Studies on pneumolysin and on pneumococcal gene expression under aerobiosis

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at the University of Leicester

By

Magda Elizabeth Bortoni Rodriguez

Department of Infection Immunity and Inflammation

University of Leicester

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Para Mama y Papa
Alberto y Yohann

Statement of Originality

The accompanying thesis submitted for the degree of Ph.D. entitled "Studies on pneumolysin and identification of the pneumococcal gene expression under aerobiosis" is based on work conducted by the author in the Department of Infection Immunity and Inflammation of the University of Leicester mainly during the period between September 2002 and March 2006.

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

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

Signed: _____ Date: _____

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Abstract

In this thesis two aspects of *Streptococcus pneumoniae* were studied. First, studies were done on the pneumolysin toxin of the pneumococcus, which has been shown to play an important role in the development of disease. This toxin is known to possess cytolytic and complement activation activities, which contribute to virulence in different ways. However, mutational studies showed that pneumolysin also contributes to virulence by a still unknown function. Following *in vitro* studies with a truncated pneumolysin it was reported that independent of its cytolytic activity, pneumolysin induces the production of interferon-γ and nitric oxide from spleen cells. It was hypothesised that this could be the third contribution to virulence of pneumolysin. In the first project, this hypothesis was tested by studying the behaviour and virulence, in mouse models of disease, of a pneumococcal mutant that expressed a truncated pneumolysin. The hypothesis was not confirmed and the production of interferon-γ and nitric oxide in the infected mice was found independent of the presence of pneumolysin. Interestingly, it was also found that the pneumococcal mutant was less virulent than a mutant that completely lacked the production of pneumolysin.

The pneumococcus is an aerotolerant anaerobe that usually infects through the nasopharynx. There it must survive in the oxygen rich environment to persist in the infected host. The second project of this thesis aimed to understand how the pneumococcus is capable of adapting to the presence of oxygen. This was done by the use of microarrays to compare the gene expression of aerobically and anaerobically grown bacteria. The results showed 55 genes up-regulated and 14 genes down-regulated when *S. pneumoniae* was subject to aerobiosis. Gene *rgg*, which was up-regulated during aerobiosis, has been found in other bacteria to be involved in biofilm formation, energy metabolism and oxidative and thermal stress. This gene was chosen for further study. A pneumococcal *rgg* knockout mutant was constructed and *in vitro* growth under aerobic and anaerobic conditions studied.

Preamble – the structure of the thesis

This thesis reports two projects. The first, which involves studies on the pneumolysin toxin of *S. pneumoniae*, was originally meant to be a short project that would provide the necessary skills for the development of the second project. The second project was aimed at understanding how the pneumococcus adapts to the presence of oxygen. In the thesis, which consists of six chapters, the first and second chapters include the introduction for both of the projects and the materials and methods used in both projects. Then the pneumolysin project is described first, with the third chapter dedicated to the results, and the fourth to the discussion, conclusions and further work. The fifth and sixth chapter are dedicated to the study of the pneumococcal gene expression under aerobiosis, with the fifth chapter giving the results and the sixth the discussion, conclusions and further work of the project.

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Chapter 1

Introduction

1.1 *Streptococcus pneumoniae*: microbiology, disease and therapy

The discovery of *S. pneumoniae* was made more than a hundred years ago when George Miller Sternberg injected his own saliva into a rabbit and recovered pneumococci from the sick animal (Sternberg, 1881). Since then, the importance of the pneumococcus as a major pathogen has become evident.

S. pneumoniae is a Gram-positive encapsulated bacterium (Figure 1.1-1A and D) commonly referred to as the pneumococcus. It is a fermentative aerotolerant organism of approximately 1μm in size that has a lancet shape (Figure 1.1-1B). It can normally be seen as diplococci or forming chains (Figure 1.1-1 A and B) (Stevens and Kaplan, 2000). The pneumococcus is a α-haemolytic bacterium (Figure 1.1-1C), it does not form spores, it is non-motile and it is catalase negative (Stevens and Kaplan, 2000). It is a fastidious bacterium that grows best in complex medium containing a source of catalase, such as blood or serum when incubated under 5-10% CO_2 at 37°C (Mims, 1998). *S. pneumoniae* is sensitive to ethyl hydrocupreine hydrochloride (optochin), which is used for the presumptive identification of *S. pneumoniae* (Stevens and Kaplan, 2000). In Figure 1.1-1C, an example of this pneumococcal sensitivity is shown, where a growth inhibition halo can be observed around the optochin disc.

Based on the antigenicity of its capsule, 90 different serotypes of pneumococcus have been identified. These can be distinguished with the use of capsular antiserum in the Quellung reaction. Figure 1.1-1D shows an example of Quellung reaction in the pneumococcus. As it can be seen from the Figure, the pneumococcal capsule becomes visible under the microscope when capsular antibodies bind to its surface (Stevens and Kaplan, 2000).

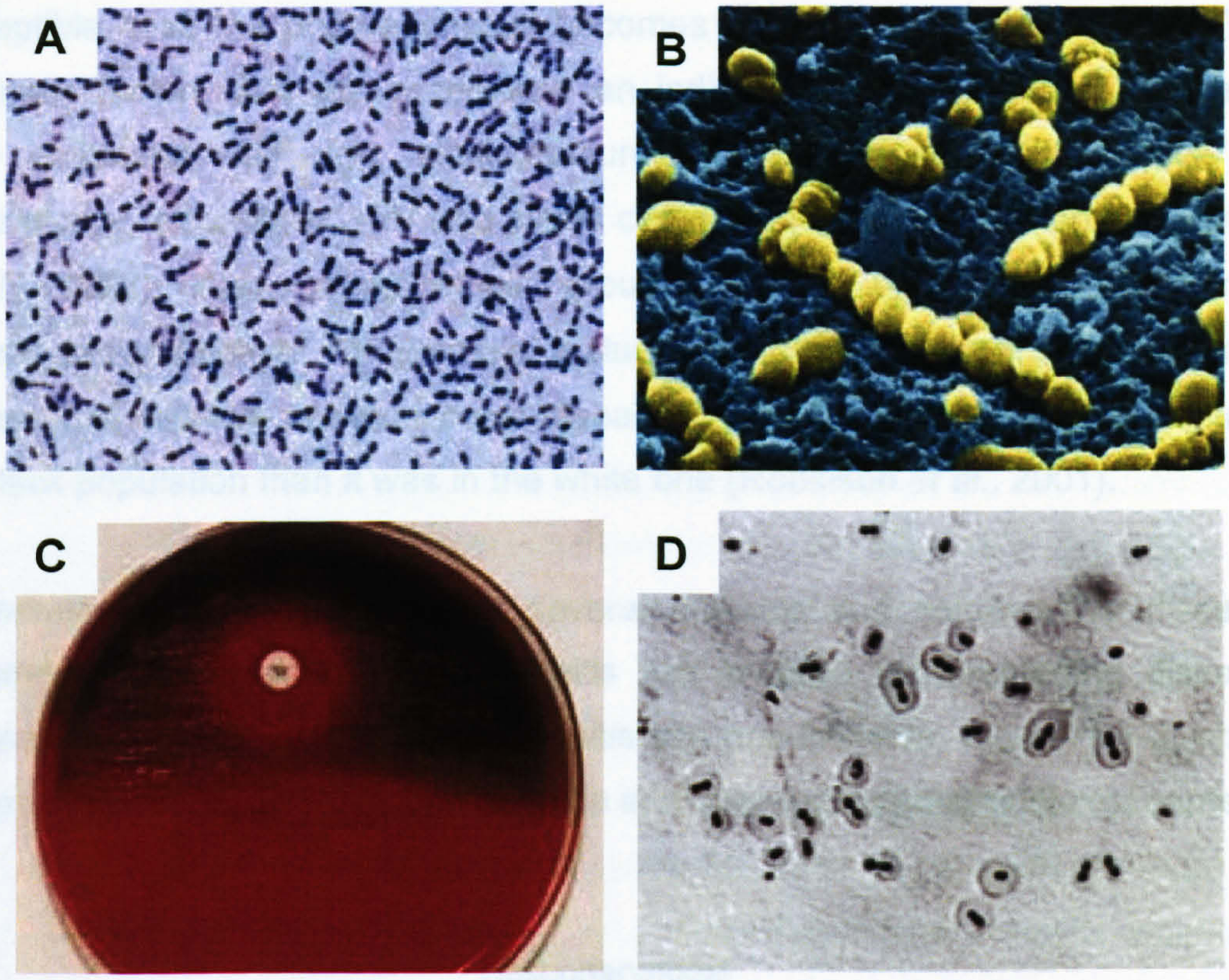


Figure 1.1-1 A.- Gram positive stained pneumococci (Skvara 2006). B.- Coloured electron microscopy of *S. pneumoniae* (webpage-1), note the lancet shape of the bacterium. C.- Pneumococcal growth on a blood agar plate. α-haemolysis can be seen with the dark green colour on the areas of bacteria growth, a halo of growth inhibition is visible around the optochin disc. (Duckworth *et al.*, 2006). D.- Visible pneumococcal capsule after the addition of pneumococcal antiserum (Quellung reaction) (webpage-2).

The pneumococcus is a common commensal of the upper respiratory tract and can colonise the nasopharynx without causing disease (Mulholland, 1999), but in a susceptible host, the pneumococcus becomes a very important pathogen. There are many factors that can predispose an individual to pneumococcal diseases. Early childhood, old age, skull fractures, malformations of the inner ear, splecnotomy, alcoholism, HIV and sickle cell disease are some examples (Obaro *et al.*, 1996). Also, some ethnic groups have a higher predisposition to pneumococcal disease. For example, a study in the United States, showed that the incidence of invasive pneumococcal diseases was more than two times higher in the black population than it was in the white one (Robinson *et al.*, 2001).

S. pneumoniae is responsible for several invasive and non-invasive diseases. Pneumonia, bacteraemia and meningitis are invasive life-threatening diseases, whereas middle-ear infection and sinusitis are non-invasive. Figure 1.1-2, shows how pneumococcal diseases can develop in a susceptible pneumococcal carrier.

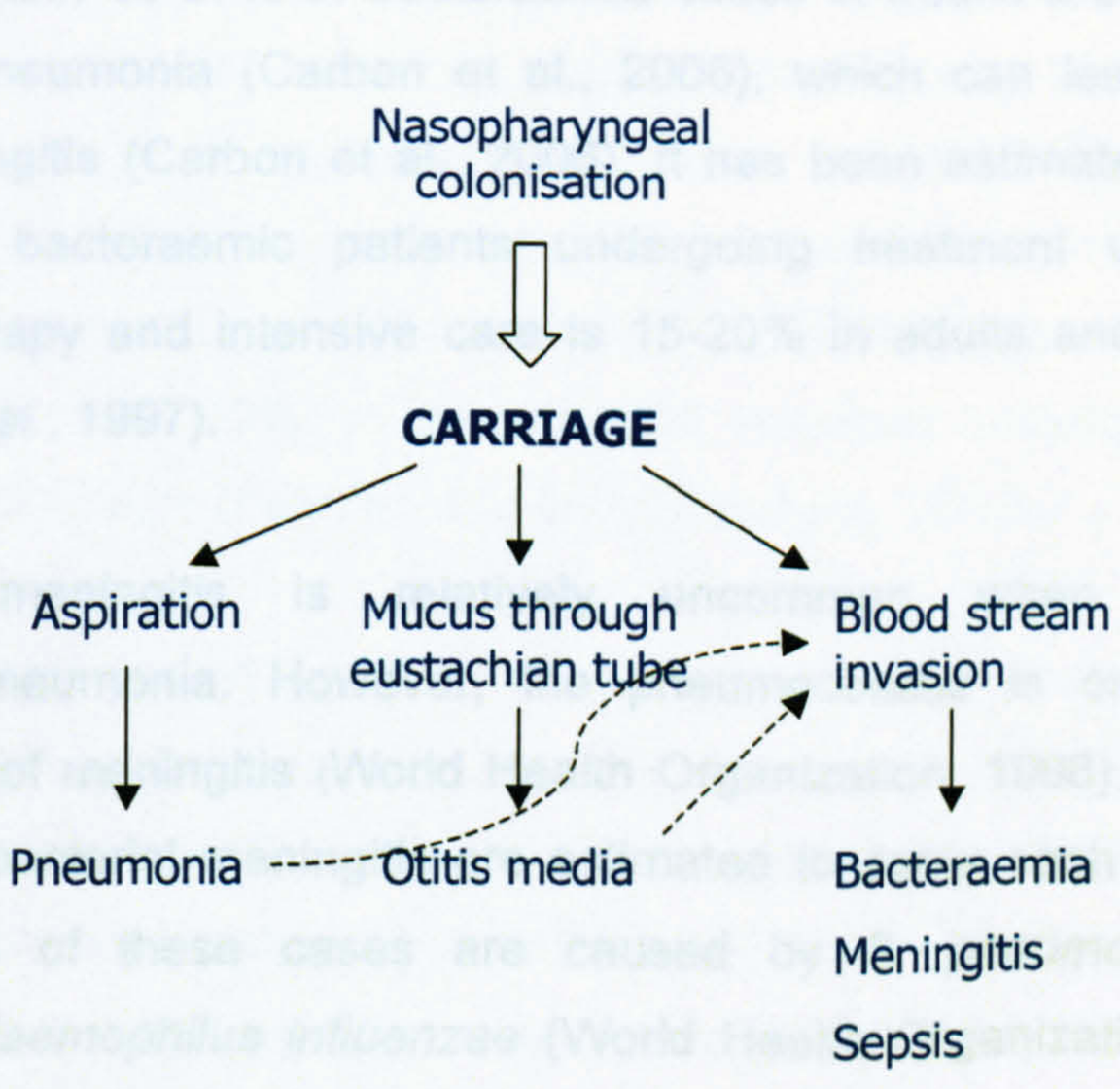


Figure 1.1-2 Possible development of disease in a pneumococcal carrier (figure modified from Briles *et al.*, 2000)

Pneumococcal diseases represent a major concern to public health, as they form a leading cause of morbidity and mortality worldwide (Obaro, 2000), representing the fifth leading cause of death worldwide from invasive infections (Kadioglu and Andrew, 2004). Otitis media is the most common cause of all pneumococcal diseases. In the United States alone, 7 million cases of otitis media caused by the pneumococcus are reported each year (World Health Organization 1999), however, it rarely represents a life-threatening disease (Mulholland, 1999). In contrast, pneumococcal pneumonia represents a serious life-threatening condition. It is estimated that 2.7 million children die of pneumonia each year, with the majority of cases attributed to the pneumococcus (Mulholland, 1999). Most of these deaths are found in underdeveloped countries (Obaro, 2000), but in economically developed regions pneumococcal pneumonia is still very important, claiming a 50% of mortality in the high-risk population and from 10-20% mortality in adults (World Health Organization 1999).

Progression to bacteraemia is a serious complication of pneumococcal pneumonia. In the United States, 60-87% of bacteraemia cases in adults are associated with pneumococcal pneumonia (Carbon et al., 2006), which can lead to respiratory failure and meningitis (Carbon et al., 2006). It has been estimated that the case fatality rate for bacteraemic patients undergoing treatment with appropriate antimicrobial therapy and intensive care is 15-20% in adults and 30-40% in the elderly (Nuorti *et al.*, 1997).

Pneumococcal meningitis is relatively uncommon when compared to pneumococcal pneumonia. However, the pneumococcus is one of the most common causes of meningitis (World Health Organization, 1998). Worldwide, 1.2 million cases of bacterial meningitis are estimated to occur each year (excluding epidemics). 80% of these cases are caused by *S. pneumoniae*, *Neisseria meningitidis* or *Haemophilus influenzae* (World Health Organization, 1998). With the introduction of a conjugate vaccine against *H. influenzae*, the pneumococcus has become the most important pathogen of bacterial meningitis where the *H. influenzae* vaccine is available (Arditi *et al.*, 1998). For example, in the United States 47% of bacterial meningitis cases have been attributed to the pneumococcus (Morton and Swartz, 2004). Pneumococcal meningitis has a

mortality rate of 20-30% in patients with antibiotic treatment (Morton and Swartz, 2004).

To combat pneumococcal infections, penicillin has been the treatment of choice for many years. With the use penicillin, the mortality rate for pneumococcal meningitis was reduced from 100% in the 1920 to 49% by mid 1940 (Morton and Swartz, 2004). Currently the use of antibiotics has reduced the mortality rate to 20-30% (Morton and Swartz, 2004). Similarly, the mortality rate for pneumococcal pneumonia was 30-35% before the introduction of penicillin, which decreased to 5-8% with its use (Peterson, 2006). Hence, the rapid development of antibiotic resistance observed in the later years by the pneumococcus is an issue of major concern. The first report of pneumococcal resistance to penicillin was from Australia in 1967 (Goldsmith *et al*, 1997). In the late 1970's, penicillin resistance was reported in South Africa and Spain (Lynch and Zhanel, 2005). By the early 1990's, strains resistant to penicillin became a worldwide issue, pneumococcal strains were also showing resistance to macrolides, and multi-drug resistant strains had appeared (Lynch and Zhanel, 2005). Currently, it is estimated that 20-30% of pneumococcal isolates are multi-drug resistant (Lynch and Zhanel, 2005). For example, information from the Alexander Project 1996-1997 has reported that approximately 50% of pneumococcal penicillin resistant isolates were also resistant to macrolides, doxycycline and chloramphenicol (Felmingham and Gruneberg, 2000). Also, more than 90% of the penicillin resistant isolates were reported resistant to co-trimoxazole (Felmingham and Gruneberg, 2000).

To obtain a general view of the distribution of pneumococcal strains resistant to antibiotics and the rapid increase in antibiotic resistant strains, Figure 1.1-3 and Figure 1.1-4 are shown. In Figure 1.1-3, the percentage of pneumococcal strains resistant to penicillin and erythromycin in Europe (Figure 1.1-3A) and in the rest of the world (Figure 1.1-3B) are shown per country according to the information published from the Alexander Project 1998-2000 (Jacobs *et al.*, 2003). The graph in Figure 1.1-4 from Felmingham *et al.* (2005) shows the increase in reports of pneumococcal resistant isolates from 1992 to 2001 from six different countries.

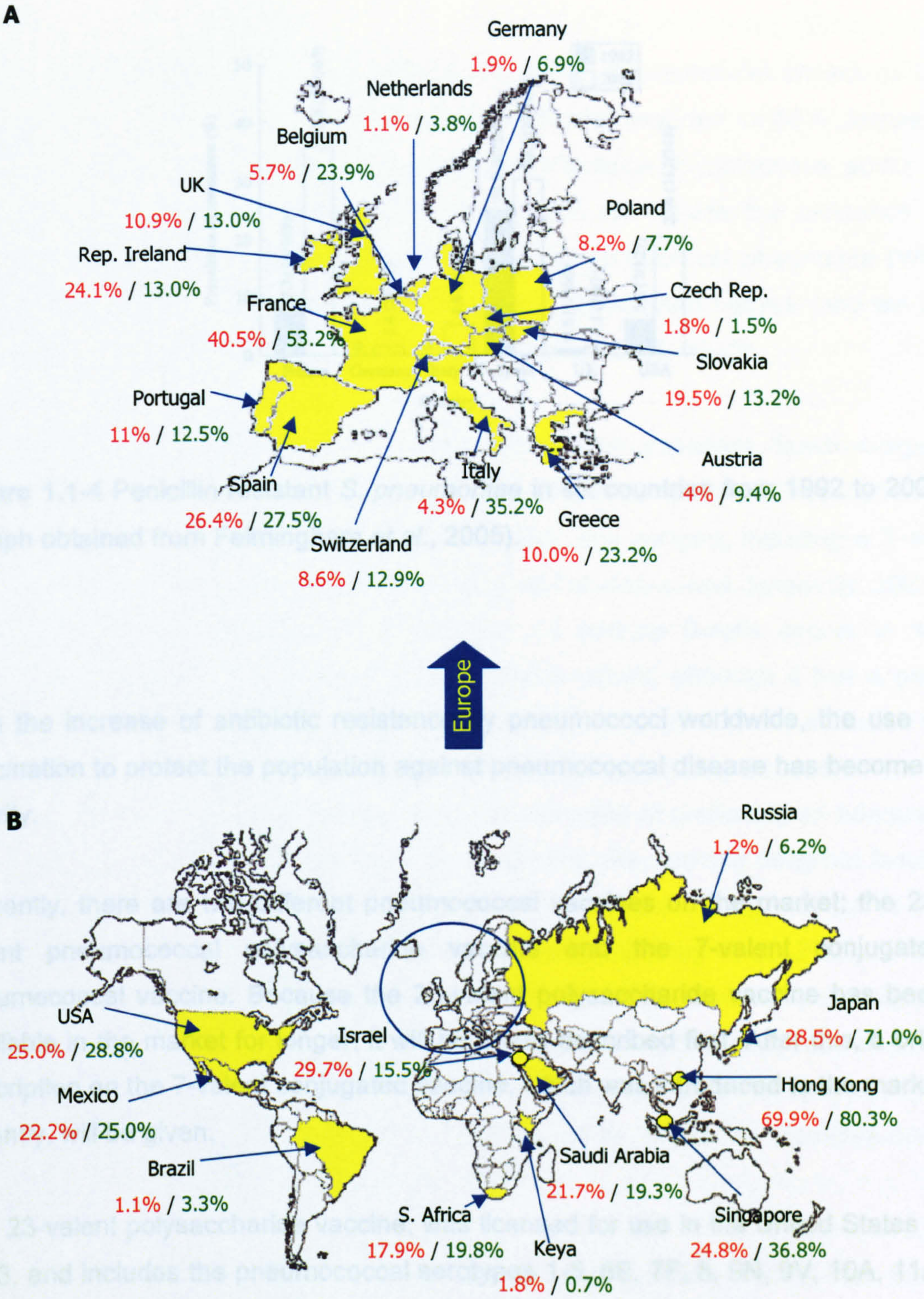


Figure 1.1-3 Percentage of pneumococcal isolates resistant to penicillin and erythromycin reported in (A) Europe and (B) rest of the world from 1998 to 2000. Resistance to penicillin is shown in red numbers and resistance to erythromycin in green.

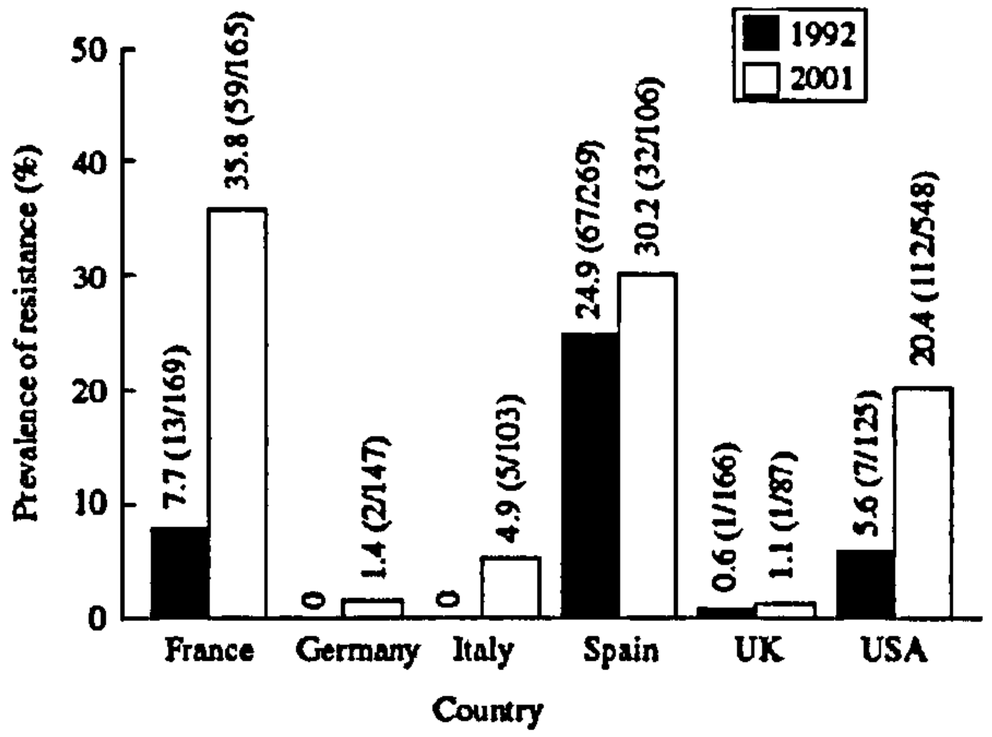


Figure 1.1-4 Penicillin resistant *S. pneumoniae* in six countries from 1992 to 2001 (Graph obtained from Felmingham *et al.*, 2005).

With the increase of antibiotic resistance by pneumococci worldwide, the use of vaccination to protect the population against pneumococcal disease has become a priority.

Currently, there are two different pneumococcal vaccines on the market; the 23-valent pneumococcal polysaccharide vaccine and the 7-valent conjugated pneumococcal vaccine. Because the 23-valent polysaccharide vaccine has been available in the market for longer, it will be briefly described first. After this, a brief description on the 7-valent conjugated vaccine, which was introduced to the market recently, will be given.

The 23-valent polysaccharide vaccine, was licensed for use in the United States in 1983, and includes the pneumococcal serotypes 1-5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19A, 19F, 20, 22F, 23F and 33F. This vaccine is produced by two pharmaceutical companies: Merck and Company Inc, which uses the commercial name of "Pneumovax23" and by Wyeth Lederle Laboratories, with the name "Pnu-Immune". The vaccine is recommended for use in elders over the age of 65 and in persons from 2-65 years of age suffering of chronic organ failure, diabetes and asplenia (Schrag *et al.*, 2001).

Since its introduction, there has been evidence of the beneficial impact of the vaccine. For example, Hanna and collaborators (2006) reported an 86% decrease in vaccine preventable invasive pneumococcal infections in indigenous adults in Australia. In Spain, vaccination of the elderly did not reduce the incidence of disease, but did reduce the mortality caused by pneumococcal pneumonia (Vila-Corcoles et al., 2005), and a study done in alcoholic Alaskan natives reported an adequate antibody response to the vaccine (McMahon et al., 1993).

Unfortunately, the polysaccharide vaccine has several important disadvantages, mainly involved with the way by which it confers protection. Polysaccharide vaccines contain bacterial polysaccharide as the only antigen, inducing a T-cell independent type-2 immune response in the host (Jakobsen and Jonsdottir, 2003). In this response the production of antibody by mature B-cells occurs in the absence of T-helper cells. It is efficient in healthy adults, although it has a poor induction of memory cells and it produces mainly IgM, without the induction of isotype switching (Jakobsen and Jonsdottir, 2003; Eskola, 2000). As most of the B-cells in young children are immature, they are incapable of producing an adequate response to this kind of stimulus. As a consequence, this vaccine does not induce an effective immune response in children under two years of age, which is the population at greatest risk of pneumococcal diseases (Schrag *et al.*, 2001). Other high-risk groups, such as patients with leukemia and AIDS also have a diminished antibody response to the vaccine (Nuorti *et al.*, 1997). In addition, the vaccine does not prevent pneumococcal carriage and it does not protect against non-bacteraemic pneumococcal disease or common upper respiratory diseases such as otitis media and sinusitis (Schrag *et al.*, 2001).

Because of these problems, the development of a conjugated vaccine has been the focus of recent research. Conjugated vaccines consist of purified polysaccharide capsule covalently attached to a protein carrier. They also contain an adjuvant to enhance immunogenicity (Eskola and Anttila, 1999). After processing, protein antigens are presented to T-helper cells producing a T-cell dependant response (Eskola and Anttila, 1999). T-helper cells induce B-cells to mature into plasma cells producing high levels of antibody and isotype switching

into IgG. This kind of response also stimulates B-cells to mature into memory cells (Eskola and Anttila, 1999) providing long term protection. In contrast to polysaccharides vaccines, the T-cell dependent immune response that conjugated vaccines induce can occur in very young children (Eskola, 2000).

The currently licensed 7-valent conjugated vaccine is manufactured by Wyeth, with the commercial name of Prevnar. It was licensed for use in children in 2000 in the United States, and has been introduced for use in the United Kingdom in 2006. It includes the polysaccharides of serotypes 4, 6B, 9V, 14, 18C, 19F and 23F, each conjugated to CRM197 protein, which is a non-toxic variant of the diphtheria toxin (Biagini et al., 2003). The manufacturer's recommendation is for vaccination at 2, 4, 6 and 12-15 months of age. In the UK, the vaccine is being introduced in the children immunisation program at 2, 4 and 13 months (Department of health, 2005).

This vaccine has proved to be effective in young children and to significantly reduce the risk of invasive pneumococcal diseases. Haddy *et al.* (2005) reported from a study done in Louisville, Kentucky, a 67% reduction in the number of pneumococcal invasive diseases in children under the age of 2 after the vaccine was introduced. Another study from Northern California reported a 90.9% reduction in invasive pneumococcal diseases in children under the age of 2 when comparing the four years before the license of the vaccine against the second year after the introduction of the vaccine (Black *et al.*, 2004). This group also reported a statistical decrease in pneumococcal diseases among persons from 20-39 years and above 60 years. They hypothesised an indirect effect of the vaccine caused by a reduction of nasopharyngeal carriage in children. Reduction of nasopharyngeal carriage by conjugated vaccines has been demonstrated. For example, in a trial done in Israel with a 9-valent conjugated vaccine, there was a 39% reduction in the carriage of vaccine serotypes in the test group compared to the control group (Givon-Lavi *et al.*, 2003).

Despite the success of the new heptavalent conjugated polysaccharide vaccine, there are issues of major concern. Firstly, the limited number of pneumococcal serotypes for which the vaccine confers protection. In Asia and Latin America, the serotypes included in the heptavalent conjugated vaccine, represent only 50% or

less of those responsible for invasive diseases (Hausdorff *et al.*, 2000). Another issue is serotype replacement. There is evidence that where the number of invasive pneumococcal diseases has decreased, there is an increase in the amount of invasive diseases caused by serotypes not included in the vaccine. For example, Schutze *et al.* (2004) reported an increase in non-vaccine isolates from children of up to 24 months from 1.3/100 cases 30.5/100 cases. They also described data from the United States Multicenter Pneumococcal Surveillance Group, that suggested that the rates of non-vaccine serotypes causing invasive diseases increased from ~6% to 37.6% after the introduction of the vaccine in children of 24 months or younger. Kaplan *et al.* (2004) also mentioned an apparent increase in penicillin resistance amongst non-vaccine serotypes. Finkelstein *et al.* (2003) reported serotype substitution in nasopharyngeal carriage after studies in Massachusetts. They observed a decrease in the carriage of serotypes included in the vaccine and an increase in non-vaccine serotypes. Consequently, rates of pnemococcal nasopharyngeal carriage remained unchanged before and after the introduction of the heptavalent vaccine. Similar findings were made after a trial of a 9-valent conjugated pneumococcal vaccine in Israel (Givon-Lavi *et al.*, 2003).

Another big disadvantage of this vaccine is the cost. In a report on economics of vaccine development and implementation from the WHO, it is reported that the cost for a full 4 dose Prevnar immunisation program is 232 U.S. dollars per child. This price is considered to be cost-effective in developed countries, but in the underdeveloped world, which is the most affected with pneumococcal diseases, a routine immunisation program with Prevnar is unaffordable (Richmond, 2004).

With all this evidence, the pneumococcal problem has not been solved. The ideal pneumococcal vaccine could be a protein vaccine that can confer protection to all pneumococcal serotypes and that can be produced at a very low cost. Currently, studies where different pneumococcal virulence factors are being evaluated for their potential use as a vaccine are being done. These include the pneumococcal surface proteins PspA and PspC, the pneumococcal surface antigen PsaA, and the pneumococcal haemolytic toxin pneumolysin (Anonymous 2001).

1.2 Pneumococcal virulence factors

There are many virulence factors of *S. pneumoniae* that are essential for its interaction with the host. Pneumococcal virulence factors play different roles in the maintenance of colonisation and in the production of invasive disease (Hava *et al.*, 2003). Figure 1.2-1 shows some important identified virulence factors in the pneumococcus. Because the first project described in this thesis studies pneumolysin, one of the most prominent virulence factors of the pneumococcus, it will be described in more detail. However, Table 1.2-1 shows a summary of the properties that are attributed to the different virulence factors shown in Figure 1.2-1. The information used in the Table 1.2-1 was obtained from Alonso De Velasco *et al.*, 1995; Bergmann and Hammerschmidt, 2006; Jedrzejas, 2001; Mitchell, 2000, Manco *et al.*, 2006 and Jounblat *et al.*, 2003.

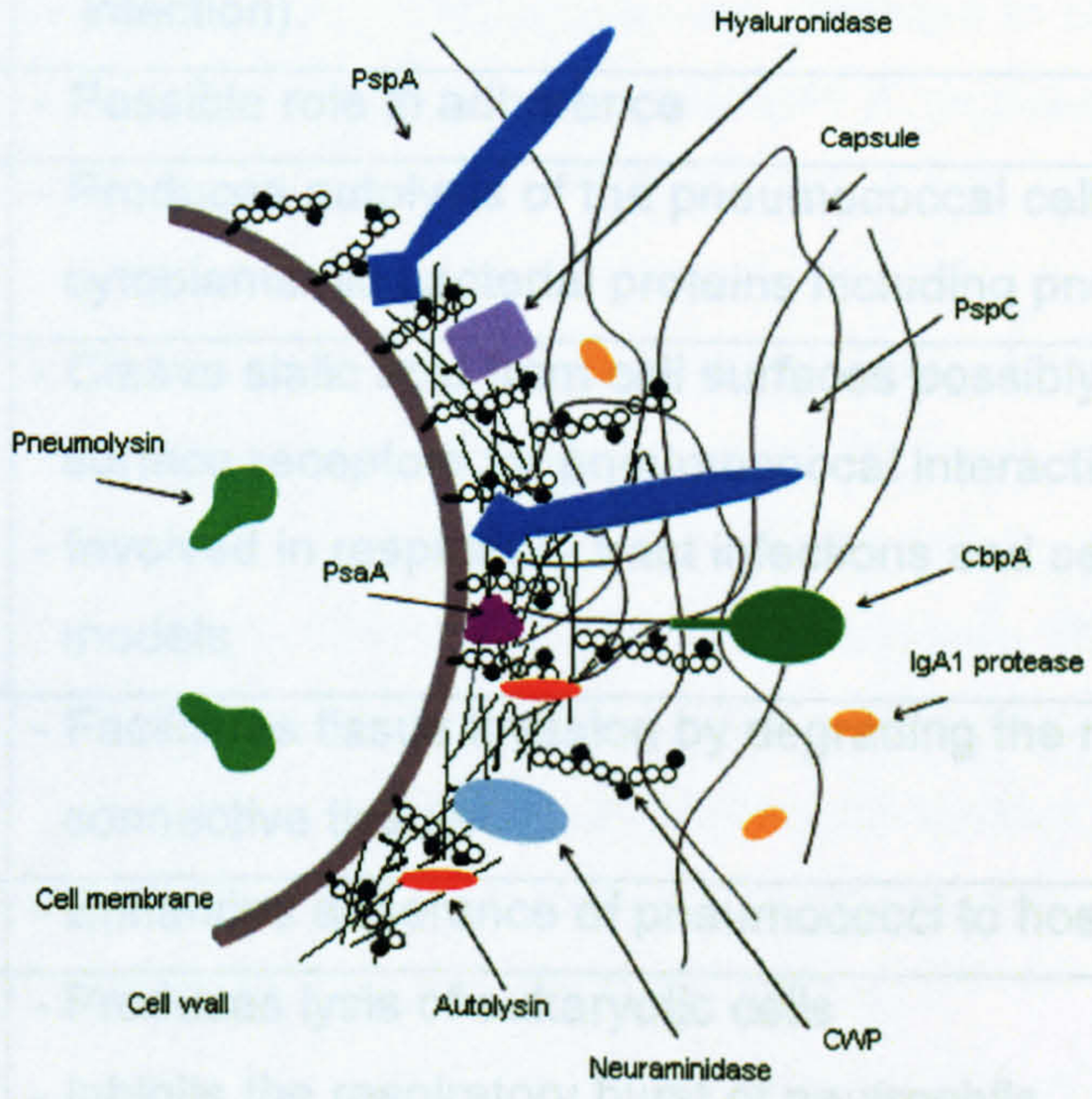


Figure 1.2-1 Pneumococcal virulence factors. (CW) cell wall; (CWP) cell wall polysaccharide; (PspA and PspB) pneumococcal surface protein A or B; (PsaA) pneumococcal surface antigen A; (CbpA) choline binding protein A. (Modified from Briles *et al.*, 2000 and Jedrzejas, 2001)

Table 1.2-1 Summary on the virulence properties attributed to different virulence factors in the pneumococcus. (CW) cell wall; (CWP) cell wall polysaccharide; (PspA and PspB) pneumococcal surface protein A or B; (PsaA) pneumococcal surface antigen A; (CbpA) choline binding protein A.

Virulence factor	Virulence properties
Capsule	- Provides resistance to phagocytosis - Does not activate the complement pathway - Does not cause inflammation
CW, CWP	- Produces strong inflammatory effects
PspA	- Prevents bactericidal activity of apolactoferrin - Reduces phagocytosis and complement mediated clearance
PspC	- Promotes pneumococcal adherence to epithelial cells
PsaA	- Transport of Mn^{2+} and Zn^{2+} into the cytoplasm of the bacteria (possibly needed as a growth factor during infection).
CbpA	- Possible role in adherence
Autolysin	- Produces autolysis of the pneumococcal cell releasing cytoplamsmic bacterial proteins including pneumolysin.
Neuraminidases	- Cleave sialic acid from cell surfaces possibly exposing cell surface receptors for pneumococcal interaction. - Involved in respiratory tract infections and sepsis in mouse models
Hyaluronidase	- Facilitates tissue invasion by degrading the matrix of connective tissue.
IgA1 protease	- Enhances adherence of pneumococci to host cells
Pneumolysin	- Produces lysis of eukaryotic cells - Inhibits the respiratory burst of neutrophils - Activates the complement pathway

1.2.1 Pneumolysin

Pneumolysin is a multifunctional cytolytic toxin that contributes widely to pneumococcal virulence and is present in virtually all pneumococcal clinical isolates (Rubins and Janoff 1998). This toxin is considered a key virulence factor of the pneumococcus (Kadioglu *et al.*, 2002). In fact, it has been shown that pneumococcal mutants that lack the production of this protein have a considerably reduced virulence in mouse models of pneumonia and sepsis (Kadioglu *et al.*, 2002; Benton *et al.*, 1995). Also, the pneumolysin protein is capable of producing the signs of pneumonia when introduced into the lung (Feldman *et al.*, 1990).

Pneumolysin is a 53kD protein that contains 471 amino acids and belongs to the family of cholesterol-binding pore-forming toxins (Rubins and Janoff 1998; Gilbert *et al.*, 1999). This family can recognise cholesterol in the membrane of eukaryotic cells and form transmembrane pores. Gram-positive bacteria from the family of *Streptococcus*, *Bacillus*, *Clostridium* and *Listeria*, have been found to produce cholesterol-binding toxins (Palmer, 2001). However, different to other members of this family, pneumolysin is not a secreted protein and it requires the lysis of the pneumococcal cell for its release (Palmer, 2001).

In the pathogenesis of pneumococcal disease, pneumolysin lyses eukaryotic cells by forming pores in their membranes. During infection, the lysis of eukaryotic cells in the host tissue may facilitate the spreading of bacterial cells to other parts of the organism (Alouf, 2001). Also, damaged cells may provide the bacteria with essential nutrients to survive in the host (Alouf, 2001). At sublytic concentrations, pneumolysin can modulate the activity of cells of immune system, for example, by inducing the release of cytokines, which in consequence, produce more inflammation and damage to the host tissue (Alouf, 2001).

To date, it has not been possible to obtain the crystal structure of pneumolysin. However, the pneumolysin amino acid sequence has a 48% identity and 60% similarity to the sequence of perfringolysin, a cholesterol-binding toxin from *Clostridium perfringens*, whose crystal structure has been resolved (Gilbert *et al.*,

1999). Because of the sequence similarity between pneumolysin and perfringolysin, a homology model of a pneumolysin monomer has been made based on the crystal structure of perfringolysin (Rossjohn *et al.*, 1997). Based on this homology model it was found that pneumolysin is an enlongated protein that consists of four domains (Figure 1.2-2) (Morgan *et al.*, 1994).

Figure 1.2-2 Homology model of pneumolysin based on the crystal structure of perfringolysin. The tryptophan rich loop of the protein is indicated at the bottom right of the fourth domain (Gilbert *et al.*, 1999).

Each of these domains has a different role in the formation of transmembrane pores. Figure 1.2-3 shows a representation of the suggested mechanism for pore formation (Tilley *et al.*, 2005). First, pneumolysin monomers individually bind the membrane through domain 4 (Figure 1.2-3A). Upon binding, oligomerisation of the pneumolysin monomers is induced. Domains 1 and 3 provide the contacts for oligomerisation to occur, causing a slight bending of domain 2 and producing a pre-pore, which involves the assembly of 38 or 44 monomers (Figure 1.2-3B). After formation of a pre-pore, domains 2 and 3 undergo a complete dissociation where domain 2 "collapses" bringing domain 3 close to the membrane of the cell (Figure 1.2-3C). In this process, alpha helices contained in domain 3 refold into β–hairpins that insert into the cell membrane to form a transmembrane pore (Figure 1.2-3C) (Tilley *et al.*, 2005).

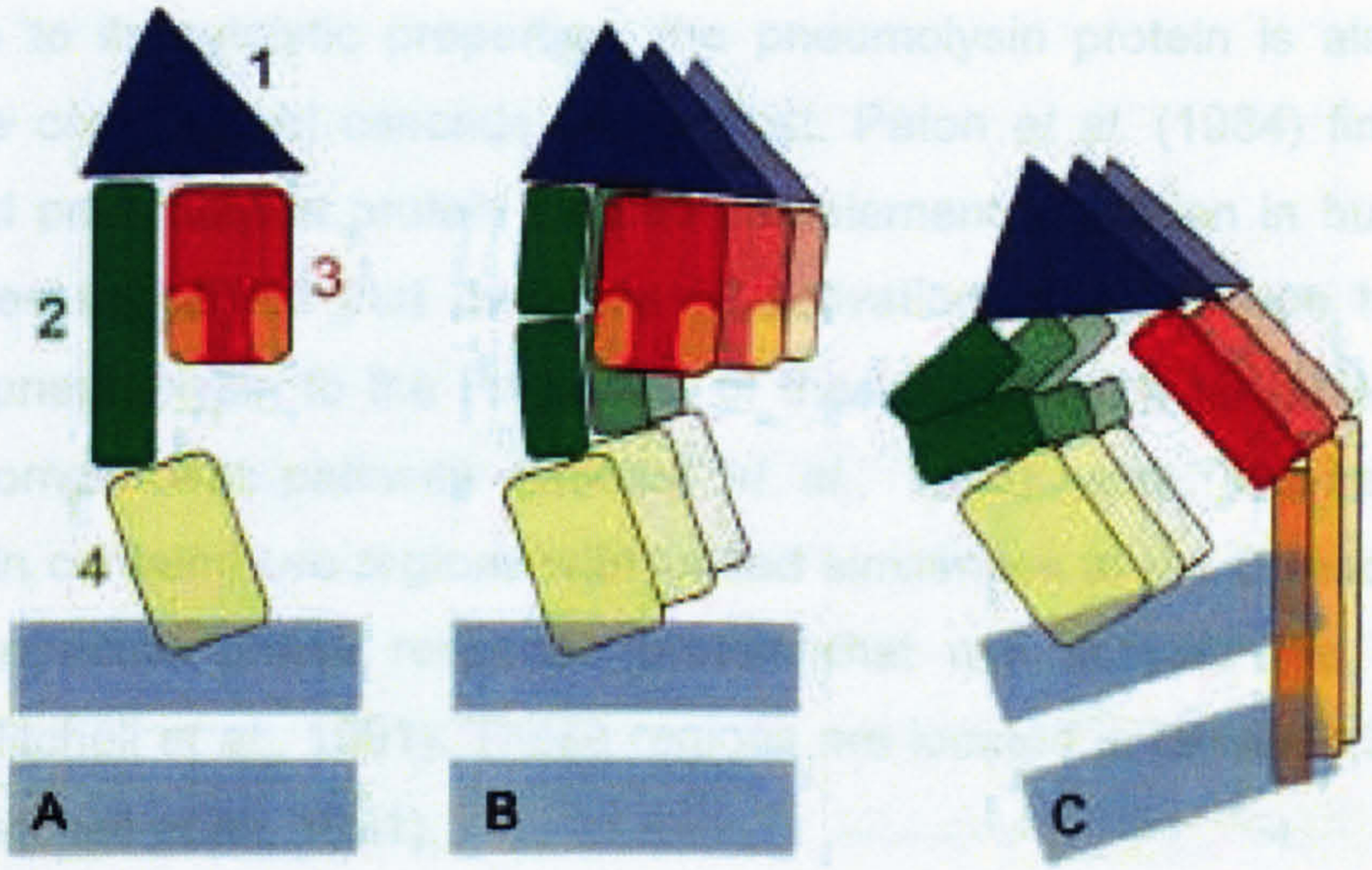


Figure 1.2-3 Cartoon representing the suggested mechanism for pore formation. (A) Pneumolysin monomer binds to membrane surface. (B) Oligomerization and formation of a pre-pore. (C) Pore formation. (Tilley *et al.*, 2005).

The main area of interest in the first part of this thesis, was the domain 4 of pneumolysin. As has been mentioned, domain 4 provides the binding ability of the toxin to cholesterol-containing cells (Rossjohn *et al.*, 1998). This domain, which include residues 360-469, contains an 11-residue sequence known as the tryptophan-rich loop, which is highly conserved between the cholesterol-binding toxins (Gilbert *et al.*, 1999B). In pneumolysin, this domain is located between residues 426 and 437, found at the base of domain four (shown in Figure 1.2-2) (Gilbert *et al.*, 1999B; Rossjohn *et al.*, 1998). During pore formation, this tryptophan-rich loop is associated with the binding of the toxin to the membrane of the eukaryotic cell. Indeed, it has been shown that domain 4 on its own is capable of binding to eukaryotic cells, and that this binding can not be performed by domains 1-3 (Baba *et al.*, 2001). However, although it is known that the fourth domain is essential for the binding of the toxin to the cell, it is not well understood how it interacts with cholesterol.

In addition to its cytolytic properties, the pneumolysin protein is also known to activate the complement cascade in the host. Paton *et al.* (1984) first described that purified pneumolysin protein caused complement activation in human serum. Later studies suggested that complement activation might be due to unspecific binding of pneumolysin to the Fc portion of the IgG, causing the activation of the classical complement pathway (Mitchel *et al.*, 1991). Also, it was found that pneumolysin contains two regions with limited similarities to the C-reactive protein, which is an acute phase response protein that can activate the complement pathway (Mitchell *et al.*, 1991). These regions are located in residues 257-297 and 368-397 (Mitchell *et al.*, 1991).

Mutagenesis studies have revealed specific amino acids responsible for maintaining the cytolytic (commonly referred to as haemolytic) and complement activation activities of the protein. For example, Mitchell and collaborators (1991) showed that point mutation of residue 385 (Asp385), which is located in one of the regions with limited homology to the C-reactive protein, caused a complete inability of pneumolysin to cause complement activation and had a reduced ability to bind to IgG. However, this point mutation did not affect the cytolytic activity of

pneumolysin (Mitchell *et al.*, 1991). In regard to its cytolytic activity, it has been found that point mutations on the conserved undecapeptide of domain four, considerably reduced the cytolytic activity of pneumolysin, in particular point mutations of residues 428 (Cys428) and 433 (Trp433). It has been found, that point mutation of both of these residues, inhibits the haemolytic activity of pneumolysin to 0.001% (Berry *et al.*, 1995; Alexander *et al.*, 1998). Also, residues involved in the oligomerization of pneumolysin have an important role in maintaining its cytolytic activity, for example, point mutation of residue 367 (His367) which has a role in oligomerization, inhibited the haemolytic activity of pneumolysin to only 0.02% of its wild type activity (Berry *et al.*, 1995; Alexander *et al.*, 1998). All of these point mutations that can inhibit the cytolytic activity of pneumolysin, do not affect its complement activation activity (Alexander *et al.*, 1998).

The finding of residues responsible for maintaining each of the known activities of pneumolysin has allowed the study of their contribution to the pathogenesis of pneumolysin during pneumococcal infections. Several studies using pneumococcal mutants that lack the cytolytic and/or complement activation activities of their pneumolysin protein have been done expecting to understand pneumolysins' full contribution to virulence *in vivo*.

Alexander and collaborators (1998) found that each, the cytolytic and complement activation activities of pneumolysin have different roles during pneumococcal pneumonia. They observed that at early hours of infection (up to 24 hours post-infection), complement activation was responsible for the increase of bacteria in the lungs, whereas cytolytic activity had a more important role after 24 hours post-infection. However, they also observed that a pneumococcal mutant lacking both, the cytolytic and complement activating activities was less virulent than the wild type as judged by survival times of mice after infection, but more virulent than the pneumolysin negative mutant (Alexander *et al.*, 1998). For example, when they performed intranasal infections, mice infected with wild type pneumococci had a median survival time of 48 hours, with 0% survivors by the end of their experiment (14 days). By contrast, all of the mice infected with a pneumococcal mutant that lacked the complete production of pneumolysin survived (Alexander *et al.*, 1998). To analyse the effect of the haemolytic and complement activation activities of

pneumolysin, they performed infection with pneumococcal mutants containing different point mutations in their pneumolysin. Infections with a pneumococcal mutant whose pneumolysin protein showed only 0.001% of wild type haemolytic activity but maintained 100% of complement activation activity (obtained by point mutation of residues 428 and 433) had a median survival time significantly higher than after infection with wild type bacteria (74 hours). However, none of the mice were alive by the end of their experiment (Alexander *et al.*, 1998). Infection with a mutant whose pneumolysin toxin exhibit 0% complement activation but retained 100% haemolytic activity (obtained by point mutation of residue 385) increased the median survival time to 84 hours and 30% of the mice survived the experiment. Surprisingly, infections with a pneumococcal mutant whose pneumolysin had only 0.001% of its haemolytic and lacked complement activation activity (obtained by point mutation of residues 385, 428 and 433) had an increased median survival of 105 hours, but still only 30% of the mice survived the experiment. These results lead the authors to hypothesise that pneumolysin possess another unidentified activity that contributes to virulence during pneumococcal infections.

Other studies have also shown that, the haemolytic and complement activation activities of pneumolysin contribute to virulence in different ways, and have also observed that pneumolysin contributes to virulence by another, still unidentified, function. For example, Berry *et al.* (1995) in their experiments, showed that after intraperitoneal infections of mice, the haemolytic activity of pneumolysin had a more important contribution to virulence, as deletion of this function reduced pneumococcal virulence. However, they observed that the complement activation activity of pneumolysin did not contribute to virulence in their model of infection, as deletion of this function, did not affect pneumococcal virulence (Berry *et al.*, 1995). Also, they observed that a pneumococcal mutant that lacked both, cytolytic and complement activation activities, was still more virulent than a pneumolysin negative mutant after intraperitoneal infections of mice (Berry *et al.*, 1999). Similarly, Benton *et al.* (1997) studied the effect of pneumococcal mutants that lacked either or both, the haemolytic and the complement activating activity of pneumolysin in mice after intravenous infections. Although they described that the deletion of the individual properties of pneumolysin did not have a major effect in virulence, they found that deletion of both activities increased the survival time of

mice, however, they also found that the mutant was still more virulent than a pneumolysin negative pneumococcal mutant (Benton *et al.*, 1997). In regard to the different contributions of the cytolytic and complement activation activities of pneumolysin to virulence, Jounblat and collaborators (2003) observed that after intranasal infection, the complement activation activity of pneumolysin caused an initial increase in bacteria killing by the host and a delay in the pneumococcus to enter the blood stream. They also observed that the lack of haemolytic activity impeded the mutant in reaching the high bacterial levels in the blood that the wild type reached. In addition, they observed that the haemolytic activity was responsible of causing a heavier cellular infiltration in the lungs that complement activation activity. Instead, complement activation activity caused less cellular infiltration but higher T-cells recruitment into the lungs of infected mice (Jounblat *et al.*, 2003).

In conclusion, these *in vivo* experiments with mutants lacking the cytolytic and/or complement activating activities of pneumolysin have shown that each of these activities contribute in different ways to pneumococcal pathogenesis. However, pneumococcal mutants lacking the whole pneumolysin protein were still less virulent than mutants lacking both the cytolytic and complement activation activities of the toxin, indicating that there is still an unknown factor in the pneumolysin protein that is also responsible for its pathogenesis. Interestingly, *in vitro* studies from Baba and collaborators (2002) suggested a possible third role of the pneumolysin protein. In their studies they analysed the production of interferon-γ and nitric oxide by purified pneumolysin truncates in which the anti-cellular and cholesterol-binding abilities of the protein were abolished. These truncates consisted of a pneumolysin truncated protein that only contained the first 426 amino acids of pneumolysin (PLY426), and consequently, excluded the conserved undecapeptide of the fourth domain; and a truncated pneumolysin protein that contained 437 amino acids of the pneumolysin protein (PLY437), this included the conserved undecapeptide of the fourth domain (Baba *et al.*, 2002). In their studies, they found that when stimulating spleen cells with these truncates, or with a full pneumolysin protein that had been treated with cholesterol to block its haemolytic activity, the production of interferon-γ was induced and that this induction was

positively related to the production of nitric oxide. On the basis of their results, they proposed that during *in vivo* infections with *S. pneumoniae*, pneumolysin would induce the production of interferon-γ in an independent way to its cytolytic activity. This induction, would lead to the production of high levels of nitric oxide. Because high levels of nitric oxide can produce organ failure and toxic shock in the infecting host, this could contribute to the pathogenesis of pneumococcal infections (Baba *et al.*, 2002). However, this hypothesis has not been tested in *in vivo* models of disease with the use of pneumococcal mutants.

1.2.1.1 Aim of the "studies on pneumolysin" project

The first project of this thesis aimed to demonstrate whether the hypothesis proposed by Baba and collaborators (2002), explained a the unidentified function of pneumolysin described by Alexander and collaborators (1998). Baba *et al.* (2002) suggested that pneumolysin could contribute to the pathogenesis of pneumococcal disease by inducing the production of interferon-γ and nitric oxide independently of the previously described anticellular/cytolytic activity of pneumolysin. To test this hypothesis, a pneumococcal mutant would be constructed that would produce a truncated pneumolysin protein similar to that described by Baba and collaborators (2002). With the mutant, *in vivo* infections could be performed in a mouse model of pneumonia and sepsis to assess its virulence compared to the wild type pneumococcus and to a pneumococcal mutant that lacked the production of pneumolysin. By doing this and by measuring the levels of interferon-γ and nitric oxide in the serum and broncheo-alveolar lavage fluid of mice infected with these pneumococcal strains, it would be possible to test the hypothesis of Baba and collaborators (2002).

1.3 *Streptococcus pneumoniae*, a fermentative bacterium

S. pneumoniae is an aerotolerant pathogenic bacterium that is constantly challenged by its need to survive under oxidative stress conditions. For example, its normal site of colonisation is the nasopharynx, where a highly oxygenated environment is present. Curiously, as it will be analysed in this section, the pneumococcus lacks most of the defenses other bacteria use against oxidative stress and keeps a fermentative metabolism even in the presence of oxygen.

As with other lactic acid bacteria, and regardless of its frequent exposure to aerobic environments, *S. pneumoniae* continues to ferment in the presence of oxygen. Analysis of the partial genome sequence available in 2000, revealed that the pneumococcus lacks 29 genes that are known in *E. coli* to be involved with aerobic respiration and 18 of the genes for the TCA cycle enzymes (Baltz *et al.*, 2000). Table 1.3-1 shows these genes with their cellular role and putative identification according to the TIGR website. In the Table, wherever TIGR uses a synonym of a gene mentioned by Baltz and collaborators (Baltz *et al.*, 2000), the original name used by Baltz is shown in parenthesis; the synonyms of the genes were searched in the EchoBASE website.

The findings by Baltz and collaborators (Baltz *et al.*, 2000) explained genetically the lack of ability of the pneumococcus to switch mode of ATP generation in the presence of oxygen. Hoskins and collaborators (Hoskins *et al.*, 2001) confirmed the previous findings of Baltz, when they published the complete genome sequence of *S. pneumoniae* R6, an avirulent pneumococcal strain. They also reported a lack of genes for anaerobic respiration (Hoskins *et al.*, 2001). Also they confirmed the lack of an electron transport chain in the pneumococcus. Panek and O'Brian (Panek and O'Brian., 2002) showed that the pneumococcus lacks the genes involved in the biosynthesis of haem, and lacks genes for the biosynthesis cytochromes, which utilize haem as a prosthetic group. Instead, it is apparent that the pneumococcus obtains its energy by the conversion of carbohydrates into pyruvate through glycolysis and the conversion of pyruvate into lactic acid to regenerate NADH (Hoskins *et al.*, 2001). However, the pneumococcal genome

does contain genes for the synthesis of enzymes such as phosphotransacetylase, acetokinase and NADH oxidase, which suggest the conversion of pyruvate into acetate via acetyl-phosphate (see Figure 1.3-1), yielding an extra ATP and reoxidation of NADH (Hoskins *et al.*, 2001), which would improve its efficiency of energy production. Evidence for this hypothesis on the conversion of pyruvate into acetate has been provided by the investigations of Spellerberg and coworkers (Spellerberg *et al.*, 1996) and more recently by the studies of Pericone and colleagues (Pericone *et al.*, 2003). Spellerberg *et al.* (1996) reported that a pneumococcal *spx*B-negative mutant was unable to grow in a chemically defined medium in micro-aerophillic conditions unless supplemented with acetate. Similarly, Pericone *et al.* (2003) found that a pneumococcal *spx*B-negative mutant was unable to survive a challenge with H_2O_2, and found that during the challenge, ATP levels decreased more rapidly in the mutant than in the wild type. They also found that the mutant produced lower levels of acetyl-phosphate (Figure 1.3-1), suggesting that the pneumococcus converts pyruvate into acetate to increase the levels of ATP during oxidative stress (Pericone *et al.*, 2003). However, Chapuy-Regaud and collaborators (Chapuy-Regaud *et al.*, 2001) observed only minor traces of acetate in the medium of aerobically and anaerobically growing pneumococcal cells. This, and failure to produce a loss of function mutation on the *ldh* gene, which produces the enzyme lactate dehydrogenase that converts pyruvate into lactate (Figure 1.3-1) led the authors to suggest that the pneumococcus behaves constantly as a homolactic fermentative bacterium despite the presence of oxygen.

Regardless of its fermentative metabolism, the fact that pneumococcus is commonly subject to oxidative stress, makes it interesting to see that it also lacks many of the mechanisms that are known to be used by bacteria, such as *E. coli*, in protection against oxidative stress. These issues will be considered in more detail within this chapter.

Table 1.3-1 Genes involved in the aerobic respiration and TCA cycle of *E. coli* found missing in the sequence of the pneumococcus (Baltz *et al.*, 2000). The cellular role and putative identification of the genes were searched in this project from the *E. coli* K12 database on the TIGR website. When a synonym (as searched in the EchoBASE website) of a gene mentioned by Baltz and collaborators was used by the TIGR website, the name of the gene as used by Baltz is shown in parenthesis.

Genes involved in the aerobic respiration of *E. coli* found missing in the pneumococcus by Baltz *et al.* (Baltz *et al.*, 2000)		
Gene symbol	**TIGR Cellular role**	**TIGR putative identification**
*cyo*A	Energy metab: Electron transport	Cytochrome o ubiquinol oxidase subunit II
*cyo*B	Energy metab: Aerobic	Cytochrome o ubiquinol oxidase subunit I
*cyo*C	Energy metab: Electron transport	Cytochrome o ubiquinol oxidase subunit III
*cyo*D	Energy metab: Aerobic	Cytochrome o ubiquinol oxidase subunit IV
*cyo*E	Unclassified: Role category not yet assigned	Protoheme IX farnesyltransferase (haeme O biosynthesis)
*ddl*A (*ddl*)	Cell envelope: biosynthesis and degradation of murein sacculus and peptidoglycan	D-alanine-D-alanine ligase A
*ddl*B (*ddl*)	Cell envelope: biosynthesis and degradation of murein sacculus and peptidoglycan	D-alanine-D-alanine ligase B, affects cell division
*hya*A	Energy meta: Aerobic	Hydrogenase-1 small subunit
*hya*B	Energy metab: Aerobic	Hydrogenase-1 large subunit
*hya*C	Energy metab: Aerobic	Probable Ni/Fe-hydrogenase 1 b-type cytochrome subunit

*hya*D	Energy metab: Aerobic	Processing of HyaA and HyaB proteins
*hya*E	Energy metab: Electron transport	Processing of HyaA and HyaB proteins
*hya*F	Unknown function: General	Nickel incorporation into hydrogenase-1 proteins
ndh	Energy metab: Aerobic, electron transport	Respiratory NADH dehydrogenase
*lld*D (*lct*D)	Energy metab: Glycolysis/gluconeogenesis	L-lactate dehydrogenase
*lld*R (*lct*R)	Hypothetical proteins: conserved	Transcriptional regulator
*nuo*A	Energy metab: Electron transport	NADH dehydrogenase I chain A
*nuo*B	Unclassified: Role category not yet assigned	NADH dehydrogenase I chain B
[1]*nuo*C	Energy metab: Aerobic, electron transport	NADH dehydrogenase I chain C, D
*nuo*E - *nuo*N	Energy metab : Aerobic, electron transport	NADH dehydrogenase I chain E – chain N respectively.
Genes involved in the TCA cycle of *E. coli* found missing in the pneumococcus by Baltz *et al.* (Baltz *et al.*, 2000)		
*acn*A	Energy metab: TCA cycle	Aconitate hydrase 1
*acn*B	Unclassified: Role category not yet assigned	Acotinate hydrase B
*cit*D	Energy metab: Fermentation	Citrate carrier protein (gamma chain)
*cit*E	Energy metab: Fermentation	Citrate lyase beta chain (acyl lyase subunit)
*cit*F	Energy metab: Fermentation	Citrate lyase alpha chain
*fum*A	Energy metab: TCA cycle	Fumarase A = fumarate hydratase Class I; aerobic isozyme
*fum*B	Energy metab: TCA cycle	Fumarase B = fumarate hydratase Class I; anaerobic isozyme

gltA	Energy metab: TCA cycle	Citrate synthase
[2]*icdC*	Function: C-terminal fragment of isocitrate dehydrogenase.	
icdA (*icdE*)	Energy metab: TCA cycle	Isocitrate dehydrogenase, specific for $NADP^+$
mdh	Energy metab: TCA cycle	Malate dehydrogenase
sdhA	Energy metab: Electron transport	Succinate dehydrogenase, flavoprotein subunit
sdhB	Energy metab : TCA cycle	Succinate dehydrogeanse, iron sulfur protein
sdhD	Unclassified: Role category not yet assigned	Succinate dehydrogenase, hydrophobic subunit
sucA	Energy metab: TCA cycle	2-oxoglutarate dehydrogenase (decarboxylase component)
sucB	Energy metab: TCA cycle	2-oxoglutarate dehydrogenase (dihydrolipoyltranssuccinase E2 component)
sucC	Energy metab: TCA cycle	Succinil-CoA synthetase, beta subunit
sucD	Energy metab: TCA cycle	Succinil-CoA synthetase, alpha subunit

[1] Baltz and collaborators (Baltz *et al.*, 2000) mention gene *nuo*C and *nuo*D, however, in the TIGR and EchoBASE websites, these two genes appear to be the same gene, and it is referred to as *nuo*C.

[2] Gene *icd*C was not found in the *E. coli* K12 database in the TIGR website, the description given is that of the EchoBASE website.

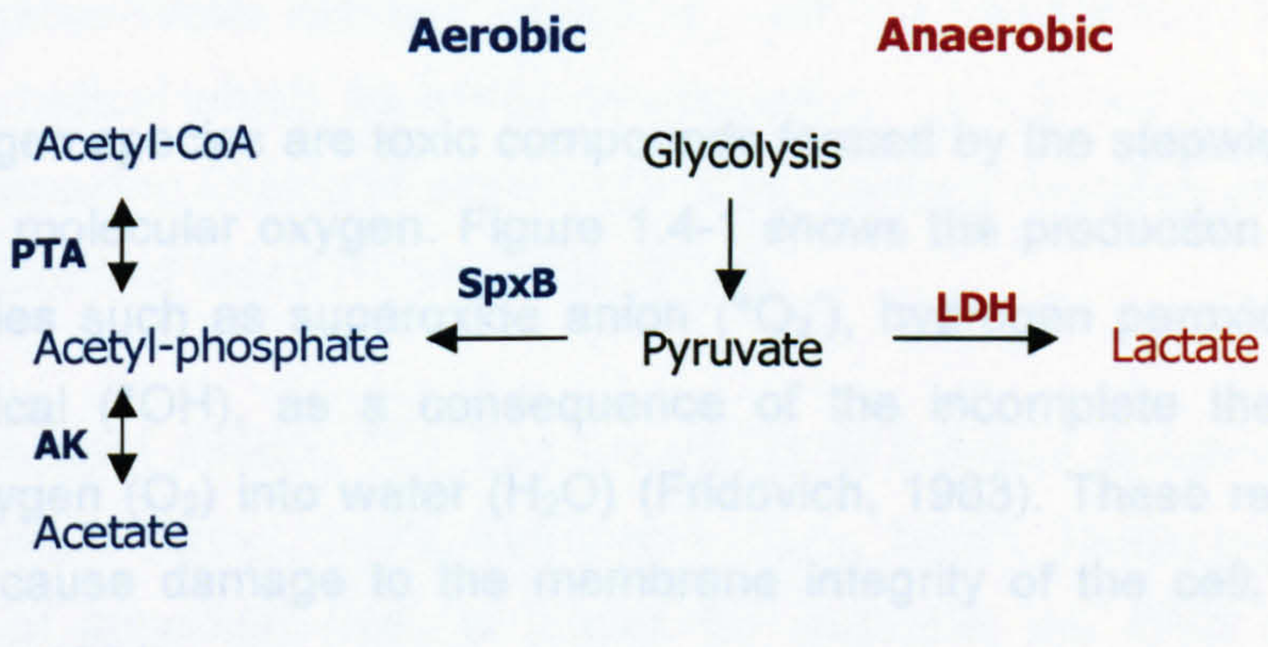


Figure 1.3-1 (modified from Spellerberg *et al.*, 1996). Pathways for metabolism of pyruvate via lactate dehydrogenase (LDH) in anaerobiosis or pyruvate oxidase (SpxB) in aerobiosis. PTA, phosphotransacetylase; AK, acetate kinase.

1.4 Bacteria and oxidative stress

1.4.1 Reactive Oxygen Species

Bacteria grown in the presence of oxygen are constantly challenged by the oxidative stress caused by reactive oxygen species, which are inevitably produced as a by-product of bacterial metabolism under aerobiosis, and which they also encounter during infection as an anti-bacterial response from the host (Storz and Imlay, 1999).

Reactive oxygen species are toxic compounds formed by the stepwise reduction of the non-toxic molecular oxygen. Figure 1.4-1 shows the production of some toxic oxygen species such as superoxide anion ($^{\bullet}O_2^-$), hydrogen peroxide (H_2O_2) and hydroxyl radical ($^{\bullet}OH$), as a consequence of the incomplete the reduction of molecular oxygen (O_2) into water (H_2O) (Fridovich, 1983). These reactive oxygen species can cause damage to the membrane integrity of the cell, proteins, and DNA (Demple, 1991).

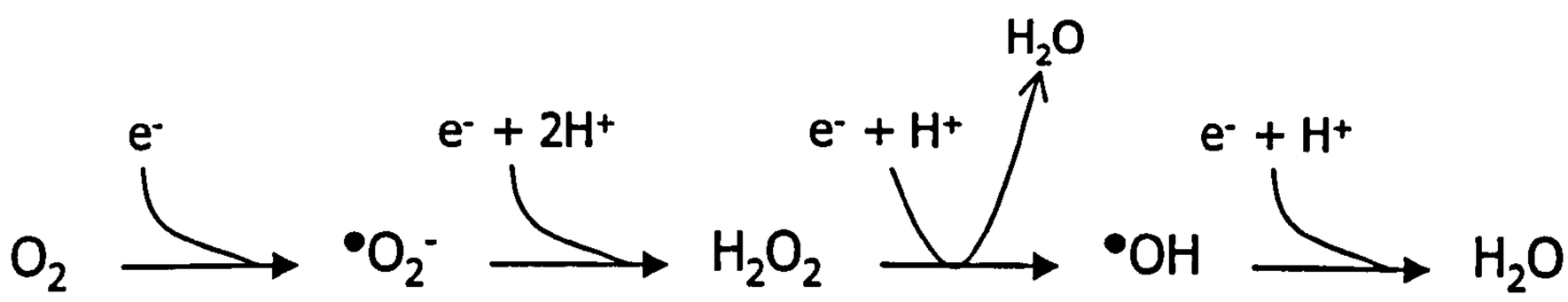


Figure 1.4-1 (Modified from Imlay, 2003). Production of reactive oxygen species consequence of an incomplete reduction of O_2 to H_2O.

1.4.1.1 Superoxide anion

Superoxide anion can cause bacterial damage either directly or indirectly (Fridovich, 1986). One of the main pathogenic effects of superoxide is actually an indirect effect that leads to the production of H_2O_2 and $^{\bullet}OH$. When $^{\bullet}O_2^-$ is scavanged by dismutation, it produces H_2O_2 (Imlay, 2002) (see 1.5.1 for dismutation of H_2O_2 by superoxide dismutase). This H_2O_2 has toxic effects in the cells (described in 1.4.1.2), and it can react with $^{\bullet}O_2^-$ to generate $^{\bullet}OH$ via the Fenton and Haber-Weiss reaction (Fridovich, 1986) (commented in 1.4.1.3). $^{\bullet}OH$ is a highly toxic radical which, as will be mentioned in 1.4.1.3, has a degrading effect on cells by damaging DNA and other molecules, causing mutation and death of the bacterium (Storz and Imlay, 1999; Imlay, 2003).

Aside of its indirect effects, $^{\bullet}O_2^-$ has the ability to damage enzymes that contain iron-sulfur clusters (Imlay, 2003; Stortz and Imlay, 1999). These enzymes have a wide variety of important roles in a cell, for example, the DNA repair enzyme endonuclease III of *E. coli* (Beinert *et al.*, 1997), the enzyme amidotransferase involved in the biosynthesis of nucleotides in *B. subtilis* (Beinert *et al.*, 1997), and enzymes involved in the aerobic metabolism of cells such as dehydratases are part of the iron-sulfur cluster containing enzymes (Imlay, 2003; Stortz and Imlay, 1999). Genomic analysis of *S. pneumoniae* has also shown that the pneumococcus contain enzymes with iron-sulfur clusters (Pericone *et al.*, 2003) which can also be damaged by $^{\bullet}O_2^-$; and in *S. thermophilus*, also a lactic acid bacterium, it is apparent that failure in the repair mechanism of these clusters leads to cell death after exposure to $^{\bullet}O_2^-$ (Thibessard *et al.*, 2004). The degradation of iron sulfur clusters occurs by the attraction of $^{\bullet}O_2^-$ to the iron in the cluster (Imlay, 2003). Figure 1.4-2 shows an example of a 4Fe-4S iron-sulfur cluster. These clusters contain a "free" iron atom that gets oxidized upon binding of $^{\bullet}O_2^-$, causing the oxidized cluster to become unstable and degrade losing its catalytic iron (Figure 1.4-2). Oxidized clusters can be repaired *in vivo*, but the mechanism by which this occurs is not known, Figure 1.4-2 shows the suggested steps for reparation (Imlay, 2003).

Superoxide can also inhibit the production of aromatic amino-acids causing aromatic auxotrophy, and it can also lead to the production of dicarbonyl by oxidizing short-chain sugars (Imlay, 2002; Imlay, 2003). Dicarbonil is a compound that exhibits toxicity by reacting with the amino groups of proteins and nucleic acids (Imlay, 2003).

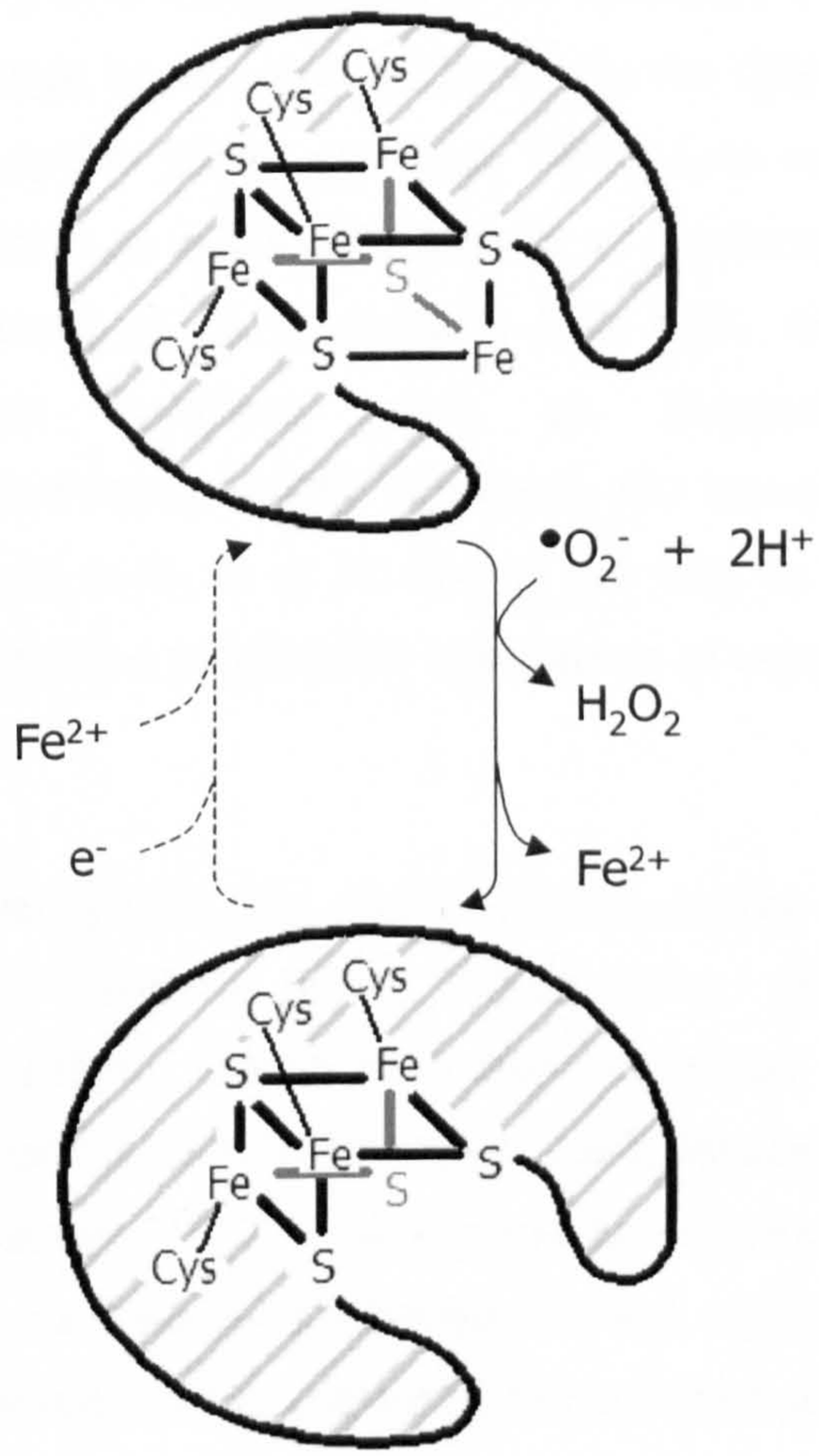


Figure 1.4-2 (Imlay, 2003). Damage caused to the iron-sulfur clusters by $^{\bullet}O_2^-$. The plain arrows indicate the process of oxidation of the catalytic iron of the cluster. The dashed arrows show the proposed steps for enzyme repair.

1.4.1.2 Hydrogen peroxide

Independently to the role of H_2O_2 in the production of $^{\bullet}OH$ radicals (see 1.4.1.3), the toxicity of H_2O_2 in the prokaryotic cell is not completely understood (Imlay, 2002). Imlay and Linn (1986) proposed two methods of damage caused by H_2O_2. In one the damage is produced by low concentrations of H_2O_2 and might be attributed to the formation of $^{\bullet}OH$ radicals and consequent damage to DNA (see 1.4.1.3). In the second one, killing is produced by the exposure of bacteria to high doses of H_2O_2 (~20mM) and is independent to the formation of $^{\bullet}OH$ radicals. In this mode of killing, the damage is not caused directly on the DNA, but possibly on the cell membrane or on enzymes (Imlay and Linn, 1986). Low concentrations of H_2O_2 (~50μM) have been shown to inhibit the growth of bacteria, which can resume when the H_2O_2 is scavanged. It is possible that H_2O_2 oxidizes the cysteinyl residues of important enzymes such as glyceraldehyde-3-phosphate dehydrogenase (Storz and Imlay, 1999). However, the amount of time needed to oxidize cysteine with 1μM H_2O_2 is of 10 hours, too long to explain the effect of H_2O_2, but it is possible that the polypeptide complexes of certain proteins are more suceptible (Imlay, 2003).

Although with less efficiency than $^{\bullet}O_2^-$, H_2O_2 can also oxidize iron-sulfur clusters of enzymes, impairing their function (Imlay, 2002; Imlay, 2003). H_2O_2 can also carbonylate proteins (a protein modification that cannot be repaired) and cause damages to lipids in the cell (Imlay, 2002; Imlay, 2003; Mostertz and Hecker, 2003; Gonzalez-Flecha and Demple, 1997). It also plays an important role in the killing of bacteria by phagocytes, as it will be described in 1.4.2, H_2O_2 can interact with the NO produced by macrophages and neutrophils to mediate bacteria killing.

1.4.1.3 Hydroxyl radical

Hydroxyl radical is a very powerful oxidant that can react with most biological molecules, but it is particularly hazardous for DNA, as minimum damage on this molecule can mutagenise or kill bacteria (Storz and Imlay, 1999; Imlay 2003). Because of its high reactivity, $^{\bullet}OH$ oxidizes substrates in the proximity of where it is formed (Miller and Britigan, 1997). Therefore, to cause DNA damage, it must be produced in close proximity to the DNA. Due to the high affinity of DNA for Fe^{2+}, and the need of Fe^{2+} to catalyze the production $^{\bullet}OH$, DNA is a main target of damage by $^{\bullet}OH$ (Floyd, 1981).

As mentioned above, for the production of $^{\bullet}OH$, Fe^{2+} is essential to catalyze and produce a rapid reaction that would be of importance in a biological system (Imlay 2002). The production of $^{\bullet}OH$ greatly depends on the presence of the ROS species $^{\bullet}O_2^-$ and H_2O_2. $^{\bullet}O_2^-$ can be a source of free Fe^{2+} by its capability to degrade iron-sulfur clusters (Figure 1.4-2), which is represented in Equation-1 below (Imlay 2002; Imlay 2003). Also, $^{\bullet}O_2^-$ can reduce Fe^{3+} to produce Fe^{2+} as shown in Equation-2 (Cadenas 1989). At this point, H_2O_2 enters into play by reacting with Fe^{2+} to produce $^{\bullet}OH$ as shown in Equation-3; this is the Fenton reaction (Cadenas 1989). The actions of Equation-2 and Equation-3 give as net balance what is known as the Haber-Weiss reaction, shown in Equation-4 (Cadenas, 1989; Miller and Britigan, 1997). Finally, as shown in Equation-5 (Imlay, 2003), $^{\bullet}OH$ can oxidize DNA and cause damage (Imlay, 2002; Imlay, 2003).

