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

Thesis submitted for the degree of Doctor of Philosophy at the University of Leicester

by

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Statement of Originality

The accompanying thesis submitted for the degree of Ph.D. entitled "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" is based on work conducted by the author in the Department of Microbiology and Immunology of the University of Leicester mainly during the period between September 1994 and March 1998.

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.

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ABSTRACT

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Title: 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

Infections caused by *Streptococcus pneumoniae*, pneumonia, bacteraemia, otitis media and meningitis, are among the leading causes of illness and death. Increasing antibiotic resistance and drawbacks of the current vaccine have increased the need for a better understanding of the pathogenesis of pneumococcal diseases. Two aspects related to virulence factors were investigated.

The major point of the study was into how the pneumococcus copes with reactive derivatives of oxygen. Superoxide dismutase is one of the enzymes that provides protection against deleterious effects of superoxide radical by conversion of superoxide to hydrogen peroxide and molecular oxygen. In this study, it was shown that *S. pneumoniae* contains two types of superoxide dismutase, MnSOD and FeSOD. While the level of MnSOD increased during growth in an aerobic environment, the amount of FeSOD remained unchanged. An isogenic *sodA* insertion-mutant strain of *S. pneumoniae* was constructed to study the role for the enzyme *in vitro* and *in vivo*. Aerobically the mutant strain of *S. pneumoniae*, designated as D39HY1, had a lower growth rate than the wild type and exhibited susceptibility to the redox active compound, paraquat. Anaerobic growth of D39HY1 was identical to the wild-type. Virulence studies showed that the median survival time of mice infected intranasally with D39HY1 was significantly longer than mice infected with the wild type pneumococcus. In contrast to the wild-type, D39HY1 did not multiply in lungs during the first 24h but thereafter grew at the same rate as the wild-type. Appearance in the bloodstream also was delayed but growth in the blood was unimpaired by the *sodA* mutation.

The aim of the second project was to assess whether IFN-γ may enhance resistance of macrophages to the deleterious effects of bacterial products. To assess this possibility, the effects of interferon gamma on sensitivity of phagocytes to the toxin pneumolysin, an important virulence determinant of *S. pneumoniae*, was tested by employing respiratory burst as an assay of sublytic effects. It was observed that IFN-γ can alter the susceptibility of cells to the lytic effect of pneumolysin. Also the respiratory burst of IFN-γ treated cells was insensitive to sublytic concentrations of pneumolysin.

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Abbreviations

BAB	Blood Agar Base plates
BHI	Brain Heart Infusion broth
bp	Base pair
BSA	Bovine serum albumin
CIP	Calf intestinal phosphatase
CRP	C-Reactive protein
CPS	Capsular polysaccharide
EDTA	Diaminoethantetra-acetic acid
GlcNAcβ1-3Gal	*N*-acetyl-D-glucosamine β1-3 galactose
GalNAcβ1-3Gal	*N*-acetyl-D-galactosamine β1-3 galactose
GalNAcβ1-4Gal	*N*-acetyl-D-galactosamine β1-4 galactose
HU	Haemolytic unit
IFN	Interferon
IL	Interleukin
Ig	Immunoglobulin
kb	Kilobase
kDa	Kilodaltons
LA	Luria agar
LB	Luria broth
LD	Lethal dose
MPO	Myeloperoxidase
NANA	N-acetylneuraminic acid
ORF	Open reading frame
PAGE	Polyacrylamide gel electrophoresis
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PEG	Polyethylene glycol
PMNL	Polymorphonuclear leukocytes
PMA	Phorbol Myristate Acetate
PspA	Pneumococcal surface proteinA
ROS	Reactive oxygen species
SOD	Superoxide dismutase
SD	Shine Dalgarno
SDS	Sodium dodecyl sulphate
SSC	Saline Sodium Citrate
TE	Tris-EDTA
TEMED	N, N, N, N'-tetramethylethyleethylenediamine
UV	Ultraviolet
X-Gal	5-bromo-4-choloro-3-inodyl-b-D-galactopyranoside

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Chapter 1

General Introduction and Methods

Part 1 General Introduction

1.1 General characteristics of *Streptococcus pneumoniae*

Streptococcus pneumoniae, alternatively known as 'the pneumococcus', is one of the most important causative agents of pneumonia, bacteraemia, otitis media and meningitis. The bacterium was first isolated simultaneously by both Pasteur (1881) and Sternberg (1881) in 1880 following development of fatal septicaemia in rabbits injected with human salvia. After this initial discovery, the pneumococcus was identified as an important pathogen of man (reviewed by Austrian, 1984). Recently the horse was also identified as a second host for the organism after isolation of a type 3 strain in tracheobronchial aspirates of horses. However no direct evidence has been found relating the presence of pneumococcus to a specific disease syndrome in the horse (Chanter, 1997).

S. pneumoniae belongs to the genus *Streptococcus*, in the family *Streptococcaceae*, which is composed of Gram-positive, coccoidal bacteria. The organism grows as lancet shaped diplococci in pairs and short chains (Sneath *et al.*, 1986). When grown on the surface of blood agar, the bacterium forms α-haemolytic mucoid colonies under aerobic conditions (Parker, 1983). Anaerobically the organism exhibits β-haemolysis and this is independent of hydrogen peroxide and pneumolysin (Canvin *et al.*, 1996). The pneumococcus is sensitive to ethylhydrocupreine HCl (optichin), ferments insulin and autolyses rapidly when exposed to detergents (Austrian, 1984). The optimum pH range for the organism is 7-7.8 and it can grow in the temperature range 25-42°C. Metabolically, *S. pneumoniae* is a fermentative facultative anaerobic organism and grows better in the presence of 5% CO_2. It cannot synthesise haem compounds and therefore it is catalase negative. For this reason, a source of catalase such as red blood cells should be added into culture media (Rotta, 1986).

1.2 The impact of *S. pneumoniae* on human health

Pneumococcal infections are among the leading causes of illness and death for young children, the elderly and people with underlying debilitating medical conditions worldwide (Table 1.1) (Breiman *et al.*, 1994). *Streptococcus pneumoniae* is one of the main pathogens of acute lower respiratory tract infections in children under the age of five, in which the devastating effect of pneumococcal diseases is reflected by the deaths of almost five million children per year in third world countries (reviewed by Andrew *et al.*, 1994). However, pneumococcal diseases are not just a problem of poor countries, people in developed countries also suffer from a high rate of incidence and increasing costs of treatment. Estimated figures from the United States suggest that *S. pneumoniae* is responsible for 3,000 cases of meningitis, 50,000 cases of bacteraemia, 500,000 cases of pneumonia, 7,000,000 cases of otitis media, each year (MMWR, 1996).

Table 1.1: The etiologic causes of community acquired pneumonia (Macfarlane, 1994)

Etiologic Agents	Percentage
Streptococcus pneumoniae	36%
Legionella pneumophila	28%
Stapylococcus aureus	9%
Haemophilus influenzae	6%
Chlamydia psittaci	1%
Viral	10%
Varicella	6%
Other	3%

Although the pneumococcus is an important cause of the disease, it is also a component of the human upper respiratory tract normal flora (Austrian, 1984). Colonisation by the organism starts so early that 59% of the population will have carried the organism by the twelfth postnatal day (Riley and Douglas, 1981). The organism can be detected in 2% to 35%

of the population, depending on the age of the individual (Hendley *et al.*, 1975). However, it is thought that the rate of carriage is underestimated due to the lack of sensitive detection methods, otherwise carriage rates could be as high as 60% of the population (Kertulla *et al.*, 1987; Farrington and Rubenstein, 1991). Little is known about why an organism which can have a commensal relationship with humans, can at the same time cause such morbidity and mortality (Boulnois, 1992). What is known is that there are predisposing factors which damage the balance of commensalism.

1.3 Predisposing factors for pneumococcal diseases

Immunocompetent adults who carry the pneumococcus are at much lower risk of contracting pneumococcal diseases (Austrian, 1986). The factors that make one prone to pneumococcal diseases are numerous. Broadly, these are either related to environment or host. Environmental factors such as poor living conditions and seasonal changes, high especially in winter were linked to high incidence rate (Austrian, 1984; Gray *et al.*, 1982). Nasopharyngeal accusation of new strains of pneumococci as a result of exposure to smoke and nasal discharge also lead to increased risk of pneumococcal diseases (Austrian, 1986; Riley and Douglas, 1981).

Predisposing factors that are related to host cover wide range of disorders including inefficient pulmonary clearance mechanisms due to smoking, chronic bronchitis, asthma, chronic obstructive pulmonary disease, and lung cancer (Musher, 1992). In addition, there are several host factors that enhance the occurrence of pneumococcal diseases, such as extremes of age, sickle cell disease, cardiopulmonary disease (Landesman and Schiffman, 1981), Hodgkin's disease (Amman *et al.*, 1981), splenectomi, cirrhosis of the liver (Johnston, 1981), chronic alcoholism (Burmen *et al.*, 1985), renal transplantation, diabetes mellitus (Schwartz, 1982) congenital deficiencies in either immunoglobulin or complement (Johnston, 1981; Gucikan *et al.*, 1980) and defects of phogocytic function (Johnson, 1991).

The presence of viral infection that breach the integrity of the lower respiratory epithelium such as, influenzae A infection was shown to effect the development of pneumococcal diseases in mice (Harford *et al.*, 1949; Harford and Hara, 1950). Therefore, intact respiratory mucosa have

an important protective role against pneumococcal diseases. It is also well known that there is a strong correlation between acquired immunodeficiency syndrome (AIDS) and pneumococcal disease, such that level of pneumococcal diseases was used to estimate the size of the immunosuppressed HIV-infected population in New Jersey (Schuchat, 1991).

1.4 Pathologic development of the disease

Pneumonia. Pneumococcal pneumonia develops as a result of the migration of the bacteria from the nasopharynx to alveoli (Rake, 1936). In pneumococcal pneumonia, the pathologic pattern may proceed either towards lobar or bronchopneumonia (Kumar *et al.*, 1992).

Lobar pneumonia has four overlapping characteristic stages (Mac Sween and Whaley, 1992). In the first stage, alveoli are filled with serous exudate containing bacteria, serum, and phagocytic cells. It is known that the autolysis of bacteria is necessary for the initiation of inflammation, since pneumococcal sonicate, not whole viable organisms can initiate chemotactic activity in a dog model of pneumonia (Jutila *et al.*, 1992). The second phase is characterised by congestion of capillaries which gives the lungs a red appearance (red hepatization). It is at this stage that erythrocytes, neutrophils and macrophages infiltrate into alveolar spaces and fibrin accumulates in the alveoli. As infection progresses, the number of erythrocytes decreases and the number of phagocytic cells increases because the excessive amount of exudate in alveoli pressurises the capillaries and stops the entry of red blood cells (Loosli and Baker, 1962). Due to the change in the composition of inflammatory cells, lungs change colour from red to grey (grey hepatization). In the final stage the disease may proceed in two directions: a) Removal of debris from alveoli and dispersion of infiltrating cells by enzymatic activity and macrophages in which case lung tissue remains intact and resolution of inflammation and restoration of the lung occur (Johnston, 1991) or b) if bacteria are not removed by host defence mechanisms, then the proliferation and spread of the bacteria within lungs may occur via the pores of Kohn leading to the formation of bacteraemia, meningitis, arthritis, infective endocarditis and death (Kumar *et al.*, 1992).

In bronchopneumonia, on the other hand, the lesions are centred around bronchioles in patches throughout one or several lobes (Kumar *et al.*, 1992). The inflammatory areas of tissue are surrounded by healthy tissue sections. In severe cases, the inflammatory areas become larger and covers the whole lobe to produce the lobar pneumonia (Mac Sween and Whaley, 1992). Microscopically, bronchopneumonia consists of focal suppurative exudate rich in neutrophils which fills the bronchi, bronchioles, and adjacent alveolar spaces (Kumar *et al.*, 1992).

Bacteraemia. Pneumococcal bacteraemia often develops as a complication of pneumonia (Boulnois, 1992). Although it is not known exactly where bacteria gain access to the blood, highly vascular nasopharyngeal mucosa and lower respiratory lymphatics were suggested as the possible entry sites (Rake, 1936; Schulz *et al.*, 1938). The outcome of pneumococcal bacteraemia is poor and antibiotic administration often fails to reverse this poor prognosis (Johnston, 1991; Dick & Gemmell, 1971). Events leading to death in pneumococcal bacteraemia involve cardiopulmonary dysfunction with leukopenia, pulmonary leukostasis, and shock (Reed *et al.*, 1984).

Meningitis. Pneumococcal meningitis arises as a consequence of transmigration of bacteria from blood across the blood brain barrier into the cerebrospinal fluid (Tuomanen, 1996). However, meningitis rarely follows after intravenous inoculation of animals (Moxon, 1982). It was observed that the pneumococci rapidly appear in the subarachnoid space after intra nasal infection of the mouse probably via the olfactory mucosa (Rake, 1937) involving the perineural space around olfactory neurones (Moxon, 1982). Head traumas damaging the integrity of dura mater also lead to direct entry of the organism into the central neural system (Musher, 1992).

Initiation of inflammation requires significant bacterial growth in cerebrospinal fluid after which the onset of symptoms occur (Tuomanen, 1996). Inflammatory reactions in cerebrospinal fluid involve the generation of chemotactic factors, appearance of complement mediated opsonic activity, immunoglobulins and polymorphonuclear neutrophils (PMNs) (Tuomanen *et al.*, 1985). In addition, an excessive host response also contributes to inflammatory reactions by creating oedema which

ultimately causes loss of neuroglial function (Dodge and Swartz, 1965) and impaired cerebral blood flow (McMenamin and Volpe, 1984). This is the reason why clinicians simultaneously apply steroids to impair host defence systems during the early phase of antibiotic therapy, particularly in childhood meningitis (Lebel *et al.*, 1988).

Otitis Media. It is speculated that the pneumococcus emanates from upper pharyngeal mucosa into middle ear cavity through the Eustachian tube (Andersson *et al.*, 1981). Bacterial invasion of middle ear can also be the part of a complex infection. As evidenced by Giebnik (1981), pneumococcal otitis media infections increase in a chinchilla model of otitis media when there is a simultaneous influenza A virus infection. The influenza infection apparently generates a negative pressure in the middle ear cavity by the obstruction of normal drainage of Eustachian tube (Giebnik, 1981). The inflammatory changes in otitis involve increased vascular permeability followed by neutrophil influx, increasing vascular permeability for larger proteins and lysozyme and arachidonic acid metabolites in middle ear fluid (Nonomura *et al.*, 1991a; Nonomura *et al.*, 1991b).

1.5 Host defence against *S. pneumoniae*

The primary defence line against the pneumococcus is formed by alveolar macrophages and recruited polymorphonuclear leukocytes (PMNL) (Perry *et al.*, 1994). The pneumococcus is very sensitive to antimicrobial action of phagocytes and it is efficiently killed once ingested (Johnston, 1991). However, efficient killing relies on opsonization processes in which opsonins (C-reactive protein, complement and antibody) interact with particles (such as bacteria) to render them more readily ingested and killed by phagocytes (Fine *et al.*, 1988). It has been suggested that in the absence of opsonins the pneumococcus is resistant to phagocytosis (Brown *et al.*, 1982). This ability was attributed to the presence of a hydrophobic capsular polysaccharide which interferes with direct cell to cell contact (Van Dam *et al.*, 1990). The bactericidal function of phagocytic cells is initiated after attachment of phagocytes to the opsonized pneumococci via immunglobulin or complement receptors (Bruyn *et al.*, 1992).

C-reactive protein (CRP), produced in large amounts during inflammation, is an acute phase protein and can bind to *S. pneumoniae* (Mold *et al.*, 1982; Horowitz *et al*, 1987). The protection provided by CRP was demonstrated using human CRP-transgenic mice (CRP is not an acute-phase protein in mice) (Szalai *et al.*, 1996). It was shown that human CRP-transgenic mice capable of mounting an acute-phase CRP response exhibited reduced bacteraemia, increased life span and reduced mortality after infection with *S. pneumoniae* (Szalai *et al.*, 1996). The mechanism of action of CRP is related to its ability to activate the classical complement pathway by interacting with pneumococcal cell wall phosphorylcholine or, in some serotypes, with the capsule (Horowitz *et al.*, 1987; Briles *et al.*, 1989). However, a study carried out with BALB/c mice showed that CRP protected decomplemented mice from fatal pneumococcal infection, indicating an opsonic activity of CRP that is independent of complement (Nakayama *et al.*, 1983). The presence of an intact complement system, nevertheless, enhances full expression of CRP-mediated protection, as shown by comparison of the course of *S. pneumoniae* infection in CRP-transgenic mice with a functional complement system and nontransgenic mice to that of infection in mice decomplemented with cobra venom factor (Szalai *et al.*, 1996). The results revealed that the transgene significantly reduced bacteraemia regardless of the presence of the complement system, but only mice with a functional complement system exhibited significantly longer median survival time and lower mortality.

Antibody production against pneumococci is initiated by infection or vaccination with polyvalent pneumococcal vaccine (Bruyn *et al.*, 1991). In addition, in healthy carriers antibodies to capsular polysaccharide (CPS) can be detected (Rynnel-Dagoo *et al.*, 1986). Type specific anti-CPS antibodies have been shown to protect various animal species and humans from pneumococcal infection (MacLeod *et al.*, 1945; Heidelberger *et al.*, 1946). Type specific antibodies to CPS exhibit their effect partly by their own opsonic activity and partly by increasing the deposition of C3b on the pneumococci (Brown *et al.*, 1983). These antibodies mainly belong to IgG2 subclass in adults but in children they are predominantly IgG1 subclass (Freijd *et al.*, 1984). Phagocytes require IgG1 and IgG2 antibodies for effective opsonization (Lortan *et al.*, 1993).

In addition to type specific antibody (IgG1 and IgG2) efficient opsonisation requires the involvement of complement (Guckian *et al.*, 1980; Winkelstein, 1981). *S. pneumoniae* is able to activate the complement cascade both through alternative and classical pathway. Regardless of the pathway, activation results in the formation of bound C3b and its major degradation product, iC3b. Both of them are capable of forming a ligand for phagocytic receptors (Winkelstein *et al.*, 1981). It has been demonstrated that activation and fixation of C3 can occur more rapidly through the classical pathway than through the alternative pathway (Winkelstein, 1984). Classical complement pathway activation necessitates the presence of IgG and IgM antibodies (Brown *et al.*, 1982). On the other hand, the alternative complement pathway is activated in the absence of type specific antibody and is very important in a non-immune host (Winkelstein *et al.*, 1972). The pneumococcal cell wall appears to play a major role for this type activation (Fischer *et al.*, 1997). However, neutrophil complement receptors can not bind easily to C3b molecules on the cell wall, since the capsule makes this interaction difficult (Brown *et al.*, 1983).

1.6 Antimicrobial therapy and vaccination

1.6.1 Therapy

Penicillin has been widely used against pneumococcal infection since the 1960s. However, since then penicillin resistant strains emerged worldwide, so much so that the penicillin dose required to treat pneumococcal infections has increased from <0.02 mg/ml to 0.05-0.1 mg/ml from the early antibiotic era to present day (Musher, 1992). Moreover, some clinical isolates are resistant to multiple antimicrobial drugs and some are susceptible only to vancomycin (MMWR, 1996). Penicillin exhibits its microbicidal ability by acting on penicillin binding proteins. Therefore, any alterations in the composition of penicillin-binding protein leads to the development of resistance to the drug (Tomasz, 1982). Attempts are being made to control the rapid emergence of drug resistant strains by implementation of a laboratory based surveillance system for reporting invasive drug resistant infections (MMWR, 1996).

1.6.2 Vaccine

The present pneumococcal vaccines are composed of 23 different capsular polysaccharides selected from the 90 capsular serotypes of *S. pneumoniae* (Andrew *et al.*, 1994; Feldman and Klugman, 1997). Despite the recommended use for individuals who are at high risk for pneumococcal infections e.g. splenectomised individuals, patients suffering from chronic pulmonary, renal or cardiac disease, immunocompromised persons, and persons over 65 years of age receiving regular medical care, there are some limitations associated with the current vaccine (Andrew *et al.*, 1994). These vaccines were found to be protective, particularly in immunocompetent adults, but not in immunocompromised, elderly and children under two years of age (Sims *et al.*, 1988). As proved by clinical trials, the vaccine is effective in 93% of under-55s, whereas antibody levels generally decreased with increasing age independently of gender (Shapiro *et al.*, 1991; Kurtti *et al.*, 1998). The hyporeponsiveness of the aged to the vaccine was shown to be due to a lack of accessory cell function in the aged mouse model, since the response of the aged mouse spleen cells was restored by supplementation with accessory cells from young mice or a variety of cytokines such as IL-1, IL-4 and IL-5 (Garg *et al.*, 1996).

The serotypes included in the vaccine are the most frequent cause of disease in the USA, Canada and Europe, covering 85% to 91% of isolates (Smart *et al.*, 1987). However, the present vaccine represents only 63% of clinical isolates from Taiwan and less than 28% from Japan (Lee *et al.*, 1991). Therefore, because of the serotype specificity of its action the effectiveness of the vaccine may not be geographically uniform. It is also known that serotypes causing disease can differ over time (Austrian, 1984). The suppression of the immune system in, for example, HIV and sickle cell disease patients, also limits the response to vaccine (Janoff *et al.*, 1992; Barrett and Ammann, 1981). Ineffectiveness of the vaccine in children under two years of age was related to the immunogenic properties of polysaccharides (Andrew *et al.*, 1994). Polysaccharide antigens are type 2, thymus-independent antigens. This group of antigens induce poor memory and are poor immunogens, especially in infants under two years of age (Mosier and Subbarao, 1982; reviewed by Peeters *et al.*, 1991). Immunogenicity of polysaccharide in infants varies with serotype. Some polysaccharides are good immunogens regardless of

the age of the recipient, such as type 3, whereas type 6 and 19, some of the important pediatric pneumococcal types, are particularly poor immunogens over a wide age range (Cowan *et al.*, 1978; Douglas *et al.*, 1983).

In order to increase the effectiveness of the current vaccine, researchers have focused on polysaccharide-protein conjugates because unlike carbohydrate immunogens, proteins are thymus-dependent, memory-inducing immunogens (Scheerson *et al.*, 1986). Therefore, conjugation of polysaccharides to proteins enhances the immunogenicity of polysaccharides and induces immunological memory (Mitchell *et al.*, 1998). The current successes of *Haemophilus influenzae* type b polysaccharide-protein conjugate vaccines in preventing disease in infants led pneumococcal researchers to take a similar approach (Andrew *et al.*, 1994). For example, 19F polysaccharide, a poor immunogen in infant mice and children, has been conjugated with genetically engineered derivatives of pneumococcal cytolytic toxin, pneumolysin (Paton *et al.*, 1991). This conjugate induced higher levels of anti-polysaccharide antibodies in mice than polysaccharide alone. In addition, immunisation of mice with this conjugate resulted in a booster effect and protection, unlike capsular polysaccharide alone.

Despite the improved effectiveness of conjugate vaccines some researchers still believe that linking protein carriers to capsular polysaccharide will not solve all the problems of current vaccine. Support of this conclusion comes from the variations among disease-causing serotypes, in terms of geographic, and temporal variation (Andrew *et al.*, 1994). This means that the disease causing serotypes of the pneumococcus change not only from one country to another but it changes in the one country over time. Therefore, there is still a need for other antigens that can provide cross-serotype protection (Andrew *et al.*, 1994), and ideally have a role in the pathogenesis, so that antibodies produced against that specific molecule could neutralise the activity of the molecule associated with pathogenesis. It was reported that pneumococcal surface-proteinA and pneumolysin fit these criteria (Paton, 1998).

Part 2 Virulence Determinants

2.1 Description of Virulence

The term virulence or pathogenity refers to the ability of a bacterium to cause disease (Abbas *et al.*, 1994). 'Virulence factor' or 'mechanism of pathogenesis' is used to describe a bacterial product or a strategy that contributes to virulence. Despite the simple description of virulence factors, some researchers believe that it is not always easy to decide what bacterial features or products directly connect with the infection process (Gordon and Andrew, 1996). In this regard some bacterial characteristics, such as the ability of the bacteria to adhere to mucosal cells or to produce toxic products can be clearly associated with the infectious process. However, in the case of some other bacterial traits, such as DNA replication or ATP generation, which are essential for bacterial survival in the host are described as 'house-keeping' functions rather than virulence factors (Abbas *et al.*, 1994).

In the 1880s Koch published a set of principles, known as Koch's postulates (Gordon and Andrew, 1996). These postulates attempted to show the connection between a bacterial pathogen and a particular disease. The current advances in molecular biology resulted in an analogous set of principles for defining virulence factors. These are known as Molecular Koch's Postulates (Falkow, 1988). Thus, there are some criteria defining a particular gene or its product as a virulence factor. First, the gene (or its product) should always be found in virulent strains of the bacterium that causes the disease. Second, disrupting the gene should reduce the virulence, and the introduction of an intact copy to the mutated organism should reinstate the virulence. Third, the gene must be expressed in the host organism *in vivo*. Finally, immunisation with the gene product should elicit a protective immune response (Finlay and Falkow, 1989).

The pneumococcus possesses several virulence factors that are assumed to contribute to the pathogenicity of pneumococci in terms of promotion and maintenance of the carrier state, and the development of disease. Some of these are discussed below.

2.2 Adhesion

It has been suggested that bacterial adherence to mucosal surfaces is an important determinant of colonisation and the pathogenesis of most infections (Rayner *et al.*, 1995). Nasopharyngeal epithelium is the first colonisation site for the pneumococcus (Yin Wu *et al.*, 1997). However, the pneumococcus binds poorly to ciliated epithelial cells of respiratory mucosa unless there is a damage caused by pneumolysin or a viral infection (Rayner *et al.*, 1995; Plotkowski *et al.*, 1986).

S. pneumoniae binds to receptors containing glycoconjugate, on the host cell surface. It is presumed that nasopharengyeal attachment is mediated by *N*-acetyl-D-glucosamine β1-3 galactose (GlcNAcβ1-3Gal) receptors (Andersson *et al.*, 1983) while preferential attachment to type II pneumocytes and resident epithelial cells is mediated by *N*-acetyl-D-galactosamine β1-3 galactose (GalNAcβ1-3Gal) or *N*-acetyl-D-galactosamine β1-4 galactose (GalNAcβ1-4Gal) (Cundell and Tuomanen, 1994; Cundell *et al.*, 1995a; Cundell *et al.*, 1995b).

The nature of pneumococcal adhesins is thought to be protein, since attachment is destroyed after treatment of whole bacteria with heat, trypsin, pepsin or formalin (Andersson *et al.*, 1988). Some researchers suggested that pneumococcal capsule also plays a role in adhesion (Cundell and Tuomanen, 1994; Talbot *et al.*, 1996). In a recent report it was shown that capsule significantly attenuates invasion and adherence of the pneumococcus to epithelial A549 cells (Talbot *et al.*, 1996). But this finding contrasts with the previous report published by Cundell and Tuomanen (1994) stating that there is no difference in adherence of encapsulated and nonencapsulated pneumococci to primary rabbit lung or A549 cells. The conflicting results may be related to the use of different serotypes in these studies (Talbot *et al.*, 1996)

The expression of pneumococcal adhesins is regulated by pneumococcal proteins, including pyruvate oxidase and peptide permeases. A mutant deficient in *spxB* gene, which encodes for pyruvate oxidase, showed 70% loss in the ability to attach to all cell types and decreased adherence to GlcNAcβ1-3Gal, GalNAcβ1-4Gal and carbohydrate GlcNAc receptors (Spellerberg *et al.*, 1996). The decreased adhesion of *spxB* mutant was related to its reduced production of intracellular messenger acetyl

phosphate. Peptide permeases were also shown to modulate pneumococcal adherence to epithelial and endothelial cells either by acting directly as adhesins or by modulating the expression of adhesins on the pneumococcal surface during the initial stages of colonisation of the lung or the vascular endothelium (Cundell *et al.*, 1995c). PsaA which has sequence similarity to other streptococcal peptide permeases was shown to be involved in bacterial coaggregation and adherence to teeth, but their direct role as adhesins for eukaryotic cells is unknown (Tuomanen, 1996). In addition, peptide methionine sulfoxide reductase, a repair enzyme, was suggested to have a role in the proper expression or maintenance of functional adhesins on the surface of *S. pneumoniae,* since a strain deficient in this enzyme exhibited 75% reduction in its ability to bind to GalNacβ1-4Gal containing eukaryotic cell receptors that are present on type II lung cells and vascular endothelial cells (Wizemann *et al.*, 1996).

The role of neuraminidase and IgA1 protease in colonisation will be separately discussed.

2.3 Capsule

The basis for the serological typing of *S. pneumoniae* comes from 90 different capsular polysaccharides (CPS) that form hydrophilic gels on the surface of the organism (Bruyn *et al.*, 1992; Paton, 1998). Capsule, despite its lack of toxicity to animals or humans (Tuomanen *et al.*, 1987), is the key determinant of the virulence of the pneumococcus (Austrian, 1984). In the absence of capsule, the LD_{50} increased by a factor of 10^6 (Watson and Musher, 1990). Once capsule is removed by the administration of an enzyme which depolymerizes the polysaccharide, the nonencapsulated mutant strain can be easily eliminated by the infected host's defence mechanism (Avery and Dubos, 1931).

Different encapsulated strains exhibit different biological characteristics. Variations among encapsulated strains of pneumococci have been observed in relation to their resistance to phagocytosis, stimulation of antibody response, activation of the alternative pathway of complement, deposition and degradation of complement fragment C3b and penetration into tissues (reviewed by Bruyn *et al.*, 1992). Investigations carried out to determine any correlation between virulence and the

amount of capsule produced by each serotype indicated that chemical composition, rather than quantity, is important for virulence (Kenecht *et al.*, 1970).

The capsule's role in the virulence of organism is attributed to its ability to prevent complement depositing on the cell wall. Also the pneumococcal capsule is antiphagocytic by itself. As a result of this, pneumococci are not efficiently opsonised, since interaction of bound complement with receptors on phagocytic cells does not materialise (Briles *et al.*, 1989). In addition, capsule may shield the surface-associated virulence proteins from circulating antibodies (Watson *et al.*, 1995).

2.4 IgA1 protease

Bacterial IgA1 proteases specifically cleave human IgA1 in the hinge region (Reinholdt *et al.*, 1990). IgA1 is an important protective element of mucosal membranes of the upper respiratory tracts in humans (Gilbert *et al.*, 1988). Therefore, cleavage of this antibody may contribute to the virulence of some pathogenic bacteria (Plout, 1983). It is known that IgA1 protease activity is present in bacteria that colonise mucosal membranes including *H. influenzae, Neisseria meningitidis, Neisseria gonorrhoeae, Ureaplasma urealyticum,* and *S. pneumoniae* (Poulsen *et al.*, 1996).

The pneumococcal IgA1 protease gene was cloned by independent groups and the presence of the enzyme was shown in different pneumococcal strains (Poulsen *et al.*, 1996; Wani *et al.*, 1996). Comparison of the deduced amino acid sequence with other known IgA1 proteases indicated that the pneumococcal IgA1 protease is a zinc-containing metalloprotease of approximately 200 kDa. An isogenic mutant strain of *S. pneumoniae* deficient of IgA protease was made (Wani *et al.*, 1996). However, since the protease is specific to human IgA1 the mutant strain can not be tested for the biological function of the enzyme because of the lack of a suitable animal model.

2.5 Neuraminidase

Neuraminidase is an enzyme which catalyses the removal of sialic acid from mucins, glycoproteins and gangliosides, which are components of cell surfaces and body fluids (Scanlon, 1989). Since sialic acids contribute

to the structure of several biological molecules, the source of targets for neuraminidase is abundant (Camara, 1992).

The number of neuraminidases produced by the pneumococcus is disputed. Earlier data indicated the presence of several pneumococcal neurominidase isoenzeymes (Tanenbaum *et al.*, 1970). However some other researchers suggested the existence of a single enzyme and the reason for molecular weight heterogeneity was thought to be due to the susceptibility of the enzyme to proteolytic cleavage during purification (Berry *et al.*, 1988). To date two distinct genes, *nanA* and *nanB*, encoding for neuraminidase were cloned (Berry *et al.* 1988; Camara *et al.*, 1991), and both genes were found in all isolates tested (Camara *et al.*, 1991).

The mechanism of virulence associated with neuraminidase was related to its ability to decrease the viscosity of mucus, which may compromise the protective role of mucosal surface (Scanlon *et al.*, 1989). In addition, it was postulated that neuraminidase shows its effect as a result of exposure of receptors that allow pneumococcal attachment (Anderson *et al.*, 1983)

Several points of indirect evidence suggest a role for neuraminidase in disease. The first indication that neuraminidase might contribute to the virulence of the pneumococcus came with the discovery of high levels of N-acetylneuraminic acid (NANA), a product produced as a result of catalytic activity of the neuraminidase, in the cerebrospinal fluid of patients with pneumococcal meningitis (O'Toole, 1971). The level of NANA directly correlated with poor prognosis (O'Toole and Stahl, 1975). Recently, an isogenic *nanA* mutant strain of *S. pneumoniae* was constructed by insertion duplication method (Dr. J. Hill, unpublished data) and its virulence in mice was studied (Winter *et al.*, 1997). Analysis of the mutant strain in a mouse model of pneumonia showed that the NanA-negative mutant was less virulent than the wild type strain. In addition, the numbers of mutant organisms in the lung remained the same over 3 days after infection whereas the number of wild-type organisms increased by 4 logs over 2 days, after which the animals had died (Dr. J. Hill, unpublished data). These results suggest that neuraminidase is a virulence factor in mice. Neuraminidase's role in the virulence of the organism combined with its expression in all

serotypes and its cell surface location (Camara, 1992) makes the enzyme a potential vaccine candidate.

2.6 Pneumococcal surface proteinA (PspA)

Pneumococcal surface proteinA (PspA) is a cell surface molecule, and is found in many clinical and laboratory isolates (Crain *et al.*, 1990). Although not required for bacterial growth, it is essential for full virulence of the organism (McDaniel *et al.*, 1987). For example, PspA negative pneumococci are cleared more efficiently from the blood of non-immune mice and mice challenged with a PspA negative strain survived longer than the mice infected with parent strain (McDaniel *et al.*, 1987). The mechanism of action of PspA has not been clearly defined but it is thought that it interferes with complement fixation (Briles *et al.*, 1996). PspA shows molecular weight heterogeneity between strains, ranging from 60 to 200 kDa, and it is also serologically very variable (Crain *et al.*, 1990). However, despite the serologic variation, antibodies elicited for one serotype are protective against infection caused by other serotypes, as immunisation with purified PspA protected CBA/N mice from subsequent iv challenge with 10^4 cfu of six different serotypes (McDaniel *et al.*, 1991). It has been proposed that a vaccine composed of a carefully chosen mixture of PspA molecules will be able to elicit protective immunity to virtually all pneumococci (Briles *et al.*, 1996). A recent study showed that intranasal immunisation of mice with PspA induced mucosal and systemic immunity and provided long-lasting protection against carriage of *S. pneumoniae* (Wu *et al.*, 1997). Furthermore, resistance to carriage was suggested to be due to mucosal rather than systemic immunity and was effective against heterologous PspA types (Wu *et al.*, 1997).

2.7 Autolysin

The pneumococcus produces cell wall hydrolysing enzymes that are called N-acetylmuramyl-L-alanine amidase or autolysins (Holtje and Tomasz, 1976). Normally this enzyme is inactive since it is thought to be bound to Forssman antigen in the cell wall (Briese and Hakenbeck, 1985). However, under some special conditions in which the cell wall biosynthesis is disrupted, such as nutrient starvation or treatment with antibiotics, autolysin is activated and causes cell wall degradation

resulting in the release of pneumolysin, and highly inflammatory cell wall breakdown products (Chetty and Kreger, 1980; Berry *et al.*, 1989).

The most precise data about autolysin's role in virulence of *S. pneumoniae* was obtained with the mutant strain of the pneumococcus carrying insertional mutation in autolysin gene. When mice were infected with an autolysin deficient strain of *S. pneumoniae*, the mice infected with the mutant strain survived significantly longer than the wild type infected group (Berry *et al.*, 1989). The protective effect of autolysin was shown when mice immunised with purified autolysin had significantly longer survival time as compared to nonimmunised mice after intranasal challenge with wild type (Berry *et al.*, 1989). Nevertheless, these findings contrasted with Tomasz and coworkers' (1988) report which suggested that an autolysin mutant strain demonstrated the same degree of virulence as the parent strain. Differences between the findings were thought to be the result of the pneumococcal strain used (Berry *et al.*, 1989). In a separate study the rapid clearance of an autolysin-negative mutant from the lungs of mice and absence of the mutant in the blood was shown (Canvin *et al.*, 1995). When these researchers examined the lungs and blood of mice infected with the mutant strain, no inflammation or hematologic changes could be found, suggesting the importance of autolysin in pathologic changes that are taking place during pneumococcal infections.

2.8 Hyaluronidase

Hyaluronidase is an enzyme whose substrate is hyaluronic acid, a component of mammalian connective tissue and the extracellular matrix (Humphrey, 1944). This enzyme is produced by a number of wound and mucosal pathogens, as well as *S. pneumoniae* (Humphrey, 1944). The enzyme is believed to facilitate the migration of the organism between tissues (Russel and Sterwood, 1948). As a matter of fact hyaluronidase activity was found to be high in strains causing meningitis in Russia (Kostyukova *et al.*, 1995). It was found that 39 strains isolated from human meningitis cases expressed high levels of hyaluronidase, whereas only 15 of 130 isolates from carriers had hyaluronidase expression. Furthermore, when the isolates from meningitis cases were used for intranasal infection of mice, they could be detected in the brain, unlike strains not producing hyaluronidase (Kostyukova *et al.*, 1995).

Virulence studies carried out with a hyaluronidase-negative pneumococcal strain also provided a direct evidence for hyaluronidase's involvement in pneumococcal pathogenesis (Mitchell *et al.*, 1997). When the mutant strain was used for intranasal infection of mice, the group infected with the mutant strain survived significantly longer than the wild type group. Analysis of bacterial growth characteristics showed that the mutant strain grew poorly compared to the wild type in the lung of infected mice. Also a dramatic decrease in blood bacterial counts was noted, although both the strains appeared in blood at the same time post infection (Mitchell *et al.*, 1997).

2.9 Pneumolysin

As part of this thesis contains a work related to pneumolysin (Chapter 3), a more extensive review will be given on this toxin

Pneumolysin is one of the thiol-activated cytolisins, which are produced by four different genera of Gram positive bacteria. It consists of an approximately single 53 kDa polypeptide chain (Alouf and Geoffroy, 1991). Pneumolysin has an intracytoplasmic location in *S. pneumoniae* and is released during the autolysis process (Johnson, 1977). It is susceptible to oxidation, losses its activity with cholesterol treatment, and serologically cross reacts with other thiol-activated toxins (Alouf and Geoffroy, 1991). All clinical isolates of *S. pneumoniae* strains produce pneumolysin (Kanclerski and Mollby, 1987) and it is expressed *in vivo* since antibody levels against pneumolysin increase during pneumococcal infection in humans (Jalonen *et al.*, 1989; Kalin *et al.*, 1987). Pneumolysin's mechanism of action was related to its ability to create pores in the target membrane cells. The pore formation starts with the toxin's interaction with cholesterol in the cell membrane (Morgan *et al.*, 1997). Then toxin is concentrated by lateral diffusion of toxin monomers in the membrane. The monomers undergo self-association to form large oligomeric structures which appear as ring and arc shapes on the surface of the membranes (Morgan *et al.*, 1997)

Pneumolysin elicits protective antibodies. When inactivated purified pneumolysin was used to immunise mice which then were challenged intranasally with virulent *S. pneumoniae*, immunised mice survived

longer than non-immunised mice (Paton *et al.*, 1983). Furthermore, the protection provided by pneumolysin is independent of the serotype (Alexander *et al.*, 1994). It has been pointed out that genetically engineered pneumolysin toxoids could be a useful protein carrier of pneumococcal capsular polysaccharide in a pneumococcal vaccine, in order to enhance the immunogenicity of the polysaccharide by inducing immunological memory (Mitchell *et al.*, 1997).

The first direct evidence of the involvement of pneumolysin in the pathogenicity of pneumococci was obtained from a study in which a pneumolysin-negative isogenic mutant was used (Berry *et al.*, 1989). Intranasal challenge of mice with the mutant strain resulted in prolonged survival time and 10 to 100 fold increase in intravenous and intraperitoneal LD_{50}. The mutant strain grew slower than the wild type in the lung and bacteraemia occurred later than with the parent strain (Canvin *et al.*, 1995).

Besides its role in pneumonia, pneumolysin takes part in other diseases caused by *S. pneumoniae*. A study set up to investigate the role of pneumolysin in meningitis-associated deafness by the pneumococcus demonstrated that pneumolysin was the principal cause of cochlear damage (Winter *et al.*, 1997). In addition, pneumolysin contributes to the inflammation process of ocular infections caused by *S. pneumoniae*, probably via its complement activating property (Johnson *et al.*, 1995). However, a limited role was attributed to pneumolysin in a rabbit model of pneumococcal meningitis, since a pneumolysin-deficient strain of *S. pneumoniae* caused meningeal inflammation in rabbits indistinguishable from that induced by the parent pneumolysin-producing strain (Freidland *et al.*, 1995). Also, studies carried out in the chinchilla otitis media model showed that the pneumolysin negative strain grew at the same rate as the wild type and both strains induced similar inflammatory cell influx (Sato *et al.*, 1996). In the chinchilla otitis media model, inflammatory changes were triggered mainly by cell wall products which were released upon treatment with penicillin.

One of the means by which pneumolysin contributes to the virulence of the organism was shown to be interference with the bactericidal function of phagocytes. Pneumolysin is capable of lysing human phagocytes

(Johnson *et al.*, 1981). Exposure of phagocytes to sublethal doses of toxin, inhibit migration and increase the lysozyme release from phagocytes (Johnson *et al.*, 1981). Pneumolysin at very low concentrations, without effecting phagocyte viability, can interfere with respiratory burst, chemotaxis and antimicrobial activities of human phagocytes (Paton and Ferrante, 1983; Nandoskar *et al.*, 1986; Saunders *et al.*, 1989; Mitchell *et al.*, 1989). In addition, pneumolysin is responsible for slowing cilliary beating and disruption of the surface integrity of human respiratory epithelium in organ culture (Steinfort *et al.*, 1989). It was demonstrated that injection of purified recombinant pneumolysin alone into a ligated apical lobe of the rat lung induces the histological changes of pneumonia seen when live virulent pneumococci are injected (Feldman *et al.*, 1990). However, analysis of pneumolysin content of different strains showed that differences in virulence were not attributable to differences in pneumolysin production (Benton *et al.*, 1997).

The activation of the complement system by pneumolysin contributes to the pneumococcal virulence. The toxin activates the complement system via the classical pathway in the absence of specific antibody (Paton *et al.*, 1984). As a result, complement components are believed to be consumed and this may compromise effective opsonisation of bacteria (Paton *et al.*, 1984). It was also suggested that membrane bound pneumolysin also activates complement, causing a complement attack on these membranes which eventually leads to a generation of an inflammatory response (reviewed by Paton *et al.*, 1993). To study the contribution of the toxin's lytic and complement activating abilities to pneumococcal pathogenesis, isogenic mutant strains deficient in lytic and complement activities have been constructed (Berry *et al.*, 1995). The result of these studies indicated that both lytic and complement activities of toxin are important depending on the route of infection (Berry *et al.*, 1995; Alexander *et al.*, 1998). For example, intranasal challenge of mice with the strains lacking either complement or lytic activity caused an increase in survival time compared with the wild type infected mice. However, these strains were still more virulent than the pneumolysin negative strain. On the other hand, when the intraperitoneal route was used to challenge mice, the complement activating feature of the toxin was found to be irrelevant for the virulence, as complement negative mutants were as virulent as wild type organisms (Berry *et al.*, 1995). However, it has been recently reported

that the haemolytic and complement-activating properties of pneumolysin do not contribute to the pneumolysin dependent difference in blood levels of pneumococci in the mouse bacteraemia model (Benton *et al.*, 1997). It was speculated that there may be another activity of the toxin which is different from complement activating and lytic activities (Alexander *et al.*, 1998).

2.10 Aims

Information presented so far indicates that despite vaccination and treatment, the incidence of pneumococcal diseases remains unacceptably high. Therefore, there is still a need for further pneumococcal research. Current pneumococcal research has concentrated mainly on two areas: firstly studies on the development of high efficiency vaccines, and secondly the investigation of new virulence factors for better understanding of the pathologic mechanism of pneumococcal diseases. It is the latter area that will be the subject of my thesis. Two aspects related to virulence factors were to be investigated. These were,

A. Studies on pneumococcal superoxide dismutase(s) (SOD). In this context, studies on

i. the characterisation of SOD(s) in pneumococci

ii. the isolation of a gene coding for SOD in *S. pneumoniae,*

iii. the construction of an isogenic mutant strain

iv. the *in vitro* and *in vivo* characterisation of the mutant strain,

will be highlighted.

B. Effects of interferon-γ on sensitivity of cells to pneumolysin

Part 3 Methods

3.1 Chemicals

Unless otherwise stated all chemicals used in this study were obtained from Sigma Biochemicals, Fisons or BDH limited.

3.2 Bacterial strains and plasmids

The bacterial strains and plasmids used in this study are listed in Tables 3.1 and 3.2, respectively.

3.3 Growth conditions and media

All media were prepared using distilled water and sterilised by autoclaving at 121°C at 15 psi (pounds per square inch) for 15 min. *Escherichia coli* strains were grown in Luria broth (LB; 10 g/l NaCl, 5 g/l yeast extract and 10 g/l trypticase peptone) with shaking at 37°C or on Luria agar (LA) plates (LB with added 1.5% w/v bacteriological agar). For soft top agar, 0.6% w/v agar was added to LB.

M9 minimal medium was made by the addition of 6 g/l Na_2HPO_4, 3 g/l KH_2PO_4, 0.5 g/l NaCl, and 1 g/l NH_4Cl, pH 7.4. After autoclaving, it was cooled to 50°C. Then, 2 ml 1 M $MgSO_4$, 0.1 ml 1 M $CaCl_2$, 10 ml 20% w/v glucose and 1 ml 1 M thiamine HCl were added, after filtering through a 0.2 μm filter unit. When M9 minimal medium agar plates were required 1.5% w/v agar was added into the medium.

Streptococcus pneumoniae strains were grown in Brain Heart Infusion broth (BHI, Oxoid) or on Blood Agar Base plates (BAB, Oxoid) without shaking at 37°C in a gas jar containing a candle (candle jar) to remove the oxygen for anaerobic growth. When required, aerobic growth was maintained on a constantly shaking platform (220 revolution per minute). Where appropriate, defined medium, containing 0.4% w/v sucrose, originally described by Lacks (1966), was used. The defined medium contained the following components: 400 ml pre C solution, 13 ml supplement solution, 10 ml 1 mg/ml glutamine (filter sterilised using 0.2 μm filter unit), 10 ml Adams III solution, 5 ml 2% w/v pyruvate (filter sterilised using 0.2 μm filter unit) and 15 ml 1 M phosphate buffer (pH 8.0), (940 ml 1 M KH_2PO_4 60 ml, 1 M $K_2HPO_4.3H_2O$). 1 l pre C solution

Table 3.1 List of bacterial strains

Strains	Genotype	Source
E. coli DH5α	*supE44 ΔlacU169 (Ø80lacZ ΔM15) hsdR 17 recA1 endA1 gyrA96 thi-1 rel*A1	Hanahan, 1983
E. coli XL1 Blue	F::Tn10*proA*$^+$*B*$^+$ *lacI*^{q}*(lacZ)M15/recA1, endA1, gyrA96 (Nal*^{r}*) thi hisdR17 supE44, relA1 lac*	Stratagene, La Jolla, CA, USA
E. coli QC774	F$^-$ *Dlac4169 rpsl f(sodA-lacZ)49 f(sodB-kan)1-D*$_2$ Cm^r Km^r	Carlioz and Touati, 1986
E.coli QC771	F$^-$ *ΔlacU169 rpsL*	Carlioz and Touati, 1986
S. pneumoniae SV1 (NTCC 7465)	Virulent type 1 strain	The National Collection of Type Cultures, London, UK; Strain NTCC 7465
S. pneumoniae D39 (NTCC 7466)	Virulent type 2 strain	The National Collection of Type Cultures, London, UK; Strain NTCC 7466
S. pneumoniae D39HY1	Derivative of *S. pneumoniae* D39 (NTCC 7466) but *ΔsodA ::aad9*	This study

Table 3.2 List of plasmids

Plasmid	Characteristics	Source
pTTQ18	High copy number *E. coli* vector, ampicillin resistance; blue-white selection	Stark, 1987
pBlueScript	As above	Stratagene, La Jolla, CA, USA
pCRScript Amp SK(+)	As above	Stratagene, La Jolla, CA, USA
pCRScript Amp SK(-)	As above	Stratagene, La Jolla, CA, USA
pDL278	The source of spectinomycin resistance cassette	Dunny *et al.*, 1991
pHYSOD1	pCRScript Amp SK (-) carrying 2.8 kb *Eco*RI insert derived from the *S. pneumoniae* type 1 lambda ZAP II library, contained 3'-end of pneumococcal *sodA* gene	This study
pHYSOD2	pCRScript Amp SK (-) containing 1.6 kb *Eco*RI insert derived from the *S. pneumoniae* type 1 lambda ZAP II library	This study
pHYSOD3	pCRScript Amp SK (+) carrying 900 bp insert derived from the *S. pneumoniae* type 1 lambda ZAP II library through VA-PCR	This study
pHYSOD4	pCRScript Amp SK (+) carrying 300 bp insert derived from the *S. pneumoniae* type 1 lambda ZAP II library through VA-PCR	This study
pHYSOD5	Same as pHYSOD4	This study
pHYSOD6	pCRScript Amp SK (+) carrying 200 bp insert derived from the *S. pneumoniae* type 1 lambda ZAP II library through VA-PCR	This study
pHYSOD7	Same as pHYSOD6	This study
pSOD1	pCRScript Amp SK (+) carrying 480 bp sod_{int} derived from *S. pneumoniae* type 2. The insert was cloned into *Srf*I site of pCRScript Amp SK (+)	This study
pSOD2	Same as pSOD1 but contains an unique *Asc*I site within the sod_{int}	This study
pHY2	Derivative of pSOD2 carrying a spectinomycin resistance cassette inserted into*Asc*I site in sod_{int}	This study
pPSTSOD	pCRScript Amp SK (+) carrying 2.3 kb *Pst*I insert derived from the *S. pneumoniae* type 2 containing 5'-end and upstream sequences of pneumococcal *sodA* gene	This study

contained 1.2 g C_2H_3ONa, 5 g acid-hydrolysed casein (vitamin free) (Difco), 5 mg L-tryptophan, 50 mg L-cysteine. The pH of pre C was adjusted to 7.4-7.6 with 10 M NaOH. For the final pH adjustment, 1 M NaOH was used. The solution was then autoclaved at 121°C at 15 psi for 15 min and stored at room temperature. Adams III solution consisted of the following components in 200 ml: 32 ml Adams I solution, 8 ml Adams II solution, 400 mg aspargine, 40 mg choline chloride, 320 µl 0.1 M $CaCl_2$. To make Adams I solution, 30 µg biotin, 30 mg nicotinic acid, 35 mg pyridoxine.HCl, 120 mg calcium pantothenate, 32 mg thiamine.HCl and 14 mg riboflavine were added to 200 ml distilled water and the solution was kept in the dark following filter sterilisation using 0.2 µm filter unit. Adams II solution was made up of the following constituents in 100 ml distilled water: 50 mg $FeSO_4.7H_2O$, 50 mg $CuSO_4.5H_2O$, 50 mg $ZnSO4.7H_2O$, 20 mg $MnCl_2.4H_2O$ and 1 ml concentrated HCl. Adams II solution was filtered through a 0.2 µm filter unit before use. Supplement solution was made by the addition of 30 ml salt solution (salt solution contained, 50 g $MgCl_2.6H_2O$, 250 mg $CaCl_2$, 0.1 ml 0.1 M $MnSO_4.4H_2O$ in 500 ml distilled water, autoclaved prior to use), 60 ml adenosine from 2 mg/ml stock, 60 ml uridine from 2 mg/ml stock in 150 ml solution.

Blood agar base (Oxoid) was made according to the manufacturer's instructions, sterilised and allowed to cool to 45°C before the addition of 7% v/v horse blood (TCF Micribiology, Buckingham, England). Brain Heart Infusion (Oxoid) also was prepared according to the manufacturer's instructions. Where necessary, media were supplemented with appropriate antibiotics at the following concentrations: 100 µg/ml ampicillin, 25 µg/ml kanamycin, 200 µg/ml spectinomycin for *E. coli*, 1 mg/ml for *S. pneumoniae*. 0.2 mg/ml X-gal (Novabiochem) and 1 mM IPTG were added to cooled molten LA where required. Where appropriate, varying concentrations of paraquat was added to M9 minimal medium agar plates.

3.4 Quellung reaction

This test was used to check the presence of type-specific polysaccharide capsule on pneumococci. The test is based on the assumption that type specific antibodies bind to the capsule and increase the refractive index of the cells, enabling recognition under the light microscope (Pelczar *et al.*, 1982). A loop of overnight culture was smeared onto a slide and air

dried. On a coverslip, 10 µl of anti-type capsule antiserum (Statens Serum Institut, Copenhagen) and 10 µl of 1% w/v methylene blue were mixed. This was put onto the smear and slide was examined by X1000 oil immersion microscopy. Positive reactions were seen if the capsule was outlined around the blue stained cells.

3.5 Studies with *S. pneumoniae* type 1 lambda ZAP II library

This library, kindly provided by Dr V. Clarke (Clarke, 1994), had high number of recombinants, $5X10^6$ (with 2 kb average insert size), before its amplification in the *E. coli* host strain XL1-blue. The library had been constructed following digestion of pneumococcal chromosomal DNA with the restriction endonuclease, *Alu*I. Fragments ranging between 1-7 kb had been extracted from agarose and the ends of the size-selected DNA treated with Klenow and T4 DNA polymerase. *Eco*RI linkers were then ligated to these ends, and finally the genomic DNA was ligated into *Eco*RI digested lambda ZAP II vector arms to generate a lambda bacteriophage expression library.

Plating of S. pneumoniae type 1 Lambda ZAP II library. Plating of the library was performed according to instructions accompanying the vector (Stratagene). *Escherichia coli* XL-1 Blue cells were grown to mid-logarithmic phase at 37°C with shaking at 200 rpm. 200 µl of this culture was incubated with 100 µl of severally diluted library in a 3 ml capped test tube at 37°C for 20 min. Then each sample of infected bacteria was added into 5 ml top agar which had been melted at 48°C. This was gently mixed and overlaid onto LA plates containing the appropriate antibiotics. Plates were allowed to set prior to incubation at 37°C overnight.

