STUDIES OF PNEUMOLYSIN, THE MEMBRANE DAMAGING TOXIN OF *STREPTOCOCCUS PNEUMONIAE*

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

by

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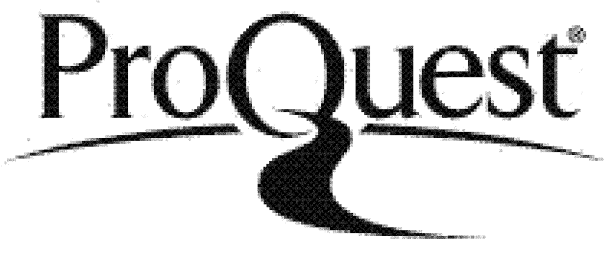

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STATEMENT

The accompanying thesis, submitted for the degree of Ph.D. and entitled " Studies of pneumolysin, the membrane damaging toxin of *Streptococcus pneumoniae* ", is based on work conducted by myself, or where noted in collaboration with others, in the Department of Microbiology of the University of Leicester during the period between October 1984 and September 1987.

All the work in this thesis is original unless otherwise acknowledged. None of this work has been submitted for another degree at this or any other university.

Signed..... Date. 8.VI.88

ABSTRACT

A recombinant phage that produced a polypeptide possessing the characteristics of pneumolysin, the membrane damaging toxin of the pneumococcus, was isolated from a bank of pneumococcal sequences in λgt10. Subclones carrying the pneumolysin gene in various plasmids were haemolytic regardless of the orientation of the insert.

The nucleotide sequence of a 5 kb fragment carrying the pneumolysin gene was determined. An open reading frame 1413 bp long was identified that when translated encoded a polypeptide with 471 amino acids and a molecular weight 52.8 kD. The N-terminal amino acid sequence of the predicted protein was identical to that of native pneumolysin. A single cysteine residue was present at position 428 in the amino acid sequence. Comparison of the DNA and amino acid sequences of pneumolysin with streptolysin O (SLO) revealed extensive homology in the amino acid sequence. The longest region of identity was a sequence of 12 amino acids surrounding the unique cysteine.

A hybrid gene consisting of the 5' region of the pneumolysin gene and the 3' end of the SLO gene was constructed. The fusion polypeptide was made in *E. coli*, but possessed a very low haemolytic activity.

Using the technique of oligonucleotide-mediated site-directed mutagenesis, two mutant genes were constructed in which the cysteine codon was changed to either a glycine or serine codon. Modified toxins when purified from *E. coli* had a specific activity of about 1-2 % that of wild type pneumolysin.

DEDICATION

This thesis is dedicated to Joan Walker (nee Thompson)

1930-1962

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ABBREVIATIONS

AIB - α-aminoisobutyric acid

APS - Ammonium persulphate

bp - Base-pairs

BSA - Bovine serum albumin

CCC - Covalently closed circle

ConA - Concanavalin A

DEPC - Diethyl pyrocarbonate

DMF - Dimethylformamide

DTT - Dithiothreitol

EDTA - Ethylenediaminetetracetic acid

ELISA - Enzyme linked immunoabsorbant assay

EMS - Ethylmethane sulphonate

IPTG - Isopropylthio-β-galactosidase

kb - Kilo base-pairs

kD - Kilo daltons

LMM - Low molecular weight mix

OD_{650} - Optical density at 650 nm

PBS - Phosphate buffered saline

PHA - Phytohaemagglutinin

PMNL - Polymorphonuclear leukocyte

PVP - Polyvinylpyrrolidone

PWM - Pokeweed mitogen

RF - Replicative form

SDS - Sodium dodecyl sulphate

SDS-PAGE- Sodium dodecyl sulphate polyacrylamide gel electrophoresis

SDW - Sterile distilled water

TEMED - N,N,N','N'-tetramethylethylenediamine

X-gal - 5-bromo-4-chloro-3-indolyl-β-D-galactosidase

PUBLICATIONS

Part of the work contained within this thesis has been included in the following publications.

i. Walker, J. A., Allen, R. L. and Boulnois, G. J. (1986)
Towards a genetic analysis of the virulence of *Streptococcus pneumoniae,* 165-171. *In* Streptococcal Genetics, Ferretti, J. J. and Curtiss, R. III (ed). A.S.M., Washington.

ii. Walker, J. A., Allen, R. L., Falmagne, P., Johnson, M. K. and Boulnois, G. J. (1987)
Molecular cloning, characterization, and complete nucleotide sequence of the gene for pneumolysin, the sulfhydryl-activated toxin of *Streptococcus pneumoniae.* Infect. Immun. 55, 1184-1189

iii. Kehoe, M. A., Miller, L., Walker, J. A. and Boulnois, G. J. (1987)
Nucleotide sequence of the streptolysin O (SLO) gene: Structural homologies between SLO and other membrane damaging thiol-activated toxins. Infect. Immun. 55, 3228-3232

iv. Mitchell, T., Walker, J., Saunders, K., Kehoe, M., Andrew, P. and Boulnois, G. (1988)
Expression of the pneumolysin gene in *E. coli*: Purification of recombinant pneumolysin and some biological properties, 241-242. *In* Bacterial Protein Toxins, Feherenbach *et al* (eds) Fischer, Stuttgart.

v. Kehoe, M., Walker, J., Boulnois, G., Shields, J. and Miller, L. (1988)

Genetics and structure of thiol-activated toxins. *In* Bacterial Protein Toxins, Feherenbach *et al* (eds) Fischer, Stuttgart.

CONTENTS

CHAPTER 1

INTRODUCTION

A. THE PNEUMOCOCCUS

A.1 GENERAL INTRODUCTION

Streptococcus pneumoniae or the pneumococcus is a major pathogen of man. Consequently the organism and the diseases it causes have been the subject of intense investigation, especially in the early years of this century (reviewed by White 1938). These studies, carried out in the pre-antibiotic era, made important contributions to many areas of biology. These include antibody-antigen interactions, the use of type-specific vaccines, resistance to anti-bacterial substances, and perhaps the most significant the ability to transform pneumococci with exogenous DNA. This latter observation lead to the discovery that DNA was the carrier of genetic information and led ultimately to the study of molecular genetics (reviewed by Finland 1981, Austrian 1981).

Improved standards of health and hygiene, together with the introduction of penicillin-based antibiotics, have resulted in a significant reduction in the mortality of infections caused by the pneumococcus. As a consequence of this, study of the pneumococcus was for some years out of favour. However, the recent isolation of penicillin resistant-pneumococci, together with the poor efficacy of the polyvalent polysaccharide vaccine, has given a fresh impetus to studies aimed at understanding the basic mechanisms involved in pneumococcal virulence.

A.2 DISEASES CAUSED BY THE PNEUMOCOCCUS

The pneumococcus is a major cause of pneumonia, meningitis and otitis media (Austrian 1984, Roberts 1979). Pneumococcal septicaemia, predominant in post-splenectomy infections (Wara 1981), can also arise as a sequala to pneumonia (Roberts 1979). Bacteraemia leads to a dissemination of the organism throughout the host and is often the route of infection of the meninges (Scheld 1986). Complications of the aforementioned diseases include pericarditis, endocarditis, empyema and sinusitis. These complications rarely exist as a primary pneumococcal infection (Roberts 1979), and now are relatively rare due to the routine use of penicillin-based antibiotics. Pneumococcal infections generally arise after colonisation of the nasopharynx and subsequent dissemination throughout the body, although they can be invoked by physical damage or trauma. The major infections are discussed in greater detail in the following sections.

A.2.i Pneumonia

Lobar pneumonia is characterised by the involvment of one or more complete lobes of the lung. Pneumococci, of the more virulent serotypes, are the causative agent in over 95% of cases of this type of pneumonia. The usual course of the disease involves the onset of a high fever within 1-2 days of the start of infection. An acute inflammatory response occurs resulting in the infiltration of PMNL and erythrocytes into the lobes of the lung. This is followed by the spread of exudate, bacteria and PMNL throughout the whole lobe. This state of lung consolidation precedes a period of crisis where the temperature of the patient drops dramatically, and in fatal cases,

death usually occurs at this point. The resolution of the infection is achieved by the removal of bacteria and cell debris by macrophages. Permanent lung damage, as a result of this form of the disease, is rare. Lobar pneumonia may be considered the major form of pneumococcal pneumonia in healthy adults in the absence of primary disease (Taussig 1984).

In contrast to lobar pneumonia, bronchopneumonia is characterised by the occurence of discrete foci of infection in the alveoli of the lower lung. In bronchopneumonia recovery is more gradual than in lobar pneumonia and lung damage can occur due to extensive fibrinisation of lung tissue. The less virulent pneumococcal serotypes are involved and infection usually occurs following a preceding infection, such as influenza, or physical damage to the lung. It is the predominant form of pneumococcal pneumonia in the young and the elderly (Roberts 1979, Taussig 1984).

A.2.ii Meningitis

Pneumococcal infection of the meninges surrounding the brain and spinal cord can be regarded as an infection of an area of impaired host defence. Complement, antibody and PMNL are all present at lower levels in cerebro-spinal fluid than in serum (Scheld 1986). Pneumococci gain access to the meninges directly, as a result of trauma, or more commonly, indirectly via the blood-brain barrier or the sub arachnoid space (Moxon 1981). In situations where the immune system is not fully developed, as in children, infection of the meninges leads to rapid multiplication of bacteria but results in limited damage to the meninges (Moxon 1981). In contrast, the immune response in immunocompetent individuals, although efficient in

eliminating pneumococci, leads to a greater damage to the meninges and brain parenchyma (Moxon 1981). The pneumococcus together with *Haemophilus influenzae* and *Neisseria meningitidis* account for the majority of cases of non neonatal bacterial meningitis (Austrian 1984).

A.2.iii Otitis Media

Otitis media caused by the pneumococcus is rarely fatal, though such infections can result in secondary meningitis, pneumonia or septicaemia (Roberts 1979). It has been calculated that approximately 30% of all children under five years suffer one episode of otitis media attributable to the pneumococcus (Roberts 1979). The predisposition of young children to otitis media may be due to the paucity of anti-polysaccharide antibody and/or the physiology of the middle ear in infancy (Giebank and Quie 1978). Experimental infections in chinchillas have indicated that obstruction of the eustacian tube predisposes to pneumococcal infection (Giebank 1981).

A.2.iv Septicaemia

This form of disease caused by the pneumococcus is usually a sequala to pneumococcal pneumonia, and results in a higher mortality than the uncomplicated disease. Septicaemia occuring as a primary focus of infection has a low rate of mortality (Roberts 1979), except in splenectomised individuals (reviewed Wara 1981).

A.3 HOST DEFENCE AGAINST THE PNEUMOCOCCUS

Maximum protection against pneumococcal infections requires the presence of anti-polysaccharide antibody, complement, PMNL and macrophages. Deficiencies in any part of the immune system can predispose individuals to pneumococcal infections, although the relative importance of each component of the immune system has been difficult to define. Cell mediated immunity is thought to play little part in the protection of the host against the pneumococcus (Johnston 1981).

A.3.i Physical Defences against the Pneumococcus

Pneumococci present in the nasopharynx are continually carried into the lung. In the normal host these pneumococci are rapidly cleared by the cough reflex and ciliary action (Johnston 1981). The importance of such physical defence systems can be seen in the predisposition to pneumococcal infections of individuals with conditions, such as alcohol intoxication, which compromise these defence mechanisms (Johnston 1981). Pneumococci not removed by these systems, are usually cleared by alveolar macrophages (Goldstein *et al* 1974).

A.3.ii Antibody Responses to the Pneumococcus

The host can mount antibody responses to many surface components of the pneumococcus, especially the polysaccharide capsule (Ammann 1981), cell wall constituents (Szu *et al* 1986) and surface proteins (McDaniel and Briles 1986). Capsule-specific antibodies of the IgM

or IgG class form the main protective antibody in immune hosts (Brown 1985). Anti-phosphocholine antibodies have been shown to offer some passive protection to mice in experimental pneumococcal infections (Yother *et al* 1982, Briles *et al* 1981, McDaniel *et al* 1984), as have monoclonal antibodies raised against certain surface proteins of the pneumococcus (McDaniel and Briles 1986). However, antibodies raised against cell wall components may not be protective (Szu *et al* 1986). Secretory IgA antibodies may prevent adhesion of pneumococci to mucosal surfaces in the nasopharynx and thus prevent colonisation (Austrian 1986).

