enzymes of the glycolytic pathway, α-enolase (Eno) and glyceraldehyde-3-phosphate dehydrogenase (GAPDH) may have important roles for *S. pneumoniae* virulence (Bergmann *et al.*, 2003). Both Eno and GAPDH are surface exposed and are displayed on the cell wall of *S. pneumoniae* and are proteolytic in nature. These enzymes lack the signal peptide and cell wall anchoring motifs and their mechanism of secretion is not known. Eno exhibits affinity to both plasminogen and plasmin and GAPDH to plasmin (Bergmann *et al.*, 2004; Kolberg *et al.*, 2006). Plasmin is an enzyme which is essential for the degradation of extracellular matrix by

fibrinolysis for vessel formation and angiogenesis. *S. pneumoniae* and other pathogens are known to bind to host plasmin which might facilitate invasion and further dissemination by leading to degradation of the extracellular fibrin matrix deposited at sites of infection. Plasminogen however is a pro-enzyme form of plasmin and its conversion to the active form is mediated by the host's physiological plasminogen activators (PA) such as tissue-type PA or urokinase PA which hydrolyse plasminogen. However several streptococcal and staphylococal bacterial pathogens are known to secrete PAs which bind to plasminogen, including *S. pneumoniae* enolase and GAPDH (Ehinger *et al.*, 2004). The inability of eno mutant strains of *S. pneumoniae* to dissolve fibrin and their attenuation in pulmonary mouse models of infection suggest the role of *S. pneumoniae* enolase in plasmin-mediated invasion of host tissues (Bergmann *et al.*, 2005).

(c) PC

PC contributes to the invasion by facilitating the *S. pneumoniae* transmigration across the host cell barriers. *In vitro* transmigration studies were performed using monolayers of rat and human brain microvascular endothelial cells (BMEC) to understand the role of *S. pneumoniae* PC in tissue invasion. Transparent variants were able to transcytose from the apical surface to the basal surface of rat and human BMEC via binding of PC to the PAFr, even though both the transparent and opaque variants of *S. pneumoniae* were able to enter into the endothelial cells. This study demonstrates the selective preference of the *S. pneumoniae* transparent variants which exhibit higher levels of PC, therefore specific binding to the host's cell-surface associated PAFr during tissue invasion when compared to *S. pneumoniae* opaque variants (Ring *et al.*, 1998).

The *in vivo* models of *S. pneumoniae* invasion demonstrated reduced mortality and delayed progression to pneumonia in $PAF^{-/-}$ mice than the wild-type mice, following the intranasal inoculation of *S. pneumoniae*, suggesting the PAFr is exploited by *S. pneumoniae* to progress from adhesion to invasion (Rijneveld *et al.*, 2004).

(d) Pav A

Pav A has been shown to contribute not only to the adhesion but also invasion, as the $PavA^{-}$ strains were attenuated in virulence both in murine pulmonary and also septicaemia models of *S. pneumoniae* infection. *In vitro* invasion studies demonstrated decreased internalisation of $PavA^{-}$ strains of *S. pneumoniae* by human brain-derived microvascular endothelial cells and human umbilical vein derived endothelial cells. These results suggest that PavA could be necessary for translocation of *S. pneumoniae* across cellular layers, although the mechanisms involved are not described (Pracht *et al.*, 2005).

(e) Pneumolysin (Ply)

Pneumolysin is a toxin present in the bacterial cytoplasm with a molecular weight of 35 kDa, produced by all the clinically important serotypes of *S. pneumoniae*. Pneumolysin belongs to the thiol-activated cytolysin family, which are cholesterol binding toxins that permeabilize the cell membrane, aggregate and fuse the cells to form a large vacuole (Tilley *et al.*, 2005). Pneumolysin lacks the N-terminal signal sequence required for secretion and is known to be secreted during the late logarithmic growth phase and the release of pneumolysin is dependent on autolysin causing *S. pneumoniae* autolysis (Cockeran *et al.*, 2002). Contradictory reports have

shown that the pneumolysin is also produced during the early logarithmic growth independent of autolysin and the mechanism may vary between strains of *S. pneumoniae* (Balachandran *et al.*, 2001).

Pneumolysin contributes to the host tissue invasion by disrupting the alveolar capillary boundary and cell junctions in the lungs. This in turn provides the required nutrients for *S. pneumoniae* and helps the penetration of the bacteria into the epithelium to obtain access to the blood stream (Jedrzejas, 2001). During acute otitis media, the hair cells of cochlea are highly sensitive to the cytotoxic effects of pneumolysin (Tuomanen, 2000). *In vitro* studies have demonstrated that exogenous pneumolysin and *S. pneumoniae* secreting pneumolysin causes severe damage to the human brain microvascular endothelial cell line representing an *in vitro* blood brain barrier suggesting its possible role during meningitis (Zysk *et al.*, 2001). *In vitro* studies have also demonstrated that *S. pneumoniae* culture filtrates containing pneumolysin and pneumolysin alone are both capable of damaging the respiratory epithelium and slowing its ciliary function (Feldman *et al.*, 1990).

1.4.3 Evasion of host immunity

For successful colonisation of the nasopharynx and / or the invasion of host's tissue and blood *S. pneumoniae* will also have to evade the host's immune response to infection. Clearance of bacterial pathogens from the host requires the innate and adaptive immune responses and the components of complement system are important in both these responses. The complement system consists of around 30 serum and cell surface proteins organised into three different enzyme cascades termed the classical, alternative and mannose binding lectin (MBL) pathways. Each pathway is activated by different mechanisms but all result in opsonisation of bacteria with breakdown

products (C3b and iC3b) of the central complement component C3 (Walport, 2001). Complement, in particular (Alper *et al.*, 1970; Gross *et al.*, 1978; Winkelstein, 1981; Sampson *et al.*, 1982; Mold *et al.*, 2002) the classical and alternative pathways, and phagocytosis (Giebink *et al.*, 1977) are both vital for immunity to *S. pneumoniae*, and it is not surprising that *S. pneumoniae* has several mechanisms which affect these elements of host immunity as discussed below.

(a) Polysaccharide capsule and cell wall

The polysaccharide capsule is an important virulence determinant of *S. pneumoniae* and is thought to aid the bacterium by inhibiting complement activity and phagocytosis, thereby allowing the *S. pneumoniae* to multiply in the blood stream and possibly cause septicaemia. The capsule consists of high molecular weight polymers made up of repeating units of oligosaccharides containing 2-8 monosaccharides. Oligosaccharides such as glucuronic acid, arabinitol, ribitol and phosphorylcholine may be present in the capsule. More than 90 capsular serotypes have been identified based on the sugar composition and the linkages (Hardy *et al.*, 2000). The degree of virulence of a particular serotype is dependent on the chemical composition rather than the thickness of the capsule, and perhaps reflects the varying ability of different serotypes to evade complement-dependent and independent opsonophagocytosis and hence to establish invasive infection (AlonsoDeVelasco *et al.*, 1995).

As well as conferring protection against phagocytosis and complement deposition, the capsule of *S. pneumoniae* inhibits neutrophil extracellular traps (NETs)-mediated killing. NETs are extracellular fibrous structures consisting of DNA backbone, histones and neutrophil granular proteins produced by activated neutrophils in the host as the first line defense against bacterial pathogens (Wartha *et al.*, 2007). It

has been demonstrated that NETs capture *S. pneumoniae* thereby confining the infection, and reducing the spread of the bacteria to other parts of the host (Beiter *et al.*, 2006). However encapsulated *S. pneumoniae* serotypes have reduced trapping by NETs and also exhibited resistance to NET-mediated killing. In addition to the polysaccharide capsule, D-alanylation of another surface- exposed polymer, the LTA of *S. pneumoniae* also confers resistance to NET-mediated killing. Incorporation of D-alanine residues into LTA imparts positive charge on the bacterial surface thereby repelling the antimicrobial peptides (AMPs) secreted by neutrophils which are used to recognise and kill the bacteria. D-alanylation of LTA has been shown to contribute to the virulence of *S. pneumoniae* during the early stages of invasive infection when capsule expression is low (Wartha *et al.*, 2007)

(b) Pneumococcal surface protein A (PspA)

Pneumococcal surface protein A (PspA) is a CBP which is anchored to the *S. pneumoniae* cell wall and has a molecular weight of 84 KDa. PspA is immunogenic and elicits protective antibodies (McDaniel *et al.*, 1994). PspA prevents both classical and alternative pathway mediated C3b deposition on *S. pneumoniae* (Tu *et al.*, 1999) and *pspA*$^{-}$ strains are reduced in virulence during systemic infection (Yuste *et al.*, 2005; Briles *et al.*, 2000). Addition of anti-PspA antibodies enhanced the deposition of C3 on the surface of PspA+ strains of *S. pneumoniae*.

PspA also interacts with human lactoferrin, an iron-binding glycoprotein present in milk and mucosal secretions that is also released by polymorphonuclear leukocytes during inflammation. Lactoferrin is bactericidal in nature and inhibits cytokine and complement activation, as well as potentially inhibiting bacterial growth by sequestering iron. Lactoferrin is formed of hololactoferrin, containing the binding

site for Fe^{2+} or Fe^{3+}, and apolactoferrin. Apolactoferrin, is thought to be bactericidal by membrane destabilisation upon interaction with *S. pneumoniae*, and PspA is known to bind and prevent apolactoferrin-mediated killing of *S. pneumoniae*. Interaction of PspA with human lactoferrin has been demonstrated in fresh human saliva suggesting that PspA may contribute to carriage by preventing apolactoferrin-mediated killing of *S. pneumoniae* (Shaper *et al.*, 2004).

(c) Pneumolysin

In addition to the inhibition of the mucocliliary clearance (ie physical defences) by pneumolysin which has been discussed in the invasion of host tissues, pneumolysin also contributes to the evasion of host's immunity. Pneumolysin is known to activate C1q directly or indirectly by binding to the Fc receptor of IgG *in vitro* thereby activating the complement by classical pathway (Rossjohn *et al.*, 1998; Mitchell *et al.*, 1991). Using unvaccinated human sera and mice sera with no prior exposure to *S. pneumoniae* Yuste and collegues have demonstrated an increased *in vitro* C3 deposition on the *ply*$^-$ mutant compared to wild-type *S. pneumoniae*. The above mentioned *in vitro* result was supported by *in vivo* mixed infection experiments in wild-type and complement-deficient mice demonstrating that pneumolysin affects classical pathway-mediated complement immunity against *S. pneumoniae*. Yuste and collegues have also showed that the PspA and pneumolysin interactions with complement are synergistic and allows the progress of infection from lungs to the blood (Yuste *et al.*, 2005). However, it is not clear how pneumolysin prevents the complement deposition on *S. pneumoniae*. Extracellularly released pneumolysin may divert complement activity away from the bacterial surface and also consume the available C3 upon binding to C1q (Paton, 1996).

As well as preventing complement activity, pneumolysin has been shown to have direct inhibitory effects on white cells. *In vitro* results have shown that highly purified pneumolysin is capable of interfering with the bactericidal function of polymorphonuclear leukocytes (PMNL) and is shown to inhibit the PMNL antimicrobial function at an extremely low dose thereby enhancing the virulence of *S. pneumoniae* (Paton and Ferrante, 1983)**.**

(d) CbpA

S. pneumoniae evades complement-mediated innate immunity by recruiting complement factor H (FH), a complement alternative pathway inhibitor. *S. pneumoniae* CbpA and its allelic variants (Janulczyk *et al.*, 2000; Dave *et al.*, 2001) have been demonstrated to bind to human FH. FH is a 155-kDa plasma glycoprotein, which inhibits the alternative pathway of the complement system by preventing the binding of factor B to C3b, enhancing the decay of the C3-convertase (C3bBb) and acting as a cofactor for the cleavage of C3b by complement factor I (Lu *et al.*, 2006). Deposition of FH on host tissue and cellular surfaces prevents nonspecific damage and avoids the wasteful consumption of complement components (Lu *et al.*, 2006). Interaction of CbpA with FH leads to the degradation of C3b, which further prevents the opsonisation of *S. pneumoniae* by the components of the alternative complement pathway (Shaper *et al.*, 2004; Tai, 2006). Receptors similar to CbpA are expressed in other pathogenic bacteria that bind to FH such as M6 of group A streptococci (Kotarsky *et al.*, 1998), YadA of *Yersinia enterocolitica* ((Roggenkamp *et al.*, 1996), Por1A of *N. gonorrhoea* (Ram *et al.*, 1998), and CRASP1 of *Borrelia burgdorferi* (Rossmann *et al.*, 2007). Using an *in vivo* mouse model, mice intranasally infected with *S. pneumoniae* preincubated with FH had increased bacteraemia and lung

invasion compared to that of mice challenged with D39 alone (Quin *et al.*, 2007). As discussed above, using *in vitro* adherence assays, Quin and collegues have also demonstrated that *S. pneumoniae* preincubated with FH showed increased adherence to human cell lines such as endothelial (HUVEC) and lung epithelial cells (SK-MES-1).

(e) Endonuclease A

One of the characteristic features of *S. pneumoniae* pneumonia is the neutrophil infiltration to the site of infection. Recently it has been shown that the activated neutrophils release neutrophil extracellular traps (NETs) which are antimicrobial proteins bound to DNA scaffold. NETs not only function by killing the bacteria extracellularly but may confine the trapped bacteria in lungs during pneumonia thereby reducing the possible spread of the pathogen to cause septicaemia. Bacterial pathogens such as *S. enterica*, serovar Typhimurium, *Shigella flexneri*, *S. aureus* and the yeast *Candida. albicans* are trapped and killed by NETs. Although NETs are unable to kill *S. pneumoniae*, they prevent the rapid dissemination of *S. pneumoniae* to other compartments of the host. EndA produced by *S. pneumoniae* have the ability to degrade the extracellular DNA present in NETs (Beiter *et al.*, 2006). *endA* mutant strains of *S pneumoniae* are unable to degrade NETs and after intranasal challenge in mice are out-competed by wild-type *S. pneumoniae* both in the lungs and the bloodstream (Beiter *et al.*, 2006).

1.4.4 Inflammation, tissue damage and the induction of septic shock

Powerful inflammatory response may be mediated by immune cells such as macrophages during the bacterial clearance from the host or may also be triggered

directly by *S. pneumoniae* virulence determinants upon interaction with host cells. The inflammatory response consists of the release of cytokines in response to cytotoxic metabolites released upon recognition of the *S. pneumoniae* virulence determinants which are also called as pathogen associated molecular patterns (PAMPs). Inflammatory response may also occur as the result of *S. pneumoniae* mediated injury to host cells, activation of complement cascade, and the release of nitric oxide and H_2O_2 produced by the host and / or *S. pneumoniae*. The inflammatory response may lead to tissue injury and have detrimental effects on the host like septic shock. *S. pneumoniae* virulence determinants such as pneumolysin, cell wall components, autolysin and pyruvate oxidase that are known mediators of inflammation and tissue injury are discussed in this section.

(a) Pneumolysin

Pneumolysin by itself can reproduce the acute lung injury associated with fatal *S. pneumoniae* pneumonia (Feldman *et al.*, 1991). Treatment of a mouse model of disease with exogenous pneumolysin can cause increased lung vascular permeability, decreased resident alveolar macrophage population and recruitment of neutrophil and monocytes in the alveolar space (Maus *et al.*, 2004). Pneumolysin is known to injure immune (Paton and Ferrante, 1983; Nandoskar *et al.*, 1986) and respiratory cells (Rubins *et al.*, 1993; Rubins *et al.*, 1992) *in vitro* and is shown to activate the phospholipase A in the pulmonary artery endothelium. The activated phospholipase A breaks down different phospholipids present in the cell membrane and releases free fatty acids and lysophosphatides. The metabolites released due to lysophosphatides are cytotoxic and recruit activated neutrophils causing more lung injury and inflammation. The inflammation is further enhanced by pneumolysin as it is capable

of activating the classical complement pathway without the need of specific antibody (Paton *et al.*, 1984). Pneumolysin is also known to induce the release of nitric oxide and inflammatory cytokines such as tumour necrosis factor-α (TNF- α), interleukin-6 (IL-6) (Malley *et al.*, 2003), and IL1β (Shoma *et al.*, 2008; Malley *et al.*, 2003; Houldsworth *et al.*, 1994) by macrophages. Pneumolysin in combination with H_2O_2 induces apoptosis of brain cells during experimental *S. pneumoniae* meningitis (Braun *et al.*, 2002). Protection against *S. pneumoniae* infections is mediated by TLR-4 mediated pneumolysin induced apoptosis (Srivastava *et al.*, 2005; Malley *et al.*, 2003). Recently it has been demonstrated that *S. pneumoniae* pneumolysin activates nuclear factor of activated T cells (NFAT) signalling pathway independently of TLR which in turn upregulates the expression of inflammatory mediators (Koga *et al.*, 2008). Overall, pneumolysin has a variety of mechanisms which induce inflammation and seems to make a significant contribution to the strong pro-inflammatory nature of *S. pneumoniae* infection.

(b) Cell wall and cell wall polysaccharides and Autolysin (LytA)

Cell wall components of *S. pneumoniae* are mainly composed of peptidoglycan, TA and LTA induce acute inflammation during *S. pneumoniae* otitis media, septicaemia, pneumonia and meningitis. Cell wall components have been shown to mimic the symptoms of infection in animal models of otitis media (Ripley-Petzoldt *et al.*, 1988), pneumonia (Tuomanen *et al.*, 1987) and meningitis (Tuomanen *et al.*, 1986). The majority of the inflammation associated with host defence could be driven by TA and LTA, which also can activate the alternative complement pathway (Winkelstein and Tomasz, 1978). LTA and TA bind to the acute phase reactant C-reactive protein (CRP) via their PC component and so can also activate the classical complement

pathway (Winkelstein and Tomasz, 1978). TA containing cell wall fragments have been shown to induce leukocytosis and increase vascular permeability in pneumonia (Tuomanen *et al.*, 1987) and meningitis (Tuomanen *et al.*, 1985) in animal models of infection. The *S. pneumoniae* cell wall is also responsible for the induction of cytokines such as IL-1, IL-6, TNFα and PAF upon binding to epithelia, endothelia and macrophages (Riesenfeld-Orn *et al.*, 1989; Heumann *et al.*, 1994; Cabellos *et al.*, 1992) and induce procoagulant activity on the surface of endothelial cells (Geelen *et al.*, 1992).

Autolysin (Lyt A) is an enzyme that lyses *S. pneumoniae* by degrading the cell wall components. Autolysin is a surface protein and belongs to CBPs anchored non-covalently to the choline present in the cell wall instead of the usual type of anchorage using the LPXTG motif. Autolysin is produced during the late logarithmic growth phase and contributes to the inflammation by cell wall degradation and *S. pneumoniae* lysis as this releases LTA- and TA-containing cell wall products as well as pneumolysin (Gillespie and Balakrishnan, 2000).

(c) Pyruvate oxidase (Spx)

S. pneumoniae is a facultative anaerobe and due to its inability to produce catalase fails to neutralise reactive oxygen intermediates (ROI) and so produces large amounts of hydrogen peroxide (H_2O_2). In addition to the production of H_2O_2, *S. pneumoniae* infection also induces the production by host cells such as phagocytes, eosinophilic granulocytes and mononuclear phagocytes of anti-bacterial reactive nitrogen intermediates (RNI) and nitric oxide (NO). Although ROI and RNI produced by host cells are antimicrobial in nature, they also damage host cells and peroxynitrite, a toxic oxidant produced by combination of ROI and RNI compounds, cause cell death.

Although cytotoxicity due to the production of reactive intermediates by host cells is not completely understood, H_2O_2 produced by *S. pneumoniae* is apoptotic in nature (Hoffmann *et al.*, 2006).

1.4.5 Replication and growth *in vivo*

As well as adhering to host tissues and avoiding immune responses, all pathogenic bacteria have to be able live and replicate in the physiological conditions found in the host, that is at a temperature of 37°C, an osmolality of approximately 290 milliosmolality per kg, high oxygen tension, low availability of iron and other micronutrients and restricted nutritional opportunities. This has been emphasised by signature tagged mutagenesis (STM) screens for identifying genes involved in the virulence of micro-organisms, which usually identify large numbers of genes involved in growth and replication under stress conditions (Polissi *et al.*, 1998; Lau *et al.*, 2001; Hava and Camilli, 2002). One such group were sugar uptake and metabolism genes which included phosphotransferase system (PTS) and ABC transporters (Tettelin *et al.*, 2001)

Investigation of PPI1 (pathogenicity island 1) by Brown *et al* led to the identification of a three gene operon *phgABC* also required for the full virulence both in pulmonary and systemic models of infection. The disruption of this operon impairs the growth of *S. pneumonaie* under high osmotic and oxidative conditions (Brown *et al.*, 2004). Alkyl hydroxyl peroxidase D (ahpD) is important for resistance to killing by H_2O_2 *in vitro*, suggesting that the operon is responsive to oxidative stress, and is required for *S. pneumoniae* virulence in pneumonia and bacteraemia models of infection in mice (Paterson *et al.*, 2006). PcsB is an essential hydrolase which aids in the separation and cell division of *S. pneumoniae* and the *in vitro* results have

demonstrated that the *pcsB* expression is upregulated during stress such as the presence of high temperature and osmolarity. However, *in vivo* data to demonstrate the role of *S. pneumoniae* PcsB in virulence is not yet available (Mills *et al.*, 2007).

Another large group of proteins that are important for the replication and survival of *S. pneumoniae in vivo* are ABC transporters which will be discussed in detail in the section on ATP binding cassette transporters.

1.4.6 Regulation of *S. pneumoniae* virulence

STM screening of 3 different serotypes of *S. pneumoniae* (serotypes 3, 4, 19F) have identified transcriptional regulators necessary for the regulation of virulence genes during various stages of disease establishment and also in response to the host's (external) stimuli. Progression from colonisation to invasive disease involves the adaptation of *S. pneumoniae* to different environmental niches in the host, during which many virulence factors are differentially expressed. It is therefore likely that *S.pneumoniae* undergoes controlled changes in the expression of various virulence factors during different stages of disease development and in different sites of infection. This hypothesis is supported by microarray data of transcriptional factors from mice models showing variations in gene expression (Orihuela *et al.*, 2004). There are 33 identified *S. pneumoniae* transcriptional regulators 20 transcription factors (Tettelin et al., 2001) (Hava *et al.*, 2003) and 13 two-component signal transduction systems (TCSTS) (Throup *et al.*, 2000) (Lange *et al.*, 1999) whose role in the regulation of virulence were either demonstrated or described as putative based on the sequence data. However, unlike other major pathogens such as *S. pyogenes*, as yet no regulator has been shown to control an invasive virulence phenotype for *S.*

pneumoniae. Several regulators are known to influence virulence and are discussed below but the exact mechanisms involved are often not clear.

CtsR is a transcription factor that negatively regulates the expression of the *S. pneumoniae* Clp ATP-dependent protease, which in turn regulates the expression of LytA, CbpA, CbpE, CbpF and CbpJ and also contributes to the genetic transformation of *S. pneumoniae* (Chastanet *et al.*, 2001), colonisation in the nasopharynx and survival in the lungs after intranasal challenge thereby contributing towards *S. pneumoniae* virulence (Kwon et al., 2004)**.** Another transcriptional regulator termed carbon catabolite repression (CCR) allows the utilisation of preferred sugars by silencing the genes specific for nonpreferred sugars. Catabolite control protein A (CcpA), another regulator, regulates sugar metabolism and is required for *S. pneumoniae* colonisation and survival in lungs (Iyer *et al.*, 2005). RegM, which is a homologue of CcpA, is required for the growth of *S. pneumoniae* in blood and hence contributes to virulence. Mutation of RegM results in the reduced transcription of the capsular polysaccharide biosynthesis locus (Giammarinaro and Paton, 2002). RegR, another transcriptional regulator has been shown to regulate hyaluronidase activity of *S. pneumoniae* and controls competence. It also regulates the *S. pneumoniae* adherence to A459 epithelial cells *in vitro* and also contributes to *S. pnemoniae* virulence *in vivo* (Chapuy-Regaud *et al.*, 2003).

TCSTS or TCS of *S. pneumoniae* consists of 13 histidine kinases (HK) paired with response regulators (RR) and an orphan unpaired RR. Of the 13 TCS, 10 have been shown to play role in the virulence of *S. pneumoniae* including the orphan RR. TCS12 consists of a HK encoded by comD and an RR encoded by comE and responds to competence-stimulating peptide (CSP), activates competence and also contributes to *S. pneumoniae* virulence. TCS05 also called CiaRH, was the first TCS to be

identified and is known to affect virulence, competence and antibiotic resistance. TCS13 (also called *blp*TCS for bacteriocin-like peptide) controls a 16-gene quorum-sensing regulon which regulates the synthesis and export of bacteriocin-like peptides and immunity proteins. Bacteriocins are bacterial products which inhibit the growth of related strains or species of bacteria and immunity proteins protect the bacteriocin-producing bacteria. The role of the *blp* product of *S. pneumoniae* in lung is unclear but they might be cytotoxic to host cells as well as other bacteria (Paterson *et al.*, 2006).

In conclusion, there is some evidence for several regulators that are important for virulence but little is understood about the role of different transcriptional regulators within specific sites during infection such as lungs and blood. At present the research into the regulation of *S. pneumoniae* gene expression is confused as various regulators have significant differences in their regulons when two different strains have been investigated. Further studies are required to understand the regulation of virulence-associated genes in *S. pneumoniae* (McCluskey et al., 2004).

1.5 ATP BINDING CASSETTE TRANSPORTERS (ABC TRANSPORTERS)

ATP binding cassette transporters are proteins that are widespread among both prokaryotic and eukaryotic organisms. In bacteria, ABC transporters are involved in the import of wide variety of substrates such as sugars, amino acids, peptides, polyamines, sulphate and metal ions such as iron, molybdate. They are also responsible for the export of capsular polysaccharide in Gram-negative bacteria, secretion of antibiotics by antibiotic resistant bacteria and drug -resistant bacteria and secretion of extracellular toxins, role in translational regulation and DNA repair.

Table 1.3: Role of *S. pneumoniae* virulence factors in

Class	Virulence factor	Role *in vivo*				
		Adhesion Replication	Invasion	Immune	Inflammation	
Carbohydrate	Capsule	✓				
	PC	✓	✓	✓		
	LTA,				✓	
CBPs	CbpA	✓	✓	✓		
	PspA			✓		
Cell wall		✓	✓			
PavA		✓				
Enzymes /	IgA1 protease	✓				
Toxins	Nan A, B, C	✓				
	Exoglycosidases	✓				
	Hyl		✓			
	PLGs-α enolase		✓			
	-GAPDH		✓			
	Ply		✓	✓	✓	
	EndA			✓		
	LytA				✓	
	Pyruvate				✓	
Lipoproteins	PsaA	✓				✓
	PiuA					✓
	PiaA					✓
	PitA					✓
	SlrA	✓				
	Adc					✓

Class	Virulence factor	Role *in vivo*				
		Adhesion	Invasion	Immune evasion	Inflammation	Replication / survival
Others	PhgABCD					✓
	AhpD					✓
	PcsB					✓
Transcriptional regulators of virulence gene expression	CcpA					✓
	RegM					✓
	RegR					✓
	Lsp					✓
Two-component systems	TCS02					✓
	TCS04					✓
	TCS05					✓
	TCS06					✓
	TCS09					✓
	TCS12					✓
	TCS13					✓

Approximately 5% of the *E. coli* and *B. subtilis* genomes are encoded by the components of ABC transporters (Garmory and Titball, 2004).

1.5.1 Structure of ABC transporters

The structure of ABC transporters differ in both Gram positive and Gram negative bacteria. A typical ABC transporter consists of four membrane-associated domains also known as transmembrane domains or integral membrane domains. Integral membrane domain consists of two ATP binding domains (ATPase) and two membrane spanning domains (MSD / permeases) which may be fused in many ways to form multidomain polypeptides. A typical MSD consists of six putative α-helical transmembrane segments which act as a channel through which the substrates are transported into the cell. Permeases or MSDs are also known as bacterial importers and are bound to the solute binding proteins (SBPs) in both Gram positive and Gram negative bacteria. In Gram negative bacteria, SBPs are also known as periplasmic solute binding proteins as they bind to the substrates and import them into the inner membrane. Outer membrane proteins (OMPs), such as porins, import the substrates across the outer membrane from the external environment. In contrast, as the Gram positive bacteria lacks the periplasmic space and outer membrane, the solute binding proteins are anchored to the outer surface of the cell with the help of lipid moiety (lipoproteins) aiding in the transport of substrates (Garmory and Titball, 2004; Davidson *et al.*, 2008) (Fig 1.3).

1.5.2 Processing of lipoproteins

Lipoproteins are a group of surface proteins performing important functions such as antibiotic resistance, substrate binding, adhesions, protein secretion, sensing, spore

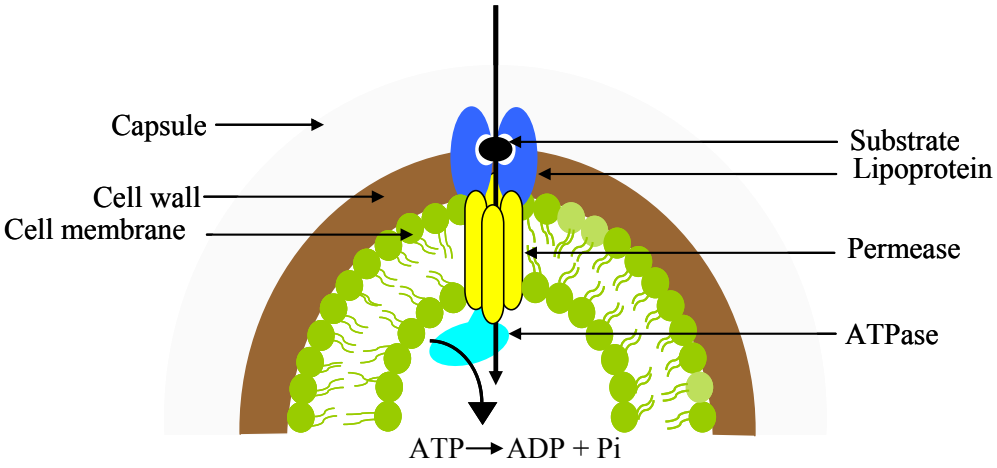


Figure 1.3: Schematic diagram of Gram positive ABC transporter.

formation, conjugation etc (Sutcliffe and Russell, 1995). The annotated genome sequence of *S. pneumoniae* has 47 lipoproteins in TIGR4 strain (Bergmann and Hammerschmidt, 2006). Lipoproteins in various Gram positive and Gram negative bacterial pathogens are known to be important during infection (Pennini *et al.*, 2006; Reglier-Poupet *et al.*, 2003), while some of them can induce protective immune responses by the host (Sadziene and Barbour, 1996). The investigated *S. pneumoniae* lipoproteins that are important in virulence and known to induce protection against *S. pneumoniae* are PiuA, PiaA (Jomaa *et al.*, 2006; Brown *et al.*, 2001) and PsaA (Seo *et al.*, 2002; Miyaji *et al.*, 2001).

Lipoprotein processing in bacteria is of 'Braun' type and is conserved among prokaryotes (Sutcliffe and Harrington, 2002). Three different enzymes are required for the processing of the lipoproteins: Lgt (prolipoprotein diacylglyceryl transferase), Lsp (prolipoprotein signal peptidase) and Lnt (apolipoprotein N-acyltransferase) in Gram negative bacteria such as *E. coli* and *S. typhimurium* Lgt and Lsp are known to be involved in the lipoprotein processing of low G+C content Gram positive bacteria such

as *Bacillus subtilis* (Fig 1.4) (Sutcliffe and Russell, 1995; Sankaran *et al.*, 1995). Various bioinformatics analyses have been used to predict bacterial lipoproteins (G+LPP (Sutcliffe and Harrington, 2002), LipoP (Juncker *et al.*, 2003) and DOLOP (Madan and Sankaran, 2002). In Gram positive bacteria, the N-terminal amino acid sequence of the unmodified prolipoprotein (signal peptide sequence) is followed by a lipobox which is a unique amino acid sequence [LVI] [ASTVI] [GAS] C where leucine is highly conserved and cysteine is strictly conserved. It is to this cysteine residue that the cell membrane lipid is anchored through a disulphide bridge (prolipoprotein). Following the lipid attachment, the enzyme Lsp cleaves the prolipoprotein removing the signal peptide amino acid sequence. In *E. coli* it has been shown that the localization of lipoproteins in the inner membrane is directed by aspartic acid at the +2 position (Masuda *et al.*, 2002) and the translocation of the processed lipoprotein is known to occurs through the SRP/Sec/YidC secretory pathway (Froderberg *et al.*, 2004). However, the mechanisms controlling the lipoprotein localization and translocation in Gram positive bacteria are not known and need to be investigated. Recent studies in *Listeria monocytogenes*, *Streptococcus agalactiae* and *S. uberis* have identified that Lsp can still cleave the unlipidated prolipoprotein (Baumgartner *et al.*, 2007; Henneke *et al.*, 2008; Denham *et al.*, 2009) indicating that the processing of lipoproteins may not follow a sequential pattern. However, Denham *et al* have identified that the full length unprocessed prolipoproteins were shed from the *S. uberis* cells independent of Lsp (Denham *et al.*, 2009).

1.5.3 Roles of ABC transporters in *S. pneumoniae* and other bacterial pathogens

ABC transporters in pathogenic bacteria are known to play important roles in the nutrient acquisition, antibiotic resistance and other functions essential for the survival in

the host, thereby contributing to the virulence of the bacteria. Such ABC transporters from various pathogenic bacteria have been identified by *in vivo* screening techniques such as signature tagged mutagenesis (STM) which allows the large scale identification of attenuated phenotype of mutant strains. STM of *S. pneumoniae* has revealed the virulence associated ABC transporter genes homologous to polyamine transport in *E. coli* (*potA* and *potF*), glutamine transport in *Bacillus subtilis* (*glnH and glnQ*) and sugar transport in *S. mutans* (*msmK*) (Polissi *et al.*, 1998). Similarly, STM performed in *Yersinia* spp. identified phosphate and nitrogen uptake ABC transporters (Darwin and Miller, 1999). In *Staphylococcus aureus*, STM identified oligopeptide transporter genes which formed the largest class of genes required for full virulence (Coulter *et al.*, 1998). Other ABC transporters known to contribute to the virulence in the infection models are metal ion ABC transport systems such as iron, zinc, manganese as metal ions act as important cofactors for various metabolic pathways. Iron-binding siderophores, haemoproteins or glycoproteins used to acquire iron have been identified in *Yersinia. pestis*. These are also known as yersiniabactin (Ybt) (Fetherston *et al.*, 1999), FeoABC (Boyer *et al.*, 2002), iron uptake ABC transporter, SitABCD which transports both manganese and iron are required for the full virulence in *Salmonella enterica* serovar Typhimurium (Janakiraman and Slauch, 2000). Piu and Pia are iron uptake ABC transporters required for the full virulence of *S. pneumoniae* (Brown *et al.*, 2001). The PsaABC manganese transporter is known to transport manganese and is also acts as an adhesin

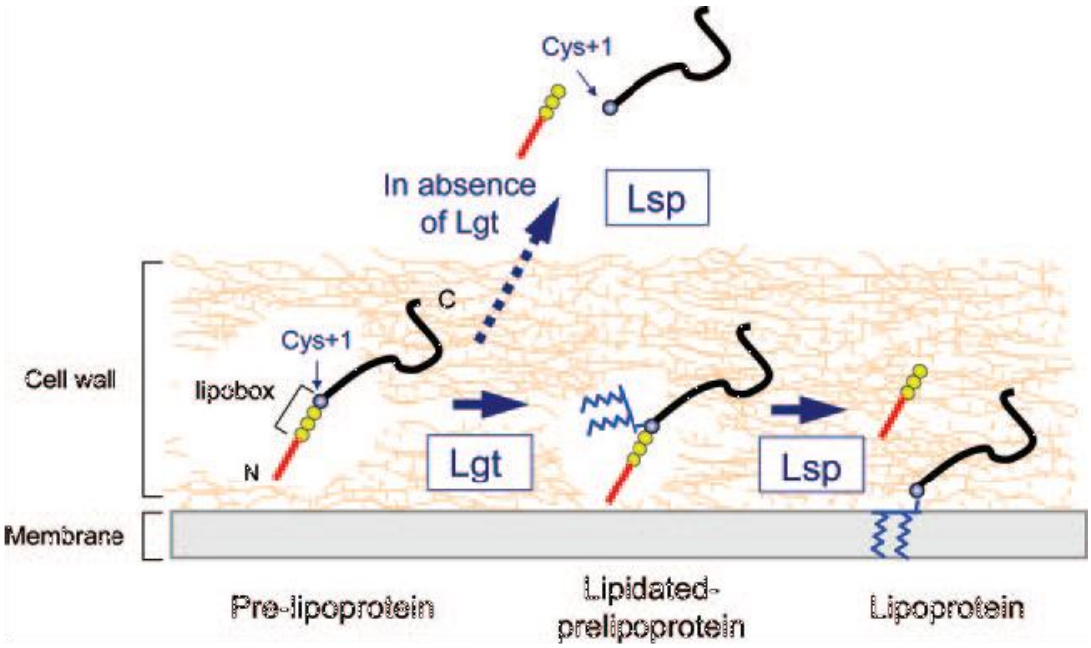


Figure 1.4: Processing of the lipoproteins in Gram positive bacteria described by (Garcia-del and Cossart, 2007; Sutcliffe and Harrington, 2002)

for pneumocytes (Berry and Paton, 1996). MtsABC of *Streptococcus pyogenes* (Janulczyk *et al.*, 2003) and SloABC of *Streptococcus mutans* transport both iron and manganese (Paik *et al.*, 2003).

The annotated *S. pneumoniae* TIGR 4 genome contains 73 ABC transporters (Harland *et al.*, 2005) of which 33 genes encode putative lipoproteins, 24 of which are organised as operons with genes encoding other components of ABC transporter proteins (ATPases, permeases and or additional lipoproteins). Eleven of these have been previously described, (Table 1.4). However the roles of the remaining ABC transporters in *S. pneumoniae* virulence need to be investigated.

1.5.4 Role of components of bacterial ABC transporters as vaccine candidates

Components of ABC transporters have shown to be protective in animal models as protein vaccine candidates against various pathogenic bacteria. In *S. pneumoniae*, solute binding components (SBP / lipoproteins) that have been demonstrated to be protective against both systemic and pulmonary infections are pneumococcal iron acquisition (Pia) and pneumococcal iron uptake (Piu) ABC transporters. PsaA is a

Table 1.4: ABC transporters investigated before.

Operon	Given name	Function	Reference
Sp0042	*comABCDE*	Competence	Havarstein *et a*l
Sp0241-243	*pitADBC*	Iron uptake	J.S.Brown *et al.*
Sp0366	*aliA*	Oligopeptide	A.R.Kerr
Sp0601-604	*vex123-pep27-vncRS*	Vancomycin tolerance	E.I. Toumanen *et al*
Sp1032-35	*piuA*	Iron uptake	J.S.Brown *et al*
Sp1396-1400	*pstSCAB*	Phosphate uptake	E.I.Toumanen *et al*
Sp1648-50	*psaABC*	Manganese uptake	J.P.Claverys *et al*
Sp1869-72	*piaA*	Iron uptake	J.S.Brown *et al*.
Sp1887-91	*amiACDEF*	Oligopeptide	A.R.Kerr *et al*
Sp2169-71	*adcABC*	Zinc uptake	J.P.Claverys *et al*
Sp1526-27	*aliB*	Oligopeptide	A.R.Kerr *et al*

manganese uptake ABC transporter present in *S. pneumoniae*. Although, the degree of protection afforded by PsaA against systemic infection is not good, it does provide protection against the nasopharyngeal carriage of *S. pneumoniae*. The vaccine against Lyme disease caused by *Borrelia burgdorferi* is based on the outer-surface lipoprotein A (Steere *et al.*, 1998). Three conserved lipoproteins identified from the genome sequence of serogroup B of *Neisseria meningitidis* were able to induce bactericidal activity (Pizza *et al.*, 2000), and active immunisation with 5 *S. pyogenes* lipoproteins are protective (Lei *et al.*, 2004). PstS, a surface exposed SBP of phosphate transport system on mycobacteria is highly immunogenic when administered to mice through the intramuscular route as DNA vaccine and exhibits protection against intravenous challenge with *M. tuberculosis* (Garmory and Titball, 2004).

1.5.5 Lipoprotein components of ABC transporters investigated as vaccine candidates in *S. pneumoniae*

Immunisation of lipoprotein components of *S. pneumoniae* ABC transporter proteins such as PsaA, PiaA, PiuA and PotD, lipoprotein component of the polyamine ABC transporter have been shown to protect against *S. pneumoniae* infections. Anti-PsaA antibodies naturally developed in humans or elicited by recombinant PsaA in animals reduce the adherence of *S. pneumoniae* to nasopharyngeal epithelial cells *in vitro*. (Romero-Steiner *et al.*, 2003) and Intranasal (IN) immunisation with recombinant PsaA provides protection against the nasopharyngeal carriage of *S. pneumoniae* and has little effect on the remaining microflora (Pimenta *et al.*, 2006). IN immunisation with combination of PsaA with other proteins such as PspC has shown to protect against naopharyngeal carriage in mice (Briles *et al.*, 2000). PiaA and PiuA are the lipoprotein components of two separate iron uptake ABC transporters required for the full virulence

of *S. pneumoniae*. Systemic immunisation (intraperitoneal, IP) (Brown *et al.*, 2001) and mucosal immunisation (IN) (Jomaa *et al.*, 2006) with PiuA and PiaA induced protective antibody response in mice against IP and IN challenge with *S. pneumoniae*. Flow cytometry analysis of *in vitro* opsonophagocytosis demonstrated that anti-PiaA and anti-PiuA aids in the complement-dependent and complement-independent clearance of *S. pneumoniae* (Jomaa *et al.*, 2005). Amplification of *piaA* and *piuA* genes have shown that *piaA* is strictly conserved among typical *S. pneumoniae* and completely absent in oral streptococci and *S. mitis* group whereas *piuA* is conserved in typical *S. pneumoniae*, and it is also present in atypical *S. pneumoniae* plus closely related species of *S. mitis* and *S. oralis* group. PotD is yet another lipoprotein component investigated as potential vaccine candidate and has demonstrated protection against *S. pneumoniae* septicaemia in mice upon immunisation with the recombinant PotD and S. pneumoniae challenge intraperitoneally (Shah and Swiatlo, 2006). Recently Shah and collegues have also demonstrated that the IN immunisation with recombinant PotD (rPotD) protects against nasopharyngeal carriage of *S. pneumoniae* serotypes (TIGR4 and 19F) in mice, and that the rPotD immunised mice clear *S. pneumoniae* from target organs such as brain, lungs, olfactory bulbs compared with the control mice (Shah *et al.*, 2009). Although other groups of surface exposed proteins such as putative proteinase maturation protein A (PpmA) and streptococcal lipoprotein rotamase A (SlrA) are known to be immunogenic, their efficacy as vaccine candidates needs to be investigated (Bergmann and Hammerschmidt, 2006).

Of the *S. pneumoniae* lipoproteins investigated, PsaA (Sampson *et al.*, 1997) PiaA and PiuA (Whalan *et al.*, 2006) have been demonstrated to be conserved between *S. pneumoniae* strains. However, these lipoproteins are less likely to be surface exposed probably because the lipoproteins are likely to be membrane bound underneath the

peptidoglycan and capsule layers (Tai *et al.*, 2003; Lawrence *et al.*, 1998). Nevertheless their functional importance in the acquisition of nutrients and therefore survival of *S. pneumoniae* in the host makes it less likely for these genes to be lost by mutation as a *S. pneumoniae* adaptation to vaccine-induced immunity.

1.5.6 Conclusion

S. pneumoniae is a major worldwide cause of morbidity and mortality especially amongst children and the elderly. Currently, capsular polysaccharide vaccines and conjugate vaccines are administered to prevent *S. pneumoniae* diseases. The most unconjugated capsular polysaccharide vaccines does not stimulate adequate immunity in infants, elderly and immunocompromised patients, and this drawback has been overcome by conjugating the capsular polysaccharide antigens to a protein carrier to make a vaccine that is effective in infants. However the conjugated vaccine has only limited serotype coverage and is expensive. Therefore conserved *S. pnemoniae* surface proteins such as ABC transporter lipoproteins are being evaluated for their potential role in future vaccination against *S. pneumoniae* diseases. PsaA, PiaA, PiuA and PotD are among the many lipoproteins of the *S. pneumoniae* ABC transporters that have been successfully investigated as potential vaccine candidates. However these are only a handful of the *S. pneumoniae* ABC transporter SBP components identified by genome sequencing, and the uninvestigated lipoprotein components may be important for the *S. pneumoniae* virulence and could be additional potential vaccines candidates.

1.6 HYPOTHESIS

'Previously uninvestigated ABC transporters may be required for the *in vivo* growth and full virulence of *S. pneumoniae*, and their lipoprotein components could be potential novel vaccine candidates'.

AIMS

- To identify previously uninvestigated *S. pneumoniae* ABC transporters required for *S. pneumoniae in vivo* growth and virulence in mouse models
- To characterise the function of previously uninvestigated ABC transporters that are important for *S. pneumoniae* virulence
- To investigate the potential of the corresponding lipoprotein(s) components of ABC transporters found to be important for virulence as vaccine candidates using mouse models of *S. pneumoniae* septicaemia and pneumonia.

Chapter 2
Materials and methods

2.1 BACTERIAL STRAINS

The *E. coli* strains DH5α, Novablue competent cells (Novagen), JM109 (Promega) and M15 (Qiagen) were used for cloning procedures. The capsular serotype 3 *S. pneumoniae* strain 0100993, originally isolated from a patient with pneumonia and obtained from Smithkline Beecham PLC (Lau *et al.*, 2001), was used to construct *S. pneumoniae* mutant strains for the *in vitro* and *in vivo* phenotype analysis. While most of the *in vitro* and *in vivo* phenotype analyses were performed in 0100993 strain, some experiments such as radioactive uptakes assays were performed using capsular serotype 2 strain D39 since mucoid colonies of 0100993 strain prevented effective pelleting of bacteria. Vaccination studies were mostly performed using the capsular serotype 2 strain D39 since this strain has been previously used for these experiments (Brown *et al.*, 2001b). *S. pneumoniae*, *E. coli* mutant strains and plasmids constructed and primers used for this thesis are listed in the tables 2.2, 2.3 and 2.4.

2.2 MEDIA AND GROWTH CONDITIONS

E. coli DH5α was cultured using Luria Bertani (LB) medium (Sambrook J. *et al.*, 1989) at 37ºC. Liquid cultures were grown with continuous shaking at 200 rotations per minute (rpm) and colonies were grown on LB agar plates. Plasmids pID701 was selected using chloramphenicol (10 μg ml^{-1}) and pST-1, pGEM-Teasy, pQE30, pQE30UA plasmids were selected using appropriate antibiotics according to manufacturer's instructions. For long term storage, strains were cultured overnight in

LB broth with appropriate antibiotic selection and stored at -70ºC in 10% glycerol. *S. pneumoniae* strains were cultured at 37ºC in the presence of CO_2 on Columbia agar (Oxoid) supplemented with 5% horse blood (TCS Biosciences), or in Todd-Hewitt broth supplemented with 0.5% yeast-extract (Oxoid). Mutant strains were grown on blood agar plates in the presence of appropriate antibiotic (10 μg ml^{-1} chloramphenicol and 0.2 μg ml^{-1} erythromycin). Broth culture growth was monitored by measuring optical density (OD) at 580nm. Single use 0.5 ml aliquots of THY broth culture ($O.D_{580}$ 0.3 - 0.4) of different *S. pneumoniae* strains were stored at -70ºC in 10% glycerol.

2.3 DNA METHODS

2.3.1 Extraction of plasmid DNA from *E. coli*

Plasmid DNA was isolated from *E. coli* using the Qiaprep® Spin Miniprep kit (50 μl at 400 ng ul^{-1}) or the Eppendorff Fast Plasmid™ Miniprep kit (50 μl at 400 ng ul^{-1}). Plasmid concentrations were quantified by comparing band intensity to the Bioline Hyperladder 1 after electrophoresis in a 0.8-1% agarose gel.

2.3.2 Extraction of genomic DNA from *S. pneumoniae*

4-6 ml of *S. pneumoniae* culture (OD_{580} ~ 0.4) was resuspended in 200 μl of 50 mM EDTA and 0.1% deoxycholate and the genomic DNA extracted using Wizard genomic DNA kit (Promega) according to the manufacturer's instructions.

2.3.3 Gel extraction and purification of DNA

Specific DNA fragments obtained by restriction digestion or PCR were fractionated using 1% agarose gels and purified using the QIAquick gel extraction kit. Restriction digests or PCR were purified by using Qiagen® QIA quick columns. 5 volumes of PB

buffer (Qiagen®) were added to the reaction product, mixed and transferred to Qiagen® QIAquick columns and centrifuged at 13000 rpm for 1 minute. The membrane bound DNA was washed 2 times with 750 µl of PE buffer (Qiagen®) and centrifuged for 2 minutes to remove traces of PE buffer, then eluted in 30 µl of distilled water or EB buffer (10 mM Tris-Cl pH8.0) (Qiagen®).

2.3.4 PCR

Templates for PCR were either genomic DNA, plasmid DNA or *E. coli* colonies picked directly from transformation plates. In a reaction volume of 200 µl, 100 pmoles of primers (Invitrogen), 200 µM of dNTPs (Promega) and 0.5 units (U) of Taq DNA polymerase (Sigma) were used. The standard PCR cycle was an initial denaturing step at 94ºC for 4 minutes, 94ºC for 30 seconds, followed by an annealing step at 50ºC for 30 seconds and an extension step at 72ºC for 45 seconds, for 35 cycles, with a final extension at 72ºC for 10 minutes. PCR products were purified by Qiagen® QIAquick columns (see above) and visualised on 1% agarose electrophoresis gels.

2.3.5 Restriction digestion

When necessary, PCR products and plasmids were digested with appropriate restriction enzymes. 4 µl of PCR products / plasmid preparations were digested by 2-4 µl of restriction enzymes at 37ºC. For ligations, the digested plasmid products were dephosphorylated by the addition of 5 U of calf intestinal alkaline phosphatase (MBI Fermentas, 10 U μl^{-1}) and incubating for up to 30 minutes at 37ºC. Once digested and dephosphorylated, PCR products and plasmids were cleaned using a Qiagen® spin kit (see above) and eluted in 20 µl or 40 µl of distilled water or EB buffer respectively (10 mM Tris-Cl pH 8.0) respectively.

2.3.6 Ligation

Ligations were performed in a total volume of 20 µl of 1x ligation buffer (50 mM Tris-Cl pH 7.6, 1 mM ATP, 1 mM DTT, 5% w/v PEG 8000) with 1 U of T4 DNA ligase (Gibco RL). Ligation reactions were performed overnight at 16ºC with variable ratios of prepared plasmid DNA and insert DNA (3:1 to 1:3). Negative controls included plasmids ligated without insert DNA and reactions with no added T4 DNA ligase.

2.3.7 Overlap extension PCR (OEP)

The deletion construct of Sp0149 was obtained by flanking the erythromycin gene (antibiotic resistance) between Sp0148 and Sp0150 genes which are upstream and downstream to Sp0149 respectively. To obtain the Sp0149 deletion construct, fragments of Sp0148 and Sp0150 genes were amplified from the genomic DNA of *S. pneumoniae* (0100993 strain). The *erm* gene was amplified from the erm cassette of pACH74 (a suicide vector carrying *erm* for selection in *S. pneumoniae*, a kind gift from J. Paton). Similarly the deletion construct of Sp0750-53 was obtained by flanking the erythromycin gene (antibiotic resistance) between Sp0749 and Sp0754 genes which are upstream and downstream of Sp0750-53 respectively. To obtain the Sp0750-53 deletion construct, fragments of Sp0749 and Sp0754 genes were amplified from the genomic DNA of *S. pneumoniae* (0100993 strain). All the primers (table 2.2) were designed from 5'to 3' direction but the reverse primer of the Sp0148 and Sp0749 and the forward primer of the Sp0150 and Sp0754 had linkers that were complementary to the 5' and 3' portion of the *erm* gene at their 5' ends (Shevchuk *et al.*, 2004).

(a) Initial PCR

The individual PCR products were amplified in a reaction volume of 200 µl, 100 pmoles of primers, 200 µM of dNTPs (Bioline) and 0.5 U of Taq DNA polymerase (Sigma) using a PCR cycle of an initial denaturing step at 94ºC for 4 minutes, 94ºC for 30 seconds, followed by an annealing step at 55ºC for 45 seconds and an extension step at 72ºC for 1 minute, for 30 cycles, with a final extension at 72ºC for 10 minutes. PCR products were purified by Qiagen® QIAquick columns (see above) and visualised on 1% agarose gels.

(b) Fusion of the initial PCR products without primers

Purified individual PCR products were pooled in a reaction volume of 20 µl which contained 8.7 µl of nuclease free water, 1 µl of buffer, 1 µl of 2 mM dNTPs, 0.4 µl of 50 mM $MgSO_4$, 2 µl (approximately 50 ng) of fragment 1 and 3, 5 µl (approximately 50 ng) of fragment 2 and 0.2 µl of Taq polymerase (Bioline). The PCR cycle was an initial denaturing step at 94ºC for 2 minutes, 94ºC for 20 seconds, followed by an annealing step at 50ºC for 30 seconds and an extension step at 72ºC for 1 minute, for 10 cycles, and a final hold at 4ºC.

(c) Amplification of fused PCR products

Final amplification was performed in a reaction volume of 100 µl which contained 68.2 µl of nuclease free water, 10 µl of buffer, 10 µl of 2 mM dNTPs, 4 µl of 50 mM $MgSO_4$, 100 pmoles of forward primer of the fragment 1 and reverse primer of fragment 3, 3 µl of unpurified PCR product from the step (b) and 0.8 µl of Taq polymerase. The PCR cycle was an initial denaturing step at 94ºC for 2 minutes, 94ºC for 20 seconds, followed by an annealing step at 50ºC for 30 seconds and an extension step at 72ºC for

2.30 minutes, for 35 cycles, with a final extension at 72ºC for 3 minutes and a final hold at 4ºC. The fusion PCR products were then analysed on 1% agarose gel, desired DNA bands excised and purified using Qiagen® QIAquick columns and the resulting deletion constructs were used to transform *S. pneumoniae* serotype 3 strain (0100993).

2.3.8 Transformation of *E. coli*

Transformation of *E. coli* was performed by mixing 5 µl of ligation reaction with 100 µl of competent *E. coli* DH5α cells (prepared by the rubidium chloride method, Sambrook *et al*. 1989) and using heat shock at 42ºC (Sambrook *et al*. 1989). The transformation mix was then plated on LB agar containing the required antibiotic. (10 µg ml^{-1} chloramphenicol or 100 µg ml^{-1} carbenicillin) and incubated overnight at 37ºC. Correct inserts in the plasmids from the transformants were confirmed by colony PCR, restriction digests and / or sequencing.

2.3.9 Transformation of *S. pneumoniae*

A single *S. pneumoniae* colony from an overnight incubation on a blood agar plate was inoculated into THY pH 6.8 broth and cultured until the $O.D_{580}$ reached 0.015. The culture was pelleted at 15,000 rpm at 4ºC for 10 minutes and the pellet resuspended in 1 ml of pre-warmed THY pH 8.0 broth containing 1 mM $CaCl_2$ and 0.2% BSA. The resuspended solution was transferred into Falcon tubes and competence stimulating peptide-I (CSP1) (kind gift from D. Morrison) was added to a final concentration of 200 ng ml^{-1} (Håvarstein *et al*., 1995; Lau *et al*., 2001). The transformation mix was incubated at room temperature for 5 minutes before the addition of transforming DNA (circular plasmid or linear constructs) followed by incubation at 37ºC for 2 hours in the presence of CO_2. 200 µl of the above transformation mixture was spread onto antibiotic

containing blood agar plates and the identity of successful transformants confirmed by PCR and / or sequencing.

2.3.10 Nucleotide sequencing

Mutant identities were confirmed by PCR followed by sequencing of the PCR products obtained using mutant genomic DNA, a target gene specific forward primer and a vector specific reverse primer. DNA sequencing was performed by Lark Technologies Inc. UK or UCL sequencing services using the Big Dye™ Terminator technique and gene specific PCR primers.

2.4 RNA METHODS

2.4.1 RNA extraction from *S. pneumoniae*

RNA was extracted from *S. pneumoniae* using the SV total RNA extraction kit (Promega). *S. pneumoniae* colonies from overnight grown blood agar were inoculated into 20 ml of THY and incubated at 37ºC in the presence of CO_2. Once OD_{580} reached 0.2-0.3, 2-5 ml of the culture was centrifuged at 13,000 rpm for 10 minutes at room temperature. The pellet was resuspended in 100 µl of TE (50 mM Tris, 5 mM EDTA) and 0.01% DOC (deoxycholate) and incubated for 10 minutes. Further steps of the extraction were performed according to the manufacturer's instructions. To inhibit RNA degradation, 0.5-1 µl of RNAsin was added to the completed RNA preparation and aliquots of 5-8 µl were made and stored at -70ºC until use.

