Streptococcus pneumoniae: involvement of neuraminidase, autolysin and superoxide dismutase in respiratory tract infections

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By

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To you

“By sealing our work with our blood, we may see at least the bright dawn of universal happiness”

Robespierre, 1794

Abstract

Sonia Cruz Manco

"*Streptococcus pneumoniae*: involvement of neuraminidase, autolysin and superoxide dismutase in respiratory tract infections"

This study focuses on the *Streptococcus pneumoniae* virulence factors neuraminidases A and B, autolysin and superoxide dismutase, with the aim of improved understanding of their roles in the pneumococcal infections. Understanding the pathogenesis of pneumococcal infection is important because this bacterium is one of the most common bacterial causes of human disease and death and because of the emergence of multidrug-resistant strains and issues with the current vaccines.

Using a murine model of bronchopneumonia it was shown that NanA and NanB have involvement in colonisation of the upper respiratory tract and in systemic spread. Lack of either of these proteins enhanced bacterial clearance in the nasopharynx, lungs and blood. Furthermore, this study demonstrated distinct roles for each of the neuraminidases, but also suggests synergistic action. Infection data clearly show that the absence of one neuraminidase is not compensated by the presence of the other. This study identified, for the first time, a role for NanB in pathogenesis and in multiplication at mucosal sites.

The major *S. pneumoniae* autolysin LytA has been implicated in pathogenicity by releasing virulence factors and components of the cell wall. In this study, the avirulence of a pneumococcal autolysin-deficient mutant was confirmed. Moreover, the results show that early after infection, LytA is crucial to pneumococcal survival and growth, both in the upper and lower respiratory tract.

The major pneumococcal enzyme for detoxifying superoxide is MnSOD, encoded by *sodA*. This study showed that absence of *sodA* impaired survival in the respiratory tract, particularly early after infection, but its absence was not lethal, suggesting that other mechanisms are involved in superoxide detoxification.

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Statement of Originality

The accompanying thesis submitted for the degree of PhD entitled "*Streptococcus pneumoniae*: involvement of neuraminidase, autolysin and superoxide dismutase in respiratory tract infections" is based on work conducted by the author in the Department of Infection, Immunity and Inflammation at the University of Leicester mainly during the period between April 2002 and September 2006.

All the work recorded in this thesis is original unless otherwise acknowledged in the text or by references.

None of the work has been submitted for another degree in this or any other University.

Signed: _____ Date: _____

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Abbreviations

μg	Microgram	**EDTA**	Ethylenediaminoetetra-acetic acid
μg/ml	Microgram per millilitre	**FeSOD**	Iron superoxide dismutase
μl	Microlitre	***g***	Gravity force
μm	Micrometre	**GalNAc**	N-acetylgalactosamine
AOM	Acute otitis media	**GFP**	Green fluorescent protein
BAB	Blood Agar Base	**Glc**	Glucosamine
BHI	Brain Heart Infusion	**GlcNAc**	*N*-acetylglucosamine
bp	Base pair	**Glu**	Glutamine
BSA	Bovine serum albumin	**G_M**	Monosialoganglioside
CBD	Choline-binding domain	**GSH**	Glutathione
CBP	Choline-binding protein	**h**	Hours
CBR	Choline-binding region	**HPSF**	High purity salt free
COG	Cluster of orthologous group	**HSA**	Human serum albumin
cfu	Colony forming units	**IgA**	Immunoglobulin A
cfu/ml	Colony forming units per millilitre	**IgG**	Immunoglobulin G
CSF	Cerebrospinal fluid	**i.n.**	Intranasal
Cu/ZnSOD	Cupper-Zinc superoxide dismutase	**i.p.**	Intraperitioneal
CWH	Cell wall hydrolases	**i.v.**	Intravenous
dH_2O	Distilled water	**kb**	Kilobase
DNA	Deoxyribonucleic acid	**kDa**	Kilo Dalton

LA	Luria-Bertani medium with agar
LB	Luria-Bertani medium
LNnT	Lacto-*N*-neotetraose
LRT	Lower respiratory tract
LytA	Autolysin (*N*-acetyl-muramoyl-L-alanine amidase)
LytB	Autolysin (β-*N*-acetylglucosamidase)
LytC	Autolysin (β-*N*-acetylmuramidase)
M	Mole
ml	Millilitre
mm	Millimetre
mM	Millimole
MnSOD	Manganese superoxide dismutase
MOPS	3-(*N*-Morpholino) Propane Sulfonic Acid
MPC	murine peritoneal cultured
MUAN	2'-(4-methylumbelliferyl)-α-D-*N*-acetylneuraminic acid
NADH	β-Nicotinamide adenine dinucleotide, oxidised
NADPH	β-Nicotinamide adenine dinucleotide phosphate, reduced
NAG	*N*-acetylglucosamine
NAMA	*N*-acetylmuramic acid
NanA	Neuraminidase A
NanB	Neuraminidase B
NCS	Newborn calf serum
Neu2en5Ac	2-deoxy-2,3-dehydro-N-acetylneuraminic acid
NeuNAc	N-acetylneuraminic acid
ng	Nanogram
ng/ml	Nanogram per millilitre
ng/μl	Nanogram per microlitre
nH_2O	Nanopure water
nm	Nanometers
OD	Optical density
OM	Otitis media
OMP	Outer membrane protein
ORF	Open reading frame
PAF	Platelet-activating factor
PAFr	Platelet-activating factor receptor
PBP	Penicillin-binding protein
PBS	Phosphate buffer saline
PCR	Polymerase chain reaction
PCV-7	7-valent pneumococcal conjugate vaccine
pM	Pico mole
pNP-NANA	2-O-(p-nitrophenyl)-α-D-*N*-acetylneuraminic acid
PPV	23-valent pneumococcal polysaccharide vaccine

rev/min	Revolutions per minute
ROI	Reactive oxygen intermediates
ROS	Reactive oxygen species
SA	Sialic acid
SDS	Sodium dodecyl sulphate
SOD	Superoxide dismutase
TE	Tris – EDTA
TSA	Tryptone Soya agar
TSB	Tryptone Soya broth
U	Units
URT	Upper respiratory tract
v/v	Volume per volume
w/v	Weight per volume

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INTRODUCTION

1. Introduction

1.1 History and general characteristics of *Streptococcus pneumoniae*

Streptococcus pneumoniae (Fig. 1.1) was first identified as a major respiratory pathogen shortly after its isolation by Pasteur in 1881. It was originally named *Diplococcus pneumoniae* in 1926 but later renamed *Streptococcus pneumoniae* (in 1974) because of its growth in chains in liquid media. Due to its role as the etiologic agent of pneumonia, it has long been known informally as the pneumococcus.

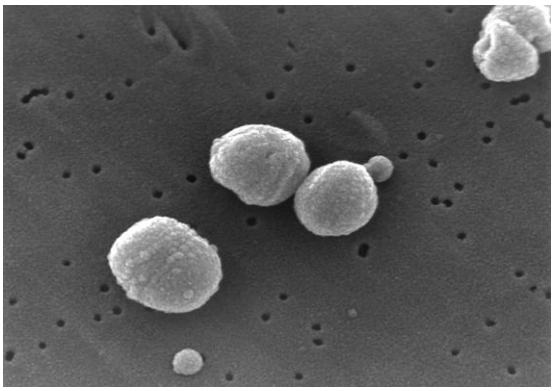


Figure 1.1 Scanning electron micrograph of *S. pneumoniae* (from CDC)

This Gram-positive organism has a distinctive morphology on Gram staining, the so-called, “lancet shape”, an elongated cocci with a slightly pointed outer curvature. Individual cells are between 0.5 and 1.5μm in diameter. The bacterium is usually seen as pairs (diplococci) but may also occur as short chains or single cells.

Pneumococci do not form spores and are nonmotile. They are fermentative aerotolerant anaerobes, growing best in 5 percent carbon dioxide. Like other streptococci, they lack catalase and are usually cultured in media that contain blood.

The organism can be identified in the laboratory by α-haemolysis on blood agar, which is characterised by an incomplete, green haemolysis (as compared to a clear and complete haemolysis in β-haemolytic streptococci and no haemolysis in γ-haemolytic cells), and by the use of optochin test. Pneumococci are optochin sensitive (Moore, 1915) and typically they form a 16 mm zone of inhibition around a 5 mg optochin disc. Other identifying characteristics are catalase negativity, inulin fermentation and bile or deoxycholate solubility. In complex media containing blood, at 37°C, the bacterium has a doubling time of 20-30 minutes. On agar, pneumococci grow as shiny colonies, about 1 mm in diameter.

Pneumococci spontaneously undergo a genetically determined, phase variation from opaque to transparent colonies at a rate of 1 in 10^5 (Weiser *et al*., 1994). The transparent colony type is reported to be adapted to colonisation of the nasopharynx, whereas the opaque variant is suited for survival in blood. The genetic mechanisms that contribute to each phenotype have not been determined (Hava *et al*., 2003; López & García, 2004) but significant difference in surface protein expression between the two types has been shown (Weiser *et al*., 1994).

The pneumococcal outer surface consists of a cell wall covered by a polysaccharide capsule. The capsule completely envelopes the pneumococcal cells and protects from phagocytosis. The idea that "the pneumococcal capsule is a gel of low density immediately surrounding the cell-wall and this, in turn, is distinct from the inner bacterial protoplasm with its limiting protoplasmic membrane" as concluded by Mudd *et al*. in 1943, has long gone. The pneumococcal capsules are polysaccharides excreted outside the cell and are composed, generally, of repeating units of simple sugars that remain attached to the outer surface of the bacterium, possibly in a covalent form (López & García, 2004). Capsule polysaccharides are highly heterogeneous. By 1940, 80 serotypes, defined by different capsular polysaccharides, had been described (CDC, 2005), and the number has now risen to over 100 different capsule types that now form the basis of antigenic serotyping of the organism (Bogaert *et al*., 2004a). These serotypes are grouped in 46 serogroups, based on immunological similarities (Hausdorff *et al*., 2005). The Quellung reaction (swelling reaction) is the simplest form of serotyping and relies on the swelling of the capsule upon binding of homologous antibody. The test consists of mixing a loopful of colony with an equal

quantity of specific antiserum and then examining microscopically at 1000X for capsular swelling. *Streptococcus pneumoniae* exists in encapsulated and unencapsulated forms but only the encapsulated form has been isolated from clinical material (Catterall, 1999). Another characteristic of capsular polysaccharides is that they have been shown to be immunogenic and antibodies against them provide type-specific protection against challenge with virulent pneumococci (Paton *et al*., 1993).

The next layer below the capsule, the cell wall, consists of polysaccharides, teichoic and lipoteichoic acid and several cell wall-associated surface proteins (Bogaert *et al*., 2004b). The cell wall of *S. pneumoniae* consists of covalently linked polysaccharide and polypeptide chains. This framework is known as peptidoglycan (or murein). Its polysaccharide component consists of linear chains of alternating β-(1-4)-linked *N*-acetylglucosamine (NAG) and *N*-acetylmuramic acid (NAMA, which consists of *N*-acetyl-D-glucosamine in an ether linkage with D-lactic acid). The NAMA lactic acid residue forms an amide bond with a D-amino acid-containing tetrapeptide to form the peptidoglycan-repeating unit. Neighbouring parallel peptidoglycan chains are covalently cross-linked through their tetrapeptide side chains. In *S. pneumoniae*, whose tetrapeptide has the sequence L-Ala- Glu- Lys- D-Ala-, this cross link consists of branched stem peptides that contain short Ala-Ala or Ala-Ser substituents that extend from the terminal carboxyl group of one tetrapeptide to the ε-amino group of the Lys in a neighbouring tetrapeptide (Fig. 1.2).

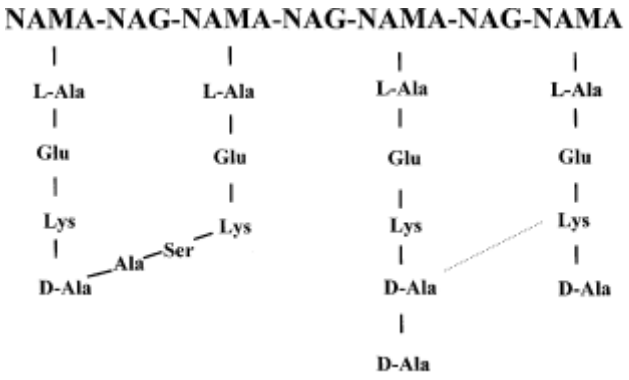


Figure 1.2 Schematic representation of the pneumococcal peptidoglycan. The variable presence of branched stem peptides is indicated on the left. (Adapted from Normark & Normark, 2002).

The surfaces of Gram-positive bacteria are covered by teichoic acids (Fig. 1.3). They are polymers of glycerol or ribitol linked by phosphodiester bridges. The hydroxyl groups of this sugar-phosphate chain are substituted by D-Ala residues and saccharides such as glucose or *N*-acetylglucosamine. Teichoic acids are anchored to the peptidoglycans via phosphodiester bonds to C6OH groups of their NAG residues. Lipoteichoic acid is chemically identical to the teichoic acid but is attached to the cell membrane by a lipid moiety (Voet, 1995). Both the teichoic acid and the lipoteichoic acid contain phosphorylcholine (Tomasz, 1967), a very unusual component in bacteria (Catterall, 1999). Phosphorylcholine is not only targeted by the choline-binding domain (CBD) of choline-binding proteins (CBPs) but functions itself as an adhesin by recognising the platelet-activating factor receptor (PAFr) of host cells (Cundell *et al*., 1995a).

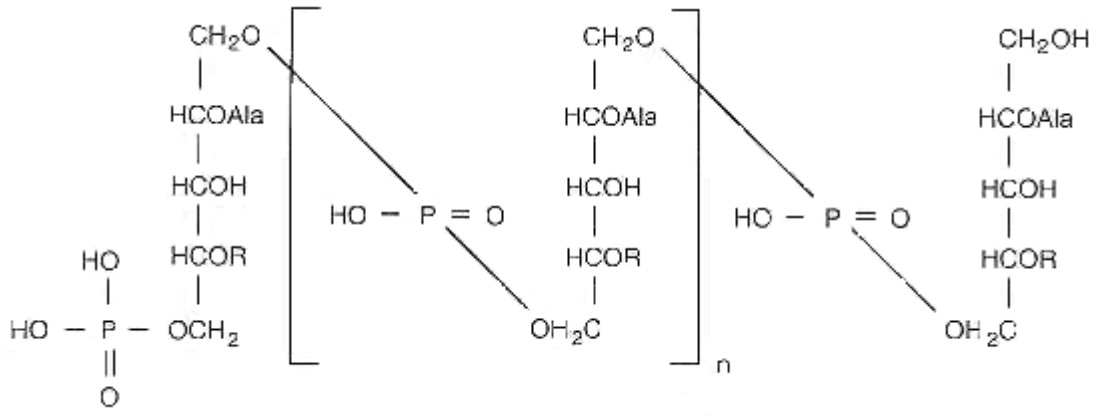


Figure 1.3 Schematic representation of ribitol teichoic acid with repeating units of 1,5-phosphodiester linkages of D-ribitol and D-alanyl ester on position 2 and glycosyl substituents (R) on position 4.

On the basis of genomic analysis (*Streptococcus pneumoniae* R6 GenBanK accession number AE007317; *Streptococcus pneumoniae* TIGR4 GenBanK accession number AE005672), it is estimated that the pneumococcus contains more than 500 surface proteins (Todar, 2003). Some are membrane-associated lipoproteins and others are physically associated with the cell wall (Fig.1.4).

As mentioned above, a unique group of proteins on the pneumococcal surface is the family of choline-binding proteins. CBPs are noncovalently bound to the choline moiety of the cell wall and are used to anchor various different functional elements onto the bacterial surface (Rosenow *et al*., 1997).

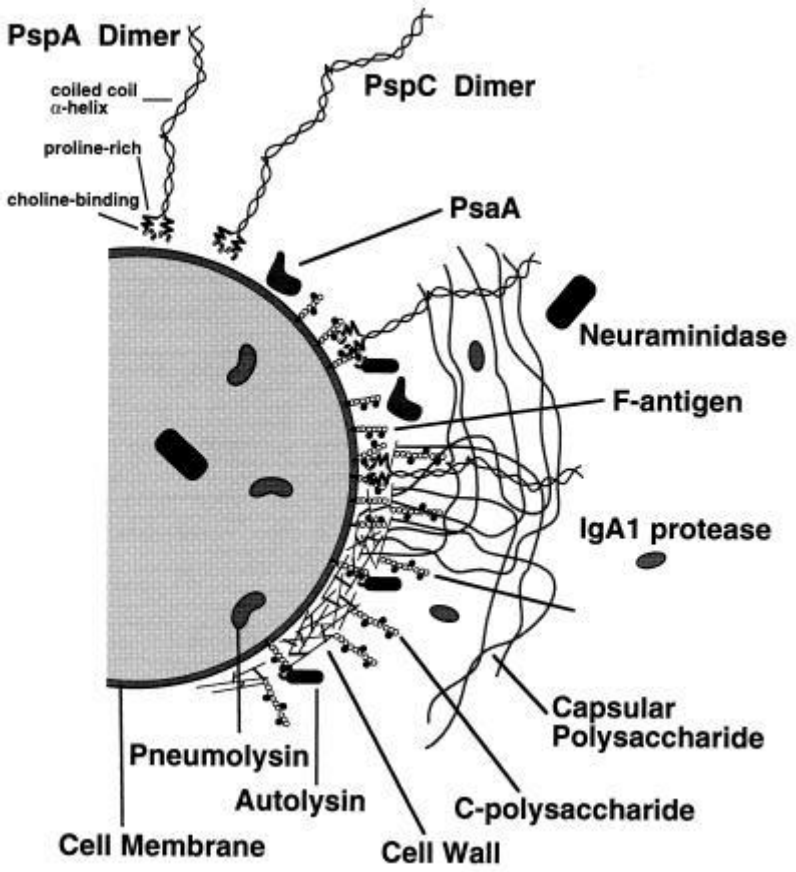


Figure 1.4 Hypothetical representation of the pneumococcal surface depicting several noncapsular antigens. C-polysaccharide (teichoic acid); F-antigen (lipoteichoic acid). Neuraminidase has been depicted both in the cytoplasm and beyond the capsule, since it is thought to be secreted by pneumococci. Pneumolysin is depicted in the cytoplasm of the cell; PspA extends from the surface to the thickness of the cell wall and capsule. The location of PsaA with respect to other cell surface structures is unknown, so its depiction here is completely hypothetical (from Briles *et al*., 1998).

1.2 Colonisation

Immediately after birth, the neonate rapidly acquires commensal bacteria that colonise mucosal sites such as the upper airway, which becomes a major reservoir of bacterial species (Rotimi & Duerden, 1981). In children the nasopharyngeal flora is established during the first year of life (Faden *et al*., 1995) becoming densely colonised by a broad variety of microorganisms, including commensal bacteria as well as potential pathogens such as *S. pneumoniae*, *H. influenzae* and *M. catarrhalis* (García-Rodríguez & Martínez, 2002). All humans are likely to be colonised with these pathogens at least once early in life, and generally, simply asymptomatic carriage is observed. Colonisation is commonly followed by horizontal dissemination of the pathogens to individuals in the direct environment, leading to spread within the community (Bogaert *et al*., 2004a). Nasopharyngeal colonisation precedes invasion but disease occurs in only a small percentage of persons who are colonised (García-Rodríguez & Martínez, 2002). However, when the condition of the host is altered, microorganisms may invade adjacent sites and/or invade the bloodstream, causing disease. Adhesion is the initial event in the colonisation process and implies the attachment of bacterial surface proteins to host cell carbohydrate receptors (Austrian, 1997). In *H. influenzae*, determinants of adhesion include fimbriae, outer membrane proteins (OMP) and lipopolysaccharide, each of which has its own specificity for host molecules. For example, P2 and P5 OMPs of *H. influenzae* adhere to mucin and fimbriae bind to carbohydrate residues of glycoproteins or glycolipids (Reddy *et al*., 1996).

Little is known about the adherence of *M. catarrhalis*. Aebi *et al*. (1998) have shown that the outer membrane protein UspA1 is essential for the attachment of these bacteria to host epithelial cells by binding to the extracellular matrix proteins fibronectin and vitronectin. Furthermore, McMichael (2000) suggested that haemagglutinin OMP 106 might play a role in adherence by *M. catarrhalis* by binding to the glycolipids globotetraosylceramid.

Typically, the pathogens remain in the airway for several months before disappearing or being replaced by a different strain of the same pathogen. The local host immune response has an important regulatory role in the trafficking of pathogens in the upper respiratory tract. A poor mucosal immune response might

lead to persistent and recurrent colonisation and consequently infection (García-Rodríguez & Martínez, 2002). Although the host factors responsible for elimination of the pathogens are not well understood, data suggests that a rapid local immune response to an organism would prevent colonisation and limit its duration, whereas a poor immune response would result in more prolonged carriage (Faden *et al*., 1995). Nasopharyngeal colonisation stimulates the production of local antibodies like IgA in response to colonisation by *H. influenzae* and systemic IgG in response to *M. catarrhalis* (Harabuchi *et al*., 1994; Samukawa *et al*., 2000).

Another aspect regarding nasopharyngeal colonisation involves the dynamic microbiota encountered. Interspecies competition and bacterial antagonism are thought to occur and to interfere with the composition of the nasopharyngeal flora hence maintaining the balance between components of the resident flora and the transient invaders. The resident flora, including α-haemolytic streptococci, inhibit colonisation by *S. pneumoniae*, *H. influenzae*, *S. aureus* and *M. catarrhalis* (García-Rodríguez & Martínez, 2002). In healthy children, α-haemolytic streptococci are able to inhibit 92% of the *S. pneumoniae* isolates, 74% of non-typable *H. influenzae* isolates, and 89% of *M. catarrhalis* isolates. In children with both secretory and recurrent otitis media, α-haemolytic streptococci are significantly less able to inhibit *S. pneumoniae* and *H. influenzae* but no difference against *M. catarrhalis* was observed (Tano *et al*., 2000).

Many factors have been shown to influence nasopharyngeal carriage rates and these include age, race, gender, socio-economic status, diet, season, smoking history, exposure to other children, sleeping position, upper respiratory tract infections, otitis media, sickle cell disease, HIV infection, allergic constitution, antibiotic therapy and immunisation (García-Rodríguez & Martínez, 2002).

Pneumococcal colonisation

Streptococcus pneumoniae is part of the commensal flora of the upper respiratory tract. Colonisation of man with pneumococci may occur on the day of birth and as many as four capsular types may be carried simultaneously in childhood (Bogaert *et al*., 2004a). Human nasopharyngeal carriage is the major reservoir of

pneumococci infecting man, and the organisms are spread through aerosols and by direct contact (Wu *et al*., 1997). Although colonisation with pneumococci is mostly symptomless, it can progress to respiratory or even systemic disease. Essential to its successful survival in the nasopharynx is the ability to produce one of the extracellular capsular polysaccharides, which render it significantly less susceptible to phagocytosis (Magee & Yother, 2001). An important feature is that pneumococcal disease will not occur without preceding nasopharyngeal colonisation with the homologous strain (Austrian, 1997). Colonisation with capsulated pneumococci is influenced by the presence or absence of homotypic anticapsular antibody. If an individual has an antibody to a given pneumococcal type, the likelihood of becoming colonised with organisms of that type is reduced by approximately half (Austrian, 1997). Ghaffaar *et al*. (1999) have shown that serotypes of *S. pneumoniae* that are poorly immunogenic tend to colonise for longer periods. Recently, in agreement, Hammerschmidt *et al*. (2005) have shown by electronic microscopy that the amount of capsule expressed by pneumococci following adherence to epithelial cells is reduced. However, the authors state that reducing its amounts of capsule during close contact with host cells is a double-edge sword for pneumococci, since in one hand it would enhance adherence and uptake but on the other hand it would also convert the pneumococcus into a more apathogenic state in terms of its ability to escape from the immune system.

As mentioned above, resident nasopharyngeal viridans streptococci can antagonise colonisation by other streptococci, including *S. pneumoniae* (García-Rodríguez & Martínez, 2002). Moreover, different pathogenic species show a competitive relationship *in vitro*, as shown by Pericone *et al*. (2000) for *N. meningitidis* and *S. pneumoniae*. This inhibitory effect of *S. pneumoniae* was also observed in co-cultures with *H. influenzae* and *M. catarrhalis*. Furthermore, *S. pneumoniae* can interfere with the growth of *S. aureus* (Bogaert *et al*., 2004a).

Colonisation by *S. pneumoniae* requires adhesion to the epithelial lining of the respiratory tract. Attachment of pneumococci is mediated through specific bacterial surface-associated molecules that bind cell-surface carbohydrates on non-inflamed resting epithelium (García-Rodríguez & Martínez, 2002). As shown by Cundell *et al*. (1995a), the cell wall phosphorylcholine binds to receptor for platelet-activating factor, which adheres pneumococci to lung epithelial cells. This occurs following activation of human epithelial and endothelial cells by interleukin

(IL)-1α and tumor necrosis factor (TNF)-α produced in inflamed sites. PsaA has also been shown to enhance adhesion to nasopharyngeal epithelial cells (Berry & Paton, 1996). In addition, Rosenow *et al*. (1997) have shown that pneumococcal CbpA is also required for nasal colonisation and lung infection.

Nasopharyngeal colonisation by pneumococci stimulates the production of local IgA and systemic IgG responses (Sun *et al*., 2004). A higher proportion of IgA-positive nasopharyngeal samples and higher antibody concentration were observed in children colonised by pneumococci than in children with cultures negative for pneumococci (García-Rodríguez & Martínez, 2002).

Tacking all these evidence into account, nasopharyngeal carriage is important, since it is related to both development of disease and spread of pathogens. However, factors that influence colonisation are multiple and not entirely clear. Moreover, the approach used to eradicate colonisation is in contrast with targeting only invasive infection and raises concerns about its effect on the natural balance between pneumococci and co-colonising species (Bogaert *et al*., 2004a).

1.3 Impact of *S. pneumoniae* on human health

Streptococcus pneumoniae is a bacterial pathogen that affects children and adults worldwide. Those most commonly at risk of pneumococcal disease are children between 6 months and 4 years of age and adults over 60 years of age (ACIP, 1997).

The risk of pneumococcal infection is much increased in persons with impaired IgG synthesis or decreased responsiveness to polysaccharide antigens due to immunosuppressive conditions such as human immunodeficiency virus infection or chronic lymphocytic leukaemia (ACIP, 1997). Also susceptible are persons with impaired phagocytosis, or defective clearance of pneumococci. In particular, the absence of a functional spleen, through congenital asplenia, splenectomy, or sickle-cell disease predisposes one to a more severe course of pneumococcal infection (WOIN, 2004).

The bacterium is currently the leading cause of invasive bacterial disease in children and the elderly (Todar, 2003). *Streptococcus pneumoniae* is the most

commonly identified bacterial pathogen causing community-acquired pneumonia (CAP) (Catterall, 2004) and mortality rates in CAP can be alarming. However, despite the name, *S. pneumoniae* causes many types of disease in addition to pneumonia. These include upper respiratory diseases such as acute sinusitis, otitis media and tracheobronchitis (WOIN, 2004). The pneumococcus also causes a broad-spectrum of invasive diseases such as meningitis, osteomyelitis, septic arthritis, endocarditis, peritonitis, pericarditis, cellulitis and brain abscess, all of which account for substantial hospitalisation and death worldwide (Sinave, 2004).

Streptococcus pneumoniae is the most common cause of bacterial meningitis in adults, and is one of the top two isolates found in otitis media (Ryan & Ray, 2004). In countries where the incidence of *N. meningitidis* and *H. influenzae* diseases has drastically decreased through the introduction of vaccines against meningococci group C and *H. influenzae* type B, *S. pneumoniae* has become the major cause of meningitis and septicemia in children. Neurological sequela and/or learning disabilities can occur in meningitis patients, and hearing impairment can result from recurrent otitis media. Virtually every child will experience pneumococcal otitis media before the age of 5 years (Bogaert *et al*., 2004b).

Pneumococcal disease causes an estimated 40,000 deaths annually in the USA, accounting for more deaths than any other vaccine-preventable bacterial disease (ACIP, 1997). Around 5 million children worldwide younger than 5 years die each year from pneumonia, with *S. pneumoniae* being the main causative agent. In the United States alone, more than half a million cases of pneumococcal pneumonia are reported each year, with 5 to 7% of them being fatal (No authors, 1985).

The World Health Organisation report on child deaths (Bryce *et al*., 2005) shows that, worldwide, 73% of deaths in children younger than age 5 years are attributable to six causes, with pneumonia accounting for 19% of the burden (Fig. 1.5). Furthermore, the report indicates that sepsis or pneumonia in neonates and pneumonia in older children constitutes 26% of all deaths (Fig. 1.5).

In the United Kingdom, the pneumococcus is responsible for 30-50% of community and 8% of nosocomial pneumonia, and it may be the cause of most cases of pneumonia with no identified causative organism (Sinave, 2004). In Ireland, there are approximately 8,000 hospital admissions from pneumonia annually and almost 2,000 people die from the disease – 90% of these are over 65 years old (WOIN, 2004).

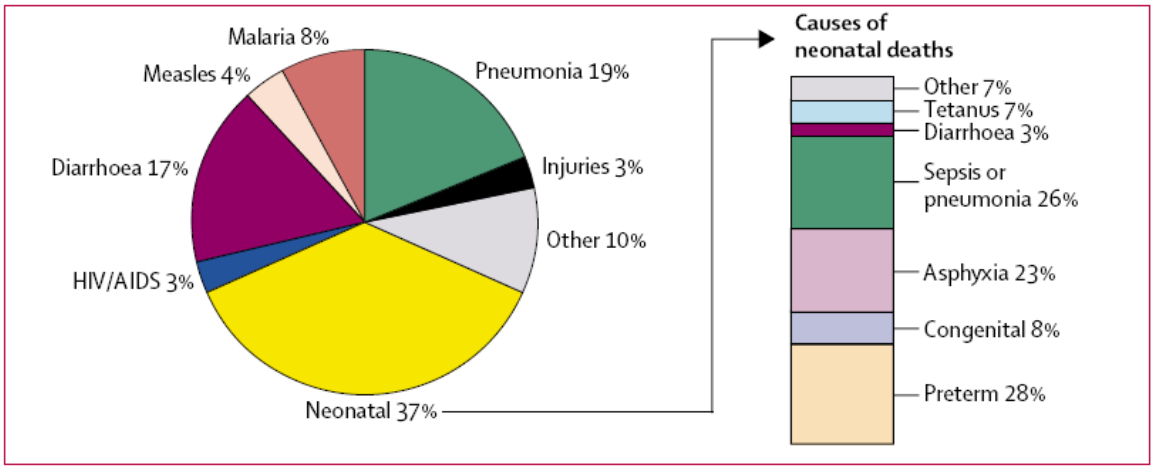


Figure 1.5 Worldwide major causes of death in children younger than age 5 years and in neonates (yearly average for the period between 2000-2003) (from Bryce *et al*., 2005).

In the USA, *S. pneumoniae* infections caused 100,000 to 135,000 hospitalisations for pneumonia, 6 million cases of otitis media, and 60,000 cases of invasive disease, including 3300 cases of meningitis until the year 2000 (CDC, 2005). Death occurred in 14% of hospitalised adults with invasive disease.

In children living in the developing world, the incidence of invasive pneumococcal disease is several times higher than the incidence in industrialised countries (No authors, 1985). It is estimated that 60-90% of lower respiratory tract infections in Gambian children younger than 5 years are caused by pneumococcal disease (Adegbola, 2006, personal communication).

1.4 Antimicrobial therapy and vaccination

Antimicrobial therapy

Treatment of pneumococcal infections is usually with β-lactam antibiotics (Normark & Normark, 2002; Catterall, 2004), which include benzyl-penicillin (penicillin G), ampicillin, cephalosporin C, ceftriaxone and aztreonam. These compounds are bactericidal and inhibit penicillin-binding proteins (PBPs) (Charpentier & Tuomanen, 2000). PBPs catalyse the trans-peptidation of the stem peptides required for cross-linking the peptidoglycan cell wall, as well as the DD-carboxy-peptidation step required to remove terminal D-alanine from the stem peptides (Normark & Normark, 2002). The β-lactam antibiotics also stimulate the activity of pneumococcal autolysins. The mechanism of penicillin resistance in clinical isolates of *S. pneumoniae* involves the alteration of PBPs (see §1.5) so as to reduce their affinity for the antibiotic molecule (Charpentier & Tuomanen, 2000).
In the 1960s, nearly all strains of *S. pneumoniae* were susceptible to penicillin, but since that time, there has been an increasing prevalence of resistance, especially in areas of high antibiotic use. The first reports on resistance to penicillin were reported in 1967 in Australia and New Guinea (Hansman *et al*., 1971). Currently, penicillin resistance in *S. pneumoniae* varies from 0% resistance in the Netherlands to 71.5% in South Korea (Walsh & Amyes, 2004). The rate of decreased penicillin susceptibility is expected to be as high as 30% in some communities in the USA (Sinave, 2004).
Macrolides are a group of drugs (typically antibiotics) which belong to the polyketide class of natural products. The mechanism of action of the macrolides is inhibition of bacterial protein synthesis by binding reversibly to the bacterial ribosome, thereby inhibiting translocation of peptidyl-tRNA. Pneumococcal resistance to macrolides occurs by alteration of the structure of the bacterial ribosome (Charpentier & Tuomanen, 2000).
In many countries macrolides resistance is higher than penicillin resistance and does not appear to be arresting. For example, in Hungary and Italy, where penicillin resistance is estimated to range 2 to 10%, erythromycin resistance can be is as high as 42% (Walsh & Amyes, 2004).

Isolates that are susceptible to penicillin also are susceptible to nearly all other antibiotics (excluding the macrolide group). However, if penicillin minimum inhibitory concentration (MIC) rises, the MIC of the most active β-lactam drugs cefotaxime, ceftriaxone (both broad-spectrum third generation cephalosporins) also rises.

Multiple antibiotic resistant strains of *S. pneumoniae* first emerged in the early 1970s in Papua New Guinea and South Africa but now cover the globe and have rapidly increased since 1995 (Todar, 2003). The cause of this international spread was mostly due to a few multidrug-resistant clones of serotypes 6B, 9V, 14, 19A, 19F, and 23F (Bogaert *et al*., 2004b). Genotyping studies on *S. pneumoniae* have identified a group of 16 multi-drug resistant clones circulating around the world (Walsh & Amyes, 2004).

In Portugal, the nationwide Viriato study, analysing the antimicrobial susceptibility of bacterial pathogens commonly associated with community-acquired respiratory tract infections during the period 2003 to 2004, recovered 18.4% of *S. pneumoniae* isolates that were resistant to penicillin, 7.1% to cefuroxime, 0.5% to amoxicillin and amoxicillin/clavulanate, 18.8% to erythromycin, clarithromycin and azithromycin, 14.9% to tetracycline, 16.5% to co-trimoxazole, and 0.4% to levofloxacin (Melo-Cristino *et al*., 2006).

The fluoroquinolones group of broad-spectrum antibiotics (a quinolone subset) remains active. Among the respiratory quinolones, moxifloxacin is currently the most active against pneumococci, followed by gatifloxacin (Sinave, 2004). Quinolones and fluoroquinolones are related to nalidixic acid and target DNA gyrase, that catalyses DNA supercoiling during replication, and topoisomerases IV complex C_2E_2, which is essential for chromosome segregation (Charpentier & Tuomanen, 2000). However, the excessive use of this class of antimicrobial agents is resulting in developing resistance that is of major concern.

So what are the antibiotics of last resort for the treatment of macrolide resistant, penicillin and multi-drug resistant *S. pneumoniae*? Currently they are linezolid and vancomycin (oxazolinidone and glycopeptide class of antibiotics, respectively) (Walsh & Amyes, 2004).

Most pneumococcal strains remain susceptible to vancomycin (Sinave, 2004), but this is a less desirable antibiotic because of dosing and tissue penetration issues. Vancomycin is another lytic antibiotic inhibiting peptidoglycan synthesis, but it

does so at an earlier step than penicillin. It exerts its antibacterial activity by binding to D-Ala- termini of peptidoglycan precursors, preventing these from being incorporated into the growing peptidoglycan wall (Normark & Normark, 2002). Recently, however, vancomycin tolerance emerged in pneumococci (Novak *et al*., 1999), both as mutants of laboratory strains and as naturally occurring clinical isolates (Normark & Normark, 2002).

Even when antimicrobial therapy is effective, pneumococcal diseases remain associated with significant mortality. Austrian and Gold (1964) compared data from the pre-antibiotic and post-antibiotic eras and demonstrated similar mortality rates during the first few days of the disease in patients with bacteremic pneumonia. This reinforces the need for an effective vaccine and/or effective new drugs against pneumococcal infection and disease. Many research groups have focused on the development of effective vaccines to be used in particular risk groups, including immunocompromised patients and children.

Vaccination

An approved vaccine composed of polysaccharides from multiple serotypes has been available for the prevention of pneumococcal disease since 1977. Since 1983 to date, a 23-valent vaccine has been licensed. This polysaccharide vaccine (effective in adults and children above 2 years old) is comprised of capsular polysaccharide antigens purified from the 23 serotypes (1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F) accounting for 85-90% of pneumococcal diseases in the UK (Catterall, 2004). The vaccine confers serotype-specific protection in adults and induces exclusively humoral immune response (Brown *et al*., 2001).

However, the polysaccharide vaccine remains one of the most controversial of the currently used vaccines because of its variable efficacy against all manifestations of pneumococcal infections. The vaccine is about only 60% effective in preventing invasive diseases and is less effective at preventing pneumonia and other localised respiratory tract infections (McDaniel & Swiatlo, 2004). Furthermore, there are major disadvantages in the use of the polysaccharide vaccines 1) the lack of efficacy in children under 2 years of age (that fail to respond

immunologically to purified polysaccharides) and in immunocompromised patients (WHO, 1999), 2) they are not effective against acute otitis media (Wadwa & Feigin, 1999) and, 3) they do not induce a T-cell dependent immune response (Bogaert *et al*., 2004b). Polysaccharides are T-independent antigens, and their effect is mediated by B cells without the involvement of T cells, and as such have very low immunogenicity in children under two. This class of antigens also induce poor memory immune responses, so boosting does not result in significantly higher antibody titres than a single immunisation (Swiatlo & Ware, 2003).

However, these weaknesses can be overcome by covalently linking polysaccharides with a carrier protein (de Roux & Lode, 2005), a technique that has been successful in the *Haemophilus influenzae* Type b and group C meningococcal vaccines (Booy *et al*., 1994; Girard *et al*., 2006).

In 2000, a heptavalent protein-polysaccharide conjugate vaccine (Prevnar - Wyeth, USA) was licensed for use in infants (less than 5 years of age) in the USA. This vaccine, that contains polysaccharides of serotypes 4, 6B, 9V, 14, 18C, 19F and 23F conjugated to diphtheria CRM197 protein (Shinefield *et al*., 1999) has been approved by the Food and Drug Administration (USA) and the Committee on Proprietary Medicinal Products (Europe) for the prevention of invasive disease in children (Bogaert *et al*., 2004b). Since the polysaccharide is conjugated to a protein carrier, infants' immune systems respond to the polysaccharide in a T-dependent manner and produce memory B cells (McDaniel & Swiatlo, 2004). In fact, it has been shown capable of inducing antibody production and immunological memory in very young children with an immature immune system (de Roux & Lode, 2005). The vaccine can be administered with other childhood vaccines and is generally well tolerated (McDaniel & Swiatlo, 2004).

In a study conducted by Black *et al*. (2000) the vaccine was shown to be highly effective (>90%) in preventing invasive disease in infants caused by the 7 serotypes included in the vaccine. This vaccine also showed to significantly reduce pneumococcal carriage and to decrease episodes of otitis media (López & García, 2004). In addition, several studies have shown a significant reduction in nasopharyngeal carriage of vaccine type pneumococci in infants (Ghaffar *et al*., 2004).

Unfortunately, this vaccine is expensive to produce and will be difficult to implement on a large scale in developing countries (Swiatlo & Ware, 2003;

Bogaert *et al*., 2004b). Another added complication with the conjugated 7-valent vaccine is the replacement of vaccine types with non-vaccine types, and the potential of these non-vaccine types to also cause invasive disease (Jomaa *et al*., 2005). Also the limited number of various capsular polysaccharides that can be included in the vaccine is considered a drawback (Ling *et al*., 2004). Furthermore, the need for varied formulations including different serotypes for different regions of the world is another issue that will affect the global impact of this vaccine (McDaniel & Swiatlo, 2004).

The current pneumococcal vaccines elicit immune responses to the polysaccharide capsules. However, there are two major disadvantages associated with these capsular-based vaccines. First, the requirements to conjugate a number of polysaccharide types to a protein carrier to give efficacy in young children who are unable to mount protective immune responses to polysaccharides (as mentioned above); and secondly, conjugation restricts the number of polysaccharide types that can be included in any given formulation (Jomaa *et al*., 2005).

An alternative approach that may offer broader coverage, be less costly and can have the potential to be used in all age groups is a vaccine based on one or more proteins common to all serotypes (McDaniel & Swiatlo, 2004). Protein-based vaccines have the advantages of being antigenically conserved across capsular types, comparatively inexpensive to produce by recombinant DNA techniques and able to induce memory responses which are long lasting (Swiatlo & Ware, 2003). Several studies performed with animals have demonstrated the ability of protein-based vaccines to protect against experimental pneumococcal disease (Jomaa *et al*., 2005). However, there is a need for a better understanding in the role in pathogenicity of several pneumococcal proteins that are being studied for their potential to induce protective immune responses.

1.5 Virulence factors of *Streptococcus pneumoniae*

This bacterium synthesises several factors that are thought to play a role in virulence. An important feature of *S. pneumoniae* is its capacity to produce capsular polysaccharide (Fig. 1.4), which is essential for pneumococcal virulence

(Morona *et al*., 2006). However, pneumococcal capsular serotypes differ significantly in their virulence for man (Austrian, 1997), as certain types are strongly associated with human disease. The capsule itself is not inflammatory (Mitchell *et al*., 1997), in contrast to unencapsulated pneumococci, which have strong inflammatory properties. Capsular polysaccharide, therefore, is not necessary for inflammation (Tuomanen *et al*., 1995). The capsule is believed to function primarily to help the pneumococcus evade phagocytosis (McDaniel & Swiatlo, 2004). Moreover, they are usually associated with increased virulence as they may function as adhesins and/or recognition molecules (López & García, 2004). In addition to protection from host humoral immune components, the pneumococcal capsule may inhibit interactions of surface proteins with phagocytes during invasive disease (Swiatlo *et al*., 2002). The polysaccharide capsules of pathogens such as *S. pneumoniae* are generally negatively charged polymers, which are thought to protect the bacterial cell from humoral immune mechanisms. This net negative charge of pneumococcal capsular polysaccharide may also function to electrostatically repel the bacteria from phagocytic cells, which usually have a net negative charge of -18 to -12 mV at their surface (Magnusson, 1989).

In contrast to the capsule, the cell wall (Fig. 1.4) is a potent inducer of inflammation, probably via the activation of complement and the induction of cytokines (Catterall, 1999). Cell wall components released by enzymatic degradation are more potent chemotatic factors than are intact cell walls, a finding relevant to the consequences of bacterial lysis induced by antibiotics (Tuomanen *et al*., 1995) and/or autolysis. The components of pneumococcal cell walls stimulate the recruitment of leukocytes into the lung and subarachnoid space, enhance the permeability of cerebral endothelia and pulmonary alveolar epithelia, initiate the procoagulant cascade, stimulate the production of platelet activating factor (PAF), cause direct damage to neurons and alter cerebral blood flow and vascular perfusion pressure (Tuomanen *et al*., 1995). The presence of teichoic acid in the cell wall of the pneumococcus enhances the inflammatory activity of the cell wall components (Mitchell *et al*., 1997).

In addition to surface polysaccharides, the pneumococcus contains a number of proteins that have been shown to contribute to virulence (Fig. 1.4).

Some of the pneumococcal proteins that are physically associated with the cell wall include five penicillin-binding proteins (PBPs), neuraminidase A (NanA) (see §1.5.1 below), and an IgA1 protease (Todar, 2003).

Three groups of surface proteins can be distinguished by genome analysis: the lipoproteins, the choline-binding proteins (CBP) family, and proteins with an LPxTG motif (leucine, proline, x, threonine, and glycine, where x is any amino acid). The later are covalently anchored in the cell wall after cleavage of the LPxTG sequence by a transpeptidase, designated a sortase (Bergmann & Hammerschmidt, 2006).

PBPs are membrane-associated molecules present in all eubacteria that are involved in the last steps of the peptidoglycan synthesis (Pagliero *et al*., 2005). This class of proteins are the classical target enzymes for β-lactam antibiotics (see §1.4). In the pneumococcus five PBPs of high molecular weight (PBPs 1a, 1b, 2x, 2a and 2b) and one PBP of low molecular weight have been described. Alterations in PBP2x and PBP2b confer low-level β-lactam resistance and are the prerequisite for high-level resistance mediated by mutations in other PBPs, like PBP1a. Resistance in many pneumococcal clinical isolates (see §1.4) is due to changes in only these three PBPs (Charpentier & Tuomanen, 2000).

IgA1-protease (Fig. 1.4) is part of the group of pneumococcal zinc metalloproteases that also include ZmpB, ZmpC and ZmpD (Oggioni *et al*., 2003). Virtually all *S. pneumoniae* strains produce IgA1-protease, which is anchored to the cell wall by an N-terminal LPxTG motif (Bergmann & Hammerschmidt, 2006). IgA1-protease cleaves human IgA1 at a specific point within the hinge region (Paton *et al*., 1993). It is believed that IgA1-protease may allow invading pneumococci to escape the local mucosal defence system, since IgA1 is the principal mediator of specific immunity in the upper airways (Mitchell *et al*., 1997). Recently, Weiser *et al*. (2003) have demonstrated that cleavage of surface-bound serotype-specific IgA1 by IgA1-protease markedly enhances adherence of pneumococci to host cells.

Pneumococci express a family of choline-binding proteins (CBPs), which are surface-exposed and possibly function as specific adhesins for glycoconjugates on eukaryotic host cells. Some members of this family specifically bind the secretory component of secretory immunoglobulin A (S-IgA) (Hammerschmidt *et al*., 1997) and complement components, like human complement factor H (Dave *et al*.,

2001). Pneumococcal CBPs share an N- or C- terminal choline-binding domain made up of homologous repeats of about 20 amino acid residues, allowing them to anchor to the cell envelope through a non-covalent interaction with cell surface-located choline residues. Analysis of the pneumococcal genome revealed 13 to 16 different CBPs, depending on the sequenced strain, whose functional role remains unknown in many cases (García *et al*., 2005). The CBP family includes several important molecules like PspA (pneumococcal surface protein A, see Fig. 1.4) a protective antigen, four cell wall hydrolases: the major autolysin LytA amidase (*N*-acetyl-muramoyl-L-alanine amidase; see Fig. 1.4 and §1.5.2) (García *et al*., 1985), a β-*N*-acetylglucosamidase (LytB) (De Las Rivas *et al*., 2002), a β-*N*-acetylmuramidase (LytC; lysozyme) (García *et al*., 1999b) and a phosphorylcholine esterase Pce (or CbpE) (Vollmer & Tomasz, 2001), and CbpA (choline binding protein A) a major adhesin (Tuomanen, 1999).

CbpA (also known as PspC, pneumococcal surface protein C [Fig. 1.4], or SpsA) is a predominant protein in the mixtures of CBPs isolated from pneumococci (Rosenow *et al*., 1997) and it has received attention from several laboratories as a potential vaccine candidate (Tuomanen, 1999). The function of CbpA in adherence to host tissues and in colonisation was confirmed by studies of $CbpA^-$ mutant pneumococcal cells. The *cbpA*-deficient mutant cells lost the ability to interact with cytokine-activated type II host cells, with immobilised 6'-siallylactose–human serum albumin (HSA) (6'SL), and with lacto-N-neotetraose-HSA (LnNT) (Rosenow *et al*., 1997), suggesting that this protein plays a role in adherence (Mitchell, 2000).

Studies have shown that PspA (Fig. 1.4) attaches itself to *S. pneumoniae* by noncovalent binding to the choline of both lipoteichoic and teichoic acids via its C-terminal end, consisting of the repeat region, also called the choline binding region (CBR) (McDaniel *et al*., 1992). This highly variable (both at the DNA and protein sequence levels [Baril *et al*., 2006]) pneumococcal surface protein is expressed by virtually all important clinical serotypes and has a significant immune protective potential (Bergmann & Hammerschmidt, 2006). Studies have shown that PspA can induce an antibody response against both a challenge dose in an animal model (Briles *et al*., 2000c; Briles *et al*., 1996) and in humans (Baril *et al*., 2006).

Pneumococcal lipoproteins, including peptide permeases, have been shown to be essential for substrate transport and bacterial fitness (Bergmann & Hammerschmidt, 2006).

Pneumococcal surface adhesin A (PsaA, see Fig. 1.4) is a membrane-anchored virulence factor, possibly involved in Mn^{2+} and Zn^{2+} transport (Lawrence *et al*., 1998). PsaA deletion mutants display low ability to adhere to mucosal cells and consequently are less pathogenic (Berry & Paton, 1996). This characteristic may be due to differences in the modulation of pneumococcal adhesins caused by the absence of Mn^{2+} or Zn^{2+} in the cell (Jedrzejas, 2001). PsaA is produced by all pneumococci and is also immunogenic, which makes it a good candidate for vaccine formulations (Baril *et al*., 2006). Antibodies produced against PsaA have been shown to protect mice against nasopharyngeal colonisation by *S. pneumoniae* (Briles *et al*., 2000a). This protection can be further increased by the co-administration of PsaA with the pneumococcal surface protein A (PspA) (Briles *et al*., 2000b).

Pneumolysin (Ply, see Fig. 1.4) is another pneumococcal protein that plays an important role in the virulence of *S. pneumoniae*. Pneumolysin is a 53-kDa protein produced by all clinical isolates of the pathogen. Unlike other pneumococcal antigens, this molecule is not surface exposed. It is a cytoplasmic protein thought to be released due to the action of surface pneumococcal autolysin (Canvin *et al*., 1995). This haemolytic toxin lysis cholesterol-containing cell membranes and activates complement (Mitchell *et al*., 1991). The first direct evidence for the involvement of pneumolysin in pathogenesis was the finding that immunisation with highly purified toxin significantly increased the survival time of mice challenged intranasally with virulent pneumococci (Paton *et al*., 1983). Previous studies in our laboratory have showed that pneumolysin is crucial in the pathogenesis of pneumococcal bronchopneumonia and septicemia (Canvin *et al*., 1995; Kadioglu *et al*., 2000). More recently, Jounblat *et al*. (2003) have shown that both pore formation and complement activation by pneumolysin are involved in inflammation and cellular influx to the lung seen in pneumonia, however these activities of the toxin contribute differently to the level of host immune response.

1.5.1 Neuraminidase

General characteristics

Neuraminidases, also known as sialidases, are a family of enzymes present in microbes, parasites and mammalian tissue, which catalyse the hydrolytic removal of terminal sialic acid residues (see below), usually present at nonreducing terminal position, from mucin and a wide variety of glycolipids, glycoproteins and oligosaccharides on cell surfaces or in body fluids. In animals, neuraminidases play an important role in the turnover of sialoglycoconjugates, influencing the development of cells (Schauer, 2000), whereas in microorganisms the function of this enzyme has not yet been finally clarified (Abrashev & Dulguerova, 2000). Remarkably, neuraminidases are often found in viruses, parasites and bacteria, which live in close contact with an animal host, where they are thought to be important in nutrition, colonisation and pathogenesis (Corfield, 1992).

The term 'sialic acid' (SA) is a generic term for a large family of nine-carbon monosaccharides (approx. 40 members) of naturally occurring analogues of *N*-acetylneuraminic acid (*N*-acyl derivative of neuraminic or acid amino sugar derivative, derived from *N*-acetylmannosamine and pyruvic acid, see Fig. 1.5). The appearance of various analogues is correlated with cell type, cell age, tissue type and species, with some analogues protecting glycoconjugates from attack by neuraminidases (Taylor, 1996). The diversity of sialic acids lies in the various substitutions at different carbon positions (carbon- 4,5, 7, 8 and 9), in addition to various linkages from carbon-2 to other sugar chains (Fig. 1.6). The predominant linkages that have been observed for sialic acid in glycoconjugates are SA-α-(2$\rightarrow$3)-Gal-R, SA-α-(2$\rightarrow$3)-GalNAc-R, SA-α-(2$\rightarrow$6)-Gal-R, SA-α-(2$\rightarrow$6)-GalNAc-R, SA-α-(2$\rightarrow$8)-SA-R where R is the carbohydrate core structure (Veluraja & Rao, 1984). It is generally accepted that the recognition of a particular sialic acid is based on its specific structure and that any substitution or change in linkage will also alter the recognition of a particular sialic acid (Varki, 1997). These monosaccharides do not occur randomly; they are expressed both quantitatively and qualitatively depending on the microorganism or animal species, as well as on the kind of tissue or cell and on the function or developmental stage (Schauer,

2000). Sialic acid is quantitatively the major carbohydrate-derived molecule in vertebrates where it is terminally linked to cell surface sialoglycolipids and sialoglycoproteins, providing a barrier of negatively charged sialic acid over the entire surface of the cellular plasma membrane (Rosenberg, 2004). Due to their negative charge, sialic acids are involved in the binding and transport of positively charged molecules, as well as in the attraction and repulsion of cells and molecules (Cross *et al*., 2003). In this way, as components of glycoproteins, they contribute to the high viscosity of mucins lining and protecting endothelia, for instance in the respiratory tract (Ryan *et al*., 2001). This anti-recognition effect of sialic acids, exerted by the negative charge, is an important field comprising the masking of the penultimate sugars like galactose which can be recognised by receptors (Schauer, 2000). This is the case with complement regulatory factor H, a soluble factor in serum that binds to sialic acid on cell surfaces and restricts alternative pathway activation (Varki, 1997). Sialic acid on a surface has been shown to prevent activation of the alternative pathway by increasing the affinity of H for C3b bound to the surface (Koistinen, 1992).

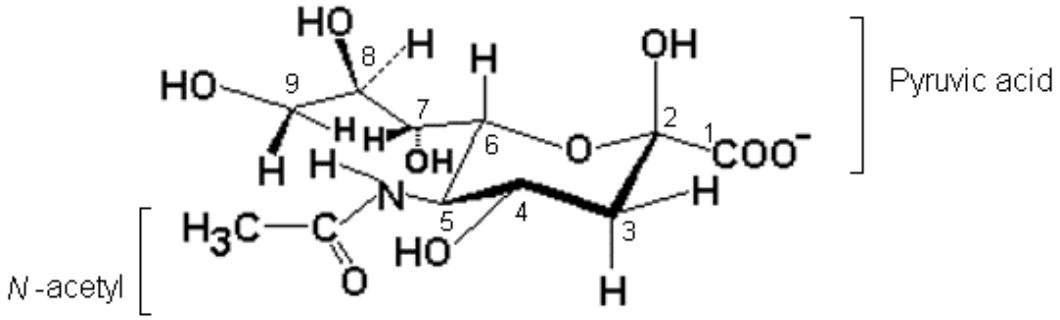


Figure 1.6 Structure of *N*-acetylneuraminic acid ($C_{11}H_{19}NO_9$) showing the pyruvic acid residue (-$CH_2COCOOH$) and *N*-acetyl group (Adapted from Schauer, 2000).

In contrast, the importance of sialic acids has also been demonstrated in both immunological and recognition processes. Its presence on the cell surface is an important determinant of that cell's interaction with other cells (Cross *et al*., 2003). An example of the importance of SA in recognition processes is shown with the trafficking of leukocytes in endothelial tissues. In this case, selectins (sialic acid-binding lectins), which are found on mammalian endothelial cells, participate in the initial stage of adhesion of leukocytes to endothelia, along which they eventually evade to the underlying tissues (Schauer, 2000). The importance of sialic acids in immunological processes is shown by the activation of T cells in an immune response, which is accompanied by a decrease in the sialylation of glycoproteins on the surface of activated T cells. This is necessary to render the cells responsive to antigen presenting cells, such as B cells (Taira & Nariuchi, 1988). Another example is the desialidation of immunoglobulin G, which results in decreased capacity to bind complement (Moncla *et al*., 1990).

The location of sialic acids at the termini of various carbohydrate complexes associated with animal cells is exploited by a broad spectrum of microbial pathogens. Certain pathogens have proteins that recognise sialic acid for cell attachment, and many of these pathogens have acquired neuraminidases to aid in their pathogenesis and/or nutritional requirements. Neuraminidases have been shown to decrease collagen synthesis in rat granulation tissues and fibroblasts, directly stimulate lymphocytes, inhibit macrophage inhibitory factor and interleukin 2, and destroy cell receptors such as G_{M1}. There is some evidence that the oxidative burst in human blood leukocytes is decreased in response to bacterial neuraminidases; however, there are conflicting data on this point (Moncla *et al*., 1990).

Bacterial neuraminidases have been considered virulence factors in many pathogenic organisms, which colonise mucosal surfaces, such as *Helicobacter pylori* (Dwarakanath *et al*., 1995), *Vibrio cholerae* (Galen *et al*., 1992), *S. pneumoniae* (Paton *et al*. 1997; Berry & Paton, 2000), and group B streptococci (Davis *et al*., 1979). The influenza type A and B viruses also posses two surface glycoproteins, hemagglutinin and neuraminidase, which recognise sialic acid for attachment (Colman, 1994).

The structure and active site of the influenza-virus neuraminidase have been shown to be well conserved, despite sequence identities down to 40% (Colman,

1994). The sequence identity between the influenza enzymes and the bacterial enzymes is very low, at 15%, and even between the non-viral enzymes the sequence identities are only 30% (Roggentin *et al*., 1989). Nevertheless, the topology of the catalytic domain is conserved, and the active sites share many features (Taylor, 1996). Work in several laboratories revealed two motifs in the neuraminidase primary structures, one a so-called aspartic box (Asp), which is a stretch of amino acids of the general formula -Ser-x-Asp-x-Gly-x-Thr-Trp where x represents variable residues. This motif is found four to five times in all microbial sequences studied, with the exception of viral neuraminidases, where it is found only once or twice or is even absent (Taylor, 1996). It also occurs in the neuraminidases from eukaryotic origin, that is, from mammals, including man. In contrast to the Asp boxes, which are found throughout the sequence, another motif, F(Y)RIP, is located in the N-terminal part. It encompasses the amino acids x-Arg-x-Pro with the arginine and proline residues absolutely conserved. While this region, *via* arginine, is directly involved in catalysis by binding of the substrate molecule, the Asp boxes are believed, based on X-ray analysis, to play a role in maintaining the enzyme structure (Roggentin *et al*., 1989).

Despite these similarities between the active sites of neuraminidases, they also exhibit differences that reflect differences in kinetics (turnover of bacterial neuraminidases is typically 1000 times that of the influenza enzyme), binding affinities (bacterial enzymes do not bind glycans with *N*-acetylneuraminic acid *O*-acetylated at carbon- 4, in contrast to viral enzymes) and substrate preferences (Taylor, 1996). Indeed, neuraminidase substrate specificity may give clues as to the physiological function of the enzyme and its primary site of action; therefore multiple substrate analysis should be made for complete characterisation of the enzyme. Some features of neuraminidase substrate specificity include the nature of the glycoconjugate, the type of glycosidic linkage (*e.g.* α-2$\rightarrow$3, α-2$\rightarrow$6, α-2$\rightarrow$8) and the substitution of hydroxyl groups at carbon 7-9 (neuraminidase activity is reduced by increasing *O*-acetylation of these hydroxyl groups). Neuraminidase enzymes of bacterial origin are non-specific for the type of sialyl-oligosaccharides, which serve as substrates, *i.e.* their activity does not depend on whether the SA is linked to the carbon-3 of Gal or GalNAc, carbon-6 of Gal or GalNAc or carbon-8 of another SA. On the other hand, virus neuraminidases (Newcastle disease virus,

Fowl plague virus and Influenza A2 virus neuraminidases) cleave α-(2→3) but not α-(2→6) linkages (Veluraja & Rao, 1984).

In contrast to bacterial neuraminidases, potent inhibitors are now available against influenza virus neuraminidase. Based on the knowledge that Neu5Ac2en (2-deoxy-2,3-dehydro-*N*-acetylneuraminic acid) is a natural inhibitor of most neuraminidases, including viral ones, as well as on the known shape and distribution of charges in the catalytic pocket of viral neuraminidases, which allows hydrolysis of sialic acid even with substituents at carbon-4, in contrast to non-viral neuraminidases (Kleineidam *et al*., 1990), the 4-amino- and 4-guanidino derivatives of Neu5Ac2en were synthesised. The latter compound especially was found to be a very strong inhibitor of influenza A and B virus neuraminidases (Woods *et al*., 1993). This inhibitor is now in medical use and weakens virus propagation, and consequently the pathological symptoms of influenza (von Itzstein *et al*., 1993). Unfortunately, an inhibitor with a high inhibitory constant is not yet available for neuraminidases from virulent bacteria.

Neuraminidases of the pneumococcus

Research investigating deglycosylation of human glycoconjugates by *S. pneumoniae* has centred on neuraminidase activity and its contribution to desialylation in pneumococcal colonisation and virulence. Neuraminidase is expressed by all clinical isolates of *S. pneumoniae* (Kelly *et al*., 1967; O'Toole *et al*., 1971). Two families of bacterial neuraminidases have been identified on the basis of their molecular size (Roggentin *et al*., 1993). The family of small enzymes have molecular sizes of around 42kDa, whereas the large neuraminidases have molecular sizes greater than 60kDa. Pneumococcus has been shown to express a major 115kDa form of neuraminidase, NanA (Berry *et al*., 1988), and a minor form, NanB with 78kDa (Camara *et al*., 1994; Berry *et al*., 1996), both enzymes belonging to the large family of bacterial neuraminidases. A third neuraminidase gene, *nanC* with 82kDa, found in the *S. pneumoniae* TIGR4 genome (GenBank AE005672) but not in R6 (GenBank AE007317), remains to be characterised (Shakhnovich *et al*., 2002; Tettelin *et al*., 2001). Pneumococcal neuraminidase was reported to hydrolyse α-(2→3)-, α-(2→6)-, α-(2→8)- glycosidic linkages of

terminal sialic acid residues in oligosaccharides, glycoproteins and glycolipids (Schauer, 1982). Scanlon *et al*. (1989) also found that pneumococcal neuraminidase acted on at least three linkage classes of substrates, α-(2$\rightarrow$6) and α-(2$\rightarrow$3) linkages of *N*-acetylneuraminic acid to galactose, and α-(2$\rightarrow$6) linkages to *N*-acetyl-galactosamine. But no attempt was made in these studies to separate different neuraminidases.

Pneumococcal neuraminidases seem to play a role in pathogenesis. However, the precise role for these enzymes in disease has not yet been established. *Streptococcus pneumoniae* neuraminidases might aid colonisation by revealing receptors for adherence, provide a carbon source for the bacteria or by modifying the surface of competing bacteria within the same niche (Kelly *et al*., 1967; Schauer, 2000; Shakhnovich *et al*., 2002; Tong *et al*., 2000; 2002). Neuraminidase might unmask potential cell-surface receptors for pneumococcal adhesins (Andersson *et al*., 1983). It has been suggested that the release of *N*-acetylneuraminic acid from gangliosides may contribute to morbidity and mortality of pneumococcal meningitis (O'Toole *et al*., 1971). In addition, in patients with pneumococcal meningitis there was a direct relationship between the level of *N*-acetylneuraminic acid in cerebrospinal fluid and the development of coma and bacteremia (O'Toole *et al*., 1971). Neuraminidase treatment of mucus could decrease its viscosity, thereby enhancing the colonisation of the underlying tissues by the pneumococcus (Scanlon *et al*., 1989). Another study investigating the histochemistry of organs from mice dying after the intraperitoneal administration of partially purified pneumococcal neuraminidase has indicated marked decreases in sialic acid content of the kidney and liver when compared with controls (Kelly & Greiff, 1970). In contrast, Paton *et al*. (1997) and Berry & Paton (2000) found no role for pneumococcal neuraminidases in the host-bacterial interaction beyond the mucosal surface as determined in intraperitoneal infection models. Further assessment of the contribution of neuraminidase to pneumococcal pathogenicity showed that purified neuraminidase is toxic for mice, but immunisation with the inactivated protein partially protected mice from challenge with virulent *S. pneumoniae* (Lock *et al*., 1988). Conversely, intrathecal inoculation of the enzyme in dogs did not produce any symptoms (O'Toole & Stahl, 1975).

Neuraminidase A (NanA)

Camara *et al*. (1994) cloned the gene that codes for one of the enzymes with neuraminidase activity (*nanA*) and showed its product to be a surface protein. They found that NanA (approx. 115 kDa in size) has an N-terminal signal peptide, while the C-terminus has a sequence with features of the anchor motif LPETG (see §1.5) found in surface proteins of Gram-positive bacteria. The activity of NanA (when assayed at its pH optimum of 6.5) is 100 times greater than that of NanB against a fluorogenic substrate (Lock *et al*., 1988; Berry *et al*., 1996). Availability of the gene that codes for NanA activity in *S. pneumoniae* allowed the construction of an isogenic mutant that does not express *nanA*. Mitchell *et al*. (1997) reported the construction of a NanA-negative mutant of *S. pneumoniae* constructed by insertion-deletion mutagenesis and found that this mutant was less virulent than the wild type parent in a murine model of pneumonia. Furthermore, Tong *et al*. (2000) showed that loss of NanA impaired pneumococcal persistence in the nasopharynx and middle ear in a chinchilla infection model. Moreover, in 2002 the same group also reported that a *nanA* knockout has diminished ability to adhere to the chinchilla tracheal epithelium and to alter the cell surface carbohydrate architecture (Tong *et al*., 2002). In contrast, Winter *et al*. (1997) saw no distinguishable differences between wild type *S. pneumoniae* D39 and its neuraminidase-deficient derivative in terms of meningeal inflammation, growth in the cerebrospinal fluid (CSF), bacteremia, or cochlear damage using a model of experimental meningitis, where guinea pigs were infected intracranially. In addition, when using the $NanA^-$ negative mutant in an intraperitoneal infection model, Berry & Paton (2000) reported that the mutant was fully virulent compared with the wild type parent.