A.3.iii Complement

Complement has a central role in the host defence against pneumococcal infection (Brown 1985, Winklestein 1981, Hosea *et al* 1980). The classical and alternative complement pathways can be activated by a variety of pneumococcal products. Anti-capsular polysaccharide antibodies bind to the capsule of the pneumococcus and results in the activation of the classical pathway of complement (Brown *et al* 1983). The alternative pathway can also be activated by certain pneumococcal capsular polysaccharide serotypes (Winkelstein *et al* 1976). Activation of either pathway leads to the deposition of C3b on the surface of the pneumococcus which facilitates phagocytosis by PMNL or macrophages. In the non-immune host, the alternative pathway of complement can be activated by cell wall components (Fine 1975, Stephens *et al* 1977, Winkelstein and Tomasz 1978), which results in the deposition of C3b onto the cell surface of the pneumococcus. How the deposition of C3b on pneumococcal cell surfaces other than the outer capsular surface promotes phagocytosis

is as yet undefined. The virulence of certain capsular types may be linked to the amount of degradation of C3b bound to the capsular surface (Hostetter 1986). The importance of the early components of the complement pathway is demonstrated by the predisposition to pneumococcal infections in those people with C1, C2, C4 deficiencies, but not in those with deficiences in the C5-C9 components (reviewed by Gorden and Hostetter 1986).

A.3.iv C-Reactive Protein

C-reactive protein (CRP) is one of a group of proteins, termed acute phase proteins, which are present at low levels in normal sera, but which rise rapidly in concentration during infection. CRP was discovered by its ability to bind to cell wall polysaccharides of the pneumococcus. CRP bound to the cell wall polysaccharide of the pneumococcus activates the classical pathway of complement, and inhibits alternative pathway activation (Mold and Gewurz 1981). CRP has been shown to promote opsonisation of pneumococci via C3b deposition on the cell surface (Holzer *et al* 1984). Both human and rabbit CRP have been shown to offer some protection to mice in experimental pneumococcal infections, though the actual mechanism is unknown (Mold *et al* 1981, Yother *et al* 1982). CRP has also been implicated in the clearance of pneumococci from the bloodstream in the absence of anti-polysaccharide antibodies (Horowitz *et al* 1987) and complement (Nakayama *et al* 1983). These processes may be important in the control of an infection before the production of a specific antibody response.

A.3.v The Role of the Spleen

Patients with functional asplenia, as a result of sickle cell anaemia, or trauma, are predisposed to pneumococcal septicaemia (Wara 1981). Asplenic children are at even greater risk to pneumococcal infections probably due to their inability to mount antibody responses against the polysaccharide capsule (Bohnsack and Brown 1986). The importance of the spleen in effective host defence can be appreciated when considering that certain functions of the spleen such as antibody production, activation of the alternative pathway of complement and clearance of bacteria in non-immune hosts, are just those components of the immune system that are implicated in the protection against pneumococcal infection (Bohnsack and Brown 1986). Antibody production in the spleen is thought to be important in the clearance of bacteria early in infection (Cohn and Schiffman 1987).

A.3.vi The Role of PMNL and Macrophages

The alveolar macrophage may be considered the first line of defence following inhalation of pneumococci into the lung (Goldstein *et al* 1974). The macrophages in the spleen also contribute extensively to the clearance of pneumococci from the blood, especially in the non immune host (Wara 1981), or in experimental pneumococcal infections in mice following prior injection with CRP (Nakayama *et al* 1983). In the immune host however, hepatic clearance of pneumococci from the blood is more important. The importance of PMNL in the clearance of pneumococci during pneumococcal pneumonia is unclear, due to the impairment of lung defences within 24 hours of the onset of infection (Ansfield *et al* 1977). The role of PMNL in meningitis has also not

been established (Ernst *et al* 1983), though they may have a significant role to play in the the clearance of pneumococci from the bloodstream (Ernst *et al* 1983).

A.4 THE PNEUMOCOCCUS

The pneumococcus produces many substances that have been implicated as potential virulence factors. With the exception of the polysaccharide capsule the importance of these substances in pneumococcal infections has yet to be determined. Those pneumococcal products that have received the most attention are discussed below.

A.4.i The Polysaccharide Capsule

The presence of a polysaccharide capsule is necessary for virulence of the pneumococcus and probably functions by preventing the phagocytosis of pneumococci by macrophages and PMNL. Pneumococci lacking a capsule are rapidly removed by PMNL and macrophages even in non-immune hosts. Encapsulated pneumococci or the polysaccharide capsule alone evokes an antibody response in immunocompetent individuals which provides long lasting protection against further infection, but only against those pneumococci of the same serotype to which the initial response was raised. There is serological cross reactivity between some of the 83 different pneumococcal polysaccharide capsules indicating a degree of structural similarity (Austrian 1984). There is also cross reactivity between pneumococcal capsules and those of other bacteria, such as the type 3 capsule of the pneumococcus and the K87 antigens of *Escherichia coli* (Amman 1981). The immunogenicity of a particular capsular serotype is a

combination of the chemical composition of the capsule and the amount of the capsule produced. The lack of immunogenicity of a particular capsule is not related to the virulence of that serotype, for example, Type 3 pneumococci are extremely virulent, yet the type 3 capsule is very immunogenic (Amman 1981).

The capsule is the basis of the current vaccine against the pneumococcus. Recently a 23-valent vaccine has been developed containing those serotypes which account for nearly 92% of isolates of the pneumococcus in the USA (Smart *et al* 1987). The use of polysaccharide based vaccines has failed to confer complete protection to individuals of all ages for all pneumococcal infections. The vaccine has been used to vaccinate groups of adults at highest risk.

A.4.ii IgA1 Protease

The IgA1 protease of the pneumococcus specifically cleaves human IgA1. The protease is one of a family of enzymes, produced by a variety of bacterial genera, which cleave IgA1 in the hinge region of the antibody, resulting in the production of Fab_{α} and Fc_{α} fragments. Each of these enzymes cleaves the IgA1 molecule at a specific site in the hinge region. There is little direct evidence that the secreted protease is directly involved in the virulence of the pneumococcus. However, the observation that related, non-pathogenic species do not possess this enzyme has lead to speculation that these enzymes are important virulence determinants (Plaut 1983). The ability to reduce mucosal immunity mediated by IgA1 could be of importance in colonisation of the host, and is an interesting hypothesis on which to base further study.

A.4.iii Hyaluronidase and Neuraminidase

Hyaluronidase depolymerases hyaluronic acid, a component of connective tissue and the extracellular matrix. Hyaluronidase, also called the spreading factor, may facilitate the spread of pneumococci throughout host tissues. It is produced by the pneumococcus (Humphrey 1944), though as yet its contribution to virulence has not been determined.

Neuraminidase cleaves α2-3 and α2-8 linked *N*-acetyl neuraminic acid from glycoproteins and gangliosides (Kelly *et al* 1966). Though no firm evidence has been established for a role for neuraminidase in virulence, rises in *N*-acetyl neuraminic acid concentrations in cerebrospinal fluid correlates with a poor prognosis in cases of meningitis (O'Toole *et al* 1971). In addition, purified pneumococcal neuraminidase injected into mice gave rise to neurological symptoms similar to meningitis and indicated a role for this enzyme in pneumococcal meningitis (Kelly and Greiff 1970).

A.4.iv Pneumolysin

Pneumolysin is a membrane damaging, cytolytic toxin produced by the pneumococcus. It is a member of a group of similar proteins, the sulphydryl activated cytolysins produced by diverse genera of Gram positive bacteria. Its biological properties indicate that it may play a role in pneumococcal infection. Since it appears to be produced by nearly all clinical isolates of the pneumococcus its potential as an immunogen for inclusion in the pneumococcal vaccine has been noted. Since pneumolysin is the subject of the work contained in this thesis, it will be discussed in detail.

B. SULPHYDRYL-ACTIVATED CYTOLYSINS

B.1 INTRODUCTION

The sulphydryl-activated cytolysins, formally the thiol-activated toxins or oxygen-labile haemolysins, are a group of 16 heat labile, haemolytic and cytotoxic proteins produced by a variety of Gram-positive bacterial genera (Table 1). They have been the subject of extensive reviews (Bernheimer 1976, Smyth and Duncan 1978, Alouf 1980). The physical characteristics of these proteins are listed in Table 2.

The sulphydryl-activated cytolysins have been grouped together on the basis of their biological and physical properties. They are lytic to all cells that contain cholesterol in their membranes, whilst at sublytic concentrations they cause significant inhibition of many activities of eukaryotic cells. At high doses they are cardiotoxic and cause rapid death when injected into animals. Their biological activities are lost in the presence of cholesterol and certain related sterols. The haemolytic activity is lost on oxidation, but can be regained after incubation with reducing agents. Hyper-immune serum raised against one toxin is cross reactive with other toxins of the group. Pneumolysin, differs from the other sulphydryl-activated cytolysins in that it is not secreted from the bacterium (Johnson 1977).

Streptolysin O (SLO), the sulphydryl-activated toxin of *Streptococcus pyogenes*, has been the subject of the most extensive study and can be regarded as the type protein of this group of toxins. The similarity in function of the sulphydryl-activated cytolysins and their

immunological relatedness has led to the assumption that these proteins have a similar structure and mode of action. The lack of structural information on these toxins has prevented clarification of this hypothesis. Amino acid compositions have been determined for some proteins (reviewed Smyth and Duncan 1978, Geoffroy and Alouf 1983, Alouf 1980, Yamakawa *et al* 1977, Bhakdi *et al* 1984), but comparison of these indicates that amino acid composition are dissimilar.

The observation that many of the bacteria listed in Table 1 possessed haemolytic activity predated, by many years, the characterisation of the haemolytic product (Bernheimer 1976). Both pneumolysin and tetanolysin were discovered around the turn of the century, and the inhibition of haemolytic activity by cholesterol was first noted about 1914 (Bernheimer 1976). The effect of oxidation and reduction on the haemolytic activity of the sulphydryl-activated cytolysins was not recognised until some years later initially by Avery and Neill in 1924. This property was later extensively characterised (Neill 1926 a, b, c). The antigenic relationships between the sulphydryl-activated cytolysins was also recognised around this time (reviewed by Bernheimer 1976). Using these criteria, SLO was fully characterised at this time (Neill 1926 b). Later, listeriolysin (Girard *et al* 1963, Njoku-obi *et al* 1963, Geoffroy *et al* 1987), alveolysin, cereolysin and laterosporulysin (Bernheimer and Grushoff 1967), thuringiolysin (Pendleton *et al* 1973, Rakatobe and Alouf 1984) and certain clostridial sulphydryl-activated cytolysins (reviewed Bernheimer 1976) were defined using these properties.

B.2. HAEMOLYSIS

The haemolytic activity of the sulphydryl-activated cytolysins is one of their most prominent biological properties. Since the mechanism of haemolysis is considered central to many of the other biological properties of these toxins, much of the analysis of these toxins has focussed on this process. Characterisation of the haemolytic process may be invaluable in the understanding of those other properties of the toxins, such as sublytic effects, that as yet have been difficult to investigate. Haemolysis has been separated into two distinct stages (Alouf 1980, Alouf and Raynaud 1968). The first stage involves the binding of toxin to the erythrocyte membrane, a process independent of temperature, pH and ionic strength of the buffer, but dependent on the state of oxidisation of the toxin. Binding of toxin to membrane was also sensitive to cholesterol. The subsequent lytic process was dependent on temperature, pH and ionic strength, but was not inhibited by cholesterol. The following sections will cover those aspects of haemolysis that have contributed to our understanding of the mode of action of these toxins.

B.2.i Thiol-Activation of Sulphydryl-Activated Cytolysins

The spontaneous loss of the haemolytic activity of crude preparations of pneumolysin after storage was first noted by Avery and Neill in 1924. That this was due to oxidation was suggested by the sensitivity of the haemolytic activity to treatment with H_2O_2 (reviewed by Bernheimer 1976). The haemolytic activity of perfringolysin, tetanolysin and SLO was also lost after incubation with inorganic oxidising agents (Neill 1926 a, b, c, Neill and

Mallory 1926). Loss of activity upon oxidation has been shown for listeriolysin (Jenkins *et al* 1964), cereolysin and alveolysin (Bernheimer and Grushoff 1967). Following oxidation, haemolytic activity of pneumolysin was regained upon the addition of sodium thiosulphate (Neill and Fleming 1927, Cowan 1934) or upon treatment with cysteine or thioglycollate (Scwachmann *et al* 1934).