2.4.2 RNA extraction from *S. pneumoniae* by acid-phenol method

Total RNA from *S. pneumoniae* grown in human and mouse blood was extracted as described by Ogunniyi *et al*, (2002). *S. pneumoniae* was harvested from the human and

mouse blood by brief centrifugation at 825 *g* at 4ºC for 5 minutes to pellet the erythrocytes and leucocytes. The resulting supernatant which is a mixture of plasma and thrombocytes along with the bacteria was centrifuged further at 15500 *g* at 4ºC for 5 minutes. The supernatant was discarded and the bacterial pellet was then used to extract the total RNA.

The bacterial pellet was resuspended in 300 µl of pre-warmed (65ºC) acid-phenol (Ambion) and incubated for 5 minutes at 65 C followed by further addition of 300 µl of pre-warmed NAES buffer and incubation at 65 C for 5 minutes with intermittent mixing. The mixture was cooled on ice for 1 minute and centrifuged at 15500 *g* for 1 minute to separate the phases. The aqueous phase was re-extracted twice with acid-phenol and NAES buffer and later extracted twice with 300 µl of chloroform. To this mixture, sodium acetate was added at a final concentration of 300 mM followed by addition of 2 volumes of ethanol and RNA was precipitated at -20 C overnight. The precipitated RNA was centrifuged at 6000 rpm for 5 minutes and the resulting pellet was washed in 70% ethanol and resuspended in 50 µl of nuclease-free water. To the resulting RNA, recombinant RNasin ribonuclease inhibitor (Promega N251A) was added to a final concentration of 1 U μl^{-1} and then treated with 0·5 U μl^{-1} RQ1 RNase-free DNase (Promega M610A) at 37 C for 40 minutes. Aliquots of this RNA were stored at -70 C until use. An aliquot was used to check the purity by RT-PCR as described in section 2.4.3 with and without reverse transcriptase using gene specific primers.

2.4.3 RT-PCR

RT-PCR was performed using the Access RT-PCR system (Promega). In the final volume of 25 µl, 5 µl AMV buffer, 0.5 µl of 5 mM dNTPs (Promega), 1 µl of 25 mM

$MgSO_4$, 1.5 U each of AMV reverse transcriptase and Tfl DNA polymerase, 1 µl of RNA with 0.1 µl stop solution and primers at 120 nanomoles final concentration were added and the volume was made up with nuclease free water. Amplification of target specific DNA was performed at 48ºC for 45 minutes, followed by PCR amplification of the cDNA with the following parameters of 94ºC for 2 minutes, 94ºC for 30 seconds, 55ºC for 30 seconds, 72ºC for 45 seconds, 72ºC for 10 minutes for 35 cycles.

2.5 PROTEIN METHODS

2.5.1 Induction of positive clones for protein expression

Protein induction was performed according to The QIAexpressionist™ manual. Colonies of *E. coli* M15 strain were confirmed for the insert and the orientation of the lipoprotein gene by colony PCR using the vector specific forward primer (pQE30F) and insert specific reverse primers (Sp0149Rev, Sp0749Rv, PiaARv and PiuARv). These colonies were picked and inoculated in 1.5 ml LB broth containing carbenicillin (100 µg ml^{-1}) and kanamycin (25 µg ml^{-1}). An extra culture was inoculated to serve as a non-induced control. These cultures were grown overnight at 37ºC at 220 rpm (rotations per minute). 500 µl of the above overnight culture was inoculated into 10 ml LB containing carbenicillin (100 µg ml^{-1}) and kanamycin (25 µg ml^{-1}) and grown at 37ºC at 220 rpm until the OD_{580} reached 0.5-0.7. Protein expression by the cultures was induced by adding isopropyl ß thiogalactosidase (IPTG) to a final concentration of 1 mM and incubation for an additional 4-5 hours. The cells were harvested by centrifugation at 4800 *g* for 5 minutes and supernatant was discarded. The harvested cells were resuspended in 400 µl of buffer B (appendix) and lysed by gentle vortexing until the solution became translucent. The lysate was centrifuged to remove the cell debris (termed pellet), and the supernatant was transferred to a fresh tube. 2.5 µl of 5x SDS-

PAGE sample buffer (appendix) was added to 10 µl of the supernatant and boiled at 95ºC for 5 minutes to denature the proteins for analysis by SDS-PAGE.

2.5.2 Purification of 6xHis-tagged lipoproteins by Ni-NTA affinity chromatography under native conditions

Purification of His-tagged lipoproteins was performed according to the QIAexpressionist^{TM} manual. 10 ml of LB containing 100 µl ml^{-1} of carbenicillin and 25 µl ml^{-1} of kanamycin was inoculated with a positive clone cultured on LB agar containing 100 µl ml^{-1} of carbenicillin and cultured at 37ºC with vigorous shaking at 220 rpm overnight. 100 ml of pre-warmed LB medium with 100 µl ml^{-1} of carbenicillin and 25 µl ml^{-1} of kanamycin was inoculated with 5 ml of the overnight culture and cultured until the OD_{580} reached 0.6. The culture was then induced with IPTG at a final concentration of 1 mM and cultured for an additional 4-5 hours. The bacteria were pelleted by centrifugation at 4000 *g* for 20 minutes and the pellets stored at -20ºC until use. The cell pellets were thawed for 15 minutes on ice and resuspended in 2-5 ml per gram of wet weight in cell lysis buffer (appendix) and sonicated on ice using six 10 second bursts at 200-300W, with 10 seconds cooling between each burst. The lysates were centrifuged at 10,000 *g* for 20-30 minutes at 4ºC to pellet the cellular debris and the supernatant (CL) was saved for further use. 5 µl of 2x SDS-PAGE sample buffer was added to 5 µl of cell lysate and stored at -20ºC for analysis. 1 ml of 50% Nickel tagged Nitrilotriacetic acid (Ni-NTA) agarose slurry was added to 4 ml of the cleared cell lysate and mixed by shaking at 200 rpm at 4ºC for 1 hour. The lysate-Ni-NTA slurry was then loaded into the protein purification columns with the bottom outlet capped. After loading, the outlet caps were removed to collect the flow-through (FT). 5 µl of 2x SDS-PAGE sample buffer was added to 5 µl of flow-through (FT) and were

stored at -20ºC for the analysis by SDS-PAGE. The columns were washed twice with 4 ml of wash buffer (appendix) and the successive fractions (W1 &W2) were saved for SDS-PAGE analysis. Finally the protein was eluted with four 0.5 ml volumes of elution buffer (appendix), and the eluate collected in 4 tubes and analysed by SDS-PAGE.

2.5.3 Preparation of dialysis tubing

Dialysis tubings were prepared as follows. The tubings were first boiled in 500 mls of 2% w/v sodium bicarbonate and 1 mM EDTA pH 8.0 for 10 minutes, rinsed in distilled water, boiled again in 1 mM EDTA pH 8.0 for 10 minutes, cooled and finally stored submerged in ethanol at 4ºC. The tubings were washed with distilled water before use.

2.5.4 Dialysis of purified lipoproteins

One end of the treated dialysis tubing was tied with a thread, the purified protein sample was loaded carefully and then the other end was also tied. The tubing was tied to a rod, placed in 10 mM NaH_2PO_4 and dialysed extensively overnight at 4 ºC. The dialysed protein samples were removed and transferred into a fresh tube and an aliquot was used to determine the protein concentration. The proteins were mixed in a final concentration of 50% sterile glycerol and stored at -20ºC.

2.5.5 Protein concentration determination by Bicinchonic acid (BCA) method (Pierce)

2 mg ml^{-1} bovine serum albumin (BSA) was diluted in 10 mM NaH_2PO_4 to 25, 125, 250, 500, 750, 1000, 1500 and 2000 µg ml^{-1} and used as protein standards. The negative control consisted of 10 mM NaH_2PO_4. BCA working reagent was prepared by mixing 50 parts of BCA reagent A with 1 part of BCA reagent B. 10 µl of each diluted standard

and the unknown samples were added into a microtitre well and 200 µl of BCA working reagent added to each well and mixed for 30 seconds. The plate was incubated for 30 minutes at 37ºC and cooled after incubation to room temperature. Absorbance was measured at 562 nm on a plate reader. The protein concentration was determined by plotting a standard curve with the BSA values on the X-axis and the absorbance at 562 nm on the Y-axis. The concentrations of the unknown protein samples were determined using the albumin values as standard.

2.5.6 Whole cell lysate preparation from *S. pneumoniae*

Different serotypes of *S. pneumoniae* were grown in THY medium until the OD_{580} reached 0.6 and the cells were centrifuged for 10 minutes at 4800 *g*. The supernatant was discarded and the pellet resuspended in sterile phosphate buffered saline (PBS) and sonicated on ice using six 10 second bursts at 200-300W with 10 seconds of cooling between each burst. The cell lysate was centrifuged for 30 minutes at 4800 *g*, the supernatants saved for further analysis and the pellet containing cell debris discarded. 5 µl of SDS-PAGE sample buffer (appendix) was added to 30 µl of the supernatant and boiled at 95ºC for 5 minutes to denature the proteins for analysis by SDS-PAGE and Western blotting.

2.5.7 Extraction of *S. pneumoniae* membrane proteins by Triton X-114

Membrane proteins were extracted from *S. pneumoniae* according to Khandavilli *et al*. (2008). *S. pneumoniae* grown in THY to mid-logarithmic phase were pelleted at 3000 *g* for 20 minutes at 4ºC and then resuspended in 0.1% DOC. This mixture was cooled for 2 minutes and then sonicated for 15 seconds with 10 seconds cooling between each sonication. 800 µl of PBS and 100 µl of 10% Triton X-114 was added to this mixture

and incubated at 4ºC for 2 hours, and then centrifuged at 13,000 rpm for 10 minutes at 4ºC. The resulting supernatant was incubated at 37ºC for 30 minutes, followed by centrifugation at 13,000 rpm for 5 minutes at room temperature to separate the phases. The upper phase was removed and 1 ml of PBS was added to the lower phase (Triton X-114 phase) and incubated at 4ºC for an hour, followed by incubation at 37ºC for 30 minutes and further centrifugation at 13,000 rpm for 5 minutes at room temperature. The upper phase was removed and the Triton X-114 phase was then diluted at 1:2 with PBS and stored at -20ºC until use.

2.5.8 SDS-PAGE

SDS-PAGE gels were prepared using SDS-PAGE cassettes (Invitrogen) according to Sambrook *et al*. 5 µl of SDS-PAGE sample buffer (appendix) was added to 30 µl of the supernatant, heated at 95ºC for 5 minutes before loading and electrophoresis using 1x Tris-glycine electrophoresis buffer (appendix) at a constant 120 volts for $1^1/_2$ hours. The gels were stained with 0.1% Coomassie Brilliant Blue R250 stain (appendix) for 2 hours, and then destained using 40% methanol and 10% acetic acid until the background staining had disappeared.

2.5.9 Western blotting

After electrophoresis, the SDS-PAGE gels were transferred to HyBond Nitrocellulose membranes (Amersham Biosciences) using a constant current of 40 mA per membrane for 2 hours and the semi-dry blotting method (Sambrook *et al*). Transferred membranes were washed for 5 minutes in 1x transfer buffer (appendix), and stained in Ponceau S solution (Sigma) to confirm the transfer of proteins. After washing to remove the stain, the membranes were incubated overnight at 4ºC in 5% milk in 1x TBS-TWEEN (TBS-

T, appendix) to block any non-specific binding of antibodies. The membranes were incubated with primary antibody (polyclonal mouse serum at 1/2000 dilution) at room temperature for 2 hours, washed 2-3 times with 1x TBS-T for one hour, and then incubated with secondary antibody (goat anti-mouse IgG conjugated with HRP [Dako Cytomation] at 1/2000 dilution) at room temperature for 2 hours. After washing 3 times with 1x TBS-T for 10 minutes, the membranes were analysed using the ECL^{TM} Western blotting analysis system according to the manufacturer's instructions and exposed to photographic film for up to 2 minutes before developing.

2.5.10 Tryptophan fluorescence spectroscopy

The protein concentration of purified lipoprotein was estimated and the protein dialysed in 10 mM NaH_2PO_4 and then used for the tryptophan fluorescence spectroscopy. The assay was performed using a Hitachi F-2500 spectrofluorimeter at an excitation wavelength of 280 nm (slit width 3 nm) and an emission wavelength of 309 nm (slit width 3 nm). Ligands (individual amino acids) were solubilised in 10 mM NaH_2PO_4. The assay was performed by mixing 0·5 μM protein in 1·5 ml 50 mM Tris/HCl pH 8 in the sample cuvette maintained at 25ºC in the spectrofluorimeter with continous stirring. The slit width of the spectrofluorimeter was adjusted to avoid photobleaching of the protein and fluorescence changes upon addition of ligands were recorded (Thomas *et al.*, 2006).

2.5.11 Radioactive substrate binding assay

100 μl of purified lipoprotein was first incubated on ice for 15 minutes. A mixture of ^{14}C-labelled and non-radioactive ligand (GE Healthcare, United Kingdom) at 5 μM concentration was added to the protein and further incubated on ice for 10 minutes. To

this mixture, 1 ml of saturated ammonium sulphate was added and incubated on ice for 20 minutes, then filtered using glass fibre filter papers (GF/F; Whatman, United Kingdom), the filters washed with 4 ml of saturated ammonium sulphate and dried for 5 minutes. The filters were allowed to dissolve in the Ready safe scintillation cocktail (Beckman Coulter, United Kingdom) for 20 minutes and the radioactivity was determined using Wallac 1214 RackBeta liquid scintillation counter after brief vortexing of the samples.

2.5.12 Streptonigrin assay

For streptonigrin assay (Brown *et al.*, 2001a), frozen *S. pneumoniae* strains were thawed and pelleted by centrifugation at 13,000 pm at 4ºC, then resuspended in 2.5 µg ml^{-1} or 5 µg ml^{-1} of streptonigrin (Sigma) and incubated at 37ºC. The reaction culture was serially diluted and appropriate dilutions were plated for cfu before (0 minute) and after (20 and 40 minutes) the addition of streptonigrin. Results are represented as the percent survival of wild-type and mutant strains of *S. pneumoniae* on exposure to streptonigrin.

2.5.13 Radioactive substrate uptake assays

Radioactive uptake assays were performed as described by (Webb *et al.*, 2008) with some modifications. Wild-type and mutant strains of *S. pneumoniae* were inoculated in THY medium until the $O.D_{620}$ reaches 0.2-0.4. Bacteria were harvested at 6000 rpm for 20 minutes and resuspended in potassium phosphate buffer (appendix) to an approximate $O.D_{620}$ between 0.8-1.1. The radioactive uptake assay was performed in a total volume of 1 ml which contained 0.85 ml of bacteria, 50 µl of 0.5 mM non-radiolabelled test substrate and 100 µl of 2.5 µCi radiolabelled test substrate. 150 µl of sample was taken immediately after the addition of non-radiolabelled substrate followed

by the addition of radiolabelled substrate at time 0. Equal volumes of samples were taken at 1, 2 and 3 minutes, and immediately filtered through glass fibre filters, then washed with potassium phosphate buffer. 50 µl of total isotope control was directly added into a scintillation vial immediately after the 3rd minute. Washed filters were placed in scintillation vials, 5 ml of Ready safe scintillation cocktail (Beckman Coulter) was added to all the vials and radioactivity was counted using a Wallac 1214 RackBeta liquid scintillation counter.

2.8 *IN VIVO* METHODS

2.8.1 *In vivo* studies of *S. pneumoniae* in mouse models

Male outbred white mice (strain CD1; Charles River Breeders) which were 4-8 weeks old were used for the *in vivo* experiments as they have been previously used in our laboratory for competitive indicies experiments. The use of different mouse strains for different *in vivo* experiments is listed in Table 2.1. The colony forming units per ml (cfu) from the frozen stocks of *S. pneumoniae* were first determined by plating serial dilutions, and diluted in 0.9 ml PBS to the cfu concentration required for inoculation. For mixed infections, equivalent numbers of bacteria from the wild-type and mutant strains were used. To determine the equivalent numbers of bacteria from the wild-type and mutant strains, the frozen THY stocks of the wild-type and mutant strains whose cfu has been previously determined were thawed, calculated to obtain equivalent numbers and mixed. The mixed strains were then centrifuged to remove THY and resuspended in sterile PBS, serially diluted and appropriate dilutions were plated on plain media and media containing appropriate antibiotic to enumerate the wild-type and mutant bacterial cfu. For the systemic models of infection, 100 µl containing $1x10^3$ cfu of bacteria were injected intraperitonially (IP) per mouse. For pneumonia models of

infection, mice were deeply anaesthetized by inhalation of halothane and 40 µl containing 5 x 10^6 cfu of bacteria per mouse inoculated intranasally (IN). After 24 hours (IP) or 48 hours (IN), mice were sacrificed and the target organs (spleens for IP inoculations, and lungs and spleen for IN inoculations) were homogenised in 0.5 ml of PBS. Appropriate dilutions of organ homogenates were plated on plain and antibiotic blood agar plates and incubated overnight (Brown *et al*., 2001). Bacterial cfu were enumerated and the competitive index (CI) calculated using the formula: ratio of mutant to wild-type strain recovered from mice divided by the ratio of mutant to wild-type strain in the inoculum. The CI technique is useful in analysing the role of surface proteins such as ABC transporters, however this technique cannot be used to analyse the role of secreted proteins (Holden *et al*, 2000). All the *in vivo* CI and survival experiments using the wild-type and mutant strains were performed using 0100993 *S. pneumoniae* strain. To recover blood, mice were deeply anaesthetized by IP injection of pentobarbital (100 µl) and the blood obtained by cardiac puncture or from the femoral artery. The blood was stored on ice during transport and allowed to clot for 2-3 hours at 4ºC and then centrifuged for 10 minutes at 10,000 rpm to obtain serum. Aliquots of sera were stored at 4ºC for ELISAs and the remaining aliquots stored at -70ºC for C3 binding and opsonophagocytosis assays.

2.8.2 IP vaccination schedule and challenge experiment

Purified proteins were prepared for vaccination at 100 µg ml^{-1} in 50% glycerol and 10% alum (Pierce) as adjuvant. Alum alone was used as the negative control. For the vaccination experiments, proteins were mixed with 10% alum at room temperature for 2 hours, and 100 µl (10 µg of protein) injected intraperitonially into groups of 24 mice. 2 booster doses of proteins were prepared as above and were given at 7 to 10 day intervals

Table 2.1: Mouse strains used for different *in vivo* experiments

Mouse strain	Experiment	Reasons
CD1	CI, survival studies	Established model for phenotype analysis
	IP and IN immunisation	Less expensive, greater relevance if positive results were obtained as this mouse strain is outbred and genetically variable
BALB/C	IP immunisation	Inbred mouse strain and therefore immunologically less variable
CBA/Ca	IN immunisation	Inbred mouse strain, susceptible to intranasal infection and immunologically less variable

after the initial vaccination. After 2 weeks, serum was recovered from 5 mice from each group, and the remaining 19 mice challenged intraperitonially with 100 µl containing 1 x 10^5 or 1 x 10^3cfu of *S. pneumoniae* D39 strain. Mice were routinely observed every 3-4 hours from 2 days to 5 days post-challenge and then twice a day for a further 9 days. When they exhibited hunched posture and poor mobility, mice were sacrificed and the survival rates of each vaccination group recorded (Brown *et al*., 2001).

2.8.3 IN vaccination schedule and challenge experiment

Intranasal vaccination was performed by mixing the purified proteins and cholera toxin (CT) (Sigma) as the adjuvant at 2.5 µg ml^{-1} in a final volume of 10 µl. Groups of 20 mice were deeply anaesthetized by inhalation of halothane and 10 µl of the

prepared proteins were intranasally administered. 2 booster doses of proteins were prepared as above and were given at 7 intervals after the initial vaccination. After 2 weeks, serum, bronchoalveolar lavage fluid (BALF) and lungs was recovered from 5 mice from each group for immune assays, and the remaining 15 mice challenged intranasally with 50 µl of PBS containing 1 x 10^5 or 1 x 10^6 cfu of *S. pneumoniae* D39 or 0100993 strain. Mice were routinely observed every 3-4 hours from 2 days to 5 days post-challenge and then twice a day for further 9 days. When they exhibited hunched posture and poor mobility, mice were sacrificed and the survival rates of each vaccination group recorded (Jomaa *et al.*, 2006).

Survival curves were further refined by quantitative analysis of the *S. pneumoniae* cfu from the target fluids and organs such as bronchoalveolar lavage fluid, lungs and blood after IN immunisation and *S. pneumoniae* challenge.

2.8.4 Immune cell surface marker staining recovered from target organs

Immune cell surface markers such as CD4, CD8, B220, CD45RB, macrophage surface markers such as CD80 and I-A-I-E (MHC class II antigens) were stained on the recovered cells from the lungs and BALF of mice. Aseptically the lungs were homogenised by passage through a nylon sieve and washing with 3 ml of RPMI (containing L-glutamine) directly into a Falcon tube and centrifuged at 1200 rpm for 5 minutes. The resulting supernatant was discarded and the pellet was resuspended in 3 ml RBC lysis buffer (appendix) for 3 minutes at room temperature, followed by addition of 3 ml RPMI to gently resuspend the pellet, which was then centrifuged at 1200 rpm for 5 minutes. The resulting pellet was resuspended in 1 ml RPMI and an aliquot of 50 µl of cells was stained with trypan blue (Sigma) and total cell counts

were determined using a haemocytometer. Approximately 500,000 cells were added into each well of a round bottomed microdish plate (Nunc) based on the total cell counts, centrifuged for 2 minutes at 2000 rpm and the supernatant aspirated. To the pellet, 30 µl of antibody solution (appendix) prepared in PBS / 1% BSA / 0.1% azide (appendix) was added and incubated in dark for 20 minutes at room temperature. This incubation was followed by addition of 100 µl of PBS / 1% BSA / 0.1% azide, centrifuged at 2000 rpm for 2 minutes. The supernatant was aspirated and the pellet washed with 200 µl of PBS followed by another wash in PBS / 1% BSA / 0.1% azide. The pellet was fixed in 100 µl of 3% paraformaldehyde (appendix) and finally analysed by flow cytometry (Brown *et al.*, 2002). Immune cell surface marker staining in mice BALF was performed by determining the total cell counts in an aliquot of 50 µl stained with trypan blue using a haemocytometer followed by staining the immune cell surface marker as described above.

2.8.5 Cytological analysis of BALF

500,000 cells from the BALF were centrifuged onto poly-l-lysine microscope slides (Cytospin 3; Shandon, UK). The slides were air dried overnight and then stained using DiffQuik (DADE AG, Switzerland). 100 cells per sample were counted and the number of macrophages, monocytes and neutrophils were recorded based on the cellular morphology (Hodges *et al.*, 2004).

2.8.6 Histological analysis of lung sectioning

The degree of inflammation of lungs before and after the intranasal vaccination was analysed by histological observation. The left lung from each mouse was fixed in 4% neutral buffered formalin, processed to paraffin wax, and stained using haematoxylin

and eosin. The degree of inflammation during *S. pneumoniae* infection was determined by a previously described scoring system (Bergeron *et al.*, 1998;Yuste *et al.*, 2007b). Microscopic observation of the lung cross section at x10 magnification was first estimated to determine the extent of inflammation. Later, at x200 magnification, six fields were scored as: 1 (no visible inflammatory change), 2 (minimal swelling of alveolar walls with slight change in architecture), 3 (increased swelling with presence of erythrocytes and inflammatory cells and an increase in type II pneumocytes), and 4 (considerable haemorrhage with inflammatory cell influx, widespread alveolar disorganisation with interstitial swelling and pneumocyte proliferation). A total score for each mouse was obtained by multiplying the percentage of the involved lung by the mean score for the areas analysed, and data presented as medians with IQRs (Yuste *et al.*, 2007).

2.9 IMMUNE ASSAYS

2.9.1 Enzyme Linked Immunosorbant assay (ELISA)

Microtitre ELISA plates were coated with 100 µl of antigen at a concentration of 5 µg ml^{-1} in TSA buffer (appendix) and incubated overnight at 4ºC. The plates were washed 5 times with ELISA wash buffer (appendix) and soaked in 150 µl of 2% BSA-Tween (appendix) for 2-4 hours at 37ºC and washed again for 3-4 times with ELISA wash buffer. A 1/1000 dilution of mouse serum was made in BSA-Tween diluent buffer and added to the first well, and then 2 fold dilutions transferred to subsequent wells and incubated at 37ºC for 4 hours. The wells were then washed 6 times with ELISA wash buffer and incubated overnight at 4ºC in 100 µl of 1/15000 dilution of goat anti-mouse IgG conjugated to alkaline phosphate (Sigma) diluted in enzyme diluent (appendix). The ELISA plate was then washed 5 times in ELISA

wash buffer and 100 µl of dinitrophenol (dNP) substrate solution added to each well and incubated for 1 hour at 37ºC (dNP substrate solution was prepared by adding 1 tablet of dNP (Sigma) in 5 ml of water). The absorbance was read at 405 nm and antibody titre was calculated as the lowest dilution at 405 nm giving an OD equal to or greater than 0.3.

2.9.2 C3 deposition and IgG binding assay

To analyse C3 deposition on *S. pneumoniae*, stocks were thawed and washed with PBS to remove THY and glycerol and the bacteria diluted in PBS to obtain 5 x 10^6 cfu in 50 µl. To these aliquots of bacteria, 10 µl of mouse serum was added and the mix incubated for 20 minutes at 37ºC, washed twice with 300 µl of ice cold PBS-Tween 20 (0.1%) by centrifugation at 10,000 rpm for 5 minutes and resuspending the bacterial pellet in 50 µl of 1:300 dilution of fluorescein isothiocyanate-conjugated polyclonal goat anti-mouse C3 antibody (ICN Cappel) followed by incubation on ice for 30 minutes. The labelled bacteria were then washed twice in 500 µl of PBS-Tween 20 (0.1%), the bacterial cells were fixed using 3% paraformaldehyde and finally analysed by flow cytometry. IgG binding to the bacteria in different sera was analysed using the same method but incubating the bacteria with a 1:100 dilution of phycoerythrin-conjugated goat anti-mouse IgG (Jackson Immunoresearch).

2.9.3 Opsonophagocytosis

Opsonophagocytosis assay is a method to determine the serum opsonic activity following immunisation. However, in this assay the functional ability of anti-Sp0149 and anti-Sp0749 antisera to aid in *S. pneumoniae* association with the phagocytes is analysed using flow cytometry.

(a) FAMSE labelling of S. pneumoniae for opsonophagocytosis

The frozen bacterial stocks were streaked on to plain blood agar and incubated overnight at 37ºC in the presence of 5% CO_2. A loopful of bacteria was inoculated in 15 ml THY and cultured until the OD_{600} reached 0.6-0.7. The bacteria were centrifuged at 4000 rpm for 10 minutes, washed once with 0.1 M sodium bicarbonate buffer and 50 µl of 10 mg ml^{-1} FAM-SE solution (5,6-carboxyfluorescein-succinidyl ester; Molecular Probes, Eugene, Oreg.) prepared in DMSO was added. After incubation at 37ºC for 1 hour without shaking, the labelled bacteria washed 5 times in opsonophagocytosis buffer (HBSS + Ca + Mg) (Invitrogen) until no free dye was present. Aliquots of labelled *S. pneumoniae* were prepared containing 10% glycerol and serial dilutions of an aliquot were plated on plain blood agar plate to determine the bacterial counts and the remaining aliquots stored at -70ºC until use.

(b) Tissue culture of HL60 for opsonophagocytosis

HL60 (Human promyelocytic leukemia cells; CCL240; American Type Culture Collection, Rockville, Md.) were cultured in RPMI medium (Invitrogen) containing 20% foetal bovine serum (Invitrogen), 1% glutamine (Invitrogen) and 1% penicillin / streptomycin (Invitrogen) to a cell density of 5 x 10^5 cells ml^{-1}. Addition of 100 mM *N*,*N*-dimethylformamide (Sigma) and culturing for 5 days stimulated differentiation of the HL60 cells into granulocytes. The undifferentiated HL60 cell line was passaged daily for maintenance and propagation. Differentiated HL60 cells were washed twice by centrifuging at 1000 *g* for 7 minutes with HBSS (Hanks balanced salt solution) with 0.1% BSA, without Ca^{2+} and Mg^{2+}. Cells were washed once in

opsonophagocytosis buffer (HBSS + Ca^{2+} + Mg^{2+}), resuspended in 5 ml of opsonophagocytosis buffer and counted using haemocytometer.

(c) Opsonophagocytosis

Different dilutions of mouse sera were prepared in opsonophagocytosis buffer and 10 µl of the diluted serum was added to a 96 well plate. To obtain a multiplicity of infection (MOI) of 10 bacteria per HL60, 10^6 cfu of FAM-SE labelled bacteria were added to each well and the plate was incubated for 20 minutes at 37ºC with gentle shaking at 150 rpm. When necessary 10 µl of 1/300 dilution of baby rabbit serum (Sigma) was added as a source of exogenous complement and the plate was incubated at 37ºC with gentle shaking at 150 rpm for 15 minutes. 10^5 washed and differentiated HL60 cells were added to each well and the plate incubated at 37ºC with gentle shaking at 150 rpm for 30 minutes. The cells were fixed by adding 50 µl of 3% paraformaldehyde to all the wells and analysed by flow cytometry.

2.10 FLUORESCENCE-ACTIVATED CELL SORTING (FACS) ANALYSIS

A minimum of 25,000 bacteria and 6,000 HL60 cells were analysed for the C3b deposition, IgG binding and opsonophagocytosis asays using a FACSCalibur flow cytometer (BD Biosciences). Cell Quest/Pro software (BD Biosciences) was used to acquire and analyse the data. Bacteria and the cells that were positive or negative for the above mentioned assays were sorted using FL-1 histogram, with the X-axis representing the intensity of fluorescence and the number of cells of varying intensity represented in the Y-axis. The left peak in the histogram determines the bacteria and cells with negative results, while the right peak determines the bacteria and cells with positive results for the assays. The percentage of positive bacteria and cells were

determined by creating a gate (M1 marker) around the positive peak which represents the proportion of bacteria positive for C3b / iC3b or IgG and the proportion of HL60 cells associated with FAMSE-labelled bacteria. For the immune cell surface marker staining from BALF and lungs, lymphocytes were determined using their forward and side scatter properties and the identity and proportion of different sub-populations confirmed using antibodies to the cell surface markers CD4, CD8 (T cell subsets) and B220 (B cells). Activated T cells were identifed by double staining with anti-CD4 and -CD8 and anti-CD45RB, with activated cells recognised by loss of the CD45RB expression. Lymphocyte counts are presented as the percent of CD4, CD8 and B220 positive cells present in the lymphocyte gate, with a minimum of 10,000 (lungs) or 1000 (BALF) cells counted (Thorpe *et al.*, 2007).

2.11 COMPUTER ANALYSIS

The genomic DNA sequence of *S. pneumoniae* serotype 4 strain was obtained from The Institute for Genomic Research website (http://www.tigr.org). Blast searches and alignments of the available complete and incomplete bacterial nucleotide and protein databases were performed using NCBI website (http:// www.ncbi.nlm.nih.gov/blast). Primers for both PCR and RT-PCR were designed using ARTEMIS software.

2.12 STATISTICAL ANALYSIS

Competitive indices of the mutant strains were compared to a theoretical value of 1.0 and the significance were determined using Student's *t*-test. All the *in vitro* growth curves were performed in triplicates and represented as means and standard deviations. Results of growth curves, radioactive uptakes, radioactive substrate binding assays, ELISA, OP assay, C3b deposition and IgG binding assays and

cytospins were analysed using two-tailed *t*-tests. Results of survival experiments were compared using the log rank method. Non-parametric data (eg target organ CFU, proportions of lymphocyte and macrophages and lung histology results) were analysed using the Kruskal Wallis (if three or more groups) or Mann Whitney U (if only two groups) tests.

Table 2.2: Primers used during this study

Primer name	Sequence
Sp0090.1	GCT CTA GAC ATT GAG AGA GAC AAC TGG
Sp0090.2	CGC TCT AGA CAA GAA GTA AGG GAA C
Sp0149.1	GCT CTA GAG GCT CTT GCA GCT TGC GG
Sp0149.2	CGC TCT AGA GGC TTT CGT TTG TAG CGT C
Sp0610.1	GCT CTA GAT TAC GGA GAC TAC CAC G
Sp0610.2	CGC TCT AGA TCC ACC AGA TAG CAT GCC
Sp0710.1	GCT CTA GAT TGG GCG TTA CGA TTG
Sp0710.2	CGC TCT AGA GTG CCT GTC CAC TTT C
Sp0750.1	GCT CTA GAC GCG CTG TTA GCC CTA GG
Sp0750.2	GCT CTA GAT GAT ACT GCA CGC ATG GC
Sp846.1	GCT CTA GAT GTT AGC AGG CCT TCT TG
Sp846.2	CGC TCT AGA GCC CCT GCA ATT TCA AC
Sp1690.1	GCT CTA GAT TGC GCT AGC GGC TGT TG
Sp1690.2	GCT CTA GAG CTT CCT CCA CCA CTA CG
Sp1798.1	GCT CTA GAT GAC TGT CCC CGG TTT AG
Sp1798.2	CGC TCT AGA TTG TTG ATT GGT CCT CCC
Sp1826.1	GCT CTA GAC GAC TGC TTC TTC ATC TG
Sp1826.2	CGC TCT AGA ATT GCC CGT CCT GTA CC
Sp2084.1	GCT CTA GAT TTG GGC TTG TTG CCT G
Sp2084.2	CGC TCT AGA TGT GAC CAC TTG TTG ACC
Sp2108.1	GCT CTA GAA CTG CTA CAC TTG CTA G
Sp2108.2	CGC TCT AGA ACC AAG GCT ACC TAC
Cm.1	GCT CTA GAT TAT AAA AGC CAG TCA AT
Cm.2	GCT CTA GAT TGA TTT TTA ATG GAT AA
Sp1	TCG AGA TCT ATC GAT GCA
Sp3	GGA TCC ATA TGA CGT CGA
R-20	CAG CTA TGA CCA TGA TTA CG
Sp0090.3	CCT GCA AAT AGG AGT ATA C
Sp0149.3	GGT TTC TCA GTT TTT AGG
Sp0610.3	GAT TTG ACT GTC CCG ATC G
Sp0610.4	GCT CTA GAG AGG AGG AGT TCC GGA TG
Sp0710.3	CTC CGC CAT GCA GGA TTC
Sp0710.4	GCT CTA GAG GTC AGG CGA TTG CTA TCG
Sp0750.3	GTG AGG GAA AAC CCT CGG
Sp0846.3	GTG GAT TTG TTG CCA ACG
Sp1690.3	CGT GCG AAT GCC TCA TCA C
Sp1798.3	GGA GGT TGC TAT GAA TAG
Sp1826.3	CCA ACT ATT ACT GTA AAC
Sp2084.3	GTA GTA AGC CTA CAC AAG
Sp2108.3	CTA TTC TTT AGG AGG AAT AC
Sp090RT1	GAG TGA CGC CAT CAC TGG
Sp090RT2	GAC CAT CCT TGC AGT CGG
Sp091RT1	CCG TGA AAT AGC GTA GGC
Sp091RT2	GCC AAA GCG GTC GGC TTG
Sp092RT1	GAG CGA CTA CAC TAG CAG

Primer name	Sequence
Sp092RT2	CAC TGG TAC TGT AGA CCC
Sp95RT1	GTG GTC TGC AGC AAA TTG
Sp095RT2	CGA GTG CCG TGT TCA CCC
Sp096RT1	CAA ACC GTC TAG CAA GGC
Sp0090RT1.1	CAC ACA ATG CTG GTC AGC
Sp0091RT2.1	CCA TTG GAA CAA TCA AGG C
90rt1.2	CTG GGC ATC TGG CCC TAT C
91rt2.2	GGT AGG ATA CAA GAG AGG
Sp148RT1	GCT CAG GGT CAA GAT GAG
Sp149RT1	GGT TGT GAG TAG TCT GTG
Sp149RT2	GGG AAA CAT CAC CTA AGG
Sp150RT1	GTG CCA TGA CAA AGG GCG
Sp150RT2	CCA CAG GGC GTT TCA GTC
Sp151RT1	CCT CTG CTG TCT TTT GGC
Sp151RT2	CAG CGT TGG CAG GTG CCC
Sp152RT1	CTG ACG GGC AAA GAA GGC
Sp152RT2	GTG ACG ACT GTG ACC TTG
Sp153RT1	GCG CCC AAA GCC TAT ATG
Sp0151RT1.1	CGC TGG AGC TTC AAC AGA C
Sp0152RT2.1	CAG AAA GTC GCT CCG CTC
Sp606RT1	GCT AGT CCT GGA ATC TAG
Sp607RT2	CTC TAC TCT GTT ATC GCC
Sp607RT1	CCA GAG AAG GTT GGG AAC
Sp608RT2	CTC CCT CCA TTG ACC AAC C
Sp608RT1	GGT CAC TGA TAA GAG GTA G
Sp609RT2	CTT CCC TGC ACG CTC ATC
Sp609RT1	CCA GAA TAA GTA CCG GTC
Sp610RT2	CCT CCT CTT TGA TGA ACC
Sp610RT1	CAG GAC AAC AAC TTG TCC
Sp611RT1	CAA TAG CCT GAC GAA TCC
Sp0749RT1	CGA TGC AGA CCA CAA CAC
Sp750RT2	CCT AGG GCT AAC AGC GC
Sp0750RT1	GGT GCG GCT CTT GGT GG
Sp0751RT2	CAC CAA TCG CCA TGA AAC C
Sp751RT1	GAC CAG GTG GAC TCC TTG G
Sp0752RT2	GTC CAA GTC CCA AAG AGG
Sp0752RT1	GGC CGT TTA ATC GCT CAA G
Sp0753RT2	GGG CGC GTC CCA TGG CAA G
750RT1.1	GAT GGG GGT TAC TCC AGG
751RT2.1	CCC AGA GTT GCT ACC GC
751RT1.1	GGT GCG ATT GTT TCG G
752RT2.1	GGT TCC GTA GCA AGG G
753RT1.1	GGA GAA TCG TCC TAT CAG
754RT2.1	CAG GCA GACGGT GCA AAC C
Sp845RT1	CCC TGG CGG TCA AGT GAT C
Sp846RT2	CAC CTT GGT ATT ATC ACG C

Primer name	Sequence
Sp846RT1	GTG GGT TGG ATG TCG GTG
Sp847RT2	GCA GAC TGC CAA AGG CTG
Sp847RT1	GGT ATT TCT GCC AAG CGG
Sp848RT2	GTA AAG ATG AGA GGT GCT G
Sp1686RT2	CGC ATC TAC CAT CTC GCG AC
Sp1687RT1	GAA GTG GCC GCA AGG GAG
Sp1687RT2	CAG TTG ATA TGA ACC TCC
Sp1688RT1	GTT GTG CTA CCG ATT GTA G
Sp1688RT2	CTG CAA CGA TGG TCG CAC
Sp1689RT1	GGT GCT TCA AGT TGG CAG
Sp1689RT2	GGC GTA CTC TGT GAA GAG
Sp1690RT1	GGG CCA AGT GTA CAA GCT G
Sp1690RT2	CCT TGA GTA AAG GAG TGC C
Sp1691RT1	CCA TGG CTG TTA CAT CGC C
Sp1794RT1	GGC AAT TGC CGG GCA ACG
Sp1795RT1	CCA AGA ACA CAA GTG GGC
Sp1795RT2	CAA GTG CTC CCA AGT CAC
Sp1796RT1	CCA AAT GGC GTT TGG ATC
Sp1796RT2	GGA GAT AAC GGT GAA CTG
Sp1797RT1	GCT TTT GTA GGA CAG TGG
Sp1797RT2	GCT GGT AAC CTT CTA CAG
Sp1798RT1	GCT TCA ACG CTC TAC ACG
Sp1798RT2	GTG CGT ATA AAA TGA GCT G
Sp1799RT1	GCA AAC GTA TTC CTC ATG
Sp1822RT1	CAG GAG GTA AAT GAA AGG C
Sp1823RT1	GCA TTT GGG CTT CGG CAG
Sp1823RT2	CCA GCG CCT AGA AAA CCC
Sp1824RT1	GGA AGA TGC AGC AAG AAG
Sp1824RT2	CAG TCG CTA AAA CCA CTC
Sp1825RT1	GTC GGT ACA CCA GTA GAG
Sp1825RT2	CCA CGC TTT TCA GGT TCC
Sp1826RT1	GGG GTT CTG GTG CAT TAG
Sp1826RT2	CAC GGT CTG CTA ATT GAG C
Sp1827RT1	GAA GAG GAG GAA GAA GAG
Sp1827RT2	CTC CGT TTC ACG ATT TTC
Sp1828RT1	GAC ATG TGT AGA GAT ACA TG
Sp1824RT2.1	GAT GTA CAG CAT CAA GGG
Sp1825RT1.1	GAC CGA TGA AAC AGT CCA CG
Sp1825RT1.1	CCA CGC TTT TCA GGT TCC
Sp1826RT2.1	GCC CTC AAA GAT CTA CCT G
Sp1827RT1.1	GCT ACA ACT GTA TTC CCA G
Sp1828RT2.1	CTA GGC TCG GGA GAA GGC
Sp1825RT1.2	ACC ACA TCC TGA AGG CCA
Sp1826RT2.2	GAA TGA TGT GGG GTT CTG
Sp2108RT1	GTC TGC AGT TTG GGA TCC AG
Sp2109RT2	GGA ATG ATA GAC AGC AGG G

Primer name	Sequence
Sp2109RT1	CAA TGG CGG CAG CTG TTA C
Sp2110RT2	GTT GAG GTA CCA AGT ACC G
2107RT1.1	CAA CGA AGT CGT AAG CAC
2108RT2.1	CCA AGC ACC GCA GCG CTC
2108RT1.1	CCC AGC TAA TAC TGA GGC
2109RT2.1	GGT TGC TCA ATT CAG GGG
2110RT1.1	CCG TAG GTC TCC AAA CC
2111RT2.1	GGA TAG GTC TCC TGG GAG
16s.1	GGT GAG TAA CGC GTA GGT AA
16s.2	ACG ATC CGA AAA CCT TCT TC
PsaA.1	CGT TCC GAT TGG GCA AGA C
PsaA.2	GCA CTT GGA ACA CCA TAG
Sp0149.1	G GCT CTT GCA GCT TGC GG
Sp0149.2	GGC TTT CGT TTG TAG CGT C
Sp0749.1	T GTG GAG AAG TGA AGT CTG GA
Sp0749.2:	GCA CGG TAA GCG TCA AGG AAGG
Sp0148F	CAC CAA TTG CCC AAA ATC C
Ery-**Sp0148R**	TATT TTAT ATT TTT GTT CAT **GAT TCT TTC TCC TTA AAA ATA**
Ery-**Sp0150F**	ATT ATT TAA CGG GAG GAA ATA A **TAA GAA ACA GGG AGG TGG GAG**
Sp0150R	CCA AGG CAT TTT TGG TCC C
Sp0749F	CAC TGA CAA TGC CAG TGA CTA TGC
Ery-**Sp0749R**	TAT TT TAT ATT TTT GTT CAT **AAG ATT CAC TCT TTC TAT TTA TAA**
Ery- **Sp0753F**	ATT ATT TAA CGG GAG GAA ATA A **AAC ATT CCA GTG GAT TGT TTT AG**
Sp0754R	GC GGA ATA TTG ACT GTA TGG GAG
Sp0149 For	TGC GGA AAC TCA GAA AAG AAA GCA
Sp0149 Rev	TTA CCA AAC TGG TTG ATC CAA ACC A
Sp0749Fwd	CGG GAT CCT GTG GAG AAG TGA AGT CTG GA
Sp0749Rv	CGG GAT CCT TAT GGT TTT ACA ACT TCT GC
Sp1032 (piaA) Fwd	TGT TCT TCT AAT TCT GTT AAA AAT G
Sp1032 (piaA) Rv	TTA TTT CGC ATT TTT GCA TG
Sp1872 (PiuA) Fwd	TGT AGT ACA AAC TCA AGC AC
Sp1872 (PiuA) Rv	TTA TTT CAA AGC TTT TTG TAT G
pQE30F	CCC GAA AAG TGC CAC CTG

Table 2.3: Plasmids constructed during the study

Plasmid name	*E. coli* strain	Target gene (bp)	Insert size	Restriction site	Plasmid backbone	Abc ^{r}
pPC110.1	DH5α	|Sp0090	337	5' *Xba*I 3'	pID701	Cm^{r}
pPC111.1	DH5α	Sp0149	392	5' *Xba*I 3'	pID701	Cm^{r}
pPC112.1	DH5α	Sp0610	315	5' *Xba*I 3'	pID701	Cm^{r}
pPC114.1	DH5α	Sp1798	357	5' *Xba*I 3'	pID701	Cm^{r}
pPC115.1	DH5α	Sp1826	425	5' *Xba*I 3'	pID701	Cm^{r}
pPC116.1	DH5α	Sp0750	462	5' *Xba*I 3'	pID701	Cm^{r}
pPC117.1	DH5α	Sp1690	657	5' *Xba*I 3'	pID701	Cm^{r}
pPC118.1	DH5α	Sp0846	590	5' *Xba*I 3'	pID701	Cm^{r}
pPC119.1	DH5α	Sp2084	500	5' *Xba*I 3'	pID701	Cm^{r}
pPC120.1	DH5α	Sp2108	450	5' *Xba*I 3'	pID701	Cm^{r}
ΔSp0149	JM109	Sp0149	2040	5'^{a} ; 3'^{a}	pGEM-T easy	Amp^{r}
ΔSp0750-53	JM109	ΔSp0750-53	2454	5'^{a} ; 3'^{a}	pGEM-T easy	Amp^{r}
pPC749	Novablue	Sp0749	1200	5' *Bam*HI; 3' *Sal*I	pST-1	Amp^{r}
pPc138	M15	Sp0149	800	5'^{a} ; 3'^{a}	pQE30UA	Amp^{r} / Kan^{r}
pPC139	M15	Sp0749	1200	5' *Bam*HI ; 3' *Sal*I	pQE30	Amp^{r} / Kan^{r}
Sp1032-pQE30UA	M15	Sp1032	969	5'^{a} ; 3'^{a}	pQE30UA	Amp^{r} / Kan^{r}
Sp1872-pQE30UA	M15	Sp1872	900	5'^{a} ; 3'^{a}	pQE30UA	Amp^{r} / Kan^{r}

Abc^{r} : Antibiotic resistance

Amp^{r} : Ampicillin resistant

Kan^{r} : Kanamycin resistant

[a] No restriction enzyme utilized during ligation.

Table 2.4: *S. pneumoniae* mutant strains constructed during the study

Strain	Target gene	ABC transporter	Description
110.1	Sp0090	Sp0090-92	0100993 containing an insertion made with plasmid pPC 110.1: Cm^r
111.1	Sp0149	Sp0149-152	0100993 containing an insertion made with plasmid pPC 111.1: Cm^r
111.1^{D39}	Sp0149	Sp0149-152	D39 containing an insertion made from 111.1 genomic DNA
112.1	Sp0610	Sp0607-610	0100993 containing an insertion made with plasmid pPC 112.1: Cm^r
114.1	Sp1798	Sp1796-98	0100993 containing an insertion made with plasmid pPC 114.1: Cm^r
115.1	Sp1826	Sp1824-26	0100993 containing an insertion made with plasmid pPC 115.1: Cm^r
116.1	Sp0750	Sp0749-753	0100993 containing an insertion made with plasmid pPC 116.1: Cm^r
116.1^{D39}	Sp0750	Sp0749-753	D39 containing an insertion made from 116.1 genomic DNA
117.1	Sp1690	Sp1689-90	0100993 containing an insertion made with plasmid pPC 117.1: Cm^r
118.1	Sp0846	Sp0844-848	0100993 containing an insertion made with plasmid pPC 118.1: Cm^r
119.1	Sp2084	Sp2084-87	0100993 containing an insertion made with plasmid pPCPC 119.1: Cm^r
120.1	Sp2108	Sp2108-10	0100993 containing an insertion made with plasmid pPC 120.1: Cm^r
ΔSp0149	Sp0149	Sp0149-53	0100993 containing the Sp0149 deletion construct: erm^r
ΔSp0750-53	Sp0750-53	Sp0749-53	0100993 containing the Sp0750-53 deletion construct: erm^r
ΔSp0149^{D39}	Sp0149	Sp0149-53	D39 containing the Sp0149 deletion construct from 0100993: erm^r
ΔSp0750-53^{D39}	Sp0750-53	Sp0749-53	D39 containing the Sp0750-53 deletion construct from 0100993: erm^r

Chapter 3
Screening of *S. pneumoniae* ABC transporters

Eleven *S. pneumoniae* ABC transporters were chosen from the annotated completely sequenced genome of serotype 4 strain (TIGR4) for the investigation. Amino acid homologies of the chosen TIGR4 ABC transporters with other bacterial species were identified using the Basic Local Alignment Search Tool (BLAST). Genetic organisation studies of the chosen ABC transporters were analysed by RT-PCR in the serotype 3 (0100993 strain) using the available TIGR4 genome information. Nine disruption mutants were then constructed in 0100993 strain and their *in vitro* and *in vivo* phenotypes were analysed. These results are presented in this chapter.

3.1 IDENTIFICATION AND SELECTION OF ABC TRANSPORTER GENES

The ABC transporters chosen for these studies were identified from the annotated completely sequenced genome of the capsular serotype 4 strain of *S. pneumoniae* (TIGR4) (Tettelin *et al*., 2001). The genome contains 73 ABC transporters (Harland *et al.*, 2005), 33 genes encoding putative lipoproteins, 24 of which are organised as operons with genes encoding other components of ABC transporter proteins (ATPases, permeases and or additional lipoproteins). Eleven of these have been previously described, (Table 1.3). Of the remaining 13 candidates, we selected 11 ABC transporters from the TIGR4 genome of *S. pneumoniae* for further investigation (Sp0090-0092, Sp0149-52, Sp0607-10, Sp0707-11, Sp0749-53, Sp0846-48, Sp1689-90, Sp1796-98, Sp1826-28, Sp2084-87 and Sp2108-2110). The genetic organisation of these selected ABC transporters are shown in figure 3.1.

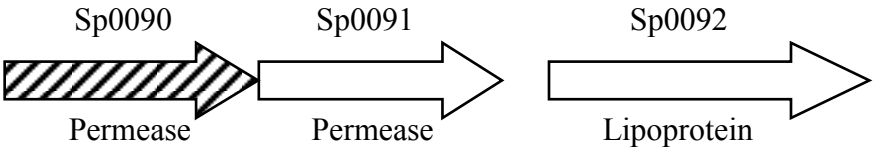
3.2 AMINO ACID HOMOLOGY OF TIGR4 ABC TRANSPORTER GENES WITH OTHER BACTERIAL SPECIES

To identify the close homologues of the *S. pneumoniae* ABC transporters, the derived amino acid sequence of each gene within the putative ABC transporter operons of TIGR4 genome were aligned against the amino acid sequence of completely sequenced R6 genome (an avirulent serotype 2 strain of *S. pneumoniae*) and other bacterial genomes using BLAST and the NCBI website (http://www.tigr.org). Six of the TIGR4 ABC transporter proteins (Sp0090-92, Sp0148-52, Sp0707-11, Sp0749-53, Sp0846-48, Sp2108-10) had >90% identity to the proteins encoded by genes in the R6 strain. The exceptions were: Sp0607 with 85% identity and 86% similarity; Sp1826 with 89% identity and 90% similarity and Sp0610, Sp1796, Sp1797, Sp1798, whose amino acid sequences were completely absent in the R6 strain of *S. pneumoniae*. Other bacterial species which contained genes encoding proteins exhibited homology of at least 50% identity and 60% similarity to the genes encoding proteins present in the TIGR4 genome included *S. suis, S. agalactaie, S. pyogenes, S. mutans* and *S. thermophilus*. In contrast, proteins encoded by the Sp1688-90, Sp1824-26 and Sp2084-87 operon had no close homologues amongst streptococci probably suggesting that these genes may not be horizontally transferred. Analysis of the *S. pneumoniae* ABC transporter genes, their homology to other bacterial species and their probable function according to BLAST similarities are listed in the Table 3.1.

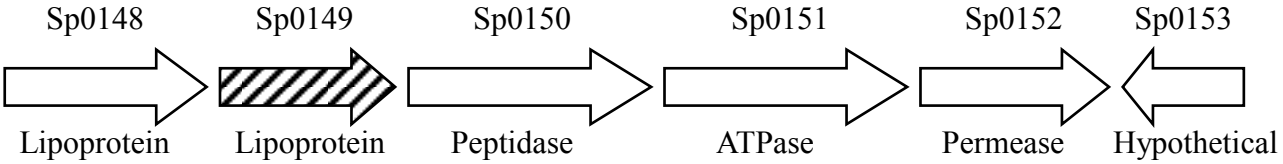
3.3 CONSTRUCTION OF THE DISRUPTION MUTANTS OF *S. PNEUMONIAE* BY INSERTIONAL DUPLICATION MUTAGENESIS

To investigate the role of these ABC transporters in virulence in mouse models of

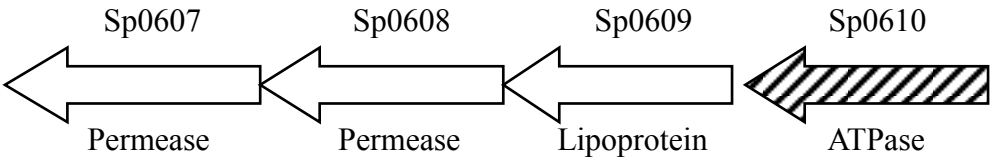
Sp0090-92 : Putative ABC transporter



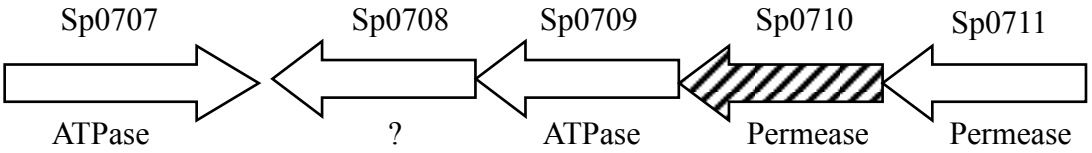
Sp0149-0153 : Metal ion ABC transporter, unknown function



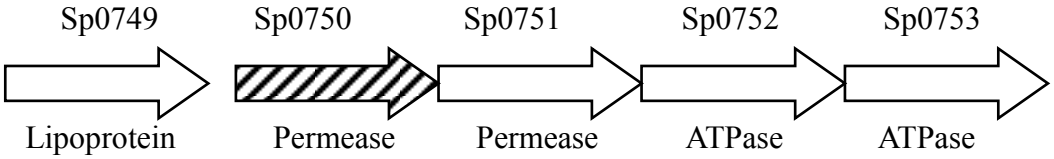
Sp0607-0610 : Putative amino acid ABC transporter



Sp0708-711: Putative glutamine ABC transporter



Sp0749- 0753 : Putative branched chain amino acid ABC transporter



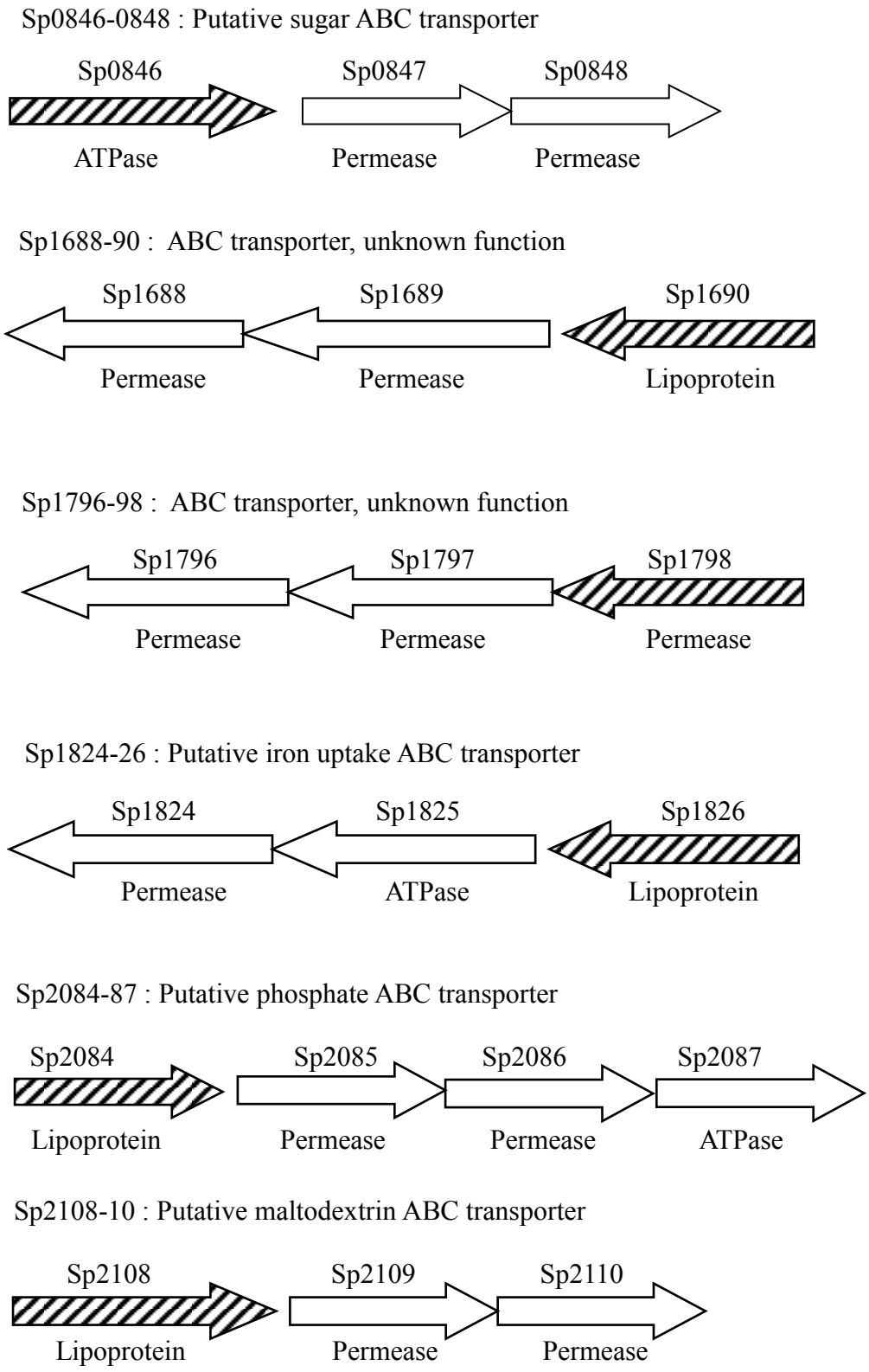


Fig 3.1 The genetic organisation of TIGR4 *S. pneumoniae* ABC transporters chosen for the phenotype studies. Shaded arrows indicate the genes disrupted by insertional duplication mutagenesis

Table 3.1 BLAST alignments of the derived amino acid sequences of the investigated *S. pneumoniae* (TIGR4) ABC transporters.