Equation-1:

$$[4Fe\text{-}4S]^{3+} \rightarrow [3Fe\text{-}4S]^{1+} + Fe^{2+}$$

Equation-2:

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

Equation-3:

$$Fe^{2+} + H_2O_2 \rightarrow Fe^{3+} + OH^- + {}^{\bullet}OH \text{ (Fenton reaction)}$$

Equation-4:

$$^{\bullet}O_2^- + H_2O_2 \rightarrow O_2 + OH^- + {}^{\bullet}OH \text{ (Haber-Weiss reaction)}$$

Equation-5:

$$^{\bullet}OH + DNA \rightarrow H_2O + \text{DNA lesion}$$

$^{\bullet}OH$ can also initiate lipid peroxidation, with damage to the membrane integrity of the cell as a consequence (Farr and Kogoma, 1991). This damage can affect the transport of nutrients into the cell and can cause an osmotic imbalance, which will eventually lead to cell death (Farr and Kogoma, 1991). The end-products of lipid peroxidation, such as alkanes, ketones, epoxides and aldehydes are also mutagenic, and can react directly with DNA or inactivate proteins (Farr and Kogoma, 1991).

1.4.2 Biological sources of Reactive Oxygen Species

In this section, and although not relevant to the pneumococcus, the way in which bacteria are subject to Reactive Oxygen Species (ROS) as a by-product of their metabolism when using the electron transport chain will first be described. After, other sources of ROS also caused by metabolic activity and other enzymatic reactions including those that are of relevance to *S. pneumoniae* will be described. Finally, the oxidative stress that phagocytes such as macrophages and neutrophils inflict on bacteria during infection will also be described.

As a consequence of a respiratory metabolism, bacteria can be subject to oxidative stress. For example, Gonzalez-Flecha and Demple (2000), found that molecules of $^{\bullet}O_2^-$ can be produced at complexes I and III of the respiratory chain during aerobic growth (Figure 1.4-3). They discovered this with the use of rotenone, which inhibits NADH dehydrogenase in complex I, and antymicin, which inhibits the transfer of electrons between ubiquinone and cytochrome b between complexes III and IV of

the electron transport chain (Figure 1.4-3) (Gonzalez-Flecha and Demple, 2000). $^{\bullet}O_2^-$ can also be produced spontaneously by the autoxidation of molecules such as reduced cytochromes and reduced ferredoxins (Frivodich, 1983).

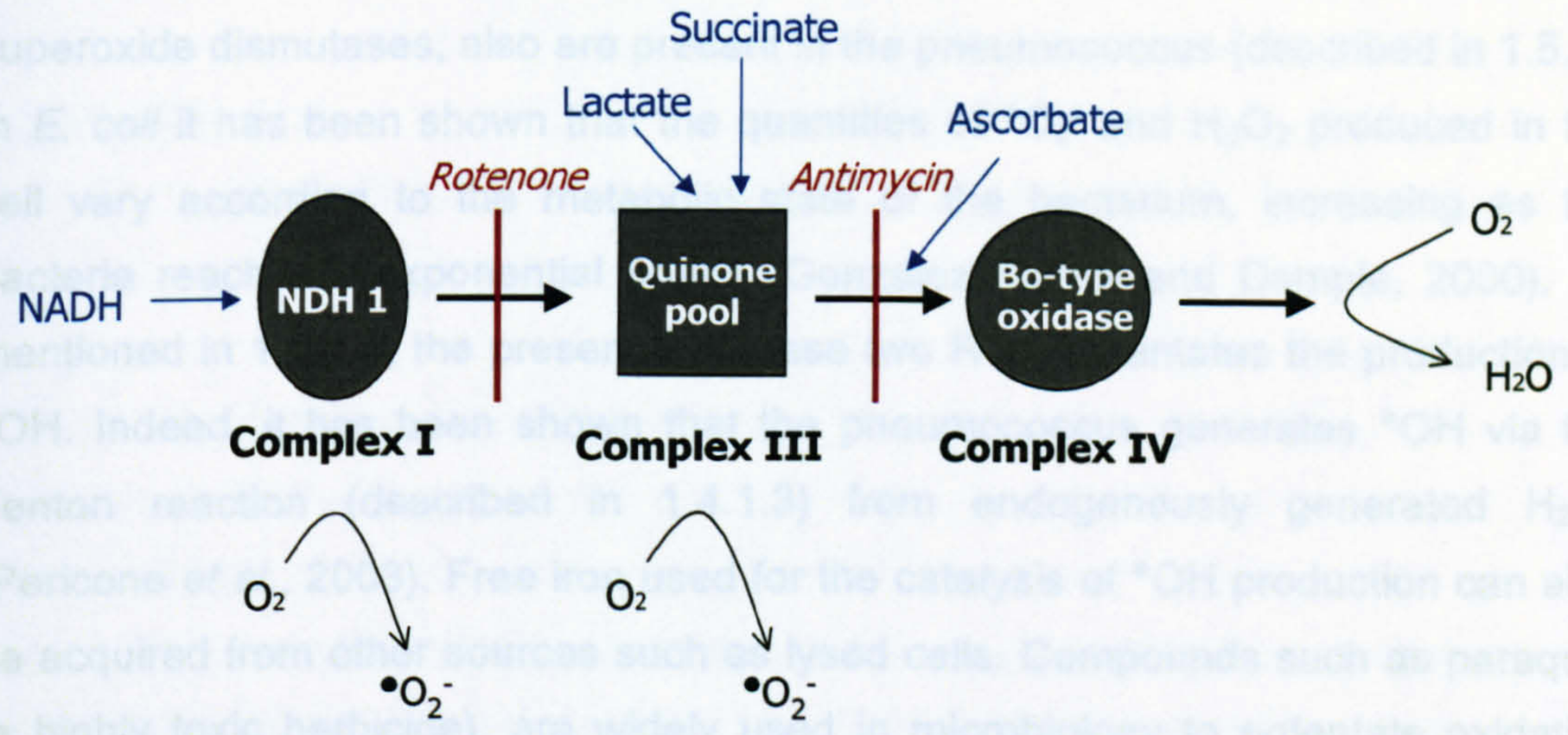


Figure 1.4-3 (from Gonzalez-Flecha and Demple, 2000). Representation of a bacterial respiratory chain showing the places in which $^{\bullet}O_2^-$ can be produced. In blue it is shown the site of entry of the reducing equivalents of different substrates. The sites of inhibition of the respiratory chain by rotenone and antimycin are shown in red. NDH 1: NADH dehydrogenase 1; Quinone pool: mostly ubiquinone; bo-type oxidase: cytochrome oxidase O.

In the metabolism of lactic acid bacteria, it has been reported that *S. mutans* contains an NADH oxidase called Nox-1 that can reconstitute NAD+ with the concomitant production of H_2O_2 (Higuchi *et al.*, 2000). *S. pneumoniae* also contains an NADH oxidase, known as Nox-2 or Nox, however, this enzyme can reduce O_2 into H_2O in the reconstitution of NAD+, therefore not representing a source of ROS, and apparently contributing in the adaptation of the pneumococcus

to oxidative stress (Higuchi *et al.*, 2000; Pericone *et al.*, 2003). *S.pneumoniae* also contains another oxidase, called pyruvate oxidase (SpxB), which decarboxylates pyruvate into acetylphosphate, producing the potentially hazardous H_2O_2 and also CO_2 (Pericone *et al.*, 2003; Spellerberg *et al.*, 1996). Moreover, it has been shown that SpxB is responsible for >99% of the H_2O_2 that the pneumococcus produces (Pericone *et al.*, 2000).

Other enzymatic reactions in bacteria, such as the dismutation of $^{\bullet}O_2^-$ to H_2O_2 by superoxide dismutases, also are present in the pneumococcus (described in 1.5.3), In *E. coli* it has been shown that the quantities of $^{\bullet}O_2^-$ and H_2O_2 produced in the cell vary according to the metabolic state of the bacterium, increasing as the bacteria reach mid-exponential phase (Gonzalez-Flecha and Demple, 2000). As mentioned in 1.4.1.3, the presence of these two ROS potentates the production of $^{\bullet}OH$. Indeed, it has been shown that the pneumococcus generates $^{\bullet}OH$ via the Fenton reaction (described in 1.4.1.3) from endogenously generated H_2O_2 (Pericone *et al.*, 2003). Free iron used for the catalysis of $^{\bullet}OH$ production can also be acquired from other sources such as lysed cells. Compounds such as paraquat (a highly toxic herbicide), are widely used in microbiology to potentate oxidative stress in bacterial cultures. Paraquat can diffuse into the bacteria and increase the rate of production of $^{\bullet}O_2^-$ (Stortz and Imlay, 1999). It can also react with H_2O_2 to produce $^{\bullet}OH$ radicals (Winterbourn, 1981). The increased production of $^{\bullet}O_2^-$ and $^{\bullet}OH$ augment oxidative stress in the bacterium.

During infection, phagocytes that phagocytose bacterial cells transport more O_2 into the cell, which they transform into $^{\bullet}O_2^-$ with the use of NADPH oxidase (the respiratory burst) (Fridovich, 1983; Miller and Britigan, 1997). This $^{\bullet}O_2^-$ is secreted into the phagosome, where is converted into H_2O_2. (Miller and Britigan., 1997). This is an important part of the antimicrobial repertoire of phagocytes. Macrophages and neutrophils, can also contribute to the production of oxidative stress and killing of bacteria by producing the reactive nitrogen intermediate, nitric oxide ($^{\bullet}NO$) (Miller and Britigan, 1997; Storz and Imlay, 1999; Hogg *et al.*, 1992). $^{\bullet}NO$ can block bacterial respiration *in vitro*, and can react with $^{\bullet}O_2^-$ to produce peroxynitrite ($ONOO^-$) (Equation-6) that can oxidize iron-sulfur clusters and protein thiols (Storz and Imlay, 1999; Hogg *et al.*, 1992). Peroxynitrite can be protonated

and produce peroxynitrous acid (HOONO) (Equation-7), which will endure homolytic cleavage and produce •OH and nitrogen dioxide (•NO_2) (Equation-8) (Hogg *et al.*, 1992). The effects of •OH have been discussed in 1.4.1.3, whereas •NO_2 can produce protein damage (van der Vliet *et al.*, 1997).

Equation-6:

$$^{\bullet}NO + {}^{\bullet}O_2^- \rightarrow ONOO^-$$

Equation-7:

$$ONOO^- + H^+ \rightarrow HOONO$$

Equation-8:

$$HOONO \rightarrow {}^{\bullet}OH + {}^{\bullet}NO_2$$

Nitric oxide can also form the reactive nitrogen intermediates nitrate (NO_3^-) and nitrite (NO_2^-), the later having various toxic effects (Klebanoff, 1993). NO_2^- can be produced during the reaction of $ONOO^-$ or ONOOH with the biomolecules they might affect, and it can also be produced by the autooxidation of •NO with O_2. However this reaction might not be significant in a biological system, as it might occur very slowly (van der Vliet *et al.*, 1997). The reaction of NO_2^- with H_2O_2 can lead to the production of ONOOH at acid pH and, in the neutrophils, it can react with myeloperoxidase or hypochlorous acid (described below) in the presence of H_2O_2 to produce reactive nitrogen intermediates such as •NO_2 (van der Vliet *et al.*, 1997).

In the neutophils, H_2O_2 plays a crucial role in the microbicidal activity of this cell, and, therefore, in the host defense against bacteria (Klebanoff, 1980). Neutrophils, contain in their granules the haem-containing peroxidase, myeloperoxidase (Klebanoff, 1980, van der Vliet *et al.*, 1997). This enzyme, in the presence of H_2O_2 and a halide, such as chloride or iodide, produces very potent microbicidal agents. For example, myeloperoxidase with Cl^- and H_2O_2 produces hypochlorous acid, a potent antimicrobial (Klebanoff, 1980). Also, neutrophils can produce NO_2^- in the

presence of H_2O_2 without requiring the production of •NO (Klebanoff and Nathan, 1993). The H_2O_2 needed for these reactions, is normally produced by the neutrophils themselves, but also, the H_2O_2 produced by the phagocytised bacteria, as is the case for the streptococcal species, can be used. The importance of H_2O_2 for the neutrophils and the immune system can be observed with the genetic disease Chronic Granulomatous Disease, in which the phagocytes are defective in NADPH oxidase and cannot produce a respiratory burst and therefore H_2O_2 (Klebanoff, 1980). Persons suffering from this disease, acquire constant infections with bacteria such as staphylococci, which contain catalase (see 1.5.1 for catalase description) capable of destroying any bacterial H_2O_2 produced. In these cases, phagocytes are capable of phagocytosis, but incapable of killing due to the lack of H_2O_2. However, if an infection occurs with an H_2O_2-producing bacteria, such as a streptococcus, the phagocytes can utilise this H_2O_2 and recover their killing ability (Klebanoff, 1980). Myeloperoxidase is also important for the killing of bacteria, however, in its absence, neutrophils are still capable of killing using the respiratory burst and ROS production (Klebanoff, 1980).

To summarise, Figure 1.4-4 shows the ways in which bacterial cells can be damaged by the oxidative stress they encounter during aerobic growth or during phagocytosis.

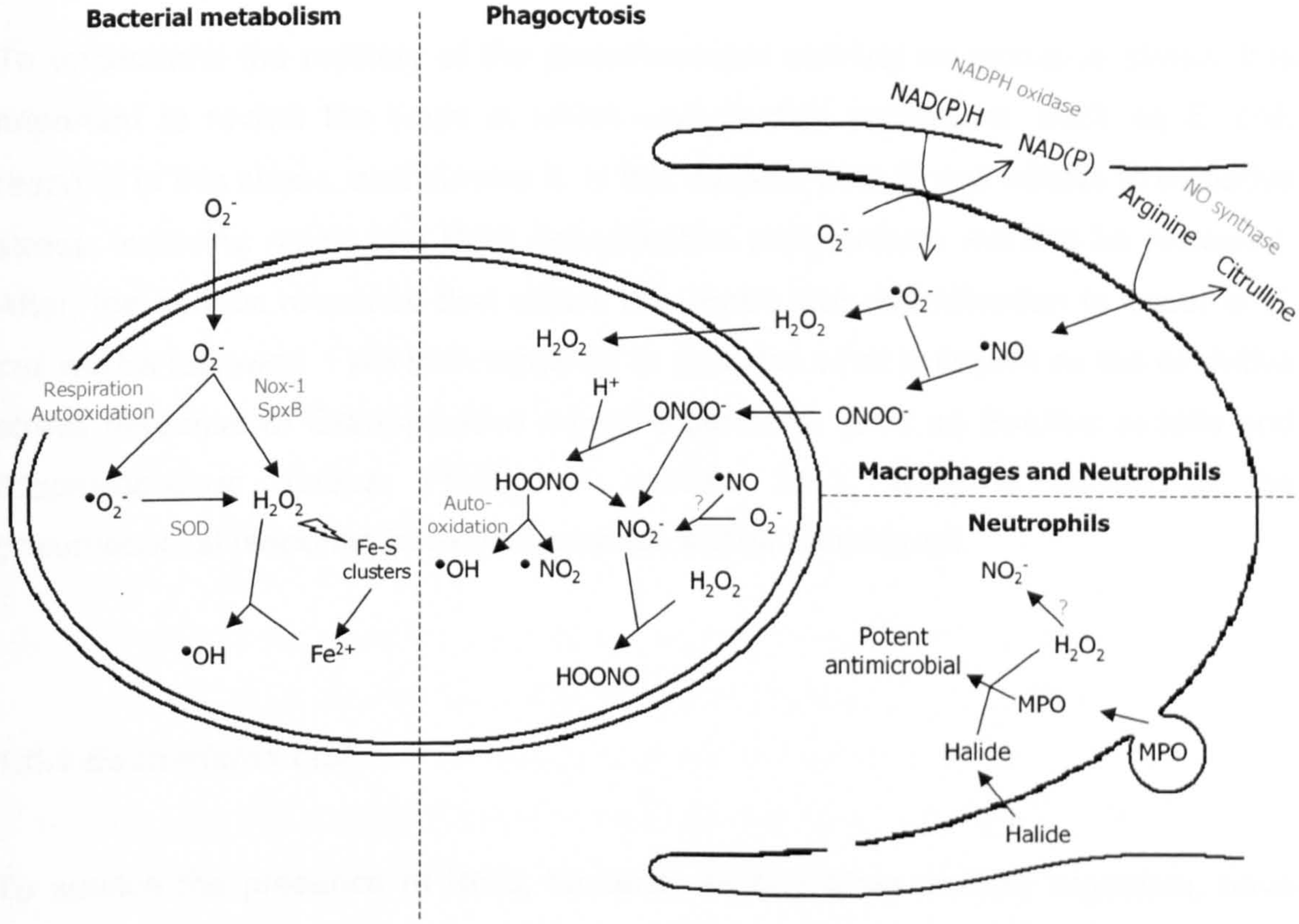


Figure 1.4-4 Sources of oxygen stress as a result of bacterial metabolism or phagocytosis. During aerobic growth, bacterial metabolism can lead to the production of toxic forms of oxygen such as H_2O_2 and $^{\bullet}OH$ from O_2^-. During phagocytosis, macrophages and neutrophils can produce ROS to aid in the killing of bacteria. Nitric oxide is also produced and leads to the production of $ONOO^-$ and HOONO, causing toxicity to bacterial cells. Neutrophils also contain MPO in their granules, which is "poured" into the phagosome and contributes to bacteria killing.

1.5 Bacterial defences to oxidative stress

To understand the mystery of the pneumococcal survival on oxidative stress, it is important to review the ways in which well studied organisms, such as *E. coli*, respond to this stress, and survive it. In this section, how *E. coli* adapts to oxidative stress, including repair and ROS detoxification mechanisms will first be reviewed. After, the genetic response that allows this repair and detoxification to occur in *E. coli* will be reviewed. I will then move on to describe what is known on the oxidative stress response of Gram-positive model organisms, such as *Bacillus subtilis* and *Staphylococcus aureus*. Finally, in section 1.5.3, what is known of the pneumococcal response to oxidative stress will be considered.

1.5.1 *Escherichia coli*

To survive the presence of ROS, bacteria, as any other aerobic organism, have developed a network of detoxification responses to convert ROS into non-toxic forms of oxygen, and of repair, to overcome the damage caused by ROS that escaped the detoxification response. Unless stated otherwise, this section reviews the repair and detoxification responses in *E. coli.*

E. coli contain several DNA repair mechanisms that it utilises in response to damage by ROS. One of them is the base excision repair system, where DNA lesions can be removed by the action of DNA glycosylases, which can detect aberrant bases, and of endonucleases. The "gap" in the DNA strand left by the action of glycosylases and endonucleases, can then be repaired by the further action of DNA polymerase and DNA ligase (Kow, 2002; Katafuchi *et al.*, 2004). Other mechanisms of repair include a DNA recombination system formed by the Rec proteins. The protein RecA, which has been shown to be induced under oxidative stress, (Farr and Kogoma, 1991) is part of this system, which involves

other Rec and also Ruv proteins (Heyer, 2004). There are two pathways by which the Rec system promotes recombination. The RecF pathway, which promotes recombination in DNA which lacks a double strand DNA break (including gaps and nicks) and the RecBCD pathway, which is initiated by a double-strand break (Heyer, 2004). In both pathways of repair, the DNA repair is initiated by the removal of nucleotides on the damaged DNA strand, leaving an area of single stranded DNA (ssDNA) (Heyer, 2004; Hishida *et al.*, 2004). RecA can then bind the ssDNA (Heyer, 2004; Hishida *et al.*, 2004), and this initiates the action by RuvABC in mediating the recombinational process (Cox, 2001). Very importantly, RecA also induces the SOS response, which involves the induction of DNA repair genes and a stop on cell division until the response is alleviated (Hishida *et al.*, 2004). RecA induces this response by mediating the auto-digestion of LexA, a SOS repressor (Maul and Sutton, 2005). Chaperone proteins also provide other sources of protection against oxidative stress. For example, the chaperones DnaK/DnaJ and GroEL/GroES, which are heat shock proteins, have been shown to reduce protein carbonylation as a result of oxidative stress (Fredriksson *et al.*, 2005). In fact, the levels of DnaK, GroEL and GroES are proven to increase during oxidative stress (Farr and Kogoma, 1991). Proteases such as ClpP and Lon can degrade proteins that did get carbonylated (Fredriksson *et al.*, 2005).

The detoxification system of bacteria such as *E. coli*, includes enzymes that can convert toxic forms of oxygen into non-toxic forms. Superoxide dismutases, catalases and peroxidases form the main antioxidants in a wide range of bacteria (Farr and Kogoma, 1991). A brief description of these antioxidants is given below.

There are three types of superoxide dismutases in *E. coli*: FeSOD and MnSOD, which are cytosolic proteins; and Cu/ZnSOD, which is a periplasmic protein (Jakubovics and Jenkinson, 2001). SOD converts the toxic $^{\bullet}O_2^-$ into H_2O_2 (Fridovich, 1999). This is done in two steps involving the oxidation and reduction of the metal by two molecules of $^{\bullet}O_2^-$ as shown in Equation-9 and Equation-10 (Hunter *et al.*, 2002; Hough and Hasnain, 1999). Equation-11 shows the net reaction for the dismutation of superoxide (Hogg *et al.*, 1992). This reduction of $^{\bullet}O_2^-$ prevents it from reacting with free iron to produce $^{\bullet}OH$. Experiments in SOD negative *E. coli* mutants have shown that the lack of both cytosolic SODs is

reflected by an increase of free iron, possibly caused by the damage of $^{\bullet}O_2^-$ to proteins with iron-sulfur clusters (see 1.4.1.1) (Keyer and Imlay, 1996). McCormick (McCormick *et al.*, 1998) showed that this FeSOD and MnSOD-negative mutant presents high levels of $^{\bullet}OH$ produced via the Fenton reaction (see 1.4.1.3), supporting the findings of increased iron levels in the mutant. To some extent, it can be assumed that the concentrations of $^{\bullet}O_2^-$ are inversely proportional to the concentration of SOD (Gort and Imlay, 1998), which increases the production of $^{\bullet}OH$ (McCormick *et al.*, 1998) and causes SOD-negative mutants to be more susceptible to oxidative stress (Keyer *et al.*, 1995). This oxidative stress is reflected by higher rates of mutagenesis caused by the effect of $^{\bullet}OH$ on the bacterial DNA (Gort and Imlay, 1998). Indeed, Frivodich (Frivodich, 1998) reported that in *E. coli*, SOD provides ~95% of protection from all the damage that could be caused by $^{\bullet}O_2^-$ to susceptible targets.

Equation-9

$$M^o + {}^{\bullet}O_2^- \rightarrow M^r + O_2$$

Where: M^o = iron or manganese or cupper in oxidized state

M^r = iron or manganese or cupper reduced

Equation-10

$$M^r + {}^{\bullet}O_2^- + 2H^+ \rightarrow M^o + H_2O_2$$

Where: M^r = iron or manganese or cupper reduced

M^o = iron or manganese or cupper re-oxidized

Equation-11

$$2H^+ + {}^{\bullet}O_2^- + {}^{\bullet}O_2^- \rightarrow H_2O_2 + O_2$$

The H_2O_2 produced by the reduction of $^{\bullet}O_2^-$ is eliminated by the action of catalases and peroxidases (Fridovich, 1999). *Escherichia coli* contain two different catalases: a periplasmic catalse which is known as hydroperoxidase I (HPI), and a cytoplasmic catalase known as hydroperoxidase II (HPII) (Miller and Britigan, 1997; Fridovich, 1998). Catalases are haem-containing enzymes that can convert two

molecules of H_2O_2 into $2H_2O$ and O_2 (Fridovich, 1999). Catalase can protect against endogenous and exogenous H_2O_2, therefore, it can provide protection from the oxidative stress caused during infection. It has been proposed that catalase and alkyl hydroperoxide (Ahp) play complementary roles (Seaver and Imlay, 2001). During *E. coli* growth, Ahp appears to scavenge low levels of H_2O_2 more effectively than HPI, and as the role of HPII appears significant only during stationary phase, it is apparent that Ahp has a main role dealing with endogenously produced H_2O_2 as a consequence of growth (Seaver and Imlay, 2001). However, HPI is more efficient in scavenging high levels of H_2O_2 than Ahp is, suggesting that HPI might play a more important role in scavenging exogenous H_2O_2 (Seaver and Imlay, 2001).

Glutathione (GSH), another important antioxidant, co-works with glutathione peroxidase in removing H_2O_2. In this process, two molecules of glutathione become oxidized per molecule of H_2O_2 by the action of glutathione peroxidase to produce $2H_2O$. Glutathione reductase will then transfer electrons from 2NADPH to reduce the oxidized glutathiones and continue with the cycle (Farr and Kogoma, 1991; Miller and Britigan, 1997).

E. coli also possess a thiol peroxidase (Tpx). This enzyme is located in the periplasmic space and has been shown to function as an H_2O_2 scavenger (Kim *et al.*, 1999). However, it is apparent that the main role of Tpx is that of reducing organic hydroperoxides (Baker and Poole, 2003) that can be formed, for example, by the damage of ROS to bacterial membranes (Cha *et al.*, 2004). Thiol peroxidase has also shown to inhibit bacterial membrane oxidation by ROS (Cha *et al.*, 2004).

1.5.1.1 Regulation of the oxidative stress response

The response to oxidative stress in *E. coli* involves the induction of many different genes. For example, upon exposure to H_2O_2, *E. coli* and *Salmonella typhimurium* induce the expression of 30 to 40 genes, and upon exposure to paraquat ($^{\bullet}O_2^-$ generator) approximately 40 genes are induced (Demple, 1999). The regulators

SoxRS and OxyR have been shown to modulate part of this response (Greenberg *et al.*, 1990; Christman *et al.*, 1985). Figure 1.5-1 shows a schematic representation of the toxicity caused by ROS and the response modulated by the regulators SoxRS and OxyR. In the Figure, some of the genes activated by these two regulators are shown. Figure 1.5-1 is based on the information published by Demple, (1991, 1999); Zheng *et al.* (2001); Zheng *et al.* (1999); Farr and Kogoma, (1991) on *S. typhimurium* and *E. coli*.

The *sox*RS response can be induced by the presence of $^{\bullet}O_2^-$ or of NO (Iuchi and Weiner, 1996). For the activation of the response, the SoxR protein acts as the sensor of the oxidative stress, possibly by the oxidation of its iron-sulfur cluster (Iuchi and Weiner, 1996). The "activated" SoxR protein stimulates the production of SoxS, which stimulates the transcription of at least 10 target genes (Iuchi and Weiner, 1996). As shown in Figure 1.5-1, the SoxRS regulator induces the expression genes such as the *sod*A gene, which encodes MnSOD; *nfo*, which encodes the DNA repair enzyme endonuclease IV; *zwf*, which encodes a glucose-6-phosphate dehydrogenase that can supply the NADPH needed for the action of peroxides; *mic*F, a regulatory RNA that that represses the production of the membrane porin OmpF; the fur gene, and others (Storz and Imlay, 1999, Farr and Kogoma, 1991; Demple, 1999). The *fur* gene, whose expression is also induced by OxyR (described below), encodes the Fur regulator (Storz and Imlay, 1999). This regulator represses the transcription of iron transport systems, maintaining low levels of intracellular iron (Iuchi and Weiner, 1996) and therefore protecting against the formation of $^{\bullet}OH$. The genes *sod*B and *sod*C, which encode Fe-SOD and CuZn-SOD respectively, also have a role in protection against $^{\bullet}O_2^-$, however, they are not induced by SoxRS (Storz and Imlay, 1999). Gene *sod*B appears to be constitutively expressed in all conditions (Iuchi and Weiner, 1996), and *sod*C gene appears to be over-expressed during aerobic stationary phase, probably under the induction of the starvation response regulator *kat*F, also known as *rpo*S (Lynch and Kuramitsu, 2000).

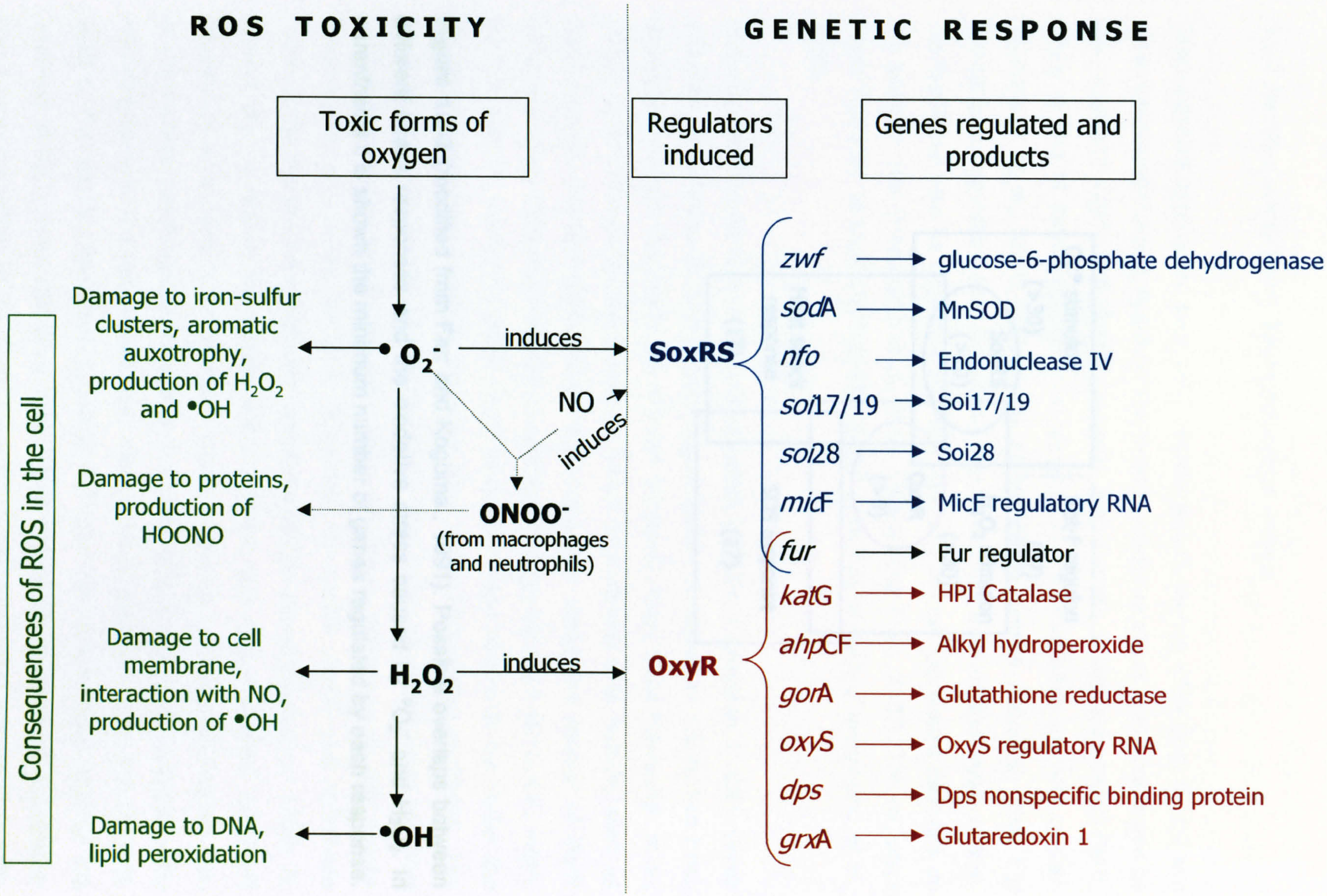
The OxyR regulator is activated in response to the presence of H_2O_2. It has been proposed that the reduced OxyR protein becomes oxidized upon exposure to H_2O_2, leading to a conformational change in the protein and subsequent activation of the

expression of at least 9 genes (Farr and Kogoma, 1991; Iuchi and Weiner, 1996). As shown in Figure 1.5-1, the OxyR regulator induces, among others, the expression of *kat*G, which encodes HPI Catalase; *ahp*CF, which encodes alkyl hydroperoxide; *gor*A, which encodes glutathione reductase; *fur* regulator (described above); and *dps* and *oxy*S, whose products might have a role in protection against mutagenesis (Stortz and Imlay, 1999; Farr and Kogoma, 1991; Demple, 1999). It is possible that the Dps protein, encoded by *dps*, might have a role in sequestering iron and protection against DNA damage (Stortz and Imlay, 1999). Other genes that are expressed under stress by H_2O_2 and are not induced by the OxyR regulator, include *kat*E, which encodes for HPII catalase; and *xth*A, *pol*A and *rec*A, whose products, exonucleaseIII, DNA polymerase I and RecA respectively, are involved in DNA repair. The gene encoding for the chaperone Hsp33 (gene *hsl*O) is also induced by H_2O_2 (Stortz and Imlay, 1999).

Although the expression of SoxRS and OxyR regulators is known to be induced by different stresses, Zheng and collaborators showed that high levels of $^{\bullet}O_2^-$ could partially induce *sox*RS, and high levels of H_2O_2 could cause an induction of *oxy*R (Zheng *et al.*, 2001).

The response to a particular stress, however, is not an independent response regulated solely by a particular set of genes. Different mechanisms and regulators can influence the same genes, and the response to a particular stress involves genes also induced by other stress stimuli. For example, it has been shown that in the response to oxidative stress, genes involved with heat shock and SOS responses are also induced (Zheng *et al.*, 2001). Genes *gro*ES and *gro*EL, which are induced by $^{\bullet}O_2^-$ and H_2O_2, are genes from the heat shock response that can also be activated during starvation and SOS. The gene *dna*K, which is induced by treatment with H_2O_2, is also induced by UV irradiation and starvation, but not by heat shock (Farr and Kogoma, 1991). Other genes involved in the oxidative stress, such as *kat*E and *xth*A can be regulated by *kat*F as part of a starvation response (Demple, 1991; Farr and Kogoma, 1991). Figure 1.5-2 modified from Farr and Kogoma (1991) shows an example of overlaps between the responses to oxidative stress ($^{\bullet}O_2^-$ and H_2O_2 stimulation), heat shock, SOS, and the KatF regulon.

Figure 1.5-1 Schematic representation of the detoxification response to ROS. The effects of ROS in the cell is shown in green, for more details on the toxic effect of ROS see section 1.4.1. The soxRS regulon can be induced by $^{\bullet}O_2^-$ and by the NO produced by macrophages. The SoxRS regulator and some examples of the genes or proteins it controls are shown in blue. The oxyR regulon can be induced by H_2O_2. The OxyR regulator and some examples of the genes or proteins it controls are shown in red. The *fur* gene is shown in black, as both, the SoxRS and OxyR regulators, can regulate it.


ROS TOXICITY
GENETIC RESPONSE
Toxic forms of oxygen
Regulators induced
Genes regulated and products
Consequences of ROS in the cell
Damage to iron-sulfur clusters, aromatic auxotrophy, production of H_2O_2 and •OH
O_2^-
induces
SoxRS
NO
induces
Damage to proteins, production of HOONO
ONOO⁻ (from macrophages and neutrophils)
Damage to cell membrane, interaction with NO, production of •OH
H_2O_2
induces
OxyR
Damage to DNA, lipid peroxidation
•OH
zwf → glucose-6-phosphate dehydrogenase
sodA → MnSOD
nfo → Endonuclease IV
soi17/19 → Soi17/19
soi28 → Soi28
micF → MicF regulatory RNA
fur → Fur regulator
katG → HPI Catalase
ahpCF → Alkyl hydroperoxide
gorA → Glutathione reductase
oxyS → OxyS regulatory RNA
dps → Dps nonspecific binding protein
grxA → Glutaredoxin 1

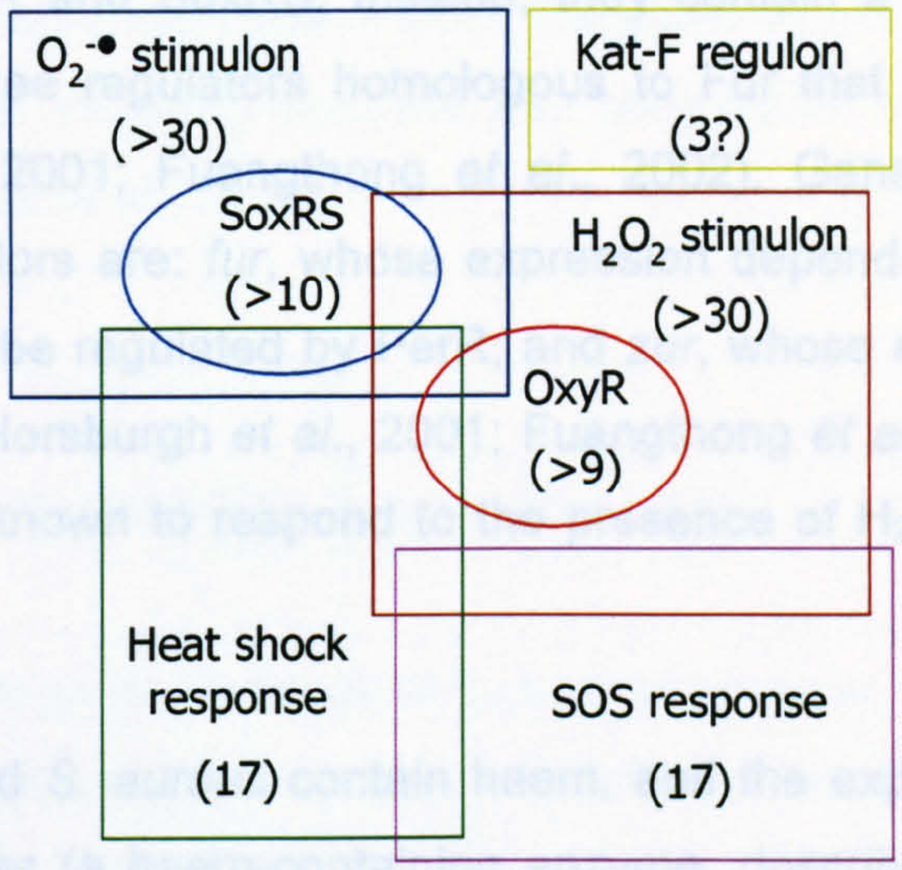


Figure 1.5-2 (modified from Farr and Kogoma., 1991). Possible overlaps between different stress responses and the oxidative stress caused by $^{\bullet}O_2^-$ and H_2O_2. In parenthesis it is shown the minimum number of genes regulated by each response.

1.5.2 *Bacillus subtilis* and *Staphylococcus aureus*

Gram-positive organisms, such as *B. subtilis* and *S. aureus* differ from *E. coli* and other Gram-negatives in that their response to oxidative stress is not controlled by the regulators OxyR and SoxRS. Instead, they contain a regulator called PerR, which is one of three regulators homologous to Fur that these bacteria contain (Horsburgh *et al.*, 2001; Fuangthong *et al.*, 2002). Genes encoding other Fur homologous regulators are: *fur*, whose expression depends on the levels of iron, but that it can also be regulated by PerR; and *zur*, whose expression depends on the levels of zinc (Horsburgh *et al.*, 2001; Fuangthong *et al.*, 2002). The regulator PerR, however, is known to respond to the presence of H_2O_2 (Fuangthong *et al.*, 2002).

Both, *B. subtilis* and *S. aureus* contain haem, and the expression of *kat*A, which encodes for catalase (a haem-containing enzyme, described in 1.5.1), has been shown to be under the regulation of PerR (Mostertz *et al.*, 2004 Horsburgh *et al.*, 2001). In fact, the genes necessary for haem biosynthesis in *B. subtilis*, such as *hem*A, *hem*X, *hem*C, *hem*D, *hem*B and *hem*L are under the control of Per*R* (Mostertz *et al.*, 2004; Helmann *et al.*, 2003). Other genes such as *ahp*CF, *mrg*A, the *fur* regulon, *zos*A and *per*R itself have also been shown to be under the regulation of PerR in *B. subtilis* (Mostertz *et al.*, 2004; Helmann *et al.*, 2003). The gene *mrg*A encodes MrgA, a member of the Dps family of proteins that can bind DNA and protect against oxidative stress, and *zos*A belongs to a zinc uptake system (Helmann *et al.*, 2003). In *B. subtilis*, the genes *ahp*CF and *kat*A have shown to be important for protection against oxidative stress (Bsat *et al.*, 1996). *S. aureus* also contains the genes *ahp*CF, which are also regulated by PerR (Horsburgh *et al.*, 2001). Other PerR regulated genes in *S. aureus* include *bcp*, which encodes the thiolperoxidase Bcp; *trx*B, encoding a thioredoxin reductase; *ftn*, encoding an iron storage protein; and also *mrg*A (Horsburgh *et al.*, 2001). In *B. subtilis*, the stress dependant regulator, σ^B also appears to form part of the response to H_2O_2 stress (Mostertz *et al.*, 2004). However, its influence in oxidative stress requires further study. Superoxide dismutase is present in both *B. subtilis*

and *S. aureus*. *B. subtilis* contains a *sod*A gene, which encodes for MnSOD and has shown to be essential for resistance to oxidative stress (Inaoka *et al.*, 1999). *S. aureus* contains two genes encoding SOD, which are *sod*A and *sod*M, both of which encode an MnSOD. However, it is the *sod*A gene that appears to have a major role in resistance to oxidative stress (Valderas and Hart, 2001). In *B. subtilis*, it has also been reported that H_2O_2 induces the expression of genes from the SOS response, such as *rec*A, *lex*A and *uvr*ABC (Mostertz *et al.*, 2004). Genes from the heat-shock response, including *clp*L, are also upregulated by the presence of H_2O_2 or paraquat (Mostertz *et al.*, 2004).

1.5.3 Pneumococcal adaptation to oxidative stress

S. pneumoniae belongs to the family of lactic acid bacteria. As with all of these, the pneumococcus cannot synthesize haem (Higuchi *et al.*, 2000), and indeed, the pneumococcus lacks most of the *hem* genes involved in the biosynthesis of haem (*hem*AXCDBL) (homology search done on the TIGR website in this work). Two genes involved in the last steps of the haem biosynthesis, *hem*H (sp1009) and *hem*K (sp1021), which encode a ferrochelatase and a putative protoporphyrinogen oxidase respectively, were the only genes found involved in this pathway (search done on the TIGR website in this work). The pneumococcus also lacks the genes *kat* (encoding catalase) and the oxidative stress regulators *oxy*R, *sox*RS and *per*R (Pericone *et al.*, 2003; homology search in the TIGR website in this work). This lack of genes, and especially, the lack of the known regulators *oxy*R, *sox*RS and *per*R, makes it unclear how the adaptation to oxidative stress occurs in the pneumococcus. However, *S. pneumoniae* does contain some of the genes that are regulated by *oxy*R or *sox*RS in *E. coli*, for example, genes encoding for gluthatione reductase (sp0784), MnSOD (*sod*A or sp0766), glucose-6-phosphate 1-dehydrogenase (*zwf* or sp1243), and a gene with 54.3% similarty to *dps* (sp1572) (Search done in the TIGR website in this work). Other genes such as *psa*D (sp1651), which encodes a Tpx; sp0313 which encodes a glutathione peroxidase; *nth* (sp1279), which encodes an endondonuclease III; *gro*EL (sp1906); *gro*ES (sp1907); and *dna*K (sp0517); which have been mentioned before as playing a role

on the adaptation to oxidative stress in *E. coli*, are also present in the pneumococcal genome (search done on the TIGR website in this work). However, not all of these genes have been studied in response to oxidative stress in the pneumococcus. In this section what it is known on the pneumococcal adaptation to oxidative stress will be described. The two pneumococcal oxidases Nox and SpxB, the products of some of the genes that have been mentioned above and other important characteristics of the pneumococcus will also be described.

S. pneumoniae contains two types of oxidases that can be of use for its adaptation to oxidative stress, that is, NADH oxidase, known as Nox or Nox-2, and pyruvate oxidase, known as SpxB (Pericone *et al.*, 2003; Claiborne *et al.*, 1992; Higuchi *et al.*, 2000). Nox can convert O_2 into H_2O without the production of ROS, possibly contributing to the oxygen tolerance of this bacterium (Higuchi *et al.*, 2000). Deletion of the functionality of Nox was reported by Yu *et al.* (Yu *et al.*, 2001) to result in a mutant more susceptible to oxidative stress caused by aeration than the wild type. Interestingly, Auzat *et al.* (Auzat *et al.*, 1999) reported that a Nox mutant had a reduced ability for genetic competence, suggesting that Nox is involved in the mechanism of DNA repair by transformation in the pneumococcus.

The second oxidase is a pyruvate oxidase, which performs a two-electron reduction of O_2 into H_2O_2, with the concomitant production of acetyl-phosphate and CO_2 (Pericone *et al.*, 2003). Deletion of its encoding gene, *spx*B, result in a mutant that produces less than 1% of the H_2O_2 made by the wild type (Pericone *et al.*, 2000). Also, the mutant is more susceptible to killing by exogenous H_2O_2 (Pericone *et al.*, 2000; Pericone *et al.*, 2003; Spellerberg *et al.*, 1996). This increased susceptibility of the mutant to H_2O_2 was found related to a metabolic role of spxB (Pericone *et al.*, 2003; Spellerberg *et al.*, 1996), as it will now be explained. As it was mentioned in 1.3, Chapuy-Regaud and collaborators (2001) found evidence that the pneumococcus behaves only as a homolactic bacterium, that is, that it can only convert pyruvate into lactate via lactate dehydrogenase. However, Spellerberg and collaborators (1996) and Pericone and collaborators (2003) found *spx*B related to the production of acetate from pyruvate in the presence of oxygen. As mentioned above, in the reduction of O_2 into H_2O_2, spxB produces acetyl-phosphate from pyruvate. Acetyl-phosphate can be converted into acetate via

acetate kinase, with the production of ATP (Pericone *et al.*, 2003; Spellerberg *et al.*, 1996). Therefore, conversion of pyruvate into acetate allow the bacteria to gain more ATP than with the conversion of pyruvate into lactate. Indeed, spxB mutants showed decreased levels of ATP under aerobiosis (Pericone *et al.*, 2003). This loss of ATP could possibly affect the ATP-dependant DNA repair mechanism by proteins such as HexA and RecA (Pericone *et al.*, 2003) leading to death of the bacterium due to damage by ROS under aerobiosis.

S. pneumoniae contains a manganese-dependent superoxide dismutase (MnSOD) encoded by *sod*A. Deletion mutation of *sod*A resulted in a pneumococcal mutant that is more susceptible to the oxidative stress induced by paraquat, and which is less virulent in a mouse model of pneumonia (Yesilkaya *et al.*, 2000). The mutant showed the same level of production of H_2O_2 as the wild type parent, suggesting its role was in protection against external oxidative stress, rather than endogenously produced H_2O_2 (Yesilkaya *et al.*, 2000).

A *psa* operon present in the pneumococcus, has also shown to be of importance in its adaptation to oxidative stress (Tseng *et al.*, 2002). The protein PsaA, which is part of an ATP-binding cassette formed by *psa*ABC has been shown to be involved in the transport of Mn, possibly related to the production of MnSOD (Tseng *et al.*, 2002). Also as part of the *psa* operon, is *psa*D, which encodes a putative thiol peroxidase. Deletion mutants of *psa*A were shown to be highly susceptible to killing by paraquat. Deletions of *psa*A or *psa*D, resulted in mutants more susceptible to killing by H_2O_2, but the addition of Mn(II) conferred some degree of protection to both mutants (Tseng *et al.*, 2002). Tseng and collaborators (2002) also reported that the SOD activity in both PsaA and PsaD mutants was reduced by 30% and 40% respectively in comparison to the wild type. From this, and because the mutants show an increased level of *nox* expression, the authors suggest that *psa*A and *psa*D might be components of a regulatory pathway involved in the response oxidative stress (Tseng *et al.*, 2002)

Production of $^{\bullet}OH$ is endogenously produced in the pneumococcus via the Fenton reaction (Pericone *et al.*, 2003). However, genomic analysis made it apparent that the iron-sulfur clusters of the pneumococcal proteins are smaller, consisting of

$[2Fe2S]^{2+}$ rather than $[4Fe4S]^{2+}$ that bacteria such as *E. coli* have (Pericone *et al.*, 2003). $[2Fe2S]^{2+}$ clusters are more resistant to degradation by oxidative stress, this quality, might contribute to the pneumococcal resistance to oxidative stress (Pericone *et al.*, 2003). This finding, is also supported by the observation that iron chelators did not confer protection to H_2O_2 toxicity in the pneumococcus, suggesting that free Fe^{2+} is not an issue for pneumococcal cells (Pericone *et al.*, 2003). Still, the pneumococcus does contain protein homologues to Dpr (Dps related protein) (sp1772), which as has been mentioned before, has iron-binding ability. It is possible that Dpr might also play a role in protection against oxidative stress in the pneumococcus (Higuchi *et al.*, 2000; Pericone *et al.*, 2003).

In the pneumococcus, the expression of genes such as *gro*EL and *dna*K was found regulated by heat-shock stress, but not by oxidative stress (Choi *et al.*, 1999). Research into other genes such as *gro*ES and *clp* has focused on their role in heat-shock stress (Kim *et al.*, 2001; Kwon *et al.*, 2003), but little or no information is available on the role these genes might have in response to oxidative stress.

In spite of these observations, it is unclear how the pneumococcus adapts to the oxidative stress that it encounters during aerobic growth. Deletion mutations of different genes, and the consequences of these mutations have shown that the pneumococcus must contain different pathways for adaptation to oxidative stress, but these pathways remain to be elucidated, as do, their regulatory mechanisms.

1.5.3.1 Aim of the "pneumococcal gene expression under aerobiosis" project

The aim of this project was to study the genetic response of the pneumococcus when it is subject to aerobic conditions. This was done by comparing with the use of microarrays the gene expression of bacteria grown under aerobic or anaerobic conditions. In order to obtain the information when the bacteria were metabolically active, and before a starvation response was produced, it was decided to extract the RNA when the bacteria reached mid-exponential phase in either condition. Also, in an attempt to create an aerobic environment similar to that that *S. pneumoniae* might experience during colonisation, chemicals such as paraquat or H_2O_2 were not used. Instead, the culture medium was kept under aerobic conditions using a flow of filtered environmental air. With the parameters used in this project, it was expected that the genes the pneumococcus needed for its survival on an aerobic condition such as that of the nasopharynx, would be observed, allowing further understanding on the pneumococcal survival to oxidative stress.

Chapter 2

Materials and Methods

2.1 General Information

Whenever autoclaving was required, this was performed at 121°C for 15 minutes at 1.5bar pressure.

2.1.1 Materials

Unless otherwise specified, all chemicals were obtained from Sigma-Aldrich,Dorset,UK or Fisher Scientific, Leicestershire, UK and the growth media from Oxoid Limited, Hampshire, UK. Primers were obtained from TAG Newcastle, Gateshead, UK and enzymes from New England BioLabs, Hertfordshire, UK or Invitrogen, Paysley, UK.

2.1.2 Growth media

The bacteria culture medium was prepared in deionised water, autoclaved, and stored at room temperature until use. All growth media used are listed in Table 2.1-1.

Table 2.1-1 Media composition for bacterial cultures

Media	Composition	
Luria Broth (LB)	Tryptone	1.0% (w/v)
	Yeast extract	0.5% (w/v)
	NaCl	86.2mM
Luria Agar (LA)	Luria Broth	
	Agar No.1	1.5% (w/v)
Brain Hearth Infusion (BHI)	Brain Heart Infusion	3.7% (w/v)
Blood Agar Base (BAB)	Blood Agar Base	4.0% (w/v)
Blood Agar[1] (BA)	Blood Agar Base[2]	95% (v/v)
	Defibrinated horse blood (Oxoid)[3]	5% (v/v)
Tryptone Soya Broth (TSB)	Tryptone Soya Broth	3% (wv)
Tryptone Soya Agar (TSA)	Tryptone Soya Agar	4% (w/v)
TSB/GT	TSB	100ml
	Glucose 20% (w/v)	1ml
	K_2HPO_4 0.5M	3ml
Serum broth	BHI	80% (v/v)
	Foetal Calf Serum (filtered sterilised)	20% (v/v)

[1]Once prepared, it was used immediately for the preparation of BA plates (see 2.1.2.1)

[2]Previously autoclaved

[3]The BAB was cooled to ~48°C before the defibrinated horse blood was added.

2.1.2.1 Preparation of LA or BA plates

For the preparation of LA or BA plates, 90mm single vent Petri dishes were used (see Table 2.1-1 for media composition). LA or BA was melted and left to cool to ~48°C. If plates with antibiotic were required, the appropriate concentration was then added (see Table 2.1-2). The agar was poured into the Petri dishes (~15ml) and left to set at room temperature. Once set, LA plates were incubated overnight at 37°C before storage at 4°C. BA plates were left overnight at room temperature, and stored at 4°C.

2.1.3 Antibiotics

The antibiotics used are shown in Table 2.1-2. Those prepared with nano-pure H_2O, were filter-sterilised through an Acrodisc 25μm Syringe Filter (Pall Corporation), divided into stocks of 500μL, and stored at –20°C. Erythromycin, which was prepared in ethanol, did not require sterilisation and was stored at 4°C and used within one week of preparation or before crystals began to form.

Table 2.1-2 Antibiotics and concentrations used.

Antibiotic	Stock concentration	Working concentration	
		For *E. coli*	For *S. pneumoniae*
Ampicillin	100mg/ml in nano-pure H_2O	100μg/ml	----------
Spectinomycin	100mg/ml in nano-pure H_2O	100μg/ml	200μg/ml
Erythromycin	100mg/ml in ethanol	----------	100μg/ml

2.1.4 Bacterial strains

In Table 2.1-3 the bacterial strains used in this study are listed. Bacterial stocks were prepared for each strain (see 2.2.2) and kept at –70°C until needed. As cryoprotectant, glycerol to a concentration of 15% (v/v) was added when freezing *E. coli* cells and foetal calf serum to a concentration of 20% (v/v) was added when freezing pneumococcal cells.

Table 2.1-3 Bacterial strains used.

Bacteria strain	**Description**	**Source**
E. coli DH5α	Laboratory *E. coli* strain used for transformation of plasmids.	Lab 227 Bead collection 3I Department University of Leicester
S. pneumoniae D39	Wild type laboratory strain. Capsulated, type 2 pneumococci. Virulent.	Lab 227 Bead collection 3I Department University of Leicester
S. pneumoniae R6	Wild type laboratory strain. Un-encapsulated. Non virulent.	Lab 227 Bead collection 3I Department University of Leicester
S. pneumoniae *PLN-A*	Isogenic mutant of *S. pneumoniae* D39. Contains an insertion mutagenesis of the *ply* gene. Erythromycin resistant.	Lab 227 Bead collection 3I Department University of Leicester
S. pneumoniae Δ4	Isogenic mutant of *S. pneumoniae* D39. Contains a truncation on its *ply* gene. Spectinomycin resistant.	Constructed during this study.
S. pneumoniae Δrgg	Isogenic mutant from *S. pneumoniae* R6. Contains an insertion deletion of its *rgg* gene. Spectinomycin resistant.	Constructed during this study.

2.1.5 Plasmids

A list of the plasmids used during this study is shown in Table 2.1-4.

Table 2.1-4 List of plasmids used during this study

Plasmid	Description	Reference
pUC 19	2.6kb High copy *E. coli* cloning vector. Ampicillin resistance. Multiple cloning site on a lacZα gene.	Yanisch-Perron *et al.*, 1985
pDL278	6.7kb Gram positive - Gram negative shuttle vector. Spectinomycin resistant.	Le Blanc *et al.*, 1992
pMBR1	5.9kb Ampicillin resistant. Developed from pUC19. Contains the full *ply* gene flanked by 1kb of its adjacent DNA.	This study
pMBR2	7.1kb Ampicillin and Spectinomycin resistant. Developed from pMBR1. A spectinomycin cassette was inserted between nucleotides 1520 and 1521 from the *ply* gene.	This study

2.1.6 Primers

A table with the list of all the primers used during this study is shown in Table 2.1-5.

Table 2.1-5 List of primers used during this study

Name	Primer sequence (5' to 3')	Tm(°C)
03-4127-1	CTAGTCATTTTCTACCTTATCCTC	50.8
A-SOE-F[1,4]	*AA**GGATCC***AAGAGGCATACGCAGGCCAC	60.7
B-SOE-R[1,2]	*GTATTCACGAACGAAAATCGATC*ACGACCGAATGTTGAATAT CTGC	55.9
C-SOE-F[1,3]	*GCATAACTTTCTCGTCCATATCG*GACTTTTCCAATCTGCTCC ATAG	54.3
D-SOE-R[1,4]	*AA**GGATCC***AAGTGATTGGCCTGCTTAACGAG	58.1
E170Q-F	GTTTGGTTCTGACTTTCAGAAGACAGGGAATTCT	61.1
E170Q-R	GAGAATTCCCTGTCTTCTGAAAGT	55
Ply1kbF[1,4]	*CGC**GGATCC***CTCTGGTTAAAAAAAGAAGCCGATAAG	55
Ply1kbR[1,5]	*CGG**GGTACC***TGGATCACCTTTTTTAGCTGCTAC	53.8
PneuF	ATGGCAAATAAAGCAGTAAATG	50.7
PneuR	CTAGTCATTTTCTACCTTATCC	48.6
RER1	GATGGCAAATAAAGCAGTAAATGA	52.7
sp0206F	ACGGGGAAAGTCAGGACACTGG	61.5
sp0206R	TGGGTACTACCAGATGGCGTCC	60.8
sp0318F	ACCGCACTCCCTGCCAACGAG	64.6
sp0318R	ACTTTGACGACAACCAAAGCCGTTC	60.4
sp0732F	TGGGATCTTGTTGGGCTCCA	59.2
sp0732R	AAAGGCGTTCATCCACTCAG	55.9
sp0766F	TGGCGGACACTTGAACCACG	60.8
sp0766R	ACGAGTTGTTGCTGCTGCAGTG	61.0
sp0806F	TCGTGTGGCTGCCAAGCGTG	63.6
sp0806R	GGCTGATCCACCAGCTGAGTC	60.7
sp1250F	CCTACGCAACGAGAGAGTTCACCT	60.4
sp1250R	AGGGCGTTACACGCACGATTT	59.9
sp1314F	TGTAGAGATTCCACAATCTCGTCG	56.4
sp1314R	GCAACACCTTATCCAAAAGCTG	55.7

sp1325F	CTGCTCGTACAGGTTGGGAAGAAC	60.1
sp1325R	ACATTGCCTCCTACCATTGTCGCT	60.7
sp1326F	TGACGCTCGTTACGGTGGAACTC	61.1
sp1326R	AGGCCATTCTAATGGTACTGGCGCA	62.4
sp1327F	TCTAGCTGATGGAGTTCCAGGAGA	58.7
sp1327R	CTTCGCTAAAAGGTACACGTGACTG	58.1
sp1332F	GGTCAACGAAAACAAGTGGTCAAGG	59.2
sp1332R	CCAAGGAACTTGATGTTGAAGGCCA	60.1
sp1438F	AAGAGATGGACAGGCGTTTG	55.9
sp1438R	AATTCGATGAAGGCTCATCC	53.4
sp1496F	ACCTGCTTTGAAGACACCTCATGT	58.8
sp1496R	TCAAGATAGCCCAAGCTTGCTCA	58.8
sp1515F	TGCTGAGGTGGTCAATCAGGCTCT	62.1
sp1515R	AGTTCCCCTTTCCCAAGAGGCA	60.9
sp1651F	AGTCGGCGACAAGGCGCTTG	63.3
sp1651R	CGACGTGTTTGAGTTGAGCAGATGC	60.9
sp1869F	ACAAGCGTCACCATGATTACCGTGG	61.5
sp1869R	TCGCAAGCCAGAACTAGATTGGCAC	61.6
sp1870F	TGCCTTGGTAGGACCTATGGCCT	62.0
sp1870R	ATCAAGGCTTGCCCCAAGGT	60.1
sp2123F	TCACTGAGACAAGCAACCGGAGG	61.3
sp2123R	ACCTCTTGCCAGAAAGAGGATCTCC	60.4
sp2207F	TCGTTCAGAGCGCTTGGGGACAG	63.5
sp2207R	ACATCCTTAGCACCAGCTTCTTCC	59.3
sp2211F	TCGCCAAGAAGAAGATTGGGCTAGG	60.9
sp2211R	TCCGCATGAAGTAAGGGCTGAGTC	61.0
SpecF[1,6]	*AAA**CGTACG***ATCGATTTTCGTTCGTGAATAC	51.8
SpecR[1,6]	*AAA**CGTACG***TATGCAAGGGTTTATTGTTTTC	50.3
specSOE-F	GATCGATTTTCGTTCGTGAATAC	52.8
specSOE-R	CGATATGGACGAGAAAGTTATGC	54.1

[1]Sequence shown in italics does not correspond to the annealing sequence of the primer

[2]Underlined sequence is complementary to primer specSOE-F

[3]Underlined sequence is complementary to primer specSOE-R

[4]Sequence shown in bold letters corresponds to restriction endonuclease *BamHI*

[5]Sequence shown in bold letters corresponds to restriction endonuclease *KpnI*

[6]Sequence shown in bold letters corresponds to restriction endonuclease *BsiWI*

The software FastPCR v.3.6.21 from the Institute of Biotechnology of the University of Helsinki was used as an aid to design the primers. When there was a specific area to locate a primer, this was designed manually and the FastPCR program was used to check for the presence of primer dimers and to assess the melting temperature of the primers. If there was no specific site to locate the primers, the DNA sequence was analysed by FastPCR for the best set of primers. Specifications on the primer design such as the number of nucleotides, preferred melting temperature, length of the PCR product and others could be given to the program, following the instructions in the electronic user manual.

All nucleotides in the primers that did not correspond to the annealing sequence, such as those for restriction endonucleases, were added at the 5' end of the primers. These sequences were not considered when assessing for primer dimer formation or for the melting temperature of the primer.

2.2 Bacteriological Techniques

2.2.1 Bacterial growth

2.2.1.1 Growth of *Escherichia coli*

When grown in broth, *E. coli* was inoculated into LB medium (Table 2.1-1) using no more than 20% of the flask capacity. The culture was incubated at 37°C on a shaking platform at 200rev/minute. When grown on agar, LA plates were used (see 2.1.2.1) and incubated at 37°C overnight.

2.2.1.2 Growth of *Streptococcus pneumoniae*

Unless stated otherwise, *S. pneumoniae* was grown in BHI broth (see Table 2.1-1) or on BA plates (see 2.1.2.1), at 37°C in micro-aerobic conditions (see below).

Anaerobic growth

BA plates or flasks inoculated with pneumococci were placed in anaerobic jars containing a platinum catalyst chamber (BBL GasPack system). An anaerobic envelope (BD BBL GasPak Anaerobic System Envelopes) and an anaerobic indicator (BBL Dry Anaerobic Indicator Strips) were placed into each jar. The anaerobic envelope was activated according to the manufacturer's instructions and the tightly closed jar incubated at 37°C.

Micro-aerobic growth

For pneumococci culture in broth, the container was tightly closed and incubated at 37°C for the required amount of time. When the culture was on BA plates (see 2.1.2.1), the inoculated plates were placed inside a CO_2 jar (BBL GasPack system). A lighted candle was placed inside the jar before closing it tightly and incubating at 37°C overnight. This system produces ~5% CO_2 (Isenberg, 1998).

Aerobic growth

Aerobic growth of pneumococci in broth was done with the use of a flask placed in a water bath at 37°C where a stream of filtered air was bubbled through the culture. The apparatus for the aerobic growth is shown in Figure 2.2-1.

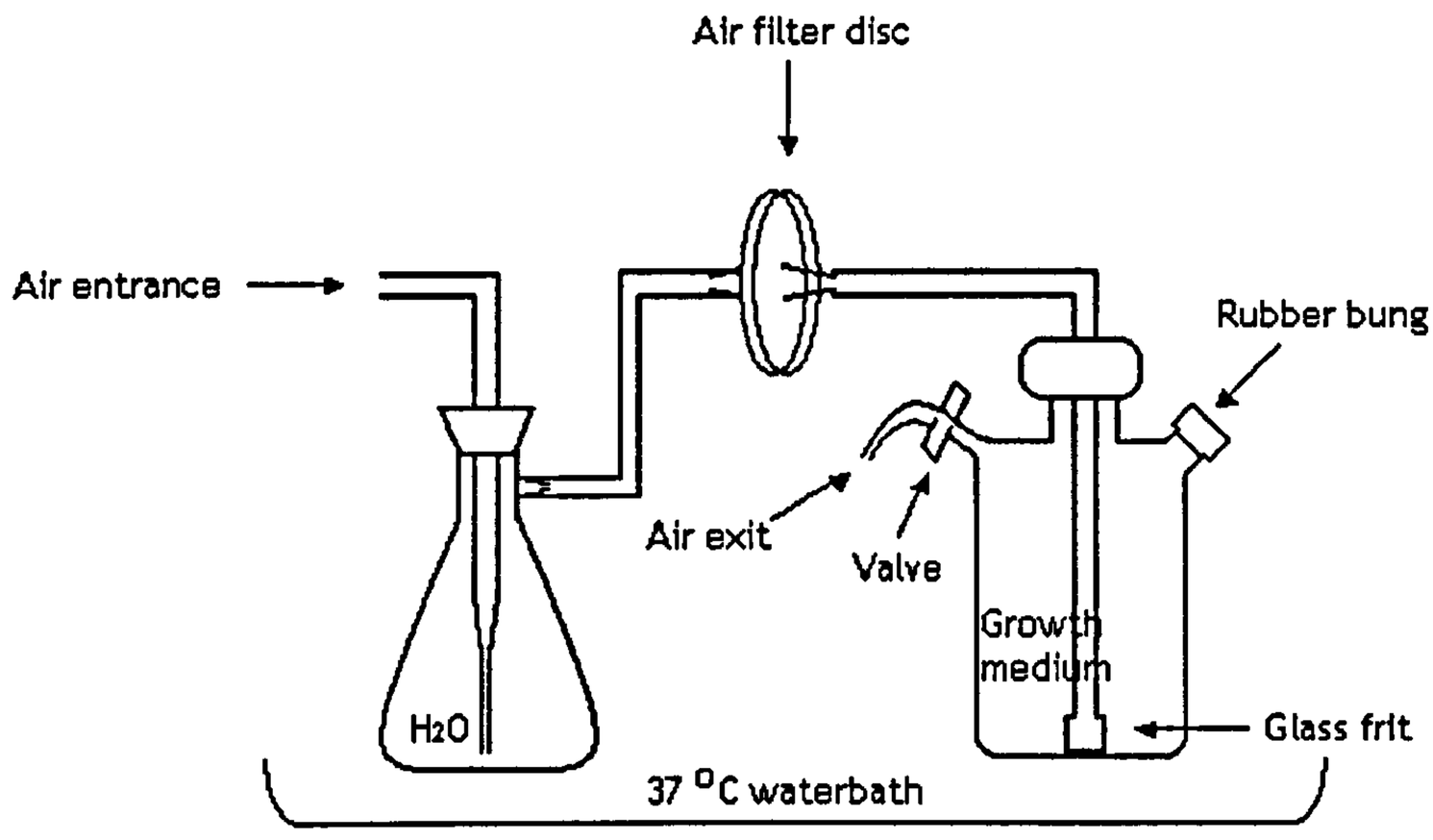


Figure 2.2-1 Apparatus used for aerobic growth

2.2.1.3 Multilayer plating technique for pneumococci (Iannelli and Pozi, 2004)

Before starting, all the media used were melted and placed into a water bath at 48°C. 90mm single vent Petri dishes were used. 15ml of TSA (see Table 2.1-1) were poured into each Petri dish, and left to solidify. Bacterial cells were added into 2ml of TSB (see Table 2.1-1) supplemented with 10%(v/v) horse blood (Oxoid, UK). The mixture was added to 6ml of TSA and poured over the initial TSA layer. Once solidified, the plate was incubated under microaerobic conditions (see 2.2.1.2) for 90 minutes at 37°C. After the incubation, the required antibiotic (see Table 2.1-2) was added to 8ml of TSA and poured over the bacterial layer. The antibiotic layer was left to solidify and the plate was incubated under microaerobic conditions at 37°C overnight.

2.2.2 Preparation of bacterial stocks

For the production of *E. coli* stocks, 15% (v/v) sterile glycerol was added into a log phase *E. coli* culture in LB medium (see Table 2.1-1). The culture was separated into microcentrifuge tubes and stored at –70°C.

For the preparation of pneumococcal stocks, 10ml of an overnight culture in BHI broth (see Table 2.1-1) were centrifuged at 3000g for 10 minutes and the pellet re-suspended in 1ml of serum broth (see Table 2.1-1). 1ml of this suspension was added to 15ml of serum broth and incubated at 37°C. After 5 hours, 500μL aliquots were made in microcentrifuge tubes and stored at –70°C.

2.2.3 Determination of colony forming units (CFU)

The Miles and Misra (Miles and Misra, 1938) method was used whereby 20μL of bacteria culture was serially diluted in 180μL of PBS (136mM NaCl, 2.68mM KCl, 10.14mM Na_2HPO_4, 1.76mM KH_2PO_4, pH7.4, autoclaved). Normally, dilutions of up to 10^6 were done. Six 20μL drops from each dilution were placed onto an agar plate, left to dry, and incubated overnight. The next day, the number of colonies was counted on the appropriate dilution. The average number of colonies per 20μL drop was calculated and multiplied times 50 to obtain the number of colonies per millilitre of dilution. That number was then multiplied by the dilution factor, to obtain the number of colonies per millilitre of culture.

2.2.4 Preparation of competent bacterial cells

2.2.4.1 Production of competent *E. coli* (Sambrook *et al.*, 1989)

E. coli competent cells were produced for electroporation, as well as for heat-shock transformation (calcium competent cells). When transforming ligation reactions (see 2.3.10 for cloning of DNA fragments) calcium competent cells were preferred (see 2.2.5.1 for transformation of *E. coli* cells). Electro-competent cells were preferred when plasmid transformations were done.

Method for the production of electro-competent *E. coli*

The protocol followed was based on the method described by Dower *et al.*, (1988). One colony of *E. coli* DH5α was grown overnight in 10ml of LB medium (see 2.1-1). The overnight culture was poured in 400ml of LB medium and incubated until the bacteria reached an OD_{550nm} of 0.6 using the Ultrospec 3000 spectrophotometer from Pharmacia Biotech. 50ml of culture were transferred into an ice-cold falcon tube and left on ice for a minimum of 15 minutes. A series of four centrifugations and gentle re-suspensions of the bacterial pellet followed. All centrifugations were done at 18000g for 10 minutes at 4°C. After the first and the second centrifugations, the bacterial pellet was re-suspended in 50ml of sterile ice-cold nano-pure H_2O. Following the third centrifugation, it was re-suspended in 5ml of ice-cold sterile 10% (v/v) glycerol in nano-pure H_2O. When the final centrifugation was done, the pellet was re-suspended in 500μL of the sterile ice-cold 10% (v/v) glycerol in nano-pure H_2O and separated into 40μL aliquot stocks in ice-cold microcentrifuge tubes. The bacteria were then frozen and stored at –70°C. Special care was taken to keep the bacterial cells cold at all times.

Method for the production of calcium-competent *E. coli* (Sambrook *et al.*, 1989)

One millilitre of an overnight *E. coli* DH5α culture was inoculated into 100ml of LB medium (Table 2.1-1) and incubated until the OD_{550nm} reached 0.6 (an Ultrospec 3000 spectrophotometer from Pharmacia Biotech was used). After incubation, the bacteria were kept on ice for at least 15 minutes, and centrifuged at 18000g for 10 minutes at 4°C. The bacterial pellet was re-suspended in 20ml of ice-cold 0.1M $CaCl_2$ and kept on ice for 30 minutes. The bacterial suspension was centrifuged as before, and the pellet re-suspended in 4ml of ice-cold 0.1M $CaCl_2$ containing 15%(v/v) glycerol. 50μL aliquots were made in ice-cold microcentrifuge tubes and stored at –70°C.

2.2.4.2 Production of competent pneumococci (Ianelli and Pozzi, 2004)

Pneumococcal cells were grown in 10ml of BHI broth (Table 2.1-1) at 37°C under microaearobic conditions (see 2.2.1.2) until early exponential phase (OD_{590} 0.03-0.06). The cells were then diluted 1:100 with 40ml of pre-warmed TSB broth (Table 2.1-1) at 37°C and the incubation was continued. After 30 minutes and every 15 minutes afterwards during at least two hours, 4x 800μL samples of bacterial culture were transferred into microcentrifuge tubes containing 200μL of 50% (v/v) glycerol in TSB/GT (Table 2.1-1). The microcentrifuge tubes containing the sample were left to cool down on ice for 2 minutes and frozen at –70°C until further use.

2.2.5 Bacterial transformation

2.2.5.1 Transformation of *E. coli*

Electroporation method (modified from Dower *et al.*, 1988)

The electroporation cuvette (Gene Pulser Cuvette, 0.2cm from Bio-Rad), transforming DNA and electro-competent cells (see 2.2.4.1) were placed on ice. When the cells thawed they were transferred into the electroporation cuvette, the transforming DNA was also added and left on ice for 5 minutes. After this, an electric pulse using a Gene Pulser electroporator from Bio-Rad was given. The settings for the electroporator were: 1000 Ohms, 25μfaradays and 1.5Kvolts. Immediately after the electric pulse was given, the cells were placed on ice for 2 minutes. The cells were transferred into a microcentrifuge tube containing 1ml of LB medium (Table 2.1-1) and incubated at 37°C on a shaking platform (200rev/minute). After 30 minutes, the cells were plated on LA plates (see 2.1.2.1) containing the required antibiotic, and incubated overnight for the selection of transformant colonies.

Heat-shock transformation (Sambrook *et al.*, 1989)

Calcium competent cells (see 2.2.4.1) were placed on ice until thawed. The transforming DNA was added to a 50μL aliquot of competent cells and left on ice for 30 minutes. A water bath was set at 42°C. After the 30 minutes, the aliquot was quickly placed into the water bath for exactly 90 seconds and returned to ice for 2 minutes. One millilitre of LB medium (Table 2.1-1) was added into the aliquot and incubated at 37°C for 30 minutes on a shaking platform (200rev/minute). After this time, the cells were plated on LA plates (see 2.1.2.1) containing the appropriate antibiotic (see Table 2.1-2 for antibiotic concentrations) and/or IPTG and X-gal if required (see 2.3.10 for selection of clones), and incubated overnight for the selection of transformant colonies.

2.2.5.2 Transformation of *Streptococcus pneumoniae* (Ianelli and Pozzi, 2004)

Three aliquots corresponding to pneumococcal competent cells from different time points of the growth curve (see 2.2.4.2) were thawed on ice. 200μL of each sample were placed into 1.5ml microcentrifuge tubes. The transforming DNA (circular or linear) to a final concentration of 1μg/ml, and competence stimulating peptide (Obtained from D. Morrison, University of Chicago) to a final concentration of 100ng/ml were added. The reaction was placed at 37°C for 45 minutes and then plated using the multilayer plating technique for pneumococci (see 2.2.1.3).

2.2.6 *Streptococcus pneumoniae* lysis by sonication (Kadioglu *et al.*, 2004)

S. pneumoniae was grown in 10ml of BHI broth (Table 2.1-1) to mid log phase and centrifuged for 10 minutes at 3000g. The supernatant was discarded, and the pellet re-suspended in 1.5ml of PBS (136mM NaCl, 2.68mM KCl, 10.14mM Na_2HPO_4, 1.76mM KH_2PO_4, pH7.4, autoclaved). The bacterial suspension was transferred into a microcentrifuge tube and left on ice for 10 minutes. The sonicator (Soniprep 150) was set to an amplitude of 8 microns. Sonication pulses of 15 seconds, every 30 seconds, were given to the bacterial suspension. The cells were kept on ice at all times and the sonication pulses were given until the bacterial suspension acquired a transparent colour. The cell lysate was centrifuged at 18000g for 1 minute and the supernatant filtered thorough a 25μm Acrodisc Syringe Filter (Pall Corporation) into clean microcentrifuge tubes. The filtered lysate was stored at –70°C until further use.

To estimate the number of bacterial cells lysed per millilitre of culture, CFU determinations (see 2.2.3) before and after sonication were done. The difference represented the number of bacteria cells lysed.

2.2.7 Haemolytic assay

The protocol followed was based on that described by Owen *et al.*, (1994). 4% (v/v) sheep blood solution was prepared by centrifugation of 2ml of sheep blood (Oxoid) at 2800g for 5 minutes. After centrifugation, the serum was carefully removed with a pipette and 400μL of the pellet were transferred to a Universal tube containing 10ml of PBS (136mM NaCl, 2.68mM KCl, 10.14mM Na_2HPO_4, 1.76mM KH_2PO_4, pH7.4, autoclaved).

Serial two-fold dilutions with 50μL of sonicated bacteria (see 2.2.6) were done in 50μL of PBS and placed on a 96-well round bottom micro titre plate (Nunc). 50μL of 4% (v/v) sheep red blood cell suspension (Oxoid) were added to the samples and incubated at 37°C for 30 minutes. After this time, the plate was placed on a light box and the dilution at which the sample showed 50% lysis of erythrocytes was recorded and multiplied by 10 to give the number of haemolytic units per millilitre.

2.3 Nucleic Acid Manipulation Techniques

2.3.1 Precipitation of DNA (based on Sambrook *et al.*, 1989)

To precipitate DNA, 0.1 volume of 3M sodium acetate pH4.6 and 0.6 volumes of isopropanol were added to the DNA solution. If the DNA concentration was known to be low (<50ng), 1μL of 1μg/ml tRNA (Sigma) were added per ml of initial solution to aid the precipitation. The solution was incubated at –20°C for 30 minutes if the volume was below 1ml, or for more than one hour if the volume was greater than 1ml. After incubation at –20°C, the solution was centrifuged at 36000g for 10 minutes at 4°C if it was contained in microcentrifuge tubes, or alternatively at 29900g for 30 minutes at 4°C if contained in Corex tubes. After centrifugation, the pellet was retained and 200μL or 3ml of 70% (v/v) ethanol were added (the volume

added was dependant on the size of the container). Centrifugation was carried out as before but this time at room temperature. Once finished, the supernatant was removed and the pellet dried at 37°C until no water was visible. The pellet was re-suspended in the desired volume of nano-pure H_2O.

2.3.2 DNA purification using the QIAquick PCR purification kit (Qiagen)

This kit was used for the purification of DNA fragments from reactions such as PCR (see 2.3.11), restriction digestion (see 2.3.8) and DNA ligation reactions (see 2.3.10) between others. This kit was also used for the concentration of DNA fragments from volumes of up to 200μL into final volumes of 30μL. When the DNA to concentrate was contained in larger volumes, or when the elution volume should be less than 30μL, the protocol for precipitation of DNA (see 2.3.1) was preferred.

The protocol used for the purification of DNA fragments with this kit, was that given by the manufacturer. All steps were performed at room temperature.