Isolation of positive clone. This procedure was performed according to the vector manufacturer's instructions (Stratagene). The clones that hybridised with specific radiolabelled probes (section 3.11.4) were transferred from agar plates to sterile eppendorf tubes containing 500 µl of SM buffer (5.8 g NaCl, 2 g $MgSO_4$, 50 ml 1 M Tris-Cl, pH 7.5, 5 ml 2% w/v gelatine (from bovine skin) (Sigma), in 1 l) and 20 µl of chloroform. The tube was vortexed to release the lambda ZAP II phage particles into the SM buffer and incubated at 4°C overnight. This phage stock was kept at 4°C.

Excision of pBlueScript SK (-) from the lambda ZAP II. To further analyse the cloned insert in lambda ZAP II vector, the cloned insert was excised within the pBlueScript SK (-) and recircularized to form a plasmid (pBlueScript SK (-)) containing the cloned insert according to manufacturer's instructions (Stratagene). To do this 200 µl of *E. coli* XL-1 Blue cells, grown to mid-logarithmic phase, 200 µl of phage stock of clone of interest prepared as described above, and 1 µl of R408 helper phage suspension (Stratagene) were mixed in a 50 ml conical tube and the mixture was incubated at 37°C for 15 min. This was followed by the addition of 5 ml of 2XYT medium (10 g NaCl, 10 g yeast extract, 16 g bacto-tryptone in 1 l) and then incubated at 37°C for 3 h with shaking. Then the mixture was kept at 70°C for 20 min prior to centrifugation at 4,000 g for 5 min and the supernatant containing pBlueScript SK(-) was transferred to a sterile tube. 10 µl and 20 µl of a 10^{-2} dilution of the rescued plasmid was mixed with 200 µl of *E. coli* XL1 Blue cells in 15 ml tubes and incubated at 37°C for 15 min. Then 1 µl to 100 µl of each dilution (dilutions were made in sterile nanopure water) was plated on LB containing ampicillin and incubated overnight at 37°C. Colonies appearing on the plate contained the double stranded pBlueScript SK(-) whereas bacteria infected with helper phage alone did not contain grow because they do not contain ampicillin resistance genes.

3.6 Extraction of *E. coli* plasmid DNA

Small and large scale plasmid DNA extraction was performed as described by Sambrook *et al.* (1989).

3.6.1 Small scale extraction of plasmid DNA

Small scale extraction of plasmid DNA was performed with 1.5 ml of stationary phase bacterial cells. Cells were collected by centrifugation at 13000 rpm for 1 min in a MicroCentaur bench-top microfuge and the resulting pellet was resuspended in 100 µl of solution I (50 mM glucose, 25 mM Tris-HCl pH 8.0, 10 mM EDTA pH 8.0, 10 µg/ml lysozyme). This mixture was incubated at room temperature for 10 min. After incubation, 200 ml solution II (0.2 M NaOH, 1% w/v SDS) was added, mixed gently by inversion, and incubated on ice for 5 min. This was followed by the addition of 150 µl ice-cold solution III (11.5% v/v glacial acetic acid, 3 M potassium acetate). The mixture was gently mixed and a precipitate was allowed to form by incubation on ice for 20 min. The

supernatant was recovered by centrifugation at 13,000 rpm for 10 min in a MicroCentaur microfuge. Phenol-chloroform extraction of the supernatant was performed by the addition of an equal volume (approximately 400 µl) of phenol:chloroform (1:1) followed by vigorous mixing for 15 seconds using a bench vortex (Gallenkamp). The clear aqueous phase was collected by centrifugation for 2 min at 13000 rpm and the supernatant was extracted once more with phenol-chloroform as before. To precipitate the DNA, 0.1 volume of 3 M sodium acetate (pH 5.2) and 2.5 volumes of 100% ethanol were added to the supernatant. After 20 min incubation at -20°C, plasmid DNA was collected by centrifugation at 13,000 rpm for 15 min in a MicroCentaur microfuge. The pellet was washed in 70% v/v ethanol and resuspended in 50 µl of sterile TE (10 mM Tris-HCl, pH 8.0, 1 mM EDTA) and stored at -20°C.

3.6.2 Large scale extraction of plasmid DNA

3.6.2.1 Polyethylene glycol (PEG 8000) precipitation

Large scale plasmid extraction was done with 400 ml of stationary phase cultures. Cells were harvested by centrifugation at 4°C for 15 min at 4,000 g and the resulting cell pellet was resuspended in ice-cold 10 ml of Solution I (section 3.6.1). After incubation at room temperature for 10 min, 20 ml freshly prepared solution II (section 3.6.1) was added and gently mixed to obtain a clear lysate. This was followed by the addition of 15 ml ice-cold solution III (section 3.6.1) and incubation on ice for 10 min. The cell debris was removed by centrifugation at 10,000 g for 30 min at 4°C. The supernatant was mixed with 0.6 volumes of propan-2-ol and incubated at room temperature for 10 min. The precipitated nucleic acid was collected by centrifugation at 10,000 g for 10 min at 20°C and resuspended in 3 ml of TE buffer (10 mM Tris-HCl pH 8.0, 1 mM EDTA). To remove high-molecular weight RNA, 3 ml of ice-cold 5 M $LiCl_2$ was added and the pellet was recovered by centrifugation at 10,000 g for 10 min. Then the supernatant was mixed with an equal volume of propan-2-ol and the DNA was pelleted by centrifugation at 10,000 g for 10 min. The pellet was resuspended in 500 µl TE buffer containing DNase-free pancreatic RNase (20 mg/ml) and incubated at 37°C for 30 min. Then, 500 µl of 1.6 M NaCl containing 13% w/v PEG8000 (Sigma) was added, mixed well, incubated on ice for 10 min and DNA was recovered by centrifugation at 13,000 rpm for 10 min in a MicroCentaur bench-top

microfuge. The DNA pellet was dissolved in 400 µl of TE and extracted with phenol:chloroform twice, as described in section 3.6.1. The DNA was precipitated by the addition of 0.1 volume of 3 M sodium acetate (pH 5.2) and 2 volumes of 100% ethanol. After incubation at -20°C for 20 min, the recovery was performed by centrifugation at 13,000 rpm for 10 min in a MicroCentaur bench-top microfuge. The chromosomal pellet was washed in 70% v/v ethanol and dissolved in 500 µl of TE buffer and stored at -20°C.

3.6.2.2 Qiagen column extraction of plasmid DNA

A Qiagen DNA purification kit (Qiagen, Hilden, Germany) was also used for large scale DNA preparation as an alternative to PEG precipitation. The preparation was performed according to manufacturer's instructions. 500 ml of stationary phase cells were sedimented at 6,000 g for 15 min at 4°C and the pellet was resuspended in 10 ml of resuspension buffer, P1, (50 mM Tris-HCl, pH 8.0; 10 mM EDTA, 100 µg/ml DNase free RNaseA) and mixed thoroughly. This was followed by the addition of 10 ml of lysis solution, P2, (200 mM NaOH, 1% w/v SDS) mixed gently and incubated at room temperature for 5 min. After the incubation, ice-cold neutralisation buffer, P3 (3M potassium acetate, pH 5.5) was added, gently mixed by inverting the tube and then the reaction was incubated on ice for 20 min. Cell debris were removed by centrifugation at 4°C for 30 min at 30,000 g and the clear supernatant was recovered. A Qiagen-tip 500 column was equilibrated with 10 ml of buffer QBT (750 mM NaCl, 50 mM MOPS, pH 7.0, 15% v/v propan-2-ol, 0.15 w/v TritonX-100) and the supernatant was applied to the column. To remove any contaminants, 2 X 30 ml of wash buffer, QC, (1 M NaCl; 50 mM MOPS, pH 7.0, 15% v/v propan-2-ol) was allowed to pass through the column by gravity flow. The DNA in the column was then eluted by the addition of 10 ml of elution buffer, QF, (1.25 M NaCl; 50 mM Tris-HCl, pH 8.5, 10% v/v propan-2-ol) and elute was mixed with 10.5 ml of propan-2-ol to precipitate DNA from the aqueous phase. Following centrifugation at 15,000 g for 30 min at 4°C, the pellet was washed twice with ice-cold 70% v/v ethanol, air-dried and dissolved in 1 ml of TE buffer. Until needed DNA was stored at -20°C.

3.7 Pneumococcal chromosomal DNA preparation

Pneumococcal chromosomal DNA preparation was performed according to the method of Saito and Maiura (1963). 200 µl of overnight culture of bacteria (OD_{600}, 0.9-1.1) were used to inoculate 10 ml of BHI and this was grown at 37°C for 6-8 hours. The cells were pelleted at 2,000 g for 10 min at 4°C. The pellet was resuspended in 200 µl TE buffer (pH 8.0) containing 25% w/v sucrose. 30 µl of 500 mM EDTA, 20 µl of 10% w/v SDS, and 1 µl of ProteinaseK (12.5 mg/ml) were also added to the bacterial cell suspension. Cells were incubated at 37°C overnight to obtain a clear lysate. The cell debris were removed by centrifugation in a MicroCentaur bench-top microfuge at 6,000 rpm for 5 min. The supernatant was transferred to a fresh tube and an equal volume of liqudified phenol washed in Tris buffer, pH 7.6 (Fisher Scientific) was added to the mixture and gently mixed until an emulsion formed; then centrifuged in a MicroCentaur bench microfuge at 6,000 rpm for 10 min. The upper aqueous phase was transferred to a fresh tube without disturbing the white protein layer and an equal volume of chloroform:iso-amylalcohol (24:1) was added and centrifuged as before. This procedure was repeated several times until no white protein residue was seen at the interface of the two phases. The upper aqueous phase was retained and mixed with 2.5 volumes of 100% ethanol and 0.1 volume of 3 M sodium acetate (pH 5.2). The precipitated DNA was collected with a glass rod. The rod was carefully washed in 70% v/v ethanol and briefly dried around a Bunsen flame, before dissolving in 200 µl of sterile TE buffer. Chromosomal DNA was kept at 4°C until needed.

3.8 Transformation of bacterial cells

3.8.1 Transformation of *E. coli* using electrotransformation

3.8.1.1 Preparation of electrocompetent *E. coli*

The method used was originally described by Dower *et al.* (1988). A 10 ml fresh overnight broth culture was used to inoculate 1 l LB and bacteria were grown with vigorous shaking to mid-log phase (OD_{600} 0.5 to 1). The bacterial growth was stopped by placing the cells on ice for 30 min, after which cells were harvested by centrifugation at 4,000 g for 15 min at 4°C. The resulting cell pellet was resuspended in ice-cold sterile nanopure water and cells were collected at 4,000 g for 20 min at 4°C. This step was

repeated twice more. Then the bacterial pellet was resuspended in 25 ml ice-cold 10% v/v glycerol and centrifuged at 5,000 rpm in a Hereaus-Christ centrifuge for 15 min at 4°C. The final cell pellet was resuspended in 1 ml of ice-cold 10% v/v glycerol and 40 µl aliquots were stored at -70°C for up to 6 months.

3.8.1.2 Electrotransformation with plasmid DNA

40 µl samples of electrocompotent cells suspension were thawed on ice immediately before use. Electrocompotent cells were mixed with 1-2 µg of plasmid DNA and transferred into a pre-chilled 0.2 cm electroporation cuvette (Bio-Rad). A Bio-Rad Gene Pulser and Pulse Controller Apparatus were used to deliver a high voltage through the sample. The settings were being 25 µF capacitance, 2.5 kV voltage and 200 ohm resistance (Dower *et al.*, 1988). After giving a single pulse, 1 ml of SOC recovery medium at room temperature (2% w/v tryptone, 0.5% w/v yeast extract, 10 mM NaCl, 10 mM magnesium chloride, 10 mM magnesium sulphate, 20 mM glucose, 2.5 mM potassium chloride) was added to the cell suspension and cells were incubated at 37°C for 1 h. The transformants were selected on agar plates containing appropriate antibiotics.

3.8.2 Transformation of pneumococci using competence stimulating peptide (CSP)

Competence stimulating (CSP) peptide, a 17 amino acid residue peptide which increases the competence of the pneumococcus in exponentially growing cells suspension, was used to aid transformation of encapsulated *S. pneumoniae* (Haverstein *et al*, 1995; Pozi *et al.*, 1996). The CSP used in this study was described by Haverstein and coworkers (1995). 10 µl of overnight anaerobically grown pneumococcal culture was used to inoculate 10 ml of BHI so that 1:1000 dilution was obtained. The culture was incubated at 37°C for 1.5 to 3 h under anaerobic conditions. At different time points, 1 ml culture was transferred into 1.5 ml eppendorf tubes and mixed with 100 ng of CSP and 5 µg of DNA to be transformed. The tube then was incubated at 37°C for 1.5 h. All of the transformation mixture was spread on blood agar plates with appropriate antibiotic selection and incubated anaerobically at 37°C overnight.

3.9 Routine DNA manipulation techniques

DNA fragments were analysed by agarose gel electrophoresis using 0.7 or 1.5% w/v agarose (SeaKem) (Sambrook *et al.*, 1989). 1.5% w/v agarose was employed to separate small DNA fragments, whereas 0.7% agarose was used to separate larger DNA fragments. Agarose was solubilised in TAE buffer, pH 7.7, (40 mM Tris-acetate, 1 mM EDTA) containing 0.5 μg/ml ethidium bromide and then boiled in a microwave to bring about the polymerisation. Electrophoresis was performed in TAE buffer containing 0.5 μg/ml ethidium bromide. DNA samples were mixed with 6X gel loading buffer (6X gel loading buffer is: 0.25% w/v bromophenol blue, 0.25% w/v xylene cyanol FF, 15% w/v Ficoll Type 400 (Sigma) in water) prior to loading into gel wells, and DNA was visualised using a long wave UV transilluminator. DNA markers (1 kb ladder, Gibco/BRL) were used as standards to determine the size of the fragments.

DNA concentration was measured by determination of the A_{260} of the DNA in water using a Phillips PU 8720 UV/VIS spectrophotometer. Concentration was calculated based on an A_{260} of 1 representing a concentration of 50 μg/ml for double stranded DNA, and 33 μg/ml for linear DNA (Sambrook *et al.*, 1989).

Restriction endonucleases were used according to manufacturers' instructions (Gibco/BRL or New England Bio Lab). Routinely, reactions were set up in 50 μl at 37°C with 1 unit of enzyme for one μg of DNA.

To create blunt ended DNA fragments, modification enzymes Klenow fragment (Gibco/BRL), for filling in 5'-overhangs, and T4 DNA polymerase (Gibco/BRL), for removal of 3'-overhangs, were used according to the method described by Sambrook *et al.* (1989). The fragment containing spectinomycin cassette was separated by double digestion with *Sca*1 and *Bgl*1, from 40 μg of pDL278 (Dunny *et al.*, 1991). A small volume of digestion mixture was electrophoresed as above to assess the separation of fragment carrying spectinomycin gene. Then the whole digestion mixture was electrophoresed through 0.6% w/v low melting agarose (BRL) and the gel slice containing the spectinomycin gene was removed for purification as described below. The purified DNA was treated with 10 U T4 DNA polymerase at 11°C for 15 min in 100 μl. When Klenow fragment was used, the purified DNA was treated with 10

U enzyme and incubation carried out at room temperature for 1 h. The reaction mixtures were phenol-chloroform extracted and DNA ethanol precipitated (section 3.6.1).

Plasmid DNA with cohesive ends was dephosphorylated in order to remove the phosphate groups from the 5' ends to prevent self-ligation of the vector (Sambrook *et al.*, 1989). Initially, DNA was digested with suitable restriction endonucleases in 50 µl of reaction volume. After phenol/chloroform extraction and ethanol precipation (see section 3.6.1), the digested DNA was resuspended in 45 µl of sterile nanopure water and 5 ml of 10X calf intestinal phosphatase (CIP) buffer and 0.1 unit CIP (Gibco/BRL) were added. Following incubation at 37°C for 30 min, a further 0.1 unit of CIP was added and the reaction allowed to proceed for a further 30 min at 37°C. To stop the reaction, the mixture was phenol:chloroform extracted and then ethanol precipitated (section 3.6.1).

Ligation reactions were set up using 400 ng vector in 20 µl using 2 U T4 DNA ligase (Gibco/BRL) and 5X ligase buffer (50 mM Tris-HCl pH 7.5, 10 mM $MgCl_2$ and 1 mM freshly added ATP). The ratio of vector to insert determined essentially as described by Sambrook *et al.* (1989). Sticky end ligations were performed at 4°C by overnight incubation. Blunt end ligation reactions, on the other hand, were carried out by overnight incubation at 22°C (Sambrook *et al.* 1989).

DNA fragments were purified from agarose gels as described by Sambrook *et al.* (1989). The DNA fragments separated by agarose gel electrophoresis using 0.6% w/v low melting agorose (BRL). The gel slices containing the desired DNA fragment were excised from the gel and placed into a 1.5 ml eppendorf tube. The agarose slice was melted in nanopure water (for each volume of agarose, 3 volume nanopure water) at 65°C for 10 min and the solution was extracted once with Tris-HCl ,pH 7.5, saturated phenol, once with phenol-chloroform (1:1) and once with chloroform. The DNA was then ethanol precipitated and washed twice with 70% v/v ethanol and dissolved in sterile TE buffer.

In addition, QIAEX II gel extraction columns (Qiagen, Hilden, Germany) were also used to purify DNA from agarose gel, according to the manufacturer's instructions. The principle of this method was based on

solubilization of agarose and selective, quantitative adsorption of nucleic acids to the silica gel particles in the presence of high salt. Elution of the DNA was accomplished with a low salt solution, such as 10 mM Tris.Cl, pH 8.5 buffer (Sambrook *et al.*, 1989).

3.10 Genomic library construction

S. pneumoniae genomic DNA (30 µg) was partially digested with various amounts of *Sau*3A, ranging from 0.05 to 0.4 U, to generate a range of fragments at 37°C for 35 min. The fragments were separated by agarose gel electrophoresis. The agarose gel slices containing the desired sizes 2-3, 1.5-6, 2-7 kb were removed and purified from these gel slices (section 3.9).

10 µg plasmid pTTQ18, was digested to completion with *BamH*1. The level of digestion was assessed by electrophoresis of 1-2 µg digested plasmid DNA. The vector was then dephosphorylated as described in section 3.9.

The size selected pneumococcal DNA fragments (1200 ng) then ligated to the dephosphorylated pTTQ18 (400 ng) using a routine ligation process (section 3.9). After incubation, ligation reactions were ethanol precipitated and resuspended in 6 µl of sterile nanopure water. 2 µl of this was then electroporated into *E. coli* DH5α cells (section 3.8.1.2). Transformants were selected on LA containing 100 µg/ml ampicillin, 0.2 mg/ml X-gal and 1 mM IPTG.

3.11 DNA hybridisation

3.11.1 Transfer of DNA to nylon filters by Southern blotting

The method of Southern (1975) was used to transfer DNA samples from agarose gels to nylon membranes. DNA samples were separated by agarose gel electrophoresis and photographed alongside a ruler as a reference. To increase the transfer efficiency, the gel was partially depurinated by soaking in 0.25 M HCl with gentle shaking for 7 min. The gel was rinsed in distilled water and soaked in denaturing solution (0.5 M NaOH, 1.5 M NaCl) for 30 min with gentle shaking. The gel was rinsed again with distilled water and placed in neutralising solution (0.5 M Tris-HCl, 1.5 M NaCl, 1 mM EDTA, pH 7.5) for 30 min with gentle rocking, after which it was rinsed in distilled water. The transfer of DNA was

performed in a tray containing 20XSSC (20XSSC: 3 M NaCl, 0.3 M tri-sodium citrate, pH 7.0). A gel casting plate covered with presoaked three sheets of Whatmann 3MM paper. The gel was placed on the plate, and a nylon membrane (BDH) pre-wetted in 3XSSC was put on the gel, ensuring that no air bubbles were trapped between the gel and nylon membrane. Three sheets of Whatmann 3MM paper, pre-wetted in 3XSSC, were placed on the nylon filter followed by a stack of paper towels. Finally, a glass plate was placed over the blotting pads along with a water filled 250 ml bottle as a weight. After the overnight capillary transfer, the blotting assembly was disassembled and dried on filter paper. The nylon filter was wrapped in Saran wrap (Dupont) and the DNA was cross-linked to the membrane by exposing to UV from a long wave transilluminator for 5 min. The filter was stored at 4°C until needed.

3.11.2 Transfer of DNA to nylon filters by dot blotting

Transfer of samples to nylon membranes was performed as described by Reed and Mann (1985). The DNA sample to be blotted was denatured by boiling for 5 min in 20 µl water containing salmon sperm DNA at a concentration of 1 ng/ml. The tube was snap cooled on ice for 2 min and a 2 µl sample was applied to nylon membrane at a pre-marked position. The membrane was allowed to dry completely before spotting a further 2 µl of sample. The DNA was cross-linked to the membrane by baking in an oven at 80°C for 1 h. The prehybridisation and hybridisation steps were performed as described below (section 3.11.4).

3.11.3 Transfer of colonies/plaques to nylon filters for colony/plaque hybridisation

Transformed bacteria were plated onto grids on duplicate agar plates. One of the plates was kept at 4°C as a master and the other was used in the hybridisation reaction. Before placing the round hybridisation transfer membranes (Dupont) on the transformants, plates were chilled at 4°C for 1 h. The membrane was allowed to sit on the agar plate for 2-3 min. The position of the membrane was marked by stabbing a needle through the membrane and agar. The membrane was carefully removed and was placed, colony side up, onto a 0.75 ml pool of 0.5 M NaOH on a Saran wrap. After 2 min, the membrane, colony side up, was placed onto 3MM Whatmann paper and denatured once more with 0.5 M NaOH. This was followed by treatment with 0.75 ml 1 M Tris-HCl, pH 7.5

solution on a plastic wrap for 2 min and then the membrane was dried on 3MM Whitmann paper. This procedure was repeated once more. The excess buffer was removed and the membrane was air dried before fixing the DNA at 80°C for 1 h. The prehybridisation and hybridisation steps were carried out as described below (section 3.11.4).

3.11.4 Preparation of a radiolabelled DNA probe

Radio-labelling of the DNA probe was carried out by random priming using the Pharmacia Ready-To-Go labelling kit. 20 µl of sterile nanopure water was added into a tube containing the lyophilised components (dATP, dTTP, dGTP, Klenow fragment of DNA polymerase I and random 9-base oligonucleotides) and the reconstituted mix was kept on ice for 10-15 min. 50 ng probe DNA was denatured by boiling for 5 min and then it was cooled on ice for 5 min. The denatured probe DNA was added into the reconstituted mix, along with 10 mCi of [α-^{32}P] dCTP (specific activity of 220 MBq/mmol, Amersham International) and the total volume was made up to 50 µl with sterile nanopure water. The mixture was incubated at 37°C for 30 min. Finally, prior to use, the radiolabelled probe DNA was denatured by boiling for 5 min and cooling rapidly on ice.

3.11.5 DNA-DNA hybridisation

Southern blot membranes were placed into cylindrical canisters containing 25 ml prehybridisation solution (5XSSC; 5X Denhardt's solution (50X Denhardt's: 1% w/v Ficoll Type 400, 1% w/v BSA, 1% w/v polyvinolpyrollidine, 0.5% w/v SDS and 100 mg/ml denatured salmon sperm DNA) and incubated in a rotary hybridisation oven (Hybaid) at 65°C for 2 h (Sambrook *et al.*, 1989). This was followed by the addition of 50 ng radiolabelled probe and incubation overnight.

After hybridisation, the filters were washed three times in 25 ml high stringency solution (0.1XSSC, 0.1 % w/v SDS) at 65°C for 15 min. The washed filters then were air dried and wrapped in cling film and exposed to Cronex (DuPont) film in an autoradiography cassette. The cassette was kept at -70°C before developing the film in an Agfa-Geveart automatic film processor.

3.11.6 Stripping DNA probes from nylon membranes

When needed, hybridised radiolabelled probe was removed in order to use the membrane with a different radiolabelled probe (Reed and Mann, 1985). This was done by washing the membrane with 0.1XSSC at room temperature for 10 min, followed by three washes with 1% w/v SDS at 65°C. The efficiency of process was determined by exposing the membrane to X-ray film.

3.12 DNA sequencing

3.12.1 Manual sequencing

3.12.1.1 Sequencing reactions

Manual DNA sequencing method was performed according to chain termination method of Sanger *et al.* (1977) in which DNA synthesis from deoxynucleotide triphosphates is terminated by the addition of dideoxynucleotide triphoshates. Sequencing reactions were set up with Sequenase Version 2.0 kit supplied by United States Biochemical Corporation. Double stranded plasmid DNA was sequenced either with pUC/M13 forward (-47) or reverse sequencing primers (New England Biolab) or primers specifically ordered from Gibco/BRL for the purpose of the work. The oligonucleotide primers used in this study are listed in Table 3.3.

5 µg double stranded DNA was denatured by addition of 2 µl of fresh denaturing solution (2 M NaOH, 2 mM EDTA) in a total volume of 16 µl, and incubating at 37°C for 20 min. Following incubation, DNA was precipitated by adding 2 µl of 2 M ammonium acetate (pH 4.6), 75 µl of 100% ethanol and incubating at -20°C for 30 min. The DNA was collected by centrifugation at 13,000 rpm in a MicroCentaur microfuge for 15 min. The pellet was washed twice with 70% v/v ethanol, air dried and resuspended in 7 µl of sterile nanopure water. Following denaturation, 1 µl primer (0.5-1 pmole) and 2 µl sequenase reaction buffer, provided in the kit, were added to tube containing DNA for annealing. The tube was placed into a 65°C water bath for 2 min then the mixture was allowed to cool slowly to <35°C over 15-30 min. DNA was radiolabelled by incorporating 0.5 µl [α-^{35}S] dATP (370 MBq/ml; Amersham) in the extension reactions. The reaction was stopped by addition of 4 µl of stop

Table 3.3: List of primers

Primer name	Sequence (5'-3')
HYK1	CCGTA(CT)GCGTA(CT)GCG(CT)TGGA(AG)CC
HYK2	A(AG)(AG)TA(AG)TAGGC(AG)TG(CT)TCCCAGAC(AG)TC
SOD1	TTGAAGTGACTTCAACAGCA
SOD2	TCTGACGATTTTGTCAGAC
SOD3	TTTTCTCGGGAGTCATCAA
SOD6	TAGCAGCTGCGTACAATTCAT
SOD9	CTGCATTGGCATTGTTGAC
SOD12	AGTATGTCAGTGATTTCC
Universal Forward	CGCCAGGGTTTTCCCAGTCACGA
Universal Reverse	AGCGGATAACAATTTCACACAGGA

solution provided in the kit. Samples were heated to 75°C for 2 min immediately before loading onto a sequencing gel and 2-3 µl was loaded in each lane.

3.12.1.2 Gradient gel electrophoresis of reaction products

The radiolabelled DNA fragments from the sequencing reactions were separated by gradient gel electrophoresis (Biggin *et al.* 1983). To prepare the gel, two glass plates (20X50 cm) were cleaned vigorously with ethanol to remove the grease. The surface of the one of the glass plates was siliconised by smearing dimethyldichlorosaline (BDH) evenly on a surface. To create the sequencing cell, the plates were assembled with 0.4 mm spacers. The solutions below then were prepared to cast the gel.

Solution 1	7 ml 2.5XTBE acrylamide/urea mix (6%)
	90 µl 10% w/v ammonium persulphate (Sigma)
	5 µl TEMED (Sigma)
Solution 2	
	40 ml 0.5X TBE acrylamide/urea mix (6%)
	360 µl 10% w/v ammonium persulphate
	15 µl TEMED
0.5XTBE acrylamide/urea mix (6%)	
	430 g urea
	200 ml 30% v/v Acrylamide (National Diagnostic)
	50 ml 10X TBE
	Distilled water to 1 l
2.5XTBE acrylamide/urea mix (6%)	
	430 g urea
	200 ml 30% v/v Acrylamide
	250 ml 10X TBE
	50 g sucrose
	50 mg Bromophenol blue
	Distilled water to 100 ml

10X TBE is 109 g Tris, 55 g boric acid, 9.3 g EDTA pH 8.3 in a final volume of 1 l.

First 10 ml solution I and then 7 ml solution II were drawn into a 25 ml pipette. In order to form a rough gradient some air bubbles were also drawn into the same pipette. Immediately, the content of the pipette was introduced into the sequencing cell avoiding formation of air bubbles. The remaining cavity between the glass plates was filled with solution II. The comb was inserted and the gel was allowed to set for at least one hour. The gel was sandwiched between aluminium sheets of the same size for even heat removal. The upper buffer reservoir was filled with 0.5X TBE and lower with 1X TBE. When the gel had set, the wells were washed with running buffer and the gel was pre-run for 30 min at 30 W. Samples were loaded and electrophoresis was performed at constant power of 30 W for 3 or 6 h. On completion of electrophoresis the assembly was dismantled but the gel was retained on the nonsiliconised plate. The gel was fixed with 10% v/v methanol:10% v/v acetic acid (1:1) for 10 min. The gel was transferred to damp 3MM Whitmann filter paper and dried under vacuum at 80°C for 1 h. The gel was exposed to Cronex (DuPont) film in an autoradiography cassette at room temperature and developed in an Agfa-Geveart automatic film processor.

3.12.2 Automated sequencing

Automated sequencing is based on the chain termination, where four dye-labelled dideoxy nucleotides, GATC DyeDeoxy terminators replaces the standard dideoxy nucleotides in the enzymatic sequencing reaction. Double stranded DNA sequencing was performed using the ABI PRISM™ dRhodamine Terminator Cycle Sequencing Ready Reaction Kit according to manufacturer's instructions (Perkin Elmer). The terminator premix contains 1.58 µM A-DyeDeoxy, 94.74 µM T-DyeDeoxy, 0.42 µM G-DyeDeoxy, 47.37 µM C-DyeDeoxy, 78.95 µM dITP, 15.79 µM dATP, 15.79 µM dCTP, 15.79 µM dTTP, 168.42 mM Tris HCl pH 9.0, 4.21 µM $(NH_4)_2SO_4$, 42.10 mM $MgCl_2$ and 0.42 U/µl AmpliTaq DNA polymerase. Reactions consisted of 8 µl of terminator premix, 0.4 to 0.6 µg DNA template, 3 pmol primer in a reaction volume of 20 µl. Reaction mixture was overlaid with 20 µl of mineral oil (before use, it was exposed to UV for 5 min). To sequence the DNA, the tube was placed in a Hybaid Omnigene TR3 CM220 thermal cycler. The settings for reactions were a rapid thermal ramp to 96°C and hold at 96°C for 10 seconds, annealing at 50°C for 5 seconds and extension at 60°C for 4 min. Extension products were purified by ethanol precipitation (section 3.6.1) and dry pellet was

sent to the Protein and Nucleic Acid Laboratory, University of Leicester for gel analysis.

3.12.3. Computer analysis of sequence data

The DNA sequence data obtained in this study was analysed using a Molecular Biology Package (Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wisconsin). This software contains several programs for various purposes. Among these, MAP was used to identify ORFs and restriction sites, TRANSLATE was used to convert nucleotide sequence to protein sequence, BESTFIT was used to compare two nucleotide sequence for similarity. PILEUP was used for multiple alignment of protein sequences. Similarity searches with known nucleotide and deduced protein sequences were performed with BLAST (Pearson and Lipman, 1988) or FASTA (Altschul *et al.*, 1990), respectively. When using the programmes in Molecular Biology Package, the default parameters were employed. Database used were the Genbank/European Molecular Biology Laboratory (Gen_EMBL) and the SWISSPORT.

Pneumococcal genomic sequence data were obtained through release from The Institute for Genomic Research at www.tigr.org and/or through National Centre for Biotechnology Information (NCBI) in Bethesda, Maryland, USA, at www.ncbi.nlm.nih.gov. The pneumococcal genome was searched for the presence of oxidative stress response genes. For the analysis, the consensus sequences of bacterial genes that are known to involve in oxidative stress response were used. The consensus sequences were obtained using PILEUP and ALIGN programmes, which are available in Molecular Biology Package (Wisconsin Package Version 9.1, Genetics Computer Group (GCG), Madison, Wisconsin). Also ProDom BLAST program (Altschul *et al.*, 1997) available at http://www.toulouse.inra.fr, was used to obtain well conserved protein domains available in databases. In addition to consensus sequences, the genome was searched using complete gene sequences from other organisms, such as from *E. coli* and *Clostridium perfringens.* The relevance of any identity in the pneumococcal genome was further investigated by comparing the pneumococcal sequences that had matched with the consensus sequences against databases maintained at the NCBI for similar sequences by employing tBLASTn program. Unless otherwise stated, default settings were used for the analysis.

3.13 Polymease chain reaction (PCR)

Polymerase chain reactions were performed using a thermal cycler (Hybaid Omnigene TR3 CM220) in a volume of 100 µl containing 50 ng of genomic or plasmid DNA, 0.1 mM of each primer, 200 mM of each dNTP, and 2 U of *Taq* DNA polymerase in a 1X amplification buffer (10 mM Tris-HCl pH 8.3, 50 mM KCl, 1.5 mM $MgCl_2$). The reaction mixtures were overlaid with 40 µl of molecular biology grade mineral oil (exposed to UV light for 15 min to destroy any contaminating DNA before use). The PCR mixture was submitted to a denaturation step (3 min at 95°C), followed by 35 cycles of amplification (2 min of annealing at 50°C, 90 seconds of elongation at 72°C, and 30 seconds of denaturation at 95°C), and finally annealing (4 min at 37°C) and elongation (12 min at 72°C) steps. Reaction products were recovered by pipetting from under the oil and a 10 µl sample was analysed by agarose gel electrophoresis.

DNA was also amplified using phage library mixture as a template. To prepare template from a phage library, 20 µl of library mixture was boiled and centrifuged at 13,000 rpm for 10 min in a MicroCentaur bench-top microfuge. Then, the desired concentration of DNA was added into the reaction mix given above.

3.13.1 Subcloning PCR products

When needed, PCR products were cloned into pCRScript Amp SK(+) cloning kit (Stratagene). pCRScript Amp SK(+) cloning vector allows efficient blunt ended cloning of PCR products. Before cloning PCR products were ethanol precipitated and resuspended in 10 µl nanopure water. In order to increase the number of transformants, the ends of purified PCR products were 'polished' in a 0.5 ml eppendorf tube by mixing 10 µl of the PCR product, 1 µl of 10 mM dNTP mix (2.5 mM each), 1.3 µl of 10X (10X polishing buffer: 200 mM Tris (pH 8.8), 100 mM KCl, 100 mM NH_2SO_4, 20 mM $MgSO_4$, 1% w/v TritonX100, 1 mg/ml bovine serum albumin) 1 µl of *Pfu* DNA polymerase (0.5 U) according to instructions provided by the manufacturer (Stratagene). The tube was gently mixed, overlaid with 20 µl of mineral oil and incubated at 72°C for 30 min. 2-4 µl of this blunt-end PCR product was then cloned into the *Srf*I site of pCR-Script Amp SK(+) cloning vector following the instructions provided by the manufacturer. The cloning reaction was transformed into the *E. coli* XL-1 Blue supercompetent cells (Stratagene)

with heat pulse following the instructions in the kit. To do this, 40 µl of supercompetent cells were thawed on ice. The cells were transferred to prechilled 15-ml Falcon 2059 polypropylene tube containing 0.7 µl 25 mM β-mercaptoethanol. The cells were incubated on ice for 10 min, swirling every 2 min. 2 µl of cloning reaction was added to the transformation reaction, swirled gently and incubated on ice for 30 min. This was followed by 45 seconds heat pulse in a 42°C water bath and incubation on ice for 2 min. Then transformants were recovered in SOC medium (see section 3.8.1.2) and incubated at 37°C for 1 h before selecting on agar plates containing 100 µg/ml ampicillin. 0.2 mg/ml X-gal and 1 mM IPTG were also added for blue-white selection as multiple cloning site of pCRScript Amp SK (+) is located in the middle of the *lacZ* gene, that encodes β-galactosidase. When a gene fragment is cloned into the multiple cloning site of pCRScript Amp SK (+), this results in insertional inactivation of the *lacZ'* gene. This feature enables the identification of recombinants as white colonies, since they are unable to hydrolyse X-gal, while the non-recombinants appear as blue colonies.

3.14 Superoxide dismutase assays

3.14.1 Preparation of bacterial cell protein

All manipulations were carried out at 4°C. Cell pellets of late log phase cultures were collected by centrifugation at 2,000 g for 15 min, and resuspended in 50 mM phosphate buffer, pH 7.0 (Kroll *et al.*, 1993). This cell suspension was sonicated using a Sanyo soniprep model 150 sonicator (power setting was 7 amplitude micron). In order not to damage the activity of the enzyme by the heat generated during sonication, samples were sonicated in ice. In addition, the cells were not exposed to sonication continuously; instead, after every 15 seconds 45 seconds breaks were given to allow the cell suspension to cool down. This process was repeated for at least five times. Then, the cell debris was removed by centrifugation at 22,000 *g* for 30 min. The supernatant was retained and kept at -20°C for up to 2 weeks.

3.14.2 Determination of protein concentration

Total protein concentration of the lysate was determined by the assay developed by Bradford (1976). Prior the assay, Bradford dye concentrate was prepared (100 mg of coomassie brilliant blue G-250 (BDH) dissolved

in 50 ml of 95% v/v ethanol, 100 ml of concentrated phosphoric acid and the volume was made up to 200 ml with distilled water) and this was diluted to a working concentration of 1 in 5 with distilled water. Samples prepared in 100 µl PBS (pH 7.0) were mixed with 5 ml of diluted dye solution and were kept at room temperature for at least 10 min to allow colour to develop. A standard curve was also prepared with known amounts of bovine serum albumin to calculate the amount of protein in the sample. The absorbance was read at 595 nm using a Philips PU 8720 UV/VIS spectrophotometer.

3.14.3 Enzyme characterisation by polyacrylamide gel electrophoresis

Polyacrylamide gel electrophoresis (PAGE) of whole cell extracts was performed under nondenaturing conditions in the discontinuous Tris-glycine buffer system of Davis (1964) (see below for 5X Tris-glycine buffer) except that the pH of the lower and upper gel was raised to 8.9 and 8.3, respectively, with 10 M NaOH for sharper zones as described by Steinman (1985). The gel conditions are given below.

Separating gel (10%)	4.1 ml distilled water
	3.3 ml 30% w/v acrylamide
	2.5 ml 1.5 M Tris pH 8.8
	100 µl 10% w/v amonium persulphate
	4 µl TEMED
Stacking gel (5%)	3.45 ml distilled water
	830 µl 30% w/v acrylamide
	630 µl 1.5 M Tris pH 8.8
	50 µl 10% w/v amonium persulphate
	4 µl TEMED
5X Tris-glycine buffer	15.1 g Tris base
	94 g glycine, pH 8.3
	distilled water to 1 l
5X sample loading buffer	4 ml distilled water
	1 ml 0.5 M Tris-HCl pH 6.8
	0.8 ml glycerol
	400 µl β-mercaptoethanol
	200 µl 0.05% w/v bromophenol blue

Protein extracts prepared as described above (section 3.14.1) were separated by PAGE under nondenaturing conditions. Polyacrylamide gel electrophoresis was performed as described by Laemmli (1970) using a mini PROTEAN™ II system (Biorad) according to the manufacturer's instructions. To cast the gel, the separating gel was first poured and allowed to set. The stacking gel was then poured onto the separating gel and a comb was immediately inserted. Before loading the samples, the comb was removed and wells were washed with 1X Tris-glycine buffer, pH 8.3. Samples were loaded onto the gel in 5X loading buffer in 50 µl and electrophoresis was performed at 80 to 120 V until the bromophenol blue dye front had migrated to the bottom of the gel.

3.14.4 Staining gels for SOD activity

Qualitative SOD activity in polyacrylamide gels was determined by the method of Beauchamp and Fridovich (1971). The principle of this method relies on photochemical reduction of flavins (riboflavin) in the presence of an oxidizable substance (TEMED). Reduced flavins, in turn, reoxidise in air and generate superoxide radical. Photochemically generated superoxide radical then converts (reduces) nitro blue tetrazolium (colourless) to insoluble formazan (insoluble blue dye) and SOD activity is detected as an achromatic zone because the enzyme inhibits the conversion of nitroblue tetrazolium by removal of superoxide.

Immediately after termination of the electrophoresis, the gel was soaked in 2.45 mM nitro blue tetrazolium solution for 20 min. Following this, the gel was placed into a container and covered with a solution containing 28 µM TEMED, 28 mM riboflavin, and 36 mM potassium phosphate, pH 7.8. Then the gel was exposed to a light source (sun or light box) until the maximum contrast between achromatic zones (SOD bands) and the uniform blue background was achieved. To determine the type of the superoxide dismutase, hydrogen peroxide or potassium cyanide were added to the riboflavin-TEMED solution to final concentrations of 5 mM (Crapo *et al.*, 1978).

3.14.5 Quantitative SOD assay

SOD activity was assayed quantitatively using the xanthine-xanthine oxidase-cytochrome *c* method described by McCord and Fridovich (1969).

The solutions used in the assay are given below. In this assay, cytochrome *c* is reduced by superoxide generated by the xanthine-xanthine oxidase system. SOD competes for superoxide and hence decreases the reduction rate of cytochrome *c* which could be monitored at 418 nm using a Phillips PU 8720 UV/VIS scanning spectrophotometer.

Solution A: 5 µmol xanthine and 2 µmol cytochrome *c* were prepared in 10 ml 0.001 M sodium hydroxide. This was then added to 100 ml 50 mM phosphate buffer (pH 7.8) containing 0.1 mM EDTA.

Solution B: 2 U/ml xanthine oxidase (Sigma) in 0.1 mM EDTA, pH 7.5. This solution was prepared freshly, just before use.

Solution A was kept at 25°C; the temperature of the cell compartment of the spectrophotometer. Solution B was kept on ice. 2.9 ml of solution A was pipetted into a 3 ml cuvette. Then, 50 µl of the sample of interest or SOD standard (pure bovine erythrocyte CuZnSOD) (Sigma) was added to same cuvette after which the reaction was started with 50 µl of solution B. The reaction components were immediately mixed and absorbance change at 418 nm was recorded every 30 seconds over 5 min.

A calibration curve was made using the readings obtained with increasing amounts of SOD standards (5, 10, 15, 20 ng), by plotting as the reciprocal absorbance change per minute versus concentration of SOD standards. The SOD activity in the sample was determined from the linear part of the reaction. One unit of SOD activity was defined as the amount of enzyme required to inhibit the rate of cytochrome *c* reduction by 50% (McCord and Fridovich, 1969).

3.15 Virulence studies

The infection of animals during virulence studies in this study were carried out kindly by Dr Peter W. Andrew, Dr Janet Alexendar, Neil Gingles, and Dr Aras M. Kadioglu, Leicester University, Leicester, UK. All the virulence studies were performed according to the standard operating procedures (SOP) developed by Dr Peter W. Andrew's laboratory in Leicester University.

3.15.1 Mice

Female MF1 outbred mice, weighing, 30-35 g, were obtained from Harlan Olac (Bicester, UK).

3.15.2 Preparation of standard inoculum

Prior to actual virulence studies, pneumococci were passaged through mice to prepare a standardised inoculum. Mice were lightly anaesthetised with 2.5% v/v fluothane (Zeneca, Macclesfield, UK) over oxygen (1 l/min). The efficiency of anaesthesia was confirmed by pinching joints and observing no reflex reaction from the animal. Then, 200 µl of pure culture of pneumococcal strains were injected into the peritoneal cavity of mice and recovered 24 h later from cardiac blood of anaesthetised animals using a syringe with a 23G needle. 50 µl of blood was used to inoculate 10 ml of BHI broth, which then was incubated overnight at 37°C. This was followed by collection of cells by centrifugation at 2,000 g for 15 min. The bacterial cell pellet was resuspended in 1 ml BHI containing 20% v/v foetal calf serum (BHI-FCS), and then diluted with fresh BHI-FCS to give an OD_{600} of 0.7. The culture was incubated at 37°C for 4 to 5 h (until it had OD_{600} of 1.6) following which 500 µl aliquots of the suspension were prepared and stored at -70°C. The viability of the frozen cells was determined 24 h after freezing the cells by ten-fold serial dilution in 200 µl sterile nanopure water and plating 20 µl on blood agar plates, in duplicate. In these conditions, the viability of strains was unaffected for at least 3 months. When needed, an aliquot was thawed at room temperature, and bacteria were harvested by centrifugation in a MicroCentaur bench-top microfuge at 13,000 rpm for 1 min. The pellet was kept and resuspended in equal volume of 0.1 M sterile phosphate buffered saline (PBS), pH 7.3.

3.15.3 Infection of mice

Intranasal infection. To determine the virulence of pneumococcal strains, mice were lightly anaesthetised with 2.5% v/v fluothane (Zeneca, Macclesfield, UK) over oxygen (1.5-2 l/min). 50 µl of PBS containing $1X10^6$ colony forming units (cfu) of bacteria were placed dropwise onto the entrance of the nostril and each drop was inhaled before the next added. Mice were placed on their backs to recover before they were placed in an isolator. The clinical symptoms, hunched, starry coat, lethargic or moribund states were recorded as mild (+) or severe (++) for 14 days.

Mice alive at the end of this period were considered survivors. Mice that became moribund during the 14-day period were accepted to have reached the end point of the assay and were killed humanely.

Intravenous infection. Intravenous infections were performed by administration of desired number of bacteria in 100 µl PBS (pH 7.3) into a tail vein. In order to enhance the tail veins, the mice were kept in an incubator at 37°C for 15 min prior to the infection. Infections were carried out placing mouse in a plastic tube (a 50 ml syringe case) with a hole that allowed the tail out. Blood samples were collected by tail bleeding and the bacterial growth in blood at selected time points was investigated by plating serial dilutions as before (section 3.15.2).

3.15.4 Bacterial count in host tissue

When the *in vivo* growth of bacteria was examined preselected mice were taken at pre-chosen intervals following infection. Blood samples were first taken by tail bleeding and then the mice were killed by cervical dislocation. In order to prevent clotting, the blood samples were collected in the tubes containing 4% w/v sodium citrate solution (for 9 part of blood 1 part of solution was used) (Sigma). The lungs were removed aseptically into 10 ml of sterile water in preweighed universal tubes and the net weight of the lungs was determined. Then the lungs were homogenised in the water in a Stomacher-Lab blender (Seward Medical, London, UK) for 90 seconds. Viable counts in homogenates and in blood were determined as above (section, 3.15.2).

3.16 Histology

3.16.1 Preparation of lung tissue for cryostat sectioning

In order to assess the pathological changes that had been taking place, lungs were taken at prechosen time points following infection. Lungs were excised and immediately transported from the post mortem room to the laboratory in sterile PBS (pH 7.3) on ice. To freeze the lung tissue samples, cylindrical aluminium foil moulds were made by wrapping aluminium foil around 4 ml Bijoux tubes. These moulds were filled up to an depth of 1 cm with Tissue Tek OCT tissue embedding compound (Miles). The lungs were covered in this compound and were frozen by placing the moulds immediately in a cup containing iso-pentane (BDH)

in liquid nitrogen. The moulds were then wrapped in 20 ml plastic tubes and kept in -70°C until needed.

3.16.2 Cryostat sectioning and haematoxylin-eosin staining

Frozen samples were transferred from -70°C to -20°C freezer a day before sectioning. The excess tissue embedding compound was removed before placing the tissue samples in a Bright microtome. 20 micron longitudinal sections were prepared at -18°C to -25°C and sections were left at room temperature for 1 h before haematoxylin-eosin staining (Bancroft and Stevens, 1996). A minimum of 4 sections of lung from each mouse was examined. For staining, the slides were covered with Harris's haematoxylin (mercury free) (BDH) for 30 seconds and washed briefly with water prior to repeating same process with 1% w/v eosin solution. Then, the slides were washed in turn with 70% v/v, 90% v/v and 100% v/v ethanol for 30 seconds. The sections were mounted with xylene (BDH) in a fume cupboard. A coverslip was placed on stained sections, which were analysed with a light microscope connected to a camera using X125 to X400 magnifications (Leitz/Diaplan, Germany). The photographs of the magnified images were taken and the films were processed by Leicester University Central Photographic Unit, Leicester, UK. The severity of inflammatory changes around bronchioles, thickness of bronchiole walls, and cellular infiltration over the section as a whole were scored separately by two observers, by Dr. Aras Kadioglu and myself, blind to the identity of samples, on a semiquantitative scale ranging from 0 (no inflammation), 1 (slight), 2 (mild), 3 (moderate), 4 (substantial) to 5 (severe). Then, the level of pathologic changes were assessed separately as the average of each pathologic conditions.

3.17 Cell culture techniques

3.17.1 Materials

Unless otherwise stated tissue culture media were obtained from Gibco BRL, and other chemicals from Sigma.

3.17.2 Tissue culture medium and buffer

In order to inactivate complement, foetal calf serum (FCS) was incubated at 56°C for 30 min in a water bath prior to use. RPMI 1640 medium was supplemented with 2 mM glutamine and 6% w/v heat-inactivated foetal

calf serum (complete RPMI 1640 medium). The stock of complete RPMI 1640 medium was kept in 4°C Hank's Balance Salt Solution, without phenol red, with magnesium (HBSS), was buffered with 2.5 ml 1 M Hepes, pH 7.4 (HBSS-Hepes). Prior to use complete RPMI 1640 medium was preincubated at 37°C.

3.17.3 Cell line maintenance

U937 human monocytic cell line (Ralph *et al.*, 1976) was purchased from European Collection Animal Cell Culture (ECACC), UK. The cell line was maintained by subculturing 10 ml of the U937 suspension into 20 ml (*i.e.*, 1 in 3) of fresh complete RPMI 1640 medium in 250 ml tissue culture flasks every two days. Growth was maintained at 37°C in the presence of 5% CO_2. Using an inverted microscope, the cells were examined for monodispersed growth and presence of pseudopodia: if they were clumped and/or became rounded, they were discarded. When required for experiments cells were harvested by centrifugation at 160 g for 10 min a day after subculturing and resuspended in HBSS-Hepes. To complete removal of medium, centrifugation and resuspension was repeated once more.

3.17.4 Activation of U937 cells with interferon gamma (IFN-γ)

When activation of cells required, 100 U/ml recombinant IFN-γ (a gift from G.R. Adolf, Boehringer Ingelheim, Austria) was added into the medium containing $1X10^5$ cells/ml, from the stock of IFN-γ, stored at -70°C $1X10^6$ U/ml. If the cells were not activated after the first 24 h incubation, the medium was removed by centrifugation at 160 g for 10 min and cells were incubated with fresh IFN-γ for further 24 h.

3.17.5 Trypan blue exclusion assay

20 µl of cell suspension to be counted was mixed with equal volume of 0.2% v/v trypan blue in HBSS, without phenol red. 20 µl of this mixture was then loaded into a haemocytometer. When observed under bright field microscopy using X400 magnification, dead cells appeared blue and any viable cells remained white (Metcalf *et al.*, 1986)

3.17.6 Preservation of cell line

In case of contamination or death of the cells, the main stock kept in liquid nitrogen in the Department of Microbiology and Immunology,

University of Leicester To preserve the cell line, U937s were allowed to grow until they reached a dense culture and were then harvested as described above (section 3.17.3). The number of viable cells was determined by trypan blue exclusion assay (section 3.17.5) and the concentration adjusted to $1X10^7$ cells/ml in complete RPMI supplemented with 20% v/v glycerol (cryoprotectant). Then 1 ml cell suspension samples in sterile cryotubes were placed inside a cryo 1°C freezing container (Nalgene, UK) containing propan-2-ol. The container was then kept overnight at -70°C to allow the cell suspensions to freeze. Once frozen, the cryotubes were removed and transferred to liquid nitrogen for storage.

3.17.7 Recovery of cell line from liquid nitrogen

The tubes were removed from liquid nitrogen and left at room temperature prior to transfer to a 37°C water bath for 1-2 min until the cells were fully thawed. The content of tube was placed in 9 ml of prewarmed complete 1640 RPMI medium and grown as described earlier except that the first subculturing was done 4-5 days after recovery (section 3.17.3).

3.17.8 Respiratory burst assay

The respiratory burst was detected by measuring the release of hydrogen peroxide (H_2O_2) from cells. H_2O_2 was quantified fluorimetrically using the method of Jackett and coworkers (1981). This assay is based on the fact that when p-hydroxphenyl acetic acid is oxidised by H_2O_2, in a reaction catalysed by horse radish peroxidase, it fluorescences. By measuring the fluorescence, the amount of H_2O_2 can be quantified because the level of fluorescence is directly proportonial to the amount of H_2O_2 generated.

The cells were harvested as described before (section 3.17.3). $5X10^5$ cells were added to sterile LP2 tubes and the volume was made up to 700 µl with HBSS-Hepes. 50 µl of freshly prepared, substrate, p-hydroxphenyl acetic acid (7.4 mg/ml) and 0.5 U horse radish peroxidase in 50 µl were added to the cell mixture (before use enzyme and substrate were prewarmed for 5 min at 37°C). If required, dilutions of pneumolysin in 100 µl HBSS-Hepes were also added to the cell suspension; tubes containing 100 µl of HBSS-Hepes were used as controls. Then, this mixture was incubated for 15 min at 37°C in 5% CO_2 prior to addition of

100 µl 10 µg/ml (Sigma) phorbol myristate acetate to give a 1 ml final volume; tubes containing 100 µl of HBSS-Hepes were used as controls. All the tubes were incubated for a further 45 min at 37°C in 5% CO_2. After the incubation period, the reaction was arrested by the addition of ice cold 1 ml 0.1 M borate buffer, pH 10.4. The tubes were centrifuged at 1,000 g for 10 min and the supernatants were put into fresh LP2 tubes. The samples were kept in the dark for 10 min at room temperature. The fluoresence was measured using an excitation wavelength 313 nm and emission wavelength 414 nm with a Shimadzu RF-1501 model spectrofluorophotometer. The machine was calibrated with 0.1 M borate buffer, pH 10.4 before use.

3.17.9 Hydrogen peroxide standard curve

For each individual experiment, a standard calibration curve with different concentrations of H_2O_2 (0 to 100 nM) was set up in duplicate using a stock solution of 200 µM. The final volume was brought to 900 µl with HBSS-Hepes. The volume was made up to 1 ml by addition of 50 µl (7.4 mg/ml) p-hydroxphenyl acetic acid and 0.5 U horse radish peroxidase in 50 µl. The same preparations of p-hydroxphenyl acetic acid and horse radish peroxidase were also used for cellular assay. The standard tubes were incubated jointly with the cells for 1 hour at 37°C in 5% CO_2. The reaction was stopped by the addition of ice cold 1 ml 0.1 M borate buffer, pH 10.4. Before use the machine was calibrated with 0.1 M borrate buffer, pH 10.4 and fluorescence was measured as described in section 3.17.8. A calibration curve was constructed by plotting fluorescence against the concentration of H_2O_2.

3.17.10 Measurement of H_2O_2 production in *S. pneumoniae* cultures

Hydrogen peroxide generated by pneumococcal cultures was measured fluorimetrically from mid-log phase cultures grown in a medium anaerobic jar (Spellberg *et al.*, 1996). The cells were harvested by centrifugation at 4000 rpm for 10 min in a Hereaus-Christ centrifuge. The number of cells was determined using a haemocytometer. In addition, the bacterial count was confirmed by growing 10 fold dilutions of culture on blood agar as described earlier (section 3.15.2).