The ability of NanA to provide protection against nasopharyngeal colonisation and otitis media in chinchilla models (Long *et al*., 2004) has emphasized the role of this enzyme in colonisation. NanA has been shown to be implicated in *in vivo* desialylation of human proteins exhibiting sialic acid, including the secretory component of IgA (hSC), where NanA acts on α-(2$\rightarrow$3) and α-(2$\rightarrow$6) linked sialic acid residues, lactoferrin and hIgA2 (King *et al*., 2004). Furthermore, recently the same group also indicated that NanA might provide the first enzymatic step in the

exposure of mannose on human glycoconjugates, and that this activity may be a factor contributing to the ability of *S. pneumoniae* to colonise the human nasopharynx (King *et al*., 2006).

Neuraminidase B (NanB)

Berry *et al*. (1996) cloned and sequenced a second pneumococcal neuraminidase gene, *nanB*. They showed that in *S. pneumoniae* R6, this gene is located on the pneumococcal chromosome approx. 4.5kb downstream of *nanA* (Fig. 1.7). The product of this gene (NanB) was shown to be much smaller that NanA, having a mass of 78kDa. NanB is 698 amino acids long, including a putative 29-residue signal peptide. Comparison of the NanB sequence with that of NanA, using TIGR4 protein alignment sequence tool (http://cmr.tigr.org), revealed only 43% amino acid sequence similarity.

However, pneumococcal NanB has a limited degree of homology with the neuraminidase of *Clostridium septicum*, and the region of greatest similarity includes an F(Y)RIP motif that is found in the active site of other bacterial neuraminidases (Roggentin *et al*., 1989). Moreover, NanB from *S.pneumoniae* R6 has 99% homology with the sequence deposited in GenBank for NanB from *S. pneumoniae* TIGR4. NanB also contains three copies of the aspartic box consensus motif that is common to other neuraminidases (see §1.2.1 General characteristics, above). However, unlike NanA, NanB does not contain an L-P-x-T-G motif found in surface proteins (Berry *et al*., 1996).

Berry *et al*. (1996) also have assayed the pH optimum for NanB and found it to be approx. 4.5, which is considerably lower than that for NanA (see above), suggesting that they may act at different sites or stages during an infection.

The benefits to the pneumococcus of production of two (or more) distinct neuraminidases are unclear. The contribution of *nanB* to the pathogenesis of *S. pneumoniae* remains to be investigated. Berry *et al*. (1996), however, have suggested that the production of distinct neuraminidases may assist exploitation and invasion of distinct anatomical sites by *S. pneumoniae*.

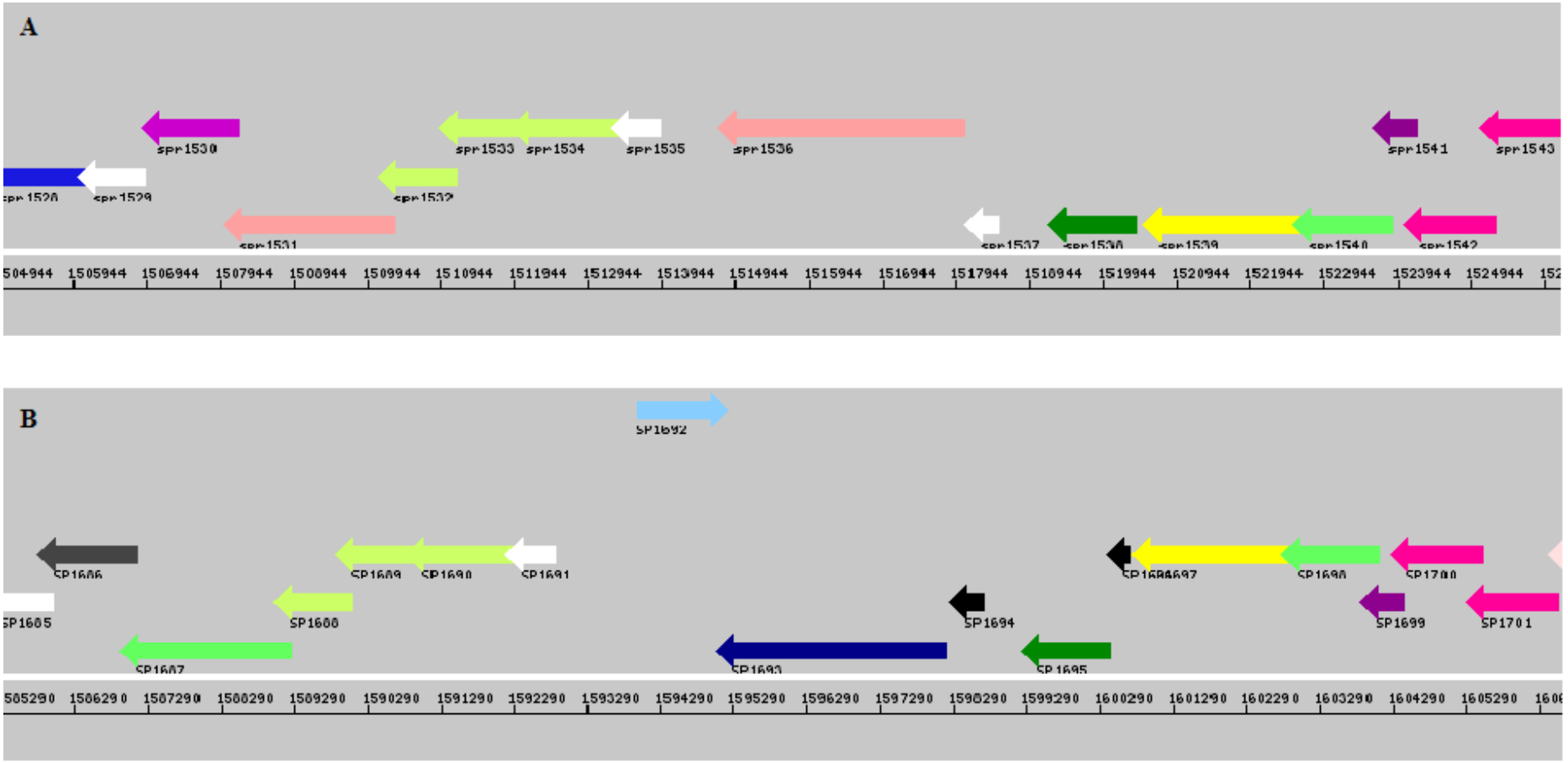


Figure 1.7 Graphical view of a small region of *S. pneumoniae* DNA surrounding neuraminidase genes *nanA* and *nanB*. Panel A: (in pale pink) genes spr1531 (*nanB*) and spr1536 (*nanA*) in the genome of strain R6 (coordinates 1504944 - 1528051); Panel B: genes sp1693 (*nanA*) (in navy blue) and sp1687 (*nanB*) (in light green) in strain TIGR4 (coordinates 1585291 – 1608177) (from http://www.tigr.org/ using the Annotation tool: Region view).

1.5.2 Autolysin

General characteristics of Cell Wall Hydrolases

The cell wall of Gram-positive bacteria is host to a wide variety of molecules and serves a multitude of functions, most of which are critical to the viability of the cell. Although the primary function of the cell wall is to provide a rigid exoskeleton for protection against both mechanical and osmotic lysis (Salton, 1953), the cell wall of Gram-positive bacteria also serves as an attachment site for proteins that interact with the bacterial environment (Navarre & Schneewind, 1999). The cell wall of Gram-positive bacteria is a peptidoglycan macromolecule, which is covalently and noncovalently decorated with teichoic acids (see §1.1, Fig.1.3), lipoteichoic acids, polysaccharides, and proteins (Hancock, 1997).

Murein hydrolases are enzymes that cleave covalent bounds of the cell wall peptidoglycan; some can eventually cause cell lysis and are called autolysins. Murein hydrolases have been found in practically all known microorganisms, and their wide distribution in bacteria, especially autolysins, has led to the idea that these enzymes participate in a variety of fundamental biological roles, such as the synthesis of cell wall, separation of the daughter cells at the end of the cell division and genetic transformation (Navarre & Schneewind, 1999). Moreover, lytic enzymes are responsible for the irreversible effects of β-lactam antibiotics (Charpentier & Tuomanen, 2000).

Bacterial cell wall hydrolases (CWH) are endogenous enzymes that specifically cleave covalent bounds of the cell wall and that can either degrade or remodel it (López *et al*., 2000). These enzymes show both substrate and bond specificities. The latter allows the classification of cell wall hydrolases as: (1) glycosidases (lysozymes) which hydrolyze the β-(1-4)- glycosidic bound between NAMA (*N*-acetylmuramic acid) and NAG (*N*-acetylglucosamine), and transglycosilases, which cleave the same bound as lysozymes but do not transfer the glycosyl moiety onto water but onto C-6 hydroxyl group of muramic acid (Holtje *et al*., 1975); (2) amidases, which cleave the amide bond between the lactyl group of NAMA and the α-amino group of L-Ala; and (3) endopeptidases that cut within the peptide moiety of the peptidoglycan (López & García, 2004).

Sequence alignment of murein hydrolases of Gram-positive bacteria revealed that most of these enzymes' amino acid sequences display a domain structure. In general, murein hydrolases harbour an N-terminal signal peptide followed by a second domain containing the enzymatic activity. In addition, these proteins harbour repeat structures that flank either the N- or C-terminal side of the enzymatic domain (Joris *et al*., 1992). Although the enzymatic domains are often conserved between enzymes from different species, the repeat domains are not. In *S. pneumoniae*, investigations on LytA (autolytic amidase) revealed that the C-terminally localised choline-binding domain is responsible for the specific recognition of choline residues of teichoic and lipoteichoic acids (Fernandez-Tornero *et al*., 2001). The choline-binding domain is constituted of several imperfect repeats of 20 amino acids each and characterised by the presence of conserved aromatic residues. Such repeats (choline-binding repeats, CBRs) are classified under the accession number PF01473 (http://www.sanger.ac.uk/Software/Pfam/) (Bateman *et al*., 2004).

The proposal of this domain-like organisation was extended to many other murein hydrolases, as was the case of the clostridial Lyc muramidase and lytic enzymes of phages infecting *Streptococcus thermophilus* (López *et al*., 2000). In contrast, Margot *et al*. (1999) showed that in lytic enzymes of *Bacillus subtilis* the N-terminal domain contains a five-fold repeated motif attributed to cell wall binding, whilst the C-terminal domain is probably endowed with the catalytic activity.

Cell Wall Hydrolases of the pneumococcus

In *S. pneumoniae*, choline residues of the cell wall (see §1.1) act as binding ligands for choline-binding proteins, including the cell wall hydrolases (López & García, 2004). To date, four pneumococcal murein hydrolases, represented in Figure 1.8, have been described in *S. pneumoniae* (Tettelin *et al*., 2001): an amidase LytA (see below) which is the major pneumococcal autolysin (García *et al*., 1985); two glycosidases LytB (García *et al*., 1999a) a glucosaminidase responsible for cell separation at the end of cell division (De Las Rivas *et al*., 2002) and LytC a lysozyme which acts as an autolysin at 30ºC (García *et al*., 1999b); and the phosphorylcholine esterase Pce (also designated CbpE), which

possibly regulates the number of choline residues available at the pneumococcal cell surface (Vollmer & Tomasz, 2001). Murein hydrolases of pneumococcal bacteriophages have also been described (García *et al*., 1987).

The isolation of the *lytB* gene in *S. pneumoniae* type 4 genome (strain TIGR4) showed that it codes for a 76kDa protein with 658 amino acid residues (García *et al*., 1999a); however the sequence of *lytB* gene in strain R6 codes for a larger protein LytB (81kDa) than the *lytB* gene reported for strain TIGR4 (De Las Rivas *et al*., 2002). LytB, together with LytC, displays a modular organisation different from all the CBD proteins reported in the pneumococcal system (López *et al*., 2000). The CBD was found in the N-terminal region, whereas the active site of the enzyme was localised in the C-terminal domain. In addition, both enzymes contain cleavable signal peptides, in contrast to other pneumococcal and phage cell wall lytic enzymes (García *et al*., 1999b). The enzymes (LytB, LytC) contain a 23 amino acid-long cleavable signal peptide, close to which (in LytB) an 18 repeat motif has been identified (García *et al*., 1999b; De Las Rivas *et al*., 2002).

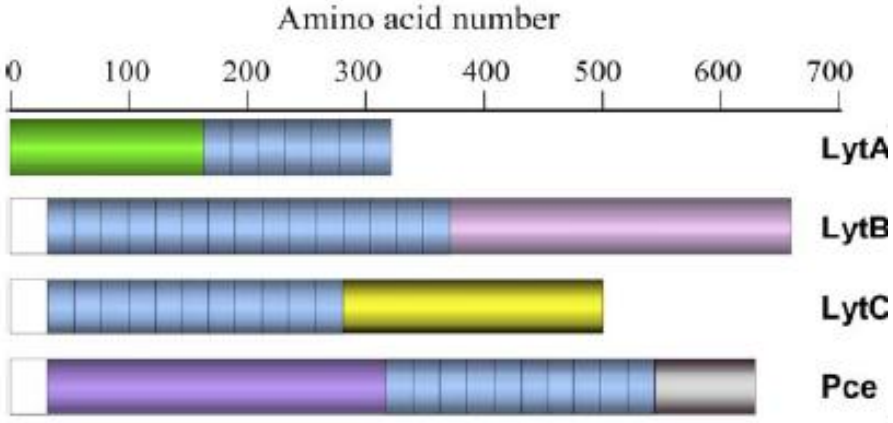


Figure 1.8 Diagram representing the structures of the pneumococcal LytA, LytB, LytC and Pce cell wall hydrolases. The domain containing the active centre of the enzymes is represented in light green (amidases), yellow (lysozymes), violet (glucosaminidase), or purple (phosphorylcholine esterase). The CBD (blue) and the CBRs are also shown. Signal peptides of LytB, LytC and Pce are depicted as open rectangles. Homology between CWHs is indicated by identical colors. (Adapted from López & García, 2004).

When analysing the biological properties of LytB, De Las Rivas *et al*. (2002) found that the protein is capable of degrading choline-containing pneumococcal cell walls *in vitro*, but the rate of choline degradation and release was very slow, in contrast to the activity reported for LytA and LytC. In fact, LytB appears to be the first non-autolytic murein hydrolase of the pneumococcus. Moreover, they found no role for LytB in competence (defined as the ability of cells to take up DNA). *lytB* mutants were genetically transformable at rates similar to those of the parental strain (De Las Rivas *et al*., 2002). A role for LytB was suggested by García *et al*. (1999a) in separation of daughter cells at the end of cell division. They showed, using a *lytB* mutant, that pneumococcal cells exhibited formation of long chains of more than 100 cells. Another group, Gosink *et al*. (2000), investigating the role of LytB, LytC and Pce in virulence of *S. pneumoniae*, suggested that the significant loss of ability of these mutant strains to colonise the nasopharynx of infant rats raises the possibility that these enzymes could play a role in toxin release. However, no changes in virulence were observed when the mutants were tested in a sepsis model.

The pneumococcal gene *lytC* encodes a protein (lysozyme) of 501 amino acid residues with a mass of 58kDa. As mentioned above, in contrast to LytA amidase, LytC has a cleavable signal peptide of 33 amino acid residues (García *et al*., 1999b). The N-terminal part of LytC revealed the presence of 11 motifs showing significant similarity with those of the CBD of several pneumococcal proteins. The C-terminal domain revealed similarities with several lysozymes, in particular, to the catalytic domain of the Cpl1 muramidase, from the Cp-1 pneumococcal phage (López *et al*., 2000). Combined cell fractionation and western blot analysis showed that the unprocessed, primary product of *lytC* is located in the cytoplasm, whereas the processed, active form is tightly bound to the cell envelope (García *et al*., 1999b). This group also showed that the inactivation of *lytC* in the *lytA* mutant strain (M31) makes the cells resistant to autolysis upon incubation at 30ºC suggesting that LytC behaves as autolysins at this temperature (García *et al*., 1999b). Besides, the *lytC* mutants either derived from strain R6 or M31 exhibited a normal growth rate and an average chain length similar to that of the parental strains. In this regard, it was suggested that LytC possibly plays a role in the pneumococcal virulence (López & García, 2004). DNA exchanges have been shown to take place in the natural environments (Ottolenghi-Nightingale, 1972),

and LytC might contribute to the liberation of DNA in habitats like the upper respiratory tract that is highly ventilated (Moscoso *et al*., 2005). In addition, as mentioned above, LytC also plays a role in the colonisation of the rat nasopharynx by the pneumococcus (Gosink *et al*., 2000).

The *pce* gene of pneumococcal strain R6 encodes a protein with 627 amino acid residues (Vollmer & Tomasz, 2001). Pce shows a modular organization of the enzyme where the CBD, involved in cell wall substrate recognition and the catalytic domain are located at the C- and N-terminal moieties of the protein, respectively. The C-terminal moiety of Pce is assembled by 10 repeated motifs, and the protein has also a cleavable signal peptide of 25 amino acids that renders after its cleavage a mature protein of 69KDa (De Las Rivas *et al*., 2001). Pce belongs to the metallo-β-lactamase family, and structural evidence shows that only phosphorylcholine residues that are located at the end of the teichoic acid chains are available to the catalytic centre (De Las Rivas *et al*., 2001). Studies investigating the role of Pce in virulence by *S. pneumoniae* have been performed. Gosink *et al*. (2000) showed that the inactivation of CbpE resulted in decreased colonisation of the nasopharynx of infant rats and decreased adherence to Detroit cells at 30ºC. Vollmer & Tomasz (2001) have reported the unusual property of the *pce* mutant strain, which possessed increased virulence for mice compared to the parental strain following i.p. infection. Pce might have a dual function both in colonisation and virulence by regulating the number of choline residues available at the pneumococcal cell surface (Vollmer & Tomasz, 2001).

N-acetylmuramoyl-L-alanine amidase: LytA

In the *S. pneumoniae* type 4 genome (strain TIGR4) the *lytA* gene encodes a predicted 318-aa protein that is the major *S. pneumoniae* autolysin. Cloning and sequencing of the *lytA* gene revealed an open reading frame specifying a 31kDa polypeptide (García *et al*., 1988). The open reading frame displays sequence homology to the gene sequences encoding enzymatic domains of many other known amidases (Romero *et al*., 1990). However, seven repeat domains located at the C-terminal end of LytA are not found in the amidase sequences from other bacterial species but are present in a variety of phage-encoded murein hydrolases

of *S. pneumoniae* (López *et al*., 2000). Pneumococcal LytA has been studied in detail and it is thought to exist in two states, a cytoplasmic inactive form (E-form) that is combined to the membrane-associated active counterpart (C-form) (García *et al*., 1985). The activity of this amidase is absolutely dependent on the presence of choline residues in the cell wall teichoic acids of this bacterium (García *et al*., 1985). The common repeat structure of pneumococcal cell wall teichoic acid and lipoteichoic acid [Glc-(β-1-3)-AAT-Gal-(α-1-4)-GalNac-(6-Cho-P)(α-1-3)-GalNac (6-Cho-P)(β-1-1)ribitol-P] suggests that LytA can bind to both structures. The *lytA* open reading frame did not reveal an N-terminal signal peptide (García *et al*., 1988) or a cell wall anchoring motif LPxTG (see §1.2) characteristic of many surface proteins of Gram-positive bacteria (Romero *et al*., 1990). So, the mechanism by which LytA is transported to the surface peptidoglycan is unknown. Either LytA is not exported under physiological conditions or its export may occur by another pathway. Díaz *et al*. (1989) showed that, when expressed in *E. coli*, pneumococcal LytA appears to be translocated across the cytoplasmic membrane being detected on the periplasmic side. Furthermore, immunogold labelling of *S. pneumoniae* revealed LytA staining on the cell surface, indicating that the enzyme is also translocated and surface displayed (Díaz *et al*., 1989). In this regard, future work is needed to further characterise the export mechanism of LytA.

Despite the prevalence of LytA in *S. pneumoniae*, the biological role of this enzyme, at least *in vitro*, seems to be quite limited. Previous studies by Sanchéz-Puelles *et al*. (1986) demonstrated that the primary consequences of the *lytA* gene deletion were the formation of small chains of six to ten cells and the absence of lysis at the stationary phase of growth (or upon addition of detergents or β-lactam antibiotics). So, LytA appears to have only a minor role in cell separation but is responsible for cellular autolysis at the end of the log phase. A role for LytA amidase in DNA release has been proposed, probably by cell lysis. This hypothesis was favoured since *lytA* mutants exhibited a two- to four-fold decrease in the amount of DNA available in the medium (Moscoso *et al*., 2005). LytA has been implicated in the pathogenicity of the pneumococcus. Berry *et al*. (1989b) showed that, following intranasal infection, autolysin-negative mutants had markedly reduced virulence for mice compared with that of the parental strain D39. Then, they demonstrated that mice immunised with the purified autolysin protein

survived significantly longer than control mice following intranasal challenge with strain D39 (Berry *et al*., 1989b). In the pneumococcus, the indirect implication of LytA in pathogenesis also is believed to involve the release of cytoplasmic proteins like pneumolysin and other intracellular toxins (Berry & Paton, 2000; Ng *et al*., 2002). The release of components of the cell wall following bacterial cell death has been previously shown to be highly inflammatory in some animal models (Sato *et al*., 1996). However, a study conducted by Balachandran *et al*. (2001) using strain WU2, which unlike strain D39 expresses extracellular pneumolysin (Ply) prior to stationary phase found that the release of Ply into the extracellular environment did not require the activity of any known pneumococcal autolysin. In 2000, Berry & Paton showed that the *lytA* mutant had significantly lower virulence for mice than did the wild type D39, as judged by both survival time and survival rate following intraperitoneal (i.p.) challenge. Recently, Kharat and Tomasz (2006) showed that deletion of *lytA* and *lytB* significantly reduces the lethality of strain D39 in a mouse model of i.p. infection.

1.5.3 Superoxide dismutase

Reactive oxygen species (ROS) such as superoxide, hydrogen peroxide, hydroxyl radical and nitric oxide have a broad-spectrum toxicity and are crucial to host defences for the optimal microbicidal activity of neutrophils and other phagocytes (Fridovich, 1997). In response, microorganisms have developed multifaceted strategies to defend themselves from oxidative stress induced endogenously or exogenously (Miller & Britigan, 1997).

Toxicity of oxidants in biological systems

Reactive oxygen intermediates (ROI) are a set of related molecules with individually distinct chemical and biological properties. ROI refers to all oxidation and excitation states of O_2, from superoxide ($^{\cdot}O_2^-$) up to but excluding water, that

arise in physiological environments, including singlet oxygen ($^1O_2$), ozone (O_3), hydrogen peroxide (H_2O_2), hypohalites, and hydroxyl radical ($^{\cdot}OH$) (Nathan, 2003). Oxidative stress, as part of the biological actions of ROI, does not exclusively mean reactions that cause harm. Indeed, ROI are routinely produced throughout the aerobic organisms and are often used as signalling molecules, including in the special case of host defence (Nathan, 2003).

The dangers imposed by O_2 arise from its electronic structure, which predisposes it to reduction (Fridovich, 1999). Many biochemical reactions vital to normal aerobic metabolism require the transfer of four electrons to reduce molecular oxygen to two molecules of H_2O (Miller & Britigan, 1997). This transfer usually occurs without the formation of toxic intermediates; however there are respiratory enzymes that reduce O_2 on the cytosolic face of the cytoplasmic membrane to H_2O (Lynch & Kuramitsu, 2000) producing intermediates, such as $^{\cdot}O_2^-$, H_2O_2 and $^{\cdot}OH$, as shown below:

$$O_2 \xrightarrow{e^-} {}^{\cdot}O_2^- \xrightarrow[2H^+]{e^-} H_2O_2 \xrightarrow{e^-} OH^- + HO^{\cdot} \xrightarrow[2H^+]{e^-} 2H_2O$$

Superoxide ($^{\cdot}O_2^-$) can act both as a reductant and as an oxidant. In the reducing environment of the cell interior it is primarily an oxidant, and among its targets are catecholamines, thiols, ascorbate and flavins (Fridovich, 1999). Bacterial enzymes such as dehydratases containing [4Fe-4S] are also included in these target molecules. Inactivation of [4Fe-4S] by $^{\cdot}O_2^-$ clusters releases iron (reaction 1) in a form that mediates production of hydroxyl radicals (Fridovich, 1999).

$$^{\cdot}O_2^- + Fe^{3+} \rightarrow O_2 + Fe^{2+} \qquad (1)$$

At physiologic pH, superoxide rapidly reduces itself to form the divalent oxygen reduction product, hydrogen peroxide (Miller & Britigan, 1997). In environments of low pH, such as at sites of inflammation, superoxide becomes protonated to form $HO_2^{\cdot}$, which because of its neutral charge, is more membrane permeable that superoxide and more likely to react with itself to form H_2O_2 (Miller & Britigan, 1997).

Hydrogen peroxide is a more reactive oxidant than superoxide, and promptly diffuses across cell membranes (Miller & Britigan, 1997). H_2O_2 reacts with iron released from oxidised [4Fe-4S] clusters to generate hydroxyl radical ($^{\cdot}OH$) (Liochev & Fridovich, 1999). This is known as the Fenton reaction (2):

$$H_2O_2 + Fe^{2+} \rightarrow Fe^{3+} + OH^- + {}^{\cdot}OH \qquad (2)$$

The potential effects of H_2O_2-mediated damage to cellular constituents include the oxidation of cellular membranes and enzymes, DNA damage and mutagenesis and the inhibition of membrane transport processes (Miller & Britigan, 1997).

$^{\cdot}O_2^-$ and H_2O_2, in the presence of free iron, can form the hydroxyl radical ($^{\cdot}OH$), a highly reactive molecule that will react at diffusion-limited rates with various biomolecules, including lipids, proteins and DNA (Miller & Britigan, 1997).

$^{\cdot}OH$ can also be produced by the reduction of H_2O_2 by Cu(I) (Fridovich, 1998). Superoxide reacts with H_2O_2 in producing $^{\cdot}OH$ within cells (reaction 3) and it does so by oxidising [4Fe-4S] clusters of dehydratases:

$$H_2O_2 + O_2^- \rightarrow O_2 + OH^- + {}^{\cdot}OH \qquad (3)$$

Sources of oxidants in biological systems

Aerobic organisms are continuously exposed to both endogenous and exogenous sources of ROI. Endogenous oxidants are a consequence of aerobic metabolism. As mentioned above, toxic oxygen species arise by sequential univalent electron reduction of O_2 to generate $^{\bullet}O_2^-$, H_2O_2 and $^{\bullet}OH$. Another example comes from the generation of extracellular $^{\bullet}O_2^-$ and H_2O_2 by a number of microorganisms occupying the same habitat, as shown by inhibition of *Neisseria gonorrhoeae* by H_2O_2-producing *Lactobacillus* spp. (Zheng *et al*., 1994). The primary source of exogenous oxidative stress for pathogenic bacteria during the process of infection is their attack by phagocytic cells. The respiratory burst occurs in stimulated phagocytes when their rate of O_2 consumption is increased. This is due to the activation of the membrane-bound NADPH oxidase which reduces O_2 to $^{\bullet}O_2^-$ (Curnutte *et al*., 1974). The $^{\bullet}O_2^-$ produced during the respiratory burst is then converted to H_2O_2, which in turn oxidises Cl^- to bactericidal hypochlorite (Fridovich, 1998). In addition, stimulated endothelial cell- or epithelial cell- derived oxidants, like $^{\bullet}O_2^-$ and H_2O_2, have also been shown to exert microbial oxidant stress, either alone or via their reaction with by-products in the extracellular space (Kinnula *et al*., 1992; Moncada & Higgs, 1993). These oxidants can also interact with phagocyte-derived oxidants, cytokines and other compounds to enhance microbial oxidant stress (Miller & Britigan, 1997). Moreover, treatments that artificially generate large amounts of ROI inside living cells, including administration of hyperoxia, redox-cycling drugs or authentic H_2O_2 can disrupt metabolism and cause mutagenesis (Seaver & Imlay, 2004).

Defence strategies specific for oxidants

One of the major challenges facing bacteria growing in oxygenated environments is to efficiently resist or repair damage caused by ROI such as $^{\bullet}O_2^-$, H_2O_2 and $^{\bullet}OH$. Oxidative stress may be induced endogenously, for example by the oxidation of flavo-proteins during respiration, or exogenously by phagocytic production of ROI

(Nathan, 2003). Therefore, enzymes that protect against reactive oxygen, including superoxide dismutases (SODs), catalases and peroxidases, and the tripeptide glutathione are ubiquitous in bacteria (Jakubovics & Jenkinson, 2001).
Glutathione (GSH, reduced form) serves as a substrate for the H_2O_2-removing enzyme glutathione peroxidase (reaction 4) and is also an $^{\cdot}OH$ scavenger (Meister & Anderson, 1983).

$$2GSH + H_2O_2 \rightarrow GS\text{-}SG + 2H_2O \qquad (4)$$

Glutathione reductase can then reduce the oxidised glutathione (GS-SG) to complete the cycle (reaction 5).

$$GS\text{-}SG + 2NADPH + H^+ \rightarrow 2GSH + 2NADP^+ \qquad (5)$$

The importance of this anti-oxidant system in bacteria has not been clearly established, however there are data suggesting that GSH may facilitate the deactivation of *E. coli* dehydratases' that have been inactivated by superoxide (Fridovich, 1998).
Microbial catalases and peroxidases eliminate H_2O_2. Catalases carry out the decomposition of H_2O_2 to O_2 and H_2O (Fridovich, 1999). Most catalases are heme-containing enzymes, however it has been shown that bacteria that cannot synthesize heme can produce a very effective catalase (Fridovich, 1999). Peroxidases use a variety of electron donors to reduce H_2O_2 to $2H_2O$. Accordingly, peroxidases can utilise glutathione, ascorbate, ferrocytochrome *c,* NADH and even iodide and chloride (Fridovich, 1998).
Another mechanism of oxidant inactivation used by microorganisms involves SOD. The production of SOD is the primary defence against $^{\cdot}O_2^-$ (Fridovich, 1997). SOD catalyses the conversion of $^{\cdot}O_2^-$ to H_2O_2 and O_2. During dismutation the active site metal of the SOD is reduced by one $^{\cdot}O_2^-$ and then re-oxidised by the next

(Fridovich, 1999). Elimination of $^{\cdot}O_2^-$ not only reduces the possibility of $^{\cdot}O_2^-$-mediated toxicity but also prevents $^{\cdot}O_2^-$-mediated reduction of iron and subsequent generation of $^{\cdot}OH$ (Miller & Britigan, 1997). A detailed description of SOD enzymes is given below.

The SoxRS regulon is a group of approximately 12 well-regulated genes that is turned on in *E. coli* in response to increased intracellular $^{\cdot}O_2^-$ production under stress conditions (Demple, 1996). The SoxR protein is the sensor. The group includes MnSOD, which removes $^{\cdot}O_2^-$, glucose-6-phosphate dehydrogenase, to ensure a supply of NADPH, endonuclease IV, to repair damaged DNA, fumarase *c*, to replace $^{\cdot}O_2^-$ sensitive fumarases *a* and *b*, aconitase A, to replace aconitases damaged by $^{\cdot}O_2^-$, and others. The SoxRS is, however, only half of the organised defence against oxidative stress. Another independent regulon turned on in response to H_2O_2 exists and is referred to as OxyR regulon (Storz *et al*., 1990).

General characteristics of SODs

Superoxide dismutases (SODs) are ubiquitous metalloenzymes that have been isolated in virtually all aerobic organisms from bacteria to humans, including anaerobes (Lynch & Kuramitsu, 2000). A variety of SODs have been identified, each of them dependent for their activity on metal atoms at the active site. There are two major forms of SOD; one employs manganese (MnSOD) or iron (FeSOD) as the catalytic metal and is generally found in the cytoplasm. All bacteria, including obligate anaerobes, produce MnSOD, FeSOD or both (Lynch & Kuramitsu, 2000). These enzymes exist as subunits linked as dimers in FeSOD and tetramers in MnSOD. The major form of SOD consists of two distinct genes, *sodA* encoding MnSOD and *sodB* encoding FeSOD (Fridovich, 1998). Although variations in the FeSOD content have been observed in bacteria producing both enzymes, it is the control of MnSOD that is usually responsible for modulating the total level of SOD in bacteria (Miller & Britigan, 1997). The active site of the second major SOD contains copper and zinc (Cu/ZnSOD), and is encoded by the *sodC* gene (Puget & Michelson, 1974). It was long believed that this form of SOD

was exclusive to eukaryotes (one form found in the cytoplasm and another in the extracellular spaces, designated EC-Cu/ZnSOD) (Fridovich, 1997). However, Cu/ZnSODs have been increasingly described in bacteria and are likely to occur in most Gram-negative bacteria (Fridovich, 1997), but also in Gram-positive ones, like *Clostridium* sp. (Bruggeman *et al*., 2003) and *Bacillus* sp. (Rey *et al*., 2004). Interestingly, each Cu/ZnSOD subunit contains an atom of each metal, the copper atom being cyclically reduced and oxidized and the zinc atom likely acting to confer structural stability to the active site of the protein (Bordo *et al*., 1994). Another form of SOD containing nickel has been identified in fungi (NiSOD) (Youn *et al*., 1996).

The construction of *E. coli* mutants lacking the cytosolic SODs gave the first insight into the significance of intracellular $^{\cdot}O_2^-$. The consequences of the lack of both the constitutive FeSOD and the inducible MnSOD in *E. coli* include oxygen-dependent decrease of growth rate, nutritional auxotrophy, increase in the rate of spontaneous mutagenesis and, susceptibility to phagocyte-mediated killing (Touati, 1989). Amino acid biosynthesis and membrane integrity also appear to be affected (Imlay & Fridovich, 1992). However, with the discovery of the periplasmic *E. coli* Cu/ZnSOD (Benov & Fridovich, 1994) further studies await to be done. It has been estimated that the SODs in *E. coli* provide approximately 95% protection for all targets susceptible to $^{\cdot}O_2^-$ attack in that cell (Liochev & Fridovich, 1999). In fact, the concentration of SOD *in vivo* exceeds that of $^{\cdot}O_2^-$ by about 100,000 to 1, however Gort & Imlay (1998) have shown that enough $^{\cdot}O_2^-$ is made to require such an abundant synthesis of SOD in *E. coli*.

Besides the enzyme relatively well described defensive functions within bacteria, it is thought to play an important role in bacterial survival and pathogenesis within the host. Several studies, using isogenic *sod* mutants, have shown the involvement of SOD in the pathogenesis of both Gram-positive and -negative organisms, including *Helicobacter pylori* (Seyler *et al*., 2001), *Salmonella enterica* (Farrant *et al*., 1997), *Campylobacter coli* (Purdy *et al*., 1999), *Neisseria meningitidis* (Wilks *et al*., 1998), *Haemophilus influenzae* (D'Mello *et al*., 1997), *Nocardia asteroides* (Beaman *et al*., 1985), *Streptococcus gordonii* (Jakubovics *et al*., 2002), *S. agalactiae* (Poyart *et al*., 2001), *Burkholderia cepacia* (Lefebre &

Valvano, 2001), *Pseudomonas aeruginosa* (Hassett *et al*., 1995) and *Moraxella catarrhalis* (Luke *et al*., 2002).

Pneumococcal resistance to oxidative stress

Streptococcus pneumoniae is a fermentative organism that nevertheless exists in oxygenated environments, like the nasopharynx of humans. Consequently, the mechanisms that it employs to detoxify ROI and the role of these mechanisms *in vivo* is of interest. *Streptococcus pneumoniae* lacks the cytochromes and heme-containing proteins involved in aerobic respiration (Pericone *et al*., 2003). It also lacks many proteins that have been shown to protect against oxidative stress in other bacterial species, such as the regulators SoxRS and OxyR or the H_2O_2 scavengers catalase and NADH peroxidase (Tettelin *et al*., 2001). Since pneumococci lack catalase, most H_2O_2 generated by oxidases and MnSOD is released by the cells, causing heamolysis or tissue damage (Hirst *et al*., 2000). Internal protection against H_2O_2 is afforded by peroxidases such as thiol peroxidase, glutathione peroxidase or alkyl hydroperoxidase (Jakubovics & Jenkinson, 2001). *Streptococcus pneumoniae* contains two oxidases that were shown to have a role *in vivo*, NADH oxidase, which reduces O_2 to H_2O, and pyruvate oxidase, which catalyses the reduction of O_2 to the potentially dangerous H_2O_2 (Auzat *et al*., 1999; Spellerberg *et al*., 1996). Production of H_2O_2 by *S. pneumoniae* has been shown to have cytotoxic effects on human epithelial cells in culture and host tissue in animal models (Duane *et al*., 1993; Hirst *et al*., 2000). The major enzyme capable of detoxifying $^{\cdot}O_2^-$ in pneumococci is MnSOD, encoded by *sodA*. In addition, the pneumococcus also produces FeSOD (Yesilkaya *et al*., 2000), but the gene encoding *sodB* has not been found in the *S. pneumoniae* genome. A role for MnSOD in virulence of *S. pneumoniae* has been previously reported in our laboratory (Yesilkaya *et al*., 2000). Construction of an isogenic mutant lacking *sodA* was used in an animal model of bronchopneumonia. ΔSodA was less virulent than the wild type parent following intranasal infection. Moreover, mutant cells were significantly impaired in ability to replicate in the lungs of mice (Yesilkaya *et al*., 2000). Here, work reporting the behaviour of this mutant in the

nasopharynx *in vivo* and how the lack of SodA affects the host immune response to this bacterium is shown.

1.6 Animal models of pneumococcal disease

Animal experimentation is crucial to study the pathogenesis of infectious diseases. At present, several animal models of diseases caused by *S. pneumoniae* are readily available to clarify mechanisms of pathogenesis and characterise the role of bacterial and host factors. Currently, the well-standardised models used to study pneumococcal pathogenesis are the sepsis, pneumonia, meningitis, otitis media and colonisation models.

Experimental pneumococcal sepsis has been used by researchers as a mean to investigate the role of pneumococcal virulence factors during sepsis and in studies aimed at analysing the efficacy of vaccines (Briles *et al.*, 1996, 2000a). Disease can be generated either by the i.v. or i.p. route of infection and, commonly, the mouse is the most used animal for inducing sepsis by *S. pneumoniae*. The occurrence of murine sepsis has been analysed by determining the presence of bacteria in the blood (bacteremia) and by observing animal survival after infection. Sepsis induced by the i.v. route is a more direct method since bacteria are directly inoculated into the bloodstream while sepsis induced by the i.p. method is secondary to peritoneal infection (Chiavolini *et al.*, 2008).

Varied models of pneumococcal pneumonia have been employed that allow the analysis of different parameters, like animal survival, presence of bacteria in lungs and blood, levels of inflammation and histology of the lungs. Moreover, quantification of antibody titres performed in vaccine studies and antimicrobial analysis in drug pharmacokinetic studies are also possible. As for the sepsis model, the mouse is the most commonly used animal for inducing pneumonia by *S. pneumoniae*. Two main routes of infection are currently used however both present advantages and disadvantages. The intratracheal (i.t.) model is a more complex and invasive technique for disease induction but has the advantage of allowing 99% delivery of the bacterial inoculum to the lungs (Rubins *et al.*, 1995).

The intranasal (i.n.) route which includes both the standard aspiration method (Canvin *et al.*, 1995) and the aerosol nebuliser system (Nuermberger *et al.*, 2005) has the advantage of being faster and easier to perform without invasive surgical procedures. Moreover, the model of infection through i.n. aspiration mimics the natural route of infection in humans. However, both i.n. aspiration models need anaesthesia and simultaneous infection of many mice is not feasible within the i.n. aerosol model since it requires the use of an exposure chamber with a nebuliser. i.t. models generally cause lobar pneumonia (Azoulay-Dupuis *et al.*, 1991), while the i.n. aspiration model induces bronchopneumonia (Canvin *et al.*, 1995). In both cases, there are simultaneous pneumonia and sepsis: infection readily leads to death of animals, which most likely succumb to septic shock rather than to pulmonary disease (Chiavolini *et al.*, 2008).

Canvin *et al.* (1995) have introduced the practice of infecting animals with mouse-passaged pneumococci. Pneumococci recovered from the blood of i.p. infected animals were inoculated into the nostrils of anesthetised mice, which rapidly developed bronchopneumonia with bacteremia. This model has been employed in the present study and in other studies on the role of virulence determinants in pneumonia (Kadioglu *et al.*, 2002; Jounblat *et al.*, 2003), host immune response to pneumococcal lung infection (Kadioglu *et al.*, 2000; Dallaire *et al.*, 2001; Kadioglu & Andrew, 2004), mouse susceptibility and resistance to the disease (Gingles *et al.*, 2001) and *S. pneumoniae* invasion of cells *in vivo* (Kadioglu *et al.*, 2001). In addition, many investigations on protection from i.n. challenge after immunization with vaccine candidates such as pneumolysin (Alexander *et al.*, 1994), PspA (Briles *et al.*, 1996) and PspC (Balachandran *et al.*, 2002) have been successfully performed.

The experimental meningitis models employed to study disease by *S. pneumoniae* allow the examination of different parameters such as animal survival, measurement of clinical scores, viable bacterial counts in the cerebrospinal fluid (CSF) and brain, histological analysis of brain tissue and assessment of leukocyte and cytokine levels in CSF and serum. For the study of experimental meningitis the key laboratory animals used for the induction of disease are rabbits and rats. The most commonly used method, which employs these animals, relies on the induction of disease via the intracisternal (i.cist.) route. Although this method does

not mimic the natural route of infection occurring in humans, it enables the study of disease once infection is established in the meninges, like it was shown by the work of Hirst and colleagues (2003). Another consideration is that the rabbit and rat models need more-sophisticated surgical procedures. However, their size has the advantage of allowing large samples of CSF and blood compared to what is possible with the mouse model.

The need to study pneumococcal interaction with more than one tissue (*in vivo* or *ex vivo*) is important because the contribution of each virulence factor, individually and collectively, varies according to the location of the pneumococcus. Evidence that individual virulence factors exert a tissue-specific effect on virulence came from a study using isogenic mutants of a type 2 pneumococcus in three murine infection models (Orihuela *et al.*, 2004). Recently Oggioni and co-workers (2006) showed that there is tissue-specific pneumococcal gene expression. Within a type 4 strain, distinct groups of genes important in pneumonia, bacteraemia and in the nasopharynx were identified. Two patterns of *in vivo* gene expression were observed: one characteristic of pneumococci in the blood and the other of pneumococci in the lungs and brain. Their study also provided a perspective on how *in vitro* growth states provide representations of different *in vivo* pneumococcal populations and finally the observation that CSP induced pneumococcal biofilm *in vitro* and that the pattern of pneumococcal gene expression in the biofilm matched that in the lungs and brain. However, although this study (Oggioni *et al.*, 2006) showed the same expression pattern in the two pneumococcal strains studied (D39 and TIGR4), work by Cron *et al.* (2009) indicates that gene expression can be strain dependent *in vitro* and *in vivo*.

The mouse represents the most commonly exploited animal model to investigate experimental pneumococcal disease. Inbred mouse strains, including BALB/c, C57BL/6, DBA, CBA and CBA/N strain (carries the Xid-linked immune deficiency) mice have largely been utilised for the analysis of pneumococcal pneumonia, sepsis, meningitis and otitis media (Azoulay-Dupuis *et al.*, 1991; Briles *et al.*, 1996; Balachandran *et al.*, 2002; Orihuela *et al.*, 2004; Nuermberger *et al.*, 2005;). Inbred strains afford more-uniform responses to treatments since their immune system is tightly controlled. On the other hand, outbred strains such as MF1, CD-1

(Swiss), Swiss-Webster and NMRI, generated to maintain maximum heterozygosity, mimic the natural variation in response to infection. The latest's have been extensively used for analysing mechanisms of disease in pneumonia, sepsis and meningitis (Canvin *et al.*, 1995; Kadioglu *et al.*, 2002; Jounblat *et al.*, 2003; Chiavolini *et al.*, 2004). Other different mammals have also been employed, like rats, chinchillas (only outbred strains are available), gerbils and guinea pigs (commonly used in experimental models of otitis media, meningitis and colonisation) (Long *et al.*, 2004; Tong *et al*., 2000) and the New Zealand White rabbit strain generally used to study pneumococcal pneumonia, sepsis and meningitis (Tuomanen *et al.*, 1985). Their advantage is the collection of more substantial samples due to their larger body size, but one limitation is that experimental groups are normally smaller consequently statistical significance can be compromised.

1.7 Aim of the project

The aim of this study was to investigate the individual contribution of neuraminidases A and B, autolysin and superoxide dismutase in the virulence of the respiratory pathogen *S. pneumoniae* by testing mutants with defects in each of these loci in a mouse model of bronchopneumonia. This was achieved by performing bacterial counts in host tissues of the upper (nasopharynx) and lower (trachea and lungs) respiratory tract and in blood following intranasal infection with wild type pneumococci or isogenic mutants. In addition, histological analysis of infected-lung tissue sections and leukocyte recruitment to these lungs was also accomplished, in order to get a better picture of the role of these enzymes in pneumococcal survival and resistance to the host immune defences.

Furthermore, another purpose of this project was the construction of a novel plasmid, containing *gfp*, erythromycin- and spectinomycin-resistance cassettes to be used to confer fluorescence to a wide range of D39 pneumococcal mutant strains as a mean to investigate the role of pneumococcal virulence factors in adherence and invasion *in vivo* (this work is described in Appendix C).

MATERIAL AND METHODS

2. Material & Methods

Studies on pneumococcal virulence factors

In vitro and *in vivo* studies were performed to investigate the contribution of the pneumococcal virulence factors neuraminidase, superoxide dismutase and autolysin to the pathogenesis of *S. pneumoniae* and are described below.

2.1 Chemicals

All the chemicals used in this work were purchased from Sigma-Aldrich Ltd (UK), VWR International (UK) or Fisher Scientific (UK) unless otherwise stated. Primers were ordered from MWG (UK) or TAGN (UK). Restriction enzymes were purchased from Invitrogen Ltd (UK) or New England Biolabs Inc. (UK), modifying enzymes from New England Biolabs Inc. (UK) and DNA polymerase was purchased from Bioline (Accuzyme) or New England Biolabs Inc. (*Taq*).

2.2 Antibiotics

The list of antibiotics used in this study is shown in Table 2.1

Table 2.1 Antibiotic solutions

Antibiotic	Stock solution		Working concentration
	Concentration	Storage	
Erythromycin	100mg/ml in ethanol[a]	-20^{o}C	1mg/ml
Spectinomycin	100mg/ml in H_2O[b]	4 ^{o}C	200μg/ml

[a]Antibiotics dissolved in 100% (v/v) ethanol
[b]Antibiotics prepared in distilled water (dH_2O) were sterilised by filtration through a 0.22μm filter.

2.3 Bacterial strains

Pneumococcal strains used in this study are listed in Table 2.2. All the *S. pneumoniae* mutant strains used in this study were made in the virulent serotype 2 strain D39. The strains used were all obtained from our laboratory collection (Collection of Professor Peter W. Andrew, Laboratory 227 Department of Infection, Immunity and Inflammation of the University of Leicester, UK).

Bacteria were identified as pneumococci prior to use by Gram stain, catalase test, α-hemolysis on blood agar plates and by optochin sensitivity.

The two neuraminidase mutants, ΔNanA an insertion duplication mutant (Berry & Paton, 2000) and ΔNanB constructed by deletion of the *nanB* gene (Berry *et al*., 1996) (for detailed construction of this mutant see Appendix A), the superoxide dismutase-negative mutant ΔSodA (Yesilkaya *et al*., 2000), constructed by insertion mutation of *sodA* gene, and the autolysin-negative mutant ΔAL2 (Berry *et al*., 1989b), constructed by insertion mutagenesis, were used to study the role of each of these pneumococcal virulence factors in pneumococcal colonisation and inflammation of the nasopharynx and lungs, invasion of the lower respiratory tract and septicaemia.

2.4 Growth conditions and media

Streptococcus pneumoniae strains were grown on blood agar plates (see below) that were placed, inverted, in a candle jar and incubated overnight, without shaking, at 37^{o}C. The following day, using a sterile loop, bacteria were inoculated into a 30 ml universal tube (Falcon) containing 10 ml of BHI liquid medium (see below) and grown overnight at 37^{o}C, statically. For the mutant strains, the appropriate antibiotic was added to the culture, at the concentration shown in Table 2.1 (§2.2).

For *in vitro* growth experiments, pneumococci were cultured overnight at 37^{o}C in BHI broth containing 20% (v/v) heat-inactivated newborn calf serum (NCS). Medium for mutants contained the appropriate antibiotic (Table 2.1 §2.2).

Table 2.2 *Streptococcus pneumoniae* strains used in this study

Strain	Characteristics	Reference
D39	Virulent wild type *S. pneumoniae* type 2	National Collection Type Culture 7466 London, UK
ΔNanA	Neuraminidase A-negative *S. pneumoniae*; Erythromycin resistant	Berry & Paton (2000)
ΔNanB	Neuraminidase B-negative *S. pneumoniae*	Kindly provided by Prof. James Paton
ΔAL2	Autolysin-negative *S. pneumoniae*; Erythromycin resistant	Berry *et al*. (1989b)
ΔSodA	Superoxide dismutase-negative *S. pneumoniae*; Spectinomycin resistant	Yesilkaya *et al*. (2000)

The next day, samples were centrifuged, and pellets were re-suspended in fresh BHI-NCS (with or without antibiotic) and adjusted to an optical density (OD) at 500nm of 0.2. Each *in vitro* growth experiment was repeated twice, with each time point sample within one experiment done in triplicate.

Streptococcus pneumoniae culture media were prepared as follow:

- Brain Heart Infusion broth (BHI) – 14.8g of BHI powder were dissolved into 400 ml of dH_2O and sterilised by autoclaving.

- Blood Agar Base (BAB) – 16g of BAB powder were dissolved into 400 ml of dH_2O and sterilised by autoclaving; for blood agar plates, the medium was cooled to around 50^oC, Defibrinated Horse Blood (Oxoid, UK) was added to 5% (v/v), then gently mixed and poured into Petri dishes. When required, the appropriate antibiotic was added to the medium at the concentration described in Table 2.1 (§2.2), before pouring into the plates.

Bacteriological media were purchased from Oxoid Ltd. (UK). The sterilisation by autoclaving was at 121^oC for 15 minutes.

2.5 Primers

To confirm the mutations of each isogenic mutant of *S. pneumoniae* used in this study, a set of forward and reverse complementary primers was designed on the basis of the published gene sequence and respective mutagenesis procedure used (the gene sequence's and location of primers is shown in Appendix A).
The primers used in this study were between 18 and 30 bases in length and were supplied by the manufacturer HPSF (high purity salt free) purified and lyophilised. On arrival, primers were centrifuged at 9460*g* for 30 seconds and 100µl nH_2O (autoclaved) was added to the aliquot and kept on ice. For use, a stock of 5pM of each primer was prepared. The primers used in this study are described in detail below and are shown in Table 2.3.

Neuraminidase primers:

Streptococcus pneumoniae neuraminidase A-negative mutant ΔNanA (Table 2.2 §2.3) was constructed by insertion duplication mutagenesis using the vector pVA891 (5.4Kb; Macrina *et al.*, 1983), which encodes chloramphenicol and erythromycin resistance and can replicate in *E. coli* but not *S. pneumoniae*. Part of the *nan*A gene (*Hind*III 1210nt – *Sph*I 1847nt, 637bp fragment) was cloned into pVA891 and the construct was transformed into strain D39. A single cross-over mutation occurred which incorporated the entire plasmid into the chromosomal *nan*A gene, thereby disrupting the *nan*A gene and rendering the recombinant erythromycin resistant. In this regard and to confirm the mutation of this strain, a set of primers, previously designed in our laboratory on the basis of the *nan*A gene sequence (4500bp in length, GenBank Accession number X72967), NanAF and NanAR, and primer ErmF, designed on the basis of the erythromycin resistance gene *erm (*from shuttle vector pVA838 [Macrina *et al.*, 1982] GenBank Accession number AB057644 region 5167 to 5904) were used (Table 2.3).

Streptococcus pneumoniae neuraminidase B-negative mutant ΔNanB (Table 2.2 §2.3) was constructed by deletion of the *nanB* gene. The mutant contains only the 5' end, from nucleotide 0 to 253 and the 3' end, from nucleotide 2048 to 2093 from the complete *nanB* gene. Therefore, to confirm the mutation in this strain, primers NanBF and NanBR (Table 2.3) were designed. These primers amplify a 1098bp fragment of the *nanB* gene (GenBank Accession number U43526) with 2093bp in the wild type parent, from nucleotide 709 to 1825.

Autolysin primers:

Streptococcus pneumoniae autolysin-negative mutant ΔAL2 (Table 2.2 §2.3) was constructed by insertion deletion mutagenesis using the vector pVA891 (5.4Kb; Macrina *et al.*, 1983).

The primers LytA-For, LytA-Rev (Table 2.3) were designed on the basis of the published *lyt*A gene sequence (GenBank Accession number M13812) and amplify a 957bp fragment of the *lyt*A gene in the wild type parent, from nucleotide 584 to 1540.

Superoxide dismutase primers:

Streptococcus pneumoniae superoxide dismutase-negative mutant ΔSodA (Table 2.2 §2.3), constructed by double crossover mutation of *sodA* gene has a 'spectinomycin resistance cassette' disrupting *sod*A. The primers SODF, SODR (Table 2.3) were designed on the basis of the published *sod*A gene sequence with 606bp (gene SP0766) and amplify a 586bp fragment of the *sod*A gene in the wild type parent, from nucleotide 15 to 601.

Table 2.3 List of primers used in this study

Primer	Sequence	Product size
NanAF	5' - CTA CGA TGA ACA ATA GAC GTG CGC - 3'	604bp
NanAR	5' - TAT CAT ACT GGG TCA TGA AGC GTG C - 3'	
ErmF	5' - AGT AAC GGT ACT TAA ATT GTT TAC - 3'	-
NanBF	5' - GAT TCT ACT CAA GCT AAC - 3'	1098bp
NanBR	5' - GAC TGC TTC TTT TCC ATC - 3'	
SODF	5' - ACC AGA ACT TCC ATA TGC - 3'	586bp
SODR	5' - TAG CAG CTG CGT ACA ATT - 3'	
LytA-F	5' - ATG GAA ATT AAT GTG AGT AAA TTA AG - 3'	957bp
LytA-R	5' - TTA TTT TAC TGT AAT CAA GCC - 3'	

2.6 Polymerase Chain Reaction

Polymerase chain reaction (PCR) was performed (Saiki *et al*., 1988) to confirm the mutations in each of the *S. pneumoniae* D39 isogenic mutants used in the present study (Table 2.2 §2.3). Chromosomal DNA from each mutant was amplified by PCR using a set of forward and reverse primers (Table 2.3 §2.5) designed on the basis of the published gene sequence and respective mutagenesis procedure used.

To obtain chromosomal DNA, colonies were picked from a blood agar plate with a sterile toothpick and vigorously mixed in 20µl nanopure water in a small eppendorf

tube. A hole was made in the cap with a needle and the eppendorf tube was placed in a polystyrene rack in a boiling water bath for 5 minutes. 5µl of this suspension was used as a template for PCR. The PCR reaction mixture was prepared in a final volume of 20µl containing the following reagents:

10x AccuBuffer[a]	2µl
dNTPs (2mM stock)	2µl
$MgCl_2$ (50mM stock)[b]	0.2µl
5' primer (5pM stock)	1µl
3' primer (5pM stock)	1µl
Template DNA (50ng/µl)	2µl
Accuzyme DNA polymerase[a]	1µl/100µl
NH_2O	To 20µl

[a] For ΔNanA, Bio-X-Act DNA polymerase and Optibuffer, Bioline
[b] Supplied by the manufacturer

The cycling conditions used for each mutant are shown below.

ΔNanA

Cycle number	Denaturation	Annealing	Extension	Final
1	5 min at 95°C	1 min at 55°C		
2-30	30 sec at 95°C	30 sec at 60°C	5 min at 68°C	
31			10 min at 68°C	
Hold				4°C

ΔNanB

Cycle number	Denaturation	Annealing	Extension	Final
1	5 min at 95°C	1 min at 42°C		
2-34	1 min at 94°C	1 min at 42°C	2.30 min at 72°C	
35			10 min at 72°C	
Hold				4°C

ΔSodA

Cycle number	Denaturation	Annealing	Extension	Final
1	5 min at 95°C	1 min at 45°C		
2-34	1 min at 94°C	1 min at 45°C	1.30 min at 72°C	
35			2 min at 72°C	
Hold				4°C

ΔAL2

Cycle number	Denaturation	Annealing	Extension	Final
1	5 min at 95°C	1 min at 50°C		
2-34	1 min at 94°C	1 min at 50°C	1.30 min at 72°C	
35			10 min at 72°C	
Hold				4°C

A control reaction, omitting the template DNA, was used in each PCR reaction to confirm the absence of contamination.

The result of the PCR reaction was observed by agarose gel electrophoresis (see below).

2.7 Agarose gel electrophoresis

Agarose gel electrophoresis was used to visualise DNA fragments, as described in Sambrook (1989). Agarose at a concentration of 0.7 or 1% (w/v) was prepared in 100 ml 1X TAE (40mM Tris-acetate pH 7.7, 1mM EDTA, see Appendix A). The suspension was heated in a microwave until the agarose had dissolved and then allowed to cool to around 55^{o}C. At this stage ethidium bromide (from a stock solution of 10 mg/ml in water) was added to a concentration of 0.5μg/ml and the solution was poured into a mould and allowed to harden. The mould was placed in a tank containing TAE buffer. The DNA samples were mixed with 3μl of loading dye (see Appendix A) and carefully transferred to wells of the agarose gel using a disposable micropipette. A 1Kb DNA ladder was loaded into one well of the agarose gel. Electrophoresis was achieved by applying a constant voltage of around 70mV and the DNA fragments were visualised under UV light using a long-wave UV transluminator.

2.8 Bradford assay

To determine the concentration of protein in sonicated *S. pneumoniae* cells, the Bradford protocol was followed (Bradford, 1976). The Bradford Reagent (product number B6916, Sigma) consists of Brilliant Blue G in phosphoric acid and methanol. The procedure is based on the formation of a complex between the dye, Brilliant Blue G, and proteins in solution. The protein-dye complex causes a shift in the absorption maximum of the dye from 465 to 595nm. The degree of absorption at 595nm is proportional to the protein present. The protein concentration was determined by comparison to a standard curve. All the assays were run in triplicate on two independent occasions.

The assay was performed in a 96 well microplate. The Bradford Reagent (stored at 4^{o}C) was brought to room temperature and gently mixed by inverting the bottle several times. 5μl of sample (previously sonicated pneumococcal cells, see §2.9) was added to the wells in triplicate. To each well being used, 250μl of Bradford Reagent was added and the plate was mixed on a shaker for approximately 30 seconds. The plate was left to incubate at room temperature for around 20

minutes. The absorbance was measured at 595nm using a BioRad model 680 Plate Reader (BioRad, UK). A standard curve using known concentrations of BSA (First Link Ltd., UK) ranging from 0.1 to 1 mg/ml (in $_nH_2O$) was used.

2.9 Neuraminidase assay

The total level of neuraminidase activity in *S. pneumoniae* wild type and isogenic mutants, ΔNanA and ΔNanB, was determined by using a quantitative assay utilising the substrate 2-O-(p-nitrophenyl)-α-D-*N*-acetylneuraminic acid (pNP-NANA, Sigma). pNP-NANA is cleaved by neuraminidase with the release of free p-nitrophenol (pNP, Sigma) which can be assayed by its absorbance at 405nm. Stocks of cells with approximately 10^8 cfu/ml were kept in aliquots at -80°C. When required, the cells were thawed on ice and centrifuged at 9460*g* for 1 minute at 4°C. The pellet was re-suspended in 250μl 40mM Tris (pH 7.4) and the samples were kept on ice before the sonication procedure. Bacterial suspensions were sonicated on ice using a Sanyo soniprep model 150 sonicator (amplitude 8 microns); the cells were sonicated for 15 seconds with 45 seconds intervals. This process was repeated for a total of 8 minutes. The samples were then centrifuged at 9460*g* for 1 minute at 4°C and the supernatant was stored on ice. 25μl of sample was added in triplicate to each well of a 96 well plate (note that the assays were run using triplicates of each strain on two independent occasions). 25μl 0.3mM pNP-NANA (dissolved in a dilution buffer at pH 6.6 [see Appendix A] and kept in 500μl aliquots at -20°C) previously thawed on ice, was added to the wells. The microplate was incubated statically for 2 hours at 37°C. The reaction was stopped by adding to each well 100μl of ice cold 0.5M Na_2CO_3 (pH 9.6). Absorbance at 405nm was measured in a BioRad model 680 Plate Reader (BioRad, UK). A reaction blank containing 25μl of 40mM Tris (pH 7.4) or 25μl of dilution buffer, instead of the sample, was included. To determine the activity of the neuraminidase enzyme, a standard curve was prepared using known concentrations of p-nitrophenol.

2.10 *In vivo* virulence studies

Virulence studies were conducted using a murine model of bronchopneumonia described by Kadioglu *et al*. (2000). The model consists in comparing the outcome of disease after intranasal infection with wild type *S. pneumoniae* type 2 strain D39 or its isogenic mutants neuraminidase A-negative, ΔNanA, neuraminidase B-negative, ΔNanB, superoxide dismutase-negative, ΔSodA and autolysin-negative ΔAL-2 (see Table 2.2 §2.3). Virulence studies were aimed at determining bacterial numbers in tissues of the upper and lower respiratory tract and in blood induced by isogenic mutants and their parent strain and the ability of these strains to colonise the respiratory tract and to cause bacteremia.
All experimental protocols were approved by appropriate UK Home Office licensing authorities and by the University of Leicester Ethical Committee.

2.10.1 Mice

Female, specific pathogen free outbred mouse strain MF1 were obtained from Harlan Olac Ltd., UK and were used throughout this study. They were at least 9 weeks old and weighing 30 to 35g at the beginning of each experiment. Before being used for experimental virulence studies, mice were acclimatised for one week in groups of five, under standard conditions followed by the Biomedical Services of University of Leicester, with regulated day length, temperature and humidity, and were given tap water and pelleted food *ad libitum*.

2.10.2 Animal-passaged *S. pneumoniae*

To obtain virulent pneumococci, bacteria were cultured and passaged through mice as described by Kadioglu *et al*. (2000).
Streptococcus pneumoniae was grown on blood agar plates overnight, as previously described in §2.4. The following day, a streak of bacteria was removed with a sterile loop, inoculated into a 30 ml universal tube with 10 ml of BHI and

grown overnight at 37^{o}C. The next day, bacteria were centrifuged and the pelleted cells were resuspended in 5 to 10 ml PBS. To passage pneumococci, 100μl of this suspension was injected intraperitoneally in female MF1 mice, using a needle attached to a syringe. The injection was given just above the mouse's crotch, at an angle of approximately 30 degrees to the skin surface and at an angle of approximately 45 degrees to the mouse's midline. At 24h post-infection, mice were deeply anaesthetised, in a cabinet, with 2.5% (v/v) fluothane (Zeneca, Macclesfield, UK) over oxygen (2 litres/min) using a Fluotec 3 calibrated vaporiser (Cyprane). Cardiac puncture was performed for removal of blood that was collected to an eppendorf tube and placed on ice as described previously (Canvin *et al.* 1995). 100μl or 200μl of blood was inoculated into 10 ml of BHI, in duplicate and grown overnight at 37^{o}C statically. The next day, bacterial growth was carefully removed without disrupting the pelleted blood cells, to a fresh 30 ml universal tube, using a disposable plastic pipette. The bacteria were spun for 15 minutes at room temperature and the pellet was resuspended in 1 ml serum broth (BHI supplemented with 20% (v/v) newborn calf serum). 660μl (1/3) of this suspension was removed to a fresh tube containing 10 ml serum broth and incubated for 5h at 37^{o}C statically. When OD_{500} was at 1.6, aliquots containing 500μl of culture were prepared and frozen at -80^{o}C. The viability of the bacterial stocks was checked using the Miles-Misra plating method on blood agar. The viability of the stocks was checked using the Miles-Misra plating method as follows: ten-fold serial dilutions were prepared in sterile PBS in a microtitre plate. Starting with the highest dilution, a 20μl drop of the dilution was placed on the relevant quadrant of a blood agar plate and viable counts were calculated as an average colony forming units (cfu) formed on duplicate plates.

2.10.3 Preparation of standard inoculum

To prepare the standard inoculum to be used for *in vivo* virulence studies, an aliquot of passaged pneumococci (see above) was thawed at room temperature. The cells were spun for 1 minute at 9460*g* and the pellet was resuspended in

400μl sterile PBS. This suspension was regarded as the standard inoculum. To administer a dose of 10^6 cfu in 50μl PBS used for intranasal infection or 10^5 cfu in 100μl PBS used for intravenous infection, a dilution was done in accordance with the previously determined viable count (see above). The inoculums' dose was confirmed by colony counting on blood agar, after administration of the dose.

2.10.4 Infection of mice

Challenge of female MF1 mice with passaged pneumococci was via the nasal route. Mice were anaesthetised, in a cabinet, with 2.5% (v/v) fluothane (Zeneca, Macclesfield, UK) over oxygen (2 litres/min) using a Fluotec 3 calibrated vaporiser (Cyprane). 50μl of PBS containing 10^6 cfu of *S. pneumoniae* (wild type or isogenic mutant, see Table 2.2 §2.3) was administrated into the nostrils of the mouse. The mouse was then laid on its back inside the cage to allow for the inoculum to reach the lungs. Following infection, the cages containing infected mice were placed inside an isolator dedicated to the purpose. Mice were monitored for signs of illness throughout the course of infection. A scale was used to score signs of disease as follows: 1- normal; 2,3- hunched position; 4,5- starry coat; 6,7- lethargic. Mice were culled at intervals or when they reached a very lethargic state. The intravenous (i.v.) route of infection was also employed in this study. 100μl of PBS containing 10^5 cfu of *S. pneumoniae* (wild type or isogenic mutant) was administrated using an insulin syringe via the dorsal tail vein of the mouse. Following infection, the cages were placed inside an isolator, as above.