Five volumes of buffer PB (provided) were mixed with one volume of sample. The mixture was poured into a QIAquick spin column sitting on a 2ml collection tube and centrifuged for 30 seconds at 18000g. If the volume of the mixture was higher than that of the column, the flow-through was discarded and the resting sample was loaded into the same column and centrifuged as before. After centrifugation, the flow-through was discarded and the column placed back on the collection tube. 750μL of buffer PE (provided) were added into the column and centrifuged for 30 seconds at 18000g. The flow-through was discarded, the column placed back over the collection tube and a further 1 minute centrifugation at 18000g was performed. The column was placed into a clean 1.5ml microcentrifuge tube and 30μL of nano-pure H_2O were added to the centre of the column. It was left to stand for 1 minute and centrifuged at 18000g for a further minute. The DNA, which was contained on the eluate, was stored at –20°C until needed.

2.3.3 Plasmid extraction from *Escherichia coli* by Alkaline Lysis (based on Sambrook *et al.*, 1989)

In Table 2.3-1 the solutions used for plasmid extraction and their composition are shown.

Table 2.3-1 Solutions used for plasmid extraction by alkaline lysis.

Solution	Composition	
AL-solution I[1,2]	Glucose	50mM
	Tris.Cl (pH 8.0)	25mM
	EDTA (pH 8.0)	10mM
AL-solution II[3]	NaOH	0.2N
	SDS	1.0% (w/v)
AL-solution III[2]	5M KAc	60 ml
	glacial acetic acid	11.5ml
	H_2O	28.5ml
Extraction solution[4]	Phenol	25 volumes
	Chloroform	24 volumes
	iso-Amyl alcohol	1 volume
Chloroform[2]	Self descriptive	

[1] Autoclaved.

[2] Stored at room temperature.

[3] Freshly prepared from a 2N NaOH stock solution that was autoclaved and stored at room temperature, and from a 10% w/v SDS solution stored at room temperature.

[4] A 50ml batch was prepared and stored at 4°C, when the solution acquired a yellow-orange colour, it was discarded and fresh batch prepared.

2.3.3.1 Small scale plasmid extraction

1.5ml of overnight *E. coli* culture in LB medium (Table 2.1-1) containing the desired plasmid were centrifuged in a microcentrifuge tube at 18000g for 1 minute at room temperature. The supernatant was discarded and the pellet re-suspended by vortexing in 150μL of AL-solution I (Table 2.3-1), and placed on ice. After 10 minutes, 150μL of AL-solution II (Table 2.3-1) were added and mixed by inversion. The tube was placed on ice for 10 minutes, and 150μL of AL-solution III (Table 2.3-1) were added and gently mixed by inversion. The sample was placed on ice for further 10 minutes. After this period, the sample was centrifuged at 18000g for 10 minutes at 4°C and the supernatant transferred into a clean microcentrifuge tube. The protocol for precipitating DNA (see 2.3.1) was then followed without the addition of sodium acetate and the DNA was re-suspended in 30μL of nano-pure H_2O.

2.3.3.2 Medium scale plasmid extraction

25ml of overnight *E. coli* culture in LB medium (Table 2.1-1) containing the desired plasmid were centrifuged in a Corex tube at 18100g for 10 minutes. The supernatant was discarded and the pellet re-suspended by vortexing in 5ml of AL-solution I (Table 2.3-1), and placed on ice for 10 minutes. 5ml of AL-solution II (Table 2.3-1) were added and mixed by inversion. The tube was placed on ice for 10 minutes, and 5ml of AL-solution III (Table 2.3-1) were added and gently mixed by inversion. The sample was placed on ice for further 10 minutes, and centrifuged at 18100g for 10 minutes at 4°C. The supernatant was filtered to a clean Corex tube. To extract the DNA, 2ml of extraction solution (Table 2.3-1) were added to the supernatant and it was carefully vortexed to avoid spillage. The sample was centrifuged at room temperature at 18000g for 10 minutes, and the upper phase was transferred to a clean Corex tube. 1 volume of chloroform was added to the upper phase, and mixed by careful vortexing. The sample was again centrifuged at room temperature, at 18000g for 10 minutes and the upper phase was transferred

to a clean Corex tube. DNA was then precipitated as explained in 2.3.1 but without the addition of sodium acetate, and re-suspended in 500µL of nano-pure H_2O.

2.3.4 Plasmid extraction from *E. coli* using QIAprep Spin Miniprep Kit (Qiagen)

A QIAprep Spin miniprep kit was used when plasmid DNA was to be sent for sequencing or when high DNA purity was required. The protocol used was that given by the kit manufacturer. All centrifugation steps were done at room temperature.

1.5ml of an overnight bacterial culture were placed in a microcentrifuge tube and centrifuged at 18000g for 1 minute. The supernatant was discarded and the pellet re-suspended in 250µL of buffer P1 (provided). 250µL of buffer P2 (provided) were added and mixed by inverting the tube several times. After this, 350µL of buffer N3 (provided) were added and the solutions mixed immediately by inverting the tube several times. The microcentrifuge tube was centrifuged for 10 minutes at 18000g, and the supernatant transferred to a QIAprep spin column sitting on its collection tube. Then the column assembly was centrifuged for 30 seconds at 18000g. The eluate was discarded and the column placed back onto the collection tube. 750µL of buffer PE (provided) were added and centrifuged for 30 seconds at 18000g. The eluate was discarded and a further centrifugation of 1 minute at 18000g was performed to remove any remaining buffer PE. To elute the DNA, the column was placed on a 1.5ml microcentrifuge tube and 50µL of nano-pure H_2O were added to the centre of the column, left to stand for 1 minute and centrifuged at 18000g for a further minute. The eluted DNA was stored at –20°C until further use.

2.3.5 Chromosomal DNA extraction from *Streptococcus pneumoniae*

The protocol used was based on Dillard and Yother, (1994). An overnight 10ml culture of *S. pneumoniae* in BHI broth (Table 2.1.2) was centrifuged at 4000g for 10 minutes. The pellet was re-suspended in 2.5ml of TE (10mM Tris HCl, 1mM EDTA, pH8.0) and 250μL of 10% (w/v) SDS was added to obtain a final SDS concentration of 1% (w/v). The mixture was incubated at 65°C for 15 minutes. 0.2 volumes of 5M potassium acetate (pH 8) were added and incubated at 65°C for 15 minutes. The lysate was then incubated at 4°C for one hour and centrifuged at 11000g for 10 minutes. The supernatant was transferred to a clean tube and two volumes of ethanol were added to precipitate the DNA. The tube was placed at –20°C for 30 minutes and centrifuged at 11000g for 20 minutes at 4°C. The supernatant was discarded and the DNA pellet left dried at 37°C. Once dried, the pellet was re-suspended in 200μL of nano-pure H_2O.

2.3.6 Agarose gel electrophoresis (Sambrook *et al.*, 1989)

Agarose gel electrophoresis was used for the separation and/or purification of DNA fragments. 1.8% (w/v) agarose gels (Table 2.3-2) were preferred when running fragments under 500bp. For DNA fragments above 500bp, circular plasmids, or chromosomal DNA, 0.8% (w/v) agarose gels (Table 2.3-2) were preferred.

Electrophoresis chambers, as described in Sambrook *et al.* (1989), were used. The chambers were filled with an amount of TAE buffer (Table 2.3-2) sufficient to cover the agarose gel by a few millimetres.

For the preparation of DNA agarose gel, the gel tray was assembled by sealing its edges and placing the comb as described by Sambrook *et al.* (1989). The required percentage of agarose gel mix was melted and left to cool to approximately 55°C. Once cooled, it was poured into the assembled gel tray to form a thickness of ~5mm and left at room temperature until set. The comb and the seal from the

edges of the gel tray were carefully removed, and the gel placed into the electrophoresis chamber in its tray.

The DNA samples were individually mixed with the loading buffer, and carefully placed into the wells of the agarose gel. Normally, a 1kb or a 100bp NEB ladder (New England Biolabs) or GeneRuler ladder (Fermentas) was also loaded as an electrophoresis control and to measure the approximate concentration and/or size of the DNA band. The electrophoresis was normally performed at 110mV.

When the electrophoresis was done, the gel was placed under UV light transilluminator at 360nm (Gel Doc Mega, BioSystematica) to visualise the DNA.

Table 2.3-2 Solutions for agarose gel electrophoresis

Solution	Composition	
Tris-acetate/EDTA electrophoresis buffer (TAE)	Tris-base Glacial acetic acid EDTA pH7	40mM 1.142ml per litre 1mM
5x Loading buffer	TAE Glycerol Orange G	2ml 2ml until strong orange colour
0.8%(w/v) Agarose gel mix	Multipurpose agarose (Bioline) in TAE Ethidium Bromide	0.8% w/v 1.8μg per 100ml
1.8%(w/v) Agarose gel mix	Multipurpose agarose (Bioline) in TAE Ethidium Bromide	1.8% w/v 1.8μg per 100ml

2.3.7 DNA recovery from agarose gels

A Fast 'n' Easy Spin Agarose Gel DNA Purification Kit from Anansa (Totam Biologicals LTD, Peterborough, UK) was used for the recovery of DNA from 0.8% (w/v) and 1.8% (w/v) agarose gels (Table 2.3-2). The protocol followed was that given by the supplier.

After DNA agarose gel electrophoresis (see 2.3.6), the gel was visualised under the UV light of a long-wave transilluminator. The band of interest was excised from the gel with a clean scalpel, taking care to remove as much agarose as possible without interfering with the DNA band. It was important to do this as quickly as possible to avoid possible mutations of the DNA due to exposure to UV light. The band was placed into a pre-weighted microcentrifuge tube and weighted. For every 0.1g of agarose, 0.3ml of Spin Buffer (provided) were added into the microcentrifuge tube. This was tightly closed and incubated for 2 minutes at 55°C. After this time, the tube was inverted once and incubated for one more minute or until the gel melted. After incubation, the solution was mixed once by inversion and placed into the spin filter sitting on its collection tube. The sample was centrifuged for 10 seconds at 10000g. The spin filter was removed and the flow through mixed by vortexing for 5 seconds, re-loaded into the spin filter and centrifuged as before. The flow through was discarded and the spin filter placed back onto the collection tube. 300μL of SpinWash Buffer (provided) were added into the spin filter and centrifuged for 10 seconds at 10000g. The flow through was discarded and the spin filter centrifuged for further 30 seconds at 10000g. The filter basket was transferred to a clean 1.5ml microcentrifuge tube. To elute the DNA, 50μL of nano-pure H_2O were added onto the centre of the spin filter and centrifuged for 30 seconds at 10000g. The spin filter was discarded and the eluted DNA was frozen at –20°C.

2.3.8 DNA restriction digestion

Restriction endonucleases were obtained from New England Biolabs or Invitrogen. Even though one unit of enzyme should have been sufficient to digest 1μg of DNA in one hour, no more than 800ng of DNA were incubated for digestion per unit of enzyme.

For a restriction digestion, the DNA was first mixed with the required amount of nano-pure H_2O and the appropriate buffer (provided by the manufacturer) to obtain a final buffer concentration of 1x, once the restriction endonuclease was added. The restriction endonuclease was added last, and the reaction incubated for a minimum of one and a half hours at the temperature required for optimum enzyme activity (usually 37°C). The volume of the digestion reaction varied according to the amount of DNA to digest, but normally a reaction containing from 200 to 400ng of DNA had a final volume of 20μL.

When double digestions were required, the efficiency of the enzymes if incubated together and their compatible buffer was determined from the New England Biolabs catalogue (2002). If the enzymes were compatible, the reaction was set up with one unit of each enzyme per 400ng of DNA to digest. If the enzymes were not compatible, two separate digestions were done.

After incubation of the restriction digestion for one and a half hours, a sample of the reaction was run on a DNA electrophoresis gel (see 2.3.6). The appropriate ladder and undigested DNA as control (if applicable) were run together with the sample to confirm the digestion. If partial digestion was detected, the incubation was left for longer, and if considered necessary, more restriction enzyme was added to the reaction.

If the restricted DNA was required for further reactions, it was purified using the QIAquick PCR purification kit (see 2.3.2), and the restricted DNA was stored at –20°C.

2.3.9 Blunt end generation (based on Ausubel *et al.*, 1995)

T4 DNA polymerase was obtained from New England Biolabs. This enzyme catalyses the synthesis of DNA from 5' to 3' in the presence of template, primer and dNTP. It also has exonuclease activity from 3' to 5'.

To set up the reaction 1μg of dsDNA, 2mM dNTP, 3μL of 10x T4 DNA Polymerase Buffer (supplied with the enzyme), one unit of T4 DNA polymerase, and the required amount of nano-pure H_2O to obtain a final volume of 30μL were mixed. The reaction was incubated for 15 minutes at 12°C and inactivated by the addition of EDTA to a final concentration of 10mM, and incubation at 75°C for 20 minutes.

2.3.10 Plasmid cloning of DNA fragments

Once the linearised plasmid and the insert DNA had compatible cohesive ends or blunt ends after restriction digestion (see 2.3.8) or blunt end generation (see 2.3.9), ligation reactions were set up with three different insert to vector ratios: 3:1, 1:1 and 1:3. This was calculated with the following formula (obtained from the pGEM-T easy vector systems technical manual of Promega), using 50ng of vector in all cases:

$$\frac{\text{ng of vector} \times \text{kb size of insert}}{\text{kb size of vector}} \times \text{insert:vector molar ratio} = \text{ng of insert}$$

The ligation reactions were done in volumes of 10μL containing 50ng of vector DNA and the required amount of insert DNA according to the insert:vector ratio. 5μL of 5X DNA Ligase Buffer (provided with the enzyme) and one unit of T4 DNA Ligase (Gibco BRL) were added. The reactions were incubated at room temperature for one hour and half of each reaction was transformed into calcium-

competent *E. coli* cells (see 2.2.5.1). The remaining reaction was left overnight at 17°C and transformed the next day.

2.3.10.1 Selection of clones

When cloning into plasmids containing the lacZ phenotype, the transformed ligation (see 2.2.5.1) was plated onto LA plates (see 2.1.2.1) where 20μL of 50mg/ml X-gal in dimethyl formamide and 100μL of 100mM filtered sterilised IPTG in water had been spread over the surface. With this selection, most of the colonies containing a plasmid where the *lac*Z gene had not been interrupted (without an insert) obtained a blue colour. Otherwise, if the *lac*Z gene was interrupted (colonies containing a plasmid with insert) the colonies had a white colour.

Possible clones were individually picked with a sterile pipette tip under sterile conditions. A small patch was made with the pipette tip on a labelled LA plate containing the required antibiotic (Table 2.1-2) and the tip was placed into an equally labelled 15ml Falcon tube containing 5ml of LB medium (Table 2.1-1) and the required antibiotic. The next day, small scale plasmid extraction (see 2.3.3.1) was done from each of the LB cultures and clones were identified by restriction digestion (see 2.3.8) and/or PCR (see 2.3.11.1).

When the insert was an antibiotic resistance cassette, the selection of clones was done by plating the transformed ligation into LA plates (see 2.1.2.1) that contained the antibiotic corresponding to the resistance cassette. Colonies growing on the plates were picked and identified as explained above.

2.3.11 Polymerase Chain Reaction techniques

2.3.11.1 Standard Polymerase Chain Reaction (PCR)

PCR was used for the amplification of DNA fragments either to confirm the presence of a gene, assess the construction of plasmids after cloning (see 2.3.10), confirm the construction of pneumococal mutants after transformation (see 2.2.5.2), or to obtain DNA fragments for cloning (see 2.3.10).

PCR Using proof-reading polymerase Accuzyme (Bioline)

When the fidelity of the DNA amplification was important, as when amplifying DNA fragments for cloning, the proof reading DNA Polymerase Accuzyme (Bioline) was used. This enzyme possess up to 47-fold higher fidelity than regular Taq Polymerase. For amplification with this enzyme, 20μL reactions containing 2pmol of forward and reverse primers (Table 2.1-5); 0.2mM dNTP; 2μL of 10X Accuzyme buffer (containing 20mM $MgSO_4$); 1.2 units of enzyme and approximately 3ng of DNA were put into a 0.5μL PCR tube (Axygen). The reaction was placed in a PCR machine (MJ Research PTC-200) and the following settings were used considering the recommendations of the enzyme supplier:

1st segment	5 minutes at 95°C (initial denaturation step)
2nd segment (repeated 34 times)	a) 1 minute at 94°C (denaturation step) b) 1 minute at the appropriate annealing temperature for the primers in use (see 2.3.11.2) (annealing step) c) 2 minutes per kb of expected fragment size at 72°C (extension step)
3rd segment	72°C for 15 minutes (final extension step)

PCR Using non-proof reading polymerise BioTaq (Bioline)

If the fidelity of the DNA amplification was not critical, DNA polymerase BioTaq (Bioline) was used. 20μL reactions containing 2pmol of forward and reverse primers; 0.2mM dNTP; 2μL of 10X BioTaq DNA polymerase buffer; 2mM $MgCl_2$; 0.5 units of enzyme; and approximately 3ng of DNA were prepared in 0.5μL PCR tubes (Axygen). The tubes were placed in the PCR machine (MJ Research PTC-200) and the following settings used considering the recommendations of the enzyme supplier:

1st segment	5 minutes at 95°C (initial denaturation step)
2nd segment (repeated 30 times)	a) 1 minute at 94°C (denaturation step) b) 1 minute at the appropriate annealing temperature for the primers in use (see 2.3.11.2) (annealing step) c) 1 minute per kb of expected fragment size at 72°C (extension step)
3rd segment	72°C for 10 minutes (final extension step)

2.3.11.2 Gradient PCR

Gradient PCR was used to establish the ideal annealing temperature when that given by the substraction of 5°C from the melting temperature of the primers gave poor results, or when primers with different melting temperatures were used.

Twelve PCR reaction tubes were prepared as explained in 2.3.11.1, using BioAct DNA polymerase (Bioline). The tubes were placed across the twelve columns of the PCR machine (MJ Research PTC-200). The settings used were as those shown in 2.3.11.1 when using BioTaq, except for the annealing temperature (2nd segment, step b). Instead, a range of temperatures no lower than 40°C nor higher than 70°C, was given across the columns of the PCR machine (Only 20°C of difference between the first and the last column was allowed by the available PCR

machine). This way, each PCR tube had a different annealing temperature. When the PCR was complete, 10μL of each reaction were run on a DNA electrophoresis gel (see 2.3.6). The temperature at which the band of interest was clearest, was chosen for all further PCR made with that set of primers.

2.3.11.3 Gene Splicing by Overlap Extension PCR (SOEing PCR)

This technique was used for the construction of mutated DNA sequences as an alternative way of mutation by cloning (see 2.3.10). The protocol used was based on that described by Horton (1995).

The constructed DNA fragments using SOEing PCR were directly transformed into *S. pnuemoniae* (see 2.2.5.2) for the construction of pneumococcal mutants. An illustration of the strategy is shown in Figure 2.3-1.

During the first step, the sequence to mutate or interrupt was amplified as two fragments, separated at the position where the insert fragment would be joined (Figure 2.3-1A left). These two fragments, as well as the insert fragment, were individually amplified using the proof reading polymerase Accuzyme from Bioline (see 2.3.11.1). Two sets of primers were therefore constructed for the DNA sequence to be interrupted (see Figure 2.3-1A left), one set for each of the fragments. The reverse primer that would amplify the first fragment (fragment a), contained a 3' sequence complementary to the forward primer of the insert sequence, and the forward primer that would amplify the second fragment (fragment b) contained a 5' sequence complementary to the reverse primer of the insert sequence (Figure 2.3-1A left). This gave three PCR products of which "fragment a" and "fragment b" have ends complementary to the fragment to be inserted (Figure 2.3-1A right). The fragments were purified using the QIAquick PCR purification kit from Qiagen (see 2.3.2), and used for the second step.

For the second step (see Figure 2.3-1B), the three PCR products were mixed together for another PCR. As "fragment a" and "fragment b" had complementary

sequences to that of the insert, they were capable of being annealed with it. Also when using the forward primer of "fragment a" and the reverse primer of "fragment b", the whole construction could be amplified.

To set up this reaction, approximately equal concentrations of each PCR product (~3ng of each) were added into a 0.5μL PCR tube. The forward primer used to amplify "fragment a" and the reverse primer used for the amplification of "fragment b", were added to a concentration of 2pmol each. 0.2mM dNTP, 2μL of 10X Accuzyme buffer (containing 20mM $MgSO_4$), 0.5mM $MgCl_2$ and 1.2 units of Accuzyme enzyme (Bioline) were also added and the volume of the reaction adjusted to 20μL with nano-pure water. The reaction was put into a PCR machine, and the settings used were as those explained in 2.3.11.1 when using Accuzyme DNA polymerase (Bioline), with an exception of the extension time. Here 3 minutes were given per kilobase of the expected sequence. The reaction was run on a DNA electrophoresis gel (see 2.3.6) and the band of interest was purified from the gel using the Fast 'n' Easy Spin Agarose Gel DNA Purification Kit from Anansa (see 2.3.7).

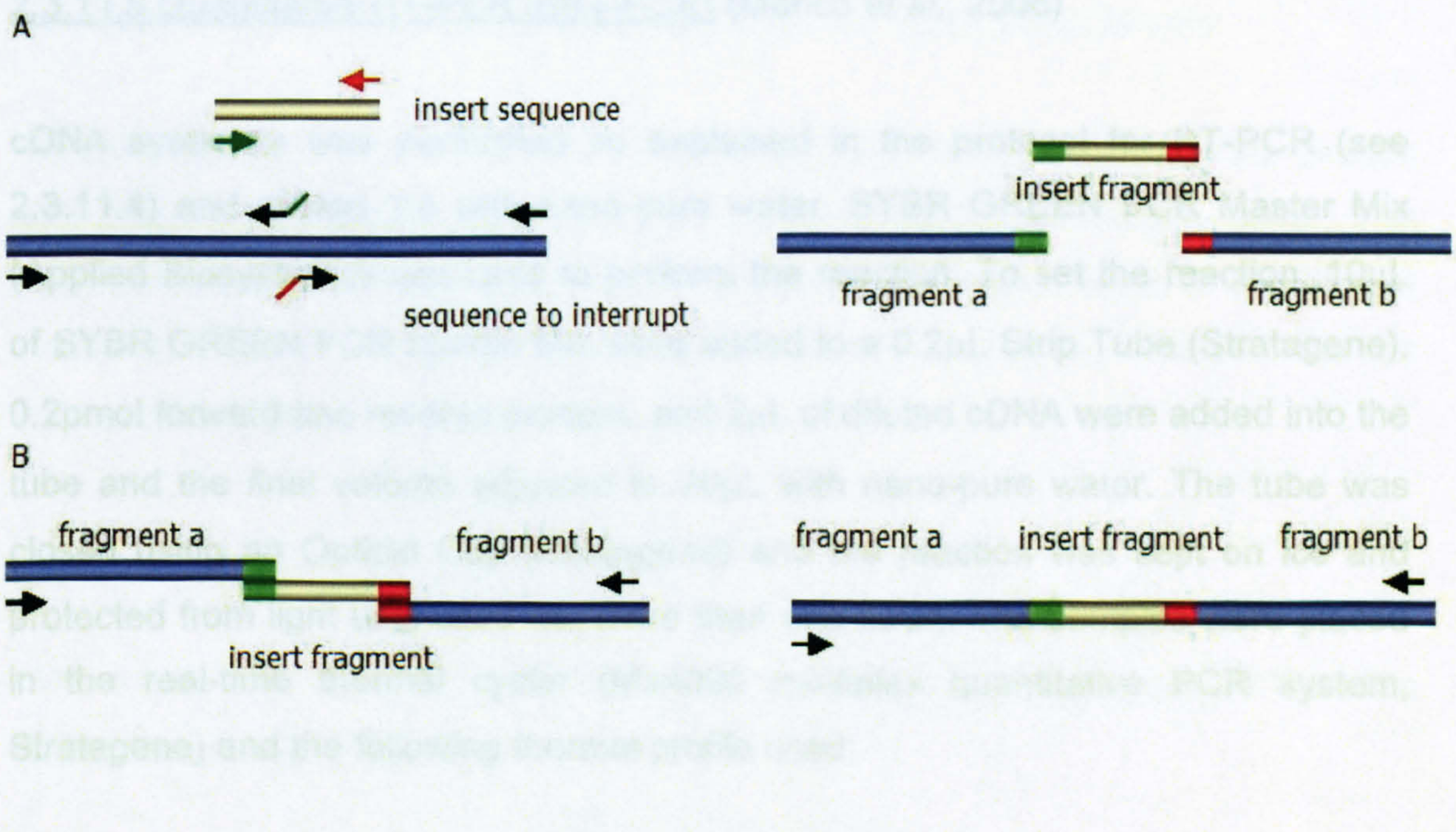


Figure 2.3-1 Illustration of the SOEing PCR technique used.

2.3.11.4 Reverse Transcriptase PCR (RT-PCR)

First strand cDNA synthesis was done using SuperScript II Reverse Transcriptase (Invitrogen). The protocol used was similar to that given by the supplier. 40μL (~900ng) of DNase-treated RNA (see 2.3.12) were mixed with 4μL of 2mM dNTP, 0.5μL of random primers (Invitrogen, 3μg/μL) and nano-pure H_2O to a final volume of 48μL. The reaction was incubated at 65°C for 5 minutes and then chilled on ice. 16μL of 5x first-strand buffer and 8μL of 0.1M DTT (both supplied with the enzyme) were added to the reaction and incubated at 25°C for 2 minutes. 4μL of Super Script II Reverse Transcriptase were added to the reaction and incubated at 25°C for 10 minutes. The reaction was further incubated at 42°C for 50 minutes, and a final incubation at 70°C for 15 minutes was done. At this point, the cDNA could be frozen at –20°C until required.

2μL of cDNA were used for PCR using the standard PCR protocol (see 2.3.11.1).

2.3.11.5 Quantitative RT-PCR (qRT-PCR) (Manco *et al.*, 2006)

cDNA synthesis was performed as explained in the protocol for RT-PCR (see 2.3.11.4) and diluted 1:5 with nano-pure water. SYBR GREEN PCR Master Mix (Applied Biosystems) was used to perform the reaction. To set the reaction, 10μL of SYBR GREEN PCR Master Mix were added to a 0.2μL Strip Tube (Stratagene). 0.2pmol forward and reverse primers, and 2μL of diluted cDNA were added into the tube and the final volume adjusted to 20μL with nano-pure water. The tube was closed using an Optical Cap (Stratagene) and the reaction was kept on ice and protected from light until used (no more than one hour). The samples were placed in the real-time thermal cycler (Mx4000 multiplex quantitative PCR system, Stratagene) and the following thermal profile used:

1st segment.- activation step (1 cycle)	10 minutes at 95°C (initial denaturation step)
2nd segment.- amplification step (40 cycles)	a) 30 seconds at 95°C b) 1 minute at 44°C c) 30 seconds at 72°C
3rd segment.- DNA melting step (1 cycle)	1 minute at 95°C
4th segment.- dissociation curve (41 cycles)	30 seconds at 55°C with 1°C increment per cycle (dissociation curve)

Information on the fluorescence was collected at the 44°C and 72°C plateaux of the 2nd segment, as well as on the plateaux of the 4th segment. The ROX filter path was selected for the passive reference dye, and the FAM filter path was used for the collection of SYBR Green data.

The results were obtained using the Mx4000 analysis software (Stratagene) and interpreted using the comparative C_T method described in the "User Bulletin #2" from ABI Prism 7700 Sequence Detection System, Applied Biosystems.

To describe briefly, the comparative C_T method consists on normalising the amount of a target gene (sample) to an endogenous reference (house keeping gene). This normalised value is then compared to the equally normalised value of a calibrator (value to which the target gene will be compared). This is represented with the following formula:

$$2^{-\Delta\Delta CT}$$

Where $\Delta\Delta C_T$ is equal to the ΔC_T of the sample ($\Delta C_{T,q}$) minus the ΔC_T of the calibrator ($\Delta C_{T,cb}$). That is:

$$\Delta\Delta C_T = \Delta C_{T,q} - \Delta C_{T,cb}$$

The ΔC_T value of the sample or of the calibrator is obtained by subtracting the C_T value of the reference or house keeping gene ($C_{T,R}$) from the C_T value of the sample or calibrator ($C_{T,X}$). That is:

$$\Delta C_T = C_{T,X} - C_{T,R}$$

This is done assuming that the amplification efficiency of the reference gene (house keeping gene) is equal to the amplification efficiency of the sample and of the calibrator.

2.3.12 RNA extraction from *S. pneumoniae*

The method described by Stewart *et al.*, 2002 for RNA extraction from *Mycobacterium tuberculosis* was used for extracting RNA from *S. pneumoniae*. Bacteria were grown in BHI broth (Table 2.1-1) under aerobic or anaerobic conditions until mid log phase (OD_{500nm} ~0.6). To stop RNA synthesis, 10ml of the culture were immediately poured into 40ml of GTC solution (5M guanidine isothiocyanate, 0.5%(w/v) sodium N-lauryl sarcosine, 25mM tri-sodium citrate pH7.0, 100mM 2-mercaptoethanol and 0.5% (v/v) Tween 80) in a Falcon tube and mixed rapidly by inversion and vortexing.

The bacteria were centrifuged at 5000g for 20 minutes, after which the supernatant was discarded and the pellet re-suspended in 1ml of GTC solution (see before). The bacterial suspension was transferred to a microcentrifuge tube and centrifuged at 12000g for 30 seconds in a microcentrifuge. The supernatant was discarded and the pellet re-suspended in 1.2ml of RNAzol (Biogenesis). The bacteria suspension was quickly transferred to a RiboLyser blue matrix tube (Haybaid) and processed in a RiboLyser (Hybaid Ribolyser) at 6.5 power setting for 45 seconds. After this, the tube was left for 5 minutes at room temperature and 200μL of chloroform were added. The suspension was vortexed for 15 seconds and left to stand at room temperature for a further 3 minutes. After this time, the sample was centrifuged at

12000g for 15 minutes at 4°C, and the upper phase was carefully transferred to a clean microcentrifuge tube. An equal volume of chloroform was added to the tube and vortexed for 15 seconds. The tube was again centrifuged at 12000g for 15 minutes at 4°C and the upper phase was carefully transferred to a clean microcentrifuge tube. 500μL of isopropanol were added to precipitate the RNA and the mixture was left to stand at room temperature for 15 minutes. After this time, it was centrifuged at 12000g for 10 minutes at 4°C. The supernatant was carefully removed and the pellet re-suspended in 100μL of RNase free nano-pure water.

The RNA was cleaned using the RNeasy Mini Kit from Qiagen, following the manufacturers' instructions. 10μL of β-Mercaptoethanol were added to 1ml of buffer RLT (provided). 350μL of this solution were added to the 100μL of RNA solution and mixed thoroughly by pipetting. 250μL of ethanol were also added and mixed with the sample, which was then transferred to an RNeasy mini column sitting on a 2ml collection tube. The tube was closed and centrifuged for 15 seconds at 8000g. The eluate was discarded, and the column placed on a new 2ml collection tube (provided with the kit). 500μL of buffer RPE (provided) were added onto the column, which was centrifuged for 15 seconds at 8000g. The eluate was discarded and the column placed into the same collection tube. 500μL of buffer RPE were again added and the column centrifuged for 2 minutes at 8000g. The eluate and collection tube were discarded and the column carefully transferred onto a clean RNase-free microcentrifuge tube. To elute the RNA, 30μL of RNase free water were added into the centre of the column, which was left to stand for one minute. The column was then centrifuged at 8000g for 1 minute. The column was discarded and the eluted RNA frozen at –70°C until further use.

To calculate the concentration of RNA, the RNA concentration program of the Ultrospec 3000 UV spectrophotometer from Pharmacia Biotech, which calculates the ratio of absorbance at 260 and 280nm was used.

Treatment of RNA with DNase I

If the RNA was required for further protocols such as RT-PCR (see 2.3.11.4) or qRT-PCR (see 2.3.11.5), the RNA was treated with the Amplification Grade DNase I from Invitrogen following the manufacturers instructions. 5μg of RNA were mixed with 5μL of 10x DNase buffer (provided with the enzyme) and 5μL of Amplification Grade DNase I (1unit/μL) in a final reaction volume of 50μL. The reaction was incubated at room temperature for 15 minutes. After this time, 5μL of 25mM EDTA pH8.0 were added and the reaction was heated for 10 minutes at 65°C to inactivate the enzyme. The reaction was placed on ice for 1 minute and used immediately for RT-PCR or qRT-PCR.

2.3.13 cDNA vs cDNA Microarray

Spotted microarray slides containing DNA fragments, obtained by PCR, representative of the 2,236 genes present in the genome of *S. pneumoniae* TIGR4 plus DNA fragments of 117 genes present in the genome of *S. pneumoniae* R6 and absent in *S. pneumoniae* TIGR4 were used. The arrays were obtained from the Bacterial Microarray Group at St. George's hospital, London. Data files obtained from the Microarray Group at St. George's hospital, containing information on each spot and on the pneumococcal genome were loaded onto the GenePix and GeneSpring softwares for the analysis of the spots (see "scan and analysis of the microarray slide" 2.3.13.7).

All rinsing and washing steps were performed with the use of dedicated staining troughs and racks, and with constant manual agitation of the staining rack.

2.3.13.1 Production of Cy3/Cy5 labelled cDNA (Stewart *et al.*, 2002)

RNA of pneumococci grown under aerobic and anaerobic conditions (see 2.2.1.2) was obtained as explained before (see 2.3.12). For the conversion of RNA into cDNA, 2-10μg of each sample of RNA were individually mixed with 3μg of random primers (Invitrogen) RNase DNase free water to make a final volume of 11μL in PCR tubes. The tubes were heated at 95°C for 5 minutes and snap-cooled on ice. 5μL of 5xFirst Strand Buffer and 2.5μL of DTT (both supplied with the SuperScript II enzyme) were added to each tube together with 2.3μL of dNTPs containing 2mM of dCTP and 5mM dATP, dGTP and dTTP. 1.5μL of either Cy3 or Cy5 dCTP (Invitrogen) were added to each tube, which was mixed gently by pippetting. 2.5μL of 200u/μL of SuperScript II (Invitrogen) were also added to each tube, mixed by pippetting and incubated in the dark at 25°C for 10 minutes followed by incubation at 42°C for 90 minutes.

2.3.13.2 Prehybridisation of microarray slide (Stewart *et al.*, 2002)

During the final incubation period to convert the RNA into cDNA, 50ml of freshly prepared prehybridisation solution that consisted of 8.75ml of 20xSSC (3M NaCl, 300mM sodium citrate, pH 7.0, autoclaved), 250μL of 20% (v/v) SDS and 5ml of 100mg/ml BSA were poured into a Coplin jar and incubated at 65°C for 45 minutes. The microarray slide was carefully placed into the Coplin jar containing the preheated prehybridisation solution and incubated for 20 minutes at 65°C. The slide was thoroughly rinsed in 400ml of filtered nano-pure water for one minute, followed by a rinse in 400ml of isopropanol for a further minute. The slide was place into a 50ml Falcon tube and centrifuged at 500g for 5 minutes. The slide was placed into a dust-free box in the dark until hybridisation (no more than one hour).

2.3.13.3 Purification of Cy3/Cy5 labelled DNA (Stewart *et al.*, 2002)

Once the RNA was converted into labelled cDNA, both, Cy3 and Cy5 labelled cDNA were mixed together in one same microcentrifuge tube and purified using the MiniElute Purification kit from Qiagen as follows. Cy3 and Cy5 labelled cDNA samples were mixed in a microcentrifuge tube and 250μL of buffer PB (provided with the kit) were added. The sample was transferred into a MiniElute column sitting on a collection tube, and centrifuged at 15,000g for 1 minute. The eluate was discarded and the column placed back into the collection tube. 500μL of buffer PE (provided with the kit) were added and centrifuged at 15,000g for 1 minute. The eluate was discarded, the column placed back into the collection tube and 250μL of buffer PE were added. The column was centrifuged at 15,000g for 1 minute. The eluate was discarded and the column centrifuged for an additional minute at 15,000g. The column was transferred into a 1.5ml microcentrifuge tube and 15.9μL of DNase RNase free water were added unto the centre of the column, which was left to stand for one minute, and then centrifuged at 15,000g for 1 minute.

2.3.13.4 Hybridisation of microarray slide with labelled cDNA (Stewart *et al.*, 2002)

The hybridisation solution was prepared by mixing 14.9μL of purified Cy3/Cy5 labelled samples with 4.6μL of filtered 20xSSC and 3.5μL of filtered 2% (v/v) SDS. The mixture was heated to 95°C for 2 minutes and left to cool slightly at room temperature. 15μL aliquots of nano-pure water were then added into the wells of the hybridisation cassette (see Figure 2.3-2). The prehybridised microarray slide was placed into the hybridisation cassette and a 22x22mm LifterSlip (VWR international) was placed over the spotted area of the microarray slide. The slightly cooled hybridisation solution was carefully pipetted under one corner of the LifterSlip allowing the solution to cover completely the array area by capillary action. The hybridisation cassette was closed tightly and incubated at 65°C in the dark for 16-20 hours.

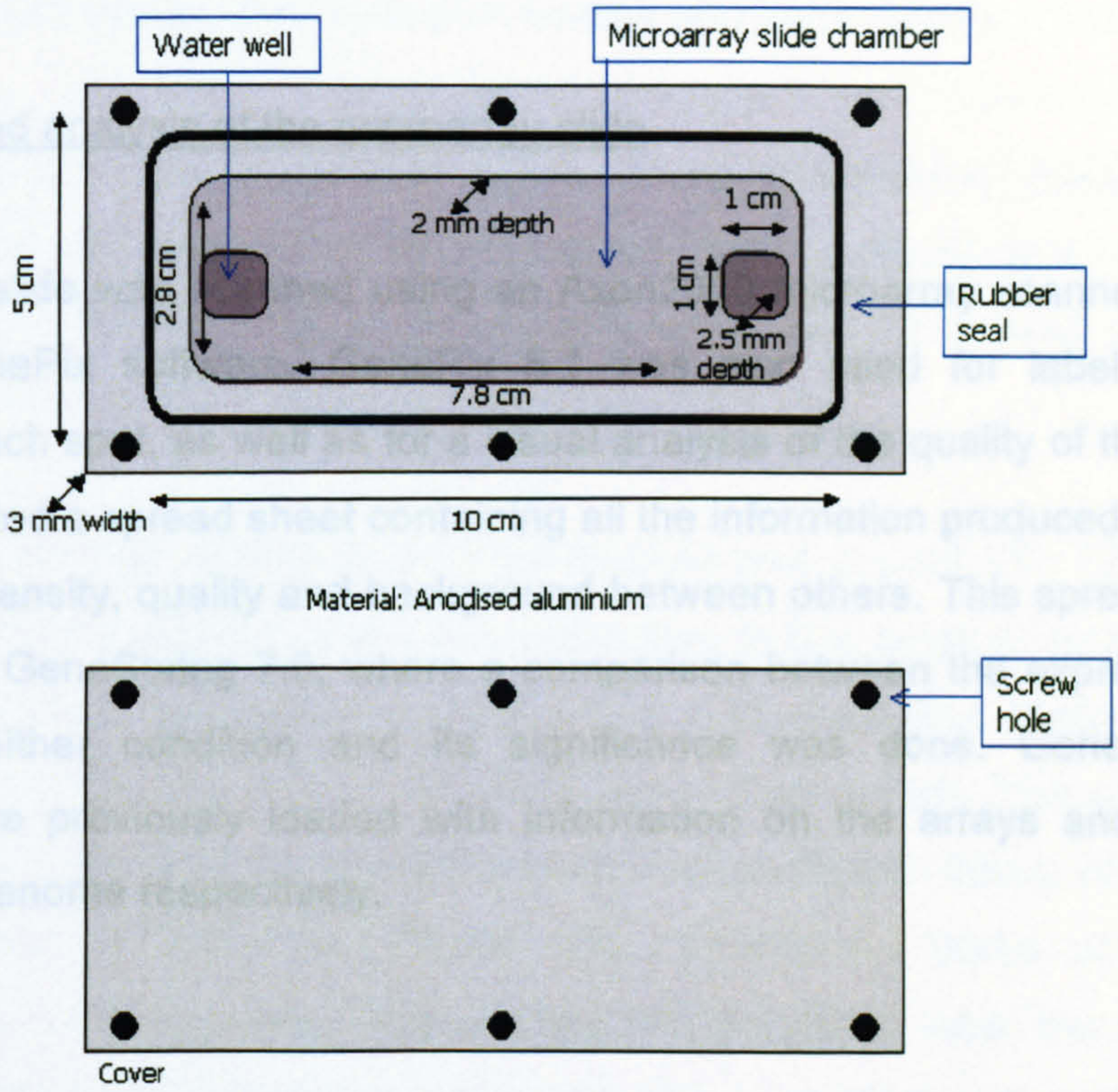


Figure 2.3-2 Microarray hybridization chamber

2.3.13.5 Washing of hybridised microarray slide (Stewart *et al.*, 2002)

The microarray slide was removed from the hybridisation cassette and gently immersed into Wash A solution (20ml of 20xSSC, 1ml of 20% (v/v) SDS, nano-pure water to 400ml) to remove the LifterSlip. Once the LifterSlip was displaced the slide was quickly placed on a staining rack and further washed on the preheated Wash A for 2 minutes. The slide was transferred into a clean staining rack and washed for 2 minutes in Wash B (1.2ml of 20xSSC and nano-pure water to 400ml). After 2 minutes, a second 2 minutes wash in fresh Wash B solution contained in a fresh staining rack was done. The slide was placed into a 50ml Falcon tube and centrifuged for 5 minutes at 500g.

2.3.13.6 Scan and analysis of the microarray slide

The microarray slide was scanned using an Axon2000 microarray scanner, which utilises the GenePix software. GenePix 5.1 was also used for labelling and localisation of each spot, as well as for a visual analysis of the quality of the spots. GenePix generated a spread sheet containing all the information produced on each spot, such as intensity, quality and background between others. This spread sheet was loaded into GeneSpring 7.0, where a comparison between the expression of the genes in either condition and its significance was done. GenePix and GeneSpring were previously loaded with information on the arrays and on the pneumococcal genome respectively.

2.4 Protein related techniques

2.4.1 Protein estimation

The Bradford assay (Bradford, 1976) was used to measure protein concentration. For the standard curve 100μL containing 0.2, 0.4, 0.6, 0.8 and 1.0mg Bovine Serum Albumin in water was prepared in microcentrifuge tubes. 5x Bradford Reagent (Bio-Rad) was diluted to 1x with water and filtered by gravity through a Whatman No.1 filter paper. 10μL of standards and samples at various dilutions were plated in triplicate into wells of a microtitre plate. 200μL of the filtered 1x Bradford reagent was added to each sample and left for 5 minutes at room temperature. The absorbance was read at 595nm in a MRX ELISA Plate Reader.

2.4.2 Anti-pneumolysin ELISA (based on Harlow and Lane, 1988)

Purified pneumolysin, kindly provided by Dr. Rana El-Rachkidy (University of Leicester), at different dilutions in PBS (136mM NaCl, 2.68mM KCl, 10.14mM Na_2HPO_4, 1.76mM KH_2PO_4, pH7.4, autoclaved) containing 0.5% (w/v) BSA was used as a positive control.

A MaxiSorp 96 well plate (Nalge Nunc International) was coated with 100μL of a sonicated (see 2.2.6) overnight pneumococcal culture in BHI broth or 100μL of the positive control. Control and samples were plated in triplicate and dilutions of the samples in PBS containing 0.5% (w/v) BSA were also added. The plate was incubated for two hours at 37°C. The wells were washed 5x with 300μL of washing buffer (PBS containing 0.05% (v/v) Tween 20). For blocking, 300μL of 3%(w/v) BSA in PBS were added and incubated for two hours at 37°C. After this time, the wells were washed 5x with washing buffer and 100μL of rabbit anti-pneumolysin polyclonal antibody (kindly provided by Prof. Timothy Mitchell, Glasgow University) at a 1:1000 dilution in PBS containing 0.5%(w/v) BSA was added. The plate was incubated for 90 minutes at 37°C and the wells were washed 5x with 300μL of washing buffer. 100μL of goat anti-rabbit antibody conjugated with Horse Radish Peroxidase (HRP) at a 1:2000 dilution in 0.5% (w/v) BSA in PBS was added and incubated at 37°C for one hour. Wells were washed 5x with 300μL of washing buffer and 100μL of Tetra Methyl Benzidine (TMB) substrate (Bio-Rad) was added and incubated at 37°C for 15 minutes. 50μL of 1M sulphuric acid were added to stop the reaction and the absorbance was read at 450nm in a MRX ELISA Plate Reader.

2.4.3 Anti-interferon-γ ELISA

Mouse interferon-γ was assayed using an ELISA kit purchased from Bender MedSystems. The protocol used was that provided by the manufacturer. All washing steps were performed with a washing buffer (PBS (136mM NaCl, 2.68mM

KCl, 10.14mM Na_2HPO_4, 1.76mM KH_2PO_4, pH7.4, autoclaved) containing 0.05% (v/v) Tween 20). The contents of the plates were first removed by inversion and by tapping the inverted plate over paper towels. For each washing, approximately 300μL of washing buffer were added and aspirated from each well using an ELISA plate washer (Nunc-Immuno). After washing, the plate was inverted and tapped over paper towels before continuing to the next step.

A MaxiSorp 96 well plate (Nalge Nunc International) was coated with 100μL of coating buffer, which contained 5μg/ml of coating antibody (provided) in PBS. The plate was covered and placed at 4°C overnight. The next day, the plate was washed once and 250μL of assay buffer (0.5% (w/v) BSA and 0.05% (v/v) Tween 20 in PBS) were added to each well and incubated at room temperature for two hours for blocking. The plate was washed twice and 100μL of standards or diluted samples were added into the wells of the plate in duplicate. For the preparation of the standards, 100μL of Standard Protein (2000pg/ml of mIFN-γ (provided) in assay buffer) were serially diluted by two-fold dilutions through 7 wells containing 100μL of assay buffer to obtain protein concentrations ranging from 1000pg/ml to 16pg/ml. Blank wells containing 100μL of assay buffer were also prepared. 50μL of 1:1000 of biotin-conjugate (supplied) in assay buffer were then added to each well and the plate was covered and incubated for two hours at room temperature on a platform rocker (Denley A600 rocker). After this time, the plate was washed 4 times and 100μL of 1:5000 dilution of Streptavidin-HRP (provided) in assay buffer were added to all the wells. The plate was covered and incubated at room temperature for 1 hour on a platform rocker. 100μL of freshly prepared TMB Substrate Solution (Bio-Rad) were added to each well and incubated at room temperature for 10 to 20 minutes on a platform rocker protected from direct light. The reaction was stopped by adding 100μL of 1M sulphuric acid into each well and the absorbance read at 450nm on a MRX ELISA Plate Reader.

2.4.4 Protein electrophoresis (Based on Sambrook *et al.*, 1989)

Protein electrophoresis was done in SDS polyacrylamide gels (SDS PAGE), and was used for Western blotting (see 2.4.5). In Table 2.4-1 the solutions used for SDS PAGE are shown.

Table 2.4-1 Solutions for SDS PAGE

Solution	Composition	
4x Tris.HCl/SDS pH 6.8	Tris base	1.5M
	SDS	4g per litre
6x SDS sample buffer	4x Tris.HCl/SDS pH 6.8	7ml
	Glycerol	3ml
	SDS	1g
	DTT	0.93g
	Bromophenol blue	1.2mg
	H_2O	to 10ml
1x Running buffer	Tris HCl pH 8.3	25mM
	Glycine	192mM
	SDS	0.1% (w/v)
10% Separating gel	H_2O	4.0ml
	30% (w/v) Acrylamide pure	3.3ml
	1.5M Tris pH 8.8	2.5ml
	10% (w/v) SDS	100µL
	14% (w/v) Ammonium Persulfate (APS)	100µL
	TEMED	4.0µL
5% Stacking Gel	H_2O	680µL
	30% (w/v) Acrylamide pure	170µL
	1.0M Tris pH 6.8	130µL
	10% (w/v) SDS	10µL
	14% (w/v) APS	10µL
	TEMED	1.0µL

The gel mould was assembled as described in Sambrook *et al.* (1989). Freshly prepared 10% separating gel (Table 2.4-1) was poured between the glass plates to approximately 2cm below the top of the small glass rim. A thin layer of water was carefully poured on top of the separating gel to allow oligomerization. After 5 minutes the water was removed by inverting the mould and freshly prepared 5% stacking gel (Table 2.4-1) was added to the edge of the small glass rim. A comb was carefully placed, taking care that no air bubbles were formed under the wells, and the gel left for 15 minutes to oligomerize. After this, the comb was removed and the wells carefully washed with distilled water. The gels were transferred into an electrophoresis chamber and 1x running buffer (Table 2.4-1) was added to cover the gel as described in Sambrook *et al.* (1989).

20μL of sonicated bacterial samples (see 2.2.6) were mixed with 4μL of 6x SDS sample buffer. The samples were loaded into the wells and the electrophoresis performed at 20mA/gel. Once the loading buffer was at the end of the gel, the electrophoresis was stopped. The gel carefully removed and used for western blotting (see 2.4.5).

2.4.5 Western blotting (Based on Harlow and Lane, 1988)

Immunoblotting was performed for the visualisation of pneumolysin. In Table 2.4-2 a list of the solutions used for western blotting is shown.

Table 2.4-2 Solutions used for western blotting

Solution	Composition	
Transfer buffer	Tris-HCl pH 8.3	12mM
	Glycine	96mM
	Methanol	20% (v/v)
Nitro Blue Tetrazolium (NBT)	NBT	0.5g
	dimethylformamide	70% (v/v)
BCIP	BCIP (disodium salt)	0.5g
	dimethylformamide	10ml
Alkaline phosphatase buffer	NaCl	100mM
	$MgCl_2$	5mM
	Tris pH 9.5	100mM
Phosphate-buffered saline (PBS)	NaCl	136nM
	KCl	2.68mM
	Na2HPO4	10.14mM
	KH2PO4	1.76mM
	pH 7.4, autoclaved	

After the sonicated samples were run on an SDS PAGE gel (see 2.4.4), the gel was placed over a 3mm Whatman filter paper. A nitrocellulose sheet was placed over the gel and another 3mm Whatman filter paper was placed over the nitrocellulose sheet. A scotch pad was finally placed on each side of the "sandwich". The filter papers, as well as the nitrocellulose sheet and the scotch pad, had been previously cut to the size of the gel and soaked in transfer buffer (Table 2.4-2).

The whole "sandwich" was placed in the blotting cassette, making sure the nitrocellulose membrane was on the positive side of the blotter and the SDS PAGE gel on the negative side. The blotting cassette was placed into the blotting tank and filled with ice-cold transfer buffer and placed in an ice bucket. The transfer was performed at 250mA of one and a half hours. Once the transfer was completed, the nitrocellulose membrane was removed and stained with Ponceau S solution to assess the protein transfer, which appeared as pink bands. The Ponceau staining was removed by washing the membrane with PBS (Table 2.4-2). The membrane was blocked overnight in 5% (v/v) skimmed milk in PBS at 4°C. The nitrocellulose was washed 3x for 5 minutes with PBS containing 0.01% (v/v) Tween 20 on a platform rocker (Denley A600 rocker). A 1:2000 dilution of rabbit anti-pneumolysin polyclonal antibody was added to cover the nitrocellulose sheet and incubated for one hour at 37°C on a shaking platform at 80 rev/minute. The membrane was washed 3x for 5 minutes with PBS containing 0.01% (v/v) Tween 20 and the secondary antibody (antirabbit conjugated with Alkaline Phosphatase (ALP), (Sigma)) in a 1:5000 dilution was added and incubated for one hour at 37°C on a shaking platform at 80 rev/minute. The membrane was washed as before and 660µL of NBT (Table 2.4-2) in 10ml of alkaline phosphatase buffer (Table 2.4-2) were added. 33µL of BCIP substrate were added and gently shaken until the bands were visible. The substrate was poured off, and the nitrocelulose sheets were washed twice with PBS to stop the reaction.

2.5 Nitrate determination from biological fluids

The method used was based on the protocol provided by Bories and Bories (1995).

62.5μL of 100mM potassium phosphate buffer (pH 7.5), 12.5μL of nano-pure H_2O, 12.5μL of 0.2mM FAD and 2.5μL of β-NADPH were mixed with 25μL of sample and left for 15 minutes to equilibrate at room temperature. 10μL of nitrate reductase from *Aspergillus* sp. (Sigma) at a concentration of 0.5u/ml were added and mixed immediately. The reaction was left to develop in the dark. After exactly 45 minutes, the absorbance was read at 340nm using a Ultrospec 3000 spectrophotometer from Pharmacia Biotech.

Each sample was done in duplicate, and for each, a control containing sample but with water replacing nitrate reductase was done. Controls on the reaction with and without nitrate reductase were also done. For these, the sample was substituted with nano-pure water.

To measure the amount of nitrate in the samples, standard curves with concentrations of sodium nitrate ranging from 0 to 150 micromols were also done. When the samples were serum samples, the standard curves were done by adding known concentrations of nitrate to serum of uninfected mice. If nitrate concentrations were measured from broncheo-alveolar lavage fluid (see 2.6.9), the standard curves were done in HBSS (Gibco).

2.6 Protocols for *in vivo* mice experiments

All the mice work performed in this project was done following the strict ethical regulations from the Animal Scientific Procedures ACT 1986 of the United Kingdom Home Office. All the mice used were pathogen free outbreed MF1 females weighing 30-35g obtained from Harlan Olac (Bicester, UK). They were housed under standard conditions in groups of five with regulated day length, temperature and humidity. The mice were given *ad libitum* tap water and pelleted food.

2.6.1 Preparation of passaged bacteria (modified from Alexander *et al.*, 1994)

10ml of an overnight pneumococcal culture in BHI broth (see Table 2.1-1) were centrifuged at 3000g for 10 minutes and re-suspended in 10ml of PBS (136mM NaCl, 2.68mM KCl, 10.14mM Na_2HPO_4, 1.76mM KH_2PO_4, pH7.4, autoclaved). 200μL of the bacteria suspension were injected intraperitoneally (see 2.6.3.1) into a mouse. After 24hours, blood was obtained form the mouse by cardiac puncture (see 2.6.4.1) and the spleen was dissected and placed into a universal tube containing 5ml of PBS. 200μL of blood were added to 10ml of BHI broth and incubated overnight. The spleen contained in PBS was homogenised and 200μL of the homogenate were transferred into a universal tube containing 10ml of BHI broth and was incubated overnight.

If passaged bacterial stocks were to be done from the blood inoculation, the bacterial suspension was transferred into a clean universal tube taking care not to transfer the red blood cells that settled at the bottom of the tube during the overnight growth. Both, blood and spleen cultures were individually centrifuged at 3000g for 15 minutes. The supernatant was carefully removed by pippetting and the pellets individually re-suspended in 1ml of serum broth (see Table 2.1-1). 600μL of each bacterial suspension were transferred into separate tubes containing 30ml of serum broth and incubated at 37°C until an OD_{500} of 1.6 was obtained (approximately 5 hours). Once the optical density was reached, the

bacteria cultures were placed on ice for 5 minutes and 500μL aliquots were dispensed into ice-cold microcentrifuge tubes. The aliquots were labelled and stored at –70°C until further use.

To evaluate the quality of the culture, one aliquot was thawed the next day and CFU (see 2.2.3) were done. The culture was also streaked onto two blood agar plates and an optochin disc (Difco), which inhibits pneumococcal growth, was placed onto an area of the streak. One plate was incubated under microaerobic conditions (see 2.2.1.2) and the other under aerobic conditions (see 2.2.1.2) to assess the purity of the stock.

When passaging pneumococcal mutants, the appropriate antibiotic was added to the culture for overnight incubation.

2.6.2 Preparation of the infecting dose

Once the CFU of the stock of passaged bacteria (see 2.2.3 and 2.6.1) was known, one 500μL aliquot was thawed and centrifuged for 2 minutes at 9000g. The supernatant was carefully removed and the pellet was re-suspended in 400μL of PBS (136mM NaCl, 2.68mM KCl, 10.14mM Na_2HPO_4, 1.76mM KH_2PO_4, pH7.4, autoclaved) to make up for the number of bacteria lost with the supernatant. The CFU was then underestimated by 2 units. For example, if the CFU of the frozen stock was $3x10^8$/ml, after centrifugation and re-suspension in PBS, the CFU were considered to be $1x10^8$/ml. The required volume from the bacterial suspension was calculated by simple proportion given the desired infecting dose. The final volume of the dose was adjusted to 50μL with PBS.

For intranasal infections (see 2.6.3.2) an infecting dose of $1x10^6$ in 50μL was used, whereas the dose used for intravenous infections (see 2.6.3.3) was of $1x10^5$ in 50μL. Preferentially, a dose test was done a day or two previous to the infection of mice to evaluate if the expected dose would be obtained.

2.6.3 Mice Infections

2.6.3.1 Intraperitoneal infections

Intraperitoneal infections were done for the production of passaged bacteria. For the infections, each mouse was handled by grasping the scruff of the neck with the thumb and the index fingers to prevent it from biting. The tail was hold with the fourth and fifth fingers of the same hand against the palm in order to stretch the mice gently. The hand was turned to show the ventral side of the mouse and dropped forward, so that the head of the mouse is at a lower level than the tail, this was done to slightly displace the organs forward and avoid their puncture. The infecting dose contained in an insulin syringe was injected by penetration of the needle into the lower right quadrant of the abdominal cavity at an approximate 45° angle.

2.6.3.2 Intranasal infections (Kadioglu *et al.*, 2000)

Mice were lightly anaesthetised with 2.5% (v/v) fluothane (Zenca, Macclesfield) over oxygen (1.5-2 L/minute). The mouse was hold by the scruff of the neck in a vertical position with its nose upward. 50μL of PBS (136mM NaCl, 2.68mM KCl, 10.14mM Na_2HPO_4, 1.76mM KH_2PO_4, pH7.4, autoclaved) containing $1x10^6$ bacteria (see 2.6.2) were given drop by drop through the nostrils for the mouse to inhale. After the whole dose was given, the mouse was placed inside a cage on its back to recover.

2.6.3.3 Intravenous infections

Prior to infection, the mice were placed in an incubator at 37°C for 30 minutes to dilate their veins. The tail of a mouse was passed through a hole in the lid of a plastic cylinder, the mouse was then placed inside the cylinder and the lid closed, leaving the tail of the mouse exposed. An infecting dose (see 2.6.2) of $1x10^5$ bacteria in 50μL of PBS (136mM NaCl, 2.68mM KCl, 10.14mM Na_2HPO_4, 1.76mM KH_2PO_4, pH7.4, autoclaved) was given through one of the lateral tail veins with the use of an insulin syringe.

2.6.3.4 Scoring of the signs of disease after infection

After infection, the signs of disease were constantly monitored and scored until the end of the experiment based on the scheme of Morton *et al.* (1985). For this, a mouse was given a score of 2 if it was found hunched, a score of 4 if it had a starey coat and a score of 6 if it was lethargic. The mice were not allowed to reach a moribund state, so if a mouse was found to have a score of 6, it was culled by cervical dislocation and the experiment for the particular mouse reached its end.

2.6.4 Collection of blood

2.6.4.1 By cardiac puncture (Canvin *et al.*, 1995)

The mice were deeply anaesthetised with 2.5% v/v fluothane (Zenca, Macclesfield) over oxygen (1.5-2 L/minute). When they were deeply anaesthetised, a mouse was placed on its back making sure it had a constant supply of anaesthetic. The base of the ribcage was compressed slightly on each side with the thumb and forefinger. The needle of a syringe was introduced parallel to the sternum through the diaphragm line, approximately 4mm below the sternum to penetrate the heart.

Normally it was possible to obtain by this method 1ml of blood. The mouse was immediately culled by cervical dislocation after the blood was obtained.

2.6.4.2 By tail-bleeding

As with intravenous infections, the mice were placed in an incubator at 37°C for 30 minutes to dilate their veins. The tail of a mouse was passed through a hole in the lid of a plastic cylinder, the mouse was then placed inside the cylinder and the lid closed, leaving the tail of the mouse exposed. The needle of a syringe was placed parallel to the tail vein and introduced with a very slight angle. Blood was obtained with a very gently vacuum. Alternatively, the vein was punctured with the syringe needle and the blood collected from the bleeding.

2.6.5 Collection of serum

Blood obtained by cardiac puncture (see 2.6.4.1) was placed into a 1.5ml microcentrifuge tube. The tube was incubated at 37°C for 30 minutes and centrifuged at maximum speed on a bench centrifuge for 20 minutes. 30-50μL of serum, were carefully transferred into several 0.5ml microcentrifuge tubes and frozen at –70°C until further use.

2.6.6 Calculation of CFU from blood

Blood from mice was obtained as described in 2.6.4. 20μL of blood were serially diluted in 180μL of PBS (136mM NaCl, 2.68mM KCl, 10.14mM Na_2HPO_4, 1.76mM KH_2PO_4, pH7.4, autoclaved). Dilutions to 10^{-6} were done. Six 20μL drops from each dilution were placed onto an agar plate, left to dry, and incubated overnight on microaerobic conditions (see 2.2.1.2). The next day, the number of colonies

was counted on the appropriate dilution and the average number of colonies per 20μL spot was calculated. From this the CFU per millilitre of blood was calculated.

2.6.7 Calculation of CFU from lungs

The mice were culled by cervical dislocation and the lungs aseptically removed and placed into pre-weighted universal tubes containing 10ml of PBS. The tubes containing the lungs were weighted again to determine the weight of the lungs. The lungs were homogenised with a tissue homogeniser (Ultra-Turrax T8 from Ika-werke) and 20μL of homogenate were serially diluted in 180μL of PBS. Dilutions of up to 10^{-6} were done. Six 20μL drops from each dilution were placed onto an agar plate, left to dry and incubated overnight. The next day, the number of colonies was counted on the appropriate dilution. From this value the number of cfu/mg of tissue was calculated.

2.6.8 Broncheo-alveolar lavage

Following the cardiac puncture procedure and after death, the mouse was placed in a ventral position and the skin of the neck was cut to expose the trachea. All of the tissue around the area of the trachea was removed and a small incision between two cartilage discs was made. A 22G catheter (without needle) was carefully inserted into the trachea through the incision and towards the lungs and secured with a nylon thread. 300μL of HBSS (Gibco) were carefully injected inside the lungs with the use of a 1ml syringe without a needle (BDH). The fluid was carefully aspirated and placed into a 1.5ml microcentrifuge tube on ice. The lavage was repeated with 200μL of HBSS and collected into the same microcentrifuge tube. The lavage fluid was separated into several ice-cold 0.5ml microcentrifuge tubes and frozen at –70°C until further use.

2.6.9 Lung histology

Freezing of lung tissue for cryostat sectioning (Based on Jounblat *et al.*, 2003)

After culling the mice by cervical dislocation, lungs were aseptically removed and placed into Bijoux tubes containing 3ml of M13 cell culture medium (Gibco). Cylindrical moulds of aluminium in which the lung samples were to be frozen, were made by tightly wrapping the body of a Bijoux tube with foil and removing it afterwards. An aluminium mould was surrounded with dry-ice and approximately 1cm of Tissue Tek OCT tissue embedding compound (Miles) was poured into it. The right and left lungs were carefully separated and placed one on top of the other inside the mould, sitting in the embedding compound before it froze. A few more drops of the compound were added on top of the lungs which were then left to freeze. Once frozen, enough embedding compound to cover the lungs completely was added and left to freeze. The frozen lungs were kept at –70°C until required.

Cryostat sectioning and staining of lung sections with haematoxylin-eosin staining (Wheater *et al.*, 1993)

The tissue samples were placed on a Bright microtome. 20micron longitudinal sections of the lungs were taken at a chamber temperature of –25°C and a specimen temperature of –17°C and placed onto microscope slides. A minimum of four sections of different areas of the lungs was prepared per mice. The sections were left overnight at room temperature to dry. For the staining of the sections, staining troughs and racks were used. The slides were submerged on Harris's haematoxylin (mercury free) (BDH) for 30 seconds and washed briefly with water. The slides were then submerged in 1% (w/v) eosin solution and rinsed with water. After this, the slides were washed for 30 seconds with 70% (v/v) ethanol, followed by 90% (v/v) ethanol and finally with 100% ethanol. The stained slides were submerged in xylene, for clearing and dehydration of the tissue, in a fume cupboard. A drop of DPX mountant for microscopy (BDH) was placed on a circular

cover slip (BDH), a slide was taken out of the xylene and the area containing the stained tissue pressed against the coverslip for mounting.

Analysis of the lung sections

The lung sections were analysed at a magnification of 200X in a light microscope connected to a digital camera (NikonE4500). Pictures of the sections were loaded into a computer and the pathologic changes were scored separately by two observers, Dr. Aras Kadioglu, who was blind to the identity of the samples and myself. The sections were given a score from 1 to 5 to represent the severity of the lung damage, where 1 represented mild damage or inflammation and 5 represented severe damage or inflammation.

2.7 Statistical analysis

For analysis of data from *in vitro* experiments such as growth curves, the student t-test was used using the Minitab software was used.

Data on signs of disease were analysed using the non-parametric Kruskal-Wallis test and the Dunns post-analysis test for comparing the differences between samples (PRISM 4.0 (GraphPad) software). Statistical analysis of the survival experiments was done using the Kaplan Meier survival curve analysis (PRISM 4.0 (GraphPad) software).

Levels of bacteria in the blood and lungs of mice after intranasal infections, as well as the levels of nitrate and interferon-γ from the serum and broncheoalveolar-lavage fluid of mice after intranasal or intravenous infection were analysed by a one-way ANOVA combined with the Scheffé correction test using the SPSS 14.0 software. For analysis of the levels of bacteria obtained from the blood of mice after intravenous infection, where the same group of mice were used throughout

the experiment, a repeated measure ANOVA with Sheffé correction test was done using the SPSS 14.0 software.

To analyse the statistical significance of the microarray results, a student t-test where the Benjamini and Hochberg false discovery rate correction test was applied, was done using the GeneSpring 7.0 software.

Chapter 3

Studies on Pneumolysin

Results

Pneumolysin is a haemolytic toxin known to be a very important virulence factor of *S. pneumoniae*. Its absence has been determined to have a crucial impact in reducing pneumococcal virulence (Kadioglu *et al.*, 2002; Benton *et al.*, 1995). Experiments have shown that the haemolytic and complement activation activities of pneumolysin are the main contributors to its virulence (Berry *et al.*, 1995, Jounblat *et al.*, 2003). But other experiments in which the toxin's haemolytic and complement activation activities had been deleted or diminished from the toxin have suggested that pneumolysin could contribute to virulence by another "unidentified" function (Alexander *et al.*, 1998). This conclusion was made after observing that a pneumococcal mutant whose pneumolysin lacked both, haemolytic and complement activation activities, presented a reduced virulence when compared to wild type pneumococcus, but was more virulent than a pneumolysin negative mutant (Alexander *et al.*, 1998). In 2002, Baba and collaborators published results of an *in vitro* study done with purified pneumolysin mutants (Baba *et al.*, 2002). In their experiments they showed that independent to its haemolytic activity, pneumolysin could induce, in spleen cells, the production of high levels of interferon-γ and of nitric oxide. Based on their findings, they raised the hypothesis that during a pneumococcal infection, pneumolysin could contribute to virulence by the induction of high levels of interferon-γ and nitric oxide, which can cause septic shock in the infected host.

To test whether the activity of pneumolysin observed by Baba *et al.* (2002) could explain the findings of Alexander *et al.* (1998) and be the "unknown" function of pneumolysin, it was decided to construct a pneumococcal mutant featuring a truncated pneumolysin toxin similar to that described by Baba *et al.* (2002). This mutant was used for intranasal and intravenous infection of the mice. Survival times, bacterial levels in lungs and/or blood after infections, levels of interferon-γ and nitric oxide in serum and/or brocheoalveolar lavage were determined. The results were compared with the behaviour and virulence of a wild type pneumococcus and to a pneumococcal mutant that lacks the production of the whole pneumolysin protein (PLN-A).

3.1 Construction of *S. pneumoniae* Δ4

Streptococcus pneumoniae D39, a virulent type 2 laboratory strain (see Table 2.1-3) was used for the construction of *S. pneumoniae* Δ4 (see Table 2.1-3). This mutant was constructed so that it expresses a pneumolysin protein similar to the truncated version of pneumolysin that Baba *et al.* (2002) used to perform their *in vitro* experiments. In Figure 3.1-1 the protein sequence of pneumolysin is shown with an indication of the site of truncation used by Baba *et al.* (2002) and the target site of the deletion for the construction of *S. pneumoniae* Δ4.

```
1   M A N K A V N D F I L A M N Y D K K K L L T H Q G E S I E N
31  R F I K E G N Q L P D E F V V I E R K K R S L S T N T S D I
61  S V T A T N D S R L Y P G A L L V V D E T L L E N N P T L L
91  A V D R A P M T Y S I D L P G L A S S D S F L Q V E D P S N
121 S S V R G A V N D L L A K W H Q D Y G Q V N N V P A R M Q Y
151 E K I T A H S M E Q L K V K F G S D F E K T G N S L D I D F
181 N S V H S G E K Q I Q I V N F K Q I Y Y T V S V D A V K N P
211 G D V F Q D T V T V E D L K Q R G I S A E R P L V Y I S S V
241 A Y G R Q V Y L K L E T T S K S D E V E A A F E A L I K G V
271 K V A P Q T E W K Q I L D N T E V K A V I L G G D P S S G A
301 R V V T G K V D M V E D L I Q E G S R F T A D H P G L P I S
331 Y T T S F L R D N V V A T F Q N S T D Y V E T K V T A Y R N
361 G D L L L D H S G A Y V A Q Y Y I T W D E L S Y D H Q G K E
391 V L T P K A W D R N G Q D L T A H F T T S I P L K G N V R N
421 L S V K I R E C T G L A W E W W R T V Y E K T D L P L V R K
451 R T I S I W G T T L Y P Q V E D K V E N D
```

Figure 3.1-1 Protein sequence of pneumolysin. The tryptophan-rich motif of the fourth domain is shown in green. The red arrow head indicates the site of deletion for the truncated pneumolysin protein used by Baba *et al.* (2002). The blue arrow head indicates the targeted deletion site for the construction of *S. pneumoniae* Δ4.

3.1.1 Strategy

It was found that the pneumolysin gene contains a unique recognition sequence for restriction endunuclease *Bsi*WI in the triplets coding for amino acids 438 and 439. Because of its proximity to the truncation site used by Baba *et al.* (2002) (Figure 3.1-1), it was decided to interrupt the pneumolysin gene at this point with the insertion of a gene encoding for spectinomycin resistance, which also would facilitate the selection of mutants. The construction was made in plasmid pUC19, which can act as a suicide vector when transformed into pneumococcus. As a double homologous recombination was desired upon transformation, nucleotides flanking the pneumolysin gene were included in the construction. Two plasmids were therefore constructed. First, pMBR1, in which the wild type sequence of interest was inserted into pUC19 and which served for the development of the second plasmid (pMBR2), in which the pneumolysin gene was interrupted. This second plasmid (pMBR2) was used for transformation into *S. pneumoniae* D39.

3.1.2 Construction of pMBR1

This plasmid was constructed as an initial step for the construction of plasmid pMBR2, which was used for the transformation of the pneumococcus. pMBR1 contained the pneumolysin sequence of pneumococci with approximately 1kb of flanking nucleotides on both sides that would allow a double homologous recombination when transformed into *S. pneumoniae* D39. In Figure 3.1-2 the strategy for the construction of pMBR1 and plasmid map are shown. Firstly, genomic DNA was extracted from *S. pneumoniae* D39 and used as a PCR template. Primers Ply1kbF and Ply1kbR (see Table 2.1-5) were designed for the amplification of the pneumolysin gene with ~1kb of flanking nucleotides on each side of the gene. As shown in Table 2.1-5 each of these primers contained a restriction recognition sequence on its 5' end to facilitate the cloning of the PCR product into pUC19. These restriction sequences (*Kpn*I and *Bam*HI) were chosen because they were found once in pUC19 but not found in the pneumolysin gene or in its flanking sequences, according to the available sequenced genome of *S. pneumoniae* R6.

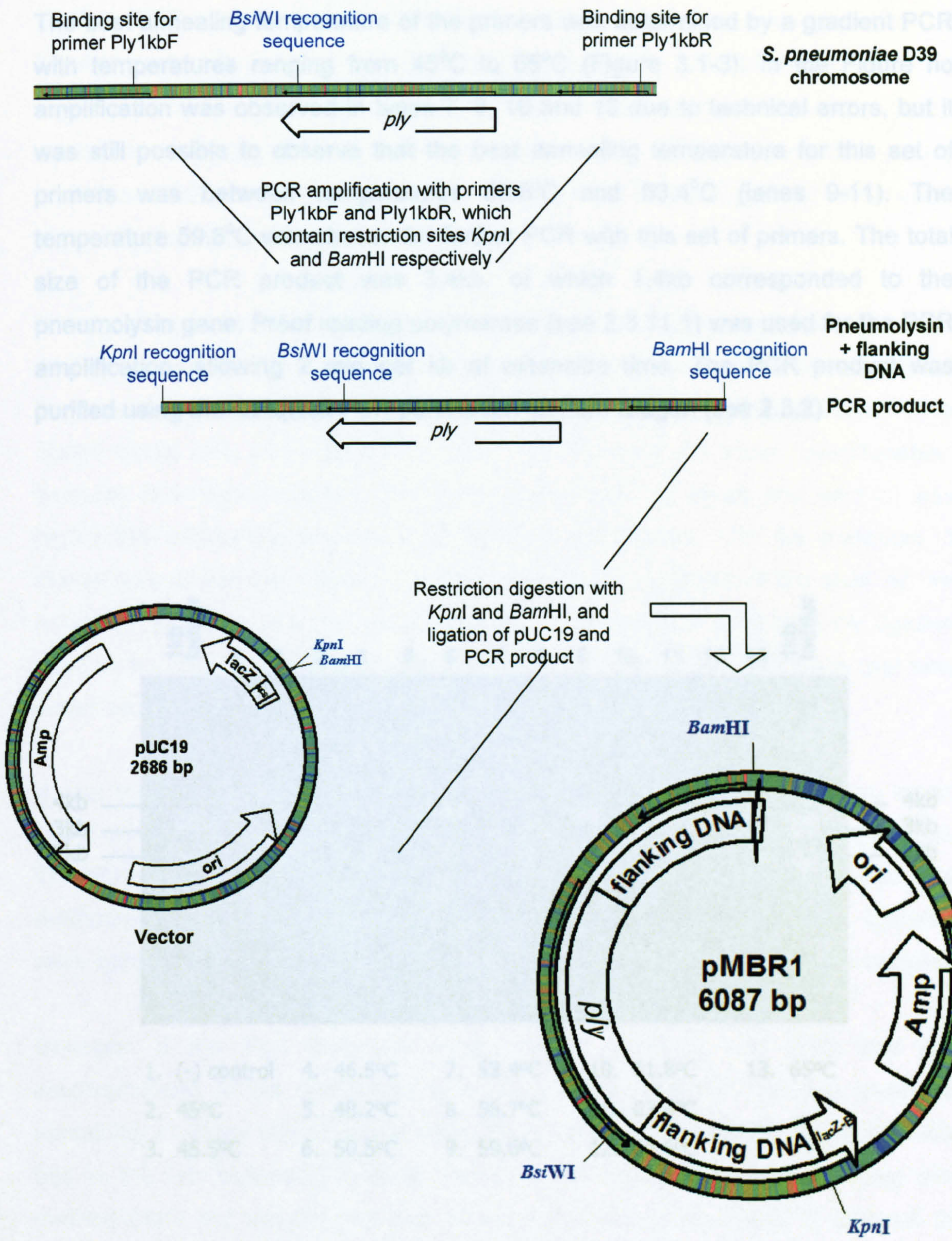
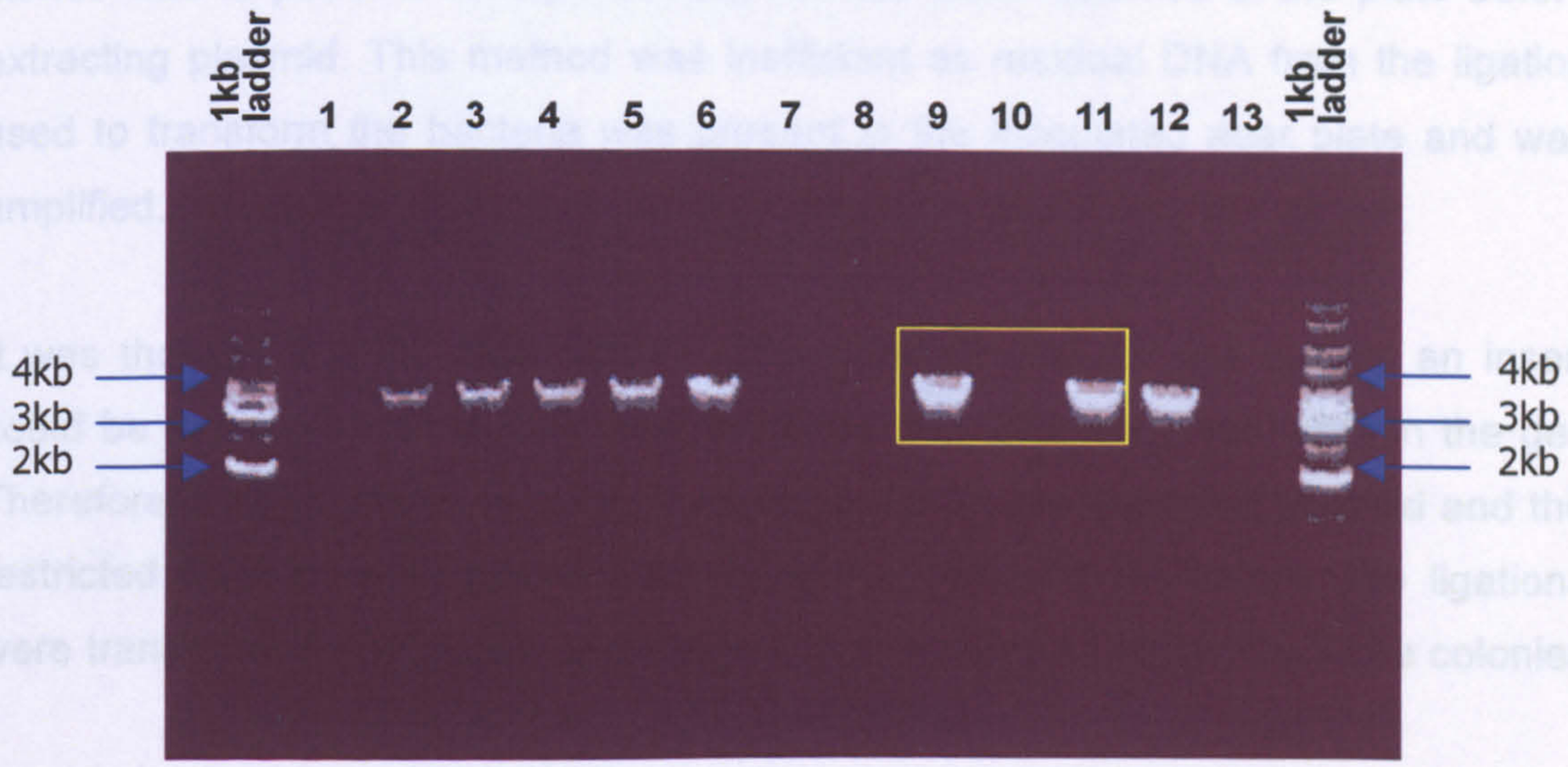


Figure 3.1-2 Strategy for the construction of pMBR1 and a plasmid map.