The cell pellet was resuspended in an equal volume of HBSS-Hepes, gently mixed and centrifuged as above to remove the any traces of BHI

medium, as the medium could interfere with the assay. $4X10^7$ cells were diluted in 900 µl of HBSS-Hepes in a LP2 tube. Following incubation in 37°C for 5 min, 0.5 U horse radish peroxidase in 50 µl and 50 µl p-hydroxphenyl acetic acid (from 7.4 mg/ml stock) were added to bacterial cell suspension. The reaction was incubated at 37°C for 60 min. Then, the reaction was stopped by the addition of ice cold 1 ml 0.1 M borate buffer and the fluorescence was measured as described above (section 3.17.8)

3.17.11 Viability test

To assess the effect of recombinant pneumolysin on cell viability, cells were harvested as before (section 3.17.3). Then, various amounts of recombinant pneumolysin (0 to 1 HU), a gift from Robert Gilbert, Department of Biochemistry, Leicester University, in 100 µl HBSS-Hepes were added into LP2 tubes containing $1X10^5$ U937 cells. After one hour incubation at 37°C in 5% CO_2, the cell viability was assessed by the trypan blue exclusion assay (section 3.17.5).

3.17.12 Haemolytic assay

The haemolytic activity present in pneumococcal whole cell extracts or of purified pneumolysin was assayed by serial two-fold dilutions of 50 µl of samples in PBS in a microtitre plate as described by Owen *et al.* (1994). 50 µl of 2% v/v sheep erythrocytes (TCF Microbiology, Buckingham, England) prepared in PBS (pH 7.3) was added to each well. The plate was incubated at 37°C for 30 min. The haemolytic unit present in 1 ml of pneumolysin was expressed as the amount of the toxin lysing 50% of a 2% v/v suspension of sheep erythrocytes in 30 min at 37°C.

3.18 Statistical analysis

Data were analysed by student-t test, ANOVA followed by Tukey-Kramer multiple comparisons test or by Mann-Whitney U test, as appropriate and as stated in the relevant section of the results. Analysis of *in vivo* growth rates was performed by comparison of regression slopes. I am grateful to John Beckett, Computer Centre, University of Leicester, for his help to compare regression slopes of my data.

Chapter 2

Studies on Pneumococcal Superoxide Dismutases

Part 4 Introduction

4.1 Formation of reactive oxygen species

Oxygen is a vital element for aerobic life (Halliwell and Gutteridge, 1984). However, it also has many toxic effects; at concentrations greater than those found in the normal atmosphere (21%), it is well known to damage plants, animals, and microorganisms (Balentine, 1982). The toxic effects of oxygen on microorganisms vary from growth inhibition to loss of viability.

Molecular oxygen is not toxic directly, but it is capable of undergoing a series of reductions to produce reactive oxygen species (ROS) including superoxide ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radical ($^{\bullet}OH$) which are responsible for many of the damaging effects of oxygen (Gerschman *et al.* 1954). The reactivity of oxygen increases, when it accepts one, two, or three electrons to form a superoxide radical ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2), and hydroxyl radicals ($OH^{\bullet}$) (Cadenas, 1989) (Figure 4.1).

$$O_2 \xrightarrow{e^-} {}^{\bullet}O_2^- \xrightarrow{e^-,\,2H^+} H_2O_2 \xrightarrow{e^-,\,H^+} {}^{\bullet}OH \xrightarrow{e^-,\,H^+} H_2O$$

Figure 4.1 Formation of ROS by incomplete reduction of O_2 to water.

4.2 The biological sources of ROS

Normally, O_2 is enzymatically (e.g., by cytochrome oxidase) reduced without the release of ROS in the cell. Nevertheless, $O_2^{\bullet -}$ is generated spontaneously and by enzymatic oxidations due to electrons that escape from the electron transport chain during aerobic metabolism (Fridovich, 1998). ROS are also generated as a result of auto-oxidation of many biological molecules, such as oxyhaemoglobin, oxymyoglobin, flavins, and thiols (Fridovich, 1978).

As a part of the body's defence mechanisms, phagocytic cells produce ROS when they encounter an invading microorganism (Andrew *et al.*, 1985). The pathway through which ROS are formed is called the respiratory burst. In this, oxygen is reduced to superoxide radical by the catalytic activity of NADPH oxidase which is associated with the phagocyte membrane (Andrew *et al.*, 1985). Superoxide is then reduced by SOD or by spontaneous dismutation to form H_2O_2. In the presence of cerain halide ions (I^-, Br^-, Cl^-) and myeloperoxidase (MPO), H_2O_2 generates chloramines which have microbicidal activities due to aldehyde formation and peptide cleavage (Klebanoff, 1996). There is substantial evidence suggesting the contribution of this system as an effective killing mechanism of neutrophils. For example, hydrogen peroxide at a concentration of 0.5 mM killed 34% of an *E. coli* inoculum, but same amount of H_2O_2 combined with neutrophil granule extracts increased the killing rate to 99.9% (McRipley and Sbarra, 1967). Superoxide radical can also react with nitric oxide ($^{\bullet}NO$), which is a short-lived free radical produced by phagocytes (Liu and Hotchkiss, 1995; Tsukahara *et al.*, 1998). The reaction between $O_2^{\bullet -}$ and $^{\bullet}NO$ can lead to the formation of peroxynitrite and $^{\bullet}OH$, both of which can modify proteins, oxidise DNA and initiate lipid peroxidation (Hogg *et al.*, 1992).

Superoxide anion is not as reactive as other ROS, particularly with lipids, carbohydrates or nucleic acids, but it does exhibit limited reactivity with certain proteins that contain transition-metal prosthetic groups, such as haem moieties or iron-sulphur clusters (Davies, 1995). Superoxide radical can not cross biological membranes, unless it passes through a specific channel, such as anion channels (Fridovich, 1995).

Hydrogen peroxide (H_2O_2) is produced by multiple oxidation pathways, one of which is dismutation of superoxide which generates molecular oxygen plus H_2O_2 (Beyer *et al.*, 1991). H_2O_2 easily crosses biological membranes (Davies, 1995) and exhibits its toxic effects by acting on many biological molecules, particularly those containing sulphydryl groups, iron-sulphur clusters, reduced haem moieties and copper prosthetic groups (Farr and Kogoma, 1991). Although H_2O_2 is not a free radical, it is a ROS and is a fairly stable oxygen intermediate. However, when it interacts with transition metals, such as Fe(III) or Cu(II), a very powerful

oxidant, $^{\bullet}OH$, is formed (Figure 4.2.a). This pathway was first described by Fenton in 1894 and is known as Fenton reaction (Storz *et al.*, 1990).

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a) $^{\bullet}O_2^- + {}^{\bullet}O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$

$^{\bullet}O_2^- + Fe^{3+} \longrightarrow O_2 + Fe^{2+}$

$H_2O_2 + Fe^{2+} \longrightarrow {}^{\bullet}OH + OH^- + Fe^{3+}$

Net reaction: $^{\bullet}O_2^- + H_2O_2 \longrightarrow {}^{\bullet}OH + OH^- + O_2$

b) $^{\bullet}O_2^- + H_2O_2 \longrightarrow O_2 + {}^{\bullet}OH + OH^-$

Figure 4.2 Generation of hydroxyl radical through the Fenton reaction (a) and Haber-Weiss reaction (b).

Evidence for the availability of iron and copper necessary for Fenton reaction *in vivo* has been the matter of debate since there is no free iron or copper in biological systems because they are bound to proteins, such as haemoglobin or myoglobin (Rice-Evans *et al.*, 1995). However, there are number of proposed ways that iron can be available *in vivo* for the Fenton reaction, for example during cell damage (Rice-Evans *et al.*, 1995).

Hydroxyl radical is also formed through the iron-catalysed, Haber-Weiss reaction (Figure 4.2b). The Haber-Weiss reaction requires traces of transition metal ions as a catalyst since the rate constant for the reaction in aqueous solution has been shown to be zero (Stadtman, 1993). The $^{\bullet}OH$ radical is extremely reactive with a wide range of compounds including proteins, lipids, carbohydrates, DNA and RNA (Davies, 1995). The $^{\bullet}OH$ radical never passes through biological membranes, since it reacts with the first biological component it encounters (Halliwell and Gutteridge, 1984). The *in vivo* generation of $^{\bullet}OH$ was demonstrated by Mello-Filho and Meneghini (1984). They showed that DNA damage in human fibroblasts caused by H_2O_2 was preventable with iron chelators,

such as phenanthroline and bipyridine, suggesting *in vivo* formation of $^{\bullet}OH$.

4.3 Damage caused by reactive oxygen species

The administration of ROS to a cell can cause severe metabolic dysfunctions through their adverse effects on all the major biological compounds (Halliwell and Gutterdidge, 1989). Exposing cells to H_2O_2, superoxide-generating compounds, organic hydroperoxides, singlet oxygen, or ozone results in several types of DNA damage (Imlay and Linn, 1988). Reactive oxygen species block DNA replication, causes base damage and strand breaks, single or double, which is due to oxidative cleavage of deoxyribose (Halliwell and Aruoma, 1991).

All the reactive oxygen species can cause peroxidation of lipids both *in vivo* and *in vitro*. Lipid peroxidation increases membrane fluidity and results directly in a loss of structural integrity, which eventually leads to cell lysis. Peroxidation intermediates and end products of lipid peroxidation (aldehyde, epoxide, hydroxy, carboxy, and peroxy groups, alkanes, and alkenes) also are mutagenic and inactivate proteins (reviewed by Farr and Kogoma, 1991). The oxidative damage to proteins is characterised by oxidised amino acids. Oxidative damage inactivates enzymes, such as dihydroxy acid dehydratase, an enzyme that has a role in the biosynthetic pathway of branched chain amino acids, and aconitase, an iron-regulatory protein that has a role in oxidative metabolism. All these enzymes contain $(Fe\text{-}S)_4$ clusters which are known to be main target of the oxidative attack (Gardner and Fridovich, 1991). The inhibition of NAD biosynthesis and reduction in thiamine content are some other effects originating from oxidative damage (reviewed by Farr and Kogoma, 1991).

4.4 Defence systems against reactive oxygen species

As mentioned above (section 4.3), reactive oxygen species cause damage and are stress factors for living organisms (Crawford *et al.*, 1994). The detoxification of oxygen radicals therefore is of great importance. Defence systems against oxygen include enzymatic and nonenzymatic antioxidant systems. Nonenzymatic antioxidant defences include glutathione, vitamin C and E, urates, beta-carotene and transferrin-lactoferrin (Camhi *et al.*, 1995). However most of oxygen radicals are removed by enzymatic

antioxidant defences by the action of four enzymes: superoxide dismutase, catalase, glutathione peroxidase or glutathione reductase (Camhi *et al.*, 1995). Enzymes that remove or repair damaged molecules are also part of the antioxidant defence system. These include endonucleases, exonucleases and DNA polymerase, which repair single-stranded breaks and modified bases in DNA caused by oxidative stress and methionine sulfoxide reductase, which repairs methionine residues in proteins that have been damaged by $OH^{\bullet}$, (Davies, 1995).

4.5 Glutathione peroxidase

Glutathione peroxidase is important for the elimination of H_2O_2. Cycling of glutathione between reduced and oxidised forms serves to remove active oxygen species (Fridovich, 1998). Two molecules of glutathione (GSH) are oxidised by one molecule of H_2O_2 to form the disulphide-bonded oxidised glutathione, which is catalysed by glutathione peroxidase. Glutathione reductase then catalyses the reduction of oxidised glutathione yielding two molecules of GSH by utilisation of NADPH as a source of reducing power.

4.6 Catalase

The catalases belong to the family of enzymes which contains the hydroperoxidases and peroxidases. Catalase (E.C. 1.11.1.6) is the enzyme that converts hydrogen peroxide into oxygen and water. Catalases isolated from a wide variety of animals, plants and microorganisms are regarded as typical catalases as they resemble each other very closely (Nadler *et al.*, 1986). Typical catalase enzymes are composed of four subunits of equal size, each containing a ferric haem IX prosthetic group. They also share common physicochemical characteristics including a molecular weight varying between 225-270 kDa, wide pH range of activity between pH 5 and 10.5 and inhibition of activity by aminotriazole (Hochman and Shemesh, 1987). On the other hand, peroxidases (E.C. 1.11.1.7) are a heterogeneous group and require a reducing agent for their activity, such as dianisidine or NADPH (Claibourne and Fridovich, 1979). In contrast to catalases, peroxidases constitute a heterogeneous group of enzymes but they are mainly monomers with a wide range of molecular weights (Hochman and Shemesh, 1987).

In addition to these two classes, the presence of another group of heme containing hydroperoxidases, called catalase-peroxidases (E.C. 1.11.1.6) which possess both catalytic and peroxidatic activities, has been described. Unlike typical catalases, they have a narrow range of pH activity, are relatively heat labile and are resistant to aminotriazole (Nadler *et al.*, 1986, Hochman and Shemesh, 1987). This group so far has been isolated only in microorganisms, including *E. coli* (Clairborne and Fridovich, 1979), *Mycobacterium tuberculosis* (Wayne and Diaz, 1982) *Rhodopseudomonas capsulata* (Hochman and Shemesh, 1987).

Catalase's role in virulence was first noted by Middlebrook and Cohen (1953) who discovered that a *Mycobacterium tuberculosis* strain devoid of catalase exhibited reduced virulence in the guinea pig. Catalase's role in virulence was attributed to its ability to remove peroxide when the bacteria encounter phagocytes (Beaman and Beaman, 1984). For example, a catalase-negative mutant strain of *Staphylococcus aureus* was more sensitive than the wild-type to killing by neutrophils (Mandell, 1975). Also studies performed with a catalase-negative strain of *Neisseria gonorrheae* showed that the mutant strain was extremely sensitive to exogenous hydrogen peroxide, in contrast to wild type (Johnson *et al.*, 1993). However, studies carried out with a number of other organisms were not able to trace a role for catalase as a virulence factor. For example, catalase-negative mutants of *Listeria monocytogenes* and *Shigella flexneri* do not show a significant reduction in the virulence (Gaillard *et al.*, 1986; Franzon *et al.*, 1990).

4.7 Superoxide Dismutase (SOD)

Superoxide dismutases (EC 1.15.1.1.) are metalloenzymes that are responsible for conversion of superoxide to hydrogen peroxide and oxygen: $^{\bullet}O_2^- + {}^{\bullet}O_2^- + 2H^+ \longrightarrow H_2O_2 + O_2$ (rate constant: $1.9 \times 10^9\ M^{-1}\ sec^{-1}$ at 25°C) (Fridovich, 1986). As far as is known, dismutation of $^{\bullet}O_2^-$ is the only biological function of superoxide dismutase (Hassan, 1989). Studies leading to the discovery of SOD started more than a half century ago with the isolation of a copper protein from blood and ox liver by Mann and Keilin (1939). These proteins were named as hemocuprein and hepatocuprein, respectively. However no function was attributed to these proteins and they were thought to act as copper storage proteins. Eventually a defined role for the function of these proteins was identified

with the work of McCord and Fridovich (1969) who discovered milk xanthine oxidase as an enzymatic source of $^{\bullet}O_2^-$ which provided an assay system for SOD.

4.7.1 Types of SODs in bacteria

There is more than one type of SOD enzyme according to the metal ion at the catalytic centre of the enzyme (Fridovich, 1986). These are copper-zinc SODs (CuZnSOD), manganese SODs (MnSOD) and iron SODs (FeSOD). In addition to these three types, the presence of nickel containing SOD (NiSOD) in several *Streptomyces* spp has been reported (Kim *et al.*, 1998; Youn *et al.*, 1996). The presence of SODs has been shown in many different microbial species including various aerobic and anaerobic bacteria (Figure 4.3), algae, protozoa and fungi. SODs are also found in invertebrates, vertebrates and plants (reviewed by Bannister *et al.*, 1987). Comparison of amino acid sequences indicates that there are two distinct phylogenetic families present, the CuZnSOD and Fe, MnSODs (Figure 4.4) (Beyer *et al.*, 1991). Neither sequence nor secondary structure homology have been found between these two families (Harris and Steinman, 1977) However, the FeSOD and MnSOD show a high degree of amino acid sequence (Figure 4.4) and structural homology (Steinmann and Hill, 1973). There are 38 residues distributed throughout the entire length of Mn and FeSODs that are invariant (Figure 4.4). Based on an examination of the crystal structure of the known SODs, a role was postulated for most of these invariant residues i.e., His 82, Trp 173 and Ala 177, which act as ligands for the metal cofactor (Parker and Blake, 1988).

The explanation for the question of why does more than one family exists can be related to geochemical history of earth (Beyer *et. al.*, 1991). It was suggested that the oxygenation of the earth imposed a selection pressure on the first organisms which were anaerobic heterotrophs. FeSOD was first SOD prototype because the primitive earth contained an abundant amount of iron which was in a soluble form, Fe^{2+} (Asada *et al.*, 1980). Thus, obligate anaerobes and facultative anaerobes, primarily, contain FeSOD. MnSOD, on the other hand, was speculated to have evolved from FeSOD as a result of either gene duplication or unavailability of soluble Fe^{2+} (Hassan and Schiavone, 1988). The formation of CuZnSOD followed a totally different route and coincided

with the oxygenation of earth, when copper became available in the more soluble oxidised state (Ochiai, 1983).

Initially, it was thought that SOD was unique to aerobes. On the basis of SOD production, McCord and coworkers (1981) even pioneered an enzyme-based hypothesis of obligate anaerobiosis. According to this hypothesis, SOD was crucial for aerobiosis and the aerobic existence of an organism was thought to be dependent upon its ability to produce SOD.

However, the presence of SOD in some strains of anaerobic sulfate-reducing bacteria was reported and so undermined the hypothesis (Bell, 1973). The reason for the presence of SOD activity in some anaerobes is thought to be as a result of an evolutionary response mechanism to oxygen which they encounter during transient exposures while passing from one anaerobic niche to another (Privalle and Gregory, 1979). There is no general rule to predict one given organism's SOD type, and no distinct correlation was found between Gram reaction and the type of SOD (Britton *et. al.*, 1978).

However, broadly speaking the general tendency for the type of SOD among bacteria, if they exhibit activity, is this: anaerobic organisms contain FeSOD, whereas most obligate aerobic organisms have MnSOD (Bannister *et al.*, 1987). Facultative anaerobic species contain either the iron or the manganese enzyme or both, some having a hybrid form of these two enzymes as well, such as *E. coli.*

The mechanism of action of SODs is based on alternate reduction and reoxidation of the metal at the active centre during successive interactions with $^{\bullet}O_2^-$. When CuZnSOD encounters $^{\bullet}O_2^-$, copper undergoes a cycles of reduction and reoxidation, oscillating between cupric and cuprous state (Fridovich, 1974). On the other hand, zinc has a structural role and also maintains the stability of the enzyme (Forman and Fridovich, 1973). The mechanism of action of FeSOD or MnSOD resembles that of CuZnSOD: the iron and manganese in FeSOD and MnSOD changes between the trivalent and the divalent states (Beyer *et al.*, 1991).

FeSOD	**Reference**
Escherichia coli	Yost and Fridivich, 1973
Mycobacterium tuberculosis	Kusunose *et al.*, 1976
Pseudomonas ovalis	Yamakura, 1976
Thiobacillus denitrificans	Baldensperger, 1978
Bacillus megaterium	Anastasi *et al.*, 1976
Photobacterium sp.	Puget and Michelson, 1974
Legionella pneumophila	Sadosky *et al.*, 1994
Shigella flexneri	Franzon *et al.*, 1990
Campylobacter jejuni	Pesci *et al.*, 1994
MnSOD	
Escherichia coli	Keele *et al.*, 1970
Streptococcus mutans	Nakayama, 1992
Streptococcus faecalis	Briton *et al.*, 1978
Bacillus stearothermophilus	Harris, 1977
Thermus acquaticus	Sato and Harris, 1977
Thermus thermophilus	Sato and Nakazawa, 1978
Paracoccus denitrificans	Terech and Vugnais, 1981
Bacillus subtilis	Tsuduka *et al.*, 1983
Bordetella pertussis	Graeeff *et al.*, 1997
Lactococcus lactis	Sanders *et al.*, 1995
Bacillus sreaothermophilus	Harris, 1977
Haemophilus influenzae	Kroll *et al.*, 1993
Salmonella typhimurium	Tsolis *et al.*, 1995
CuZnSOD	
Haemophilus sp.	Kroll *et al.*, 1995
Caulobacter crescentus	Steinman, 1982
Pseudomonas dimunata	Steinman, 1985
Pseudomonas maltophilia	Steinman, 1985
Brucella abortus	Bricker *et al.*, 1990
Actinobacillus pleuropneumoniae	Langford *et al.*, 1996
Escherichia coli	Imlay and Imlay, 1996
Photobacterium leeiognathi	Puget and Michelson, 1974
Neisseria meningitidis	Wilkis *et al.*, 1998
Salmonella typhimurium	Farrant *et al.*, 1997
NiSOD	
Stretomyces spp.	Youn *et al.*, 1996

Figure 4.3 Type distribution of SODs among bacteria

Figure 4.4 Aligned amino acid sequences of Fe, Mn, CuZnSODs. Amino acid sequences from a range of bacteria were compared for similarity using the PILEUP programme in the GCG molecular biology package. Superoxide dismutase sequences were extracted from the EMBL sequence database. In the figure "A" represents MnSODs and these SODs were from (accession numbers): *Pseudomonas putida* (U64799), *Bordetella pertussis* (X84800), *Streptococcus mutants* (S39782), *Salmonella typhimurium* (U20645); "B" represents FeSODs and these SODs were from (accession numbers): *Pseudomonas aeruginosa* (L25675), *Bacteroides fragilis* (M96560), *Pseudomonas putida* (U64798), *Bordetella pertussis* (M83095); "C" represents CuZnSODs and these SODs were from (accession numbers): *Neisseria meningtidis* (X83126), *Escherichia coli* (X97766), *Legionella pneumophila* (S82042), *Caulobacter crescentus* (M55259). Residues which are present in 8/8 Fe and MnSOD sequences are marked with "*", residues which are present in 7/8 Fe and MnSOD sequences are marked with "+". Identical residues in CuZnSOD sequences are underlined.

```
                    *   ** *+*   ** * +   * + *+   * ***
A  P.putida         MPHTLPALPYAYDALEPHIDAQTMEIHHTKHHQTYVNGLNAAIEGTEW.A
A  B.pertussis      MPYVLPALSYAYDALEPHIDARTMEIHHTRHHQTYVNGLNAALEGAGLDS
A  S.mutants        MAILLPDLPYAYDALEPYIDAETMTLHHDKHHATYVANANAALEKHPEIG
A  S.typhimurium    MSYTLPSLPYAYDALEPHFDKQTMEIHHTKHHQTYVNNANAALENLPEFA
B  P.aeroginosa     MAFELPPLPYEKNALEPHISAETLEYHHDNNHNTYVVNLTNLIPG..TEF
B  B.fragilis       MAFELPPLPYEKNALEPHISAETLEYHHDNNHNTYVVNLTNLIPG..TEF
B  P.putida         MAFELPPLPYAHDALQPHISKETLEFHHDKHHNTYVVNLNNLVPG..TEF
B  B.pertussis      MAHTLPPLPYALDALAPRISKETLEFHYGKHHQTYVTNLNNLVPGFTTEF
C  N.meningitidis  ~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~~
C  E.coli          ~~~~~~~~~~~~~~~~~~~MASEKVEMNLVTSQGVGQSIGSVTITETDKG
C  L.pneumophila  ~~MNKSGIILIGTILFSSMAIADDLTAPIYTTGPKPVAIGKVTFTQTPYG
C  C.crescentus    MIRLSAAAALGLAAALAASPALAQTSATAVVKAGDGKDAGAVTVTEAPHG

                    +                          **     **   *    +       +
B  P.aeroginosa     EG......KSLEEIVKSSSGGIFNNAAQVWNHTFYWNCLSPNGGGQPTGG
B  B.fragilis       EG......KSLEEIVKSSSGGIFNNAAQVWNHTFYWNCLSPNGGGQPTGG
B  P.putida         EG......KTLEEIVKTSSGGIFNNAAQVWNHTFYWNCLAPNAGGQPTGA
B  B.pertussis      EN......LSLEEIVKKSSGGVFNNAAQVWNHTFYWNSLSPNGGGEPSGA
A  P.putida         EWPVEKLVGAVKQLPESLRGAVTNHGGGHANHSLFWTVMSPQGGGEPHGQ
A  B.pertussis      EEPVEQLLRRIPALPPGIHGAVRNHGGGHANHSLLWTVMSPSGGGRPDGR
A  S.mutants        E.NLEVLLADVEQIPADIRQSLINNGGGHLNHALFWELLSPE.KTKVTAE
A  S.typhimurium    SLPVEELITKLDQVPADKKTVLRNNAGGHANHSLFWKGL..KTGTTLQGD
C  N.meningitidis   ~~~~~~~~~~~~~~~~~~~~~~~~~EPKEKEGKLTAGLGAGGHWDPKGAK
C  E.coli          LEFSPDLKALPPGEHGFHIHAKGSCQPATKDGKASAAESAGGHLDPQNTG
C  L.pneumophila  VLITPDLTNLPEGPHGFHLHKTADC........GNHGMHAEGHYDPQNTN
C  C.crescentus    VLLKLELKGLTPGWHAAHFHEKGDCGTPDFK.......SAGAHVHTAATT

                    ++ +*    *    * *+  *              **       *   +
B  P.aeroginosa     LADAINAGFGSF..DKFKEEFTKTSVGHLRFRSGWLVKKPDGSLALASTI
B  B.fragilis       LADAINAGFGSF..DKFKEEFTKTSVGHLRFRSGWLVKKPDGSLALASTI
B  P.putida         LADAINAAFGSF..DKFKEEFTKTSVGTFGSGWGWLVKKADGSLALASTI
B  B.pertussis      LADAIKAKWGSVFTDAFKEAFNKSAAGNFGSGWTWLVKKADGTLDIVNTS
A  P.putida         LAQAIASQLGGF..DAFKEAFTKAALTRFGSGWAWLSVTPQKTLVVESSG
A  B.pertussis      LAADIQAQLGGH..DAFQAAFTQAALGRFGSGWAWLTVTPAGRLRVDSSA
A  S.mutants        VAAAINEAFGSF..DDFKAAFTAAATTRFGSGWAWLVVDKEGKLEVTSTA
A  S.typhimurium    LKAAIERDFGSV..DNFKAEFEKAAATRFGSGWAWL.VLKGDKLAVVSTA
C  N.meningitidis  QHGYPWQDDAHLGDLPALTVLHDGTATNPVLAPRLK........HLDDVR
C  E.coli          KHEGP.EGAGHLGDLPALVVNNDGKATDAVIAPRLK........SLDEIK
C  L.pneumophila  SHQGPY.GNGHLGDLPVLYVTSNGKAMIPTLAPRLK.........LSDMH
C  C.crescentus    VHGLLNPDANDSGDLPNIFAAADGAATAEIYSPLVSLKGAGGRPALLDAD

                        ++           +++  *******  *  +          *
B  P.aeruginosa     GAGNPLTS......GDTPLLTCDVWEHAY..YIDYRT.ASEVRRAFWNLV
B  B.fragilis       GAGNPLTS......GDTPLLTCDVWEHAY..YIDYRT.ASEVRRAFWNLV
B  P.putida         GAGCPLTS......GDTPLLTCDVWEHAY..YIDYRNLRPKYVEAFWNLV
B  B.pertussis      NAATPLTT......ADKALLTCDVWEHAYFTYIDYRNARPKYLENFWALV
A  P.putida         NQDSPLM......FGNTPIFGLDVWEHAY..YLKYQNRRPEYIGAFYNVI
A  B.pertussis      NQDSPLM......EGNTPILGLDVWEHAY..YLQYQNRRPEYIEAFYRVV
A  S.mutants        NQDTPIS......QGLKPILALDVWEHAY..YLNYRNVRPNYIKAFFEVI
A  S.typhimurium    NQDSPLMGEAISGASGFPILGLDVWEHAY..YLKFQNRRPDYIKEFWNVV
C  N.meningitidis  GHSIMIHTGGDNHSDHPAPL
C  E.coli          DKALMVHVGGDNMSDQPKPLGGGGERYACGVIK
C  L.pneumophila  NLAVMIHANGDTYSDNP.PQGGGGDRIACGVIK
C  C.crescentus    GSSIVVHANPDDHKTQ..PIGGAGARVACGVIK
```

Copper-zinc superoxide dismutases primarily are found in the cytosol of many eukaryotic organisms. However, in recent years CuZnSODs have been identified in the periplasms of Gram-negative bacteria (Table 4.3), in the plastids of plants and in the extracellular spaces of mammals (Fridovich, 1998). The mammalian extracellular CuZnSODs differ from cytoplasmic CuZnSODs by being tetrameric and glycosylated (Marklund, 1982). In addition to mammals, extracellular CuZnSODs have been found in several non-mammalian species including *Schistosoma mansoni* and *Onchocerca volvulus* (Fridovich, 1995).

Replacement of copper with another metal causes the loss of activity of CuZnSOD, whereas the substitution of zinc with mercury does not result in any change in activity, indicating the crucial role of copper for the catalytic activity (Forman and Fridovich, 1973). CuZnSODs are reversibly inhibited by cyanide and azide, both of which exhibit their activity by binding to copper (Ratillio *et al.*, 1972). Hydrogen peroxide, however, irreversibly inactivates CuZnSODs which is associated with the oxidation of one histidine residue per subunit (Bray *et al.*, 1974).

Iron and manganese co-factored SODs have been found mainly in the cytosol of prokaryotes. Following SODs, both FeSOD and MnSOD, discovery in *E. coli*, it has been shown to exist in various prokaryotes (Table 4.3). MnSODs are also found in the matrix of mitochondria (Beyer *et al.*, 1991). The presence of extracellular MnSOD from *Mycobacterium avium* and *Streptococcus pyogenes* has been recently reported (Escuyer *et al.*, 1996; Gerlach *et al*, 1998). FeSOD and MnSOD show a high degree sequence and structural homology which indicates a common ancestral protein for these enzymes (Harris *et al.*, 1980). Nonetheless, despite the relatedness of these enzymes, the two types are immunologically distinct from each other (Touati, 1983). Structurally they are in a homodimeric form containing one atom of metal per subunit and generally have a subunit molecular weight of about 20,000 (Sato and Nakazawa, 1978). However, tetrameric forms of MnSODs were isolated from various microorganisms including *Thermus thermophilus* and *Mycobacterium tuberculosis* (Sato and Nakazawa, 1978; Kusunose *et al.*, 1976). The presence of metal is very crucial for catalytic activity of Mn and FeSODs. Metal substitution in different bacterial species resulted in the loss of activity both for MnSODs and FeSODs suggesting strict metal co-factor

specificity of these enzymes (Kirby *et al.*, 1980). Unlike CuZnSODs, Fe and MnSODs are not inhibited with cyanide. Hydrogen peroxide, on the other hand, irreversibly inactivates FeSODs but has no effect on MnSODs (Bannister *et al.*, 1987; Beyer *et al.*, 1991).

4.8 Function of SODs

Superoxide dismutase is one of the superoxide-induced proteins and its main role is the removal of $^{\bullet}O_2^-$ (Fridovich, 1995). Although $^{\bullet}O_2^-$ is not as reactive as $^{\bullet}OH$ or H_2O_2, it acts as a precursor for the formation of the latter (section 4.2).

If ROS are not adequately removed, either because of depleted levels of antioxidants or an increased formation of ROS, oxidative stress occurs resulting in damage of almost all cellular components by causing mutagenesis, enzyme inactivation and membrane damage (Sies 1991). Studies carried out with *E. coli* and *S. typhimurium* revealed that when bacteria are exposed to ROS, the level of several proteins is increased. However, the nature of the response varies according to the particular ROS (Farr and Kogoma, 1991). In response to H_2O_2 or organic peroxides, such as *tert*-butyl hydroperoxide and cumene peroxide, the cellular level of at least 30 proteins rises (Greenberg and Demple, 1989). However, the enzymatic functions of many of these proteins are not known. So far, nine of the proteins with known enzymatic functions have been identified and these are coregulated by a common regulatory gene called *oxyR* (Demple, 1991) (Table 4.1). Hence, *oxyR*-deleted strains of *E. coli* and *Salmonella typhimurium* failed to induce these proteins (Storz *et al.*, 1990).

On the other hand, when these bacteria are exposed to a superoxide source, such as paraquat or menadione, the production of over 40 proteins is induced (Demple, 1991). The proteins synthesised upon exposure to paraquat or menadione include all the proteins in the *oxyR* regulon in addition to a total of 33 proteins that are not seen with hydrogen peroxide treatment, including three heat shock proteins, MnSOD, endonuclease IV. Expression of twelve superoxide response proteins are regulated by *soxRS* gene locus and function of many of these proteins is known (Table 4.2) (Demple, 1996). However, there has not

been any functions attributed to the other 21 proteins synthesised upon exposure to superoxide generating agents (Greenberg and Demple, 1989).

In addition to *oxyR* and *soxRS*-regulated responses, oxidative stress induces some of the proteins that are also induced under other stress conditions, such as heat shock response, carbon starvation stress, acid stress and the SOS response (VanBogalen *et al.*, 1987; Farr and Kogoma, 1991). It was demonstrated, for example, that *Porphyromonas gingivalis* expressed elevated levels of SOD in response to heat stress (Amano *et al.*, 1994). The organism's ability to induce increased levels of SOD under heat stress was thought to be due to its defence mechanism against superoxide production by neutrophils in inflamed periodontal pockets (Amano *et al.*, 1994). Sanders and coworkers (1995) reported induction of MnSOD in response to low pH culture conditions suggesting a role for MnSOD in resistance to low pH, but how MnSOD functions at acidic conditions is unclear.

4.8.1 The role of Mn and Fe co-factored SOD in bacteria

For the sake of clarity, this section will be reviewed under two subheadings. In section 4.8.1.1 a review of *in vitro* effects of SOD in bacterial survival; in section 4.8.1.2 mainly the *in vivo* effect of SOD will be discussed.

4.8.1.1 Protection against reactive oxygen species

The cytosolic SODs are principally responsible for protecting cytosolic constituents from free radical damage (Kroll *et al.*, 1993). However, physiologically, the protection provided by FeSOD and MnSOD are not equivalent to each other. Using isogenic strains of *E. coli* containing either Mn or FeSOD encoded on a plasmid under the control of a *tac* promoter, it was revealed that MnSOD was more effective than FeSOD in preventing damage to DNA, as assessed by mutation rate or toxicity of paraquat (Hopkin *et al.*, 1992). FeSOD appeared to be more effective in protecting a superoxide-sensitive enzyme, 6-phosphogluconate dehydratase.

Early studies carried out with *E. coli* showed that increased levels of MnSOD, induced by a variety of physiological conditions, provided an

Table 4.1: Genes of the *oxyR* regulon and their proposed function in *E. coli* (Camhi *et al.*, 1995; Farr and Kogoma, 1991).

Gene	Product	Role
katG	HPI catalase	Removal of H_2O_2 or organic peroxides
ahpF	Alkyl hydroperoxidase F	Removal of H_2O_2 or organic peroxides
ahpC	Alkyl hydroperoxidase C	Removal of H_2O_2 or organic peroxides
gor	Glutathione reductase	Reduction of oxidised glutathione
groES	GroES protein	Heat shock protein
groEL	GroEL protein	Heat shock protein
dnaK	DnaK protein	Heat shock protein
dps	DNA-binding protein	Protects DNA from oxidative damage
recA	RecA gene product	DNA repair protein

Table 4.2: The genes of *soxRS* regulon and their proposed functions in *E. coli*. The genes that have been cloned and whose function has been biochemically analysed are shown (Demple, 1996).

Gene	Product	Role
sodA	MnSOD	Keeps O_2^{-} •levels low
nfo	Endonuclease IV	By initiating repair of oxidative DNA damages provides viability and genetic stability
zwf	Glucose-6-phosphate dehydrogenase, generates NADPH	Provides reducing power for antioxidant enzymes
fumC	Fumarase C, heat-stable fumarase activity	Replace O_2^{-}• -sensitive fumarases
acn	Aconitase	Activity in citric acid cycle replace aconitase inactivated by O_2^{-}•
micF	Antisense RNA for *ompF* mRNA, blocks OmpF synthesis)	Diminishes uptake of antibiotics and other compounds
acrAB	AcrA and AcrB, activity in efflux pump(s) in inner membrane)	Maintains low levels of antibiotics and other compounds
fpr	Fpr, ferredoxin: NADPH oxidoreductase	Reduce FeS clusters

increased resistance against oxygen toxicity and the mutagenicity of oxygen radicals (Hassan and Fridovich, 1977; Moody and Hassan, 1982). However, the direct contribution of SOD in protection against oxygen toxicity was obtained by studies conducted with several *sod* negative mutant strains of *E. coli* by Carlioz and Touati (1986). It was shown that a single mutation in either FeSOD or MnSOD did not effect aerobic growth of the strains in neither complex nor minimal medium, as compared to wild type. The strain carrying a mutation in both *sodA* and *sodB*, on the other hand, could not grow aerobically in minimal medium and growth in rich medium was inhibited by the presence of paraquat or hydrogen peroxide. The mutant strain, which could grow well under anaerobic conditions, exhibited oxygen-dependent nutritional auxotrophies, and enhanced mutagenesis. In another study, analysis of a *sodA* mutant strain of *Pseudomonas aeruginosa* in rich and in glucose minimal medium under the aerobic conditions did not indicate any growth impairment (Hassett *et al.*, 1995). In contrast, a *sodB* insertion mutant grew much more slowly than the *sodA* mutant or wild type bacteria in both media. However, a mutation in both genes resulted in very slow growth in rich medium, which was slower than the *sodB* mutation alone, and required approximately 48 h to attain saturated growth in minimal medium under aerobic conditions (Hassett *et al.*, 1995). These results suggest the importance of SOD for *in vitro* growth of bacteria

Protection provided by SOD may vary from one bacterium to another. A *sodA* mutant of *S. pyogenes*, for example, could not maintain growth on solid medium under aerobic conditions and the growth rate of the mutant strain was lower than the parent strain in liquid culture (Gibson and Caparon, 1996). Also, a *sodA* negative mutant of *Haemophilus influenzae* Eagan strain could not survive in the presence of paraquat at any of the tested concentrations unlike wild type which could tolerate 10 to 20 mM of paraquat and sustain normal growth on solid medium (D'mello *et al.*, 1997). On the other hand, a *sodA* negative mutant of *Streptococcus mutant* was able to grow under aerobic conditions on solid medium albeit more slowly than the parent strain (Nakayama, 1992).

The degree of protection afforded by Fe and MnSODs can be vital for some organisms. For example, *Legionella pneumophila* contains a CuZnSOD and an FeSOD (Sadosky *et al.*, 1994). When an attempt was

made to replace the *sodB* gene with a *sodB::lacZ* gene fusion, it was only possible after provision of a second wild type copy. This result suggests that CuZnSOD cannot replace the FeSOD and FeSOD is required for the viability of *L. pneumophila* (Sadosky *et al.*, 1994). A similar observation was also made with *Bordetella pertussis* and *Bordetella bronchiseptica* by Graeff-Wohlleben and coworkers (1997). Both *B. pertussis* and *B. bronchiseptica* contain MnSOD and FeSOD activities. The *sodA* negative mutant of these bacteria did not show any obvious phenotype change. However, these researchers failed to construct stable *sodB* negative mutant strains of *B. pertussis* and *B. bronchiseptica* indicating an essential function of the *sodB* gene for viability of the bacteria under normal growth conditions.

4.8.1.2 Superoxide dismutase as a virulence factor

Phagocytes kill microorganisms through oxygen dependent and oxygen independent mechanisms. Oxygen dependent microbicidal mechanisms rely on production of ROS through a pathway called the respiratory burst (see section 4.2). The importance of the oxygen dependent killing mechanism was most vividly demonstrated with chronic granulamatous disease (CGD). Phagocytes of CGD patients are unable to produce ROS and the patients suffer from severe infections with catalase positive microorganisms (Mouy *et al.*, 1989).

Microorganisms possess several counter defence mechanisms to circumvent the oxygen dependent microbicidal activity of phagocytes, such as the ability to produce specific enzymes that destroy or neutralise the toxic ROS (Beaman and Beaman, 1984). Based on this assumption, some researchers suggested that SOD might be an important virulence factor for pathogenic bacteria (Beaman and Beaman, 1984). There is evidence in favour and against this hypothesis.

Franzon and coworkers (1990) reported extreme sensitivity of a *sodB* negative mutant of *Shigella flexneri* to killing by phagocytes, as compared to wild type parent. When this mutant was used to infect rabbit ileal loops, it caused very little detectable damage to intestinal villi. This result suggested that FeSOD plays an important role in protecting the bacterial cell against phagocytic killing. However, in this study, the mutant strain was not examined for its sensitivity to oxygen with one of

the test systems that are used to examine *sod* mutant strains; such as exposure to paraquat or oxygenyzation of the culture. Thus, the effect of the *sod* mutation on the cell's physiology was not known before examining the impact of *sod* mutation in interaction with phagocytes.

The contribution of SOD to virulence of *Yersinia enterocolitica* was recently demonstrated using a *sodA*-negative mutant strain of this organism in a mouse infection model. It was shown that virulence of the Δ*sodA* mutant strain was markedly reduced in terms of survival and multiplication in the spleen and liver, when compared to its parental strain, after intravenous infection (Roggenkamp *et al.*, 1997). However, the diminished virulence was dependent on the route of infection because no major effect was seen after orogastric infection model. Further analysis of the mutant strain indicated that the *sodA*-negative mutant was less resistant to the bactericidal activity of polymorphonuclear leukocytes (Roggenkamp *et al.*, 1997).

Salmonella typhimurium can persist within macrophages in the liver and spleen of mice, suggesting the microorganism's ability to intervene against the oxidative killing mechanism (Garcia-del Portillo *et al.*, 1992). When SOD's role in virulence of the organism was tested using a *sodA*-negative mutant of *S. typhimurium*, it was seen that high levels of MnSOD protected the organism against early killing by J774 macrophages. However, when a *sodA*-negative mutant of *S. typhimurium* was used in the murine typhoid model, the *sodA* mutant was found to be only slightly attenuated in mice after intragastric infection. It was concluded that resistance to early oxygen-dependent microbicidal mechanisms *in vivo* does not play a major role in the pathogenesis of this organism (Tsolis *et al.*, 1995).

As a consequence of its ability to remove ROS, Fe-MnSODs may assist colonisation and invasion of some microorganisms. In order to test if MnSOD had a role in colonisation of *H. influenzae*, which colonises tissues where highly divergent levels of oxygen tension is present during infection, a mutant *H. influenzae* strain devoid of MnSOD was constructed and tested in an infant rat infection model (D'Mello *et al.*, 1997). It was found that the mutant strain exhibited an impaired colonisation in the nasopharynx in comparison to the wild type strain.

Nevertheless, the mutant was still as virulent as the wild type. The researchers suggested that after invasion, the growth disadvantage imposed by the sodA-negative phenotype was not limiting (D'Mello *et al.*, 1997).

The ability to invade is an important component of *Camplyobacter jejuni* pathogenesis. When SOD's contribution to invasiveness of *C. jejuni* was investigated using a *sodB*-negative mutant strain of the organism, it was demonstrated that *C. jejuni* exhibited an approximately 12-fold decrease in its invasion capability in INT407 (human embryonic intestinal cell line) cells as compared to the parent strain (Pesci *et al.*, 1994).

However, although it may seem attractive to suggest that SOD is a necessary factor for virulence, there are reports contradicting this. For example, when a *sodB* mutant strain of *E. coli* K-12 was tested for phagocytic killing, both wild type and mutant strains showed similar sensitivity to human neutrophils (Papp-Szabo *et al.*, 1993). *SodA* deletion mutation of *B. brochiseptica* and *B. pertussis* did not effect the virulence of these strains in mice following intranasal infection, as compared to the wild type strains (Graeff-Wohlleben *et al.*, 1997). In addition, the survival of the mutant strains was not effected after 2 and 24 h in both murine J774 and MH-S macrophage cell lines (Graeff-Wohlleben *et al.*, 1997).

4.8.2 The role of CuZnSOD in bacteria

The cytosolic CuZnSOD in eukaryotic cells provides protection against superoxide radicals produced in the cytoplasm (Lynch and Fridovich, 1978). However, CuZnSOD in bacteria is located in the periplasm but it has been found only in Gram negative bacteria. It was thought that periplasmic SOD in bacteria was an important defence factor against extracellularly or periplasmically produced superoxide (Imlay and Imlay, 1996). In a recent report, Schnell and Steinman (1995) demonstrated the protective effect of CuZnSOD against environmentally produced superoxide in a *sodC* -negative mutant of *Caulobacter crescentus*. The same degree of tolerance was observed against intracellular superoxide generated with paraquat, a redox cycling drug that produces superoxide in the cytosol, both for wild type and the mutant strain. However, when exposed to an extracellular source of superoxide, generated by the action of xanthine oxidase on purines, the wild type survival was 20-fold higher

than the mutant, indicating protective effect of periplasmic CuZnSOD (Schnell and Steinman, 1995). Despite this, some researchers still find the protective effect of periplasmic CuZnSOD controversial (Imlay and Imlay, 1996). One reason for this is the absence of targets for superoxide radicals in the periplasm, such as a subclass of dehydratases that contain $(Fe\text{-}S)_4$ clusters; the main targets of $^{\bullet}O_2^-$ in any organism (Flint *et al.*, 1993). Another reason is that, as far as is known, there is no superoxide source in the periplasm (Imlay, 1996).

The presence of CuZnSOD in some pathogenic bacteria suggests that the enzyme might contribute to the virulence of these organisms by protecting bacterial cells against superoxide generated from the host phagocytes. However, research carried out by Tatum and coworkers (1992) showed that CuZnSOD deletion mutants of *Brucella abortus* S2308, a virulent strain, and S19, a vaccine strain, exhibited the same survival and growth pattern as the parental strains in HeLa epithelioid carcinoma and J774 mouse macrophage-like cell line. Nevertheless when these mutant strains were used to infect BALB/c mice intraperitoneally, the CuZnSOD negative mutant of S19 strain had 10-fold less bacterial count in the spleen than the wild type, 26 days post-infection. In a recent report Wilks and coworkers (1998) reported that a CuZnSOD mutant strain of *Neisseria meningitidis* was 1000-fold more sensitive to exogenous superoxide but not to paraquat, which generates superoxide within the cytosol. This result shows the important protective effect of CuZnSOD against extracellular $^{\bullet}O_2^-$. This mutant strain was also less virulent in a mouse intraperitoneal infection model compared to the wild type strain, suggesting the contribution of CuZnSOD to virulence of *N. meningitidis* (Wilks, *et al.*, 1998). CuZnSOD was also found to be important in the pathogenesis of systemic salmonellosis, as *sodC* negative mutants of *S. typhimurium, S. choleraesuis* and *S. dublin* showed reduced lethality in a mouse model of oral infection in comparison to parent strains, and persisted in significantly lower numbers in liver and spleen after intraperitoneal infection (Farrant *et al.*, 1997). Nonetheless, there was no detectable difference compared with wild type in the interaction of *sodC* mutants with porcine pleural, mouse peritoneal or J774 macrophages *in vitro* (Farrant *et al.*, 1997). The researchers suggested that there might be a difference in the capacity of different macrophage lines to kill *Salmonella*.

4.9 Regulation of *sod* in bacteria

In prokaryotes most of the regulation studies have been carried out with *E. coli* which possesses four isozymic forms of SOD. These are MnSOD, FeSOD, hybrid SOD which consists of one subunit each of the iron and manganese isoenzymes (Fridovich, 1995) and CuZnSOD (Imlay and Imlay, 1996). FeSOD is considered as a constitutive enzyme, and is present under both aerobic and anaerobic conditions, (Hassan and Fridovich, 1977), whereas, the synthesis of MnSOD, CuZnSOD and the hybrid form is induced upon exposure to air (Fridovich, 1995).

It has been shown that exposure to the high concentration of oxygen induces MnSOD and CuZnSOD biosynthesis in several microorganisms including, *H. influenzae*, *S. pyogenes*, *E. coli*, and *C. crescentus* (D'mello *et al.*, 1997; Gibson and Caparon, 1996; Benov and Fridovich, 1995; Schnell and Steinman, 1995). When a *H. influenzae sodA::lacZ* mutant was exposed to an oxygen flux of 3 and 36 mmol/l/h, the expression of β-galactosidase activity was 3.4-fold higher in the latter concentration than the former, correlating increase in MnSOD activity with oxygen concentration (D'mello *et al.*, 1997). Similar findings were reported for *S. pyogenes* MnSOD (Gibson and Caparon, 1996). It was found that SOD specific activity was higher in whole cell extracts of *S. pyogenes* prepared from aerobic cultures than the anaerobic ones, as assessed by activity gel staining. Induced levels of CuZnSOD in *E. coli* and *C. crescentus* was observed during the stationary phase when generation of $O_2^{\bullet -}$ is high due to active respiration (Benov and Fridovich, 1995; Schnell and Steinman, 1995). In addition, anaerobic oxidants, such as nitrate or ferricyanide, or iron chelators, can induce anaerobic MnSOD expression (Privalle *et al.*, 1989).

Besides MnSOD, *E. coli* expresses over 30 proteins upon exposure to superoxide-generating drugs. Analysis of the genes encoding for oxidative stress proteins indicated a tendency toward clustering on the *E. coli* chromosome (Farr and Kogoma, 1991). In *E. coli*, twelve genes induced in response to $^{\bullet}O_2^-$ are coregulated under the control of an *E. coli* locus called *soxRS* (superoxide response) regulon, one of which is *sodA* as mentioned earlier in section 4.8. Acute exposure to pure nitric oxide also can activate the *soxRS* regulon in a manner independent from $^{\bullet}O_2^-$. The biological significance of activation of the *soxRS* regulon by nitric

oxide was linked to resistance provided by *soxRS* response to nitric oxide mediated toxicity (Nathan and Xie, 1994). Gene activation by *soxRS*-regulon mediated by genes called *soxR* and *soxS* which code for two polypeptides, 17 and 13 kDa in size named SoxR and SoxS, respectively. These genes are essential for the inducibility of *SoxR*-regulon and code for a two-stage regulatory system. Upon activation by superoxide, SoxR stimulates the transcription of *soxS* the product of which activates in turn the transcription of the various *soxRS* regulon genes, including *sodA* (Nunoshiba *et al.*, 1992). In *E. coli* the target sequence for *SoxS* resides in the *sodA* promoter, between positions -74 and -33. This conclusion was based on a strain carrying deletion in this region being unable to induce expression of sodA in response to paraquat (Compan and Touati, 1993). Moreover, comparison of the *sodA* promoter sequence between -74 and -33, with the promoter sequence of *zwf*, another gene of the *soxRS* regulon revealed an 61% sequence identity. Nevertheless no sequence homology was identified in the other promoters of genes of the *soxRS* regulon (Compan and Touati, 1993).

In addition to the *soxRS* control, *sodA* expression is transcriptionally and posttranscriptionally controlled by five other global regulators: *fur* (ferric uptake regulon), *fnr* (fumarate nitrate reductase), *arc* (aerobic regulation control), *ihf* (integration host factor), and *soxQ* (redox stress and antibiotic resistance). Unlike *soxRS* and *soxQ* gene products, the products of other genes repress the expression of *sodA* in *E. coli* (Hassan and Schrum, 1994).

Iron (Fe^{2+}) plays an important regulatory role in the biosynthesis of MnSOD both at transcriptional and posttranslational levels in *E. coli* (Fridovich, 1995). At the transcriptional level Fe^{2+} is required for repressor activities of Fur and Fnr, the products of *fur* and *fnr* genes, respectively (Hassan and Schrum, 1994). The Fur protein in the presence of a divalent metal, likely to be Fe^{2+} *in vivo*, represses the transcription of genes involved in iron assimilation and negatively regulates *sodA* expression under both aerobic and anaerobic conditions (Niederhoffer *et al.*, 1990). Fnr protein, on the other hand, acts as a repressor of *sodA* expression primarily under anaerobic conditions (Hassan and Sun 1992). The Fur and Fnr regulation prevents the formation of $^{\bullet}OH$ via the Fenton reaction by minimising the level of intracellular Fe^{2+} (Hassan and

Sun 1992; Compan and Touati, 1993). The presence of consensus sequences have been reported in the promoter region of *sodA* gene for Fur, called an 'iron box', and Fnr proteins (Naik and Hassan, 1990; Beaumont and Hassan, 1993). The activity of SoxR protein was also effected by Fe^{2+}, at transcriptional level, as shown by a *soxS::lacZ* fusion which is stimulated by the iron chelator, 1,10-phenanthroline (Privalle *et al.*, 1993). It was speculated that in the reduced state, SoxR-Fe^{2+} may be inactive, and oxidation of the bound Fe^{2+} or removal of the Fe^{2+} by chelators may activate *soxR* (Hassan and Schrum,1994).

At the posttranslational level, Fe^{2+} shows its effect on MnSOD biosynthesis by competing against Mn^{2+} for the metal site in nascent SodA protein (Privalle and Fridovich, 1992). This competition results in fully, or partially active, or inactive forms of MnSOD. It was shown that anaerobic conditions favour the insertion of Fe^{2+} into the nascent sodA, which leads to formation of inactive enzyme (Privalle and Fridovich, 1992).

4.10 Defence mechanism against ROS in *S. pneumoniae*

Before evaluation of oxidative defence systems of *S. pneumoniae*, the possible sources of oxidative stress to which the pneumococci may be exposed will be discussed.

As discussed previously (section 4.2), aerobic metabolism was shown as the main origin for ROS production. However *S. pneumoniae* is a facultative anaerobic microorganism and cannot synthesise heme (Sneath *et al.*, 1986). Therefore, the pneumococcus does not have the respiratory cytochromes that are crucial to energy-linked oxygen metabolism in other bacteria, such as in *E. coli.* Consequently, facultative anaerobes depend primarily on glycolysis for ATP synthesis (Claiborne *et al.*, 1992). However when grown aerobically, many of facultative anaerobes can eliminate O_2 through flavoproteins such as NADH oxidase, which reduces O_2 by direct tetravalent reduction to water, or NADH peroxidase, which catalyses the reduction of H_2O_2 to H_2O (Thomas and Pera, 1983). Thus the production of H_2O_2 by *S. pneumoniae* was described as early as 1924 by Avery and Neill. The amount of H_2O_2 produced was greater under aerobic conditions than anaerobic conditions. In *S. pneumoniae*, lactate, pyruvate and oxygen were shown as substrates

for *S. pneumoniae* H_2O_2 formation, which is catalysed by lactate oxidase and pyruvate oxidase (Udaka *et al.*, 1959; Spellerberg *et al.*, 1996). In conclusion, O_2 utilisation can be a source of ROS production of *S. pneumoniae*.

Secondly, PMNL-derived ROS pose another threat to the pneumococcus, since oxygen dependent phagocytic killing has a key role in the defence against *S. pneumoniae* (Braconier *et al.*, 1982; Fine *et al.*, 1988). Thirdly, *S. pneumoniae* naturally resides in the nasopharynx where there is a high oxygen tension and therefore this may also be the source of oxidative stress (Sneath *et al.*, 1986).