Gene[a]	Size (amino acids)	Protein name	Organism	% Identity / Similarity	Possible substrate (transport classification)[b]
Sp0090	319	EfaeDRAFT_2526	*Enterococcus faecium*	61/80 (311)	
Sp0091	307	EfaeDRAFT_2527	*E. faecium*	60/79 (300)	***Sugar***
Sp0092	491	EfaeDRAFT_2538	*E. faecium*	53/70 (486)	(CUT1 3.A.1.1)
Sp0148	276	SMU.1942c	*Streptococcus mutans*	55/76 (237)	Methionine
Sp0149	284	SPs1626	*Streptococcus pyogenes*	67/81 (278)	(MUT 3.A.1.24)
Sp0150	457	SMU.1940c	*S. mutans*	70/85 (457)	
Sp0151	353	SsiuDRAFT_0064	*Streptococcus suis*	80/90 (352)	
Sp0152	230	SPs1624	*S. pyogenes*	71/89 (230)	
Sp0607	219	SMU.1522	*S. mutans*	74/85 (213)	Amino acid
Sp0608	219	SMU.1521	*S. mutans*	62/81 (222)	(PAAT 3.A.1.3)
Sp0609	254	StheL01000593	*Streptococcus thermophilus*	60/73 (232)	
Sp0610	252	StheL01000592	*S. thermophilus*	82/90 (251)	
Sp0707	215	SsuiDRAFT_0032	*S. suis*	60/77 (213)	Amino acid
Sp0709	252	SGO_0983	*Streptococcus gordonii*	88/94 (252)	(PAAT 3.A.1.3)
Sp0710	225	SGO_0984	*S. gordonii*	89/95 (225)	
Sp0711	206	SGO_0985	*S. gordonii*	87/94 (226)	
Sp0749	386	SAG1582	*Streptococcus agalactiae*	53/73 (390)	BCAA[c]
Sp0750	289	SAK_1597	*S. agalactiae*	83/93 (289)	(HAAT 3.A.1.4)
Sp0751	318	gbs1630	*S. agalactiae*	73/88 (252)	
Sp0752	254	SsuiDRAFT_0078	*S. suis*	85/93 (254)	
Sp0753	236	SsuiDRAFT_0077	*S. suis*	87/96 (236)	
Sp0846	511	Spy1227	*S. pyogenes*	81/91 (508)	Ribonucleoside
Sp0847	352	SAK_1051	*S. agalactiae*	77/88 (353)	(CUT2 3.A.1.2)
Sp0848	318	Spy0928	*S. pyogenes*	80/92 (318)	
Sp1688	277	PM1760	*Pasteurella multocida*	80/91 (277)	Sugar
Sp1689	294	PM1761	*P. multocida*	79/93 (291)	(CUT1 3.A.1.1)
Sp1690	445	PM1762	*P. multocida*	70/85 407	
Sp1796	538	SsuiDRAFT_0524	*S. suis*	78/89 (537)	Sugar
Sp1797	305	SsuiDRAFT_0525	*S. suis*	82/94 (294)	(CUT1 3.A.1.1)
Sp1798	305	SsuiDRAFT_0526	*S. suis*	84/95 (303)	
Sp1824	563	Lxx14070	*Leifsonia xyli*	31/48 (539)	Cation
Sp1825	336	STH2752	*Symbiobacterium thermophilum*	45/59 (343)	(BIT 3.A.1.20)
Sp1826	355	Lxx14040	*L. xyli*	30/48 (323)	
Sp2084	291	RUMOBE_00498	*Ruminococcus. obeum*	46/65 (287)	Phosphate
Sp2085	287	Cthe_1604	*Clostridium thermocellum*	61/98 (284)	(PhoT 3.A.1.7)
Sp2086	271	DORLON_00312	*Dorea longicatena*	59/80 (287)	
Sp2087	250	BACCAP_00261	*Bacteroides capillosus*	72/86 (253)	
Sp2108	423	M_28Spy1048	*S. pyogenes*	53/67 (420)	maltodextrin
Sp2109	435	Spy1301	*S. pyogenes*	66/82 (430)	(CUT1 3.A.1.1)
Sp2110	280	SsuiDRAFT_0440	*S. suis*	85/93 (280)	

[a]TIGR4 genome Sp number.

[b]Transport classification and subfamily and number according to the transport classification system based on sequence similarity (32,45) (http://www.tcdb.org/).
[c]BCAA Branched-chain amino acid.

S. pneumoniae septicaemia and pneumonia, and to identify their growth defects in *in vitro* conditions,, mutant strains were constructed containing a disrupted copy of one of the genes encoding each of the ABC transporters using insertional duplication mutagenesis (IDM) in 0100993 strain of the capsular serotype 3 *S. pneumoniae*. The 0100993 strain was used to construct IDM mutants of the chosen ABC transporters because previous virulence studies of *S. pneumoniae* ABC transporters were performed by constructing IDM mutants in 0100993 strain in our laboratory. In general, the first gene of the probable operon was disrupted to prevent the transcription of the downstream genes and therefore ensure the maximum loss of function of the putative ABC transporter operon.

To construct *S. pneumoniae* mutant strains by IDM, forward and reverse primers were designed (Table 2.1**)** to amplify an internal portion of the target gene, which was amplified by PCR using 0100993 strain of serotype 3 genomic DNA as the template (Fig 3.2 A**).** An *Xba*1 restriction enzyme site was linked to the primers at 5'and 3'end and the amplified PCR products were digested with *Xba*I and ligated into pID701 (Fig 3.2 B). pID701 is a suicide vector derived from pEVP3 which carries chloramphenicol for selection in *S. pneumoniae* and *E. coli* (Claverys *et al*., 1995; Lau *et al*., 2001). After transformation into *E. coli* DH5α, positive clones of were identified by colony PCR using insert specific forward and reverse primers and plasmid DNA was extracted. The 0100993 strain of *S. pneumoniae* was then transformed with recombinant pID701 containing the target gene as described in chapter 2, plasmid constructs were confirmed by sequencing the PCR products generated by plasmid specific primer Sp1 or Sp3, upstream and downstream primers from the target region using the genomic DNA extracted from the mutant strains of *S. pneumoniae* as the template (Fig 3.3 A and B). Of the chosen 11 ABC transporters,

mutants were successfully constructed for 10 of them (Table 3.2) Despite repeated attempts and confirmation by sequencing that the plasmid was correctly constructed, no corresponding mutants were obtained for Sp0710 possibly because some regions of *S. pneumoniae* genome may be resistant to transformation or because the disruption of this gene may prove to be lethal for the bacteria. The *Sp0090*$^{-}$, *Sp0149*$^{-}$, *Sp0610*$^{-}$, *Sp1796*$^{-}$ and *Sp1824*$^{-}$ mutant strains were constructed by Suneeta Khandavilli.

Table 3.2: List of mutants constructed in 0100993 strain with the Sp number, possible substrate specificity and disruption site.

Mutant name	Plasmid name	ABC transporter gene	Putative substrate	Site of disruption
Sp0090^{-}	pPC110.1	Sp0090-92	Sugar	Sp0090
Sp0149^{-}	pPC111.1	Sp0149-152	Metal ion	Sp0149
Sp0610^{-}	pPC112.1	Sp0607-610	Amino acid	Sp0610
Sp0750^{-}	pPC116.1	Sp0749-753	BCCA[a]	Sp0750
Sp0846^{-}	pPC118.1	Sp0846-848	Sugar	Sp0846
Sp1690^{-}	pPC117.1	Sp1689-90	Sugar	Sp1690
Sp1796^{-}	pPC114.1	Sp1796-98	Sugar	Sp1798
Sp1824^{-}	pPC115.1	Sp1824-26	Iron	Sp1826
Sp2084^{-}	pPC119.1	Sp2084-87	Phosphate	Sp2084
Sp2108^{-}	pPC120.1	Sp2108-2110	Maltose	Sp2108

[a]Branched chain amino acids

3.4 STABILITY OF THE MUTATION

All the isogenic mutants listed in the table 3.2 were checked for the stability of the

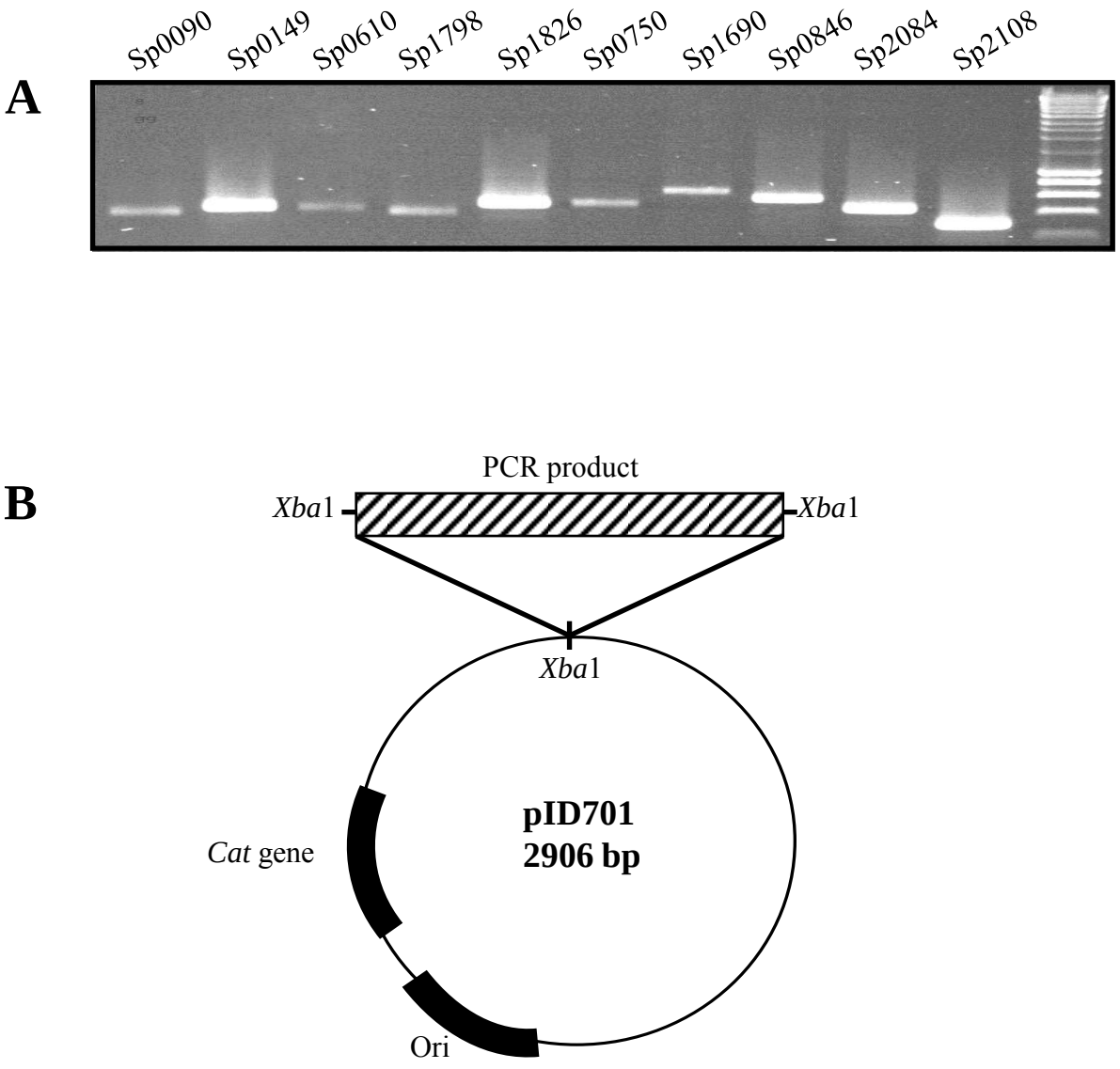


Figure 3.2 Schematic diagram of pID701 plasmid used to construct *S. pneumoniae* mutants in 0100993 strain using insertional duplication mutagenesis.

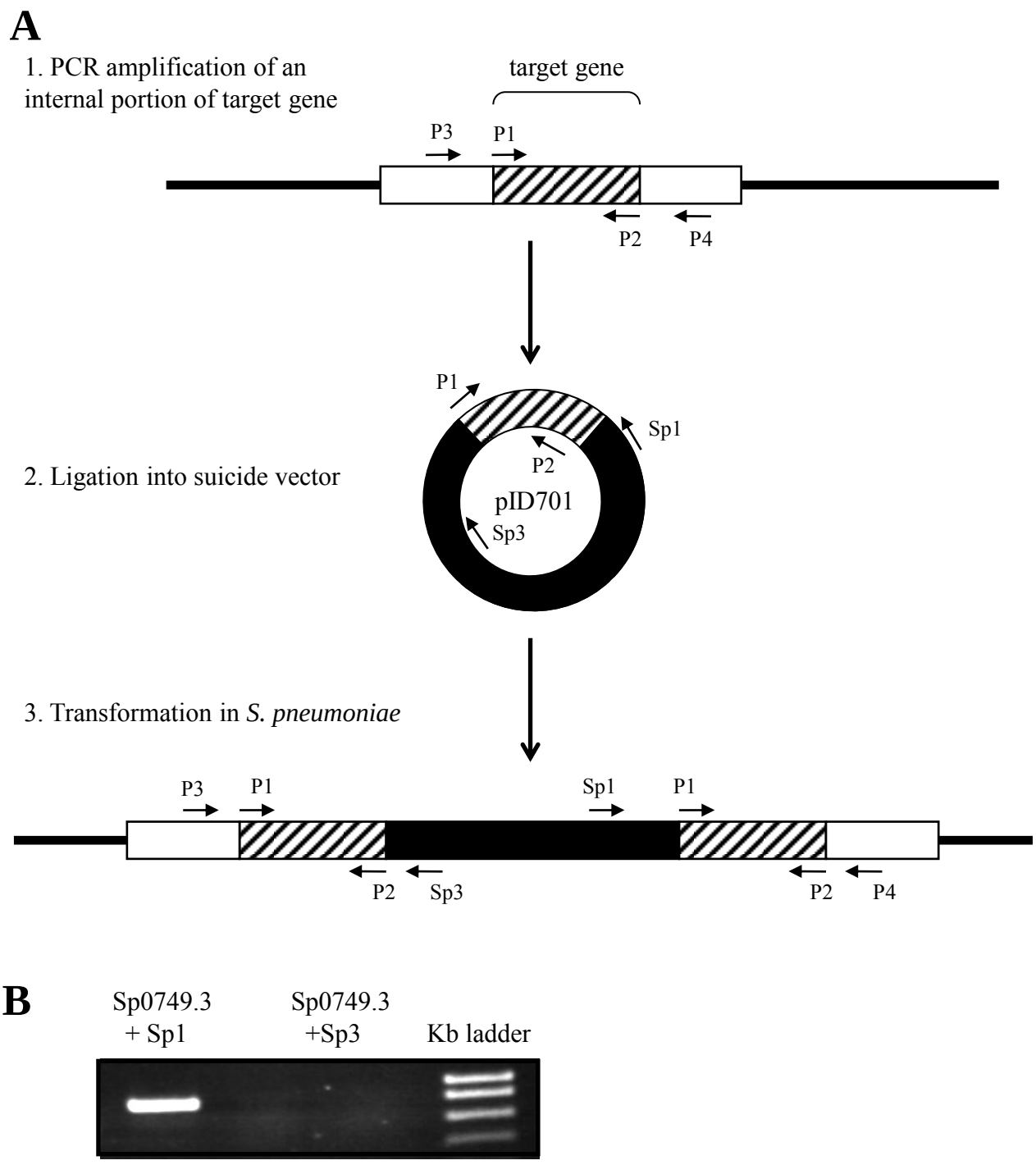


Fig 3.3 Diagram of insertional duplication mutagenesis, showing the integration of the entire plasmid into the target gene flanked by a duplication of the insert targeting DNA. (1 and 2) Successful ligation of the PCR product into pID701 was confirmed by PCR with primers Sp1 and P1 or P2 (depending on the insert orientation). (3 and B) Successful transformation into *S. pneumoniae* (0100993 strain) was confirmed by PCR using P3 or P4 and Sp1 or Sp3 (depending on the orientation of the insert).

mutation by culturing the mutant strains in THY broth in the absence of antibiotic (and therefore with no selective pressure for the mutation) for two 8 hours growth cycles followed by plating onto plain and antibiotic containing Columbia agar plates. *S. pneumoniae* mutant strains *Sp0090*$^{-}$, *Sp0149*$^{-}$, *Sp0610*$^{-}$, *Sp0750*$^{-}$, *Sp0846*$^{-}$, *Sp1690*$^{-}$, *Sp1796*$^{-}$, *Sp1824*$^{-}$, and *Sp2108*$^{-}$ had similar number of colonies on the antibiotic-free and antibiotic-supplemented plates confirming the mutation was stable over these time periods. However, *Sp2084*$^{-}$ had an unstable mutation, as the number of colonies recovered from these mutant strains were very low on the blood agar containing the antibiotic (19 cfu) compared to the number of colonies recovered on the unsupplemented blood agar (>1000 cfu) when a 10^{-4} dilution of the second 8 hour THY culture was plated.

3.5 GROWTH CURVES IN THY

The *in vitro* phenotypes of the mutants strains were investigated by comparing the growth rates of the mutant strains to the wild-type strains in the undefined complete medium (THY). Equal numbers of the wild-type and the mutant bacteria obtained from thawed stocks were inoculated into THY, cultured at 37°C in the presence of 5% CO_2 and the optical density recorded at one hourly intervals for 8 hours. All the mutant strains exhibited good growth in THY, similar to the wild-type strain (Fig 3.4 and 3.5), suggesting that the disruption of the target genes of the ABC transporters had little effect on the growth of *S. pneumoniae* in a complete laboratory medium.

3.6 PHENOTYPE ANALYSIS BY COMPETITIVE INDEX

To investigate the role of the selected ABC transporters in mouse models of pulmonary (IN inoculation) and systemic (IP inoculation) infection, mixed inocula of

wild-type and mutant strains were inoculated into mice by either intraperitoneal or intranasal routes and the competitive indices (CI) determined by plating the bacteria recovered from the target organs (spleen and lungs) on plain and chloramphenicol containing medium. The *in vitro* phenotypes of the mutant strains were also investigated using CIs to identify any subtle growth defects in complete medium (THY), high osmotic stress medium (THY + 100mM NaCl) and normal physiological fluid (human blood) and relate these to the *in vivo* phenotype. The cfu of wild-type and mutant strains of bacteria recovered from various target organs and media were enumerated by plating and CIs calculated using the formula described in Chapter 2. A CI of 1 shows there is no difference in virulence or growth between wild-type and mutant strains whereas CIs less than 1 suggest a degree of attenuation in virulence or growth. The CIs of the mutants in different *in vitro* growth conditions and mouse models of septicaemia (IP) and pneumonia (IN) infection are shown in the table 3.3.

Most of the mutants had CIs close to 1.0 in both complete medium (THY) and high osmotic stress medium (THY + 100mM NaCl), suggesting that the disruption performed in the target gene of the ABC transporters had no effect on growth in rich laboratory medium or in the presence of high osmotic stress. The exceptions were *Sp0610*$^{-}$which had impaired growth in high osmotic media and *Sp0750*$^{-}$ which had mildly impaired growth in THY. In contrast, in normal physiological fluid, that is human blood, several mutants (*Sp0090*$^{-}$, *Sp0149*$^{-}$, *Sp1824*$^{-}$, *Sp0750*$^{-}$), and to a lesser extent *Sp0610*$^{-}$, exhibited decreased CIs. This suggests these mutants have a particular problem growing under the greater stress conditions (presence of immune cells, restricted nutrient availability) found in physiological fluid compared to THY. In both systemic and pulmonary infection, *Sp0149*$^{-}$ and *Sp0750*$^{-}$ were markedly attenuated in

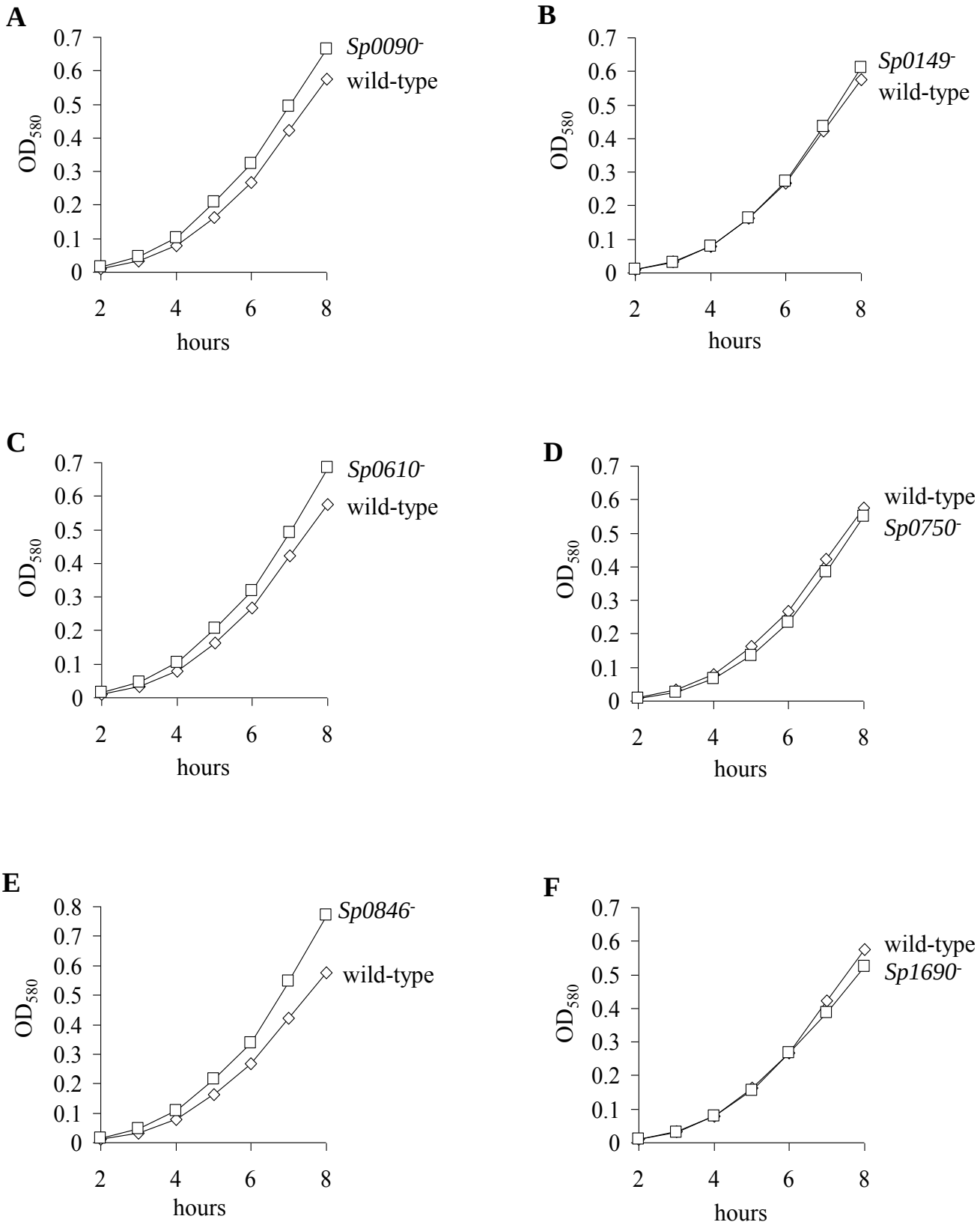


Fig 3.4 Growth of mutant strains (squares) of ABC transporters and the wild-type (diamonds) of *S. pneumoniae*,ST3 (0100993 strain) measured in THY medium. Results presented are the mean of triplicate samples for each strain.

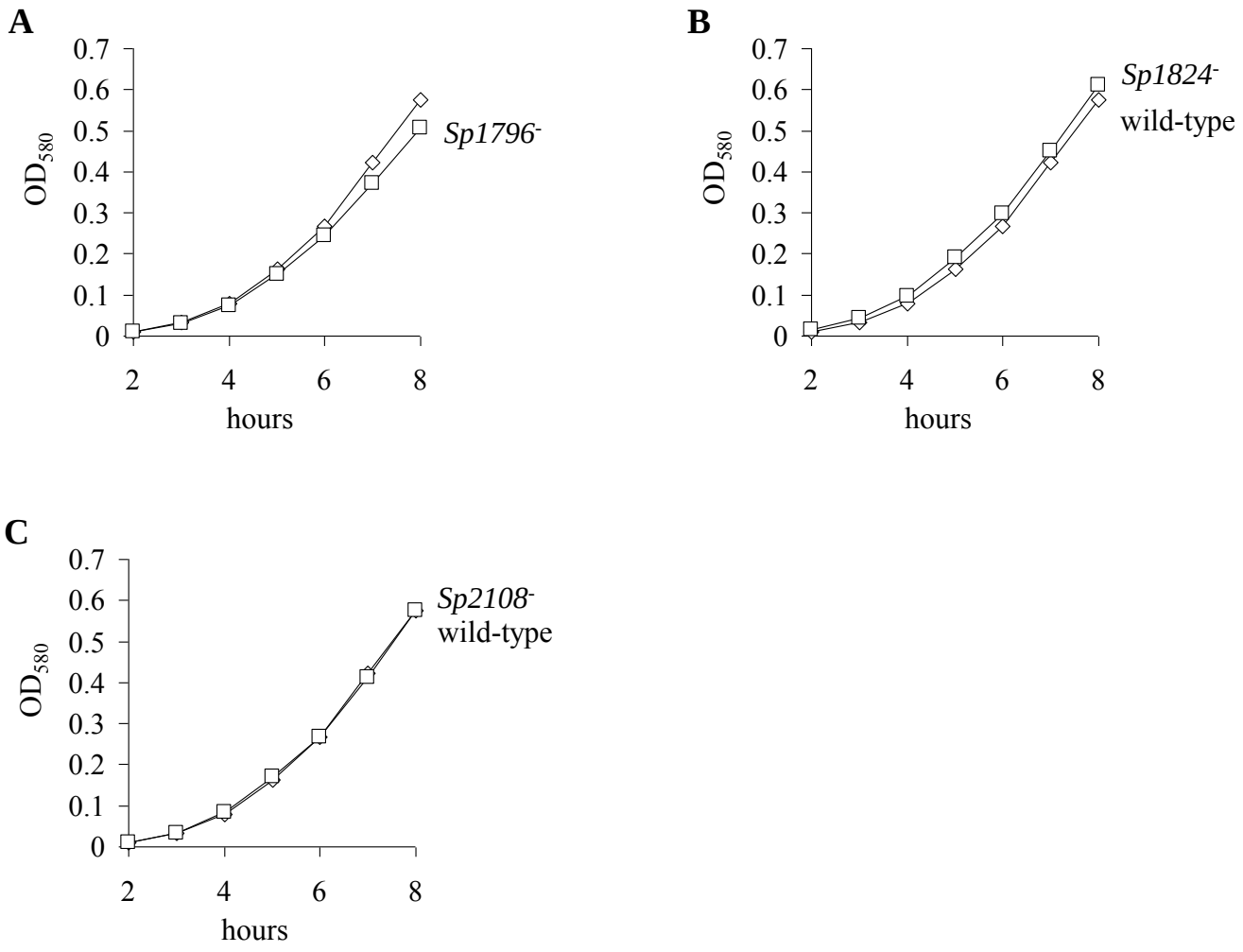


Fig 3.5 Growth of mutant strains (squares) of ABC transporters and the wild-type (diamonds) of *S. pneumoniae*,ST3 (0100993 strain) measured in THY medium. Results presented are the mean of triplicate samples for each strain.

Table 3.3 *In vitro* and *in vivo* phenotype analysis of *S. pneumoniae* ABC transporter mutant strains (0100993) by competitive index (CI).

Strain	THY	THY + 100mM NaCl	Blood	IP	IN
Sp0090^{-}	1.06 ± 0.31 n=7	1.17 ± 0.21 n=3	0.40 ± 0.08 n=6	0.50 ± 0.10 n=5	0.43 ± 0.13 n=4
Sp0149^{-}	0.80 ± 0.27 n=13	0.85 ± 0.26 n=6	0.46 ± 0.13 n=6	0.067 ± 0.028 n=5	0.023 ± 0.019 n=6
Sp0610^{-}	0.94 ± 0.32 n=17	0.65 ± 0.13 n=6	0.71 ± 0.12 n=6	0.70 ± 0.13 n=5	0.64 ± 0.30 n=4
Sp0750^{-}	0.68 ± 0.17 n=9	0.94 ± 0.30 n=3	0.38 ± 0.15 n=6	0.17 ± 0.12 n=10	0.016 ± 0.018 n=8
Sp0846^{-}	1.07 ± 0.22 n=8	0.88 ± 0.26 n=8	0.93 ± 0.17 n=6	0.61 ± 0.37 n=6	0.45 ± 0.18 n=4
Sp1690^{-}	0.97 ± 0.23 n=12	1.27 ± 0.52 n=3	1.61 ± 0.35 n=6	0.89 ± 0.39 n=5	ND
Sp1796^{-}	0.91 ± 0.28 n=7	0.91 ± 0.30 n=3	0.99 ± 0.29 n=6	1.34 ± 0.48 n=6	ND
Sp1824^{-}	0.95 ± 0.23 n=7	0.85 ± 0.14 n=3	0.49 ± 0.008 n=6	0.59 ± 0.10 n=3	0.40 ± 0.17 n=3
Sp2108^{-}	1.06 ± 0.076 n=4	0.81 ± 0.18 n=8	0.94 ± 0.14 n=6	0.37 ± 0.18 n=8	3.33 ± 3.5[a] n=5

ND: Not Done

[a]: Highly variable results were obtained even after the experiment was repeated several times

virulence, while *Sp0090*$^{-}$, *Sp0610*$^{-}$, *Sp1824*$^{-}$ and *Sp2108*$^{-}$ had partial attenuation of their virulence. *Sp1796*$^{-}$ and *Sp1690*$^{-}$ did not show any impairment in virulence. *Sp0750*$^{-}$ showed a greater attenuation of virulence in pulmonary infection than in systemic infection suggesting a possible greater role for the corresponding ABC transporters during pneumonia compared to septicaemia These *in vivo* CIs mirrored the CIs for blood for most mutant strains, with those strains showing no impairment in blood also being fully virulent after IP inoculation (*Sp0846*$^{-}$*, Sp1690*$^{-}$ and *Sp1796*$^{-}$) and those strains with impaired CIs in blood having a similar CI or lower during infection. *Sp2108*$^{-}$ is an exception as this particular mutant strain exhibited attenuation in systemic models of infection although there was no impairment in human blood. Even though the CIs of the mutant strains in human blood mirrored the CIs in *in vivo* infection models, there was no marked attenuation as observed in IP and IN models of infection. One explanation could be that the natural exposure of the humans to *S. pneumoniae* would have elicited antibodies and therefore may have cleared both the wild-type and mutant strains of bacteria. The CI for pulmonary infection for *Sp2108*$^{-}$ was too variable for easy interpretation. The similar CI for the *Sp0610*$^{-}$ *strain* in THY+NaCl, blood and *in vivo* suggests the mildly impaired virulence of this strain is due to poor growth under the osmotic conditions found *in vivo*. The impairment in virulence of *Sp0090*$^{-}$, *Sp0610*$^{-}$, *Sp1824*$^{-}$ and *Sp2108*$^{-}$ were relatively mild and these strains were not investigated further. However, as the *Sp0149*$^{-}$ and *Sp0750*$^{-}$ were more significantly attenuated in virulence they were chosen for further investigation and as potential vaccine candidates.

3.7 ANALYSIS OF THE GENETIC ORGANISATION OF THE ABC TRANSPORTER OPERONS BY RT-PCR

To confirm the operon structure of the ABC transporters and identify genes whose transcription will be disrupted in the mutant strains, RT-PCR was performed with primers designed to amplify a product that spans the junctions of the genes present in the possible operon. Total RNA from 0100993 strain of *S. pneumoniae* serotype 3 was used as the template for RT-PCR, its purity being demonstrated by the absence of DNA contamination using appropriate RT-PCR negative control reactions containing no reverse transcriptase. As controls for the target specific PCR, the same primers were used with the genomic DNA as the template. Since the ABC transporters were selected using the TIGR4 genome sequence, cDNA products obtained from the RT-PCR not only gave the information about the organisation of genes, but also, confirmed that the genes were organised in the serotype 3 strain in an identical way to the TIGR4 genome. In addition, deducing the genetic organisation of these operons in serotype 3 genetic background will also provide information on the possible polar effect due to IDM. Amplification of cDNA failed if the primers spanned junctions of genes of the ABC transporter operon were not co-transcribed.

(a) Sp0090-0092:

RT-PCR analysis of the transcriptional structure for these three genes suggested that Sp0090, Sp0091 and Sp0092 are all co-transcribed with transcription terminating after Sp0092 in serotype 3 (Table 3.4).

(b) Sp0148-0152

Using serotype 3 RNA, RT-PCR analysis of the transcriptional structure for Sp0148-0152 suggested that Sp0148, Sp0149 and Sp0150 are not transcriptionally linked in serotype 3. However, Sp0150, Sp0151 and Sp0152 are all co-transcribed with

transcription terminating after Sp0152 (Fig 3.6). Hence the RT-PCR results of Sp0148-52 region in serotype 3 strain correlated with the operon structure of the TIGR4 genome sequence.

(c) Sp0607-10

RT-PCR across the junction of Sp0607/Sp0608, Sp0609/610 and Sp0610/611 suggested that these junctions may be linked in serotype 3. However the junction of Sp0608/Sp0609 is not transcriptionally linked in serotype 3(Table 3.4).

(d) Sp0749-53

RT-PCR analysis of the transcriptional structure for Sp0749-53 suggested that Sp0749 and Sp0750 are not transcriptionally linked in serotype 3. However, Sp0750, Sp0751 and Sp0752 are all co-transcribed with transcription terminating after Sp0753 (Fig 3.7).

(e) Sp0846-48

RT-PCR analysis of the transcriptional structure for Sp0846-48 suggested that Sp0846, Sp0847 and Sp0848 are co-transcribed (Fig 3.8) in serotype 3 genome.

(f) Sp1686-90

RT-PCR analysis of the transcriptional structure for Sp1686-90 suggested that Sp1686, Sp1687 and Sp1688 are co-transcribed. However, from the RT-PCR results it was not clear if Sp1688, Sp1689 and Sp1690 are transcriptionally linked (Table 3.4) in serotype 3.

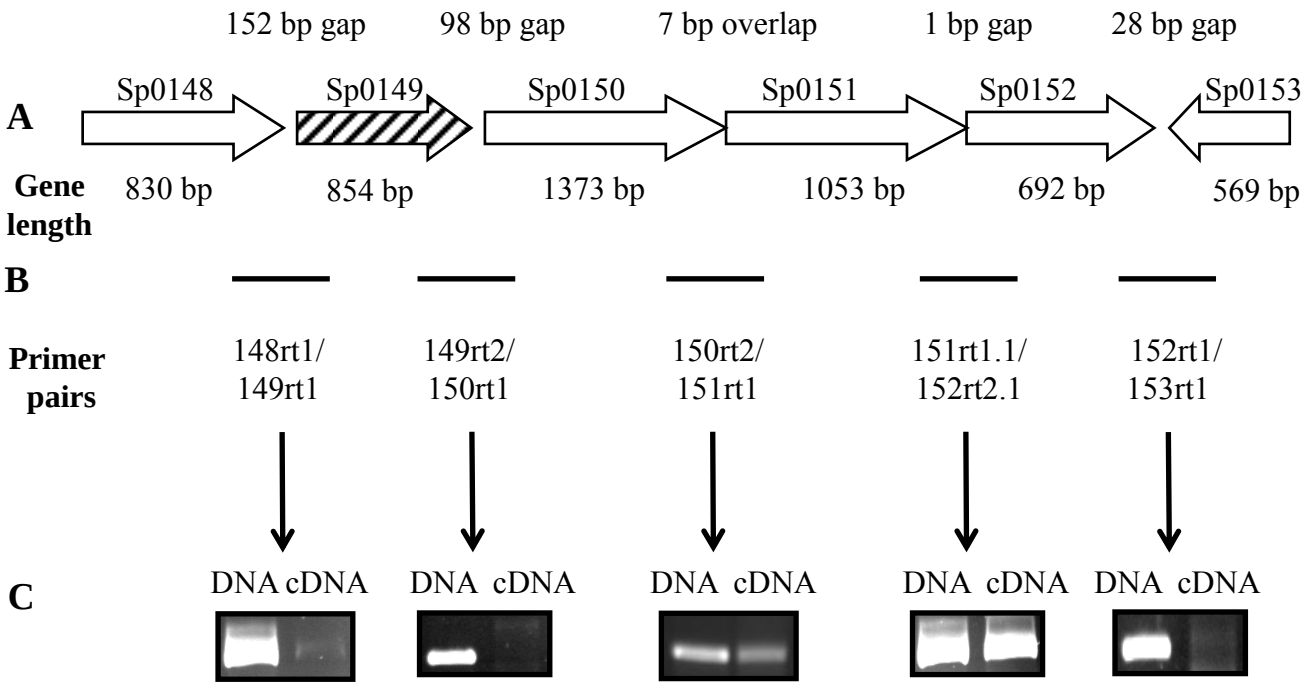


Fig 3.6 (A) Operon structure of Sp0148-0153. Open arrows: ORFs of genes present in the operon (Sp0148: lipoprotein, Sp0149: lipoprotein, Sp0150: hypothetical protein, Sp0151: ATPase, Sp0152: permease, Sp0153: hypothetical protein) in the chromosomal DNA of *S. pneumoniae*. Diagonally hatched arrow: site of disruption in the mutant strain using IDM. (B) Horizontal line: PCR products generated using the primer pairs. (C) Ethidium bromide stained agarose gel containing PCR products generated by primer pairs (mentioned in B) using *S. pneumoniae* genomic DNA as the template and RT-PCR products generated using *S. pneumoniae* RNA as the template.

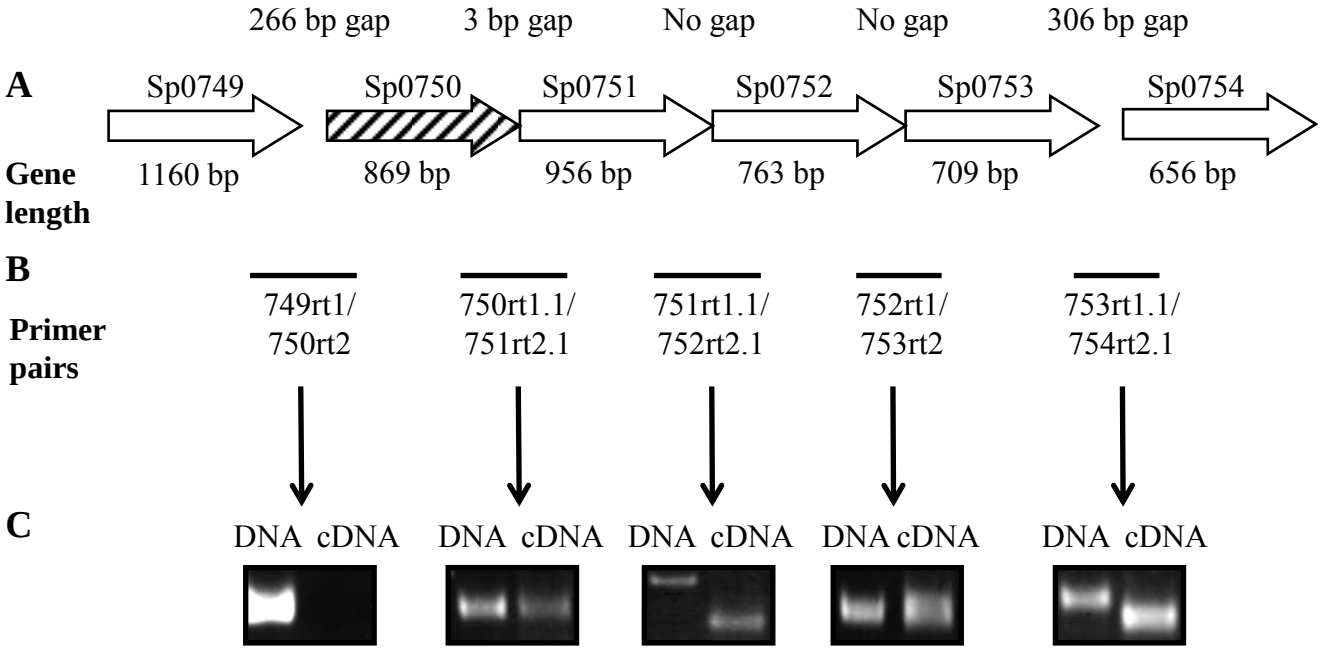


Fig 3.7 (A) Operon structure of Sp0749-0753. Open arrows: ORFs of genes present in the operon (Sp0749: lipoprotein, Sp0750: permease, Sp0751: permease, Sp0752: ATPase, Sp0753: ATPase, Sp0754: putative acetion utilization protein) in the chromosomal DNA of *S. pneumoniae*. Diagonally hatched arrow: site of disruption in the mutant strain using IDM. (B) Horizontal line: PCR products generated using the primer pairs. (C) Ethidium bromide stained agarose gel containing PCR products generated by primer pairs (mentioned in B) using *S. pneumoniae* genomic DNA as the template and RT-PCR products generated using *S. pneumoniae* RNA as the template. cDNA band generated from the junction of Sp0751/Sp0752 was sequenced and then aligned against TIGR4 genome which matched the Sp0751 gene.

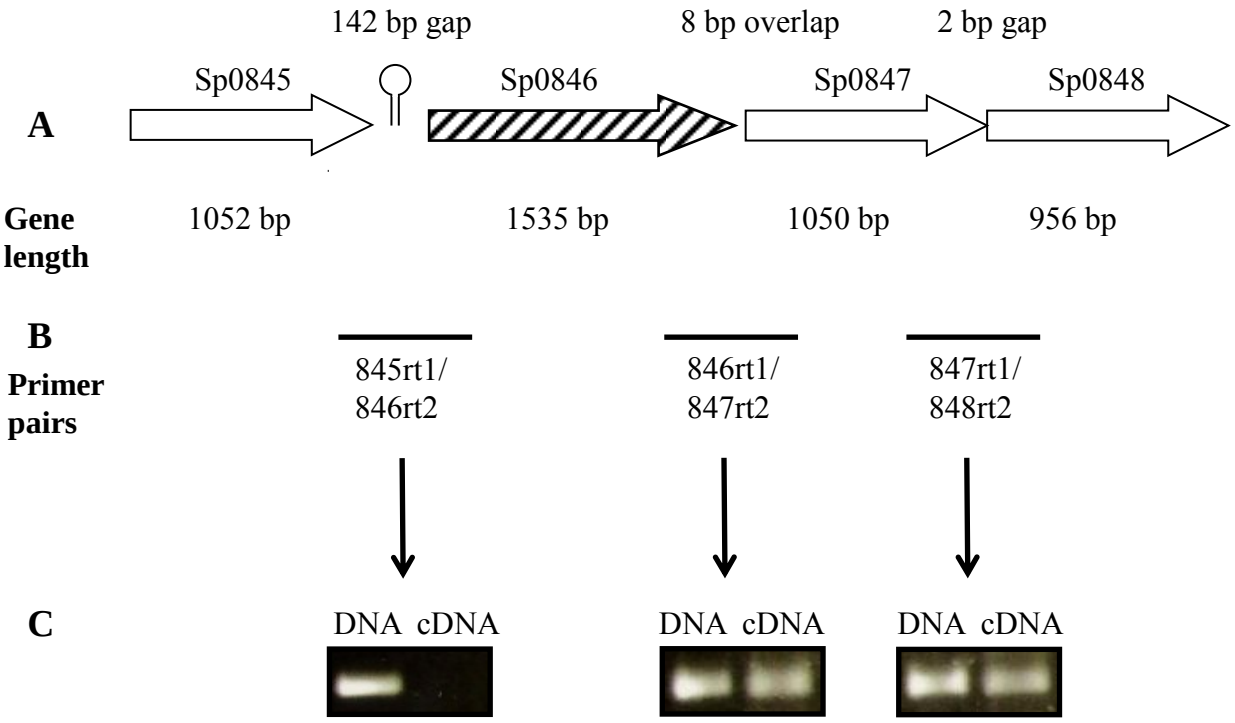


Fig 3.8 (A) Operon structure of Sp0846-0848. Open arrows: ORFs of genes present in the operon (Sp0845: lipoprotein, Sp0846: ATPase, Sp0847: permease, Sp0848: permease) in the chromosomal DNA of *S. pneumoniae*. Diagonally hatched arrow: site of disruption in the mutant strain using IDM. (B) Horizontal line: PCR products generated using the primer pairs. (C) Ethidium bromide stained agarose gel containing PCR products generated by primer pairs (mentioned in B) using *S. pneumoniae* genomic DNA as the template and RT-PCR products generated using *S. pneumoniae* RNA as the template.

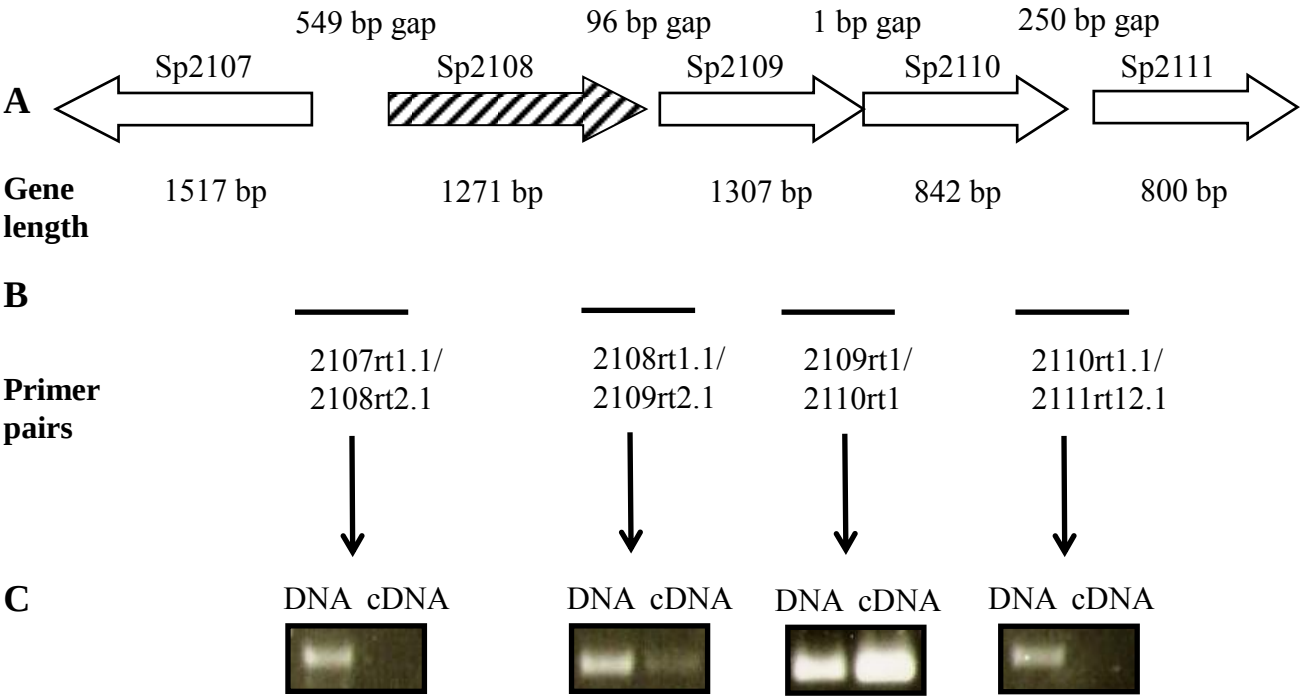


Fig 3.9 (A) Operon structure of Sp2108-2110. Open arrows: ORFs of genes present in the operon (Sp2107: 4-alpha-glucanotransferase, Sp2108: lipoprotein, Sp2109: permease, Sp2110: permease, Sp2111: malA protein) in the chromosomal DNA of *S. pneumoniae*. Diagonally hatched arrow: site of disruption in the mutant strain using IDM. (B) Horizontal line: PCR products generated using the primer pairs. (C) Ethidium bromide stained agarose gel containing PCR products generated by primer pairs (mentioned in B) using *S. pneumoniae* genomic DNA as the template and RT-PCR products generated using *S. pneumoniae* RNA as the template.

Table 3.4 Analysis of ABC transporter operon structure in serotype 3 by RT-PCR

Operon	Junction between genes	Gap / overlap between junction in base pairs (bp)	Primer pairs used	DNA (bp)		cDNA (bp)	
				Expected	Obtained	Expected	Obtained
Sp0090-96	Sp0090 / Sp0091	13 bp	Sp0090RT2 / Sp0091RT1 Sp0090rt1.2 / Sp0091rt2.2	605 bp	~600 bp	NP	600 bp
	Sp0091 / Sp0092	256 bp	Sp0091RT2 / Sp0092RT1	467 bp	~500 bp	NP	~500 bp
	Sp0092 / Sp0095	270 bp	Sp0092RT2 / Sp0095RT1	600 bp	600 bp	NP	NP
	Sp0095 / Sp0096	273 bp	Sp0095RT2 / Sp0096RT1	500 bp	500 bp	NP	NP
Sp0607-11	Sp0607 / Sp0608	9 bp	Sp0607RT1 / Sp0608RT2	509 bp	500 bp	NP	~500 bp
	Sp0608 / Sp0609	12 bp	Sp0608RT1 / Sp0609RT2	415 bp	~400 bp	NP	NP
	Sp0609 / Sp0610	38 bp	Sp0609RT1 / Sp0610RT2	436 bp	~400 bp	NP	~400 bp
	Sp0610 / Sp0611	575 bp	Sp0610RT1 / Sp0611RT2	578 bp	~600 bp	NP	~600 bp
Sp1686-91	Sp1686 / Sp1687	11 bp	Sp1686RT2 / Sp1687RT1	540 bp	>500 bp	NP	>500 bp
	Sp1687 / Sp1688	17 bp	Sp1687RT2 / Sp1688RT1	400 bp	400 bp	NP	400 bp + other bands
	Sp1688 / Sp1689	No gap	Sp1688RT2 / Sp1689RT1	350 bp	350 bp	350 bp	NP
	Sp1689 / Sp1690	77bp	Sp1689RT2 / Sp1690RT1	600 bp	600 bp	NP	NP

Operon	Junction between genes	Gap / overlap between junction in base pairs (bp)	Primer pairs used	DNA (bp)		cDNA (bp)	
				Expected	Obtained	Expected	Obtained
	Sp1690 / Sp1691	18 bp	Sp1690RT2 / Sp1691RT1	350 bp	350 bp	NP	Non-specific bands
Sp1794-99	Sp1794 / Sp1795	400 bp	Sp1794RT1 / Sp1795RT1	714 bp	700 bp	NP	NP
	Sp1795 / Sp1796	9 bp	Sp1795RT2 / Sp1796RT1	544 bp	NP	NP	NP (obtained 544 bp DNA from TIGR4)
	Sp1796 / Sp1797	27 bp	Sp1796RT2 / Sp1797RT1	485 bp	485 bp	NP	NP
	Sp1797 / Sp1798	13 bp overlap	Sp1797RT2 / Sp1798RT1	583 bp	>550 bp	583 bp	NP
	Sp1798 / Sp1799	149 bp	Sp1798RT2 / Sp1799RT1	734 bp	>700 bp	NP	NP
Sp1822-28	Sp1822 / Sp1823	95 bp	Sp1822RT1 / Sp1823RT1	626 bp	~600 bp	NP	~600 bp
	Sp1823 / Sp1824	2 bp	Sp1823RT2 / Sp1824RT1	571 bp	~600 bp	571 bp	~600 bp
	Sp1824 / Sp1825	11 bp	Sp1824RT2.1 / Sp1825RT1.1	431 bp	~400 bp	NP	NP
	Sp1825 / Sp1826	101 bp	Sp1825RT1.1 / Sp1826RT2.1	514 bp	NP	NP	NP
	Sp1826 / Sp1827	23 bp	Sp1826RT2 / Sp1827RT1	426 bp	~400 bp	NP	NP
	Sp1827 / Sp1828	179 bp	Sp1827RT1.1 / Sp1828RT2.1	469 bp	NP	NP	NP

NP: No product

(g) Sp1796-98

RT-PCR analysis of the transcriptional structure for Sp1796-98 was not clear as the expression level of this region was poor in serotype 3 (Table 3.4).

(h) Sp1824-26

RT-PCR analysis of the transcriptional structure for Sp1824-26 suggested that Sp1823, Sp1824 may be transcriptionally linked in serotype 3. However It is not clear from the RT-PCR if other genes of this region are transcriptionally linked as the expression level of this region was poor (Table 3.4).

(i) Sp2108-10

RT-PCR analysis of the transcriptional structure for Sp2108-10 suggested that Sp2108, Sp2109 and Sp2110 are transcriptionally linked with transcription terminating after Sp2110 in serotype 3 (Fig 3.9).

3.8 SUMMARY

For this study, 11 ABC transporters (Sp0090-0092, Sp0149-0152, Sp0607-0610, Sp0707-0711, Sp0846-0848, Sp1689-90, Sp1796-98, Sp1826-28, Sp2084-87, Sp2108-2110) were chosen for investigation from the annotated completely sequenced genome of serotype 4 strain (Tettelin *et al.*, 2001). Of the 11 ABC transporters operons, four of the operons (Sp0090-92, Sp0148-52, Sp0846-48 and Sp2108-2110) from serotype 3 strain are genetically organised in a similar fashion to that of annotated serotype 4 strain. Mutant strains were successfully constructed in a serotype 3 (0100993 strain) of *S. pneumoniae* for 10 of these ABC transporters (*Sp0090*$^{-}$*, Sp0149*$^{-}$*, Sp0610*$^{-}$*, Sp0750*$^{-}$*, Sp0846*$^{-}$*, Sp1690*$^{-}$*, Sp1798*$^{-}$*, Sp1826*$^{-}$*, Sp2084*$^{-}$*, Sp2108*$^{-}$) by disrupting the first gene of the probable operon using insertional

duplication mutagenesis. 9 mutant strains (*Sp0090*$^{-}$, *Sp0149*$^{-}$, *Sp0610*$^{-}$, *Sp0750*$^{-}$, *Sp0846*$^{-}$, *Sp1690*$^{-}$, *Sp1798*$^{-}$, *Sp1826*$^{-}$, *Sp2108*$^{-}$) were confirmed to have a stable mutation, with one mutant strain (*Sp2084*$^{-}$*)* containing an unstable mutation. Despite the mutations generally having weak or no effect in culture media, *in vivo* phenotype analysis using CIs suggested a strong attenuation of virulence for *Sp0149*$^{-}$ and *Sp0750*$^{-}$ in both systemic and pulmonary models of infection, while *Sp0090*$^{-}$, *Sp0610*$^{-}$, *Sp0846*$^{-}$ *Sp1826*$^{-}$ and *Sp2108*$^{-}$ had partial attenuation in virulence. These data confirm that ABC transporters are frequently required for full virulence and identified the Sp0149 and Sp0749 lipoproteins for further investigation.