The best annealing temperature of the primers was determined by a gradient PCR with temperatures ranging from 45°C to 65°C (Figure 3.1-3). In the Figure no amplification was observed in lanes 7, 8, 10 and 13 due to technical errors, but it was still possible to observe that the best annealing temperature for this set of primers was between temperatures 59.6°C and 63.4°C (lanes 9-11). The temperature 59.6°C was chosen for further PCR with this set of primers. The total size of the PCR product was 3.4kb, of which 1.4kb corresponded to the pneumolysin gene. Proof reading polymerase (see 2.3.11.1) was used for the PCR amplification, allowing 2 min per kb of extension time. The PCR product was purified using the QIAquick PCR purification kit from Qiagen (see 2.3.2).



1. (-) control
2. 45°C
3. 45.5°C
4. 46.5°C
5. 48.2°C
6. 50.5°C
7. 53.4°C
8. 56.7°C
9. 59.6°C
10. 61.8°C
11. 63.4°C
12. 64.6°C
13. 65°C

Figure 3.1-3 Gradient PCR using primers Ply1kbF and Ply1kbR for the amplification of the pneumolysin gene with 1kb of flanking DNA on each side. The yellow square indicates the PCR products where the optimum annealing temperature was obtained.

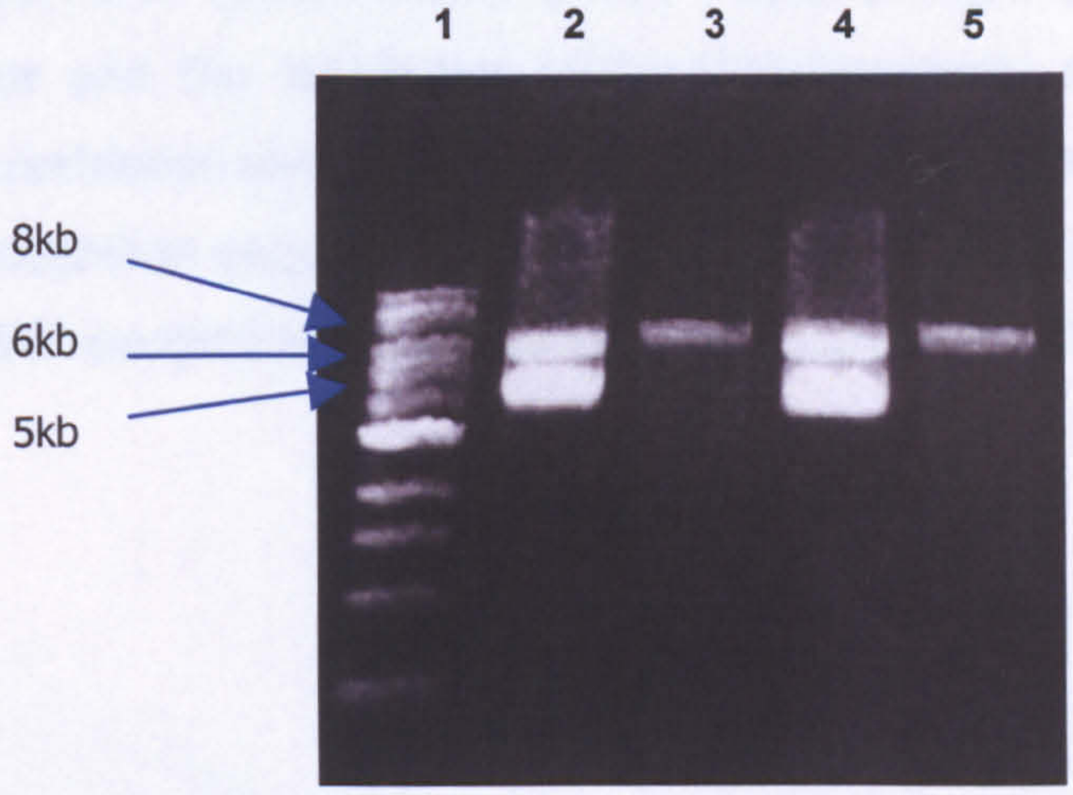
Plasmid pUC19 was obtained by medium scale alkaline lysis and both plasmid and purified PCR product were digested with restriction enzymes *Kpn*I and *Bam*HI. Ligations were done with the restricted vector and PCR product and transformed into *E. coli* DH5α. The transformed ligations were plated onto LA agar plates containing ampicilin, IPTG and X-gal (see 2.3.10.1) for blue and white colony selection. White colonies were selected and grown in 5ml of LB for small-scale plasmid extraction and further analysis.

Initially, plasmid obtained from all white colonies was digested with enzymes *Kpn*I or *Bam*HI and *BsiW*I individually. It was expected that *Kpn*I or *Bam*HI would linearise the plasmid with or without insert and give the appropriate size, while *BsiW*I would only linearise the plasmid if it contained an insert. Unfortunately, because of a high amount of white colonies without an insert, this method was highly time consuming and non-practical. The next approach for the screening of clones was to perform colony PCR from all the white colonies of the plate before extracting plasmid. This method was inefficient as residual DNA from the ligation used to transform the bacteria was present in the inoculated agar plate and was amplified.

It was thought that the high rate of white colonies that did not contain an insert could be caused by unspecific PCR products that were not detectable in the gel. Therefore it was decided to purify from the gel both the restricted plasmid and the restricted PCR product before performing the ligations. As before, the ligations were transformed and plated, and plasmid was obtained from all the white colonies.

Because of the high molecular length of the insert, it was likely that the electrophoresis of the undigested plasmid would be different between plasmids containing an insert and plasmids without an insert. It was therefore decided to use this as the first screening method. Three transformants were selected using this method (data not shown) and two of them (transformants 4 and 9) showed to contain an insert. The plasmids of these transformants were linearised with *BsiW*I, which could only linearise a plasmid if the insert was present (see Figure 3.1-2 for

cloning strategy and plasmid map). The digestions are shown in Figure 3.1-4. As expected, the linearised plasmids had a size of ~6kb.



1. 1kb ladder
2. Transformant 4 plasmid
3. Transformant 4 + BsiWI
4. Transformant 9 plasmid
5. Transformant 9 + BsiWI

Figure 3.1-4 Digestion with *Bsi*WI and electrophoresis of plasmids containing an insert to confirm the size of the linearised plasmids. The digested transformants in lanes 3 and 5 gave the expected size of 6kb.

The clones were sent to the PNACL division of the University of Leicester, UK, for sequencing of the pneumolysin gene. The pneumolysin sequence of both plasmids was identical to the original pneumolysin sequence (data not shown) and one of the clones was selected to continue with the construction. This clone was called pMBR1 (see Figure 3.1-2 for plasmid map).

3.1.3 Construction of plasmid pMBR2

Plasmid pMBR2 was constructed by inserting a spectinomycin resistance cassette into the pneumolysin gene contained in plasmid pMBR1. In Figure 3.1-5, the original strategy for the construction of pMBR2 is shown. The spectinomycin resistance gene was amplified from plasmid pDL278 (see Table 2.1-4) using primers SpecF and SpecR (Table 2.1-5). These primers were designed to include the promotor and the terminator of the spectinomycin resistance gene, and to contain the restriction recognition sequence for *Bsi*WI on their 5' ends. The addition of *Bsi*WI recognition sequence would allow digestion with this enzyme and cloning into the *Bsi*WI recognition sequence of pMBR1 to interrupt the pneumolysin gene.

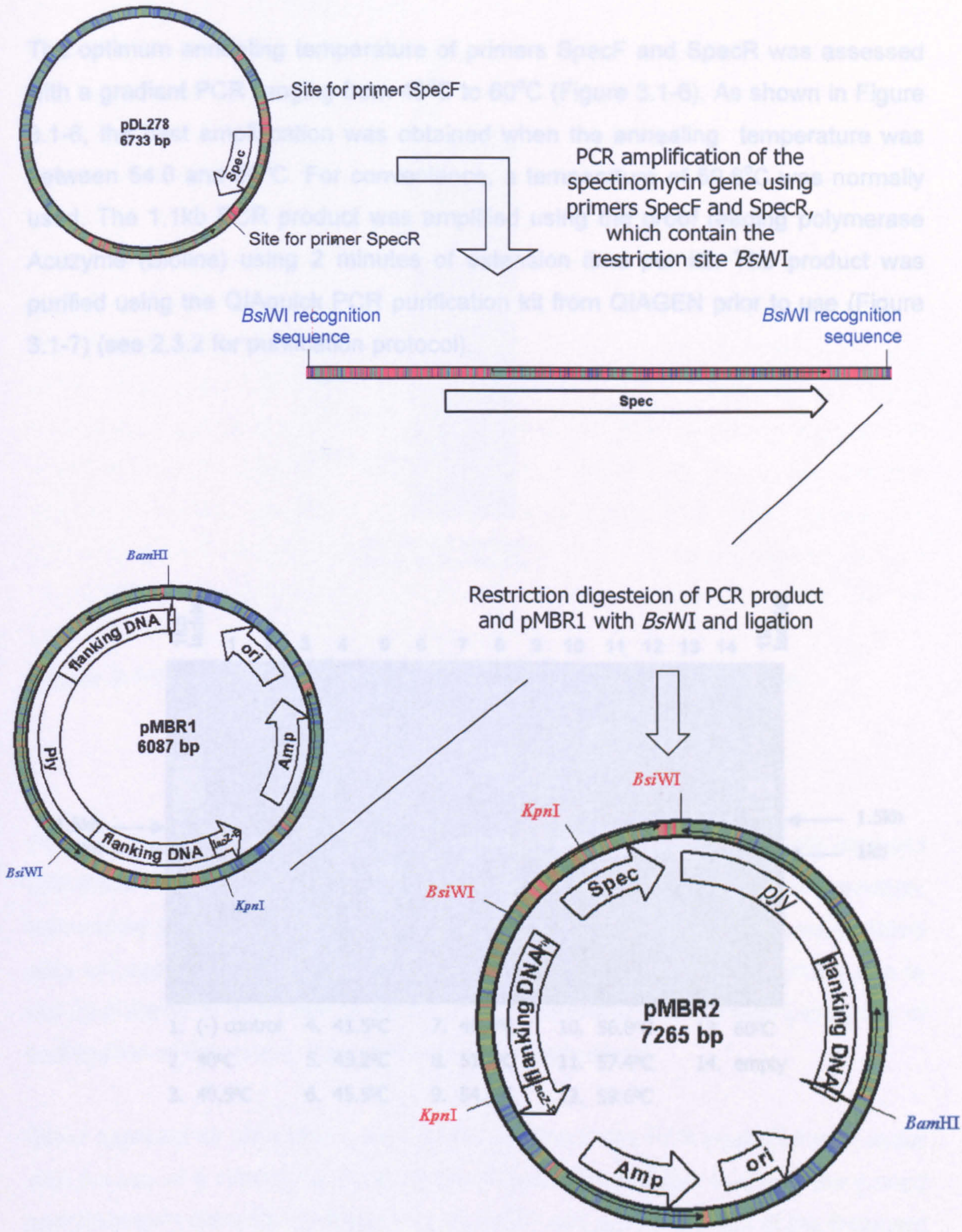


Figure 3.1-5. Strategy for the construction of plasmid pMBR2 and a plasmid map.

The optimum annealing temperature of primers SpecF and SpecR was assessed with a gradient PCR ranging from 40°C to 60°C (Figure 3.1-6). As shown in Figure 3.1-6, the best amplification was obtained when the annealing temperature was between 54.6 and 60°C. For convenience, a temperature of 59.6°C was normally used. The 1.1kb PCR product was amplified using the proof reading polymerase Acuzyme (Bioline) using 2 minutes of extension time per kb. The product was purified using the QIAquick PCR purification kit from QIAGEN prior to use (Figure 3.1-7) (see 2.3.2 for purification protocol).

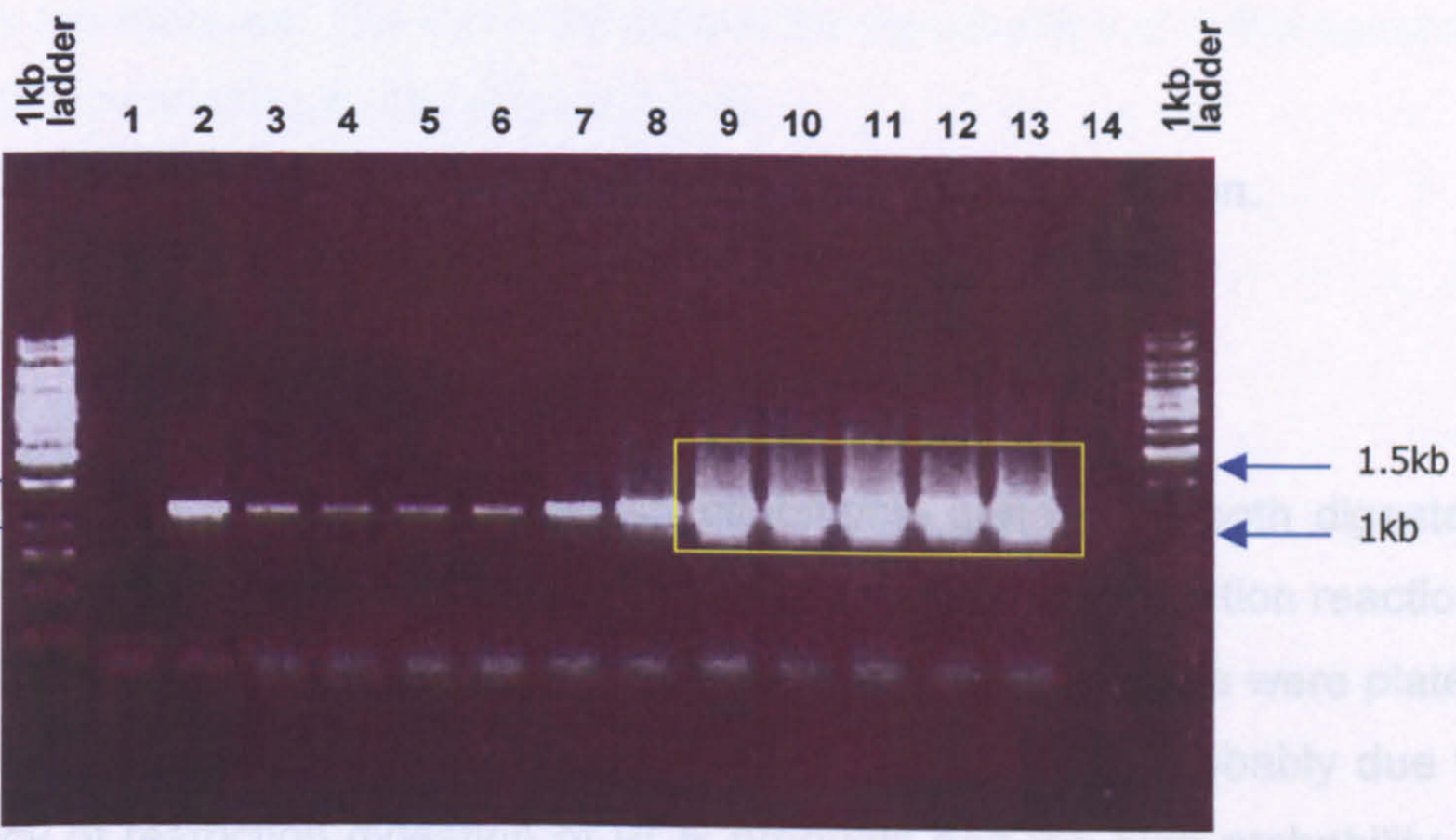
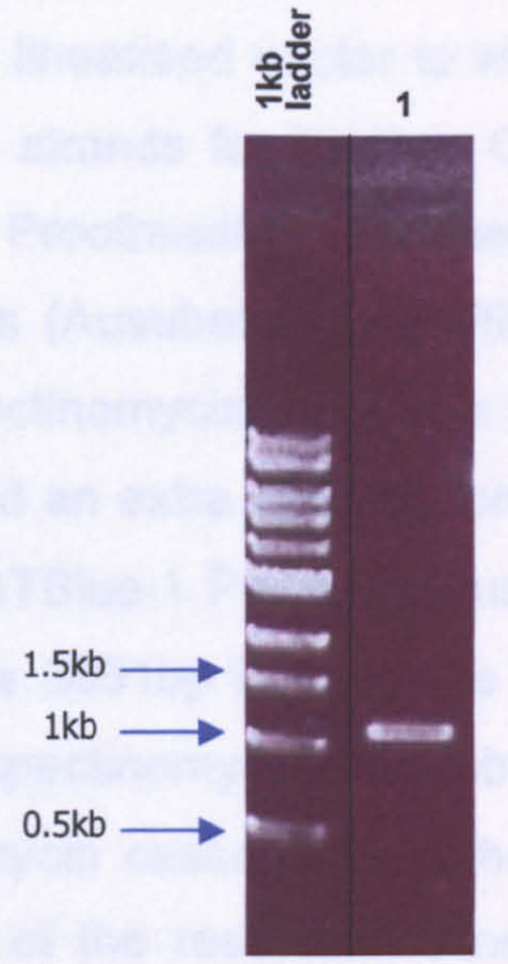


Figure 3.1-6 Gradient PCR using primers SpecF and SpecR for the amplification of the spectinomycin gene. The yellow square indicates the PCR products where the optimum annealing temperature was obtained.



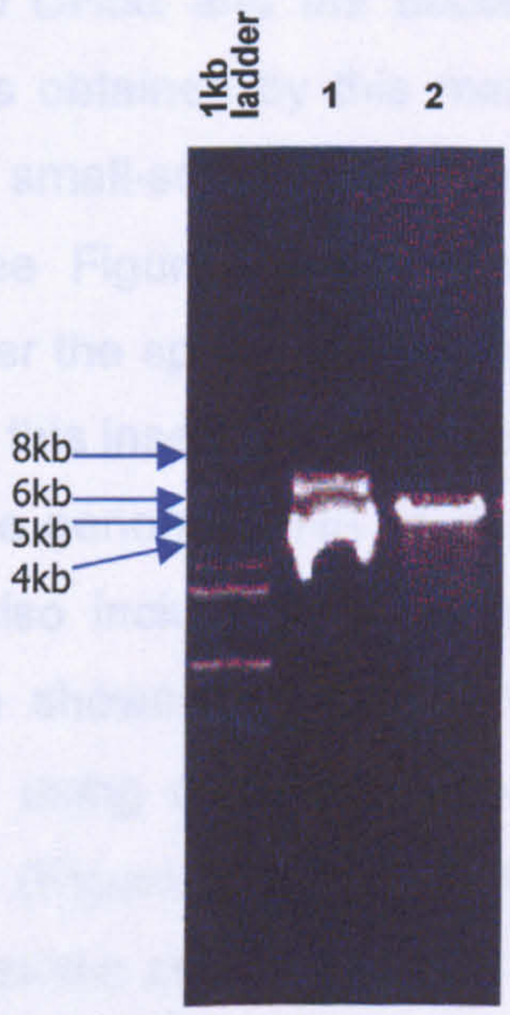
1. Purified PCR amplified spectinomycin cassette

Figure 3.1-7 Purified spectinomycin cassette after its PCR amplification.

Plasmid pMBR1 and the PCR amplified spectinomycin gene were both digested with the restriction enzyme *Bsi*WI and purified before mixing into a ligation reaction, followed by transformation into *E. coli* DH5α. The transformed bacteria were plated onto LA agar plates containing spectinomycin (see Table 2.1-2). Probably due to the inefficiency of restriction digestion of PCR products and the high probability of self-ligation of the vector, this approach was unsuccessful.

Other approaches were taken, such as the ligation of the PCR product into a vector with the use of a cloning kit. By doing this, it would be possible to obtain the cloned spectinomycin cassette by digestion with *Bsi*WI and gel purification of the fragment, which would solve the problem of inefficient digestion of PCR products. Two types of PCR cloning kits are mainly available commercially. One of them is for the ligation of PCR products that contain an adenine on their 5'ends. Regular Taq

polymerases have the characteristic of adding this base at the beginning and at the end of the amplification product (Ausubel, *et al*., 1995). These kits make use of this characteristic and use a linearised vector to which a thymidine has been added at the 3'ends of both DNA strands for ligation. Other types of kits are for cloning of blunt PCR products. Proofreading polymerases have the characteristic of producing blunt products (Ausubel *et al*., 1995) and these kits consist of a blunt vector. Because the spectinomycin gene was being amplified with a proof reading polymerase, and to avoid an extra step to cloning by the addition of an adenine to the end product, the "pSTBlue-1 Perfectly Blunt Kit" from Novagen was used. The vector used on this kit is 3851bp in site. The spectinomycin cassette was ligated and clones resistant to spectinomycin were obtained. However, it was not possible to extract the spectinomycin cassette from the plasmid with the *Bsi*WI restriction site, as apparently one of the restriction sites was lost. When this enzyme was used for digestion of the transformant plasmid, this was only linearised and the fragment was not released. The expected size of the construction of 4.8kb could be observed in the linearised plasmid (Figure 3.1-8).



1. Undigested clone
2. Plasmid + *Bsi*WI

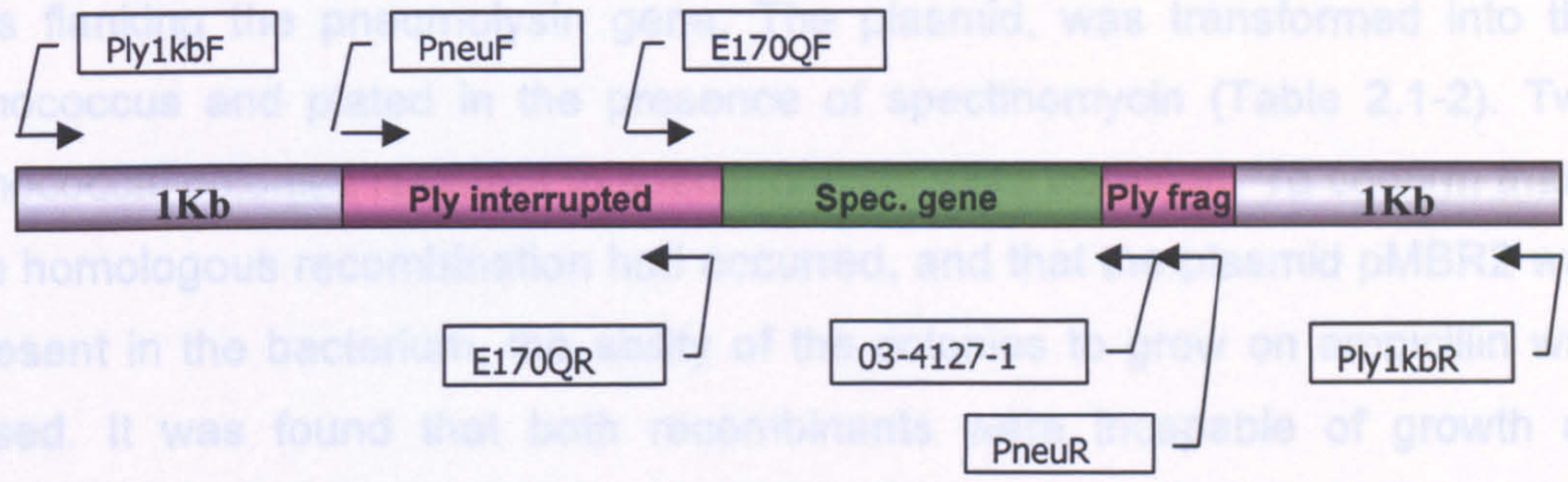
Figure 3.1-8 Transformant obtained with the use of "pSTBlue-1 Perfectly Blunt Kit" from Novagen digested with restriction enzyme *Bsi*WI.

As no mistake had been made with the sequence of the primers when ordering, there were several explanations for the lack of release of the fragment. One possibility was that the reagents contained in the kit which also convert non-blunt products into blunt-end products might have degraded double stranded DNA leading to the lost of the restriction site. However, most of the blunting enzymes used for cloning only resect single stranded DNA and not double stranded DNA. Also, technical services from Novagen suggest the problem is not within the kit. Another possibility was a mistake in the synthesis of the primers. Sequencing of the PCR product could have identified if this was the case, but another strategy was taken and sequencing was not done.

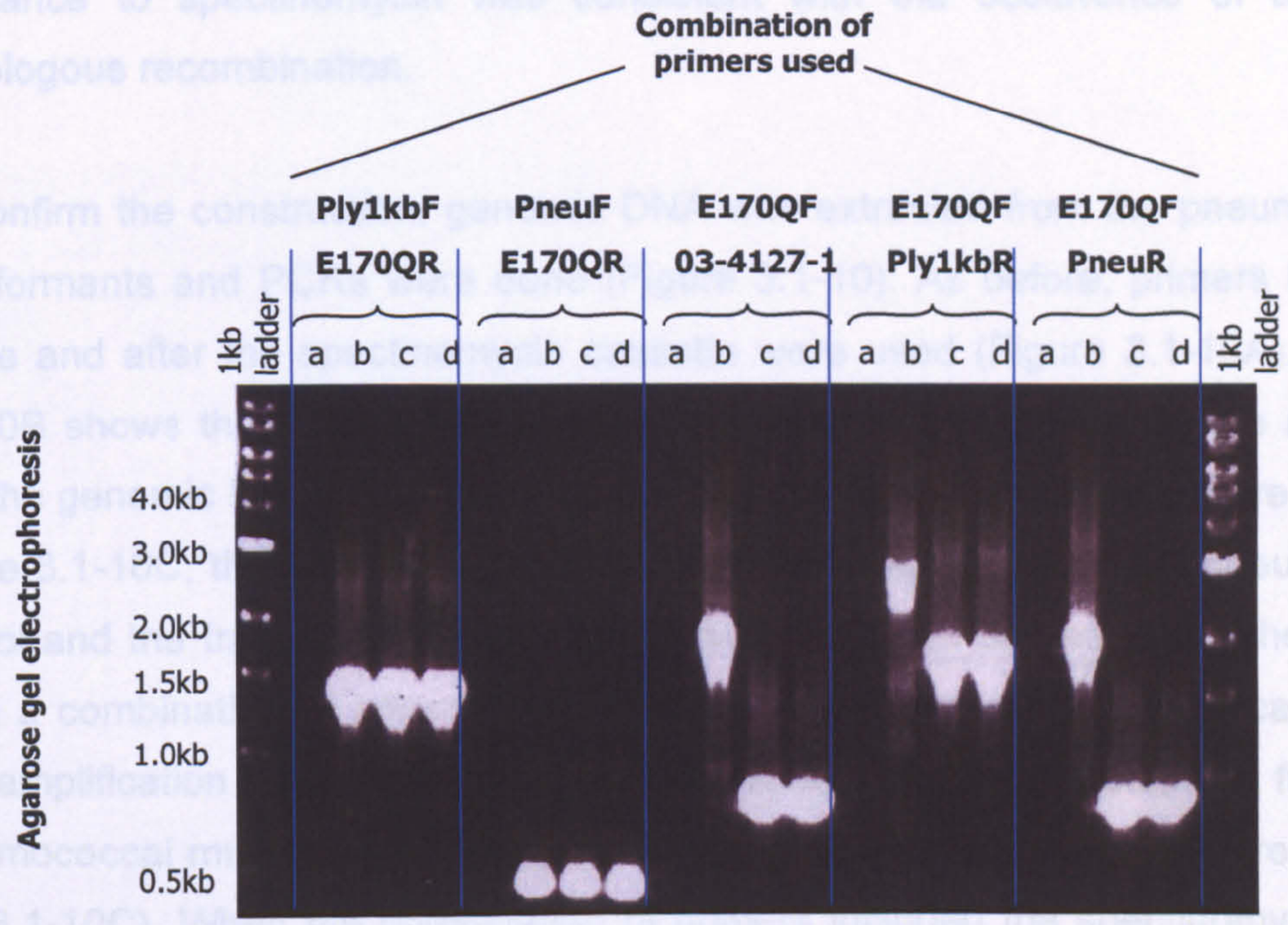
As the PCR product was a blunt product, it was decided to blunt pMBR1 after restriction digestion with *Bsi*WI and to use the purified PCR product without further manipulation for ligation. Because resistance to antibiotic provided by the insert was used for the selection of transformants, avoiding self-ligation of the vector by dephosphorylation was not a priority. As before, the ligation mixture was transformed into *E. coli* DH5α and the bacteria plated into LA plates containing spectinomycin. Colonies obtained by this method were inoculated into 5ml of LB with spectinomycin for small-scale plasmid extraction and the construction was confirmed by PCR (see Figure 3.1-9). As shown in Figure 3.1-9A, primers localised before and after the spectinomycin gene in the construction were used to confirm the presence of this insert. The gel from the PCRs are shown in Figure 3.1-9B, with pMBR1 and the genomic DNA of *S. pneumoniae* D39 used as control. A negative control was also included. The expected and obtained band sizes for each of the PCRs are shown in Figure 3.1-9C. As expected, when the PCR amplification was done using a combination of primers that did not include the spectinomycin cassette (Figure 3.1-9A), the band from the plasmid being tested showed the same size as the controls pMBR1 or the genomic DNA (Figure 3.1-9B and 3.1-9C). When a set of primers that include the spectinomycin gene was used for the PCR (Figure 3.1-9A), the sizes of the fragments were 1kb more than the controls (Figure 3.1-9B and 3.1-9C). Once the construction was confirmed, the plasmid was called pMBR2. A map of pMBR2 is shown in Figure 3.1-5.

Figure 3.1-9. PCRs done to confirm the insertion of the spectinomycin cassette into pMBR1. A) Localisation of the primers used to confirm the presence of the spectinomycin cassette in the construction. B) Gel from the PCRs done using different combination of primers. pMBR1 and the genomic DNA of *S. pneumoniae* D39 were used as positive controls for the PCR, and a negative control was also included. C) Expected and obtained band sizes from the different PCRs performed.

A **Localization of primers in the construction**



B



a. (-) control
b. plasmid
c. (+) control (pMBR1)
d. (+) control (genomic DNA from *S.pneumoniae* D39)

C

Primer combination →	Ply1kbF E170QR		PneuF E170QR		E170QF 03-4127-1		E170QF Ply1kbR		E170QF PneuR	
Lane	**b**	**c, d**	**b**	**c, d**	**b**	**c, d**	**b**	**c, d**	**b**	**c, d**
Expected size (kb)	1.5	1.5	0.5	0.5	2.0	0.85	3.0	1.85	2.0	0.85
Obtained size (kb)	~1.5	~1.5	~0.5	~0.5	~2.0	~0.8	~3.0	~2.0	~2.0	~0.8

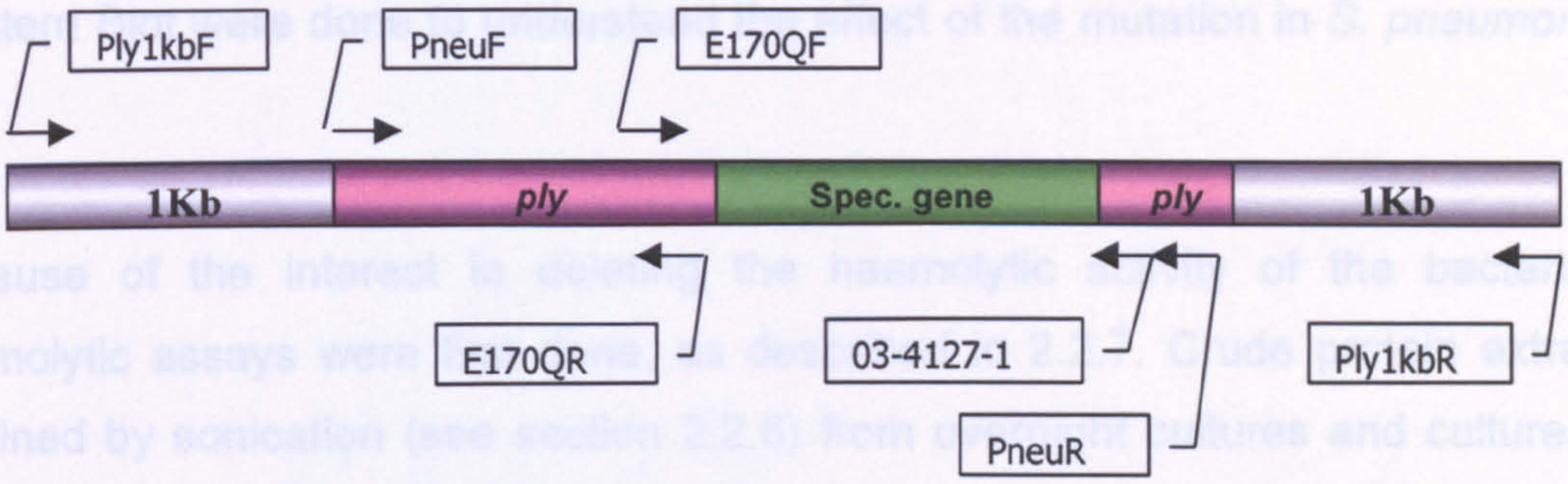
3.1.4 Transformation of pMBR2 into *S. pneumoniae* D39

Plasmid pMBR2 was used for the transformation of *S. pneumoniae* D39, with the expectation that a double homologous recombination would occur within the DNA regions flanking the pneumolysin gene. The plasmid, was transformed into the pneumococcus and plated in the presence of spectinomycin (Table 2.1-2). Two pneumococcal colonies resistant to spectinomycin were obtained. To confirm that a double homologous recombination had occurred, and that the plasmid pMBR2 was not present in the bacterium, the ability of the colonies to grow on ampicillin was assessed. It was found that both recombinants were incapable of growth on ampicillin, confirming the absence of pMBR2. The susceptibility to ampicillin and resistance to spectinomycin was consistent with the occurrence of a double homologous recombination.

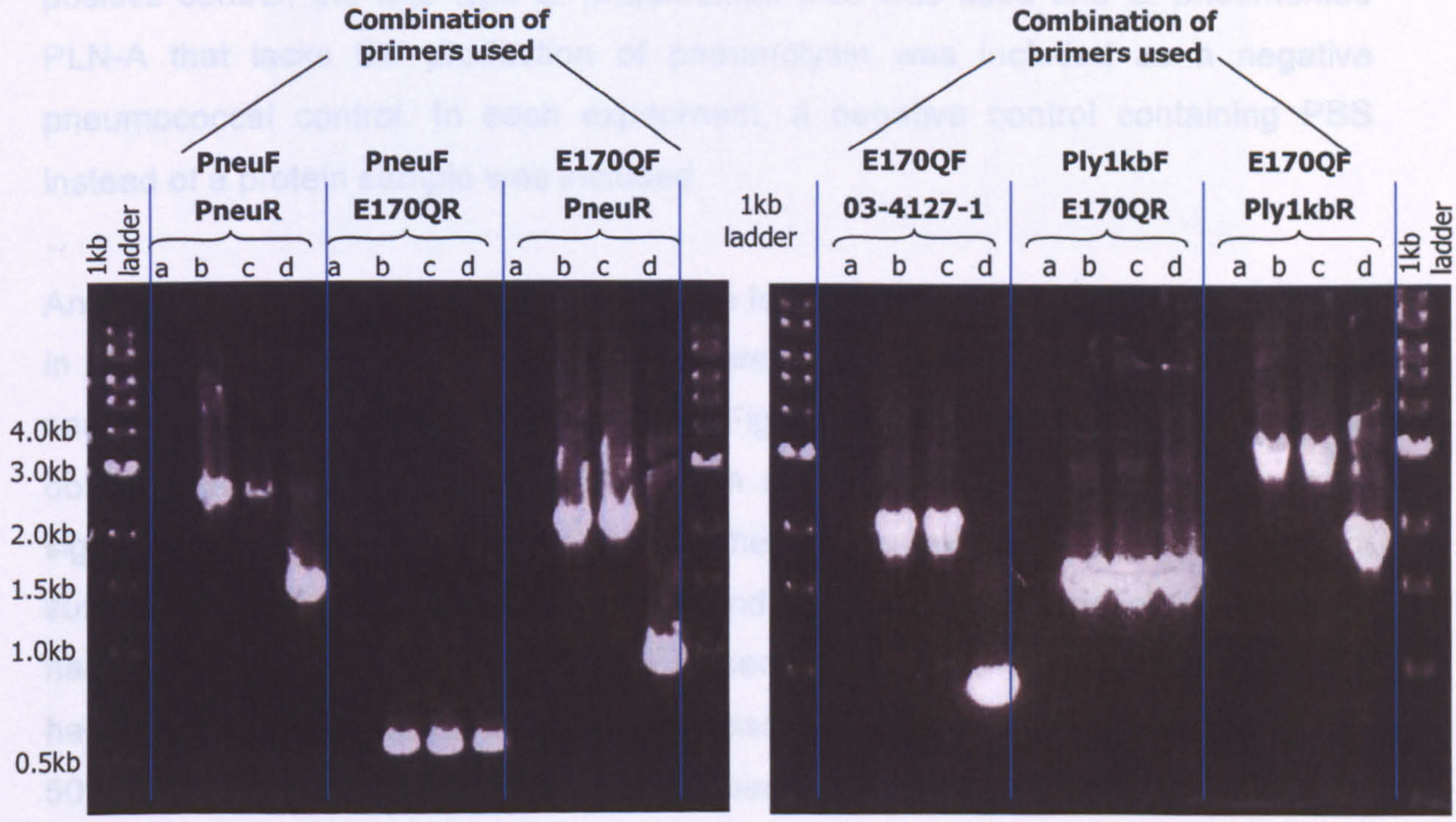
To confirm the construction, genomic DNA was extracted from the pneumococcal transformants and PCRs were done (Figure 3.1-10). As before, primers localised before and after the spectinomycin cassette were used (Figure 3.1-10A). Figure 3.1-10B shows the PCRs using different combination of primers, where a control with the genomic DNA of *S. pneumoniae* D39, and a negative control were used. In Figure 3.1-10C, the expected and obtained PCR sizes for each, the pneumococal control and the transformants are also shown. As it can be seen from the Figure, when a combination of primers that did not include the spectinomycin cassette in the amplification was used (Figure 3.1-10A), the size obtained from the pneumococcal mutants was the same as the D39 wild type strain (Figure 3.1-10B and 3.1-10C). When the combination of primers included the spectinomycin gene (Figure 3.1-10A), the amplicon obtained from the pneumococcal mutants was 1kb more than the D39 wild type (Figure 3.1-10B and 3.1-10C). As it can be seen from the Figure, both pneumococcal transformants contained the desired mutation (Figure 3.1-10B and 3.1-10C). One of them was selected to continue with the experiments and was called *Streptococcus pneumoniae* Δ4.

Figure 3.1-10 PCRs done to confirm the construction of pneumococcal mutants transformed with plasmid pMBR2. A) Localisation of the primers used to perform the PCRs in the construction. B) Gel from the PCRs done where different combination of primers were used. The genomic DNA of *S. pneumoniae* D39 was used as a positive PCR control, and a negative control was also included. C) Expected and obtained band sizes from the different PCRs performed.

A **Localization of primers in the construction**



B **Agarose gel electrophoresis**



a. (-) control
b. pneumococcal transformant 1
c. pneumococcal transformant 2
d. (+) control (genomic DNA from *S.pneumoniae* D39)

C

Primer combination →	PneuF PneuR		PneuF E170QR		E170QF PneuR		E170QF 03-4127-1		Ply1kbF E170QR		E170QR Ply1kbR	
Lane	**b**	**c, d**	**b**	**c, d**	**b**	**c, d**	**b**	**c, d**	**b**	**c, d**	**b**	**c, d**
Expected size (kb)	2.4	1.4	0.5	0.5	2.0	0.85	2.0	0.85	1.5	1.5	3.0	1.85
Obtained size (kb)	~2.5	~1.5	~0.5	~0.5	~2.0	~1.0	~2.0	~1.0	~1.5	~1.5	~3.0	~2.0

3.2 *In vitro* characterisation of *S. pneumoniae* Δ4

Several experiments such as haemolytic assays, ELISA, growth curves and Western Blot were done to understand the effect of the mutation in *S. pneumoniae* Δ4.

Because of the interest in deleting the haemolytic activity of the bacterium, haemolytic assays were first done, as described in 2.2.7. Crude protein extracts obtained by sonication (see section 2.2.6) from overnight cultures and cultures in mid-log phase were used for the experiments, using equal numbers of lysed bacteria as assessed by the CFU counts before and after the sonication. As a positive control, the wild type *S. pneumoniae* D39 was used and *S. pneumoniae* PLN-A that lacks the production of pneumolysin was included as a negative pneumococcal control. In each experiment, a negative control containing PBS instead of a protein sample was included.

An example of the results obtained with the total protein of $1x10^4$ bacteria is shown in Figure 3.2-1. Dilutions of total protein were done across the rows for each of the samples. As it can be observed from Figure 3.2-1, only the protein sample obtained from *S. pneumoniae* D39, which served as the positive control, show signs of haemolysis. This can be distinguished by the red colour of the supernatant surrounding the pellet of red blood cells and the absence of a pellet where 100% haemolysis took place. As explained in section 2.2.7, to assess the amount of haemolytic units in the sample, it is necessary to identify the dilution that shows 50% of haemolysis. In this case, the *S. pneumoniae* D39 positive control showed 50% of haemolysis in well number 4, where it can be observed a pellet size smaller than that in well number 5, and where well number 5 still presented haemolysis. This result corresponded to 160HU in $1x10^4$ for *S. pneumoniae* D39. In contrast, *S. pneumoniae* Δ4 showed no haemolytic activity (Figure 3.2-1). These results showed the haemolytic activity of *S. pneumoniae* D39 was abolished with the construction of *S. pneumoniae* Δ4.

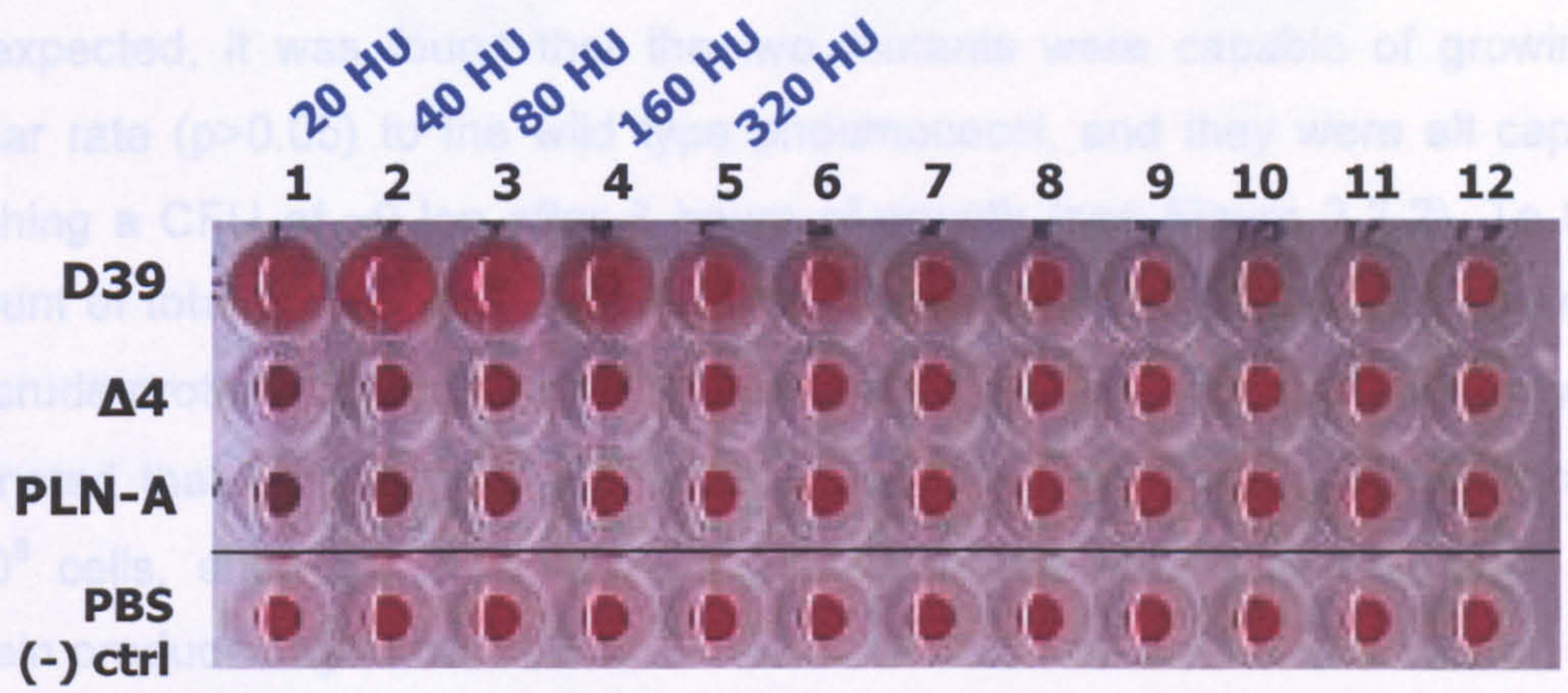


Figure 3.2-1 Comparison of the haemolytic activities of *S. pneumoniae* Δ4, *S. pneumoniae* D39 and *S. pneumoniae* PLN-A. Serial two-fold dilutions of crude protein extract were done across the columns, from less diluted in column number 1 to the most diluted in column number 12. The amount of haemolytic units corresponding to each dilution is shown in blue.

To assess the impact of the mutation on the growth of the mutant *S. pneumoniae* Δ4, growth curves under microaerobic conditions were done comparing the mutant with the wild type *S. pneumoniae* D39. The mutant *S. pneumoniae* PLN-A (see Table 2.1-3 of the Materials and Methods section), that lacks the production of the whole pneumolysin protein, was also included to assess its growth. This was done because *S. pneumoniae* PLN-A was used as a control for all *in vivo* experiments. As expected, it was found that the two mutants were capable of growing at a similar rate ($p>0.05$) to the wild type pneumococci, and they were all capable of reaching a CFU of ~9 log after 8 hours of growth (see Figure 3.2-2). To test the amount of total protein produced by both *S. pneumoniae* D39 and *S. pneumoniae* Δ4, crude protein extract from $5x10^8$ bacteria were used in a Bradford assay. It was estimated that both the mutant and the wild type had ~800μg of total protein in $5x10^8$ cells, showing no significant differences ($p>0.05$) in the amount of total protein produced by either strain.

To assess whether the *S. pneumoniae* Δ4 mutant was able to produce pneumolysin protein in a similar quantity to the wild type, an anti-pneumolysin ELISA was done with the crude protein extracts obtained from both *S. pneumoniae* D39 and *S. pneumoniae* Δ4 (Figure 3.2-2). For this, a total of $5x10^8$ of sonicated bacteria were used for each, *S. pneumoniae* D39 and *S. pneumoniae* Δ4. Equal numbers of sonicated bacteria of *S. pneumoniae* PLN-A were used as a pneumolysin negative pneumococcal control and PBS as a negative control. It was found that the PLN-A control produced no unspecific binding and its absorbance was similar to the negative PBS control. In Figure 3.2-3, the concentration of pneumolysin protein obtained from *S. pneumoniae* D39 and *S. pneumoniae* Δ4 is given by the absorbance at 450nm. As it can be seen from the Figure, no significant differences were found ($p>0.05$), indicating that *S. pneumoniae* Δ4 is able to produce the same amount of pneumolysin protein as the wild type *S. pneumoniae* D39.

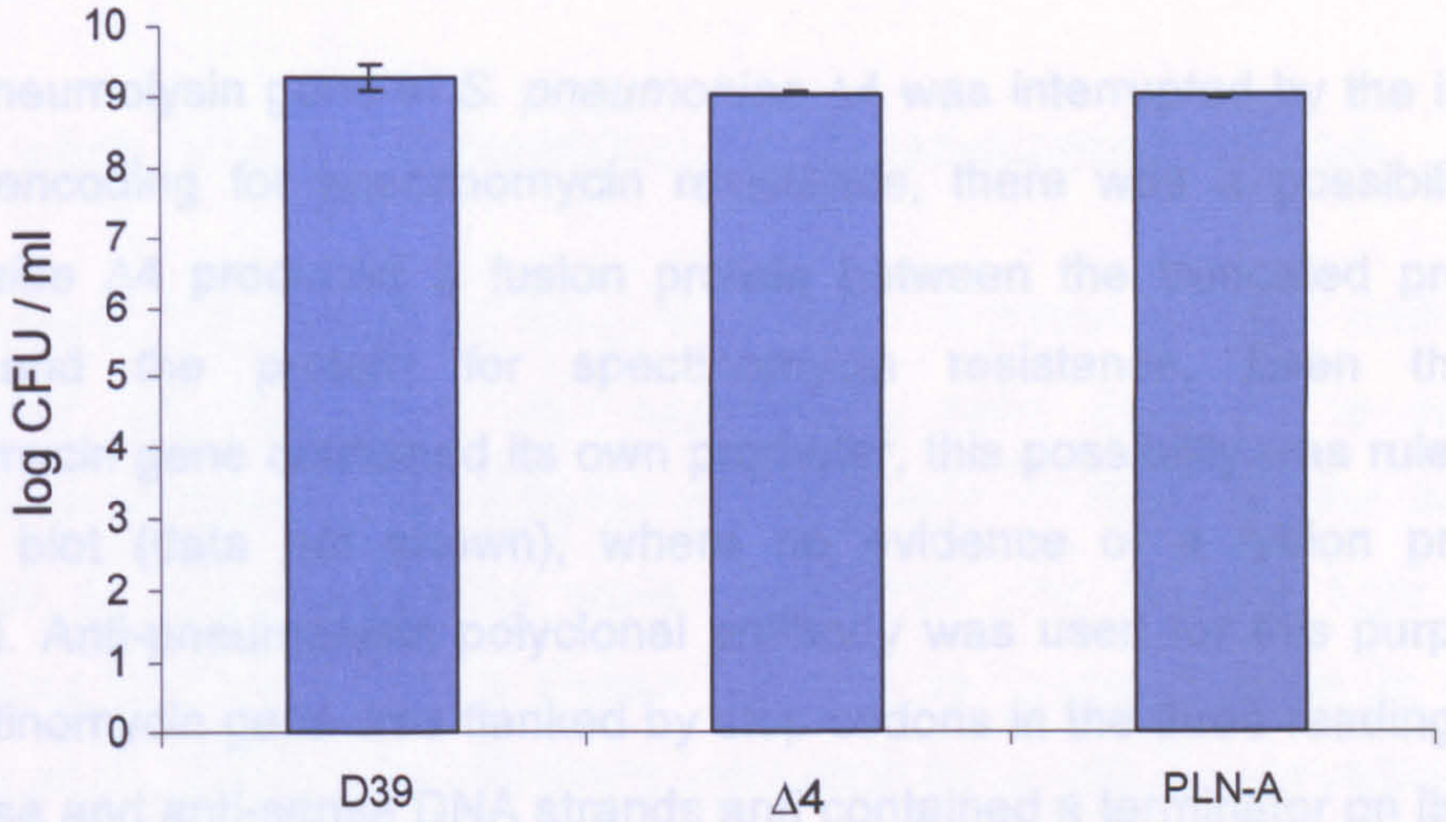


Figure 3.2-2. Log CFU/ml with wild type pneumococci and the mutants *S. pneumoniae* Δ4 and *S. pneumoniae* PLN-A after 8 hours of growth. N = 2 experiments with 3 repetitions each.

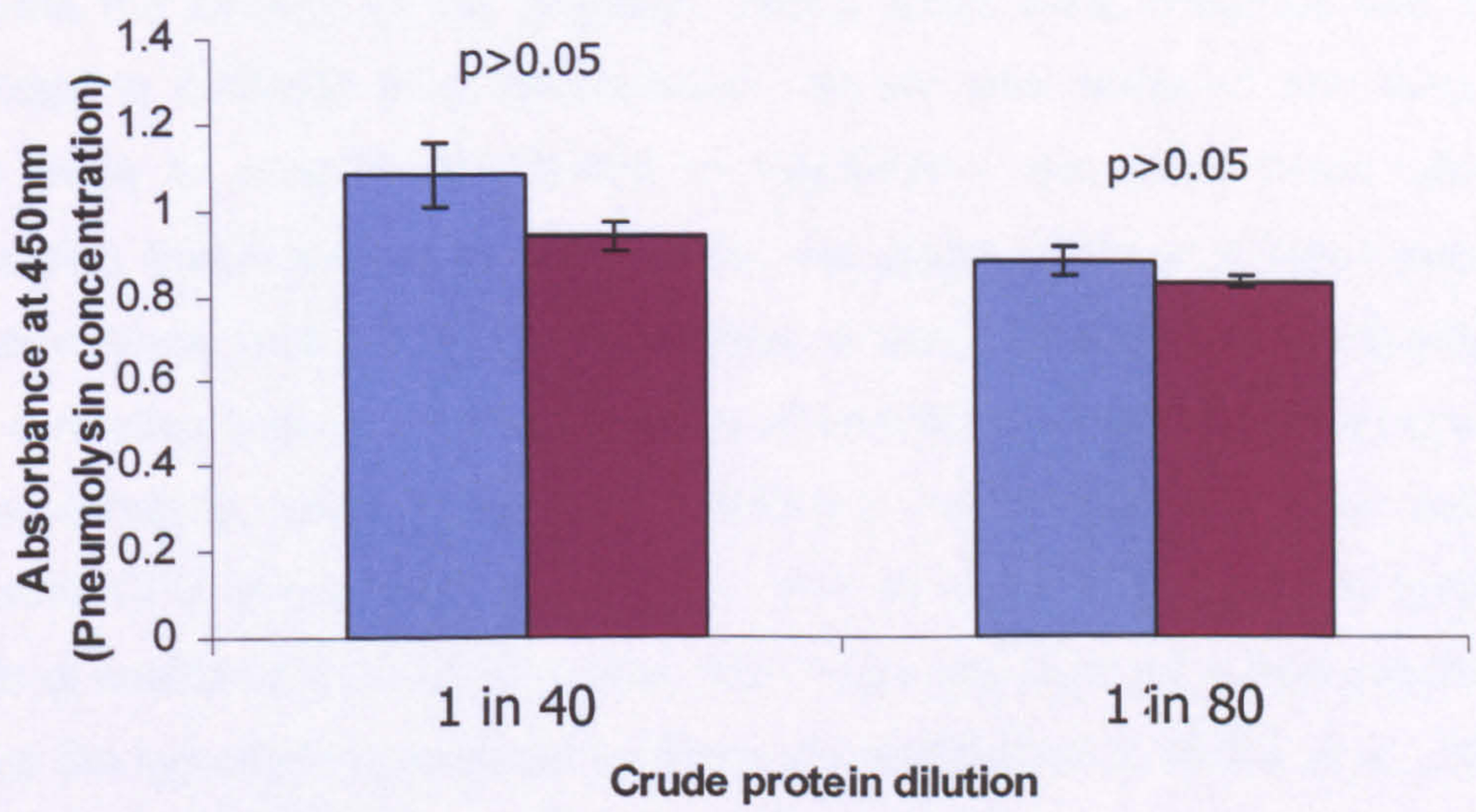


Figure 3.2-3 Comparison of the concentrations of pneumolysin protein contained in crude protein extracts from $5x10^8$ *S. pneumoniae* D39 and *S. pneumoniae* Δ4, as measured with an anti-pneumolysin ELISA. The blue colour represents the pneumolysin protein present in the samples of *S. pneumoniae* D39 and the red colour represents that present in *S. pneumoniae* Δ4. The bar shows the standard error of the mean. For each data point n=3.

As the pneumolysin gene in *S. pneumoniae* Δ4 was interrupted by the insertion of a gene encoding for spectinomycin resistance, there was a possibility that *S. pneumoniae* Δ4 produced a fusion protein between the truncated pneumolysin protein and the protein for spectinomycin resistance. Even though the spectinomycin gene contained its own promoter, this possibility was ruled out by a Western blot (data not shown), where no evidence of a fusion protein was observed. Anti-pneumolysin polyclonal antibody was used for this purpose. Also, the spectinomycin gene was flanked by stop codons in the three reading frames at both sense and anti-sense DNA strands and contained a terminator on its 3' end.

3.3 *In vivo* characterisation of *S. pneumoniae* Δ4

To understand the impact of the pneumolysin mutation on the virulence of *S. pneumoniae* Δ4, various *in vivo* experiments were done. The experiments consisted on intranasal and intravenous infections of mice. All the experiments done with *S. pneumoniae* Δ4 were also done with its wild type parent *S. pneumoniae* D39, which was known capable to cause disease in MF1 mice (Kadioglu *et al.*, 2000). The infections were also done with *S. pneumoniae* PLN-A, which is an isogenic mutant of *S. pneumoniae* D39 that lacks the production of the whole pneumolysin protein and is known to have a considerably reduced virulence in mice (Kadioglu *et al.*, 2000).

In Figure 3.3-1 a diagram of the experiments done is shown. After intranasal infections, CFU were measured from blood and from lung homogenates to evaluate the kinetics of the infection. Whole lungs were collected and frozen for histology to evaluate lung inflammation. Serum was obtained and lung lavages were done to quantify the levels of interferon-γ and nitric oxide present. By measuring interferon-γ and nitric oxide, we could observe if there was relation between these and the levels of disease. It was also possible to identify if there was a relation between the production of interferon-γ and the production of nitric oxide. Similarly, after intravenous infections, blood samples were collected to measure CFU throughout the infection, and serum was obtained to measure the levels of interferon-γ and nitric oxide. With these experiments it was possible to test *in vivo* the hypothesis proposed by Baba and collaborators (Baba *et al.*, 2002) after their *in vitro* observations. According to their hypothesis, a pneumolysin that lacks haemolytic activity can induce the production of high levels of interferon-γ, which can positively regulate the production of nitric oxide. This increase in interferon-γ and nitric oxide levels could contribute to septic shock. Wild type pneumolysin that has haemolytic activity would not induce the production of interferon-γ or nitric oxide.

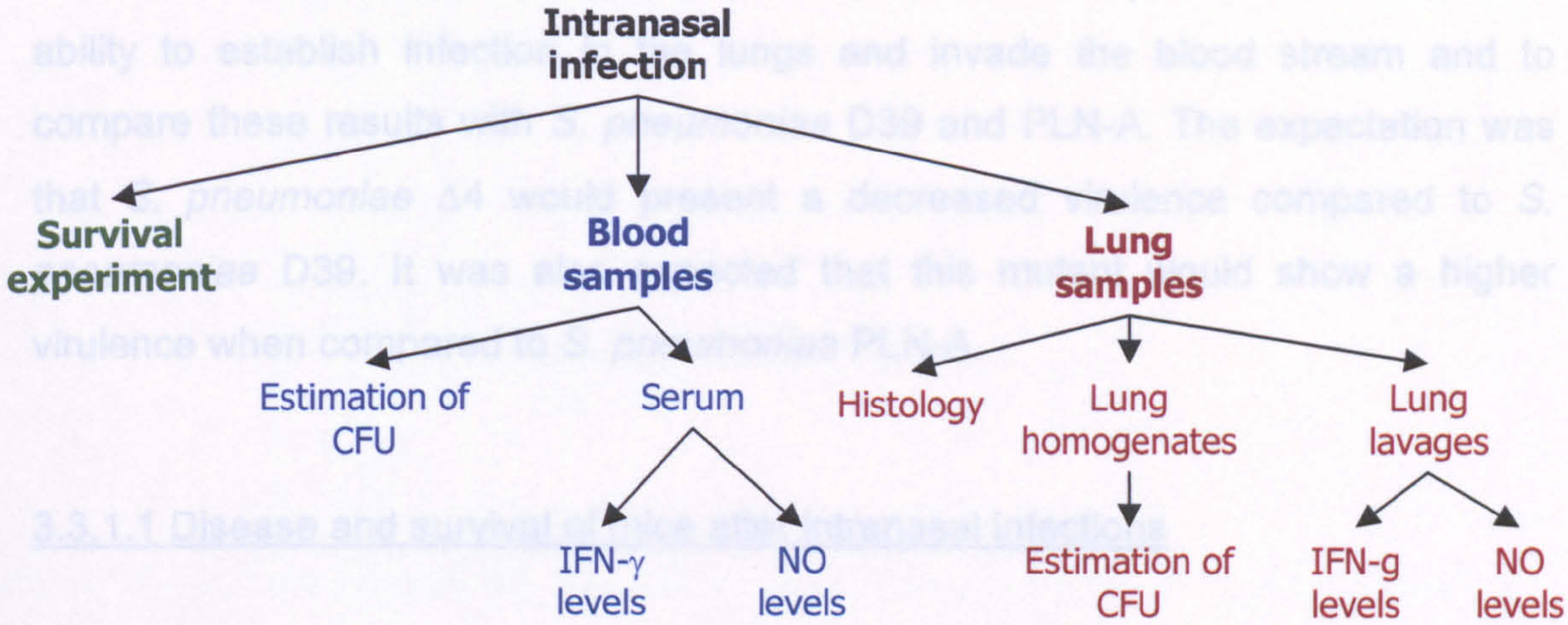


Figure 3.3-1 Diagram of experiments after intranasal and intravenous infections.

3.3.1 Intranasal infections: disease, survival and bacteria growth in lungs and blood after infection.

The aim of these studies was to assess the virulence of *S. pneumoniae* Δ4 and its ability to establish infection in the lungs and invade the blood stream and to compare these results with *S. pneumoniae* D39 and PLN-A. The expectation was that *S. pneumoniae* Δ4 would present a decreased virulence compared to *S. pneumoniae* D39. It was also expected that this mutant would show a higher virulence when compared to *S. pneumoniae* PLN-A.

3.3.1.1 Disease and survival of mice after intranasal infections

In total 14 mice were infected with *S. pneumoniae* Δ4, ten with *S. pneumoniae* D39 and seven with *S. pneumoniae* PLN-A. The infections reported here were done in two independent experiments. After infection, the signs of disease of the mice were frequently evaluated and scored as explained in 2.6.3.4. Briefly, a mouse was given a score of 0 if it showed no signs of disease; 2 if it was hunched, 4 if it had a starey coat and 6 if it was lethargic, at which point the mice was culled. For description purposes, if a mouse reached a score of 6 and was consequently culled before the end of the experiment (72 hours), this score continued to be used to represent the culled mouse.

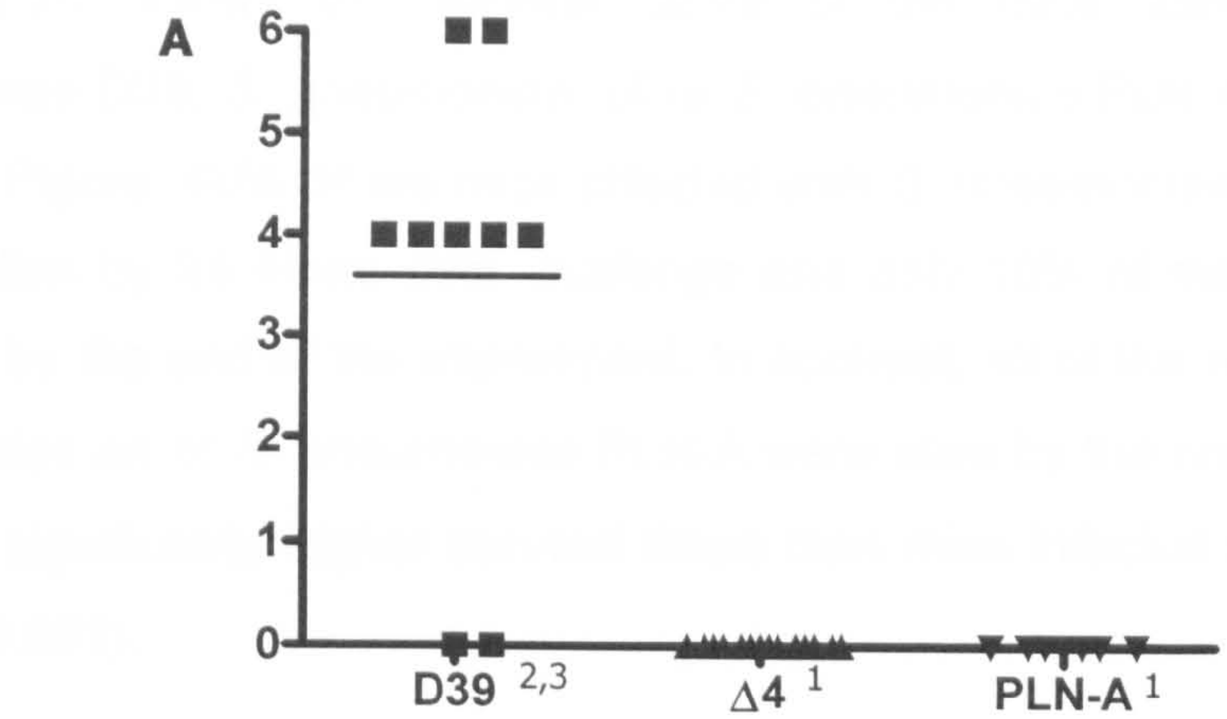
Figure 3.3-2 shows a comparison on the signs of disease of mice recorded after 24, 48 and 72 hours post infection with either strain of bacteria (*S. pneumoniae* D39, *S. pneumoniae* Δ4 or *S. pneumoniae* PLN-A). In Figure 3.3-2A it can be observed that at 24 hours post-infection only mice infected with *S. pneumoniae* D39 were showing signs of disease ($p<0.05$ versus Δ4 and PLN-A). At 48 hours (Figure 3,3-2B) some of the mice infected with Δ4 and PLN-A showed signs of disease, however their scores were significantly lower ($p<0.05$) than those of mice infected with *S. pneumoniae* D39. At 72 hours (Figure 3.3-2C), mice infected with *S. pneumoniae* Δ4 showed significantly lower ($p<0.05$) signs of disease than mice infected with either D39 or PLN-A.

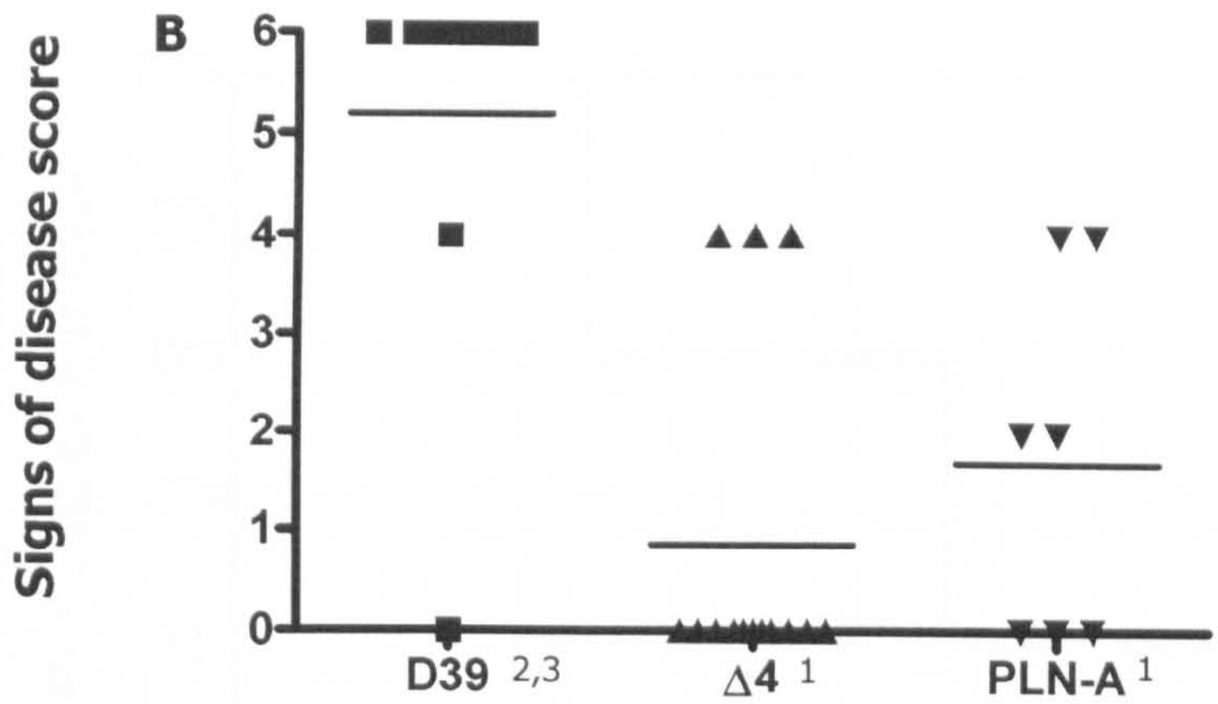
Figure 3.3-2 Signs of disease of mice after (A) 24, (B) 48 and (C) 72 hours of intranasal infections with *S. pneumoniae* D39 (squares) *S. pneumoniae* Δ4 (triangles) or *S. pneumoniae* PLN-A (inverted triangles). Each symbol represents one mouse, where n=10 for mice infected with D39; n=14 for mice infected with Δ4; and n=7 for mice infected with PLN-A. The infections were done in two independent experiments. A continuous line represents the average sign of disease for each group of mice at each time point.

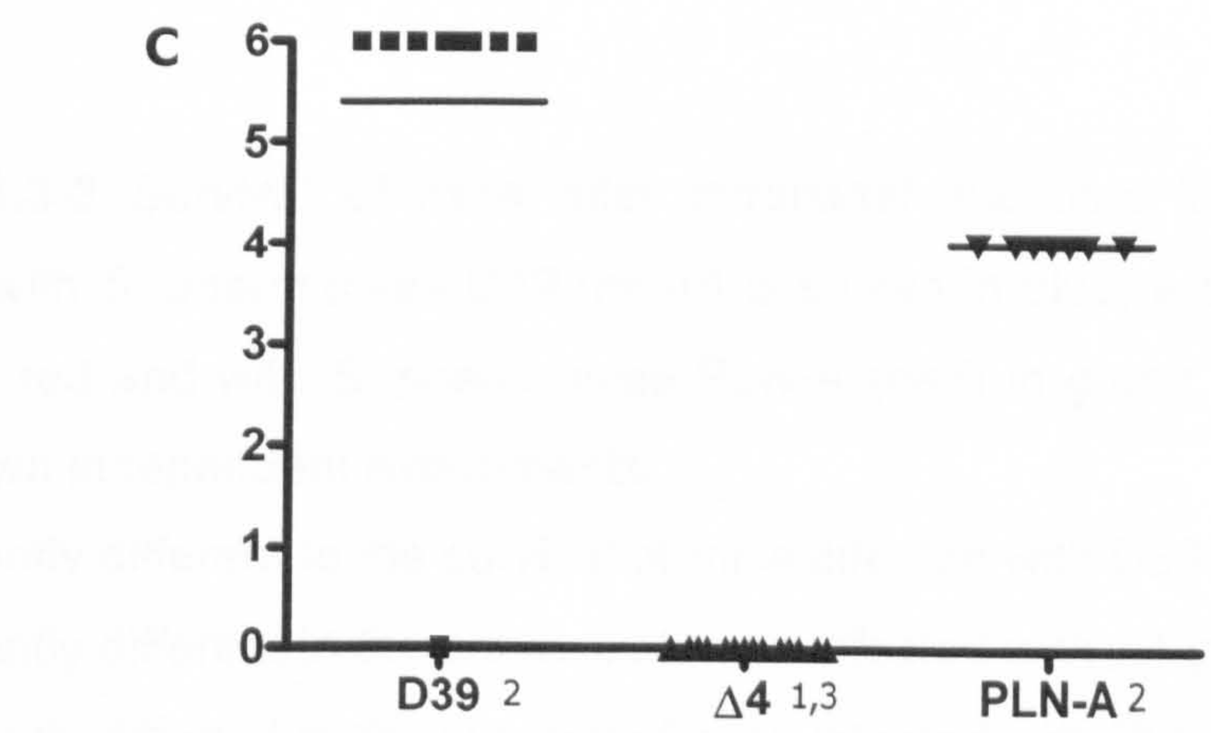
1 Significantly different to *S. pneumoniae* D39 ($p>0.05$)

2 Significantly different to *S. pneumoniae* Δ4 ($p>0.05$)

3 Significantly different to *S. pneumoniae* PLN-A ($p>0.05$)







Pneumococcal strain used

Figure 3.3-3 shows the survival curve of the mice after infection with *S. pneumoniae* D39, *S. pneumoniae* Δ4 or *S. pneumoniae* PLN-A. As it can be seen from the Figure, 40% of the mice infected with *S. pneumoniae* D39 succumbed to the infection by 24 hours post challenge and only 10% of the mice (1 out of 10) survived by the end of the experiment. In contrast, all of the mice infected with *S. pneumoniae* Δ4 or *S. pneumoniae* PLN-A were alive by the end of the experiment, showing significantly higher survival times than mice infected with *S. pneumoniae* D39 ($p<0.001$).

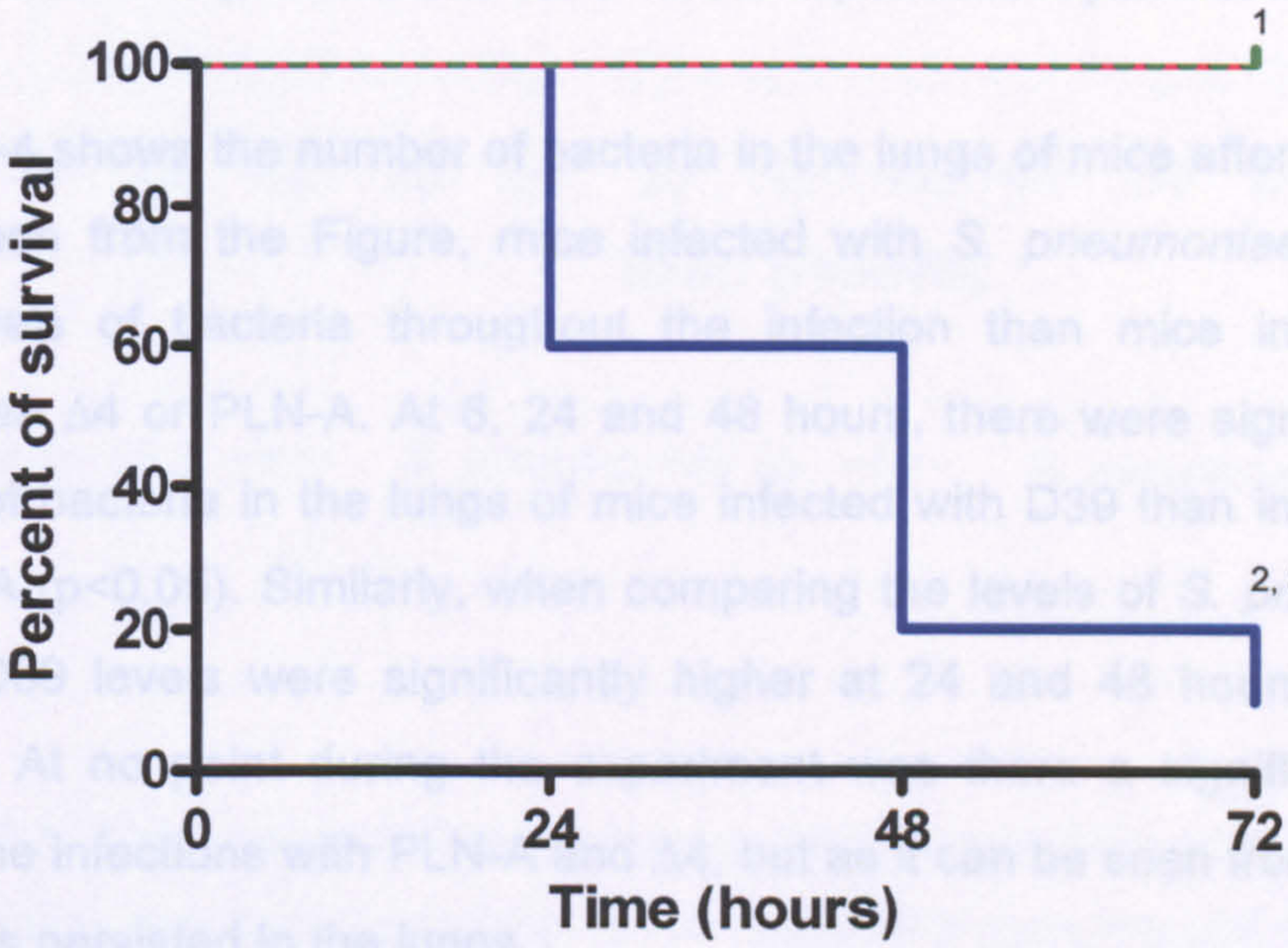


Figure 3.3-3 Survival of mice after intranasal infection. The survival of mice infected with *S. pneumoniae* D39 (n=10) is shown in blue, with *S. pneumoniae* Δ4 (n=14) in red and with *S. pneumoniae* PLN-A (n=7) in green. The infections were done in two independent experiments.

[1]Significantly different to the survival of mice infected with D39 ($p<0.001$).

[2]Significantly different to the survival of mice infected with Δ4 ($p<0.001$).

[3]Significantly different to the survival of mice infected with PLN-A ($p<0.001$).

3.3.1.2 Estimation of CFU from lungs and blood after intranasal infections

To measure the ability of the *S. pneumoniae* Δ4 mutant to replicate in the lungs of the infected host and its ability to enter and replicate in the blood stream, the number of bacteria from the lungs and blood of infected mice were assessed. Infections with *S. pneumoniae* D39 and PLN-A were also done.

The experiment with mice infected with *S. pneumoniae* Δ4 and *S. pneumoniae* PLN-A lasted 72 hours, whereas with *S. pneumoniae* D39 all the experiment finished by 48 hours, as experiment endpoints were reached before 72 hours. Five mice were used for each time point with each of the pneumococcal strains except for the 48 hours time point, where nine mice were infected with *S. pneumoniae* D39 and ten with *S. pneumoniae* Δ4 in two independent experiments.

Figure 3.3-4 shows the number of bacteria in the lungs of mice after infection. As it can be seen from the Figure, mice infected with *S. pneumoniae* D39, showed higher levels of bacteria throughout the infection than mice infected with *S. pneumoniae* Δ4 or PLN-A. At 6, 24 and 48 hours, there were significantly higher numbers of bacteria in the lungs of mice infected with D39 than in those infected with PLN-A ($p<0.05$). Similarly, when comparing the levels of *S. pneumoniae* D39 with Δ4, D39 levels were significantly higher at 24 and 48 hours post-infection ($p<0.05$). At no point during the experiment was there a significant difference between the infections with PLN-A and Δ4, but as it can be seen from Figure 3.3-4, both strains persisted in the lungs.

In Figure 3.3-5 the numbers of bacteria found on the blood of intranasally infected mice are shown. As it can be seen from the Figure, the three pneumococcal strains were able to cause bacteraemia after the infection. All of them had entered the blood stream by 24 hours. As expected, *S. pneumoniae* D39 was more aggressive than the mutant Δ4 and PLN-A. At 24 and 48 hours post infection, significantly higher numbers of D39 bacteria were in the blood stream when compared to Δ4 or PLN-A ($p<0.05$). As before, no significant differences were observed at any point of the infection between Δ4 and PLN-A ($p>0.05$).