2.10.5 Bacterial counts in host tissues

At pre-chosen times following infection, groups of mice were deeply anaesthetised as before (see above). Blood was collected by cardiac puncture and placed in an eppendorf tube on ice. Mice were immediately culled by cervical dislocation and the nasopharynx, trachea and lungs were removed separately to 10 ml sterile PBS in a universal tube and weighed. All the tissues were kept on ice while processing

and were homogenised using an Ultra-Turrax T8.01 (Ika-Werke, Germany) electric homogeniser. Viable counts in tissue homogenates and in blood were determined by serial dilution in sterile PBS.
For intravenous infections, mouse's tail bleeds were done at 2h, 24h and 48h post-infection and viable counts in blood were performed as described above.
To ensure data normality, the colony counts (cfu/mg of tissue or cfu/ml of blood) were transformed to log counts (log_{10} cfu/mg of tissue or log_{10} cfu/ml of blood).

2.11 Histology

At pre-selected times following intranasal infections, animals were culled and whole-lung tissue samples were collected to be processed for histology examination.

2.11.1 Preparation of frozen lung tissue

At intervals following infection, lungs of infected mice were excised and collected to a universal tube containing chilled PBS. The lungs were subsequently dissected into two lobes. Molds previously prepared using aluminium foil moulded around a bijoux tube were partially filled with tissue matrix OCT® (Sakura, Finetek, The Netherlands) and carefully placed inside a bucket of dry ice. The tissue was placed in the matrix near the bottom (so it is easily exposed when sections are cut) and allowed to solidify completely. Each mold was then placed inside a 30 ml universal tube that was labelled accordingly and stored at -20°C until ready for sectioning.

2.11.2 Sectioning of frozen tissues

To prepare sections from frozen lungs, a Bright cryostat was used. The tissue block was placed on the cryostat specimen disk and sections of the desired thickness (usually between 10 to 15µm) were cut. The sections were placed on a

BDH Superfrost slide and dry overnight at room temperature. At least thirty sections from throughout the lung were taken for examination.

2.11.3 Haematoxylin and Eosin staining method

For the Haematoxylin and Eosin staining of tissue sections, the protocol described in Wheater's Functional Histology (Burkitt, 1993) was followed. The slides, with tissue sections to be stained, were placed on a slide rack, with one space between each slide, and submersed in acetone for approximately 1 minute. Following this, the rack was left on top of tissue paper to allow the sections to dry completely. The rack was then immersed in Harris haematoxylin solution (see Appendix A) and left for 1 minute. It was washed under running tap water, and for counterstaining, placed in eosin Y (see Appendix A) for approximately 2 minutes; again the slides were washed with running tap water. Both the stain solutions were filtered prior to use. Following this, the rack was placed, for approximately 30 seconds in each solution, on a series of alcohol washes starting with 70% (v/v) solution, 90% (v/v) and finally 100% (v/v). After dehydrating the sections in absolute ethanol, the rack was placed in xylene inside a fume hood and left for approx. 10 minutes. Coverslips were placed on top of tissue paper and one drop of synthetic resin mountant DPX was placed in the centre of the coverslip. The slides were taken out of the xylene and placed on top of a sheet of tissue paper, inside the fume hood, to drain all traces of xylene. Carefully, the slide containing the section was lowered onto the drop of DPX so that the section was sandwiched between its slide and the coverslip. The slide was then turned over and the DPX was allowed to spread between the section and coverslip. Sections were examined using a light microscope. Harris haematoxylin stains nuclei blue and eosin stains the cytoplasm pink to red.

2.11.4 Lung homogenate cell preparation

Lungs from sacrificed animals were placed in a 30 ml universal tube containing 5ml RPMI 1640 and placed on ice until required. Lungs were transferred to a fresh

tube containing 5ml RPMI 1640 with 5% (v/v) NCS and homogenised using an Ultra-Turrax T8.01 (Ika-Werke, Germany) electric homogeniser. Following this, 500μl of digestion buffer (5% [v/v] NCS in RPMI1640 with 0.5 mg/ml [207 collagen digestion units] Collagenase (Sigma) and bovine pancreas DNase I (Sigma) at 30μg/ml [87 units]) was added to the homogenised tissue and the mixture was transferred to a 15 ml conic tube (Falcon) and incubated for 30 minutes at 37^{o}C in a water bath. Columns for filtering the cell extracts were prepared, using approximately 1 cm of non-absorbent cotton wool in a glass Pasteur pipette that was placed in a round bottom Falcon 2052 tube (Becton Dickinson). Following incubation, digested tissue was carefully removed out of the conical tube using a plastic Pasteur pipette and cells were filtered through the column to remove large pieces of cell debris. The filtered cells were spun at 1200rpm for 6 minutes at 4^{o}C (MSE Mistral 3000i centifuge). The pellet was resuspended in 1 ml lysis solution (Pharmingen, San Diego, USA) and incubated for 5 minutes at room temperature to allow lysis of red blood cells. The tubes were filled with ice-cold PBS and centrifuged at 1200rpm for 6 minutes at 4^{o}C. The cells were resuspended in 1 ml 5% (v/v) NCS in RPMI1640. For differential analysis, 50μl of cell suspension was centrifuged onto cytospin slides (Shandon) using a cytocentrifuge (Cytospin 2, Shandon) at 1100rpm for 3 minutes. Following centrifugation, the slides were left overnight to air dry.

2.11.5 Differential staining of cells with Giemsa's stain

Differential staining of cells onto cytospin slides (see above) was achieved using Giemsa's stain. The slides were placed on a slide rack, with one space between each slide, and submersed in acetone for 15 minutes. Following this, the rack was left on top of tissue paper to allow the sections to dry completely. Staining of the cells was accomplished by flooding the slides with one part in eight in Giemsa's stain to tap water for 20 minutes before rinsing in running tap water. Appropriate size coverslips were placed on a sheet of tissue paper and a drop of DPX was applied to the coverslip as described previously (see §2.11.3). The slides were quantified at x400 magnification with a graticule-equipped eyepiece and

lymphocytes, neutrophils and macrophages were identified and counted. At least 100 cells were counted on each slide in total.

2.12 Immunohistochemistry

2.12.1 APAAP staining method

Leukocyte recruitment into the lung was analysed by alkaline phosphatase anti-alkaline phosphatase (APAAP) staining method described in Kadioglu & Sheldon (1998). Rat anti-mouse monoclonal antibodies to T cells (anti-CD3), B cells (anti-CD19), macrophages (anti-F4/80) and neutrophils (anti-7/4) (Serotec, UK) were used. A minimum of four whole-lungs, collected at 0, 24 and 48h post infection, were sectioned completely and assessed for wild type and ΔSodA. Four sections from throughout the lung, picked randomly, were used for each antibody to be tested.

First, lung sections were fixed with acetone for 10 minutes at 4^{o}C. Slides were air dried, followed by a wash in PBS for 5 minutes. Normal rabbit serum (diluted 1 in 5) was added onto each slide for 5 minutes. Excess serum was tapped off and the primary antibody (rat anti-mouse monoclonal antibodies – see above) was added to tissue sections for 30 minutes. Sections were than washed with PBS for 5 minutes and incubated with rabbit anti-mouse monoclonal antibody, diluted 1:20, at room temperature, for 30 minutes. Sections were again washed with PBS for 5 minutes and a third antibody, APAAP rat monoclonal (diluted 1 in 50) was added for 30 minutes. Sections were washed in PBS for 5 minutes. Finally, Fast Red TR/Naphtol AS-MX (Sigma F4523) was added (to prepare this solution, a tablet of Sigma Fast Tris buffer [pH 7.4] was dissolved in 10 ml distilled water and a tablet Sigma Fast Red/Naphtol was than dissolved; to inhibit endogenous phosphatase activity, 10μl 1M Levamisole was added to the prepared Fast Red solution) generously to each slide for approximately 20 minutes. Next, sections were washed in distilled water and counterstained briefly with haematoxylin. Finally, slides were washed with tap water, briefly left to dry and subsequently fixed with Aquamount mounting medium (BDH). Once stained, the sections were analysed

and quantified independently by two observers at x200 magnification with a graticule-equiped eyepiece. Positively red-stained leukocytes within the vicinity of inflamed bronchioles were enumerated. At least 10 fields were counted and the number of cell per mm^2 was calculated.

2.13 Statistical analysis

Data were expressed as means ± standard deviations (SD). Groups were compared by analysis of variance (ANOVA) followed by Scheffe's multiple-comparison test. Statistical significance was assumed for $P < 0.05$.

RESULTS

3. Results

Streptococcus pneumoniae is a commensal of the human nasopharynx and a major human pathogen. Nasopharyngeal acquisition may manifest as asymptomatic colonisation or can be the first step to disease (Jones *et al*., 2005). In the initial stages of infection the bacterium is thought to adhere to and enter human nasopharyngeal epithelial cells and subsequently escape to the bloodstream (O'Connell, 2005). The attachment of *S. pneumoniae* to host cell carbohydrate receptors is mediated by specific surface adhesin molecules that form a bridge between bacterial surface components and epithelial cell receptors (García-Rodríguez & Martínez, 2002). However, the molecular details of the interactions of *S. pneumoniae* with human cells are still under investigation. Many questions concerning the site of attachment in the lower respiratory tract, the mechanisms of attachment and the site of invasion remain unanswered. Furthermore, the *in vitro* studies on pneumococcal attachment and invasion need to be corroborated *in vivo*.

Section 3.1 focuses on the attempt to achieve a better understanding of the four pneumococcal factors that play fundamental roles in virulence. The contribution of pneumococcal neuraminidases A and B (§3.1.1), autolysin (§3.1.2) and superoxide dismutase (§3.1.3) to the pathogenesis of *S. pneumoniae* was assessed individually, using a panel of isogenic mutants lacking these enzymes together with a murine model of bronchopneumonia, previously developed in our laboratory.

At the beginning of this project, pneumococcal neuraminidase had been shown to contribute to the virulence of this bacterium. Neuraminidase might aid colonisation by revealing receptors for adherence, providing a carbon source for the bacteria, modifying the surface of competing bacteria within the same niche and/or modifying the function of host clearance glycoproteins (Kelly *et al*., 1967; Schauer, 2000; Shakhnovich *et al*., 2002; Tong *et al*., 2000; 2002). However, the precise role for this enzyme in disease had not yet been established. Furthermore, the importance of the different pneumococcal neuraminidases in the development of upper and lower respiratory tract infections had not been investigated. Contribution

of both NanA and NanB in survival and virulence of pneumococci was investigated in this work.

The *lytA* gene encodes the major *S. pneumoniae* autolysin (amidase). LytA plays a role in autolysis, cell division and peptidoglycan remodelling. Autolysin had been implicated in the pathogenicity of the pneumococcus by catalysing the release of intracellular toxins like pneumolysin, and generating pro-inflammatory cell wall fragments (Ng *et al*., 2002). However, the precise role of LytA in virulence of *S. pneumoniae* is still under debate and was further investigated in this study.

Work previously done in our laboratory has shown that superoxide dismutase (SOD) is a virulence factor for *S. pneumoniae*. An isogenic mutant ΔSodA was significantly impaired in ability to replicate in the lungs of mice (Yesilkaya *et al*., 2000). In this study, results regarding the contribution of SOD in the nasopharynx *in vivo* and how the lack of *sodA* affects the host immune response to this bacterium are presented.

3.1 Studies on pneumococcal virulence factors

In this section, the contributions of pneumococcal neuraminidases A and B (§3.1.1), autolysin (§3.1.2) and superoxide dismutase (§3.1.3) in virulence were assessed individually using a murine model of bronchopneumonia. In this regard, mice were infected intranasally with either *S. pneumoniae* D39 wild type or its isogenic mutants and were culled at pre-chosen time intervals or when they reached a very lethargic state. Bacterial growth in the upper and lower respiratory tract and in blood, histological changes in lungs and host immune responses to pneumococci lacking each of the enzymes were investigated and compared to the parental wild type strain.

Data regarding the intranasal infection with wild type strain were collected from two independent sets of experiments. These data are repeated in each section (§3.1.1, §3.1.2, §3.1.3) together with data for each mutant, in order to compare the outcomes of the parent and mutant strains.

3.1.1 Neuraminidase

Wild type *S. pneumoniae* serotype 2 strain D39 and the isogenic neuraminidase-deficient mutants, ΔNanA and ΔNanB, were used in this study to assess the effect of neuraminidase on pneumococcal survival in tissues of the respiratory tract and onset of disease.

3.1.1.1. Confirmation of the mutation by PCR

Both isogenic neuraminidase-deficient mutants used in this study, ΔNanA and ΔNanB (Table 2.2 §2.3), were constructed previously. ΔNanA (Berry & Paton, 2000) was constructed by insertion duplication mutagenesis rendering the recombinant erythromycin resistant (see §2.5); Prof. J. Paton (School of Molecular and Biomedical Science, University of Adelaide, Australia) constructed ΔNanB by deletion of the *nanB* gene (Berry *et al*., 1996).

To confirm the mutation of ΔNanA and ΔNanB, a set of forward and reverse complementary primers was designed on the basis of the published gene sequence and respective mutagenesis procedure used (see §2.5 for details on primers used). PCR (see §2.6) was then performed to confirm the mutations. The PCR products obtained were analysed on a 0.7% (w/v) agarose gel (see §2.7).

ΔNanA was constructed by insertion duplication mutagenesis using the vector pVA891 (5.4Kb) which encodes chloramphenicol and erythromycin resistance. The first step of the mutagenesis procedure involves cloning an internal fragment of the *nanA* gene (637bp) *Hind*III - *Sph*I fragment corresponding to nucleotides 1210 to 1847 of the *nanA* ORF into *Hind*III - *Sph*I digested pVA891 (Fig. 3.1).

The primers NanAF and NanAR (previously available in the laboratory at the time of the PCR experiment) could not be used to confirm the mutation in ΔNanA mutant strain since they ‘read’ outside the *nanA* fragment cloned into plasmid pVA891, as shown in Figure 3.1. These primers amplify a fragment of 604bp both in the wild type strain and isogenic mutant ΔNanA.

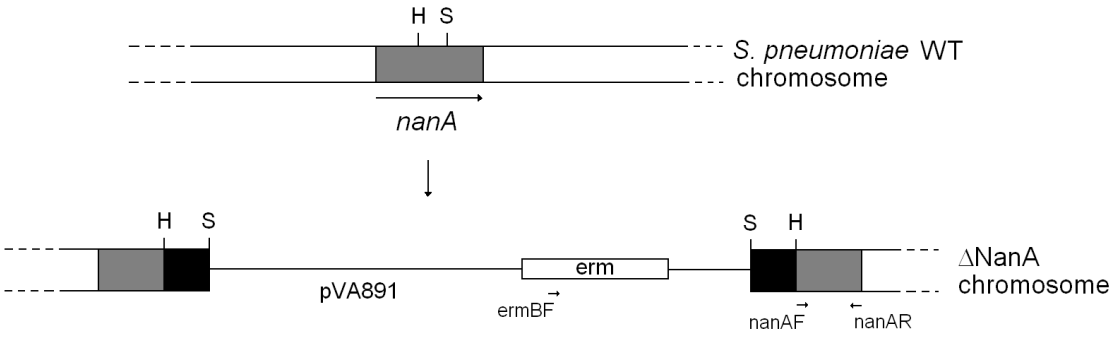


Figure 3.1 Scheme for insertion duplication mutagenesis of the *S. pneumoniae* chromosomal neuraminidase A gene. The grey box (denoted as *nanA*) with 3Kb shows the complete structural gene for neuraminidase A. The open wide box in pVA891 (denoted as erm) indicates the location of the erythromycin resistance gene. H, *Hind*III; S, *Sph*I. NanAF, NanAR and ErmF, primers used to confirm ΔNanA mutant.

Consequently, the primers ErmF and NanAR (Fig. 3.1) that amplify a fragment of around 3Kb in this mutant were chosen to confirm the mutation in the strain ΔNanA. Figure 3.2A (Lanes 3, 6, 7, 8 and 9) shows the PCR amplified fragment from neuraminidase-negative mutant ΔNanA of approx. 3Kb, confirming the presence of an erythromycin cassette disrupting *nanA*. However, the presence of a band at around 1.5Kb when using the wild type parent as a template was unexpected, as seen in Figure 3.2A lane 2.

ΔNanB mutant strain was confirmed using the primers NanBF, NanBR, that amplify a fragment of 1098bp in the wild type parent strain and do not amplify any fragment in ΔNanB (consistent with the deletion of the *nanB* gene, see Appendix A), as shown in Figure 3.2B.

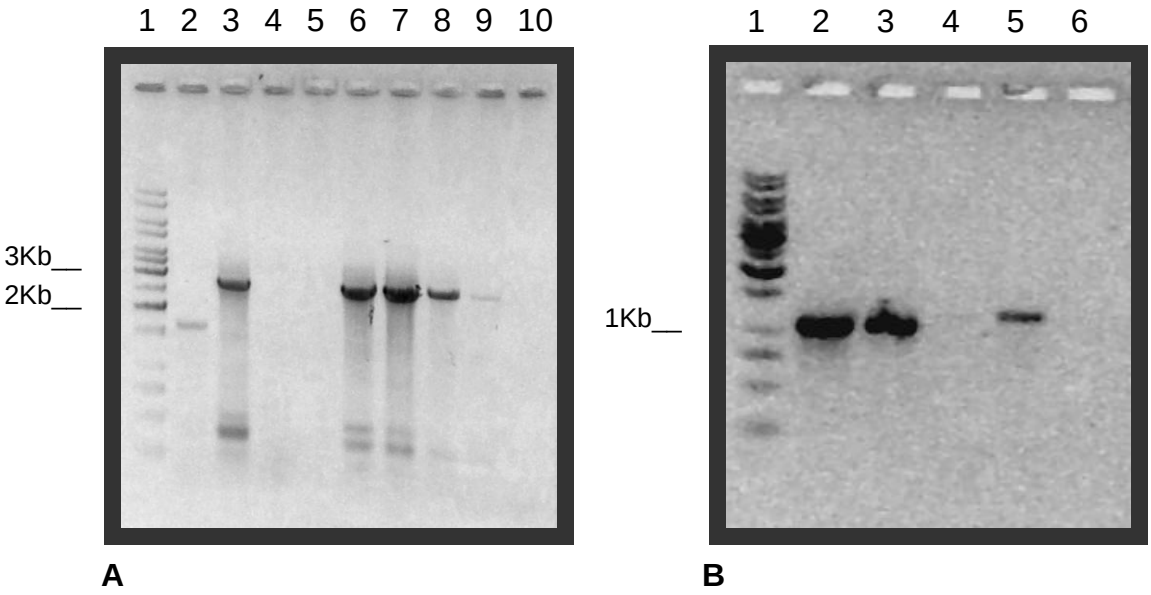


Figure 3.2 PCR confirmation of the mutation of the neuraminidase mutants:
(A) ΔNanA, using the primers ErmBF and NanAR; Lane 1: 1Kb ladder (GeneRuler); Lane 2: wild type strain showing a band just above 1.5Kb in size; Lanes 3-9: neuraminidase-mutant ΔNanA showing the expected band at around 3Kb; Lane 10: nanopure water (negative control)
(B) ΔNanB, using the primers NanBF and NanBR; Lane 1: 1Kb ladder (GeneRuler); Lanes 2 & 3: wild type strain and ΔNanA (respectively) showing a band at around 1098bp (positive controls); Lane 4: neuraminidase-negative mutant ΔNanB showing no band, consistent with the mutation in the *nanB* gene; Lane 6: nanopure water (negative control)

3.1.1.2 *In vitro* growth curve

For *in vitro* growth experiments, wild type pneumococci and the neuraminidase-deficient mutants, ΔNanA and ΔNanB, were grown from an optical density at 500nm of 0.2 in BHI-NCS, as described in §2.4. Deletion of either *nanA* or *nanB* did not affect the fitness of the mutants, as growth curves of the wild type, ΔNanA and ΔNanB strains, in rich liquid medium, aerobically without agitation, were comparable, as shown in Figure 3.3. Moreover, the rates of increase in optical density were similar for wild type, ΔNanA and ΔNanB.

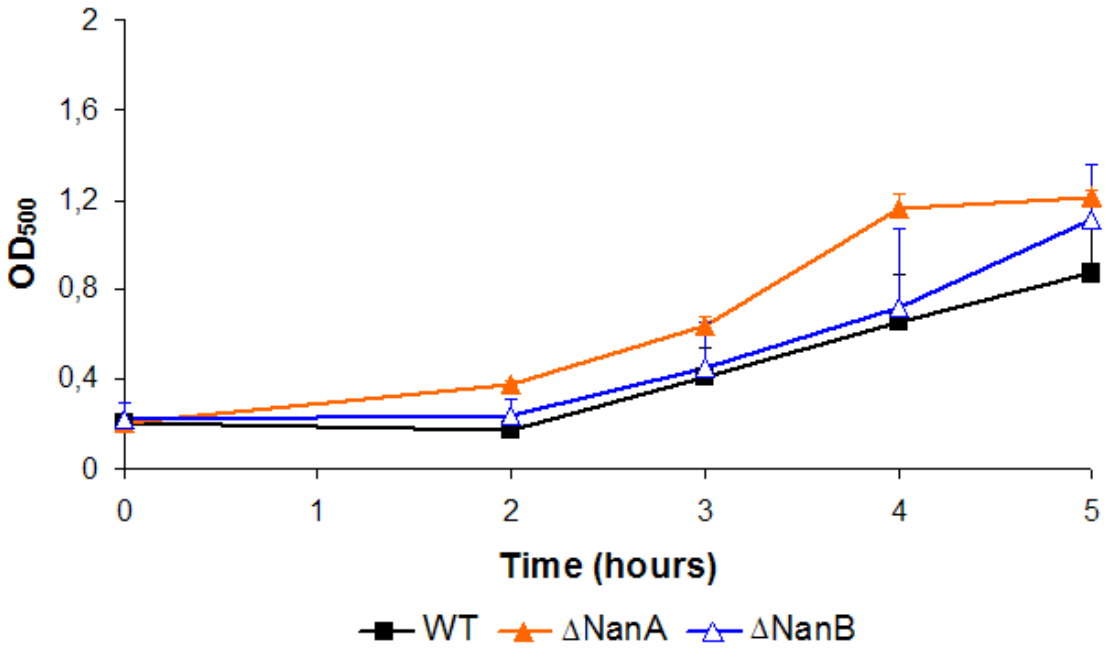


Figure 3.3 Growth of *S. pneumoniae* D39 wild type, ΔNanA and ΔNanB in BHI-NCS broth. N= 3 for each point. Bars indicate standard deviation (SD). OD_{500}, optical density at 500nm.

3.1.1.3 Neuraminidase activity

The neuraminidase activity of *in vitro* grown and *in vivo* grown (passaged) wild type, ΔNanA and ΔNanB pneumococci was assayed at pH 6.6 using the chromogenic pNP-NANA substrate, as described in §2.9. The results are shown in Table 3.1.

Neuraminidase activity for *in vitro* grown wild type pneumococci was significantly greater ($P<0.05$) than its isogenic neuraminidase mutants grown in the same conditions, confirming the phenotypes of the mutants. A significant difference ($P<0.05$) was also observed between the neuraminidase activities of ΔNanA and ΔNanB, both *in vitro* and *in vivo*. The level of neuraminidase activity was lower in passaged pneumococci compared to non-passaged bacteria for all the strains, however the changes did not reach statistical significance ($P>0.05$). These results show that, using this quantitative method, small differences in neuraminidase activity could be detected between murine peritoneal cultured pneumococci and non-passaged bacteria.

For *in vivo* passaged bacteria, the level of neuraminidase activity was greater in wild type when compared to ΔNanA ($P<0.05$) but not when compared to ΔNanB pneumococci ($P>0.05$). These results show a considerably lower neuraminidase activity in both *in vitro* and *in vivo* grown ΔNanA pneumococci, at pH 6.6 (pH optimum for NanA).

3.1.1.4 Transcriptional analysis of neuraminidase gene expression

Levels of *nanA* and *nanB* gene expression were quantified during *in vitro* growth (this work was performed by Dr. Yesilkaya, Department of Infection, Immunity and Inflammation, University of Leicester; for Methods see Manco *et al*., 2006). Both *nanA* and *nanB* genes were transcribed *in vitro*; however, the level of *nanA* gene transcription in mid-log phase was 10-fold higher than that of *nanB* (there was no difference in bacterial growth *in vitro* between the mutants and the wild type pneumococci [data not shown]).

Table 3.1 Neuraminidase activity in *S. pneumoniae*. Neuraminidase activity was assessed in *in vitro* grown and *in vivo* passaged (denoted as pass.) wild type (WT), ΔNanA and ΔNanB pneumococci, at pH 6.6. Average values are expressed as nMole PNP released per minute per μg total protein, and represent the average of two separate experiments, using triplicates in each experiment. SD, standard deviation.

Strain	Average	SD
WT	43.5	11.2
WT pass.	29.7	12.5
ΔNanA	4.4	1.2
ΔNanA pass.	1.4	1.5
ΔNanB	22.7	7.8
ΔNanB pass.	18.4	3.2

The mean Ct value for *nanA* expression in the wild type strain was 28.33 ± 0.26, corresponding to 114 ± 2.64 pg *nanA*-specific mRNA transcript, whereas the mean Ct value for *nanB* was 32.59 ± 0.28 corresponding to 11.3 ± 0.36 pg *nanB*-specific mRNA transcript. Analysis of both neuraminidase mutants also revealed that the mutation of neither the *nanA* nor *nanB* gene was compensated by the increased transcription of the other. The mean Ct values for *nanB* expression in the ΔNanA mutant or *nanA* expression in the ΔNanB mutant were similar to those for the wild type (31.71 ± 0.24 and 27.81 ± 0.08, respectively). PCR efficiency with both *nanA* and *nanB* primer sets was also comparable. In addition, we measured the transcriptional expression of SPR1535 (the gene downstream of *nanA*) in both the wild type and in the *nanA* isogenic mutant strain to see whether or not there was a polar effect of the insertion mutagenesis method used for the ΔNanA mutant. We found no significant difference in the transcriptional level of SPR1535 in the *nanA* mutant strain compared to the parent wild type strain (data not shown). Therefore, this rules out the possibility of a polar effect of the mutation.

3.1.1.5 Virulence following intranasal infection

MF1 mice infected intranasally with10^6 cfu *S. pneumoniae* wild type, ΔNanA or ΔNanB were monitored for clinical signs throughout the experiment, in order to assess the virulence of these strains. Clinical signs were registered in a score sheet throughout the course of each experiment. A scale was used to score signs of disease as follows: 1- normal; 2,3- hunched position; 4,5- starry coat; 6,7- lethargic. Mice were culled at intervals or when they reached a very lethargic state (note that even if mice presented no signs of disease they were culled at a given time point in order to register the number of cfu or for histological analysis at that time point).

By 12h post-infection 8 out of 10 mice challenged with wild type strain were reasonably ill (*i.e.* they were hunched and with severe starry coat) and the average score for these mice was 4 (Fig. 3.4). All mice were very lethargic by 24h post-infection (except for one mouse that was normal) at which point they were culled. Noteworthy is the fact that, in this particular experiment, the batch of wild type-passaged pneumococci used for infection seemed particularly virulent. Usually, following intranasal infection with *S. pneumoniae* type 2 strain D39, mice are moderately ill by 24h post-infection and become very lethargic by 48h (results obtained from other animal experiments done in our laboratory, see Gingles *et al*., 2001).

In contrast, when mice were infected with ΔNanA, none (32 mice in total) showed signs of disease by 12h post-infection (Fig. 3.4). It was not until 24h post-infection that 10% of the mice started to show slight symptoms of disease and by 48h the percentage of mice with moderate signs of disease (score between 2 and 3) increased to 25% (Fig. 3.4).

When mice were infected with ΔNanB mutant strain, no signs of disease were observed throughout the 7 days - time course of the experiment (Fig. 3.4 shows data to 48 hours).

Overall, in this mouse model of bronchopneumonia, the *nanA* and *nanB* knockouts, ΔNanA and ΔNanB, caused a significant difference ($P<0.05$) in the survival curves of mice compared with the isogenic wild type strain.

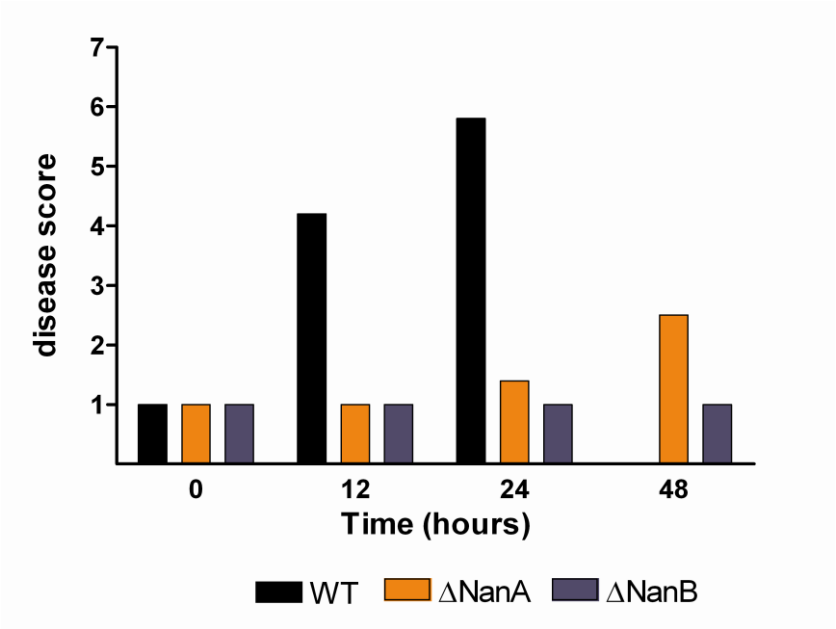


Figure 3.4 Effect of neuraminidase in virulence of *S. pneumoniae*. The virulence of *S. pneumoniae* neuraminidase mutants ΔNanA and ΔNanB was tested in a murine model of bronchopneumonia by scoring the signs of disease of each mouse. 10^6 cfu of wild type, ΔNanA or ΔNanB pneumococci were administered through the nostrils of mice. Signs of disease were recorded on a score sheet through the course of each experiment. A scale was used to score signs of disease as follow: 1- normal; 2, 3- hunched position; 4, 5- starry coat; 6, 7- lethargic. Mice were culled at intervals or when they reached a very lethargic state (score 6). The graph shows the average disease score for all the mice at each time point.

3.1.1.6 Growth of wild type, ΔNanA and NanB in nasopharyngeal tissues and trachea

Streptococcus pneumoniae D39 wild type was present in the nasopharynx of mice in significantly greater numbers ($P<0.05$) than ΔNanA or ΔNanB by 24h post-infection (Fig. 3.5A). However, there were significantly more ($P<0.05$) ΔNanB in the nasopharynx than ΔNanA at each time point (Fig. 3.5A) from 2h post-infection onwards. When ΔNanB pneumococci were administered i.n. at a lower dose ($1x10^3$ cfu) than here ($1x10^6$ cfu), bacterial numbers still persisted in the

nasopharynx of infected mice by 48h post-infection (log_{10} cfu 1.15 +/- 1.6 at T=0, and log_{10} cfu 1.08 +/- 1.5 at T=48).

In contrast to ΔNanB, which persisted in the nasopharynx, ΔNanA was rapidly cleared from this niche and was not detected at 12h post-infection (Fig. 3.5A). Even when a higher dose of ΔNanA pneumococci was administrated i.n. ($1x10^7$ cfu), bacteria were cleared from this site by 24h post-infection (log_{10} cfu 3.3 +/- 0.007 at T=0; log_{10} cfu 2.5 +/- 0.4 at T=6 and log_{10} cfu 0 +/- 0 at T=24).

In the early hours following infection (*i.e.* 2 to 6h), the numbers of wild type and ΔNanB pneumococci in the nasopharynx were not statistically different in contrast with the numbers of ΔNanA that sharply declined during this period of time. However, from 6h post-infection, numbers of wild type pneumococci started to increase significantly in the nasopharynx ($P<0.05$ between 6h and 24h post-infection) whereas the numbers of ΔNanB bacteria did not change ($P>0.05$ between 6h and 24h post-infection).

Wild type pneumococci infected the trachea (Fig. 3.5B) in significantly greater numbers than both ΔNanA and ΔNanB by 24h post-infection ($P<0.05$). Wild type pneumococci increased significantly in the trachea over this time ($P<0.05$). However, the behaviour of the two mutants was different (Fig. 3.5B). Whereas ΔNanB numbers remained constant over this period, ΔNanA was rapidly cleared. By 4h post-infection no ΔNanA could be detected in the trachea of mice. Similar results were obtained when ΔNanA was administrated at a higher dose (see above). In this case the mutant was not recovered from this niche by 6h post-infection (log_{10} cfu 2.4 +/- 0.3 at T=0 and log_{10} cfu 0 +/- 0 at T=6 and T=24). In contrast, ΔNanB persisted in the trachea in significantly greater numbers than ΔNanA, as mentioned above. Even at a lower dose, ΔNanB persisted in the trachea over 48h post-infection (log_{10} cfu 0.9 +/- 1.3 at T=0; log_{10} cfu 3.7 +/- 0.09 at T=6; log_{10} cfu 3.1 +/- 0.6 at T=24 and log_{10} cfu 1.7 +/- 1.6 at T=48).

As ΔNanB persisted in the upper respiratory tract of mice over a 24h period without causing any symptoms of disease, the outcome of this mutant in the nasopharynx and trachea was investigated over a period of 4 days. ΔNanB was still recovered from these niches at the end of the experiment (log_{10} cfu 1.1 +/- 1.3 in the nasopharynx at T=96; log_{10} cfu 1.4 +/- 1.0 in the trachea at T=96). The timescale of the experiment was then increased to 7 days. At the end of this

experiment ΔNanB was still recovered from the trachea of infected mice (Fig. 3.5B) in contrast with the nasopharynx where the bacteria were only recovered for up to 5 days (Fig. 3.5A). The average number of bacteria recovered from the nasopharynx beyond the 24h period varied between $\log_{10}$ cfu 1.95 +/- 0.8 at 48h post-infection and $\log_{10}$ cfu 0.97 +/- 1.15 at 120h post-infection.

3.1.1.7 Growth of wild type, ΔNanA and ΔNanB in lung tissue and blood

There were clear differences in the numbers of the wild type and neuraminidase-negative mutant strains in the lungs (Fig. 3.6) and blood.

As was the case for the upper respiratory tract, there were significantly greater numbers of wild type pneumococci in the lungs by 24h post-infection (Fig. 3.6) than either ΔNanA or ΔNanB pneumococci ($P<0.05$). ΔNanA was completely cleared from the lungs by 12h post-infection (Fig. 3.6). When administered at a higher dose ($1x10^7$ cfu) ΔNanA was recovered from the lungs by 6h post-infection in very low numbers ($\log_{10}$ cfu 0.8 +/- 0.94) and was cleared from this tissue at 24h post-infection. Both wild type and ΔNanB were present in the lungs in significantly greater numbers than ΔNanA during the 2 to 12h post-infection period ($P<0.05$). However ΔNanB was cleared from the lungs by 72h post-infection (Fig. 3.6), in contrast to its ability to persist both in the nasopharynx and trachea for longer periods. Interestingly, when ΔNanB was administered i.n. at a significantly lower dose ($1x10^3$ cfu), the mutant was still recovered from the lungs of mice by 48h post-infection, however at a low number ($\log_{10}$ cfu 1.6 +/- 0.97).

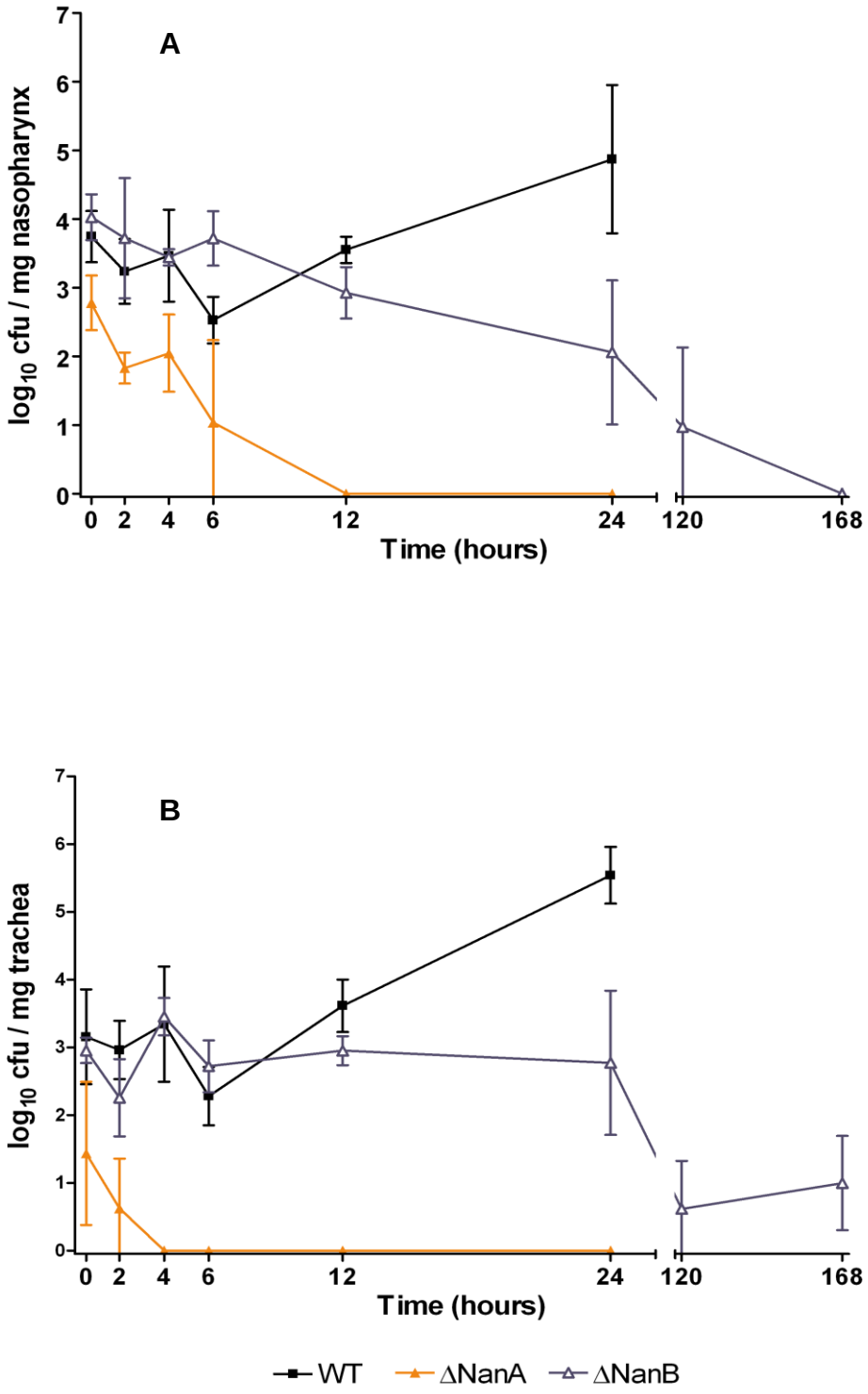


Figure 3.5 Effect of neuraminidase in nasopharyngeal tissues and trachea. *S. pneumoniae* numbers in the nasopharynx (A) and trachea (B) of mice challenged i.n. with 10^6 cfu of wild type, ΔNanA or ΔNanB. For cfu determination pre-selected groups of 4 to 8 mice were culled at the time points shown. Data points and error bars represent the mean and SD of the $\log_{10}$ bacteria per mg of organ.

Wild type pneumococci caused severe bacteremia following intranasal infections. A rapid rise in wild type pneumococcal numbers in the blood was observed during the period 24h post-infection (log_{10} cfu 4.3 +/- 0.58 at 12h to log_{10} cfu 9.3 +/- 0.36 at 24h post-infection). All mice were severely lethargic by this stage and were culled.

Interestingly however, ΔNanA and ΔNanB were never isolated from the blood at any time point following intranasal infection. These results were also true when ΔNanA or ΔNanB were administrated i.n. at a higher dose ($1x10^7$ cfu). To determine whether or not this was due to an inability to seed from lungs to blood or a failure to survive in blood, both ΔNanA and ΔNanB were used in intravenous infections (i.v.). These i.v. infections were performed by Dr Kadioglu (Department of Infection, Immunity and Inflammation, University of Leicester).

When ΔNanA, ΔNanB and wild type pneumococci were infected directly into the blood, ΔNanA and ΔNanB numbers declined over 24h and 48h post-infection, respectively (Fig. 3.7) and were not recovered from blood beyond this time point. Moreover, mice did not show any signs of disease during the time course of the experiment. On the other hand wild type pneumococci increased rapidly until 48h post-infection (Fig. 3.7), by which time mice became severely lethargic and the experiment was ended.

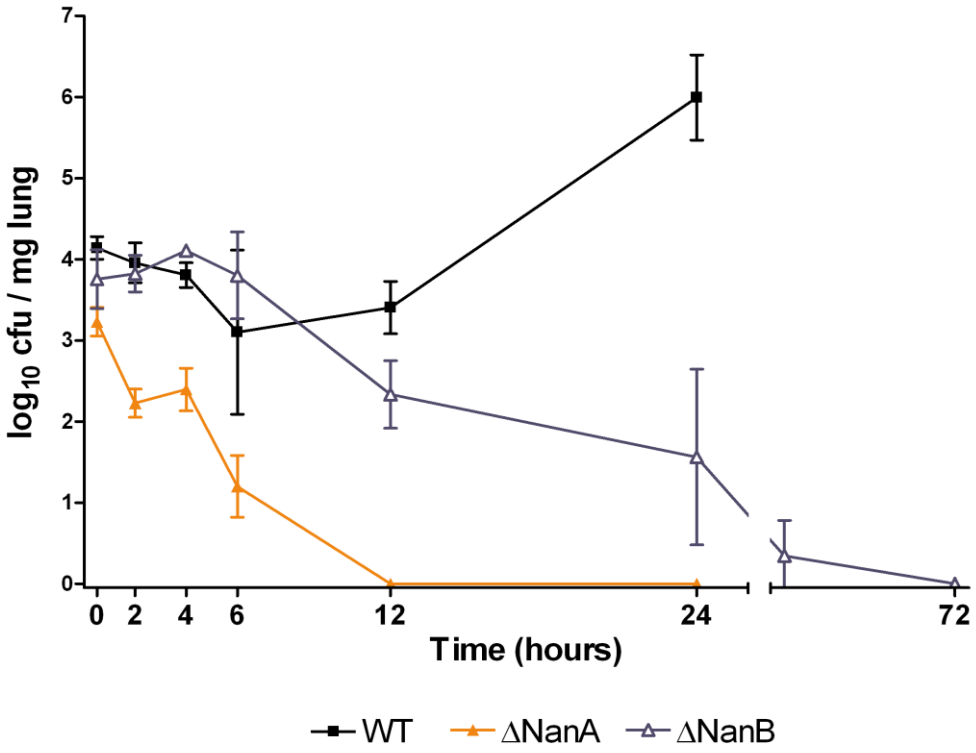


Figure 3.6 Effect of neuraminidase in the lungs. *S. pneumoniae* numbers in the lungs of mice challenged i.n. with 10^6 cfu of wild type, ΔNanA or ΔNanB. For cfu determination pre-selected groups of 4 to 8 mice were culled at the time points shown. Data points and error bars represent the mean and SD of the $\log_{10}$ bacteria per mg of organ.

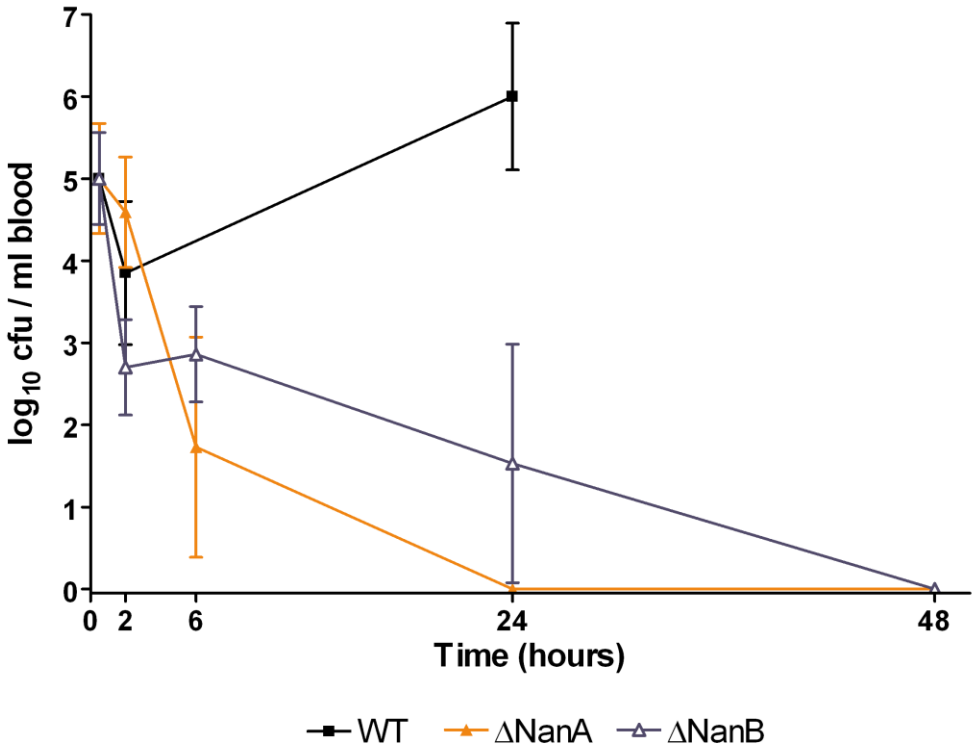


Figure 3.7 Effect of neuraminidase in bacteremia. *S. pneumoniae* numbers in blood of mice challenged i.v. with 10^5 cfu of wild type, ΔNanA or ΔNanB. For cfu determination tail bleeds from 10 mice were performed at the time points shown. Data points and error bars represent the mean and SD of the $\log_{10}$ bacteria per ml of blood.

3.1.1.8 Histological analysis of wild type-, ΔNanA- and ΔNanB-infected lung tissue

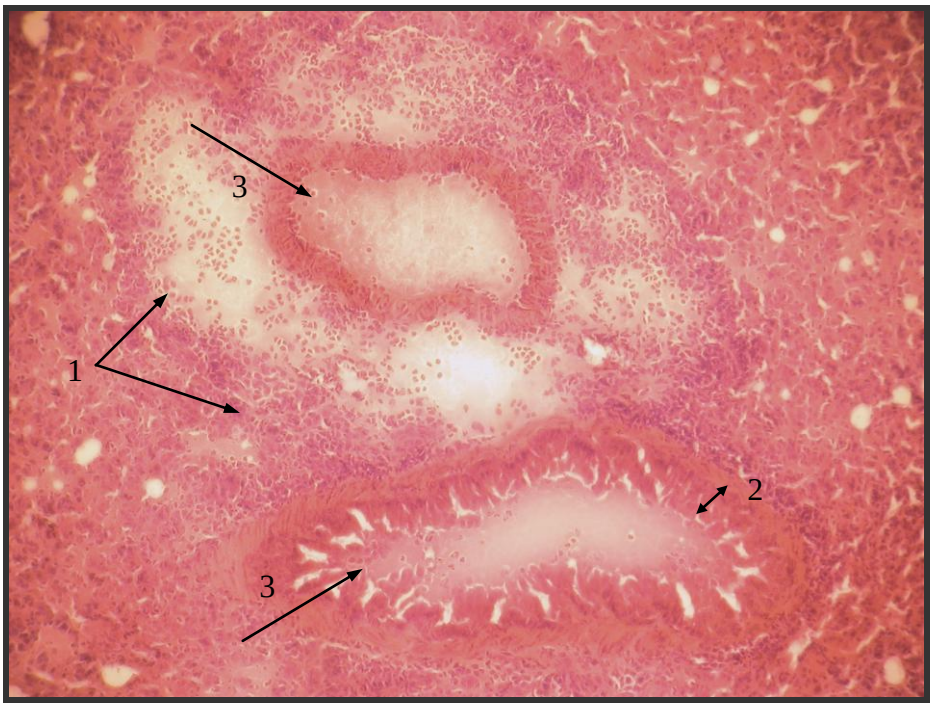
At intervals following intranasal infection, mice were culled for histological examination of lung tissue sections. Mice were infected with wild type pneumococci, ΔNanA or ΔNanB and lung sections were stained with haematoxylin and eosin, as described in §2.11.1 to §2.11.3.

(Note that in this experiment, the infection with wild type pneumococci was using a different batch of passaged bacteria than the one used for infections to determine colony counts. Here mice only became very lethargic by 48h post-infection, as opposed to 24h post-infection in the cfu counts experiment. For this reason, lungs infected with wild type pneumococci were collected at intervals up to 48h post-infection for histological examination).

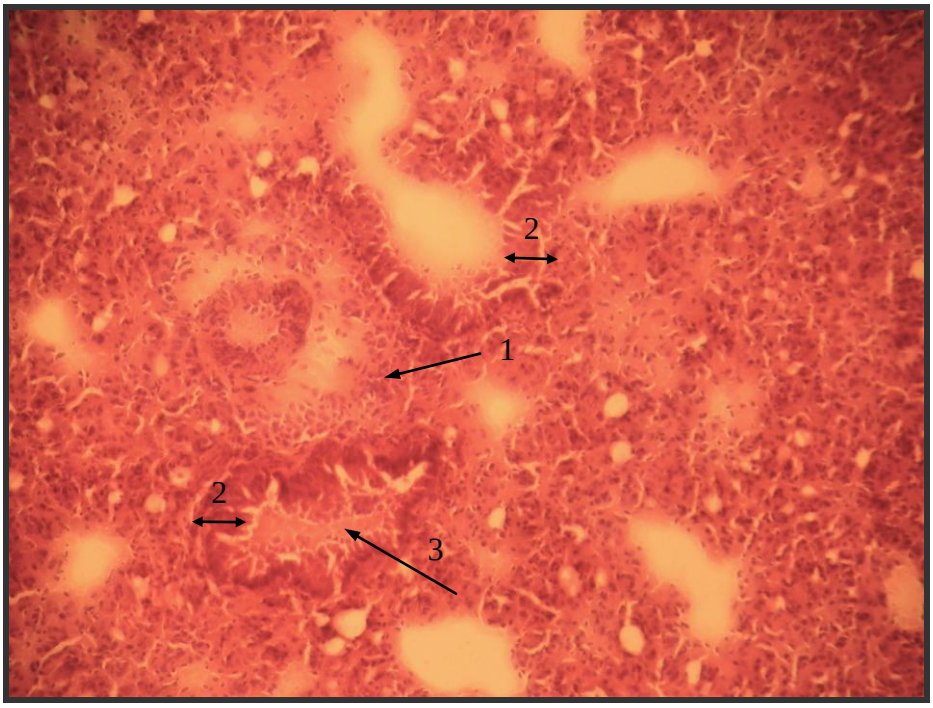
By 24h after infection, histology of lung sections of mice infected with wild type pneumococci showed inflammation and heavy cellular infiltration centred around bronchioles and perivascular areas (Fig. 3.8 A&B, arrow-1). This inflammation was however restricted to the bronchioles that exhibited infiltration. Inflammation presented itself as slight hypertrophy of bronchial walls (Fig. 3.8 A&B, arrow-2) and some exudate filling those infected bronchioles (Fig. 3.8 A&B, arrow-3). The parenchyma was not involved in inflammation and interstitial alveolitis was not detected at this time point.

By 48h post-infection with wild type, bronchiole wall thickening had increased (Fig. 3.9 A&B, arrow-2) and exudate was filling the bronchioles (Fig. 3.9 A&B, arrow-3) and alveolar spaces. Additionally, cellular infiltration had increased, with extension of inflammation into the lung parenchyma (Fig. 3.9 A&B, arrow-1). Intensive tissue exudate was now visible. Overall, by this time inflammation had covered nearly the entire lung surface.

When ΔNanB-infected lungs were examined at 24h after infection, mild peribronchial cellular infiltration was observed (Fig. 3.10A, arrow-1) together with

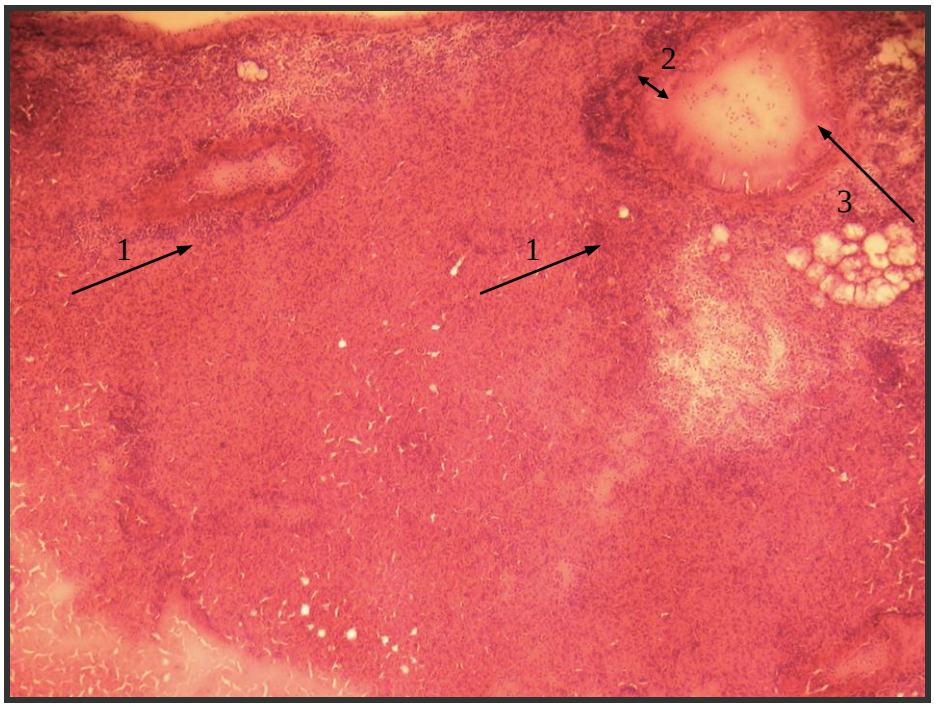


A



B

Figure 3.8 Inflammation in the lung following infection with wild type. Light microscopy of H&E stained lung tissue collected from MF1 mice infected with 10^6 cfu of *S. pneumoniae* wild type at 24h post-infection. Magnification: Panel (A) 40x; Panel (B) 100x. Arrow-1 indicates cellular infiltrate; Arrow-2 indicates bronchial wall hypertrophy; Arrow-3 indicates exudate.



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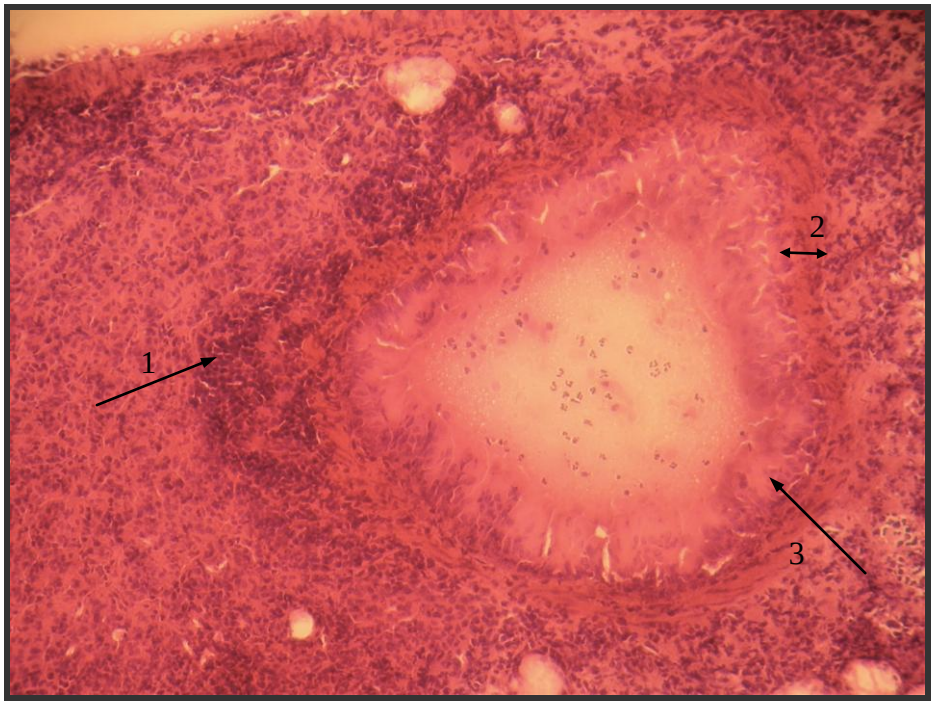
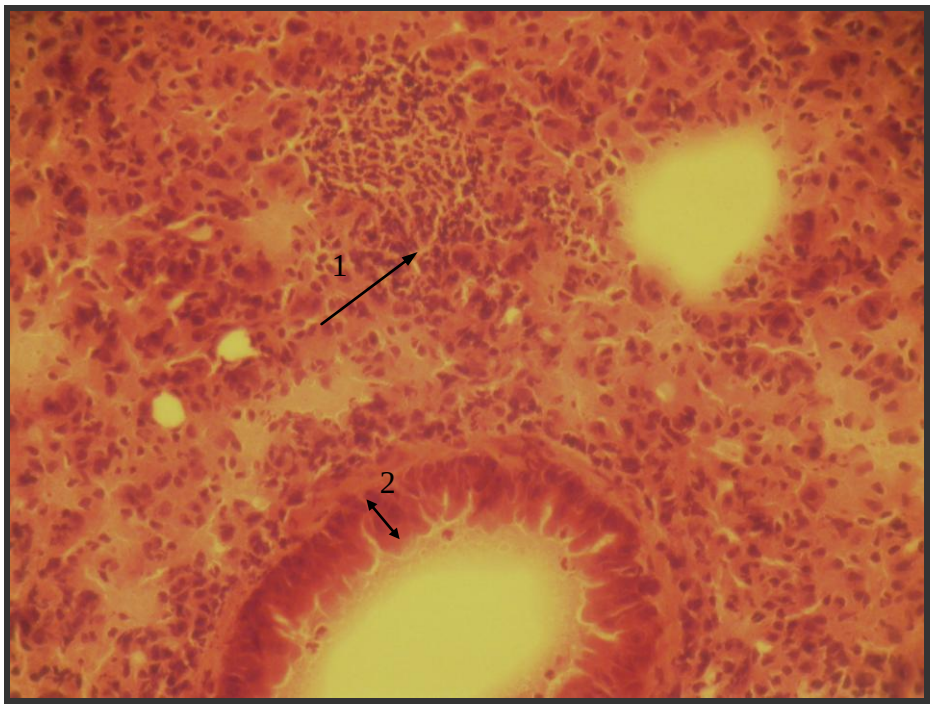


B

Figure 3.9 Inflammation in the lung following infection with wild type. Light microscopy of H&E stained lung tissue collected from MF1 mice infected with 10^6 cfu of *S. pneumoniae* wild type at 48h post-infection. Magnification: Panel (A) 40x; Panel (B) 100x. Arrow-1 indicates cellular infiltrate; Arrow-2 indicates bronchial wall hypertrophy; Arrow-3 indicates exudate.

mild levels of bronchial wall hypertrophy (Fig. 3.10A, arrow-2). Lung parenchyma in these lungs was generally not involved in inflammation, with no clear diffusion of cellular infiltrates into these areas. Moreover, there was no interstitial alveolitis apparent. ΔNanB-infected lungs showed an increase in the extension of inflammation when examined at 48h post-infection, both in bronchial wall hypertrophy (Fig. 3.10B, arrow-2) and cellular infiltration (Fig. 3.10B, arrow-1). To a certain extent, tissue exudate was also observed (Fig. 3.10B, arrow-3). Overall, however, ΔNanB-infected lungs exhibited less severe inflammation than wild type at 24h and 48h post-infection.

The histological changes seen at 24h post-infection in the lungs infected with ΔNanA (Fig. 3.11B) were substantially diminished when compared with both the wild type parent (Fig. 3.8) and ΔNanB (Fig. 3.10A). There was little general involvement or consolidation of the lung parenchyma and minimal hypertrophy of bronchiole walls, indeed the vast majority of bronchiole walls did not appear to be inflamed. Moreover, there were no major areas of cellular infiltration, either peribronchial or perivascular, and there was an absence of exudate in bronchioles and alveoli.



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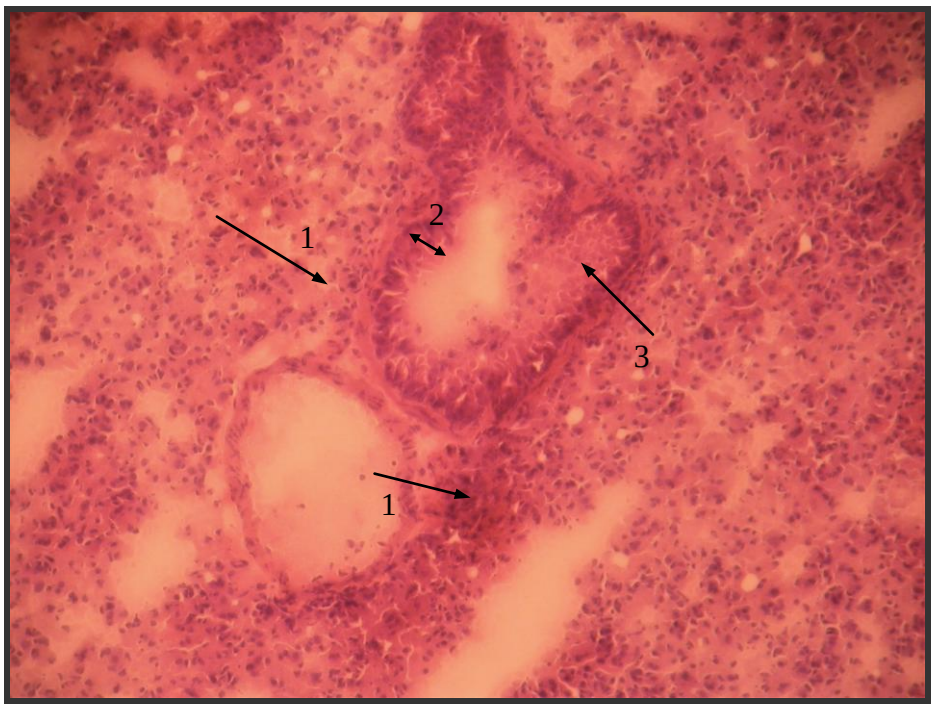
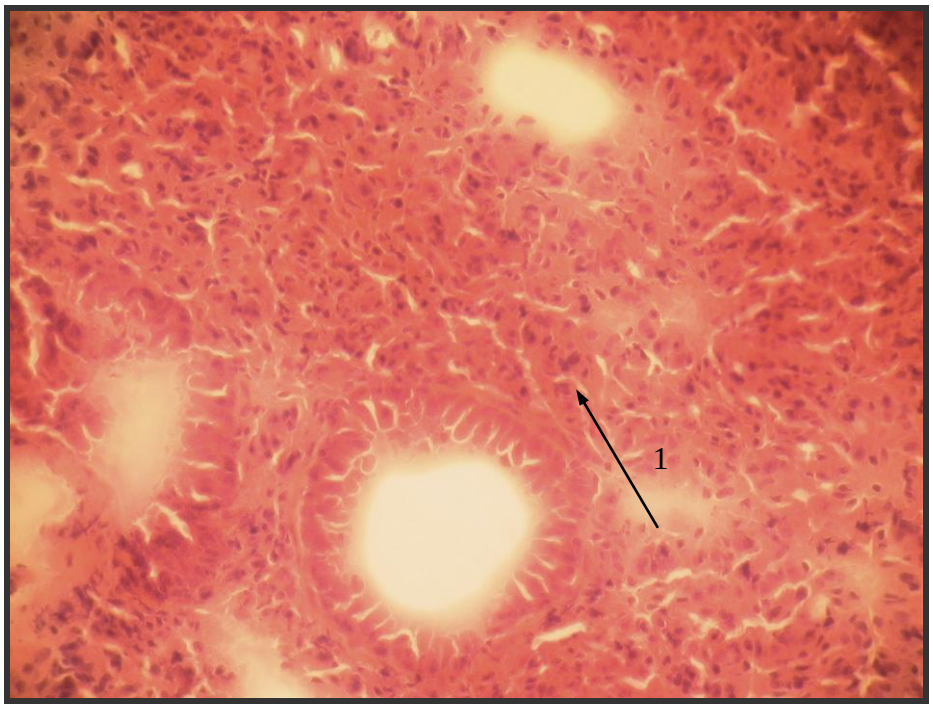


B

Figure 3.10 Inflammation in the lung following infection with ΔNanB. Light microscopy of H&E stained lung tissue collected from MF1 mice infected with 10^6 cfu of *S. pneumoniae* ΔNanB at 24h post-infection (panel A) or at 48h post-infection (panel B). Magnification: Panel (A) 200x; Panel (B) 100x. Arrow-1 indicates cellular infiltrate; Arrow-2 indicates bronchial wall hypertrophy; Arrow-3 indicates exudate.