Chapter 4

Detailed phenotype analysis of the effects of mutation of *Sp0149* and *Sp0750-53*

Results from chapter 3 showed that *Sp0149-52* and *Sp0749-53* are important for *S. pneumoniae* survival in *in vivo* models of pneumonia and septicaemia. Therefore, detailed *in vitro* and *in vivo* phenotype analysis of the effects of deletion of *Sp0149* and *Sp0750-53* were performed mostly using 0100993 strain. However, for the ease of performing certain experiments such as uptake assays, the deletion mutation was transferred from the serotype 3 (0100993) to serotype 2 (D39) strain. These results are presented in this chapter.

4.1 GENE EXPRESSION OF *SP0149* AND *SP0749* BY SEMI-QUANTITATIVE RT-PCR

To investigate whether the *Sp0149* and *Sp0749* lipoprotein genes are expressed during infection, semi-quantitative RT-PCR was performed on total RNA extracted from *S. pneumoniae* recovered from mice, or after incubation of the bacteria in blood or THY. The abundance of the *Sp0149* and *Sp0749* lipoprotein mRNA transcripts were compared with the mRNA expression of lipoprotein component (*Sp1386*) of polyamine ABC transporter (ORFs 1386-89) (Shah *et al.*, 2008) and another well-characterized ABC transporter lipoprotein, *psaA*, both of which are known to be expressed during infection (Ogunniyi *et al.*, 2002; Shah *et al.*, 2008). To analyse the relative abundance of *Sp0149*, *Sp0749* and *Sp1386*, primer pairs (Table 2.1) were designed to amplify cDNA products of comparable sizes, and the amplification efficiency of the primer pairs was analysed using *S. pneumoniae* genomic DNA (0100993) as the target for

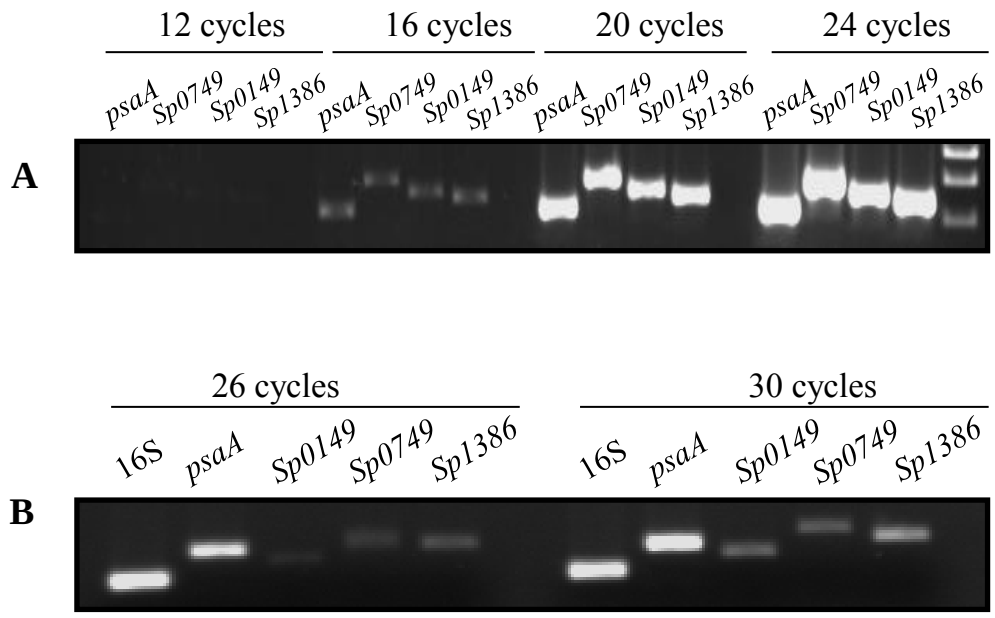


Fig 4.1 (A) Amplification efficiency of primer pairs using *S. pneumoniae* 0100993 genomic DNA as template for the PCR. (B) cDNA products generated by RT-PCR after 26 and 30 cycles using *S. pneumoniae* 0100993 total RNA extracted from the blood of the infected mice . 8 µl of the PCR and cDNA products were electrophoresed on the agarose gel.

PCR. After 12, 16, 20 and 24 cycles *Sp0149*, *Sp0749*, *Sp1386* and *psaA* PCR products were electrophoresed on prestained ethidium bromide agarose gels, loading the same volume of the PCR reaction for each gene. Using DNA as the target, similar intensities of *Sp0149*, *Sp0749*, *Sp1386* and *psaA* PCR products were obtained for each cycle length, indicating that the PCR primers have similar amplification efficiencies for each of these genes (Fig 4.1 A).

To extract total RNA from bacteria grown in human blood and THY, the 0100993 wild-type strain was first inoculated in THY and grown until the OD_{580} reached 0.2-0.3. The culture was divided into two tubes, the resulting bacterial pellets were washed in sterile PBS and each pellet was resuspended in 3 mls of THY or in freshly extracted heparinised human blood. An aliquot from this culture was removed, serially diluted and plated onto blood agar plates to determine the approximate *S. pneumoniae* cfu immediately after the inoculation. The culture was incubated for an hour at 37ºC in the presence of CO_2 followed by simultaneous extraction of the total RNA from *S. pneumoniae* grown in THY or in human blood. To extract total RNA from *S. pneumoniae* during infection, 3 to 5 CD1 mice were intraperitoneally injected with approximately 4 x 10^6 cfu/ mouse of 0100993 strain of *S. pneumoniae* and blood was obtained from the mice 12 hours later and pooled before total RNA was extracted using acid-phenol as described in chapter 2. Expression of the lipoprotein genes in the THY was used only as baseline for the comparison of gene expression in human blood and septicaemia model of infection. The extracted total RNA from these different conditions were subjected to RT-PCR with and without reverse transcriptase to ensure that DNA contamination was not present as described in chapter 2 and the RNA concentration was determined using a nanospectrophotometer. Equal quantities of total RNA (0.04 ng μl^{-1}) from each condition were then used for semi-quantitative RT-PCR

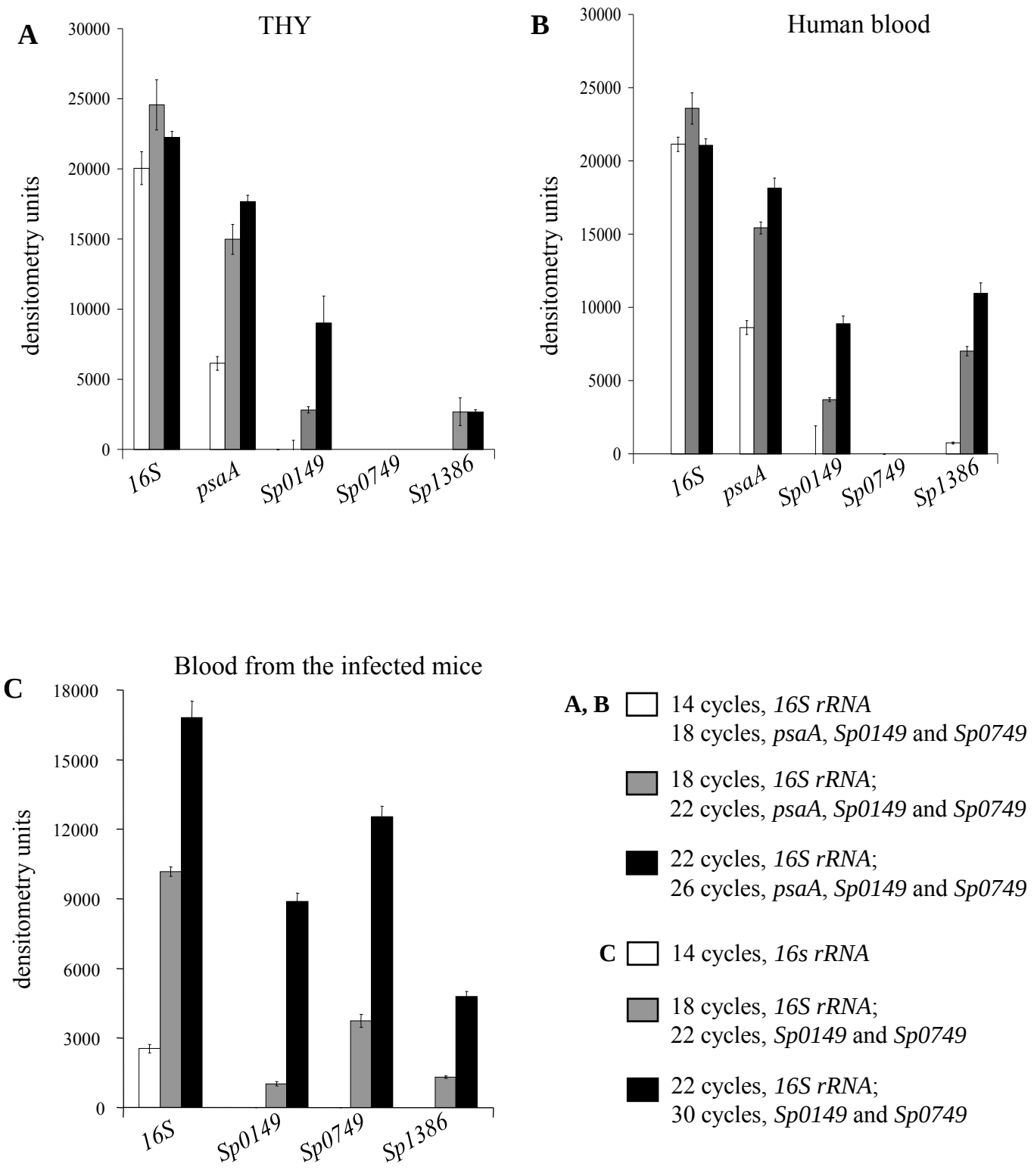


Fig 4.2 Densitometry analysis of *16S, psaA, Sp0149, Sp0749* and *Sp1386* cDNA product generated by RT-PCR after different cycles using *S. pneumoniae* 0100993 total RNA extracted from the (A) THY, (B) human blood and (C) infected mouse blood.

to assess mRNA expression levels of the *Sp0149, Sp0749, Sp1386* and *psaA* genes. To minimise sample variation a master mix was prepared for all RT-PCR reactions and these were performed simultaneously by mixing all the RT-PCR constituents except the primer pairs and the total RNA before aliquoting into individual tubes. cDNA products generated after 10, 14, 18, 22, 26 and 30 cycles for each primer pair were analysed in triplicate. Since the *16S rRNA* level is constantly maintained under *in vitro* and *in vivo* conditions, *16S rRNA* was used as internal control in all of these experiments. In each experiment 8 μl of the cDNA products generated from each triplicate sample was electrophoresed on agarose gel and the intensity of the bands corresponding to the lipoprotein genes were analysed by densitometry. The results are shown in Fig 4.2. The band intensities at 10, 14, 18, 22, 26 and 30 cycles demonstrated that there was differential expression of these genes when bacteria were grown in all three conditions. From the densitometry analysis (Fig 4.2 A, B, C), in THY 16S rRNA was the most abundantly present cDNA followed by those of *psaA*, *Sp0149* and *Sp1386* with no detectable levels of *Sp0749* cDNA observed (Fig 4.2 A). In human blood, the cDNA level of *Sp1386* was similar to that of *Sp0149* but again both were lower than the cDNA levels of *psaA* and *Sp0749* cDNA was not detected (Fig 4.2 B). However, in bacteria recovered from infected mice (Fig 4.2 C), the results were quite different, with greater expression of *Sp0749* compared to *Sp0149* and *Sp1386*. Expression of *psaA* was not investigated in this infection model. These data suggest that mRNA of *Sp0749* and *Sp1386* are differentially expressed under different conditions, with expression of *Sp0749* specifically induced during septicaemia despite low levels of expression after culture in THY or incubation in blood.

4.2 CONSTRUCTION OF *SP0149* AND *SP0750-53* DELETION MUTANT STRAINS IN *S. PNEUMONIAE*

In order to investigate the *in vitro* and *in vivo* phenotypes of the *Sp0148-53* and Sp0749-53 ABC transporters in detail, deletion mutant strains were constructed in which the *Sp0149* lipoprotein gene and the *Sp0750-53* genes (encoding permeases and ATPases) were replaced with the erythromycin resistance cassette (*erm*) by the overlap extension PCR (OEP) method (Fig 4.3 A-D and 4.4 A-D) as described in chapter 2. The role of these two operons during *in vivo* virulence and *in vitro* growth had initially been investigated using IDM mutants (chapter 3), and the deletion mutants were designed to affect the genes disrupted within the IDM mutant strains (Fig 4.5 A-C). The *ΔSp0149* and *ΔSp0750-53* deletion constructs were then transformed in to the 0100993 strain of *S. pneumoniae*. *ΔSp0149* and *ΔSp0750-53* deletion mutant strains were successfully obtained, and the deletion construct confirmed using the genomic DNA of the mutant strain as template to amplify the junctions of the upstream and downstream regions surrounding the deleted target gene (Fig 4.6 A-D). Fig 4.6 B shows the confirmation of *Sp0149* deletion by PCR using different primer pairs. To demonstrate that *erm* gene is flanked by *Sp0148* and *Sp0150* genes in the *ΔSp0149* mutant strain, PCR was performed using the Sp0148F + ermR and ermF + Sp0150R primers. This generated correct sized products only in the mutant strain. PCR across *Sp0148* to *Sp0150* genes using Sp0148F and Sp0150R primers generated similar sized bands in the wild-type and *ΔSp0149* mutant strain because the *erm* gene and *Sp0149* genes are of similar size. A non-specific band of smaller size was also obtained in the mutant strain. The absence of *Sp0149* in the *ΔSp0149* mutant strain was confirmed by PCR across the *Sp0149* gene (Sp0149F and R) in the wild-type and *ΔSp0149* mutant

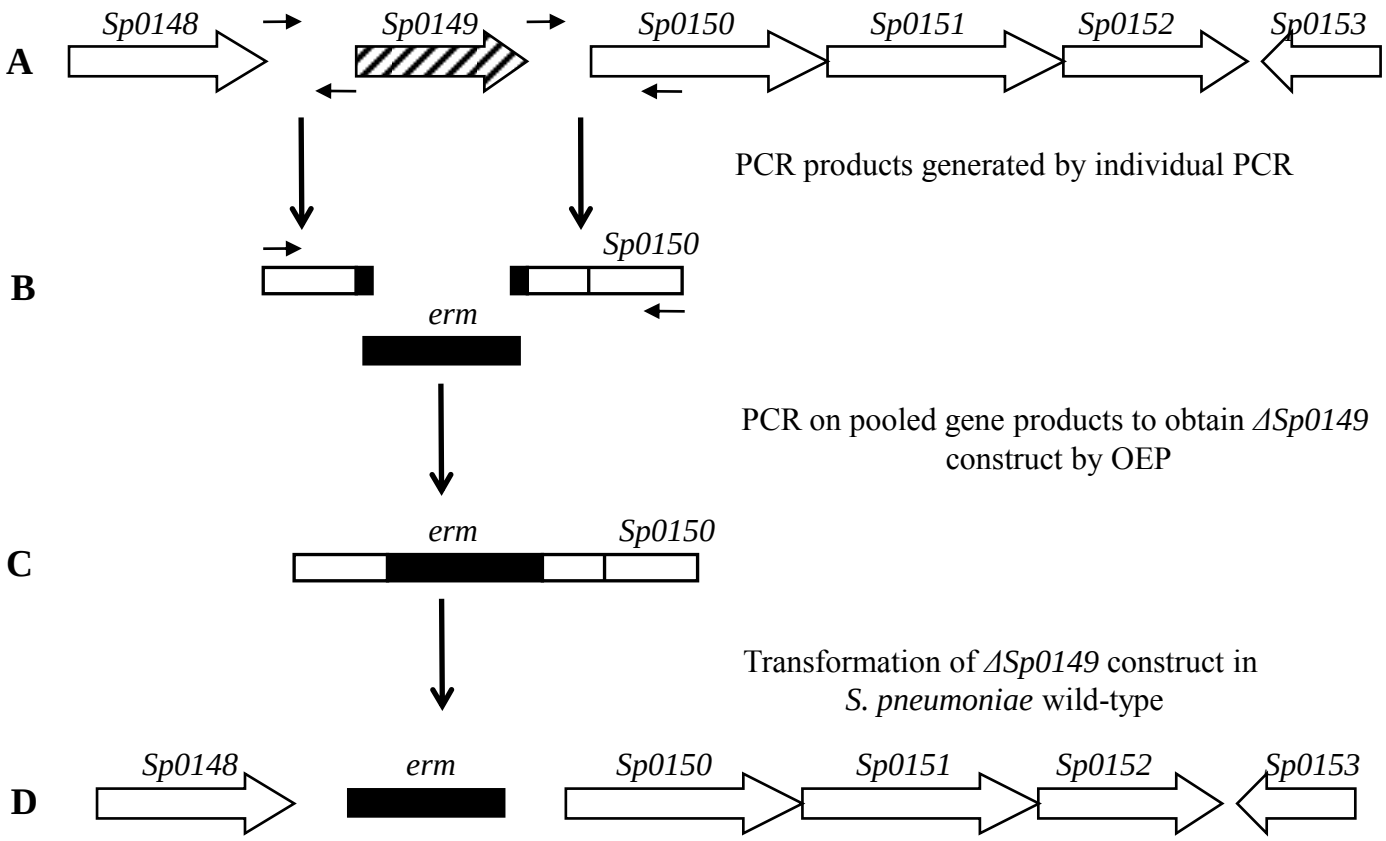


Fig 4.3 Schematic diagram of the deletion of *Sp149* by overlap extension PCR (OEP). (A) Arrows indicate the transcription direction of genes of *Sp0148-53* operon in the TIGR4 genome. The *Sp0149* gene, shaded with diagonal lines, was chosen for deletion. Small black arrows indicate the forward and reverse primers used for OEP. (B and C) OEP to obtain the *Sp0149* deletion construct by replacing *Sp0149* with the erythromycin gene (*erm*) (D) Representation of the product of transformation of the *ΔSp0149* construct in to the 0100993 *S. pneumoniae* strain.

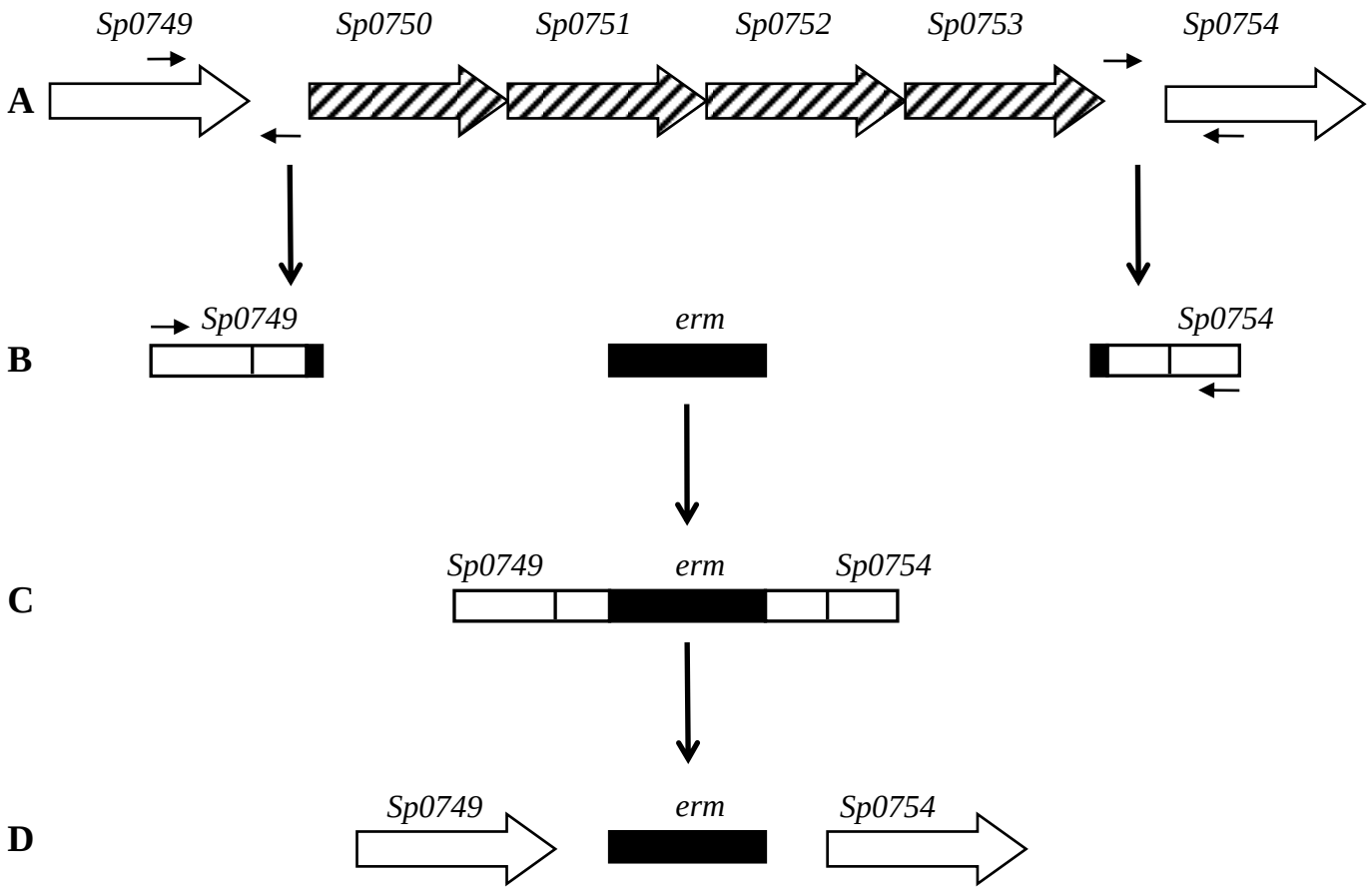


Fig 4.4 Schematic diagram of the deletion of *Sp750-53* by overlap extension PCR (OEP). (A) Arrows indicate the transcription direction of genes of *Sp0749-53* operon in the TIGR4 genome. The *Sp0750-53* genes are shaded with diagonal lines, were chosen for deletion. Small black arrows indicate the forward and reverse primers used for OEP. (B and C) OEP to obtain the *Sp0750-53* deletion construct by replacing *Sp0750-53* genes with the erythromycin gene (*erm*) (D) Representation of the product of transformation of the *ΔSp0750-53* construct in to the 0100993 *S. pneumoniae* strain.

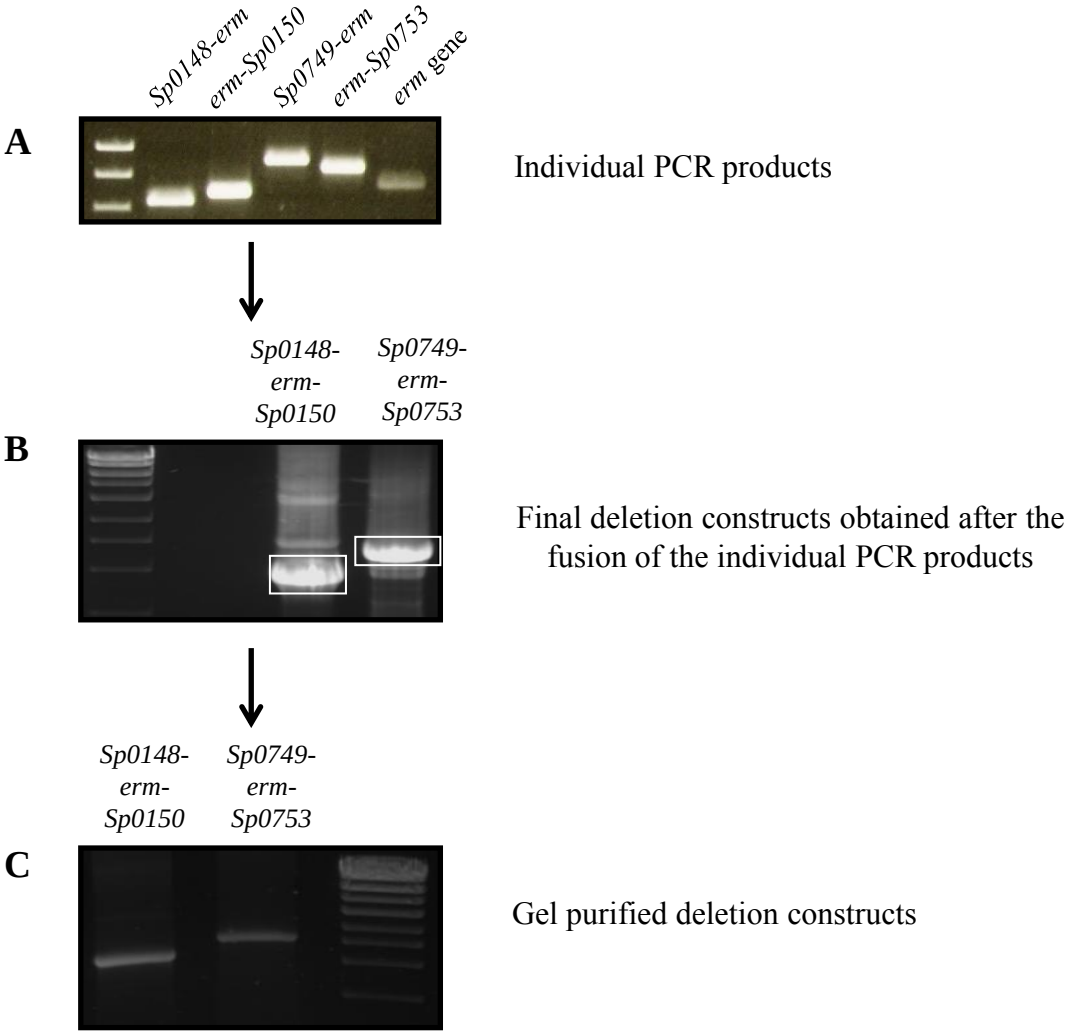


Fig 4.5 Generation of *ΔSp0149* and *ΔSp0750-53* deletion constructs. (A) Amplification of *Sp0148* (630 bp), *Sp0150* (672 bp), *Sp0749* (883 bp), *Sp0754* (833 bp) using *S. pneumoniae* 0100993 genomic DNA as the template and the *erm* gene (738 bp) using pACH74 plasmid DNA as the template (B) *ΔSp0149* (2040 bp) and *ΔSp0750-53* (2454 bp) deletion constructs generated by OEP after using the individual PCR products generated from A (C) Gel purified *ΔSp0149* and *ΔSp0750-53* deletion constructs used for *S. pneumoniae* transformation.

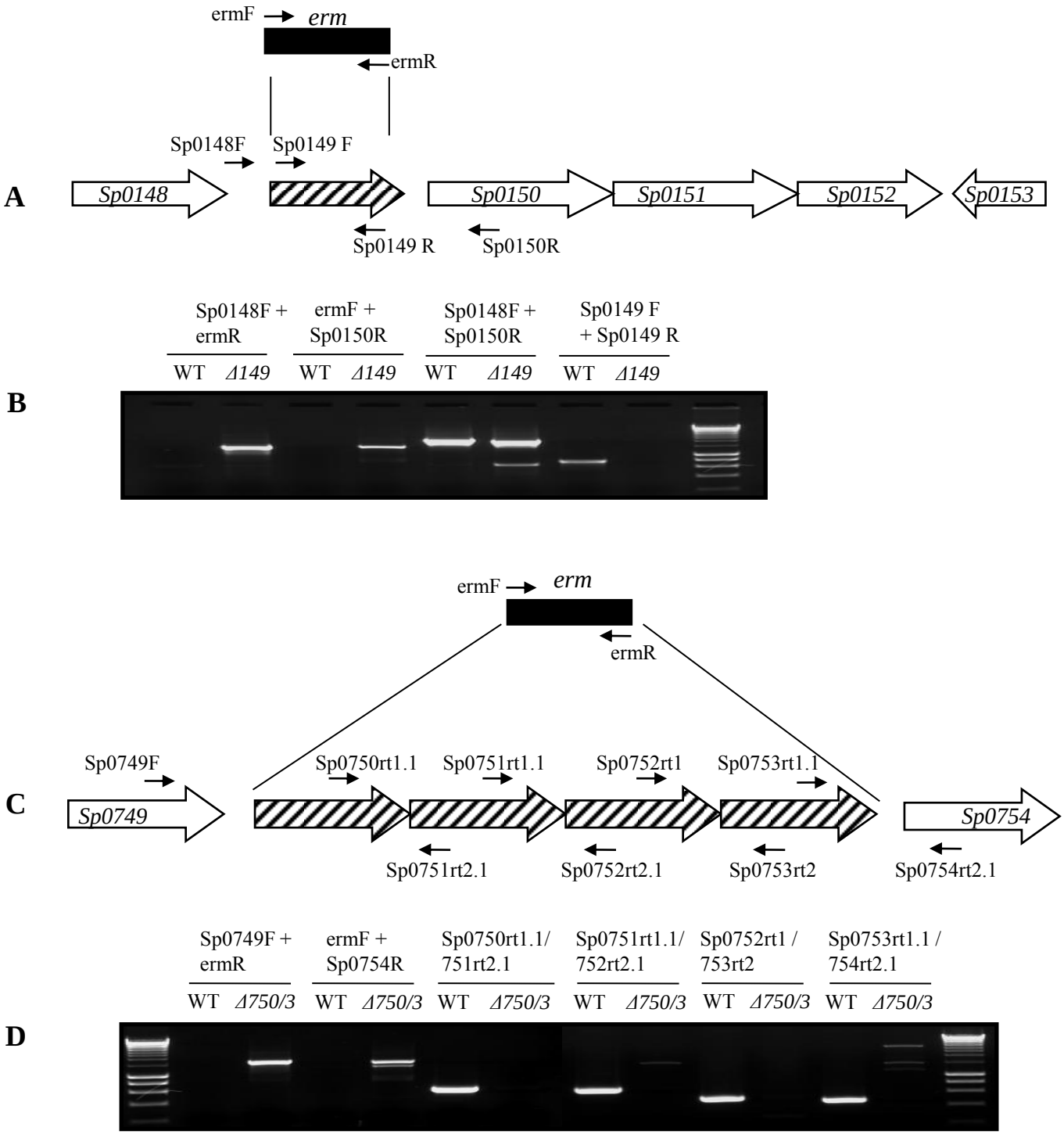


Fig 4.6 (A + C) Schematic diagram showing the deletion of *Sp0149* gene from the *Sp0148-53* operon and *Sp0750-53* genes from *Sp0749-53* operon. Small black arrows indicate the forward and the reverse primers used for the confirmation of deletion of target gene(s) Gel pictures (B and D) demonstrating the confirmation of the deletion of *Sp0149* and *Sp0750-53* genes by PCR using the 0100993 wild-type, *ΔSp0149*, *ΔSp0750-53* genomic DNA as templates.

strain, which generated a correct sized product only in the wild-type. Fig 4.6 D demonstrates the confirmation of the *Sp0750-53* deletion in the *ΔSp0750-53* mutant strain by PCR and later by sequencing. To demonstrate the replacement of *Sp0750-753* genes by *erm* in the *ΔSp0750-53* mutant strain, PCR using Sp0749F + ermR and ermF + Sp0754R primers was performed. This generated correct sized product in the mutant strain but no product was obtained in the wild-type. Similarly, to confirm the deletion of *Sp0750-53* genes, primers designed to amplify the junction of genes in the *Sp0749-754* operon were used (chapter 3). PCR using these primers spanning the junction of *Sp0750-54* did not generate a PCR product in the *ΔSp0750-53* mutant strain but generated correct sized bands in the wild-type suggesting that *Sp0750-53* genes were deleted in the mutant strain.

Efforts to obtain *Sp0149* and *Sp0750-53* complemented mutants were not successful as the *Sp0149* complementation construct did not transform into the *ΔSp0149* deletion mutant and several efforts to obtain the *Sp0750-53*^{c} construct failed.

4.3 LOCALISATION STUDIES OF SP0149 AND SP0749 LIPOPROTEINS

To investigate whether Sp0149 and Sp0749 lipoproteins are localised to the *S. pneumoniae* cell membrane, the Triton X-114 extracts of the membrane bound proteins were probed with the polyclonal mouse anti-Sp0149 and anti-Sp0749 antibodies (obtained as described in chapter 5) and analysed by Western blotting (Khandavilli *et al.*, 2008). To demonstrate whether Sp0149 and Sp0749 are, as predicted, lipoproteins, extracts from the *lsp* mutant strain as well as the wild-type and the complemented *lsp*^{c} strain were probed. *Δlsp* and *lsp*^{c} strains were constructed by Suneeta Khandavilli (Khandavilli *et al.*, 2008). Previous studies have demonstrated that lipoprotein signal peptidase (Lsp) is employed to process the prelipoproteins into mature lipoproteins in

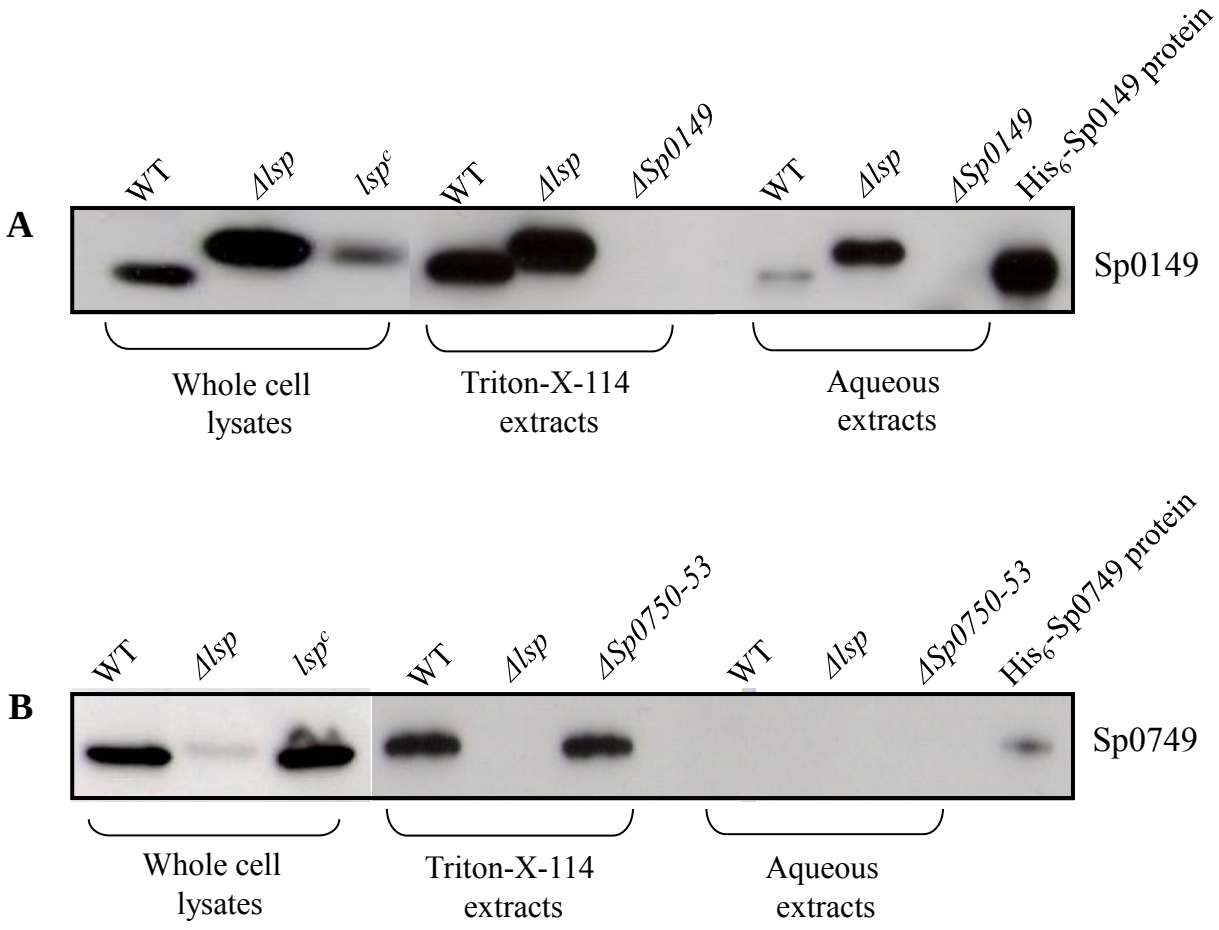


Fig 4.7 Western blots of the whole cell lysates, Triton X-114 and aqueous extracts of *S. pneumoniae* wild-type (WT), *ΔSp0149*, *ΔSp0750-53*, *Δlsp* and *lsp*^{c} strains probed with anti-Sp0149 and anti-Sp0749 antibodies to demonstrate the localisation of Sp0149 (A) and Sp0749 (B) lipoproteins. Purified His_6-Sp0149 and His_6-Sp0749 were used as controls. The sizes of Sp0149 and Sp0749 are 1-2 kDa larger in the *Δlsp* strain than in the wild-type, *lsp*^{c} strains and the purified lipoproteins.

various bacteria (Sutcliffe and Russell, 1995) (described in chapter 1). Recently, Khandavilli and collegues have demonstrated that Lsp (encoded by the Sp0928 gene) is responsible for the processing of prelipoproteins by enzymatic cleavage of the N-terminal signal sequence from the prelipoproteins to form mature lipoproteins in *S. pneumoniae*. They have also demonstrated that the deletion of Sp0928 prevents the formation of the mature lipoproteins and therefore the lipoproteins in the Δ928 mutant strain (*Δlsp*) have slightly higher molecular masses. Complementation of Sp0928 in 928^c (lsp^c) strain restores normal processing of lipoproteins. Fig 4.7A shows that the molecular weight signals from the whole cell lysates of wild-type, *Δlsp*, and lsp^c strains probed with anti-Sp0149 or anti-Sp0749. The *Δlsp* gave signal at higher molecular weight when probed with anti-Sp0149, but it was difficult to determine the size difference of Sp0749 lipoprotein in *Δlsp* strain when probed with anti-Sp0749 as this antibody only gave a weak signal with this strain. Surprisingly, the band identified by probing with anti-Sp0149 in the lsp^c strains was not restored to the size found in the wild-type strain. However similar sized molecular weight signals were obtained when the whole cell lysates of lsp^c and wild-type strains were probed with anti-Sp0749. A strong signal was obtained when the Triton X-114 extracts of the wild-type and *Δlsp* strains were probed with anti-Sp0149 antibodies, where as *ΔSp0149* strain did not give any signal due to the deletion of Sp0149 and the band obtained with the *Δlsp* strain had a slightly higher molecular weight as the result of incomplete processing of the lipoprotein.

The presence of same sized signal in the aqueous extract (Fig 4.7 A) of wild-type, *Δlsp* and *ΔSp0149* probably occurred as a result of the contamination of Triton X-114 extract in to aqueous extract during the extraction procedure. Alternatively it is also possible that the loss of lsp may result in the release of significant quantities of

Sp0149 lipoprotein from the cell surface into the culture medium. When probed with anti-Sp0749 antibodies a strong signal was obtained for the whole cell lysates of the wild-type and *lsp*^{c} strains but only a weak signal of slightly higher molecular mass for the whole cell lysate of the *Δlsp* strains. Furthermore in the Triton X-114 extracts, the wild-type and *ΔSp0750-53* strain, but not the *Δlsp* strain gave a strong signal for the Sp0749 lipoprotein suggesting that Sp0749 lipoprotein is membrane bound but expression on the membrane is lost in the absence of Lsp. However, there was no Sp0749 signal in the aqueous extracts indicating that loss of expression of the Sp0749 in the *Δlsp* strain was not accompanied by shedding of the protein from the cell surface (Fig 4.7 B).

Overall the Western blots indicated that Sp0149 and Sp0749 are both membrane bound, and that the Sp0149 exists as a prelipoprotein that is processed by Lsp. The reduction of expression of Sp0749 in the absence of Lsp was unusual and differs from the effect of the *Δlsp* mutation on other lipoproteins. However the localisation of Sp0749 within the membrane and the fact that this is affected by Lsp does indicate it is also a lipoprotein.

4.4 *IN VITRO* PHENOTYPE ANALYSIS OF THE *S. PNEUMONIAE ΔSP0149 AND ΔSP0750-53* DELETION MUTANTS

4.4.1 Growth curve of *ΔSp0149* and *ΔSp0750-53* mutants in THY

The *in vitro* phenotypes of the *ΔSp0149* and *ΔSp0750-53* mutants strains were investigated by comparing the growth rates of the deletion mutant strains to the wild-type strains in the THY medium. Equal numbers of the wild-type and the mutant bacteria obtained from thawed stocks were inoculated into THY, cultured at 37°C in the presence of 5% CO_2 and the optical density recorded at one hourly intervals for 8

hours. Both the deletion mutant strains exhibited good growth in THY, similar to the wild-type strain (Fig 4.8 A), suggesting that the deletion of the *Sp0149* and *Sp0750-53* genes of the ABC transporters had little effect on the growth of *S. pneumoniae* in a rich laboratory medium that is THY.

4.4.2 Growth curve of *ΔSp0149* and *ΔSp0750-53* mutants in cation depleted media

From the BLAST search, Sp0149-52 encodes a putative metal ion ABC transporter, hence the role of Sp0149-52 and Sp0749-53 operon in a cation depleted environment was investigated by analysing growth of the mutant strain in THY that has been treated with Chelex-100 to remove cations from the medium (Brown *et al.*, 2001). Fig 4.8.B showed no differences in the growth pattern of *ΔSp0149, ΔSp0750-53* mutant strains and the wild-type *S. pneumoniae* in THY-chelex supplemented with 100 μM $CaCl_2$ and 2 mM $MgSO_4$. Therefore cations such as zinc, ferric iron and cobalt were supplemented in THY-Chelex at a final concentration of 100 μM and the growth defect of *ΔSp0149* in the presence and absence of these cations were compared with the wild-type. Fig 4.9 A demonstrates that the wild-type and *ΔSp0149* strain had impaired growth in Chelex-THY medium alone. Both the wild-type and *ΔSp0149* showed better growth when supplemented with 100 μM ferric citrate (Fe^{3+}) as the source of iron suggesting that the depletion of iron from the medium may be responsible for this growth defect, but there was no difference between the strains indicating iron was not a major substrate for Sp0149 (Brown *et al.*, 2001). However, when the Chelex-THY medium was supplemented with 100 μM cobalt or 100 μM zinc chloride, the growth of wild-type and *ΔSp0149* strains was inhibited, suggesting these concentration of cobalt or zinc were relatively toxic. The growth of the *ΔSp0149* strain was actually better than the wild-type strain in zinc supplemented THY-chelex, and this may indicate that this

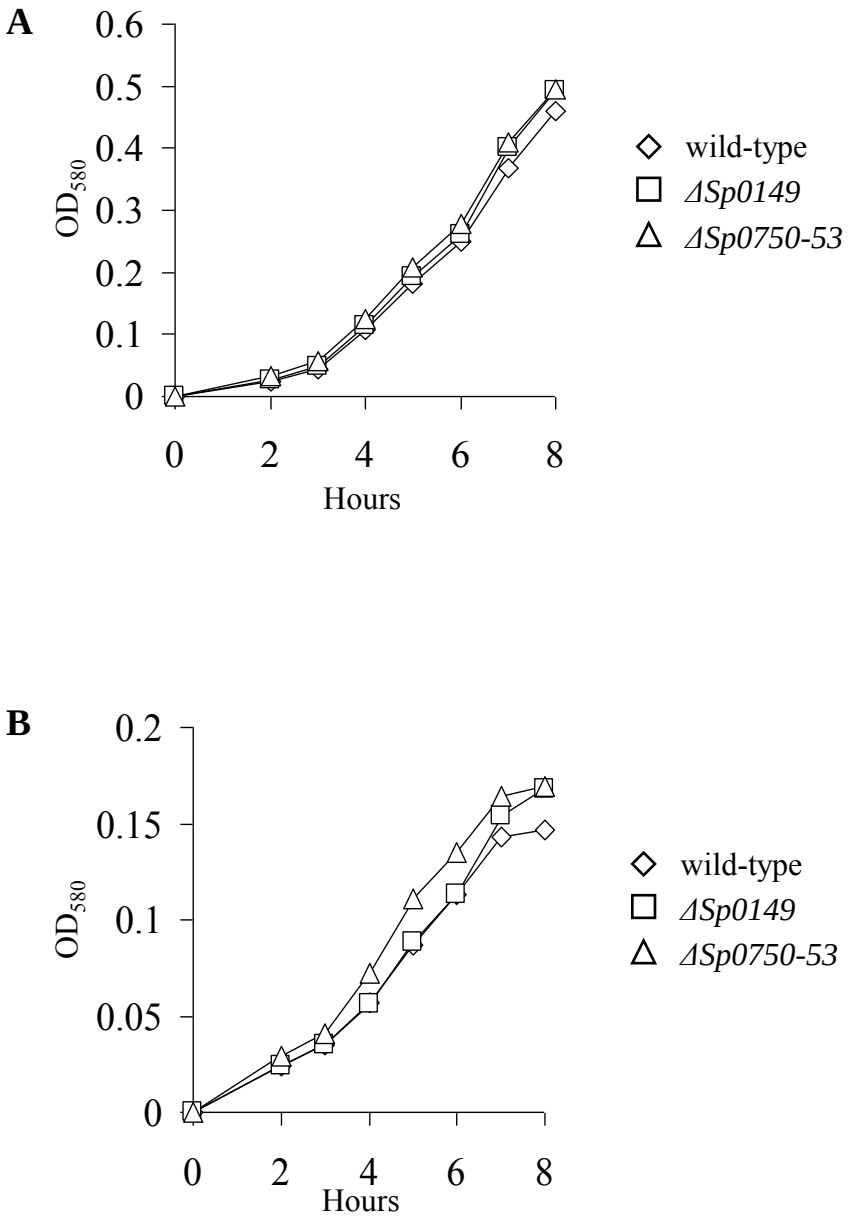


Fig 4.8 (A) Growth of the wild-type, *ΔSp0149* and *ΔSp0750-53* deletion mutant strains of *S. pneumoniae* (0100993) measured in THY medium (B) Growth of the wild-type, *ΔSp0149* and *ΔSp0750-53* deletion mutant strains of *S. pneumoniae* (0100993) in Chelex-THY medium Results presented are the mean of triplicate samples for each strain

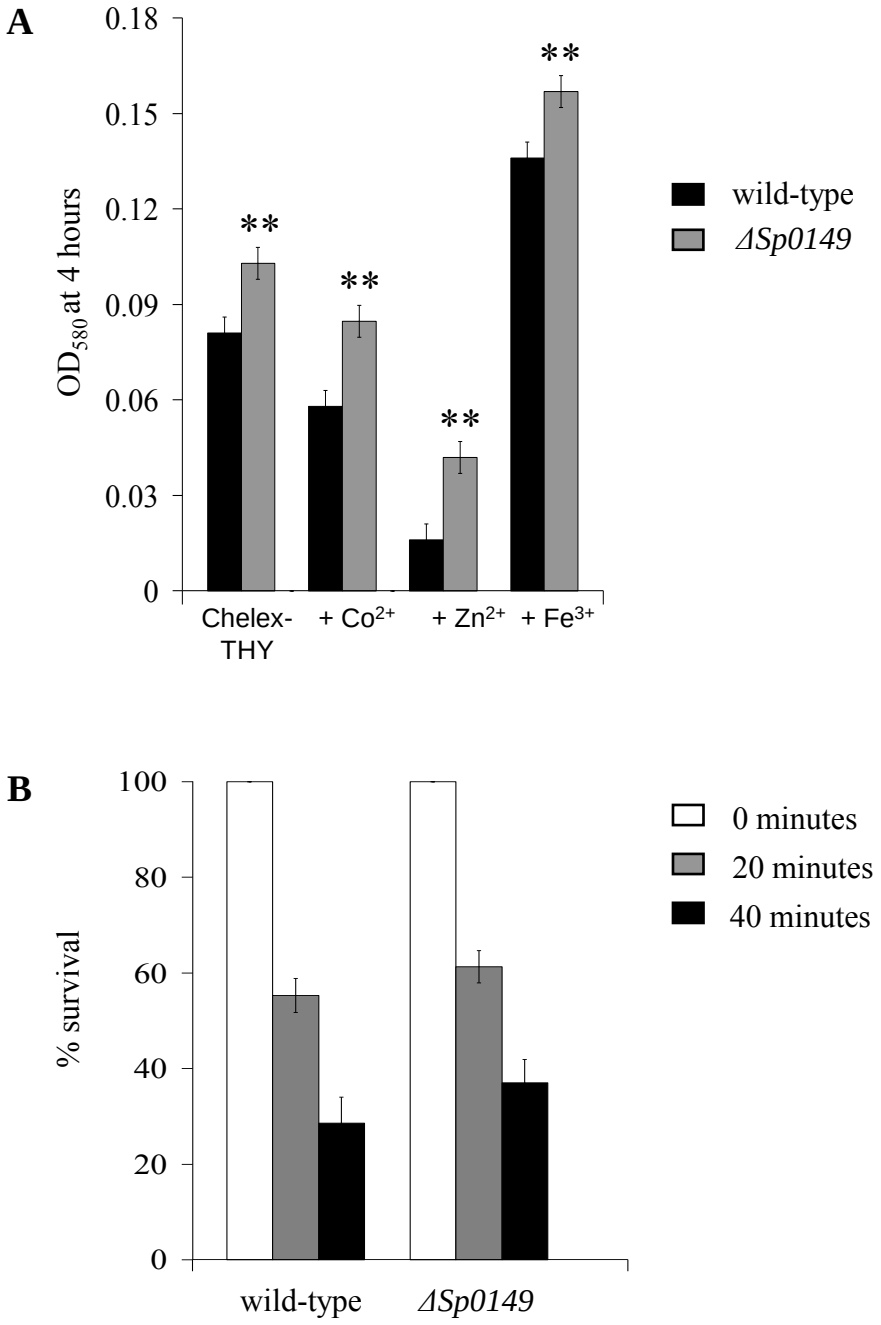
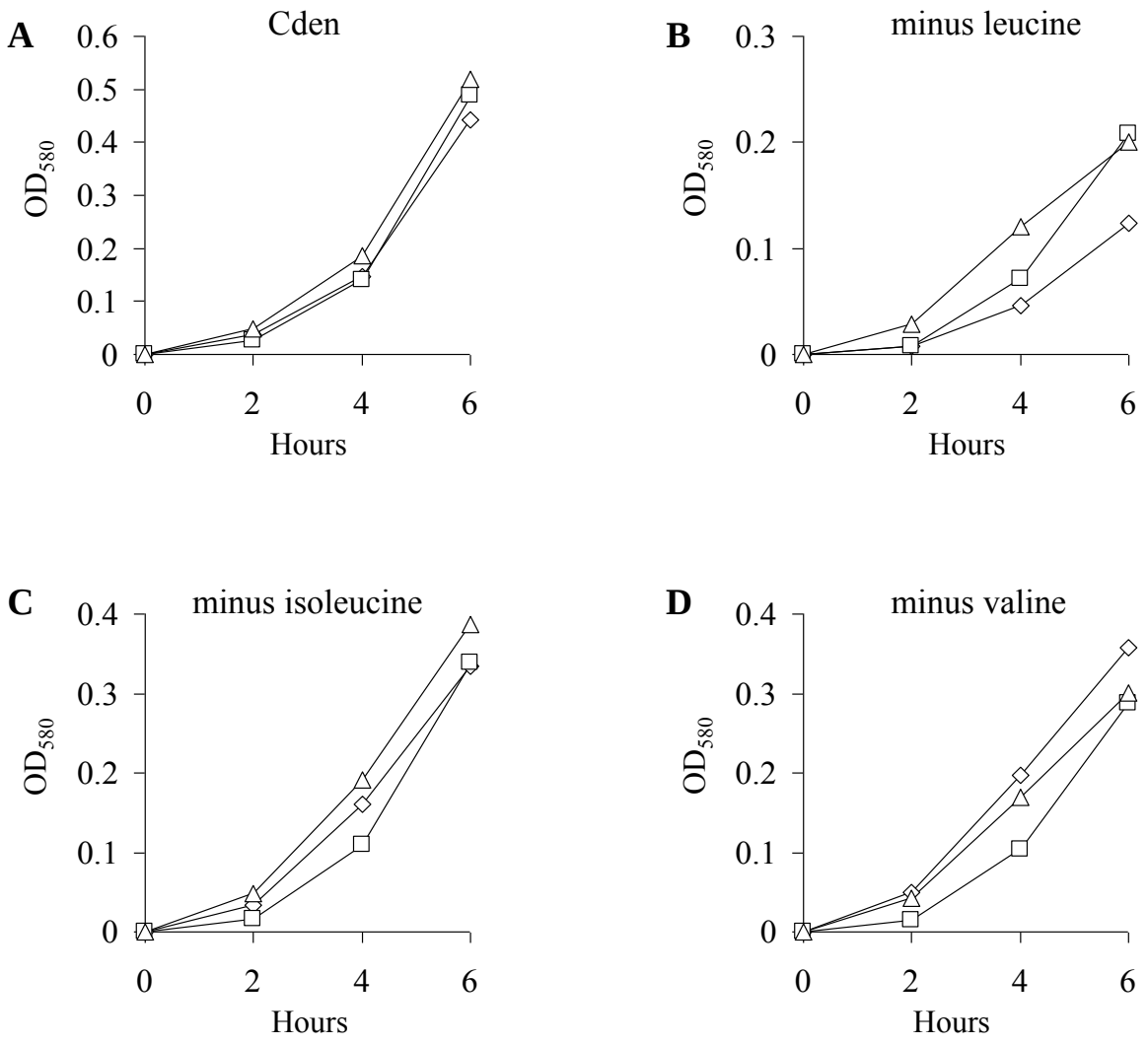


Fig 4.9 (A) Growth of wild-type and *ΔSp0149 S. pneumoniae* strains at 4 hours in Chelex-THY medium supplemented with and without individual cations (100 μM) such as cobalt, zinc and ferric iron. *p* values were obtained using Student's-*t*-test by comparing the wild-type with *ΔSp0149*. (B) Sensitivity to streptonigrin of the wild-type and *ΔSp0149 S. pneumoniae* strains. Results are expressed as mean percent survival (triplicate samples) of the wild-type and *ΔSp0149* mutant strains surviving the exposure to 5 μg ml^{-1} streptonigrin for 20 and 40 minutes.

strain has had less toxic effects due to loss of cation transport. However this data requires further development before the significance of any differences in growth between strains can be correctly interpreted.

4.4.3 Streptonigrin sensitivity test

Streptonigrin is an antibiotic which is bactericidal in nature and requires intracellular iron for its activity. Mutations in iron uptake systems are associated with streptonigrin resistance due to the reduced levels of intracellular iron preventing streptonigrin activity. In *S. pneumoniae*, mutations in iron uptake ABC transporters such as Piu, Pia and Pit have demonstrated increased resistance to streptonigrin (Brown *et al.*, 2001). The streptonigrin sensitivity test was performed (as described in chapter 2) to identify whether streptonigrin has any effect on the *ΔSp0149* mutant strain of *S. pneumoniae*. Both the wild-type and the *ΔSp0149* mutant strain were incubated with 5 μg ml^{-1} streptonigrin and serial dilutions were plated on Columbia blood agar after 0, 20 and 40 minutes of streptonigrin exposure. Bacterial cfu were represented as percentage survival after each time point and the percentage survival of the wild-type and the *ΔSp0149* mutant strains were compared. Fig 4.9 B shows no difference between the wild-type and the *ΔSp0149* mutant strain after 20 and 40 minutes of exposure to streptonigrin. The above result suggests that the intracellular concentration of iron was adequate for the streptonigrin activity in both the wild-type and *ΔSp0149* mutant strain of *S. pneumoniae*, and that the deletion of Sp0149 gene has little or no effect on the streptonigrin sensitivity. Hence Sp0149 is either not involved in iron uptake or is redundant and can be compensated for by other iron transporters.



Keys: ◇ wild-type; □ *ΔSp0149*; △ *ΔSp0750-53*

Fig 4.10 Growth of *S. pneumoniae* wild-type, *ΔSp0149* and *ΔSp750-53* strains in Cden medium in the presence and absence of branched chain amino acids, leucine, isoleucine and valine. *ΔSp0149* mutant strain was used as negative control in these experiments. Growth of the wild-type and mutant strains in (A) complete Cden medium, (B) Cden medium without leucine, (C) Cden medium without isoleucine (D) Cden medium without valine. Results presented are the mean of triplicate samples.