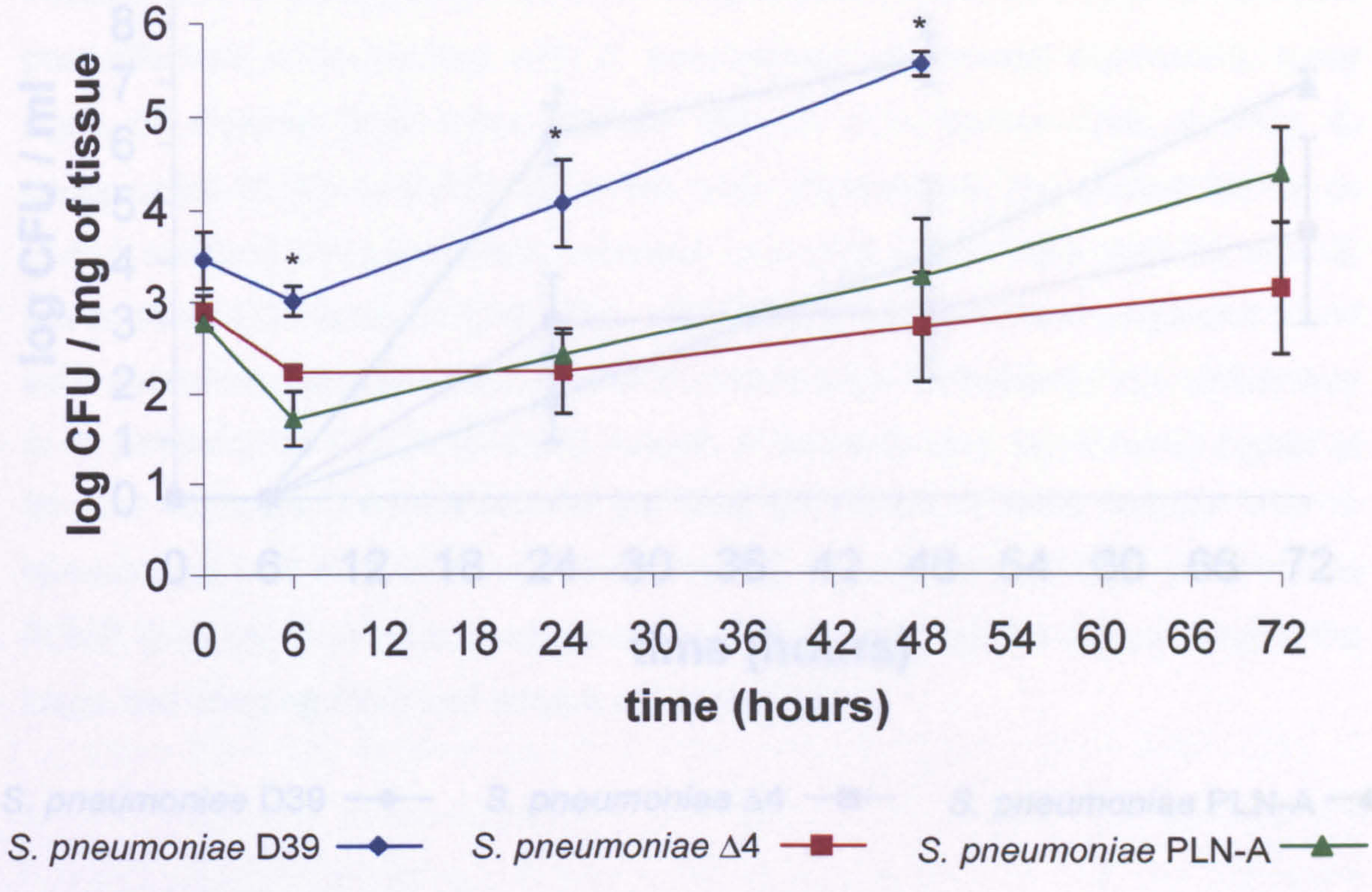


Figure 3.3-4 Time course of change in pneumococcal CFU in the lungs of mice after intranasal infection. Each point is the mean of 5 mice, except for the 48 hours time-point, where n=9 for *S. pneumoniae* D39 and n=10 for *S. pneumoniae* Δ4 (the 48 hours time point was done in two independent experiments). The bars at each data point show the standard error of the mean. The (*) symbol over each time point represent that significant differences were found ($p<0.05$). At 6, 24 and 48 hours, *S. pneumoniae* D39 is significantly higher than *S. pneumoniae* PLN-A ($p<0.05$). At 24 and 48 hours, *S. pneumoniae* D39 is also significantly higher than *S. pneumoniae* Δ4 ($p<0.05$).

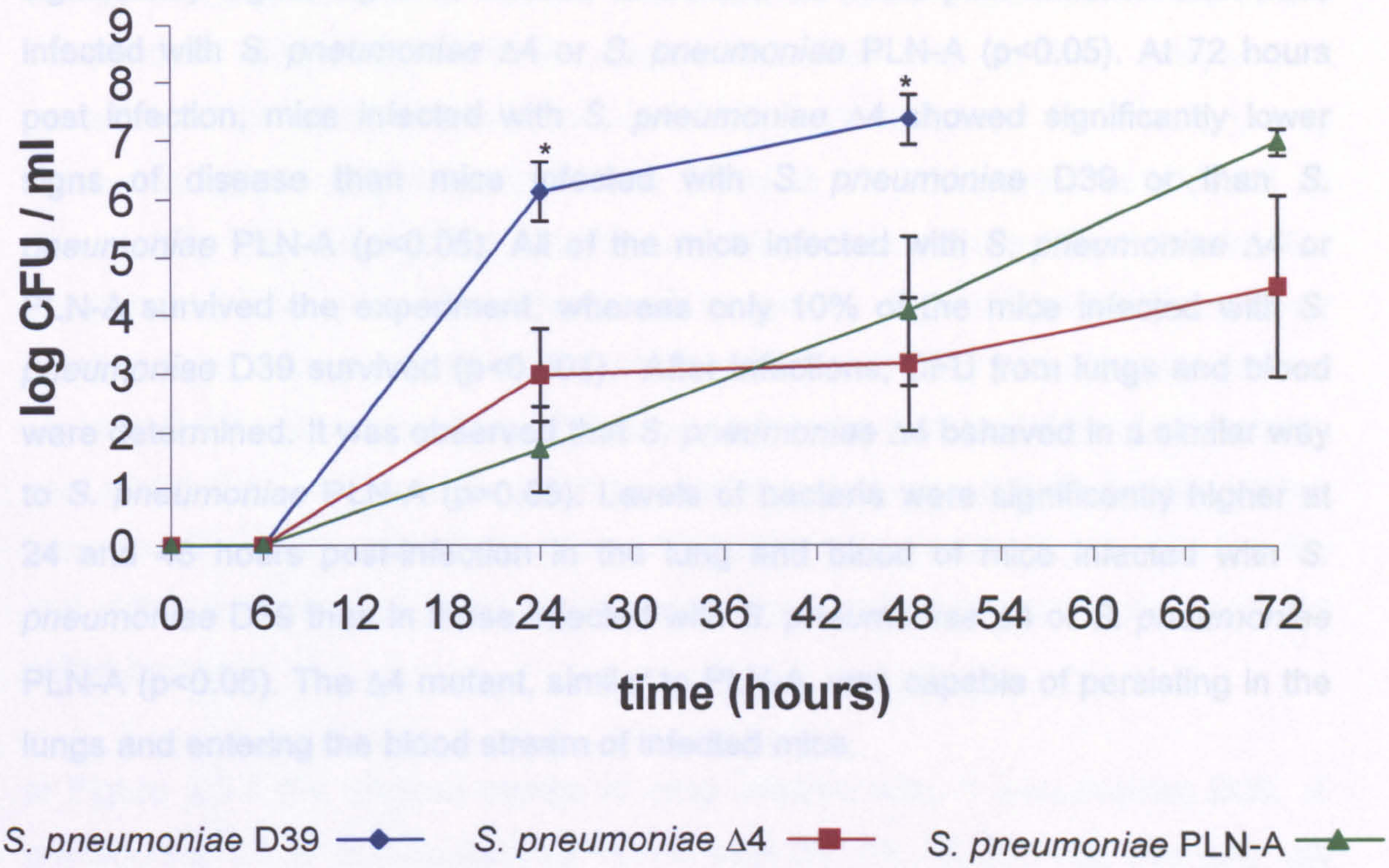


Figure 3.3-5 Time course of pneumococcal CFU in blood of mice after intranasal infections. Each point is the mean of 5 mice, except for the 48 hours time-point, where n=9 for *S. pneumoniae* D39 and n=10 for *S. pneumoniae* Δ4 (The 48 hours time point was done in two independent experiments). The bars at each data point show the standard error of the mean. The (*) symbol over each time point represent that significant differences were found ($p<0.05$). At 24 and 48 hours, *S. pneumoniae* D39 is significantly higher than *S. pneumoniae* Δ4 and *S. pneumoniae* PLN-A ($p<0.05$).

Summary of observations from 3.3.1

In the survival experiment, mice infected with *S. pneumoniae* D39 showed significantly higher signs of disease at 24 and 48 hours post infection than mice infected with *S. pneumoniae* Δ4 or *S. pneumoniae* PLN-A ($p<0.05$). At 72 hours post infection, mice infected with *S. pneumoniae* Δ4 showed significantly lower signs of disease than mice infected with *S. pneumoniae* D39 or than *S. pneumoniae* PLN-A ($p<0.05$). All of the mice infected with *S. pneumoniae* Δ4 or PLN-A survived the experiment, whereas only 10% of the mice infected with *S. pneumoniae* D39 survived ($p<0.001$). After infections, CFU from lungs and blood were determined. It was observed that *S. pneumoniae* Δ4 behaved in a similar way to *S. pneumoniae* PLN-A ($p>0.05$). Levels of bacteria were significantly higher at 24 and 48 hours post-infection in the lung and blood of mice infected with *S. pneumoniae* D39 than in those infected with *S. pneumoniae* Δ4 or *S. pneumoniae* PLN-A ($p<0.05$). The Δ4 mutant, similar to PLN-A, was capable of persisting in the lungs and entering the blood stream of infected mice.

3.3.2 Intravenous infections: disease survival and bacteria levels in blood after infection

To assess the impact of the pneumolysin mutation in *S. pneumoniae* Δ4 in a bacteraemia model, intravenous infections of mice were done. With this model, in which the bacteria are directly inoculated into the blood stream, a different kinetics of infection could be observed. It was therefore expected that *S. pneumoniae* Δ4 might behave in a similar way to *S. pneumoniae* D39 and show more virulence than *S. pneumoniae* PLN-A.

3.3.2.1 Disease and survival of mice after intravenous infections

In total, 13 mice were infected with *S. pneumoniae* D39, 20 with *S. pneumoniae* Δ4 and 23 with *S. pneumoniae* PLN-A. The infections of mice were performed in two independent experiments for *S. pneumoniae* D39 and in four independent experiments for *S. pneumoniae* Δ4 and PLN-A. As a control for infection, only those mice presenting bacteria in their blood at six hours were considered as successfully infected and the information obtained from them was used for the experiments. The mice were monitored for seven days (168 hours) and their disease signs were recorded as explained in 2.6.3.4. Briefly, a mouse was given a score of 0 if it showed no signs of disease; 2 if it was hunched, 4 if it had a starey coat and 6 if it was lethargic, at which point the mice was culled. For description purposes, if a mouse reached a score of 6 and was consequently culled before the end of the experiment (168 hours), this score continued to be used to represent the culled mouse.

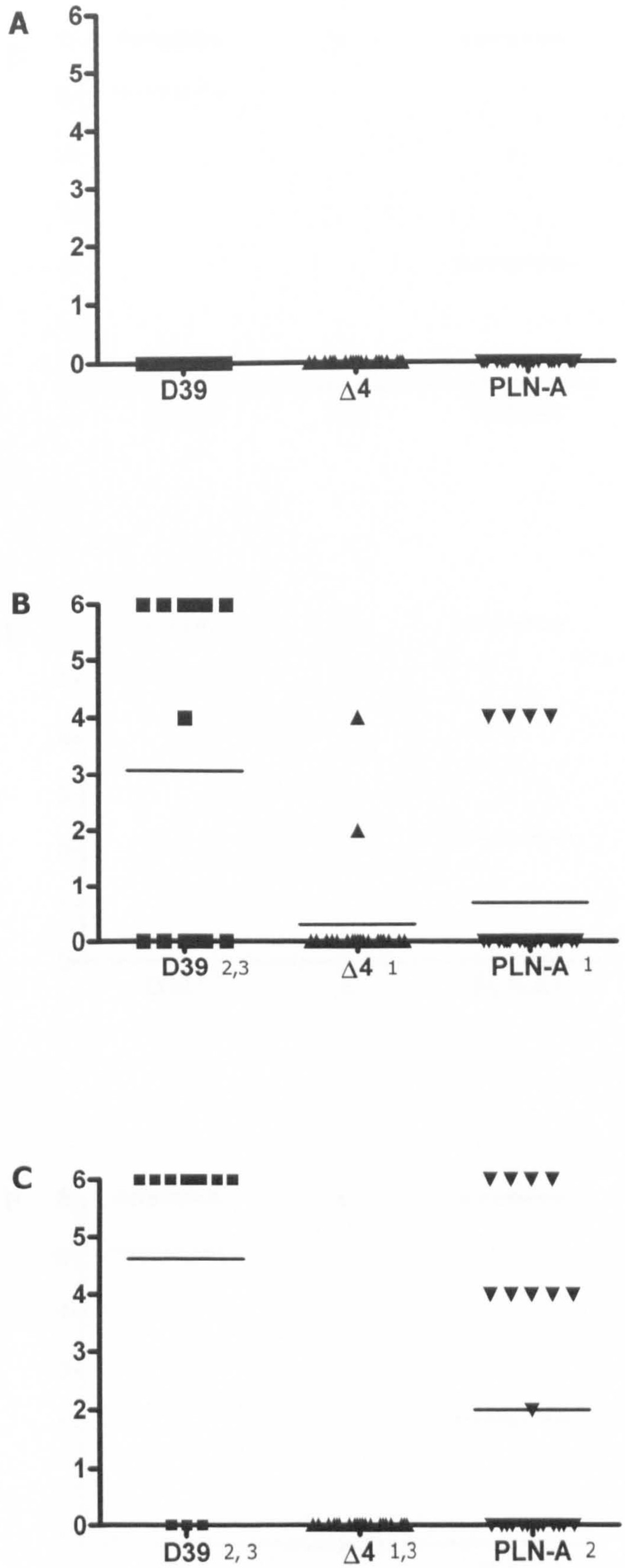
In Figure 3.3-6 the disease scores of mice infected with *S. pneumoniae* D39, *S. pneumoniae* Δ4 or *S. pneumoniae* PLN-A after 24, 48, 72, 96, 120, 144 and 168 hours of infection are shown. Because the graphs at the 144 and 168 hours are exactly the same, only one graph is shown representing these two time points (Figure 3.3-6F). From Figure 3.3-6, it can be observed that none of the mice showed signs of disease at 24 hours post intravenous infection regardless of the bacterial strain used (Figure 3.3-6A). However, nearly half of the mice infected with *S. pneumoniae* D39 had severe signs of disease by 48 hours (Figure 3.3-6B). At this time point mice infected with D39 showed significantly higher signs of disease ($p<0.05$) than mice infected with Δ4 of PLN-A. By 72 hours (Figure 3.3-6C) mice infected with *S. pneumoniae* Δ4 show significantly lower signs of disease than mice infected with *S. pneumoniae* D39 or *S. pneumoniae* PLN-A. The signs of disease for mice infected with PLN-A were also significantly lower than that of mice infected with D39. For 96, 120, 144 and 168 hours post infection (Figure 3.3-6D, E and F), mice infected with *S. pneumoniae* D39 showed significantly higher signs of disease than mice infected with *S. pneumoniae* Δ4 or *S. pneumoniae* PLN-A.

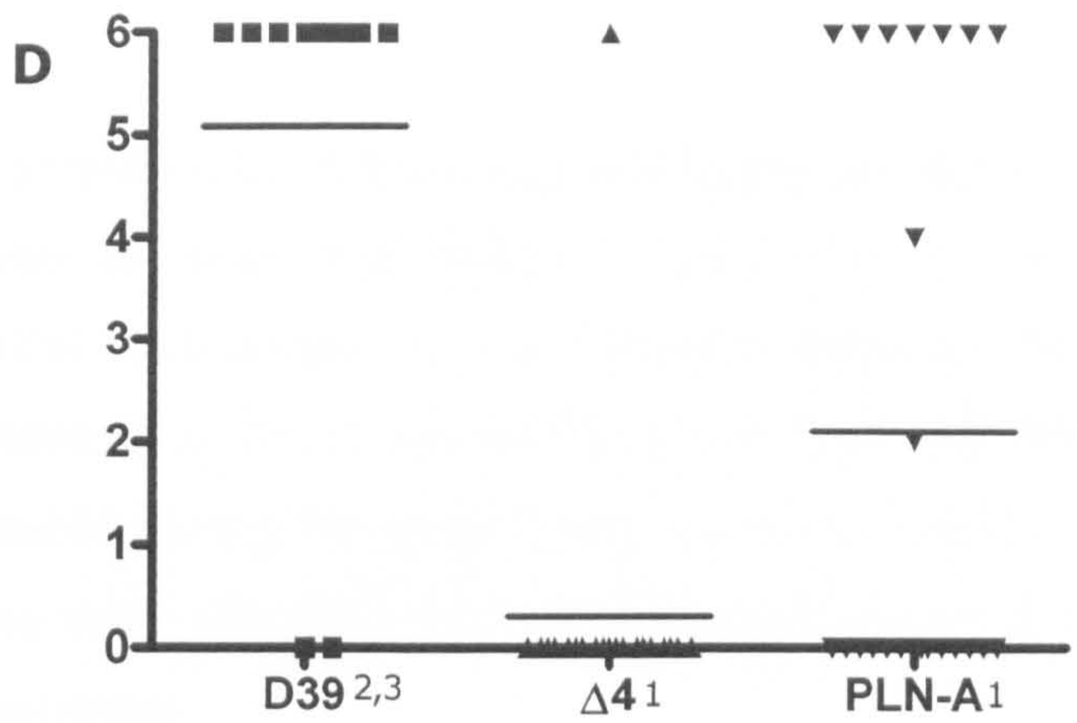
Figure 3.3-6 Signs of disease of mice after (A) 24, (B) 48, (C) 72, (D) 96, (E) 120 and (F) 144 and 168 hours of intravenous infections with *S. pneumoniae* D39 (squares), *S. pneumoniae* Δ4 (triangles) or *S. pneumoniae* PLN-A (inverted triangles). Because the graphs corresponding to 144 and 168 hours post-infection are identical, graph F is being used to represent both. Each symbol represents one mouse, where n=13 for mice infected with D39; n=20 for mice infected with Δ4; and n=23 for mice infected with PLN-A. The infections were done in two independent experiments for mice infected with *S. pneumoniae* D39 and in four independent experiments for mice infected with *S. pneumoniae* Δ4 and *S. pneumoniae* PLN-A. A continuous line represents the average sign of disease for each group of mice at each time point.

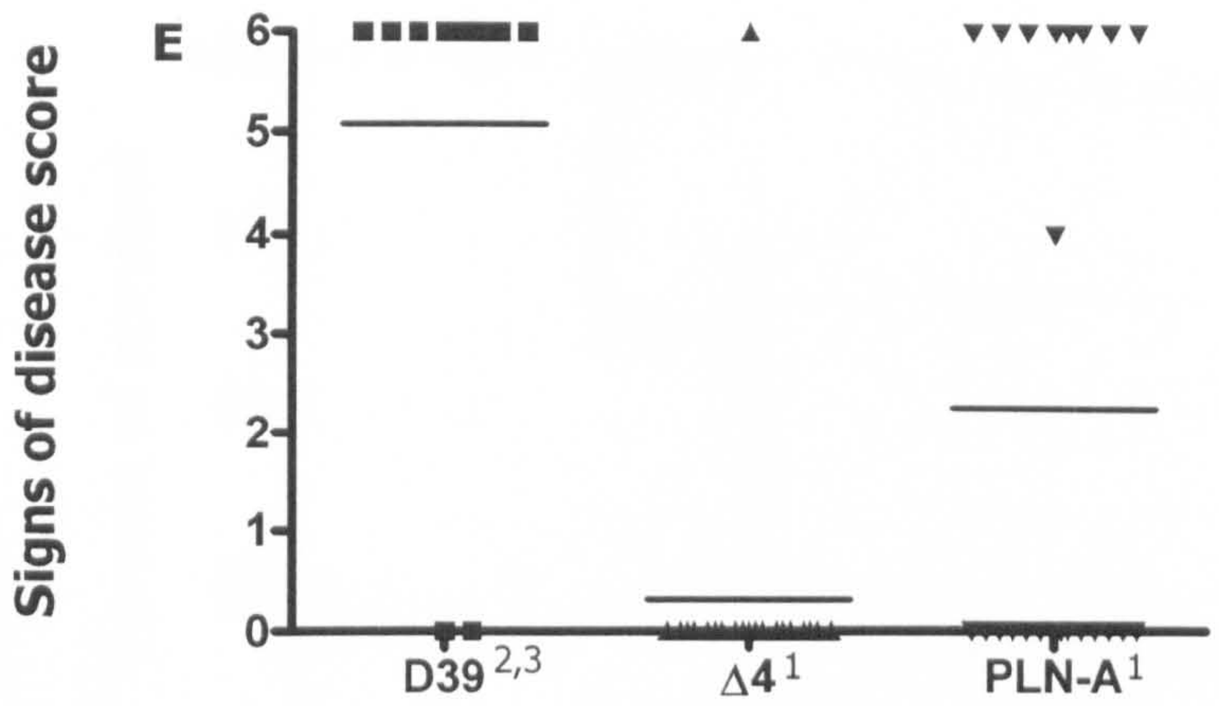
[1]Significantly different to *S. pneumoniae* D39 ($p<0.05$)

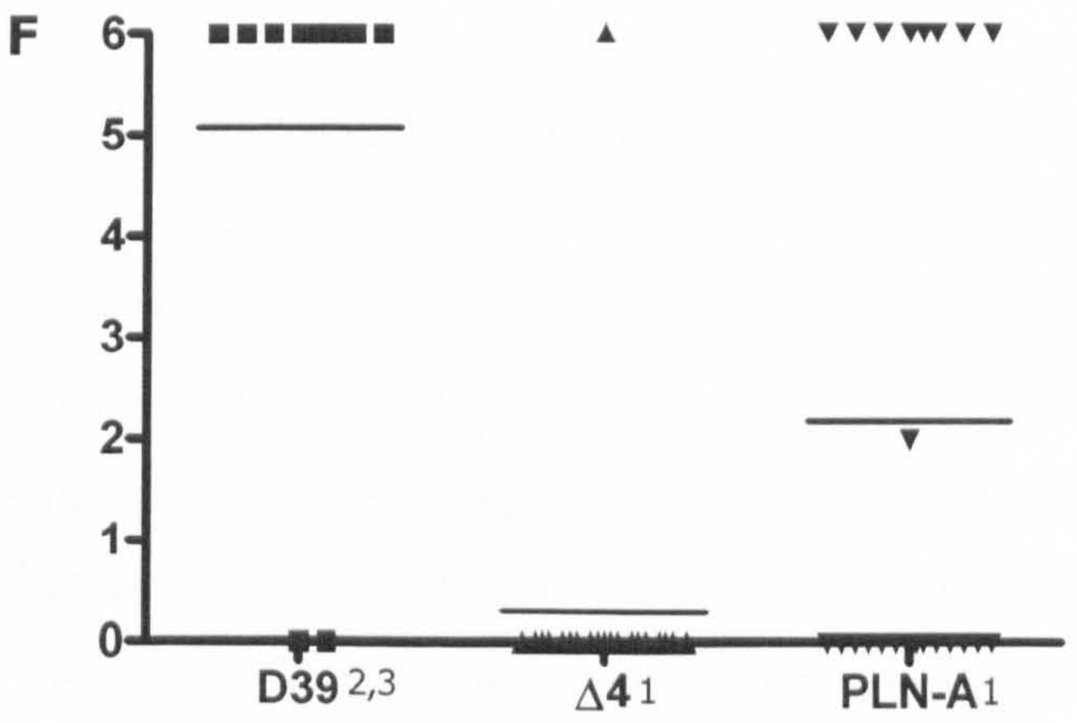
[2]Significantly different to *S. pneumoniae* Δ4 ($p<0.05$)

[3]Significantly different to *S. pneumoniae* PLN-A ($p<0.05$)


A
6
5
4
3
2
1
0
D39
Δ4
PLN-A
B
Signs of disease score
6
5
4
3
2
1
0
D39 2,3
Δ4 1
PLN-A 1
C
6
5
4
3
2
1
0
D39 2, 3
Δ4 1,3
PLN-A 2
Pneumococcal strain used







Pneumococcal strain used

The survivals of mice after intravenous infections are shown in Figure 3.3-7. From the Figure it can be seen that 85% (11 out of 13) of the mice infected with *S. pneumoniae* D39 succumbed to the infection between 24 and 80 hours post-infection, whereas only 5% (1 out of 20) of the mice infected with *S. pneumoniae* Δ4 had to be culled during the experiment. It was surprising to observe that 35% (8 out of 23) of the mice infected with PLN-A became severely ill and had to be culled during the experiment.

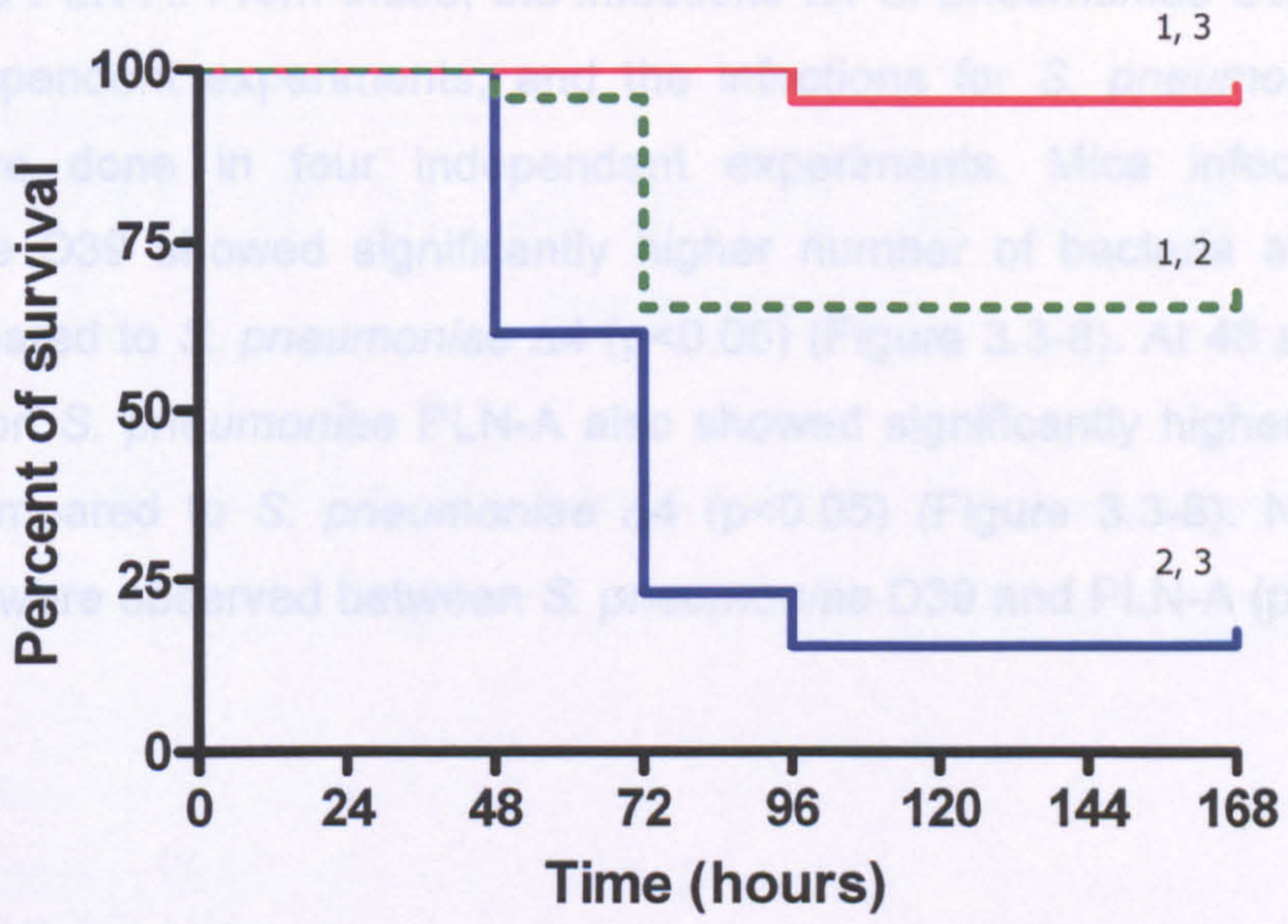


Figure 3.3-7 Survival of mice after intravenous infections. The survival of mice infected with *S. pneumoniae* D39 (n=13) is shown in blue; with *S. pneumoniae* Δ4 (n=20) in red; and with *S. pneumoniae* PLN-A (n=23) in green. The infections for *S. pneumoniae* D39 were done in two independent experiments, while the infections for *S. pneumoniae* Δ4 and PLN-A were done in four independent experiments.

[1]Significantly different to the survival of mice infected with D39 ($p<0.05$).

[2]Significantly different to the survival of mice infected with Δ4 ($p<0.05$).

[3]Significantly different to the survival of mice infected with PLN-A ($p<0.05$).

3.3.2.2 Estimation of CFU from blood after intravenous infections

Because of the small amount of blood required for the calculation of CFU and because this amount could be obtained by tail bleeding of the mouse (see 2.6.4.2) without compromising its health, blood samples were obtained at different time points from the mice used for the survival experiment. As in the survival experiment, only mice that presented bacteria at 6 hours post infection were considered successfully infected and used for the experiment. Therefore, a total of 13 mice were infected with *S. pneumoniae* D39, 20 with *S. pneumoniae* Δ4 and 23 with *S. pneumoniae* PLN-A. From these, the infections for *S. pneumoniae* D39 were done in two independent experiments, and the infections for *S. pneumoniae* Δ4 and PLN-A were done in four independent experiments. Mice infected with *S. pneumoniae* D39 showed significantly higher number of bacteria at 24 and 48 hours compared to *S. pneumoniae* Δ4 ($p<0.05$) (Figure 3.3-8). At 48 and 72 hours post infection *S. pneumoniae* PLN-A also showed significantly higher numbers of bacteria compared to *S. pneumoniae* Δ4 ($p<0.05$) (Figure 3.3-8). No significant differences were observed between *S. pneumoniae* D39 and PLN-A ($p>0.05$).

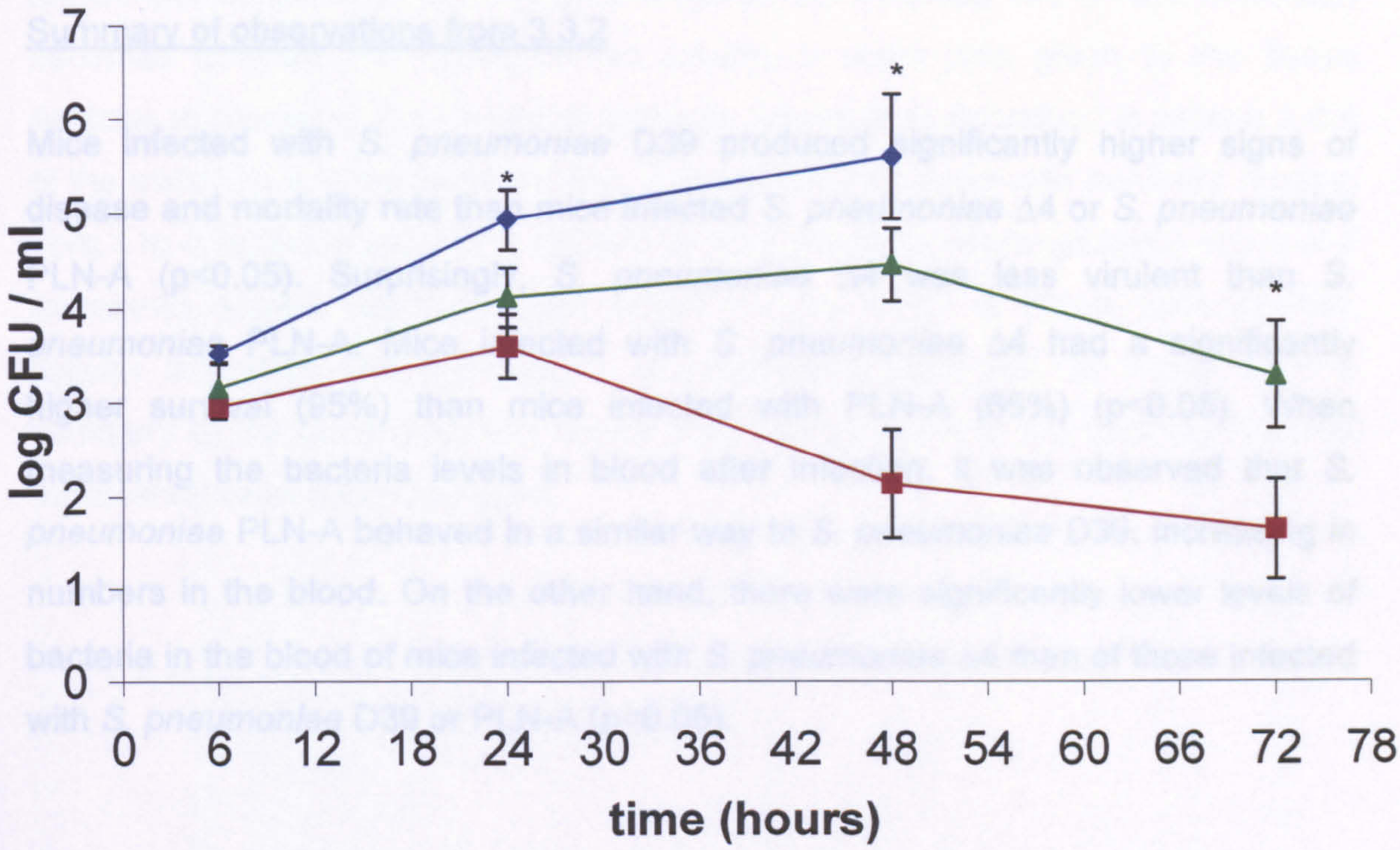


Figure 3.3-8 Time course of pneumococcal CFU in the blood of mice after intravenous infections. Each point for *S. pneumoniae* D39 is the mean of 13 mice, for *S. pneumoniae* Δ4 of 20 mice and for *S. pneumoniae* PLN-A of 23 mice (The infections for *S. pneumoniae* D39 were done in two independent experiments, whereas the infections for *S. pneumoniae* Δ4 and PLN-A were done in four independent experiments). The bars at each data point show the standard error of the mean. The (*) symbol over each time point represent that significant differences were found ($p<0.05$). At 24 and 48 hours, *S. pneumoniae* D39 was significantly higher than *S. pneumoniae* Δ4 ($p<0.05$). At 48 and 72 hours, *S. pneumoniae* PLN-A was significantly higher than *S. pneumoniae* Δ4.

Summary of observations from 3.3.2

Mice infected with *S. pneumoniae* D39 produced significantly higher signs of disease and mortality rate than mice infected *S. pneumoniae* Δ4 or *S. pneumoniae* PLN-A ($p<0.05$). Surprisingly, *S. pneumoniae* Δ4 was less virulent than *S. pneumoniae* PLN-A. Mice infected with *S. pneumoniae* Δ4 had a significantly higher survival (95%) than mice infected with PLN-A (65%) ($p<0.05$). When measuring the bacteria levels in blood after infection, it was observed that *S. pneumoniae* PLN-A behaved in a similar way to *S. pneumoniae* D39, increasing in numbers in the blood. On the other hand, there were significantly lower levels of bacteria in the blood of mice infected with *S. pneumoniae* Δ4 than of those infected with *S. pneumoniae* D39 or PLN-A ($p<0.05$).

3.3.3 Lung histology: Comparison of infected lungs

To compare tissue inflammation caused by each of the pneumococcal strains, whole lungs obtained at different time points after intranasal infections were collected and frozen for histology as explained in 2.6.9. The lungs of two mice were analysed for each time point, except for the 6 hours time point of *S. pneumoniae* D39 infections, where only one mouse was analysed

It is already known that *S. pneumoniae* D39 induces severe lung tissue inflammation with heavy cellular infiltration and that the level of inflammation induced by *S. pneumoniae* PLN-A is less severe (Kadioglu *et al.*, 2000). In this experiment, tissue sections of the lungs of mice infected via intranasal route with either *S. pneumoniae* D39, *S. pneumoniae* PLN-A or *S. pneumoniae* Δ4 were analysed at 6, 24 and 48 hours; for *S. pneumoniae* Δ4 and *S. pneumoniae* PLN-A a 72 hours time point was also done.

Levels of inflammation in the lungs were observed by consolidation of the tissue, where the spaces corresponding to the alveolar sacs become smaller or undistinguishable. Also, the bronchial walls appear inflamed and cellular infiltration becomes obvious. For clarity of the results, a score was given to the tissue sections to represent the degree of damage observed as described in section 2.6.9 of the materials and methods section. The scores are shown with numbers ranging from 1 to 4, where a score of 1 represents very low tissue damage and a score of 4 represents severe tissue damage based on the levels of tissue consolidation, cellular infiltration and hypertrophy of bronchioles observed. In Table 3.3-1 a summary of the observations is shown and Figures 3.3-9 to 3.3-13 show pictures representative of each time point.

Table 3.3-1. Summary of the lung tissue damage observed after infections with *S. pneumoniae* D39, *S. pneumoniae* Δ4 and *S. pneumoniae* PLN-A.

	Infection with *S. pneumoniae* D39 Observed lung damage				**Infection with S. pneumoniae Δ4** Observed lung damage				**Infection with S. pneumoniae PLN-A** Observed lung damage			
	HB	**CI**	**GC**	**score**	**HB**	**CI**	**GC**	**score**	**HB**	**CI**	**GC**	**score**
6 hours												
mouse 1	yes	low	medium	2	yes	low	low	1	yes	none	low	1
mouse 2	Not done				yes	low	low	1	yes	low	low	1
24 hours												
mouse 1	yes	heavy	high	4	yes	low	medium	2	yes	none	low	1
mouse 2	yes	moderate	high	3	yes	low	high	2	yes	none	low	1
48 hours												
mouse 1	yes	heavy	high	4	yes	moderate	medium	2	yes	moderate	low	2
mouse 2	yes	heavy	medium	3	yes	heavy	medium	3	yes	low	low	1
72 hours	Not done											
mouse 1					yes	heavy	high	3	yes	low	medium	2
mouse 2					yes	heavy	medium	3	yes	low	medium	2

HB.- Hypertrophy of bronchial walls. CI.- Cellular infiltration. GC.- General consolidation of the lung tissue.

Figure 3.3-9 shows a control picture of a non-infected mouse lung section. As it can be seen in this Figure, healthy lungs exhibit large respiratory spaces that correspond to alveolar sacs, which because of the methodology used for freezing and cutting the lungs, loose their fragile reticulated appearance. In Figure 3.3-9 a healthy bronchiole is also shown.

Figures 3.3-10, 3.3-11, 3.3-12 and 3.3-13 show an example of the comparisons of infected lungs with each pneumococcal strain at each of the time points studied. As it can be seen from Figure 3.3-10 at 6 hours post-infection the lungs of mice infected with Δ4 (Figure 3.3-10B) and PLN-A (Figure 3.3-10C) showed a lower degree of damage (scored 1) than the lungs of mice infected with *S. pneumoniae* D39 (scored 2) (Figure 3.3-10A), which showed higher levels of tissue consolidation. The lung of mice infected with *S. pneumoniae* D39 (Figure 3.3-10A) or *S. pneumoniae* Δ4 (Figure 3.3-10B) also showed low levels of cellular infiltrate, which was not present in the lungs of mice infected with PLN-A (Figure 3.3-10C). Hypertrophy of bronchial walls was observed in the lungs of mice infected with either strain of bacteria. By 24 hours, the lungs of mice infected with *S. pneumoniae* D39 (Figure 3.3-11A) showed high consolidation of the lung parenchyma with heavy cellular infiltration in different areas of the lungs and hypertrophy of bronchial walls (scored 3). By contrast, the lungs of mice infected with PLN-A (Figure 3.3-11C) showed only low levels of consolidation in the lung parenchyma and also hypertrophy of bronchial walls (scored 1). The lungs of mice infected with Δ4 (Figure 3.3-11B) showed a degree of disease between PLN-A and D39, where medium consolidation of the lung parenchyma was observed with localised low cellular infiltrate and hypertrophy of bronchial walls (scored 2). At 48 hours post-infection, the lungs of mice infected with *S. pneumoniae* D39 (Figure 3.3-12A) were severely affected (scored 4). Heavy cellular infiltration was observed throughout the lung and the lung parenchyma had heavy consolidation. Usually mice infected with D39 do not survive beyond 48 hours post-infection. Mice infected with PLN-A (Figure 3.3-12C) showed heavy cellular infiltration in some areas of the lung, some of the bronchioles showed hypertrophy, but the lung parenchyma appeared with low consolidation (scored 2). The lungs of mice infected with Δ4 (Figure 3.3-12B) showed similar degree of damage than mice

infected with PLN-A, however, tissue consolidation was more apparent with Δ4 infections. At 72 hours post-infection, the lung tissue of mice infected with *S. pneumoniae* PLN-A (Figure 3.3-13B) showed medium consolidation of the lung parenchyma with low cellular infiltrate in localised areas and hypertrophy of bronchial walls (scored 2). By contrast, mice infected with *S. pneumoniae* Δ4 (Figure 3.3-13A), showed high tissue consolidation throughout the lung with heavy cellular infiltrate and hypertrophy of bronchial walls (scored 3).

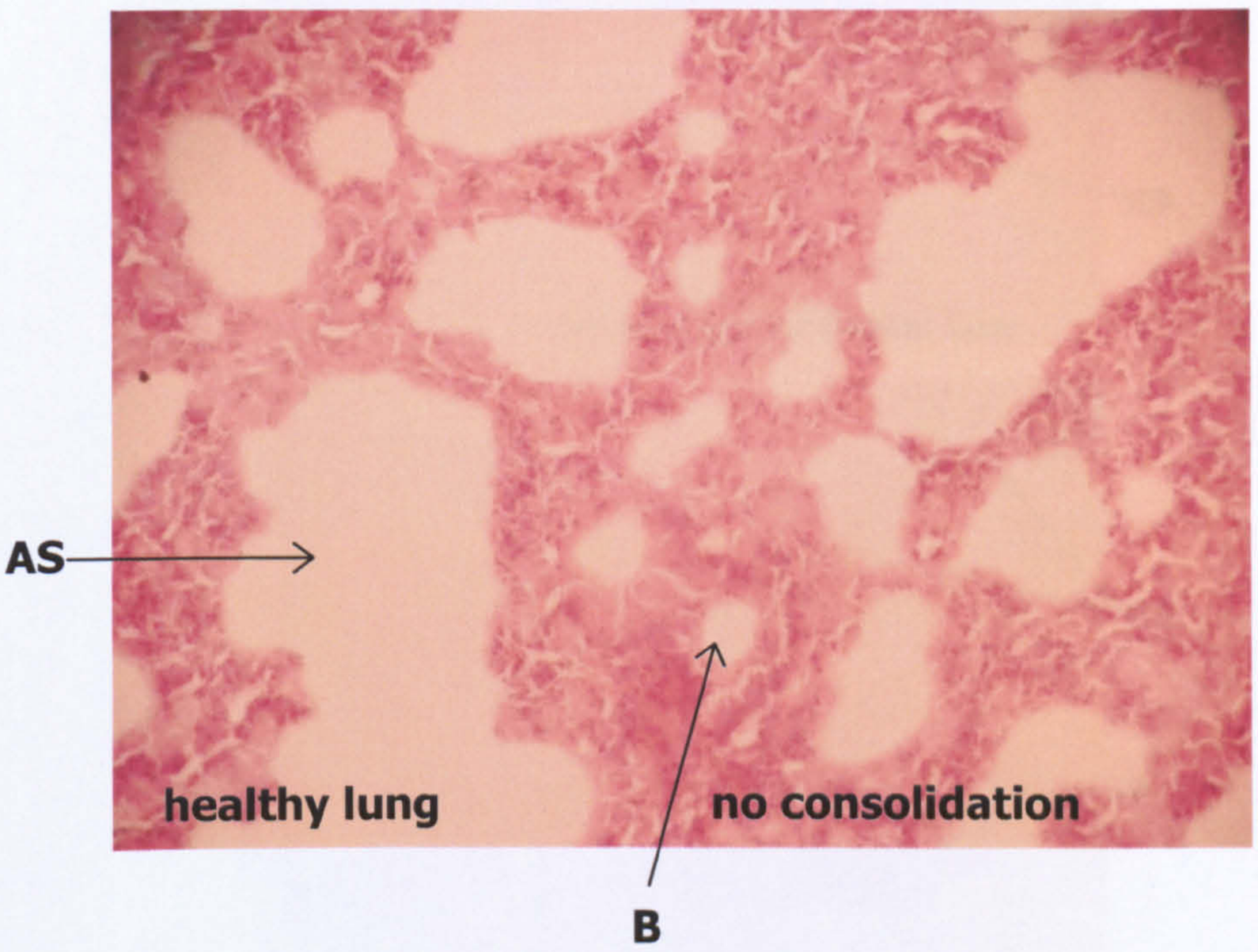


Figure 3.3-9 Lung section of a healthy mouse. AS.- Space corresponding to alveolar sacs. B.- Bronchiole. Magnification: 200x. The section was prepared by cryostat sectioning and haematoxylin-eosin staining as explained in 2.6.9.

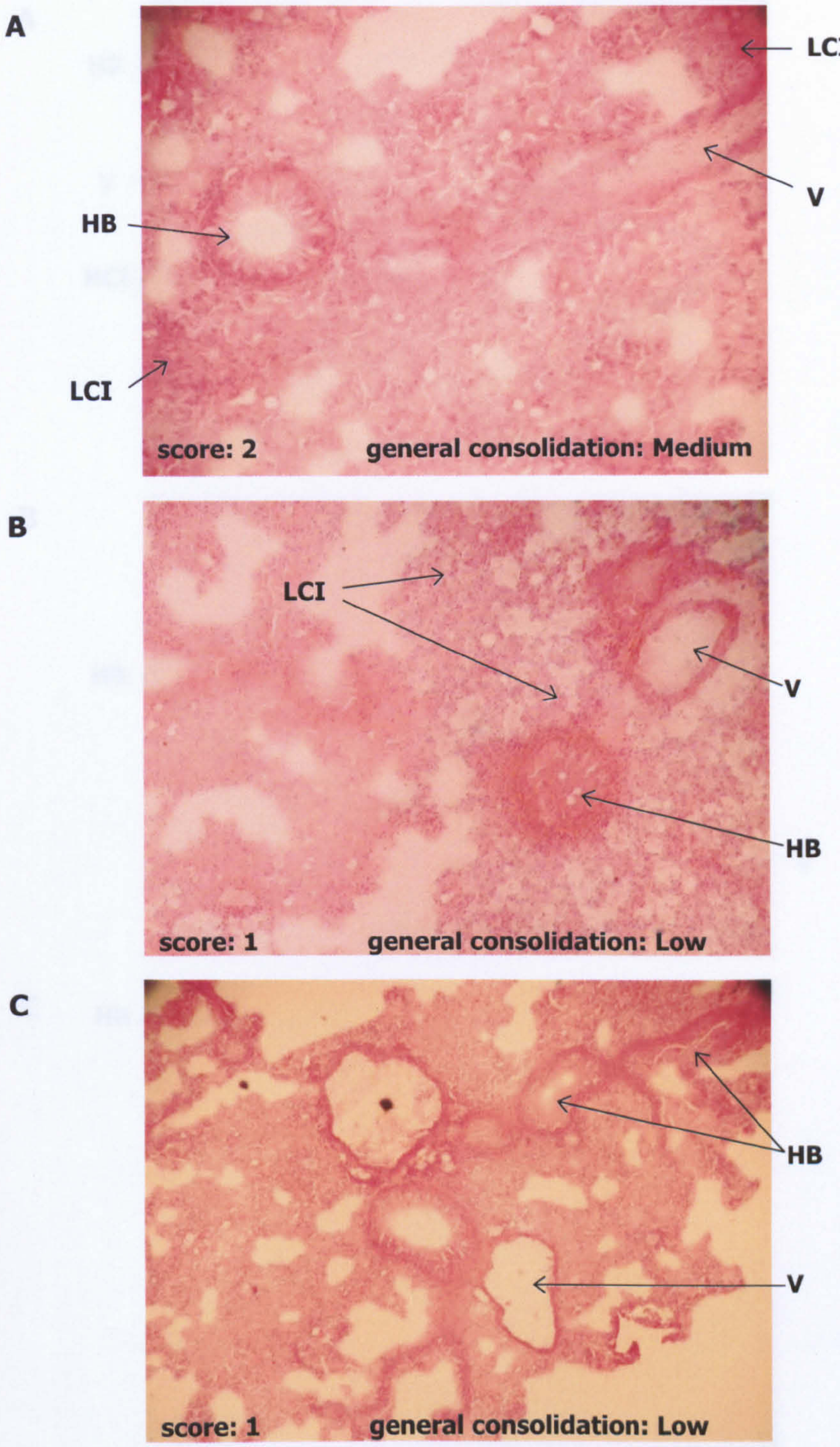


Figure 3.3-10 Lung sections of mice after 6 hours of IN infection with (A) *S. pneumoniae* D39, (B) *S. pneumoniae* Δ4 and (C) *S. pneumoniae* PLN-A. LCI.- Light cellular infiltration. HB.- Hypertrophy of bronchial wall. V.- Blood vessel. The damage score and the general consolidation of the tissue are also shown. Magnification: 200x. The sections were prepared by cryostat sectioning and haematoxylin-eosin staining as explained in 2.6.9.

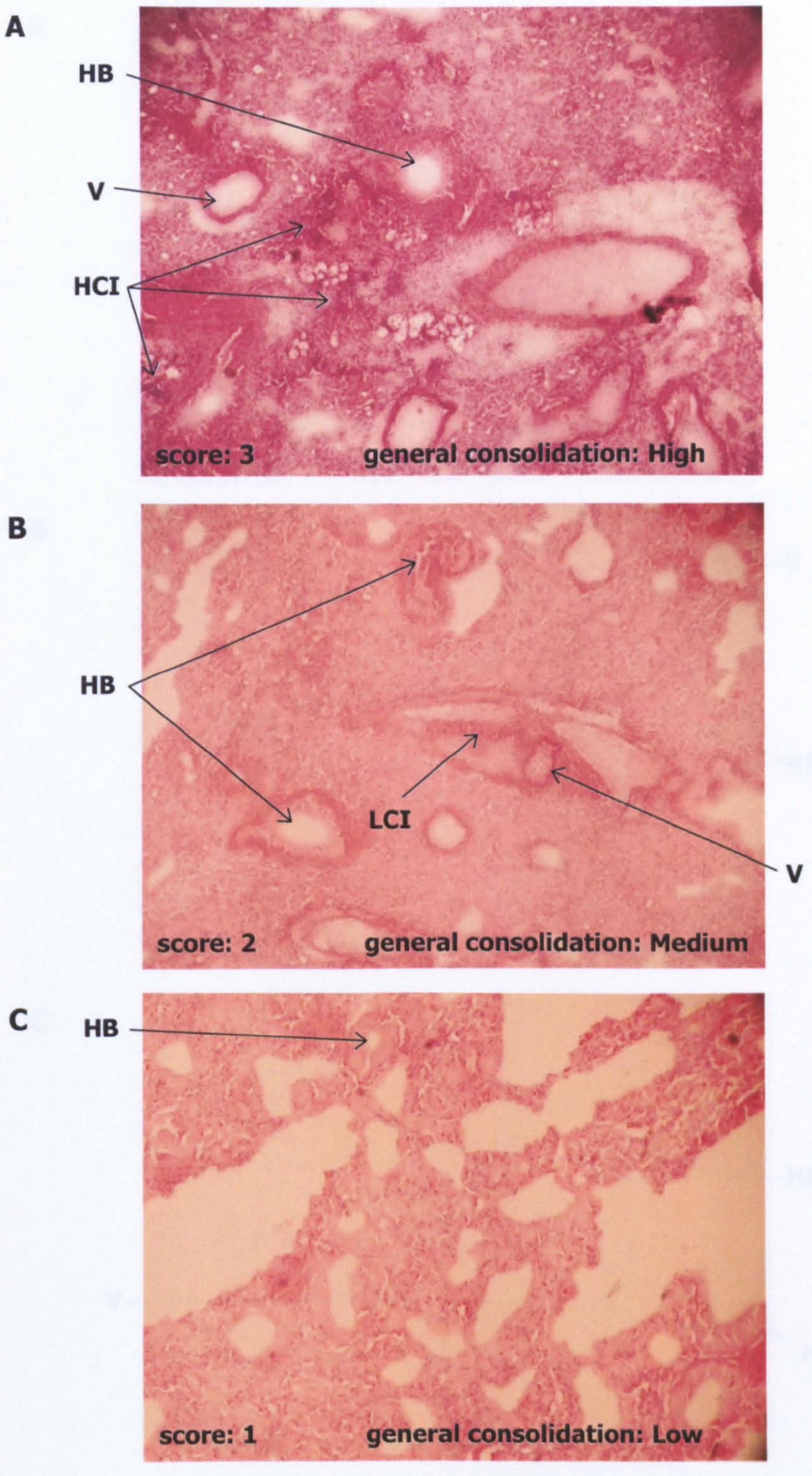


Figure 3.3-11 Lung sections of mice after 24 hours of IN infection with (A) *S. pneumoniae* D39, (B) *S. pneumoniae* Δ4 and (C) *S. pneumoniae* PLN-A. HCI, LCI.- Heavy or light cellular infiltration respectively. HB.- Hypertrophy of bronchial wall. V.- Blood vessel. The damage score and the general consolidation of the tissue are also shown. Magnification: 200x. The sections were prepared by cryostat sectioning and haematoxylin-eosin staining as explained in 2.6.9.

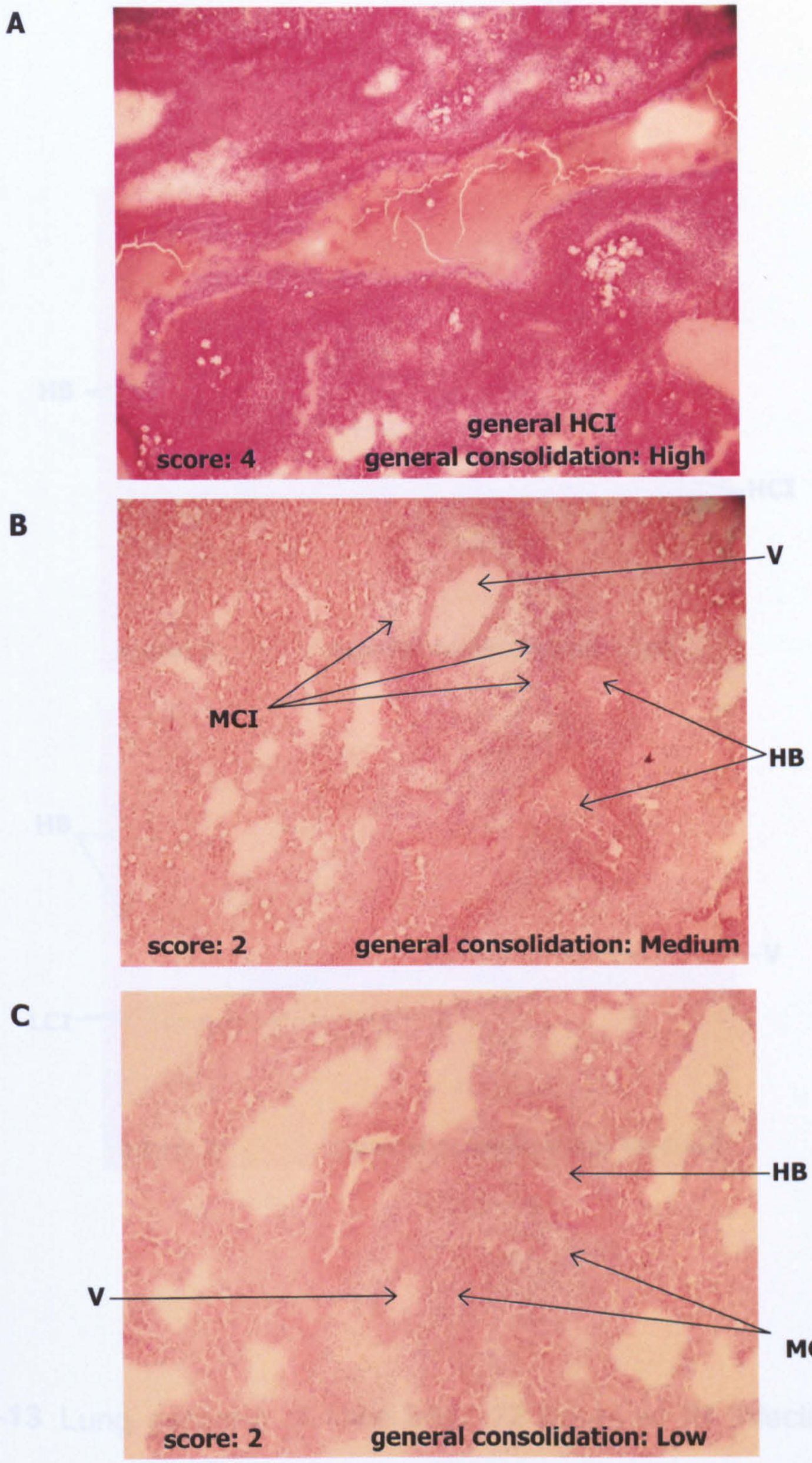


Figure 3.3-12 Lung sections of mice after 48 hours of IN infection with (A) *S. pneumoniae* D39, (B) *S. pneumoniae* Δ4 and (C) *S. pneumoniae* PLN-A. HCI, MCI.- Heavy and moderate cellular infiltration respectively. HB.- Hypertrophy of bronchial wall. V.- Blood vessel. The damage score and the general consolidation of the tissue are also shown. Magnification: 200x. The sections were prepared by cryostat sectioning and haematoxylin-eosin staining as explained in 2.6.9.

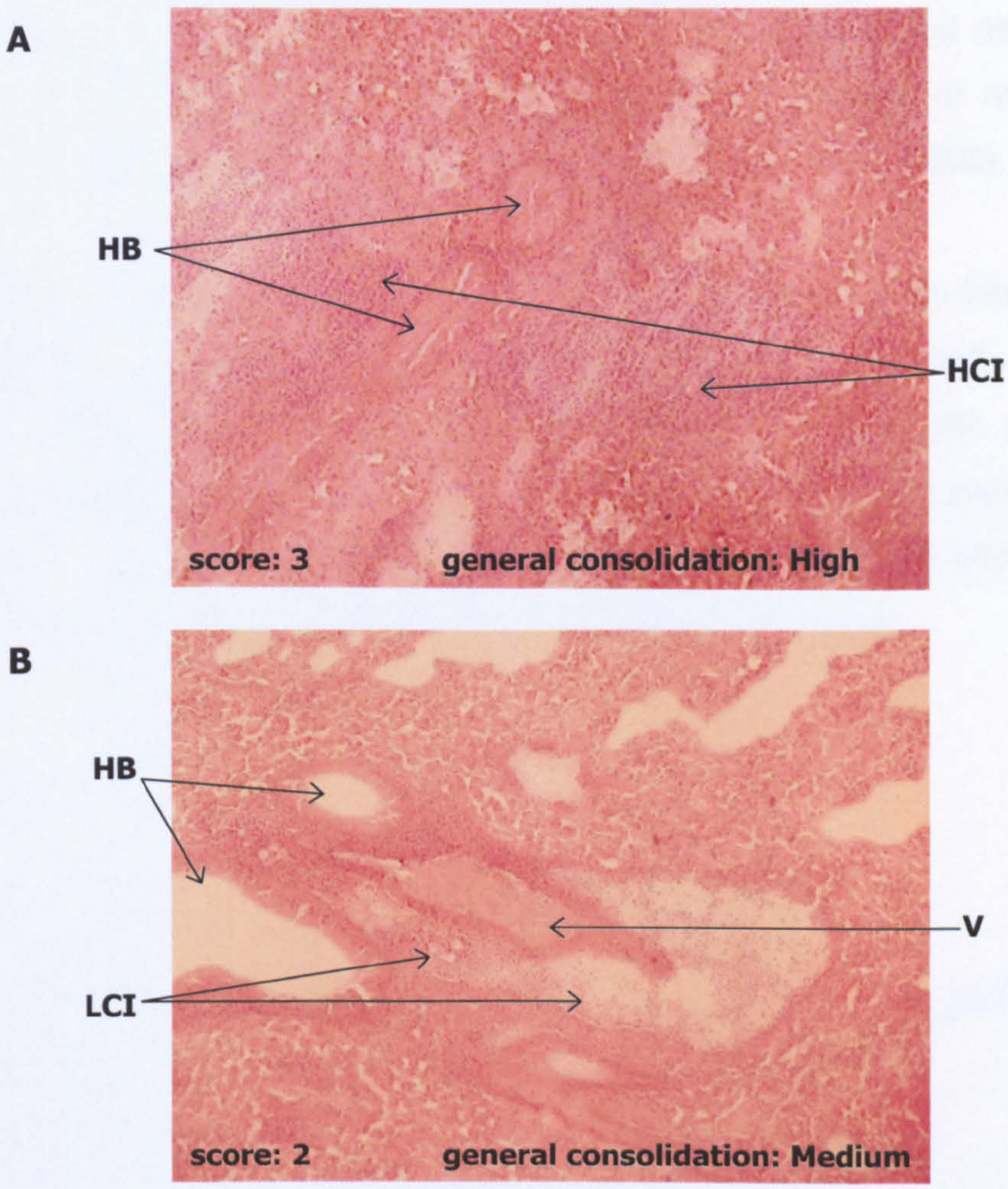


Figure 3.3-13 Lung sections of mice after 72 hours of IN infection with (A) *S. pneumoniae* Δ4 and (B) *S. pneumoniae* PLN-A. HCI, LCI.- Heavy or light cellular infiltration respectively. HB.- Hypertrophy of bronchial wall. V.- Blood vessel. The damage score and the general consolidation of the tissue are also shown. Magnification: 200x. The sections were prepared by cryostat sectioning and haematoxylin-eosin staining as explained in 2.6.9.

3.3.4 Interferon-γ and nitrate levels after intranasal and intravenous infections

As mentioned at the beginning of this chapter, interferon-γ and nitric oxide levels were measured after intranasal and intravenous infections of mice to test the hypothesis proposed by Baba and collaborators (Baba *et al.*, 2002).

An ELISA kit was used to measure the levels of interferon-γ as described in 2.4.3. A standard curve was included in each ELISA plate and the interferon-γ concentration in the samples was calculated according to the equation of the trendline obtained from the standard curve of its corresponding plate. In Figure 3.3-14, an example of a standard curve is shown. The example shows the desired linear trendline (Figure 3.3-14) with an R^2 value close to one.

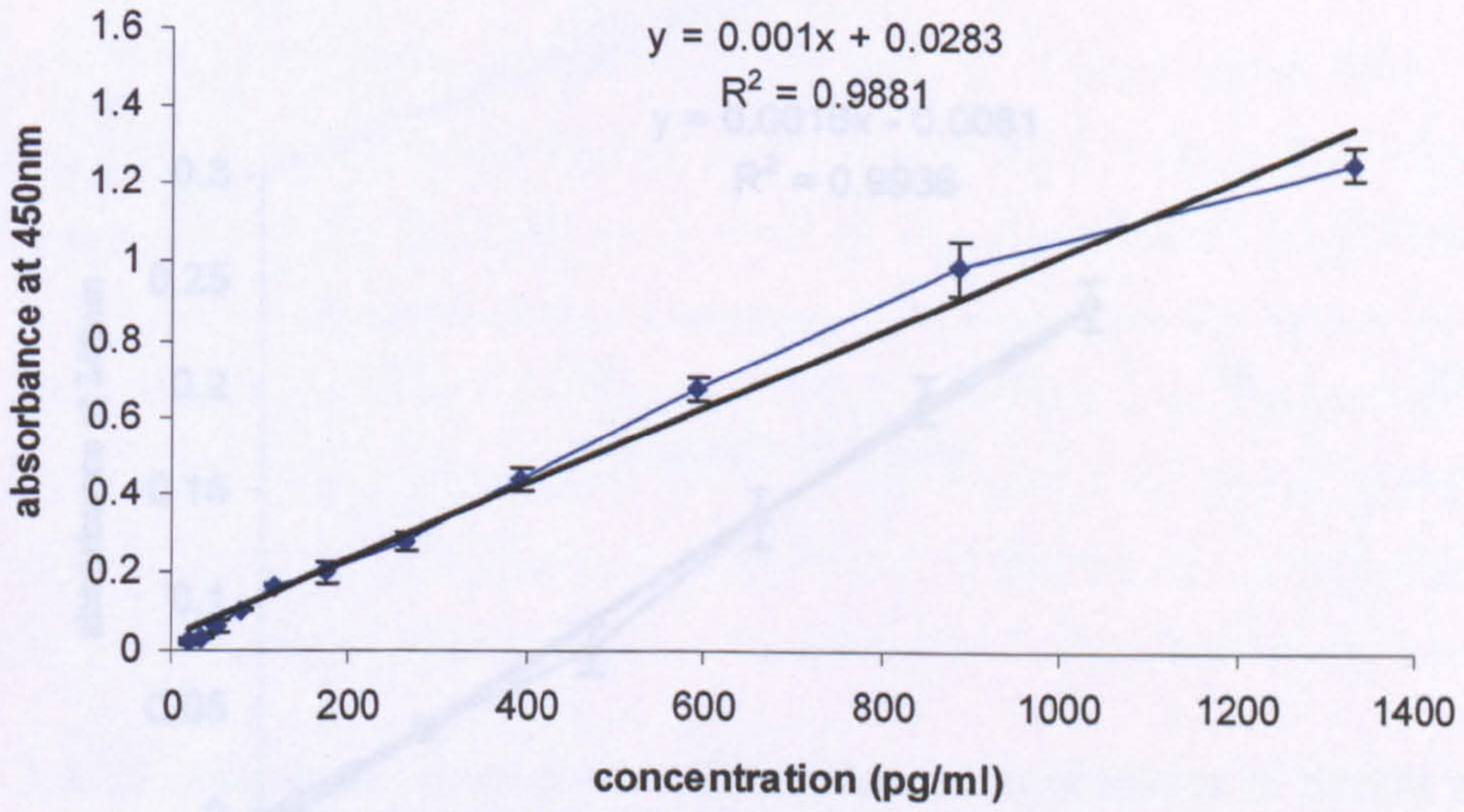


Figure 3.3-14 Example of a standard curve used to measure the concentrations of interferon-γ. The standard error of the mean is represented with a bar on each data point. The trendline (in bold), its equation and the R^2 are also shown. N=2

To measure the concentration of nitric oxide from biological samples, quantification of nitrate was done as explained in section 2.5. This was done because any nitric oxide produced is very unstable and quickly degrades into nitrate and nitrite (Boris and Bories, 1995). Because of the large number of samples and the limited amount of samples that could be tested per experiment with the available equipment, it was decided to prepare standard curves that could be used for all the samples.

As mentioned in section 2.5, the standard curves used to measure the concentration of nitrate in lung lavages were made using known amounts of nitrate in HBSS. HBSS was chosen because the lung lavages were done with this solution. In Figure 3.3-15 the standard curve used is shown. This curve is an average of four standard curves, each with duplicate samples, and done in separate experiments. As it can be seen from Figure 3.3-15, a linear trendline with an R^2 value very close to one was obtained, indicating a good standard curve.

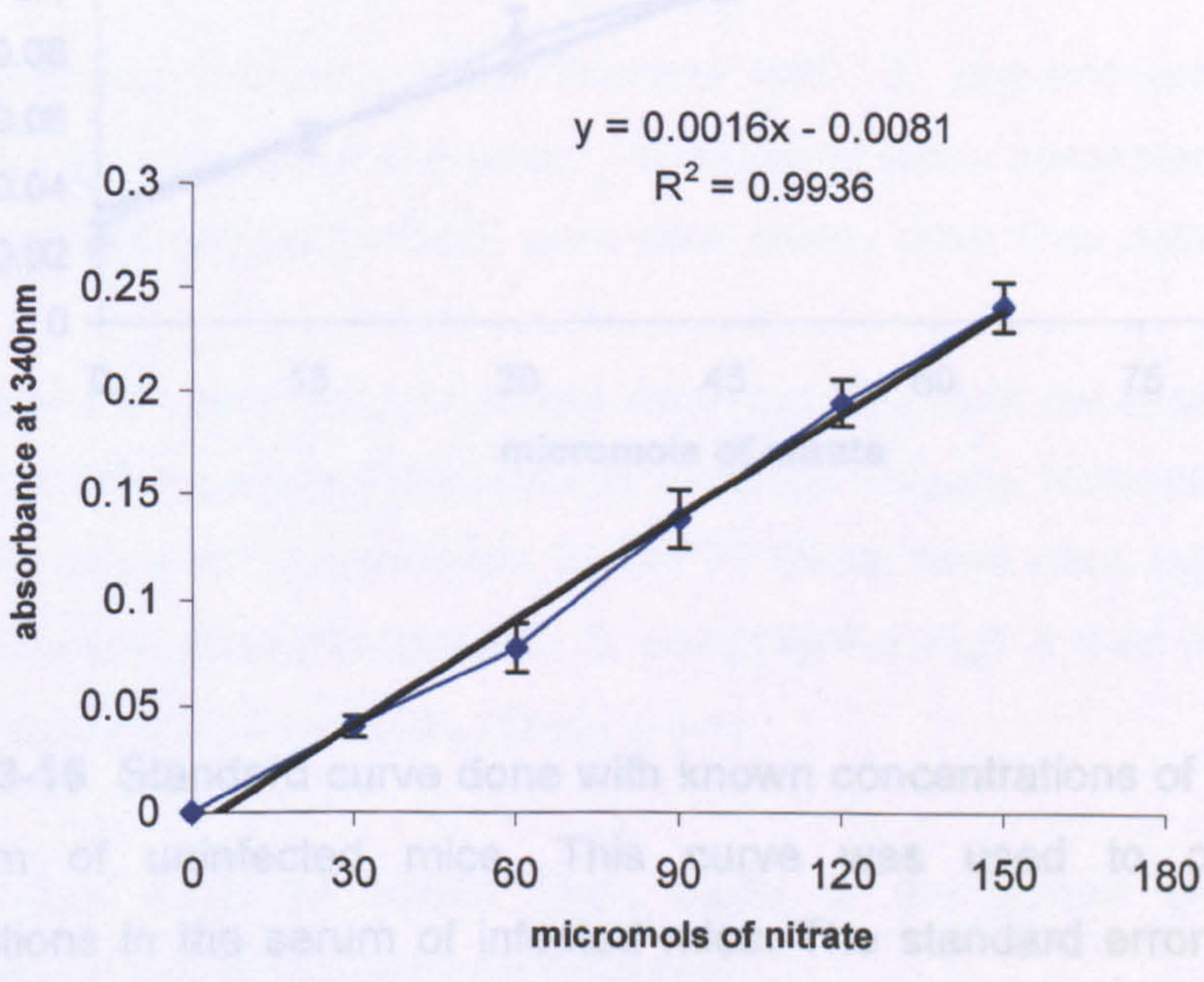


Figure 3.3-15 Standard curve of nitrate in HBSS used to calculate nitrate concentrations in lung lavages. The standard error of the mean is represented by a bar on each datum point. The trendline (shown in bold), its equation and the R^2 value are also shown. N= 4 experiments with 2 duplicates each.

For the estimation of nitrate concentrations in serum, the standard curve was made with known amounts of nitrate added to serum of uninfected mice. In Figure 3.3-16, the standard curve is shown. This standard curve is the average of five curves done in separate experiments, each with samples in duplicate. As it can be seen from the Figure, a good standard curve with a linear trendline and an R^2 value close to one was obtained. The equation of this trendline (also shown in the Figure) was used to determine nitrate concentrations from the serum of infected mice.

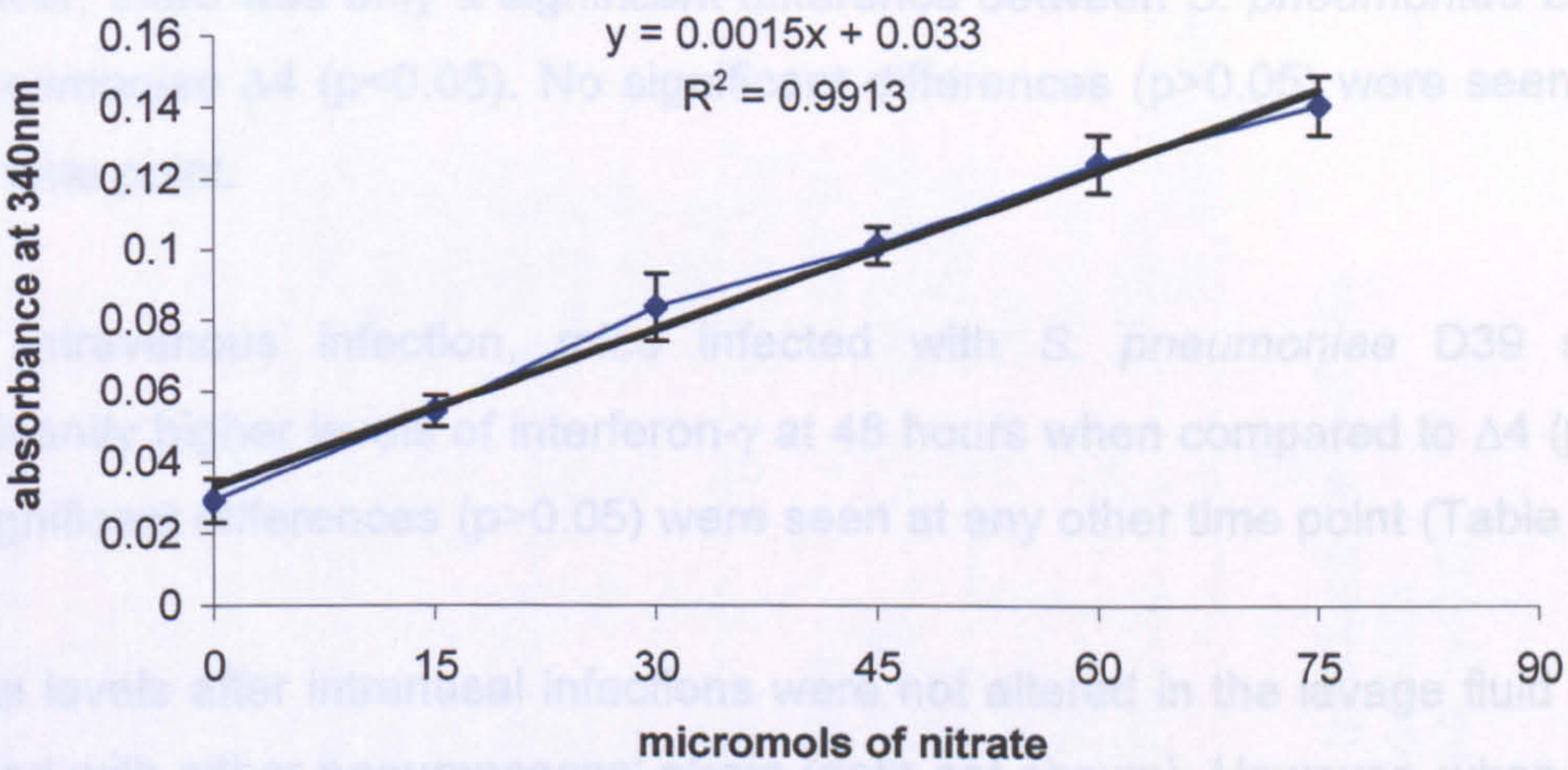


Figure 3.3-16 Standard curve done with known concentrations of nitrate added to the serum of uninfected mice. This curve was used to calculate nitrate concentrations in the serum of infected mice. The standard error of the mean is represented with a bar on each data point. The trendline (shown in bold), its equation and the R^2 value are also shown.

In Table 3.3-1 the levels of interferon-γ and nitric oxide in serum and lung lavages after intranasal infections, and in serum after intravenous infections, are shown. Because of the way in which the experiment and the standard curves were done, these values should be considered as a reference, rather than as absolute values.

As shown in Table 3.3-1, it was found that at 24 hours after intranasal infection, there were significantly higher levels of interferon-γ in the lavage fluid of mice infected with *S. pneumoniae* D39 or Δ4, compared to the lavage fluid of mice infected with PLN-A ($p<0.05$) but no differences at other time points were found ($p>0.05$). Also, by 24 hours when serum concentrations of interferon-γ were measured after intranasal infections, mice given *S. pneumoniae* D39 and PLN-A appeared to have higher levels of interferon-γ than those given *S. pneumoniae* Δ4. However, there was only a significant difference between *S. pneumoniae* D39 and *S. pneumoniae* Δ4 ($p<0.05$). No significant differences ($p>0.05$) were seen at any other time point.

After intravenous infection, mice infected with *S. pneumoniae* D39 showed significantly higher levels of interferon-γ at 48 hours when compared to Δ4 ($p<0.05$). No significant differences ($p>0.05$) were seen at any other time point (Table 3.3-1).

Nitrate levels after intranasal infections were not altered in the lavage fluid of mice infected with either pneumococcal strain (data not shown). However, when looking at the levels on serum, it was found that by 72 hours there were significantly higher levels of nitrate in mice infected with *S. pneumoniae* PLN-A than in those infected with *S. pneumoniae* Δ4 ($p<0.05$) (Table 3.3-1).

No detectable levels of nitrate were found in the serum of mice infected intravenously at any time point after infection.

According to these results, the induction of interferon-γ does not seem independent on the haemolytic activity of pneumolysin, nor do the levels of nitrate seem to be related to interferon-γ concentrations.

Table 3.3-1 Interferon-g and nitrate levels from lung lavage and/or serum after intranasal and intravenous infections. N=5 at all points.