In crude preparations of sulphydryl-activated cytolysins loss of haemolytic activity upon oxidation may be a result of the interaction of the toxins with other proteins, since purified SLO did not undergo this loss of activity. Oxidation of purified SLO was accompanied by irreversible loss of activity (Van epps and Andersen 1971, Alouf 1980). The corollary to this is that the haemolytic activity of purified SLO was not increased by incubation of the toxin with thiol compounds (Alouf 1980). However, this was not the case for pneumolysin (Kanclerski and Mollby 1987) or listeriolysin (Jenkins *et al* 1964)

The reactivation of the haemolytic activity of oxidised sulphydryl-activated cytolysins by thiol compounds led to the hypothesis that sulphydryl groups play an important role in the mechanism of action of these toxins. Subsequent studies with compounds specifically reactive for sulphydryl groups have strengthened this hypothesis. Tosyllysine chloromethyl ketone (TLCK) has been used to study the activity of alveolysin (Geoffroy and Alouf 1982). TLCK only reacts with highly reactive sulphydryl groups, and the kinetics of its reactivity indicated a single highly reactive sulphydryl group in alveolysin (Geoffroy and Alouf 1982). This is of significance considering the presence of four cysteine residues in alveolysin (Geoffroy *et al* 1981) and was taken to imply that only one

of the cysteines was essential for the activity of the toxin. TLCK does not react with listeriolysin (Geoffroy *et al* 1987) and its interaction with other sulphydryl-activated cytolysins has not been reported. Incubation of sulphydryl-activated cytolysins with iodoacetic acid, iodoacetamide and N-ethylmaleimide results in irreversible loss of haemolytic activity. However, the loss of haemolytic activity of sulphydryl-activated cytolysins upon incubation with mercurial compounds is reversed by the addition of excess thiol reagents (Scwachmann *et al* 1934, Alouf 1980, Geoffroy and Alouf 1983, Geoffroy *et al* 1987).

It has been suggested that activation by thiol reagents and inactivation by sulphydryl reactive compounds results from the particular oxidation-reduction potentials (E_0) of the compounds used (Mitsui and Hase 1979). Therefore the ability of compounds to activate these toxins results from their low E_0.

B.2.ii Interaction with Cholesterol

The irreversible loss of haemolytic activity of pneumolysin after incubation with cholesterol was first demonstrated by Cole in 1914, and subsequently demonstrated for all other sulphydryl-activated cytolysins (reviewed by Smyth and Duncan 1978). Not all sterols inhibited the haemolytic activity of these toxins with equal efficiency. *In vitro* studies on SLO demonstrated the basic structural requirements for sterols to inhibit the haemolytic activity of this toxin (Howard *et al* 1953, Watson and Kerr 1974, Prigent and Alouf 1976). All inhibitory sterols had a 3β-OH group on the A ring (cyclo pentanoperhydrophenanthrene nucleus). Sterols containing a 3α-OH

group (Epicholesterol), or any other modification to the 3-carbon atom (cholesterol-acetate, thiocholesterol) are non-inhibitory (reviewed by Alouf 1980, Alouf and Geoffroy 1979). An intact B ring is required for inhibition of sulphydryl-activated cytolysins, though cis-trans changes in configuration (Coprestanol) or a saturated B ring (7-dehydrocholesterol), exert no effect on the inhibitory potential of the sterol. The presence of methyl groups (lanosterol) or hydroxyl groups (11-αOH cholesterol) on the B ring, result in a reduction of inhibitory capacity. A further consideration is the nature of the side chains attached to C-17. Removal of the side chain, its substitution with larger groups or carbon chains carrying reactive groups results in a reduced inhibitory effect of these analogues compared to cholesterol (reviewed Alouf 1980). Similar results were observed for other sulphydryl-activated cytolysins tested (Geoffroy and Alouf 1983, Geoffroy *et al* 1987, Mitsui *et al* 1980, Johnson *et al* 1980, Hase *et al* 1976).

Attempts to determine the stoichiometry of the reaction between cholesterol and sulphydryl-activated cytolysins have been largely unsuccessful (reviewed by Smyth and Duncan 1978). This is, in part, due to the physical nature of the sterols used. They are almost insoluble in water and form micelles that are non inhibitory. In addition, cholesterol has a tendency to adhere to glassware used in the experiments, thus making accurate determination of the amount of sterol present very difficult. Two lines of evidence, however, indicate that the stoichiometry of the reaction may be 1:1. Firstly, a study of the binding of pneumolysin to C^{14}-labelled cholesterol (Johnson *et al* 1980) indicated a linear relationship between the binding of cholesterol and toxin. A similar conclusion was reached for alveolysin (Geoffroy and Alouf 1983). Secondly, the specific

structural requirements of sterols for inhibition of haemolytic activity (Alouf 1980) implies that the interaction between toxin and sterol occurs via a particular domain of the protein.

It has been noted that addition of serum to SLO and pneumolysin causes an inhibition of haemolytic activity (Bernheimer 1947, Paton *et al* 1983). It has been assumed that this inhibition by serum is due to cholesterol (Paton *et al* 1983), though the availability of free cholesterol in serum has been questioned (Bernheimer 1976). This point will be discussed later in section B.6.

B.2.iii Toxin Binding to Erythrocytes

There has accumulated a great deal of indirect evidence to support the hypothesis that membrane cholesterol is the receptor for sulphydryl-activated cytolysins (reviewed by Smyth and Duncan 1978, Alouf 1980).

Indirect analyses of the interaction between sulphydryl-activated cytolysins and membranes have indicated the absolute requirement for cholesterol in the binding process. Only those cells that contain cholesterol in their membrane were found to bind these toxins. Bacteria and streptococcal L-forms are resistant to damage by SLO (Bernheimer 1974). Mycoplasma do not normally contain cholesterol in their membranes, however cholesterol is incorporated when these organisms are grown on media containing cholesterol. Only mycoplasma containing cholesterol in their membrane are susceptible to the action of tetanolysin (Rottem *et al* 1976).

In a series of experiments using ferritin labelled cereolysin or SLO (Shany *et al* 1974) toxin bound to erythrocytes was visualised using electron microscopy. After erythrocytes had been treated with compounds that interact with cholesterol such as alfalfa saponin or filipin, the erythrocyte membranes had a reduced capacity to bind labelled SLO or cereolysin. This was thought to reflect a competition between sulphydryl-activated cytolysins and other cholesterol-binding compounds for membrane cholesterol, and implied that cholesterol was the binding site for sulphydryl-activated cytolysins. In a similar series of experiments, cereolysin bound to erythrocyte membranes was detected using anti-tetanolysin antibodies labelled with ferritin (Pendleton *et al* 1972).

Fractionation of cell membranes was undertaken in an attempt to define membrane constituents capable of binding sulphydryl-activated cytolysins. The haemolytic activity of SLO was only inhibited by those fractions containing cholesterol (Thiele *et al* 1965). In addition, it was noted that inhibition of SLO was greater in the presence of phospholipid, though phospholipid alone had no inhibitory effect. This observation suggested that the susceptibility of cell membranes to attack by sulphydryl-activated cytolysins does not depend solely on the presence of cholesterol in the membrane.

In a set of experiments using artificial membranes it has been shown that binding of sulphydryl-activated cytolysins to these membranes is dependent on the ratio of cholesterol to phospholipid. In addition, the nature of the phospholipid can alter the amount of cholesterol required for maximal binding (Delattre *et al* 1979, Alving *et al* 1979, Blumenthal and Habig 1984). Membranes with ratios of cholesterol to phospholipid of 1:2 were capable of maximal binding of SLO.

A similar observation was made using HeLa cells. When grown in the presence of 20-αOH cholesterol or 25-αOH cholesterol these cells were found to contain less cholesterol in their membranes and were less susceptible to SLO. Hela cells containing reduced amounts of cholesterol in their membranes were also less susceptible to the action of saponin and digitonin, compounds known to react with membrane cholesterol. Susceptibility to SLO was restored by the addition of serum or exogenous cholesterol to the cells, treatments which resulted in an increase in the amount of cholesterol in the membrane (Duncan and Buckingham 1978). Using a slightly different approach, it was found that treatment of human erythrocytes with DMSO resulted in changes in the molar ratio of cholesterol to phospholipid in the cell membrane. When cholesterol was increased, the cells became more sensitive to lysis by SLO but more resistant to SLO-induced lysis when the amount of cholesterol was reduced (Linder and Bernheimer 1984). Availability of membrane cholesterol may explain the increased resistance of mouse erythroctes to SLO action (Feherenbach and Templefield 1979), since it is known that these erythrocytes contain more cholesterol in their membranes than other erythrocytes that are more susceptible to lysis.

The binding of sulphydryl-activated toxin to cell membranes does not involve interaction with proteins, since enzymatic removal of surface proteins does not alter the susceptibility of cells to SLO (Oberley and Duncan 1971).

Binding of sulphydryl-activated cytolysins to membranes may be considered a specific process involving a specific receptor, probably cholesterol, in the erythrocyte membrane. The nature of this toxin-cholesterol interaction has so far eluded characterisation, though

the sulphydryl groups of cysteine residues have been implicated in the process (reviewed Smyth and Duncan 1978). In a recent study of perfringolysin, modification of the sulphydryl group by 5,5'-dithio-bis(2-nitrobenzoic acid) [NBS], resulted in a 100-fold reduction of binding relative to native perfringolysin (Iwamoto *et al* 1987). Therefore free, reactive, sulphydryl groups are thought to be required for maximal binding of toxin to cell membranes.

Attempts to determine the mechanism of toxin binding to cell membranes have so far failed. Even the conditions required for binding have been found to vary from experiment to experiment. For example, the binding of SLO to erythrocytes was considered an irreversible event requiring toxin in the reduced form (Alouf 1980). However, oxidised SLO was found to bind to erythrocyte membranes, albeit at a slower rate (Fehrenbach 1977), and the binding of SLO was also found to be reversible at 0°C (Kanbayashi *et al* 1977). These conflicting findings imply that binding of toxin to membrane may be a two step process; a non specific binding to the cell surface which is reversible and a second, cholesterol-dependent phase that involves insertion of the toxin into the membrane, a process which is irreversible and thought to require the reactive and essential sulphydryl group of the toxin.

B.2.iv Cytolysis

The lysis of erythrocytes by sulphydryl-activated cytolysins has been intensively studied, yet the processes involved remain as poorly defined as the binding process. Lysis is dependent on many factors such as temperature, concentration of toxin, and many other conditions, that do not apply to the binding process. There have

been many hypotheses to explain the various observations of erythrocyte lysis, yet none of them encompass the wide spectrum of effects observed during lysis. Currently accepted hypotheses are dealt with below.

Integration of sulphydryl-activated toxin into the cell membrane with concomitant sequestration of cholesterol has been suggested to lead to an increased fluidity of the membrane resulting in increased membrane fragility (Alouf 1980). However, the capacity of erythrocytes to bind SLO without ensuing lysis is lower than the capacity to bind other molecules, such as filipin and saponin, known to cause membrane damage via interaction with cholesterol (Alouf 1980). This may indicate that some other mode of lysis, other than sequestration of cholesterol, occurs after treatment with SLO. The binding of SLO to erythrocyte or artificial membranes does not appear to cause a loss of cholesterol from the membrane (Alouf *et al* 1984, Bhakdi *et al* 1985), so that increased fragility due to the loss of cholesterol is not likely to be important for the lytic process.

In a study of the kinetics of SLO-induced haemolysis (Kanbayashi *et al* 1972, Inoue *et al* 1976), it has been calculated that aggregation of SLO molecules is a prerequisite for cell damage. In the mathematical model of Inoue *et al* (1976), multiple "hits" or lesions were required for lysis.

An attractive hypothesis for erythrocyte lysis was proposed from studies on the loss of haemoglobin from SLO-treated erythrocytes (Duncan 1974). During haemolysis, the rate of loss of haemoglobin was similar to that of cytoplasmic components as judged by the loss of ^{86}Rb. This loss of haemoglobin could not be retarded by the

addition of BSA to the media, which indicated that membrane lesions were produced large enough for haemoglobin to pass through. This suggested that lysis did not occur by a colloid osmotic process. Lysis by a colloid osmotic process is characterised by the influx of water through small pores leading to a swelling of the cell and eventual lysis. Earlier studies of the haemolysis induced by SLO (Bernheimer 1947) indicated that swelling of the erythrocytes occurred prior to lysis. Later studies involving measurement of mean cell volume supported this contention, and indicated that loss of haemoglobin from erythrocytes could be retarded by the presence of large molecular weight dextrans in the supernatant (Fehrenbach *et al* 1982). This observation indicates that a colloid osmotic form of haemolysis by sulphydryl-activated cytolysins may occur, though the lesions produced must be very large. The size of SLO induced lesions has been determined to be greater than 13nm (Buckingham and Duncan 1983), and this observation will be discussed below.

The presence of large holes in toxin-treated cell membranes that may account for haemolysis were first observed after electron microscopic examination of SLO-treated membranes (Dourmashkin and Rosse 1966). Similar holes were observed when rabbit erythrocytes were incubated with cereolysin, SLO (Pendleton *et al* 1972) or perfringolysin (Smyth *et al* 1975). Arc shaped structures were also observed on toxin treated membranes, and it was assumed that they represented incomplete rings. The nature of these "arc and ring" structures as they were called, was at the time unclear but their possible involvement in haemolysis was duly noted (Smyth *et al* 1975). The first of many investigations to determine the nature of these arc and ring structures was undertaken when various natural and artificial membranes were incubated with SLO and the resulting effects examined

by electron microscopy (Duncan and Schlegel 1975). Arc and ring structures were only apparent when haemolytically active toxin was incubated with either cholesterol-containing membranes or cholesterol alone. No arcs or rings were observed at 0°C, when inactive toxin was used, or when toxin alone was examined. The appearance of arc and ring structures was attributed to the unfolding of SLO due to interaction with cholesterol. SLO-mediated sequesteration of membrane cholesterol was postulated to lead to membrane disruption and eventual lysis (Duncan and Schlegel 1975).