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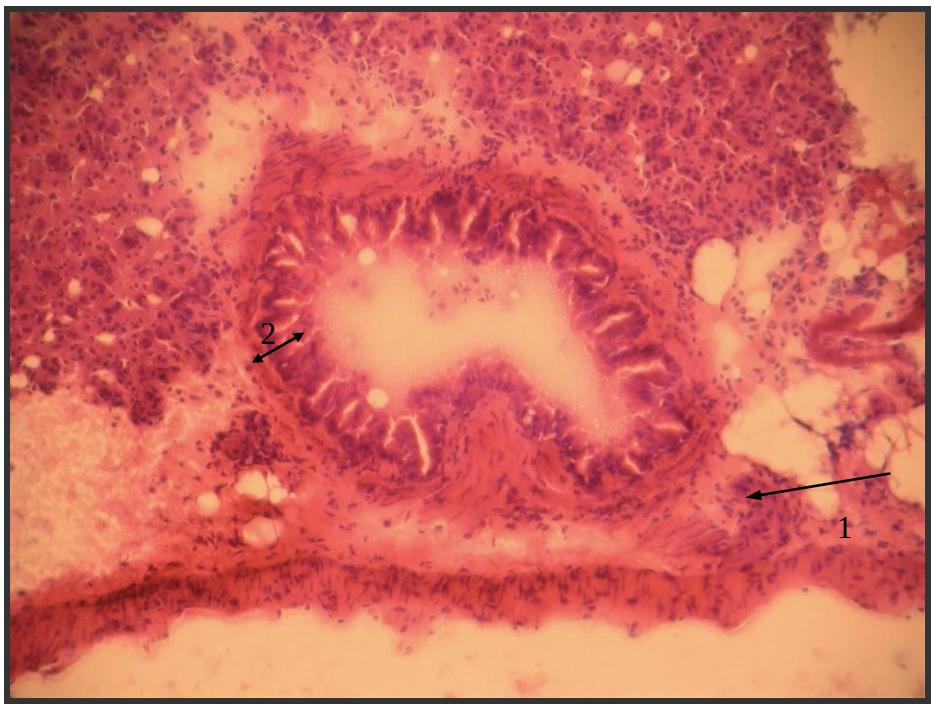


B

Figure 3.11 Inflammation in the lung following infection with ΔNanA. Light microscopy of H&E stained lung tissue collected from MF1 mice infected with 10^6 cfu of *S. pneumoniae* ΔNanA at 12h post-infection (panel A) or at 24h post-infection (panel B). Magnification: Panel (A) 100x; Panel (B) 200x. Arrow-1 indicates cellular infiltrate; Arrow-2 indicates bronchial wall hypertrophy.

3.1.1.9 Analysis of leukocyte infiltration in wild type-, ΔNanA- and ΔNanB-infected lung tissue

Total leukocyte numbers (Fig. 3.12) and individual lymphocytes, macrophages and neutrophils cell numbers were enumerated (Table 3.2) over the time course of infection with *S. pneumoniae* wild type, ΔNanA and ΔNanB in lungs of mice infected intranasally, as described in §2.11.4 and §2.11.5. Total and individual leukocyte counts were also enumerated in lungs from two pathogen-free mice and were used as a control.

The results presented in this section for wild type-infected mice were provided by Dr Kadioglu (Department of Infection, Immunity and Inflammation, University of Leicester), from experiments done in parallel. (Note that these mice became very lethargic by 48h post-infection, so data were collected up to this time point).
Total leukocyte levels in wild type-infected lung tissue homogenates reached significant increased values by 24h post-infection, compared with time zero levels ($P<0.05$) (Fig. 3.12). These numbers remained unchanged at 48h post-infection. As can be seen from Table 3.2, this rise in total leukocyte cells was due to a major increase in the number of neutrophils present in the lungs of infected mice (4.6-fold increase). Indeed, the numbers of macrophages in the lungs decreased by 24h post-infection ($P<0.05$). Moreover, the table shows no significant changes in the numbers of lymphocytes throughout the time course of this experiment.

When mice were infected with ΔNanA, again as seen with the wild type counts, total leukocyte levels reached significant increased values by 24h post-infection, compared with time zero levels ($P<0.05$) (Fig. 3.12). Table 3.2 shows that this increase in total leukocyte numbers appeared to be due to a rise in all types of cells counted, *i.e.*, neutrophils (3.2-fold increase), lymphocytes (2.3-fold increase) and macrophages (2.3-fold increase). However, statistical significance ($P<0.05$) was only observed for the increase in lymphocyte levels, when comparing values at time zero and 24h post-infection.

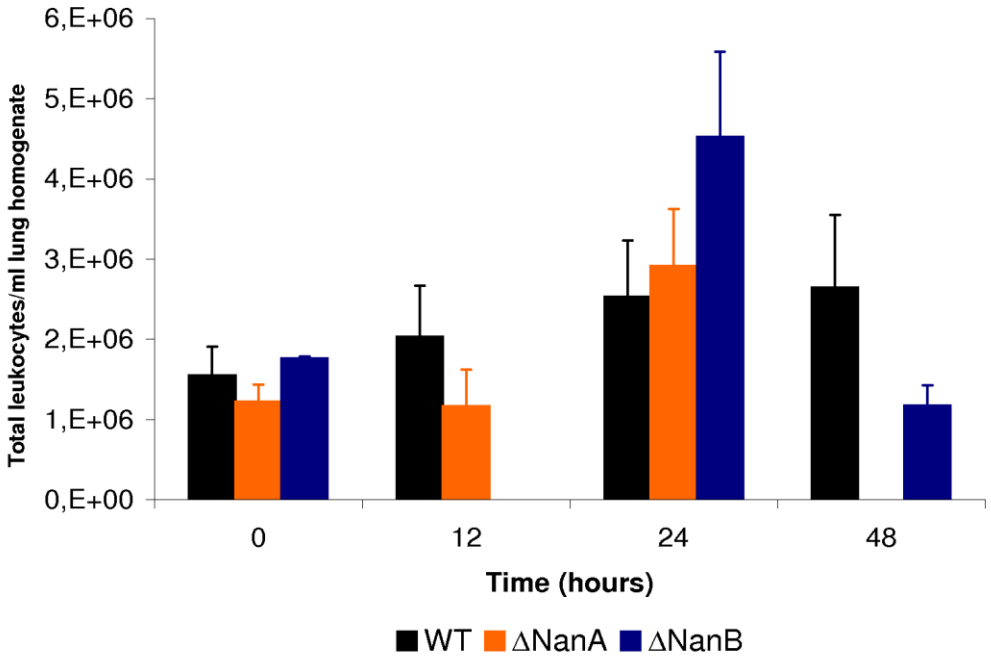


Figure 3.12 Effect of neuraminidase on recruitment of leukocytes to the lung. Total leukocyte counts in cytospin preparations of whole lung homogenates from MF1 mice infected i.n. with 10^6 cfu of *S. pneumoniae* wild type, ΔNanA or ΔNanB. Data are the mean of 4 mice. ΔNanB was not checked at 12h and ΔNanA was not checked at 48h. Bars indicate SD.

Differences between leukocyte counts in lungs infected with ΔNanA compared to control mice lungs (pathogen-free; lymphocytes= 9.0E5 cells/ml, neutrophils= 5.0E4 cells/ml, macrophages= 4.6E4 cells/ml) were statistically significant at time zero for neutrophils, at 12h for neutrophils and macrophages and at 24h for all leukocyte types.

Total leukocyte levels in ΔNanB-infected lungs registered its highest level by 24h post-infection ($P<0.05$ when compared with time zero) however by 48h post-infection these numbers than returned to time zero values ($P>0.05$ when compared with time zero) (Fig. 3.12). This rise seen at 24h was caused by the increase in numbers of neutrophils, lymphocytes and macrophages, which had a 2.3- to 2.7-fold increase (Table 3.2). The numbers of each leukocyte cells were significantly greater by 24h post-infection than at time zero ($P<0.05$). Statistical differences were also observed when comparing this result with individual cell counts in lungs of pathogen-free mice, namely at time zero for neutrophils and macrophages, at 24h for all leukocyte types and at 48h for neutrophils.

Table 3.2 Individual leukocyte counts in cytospin preparations of whole lung homogenates from MF1 mice infected i.n. with 10^6 cfu of *S. pneumoniae* wild type, ΔNanA or ΔNanB.

Neutrophils	WT		ΔNanA		ΔNanB	
Time	Cells/ml	Fold increase	Cells/ml	Fold increase	Cells/ml	Fold increase
0	3.0E5		1.6E5		3.5E5	
12	7.2E5	2.4	2.7E5	1.8	-	-
24	1.4E6	4.6	4.9E5	3.2	9.5E5	2.7
48	1.3E6	4.4	-	-	2.9E5	0.8
Lymphocytes	WT		ΔNanA		ΔNanB	
Time	Cells/ml	Fold increase	Cells/ml	Fold increase	Cells/ml	Fold increase
0	5.6E5		9.7E5		1.3E6	
12	5.5E5	1.0	7.2E5	0.7	-	-
24	6.2E5	1.1	2.2E6	2.3	3.2E6	2.5
48	6.5E5	1.1	-	-	7.7E5	0.6
Macrophages	WT		ΔNanA		ΔNanB	
Time	Cells/ml	Fold increase	Cells/ml	Fold increase	Cells/ml	Fold increase
0	6.8E5		9.3E4		1.4E5	
12	7.5E5	1.1	1.7E5	1.8	-	-
24	4.9E5	0.7	2.1E5	2.3	3.2E5	2.3
48	7.3E5	1.1	-	-	1.1E5	0.8

Data are the mean of 4 mice. Leukocytes were quantified at x400 magnification with a graticule-equipped eyepiece. Fold increases in leukocyte populations are relative to time zero values.

In summary:

- *In vitro* experiments show that the growth of *S. pneumoniae* D39 wild type is similar to the growth of ΔNanA and ΔNanB.
- The neuraminidase activity of ΔNanA was significantly lower than the wild type or ΔNanB activity when assayed at pH 6.6.
- Both ΔNanA and ΔNanB are considerably less virulent than the wild type parent following i.n. or i.v. infections.
- ΔNanA but not ΔNanB were significantly impaired of growth in the nasopharynx; ΔNanB survived in this site up to one week.
- In the lungs, ΔNanA and ΔNanB were cleared from this site up to 72h post-infection.
- After i.n. infection neither ΔNanA nor ΔNanB caused bacteremia. After i.v. infection both the mutants were cleared from the blood by 48h.
- Both ΔNanA and ΔNanB caused less inflammation of the lungs than the wild type parent.
- Leukocyte infiltration into the lungs infected with ΔNanA or ΔNanB was increased by 24h due to an increase in neutrophil numbers, however smaller than the wild type.

3.1.2 Autolysin

Wild type *S. pneumoniae* serotype 2 strain D39 and the isogenic autolysin-deficient mutant ΔAL2 were used in this study to assess the effect of autolysin (LytA) on pneumococcal survival in tissues of the respiratory tract and onset of disease.

3.1.2.1 Confirmation of the mutation by PCR

The autolysin-deficient mutant used in this study was constructed previously (Berry *et al*., 1989b) by insertion mutagenesis using the vector pVA891 (which confers erythromycin resistance to this mutant).
The primers LytA-For, LytA-Rev (Table 2.3 §2.5) were designed on the basis of the published *lytA* gene sequence (GenBank M13812) and amplify a 957bp fragment of the *lytA* gene in the wild type parent, from nucleotide 584 to 1540. PCR reaction was performed (see §2.6) on *S. pneumoniae* D39 wild type colonies (used as positive control) and the mutant strain ΔAL2; nanopure water was used as a negative control. Using these primers, the mutation of strain ΔAL2 was confirmed by analysing the products obtained on a 0.7% agarose gel (result not shown).

3.1.2.2 Virulence following intranasal infection

MF1 mice infected intranasally with10^6 cfu *S. pneumoniae* wild type or ΔAL2 were monitored for clinical signs throughout the experiment, in order to assess the virulence of these strains. Clinical signs were recorded on a score sheet throughout the course of each experiment (using a scale from 1 to 7, as described in detail in §3.1.1.5). Mice were culled at intervals in order to register the number of cfu or for histological analysis.
Mice challenged with ΔAL2 pneumococci did not show any signs of disease throughout the 8 days of the experiment, except for 1 out of 8 mice that was fairly

hunched and presented slight starry coat by 7 days post-infection. All the mice infected with ΔAL2 survived infection, whereas mice infected with wild type strain became very lethargic by 24h post-infection, at which point they were culled (as mentioned in detail in §3.1.1.5; see Fig. 3.4). Overall, in this mouse model of bronchopneumonia, after infection with the *lytA* knockout ΔAL2 there was a significant difference ($P<0.05$) in the survival times of mice compared with the isogenic wild type strain.

3.1.2.3 Growth of wild type and ΔAL2 in nasopharyngeal tissues and trachea

The ability of pneumococci to undergo autolysis did not influence the growth of bacteria in the nasopharynx in the early hours of infection (*i.e.* 2 to 6h). As depicted in Figure 3.13A, the survival of wild type pneumococci and ΔAL2 were not significantly different during this period of time. In contrast, by 12h and 24h post-infection, there were significantly greater numbers of *S. pneumoniae* wild type bacteria in the nasopharynx of infected mice than ΔAL2 ($P<0.05$, at both time points) (Fig. 3.13A). Moreover, ΔAL2 bacteria persisted in the nasopharynx of mice for up to six days without causing signs of disease.

As in the nasopharynx, the growth of wild type pneumococci and ΔAL2 in the trachea of infected mice in the early hours of infection was not different (Fig. 3.13B). However, by 12h and 24h post-infection wild type bacteria had significantly better survival in this niche than ΔAL2 ($P<0.05$) (Fig. 3.13B). In contrast with the observations in the nasopharynx, numbers of ΔAL2 cells in the trachea started to decrease by 12h post-infection and were cleared from this niche by 48h post-infection (Fig. 3.13B).

3.1.2.4 Growth of wild type and ΔAL2 in lung tissue and blood

As reported for the nasopharynx and trachea, the growth of wild type pneumococci and ΔAL2 in the lungs of infected mice was not significantly different up to 12h post-infection. However, from this time onwards, wild type numbers increased

while ΔAL2 cells started to decline and were cleared from the lungs of mice by 72h post-infection (Fig. 3.14). By 24h post-infection there was a significant difference in the numbers of wild type bacteria and ΔAL2 in the lungs of infected mice ($P<0.05$).

Following intranasal infection, ΔAL2 pneumococci were unable to survive in the blood of mice, in contrast to the wild type parent. Throughout the 8 days of the experiment, ΔAL2 mutant strain was not, at any time (T=0h, 12h, 24h, 48h, 72h, 96h, 192h post-infection) recovered from the blood of infected mice.

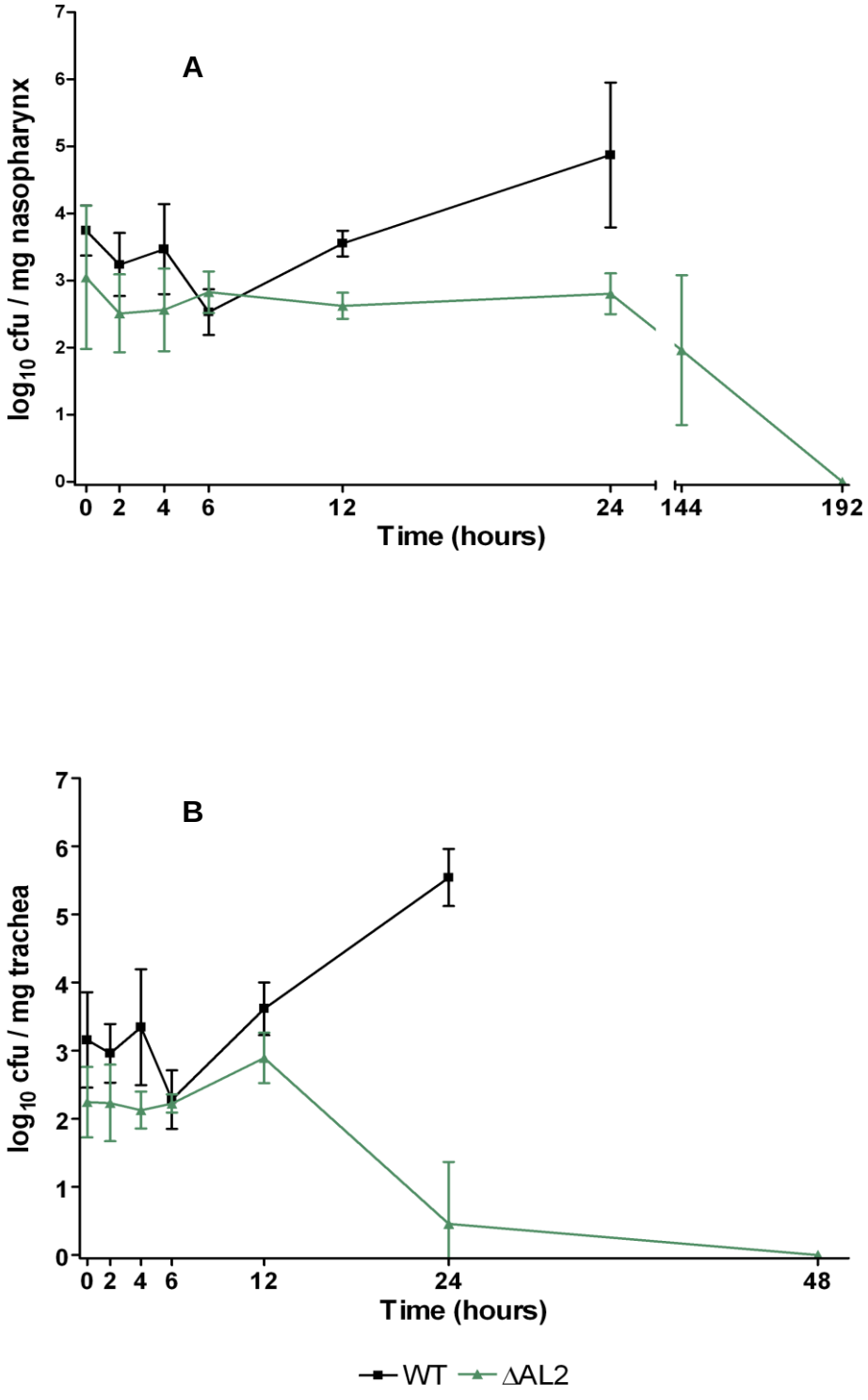


Figure 3.13 Effect of autolysin in nasopharyngeal tissues and trachea. *S. pneumoniae* numbers in the nasopharynx (A) and trachea (B) of mice challenged i.n. with 10^6 cfu of wild type or ΔAL2. For cfu determination pre-selected groups of 4 to 8 mice were culled at the time points shown. Data points and error bars represent the mean and SD of the $\log_{10}$ bacteria per mg of organ.

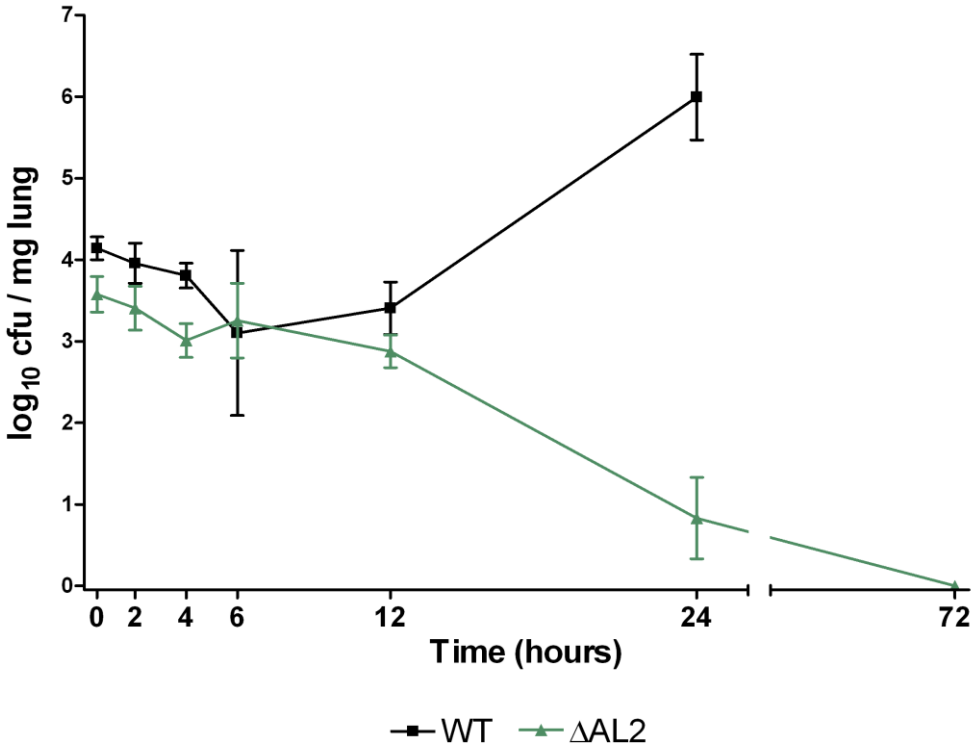


Figure 3.14 Effect of autolysin in the lungs. *S. pneumoniae* numbers in the lungs of mice challenged i.n. with 10^6 cfu of wild type or ΔAL2. For cfu determination pre-selected groups of 4 to 8 mice were culled at the time points shown. Data points and error bars represent the mean and SD of the $\log_{10}$ bacteria per mg of organ.

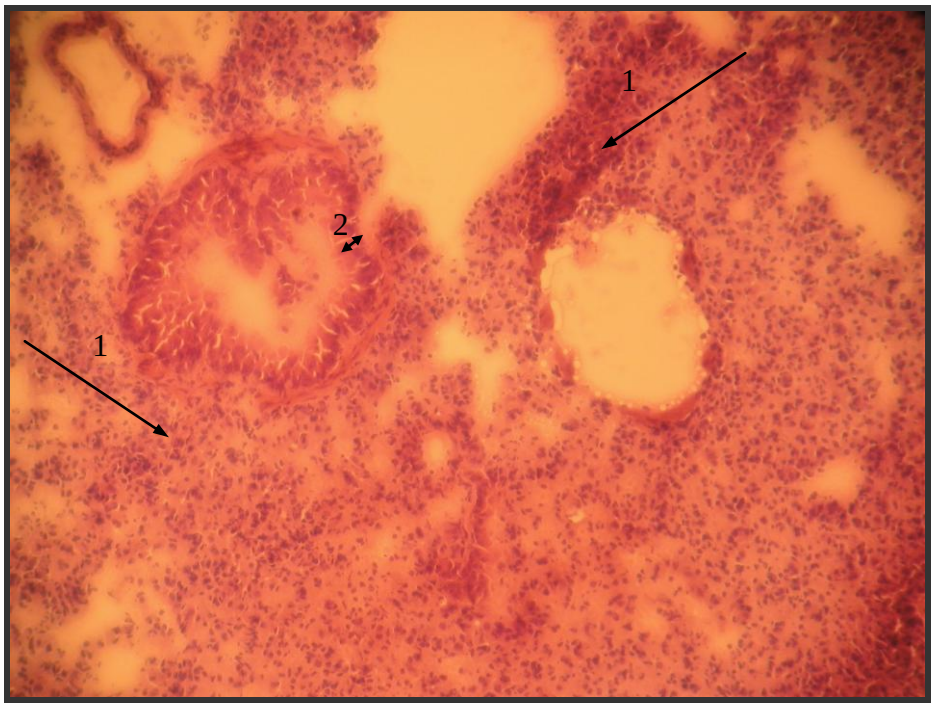
3.1.2.5 Histological analysis of wild type- and ΔAL2-infected lung tissue

At intervals following intranasal infection, mice were culled for histological examination of lung tissue sections. Mice were infected with wild type pneumococci or ΔAL2 and lung sections were stained with haematoxylin and eosin, as described in §2.11.1 to §2.11.3.

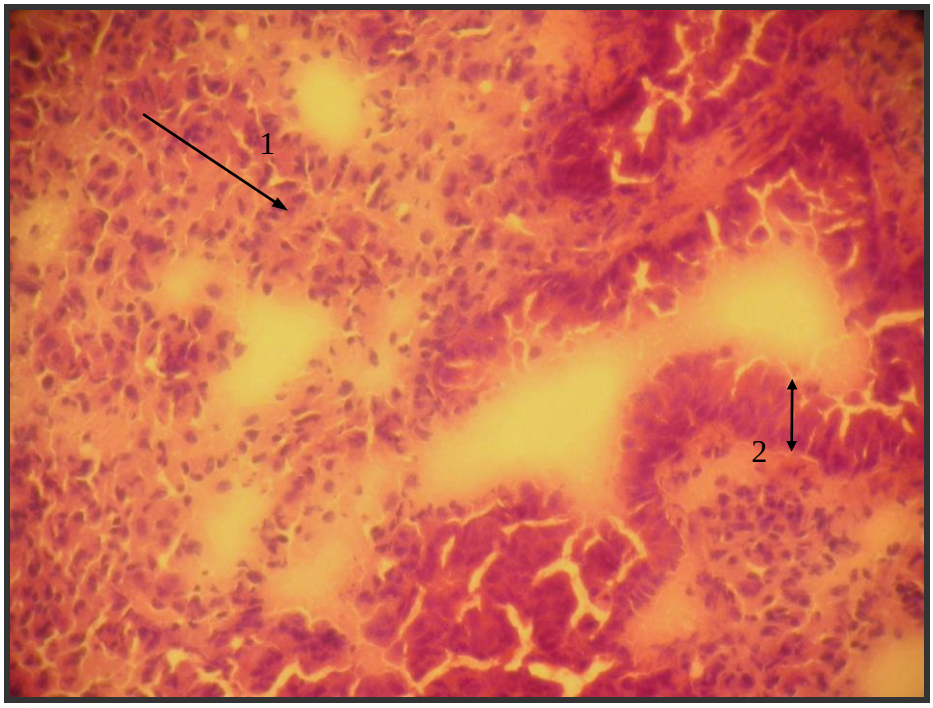
For a detailed description of the histology of the lungs infected with wild type pneumococci see §3.1.1.8. In summary, inflammation was present by 24h post-infection (Fig. 3.8 A&B), with slight hypertrophy of bronchial walls and some exudate filling inflamed bronchioles. By 48h post-infection (Fig. 3.9 A&B), hypertrophy of bronchiole walls had increased and exudate was filling the bronchioles and alveolar spaces. Additionally, cellular infiltration had increased, with extension of inflammation into the lung parenchyma.

By 24h post-infection, mice infected with ΔAL2 showed less tissue inflammation and cellular infiltration into perivascular areas between infected bronchioles than wild type-infected mice (Fig. 3.15A, arrow-1). Bronchiole wall thickening was comparably less than that seen in wild type-infected lungs at the same time point (Fig. 3.15A, arrow-2). Overall, at 24h, lungs infected with ΔAL2 showed minor levels of inflammation and cellular infiltration, which were generally less severe than that seen in lungs infected with the wild type.

ΔAL2-infected lungs showed an increase in the extent of inflammation when examined at 48h post-infection, both in bronchial wall hypertrophy (Fig. 3.15B, arrow-2) and cellular infiltration (Fig. 3.15B, arrow-1). However, in general ΔAL2-infected lungs exhibited much less inflammation than wild type at 24h and 48h post-infection.



A



B

Figure 3.15 Inflammation in the lung following infection with ΔAL2. Light microscopy of H&E stained lung tissue collected from MF1 mice infected i.n. with 10^6 cfu of *S. pneumoniae* ΔAL2 at 24h post-infection (panel A) or at 48h post-infection (panel B). Magnification: Panel (A) 100x; Panel (B) 200x. Arrow-1 indicates cellular infiltrate; Arrow-2 indicates bronchial wall hypertrophy.

3.1.2.6 Analysis of leukocyte infiltration in wild type- and ΔAL2-infected lung

Total leukocyte numbers (Fig. 3.16) and individual lymphocytes, macrophages and neutrophils cell numbers were enumerated (Table 3.3) over the time course of infection with *S. pneumoniae* wild type and ΔAL2 in lungs of mice infected intranasally, as described in §2.11.4 and §2.11.5.

A detailed description of the leukocyte population in the lungs of mice infected with wild type pneumococci has been given in §3.1.1.9. Briefly, total leukocyte levels in wild type-infected lung tissue homogenates reached significant increased values by 24h post-infection, compared with time zero levels ($P<0.05$) (Fig. 3.16). These numbers remained unchanged by 48h post-infection. This rise in total leukocyte cells was mainly due to a significant increase in the number of neutrophils from time zero to 24h (4.6-fold increase), as shown in Table 3.3.

As seen with the wild type, total leukocyte counts in ΔAL2-infected lungs were significantly greater by 24h post-infection compared to time zero numbers, and presented a small but not statistically significant decrease by 48h post-infection (Fig. 3.16). Table 3.3 shows that this increase seen at 24h post-infection for leukocytes enumerated in the lungs of ΔAL2-infected mice was due to a significant increase in the numbers of neutrophils, lymphocytes and macrophages ($P<0.05$ compared with time zero).
Reduction of total leukocytes at 48h post-infection was due to a significant decrease in the number of neutrophils at this time point when compared to values at 24h ($P<0.05$). There was no significant change in the number of lymphocytes between 24h and 48h post-infection and macrophages slightly increase in numbers in this period of time.

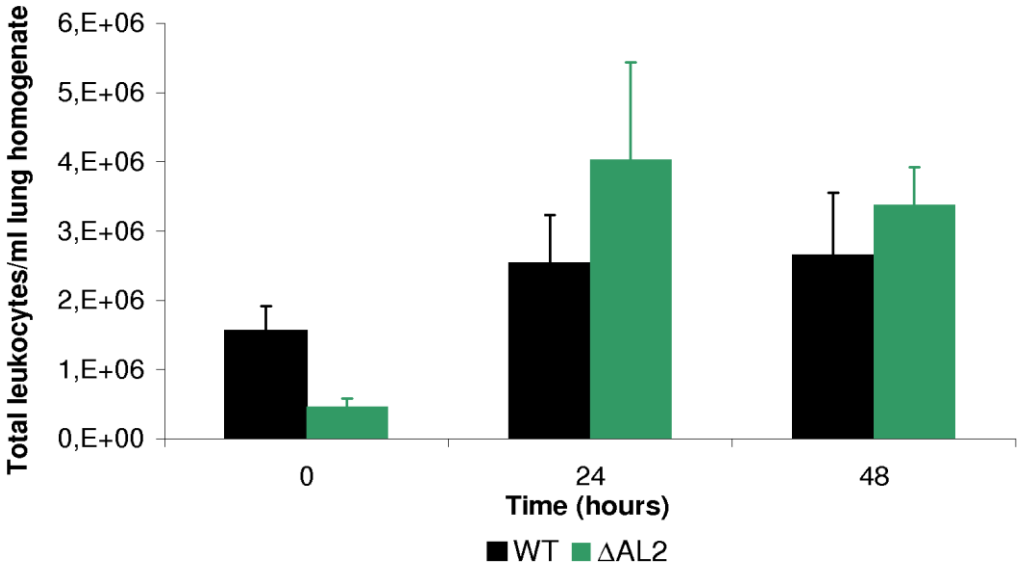


Figure 3.16 Effect of autolysin in recruitment of leukocytes to the lung. Total leukocyte counts in cytospin preparations of whole lung homogenates from MF1 mice infected i.n. with 10^6 cfu of *S. pneumoniae* wild type or ΔAL2. Data are the mean of 4 mice. Bars indicate SD.

Table 3.3 Individual leukocyte counts in cytospin preparations of whole lung homogenates from MF1 mice infected i.n. with 10^6 cfu of *S. pneumoniae* wild type or ΔAL2.

Neutrophils	WT		ΔAL2	
Time	Cells/ml	Fold increase	Cells/ml	Fold increase
0	3.0E5		1.3E4	
24	1.4E6	4.6	8.2E5	65.8
48	1.3E6	4.4	3.4E5	27.5
Lymphocytes	WT		ΔAL2	
Time	Cells/ml	Fold increase	Cells/ml	Fold increase
0	5.6E5		4.2E5	
24	6.2E5	1.1	3.0E6	6.9
48	6.5E5	1.1	2.8E6	6.5
Macrophages	WT		ΔAL2	
Time	Cells/ml	Fold increase	Cells/ml	Fold increase
0	6.8E5		2.8E4	
24	4.9E5	0.7	2.5E5	8.9
48	7.3E5	1.1	2.7E5	9.9

Data are the mean of 4 mice. Leukocytes were quantified at x400 magnification with a graticule-equipped eyepiece. Fold increases in leukocyte populations are relative to time zero values.

In summary:

- The *lytA* knockout significantly increased the survival times of mice compared with the wild type.
- Up to 12h post-infection there was no difference in the numbers of ΔAL2 and wild type pneumococci in the upper and lower respiratory tract.
- From 12h post-infection significantly more wild type bacteria than ΔAL2 were recovered from the upper and lower respiratory tract.
- ΔAL2 bacteria were unable to cause bacteremia following intranasal infection.
- ΔAL2-infected lungs exhibited generally less severe inflammation than the wild type.
- Leukocyte infiltration into lungs infected with ΔAL-2 was significantly increased by 24h and 48h post-infection due to an increase in all types of cells but specially neutrophils.

3.1.3 Superoxide dismutase

Wild type *S. pneumoniae* serotype 2 strain D39 and the isogenic superoxide dismutase-deficient mutant ΔSodA were used in this study to assess the effect of superoxide dismutase on pneumococcal survival in tissues of the upper and lower respiratory tract and on the onset of disease.

3.1.3.1 Confirmation of the mutation by PCR

The mutant ΔSodA used in this study was constructed previously in our laboratory (Yesilkaya *et al*., 2000). ΔSodA has a 'spectinomycin resistance' cassette (from plasmid pDL278) disrupting *sod*A. The primers SODF, SODR (Table 2.3 §2.5) were used to amplify a 586bp fragment of the *sod*A gene in the wild type parent, from nucleotide 15 to 601. These same primers also amplify a fragment with 1737bp in the mutant, corresponding to 586bp from the *sodA* gene and 1151bp from the 'spectinomycin resistance' cassette. Figure 3.17 shows the PCR (§2.6) results obtained, confirming the mutation of strain ΔSodA, that were visualised on a 0.7% (w/v) agarose gel (§2.7).

3.1.3.2 *In vitro* growth curve

For *in vitro* growth experiments, wild type pneumococci and the superoxide dismutase-deficient mutant ΔSodA were grown from an optical density at 500nm of 0.2 in BHI-NCS as described in §2.4. Figure 3.18 shows that in rich liquid medium, aerobically without agitation, deletion of *sodA* markedly impaired the growth of pneumococci as shown by the considerably longer lag phase of the mutant compared to the wild type. However, following this lag period, rate of increase in the optical density was similar for the two strains. The results indicate that *sodA* is required for optimal aerobic *in vitro* growth of *S. pneumoniae*.

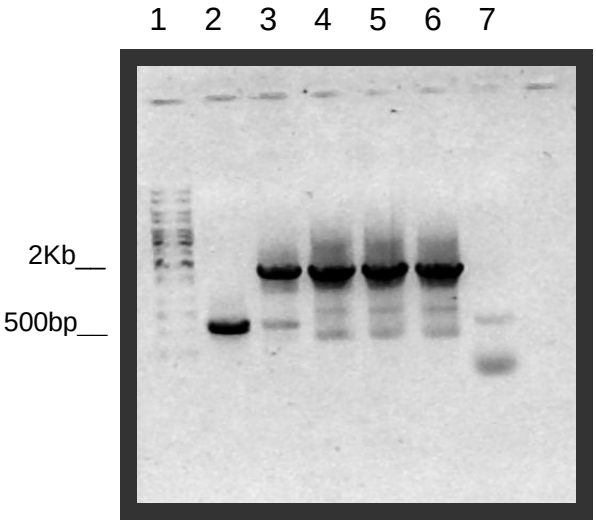


Figure 3.17 PCR confirmation of the mutation in the superoxide dismutase-negative mutant ΔSodA using the primers SODF, SODR; Lane 1: 1Kb ladder (GeneRuler); Lane 2: wild type strain showing the expected band at around 586bp in size (positive control); Lanes 3-6: superoxide dismutase-negative mutant ΔSodA showing the expected band at around 1.7Kb; Lane 3: passaged stock of ΔSodA used for infections; Lane 4 & 5: ΔSodA used for growth curves experiments; Lane 6: ΔSodA from laboratory bead collection; Lane 7: nanopure water (negative control)

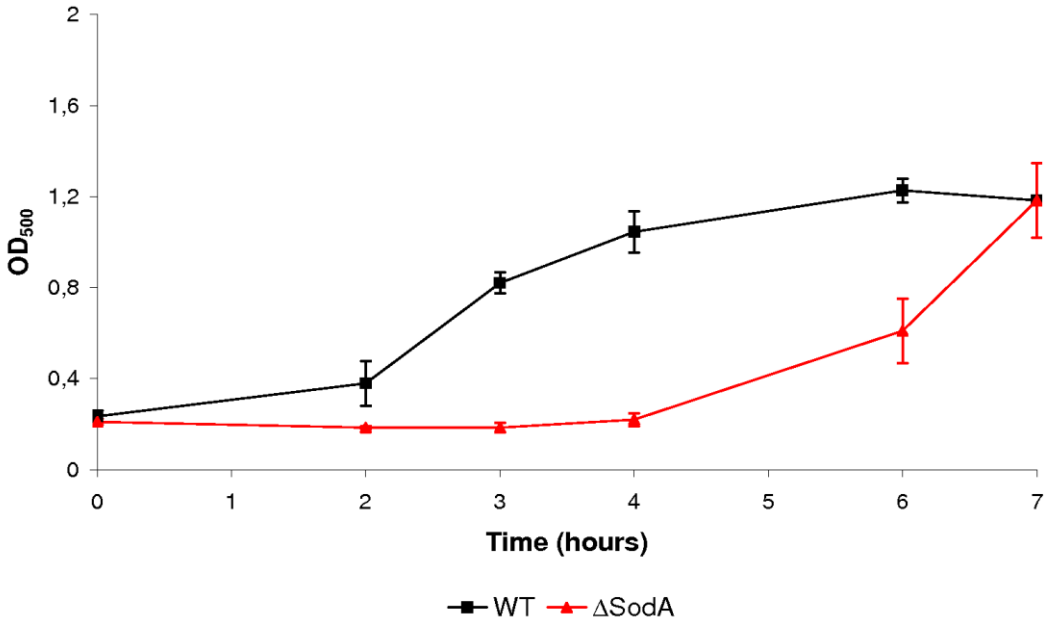


Figure 3.18 Growth in BHI-NCS broth of *S. pneumoniae* D39 wild type and ΔSodA. N= 3 for each time point. Bars indicate SD. OD_{500}, optical density at 500nm.

3.1.3.3 Virulence following intranasal infection

Figure 3.19 shows the signs of disease in mice following intranasal infection with wild type or ΔSodA pneumococci. Clinical signs were registered in a score sheet throughout the course of each experiment (using a scale from 1 to 7, as described in detail in §3.1.1.5). Mice were culled at pre-determined intervals in order to register the number of cfu or for histological analysis.

None of the 50 mice (used in total from 7 independent experiments) infected intranasally with ΔSodA presented signs of illness by 12h after infection. It was not until 24h post-infection that 7 out of 46 mice started to show slight clinical signs (disease score between 1 and 2). By 48h post-infection, 50% of mice (out of 22) had moderate to severe signs of disease (disease score 3 or higher) (Fig. 3.19). Only one mouse out of four had survived infection by 72h, however with acute signs of disease (score 6; result not shown in Fig. 3.19 for this time point).

In contrast, as mentioned previously (§3.1.1.5), mice infected with wild type strain became very lethargic by 24h post-infection at which point they were culled.

Overall, the lack of *sodA* significantly ($P<0.05$) changed the course of disease in mice infected with mutant pneumococci compared with the isogenic wild type strain, suggesting that resistance to oxidative stress is a factor involved in pneumococcal pathogenesis.

3.1.3.4 Growth of wild type and ΔSodA in nasopharyngeal tissues and trachea

There was a clear difference between the growth of wild type pneumococci and the ΔSodA mutant strain in the upper respiratory tract and in the trachea of infected mice (Fig. 3.20). The lack of superoxide dismutase visibly influenced the growth of pneumococci in the nasopharynx and trachea, in particular in the initial 4 hours following infection ($P<0.05$ at 4h post-infection compared to the wild type), where the mutant appears to be unfit for survival within the host.

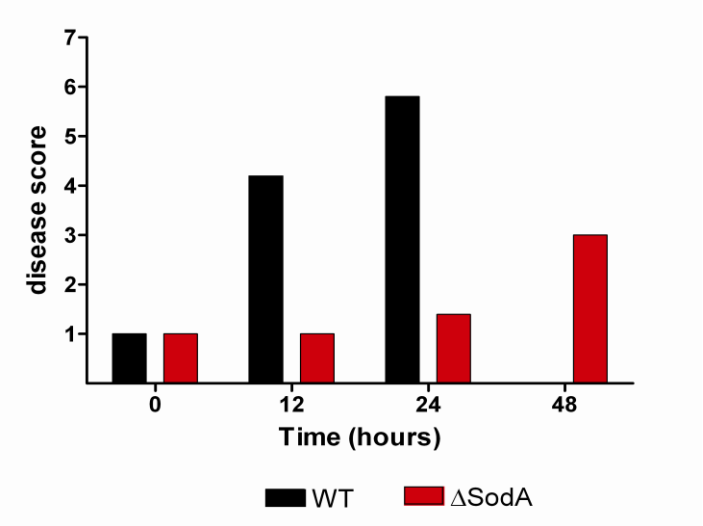


Figure 3.19 Effect of superoxide dismutase in virulence of *S. pneumoniae*. The virulence of *S. pneumoniae* superoxide dismutase mutant ΔSodA was tested in a murine model of bronchopneumonia by scoring the signs of disease of each mouse. 10^6 cfu of wild type or ΔSodA pneumococci were administered into the nostrils of mice. Signs of disease were recorded on a score sheet through the course of each experiment, using the scale signs of disease described before (see Fig. 3.4, §3.1.1.5). Mice were culled at intervals or when they reached a very lethargic state (score 6). The graph shows the average disease score for all the mice at each time point.

Note that the difficulty in growth during this period of time had already been observed during aerobic *in vitro* growth (Fig. 3.18), which suggests that early events in aerobic metabolism are influencing the mutant's growth.

Although numbers of ΔSodA bacteria increased by 6h post-infection to levels comparable with the wild type strain ($P>0.05$), they were again significantly lower than the parent strain at 12h and 24h post-infection ($P<0.05$), in both niches. (In the trachea at 12h post-infection, all the 4 mice used for estimation of bacterial counts were clear of pneumococci [cfu = 0]).

From these results, it seems that following an "adaptation" period of the mutant cells to an oxygenated environment, they are then able to grow. Therefore, SodA appears to play a role in early colonisation of the upper airways by pneumococci, allowing the bacteria to successfully encounter reactive oxygen species from the respiratory burst of phagocytes or, endogenously from aerobic metabolism. In facultative anaerobic bacteria such as *S. pneumoniae*, prompt and effective oxidative stress response mechanisms are necessary to allow growth and survival under aerobic challenges.

3.1.3.5 Growth of wild type and ΔSodA in lung tissue and blood

Reflecting the events observed in the nasopharynx and trachea, there was a clear difference between the growth of wild type pneumococci and ΔSodA in the lungs of mice (Fig. 3.21). Again, a critical stage for the growth of ΔSodA mutant cells was apparent in the 4 hours following infection ($P<0.05$ at 4h post-infection compared to the wild type). At 6h post-infection an apparent increase in the number of ΔSodA bacteria was also observed.

Significantly higher numbers of wild type bacteria than ΔSodA ($P<0.05$) were present at 12h and 24h post-infection in the lungs of infected mice. As in the upper respiratory tract, SodA seems to be important for pneumococcal growth in the lungs.

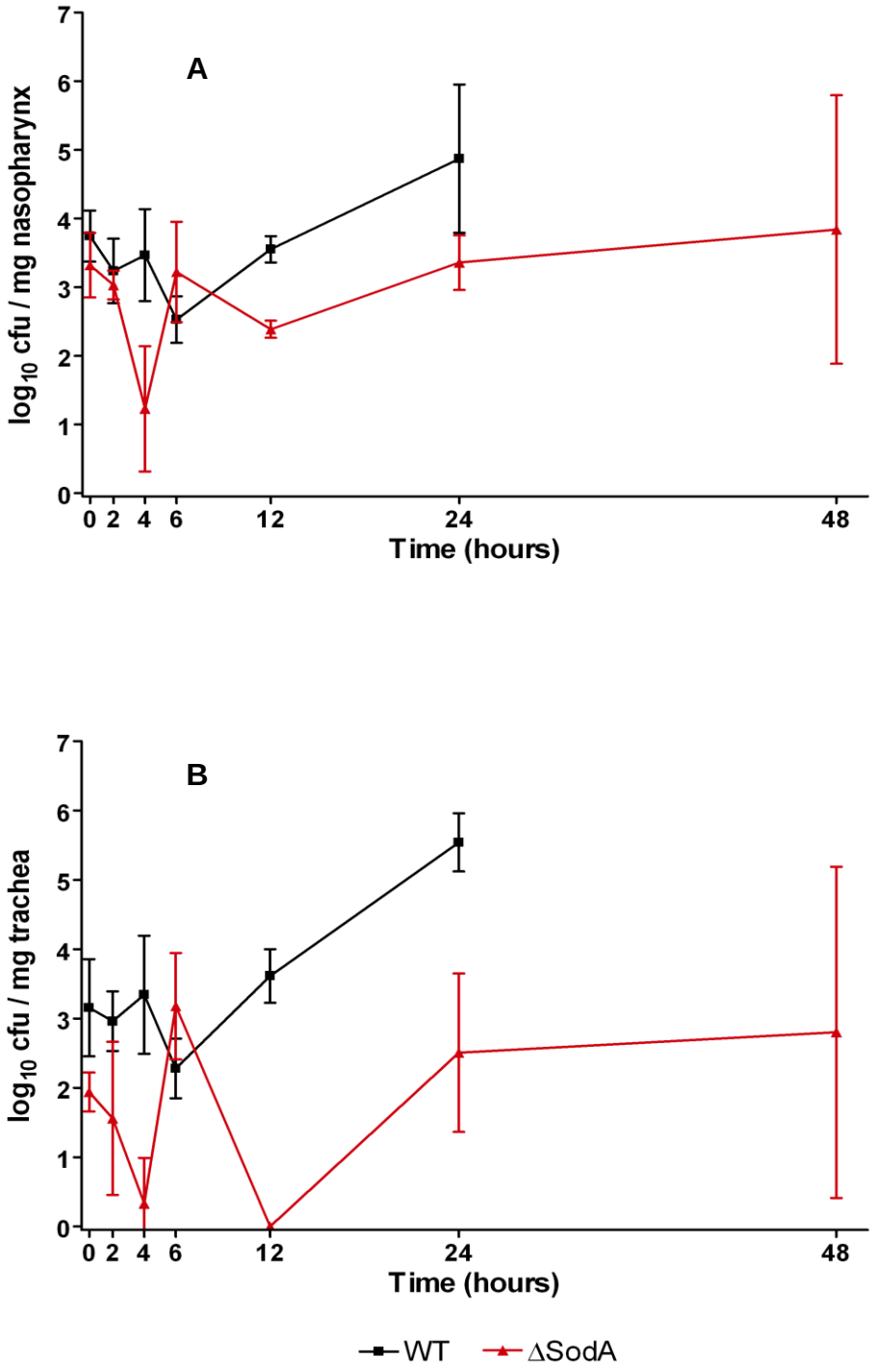


Figure 3.20 Effect of superoxide dismutase in nasopharyngeal tissues and trachea. *S. pneumoniae* numbers in the nasopharynx (A) and trachea (B) of mice challenged i.n. with 10^6 cfu of wild type or ΔSodA. For cfu determination pre-selected groups of 4 to 8 mice were culled at the time points shown. Data points and error bars represent the mean and SD of the $\log_{10}$ bacteria per mg of organ.

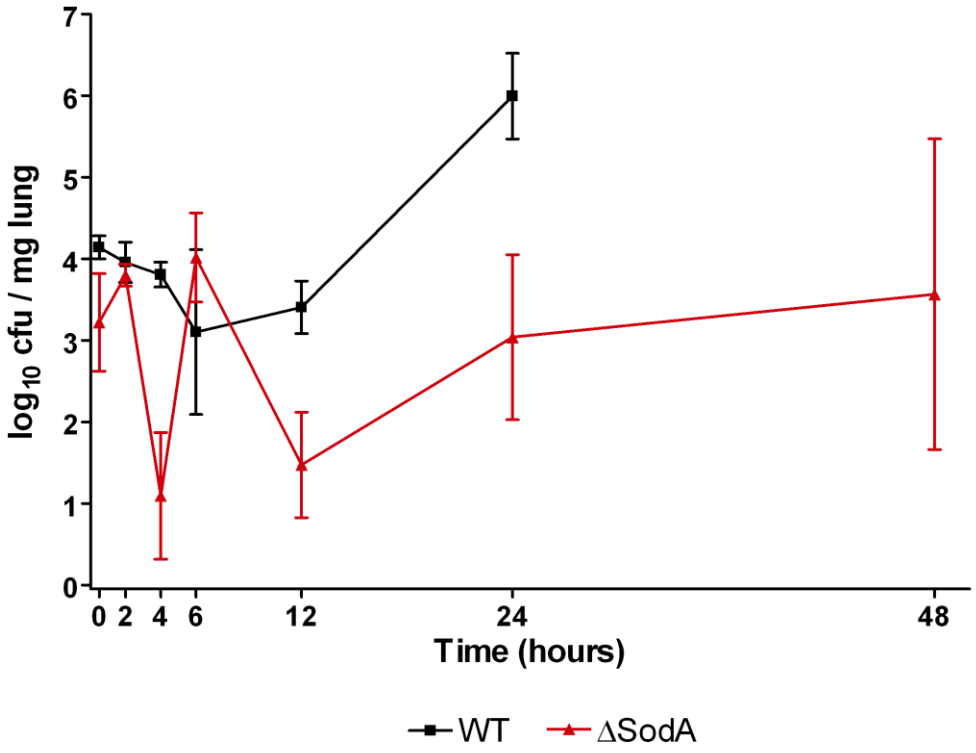


Figure 3.21 Effect of superoxide dismutase in the lungs. *S. pneumoniae* numbers in the lungs of mice challenged i.n. with 10^6 cfu of wild type or ΔSodA. For cfu determination pre-selected groups of 4 to 8 mice were culled at the time points shown. Data points and error bars represent the mean and SD of the $\log_{10}$ bacteria per mg of organ.

After intranasal challenge, ΔSodA appears to be less invasive than the wild type parent. ΔSodA pneumococci were not detected in the blood of mice until 24h post-infection (Fig. 3.22), at which time bacteria were isolated from 70% of infected mice (cfu's in blood were analysed in a total of 20 infected mice for this time point). There was a clear difference in numbers of bacteria isolated from the blood at 24h post-infection between wild type and ΔSodA ($P<0.05$). By 48h post-infection, the mutant strain was still recovered from the blood of 60% of the infected mice (21 mice analysed in total; Fig. 3.22).

The error bars (showing the standard deviation) represented in the graph in Figure 3.22 reflect the variability of the results observed, where, at each time point (24h and 48h post-infection) a proportion of mice were not bacteremic. Indeed, considering only bacteremic mice, the results from intranasal infections with the ΔSodA mutant strain show that these 14 mice (out of 20) had an average $\log_{10}$ cfu of 4.3 (SD 1.8) in the blood by 24h post-infection and by 48 hours the numbers of bacteria in the blood of these mice had increased to $\log_{10}$ cfu 7.2 (SD 1.8). Moreover, if the same division (bacteremic *versus* non-bacteremic mice) was done for the results obtained in the respiratory tract tissues, a similar picture could be observed. For instance, by 24h post-infection, an average $\log_{10}$ cfu of 3.5 (SD 0.9) was counted in the lungs of the 8 bacteremic mice whereas there was an average $\log_{10}$ cfu of 2.1 (SD 0.3) in the 4 non-bacteremic mice. By 48h post-infection, this difference between groups was even more pronounced, with bacteremic mice presenting $\log_{10}$ cfu 5.2 (SD 0.6) and non-bacteremic having $\log_{10}$ cfu 1.6 (SD 0.3) in the lungs. In Appendix B, *S. pneumoniae* numbers in the nasopharynx, trachea, lungs and blood of mice challenged with wild type or ΔSodA are given divided between bacteremic *versus* non-bacteremic mice (note that this division was only possible starting at 12h post-infection, time where bacterial counts in blood were done following intranasal infections).

Given the nature of the results obtained when using the ΔSodA mutant in murine infections, a series of additional experiments was done in order to better understand the observations.

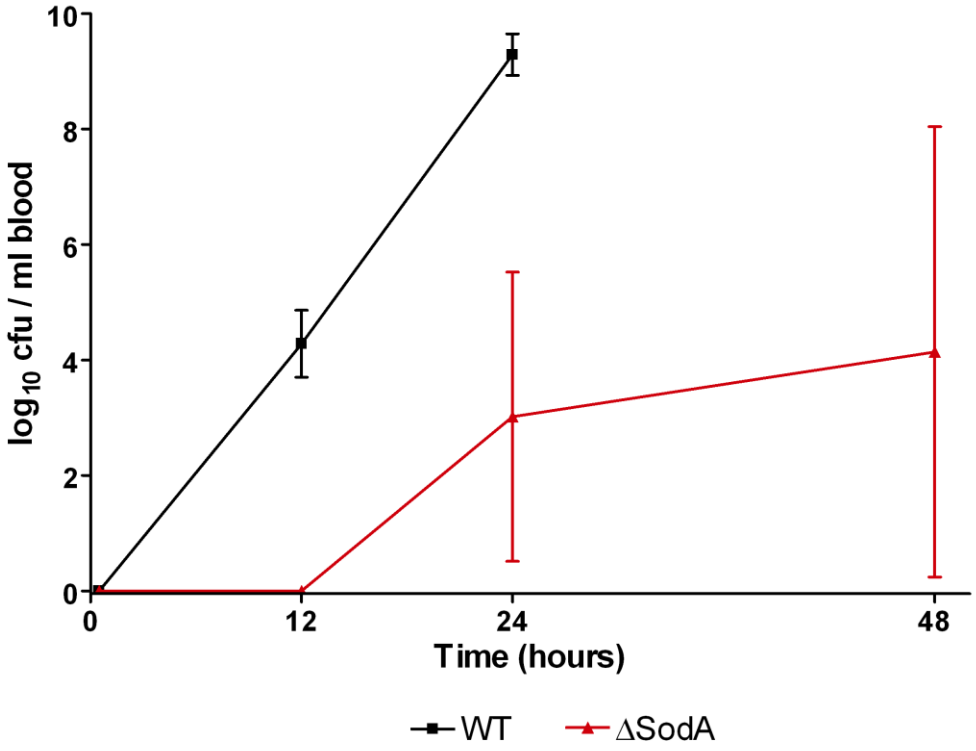


Figure 3.22 Effect of superoxide dismutase in bacteremia. *S. pneumoniae* numbers in blood of mice challenged i.n. with 10^6 cfu of wild type or ΔSodA. For cfu determination pre-selected groups of at least 4 mice (see text above for ΔSodA) were culled at the time points shown. Data points and error bars represent the mean and SD of the $\log_{10}$ bacteria per mg of organ.

Note that, the variability of the results after intranasal infection was observed in all 7 independent experiments, and also in a separate infection done using a low dose ($4x10^4$ cfu i.n.) of ΔSodA pneumococci (result not shown), which discounts any explanation of variability due to technical failure.

Firstly, in order to eliminate the possibility of reversion of the *sod* mutation had taken place *in vivo*, the number of colonies on selective or non-selective medium from lung and blood samples at 24h and 48h post-infection was counted (data not shown). The results revealed no difference between colony counts, showing no evidence for appearance of revertants.
Moreover, PCR reactions were set up to confirm the mutation of strain ΔSodA, using the primers SODF and SODR in stocks used for infection experiments, and the results observed (Fig. 3.17 above) showed that the spectinomycin gene remained in ΔSodA cells during murine peritoneal culture.

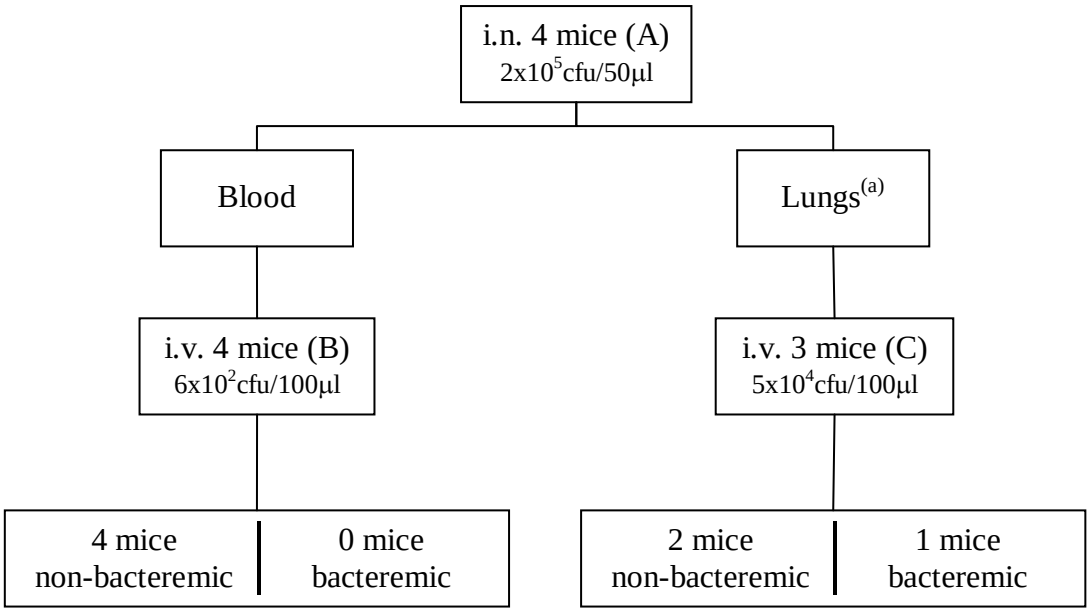
In addition to intranasal challenge, the survival of ΔSodA pneumococci in blood was tested using the intravenous (i.v.) route for infection, in order to assess: i) if the diminished virulence of ΔSodA pneumococci was dependent on the route of infection, ii) if the variability in bacteremia observed following intranasal infection was also seen following i.v. infection. These studies were done in collaboration with Dr Kadioglu, Dpt. Infection, Immunity and Inflammation, University of Leicester.
Reduction in virulence in this mutant appears to be related to the route of infection. All the five mice infected in the tail vein with $1x10^5$ cfu of ΔSodA survived the experiment and were clear of bacteria in the blood at 24h and 48h post-infection. This was in contrast to the wild type results which showed $\log_{10}$ cfu of 5.8 and of 6.7 in the blood at 24h and 48h, respectively. This inability of ΔSodA pneumococci to survive in blood suggests that following i.n. infection, bacteria recovered from the blood of infected mice are those seeded from the lungs. However, the variability in bacteremia caused by the mutant following intranasal infections was also observed after i.v. infection. By 2h post-infection there were two bacteremic mice (out of five) with cfu numbers varying between $\log_{10}$ cfu 1.9 and 2.7.

Another experiment was set up, using female inbred C57BL/6 mice (Harlan Olac Ltd., UK), to determine whether the observed variability in bacteremia due to ΔSodA, following i.n. infection, was due to a genetic difference between individual outbred MF1 mice. The C57BL/6 mice are known to have an intermediate susceptibility to infection with D39 (Gingles *et al*., 2001). Following i.n. infection with 10^6 cfu ΔSodA pneumococci, one out of four mice was bacteremic at 24h post-infection (log_{10} cfu 3.4), repeating the results obtained when using MF1 outbred mice for infection, and therefore suggesting that the observed variability in bacteremia was not due to variability of individual or due to an effect of mouse strain.

A final set of experiments, which were done twice (experiment 1 & 2, Fig. 3.23), was performed to test the possibility of appearance of "two different phenotypes" of ΔSodA mutant cells following infection. In these experiments, bacteria recovered from tissues of bacteremic and non-bacteremic mice after an initial intranasal infection were used to infect a second set of mice intravenously. The hypothesis was that this second infection would lead to the same variability in the results as observed before.

Four mice were infected intranasally with $2x10^5$ cfu (experiment 1, group A) or $2x10^6$ cfu (experiment 2, group D) of ΔSodA and lungs and blood were collected 24h post-infection. 100μl or 200μl of homogenised lungs (in 10ml sterile PBS) or 50μl of blood were inoculated into 10ml of BHI or BHI with spectinomycin (Table 2.1, §2.2), in duplicate and grown overnight at 37°C statically. Viable counts in tissue homogenates and in blood were determined by serial dilution in sterile PBS as described before (see §2.10.2) and plating was done on medium with and without spectinomycin.
The next day, two out of four mice from experiment 1 were found to have bacteria in the blood and in experiment 2 all the mice were bacteremic. Bacterial counts from lung homogenates and blood were the same in medium with or without antibiotic for both experiments.

EXPERIMENT 1



EXPERIMENT 2

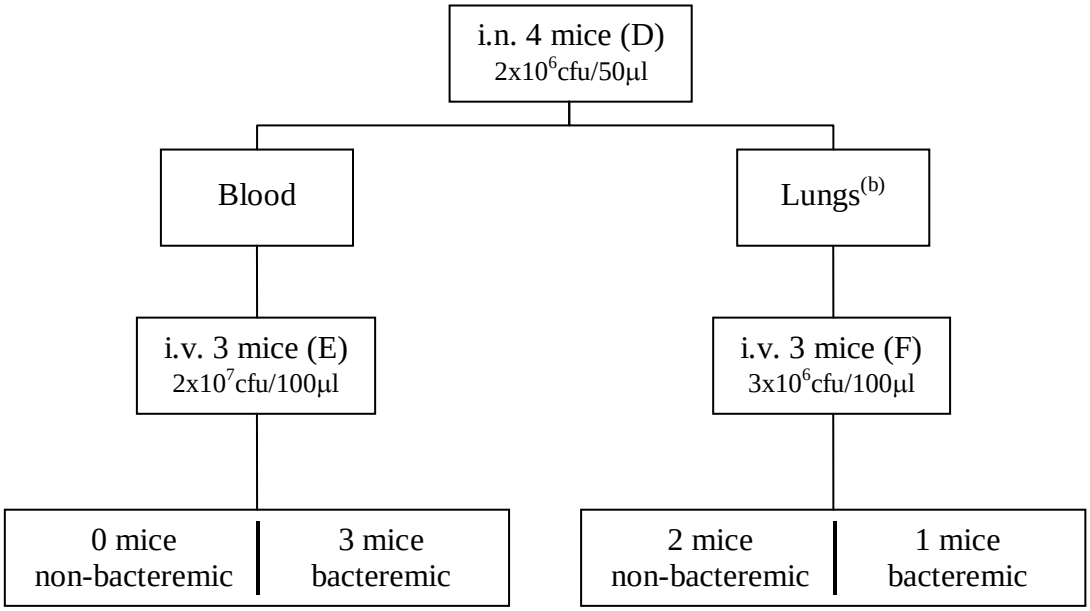


Figure 3.23 Diagram showing two independent experiments performed to test the possibility of appearance of "two different phenotypes" of ΔSodA mutant cells following infection. Bacteria recovered from tissues of bacteremic and non-bacteremic mice after an initial intranasal infection were used to infect intravenously a second set of mice. (a) two out of four mice were found to have bacteria in blood; (b) all the mice were bacteremic. (A), (B), (C), (D), (E), (F) refers to groups of mice (see text).

From experiment 1, bacterial growth from lungs from mouse 1 (not bacteremic, $\log_{10}$ cfu 1.1 in lungs) and from blood from mouse 2 ($\log_{10}$ cfu 5.7 in blood and $\log_{10}$ cfu 3.3 in lungs) were chosen to intravenously infect four (group B) and three mice (group C), respectively.
From experiment 2, bacterial growth from lungs from mouse 4 ($\log_{10}$ cfu 3.2 in blood and $\log_{10}$ cfu 1.8 in lungs) and from blood from mouse 1 ($\log_{10}$ cfu 5.5 in blood and $\log_{10}$ cfu 2.5 in lungs) were chosen to infect three mice intravenously (groups E and F).

For intravenous infections, selected overnight bacterial growth from intranasally infected animal tissues was spun down and re-suspended in 10ml sterile PBS. The dose for infection was calculated assuming a bacterial count of 10^6 cfu/ml for lungs from mouse 1 (experiment 1), from lungs from mouse 4 (experiment 2) and from blood from mouse 1 (experiment 2) and a bacterial count of 10^8 cfu/ml from blood from mouse 2 (experiment 1). Viable counts in bacterial pellets used for infection were determined by serial dilution in sterile PBS and plating was done in medium with or without spectinomycin.

Mice were infected in the tail vein with $1x10^5$ cfu of ΔSodA in 100μl of PBS. At 2h post-infection, blood was collected by tail bleeding for colony count on selective and non-selective agar. The next day, results from bacterial counts at 2h post-infection showed that, in experiment 1, none of the four mice infected i.v. (group B; dose for infection $6x10^2$ cfu/100μl) with bacteria recovered from blood from mouse 2 (bacteremic) were bacteremic and one (out of three mice, group C; dose for infection $5x10^4$ cfu/100μl) infected with bacteria recovered from the lungs from mouse 1 (non-bacteremic) had bacteria in the blood. In experiment 2, all three of the mice infected i.v. (group E; dose for infection $2x10^7$ cfu/100μl) with bacteria recovered from the blood from mouse 1 (bacteremic) were bacteremic and only one (out of three mice, group F; dose for infection $3x10^6$ cfu/100μl) infected with bacteria recovered from lungs from mouse 4 (bacteremic) had bacteria in blood at that time point.

Experiment 1 was ended at 24h post-infection and lungs and blood were collected for colony counts in these tissues. Results showed no bacterial growth at any site sampled.

In experiment 2, one mouse (dose for infection $2x10^7$ cfu/100μl) was severely lethargic at 24h, at which time it was culled. Blood and lungs were collected for cfu counts. Tail bleeding was done with the 5 remaining mice (two infected with $2x10^7$ cfu/100μl and three infected with $3x10^6$ cfu/100μl; see above) for cfu analysis. Colony counts revealed that the severely lethargic mouse was bacteremic and had $\log_{10}$ cfu 9.6 in the blood and $\log_{10}$ cfu 6.7 in the lungs. The other two mice from this group ($2x10^7$ cfu/100μl dose) had bacterial loads of $\log_{10}$ cfu 3.7 and $\log_{10}$ cfu 8.9 in the blood. In the other group ($3x10^6$ cfu/100μl dose) one mouse was not bacteremic and the other two had bacterial loads of $\log_{10}$ cfu 6.2 and $\log_{10}$ cfu 3.1 in the blood.

The next day, one mouse infected with $2x10^7$ cfu/100μl was found dead (no results) and the other one was still bacteremic with bacterial loads of $\log_{10}$ cfu 6.1 in blood and $\log_{10}$ cfu 3.0 in lungs. From the other group ($3x10^6$ cfu/100μl dose), by 48h post-infection, one mouse remained with no bacteria recovered from the blood and the other two had $\log_{10}$ cfu 6.3 in the blood and $\log_{10}$ cfu 3.5 in the lungs and $\log_{10}$ cfu 2.4 in the blood and $\log_{10}$ cfu 1.4 in the lungs.