		Time post-infection			
Intranasal infections		**6 hours**	**24 hours**	**48 hours**	**72 hours**
IFN-γ lavage (log pg/ml)	Wild type	2.9±0.1	3.1±0.0	3±0.2	not done
	Δ4	2.9±0.1	3.1±0.1	3.1±0.1	3±0.3
	PLN-A	2.8±0.1	2.1±0.1[A,B]	2.8±0.1	2.1±0.0
IFN-γ serum (log pg/ml)	Wild type	0.6±0.5	1.9±0.3[B]	2.8±0.3	not done
	Δ4	0.8±0.4	0.3±0.3[A]	2.1±0.1	2.6±0.2
	PLN-A	0.3±0.3	1.6±0.5	2.6±0.1	2.6±0.1
Nitrate serum (μM)	Wild type	3.8±1.1	26.5±13.6	78.5±44.3	not done
	Δ4	12.6±5.8	10.6±3.6	30.1±13.8	38.8±10.9[C]
	PLN-A	54.2±25.8	26.3±10.3	58.4±28	233.6±68.4[B]
Intravenous infections		**0 hours**	**24 hours**	**48 hours**	
IFN-γ serum (log pg/ml)	Wild type	0.7±0.7	2±0.4	3±0.3[B]	
	Δ4	1.1±0.6	1.2±0.4	1.7±0.4	
	PLN-A	0.8±0.8	1.8±0.4	2±0.1	
Nitrate serum	No detectable				

[A] Data significantly different to infections with wild type bacteria ($p<0.05$)
[B] Data significantly different to infections with D4 ($p<0.05$)
[C] Data significantly different to infections with PLN-A ($p<0.05$)

Chapter 4

Studies on Pneumolysin

Discussion, Conclusions and Further Work

4.1 To recapitulate

The pneumolysin toxin of *S. pneumoniae* is known to contribute to virulence by two different mechanisms, its cytolytic activity, which can cause lysis of the host cells and at sublytic concentrations can damage cells of the immune system; and by its ability to activate the complement pathway. Mutagenesis studies have shown that different regions of the toxin are responsible for maintaining each of these activities (Alexander *et al.*, 1998) and *in vivo* studies have shown that each, the cytolytic and complement activation activities of the toxin contribute to virulence in different ways (Alexander *et al.*, 1998; Berry *et al.*, 1995; Benton *et al.*, 1997; Jounblat *et al.*, 2003). However, *in vivo* studies where a pneumococcal mutant lacking both the cytolytic and complement activation activities of its pneumolysin toxin was used, showed that the pneumolysin protein contributes to virulence by a still unidentified function (Alexander *et al.*, 1998; Benton *et al.*, 1997; Berry *et al.*, 1999). Baba and collaborators (2002) showed with the construction and purification of pneumolysin truncates, that *in vitro* pneumolysin was capable of inducing the production of interferon-γ and nitric oxide from spleen cells in an independent way to the protein's anticellular activity. They also found that the nitric oxide produced was dependent on the production of interferon-γ. Their results lead the authors to hypothesise that during *in vivo* infections pneumolysin could induce the production of interferon-γ and nitric oxide (Baba *et al.*, 2002). Because high levels of nitric oxide can produce organ failure and toxic shock in the infecting host, this could contribute to the pathogenesis of pneumococcal infections and be the unknown function of pneumolysin.

The aim of this project was to test if the ideas of Baba and collaborators (2002) could explain, *in vivo*, the unidentified function of pneumolysin observed by Alexander and collaborators (1998). A pneumococcal mutant (*S. pneumoniae* Δ4) that produced a pneumolysin protein lacking 34 amino acids from its C-terminus, that is, one amino acid longer than a truncation used by Baba *et al.* (2002) was constructed for this purpose. Virulence studies were done by intranasal and intravenous infection of mice and the levels of interferon-γ and nitrate in serum and lung lavages where appropriate were determined to test the hypothesis. All the

experiments were done with multiple replicates (see Chapter 3 for number of replicates and independent experiments) comparing the results from *S. pneumoniae* Δ4 to those of the wild type *S. pneumoniae* D39 and of the pneumolysin-negative pneumococcal mutant *S. pneumoniae* PLN-A. The results obtained in this project were highly reproducible as assessed by the number of experiments and/or of replicates done.

4.2 Observations from the project

4.2.1 Could the mutation of *S. pneumoniae* Δ4 have caused down-stream effects?

When constructing bacterial mutants by insertion deletion, that is, by the insertion of a foreign DNA sequence to interrupt a gene, there is always the possibility of affecting down-stream genes. In this project, that possibility was considered highly unlikely for the following reasons.

First, a similar mutation strategy on the pneumolysin gene of the pneumococcus had been used previously, for example, with the construction of *S. pneumoniae* PLN-A where the pneumolysin gene was interrupted by the insertion of an erythromycin resistance cassette (Berry *et al.*, 1989). This mutant (*S. pneumoniae* PLN-A) was shown to have the same *in vitro* growth than its wild type parent (Berry *et al.*, 1989). Also, although the mutant showed to be significantly less virulent *in vivo* than its wild type parent, its reduced virulence could be restored by co-infection with wild type pneumococci (Benton *et al.*, 1995), suggesting that the reduce virulence was due to the lack of a virulence factor rather than to a growth deficiency that polar effects of the mutation could have caused. In the study done in this thesis, the growth of the mutant *S. pneumoniae* Δ4, *S. pneumoniae* PLN-A and of their wild type parent *S. pneumoniae* D39 was assessed and no differences were found between their *in vitro* growths.

Another reason to expect that the truncation performed in the pneumolysin gene did not affect any downstream genes is the fact that the pneumolysin gene is not contained in an operon (see Figure 4.2-1). Also the gene immediately downstream the pneumolysin gene is >800bp away, and indeed, according to the software TransTermHP (a transcriptional terminator predictions software from The Institute of Genome Research), a terminator is present between the pneumolysin gene and the gene immediately downstream (see Figure 4.2-1).

Because of this reasons, the possibility that the mutation in *S. pneumoniae* Δ4 could have caused any downstream effects that could affect the results observed in this project was considered highly unlikely.

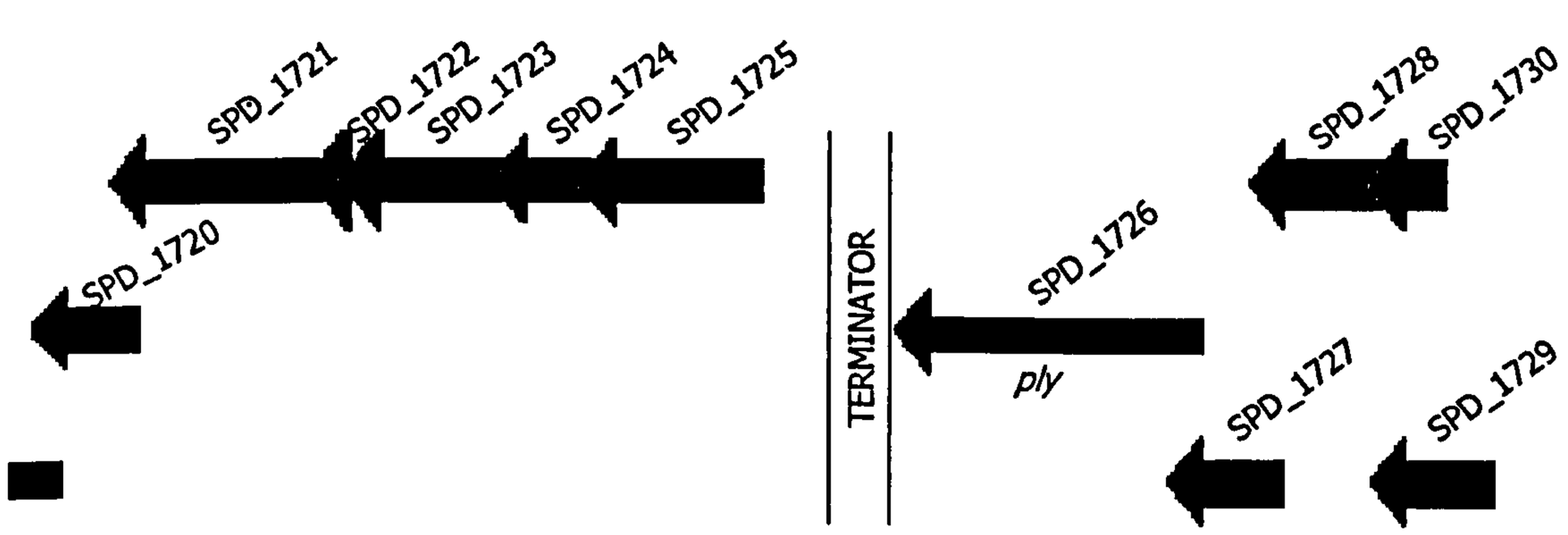


Figure 4.2-1. Region in the chromosome of *S. pneumoniae* D39 where the pneumolysin gene (*ply*) is present. A terminator was predicted between the *ply* gene and the downstream gene (SPD_1725) (see text for more information). The label of the genes shown is that of *S. pneumoniae* D39.

4.2.2 Production of interferon-γ by the infecting host was independent to pneumolysin

In the project, the levels of interferon-γ produced during pneumococcal disease in mice did not appear related to the cytolytic activity of pneumolysin. In fact, it did not appear related to the presence of the pneumolysin protein. In these studies, except for one time point, the levels of interferon-γ induced by the pneumolysin negative mutant PLN-A were not significantly different to those induced by *S. pneumoniae* D39 or by *S. pneumoniae* Δ4. Also, the levels of interferon-γ induced by *S. pneumoniae* Δ4 were not higher than those induced by the wild type bacteria, showing that in the *in vivo* model used, the cytolytic activity of the protein had no effect over the induction of interferon-γ. Baba and collaborators had cited the work of Benton *et al.*, 1995 to support their hypothesis. In that study, it had been reported by the use of *in vivo* experiments that PLN-A did not induce the production of interferon-γ and that detectable levels of interferon-γ were only found in mice infected with wild type pneumococcus just before death (Benton *et al.*, 1995). The reason for the discrepancy between the results of this project and those of Benton *et al.* (1995) is not clear. However, a difference could be that the mice used in the experiments of Benton *et al.* (1995) were immunodeficient mice unable to produce antibodies to polysaccharides. It is likely that the response of these mice to bacterial infections is different to the MF1 outbred mice used in this study. In their experiments, they also mentioned that interferon-γ levels were only detected immediately before death of the mouse (Benton *et al.*, 1995). In the experiments performed in this project, strict ethical regulations were applied and mice were not allowed to reach a moribund state, so it was not possible to assess if the interferon-γ levels would have increased significantly at that stage of disease. The studies of another group reported detectable levels of interferon-γ after intranasal infections with PLN-A (Kerr *et al.*, 2005) however, they found that *S. pneumoniae* wild type induced higher interferon-γ levels than a pneumolysin negative mutant. In their studies, they used BALB/c mice, which are more resistant to pneumococcal infection than the MF1 outbred mice used in this study.

Other bacterial factors could have contributed to the interferon-γ induction observed. For example, Hessle *et al.* (2000) in their experiments with seven different species of Gram-positive bacteria showed that they were able to induce the production of interferon-γ from mononuclear blood cells via the induction of IL-12 at significantly higher rates that Gram-negative bacteria. They concluded that the cell wall of Gram-positive bacteria could have a role on this but with the experiments they performed they were unable to prove this hypothesis (Hessle *et al.*, 2000).

4.2.3 Nitric oxide produced does not appear related to the levels of interferon-γ, was independent to the presence of pneumolysin and was not indicative of severe disease

For clarity in the discussion, a reminder of the findings at specific time points of the intranasal infections will now be made. At 72 hours post-infection, mice infected with PLN-A had significantly higher levels of nitrate in serum than mice infected with Δ4, but their levels of interferon-γ in serum and in the lung lavage fluid were not significantly different. At this time point, the bacteria levels of the infected mice were not significantly different, however, mice infected with PLN-A had on average more than one log/mg tissue of bacteria in the lungs and more than two logs/ml in the blood compared to the mean of mice infected with Δ4. At 48 hours post infection, most of the mice infected with D39 were severely ill, but their levels of interferon-γ and nitric oxide were not significantly different to those of mice infected with Δ4 and PLN-A at the same time point. In addition, after intravenous infections, no nitrate levels were detected even if interferon-γ levels were similar to those observed after intranasal infections.

From these results, three observations can be drawn. First, nitric oxide levels were not positively related to the production of interferon-γ as measured by the concentration of nitrate in the samples. This does not necessarily mean that interferon-γ was not required for the production of nitric oxide, but perhaps that

higher or lower levels of interferon-γ might not affect the production of nitric oxide as long as some is present. Also, in the case of intravenous infections interferon-γ did not seem to stimulate the production of nitric oxide, as no detectable levels of nitrate were found in the serum of infected mice.

Second, the levels of nitrate were not related to the presence of pneumolysin in the pneumococcus as observed by the significantly high levels of nitrate from serum samples of mice infected with PLN-A. It has previously been reported that pneumococcal mutants that lacked the production of pneumolysin did not induce the production of nitric oxide from macrophages (Braun *et al.*, 1999). However, other studies have shown that pneumococcal cell wall on its own is capable of inducing the production of nitric oxide in macrophages (Orman *et al.*, 1998) and in astrocytes and microglia (Kim and Tauber, 1996).

The final observation was that the levels of nitrate found in the sample were not necessarily an indicative on the degree of disease caused by *S. pneumoniae*. In the studies of Baba and collaborators (2002) it was proposed that high levels of nitric oxide could provoke shock and death of the infecting host. Indeed, it is well known that high levels of nitric oxide can contribute to multiple organ failure, and that in some situations the production of nitric oxide by the host defence system appears rather detrimental (Kerr *et al.*, 2004). However, in this project, mice that were severely ill after 48 hours of intranasal infection with *S. pneumoniae* D39 did not have significantly higher levels of nitrate in their serum compared to mice infected with D39 or PLN-A, which at this time post infection were not showing signs of disease. In contrast, the high levels of nitric oxide observed after 72 hours of infection with PLN-A bacteria came from mice that only showed mild signs of disease.

4.2.4 *S. pneumoniae* Δ4 is less virulent than *S. pneumoniae* PLN-A

It was surprising to observe that *S. pneumoniae* Δ4 was less virulent in mice than *S. pneumoniae* PLN-A as judged by mortality rates and signs of disease after infections. Also surprising was the fact that although less virulent, *S. pneumoniae* Δ4 caused more lung damage after intranasal infections than PLN-A. The reason for this has not yet been studied. Baba and collaborators (2001) reported that the truncated pneumolysin was unable to bind to cell membranes. It is tempting to speculate that the presence of most of the pneumolysin protein in *S. pneumoniae* Δ4 in the absence of its cytolytic and binding activity might produce a better immune response without the overwhelming consequences of the cytolytic activity of the protein.

Pneumolysin has shown to be immunogenic and to confer certain degree of protection to pneumococcal disease in mice (Paton *et al.*, 1983), and indeed has a huge potential for vaccine development. It is possible, that the lack of pneumolysin in *S. pneumoniae* PLN-A caused a slow immune response to the infection compared to *S. pneumoniae* Δ4. This could be observed with the delayed cellular infiltrate observed in the lungs of mice infected with PLN-A compared to those infected with Δ4 or with the wild type *S. pneumoniae* D39. In fact, previous studies have also shown that the lungs of mice infected intranasally with *S. pneumoniae* PLN-A show less levels of inflammation than mice infected with wild pneumococcus (Kadioglu *et al.*, 2000). Interestingly, it has also been found that mice infected with wild type pneumococci have a greater infiltration of T-cell into the lungs than mice infected with PLN-A (Kadioglu *et al.*, 2000). Recent studies have suggested that pneumolysin could be responsible for this T-cell migration into the lungs, as it was shown that *in vitro*, pneumococcal cells containing pneumolysin could induce CD4-Tcells migration, but pneumococcal cells lacking pneumolysin could not (Kadioglu *et al.*, 2004). All this previous observations on T-cells could be an explanation for the reduced virulence of *S. pneumoniae* Δ4 compared to *S. pneumoniae* PLN-A, supporting the previously mentioned hypothesis that *S. pneumoniae* Δ4 could be inducing a better immune response

than *S. pneumoniae* PLN-A without the detrimental effects of wild type pneumococci.

Theoretically, the pneumolysin protein of *S. pneumoniae* Δ4 still contains complement activation activity, as the residues 368-397 which have been related to this activity of pneumolysin (Mitchell *et al.*, 1991) are still present. It is also possible that this activity could be responsible for the difference in cellular infiltration and inflammation of the lung tissue observed.

Because of the huge potential of pneumolysin as a pneumococcal vaccine candidate, it would be well worth to further investigate this finding.

4.3 Conclusions and further work

The experiments performed in this project did not support the hypothesis proposed by Baba and collaborators (2002). According to this project, the unknown factor that contributes to the virulence of pneumolysin *in vivo*, is not related to the production of interferon-γ and nitric oxide, whose production appeared independent to the presence of pneumolysin. The discrepancies between the findings of Baba and collaborators and the findings of this project could be due to the huge differences between *in vitro* and *in vivo* models. This could be assess by challenging spleen cells with crude protein extracts of *S. pneumoniae* Δ4, using as controls *S. pneumoniae* D39 and *S. pneumoniae* PLN-A. The levels of interferon-γ and nitric oxide produced by the cells could then be measured, expecting that the results obtained would be similar to those observed by Baba and collaborators (2002).

This project was not designed to understand how or why interferon-γ and nitric oxide are produced during pneumococcal infections. As a consequence, questions that arose regarding these issues remained unanswered.

A surprising and interesting finding in the project was the fact that *S. pneumoniae* Δ4 was less virulent than *S. pneumoniae* PLN-A, but still, caused more lung tissue damage. Because the pneumolysin protein of *S. pneumoniae* Δ4 still has the region responsible for the complement activation activity of the protein, it could be assessed if this could be responsible for the difference in tissue damage observed. For this, antibodies against a protein from complement system could be used to assess complement deposition over the infected tissue. Because of the potential of pneumolysin as a vaccine candidate, it would be interesting to perform further experiments to assess if the pneumolysin protein from *S. pneumoniae* Δ4 could have a role inducing a better immune response in the infected host. For example, it could be analysed at different time points post-infection if cells from the immune system such as T-cells or B-cells are higher in mice infected with Δ4 than in mice infected with PLN-A. Also, it could be assessed if mice infected with *S. pneumoniae* Δ4 produce antibodies against pneumolysin protein. It could also be very interesting to test if a recombinant pneumolysin protein with a truncation as that contained in *S. pneumoniae* Δ4 could immunise mice against pneumococcal infections. In fact, during this project, the pneumolysin DNA encoding for the first 438 amino acids of the pneumolysin protein was successfully inserted into pRSET-A. However, due to time restrictions it was not possible to continue with these experiments.

Chapter 5

Pneumococcal Gene Expression under Aerobiosis

Results

As mentioned in the introduction of this thesis, *S. pneumoniae* is an aerotolerant anaerobe that continues to ferment in the presence of oxygen. Interestingly, although the pneumococcus is a bacterium that is constantly challenged by aerobic environments, it lacks the regulatory genes that are known in other bacteria to regulate their adaptation to oxygen environments and the consequent oxidative stress, such as *oxy*R and *sox*RS in *E. coli* or *per*R in *B. subtilis* and *S. aureus* (Pericone *et al.*, 2003). Also, the pneumococcus does not produce catalase, an important enzyme used by many bacteria in their adaptation to oxidative stress (Fridovich, 1998). Other genes present in the pneumococcus, such as *gro*EL and *dna*K, are known to be used by *E. coli* in the response to oxidative stress (Farr and Kogoma, 1991), however, there is no evidence that the pneumococcus utilises these genes as a response to oxygen. All this indicates that the pneumococcus possess a different mechanisms of adaptation to oxidative stress than that known in other microorganisms. However, these pneumococcal mechanisms are not yet known.

The aim of this project was to study the genetic response of the pneumococcus when it is subject to aerobic conditions. This was done by comparing with the use of microarrays the gene expression of bacteria grown under aerobic or anaerobic conditions as explained in 2.2.1.2 of the materials and methods section. In order to obtain the information on the genetic response when the bacteria were metabolically active, and before a starvation response was produced, it was decided to extract the RNA when the bacteria reached mid-exponential phase in either condition. Also, in an attempt to create an aerobic environment similar to that that *S. pneumoniae* might experience during colonisation, chemicals such as paraquat or H_2O_2 were not used. Instead, the culture medium was kept under aerobic conditions using a flow of filtered environmental air (explained in 2.2.1.2). With the settings used in this project, it was expected that the genes the pneumococcus need for its survival in an aerobic condition such as that of the nasopharynx, would be observed, allowing further understanding on the pneumococcal survival of oxidative stress.

The expression results obtained by microarray of some of the genes was confirmed by quantitative RT-PCR and gene *rgg* was selected to produce a pneumococcal

knockout mutant, expecting to evaluate its importance in the pneumococcal adaptation to oxidative stress.

The microarray slides used for these experiments (SPv1) were obtained from the Bacterial Microarray Group at St. Georges Hospital, in London. The slides contained the spoted PCR products of 2236 target genes from the sequenced genome of *S. pneumoniae* TIGR4, plus the PCR products of 117 genes that were not found in TIGR4 but were found in *S. pneumoniae* R6 (Bacterial Microarray Group at St. George's). Because *S. pneumoniae* R6 is an un-encapsulated non-virulent strain similar to *S. pneumoniae* D39 (a virulent capsulated bacterium), it was decided to use *S. pneumoniae* R6 for the experiments.

5.1 Establishment of aerobic and anaerobic conditions

The first stage was to establish aerobic and anaerobic conditions in which to grow the bacteria while minimising other stresses. For example, it was decided not to shake the cultures to produce an aerobic environment because of shear stress. Also, as it had been decided to extract RNA from the bacterial culture when it reached mid-exponential phase (OD_{500} of ~600), it was necessary that at this stage of growth, the percentage of dissolved oxygen was higher in the medium of the aerobic condition compared to the anaerobic one. In these experiments, the percentage of dissolved oxygen was measured using an oxygen electrode, where 100% of dissolved oxygen saturation was obtained when the medium was fully saturated with air, therefore containing 20% (v/v) oxygen. To measure 0% of dissolved oxygen, the oxygen electrode was calibrated using water or medium into which oxygen-free nitrogen was bubbled for at least one hour to create anaerobiosis.

The first attempt to obtain these characteristics was to grow the bacteria in static culture. For this, a filter caped 25cm tissue culture flask containing 5ml of culture was placed under anaerobiosis as explained in section 2.2.1.2 and growth followed. For the aerobic growth, the same set-up was used, but the flasks were not placed in an anaerobic jar. The optical density and the concentration of dissolved oxygen as measured with an oxygen electrode, were assessed through the growth. As it can be seen in Figure 5.1-1A, aerobic growth commenced with 100% of dissolved oxygen saturation, but by the time it reach mid exponential phase, between six and seven hours (OD_{500} of ~0.6) it had dropped to only ~18% of dissolved oxygen saturation. Under "anaerobic" growth, the concentration of dissolved oxygen commenced at 20% of saturation and similarly to the aerobic growth, when the culture reached mid exponential phase, at ~6 hours, the concentration was of ~16% of dissolved oxygen saturation. In these conditions it was also apparent that bacteria grown under anaerobiosis had a faster generation time than bacteria grown under aerobiosis, however, this was not further studied.

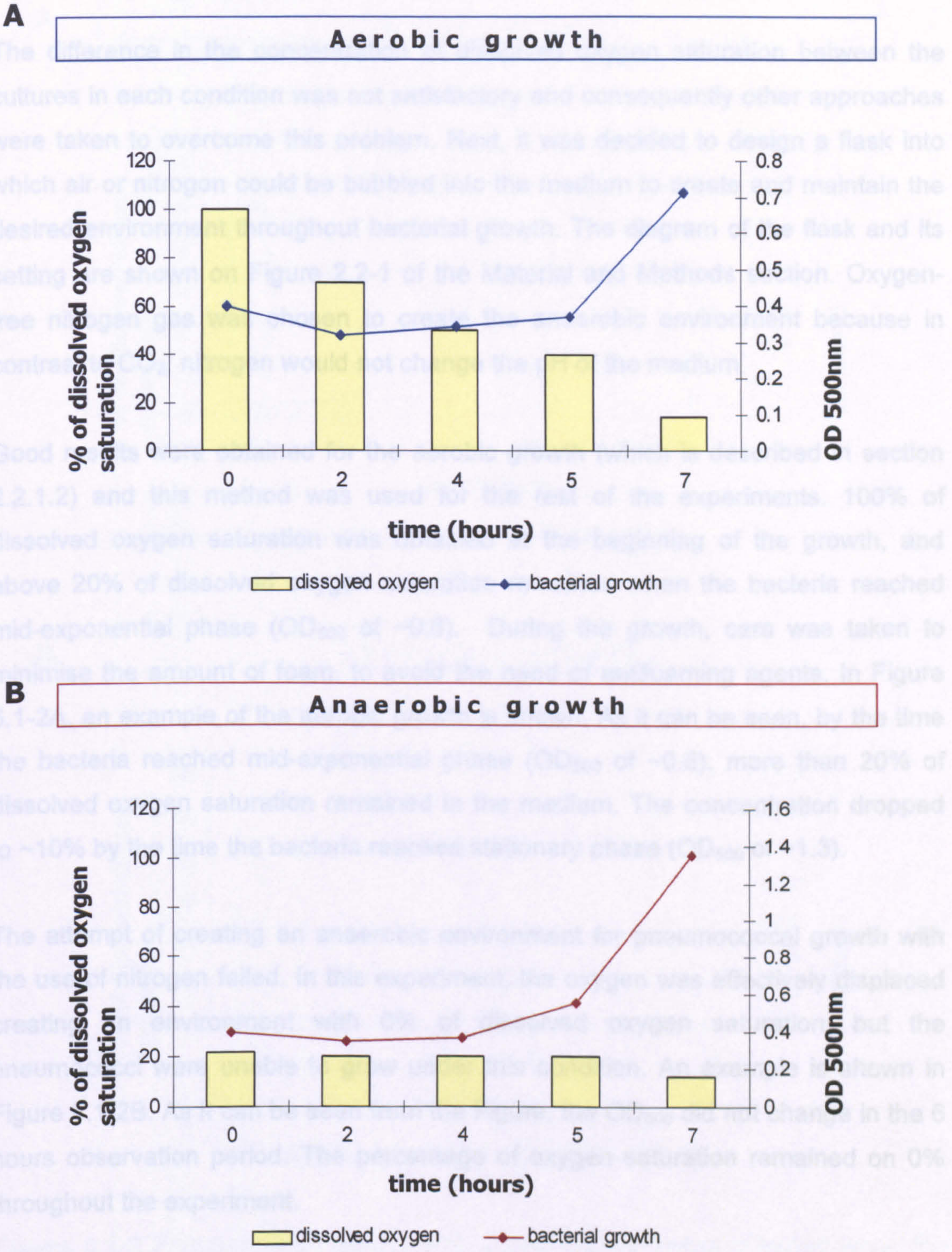


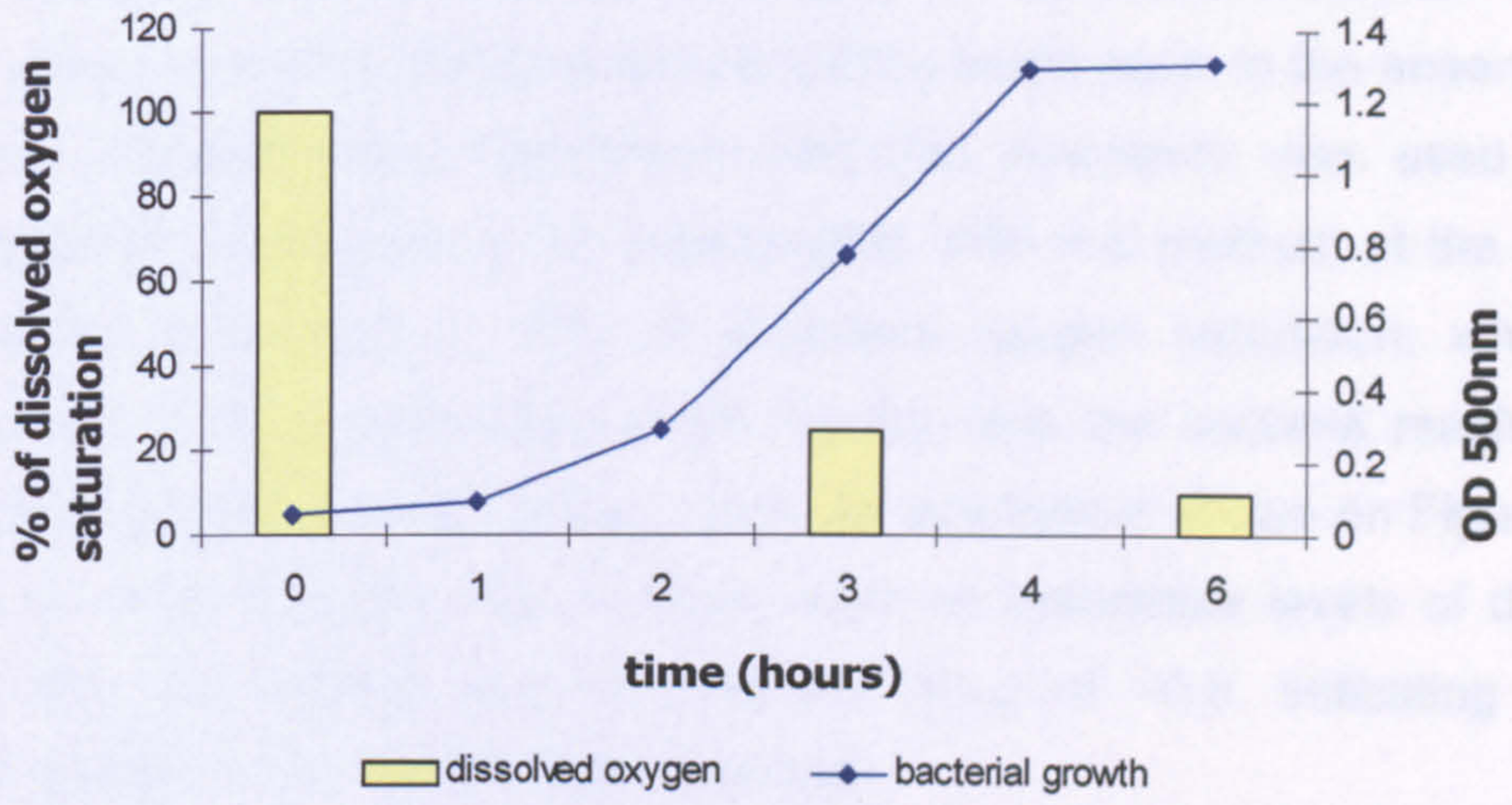
Figure 5.1-1 Pneumococcal growth and oxygen concentration obtained when the bacteria were grown on cell culture flasks with filter cap expecting to obtain an aerobic environment (A), and on cell culture flasks with filter cap in anaerobic jars expecting to obtain an anaerobic environment (B). For each point n=2.

The difference in the concentration of dissolved oxygen saturation between the cultures in each condition was not satisfactory and consequently other approaches were taken to overcome this problem. Next, it was decided to design a flask into which air or nitrogen could be bubbled into the medium to create and maintain the desired environment throughout bacterial growth. The diagram of the flask and its setting are shown on Figure 2.2-1 of the Material and Methods section. Oxygen-free nitrogen gas was chosen to create the anaerobic environment because in contrast to CO_2, nitrogen would not change the pH of the medium.

Good results were obtained for the aerobic growth (which is described in section 2.2.1.2) and this method was used for the rest of the experiments. 100% of dissolved oxygen saturation was obtained at the beginning of the growth, and above 20% of dissolved oxygen saturation remained when the bacteria reached mid-exponential phase (OD_{500} of ~0.6). During the growth, care was taken to minimise the amount of foam, to avoid the need of antifoaming agents. In Figure 5.1-2A, an example of the aerobic growth is shown. As it can be seen, by the time the bacteria reached mid-exponential phase (OD_{500} of ~0.6), more than 20% of dissolved oxygen saturation remained in the medium. The concentration dropped to ~10% by the time the bacteria reached stationary phase (OD_{500} of ~1.3).

The attempt of creating an anaerobic environment for pneumococcal growth with the use of nitrogen failed. In this experiment, the oxygen was effectively displaced creating an environment with 0% of dissolved oxygen saturation, but the pneumococci were unable to grow under this condition. An example is shown in Figure 5.1-2B. As it can be seen from the Figure, the OD_{500} did not change in the 6 hours observation period. The percentage of oxygen saturation remained on 0% throughout the experiment.

A **Aerobic growth**



B **Anaerobic growth**

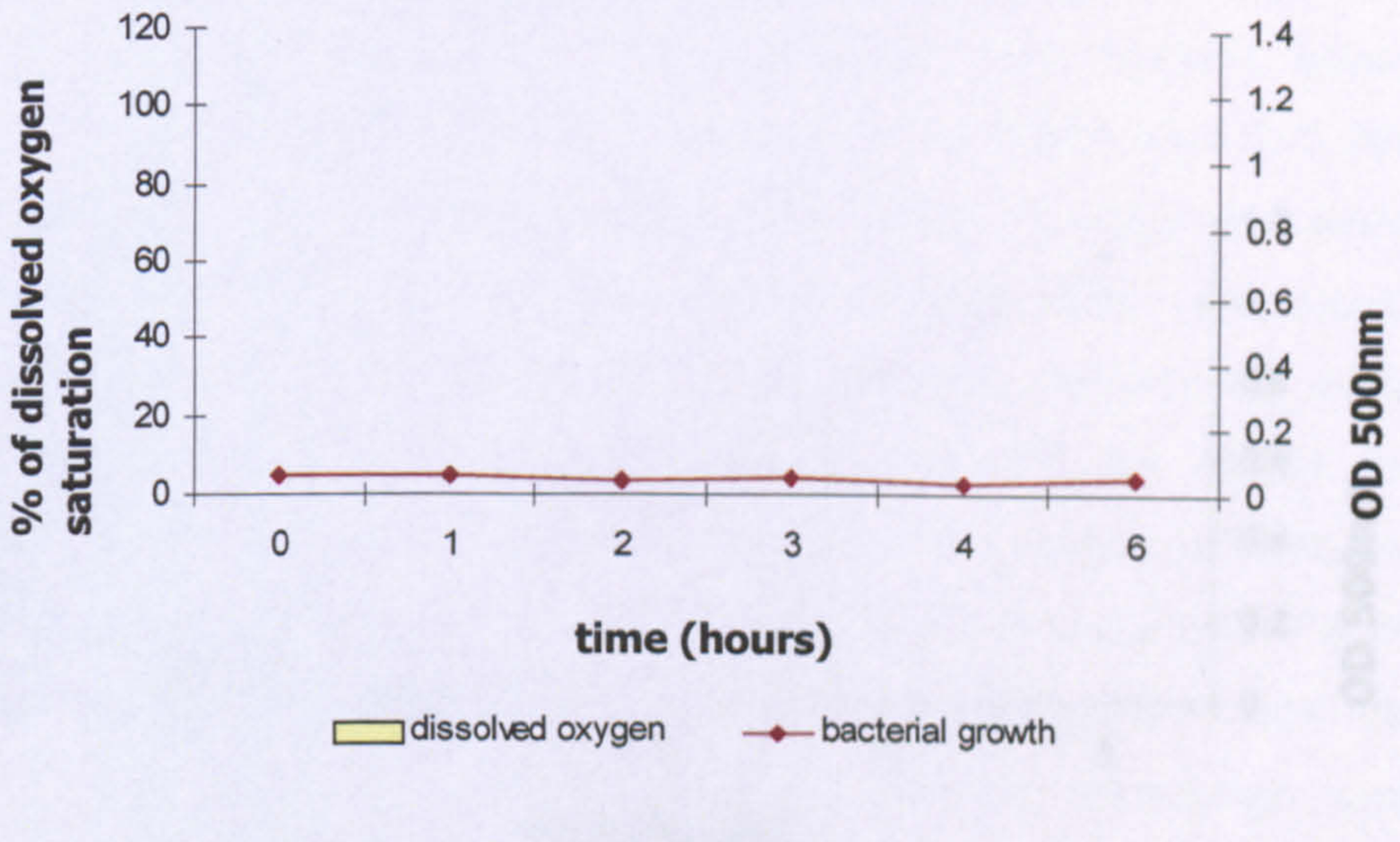


Figure 5.1-2 Pneumococcal growth and oxygen concentration obtained when the bacteria were grown in a flask where air was being bubbled expecting to obtain an aerobic environment (A); and where nitrogen was being bubbled expecting to obtain an anaerobic environment (B). The graph shown as graph A is representative of two experiments, and the graph shown as graph B is representative of three experiments.

As an alternative approach to the anaerobic growth, it was decided to use universal tubes filled with culture medium. The cap of the tubes was loosely fitted and the tubes were placed in anaerobic jars overnight, as described in 2.2.1.2. The next day, they were inoculated with pneumococci and placed again in the anaerobic jars. The results obtained were satisfactory and this procedure was used for the anaerobic growth in the rest of the experiments. With this method, at the moment of inoculation, there was a 10% of dissolved oxygen saturation, which had decreased to 2% by 2 hours and to 0% by the time the bacteria reached mid-exponential phase at ~4 hours (OD_{500} ~0.6). An example is shown on Figure 5.1-3. As it can be seen from the Figure, there were no detectable levels of dissolved oxygen when the bacteria had reached an OD_{500} of ~0.5, indicating that an anaerobic condition was successfully obtained.

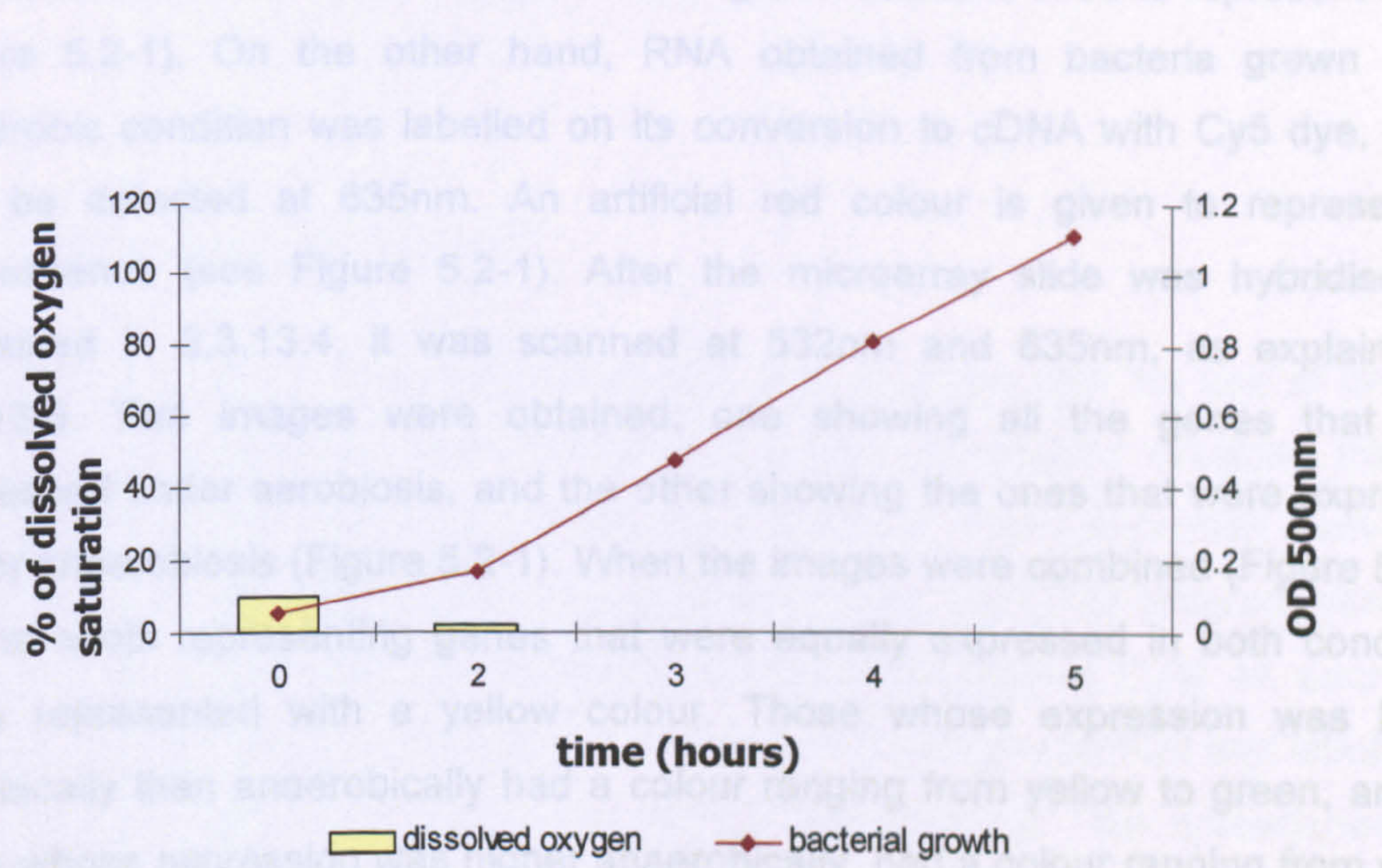


Figure 5.1-3 Pneumococcal growth and oxygen concentration obtained when the bacteria were grown in a universal tube and incubated in an anaerobic jar as described in section 2.2.1.2. The graph is representative of two experiments.

In Figure 5.1-2A and Figure 5.1-3, where satisfactory aerobic and anaerobic conditions were obtained, pneumococcus grown under aerobiosis showed a faster generation time (0.60 hours) than pneumococcus grown under anaerobiosis (0.95 hours). Although this was not further investigated, it was observed in experiments for the collection of samples for RNA extraction, that at approximately four hours after the initiation of growth, the aerobic and the anaerobic growth was in the exponential phase and had a similar optical density. The average OD_{500} at this time was 0.74 ± 0.05 and 0.74 ± 0.06 for the aerobic and anaerobic samples respectively (Data shown ± standard error of the mean. n=4).

5.2 Microarray experiments

Microarrays were performed as explained in 2.3.13. A total of 5 independent microarrays were performed. Total RNA obtained from bacteria grown under aerobic condition was labelled with Cy3 dye when converted to cDNA. Cy3 fluorescence can be read at 532nm and a green colour is used to represent it (see Figure 5.2-1). On the other hand, RNA obtained from bacteria grown under anaerobic condition was labelled on its conversion to cDNA with Cy5 dye, which can be detected at 635nm. An artificial red colour is given to represent its fluorescence (see Figure 5.2-1). After the microarray slide was hybridised as explained in 2.3.13.4, it was scanned at 532nm and 635nm, as explained in 2.3.13.6. Two images were obtained, one showing all the genes that were expressed under aerobiosis, and the other showing the ones that were expressed under anaerobiosis (Figure 5.2-1). When the images were combined (Figure 5.2-1), all the spots representing genes that were equally expressed in both conditions were represented with a yellow colour. Those whose expression was higher aerobically than anaerobically had a colour ranging from yellow to green, and the ones whose expression was higher anaerobically, had a colour ranging from yellow to red (Figure 5.2-1).

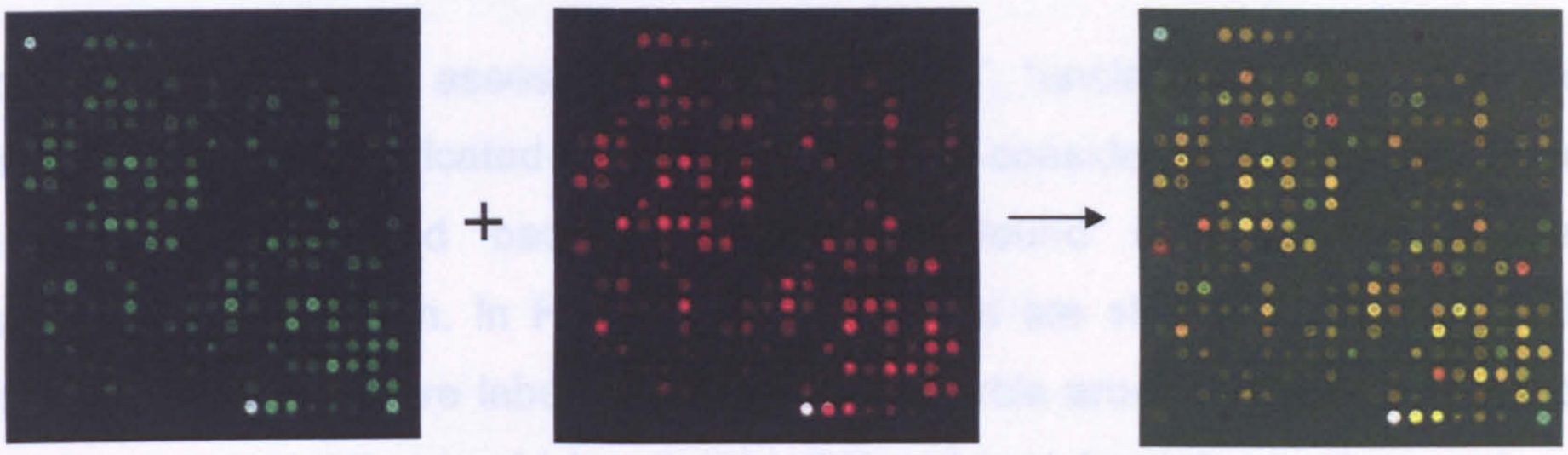


Figure 5.2-1 Microarray hybridization scan. Cy3 labelled cDNA is represented by green colour whereas Cy5 is shown as red. When the images are combined, yellow spots represent equal expression. Spots with a range of green or red colour represent over-expression in their corresponding condition.

5.2.1 Microarray data analysis

As mentioned in 2.3.13.6, each array was first analysed with the use of GenePix software. This software was initially loaded with information obtained from the Bacterial Microarray Group at St. George's Hospital. This information allowed the program to identify which gene corresponds to each spot. The program generates a spreadsheet indicating the intensity of each dye (Cy3 or Cy5) on each spot, background intensity, and the "flags", which is a visual assessment and labelling on the quality of each spot (described later in this section). This spreadsheet was loaded into GeneSpring software for further analysis and interpretation of the array. The spreadsheet generated by GenePix includes data for each spot in the array regardless the quality or the presence of hybridisation. For this reason, "flagging"

each spot was necessary, as this allowed the exclusion of spots that were considered un-hybridised or with artefacts.

Each spot was visually assessed as "good", "bad", "unclassified" or "not found", where "unclassified" indicated a spot that was not considered particularly "good", but was not considered "bad" either, and "not found" indicated the apparent absence of hybridisation. In Figure 5.2-2 examples are shown. As shown in the Figure, "good" spots were labelled with a double circle around the spot. This label was given to spots that could be clearly distinguished from the background, and that had an "average" size. "Bad" spots were labelled with an X on top of the spot. Spots that were affected by artefacts, such as lines of dye across the array, or that were very small or too big, were labelled as "bad" spots. The "unclassified" spots were those spots that could be distinguished from the background but were not especially good. As its name indicates, the "not found" label was used when there was no distinguishable spot at the place where it was expected. A line crossing the area of the spot was used to represent this label (Figure 5.2-2).

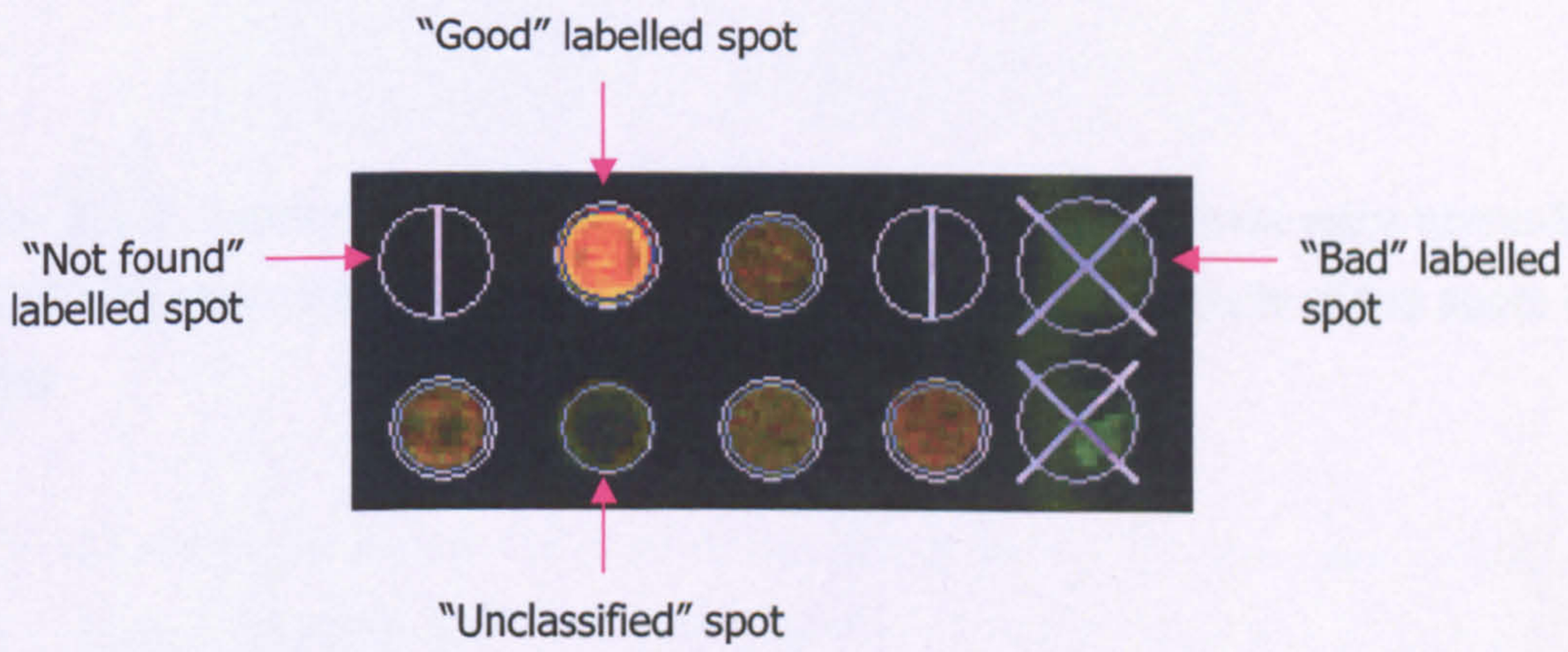


Figure 5.2-2 Example of the visual assessment of the quality of each spot present on the microarray slide after hybridization and scanning of the array.

The GenePix analysis of five replicate microarrays was loaded into GeneSpring. Because the aim of the experiment was to identify genes that are over-expressed under aerobiosis and select a gene for directed mutation, a strict selection of genes was done.

The data were first normalised using LOWESS normalisation. This normalisation method is commonly used for two colour experiments and corrects for dye incorporation artefacts (GeneSpring user manual). Also, a cut-off value of 10log for the intensity of fluorescence of the spot was used. This was done to eliminate weak spots with intensity close to the background. This selection excluded 138 genes of the 2,353 present in the array, leaving 2,215 genes in the experiment. Figure 5.2-3 shows the scatter plot of these genes. In the scatter plot, the "x" axis corresponds to the log intensity of fluorescence of the spots from the aerobic sample; and the "y" axis, to the ones of the anaerobic sample. The three parallel green lines indicate the difference of expression from one condition to the other. The middle line represents equal expression under both conditions, and the flanking lines, a two-fold difference from equal expression.

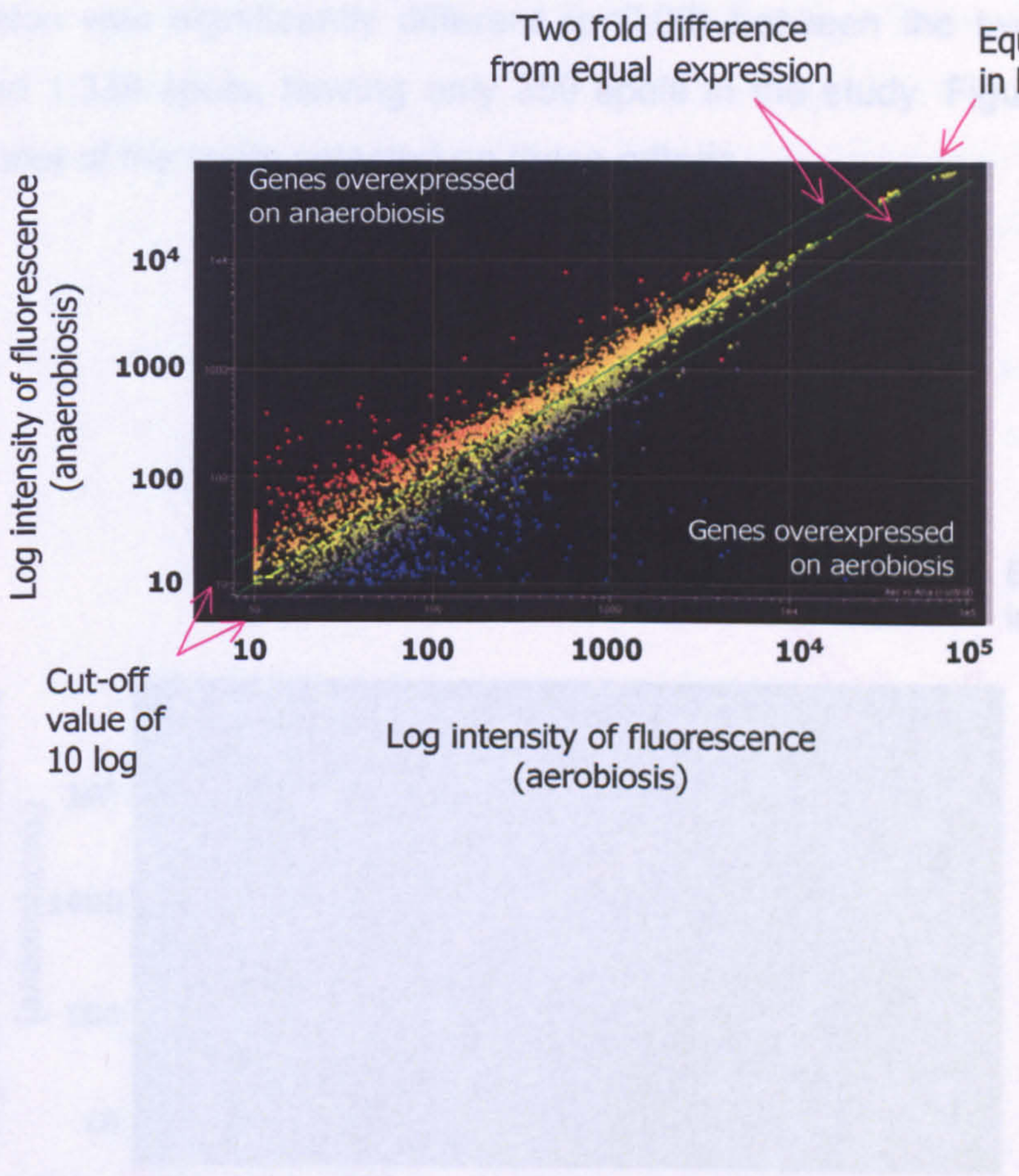


Figure 5.2-3 Scatter plot of the microarray experiments. The data were normalised by LOWESS normalisation. A cut-off value of 10log in the intensity of the spots was applied.

As mentioned before, assessment of the quality of the spots was done with the use of the GenePix program. Therefore, the next selection excluded genes corresponding to spots that were labelled as bad or not found in at least two out of the five microarray repetitions. This selection excluded 517 genes, leaving a total of 1698 genes in the study. Next, a selection was done only for the genes whose expression was significantly different ($p<0.05$) between the two conditions. This excluded 1,339 spots, leaving only 359 spots in the study. Figure 5.2-4, shows a scatter plot of the spots selected on these criteria.

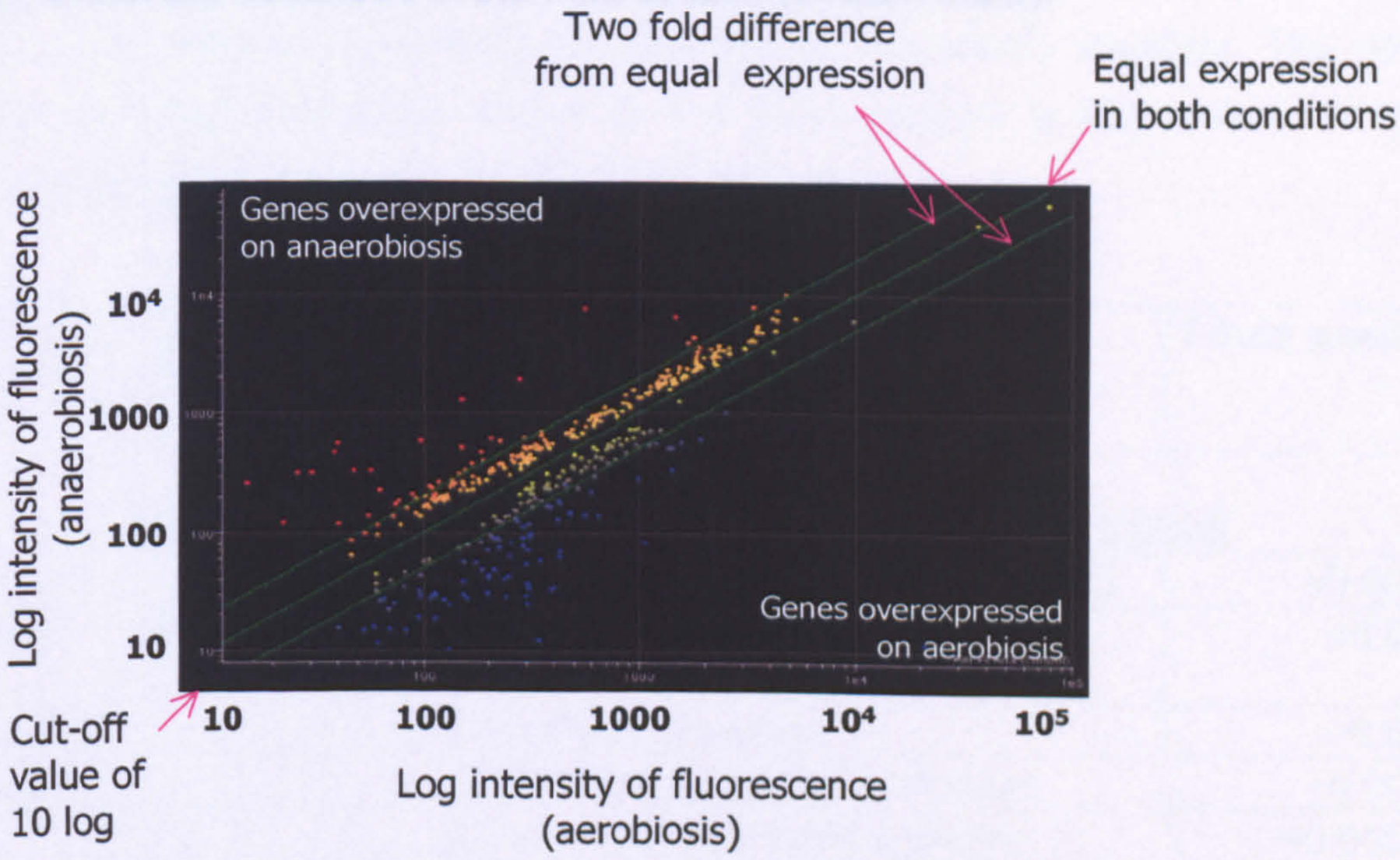


Figure 5.2-4 Scatter plot of the microarray experiments. The data were normalised by LOWESS normalisation and a cut-off value of 10log in the intensity of the spots was applied. Spots representing genes that were labelled as "bad" or "not found" in more than two microarray experiments were excluded. Spots representing genes whose expression was not significantly different ($p>0.05$) in one condition compared to the other one were also excluded.

In a further round of analysis, even though a selection of genes whose expression was significantly different under one condition compared to the other one had been done, only genes whose expression was at least two-fold different from equality were selected. This excluded 240 genes, leaving 119 genes in the experiment. Because a further selection was done, the appendix (Table A.1 and Table A.2) shows the list of these 119 genes grouped according to their fold over-expression in aerobiosis (Table A.1) or anaerobiosis (Table A.2). Their p-value, common name and annotation are also shown. Finally, a gene was only selected if this characteristic of at least two-fold difference from equality remained constant through all the individual experiments in which data for a particular gene were present. This final selection excluded 50 genes, leaving a final selection of only 69 genes, which are described in the next section (section 5.2.2).

5.2.2 Microarray results

In Table 5.2-1 and 5.2-2 the genes that were selected as over-expressed under aerobiosis and anaerobiosis respectively are shown. The list of genes is divided according to their fold over-expression from equality. The systematic and common names of the genes, as well as their product and p-value are shown. From the Tables, it can be seen that 55 genes appeared over-expressed under aerobic condition and 14 under the anaerobic condition.

Table 5.2-1 List of genes that were over-expressed when *S. pneumoniae* R6 were grown under aerobic conditions as selected by microarray analysis. The gene number and the information shown in the gene product is also based on that provided by the TIGR institute.

Systematic name	Synonyms or R6 systematic name	Product	T-test p-value
BETWEEN TWO AND FOUR-FOLD OVER-EXPRESSION			
spr0957		SpR6: Tn5252, relaxase, truncation	<0.0001
spr0960	*mut*R	Similar to positive transcriptional regulator MutR.	<0.001
spr0965		Hypothetical protein	<0.002
spr0966		Conserved hypothetical protein	<0.0002
spr0967		Conserved hypothetical protein	<0.00008
sp0731	spr0643	Conserved domain protein; hypothetical protein	<0.0003
sp0732	spr0644	Degenerate transposase	<0.00005
sp0992	spr0895	Conserved hypothetical protein	<0.003
sp1013	*asd*; spr0918	Aspartate beta-semialdehyde dehydrogenase	<0.0002
sp1314		IS66 family element, Orf1	<0.003
sp1346	spr1206	Conserved hypothetical protein; hypothetical protein	<0.00002
sp1382	*amy*; spr1239	Alpha-amylase precursor	<0.002
sp1435	ABC-N/P; spr1290	ABC transporter, ATP-binding protein	<0.00007

sp1459	spr1313	Hypothetical protein	<0.0002
sp1496	spr1349	Transposase, IS630-Spn1 related, Orf2; degenerate transposase	<0.005
sp1515	spr1367	Transposase	<0.00003
sp1694	spr1537	Hypothetical protein	<0.001
sp1728	spr1572	Hypothetical protein	<0.00001
sp1802	spr1623	Hypothetical protein	<0.0003
sp2004		Hypothetical protein	<0.0002
sp2017	spr1830	Membrane protein; conserved hypothetical protein	<0.00002
sp2104	spr1914	Hypothetical protein	<0.0009
sp2181	spr1985	Transposase, truncation	<0.008
BETWEEN FOUR AND EIGHT-FOLD OVER-EXPRESSION			
sp0095	spr0084	Conserved hypothetical protein	<0.002
sp0718	*thi*E; spr0630	Thiamin-phosphate pyrophosphorylase	<0.003
sp1326		Neuraminidase, putative	<0.005
sp1424	spr1280	Hypothetical protein	<0.00001
sp1426	ABC-NBD; spr1281	ABC transporter ATP-binding protein - multidrug efflux	<0.001
sp1434	ABC-N/P; spr1289	ABC transporter, ATP-binding/permease protein	<0.007
sp1441	spr1294	Transposon and IS element	<0.0006
sp1495	spr1348	Hypothetical protein	<0.006
sp1691	spr1535	Conserved hypothetical protein; hypothetical protein	<0.0007
sp1759		preprotein translocase, SecA subunit	<0.004
sp1767		Glycosyl transferase, family 8	<0.007
sp1919	spr1735; spr1736	ABC transporter, permease protein	<0.0004
sp2032	spr1843	Transcriptional regulator, BglG family; conserved hypothetical protein	<0.003
sp2034	*sga*; spr1845	Hexulose-6-phosphate isomerase, putative	<0.0008
sp2035	*sgh*; spr1846	Hexulose-6-phosphate synthase, putative	<0.0002
sp2036	PTS-EII; spr1847	PTS system, IIA component	<0.003
sp2038	PTS-EII; spr1849	PTS system, membrane component, putative	<0.0005
sp2046	spr1856; spr1857	Hypothetical protein	<0.0002
sp2047	spr1858	Conserved domain protein; hypothetical protein	<0.00009
sp2048	spr1859	Conserved hypothetical protein	<0.0007
sp2050	*cgl*D; spr1861	Competence protein CglD	<0.00001

sp2051	*cgl*C; spr1862	Competence protein CglC	<0.0003
sp2178	spr1983	Conserved hypothetical protein	<0.001
BETWEEN EIGHT AND SIXTEEN-FOLD OVER-EXPRESSION			
Spr0968		Hypothetical protein	<0.002
sp0766	*sod*A; spr0674	Superoxide dismutase, manganese-dependent	<0.002
sp1332	spr1206	Conserved domain protein; hypothetical protein	<0.007
sp1438	ABC-NBD; spr1293	ABC transporter, ATP-binding protein	<0.00009
sp1651	*tpx*; spr1495	Thiol peroxidase	<0.004
sp2123	*rgg*; spr1933	Positive transcriptional regulator of glucosyltransferase and Spp phenotype	<0.0003
sp2207	*com*FC; spr2012	Competence protein ComF, putative	<0.008
sp2211	spr2016	Transposase, uncharacterised, truncation	<0.00002
MORE THAN SIXTEEN-FOLD OVER-EXPRESSION			
sp1250	spr1129	Conserved domain protein; hypothetical protein	<0.00006

Table 5.2-2 List of genes that appeared over-expressed when *S. pneumoniae* R6 were grown under anaerobic condition as selected by microarray analysis. The gene number and the information shown in the annotation is also based on that provided by the TIGR institute.

Systematic name	Synonyms or R6 systematic name	Product	T-test p-value
BETWEEN TWO AND FOUR-FOLD OVER-EXPRESSION			
sp1179	*nrd*E; spr1065	Ribonucleoside-diphosphate reductase, alpha subunit	<0.00001
BETWEEN FOUR AND EIGHT-FOLD OVER-EXPRESSION			
sp0202	*nrd*D; spr0183	Anaerobic ribonucleoside-triphosphate reductase	<0.00001
sp0204	spr0184	Acetyltransferase, GNAT family; hypothetical protein	<0.00003
sp0205	*nrd*G; spr0185	Anaerobic ribonucleoside-triphosphate reductase activating; NrdD activating enzyme, generating glycyl radical	<0.00002
sp0338	*clp*L; spr0307	ATP-dependent Clp protease, ATP-binding subunit, putative	<0.0002
sp0482	spr0429	Conserved hypothetical protein	<0.008
sp1871	*fec*E; sp1686	Iron-compound ABC transporter, ATP-binding protein	<0.002
BETWEEN EIGHT AND SIXTEEN-FOLD OVER-EXPRESSION			
sp0206	spr0186	Hypothetical protein	<0.003
sp0319	spr0289	Conserved domain protein; hypothetical protein	<0.00008
sp0320	*gno*; spr0290	Oxidoreductase, short chain dehydrogenase/reductase family; 5-keto-D-gluconate 5-reductase	<0.0005
sp1869	*fat*D; spr1684	Iron-compound ABC transporter, permease protein	<0.004
sp1870	*fat*C; spr1685	Iron-compound ABC transporter, permease protein	<0.002
sp1872	*fat*B; spr1687	Iron-compound ABC transporter, iron-compound-binding protein	<0.003
MORE THAN SIXTEEN-FOLD OVER-EXPRESSION			
sp0318	*kdg*K; spr0288	Carbohydrate kinase, PfkB family	<0.003

5.2.2.1 Selection of genes from microarray results for confirmation of their expression by RTqPCR

From the genes selected by microarray for their over-expression under aerobic conditions, all those that showed >8-fold over-expression and that were present in the genome of both *S. pneumoniae* R6 and *S. pneumoniae* TIGR4 were confirmed by RTqPCR. Other genes, such as those involved with transposon activities were also selected. Table 5.2-3, shows the list of selected genes.

Only four genes were selected for their over-expression under anaerobiosis; one that showed more than 16-fold over-expression under this condition, two genes that belong to an iron-compound ABC transporter operon, and a hypothetical protein that showed more than 8-fold over-expression. The list of genes selected for their confirmation by RTqPCR is also shown in Table 5.2-3. The annotation of the selected genes can be found in Tables 5.2-1 and 5.2-2.

Table 5.2-3 Genes selected from the microarray experiments for their confirmation by RTqPCR. Genes that appeared over-expressed under aerobiosis in the microarray experiments are shown in blue, the ones that appeared over-expressed under anaerobiosis are shown in red.

sp0732	sp0766	sp2123	sp0318
sp1314	sp1332	sp2207	sp1869
sp1496	sp1438	sp2211	sp1870
sp1515	sp1651	sp1250	sp0206

5.3 RTqPCR: Confirmation of microarray results

To evaluate the expression of the selected genes (Table 5.2-3) by RTqPCR, it was necessary to normalise their gene expression to a control gene whose expression remains constant regardless the stress to which the bacteria is subject. Following a personal communication with Dr. Marco Oggioni from the University of Siena, Italy, the gene sp0806 (*gyrB*), which is a DNA gyrase, was recommended for this purpose. Therefore, the expression of the genes in question was normalised to the expression *gyrB* in their corresponding condition. For example, if RTqPCR was done for gene x from RNA obtained from bacteria grown under aerobiosis and under anaerobiosis, simultaneously, the expression of *gyrB* in each condition was also measured. The expression of gene x under aerobiosis was normalised with the expression of *gyrB* under aerobiosis, and the same was done for the anaerobic expression. This normalisation allowed comparison of the expression of gene x from one condition to the other regardless of a difference in the initial concentration of RNA.

Also, to perform the RTqPCR experiments, the RNA samples were treated with DNase as explained in 2.3.12. The RNA free of DNA was then converted into cDNA, and qPCR performed on the cDNA samples. To confirm that no DNA contamination was present, PCR was done from the RNA samples before and after DNase treatment and on the cDNA to confirm a successful RT. The results are shown in Figure 5.3-1. As it can be seen from the Figure, the contaminating DNA present after RNA extraction was eliminated with the DNase treatment. The conversion to cDNA was successful, as amplification was observed on the samples after RT.

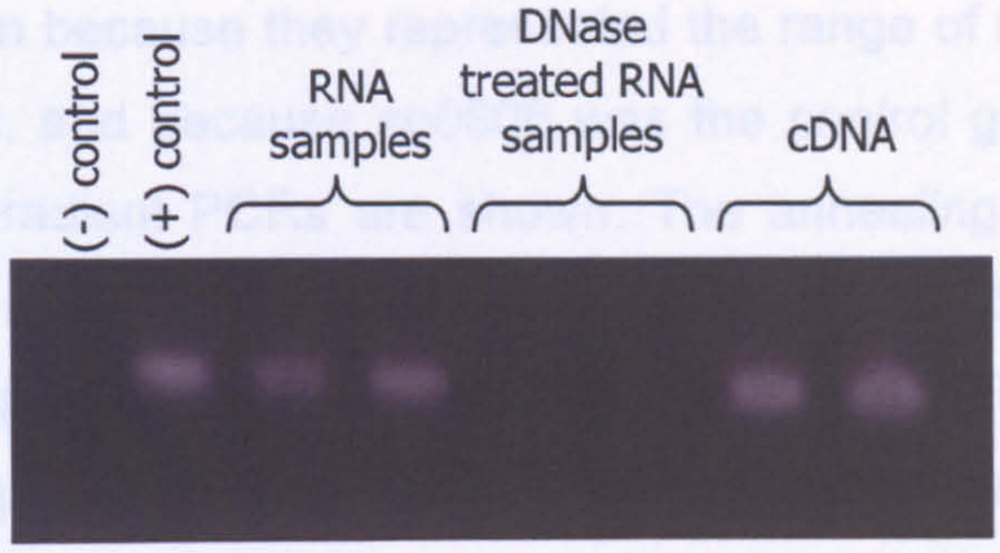


Figure 5.3-1 Confirmatory gel of DNA-free RNA samples and their conversion to cDNA for use in qPCR.

5.3.1 Primer design for RTqPCR and primer analysis

Primers were designed for each of the selected genes, as well as for *gyrB*, with the program FastPCR, as explained in 2.1.6. The program chose primers with a size ranging from 20 to 25 nucleotides that would give a product size between 140 to 160bp. This size of product was chosen following a personal communication from Dr. Marco Oggioni from the University of Siena, Italy. He recommended a size of 150bp, as he had found consistent amplification results using this product size. Also, for convenience, the program determined the melting temperature of the primers, preferably ranged between 60°C and 75°C, expecting that a common annealing temperature could be found for all the set of primers. The primers were named with the systematic name of the gene followed by the letter F for a forward primer or with the letter R for a reverse primer. The list of primers is shown on Table 2.1-5 of the materials and methods section.

Gradient PCRs were done using chromosmal DNA of *S. pnuemoniae* R6 with the primers corresponding to genes sp0766, sp0806 (*gyrB*) and sp1438. These primers were chosen because they represented the range of melting temperatures from all the primers, and because sp0806 was the control gene. In Figure 5.3-2, the results of the gradient PCRs are shown. The annealing temperature ranged from 45-65°C, and as it can be seen from the Figure, the desired product sizes were obtained for sp0766 and sp0806 at all temperatures. For the set of primers corresponding to sp1438 (Figure 5.3-2C) the annealing temperature of 53.4°C appeared to be the best. As amplification at this temperature with the set of primers corresponding to sp0766 (Figure 5.3-2A) and sp0806 (Figure 5.3-2B) was adequate, this temperature was chosen as the best common annealing temperature as judged by visualisation of the gel.

To confirm that this temperature would be adequate for the complete set of primers, PCRs were done with each pair using the annealing temperature of 53.4°C. These PCRs also allowed assessment of unspecific binding of the primers. The PCRs are shown in Figure 5.3-3. As it can be seen from the Figure, all the primers amplified at the chosen annealing temperature. The PCRs in lanes number 4, 11 and 15 appear to have lower amplification rates, but an amplification product was still visible. Also, no non-specific amplification bands were observed.

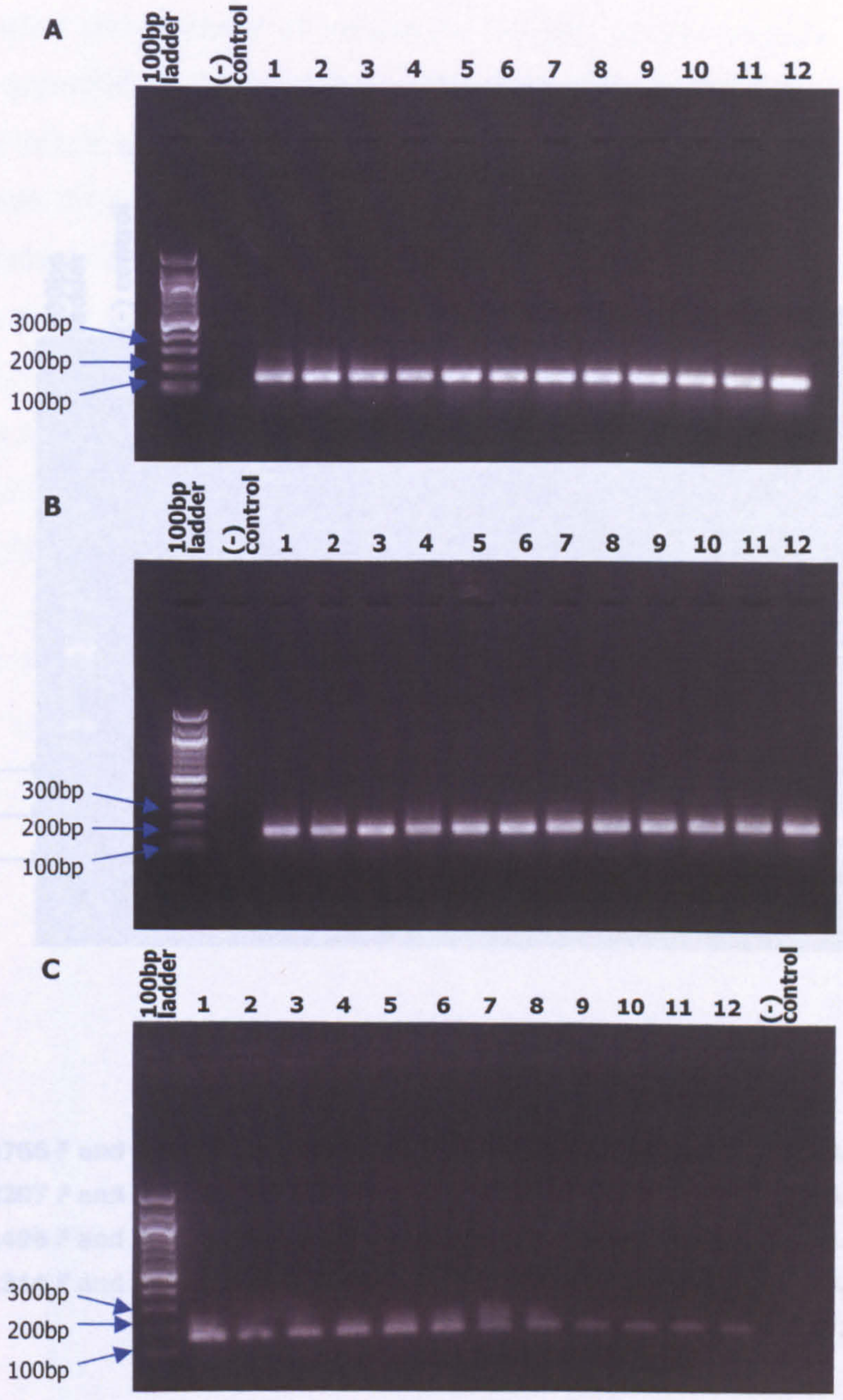
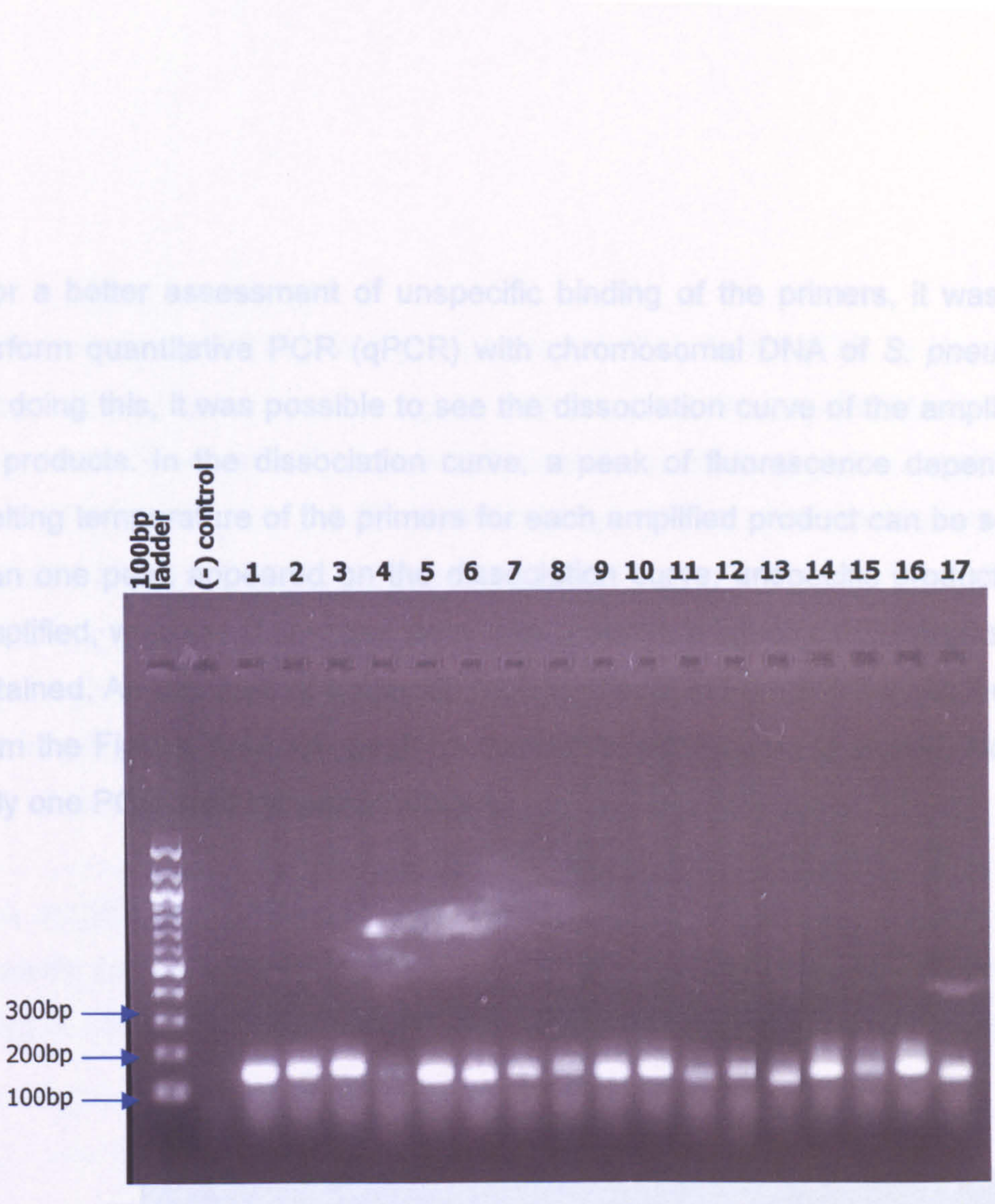


Figure 5.3-2 Gradient PCRs for primers (A) sp0766F and sp0766R; (B) sp0806F and sp0806R; and (C) sp1438F and sp1438R.