4.4.4 Growth curve of *ΔSp0149* and *ΔSp0750-53* mutants in chemically defined medium (Cden)

From the BLAST search prediction, Sp0749-53 operon encodes a putative branched chain amino acid transporter. In order to investigate whether the deletion of Sp0750-53 genes has any effect on the growth of the *ΔSp0750-53* mutant strain, a chemically defined medium, Cden (Tomaz., 1964) was used to compare the growth patterns of the wild-type and *ΔSp0750-53* strain in the absence of branched chain amino acids such as leucine, isoleucine and valine. *ΔSp0149* was used as a negative control in these experiments. The *ΔSp0149*, *ΔSp0750-53* mutant and the wild-type strains did not exhibit growth pattern differences in the Cden medium alone (Fig 4.10 A). Growth patterns of *ΔSp0149*, *ΔSp0750-53* mutant and the wild-type strains in Cden medium were impaired by the absence of any of the branched chain amino acids with a maximum optical density attained at 6 hours of approximately 0.2 to 0.4 compared to 0.6 for complete Cden (Fig 4.10 B, C, D). However, there were no significant differences in the growth of the mutant or wild-type strains in the absence of branch chain amino acids.

4.4.5 Azaleucine toxicity test

Azaleucine is a toxic analogue of leucine that is taken up by branched chain amino acid transporters. Hence the relative level of toxicity of azaleucine indicates whether a bacterial strain has impaired transport of branched chain amino acids. The azaleucine sensitivity test was performed using the wild-type and the *Sp0750*$^{-}$ IDM mutant strain of *S. pneumoniae* (0100993 strain). Azaleucine at concentrations of 10, 20, 30, 40, 50, 60, 70, 80, 90 and 100 μg ml^{-1} in THY were used to grow the bacteria and the growth

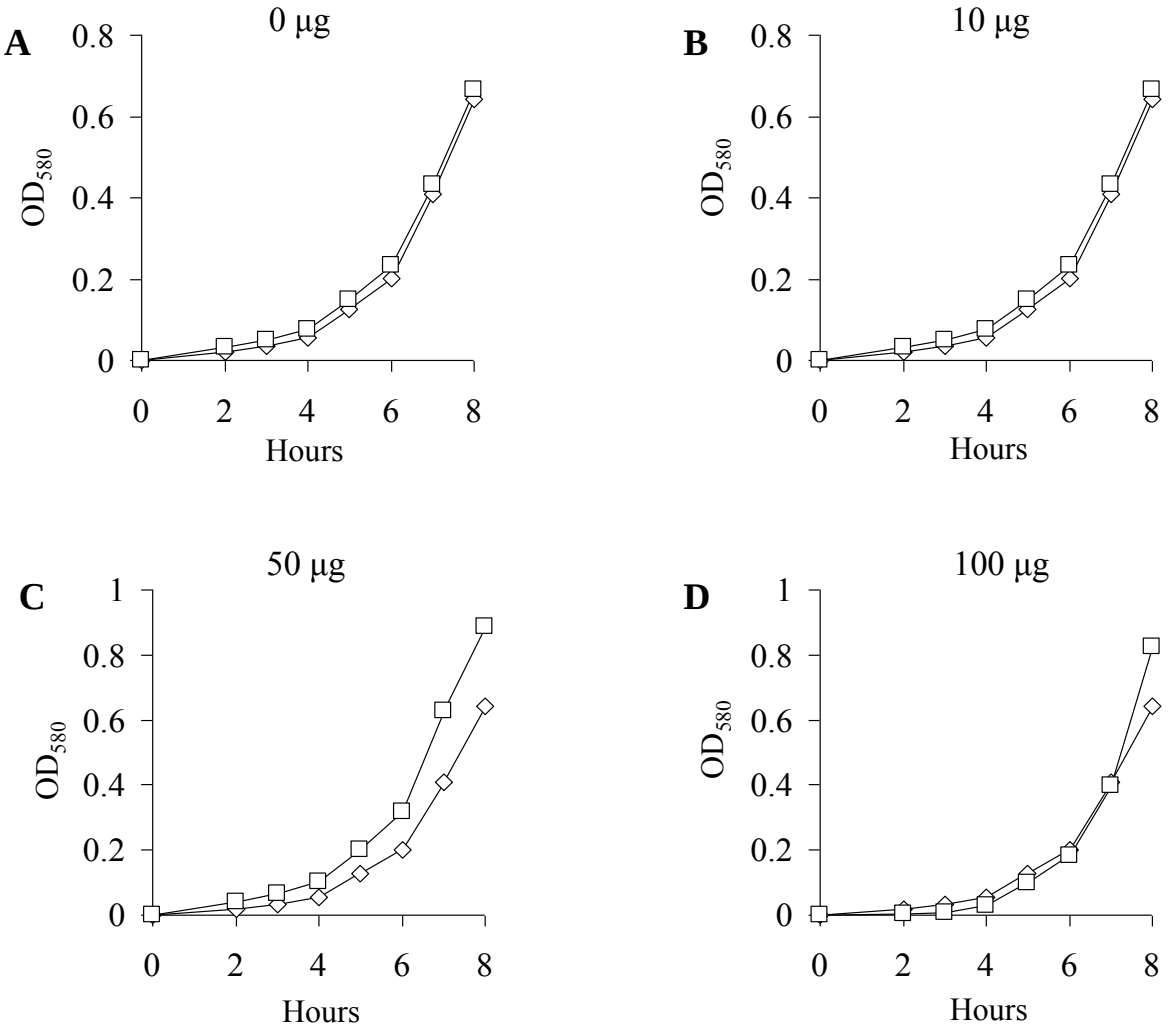


Fig 4.11 Growth of *S. pneumoniae* (0100993) wild-type and *Sp0750*$^{-}$ IDM mutant strains in THY medium at different concentrations of azaleucine. Growth of the wild-type and the *Sp0750*$^{-}$ strain in (A) THY medium, (B) THY + 10 µg azaleucine, (C) THY + 50 µg azaleucine and (D) THY + 100 µg azaleucine. Results presented are the mean of triplicate samples.

patterns of wild-type and *Sp0750*$^{-}$ mutant strains were recorded (shown in Fig 4.11 A-D; similar growth patterns were observed in the other concentrations tested). There was no evidence of toxicity due to azaleucine in the wild-type and *Sp0750*$^{-}$ IDM mutant strains of *S. pneumoniae* at all the tested concentrations. One of the reason could perhaps be that the azaleucine toxicity assay was performed in THY which is a rich undefined medium with leucine as one of the amino acid component. Therefore even in the presence of azaleucine, leucine may be the substrate preferred by Sp0749-0753 ABC transporter. Therefore repeating the experiment with modifications, such as using chemically defined medium in which leucine could be replaced with azaleucine to identify the differences between wild-type and *Sp0750*$^{-}$ mutant strains may provide information regarding the azaleucine toxicity.

4.4.6 Uptake assays

To try and confirm that Sp0749-0753 does encode a branched chain amino acid ABC transporter, uptake assays of ^{14}C-labelled leucine were performed with the wild-type and *ΔSp0750-53* mutant strains (experiments performed under Dr Hosie's supervision at King's College London) (Webb et al., 2008). For the ease of performing the uptake assays, deletion mutation from the serotype 3 (0100993) was transferred into serotype 2 (D39) because the mucoid serotype 3 strain would prevent efficient pelleting. Preliminary ^{14}C-labelled leucine uptake experiments were performed as described in chapter 2 by inoculating the wild-type and *ΔSp0750-53* mutant strains of *S. pneumoniae* in CDM medium alone containing branched chain amino acids and CDM medium without branched chain amino acids (Fig 4.12 A, B, C, D). No significant detectable leucine uptake was observed in either the wild-type or *ΔSp0750-53 S. pneumoniae* strains in the presence (Fig 4.12 A) and absence (Fig 4.12 B) of

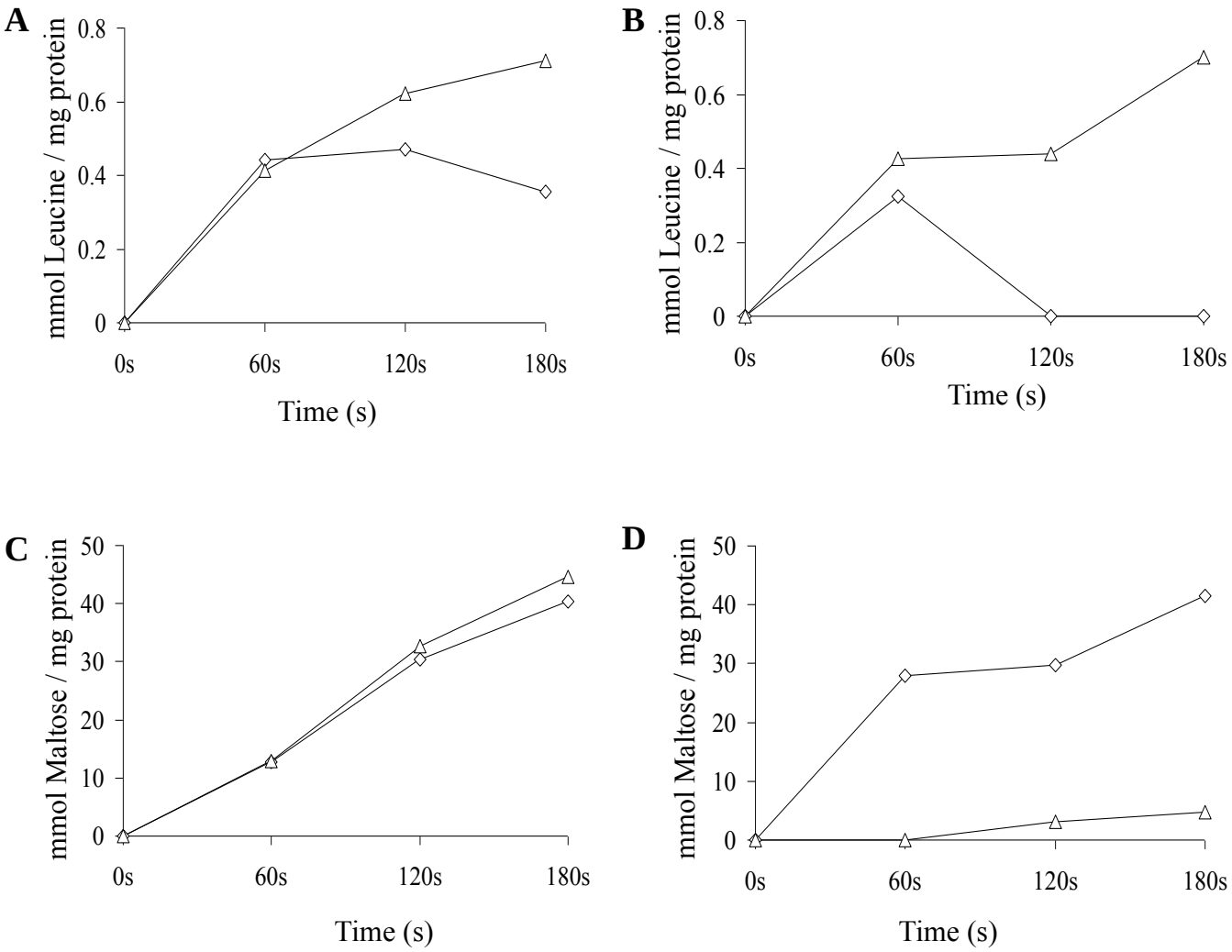


Fig 4.12 Radioactive leucine and maltose uptake using serotype 2 (D39) *S. pneumoniae* wild-type (diamonds) and *ΔSp0750-53* (triangle) strains in CDM medium containing isoleucine, leucine and valine (I, L, V) (A and C) and CDM without isoleucine, leucine and valine (B and D). (A) ^{14}C-leucine uptake by wild-type and *ΔSp0750-53 S. pneumoniae* strains in CDM medium (B) ^{14}C-leucine uptake by wild-type and *ΔSp0750-53 S. pneumoniae* strains in CDM medium without I, L, V (C) ^{14}C-maltose uptake by wild-type and *ΔSp0750-53 S. pneumoniae* strains in CDM medium (D) ^{14}C-maltose uptake by wild-type and *ΔSp0750-53 S. pneumoniae* strains in CDM medium without I, L, V.

branched chain amino acids. To ensure that the experimental conditions for the uptake were correct, ^{14}C-labelled maltose uptake assays were also performed as a positive control. There was good ^{14}C-labelled maltose uptake in the *S. pneumoniae* wild-type both in the presence (Fig 4.12 C) and absence (Fig 4.12 D) of branched chain amino acids and by the *ΔSp0750-53* mutant strain in the CDM medium. However, in the CDM medium without branched chain amino acids, there was markedly reduced maltose uptake by the *ΔSp0750-53* mutant strain, the reasons are unclear. ^{14}C-labelled isoleucine, leucine and maltose uptake was also performed, and again showed negligible uptake both amino acids. Hence, the above results indicate that there is no significant detectable uptake of BCAA by *S. pneumoniae* under the *in vitro* conditions tested, preventing effective assessment of whether *Sp0749-0753* does encode a BCAA ABC transporter using these assays.

4.4.7 Analysis of substrate specificity by tryptophan fluorescence spectroscopy

In order to investigate whether the Sp0749 lipoprotein binds to branched chain amino acids (BCAAs), tryptophan fluorescence spectroscopy was performed using purified and dialysed recombinant His_6-Sp0749 lipoprotein (Thomas *et al.*, 2006). Tryptophan fluorescence spectroscopy analyses the fluorescence emitted as a result of excitation of electrons by the tryptophan residues present in the protein. In the absence of tryptophan residues, as is the case for the Sp0749 lipoprotein, this method uses tyrosine residues to analyse the fluorescence in the proteins. Fig 4.13 A demonstrates that the maximum emission of fluorescence for the purified His_6-Sp0749 lipoprotein occurs at the wavelength of 309 nm. To test which amino acids induce fluorescence changes in His_6-Sp0749 lipoprotein, branched chain amino acids such as isoleucine, leucine, valine and other amino acids such as proline, glycine, alanine and threonine were incubated with

the recombinant Sp0749 protein. Fig 4.13 B demonstrates that the addition of amino acids such as proline, glycine, alanine and threonine and valine at a concentration of 50 μM to His_6-Sp0749 lipoprotein soon after its stabilization in the spectrofluorimeter had minor effects on fluorescence. However, the addition of the branched chain amino acid isoleucine at the concentration of 3.2 μM resulted in a marked change in Sp0749 fluorescence. Further addition of 3.2 μM isoleucine did not result in any quenching of the changes in fluorescence (data not shown). Therefore to identify the minimum concentration of the ligand required to obtain maximum fluorescence change, isoleucine concentrations of 0.64 μM, 0.32 μM (Fig 4.14 A) and 0.16 μM (Fig 4.14 B) were tested. Addition of 0.16 μM isoleucine did not induce significant changes in fluorescence of His_6-Sp0749, whereas significant changes did occur with the addition of 0.32 or 0.64 μM isoleucine. No further quenching following the addition of a further 0.64 μM of isoleucine (data not shown). These data suggest that Sp0749 binds to isoleucine with a high affinity.

To investigate whether His_6-Sp0749 lipoprotein binds to leucine, another potential branched chain amino acid ligand, fluorescence changes upon addition of leucine at different concentrations were measured. After the addition of leucine at the final concentration of 3.2 μM, change in fluorescence occurred after a small delay as shown in Fig 4.15 A, and addition of leucine at the final concentration of 16 μM did not result in further quenching of fluorescence. When a lower concentration of leucine (0.64 μM) was tested, only a slow change in fluorescence was observed and further addition of leucine at 3.2 μM did not result in any fluorescence change. These data suggest that the His_6-Sp0749 lipoprotein binds to leucine with low affinity.

Fluorescence changes upon addition of valine, another branched chain amino acid ligand was also tested. A marked fluorescence change was observed when valine

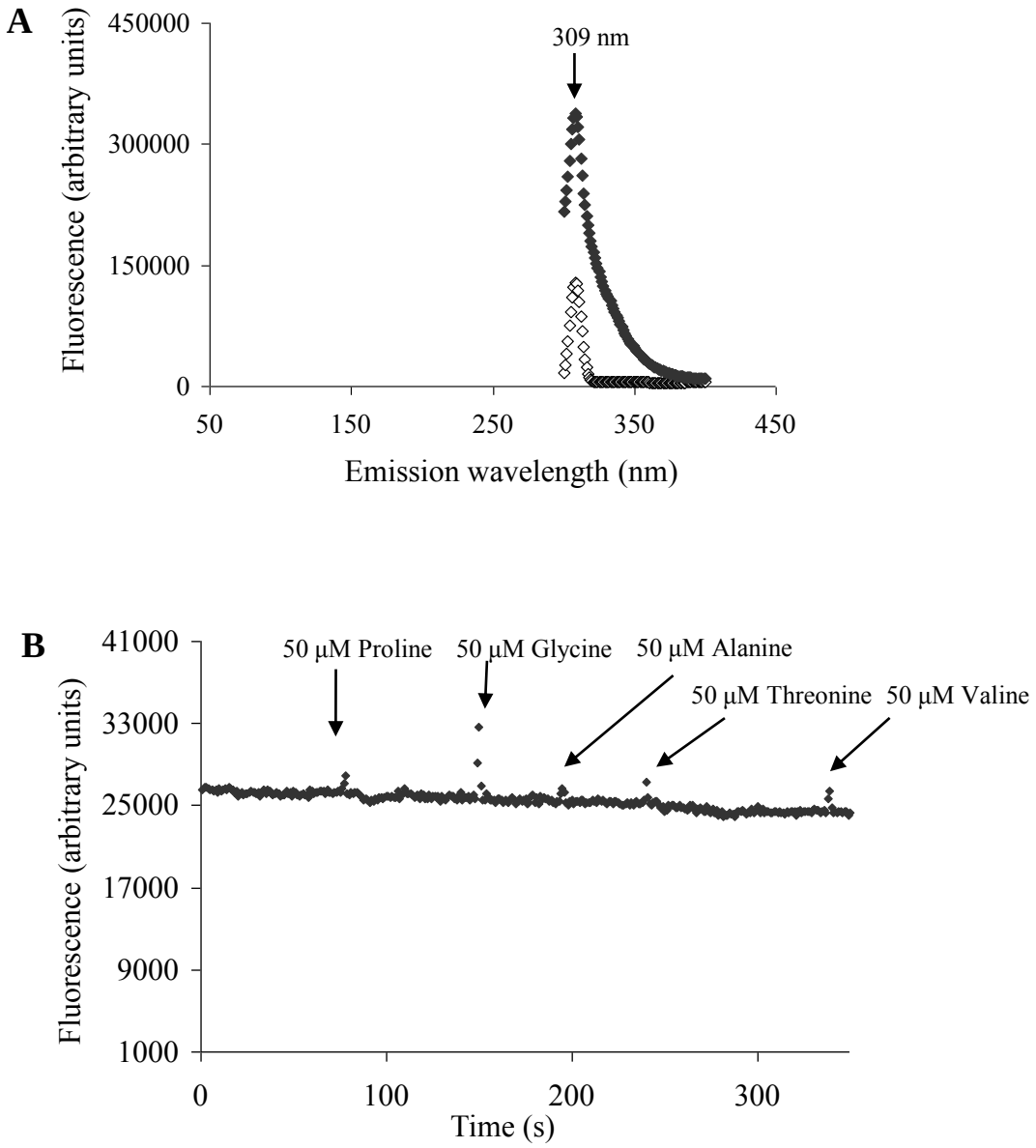


Fig 4.13 Flourescence spectroscopy analysis of the purified His_6-Sp0749 lipoprotein (A) Emission scan of His_6-Sp0749 lipoprotein (0.5 µM) demonstrating the maximum flourescence emission at 309 nm (indicated by an arrow) when excited at the 280 nm wavelength. (B) Measurement of flourescence change upon addition of 50 µM proline, glycine, alanine, threonine and valine to 2 µM His_6-Sp0749 lipoprotein (point of addition of the amino acid ligands are indicated by arrows).

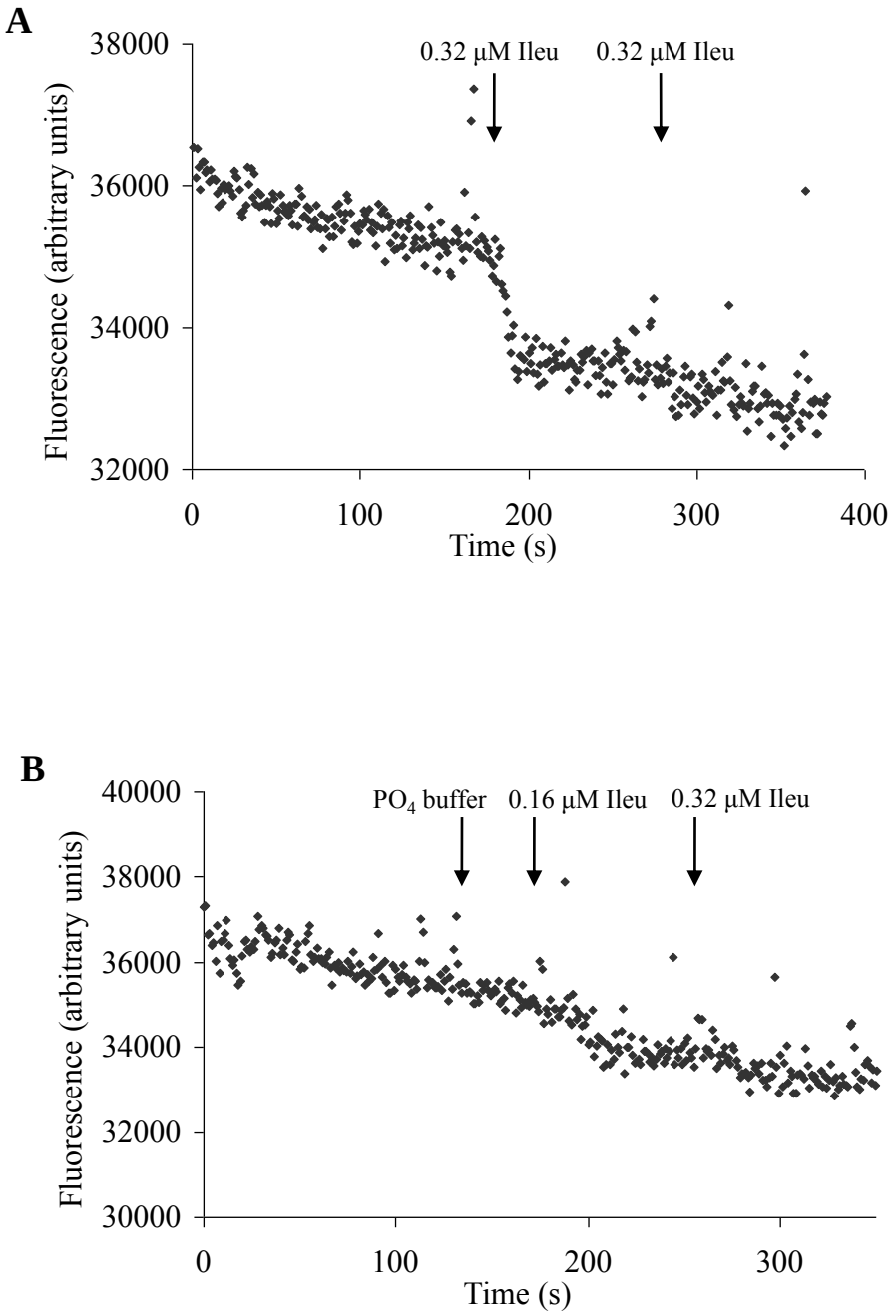


Fig 4.14 Flourescence change recorded upon addition of (A) 0.32 µM isoleucine, (B) 0.16 and 0.32 µM isoleucine to 2 µM purified His_6-Sp0749 (point addition of ligands are indicated by arrows).

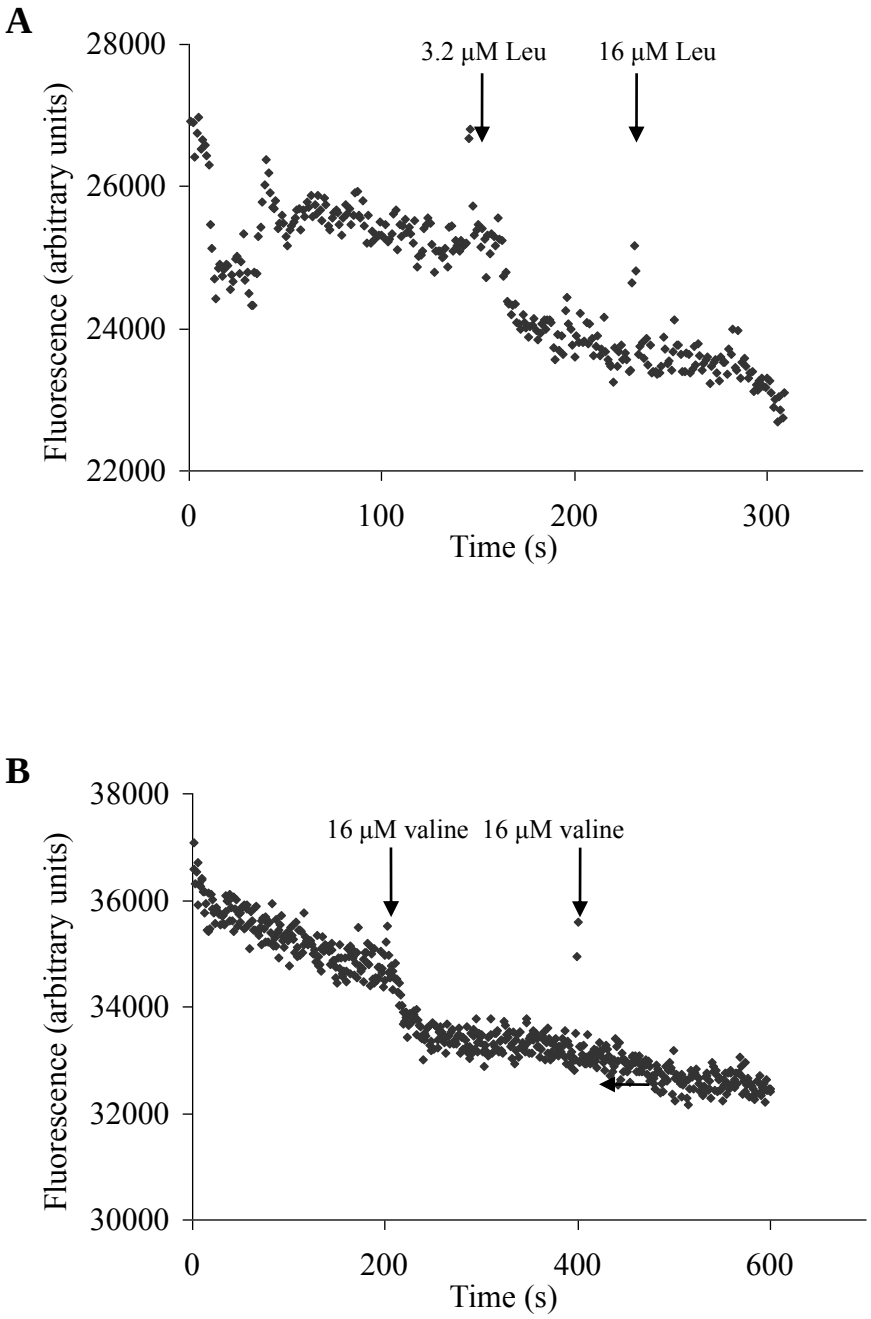


Fig 4.15 Flourescence change recorded upon addition of (A) 3.2 and 16 µM leucine (B) 16 µM valine to 2 µM purified His_6-Sp0749 (point addition of ligands are indicated by arrows).

was added to His_6-Sp0749 lipoprotein at the final concentration of 16 μM but additional valine at the same concentration did not quench the fluorescence change as shown in Fig 4.15 B. To assess the minimum concentration required for maximum fluorescence change, 3.2 μM valine was added to the His_6-Sp0749 lipoprotein. This induced only a small fluorescence change. Addition of 50 μM valine in the presence of 50 μM each of a variety of other amino acids failed to induce any changes on fluorescence, probably due to the large amounts of other amino acids inhibiting binding the His_6-Sp0749 lipoprotein. Overall, the results of the fluorescence binding studies suggest that the His_6-Sp0749 lipoprotein binds to BCAA exhibiting its highest affinity towards isoleucine, moderate affinity to valine and least affinity towards leucine.

4.4.8 Radioactive substrate binding assay

To further determine the specific binding affinity of the Sp0749 lipoprotein to isoleucine, leucine and / or valine, radioactive ligand binding assays were performed using the purified His_6-Sp0749 putative lipoprotein. The results suggest that the His_6-Sp0749 lipoprotein bound to ^{14}C-isoleucine, and to a lesser degree to ^{14}C-leucine ($p < 0.05$) but not to the negative control, ^{14}C-amino isobutyric acid (AIB) (Fig 4.16 A). Branched chain amino acid transporters (LIV transporters) have been well characterised in *E. coli* and *P. aeruginosa* and have been shown to bind to amino acids such as L-threonine, L-alanine, L-serine in addition to L-leucine, L-isoleucine and L-valine (Rahmanian *et al.*, 1973; Hoshino *et al.*, 1992). Therefore His_6-Sp0749 lipoprotein was used to investigate if Sp0749 exhibits broader specificity towards amino acids other than isoleucine, leucine and valine, such as L-threonine. Competitive ligand binding with leucine, valine, threonine and AIB (negative control) was performed in the presence of ^{14}C-isoleucine. Fig 4.16 B shows that His_6-Sp0749

lipoprotein strongly binds to ^{14}C-isoleucine alone and does not bind to ^{14}C-AIB. However, valine, leucine and threonine inhibited the ^{14}C-isoleucine binding to His_6-Sp0749 lipoprotein and that the His_6-Sp0749 lipoprotein bound to valine and threonine inhibited the ^{14}C-isoleucine binding at similar affinities than leucine. These results confirm that His_6-Sp0749 lipoprotein has greatest affinity towards isoleucine and also indicates that His_6-Sp0749 lipoprotein probably exhibits preferential binding to amino acid ligands in decreasing order of isoleucine > valine > threonine > leucine. Although the preferential binding experiment should have been performed with other amino acids such as serine and alanine in the presence of ^{14}C-isoleucine, it could not be done due to the lack of purified His_6-Sp0749 lipoprotein and time constraints.

4.5 *IN VIVO* PHENOTYPE ANALYSIS OF THE *S. PNEUMONIAE ΔSP0149 AND ΔSP0750-53* DELETION MUTANTS

4.5.1 Competitive index

To investigate the effect on the virulence of *S. pneumoniae* as the result of *Sp0149* and *Sp0750-53* deletion during the pulmonary (IN) and systemic (IP) infection in mouse models, CIs were performed. Mixed inocula of *S. pneumoniae* wild-type, *ΔSp0149* and *ΔSp0750-53* strains were inoculated in mice by IN and IP routes and the CIs were determined as described in chapters 2 and 3. Deletion of *Sp0149* and *Sp0750-53* affected the *S. pneumoniae* virulence with markedly reduced CIs for the *ΔSp0149* and *ΔSp0750-53* strains in both pulmonary (median CIs in lungs: *ΔSp0149*: 0.028, *ΔSp0750-53*: 0.109) and systemic (median CIs in spleen: *ΔSp0149*: 0.028, *ΔSp0750-53*: 0.077) models of infection (Fig 4.17). These results are similar to those obtained with *Sp0149*$^-$ and *Sp0750*$^-$ IDM mutant strains of *S. pneumoniae* (mean CI in lungs: *Sp0149*$^-$:

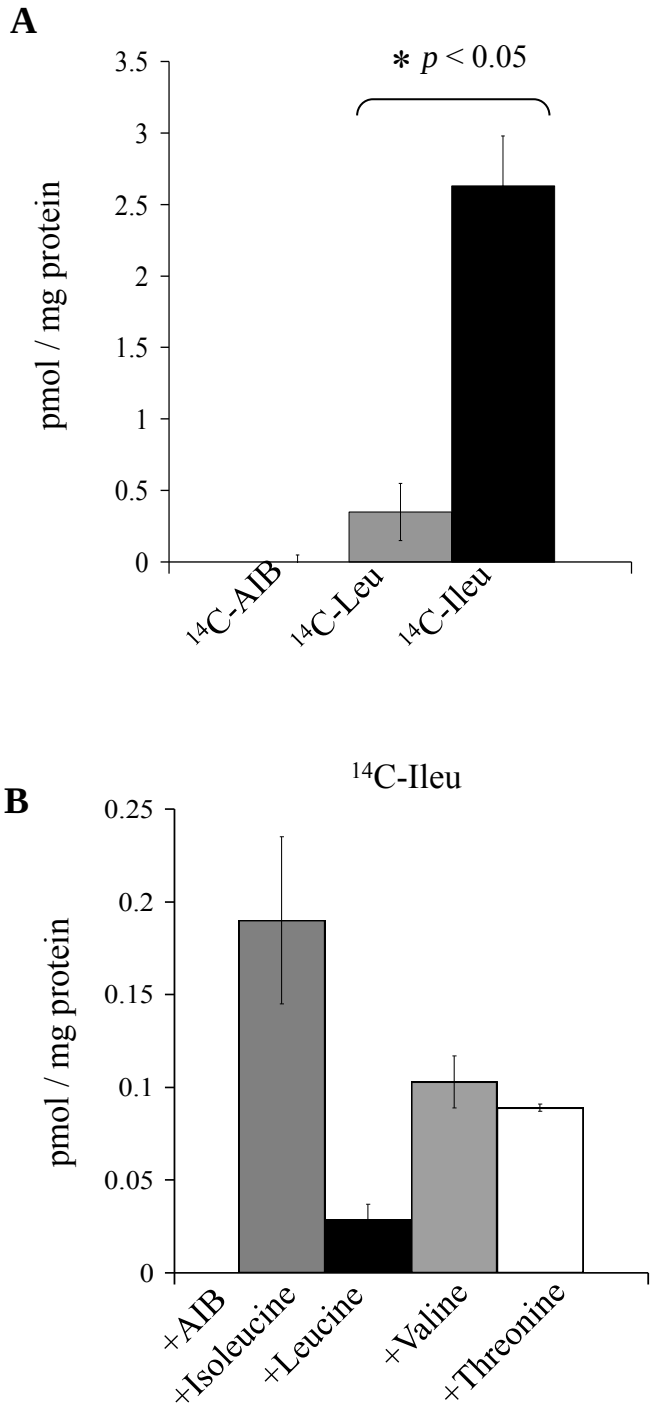


Fig 4.16 Radioactive substrate binding assay expressed as pmoles of amino acid bound per mg of purified His_6-Sp0749 lipoprotein (A) Binding affinities of His_6-Sp0749 towards ^{14}C-Labelled isoleucine (Ileu), leucine (Leu) and amino isobutryic acid (AIB) (B) Competitive amino acid ligand binding of His_6-Sp0749 to ^{14}C-AIB (negative control), ^{14}C-isoleucine, AIB, leucine, valine and threonine in the presence of ^{14}C-isoleucine.

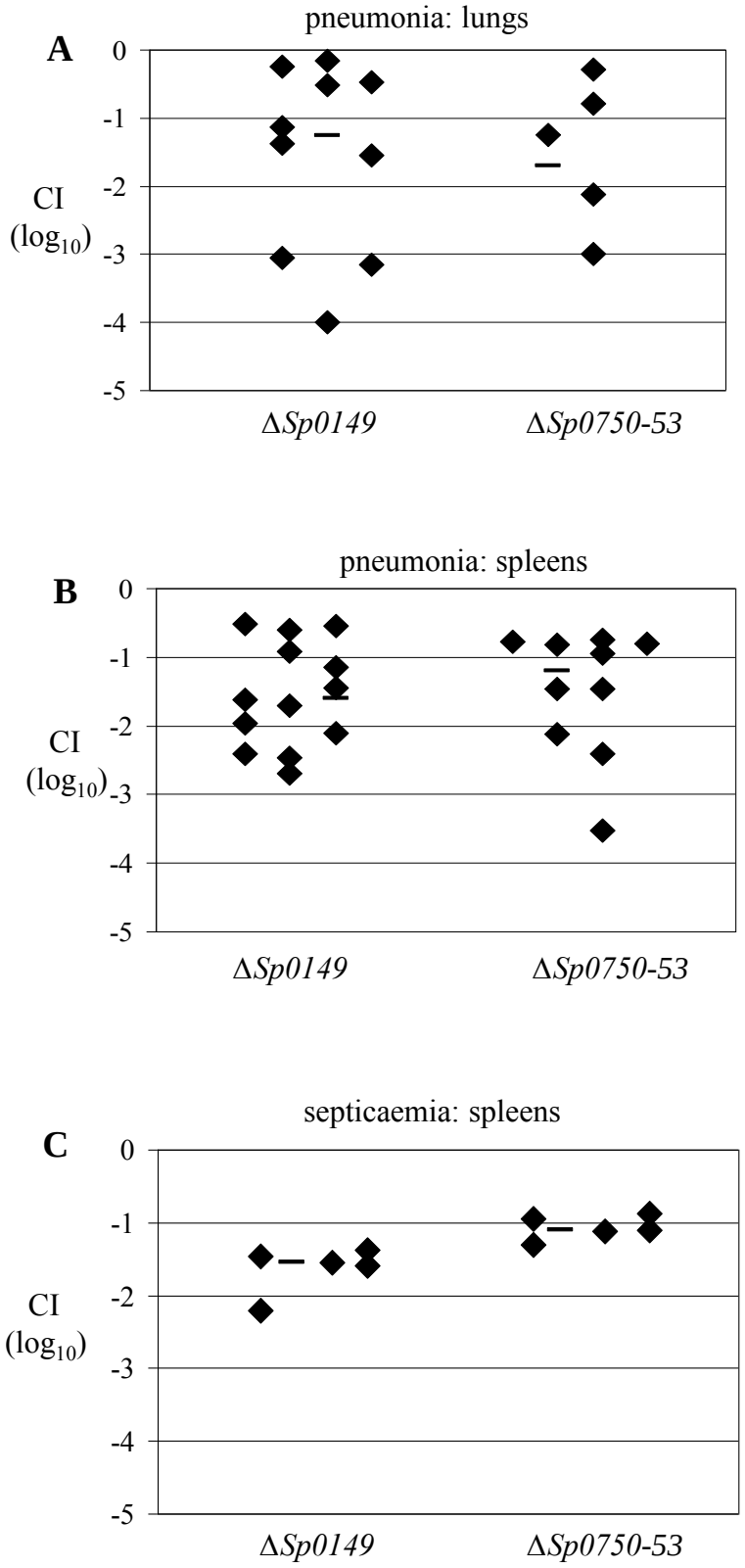


Fig 4.17 *In vivo* phenotype analysis of *ΔSp0149* and *ΔSp0750-53* deletion mutants measured by CIs, expressed as $\log_{10}$ results for the *ΔSp0149* and *ΔSp0750-53* deletion mutants compared to the wild-type strain (0100993) in mouse models of pneumonia (A and B), septicaemia (C). In both the models, each symbol represents the CI obtained from an individual animal.

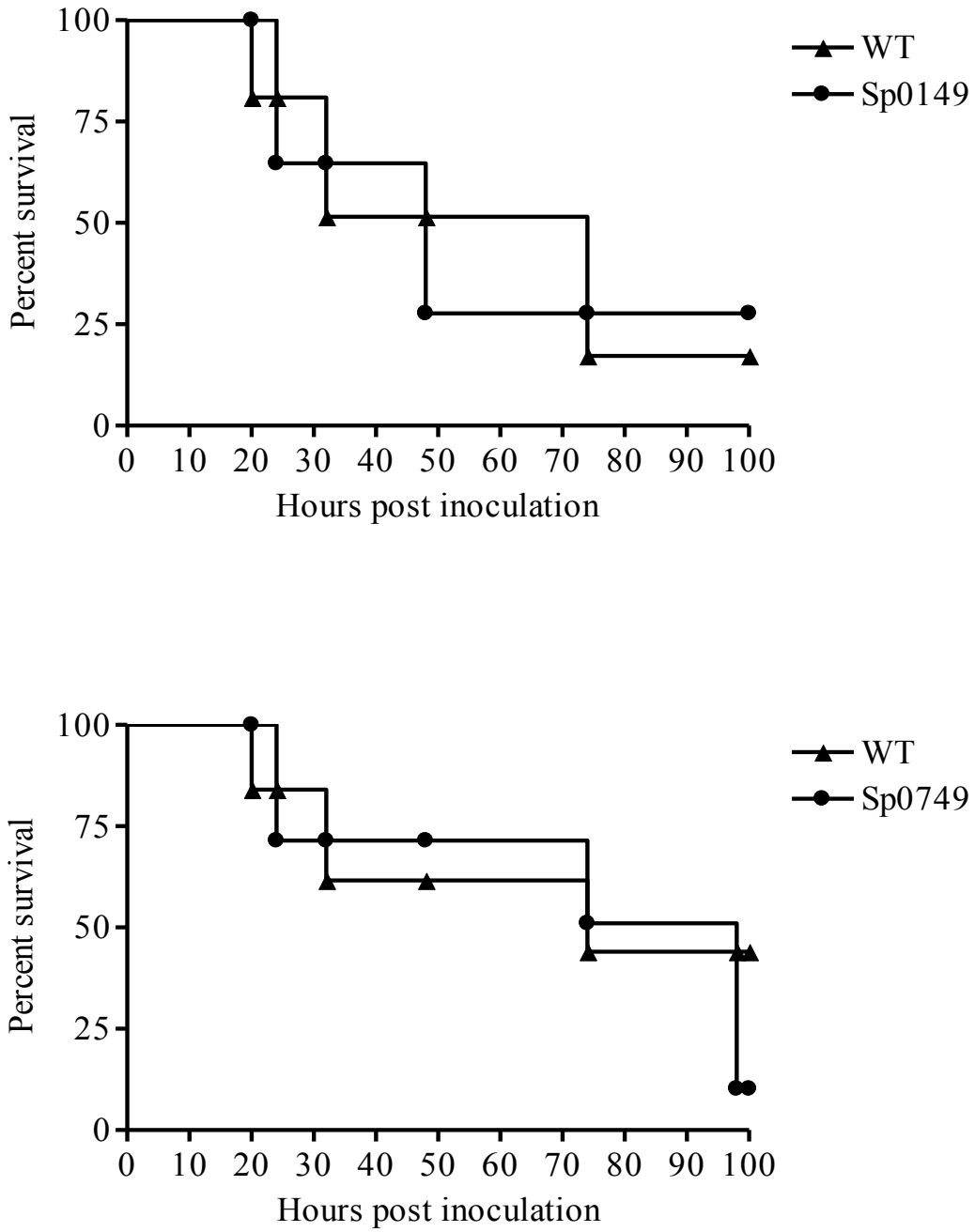


Fig 4.18 Survival of groups of 10 mice were intranasally inoculated with 10^7 cfu of *ΔSp0149* (A) and *ΔSp0750-53* (B) deletion mutant strains compared to mice inoculated with the wild-type *S. pneumoniae* strains (0100993).

0.023, *Sp0750*$^{-}$: 0.016) (mean CI in spleens: *Sp0149*$^{-}$: 0.067, *Sp0750*$^{-}$: 0.017) models of infection in mice (discussed in chapter 3).

4.5.2 Survival studies

In order to asses the ability of *S. pneumoniae* strains to cause fatal disease, group of 10 CD1 mice strain were intranasally inoculated with *S. pneumoniae* (0100993) wild-type, *ΔSp0149* and *ΔSp0750-53* strains and their survival curves were determined (Brown *et al.*, 2001). The mice were inoculated intranasally with the challenge dose of 10^7 cfu of the wild-type, *ΔSp0149* and *ΔSp0750-53* strains and monitored for the progress of infection. Fig 4.18 demonstrates that there was no difference in the survival of mice inoculated with the wild-type, *ΔSp0149* and *ΔSp0750-53* strains. Only 50% of mice cleared infection and survived after inoculation with the wild-type strain. This was an unexpectedly high level of clearance, as disease usually progresses into fatal infection in 80 to 90% of mice when the wild-type *S. pneumoniae* is inoculated Therefore this experiment should be repeated to ensure a small survival benefit in mice given the mutant strains has not been missed.

4.6 SUMMARY

The Sp0148-53 and Sp0749-53 ABC transporters of *S. pneumoniae* were investigated in detail for their *in vitro* and *in vivo* phenotypes. The relative mRNA expression of *Sp0149* and *Sp0749* lipoprotein genes in *S. pneumoniae* were analysed in THY, human blood and during infection in mice after IP infection. The results demonstrated that *Sp0149* and *Sp0749* genes are expressed during infection in mice, and suggest that the expression of *Sp0749* is actually increased during infection compared to culture in either THY or blood in the laboratory.

From the localisation studies of Sp0149 and Sp0749, Western blot analysis demonstrated that both Sp0149 and Sp0749 are associated with the membrane compatible with being lipoproteins. Western blot analysis using the *Δlsp* and *lsp*^{c} strains showed that Sp0149 is enzymatically processed by Lsp. Loss of Lsp resulted in an unusual phenotpye for Sp0749, with reduced band strength in the *Δlsp* strain, suggesting Lsp activity is required for efficient attachment of Sp0749. *ΔSp0149* and *ΔSp0750-53* deletion mutants were successfully constructed by OEP. *In vitro* phenotypes of these strains did not detect any consistent phenotype in growth in specialised media or laboratory assays associated with the predicted functions of the corresponding ABC transporter. Uptake assays failed to demonstrate the uptake of amino acids. However, using the purified His_6-Sp0749 lipoprotein, tryptophan fluorescence spectroscopy and radioactive ligand binding assays demonstrated that purified Sp0749 binds to BCAAs with a higher affinity towards isoleucine at submicromolar concentrations.

The *in vivo* phenotype of *ΔSp0149* and *ΔSp0750-53* mutant strains was assessed using CIs, demonstrated a marked decrease in the virulence in pulmonary and septicaemia models of infection in mice for both strains. However contrasting results were obtained when survival studies were performed, with no impaired ability of the *ΔSp0149* and *ΔSp0750-53* mutant strains to cause fatal disease in a pulmonary model of infection in mice. Hence loss of these ABC transporters results in a significant impairment of the mutant strains to compete with the wild-type strain during infection but the mutants are still able to cause fatal infection.

Chapter 5
Investigation of Sp0149 and Sp0749 vaccine potential against systemic *S. pneumoniae* disease

The data from chapters 3 and 4 have shown that Sp0149-52 and Sp0749-53 ABC transporters have role in *S. pneumoniae* virulence and that the Sp0749 lipoprotein is a branched chain amino acid binding protein. In this chapter, the potential of the recombinant Sp0149 and Sp0749 lipoproteins as vaccine candidates are investigated against *S. pneumoniae* septicaemia in two different mouse models (outbred CD1 and inbred BALB/c) using a virulent serotype 2 (D39) as the *S. pneumoniae* challenge strain. The immune responses that may be elicited due to intraperitoneal immunisation of Sp0149 and Sp0749 lipoproteins are also presented.

5.1 Expression and purification of Sp0149 and Sp0749 lipoproteins

The phenotype analysis of Sp0149-52 and Sp0749-53 ABC transporters using *Sp0149*$^{-}$ and *Sp0750*$^{-}$ mutant strains demonstrated marked attenuation in murine models of septicaemia and pneumonia models compared to the parental wild-type strain. Hence the lipoprotein components of these ABC transporters were chosen for investigation as potential novel *S. pneumoniae* vaccine candidates. This required the expression and purification of the recombinant lipoproteins of both ABC transporters. The genes encoding the lipoproteins, Sp0149 for Sp0149-0153 and Sp0749 for Sp0749-0753, were amplified with the primers listed in chapter 2 using *S. pneumoniae* strain 0100993 genomic DNA as the template. To design the primers, the nucleotide and derived amino acid sequence of Sp0149 and Sp0749 genes were

analysed from the available complete genome sequence of a serotype 4 strain of *S. pneumoniae* (Tettelin *et al.*, 2001) to identify the lipobox, the conserved amino acid sequence where the N-terminal signal sequence of the prolipoprotein is enzymatically cleaved after the attachment to the membrane (Khandavilli *et al.*, 2008; Sutcliffe and Russell, 1995, Hutchings *et al.*, 2009). The lipobox has the consensus amino acid sequence [LVI] [ASTVI] [GAS] C, where the leucine is highly conserved and the cysteine (a site of covalent attachment to the cell membrane phospholipid) is strictly conserved (Sutcliffe and Russell, 1995). To avoid potential secretion of recombinant lipoproteins by *E. coli*, primers were designed to amplify Sp0149 and Sp0749 excluding the N-terminal lipoprotein signal sequence (Table 2.1 and 2.2). Sp0149 primers were designed without flanking restriction sites, and the amplified PCR product (Fig 5.2 A) ligated into the pQE30UA expression vector (a TA cloning vector that relies on the addition of terminal thymidine and adenosine residues to the product of PCR for ligation) (Fig 5.1 A). The Sp0749 gene was amplified using the forward primer with additional nucleotides encoding a *Bam*HI recognition site, and the Sp0749 reverse primer with an additional *Sal*I restriction site (Fig 5.2 A). The amplified PCR product of Sp0749 was ligated into a TA cloning vector (pGEM-T easy) for efficient restriction digestion, the digested PCR product was gel extracted, and ligated into the dephosphorylated *Bam*HI and *Sal*I digested pQE30 vector (Fig 5.1 B). The ligation mixture was digested with *Sac*I to linearize the uncut pQE30 vector (*Sac*I is present between the *Bam*HI and *Sal*I restriction sites) and transformed into the M15 strain of *E. coli*. The presence of plasmids containing the correct inserts in the right orientation was confirmed by PCR using the vector-specific forward primer (pQE30F) and an insert-specific reverse primer (149Rev and 749Rev) (Fig 5.2 B and C). The sizes of the PCR products were confirmed by gel electrophoresis. For final

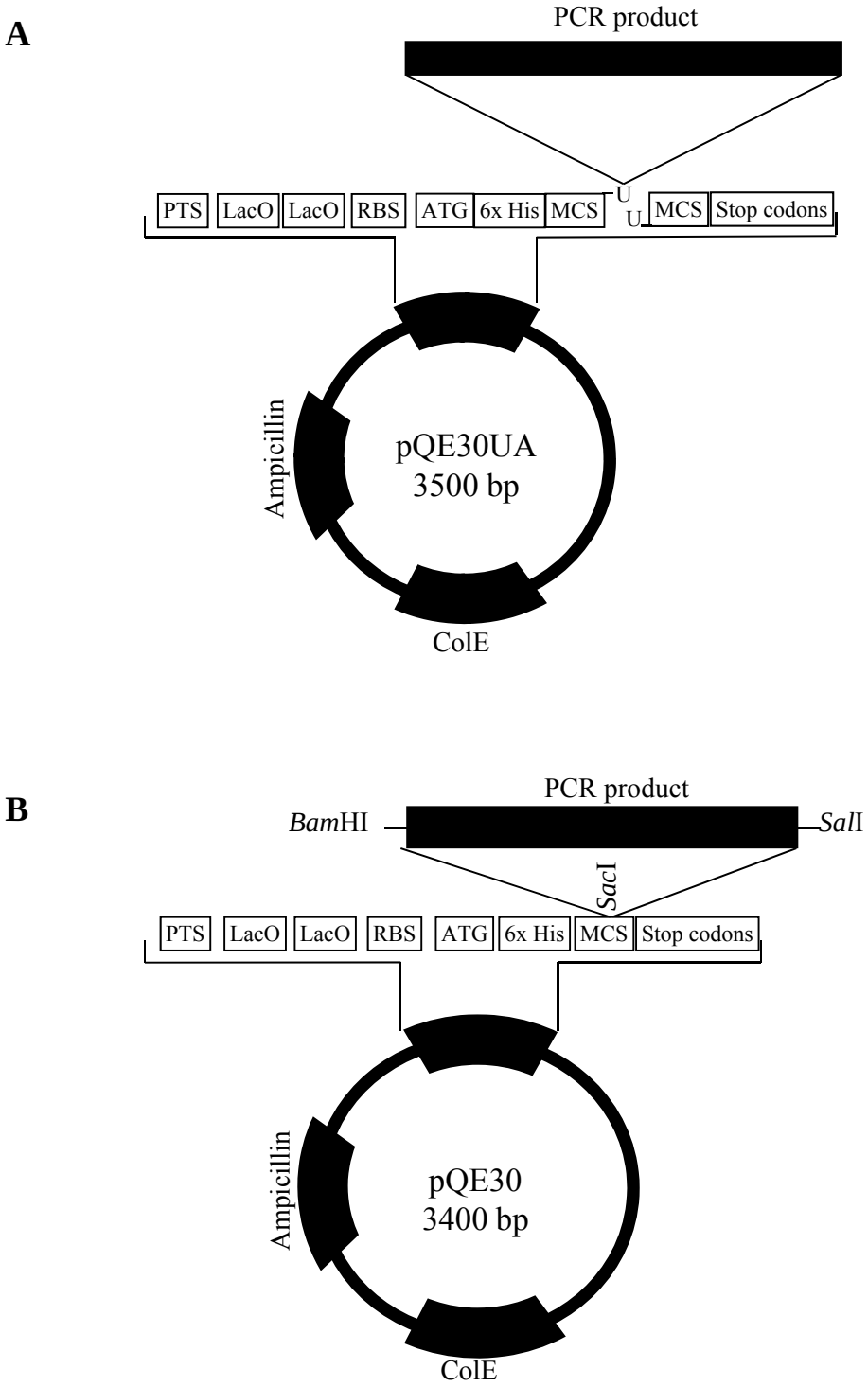


Fig 5.1 (A) Map of Sp0149 lipoprotein gene (800 bp) ligated into prelinearised pQE30UA expression vector. (B) Map of Sp0749 lipoprotein gene (1000 bp) ligated into *Bam*HI and *Sal*I restriction sites of the pQE30 expression vector.

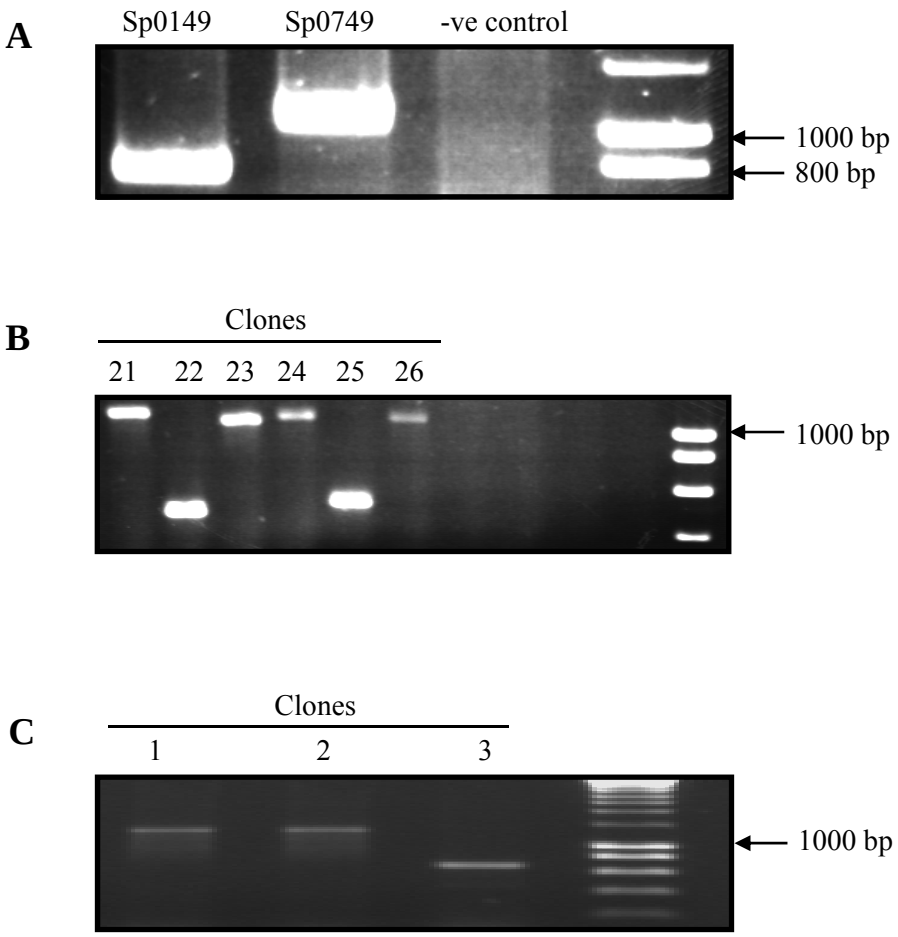


Fig 5.2 (A) Amplified PCR product of Sp0149 (0.8 kb) and Sp0749 (1 kb) lipoprotein genes from *S. pneumoniae* (0100993 strain) genomic DNA used for the ligation into pQE30UA and pQE30 expression vector. (C) Colony PCR of Sp0749 lipoprotein gene using pQE30F and Sp0749 Rev primers to confirm the orientation of Sp0749 gene after the ligation into pQE30 expression vector. M15 strain of *E. coli* clones 1 and 2 showing the correct orientation of Sp0749 gene. (B) *E. coli* M15 colony PCR for amplification of inserts containing the Sp0149 lipoprotein gene in the expression plasmid pQE30UA, using the forward primer pQE30F and the reverse primer Sp0149Rev to confirm the correct orientation of Sp0149. Clones 21, 23, 24, 26 contain plasmids carrying Sp0149 in the correct orientation.