These data suggest that the observed variability obtained after intranasal infection with *S. pneumoniae sodA* mutant cells is i) not dependent on the route of infection, ii) not dependent on the dose, iii) not dependent on the site from which bacteria are collected and iv) not dependent on emergence of bacteremia.

3.1.3.6 Histological analysis of wild type- and ΔSodA-infected lung tissue

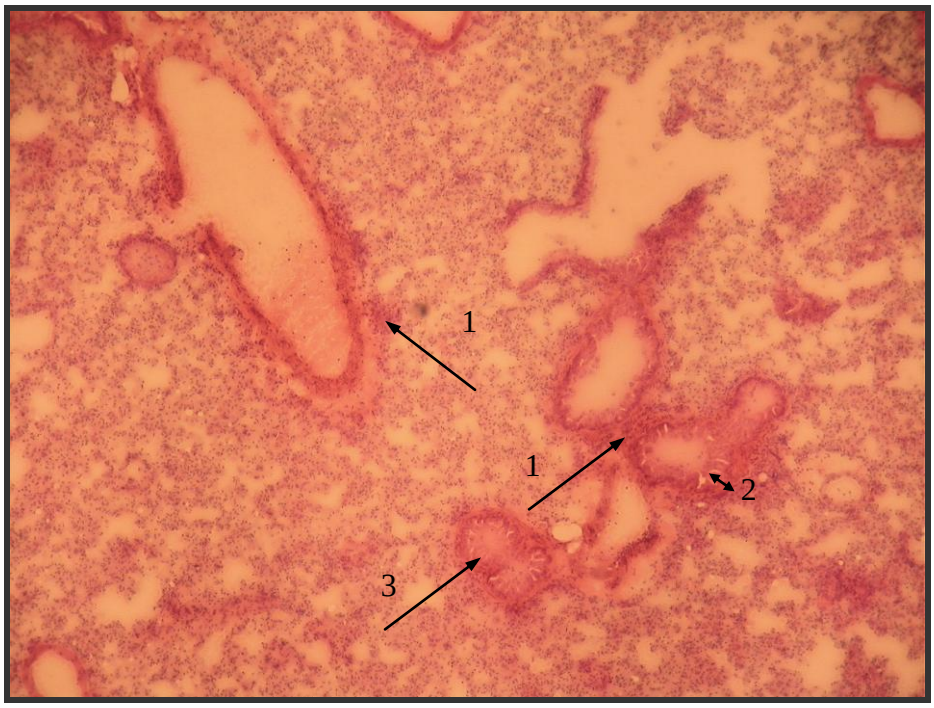
At intervals following intranasal infection, mice were culled for histological examination of lung tissue sections. Mice were infected with wild type pneumococci or ΔSodA and sections were stained with haematoxylin and eosin, as described in §2.11.1 to §2.11.3.

Note that, as for the experiments for colony counts in tissues (see §3.1.3.5), only half the mice at each time point examined for histological features were

bacteremic following infection. Namely, by 24h post-infection, two out of four mice were bacteremic (log_{10} cfu 3.3; log_{10} cfu 1.9) and at 48h, three out of six mice were bacteremic (log_{10} cfu 7.6; log_{10} cfu 6.3; log_{10} cfu 2.8). Despite this, at 24h post-infection there was no difference between the extent of inflammation in the lungs of bacteremic and non-bacteremic mice. However, by 48h post-infection, lungs of mice with no bacteria in blood were, overall, considerably less inflamed than lungs of bacteremic mice (results not shown).

For a detailed description of the histology of the lungs infected with wild type pneumococci, see §3.1.1.8. In summary, inflammation was present by 24h post-infection (Fig. 3.8 A&B), with slight hypertrophy of bronchial walls and some exudate around those infected bronchioles. By 48h post-infection (Fig. 3.9 A&B), hypertrophy of bronchiole walls had increased and exudate was filling the bronchioles and alveolar spaces. Additionally, cellular infiltration had increased, with extension of inflammation into the lung parenchyma.

The same extent of bronchiole wall thickening was seen in the lungs infected with ΔSodA (Fig. 3.24 A&B Arrow-2) or the wild type parent (Fig 3.8 A&B, §3.1.1.8) at 24h post-infection but there was considerably less cellular infiltration into peribronchial and perivascular areas of ΔSodA-infected lungs (Fig. 3.24 A&B Arrow-1) when compared to wild type-infected lungs. Overall, inflammation in ΔSodA-infected lungs was much less severe when compared to the wild type.
By 48h post-infection, histologic evidence of infection was apparent (Fig. 3.25 A&B), and the lungs appeared more inflamed than at 24h (Fig. 3.24 A&B). Nonetheless, mice infected with ΔSodA showed less tissue inflammation and cellular infiltration into perivascular areas between infected bronchioles (Fig. 3.25 A&B Arrow-1) than wild type-infected mice (Fig. 3.9 A&B). Yet, despite these lower levels of pathologic tissue damage, some bronchioles exhibited levels of inflammation comparable to the wild type-infected lungs at 48h (Fig. 3.25 A&B Arrow-2 and Fig. 3.9 A&B). Bronchioles also exhibited substantial levels of cellular infiltration at 48h post-infection. Compared to wild type-infected mice, the cellular infiltration around such bronchioles appeared to be more intense and localised and did not extend into the perivascular areas (Fig. 3.25 A&B Arrow-1∗).



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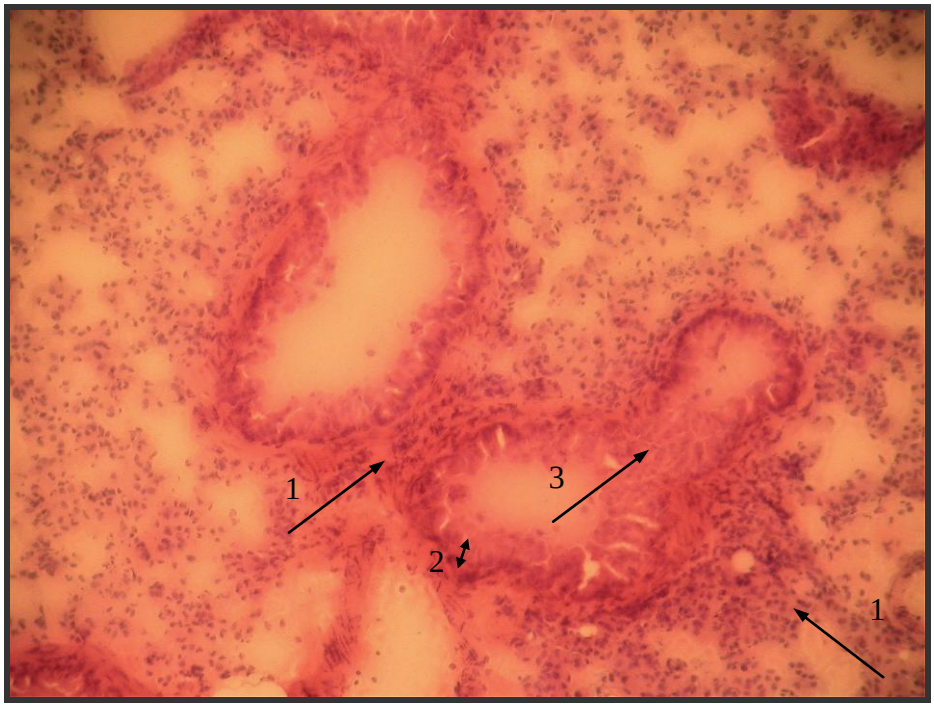
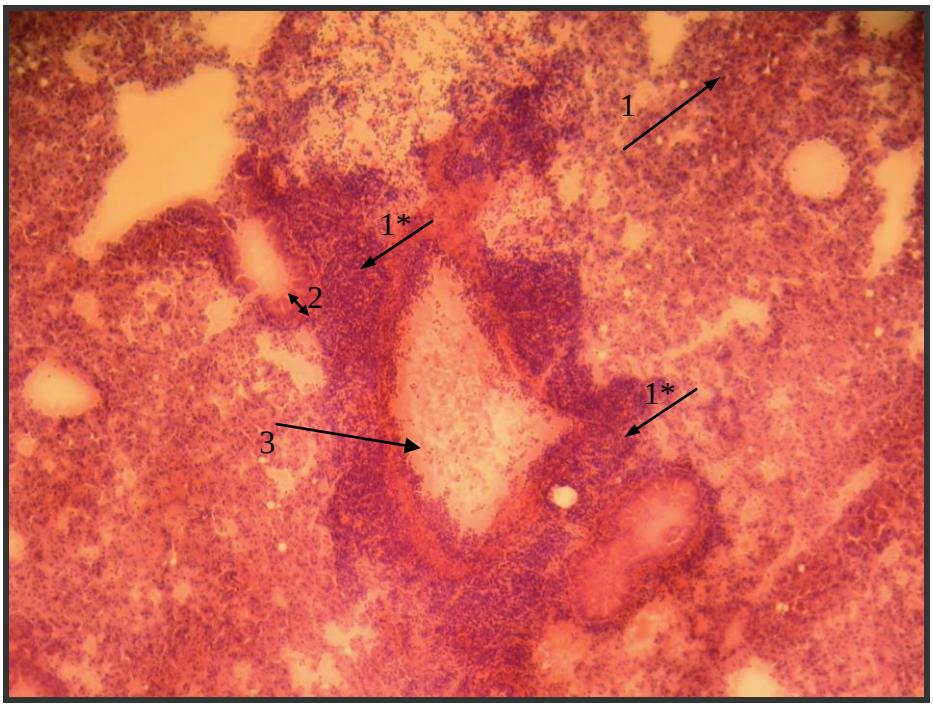
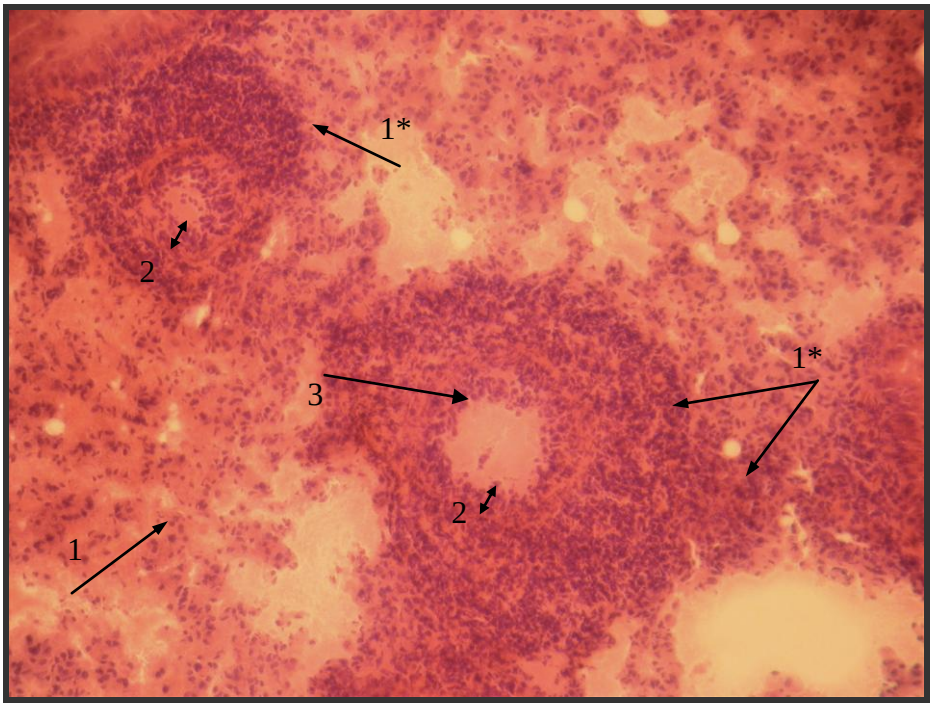


B

Figure 3.24 Inflammation in the lung following infection with ΔSodA. Light microscopy of H&E stained lung tissue collected from MF1 mice infected i.n. with 10^6 cfu of *S. pneumoniae* ΔSodA at 24h post-infection. Magnification: Panel (A) 40x; Panel (B) 100x. Arrow-1 indicates cellular infiltrate; Arrow-2 indicates bronchial wall hypertrophy; Arrow-3 indicates exudate.





B

Figure 3.25 Inflammation in the lung following infection with ΔSodA. Light microscopy of H&E stained lung tissue collected from MF1 mice infected i.n. with 10^6 cfu of *S. pneumoniae* ΔSodA at 48h post-infection. Magnification: Panel (A) 40x; Panel (B) 100x. Arrow-1 indicates cellular infiltrate; Arrow-2 indicates bronchial wall hypertrophy; Arrow-3 indicates exudate.

3.1.3.7 Analysis of leukocyte infiltration in wild type- and ΔSodA-infected lungs

Total leukocyte numbers (Fig. 3.26) and individual lymphocytes, macrophages and neutrophils cell numbers were enumerated (Table 3.4) over the time course of infection with *S. pneumoniae* wild type or ΔSodA in lungs of mice infected intranasally, as described in §2.11.4 and §2.11.5.

Note that, following infection with ΔSodA, all the four mice analysed were bacteremic at 24h post-infection but, at 48h post-infection, only three out of four mice were bacteremic. As for histological analysis (§3.1.3.6) at 48h post-infection, lungs of mice with no bacteria in the blood had considerably less leukocytes than lungs of bacteremic mice (results not shown).

A detailed description of the leukocyte population in the lungs of mice infected with wild type pneumococci has been given in §3.1.1.9. Briefly, total leukocyte levels in wild type-infected lungs were significantly higher by 24h post-infection ($P<0.05$) and remained unchanged by 48h post-infection (Fig. 3.26). This rise in total leukocyte cells was due to a significant increase in the number of neutrophils from time zero to 24h (4.6-fold increase, Table 3.4).

When mice were infected with the ΔSodA mutant, total leukocytes counts were higher at 24h post-infection (Fig. 3.26) when compared with time zero ($P<0.05$), as seen in the wild type parent. However, the leukocytes counts for ΔSodA mutant were lower at 48h post-infection than at 24h ($P<0.05$), as shown in Figure 3.26.
A possible explanation for this is the fact that, in lungs analysed at 48h post-infection, one lung was from a non-bacteremic mouse and had significantly less ($P<0.05$) neutrophils than bacteremic lungs ($3.3x10^5$ neutrophil cells *versus* approx. $1.4x10^6$ neutrophil cells, respectively) in contrast with the lungs analysed at 24h, which were all from bacteremic mice. Moreover, as mentioned above (§3.1.3.6), by 48h post-infection, lungs of non-bacteremic mice were considerably less inflamed than lungs of bacteremic ones, which may explain the decrease seen in the number of leukocyte counts.

Table 3.4 shows that the increase seen at 24h post-infection for leukocytes enumerated in the lungs of ΔSodA-infected mice was due to a significant increase in the numbers of neutrophils ($P<0.05$ compared with time zero), as seen for the wild type. The numbers of lymphocytes and macrophages remained unchanged at this time point ($P>0.05$ compared with time zero). The macrophage levels were still unchanged at 48h post-infection (Table 3.4). However, the numbers of lymphocytes were significantly lower at this time point of the infection ($P<0.05$ compared with time zero and time 24h). By 48h post-infection, the levels of neutrophils in the lungs had decreased compared with the levels at 24h ($P<0.05$) but neutrophil numbers were nevertheless higher than those at time zero ($P=0.057$). At this point, neutrophil cell numbers were still larger than those of macrophages or lymphocytes.

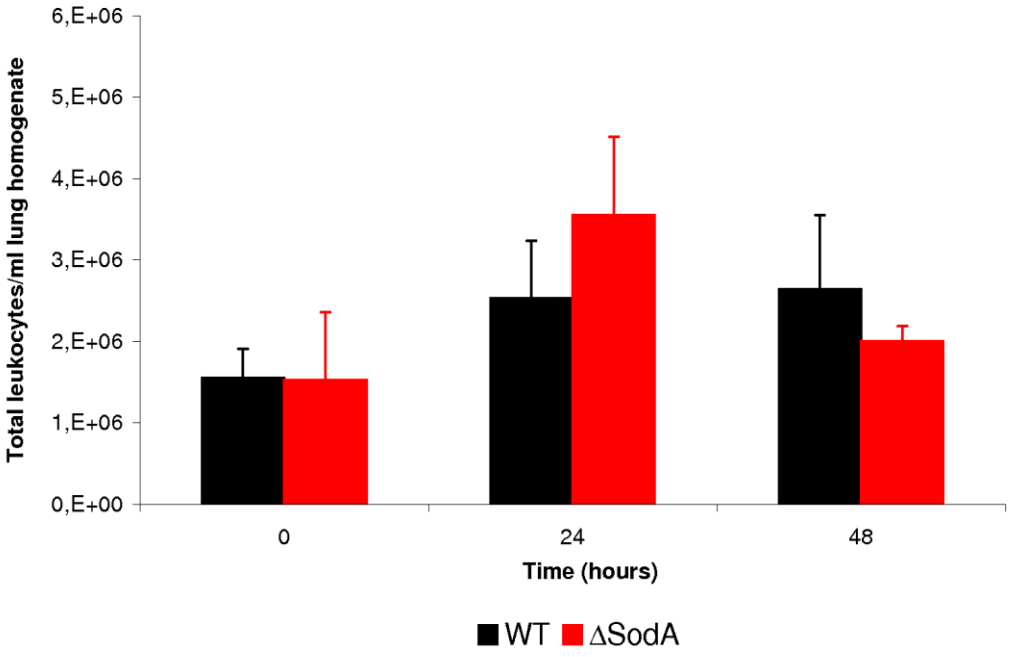


Figure 3.26 Effect of superoxide dismutase on recruitment of leukocytes to the lung. Total leukocyte counts in cytospin preparations of whole lung homogenates from MF1 mice infected i.n. with 10^6 cfu of *S. pneumoniae* wild type or ΔSodA. Data are the mean of 4 mice. Bars indicate SD.

Table 3.4 Individual leukocyte counts in cytospin preparations of whole lung homogenates from MF1 mice infected i.n. with 10^6 cfu of *S. pneumoniae* wild type or ΔSodA.

Neutrophils	WT		ΔSodA	
Time	Cells/ml	Fold increase	Cells/ml	Fold increase
0	3.0E5		2.9E5	
24	1.4E6	4.6	2.3E6	7.8
48	1.3E6	4.4	1.1E6	3.8
Lymphocytes	WT		ΔSodA	
Time	Cells/ml	Fold increase	Cells/ml	Fold increase
0	5.6E5		1.1E6	
24	6.2E5	1.1	1.1E6	1.0
48	6.5E5	1.1	5.2E5	0.5
Macrophages	WT		ΔSodA	
Time	Cells/ml	Fold increase	Cells/ml	Fold increase
0	6.8E5		1.1E5	
24	4.9E5	0.7	1.3E5	1.1
48	7.3E5	1.1	1.4E5	1.2

Data are the mean of 4 mice. Leukocytes were quantified at x400 magnification with a graticule-equipped eyepiece. Fold increases in leukocyte populations are relative to time zero values.

3.1.3.8 Immunohistochemical analysis of inflammatory cell infiltrates

To further analyse leukocyte infiltration in the lung and to assess the number of leukocytes in inflamed areas of the lung tissue, immunohistochemistry was performed, as described in §2.12. Positively stained cells were enumerated only in inflamed areas of sectioned lung tissue taken at 0h, 24h and 48h post-infection from mice infected i.n. with wild type or ΔSodA pneumococci (Table 3.5). The results presented in this section for wild type-infected mice were provided by Dr Kadioglu (Dpt. Infection, Immunity and Inflammation, University of Leicester), from experiments done in parallel. Also note that the ΔSodA-infected lungs were the same as the ones used for histological examination (§3.1.3.6) and that only half of the mice culled at each time point were bacteremic following infection. Moreover, at 24h post-infection there was no difference between lungs of bacteremic and non-bacteremic mice, but by 48h post-infection, lungs of mice with no bacteria in blood were considerably less inflamed than lungs of bacteremic mice (results not shown).

In inflamed areas of wild type-infected lungs, the numbers of neutrophils (Table 3.5) showed a significant increase at 24h post-infection compared to time zero ($P<0.05$). This also reflected the equivalent increase seen in total lung homogenate counts of neutrophils (Table 3.4). Neutrophils were observed within inflamed bronchioles and in bronchiole walls but also to a greater extent in the perivascular areas surrounding inflamed bronchioles and in alveolar spaces. However, the numbers of neutrophils counted in tissue, especially in and around inflamed bronchioles, decreased significantly by 48h post-infection (Table 3.5) compared to 24h ($P<0.05$).

As observed for the wild type, in the inflamed areas of ΔSodA-infected lung tissue, neutrophils showed a significant increase at 24h post-infection (Table 3.5) compared to time zero ($P<0.05$). This reflected the increase seen in total lung homogenate counts of neutrophils (Table 3.4). In ΔSodA-infected lungs, heavy infiltration of neutrophils was detected in areas surrounding inflamed bronchioles and was also observed to a smaller extent in inflamed bronchiole walls and within

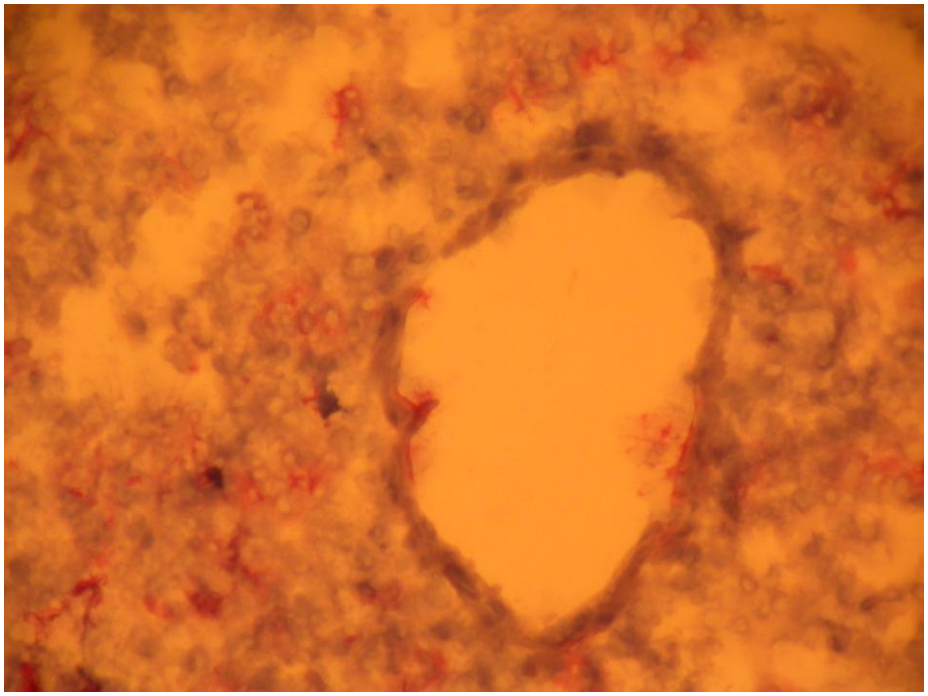
inflamed bronchioles. However, neutrophils were not detected in the alveolar spaces (Fig. 3.27A). By 48h post-infection, again as for the wild type, numbers of neutrophils in tissue decreased significantly compared to 24h ($P<0.05$). However, the number of cells at this time point appeared to be underestimated maybe due to difficulties in differentiating individual cells, since substantial levels of cellular infiltration were observed around inflamed bronchioles, as can be seen in Fig. 3.27B.

Both in wild type and ΔSodA-infected lungs, the numbers of neutrophils were still larger than those of macrophages or lymphocytes at equivalent time points, as was also the case for the numbers of total-lung homogenate. In wild type-infected lungs, T lymphocyte numbers in inflamed areas increased significantly by 24h and 48h post-infection compared to time zero ($P<0.05$). The numbers of T cells were large in tissue surrounding inflamed bronchioles and perivascular tissue by 24h and 48h post-infection. The number of B cells in lung tissue increased by 24h and 48h post-infection. This increase was observed in tissue around inflamed bronchioles and to a lesser extent within alveolar spaces. By 48h, B cells were also observed within inflamed bronchioles. The number of T cells and B cells in lung tissue sections infected with ΔSodA pneumococci also increased over the 48h following infection however this change was not statistically significant. Moreover, the T cell increase in ΔSodA infected lungs was 2-fold less than the wild type.
The total number of macrophages in inflamed areas remained constant at 0h, 24h and 48h post-infection in lung tissue sections infected with both wild type and ΔSodA pneumococci. For both strains, macrophages were seen inside inflamed bronchioles, in perivascular areas surrounding these bronchioles and in alveolar spaces.

Table 3.5 Lung leukocyte sub-populations (neutrophils, T cells, B cells and macrophages) numerated in the vicinity of inflamed bronchioles in lungs of mice infected i.n. with 10^6 cfu of *S. pneumoniae* wild type or ΔSodA.

Neutrophils	WT		ΔSodA	
Time	Cells/mm^2	Fold increase	Cells/mm^2	Fold increase
0	17		124	
24	66	3.9	279	2.3
48	37	2.2	164	1.3
T cells	WT		ΔSodA	
Time	Cells/mm^2	Fold increase	Cells/mm^2	Fold increase
0	9		20	
24	40	4.4	42	2.1
48	40	4.4	38	1.9
Macrophages	WT		ΔSodA	
Time	Cells/mm^2	Fold increase	Cells/mm^2	Fold increase
0	20		12	
24	13	0.65	13	1.1
48	19	0.95	9	0.75
B cells	WT		ΔSodA	
Time	Cells/mm^2	Fold increase	Cells/mm^2	Fold increase
0	6		21	
24	15	2.3	50	2.4
48	24	4.0	23	1.1

Data are the mean of 4 mice. Leukocyte sub-populations expressed as the percentage of total leukocytes are shown in parenthesis. Fold increases in leukocyte sub-populations compared to time zero values.



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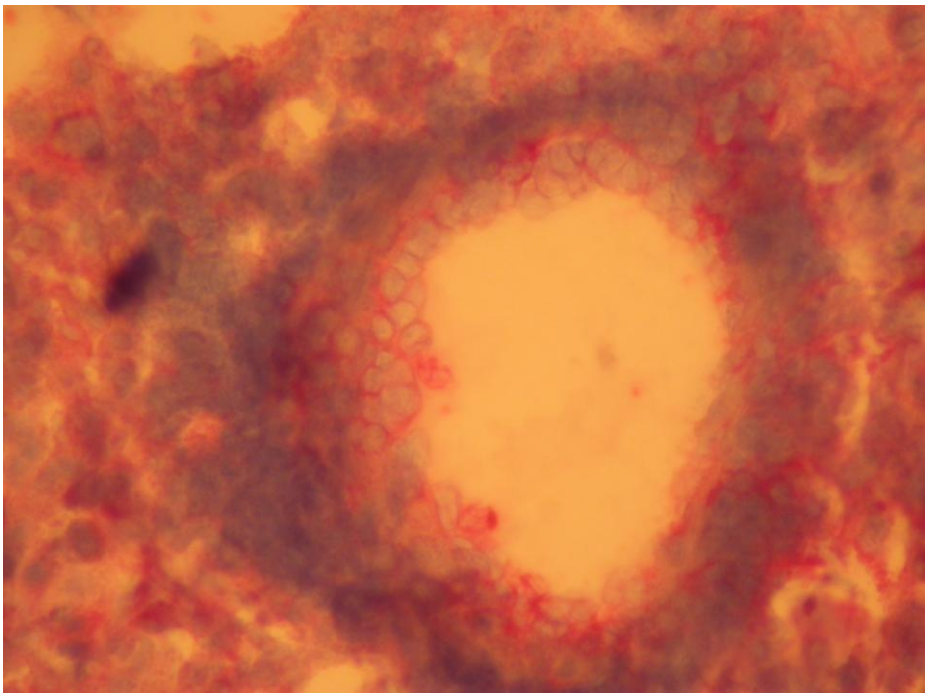

B

Figure 3.27 Light microscopy of APAAP-stained neutrophils (red stained cells) in lung tissue infected with 10^6 cfu of *S. pneumoniae* ΔSodA at 24h post-infection (panel A) or at 48h post-infection (panel B). Magnification: Panel (A)100x; Panel (B) 200x.

In summary:

- *In vitro* experiments show a 4h lag phase in the aerobic growth of ΔSodA that was not seen in the wild type. After this time, the growth of ΔSodA was comparable with the wild type.
- ΔSodA was significantly impaired of growth in the upper and lower respiratory tract in the initial hours following intranasal infection, as was seen in the *in vitro* growth.
- ΔSodA is less invasive than the wild type with only 70% of bacteremic mice by 24h post-infection and 60% by 48h post-infection.
- Deletion of *sodA* from pneumococci appears to result into the appearance "two different phenotypes" of ΔSodA mutant cells.
- ΔSodA caused less inflammation of the lungs than the wild type yet a major feature was noted following immunohistochemistry, corresponding to substantial levels of cellular infiltration around inflamed bronchioles
- Leukocyte infiltration into the lungs of mice infected with ΔSodA was increased by 24h post-infection due to a significant increase in the numbers of neutrophils.
- Immunohistochemistry in lungs infected with ΔSodA revealed that the cells packed around inflamed bronchioles were mainly neutrophils. The T cell increase was lower than that seen for the wild type, at both 24h and 48h post-infection.

DISCUSSION

4. Discussion

Streptococcus pneumoniae is a bacterial pathogen that affects humans worldwide. The bacterium is currently the leading cause of invasive bacterial disease in children and the elderly (Todar, 2003) and is also the most commonly identified bacterial pathogen causing community-acquired pneumonia (Catterall, 2004). Despite being part of the commensal flora of the upper respiratory tract (URT) of humans, infection can progress to respiratory or systemic disease.

A better understanding of the mechanisms of pneumococcal infection is a pre-requisite for development of effective vaccines and for designing novel strategies for the prevention and treatment of these infections. Given the very large number of factors that have been identified as potential virulence determinants, the underlying mechanisms of these virulence factors are of utmost importance for designing novel therapeutic strategies. Bacterial surface molecules can be used as vaccine candidates. However, not all surface proteins genes are present in every strain and environment factors can affect expression of these genes. Effective drugs should therefore contain a cocktail of various inhibitors.

A number of pneumococcal proteins have been suggested as putative virulence factors, among them neuraminidase A and B, the major pneumococcal autolysin LytA and the detoxifying superoxide dismutase MnSOD. These proteins were the subject of this study.

4.1 Neuraminidase

Research investigating deglycosylation of human glycoconjugates by *S. pneumoniae* has focused on neuraminidase activity and the contribution of desialylation to pneumococcal colonisation and pathogenesis.

Prior to the work presented herein, Mitchell *et al*. (1997) reported that a deficiency of neuraminidase A led to decreased virulence in a model of pneumococcal pneumonia. Furthermore, animal studies investigated after that, indicate that NanA plays an important role in pneumococcal pathogenesis (Tong *et al.*, 2000; 2002). In contrast, Paton *et al*. (1997) and Berry & Paton (2000) found no role for

pneumococcal NanA in the host-bacterial interaction beyond the mucosal surface as determined in intraperitoneal infection models.

Streptococcus pneumoniae neuraminidases' have also been implicated in colonisation by revealing receptors for adherence, providing a carbon source for the bacteria or by modifying the surface of competing bacteria within the same niche (Kelly *et al*., 1967; Schauer, 2000; Shakhnovich *et al*., 2002; Tong *et al*., 2000; 2002). Recently, in accordance with the suggestions above, our group has shown a role for NanA in mucin utilisation (Yesilkaya *et al.,* 2008).

The contribution of pneumococcal neuraminidase B to respiratory disease had not yet been investigated before this study, although it had been reported that in a murine meningitis model the absence of NanB did not affect the course of bacterial spread and multiplication compared to wild type pneumococci (Wellmer *et al*., 2002).

The benefits to the pneumococcus of production of two (or more) distinct neuraminidases are unclear. Their different pH optima and difference in size suggests that the production of distinct neuraminidases may assist occupancy and invasion of distinct anatomical sites by *S. pneumoniae*. In accordance, Xu *et al.* (2008a) and Gut *et al.* (2008) have just recently revealed, by analysis of substrate binding, that NanB has an increased preference for SA-α-(2$\rightarrow$3) over SA-α-(2$\rightarrow$6) and SA-α-(2$\rightarrow$8) whereas NanA shows little discrimination between the different linkage and is over ten times more active against each than NanB.

Given these findings, the hypothesis tested in this work was that pneumococcal neuraminidases are important for the initiation of infection in the upper airways, probably by removing important receptors on mucins, which would enable pneumococcal access to functional receptors on target cells and enhance colonisation. A murine model of bronchopneumonia was used to assess the roles of neuraminidase A and B in pneumococcal survival, infection of the nasopharynx and invasion of the lower respiratory tract using *S. pneumoniae* type 2 strain D39 deficient mutants in these putative virulence factors.

4.1.1 Characterisation of *S. pneumoniae* neuraminidase-negative mutants ΔNanA & ΔNanB

4.1.1.1 *In vitro* growth

To verify if the mutations of Δ*nanA* and Δ*nanB* affected the *in vitro* growth of *S. pneumoniae*, the wild type parent and isogenic derivatives were inoculated into serum broth and incubated aerobically at 37ºC for 5 hours. There were no differences ($P>0.05$) in the growth of the wild type parent strain compared to the growth of ΔNanA and ΔNanB strains (Fig. 3.3, §3.1.1.2). In addition, the rates of increase in OD of the two mutants were comparable ($P>0.05$). Given these findings, the discussion below presumes no differences in the fitness of the three bacterial strains used in each experiment.

4.1.1.2 Neuraminidase activity

To assess the phenotypes of the *S. pneumoniae* derivatives ΔNanA and ΔNanB, a quantitative enzyme assay was performed at pH 6.6. Lysates of fresh cultures (§2.4) or murine peritoneal cultured (MPC) pneumococci (§2.10.2) were used together with the pNP-NANA substrate (§2.9) and the results were compared to the wild type parent (Table 3.1, §3.1.1.3). Neuraminidase activity for *in vitro* grown wild type pneumococci was significantly greater ($P<0.05$) than its isogenic neuraminidase mutants grown in the same conditions, confirming the phenotypes of the mutants. Moreover, the neuraminidase activity of NanA (observed in the ΔNanB strain) was significantly higher ($P<0.05$) than that of NanB (observed in the ΔNanA strain) in both passaged and non-passaged pneumococci, as expected, since the assay was performed at the NanA (and not NanB) pH optimum (as described by Berry *et al.*, 1996). In the present study, the lower activity of NanB observed in the *in vitro* grown cells is in agreement with Berry and colleagues (1996) who had previously reported a reduced specific activity for NanB (37 U/mg of protein when assayed at its pH optimum 4.5) (Berry *et al.,* 1996) compared to

NanA (3500 U/mg of protein when assayed at pH 6.6, optimum for NanA) (Lock *et al.,* 1988) using the fluorogenic MUAN substrate (2'-4-methylumbelliferyl-α-D-*N*-acetylneuraminic acid). However, the difference observed between the two enzymes regarding their specific activity might be related to the type of substrate used in these assays. As mentioned above, NanB has a preference for α-(2→3) linkages as opposed to NanA which shows little discrimination between different linkages (Xu *et al.,* 2008a; Gut *et al.,* 2008). Indeed, the use of pNP-NANA or MUAN substrates for enzyme detection could induce the observation described in this study [and by Berry *et al.* (1996)] in that NanA is more active than NanB. To further investigate this uncertainty, levels of *nanA* and *nanB* gene expression were quantified during *in vitro* growth, in our laboratory, by Dr. Yesilkaya (Department of Infection, Immunity and Inflammation, University of Leicester; see below). Both *nanA* and *nanB* genes were transcribed *in vitro* but *nanA* mRNA levels at mid-log phase were significantly higher than *nanB* mRNA, showing that the results obtained on the neuraminidase assay are valid. Moreover, data presented by Oggioni *et al*. (2006) are in agreement with the findings presented herein in that NanA is more active than NanB. They have measured by quantitative real time RT-PCR gene expression during infection in mice. In lung-infected tissue the *nanA* gene showed a 16.6-fold increase compared to 3.2 for *nanB* (Oggioni *et al*., 2006). Neuraminidase detection at NanB pH optimum was not done in the present work. It would be of interest to check the neuraminidase activity of these strains at pH 4.5. This lower pH can occur at sites of inflammation, inside phagosomes or the external auditory canal. It may be that NanB provides an advantage for survival of pneumococci at these sites. Tong *et al.* (2001) has shown that sialic acid can be removed from the Eustachian tube epithelia of chinchillas infected with NanA-deficient *S. pneumoniae* strain, suggesting that NanB is important for infection of the ear canal.

The results presented here for *in vivo* passaged cells show that, following murine peritoneal culture, neuraminidase activity of the strains examined was not significantly altered when compared to the *in vitro* results. The level of neuraminidase activity at pH 6.6 from *in vitro* grown bacteria was slightly higher but not statistically different ($P>0.05$) to MPC pneumococci for all the strains examined. The results show that pneumococci appear to respond to the *in vivo* MPC environment, and that host factors have the ability to alter neuraminidase

activity of the pneumococcus. Changes in expression of known pneumococcal virulence genes have been previously detected by Orihuela *et al.* (2000). They have looked at the differences in gene expression using total RNA isolated from pneumococci grown in MPC *versus* those grown *in vitro* and found that two (*ply*, *capA3*) out of four genes examined (*lytA*, *pspA*) showed changes in expression following MPC. On the other hand, it may also be that, has found by Oggioni and colleagues (2006), gene expression of MPC pneumococci that were recovered from the blood of mice resembles the pattern of gene expression of bacteria grown in liquid medium, therefore explaining the minimal change observed in the present work, between the neuraminidase activity of MPC strains compared to the *in vitro* results.

Neuraminidase activity of MPC wild type strain was higher than that of ΔNanA strain but not statistically different from the activity of the ΔNanB strain, suggesting that the mutation of *nanA* significantly reduces total neuraminidase activity in MPC pneumococci. In fact, the result of the second replication of the neuraminidase assay using ΔNanA-passaged was zero for all the three samples tested, which shows that the activity of NanB is very low. In order to investigate whether molecules like mucin or sialic acid may inhibit neuraminidase *in vivo* the use of ELISA could be done. Or, maybe one could look for patterns of gene expression using, for example, quantitative real time RT-PCR. It has been confirmed for many bacterial pathogens that they are different when gown *in vitro* or *in vivo*, suggesting that behaviour *in vivo* must be explored to increase knowledge of bacterial pathogenicity (Smith, 2000).

4.1.1.3 Quantitative reverse transcription-PCR

In addition to the enzyme assay, levels of *nanA* and *nanB* gene expression were quantified during *in vitro* growth (this work was performed by Dr. Yesilkaya, Department of Infection, Immunity and Inflammation, University of Leicester). Both *nanA* and *nanB* genes were transcribed *in vitro*; however, *nanA* mRNA levels at mid-log phase were significantly higher than *nanB* mRNA. Transcript analysis also showed that inactivation of one gene was not compensated by the transcription

level of the other. The transcript quantification also clearly showed that the mutations in the *nanA* and *nanB* genes had no polar effect upon each other. These results are in keeping with the neuraminidase activity data that also showed significant differences ($P<0.05$) between the neuraminidase activities of ΔNanA and ΔNanB strains, both *in vitro* and *in vivo*. As previously described by Berry *et al.* (1996) using the clinical isolate serotype 6 for analysis, *nanB* is located on the pneumococcal chromosome approximately 4.5kb downstream of *nanA* and the genes seem to be part of a different operon, given that a strong transcription termination sequence is observed immediately downstream of *nanA* (Camara *et al.,* 1994). In addition, King *et al*. (2004) when looking at transcriptional profiling of opacity variants by microarray analysis using serotype 6A and 6B strains have also demonstrated that *nanA* and *nanB* are in separate regulons, consistent with these genes having distinct roles in the infection process. However, note that the genomic location of the *orfs* analysed in King's *et al*. (2004) work was based on the published genome sequence of a serotype 4 strain (Tettelin *et al*., 2001).
Furthermore, transcriptional expression of SPR1535 (the gene downstream of *nanA* in the genome of strain R6 [Fig. 1.7 §1.5.1], which is believed to be a hypothetical protein from a COG [cluster of orthologous group] of proteins with carbohydrate transport and metabolism function) in both the wild type and in the *nanA* isogenic mutant strain was not affected by the mutation in the *nanA* gene. Therefore, this rules out the possibility of a polar effect of the mutation.

4.1.1.4 *In vivo* virulence studies

Neuraminidase A-deficient pneumococci showed reduced virulence in the murine model of bronchopneumonia used in this study. Following intranasal infection, none of the mice infected with this mutant showed signs of disease at 12h post-infection (Fig. 3.4, §3.1.1.5). This was in contrast with the wild type-infected mice that at this time already had an average disease score of 4 in a scale from 1 to 7. Moreover, the *nanA* knockout caused a significant difference ($P<0.05$) in the survival of mice compared with the isogenic wild type strain.

The reduced virulence of ΔNanA in this study is in agreement with the findings of Mitchell *et al*. (1997) which showed that a *nanA*-negative mutant was less virulent that the wild type parent in a murine model of pneumonia. However, the authors have only assessed the number of cells in the lungs of infected animals, which leaves unchecked what is happening in the upper respiratory tract. The results presented herein showed that the lack of *nanA* increased the clearance of pneumococci from both the upper and lower respiratory tract. The bacterial counts in the nasopharynx and trachea (Fig. 3.5 A&B, §3.1.1.6) suggest that the lack of *nanA* significantly impairs *S. pneumoniae* infection of these niches. ΔNanA pneumococci were not detected in these sites beyond 12h post-infection, in contrast to the wild type parent. Further confirmation for a role for pneumococcal neuraminidase A in the nasopharynx is given by the results obtained in this study when using ΔNanB mutant cells (which have *nanA*). In this case, the number of cells was not statistically different from wild type numbers up to 12 hours post-infection (Fig. 3.5A, §3.1.1.6), in contrast to ΔNanA cells which declined rapidly, emphasising a role for NanA in pneumococcal survival in the upper airways during this time. These results also suggest that neuraminidase activity from NanA may be involved with the pathogenesis of *S. pneumoniae* or that it may be important to the ability of this organism to colonise mucosal surfaces of the respiratory system. Given the rapid elimination of ΔNanA from these sites and since challenge doses play a decisive role in determining the outcome observed, a higher dose ($1x10^7$ cfu) was tested to check whether an increase in the number of cells administered through the nostrils of mice would change the result observed. Even when a higher dose of ΔNanA was given, bacteria were cleared from these sites within a 24h period, confirming that the lack of *nanA* significantly impairs survival of pneumococci in the host. Further support for a role for NanA in pneumococcal survival in the host comes from the recent published work of Pettigrew *et al*. (2006), who analysed the distribution of neuraminidase genes among 342 pneumococcal paediatric clinical and carriage isolates and found that the *nanA* gene was present in 100% of the *S. pneumoniae* strains. This high prevalence of *nanA* suggests that it is an essential gene for survival and pathogenesis in all pneumococcal strains (Pettigrew *et al*., 2006).

However, it is not clear what sequence of events triggered the abrupt elimination of ΔNanA from the host. Kelly *et al*. (1967) have suggested that neuraminidase cleavage of sialic acid glycoprotein conjugates of cell membranes alters the charge at the surface of the plasma membrane and modifies or inhibits active cation transport, mechanisms that might be used by the pneumococcus to cause injury to cells in infected tissues. Or it may be that neuraminidase alters certain host proteins. In 1983 Andersson *et al*. had already suggested that neuraminidase might unmask potential cell-surface receptors for pneumococcal adhesins. NanA could act directly by binding to sialic acid or indirectly by removing sialic acid to reveal other ligands. Pneumococcal NanA has been shown to be implicated in desialylation of human proteins exhibiting sialic acid, including the secretory component of IgA (hSC), where NanA acts on α-(2→3) and α-(2→6) linked sialic acid residues, lactoferrin and hIgA2 (King *et al*., 2004). Furthermore, recently the same group also indicated that NanA might provide the first enzymatic step in the exposure of mannose on human glycoconjugates, and that this activity may contribute to the ability of *S. pneumoniae* to colonise the human nasopharynx (King *et al*., 2006). Mucins of the nasopharyngeal mucus or other glycoconjugates at the surface of cells may function as receptor molecules for *S. pneumoniae* and thus play an important role in the colonisation of the nasopharynx. Upon entering the nasal cavity the pneumococcus first encounters the mucus which coats the mucosal epithelium. Solubilisation of mucin (the major glycoprotein component of respiratory tract mucus) and pneumococcal adherence should occur quickly or the organism will be swept into the gut and eliminated. So, perhaps, as shown by the results presented here using ΔNanA pneumococci, NanA is required for the rapid pneumococcal adherence to receptors on the epithelial surface, after cleavage of terminal sialic acids and exposure of suitable receptors. Our group has shown that the pneumococcus adhered to mucus during co-culture with human respiratory mucosa *in vitro* (Feldman *et al*., 1992). We have now recently demonstrated the inability of ΔNanA to utilise mucin and the marked increase in *nanA* transcription level in the presence of mucin, which underlines the prominent role of this enzyme in mucin utilisation (Yesilkaya *et al*., 2008). The mechanism of action of NanA is believed to be through the interference with the protective role of mucus, by reducing viscosity. A study carried out by Puchelle *et al*. (1975) on the action of

purified *S. pneumoniae* neuraminidase on mucins showed a significant reduction in the mucin viscoelastic properties, which lead to important physiological changes in the mucus transport along the tracheo-bronchial epithelium and thus contributing to the pathologic mechanisms of infection. Many bacterial pathogens have been shown to bind to mucin. Ryan and colleagues (2001) have studied the interaction between group A streptococci (*S. pyogenes*) and mucus. By determining which monosaccharide mucin component (GlcNAc, GalNAc, galactose, fucose and NANA) was the M protein receptor, using an inhibition assay, they showed that the streptococcal M6 protein binds to bovine mucin and have identified sialic acid as the monosaccharide ligand on mucin. Indeed, at 30ºC, sialic acid decreased adherence to bovine mucin by approximately 70%. Additionally, they found that sialic acid plays a critical role not just in the binding of streptococci to mucin but in the adherence of this organism to *in vitro* cultured Detroit 562 pharyngeal cells.

Neuraminidase has been thought to play an important role in pneumococcal colonisation and pathogenesis by allowing bacterial adhesion to terminal galactose residues after removal of sialic acids. The contribution of NanA to prolonged nasopharyngeal colonisation has been emphasised by the work of Long and colleagues (2004) that showed the ability of NanA to provide protection against nasopharyngeal colonisation and otitis media in chinchilla models. Adhesion is the initial event in the colonisation process and implies the attachment of bacterial surface proteins to host cell carbohydrate receptors (Austrian, 1997). In *Haemophilus influenzae*, for example, P2 and P5 OMPs adhere to mucin and, in addition, high molecular weight surface-exposed proteins HMW1 and HMW2 recognise a glycoprotein receptor containing *N*-linked oligosaccharide chains with sialic acid and an uncharacterised carbohydrate moiety, respectively (Reddy *et al*., 1996).

Evidence for adherence of *S. pneumoniae* to the human airway via carbohydrate receptors on respiratory epithelial cells was first presented by Andersson *et al*. (1983) which reported pneumococcal adherence to the naturally occurring milk oligosaccharide lacto-*N*-neotetraose (LNnT) (Galβ1-4GlcNAcβ1-3Galβ1-4Glc), followed by Krivan *et al*. (1988) who showed adherence to a number of different glycoconjugates including GalNAcβ1-4Gal found in gangliosides G_{M1} and G_{M2}.

Also Cundell *et al*. (1994, 1995b) found that the glycolipid globoside (GalNAcβ1-3Galα1-4Galβ1-4Glc-Cer) could inhibit adherence of *S. pneumoniae* to lung and endothelial cells *in vitro* and finally Barthelson *et al*. (1998) who showed inhibition of adherence by sialylated oligosaccharides.

In this study the results presented when using ΔNanB for infection further support a role for NanA in prolonged colonisation of the upper airways. ΔNanB mutant cells were recovered from the nasopharynx and trachea of mice five and seven days post-infection, respectively, showing that the presence of *nanA* in the pneumococcal cells allowed the organism to colonise these niches. The mechanism of this process could therefore be related with the action of neuraminidase on mucin resulting in an increase in the number of available receptors for *S. pneumoniae*, as discussed above.

The results herein regarding the contribution of NanA to prolonged colonisation are similar to those obtained in a study by Tong *et al*. (2000), in which a D39 isogenic neuraminidase A-negative mutant ΔNA1 was significantly impaired in the ability to colonise and to persist in the nasopharynx in a chinchilla model of otitis media, following intranasal challenge. This group also showed that adherence of *S. pneumoniae* to tracheal epithelium *ex vivo* was decreased when using the mutant ΔNA1 (Tong *et al*., 2002). Accordingly, Orihuela *et al*. (2004) reported that NanA contributed to prolonged nasopharyngeal colonisation following intranasal infection of inbred BALB/cj mice.

As observed for ΔNanA following intranasal infection, ΔNanB mutant strain was completely avirulent for infected mice, in contrast to the wild type (Fig. 3.4, §3.1.1.5). No signs of disease were observed throughout the seven days of the experiment and all the animals survived infection. This was despite the fact that ΔNanB cells were recovered from the nasopharynx and trachea of mice up to five and seven days post-infection, respectively, and also from the lungs up to 48h, although at lower numbers than the wild type parent. Furthermore, no bacteremia was detected in these mice at any time following infection. In addition, when ΔNanA or ΔNanB pneumococci were injected directly into the blood (i.v. infection, Fig.3.7, §3.1.1.7) mice did not show any signs of disease during the 48h post-infection.

These results show for the first time, that the lack of *nanB* completely reduces pneumococcal virulence *in vivo*, indicating a role for NanB in pathogenesis following intranasal and intravenous infections. In support of this idea, recently Pettigrew and colleagues (2006) found that *nanB* is present in 96% of 342 carriage, middle ear, blood and cerebrospinal fluid pneumococcal strains from young children, suggesting the involvement of NanB in pathogenicity.

The lack of NanB did not impair pneumococcal colonisation of the nasopharynx and trachea (Fig. 3.5, §3.1.1.6). Even at a lower dose ($1x10^3$ cfu), by 48h post-infection, ΔNanB bacterial cells were recovered from these sites. However, despite these cells being able to survive in the upper respiratory tract they were unable to multiply in these niches. There were no differences in numbers of ΔNanB cells observed from 6h to 24h post-infection. Moreover, from that time onwards, bacterial numbers start to decline until cells were not detected seven days post-infection. The presence of NanB appears to be related to the ability of the pneumococcus to grow in the mucosal surfaces of the URT. The results show that NanB is important for pneumococcal multiplication in the upper airways. The lack of growth of ΔNanB cells in these niches could be attributed to a decreased ability of the pneumococcus to obtain energy from carbohydrates present in mucin due to the lack of NanB. Up to 85% of the dry weight of mucin is carbohydrate (Wiggins *et al.*, 2001), the principal energy source for the pneumococcus (Sicard, 1964; Hoskins *et al.,* 2001). We have recently demonstrated that *S. pneumoniae* can utilise mucin as a source of carbon and nitrogen to sustain growth (Yesilkaya *et al*., 2008). Therefore, it is possible that NanB has a role to play in pneumococcal growth (nutrition) *in vivo*, enabling *S. pneumoniae* to acquire energy during colonisation by desialylation of glycoconjugates. A nutritional role for bacterial neuraminidases has been previously demonstrated in enteric bacteria by Corfield *et al*. (1992). Furthermore, Burnaugh *et al*. (2008) have recently found that NanB can partially support the growth of pneumococci on glycoconjugates *in vitro*. They first observed the ability of the *S. pneumoniae* clinical isolate 6A-T to utilise human α-1 acid glycoprotein (AGP) as a source of carbon to sustain growth and that it was dependent on the presence of glycans on AGP. Than, they found a NanA-

independent desialylation of SA from AGP and demonstrated that, in the absence of NanA, NanB could partially support growth of pneumococci on glycoconjugates.
The contribution of pneumococcal neuraminidase B to respiratory disease has not been investigated before, and the data presented in this work are the first to be available on the behaviour of a pneumococcal neuraminidase B-deficient mutant in the respiratory tract. A study by Wellmer *et al*. (2002) has been done before, using $NanA^-$ or $NanB^-$ mutants in a murine meningitis model and the results showed that the absence of neuraminidase did not affect the course of bacterial spread and multiplication compared to wild type pneumococci. However, it was previously found by O'Toole *et al*. (1971) that, in patients with pneumococcal meningitis, there was a direct relationship between the level of *N*-acetylneuraminic acid in cerebrospinal fluid and the development of coma and bacteremia. Moreover, Andersson *et al*. (1981) observed that pneumococcal isolates from the nasopharynx of patients with otitis media or healthy carriers adhered more avidly to human nasopharyngeal epithelial cells than isolates from patients with septicemia or meningitis, suggesting that adhesive capacity may thus localise pneumococci to that site (Andersson *et al*., 1983).
The results presented here show for the first time that pneumococcal neuraminidases NanA and NanB play a role in colonisation of the upper respiratory tract, but the enzymes are acting separately in the course of infection as shown from the analysis of cfu in these sites, *ie*, NanA appears to be crucial in the early hours following administration of bacteria intranasally presumably for a successful attachment of cells to the mucosa than NanB is required for optimal growth and multiplication.

In the lower respiratory tract, ΔNanA cells were cleared from the lungs of mice by 12h post-infection, increasing the time for clearance to 24h when a higher dose ($1x10^7$ cfu) was administered. This result is in agreement with the work by Orihuela *et al*. (2004) who also observed reduced numbers of pneumococci deficient in *nanA* in the lungs of mice when compared to the wild type, after intranasal infection. However, the possibility that reduced numbers of bacteria in the lungs are due to reduced numbers of bacteria present in the nasopharynx still remains, since pneumococci could presumably be aerosolised from the nasopharynx to the lungs, contributing in this way to a constant supply of bacteria

to this site. To further investigate the contribution of NanA to pneumococcal spread to the lower respiratory tract and growth in the lungs, ΔNanA could be administered intratracheally, bypassing the nasopharynx. Orihuela *et al*. (2004) found that, when a *nanA*-deficient mutant was administered in this way, the mutant replicated in the lungs at levels comparable to those of the wild type, suggesting that NanA is not required after lower respiratory tract infection is established. However, the authors have used a different mouse strain for infections (inbred BALB/cj) than the one used in my study and differences in susceptibility to proliferation of D39 pneumococci in the lungs has been reported before (Gingles *et al*., 2001). Nonetheless, results similar to the ones presented by Orihuela *et al*. (2004) had been obtained before by Berry & Paton (2000) when looking at survival times following i.p. infection of BALB/cj. They found no role for NanA in virulence. Yet, the interpretation of these findings can be complicated by the presence of NanB which could compensate for the absence of NanA, and these authors did not test the virulence of the $NanB^-$ mutant strain in their model of infection. These different results when using $NanA^-$ mutants point to a site- and route-specific role for NanA, whereby the tissue-specific characteristics of the host site dictate the relative role of NanA. This was also suggested by Tong and co-workers (2005) when using recombinant *S. pneumoniae* neuraminidase A in immunisations of young chinchillas. Their studies (Long *et al*., 2004; Tong *et al*., 2005) support a role for neuraminidase in the prevention of pneumococcal diseases involving the respiratory tract.

Analysis of cfu counts in lungs infected with ΔNanB together with the results obtained with ΔNanA suggests a role for NanA in pneumococcal survival in the lower respiratory tract up to 12 hours post-infection, following intranasal infection. Again, as in the URT, the number of ΔNanB mutant cells was not statistically different from wild type numbers up to this time point, in contrast to ΔNanA cells, which declined rapidly. Nonetheless, the results presented herein show that a defect in NanA is not compensated by NanB or *vice-versa*, in agreement with the pH optima of the two neuraminidases and the suggestion that enzyme activity of NanA and NanB is probably required in different host environments.

To further investigate the role of pneumococcal neuraminidases in the lower respiratory tract, histological examination of lungs infected with wild type, ΔNanA or ΔNanB mutant cells was performed. Results suggest that neuraminidase A is involved in the induction of inflammation. There was a clear difference in inflammation and cellular infiltration patterns between wild type and neuraminidase A-negative mutant at 24h post-infection. Lungs infected with ΔNanA presented minimal hypertrophy of bronchiole walls, no major areas of cellular infiltration and there was an absence of exudate in bronchioles and alveoli. This was in contrast with the wild type-infected lungs, which exhibited inflammation and heavy cellular infiltration centred around bronchioles and perivascular areas, slight hypertrophy of bronchial walls and some exudate. On the other hand, ΔNanB-infected lungs showed a degree of inflammation that was in between that observed using ΔNanA and wild type, where medium consolidation of the lung parenchyma was observed with localised mild cellular infiltrate and hypertrophy of bronchial walls.

However, a relation between the degree of inflammation and the number of mutant cells recovered from the lungs at equivalent time points following infection with ΔNanA was apparent, suggesting that the reduced level of inflammation might be due to the reduced number of ΔNanA cells present in the tissue. To further investigate this hypothesis, leukocyte infiltration (which can be related to tissue inflammation) into the lungs of mice infected with wild type, ΔNanA or ΔNanB mutant cells was analysed. Our group has previously shown (Kadioglu *et al*., 2000), from the analysis of inflammatory cell influx into wild type infected lungs that a sequential infiltration of cells occurs but fails to eliminate wild type pneumococci. It was reported that an early neutrophil influx was followed by an increase in the number of lymphocytes but the number of macrophages remained unchanged throughout the 48h time course (Kadioglu *et al*., 2000). In the present study, the same analysis was carried out using ΔNanA or ΔNanB mutant strains.

When individual cell types were analysed in ΔNanA-infected total lung homogenates, an early neutrophil influx was also observed although at a slower rate than in the wild type, followed by an increase in macrophage numbers and a later increase in lymphocytes. In contrast to the wild type, this sequential infiltration of cells resulted in elimination of pneumococci from the lungs by 12h post-infection and survival of the host. These results are in agreement with the minimal

inflammation observed following H&E staining of the lungs. Indeed, in wild type-infected lungs, the inflammatory response to infection was associated with a major increase in neutrophils, which may possess the ability to damage tissues and may also generate chemotactic factors with the potential to amplify leukocyte infiltration (Henson & Johnston, 1987). Therefore, NanA could presumably be directly stimulating lymphocytes maybe by indirect role *via* tissue damage in toxaemia associated with pneumococcal disease and inhibiting macrophage inhibitory factor and interleukin 2, as shown by Moncla *et al*. (1990).

NanB activity also seems to be important for pneumococcal survival in the lungs, as judged by cfu counts in this site when using ΔNanB mutant cells for infection. After 12h post-infection, reduced numbers of *nan*B-deficient cells were seen in the lungs of mice compared to the wild type. Also, opposite to what was observed in the nasopharynx, ΔNanB pneumococci were rapidly cleared from the lungs of mice, with very few cells being recovered by 48h post-infection, suggesting a role for NanB in pneumococcal survival in the lungs. Histological examination of these lungs revealed less severe inflammation and cellular infiltration compared to lungs infected with the wild type parent. There was a significant increase in leukocyte infiltration into lungs infected with ΔNanB by 24h post-infection, corresponding to the recruitment of phagocytic cells (neutrophils and macrophages) and also lymphocytes. The numbers eventually decreased by 48h when the host was cleared from invading pneumococci. However, lungs infected with ΔNanB were slightly more inflamed than the ones infected with ΔNanA at equivalent time points, suggesting that NanB is less required than NanA in the induction of inflammation.
The results obtained when using ΔNanB showed an increase in neutrophils and macrophages in the early hours following infection that was followed by an increase in lymphocytes. In this case, the influx of leukocytes to the lungs of infected mice did eventually eliminate pneumococci by 72h post-infection. Taken together, these results suggest a relation between the degree of inflammation and the number of mutant cells recovered from the lungs. The presence of an increased number of pneumococci could explain the severity of the inflammation observed in these lungs. Indeed, an effective mechanism seems to exist to clear bacteria defective in neuraminidase from the lungs of mice infected with

pneumococci. Moreover, NanA seems to play a role in induction of inflammation. In the absence of neuraminidase A, phagocytic cells seem to be more effective in clearing bacteria from the lungs. One possible explanation is that NanA is directly affecting neutrophil's function. Neuraminidase could be unmasking host cell surface sialoglycoconjugates, resulting in a potentially depressed immune response. Another scenario is that the presence of free sialic acid released by the action of NanA from the pneumococcus could trigger bacteria to express more virulence factors that in turn are able to neutralise phagocytic cells (P.W. Andrew, personal communication). Finally, a third possibility is that the two hypothesis mentioned above are functioning together. In support of these propositions, data presented by Soong *et al*. (2006) suggest that neuraminidase production by *Pseudomonas aeruginosa* is involved in cell-cell interactions that are necessary for colonisation and persistence of the bacteria in the airway.

Wild type pneumococci caused severe bacteremia following intranasal infections. Interestingly, neither ΔNanA nor ΔNanB were isolated from the blood at any time following intranasal infection. This result was also true when ΔNanA was administered i.n. at a higher dose ($1x10^7$ cfu). Two possible explanations for this can be proposed: (i) it may be due to an inability of the mutant cells to seed from the lungs to blood or (ii) a failure of the mutants to survive in blood due to the lack of an essential process that involves neuraminidases.

To test these hypotheses, ΔNanA and ΔNanB were used in intravenous infections, done by Dr Kadioglu, Department of Infection, Immunity and Inflammation, University of Leicester. Results obtained show that, in contrast to the lack of bacteremia following intranasal infection, ΔNanA and ΔNanB cells survived in blood immediately after intravenous administration but were eventually cleared by 24h and 48h post-infection, respectively, in contrast to the wild type pneumococci which increased exponentially in numbers until 48h post-infection, by which time the experiment was ended. This result shows that a deficiency in neuraminidase affects pneumococcal survival in blood suggesting that the absence of mutant cells in blood following intranasal infection is the result of a failure of neuraminidase mutants to survive in blood. Moreover, both neuraminidases A and B, appear to have an essential role in blood in the early hours following infection.

The reasons for this are less clear, however. Whereas proposals on the role of neuraminidase in the colonisation of mucosal site are well known, its role in a non-mucosal site, such as blood, seems less obvious. However, previous evidence has shown that the pneumococcus has the ability to bind to its surface several glycosylated protein components of the host immune response implicated in bacterial clearance. These host proteins include C-reactive protein, complement components, immunoglobulin and lactoferrin (Hammerschmidt *et al*., 1997; Jarva *et al*., 2003; Smith & Hostetter, 2000; Wani *et al*., 1996). Although it is not clear whether or not neuraminidase interact directly with these components, recent evidence suggests that NanA-dependent desialylation of immune components such as lactoferrin, secretory component and IgA1, does indeed occur (King *et al*., 2004). Glycosylation is known to affect the function of many proteins and hence, it is conceivable that NanA may be contributing to a mechanism of modification of host protein components involved in bacterial clearance, thereby diminishing their protective functions and consequently promoting pneumococcal persistence (King *et al*., 2004). Moreover, as suggested by Moncla *et al*. (1990), neuraminidases might also be responsible for a decreased oxidative burst in human blood leukocytes.

Another interesting possibility is that neuraminidase activity may help the pneumococcus to scavenge sugars from host macromolecules as a source of carbon and possibly as capsule precursors. The lack of neuraminidase may thus inhibit the ability of the pneumococcus to successfully resist phagocytosis in the blood due to poor capsule formation, although based on colony appearance and capsule size, as judged by Quellung reaction, no obvious differences were apparent. Recently, it has been proposed that NanB could partially support growth of pneumococci on glycoconjugates (Burnaugh *et al*., 2008), suggesting that the enzyme could presumably have a nutritional role in blood, as predicted for the respiratory tract.

The findings presented in this work using outbred MF1 mice on the importance of NanA in blood following intravenous infection do, however, differ from those reported with neuraminidase A-deficient mutants using inbred BALB/cj mice (Orihuela *et al*., 2004). One explanation for this could be the differences in mouse strains used between the two studies. While it is known that BALB/cj do not

develop sepsis following intranasal challenge (Gingles *et al*., 2001), MF1 mice develop lethal septicaemia following intranasal infection with the same dose and strain of pneumococci (Gingles *et al*., 2001; Kadioglu *et al*., 2000). Thus, from the results obtained in the two studies it may be suggested that NanA confers a particular trait to the pneumococcus that, in case of absence of the gene, certain mouse strains are able to benefit from. Nonetheless, further studies should be carried out using different mouse strains and even different host species in order to asses the response of pneumococcal mutants in each model.

Collectively, the findings presented in this work seem to indicate a role for NanA in (i) virulence following intranasal infection, (ii) survival and (prolonged) colonisation in the URT, (iii) spread of the pneumococcus to the LRT, (iv) survival in the lungs, (v) induction of inflammation in the lungs (vi) survival in blood. Moreover, the results seem to indicate a putative role for neuraminidase B in (i) pathogenesis following intranasal infection, (ii) growth/multiplication/nutrition in the upper airways, (iii) the spread of the pneumococcus from the nasopharynx to the lungs, (iv) survival in the lungs, (v) survival in blood. However, the mechanisms by which NanB contributes to these events have not been investigated here. Nonetheless, the infection data show that the absence of one neuraminidase is not compensated for by the presence of the other neuraminidase, in keeping with the lack of compensatory expression of the *nan*B gene in mutants lacking *nan*A gene and *vice-versa*. Moreover, the results obtained clearly suggest that NanA and NanB assist exploitation and invasion of distinct anatomical sites by *S. pneumoniae*. Berry *et al*. (1996) had already suggested that differences in pH optimum for NanB and NanA may indicate that the enzymes act at different sites or stages during the infection. The results presented here show that, while NanA seems to have a major role in infection of the nasopharynx, NanB appears to play a less important role in this niche. Disruption of the *nan*A gene had already been shown to result in a diminished ability of the bacteria to alter the cell surface carbohydrates in the Eustachian tube, resulting in a diminished ability to colonise that site (Tong *et al*., 2001).