Pair of primers used

1. sp0766 F and R
2. sp2207 F and R
3. sp1496 F and R
4. sp1314 F and R
5. sp1870 F and R
6. sp0318 F and R
7. sp2211 F and R
8. sp0732 F and R
9. sp0806 F and R
10. sp1332 F and R
11. sp1869 F and R
12. sp1250 F and R
13. sp1651 F and R
14. sp0206 F and R
15. sp1515 F and R
16. sp1438 F and R
17. sp2123 F and R

Figure 5.3-3 PCR amplification using a common annealing temperature of 53.4°C with the complete set of primers designed for use in RTqPCR.

For a better assessment of unspecific binding of the primers, it was decided to perform quantitative PCR (qPCR) with chromosomal DNA of *S. pneumoniae* R6. By doing this, it was possible to see the dissociation curve of the amplified product or products. In the dissociation curve, a peak of fluorescence dependent on the melting temperature of the primers for each amplified product can be seen. If more than one peak appeared on the dissociation curve, unspecific products had been amplified, whereas if only one peak was present, a specific PCR product had been obtained. An example of a specific PCR is shown in Figure 5.3-4. As it can be seen from the Figure, only one peak on the dissociation curve is shown, indicating that only one PCR product was produced.

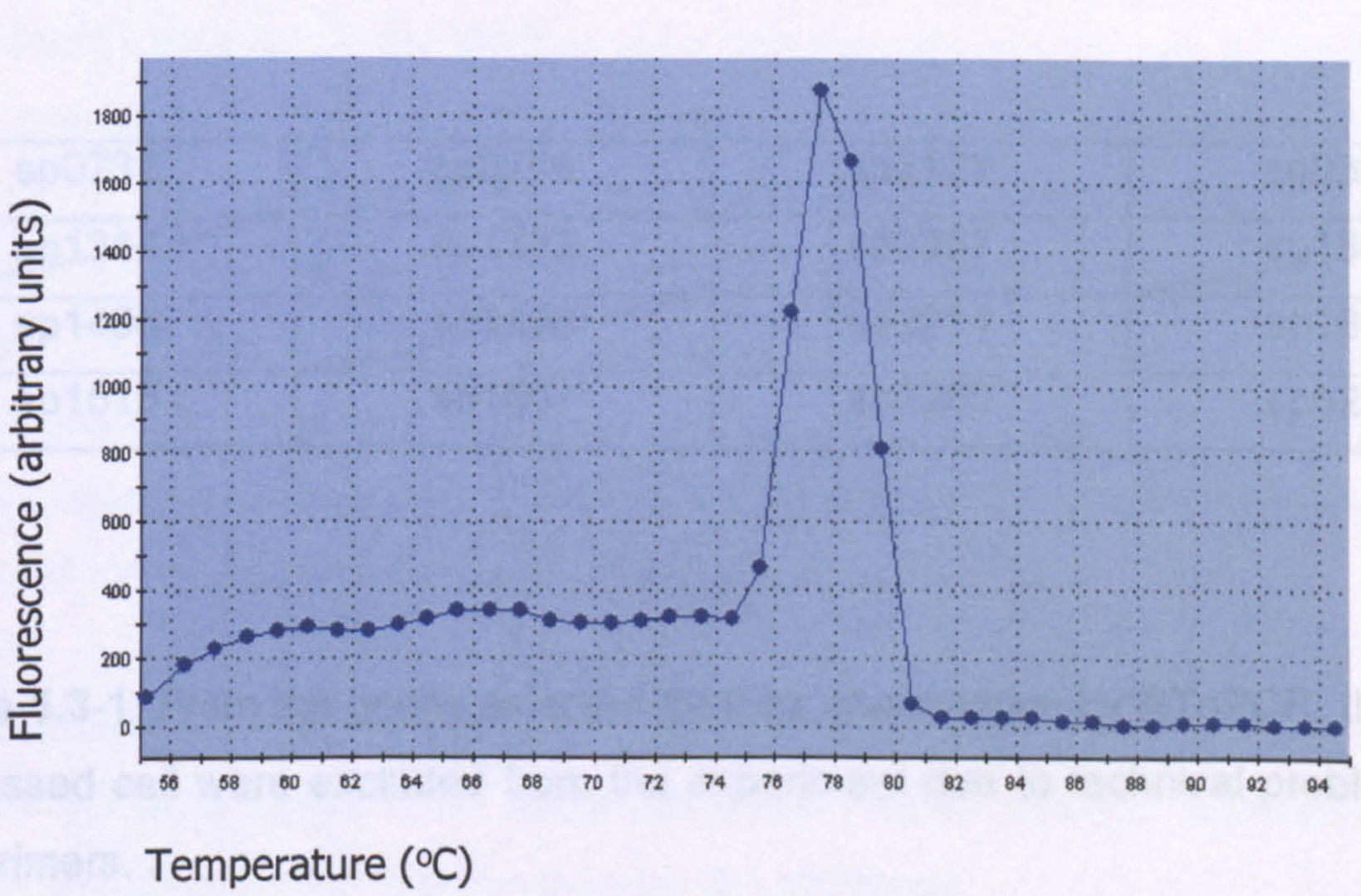


Figure 5.3-4 Example of a dissociation curve where only one dissociation peak is observed, indicating that no unspecific primer binding had occurred.

However, the qPCR in which primers Sp1314F/R, Sp1869F/R, or Sp1438F/R were used did not give enough signal and no peak in the dissociation curve was observed. These pair of primers had already shown to give low amplification rates by PCR (see Figure 5.3-3). Unfortunately, due to time restrictions it was not possible to standardise a common temperature or re-design primers for the qPCR of these genes. For this reason, genes sp1314, sp1869 and sp1438 were left out of the analysis. Table 5.3-1 shows from the list of genes selected for their confirmation by RTqPCR those that were excluded from the analysis, and the ones that remained in the study.

The amplification efficiency of the primers was also analysed using the MX4000 analysis software from Stratagene and DNA concentrations ranging from 1.25ng to 20ng of DNA. The primer efficiencies are shown on Table A-3 of the appendix.

sp0732	sp0766	sp2123	sp0318
~~sp1314~~	sp1332	sp2207	~~sp1869~~
sp1496	~~sp1438~~	sp2211	sp1870
sp1515	sp1651	sp1250	sp0206

Table 5.3-1 From the genes selected for their confirmation by RTqPCR, those with a crossed cell were excluded from the experiment due to technical problems with the primers.

5.3.2 RTqPCR Results

RTqPCR was performed for each gene in at least two independent experiments, each with duplicate samples. The data were analysed using the Comparative C_T Method ($2^{-\Delta\Delta CT}$) as explained in 2.3.11.5, where the expression of each gene was normalised against the expression of the housekeeping gene *gyr*B (sp0806) in the same sample. Because the expression of each gene was analysed against itself in a different condition and a comparison of expression between different genes was not done, differences in the primer efficiencies were not considered a major issue. The normalised expression of each gene in the anaerobic condition was arbitrarily considered as the basal gene expression. As a consequence, the $2^{-\Delta\Delta CT}$ value of each gene in anaerobiosis was 1 (see Table 5.3-2). The normalised expression of each gene in aerobiosis was "dependent" on its basal expression; in consequence, its $2^{-\Delta\Delta CT}$ value was a real number above or below one, depending if its expression was higher or lower than its basal expression (see Table 5.3-2). The difference in expression according to the $2^{-\Delta\Delta CT}$ values was then represented as a fold difference in expression (see Table 5.3-2). In Table 5.3-2, which shows representative data where n=2, the C_T value of each gene and of the housekeeping gene in each condition and their standard deviations are shown. Figure 5.3-5 shows a graph displaying the fold difference of expression that each gene had under aerobiosis when anaerobic expression was taken as the basal expression. The expression of all the genes that are shown in Table 5.3-2 and Figure 5.3-5 was confirmed with at least one more experiment. For each experiment, n=2.

The expression of genes sp0732, sp1332 and sp1250, which are not shown in Table 5.3-2 or in Figure 5.3-5, gave discrepant results between experiments. Due to time restrictions, their expression was not confirmed.

Table 5.3-2 Representative data of the RTqPCR experiments, where n=2. For each gene, the C_T value and the standard deviation (SD) of the gene and of the housekeeping gene (Hk) in each condition (anaerobic and aerobic expression) is shown. The $2^{-\Delta\Delta CT}$ value is also shown, considering the anaerobic expression as the basal expression and the aerobic expression as the "dependent" expression. The fold difference column represents the difference of expression of each gene under aerobiosis, when its anaerobic expression was considered as the basal level. The fold difference value was calculated from the aerobic $2^{-\Delta\Delta CT}$ value of each gene. (see section 5.3.2 of the text for further information on the table).

Gene	Anaerobic expression (basal expression)					Aerobic expression (dependent expression)					Fold difference
	Hk C_T	Hk SD	C_T	SD	$2^{-\Delta\Delta CT}$	Hk C_T	Hk SD	C_T	SD	$2^{-\Delta\Delta CT}$	
sp0206	19.22	0.19	18.23	0.03	1.00	19.78	0.22	20.20	0.09	0.38	-2.63
sp0318	21.34	0.00	23.59	0.05	1.00	20.86	0.00	25.32	0.13	0.22	-4.55
sp0766	19.22	0.19	19.47	0.02	1.00	19.78	0.22	18.16	0.00	3.66	3.66
sp1496	19.22	0.19	27.52	0.26	1.00	19.78	0.22	26.99	0.18	2.13	2.13
sp1515	19.79	0.01	26.14	0.05	1.00	19.26	0.08	24.61	0.01	2.00	2.00
sp1651	19.22	0.19	23.59	0.11	1.00	19.78	0.22	20.92	0.20	9.38	9.38
sp1870	19.79	0.01	21.91	0.04	1.00	19.26	0.08	24.43	0.01	0.12	-8.33
sp2207	19.79	0.01	30.30	0.12	1.00	19.26	0.08	30.47	0.09	0.62	-1.61
sp2123	19.79	0.01	26.80	0.04	1.00	19.26	0.08	25.92	0.00	1.27	1.27
sp2211	19.22	0.19	28.63	0.01	1.00	19.78	0.22	28.47	0.29	1.65	1.65

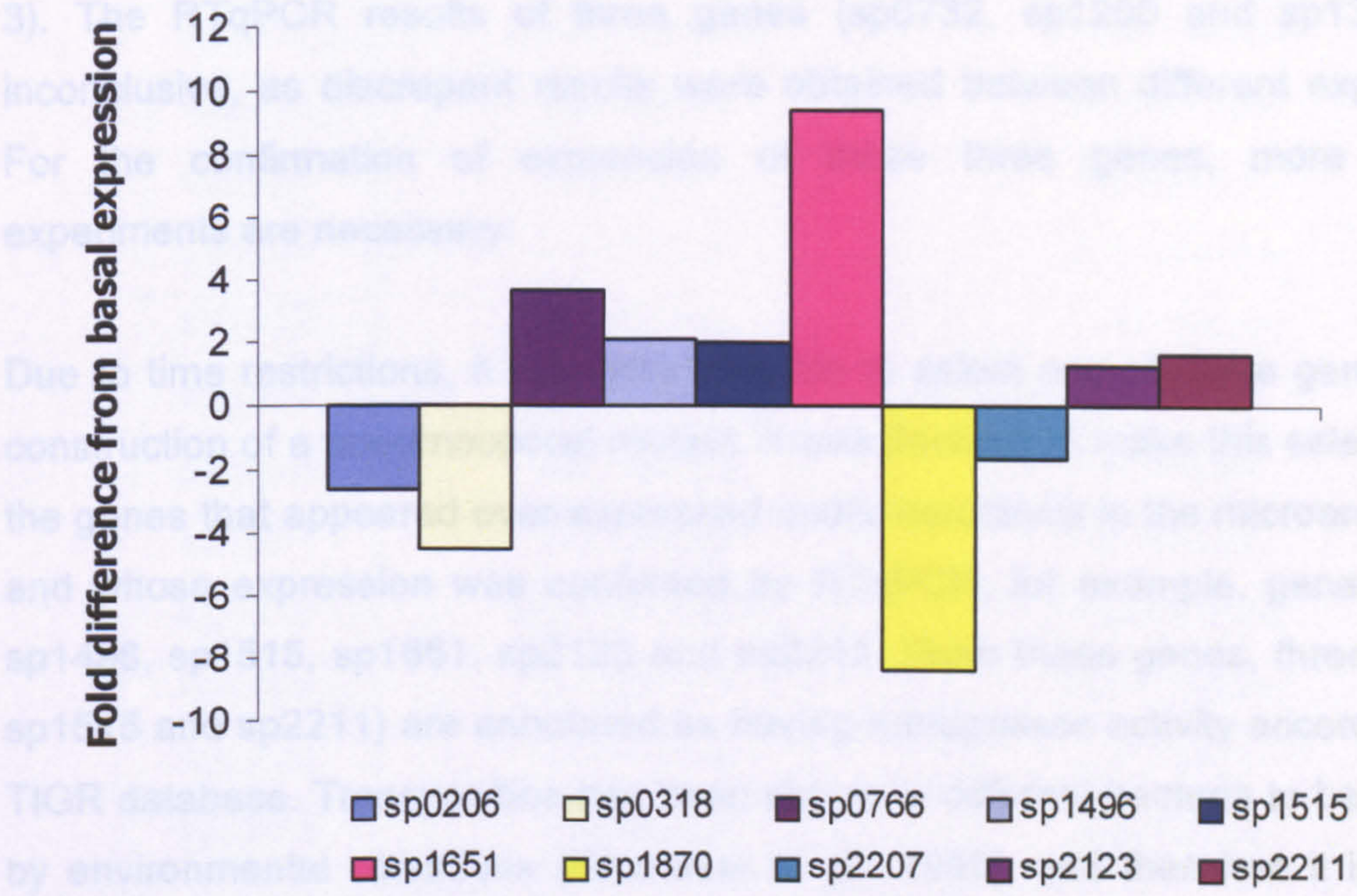


Figure 5.3-5 Fold difference of expression of each gene in aerobic conditions. In this graph, the anaerobic expression of each gene was considered as the basal expression level for that particular gene.

Table 5.3-3 shows a summary of the findings from the RTqPCR experiments, and if the RTqPCR confirmed the microarray results. In Table 5.3-3 the condition in which each gene was observed as over-expressed by microarray is shown in one column, and in the next column, the condition at which each gene appeared over-expressed by RTqPCR is shown. As it can be seen from the Table, RTqPCR confirmed the microarray results of nine of the genes analysed. The RTqPCR of one gene (sp2207) indicated opposite expression to that observed by microarray (Table 5.3-3). The RTqPCR results of three genes (sp0732, sp1250 and sp1332) were inconclusive, as discrepant results were obtained between different experiments. For the confirmation of expression of these three genes, more RTqPCR experiments are necessary.

Due to time restrictions, it was only possible to select one of these genes for the construction of a pneumococcal mutant. It was decided to make this selection from the genes that appeared over-expressed under aerobiosis in the microarray results and whose expression was confirmed by RTqPCR, for example, genes sp0766, sp1496, sp1515, sp1651, sp2123 and sp2211. From these genes, three (sp1496, sp1515 and sp2211) are annotated as having transposase activity according to the TIGR database. Transposition has been shown in different bacteria to be regulated by environmental conditions (Ghanekar *et al.*, 1999), and therefore it is possible that the expression of these genes might have occurred as a general response to stress, rather than being uniquely related to the stress suffered because of aerobiosis. Genes sp0766 (*sod*A) and sp1651 (*psa*D or *tpx*), which are known to be related to oxidative stress, have been previously studied by Yesilkaya *et al.* (2000) and Tseng *et al.* (2002) respectively, so they were not considered for the construction of a mutant. No literature was found relating to gene sp2123 (rgg) in the pneumococcus, however, studies in other streptococci have related the role of this gene to bacterial adaptation to oxidative stress and to colonisation (Chaussee *et al.*, 2004; Kreikemeyer *et al.*, 2003). It was therefore decided to select this gene for the construction of a pneumococcal mutant.

Gene	Condition of gene over-expression as evaluated by microarray	Condition of gene over-expression as evaluated by RTqPCR
sp0206	Anaerobiosis	Anaerobiosis
sp0318	Anaerobiosis	Anaerobiosis
sp0732	Aerobiosis	Inconclusive
sp0766	Aerobiosis	Aerobiosis
sp1250	Aerobiosis	Inconclusive
sp1332	Aerobiosis	Inconclusive
sp1496	Aerobiosis	Aerobiosis
sp1515	Aerobiosis	Aerobiosis
sp1651	Aerobiosis	Aerobiosis
sp1870	Anaerobiosis	Anaerobiosis
sp2207	Aerobiosis	Anaerobiosis
sp2123	Aerobiosis	Aerobiosis
sp2211	Aerobiosis	Aerobiosis

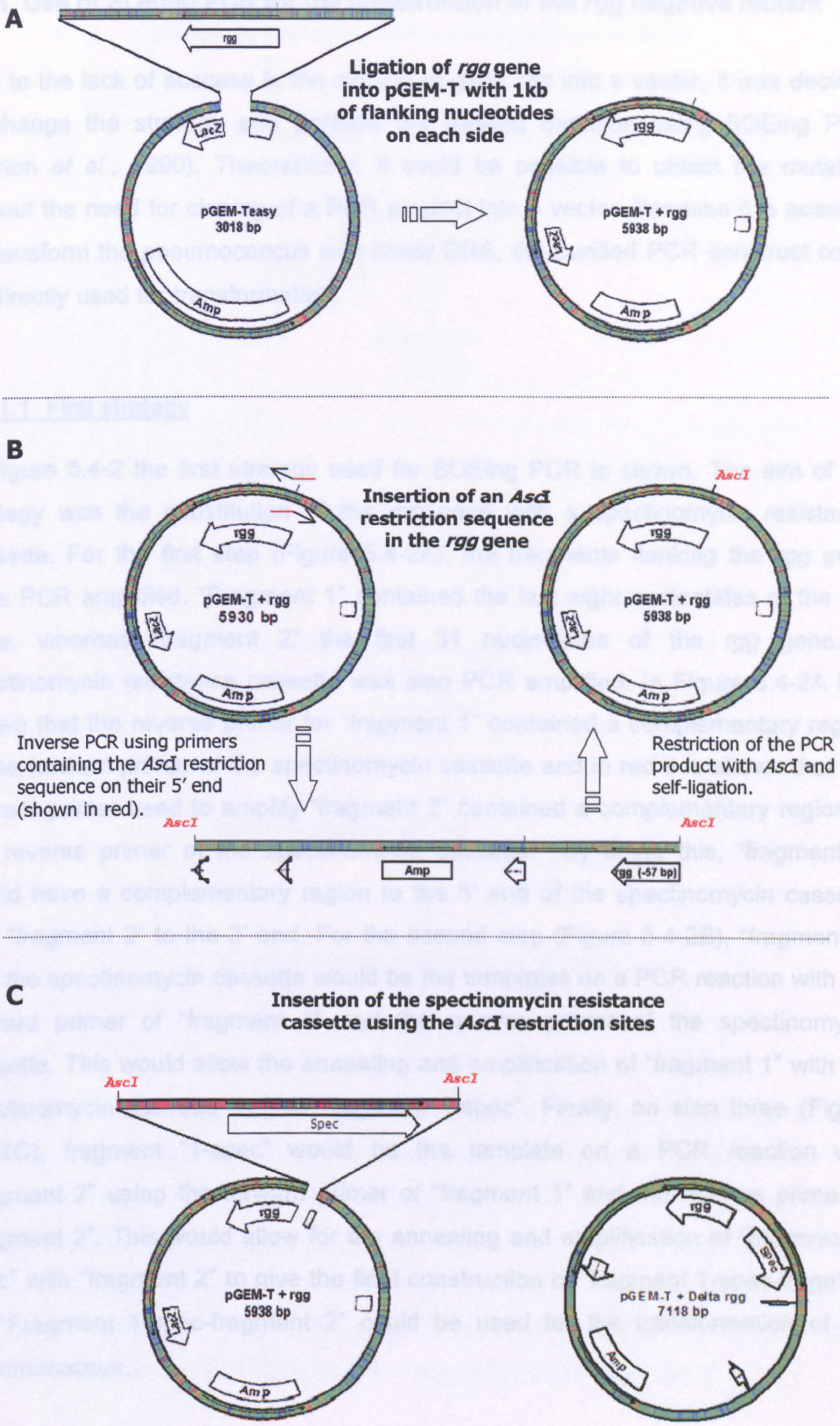
Table 5.3-3 Genes analysed by RTqPCR for confirmation of their microarray results. The systematic name of each gene analysed is given in the first column. Next, the condition in which each gene was observed over-expressed as evaluated by microarray is shown. In the third column, it is shown the condition of over-expression as evaluated by RTqPCR. Highlighted in yellow are the nine genes whose microarray expression was confirmed by RTqPCR.

5.4 Construction of an *rgg* negative mutant

Initially, to obtain an *rgg* negative mutant, the strategy shown in Figure 5.4-1 was used. The *rgg* gene with 1kb of flanking nucleotides on each side would be PCR amplified and ligated into pGEM-Teasy vector (Figure 5.4-1A), which is a suicide vector when transformed into the pneumococcus. An inverse PCR of the plasmid obtained would be done to insert the restriction site *Asc*I into the *rgg* gene (Figure 5.4-1B). This restriction site would be used for the insertion of a spectinomycin cassette to delete the function of the gene (Figure 5.4-1C). The spectinomycin cassette would be obtained by restricting plasmid that contained the spectictinomycin cassette flanked by *Asc*I restriction sites. The final construction would be transformed into the pneumococcus expecting a double homologous recombination to occur, and resistance to spectinomycin used to select the pneumococcal mutants.

The insertion of the *rgg* gene with its flanking nucleotides using the pGEM-Teasy vector failed. Many attempts were done varying the insert : vector ratios as 5:1, 3:1, 1:1, 1:3, 1:5 but no successful construction was obtained. Because the primers used to amplify the *rgg* gene with its flanking nucleotides contained restriction sites compatible with pUC19, the same strategy using plasmid pUC19 instead of pGEM-Teasy was attempted. This approach was also unsuccessful and so was the attempt to ligate the insert using the cloning vector pCRscript. During all the experiments high rates of false positives as judged by blue and white colony selection were seen.

Figure 5.4-1 Initial strategy for the construction of an *rgg* negative pneumococcal mutant. A: The *rgg* gene and 1kb of its flanking nucleotides on each side would be ligated into pGEM-Teasy. B: Inverse PCR would be done for the insertion of the *Asc*I restriction sites into the *rgg* gene. C: This restriction site would be used for the inserting a spectinomycin resistance cassette into the *rgg* gene, deleting this way its function.


A
rgg
LacZ
pGEM-Teasy
3018 bp
Amp
Ligation of rgg gene into pGEM-T with 1kb of flanking nucleotides on each side
rgg
LacZ
pGEM-T + rgg
5938 bp
Amp
B
rgg
LacZ
pGEM-T + rgg
5930 bp
Amp
Insertion of an AscI restriction sequence in the rgg gene
AscI
rgg
LacZ
pGEM-T + rgg
5938 bp
Amp
Inverse PCR using primers containing the AscI restriction sequence on their 5' end (shown in red).
Restriction of the PCR product with AscI and self-ligation.
AscI
AscI
Amp
gg (-57 bp)
C
Insertion of the spectinomycin resistance cassette using the AscI restriction sites
AscI
AscI
Spec
rgg
LacZ
pGEM-T + rgg
5938 bp
Amp
rgg
Spec
pGEM-T + Delta rgg
7118 bp
Amp

5.4.1 Use of SOEing PCR for the construction of the *rgg* negative mutant

Due to the lack of success in the cloning of gene *rgg* into a vector, it was decided to change the strategy and perform the desired mutation using SOEing PCR (Horton *et al.*, 1990). Theoretically, it could be possible to obtain the mutation without the need for cloning of a PCR product into a vector. Because it is possible to transform the pneumococcus with linear DNA, the purified PCR construct could be directly used for transformation.

5.4.1.1 First strategy

In Figure 5.4-2 the first strategy used for SOEing PCR is shown. The aim of the strategy was the substitution of the *rgg* gene with a spectinomycin resistance cassette. For the first step (Figure 5.4-2A), the fragments flanking the *rgg* gene were PCR amplified. "Fragment 1" contained the last eight nucleotides of the *rgg* gene, whereas "fragment 2" the first 31 nucleotides of the *rgg* gene. A spectinomycin resistance cassette was also PCR amplified. In Figure 5.4-2A it is shown that the reverse primer for "fragment 1" contained a complementary region to the forward primer of the spectinomycin cassette and in red it is shown that the forward primer used to amplify "fragment 2" contained a complementary region to the reverse primer of the spectinomycin cassette. By doing this, "fragment 1" would have a complementary region to the 5' end of the spectinomycin cassette and "fragment 2" to the 3' end. For the second step (Figure 5.4-2B), "fragment 1" and the spectinomycin cassette would be the templates on a PCR reaction with the forward primer of "fragment 1" and the reverse primer of the spectinomycin cassette. This would allow the annealing and amplification of "fragment 1" with the spectinomycin cassette to give "fragment 1-spec". Finally, on step three (Figure 5.4-2C), fragment "1-spec" would be the template on a PCR reaction with "fragment 2" using the forward primer of "fragment 1" and the reverse primer of "fragment 2". This would allow for the annealing and amplification of "fragment 1-spec" with "fragment 2" to give the final construction of "fragment 1-spec-fragment 2". "Fragment 1-spec-fragment 2" could be used for the transformation of the pneumococcus.

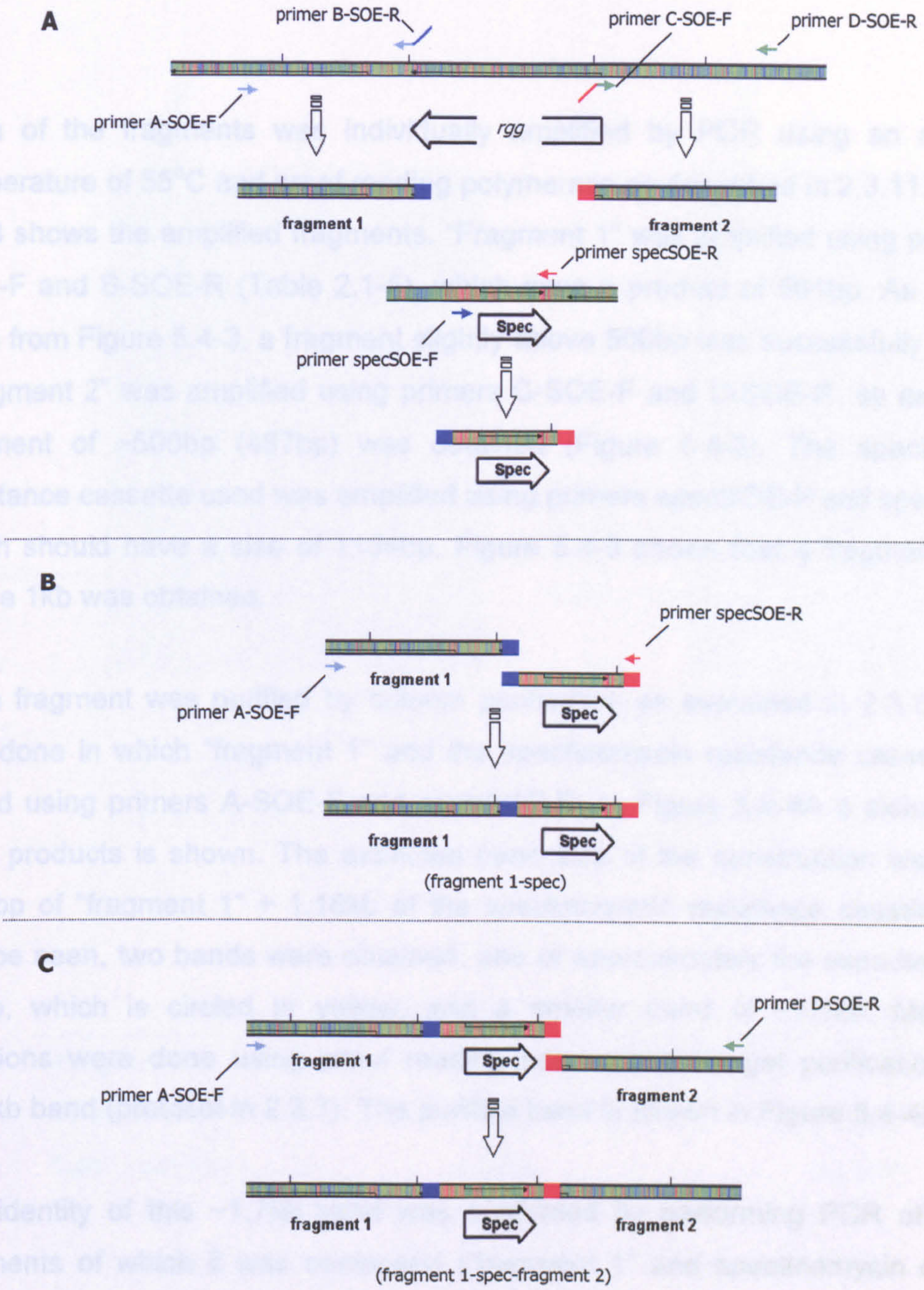
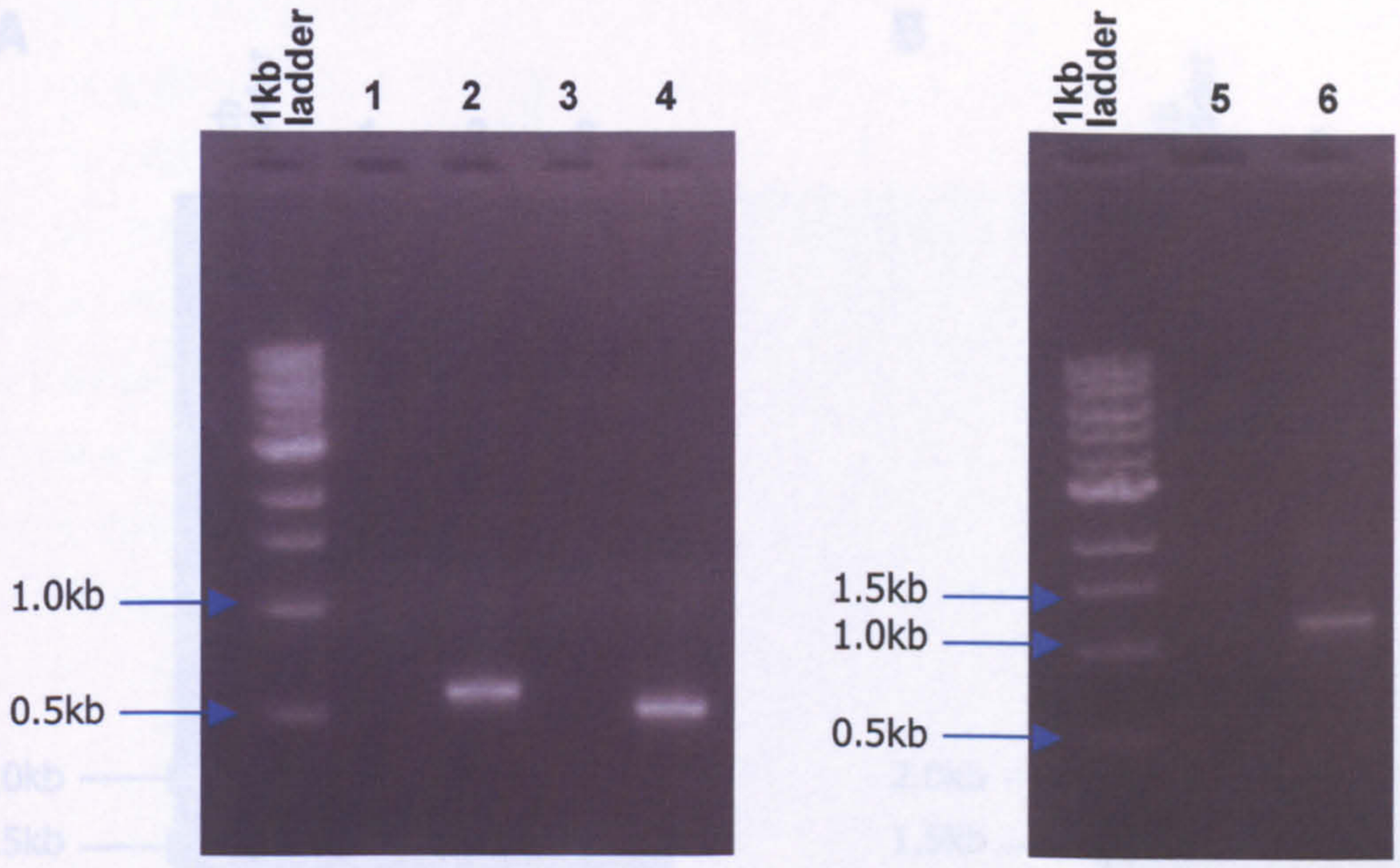


Figure 5.4-2 First strategy used for the obtaining of the Δrgg construct by SOEing PCR. A: The fragments flanking the *rgg* gene and a spec cassette are PCR amplified. Primers B-SOE-R and C-SOE-F contained regions homologous to the primers specSOE-F and specSOE-R. B: "fragment 1" and the spec cassette are joined and amplified by PCR using primers A-SOE-F and spec-SOE-R to give "fragment 1-spec". C: "fragment 1-spec" is joined to "fragment 2" by PCR using primers A-SOE-F and D-SOE-R to give "fragment 1-spec-fragment 2".

Each of the fragments was individually amplified by PCR using an annealing temperature of 55°C and proof reading polymerase as described in 2.3.11.1. Figure 5.4-3 shows the amplified fragments. "Fragment 1" was amplified using primers A-SOE-F and B-SOE-R (Table 2.1-5), which gave a product of 561bp. As it can be seen from Figure 5.4-3, a fragment slightly above 500bp was successfully obtained. "Fragment 2" was amplified using primers C-SOE-F and D-SOE-R, as expected a fragment of ~500bp (487bp) was obtained (Figure 5.4-3). The spectinomycin resistance cassette used was amplified using primers specSOE-F and specSOE-R, which should have a size of 1184bp. Figure 5.4-3 shows that a fragment slightly above 1kb was obtained.

Each fragment was purified by column purification as explained in 2.3.2. A PCR was done in which "fragment 1" and the spectinomycin resistance cassette were joined using primers A-SOE-F and specSOE-R. In Figure 5.4-4A a picture of the PCR products is shown. The expected band size of the construction was ~1.7kb (560bp of "fragment 1" + 1.18kb of the spectinomycin resistance cassette). As it can be seen, two bands were obtained, one of approximately the expected size of 1.7kb, which is circled in yellow, and a smaller band of ~1.1kb. More PCR reactions were done using proof reading polymerase for gel purification of the ~1.7kb band (protocol in 2.3.7). The purified band is shown in Figure 5.4-4B.

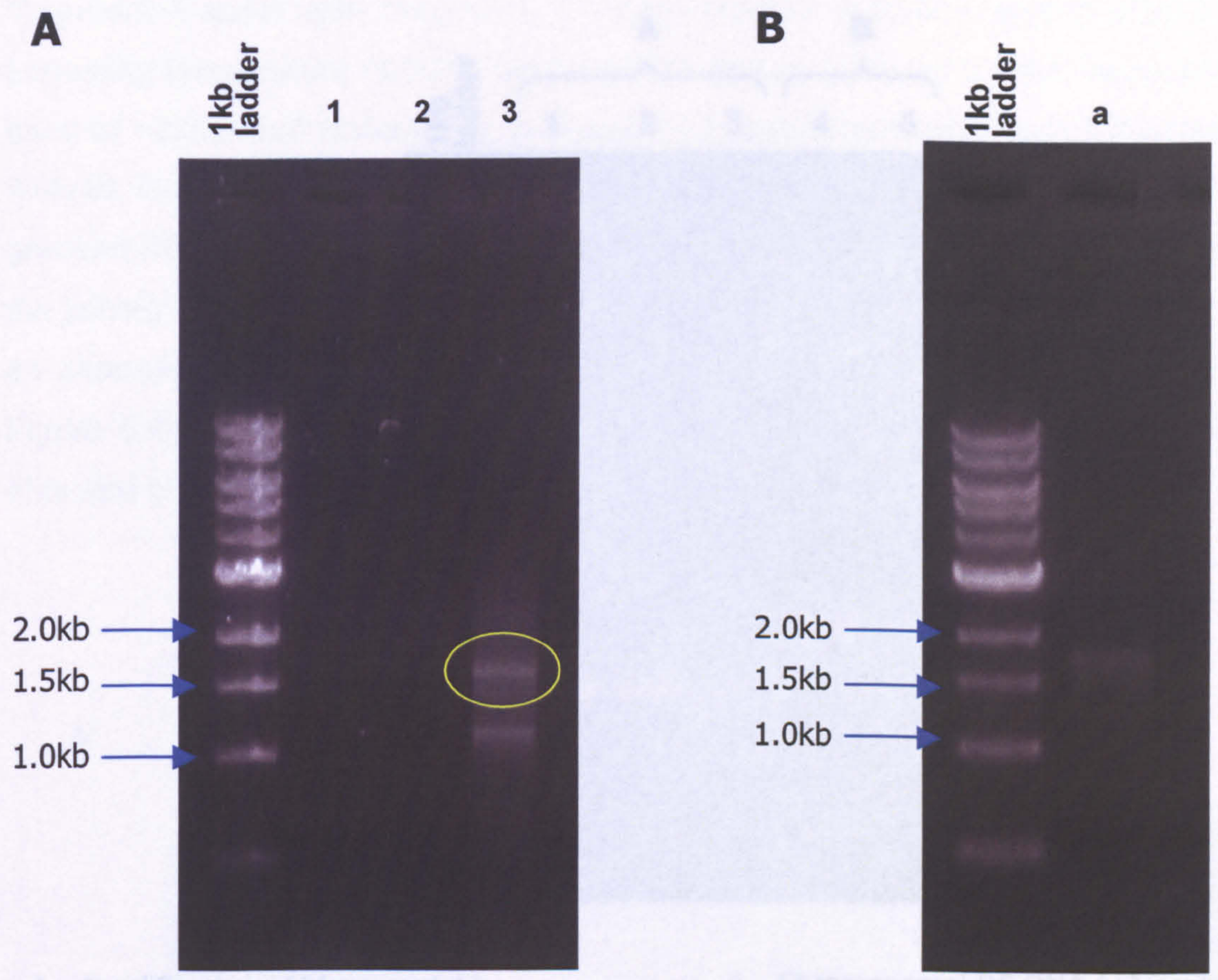
The identity of this ~1.7kb band was confirmed by performing PCR of the two fragments of which it was composed ("fragment 1" and spectinomycin cassette) using the purified ~1.7kb band as a template. Figure 5.4-5, shows the results, where it can be seen that both "fragment 1" and the spectinomycin cassette (lanes three and five respectively) were successfully amplified from the ~1.7kb band, confirming it was the expected "fragment 1-spec".



1. Fragment 1 (-) control
2. Fragment 1
3. Fragment 2 (-) control
4. Fragment 2
5. Spec (-) control
6. Spec cassette

	Expected size	Size obtained
Fragment 1	560bp	>0.5kb
Fragment 2	487bp	~0.5kb
Spec cassette	1.18kb	>1.0kb

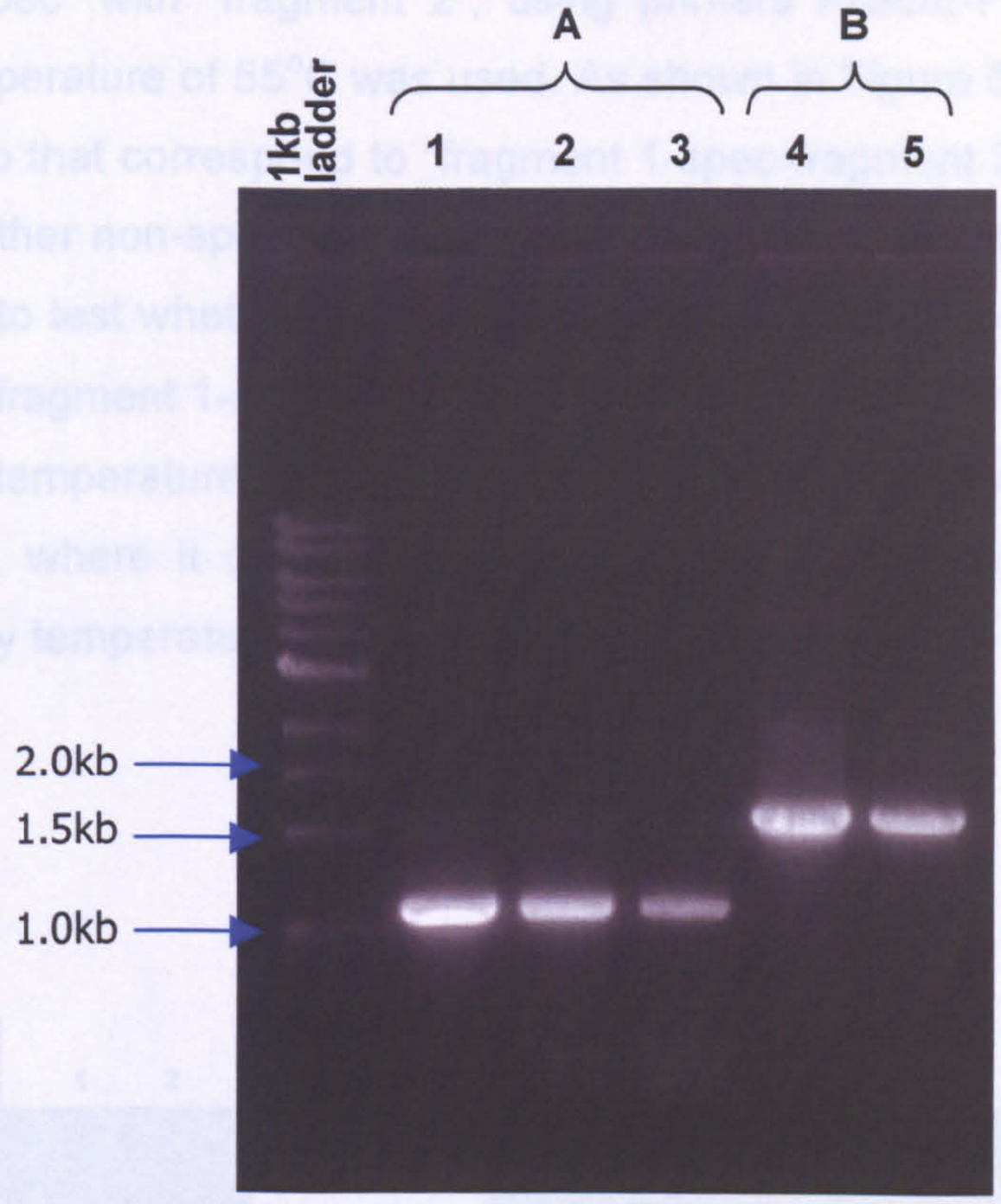
Figure 5.4-3 PCR amplification of "fragment 1", "fragment 2" and of the spectinomycin resistance cassette. The expected and obtained size for the amplification of each fragment is also shown.



1. No fragments (-) control
2. No enzyme (-) control
3. Fragment 1 + spec resistance cassette

a. Purified band

Figure 5.4-4 A: Annealing and amplification by PCR of "fragment 1" and the spectinomycin cassette. Circled in yellow is the band with the expected size of ~1.7kb that "fragment 1-spec" would have. B: Gel purification of the ~1.7kb band.



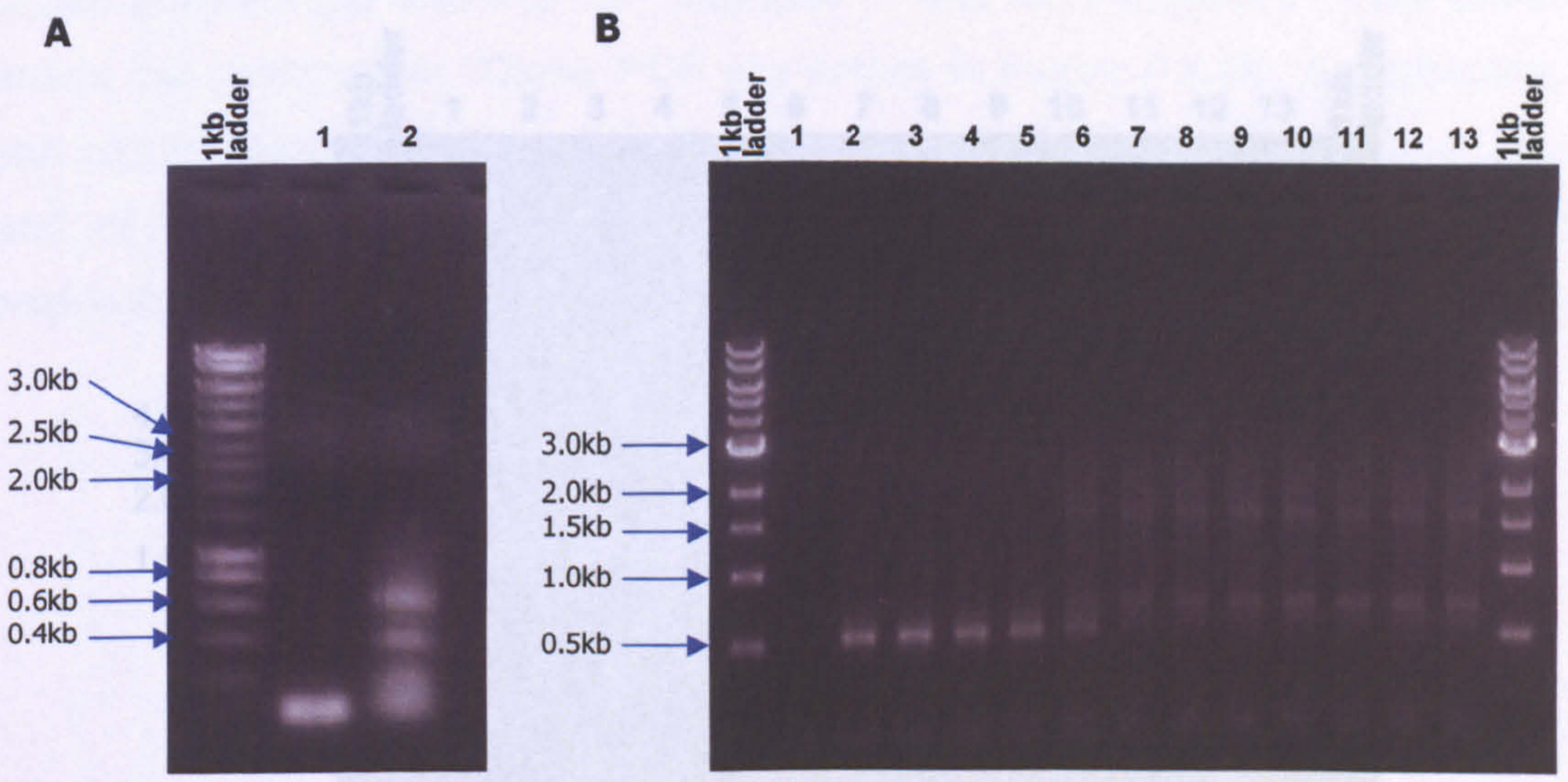
A. Amplification of "fragment 1"

B. Amplification of the spectinomycin resistance cassette

1. Chromosomal R6 DNA (+) control
2. Fragment 1 (+) control
3. Fragment 1-spec
4. Plasmid pDL278 (+) control
5. Fragment 1-spec

Figure 5.4-5 PCR done to confirm that the purified ~1.6kb fragment shown in Figure 5.3-4B had the expected construction "fragment 1-spec". The letters A and B design the fragment that was being amplified and the numbers 1-5 the template DNA used for the amplification.

Once the construction of "fragment 1-spec" was confirmed, a PCR was done to join "fragment 1-spec" with "fragment 2", using primers A-SOE-F and D-SOE-R. An annealing temperature of 55°C was used. As shown in Figure 5.4-6A, the expected band of ~2.2kb that correspond to "fragment 1-spec-fragment 2" was not obtained. Instead, two other non-specific bands were observed. It was decided to perform a gradient PCR to test whether a better annealing temperature could be obtained for the joining of "fragment 1-spec" with "fragment 2". The gradient PCR was done with an annealing temperature ranging from 45°C to 65°C. The results are shown on Figure 5.4-6B, where it can be seen that the expected band of 2.2kb was not obtained at any temperature in the gradient.



1. (-) control (no primers)
2. PCR of Fragment 1-Spec + Fragment 2

1. (-) control (no primers)	4. 46.7°C	8. 56.7°C	12. 64.6°C
2. 45.0°C	5. 48.2°C	9. 59.6°C	13. 65.0°C
3. 45.5°C	6. 50.5°C	10. 61.8°C	
	7. 53.4°C	11. 63.4°C	

Figure 5.4-6 A: PCR to obtain "fragment 1-spec-2. The expected band size of 2.2kb was not obtained. B: Gradient PCR to obtain "fragment 1-spec-fragment 2". At no temperature was the expected band of 2.2kb obtained.

As the gradient done to join and amplify "fragment 1-spec" with "fragment 2" was unsuccessful, it was decided to test if primers A-SOE-F and D-SOE-R were compatible and whether unspecific binding was observed when using these primers together. To test this, a gradient PCR with chromosomal DNA from *S. pneumoniae* R6 was done. Annealing temperatures ranging from 50°C to 70°C were used, expecting a band size of 1.9kb. The results are shown on Figure 5.4-7. It can be observed from the Figure, that a very strong band of ~1.9kb was obtained at all temperatures. A weak non-specific band of ~3kb was also observed at all of the temperatures, indicating some non-specific binding of the primers that might be affecting the SOEing PCR of "fragment 1-spec" with "fragment 2".

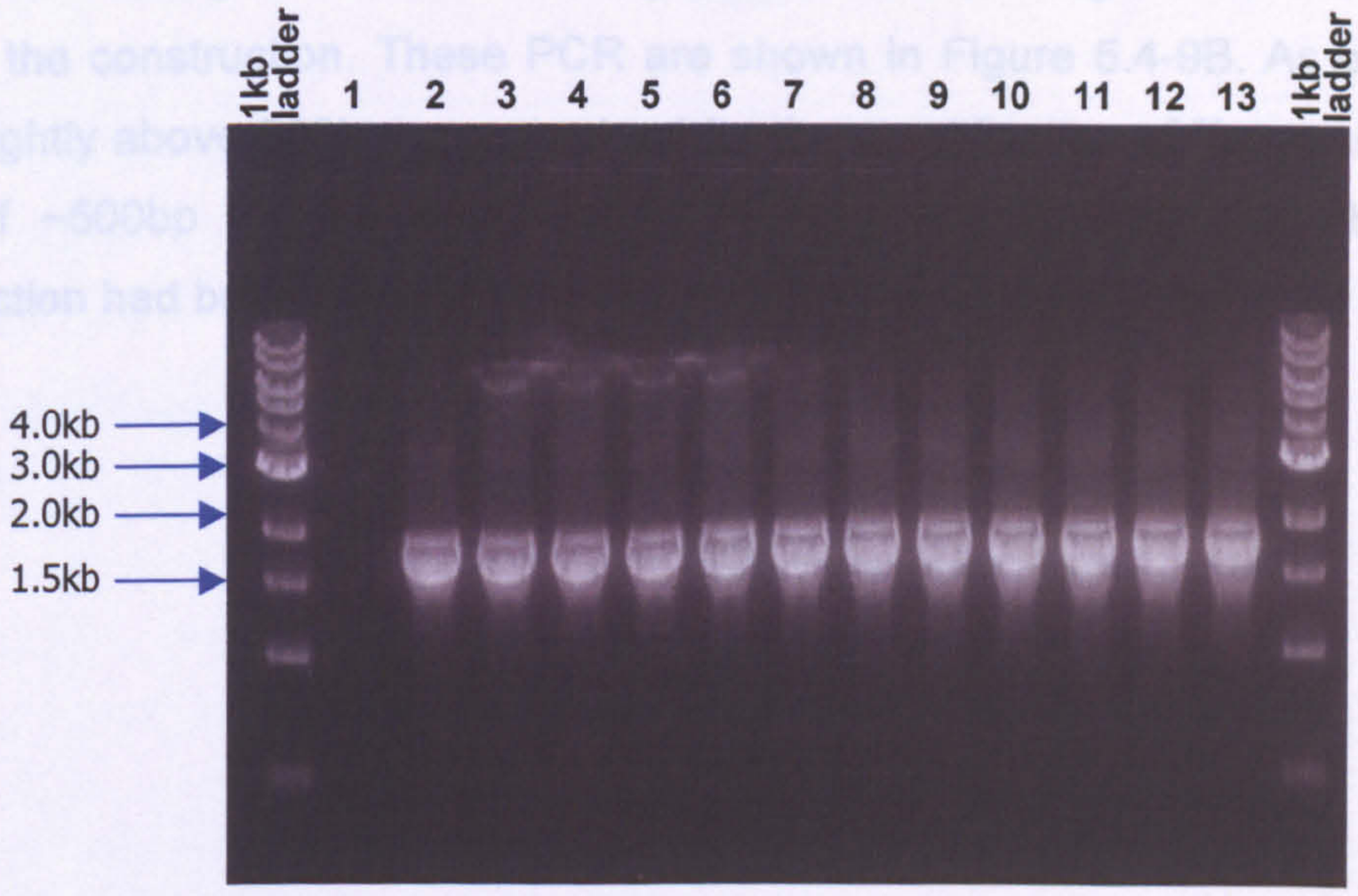


Figure 5.4-7 Gradient PCR using chromosomal DNA from *S. pneumoniae* R6 to test for the optimum annealing temperatures of primers A-SOE-F and D-SOE-R. Good amplification was observed at all temperatures.

5.4.1.2 Second strategy

From the first strategy for SOEing PCR shown on Figure 5.4-2, it was decided to merge steps two and three. With this strategy, the three PCR fragments would be placed together in a PCR reaction with primers A-SOE-F and D-SOE-R, with the expectation the three fragments could be fused in a one-step reaction. The new strategy is shown on Figure 5.4-8.

The PCR to merge "fragment 1", the spectinomycin resistance cassette, and "fragment 2" in a one-step reaction is shown in Figure 5.4-9A. An annealing temperature of 55°C and proof reading polymerase were used. From Figure 5.4-9A it can be observed that two bands were obtained. One band showed the expected size of ~2.2kb, and the second band had a size of ~1.6kb. The 2.2kb band was purified from the gel and PCR of "fragment 1" and of "fragment 2" were done to confirm the construction. These PCR are shown in Figure 5.4-9B. As expected, a band slightly above 500bp was obtained for the amplification of "fragment 1" and a band of ~500bp for the amplification of "fragment 2", indicating the desired construction had been made.

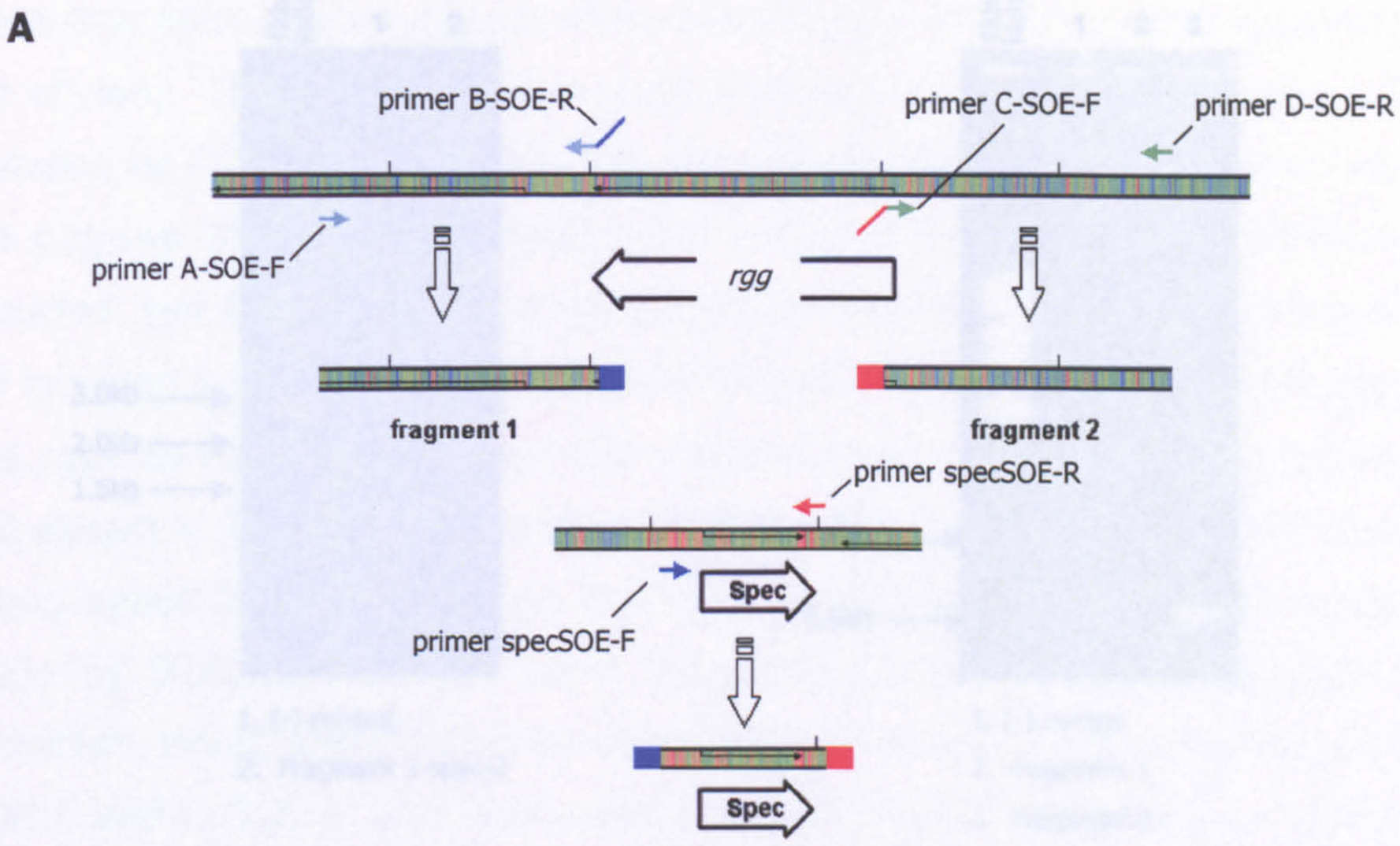


Figure 5.4-8 Second strategy used to obtain the Δrgg construct by SOEing PCR. A: The fragments flanking the *rgg* gene and the spectinomycin resistace cassette (spec) were PCR amplified. Primers B-SOE-R and C-SOE-F contained regions homologous to the primers specSOE-F and specSOE-R. B: The three fragments were joined in one PCR amplification reaction using primers A-SOE-F and D-SOE-R, to give "fragment 1-spec-fragment 2".

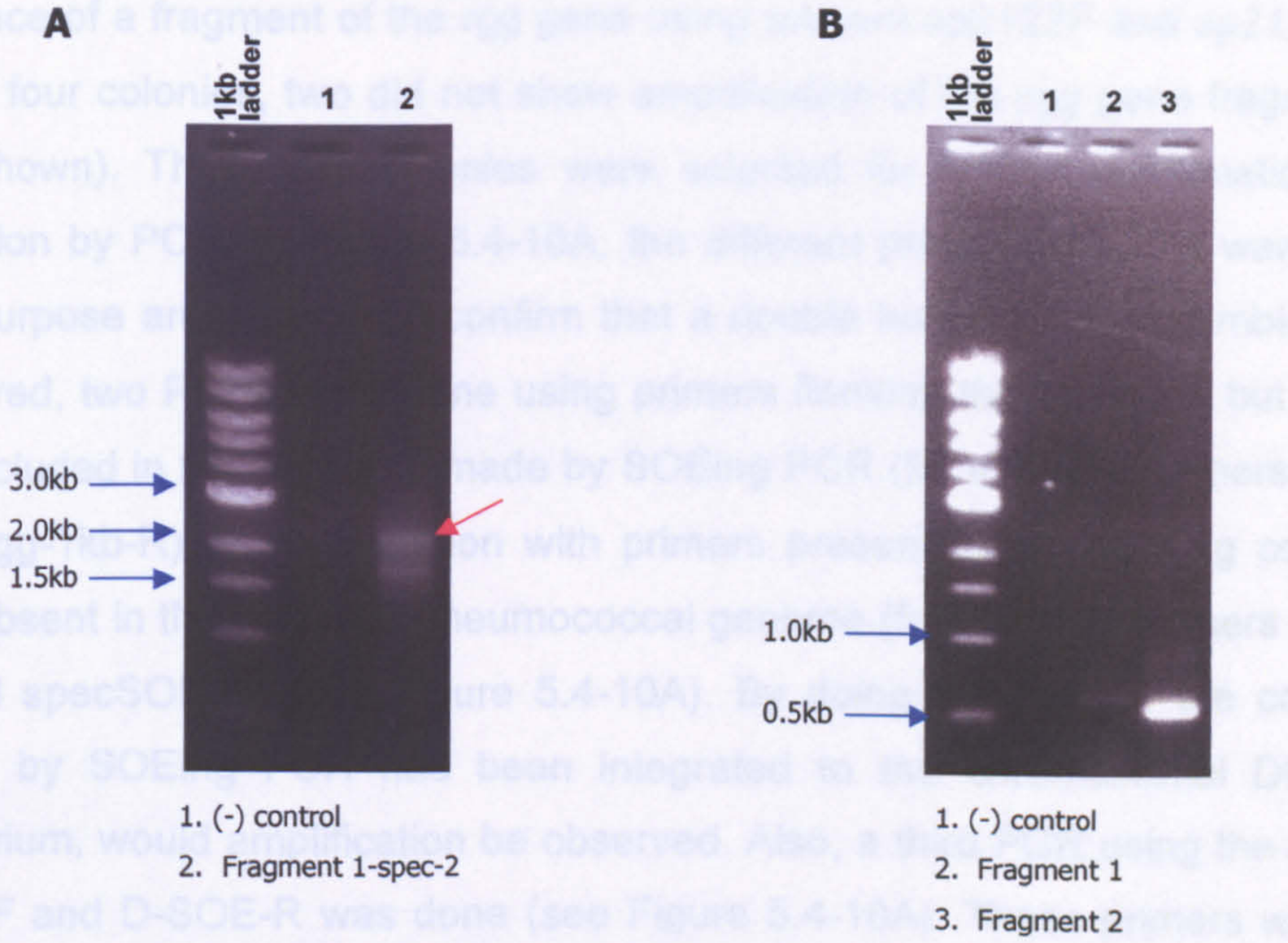


Figure 5.4-9 A: PCR for the annealing and joining of "fragment 1", the spectinomycin cassette, and "fragment 2". The red arrow shows a band with the expected size of ~2.2kb. B: PCR from the purified ~2.2kb band to confirm the presence of "fragment 1" and "fragment 2", indicating that the construction of "fragment 1-spec-fragment 2" was successful.

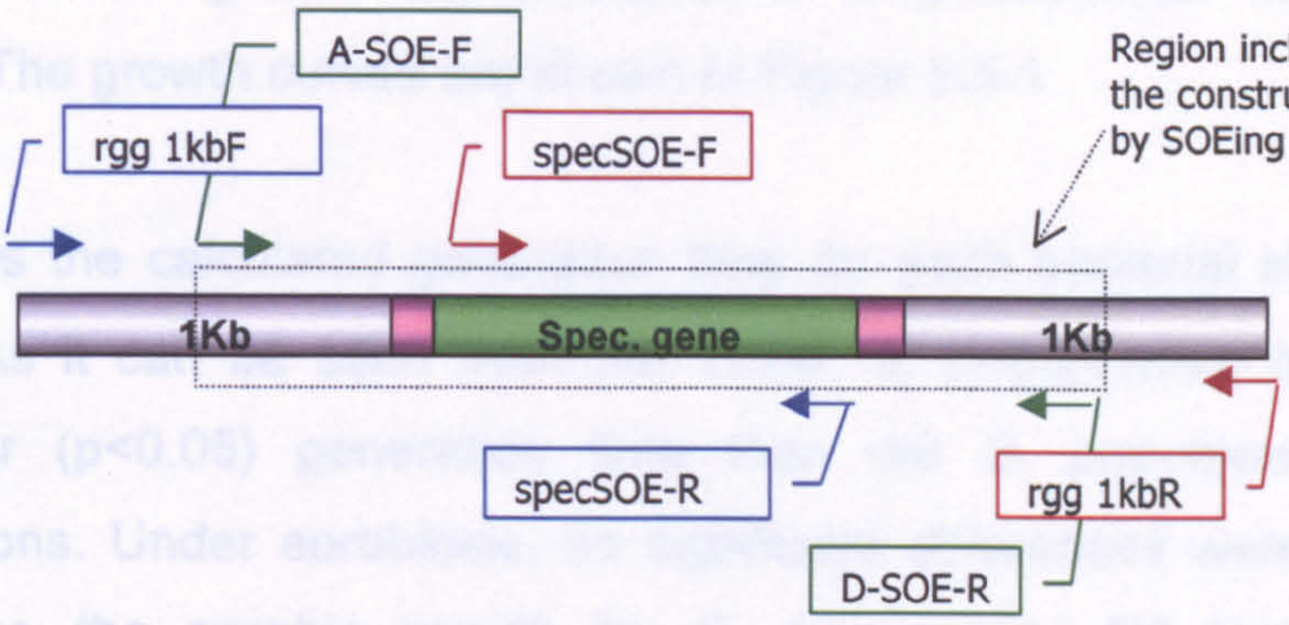
5.4.2 Transformation of "fragment 1-spec-fragement 2" into *S. pneumoniae* and selection of mutant *S. pneumoniae* Δrgg

Competent *S. pneumoniae* D39 and *S. pneumoniae* R6 were prepared as explained in section 2.2.4.2, and were transformed with purified "fragment 1-spec-fragment 2". The transformants were selected by growth on spectinomycin. No colonies were obtained after the transformation of *S. pneumoniae* D39, but pneumococcal colonies resistant to spectinomycin were obtained from the transformation of *S. pneumoniae* R6. Four colonies resistant to spectinomycin were selected for confirmation of their mutation by PCR. Chromosomal DNA was

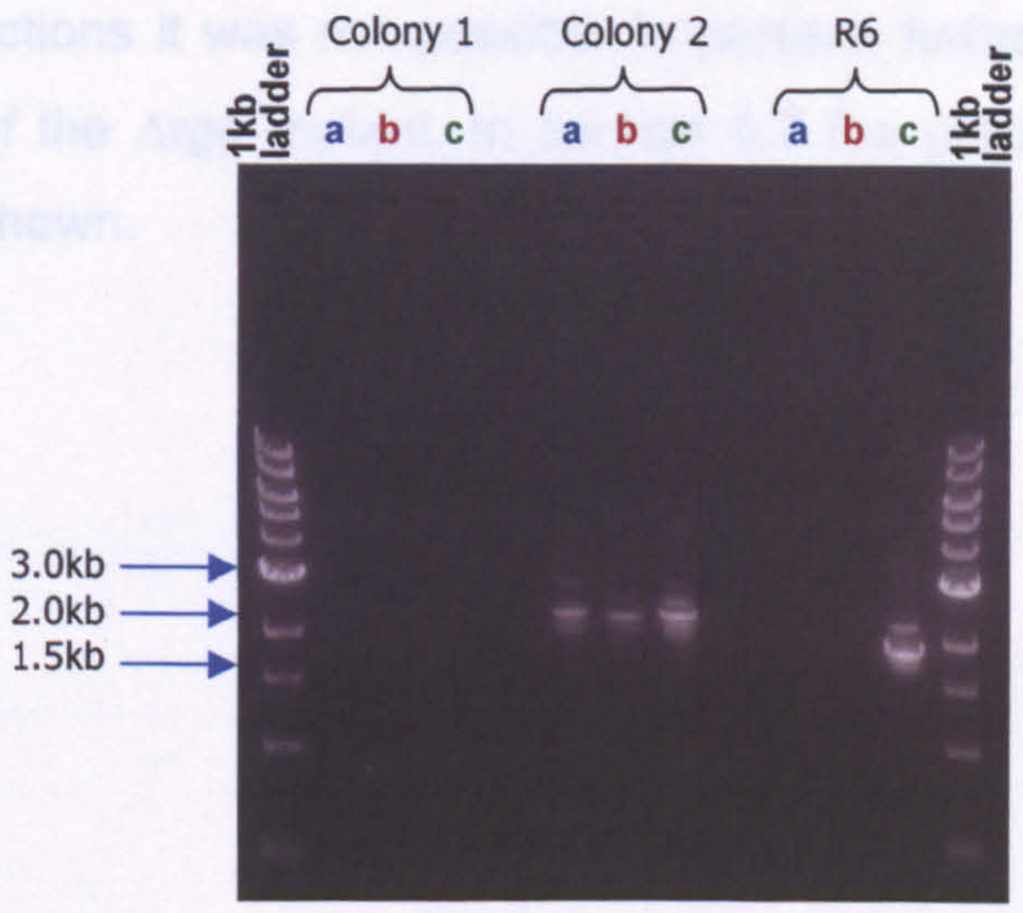
extracted from the colonies and PCR was first done to confirm for the presence or absence of a fragment of the *rgg* gene using primers sp2123F and sp2123R. From these four colonies, two did not show amplification of the *rgg* gene fragment (data not shown). These two colonies were selected for further confirmation of their mutation by PCR. In Figure 5.4-10A, the different primer pairs that were used for this purpose are shown. To confirm that a double homologous recombination had occurred, two PCRs were done using primers flanking the *rgg* gene but in regions not included in the construct made by SOEing PCR (for example primers rgg-1kb-F and rgg-1kb-R), in combination with primers present in the SOEing construction and absent in the wild type pneumococcal genome (for example primers specSOE-F and specSOE-R) (see Figure 5.4-10A). By doing this, only if the construction made by SOEing PCR had been integrated to the chromosomal DNA of the bacterium, would amplification be observed. Also, a third PCR using the primers A-SOE-F and D-SOE-R was done (see Figure 5.4-10A). These primers would allow PCR amplification on the mutant as well as in the wild type *S. pneumoniae* R6 control. The results from the PCRs are shown in Figure 5.4-10B, where it can be observed that no PCR amplification with any of the three PCR's was observed with colony 1. Colony 2 showed the expected amplification with all of the combinations of primers, indicating that the construction had been integrated on the chromosomal DNA of the pneumococcus. The *S. pneumoniae* R6 DNA control showed, as expected, no amplification when one of the primers bound to the spectinomycin cassette and amplification of the expected size was observed when primers A-SOE-F and D-SOE-R were used. Figure 5.4-10B shows the amplification bands, and Figure 5.4-10C the expected and obtained band sizes for all the PCRs done. The results indicated that the expected mutant was successfully obtained (colony 2), and it was named *S. pneumoniae* Δrgg.

Figure 5.4-10 PCR to confirm the construction of a pneumococcal mutant lacking the rgg gene. Figure A shows the localisation of the different primers used to confirm the construction of a pneumococcal mutant; the section shown in the dashed square (Figure A) represents the fragment made by SOEing PCR, which was then used to transform the pneumococcus. In A, the primers are indicated in colour. Primers of the same colour were used together in the PCR reactions. Figure B shows the agarose gel electrophoresis done after the PCRs with two colonies and with the wild type *S. pneumoniae* R6. The letters a, b and c indicating the lanes, are colour coded to show which pair of primers were used for the reaction (see Figure A). Figure C shows the expected and obtained sizes of the PCR bands. From the results, colony 2 was shown to contain the expected mutation.

A



B



C

	Colony 1			Colony 2			R6		
Lane	**a**	**b**	**c**	**a**	**b**	**c**	**a**	**b**	**c**
Expected size (Kb)	2.27	2.18	2.2	2.27	2.18	2.2	no amplification	no amplification	1.89
Obtained size (Kb)	did not amplify	did not amplify	did not amplify	~2.0	~2.0	~2.0	no amplification	no amplification	<2.0

5.5 *In vitro* growth curves of *S. pneumoniae* Δrgg

Growth curves under aerobic and anaerobic conditions were performed using BHI medium to compare the growth characteristics of *S. pneumoniae* Δrgg and *S. pneumoniae* R6. The growth curves are shown in Figure 5.5-1.

Table 5.5-1 shows the calculated generation time for each bacterial strain under each condition. As it can be seen from the Table, *S. pneumoniae* Δrgg had a significantly lower ($p<0.05$) generation time than did *S. pneumoniae* R6 in anaerobic conditions. Under aerobiosis, no significant differences were observed ($p>0.05$), however, the aerobic growth for *S. pneumoniae* R6 had only two replicates and more experiments are necessary to confirm this result.

Due to time restrictions it was not possible to perform further experiments for the characterisation of the Δrgg mutant. In section 6.7 the proposed experiments for this purpose are shown.

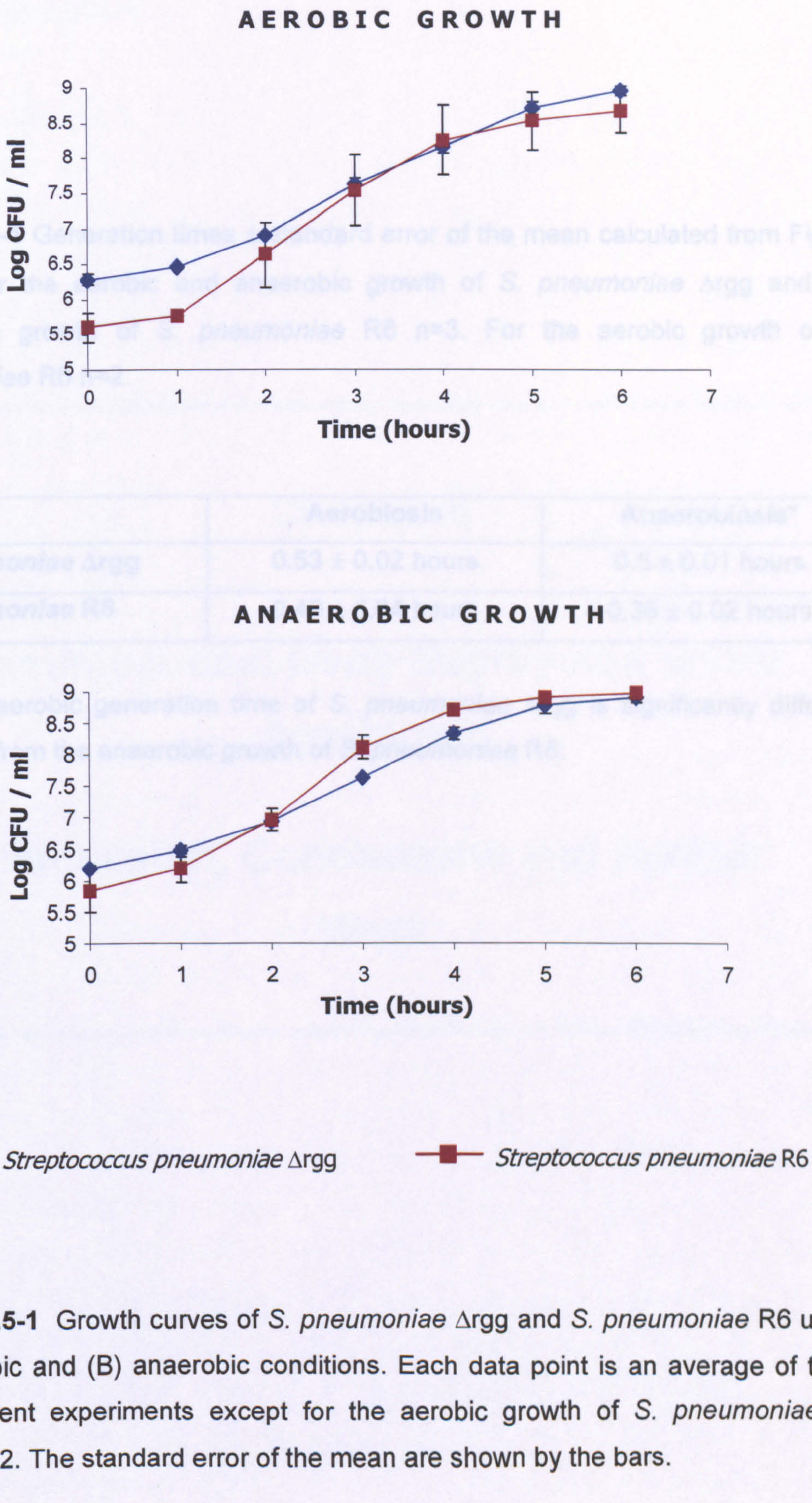


Figure 5.5-1 Growth curves of *S. pneumoniae* Δrgg and *S. pneumoniae* R6 under (A) aerobic and (B) anaerobic conditions. Each data point is an average of three independent experiments except for the aerobic growth of *S. pneumoniae* R6 where n=2. The standard error of the mean are shown by the bars.

Table 5.5-1 Generation times ± standard error of the mean calculated from Figure 5.5-1. For the aerobic and anaerobic growth of *S. pneumoniae* Δrgg and the anaerobic growth of *S. pneumoniae* R6 n=3. For the aerobic growth of *S. pneumoniae* R6 n=2.

	Aerobiosis	**Anaerobiosis***
***S. pneumoniae* Δrgg**	0.53 ± 0.02 hours	0.5 ± 0.01 hours
***S. pneumoniae* R6**	0.40 ± 0.04 hours	0.35 ± 0.02 hours

* The anaerobic generation time of *S. pneumoniae* Δrgg is significantly different ($p<0.05$) from the anaerobic growth of *S. pneumoniae* R6.