Pneumococcal defences against ROS can be evaluated in two groups: direct antioxidative defences which contain enzymatic and nonenzymatic systems (see section 2.4) and indirect antioxidative defences which are composed of some pneumococcal metabolism bi-products and cell components. Regarding the enzymes that are responsible for direct inactivation of ROS, SOD has been shown in *S. pneumoniae* samples and supernatants, but was not further characterised (Perry *et al.*, 1993). As stated earlier, *S. pneumoniae* does not have any catalase activity, because it cannot synthesise heme (Sneath *et al.*, 1986). In addition to catalase, members of the genus *Streptococcus* also lack the moderate-to-high levels of intracellular glutathione which provides protection against ROS by interacting with free radicals (Claiborne *et al.*, 1992). However, there is no report, to my knowledge, specifically on the content of glutathione in the pneumococcus.

S. pneumoniae also contains some of the proteins which are known to be induced upon exposure to $^{\bullet}O_2^-$. One of them is the RecA protein which plays a role in the promotion of homologous recombination (Pearce *et al.*, 1995). The other one is the heat shock protein induced upon exposure to heat (HSP72) (Hamel *et al.*, 1997). However, it is not known yet if these proteins afford any direct or indirect protection against oxygen toxicity.

Indirect antioxidative defences include some pneumococcal proteins and capsule. The interference of polymorphonuclear leukocyte's (PMNL) respiratory burst by pneumolysin, even at sublytic concentrations, has been mentioned earlier (section 2.8). In addition to pneumolysin, the

presence of a product that depresses PMNL H_2O_2 production was identified in autolysis phase supernatants (Perry *et al.*, 1994). The analysis of the inhibitory product revealed that this was distinct from pneumolysin, heat sensitive and present in >10 kD and <10kD fractions of autolysis phase supernatant. Capsule also protects the pneumococcus from phagocytic killing. Neutrophils preincubated with serum containing ≥1 μg/ml purified capsular polysaccharide exhibited an impaired bactericidal activity against type 3 *S. pneumoniae* (Esposito and Clark, 1990). It is known that encapsulated strain of *S. pneumoniae* type 3 is completely resistant to phagocytosis by alveolar macrophages, whereas rapid intracellular killing of unencapsulated strain of same serotype by alveolar macrophages was observed (Jonsson *et al.*, 1985).

Part 5 Results

Isolation of a Pneumococcal *sod* Gene

5.1 Experimental strategy

The first stage involved the isolation and sequencing of a pneumococcal *sod* gene. The sequencing data could then be used to make an isogenic mutant strain of *S. pneumoniae*. In order to isolate a pneumococcal *sod* gene, several approches were tested. Initially, genetic complementation of *E. coli* QC774 Δ*sodA*, Δ*sodB* mutant strain with genomic libraries of *S. pneumoniae* was attempted. This *E. coli* mutant is unable to grow aerobically in minimal media unless complemented with a plasmid expressing either *sodA*, *sodB* or both.

While this work was underway, a sequence of an internal fragment of *sod* (sod_{int}) obtained using degenerate primers from *S. pneumoniae* was published (Poyart *et al.*, 1995). This gene fragment was used in two ways for the work in this thesis. Firstly, for the isolation of the entire pneumococcal *sod* gene either employing sod_{int} as a probe to screen genomic libraries, or designing oligonucleotide primers to isolate the gene by vector anchored PCR (VA-PCR). Secondly, sod_{int} was used to make an isogenic *sod* insertion mutant strain of *S. pneumoniae*. Once the mutant strain was made, it was tested under *in vitro* and *in vivo* conditions.

5.2 Isolation of a pneumococcal *sod* gene

5.2.1 Construction of partial genomic libraries

Plasmid pTTQ18 (Stark, 1987) was chosen as a vector for library constructions. This vector is suitable for blue-white selection because the cloning site for recombinant DNA is located in the middle of the *lacZ* gene that encodes β-galactosidase. When a gene fragment is cloned into the multiple cloning site of pTTQ18, this results in insertional inactivation of the *lacZ'* gene. This feature enables the identification of recombinants as white colonies, since they are unable to breakdown X-gal, while the nonrecombinants appear as blue colonies.

10 µg of circular plasmid DNA was digested to completion with *BamH*1. In order to achieve high frequency recombination between vector and insert DNA, the cleaved vector was treated with calf intestinal phosphotase to remove the 5' terminal phosphate, as described earlier in section 3.9. The efficiency of the process was measured by transformation of equal amount of self-ligated phosphatased and self-ligated unphosphatased vector into *E. coli* DH5α cells. It was found that the number of transformants with unphosphatased vector was 14 times more than with phosphatased samples.

To determine the enzyme concentration that would create DNA fragments in the range of 0.1 to <12 kb, a set of restriction digests were prepared using 0.05 , 0.1 , 0.2 or 0.4 U of restriction endonuclease *Sau*3A at 37°C for 35 min (Figure 5.1). Fragments were analysed by agarose gel electrophoresis and the results showed that 0.1 U of enzyme provided the desired range of DNA fragments (Figure 5.1, lane 4). Following this result, the reaction components were scaled up and 30 µg pneumococcal genomic DNA samples were cleaved with 0.6 U *Sau*3A for 35 min for the construction of genomic libraries. The partially cleaved DNA was electrophoresed using 0.6% LMT agarose and gel slices containing DNA fragments from 2 to 3, 1.5 to 6 and 2 to 7 kb were removed and purified as described previously (section 3.9). The reason for choosing a large insert size was to increase the chance of isolating the gene with its promoter and upstream sequences, so that a study on regulation and expression of gene could be conducted, if desired.

Ligation reactions were set up in a final volume of 20 µl using approximately 400 ng dephosphorylated vector DNA and 1200 ng of each isolated DNA fragment range; 400 ng dephosphorylated vector was self-ligated as a control. Plasmid DNA from 10 transformants of each library was isolated and the presence of insert DNA confirmed by restriction analysis with *Eco*RI (*Eco*RI was chosen because this enzyme linearised the vector). The presence of insert in library transformants was shown when restriction digest with *Eco*RI digest produced larger sizes than *Eco*RI restricted empty plasmid. When, however, the insert had at least one *Eco*RI site, then the multiple restriction fragments were appeared after analysis of digested bands by agarose gel electrophoresis as described in section section 3.9.

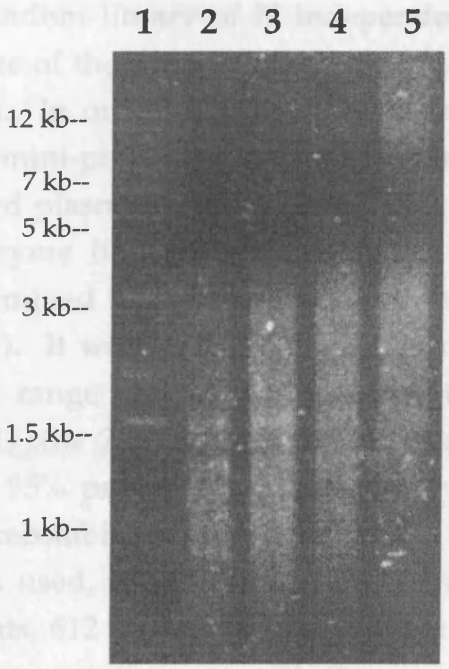


Figure 5.1: Generation of partially cleaved pneumococcal DNA. 5 μg *S. pneumoniae* type 2 chromosomal DNA was cleaved at 37°C for 35 min with ranging amounts of *Sau*3A to produce DNA fragments in the range of 0.1 to <12 kb (lane 4). Lane 2, 3, 4 and 5 represent chromosomal DNA digested with 0.4, 0.2, 0.1 and 0.05 U *Sau*3A, respectively. Lane 1 represents 1 kb ladder DNA size markers (Gibco/BRL) with fragment sizes shown in kilobases (kb).

A representative library of any organism should contain a complete collection of all or nearly all of the DNA sequences in the entire genome (Watson *et al.*, 1992). In order to assess if the libraries made were representative, a formula developed by Clarke and Carbon (1976) was used. This formula relates the probability (P) of including any DNA sequence in a random library of N independent recombinants, where n represents the size of the genome relative to a single cloned fragment, N= ln(1-P)/ln(1-1/n). In order to assess how many recombinants had been obtained, first a mini-prep plasmid preparation of 10 transformants was made and isolated plasmids were cleaved with *Eco*R1 (*Eco*R1 was chosen because this enzyme linearised the vector). The average size of the inserts was determined by calculating the geometric means of the inserts (Table 5.1, row 4). It was found that the library constructed with 2-3 kb (library 1) insert range had 1590 transformants, 950 of them contained inserts (Table 5.1, row 2, 3 and 5). To achieve the inclusion of a single fragment with a 95% probability in this library, with the average insert size used, 3476 recombinants were required. When 1.5-6 kb (library 2) insert range was used, the total number of transformants was 1530; of 1530 transformants, 612 contained insert (Table 5.1, row 2 and 3). If this library was a representative one with the average insert size used, 1993 recombinants would be required. On the other hand, the total number of transformants in the library constructed with 2-7 kb (library 3) was 3600, and 2520 of them contained insert. In order to designate library three as a representative one, 2902 recombinants were needed with the insert range used (Table 5.1, row 2, 3 and 5).

Collectively, the number of recombinants in library 1 and 2 were not enough for a representative library (Table 5.1, row 5). Library 3, on the other hand, had 87% of the number required (Table 5.1, row 5). Regardless of the efficiency, all libraries were screened because of the convenient screening method available.

5.2.2 Screening plasmid libraries with *E. coli* QC774 strain

Escherichia coli QC774 strain has both *sodA* and *sodB* genes insertionally inactivated (Carlioz and Touoti, 1986) and as stated earlier is unable to grow aerobically in minimal medium, unless complemented with an active *sod* gene. However, it maintains a normal growth in a complex medium. Therefore, if the libraries contained an active copy of a *sod*

Table 5.1: Analysis of genomic libraries.

size of insert used	2-3 kb	1.5-9 kb	2-7 kb
Total number of transformants	1590	1530	3600
Number of tested transformants containing insert	6 out of 10	4 out of 10	7 out of 10
Average insert size	2 kb	3.5 kb	2.5 kb
Number of transformants containing insert	950	612	2520

gene were transferred to *E. coli* QC774 cells, the clone(s) carrying an intact *sod* gene would grow in minimal medium, assuming that the gene has its promoter sequence is functional in *E. coli* QC774.

Large scale plasmid DNA preparations (separate for each library) were made from the transformants on the original *E. coli* DH5α library transformation plates. The size distribution of the inserts was shown by digestion of 3-5 μg maxi-prep plasmid DNA with *Eco*RI (Figure 5.2A, B and C). Also 3-5 μg undigested maxi-prep plasmid DNA for each library was electrophoresed along side the digested sample as the controls (Figure 5.2A, B and C, lanes 3, 2 and 2, respectively). As *Eco*RI digestion linearised the plasmid, plasmids containing insert appeared to have larger sizes (Figure 5.2A, B and C, lanes 4, 3 and 3, respectively) than the *Eco*RI-cleaved empty plasmid (Figure 5.2A lane 2). In addition, some bands appeared to be smaller than *Eco*RI-cleaved empty plasmid, indicating that some of the inserts in recombinant plasmids contained at least one *Eco*RI site.

4-5 μg of DNA from each library was then used to transform *E. coli* QC774 strain, selecting for kanamycin as *E. coli* QC774 cells are resistant to this antibiotic, and ampicillin as selection for pTTQ18. *E. coli* QC771 strain which contains three intact copies of *sod* genes (*sodA* encodes a MnSOD, *sodB* encodes a FeSOD and *sodC* encodes, a CuZnSOD) was included as a positive control. In order to rule out the possibility that any SOD activity was originating from the vector, 2 μg pTTQ18 was also electrotransformed into *E. coli* QC774 cells as a control. All the tranformants were then plated out on LA containing ampicillin to propagate recombinants before selecting in minimal medium (Dr D. Touati, personal communication). Following overnight growth at 37°C, the confluently grown transformants on LA were pooled and washed in PBS (pH 7.3) to remove any traces of complex medium. Thereafter, all the transformants were grown on solidified minimal medium in the presence of appropriate antibiotics and $1X10^{-9}$ M paraquat to prevent growth of spontaneous mutants (Dr D Touati, personal communication; Imlay and Fridovich, 1991). Plates were incubated at 37°C for 2 days. Unfortunately, no growth was observed on plates containing *E. coli* QC774 cell transformed with library DNA. On the other hand, *E. coli* QC771 strain, as expected, grew on minimal medium, but no growth was

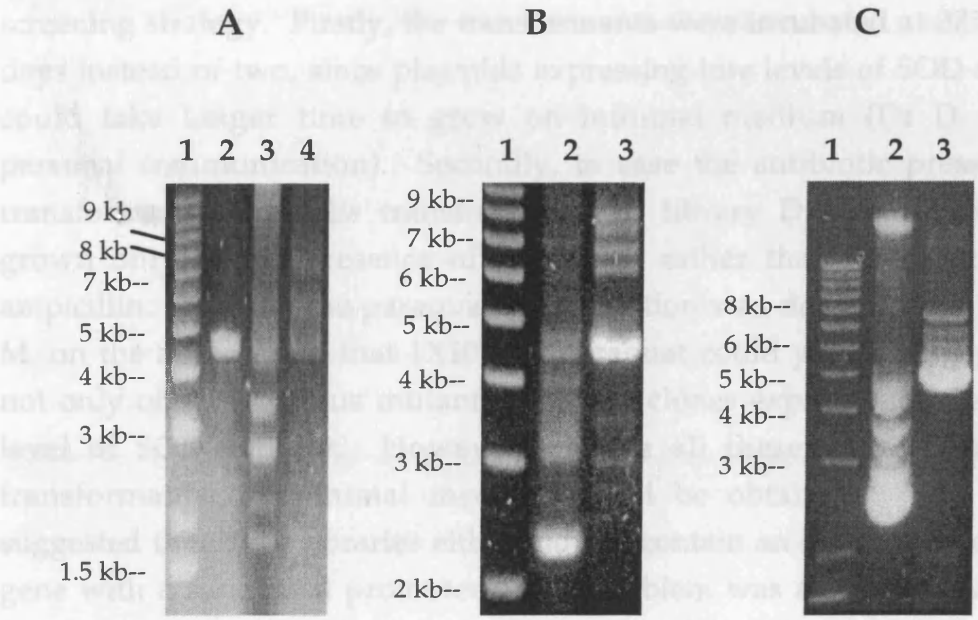


Figure 5.2: Large scale DNA preparation from genomic libraries. Plates containing the original *E. coli* DH5α library transformants were washed off with 2-3 ml sterile PBS and the pool of transformants was then used to inoculate 1 l of LB containing 100 µg/ml ampicillin. After an incubation period of 2-3 h at 37°C, plasmid DNA was purified from the cultures. 3-5 µg DNA from each library was cleaved with *Eco*RI. **A** represents the library constructed with 2-7 kb fragment size. Lane 2 is linear vector. Lane 3 is uncut and lane 4 is cut library DNA. **B** represents the library constructed with 1.5-6 kb insert size and **C** represents the library constructed with 2-3 kb insert size. In **B** and **C**, lane 2 represents uncut, lane 3 represents cut library DNA. In **A**, **B** and **C**, lane 1 represents 1 kb DNA size markers (Gibco/BRL) with fragment sizes shown in kilobases (kb).

observed with QC774 transformed with vector pTTQ18, confirming that the vector did not code for any SOD activity. The complete process was repeated but with the same negative result.

As this first attempt was unsuccessful, several changes were made to the screening strategy. Firstly, the transformants were incubated at 37°C for 4 days instead of two, since plasmids expressing low levels of SOD activity could take longer time to grow on minimal medium (Dr D. Touati personal communication). Secondly, to ease the antibiotic pressure on transformants, the cells transformed with library DNA aliquots were grown only in the presence of ampicillin rather than kanamycin and ampicillin. Thirdly, the paraquat concentration was decreased to $1X10^{-10}$ M, on the assumption that $1X10^{-9}$ M paraquat could prevent the growth not only of spontaneous mutants, but also clones expressing a very low level of SOD activity. However, despite all these modifications no transformants on minimal medium could be obtained. The results suggested that these libraries either did not contain an intact copy of a *sod* gene with a functional promoter or the problem was associated with the expression of the gene in the vector system and host strain used (see Part 8).

5.3 Studies with *S. pneumoniae* type 1 lambda bacteriophage expression library

Having failed to isolate a *sod* gene by genetic complementation of *E. coli* QC774 strain with genomic plasmid libraries, a *S. pneumoniae* type 1 lambda bacteriophage expression library, kindly provided by Dr Valerie Clarke (Clarke, 1994), was screened with the hope that this library contained a functioning copy of a pneumococcal *sod* gene. Three strategies were employed to screen *S. pneumoniae* type 1 lambda ZAP II library: a) complementation of *E. coli* QC774 cells, b) plaque hybridisation with the sod_{int} fragment, c) use of the library as the template for VA-PCR.

To screen the whole library by complementation of *E. coli* QC774 strain, inserts within the Lambda ZAP II vector were cloned into pBlueScript SK (-) (section 3.5). 2-3 µg of library DNA was electrotransformed into *E. coli* QC774 cells and the transformants were selected as above (section 5.2.2). However, after 4 days incubation, no growth was detected on minimal medium containing paraquat. It was concluded that this library did not

have a complete copy of a *sod* gene. However, it still might have contained the 5' or/and 3' of the gene and therefore the library was screened using radiolabelled sod_{int} fragment as the probe.

5 3.1 Screening type 1 library with sod_{int} gene fragment

To isolate 5' and 3'-end of pneumococcal *sod* gene, the *S. pneumoniae* type 1 library was screened by plaque hybridisation using radiolabelled sod_{int} gene fragment (Poyart *et al.*, 1995) as a probe. The sod_{int} gene had already been amplified by PCR, cloned and sequenced from *S. pneumoniae* type 2 as a part of the work to construct an isogenic *sod* mutant of the type 2 strain (section 6.1). Before using this fragment as the probe to screen the *S. pneumoniae* type 1 library, chromosomal DNA prepared from *S. pneumoniae* type 1 strain was dot blotted to membrane, as described in section 3.11.2, and probed with radiolabelled sod_{int} gene fragment as there could be genetic variation between strains. The positive hybridisation signal (data not shown) indicated that sod_{int} could be used as a probe to screen *S. pneumoniae* type 1 library.

The phage library was plated out as described earlier (section 3.4) and plates containing approximately 150 to 200 plaques were lifted onto round nylon membranes (section 3.11.3). 2000 plaques were screened and one plaque was identified as positive (Figure 5.3A). To confirm the positive result, the hybridised plaque was isolated and plated (section 3.5) for reprobing with radiolabelled sod_{int} gene. The result showed that all plaques in the second hybridisation experiment strongly bound to the probe, confirming the result obtained in the first hybridisation experiment (Figure 5.3B). For detailed analysis, the insert within the lambda phage vector excised into pBlueScript SK(-) (section 3.5) and the recombinant plasmid was transformed into *E. coli* XL-Blue cells (section 3.5). The plasmid DNA isolated from seven random transformants was cleaved by *Eco*RI, as the insert was cloned into *Eco*RI-cut arms of the vector. The restriction analysis indicated the presence of an approximately 2.8 kb insert. One of the positive clones, designated as pHYSOD1 was chosen for large scale plasmid preparation. *Eco*RI digestion of this clone is shown in Figure 5.4, lane 3.

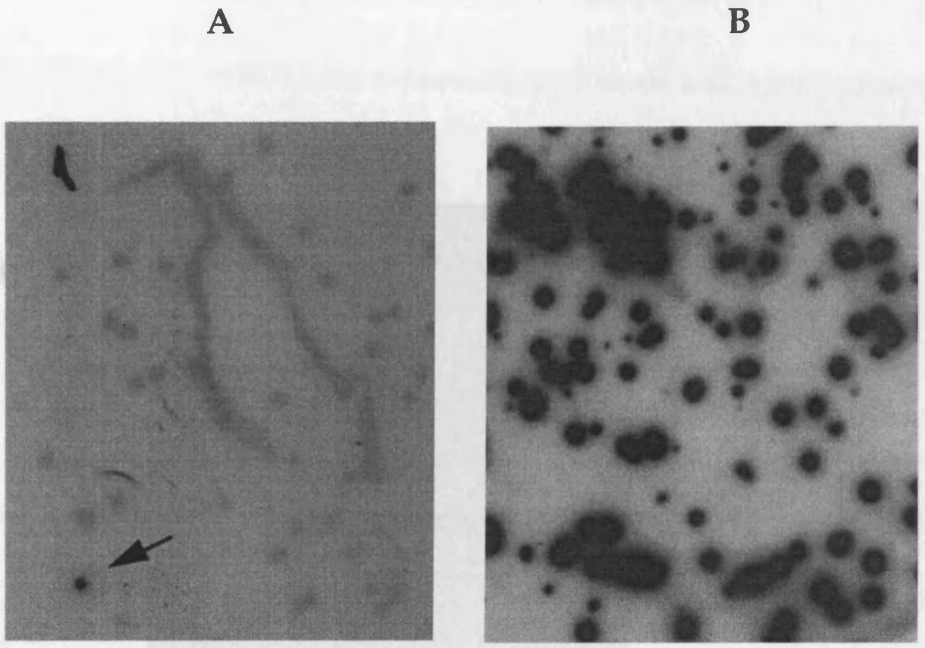


Figure 5.3: Isolation of pHYSOD1. **A.** Membranes containing 150-200 plaques were hybridised with radiolabelled sod_{int}. The positive plaque is indicated by an arrow. **B.** To confirm the result in A, the positive plaque was sucked into a Pasteur pipette, placed in 1 ml SM buffer containing 20 µl chloroform, vortexed and incubated overnight at 4°C to release the lambda ZAP II phage particles. Then 200 µl of this was used to infect 200 µl of *E. coli* XL-Blue cells. After 8 h incubation, the plaques were lifted to nylon membrane and hybridised with radiolabelled sod_{int} gene. As a result, all the plaques hybridised strongly with the probe, confirming the result of the first hybridisation.

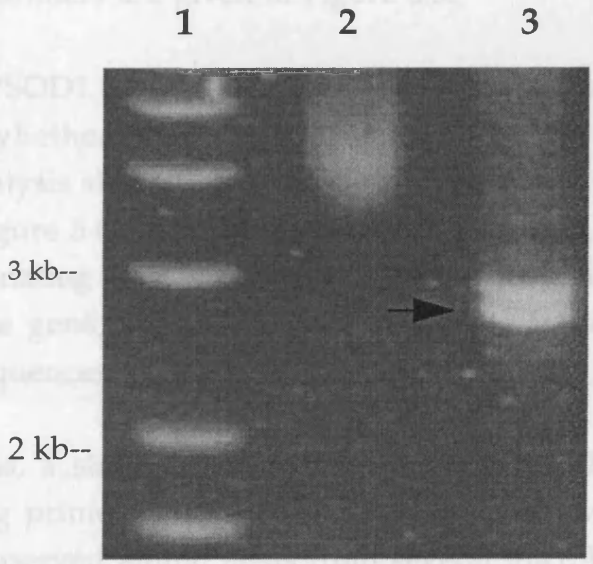


Figure 5.4: Restriction analysis of pHYSOD1. 3 µg DNA prepared from pHYSOD1 was digested with *Eco*RI (lane 3). This digestion separated the insert from the vector (insert indicated with an arrow). Lane 2 represents uncut pHYSOD1. Lane 1 is 1 kb DNA size markers (Gibco/BRL), fragment sizes are shown in the left hand column in kilobases (kb).

5.3.2 Sequencing strategy and sequence determination of pHYSOD1

As will be presented in the coming sections, the 5'-end and internal part of a pneumococcal *sod* was isolated from *S. pneumoniae* type 2 (Figure 5.5) and the 3'-end from *S. pneumoniae* type 1 lambda ZAP II library (Figure 5.5). The whole gene was then amplified from *S. pneumoniae* type 2 using specific primers and resequenced (Figure 5.5). The relative positions of primers are given in Figure 5.5.

Plasmid pHYSOD1 was used as a template to sequence the 2.8 kb insert to determine whether the insert contained the *sod* gene. Nucleotide sequence analysis showed that the start of the insert was within the sod_{int} sequence (Figure 5.6). In addition the insert appears to contain the entire 3'-end, terminating with a putative TAA stop codon. Having found the 3′ end of the gene, all attention was given to isolating the 5'-end and upstream sequences.

To begin this, a short fragment of sod_{int} was amplified by PCR from pSOD1 using primers HYK1, a degenerate primer whose sequence was based on conserved amino acids from several microbial SODs (Poyart *et al*, 1995), and SOD3 (SOD3 binds to nucleotides 219 to 238 within the sod_{int} fragment). This reaction yielded a 250 bp fragment which was presumed to contain 5'-end of sod_{int} (Figure 5.7). Therefore, when used as a probe, it would be more specific to the 5'-end of the *sod* gene, than the complete sod_{int}. This short fragment of sod_{int} was radiolabelled and approximately 5200 plaques of *S. pneumoniae* type 1 library were screened as described in section 5.3.1 and four plaques were identified as positives (Figure 5.8A). However, when the four plaques were plated out separately and re-probed, only one of the four hybridised with the 250 bp sod_{int} fragment (data not shown).

Following this result, the hybridisation experiment was repeated using the membranes containing the positive plaques from both the first and second hybridisations. After stripping the old probe (section 3.11.6), membranes were hybridised as before. It was seen yet again that only one plaque was positive (Figure 5.8C) and no hybridisation signal was detected for the three plaques identified as positives in the first hybridisation (Figure 5.8B). It was concluded that the hybridisation signal

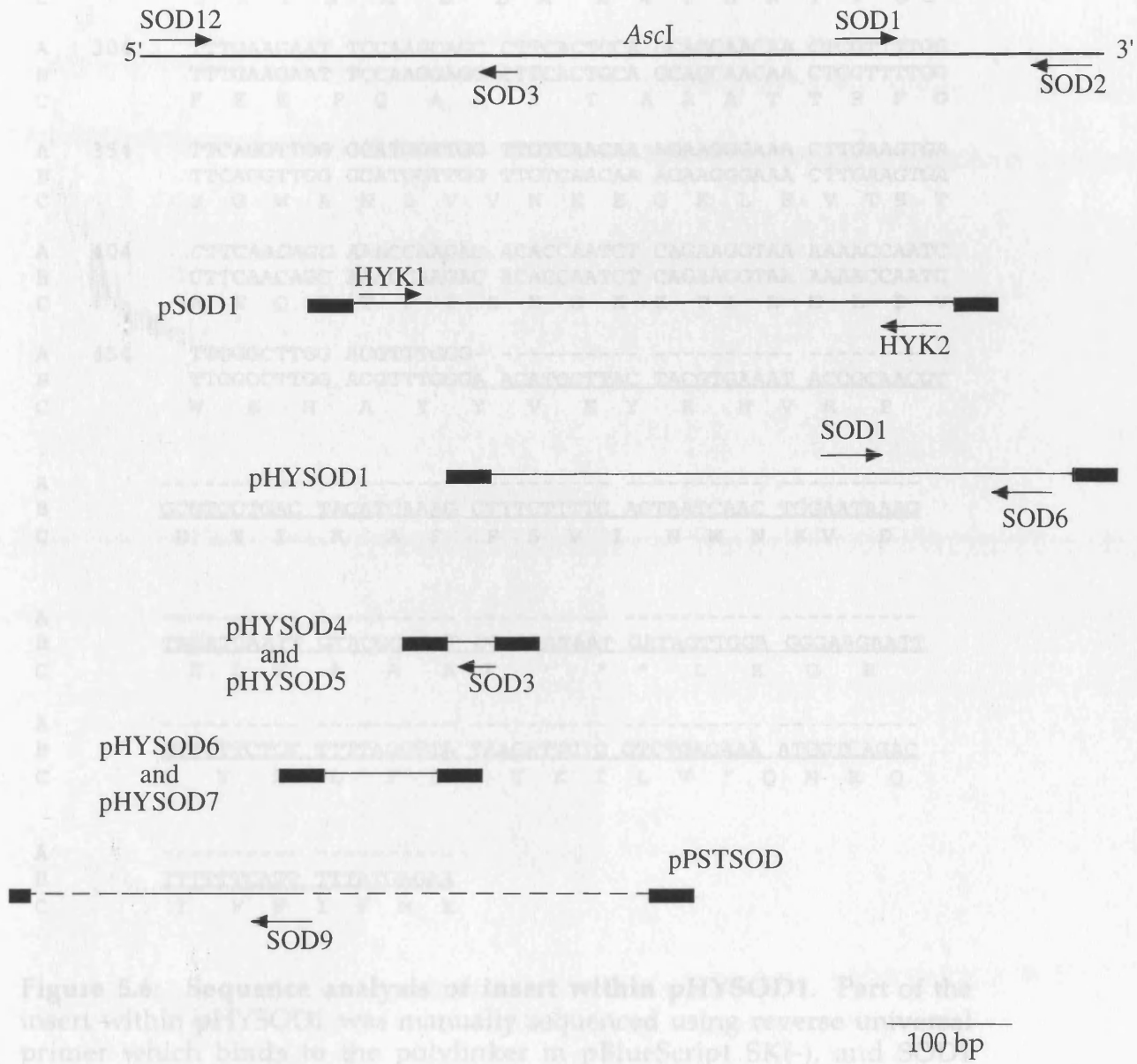


Figure 5.5: DNA sequencing strategy of pneumococcal *sodA* gene. In the diagram solid rectangles represent the vector sequence, while the insert is represented by a thin solid line. The completed sequence of *sodA* gene is represented with a solid thin line at the top of diagram. The position of insertion of *Asc*I site, which was used to ligate the spectinomycin resistance gene, is indicated. The 5' and 3' end of the insert within pPSTSOD is represented with a dashed line, while the fragment containing 5'-end of *sodA* gene was indicated with a solid thin line. Arrows indicate the direction of the primer extension. The oligonucleotides primers used for sequencing detailed in Table 3.3, are indicated over arrows.

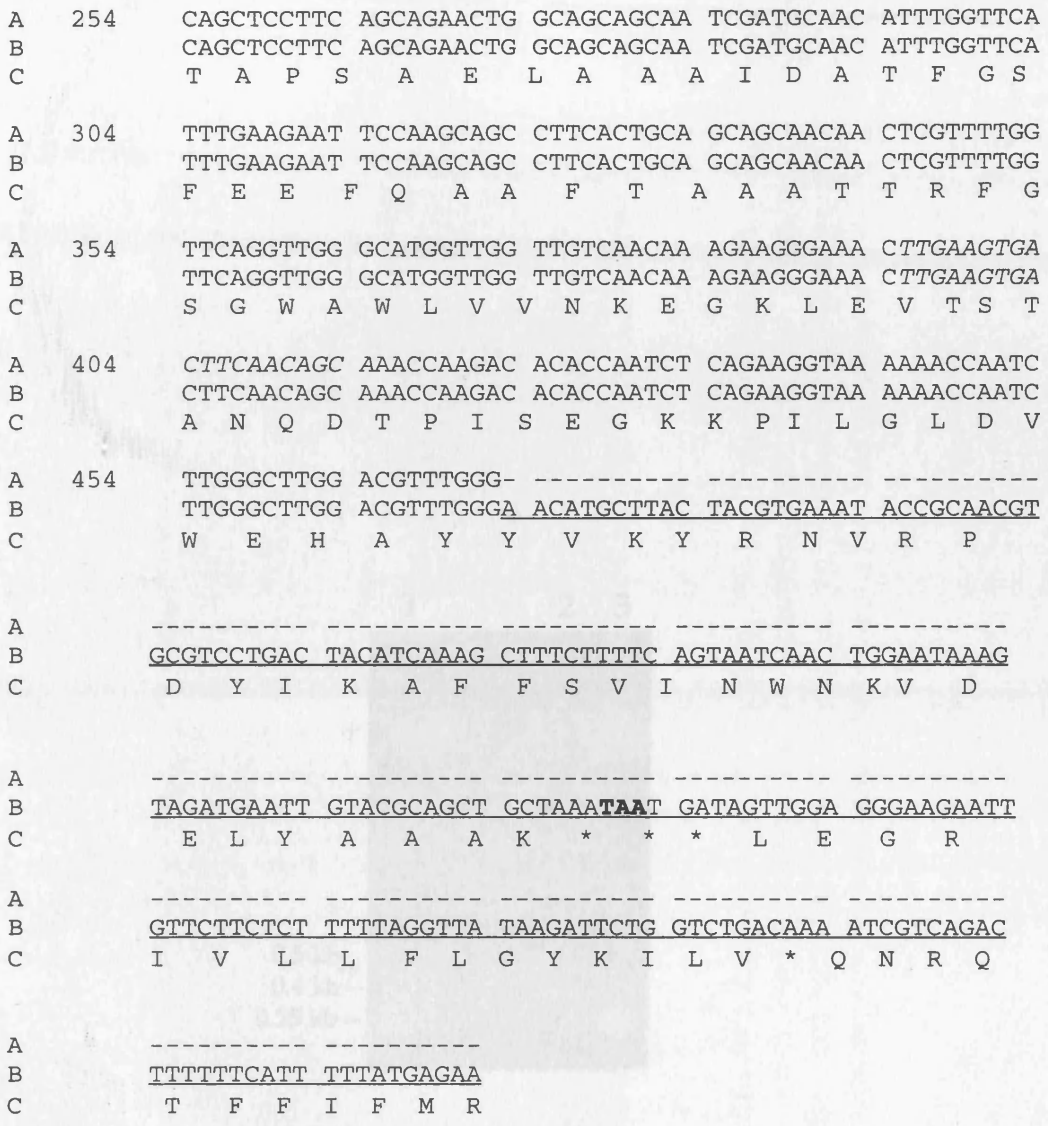


Figure 5.6: Sequence analysis of insert within pHYSOD1. Part of the insert within pHYSOD1 was manually sequenced using reverse universal primer which binds to the polylinker in pBlueScript SK(-), and SOD1 primer which anneals to nucleotides 395 to 411 within sod_{int} (shown as italic). The sequence data belonging to pHYSOD1 (B) was aligned with sod_{int} (A). The new sequence, representing 3'-end of the *sod* gene, obtained from the insert within pHYSOD1 is shown with a solid line. The putative stop codon TAA is indicated with bold face. Predicted protein sequence is given under the nucleotide sequence (C). The numbers represent the sequence data within sod_{int}.

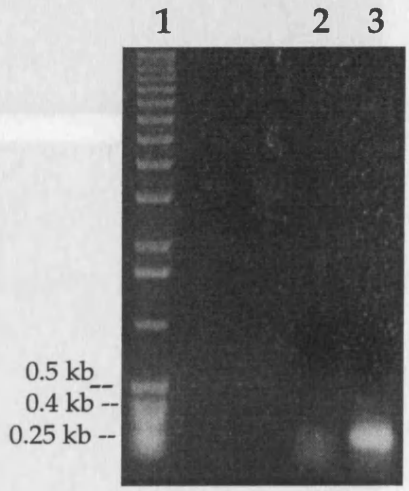


Figure 5.7: Amplification of 250 bp long sod_{int} fragment by PCR. pSOD1 was used as the template to amplify 250 bp short fragment of sod_{int} where HYK1 and SOD3 primers were used as the primers (lane 3). Lane 2 represents negative control; reaction with no template. Lane 1 is 1 kb DNA size markers (Gibco/BRL), fragment sizes are shown on the left hand column, in kilobases (kb).

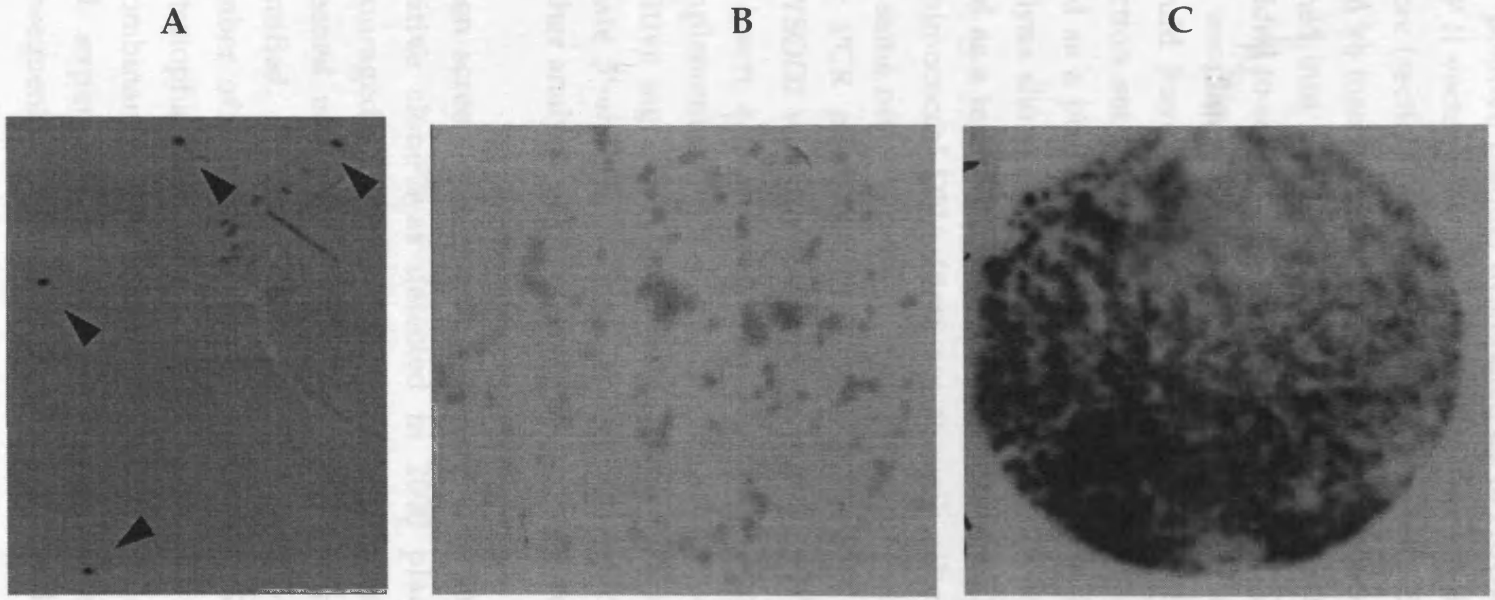


Figure 5.8: Screening *S. pneumoniae* lambda ZAPII phage library with 250 bp fragment from *sod*int. **A** shows four plaques identified as positives in the first hybridisation experiment (indicated with arrows). **B** shows one of the negative plaques after third hybridisation. **C** represents the result of third hybridisation experiment for the positive plaque hybridised in all hybridisation experiments (see text for details).

detected for the three other clones was a result of unspecific hybridisation with the probe in the first hybridisation.

The positive plaque, designated as pHYSOD2, was excised from lambda ZAP II vector into pBlueScript SK(-) for mini-prep plasmid preparation as before (section 3.5). Restriction digest with *Eco*RI revealed the presence of a 1.6 kb insert (Figure 5.9). *Eco*RI was chosen because the insert had been cloned into *Eco*RI site of the vector. Before sequencing pHYSOD2, it was decided to set up a PCR using HYK1 and SOD3 primers, with pHYSOD2 as the template. If the insert contained the *sod* sequence, these primers should have amplified a PCR product of approximately 250 bp. A reaction set up with pneumococcal chromosomal DNA (type 1 strain) used as a positive control. Examination of PCR products by agarose gel analysis showed that no product was obtained when pHYSOD2 DNA was used as a template, whereas an expected 250 bp insert was obtained with pneumococcal DNA (data not shown). The PCR was repeated but with the same negative result. It was thought that the reason for not obtaining any PCR product but detecting a positive hybridisation signal for pHYSOD2 was due to the absence of the HYK1 primer recognition site in the insert, although the insert would have contained part of the sequence complementary to the 250 bp sod_{int} probe which was the reason for the positive signal in the hybridisation experiments. As the aim was to isolate 5'-end of the gene and it was concluded that this was absent, further analysis of pHYSOD2 was not considered.

When screening the type 1 library with the sod_{int} gene fragment, the positive clone was detected in 2000 plaques. The early successes encouraged a continuation of this method and 5200 more plaques were screened using the 250 bp *sod* gene fragment, but no positives were identified. At this stage a change in the strategy was needed because the number of primary recombinants for the *S. pneumoniae* type 1 lambda bacteriophage expression library was $5X10^6$. Considering the number of recombinants to be screened, plaque hybridisation would be laborious and expensive in terms of membrane cost and hybridisation. Consequently, a change in the strategy to isolate the gene was made.

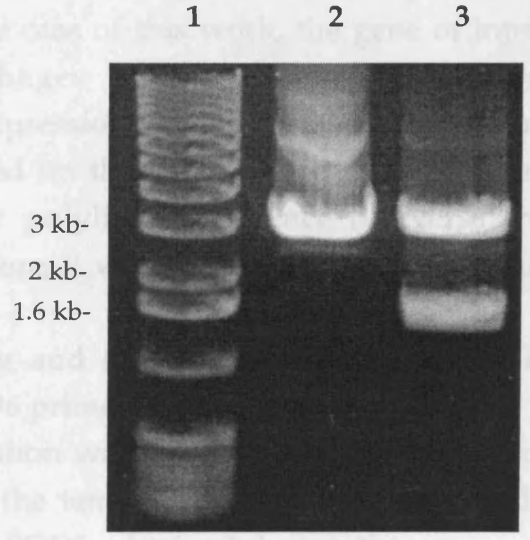


Figure 5.9: Restriction analysis of pHYSOD2. pHYSOD2 was restricted with *Eco*RI, as the insert was cloned into *Eco*RI arms of the vector, indicated the presence of approximately 1.6 kb insert (lane 3). Lane 2 contains uncut pHYSOD2. Lane 1 is 1 kb DNA size markers (Gibco/BRL), fragment sizes are shown in the left hand column in kilobases (kb).

5.3.3 Isolation of the gene by vector anchored PCR method

Vector anchored PCR (VA-PCR) was considered as an alternative method to plague hybridisation as it was faster, and less laborious, since there was no need for manipulations involving plaque lifting and hybridisation, and it was cheaper because of the elimination of the membrane cost and radiolabelling. This method is based on the fact that when a sequence of a part of a gene cloned in a plasmid or phage library is known, upstream or downstream sequences could be obtained by using PCR with a primer which binds to vector sequence and a primer to the known gene sequence. In the case of this work, the gene of interest was in a pool of recombinant phages or plasmids (*S. pneumoniae* type 1 lambda bacteriophage expression library or its excised plasmid form) and one of the primers based on the insert sequence (SOD6) and the other primers based on vector polylinker sequence (universal reverse and forward primers). Therefore, it was a feasible approach for this project.

5.3.3.1 Screening and sequencing amplicons obtained with universal reverse and SOD6 primers

A PCR amplification was performed using *S. pneumoniae* type 1 plasmid library DNA as the template with reverse universal and SOD6 primers. The sequence of SOD6, obtained during the course of this study (section 5.3.2), starts 105 bp downstream of HYK2 primer (see Figure 5.5). It was hoped that by employing SOD6 primer in combination with reverse universal primer, a large portion of the gene could be isolated. Analysis of amplicons by agarose gel electrophoresis indicated that fragments ranging from 100 bp to 3 kb had been amplified (Figure 5.10A). These products were then Southern blotted to nylon membrane and hybridised with radiolabelled 250 bp *sod* fragment. Two hybridisation signals were detected: one was approximately 600 bp and the other was 800 bp (Figure 5.10B). The gel slices containing 600 bp and 800 bp gene fragments were removed, purified from the agarose (section 3.9) and cloned into pCRScript SK(+). To demonstrate that the correct bands had been cloned, DNA prepared from these clones was digested with *BamH*I and *Not*I to release the insert (Figure 5.10C and 5.10D). These enzymes have a recognition site on both sides of the multiple cloning site and double digestion with these enzymes released 600 bp (Figure 5.10C, lane 3) and 800 bp inserts (Figure 5.10D, lane 3). Then, DNA was Southern blotted and probed with the radiolabelled 250 bp sod_{int} fragment. However no

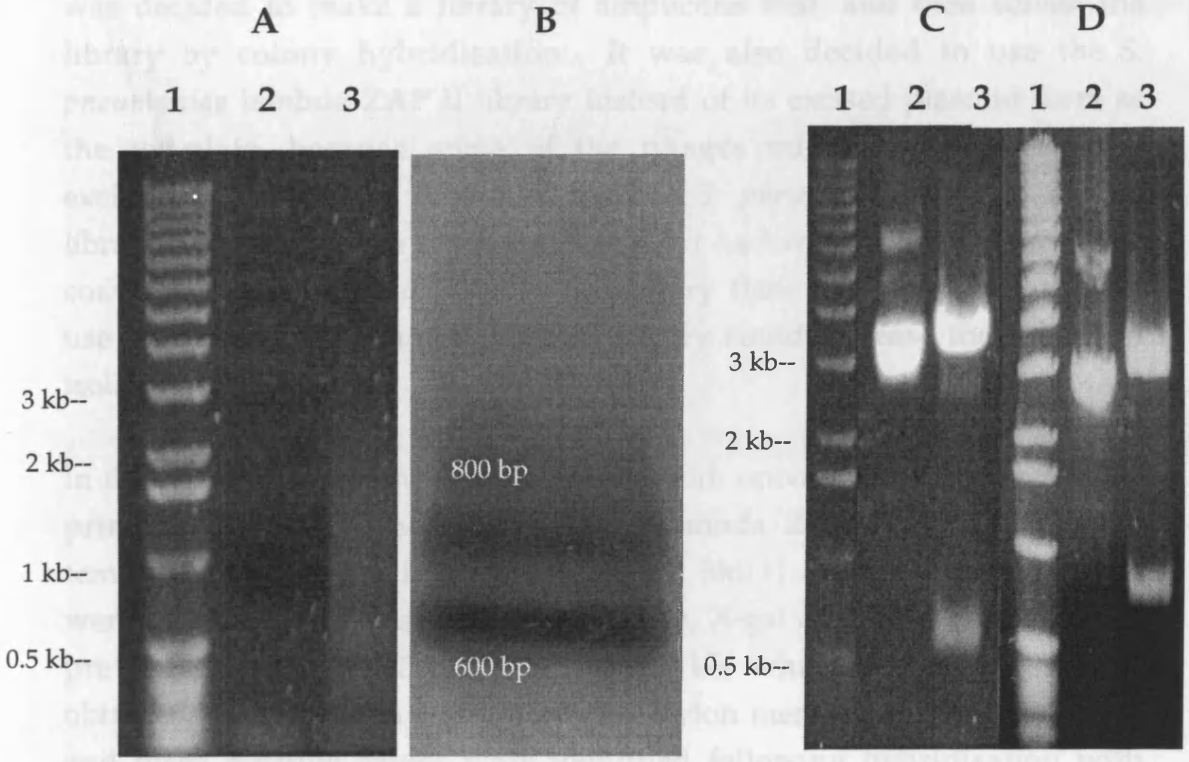


Figure 5.10: Isolation of *sod* gene by VA-PCR. A. Using HYK1 and SOD6 primers and *S. pneumoniae* type 1 library DNA as the template, amplicons ranging from 0.1 kb to 3 kb were obtained (lane 3). Lane 2, negative control (no template added to reaction). **B** Two hybridisation signals were detected using radiolabelled 250 bp sod_{int} gene fragment as the probe. Fragments that hybridised to the probe (approximate size of positive bands indicated with white face) were purified from agarose gel slices and cloned into pCRScript SK(+). **C** and **D** The presence of cloned inserts was shown by restriction analysis with *BamHI* and *Not*I (lane 3 in **C** and **D**), as the recognition sites for these enzymes are located at either ends of the cloning site in the vector. Lane 2 in **C** and **D** contains uncut plasmid. Lane 1 in **A**, **C** and **D** is 1 kb DNA size markers (Gibco/BRL), fragment sizes are shown in the left hand column in kilobases (kb).

hybridisation signal was detected (data not shown). This blot was repeated but with the same negative result.

The reason for this negative result was thought to be due to the extraction and cloning of the wrong bands. In order to prevent this, it was decided to make a library of amplicons first, and then screen the library by colony hybridisation. It was also decided to use the *S. pneumoniae* lambda ZAP II library instead of its excised plasmid form as the template, because some of the phages might have inefficiently excised. As shown in Figure 5.11, when *S. pneumoniae* lambda ZAP II library was used as the template (lane 4), it had wider range of inserts as compared with plasmid form of the library (lane 3). Consequently, the use of *S. pneumoniae* lambda ZAP II library could increase the chance of isolating the gene.

In the new strategy, amplicons obtained with universal reverse and SOD6 primers by PCR, with *S. pneumoniae* lambda ZAP II library was the template, were cloned first into pCRScript SM(+) and then transformants were selected on LA containing ampicillin, X-gal and IPTG, as described previously in section 3.13.1. A total of 176 white transformants were obtained. The colonies were lifted onto nylon membranes (section 3.11.3) and three positive clones were identified following hybridisation with the 250 bp sod_{int} fragment (Figure 5.12A). Restriction analysis of positive plasmids with *Bam*HI and *Not*I (chosen because recognition sites for these enzymes were located at the both side of the cloning site) revealed the presence of an approximately 900 bp insert. Restriction digest of a representative positive plasmid is shown in Figure 5.12B. Large scale plasmid preparation of one of these clones, designated as pHYSOD3 was made and insert DNA was manually sequenced using universal forward and reverse primers (section 3.12).

The results showed that the sequence data obtained with both primers was the sequence from the 3'-end of the gene. It was identical to the sequence of insert in pHYSOD1 (Figure 5.6), corresponding to region between 220 to 324 nucleotides on complementary strand (Figure 5.13).

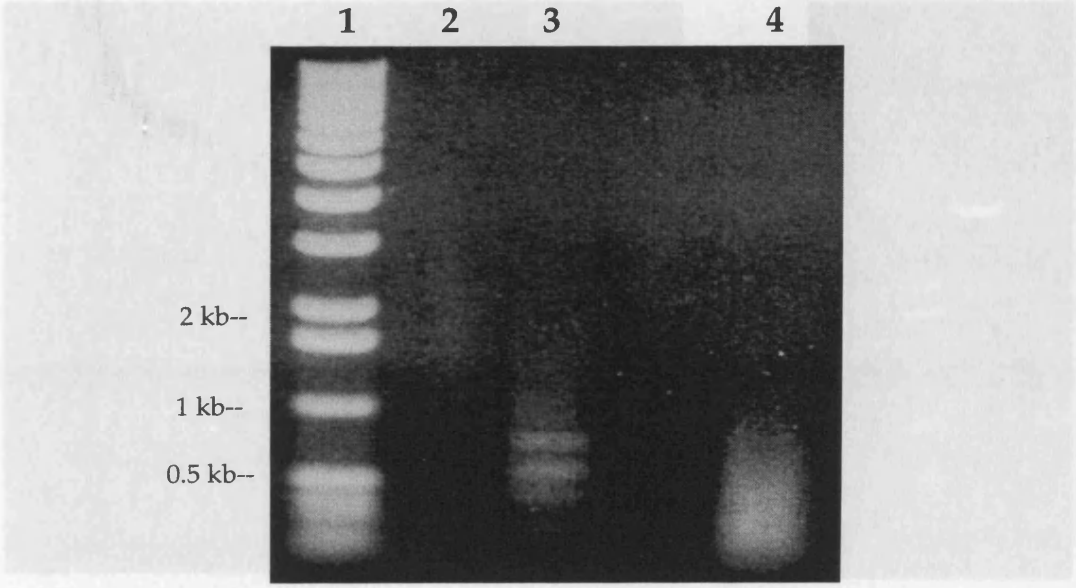


Figure 5.11: PCR products obtained with *S. pneumoniae* lambda ZAP II library. The templates used were either *S. pneumoniae* type 1 plasmid library (lane 3) or its Lamda ZAP form (lane 4). Reverse universal and SOD6 primers were used for both reactions. Lane 2 represents a negative control reaction with no template. Lane 1 is 1 kb DNA size markers (Gibco/BRL), fragment sizes are shown in the left hand column in kilobases (kb).

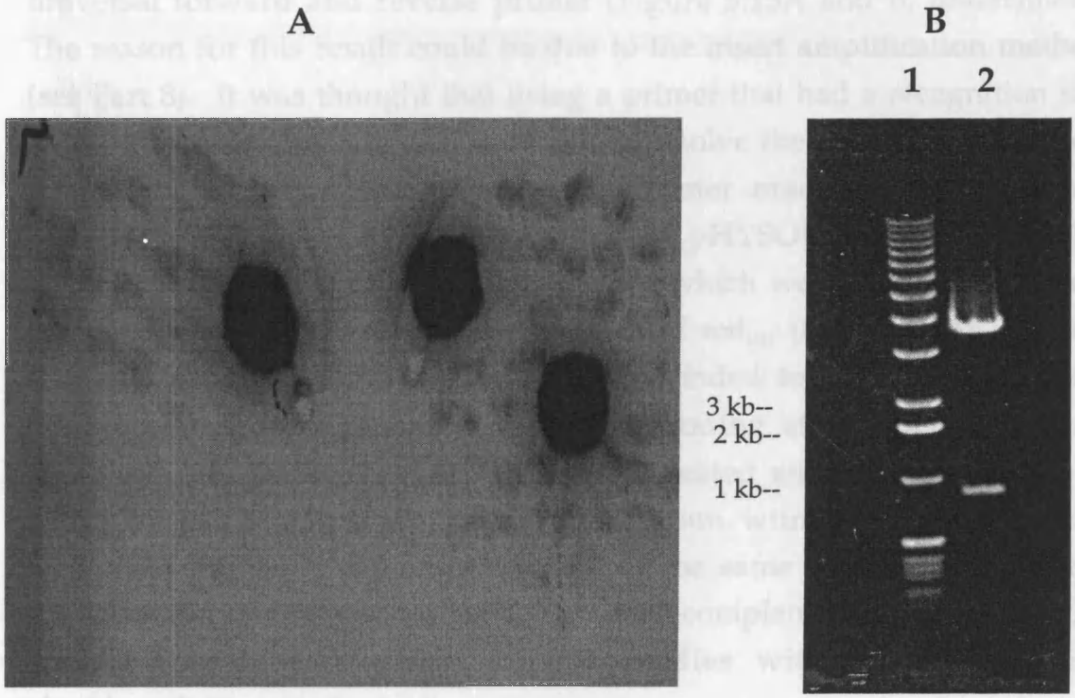


Figure 5.12: Identification and restriction analysis of pHYSOD3. **A.** Amplicons synthesised from *S. pneumoniae* type 1 lambda ZAP II library with reverse universal and SOD6 primers were cloned into pCRScript SK(+). Three clones were strongly hybridised with the probe. **B.** Restriction analysis of one of these clones, pHYSOD3, with *Not*I and *BamH*I revealed the presence of approximately 0.9 kb insert (lane 2). Lane 1 is 1 kb DNA size markers (Gibco/BRL), fragment sizes are shown in the left hand column in kilobases (kb).