More detailed investigations of arc and ring structures formed by cereolysin (Cowell *et al* 1978), perfringolysin (Mitsui *et al* 1979a, b) or SLO (Bhakdi *et al* 1985, Neidermeyer 1985) were undertaken. Attempts to correlate these structures with lysis of erythrocytes were not conclusive. For example, sealed erythrocyte ghosts incubated with cereolysin at concentrations of 5 haemolytic units (HU) of toxin per ml were sufficiently damaged to release internal markers but no arc and ring structures were observed. Arc and ring structures were only observed when concentrations of cereolysin greater than 1,000 HU/ml were used (Cowell et al 1978). Freeze-fracture analysis of membranes after treatment with either cereolysin or perfringolysin revealed no arc and ring structures on the inner surface of the membranes (Cowell *et al* 1978, Mitsui *et al* 1979 a). Although depressions and protrusions were seen on the protoplasmic and exoplasmic faces of erythrocytes lysed with perfringolysin (Mitsui *et al* 1979 a) these were few compared with the arc and ring structures observed on the surface. From these observations it was suggested that arc and ring structures were not transmembrane structures, and their role in cytolysis was not proved.

In a study of SLO (Bhakdi *et al* 1985), aggregates of SLO, with a molecular weight of several million, were isolated from membrane after solubalisation with deoxycholate (DOC) and fractionation by sucrose gradient centrifugation. These aggregates,when viewed by electron microscopy, were typical arc and ring structures and could be incorporated back into cholesterol-free liposomes to give structures similar to those observed in erythrocyte membranes. Examination of freeze-fractures of SLO treated membranes indicated the presence of similar aggregates and depressions observed on membranes following treatment with cereolysin and perfringolysin (Cowell *et al* 1978, Mitsui *et al* 1979 a). This study also indicated that the toxin aggregates spanned the membrane bilayer, although toxin aggegates on the inner face of the membrane were few compared to the number observed on the outer face. Bhakdi *et al* (1985) concluded that these represented the transmembrane pores responsible for lysis of erythrocytes.

Arc and ring structures have been isolated from erythrocytes following lysis with listeriolysin O (Parrisius *et al* 1986) and have been observed after treatment of cholesterol containing liposomes with tetanolysin (Rottem *et al* 1982).

The arc and ring structures observed for all the sulphydryl-activated cytolysins studied are remarkably similar in size. The internal diameter of rings is approximately 30nm, and the internal radii of arcs about 17nm (Cowell *et al* 1978, Mitsui *et al* 1979 a, b, Neidermeyer 1985, Bhakdi *et al* 1985). These are much larger than the pores formed by other membrane binding proteins, such as the C5-C9 complement complex (9nm) and staphylococcal α toxin (2.5 nm) (Bhakdi and Tranum-Jensen 1986). Thus the transmembrane pores produced by

sulphydryl-activated cytolysins are consistent with the size of lesion determined experimentally (Buckingham and Duncan 1983), and are sufficiently large to allow haemoglobin, with a diameter of 3.5 nm to pass through.

A mechanism for the formation of arc and ring structures by SLO has been proposed (Bhakdi *et al* 1985, Hugo *et al* 1986). In this model toxin monomers, after insertion into the membrane, aggregate via lateral diffusion in the plane of the lipid bilayer to form a structure with an outer hydrophobic face that interacts with membrane phospholipids and an internal hydrophilic face which allows the passage of ions and water. The isolation of SLO complexes from erythrocytes and their ability to re-insert into liposomes, indicates that the complex is stable (Bhakdi *et al* 1984). The formation of incomplete rings give rise to arc-shaped structures, which can still insert into liposomes (Bhakdi *et al* 1984). Membrane disruption might occur following aggregation of only a few toxin molecules, resulting in the formation of small lesions that allow the free passage of ions and water, but not large cytoplasmic molecules *i.e.* a colloid osmotic mechanism of lysis (Bernheimer 1947, Fehrenbach *et al* 1982). In support of this hypothesis was the identification of small aggregates of 3-4 SLO molecules as well as larger arc and ring structures in erythrocyte membranes (Neidermeyer 1985).

It is very probable that lysis by sulphydryl-activated cytolysins can occur by the aggregation of protein monomers either into small multimers or the larger arc and ring structures. The relative contribution of each size of aggregate to the overall lytic process, is probably dependent on such factors as protein concentration and incubation temperature.

In contrast to the above hypotheses it could also be argued that arc and ring structures are no more than artifacts and may represent inactive aggregates of toxin molecules that occur during the preparation of samples for electron microscopy. This is suggested by the observation that arc and ring structures are observed at high toxin concentrations in the absence of membranes or cholesterol (Cowell *et al* 1968, Mitsui *et al* 1979 a, b). The true nature and importance of these arc and ring structures remains unclear and is likely to be the subject of much work in the future.

B.3 CYTOLYTIC EFFECTS OF SULPHYDRYL-ACTIVATED CYTOLYSINS

Incubation of pneumolysin with nucleated cells leads to cell lysis and death, though at toxin concentrations significantly higher than required for haemolysis (Johnson *et al* 1981). Many of the interactions between eukaryotic cells and sulphydryl-activated toxins have been extensively reviewed (Smyth and Duncan 1978, Alouf 1980). SLO has been found to lyse leukocytes (Hirch *et al* 1963), macrophages (Zucker-Franklin 1965, Fauvre *et al* 1966), Ehrlich ascites tumour cells (Ginsburg and Crosswicz 1960), human diploid fibroblasts (Thelestam and Mollby 1979), kidney cells in tissue culture (Alouf 1980), HeLa cells (Duncan and Buckingham 1977) and platelets of a variety of mammalian species (Launey and Alouf 1979). Pneumolysin has been shown to affect leukocytes and platelets (Johnson *et al* 1981) and macrophages (Nandosker *et al* 1986). The effect of perfringolysin on PMNL has also been investigated (Stevens *et al* 1987).

Lysis or damage to nucleated cells is usually determined either by biochemical changes in the treated cell or visualised by microscropy. Microscopic studies have indicated that damage to organelles occurs before cell lysis is perceived. SLO treatment of PMNL and macrophages results in massive degranulation before the cells rounded up and disruption of the cell membrane was observed (Hirsch *et al* 1963, Zucker-Franklin 1965, Fauvre *et al* 1966). Degranulation of PMNL after treatment with pneumolysin has also been monitored enzymatically (Johnson *et al* 1981), where significant release of lysomal enzymes was achieved in the presence of sublytic doses of pneumolysin. Organelles, such as lysosomes and mitochondria, also release internal markers following incubation of organelles with SLO (Weissmann *et al* 1963, 1964). Lysosomes isolated from rabbit liver cells were damaged by tetanolysin (Cox *et al* 1974). The sensitivity of different organelles to sulphydryl-activated toxins is difficult to assess due to the different experimental methodologies used, but subcellular particles appeared more sensitive to lysis than the nucleated cells from which they were isolated.

Platelets from various mammalian species, have also been shown to undergo lysis when treated with SLO (Launey and Alouf 1979) and the concomitant release of organelle markers such as monoamine oxidase and serotonin suggests that lysis was associated with disruption of internal organelles. However, lysis did not appear to be accompanied by gross damage to platelet membranes, as evidenced by the retention of membrane properties by platelet ghosts following lysis with SLO (Launey and Alouf 1979).

The varied susceptibility of erythrocytes, platelets and nucleated cells to these toxins may be due to differences in membrane structure. Nucleated cells may be protected to some extent by turn over of the cell membrane which may repair damage caused by the binding and/or aggregation of these toxins. However, the ingestion of membrane-bound toxin may cause the rapid damage to organelles observed, before cell lysis occurs. The exact cause of degranulation is still undetermined.

B.4 SUBLYTIC EFFECTS

The cytolytic effects of the sulphydryl-activated cytolysins on macrophages and PMNL occur *in vitro* at high concentrations of toxin (Bernheimer and Schwartz 1960, Johnson *et al* 1981, Stevens *et al* 1987). Whether or not the concentrations of toxin required to cause damage to cells *in vitro* are achieved *in vivo* has been considered doubtful. These considerations prompted investigations into the effects of sublytic doses of SLO on PMNL (Andersen and van Epps 1972, van Epps and Andersen 1974). At doses of SLO of 1 HU and 0.12 HU per 10^6 PMNL, a significant decrease in both PMNL random migration and migration in response to chemotactic factors was observed. Similar effects on the random migration of PMNL by perfringolysin were observed (Wilkinson 1975, Stevens *et al* 1987). At low concentrations of pneumolysin (less than 0.5 HU/10^6 cells), PMNL migration was stimulated (Johnson *et al* 1981), while at slightly higher doses (0.5-1.0 HU/10^6 PMNL), both cell migration in response to chemotactic factors and random migration of PMNL were inhibited (Johnson *et al* 1981, Paton and Ferrante 1983). High concentrations of pneumolysin applied to the eyes of rabbits produced an acute inflammatory response and infiltration of PMNL (Johnson and Allen 1975). Whether

this was due directly to pneumolysin or to the host response to tissue damage was not investigated. Recent work on the effects of perfringolysin on PMNL migration (Stevens *et al* 1987), indicated that low concentrations of toxin, less than 0.06 HU/2.5x10^5 PMNL were stimulatory to random but not directional migration of PMNL whereas doses greater than 0.06 HU were inhibitory to both types of migration. The reasons for the inhibition and/or stimulation of migration after treatment with different amounts of toxin is unknown.

The ability of sublytic concentrations of sulphydryl-activated cytolysins to effect changes in the migration of PMNL prompted investigations into the possibility that these toxins may affect other PMNL activities. One area of investigation was the production of chemotactic factors by PMNL after treatment with toxin. PMNL incubated with SLO, alveolysin or perfringolysin at concentrations up to 100 HU/10^7 PMNL (Bremm *et al* 1984, Bremm *et al* 1985), resulted in the stimulation of leukotriene production. The production of LTB4, the major class of leukotriene was less than that of LTC4, whilst the later components of the arachidonic pathway, LTD4 and LTE4, were only produced at high toxin concentrations. The production of such potent chemotactic factors may explain the stimulation of PMNL migration observed at low doses of toxin, but is in conflict with the inhibition of of PMNL migration at higher toxin concentrations.

Sub-lytic doses of SLO caused a reduction in the phagocytosis of *Streptococcus pyogenes* by murine macrophages (Ofek *et al* 1972). Sub-lytic concentrations of pneumolysin, less than 2.5 HU/10^6 cells, were shown to reduce the intracellular killing of *S. pneumonia* by PMNL in a dose dependent manner (Paton and Ferrante 1983). Similar effects were observed with pneumolysin and macrophages (Nandoskar *et*

al 1986). This effect on macrophages was abrogated by the pretreatment of pneumolysin by cholesterol.

The ability of pneumolysin to inhibit the respiratory burst, as measured by the inhibition of the hexose monophosphate shunt (HMP), in both PMNL and macrophages was also investigated. The activity of the HMP in macrophages, previously stimulated with zymosan, was reduced by 50%, and the production of H_2O_2 by 60%, after the addition of 1 HU of pneumolysin per 10^6 cells (Nandoskar *et al* 1986). Similar levels of inhibition of HMP activity and H_2O_2 production were observed after the addition of pneumolysin to PMNL (Paton and Ferrante 1983). In contrast to these observations SLO, at concentrations less than 9 HU/ 10^6 PMNL, caused an increase in chemiluminescence, an indication of a higher consumption of oxygen related to the respiratory burst. Higher levels of chemiluminescence were observed at higher doses of toxin but were accompanied by a decreased cell viability (Andersen and Duncan 1980). Low doses of perfringolysin (less than 4 HU/ml) were also found to stimulate PMNL chemiluminescence, though higher concentrations of perfringolysin resulted in a reduction of this response consistent with the results for pneumolysin (Stevens *et al* 1987).

Degranulation of PMNL as measured by the release of lysozyme was also observed after treatment with sub lytic doses of pneumolysin (Johnson *et al* 1981). This complements the observation of degranulation using electron microscopy (Hirsch *et al* 1963, Zucker-Franklin 1972, Fauvre *et al* 1966). In contrast, however, macrophages activated by zymosan gave a lower lysosyme release after treatment with pneumolysin (Nandoskar *et al* 1986).