A considerable amount of work has been done recently in the study of structure-function of pneumococcal neuraminidases in the perspective of finding specific inhibitors as possible therapeutic agents. Sequencing of *nanA* and *nanB* has

shown that they both posses a signal sequence followed by a lectin domain and a catalytic domain with four bacterial neuraminidase repeats common to all neuraminidases (Taylor, 1996). The 115kDa NanA is the largest of the three pneumococcal neuraminidases identified so far. NanA is anchored to the pneumococcal membrane via its C-terminal domain containing an LPETG motif (Camara *et al.*, 1994) in a sortase-dependent manner (Paterson & Mitchell, 2006). Although both NanA and NanB are exported proteins with typical signal peptides, NanB does not contain an L-P-x-T-G motif found in surface proteins (Berry *et al*., 1996). Recently, Xu *et al.* (2008b) have shown that the structure of the catalytic domain of *S. pneumoniae* NanA has the canonical six-bladed β-propeller fold common to all neuraminidases. They have also observed that the active site contains the predicted catalytic residues with three arginines that interact with sialic acid, a tyrosine which possibly forms a covalent intermediate (Watts *et al.*, 2006) and a glutamic acid and aspartic acid that accommodate the acetamidomethyl group of sialic acid (Xu *et al.*, 2008b).

Investigation into the classes of substrates hydrolysed by neuraminidase B would give insights on the kind of host components targeted by NanB. Analysis of the amino acid sequence of NanB revealed no significant homology with that of NanA but NanB does contain the F(Y)RIP motif involved in catalysis by binding of the substrate molecule (Roggentin *et al*., 1989). NanB also contains three copies of the aspartic box consensus motif that is believed to play a role in maintaining the enzyme structure (Berry *et al*., 1989). More recently, Xu *et al.* (2008a) and Gut *et al.* (2008) have studied in detail de structure-function of *S. pneumoniae* neuraminidase B. They showed, based on substrate specificity and catalytic mechanisms that NanB acts as an intramolecular *trans*-sialidase producing 2,7-anhydro-α-*N*-acetylneuraminic acid. *Trans*-sialidases transfer the cleaved sialic acid to other glycoconjugates rather than releasing free sialic acid. Moreover, Xu *et al.* (2008a) found that the electrostatic surface potential of NanB shows a distinct asymmetry typical of secreted proteins. These studies further suggest different modes and sites of action for these two enzymes.

The precise function of NanB is not known, but analysis of *S. pneumoniae* R6 genome seems to show that it is part of a large operon consisting of at least six ORFs (Berry *et al*., 1996). ORF3 and ORF4 have a degree of homology with MsmF and MsmG of *Streptococcus mutans*. These are membrane proteins, which

form part of a transport system responsible for metabolism of multiple sugars (Russel *et al*., 1992). ORF2 contains a typical lipoprotein signal sequence and has a limited degree of homology with MsmE (Berry *et al.,* 1996), the putative sugar-binding protein of the *msm* locus.

The assessment of the involvement of neuraminidase A and B in pneumococcal pathogenesis presented here has some limitations, since the mutants used have residual enzymatic activity. Construction of pneumococci carrying double mutations in *nanA* and *nanB* would be of great use in the study of the contribution of pneumococcal neuraminidase to disease. This idea is further supported by a recent survey indicating that the pneumococcus requires at least two genes coding for neuraminidase to be able to survive, colonise and infect the host. Distribution of neuraminidase genes in *S. pneumoniae* isolates from paediatric patients (recovered from carriage, middle ear, blood and CSF) indicated that only 1% of isolates had just *nanA*, 49% had *nan*A and *nan*B only and 47% had *nan*A, *nan*B and *nan*C (Pettigrew *et al*., 2006). Investigation of strains carrying the three neuraminidases (like TIGR4) in experiments using animal models would be useful to compare the results obtained in this study.

Overall, the present study contributes to a better understanding of the role in pathogenicity of neuraminidases of the respiratory pathogen *S. pneumoniae*. These experimental data are important steps in furthering our understanding of the putative use of these enzymes to induce protective immune responses. Supplementation of vaccines with appropriate pneumococcal protein antigens could provide the answer to the disadvantages associated with capsular-based vaccines. Linkage of capsular polysaccharides to a protein carrier, either by covalent linking or through reactive groups has been proved successful in the *Haemophilus influenzae* Type b (Hib) and group C meningococcal conjugate vaccines (Booy *et al*., 1994; Girard *et al*., 2006). Protective pneumococcal protein antigens can be appealing candidates for use as carriers for polysaccharides in a conjugate vaccine. By covalently linking polysaccharides to carrier proteins, vaccines might be improved further, as increased immunogenicity of the polysaccharide component is achieved together with non-serotype-specific protection.

The currently used 7-valent pneumococcal conjugate vaccine (PCV-7; a sterile solution of saccharides of the capsular antigens of *S. pneumoniae* serotypes 4, 6B, 9V, 14, 18C, 19F and 23F individually conjugated to nontoxic diphtheria toxin mutant protein CRM197) has been shown to greatly reduce invasive pneumococcal infections in children under the age of 2 years (de Roux & Lode, 2005). Moreover, it produces transient reductions in nasopharyngeal carriage of vaccine-type pneumococci (Ghaffar *et al*., 2004), but does not reduce overall nasopharyngeal *S. pneumoniae* colonisation because of serotype "substitution" with non-vaccine types (Revai *et al*., 2006). Pneumococcal nasopharyngeal carriage is important, since it is related to both development of disease and spread of pathogens. Therefore, to induce herd immunity against *S. pneumoniae* it is necessary to elicit protection against carriage.

The Hib conjugate vaccine has been successful in reducing nasopharyngeal carriage of this organism in infants and children, which has significantly contributed to the success of immunisation programs, by providing large herd effect (Booy *et al.,* 1994). Revai and colleagues (2006) have studied the impact of PCV-7 on nasopharyngeal colonisation with bacterial pathogens during acute otitis media (AOM) in 417 children aged between 6 months and four years. Of these, 200 were enrolled before vaccine use (controls) and the remaining after initiation of PCV-7 vaccination. They found that the nasopharyngeal colonisation rate with *S. pneumoniae* was not different between groups, but a significantly higher proportion of PCV7-immunised children with AOM were colonised with *Moraxella catarrhalis* and significantly more pathogenic bacteria types were isolated from immunised children compared to controls. This result is challenging in the sense that the approach used to eradicate colonisation can possibly have a negative effect on nasopharyngeal colonisation in children and raises concerns about its outcome on the natural balance between pneumococci and co-colonising species.

Given these difficulties, added to the cost of production of actual pneumococcal conjugate vaccines, the supplementation of vaccines with appropriate pneumococcal protein antigens could provide an answer. Cell wall-associated proteins from different capsular serotypes can be cross-reactive and immunogenic in all age groups. Data presented herein strongly supports the idea that the pneumococcal neuraminidases NanA and NanB are valid targets for therapy. Assessment of the contribution of neuraminidase to the pathogenicity of the

pneumococcus showed that purified neuraminidase is toxic for mice, but immunisation with the inactivated protein partially protected mice from challenge with virulent *S. pneumoniae* (Lock *et al*., 1988). Recently, Long *et al*. (2004) and Tong *et al*. (2005) showed a significant reduction in pneumococcal nasopharyngeal colonisation and incidence of OM with effusion, in the chinchilla model, after immunisation with recombinant *S. pneumoniae* neuraminidase NanA (rNanA). More work has been done lately in our laboratory by Yesilkaya and colleagues (2006) in order to investigate neuraminidase as a candidate to include in a future pneumococcal vaccine. They looked in more detail at the structure-function relashionship of NanA and identified key amino acids required for the enzymatic activity of NanA. By mutation of residue R663 to H (CGT to CAT), they achieved drastic reduction in the catalytic ability of the enzyme and thus carried out immunisation studies in mice. The experiment demonstrated that the inactive neuraminidases produced were immunogenic and protective.

However, protein candidates to be included in a novel vaccine should be conserved among different *S. pneumoniae* strains and recent studies have shown that NanA exhibits high level of sequence diversity among pneumococcal strains (King *et al*. 2005). Using primers from the 5' and 3' regions of *nanA* the authors investigated the distribution and diversity of the gene in 106 isolates. They found up to 17.2% divergence at the nucleotide level and 14.8% at the amino acid level and have identified several causes for this sequence divergence, from mosaic blocks (probably introduced by horizontal gene transfer from oral streptococci), to point mutations and small insertions. Nonetheless, they found no amino acid alterations within the Asp boxes or active site of the enzyme, suggesting that sequence variation may be important in evading the adaptive immune response of the host (King *et al*. 2005) which further suggests that NanA is an important target of the immune system. However, these findings may limit the use of NanA as a pneumococcal protein vaccine. From the data presented herein, a solution to this problem could be the combined use of the two pneumococcal neuraminidases, NanA and NanB. Pettigrew and co-workers (2006) have also shown that *nanA* and *nanB* are present in 100% and 96% of 342 carriage, middle ear, blood and cerebrospinal fluid pneumococcal strains from young children, respectively. Thus the inclusion of the two proteins in a vaccine would provide a greater degree of coverage and reduce complications due to variability.

Bacterial neuraminidases may be useful targets to prevent infection in high-risk patients. The use of compounds optimised for activity against bacterial enzymes could be especially useful in preventing *S. pneumoniae* colonisation in susceptible individuals. Neuraminidases have been shown to be involved in the pathogenesis of a whole range of diseases. The structure-based design of a potent inhibitor of the influenza-virus neuraminidase is one of the outstanding successes of rational drug design. So perhaps the knowledge and expertise gained from the influenza story can be used in the design of other drugs, given that they all share certain structural features (Taylor, 1996). Although, McCullers & Bartmess (2003) shown that oseltamivir carboxylate (a specific inhibitor of influenza virus) has no activity against bacterial neuraminidases and pneumococcal neuraminidases, Soong and co-workers (2006) have recently demonstrated the usefulness of such drugs by blocking neuraminidase activity of the respiratory pathogen *P. aeruginosa* using viral neuraminidase inhibitors, oseltamivir and peramivir. In the study report in this thesis, no tests were done to check if these viral neuraminidase inhibitors could actually block/interact with pneumococcal neuraminidases. Nonetheless, collaborative studies are currently in progress based on the work presented here: one group is screening small molecules to find neuraminidase inhibitors. Inhibition of bacterial neuraminidases could provide a novel mechanism to prevent bacterial pneumonia. Another group (Xu *et al.*, 2008a,b) has been looking into the crystallisation of NanA and NanB in order to find drugs to fight these enzymes. They have observed that, for NanA, the hydroxyl group at C4 on Neu5Ac2en interacts with an arginine (Arg 366) and an aspartic acid (Asp 417) (Xu *et al.*, 2008b). Based on the currently used viral neuraminidase inhibitors, zanamivir and oseltamivir that were developed on the structure of Neu5Ac2en, potential elaboration of inhibitors for pneumococcal neuraminidases could be developed. Moreover, this group has also found that Neu5Ac2en inhibits NanB with a Ki~0.3mM (Xu *et al.*, 2008a).

4.2 Autolysin

Autolysin (LytA), the main pneumococcal murein hydrolase, is responsible for bacterial cellular autolysis at the end of the log phase and also plays a minor role

in cell separation (Sanchéz-Puelles *et al*., 1986). Furthermore, the enzyme might be required for DNA release (Moscoso *et al*., 2005) and competence for genetic transformation (Ronda *et al*., 1987). Although LytA is conserved among pneumococcal isolates, it is unclear what advantages autolysis confers to the organism. The enzyme has been implicated in the virulence of the pneumococcus however the mechanisms of this contribution remain unclear.

4.2.1 *In vivo* virulence studies

In accordance with the working hypothesis, the *lytA* knockout caused a significant difference in the lethality of mice compared with the isogenic wild type strain D39. The *lytA* mutant was completely avirulent for mice following intranasal infection. Mice challenged intranasally with ΔAL2 did not show signs of disease throughout the eight days of the experiment. Moreover, histological analysis of infected lungs showed reduced inflammation compared to the wild type parent. This extensive study using a pneumococcal *lytA* negative mutant in a murine model of bronchopneumonia confirms limited studies done previously by others. Berry *et al*. (1989b) had found clearly reduced virulence for mice (adult male outbred Prince Henry Hospital strain) when using the autolysin-negative mutant (ΔAL2) compared with the parental strain D39; intranasal and intraperitoneal 50% lethal doses were increased 10^2- and 10^5-fold, respectively. Canvin *et al*. (1995), using mice and the same autolysin-negative mutant (ΔAL2) as in this thesis, observed that following intranasal infection with $5x10^5$ ΔAL2, mice did not show signs of illness throughout the 48 hours of the experiment. Moreover, also in keeping with these findings, Berry & Paton (2000) showed that a *lytA* mutant had significantly lower virulence for Balb/c mice than the wild type D39, as judged by both survival time and survival rate following intraperitoneal challenge. Recently, Kharat and Tomasz (2006) showed that the double deletion of *lytA* and a second pneumococcal murein hydrolase, *lyt*B, significantly reduces the lethality of strain D39 in a mouse model of intraperitoneal infection. The authors suggested, however, that the major contributor to this effect was most likely *lytA*, because reduction in virulence of the *lyt*B deletion mutant was only marginal.

Reduced virulence of autolysin-negative mutants in mice has also been observed using pneumococci with serotype 3 capsule. Attenuation of virulence in mice challenged intraperitoneally with an autolysin-negative mutant of a type 3 strain was observed by Berry *et al*. (1992). Balachandran *et al*. (2001) have also shown reduced virulence (after i.v. or i.p. infections) with the virulent strain WU2 (capsular type 3) and its isogenic *lytA* mutant (SM121.10) using resistant Balb/c or highly susceptible CBA/N mice. Reduced virulence was also found by Ng *et al*. (2002) using a type 2 strain D39 autolysin-deficient derivative (AL-6) in a model of pneumococcal endophtalmitis and by Sato *et al*. (1996) using a type 3 strain in the chinchilla otitis media model. There is also evidence that bacterial autolysins contribute to the pathogenicity of other Gram-positive bacteria, such as *Staphylococcus aureus* (Mani *et al*., 1994) and *Listeria monocytogenes* (Milohanic *et al*., 2001).

In vitro studies have shown that LytA appears to be responsible for cellular autolysis at the end of the log phase (Sanchéz-Puelles *et al*., 1986). A possible implication of pneumococcal LytA in pathogenesis involves the release of components of the cell wall following bacterial cell death (shown to be highly inflammatory in some animal models) (Sato *et al*., 1996) and of cytoplasmic proteins such as pneumolysin (Berry & Paton, 2000; Ng *et al*., 2002). The pneumolysin protein lacks an N-terminal signal sequence for transport out of the cytoplasm, which led to the hypothesis that this enzyme is released upon the action of autolysin (Canvin *et al*., 1995). Kadioglu *et al*. (2000; 2002) investigated the role of pneumolysin (Ply) in the upper and lower respiratory tract in the same murine model of pneumonia as used in this thesis. They found that following intranasal challenge with the *ply*-negative mutant PLN-A, mice survived infections without major signs of disease, in accordance with what was observed with the ΔAL2 mutant in this study. These findings are in agreement with the hypothesis that LytA is responsible for the release of pneumolysin *in vivo*, and consequently has a role in pneumococcal pathogenesis. However, not all pneumococcal strains may require LytA for pneumolysin release. A study conducted by Balachandran *et al*. (2001) found that strain WU2 expresses extracellular pneumolysin prior to stationary phase and this did not require the activity of any known pneumococcal autolysin. But the mechanism of pneumolysin release in strain WU2 is not yet known.

The study on host morbidity and mortality described in this work suggest that the lack of *lytA* significantly reduces pneumococcal virulence, indicating a role for LytA in pathogenesis following intranasal infection. To obtain a better and more complete picture of the role of the LytA amidase in colonisation and pathogenesis, the analysis of pneumococcal survival in the nasopharynx, trachea, lungs and blood of intranasally infected mice was combined with the characterisation of inflammatory-cell influx into the lungs.

Bacterial counts in the nasopharynx of infected mice show that the lack of *lytA* in *S. pneumoniae* did not impair colonisation of the upper respiratory tract by this bacterium. The mutant strain persisted in the nasopharynx of mice without causing signs of disease for up to six days following intranasal infection. Interestingly, LytA appears to have no contribution to survival of *S. pneumoniae* in the nasopharynx until 12h post-infection, as judged by cfu counts of wild type and ΔAL2 pneumococci. One possibility is that *in vivo* LytA is not active until after 12h, and therefore survival and growth of pneumococcus in the nasopharynx is not dependent on this enzyme during this time. The results observed by Sato *et al*. (1996) following inoculation of the chinchilla's middle ear with *S. pneumoniae* type 3 wild type or an isogenic autolysin mutant are in agreement with this hypothesis; they also found no significant difference in pneumococcal numbers between the two strains at 12h.

After 12h, however, the results obtained seem to indicate that lack of LytA significantly affects the growth of pneumococcal cells in the upper respiratory tract, as judged by the rapid increase in wild type cfu numbers and lack of multiplication of ΔAL2 bacteria. On the other hand, LytA has been shown to have a role in daughter cell separation and pneumococcal lysis in stationary phase (Sanchéz-Puelles *et al*., 1986). Therefore, it is possible that the observed number of ΔAL2 cells reflects the growth of pneumococci in chains, in which case the cfu counts remain unchanged.

Persistence of *lytA* mutant cells in the upper respiratory tract for around one week could be explained by LytC (a third pneumococcal murein hydrolase) acting as an autolysin in the nasopharynx, as suggested by García *et al*. (1999b). In the absence of LytA, LytC could play a role in the release of the cytotoxin pneumolysin as well as components of the cell wall, albeit at a reduced level compared to the

lytA sufficient strain (Paton *et al*., 1983; Catterall, 1999). Gosink and colleagues (2000) have previously reported a role for LytC in colonisation of the nasopharynx of infant rats and adherence to Detroit nasopharyngeal cells. More recently, Guiral *et al*. (2005) verified, *in vitro*, that inactivation of both pneumococcal genes, *lytA* and *lytC*, resulted in a clear reduction of pneumolysin release that was not obtained with the deletion of either of the genes alone.

The clearance of ΔAL2 cells after 8 days post-infection might be explained by the increased target size of pneumococcal chains, which may increase the efficiency of phagocytic cells in eliminating the bacteria, as proposed by Kharat and Tomasz (2006).

Overall, these results indicate a role for LytA in growth of pneumococci in the nasopharynx and it is proposed this occurs by (i) facilitating the process of daughter cell separation and (ii) releasing pneumolysin and components of the cell wall during bacterial cell lysis.

Again, as in the nasopharynx, LytA appears to have no contribution to growth of *S. pneumoniae* in either the trachea or the lungs of infected mice until 12h post-infection given that there were no significant differences between the cfu counts of wild type and mutant bacteria in these niches. These results corroborate the view that *in vivo* LytA is not active until after 12h, and suggest that pneumococcal growth in the lower respiratory tract is not dependent on this enzyme until this time.

However, in contrast with what was seen in the nasopharynx, from 12h onwards the number of ΔAL2 mutant cells decreased rapidly, and bacteria were eliminated from the trachea and lungs by 48h and 72h post-infection, respectively. These results reinforce the hypothesis that *in vivo* LytA is not active until after 12h; and are in agreement with the suggestion that LytC may act as an autolysin in the nasopharynx (García *et al*., 1999b), where the temperatures are cooler, but has no role in lysis of bacteria cells in the lower respiratory tract. Furthermore, the major pneumococcal autolysin LytA amidase appears to be crucial to the survival of the bacteria cells in the lungs from 12h post-infection. Thus, LytA seems to play a direct role in the virulence of *S. pneumoniae* by promoting the infection of host tissues, probably by the action of the cell wall-containing teichoic acid, as observed by Sato *et al.* (1996). In the absence of autolysin there was a great

reduction in the severity of the inflammatory response in the lungs of infected mice. At 24h post-infection, histological analysis of the lungs of mice infected with the mutant strain showed minor levels of inflammation and cellular infiltration, which were generally less severe than that seen in lungs infected with the wild type parent or PLN-A-infected lungs (Canvin *et al*., 1995). The pattern of influx of inflammatory cells showed that, at this time, total leukocyte counts were significantly greater compared to time zero numbers. These data suggest that LytA amidase plays a direct role in the induction of inflammation. Sato *et al.* (1996) suggested that cell wall contributes more significantly to lung inflammation that does pneumolysin during the 12h of middle ear infection.

The results presented herein using LytA-negative pneumococci are consistent with a role for this enzyme in the release of the cytotoxin pneumolysin from the cell. As shown by Rubins *et al*. (1995), a pneumolysin-negative mutant PLN-A has a reduced capacity to injure the alveolar-capillary barrier and thus a reduced capacity to multiply within lung tissue. Alternatively, the autolysin LytA might contribute directly to the pathogenicity of the pneumococcus by mediating bacterial adherence. This feature has been previously reported in other Gram-positive autolysins. For instance, Heilmann *et al*. (1997) observed reduced attachment to polystyrene in a *Staphylococcus epidermidis* mutant lacking the *atlE* gene encoding a 148kDa protein similar to the autolysin Atl of *S. aureus*. Milohanic *et al*. (2001) have also reported the contribution of the autolysin Ami of *Listeria monocytogenes* to adhesion to eukaryotic cells via its cell wall anchor. They suggested that *L. monocytogenes* and some staphylococcal species appear to use the dipeptide GW repeat domain ('GW modules') of their autolysins to promote bacterial attachment to cells. Interestingly, the cell wall-anchoring domain of pneumococcal LytA is also made up of repeated modules containing the dipeptide GW. In this regard, it would be interesting to check whether the non-catalytic choline-binding domain of LytA amidase carries the adhesive properties of the molecule.

Following intranasal infection, the lack of *lytA* significantly impaired the growth of pneumococci in blood, since ΔAL2 cells were not recovered from this tissue at any time following infection. This is in agreement with the results obtained by Canvin *et al*. (1995). However, the result obtained could be due to an inability of the mutant to cross the barrier from lungs to blood, or inability to survive in blood.

Unfortunately, due to time restrictions, intravenous infection of mice using ΔAL2 cells was not performed to test the two hypotheses.

Recent efforts to develop new vaccines against pneumococci have focused on proteins that may be conserved throughout the different serotypes. The LytA amidase sequence is highly conserved among *S. pneumoniae* isolates, which has been used as a basis for the development of a PCR-based pneumococci detection test (Sheppard *et al*. 2004). Nonetheless, the use of *lytA* gene as a specific pneumococcal identification tool is a subject of great controversy, as shown by the work of two different groups (Whatmore *et al*., 2000; McAvin *et al*., 2001). Whatmore's group, studying the molecular characterisation of equine isolates of *S. pneumoniae*, have found that the use of a *lytA* probe in Southern blot analysis resulted in the presence of multiple bands with homology to that probe. They pointed out several disadvantages in utilisation of this probe, namely the frequent presence of homologous bacteriophage lytic genes and choline-binding proteins. On the other hand, McAvin *et al.* (2001) using real-time fluorescence PCR and a probe constructed from 15 *S. pneumoniae lytA* gene sequence's have successfully detected all seventy clinical *S. pneumoniae* isolates without cross-reaction to 26 negative controls.

Immunisation of mice with autolysin as been shown by Lock *et al*. (1992) to provide significant protection against intraperitoneal challenge with virulent type 2 pneumococci but not with the pneumolysin-negative mutant (PLN-A). Moreover, the degree of protection was similar to that achieved by immunisation with pneumolysin and no additional protection was observed when mice were immunised with both antigens. Berry & Paton (2000) showed significant reduction in virulence following intraperitoneal infection with a *lytA*-deletion mutant of *S. pneumoniae*, but the virulence of a mutant lacking both *ply* and *lytA* was no further attenuated than in the single *ply* mutant. Therefore, autolysin appears to elicit no further protection that pneumolysin as a protective antigen in a protein-based pneumococcal conjugated vaccine.

Results presented in this thesis suggest that pneumococcal LytA has a major role in inflammation of the lungs. Reducing the inflammation process during antimicrobial treatment of pneumonia may be a factor to take into account in order to limit the release of cell wall degradation products, pneumolysin, neuraminidase

and bacterial DNA. Perhaps, the use of products such as non-lytic antibiotics or autolysin immunisation would prove to reduce inflammation, as recommended by Sato *et al*. (1996) for treatment of AOM, in order to achieve a rapid elimination of pneumococci from the middle ear cavity and attenuate middle ear inflammatory response. Ng *et al*. (2002) also suggested that the use of bacteriolytic antibiotics in pneumococcal endophtalmitis may theoretically exacerbate intraocular inflammation by triggering the release of cytoplasmic products such as pneumolysin. In 1985, Tuomanen and co-workers had already proposed that the use of β-lactam antibiotics for the treatment of meningeal inflammation would further contribute to inflammatory tissue injury and possibly mortality (Tuomanen *et al.*, 1985). In this regard, the search for techniques to limit the release of pneumococcal products, such as pneumolysin, cell wall debris and neuraminidase or neutralise them warrants further investigation.

4.3 Superoxide dismutase

One of the major challenges facing bacteria growing in oxygenated environments is to efficiently resist or repair damage caused by ROI such as superoxide ($^{\cdot}O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\cdot}OH$), which may be formed endogenously or exogenously (Nathan, 2003). During aerobic growth, superoxide radicals are generated in the cytosol as the result of oxygen reduction. Optimally, aerobic metabolism of bacteria results in reduction of O_2 to H_2O, however a percentage of O_2 reduction occurs by one electron reduction to generate superoxide or by divalent reduction to H_2O_2. Moreover, $^{\cdot}O_2^-$ and H_2O_2, in the presence of free iron, can form the hydroxyl radical $^{\cdot}OH$ and these ROI are capable of reacting with and damaging various biomolecules, including proteins, lipids and DNA (Imlay & Fridovich, 1992). Therefore, enzymes that protect against reactive oxygen species, including superoxide dismutases (SODs), are ubiquitous in aerobes and in facultative anaerobes (Jakubovics & Jenkinson, 2001) which lack catalase.

The production of SOD by bacterial cells is the primary defence against superoxide (Fridovich, 1997), converting it to H_2O_2 and O_2. *Streptococcus*

pneumoniae is a fermentative organism that nevertheless exists in oxygenated environments, such as the nasopharynx of humans where the oxygen concentration is high. The major enzyme capable of detoxifying superoxide in pneumococci is MnSOD, encoded by *sodA*. The pneumococcus also may produce a FeSOD or SodB (Yesilkaya *et al*., 2000), but the *sodB* gene has not been found in the *S. pneumoniae* genome. It was previously reported, by using a mutant lacking *sodA* in an animal model of bronchopneumonia, that MnSOD plays a role in virulence of *S. pneumoniae* (Yesilkaya *et al*., 2000). However, the role of SOD in nasopharyngeal colonisation remained unknown. The current study focused on the pneumococcal SodA with the hypothesis that it plays a role in protecting *S. pneumoniae* from the oxidative environment within the host, especially in the upper airways, contributing to colonisation of the nasopharynx and invasion of the lower respiratory tract. This study also sought to expand on the work of Yesilkaya *et al*. (2000) in terms of the host response to SodA-sufficient and -deficient pneumococci.

4.3.1 Characterisation of *S. pneumoniae* superoxide dismutase-negative mutant ΔSodA

4.3.1.1 *In vitro* growth

To verify if the mutation of *sodA* affected the aerobic *in vitro* growth of *S. pneumoniae*, the wild type parent and isogenic derivative, ΔSodA, were inoculated into BHI liquid broth and incubated aerobically at 37ºC. The mutant showed a 4 hours-lag phase, but by seven hours post-inoculation it reached the wild type optical density. However, at this time point, the wild type was already in stationary phase in contrast to ΔSodA which reached its maximum growth. Rates of increase in OD, calculated from their growth curves, showed that the growth rate of the wild type parent was similar to the growth rate of the mutant (Fig. 3.18, §3.1.3.2). The data indicate that SodA is important for optimal *in vitro* aerobic growth of *S. pneumoniae*, particularly during the early hours following incubation.

In agreement with the results presented here, Jakubovics *et al*. (2002) reported the importance of SOD for *in vitro* growth of *Streptococcus gordonii* in the presence of oxygen. Diminished growth appears to be a general feature of bacteria deficient in cytosolic SOD, being also observed in *sodA*-deficient mutants of *H. influenzae* (D'Mello *et al*., 1997), *S. mutans* (Nakayama, 1992), *P. aeruginosa* (Hasset *et al*., 1995) and *Lactococcus lactis* (Sanders *et al*., 1995). In accordance, Imlay & Fridovich (1992) showed that the consequences of the lack of both the constitutive FeSOD and the inducible MnSOD in *E. coli*, includes oxygen-dependent decrease of growth rate.

Moreover, this result is in agreement with the enhanced expression of *sodA* under oxidative stress which was described by Yesilkaya *et al*. (2000) in the pneumococcus, suggesting that SodA plays an essential role in conferring protection against oxidative stress. Up-regulation of genes involved in resistance to aerobic oxidative stress has also been shown by Jakubovics *et al*. (2002) in *S. gordonii sodA*.

The importance of SOD during pneumococcal aerobic growth is further supported by the similarity in growth characteristics of wild type and *sodA*-negative mutant when cultured under anaerobic conditions, as shown in the work by Yesilkaya *et al*. (2000). Moreover, as mentioned above, SOD activity of wild type cells grown anaerobically was decreased in comparison to aerobically grown bacteria (2.4U/mg protein and 4.1U/mg protein respectively). In contrast, the SOD activity of the mutant strain was not statistically different in both conditions (0.5U/mg protein anaerobically and 1.0U/mg protein aerobically, $P>0.05$). The authors further characterised the *in vitro* growth of this mutant. They showed a lower rate of increase of ΔSodA cells grown aerobically as compared to anaerobic cultures. Moreover, in defined medium, anaerobically, the wild type growth was the same as the *sodA*-deficient mutant which was unable to grow in this medium aerobically. The haemolytic activity of the two strains was also compared. On BA plates grown aerobic or anaerobically, ΔSodA formed α-haemolytic colonies, as was seen in this thesis. However, ΔSodA extracts from late log phase cultures had less haemolytic activity (7.8 HU/μg protein) than the wild type parent (15 HU/μg protein) (Yesilkaya *et al*., 2000). Finally, measurement of H_2O_2 production by *S. pneumoniae* cultures

grown in anaerobic jar showed no difference between the two strains (Yesilkaya *et al*., 2000).

Given the findings that SodA is important for *in vitro* aerobic pneumococcal growth, the isogenic *sodA* mutant was used to study the contribution of this enzyme in pneumococcal colonisation of the respiratory tract and onset of bacteremia in a model of bronchopneumonia.

4.3.1.2 *In vivo* virulence studies

SodA appears to play a role in virulence following intranasal infection with *S. pneumoniae*, possibly by facilitating a high level of bacteremia in the early phase of infection. The *sodA*-deficient pneumococcus showed reduced virulence in the murine model of bronchopneumonia, as judged by the mortality and clinical signs registered from seven independent experiments. Following intranasal infection, none of the 50 mice infected with this mutant showed signs of disease at 12h post-infection (Fig. 3.19, §3.1.3.3) in contrast with the wild type-infected mice that at this time already had a disease score of 4, in a scale from 1 to 7. By 24h, 15% of ΔSodA-infected mice started to show moderate clinical signs (starry coat and hunched position) and by 48h, 50% of the mice presented severe signs of disease (disease score 4). Wild type-infected mice were very lethargic (disease score 6) by 24h post-infection. Yesilkaya *et al*. (2000) had previously reported reduced virulence for this mutant and showed that intranasally ΔSodA-infected mice survived significantly longer (107h; $P<0.001$) than the wild type-infected group (50h).

A route-dependent virulence was observed in this study for ΔSodA. Mice infected intravenously with ΔSodA did not show signs of disease throughout the 48h time course, in contrast to what was found after intranasal infection (see above). This result was surprising given the fact that, due to the reduced oxidative stress environment found in the blood, the mutant shouldn't be at disadvantage in survival. However this finding might be explained by a rapid elimination of ΔSodA from this site by circulating neutrophils due to an increased sensitivity of the mutant to the oxidative burst of these phagocytes. A route-dependent virulence

was also seen in Yesilkaya's study when comparing intranasal and intravenous routes. However in this case, in contrast to what was found in my study, mice infected intranasally survived longer than intravenously infected mice (107h and 45h, respectively) (Yesilkaya *et al*., 2000). A possible explanation for this difference could be the use of a small number of animals in Yesilkaya's study (5 mice/time point for i.n infections and 2 mice/time point for i.v. infections) and to the great variability of the mutant strain's ability to cause bacteremia, as was reported for ΔSodA in this thesis. The finding that SodA is involved in virulence fits in with the hypothesis that the enzyme plays a role in pneumococcal protection against the toxic effects of oxygen species.

The contribution of superoxide dismutase to the virulence of other human pathogens has been investigated. D'Mello *et al*. (1997) found a role for MnSod of *H. influenzae* Type b (Hib) in colonisation of the nasopharynx of infant rats but not in invasive disease or sustained bacteremia. However, in this case, no route-dependent virulence was reported since no differences regarding invasive disease between *H. influenza* type b *sodA* mutants and the wild type parent following i.n. or i.p. infections were observed.

After i.v. infection with the facultative anaerobe *Streptococcus agalactiae*, Poyart *et al*. (2001) observed impaired virulence of a *sodA* mutant when compared to the wild type parent, as judged by mortality rates and bacterial survival in tissues and blood, suggesting a role for SodA in virulence of this bacterium.

Iron-containing superoxide dismutase (SodB) and Cu/ZnSOD (SodC) have also been found to have a role in virulence of bacteria. For instance, in the microaerophilic *Campylobacter coli*, deletion of *sodB* was shown to decrease the colonisation potential of bacteria in experimental infection of chicks (Purdy *et al.,* 1999). A role for SodB in *Campylobacter jejuni* intracellular survival was shown in *in vitro* experiments (Purdy *et al.,* 1999). Wilks and coworkers (1998) detected significant differences in survival times when comparing wild type *versus sodC* mutant meningococci following i.p. infection of the mouse. In experiments with *Salmonella* spp., Farrant *et al*. (1997) found that disruption of *sodC* (Cu/ZnSOD) led to attenuation of virulence after systemic infection in mice and no difference in virulence regardless of the route of infection (oral or intraperitoneal) was observed.

As hypothesised, ΔSodA pneumococci were significantly impaired in survival in the oxygenated environment of the upper respiratory tract. The results obtained suggest that the ability to adapt to oxidative stress is part of the virulence of this pathogen. Moreover, it seems that SodA activity is necessary for optimal survival and growth of pneumococci in the early hours following infection in aerobic environments. The data collected from intranasal infection showed that in the initial hours in the nasopharynx (Fig. 3.20A, §3.1.3.4), bacterial numbers oscillated drastically until 12h post-infection. An apparent decrease (although not statistically significant compared to time zero) in bacterial numbers was seen by 4h post-infection in all the respiratory sites sampled (nasopharynx, trachea and lungs) and numbers of mutant cells were significantly lower than the wild type at this time point ($P<0.05$). However, following this decline, ΔSodA cells where able to multiply and by 6h post-infection the numbers reached wild type values in all the sites tested. This observation could reflect the time taken for the bacteria to move from an oxygen-rich environment into more micro-aerophilic areas.

A period of adaptation to the host's oxygenated environment was also observed for wild type pneumococci in the 6h following infection, where the bacterial numbers slightly decreased. However, in this case, the bacteria seem to be provided with a mechanism to survive this unfavourable oxygenated environment and grow. In contrast, it appears that in this early stage following intranasal infection, mutant cells are presented with an environment that affects their ability to survive. This impaired growth (four hours lag-phase) was also observed in *in vitro* experiments (Fig. 3.18, §3.1.3.2), suggesting that in aerobic environments, the lack of *sodA* affects the survival of pneumococci. If the FeSOD suggested by Yesilkaya *et al*. (2000) is present, it seems to play only a minor role in virulence in the model used in this thesis, at least during the early hours following infection.

However, the consequences of deletion of *sodA* on pneumococcal colonisation of the nasopharynx might not just be related to the ability of the bacteria to deal with reactive oxygen species but also perhaps, with changes in its physiology and surface phenotype. Evidence from other human pathogens and from pneumococcal studies suggests that apart from *S. pneumoniae* SodA having a role against oxidative stress, the lack of the *sodA* gene probably affects bacterial physiology. This hypothesis comes from the findings by D'Mello *et al*. (1997) using *H. influenzae*, Luke *et al.* (2002) with *Moraxella catarrhalis* and Imlay & Fridovich

(1992) with *E. coli*. Moreover, in *S. pneumoniae*, another enzyme involved in oxidative stress tolerance, pyruvate oxidase (SpxB) (Pericone *et al.*, 2003) has recently been implicated in determining colony morphology (Belanger *et al.,* 2004). D'Mello *et al*. (1997) looked at the role of Mn-cofactored SOD in oxidative stress response, nasopharyngeal colonisation and sustained bacteremia caused by *H. influenzae* Type b (Hib). They found that a *sodA* mutant of Hib was impaired in its ability to establish sustained colonisation of the nasopharynx of infant rats but not in its ability to cause initial colonisation, invasive disease or sustained bacteremia, as was the case in the study here. As suggested by these authors, the reduced colonisation observed could be due to a complex phenotypic change, like loss of adhesins. Alternatively, the decreased capacity for colonisation of the *sodA* mutant could instead be related to the oxidative conditions found in the nasopharynx which would inhibit survival of the bacteria before they can establish residency in the mucosa. A third option might be that it is a reflection of the mutant's sensitivity to the oxidative burst of the host immune responses.

The adhesive capacities of wild type Hib and isogenic *sodA* mutant have been tested by D'Mello *et al*. (1997) using Chang conjunctival epithelial cells but no differences between the two strains were found. On the other hand, Luke *et al.* (2002), who identified the gene responsible for SOD activity in *M. catarrhalis*, have shown modifications in the outer membrane protein (OMP) profile of *M. catarrhalis sodA* mutants. They first isolated the *sodA* gene in a paediatric clinical isolate, strain 7169, and constructed an isogenic *sodA* mutant. A role for SodA in detoxification of endogenous, metabolically produced oxygen radicals was demonstrated by the researchers. In addition, they looked into the effect of the loss of SodA on the OMP profile of *M. catarrhalis* and found that deletion of the *sodA* gene markedly altered the OMP profile of the mutant compared to that of wild type 7169. In particular, the OMP profile of the *sodA* mutant was characteristic of iron-starved *M. catarrhalis* cultures (Luke *et al.*, 2002). Their results also suggested that the iron-stress phenotype observed was not a strain-specific event but instead corresponded to a common phenotypic change in strains lacking SodA. However, they failed to relate this iron-stress OMP profile with environmental oxidative stress, as shown when comparing OMP profiles of 7169 grown under methyl viologen-induced (a redox-cycling agent that generates superoxide inside the cell) oxidative stress with the isogenic *sodA* mutant. In *E.*

coli, amino acid biosynthesis and membrane integrity appear to be affected by the lack of both FeSOD and MnSOD, as was reported by Imlay & Fridovich (1992).
In *S. pneumoniae*, the *spxB* gene that encodes pyruvate oxidase has been found to be related to adhesive capacities of this bacterium on the basis that a *spxB*-deficient mutant showed more than 70% loss of ability to attach to epithelial cells of the nasopharynx and lungs and to endothelial cells (Spellerberg *et al.*, 1996). Pyruvate oxidase catalyses the reduction of O_2 to H_2O_2 (Spellerberg *et al*., 1996) and has been shown to be involved in pneumococcal oxidative stress resistance (Pericone *et al.*, 2003). More recently, Belanger and co-workers (2004) have reported the involvement of SpxB in pneumococcal colony morphology by identifying *spxB* as the mutated gene in strains R36A (used by Avery, McLoed & McCarty to identify DNA in 1944) and R6 (both un-encapsulated) that gives rise to rough colony morphology. However, their findings were serotype-dependent (Belanger *et al*., 2004). Therefore, the adherence capacities of wild type pneumococci and isogenic *sodA* mutant should be tested to more precisely define a role for SodA in colonisation of the URT. Moreover, understanding the pneumococcal adherence capacities is crucial to the development of new vaccines and novel therapeutic agents based on protein components.

Data presented in this thesis show that following a period of "adaptation" of the mutant cells to the oxygenated environment of the nasopharynx they are then able to grow. From 12h post-infection, ΔSodA cells appear to increase in numbers, however there were significantly less ($P<0.001$) mutant bacteria, in all the niches sampled, than in wild type-infected animals. As mentioned above, the suggested presence of FeSOD in pneumococcus (Yesilkaya *et al*., 2000) may have contributed to this ability to grow, enabling the bacteria to be protected against superoxide. Indeed, ΔSodA bacteria collected at late log phase were not completely deprived of SOD activity (Yesilkaya *et al*., 2000) reinforcing the idea of the existence of another superoxide dismutase in the pneumococcus.
The work by Pesci *et al.* (1994) which showed that FeSOD protects the microaerobic human pathogen *Campylobacter jejuni* from oxygen-mediated intracellular killing during invasion of epithelial cells might explain the results obtained in this study. They proposed that, in order for this protection to occur, the FeSOD of *C. jejuni* should be present on its surface. Therefore, considering the

work above, it is possible that a similar mechanism occurs with pneumococcal MnSOD or FeSOD (if it exists). It has been shown by Cundell *et al.* (1995a) that *S. pneumoniae* transits from host cell vacuoles in the cytoplasm to the opposite side of mammalian cells. Hence, the presence of FeSOD might allow the bacteria to move from oxygen-rich compartments into more micro-aerophilic areas and possibly, passing from the respiratory mucosa to the blood. The use of ΔSodA in invasion assays could prove useful to determine the role of the enzyme in pneumococcal overcoming epithelial and endothelial barriers to access underlying tissues.

Another explanation for the observed growth of the *sodA*-deficient mutant cells in the host from 12h post-infection comes from the work by Inaoka *et al.* (1999) who have shown that Mn^{2+} salts can substitute the SOD activity in *sod*-deficient laboratory mutants of *Bacillus subtilis* and that both SodA and Mn^{2+} are involved in oxidative stress resistance of cells; a mechanism that might also be present in *S. pneumoniae*. However, the precise mechanism by which non-enzymic Mn^{2+} scavenges $^{\bullet}O_2^-$ in bacteria is not yet understood, but probably involves significantly higher intracellular Mn^{2+} levels than those needed for effective MnSOD-mediated protection (Jakubovic & Jenkinson, 2001). Mn^{2+} concentrations differ greatly between different sites in the human body, with values varying from 0.2 µg/g in lung tissue to 0.5 ng/ml in the blood (Versieck, 1985), which may lead to varying concentrations of this metal in the cytoplasm of *S. pneumoniae*. Dintilhac *et al.* (1997) have shown that Mn^{2+} is essential for *S. pneumoniae* optimal growth and transformation, and Lawrence *et al.* (1998) demonstrated the presence of the PsaBCA Mn^{2+} transporter system in *S. pneumoniae*. More recently, McAllister and co-workers (2004) confirmed the importance of the Psa permease in resistance to superoxide and hydrogen peroxide, by testing the sensitivity of *psa*-negative mutants to superoxide, generated by paraquat, and to H_2O_2. Orihuela *et al.* (2004) found enhanced expression of the *psa* operon, which is involved in manganese transport, when looking at bacterial gene expression in response to epithelial cell contact. In addition, downstream of the PsaBCA operon is *psaD* (Lawrence *et al.*, 1998), which may be co-transcribed and encodes a thiol peroxidase that might provide pneumococcal cells with additional protection against oxidative stress.

Therefore, these findings might suggest the involvement of Mn^{2+} in pneumococcal oxidative stress resistance.

Kolenbrander *et al.* (1998) have provided evidence for a high-affinity Mn^{2+} transport system in *S. gordonii* that is necessary for the growth of cells in low-Mn^{2+} concentration environments and which involves the ScaA lipoprotein. Their results show that Sca permease is a high-affinity mechanism for the acquisition of Mn^{2+} and is essential for growth of streptococci under Mn^{2+}-limiting conditions. The genes encoding the Mn^{2+} transporter system are found in *S. gordonii* (*scaA*), *Streptococcus parasanguis* (*fimA*) and *S. pneumoniae* (*psaA*). Furthermore, since sequences highly similar to that of the *scaA* gene are found in *Streptococcus sanguis*, *Streptococcus crista* and *Enterococcus faecalis*, it seems likely that the transporter functions in all these organisms. It is possible then that under Mn^{2+}-limiting conditions, up-regulation of *sca* transcription may result not only in increased ScaA production and Sca transporter activity but also in increased production of thiol peroxidase. Since superoxide dismutase activity in streptococci is dependent upon Mn^{2+} (Dintilhac *et al.*, 1997), increased production of thiol peroxidase under Mn^{2+}-limiting conditions might provide streptococcal cells with additional protection against oxidant stress (Kolenbrander *et al.,* 1998).

Lack of *sodA* appears to have a minor effect on prolonged colonisation of the nasopharynx, as judged by data from this study. Instead, SodA seems to have a major role to play in initial pneumococcal colonisation, as discussed above. Nonetheless, a role for SodA in prolonged colonisation of the upper airway was not possible to verify since mice infected intranasally with the mutant strain (ΔSodA) were very sick by 48h post-infection, with only one out of four mice surviving the infection by 72h. This was in part due to the challenge dose used in this model ($1x10^6$ cfu), as it was aimed at examining the transition of pneumococci from the upper to the lower respiratory tract, and not colonisation of the upper airways. In a previous study conducted in our laboratory (Yesilkaya *et al.,* 2000), mice infected with the *sodA* mutant survived longer (median survival time 107h) than in this study. In both cases, survival of ΔSodA-infected mice was significantly longer than the wild type ($P<0.05$).

As stated by Wu *et al*. (1997), to identify virulence factors required for carriage, or antigens that can protect against carriage, it is desirable to have a model in which

nasopharyngeal carriage can be established without significant bacteremia, sepsis or pulmonary infection, and this is clearly not the case in the murine model used here. So, it would be of interest to use the ΔSodA pneumococci in an asymptomatic nasopharyngeal colonisation model in order to assess the contribution of this enzyme to *S. pneumoniae* colonisation of the nasopharynx. Colonisation of the upper airways is of great importance since it is the first step towards invasive disease and is the major reservoir of pneumococci infecting man, where the organisms can be spread through aerosols and by direct contact (Wu *et al*., 1997). Pneumococcal nasopharyngeal colonisation has been studied by infecting mice with 10^{4-5} cfu which results in asymptomatic nasopharyngeal colonisation for one to eight weeks. This is in contrast with the dose of 10^6 cfu used to develop the invasive disease model (as used in this thesis). Another approach that could be followed to study the contribution of SodA in colonisation of the respiratory mucosa by pneumococci is given by the work of Briles *et al.* (2000b). These researchers have used pneumococcal strains capsular types 6B and 23 in a study conducted to investigate nasopharyngeal carriage following intranasal immunisation with pneumococcal proteins PspA or PsaA. Strains of these pneumococcal serotypes are carried on nasal tissues in high numbers following intranasal infection without causing bacteremia.

Investigating the contribution of SodA to nasopharyngeal colonisation could lead eventually to the inclusion of this antigen in a pneumococcal vaccine. However, first the precise location of MnSOD (or FeSOD) in the pneumococcal cell should be investigated. This is because prevention of pneumococcal disease mainly relies on the production of antibodies that neutralise the activity of virulence factors or function as opsonins to enhance clearance of pneumococcal cells. The study by Beaman & Beaman (1990) demonstrates that surface-associated SOD protects *Nocardia asteroides* from oxidative killing *in vivo* during all stages of infection. Using murine monoclonal antibodies specific for nocardial SOD, the authors showed the importance of SOD in protecting cells of *N. asteroides* from the oxidative killing mechanisms within the host. Cells of *N. asteroides* that were incubated with monoclonal antibody against nocardial SOD prior to administration intravenously, showed enhanced clearance from lungs, liver and kidneys of infected mice (Beaman & Beaman, 1990).

Although the host factors responsible for elimination of pathogens are not fully understood, compiled data suggest that a rapid local immune response to a microorganism would prevent colonisation and limit its duration, whereas a poor immune response would result in a more prolonged carriage (Faden *et al*., 1995). Nasopharyngeal colonisation stimulates the production of local antibodies, especially IgA, in response to colonisation by *H. influenzae* and systemic IgG in response to *M. catarrhalis* (Harabuchi *et al*., 1994; Samukawa *et al*., 2000). Nasopharyngeal colonisation by pneumococci stimulates the production of local IgA and systemic IgG responses (Sun *et al*., 2004). A higher proportion of IgA-positive nasopharyngeal samples and higher antibody concentration were observed in children colonised by pneumococci than in children with cultures negative for pneumococci (García-Rodríguez & Martínez, 2002). It would be interesting to consider antibody titres after infection with ΔSodA.

The growth characteristics of ΔSodA in the lower respiratory tract were also analysed in this study. The results presented here suggest a role for SodA in survival and virulence of pneumococci in the lower respiratory tract in the initial hours after lung infection. In the lungs, reflecting the events observed in the nasopharynx and trachea, there was a clear difference between the growth of wild type and ΔSodA pneumococci. SodA-deficient cells where detected in the lungs of mice up to 72h post-infection but always at significantly lower numbers than the wild type parent. In the 4 hours following infection, a critical stage in the growth of ΔSodA cells was observed, as was the case in the upper respiratory tract. The first 12h post-infection seem to be crucial if pneumococci are to survive oxidative stress *in vivo*. These results are in accordance with the work by Yesilkaya *et al*. (2000) who showed a lag in the growth of mutant cells in the first 24h post-infection in the lungs, which did not occur with the wild type. Kadioglu *et al.* (2000) also examined pneumococcal growth kinetics in the lungs of mice in the first 48h post-infection. They showed three phases of growth when examining wild-type or pneumolysin-deficient pneumococci: (i) an early and sharp decline in numbers of pneumococci, (ii) an increase in numbers, and (iii) a stage where pneumococcal numbers remained constant or declined, which is consistent with the wild type results presented herein (except for the last phase which was not seen). However,

the growth kinetics in the lungs when using the SodA-deficient mutant was considerably different than the wild type or PLN-A results, particularly in the first hours of infection. Colony counts in ΔSodA-infected lungs show that these bacteria are unable to maintain a sustained growth and numbers repeatedly decreased sharply until 12h post-infection. The reasons for this pattern of growth are not clear, but it may be due to i) host factors involved in clearance of pathogens or ii) events happening inside the pneumococcal cell due to the lack of SodA or iii) a combination of these two hypotheses.

So what are the host inflammatory responses to pneumococci? The lung presents varied and complex defence mechanisms in order to clear microorganisms. These include the integrity of the epithelium, the mucociliary escalator and secretions containing different antimicrobial proteins. Nonspecific innate immunological defence mechanisms consist of complement, enzymes and neutrophils and macrophages that ingest and kill microorganisms.

The role of complement in host immune defences against pneumococci has been well studied. The classical pathway appears to be the most important to activate the complement system during innate immunity to *S. pneumoniae*. The third component of complement C3 seems to play an important role in bacterial clearance. Deposition of complement proteins on the surface of *S. pneumoniae* enhances opsonisation of bacteria which are then phagocytised by neutrophils and macrophages. Killing by phagocytes is a key part of the host's defence against pneumococci. Firstly, the phagocytes are attracted to the site of infection by a wide range of inflammatory mediators and cytokines such as members of the IL-1 family, IL-8 and TNF-α. Yamamoto *et al.* (2004) showed that IL-12 also plays a critical role in the initial phase of innate host immune response to acute pulmonary infection by *S. pneumoniae* by promoting neutrophil-mediated inflammatory responses.

The role played by SodA in interactions with the host's defences is not clearly understood. SodA might indirectly affect the host response by a mechanism that involves alterations of the pneumococcal cell surface as an indirect consequence of changed oxygen metabolism. Belanger's group has recently shown a widespread effect on phenotype, specifically surface characteristics, involving pyruvate oxidase (Belanger *et al.,* 2004). More recently, Pesakhov *et al.* (2007) have also shown the importance of reactive oxygen species in the membrane

composition of *S. pneumoniae* by analysing the fatty acid and lipid composition under aerobic and anaerobic growth conditions of wild type strains of serotypes 18C, 6A and 2 (D39) and the *spxB*-mutant of D39. On the other hand, the lack of *sodA* could be affecting the susceptibility of pneumococci to the oxidative mechanism of phagocytes during the oxidative burst (Leeson *et al.,* 1985). However, the involvement of neutrophils in clearance of ΔSodA pneumococci in the lungs in the early hours after infection might not be the answer to this question. As shown by Kadioglu *et al.* (2000), neutrophil influx into the lungs of mice infected with pneumococci serotype 2 strain D39 was not seen at 6h post-infection and only started to increase by 12h. Similarly, Rosseau *et al.* (2007) analysed the inflammatory cell influx into the lungs of mice after intranasal pneumococcal infection with a serotype 3 strain and observed the emergence of predominantly neutrophils also by 12h post-infection. These findings suggest that other host mechanisms are involved in the elimination of *sodA*-deficient pneumococci.

The second possibility proposed above was the inability of ΔSodA pneumococci to survive inside the host due to the impairment of these organisms' ability to deal with superoxide produced endogenously during aerobic metabolism. This handicap would predispose the bacteria to i) death or ii) being removed by resident macrophages, the mucociliary escalator or surfactant. In addition, a diminished supply of bacterial cells from the URT to the lungs (by aspiration) might also contribute to the uneven growth of this mutant. As mentioned above, the wild type cells also experience a decline in numbers in the early hours following infection, but a continuous supply of bacterial cells from the URT in significantly higher numbers than mutant cells, and the ability to deal with superoxide, may explain the greater numbers of the wild type in the LRT, in contrast to ΔSodA bacteria.

Despite the impaired growth of SodA mutant bacteria in the early hours following infection, bacterial numbers increased in the lungs of infected mice from 24h onwards and bacteria were still recovered from this site by 72h post-infection. However, by 24h, numbers of mutant bacteria in the lungs were significantly lower than wild type numbers ($P<0.05$). This ability of *sodA*-deficient cells to grow after a lag period is in agreement with what was observed in the URT and in *in vitro*

studies, showing that after becoming accustomed to the oxidative environment of the host, pneumococci defective in *sodA* are able to survive and grow in this milieu.

The present experimental findings suggest that SodA has a role in immune responses to pneumococci, in particular regarding the localisation of immune cells in the lungs. The effect of the lack of *sodA* on the host immune responses to bacteria in the lungs has been investigated in this thesis. Histologic examination of H&E-stained lung sections at 24h and 48h from SodA-deficient pneumococci showed considerably lower levels of pathologic tissue damage compared to the wild type-infected lungs. This is in accordance with the results obtained previously by Yesilkaya *et al*. (2000) using the *sodA* mutant. Moreover, the particular observation made by this group regarding the restricted localisation of immune cells to perivascular and peribronchial sites in ΔSodA-infected lungs has also been noticed in this thesis. To my knowledge, this feature has not been seen with any other pneumococcal mutant strain. This confinement of cells was in contrast to the widespread distribution into the lung tissue observed when using wild type bacteria for infection. In this thesis this observation was further characterised by determining the types of leukocytes in the lungs of mice infected with ΔSodA. In ΔSodA-infected lungs, neutrophil recruitment was notably increased at 24h compared to time zero, whereas lymphocytes and macrophages numbers remained unchanged, as judged by counts done on total lung homogenates. Pneumococcal pneumonia is characterised by a heavy neutrophil influx, and this has been observed when wild type *S. pneumoniae* was used for infection (this study; Kadioglu *et al.,* 2000; Rosseau *et al.*, 2007). From the data presented here it appears that ΔSodA alters the distribution rather that the number of neutrophils in the lungs.

The reason why, following infection with ΔSodA, neutrophils remain localised around bronchioles is not known. Yesilkaya *et al*. (2000) suggested that this feature may be due to failure of ΔSodA to disseminate into the lungs. Consequently, inflammatory cells localisation reflects accumulation around foci of infection. However, the precise location of mutant bacteria within the lungs is not known. The use of ΔSodA-GFP-positive pneumococci could prove a useful tool to more precisely define the location of the mutant. Our group has previously shown

the value of GFP-expressing pneumococci (Kadioglu *et al*., 2001) with regard to pneumococcal adherence and invasion of broncho-epithelial cells *in vivo*.
A second possibility is related to the suggestion that lack of SodA might alter the surface phenotype of pneumococci which in turn could adjust the host's mechanisms of neutrophil influx and localisation. Neutrophils constitute the first line of defence against invading pneumococci, when they respond to chemotactic stimulation. It has been shown that neutrophil chemotaxis is mediated by cytokines, chemokines and adhesion molecules (Kadioglu & Andrew, 2004). In this context, analysis of the host immune signals such as MIP-2 (IL-8) and adhesion molecules such as Mac-1 (CD11b/CD18) after infection with *sodA*-deficient pneumococci would be of interest.

Interestingly, the data presented here suggest a role for SodA regarding T cell recruitment to sites of infection. A feature observed when looking at the inflammatory profile of lungs infected with *sodA* mutants was a smaller increase in T cell numbers in areas of inflamed lungs, in contrast to what was seen with the wild type parent. The work by Kadioglu *et al.* (2000) has shown the involvement of T cells at an early stage in the host immune response to invading pneumococci. Furthermore, Jounblat *et al.* (2003) later found the involvement of pneumolysin in T cell recruitment to inflammatory lesions, apparently due to the complement-activating activity of the toxin and not to its pore forming activity. In this regard, the possible involvement of SodA in T cell recruitment might be related to the suggested alterations at the pneumococcal surface. Analysis of SodA induction of host mediators of inflammation is necessary to obtain a better understanding of how this enzyme acts as a trigger of inflammation during pneumococcal pneumonia.

The pattern of immune cells infiltration described here, with substantial increased accumulation of phagocytic cells around bronchioles, may render this pneumonic region under-ventilated, causing hypoxemia, and allowing *sodA*-deficient cells to cross the blood-air barrier and survive in the bloodstream. The presence of mutant cells in blood was, therefore, only detected by 24h post-infection (Fig. 3.22), at which time bacteria were isolated from 70% of infected mice (20 mice in total) but in significantly ($P<0.05$) lower numbers than wild type cells. By 48h post-infection,

the mutant strain was still recovered from 60% of infected mice (21 mice analysed in total; Fig. 3.22) and the number of cells had increased. An interesting observation was made when ΔSodA bacterial counts were performed in blood. In eight independent intranasal infections, by 24h post-infection, 6 mice were not bacteremic (as mentioned above). This lack of bacteria in blood appeared to be related to a decreased number of cells in the lungs of these mice (and also in the trachea and the nasopharynx, see Appendix B) as compared to bacteremic mice. Moreover, this result was again observed by 48h post-infection, where 9 non-bacteremic mice had significantly ($P<0.05$) lower numbers of cells in the lungs, trachea and nasopharynx when compared to bacteremic mice. These results raised the possibility of the existence of genetic instability of the pneumococcus ΔSodA mutant. Touati had reported in 1989, that a consequence of the lack of both the constitutive FeSOD and the inducible MnSOD in *E. coli* was an increase in the rate of spontaneous mutagenesis (Touati, 1989). This was due to an increased flux of superoxide in SOD-deficient *E. coli* that lead to increased levels of free iron resulting from superoxide-mediated release of iron from non-sulphur proteins. When free iron is available in the cytosol, it participates in the Fenton reaction (see §1.5.3) causing oxidative damage to DNA (Keyer *et al.,* 1996). DNA mutations in pneumococcus have also been shown by Pericone *et al.* (2002) to be associated with physiologic levels of H_2O_2.

Given this possibility, a series of additional experiments were done in order to better understand the results obtained in this study using ΔSodA. However, as described in detail in the results chapter, no support for this hypothesis was found. Firstly, emergence of revertants was discarded because the number of colonies on selective (200μg/ml spectinomycin) or non-selective medium, from lung and blood samples, at each time was the same. In addition, the mutation in ΔSodA cells was confirmed using PCR reactions and the results (Fig. 3.17) showed that the spectinomycin gene remained in these cells during murine peritoneal culture. Moreover, no differences were seen regarding colony appearance, size and optochin resistance (results not shown).

Further experiments were done to check the possibility that the emergence of two different phenotypes of ΔSodA was due to i) the route of infection used in this study (intranasal) or ii) the strain of mice (MF1 outbred). However, when ΔSodA was given intravenously or i.n. to inbred C57BL/6 mice, the same variability in bacteremia was observed, suggesting that this uneven of infection by ΔSodA is not related to the host environment but with deletion of the *sodA* gene in pneumococci.
Finally, after intranasal infection, bacteria recovered from tissues of bacteremic and non-bacteremic mice were used to intravenously infect a second set of mice. The results obtained suggested that the observed variability obtained after intranasal infection with *S. pneumoniae sodA* mutant cells was i) not dependent on the route of infection, ii) not dependent on the dose, iii) not dependent on the site where bacteria are collected and iv) not dependent on emergence of bacteremia.

A possibility is that in this model, after infection with ΔSodA, unknown or stochastic events lead to two possibilities i) the bacterial load remains low and the primary lung defences result in effective clearance, ii) bacterial load is higher, local defences fail to cope and bacterial numbers increase and invade the bloodstream. In order to try to explain these outcomes, the approach has to focus in both the bacteria response and the host response. Regarding the former, the methodology used by Oggioni *et al.* (2006) to study the patterns of gene expression during *S. pneumoniae* infection could be applied here to allocate RNA to virulent or avirulent ΔSodA pneumococci. This group has already shown an increase in *sodA* gene expression in lungs of mice infected with *S. pneumoniae* in comparison to liquid broth cultured cells (Oggioni *et al.*, 2006). With regard to the host response, cytokine gene expression analyses could be done in different tissues (nasopharynx, trachea, lungs and blood) to determine whether diverse immune responses are involved in the appearance of the outcomes observed here.

Taken together, the results presented in this study show that *sodA* mutants are highly susceptible to oxygen in the environment and that the altered oxygen metabolism alters the *in vivo* behaviour, particularly during the first 12h following infection. Identifying the critical genes and proteins involved in oxidative stress

tolerance in this organism and how SodA changes the pneumococcal transcriptome and proteome may provide new targets for anti-pneumococcal therapies.

However, the suggestion that *S. pneumoniae* might have a second SOD (SodB) or SOD-like activity (Yesilkaya *et al*., 2000) has to be taken into consideration regarding the role of SOD in pneumococci. Another aspect to take into account is that the question whether SodA is protecting pneumococci from internally or externally derived superoxide is not yet understood. Yesilkaya *et al*. (2000) suggested that the enzyme protects against externally derived oxidative stress on the basis of similar peroxide production from the mutant and the wild type parent. Nonetheless, *in vitro* studies reported by these authors showed that SOD is increased upon aeration of wild type cells but not of mutant cells (Yesilkaya *et al*., 2000). This increase in MnSOD production could imply that SOD is protecting pneumococci from reactive oxygen intermediates produced inside the cell during aerobic metabolism. Moreover, the same study also showed that ΔSodA had a lower rate of aerobic increase in OD than anaerobic increase ($P<0.001$), while the rate of increase in OD of the wild type was unaltered in both conditions. In addition, when 0.001M paraquat (a redox-cycling agent which penetrates cells, including encapsulated bacteria, and that under aerobic conditions, generates a flux of superoxide inside the cell) was added, it affected the growth of ΔSodA cells but not of wild type, suggesting that the latter were protected by the cytosolic MnSOD. The finding by Gerlach *et al.* (1998) that the cytoplasmic MnSOD of *Streptococcus pyogenes* is also secreted to the cell surface, where it may interact with exogenous superoxide, suggests the existence of a similar mechanism in *S. pneumoniae*.

Gibson & Caparon (1996) have shown that inactivation of the *sodA* gene in *S. pyogenes* sensitises these cells to superoxide and aerobic conditions as observed using pneumococci (Yesilkaya *et al*., 2000). It would be of interest to find the localisation of SOD in pneumococcus perhaps by performing a quantitative SOD assay using the xanthine oxidase-cytochrome *c* method on fractionated cells or a western blot assay.

The use of tools such as the complete genome sequences and powerful bioinformatics can also help identifying proteins that contain certain sequence

motifs for secreted or surface proteins from the genome of *S. pneumoniae*. One of this tools, the SignalP 3.0 server, predicts the presence and location of a signal peptide cleavage site in an amino-acid sequence of an organism (available at http://www.cbs.dtu.dk/services/SignalP/). However, the results obtained for SodA using this tool, show that no signal peptide is present. Nonetheless, in addition to the predicted surface proteins, non-classical surface proteins that lack membrane anchoring motifs and signal peptides have been shown to be on the pneumococcal surface (Bergmann and Hammerschmidt, 2006). Yet to date, no mechanism of secretion or anchoring has been described for these proteins. These proteins include PavA (pneumococcal adherence and virulence factor A), glyceraldehyde-3-phosphate dehydrogenase (GADPH) and enolase (Bergmann and Hammerschmidt, 2006).

An additional consideration is related to the issue of whether the loss of virulence of the *sodA*-deficient pneumococcus in this study could have been observed if mutation in *sodA* had polar effects on the expression of some downstream gene. Although no attempt was made to complement the ΔSodA mutant strain, from the analysis of the published nucleotide sequence of the *sodA* gene (GenBank Accession SP0766) using a set of tools available online, it was predicted that it is very unlikely that the *sodA*-negative phenotype, resulting from the deletion of the *sodA* gene, is the consequence of a polar effect. Using the OperonDB tool the results show that this gene is monocistronic (OperonDB tool available at http://operondb.cbcb.umd.edu/cgi-bin/operondb/operons.cgi.; Pertea *et al.,* 2009). In addition, it was found that there is approximately 160bp gap between *sodA* and the downstream gene (hypothetical protein) and this stretch of DNA has two strong transcriptional terminators [GenomeNet Kegg tool available at http://www.genome.jp/en/gn_kegg.html; TransTermHP tool available at http://transterm.cbcb.umd.edu/query.php; Kingsford *et al.* (2007)]. Previously, Yesilkaya (1999) had analysed the sequence of the pneumococcal *sod* gene which consists of a 606bp ORF. He reported the existence of a TAA stop codon at the end of the ORF (Yesilkaya, 1999), in agreement with what was found here. The *sodA* gene of *S. agalactiae* is also transcribed monocistronically (Poyart *et al.,* 2001), as seen for *S. pneumoniae* (this study and Yesilkaya, 1999). Complementation of the *sodA* mutation in *S. agalactiae sodA*-deficient mutant strain fully restored the wild type characteristics of the bacteria (Poyart *et al.,*

2001). Purdy *et al.* (1999) have also used complementation of the single SOD in *C. coli sodB*, and found no differences between the wild type and complemented stains.