Obtaining same sequence data with different primers was unexpected. However the reason for this was not due to use of the same primer for each sequencing reaction, since the sequence data corresponding to the vector's polylinker was different for sequencing reactions set up with universal forward and reverse primer (Figure 5.13A and B, underlined). The reason for this result could be due to the insert amplification method (see Part 8). It was thought that using a primer that had a recognition site within the insert, such as SOD3, could help solve the problem. However, the sequence data obtained with this primer made the puzzle more difficult to resolve. Sequence analysis of pHYSOD3 showed that the insert contained a 94 nucleotides fragment which was identical to strand complementary to 270 to 364 nucleotides of sod_{int} (Figure 5.14). Then a sequence of 134 nucleotides which corresponded to the region between 259 nucleotide to 393 nucleotide on the coding strand of sod_{int} gene followed. The sequencing reaction was repeated with a fresh sample of SOD3 primer to eliminate any contamination with other primers that recognises the complementary strand, but the same result was obtained. The reason for the sequence switching from complementary strand to the coding strand were unknown and studies with pHYSOD3 were abandoned.

A.

CTGCAGCCCG GGGGATCCGC CCTAGCAGCT GCGTACAATT CATTTACTTT

ATTCCAGTTG ATTACTGAAA AGAAAGCTTT GATGTAGTCA GGACGCACGT

TGCGGTATTT CACGTAGTAA GCATGTT

B.

CCGCGGTGGC GGCCGCTCTA GCCCTAGCAG CTGCGTACAA TTCATCTACT

TATTCCAGTT GATTACTGAA AAGAAAGCTT TGATGTAGTC AGGACGCACG

TGCGGTATTT CACGTAGTAA GCATGTT

Figure 5.13: Sequence determination of pHYSOD3. Manual DNA sequencing was performed using forward and reverse universal primers. Sequence of pCRScript SK(+) obtained with universal forward primer (A) and universal reverse primer (B) were underlined. In both A and B the sequence data belonging to the insert is the same.

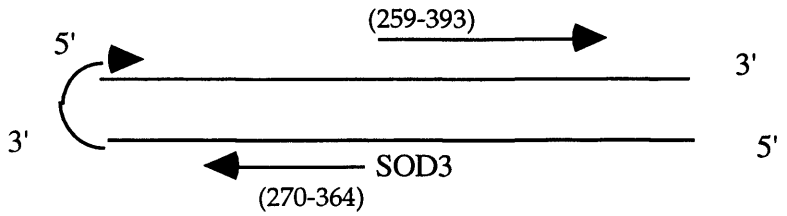


Figure 5.14: Illustration of strand switching in the sequence of insert within pHYSOD3. pHYSOD3 was sequenced with SOD3 primer. As expected 94 nucleotides fragment was sequenced on complementary strand. This was followed by the sequence of 134 nucleotides on the coding strand. The numbers in the parenthesises indicate the position of sequence data within sod_{int}. The parellel arrows indicate the relative positions of sequence data. The arrow on left hand side represents strand switching.

5.3.3.2 Screening and sequencing amplicons obtained with universal reverse and SOD3 primers

To overcome the difficulty faced in the first attempt (section 5.3.3.1), the VA-PCR was repeated but replacing SOD6 with SOD3 primer as it was closer to 5'-end of the gene (see Figure 5.5). The amplicons obtained with reverse universal and SOD3 primers were processed (cloning and hybridisation) as described above (section 5.3.3.1). Eight positive clones were detected. Restriction digests of plasmids isolated from the positive clones with *BamH*I and *Not*I (chosen because the recognition sites for these enzymes were located at the both side of the cloning site) showed the presence of a 300 bp insert. Restriction digest of one of the eight plasmids is shown in figure Figure 5.15. Despite each having the same insert size, the sequence of each insert could have been different. For this reason, two clones, designated as pHYSOD4 and pHYSOD5 were chosen for sequencing. Sequencing was done manually (section 3.12) employing SOD3 as the primer. The results showed that inserts in both pHYSOD4 and pHYSOD5, contained 57 bp nucleotides corresponding to between 99 to 155 within the sod_{int} sequence (Figure 5.16). This was followed by vector sequence, a clear indication of absence of 5'-end of the *sod* gene in this insert.

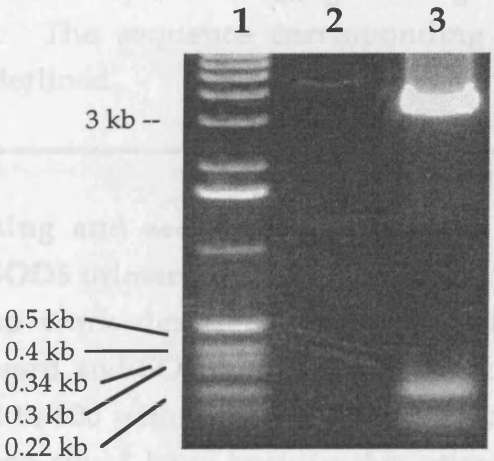


Figure 5.15: Restriction analysis of pHYSOD4. 2-3 µg DNA prepared from pHYSOD4 was digested with *BamH*I and *Not*I (lane 3). Double digestion with these enzymes separated the insert from the vector as *BamH*I and *Not*I have recognition site at both side of the cloning site. Due to either *BamH*I or *Not*I recognition site in the insert, one large, approximately 0.22 kb, and the other 0.07 kb, two fragments are seen. Lane 2 represents uncut plasmid. Lane 1 is 1 kb DNA size markers (Gibco/BRL), fragment sizes are shown in left hand column in kilobases (kb).

```
1    AGCTTTAGAAAAACACCCTGAAATCGGTGAAGACCTTGAAGCCTTGCTTG
     ||||||||||||||||||||||||||||||||||||||||||||||||||
2    AGCTTTAGAAAAACACCCTGAAATCGGTGAAGACCTTGAAGCCTTGCTTG

1    CTGATGTGCTCTAGAACTAGTGGATCCCCCGGGCTGGGAATTCGGCACG
     |||||||
2    CTGATGT
```

Figure 5.16: Sequence determination of inserts within pHYSOD4 and pHYSOD5. DNA sequence analysis of insert within pHYSOD4 and pHYSOD5 (1) was performed manually using SOD3 primer. The sequence that overlaps to sod_{int} gene fragment (2) was shown with vertical lines. The sequence corresponding to vector's polylinker is shown as underlined.

5.3.3.3 Screening and sequencing amplicons obtained with universal forward and SOD6 primers

In addition to work described above, a new PCR was set up with universal forward and SOD9 primer, whose recognition site lies between nucleotides 82 to 100 within the sod_{int} gene, because the insert carrying 5'-end of the gene could have been in this orientation in the vector. The sizes of amplicons obtained were in the range of 150-220 bp (Figure 5.17). These amplicons were procesessed as described previously in section 5.3.3.1 (cloning into pCRscript SK(+), hybridisation with radiolabelled 250 bp long internal fragment) and 22 clones were found to contain an insert of approximately 100 bp that hybridised with the probe. Two of them, designated as pHYSOD6 and pHYSOD7, were sequenced using universal reverse and forward primers. Although the inserts contained sequences identical to strand complementary to 1 to 99 nucleotides of sod_{int}, none of them had 5'-end of the gene (Figure 5.18).

The isolation of 5'-end of the *sod* gene by VA-PCR had been a feasible approach (section 5.3.3). Nevertheless, attempts to isolate the gene by this approach failed. This was apparently due to the fact that the insert carrying 5'-end of the gene was not attached to sod_{int} in the library. Meanwhile, an isogenic *sod* mutant strain of *S. pneumoniae* type 2 was being made in parellel. Once the mutant strain was constructed, it was

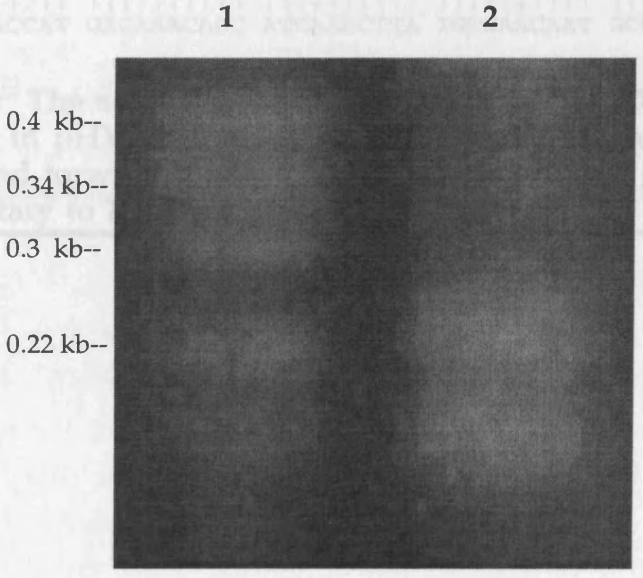


Figure 5.17: Amplicons obtained with universal forward and SOD9 primers. The size of amplicons sythesized with these fragments were between 150 to 220 (Lane 2). Lane 1 represents 1 kb DNA size markers (Gibco/BRL), fragment sizes are shown in left hand column in kilobases (kb).

decided to use the mutant strain to isolate the remaining part of *sod* gene. In part 6, following the description of the construction of the *sod* isogenic mutant strain of *S. pneumoniae,* the isolation of *sod* gene from the mutant strain will be described.

```
1   CCGTACGCGT ACGATGCGCT GGAACCATAC ATCGATGCGG AAACAATGCA
    |||||||||| |||||||||| |||||||||| |||||||||| ||||||||||
2   CCGTACGCGT ACGATGCGCT GGAACCATAC ATCGATGCGG AAACAATGCA

1   TTTGCACCAT GACAAACACC ATCAAACTTA TGTCAACAAT GCCAATGCA
    ||||||||||| |||||||||| |||||||||| |||||||||| |||||||||
2   TTTGCACCAT GACAAACACC ATCAAACTTA TGTCAACAAT GCCAATGCA
```

Figure 5.18: The sequence determination of pHYSOD6 and pHYSOD7. The inserts in pHYSOD5 and pHYSOD6 were sequenced using reverse universal and forward primers (2). The sequence was identical to strand complementary to 1 to 99 nucleotides of sod_{int} (1).

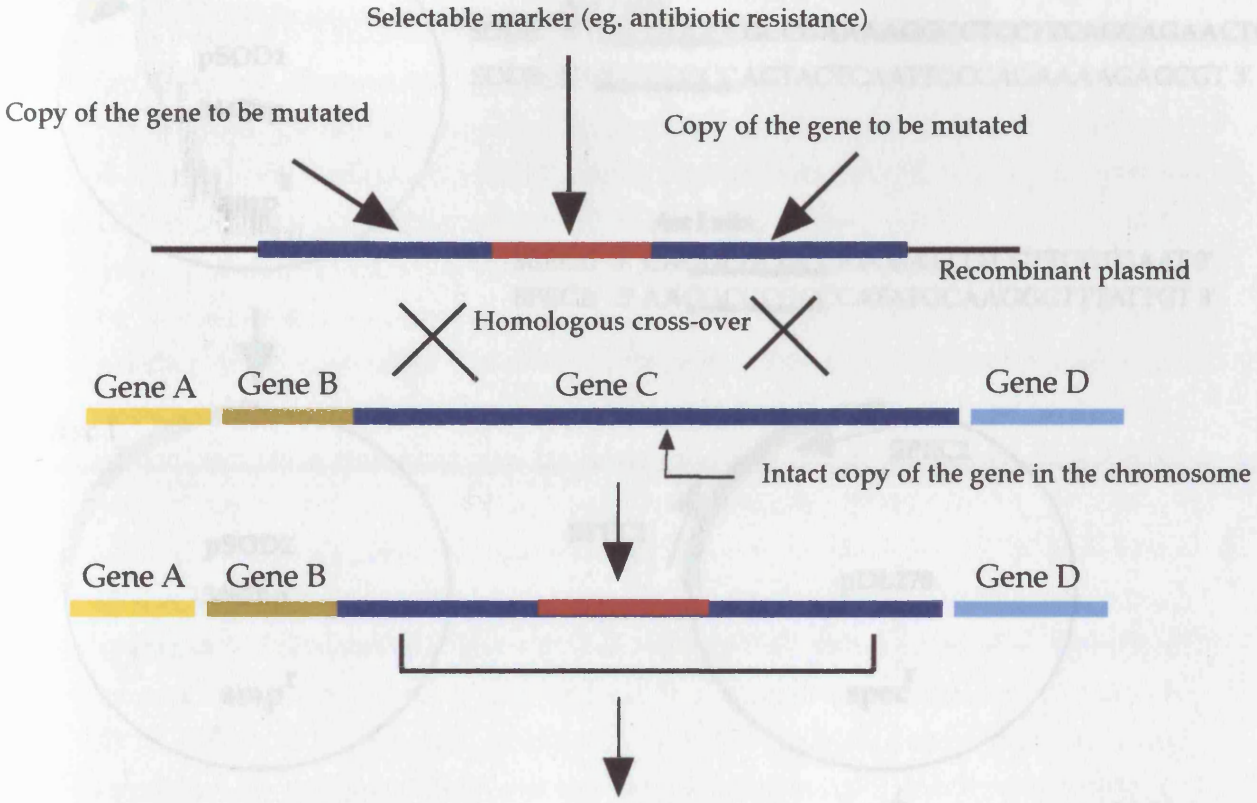
Part 6 Results

Construction of a MnSOD-Deficient Isogenic Mutant Strain of *Streptococcus pneumoniae* Type 2

Insertional inactivation of a gene by homologous recombination has been a well established method for studying gene function, because it can place foreign DNA at a precise locus in the genome. This method makes use of a recombinant plasmid that carries a part of the gene or a sequence, homologous to the gene to be knocked-out, but which has been disrupted by a selectable marker. In addition the vector must be incapable of replication in the host strain. After transformation of the recombinant plasmid into the host cell, homologous recombination results in the incorporation of the marker into the transcription unit of the target gene, allowing marker expression and the survival of the cell during selection (Figure 6.1) (Watson *et al.*, 1992).

In this study, insertional inactivation of a *sod* gene was attempted using a recombinant plasmid carrying the sod_{int} gene fragment (Poyart *et al.*, 1995) disrupted by a spectinomycin resistance gene. The spectinomycin resistance gene was originally derived from an *Enterococcus faecium* isolate, and is expressed both in *E. coli* and *S. pneumoniae* (Dunny *et al.*, 1991). When this recombinant plasmid, pHY2, was transformed into *S. pneumoniae*, spectinomycin resistant colonies could be obtained only through integration of the spectinomycin resistance cassette into the chromosome via homologous cross-over between DNA sequences flanking the spectinomycin gene on recombinant plasmid and the chromosome. This would result in insertional inactivation of *sod* (Figure 6.2). The double cross-over is preferred over single cross-over as a result of which more stable mutation event occurs (Gibson and Caparon, 1996).

Despite some of its advantages, such as total and stable knock out of the gene, insertion mutation can not be detected if the gene mutation has lethal effect (Miller, 1992). In addition, insertion mutation does not tell if the effect seen in transformants are the direct effect created by mutation or polar effect of the inactivation of downstream genes (Miller, 1992).



Disrupted gene C. Transformants carrying a disrupted copy of the gene can be selected for the presence of marker.

Figure 6.1: Insertion mutagenesis

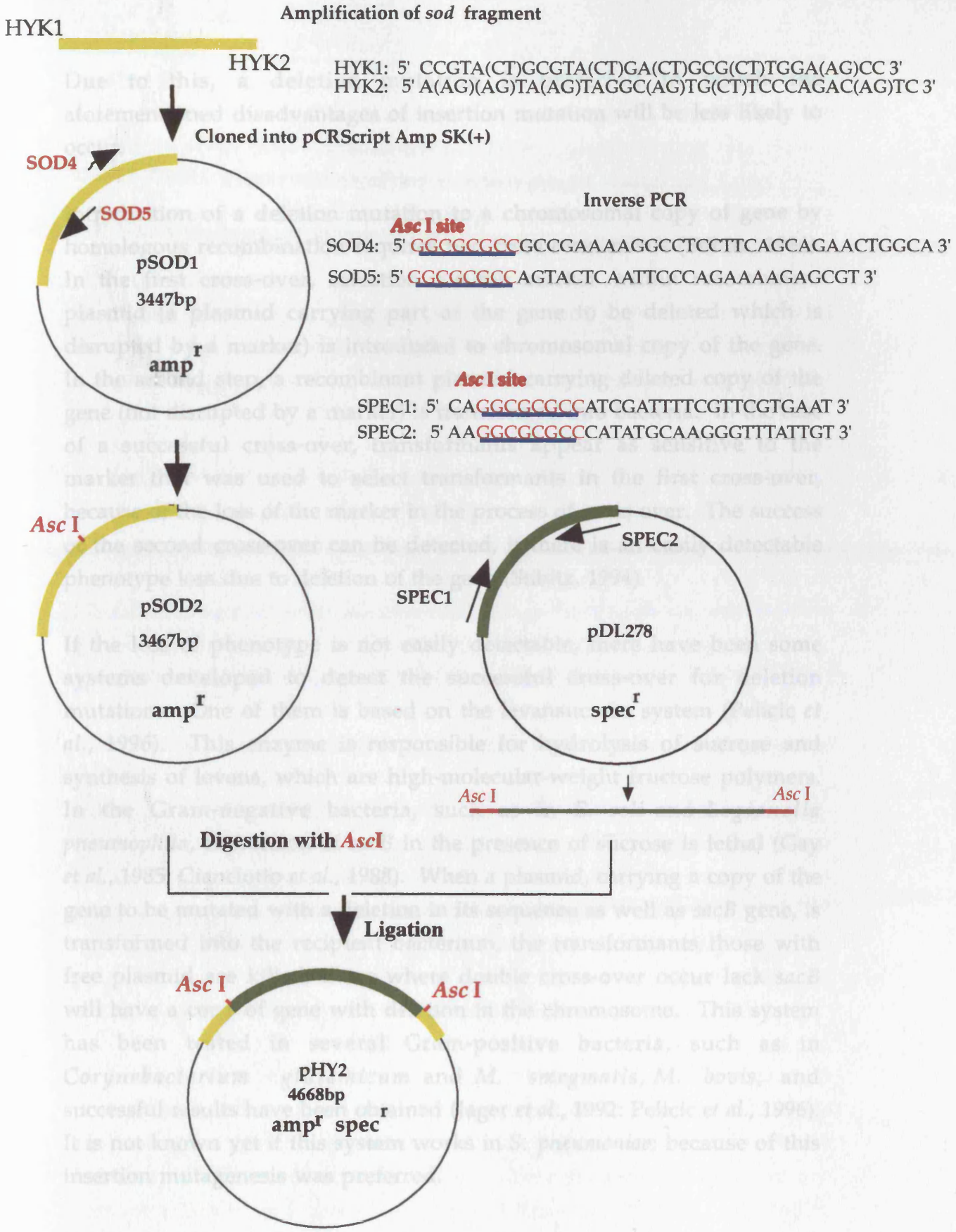


Figure 6.2: Construction of pHY2. See the text for details

Due to this, a deletion mutation is preferred in which the aforementioned disadvantages of insertion mutation will be less likely to occur.

Introduction of a deletion mutation to a chromosomal copy of gene by homologous recombination requires two cross over events (Stibitz, 1994). In the first cross-over, selection for the marker within recombinant plasmid (a plasmid carrying part of the gene to be deleted which is disrupted by a marker) is introduced to chromosomal copy of the gene. In the second step, a recombinant plasmid carrying deleted copy of the gene (not disrupted by a marker) is transformed into bacteria. In the case of a successful cross-over, transformants appear as sensitive to the marker that was used to select transformants in the first cross-over, because of the loss of the marker in the process of cross-over. The success of the second cross-over can be detected, if there is an easily detectable phenotype loss due to deletion of the gene (Stibitz, 1994).

If the loss of phenotype is not easily detectable, there have been some systems developed to detect the successful cross-over for deletion mutations. One of them is based on the levansucrose system (Pelicic *et al.*, 1996). This enzyme is responsible for hydrolysis of sucrose and synthesis of levans, which are high-molecular-weight fructose polymers. In the Gram-negative bacteria, such as in *E. coli* and *Legionella pneumophila*, expression of *sacB* in the presence of sucrose is lethal (Gay *et al.*, 1985; Cianciotto *et al.*, 1988). When a plasmid, carrying a copy of the gene to be mutated with a deletion in its sequence as well as *sacB* gene, is transformed into the recipient bacterium, the transformants those with free plasmid are killed, those where double cross-over occur lack *sacB* will have a copy of gene with deletion in the chromosome. This system has been tested in several Gram-positive bacteria, such as in *Corynebacterium glutamicum* and *M. smegmatis, M. bovis*, and successful results have been obtained (Jager *et al.*, 1992: Pelicic *et al.*, 1996). It is not known yet if this system works in *S. pneumoniae*; because of this insertion mutagenesis was preferred.

6.1 Construction of pSOD1

An internal fragment of the pneumococcal *sod* gene was amplified from *S. pneumoniae* type 2 genomic DNA using degenerate primers HYK1 and

HYK2. This reaction yielded an expected 480 bp sod_{int} fragment (Figure 6.3A). Following amplification, the *sod* gene fragment was cloned into pCRScript Amp SK(+) (Figure 6.3B). The successful cloning was shown by restriction digest of one of the positive clones with *BamH*I and *Not*I. These enzymes were chosen because the recognition site for these enzymes are located at both sides of cloning site. One of the positive clones, designated as pSOD1, was chosen for large scale plasmid preparation and it was manually sequenced using universal forward and reverse primers that hybridised on either side of the polylinker in the vector. The sequence data confirmed that the correct fragment had been cloned as it had perfect identity with the sod_{int} fragment (Figure 6.4).

In order to find out how many copies of sod_{int} are present in the pneumococcal chromosome, 3-4 µg pneumococcal genomic DNA was restricted either with *BamH*I, *Kpn*I or *Sca*I since these enzymes do not have a recognition site in sod_{int}. Then, the digested DNA was Southern blotted and probed with radiolabelled sod_{int}. The result showed that the probe hybridised with a single band indicating only one copy of *sod* gene specific to this probe was present in the pneumococcus (Figure 6.5).

6.2 Insertion of a spectinomycin resistance cassette into sod_{int} fragment in pSOD1

Initially, the disruption of the sod_{int} gene in pSOD1 was attempted by ligation of spectinomycin gene into an unique *BsaB*I (generates blunt ends) (New England Bio Lab) site within sod_{int}. To do this, a spectinomycin resistance gene cassette was separated from pDL278 by *Sca*I and *Bgl*I digestions. This fragment was then treated with T4 DNA polymerase and Klenow fragment to create blunt ends. However several attempts to ligate this fragment into the *BsaB*I site of pSOD1 failed.

PCR was used as an alternative approach to create compatible ends. An *Asc*I site was added to the sod_{int} fragment in pSOD1 and to the ends of the spectinomycin resistance cassette. An *Asc*I site was preferred, firstly because pSOD1 as well as the spectinomycin resistance cassette does not contain this site and therefore it was suitable for cloning. Secondly, unlike certain enzymes, *Asc*I cuts efficiently even when its recognition site is at the end of a DNA fragment (Moreira and Noren, 1995).

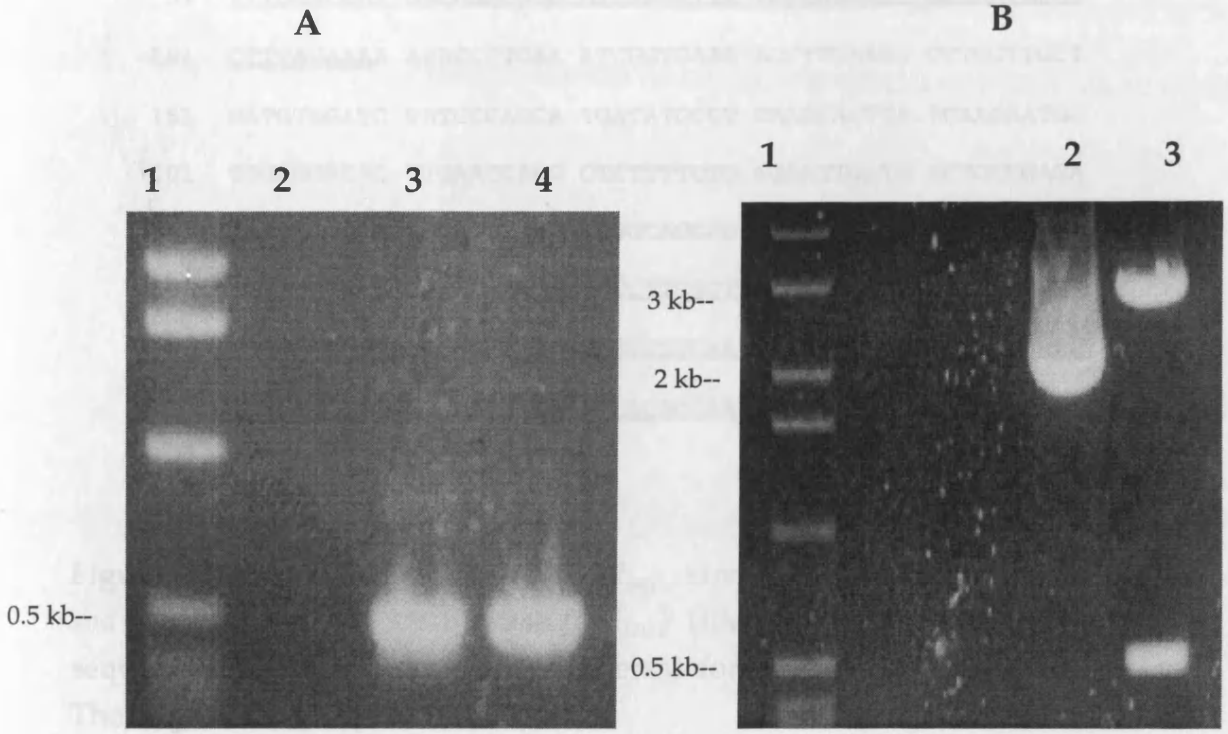


Figure 6.3: Amplification and cloning of sod_{int} fragment. A. An internal fragment of *sod* gene was amplified using the degenerate primers HYK1 and HYK2 (lanes 3 and 4). 50 ng *S. pneumoniae* type 2 chromosomal DNA was employed as template. Lane 2 represents the negative control reaction with no added template. **B. Restriction analysis of pSOD1.** pSOD1 was digested with *Bam HI* and *Not*I (lane 3). Lane 2 is uncut plasmid (pSOD1). In A and B lane 1 is 1 kb DNA size markers (Gibco/BRL); fragment sizes are shown in the left hand column in kilobases (kb).

```
  1  CCGTACGCGT ACGATGCGCT GGAACCATAC ATCGATGCGG AAACAATGCA
 51  TTTGCACCAT GACAAACACC ATCAAACTTA TGTCAACAAT GCCAATGCAG
101  CTTTAGAAAA ACACCCTGAA ATCGGTGAAG ACCTTGAAGC CTTGCTTGCT
151  GATGTAGATC TATCCCAGCA TGATATCCGT CAAGCACTTA TCAACAATGG
201  TGGCGGACAC TTGAACCACG CTCTTTTCTG GGAATTGATG ACTCCCGAGA
251  AAACAGCTCC TTCAGCAGAA CTGGCAGCAG CAATCGATGC AACATTTGGT
301  TCATTTGAAG AATTCCAAGC AGCCTTCACT GCAGCAGCAA CAACTCGTTT
351  TGGTTCAGGT TGGGCATGGT TGGTTGTCAA CAAAGAAGGG AAACTTGAAG
401  TGACTTCAAC AGCAAACCAA GACACACCAA TCTCAGAAGG TAAAAAACCA
451  ATCTTGGGCT TGGACGTTTG GG
```

Figure 6.4: Sequence analysis of sod_{int}. *Streptococcus pneumoniae* partial *sod* for superoxide dismutase (sod_{int}) (EMBL acc. no: z49246) was sequenced at both sides using universal forward and reverse primers. The sequenced section is underlined.

The primer SOD4 (Figure 6.2), anneals to nucleotides 257 to 276 within sod_{int} and generates an *Asc*I site, and the primer SOD5 binds to nucleotides 237 to 257 of the complementary strand of sod_{int} and also creates an *Asc*I site (Figure 6.2). These primers were used to introduce an unique *Asc*I site into sod_{int} fragment by inverse PCR in which pSOD1 was the template. The annealing temperature used was 50°C. The amplified fragment was then cleaved with *Asc*I in order to free the *Asc*I site from a single 3'-terminal nucleotide overhang as *Taq* DNA polymerase has a nontemplate-dependent activity which adds a single deoxyadenosine (A) to the 3'-ends of PCR products (Sambrook *et al.*, 1989). The digested PCR products were extracted with phenol-chloroform and ethanol precipitated as described in section 3.6.1. The PCR products were then self ligated to form a circular plasmid, electroporated into *E. coli* JM109 and then transformants were selected on LA containing ampicillin. This yielded 250 colonies. Mini plasmid preparation was made from 10 colonies and cleaved with *Asc*I to confirm the presence of

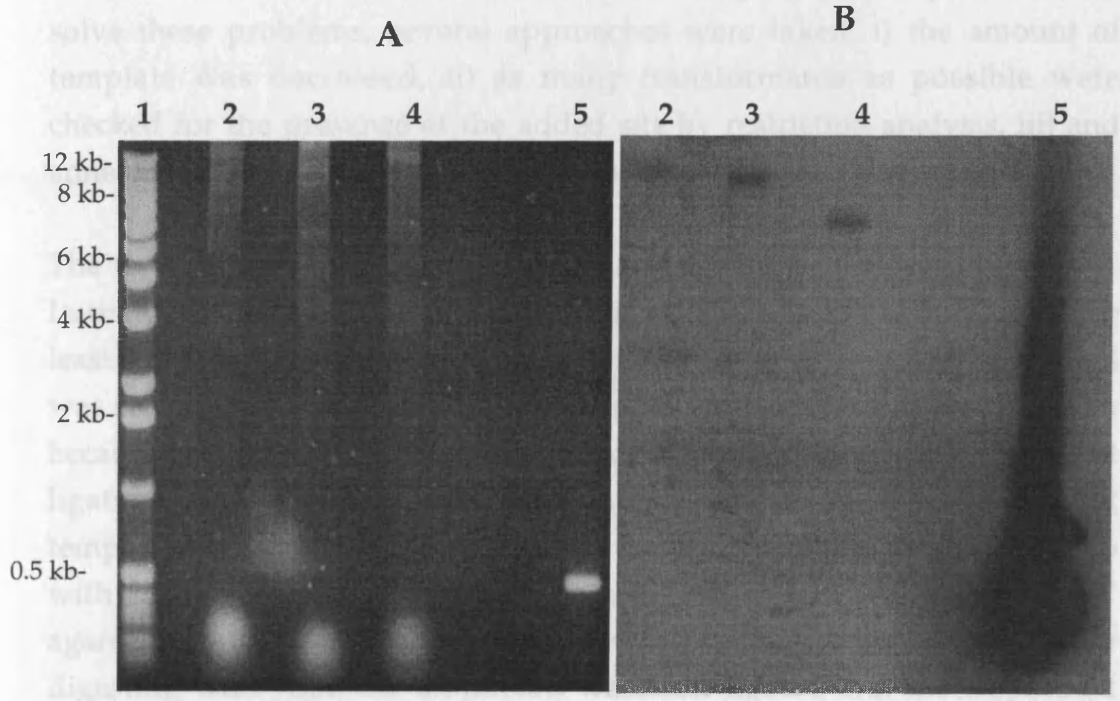


Figure 6.5: Detection of sod_{int} in *S. pneumoniae* type 2 chromosome. A. Chromosomal DNA from *S. pneumoniae* type 2 was digested with *BamH*I (lane 2), *Kpn*I (lane 3) and *Sca*I (lane 4), and analysed on 0.7% w/v agarose gel. Lane 5 is sod_{int} and lane 1 is 1 kb DNA size markers (Gibco/BRL); fragment sizes are shown in the left hand column in kilobases (kb). **B.** The DNA was transferred to nylon membrane and probed with the radiolabelled sod_{int}. Lane orders are same as in A.

the new site. The parent pSOD1 was also included as a control. However, restriction pattern of the sample was the same as the control, indicating the absence of the *Asc*I site (data not shown). This was repeated once more, but again the incorporation of *Asc*I site into pSOD1 was unsuccessful.

This might have been due to several reasons. Firstly, at an annealing temperature of 50°C, non specific amplification of the parent plasmid might have been happening. Secondly, the amount of template might have been high in the PCR. Both of these factors could lead to the an increase in the number of transformants carrying parent plasmid. To solve these problems, several approaches were taken: i) the amount of template was decreased, ii) as many transformants as possible were checked for the presence of the added site by restriction analysis, iii) and annealing temperature was raised to 55°C.

The new PCRs were performed using an annealing temperature of 55°C. Initially, to assess the optimum amplification conditions at 55°C with the least possible template, 20, 10 or 2 ng of pSOD1 were used (Figure 6.6). It was seen that 10 ng DNA (lane 4) was sufficient for efficient amplification because the product yielded was sufficient enough to set up an efficient ligation, as compared to the reaction set up with 2 ng template, and template contamination was lower as compared to the reaction set up with 20 ng template. The PCR products were separated on 0.6% LMT agarose overnight at low voltage and were extracted from agarose. After digesting with *Asc*I, the amplicons were self ligated and electroporated into *E. coli* JM109 strain. The transformation yielded 5 colonies. Mini plasmid preparation of these colonies were made and plasmids were restricted with *Asc*I. One of these colonies, designated as pSOD2, was linearised after digestion with *Asc*I, confirming the presence of an *Asc*I site (Figure 6.7).

The spectinomycin sequence obtained from the nucleotide sequence database (Accession no: SWISS-PORT;Q07448) was used to design spec1 and spec2 primers. These primers, when used with pDL278 as the template, were expected to amplify an approximately 1151 bp fragment (Figure 6.8A) and create an *Asc*I site due to the design of the primers. Prior to ligation into pSOD2, the spectinomycin gene was first cloned into

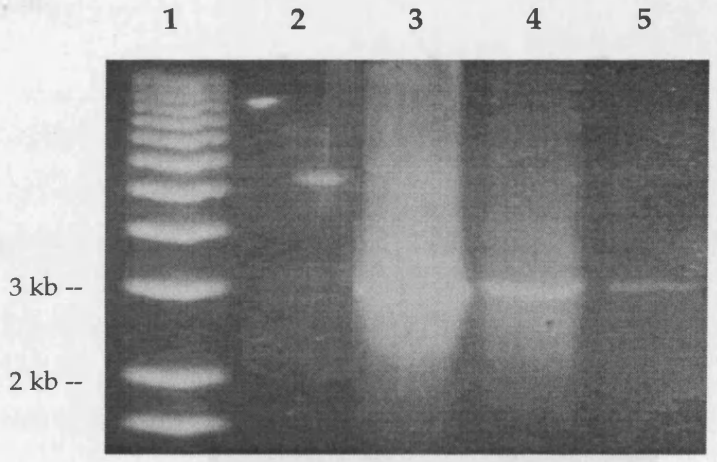


Figure 6.6: Optimisation of PCR conditions for the addition of *Asc*1 site to pSOD1. To assess the optimum amplification conditions at 55°C with the least possible template, 20 (lane 3), 10 (lane 4) or 2 ng (lane 5) pSOD1 was used as the template. PCR products were analysed by 0.7% agarose gel electrophoresis. Lane 2 is negative control with no added template DNA. Lane 1 is 1 kb DNA size marker (Gibco/BRL), fragment sizes are shown in the left hand column in kilobases (kb).

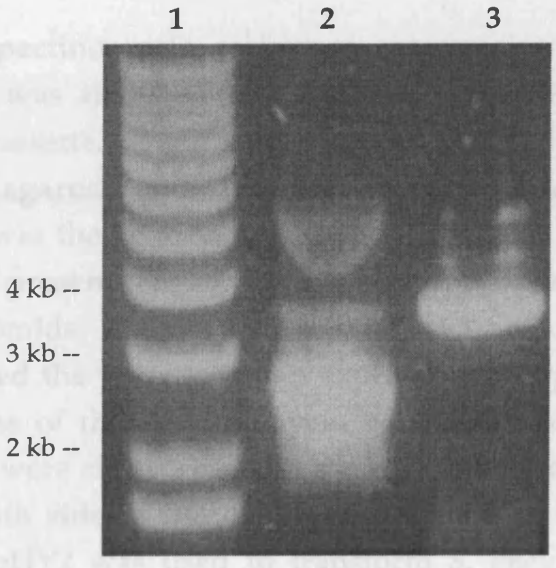


Figure 6.7: Restriction analysis of pSOD2. 4 µg pSOD2 was linearised with *Asc*I indicating that the creation of this site had been achieved (lane 3). Lane 2 represents uncut pSOD2. Lane 1 is 1 kb DNA size markers (Gibco/BRL), fragment sizes are shown in the left hand column in kilobases (kb).

pCRScript Amp SK(+) (section 3.13.1) to ensure that the *Asc*I site had been created. After cloning, the transformants were selected on LA in the presence of ampicillin and purified plasmids were digested with *Asc*I. The release of insert by *Asc*I digestion indicated the successful cloning, and ultimately the creation of the site (Figure 6.8B).

Following selection on ampicillin the transformants were also successfully grown in the presence of ampicillin and spectinomycin. One of these plasmids designated as pHY5 was chosen for large scale plasmid preparation.

To clone the spectinomycin resistance cassette into *Asc*I-cut arms of pSOD2, pHY5 was digested to completion with *Asc*I to release the spectinomycin cassette. The fragments of restricted pHY5 were separated on 0.6% LMT agarose and the spectinomycin cassette was purified (Qiagen). This was then cloned into *Asc*I-cut pSOD2. Recombinants were selected in the presence of ampicillin and spectinomycin. Restriction analysis of plasmids, prepared from random transformants by *BamH*I and *Not*I revealed the presence of an expected 1630 bp insert, which was equal to the size of the spectinomycin gene and sod_{int} gene fragment. These enzymes were chosen because a recognition site for these enzymes is located at both side of the cloning side. One of the positive clones designated as pHY2 was used to transform *S. pneumoniae,* after large scale plasmid preparation (Figure 6.9).

6.3 Transformation with pHY2 to *S. pneumoniae* type 2 encapsulated strain

Transformation of foreign DNA into encapsulated strain of *S. pneumoniae* by conventional culture and plating techniques or filter mating is known to be inefficient (Canvin *et al.*, 1994). In addition, optimum conditions for electrotransformation varied from one strain to another (Canvin *et al.*, 1994). Due to this, transformation with competence stimulating peptide (CSP) was used as an alternative way to introduce pHY2 DNA into *S. pneumoniae* (Haverstein *et al*, 1995). The transformation procedure was followed as described in section 3.8.2 and the transformants were selected on blood agar plates containing 1 mg/ml spectinomycin and overnight incubation was done at 37°C. A total of six colonies appeared on the blood agar plates: 2 from the plate transformed

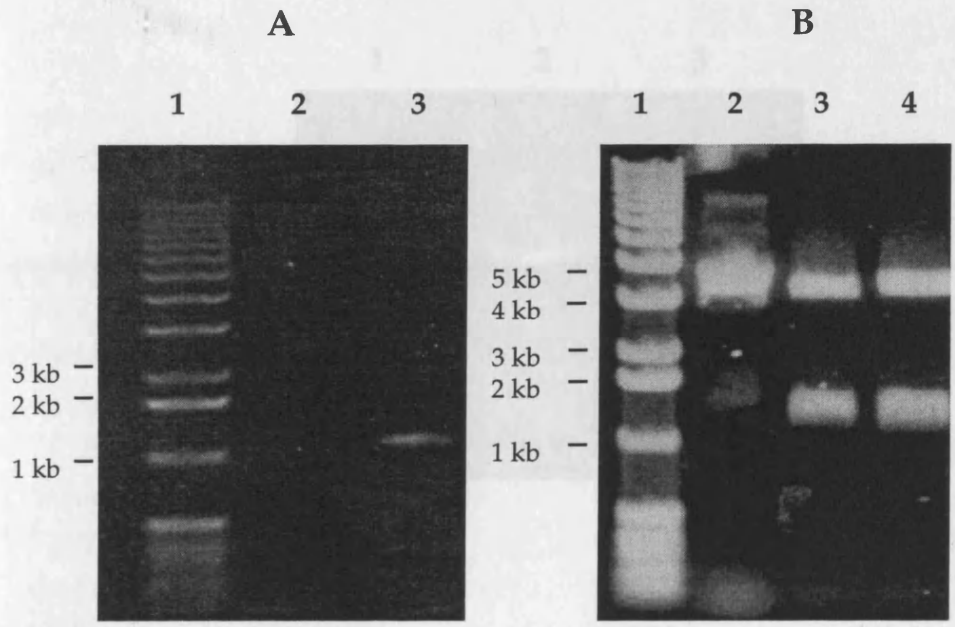


Figure 6.8: Amplification and cloning of spectinomycin resistance gene. **A.** Spectinomycin resistance gene was amplified using spec1 and spec2 primers and 50 ng pDL278 DNA (lane 3). Lane 2 is the negative control; reaction with no added template. **B.** The PCR reaction product containing the spectinomycin resistance gene was digested with *Asc*I before ligating into pCRScript Amp SK(+), and the presence of the insert was shown first by *BamH*I and *Not*I digestion (lane 3) and then *Asc*I digestion (lane 4). Lane 2 represents uncut pHY5. Lane 1 is 1 kb DNA size marker (Gibco/BRL), fragment sizes are shown in the left hand column in kilobases (kb).

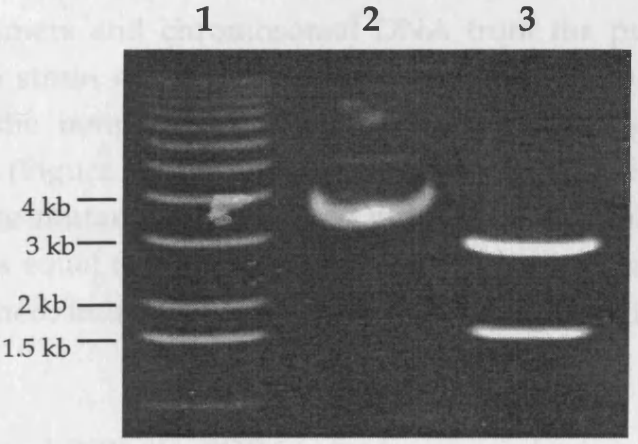


Figure 6.9: Restriction digest of pHY2. pHY2 was digested by *BamH*I and *Not*I to show the presence of 1.6 kb cloned insert (lane 3). Uncut pHY2 is shown in lane 2. Lane 1 is 1 kb DNA size marker (Gibco/BRL), fragment sizes are shown in the left hand column in kilobases (kb).

after 3 h 30 min, and 4 from the plate transformed after 4 h 30 min. Chromosomal DNA from all colonies was prepared to check whether recombination had occurred and to determine the nature of the recombination event ie, single or double cross over. This was done by PCR and Southern blot hybridisation.

6.4 Testing the transformants for chromosomal integration by PCR and Southern Blot hybridisation

The integration of the spectinomycin resistance cassette into the pneumococcal chromosome was shown by PCR, employing HYK1 and HYK2 primers and chromosomal DNA from the putative mutants and wild type strain as the templates. When wild-type genomic DNA was used as the template an expected 480 bp sod_{int} gene fragment was amplified (Figure 6.10, lane 16). However, when the chromosomal DNA of putative mutants was used as the template, a 1630 bp PCR product, which was equal the total size of sod_{int} and spectinomycin resistance gene was obtained, indicative of a successful integration (Figure 6.10, lanes 4, 6, 8, 12, 14).

In the second PCR, the SOD6 primer was used. As stated earlier (section 5.3.2.1), SOD6 primer starts 105 bp downstream of the HYK2 primer on the complementary strand. When SOD6 primer was combined with HYK1, the reaction yielded an expected 577 bp product with the wild type chromosomal DNA (Figure 6.10, lane 17) whereas transconjugants yielded approximately 1700 bp PCR product, equal to the size of sod_{int} and spectinomycin (Figure 6.10, lanes 5, 7, 9, 11, 13, 15). However, when pHY2 was employed as the template with HYK1 and SOD6 primers, as predicted, no product was obtained because sod_{int} fragment did not contain the sequence of SOD6 primer (Figure 6.10, lane 19).

The presence of pCRScript Amp SK(+) in the chromosome of putative mutants was checked to reveal information about the nature of the mutation as the presence of the plasmid would indicate a single cross-over event. This was tested by growing putative mutants in the presence of ampicillin or by attempting to detect pCRScript Amp SK(+) sequence in the chromosome of the transformants. The transformants were grown in 1, 10 ,100, or 500 μg ampicilin. In any of these concentrations of ampicillin, the transformants were unable to maintain growth. The

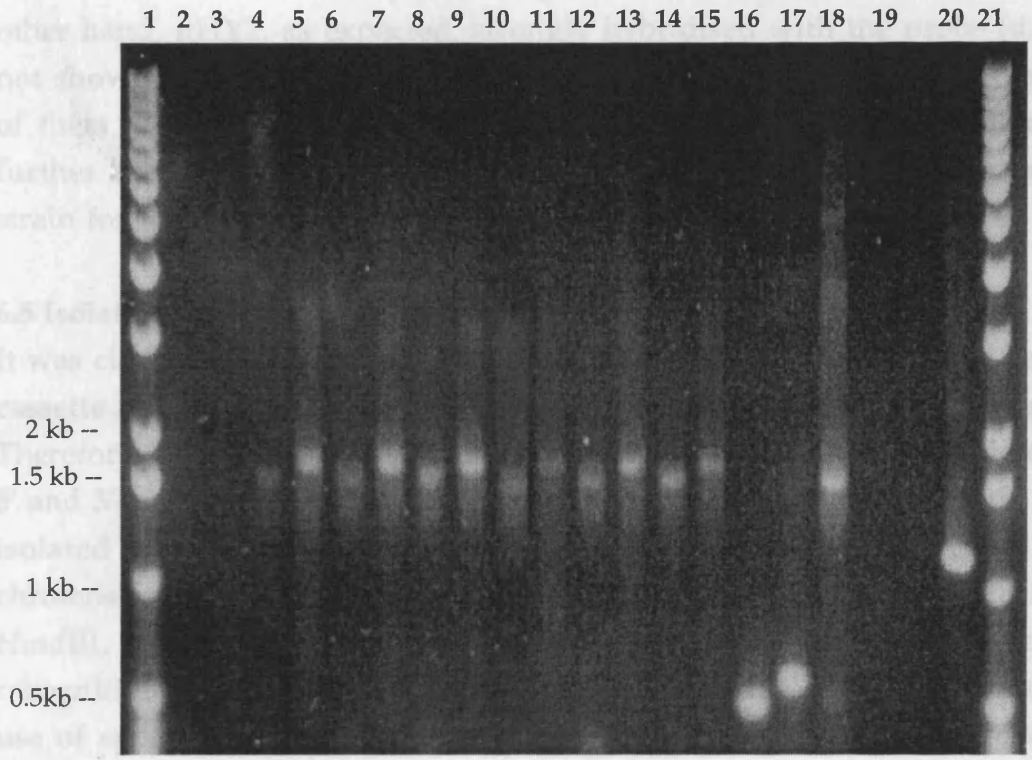


Figure 6.10: PCR confirmation of integration of the spectinomycin cassette into the *sod* gene in the pneumococcal chromosome. PCR with HYK1 and HYK2 primers which generate a 480 bp sod_{int} fragment in wild type (lane 16) produced 1.6 kb products with spectinomycin resistant recombinants (lanes 4, 6, 8, 10, 12, 14) which was equal to the total size of sod_{int} and spectinomycin resistance gene. The same size fragment was obtained with the primers when pHY2 was used as a template (lane 18). As expected, PCR with HYK1 and SOD6 produced slightly larger products for recombinants (lanes 5, 7, 9, 11, 13, 15) and the wild type (lane 17) but no product for pHY2 (lane 19). Lanes 1 and 21 are kilobase markers (BRL), lanes 2 and 3 are negative controls (no template DNA added). Lane 20 is a positive control for spec1 and spec2 primers which amplify 1.15 kb spectinomycin cassette from pHY2.

absence of vector sequence was also confirmed by Southern blot hybridisation using radiolabelled pCRScript Amp SK(+) as the probe (data not shown). *Dra*I-digested pHY2 was used as a positive control. The result showed that none of the transconjugants hybridised with pCR Script Amp SK(+) probe, indicating the absence of vector sequence in the chromosome and ultimately revealing a double cross-over event. On the other hand, pHY2, as expected, strongly hybridised with the probe (data not shown). Since all the transformants indicated the same results; one of them, designated as D39HY1, was chosen for further study. Before further characterisation of D39HY1, it was decided to use the mutant strain for the isolation of 5'-end and upstream sequences of *sod* .

6.5 Isolation of pneumococcal *sod* gene from D39HY1

It was clear from the above results that the spectinomycin resistance gene cassette had been inserted into the chromosomal copy of the *sod* gene. Therefore, the sequences flanking the spectinomycin cassette include the 5' and 3'-ends of the *sod* gene. Consequently, 5'-end of the gene could be isolated from the mutant strain. To isolate the *sod* gene, D39HY1 chromosomal DNA and wild type DNA was digested with either *Eco*RI, *Hind*III, or *Pst*I. These enzymes were chosen because they do not have recognition sites in the spectinomycin gene and this would enable the use of spectinomycin resistance as a selective marker when cloning the 5'-end of the *sod* gene that flanked spectinomycin resistance gene cassette in D39HY1.

To identify the fragments containing the *sod* gene, 12 μg separate batches of D39HY1 DNA was digested with these enzymes. Wild type DNA was used as the control. The digested DNA fragments (4 μg) were separated by agarose gel electrophoresis and Southern blotted to nylon membrane, probed with radiolabelled sod_{int}. The rest of the reaction mixtures were kept for cloning. The result showed that the probe hybridised to approximately 1 kb *Eco*RI , 8 kb *Hind*III, 1.4 kb *Pst*I fragments for wild type (Figure 6.11, lanes 1, 2, 3, respectively), whereas for D39HY1 the size of the hybridised fragments were 2.1 kb, 10.5 kb, and 2.3 kb, respectively (Figure 6.11, lanes 4, 5, 6). The size increase in the mutant provided further evidence for chromosomal integration of the spectinomycin resistance cassette. The *Pst*1 fragments were then chosen for cloning as it was a convenient size for cloning.

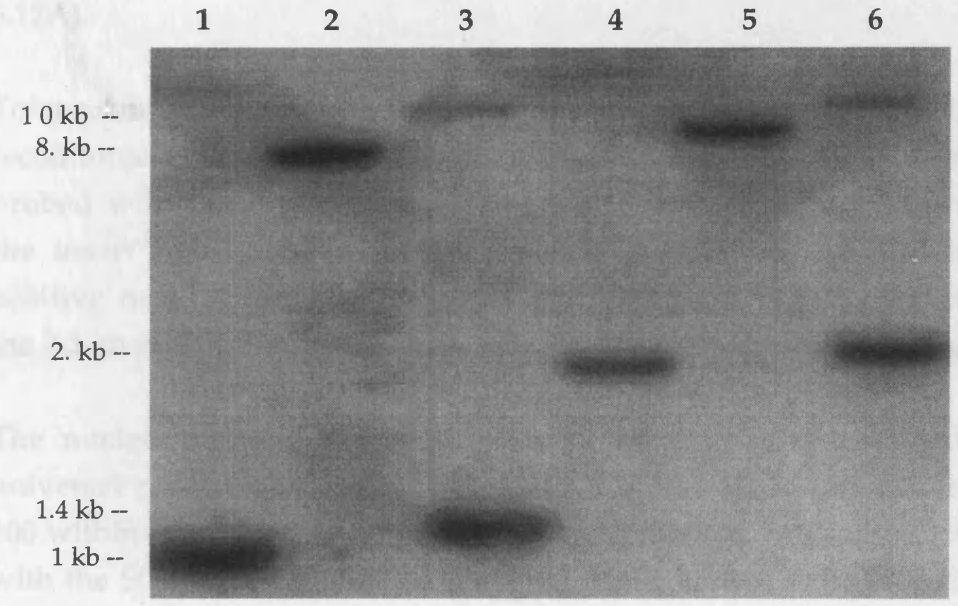


Figure 6.11: Detection of increase in size of *sod* by Southern blot analysis for putative *sodA* mutants. Chromosomal DNA of the wild type (lanes 1-3) and transformants (lanes 4 to 6) was digested with *EcoR*I (lanes 1 and 4), *Hind*III (lanes 2 and 5) or *Pst*I (lanes 3 and 5). Hybridisation with sod_{int} fragment showed an increase in the size of the fragment in the recombinants. The increase in size was expected if the spectinomycin resistance cassette had inserted into the *sod* gene.

The vector pBlueScript Amp SK(+), 300-400 ng, was linearised with *Pst*I and ligated with 2 μg *Pst*I digested D39HY1 chromosomal DNA. The standard electrotransformation procedure was followed (section 3.8.1.2) and transformants (*E. coli* DH5α cells were used for transformation) were selected on LA plates containing spectinomycin. As a result, 60 spectinomycin resistance colonies were obtained and the presence of 2.3 kb insert was shown by restriction digestion of plasmids by *Pst*I (Figure 6.12A).

To ensure that the cloned fragment hybridised with sod_{int}, *Pst*I-digested recombinant plasmid was Southern blotted onto nylon membrane and probed with radiolabelled sod_{int} fragment. The procedure showed that the insert hybridised with the probe (Figure 6.12B). Following this positive result, maxi plasmid DNA preparation of one clone containing the 2.3 kb insert, designated as pPSTSOD, was performed for sequencing.

The nucleotide sequence of the 2.3 kb insert was determined using universal reverse and SOD9 primers (SOD9 anneals to nucleotides 82 to 100 within the sod_{int}). Analysis of 180 bp of nucleotide sequence obtained with the SOD9 primer showed that part of the known sod_{int} as well as the 5'-end and upstream sequence had been obtained (Figure 6.13A). The sequence revealed with the universal reverse primer also clearly demonstrated that the insert, as expected from the design of experiment, started with *sod* sequence and this was followed by spectinomycin sequence (Figure 6.13B).

6.6 Sequence determination and analysis of *sod* from *S. pneumoniae*

Although the sequence of entire *sod* gene was revealed, it had not been uniformly isolated from one strain. The 5'-end and internal fragment was from a type 2, but the 3'-end had been isolated from a type 1 strain. When the 3'-end of the gene from type 1 and type 2 strain was compared, the 3'-end of the gene in both strains was identical (Figure 6.14). However, despite this, to determine the entire gene of the type 2, it was amplified from *S. pneumoniae* type 2 using SOD12, which anneals to nucleotides between 28 to 44 in the upstream *sod* sequence (Figure 6.15) and SOD2 primers. Approximately 700 bp fragment was recovered as expected and the products of two PCRs were then sequenced using SOD1, SOD2, SOD3 and SOD12 primers (see Figure 5.5).