B

cat cac cat cac cat cac gga tcc cac gtg ata tcc tca atc gct tct tgc gga aac tca gaa aag aaa gca gac aat
H H H H H H G S H V I S S I A S C G N S E K K A D N

gca aca act atc aaa atc gca act gtt aac cgt agc ggt tct gaa gaa aaa cgt tgg gac aaa atc caa gaa ttg gtt aaa
A T T I K I A T V N R S G S E E K R W D K I Q E L V K

aaa gac gga att acc ttg gaa ttt aca gag ttc aca gac tac tca caa cca aac aaa gca act gct gat ggc gaa gta gat
K D G I T L E F T E F T D Y S Q P N K A T A D G E V D

ttg aac gct ttc caa cac tat aac ttc ttg aac aac tgg aac aaa gaa aac gga aaa gac ctt gta gcg att gca gat act tac
L N A F Q H Y N F L N N W N K E N G K D L V A I A D T Y

atc tct cca atc cgc ctt tac tca ggt ttg aat gga agt gcc aac aag tac act aaa gta gaa gac atc cca gca aac gga
I S P I R L Y S G L N G S A N K Y T K V E D I P A N G

gaa atc gct gta ccg aat gac gct aca aac gaa agc cgt gcg ctt tat ttg ctt caa tca gct ggc ttg att aaa ttg gat gtt
E I A V P N D A T N E S R A L Y L L Q S A G L I K L D V

tct gga act gct ctt gca aca gtt gcc aac atc aaa gaa aat cca aag aac ttg aaa atc act gaa ttg gac gct agc caa
S G T A L A T V A N I K E N P K N L K I T E L D A S Q

aca gct cgt tca ttg tca tca gtt gac gct gcc gtt gta aac aat acc ttc gtt aca gaa gca aaa ttg gac tac aag aaa tca
T A R S L S S V D A A V V N N T F V T E A K L D Y K K S

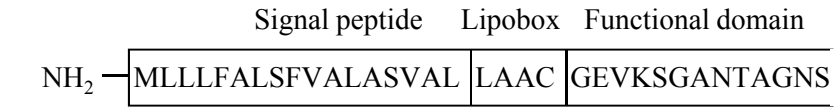
ctt ttc aaa gaa caa gct gat gaa aac tca aaa caa tgg tac aac atc att gtt gca aaa aaa gat tgg gaa aca tca cct aag
L F K E Q A D E N S K Q W Y N I I V A K K D W E T S P K

gct gat gct atc aag aaa gta atc gca gct tac cac aca gat gac gtg aaa aaa gtt atc gaa gaa tca tca gat ggt ttg gat
A D A I K K V I A A Y H T D D V K K V I E E S S D G L D

caa cca gtt tgg taa
Q P V W *

Fig 5.3 N-terminal signal sequence and the lipobox of Sp0149 (A) Sp0149 was ligated into pQE30UA by designing the forward primer from the nucleotide sequence encoding the cysteine residue of the lipobox. (B) Inframe sequence obtained from sequencing the expression vector (pQE30UA) after Ligation of Sp0149 into pQE30UA expression vector. Text in bold; 6xHis tag followed by sequence of pQE30UA.

A



B

cat cac cat cac cat cac gga tcc tgt gga gaa gtg aag tct gga gca gtc aac act gct ggt aac tca gta gag gaa aag aca att aaa
H H H H H H G F C G E V K S G A V N T A G N S V E E K T I K

atc ggg ttt aac ttt gaa gaa tca ggt tct tta gct gca tac gga aca gct gaa caa aaa ggt gcc caa ttg gct gtt gat gaa atc aat gcc gca
I G F N F E E S G S L A A Y G T A E Q K G A Q L A V D E I N A A

ggt ggt atc gat gga aaa caa atc gaa gta gtc gat aaa gat aat aag tct gaa aca gct gag gct gct tca gtt aca act aac ctt gta acc caa
G G I D G K Q I E V V D K D N K S E T A E A A S V T T N L V T Q

tct aaa gta tca gca gtc gta gga cct gcg aca tct ggt gcg act gca gct gcg gta gcg aac gct aca aaa gca ggt gtt cca ttg atc tca
S K V S A V V G P A T S G A T A A A V A N A T K A G V P L I S

cca agt gcg act caa gat gga ttg act aaa ggt caa gat tac ctc ttt att gga act ttc caa gat agc ttc caa gga aaa att atc tca aac tat gtt
P S A T Q D G L T K G Q D Y L F I G T F Q D S F Q G K I I S N Y V

tct gaa aaa tta aat gct aag aaa gtt gtt ctt tac act gac aat gcc agt gac tat gct aaa ggg att gca aaa tct ttc cgc gag tca tac aag
S E K L N A K K V V L Y T D N A S D Y A K G I A K S F R E S Y K

ggt gaa atc gtt gca gat gaa act ttc gta gca ggt gac aca gac ttc caa gca gcc ctt aca aaa atg aaa ggg aaa gac ttt gat gct atc gtt
G E I V A D E T F V A G D T D F Q A A L T K M K G K D F D A I V

gtt cct ggt tac tat aat gag gct ggt aaa att gta aac caa gcg cgt ggc atg gga att gac aaa cca atc gtt ggt ggt gat gga ttc aac ggt
V P G Y Y N E A G K I V N Q A R G M G I D K P I V G G D G F N G

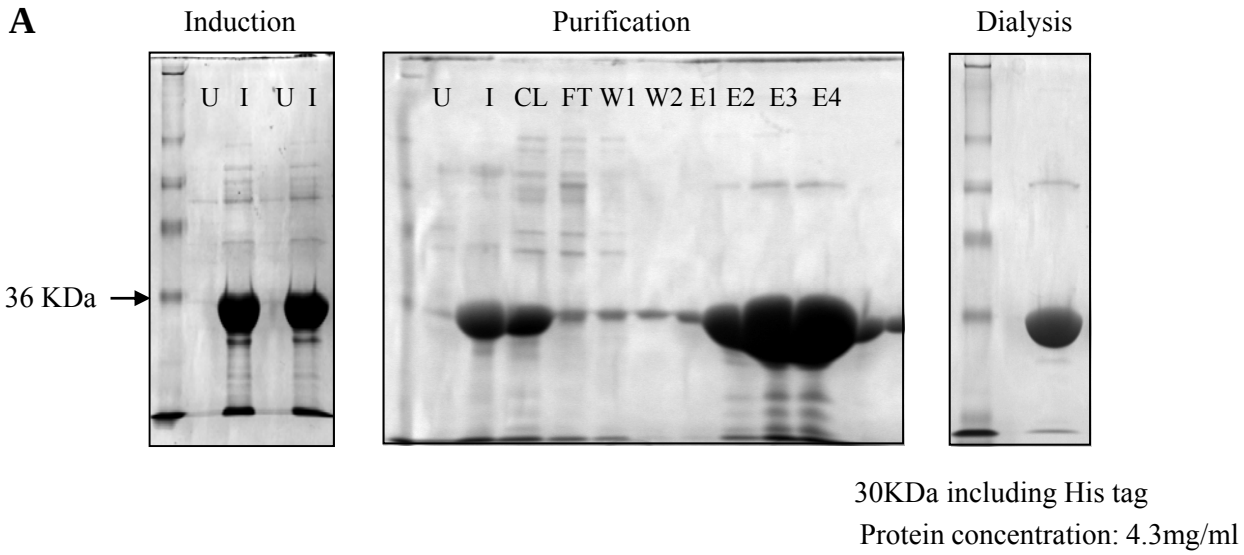
gag gag ttt gta caa caa gca act gct gaa aaa gca tca aac atc tac ttt atc tca ggc ttc tca act act gta gaa gtt tca gct aaa gct aaa
E E F V Q Q A T A E K A S N I Y F I S G F S T T V E V S A K A K

gcc ttc ctt gac gct tac cgt gct aag tac aat gaa gag cct tca aca ttt gca gcc ttg gct tat gat tca gtt cac ctt gta gca aac gca gca
A F L D A Y R A K Y N E E P S T F A A L A Y D S V H L V A N A A

aaa ggt gct aaa aat tca ggt gaa atc aag aat aac ctt gct aaa aca aaa gat ttt gaa ggt gta act ggt caa aca agc ttc gat gca gac cac
K G A K N S G E I K N N L A K T K D F E G V T G Q T S F D A D H

aac aca gtc aaa act gct tac atg atg acc atg aac aat ggt aaa gtt gaa gca gca gaa gtt gta aaa cca taa
N T V K T A Y M M T M N N G K V E A A E V V K P *

Fig 5.4 N-terminal signal sequence and the lipobox of Sp0749 (A) Sp0749 was ligated into pQE30 by designing the forward primer from the nucleotide sequence encoding the cysteine residue of the lipobox. (B) Inframe sequence obtained from sequencing the expression vector (pQE30) after ligation of Sp0749 into pQE30 expression vector. Text in bold; 6xHis tag followed by sequence of pQE30.



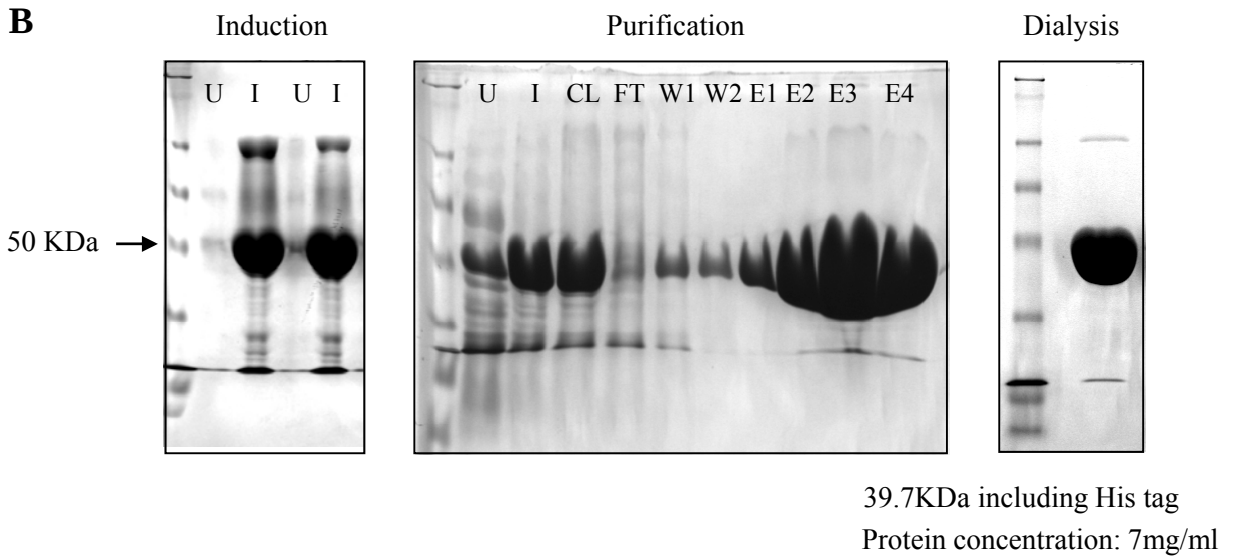


Fig 5.5 Induction, purification and dialysis of recombinant His_6-Sp0149 (A) and His_6-Sp0749 (B) lipoproteins from *E. coli*. Key: U, Uninduced; I, Induced; CL, Cell lysate; FT: Flow through; W1-2, Washes; E1-4, elutions.

confirmation of the identity of the PCR products, the PCR products were sequenced (Fig 5.3 A, B and Fig 5.4 A, B). The recombinant lipoproteins were expressed as histidine tag fusion proteins, and expressed and purified as described in chapter 2 using conventional techniques. The purity and quantity of His_6-Sp0149 and His_6-Sp0749 lipoproteins were checked by running on SDS-PAGE electrophoresis gels. The obtained molecular weight of His_6-Sp0149 and His_6-Sp0749 were 30 KDa and 40 KDa respectively, correlating closely with the expected molecular weights after excluding the N-terminal signal sequence of 29 KDa and 38 KDa respectively. The purity and concentration of Sp0149 and Sp0749 were improved by dialysis. After dialysis, His_6-Sp0149 and His_6-Sp0749 were greater than 95% pure, although faint high molecular weight bands were observed in both samples (Fig 5.5 A and B), and had protein concentrations of 4.3 mg ml^{-1} and 7 mg ml^{-1} respectively.

For systemic immunisation purposes, PspA and PsaA were used as positive controls as their efficacy as vaccine candidates against *S. pneumoniae* infection has been previously studied.

5.2 CONSERVATION OF SP0149 AND SP0749 GENES IN *S. PNEUMONIAE* STRAINS

A prerequisite for a protein based vaccine candidate is the conservation and widespread distribution of the target antigen amongst clinically important capsular serotypes of *S. pneumoniae*. Therefore whether the Sp0149 and Sp0749 lipoprotein genes were present in a range of *S. pneumoniae* strains was analysed by PCR using primers Sp0149For and Sp0149Rev, and Sp0749Fwd and Sp0749Rv. Genomic DNA obtained from strains representative of common multi-locus strain types (MLST) lineages for each vaccine capsular serotype (3, 4, 6B, 9V, 14, 18C, 19F, 23F, a kind

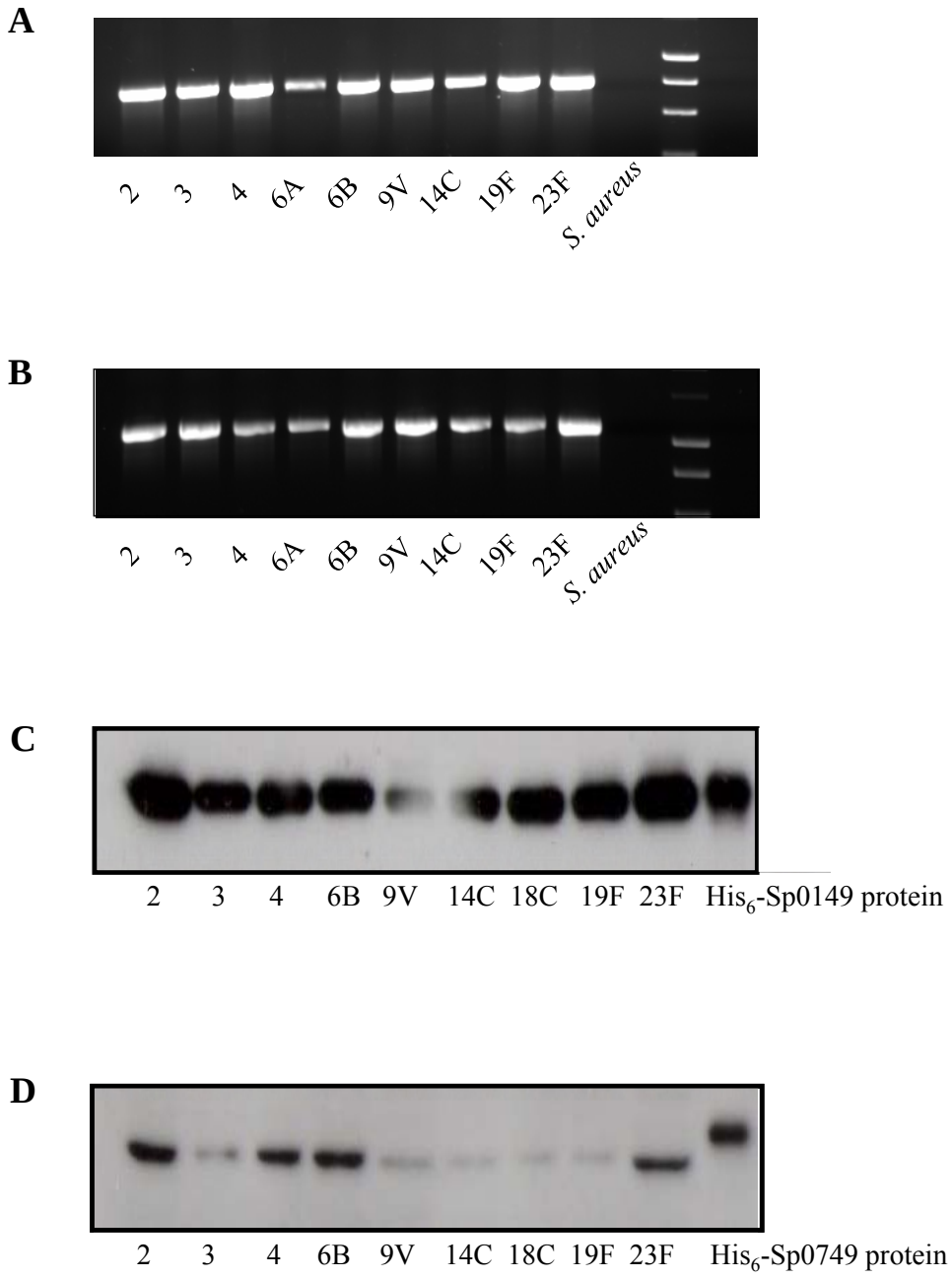


Fig 5.6 (A, B) Conservation of Sp0149 (A) and Sp0749 (B) genes in the representative strains of capsular serotypes present in the 7-valent conjugate vaccine. (C, D) Immunoblot of whole cell lysates of the representative strains of capsular serotypes from the 7-valent conjugate vaccine (plus the ST2, strain D39 and the ST3 strain 0100993 used for the laboratory studies) probed with anti-Sp0149 (C) and anti-Sp0749 (D). Identical loading of whole cell lysates were used for (C) and (D).

gift from Professor Brian Spratt) and from the laboratory strain D39 (capsular serotype 2) were used as the templates. For both Sp0149 and Sp0749 identical sized fragments were amplified from all the tested strains, indicating that there was likely to be a high degree of conservation of these genes amongst *S. pneumoniae* strains (Fig 5.6 A, B). To further explore whether these proteins are conserved amongst different *S. pneumoniae* strains, Western blots were performed against whole cell lysates of the *S. pneumoniae* strains discussed above using antisera raised in mice to recombinant Sp0749 and Sp0149 raised in mice. Anti-Sp0149 antisera gave good signals for all strains probed, whereas the anti-Sp0749 antisera gave a variable strength signal between strains, with strong signals only for serotypes 2, 4, 6B and 23F. This suggests that the expression of Sp0749 lipoprotein varied with strain background in *S. pneumoniae* (Fig 5.6 C and D).

5.3 ELISA

To analyse the antibody response after the immunization of mice with the antigens His_6-Sp0149, His_6-Sp0749, His_6-PspA, and His_6-PsaA and with alum, ELISAs were performed using sera obtained by cardiac puncture from 4 to 5 immunized CD1 or Balb/c mice 2 weeks after the third booster dose of antigens as described in chapter 2. Antibody titres of IgG from individual mouse sera were determined. Both His_6-Sp0149 and His_6-Sp0749 induced good titres of specific IgG in most mice, albeit with marked variation between mice. His_6-PspA induced IgG titres similar to those obtained with His_6-Sp0149 (median titre of 4.8) and His_6-Sp0749 (median titre of 5.4) (data not shown). Vaccination of Balb/c mice with His_6-PsaA, His_6-Sp0149 and His_6-Sp0749 also produced good titres of specific IgG antibody with the exception of the results for one mouse serum versus His_6-PsaA (Fig 5.7 A). ELISA was also used to

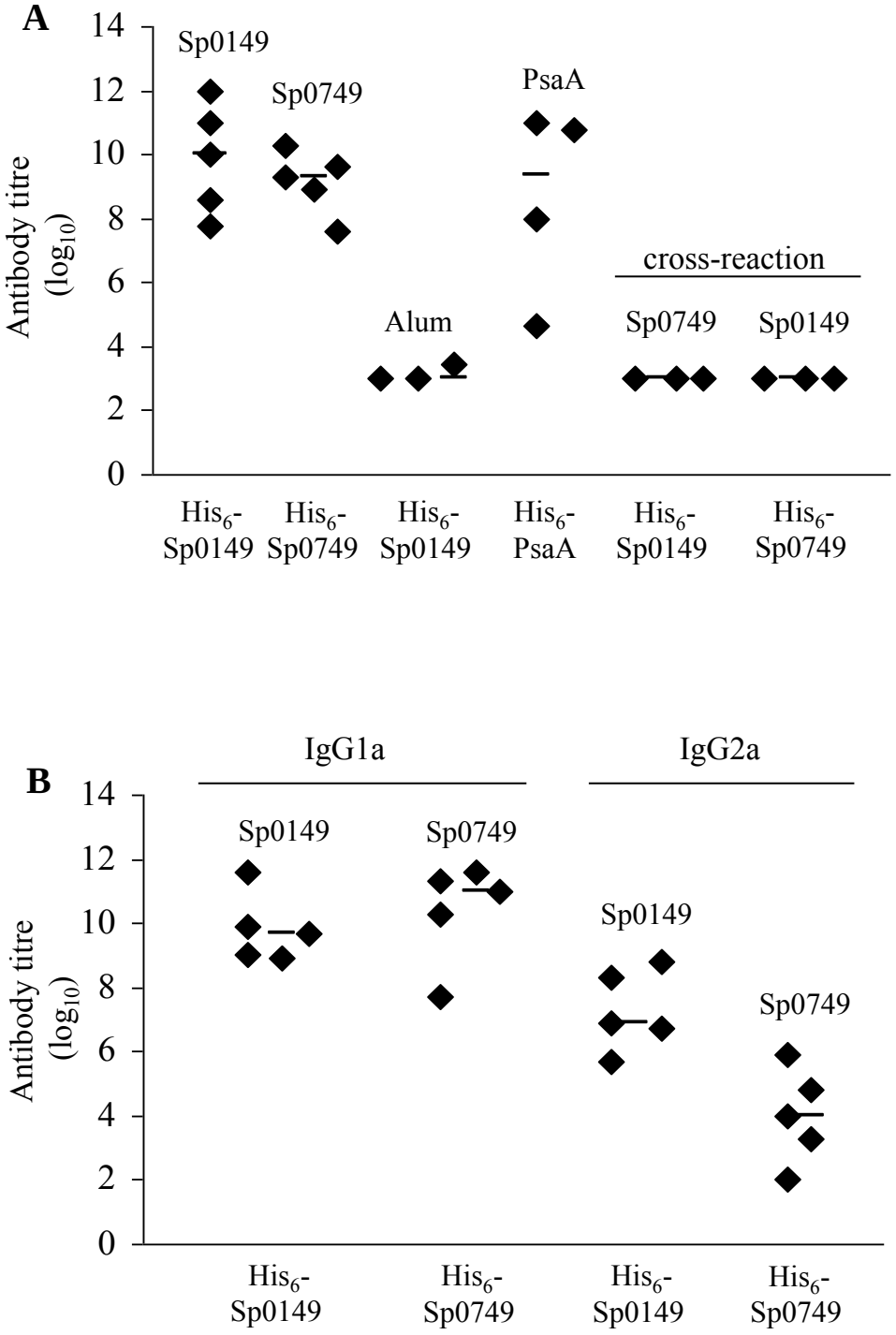


Fig 5.7 IgG antibody titres measured by ELISA using the sera obtained from Balb/c mice after three IP immunisations with purified His_6-Sp0149, His_6-Sp0749 lipoprotein, PsaA (positive control) or alum alone (negative control). (A) Total IgG titres obtained from the sera of Balb/c mice after the IP immunisation and cross-reactivity between Sp0149 and Sp0749 lipoproteins (B) IgG1a and IgG2a antibody titres in the immunised sera against purified His_6-Sp0149 and His_6-Sp0749 lipoproteins.

demonstrate that there was no cross-reaction of His_6-Sp0149 lipoprotein with anti-Sp0749 IgG antibodies and vice versa, suggesting there was very little or no antigenic similarity between the two proteins (Fig 5.7 A). As expected no detectable antibody response was observed in anti-alum antisera. To demonstrate whether the His_6-Sp0149 and His_6-Sp0749 lipoproteins elicit a Th1 or a Th2 immune response, IgG1a and IgG2a antibody titres were analysed (Jomaa *et al.*, 2005). Fig 5.7 B demonstrates that both antigens mainly stimulate an IgG1a subclass in Balb/c mice following IP immunisation, suggesting mainly a Th2 response. A similar result was obtained using CD1 mice sera following IP immunisation. The results of the ELISAs demonstrate that the purified antigens are immunogenic and induce a good IgG antibody response.

5.4 C3 DEPOSITION

To assess whether anti-Sp0149 and anti-Sp0749 were functional, their effect on the deposition of C3b/iC3b on several *S. pneumoniae* strains was analysed by a well-established flow cytometry assay (Yuste *et al.*, 2005). The pooled sera of anti-Sp0149 and anti-Sp0749 showed a small increase in the C3b/iC3b deposition (Fig 5.8 A), but the combination of pooled sera containing anti-Sp0149 and anti-Sp0749 increased the size of the effect (Fig 5.8 B). C3b deposition assay was also performed by incubating the pooled sera containing anti-Sp0149, anti-Sp0749 and anti-alum with capsular serotype 3, 6B and 9V (Fig 5.9 A, B, C). Fig 5.9 A and B showed a decrease in the C3b/iC3b deposition on serotypes 3 and 6B using anti-Sp0149 anti-Sp0749 sera when compared to anti-alum sera. Fig 5.9 C showed a significantly lower C3b/iC3b deposition on capsular serotype 9V after incubation in sera containing anti-Sp0749. Overall, the data suggest that anti-Sp0149 and anti-Sp0749 have a weak effect in

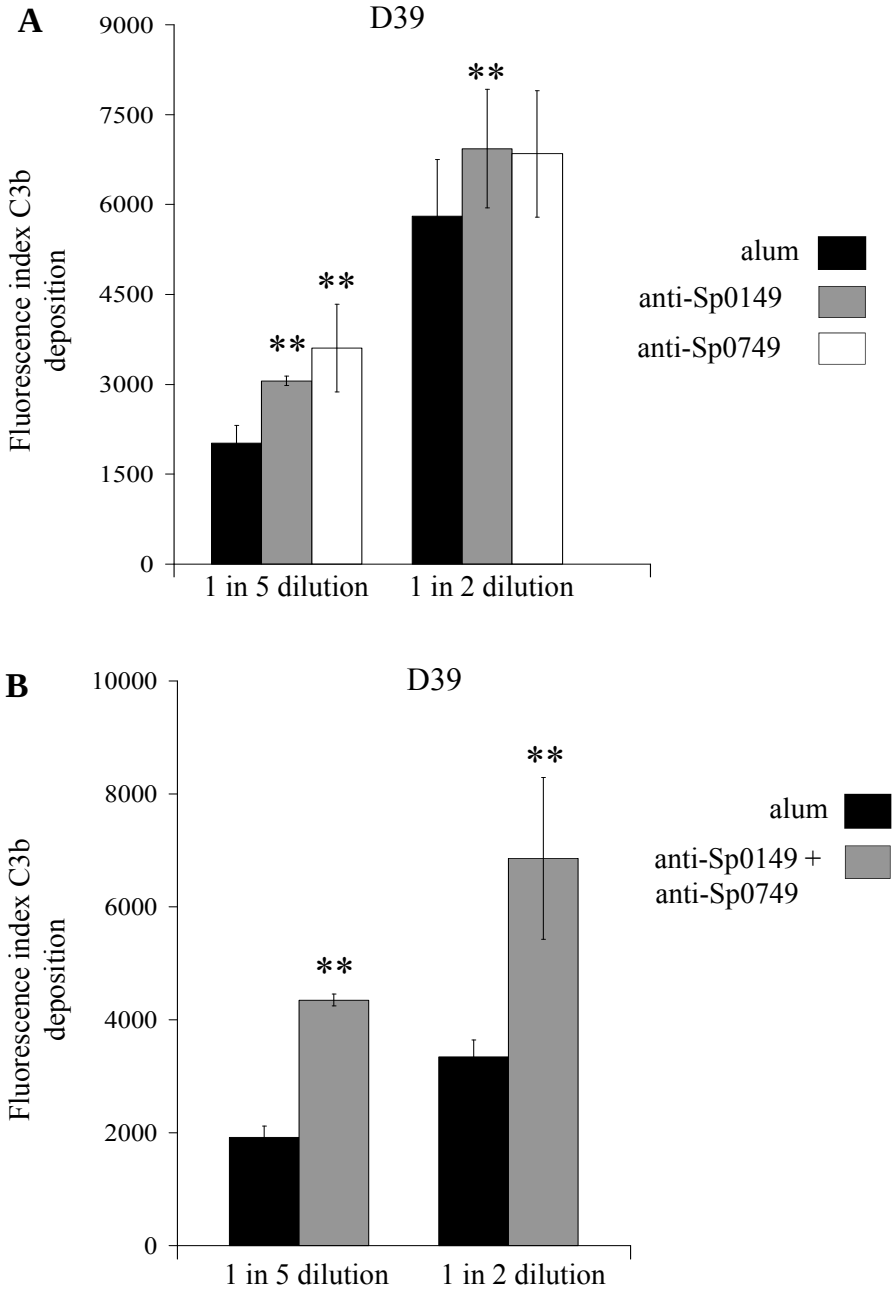


Fig 5.8 The role of anti-Sp0149 and anti-Sp0749 in the deposition of C3 on the surface of *S. pneumoniae* D39 strain using flow cytometry. The proportion of bacteria positive for the C3 after incubating with FITC-goat anti-mouse C3 antibody were analysed using Balb/c mice sera. (A) C3 deposition on D39 strain using pooled sera of anti-Sp0149 and anti-Sp0749 on D39 strain (*p* value <0.01). (B) Effect of C3 deposition on D39 strain upon mixing the pooled anti-Sp0149 and anti-Sp0749 sera (*p* value ≤ 0.01). *p* values were obtained using Student's-*t*-test by comparing alum with anti-Sp0149 and anti-Sp0749. C3 deposition on *S. pneumoniae* serotypes 3.

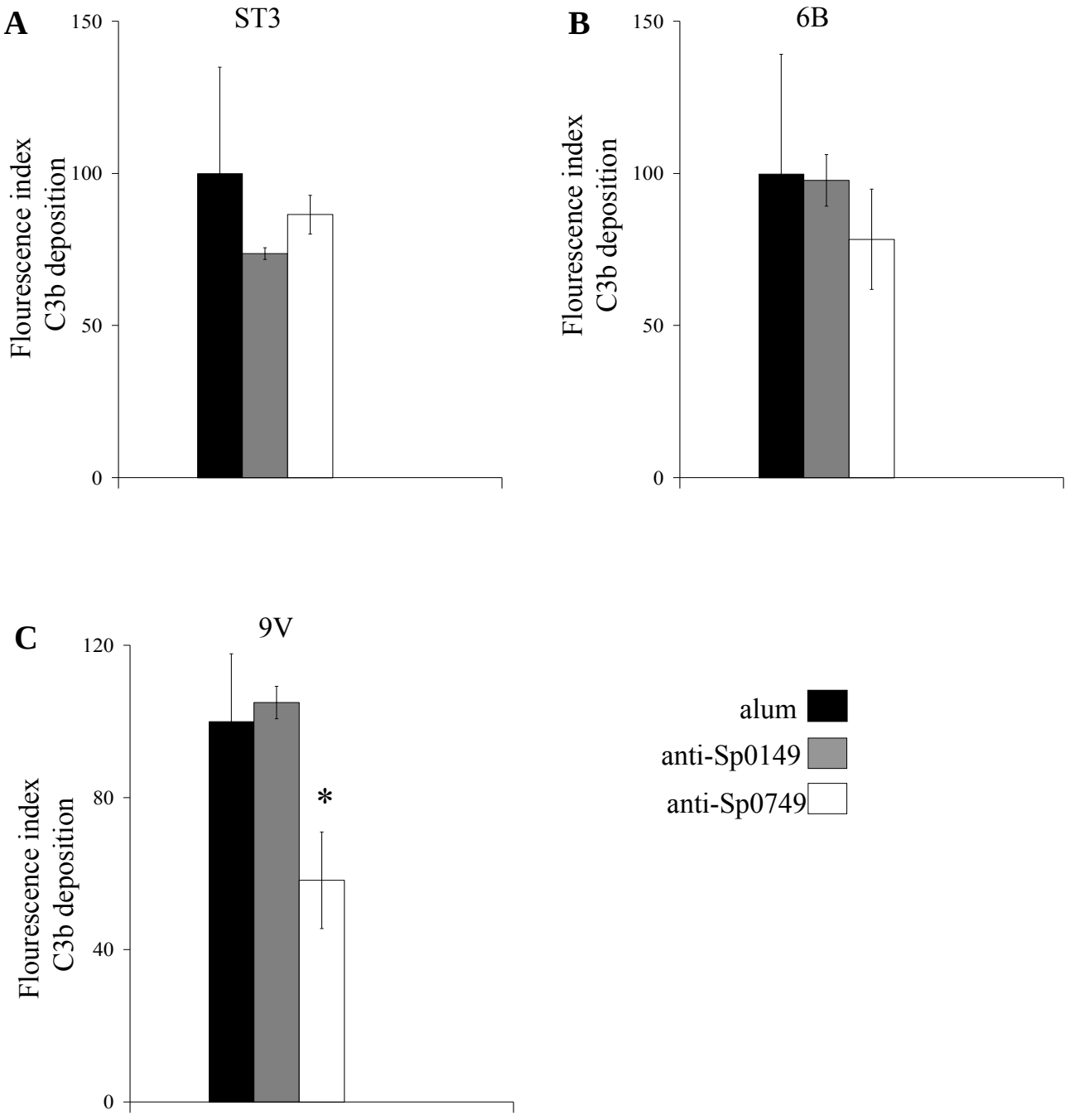


Fig 5.9 C3 deposition on *S. pneumoniae* serotypes (A) 3, (B) 6B and (C) 9V (p value < 0.05) using the pooled sera of the anti-Sp0149 and anti-Sp0749 at 1/2 dilution obtained after the second immunisation experiment in CD1 mice strain. p values were obtained using Student's t-test by comparing alum with anti-Sp0149 and anti-Sp0749.

increasing deposition of complement on the surface of some *S. pneumoniae* strains such as serotypes 3, 6B and 9V.

5.5 OPSONOPHAGOCYTOSIS

Effective antibodies may stimulate phagocytosis directly through Fcγ receptors in addition to improving opsonisation of bacteria by increasing C3b/iC3b deposition on their surface. Hence the effect of anti-Sp0149 and anti-Sp0749 on phagocytosis of *S. pneumoniae* by human neutrophils was assessed using an *in vitro* flow cytometry assay (Lehmann *et al.*, 2000) which measures the association of *S. pneumoniae* with phagocytes (Yuste *et al.*, 2008). Fluorescently-labelled bacteria were incubated with anti-Sp0149 and anti-Sp0749 at different dilutions before incubation with cells from the neutrophil cell line HL60, and then the association of bacteria and cells measured by flow cytometry. The role of complement in the *in vitro* opsonophagocytosis of *S. pneumoniae* by HL60 cells was analysed by using heat-treated sera, which inactivates complement, as a source of antibody in the presence and absence of exogenous supplementation with purified rabbit complement. Both anti-Sp0149 and anti-Sp0749 increased the proportion of serotype D39 *S. pneumoniae* associated with HL60 cells by 40% in 1 in 20 dilution of serum. Heat treatment prevented increased association of bacteria with HL60 cells for both anti-Sp0149 and anti-Sp0749 sera (Fig 5.10 B). Similarly, exogenous supplementation with purified rabbit complement also increased the proportion of *S. pneumoniae* associated with HL60 cells (Fig 5.10 A), suggesting the beneficial effect was complement-mediated. Phagocytosis assays were also performed for representative strains of various *S. pneumoniae* capsular serotypes (6B, 9V, 23F, 4, 14 and 19) (Fig 5.10 C). In the presence of anti-Sp0149, there was a statistically significant increase in the association of *S. pneumoniae* serotype 4 with

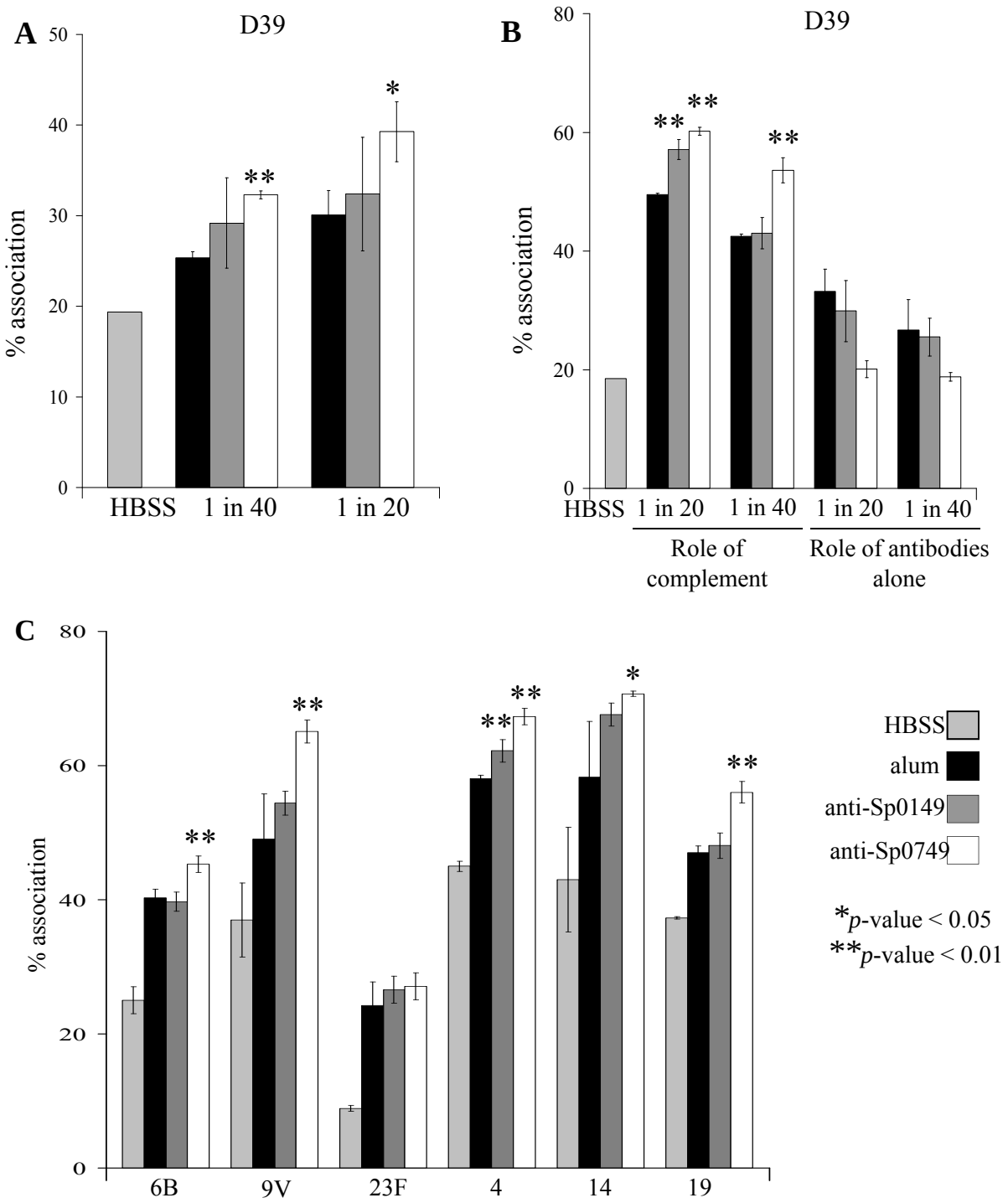


Fig 5.10 (A, B and C) *In vitro* opsonophagocytosis assay performed using the anti-Sp0149 and anti-Sp0749 sera obtained after the immunisation experiment in CD1 (A) and Balb/c (B, C) mice strain. Analysis of the role of complement in the opsonophagocytosis of *S. pneumoniae* (D39) in the presence of exogenous addition of purified rabbit complement (A), role of mouse complement and antibodies (B), and opsonophagocytosis of representative *S. pneumoniae* vaccine serotypes using anti-Sp0149 and anti-Sp0749 sera (C).

HL60 cells compared to alum sera ($p < 0.01$) but non-significant increases for serotypes 9V and 14. In the presence of anti-Sp0749, a statistically significant increase in the association of *S. pneumoniae* with HL60 cells was observed for serotypes 6B, 9V, 4, 19 ($p < 0.01$) and 14 ($p < 0.05$) compared to sera from mice vaccinated with alum alone. Overall, these results suggest that anti-Sp0149 and anti-Sp0749 could improve complement-dependent opsonophagocytosis of *S. pneumoniae* and therefore aid immunity against *S. pneumoniae* infection (Jomaa *et al.*, 2005).

5.6 ACTIVE IMMUNIZATION STUDIES

To assess the efficacy of the purified His_6-Sp0149 and His_6-Sp0749 lipoproteins as vaccine candidates, the degree of protection provided by these two lipoproteins in a septicaemia model of *S. pneumoniae* infection was evaluated. Three separate IP immunization experiments were performed using three different challenge doses of the virulent capsular strain of *S. pneumoniae* (D39) in naïve outbred CD1 and inbred Balb/c mice as described in chapter 2.

The initial immunization experiment was performed in male outbred CD1 mice. 10 µg of the purified His_6-Sp0149 and His_6-Sp0749 lipoproteins with alum as an adjuvant and alum alone as the negative control were injected intraperitoneally followed by two booster doses at 7 days intervals. After 2 weeks of immunization, mice were challenged by i.p injection of 10^5 cfu of the virulent capsular serotype 2 *S. pneumoniae* strain D39 and the progress of infection monitored over two weeks (Fig 5.11 A, B). Mice immunized with His_6-Sp0149 and His_6-Sp0749 lipoproteins exhibited only a small delay of about 4 hours in the progress of infection compared to alum alone. Of the 19 mice challenged with D39 strain of *S. pneumoniae*, 100% mortality was observed in the alum group, while only one mouse was alive in His_6-

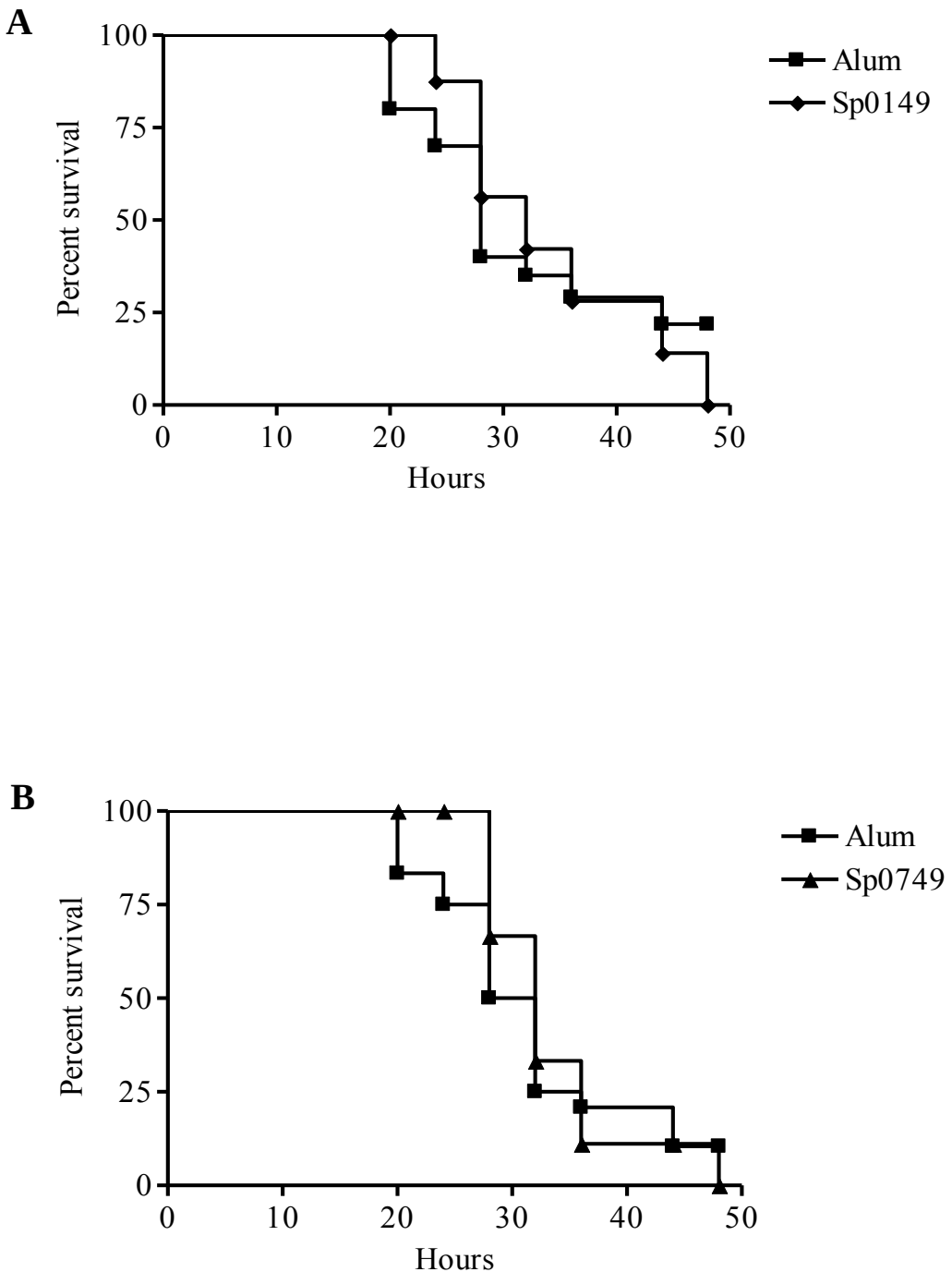


Fig 5.11 Survival curves after the first IP active immunisation in CD1 mice strain with purified (A) His_6-Sp0149, (B) His_6-Sp0749 and alum (negative control) followed by challenge with 10^5 (B) cfu of *S. pneumoniae* (D39) for a group of 19 mice.

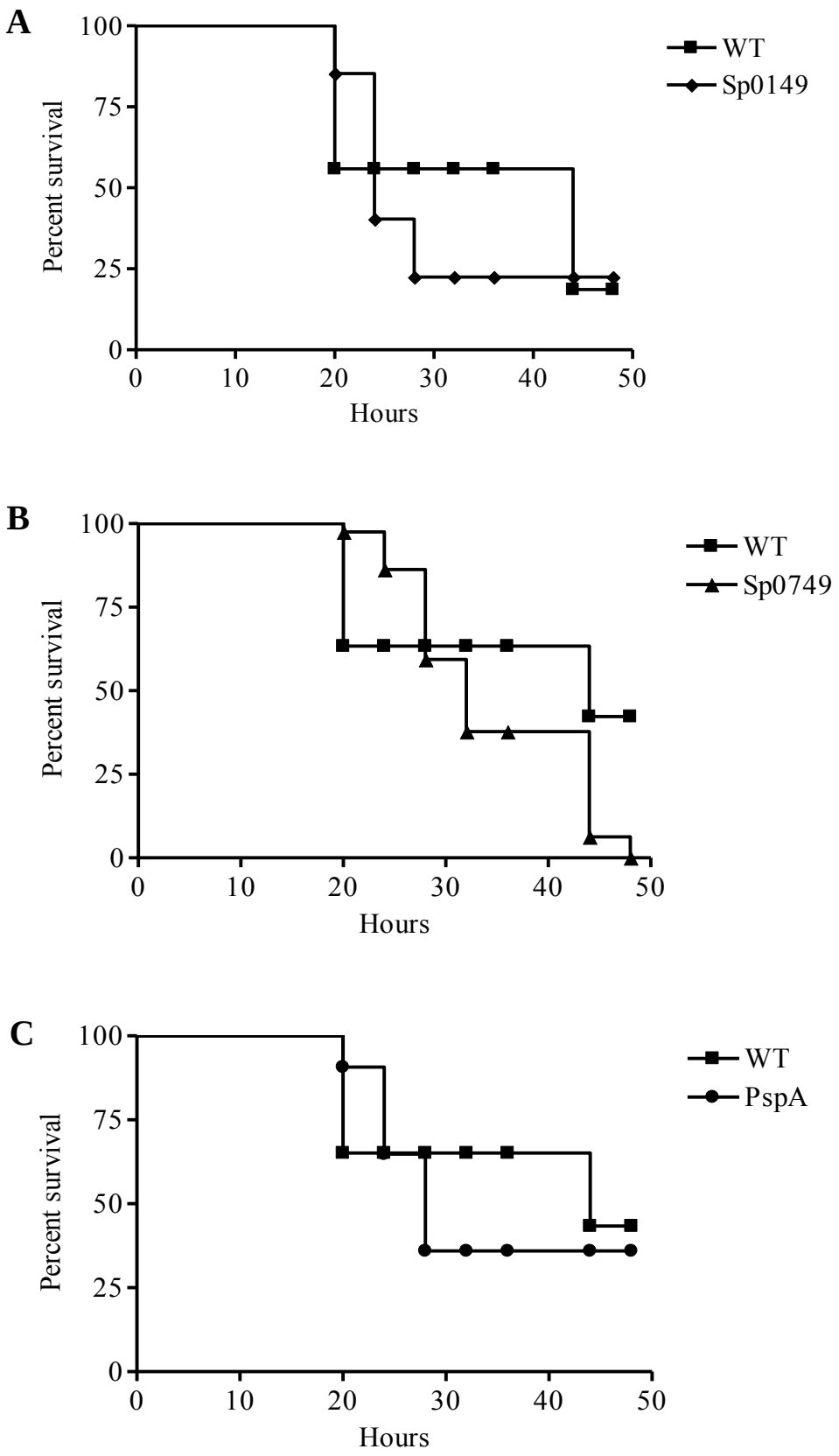


Fig 5.12 Survival curves after the second IP active immunisation in CD1 mice strain with (A) purified His_6-Sp0149, (B) His_6-Sp0749, (C) PspA (positive control) and alum (negative control) followed by challenge with 10^3 cfu of *S. pneumoniae* (D39) for a group of 19 mice.

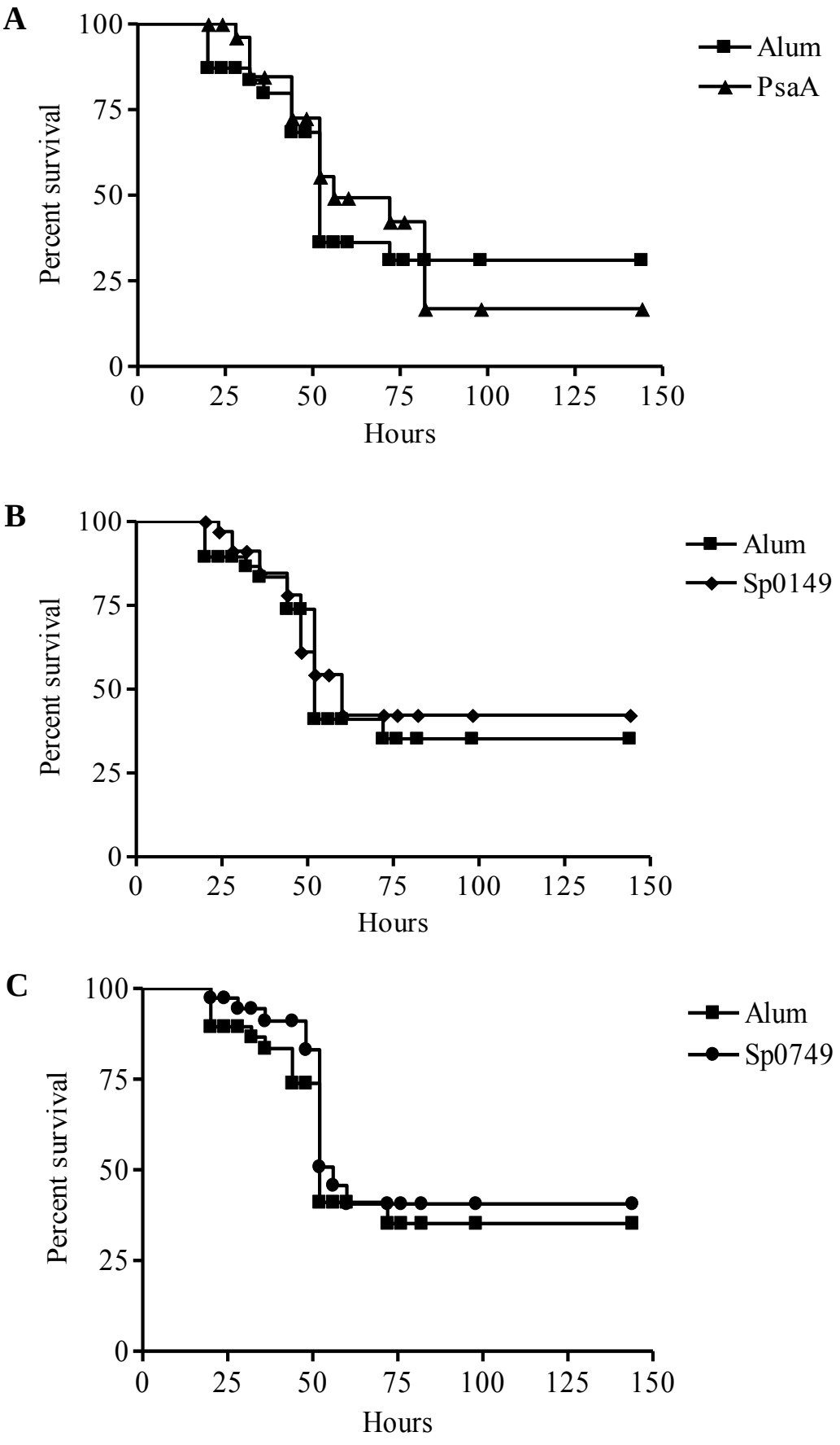


Fig 5.13 Survival curves after the third IP active immunisation in Balb/c mice strain with (A) purified PsaA (positive control), (B) His_6-Sp0149, (C) His_6-Sp0749 and alum (negative control) followed by challenge with 10^4 cfu of *S. pneumoniae* (D39) for a group of 19 mice

Sp0149 group and 3 mice were alive in His_6-Sp0749 group until the end of the experiment.

To ensure the small differences in the progress of infection in the vaccinated mice were not due to an overwhelming bacterial challenge, the immunization experiment was repeated with a lower *S. pneumoniae* (D39) challenge dose of 10^3 cfu. In the second vaccination experiment (Fig 5.12 A, B and C**),** His_6-PspA (pneumococcal surface protein) was also included as a positive control. His_6-Sp0149 and His_6-PspA failed to exhibit any protection, but mice immunised with His_6-Sp0749 exhibited a non-significant delay ($p = 0.08$) in the lethal progression of the disease of approximately 9 hours.

Since the His_6-Sp0149 failed to exhibit protection and His_6-Sp0749 only exhibited a delay in the lethal progression of the disease in CD1 mice strain, the immunisation experiment was repeated in Balb/c mice strain. Balb/c mice were chosen because they are inbred strain of mice and are relatively resistant to *S. pneumoniae* infections unlike the outbred CD1 mouse strain (Gingles *et al.*, 2001). PsaA (pneumococcal surface adhesion A), a lipoprotein component of *S. pneumoniae* manganese ABC transporter was used as positive control and a challenge dose of 10^4 cfu given by IP inoculation after immunisation with the purified lipoproteins. Both His_6-PsaA and His_6-Sp0149 failed to protect the mice from fatal infection. Again, His_6-Sp0749 seemed to delay the lethal progression of infection, although the results were not statistically significant. After 144 hours only one mouse was alive in the alum group, 3 mice alive in the His_6-PsaA and His_6-Sp0149 group, and 5 mice alive in His_6-Sp0749 group (Fig 5.13 A, B and C).

5.7 SUMMARY

The Sp0149 and Sp0749 lipoproteins were chosen for analysis of their efficacy as vaccine candidates as the mutant strains affecting the corresponding ABC transporters had marked attenuation of virulence in murine models of septicaemia and pneumonia. The PCR–amplified products of the lipoprotein genes, *Sp0149* and *Sp0749* were ligated into the expression vectors, pQE30UA and pQE30 respectively (excluding the N-terminal signal sequence) and the lipoprotein components were successfully expressed in *E. coli* and relatively high concentrations of ~~good quality~~ protein obtained. Vaccination of mice generated high titres of specific antibodies to both Sp0149 and Sp0749 and there was no evidence of cross reaction of antibodies to Sp0149 with the protein Sp0749 and vice versa. PCR and western blot analysis demonstrated that Sp0149 and Sp0749 are conserved in the serotypes present in the 7-valent conjugate vaccine. *In vitro* immune assays measuring the effects of polyclonal murine anti-Sp0149 and anti-Sp0749 on C3b deposition and phagocytosis demonstrated improvements in both complement deposition and phagocytosis for several *S. pneumoniae* strains. Active immunization with the purified Sp0149 and Sp0749 lipoproteins and challenge with D39 strain of *S. pneumoniae* indicated that there was a small but non-significant degree of protection when mice were vaccinated with recombinant Sp0749.