These observations indicate a spectrum of effects resulting from the interaction between sulphydryl-activated cytolysins and eukaryotic cells, dependent, in part, on the concentration of the toxin used (Andersen and van Epps 1972, Johnson *et al* 1981, Paton and Ferrante 1983, Nandoskar *et al* 1986, Stevens *et al* 1987). Nevertheless, it is clear that significant changes in the ability of cells of the immune system to respond to bacteria are induced by these toxins.

Other cells of the immune system such as T and B lymphocytes are also affected by sub-lytic concentrations of sulphydryl-activated cytolysins. SLO affected E rosette formation (Andersen and Amirault 1976), an indication that the toxin interfered with T-lymphocyte receptors. Haemolytically active SLO, at concentrations of 2 HU/ml, inhibited PHA-induced blast transformation of lymphocytes (Andersen and Cone 1974). A similar response was observed when lymphocytes were incubated with pneumolysin (Ferrante *et al* 1984). The inhibition of lymphocyte blast transformation by a variety of mitogens was investigated at varying concentrations of pneumolysin. At concentrations of pneumolysin greater than 1 HU/ml inhibition of lymphocyte proliferation by PHA, ConA or PWM was observed, though only if pneumolysin was added to the lymphocytes prior to the addition of mitogen. The inhibition of blast transformation was observed throughout the duration of the experiment even though mitogen was present throughout (Ferrante *et al* 1984). Pneumolysin (5HU/ml) caused nearly a complete inhibition of immunoglobulin synthesis from lymphocytes stimulated with PWM, but did not inhibit secretion of immunoglobulin when added to lymphocytes already commited to antibody synthesis. This indicates that pneumolysin irreversibly affects early signals involved in lymphocyte response.

The effect of SLO, perfringolysin (Thelestam and Mollby 1980) and alveolysin (Thelestam *et al* 1981) on human diploid fibroblasts has been investigated. This series of experiments is of interest since differences in the activity of SLO and the other toxins were observed. Although SLO, when bound to membranes at 0°C, appeared to cause no damage, both perfringolysin and alveolysin caused membrane damage at 0°C, though they did not appear to bind at this temperature. Other differences observed included the ability of fibroblasts to take up AIB from the media, and the size of functional lesions in the cell membrane. On each occasion the activity of SLO differed from that of perfringolysin and alveolysin. The findings have been summarised (Mollby *et al* 1982), and led to the conclusion that sulphydryl-activated cytolysins have at least two different mechanisms of action. However, it must be noted that the SLO used in these experiments had a specific activity of 94 HU/mg of protein, which is very much lower than that used by other workers (Alouf 1980). The specific activity of alveolysin (4.7×10^4 HU/mg) and perfringolysin (1×10^6 HU/mg), used in the experiments, are more typical of the activities of purified toxin. These experiments should be repeated using purified SLO with high specific activity before the distinction postulated by these authors can be proved.

SLO affected the transport of simple compounds into and out of HeLa cells (Duncan and Schlegel 1972). The ability of HeLa cells to take up amino acids, nucleosides and glucose was reduced after the addition of SLO at sublethal concentrations. The reduced uptake of AIB observed after SLO treatment, was considered to be due to the inability of HeLa cells to retain this compound rather than an inhibition of the transport into the cell. The ability of the cells to take up AIB was regained within a few hours of the removal of SLO.

Phospholipid methylation in macrophages was also found to be inhibited by pneumolysin (Nandoskar *et al* 1986) at concentrations of 2.5 HU/ 10^6 macrophages. The potential importance of this observation will be discussed below.

The mechanisms by which sulphydryl-activated cytolysins affect cellular processes, in the absence of lysis are unknown, although there have been various hypotheses put forward to account for these effects. For example, transport of molecules into and out of the cell is known to be affected by the concentration of membrane cholesterol (Plagemann and Erbe 1975). In contrast, the activity of ATPase in the transport of molecules through the cell membrane is thought to depend on the presence of phospholipid around the membrane-bound enzyme (Demel and deKruyff 1976, Warren *et al* 1975). Thus any interaction of sulphydryl-activated cytolysins with cholesterol that reduces the fluidity of the membrane may lead to the inhibition of membrane associated transport systems. Signal transmission through membranes is thought to be associated with the methylation of phospholipids (Hirata and Axelrod 1980). The pneumolysin mediated inhibition of phospholipid methylation in macrophages (Nandoskar *et al* 1986) may be one of the ways that pneumolysin, and by inference other toxins of this group, exert their effects on PMNL and macrophages. One additional mechanism by which sulphydryl-activated cytolysins may exert their effect on biological systems is the production of calcium channels in membranes. The influx of calcium ions is known to effect many changes in cell physiology including the stimulation of the arachidonic acid pathway (Suttorp *et al* 1985). Thus the production of leukotrienes by SLO, perfringolysin or alveolysin-treated cells (Bremm *et al* 1984, Bremm *et al* 1985) may result from the influx of calcium ions through small

pores composed of a few toxin molecules. Clearly the mechanisms by which this group of toxins exert their influence requires further investigation. Whether the inhibitory activities observed *in vitro* occur *in vivo* also remains to be determined.

B.5 LETHAL PROPERTIES OF SULPHYDRYL-ACTIVATED CYTOLYSINS

All of the sulphydryl-activated cytolysins so far tested when injected in milligram amounts into small mammals, have been shown to cause rapid death (Halbert 1970, Smyth and Duncan 1978, Hardegree *et al* 1971, Geoffroy *et al* 1987, Shumway and Klebanoff 1971). Although the LD_{50} for each animal species varies, the susceptibility on a weight for weight basis is similar (reviewed by Smyth and Duncan 1978). The cause of death after administration of SLO resulted from interaction with heart tissue (Halbert *et al* 1961). The effects on heart tissue, of high SLO doses are almost instantaneous, although smaller doses, though still lethal, also induce a wide variety of electro- cardiographic changes (Alouf 1980, Halbert 1970). The basis of the cardiotoxicity of these toxins is unknown, but may be due to the release of substances from heart cells that interrupt electrical signals to and from the heart and lead to cardiac failure (Halbert 1970).

B.6 IMMUNOLOGY OF THE SULPHYDRYL-ACTIVATED CYTOLYSINS

B.6.i Antigenic cross reactivity between sulphydryl-activated cytolysins

The current dogma that the sulphydryl-activated cytolysins share structural similarities is based on the observation that antibodies raised against one toxin can cross-react and neutralise other toxins in the group (reviewed Bernheimer 1976). Cross reactions between pneumolysin, perfringolysin, tetanolysin and SLO were first revealed by the ability of antibody raised against one toxin to neutralise the haemolytic activity of heterologous toxins (Todd 1932). This cross-reactivity, in addition to physical and biological properties has frequently been used to define new members of sulphydryl-activated cytolysin family, for example, listeriolysin (Jenkins *et al* 1964, Geoffroy *et al* 1987), cereolysin, alveolysin and latersporulysin (Bernheimer and Grushoff 1967) and other clostridial toxins (reviewed Smyth and Duncan 1978).

In a study of the relationships between sulphydryl-activated cytolysins Cowell and Bernheimer (1977) showed that antibody raised against one toxin was capable of inhibiting the haemolytic activity of other sulphydryl-activated toxins tested, but to a lesser extent than with the homologous toxin. Antibodies raised against cereolysin, perfringolysin or SLO, when used in a double immuno-diffusion (Ouchterlony) experiment, produced precipitin lines of complete or partial identity between heterologous sulphydryl-activated cytolysins. However, antibody raised against SLO, cereolysin and perfringolysin failed to precipitate tetanolysin, even though neutralisation of the haemolytic activity of tetanolysin was

achieved by all three sera. This apparent inconsistency was not explained.

Polyclonal serum raised against SLO was found to contain two distinct subgroups of antibodies (reviewed Alouf 1980). One sub-group inhibited the binding of toxin to the erythrocyte membrane and were termed the anti-fixation or anti-f antibodies. The other subgroup, termed the anti-lytic or anti-l antibodies, inhibited lysis, although they did not inhibit the binding of sulphydryl- activated toxin to membrane.

The extent of structural similarity shared by various sulphydryl-activated cytolysins has been investigated using sera containing different ratios of anti-f and anti-l antibodies (Geoffroy and Alouf 1984). The results were inconclusive, but all sulphydryl-activated toxins analysed were neutralised to a greater or lesser extent by three different anti-SLO sera of known anti-f and anti-l composition.

Monoclonal antibodies, however, provide a more sensitive means by which to investigate the structural relationships between these toxins. Ten monoclonal IgG antibodies against SLO, were isolated (Mansa and Kjems 1970) and tested for their neutralising activity against pneumolysin, tetanolysin and perfringolysin. Only one of monoclonal antibodies had the ability to neutralise heterologous toxin and then only pneumolysin (Mansa and Kjems 1970). This suggested that the other SLO-specific monoclonal antibodies either recognised epitopes that were not present on the other toxins, or that common epitopes exist which do not give rise to neutralisation.

In a similar series of experiments, monoclonal antibodies were raised against a toxoid preparation of perfringolysin, and the cross-reactivity of anti-perfringolysin antibodies was investigated (Sato 1986). Two anti-perfringolysin monoclonal antibodies were isolated that reacted with and neutralised SLO. These two monoclonal antibodies bound to tetanolysin very weakly but did not neutralise the haemolytic activity of the toxin. Binding of these monoclonal antibodies to SLO also inhibited the cardiotoxicity of the toxin (Sato 1986). The two monoclonal antibodies did not interfere with the binding of perfringolysin to erythrocyte membranes but did inhibit subsequent lysis, *ie* they were anti-1 antibodies (Sato 1986).

In a parallel study on SLO, monoclonal antibodies were raised against toxin oligomers isolated from lysed cell membranes (Hugo *et al* 1986). Only four antibodies were isolated that bound to denatured SLO in western blots. Only one of the monoclonal antibodies neutralised the haemolytic activity of SLO. This neutralising antibody did not bind to cereolysin or listeriolysin O. The implication is that the epitope present on SLO, to which the antibody was raised, is not present on the other sulphydryl-activated toxins tested. The isolation of three monoclonal antibodies that do not inhibit either binding or lysis could indicate the presence of epitopes on these toxins that are not involved in the binding to membranes or the subsequent lytic process. The use of a toxoid, as in perfringolysin, or aggregated toxin (SLO) for immunisation of mice may not represent the best way of isolating a complete battery of monoclonal antibodies since the full range of epitopes present on active toxin may not be presented to the immune system. It is to be expected that monoclonal antibodies will provide a significant insight into the molecular relationships in this family of toxins.

B.6.ii Interaction of Pneumolysin and SLO with Complement

The central role of complement in host defence against pneumococcal disease has been considered (Brown 1985, Winklestein 1981). Purified pneumolysin when added to human sera, at a concentration of 10μg/ml, resulted in conversion of C3 via the classical complement pathway. This conversion was higher when the toxin had been inactivated by cholesterol (Paton *et al* 1984). The sera used in these experiments contained no anti-pneumolysin antibody as determined by ELISA. Similar observations have been made with SLO (Bhakdi and Tranum-Jensen 1985), but in this case, there were low but detectable levels of anti-SLO antibody present in the sera. Removal of IgG from the sera abrogated the activation of complement by SLO, though the ability to activate complement was restored when the homologous IgG was returned to the assay. It was also noted that fixation of the terminal components of the complement cascade, C5-C9, to erythrocytes occurred after the binding of SLO to the erythrocytes. This led to the suggestion that membrane-bound SLO acts as a focus for complement activation, which might result in autolysis. Clearly the mechanism of the activation of complement by pneumolysin and SLO requires further study, since it is not clear how the conformational change of IgG, required for the activation of complement by the classical pathway is achieved in the above experiments. The lack of specific antibody to the toxin suggests that a non-immune binding of pneumolysin or SLO to IgG occurs, resulting in a conformational change of the antibody to allow the subsequent activation of the classical pathway of complement.

B.6.iii Antibody response to the sulphydryl-activated cytolysins

Pneumolysin is produced by nearly all clinical isolates of the pneumococcus (Kanclerski and Mollby 1987, Lorian *et al* 1973). The antibody response to pneumolysin during pneumococcal infection has been difficult to quantify (Sutliffe and Zoffuto 1968, Kalin *et al* 1987). This situation contrasts sharply with that observed for infections of *S. pyogenes*, where the antibody response to SLO is sufficiently reproducible to use the rise in anti-SLO antibody titre in the diagnosis of streptococcal disease (Todd 1932). One reason for the low antibody response to pneumolysin during pneumococcal infections may be due to the intracellular nature of this toxin (Johnson 1976).