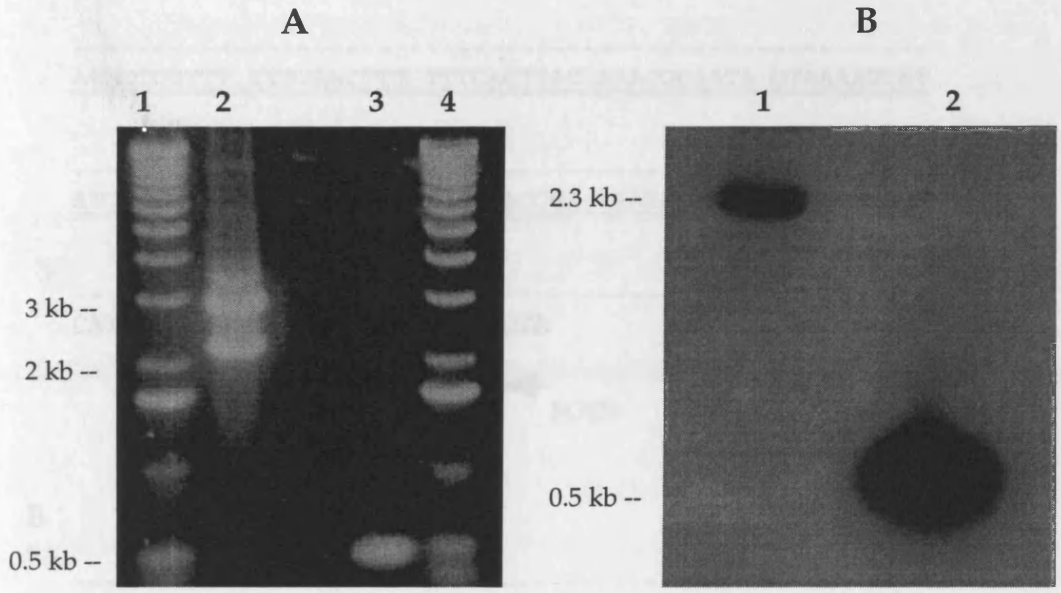


Figure 6.12: A. Cloning 2.3 kb *Pst*I fragment into pBlueScript SK(+). A. The construct containing *Pst*I fragment was restricted by *Pst*I digestion (lane 2), sod_{int} fragment (lane 3) was included as positive control for subsequent Southern blot hybridisation. Lane 1 and 4 are 1 kb DNA size marker (Gibco/BRL), fragment sizes are shown in the left hand column in kilobases (kb). B. The DNA samples in A were transferred to nylon membrane and hybridised with radiolabelled sod_{int} fragment. Lane 1 shows hybridised *Pst*I fragment, lane 2 represents 480 bp sod_{int} fragment, hybridised against itself as positive control.

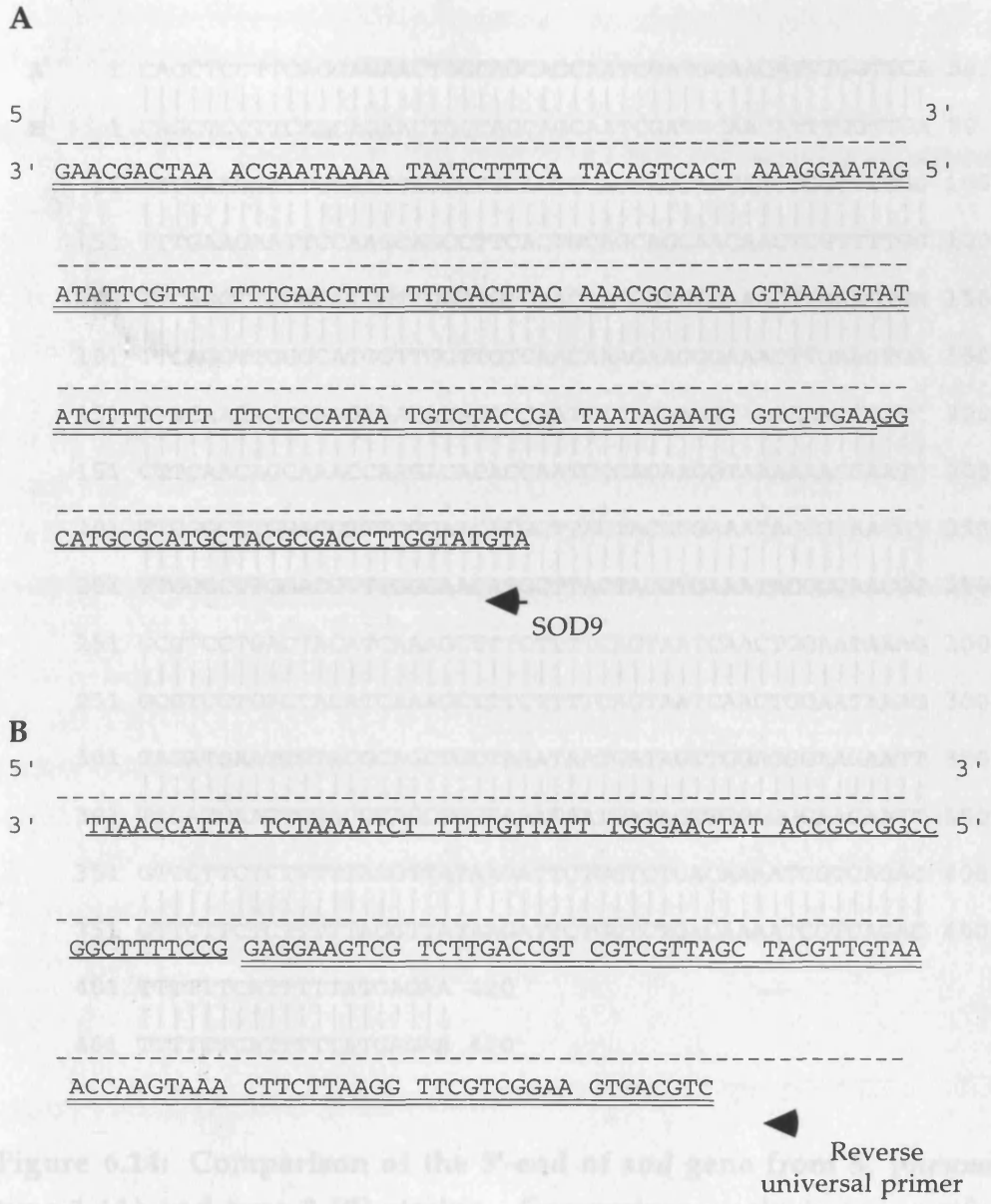


Figure 6.13: Sequence analysis of insert within pPSTSOD by SOD9 and universal reverse primers: A. Shows the sequence acquired with SOD9 primer. Singly underlined represents *sod* sequence within the insert in pPSTSOD. Doubly underlined sequence represent 5'-end and upstream sequences. **B.** Shows the sequence acquired with universal reverse primer. Single underlined is spectinomycin resistance gene, double underlined is *sod* sequence. The arrows indicate the direction of the primers.

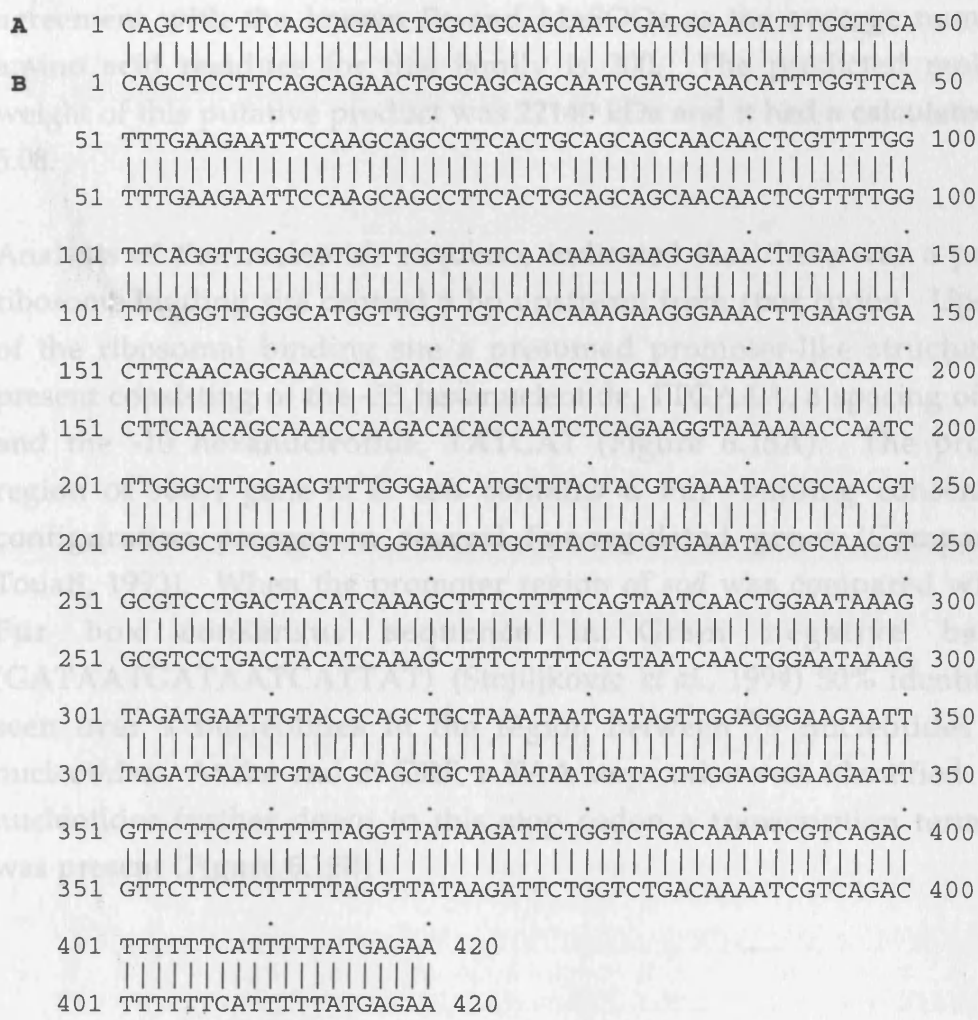


Figure 6.14: Comparison of the 3'-end of *sod* gene from *S. pneumoniae* type 1 (A) and type 2 (B) strains. Comparison analysis was performed using the BESTFIT programme in the GCG molecular biology package.

The sequence analysis of the gene showed that pneumococcal *sod* consisted of a 606 bp open reading frame that was predicted to encode 202 amino acids (Figure 6.15). The number of amino acid residues was in agreement with the known Fe and MnSODs as the average number of amino acid residues for this family is 200. The predicted molecular weight of this putative product was 22140 kDa and it had a calculated pI of 5.08.

Analysis of the nucleotide sequence indicated that there was a putative ribosome binding site centred 8 bp upstream from start codon. Upstream of the ribosomal binding site a presumed promoter-like structure was present consisting of the -35 hexanucleotide, TTGAAA, a spacing of 17 bp and the -10 hexanucleotide, TATCAT (Figure 6.15A). The promoter region of *sodA* gene in *E. coli* contains a Fur -binding consensus, a configuration present in several Fur-regulated genes (Compan and Touati, 1993). When the promoter region of *sod* was compared with the Fur box consensus sequence in Gram negative bacteria (GATAATGATAATCATTAT) (Stojiljkovic *et al.*, 1994) 50% identity was seen over 9 nucleotides in the region between 79 nucleotides to 96 nucleotides. At the end of ORF, a TAA stop codon was identified. Eight nucleotides further down to this stop codon a transcription terminator was present (Figure 6.15B)

A.

```
   1  CTTGCTGATT TGCTTATTTT ATTAGAAAGT ATGTCAGTGA TTTCCTTATC

                        -35                          -10
  51  TATAAGCAAA AAACTTGAAA AAAGTGAATG TTTGCGTTAT  CATTTTCATA

                    SD
 101  TAGAAAGAAA AAGAGGTATT ACAGATGGCT ATTATCTTAC CAGAACTTCC
                                    M  A  I  I  L  P  E  L  P
 151  GTACGCGTAC GATGCGCTGG AACCATACAT CGATGCGGAA ACAATGCATT
        Y  A  Y  D  A  L  E  P  Y  I  D  A  E  T  M  H  L
 201  TGCACCATGA CAAACACCAT CAAACTTATG TCAACAATGC CAATGCAGCT
        H  H  D  K  H  H  Q  T  Y  V  N  N  A  N  A  A  L
 251  TTAGAAAAAC ACCCTGAAAT CGGTGAAGAC CTTGAAGCCT TGCTTGCTGA
        E  K  H  P  E  I  G  E  D  L  E  A  L  L  A  D
 301  TGTAGATCTA TCCCAGCATG ATATCCGTCA AGCACTTATC AACAATGGTG
        V  D  L  S  Q  H  D  I  R  Q  A  L  I  N  N  G  G
 351  GCGGACACTT GAACCACGCT CTTTTCTGGG AATTGATGAC TCCCGAGAAA
        G  H  L  N  H  A  L  F  W  E  L  M  T  P  E  K
 401  ACAGCTCCTT CAGCAGAACT GGCAGCAGCA ATCGATGCAA CATTTGGTTC
        T  A  P  S  A  E  L  A  A  A  I  D  A  T  F  G  S
 451  ATTTGAAGAA TTCCAAGCAG CCTTCACTGC AGCAGCAACA ACTCGTTTTG
        F  E  E  F  Q  A  A  F  T  A  A  A  T  T  R  F
 501  GTTCAGGTTG GGCATGGTTG GTTGTCAACA AAGAAGGGAA ACTTGAAGTG
        G  S  G  W  A  W  L  V  V  N  K  E  G  K  L  E  V
 551  ACTTCAACAG CAAACCAAGA CACACCAATC TCAGAAGGTA AAAAACCAAT
        T  S  T  A  N  Q  D  T  P  I  S  E  G  K  K  P  I
 601  CTTGGGCTTG GACGTTTGGG AACATGCTTA CTACGTGAAA TACCGCAACG
        L  G  L  D  V  W  E  H  A  Y  Y  V  K  Y  R  N
 651  TGCGTCCTGA CTACATCAAA GCTTTCTTTT CAGTAATCAA CTGGAATAAA
        V  R  P  D  Y  I  K  A  F  F  S  V  I  N  W  N  K
 701  GTAGATGAAT TGTACGCAGC TGCTAAATAA TGATAGTTGG AGGGAAGAAT
        V  D  E  L  Y  A  A  A  K  *  *  *  L  E  G   R  I
 751  TGTTCTTCTC TTTTTAGGTT ATAAGATTCT GGTCTGACAA AATCGTCAGA
        V  L  L  F  L  G  Y  K  I  L  V  *  Q  N  R  Q  T
 801  CTTTTTTCAT TTTTATGAGA A
```

B.

Fur box consensus	5'	GATAATGATAATCATTAT 3'
		T T ATCATT T
S. pneumoniae sodA	5'	TGTTTGCGTTATCATTTT 3'

Figure 6.15: Nucleotide sequence of pneumococcal *sod* gene and deduced amino acid sequence. A. A probable Shine-Dalgarno (SD) sequence and a putative promoter (-35 and -10 regions) are shown in bold type face. The putative stem loop rho-independent terminator region is shown in italic. Double underlined indicates the data sequenced on both strands, single underlined indicates the data sequenced only on one strand. **B.** Fur box consensus sequence (upper line) was used to search promoter region of *sodA* (bottom line). Nine nucleotides in the pneumococcal *sodA* promoter region were identical to consensus (middle line).

Part 7 Results

Characterisation of D39HY1

7.1 *In vitro* characterisation of D39HY1

7.1.1 Assay of SOD activity

To assess if disruption of *sodA* resulted in reduction of SOD activity, qualitative and quantitative assays of activity were performed using D39HY1 and wild type pneumococcal whole cell extracts.

7.1.1.1 Qualitative gel assay

The qualitative gel activity assays were performed by electrophoresis of whole cell extracts under non denaturing conditions, and then negatively staining for SOD activity with nitroblue tetrazolium (sections 3.14.3 and 3.14.4). The cell extract of *E. coli* QC771 and wild type *S. pneumoniae* were used as controls. Two electrophoretically-distinct activity bands for the wild type were detected (Figure 7.1A, lane 2 and 7.1C, lane 4). As well as their mobility, the intensities of these bands were considerably different. In the mutant strain, however, only the minor activity band was seen (Figure 7.1C, lanes 1, 2 and 3). Clearly, the major band had been inactivated as a result of insertion duplication event.

7.1.1.2 Quantitative SOD assay

As discussed earlier, expression of MnSOD and CuZnSOD is induced upon exposure to superoxide radicals in *E. coli* and *S. typhimirium* (section 4.7). To assess if the level of pneumococcal SOD also was increased in high oxygen tension, pneumococcal cultures were grown on a shaking platform to expose cells to oxygen. It was observed that when wild type pneumococcus were grown aerobically the activity of the major band was significantly higher than in anaerobically grown cultures (Figure 7.2). However no difference was seen in the activity of the minor band. Clearly aerobiosis was increasing the level of the major band.

To determine the amount of pneumococcal SOD and the increase in the activity under aerobic conditions, a quantitative SOD assay (section 3.14.5) was performed with cell extracts prepared from aerobically or anaerobically grown cultures of *S. pneumoniae* wild type and D39HY1

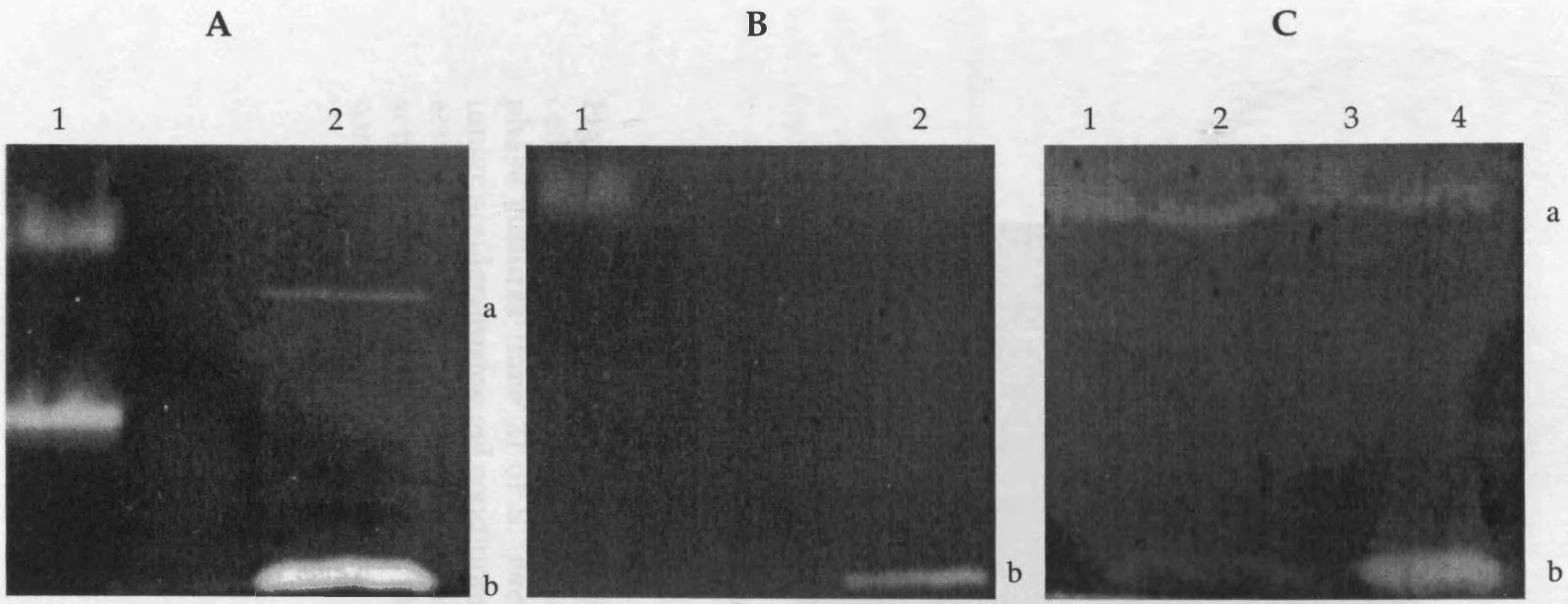


Figure 7.1: Type inhibition assay. Gels for determination of SOD activity were treated (B) or not treated (A) with 5 mM H_2O_2 . The minor activity band of WT lane (A, lane 2) was inhibited by 5 mM H_2O_2 (B, lane 2) as was FeSOD of *E. coli* (B, lane 1). *E. coli* SOD in the absence of H_2O_2 is seen in A, lane 1. **Insertional inactivation of SOD activity.** 390 µg of cell extract of wild type (WT) (C, lane 4) or mutant (D39HY1) (C, lanes 1-3) were run under nondenaturing gel conditions. WT lane had two bands of activity, a minor and a major band whereas D39HY1 had only the minor activity band. The minor activity band of WT (A, lane 2 and C, lane 4) is represented with 'a', while the major activity band is indicated with 'b' in A and B, lane 2, and in C, lane 4.

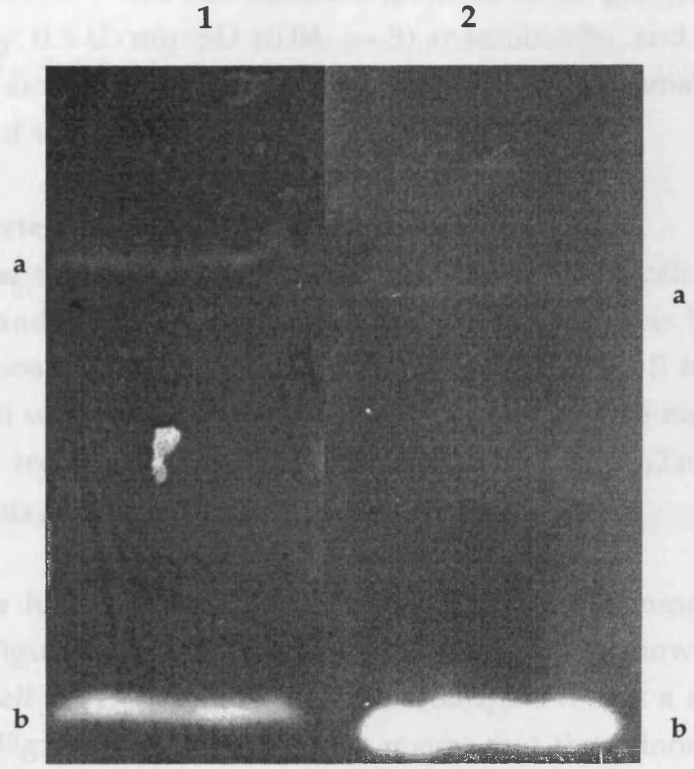


Figure 7.2: Increase in sodA activity with oxygenization. Whole cell extracts prepared from anaerobic (lane 1) or aerobic late log phase cultures (lane 2) of *S. pneumoniae* were electrophoresed under nondenaturing gel conditions and gels were stained for SOD activity. The minor activity band is indicated with **a** and the major activity band is shown with **b** by the side of the figure. Each lane contains 390 µg whole cell extract.

strains . The result showed that the SOD activity of wild type D39 grown anaerobically was 2.4 U/mg of protein (SD ±0.1: n= 3) whereas aerated cultures had 4.1 U/mg (SD ±0.49: n= 3) ($p<0.05$). In contrast, the SOD activity of D39HY1 was not different ($p>0.05$) when grown anaerobically or aerobically: 0.5 U/mg (SD ±0.04: n= 3) anaerobically, and 1.0 U/mg (SD ±0.06: n= 3) aerobically. This activity in D39HY1 presumably represents the activity of the minor band.

7.1.1.3 Characterisation of pneumococcal SOD

To determine the type of pneumococcal SODs, whole cell extracts of *S. pneumoniae* and *E. coli* QC771 were separated by PAGE as before (section 3.14.3) and soaked in the staining solution containing 5 mM KCN or 5 mM H_2O_2. It was observed that SOD activity bands were not affected by 5 mM KCN treatment, indicating the lack of CuZnSOD in the pneumococcus (Crapo *et al.*, 1978) (Figure 7.3).

On the other hand, H_2O_2 inhibited the activity of the minor band in the wild type (Figure 7.1B, lane 2) and D39HY1 (data not shown). The crude *Escherichia coli* FeSOD activity band also disappeared as a result of H_2O_2 treatment (Figure 7.1B, lane 1). This meant that the minor band was an iron co-factored SOD. The major band was partially inhibited by H_2O_2. By exclusion the major SOD in the pneumococcus appears to be MnSOD: MnSOD is not effected by KCN and is either fully or partially resistant to H_2O_2 (Crapo *et al.*, 1978; Martin *et al.*, 1984). In the mutant strain, however, only the minor activity band was seen (Figure 7.1C) confirming that D39HY1 is a *sodA* mutant.

7.1.2 *In vitro* growth characteristics of D39HY1

To investigate the effect of MnSOD inactivation on *S. pneumoniae* colony morphology, D39HY1 was grown on blood agar containing 1 mg spectinomycin under aerobic and anaerobic conditions. The comparision of colony sizes between wild type and D39HY1 showed that the colony size of D39HY1 was comparable to that of wild type under anaerobic conditions. However, aerobically D39HY1 grew slower than parent strain. Under both conditions D39HY1 formed α-haemolytic colonies.

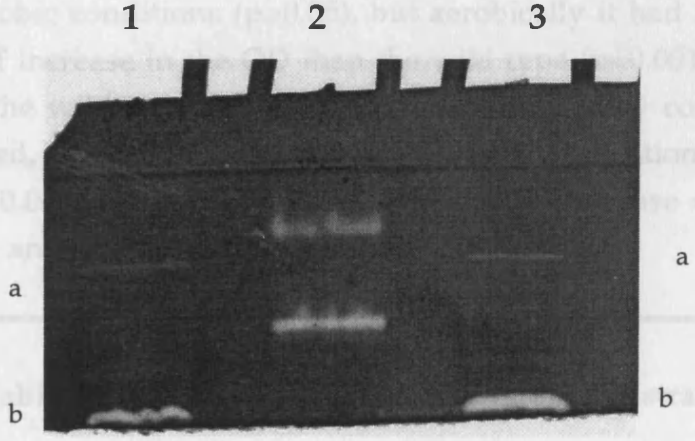


Figure 7.3: Detection of CuZnSOD in cell extract of *S. pneumoniae*. Approximately 390 µg of crude cell extracts of *S. pneumoniae*, grown anaerobically (lane 1) and aerobically (lane 3) were electrophoresed under nondenaturing gel conditions. Crude cell extract of *E. coli* QC771 was also included as a source of Mn (upper band) and FeSOD (lower band) (lane 2). When the gel was treated with 5 mM KCN, it did not effect Fe nor MnSOD activity of *E. coli* QC771 (lane 2). In addition, SOD activity bands, both minor (a) and major (b), of *S. pneumoniae* were not effected by KCN treatment indicating the absence of CuZnSOD in the pneumococcus.

To examine possible effects of the *sodA* mutation, the SOD-deficient strain was also tested in broth culture under aerobic and anaerobic growth conditions. In addition, the redox active compound paraquat was added to the culture medium as a superoxide generator to assess any effect of excess superoxide on the mutant strain.

The growth rate of the wild type strain did not change under aerobic (0.93 h^{-1}, SD ±0.05, n=3), and anaerobic conditions (1.16 h^{-1}, SD ±0.03, n=3) ($p>0.05$) (Figure 7.4A and B). However, D39HY1 had a lower rate of increase aerobically (0.46 h^{-1}, SD ±0.2, n=3) compared to anaerobically (1.06 h^{-1}, SD ±0.06, n=3) ($p<0.001$). D39HY1 had a rate similar to the wild type under anaerobic conditions ($p>0.05$), but aerobically it had a significantly lower rate of increase in the OD than the wild type ($p<0.001$). The rate of increase of the wild type strain was not affected by any concentration of paraquat used, under either aerobic nor anaerobic conditions ($p>0.05$) but addition of 0.001M paraquat affected the rate of increase of D39HY1 in both aerobic and anaerobic conditions ($p<0.001$).

Table 7.1: The growth rates of pneumococcal strains

	Anaerobic Growth		**Aerobic Growth**	
Concentration of paraquat, M	W T	D39HY1	W T	D39HY1
0	1.1 h^{-1}, ±0.03	1.0, h^{-1}, ±0.06	0.9 h^{-1}, ±0.05	0.4, h^{-1}, ±0.2
0.001	1.0 h^{-1}, ±0.06	0.4 h^{-1}, ±0.01	0.9 h^{-1}, ±0.16	0.1, h^{-1}, ±0.03
0.0001	0.9 h^{-1}, ±0.17	0.7, h^{-1}, ±0.09	0.9 h^{-1}, ±0.13	0.2, h^{-1}, ±0.09

Streptococcus pneumoniae wild type (WT) or D39HY1 were tested under aerobic and anaerobic conditions in the presence of 0.001 and 0.0001 M paraquat in BHI broth. The values represent the mean SD± of three experiments and are derived from curves shown in Figure 7.4.

In addition to growth characteristics in complex medium, the growth of the mutant strain was investigated in defined medium (Figure, 7.5), because defined medium lacks superoxide scavengers which are present

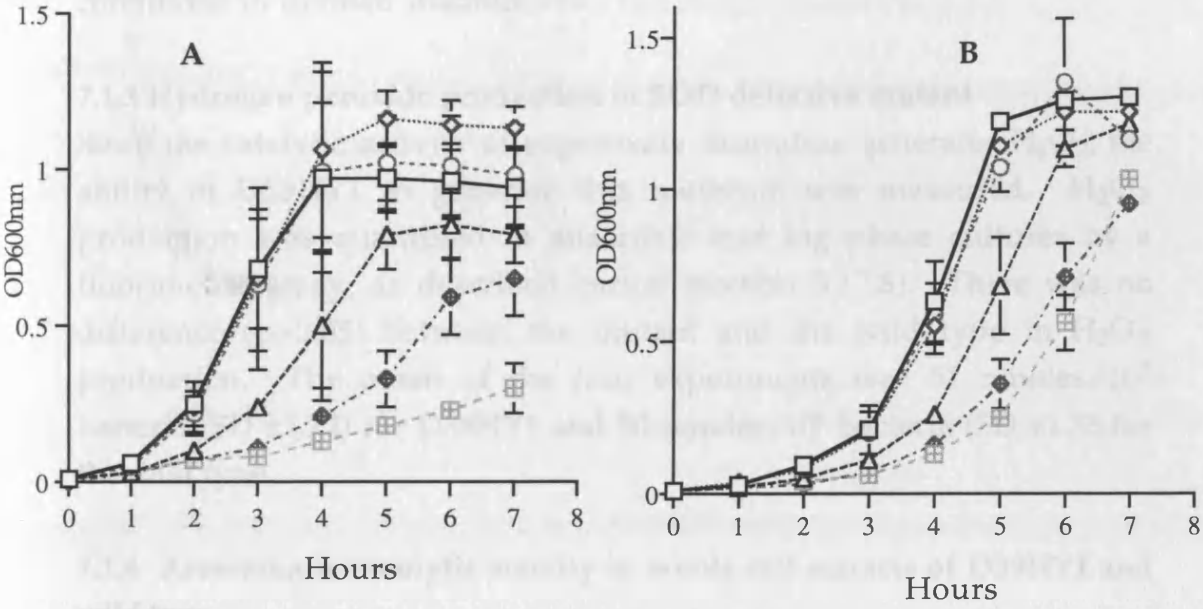


Figure 7.4: A. Aerobic growth of wild type (WT) and *sod* mutant strain (D39HY1). Strains and concentrations of paraquat are represented with symbols as follows: (—□—) WT, no paraquat, (···◇···) WT, 0.001M paraquat, (----○----) WT, 0.0001M paraquat, (----△----) D39HY1, no paraquat, (---⊞---) D39HY1, 0.001M paraquat, (---◆---) D39HY1, 0.0001M paraquat. Statistical analysis of growth rates (ANOVA followed by Tukey-Kramer multiple comparisons test) indicated that paraquat did not effect the WT growth at any concentration used ($p>0.05$). However, a significant difference was found between WT and D39HY1 even without paraquat under aerobic conditions ($p<0.001$). Paraquat at 0.001M significantly slowed the growth rate of the mutant strain further ($p<0.001$) but not at 0.0001 M. **B. Anaerobic growth.** No significant difference was found between WT and D39HY1 when grown without paraquat ($p>0.05$). Paraquat did not have any effect on anaerobic growth of WT at any concentration used ($p>0.05$). Paraquat at a concentration of 0.001M and 0.0001M inhibited the growth rate of the mutant strain ($p<0.001$ and $p<0.05$, respectively). Each curve represents the mean of three experiments. SEM is represented with bars.

in complex medium. Anaerobically, the wild type (0.68 h^{-1}, SD ±0.16, n=3) and D39HY1 (0.4 h^{-1}, SD ±0.1, n=3) showed similar rates of increase of OD_{600} ($p>0.05$), but D39HY1 strain was unable to grow under aerobic conditions in defined medium.

7.1.3 Hydrogen peroxide production in SOD defective mutant

Since the catalytic activity of superoxide dismutase generates H_2O_2, the ability of D39HY1 to generate this molecule was measured. H_2O_2 production was quantified in anaerobic mid log phase cultures by a fluorimetric assay, as described earlier (section 3.17.8). There was no difference ($p>0.05$) between the mutant and the wild type in H_2O_2 production. The mean of the four experiments was 51 nmoles/10^7 bacteria (SD ±1.72) for D39HY1 and 50 nmoles/10^7 bacteria (SD ±1.35 for the wild type.

7.1.4 Assessing haemolytic activity in whole cell extracts of D39HY1 and wild type

SOD was found to be important in the regulation of certain virulence factors in some bacteria, for example cyclase-hemolysin and pertactin in *B. pertussis* (Khelef *et al.*, 1996). In order to determine if SOD had an effect on pneumolysin, haemolytic activity in whole cell extracts of D39HY1 was measured using sheep red blood cells (section 2.8). The results showed that D39HY1 extract contained less haemolytic activity, 7.8 HU/µg protein (SD ±1.1, n=3), than wild type, 15 HU/µg protein (SD ±1.7, n=3) ($p<0.05$).

7.2 *In vivo* studies

In order to investigate the effect of the *sodA* mutation *in vivo*, the mutant strain was tested in mouse models of bacteraemia and pneumonia.

7.2.1 Determination of median survival time

The median survival time of 20 mice was determined after intranasal infection with D39HY1 or wild type, to investigate if the *sodA* mutation had any effect in the ability of *S. pneumoniae* to cause disease. Infected mice were observed for seven days and the development of the clinical symptoms were recorded (Figure 7.6). Around 19 h post infection, individual mice within the wild type infected group began to show

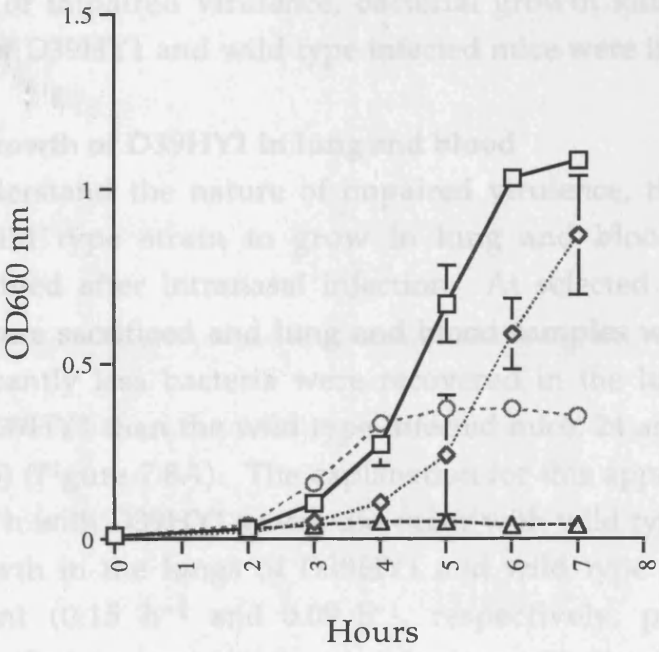


Figure 7.5: The growth of the mutant (M) and the wild type (WT) in defined medium. The mutant grew at a comparable rate to wild type, anaerobically ($p>0.05$). Under aerobic conditions, however, the mutant did not grow. Each point is the mean of three experiments. Strains and growth conditions are represented with symbols: (—□—) WT, anaerobic, (----○----) WT, aerobic, (·····◇·····) D39HY1, anaerobic, (----△----) D39HY1, aerobic.

symptoms (starry coat) and all showed symptoms by 45 h. However, none of the D39HY1-infected group showed any sign of illness until 45 h post infection at which point 3 mice in the wild type infected group were moribund. Within seven days, 14 mice became moribund (the end point of the assay) in each group. Statistical analysis showed that D39HY1 infected group survived significantly longer (107 h) than wild-type infected mice (50 h) ($p<0.0001$) (Figure 7.7). To be able to understand the nature of impaired virulence, bacterial growth kinetics in the lung and blood of D39HY1 and wild type infected mice were investigated.

7.2.2 Growth of D39HY1 in lung and blood

To understand the nature of impaired virulence, the ability of D39HY1 and wild type strain to grow in lung and blood of MF1 mice was determined after intranasal infection. At selected times after infection, mice were sacrificed and lung and blood samples were taken for culture. Significantly less bacteria were recovered in the lungs of mice infected with D39HY1 than the wild type-infected mice, 24 and 48 h after infection ($p<0.05$) (Figure 7.8A). The explanation for this appears to be a lag in the first 24 h with D39HY1 which did occur with wild type. After 24 h the rate of growth in the lungs of D39HY1 and wild type was not significantly different (0.15 h^{-1} and 0.09 h^{-1}, respectively; $p>0.05$). D39HY1 is apparently less invasive than wild type, with the mutant not recovered from blood at 24 h in contrast to the wild type (Figure 7.8B). However, by 48 h, the numbers of D39HY1 in the blood were similar to wild type and by 72 h the number of D39HY1 in blood had reached around 10^9/ml. The maximum growth rates for D39HY1 and wild type were the same (0.51 h^{-1}).

7.2.3 Intravenous infection of mice

In addition to intranasal challenge, the virulence of mutant strain was tested after intravenous infection of mice to check if the diminished virulence of D39HY1 was dependent on the route the bacteria had been administered. Initially, the number of bacteria required for an ED_{100} was assessed by following symptoms for 7 days after infection. All mice (4) survived 50 cfu but each of two mice in each group infected with 500 and 1000 cfu became moribund. As a result, it was decided to use $1X10^4$ cfu for infections. Following i.v. infection with $1X10^4$ cfu wild type or D39HY1, blood cfu in the blood of both groups were found to be similar: growth rates were 0.22 h^{-1} and 0.17^{-1}, respectively ($p>0.05$) (Figure 7.9A). There

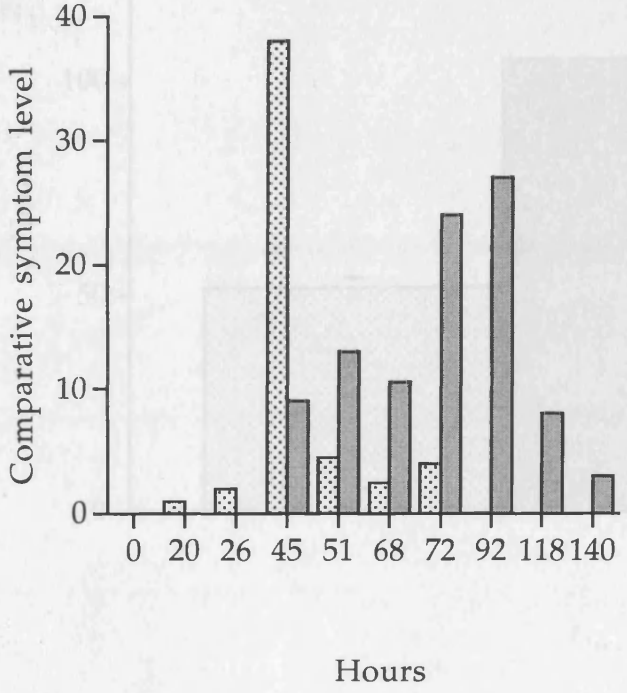


Figure 7.6: The development of symptoms during infection. The symptoms were recorded after intranasal infection of 20 mice with wild type (▩) or D39HY1 (■). The clinical symptoms were graded as follows: starry coat 1 point, hunched 2 points, lethargic 3 points, moribund 4 points. If the symptoms were mild (++) an extra 0.5 point was added, if severe (+++) an extra 1 point was added.

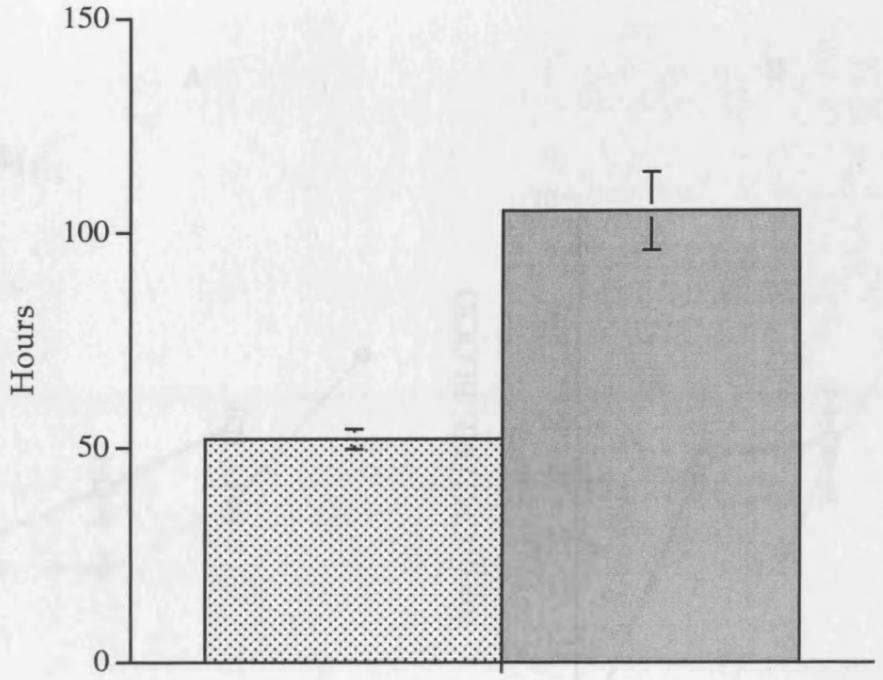


Figure 7.7: Median survival time of mice infected with D39HY1 and wild type *S. pneumoniae*. Groups of 20 mice were challenged either with $1X10^6$ D39HY1 ( ) or wild type strain ( ). The mice were observed for survival for seven days. Mice challenged intranasally with D39HY1 survived twice as long than the wild type infected mice. SEM for each group is indicated with bars.

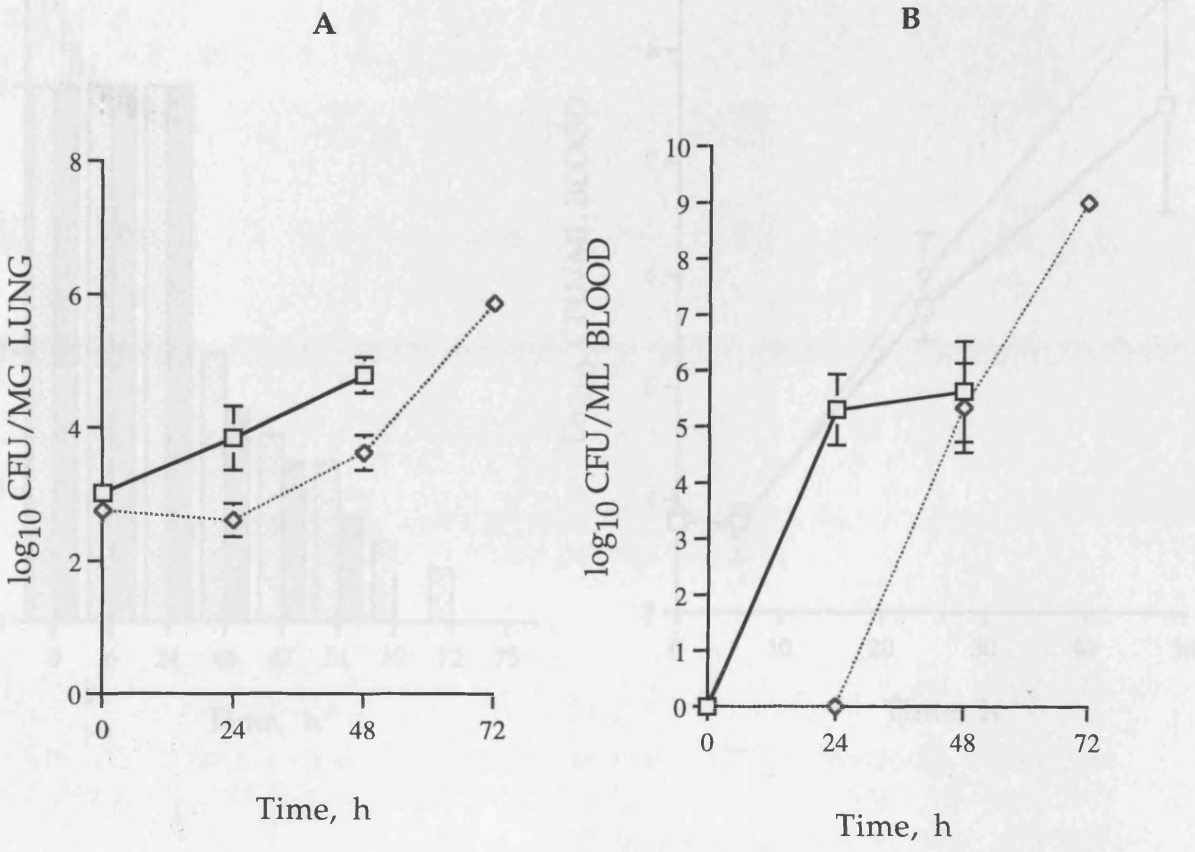


Figure 7.8: Time course of bacterial growth in the lungs (A) and blood (B) of mice infected with D39HY1 or the wild type. Each point represents mean log counts from 5 MF1 female mice, but for D39HY1 72 h is 3 mice. Symbols represent mice infected with WT (—□—) or D39HY1 (·····◇·····). SEM is represented by bars.

was also no difference seen in the median survival time of the mice infected with D39HY1 (46 h) and the wild type (45 h) ($p>0.05$) (Figure 7.9B).

7.2.4 Examination of D39HY1 for revertants

To investigate if *in vivo* reversion of the *sod* mutation was taking place through excision of the spectinomycin gene, a Southern hybridisation was performed using radiolabelled spectinomycin resistance gene cassette as the probe to identify spectinomycin resistance cassette gene in bacteria isolated from tissues. To prepare chromosomal DNA, D39HY1 recovered from lung of mice after 24 h infection and grown with and without spectinomycin. Wild type chromosomal DNA was included as the negative control. The result showed that the probe hybridised to a single band for D39HY1 with or without spectinomycin selection (Figure 7.10, lane 2 and 3) but not for wild type (Figure 7.10, lane 1), showing that the spectinomycin resistance gene remained in D39HY1 during infection.

Although it was shown by Southern blot hybridisation that mutation was stable in the majority of bacteria, a percentage of the bacteria recovered from the lungs still might have lost the spectinomycin resistance cassette during infection. To investigate this, the bacteria isolated from lung and blood samples of mice infected with D39HY1 (48 h after intranasal infection) were grown with and without spectinomycin selection. A higher number of colonies without selection would indicate the excision of spectinomycin from a proportion of the population. The results showed that the number of bacteria in blood after 48 h infection, when grown with spectinomycin was $\log_{10}$ 5.07/ml (SD ±0.9, n=5) and without spectinomycin was $\log_{10}$ 4.98/ml (SD ±0.7, n=5) ($p>0.05$). The numbers in the lung after 48 h infection were $\log_{10}$ 3.30/ml (SD ±0.36, n=5) with selection and $\log_{10}$ 3.43/ml (SD ±0.5, n=5) without selection ($p>0.05$).

7.2.5 Histopathologic examination of lung tissue

To understand the nature of the pathologic changes that were taking place during infection, lung samples of D39HY1 and wild type infected mice were analysed for macroscopic and microscopic changes. These studies were done in collaboration with Dr. Aras Kadioglu, Microbiology & Immunology Department, University of Leicester, Leicester.

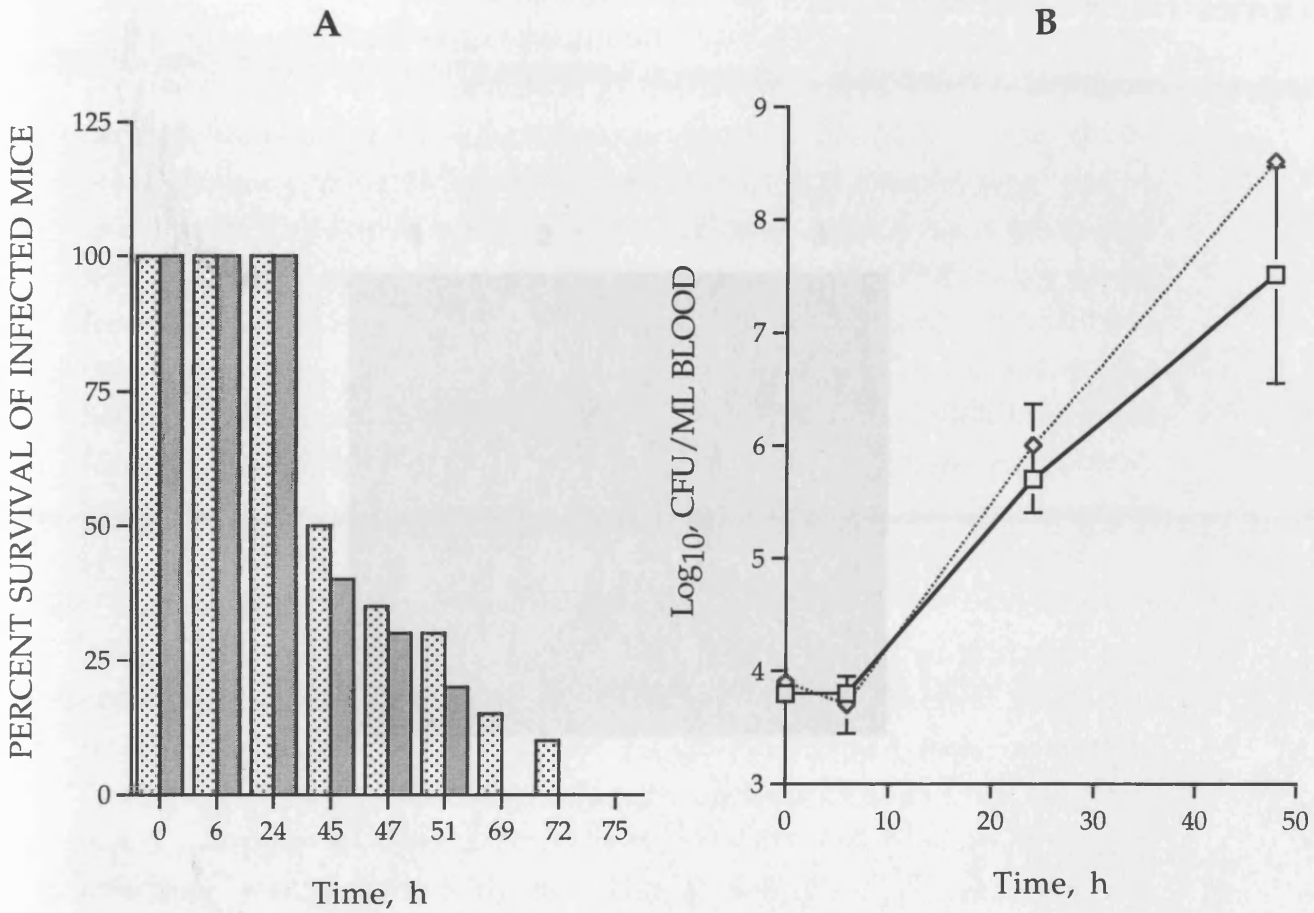


Figure 7.9: Survival and blood growth of mice infected intravenously with D39HY1 or wild type. A. The survival of twenty mice is shown for infections with 10^4 WT CFU or 10^4 D39HY1 CFU. No difference ($p>0.05$) in percentage survival was seen when data were analysed by two-sample rank test followed by Mann Whitney U test. Symbols indicate mice infected with (⊡) WT or (■) D39HY1. **B.** The growth of WT and D39HY1 in the blood of mice is shown for groups of five mice for each time point except at 48 h when D39HY1 was represented with 1 mouse, and WT was represented with 2 mice (B). Symbols indicate mice infected with (—□—) WT or (·····◇·····) D39HY1.

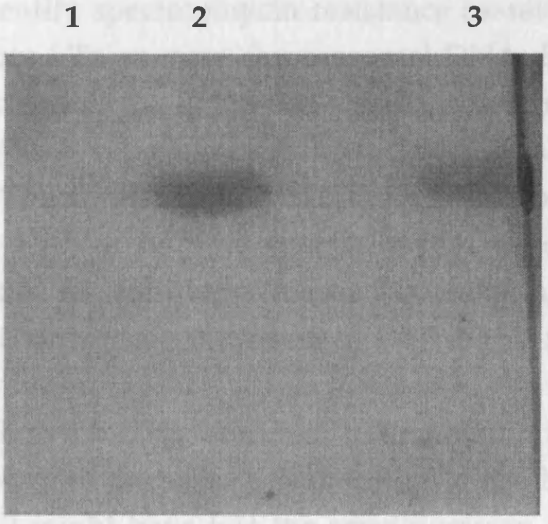


Figure 7.10: The presence of spectinomycin cassette in the chromosome of bacteria recovered from lungs 24 h after infection. Chromosomal DNA was prepared from bacteria grown with (lane 2) or without (lane 3) spectinomycin *in vitro*. Lane 1 represents wild type chromosomal DNA grown without spectinomycin. The probe used was radiolabelled spectinomycin gene.

Macroscopically no difference was observed in lung samples of D39HY1 and wild type-infected mice after 24 and 48 h infection. The lesions were of haemorhagic appearance and a few petechies.

Histopathologic changes of lung tissue during infection were examined semiquantitatively after haemotoxylin and eosin staining, as described in section 3.16 (Table 7.2). Lung sections of mice prepared immediately after infection with WT were used as the controls (Figure 7.11). The lung tissue at this time point did not show any sign of inflammatory changes. The lung sections of mice infected with wild type at 24h after infection exhibited typical bronchopneumonia, which presented itself as areas of inflammation with inflamed bronchioles and consolidation of tissue in perivascular areas, alongside non-inflamed bronchioles and relatively healty tissue (Figure 7.12A). The more detailed analysis of the tissue sections at this time point also indicated a moderate cellular infiltration around bronchioles (Figure 7.12B) as well as section as a whole (Figure 7.12A) .

Table 7.2: Histopathologic changes in the lungs of mice infected with wild type *S. pneumoniae* (WT) or D39HY1.