Chapter 6
Investigation of Sp0749 vaccine potential against *S. pneumoniae* pneumonia

In this chapter, data regarding the vaccine potential of the recombinant Sp0149 and Sp0749 lipoproteins against *S. pneumoniae* pneumonia are presented. Many variations are adopted in the immunisation-challenge methodology in this chapter compared to the methodology presented in chapter 5. These variations include mouse strains, use of adjuvant, *S. pneumoniae* challenge strains and the route of lipoprotein and S. pneumoniae administration. In this chapter, the intranasal immunisations and *S. pneumoniae* challenge (using 0100993 and D39 strains) experiments were performed in two different mouse strains (outbred CD1 and inbred CBA/Ca). Cholera toxin was used as the adjuvant for intranasal immunisation unlike alum which was used as adjuvant for the intraperitoneal immunisation in chapter 5. The role of Sp0749 alone and in combination with previously investigated lipoproteins of iron uptake ABC transporters PiaA and PiuA in eliciting protective immune response was also investigated and the data are presented in this chapter.

6.1 Expression and purification of PiaA and PiuA lipoproteins

PiaA and PiuA are lipoproteins components of iron uptake ABC transporters of *S. pneumoniae* whose vaccine potential were previously investigated by my supervisor Dr. Brown. Brown *et al* previously identified that intraperitoneal and intranasal immunisation with the recombinant PiaA and PiuA lipoprotein components protected the mice against *S. pneumoniae* septicaemia and pneumonia (Brown *et al.*, 2001,

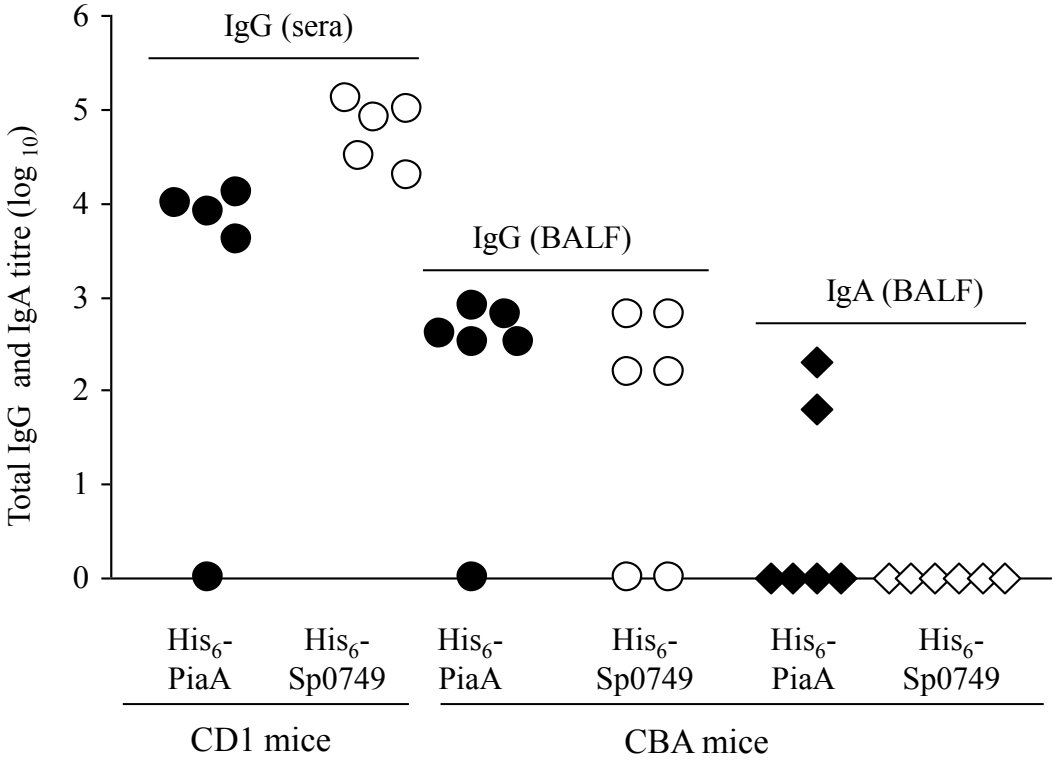


Fig 6.1 Antibody titres measured by ELISA after IN immunisations with the purified lipoproteins. Total IgG (serum) and IgA (BALF) antibody titres were determined using purified His_6-Sp0749 and His_6-PiaA. Each point represents the anti-PiaA or anti-Sp0749 antibody titre of serum or BALF from individual mouse. The titres are represented as the reciprocal of the dilution of serum which resulted in an $OD_{405} \geq 0.30$. No detectable antibody titres were obtained in sera and BALF from PiaA or PiaA-Sp0749 immunised mice when tested against cholera toxin (CT) (data not shown).

Jomaa *et al.*, 2005). Therefore the recombinant PiaA and PiuA lipoproteins were used for the intranasal immunisation and challenge experiments in mice as positive controls in order to evaluate the vaccine potential of Sp0749. The PiaA and PiuA lipoproteins were expressed and purified according to (Brown *et al.*, 2001) with some modifications. The PiaA (Sp1032) and PiuA (Sp1872) lipoprotein genes were amplified using the primers PiaA F and PiaA R for the PiaA gene and PiuA F and PiuA R for the PiuA gene and 0100993 genomic DNA as template as described in chapter 2 and 4. The primers were designed using the TIGR 4 genome to exclude the DNA sequence encoding the signal peptide as described in chapter 4. The amplified PCR products were ligated into the expression vector pQE30UA and transformed into the M15 strain of *E. coli*. The positive clones for the lipoprotein genes were confirmed by colony PCR using the vector-specific forward primer (pQE30F) and the insert specific reverse primer (PiaA R and PiuA R) and by sequencing. The positive clone for each of the lipoprotein gene was then induced for over expression of His-tagged lipoproteins and purification of the his-tagged protein performed according to the QIAexpressionist^{TM} manual. The purified his-tagged lipoproteins were dialysed and analysed by SDS-PAGE.

6.2 ELISA

In order to analyse the antibody responses after the IN immunisations of mice with purified lipoproteins (His_6-PiaA, and His_6-Sp0749), serum and bronchoalveolar lavage fluid (BALF) were obtained from 4 to 5 immunised CD1 and CBA/Ca mice 2 weeks after the second booster dose of antigens and used for ELISAs (Jomaa *et al.*, 2005). Cholera toxin (CT) was used as the adjuvant with the lipoproteins and CT alone was used as the negative control for the immunisation experiments and CT is

routinely used as an adjuvant with different *S. pneumoniae* proteins for IN immunisations (Pimenta *et al.*, 2006; Shah *et al.*, 2009). Outbred CD1 mice were used because they are less expensive where as inbred CBA/Ca mice were used because they are genetically less variable and are susceptible to IN infection (Kadioglu and Andrew, 2005; Gingles *et al.*, 2001). Purified His_6-PiaA lipoprotein was used as positive control as it has been demonstrated to induce good systemic and mucosal antibody responses upon pulmonary and systemic immunisations in mice (Brown *et al.*, 2001; Jomaa *et al.*, 2006). IN immunisation with both His_6-PiaA and His_6-Sp0749 induced significant serum IgG titres to purified PiaA and Sp0749 in the CD1 mouse strain (Fig 6.1). Titres of IgG and IgA were measured using BALF obtained from CBA/Ca mice immunised IN with combination of proteins (His_6-PiaA plus His_6-Sp0749). Fig 6.1 demonstrates that His_6-PiaA and His_6-Sp0749 induced good IgG titres in BALF, with similar levels to the IgG titres found in sera from CD1 mice. However, when the IgA titres in the BALF of the CBA/Ca mice were measured, negligible amounts of anti-Sp0749 IgA were present and anti-PiaA IgA were only elicited in two mice. These data suggest that after IN immunisation both the proteins elicit a good systemic and some local IgG response but only a poor mucosal IgA response.

6.3 BACTERIAL CFUS IN TARGET ORGANS

To further investigate whether IN immunisation of CBA/Ca mice with His_6-PiaA and His_6-PiaA plus His_6-Sp0749 of CBA/Ca mice protects against *S. pneumoniae* challenge, the cfu counts in target organs were determined from the BALF, lungs and blood. The immunised mice were intranasally challenged with 10^5 cfu of *S. pneumoniae* and the target organs were harvested 48 hours after IN inoculation. The

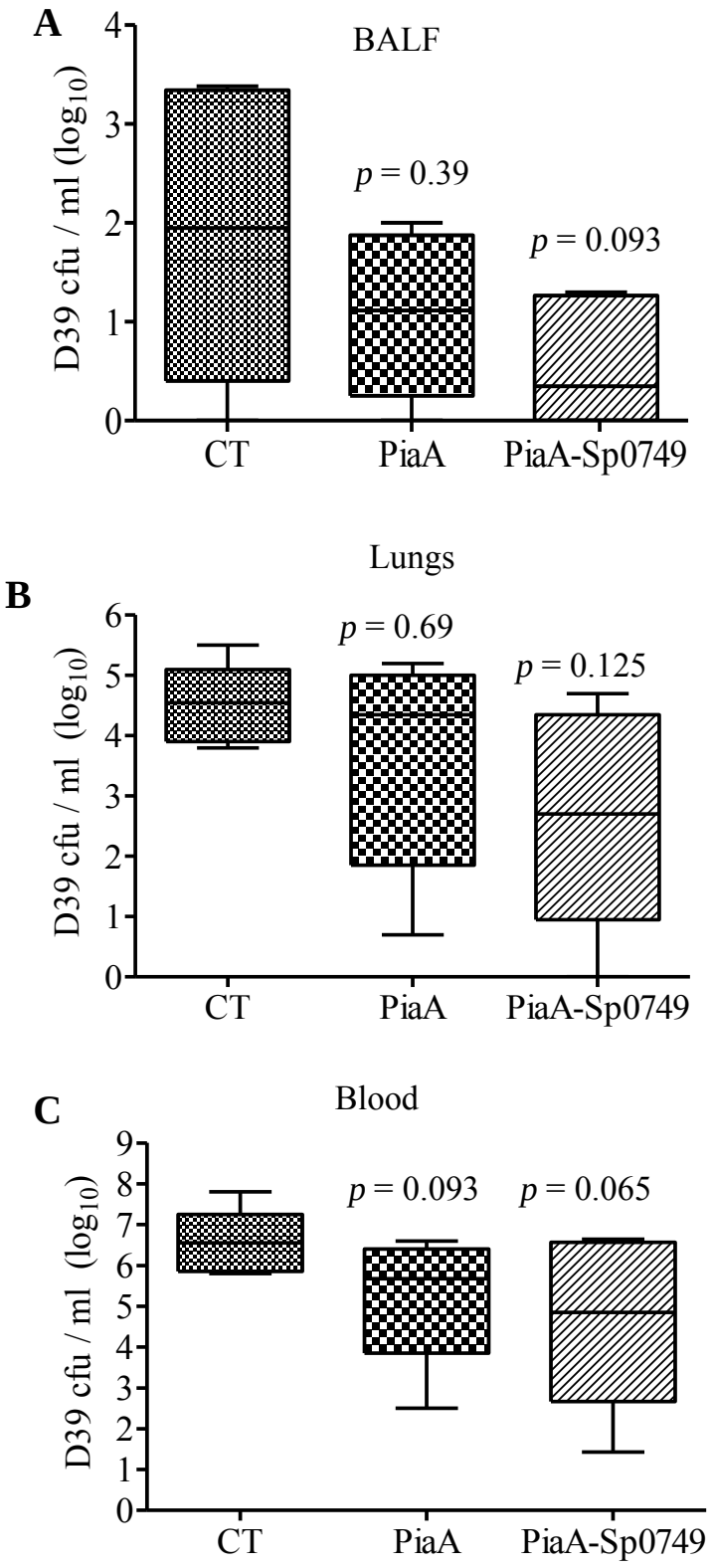


Fig 6.2 *S. pneumoniae* (D39) cfu determined from the target fluids and organs such as BALF (A), lungs (B) and blood (C) of IN immunised of CBA/Ca mice (using CT alone, CT + His_6-PiaA or CT + His_6-PiaA + His_6-Sp0749, n = 6 in each group) 48 hours after *S. pneumoniae* IN challenge with 10^5 cfu / mouse. *p*-value analysed by Mann-Whitney test.

	BALF Median (IQR)	Lungs Median (IQR)	Blood Median (IQR)
CT	1.2 (0.93-3.1)	4.5 (4.1-4.67)	6.6 (6.05-6.67)
His_6-PiaA	1.1 (0.57-1.09)	4.4 (3.3-4.7)	5.7 (5.2-6.16)
His_6-PiaA+ His_6-Sp0749	0.35 (0-1.09)	2.7 (1.9-4.0)	4.9 (3.9-6.3)

Table 6.1 Median and interquartile range (IQR) of *S. pneumoniae* (D39) cfu determined from BALF, lungs and blood after the IN immunisation of CBA/Ca mice with CT alone, CT + His_6-PiaA, CT + His_6-PiaA + His_6-Sp0749 (n = 6 in each group) followed by *S. pneumoniae* challenge 48 hours after the inoculation of 10^5 cfu / mouse.

target organs were then homogenised in sterile PBS, serially diluted and appropriate dilutions were plated to determine the cfu / ml in the target organs (Fig 6.2 and Table 6.1). Although CBA/Ca mice immunised intranasally with His_6-PiaA plus His_6-Sp0749 had reduced *S. pneumoniae* cfu in BALF (Fig 6.2 A), lung (Fig 6.2 B) and blood (Fig 6.2 C) compared to mice immunised with CT alone, and mice immunised with His_6-PiaA alone had reduced *S. pneumoniae* cfu in blood, these reductions failed to attain statistical significance. However, data were obtained from a relatively small number of mice and varied widely between mice in the same vaccine group.

6.4 CYTOSPINS OF BALF

BALF cellular content was analysed before and 48 hours after the *S. pneumoniae* challenge (D39) of the CBA/Ca mice immunised with CT alone, His_6-PiaA and His_6-PiaA plus His_6-Sp0749 proteins. Total cell counts in BALF were determined using a haemocytometer and microscopy of the cytospins was used to assess the proportion of macrophages, neutrophils and lymphocytes present as described in chapter 2. Total cell counts of BALF did not demonstrate significant differences before and after the *S. pneumoniae* challenge (Table 6.2). The BALF cytospin differential counts (Fig 6.3) demonstrated significantly higher neutrophil numbers post-challenge with *S. pneumoniae* in all three immunised groups (Fig 6.3 B), whereas the proportion of macrophages and lymphocytes were significantly reduced (Fig 6.3 A and B). Neutrophil counts were very low in all the three immunisation groups before challenge and therefore the data are not shown. A statistically significant increase in the neutrophil counts was obtained in the post-challenge mice immunised with both His_6-PiaA ($p = 0.0022$) and His_6-PiaA plus His_6-Sp0749 ($p = 0.0043$) when compared to pre-challenge groups.

	BALF Median (IQR)		Lungs Median (IQR)	
	Pre	Post	Pre	Post
CT	5.5 (5.2-5.7)	5.9 (5.7-6.1)	6.3 (6.3-7.09)	6.2 (6.0-6.6)
His_6-PiaA	5.5 (5.3-5.5)	5.8 (5.6-5.8)	6.0 (5.9-6.2)	5.9 (5.7-6.2)
His_6-PiaA+ His_6-Sp0749	5.5 (5.5-5.6)	5.6 (5.4-5.7)	6.6 (5.8-7.3)	5.9 (5.7-6.3)

Table 6.2 Median and IQRs of the total cell counts in BALF and lungs of CBA/Ca mice after the IN immunisation with CT alone, CT + His_6-PiaA and CT + His_6-PiaA + His_6-Sp0749 (n = 6) pre and post *S. pneumoniae* challenge (D39, 10^5 cfu / mouse).

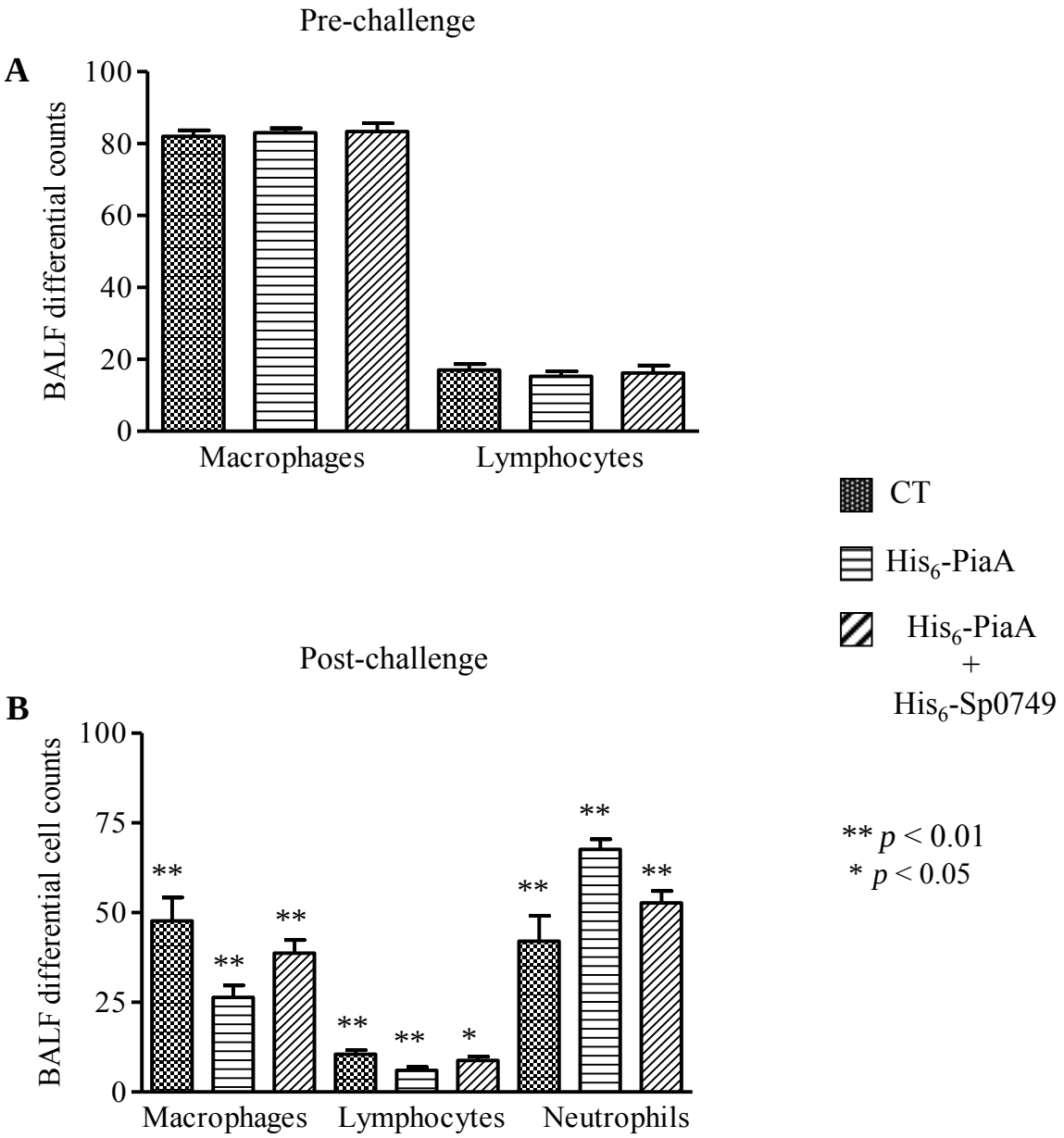


Fig 6.3 Differential cell counts of macrophages, lymphocytes and neutrophils present in the BALF of IN immunised CBA/Ca mice (n = 6 in each group) with CT alone, CT + His_6-PiaA, and CT + His_6-PiaA + His_6-Sp0749 pre (A) and post (B) *S. pneumoniae* challenge (D39, 10^5 cfu / mouse). *p* value for pre vs post-challenge comparisons analysed by Mann-Whitney test.

6.5 IMMUNE RESPONSE TO CT, HIS_6-PIAA AND HIS_6-PIAA PLUS HIS_6-SP0749 AFTER IN IMMUNISATION AND *S. PNEUMONIAE* CHALLENGE

To investigate the effects of immunisation of mice with CT, His_6-PiaA or His_6-PiaA plus His_6-Sp0749 on the host immune response, leukocytes were isolated from the BALF and lungs of CBA/Ca mice immediately before and 48 hours after challenge with *S. pneumoniae* as described in chapter 2. Total cell counts of the homogenised lung were determined using a haemocytometer, and cells incubated with fluorescently-labelled antibodies to lymphocytes and macrophage surface markers were analysed by flow cytometry to identify the proportion of cells belonging to each lymphocyte subgroup and the proportion of these cells that are activated (Brown *et al.*, 2002).

There were only small differences in lung cell counts before and after *S. pneumoniae* challenge between the vaccine groups (Table 6.2). The results of the cell surface marker experiments were highly variable between mice and it was difficult to identify clear trends. There were no clear differences in the proportions of CD4, CD8 and B cells between the vaccinated groups with the exception of activated CD4 and CD8 cells in the lungs (Fig 6.5 C, D).

In the BALF, the proportion of activated CD4 and CD8 (CD45RB –ve) cells (Fig 6.4 C, D) showed a statistically significant decrease in all the three post-challenge vaccinated groups compared to pre-challenge (for all groups $p < 0.01$ for the comparison of results pre and post-challenge of activated CD4 cells) (for all groups $p < 0.05$ for the comparison of results pre and post-challenge of activated CD8 cells).

In the lungs, there was a statistically significant increase in the proportion of

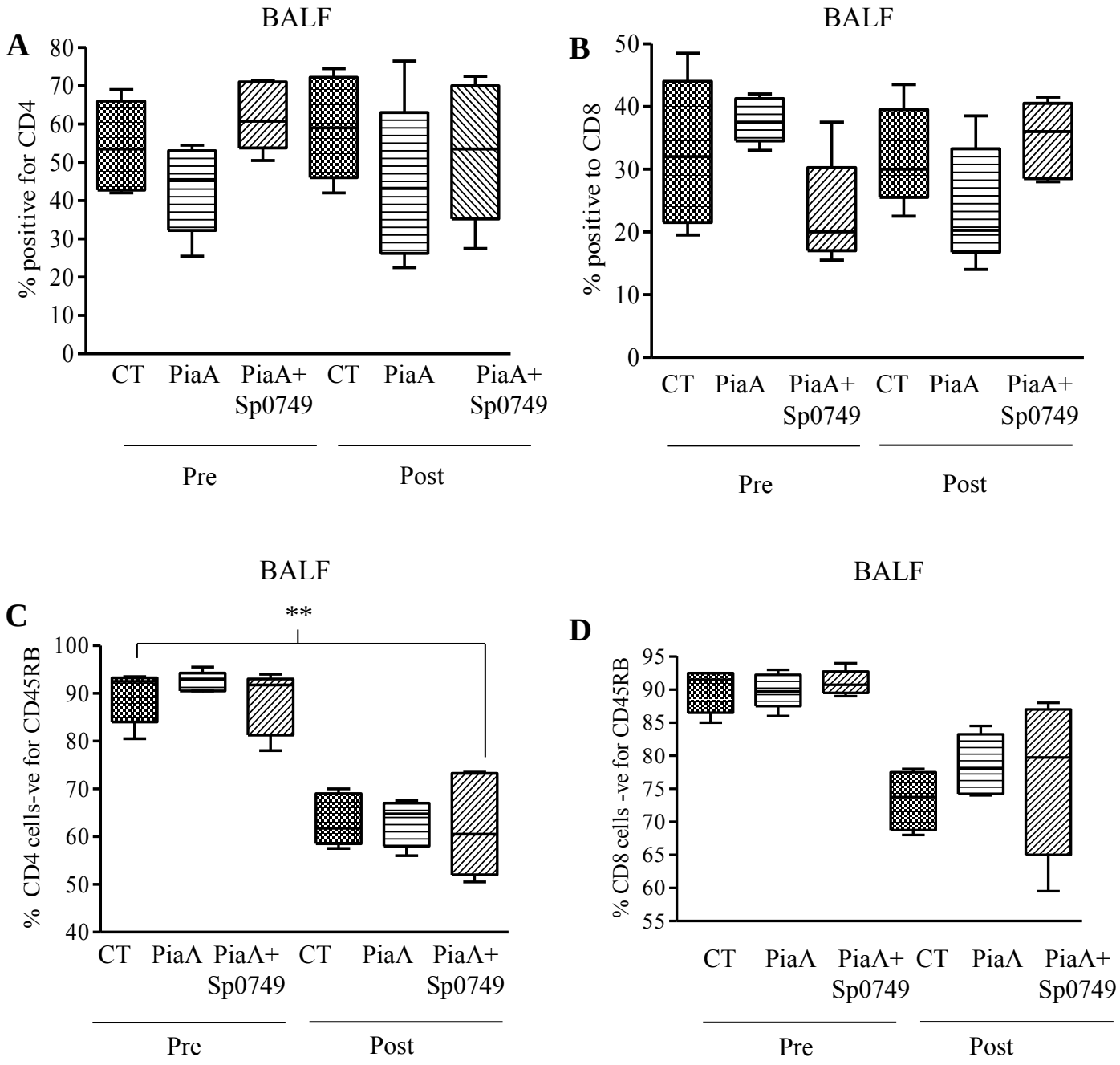


Fig 6.4 Flow cytometry analysis of the host's immune response in the BALF by immune cell surface staining following the IN immunisation (pre) and subsequent challenge (post). The proportion of (A) CD4 positive cells, (B) CD8 positive cells, (C) CD4 positive cells (CD45RB –ve) ($p < 0.01$ for comparisons pre vs post for all groups), (D) CD8 positve cells (CD45RB-ve) ($p < 0.05$ for comparisons pre vs post for all groups). p value were analysed by Kruskal-Wallis test

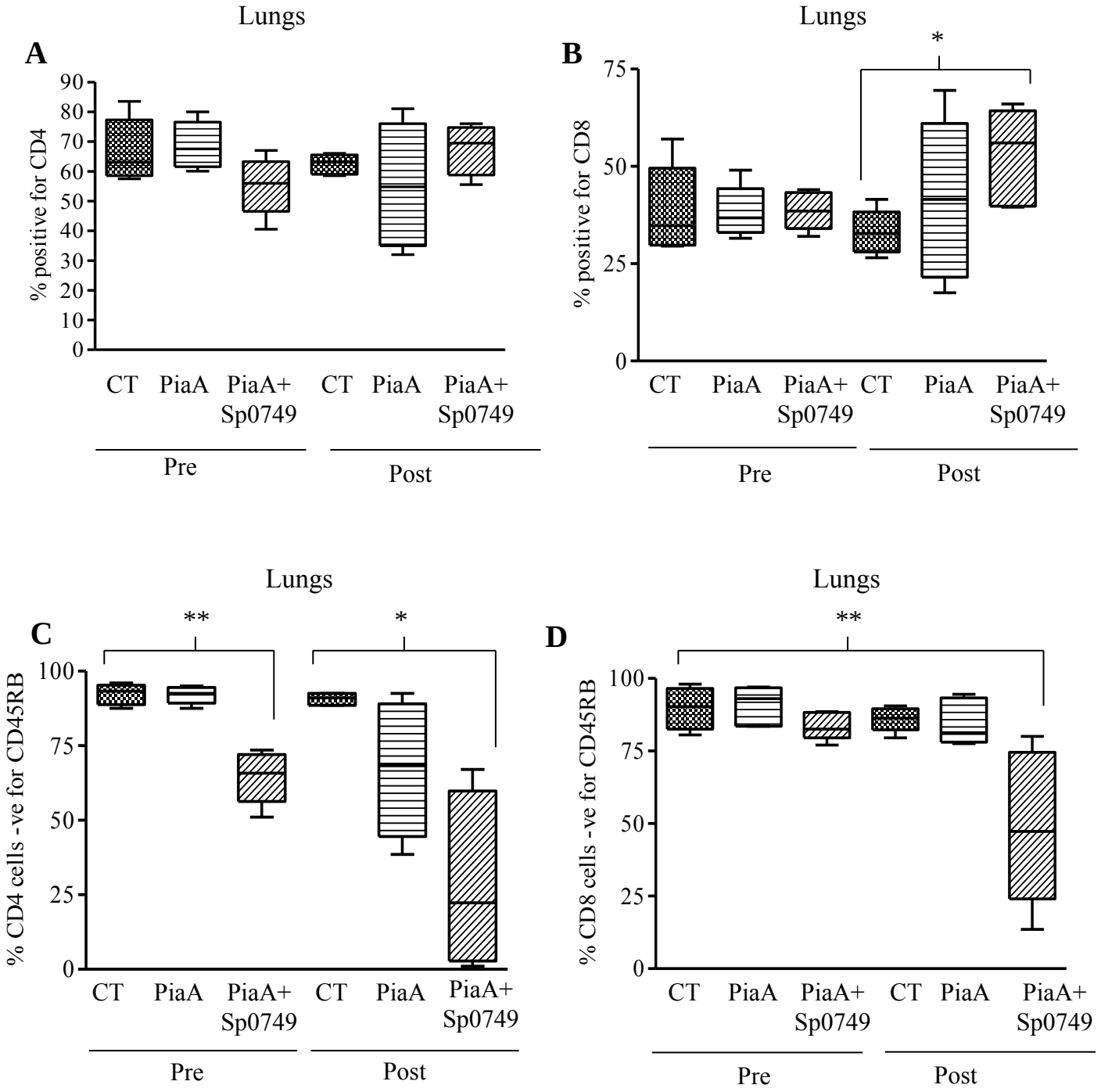


Fig 6.5 Flow cytometry analysis of the host's immune response in the lungs by immune cell surface staining following the IN immunisation and challenge. The proportion of (A) CD4 positive cells, (B) CD8 positive cells ($p < 0.05$ for comparisons pre Vs post for all groups), (C) CD4 positive cells (CD45RB –ve) ($p < 0.01$ for comparisons pre Vs post) and (D) CD8 positive cells (CD45RB -ve) ($p < 0.01$ for comparisons pre Vs post for all groups). p values were analysed using Kruskal-Wallis test

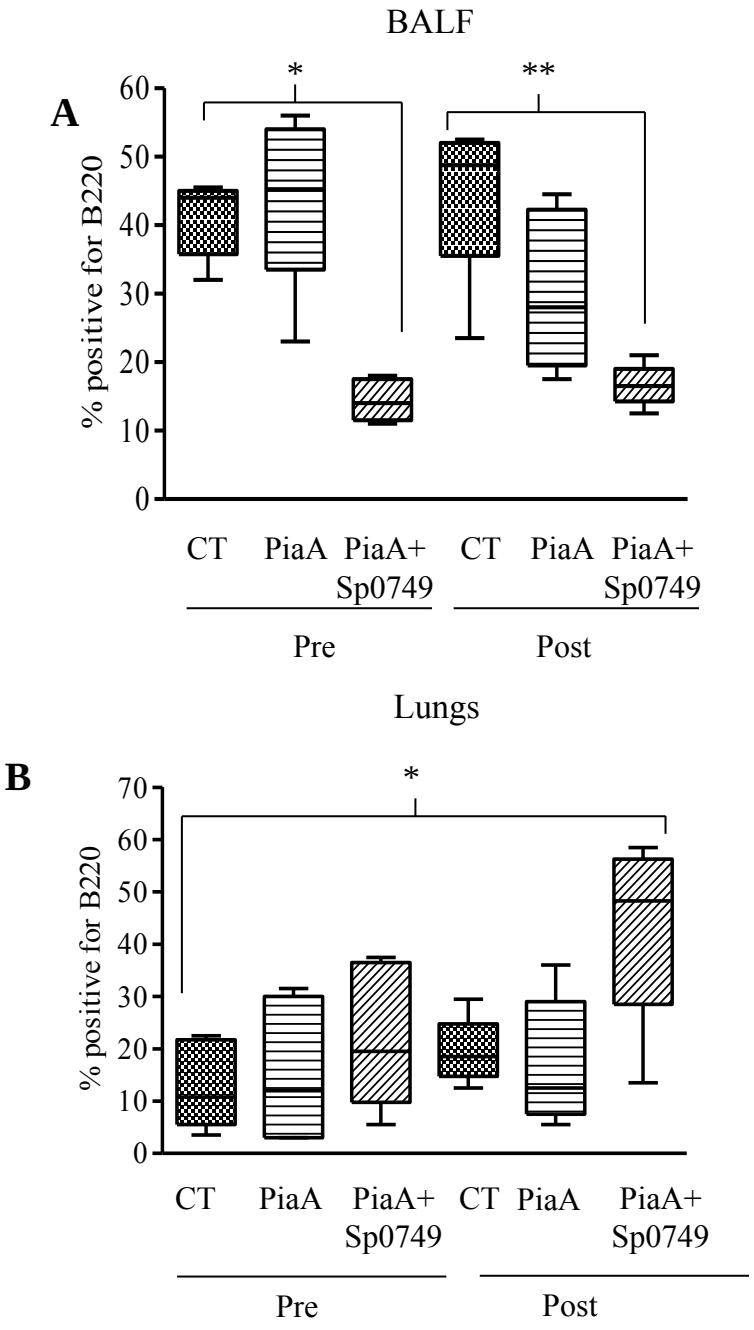


Fig 6.6 Flow cytometry analysis of the proportion of B220 positive cells in the (A) BALF and (B) lungs ($p < 0.05$ for comparisons pre vs post for all groups) by immune cell surface staining following the IN immunisation and challenge. *p* values analysed using Kruskal-Wallis test.

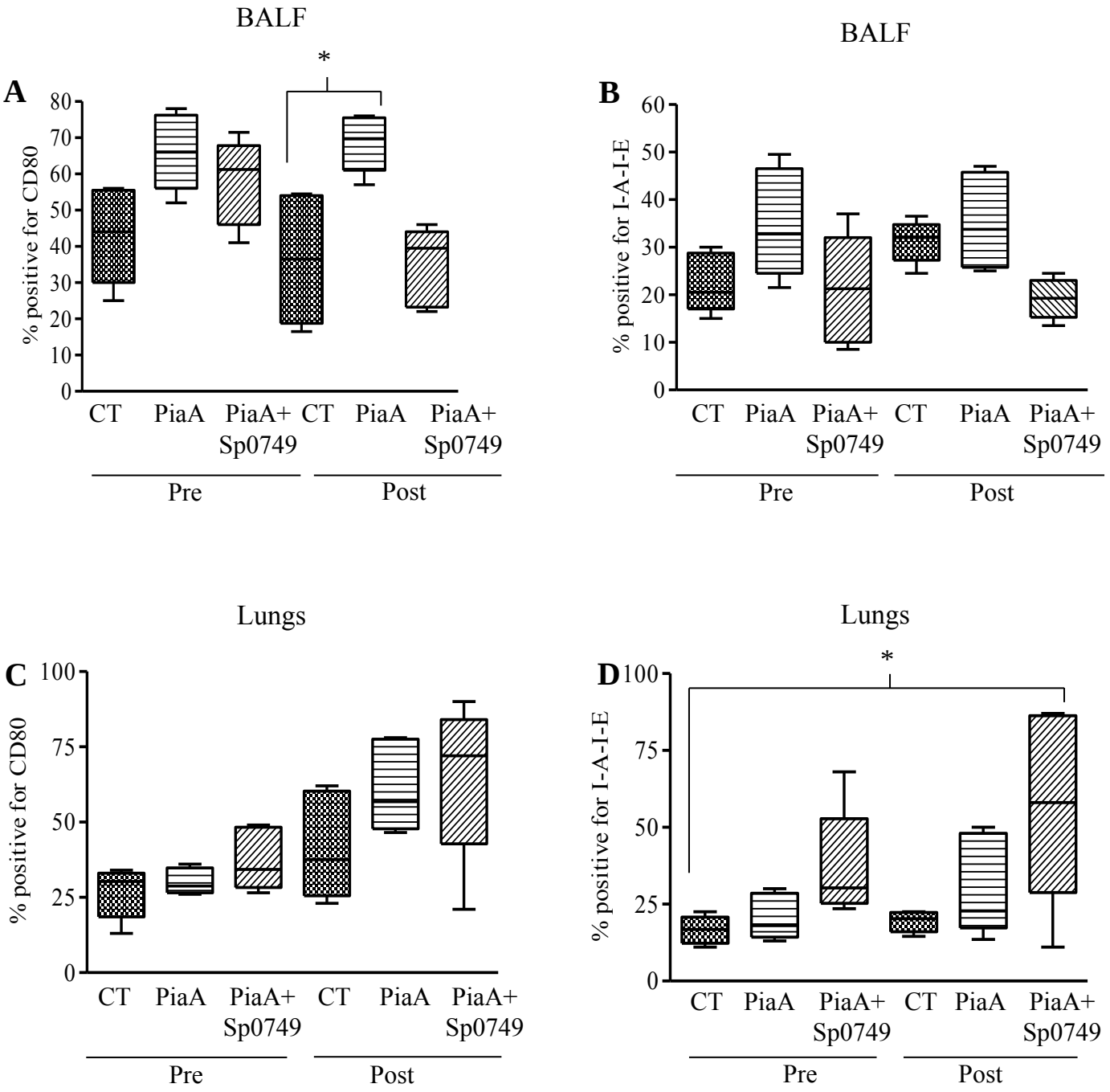


Fig 6.7 Flow cytometry analysis of the host's immune response in the BALF (A, B) and lungs (C, D) by immune cell surface staining following the IN immunisation and challenge. The proportion of CD80 positive for macrophages in the (A) BALF ($p < 0.05$ for comparisons pre vs post for all groups) and (C) lungs. The proportion of macrophages positive for MHC class II antigens (I-A-I-E) in the BALF (B) and lungs (D) ($p < 0.05$ for comparisons pre vs post for all groups). *p* values were analysed using Kruskal-Wallis test

CD8 positive lymphocytes in the His_6-PiaA plus His_6-Sp0749 compared to CT alone after *S. pneumonie* challenge (for all groups $p < 0.05$ for the comparison of results pre and post-challenge) (Fig 6.5 B). His_6-PiaA plus His_6-Sp0749 vaccinated group showed a statistically significant decrease in the proportions of CD4 and CD8 (CD45RB –ve) cells post-challenge compared to pre and post-CT and pre-PiaA (for all groups $p < 0.05$ for the comparison of results pre and post-challenge) (Fig 6.5 C, D). However, the proportion of B220 positive cells may be increased in the lungs of His_6-PiaA plus His_6-Sp0749 vaccinated group after *S. pneumoniae* challenge compared to pre-CT (for all groups $p < 0.05$ for the comparison of results pre and post-challenge) (Fig 6.6 B).

The activation of macrophages before and after the *S. pneumoniae* challenge of the immunised mice were analysed using antibodies to the cell surface marker CD80 and the MHC class II antigen I-A-I-E (Fig 6.7). In the BALF, the proportion of CD80 positive cells seemed to be similar in the CT group but decrease in the His_6-PiaA plus His_6-Sp0749 vaccinated groups upon *S. pneumoniae* challenge (Fig 6.7 A) (for all groups $p < 0.05$ for the comparison of results pre and post-challenge). In contrast in the lungs, the proportion of CD80 positive cells showed no statistically significant increase in the His_6-PiaA and His_6-PiaA plus His_6-Sp0749 vaccinated groups upon *S. pneumoniae* challenge compared to all the pre-challenge groups and post-CT (Fig 6.7 C). However, the BALF and lungs results using the (I-A-I-E) surface marker did not mirror the results for the CD80 cell surface marker (Fig 6.7 B, D). Therefore the significance of the CD80 marker results remain unconfirmed.

Overall, these results suggest that intranasal immunisation with His_6-PiaA and His_6-PiaA plus His_6-Sp0749 may affect components of the cellular immune response

to *S. pneumoniae* pneumonia. However these experiments need to be repeated to identify clear patterns.

6.6 HISTOLOGICAL ANALYSIS OF INFLAMMATION AFTER *S. PNEUMONIAE* CHALLENGE OF INTRANASALLY VACCINATED MICE

To investigate the degree of inflammation leading to lung injury of mice vaccinated IN with His_6-PiaA or His_6-PiaA plus His_6-Sp0749, lungs obtained from the pre and post, *S. pneumoniae* challenged mice (48 hours) underwent histological analysis. Each lung was scored as follows: 1 (visible inflammatory change), 2 (minimal swelling of alveolar walls and changes in architecture), 3 (increased swelling with presence of erythrocytes and inflammatory cells and an increase in type II pneumocytes), 4 (considerable haemorrhage with inflammatory cell influx, widespread alveolar disorganisation with interstitial swelling and pneumocyte proliferation**)** as described in chapter 2. A total score of the level of inflammation for each mouse was obtained by multiplying the percentage of involved lung by the mean score for the areas analysed, and data presented as medians with IQRs. Table 6.3 shows the levels of inflammation as medians and interquartile ranges of IN immunised CBA/Ca mice with His_6-PiaA or His_6-PiaA plus His_6-Sp0749 proteins followed by *S. pneumoniae* challenge. Histological analysis of lungs showed there was a statistically significant increase in the level of inflammation after *S. pneumoniae* challenge in mice vaccinated with His_6-PiaA or His_6-PiaA plus His_6-Sp0749 compared to those vaccinated with CT alone (Table 6.3). These data suggest that vaccination with these proteins may cause a more rapid inflammatory influx into lungs during subsequent *S. pneumoniae* challenge however these experiments needs to be repeated.

	Pre-challenge Median (IQR)	Post-challenge Median (IQR)
CT	11.25 (6.6-25.6)	84.2 (56.3-113.3)
His_6-PiaA	22.5 (16.6-35.6)	185.8 (147.9-375)
His_6-PiaA+ His_6-Sp0749	76.25 (45.4-94)	130.8 (103.3-300)

Table 6.3 Histological analysis of the level of inflammation in the lungs of CBA/Ca mice after the IN immunisation with CT alone, CT + PiaA and CT + PiaA + Sp0749 pre and post *S. pneumoniae* challenge (D39, 10^5 cfu / mouse). Degree of lung inflammation before and after the *S. pneumoniae* challenge is represented as medians and IQRs. *p* values were analysed using Kruskal-Wallis test (CT Vs PiaA or PiaA-Sp0749, $p < 0.001$ or $p < 0.01$ or $p < 0.05$) and Mann-Whitney test for all groups (Pre-challenge vs post-challenge, $p = 0.0022$).

6.7 SURVIVAL OF THE His_6-SP0149 AND His_6-SP0749 IMMUNISED MICE AFTER *S. PNEUMONIAE* CHALLENGE

In order to asses the efficacy of the purified His_6-Sp0149 and His_6-Sp0749 as intranasal vaccine candidates, the degree of protection provided by these two lipoproteins in the pulmonary models of infection was evaluated. Purified His_6-PiaA and His_6-PiuA lipoproteins were used as positive controls as both the lipoproteins have been investigated as vaccine candidates in the pulmonary and systemic models of infection in mice (Brown *et al.*, 2001; Jomaa *et al.*, 2006). Four intranasal immunisation experiments were performed using four different challenge doses of the virulent capsular strain of *S. pneumoniae* (D39 and 0100993) in naïve outbred CD1 mice and inbred CBA/Ca mice as described in chapter 2.

Two separate IN immunisations were performed in CD1 mice using 2.5µg of the purified His_6-PiaA, His_6-PiuA, His_6-Sp0749, His_6-PiaA plus His_6-Sp0749 and His_6-PiaA plus His_6-Sp0149 plus His_6-Sp0749 lipoproteins with CT as an adjuvant and CT alone as the negative control were injected intranasally followed by two booster doses at 7 days intervals. Two weeks after immunization, mice were challenged by intranasal injection of 10^6 cfu of the virulent capsular serotype 2 *S. pneumoniae* strain D39 and the progress of infection monitored over two weeks (Fig. 6.1 and 6.2). Fig 6.8 A and B represents pooled data of two separate experiments in which mice were immunised IN with His_6-PiaA and His_6-PiaA plus His_6-Sp0749 and D39 challenge experiments in CD1 mice strain (n = 10+10). IN immunisation with His_6-PiaA (p = 0.405) did not exhibit protection, although His_6-PiaA plus His_6-Sp0749 exhibited a statistically non-significant delay (p = 0.083) in the progression of *S. pneumoniae* infection compared to CT. However the immunisation with His_6-PiuA

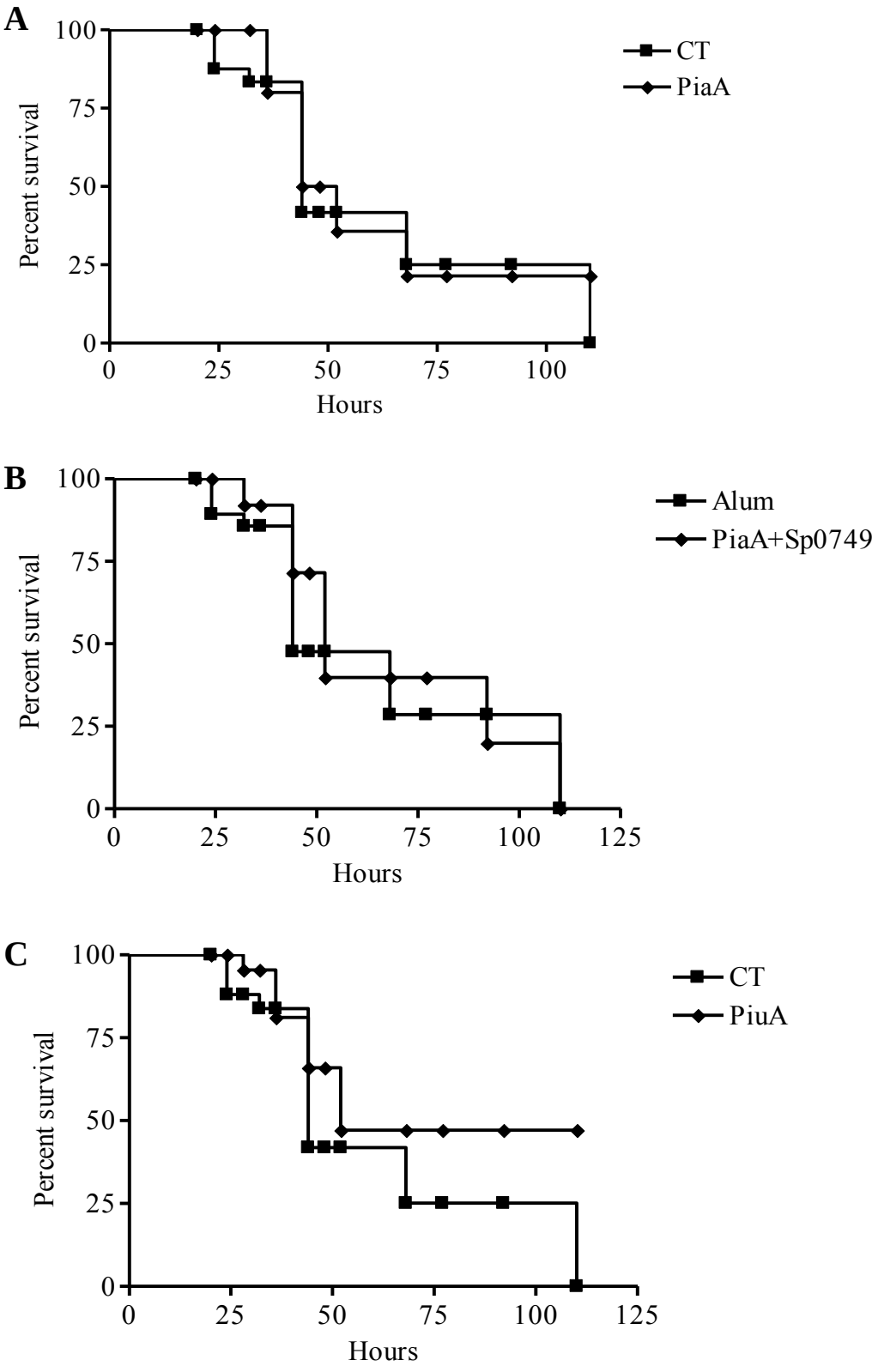


Fig 6.8 Survival curves after the intranasal (IN) immunisation of CD1 mice with cholera toxin CT (squares), PiaA, PiuA, Sp0749 and combinations of PiaA with Sp0749 (diamonds). IN immunisation of CD1 mice with CT alone, CT + PiaA (p = 0.405) (A), CT + PiaA + Sp0749 (p = 0.083) (B) and CT + PiuA (C) followed by IN challenge with *S. pneumoniae* D39 (10^6 cfu / mouse). A and B are the pooled results of two separate IN immunisation and challenge experiments (n = 10 + 10, n = number of mice) and p values are analysed by the log rank.

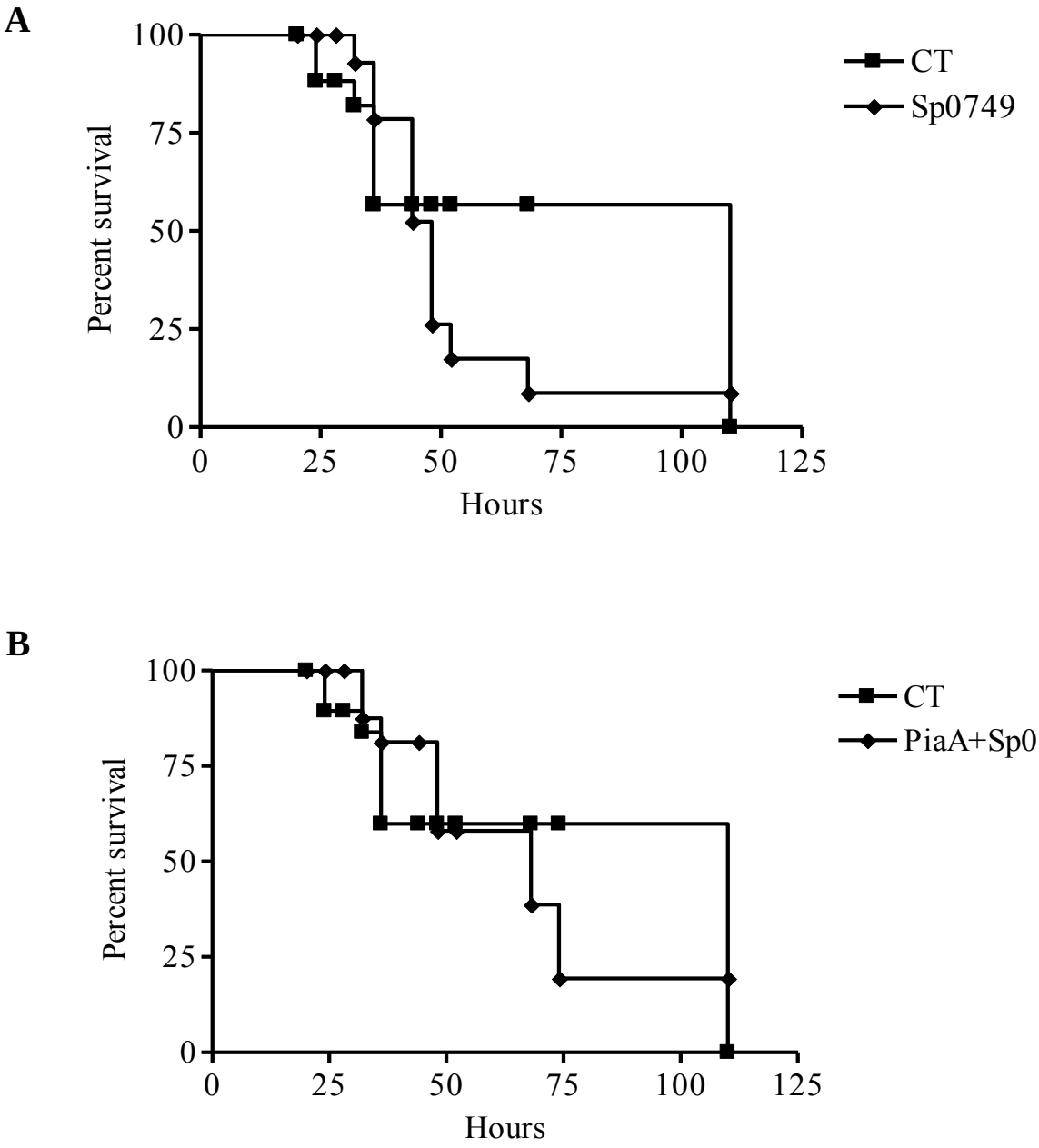


Fig 6.9 Survival curves after the IN immunisation of CD1 mice (n = 10) with CT (squares) Sp0749 and combinations of PiaA with Sp0149 and Sp0749 (diamonds). IN immunisation of CD1 mice with CT alone, CT + Sp0749 (A), CT + PiaA + Sp0149 + Sp0749 (B) followed by IN challenge with *S. pneumoniae* D39 (10^6 cfu / mouse). *p* values are analysed by the log rank test.

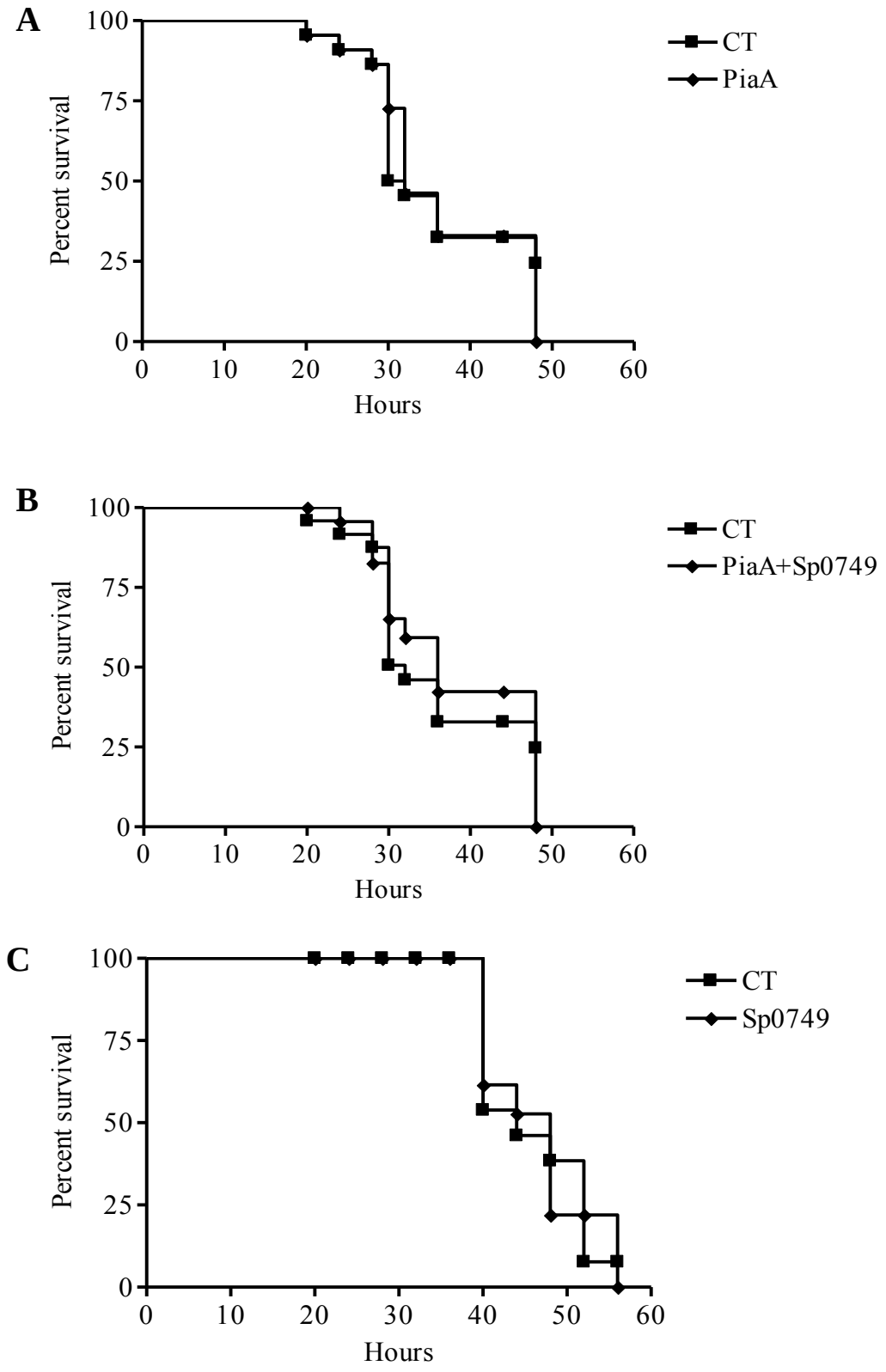


Fig 6.10 Survival curves after the IN immunisation of CBA/Ca mice (n = 15) with CT (squares), PiaA, Sp0749 and combinations of PiaA with Sp0749 (diamonds). IN immunisation of CBA/Ca mice with CT alone, CT + PiaA (A), CT + PiaA + Sp0749 (B) followed by IN challenge with *S. pneumoniae* D39 (10^5 cfu / mouse). IN immunisation of CBA/Ca mice (n = 20) with CT alone, CT + Sp0749 (C) followed by IN challenge with *S. pneumoniae* 00100993 strain (10^5 cfu / mouse). *p* values are anaysed by the log rank test.