Finally, studies with a much wider set of isolates would be useful. Almost all studies of pneumococci in models of disease (*in vivo*, *ex vivo* or *in vitro*) have been done with a very limited number of strains, and often only one strain. This may give a distorted picture because pneumococcal isolates have differing potentials to cause disease and mechanisms for evoking pathogenicity differ between strains. Different serotypes have differing propensities to be among invasive and carriage isolates and the different serotypes are isolated at differing frequencies from different age groups. Moreover, compiled data show that the role of an individual factor varies between strains emphasising the need to study more than one pneumococcal strain. Therefore, in order to get a more accurate picture in the outcome of disease following infection with ΔSodA, the use of different strains of mice is necessary, together with different challenge techniques and a set of bacterial strains.

In conclusion, in this study a role for pneumococcal SodA in virulence and colonisation of host tissues in the early hours after infection was identified. Moreover, the involvement of this enzyme in the inflammatory response to pneumococci has also been shown. Understanding the complex relationships between the participants in the acute inflammation process to pneumococcal infection is of great importance given that manipulation of host responses can improve the outcome of disease.

FINAL REMARKS

5. Final remarks

In this thesis it was shown that both pneumococcal neuraminidases NanA and NanB, the major autolysin LytA and the superoxide dismutase SodA play essential roles in the virulence of this bacterium. The results demonstrate that the use of isogenic mutants in *in vivo* systems provides insights into the capacities of these enzymes to be involved in the infection process. In addition, these experimental findings add significantly to the body of data accumulated on the effect of neuraminidases, autolysin and SOD to the capacity of the pathogen to cause disease. Moreover, these data also contribute to the better understanding of the host-bacteria interactions. It is clear that the determinants of virulence of *S. pneumoniae* are complex and that no single attribute can be defined as the mechanism of virulence. Despite the considerable amount of work gathered on pneumococcal infection we are still a long way in determining how this usable knowledge can be exploited clinically.

Streptococcus pneumoniae is a bacterial pathogen to which children between 6 months and 4 years of age and adults over 60 years are most commonly at risk of disease (ACIP, 1997). Recently, a study conducted in the USA for the "Active Bacterial Core Surveillance of the Emerging Infections Program Network" by Kyaw and colleagues (2006) has shown the efficacy of the heptavalent protein-polysaccharide conjugate vaccine Prevnar - Wyeth, USA (serotypes 4, 6B, 9V, 14, 18C, 19F and 23F) licensed in the USA in 2000. Five of the seven serotypes in this vaccine are responsible for most penicillin-resistant infections. The results presented by the group show that rates of resistant disease caused by vaccine serotypes fell 87%. Moreover, rates of antibiotic-resistant invasive pneumococcal infections decreased in young children by 81% and in older persons by 49%, after the introduction of the conjugate vaccine. However, the report shows an increase in infections caused by serotypes not included in the vaccine, mainly by serotype 19A. In the same year (2006), a talk given at the University of Leicester by Prof. Adegbola (Head of Bacteriology at the MRC Laboratories in The Gambia) reported that it was estimated that 60% to 90% of lower respiratory tract infections in Gambian children younger than 5 years were caused by pneumococci and, it is known that in children living in the developing world, the incidence of invasive

pneumococcal disease is several times higher than the incidence in industrialised countries like the USA (Lancet, 1985). Given the suggested four-dose schedule for the Prevenar vaccine and the cost of a single dose of Prevenar in Portugal of around 70 Euros (personal experience), it seems that the vaccine is too expensive and would be difficult to implement on a large scale in developing countries (Swiatlo & Ware, 2003; Bogaert *et al.,* 2004a), where it is the most needed.

The work presented in this thesis was developed with the aim of contributing to a better understanding of the pathogenesis of pneumococcal pneumonia as a mean to move forward new strategies to combat this disease. Therefore it is important that extension of the results presented here move forward. The use of new models together with advanced techniques in genomics and proteomics are of obvious interest to study the contribution of individual pneumococcal virulence factors and the response of the host to these virulence factors.

APPENDIX

Appendix A

►Construction of *S. pneumoniae* neuraminidase B-mutant ΔNanB (§ 2.3)

The ΔNanB mutant, kindly provided by Prof. James Paton, was constructed in serotype 2 strain D39. First, a single crossover mutation was made at the *nanB* gene (Berry *et al*., 1996). The plasmid pVA891 containing *nanB* sequence was incorporated into the chromosomal *nanB* gene by a single crossover mutation, thereby disrupting the *nanB* gene and rendering the strain erythromycin resistant. Secondly, to create a deletion, a second plasmid containing only 5' and 3' ends of the *nanB* gene was constructed and transformed into the erythromycin resistant *nanB* mutant. A double crossover mutation occurred at both ends of the *nanB* gene to create a deletion. To construct the second plasmid, the vector pJCP310 was used (Berry *et al*., 1996). ΔNanB contains only the 5' end, from nucleotide 0 to 253 and the 3' end, from nucleotide 2048 to 2093 from the complete *nanB* gene.

►Gene sequences and location of primers

All the gene sequences below have been taken from NCBI Sequence Viewer (v2.0) webpage.
Highlighted in yellow are the codons of the forward and reverse primers used to confirm the mutation of each mutant strain using the PCR method.

Nucleotide sequence of *nan*A gene (§ 2.5)

Gene ---------- nanA
Product ---------- neuraminidase; surface protein
Gene ---------- bases 1 to 4500
Accession ---------- X72967

1 gatcaggaca gtcaaatcga tttctaacaa tgttttagaa gtagatgtgt actattctag
61 tttcaatcta ttatatttat agaatttttt gttgctagat ttgtcaaatt gcttaaaata
121 atttttttca gaaagcaaaa gccgatacct atcgagtagg gtagttcttg ctatcgtcag
181 gcttgtctgt aggtgttaac acttttcaaa aatctcttca aacaacgtca gctttgcctt
241 gccgtatata tgttactgac ttcgtcagtt ctatctgcca cctcaaaacg gtgttttgag
301 ctgacttcgt cagttctatc cacaacctca aaacagtgtt ttgagctgac ttcgtcagtt
361 ctatccacaa cctcaaaaca gtgttttgag ctgactttgt cagtcttatc tacaacctca
421 aaacagtgtt ttgagcatca tgcggctagc ttcttagttt gctctttgat tttcattgag
481 tataaaaaca gatgagtttc tgttttcttt ttatggacta taaatgttca gctgaaacta
541 ctttcaagga cattattata taaaagaatt ttttgaaact aaaatctact atattacact
601 atattgaaag cgttttaaaa atgaggtata ataaatttac taacacttat aaaaagtgat
661 agaatctatc tttatgtata tttaaagata gattgctgta aaaatagtag tagctatgcg
721 aaataacaga tagagagaag ggattgaagc ttagaaaagg ggaataatat gatatttaag
781 gcattcaaga caaaaaagca gagaaaaaga caagttgaac tacttttgac agttttttc
841 gacagttttc tgattgattt atttcttcac ttatttggga ttgtcccctt taagctggat
901 aagattctga ttgtgagctt gattatattt cccattattt ctacaagtat ttatgcttat
961 gaaaagctat ttgaaaaagt gttcgataag gattgagcag gaagtatggt gtaaatagca
1021 taagctgatg tccatcattt gcttataaag agatatttta gtttaattgc agcggtgtcc
1081 tggtagataa actagattgg caggagtctg attggagaaa ggagagggga aatttggcac
1141 caatttgaga tagtttgttt agttcatttt tgtcatttaa atgaactgta gtaaaagaaa
1201 gttaataaaa gacaaactaa gtgcattttc tggaataaat gtcttatttc agaaatcggg
1261 atatagatat agagaggaac agtatgaatc ggagtgttca agaacgtaag tgtcgttata
1321 gcattaggaa actatcggta ggagcggttt ctatgattgt aggagcagtg gtatttggaa
1381 cgtctcctgt tttagctcaa gaaggggcaa gtgagcaacc tctggcaaat gaaactcaac
1441 tttcggggga gagctcaacc ctaactgata cagaaaagag ccagccttct tcagagactg
1501 aactttctgg caataagcaa gaacaagaaa ggaaagataa gcaagaagaa aaaattccaa
1561 gagattacta tgcacgagat ttggaaaatg tcgaaacagt gatagaaaaa gaagatgttg
1621 aaaccaatgc ttcaaatggt cagagagttg atttatcaag tgaactagat aaactaaaga
1681 aacttgaaaa cgcaacagtt cacatggagt ttaagccaga tgccaaggcc ccagcattct
1741 ataatctctt ttctgtgtca agtgctacta aaaaagatga gtacttcact atggcagttt
1801 acaataatac tgctactcta gaggggcgtg gttcggatgg gaaacagttt tacaataatt
1861 acaacgatgc acccttaaaa gttaaaccag gtcagtggaa ttctgtgact ttcacagttg
1921 aaaaaccgac agcagaacta cctaaaggcc gagtgcgcct ctacgtaaac ggggtattat
1981 ctcgaacaag tctgagatct ggcaatttca ttaaagatat gccagatgta acgcatgtgc

2041 aaatcggagc aaccaagcgt gccaacaata cggtttgggg gtcaaatcta cagattcgga
2101 atctcactgt gtataatcgt gctttaacac cagaagaggt acaaaaacgt agtcaacttt
2161 ttaaacgctc agatttagaa aaaaaactac ctgaaggagc ggctttaaca gagaaaacgg
2221 acatattcga aagcgggcgt aacggtaaac caaataaaga tggaatcaag agttatcgta
2281 ttccagcact tctcaagaca gataaaggaa ctttgatcgc aggtgcagat gaacgccgtc
2341 tccattcgag tgactggggt gatatcggta tggtcatcag acgtagtgaa gataatggta
2401 aaacttgggg tgaccgagta accattacca acttacgtga caatccaaaa gcttctgacc
2461 catcgatcgg ttcaccagtg aatatcgata tggtgttggt tcaagatcct gaaaccaaac
2521 gaatcttttc tatctatgac atgttcccag aagggaaggg aatctttgga atgtcttcac
2581 aaaaagaaga agcctacaaa aaaatcgatg gaaaaaccta tcaaatcctc tatcgtgaag
2641 gagaaaaggg agcttatacc attcgagaaa atggtactgt ctatacacca gatggtaagg
2701 cgacagacta tcgcgttgtt gtagatcctg ttaaaccagc ctatagcgac aaggggatc
2761 tatacaaggg taaccaatta ctaggcaata tctacttcac aacaaacaaa acttctccat
2821 ttagaattgc caaggatagc tatctatgga tgtcctacag tgatgacgac gggaagacat
2881 ggtcagcgcc tcaagatatt actccgatgg tcaaagccga ttggatgaaa ttcttgggtg
2941 taggtcctgg aacaggaatt gtacttcgga atgggcctca caagggacgg attttgatac
3001 cggtttatac gactaataat gtatctcact taaatggctc gcaatcttct cgtatcatct
3061 attcagatga tcatggaaaa acttggcatg ctggagaagc ggtcaacgat aaccgtcagg
3121 tagacggtca aaagatccac tcttctacga tgaacaatag acgtgcgcaa aatacagaat
3181 caacggtggt acaactaaac aatggagatg ttaaactctt tatgcgtggt ttgactggag
3241 atcttcaggt tgctacaagt aaagacggag gagtgacttg ggagaaggat atcaaacgtt
3301 atccacaggt taaagatgtc tatgttcaaa tgtctgctat ccatacgatg cacgaaggaa
3361 aagaatacat catcctcagt aatgcaggtg gaccgaaacg tgaaaatggg atggtccact
3421 tggcacgtgt cgaagaaaat ggtgagttga cttggctcaa acacaatcca attcaaaaag
3481 gagagtttgc ctataattcg ctccaagaat taggaaatgg ggagtatggc atcttgtatg
3541 aacatactga aaaaggacaa aatgcctata ccctatcatt tagaaaattt aattgggact
3601 ttttgagcaa agatctgatt tctcctaccg aagcgaaagt gaagcgaact agagagatgg
3661 gcaaaggagt tattggcttg gagttcgact cagaagtatt ggtcaacaag gctccaaccc
3721 ttcaattggc aaatggtaaa acagcacgct tcatgaccca gtatgataca aaaaccctcc
3781 tatttacagt ggattcagag gatatgggtc aaaaagttac aggtttggca gaaggtgcaa
3841 ttgaaagtat gcataattta ccagtctctg tggcgggcac taagctttcg aatggaatga
3901 acggaagtga agctgctgtt catgaagtgc cagaatacac aggcccatta gggacatccg
3961 gcgaagagcc agctccaaca gtcgagaagc cagaatacac aggcccacta gggacatccg
4021 gcgaagagcc agccccgaca gtcgagaagc cagaatacac aggcccacta gggacagctg

4081 gtgaagaagc agctccaaca gtcgagaagc cagaatttac agggggagtt aatggtacag
4141 agccagctgt tcatgaaatc gcagagtata agggatctga ttcgcttgta actcttacta
4201 caaaagaaga ttatacttac aaagctcctc ttgctcagca ggcacttcct gaaacaggaa
4261 acaaggagag tgacctccta gcttcactag gactaacagc tttcttcctt ggtctgttta
4321 cgctagggaa aaagagagaa caataagaga agaattctaa acatttgatt ttgtaaaaat
4381 agaaggagat agcaggtttt caagcctgct atctttttt gatgacattc aggctgatac
4441 gaaatcataa gaggtctgaa actactttca gagtagtctg ttctataaaa tatagtagat

<u>Nucleotide sequence of *nan*B gene (§ 2.5)</u>

Gene ---------- nanB
Product ---------- neuraminidase; surface protein
Gene ---------- bases 1 to 2093
Accession ---------- U43526

1 aatgaataaa agaggtcttt attcaaaact aggaatttct gttgtaggca ttagtctttt
61 aatgggagtc cccactttga ttcatgcgaa tgaattaaac tatggtcaac tgtccatatc
121 tcctattttt caaggaggtt catatcaact gaacaataag agtatagata tcagctcttt
181 gttattagat aaattgtctg gagagagtca gacagtagta atgaaattta aagcagataa
241 accaaactct cttcaagctt tgtttggcct atctaatagt aaagcaggct ttaaaaataa
301 ttacttttca attttcatga gagattctgg tgagataggt gtagaaataa gagacgccca
361 agagggaata aattatttat tttctagacc agcttcatta tggggaaagc ataaaggaca
421 ggcagttgaa aatacactag tatttgtatc tgattctaaa gataaaacat acacaatgta
481 tgttaatgga atagaagtgt tctctgaaac agttgataca tttttgccaa tttcaaatat
541 aaatggtata gataaggcaa cactaggagc tgttaatcgt gaaggtaagg aacattacct
601 cgcaaaagga agtattggtg aaatcagtct atttaacaaa gcaattagtg atcaggaagt
661 ttcaaatatt cccttgtcaa atccatttca gttaattttc caatcaggag attctactca
721 agctaactat tttagaatac cgacactata tacattaagt agtggaagag ttctatcaag
781 tattgatgca cgttatggtg ggactcatga ttctaaaagt aagattaata ttgccacttc
841 ttatagtgat gataatggga aaacgtggag tgagccaatt tttgctatga agtttaatga
901 ctatgaggag cagttagttt actggccacg agataataaa ttaaagaata gtcaaattag
961 tggaagtgct tcattcatag attcatccat tgttgaagat aaaaaatctg ggaaaacgat
1021 attactagct gatgttatgc ctgcgggtat tggaaataat aatgcaaata aagccgactc

1081 aggttttaaa gaaataaatg gtcattatta tttaaaacta aagaagaatg gagataacga
1141 tttccgttat acagttagag aaaatggtgt cgtttatgat gaaacaacta ataaacctac
1201 aaattatact ataaatgata agtatgaagt tttggaggga ggaaagtctt taacagtcga
1261 acaatattcg gttgattttg atagtggctc tttaagagaa aggcataatg gaaaacaggt
1321 tcctatgaat gttttctaca aagattcgtt atttaaagtg actcctacta attatatagc
1381 aatgacaact agtcagaata gaggagagag ttgggaacaa tttaagttgt tgcctccgtt
1441 cttaggagaa aaacataatg gaacttactt gtgtcctgga caaggtttag cattaaaatc
1501 aagtaacaga ttgatttttg caacatatac tagtggagaa ctaacctatc tcatttcgga
1561 tgatagtggt caaacatgga agaaatcctc agcttcaatt ccgtttaaaa atgcaacagc
1621 agaagcacaa atggttgaac tgagagatgg tgtgattaga acattcttta gaaccactac
1681 aggtaagata gcttatatga ctagtagaga ttctggagaa acatggtcga aagtttcgta
1741 tattgatgga attcaacaaa cttcatatgg cacacaagta tctgcaatta aatactctca
1801 attaattgat ggaaaagaag cagtcatttt gagtacacca aattctagaa gtggccgtaa
1861 gggaggccaa ttagttgtcg gtttggtcaa taaagaagat gatagtattg attggagata
1921 ccactatgat attgatttgc cttcgtatgg ttatgcctat tctgcgatta cagaattgcc
1981 aaatcatcac ataggtgtac tgtttgaaaa atatgattcg tggtcgagaa atgaattgca
2041 tttaagcaat gtagttcagt atatagattt ggaaattaat gatttaacaa aat

Nucleotide sequence of *sod*A gene (§ 2.5)

Gene ---------- sodA
Product ---------- superoxide dismutase, manganese-dependent
Gene ---------- bases 1 to 606
Accession ---------- SP0766

1 atggctatta tcttaccaga acttccatat gcatacgacg ctttggaacc atacatcgat
61 gcggaaacaa tgcatttgca ccatgacaaa caccatcaaa cttatgtcaa caatgccaat
121 gcagctttag aaaaacaccc tgaaatcggt gaagaccttg aagccttgct tgctgatgta
181 gaatctatcc cagctgatat ccgtcaagca cttatcaaca atggtggcgg acacttgaac
241 cacgctcttt tctgggaatt gatgactccc gagaaaacag ctccttcagc agaactggca
301 gcagcaatcg atgcaacatt tggttcattt gaagaattcc aagcagcctt cactgcagca
361 gcaacaactc gttttggttc aggttgggca tggttggttg tcaacaaaga agggaaactt
421 gaagtgactt caacagcaaa ccaagacaca ccaatctcag aaggtaaaaa accaatcttg

481 ggcttggacg tttgggaaca tgcttactac gtgaaatacc gcaacgtgcg tcctgactac
541 atcaaagctt tcttttcagt aatcaactgg aataaagtag atgaattgta cgcagctgctaaataa

Nucleotide sequence of *lyt*A gene (§ 2.5)

Gene ---------- lytA
Product ---------- autolysin
Gene ---------- 1….2376
Accession ---------- M13812

Origin 1 bp upstream of HindIII site.
1 gatccttcct ctagtttcta gctttattga actaatcgga aaaatcgttt ttgtggtttt
61 gattattcct tgggcaggat ataagggtgt tatcctttgt gaacctctta tctgggttgc
121 catgacagtt caactgtact tctcattatt ccgtcatccc ttgataaaag aaggcaaggc
181 aatcttggca accaaagtgc aatcctagtt ggatttactg aataaaatcc atttcctcta
241 gtgaaaatcg aaaaaacttg tgttctcgtc tttagtttgg tgttgaaaat agtttaacag
301 acttttgact tcttttatat gatataataa agtatagtat ttatgaaaag gacatataga
361 gactgtaaaa atatactttt gaaaagcttt ttagtctggg gtgttattgt agatagaatg
421 cagaccttgt cagtcctatt tacagtgtca aaatagtgcg ttttgaagtt ctatctacaa
481 gcctaatcgt gactaagatt gtcttctttg taaggtagaa ataaaggagt ttctggttct
541 ggattgtaaa aaatgagttg ttttaattga taaggagtag aatatggaaa ttaatgtgag
601 taaattaaga acagatttgc ctcaagtcgg cgtgcaacca tataggcaag tacacgcaca
661 ctcaactggg aatccgcatt caaccgtaca gaatgaagcg gattatcact ggcggaaaga
721 cccagaatta ggtttttct cgcacattgt tgggaacggt tgcatcatgc aggtaggacc
781 tgttgataat ggtgcctggg acgttggggg cggttggaat gctgagacct atgcagcggt
841 tgaactgatt gaaagccatt caaccaaaga agagttcatg acggactacc gcctttatat
901 cgaactctta cgcaatctag cagatgaagc aggtttgccg aaaacgcttg atacagggag
961 tttagctgga attaaaacgc acgagtattg cacgaataac caaccaaaca accactcaga
1021 ccacgttgac ccttatccat atcttgctaa atggggcatt agccgtgagc agtttaagca
1081 tgatattgag aacggcttga cgattgaaac aggctggcag aagaatgaca ctggctactg
1141 gtacgtacat tcagacggct cttatccaaa agacaagttt gagaaaatca atggcacttg
1201 gtactacttt gacagttcag gctatatgct tgcagaccgc tggaggaagc acacagacgg
1261 caactggtac tggttcgaca actcaggcga aatggctaca ggctggaaga aaatcgctga
1321 taagtggtac tatttcaacg aagaaggtgc catgaagaca ggctgggtca agtacaagga

1381 cacttggtac tacttagacg ctaaagaagg cgccatggta tcaaatgcct ttatccagtc

1441 agcggacgga acaggctggt actacctcaa accagacgga acactggcag acaggccaga

1501 attcacagta gagccagatg gcttgattac agtaaaataa taatggaatg tctttcaaat

1561 cagaacagcg catattatta ggtcttgaaa aagcttaata gtatgcgttt tcttgtggag

1621 atatttcctt caatgtttgc tactatatta aacaaaaatc agaaagcaaa ctagaaagtt

1681 atgctcaaat aaaatctaaa tttgacaatg taaaccgagt cggatagctt taagtactgt

1741 tttgaggttg aagatacgat ttttgatagg aactcatcaa ttttagattt ttaagcagca

1801 tcaataaatt gcttccttgt tttgtcataa ttttttatt taaaaaatta tgacaaaagt

1861 gtgctattct ttttatgaga ggtgtatgaa tatgataaat gtatgtgata aatgtatgtg

1921 atgttggaaa aagaataaaa gaacttagaa tatcttcaaa tcttactcaa gataagattg

1981 ctgaatattt gtctttatat ttgtctttga atcaaagcat gattgccaaa atggaaaaag

2041 gtgaaaggaa tatcacggat ggatttaagt aataaagctt caaatcttag aaaaaagttg

2101 ggagctgatg gtgaagcgcc gatagatatt tttaaattgg tacaaaagat agaaaatttg

2161 acgctggtat tttatggact cggaaagata ttagcggagt ctgttataaa ggaactcagt

2221 tcagtctcat tgcagtcaat tcagacatgt cattaggaag gtaaagattt tctttagcac

2281 atgaactgta tcatctttat tatgatgagg tgaagaagag ttcagtcagt cttatcttga

2341 ttggtgaagg agatgaaact gaaagaaaag cggatc

►Composition of solutions used for small-scale extraction of plasmid DNA from *E. coli* (§2.2.1.1)

Solution	Composition	Storage
I (Lysozyme solution)	50mM Glucose; 25mM Tris-HCl, pH 8.0; 10mM EDTA, pH 8.0; 40mg Lysozyme	$4^{o}C$
II (Alkaline solution)	0.2N NaOH; 1% SDS	Room temperature
III	5M Potassium acetate; Glacial acetic acid, nH_2O	Room temperature

►Composition of buffers for large-scale extraction of plasmid DNA from *E. coli* (§2.2.1.2)

Buffer	Composition	Storage
P1 (Resuspension buffer)	50mM Tris-HCl, pH8.0; 10mM EDTA; 100μg/ml RNaseA	4°C
P2 (Lysis buffer)	200mM NaOH; 1%SDS	Room temperature
P3 (Neutralisation buffer)	3M potassium acetate, pH5.5	Room temperature
QBT (Equilibration buffer)	750mM NaCl; 50mM MOPS, pH7.0; 15% ethanol; 0.15%Triton X-100	Room temperature
QC (Wash buffer)	1M NaCl; 50mM MOPS, pH7.0; 15% ethanol	Room temperature
QF (Elution buffer)	1.25M NaCl; 50mM Tris-HCl, pH8.5; 15% ethanol	Room temperature
TE	10mM Tris-HCl pH8.0, 1mM EDTA	Room temperature

►Composition of 10x Accubuffer (Bioline) used in PCR reaction (§2.2.2.2)

- 600mM Tris-Cl
- 60mM $(NH_4)_2SO_4$
- 100mM KCl
- 20mM $MgSO_4$

►Preparation of 2mM dNTP mixture (Bioline) used in PCR reaction (§2.2.2.2)

A dNTP set from Bioline containing four separate tubes, one for each deoxynucleotide (A, T G, C), with a concentration of 100 mM was used to prepare stocks of 2 mM dNTP mixture. For this, each deoxynucleotide was diluted 1:50. 10μl of each dNTP was pipetted to a fresh aliquot containing 460μl nH_2O and mixed by inversion. Aliquots containing 50μl of the mixture were prepared and frozen at -20^oC.

►Preparation of 50X TAE used for agarose gel electrophoresis (§2.2.2.3)

- 242 g Tris Base
- 57.1 ml Glacial Acetic Acid
- 100 ml 500 mM EDTA, pH 8.0
- 600 ml dH_2O

Mix then bring volume to 1 L and autoclave.

►Preparation of 6X Loading Dye (§2.2.2.3)

- 3 ml 100% Glycerol
- 3 ml 0.5 M EDTA, pH 8.0
- 3 mg Bromophenol Blue
- 3 mg Xylene Cyanol
- 4 ml Sterile Water
- 10 ml Total Volume

►Composition of Dilution Buffer used in Neuraminidase Assay (§2.3.2)

The dilution buffer, used to dilute the substrate pNP-NANA, was prepared as follow:

- 38.4 ml 100mM Citric-acid phosphate (prepared by mixing 18.5 ml 0.1M Citric acid [in dH_2O] and 81.5 ml 0.2M Sodium orthophosphate dehydrate [in dH_2O]; the pH was adjusted to 6.6)

- 3.12 ml 25 mg/ml BSA
- 35.5 ml dH_2O
- 160µl 10% (w/v) Sodium azide

►Haematoxylin and Eosin Solutions (§2.3.4.3)

Gills' Haematoxylin Solution:

- 6.0 g Haematoxylin
- 4.2 g Aluminium Sulphate
- 1.4 g Citric Acid
- 0.6 g Sodium Iodate
- 269 ml Glycol
- 680 ml Distilled Water
- 269 ml Ethylene Glycol

Eosin solution:

- 1.0 g Eosin Yellowish
- 100 ml Distilled Water

Place into a dark glass Duran bottle.

Appendix B

►Growth of wild type and ΔSodA mutant strain in the nasopharynx, trachea, lungs and blood (ΔSodA results were divided between bacteremic [ΔSodA$^+$] *versus* non-bacteremic [ΔSodA$^-$] mice, §3.2.3.5)

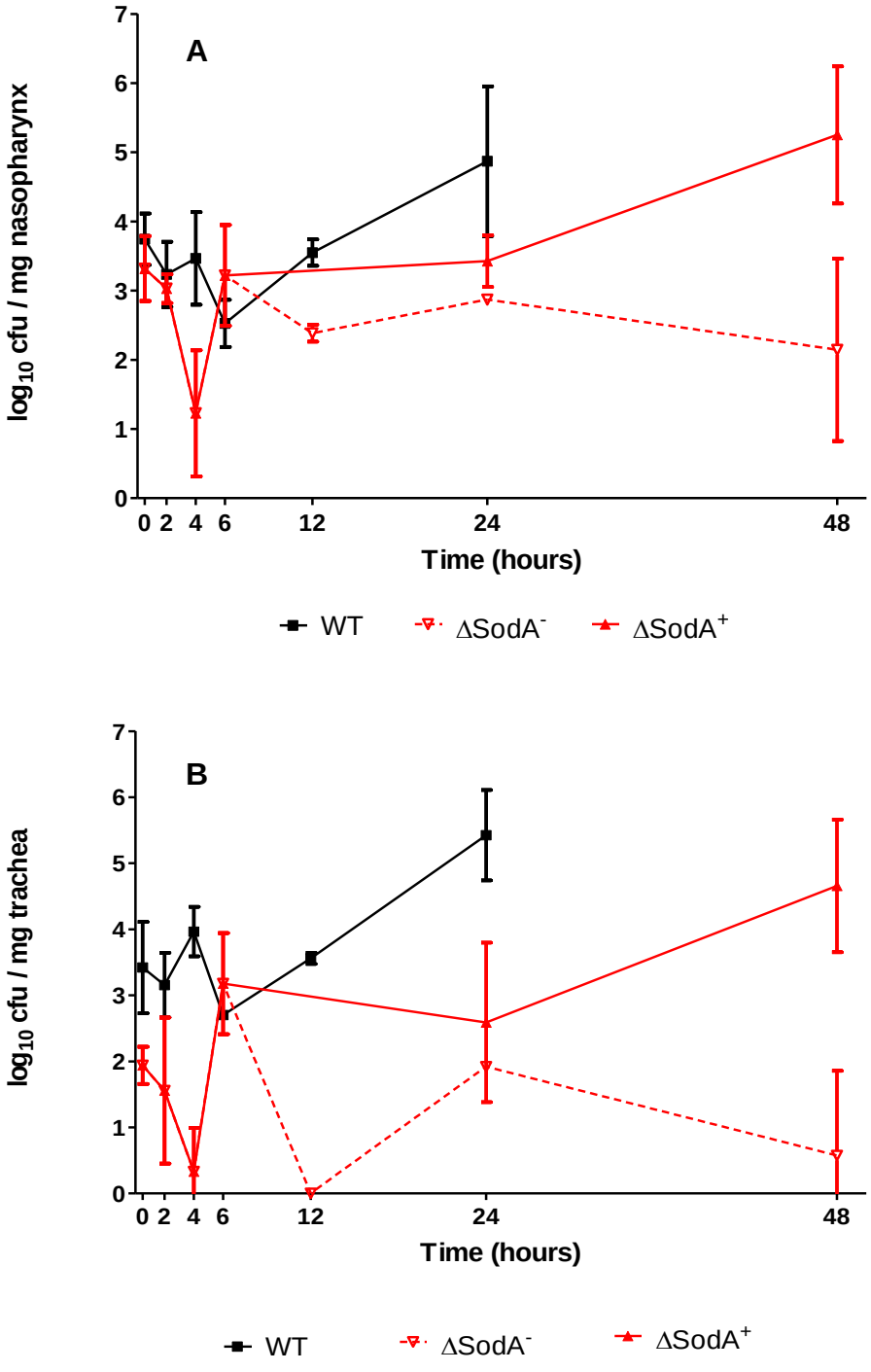

Figure B1 Effect of superoxide dismutase in nasopharyngeal tissues and trachea. *S. pneumoniae* numbers in the nasopharynx (A) and trachea (B) of mice challenged i.n. with 10^6 cfu of wild type or ΔSodA. Data points and error bars represent the mean and SD of the $\log_{10}$ bacteria per mg of organ.

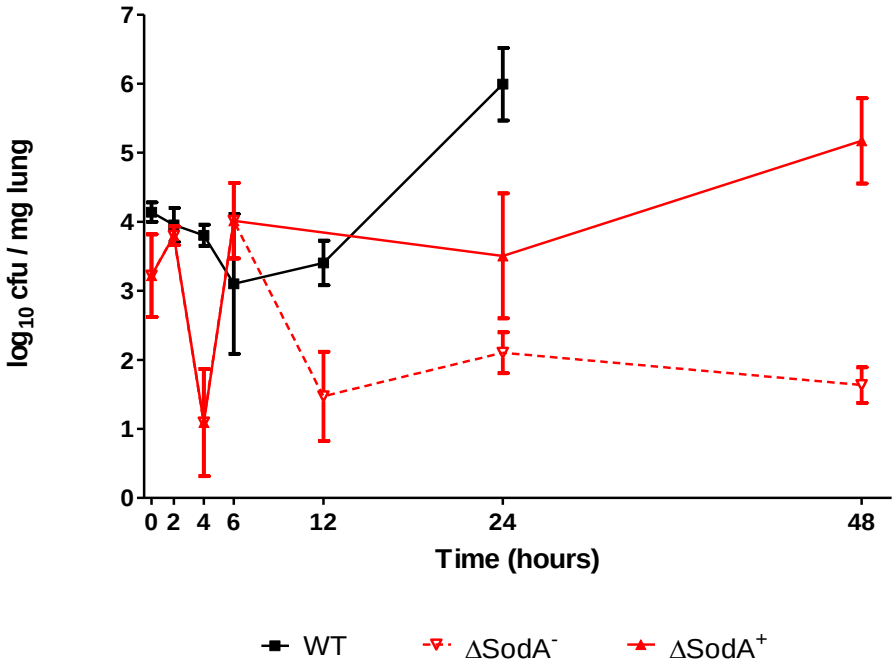

Figure B2 Effect of superoxide dismutase in the lungs. *S. pneumoniae* numbers in the lungs of mice challenged i.n. with 10^6 cfu of wild type or ΔSodA. Data points and error bars represent the mean and SD of the $\log_{10}$ bacteria per mg of organ.

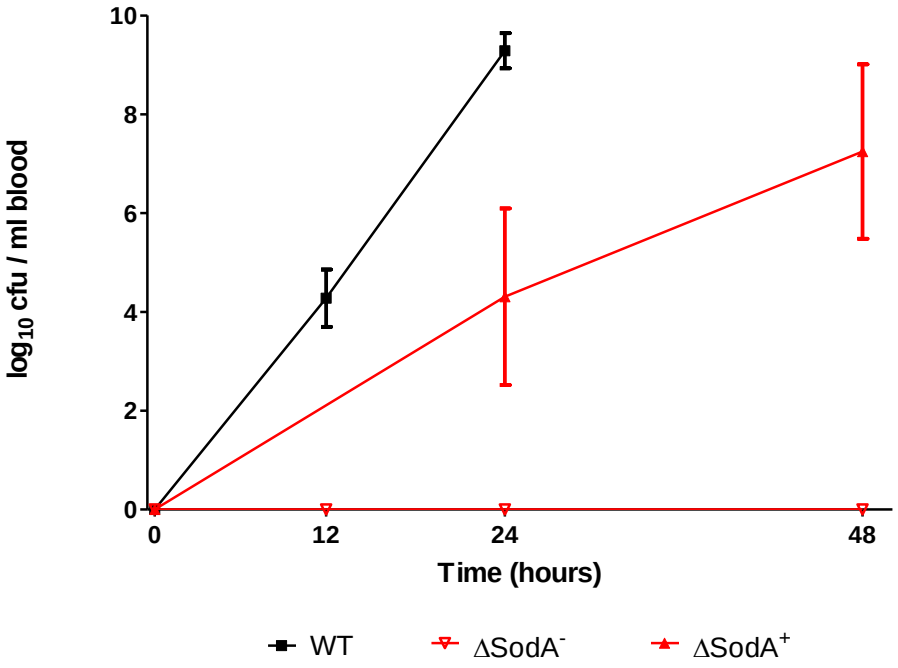

Figure B3 Effect of superoxide dismutase in bacteremia. *S. pneumoniae* numbers in blood of mice challenged i.n. with 10^6 cfu of wild type or ΔSodA. Data points and error bars represent the mean and SD of the $\log_{10}$ bacteria per mg of organ.

Appendix C

►Construction of a novel plasmid with a 'spectinomycin cassette' and *gfp*

Previous studies made in our laboratory (Kadioglu *et al*., 2001) using genetically engineered pneumococci expressing green fluorescence protein (GFP) have shown that pneumococci adhere to and invade broncho-epithelial cells *in vivo* by 4h post-infection, with increased pneumococcal invasion by 24h. Expression of GFP in D39 wild type pneumococci allowed visualisation of longer term *in vivo* interactions between bacteria and host cells with no loss of fluorescence signal.

Green fluorescent protein (GFP) is a naturally fluorescent protein first isolated from the jellyfish *Aequorea victoria* (synonyms *A. forskalea*, *A. aequorea*) (Shimomura [Nobel Prize in Chemistry in 2008] *et al*., 1962). Biologically, GFP converts the blue bioluminescence of other proteins (aequorin or luciferase) into green fluorescent light in luminous coelenterates (jellyfish, sea pens) and acts to increase the quantum yield of light emission (Ward, 2006). The GFP molecule found in the hydrozoan jellyfish *A. victoria* (λ_{max}= 395nm) has an excitation maximum in the ultraviolet (UV) region (Morise *et al*., 1974) and is the only GFP for which the gene has been cloned (Chalfie [Nobel Prize in Chemistry in 2008] *et al*., 1994). Full-length wild type recombinant GFP (also known as TU#58) is a protein containing 238 amino acids (Prasher *et al*., 1992). Sequencing of the protein from the cDNA nucleotide sequence revealed that the *p*-hydroxybenzylideneimidazolinone chromophore is formed by the autocatalytic cyclisation of Ser65, Tyr66 and Gly67 and dehydrogenation of the tyrosine (Shimomura, 1979), and requires molecular oxygen for assembly.

One of the great advantages of GFP as a reporter of gene expression is its high level of stability due to its unique three-dimensional structure (Ormö *et al*., 1996). The mature purified protein remains fluorescent up to 65ºC, pH 11, 1% (w/v) sodium dodecyl sulphate (SDS) or 6M guanidinium chloride and resists most proteases for many hours (Ward, 2006). GFP is also tolerant to fixatives such as formaldehyde (Chalfie *et al*., 1994). Retention of GFP fluorescence in this fixative provides an opportunity to view and localise GFP in preserved tissue.

GFP is stable, does not require cofactors for activity (Chalfie *et al*., 1994) and can be functionally expressed in different bacterial species (Valdivia & Cormack, 2006). In this regard, it can be used as reporter of gene expression, protein localisation in living cells, dynamic processes during bacterial development and the behaviour of single bacteria in complex environments (Valdivia & Cormack, 2006). Several properties of wild type GFP, however, limit its applications in prokaryotes: i) GFP tends to precipitate in the cytoplasm as insoluble, non-fluorescent inclusion bodies; ii) the formation of a functional chromophore occurs only two hours after synthesis of GFP (Heim *et al*., 1995); and iii) the magnitude of the fluorescence signal is low compared to that of other reporter proteins (*e.g.* β-galactosidase [LacZ], chloramphenicol acetyl transferase [CAT], luciferase [Lux]) (Valdivia & Cormack, 2006). Despite these concerns, GFP has had a significant impact in several fields and the new generations of GFP variants with increased sensitivity and solubility are widening the uses to which GFP is applied. GFP has been used to study the interactions between pathogenic bacteria and their mammalian hosts. Green fluorescent protein cDNA has been expressed in a variety of both Gram-positive and -negative bacteria such as *B. subtilis* (Lewis & Errington, 1996), *Mycobacterium* sp. (Valdivia *et al*., 1996), *E. coli* (Chalfie *et al*., 1994) and *H. pylori* (Valdivia & Cormack, 2006) with no adverse effect on the ability of these pathogenic organisms to interact with their host or cause disease (Valdivia *et al*., 1996). GFP-tagged bacteria can be visualised in the tissues of experimentally infected animals. For example, *Mycobacterium marinum* expressing *gfp* has been imaged in cryosections of chronically infected frog spleens for up to five weeks (Valdivia *et al*., 1996) and from lung sections of *M. bovis* BCG-infected mice (Kremer *et al*., 1995).

The work by Kadioglu and colleagues (2001) mentioned above was to be extended in this project by constructing pneumococci mutants expressing GFP and studying their behaviour inside the host. Therefore the work presented below shows the construction of a novel plasmid (pSCM1) containing *gfp* and resistance to erythromycin and spectinomycin, to confer fluorescence to *S. pneumoniae* D39 isogenic mutants PLN-A (Berry *et al*., 1989a), ΔNanA (Berry &

Paton, 2000) and ΔAL2 (Berry *et al*., 1989b), which are all resistant to erythromycin.

The plasmids pDL278 (Spec$^+$; LeBlanc *et al*., 1992) and pGFP1 (Ery$^+$; Kadioglu *et al*., 2001) were used to construct a novel plasmid (pSCM1) that contains *gfp* and resistance to erythromycin (Ery) and spectinomycin (Spec).

The plasmid pGFP1 (11.2Kb) has *gfp* in the streptococcal/*E. coli* shuttle vector pVA838 (Macrina *et al.*, 1982). Pneumococcal transformants containing the plasmid pGFP1 are fluorescent and resistant to erythromycin to a concentration of 1mg/ml.

An outline of the steps followed in the construction of pSCM1 is shown in Figure C1. To obtain a novel plasmid containing *gfp*, erythromycin and a "spectinomycin resistance cassette", the following approach was followed: **1)** the "spectinomycin resistance cassette" in plasmid pDL278 (6733bp) was amplified by PCR (Saiki *et al*., 1988) (see Table C1 for reaction mixture and conditions) using the primers Spec-F (5' - CGG AAT TCA TCG ATT TTC GTT CGT GAA TAC - 3') and Spec-R (5' - CGG AAT TCA TGC AAG GGT TTA TTG TTT TC - 3') (*EcoR*I site underlined with addition of 2 bases at 5') designed on the basis of the published *spc* gene sequence (LeBlanc *et al*., 1991), which amplify a fragment with 1167bp (corresponding to 1151bp plus the addition of 16bp [from the *EcoR*I sites]); **2)** the plasmid pGFP1 was digested (as described in Sambrook [1989]) with the enzyme *EcoRI* which cuts this plasmid at only one site generating 5'-protruding ends, inactivating the "chloramphenicol cassette" (Macrina *et al*., 1982); **3)** a ligation reaction was set up between the "spectinomycin resistance cassette" and the linearised pGFP1 (DNA fragments from PCR were purified using the QIAquick PCR purification kit protocol [Qiagen]; DNA dephosphorylation was used to prevent digested plasmid pGFP1 from self-ligating, using alkaline phosphatase [CIP, New England Biolabs]) to obtain plasmid pSCM1 (Fig. C2); **4)** the novel plasmid now constructed (pSCM1) was used to transform *E. coli* DH5α by the calcium-chloride protocol described in Sambrook (1989); **5)** positive clones - fluorescent under UV microscopy and Spec$^+$ and Ery$^+$ - were selected, confirmed by PCR (Fig. C3) with primers SpecF and SpecR (see above) and plasmid DNA was extracted from *E. coli*; **6)** for preparation of competent cells and transformation of *S.*

pneumoniae using donor DNA (extracted from *E. coli*; concentration 50ng/ml), the protocol described by Iannelli & Pozzi (2004) was followed. PLN-A cells collected 90 minutes (OD_{590} 0.006) following the incubation period were used in this transformation. No positive clones were retrieved and due to time constraints no further attempts were made to transform PLN-A with pSCM1.

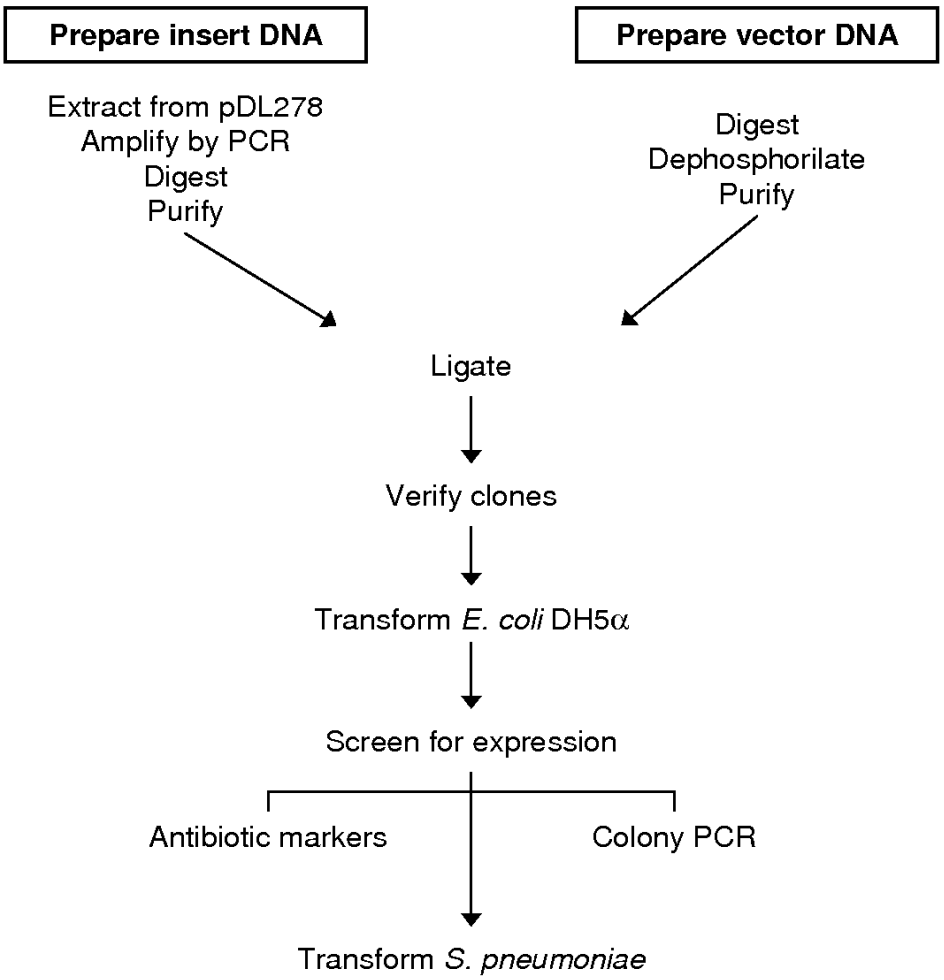

Figure C1 Outline of the steps followed in the construction the novel plasmid pSCM1. The insert DNA was the 'spectinomycin cassette' from plasmid pDL278 and the vector DNA was plasmid pGFP1.

Table C1. PCR reaction mixture used to isolate the complete spectinomycin gene *spc* of plasmid pDL278

10x Accubuffer[a]	2μl
dNTPs (2mM stock)[a]	2μl
$MgCl_2$ (50mM stock)[b]	0.2μl
5' primer (5pM stock)	1μl
3' primer (5pM stock)	1μl
Template DNA[c]	1μl
Accuzyme DNA polymerase	1.5μl/100μl
nH_2O	to 20μl

[a] For composition see Appendix A; [b] Supplied by the manufacturer
[c] Approximately 10 ng/μl

Ten samples of plasmid DNA pDL278 were used to set up the reaction. This was done in a thermal cycler (PTC-200 Peltier Thermal Cycler, MJReasearch Inc.) and the cycling conditions are described below:

Cycle number	Denaturation	Annealing	Extension	Final
1	10 min at 95^{o}C	1 min at 60^{o}C		
2-34	1 min at 94^{o}C	1 min at 60^{o}C	5 min at 72^{o}C	
35			15 min at 72^{o}C	
Hold				4^{o}C

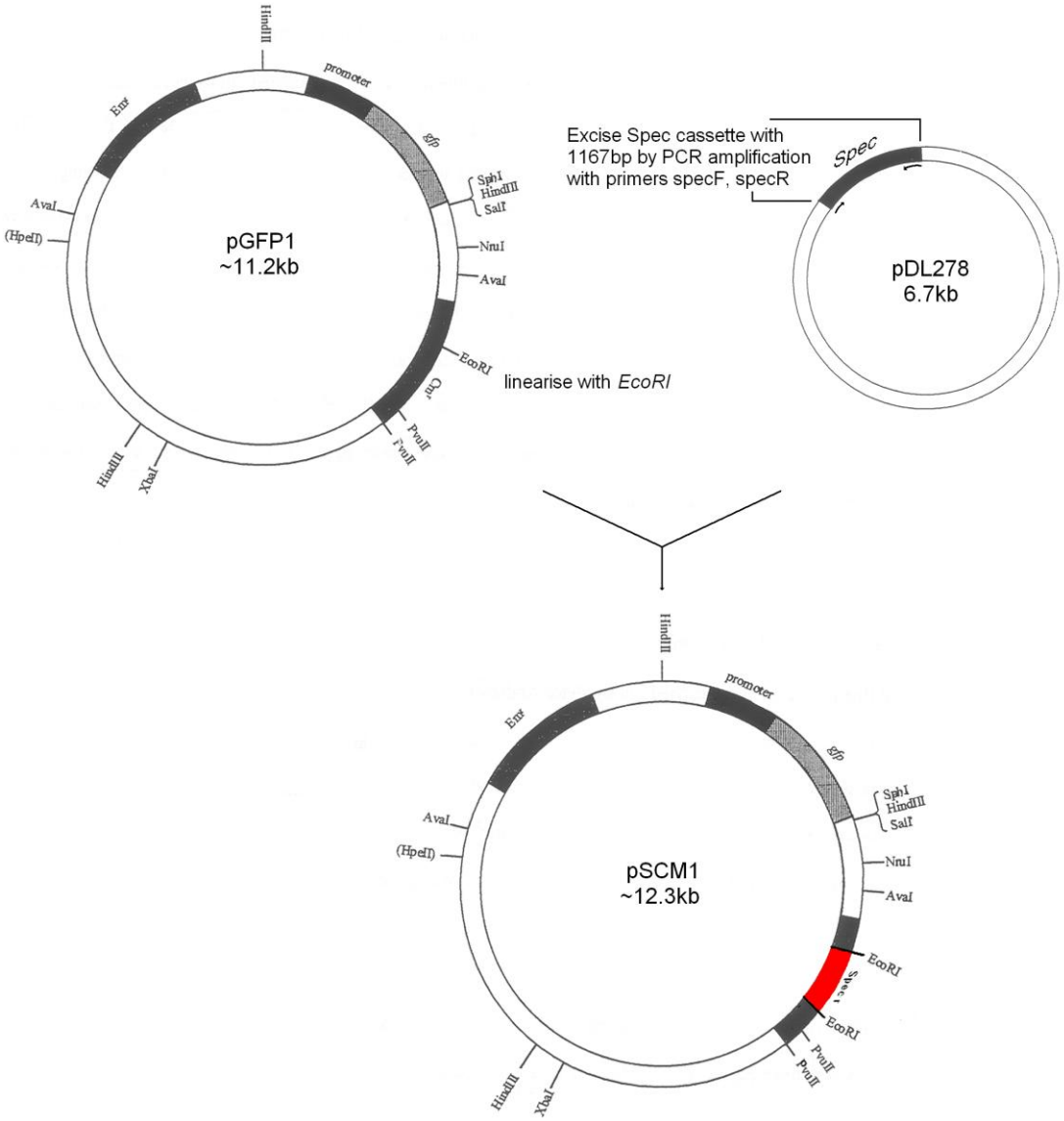

Figure C2 Diagram depicting the construction of plasmid pSCM1 of approx. 12.3Kb, showing location of the 'spectinomycin cassette' fragment (red box). Abbreviations used: Em^r, erythromycin resistance cassette; $Spec^r$, spectinomycin resistance cassette.

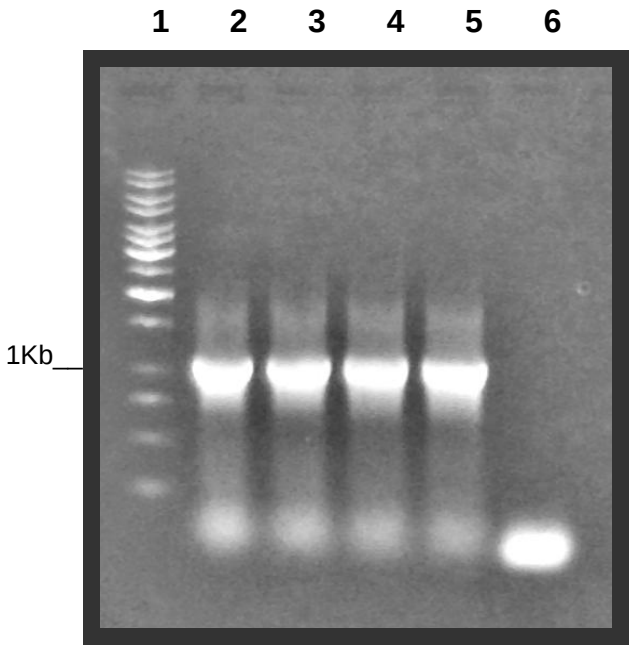

Figure C3 Colony PCR of *E. coli* clones 1, 4, 5, 6 transformed with plasmid pSCM1, using primers SpecF and SpecR to confirm the presence of the spectinomycin cassette. Lane 1: 1Kb ladder (GeneRuler); Lane 2, 3, 4, 5: 5μl of PCR reaction from each clone (1, 4, 5 and 6, respectively) showing a band at the expected size of around 1167bp; Lane 6: nanopure water (negative control showing a bright band probably corresponding to primer-dimers).

A probable explanation for the negative result obtained in this study is the fact that the pneumococcal batch used for transformation was not tested for transformability prior to use, and thus, the cells in that batch may not have been transformable (competent). The protocol followed (using competence-stimulating peptide, CSP) requires the employment of competent pneumococcal cells for transformation.

A second possibility is that the direct transformation of *S. pneumoniae* type 2 strain D39 has a very low rate of success, as mentioned by Berry *et al*. (1989b) when attempting to create an autolysin-negative *S. pneumoniae* mutant by insertion-duplication mutagenesis. These authors used a derivative of the recombinant plasmid pVA891, and observed a low rate of success of transformability, even in the presence of competence factor derived from the highly transformable D39 derivative, Rx1. Given the difficulties, they adopted a two-step approach. They first transformed the non-encapsulated strain Rx1 with the recombinant plasmid and isolated a single erythromycin resistant transformant which did not produce autolysin. In order to construct an encapsulated autolysin-negative pneumococcus, the encapsulated strain D39 was then transformed with DNA extracted from the autolysin-negative, erythromycin-resistant transformant of Rx1, originating several autolysin-negative transformants. Perhaps this approach could have been followed here in order to achieve more reliably transformation of PLN-A mutant cells with the new plasmid pSCM1.

Another option would have been the construction of a completely new, smaller plasmid, which could be transformed into pneumococcal cells more consistently. The novel plasmid created in this work (pSCM1) has around 12.3Kb in size (from the ligation of pGFP1 with approximately 11.2Kb [Kadioglu *et al*., 2001] with the 'spectinomycin cassette' from the shuttle vector pDL278 [LeBlanc *et al*., 1992]), which possibly renders it difficult to insert into the receptor's cells.

Nonetheless, since doing the experimental part of this thesis, substantial experience has been gained in the use of CSP for transformation, which leaves the prospect that future work will be continued in this area in our laboratory.

In conclusion, the recent 2008 Nobel Prize awarded for the work on GFP by Shimomura, Chalfie and Tsien leaves no doubts regarding the importance of this "tool" in the monitoring of spatio-temporal processes in living systems. Shimomura was the first person to isolate GFP and to find out which part of GFP was responsible for its fluorescence (Shimomura *et al.,* 1962). Later, Chalfie cloned *gfp* and shown that GFP is generally non-toxic and can be expressed to high levels in different organisms with minor effects on their physiology (Chalfie *et al.,* 1994). Then, Tsien investigated the mechanism of the fluorescence of GFP and developed several new GFP variants (Tsien, 1998). Despite the ability to create a novel plasmid containing GFP in this study, the expression of this plasmid was only possible in *E. coli* cells but not in pneumococci. Extension of the work developed here needs to be pursued so that new data using GFP-tagged mutant pneumococci can be obtained regarding the role of the mutated enzymes in pneumococcal localisation, adherence and invasion in the host.

BIBLIOGRAPHY

6. Bibliography

Abrashev, I. and G. Dulguerova. 2000. Neuraminidases (sialidases) from bacterial origin. *Experimental Pathology and Parasitology* 4: 35-40.

ACIP. 1997. *Prevention of pneumococcal disease: Recommendations of the advisory committee on immunization practice (ACIP).* USA: Centers for Disease Control and Prevention, MMWR Vol.46 (Nº RR-8).

Adegbola, R. May 2006. *Acute respiratory infections (ARI) research in the Gambia*. Seminar held at the University of Leicester, UK.

Alexander, J. E., R. A. Lock, C. A. M. Peeters, J. T. Poolman, P. W. Andrew, T. J. Mitchell, D. Hansman and J. C. Paton. 1994. Immunization of mice with pneumolysin toxoid confers a significant degree of protection against at least nine serotypes of *Streptococcus pneumoniae*. *Infection and Immunity* 62:5683-88.

Andersson, B., B. Eriksson, E. Falsen, A. Fogh, L. A. Hanson, O. Nylen, H. Peterson and C. Svanborg Eden. 1981. Adhesion of *Streptococcus pneumoniae* to human pharyngeal epithelial cells in vitro: Differences in adhesive capacity among strains isolated from subjects with otitis media, septicemia, or meningitis or from healthy carriers. *Infection and Immunity* 32, (1) (Apr): 311-7.

Andersson, B., J. Dahmen, T. Frejd, H. Leffler, G. Magnusson, G. Nooriand and C. S. Eden. 1983. Identification of an active disaccharide unit of a glycoconjugate receptor for pneumococci attaching to human pharyngeal epithelial cells. *The Journal of Experimental Medicine* 158, (2) (Aug 1): 559-70.

Aebi, C., E. R. Lafontaine, L. D. Cope, J. L. Latimer, S. L. Lumbley, G. H. McCracken Jr. and E. J. Hansen. 1998. Phenotypic effect of isogenic uspA1 and uspA2 mutations on *Moraxella catarrhalis* 035E. *Infection and Immunity* 66, (7) (Jul): 3113-9.

Austrian, R. 1997. The enduring pneumococcus: Unfinished business and opportunities for the future. *Microbial Drug Resistance* 3, (2) (Summer): 111-5.

Austrian, R. and J. Gold. 1964. Pneumococcal bacteremia with especial reference to bacteremic pneumococcal pneumonia. *Annals of Internal Medicine* 60, (May): 759-76.

Auzat, I., S. Chapuy-Regaud, G. Le Bras, D. Dos Santos, A. D. Ogunniyi, I. Le Thomas, J. R. Garel, J. C. Patonand M. C. Trombe. 1999. The NADH oxidase of *Streptococcus pneumoniae*: Its involvement in competence and virulence. *Molecular Microbiology* 34, (5) (Dec): 1018-28.

Avery, O. T., C. M. MacLeod and M. McCarty. 1944. Studies on the chemical nature of the substance inducing transformation of pneumococcal types. Induction of transformation by a desoxyribonucleic acid fraction isolated from Pneumococcus type III. *Journal of Experimental Medicine* 79: 137–158.

Azoulay-Dupuis, E., J. P. Be´dos, E. Vallee, D. J. Hardy, R. N. Swanson and J. J. Pocidalo. 1991. Antipneumococcal activity of ciprofloxacin, oxofloxacin and temafloxacin in an experimental mouse pneumonia model at various stages of the disease. *Journal of Infectious Diseases* 163:319-24.

Balachandran, P., A. Brooks-Walter, A. Virolainen-Julkunen, S. K. Hollingshead and D. E. Briles. 2002. Role of pneumococcal surface protein C in nasopharyngeal carriage and pneumonia and its ability to elicit protection against carriage of Streptococcus pneumoniae. *Infection and Immunity* 70:2526-34.

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

Baril, L., J. Dietemann, M. Essevaz-Roulet, L. Beniguel, P. Coan, D. E. Briles, B. Guy and G. Cozon. 2006. Pneumococcal surface protein A (PspA) is effective at eliciting T cell-mediated responses during invasive pneumococcal disease in adults. *Clinical and Experimental Immunology* 145, (2) (Aug): 277-86.

Barthelson, R., A. Mobasseri, D. Zopf and P. Simon. 1998. Adherence of *Streptococcus pneumoniae* to respiratory epithelial cells is inhibited by sialylated oligosaccharides. *Infection and Immunity* 66, (4) (Apr): 1439-44.

Bateman, A., L. Coin, R. Durbin, R. D. Finn, V. Hollich, S. Griffiths-Jones, A. Khanna, et al. 2004. The pfam protein families database. *Nucleic Acids Research* 32, (Jan 1): D138-41.

Beaman, L. and B. L. Beaman. 1990. Monoclonal antibodies demonstrate that superoxide dismutase contributes to protection of *Nocardia asteroides* within the intact host. *Infection and Immunity* 58, (9) (Sep): 3122-8.

Beaman, B. L., C. M. Black, F. Doughty and L. Beaman. 1985. Role of superoxide dismutase and catalase as determinants of pathogenicity of *Nocardia asteroides*: Importance in resistance to microbicidal activities of human polymorphonuclear neutrophils. *Infection and Immunity* 47, (1) (Jan): 135-41.

Belanger, A. E., M. J. Clague, J. I. Glass and D. J. Leblanc. 2004. Pyruvate oxidase is a determinant of Avery's rough morphology. *Journal of Bacteriology* 186, (24) (Dec): 8164-71.

Benov, L. T. and I. Fridovich. 1994. *Escherichia coli* expresses a copper- and zinc-containing superoxide dismutase. *Journal of Biological Chemistry* 269, (41) (Oct 14): 25310-4.

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

Berry, A. M., R. A. Lock, D. Hansman and J. C. Paton. 1989b. Contribution of autolysin to virulence of *Streptococcus pneumoniae*. *Infection and Immunity* 57, (8) (Aug): 2324-30.

Berry, A. M., R. A. Lock and J. C. Paton. 1996. Cloning and characterization of *nanB*, a second *Streptococcus pneumoniae* neuraminidase gene, and purification of the NanB enzyme from recombinant *Escherichia coli*. *Journal of Bacteriology* 178, (16) (Aug): 4854-60.

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

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

Berry, A. M., J. C. Paton, E. M. Glare, D. Hansman and D. E. Catcheside. 1988. Cloning and expression of the pneumococcal neuraminidase gene in *Escherichia coli*. *Gene* 71, (2) (Nov 30): 299-305.

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

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

Black, S., H. Shinefield, B. Fireman, E. Lewis, P. Ray, J. R. Hansen, L. Elvin, et al. 2000. Efficacy, safety and immunogenicity of heptavalent pneumococcal conjugate vaccine in children. Northern California kaiser permanente vaccine study center group. *The Pediatric Infectious Disease Journal* 19, (3) (Mar): 187-95.

Bogaert, D., R. De Groot and P. W. Hermans. 2004a. *Streptococcus pneumoniae* colonisation: The key to pneumococcal disease. *Lancet Infectious Diseases* 4, (3) (Mar): 144-54.

Bogaert, D., P. W. Hermans, P. V. Adrian, H. C. Rumke and R. de Groot. 2004b. Pneumococcal vaccines: An update on current strategies. *Vaccine* 22, (17-18) (Jun 2): 2209-20.

Booy, R., S. Hodgson, L. Carpenter, R. T. Mayon-White, M. P. Slack, J. A. Macfarlane, E. A. Haworth, M. Kiddle, S. Shribman and J. S. Roberts. 1994. Efficacy of *Haemophilus influenzae* type b conjugate vaccine PRP-T. *Lancet* 344, (8919) (Aug 6): 362-6.

Bordo, D., K. Djinovic and M. Bolognesi. 1994. Conserved patterns in the Cu/Zn superoxide dismutase family. *Journal of Molecular Biology* 238, (3) (May 6): 366-86.

Bradford, M. M. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. *Analytical Biochemistry* 72, (May 7): 248-54.

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

Briles, D. E., S. Hollingshead, A. Brooks-Walter, G. S. Nabors, L. Ferguson, M. Schilling, S. Gravenstein, P. Braun, J. King and A. Swift. 2000a. The potential to use PspA and other pneumococcal proteins to elicit protection against pneumococcal infection. *Vaccine* 18, (16) (Feb 25): 1707-11.

Briles, D. E., S. K. Hollingshead, J. King, A. Swift, P. A. Braun, M. K. Park, L. M. Ferguson, M. H. Nahm and G. S. Nabors. 2000c. Immunization of humans with recombinant pneumococcal surface protein A (rPspA) elicits antibodies that passively protect mice from fatal infection with *Streptococcus pneumoniae* bearing heterologous PspA. *The Journal of Infectious Diseases* 182, (6) (Dec): 1694-701.

Briles, D. E., J. D. King, M. A. Gray, L. S. McDaniel, E. Swiatlo and K. A. Benton. 1996. PspA, a protection-eliciting pneumococcal protein: Immunogenicity of isolated native PspA in mice. *Vaccine* 14, (9) (Jun): 858-67.

Briles, D. E., R. C. Tart, E. Swiatlo, J. P. Dillard, P. Smith, K. A. Benton, B. A. Ralph, et al. 1998. Pneumococcal diversity: Considerations for new vaccine strategies with emphasis on pneumococcal surface protein A (PspA). *Clinical Microbiology Reviews* 11, (4) (Oct): 645-57.

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

Bruggemann, H., S. Baumer, W. F. Fricke, A. Wiezer, H. Liesegang, I. Decker, C. Herzberg, et al. 2003. The genome sequence of *Clostridium tetani*, the causative agent of tetanus disease. *Proceedings of the National Academy of Sciences of the United States of America* 100, (3) (Feb 4): 1316-21.

Bryce, J., C. Boschi-Pinto, K. Shibuya, R. E. Black and WHO Child Health Epidemiology Reference Group. 2005. WHO estimates of the causes of death in children. *Lancet* 365, (9465) (Mar 26-Apr 1): 1147-52.

Burkitt, H. G. 1993. Wheater's functional histology: A text and colour atlas. Eds. Barbara Young, John W. Heath and Paul R. Wheater. 3rd Ed. Edinburgh: Churchill Livinstone.

Burnaugh, A. M., L. J. Frantz and S. J. King. 2008. Growth of *Streptococcus pneumoniae* on human glycoconjugates is dependent upon the sequential activity of bacterial exoglycosidases. *Journal of Bacteriology* 190, (1) (Jan): 221-30.

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

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

Catterall, J. R. 1999. *Streptococcus pneumoniae*. *Thorax* 54, (10) (Oct): 929-37.

Catterall, J. R. 2004. New developments with *Streptococcus pneumoniae*. *Respiratory Disease in Practice* 20, (1): 2-5.

CDC, Centers for Disease Control and Prevention. 2005. *Streptococcus pneumoniae* disease *in* CDC, Atlanta, USA. [cited 31/05/2006] Available from http://www.cdc.gov/ncidod/dbmd/diseaseinfo/streppneum t.htm.