Chapter 6

Pneumococcal Gene Expression under Aerobiosis

Discussion, Conclusions and Further Work

6.1 To recapitulate

It is not understood how the pneumococcus, an aerotolerant anaerobe, is capable of adapting to aerobic environments and the consequent oxidative stress which it encounters, for example, during colonisation of the nasopharynx, a common route for pneumococcal infections (Schrag *et al.*, 2000). The pneumococcus lacks the regulatory genes, such as *oxy*R and *sox*RS, present in model organisms such as *E. coli* and *Salmonella typhimurium*, or *per*R in *B. subtilis* and *Staphylococcus aureus*, which are known to have an important role in the bacterial response to oxidative stress (Pericone *et al.*, 2003; homology search of the TIGR website in this work). Moreover, no regulatory genes have been identified in the pneumococcus to act as a response to oxidative stress. Also, the pneumococcus lacks the genes for the production of catalase (homology search done on the TIGR database in this work), an enzyme known in many organisms to have an important role in the defence against oxidative stress (Seaver and Imlay, 2001).

In order to contribute to the understanding of the pneumococcal survival in aerobic environments, microarrays were done to compare the gene expression of the pneumococcus grown under aerobiosis (as explained in 2.2.1.2) to that of the pneumococcus grown under anaerobiosis (as explained in 2.2.1.2). The aerobic and anaerobic environments in which to grow the bacteria for this purpose were analysed in this project, and can be seen on section 5.1 of the results. From the microarray results, a list of 69 genes whose expression changed when the pneumococcus was subject to oxidative stress was obtained. Of these, 55 were up-regulated and 14 were down-regulated under aerobiosis. From this list, genes present in *S. pneumoniae* R6 as well as in *S. pneumoniae* TIGR4, which showed more than eight-fold over-expression on the microarray results and some genes involved in transposon activities were chosen for confirmation of their expression by RTqPCR. From the confirmed genes, gene *rgg* (sp2123) was chosen for the construction of a pneumococcal mutant. This decision was made on the basis of selecting a gene over-expressed on aerobiosis that had not been studied before on the pneumococcus. Gene *rgg* had been reported in other streptococci to have a role in the adaptation to oxidative stress and in colonisation (Kreikemeyer *et al.*,

2003; Chaussee *et al.*, 2004), but no reports on studies of this gene were found for the pneumococcus. An *rgg* negative pneumococcal mutant was constructed by SOEing PCR. Growth curves in complex medium showed that the mutant had a decreased ability to grow in anaerobic conditions compared to wild type. Due to time restrictions, it was not possible to perform further characterisation of the pneumococcal mutant.

6.2 Aerobic and anaerobic growth

To evaluate the response of the pneumococcus to oxidative stress, it was decided to use a constant flow of environmental air into the growth medium, which kept oxygen present during bacterial growth. The reason for this was an attempt to expose the bacteria to a stress similar to that they would encounter, for example, during colonisation of the nasopharynx. It is known that in the presence of oxygen, the metabolism of *S. pneumoniae* produces the toxic forms of oxygen H_2O_2 and $^{\bullet}OH$ (Pericone *et al.*, 2003), and that $^{\bullet}O_2^-$ can be metabolically produced from an incomplete reduction of O_2 to H_2O (Yu *et al.*, 2001). Hence, by the input of oxygen in the growth medium, it was expected to identify the pneumococcal genetic response to atmospheric oxygen and the subsequent toxic forms of oxygen. To compare this genetic response, bacteria grown in the absence of oxygen, were used.

The aerobic growth was performed as explained in 2.2.1.2 of the materials and methods section, however this methodology presented some limitations. For example, it was not possible to maintain a constant level of aerobiosis throughout the growth. In the first attempts to create an aerobic environment (see section 5.1) it was found that the pneumococci would commence growth only after the levels of oxygen had dropped to a nearly anaerobic environment. It was subsequently found that this observation was also reported by Johnston *et al.* (2004). They found that the pneumococcus enters exponential phase only after the levels of oxygen were cleared from the medium as assessed by methylene blue, a dye that can be used

as a reporter for anaerobiosis (Johnston *et al.*, 2004). This finding might be related to the anaerobic nature of the pneumococcus. It is possible that during the lag phase, the pneumococci adapts to the presence of oxygen and starts to metabolise it. Without the further input of oxygen, its levels might drop sharply forcing the bacteria to re-adapt to the anaerobic environment. Only at this point would the environmental condition be stable, and the bacteria fully adapted and able to enter the exponential phase. In this project, this oxygen-clearing problem was "solved" by a constant bubbling of environmental air into the batch culture. Although it was not possible to maintain a constant percentage of dissolved oxygen through the bacterial growth, this approach allowed a continuous flow of air, subjecting the bacteria to the constant presence of oxygen.

An alternative growth approach could have been the use of a chemostat, in which a continuous culture can be obtain while maintaining the percentage of dissolved oxygen stable in the medium. The use of continuous cultures has been regarded as a way of obtaining reproducible results in a controlled environment in which the condition studied can be dissociated from other variables that can occur in batch cultures (Hoskisson and Hobbs, 2005). However, continuous cultures have some potential disadvantages. For example, the bacterial response to a particular stimulus can be influenced by the chosen growth or dilution rate at which to keep the bacteria, and there is also the possibility of favouring the growth of mutants better adapted to the controlled environment thereby producing a population swap (Ferenci, 2006). It has been shown that limiting the carbon substrate in a culture (which is used to adjust the growth rate) can produce an increase in the synthesis of enzymes involved in the metabolism of the limiting nutrient, or can induce the synthesis of enzymes involved in different metabolic pathways (Harder and Dijkhuizen, 1983). Ferenci (1999) states that the expression changes that occur in response to nutrient limitation are just "short-term adaptations before longer-term mutational changes take place". Also, Harder and Dijkhuizen (1983) in their review, cite that microbial enzymatic activity is affected by the dilution rate used. For example, a lower enzymatic activity of the bacteria occurs as the dilution rate increases (Harder and Dijkhuizen 1983). Mutational changes leading to population alteration, which can occur in a chemostat as a response to a stress condition, is common. In fact, continuous cultures have been commonly used for studies

involved with evolutionary applications (Ferenci, 2006). For example, Wilkins (Wilkins, 2000) refers to the use of chemostats as advantageous over the use of batch cultures to study intestinal flora because, as in living organisms it allows the production of mutations, and a quick detection of resistant strains present in a population.

The risk of obtaining a mutant more resistant to oxidative stress or of obtaining difference in gene expression caused by standard procedures used in a chemostat represented a potential and important bias for the results. This together with the fact that establishing a continuous culture is a time consuming prodedure, made the use of batch cultures the best available option.

6.3 Use of microarray

Microarray technology is a powerful tool by which the expression of thousands of genes can be monitored at one time (Hinton *et al.*, 2004), making it possible to elucidate expression profiles under a particular condition or to elucidate regulatory networks by the comparison of mutant strains (Conway and Schoolnik, 2003). In this study, microarray technology was used with the purpose of obtaining an overall view of the gene expression of *S. pneumoniae* when it was subject to aerobic environments, in comparison to anaerobic environments.

In obtaining of the bacterial samples used to perform the microarrays, the use of an RNA stabiliser was considered of major importance. For example, it is known that if the bacteria are subject to any change of environmental condition during or after the sample collection, such as a change in temperature, agitation or centrifugation, the gene expression could be altered in within few minutes (Hinton *et al.*, 2004). Indeed, it has been estimated that in *E. coli* the initiation of transcription at any given promoter can be as fast as once per second (Conway and Schoolnik, 2003), and that only one minute is necessary for the product of a large gene such as *lacZ* to be synthesised after a signal for gene induction is received (Conway and

Schoolnik, 2003), indicating the speed at which a change of gene expression could occur. Also, it has been estimated that the average half-life of bacterial mRNA is of around five minutes (Hinton *et al.*, 2004), making essential the use of a stabiliser agent to prevent mRNA degradation, as well as to stop transcription at the moment of sample collection. Guanidium is known to be an efficient stabilising agent for Gram-positive bacteria (Hinton *et al.*, 2004), and it was used in this project at the moment of sample collection (see 2.3.12 of materials and methods section).

The microarray data were analysed with the use of GeneSpring software from Aglient Technologies. This software for data analysis was chosen as it was the recommended software from the Bacterial Microarray Group at St. George's hospital, which was the group that provided the microarray slides used in this project. However, although microarray technology is a powerful tool, the need to confirm the microarray results by another method has become evident (Kothapalli *et al.*, 2002). In this project, reverse transcriptase quantitative PCR (RTqPCR) was used for this purpose, but due to time restrictions, this was only performed in a selection of genes, as explained in section 5.2.2.1.

6.4 Microarray results

In this section, the results of the project will first be compared to other studies where the global oxidative stress response of different bacteria was also evaluated. After, in groups, the genes that were observed in this project to undergo a change in gene expression when *S. pneumoniae* was subject to aerobic conditions will be discussed. Also, comment will be made briefly on the genes that are known in the pneumococcus or in other bacteria to be involved in the oxidative stress response, but that were not identified as changing of expression in this project. In further sections, the confirmation of the microarray results by RTqPCR, the interest in mutating the *rgg* gene in *S. pneumoniae* and the results of the mutation will be discussed.

6.4.1 Overview

Table 6.4-1 summarises the oxidative stress response observed in different bacteria when they were subject to H_2O_2 or paraquat stress (a source of $^{\bullet}O_2^-$). This includes publications on the genetic response of *Staphylococcus aureus* (Chang *et al.*, 2006), *Pseudomonas aeruginosa* (Chang *et al.*, 2005), *Neisseria gonorrhoeae* (Stohl *et al.*, 2005) and *B. subtilis* (Mostertz *et al.*, 2004) to H_2O_2 and/or paraquat. In the Table, the total number of genes reported to be altered in each bacterium in response to stress (H_2O_2 or paraquat), as well as the total number of altered genes identified in this project when *S. pneumoniae* was subject to an aerobic environment is shown. For comparison purposes, the total number of genes present in the chromosome, or in the array used to study each of these bacteria is also shown, and it was used to calculate the percentage of genes that underwent a change in expression in each condition. Also shown in Table 6.4-1 are the metabolic properties of each bacterium. However, because all of them are able to adapt to the presence or absence of oxygen, discussion on this subject will not be done. Even though caution must be observed when comparing the expression results between different studies due to differences in the nature of the arrays and to technical and interpretation procedures, it is useful for obtaining a general view of the experiment's "behaviour".

Table 6.4-1 Summary of the oxidative stress response observed when different bacteria were subject to H_2O_2 and/or paraquat stress. A summary of the data obtained in this project when *S. pneumoniae* was subject to aerobiosis is also shown. *The metabolic properties of each bacterium were obtained from Masalha *et al.* (2001) for *S. aureus*; Schreiber *et al.* (2006) for *P. aeruginosa*; Knapp and Clark (1984) for *N. gonorrhoeae*; Nakano and Zuber (1998) and Ye *et al.* (2000) for *B. Subtilis*; Kadioglu *et al.* (2003) for *S. pneumoniae*. **The number of genes present in the array was not mentioned by the authors; the number shown is the total number of ORFs present in that bacteria strain according to the TIGR database.

Bacteria species	*Metabolism	Genes present in the array	Challenge	Up-regulated genes	Down-regulated genes	% of genes with altered expression	effect of the challenge in growth	Reference
Staphylococcus aureus NCTC8325	Facultative aerobe	**2892	10mM H_2O_2 for 10min	113	151	9.1	growth inhibition	Chang *et al.*, 2006
Staphylococcus aureus NCTC8325	Facultative aerobe	**2892	10mM H_2O_2 for 20min	95	24	4.1	initial growth inhibition and recovery	Chang *et al.*, 2006
Pseudomonas aeruginosa PA01	Facultative aerobe	5570	1mM H_2O_2 for 20min	116	107	4.0	growth inhibition	Chang *et al.*, 2005
Neisseria gonorrhoeae FA1090	Facultative aerobe	2704	5mM H_2O_2 for 15min	74	80	5.7	not mentioned	Stohl *et al.*, 2005
Bacillus subtilis 168	Facultative aerobe	4107	58μM H_2O_2 for 10min	92	97	4.6	reduced growth rate	Mostertz *et al.*, 2004
Bacillus subtilis 168	Facultative aerobe	4107	10mM paraquat for 10min	129	169	7.3	cessation of growth	Mostertz *et al.*, 2004
Streptococcus pneumoniae R6	Aerotolerant anaerobe	2084	growth on aerobiosis	55	14	3.3	no effect on growth	this study

From Table 6.4-1 it can be observed that the experiments that showed the highest percentage of change in gene expression were with *Staphylococcus aureus* and *B. subtilis* after challenge with 10mM H_2O_2 and 10mM paraquat respectively. The challenge used in these two experiments had a similar effect on the bacteria. It was reported by Chang *et al.* (2006) that treatment of *S. aureus* with 10mM H_2O_2 caused an initial growth inhibition, which could still be observed after 10min of the challenge. Similarly the paraquat concentration used by Mostertz *et al* (2004) in the challenge of *B. subtilis*, was reported to cause a cessation in the bacterial growth (Mostertz *et al.*, 2004). By contrast, except for the experiment with *P. aeruginosa* where growth inhibition was also observed, the authors did not report a drastic change in growth. In the *P. aeruginosa* experiment, although the percentage of genes altered by the treatment with H_2O_2 was not particularly high (4%), it can be observed that the expression of more than 200 genes was altered. The comparison of these results shows a common behaviour in that it is when the challenge to the bacteria produces a greater impact on the bacterial growth the expression of the highest number of genes becomes affected.

From Table 6.4-1 it can also be observed that the results from this project show that *S. pneumoniae* has the lowest number of genes with an altered expression, as well the lowest percentage of genes affected. This could be due to several reasons. One could have been the selection method used to identify the affected genes. As can be reviewed in section 5.2.1 of the results, after selection of genes based on normalisation, p-value, quality on the spots (flags) and fold-change was done, a final selection was done where only genes whose fold change was equal or greater to two-fold in each individual experiment were considered. Appendix 1 shows the list of genes if this last selection had not been done. This list shows a total of 119 genes with an altered expression (86 up-regulated and 33 down-regulated) representing 5.7% of the genome, making the results "comparable" to other experiments in Table 6.4-1. However for this project, the most conservative approach was taken. It is difficult to compare the selection used in this project to those used in the other studies mentioned in Table 6.4-1 due to different software used to perform the analysis, and the use of macroarrays by Mostertz *et al.* (2004). For example, Chang and collaborators (Chang *et al.*, 2005; Chang *et al.*, 2006) used the Affymetrix Gene Chip Operating Software and Data Mining Tool to

perform statistical analysis but gave only the settings entered to the program. In their experiments of 2005 (Chang *et al.*, 2005), they also mentioned a filter done on flags excluding spots that were absent in 50% or more of the experiment replicates, but no mention was made on selecting for bad spots. Stohl and collaborators (2005) stated that "spots were analysed by adaptive circle quantification, local background was subtracted for each spot and Lowess normalisation was applied (...) Results were exported to Excel and statistical analyses were performed" but gave no more detail on the selection of spots. Helmann and collaborators (2003) gave complex detail of their analysis, where a selection of spots that showed an expression equal or greater than 3-fold was done. They also mention a selection done on standard deviation, and a normalisation done using the Stanford Microarray Database. In summary, all the microarray experiments performed statistical analysis and normalisation procedures according to the software used for the analysis. Selection on the quality and/or intensity of the spots was apparently not common to all of the experiments, and none performed a final selection of genes as was done in this project. Commonly, the expression of a gene is only considered as an average of various experiments, and looking for its expression in the individual experiments is not done. As it has been mentioned before, an aim of this project was to select a gene to produce a pneumococcal knockout mutant. For this reason, it was considered of importance to perform this final selection assessing that the expression the genes was two or more fold difference from equality in the individual experiments.

Another possibility for the low number of genes identified in this project, compared to the others in Table 6.4-1, is the nature of the challenge. For example, in this project, it can be expected that in the presence of oxygen, the pneumococcus will be subject to different toxic forms of oxygen, however, the levels produced might not reach the concentrations of H_2O_2 or of $^{\bullet}O_2^-$ produced by paraquat, used to study the stress response in the other organisms. It is possible that in response to the presence of oxygen (which in this study was present since the beginning of the growth), *S. pneumoniae* would have adapted its metabolism to overcome this environmental stress, without having an "emergency" response. By constrast, the other experiments exposed mid-logarithmic phase bacteria to the stress challenge for a short period of time, which might result in a stress response closer to a shock

response, as the integrity of the bacteria might be at risk. For example, in Table 6.4-1, this can be observed with the studies of Chang *et al* (2006) on *S. aureus*, where a lower number of genes with a change in expression was observed after 20min of exposure to H_2O_2, compared to 10min of exposure. This indicates that the bacteria responded with a larger number of genes to the sudden input of stress.

As was mentioned in the introduction of this thesis, it known that *Salmonella* and *E. coli* respond with different set of genes to the stress caused by H_2O_2 or by $^{\bullet}O_2^-$. If this was the case for all bacteria, is possible that the presence of oxygen might induce some of the genes involved in these stresses, but perhaps not all of them, resulting as well, in a lower number of genes involved in aerobic stress.

The results of this project can also be compared to those of the pneumococcus under different stress conditions. Experiments by Martin-Galiano *et al.* (2005) have evaluated the genetic response of *S. pneumoniae* R6 to acid pH stress, studying the response at different time points post-challenge. In their investigation, they observed different numbers of genes over and under-expressed according to the amount of time that the bacteria were subject to low pH. This ranged from 32 genes after 5 minutes of exposure to low pH, to 78 and 67 genes after 30 and 200 minutes of exposure respectively (Martin-Galiano *et al.*, 2005). The number of genes with a change of expression identified by Martin-Galiano *et al.* (2005) at 30 and 200 minutes post-exposure is similar to the number of genes identified in this project (69 genes). In their experiments, Martin-Galiano *et al.* (2005) mention that at 30 minutes post-exposure, the bacteria were at the end of the adaptation phase to the low pH input, which was estimated by the delay in growth rate. At 200 minutes post-exposure, they mention that the bacteria had adapted to the low-pH stress, as they had recovered from the initial delay in growth. It is interesting to observe that in the study of Martin-Galiano, fewer genes with a change of expression were identified after 5 minutes of exposure to low pH compared to 30 and 200 minutes. As mentioned before, it could be expected that a larger number of genes might be altered at the initial stages of a stress input; however, the authors did not discuss this issue.

6.4.2 Genes identified by microarray to suffer a change in gene expression when the pneumococcus was subject to aerobic environment

In this project, 69 genes in the pneumococcus were identified by microarray to undergo a change in expression under aerobiosis. Of them, 55 genes were over-expressed when the bacteria were subject to aerobic conditions, and 14 genes were under-expressed. In this section, a description of these genes will be done.

Table 6.4-2 shows these genes divided into various groups for description purposes. The groups were labelled according to their predicted products into:

- Hypothetical and conserved hypothetical
- Genes involved in genome plasticiy
- ABC transporters
- Ribonucleotide reductase system
- Bgl and PTS related
- Known oxidative stress related
- Others

In the Table, genes that were over-expressed under aerobiosis are shown in blue, and genes that were over-expressed under anaerobiosis are shown in red. Through this section, genes over-expressed under anaerobiosis, are also referred to as under-expressed in aerobiosis.

In Table 5.2-1 and Table 5.2-2 of the results section, or on appendix 1, the annotation of these genes can be found.

Table 6.4-2 Genes identified by microarray to suffer a change of expression when *S. pneumoniae* was grown on aerobiosis compared to its growth on anaerobiosis. The genes are divided into groups according to their predicted products. In blue, it is indicated those genes that were over-expressed under aerobiosis, whereas in red, it is indicated those genes that were under-expressed in aerobic environment.

Hypothetical and conserved hypothetical					
sp0965	sp0966	sp0967	sp0968	sp0095	sp0731
sp0992	sp1250	sp1332	sp1346	sp1424	sp1459
sp1495	sp1691	sp1694	sp1728	sp1802	sp2004
sp2017	sp2046	sp2047	sp2048	sp2104	sp2178
sp0482	sp0206	sp0319			
Involved in genome plasticity					
Transposons and IS elements					
sp0732	sp1314	sp1441	sp1496	sp1515	sp2181
sp2211	spr0957				
Competence					
sp2050	sp2051	sp2207			
ABC transporters					
sp1426	sp1434	sp1435	sp1438	sp1919	sp1871
sp1869	sp1870	sp1872			
Ribonucleotide reductase system					
sp1179	sp0202	sp0205			
Bgl and PTS related					
sp2032	sp2034	sp2035	sp2036	sp2038	

Known oxidative stress related					
sp0766	sp1651				
Others					
sp0718	sp1013	sp1326	sp1382	sp1759	sp1767
sp2123	spr0960	sp0338	sp0204	sp0320	sp0318

6.4.2.1 Hypothetical and conserved hypothetical

A large number of genes producing hypothetical proteins were identified in this project, they accounted for 39.1% of the results. With the use of microarrays, the relevance of more genes with still unknown functions becomes evident, and their expression forms a big part of any microarray experiment. From the results of this study, genes producing the hypothetical proteins sp1802 and sp2047, which were over-expressed in aerobiosis, and sp0206, which was under-expressed, were found to be present in the results of other pneumococcal studies that used microarrays. For example, gene sp1802 was found under-expressed when the pneumococcus was subject to pH stress (Martin-Galiano *et al.*, 2005) and over-expressed in pneumococcal cells that were attached to pharyngeal epithelial cells in vitro (Orihuela *et al.*, 2004). The presence of this gene in the experiments of three different laboratories, suggest it has potentially an important role in the pneumococcal virulence. The over-expression of this gene (sp1802) found by Orihuela *et al.* (2004) when the pneumococcus was attached to pharyngeal epithelial cells could be due to several reasons: a response of a factor released by the host cell, in which case this gene might act as a stress response gene, or to the attachment to host cell in itself, in which case it might be related with colonisation.

Gene sp2047 mentioned above, was also identified by Martin-Galiano *et al.* (2005) in the pneumococcus as under-expressed at low pH, and gene sp0206 (also mentioned above) was identified as over-expressed in pneumococci recovered from blood after infection (Orihuela *et al.*, 2004). As before, their appearance in

different studies indicates a possibly important role of these genes in virulence, which might be worth studying.

6.4.2.2 Genes involved in genome plasticity

Several genes with functions such as transposon activities, insertion sequences and competence, were found over-expressed when the pneumococcus was subject to oxygen stress. This type of response, which can induce genetic diversity, can occur when bacteria are subject to a stress condition as a way of adaptation, survival and virulence. For example, antibiotic resistance in the pneumococcus can be acquired by transformation of DNA from related species, or by transposons, which are large sequences of DNA with self-transfer functions (Schrag *et al.*, 2000). It has been shown in different bacteria that transposition can be regulated by environmental conditions that affect the physiological state of the cell (Ghanekar *et al.*, 1999). For example, nutritional deprivation in *E. coli* cultures augments the transposition of IS elements (Ghanekar *et al.*, 1999). Transposon activity in *E. coli* and *S. faecalis* can be induced by sodium acetate and erythromycin respectively (Ghanekar *et al.*, 1999), and transposition in *M. tuberculosis* is induced by microaerobic environments (Ghanekar *et al.*, 1999), a stress condition for mycobacteria.

In the pneumococcus, it has been shown that oxygen can control the induction of competence (Echenique *et al.*, 2000), and that induction of competence increases the pneumococcal virulence of *in vivo* pneumonia (Oggioni *et al.*, 2006). In this project, the genes *com*F also referred to as *com*FC (sp2207); *cgl*D (sp2050); and *cgl*C (sp2051), were over-expressed when the bacteria were subject to aerobic environments. Genes *cgl*C and *cgl*D belong to the cgl locus, which has been described as crucial for the production of competence, as its absence results in a complete loss of transformability of the pneumococcus (Pestova and Morrison, 1998). Genetic analysis evaluating the competence response in the pneumococcus, have also shown the up-regulation of *cgl*C and *cgl*D during competence, however, no mention was made of *com*F (Rimini *et al.*, 2000). In *S. pneumoniae*, comF has

been poorly characterised (Song *et al.*, 2005), but microarray studies have found this gene expressed on the late stages of competence (Dagkessamanskaia *et al.*, 2004). In *B. subtilis*, *com*F is known to be a late competence gene involved on the DNA uptake machinery (Ogura *et al.*, 2002).

The observations of genes involved with competence in the project (*cgl*C, *cgl*D and *com*F), support previous findings where the production of competence was associated with the presence of oxygen (Echenique *et al.*, 2000). Consequently it is also in accordance with the statement that environmental conditions can augment transposition activities (Ghanekar *et al.*, 1999). It is possible that these mechanisms of genome plasticity involving transposon activities and competence, might help the pneumococcus during colonisation of the nasopharynx, where it might interact with other bacteria and uptake DNA that might help its survival, for example by the uptake of DNA that might provide antibiotic resistance. Also as mentioned before, it has already been shown that the lack of competence diminishes pneumococcal virulence in a mouse model of pneumonia (Oggioni *et al.*, 2006). Interestingly, competence has also been shown related to the formation of biofilm, which has an apparent role in meningitis and pneumonia (Oggioni *et al.*, 2006).

6.4.2.3 ABC transporters

From the results of this project, it was observed that the ABC transporters sp1869 (*fat*D also known as *piu*B), sp1870 (*fat*C, also known as *piu*C), sp1871 (*fec*E, also known as *piu*D) and sp1872 (*fat*B, also known as *piu*A), which form an iron transport operon (Ulijasz *et al.*, 2004), were under-expressed when the bacteria were subject to aerobic environment. These results might indicate an adaptive response of the pneumococcus to oxidative stress by reducing levels of intracellular iron, consequently preventing the production of $^{\bullet}OH$ via the Fenton reaction. To illustrate the importance of iron regulation in oxidative stress, some comparisons will be done with bacteria that contain the Fur regulon (which is

involved in the regulation of iron transport) however, this regulon is not present on *S. pneumoniae*.

In *E. coli* it is known that under oxidative stress, the repressor of iron uptake Fur is activated to maintain low levels of intracellular iron (Iuchi and Weiner, 1996). In *S. pneumoniae*, it has been found that RitR acts as a repressor of iron uptake, inhibiting the expression of *fat*B, which encodes a permease, and of *fat*D, which encodes an iron binding protein (Ulijasz *et al.*, 2004); however, in this project, its corresponding gene (*rit*R) was not found over-expressed under aerobiosis. It is possible that in the present study, these genes might be regulated by a way other than *rit*R.

Results from other projects, for example, a study done on *Pseudomonas aeruginosa* response to H_2O_2 stress, also showed a repression of the iron uptake system under this oxidative stress input (Chang *et al.*, 2005). However, this finding does not remain constant through other oxidative stress studies, for example, studies on *B. subtilis*, found that although the Fur regulator was over-expressed, some genes involved in iron uptake would be over-expressed when the bacteria were subject to H_2O_2 (Mostertz *et al.*, 2004). The author hypothesised that the Fur regulator might have a role on sequestering iron, and that the genes involved in iron uptake were expressed as a response to low iron, which might be necessary for the activity of proteins such as catalase (Mostertz *et al.*, 2004). On the other hand, studies done by Chang *et al.* (2006) on *S. aureus*, lead the authors to hypothesise that iron uptake in this bacterium was initially repressed under oxidative stress as a response to high levels of intracellular iron caused by the Fenton reaction, but that the iron uptake genes would be expressed again, once the intracellular iron levels reached equilibrium.

No information was found regarding the ABC transporters sp1426, sp1435, sp1438 and sp1919, which were over-expressed under aerobiosis, and further study of these genes is necessary to elucidate their role on the pneumococcus. Gene sp1434, also over-expressed under aerobiosis, was previously identified by Orihuela *et al.* (2004) as possibly having a role during infection in a mouse model of bacteremia, which they determined by studying the pneumococcal gene

expression during infection (Orihuela *et al.*, 2004). However, mutation of this gene did not appear to have an effect on the pneumococcal virulence (Orihuela *et al.*, 2004).

6.4.2.4 Genes involved in ribonucleotide reductase systems

Three genes that belong to two different ribonucleotide reductase systems were found over-expressed under anaerobiosis. These systems have a very important role in a cell, as it provides the deoxyribonucleoside triphosphates needed for the synthesis of DNA (Torrents *et al.*, 2001). The gene sp1179 or *nrd*E, together with gene *nrd*F (not identified in this project), and others, belong to the class I of ribonucleotide reductases, which are present in higher organisms and in many microorganisms, and are considered to be oxygen dependant (Torrents *et al.*, 2001). The presence of *nrd*E in the anaerobic condition of this project will be discussed later in this section. The genes sp0202 and sp0205, also known as *nrd*D and *nrd*G respectively, belong to the class III, which are present in microorganisms with anaerobic metabolism, and are highly sensitive to the presence of oxygen (Torrents *et al.*, 2001).

In this project, genes belonging to the class I and class III ribonucleotide reductase systems, were found over-expressed under anaerobiosis. Studies on these genes in other bacteria have also lead to discrepant results if their function is specifically active during aerobiosis or anaerobiosis respectively. For example, in *B. subtilis*, the *nrd*EF genes (which belong to the aerobic class I) are reported to be the only ribonucleotide reductase genes of this bacterium (Härtig *et al.*, 2006). Mutations on the *nrd*E gene showed that this gene was essential for the anaerobic growth of *B. subtilis* when an external source of deoxyribonucleosides was not provided (Härtig *et al.*, 2006). In *S. aureus*, which contains both class I and class III genes, it was shown that the *nrd*DG genes were essential for the anaerobic growth (Masalha *et al.*, 2001). In *Lactococcus lactis*, which as *S. pneumoniae* belongs to the family of lactic acid bacteria, it was found that an *nrd*D negative mutant grew as well as the wildtype under the anaerobic conditions created with a fermentor. This normality in

growth was attributed to the *nrd*EF genes (Jordan *et al.*, 1996). However, when sodium sulfide was added to the growth medium to eliminate remaining oxygen, the *nrd*D gene became essential for growth, indicating the lack of activity of the *nrd*EF genes under this strict anaerobic condition (Jordan *et al.*, 1996). It is possible that this last situation had occurred in this project, where traces of oxygen maybe remaining in the medium of the anaerobic gorwth caused the observed over-expression of the *nrd*E gene.

Interestingly, in a pneumococcal virulence experiment, the *nrd*D and *nrd*G genes were found over-expressed in bacteria recovered from the blood in a mouse model of bacteraemia (Orihuela *et al.*, 2004). Moreover, the *nrd*G gene was also over-expressed in bacteria recovered from the cerebrospinal fluid in a rabbit model of meningitis (Orihuela *et al.*, 2004). It would be interesting to characterise by the construction of pneumococcal mutants, the role of the *nrd*D and *nrd*G genes during anaerobic growth, and how it relates to virulence.

6.4.2.5 Bgl and PTS related genes

The Bgl system is involved in the regulation of the expression of genes that are related to sugar utilisation (Amster-Choder, 2005). This system comprises a BglF, which belongs to a family of Phosphotransferase systems (PTS) that can detect and transport carbohydrates into the cell (Amster-Choder and Wright, 1997; Amster-Choder, 2005). BglF, upon encounter of a carbohydrate, activates BglG, which acts as a transcriptional antiterminator (Amster-Choder, 2005). In this project, several genes with putative functions involving this system were over-expressed in the aerobic condition; that is, the gene sp2032, which encodes BglG, and two genes involved in the PTS system (sp2036 and sp2038). Normally, enzymes involved in the transformation of carbohydrates imported by the PTS system into the intermediate of glycolysis, fructose-6-phosphate, also form part of this system (webpage-3). It is possible that the genes sp2034 and sp2035 (also up-regulated in aerobiosis), which encode a putative hexulose-6-phosphate isomerase and a

putative hexulose-6-phosphate synthase respectively, could have a similar role to this conversion.

S. pneumoniae utilises carbohydrates as the sole sources of energy to support growth and cell division (Hoskins *et al.*, 2001), and the PTS system is an efficient way of transporting carbohydrates (Kotrba *et al.*, 2001; webpage-3). In *Lactococcus lactis*, it has been suggested that the PTS system increases the rates of consumption of sugar, and the efficiency of homolactic fermentation (Bolotin *et al.*, 2001). It is possible that in the pneumococcus, this system has an equal effect in increasing the efficiency of fermentation. The reason why these genes were over-expressed under aerobiosis is not clear. It is possible that a difference in the growth rate between the aerobic and anaerobic cultures could have triggered this response, however, samples were taken during mid-exponential phase from both cultures, and no nutrient limitation is expected at this point of growth. Studies in other bacteria such as *S. mutans* and *Listeria monocytogenes*, have found that the genes of the PTS system can be involved in bacterial attachment to surfaces (Abranches *et al.*, 2006; Gorski *et al.*, 2003). It would be interesting to test whether this could be the case of *S. pneumoniae*, and analyse if the presence of a stress condition, such as aerobiosis, could stimulate attachment, which could be of great importance during colonisation.

6.4.2.6 Known oxidative stress related genes and others

Genes *sod*A (sp0766) which codes an MnSOD and gene *tpx*, also known as *psa*D, (sp1651) which codes for a thiol peroxidase, were found over-expressed under aerobiosis. These genes have shown to be related to the oxidative stress response of the pneumococcus, and were described on section 1.5.3 of the introduction. To mention briefly, the importance of these genes in the pneumococcal survival of oxidative stress has previously been shown. For example, deletion mutations of the *sod*A gene in the pneumococcus produced a mutant that was more susceptible to oxidative stress induced by paraquat (Yesilkaya *et al.*, 2000) and deletion on the

pneumococcal *psa*D gene produced mutants more susceptible to killing by H_2O_2 (Tseng *et al.*, 2002).

From the genes over-expressed under aerobiosis, gene sp1326, which codes a putative neuraminidase, now known as nanC (Pettigrew *et al.*, 2006), was identified as over-expressed. However this gene is not present on the genome of *S. pneumoniae* R6. This bacterium (*S. pneumoniae* R6), does contains the genes from two other neuraminidases, nanA and nanB (search done on the TIGR database). It is possible that in the experiments, the product of these genes could have cross-reacted with the spot on the microarray corresponding to sp1326, but this was not studied. Another of the genes that appeared over-expressed under aerobiosis is gene spr0960, which codes for a MutR protein known in *S. mutans* to be a transcriptional regulator of a family of mutacins, which produce antimicrobial peptides also called bacteriocins (Qi *et al.*, 1999). This mutacins are known to help *S. mutans* compete with other bacteria in the community of oral biofilm (Merritt *et al.*, 2005). *S. pneumoniae* is known to have the ability to kill other bacteria *in vitro* by the production of hydrogen peroxide (Regev-Yochay *et al.*, 2006). It is possible that the pneumococcus could also utilise genes such as *mut*R (spr0960) to compete with other bacteria during colonisation.

Gene *rgg* (sp2123 or spr1933), was also over-expressed under aerobiosis, and was the gene chosen for the construction of a pneumococcal mutant in *S. pneumoniae* R6. In section 6.6, a wider description is given of what is known in other streptococci in regard to the *rgg* gene and why this gene was chosen for the construction of a mutant. Gene *clp*L, which was under-expressed under aerobiosis codes for the thermal stress protein ClpL. Under-expression of this gene might be related to the over-expression of the *rgg* gene, however, this will also be discussed later (section 6.6).

6.4.3 Genes known as oxidative stress response genes that are present in the pneumococcus but did not suffer a change of expression

From the microarray results, it was surprising that of the genes known in other bacteria to serve in the oxidative stress response, only the *sod*A and *tpx* were found over-expressed under aerobiosis. Figure 6.4-1 shows the area of localisation of most of these genes in the microarray scatter plot, as well as their p-value. In this Figure, LOWESS normalisation, a cut-off value of 10 on the intensity of the spot, and a filter on flags was done on the microarray. As it can be seen from the Figure, some genes did have a significantly different p-value between the aerobic and anaerobic expression, however, their fold-difference of expression from equality was <2-fold, and were therefore not considered as differently expressed. This was the case for genes sp1243 (*zwf*), sp0784 (*gor*), sp0313 (glutathione peroxidase), sp1907 (*gro*ES), sp1906 (*gro*EL) and sp0517 (*dna*K). As has been mentioned before, it is possible that the aerobic environment used in this project did not cause as strong a response as H_2O_2 or paraquat (used in most oxidative stress studies) might have caused.

From Figure 6.4-1, it can be seen that genes that are known in *E. coli* to be regulated by the OxyR or SoxRS regulons: *zwf* (sp1243), glutathione reductase (sp0784 or *gor*), and a *dps* homologue (sp1572 or *dpr*) were found on the array, but did not satisfied the conditions to be considered as differently expressed. Only gene *sod*A (sp0766), known in *E. coli* to be regulated by SoxRS appeared over-expressed on aerobiosis (not shown in the Figure).

Genes found in other bacteria to serve in the DNA or protein repair mechanisms, and to be expressed during oxidative stress, such as endonuclease III (sp1279 or *nth*), *gro*EL (sp1906), *gro*ES (sp1907), *dna*K (sp0517) and *clp*P (sp0746) were also considered as equally expressed in both conditions (Figure 6.4-1) (Farr and Kogoma, 1991; Fredriksson *et al.*, 2005). From these, genes *gro*EL and *dna*K had been previously reported to respond poorly to oxidative stress in the pneumococcus, and to show an increased expression in heat-shock stress (Choi *et al.*, 1999). Studies on *gro*ES and *clp*P in the pneumococcus have also shown that

these genes respond to heat-shock stress (Kim *et al.*, 2001; Kwon *et al.*, 2003), but no information was found on their response to oxidative stress.

Genes *spx*B (sp0730) and *nox* (sp1469), which had previously been described to be important for the pneumococcal survival in the presence of oxygen (Yu *et al.*, 2001; Spellerberg *et al.*, 1996), as well as glutathione peroxidase (sp0313 or *bas*A), were also considered as equally expressed under the selection criteria used in this project (Figure 6.4-1). However, it is important to consider that the lack of over-expression of a gene in a particular condition does not necessarily indicate a lack of importance of the gene under this condition. It is possible that some of these genes are constitutively expressed in the pneumococcus and play an important role in the bacteria survival to aerobiosis.

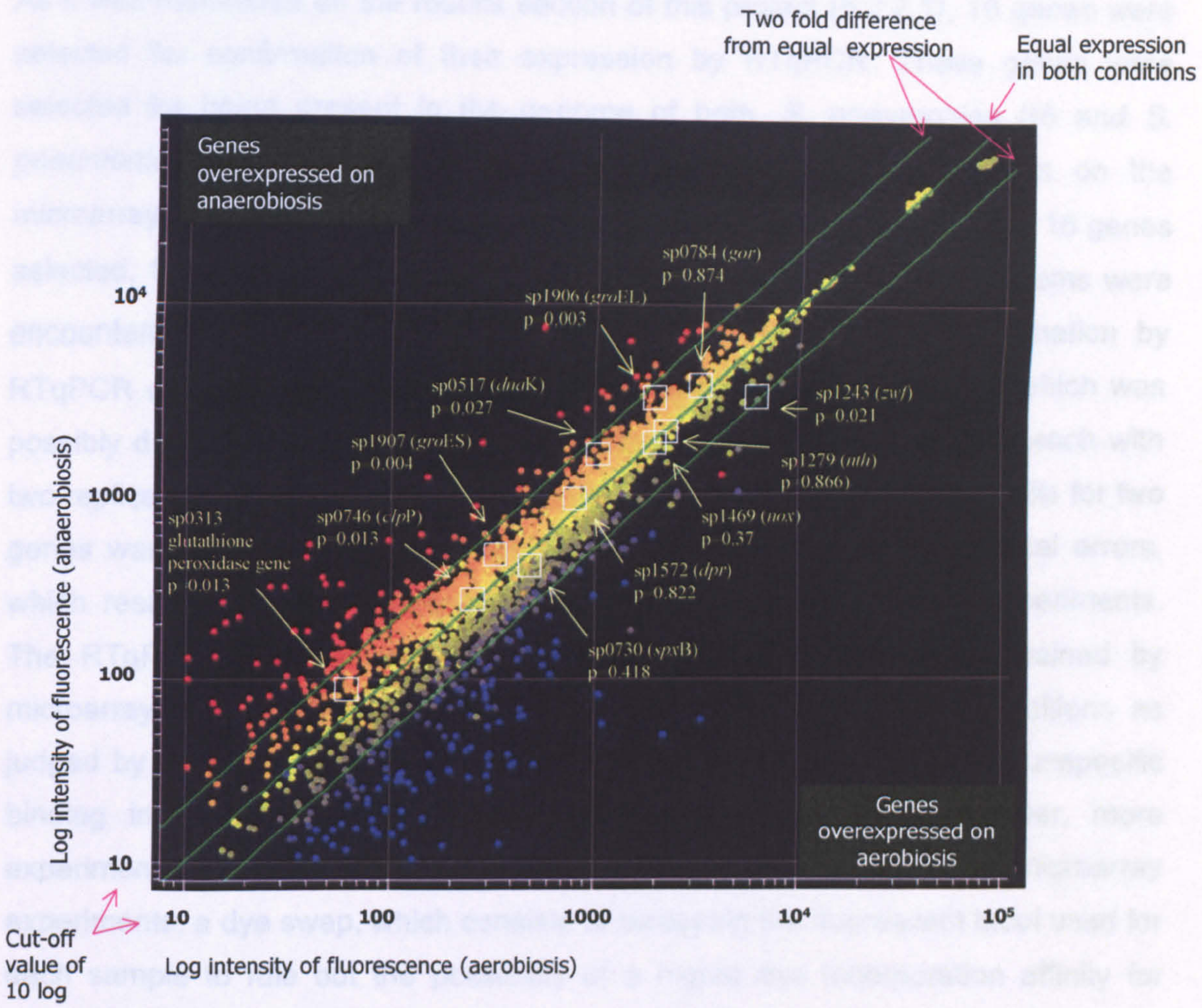


Figure 6.4-1 Genes found in the pneumococcus or in other bacteria related to the oxidative stress response, but that were not identified in this project as to suffer a change of expression in response to aerobic growth. The area of localisation of the gene in the microarray scatter plot is shown in a white square. Indicating the square is the name of the gene, whose common name is shown in within prenthesis and the p-value. To obtain the scatter plot, the data obtained from the microarrays was subject to LOWESS normalisation, cut-off value of 10 on the log intensity of fluorescence of the spot, and a filter on flags as described in section 5.2.1 of the results.

6.5 Confirmation of the microarray results by RTqPCR

As it was mentioned on the results section of this project (5.2.2.1), 16 genes were selected for confirmation of their expression by RTqPCR. These genes were selected for being present in the genome of both, *S. pneumoniae* R6 and *S. pneumoniae* TIGR4; and showing a >8-fold difference in expression on the microarray results or for having a predicted transposon activity. From the 16 genes selected, the expression of only 9 genes was confirmed. Several problems were encountered when performing these experiments. Initially, the confirmation by RTqPCR of three genes was excluded for problems with the primers, which was possibly due to low primer affinity. Two different RTqPCR experiments, each with two replicates, were performed for each of the remaining genes. The results for two genes was reported as inconclusive. This was probably due to technical errors, which resulted in huge differences between replicates or between experiments. The RTqPCR result of one gene was opposite from the result obtained by microarray, and another gene showed equal expression on both conditions as judged by RTqPCR (see Table 5.3-3 of the results). It is possible that unspecific binding in the microarray slide caused this opposite result. However, more experiments would be needed to confirm this hypothesis. Also, in the microarray experiments, a dye swap, which consists of swapping the fluorescent label used for each sample to rule out the possibility of a higher dye incorporation affinity for some spots, or on the array, was not performed. In consequence, it remains possible that the unspecific binding could be as a result of a dye incorporation affinity. Unfortunately, due to time restrictions it was not possible to perform the dye-swap experiment on the microarray, nor to repeat the RTqPCR experiments for those genes from which inconclusive data was obtained. Also, because there was a greater interest on studying the *rgg* gene, whose microarray result was confirmed by RTqPCR, it was not prioritised to reassess the inconclusive data.

6.6 The *rgg* gene

The genomes of some low G+C Gram-positive bacteria have been found to encode different members of a transcriptional regulator family known as Rgg (Dmitriev *et al.*, 2006). These include the Rgg and RggD of *S. gordonii* (see below); GadR of *Lactococcus lactis*, which is involved in glutamate-dependent acid tolerance, Rgg or RopB of *S. pyogenes* (described below) and MutR of *S. mutans*, which is involved in the production of mutacins (Chaussee *et al.*, 2003; Vickerman *et al.*, 2001). Proteins of this family contain a helix-turn-helix motif that is thought to be involved in the binding and regulation of other genes (Dmitriev *et al.*, 2006). Gene rgg has been found present in the complete published genomes of *S. pneumoniae*, that is, *S. pneumoniae* R6, *S. pneumoniae* G54 and *S. pneumoniae* TIGR4 (homology search in the TIGR database). However, in *S. pneumoniae* TIGR4, the gene contains a frameshift in its sequence, possibly leading to an inactive protein. Protein blast of the Rgg amino acid sequence from *S. pneumoniae* R6 on the NCBI database, revealed that pneumococcal Rgg has 45% of identity to the RggD of S. gordonii (search done using the BLAST service from NCBI). Because of this, what is known on the *rgg* and *rgg*D genes of *S. gordonii* will first be discussed. After, because it is believed of relevance to the *rgg* gene of *S. pneumoniae*, the *rgg* gene of *S. pyogenes* will also be described.

According to the annotation of the pneumococcal *rgg* gene on the TIGR database, this gene regulates the expression of a gene or genes that lead to the production of glucosyltransferase (Vickerman *et al.*, 2003). In *S. gordonii*, and other oral streptococci, this enzyme can produce glucans in the presence of sucrose, which allow bacterial adherence to surfaces, colonisation and biofilm formation, producing dental plaque. This characteristic is known as a sucrose promoted phenotype (spp) (Vickerman *et al.*, 2003). Vickerman and collaborators, in their study of 2003, suggested that the Rgg protein plays a direct interaction in the production of glucosyltransferase in *S. gordonii* by binding to the *gtf*G gene, which in *S. gordonii* is immediately downstream the *rgg* gene and codes for the glucosyltranferase protein. Studies have been done investigating the effect of the *rgg* gene on the production of glucosyltransferase by *S. gordonii* and *S. oralis*. For

example, in a study done by Sulavik and collaborators (1992), they showed that a functional deletion of the *rgg* gene produced a reduced amount of glucosyltransferase and an spp negative phenotype in *S. gordonii*. Similar results were observed by Fujiwara and collaborators (2000) in *S. oralis*, where they observed a reduced production of glucosyltranferase in an *rgg* negative mutant.

Vickerman *et al.* (2001), found that *S. gordonii* contained two genes immediately downstream of the *gtf*G gene, read in the opposite DNA strand from *rgg* and *gtf*G (see Figure 6.6-1B), which are *dsg* and the rgg-like gene *rgg*D (Figure 6.6-1B). In their experiments, they found that the *dsg* gene influenced the production of glucosyltransferase, and that its absence results in a mutant with only 57% of glucosyltransferase activity (Vickerman *et al.*, 2001). They speculated that the *rgg*D gene could have a function in the regulation of the *dsg* gene, however, they did not find the expression of *dsg* related to the presence of *rgg*D, and suggested this protein might regulate a distally located gene. Interestingly, the product of the *rgg* gene of *S. pneumoniae* has a 45% identity and 63% similarity to RggD in *S. gordonii*. Also, the gene immediately downstream of *rgg* in the pneumococcus (see Figure 6.6-1A), which is a hypothetical gene, encodes a protein with 42% identity and 69% similarity to Dsg of *S. gordonii* (homology search done using the NCBI BLAST protein service). In the pneumococcus, no proteins were found with significant similarity to the Rgg of *S. gordonii*, and only one protein with low identity and similarity (27% and 37% respectively) to Gtf, called PcpA was found. Also, apart for the gene encoding the conserved hypothetical protein similar to Dsg, the genes flanking the pneumococcal *rgg* had no significant similarity to the genes flanking the *rgg* or *rgg*D genes of S. gordonii (see Figure 6.6-1). The lack of homology between the peumococcus and the Rgg protein of *S. gordonii*, allows speculation that the pneumococcal Rgg might have a different than in its *S. gordonii* homologous (RggD). However, it is difficult to speculate whether this might be by regulating the *dsg* gene, which could, as in S. gordonii, have a role in surface adherence and perhaps in colonisation, or whether it might also regulate other genes. It could be possible to determine if the *rgg* gene in the pneumococcus regulates the expression of *dsg* by performing RTqPCR of an *rgg*-negative mutant and comparing it to wild type. Also, attachment studies could be done to investigate if the pneumococcal *rgg* could have a role similar to that of the *rgg* gene

of *S. gordonii*. It makes sense that when the pneumococcus is subject to aerobiosis, it can induce genes related to bacterial adherence, as this would secure its colonisation of the host. However, the pneumococcal *rgg* gene, as in other bacteria, could have a function related to other areas of oxidative stress, as it will now be discussed.

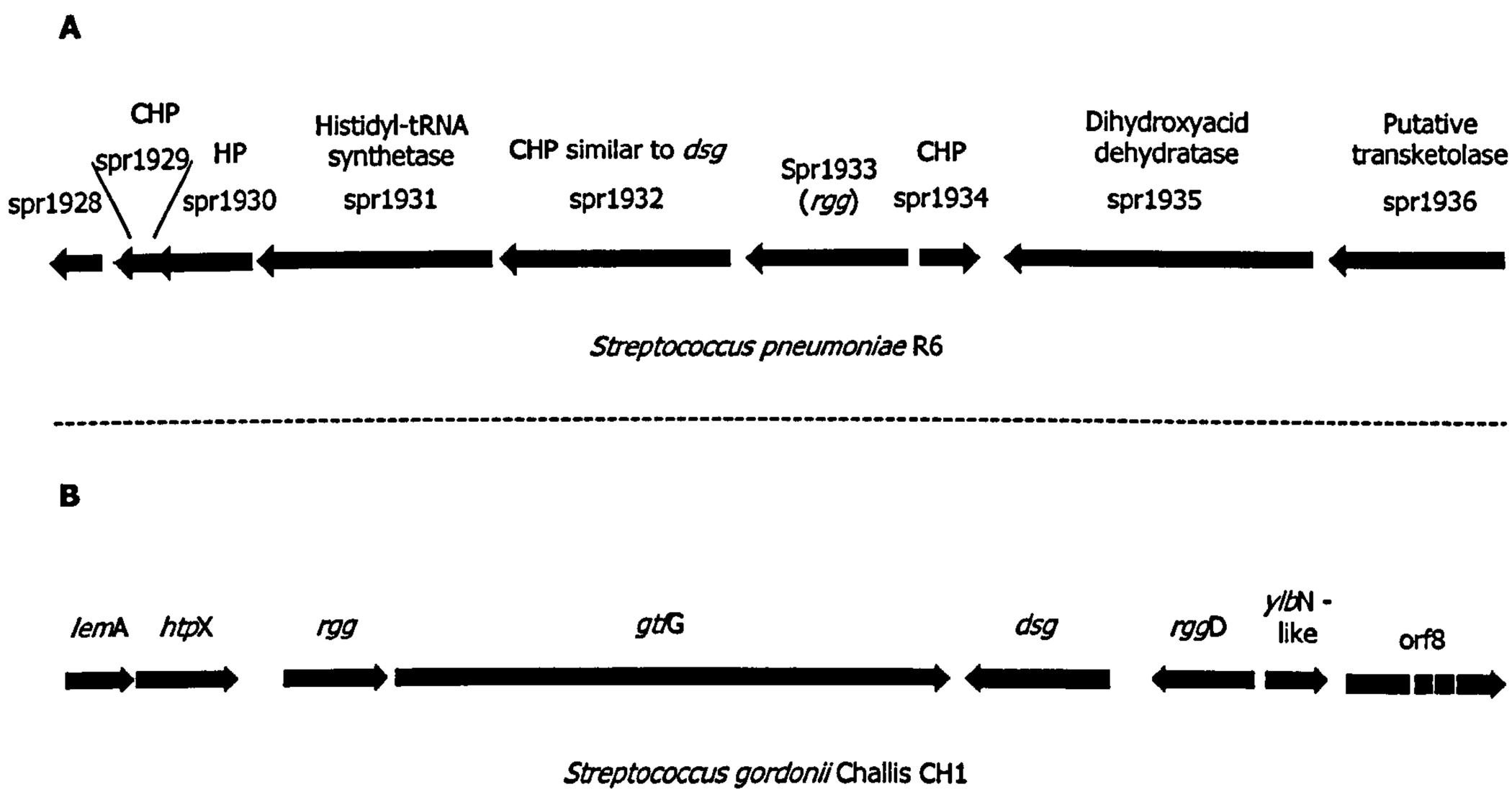


Figure 6.6-1 Graphical display of the genes present in the region of (A) the *S. pneumoniae* R6 *rgg* gene, and (B) the *S. gordonii* Challis CH1 *rgg* and *rgg*D genes. The information for the graphical display of *S. pneumoniae* R6 was obtained from the TIGR database, and for *S. gordonii* Challis CH1 from Vickerman *et al.* (2001). Because common names were not found for most of the genes in the *S. pneumoniae* R6 display, the locus name and their products are shown instead. (HP) Hypothetical Protein (CHP) Conserved Hypothetical Protein.

Chaussee and collaborators (Chaussee *et al.*, 2003) reported studies in *S. pyogenes*, in which they analysed the impact of an *rgg* negative mutation. In their studies, the relationship between the absence of *rgg* and the ability of the bacteria to survive in high temperatures and oxygen stress was observed. They found that the *rgg* negative mutant had an increased resistance to high temperatures, and that the protein ClpL (a thermal stress protein) was produced in the negative mutant whereas it was absent from the wild type. Also as a consequence of the mutation, they found the mutant was more susceptible to oxidative stress as judged by the sensitivity to paraquat (which produces $^{\bullet}O_2^-$ stress), and found that the proteins related to oxidative stress NoxI, which produces H_2O_2 from O_2, and AhpC, a scavenger of H_2O_2, were more abundant in the mutant than in the wild type (Chaussee *et al.*, 2004). The authors suggested that the absence of the *rgg* gene produces an altered response to stress in the bacteria. It was very interesting to observe that when the pneumococcus was subject to aerobic conditions, the pneumococcal *rgg* gene was over-expressed, but also the pneumococcal *clp*L gene was repressed. Although this could be a coincidental finding, it could be explained by the findings of Chaussee and collaborators (2003), and it can be hypothesised that the over-expression of the *rgg* gene observed in the pneumococcus under aerobiosis produced the repression of its *clp*L gene. In this case, pneumococcal *rgg* negative mutant should behave in a similar manner to the *S. pyogenes rgg* negative mutant of Chaussee and collaborators. Also, Chaussee *et al.* (2003) found that while wild type *S. pyogenes* preferred to ferment glucose and entered to stationary phase in its absence, the *rgg* negative mutant would ferment arginine and serine even when glucose was available, showing that Rgg is also responsible for repressing arginine fermentation. They suggested that this metabolic activity (arginine and serine fermentation) could be of importance to sustain infection, where amino acid fermentation might be a necessity. Finding that the *rgg* gene involved with the regulation of thermal and oxidative stress, and also with arginine fermentation, led the authors to conclude that Rgg is a global regulatory factor that might play an important role on the survival and adaptation of bacteria during infection (Chaussee *et al.*, 2004).

All these studies regarding the importance of *rgg* gene in different streptococcal species, the similarity of *rgg* to the *rgg*D gene of *S. gordonii*, and the repression of the *clp*L gene when the *rgg* gene was over-expressed support the interest in studying the role of the *rgg* gene in the pneumococcus. It was predicted that this gene could have a role in the pneumococcal survival in aerobic conditions and during infection. Because RTqPCR also confirmed the over-expression of the *rgg* gene under aerobiosis, it was decided to select this gene for the construction of a mutant.

6.6.1 Characterisation of the mutant *S. pneumoniae* Δrgg

Due to time restrictions, it was only possible to perform growth curves in complex media comparing the aerobic and anaerobic growth of the mutant *S. pneumoniae* Δrgg and *S. pneumoniae* R6. In the results, we observed that the mutant had a significantly slower generation time ($p<0.05$) than the wild type in anaerobic conditions. Although the aerobic growth was not significantly different ($p>0.05$), (probably due to the few replicates in the experiment), a difference of ~10 minutes in the generation time for both growth conditions (aerobiosis and anaerobiosis) was observed when comparing the mutant versus the wild type (~30 minute generation time for the mutant and only ~20 minutes for the wild-type). The growth effect seen with this experiment was not specific for aerobic growth, rather showing that the *rgg* gene in *S. pneumoniae* R6 could have a general effect in growth. The reason for this is not yet known, but it could be explained if the deletion of the *rgg* gene caused a change in the fermentation metabolism. Chaussee *et al.* (2003), reported that the growth of the *rgg* negative mutant of *S. pyogenes* was similar to the wild type until ~8 hours of growth, after which the mutant showed decreased growth levels compared to the wild type (see Figure 6.6-2). They also reported that after 25 hours of growth, the mutant had attained a higher growth than the wild type. In Figure 6.6-2, the graph published by Chaussee *et al.* (2003) regarding this experiment is shown. In the graph, although the early stages of growth can not be seen in detail, it is clear that the wild type had a higher growth rate for up to ~12 hours than what the mutant did. As it has been mentioned earlier, Chaussee and

collaborators (2003) found that the *S. pyogenes* mutant preferred to ferment arginine and serine, over glucose, which the wild type ferments. From this, it can be hypothesised that as in the experiments of Chaussee (Chaussee *et al.*, 2003) the diminished growth observed in the pneumococcal mutant could be associated with a change in its fermentative metabolism.

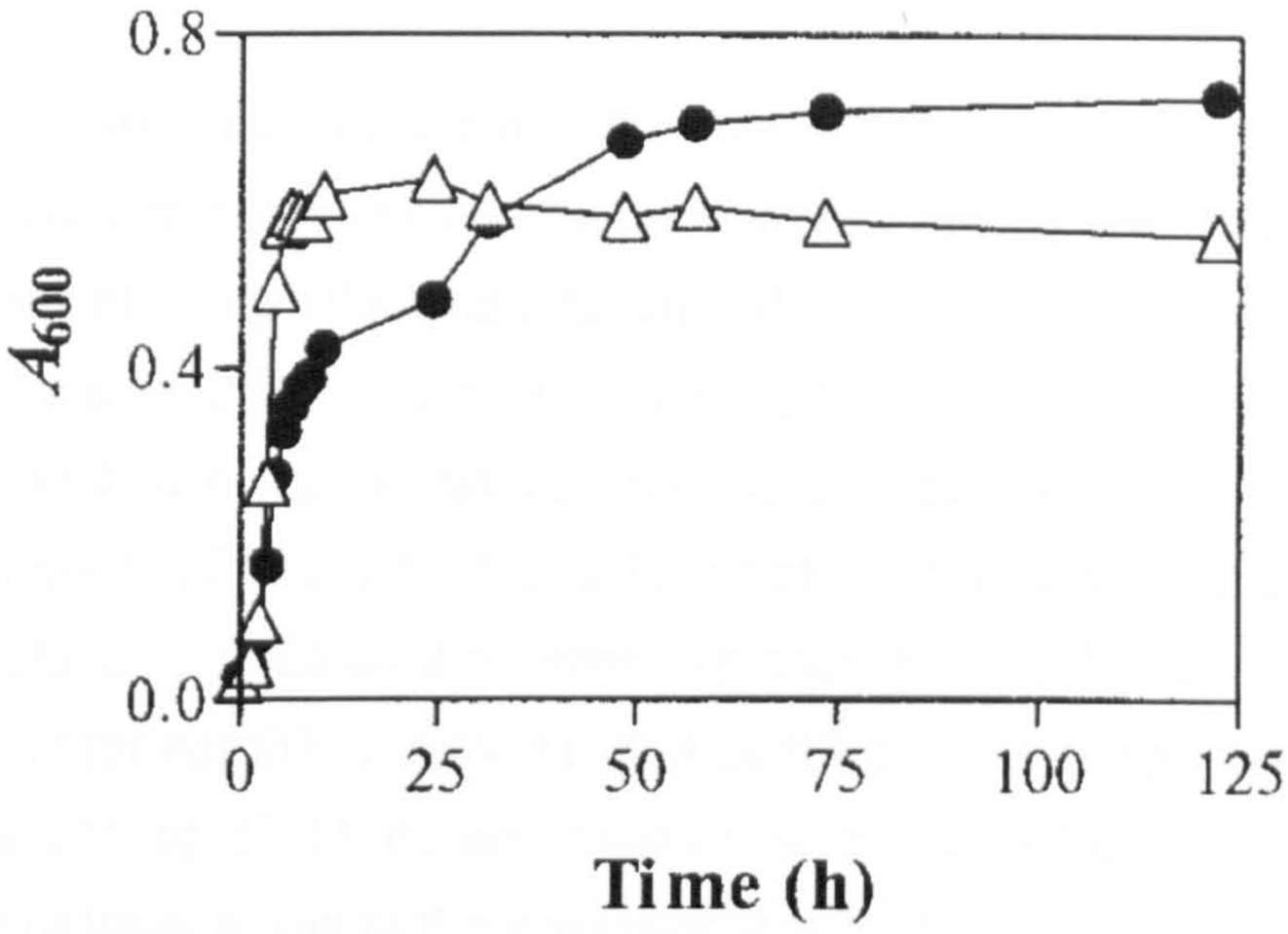


Figure 6.6-2 (Chaussee *et al.*, 2003) Growth curve comparison between the growth of a *S. pyogenes* NZ131 mutant lacking the expression of the *rgg* gene (black solid circles) and *S. pyogenes* NZ131 wild type (empty triangles). The growth curves were performed in Todd-Hewitt broth containing 0.2% (w/v) yeast extract from Difco Laboratories.

The lack of difference in growth observed between the aerobic and anaerobic growth of the mutant could be due to the use of rich medium to perform the experiments. It is possible that other genes in the bacterium might substitute the role that the *rgg* gene might have in regard to oxidative stress, thus preventing observation of an immediate consequence of the mutation during aerobic growth. For example, in a pneumococcal *spx*B negative mutant, no growth effect was observed when the mutant was grown in rich medium, however, the mutant did show a growth deficiency when grown in a chemically defined medium (Spellerberg *et al.*, 1996). To elucidate whether the mutation has an impact on the bacterial

resistance to oxidative stress, further experiments, such as growth in minimal medium, challenge with H_2O_2 and paraquat need to be done.

6.7 Conclusions from the project and future work

The genetic response observed when the pneumococcus was subject to aerobic conditions included genes involved in a wide variety of responses. These included genes that might help the adaptation of the pneumococcus to aerobic environments in three different ways: by the depletion of toxic forms of oxygen, as judged by the finding of genes sp0766 (*sod*A) and sp1651 (*tpx* or *psa*D); by the repressed transport of iron into the cell, which might prevent the formation of hydroxyl radicals, as judged by the under-expression of ABC transporters involved in iron uptake under aerobic conditions; and by the genetic adaptation to stress, as judged by the finding of 11 genes involved in genome plasticity. Interestingly, genes that might have a role in the pneumococcal colonisation and virulence were also observed. For example, genes involved in competence have been related to virulence and to the formation of biofilm by the pneumococcus (Oggioni *et al.*, 2006). Genes related to the Bgl and PTS system, have been reported in other organisms to be related to bacterial attachment to surfaces (Abranches *et al.*, 2006; Gorski *et al.*, 2003), and so has the *rgg* gene (sp2123) (Sulavik *et al.*, 1992; Fujiwara *et al.*, 2000). The results of this project indicated that the aerobic response in *S. pneumoniae*, is not only an individual response to diminish the formation and effects of reactive oxygen species, but it also triggers a network of responses that might provide the pneumococcus with the appropriate "gene combination" for causing infection, colonisation and disease.

It is evident that the role of most of the genes identified in this project are unknown in the pneumococcus. The fact that several of these genes, for example, those that encode the hypothetical proteins sp1802, sp2047 and sp0206 and the anaerobic ribonucleases sp0202 and sp0205 have been previously identified in other pneumococcal experiments related to other stresses or to virulence, increases the

interest in characterising the function of these genes. This could initially be done by assessing with the use of RTqPCR, their expression under different stress conditions, such as temperature stress, high and low pH stress and bacterial starvation. Pneumococcal knockout mutants could also be constructed and challenged *in vitro* according to the condition or conditions in which the gene was observed to change its expression, expecting that the growth of the mutants would be affected in the conditions where the expression of the deleted gene had changed.

In this project, the characterisation of the function of the *rgg* gene in the pneumococcus was attempted. Gene *rgg* has been related in other streptococci with roles in fermentative metabolism (Chaussee *et al.*, 2003), oxidative and thermal stress (Chaussee *et al.*, 2004), and attachment (Vickerman, 2001; Sulavik *et al.*, 1992; Fujiwara *et al.*, 2000). A pneumococcal *rgg* negative mutant was constructed and it was observed that the mutant had a slower generation time than did the wild type. This slower growth was observed anaerobically, indicating an effect non-specific to aerobiosis. Because the results show a general growth disadvantage, rather than an effect specific to aerobiosis, it was hypothesised that the deletion of the *rgg* gene could have caused, as in *S. pyogenes* (Chausee *et al.*, 2003), a change in the fermentative metabolism of the bacterium. Due to time restrictions it was not possible to confirm this hypothesis or to perform further characterisation of the mutant. However, to test if contrary to the wild type, the mutant prefers to ferment different substrates, experiments could be done growing the bacteria in minimal medium varying the availability of substrates to ferment, and performing growth curves of the culture.

However, the possibility that the *rgg* gene could help the pneumococcus in its adaptation to aerobic environments cannot yet be ruled out. Simple experiments could be done to evaluate the susceptibility of the mutant to oxidative stress. Challenging the bacteria with different concentrations of H_2O_2 or paraquat and analysing the percentage of bacteria that survived the challenge could do this. Because the *rgg* gene was over-expressed in the aerobic conditions of this project, it would be expected that the *rgg* negative mutant would be more susceptible to H_2O_2 and paraquat than the wild type bacteria. In *S. pyogenes* it was also reported

that the absence of *rgg* increased the mutant resistance to thermal stress (Chaussee *et al.* 2004). To test if this could be the case for the pneumococcal *rgg* negative mutant, growth curves could be performed at higher temperatures comparing its growth to that of the wild type.

In the project, it was also considered that the pneumococcal *rgg* gene could be involved in the pneumococcal attachment to surfaces, which if true, could have great implications in pneumococcal colonisation of the host and in virulence. To test whether if this could be a function of the *rgg* gene, the method described by Oggioni *et al.* (2006) to evaluate pneumococcal attachment to surface could be used.

To study the role that the *rgg* gene could have during infection, an *rgg* negative mutant could be constructed in a virulent laboratory strain such a *S. pneumoniae* D39. Intranasal and intravenous infections could be performed in a mouse model of pneumonia and septicaemia to evaluate the effect of the gene in virulence. As it has been mentioned, the pneumococcal *rgg* gene could have a role in the bacterial survival to oxidative stress, and/or it could have a role in the bacterial attachment to surfaces, which has been related to the ability of the pneumococcus to cause pneumonia (Oggioni *et al.*, 2006). It would therefore be expected that the mutation will result in a decreased ability of the bacteria to cause disease after intranasal infection, but perhaps it would be fully able to cause disease after intravenous infection.

In conclusion, the results of this project have provided the first insight on the global genetic response of the pneumococcus to aerobiosis. This response involves a network of genes with different properties that might help the bacteria not only to survive in the presence of oxygen, but also to infect and cause disease in the host. This project opened several paths of research that can contribute in the understanding of pneumococcal virulence.

Appendix

Table A-1. Genes over-expressed under aerobiosis. The data were normalized by LOWESS normalization. A cut-off value of 10log in the intensity of the spot was applied and a filter on flags was done. Spots that had a p-value <0.05 and that had >2-fold over-expression in the averaged experiment were selected.

Systematic name	Synonyms or R6 systematic name	Product	T-test p-value
		BETWEEN TWO AND FOUR-FOLD OVER-EXPRESSION	
spr0957		SpR6: Tn5252, relaxase, truncation	0.00005
spr0960	mutR	Similar to positive transcriptional regulator MutR.	0.0009
spr0965		Hypothetical protein	0.002
spr0966		Conserved hypothetical protein	0.0002
spr0967		Conserved hypothetical protein	0.00007
spr0971	mefE	ABC transporter membrane-spanning permease - macrolide efflux	0.005
sp0502	glnA; spr0444	Glutamine synthetase, type I	0.007
sp0717	thiM; spr0629	Hydroxyethylthiazole kinase	0.0005
sp0723	spr0635	Conserved domain protein; hypothetical protein	0.007
sp0731	spr0643	Conserved domain protein; hypothetical protein	0.0003
sp0732	spr0644	Degenerate transposase	0.00005
sp0735	spr0646	Phospho-beta-gluco or galactosidase, truncation	0.003
sp0825	folD; spr0729	Methylenetetrahydrofolate dehydrogenase/methenyltetrahydro	0.000001
sp0924	spr0825	Hypothetical protein	0.002
sp0961	rplT; spr0863	Ribosomal protein L20	0.0004
sp0992	spr0895	Conserved hypothetical protein	0.003
sp1013	asd; spr0918	Aspartate beta-semialdehyde dehydrogenase	0.0001
sp1050	spr0951	Transcriptional regulator, putative; hypothetical protein	0.001
sp1162	acoC; spr1049	Acetoin dehydrogenase complex, E2 component, dihydrolipoam	0.0008
sp1210	spr1091	Hypothetical protein	0.0006
sp1241	glnP;spr1120	Amino acid ABC transporter, amino acid-binding protein/per	0.002
sp1242	glnQ;spr1121	Amino acid ABC transporter, ATP-binding protein	0.0005

sp1271	spr1149	Cytidine diphosphocholine pyrophosphorylase, putative; conserved hypothetical protein	0.000005
sp1281	spr1159; spr1160	Hypothetical protein	0.002
sp1314		IS66 family element, Orf1	0.003
sp1346	spr1206	Conserved hypothetical protein; hypothetical protein	0.00002
sp1382	amy; spr1239	Alpha-amylase precursor	0.002
sp1435	ABC-N/P; spr1290	ABC transporter, ATP-binding protein	0.00006
sp1436	sp1436 spr1291	Hypothetical protein	0.004
sp1437	spr1292	Conserved hypothetical protein	0.000006
sp1459	spr1313	Hypothetical protein	0.0001
sp1496	spr1349	Transposase, IS630-Spn1 related, Orf2; degenerate transposase	0.004
sp1515	spr1367	Transposase	0.00003
sp1531	spr1385	Hypothetical protein	0.005
sp1628	spr1469	Hypothetical protein	0.0002
sp1639		IS1167, transposase	0.0001
sp1679	spr1523	Hypothetical protein	0.0001
sp1694	spr1537	Hypothetical protein	0.00001
sp1708	spr1552	Hypothetical protein	0.003
sp1728	spr1572	Hypothetical protein	0.0000003
sp1802	spr1623	Hypothetical protein	0.0003
sp1804	spr1625	General stress protein 24, putative; conserved hypothetical protein	0.001
sp1807	spr1627	Acetyltransferase, GNAT family; conserved hypothetical protein	0.0003
sp1948	spr1765	Conserved domain protein; hypothetical protein	0.008
sp1953	clyB; spr1770	Toxin secretion ABC transporter, ATP-binding/permease prot; ABC transporter ATP-binding/membrane-spanning - hemolysin secretion protein	0.0004
sp2004		Hypothetical protein	0.0001
sp2007	nusG; spr1820	Transcription antitermination factor	0.0006
sp2017	spr1830	Membrane protein; conserved hypothetical protein	0.00001
sp2084	pstS; spr1895	Phosphate ABC transporter, phosphate-binding protein	0.002
sp2087	pstB; spr1898	Phosphate ABC transporter, ATP-binding protein	0.0005
sp2102	spr1912	Hypothetical protein	0.002
sp2104	spr1914	Hypothetical protein	0.0009
sp2122	spr1932	Conserved hypothetical protein	0.005
sp2181	spr1985		0.007

BETWEEN FOUR AND EIGHT-FOLD OVER-EXPRESSION

sp0095	spr0084	Conserved hypothetical protein	0.002
sp0718	thiE; spr0630	Thiamin-phosphate pyrophosphorylase	0.002
sp1326		Neuraminidase, putative	0.004

sp1424	spr1280	Hypothetical protein	0.0000002
sp1426	ABC-NBD; spr1281	ABC transporter ATP-binding protein - multidrug efflux	0.00001
sp1434	ABC-N/P; spr1289	ABC transporter, ATP-binding/permease protein	0.007
sp1441	spr1294		0.0006
sp1495	spr1348	Hypothetical protein	0.006
sp1691	spr1535	Conserved hypothetical protein; hypothetical protein	0.0007
sp1759		preprotein translocase, SecA subunit	0.003
sp1767		Glycosyl transferase, family 8	0.006
sp1919	spr1735; spr1736	ABC transporter, permease protein	0.00004
sp2032	spr1843	Transcriptional regulator, BglG family; conserved hypothetical protein	0.002
sp2034	sga; spr1845	Hexulose-6-phosphate isomerase, putative	0.0008
sp2035	sgh; spr1846	Hexulose-6-phosphate synthase, putative	0.0002
sp2036	PTS-EII; spr1847	PTS system, IIA component	0.002
sp2038	PTS-EII; spr1849	PTS system, membrane component, putative	0.0004
sp2046	spr1856; spr1857		0.0002
sp2047	spr1858	Conserved domain protein; hypothetical protein	0.00009
sp2048	spr1859	Conserved hypothetical protein	0.0007
sp2050	cglD; spr1861	Competence protein CglD	0.000003
sp2051	cglC; spr1862	Competence protein CglC	0.0003
sp2178	spr1983	Conserved hypothetical protein	0.001

BETWEEN EIGHT AND SIXTEEN-FOLD OVER-EXPRESSION

sp1332	spr1206	Conserved domain protein; hypothetical protein	0.007
spr0968		Hypothetical protein	0.002
sp0766	sodA; spr0674	Superoxide dismutase, manganese-dependent	0.001
sp1438	ABC-NBD; spr1293	ABC transporter, ATP-binding protein	0.00009
sp1651	tpx; spr1495	Thiol peroxidase	0.003
sp2123	rgg; spr1933	Positive transcriptional regulator of glucosyltransferase and Spp phenotype	0.0003
sp2207	comFC; spr2012	Competence protein ComF, putative	0.007
sp2211	spr2016	Transposase, uncharacterized, truncation	0.00001

MORE THAN SIXTEEN-FOLD OVER-EXPRESSION

sp1250	spr1129	Conserved domain protein; hypothetical protein	0.00005

Table A2. Genes over-expressed under anaerobiosis. The data were normalized by LOWESS normalization. A cut-off value of 10log in the intensity of the spot was applied and a filter on flags was done. Spots that had a p-value <0.05 and that had >2-fold over-expression in the averaged experiment were selected.

Systematic name	Synonyms or R6 systematic name	Product	T-test p-value
BETWEEN TWO AND FOUR-FOLD OVER-EXPRESSION			
spr0121	pspA	Surface protein pspA precursor	0.0009
spr1549		Hypothetical protein	0.003
sp0041	thmA; spr0040	Bacteriocin BlpU; amphipathic pore-forming peptide precursor	0.0006
sp0110	ABC-MSP; spr0099	Hypothetical protein; ABC transporter membrane-spanning permease - amino acid transport	0.001
sp0117	pspA; spr0121	Surface protein pspA precursor	0.0003
sp0330	regR; spr0298	Sugar binding transcriptional regulator RegR	0.000003
sp0436	gatB; spr0393	Glutamyl tRNA-Gln amidotransferase subunit B	0.002
sp0459	pfl; spr0415	Formate acetyltransferase; pyruvate formate-lyase	0.004
sp0657	spr0574	Ribonuclease BN, putative; conserved hypothetical protein	0.0008
sp1043	spr0945	Hypothetical protein	0.006
sp1179	nrdE; spr1065	Ribonucleoside-diphosphate reductase, alpha subunit	0.000001
sp1258	spr1137	2-isopropylmalate synthase, putative	0.001
sp1648	psaB; spr1492	Manganese ABC transporter, ATP-binding protein	0.009
sp1649	psaC; spr1493	ABC transporter membrane-spanning permease - manganese transport	0.00007
sp1650	psaA; spr1494	ABC transporter substrate-binding protein - manganese transport.	0.000009
sp1705	spr1547	Hypothetical protein	0.009
sp1810	spr1630	Hypothetical protein	0.0004
sp1854	galR; spr1669	Galactose operon repressor	0.008
sp1916	spr1732	PAP2 family protein; conserved hypothetical protein	0.01
sp2130	spr1939	PTS system, IIB component, putative; hypothetical protein	0.004
BETWEEN FOUR AND EIGHT-FOLD OVER-EXPRESSION			
sp0202	nrdD; spr0183	Anaerobic ribonucleoside-triphosphate reductase	0.000002
sp0204	spr0184	Acetyltransferase, GNAT family; hypothetical protein	0.00002

sp0205	nrdG; spr0185	Anaerobic ribonucleoside-triphosphate reductase activating; NrdD activating enzyme, generating glycyl radical	0.00001
sp0338	clpL; spr0307	ATP-dependent Clp protease, ATP-binding subunit, putative	0.0001
sp0482	spr0429	Conserved hypothetical protein	0.007
sp1871	fecE; spr1686	Iron-compound ABC transporter, ATP-binding protein	0.002

BETWEEN EIGHT AND SIXTEEN-FOLD OVER-EXPRESSION

sp0206	spr0186	Hypothetical protein	0.003
sp0319	spr0289	Conserved domain protein; hypothetical protein	0.00008
sp0320	gno; spr0290	Oxidoreductase, short chain dehydrogenase/reductase family; 5-keto-D-gluconate 5-reductase	0.0005
sp1869	fatD; spr1684	Iron-compound ABC transporter, permease protein	0.004
sp1870	fatC; spr1685	Iron-compound ABC transporter, permease protein	0.002
sp1872	fatB; spr1687	Iron-compound ABC transporter, iron-compound-binding protein	0.002

MORE THAN SIXTEEN-FOLD OVER-EXPRESSION

sp0318	kdgK; spr0288	Carbohydrate kinase, PfkB family	0.003

Table A-3. Efficiency of the primers used for Real-Time PCR. The slope was calculated from the PCR amplification of DNA at concentrations ranging from 1.25 to 20ng.

Primers	Slope	Efficiency
sp0206F,R	-3.368	1.981126
sp0318F,R	-3.488	1.935073
sp0732F,R	-3.555	1.911146
sp0766F,R	-3.397	1.969597
sp0806R,R	-3.429	1.957177
sp1250F,R	-3.696	1.864502
sp1314F,R	-4.482	1.671532
sp1332F,R	-3.046	2.129607
sp1438F,R	-2.606	2.419524
sp0806F,R	-3.471	1.941339
sp1496F,R	-3.687	1.867339
sp1515F,R	-3.828	1.824875
sp1651F,R	-3.467	1.942826
sp1869F,R	-5.046	1.578254
sp1870F,R	-3.548	1.91359
sp2207F,R	-3.503	1.92961
sp2123F,R	-3.444	1.951462
sp2211F,R	-3.581	1.90218

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