Histopathologic changes	WT 24 h	W T 48 h	D39HY1 24h	D39HY1 48 h
Cellular infiltration over whole section	2.5	4	2	3
Cellular infiltration around bronchioles	3	3.75	3	5
Bronchiole wall thickining	2.25	3.5	2.3	3

Histopathologic changes were scored following haemotoxylin eosin staining of cryostat sections of mice lung samples. The symptoms were scored separately by one observer, blind to the identity of samples. Scoring was by a semiquantitative scale ranging from 0 (no inflammation), 1 (slight), 2 (mild), 3 (moderate), 4 (substantial) to 5 (severe). The results represent the mean value, derived from the examination of lung tissue sections (at least 4) from two animals infected intranasally with either $1X10^6$ *S. pneumoniae* (WT) or D39HY1.

By 48 h the severity of inflammatory cell infiltrate in perivascular and peribronchiole areas increased and focal areas of consolidation became larger and more diffuse (Figure 7.13A). Bronchioles were filled with exudate and wall tickening increased (Figure 7.13B). Formation of fibrin nets was also observed at this time point (Figure 7.13B).

Examination of lung tissue sections of mice infected with D39HY1 showed less inflammation and cellular infiltration in perivascular areas between infected bronchioles, as compared to wild type infected mice at both time points (Figure 7.14A, B, and 7.15A, B). However, despite this, some bronchioles still exhibited levels of inflammation comparable to wild type infected mice at 24 h (Figure 7.14A). By 48 hour the severity of inflammatory changes was increased (Figure 7.15A, B). A dense inflammatory cell infiltrate was seen particularly around peribronchiole areas such that the cell density was substantially heavier as compared to WT infected mice at the same time point (Figure 7.15A, B). Unlike wild type infected mice, this infiltration was not diffuse and did not extend into the perivascular areas (Figure 7.15A, B).

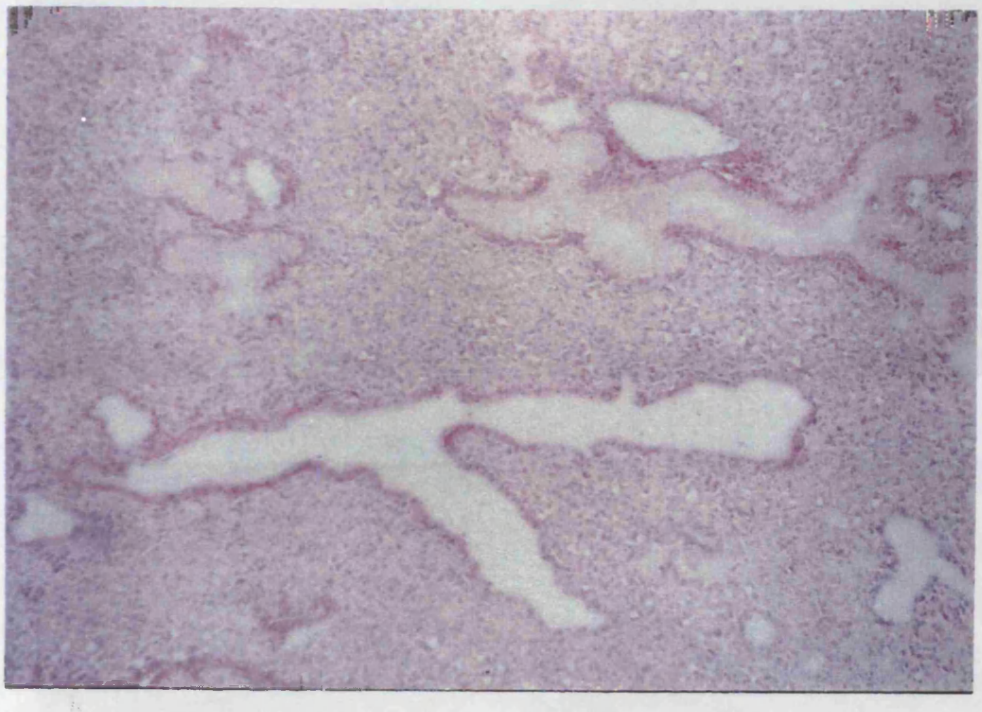
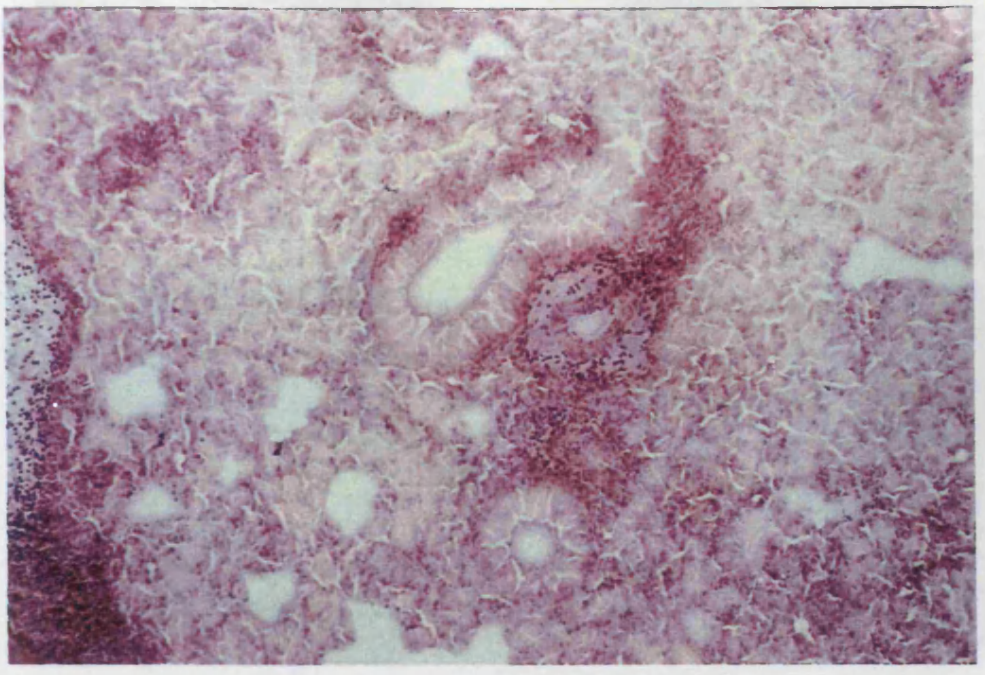


Figure 7.11: Examination of lung tissue for histopathologic changes. Haematoxylin and eosin staining of cryostat cut frozen section (20μm) of a mouse lung immediately after infection with $1X10^6$ wild type *S. pneumoniae* (magnification, x60) was analysed by light microscopy, (with permission of Dr. Aras, Kadioglu)

A



B

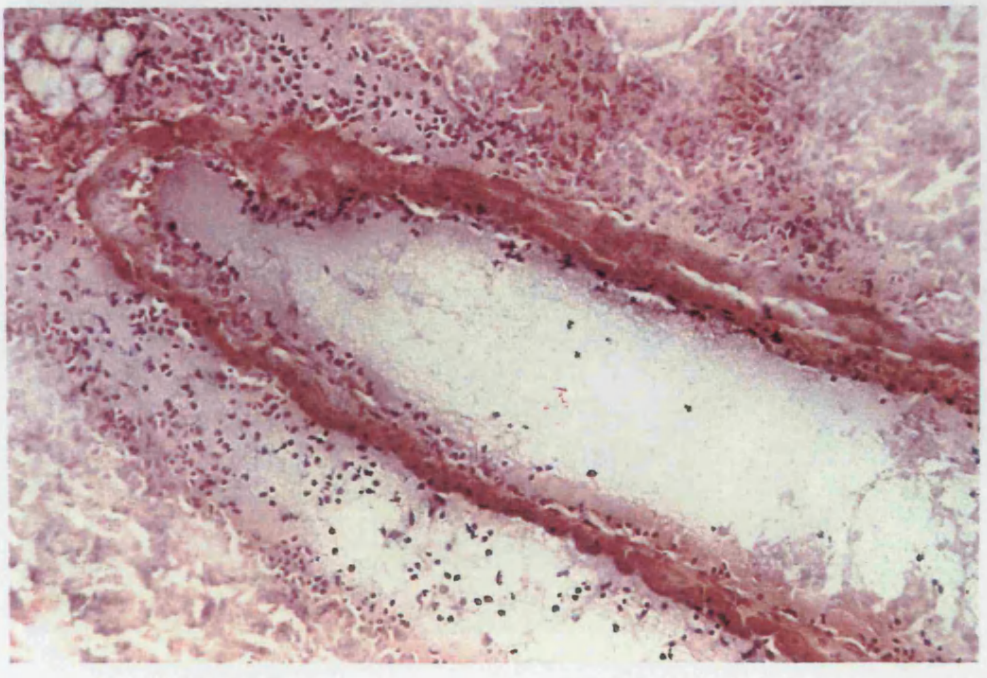
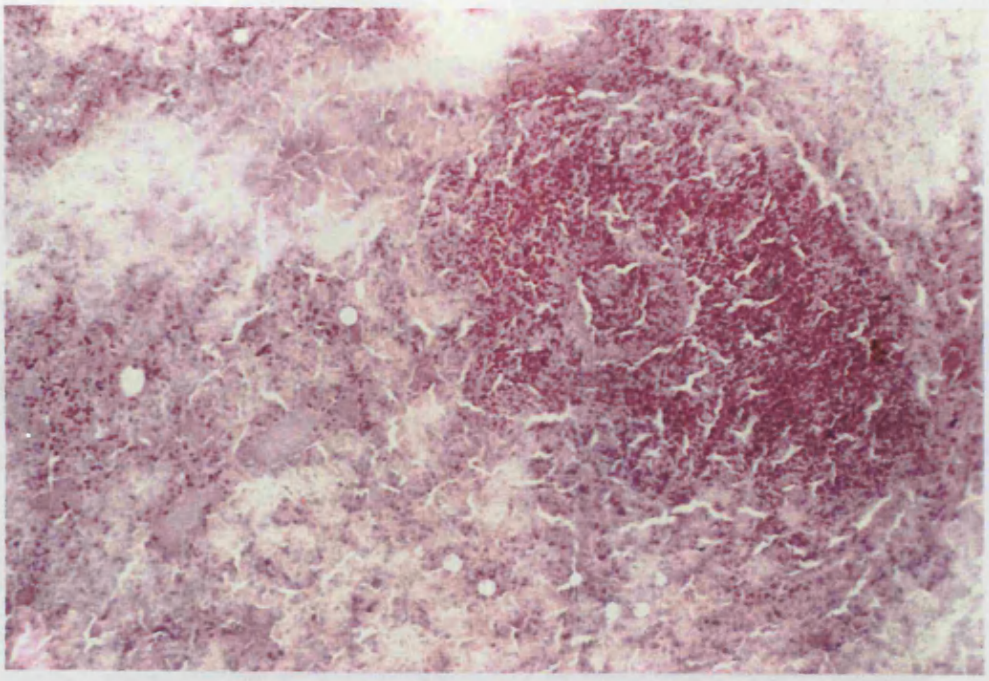


Figure 7.12: Examination of lung tissues for histopathologic changes 24 h after infection with wild type *S. pneumoniae*. Cryostat cut frozen sections (20μm) of lung tissues from mice infected with $1X10^6$ wild type *S. pneumoniae* were prepared. Sections were stained with haematoxylin and eosin and analysed by light microscopy, (magnification: A, x100 and, B, x150).

A



B

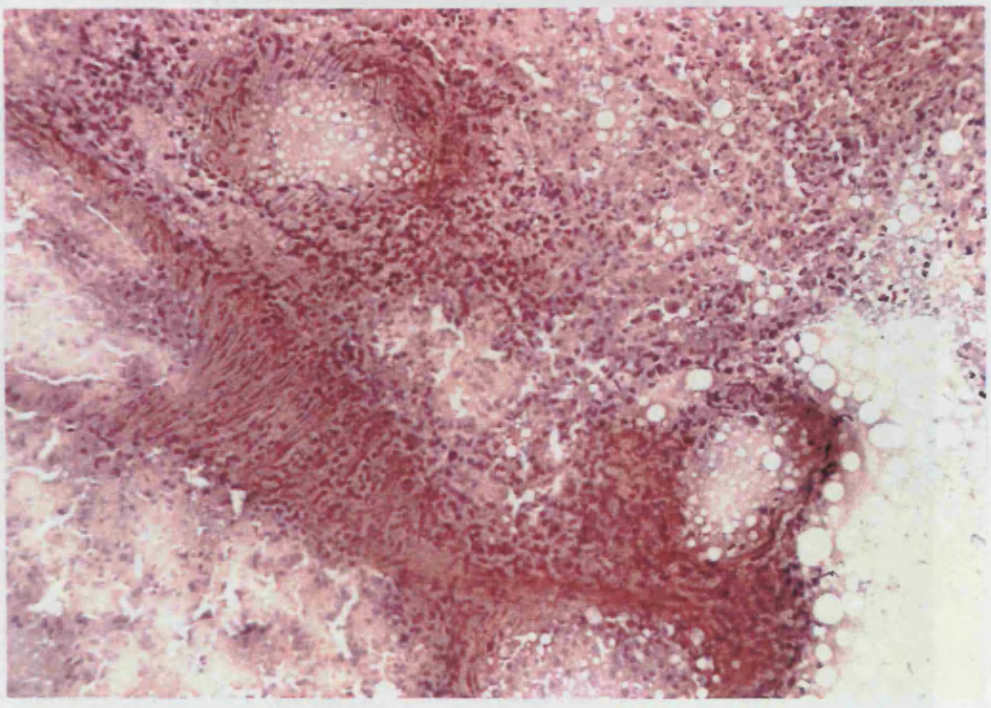
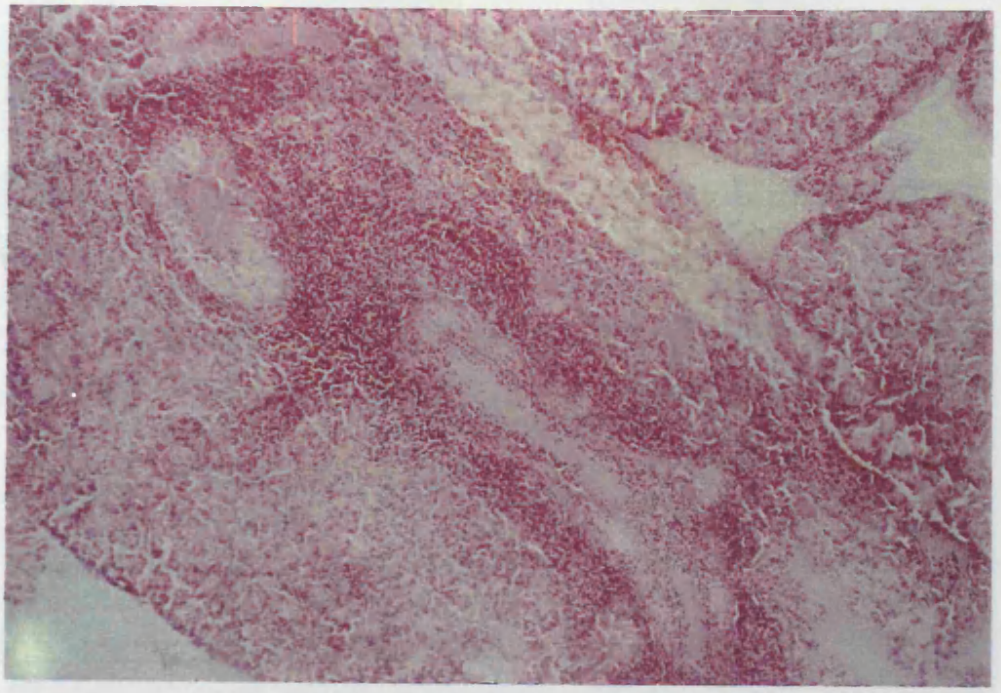


Figure 7.13: Examination of lung tissues for histopathologic changes 48 h after infection with wild type *S. pneumoniae*. Cryostat cut frozen sections (20μm) of lung tissues from mice infected with $1X10^6$ wild type *S. pneumoniae* were prepared. Sections were stained with haematoxylin and eosin and analysed by light microscopy, (magnification: A, x125 and, B, x150).

A



B

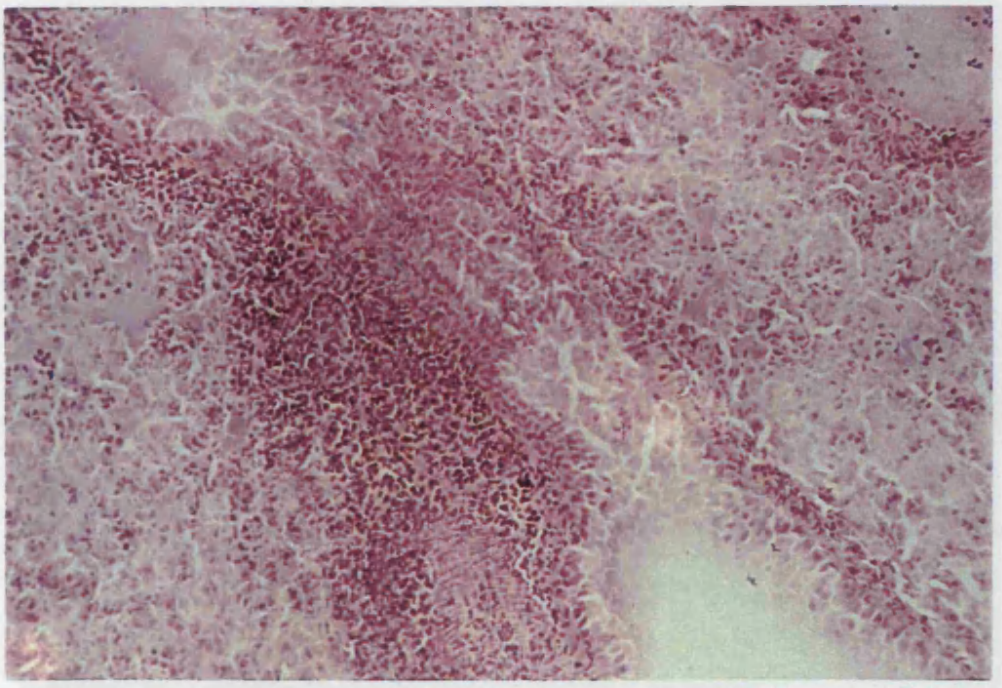
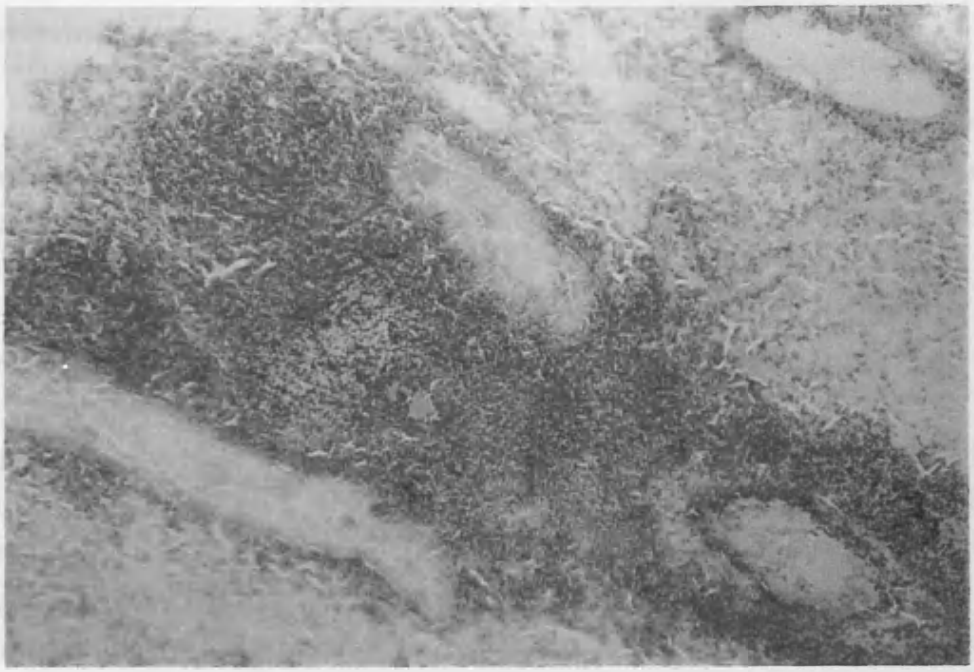


Figure 7.14: Examination of lung tissues for histopathologic changes 24 h after infection with *S. pneumoniae* D39HY1 strain. Cryostat cut frozen sections (20μm) of lung tissues from mice infected with $1X10^6$ D39 were prepared. Sections were stained with haematoxylin and eosin and analysed by light microscopy, (magnification: A, x80 and, B, x140).

A



B

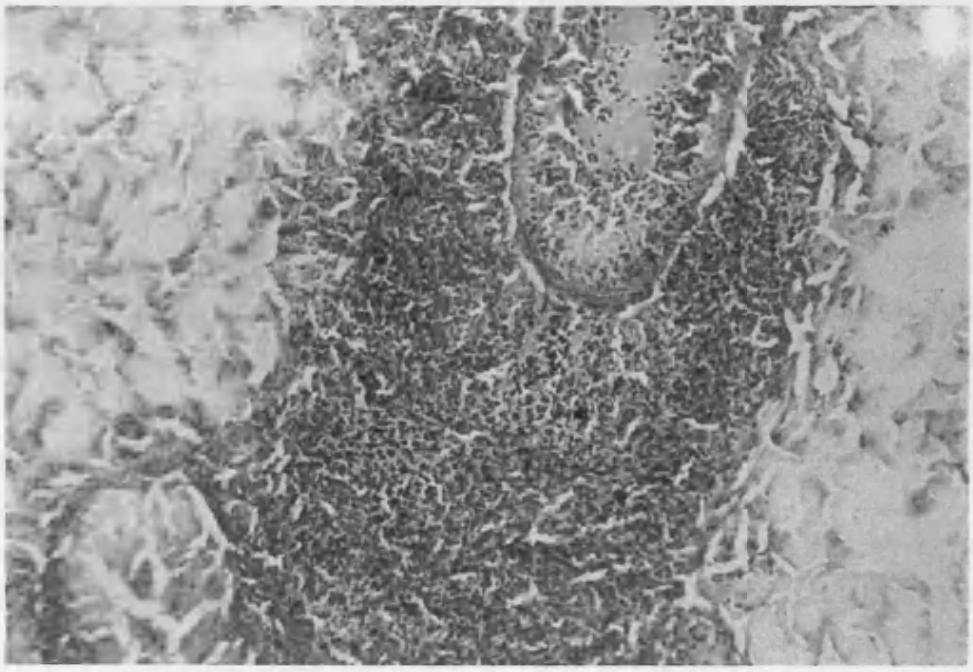


Figure 7.15: Examination of lung tissues for histopathologic changes 48 h after infection with *S. pneumoniae* D39HY1 strain. Cryostat cut frozen sections (20μm) of lung tissues from mice infected with $1X10^6$ D39HY1 strain were prepared. Sections were stained with haematoxylin and eosin and analysed by light microscopy, (magnification: A, x80 and, B, x150).

Part 8

Discussion

The aims of this study were two fold: *i.* isolation of a pneumococcal *sod* gene, *ii.* construction and characterisation of an isogenic *sod* mutant strain of the pneumococcus, to test the role of SOD in virulence of this organism.

Characterisation of pneumococcal SODs

As it was revealed by quantitative gel assays (section 7.1.1.1), the pneumococcus has two types of SOD activity, a MnSOD as the major fraction and a FeSOD as a minor fraction. This was consistent with the suggestion that facultative anaerobes contain either Mn, or Mn and Fe cofactored SODs (Bannister *et al.*, 1987). Identification of the minor band as FeSOD was on the basis its sensitivity to inhibition by H_2O_2. The occurrence of this band from D39HY1 confirmed that it was a product of a separate gene and not due to Fe co-factoring the SodA product. The major band was identified as a MnSOD because of the extent of its resistance to H_2O_2 and on the basis of the gene sequence. MnSODs are classically described as being completely resistant to H_2O_2, but the pneumococcal SOD appeared to be slightly senstive to H_2O_2. This might be because a proportion of the SOD in the major band is in the form of a hybrid SOD, co-factored in the main by Mn but with some Fe also. Such a hybrid would have limited sensitivity to H_2O_2 (Clare *et al.*, 1984). Against this idea is the expectation at a hybrid would have a different pI from the other two SODs and appear as a third band (Beyer and Fridovich, 1991).

When attempt was made to prove the presence of two genes by genetic means, either employing radiolabelled sod_{int} fragment to detect *sod* genes in pneumococcal chromosome (section 6.1) or analysis of the pneumococcal genome using a consensus sequence common in both Fe and Mn containing SODs, contradictory results were obtained.

In order to determine the number of chromosomal copies of *sod* genes in the pneumococcal genome, a Southern blot hybridisation was performed in which radiolabelled sod_{int} fragment was used as the probe. The result

showed that only one hybridisation signal was detected (section 6.1). Obtaining only one hybridisation signal was unexpected. Because, although sod_{int} by comparison to other *sod* genes exhibits the characteristics of MnSOD (Poyart *et al*, 1995), it also bears a 24-base pair consensus sequence shared by both Fe and MnSODs, which is GACGTTTGGGAACATGCTTACTAC. A similarity search indicated that the consensus is 95% identical to *Bacteroides fragilis sodB* over 23 nucleotides and 100% identical to *Campylobacter jejuni sodB* over 17 nucleotides. Therefore, the probe should have hybridised to the pneumococcal chromosome twice; one for *sodA* and the other for *sodB*, to be in line with gel activity assays.

The pneumococcal gene encoding for FeSOD was also searched by examination of the pneumococcal genomes sequence available through early release from The Institute for Genomic Research through NCBI at www.ncbi.nlm.nih.gov and Zeneca Pharmaceuticals. The common consensus sequence for both sodA and sodB, DVWEHAYY was used. In order to increase the chances of regions in the pneumococcal genome matching with the consensus sequence , search conditions were set in a way to maximise the likelihood of a match, such as decreasing the stringency conditions. The result showed that one region in the pneumococcocal genome, where *sodA* resides, had a perfect match with the probe (Figure 8.1A). In addition to this perfect match, several other regions were also found to have homology with the consensus sequence. However these regions were over short amino acid sequences; the highest match was 42% identical and 84% chemically similar over 7 amino acids (Figure 8.1B). When this 7 amino acid long sequence from the pneumococcus was used for a blast search, no known protein sequences were identified in databases.

Besides DVWEHAYY, a new consensus sequence unique for Fe cofactored SODs, FNNAAQVWNHTF, derived from nine different organisms (Figure 8.2A), was used to the search pneumococcal genome. It was expected that this sequence should have been found present in a region outside of the *sodA* gene in the pneumococcal genome. Although several regions had homology with *sodB* consensus sequence, these were over short amino acid sequences, despite the use of low stringency search settings. The best continguous region in the pneumococcal genome that

had identity with the consensus was only over 5 amino acids (Figure 8.2B).

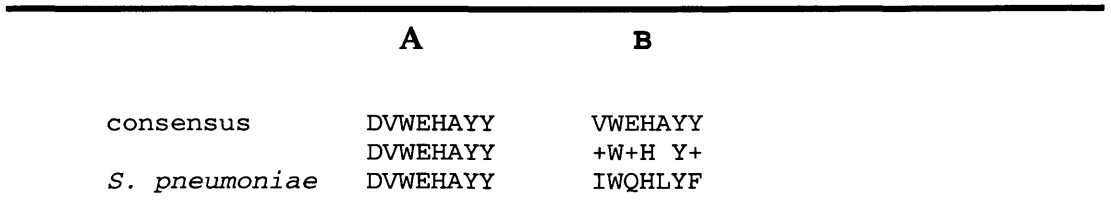


Figure 8.1: Search of the pneumococcal genome for the presence of *sodB* gene. In **A** the perfect match with the consensus is shown. In **B** the best perfect match which is in a region outside of *sod*A gene in the pneumococcal genome is indicated. The identical and chemically similar (+) residues are shown in the middle lane.

In addition, several other regions also showed homology with the probe over short amino acid sequences, e.g., 41% identity and 66% chemical similarity over 12 amino acids (Figure 8.2C) and 54% identity and 54% homolgy over 11 amino acids (Figure 8.2D). The regions in the pneumococcal genome identified to have homology with the probe were used in the blast search. However, no known proteins matched with these sequences. Therefore, the significance of the homology, particularly, for the best continguous region, WNHTF, should be checked by designing a primer based on these residues, and then this primer can be used in a PCR in combination with a primer based on DVWEHAYY. The successful amplification reaction is expected to synthesise approximately 120 bp long internal fragment. Alternatively another sequence known to be well conserved among FeSODs, FGSGWAW (Nakayama, 1991) also can be used for the isolation of *sodB* gene in the pneumococcus. When the presence of FGSGWAW was checked in the pneumococcal genome, one region, within *sodA*, showed perfect identity with the consensus (Figure 8.2E). In addition another region outside of the *sodA* gene in the pneumococcal genome was also 83% identical and 99% homologous over 6 amino acids (Figure 8.2F). As FGSGWAW consensus sequence is also present in MnSOD, the use of a primer based on FGSGWAW in combination with a sequence based on DVWEHAYY

A.

```
sodB 1    EFENLSLEEI VK....KSSG .....GVFNN AAQVWNHTFY WNSLSP....
sodB 2    EFESMTLEEI IM....KAKG .....GIFNN AAQVWNHTFY WHSMSP....
sodB 3    AFEGKSLEEI IR....SSEG .....GVFNN AAQVWNHTFY WNCLAP....
sodB 4    ELAEKSLEEI IK....TSTG .....GVFNN AAQVWNHTFY WNCLAP....
sodB 5    EFEGKSLEEI VK....SSSG .....GIFNN AAQVWNHTFY WNCLSP....
sodB 6    EFEGKTLEEI VK....SSSG .....GIFNN AAQVWNHTFY WNCLSP....
sodB 7    EFEGKTLEEI VK....TSSG .....GIFNN AAQVWNHTFY WNCLAP....
sodB 8    PMESLSLEDV IK....QSFG DSSKVGVFNN AAQVWNHTFF WNCLKA....
```

	B.	C.	D.
consensus	WNHTF	FNNAAQVWNHTF	NNAAQVWNHTF
	WNHTF	FN+ Q W+H +	NN WNH F
S. pneumoniae	WNHTF	FNSIIQFWHHFY	NNR*SEWNHIF

	E.	F.
consensus	FGSGWAW	GSGWAW
	FGSGWAW	GSGW+W
S. pneumoniae	FGSGWAW	GSGWSW

Figure 8.2: Investigation of *sodB* gene in *S. pneumoniae* genome. A. The alignment of amino acid sequences of Fe-containing SODs of *B. pertussis* (sodB 1) (SWISS-PROT: P37369), *L. pneumophila* (sodB 2) (SWISS-PROT: P31108), *E. coli* (sodB 3), (SWISS-PROT: P09157), *Photobacterium leiognathi* (sodB 4) (SWISS-PROT: P09213), *Pseudomonas ovalis* (sodB 6) (SWISS-PROT: P09223), *Pseudomonas putida* (sodB 7), (SWISS-PROT: P77928), *Pseudomonas aeroginosa* (sodB 7) (SWISS-PROT: P53641) and *Plectonema boryanum* (sodB 8) (SWISS-PROT: P50061) were performed using the GCG PILEUP program. The underlined amino acids represent the consensus sequence. The consensus in **A** used to analyse the pneumococcal genome for the presence of *sodB*, shown in **B, C**, and **D.** The search done using FGSGWAW consensus sequence is shown in **E** and **F.** The regions of homology between the consensus sequences and *S. pneumoniae* genome are given in the middle line in B, C, D, and F.

in a PCR may not give a conclusive answer for the presence of *sodB* gene in the pneumococcus.

Attempts to demonstrate the presence of *sodB* in *S. pneumoniae* by Southern blotting and analysis of the whole pneumococcal genome did not produce a clear cut answer. Therefore, the presence of the band identified as FeSOD in qualitative gel assays remains to be explained. Firstly, it can be suggested that the band in question was an artefact. However, this was unlikely, because this band was constantly present in all the gel assays performed and was not seen in the control lanes. In addition if it was an artefact it may not be expected to disappear with

H_2O_2 treatment. Secondly, it can be suggested that the minor fragment identified as FeSOD is, in fact, encoded by *sodA* and is formed as a result of some modifications to the protein that occur posttranslationally. It was reported (Hassan and Schrum, 1994) that the biosynthesis of MnSOD is influenced at the posttranslational level by both iron and manganese. These metals compete for metal site in apo-SodA, resulting in formation of three forms of protein: Mn_2-SodA (fully active), Mn-Fe-SodA (partially active) and Fe_2-SodA (totally inactive). However, this explanation is not a satisfactory one because when the gene coding for sodA was inactivated, in addition to major MnSOD, the minor activity band representing FeSOD should have disappeared.

Alternatively, the band of activity identified as FeSOD may in fact be a protein showing activity similar to SOD but encoded by a gene different from *sod*. In a recent research, Lehmann and coworkers (1996) in an attempt to isolate *sod* gene from *Clostridium perfringens* purified a protein exhibiting SOD activity, identified following gel activity assay. However, when eventually they isolated the corresponding gene it was found that the gene showed strongest homology to a gene for the protein rubrerythrin, a non-heme, non-sulfur iron protein whose biological function has not been completely described yet (Lehman *et al.*, 1996). These researchers demonstrated rubrerythrin-dependent H_2O_2 production, the product also generated as a result of SOD activity. The pneumococcal genome was searched for the presence of rubrerythrin using *C. perfringens* rubrerythrin gene sequence, as it is the only Gram positive bacterium known to possess this gene. A region of

pneumococcal genome showed 46% identity and 59% homology over 30 amino acids, the total length of gene encoding for rubrerythrin is 587 nucleotides (Figure 8.3).

```
S. pneumoniae    KEHAKRFYKFLKDDLQGEAVEINAAYPVEL
consensus        KE+ K FYKFL  DL    ++ +AA  V L
C. perfringens   KENLKLFYKFLLFDLDHTLLDFDAAEDVAL
```

Figure 8.3: Search for ruberythrin gene in the pneumococcal genome. Amino acid sequence of *C. perfringens* (EMBL acc.: X92844) (bottom lane) was used to investigate the presence of ruberythrin gene in the pneumococcal genome, upper lane. The residues that are common or chemically similar (+) are shown in the middle lane.

This result does not indicate that the pneumococcal genome contains a gene encoding for rubrerythrin, but it leaves the possibility open that the activity band identified as FeSOD can be encoded by a gene other than *sod* which has similar activity as SOD in the assay system used.

Oxygen induces the level of MnSOD

The amount of enzyme detected in crude extracts of pneumococci varied depending on aeration. Under both aerobic and anaerobic conditions the amount of FeSOD remained constant. This was the case for FeSOD of *E. coli* (Fee, 1991; Tardat and Touati, 1991) and *L. pneumophila* (Sadosky *et al.*, 1994). On the other hand, the level of MnSOD was higher under aerobic than anaerobic conditions. These findings were also consistent with the observations in bacteria that *sodA* expression is induced upon exposure to oxygen (Hassan and Fridovich, 1977; D'Mello *et al.*, 1997). It was suggested that the basal level of FeSOD provides a standby defence mechanism, while inducable MnSOD ensures a protection to harmful effects of elevated levels of ROS. This mechanism enables the cell to save energy by not producing a protein unless it is necessary (Fridovich, 1983).

Isolation of a pneumococcal sod gene

To isolate a pneumococcal *sod* gene, genetic complementation of *E. coli* QC774 strain with genomic libraries of the pneumococcus was attempted. This method had been successfully used previously to isolate *sod* genes in *S. mutants* (Nakayama, 1991) and *Methanobacterium*

thermoautotrophicum Marburg (Meile *et al.*, 1995). However, our attempt to isolate the gene using the same method failed. The reason for not isolating a gene with the method used could be due to difficulties associated with cloning or expression of the protein.

In this study, three attempts were made to construct a representative genomic library (section 5.2.1). In the first and second attempt the number of the recombinants obtained in the libraries only provided 27% and 30% of the recombinants required for a representative library, respectively. On the other hand, the number of recombinants obtained in the third attempt were sufficient enough to provide 87% of the recombinants required from a representative genomic library. Therefore, it is possible that the lack of a clone carrying *sod* gene in the libraries could be a reason for the failure to isolate the gene. The difficulties faced in constructing representative genomic libraries of *S. pneumoniae* in colE1-type *E. coli* plasmids have been reported by several investigators (Stassi and Lacks, 1982; Stassi *et al.*, 1982). It was presumed that the inability to clone certain pneumococcal genes might either relate to lethal effects of their protein products in a heterologous host or to the presence of large number of random sequences with strong promoter activity that result in instability in *E. coli* (Dillard and Yother, 1991).

It was proposed that the lethal effects of protein products, such as *amiA* and *malX*, were connected to their deleterious effects on *E. coli* protein export machinery (Martin *et al.*, 1989). The strong promoter activity, on the other hand, was related to chemical composition of pneumococcal DNA, which is AT rich (61%) (Mulligan and McClure, 1986). These AT rich regions can act as promoters as they resemble with *E. coli* σ^{70} promoter sequence in *E. coli* vectors (Mulligan and McClure, 1986). Consequently, it has been proposed that the strong promoter activity can interfere with maintenance of a recombinant plasmid in *E. coli* unless the vector is protected by efficient transcriptional terminators placed downstream from the cloned DNA fragment (Chen and Morrison, 1987). The strong promoter hypothesis was tested by Dillard and Yother (1991) by cloning random DNA fragments from *S. pneumoniae* into an *E. coli* vector containing transcription terminators, identified strong-promoter-acting sequences and subsequently removing the transcription terminators. The researchers concluded that promoters strong enough to

require transcription terminators for plasmid stability are rare in *S. pneumoniae* DNA since terminator removal did not result in altered plasmid stability even when the strongest promoter-acting fragment was cloned.

The third reason for the failure of the complementation experiments could be due to the level of expression of SOD needed for the maintenance of *E. coli* QC774 strain on minimal medium. As far as is known, plasmids expressing as low as 2 U SOD activity/mg of protein can enable the growth of *E. coli* QC774 strain on minimal medium (Dr D Touati, personal communication). In this study, pneumococcal *sodA* was not expressed in *E. coli* and therefore it is not known how much SOD activity could be obtained.

The vector used in this study, pTTQ18, is suitable for high level, regulated expression of genes in *E. coli* by virtue of a strong *tac* promoter and its own *lac* repressor (Stark, 1987). Consequently, the reason for not isolating a functioning copy of a *sod* gene could not be related to the low level expression of *sod* in *E. coli*. To initiate the expression of the gene, several modifications were done, such as easing antibiotic pressure on the selection cells and use of $1X10^{-10}$ M paraquat instead of $1X10^{-9}$ M. However, complete removal of paraquat from minimal medium was not attempted because pseudorevertants of *E. coli* QC774 strain appear on minimal medium at modest rates unless the growth medium is supplemented with paraquat (Dr D Touati, personal communication; Imlay and Fridovich, 1991).

As an alternative to genetic complementation of *E. coli* QC774 strain, VA-PCR was used, employing both plasmid and phage versions of the library of *S. pneumoniae* type 1 lambda ZAP II as the template. The analysis of the amplicons (section 5.3.2) showed that the amplicons contained the 3'-end and the internal part of the gene, but not 5'-end region of the gene. Sequence analysis of several amplicons containing part of the *sod* gene indicated that the *sod* sequence terminated at the same point towards 5'-end. It was thought that this might have been due to an *Alu*I site located at this point, because the fragments used to construct the lambda ZAP II library had been produced by *Alu*I digestion. Examination of the hypothetical restriction map of the finally completed sequence of *sodA*

gene however did not reveal any *Alu*I site at this end of the *sod* sequence. However, analysis of the finally completed sequence of *sodA* gene showed the presence of four *Alu*I sites within the gene, indicating that the *S. pneumoniae* type 1 lambda ZAP II library was unlikely to contain complete copy of the gene.

The sequence analysis of the amplicons obtained as a result of VA-PCR was also problematic. In one instance, a sequence representing both coding and complementary strand was obtained by using the same primer (section 5.3.3.1, Figure 5.14). The reason for strand switching remains unexplained.

Another anomaly was that an identical sequence was obtained when using the forward and reverse universal primers (section 5.3.3.1, Figure 5.13). The reason for this result might have been due to the insert amplification method. As can be seen in Figure 8.4a, when *S. pneumoniae* type 1 lambda ZAP II library was used as the template with reverse universal and SOD6 primers, the reaction would have amplified part of the vector sequence (shown as dashed line). Cloning of this PCR product into pCRScript Amp SK(+) created two recognition sites for the reverse universal primer but the relative position of these sites depends on the orientation of the insert within pCRScript Amp SK(+). If the orientation is as shown in Figure 8.4b then no sequence anomaly was expected (Figure 8.4b). However, if the orientation of the insert was as shown in Figure 8.4c, this would place a reverse universal primer binding site on both sides of the insert. Then during sequencing strand extension from the reverse primer, labelled R2, would give the same *sod* sequence. However, one piece of data does not fit with this explanation. The vector sequence obtained with reverse universal primer (underlined in Figure 5.13B) contains only a *Not*I site (consistent with extension from R1) and not the *Not*I, *BamH*I and *Eco*RI sites, expected if extension had been from R2. Hence the reason for the sequencing data obtained remains a puzzle.

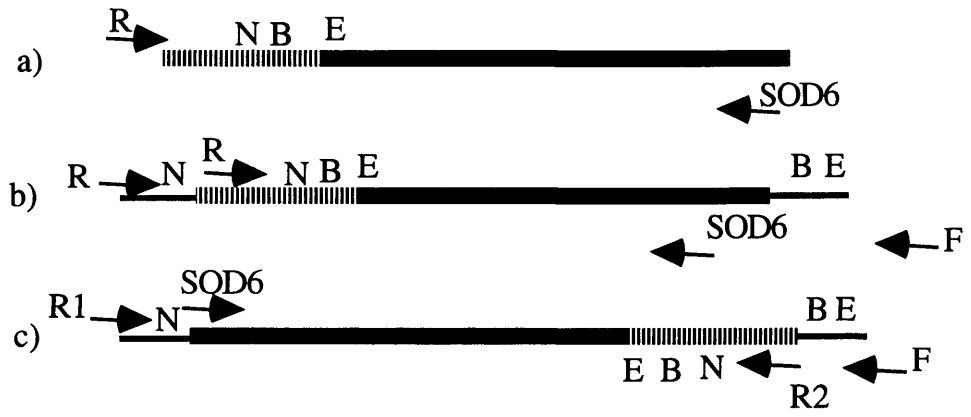


Figure 8.4: Explanation of obtaining same sequence data with different primers. **a** represents amplification of the insert from *S. pneumoniae* type 1 lambda ZAPII library by VA-PCR (see section 5.3.3), **b** and **c** represent different orientations of the insert in **a**, after cloning into pCRScript Amp SK(+) cloning vector. Dashed lines represent the vector sequence amplified from the type 1 library. Solid thick lines represent *sod* sequence. Thin solid line represents the sequence of pCRScript Amp SK(+). Relative sites of restriction enzymes are indicated with letters: N: *Not*I, B: *BamH*I, E: *Eco*RI. The direction of primers, reverse (R) and forward universal primers (F) and SOD6 primers are indicated with arrows. See text for the details.

Taken together, the failure in isolating a *sod* gene by genetic complementation of *E. coli* QC774 strain might have been either due to absence of *sod* genes from the gene libraries constructed or due to difficulties associated with cloning *S. pneumoniae* DNA fragments into colE-type *E. coli* vectors, possibility related to strong promoter activity or to lack of strong transcription terminator. VA-PCR approach could not acquire the whole gene either, indicating the possibility of the absence of 5'-end and upstream sequences from *S. pneumoniae* type 1 lambda ZAP II library.

The 5'-end and upstream sequence of *sod* gene was eventually isolated from D39HY1. Then, the complete gene was synthesised by PCR and the products from two different reactions were sequenced and analysed. The analysis of the 5'-end region of the pneumococcal *sodA* showed putative -10 and -35 hexamers of the pneumococcal *sodA* that were identical to

promoter regions of *sodA* genes from *S. mutants* and *Lactococcus lactis*, over 10 and 9 nucleotides, respectively (Figure 8.5). In addition, the promoter region was analysed for the presence of a *fur* box using a *fur* box consensus sequence, which is found in fur-regulated genes (Stojiljkovic *et al.*, 1994). The result revealed that the pneumococcal promoter region matched over 10 nucleotides to 18 nucleotides long *fur* box consensus (see Figure 6.15B). The Fur regulon is a primarily negative regulator of *sod* under both aerobic and anaerobic conditions in *E. coli* (Tardat and Touati, 1991). Although it is not known if the pneumococcus has similar regulators, the presence of this motif suggests that a similar regulatory mechanism may exist in the pneumococcus. To test whether pneumococcal *sodA* is regulated by fur, initially the presence of *fur* must be shown in *S. pneumoniae* and then a pneumococcal strain carrying a mutation in putative *fur* box region can be constructed.

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1 TCATTTGGCAATGATTTCAAAAACGACTATAATGA
2 ATAGTTGTAAGCTTTGTTTTTTTACGATATGATGA
3 AAACTTGAAAAAAGTGAATGTTTGCGTTATCATTT

Figure 8.5: Comparision of promoter regions of *sodA* genes. The -35 and -10 regions of *sodA* genes of *S. lactis* (Sanders *et al.*, 1995) (1), *S. mutants* (Nakayama, 1992) (2) and *S. pneumoniae* (3) are underlined.

The analysis of predicted amino acid sequence of pneumococcal *sodA* indicated the presence of invariant residues that are preserved in both Fe and MnSODs. These are histidine at positions 27, 82 and 176, and aspartic acid, at position 172 of pneumococcal sodA (Parker and Blake, 1988) (Figure 8.6). These residues act as ligand to metal cofactor (Parker and Blake, 1988). A comparison of the predicted amino acid sequence of pneumococcal SodA, with *S. mutants* SodA showed 84.4% similarity and 81% identity over a 193 amino acids (Figure 8.6). On the other hand, the match to *S. typhimurium* sodA was 64% similarity and 57% identity over a 198 amino acid-overlap. However this was still greater than the matches to FeSODs of *B. fragilis*, which were 52% similar, 45% identical over 190 amino acids, and *B. pertussis*, which had 51% similarity and 43% identity over 186 amino acids. Further analysis of the amino acid sequence of the pneumococcal SodA also indicated that it contained the

residues that are unique to MnSODs. These were glycine residues at positions 76 and 77, and phenylalanine, glutamine and aspartic acid residues at positions 85, 151 and 152, respectively (Figure, 8.6). These residues were proposed to act as active-site residues (Parker and Blake, 1988).

Construction of an isogenic sod mutant strain

The second aim of this study was to construct an isogenic mutant strain of the pneumococcus. Insertional duplication mutagenesis has been used to make several isogenic mutant strains of *S. pneumoniae* (Berry *et al.*, 1989; McDaniel *et al.*, 1987). This method allows the disruption of a targeted gene and therefore is a powerful tool to study a function of a particular gene. In this work *E. coli* plasmid pCRScript Amp SK (+) was used for insertion of a spectinomycin cassette into the chromosomal copy of *sod* gene. This vector is unable to replicate in *S. pneumoniae* because it does not have an origin of replication for Gram positive bacteria (Brown, 1990).

The choice of antibiotic cassette is important in insertion mutagenesis (Clavyers *et al.*, 1995). An ideal antibiotic cassette should be active in a broad range of hosts which enables propagation of the recombinant plasmid before transferring into the final host organism. The drug resistance marker used for selection must also be able to be autonomously expressed without relying on an external promoter. This is important for mutations of certain genes, particularly for those that are not active permanently (Claverys *et al.*, 1995). In this study spectinomycin resistance gene was used to select the transformants. Spectinomycin resistance gene had been shown to be active in both *S. pneumoniae* and *E. coli* (Dunny *et al.*, 1991). However, it was demonstrated that the spectinomycin resistance gene was dependant on an external promoter upon integration into the pneumococcal chromosome in a single copy, since insertion of spectinomycin resistance

```
        1                                                        50
sodA 1  MAIILPEPPY AYDALEPYID AETMHLHHDK  HQTYVNNAN AALEKHPEIG
sodA 2  MAILLPDLPY AYDALEPYID AETMTLHHDK HHATYVANAN AALEKHPEIG
sodA 3  MSYTLPSLPY AYDALEPHFD KQTMEIHHTK HHQTYVNNAN AALENLPEFA
sodB 1  MTYEMPKLPY ANNALEPVIS QQTIDYHYGK HLQTYVNNLN .SLVPGTEYE
sodB 2  MAHTLPPLPY ALDALAPRIS KETLEFHYGK HHQTYVTNLN .NLVPGTEFE

        51                                                      100
sodA 1  E.DLEALLAD VDLSQHDIRQ ALINNGGGHL NHALFWELMT PE..KTAPSA
sodA 2  E.NLEVLLAD VEQIPADIRQ SLINNGGGHL NHALFWELLS PE..KTKVTA
sodA 3  SLPVEELITK LDQVPADKKT VLRNNAGGHA NHSLFWKGLK .T..GTTLQG
sodB 1  GKTVEAIVAS APD......G AIFNNAGQVL NHTLYFLQFA PKPAKNEPAG
sodB 2  NLSLEEIVKK S.S......G GVFNNAAQVW NHTFYWNSLS PN.GGGEPSG

        101                                                     150
sodA 1  ELAAAIDATF GSFEEFQAAF TAAATTRFGS GWAWLVVNKE GKLEVTSTAN
sodA 2  EVAAAINEAF GSFDDFKAAF TAAATTRFGS GWAWLVVDKE GKLEVTSTAN
sodA 3  DLKAAIERDF GSVDNFKAEF EKAAATRFGS GWAWLVL.KG DKLAVVSTAN
sodB 1  KLGEAIKRDF GSFENFKKEF NAASVGLFGS GWAWLSVDKD GKLHITKEPN
sodB 2  ALADAIKAKW GSVDAFKEAF NKSAAGNFGS GWTWLVKKAD GTLDIVNTSN

        151                                                     200
sodA 1  QDTPI..... .SEGKKPILG LDVWEHAYYV KYRNVRPDYI KAFFSVINWN
sodA 2  QDTPI..... .SQGLKPILA LDVWEHAYYL NYRNVRPNYI KAFFEVINWN
sodA 3  QDSPLMGEAI SGASGFPILG LDVWEHAYYL KFQNRRPDYI KEFWNVVNWD
sodB 1  GSNPVR.... ..AGLKPLLG FDVWEHAYYL DYQNRRADDV NKLWEIIDWD
sodB 2  AATPLT.... ..TADKALLT CDVWEHAYYI DYRNARPKYL ENFWALVNWE

        201       212
sodA 1  KVDELYAAAK ***LEGRIVL LFLGYKILV
sodA 2  TVARLYAEAL TK
sodA 3  EAAARFALK~ ~~
sodB 1  VVEKR~~~~~ ~~
sodB 2  FAAKNFA~~~ ~~
```

Figure 8.6: Multiple alignment of the amino acid sequences of Fe and MnSODs. The alignment of amino acid sequences of Mn-containing SODs of *S. pneumoniae* (*sodA* 1), *S. mutants* (*sodA* 2) (EMBL acc.: S39782) *S. typhimurium* (*sodA* 3) (EMBL acc.: U20645), and Fe-containing SODs of *B. fragilis* (*sodB* 1) (EMBL acc.: M96560) and *B. pertussis* (*sodB* 2) (EMBL acc.: M83095) were performed using the GCG PILEUP program. The bold face residues indicate amino acids ligands to the metal cofactor. Amino acids characteristic for Mn-containing enzymes are underlined. The stop codon was shown with * symbol.

cassette in silent sites in the chromosome did not afford drug resistance, as shown in an *ami* test platform (Claverys *et al.*, 1995). However this did not cause a problem for selecting transformants in this study because it was known that the pneumococcal *sodA* gene was active under both aerobic and anaerobic selection conditions. If it is known that the promoter of gene of interest is not active, it is important to chose an antibiotic resistance cassette whose expression must be independent of local transcription (Claverys *et al.*, 1995).

Several approaches were taken by other researchers to transform plasmid DNA into the pneumococcus, including simple culture and plating techniques or filter mating. However these techniques were found to be inefficient (Canvin *et al.*, 1994). Some other researchers combined competence factor, a soluble, excreted protein that induces the transient synthesis of a group of proteins required for transformation, with chemical removal of capsule for efficient transformation. These methods reached to the efficiency level of nonencapsulated strain transformation, but only with certain serotypes (Watson and Musher, 1990). More recently, a method was developed to electroporate plasmid DNA into *S. pneumoniae* (Canvin *et al.*, 1994). However optimum conditions for electrotransformation varied from one strain to another.

In this study, natural competence of *S. pneumoniae* was exploited to allow efficient replacement of homologous region between recombinant plasmid and chromosomal copy of *sod* gene. Natural competence is the competent ability of the pneumococcus to take up free DNA from the surrounding medium with competence factor. It relies on expression of a set of genes whose products are involved in binding, uptake and integration of extracellular DNA (Havarstein *et al.*, 1997). Natural competence is induced in exponentially growing cultures at a specific cell density and lasts for less than 1 h. The initiation of competence is regulated by a 17-residue extracellular activator peptide, competence stimulating peptide (CSP) (Havarstein *et al.*, 1997). The development of competence in *S. pneumoniae* is accompanied by a drastic change in protein synthesis: synthesis of most cellular proteins is switched off and the production of at least 14 competence-specific proteins is initiated (Pestova and Morrison, 1998). The advantage of this method compared to others is that it is simple and efficient as there is no manipulation

involving filter-mating or removal of capsule, and it is cheap as it does not require the equipment needed for electrotransformation. Moreover, the availability of synthetic CSP eliminates the drawbacks of the use of crude culture extracts, which could act as a potential gene donor as reported in early studies (Haverstein *et al.*, 1995).

The parameters effecting maximum competence induction in exponentially growing cultures by the synthetic CSP are an optical density at 550 nm of 0.001, the concentration of CSP, at about 100 ng/ml; pH of the transformation mixture of 7.4-8.8; and the presence of 1 mM calcium ions (Haverstein *et al.*, 1995; Pestova and Morrison, 1998). In this study 100 ng/ml CSP was used, however, the pH and calcium level of the transformation mixture were not optimised to the level as required for an efficient transformation, because the transformants were obtained in the first shotgun experiment. Therefore, the number of transformants, 6 for 5 μg of plasmid DNA in this study, could have been higher if the optimum conditions had been prepared.

The putative mutants obtained as a result of transformation were then analysed to confirm that the *sod* gene had been inactivated. Having proven this both genetically, by Southern blot analysis and PCR, and phenotypically, by qualitative and quantitative SOD assays, one of the transformants designated as D39HY1 was analysed *in vitro* and *in vivo* to assess the effect of the inactivation of *sod*.