It has been shown that immunisation of mice with sub lethal doses of pneumolysin, affords some protection against subsequent challenge of virulent pneumococci (Paton *et al* 1983). There exists, therefore, the prospect of including pneumolysin in the the present pneumococcal vaccine. This may have certain advantages over the present vaccine since pneumolysin, being a protein antigen, may stimulate immunity in young children. The production of pneumolysin by nearly all clinical isolates of the pneumococcus (Kanclerski and Mollby 1987) may also result in protection against all serotypes. However, circulating immune complexes (CIC) of SLO and anti-SLO antibody have been implicated in the pathogenesis of acute rheumatic fever (Gupta *et al* 1986). Whether or not pneumolysin would elicit a similar response is unknown, though the *in vivo* response to SLO does not appear to generate antibodies cross reactive with pneumolysin (Kanclerski *et al* 1987).

B.7 MOLECULAR BIOLOGY OF THE SULPHYDRYL-ACTIVATED CYTOLYSINS

At the present time there exists a great potential for the application of recombinant DNA technology to the study of the structure of sulphydryl-activated cytolysins. The first reported cloning of a gene expressing one of these toxins was the isolation of the SLO gene from *S. pyogenes* (Kehoe and Timmis 1984). The cloned gene produced a 68 and 61 kD protein in minicells (Kehoe and Timmis 1984) which were similar to the sizes of native SLO (Bhakdi *et al* 1985). In a Southern blotting experiment carried out under stringent conditions of hybridisation, no homology was demonstrated between an internal restriction fragment from within the coding sequence of the cloned SLO gene and chromosomal DNA from *S. pneumoniae, Clostridium perfringens* and *Listeria monocytogenes*. The hybridisation indicated that what homology exists between these toxins must reside in the primary or secondary sequence.

The reported isolation of a recombinant plasmid carrying the cereolysin gene from *Bacillus cereus* (Kreft *et al* 1983), was subsequently shown to be erroneous (Gilmore 1985), despite the characterisation of the properties of the cloned gene product. The error was only discovered when the amino acid sequence derived from the DNA sequence was found to be dissimilar to that determined for the native toxin (Cowell and Bernheimer 1976).

Transposon mutagenesis has been used by various groups to study the role of listeriolysin in the infections caused by *L. monocytogenes*. Using either the transposon Tn916 (Kathariou *et al* 1987) or Tn1545 (Gaillard *et al* 1986), non haemolytic *L. monocytogenes* have been isolated. Some sequence data of the

listeriolysin gene was determined after cloning the region flanked by Tn1545 in *E. coli* (Mengaud *et al* 1987). A preliminary report of the cloning of the listeriolysin gene (Vicente *et al* 1985), has not been followed up by other work.

Subsequent to the work described in this thesis, the cloning and expression of the pneumolysin gene in *E. coli* was reported (Paton *et al* 1986). The significance of this will be discussed where appropriate.

B.8 ROLE OF SULPHYDRYL-ACTIVATED CYTOLYSINS IN DISEASE

All the bacteria that produce sulphydryl-activated cytolysins (Table 1) are capable of causing infections in man, animals or insects. This observation, together with the effects of these toxins on erythrocytes, PMNL, macrophages and complement strongly implies that these toxins have a role in the infections caused by their respective bacteria.

The only direct evidence at the present time of a role of these toxins in infection is that virulence of *L. monocytogenes* is dependent on the production of listeriolysin O (Kathariou *et al* 1987). However, the exact role of the toxin in virulence awaits characterisation. It is thought that the toxin supports the survival of the organism within macrophages (Kuhn *et al* 1988).

The production of a specific pneumolysin negative *S. pneumoniae* serotype 1 (J. Paton personal communication), following *in-vitro* mutagenesis of the pneumolysin gene, has indicated that pneumolysin

contributes to the virulence of the pneumococcus. Pneumolysin negative mutants had a higher LD_{50} than the parental isogenic strain, following challenge of mice by the intra-nasal route. Whether this loss of virulence observed in the experimental animal model can be extrapolated to a reduction of the virulence of the organism in human infections remains to be clarified.

Pneumolysin has been shown to induce severe abnormal ciliary beating when added to sections of rat trachea *in-vitro* (R. Wilson personal communication) though this effect was accompanied by damage to the epithelial cells. Sub-lytic doses of pneumolysin in the trachea may impair normal ciliary beating, thus preventing clearance of ingested organisms from the lung, and is an interesting consideration in assigning possible roles for pneumolysin in the virulence of the pneumococcus.

Sulphydryl-activated cytolysins may simply effect damage on the host by interaction with components of the immune system. Circulating immune complexes, as have been detected for SLO (Gupta *et al* 1986) may result in the slow release of active toxin targeted to cardiac cells, and be the cause of rheumatic heart disease, a common sequalae to streptococcal infections. The activation of the classical complement pathway by pneumolysin and SLO (Paton *et al* 1984, Bhakdi and Tranum-Jensen 1985) may have significant effects *in vivo*, diverting opsonising components of complement away from bacteria. This may reduce the overall efficiency of opsonisation and result in a higher survival of pathogenic bacteria.

B.9 SUMMARY

The membrane damaging properties of pneumolysin and other sulphydryl-activated cytolysins have been the subject of intense study. Despite the accumulation of data on their activities, our understanding of the mechanisms by which these proteins exert their effects is poor. How these toxins bind to cell membranes, the mechanism of lysis or how changes to PMNL and macrophages occur at sub-lytic concentrations of toxin remain unclear. Much of this lack of knowledge stems from the paucity of data available concerning the structure of these proteins. Recombinant DNA technology provides the most rapid route to this goal and was the approach adopted in this work.

Table 1

SULPHYDRYL-ACTIVATED CYTOLYSINS PRODUCED BY BACTERIAL SPECIES

ORGANISM	CYTOLYSIN	OTHER HAEMOLYSINS
S. pneumoniae	Pneumolysin	
S. pyogenes	Streptolysin O	Streptolysin S
L. monocytogenes	Listeriolysin O	α listeriolysin
B. alvei	Alveolysin	
B. thuringiensis	Thuringeolysin	
B. cereus	Cereolysin	Cereolysin X
Cl. perfringens	Perfringiolysin	α haemolysin
		β haemolysin
Cl. tetani	Tetanolysin	

TABLE 2

PHYSICAL CHARACTERISTICS OF SULPHYDRYL-ACTIVATED CYTOLYSINS

PROTEIN	SIZE	pI	REFERENCE
Pneumolysin	53,000	5.2	Kanclerski and Mollby 1987
SLO	69,000	6-6.4	Bhakdi *et al* 1984
	61,000	7-7.5	" " "
Alveolysin	63,000	5.1	Geoffroy and Alouf 1983
Listeriolysin	60,000	-	Geoffroy *et al* 1987
Tetanolysin	53,000	(5.3,5.6)	Mitsui *et al* 1980
	48,000	(6.1,6.6)	
Perfringolysin	51,000	-	Yamakawa *et al* 1977
Cereolysin	52,000	6.3-6.5	Cowell *et al* 1976
	55,500		
	59,000		
Thuringiolysin	55,000	-	Rakatobe and Alouf 1984

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial Strains, Plasmids and Bacteriophage: Growth and Maintenance

All strains of bacteria used are listed in Table 3. Plasmids and bacteriophage are listed in Table 4. Bacteria carrying recombinant or vector plasmids were maintained at all times in the presence of the appropriate antibiotic selection. Unless stated all strains of *E. coli* were grown on Luria media at 37°C. *E. coli* strains BHB2688, BHB2690, JM101 and TG1 were maintained on minimal media.
S. pneumoniae strains were maintained and propagated on Brain Heart Infusion broth or blood agar at 37°C. All bacteria were stored at -20°C in the appropriate media containing 10 % glycerol.

2.2 Growth Media

All media was made up with distilled water and sterilised at 15 psi for 20 minutes unless stated otherwise.

Luria Agar (L-Agar) per Litre

10 g Peptone (Difco)
5 g Yeast Extract (Oxoid)
5 g NaCl
15 g Agar (Difco)

Luria Broth (L-Broth) per Litre

10 g Peptone (Difco)
5 g Yeast Extract (Oxoid)
5 g NaCl

M 9 salts	per Litre
	24 g Na_2HPO_4
	12 g KH_2PO_4
	6 g NH_4Cl
	3 g NaCl

Minimal media	per Litre
	15 g Agar (Difco) in 750 ml water
	sterilised separately
	250 ml 4x M9 salts
	20 ml 10 % glucose (Filter sterilised)
	1 ml 20 % $MgSO_4$
	0.5 ml 1 % Thiamine (Filter sterilised)

Top agar	per 100 ml
	1 g Peptone
	0.5 g NaCl
	0.8 g Agar

Blood agarose overlay	per 100 ml
	0.5 g Agarose (Seakhem)
	100 ml PBS (Oxoid)
	Sterilise, cool to 50°C, and add
	7.5 ml Sheep erythrocytes (Gibco/BRL)

All other media: Brain Heart Infusion (Oxoid); Columbia Blood Agar Base (Oxoid) were made up according to the manufacturers instructions.

2.3 Ethanol Precipitation of DNA

To the DNA solution was added 0.1 volume of 3 M sodium acetate pH 5.6 and 2.5 volumes of ethanol. The contents were mixed well by gentle vortexing and either stored at -70°C for 10 min in a dry ice-ethanol bath or for two hours at -20°C. The precipitated DNA was collected by centrifugation for 10 min in a microfuge. The supernatant was carefully discarded, and the pellet washed with an appropriate volume of ice cold 70 % Ethanol-water. The contents were vortexed briefly and recentrifuged for 5 min. The supernatant was carefully removed by aspiration through a drawn out pasteur pipette. The DNA pellet was dried under vacuum, and resuspended in the appropriate volume of TE buffer (10 mM Tris-HCl pH8.0, 1 mM EDTA), unless otherwise specified.

2.4 Ligation of DNA Molecules

Unless stated otherwise all ligation reactions were carried out in a final reaction volume of 20 µl. The reaction mix contained.

2 µl 10x ligation buffer (10 µl 1 M Tris-HCl pH 7.4, 2 µl 1 M $MgCl_2$, 8 µl SDW)

2 µl 10 mM ATP (1/10 dilution of 100 mM stock solution)

2 µl 100 mM DTT

x µl DNA solution

SDW to 20 µl

0.5 µl T4 DNA ligase [1u] (Pharmacia)

The reactions were incubated at 16°C overnight. Where required the products of the ligation reaction were analysed by agarose gel electrophoresis.

2.5 Phenol Extraction of Protein from DNA solutions

i Preparation of phenol

Solid phenol [100 g] (Fisons) was added to 100 ml of chloroform, containing 2 ml isoamyl-alcohol (IAA), and mixed gently until all the phenol had dissolved. This solution was equilibrated with 1 M Tris-HCl pH 8.0 until the pH of the aqueous layer was greater than pH 7. To the equilibrated Phenol/chloroform/IAA, 0.2 g of 8-hydroxy quinoline was added to prevent oxidation of the phenol. The Phenol/chloroform/IAA (PCI) was finally equilibrated with 0.1 M Tris-HCl pH 8.0 and stored in the dark at 4°C.

ii Phenol extraction

To deproteinate DNA, an equal volume of PCI was added to the DNA solution, and the two phases mixed by vortexing for 10 seconds. The phases were separated by centrifugation either for 2 min in a microfuge or in a Heraeus Christ bench centrifuge (5K for 5 min), whichever was more convenient. The upper aqueous layer was retained and any residual phenol removed by extraction with either diethyl-ether or chloroform/IAA (50:1 v/v). The DNA was collected by ethanol precipitation and resuspended in an appropriate volume of TE buffer.

2.6 End Repair of DNA Fragments

DNA fragments were dissolved in SDW to a total volume of 20 µl and 2 µl 10x End repair buffer (500 mM Tris-HCl pH 7.5, 100 mM $MgSO_4$, 1 mM

DTT), 1 µl 2.5 mM dNTP and 0.5 µl Klenow fragment DNA polymerase (BRL) was added. The reaction mixture was incubated for 15 min at 37°C and the enzyme inactivated for 10 min at 70°C.

2.7 5' Phosphorylation of Oligonucleotides

To 10 µl (≙ 100 pM) of oligonucleotide was added; 2 µl 10x Kinase buffer (1 M Tris-HCl pH 8.0, 100 mM $MgCl_2$, 100 mM DTT), 2 µl 10 mM ATP, 1 µl T4 Kinase (BRL) and 4 µl SDW. The contents were mixed by gentle pipetting and incubated at 37°C for 15 min. The enzyme was inactivated by heating at 70°C for 10 min. The oligonucleotides were either used immediately or stored at -20°C until required.

2.8 Dephosphorylation of DNA

To DNA in a volume of 20 µl was added 2 µl 10x CIP buffer (500 mM Tris-HCl pH 9.0, 100 mM $MgCl_2$, 1 mM $ZnCl_2$) and 1 µl of Calf intestinal alkaline phosphatase (Boehringer). The reaction was incubated at 37°C for 30 min. The phosphatase was denatured and removed from the DNA solution by two extractions with PCI followed by extraction with diethyl ether. Where necessary the DNA was concentrated by ethanol precipitation.