Chalfie, M., Y. Tu, G. Euskirchen, W. W. Ward and D. C. Prasher. 1994. Green fluorescent protein as a marker for gene expression. *Science* 263, (5148) (Feb 11): 802-5.

Charpentier, E. and E. Tuomanen. 2000. Mechanisms of antibiotic resistance and tolerance in *Streptococcus pneumoniae*. *Microbes and Infection / Institut Pasteur* 2, (15) (Dec): 1855-64.

Chiavolini, D., G. Pozzi and S. Ricci. 2008. Animal models of *Streptococcus pneumoniae* diseases. *Clinical Microbiology Reviews* 21, (4) (Oct): 666-85.

Chiavolini, D., S. Tripodi, R. Parigi, M. R. Oggioni, E. Blasi, M. Cintorino, G. Pozzi and S. Ricci. 2004. Method for inducing experimental pneumococcal meningitis in outbred mice. *BMC Microbiology*: 22-36.

Colman, P. M. 1994. Influenza virus neuraminidase: structure, antibodies, and inhibitors. *Protein Science* 3, (10) (Oct): 1687-96.

Corfield, A. P., S. A. Wagner, J. R. Clamp, M. S. Kriaris and L. C. Hoskins. 1992. Mucin degradation in the human colon: production of sialidase, sialate O-acetylesterase, N-acetylneuraminate lyase, arylesterase, and glycosulfatase activities by strains of fecal bacteria. *Infection and Immunity* 60, (10) (Oct): 3971-8.

Corfield, T. 1992. Bacterial sialidases: roles in pathogenicity and nutrition. *Glycobiology* 2, (6) (Dec): 509-21.

Cron, L.E., H.J. Bootsma, N. Noske, P. Burghout, S. Hammerschmidt and P.W. Hermans. 2009. Surface-associated lipoprotein PpmA of *Streptococcus pneumoniae* is involved in colonization in a strain-specific manner. *Microbiology* 155 (Pt 7):2401-10.

Cross, A. S., S. Sakarya, S. Rifat, T. K. Held, B. E. Drysdale, P. A. Grange, F. J. Cassels, et al. 2003. Recruitment of murine neutrophils in vivo through endogenous sialidase activity. *Journal of Biological Chemistry* 278, (6) (Feb 7): 4112-20.

Cundell, D. R., N. P. Gerard, C. Gerard, I. Idanpaan-Heikkila and E. I. Tuomanen. 1995a. *Streptococcus pneumoniae* anchor to activated human cells by the receptor for platelet-activating factor. *Nature* 377, (6548) (Oct 5): 435-8.

Cundell, D. R., B. J. Pearce, J. Sandros, A. M. Naughton and H. R. Masure. 1995b. Peptide permeases from *Streptococcus pneumoniae* affect adherence to eukaryotic cells. *Infection and Immunity* 63, (7) (Jul): 2493-8.

Cundell, D. R. and E. I. Tuomanen. 1994. Receptor specificity of adherence of *Streptococcus pneumoniae* to human type-II pneumocytes and vascular endothelial cells in vitro. *Microbial Pathogenesis* 17, (6) (Dec): 361-74.

Curnutte, J. T., D. M. Whitten and B. M. Babior. 1974. Defective superoxide production by granulocytes from patients with chronic granulomatous disease. *The New England Journal of Medicine* 290, (11) (Mar 14): 593-7.

Dallaire, F., N. Ouellet, Y. Bergeron, V. Turmel, M. C. Gauthier, M. Simard, and M. G. Bergeron. 2001. Microbiological and inflammatory factors associated with development of pneumococcal pneumonia. *Journal of Infectious Disease* 184:292-300.

Dave, S., A. Brooks-Walter, M. K. Pangburn and L. S. McDaniel. 2001. PspC, a pneumococcal surface protein, binds human factor H. *Infection and Immunity* 69, (5) (May): 3435-7.

Davis, L., M. M. Baig and E. M. Ayoub. 1979. Properties of extracellular neuraminidase produced by group A streptococcus. *Infection and Immunity* 24, (3) (Jun): 780-6.

De Las Rivas, B., J. L. Garcia, R. Lopez and P. Garcia. 2002. Purification and polar localization of pneumococcal LytB, a putative endo-beta-N-acetylglucosaminidase: The chain-dispersing murein hydrolase. *Journal of Bacteriology* 184, (18) (Sep): 4988-5000.

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

de Roux, A. and H. Lode. 2005. Pneumococcal vaccination. *The European Respiratory Journal* 26, (6) (Dec): 982-3.

Demple, B. 1996. Redox signaling and gene control in the *Escherichia coli* soxRS oxidative stress regulons: a review. *Gene* 179, (1) (Nov 7): 53-7.

Díaz, E., E. Garcia, C. Ascaso, E. Mendez, R. Lopez and J. L. Garcia. 1989. Subcellular localization of the major pneumococcal autolysin: A peculiar mechanism of secretion in *Escherichia coli*. *Journal of Biological Chemistry* 264, (2) (Jan 15): 1238-44.

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

D'Mello, R. A., P. R. Langford and J. S. Kroll. 1997. Role of bacterial Mn-cofactored superoxide dismutase in oxidative stress responses, nasopharyngeal colonization, and sustained bacteremia caused by *Haemophilus influenzae* type b. *Infection and Immunity* 65, (7) (Jul): 2700-6.

Duane, P. G., J. B. Rubins, H. R. Weisel and E. N. Janoff. 1993. Identification of hydrogen peroxide as a *Streptococcus pneumoniae* toxin for rat alveolar epithelial cells. *Infection and Immunity* 61, (10) (Oct): 4392-7.

Dwarakanath, A. D., H. H. Tsai, D. Sunderland, C. A. Hart, N. Figura, J. E. Crabtree and J. M. Rhodes. 1995. The production of neuraminidase and fucosidase by *Helicobacter pylori*: Their possible relationship to pathogenicity. *FEMS Immunology and Medical Microbiology* 12, (3-4) (Dec): 213-6.

Faden, H., L. Duffy, A. Williams, D. A. Krystofik and J. Wolf. 1995. Epidemiology of nasopharyngeal colonization with nontypeable *Haemophilus influenzae* in the first 2 years of life. *The Journal of Infectious Diseases* 172, (1) (Jul): 132-5.

Farrant, J. L., A. Sansone, J. R. Canvin, M. J. Pallen, P. R. Langford, T. S. Wallis, G. Dougan and J. S. Kroll. 1997. Bacterial copper- and zinc-cofactored superoxide dismutase contributes to the pathogenesis of systemic salmonellosis. *Molecular Microbiology* 25, (4) (Aug): 785-96.

Feldman, C., R. Read, A. Rutman, P. K. Jeffery, A. Brain, V. Lund, T. J. Mitchell, P. W. Andrew, G. J. Boulnois and H. C. Todd. 1992. The interaction of *Streptococcus pneumoniae* with intact human respiratory mucosa in vitro. *The European Respiratory Journal* 5, (5) (May): 576-83.

Fernandez-Tornero, C., R. Lopez, E. Garcia, G. Gimenez-Gallego and A. Romero. 2001. A novel solenoid fold in the cell wall anchoring domain of the pneumococcal virulence factor LytA. *Nature Structural Biology* 8, (12) (Dec): 1020-4.

Fridovich, I. 1999. Fundamental aspects of reactive oxygen species, or what's the matter with oxygen? *Annals of the New York Academy of Sciences* 893: 13-8.

Fridovich, I. 1998. Oxygen toxicity: A radical explanation. *The Journal of Experimental Biology* 201, (Pt 8) (Apr): 1203-9.

Fridovich, I. 1997. Superoxide anion radical (O2-.), superoxide dismutases, and related matters. *Journal of Biological Chemistry* 272, (30) (Jul 25): 18515-7.

Galen, J. E., J. M. Ketley, A. Fasano, S. H. Richardson, S. S. Wasserman and J. B. Kaper. 1992. Role of *Vibrio cholerae* neuraminidase in the function of cholera toxin. *Infection and Immunity* 60, (2) (Feb): 406-15.

García, E., J. L. Garcia, P. Garcia, A. Arraras, J. M. Sanchez-Puelles and R. Lopez. 1988. Molecular evolution of lytic enzymes of *Streptococcus pneumoniae* and its bacteriophages. *Proceedings of the National Academy of Sciences of the United States of America* 85, (3) (Feb): 914-8.

García, E., J. L. Garcia, C. Ronda, P. Garcia and R. Lopez. 1985. Cloning and expression of the pneumococcal autolysin gene in *Escherichia coli*. *Molecular & General Genetics* 201, (2): 225-30.

García, J. L., E. Garcia, A. Arraras, P. Garcia, C. Ronda and R. Lopez. 1987. Cloning, purification, and biochemical characterization of the pneumococcal bacteriophage cp-1 lysin. *Journal of Virology* 61, (8) (Aug): 2573-80.

García, J. L., J. A. Hermoso, L. Lagartera, A. González, P. García, M. Martínez-Ripoll and M. Menéndez. 2005. 3D structure of choline binding proteins. Paper presented at 7th European Meeting on the Molecular Biology of Pneumococcus, Braunschweig, Germany.

García, P., M. P. Gonzalez, E. Garcia, R. Lopez and J. L. Garcia. 1999a. LytB, a novel pneumococcal murein hydrolase essential for cell separation. *Molecular Microbiology* 31, (4) (Feb): 1275-7.

García, P., M. Paz Gonzalez, E. Garcia, J. L. Garcia and R. Lopez. 1999b. The molecular characterization of the first autolytic lysozyme of *Streptococcus pneumoniae* reveals evolutionary mobile domains. *Molecular Microbiology* 33, (1) (Jul): 128-38.

García -Rodriguez, J. A. and M. J. F. Martínez. 2002. Dynamics of nasopharyngeal colonization by potential respiratory pathogens. *The Journal of Antimicrobial Chemotherapy* 50 Suppl. S2, (Dec): 59-73.

Gerlach, D., W. Reichardt and S. Vettermann. 1998. Extracellular superoxide dismutase from *Streptococcus pyogenes* type 12 strain is manganese-dependent. *FEMS Microbiology Letters* 160, (2) (Mar 15): 217-24.

Ghaffar, F., T. Barton, J. Lozano, L. S. Muniz, P. Hicks, V. Gan, N. Ahmad and G. H. McCracken Jr. 2004. Effect of the 7-valent pneumococcal conjugate vaccine on nasopharyngeal colonization by *Streptococcus pneumoniae* in the first 2 years of life. *Clinical Infectious Diseases* 39, (7) (Oct 1): 930-8.

Ghaffar, F., I. R. Friedland, K. Katz, L. S. Muniz, J. L. Smith, P. Davis, J. Reynolds and G. H. McCracken Jr. 1999. Increased carriage of resistant non-pneumococcal alpha-hemolytic streptococci after antibiotic therapy. *The Journal of Pediatrics* 135, (5) (Nov): 618-23.

Gibson, C. M. and M. G. Caparon. 1996. Insertional inactivation of *Streptococcus pyogenes* sod suggests that prtF is regulated in response to a superoxide signal. *Journal of Bacteriology* 178, (15) (Aug): 4688-95.

Gingles, N. A., J. E. Alexander, A. Kadioglu, P. W. Andrew, A. Kerr, T. J. Mitchell, E. Hopes, et al. 2001. Role of genetic resistance in invasive pneumococcal infection: Identification and study of susceptibility and resistance in inbred mouse strains. *Infection and Immunity* 69, (1) (Jan): 426-34.

Girard, M. P., M. P. Preziosi, M. T. Aguado and M. P. Kieny. 2006. A review of vaccine research and development: Meningococcal disease. *Vaccine* 24, (22) (May 29): 4692-700.

Gort, A. S. and J. A. Imlay. 1998. Balance between endogenous superoxide stress and antioxidant defenses. *Journal of Bacteriology* 180, (6) (Mar): 1402-10.

Gosink, K. K., E. R. Mann, C. Guglielmo, E. I. Tuomanen and H. R. Masure. 2000. Role of novel choline binding proteins in virulence of *Streptococcus pneumoniae*. *Infection and Immunity* 68, (10) (Oct): 5690-5.

Guiral, S., T. J. Mitchell, B. Martin and J. P. Claverys. 2005. Competence-programmed predation of noncompetent cells in the human pathogen

Streptococcus pneumoniae: Genetic requirements. *Proceedings of the National Academy of Sciences of the United States of America* 102, (24) (Jun 14): 8710-5.

Gut, H., S. J. King and M. A. Walsh. 2008. Structural and functional studies of *Streptococcus pneumoniae* neuraminidase B: An intramolecular trans-sialidase. *FEBS Letters* 582, (23-24) (Oct 15): 3348-52.

Hammerschmidt, S., S. R. Talay, P. Brandtzaeg and G. S. Chhatwal. 1997. SpsA, a novel pneumococcal surface protein with specific binding to secretory immunoglobulin A and secretory component. *Molecular Microbiology* 25, (6) (Sep): 1113-24.

Hammerschmidt, S., S. Wolff, A. Hocke, S. Rosseau, E. Muller, and M. Rohde. 2005. Illustration of pneumococcal polysaccharide capsule during adherence and invasion of epithelial cells. *Infection and Immunity* 73, (8) (Aug): 4653-67.

Hancock, I. C. 1997. Bacterial cell surface carbohydrates: Structure and assembly. *Biochemical Society Transactions* 25, (1) (Feb): 183-7.

Hansman, D., H. Glasgow, J. Sturt, L. Devitt and R. Douglas. 1971. Increased resistance to penicillin of pneumococci isolated from man. *The New England Journal of Medicine* 284, (4) (Jan 28): 175-7.

Harabuchi, Y., H. Faden, N. Yamanaka, L. Duffy, J. Wolf and D. Krystofik. 1994. Human milk secretory IgA antibody to nontypeable *Haemophilus influenzae*: Possible protective effects against nasopharyngeal colonization. *The Journal of Pediatrics* 124, (2) (Feb): 193-8.

Hassett, D. J., H. P. Schweizer and D. E. Ohman. 1995. *Pseudomonas aeruginosa sodA* and *sodB* mutants defective in manganese- and iron-cofactored superoxide dismutase activity demonstrate the importance of the iron-cofactored form in aerobic metabolism. *Journal of Bacteriology* 177, (22) (Nov): 6330-7.

Hausdorff, W. P., D. R. Feikin and K. P. Klugman. 2005. Epidemiological differences among pneumococcal serotypes. *Lancet Infectious Diseases* 5, (2) (Feb): 83-93.

Hava, D. L., J. LeMieux and A. Camilli. 2003. From nose to lung: The regulation behind *Streptococcus pneumoniae* virulence factors. *Molecular Microbiology* 50, (4) (Nov): 1103-10.

Heilmann, C., M. Hussain, G. Peters and F. Gotz. 1997. Evidence for autolysin-mediated primary attachment of *Staphylococcus epidermidis* to a polystyrene surface. *Molecular Microbiology* 24, (5) (Jun): 1013-24.

Heim, R., A. B. Cubitt and R. Y. Tsien. 1995. Improved green fluorescence. *Nature* 373, (6516) (Feb 23): 663-4.

Henson, P. M. and R. B. Johnston Jr. 1987. Tissue injury in inflammation. Oxidants, proteinases, and cationic proteins. *The Journal of Clinical Investigation* 79, (3) (Mar): 669-74.

Hirst, R.A., B. Gosai, A. Rutman, P.W. Andrew and C. O'Callaghan. 2003. *Streptococcus pneumoniae* damages the ciliated ependyma of the brain during meningitis. *Infection and Immunity* 71 (10) (Oct): 6095-100.

Hirst, R. A., K. S. Sikand, A. Rutman, T. J. Mitchell, P. W. Andrew and C. O'Callaghan. 2000. Relative roles of pneumolysin and hydrogen peroxide from *Streptococcus pneumoniae* in inhibition of ependymal ciliary beat frequency. *Infection and Immunity* 68, (3) (Mar): 1557-62.

Holtje, J. V., D. Mirelman, N. Sharon and U. Schwarz. 1975. Novel type of murein transglycosylase in *Escherichia coli*. *Journal of Bacteriology* 124, (3) (Dec): 1067-76.

Hoskins, J., W. E. Alborn Jr, J. Arnold, L. C. Blaszczak, S. Burgett, B. S. DeHoff, S. T. Estrem, et al. 2001. Genome of the bacterium *Streptococcus pneumoniae* strain R6. *Journal of Bacteriology* 183, (19) (Oct): 5709-17.

Iannelli, F. and G. Pozzi. 2004. Method for introducing specific and unmarked mutations into the chromosome of *Streptococcus pneumoniae*. *Molecular Biotechnology* 26, (1) (Jan): 81-6.

Imlay, J. A. and I. Fridovich. 1992. Suppression of oxidative envelope damage by pseudoreversion of a superoxide dismutase-deficient mutant of *Escherichia coli*. *Journal of Bacteriology* 174, (3) (Feb): 953-61.

Inaoka, T., Y. Matsumura and T. Tsuchido. 1999. SodA and manganese are essential for resistance to oxidative stress in growing and sporulating cells of *Bacillus subtilis*. *Journal of Bacteriology* 181, (6) (Mar): 1939-43.

Jakubovics, N. S. and H. F. Jenkinson. 2001. Out of the iron age: New insights into the critical role of manganese homeostasis in bacteria. *Microbiology* 147, (Pt 7) (Jul): 1709-18.

Jakubovics, N. S., A. W. Smith and H. F. Jenkinson. 2002. Oxidative stress tolerance is manganese (Mn(2+)) regulated in *Streptococcus gordonii*. *Microbiology* 148, (Pt 10) (Oct): 3255-63.

Jarva, H., T. S. Jokiranta, R. Wurzner and S. Meri. 2003. Complement resistance mechanisms of streptococci. *Molecular Immunology* 40, (2-4) (Sep): 95-107.

Jedrzejas, M. J. 2001. Pneumococcal virulence factors: Structure and function. *Microbiology and Molecular Biology Reviews* 65, (2) (Jun): 187,207.

Jomaa, M., J. M. Kyd and A. W. Cripps. 2005. Mucosal immunisation with novel *Streptococcus pneumoniae* protein antigens enhances bacterial clearance in an acute mouse lung infection model. *FEMS Immunology and Medical Microbiology* 44, (1) (Apr 1): 59-67.

Jones, V. F., C. Harrison, G. G. Stout and J. Hopkins. 2005. Nasopharyngeal colonization with heptavalent pneumococcal conjugate vaccine serotypes of *Streptococcus pneumoniae* with prolonged vaccine dosing intervals. *The Pediatric Infectious Disease Journal* 24, (11) (Nov): 969-73.

Joris, B., S. Englebert, C. P. Chu, R. Kariyama, L. Daneo-Moore, G. D. Shockman and J. M. Ghuysen. 1992. Modular design of the *Enterococcus hirae* muramidase-2 and *Streptococcus faecalis* autolysin. *FEMS Microbiology Letters* 70, (3) (Mar 15): 257-64.

Jounblat, R., A. Kadioglu, T. J. Mitchell and P. W. Andrew. 2003. Pneumococcal behavior and host responses during bronchopneumonia are affected differently by the cytolytic and complement-activating activities of pneumolysin. *Infection and Immunity* 71, (4) (Apr): 1813-9.

Kadioglu, A. and P. W. Andrew. 2004. The innate immune response to pneumococcal lung infection: The untold story. *Trends in Immunology* 25, (3) (Mar): 143-9.

Kadioglu, A., N. A. Gingles, K. Grattan, A. Kerr, T. J. Mitchell and P. W. Andrew. 2000. Host cellular immune response to pneumococcal lung infection in mice. *Infection and Immunity* 68, (2) (Feb): 492-501.

Kadioglu, A., J. A. Sharpe, I. Lazou, C. Svanborg, C. Ockleford, T. J. Mitchell and P. W. Andrew. 2001. Use of green fluorescent protein in visualisation of pneumococcal invasion of broncho-epithelial cells in vivo. *FEMS Microbiology Letters* 194, (1) (Jan 1): 105-10.

Kadioglu, A. and P. Sheldon. 1998. Steroid pulse therapy for rheumatoid arthritis: Effect on lymphocyte subsets and mononuclear cell adhesion. *British Journal of Rheumatology* 37, (3) (Mar): 282-6.

Kadioglu, A., S. Taylor, F. Iannelli, G. Pozzi, T. J. Mitchell and P. W. Andrew. 2002. Upper and lower respiratory tract infection by *Streptococcus pneumoniae* is affected by pneumolysin deficiency and differences in capsule type. *Infection and Immunity* 70, (6) (Jun): 2886-90.

Kelly, R. and D. Greiff. 1970. Toxicity of pneumococcal neuraminidase. *Infection and Immunity* 2, (1) (Jul): 115-7.

Kelly, R. T., S. Farmer and D. Greiff. 1967. Neuraminidase activities of clinical isolates of *Diplococcus pneumoniae*. *Journal of Bacteriology* 94, (1) (Jul): 272-3.

Kharat, A. S. and A. Tomasz. 2006. Drastic reduction in the virulence of *Streptococcus pneumoniae* expressing type 2 capsular polysaccharide but lacking choline residues in the cell wall. *Molecular Microbiology* 60, (1) (Apr): 93-107.

King, S. J., K. R. Hippe, J. M. Gould, D. Bae, S. Peterson, R. T. Cline, C. Fasching, E. N. Janoff and J. N. Weiser. 2004. Phase variable desialylation of host proteins that bind to *Streptococcus pneumoniae* in vivo and protect the airway. *Molecular Microbiology* 54, (1) (Oct): 159-71.

King, S. J., K. R. Hippe and J. N. Weiser. 2006. Deglycosylation of human glycoconjugates by the sequential activities of exoglycosidases expressed by *Streptococcus pneumoniae*. *Molecular Microbiology* 59, (3) (Feb): 961-74.

King, S. J., A. M. Whatmore and C. G. Dowson. 2005. NanA, a neuraminidase from *Streptococcus pneumoniae*, shows high levels of sequence diversity, at least in part through recombination with *Streptococcus oralis*. *Journal of Bacteriology* 187, (15) (Aug): 5376-86.

Kingsford, C. L., K. Ayanbule and S. L. Salzberg. 2007. Rapid, accurate, computational discovery of Rho-independent transcription terminators illuminates their relationship to DNA uptake. *Genome Biology* 8, (2): R22.

Kinnula, V. L., K. B. Adler, N. J. Ackley and J. D. Crapo. 1992. Release of reactive oxygen species by guinea pig tracheal epithelial cells in vitro. *The American Journal of Physiology* 262, (6 Pt 1) (Jun): L708-12.

Kleineidam, R. G., K. Furuhata, H. Ogura and R. Schauer. 1990. 4-methylumbelliferyl-alpha-glycosides of partially O-acetylated N-acetylneuraminic acids as substrates of bacterial and viral sialidases. *Biological Chemistry Hoppe-Seyler* 371, (8) (Aug): 715-9.

Koistinen, V. 1992. Limited tryptic cleavage of complement factor H abrogates recognition of sialic acid-containing surfaces by the alternative pathway of complement. *The Biochemical Journal* 283, (Pt 2) (Apr 15): 317-9.

Kolenbrander, P. E., R. N. Andersen, R. A. Baker and H. F. Jenkinson. 1998. The adhesion-associated sca operon in *Streptococcus gordonii* encodes an inducible high-affinity ABC transporter for Mn2+ uptake. *Journal of Bacteriology* 180, (2) (Jan): 290-5.

Kremer, L., A. Baulard, J. Estaquier, O. Poulain-Godefroy and C. Locht. 1995. Green fluorescent protein as a new expression marker in mycobacteria. *Molecular Microbiology* 17, (5) (Sep): 913-22.

Krivan, H. C., D. D. Roberts and V. Ginsburg. 1988. Many pulmonary pathogenic bacteria bind specifically to the carbohydrate sequence GalNAc beta 1-4Gal found in some glycolipids. *Proceedings of the National Academy of Sciences of the United States of America* 85, (16) (Aug): 6157-61.

Kyaw, M. H., R. Lynfield, W. Schaffner, A. S. Craig, J. Hadler, A. Reingold, A. R. Thomas, et al. 2006. Effect of introduction of the pneumococcal conjugate vaccine on drug-resistant S*treptococcus pneumoniae*. *The New England Journal of Medicine* 354, (14) (Apr 6): 1455-63.

Lawrence, M. C., P. A. Pilling, V. C. Epa, A. M. Berry, A. D. Ogunniyi and J. C. Paton. 1998. The crystal structure of pneumococcal surface antigen PsaA reveals a metal-binding site and a novel structure for a putative ABC-type binding protein. *Structure (London, England)* 6, (12) (Dec 15): 1553-61.

LeBlanc, D. J., L. N. Lee and A. Abu-Al-Jaibat. 1992. Molecular, genetic, and functional analysis of the basic replicon of pVA380-1, a plasmid of oral streptococcal origin. *Plasmid* 28, (2) (Sep): 130-45.

LeBlanc, D. J., L. N. Lee and J. M. Inamine. 1991. Cloning and nucleotide base sequence analysis of a spectinomycin adenyltransferase AAD(9) determinant from *Enterococcus faecalis*. *Antimicrobial Agents and Chemotherapy* 35, (9) (Sep): 1804-10.

Lefebre, M. and M. Valvano. 2001. In vitro resistance of *Burkholderia cepacia* complex isolates to reactive oxygen species in relation to catalase and superoxide dismutase production. *Microbiology* 147, (Pt 1) (Jan): 97-109.

Ling, E., G. Feldman, M. Portnoi, R. Dagan, K. Overweg, F. Mulholland, V. Chalifa-Caspi, J. Wells and Y. Mizrachi-Nebenzahl. 2004. Glycolytic enzymes associated with the cell surface of *Streptococcus pneumoniae* are antigenic in humans and elicit protective immune responses in the mouse. *Clinical and Experimental Immunology* 138, (2) (Nov): 290-8.

Liochev, S. I. and I. Fridovich. 1999. Superoxide and iron: Partners in crime. *IUBMB Life* 48, (2) (Aug): 157-61.

Lock, R. A., D. Hansman and J. C. Paton. 1992. Comparative efficacy of autolysin and pneumolysin as immunogens protecting mice against infection by streptococcus pneumoniae. *Microbial Pathogenesis* 12, (2) (Feb): 137-43.

Lock, R. A., J. C. Paton and D. Hansman. 1988. Comparative efficacy of pneumococcal neuraminidase and pneumolysin as immunogens protective against *Streptococcus pneumoniae*. *Microbial Pathogenesis* 5, (6) (Dec): 461-7.

Long, J. P., H. H. Tong and T. F. DeMaria. 2004. Immunization with native or recombinant *Streptococcus pneumoniae* neuraminidase affords protection in the chinchilla otitis media model. *Infection and Immunity* 72, (7) (Jul): 4309-13.

López, R. and E. García. 2004. Recent trends on the molecular biology of pneumococcal capsules, lytic enzymes, and bacteriophage. *FEMS Microbiology Reviews* 28, (5) (Nov): 553-80.

López, R., M. P. Gonzalez, E. Garcia, J. L. Garcia and P. Garcia. 2000. Biological roles of two new murein hydrolases of *Streptococcus pneumoniae* representing examples of module shuffling. *Research in Microbiology* 151, (6) (Jul-Aug): 437-43.

Luke, N. R., R. J. Karalus and A. A. Campagnari. 2002. Inactivation of the *Moraxella catarrhalis* superoxide dismutase SodA induces constitutive expression of iron-repressible outer membrane proteins. *Infection and Immunity* 70, (4) (Apr): 1889-95.

Lynch, M. and H. Kuramitsu. 2000. Expression and role of superoxide dismutases (SOD) in pathogenic bacteria. *Microbes and Infection / Institut Pasteur* 2, (10) (Aug): 1245-55.

Macrina, F. L., R. P. Evans, J. A. Tobian, D. L. Hartley, D. B. Clewell and K. R. Jones. 1983. Novel shuttle plasmid vehicles for *Escherichia-Streptococcus* transgeneric cloning. *Gene* 25, (1) (Nov): 145-50.

Macrina, F. L., J. A. Tobian, K. R. Jones, R. P. Evans and D. B. Clewell. 1982. A cloning vector able to replicate in *Escherichia coli* and S*treptococcus sanguis*. *Gene* 19, (3) (Oct): 345-53.

Magee, A. D. and J. Yother. 2001. Requirement for capsule in colonization by *Streptococcus pneumoniae*. *Infection and Immunity* 69, (6) (Jun): 3755-61.

Magnusson, K.E. 1989. Physicochemical properties of bacterial surfaces. *Biochemical Society Transactions* 17:454-458.

Manco, S., F. Hernon, H. Yesilkaya, J. C. Paton, P. W. Andrew and A. Kadioglu. 2006. Pneumococcal neuraminidases A and B both have essential roles during infection of the respiratory tract and sepsis. *Infection and Immunity* 74, (7) (Jul): 4014-20.

Mani, N., L. M. Baddour, D. Q. Offutt, U. Vijaranakul, M. J. Nadakavukaren and R. K. Jayaswal. 1994. Autolysis-defective mutant of *Staphylococcus aureus*: Pathological considerations, genetic mapping and electron microscopic studies. *Infection and Immunity* 62, (4) (Apr): 1406-9.

Margot, P., M. Pagni and D. Karamata. 1999. *Bacillus subtilis* 168 gene *lytF* encodes a gamma-D-glutamate-meso-diaminopimelate muropeptidase

expressed by the alternative vegetative sigma factor, sigmaD. *Microbiology* 145 (Pt 1) (Jan): 57-65.

McAllister, L. J., H. J. Tseng, A. D. Ogunniyi, M. P. Jennings, A. G. McEwan and J. C. Paton. 2004. Molecular analysis of the psa permease complex of *Streptococcus pneumoniae*. *Molecular Microbiology* 53, (3) (Aug): 889-901.

McAvin, J. C., P. A. Reilly, R. M. Roudabush, W. J. Barnes, A. Salmen, G. W. Jackson, K. K. Beninga, et al. 2001. Sensitive and specific method for rapid identification of *Streptococcus pneumoniae* using real-time fluorescence PCR. *Journal of Clinical Microbiology* 39, (10) (Oct): 3446-51.

McCullers, J. A. and K. C. Bartmess. 2003. Role of neuraminidase in lethal synergism between influenza virus and *Streptococcus pneumoniae*. *The Journal of Infectious Diseases* 187, (6) (Mar 15): 1000-9.

McDaniel, L. S., J. S. Sheffield, E. Swiatlo, J. Yother, M. J. Crain and D. E. Briles. 1992. Molecular localization of variable and conserved regions of PspA and identification of additional PspA homologous sequences in *Streptococcus pneumoniae*. *Microbial Pathogenesis* 13, (4) (Oct): 261-9.

McDaniel, L. S. and E. Swiatlo. 2004. Pneumococcal disease: pathogenesis, treatment, and prevention. *Infectious Diseases in Clinical Practice* 12: 93-8.

McMichael, J. C. 2000. Progress toward the development of a vaccine to prevent *Moraxella (branhamella) catarrhalis* infections. *Microbes and Infection* / Institut Pasteur 2, (5) (Apr): 561-8.

Meister, A. and M. E. Anderson. 1983. Glutathione. *Annual Review of Biochemistry* 52: 711-60.

Melo-Cristino, J., L. Santos, M. Ramirez and Grupo de Estudo Português de Bactérias Patogénicas Respiratórias. 2006. The Viriato study: Update of antimicrobial susceptibility data of bacterial pathogens from community-acquired respiratory tract infections in Portugal in 2003 and 2004. *Revista Portuguesa de Pneumologia* 12, (1) (Jan-Feb): 9-30.

Miller, R. A. and B. E. Britigan. 1997. Role of oxidants in microbial pathophysiology. *Clinical Microbiology Reviews* 10, (1) (Jan): 1-18.

Milohanic, E., R. Jonquieres, P. Cossart, P. Berche and J. L. Gaillard. 2001. The autolysin ami contributes to the adhesion of *Listeria monocytogenes* to eukaryotic cells via its cell wall anchor. *Molecular Microbiology* 39, (5) (Mar): 1212-24.

Mitchell, T. J. 2000. Virulence factors and the pathogenesis of disease caused by *Streptococcus pneumoniae*. *Research in Microbiology* 151, (6) (Jul-Aug): 413-9.

Mitchell, T. J., J. E. Alexander, P. J. Morgan and P. W. Andrew. 1997. Molecular analysis of virulence factors of *Streptococcus pneumoniae*. *Society for Applied Bacteriology Symposium Series* 26: 62S-71S.

Mitchell, T. J., P. W. Andrew, F. K. Saunders, A. N. Smith and G. J. Boulnois. 1991. Complement activation and antibody binding by pneumolysin via a region of the toxin homologous to a human acute-phase protein. *Molecular Microbiology* 5, (8) (Aug): 1883-8.

Moncada, S. and A. Higgs. 1993. The L-arginine-nitric oxide pathway. *The New England Journal of Medicine* 329, (27) (Dec 30): 2002-12.

Moncla, B. J., P. Braham and S. L. Hillier. 1990. Sialidase (neuraminidase) activity among Gram-negative anaerobic and capnophilic bacteria. *Journal of Clinical Microbiology* 28, (3) (Mar): 422-5.

Moore, H. F. 1915. The action of ethylhydrocuprein (optochin) on type strains of pneumococci in vitro and in vivo, and on some other microorganisms in vitro *Journal of Experimental Medicine* 22: 269.

Morise, H., O. Shimomura, F. H. Johnson and J. Winant. 1974. Intermolecular energy transfer in the bioluminescent system of *Aequorea*. *Biochemistry* 13, (12) (Jun 4): 2656-62.

Morona, J. K., R. Morona and J. C. Paton. 2006. Attachment of capsular polysaccharide to the cell wall of *Streptococcus pneumoniae* type 2 is required for invasive disease. *Proceedings of the National Academy of Sciences of the United States of America* 103, (22) (May 30): 8505-10.

Moscoso, M., E. Lopez, E. Garcia and R. Lopez. 2005. Implications of physiological studies based on genomic sequences: *Streptococcus pneumoniae* TIGR4 synthesizes a functional LytC lysozyme. *Journal of Bacteriology* 187, (17) (Sep): 6238-41.

Moustafa, I., H. Connaris, M. Taylor, V. Zaitsev, J. C. Wilson, M. J. Kiefel, M. von Itzstein and G. Taylor. 2004. Sialic acid recognition by *Vibrio cholerae* neuraminidase. *The Journal of Biological Chemistry* 279, (39) (Sep 24): 40819-26.

Mudd, S., F. Heinmets and T. F. Anderson. 1943. Bacterial morphology as shown by the electron microscope: VI. Capsule, cell-wall and inner protoplasm of pneumococcus, type III. *Journal of Bacteriology* 46, (2) (Aug): 205-11.

Nakayama, K. 1992. Nucleotide sequence of *Streptococcus mutans* superoxide dismutase gene and isolation of insertion mutants. *Journal of Bacteriology* 174, (15) (Aug): 4928-34.

Nathan, C. 2003. Specificity of a third kind: Reactive oxygen and nitrogen intermediates in cell signaling. *The Journal of Clinical Investigation* 111, (6) (Mar): 769-78.

Navarre, W. W. and O. Schneewind. 1999. Surface proteins of gram-positive bacteria and mechanisms of their targeting to the cell wall envelope. *Microbiology and Molecular Biology Reviews* 63, (1) (Mar): 174-229.

Ng, E. W., J. R. Costa, N. Samiy, K. L. Ruoff, E. Connolly, F. V. Cousins and D. J. D'Amico. 2002. Contribution of pneumolysin and autolysin to the pathogenesis of experimental pneumococcal endophthalmitis. *Retina* 22, (5) (Oct): 622-32.

No authors. 1985. Acute respiratory infections in under-fives: 15 million deaths a year. *The Lancet* 326, (8457) (Sep 28): 699-701.

Normark, B. H. and S. Normark. 2002. Antibiotic tolerance in pneumococci. *Clinical Microbiology and Infection: The Official Publication of the European Society of Clinical Microbiology and Infectious Diseases* 8, (10) (Oct): 613-22.

Novak, R., B. Henriques, E. Charpentier, S. Normark and E. Tuomanen. 1999. Emergence of vancomycin tolerance in *Streptococcus pneumoniae*. *Nature* 399, (6736) (Jun 10): 590-3.

Nuermberger, E., K. Helke and W. R. Bishai. 2005. Low-dose aerosol model of pneumococcal pneumonia in the mouse: utility for evaluation of antimicrobial efficacy. *International Journal of Antimicrobial Agents* 26:497–503.

O'Connell, D. 2005. Pneumococcus forks out... *Nature Reviews Microbiology* 3, (February): 99.

Oggioni, M. R., G. Memmi, T. Maggi, D. Chiavolini, F. Iannelli and G. Pozzi. 2003. Pneumococcal zinc metalloproteinase ZmpC cleaves human matrix metalloproteinase 9 and is a virulence factor in experimental pneumonia. *Molecular Microbiology* 49, (3) (Aug): 795-805.

Oggioni, M. R., C. Trappetti, A. Kadioglu, M. Cassone, F. Iannelli, S. Ricci, P. W. Andrew and G. Pozzi. 2006. Switch from planktonic to sessile life: A major event in pneumococcal pathogenesis. *Molecular Microbiology* 61, (5) (Sep): 1196-210.

Orihuela, C. J., G. Gao, K. P. Francis, J. Yu and E. I. Tuomanen. 2004. Tissue-specific contributions of pneumococcal virulence factors to pathogenesis. *The Journal of Infectious Diseases* 190, (9) (Nov 1): 1661-9.

Orihuela, C. J., R. Janssen, C. W. Robb, D. A. Watson and D. W. Niesel. 2000. Peritoneal culture alters *Streptococcus pneumoniae* protein profiles and virulence properties. *Infection and Immunity* 68, (10) (Oct): 6082-6.

Ormo, M., A. B. Cubitt, K. Kallio, L. A. Gross, R. Y. Tsien and S. J. Remington. 1996. Crystal structure of the *Aequorea victoria* green fluorescent protein. *Science* 273, (5280) (Sep 6): 1392-5.

O'Toole, R. D., L. Goode and C. Howe. 1971. Neuraminidase activity in bacterial meningitis. *The Journal of Clinical Investigation* 50, (5) (May): 979-85.

O'Toole, R. D. and W. L. Stahl. 1975. Experimental pneumococcal meningitis: effects of neuraminidase and other pneumococcal constituents on cerebrospinal fluid in the intact dog. *Journal of the Neurological Sciences* 26, (2) (Oct): 167-78.

Ottolenghi-Nightingale, E. 1972. Competence of pneumococcal isolates and bacterial transformations in man. *Infection and Immunity* 6, (5) (Nov): 785-92.

Owen, R. H., G. J. Boulnois, P. W. Andrew and T. J. Mitchell. 1994. A role in cell-binding for the C-terminus of pneumolysin, the thiol-activated toxin of *Streptococcus pneumoniae*. *FEMS Microbiology Letters* 121, (2) (Aug 15): 217-21.

Pagliero, E., O. Dideberg, T. Vernet and A. M. Di Guilmi. 2005. The PECACE domain: A new family of enzymes with potential peptidoglycan cleavage activity in Gram-positive bacteria. *BMC Genomics* 6, (1) (Feb 17): 19.

Paterson, G. K. and T. J. Mitchell. 2006. The role of *Streptococcus pneumoniae* sortase A in colonisation and pathogenesis. *Microbes and Infection / Institut Pasteur* 8, (1) (Jan): 145-53.

Paton, J. C., P. W. Andrew, G. J. Boulnois and T. J. Mitchell. 1993. Molecular analysis of the pathogenicity of *Streptococcus pneumoniae*: The role of pneumococcal proteins. *Annual Review of Microbiology* 47: 89-115.

Paton, J. C., A. M. Berry and R. A. Lock. 1997. Molecular analysis of putative pneumococcal virulence proteins. *Microbial Drug Resistance* 3, (1) (Spring): 1-10.

Paton, J. C., R. A. Lock and D. J. Hansman. 1983. Effect of immunization with pneumolysin on survival time of mice challenged with *Streptococcus pneumoniae*. *Infection and Immunity* 40, (2) (May): 548-52.

Pericone, C. D., D. Bae, M. Shchepetov, T. McCool and J. N. Weiser. 2002. Short-sequence tandem and nontandem DNA repeats and endogenous hydrogen peroxide production contribute to genetic instability of *Streptococcus pneumoniae*. *Journal of Bacteriology* 184, (16) (Aug): 4392-9.

Pericone, C. D., K. Overweg, P. W. Hermans and J. N. Weiser. 2000. Inhibitory and bactericidal effects of hydrogen peroxide production by *Streptococcus pneumoniae* on other inhabitants of the upper respiratory tract. *Infection and Immunity* 68, (7) (Jul): 3990-7.

Pericone, C. D., S. Park, J. A. Imlay and J. N. Weiser. 2003. Factors contributing to hydrogen peroxide resistance in *Streptococcus pneumoniae* include pyruvate oxidase (SpxB) and avoidance of the toxic effects of the Fenton reaction. *Journal of Bacteriology* 185, (23) (Dec): 6815-25.

Pertea, M., K. Ayanbule, M. Smedinghoff and S. L. Salzberg. 2009. OperonDB: A comprehensive database of predicted operons in microbial genomes. Nucleic Acids Research 37, (Jan): D479-82.

Pesakhov, S., R. Benisty, N. Sikron, Z. Cohen, P. Gomelsky, I. Khozin-Goldberg, R. Dagan and N. Porat. 2007. Effect of hydrogen peroxide production and the fenton reaction on membrane composition of S*treptococcus pneumoniae*. *Biochimica Et Biophysica Acta* 1768, (3) (Mar): 590-7.

Pesci, E. C., D. L. Cottle and C. L. Pickett. 1994. Genetic, enzymatic, and pathogenic studies of the iron superoxide dismutase of *Campylobacter jejuni*. *Infection and Immunity* 62, (7) (Jul): 2687-94.

Pettigrew, M. M., K. P. Fennie, M. P. York, J. Daniels and F. Ghaffar. 2006. Variation in the presence of neuraminidase genes among *Streptococcus pneumoniae* isolates with identical sequence types. *Infection and Immunity* 74, (6) (Jun): 3360-5.

Poyart, C., E. Pellegrini, O. Gaillot, C. Boumaila, M. Baptista and P. Trieu-Cuot. 2001. Contribution of Mn-cofactored superoxide dismutase (SodA) to the virulence of *Streptococcus agalactiae*. *Infection and Immunity* 69, (8) (Aug): 5098-106.

Prasher, D. C., V. K. Eckenrode, W. W. Ward, F. G. Prendergast and M. J. Cormier. 1992. Primary structure of the *Aequorea victoria* green-fluorescent protein. *Gene* 111, (2) (Feb 15): 229-33.

Puchelle, E., F. Girard, N. Houdret and V. Bailleul. 1975. Action of neuraminidases isolated from *Diplococcus pneumoniae* and *Clostridium perfringens* on the viscosity and elastic properties of bronchial secretions. *Biorheology* 12, (3-4) (Jun): 219-24.

Puget, K. and A. M. Michelson. 1974. Isolation of a new copper-containing superoxide dismutase bacteriocuprein. *Biochemical and Biophysical Research Communications* 58, (3) (Jun 4): 830-8.

Purdy, D., S. Cawthraw, J. H. Dickinson, D. G. Newell and S. F. Park. 1999. Generation of a superoxide dismutase (SOD)-deficient mutant of *Campylobacter coli*: Evidence for the significance of SOD in campylobacter survival and colonization. *Applied and Environmental Microbiology* 65, (6) (Jun): 2540-6.

Reddy, M. S., J. M. Bernstein, T. F. Murphy and H. S. Faden. 1996. Binding between outer membrane proteins of nontypeable *Haemophilus influenzae* and human nasopharyngeal mucin. *Infection and Immunity* 64, (4) (Apr): 1477-9.

Revai, K., D. P. McCormick, J. Patel, J. J. Grady, K. Saeed and T. Chonmaitree. 2006. Effect of pneumococcal conjugate vaccine on nasopharyngeal bacterial colonization during acute otitis media. *Pediatrics* 117, (5) (May): 1823-9.

Rey, M. W., P. Ramaiya, B. A. Nelson, S. D. Brody-Karpin, E. J. Zaretsky, M. Tang, A. Lopez de Leon, et al. 2004. Complete genome sequence of the

industrial bacterium *Bacillus licheniformis* and comparisons with closely related bacillus species. *Genome Biology* 5, (10): R77.

Roggentin, P., B. Rothe, J. B. Kaper, J. Galen, L. Lawrisuk, E. R. Vimr and R. Schauer. 1989. Conserved sequences in bacterial and viral sialidases. *Glycoconjugate Journal* 6, (3): 349-53.

Roggentin, P., R. Schauer, L. L. Hoyer and E. R. Vimr. 1993. The sialidase superfamily and its spread by horizontal gene transfer. *Molecular Microbiology* 9, (5) (Sep): 915-21.

Romero, A., R. Lopez and P. Garcia. 1990. Sequence of the *Streptococcus pneumoniae* bacteriophage HB-3 amidase reveals high homology with the major host autolysin. *Journal of Bacteriology* 172, (9) (Sep): 5064-70.

Ronda, C., J. L. Garcia, E. Garcia, J. M. Sanchez-Puelles and R. Lopez. 1987. Biological role of the pneumococcal amidase: cloning of the *lytA* gene in *Streptococcus pneumoniae*. *European Journal of Biochemistry / FEBS* 164, (3) (May 4): 621-4.

Rosenberg, A. 2004. Pneumococcus virulence factor sialidase: A new direction in neuro-AIDS research? *Journal of Neuroimmunology* 147, (1-2) (Feb): 33-4.

Rosenow, C., P. Ryan, J. N. Weiser, S. Johnson, P. Fontan, A. Ortqvist and H. R. Masure. 1997. Contribution of novel choline-binding proteins to adherence, colonization and immunogenicity of *Streptococcus pneumoniae*. *Molecular Microbiology* 25, (5) (Sep): 819-29.

Rotimi, V. O. and B. I. Duerden. 1981. The development of the bacterial flora in normal neonates. *Journal of Medical Microbiology* 14, (1) (Feb): 51-62.

Rubins, J. B., D. Charboneau, J. C. Paton, T. J. Mitchell, P. W. Andrew and E. N. Janoff. 1995. Dual function of pneumolysin in the early pathogenesis of murine pneumococcal pneumonia. *The Journal of Clinical Investigation* 95, (1) (Jan): 142-50.

Russell, R. R., J. Aduse-Opoku, I. C. Sutcliffe, L. Tao and J. J. Ferretti. 1992. A binding protein-dependent transport system in *Streptococcus mutans* responsible for multiple sugar metabolism. *Journal of Biological Chemistry* 267, (7) (Mar 5): 4631-7.

Ryan, K. J. and C. G. Ray, Eds. 2004. *Sherris medical microbiology*. 4th Ed. New York: McGraw Hill.

Ryan, P. A., V. Pancholi and V. A. Fischetti. 2001. Group A streptococci bind to mucin and human pharyngeal cells through sialic acid-containing receptors. *Infection and Immunity* 69, (12) (Dec): 7402-12.

Saiki, R. K., D. H. Gelfand, S. Stoffel, S. J. Scharf, R. Higuchi, G. T. Horn, K. B. Mullis and H. A. Erlich. 1988. Primer-directed enzymatic amplification of DNA with a thermostable DNA polymerase. *Science* 239, (4839) (Jan 29): 487-91.

Salton, M. R. 1953. Studies of the bacterial cell wall. IV. The composition of the cell walls of some gram-positive and gram-negative bacteria. *Biochimica Et Biophysica Acta* 10, (4) (Apr): 512-23.

Sambrook, J. 1989. *Molecular cloning: A laboratory manual*, Ed. Edward F. Fritsch. 2nd Ed. Cold Spring Harbor, N.Y. Cold Spring Harbor Laboratory.

Samukawa, T., N. Yamanaka, S. Hollingshead, K. Klingman and H. Faden. 2000. Immune responses to specific antigens of *Streptococcus pneumoniae* and *Moraxella catarrhalis* in the respiratory tract. *Infection and Immunity* 68, (3) (Mar): 1569-73.

Sanchéz-Puelles, J. M., C. Ronda, J. L. Garcia, P. Garcia, R. Lopez and E. Garcia. 1986. Searching for autolysin functions: characterization of a pneumococcal mutant deleted in the *lytA* gene. *European Journal of Biochemistry / FEBS* 158, (2) (Jul 15): 289-93.

Sanders, J. W., K. J. Leenhouts, A. J. Haandrikman, G. Venemaand J. Kok. 1995. Stress response in *Lactococcus lactis*: Cloning, expression analysis, and

mutation of the lactococcal superoxide dismutase gene. *Journal of Bacteriology* 177, (18) (Sep): 5254-60.

Sato, K., M. K. Quartey, C. L. Liebeler, C. T. Le and G. S. Giebink. 1996. Roles of autolysin and pneumolysin in middle ear inflammation caused by a type 3 *Streptococcus pneumoniae* strain in the chinchilla otitis media model. *Infection and Immunity* 64, (4) (Apr): 1140-5.

Scanlon, K. L., W. F. Diven and R. H. Glew. 1989. Purification and properties of *Streptococcus pneumoniae* neuraminidase. *Enzyme* 41, (3): 143-50.

Schauer, R. 2000. Achievements and challenges of sialic acid research. *Glycoconjugate Journal* 17, (7-9) (Jul-Sep): 485-99.

Schauer, R. 1982. Chemistry, metabolism, and biological functions of sialic acids. *Advances in Carbohydrate Chemistry and Biochemistry* 40: 131-234.

Seaver, L. C. and J. A. Imlay. 2004. Are respiratory enzymes the primary sources of intracellular hydrogen peroxide? *Journal of Biological Chemistry* 279, (47) (Nov 19): 48742-50.

Seyler, R. W.,Jr, J. W. Olson and R. J. Maier. 2001. Superoxide dismutase-deficient mutants of *Helicobacter pylori* are hypersensitive to oxidative stress and defective in host colonization. *Infection and Immunity* 69, (6) (Jun): 4034-40.

Shakhnovich, E. A., S. J. King and J. N. Weiser. 2002. Neuraminidase expressed by *Streptococcus pneumoniae* desialylates the lipopolysaccharide of *Neisseria meningitidis* and *Haemophilus influenzae*: A paradigm for interbacterial competition among pathogens of the human respiratory tract. *Infection and Immunity* 70, (12) (Dec): 7161-4.

Sheppard, C. L., T. G. Harrison, R. Morris, A. Hogan and R. C. George. 2004. Autolysin-targeted LightCycler assay including internal process control for detection of *Streptococcus pneumoniae* DNA in clinical samples. *Journal of Medical Microbiology* 53, (Pt 3) (Mar): 189-95.

Shimomura, O. 1979. Structure of the chromophore of *Aequorea* green fluorescent protein. *FEBS Letters* 104: 220-2.

Shimomura, O., F. H. Johnson and Y. Saiga. 1962. Extraction, purification and properties of aequorin, a bioluminescent protein from the luminous hydromedusan, *Aequorea*. *Journal of Cellular and Comparative Physiology* 59, (Jun): 223-39.

Shinefield, H. R., S. Black, P. Ray, I. Chang, N. Lewis, B. Fireman, J. Hackell, et al. 1999. Safety and immunogenicity of heptavalent pneumococcal CRM197 conjugate vaccine in infants and toddlers. *The Pediatric Infectious Disease Journal* 18, (9) (Sep): 757-63.

Sicard, A. M. 1964. A new synthetic medium for *Diplococcus pneumoniae*, and its use for the study of reciprocal transformations at the amia locus. *Genetics* 50, (Jul): 31-44.

Sinave, C. P. 2004. Pneumococcal infections *in* www.eMedicine.com. Omaha, Nebraska, [cited 05/05/2006] Available from http://www.emedicine.com/MED/topic1848.htm.

Smith, B. L. and M. K. Hostetter. 2000. C3 as substrate for adhesion of *Streptococcus pneumoniae*. *The Journal of Infectious Diseases* 182, (2) (Aug): 497-508.

Smith, H. 2000. Questions about the behaviour of bacterial pathogens in vivo. *Philosophical Transactions of the Royal Society of London. Series B, Biological Sciences* 355, (1397) (May 29): 551-64.

Soong, G., A. Muir, M. I. Gomez, J. Waks, B. Reddy, P. Planet, P. K. Singh, et al. 2006. Bacterial neuraminidase facilitates mucosal infection by participating in biofilm production. *The Journal of Clinical Investigation* 116, (8) (Aug): 2297-305.

Spellerberg, B., D. R. Cundell, J. Sandros, B. J. Pearce, I. Idanpaan-Heikkila, C. Rosenow and H. R. Masure. 1996. Pyruvate oxidase, as a determinant of

virulence in *Streptococcus pneumoniae*. *Molecular Microbiology* 19, (4) (Feb): 803-13.

Storz, G., L. A. Tartaglia and B. N. Ames. 1990. The OxyR regulon. *Antonie Van Leeuwenhoek* 58, (3) (Oct): 157-61.

Sun, K., F. E. Johansen, L. Eckmann and D. W. Metzger. 2004. An important role for polymeric Ig receptor-mediated transport of IgA in protection against *Streptococcus pneumoniae* nasopharyngeal carriage. *Journal of Immunology (Baltimore, Md: 1950)* 173, (7) (Oct 1): 4576-81.

Swiatlo, E., F. R. Champlin, S. C. Holman, W. W. Wilson and J. M. Watt. 2002. Contribution of choline-binding proteins to cell surface properties of *Streptococcus pneumoniae*. *Infection and Immunity* 70, (1) (Jan): 412-5.

Swiatlo, E. and D. Ware. 2003. Novel vaccine strategies with protein antigens of *Streptococcus pneumoniae*. *FEMS Immunology and Medical Microbiology* 38, (1) (Aug 18): 1-7.

Taira, S. and H. Nariuchi. 1988. Possible role of neuraminidase in activated T cells in the recognition of allogeneic Ia. *Journal of Immunology (Baltimore, Md: 1950)* 141, (2) (Jul 15): 440-6.

Tano, K., E. Grahn-Hakansson, S. E. Holm and S. Hellstrom. 2000. Inhibition of OM pathogens by alpha-hemolytic streptococci from healthy children, children with SOM and children with rAOM. *International Journal of Pediatric Otorhinolaryngology* 56, (3) (Dec 22): 185-90.

Taylor, G. 1996. Sialidases: Structures, biological significance and therapeutic potential. *Current Opinion in Structural Biology* 6, (6) (Dec): 830-7.

Tettelin, H., K. E. Nelson, I. T. Paulsen, J. A. Eisen, T. D. Read, S. Peterson, J. Heidelberg, et al. 2001. Complete genome sequence of a virulent isolate of *Streptococcus pneumoniae*. *Science* 293, (5529) (Jul 20): 498-506.

Todar, K. 2003. *Todar's online textbook of bacteriology*. University of Wisconsin-Madison Department of Bacteriology. Available from http://www.textbookofbacteriology.net/

Tomasz, A. 1967. Choline in the cell wall of a bacterium: Novel type of polymer-linked choline in pneumococcus. *Science* 157, (789) (Aug 11): 694-7.

Tong, H. H., L. E. Blue, M. A. James and T. F. DeMaria. 2000. Evaluation of the virulence of a *Streptococcus pneumoniae* neuraminidase-deficient mutant in nasopharyngeal colonization and development of otitis media in the chinchilla model. *Infection and Immunity* 68, (2) (Feb): 921-4.

Tong, H. H., M. James, I. Grants, X. Liu, G. Shi and T. F. DeMaria. 2001. Comparison of structural changes of cell surface carbohydrates in the eustachian tube epithelium of chinchillas infected with a *Streptococcus pneumoniae* neuraminidase-deficient mutant or its isogenic parent strain. *Microbial Pathogenesis* 31, (6) (Dec): 309-17.

Tong, H. H., D. Li, S. Chen, J. P. Long and T. F. DeMaria. 2005. Immunization with recombinant *Streptococcus pneumoniae* neuraminidase NanA protects chinchillas against nasopharyngeal colonization. *Infection and Immunity* 73, (11) (Nov): 7775-8.

Tong, H. H., X. Liu, Y. Chen, M. James and T. Demaria. 2002. Effect of neuraminidase on receptor-mediated adherence of *Streptococcus pneumoniae* to chinchilla tracheal epithelium. *Acta Oto-Laryngologica* 122, (4) (Jun): 413-9.

Touati, D. 1989. The molecular genetics of superoxide dismutase in *E. coli*. An approach to understanding the biological role and regulation of SOD's in relation to other elements of the defence system against oxygen toxicity. *Free Radical Research Communications* 8, (1): 1-9.

Tsien, R. Y. 1998. The green fluorescent protein. *Annual Review of Biochemistry* 67: 509-44.

Tuomanen, E. 1999. Molecular and cellular biology of pneumococcal infection. *Current Opinion in Microbiology* 2, (1) (Feb): 35-9.

Tuomanen, E., H. Liu, B. Hengstler, O. Zak, and A. Tomasz. 1985. The induction of meningeal inflammation by components of the pneumococcal cell wall. *The Journal of Infectious Diseases* 151, (5) (May): 859-68.

Tuomanen, E. I., R. Austrian and H. R. Masure. 1995. Pathogenesis of pneumococcal infection. *The New England Journal of Medicine* 332, (19) (May 11): 1280-4.

Valdivia, R. H. and B. P. Cormack. 2006. The uses of green fluorescent protein in prokaryotes *in Green fluorescent protein: Properties, applications and protocols.* Ed. M. Chalfie. 2nd Ed., p 163-178. New Jersey, USA: Wiley & Sons, Inc. 443 pp.

Valdivia, R. H., A. E. Hromockyj, D. Monack, L. Ramakrishnan and S. Falkow. 1996. Applications for green fluorescent protein (GFP) in the study of host-pathogen interactions. *Gene* 173, (1 Spec No): 47-52.

Varki, A. 1997. Sialic acids as ligands in recognition phenomena. *The FASEB Journal: Official Publication of the Federation of American Societies for Experimental Biology* 11, (4) (Mar): 248-55.

Veluraja, K. and V. S. R. Rao. 1984. Studies on the conformations of sialyloligosaccharides and implications on the binding specificity of neuraminidases. *Journal of Bioscience* 6, (5): 625-34.

Versieck, J. 1985. Trace elements in human body fluids and tissues. *Critical Reviews in Clinical Laboratory Sciences* 22, (2): 97-184.

Voet, D. 1995. Biochemistry. Eds. Judith G. Voet. 2nd Ed. New York: J. Wiley & Sons.

Vollmer, W. and A. Tomasz. 2001. Identification of the teichoic acid phosphorylcholine esterase in *Streptococcus pneumoniae*. *Molecular Microbiology* 39, (6) (Mar): 1610-22.

von Itzstein, M., W. Y. Wu, G. B. Kok, M. S. Pegg, J. C. Dyason, B. Jin, T. Van Phan, M. L. Smythe, H. F. White and S. W. Oliver. 1993. Rational design of potent sialidase-based inhibitors of influenza virus replication. *Nature* 363, (6428) (Jun 3): 418-23.

Wadwa, R. P. and R. D. Feigin. 1999. Pneumococcal vaccine: An update. *Pediatrics* 103, (5 Pt 1) (May): 1035-7.

Walsh, F. M. and S. G. Amyes. 2004. Microbiology and drug resistance mechanisms of fully resistant pathogens. *Current Opinion in Microbiology* 7, (5) (Oct): 439-44.

Wani, J. H., J. V. Gilbert, A. G. Plaut and J. N. Weiser. 1996. Identification, cloning, and sequencing of the immunoglobulin A1 protease gene of *Streptococcus pneumoniae*. *Infection and Immunity* 64, (10) (Oct): 3967-74.

Ward, W. W. 2006. Biochemical and physical properties of green fluorescent protein *in Green fluorescent protein: Properties, applications and protocols.* Ed. M. Chalfie. 2nd Ed., p 39-65. New Jersey, USA: Wiley & Sons, Inc. 443 pp.

Watts, A. G., P. Oppezzo, S. G. Withers, P. M. Alzari and A. Buschiazzo. 2006. Structural and kinetic analysis of two covalent sialosyl-enzyme intermediates on *Trypanosoma rangeli* sialidase. *The Journal of Biological Chemistry* 281, (7) (Feb 17): 4149-55.

Weiser, J. N., R. Austrian, P. K. Sreenivasan and H. R. Masure. 1994. Phase variation in pneumococcal opacity: Relationship between colonial morphology and nasopharyngeal colonization. *Infection and Immunity* 62, (6) (Jun): 2582-9.

Weiser, J. N., D. Bae, C. Fasching, R. W. Scamurra, A. J. Ratner and E. N. Janoff. 2003. Antibody-enhanced pneumococcal adherence requires IgA1 protease. *Proceedings of the National Academy of Sciences of the United States of America* 100, (7) (Apr 1): 4215-20.

Wellmer, A., G. Zysk, J. Gerber, T. Kunst, M. Von Mering, S. Bunkowski, H. Eiffert and R. Nau. 2002. Decreased virulence of a pneumolysin-deficient strain of *Streptococcus pneumoniae* in murine meningitis. *Infection and Immunity* 70, (11) (Nov): 6504-8.

Whatmore, A. M., A. Efstratiou, A. P. Pickerill, K. Broughton, G. Woodard, D. Sturgeon, R. George and C. G. Dowson. 2000. Genetic relationships between clinical isolates of *Streptococcus pneumoniae*, *Streptococcus oralis*, and *Streptococcus mitis*: Characterization of "atypical" pneumococci and organisms allied to *S. mitis* harboring *S. pneumoniae* virulence factor-encoding genes. *Infection and Immunity* 68, (3) (Mar): 1374-82.

WHO, World Health Organisation. 1999. Pneumococcal vaccines. *Weekly Epidemiological Record* 74: 177-83.

Wiggins, R., S. J. Hicks, P. W. Soothill, M. R. Millar and A. P. Corfield. 2001. Mucinases and sialidases: Their role in the pathogenesis of sexually transmitted infections in the female genital tract. *Sexually Transmitted Infections* 77, (6) (Dec): 402-8.

Wilks, K. E., K. L. Dunn, J. L. Farrant, K. M. Reddin, A. R. Gorringe, P. R. Langford and J. S. Kroll. 1998. Periplasmic superoxide dismutase in meningococcal pathogenicity. *Infection and Immunity* 66, (1) (Jan): 213-7.

Winter, A. J., S. D. Comis, M. P. Osborne, M. J. Tarlow, J. Stephen, P. W. Andrew, J. Hill and T. J. Mitchell. 1997. A role for pneumolysin but not neuraminidase in the hearing loss and cochlear damage induced by experimental pneumococcal meningitis in guinea pigs. *Infection and Immunity* 65, (11) (Nov): 4411-8.

WOIN. 2004. Vaccination: A major cause of serious illness and mortality, pneumococcal infection is fully preventable by vaccination. *World of Irish Nursing* 12, (9): 48-49.

Woods, J. M., R. C. Bethell, J. A. Coates, N. Healy, S. A. Hiscox, B. A. Pearson, D. M. Ryan, J. Ticehurst, J. Tilling and S. M. Walcott. 1993. 4-guanidino-2,4-

dideoxy-2,3-dehydro-N-acetylneuraminic acid is a highly effective inhibitor both of the sialidase (neuraminidase) and of growth of a wide range of influenza A and B viruses in vitro. *Antimicrobial Agents and Chemotherapy* 37, (7) (Jul): 1473-9.

Wu, H. Y., A. Virolainen, B. Mathews, J. King, M. W. Russell and D. E. Briles. 1997. Establishment of a *Streptococcus pneumoniae* nasopharyngeal colonization model in adult mice. *Microbial Pathogenesis* 23, (3) (Sep): 127-37.

Xu, G., X. Li, P. W. Andrew and G. L. Taylor. 2008b. Structure of the catalytic domain of *Streptococcus pneumoniae* sialidase NanA. *Acta Crystallographica.Section F, Structural Biology and Crystallization Communications* 64, (Pt 9) (Sep 1): 772-5.

Xu, G., J. A. Potter, R. J. Russell, M. R. Oggioni, P. W. Andrew and G. L. Taylor. 2008a. Crystal structure of the NanB sialidase from *Streptococcus pneumoniae*. *Journal of Molecular Biology* 384, (2) (Dec 12): 436-49.

Yamamoto, N., K. Kawakami, Y. Kinjo, K. Miyagi, T. Kinjo, K. Uezu, C. Nakasone, M. Nakamatsu and A. Saito. 2004. Essential role for the p40 subunit of interleukin-12 in neutrophil-mediated early host defense against pulmonary infection with *Streptococcus pneumoniae*: Involvement of interferon-gamma. *Microbes and Infection* / Institut Pasteur 6, (14) (Nov): 1241-9.

Yesilkaya, H. 1999. Studies on the role of superoxide dismutase (SOD) in the virulence of *Streptococcus pneumoniae* and the effects of interferon gamma on sensitivity of phagocytes to the toxin pneumolysin. PhD., University of Leicester.

Yesilkaya, H., A. Kadioglu, N. Gingles, J. E. Alexander, T. J. Mitchell and P. W. Andrew. 2000. Role of manganese-containing superoxide dismutase in oxidative stress and virulence of *Streptococcus pneumoniae*. *Infection and Immunity* 68, (5) (May): 2819-26.

Yesilkaya, H., S. Manco, A. Kadioglu, V. S. Terra and P. W. Andrew. 2008. The ability to utilize mucin affects the regulation of virulence gene expression in *Streptococcus pneumoniae*. *FEMS Microbiology Letters* 278, (2) (Jan): 231-5.

Yesilkaya, H., S. Soma-Haddrick, S. J. Crennell and P. W. Andrew. 2006. Identification of amino acids essential for catalytic activity of pneumococcal neuraminidase A. *Research in Microbiology* 157, (6) (Jul-Aug): 569-74.

Youn, H. D., E. J. Kim, J. H. Roe, Y. C. Hah and S. O. Kang. 1996. A novel nickel-containing superoxide dismutase from *Streptomyces spp. The Biochemical Journal* 318 (Pt3) (Sep 15): 889-96.

Young, B and J.W. Heath. 1993. Wheater's Functional Histology: a text and colour atlas. 3rd Ed. Elsevier Ltd. UK. 413 pp.

Zheng, H. Y., T. M. Alcorn and M. S. Cohen. 1994. Effects of H2O2-producing lactobacilli on *Neisseria gonorrhoeae* growth and catalase activity. *The Journal of Infectious Diseases* 170, (5) (Nov): 1209-15.