(Fig 6.8 C), His_6-Sp0749 (Fig 6.9 A), and His_6-PiaA plus His_6-Sp0149 plus His_6-Sp0749 (Fig 6.9 B) lipoproteins in CD1 mice strain (n = 10) did not show any protection compared to CT alone.

The third intranasal immunisation experiment was performed in the inbred CBA/Ca mouse strain .Groups of CBA/Ca mice (n = 15) were immunised with CT alone, His_6-PiaA, His_6-PiaA plus His_6-Sp0749 followed by intranasal challenge with 10^5 cfu of *S. pneumoniae* D39. Mice immunised with His_6-PiaA, His_6-PiaA plus His_6-Sp0749 showed only delayed progression of disease after *S. pneumoniae* challenge (Fig 6.10 A and B). The fourth intranasal immunisation experiment was also performed in CBA/Ca mice strain with groups of mice (n = 20) immunised with CT alone, His_6-Sp0749 followed by intranasal challenge with 10^5 cfu of *S. pneumoniae* 0100993 strain. Fig 6.10 C shows that His_6-Sp0749 failed to exhibit any protection and that the survival curve of His_6-Sp0749 was almost similar to mice immunised with CT alone.

Overall these data suggest there may be some protective effect in these models after the combined immunisation with PiaA and Sp0749 lipoproteins, but any protective effect of individual lipoproteins was too weak to be detected by the infection models used for these experiments.

6.8 SUMMARY

PiaA and PiuA his-tagged lipoproteins were expressed and purified in order to use as positive controls for the intranasal immunisation studies. Following intranasal immunisation with PiaA and Sp0749, good IgG titres were demonstrated in sera obtained from CD1 mice and BALF obtained from CBA/Ca mice. However, anti-PiaA IgA titres in BALF were variable and no detectable anti-Sp0749 IgA titres were

found. When the *S. pneumoniae* load was determined from the target organs such as BALF, lungs and blood 48 hours after challenge of vaccinated mice with *S. pneumoniae*, statistically non-significant reductions in *S. pneumoniae* cfu were demonstrated in the BALF, lungs and blood of mice vaccinated with PiaA plus Sp0749 compared to CT group. Mice immunised with PiaA alone showed non-significant reductions of *S. pneumoniae* cfu in blood only compared to CT group. The differential cell counts in the BALF of the CT, PiaA and the PiaA plus Sp0749 immunised mice demonstrated a statistically significant increase in the neutrophil infiltration in PiaA vaccinated mice upon challenge with *S. pneumoniae* compared to CT alone**.** Flow cytometry analysis of the lymphocytes in the BALF and lung homogenate of the immunised CBA/Ca mice before and after the *S. pneumoniae* challenge by cell surface marker staining was performed. Flow cytometry analysis after immune cell surface staining did not indicate a particular trend and the results were highly variable between mice. Intranasal immunisation demonstrated that PiaA in combination with Sp0749 only slightly delayed the progression of lethal *S. pneumoniae* infection. In summary, although vaccination with PiaA and Sp0749 causes evidence of an altered immune response to subsequent pneumonic infection, the protective effects of this seems limited.

Chapter 7
Discussion

There has been strong interest in *S. pneumoniae* proteins as vaccine candidates because they offer the potential to overcome the limitations of the currently available *S. pneumoniae* vaccines based on capsular polysaccharide antigens. Important features for a protein to qualify as a potential *S. pneumoniae* vaccine candidate are surface exposure, immunogenicity, conservation amongst a range of clinically important serotypes and the ability to elicit a protective immune response. A role for virulence would be beneficial as this would suggest that the protein candidate is expressed during systemic infection. The antibodies elicited against the protein in the host may inhibit the function of the protein thereby affecting the *S. pneumoniae* virulence. In addition, an important role in virulence means that the expression of the protein is necessary for the survival of *S. pneumoniae* in the host to cause disease. Genes encoding such proteins which are necessary for the *S. pneumoniae* survival in the host rarely undergo mutations. Therefore use of such proteins as vaccine antigens induces immunity to help prevent immune evasion of *S. pneumoniae*. Simultaneous immunisation with several *S. pneumoniae* surface proteins has been shown to protect mouse models against *S. pneumoniae* pulmonary and systemic infections (Briles *et al.*, 1997; Briles *et al.*, 1996; Brown *et al.*, 2001; Briles *et al.*, 2003; Ogunniyi *et al.*, 2007) and a vaccine containing several protein antigens is likely to offer advantages in efficiency and greater serotype coverage.

One group of surface proteins which are important for the survival of *S. pneumoniae* in the host, thereby playing important role as virulence determinants, are

ABC transporters (Unsworth and Holden, 2000) (Garmory and Titball, 2004) (Benton *et al.*, 2004). Bioinformatic analysis of the genome of S. *pnemoniae* has identified as many as 73 ABC transporters (Harland *et al.*, 2005), many of which are predicted to be involved in nutrient acquisition from the host (Bergmann and Hammerschmidt, 2006). Several *S. pneumoniae* ABC transporters are known to be important for virulence such as PiaA, PiuA (Brown *et al.*, 2004) and PsaA (Dintilhac *et al.*, 1997), but the role of many *S. pneumoniae* ABC transporters during virulence has yet to be investigated. I have therefore chosen 11 ABC transporters from the annotated completely sequenced genome of capsular serotype 4 of *S. pneumoniae* (TIGR4) that have previously not been characterised and investigated their role in virulence and selected two of these for assessment as potential vaccine candidates. The *S. pneumoniae* ABC transporters chosen for these studies were the majority of the remaining 18 uncharacterised ABC transporters that are organised as operons of three or more genes containing at least two of the three main components of ABC transporters (the lipoprotein, ATPase and transmembrane permeases). Disruption of these ABC transporters was thought to be more likely to give a strong phenotype as most or all of the components of that ABC transporter would be co-transcribed. Components of some of the ABC transporters chosen for this study (Sp0092, Sp0150, Sp2086 and Sp2108) have been identified as potential virulence factors by STM screening (Hava and Camilli, 2002; Lau *et al.*, 2001). Mutant strains were constructed by disruption of the predicted first gene of the operon using IDM to try and ensure the disruption of transcription of the whole operon and therefore the generation of a stronger phenotype. Nine mutant strains of the chosen 11 ABC transporter operons of *S. pneumoniae* were successfully constructed by IDM. However I failed to obtain any mutant strain for the putative amino acid transporter encoded by Sp0707-0711 even

after repeated attempts, possibly because some regions of the genome may be resistant to transformation or because that disruption of this gene may prove to be lethal for the bacterium. In another mutant strain, *Sp2084*$^{-}$, the putative phosphate transporter encoded by Sp2084-2087 had an unstable mutation, a relatively common problem for mutant strains made by IDM due to the duplicated regions flanking the inverted plasmid allowing homologous recombination to excise the plasmid containing the gene for antibiotic selection.

BLAST searches indicated that several of the chosen ABC transporters are likely to be sugar ABC transporters (Sp0090-92, Sp0846-48, Sp1796-98, and Sp2108-10), which is not surprising given the large range of sugars that *S. pneumoniae* is thought to be able to utilise (Tettelin *et al*., 2001). BLAST searches also indicated that Sp0607-10, Sp0707-11 and Sp0749-53 are putative amino acid ABC transporters. Sp0749-53 was identified a putative branched-chain amino acid (BCAA) transporter. The *S. pneumoniae* genome also contains genes encoding enzymes required for BCAA synthesis (Sp0445-50) (Hendriksen *et al.*, 2008) which may partially compensate for BCAA uptake by *S. pneumoniae*. Sp0149 was identified as a putative cation lipoprotein, but other genes of this operon did not correlate with the likely function of cation uptake. However another transport classification system (Saier, Jr., 2000) indicated Sp0148-52 as a putative methionine ABC transporter. A similar discrepancy between the BLAST alignment and the transport classification system was also observed when predicting the possible substrate for Sp0846-48. BLAST alignment predicted Sp0846-48 as a putative sugar transporter, whereas the transport classification system predicted it is a possible nucleoside ABC transporter. Detailed characterisation of these ABC transporters is needed to confirm their ligand binding properties.

Many ABC transporters seem to be highly conserved amongst different *S. pneumoniae* strains (eg PiaA, PsaA and PiuA) (Whalan *et al.*, 2006), (Sampson *et al.*, 1997). BLAST alignment of the predicted lipoprotein genes of the chosen ABC transporters to 18 other available *S. pneumoniae* genomes demonstrated a high degree of amino acid conservation between *S. pneumoniae* strains (Basavanna *et al.*, 2009). BLAST alignment of the chosen ABC transporters showed that 8 of the eleven ABC transporters have the greatest degree of amino acid homology to various streptococci (Sp0090-92, Sp0148-52, Sp0607-10, Sp0707-11, Sp0749-53, Sp0846-48, Sp1796-98 and Sp2108-10). However no close homologues amongst streptococci were found for Sp1688-90 (a putative cation ABC transporter), Sp2084-87 (a putative sugar transporter), or Sp1824-26 (a putative phosphate ABC transporter). Previously, the iron uptake ABC transporter PiaA was shown to be highly conserved in *S. pneumoniae* strains but absent in oral streptococci, including the *S. mitis* group (Whalan *et al.*, 2006)**,** suggesting horizontal gene transfer of this loci into *S. pneumoniae* from unrelated bacterial species has occurred, and a similar mechanism might explain why Sp1688-90, Sp2084-87 and Sp1824-26 are not found amongst other streptococci.

The genetic organisation of all the 9 ABC transporters were analysed using TIGR4 genome, and their organisation investigated by RT-PCR using 0100993 strain of serotype 3 RNA. The RT-PCR analysis using 0100993 strain showed that the probable transcriptional structure of the Sp0149-152, Sp0749-53, Sp0846-48, Sp2108-10 loci correlated with the TIGR4 genome. Amplification of cDNA from the RNA of 0100993 *S. pneumoniae* grown in THY for these genes was straightforward suggesting these regions are significantly expressed during growth in THY. However Sp1794-99 and Sp1822-28 ABC transporter loci were poorly expressed in THY and

therefore it was difficult to assess the transcriptional organisation of these operons using RT-PCR. The transcriptional structure of other ABC transporter loci did not match well with what would be predicted from the TIGR4 genome (Sp0090-92, Sp0607-11, Sp1689-91), and sequencing of these regions in the 0100993 *S. pneumoniae* strain may help clarify these results.

PCR amplification of the junctions of Sp1794-99 and Sp1822-28 ABC transporter loci using 0100993 genomic DNA as template showed that some genes of these ABC transporter loci may be absent. As discussed earlier, the BLAST alignment of Sp1794-99 ABC transporter loci against 18 available *S. pneumoniae* genomes showed that this region was absent in some of the *S. pneumoniae* strains (Basavanna *et al.*, 2009). However why some genes of Sp1822-28 ABC transporter loci were absent in 0100993 strain is not clear. I therefore speculate whether the expression of Sp1822-28 is induced under certain conditions such as oxidative, osmotic stress, or a minimal nutrient supply and that the genes of this region may not be constitutively expressed in THY.

Which transcriptional regulators regulate these *S. pneumoniae* ABC transporters warrants further investigation as the information is very limited at present. In other bacteria such as *S. mutans* and *S. agalactiae*, genes of methionine uptake are under the control of the transcriptional regulator MetR / MtaR respectively (Sperandio *et al.*, 2007) (Shelver *et al.*, 2003), and whether Sp0149-152 is regulated in a similar fashion needs to be identified. However, Sp0749-73 and the BCAA synthesis operon Sp0445-50 have been shown to be negatively regulated by CodY (Hendriksen *et al.*, 2008), although the relationship between CodY repression of Sp0749-53 requires further investigation to clarify its functional consequences and role during bacterial physiology.

The phenotype of each of the mutant strains was analysed using *in vitro* and *in vivo* CIs. CI tests the ability of the mutant strain to replicate compared to the wild-type parental strain in the test condition (as long as the mutant phenotype cannot be complemented by the extracellular secretion of the missing protein(s) by the wild-type strain). *In vitro* CIs performed in a rich complete medium (THY) showed no growth defects of the mutant strains except a mild impairment for *Sp0750*$^{-}$. Similarly, all the mutant strains grew well in the osmotic stress media (THY + 100 mM NaCl) except strain *Sp0610*$^{-}$ which had partial attenuation thereby suggesting a role of this ABC transporter for aiding growth in high osmotic stress conditions. Osmotic stress is one physiological stress that pathogens have to overcome in order to cause invasive infection, and decreased resistance to osmotic stress has been associated with decreased virulence for some pathogens. For example, expression of the three-gene operon of *S. pneumoniae*, *phgABC,* has been reported to be important for growth during high osmotic conditions and is required for the full virulence in pulmonary and systemic infection (Brown *et al.*, 2004), and a proline ABC transporter of *S. aureus* is important for the virulence of *S. aureus* and protects against high osmolarity by accumulating proline within the cell (Schwan *et al.*, 2004).

To indicate a potential role of the selected *S. pneumoniae* ABC transporters during invasive infections, CIs were performed on human blood as an *ex vivo* culture system that closely represents at least some of the physiological conditions during infection such as limited availability of nutrients, pH, and relative hyperosmolarity. Culture on blood also replicates some of the interaction with different cell types and immune system components such as neutrophils and complement that occur during infection (Mereghetti *et al.*, 2008). In this more stringent environment, mutant strains *Sp0090*$^{-}$, *Sp0149*$^{-}$, *Sp0610*$^{-}$, *Sp0750*$^{-}$, *Sp1824*$^{-}$ were moderately impaired in growth

compared to the wild-type, suggesting that these ABC transporters may contribute to growth of *S. pneumoniae* in normal physiological fluid. These strains would therefore be predicted to have impaired virulence and in general, the CIs in human blood mirrored the *in vivo* CIs in mouse models of pneumonia and septicaemia. Unlike the *in vitro* CIs, where the mixed inocula of wild-type and the mutant strains were grown for a period of 8 hours in various growth conditions, the *in vivo* CIs performed in the mouse models of infection results in the interaction of the mutant and the wild-type strains in various cells, tissues and body compartments during a period of 24-48 hours. This allows even small differences in virulence between mutant and wild-type strains to be identified. Of the nine ABC transporter mutant strains successfully constructed, seven were impaired in full virulence in mouse models of sepsis and / or pneumonia (*Sp0090*$^{-}$*, Sp0149*$^{-}$*, Sp0610*$^{-}$*, Sp0750*$^{-}$*, Sp0846*$^{-}$*, Sp1824*$^{-}$ and *Sp2108*$^{-}$). Of the seven attenuated ABC transporter mutant strains, five were partially reduced in virulence (*Sp0090*$^{-}$*, Sp0610*$^{-}$*, Sp0846*$^{-}$*, Sp1824*$^{-}$ and *Sp2108*$^{-}$) and two were markedly attenuated (*Sp0149*$^{-}$ and *Sp0750*$^{-}$) in mouse models of sepsis and / or pneumonia. For two of the ABC transporter mutant strains (*Sp1690*$^{-}$*, Sp1796*$^{-}$) the impairment in virulence was relatively small, comparable to the effect of loss of a single iron transporter (Brown *et al.*, 2002), and this maybe because their functions are partially redundant. For example, BLAST alignments suggest several of the ABC transporters investigated encode sugar transporters, and more than 30% of *S. pneumoniae* transporters are predicted to be sugar transporters which also includes non-ABC transporter uptake systems such as phosphoenolpyruvate-dependent sugar transporters (Tettelin *et al.*, 2001). Hence disrupting the function of a single sugar ABC transporter could be compensated for by the others or by non-ABC transporter uptake mechanisms such as phosphoenolpyruvate-dependent sugar transporters (Tettelin *et*

al., 2001). Dual mutations in genes encoding components of ABC transporters with related functions may have a much more marked effect on virulence as has been shown for the PiuA and PiaA iron transporters (Brown *et al.*, 2001). Two strains *Sp0149*$^{-}$ and *Sp0750*$^{-}$ were markedly more attenuated in virulence in pneumonia and septicaemia models of *S. pneumoniae* infection compared to the other strains. The functions of proteins encoded by these genes are indicated by BLAST searches to be methionine / cation and BCAA uptake respectively. *Sp0750*$^{-}$ had greater degree of attenuation of virulence in pneumonia model than in the septicaemia model of *S. pneumoniae* infection suggesting that this ABC transporter may be particularly important for the infection of the respiratory tract. As well as ABC transporters encoded for by groups of genes investigated in this and previous studies, there are many additional ABC transporters components encoded by isolated single genes or gene pairs within the TIGR4 genome, some of which STM screens suggest affect virulence (Hava and Camilli, 2002; Lau *et al.*, 2001). These ABC transporter components also warrant further investigation, although their specific putative functions may be in general be even less apparent than those encoded by several adjacent genes in putative operons.

S. pneumoniae disease process is complex and requires the bacterium to acclimatise in different environments such as nasopharynx, lungs, blood, ear and brain. The optimal survival of *S. pneumoniae* in any of these niches would depend on the expression of virulence factors (Ogunniyi *et al.*, 2002). Since Sp0149 and Sp0749 ABC transporters have been shown to be important for *S. pneumoniae* virulence as demonstrated by *in vivo* CIs, their relative abundance in different conditions were investigated. The relative expression of Sp0149 and Sp0749 mRNA, were compared with the well-characterised virulence protein genes, *psaA* and Sp1386 (potD,

polyamine ABC transporter) (Shah *et al.*, 2008) in the THY, human blood and mice blood 12 hours after IP infection with *S. pneumoniae*. Relative mRNA expression of the virulence genes (Sp0149, Sp0749, *psaA* and Sp1386) in THY were used as baseline. *psaA* was abundantly present in the THY and human blood, therefore I did not investigate the relative abundance of *psaA* gene in mouse blood due to limited recovery of total *S. pneumoniae* RNA. Also similar work has been performed by Ogunniyi *et al* (Ogunniyi *et al.*, 2002) and they have demonstrated that *psaA* is expressed abundantly during *in vitro* conditions and during pulmonary and systemic *S. pneumoniae* infection in mice. Sp1386, a lipoprotein component of a polyamine ABC transporter demonstrated lower expression than *psaA* and Sp0149 in THY, but increased mRNA expression was demonstrated in human blood and in the blood of mice recovered 12 hours after IP inoculation of *S. pneumoniae* suggesting a role in this physiological fluid and thereby contributing to *S. pneumoniae* virulence. Shah *et al* have also demonstrated that the expression of the *S. pneumoniae* polyamine ABC transporter is up-regulated during oxidative stress and high temperature (Shah *et al.*, 2008) such results that are compatible with the lower expression of Sp1386 in favourable growth conditions such as THY.

In the mouse blood, the relative abundance of Sp0149 was increased compared to THY and human blood. However, Sp0749 was only expressed in mouse blood and no detectable levels were found in THY and human blood. Previous work by Carlos Orihuela and collegues has shown that the Sp0149 lipoprotein gene and Sp0750-53 operon are both up-regulated in a rabbit model of meningitis suggesting their gene expression may contribute towards *S. pneumoniae* virulence (Orihuela *et al.*, 2004). The genes of Sp0749-53 are under the negative control of the transcriptional regulator CodY (Hendriksen *et al.*, 2008) but the relationship between CodY repression, *in*

vitro and *in vivo* expression of Sp0749-53 and BCAA transport requires further investigation. There is also a need to repeat the experiment using more sensitive technique such as real-time PCR, which would allow more accurate quantification of the differential expression of these ABC transporter genes in both *in vitro* and *in vivo* (systemic and pulmonary murine models).

The subcellular localisation of the lipoproteins Sp0149 and Sp0749 was investigated using Triton X-114 extracts of the *S. pneumoniae* membrane associated proteins. Immunoblots identified that Sp0149 and Sp0749 are membrane localised. Reports for other Gram positive bacteria such as *Mycobacterium tuberculosis* (Sander *et al.*, 2004) *Listeria monocytogenes* (Reglier-Poupet *et al.*, 2003) and *S. suis* (De *et al.*, 2003) have shown that their lipoproteins are processed by Lsp, and that disruption of lsp in *L. monocytogenes* (Reglier-Poupet *et al.*, 2003), *S. suis* (De *et al.*, 2003) and recently by my supervisor's group in *S. pneumoniae* (Khandavilli *et al* 2008) results in retention of the N terminal signal peptide by lipopeptides. Hence I investigated the effects of deletion of *lsp* on the Sp0149 and Sp0749 lipoproteins. As expected, the Sp0149 gave a higher molecular weight signal in the whole cell lysate of *Δlsp* strain due to incomplete processing of the lipoproteins in the absence of Lsp. However in the whole cell lysate of lsp^c strain, the Sp0149 did not revert to the normal wild-type molecular weight. Whether the differences of Sp0149 is due to the rate of migration of proteins or due to incomplete Lsp function in the complementation mutant as described by Khandavilli and collegues (Khandavilli *et al* 2008) needs to be investigated. With respect to Sp0749 there was a weak signal in the whole cell lysate of the *Δlsp* strain, but the intensity of expression was similar in the wild-type and lsp^c strain. At present there is no clear explanation for why there is lower expression of Sp0749 in the absence of Lsp. However, the localisation of Sp0149 and Sp0749

within the membrane and that they are affected by Lsp does indicate that both are lipoproteins and could be investigated as potential vaccine candidates against *S. pneumoniae*. Analysis of the effect of loss of *lgt* on the processing of Sp0149 and Sp0749 would also be of interest as this would be predicted to result in loss of both lipoproteins from the cell membrane associated protein fraction.

The data obtained with the disruption mutant strains suggested that the ABC transporters encoded by Sp0149-52 and Sp0749-53 have the most crucial roles during *S. pneumoniae* infection. For further investigation of the function of the Sp0149-52 and Sp0749-53 ABC transporters, mutant strains were made in the *S. pneumoniae* 0100993 background in which Sp0149 and Sp0750-53 were deleted from Sp0149-52 and Sp0749-53 operons using constructs made by overlap extension PCR (OEP) (Shevchuk *et al.*, 2004). OEP is a relatively new technique for constructing deletion and complemented mutant stains to allow complete replacement of an operon with an antibiotic resistance cassette. Different techniques have also been used in recent years to construct mutations in *S. pneumoniae* such as IDM, *in vitro* mariner transposon mutagenesis, and gene replacement using Janus cassette and OEP. IDM however has been used to for many years to make disruption mutants in *S. pneumoniae* using plasmids as it is rapid and a relatively simple technique. However, due to homologous recombination of duplicated regions flanking the inverted plasmid, the entire plasmid may be excised allowing the plasmid containing the gene for antibiotic selection to be excised and this is a relatively common problem with IDM. The advantages of OEP are that it is a relatively quick method to obtain the desired constructs for mutations and does not involve plasmids and restriction digestion. However the main disadvantage of OEP is the induction of random mutation due to amplification by PCR into the mutant construct. Two other relatively new methods to construct *S.*

pneumoniae mutants are *in vitro* mariner transposon mutagenesis and gene replacement using Janus cassettes. *In vitro* mariner transposon mutagenesis is rapid and is particularly useful for the identification of genes required for growth and survival, but causes random insertions into the target sequence rather than a clean deletion (Akerley *et al.*, 1998). Gene replacement using Janus cassette is a method for negative selection particularly useful to construct substitutions and inframe deletions which allows the selection for gene acquisition and loss. Therefore use of Janus cassette circumvents the problem of accumulation of antibiotic markers in the mutated strains (Sung *et al.*, 2001), but is more complex than OEP and was not necessary as multiple mutations in the one strain were not planned. I therefore used OEP to construct deletion and complementation mutant strains of Sp0149 and Sp0750-53 in *S. pneumoniae* as this method is relatively quick, stable and it is possible to construct in frame mutations. For phenotype analysis of mutant strains it is important if possible to have a complemented mutant so any phenotypes observed is linked directly to the genetic manipulation of the gene of interest. However, although after several attempts I was able to construct the complementation construct of Sp0149 this construct failed to transform in *S. pneumoniae*, and I was also unable to obtain the complementation construct of Sp0750-53 possibly due to its larger size.

The role of *S. pneumonaie* Sp0149-53 and Sp0749-53 ABC transporters were investigated in detail for their possible substrate specificity and role in virulence using the *ΔSp0149* and *ΔSp0750-53 S. pneumoniae* deletion mutants. The role of Sp0149-53 ABC transporter was investigated *in vitro* by comparing the growth rates of the wild-type and the *ΔSp0149* deletion mutant strains under normal laboratory conditions. Particular phenotypes associated with the specific functions of the Sp0149-53 (requirement of particular cations, sensitivity to streptonigrin) were also investigated.

These assays did not show impairment of growth in the tested conditions such as cation depleted media, but as discussed earlier recent data suggested that Sp0149-53 is a methionine ABC transporter (Saier, Jr., 2000) rather than a cation transporter. Further investigation of this transporter is required using uptake assays and ligand binding assays. Methionine ABC transporters are functionally characterised in Gram negative bacteria such as *E. coli* (Kadner, 1974) and S. *enterica* serovar Typhimurium (Grundy and Ayling, 1992). To my knowledge, there have been no reports demonstrating a role for the methionine uptake in the virulence of Gram positive pathogens including *S. pneumoniae.* In *S. mutans*, the Sp0149-53 homologues encode AtmBDE, an ABC transporter that was identified as a likely methionine uptake transporter, with functional studies suggesting AtmBDE has a strong affinity for L-methionine but also transports other sulphur compounds such as selenomethionine, D-methionine, or homocysteine (Sperandio *et al.*, 2007). MtaR, a transcriptional regulator, was also identified which not only regulates methionine synthesis and / or uptake genes (AtmBDE) in *S. mutans* (Sperandio *et al.*, 2007) and *S. agalactiae,* but was also essential for the survival of *S. agalactiae* in a rat model of septicaemia therefore contributing towards virulence (Shelver *et al.*, 2003). It would therefore be interesting to investigate the role of Sp0149-53 as a putative methionine ABC transporter using methionine as the potentially preferred substrate.

In order to identify particular phenotypes associated with the specific functions of the Sp0749-53 putative BCAA ABC transporter, toxicity towards azaleucine, radioactive uptake assays, tryptophan fluorescence spectroscopy and radioactive binding assay were performed. Loss of Sp0749-53 ABC transporter did not result in a particular requirement for BCAA in the conditions used such as THY, and the wild-type strain was resistant to the toxicity of azaleucine, so we were unable

to identify significant uptake of the BCAA leucine by *S. pneumoniae*. Hence in the conditions used for these experiments (ie., the complete medium THY and the defined medium CDEM) there seems to be very little BCAA uptake by *S. pneumoniae*. Tryptophan fluorescence spectroscopy and radioactive binding assays using ^{14}C-leucine, isoleucine and valine demonstrated that His_6-Sp0749 binds specifically to BCAA, strongly supporting the hypothesis that Sp0749-53 encodes a BCAA ABC transporter. Both the tryptophan fluorescence spectroscopy and radioactive binding assays suggest that His_6-Sp0749 has the highest affinity for isoleucine, moderate affinity for leucine, with the least affinity for valine (Basavanna *et al.*, 2009). These data suggest strongly that Sp0749 to 0753 does encode a BCAA transporter despite the lack of evidence for BCAA uptake *in vitro*.

In mouse models of infection the *Δ750-53* and the *ΔSp0149* deletion strains were both significantly out-competed by the wild-type strain. These data suggest there is an important role for the *S. pneumoniae* BCAA and putative methionine ABC transporters specifically during *in vivo* growth, a conclusion that is supported by previous publications at least for BCAAs, with disruption of BCAA synthesis affecting virulence of the unrelated pathogens *B. pseudomallei* and *M. bovis* (Atkins *et al.*, 2002; McAdam *et al.*, 1995). However the effect of loss of Sp0750-53 and Sp0149 on virulence were only detectable in competitive infection experiments and after IN inoculation with the *ΔSp0149, ΔSp0750-53*, or wild-type strain, progression of infection was similar. Hence, Sp0149-53 and Sp0749-53 ABC transporters do not have a powerful effect on *S. pneumoniae* virulence. This could reflect redundancy associated with *S. pneumoniae* ABC transporters. For example, the *S. pneumoniae* genome contains genes encoding enzymes required for BCAA synthesis (Sp0445-50) (Tettelin *et al.*, 2001) (Hendriksen *et al.*, 2008) may partially compensate for impaired

BCAA uptake *in vivo*. Whether the loss of Sp0149 is perhaps compensated by other gene(s) involved in methionine metabolism is not known. Exactly why loss of genes encoding putative methionine and BCAA affects virulence is not clear. The most obvious explanation is a nutritional requirement for methionine and BCAA *in vivo*. However, the marked difference in CI between growth in blood and during infection for the *Sp0149*$^{-}$ and *Sp0750*$^{-}$ mutant strains indicates that the loss of virulence maybe more complex than simple impaired growth under physiological conditions. As stated above BCAA metabolism and uptake genes in various bacteria have been identified to be regulated by CodY. CodY, a nutritional repressor identified in *L. lactis* (Guedon *et al.*, 2001), *C. difficile* (Dineen *et al.*, 2007), *S. pyogenes* (Malke *et al.*, 2006), and *L. monocytogenes* (Bennett *et al.*, 2007), *S. aureus* (Majerczyk *et al.*, 2008) and *S. pneumoniae* (Hendriksen *et al.*, 2008) is known to inhibit the genes involved in amino acid metabolism. It is highly conserved in Gram positive bacteria and responds to intracellular concentrations of BCAAs and GTP (guanosine triphosphate), thereby sensing carbon, nitrogen availability and aids the bacterium to adapt to survive under harsh conditions. Therefore CodY plays a central role in the regulation of bacterial metabolism (Somerville and Proctor, 2009; Tojo *et al.*, 2008) and will influence growth and therefore virulence *in vivo*. In *S. pneumonaie*, the CodY regulon consists of genes involved in BCAA and general amino acid metabolism. Upregulation of BCAA metabolism and uptake genes including Sp0749-53 was observed in a *codY* mutant strain suggesting that CodY directly controls the intracellular BCAAs in *S. pneumoniae* (Hendriksen *et al.*, 2008).

As the Sp0149-0153 and Sp0749-53 ABC transporters are important for virulence, and their corresponding lipoprotein genes, Sp0149 and Sp0749 are surface localised, they were chosen for further investigation as vaccine candidates analogous

to other protein antigens such as PiaA, PiuA and PsaA. Western blotting of the cell lysates of clinically important serotypes responsible for *S. pneumoniae* infections with polyclonal antibodies to Sp0149 and Sp0749 demonstrated the presence of the lipoproteins Sp0149 and Sp0749 in all serotypes investigated. Furthermore, the proteins were of the same size in all strains and PCR amplified identical sized gene products. This suggests that both Sp0149 and Sp0749 are identically sized and conserved amongst *S. pneumoniae* strains, making them potentially attractive vaccine candidates. Compatible with the results of the *in vitro* assays and RT-PCR analysis for Sp0749 function, the expression of Sp0749 generally seemed to be low compared to the expression of Sp0149, with probing with anti-Sp0749 giving a variable and much weaker signal compared to probing with anti-Sp0149 serum. ELISA demonstrated that Sp0149 and Sp0749 lipoproteins are immunogenic as they elicited good IgG antibody titres comparable to that of PsaA which was used as positive control. The antibody results were predominantly IgG1 subclass, although a IgG2a subclass response was also observed. Hence the lipoproteins may induce a Th2 immune response similar to that observed upon immunisation with PiaA and PiuA (Jomaa *et al.*, 2005). No cross reactivity between antibodies to the two proteins was seen suggesting that both the lipoproteins are antigenically dissimilar. Incubation with anti-Sp0149 and anti-Sp0749 resulted in small but a significant increase in the deposition of complement on *S. pneumoniae* and aided phagocytosis. Antibodies to iron uptake ABC transporters do not inhibit ABC transporter function (Jomaa *et al.*, 2005), and although antibodies to Sp0149 and Sp0749 seems to have immunological effects, it is not known whether anti-Sp0149 and anti-Sp0749 also inhibits the function of Sp0149 and Sp0749 lipoproteins. However this would be difficult to assess due to potential

redundancy and the lack of a specific phenotype *in vitro* that could be used to assess the function of these ABC transporters (Brown *et al.*, 2001).

Immunisation with Sp0149 or Sp0749 lipoproteins and subsequent challenge by the intraperitoneal route with D39 strain to simulate a model of *S. pneumoniae* septicaemia involving experiments were performed twice in the CD1 mouse strain. In the initial immunisation experiment, CD1 mice were challenged with 10^5 cfu of D39 strain of *S. pneumoniae*. Only a minor delay in the development of fatal infection was observed in the mice immunised with the lipoprotein groups. Since the lipoproteins failed to confer strong protection, one of the questions that arose is whether these lipoproteins are unstable in the laboratory storage conditions and whether they have degraded from the time of purification to the immunisation or failed to induce good antibody responses. Western blotting suggested that this was not the case, as polyclonal antisera raised against the purified Sp0149 and Sp0749 lipoproteins produced specific signals when used to probe whole cell lysates of *S. pneumoniae* and the purified His_6-Sp0149 and His_6-Sp0749. The disappointingly weak protective effect of immunisation with His_6-Sp0149 and His_6-Sp0749 may reflect a challenge dose that may be overwhelmingly high leading to fast multiplication of *S. pneumoniae* in the host even if the vaccine antigens had elicited protective responses.

As well as a negative control group vaccinated with alum alone, the efficacy of these lipoproteins as vaccines also needs to be evaluated in comparison with a positive control whose efficacy as a vaccine candidate has been previously determined. I therefore used PspA, a surface protein known to be protective against several clinically important serotypes, as a positive control for the second immunisation experiment. Unfortunately PspA is a weak vaccine candidate against D39 (McDaniel *et al.*, 1991), but this protein was the only alternative vaccine protein

available in our laboratory at the time of second immunisation experiment. A lower challenge dose of 10^3 cfu of *S. pneumoniae* was used in the second immunisation experiment to increase the likelihood of detecting protective responses. PspA did not exhibit protection against D39 strain of *S. pneumoniae*, and the survival of mice vaccinated with Sp0149 was similar to that of mice immunised with PspA or alum alone. Sp0749 showed a statistically non-significant delay the development of fatal infection, perhaps suggesting that Sp0749 may be an alternative protein vaccine antigen. CD1 mice were used for the immunisation-challenge experiments because they are an outbred strain, as well as being cheaper, experiments using outbred mice are more likely to be of greater relevance than positive results obtained with an inbred mouse population. The immunisation experiment was therefore repeated in the inbred mice strain Balb/c. Unfortunately, no protection against *S. pneumoniae* challenge was observed after immunisation with the Sp0149 or Sp0749 lipoproteins, nor with the positive control protein PsaA. Why Sp0149, Sp0749 and PsaA did not demonstrate protection despite the *in vitro* data suggesting they induce a significant and immunologically relevant response is not clear. One probable explanation is that the intraperitoneal route may be too stringent an infection route, and that any protective response elicited by the lipoproteins may be masked by the severe *S. pneumoniae* septicaemia induced by this route of inoculation.

As *S. pneumoniae* pneumonia is a common disease affecting both the elderly and infants to which the available vaccines do not provide efficient protection, intranasal immunisation and challenge studies were performed in mice to investigate whether immunisation with the protein antigens can protect against *S. pneumoniae* pneumonia. The protective efficacy of the purified Sp0149, Sp0749 by itself or in combination with the previously investigated lipoprotein vaccine candidates PiaA and

PiuA (Jomaa *et al.*, 2006) against *S. pneumoniae* pneumonia was investigated through the intranasal route (instilling the purified lipoproteins and bacteria into mouse nostrils, IN). This simulates the natural route of infection in humans. Cholera toxin was used as the adjuvant as it is well established and used as adjuvant with different *S. pneumoniae* proteins for IN immunisation experiments (Jomaa *et al.*, 2006) (Pimenta *et al.*, 2006; Shah *et al.*, 2009). Experiments assessing survival after IN immunisation and challenge were performed in outbred CD1 mice due to the cost, whereas inbred CBA/Ca mice were used for experiments assessing immunological responses in more detail in order to minimise mouse to mouse variation in results that may compromise the results of the experiment and its analysis.

The antibody titres, types of immune cell activation and histological examination of the degree of inflammation in the lungs elicited against the immunised lipoproteins were analysed. Sp0749 in combination with PiaA elicited good IgG titres in the sera and respiratory secretions such as BALF after the IN immunisation, although greater IgG titres were demonstrated in the sera after IP immunisation with Sp0749. Perhaps surprisingly given the immunisation route Sp0749 specific IgA antibodies were not detected and PiaA specific IgA antibodies were detected in the BALF of only two mice immunised with PiaA and Sp0749. Nevertheless, ELISA demonstrated that the IN immunisation with Sp0749 and PiaA can induce specific local and systemic IgG antibodies.

To investigate the detailed host immune responses upon *S. pneumoniae* challenge, cells were isolated from the BALF and lungs of CT, PiaA and PiaA+Sp0749 immunised mice before and 48 hours after the *S. pneumoniae* challenge and analysed using labelled lymphocyte and macrophage antibody markers and flow cytometry. The total cell counts in the BALF and lungs determined using a

haemocytometer indicated only small differences before and after the *S. pneumoniae* challenge between the vaccine groups. The results of the cell surface marker experiments were highly variable between mice and it was difficult to identify clear trends. However, there was an indication of an increase in the proportion of B220 cells. As expected, differential cell counts of the BALF cytospins indicated an overwhelming increase in the neutrophil infiltration upon *S. pneumoniae* challenge in all groups. Macrophage activation in the vaccine groups upon *S. pneumoniae* challenge was also analysed by flow cytometry using two macrophage surface markers (CD80 and I-A-I-E). In the lungs, the results suggests both the active vaccine groups had an increase in CD80 positive cells suggesting a greater recruitment of activated macrophages in these mice compatible with a positive immunological benefit of vaccination with lipoproteins. However, flow cytometry using I-A-I-E surface marker did not mirror the results obtained using the CD80 surface marker, therefore the significance of the results obtained by CD80 are unconfirmed.

Interestingly the histological examination of the lung tissues from the control and vaccine groups indicated more inflammation in the vaccine groups than the control group, with greater degree of inflammation in the PiaA immunised group upon *S. pneumoniae* challenge. Why there was increased level of inflammation in the vaccine groups after the *S. pneumoniae* challenge is not immediately clear, but could indicate a more rapid inflammatory response in actively vaccinated mice which could aid protective immunity. An analysis of the cytokine response would help identify whether there are differences in the inflammatory response between vaccinated and control mice after *S. pneumoniae* challenge. Inflammatory cytokines such as IL-1, TNF and IL-6 are present in both the patients (Dehoux *et al.*, 1994) and mice with pulmonary infection caused by *S. pneumoniae* (Bergeron *et al.*, 1998). Blocking the

expression or action of cytokines such as TNF-α, and IL-6 impairs the host's immune response to *S. pneumoniae* with reduced bacterial clearance and reduced survival in experimental mice models of pneumonia (Takashima *et al.*, 1997; Van der *et al.*, 1997). However, studies have demonstrated that the host's cytokine response depends on the specific strain of *S. pneumoniae* in addition to the type of infection such as pneumonia / septicaemia and host's genetic background (Mohler *et al.*, 2003). Furthermore, differences in bacterial cfu present at different time points could make the interpretation of a direct comparison of cytokine levels between groups complex. Hence a careful selection of the mouse strain and time point for cytokine analysis will be necessary. To determine which of the observations on differences in the immune response after IN immunisation with PiaA and Sp0749 or the control group are consistent and biologically significant and assess their potential importance for control of *S. pneumoniae* infection, these experiments will need to be repeated.

Despite the evidence of some modifications of the immunological response to infection in the pneumonia model in mice vaccinated with the lipoproteins, little protection was found after intranasal vaccination with the lipoproteins. Sp0149 alone and in combination with PiaA and PiuA in the triple vaccination gave no protection. Sp0749 showed a statistically non-significant (p = 0.083) weak level of protection when combined with PiaA, but not when used alone or in combination with Sp0149 and PiaA in a triple antigen vaccination. The results obtained with PiaA and PiuA contrast to previously published data on these antigens as intranasal vaccines (Jomaa *et al.*, 2006). The reasons why both PiaA and PiuA did not protect in both CD1 and CBA/Ca mice strain when previously they had shown protection against pulmonary *S. pneumonaie* infection (Jomaa *et al.*, 2006) are not clear, but could be due to variations in the serotype of *S. pneumoniae* used (1x 10^6 cfu of serotype 1 in CBA/Ca mice).

The results of *S. pneumoniae* cfu enumeration 48 hours after the *S. pneumoniae* challenge in the BALF, lungs and blood of immunised CBA/Ca mice were compatible with the results of the survival experiments. There was a statistically non-significant decrease in the bacterial counts in BALF, lungs and blood of mice immunised with PiaA and PiaA+Sp0749 when compared to CT alone, suggesting that the immunisation with these lipoproteins may perhaps have some weak effect on the clearance of *S. pneumoniae* that might not be enough to influence overall survival in this model. These experiments require repetition to identify whether the differences in cfu are consistent and to increase the numbers for a more accurate assessment of their statistical significance.

One explanation for the weak protective effects on *S. pneumoniae* clearance from the target organs is that CBA/Ca mice are particularly susceptible to intranasal infection. Previous work by Gingles *et al*. demonstrated that the median survival time of CBA/Ca mice after challenge with 10^6 cfu of D39 was 28 hours, whereas that of Balb/c was greater than 168 hours (Gingles *et al.*, 2001). In the future it would be important to enumerate *S. pneumoniae* cfu at an earlier time point in CBA/Ca mice strain or to use Balb/c mice, although these mice may be too resistant to *S. pneumoniae* pneumonia for consistent results in the negative control group. When designing *S. pneumoniae* vaccination experiments careful consideration of a range of variables such as the quantity of the antigen, type of adjuvants, route of immunisation, strain and dose of *S. pneumoniae* and mice strain is required (Chiavolini *et al.*, 2008; Tai, 2006). However at present there is a lack of accepted standard mouse strain / bacterial strain combinations as well as positive controls for the different types of immunisation-challenge studies for investigating *S. pneumoniae* vaccine antigens. Overall the lack of efficacy of the lipoprotein vaccine candidates was disappointing

Table 7.1: Flow chart to summarise the novel results of Sp0149-52 and Sp0749-53 ABC transporters

Chosen 11 ABC transporter operons from TIGR4 genome

↓

Amino acid homology searches using BLAST

↓

Analysis of genetic organisation in 0100993 using RT-PCR

↓

Four of the eleven ABC transporter regions of 0100993 are similar to TIGR4 genome

Constructed IDM mutants for nine of the eleven ABC transporters in 0100993

In vitro phenotype screening by CIs:

↓

Four mutant strains showed partial growth defect in human blood

In vivo phenotype screening by CIs:

↓

Sp0149^{-} and *Sp0750*$^{-}$ mutant strains were markedly attenuated in systemic and pulmonary *S. pneumoniae* infection

↓

Sp0149-52 and Sp0749-53 are important for *S. pneumoniae* virulence

Detailed phenotype analysis of Sp0149-53 and Sp0749-53 using *ΔSp0149* and *ΔSp0750-53* deletion mutant strains

<u>*In vitro* phenotype analysis demonstrated that:</u>

• Sp0149 and Sp0749 are membrane bound lipoproteins
• Sp0749 lipoprotein is a branched chain amino acid binding protein.

<u>*In vivo* phenotype analysis demonstrated that:</u>

• Sp0149 and Sp0749 are differentially expressed in mice after systemic *S. pneumoniae* infection
• Deletion of Sp0149 and Sp0750-53 affects *S. pneumoniae* virulence in mixed infection models, but were able to cause fatal disease when given as pure inoculum

Investigation of vaccine potential of Sp0149 and Sp0749 lipoproteins

• Sp0149 and Sp0749 are present in *S. pneumoniae* capsular serotypes present in 7-valent vaccine
• Sp0149 and Sp0749 lipoproteins elicits good IgG immune response, predominantly Th2 type immune response after IP and IN immunisation with Sp0149 and Sp0749.
• Anti-Sp0149 and anti-Sp0749 induces small increase in *in vitro* complement deposition and opsonophagocytosis after IP immunisation with Sp0149 and Sp0749.
• IN immunisation with Sp0749 with PiaA may increase the activation of lung macrophages.
• IN immunisation with Sp0749 and PiaA increased inflammation in lung sections.
• Survival studies after IP and IN immunisation with Sp0149, Sp0749 and PiaA did not protect after *S. pneumoniae* challenge

Sp0749 is a branched chain amino acid binding lipoprotein and Sp0149 and Sp0749 do not aid protection against *S. pneumonaie* septicaemia and pneumonia.

and suggests that Sp0149 or Sp0749 are not likely to be major vaccine candidates. They may contribute as an additional antigen for a multivalent vaccine, but any benefit they might add over and above major vaccine antigens such as PiaA, PspA, or PsaA would require careful evaluation. Flowchart 7.1 summarises the novel findings of Sp0149-52 and Sp0749-53 ABC transporters.

7.1 SUMMARY

I selected 11 ABC transporters using the annotated TIGR4 *S. pneumoniae* genome, constructed disruption mutant strains for nine of the ABC transporter operons in 0100993 strain. BLAST searches showed that several of the chosen *S. pneumoniae* ABC transporters have close homologues in streptococci. However, some discrepancy in the prediction of the substrate specificity occured using BLAST searches and transport classification system (Sp0149-52, Sp0846-48). Of the nine ABC transporter mutant strains successfully constructed, six were impaired in full virulence in mouse models of sepsis and / or pneumonia (*Sp0090*$^{-}$*, Sp0149*$^{-}$*, Sp0610*$^{-}$*, Sp0750*$^{-}$*, Sp1824*$^{-}$ and *Sp2108*$^{-}$). Of these six ABC transporter mutant strains, *Sp0149*$^{-}$, and *Sp0750*$^{-}$ were highly attenuated in virulence in mouse models of sepsis and pneumonia compared to the other strains. These ABC transporters were predicted by BLAST searches to be methionine and BCAA uptake respectively. Semi-quantitative RT-PCR indicated that the lipoprotein components of these ABC transporters Sp0149 and Sp0749 are expressed in mouse blood during systemic infection, suggesting these transporters are expressed under physiological conditions and supporting a role for virulence. I also constructed *S. pneumoniae ΔSp0149* and *ΔSp0750-53* deletion mutant strains in the 0100993 background, but failed to obtain complemented strains despite several attempts. Membrane localisation studies using the polyclonal mouse

antibodies to recombinant Sp0149 and Sp0749, a lipoprotein processing mutant strain (*Δlsp*), and lipoprotein fraction triton-X-114 extracts showed that both Sp0149 and Sp0749 are membrane bound lipoproteins and therefore could be potential vaccine candidates against *S. pneumoniae*. Fluorescence spectroscopy and radioactive binding assays demonstrated that the Sp0749 lipoprotein is a BCAA binding protein. Similar experiments needs to be performed to identify if methionine is the preferred substrate for Sp0149 lipoprotein. After IN inoculation with the *ΔSp0149, ΔSp0750-53*, or wild-type strain progression of infection were similar, and the effect of loss of Sp0149 and Sp0750-53 on virulence were only detectable in competitive infection experiments. These data suggest that although Sp0149-53 and Sp0749-53 ABC transporters are required for full virulence these ABC transporters do not have a powerful effect on *S. pneumoniae* virulence, perhaps because of functional redundancy of methionine or BCAA uptake by these ABC transporters.

PCR and Western blot analysis demonstrated that Sp0149 and Sp0749 are conserved in the *S. pneumoniae* serotypes present in the 7-valent conjugate vaccine. Systemic immunisation with Sp0149 and Sp0749 lipoproteins induced good IgG antibody titres, predominantly of a Th2 type immune response. Good IgG titres were obtained in the sera and BALF after IN immunisaton with Sp0749, but IgA titres were not detected. There was a small but significant increase in *in vitro* complement deposition and opsonophagocytosis assays in the presence of polyclonal antibody to Sp0149 and Sp0749. Detailed assessment of the mouse immune response to *S. pneumoniae* pneumonia after IN vaccination with Sp0749 plus another lipoprotein vaccine candidate PiaA suggested a possible increase in the activation of lung macrophages. Histological analysis of the lung sections also indicated an increased degree of inflammation in actively vaccinated mice compared to the controls upon *S.*

pneumoniae challenge. However, despite these immunological effects of vaccination with these lipoproteins , IP and IN immunisations with Sp0149 and Sp0749 did not significantly delay the progression of infection after systemic or pulmonary *S. pneumoniae* challenge in mice, although IN immunisation with Sp0749 in conjunction with PiaA may perhaps have weak effect on the *S. pneumoniae* clearance in the BALF, lungs and blood

7.2 POTENTIAL FUTURE DIRECTIONS

- To screen for specific substrates as predicted by BLAST searches of the chosen ABC transporters using tryptophan fluorescence spectroscopy, radioactive binding and uptake assays and their detailed *in vitro* and *in vivo* characterisation.
- To investigate the ligand binding properties of Sp0149 using methionine as a potentially preferred substrate using different forms of methionine such as D, L- methionine, selenomethionine and homocysteine.
- To investigate the phenotype *in vitro* and *in vivo* of a strain containing a deletion of the Sp0749-53 BCAA ABC transporter and the Sp0445-50 BCAA synthesis enzymes
- To repeat and investigate in more detail the host's immune response and *S. pneumoniae* clearance from the target organs after intranasal immunisation with Sp0749, perhaps using mouse and bacterial strains that are more likely to identify differences between groups.
- To perform intranasal immunisation-challenge experiments with Sp0749 and different combination of potential lipoprotein vaccine candidates such as PiaA, PiuA, PsaA and PotD to identify potential optimal combinations of protein antigens for a multivalent vaccine.

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Appendix

MEDIA

Cden medium

To prepare 395 ml Cden medium

Cden base	200 ml
HYR	50 ml
Glutamine (1 mg ml^{-1})	10 ml (stock solution)
Vitamins (without choline	10 ml
Sodium pyruvate (2%)	5 ml (stock solution
SAC solution	40 ml
Supplement	13 ml
KPO_4 (1 M; pH 8.0)	15 ml
Leucine (10 mg ml^{-1})	10 ml (stock solution)
Phenylalanine (10 mg ml^{-1})	5 ml (stock solution)
Lysine (10 mg ml^{-1})	9 ml (stock solution)
Choline chloride (1 mg ml^{-1})	2 ml (stock solution)
Distilled water	26 ml

Filter sterilize before use.

Cden base

Glycine	190 mg
Alanine	350 mg
Valine	720 mg
Isoleucine	760 mg
Proline	1160 mg
Serine	590 mg
Threonine	450 mg
Methionine	310 mg
Tryptophan	140 mg
Asparagine	720 mg
Glutamic acid	2200 mg
Cysteine	150 mg

Dissolve in the final volume of 2 litres (L), adjust the pH to 7.0 and filter sterilize.

HYR (Histidine, Tyrosine, Arginine)

Histidine	640 mg
Tyrosine	122 mg
Arginine	800 mg

Vitamins without choline

Adam's I solution	12 ml
Asparagine (5 mg ml^{-1})	32 ml (stock solution)

Distilled water	36 ml

Dissolve and filter sterilize

Adam's I Cden solution

Biotin (0.5 mg ml^{-1})	60 µl (stock solution)
Nicotinic acid	30 mg
Pyridoxine	35 mg
Calcium pantothenate	120 mg
Thiamine hydrochloride	32 mg
Riboflavin	14 mg

Dissolve in final volume of 200 ml, filter sterilize and store at 4ºC in dark.

SAC solution

Sodium chloride	12 g
Anhydrous sodium acetate	12 g

Dissolve in final volume of 1 L.

Supplement

3 in 1 salts	60 ml
Glucose (20%)	120 ml (stock solution)
Sucrose (50%)	6 ml (stock solution)
Adenosine (2 mg ml^{-1})	120 ml (store at room temperature)
Uridine (2 mg ml^{-1})	120 ml (store at room temperature)

Filter sterilize

3 in 1 salts

$MgCl_2.6H_2O$	100 g
$CaCl_2$ (anhydrous)	0.5 g
MnSO4 (0.1 M)	200 µl (stock solution)

Dissolve in final volume of 1 L, autoclave or filter sterilize.

TE-DOC

Tris	50 mM
EDTA	5 mM
Sodium deoxycholic acid	0.01%

NAES buffer

Sodium acetate	50 mM, pH 5·1
EDTA	10 mM

SDS	1%

SDS-PAGE gels

10% resolving gel

30% acrylamide mix	6.7 ml
1M Tris (pH8.8)	7.5 ml
10% SDS	0.2 ml
10% ammonium persulphate	0.2 ml
TEMED	0.008 ml
Distilled water	5.4 ml

Stacking gel

30% acrylamide mix	0.83 ml
1M Tris (pH6.8)	0.63 ml
10% SDS	0.05 ml
10% ammonium persulphate-	0.05 ml
TEMED	0.005 ml
Distilled water	3.4 ml

10X running buffer for SDS-PAGE

Tris base (0.25 M)	30.3 g
Glycine (1.92 M)	144 g
SDS (1%)	10 g
Distilled water	1000 ml

Adjust the pH to 8.3

5X Laemeli buffer

Tris (312 mM, pH 6.8)	3.125 ml of 1M stock
SDS (10%)	1 g
Glycerol (20%)	2 ml
Dithiothretol (50 mM)	0.5 ml
Bromophenol blue	Add until dark blue
Distilled water	10 ml

Coomassie brilliant blue stain (CBB)

CBB R-250	0.025 g
Methanol	40 ml
Acetic acid	10 ml
Distilled water	100 ml

Filter through Whatman No 1 paper.

Destaining solution

Methanol	40 ml
Acetic acid	10 ml
Distilled water	100 ml

Transfer buffer (1x)

Glycine	2.93 g
Tris	5.81 g
SDS	0.375 g
Methanol	200 ml

Dissolve in 1 L of distilled water.

Tris buffered saline (10x TBS)

Tris	24.2 g
NaCl	80 g

Dissolve in 1 L of distilled water and adjust the pH to 7.6.

1x TBS-Tween

Dilute 10x TBS to 1x TBS and add 0.1% Tween-20.

Protein purification buffers (*E. coli*)

Buffer B

NaH_2PO_4 (100 mM)	13.8 g
TrisCl (10 mM)	1.2 g
Urea (8M)	480.5 g

Dissolve in 1 L of distilled water and adjust the pH to 8.0 using HCL.

Cell Lysis buffer

NaH_2PO_4 (50 mM)	6.90 g
NaCl (300 mM)	17.54 g
Imidazole (10 mM)	0.68 g

Dissolve in 1 L of distilled water and adjust the pH to 8.0 using NaOH.

Wash buffer

NaH_2PO_4 (50 mM)	6.90 g
NaCl (300 mM)	17.54 g
Imidazole (20 mM)	1.36 g

Dissolve in 1 L of distilled water and adjust the pH to 8.0 using NaOH.

Elution buffer

NaH_2PO_4 (50 mM)	6.90 g
NaCl (300 mM)	17.54 g
Imidazole (250 mM)	17 g

Dissolve in 1 L of distilled water and adjust the pH to 8.0 using NaOH

RBC lysis buffer

NH_4Cl	8.29 g
$KHCO_3$	1 g
EDTA	37.2 mg

Dissolve in 1 L of distilled water, adjust the pH to 7.3. Filter sterilize and store at 4ºC.

PBS / 1% BSA / 0.1% azide

Sterile PBS (1x)	100 ml
BSA	1 g
Azide	0.1 g

Store at 4ºC.

Buffers for ELISA

TSA buffer

NaCl (132 mM)	7.72 g
Tris-HCl (125 mM)	3.03 g
NaN_3	0.5 g

Dissolve in 1 L of distilled water and adjust the pH to 7.5.

10x ELISA wash buffer

NaCl	43.83 g
Tris	3.03 g
Triton-X-100	5 ml
HCl (1M)	18 ml

Dissolve in 5 L of distilled water and adjust the pH to 7.6.

BSA-Tween buffer

NaCl	8 g
Triethanolamine (0.25 M)	50 ml
Tween-20	0.5 ml
NaN_3	200 mg

BSA	200 mg

Dissolve in 1 L of distilled water and store in dark.

Enzyme diluent (for secondary antibody)

NaCl	8 g
Triethanolamine (0.25 M)	50 ml
500 x Mg/Zn	2 ml
BSA	200 mg
NaN_3	200 mg

Dissolve in 1 L of distilled water

500 x Mg/Zn (500 mM $MgCl_2$ / 1.25 mM $ZnCl_2$)

1 M $MgCl_2$ in 1 M HCL	50 ml
0.1 M $ZnCl_2$ in 1 M HCL	1.25 ml

Add 0.1 M HCL to a final volume of 100 ml.

Paraformaldehyde preparation

Paraformaldehyde	3 g
$CaCl_2$ (100 mM)	100 µl
$MgCl_2$ (100 mM)	100 µl
PBS	100 ml

Potassium phosphate buffer

50 mM potassium phosphate buffer, pH 7.2, supplemented with 1 mM magnesium chloride