2.9 Restriction Enzyme Digestion of DNA

Restriction enzyme digests for analytical purposes were set up in a total volume of 10 µl according to the manufacturers recommendations.

The digest was then stopped by the addition of 1 μl agarose gel loading buffer. For preparative purposes, the volume of the reaction depended on the amount of DNA to be digested. This volume was amended to give optimal conditions for digestion, in accordance with the manufacturers recommendations. The digest was stopped either by heating at 70°C for 10 min or by phenol extraction.

2.10 Haemolytic Assays

Fifty microlitres of PBS (Oxoid) containing 1 mM DTT was added to the wells of a microtitre plate (Nunc). Into the first well was pipetted 50 μl of the supernatant to be analysed. Serial two-fold dilutions were then carried out along the wells of the microtitre plate. Where required, cholesterol or serum containing antibodies to pneumolysin or related toxins were added and the plates incubated at room temperature for 15 min. Fresh, washed sheep erythrocytes (50 μl of a 1.7 % suspension in PBS) were added to each well. The plate was incubated at 37°C for 30 min, and then placed at 4°C to enhance the settling of the unlysed erythrocytes. The haemolytic activity present, in haemolytic units (HU), was calculated as the reciprocal of the lowest dilution giving approximately 50 % lysis. This figure was corrected to give HU/ml of original suspension.

2.11 Transformation of *E. coli* by DNA

The method of transformation used routinely was based on the method of Cohen and Chang (1973). L-broth (50 ml) was inoculated with 500 μl of an overnight culture of an appropriate *E. coli* strain and grown at 37°C with vigorous shaking until mid-log phase (OD_{650} 0.5-0.6) was

reached. The culture was transferred to sterile plastic Universal bottles (Nunc) and the cells pelleted by centrifugation (Heraeus-Christ bench centrifuge, 3.5 K for 5 min at 4°C). The supernatant was carefully discarded and the cells gently resuspended in 1 volume of ice cold $MgCl_2$ (100 mM). The cells were pelleted by centrifugation (Heraeus Christ, 2 K for 10 min at 4°C). The supernatant was discarded and the cells gently resuspended in ½ volume ice cold $CaCl_2$ (100 mM) [Sigma]. The cell suspension was kept on ice for 20 min. The cells were again pelleted as before. The supernatant was discarded and the pellet resuspended in 1/20 volume of ice cold, sterile $CaCl_2$ (100 mM). The cell suspension was kept on ice for at least 1 hour to achieve competence. An appropriate volume of plasmid DNA was added to 200 µl competent cells in a 1.5 ml microfuge tube and kept on ice for 1 hour. The tubes were then placed at 42°C for 2 min and returned to ice for at least another 20 min.

L-broth (1 ml) was added to each tube and the cells incubated for a further 45 min at 37°C, to allow expression of antibiotic resistance genes. The neat culture (100 µl) or ten-fold serial dilutions, where necessary, were then spread onto the surface of plates of the appropriate selective media. All plates were incubated in an inverted position at 37°C overnight unless otherwise stated.

M13 recombinants were identified by their ability to form clear plaques on a lawn of *E. coli*. The method for selection of M13 recombinants differs from the isolation of plasmid recombinants at the following points. At the initial harvesting of mid log phase bacteria, 50 µl of the culture was transferred to 10 ml of L-broth and incubated at 37°C to provide the indicator cells. After the

42°C heat shock, competent cells plus DNA were stood on ice for a further 30 min. This cell suspension (usually 100 µl, 50 µl and 20 µl aliquots) was added to sterile glass phage tubes. To each tube was added 200 µl of indicator cells, 25 µl X-gal (2 % in DMF), 10 µl IPTG (100 mM) and 3 ml Top agar, pre-cooled to 50°C. The contents of each tube were poured immediately onto a pre-dried minimal media agar plates, and allowed to set. All the plates were incubated overnight at 37°C in an inverted position.

2.12 Preparation of Cell Free Extracts

Bacteria from a 500 ml overnight culture of *E. coli* harbouring plasmids expressing pneumolysin were harvested by centrifugation (GSA rotor, 7 K for 10 min). The bacterial pellet was resuspended in 20 ml of PBS and frozen at -20°C. The frozen culture was thawed and sonicated (Braun, labsonic 2000) at 50W in 30 sec bursts until lysis was achieved. Bacteria and other cell debris were cleared by centrifugation (SS-34 rotor, 12 K for 20 min). Crude, cell-free supernatants were stored at -20°C. This method was also used to produce cell free extracts from smaller cultures. Variations in the above protocol are dealt with in the relevant results section.

2.13 (i) Large Scale Preparation of Plasmid DNA

Plasmid DNA was routinely prepared using the alkali lysis method of Birnboim and Doly (1979). L-broth (500 ml) containing the appropriate antibiotic selection was inoculated with *E.coli* carrying the plasmid to be purified and incubated overnight at 37°C. The

following day the cells were harvested by centrifugation (GSA rotor, 7 K for 10 min). The supernatant was discarded and each pellet was resuspended in 5 ml of lysis solution I, transferred to a 35 ml polycarbonate centrifuge tube and stood at room temperature for 5 min. To each tube was added 10 ml of lysis solution II, the contents mixed and stored on ice for a further 10 min. To this lysate was added 7.5 ml of KAc, the contents were again mixed well and stored on ice for a further 10 min. The lysate was cleared of bacterial debris by centrifugation (SS-34 rotor, 21 K for 30 min), and to 18 ml of the cleared lysate was added 12 ml of propan-2-ol in a 30 ml test tube (Corex). The contents were mixed thoroughly and allowed to stand at room temperature for 30 min. The precipitated DNA was collected by centrifugation (Kontron Hermle AS4-13 rotor, 7 K for 20 min). The supernatant was discarded and excess solvent removed under vacuum. The DNA pellet was completely dissolved in 12 ml of TE buffer. CsCl (12 g) was added and dissolved gently to avoid frothing. When the CsCl had dissolved completely, 1.2 ml of ethidium bromide (10 mg/ml) was added and mixed thoroughly. The density was adjusted to 1.3860, where necessary, by the addition of either TE buffer or a saturated CsCl solution. The lysate was carefully pipetted into a 36 ml quick seal centrifuge tube (Sorvall), and the remaining space filled with liquid paraffin (Fisons). The tubes were balanced to within 0.05 g and then sealed. The plasmid DNA was then centrifuged to equilibrium in the CsCl gradient (TV-850 rotor, 20 hr at 39 K at a temperature of 20°C).

After centrifugation the DNA was visualised under UV light. The lower band, comprising CCC plasmid DNA, was removed through the side of the tube using a syringe and needle. Ethidium bromide was extracted with propan-2-ol, equilibrated with a saturated CsCl

solution, until no pink colour was observed in the aqueous phase. The CsCl was then removed from the DNA solution by dialysis, against several changes of TE buffer at 4°C. The DNA was deproteinised by phenol extraction and finally concentrated by ethanol precipitation. Plasmid DNA pellets were routinely resuspended in 500 µl of TE buffer.

2.13 (ii) Small Scale Preparation of Plasmid DNA

An overnight culture (1 ml) of *E. coli* harbouring the required plasmid was transferred to a 1.5 ml microfuge tube, and the bacterial cells pelleted by centrifugation for 5 min in a microfuge. The bacterial pellet was resuspended in 100 µl of lysis solution I and stored at room temperature for 5 min. Lysis solution II (200 µl) was added and the contents mixed until the solution cleared and then stored on ice for 5 min. To this lysate was added 150 µl of KAc, the contents were mixed by gentle vortexing for 10 sec and again stored on ice for a further 5 min. The precipitate was collected by centrifugation for 5 min. The supernatant (400 µl) was transferred to a fresh microfuge tube to which was added 400 µl of PCI, the contents were mixed by vortexing for 10 sec. The two phases were separated by centrifugation for 2 min and the aqueous layer removed to a fresh microfuge tube. Ethanol (800 µl) at -20°C was added, the contents were mixed by vortexing and stood at room temperature for 2 min. The DNA was pelleted by centrifugation for 10 min. The supernatant was discarded and the tubes left to drain in an inverted position. The pellet was washed in ice cold 70 % ethanol and recentrifuged for 3 min. The supernatant was gently removed by aspiration through a drawn out pasteur pipette and the pellet dried

under vacuum. The DNA was resuspended in 50 µl TE buffer containing 20 ng/ml RNase.

Solutions for the preparation of DNA.

Lysis solution I

50 mM Glucose

25 mM Tris-HCl pH 8.0

10 mM EDTA

5 mg/ml of lysozyme added prior to use

Lysis solution II (made up fresh prior to use)

0.2 M NaOH

1 % SDS

KAc solution

60 ml 5M Potassium acetate

11.5 ml Glacial acetic acid

28.5 ml Water

2.14 Separation of DNA Fragments by Agarose Gel Electrophoresis

Unless otherwise stated agarose gels contained 0.8 % agarose, which is suitable for the separation of DNA fragments between 500 bp and 7 kb in length.

To 0.8 g of agarose (Seakhem, high gelling temperature) was added 100 ml of 1x Tris-Acetate-EDTA buffer (TAE) containing 0.5 µg/ml ethidium bromide, and the slurry boiled until the agar had completely dissolved. The gel was cast on gel plates (110 mm x 110 mm) the edges of which were sealed with tape to form a mould. Sufficient

agarose was poured into the mould to a depth of 5 mm. A comb was placed in the agarose to form the sample wells, and the agarose allowed to set. The gel was placed in a horizontal gel tank and 1x TAE buffer added so that the gel was completely submerged. Agarose gel loading buffer, at 1/10 volume was added to each sample, which were then loaded into the wells of the gel. DNA markers (250 ng per well) were also included on the gel to allow size estimation of DNA fragments. The DNA fragments were electrophoresed at 100 V, until the required separation had been achieved.

Minigels were run on a 70 x 70 mm lantern slide. A minigel comb with teeth of 1.5 mm x 0.5 mm was placed in contact with the surface of the glass plate, and 15 ml of agarose poured onto the slide. The agarose was retained on the plate by surface tension. Minigels were routinely run at 100 V until the bromophenol blue present in the loading buffer had migrated 3/4 of the length of the gel.

50x TAE buffer (per litre)

242 g Tris base

57.1 ml Glacial acetic acid

100 ml 500 mM EDTA

Agarose gel loading buffer

2 g Ficoll 400

25 mg Bromophenol Blue

25 mg Xylene cyanol FF

make up to 10 ml with 1x TAE.

2.15 Separation of DNA Fragments on Polyacrylamide Gels

DNA fragments less than 500 bp were separated by polyacrylamide gel electrophoresis. Two plates (200 mm x 200 mm) were assembled, separated by 1.5 mm spacers, and the bottom and sides of the plates sealed with tape. An 8 % acrylamide solution (50 ml) was degassed under vacuum and 350 µl of 10 % APS and 80 µl of TEMED added. The acrylamide was poured between the two plates, avoiding the formation of air bubbles. The wells were formed by inserting a comb into the space at the top of the gel. When the gel had polymerised the tape and comb were removed and the gel assembled into a vertical gel apparatus. The top and bottom reservoirs were filled with 1x TBE buffer. The DNA samples and appropriate standard markers were loaded into the wells at the top of the gel. Electrophoresis was carried out at 200 V until the bromophenol blue dye front had migrated to the bottom of the gel. The gel was stained in ethidium bromide solution (0.5 µg/ml) for 30 min. The DNA fragments were visualised under UV light.

Solutions

30 % Acrylamide — per 100 ml

29 g Acrylamide

1 g Bis acrylamide

SDW to 100 ml

The acrylamide solution was deionised with amberlite MB-1 (BDH), filtered and stored at 4°C

10x TBE buffer	per Litre
	108 g Tris base
	55 g Boric acid
	9.3 g EDTA

8 % Acrylamide	per 50 ml
	13.3 ml 30 % Acrylamide
	5 ml 1x TBE buffer
	700 µl 10 % APS
	32 ml SDW

2.16 Discontinuous SDS-polyacrylamide Gel Electrophoresis

Proteins were separated and visualised using the method of Laemmli 1970. Gel plates were assembled as described in the previous method. Separating acrylamide solution (40 ml) was degassed under vacuum. To this solution was added 80 µl of TEMED, mixed well and poured between the two plates to within 5 cm of the top of the notched plate. Water saturated butanol was gently poured over the surface of the acrylamide. When the acrylamide had polymerised, the butanol was discarded and the surface of the gel rinsed with water and dried. The remainder of the gel space was filled with stacking acrylamide solution, after the addition of 30 µl of TEMED. A comb was inserted at the top of the gel and the acrylamide left to polymerise. The comb and tape were removed and the gel assembly placed into a vertical electrophoresis tank. SDS-PAGE running buffer was added to the top and bottom reservoirs. An equal volume of SDS-PAGE gel loading buffer was added to the protein samples, which were boiled for 5 min prior to loading. The samples were loaded into the wells

of the gel, together with appropriate standard protein markers. The samples were run through the stacking gel at 100 V, and after the dye front had entered the separating gel further electrophoresis was carried out at 250 V until the bromophenol blue dye front had migrated to the bottom of the gel. The separated proteins were visualised by staining with coomassie stain for 10 min followed by extensive destaining (30 % methanol, 10 % acetic acid in water). Proteins were also analysed on a Biorad minigel system using glass plates of 80 mm x 100 mm separated by 1 mm spacers. Volumes of acrylamide were adjusted accordingly.