In vitro analysis of D39HY1

In vitro analysis of D39HY1 showed that the insertional inactivation of MnSOD had caused impaired ability to survive oxidative stress either from an oxygenated environment or due to a flux of superoxide from paraquat. The effect of MnSOD inactivation was lethal when the mutant strain was grown in defined medium. This was presumably due to lack of superoxide scavengers in defined medium, but which are present in complex medium in which it grew. Impaired growth appears to be a common characteristic of bacteria deficient in cytosolic SOD, being also observed in *sodA* mutants of *S. mutans* (Nakayama, 1992), *Haemophilus influenzae* (D'Mello *et al.*, 1997) *Pseudomonas aeruginosa* (Hasset *et al.*, 1995), *Lactobacillus lactis* (Sanders *et al.*, 1995), and *E. coli sodA sodB*

mutants (Carlioz and Touati, 1986). These results suggest the protective role of MnSOD is in *in vitro* growth of *S. pneumoniae.*

In vivo analysis of D39HY1

In vivo analysis of D39HY1 in a mouse model of pneumonia and bacteraemia showed that the *sodA* mutation reduced the virulence of *S. pneumoniae* after intranasal infection. Reduction in virulence was dependent, however, on the route of infection, no difference being seen between wildtype and D39HY1 when infection was intravenous. The observation that D39HY1 had attenuated virulence when administered intranasally, but not intravenously can be attributed to several factors. Firstly, there is a higher oxygen tension in the respiratory tract (21 kPa) compared with blood (4 kPa) (Lenter, 1986). The high oxygen tension in the lung may present a survival disadvantage to D39HY1 due to inactivation of MnSOD. Secondly, differences in virulence associated with infection route may be related to the differences in phagocyte function in blood and lung tissue. Although there are no available data on the functional differences between lung and blood phagocytes in pneumococcocal infections, functional differences between lung and blood phagocytes, such as impairment of their microbicidal activities was reported to contribute the high incidence of pulmonary infections seen in the adult respiratory distress syndrome (ARDS), a diffuse pulmonary disorder associated with life-threatening oedema and hypoxemia (Martin *et al.*, 1991). It was demonstrated that after intranasal infection, the phagocytes in the lungs, unlike pulmonary artery, have a reduced production of ROS (Martin *et al.*, 1991). The pathologic sequence of ARDS may not set an example for pneumococcal infection, but it reflects the fact that differences in microbicidal activity of phagocytes in lung and blood may contribute differences in behaviour of the pneumococcus after intranasal and intravenous infection.

The growth impairment of the mutant strain in the lungs was observed during the early stages of the infection after intranasal administration of the bacteria. The mutant was unable to grow in the first 24 h in the lungs in contrast to the wildtype. This could reflect the time taken for the bacteria to move from oxygen-rich environments in the alveoli and bronchioles into more microaerophilic areas of the lung. Wildtype pneumococci, with oxygen-resistance mechanisms intact, rapidly move

from the bronchioles and invade the bloodstream, whereas pneumococci without SodA remain for a time within the lungs. The difference in invasion abilities of wild type and D39HY1 can be tested using an appropriate cell culture model.

The different patterns of cellular infiltration into the lungs may be explained by different invasive properties of the pneumococci. D39HY1 remain in the bronchioles longer than the wildtype and inflammatory cells congregate around these areas of the lung whereas wildtype pneumococci spread throughout the lung tissue and therefore the distribution of neutrophils is more diffuse.

The organism's inability to elevate the level of MnSOD when needed in the oxygen rich environment of respiratory tract may be given as a reason for *in vivo* reduced virulence of D39HY1. Studies performed with other microorganisms showed that inactivation of the *sodA* gene may affect virulence of some organisms, but not others. For example, *sodA* mutant strain of *Yersinia enterocolitica* was less virulent in a mouse infection model after intravenous infection (but not after orogastric infection) (Roggenkamp *et al.*, 1997). Inactivation of *sodA* in *Shigella flexneri* resulted in severe attenuation of virulence in the rabbit ileal loop model (Franzon *et al.*, 1990). Also, SOD has been shown to protect *Nocardia asteroides* against the bactericidal effects of neutrophils during infection (Beaman and Beaman, 1984). However, mutation of *sodA* gene did not effect survival of *Bordetella* species either in cultured macrophages or in a mouse respiratory infection model. The inactivation of *sodA* also did not cause reduction in virulence of *H. influenzae*, when tested in an infant rat infection model (D'Mello *et al.*, 1997). These findings suggest that MnSOD's contribution to virulence is dependent on the pathogen and infection sequence of the pathogens. For example, under anaerobic growth conditions, such as in the gut lumen, the expression of *sodA* is down regulated and most of the metabolically produced $^{\bullet}O_2^-$ can be inactivated by FeSOD (Fee, 1991). Hence, when *sodA* mutant strain of *Yersinia enterocolitica* was used in a mouse orogastric infection model, no major reduction in virulence was observed (Roggenkamp, 1997). However, if the pathogenic sequence involves transitions from anaerobic to aerobic or iron rich to iron limited conditions then expression of MnSOD is upregulated (Touati *et al.*, 1995). In these latter conditions,

therefore, the absence of SodA can be important for bacterial survival in host and consequently in virulence. *Shigella flexneri* forms an example for this latter phenomen as it passes from the anaerobic environment of host intestine to macrophages and PMNL, where the bacteria encounter a heavy flux of ROS (Franzon *et al.*, 1990).

Studies performed with *E. coli* showed that the elevated expression of *sodA* occurs under both high oxygen tension and iron limited conditions (Touati *et al.*, 1995). As far as the biological niche of the pneumococcus is considered, the organism colonise and invade an environment that has high oxygen tension. In the natural habitat of *S. pneumoniae* there is also only a low level of iron available, as under physiological conditions iron is associated with high-affinity iron-binding proteins, such as transferrin, lactoferrin, hemoglobin and myoglobin (Rice-Evans *et al.*, 1995). When the level of oxygen tension and the amount of the free iron in the biological niche of the pneumococcus is taken into account, one can suggest that *in vivo* expression of *sodA* must be high and is required for survival and virulence of the organism.

In addition to its direct contribution, MnSOD shows its effect on virulence through effecting the expression of other important virulence genes. For example, *S. pyogenes* regulates some virulence-associated genes in response to the environmental concentrations of CO_2 and O_2 (Gibson and Caparon, 1996). One of these genes is *prtF* which encodes for F protein which enables the bacterium to bind to the host extracellular matrix protein, fibronectin. It was shown that *prtF* transcription is regulated in response to superoxide concentration (Gibson and Caparon, 1996). When a *sodA* negative strain of *S. pyogenes* was tested, it was found that the mutant showed hypersensitive induction of *prtF* in response to superoxide and expressed *prtF* under normally unfavourable O_2 limited conditions. These results suggest that the presence of SodA in *S. pyogenes*, by removing superoxide, down regulates the expression of *prtF*.

Another example of SOD's regulatory role can be derived from studies performed with *B. pertussis*. (Khelef *et al.*, 1996). Insertional mutation of *sodB* in *B. pertussis* resulted in a reduced ability to express adenylate

cyclase-hemolysin and pertactin, two factors important for *B. pertussis* pathogenesis.

In this study, to assess the reason for reduced virulence of D39HY1, H_2O_2 production and haemolytic activity of the mutant strain were determined. SOD's catalytic activity leads to formation of hydrogen peroxide. It was suggested that hydrogen peroxide can contribute to host-cell injury and progression of disease as it was found to be toxic to rat alveolar epithelial cells (Duane *et. al.*, 1993). In this study, it was demonstrated that D39HY1 H_2O_2 production was same as the wild type, therefore, attenuated virulence of D39HY1 could not be attributed to a reduced cytotoxic effect due to H_2O_2.

In addition to H_2O_2, the level of haemolytic activity as an indirect measurement of pneumolysin production was investigated with whole cell extract of D39HY1. The result showed that D39HY1 had less haemolytic activity than the wild type strain. The reduction in pneumolysin activity may be attributed to the elevated level of $^{\bullet}O_2^-$ in the cytoplasm, originating from inactivation of SOD, as pneumolysin loses its activity by exposure to oxidising agents (Mitchell, 1998). Although the difference between wild type and D39HY1 haemolytic activity was statistically significant, it was reported that even if the level of the heamolytic activity is as low as 0.1% level of wild-type, the pneumococcus still maintains its full virulence (Alexander *et al.*, 1998). Hence, diminished virulence of D39HY1 can not be attributed to the reduction in the haemolytic activity.

Despite the accumulated *in vitro* and *in vivo* evidence some researchers suggest that cytoplasmic SODs should not be considered as an element required for virulence (Imlay and Imlay, 1996). This suggestion originates from the fact that superoxide radical produced outside the cytoplasm will not be dealt with by intracytoplasmic SODs because superoxide radical cannot pass through biological membranes (Fridovich, 1995). However, this suggestion does not undervalue the role played by intracytoplasmic SOD in virulence of *S. pneumoniae*, it rather necessitates a different approach to explain how the pneumococcal MnSOD contributes to the virulence of the organism. Firstly, as stated earlier, *S. pneumoniae* can utilise oxygen, hence, during the utilisation of

oxygen some $^{\bullet}O_2^-$ can be formed and damage the cytoplasm. Therefore, MnSOD's absence may compromise the organism's ability to detoxify oxygen radicals in the cytoplasm. As it was shown during the course of this study, D39HY1 was found to be susceptible to action of paraquat, which produces $^{\bullet}O_2^-$ radical. Secondly, it is possible that MnSOD may be taking part in some of the important biological pathways that are vital for cell virulence, similar to those that is seen in *B. pertussis* and *S. mutants* (see above). Thus, the defect in SOD production compromises virulence of the organism.

As stated above, despite the reduced virulence of D39HY1, it does eventually become invasive. However, this was not due to reversion of the mutation because D39HY1 recovered from tissue samples contained similar numbers of bacteria when grown in the presence or absence of spectinomycin (section 7.2.4). Why D39HY1 should eventually begin to grow within the lungs and become invasive lead us to investigate if the pneumococcus possess any other oxygen defence mechanisms that substitute for the loss of SOD.

Analysis of pneumococcal genome for the presence of oxidative stress genes

To identify potential oxygen defence mechanisms, the genome sequence of *S. pneumoniae* was analysed using consensus sequences of the genes whose products are known to be part of oxygen defence mechanisms in other organisms. Recently, most of the pneumococcal genome DNA became available through early release from The Institute for Genomic Research through NCBI at www.ncbi.nlm.nih.gov.

The availability of genome sequence analysis provides a very powerful tool for researchers. An example of computer assisted analysis of pneumococcal genome can be derived from the work performed by Campbell and coworkers (1998) who identified a regulon involved in the development of natural competence in *S. pneumoniae.* However, it is still believed that the biological significance of computer assisted search of genome sequence must be supported with conventional experimentation (Masure, 1998).

At the moment, four main regulons and genes belonging to these regulons have been identified as being involved with response to oxidative stress in other bacteria (Table 8.1). One of these regulons is *rpoS* whose product controls expression of over 30 gene, when cells enter stationary phase, including genes encoding for catalase and glutathione reductase (Loewen and Hengge-Aronis, 1994). The presence of *rpoS* was shown in several Gram negative bacteria but, to date, no Gram positive bacteria were found to contain this regulon (Eisenstark, 1996) (Table 8.1).

In order to search the pneumococcal genome a consensus sequence derived from seven organisms was used (Figure 8.7A). This consensus sequence matched with a region in the pneumococcal genome showing 67% identity and 88% chemical similarity over 52 amino acids (Figure 8.7B). Then the region of homology in the pneumococcal genome was used for a blast search in order to see if the region of homology in the pneumococcal genome would match with any of the known *rpoS* genes in databases. The result showed that the region showing homology with the *rpoS* consensus in the pneumococcal genome was identical with already known *rpoD* gene of *S. pneumoniae*, which is the primary sigma-factor of this bacterium (Figure 8.7C). The product of *rpoD* gene is an initiation factor that promotes attachment of the RNA polymerase to specific initiation sites (Grebe *et al.*, 1997). These results suggest that *S. pneumoniae* does not have a *rpoS* regulatory gene.

The second main regulon involved in defence against ROS is *oxyR*. *oxyR* is a member of the *LysR* family of transcriptional activators which have been found in both Gram positive and Gram negative organisms (Gallegos *et al.*, 1997). The *oxyR* homologous genes have been found in several bacteria (Table 8.1). The result of an analysis of the pneumococcal genome indicated that *S. pneumoniae* may contain an *oxyR* regulatory gene.

Table 8.1: Regulators of response to oxidative stress in prokaryotes (Rosner and Storz, 1997)

Regulator	Homologue found in
oxyR	*E. coli* *S. typhimurium* *H. influenzae* *Mycobacterium*
soxR/soxS	*E. coli* *S. typhimurium*
marA	*E. coli* *S. typhimurium*
rpoS	*E. coli* *S. typhimurium* *H. influenzae* *Klebsialla pneumoniae* *Shigella flexneri* *Pseudomonas aeroginosa*

A.

```
RPOS1 EGNLGLIRAVEKFDPERGFRFSTYATWWIRQTIERAIMNQTRTIRLPIHIVKE
RPOS2 EGNLGLIRAVEKFDPERGFRFSTYATWWIRQTIERAIMNQTRTIRLPIHIVKE
RPOS3 EGNLGLIRAVEKFDPERGFRFSTYATWWIRQTIERAIMNQTRTIRLPIHIVKE
RPOS4 EGNLGLIRAVEKFDPERGFRFSTYATWWIRQTIERAIMNQTRTIRLPIHIVKE
RPOS5 EGNLGLIRAVEKFDPERGFRFSTYATWWIRQTIERAIMNQTRTIRLPIHIVKE
RPOS6 EGNLGLIRAVEKFDPERGFRFSTYATWWIRQTIERAIMNQTRTIRLPIHIVKE
RPOS7 EGNLGLIRAVEKFDPERGFRFSTYATWWIRQTIERAIMNQTRTIRLPIHVVKE

Consensus  EGNLGLIRAVEKFDPERGFRFSTYATWWIRQTIERAIMNQTRTIRLPIHIVKE
```

B.

```
1      EGNLGLIRAVEKFDPERGFRFSTYATWWIRQTIERAIMNQTRTIRLPIHIVK
2      EGN+GL++AV+KFD  +GF+FSTYATWWIRQ I RAI +Q RTIR+P+H+V+
3      EGNMGLMKAVDKFDYSKGFKFSTYATWWIRQAITRAIADQARTIRIPVHMVE
```

C.

```
1      EGNMGLMKAVDKFDYSKGFKFSTYATWWIRQAITRAIADQARTIRIPVHMVE
2      EGNMGLMKAVDKFDYSKGFKFSTYATWWIRQAITRAIADQARTIRIPVHMVE
3      EGNMGLMKAVDKFDYSKGFKFSTYATWWIRQAITRAIADQARTIRIPVHMVE
```

Figure 8.7: Investigation of *rpoS* in the pneumococcal genome. A. The alignment of amino acid sequences of *rpoS* of *E. coli* (RPOS1) (SWISS-PROT: P13445), *Salmonella dublin* (RPOS2) (SWISS-PROT: P39699), *Salmonella typhi* (RPOS3) (SWISS-PROT: Q56132), *S. typhimurium* (RPOS4) (SWISS-PROT: P37400), *Y. enterocolitica* (RPOS5) (SWISS-PROT: P47765), *S. flexneri* (RPOS6) (SWISS-PROT: P35540) and *P. aeroginosa* (RPOS7) (SWISS-PROT: P45684) were performed using the GCG PILEUP program. The consensus sequence is shown underlined. **B** represents the blast search performed with the *rpoS* consensus sequence (1) against the pneumococcal genome (3). **C** represents blast search using the pneumococcal sequence (1) that match with the *rpoS* consensus sequence against the whole database (3). In B and C 2 the region of identity and chemical similarity (+) between consensus sequence and *S. pneumoniae.*

The product of *oxyR* regulatory gene activates transcription of nine other oxidative stress response proteins upon exposure to H_2O_2 (Table 4.1). A consensus sequence, VSQPTLSGQIRKLEDELG, derived from *E. coli, H. influenzae* and *Xanthomonas campestris* OxyR was used as a consensus sequence to search the pneumococcal genome (Figure 8.8A). This consensus sequence contained some of the residues that are well conserved among the family of bacterial regulatory proteins (Christman *et al.*, 1989) (Figure 8.8A). Initially this probe was used for a blast search to confirm that it would match with some of the known *oxyR* genes. As expected the probe showed greatest homology to *H. influenzae* and *E. coli oxyR* regulatory genes (Figure 8.8B).

After this positive result, the probe used for the analysis of the pneumococcal genome for the presence of *oxyR* gene. It was found that a region in the pneumococcal genome was identical over 12 aa (66%) and chemically similar over 14 aa (77%) (Figure 8.8C). In addition to the consensus sequence, the pneumococcal genome was searched by employing the complete *E. coli oxyR* gene as the probe. The result showed that a region in the pneumococcal genome was identical over 53/187 amino acids (28%), and chemically similar over 93/187 acids (49%). When the region in the pneumococcal genome that was homologous with *E. coli* oxyR was used for a blast search, the result revealed that this region showed the greatest homology with already known *oxyR* regulatory genes and other transcriptional activators (Figure 8.9A, B, C, D).

Earlier studies showed that *S. pneumoniae* has a *recA* gene, which is regulated by *oxyR* in *E. coli.* The *recA* gene is known to encode for a heat shock protein (Farr and Kogoma, 1991). In, *S. pneumoniae* the *recA* gene was found to be responsible for promotion of homologous recombination (Martin *et al.*, 1992). It is not known yet if the gene product has any role in the oxidative stress response in *S. pneumoniae.*

The results presented above suggest that *S. pneumoniae* may contain an *oxyR* regulatory gene. There are several reports suggesting the protective role of *oxyR* in protection against ROS. It was found that expression of wild type *oxyR* protein by *H. influenzae* is essential to allow this organism to survive oxidative stress *in vitro* (Maciver and Hansen,

A.

```
oxyR1  MNIRDLEYLV ALAEHRHFRR AADSCHVSQP TLSGQIRKLE DELGVMLLER
oxyR2  MNIRDLEYLV ALSEYKHFRR AADSCNVSQP TLSGQIRKLE DELGIILLER
oxyR3  MNLRDLKYLV ALADHKHFGR AASACFVSQP TLSTQIKKLE DELGVSLVER
```

B.

```
                Consensus   VSQPTLSGQIRKLEDELG
                            VSQPTLSGQIRKLEDELG
    H. influenzae/E. coli   VSQPTLSGQIRKLEDELG
```

C.

```
                Consensus   VSQPTLSGQIRKLEDELG
                            VSQP+LS  +R LE ELG
          S. pneumoniae     VSQPSLSISVRDLEKELG
```

Figure 8.8: Analysis of the pneumococcal genome for *oxyR* regulatory gene. A. The alignment of amino acid sequences of *oxyR* belonging to *E. coli* (oxyR1) (EMBL acc: X52666), *H. influenzae* (oxyR2) (EMBL acc: U49355), and *X. campestris* (oxyR3) (EMBL acc: U94336) were performed using the GCG PILEUP program. The underlined residues indicate the consensus sequence. Amino acids that are conserved in the family of bacterial regulatory proteins in the consensus are written in italic. **B.** The region matched with the consensus sequence (upper line) in the pneumococcal genome is given in the bottom line. The middle line represents the homologous or chemically similar residues (+). **C.** represents blast search performed using *oxyR* consensus sequence. The residues homologous with the consensus sequence (upper line) in *H. influenzae* and *E. coli oxyR* gene are given in the bottom line. The middle line represents the identical residues.

A

```
1 MNIQQLRYVVAIANSGTFREAAEKMYVSQPSLSISVRDLEKELGFKIFRRTSSGTFLTRR
2 M+I+ L Y + +A   +F +A++ +YVSQP++S  +++LE+ELG ++F R      LT
3 MDIRHLTYFLEVARLKSFTKASQSLYVSQPTISKMIKNLEEELGIELFYRNGRQVELTDA

1 GMEFYEKSQELVKGFDIFQNQYANPXXXXXXXXXXFSVASQHYDFLPPTITAFSERYPDY
2 G   Y ++QE++K F    ++  +                    F P  +  F E YP+
3 GHSMYVQAQEIIKSFQNLTSELNDIMEVKKGHVRIGLPPMIGSGFFPRVLGDFRENYPNV

1 KNFRIFESTTVQILDEVAQGHSEIGIIYL
2   F++ E  ++++ + V  G  +IG++ L
3  TFQLVEDGSIKVQEGVGDGSLDIGVVVL
```

B

```
1 MNIQQLRYVVAIANSGTFREAAEKMYVSQPSLSISVRDLEKELGFKIFRRTSSGTFLTRR
2 MNI+ L Y+VA+A    FR AA+  +VSQP+LS  +R LE ELG  +  RTS     T+
3 MNIRDLEYLVALAEHRHFRRAADSCHVSQPTLSGQIRKLEDELGVMLLERTSRKVLFTQA

1 GMEFYEKSQELVKGFDIFQNQYANPXXXXXXXXXXFSVASQHYDFLPPTITAFSERYPDY
2 G+   E+++ +++   + +   +               + +     LP  I      +P
3 GLLLVEQARTVLREVKVLKEMASQQGETMSGPLHIGLIPTVGPYLLPQIIPMLHRTFPKL

1 KNFRIFESTTVQILDEVAQGHSEIGIIYLNNQNKKGIMQRVEKLGLEVIELIPFHTHIYL
2 + + + E+ T Q+L ++  G  +  I+ +  +++  I           + L      + +
3 EMY-LHEAQTHQLLAQLDSGKLDCAILAMVKESEAFI----------EVPLFDEPMKLAI

1 REGHPLAQKEELVMEDLA
2  + HP A +E + M DLA
3 YQDHPWANRERVAMSDLA
```

C

```
1 MNIQQLRYVVAIANSGTFREAAEKMYVSQPSLSISVRDLEKELGFKIFRRTSSGTFLTRR
2 MNI+ L Y+VA++    FR AA+   VSQP+LS  +R LE ELG  +  RTS     T+
3 MNIRDLEYLVALSEYKHFRRAADSCNVSQPTLSGQIRKLEDELGIILLERTSRKVLFTQS

1 GMEFYEKSQELVKGFDIFQNQYANPXXXXXXXXXXFSVASQHYDFLPPTITAFSERYPDY
2 GM   ++++ +++   + +   +N              + +     LP  +      +PD
3 GMLLVDQARTVLREVKLLKEMASNQGKEMTGPLHIGLIPTVGPYLLPYIVPMLKAAFPDL

1 KNFRIFESTTVQILDEVAQGHSEIGIIYLNNQNKKGIMQRV--EKLGLEVIELIPFHTHI
2 + F ++E+ T Q+L+++  G  +  I+    + +  I   +  EK+ L V
3 EVF-LYEAQTHQLLEQLETGRLDCAIVATVPETEAFIEVPIFNEKMLLAV----------

1 YLREGHPLAQKEELVMEDL
2    E HP AQ+ +L M  L
3 --SEHHPWAQESKLPMNQL
```

D

```
1 MNIQQLRYVVAIANSGTFREAAEKMYVSQPSLSISVRDLEKELGFKIFRRTSSGTFLTRR 60
2 MNI+ L Y+VA+A    FR AA+  +VSQP+LS  +R LE ELG  +  RTS     T+
3 MNIRDLEYLVALAEHRHFRRAADSCHVSQPTLSGQIRKLEDELGVMLLERTSRKVLFTQA 60

1 GMEFYEKSQELVKGFDIFQNQYANPXXXXXXXXXXFSVASQHYDFLPPTITAFSERYPDY 120
2 GM   ++++ +++   + +   +               + +     LP  I    + +P
3 GMLLVDQARTVLREVKVLKEMASQQGETMSGPLHIGLIPTVGPYLLPHIIPMLHQTFPKL 120

1 KNFRIFESTTVQILDEVAQGHSEIGIIYLNNQNKKGIMQRVEKLGLEVIELIPFHTHIYL 180
2 + + + E+ T Q+L ++  G  +  I+ L  ++++ I           + L      + +
3 EMY-LHEAQTHQLLAQLDSGKLDCVILALVKESERFI----------EVPLFDEPMLLAI

1 REGHPLAQKEELVMEDLA
2  E HP A +E + M DLA
3 YEDHPWANRECVPMADLA
```

Figure 8.9: The region homologous with *E. coli oxyR* gene in the pneumococcal genome (lane 1 in A, B, C, and D) was used for blast search. The greatest homology was obtained with **A.** *Bacillus subtilis ipa-24d* gene; similar to transcriptional regulator (LysR family), (Lane 3) **B.** *Erwinia carotovora* oxidative stress transcriptional regulator gene (Lane 3) **C.** *H. influenzae oxyR* homologue (Lane 3) and **D.** *E. coli oxyR* gene (Lane 3). In A, B, C, D 2 represents the homologous or chemically similar residues (+).

1996). *oxyR*'s role in virulence was demonstrated from studies performed with *S. typhimurium* carrying a mutation in the *oxyR* gene (Fields *et al.*, 1986). It was shown that a mutation in *oxyR* resulted in reduced virulence as compared to the wild type parent *in vivo*. However, in a separate study when the mechanism of reduced virulence was investigated, no protective effect of *oxyR* was seen in resistance to phagocytic killing *in vitro* (Pabb-Szabo *et al.*, 1994).

Although the genome sequence analysis of the pneumococcus indicated the presence of an *oxyR* gene, the attempts to find the genes likely to be regulated by *oxyR* were fruitless; genes for those that are involved directly in inactivation of peroxides, such as glutathione reductase, alkyl hydroperoxide reductase, both glutathione reductase and alkyl hydroperoxide reductase contain flavin adenine dinucleotide (Patel *et al.*, 1998; Calzi and Poole, 1997), and catalase. The absence of these genes suggests that *oxyR* regulatory protein plays a role in the regulation of transcription of genes that are different than those in *E. coli* and *S. typhimurium*.

To identify a consensus sequence for glutathione reductase, glutathione reductase genes from three organisms were aligned (Figure 8.10A). Then, this consensus was used as a consensus sequence to search the pneumococcal genome. It was found that two distinct pneumococcal regions were similar to the probe, exhibiting 69% identity and 92% chemical similarity over 13 amino acids for first, and 69% identity and 84% chemical similarity over 13 amino acids for the second region (Figure 8.10B).

The regions showing homology with the consensus sequence in the pneumococcal genome were used in a blast search in order to check if these regions would show homology with any known *gor* gene, encodes for glutathione reductase, in the databases. The result showed that the greatest similarity with the putative *gor* regions of pneumococcal genome was not with a known *gor* gene but it was with dihydrolipoamide dehydrogenase genes from different bacterial sources, including *Chlamydia trachomatis* and *Bacillus subtilis*. In addition to a search with the consensus sequence, the pneumococcal genome was searched with the whole *E. coli gor* gene.

A

```
gor1  EAKELGGTCV NVGCVPKKVM WHAAQIREAI HMYGPDYGFD TTINKFNWET
gor2  EGKEVGGTCV NVGCVPKKVM WYGAQVAETL HRYAGEYGFD VTINNFDFAT
gor3  ESRYLGGTCV NVGCVPKKLL VYGAHFSEDF EQ.ARAYGWS AGEAQFDWAT
```

B

```
S. pneumoniae   GGTCINIGCIPTK        GGTCLNRGCIPTK
                GGTC+N+GC+P K        GGTC+N GC+P K
Consensus       GGTCVNVGCVPKK        GGTCVNVGCVPKK
```

Figure 8.10: Investigation of glutathione reductase gene in the pneumococcal genome. A. Amino acid sequences from *E. coli* (gor1) (EMBL acc: M13141), *Streptococcus thermophilus* (gor2) (EMBL acc: L27672) and *P. aeroginosa* (gor3) (EMBL acc: X54201) were aligned using GCG PILEUP program. The consensus sequence is underlined. **B.** The identical and chemically similar residues (+) shared by the consensuses and *S. pneumoniae* are shown in the middle lane.

However, the region in the pneumococcal genome hybridising with *E. coli gor* gene was found to have great similarity to dihydrolipoamide dehydrogenase genes. These results indicate that *S. pneumoniae* does not contain the gene encoding for glutathione reductase.

Dihydrolipoamide dehydrogenase is a flavoprotein component of multi-enzyme complexes catalysing oxidative decarboxylation of α-ketoacids in the Kreb's cycle (Pullikuth and Gill, 1997). Dihydrolipoamide dehydrogenase belongs to the groups of flavin-containing pyridine nucleotide disulfide oxidoreductases to which *gor* belongs (Greer and Perham, 1986)

The pneumococcal genome was checked for the presence of alkyl hydroperoxide reductase using the complete *E. coli ahpC* gene. The result showed that a region in the pneumococcal genome exhibited 26% identity and 38% similarity to *E. coli ahpC* gene over 90 amino acids (Figure 8.11). Then the region in the pneumococcal genome that matched with *E. coli* was used for a blast search. It was found that this region of the pneumococcus was 70% identical and 76% chemically similar to genes encoding for heat shock protein, GroEL, over 89 amino

acids (data not shown). GroEL is a heat shock protein and its presence has already been known (Hamel *et al.*, 1997). The result of this search suggests that *S. pneumoniae* does not have an *ahpC* gene.

Although it is known the pneumococcus does not synthesise heme products and therefore lacks catalase, the genome was investigated for the existence of catalase gene using two consensus sequences, GGLFIRMAWHSA and ELVWLLGAHSV, obtained using ProDom BLAST program available in http://www.toulouse.inra.fr. However, the consensus sequences used showed no homology to the pneumococcal genome.

The third regulon that was investigated in the pneumococcal genome was *soxRS* which coordinates the transcriptional induction of at least 12 promoters (see Table 4.2). At the moment, the presence of *soxRS* has been shown in *E. coli* and *S. typhimurium* (Table 8.1). In *E. coli*, the *SoxRS* regulon is needed for resistance to redox-cycling agents that increase cytosolic superoxide levels, as well as for resistance to nitric oxide-dependent macrophage killing (Li and Demple, 1994).

The *soxS* regulatory gene is the member of AraC/XylS family of transcriptional regulators (Ariza *et al.*, 1995). The members of AraC/XylS family share three main regulatory functions in bacteria: carbon metabolism, stress response, and pathogenesis (Gallegos *et al.*, 1997). The pneumococcal genome was searched with the *soxS* gene of *E. coli* (Figure 8.12). Analysis of the pneumococcal genome showed that a pneumococcal chromosomal region have 29% identity and 54% chemical similarity over 101 amino acids. This similarity is enough to reach a conclusion because analyses of the structures and sequences of proteins have established that homology greater than 25% between two proteins extending for 50 amino acids is sufficient to ensure their identical teritary structure (Sander and Schneider, 1991). Moreover, the region of homology in the pneumococcal genome contained 12 out of 17 amino acids that are well conserved in members of this family (Sander and Schneider, 1991) (Figure 8.12).

```
1  HKAWHSSSETIAKIKYAMIGDPTGALTRNFDNMREDEGLADRATFVVDPQGIIQAIEVTA
2  H A    S  I ++K A +G    A T  + NM +              QGII  ++V+
3  HNAGFEGSIVIDRLKNAELGIGFNAATGEWVNMID-------------QGIIDPVKVSR

1  EGIGRDASDLLRKIKAAQYVASHPGEVCPA
2    +   AS     +     VA+ P  V PA
3  SALQNAASVASLILTTEAVVANKPEPVAPA
```

Figure 8.11: Investigation of the pneumococcal genome for the presence of *ahpC* gene. The pneumococcal genome was searched using complete amino acid sequence of *E. coli ahpC* gene (1). The region hybridised with *E. coli ahpC* gene in the pneumococcal genome is given in row 3. The identical or chemically similar residues (+) between *S. pneumoniae* and *E. coli* are given in lane 2.

```
E. coli         KIIQDLIAWIDEHIDQPLNIDVVAKKSGYSKWYLQRMFRTVTHQTLGDYI
                K ++D+  ++D+H    +N   + K S  S   L+ +F+    Q++ +Y
S. pneumoniae KALEDVARFLDDHFATNVNQGTLEKISKMSGTKLKNLFKEKYCQSITEYT

E. coli         RQRRLLLAAVELRTTERPIFDIAMDLGYVSQQTFSRVFRRQFDRTPSDYRH
                +++R+ +A   L  TE PI +IA  +GY S   FS  ++R   + PS+ R+
S. pneumoniae QRKRMNVAETLLLNTELPIKEIAESVGYSSASKFSIYYKRYKGKLPSEVRN
```

Figure 8.12: Analysis of the pneumococcal genome for *soxS* regulatory gene. The identical and chemically similar (+) residues between *E. coli* soxS (EMBL acc.: M60111) and the pneumococcal sequence are given in the middle lane. The residues typed in italic represent the residues well conserved in members of AraC/XylS family.

In *S. typhimurium*, the *in vitro* and *in vivo* role of *soxRS* was assessed by using a strain carrying a *soxS* mutation (Fang *et al.*, 1997). It was found that the mutant strain could not elevate MnSOD level when exposed to paraquat and was hyper susceptible to the effect of this compound compared with the wild type. However, the mutation in *soxS* gene did not effect the resistance of the bacterium to nitric oxide donor compounds. When tested for survival in J774.1 macrophage-like cells and gamma interferon stimulated resident macrophages, no effect of the *soxS* mutation on survival could be detected. In addition, no significant effects of the *soxS* mutation was seen in any mouse model after intraperitoneal infection. It was then concluded that compensation for the loss of *soxS* by a related transcriptional activator responding to a different environmental signal may account for the dispensability of *soxS* for *Salmonella* virulence and survival in phagocytes (Fang *et al.*, 1997).

Fawcett and Wolf (1994), using DNase I footprinting and a methylation interference assay, demonstrated the presence of a sox box in the promoter region of *zwf*, *sodA*, *nfo*, *micF*, and *fumC*, where the SoxS protein binds for the activation of these genes in *E. coli*. An eighteen nucleotide long sox box, ANNGCAYRANNRNNAAR (N: any base, Y: pyrimidine, R: purine) was well conserved GCAY motif which lies near the 5'-end of sox box consensus sequence. In *E. coli* the sox box covers the -35 hexamer of *sodA* gene (Fawcett and Wolf, 1994).

Examination of the *S. pneumoniae sodA* promoter region for a sox box did not reveal a conserved GCAY motif but a GCAA motif was identified (Figure 8.13A). In addition, 15 nucleotides were found to match with an 18 nucleotide-long consensus sequence (Figure 8.13B). Similar to *E. coli*, the sox box consensus sequence overlapped the -35 hexamer of pneumococcal *sodA* promoter (Figure 8.13A). However, for more definite answers for *soxbox* further study, similar to those that have been done by in *E. coli* by Fawcett and Wolf (1994), is needed.

SoxR is transcriptional activator of the *soxS* gene in *E. coli* (Demple, 1996). The presence of a *soxR* gene in the pneumococcal genome was investigated using the *E. coli soxR* gene (Figure 8.14). The result of this analysis showed that a region in the pneumococcal genome was 25% identical and 42% chemically similar over 106 amino to *E. coli* redox-

A.

```
  1  CTTGCTGATT TGCTTATTTT ATTAGAAAGT ATGTCAGTGA TTTCCTTATC
 51  TATAAGCAAA AAACTTGAAA AAAGTGAATG TTTGCGTTAT CATTTTCATA
                      -35                        -10
101  TAGAAAGAAA AAGAGGTATT ACAGATGGCT ATTATCTTAC CGTACGCGTA
                    SD
```

B.

```
S. pneumoniae  TAAGCAAAAAACTTGAAA
               -NNGCA-NRANN-NNAAR
consensus      ANNGCAYNRANNRNNAAR
```

Figure 8. 13: Potential sox box in *S. pneumoniae sodA* promoter region. **A.** The putative *soxbox* is indicated with italic letters. The well conserved GCA nucleotides are written in bold face. The possible -35 hexamer and -10 region are underlined. Shine-Dalgarno sequence indicated with SD is underlined. **B.** Sox box consensus sequence is aligned with *S. pneumoniae* promoter region. Shared nucleotides are given in the middle lane. '-' represents nucleotides that are not identical, N is any base.

```
1 EKEFRRNMAVFPIGSVMKLTDLSARQIRYYEDQELIKPDRNEGNRRMYSLNDMDRLLEIK
2 EK+  R  A+   G V K + ++   + +YE + LI   RN GN+R Y  + +  +  IK
3 EKKLPRIKALLTPGEVAKRSGVAVSALHFYESKGLITSIRNSGNQRRYKRDVLRYVAIIK

1 DYISEGYNIAAIXXXXXXXXXXXXXXAEREAKSKKAVSQTEVRRALH
2     G  +A I             + +E K   +  + E+ R +H
3 IAQRIGIPLATIGEAFGVLPEGHTLSAKEWKQLSSQWREELDRRIH
```

Figure 8.14: Analysis of the pneumococcal genome for the presence of *soxR* gene. Amino acid sequence of *E. coli soxR* gene (3) was used as a probe. A region in the pneumococcal genome (1) showed 25% identity and 42% chemical similarity (+) (2) to *soxR* gene of *E. coli* (3).

sensitive transcriptional activator, *soxR*. At the moment the significance of the similarity is not clear.

Studies with the pneumococcus so far demonstrated that *S. pneumoniae* has *sodA* (Poyart *et al.*, 1995 and this study) and *zwf* genes which encode for glucose-6-phosphate dehydrogenase (Coffey *et al.*, 1998). In *E. coli* and *S. typhimurium*, the *soxRS* regulon is responsible for the regulation of *sodA* and *zwf* genes (Rosner and Storz, 1997). The results presented above suggest the possibility of *soxRS* regulon. However, it is not known yet if these genes are regulated by SoxRS.

Glucose-6-phosphate dehydrogenase catalyses the NAD^+ or $NADP^+$-dependent conversion of glucose-6-phosphate to 6-phosphogluconate in which NADPH also is produced as a product (Hunt and Phibbs, 1983). When an *E. coli* strain deficient in *zwf* was tested, it was found that the mutant strain was more sensitive to paraquat as compared to the wild type (Liochev and Fridovich, 1991). It was postulated that the sensitivity was due to a reduced level of NADPH, a cofactor necessary for the activity of glutathione reductase and alkyl hydroperoxidase. A *zwf* mutant strain of *P. aeroginosa* exhibited impairment in resistance to the O_2^- generating agents (Ma *et al.*, 1998). Recently the presence of *zwf* gene was reported in *S. pneumoniae* (Enright and Spratt, 1998). The construction of a *zwf* deficient strain of the pneumococcus could illuminate the importance of this enzyme, both in organism's aerobic metabolism and in virulence.

The fourth regulon that is involved in oxygen defence is the *mar* regulon which is transcriptionally activated by MarA, which is a member of AraC/XylS transcriptional activators (Table 8.1). Expression of *marA* renders *E. coli* partially resistant to various antibiotics and superoxide-generating agents (Martin and Rosner, 1998). MarA protein was reported to be homologous to SoxS. It was reported that SoxS and MarA activate some of the same genes *in vitro*, such as *sodA, zwf, fumC* (Ariza *et al.*, 1995). A region of the pneumococcal chromosome showed 27% identity and 48% chemical similarity with *E. coli marA* gene (Figure 8.15). The homologous sequence contained some of the well-conserved amino acid sequences in the members of AraC/XylS family (Figure 8.15). As stated earlier, if the sequence homology is greater than 25% between two proteins extending for 50 amino acids is sufficient to ensure their

identical teritary structure (Sander and Schneider, 1991). For the case of transcriptional regulators, it was suggested that if the region of similarity extends for a region of 100 amino acids with an overall similarity of 20%, these proteins can be assumed to possess identical tertiary structure (Sander and Schneider, 1991). Based on this assumption, it can be suggested that *S. pneumoniae,* may have a *mar* regulon.

Although it is not a member of any of the regulons discussed above, the presence of a gene encoding for CuZnSOD was also checked in the pneumococcal genome, despite the fact that no CuZnSOD activity was detected in whole cell extracts of the pneumococcus. The rationale behind this was that CuZnSOD activity in *E. coli* was not shown until recently because of its instability in extracts and its almost complete repression during log-phase growth (Benov and Fridovich, 1995). It was thought that similar difficulties associated with assaying CuZnSOD in *E. coli* might have been the case for *S. pneumoniae.* The presence of the gene encoding for CuZnSOD was investigated using two conserved sequences identified in CuZnSODs from other organisms, which were GFHIHENGSCG and GGGGARIACGVI obtained using ProDom BLAST program available in http://www.toulouse.inra.fr. However no region in pneumococcal genome showed significant homology to the consensus sequences, indicating the absence of CuZnSOD in *S. pneumoniae.*

```
1 ESPLSLEKVSERSGYSKWHLQRMFKKETGHSLGQYIRSRKMTEIAQKLKESNEPILYLAE
2 +S  SL+ ++   G+S  +L  + KKE G     Y+   ++ +    L  ++  I  +AE
3 DSQFSLKSLASDLGFSPTYLSSLIKKELGLPFQDYLVRERVKQAKLLLLTTDLKIYEIAE

1 RYGFESQQTLTRTFKNYFDVPPHKYRMTNMQGESR
2 + GFE     T+ FK    V P +++    +GE R
3 KVGFEDMNYFTQRFKQIAGVTPRQFK----KGEDR
```

Figure 8.15: Analysis of pneumococcal genome for the presence of *marA* gene. The pneumococcal genome was investigated for the presence of *marA* by using amino acid sequence of *E. coli marA* gene (EMBL acc.: L06966) (1), as the probe. The identical and chemically similar residues (+) (2) between E. coli (1) and a pneumococcal region (3) are shown. The underlined residues represent the well conserved amino acids in members of AraC/XylS family.

As stated above, *S. pneumoniae* has some of the genes whose products are known to be important in defence against ROS, such as *sod* and *zwf*. The genome sequence analysis showed that the pneumococcus might have some of the genes that are known to involve in detoxification of the ROS, such as the genes whose products are known to possess regulatory role including *oxyR*, *soxS*, and *marA*. However, pneumococcal genome analysis did not indicate the presence of the other genes that are important in defence against ROS, such as *gor* and *ahpC*. Collectively, these results suggest that the pneumococcus has different response mechanism to oxidative stress than what is known in *E. coli* and *S. typhimurium*. As mentioned earlier, although the genome sequence analysis provide valuable information on what the organism may or may not have in terms of genes and regulons, these data must be supported with experimentation (Masure, 1998). Hence, further study is needed to highlight the mechanisms involving in oxygen defence systems of the pneumococcus. Such a study may define not only a more definite role to MnSODs in pneumococcal virulence but also it enables us to understand pneumococcal virulence in relation to its combat ability to oxidative stress.

Chapter 3

Effects of Interferon-γ on Sensitivity of Cells to Pneumolysin

Part 9 Introduction, Results and Discussion

9.1 Introduction

Interferons (IFN) are a group of cytokines that are produced by a diverse group of cells including T cells, natural killer cells, mononuclear phagocyte and cultured fibroblasts. Interferons differ according to their physicochemical properties, cellular origins, and biological properties and form three families: α, β, and γ (Abbas *et al.*, 1994). These three families are studied in two groups; type I and type II. The type I interferons are IFN-α and IFN-β which are secreted by leukocytes and fibroblasts, respectively (Morris and Zvetkova, 1995). Type I IFNs exhibit antitumor and antiviral activities. In addition, type I IFNs enhance natural killer (NK) cell activity, and increase the expression of MHC class I antigens (Belardelli, 1995; Abbas *et al.*, 1994; Morris and Zvetkova, 1995).

The type II IFN, INF-γ, is secreted by T lymphocytes and NK cells (Tyring, 1995). INF-γ is a key activator of macrophage killing activity and NK cell cytotoxicity (Morris and Zvetkova, 1995; Lei *et al.*, 1997). Also IFN-γ activates the expression of MHC class II antigens and increases the surface expression of IgG Fc receptors (Abbas *et al.*, 1994).

IFN-γ production has been shown to play a protective role against several microbial infections, as demonstrated in different animal models (Czarniecki and Sonnenfeld, 1993). Transgenic knock-out mice lacking either receptors for IFN-γ, or IFN-γ itself, have been reported to be more sensitive to bacteria and parasite infections, such as by *Listeria monocytogenes*, *Mycobacterium tuberculosis*, and *Leishmania major* (Dalton *et al.*, 1993; Flynn *et al.*, 1993).

Recently, Rubins and Pomeroy (1997) suggested a protective role for IFN-γ in the host response to pneumococcal infection. It was demonstrated by these researchers that immunocompetent mice were more resistant than INF-γ-deficient mice to *S. pneumoniae* infection. Analysis of serum IFN-

γ level after intranasal infection with virulent strains indicated that IFN-γ concentration increased significantly between 24 h to 48 h after infection.

In addition to direct demonstration of a protective effect of IFN-γ in the knock-out mice, the exogenous administration of IFN-γ has been also shown to enhance survival following subsequent challenge with virulent bacteria, in different animal models. For example, IFN-γ treatment of mice prior to infection with *S. pneumoniae* decreased the early mortality, compared with mice injected with control diluent (Weigent *et al.*, 1986). Moreover, when the mice were treated with antibody directed against IFN-γ before the infection with the pathogen, increased mortality was observed (Weigent *et al.*, 1986).

The protective effect of IFN-γ was also shown in *Francisella tularensis* infection, by demonstrating that mice treated with recombinant murine IFN-γ before the infection with *F. tularensis* exhibited a decrease in viable bacteria, in both liver and spleen (Anthony *et al.*, 1989). In addition, administration of monoclonal antibody reactive to recombinant murine IFN-γ to infected mice led to increased numbers of bacteria in liver and spleen (Anthony *et al.*, 1989).

In vitro studies performed with *L. pneumophila* and *F. tularensis* revealed that IFN-γ treatment renders macrophages able to exhibit a bacteriostatic effect towards these organisms (Bhardwaj *et al*, 1986; Fortier *et al.*, 1992). The protection provided by IFN-γ against bacterial infections is believed to originate from its ability to activate macrophages and neutrophils for the oxidative burst, resulting in formation of ROS and also for formation of nitric oxide, (see section 4.2) (Morris and Zvetkova, 1995).

The activation effect of IFN-γ on macrophages was demonstrated by Nathan and coworkers (1983). These researchers showed that recombinant IFN-γ has potent macrophage-activating factor activity, stimulating the peroxide-releasing capacity of macrophages an average of 19.8-fold at peak response, and enhancing their ability to kill *Toxoplasma gondii*.

The prevention of invasion of bacteria was also suggested to be another mechanism by which IFN-γ exhibits its protective effect (Niessel *et al.*, 1986; Bukholm and Degre, 1985). It was reported that pretreatment of HeLa cells with natural or recombinant IFN-γ protected cells from *Shigella* invasion. Antiserum against natural IFN-γ abolished the protective effect against *Shigella* invasion (Niessel *et al.*, 1986). In addition to *Shigella* invasion, pretreatment of epithelial cell line with IFN-γ protects cells against *Salmonella* invasion (Bukholm and Degre, 1985). The inhibitory effect of IFN-γ against cellular invasion was proposed to be an important defence mechanism against facultative intracellular bacteria, as virulence of facultative intracellular bacteria is dependent on the ability of these organisms to penetrate and replicate within epithelial cells (Hess *et al.*, 1989).

The examples given above clearly demonstrate that IFN-γ plays a protective role against bacterial infections by activating macrophages to kill bacteria. However, it is not known if IFN-γ also can enhance resistance of macrophages to the deleterious effects of bacteria or bacterial products. To assess whether IFN-γ elevate the resistance of macrophages against adverse effects of bacterial products which are proven to be involved with virulence of a pathogen, pneumolysin was used as a model. Pneumolysin was chosen for two reasons: Firstly, as described earlier (section 2.8), its contribution to the virulence of *S. pneumoniae* and its ability to promote inflammation is well documented and secondly one of the mechanisms by which pneumolysin exhibits its effect was attributed to pneumolysin's negative effects on immune system cell functions (Paton and Ferrante, 1983; Mitchell *et al.*, 1989; Saunders *et al.*, 1989). When used at high concentration, pneumolysin show lytic effect on phagocytes; while at concentrations which have no effect on phagocytes viability, it inhibits the respiratory burst of phagocytes. The purpose of this study, therefore, was to investigate if IFN-γ could alter the susceptibility of cells to the lytic and non-lytic effects of pneumolysin. The effect on respiratory burst was chosen as an assay of sublytic effects.

9.2 Results and Discussion

To investigate if IFN-γ could alter pneumolysin's adverse effect on immune system cell function, U937 cells were used. U937 is a frequently used model for macrophage study and has the general advantages of cell

lines, such as ease of culture, homogeneity, and it could be grown in large quantities (Andrew *et al.*, 1984).

To determine the range of toxin that was to be used, the cells were exposed to various concentrations of pneumolysin and their viability was assessed by trypan blue exclusion, after incubating the cells at 37°C for 1 hour in the presence of 5% CO_2 (Table 9.1). In the absence of IFN-γ it was seen that at 0.2 HU/ml and above, the cell viability decreased as compared to the control, when analysed by ANOVA followed by Tukey-Kramer multiple comparisons test ($p<0.05$) (Table 9.1, column 2). When the same toxin range was used with IFN-γ activated cells, activated cells exhibited a higher percent viability than the nonactivated cells after exposure to pneumolysin (Table 9.1, column 3). When the data were analysed by ANOVA followed by Tukey-Kramer multiple comparisons test, it was seen that the protective effect of IFN-γ on cell viability was significant at 0.3 ($p<0.01$), 0.5 ($p<0.01$), 0.75 ($p<0.001$), 1 ($p<0.001$) and 2.5 HU ($p<0.05$).

In the next stage, the effect of IFN-γ treatment on the respiratory burst of the U937 cell line was determined by exposing the cells to sublytic levels of pneumolysin and then measuring the respiratory burst after stimulation with PMA (Table 9.2). As a control, the respiratory burst of non-activated cells were measured in the presence and absence of PMA stimulation. The results showed that, in the absence of pneumolysin, the respiratory burst of IFN-γ treated cells were significantly higher than the cells that were not treated with IFN-γ, after PMA stimulation, indicating the successful activation of the cells ($p<0.001$). When IFN-γ activated cells were exposed to sublytic pneumolysin concentrations, the respiratory burst was not affected: the levels of peroxide was the same as in the absence of pneumolysin ($p>0.05$). Cells not treated IFN-γ did not exhibit a respiratory burst after PMA and thus there was no effect of pneumolysin exposure ($p>0.05$).

In this study, it was demonstrated that IFN-γ can alter the susceptibility of cells to the lytic effect of pneumolysin. Also the respiratory burst of IFN-γ treated cells was insensitive to sublytic concentrations of pneumolysin. It was not possible to directly compare the effect of sublytic concentrations of pneumolysin on the respiratory burst of IFN-γ treated and untreated

Table 9.1: The effect of INF-γ on the susceptibility of cells to lytic activity of pneumolysin

Haemolytic Units of pneumolysin/ml	% Viability without INF-γ [a]	% Viability with INF-γ [b]
0	95.8 ±1.6	92.9 ±1.9
0.1	91.6 ±3.8	88.5 ±4.0
0.2	76.1 ±10.7	86.3 ±0.9
0.3	64.1 ±10.5	85.6 ±2.9
0.5	46.0 ±12.2	66.4 ±8.8
0.75	19.6 ±6.2	47.1 ±7.2
1	6.7 ±1.9	45.0 ±3.7
2.5	0.5 ±1.0	19.1 ±1.4

[a]The mean ±SD of 4 experiments. Cells were grown in medium alone for 48 hours prior to exposure to various levels of pneumolysin.

[b]The mean ±SD of 3 experiments. Cells were grown in medium supplemented with 100 U/ml interferon γ for 48 hours prior to exposure to various levels of pneumolysin. The medium was removed after first 24 and cells were incubated with fresh IFN-γ.

Table 9.2: The effect of INF-γ on respiratory burst of U937 cells exposed to sublytic pneumolysin range.

	nmole/H_2O_2/10^5 cells/h			
Haemolytic Units of pneumolysin /ml	**+INF-γ, +PMA**	**-INF-γ, +PMA**	**+INF-γ, -PMA**	**-INF-γ, -PMA**
0	2.56 ±0.11	0.74 ±0.1	0.77 ±0.05	0.69 ±0.06
0.02	3.18 ±0.69	0.75 ±0.07	0.62 ±0.08	ND
0.05	2.89 ±0.05	0.67 ±0.06	0.68 ±0.003	ND
0.1	2.7 ±0.2	0.67 ±0.05	0.77 ±0.03	ND
0.12	2.58 ±0.13	0.53 ±0.09	0.54 ±0.07	ND
0.14	2.88 ±0.61	0.66 ±0.05	0.62 ±0.04	ND
0.17	2.84 ±0.15	0.73 ±0.14	0.94 ±0.11	ND

All values represent the mean ±SD of triplicate experiments. +INF-γ represents the cells grown in the presence of 100 U/ml INF-γ for 48 hours (the medium was removed after first 24 and cells were incubated with fresh IFN-γ), -IFN-γ indicates the cells grown in medium alone for 48 hours, prior to exposure to various levels of pneumolysin.

cells but the effect of sublytic concentrations of pneumolysin have been in sharp contrast to its inhibition of the respiratory burst of neutrophils (Paton and Ferrante, 1983; Mitchell *et al.*, 1989; Saunders *et al.*, 1989).

The mechanism of action by which IFN-γ treatment made cells more resistant to pneumolysin was not investigated in this study. Pneumolysin is a thiol activated toxin (Paton *et al.*, 1993). The available data suggest that different cell types exhibit differences in their susceptibility to other thiol activated toxins (Tanigawa *et al.*, 1996; Walev *et al.*, 1995). For example, immature myeloid cells were found to be more susceptible to the action of streptolysin-O, a thiol-activated toxin, than the mature cells (Tanigawa *et al.*, 1996). These researchers proposed that differences in cell susceptibility may originate as a result of changes in plasma membrane proteins as differentiation occurs. A similar change in the composition of membrane proteins of U937 cells in the activation process can not be ruled out.

In addition, effects of thiol-activated toxins may be subverted by some cell types through their ability to tolerate formation of a limited number of polymers and through shedding of nonoligomerized toxin from their surface (Walev *et al.*, 1995). This postulation came when erythrocytes, keratinocytes, monocytes and fibroblasts of various species showed different membrane lesions after streptolysin-O treatment. The results showed that despite identical binding and polymerisation of streptolysin-O, indicating membrane damage, different amount of lysis was detected for different cell types. Although it is not known if IFN-γ treatment can result in tolerance of polymers or shedding of nonoligomerized toxin from the cell membrane, studies performed with polymorphonuclear leukocytes (PMNLs) showed that PMNLs are capable of removing sublytic membrane attack complexes of complement, enabling them to resist killing (Campbell and Morgan, 1985). It was shown that the removal of sublytic membrane attack complexes of complement happens via vesiculation, probably by increasing intracytoplasmic Ca^{2+} (Campbell and Morgan, 1985). Although the model used in the aforementioned study was different to ours, a similar system might be exist in U937 cell and IFN-γ might be activating the process.

Alternatively, it can be suggested that IFN-γ shows its effect by affecting cell's ability to bind pneumolysin. A study investigating the amount of membrane-bound pneumolysin in IFN-γ activated and non-activated cells may help explain the mechanism of protection provided by IFN-γ against adverse effects of pneumolysin.

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