Solutions for SDS-PAGE

Separating acrylamide (10 %)	per gel
	15 ml 30 % Acrylamide
	4.8 ml 1 M Tris-HCl pH 8.8
	23.6 ml water
	450 µl 10 % SDS
	225 µl 10 % APS (made fresh)

Stacking acrylamide (5.3 %)	per gel
	3.5 ml 30 % Acrylamide
	5 ml 1 M Tris-HCl pH 6.8
	12.2 ml water
	200 µl 10 % SDS
	100 µl 10 % APS (made fresh)

SDS-PAGE Running Buffer	per litre
	28.8 g Glycine
	3.03 g Tris base
	5 g SDS

SDS-PAGE Loading Buffer	1.25 ml 0.5 M Tris-HCl pH 6.8
	0.2 g SDS
	2 ml Glycerol
	0.5 ml β-mercaptoethanol
	10 mg Bromophenol blue
	6.25 ml SDW

Coomassie Stain	per 100 ml
	30 ml Methanol (Analytical grade)
	10 ml Glacial acetic acid
	60 ml Water
	250 mg Coomassie blue (R 250)

2.17 Purification of Synthetic Oligonucleotides

Synthetic oligonucleotides were produced by J. Keyte, Department of Biochemistry by either a machine method (Applied Biosystems 380B DNA synthesizer) using cyanoethyl phosphoramidite chemistry or using a paper disk method (Frank *et al* 1983). Oligonucleotides made by the paper disk method were purified as follows.

Two glass plates (200 mm x 500 mm) were siliconised and assembled with 0.4mm spacers (BRL). To 50 ml of 20 % acrylamide was added 360 µl of 10 % APS and 15 µl TEMED. The solution was poured between the

plates and allowed to polymerise. The gel assembly was clamped into a vertical electrophoresis tank and 1x TBE poured into both top and bottom reservoirs. The gel was pre run at 45 watts for 15 min prior to the loading of the samples. Oligonucleotides were dissolved in 100 µl of SDW, and the DNA was ethanol precipitated and resuspended in 10 µl of SDW. To 2 µl of the oligonucleotide was added an equal volume of formamide dye mix, and the samples boiled for 2 min. The samples were loaded between the teeth of a 5.7 mm sharkstooth comb (BRL) with at least one space between the samples. The gel was electrophoresed at 40 W for about 3 hr, or until the bromophenol blue dye front was about 2/3 down the length of the gel. The plates were gently separated and the gel sandwiched between two sheets of clingfilm. Oligonucleotides were visualised by UV shadowing under short wave illumination. The required band, running at the top of the gel, was cut from the gel, transferred to a 1.5 ml microfuge tube and 200 µl of SDW added. The oligonucleotide diffused from the acrylamide during storage at 4°C overnight. The oligonucleotide was purified by passage through a spun G-25 column as described (Maniatis *et al* 1982).

Oligonucleotides produced by the machine method were purified by ethanol precipitation. The DNA pellet was resuspended in an equal volume of TE buffer. The concentration of oligonucleotide obtained was determined using absorbance at 258 nm.

2.18 Purification of DNA Fragments from Agarose and Acrylamide Gels

The required DNA fragment was located by fluorescence under UV light, and was cut from the gel with the minimum of extraneous gel material. The gel slice was placed in dialysis tubing, sealed at one end with a dialysis clip. TAE buffer (400 µl of ½ x) was added, air bubbles were carefully removed and the bag sealed with another dialysis clip. The bag was placed in a horizontal electrophoresis tank, at right angles to the flow of current. 1x TAE buffer was added to the tank and the DNA eluted from the gel by electrophoresis at 100 V. When all the DNA had been eluted from the gel slice, the buffer containing the DNA was tranferred to a sterile 1.5 ml microfuge tube. The DNA was extracted with PCI and collected by ethanol precipitation. The DNA pellet was finally resuspended in an appropriate volume of TE buffer.

2.19 Southern Blotting of DNA

The method used was based on that described by Southern 1975.
DNA fragments were separated by agarose gel electrophoresis. The gel was soaked in 0.25 M HCl at room temperature for 15 min to depurinate the DNA thus facilitating the transfer to nitrocellulose. The DNA was then denatured by soaking in 0.5 M NaOH, 1.5 M NaCl for 15 min. The gel was briefly washed in distilled water and neutralized by soaking in 0.5 M Tris-HCl pH 7.5, 1.5 M NaCl for 30 min. The gel was soaked in 6x SSC for 10 min prior to setting up the transfer. Nitrocellulose filter and Whatman 3MM paper, cut to the size of the gel were soaked in 2x SSC prior to use. The gel was placed on a clean glass plate and the wetted nitrocellulose placed over the gel

ensuring that no air bubbles were trapped between the gel and the nitrocellulose. A stack of Whatman 3MM filters and paper towels was then built up to a depth of 1.5 inches. A glass plate was then placed on this stack and a 500 g weight placed on top. Transfer of DNA fragments was complete after 2-4 hours at room temperature, or overnight at 4°C. The DNA was fixed on to the filter by baking at 80°C for two hours.

20x SSC per litre

175.3 g NaCl

88.2 g Sodium citrate

2.20 Radioactive Labelling of DNA Fragments using Random Primers

The incorporation of radioactive label into DNA fragments was achieved using the method of Feinberg and Vogelstein 1984. The DNA solution (18 µl), containing approximately 25 ng of DNA was boiled for 5 min then kept at 37°C prior to use. To the DNA solution was added 5 µl of OLB buffer, 1 µl of BSA (BRL), 1 µl ^{32}P α-dCTP (Amersham, 3000 Ci/mM) and 0.5 µl Klenow fragment of DNA polymerase 1 (BRL) [2 u]. The mixture was then incubated for at least 1 hr at room temperature. The labelled DNA was separated from unincorporated nucleotides by passage through a G-50 column. Aliquots of 100 µl were collected and counted by Cerenkow counting (Hewlett Packard Tri carb 4000 Scintillation counter). Those samples from the first peak of radioactivity, greater than 10^6, counts were collected and pooled.

Solutions

TEO Buffer 3 mM Tris-HCl pH 7.0
0.2mM EDTA

Solution O 1.25 M Tris-HCl pH 8.0
0.125 M $MgCl_2$

dNTP mix 100 mM dATP in TEO buffer
100 mM dTTP " "
100 mM dGTP " "

Solution A 1 ml Solution O
18 μl 2-β-mercaptoethanol (14.2 M)
5 μl of dATP, dTTP, dGTP

OLB buffer 10 μl Solution A
25 μl 2 M HEPES (adjusted to pH 6.6 with 4 M NaOH)
15 μl Hexadeoxynucleotides (Pharmacia) at 90_A units/ml in TEO buffer.

2.21 Hybridisation of radioactive-labelled DNA probes to bound DNA

A nitrocellulose filter carrying bound DNA fragments was wetted by soaking in 6 x SSC before transfer to a hybridisation chamber, containing 20 mls of hybridisation solution. The filter was prehybridised for 1-2 hours at 65°C. The radioactive-labelled DNA was boiled for 5 min prior to addition to fresh hybridisation mix,

pre-warmed to 65°C. The filter was transferred to the fresh hybridisation mix containing the probe. The volume of the final hybridisation solution used depended on the activity of the probe, ideally probes were used with a specific activity of greater than $5x10^5$ Cerenkow counts/ml of final hybridisation solution. The filter and probe were incubated overnight at 65°C. Filters were washed five times in 3x SSC: 0.1x SDS at 65°C for 20 min, with a final wash in 0.1x SSC: 0.1x SDS at 65°C for 20 min. Filters were dried at room temperature for 15 min and placed against X-Ray film with an intensifying screen at -70°C (Bonner and Laskey 1974)

Solutions

10x Denhardts	per 100 ml
	0.1 g Ficoll 4000
	0.1 g BSA
	0.1 g PVP

Hybridisation solution

6x SSC
1x Denhardts
0.1% SDS

2.22 Preparation of λgt10 Bacteriophage DNA

A phage stock of λgt10 was plated out on *E. coli* BNN93 to obtain single plaques. One turbid plaque was picked into 1 ml of *E. coli* BNN93 obtained from an overnight culture, that had been previously washed and the cell pellet suspended in an equal volume of λ diluent (10 mM Tris pH 7.5, 10 mM $MgCl_2$, 1 mM EDTA). The contents of the tube were mixed well and 100 μl of the cell suspension aliquoted into sterile phage tubes. Two mls of soft top agar was added to each tube and contents quickly poured onto fresh plates of L-agar (pH 7.5) containing 0.2 % glucose. The plates were incubated right side up at 37°C for about six hours until patchy growth was observed. The plates were cooled at 4°C, and 2.5 ml λ diluent containing a few drops of chloroform were added to each plate and allowed to stand at 4°C overnight. The top agar and λ diluent were carefully removed, mixed well and transferred to a 30 ml Corex tube. The agar was pelleted by centrifugation (Kontron-Hermle AS4-13 rotor, 7K for 10 min). The supernatant was removed and bacteriophage pelleted by centrifugation (AH-627 rotor, 23 K for two hr). The supernatant was carefully discarded and the bacteriophage pellets resuspended in 1 ml of λ diluent. A block gradient consisting of 4 ml of "1.7" density CsCl, overlaid with 4 ml of "1.5" density CsCl and finally overlaid with 4 ml of "1.3" density CsCl was set up. The phage suspension was gently layered on top of the block gradient and centrifuged (AH-627 rotor, 37K for one hour at 20°C). The 'phage, banded at the interface of the 1.5 and 1.3 density layers, were removed from the gradient and CsCl removed by dialysis for two hr at 37°C against 10 mM Tris-HCl pH 7.5, 10 mM NaCl, 1 mM EDTA, 0.016 % Triton X-100. The λgt10 DNA was extracted by two rounds of phenol extraction, followed by ethanol precipitation, and finally suspended in 2 ml SDW.

Block gradient solutions

Density	Saturated CsCl	λ diluent
1.7	27.96 ml	12.0 ml
1.5	17.76 ml	14.2 ml
1.3	13.3 ml	21.1 ml

2.23 Production of Streptococcal Chromosomal DNA

The method for the production of chromosomal DNA was based on the method of Saito and Miura 1963. One bead from a glycerol culture of *S. pneumoniae* was removed aseptically and used to inoculate a Columbia Blood Agar plate, and an optichin disk placed on the streak. The plate was incubated in a candle jar until growth appeared. If the colony morphology and optichin sensitivity confirmed the presence of *S.pneumoniae*, a single colony was picked into 400 ml of Brain Heart Infusion (Oxoid) which was incubated at 37°C for 40 hours without shaking. The cells were collected by centrifugation (GSA rotor, 7K for 10 min). The supernatant was discarded and the cell pellet resuspended in 2 ml of 20 % glucose, 50 mM Tris pH 8.0. EDTA (200 μl of 200 mM) was added together with 1 ml of lysozyme solution (10 mg/ml in SDW). The suspension was incubated at room temperature for five minutes. SDS (1 ml of a 10 % solution) was then added to the cell suspension and gently mixed until the solution started to clear. This mixture was deproteinated by the addition of 5 ml of PCI and the two phases mixed by gentle rolling until an emulsion was formed. Vigorous mixing was avoided to minimise the mechanical shearing of chromosomal DNA. The two phases were separated by

centrifugation (Heraeus-Christ, 5 K for 5 min.). The top, aqueous layer was removed into a fresh tube using a wide mouthed pasteur pipette. The aqueous layer was subjected to further cycles of treatment with PCI, until no white material at the interface of the two phases was seen after centrifugation. One tenth volume of 3M sodium acetate pH 5.6 and 2½ volumes of ethanol at room temperature were then added and the contents gently mixed. The precipitated DNA was collected and transferred to 2 ml of TE buffer and gently shaken until dissolved. The ethanol precipitation was repeated and the chromosomal DNA finally resuspended in 2 ml TE buffer. The size of the chromosomal DNA fragments was ascertained by minigel agarose electrophoresis. If the fragments were, on average, greater than 40 kb in length, the DNA was further purified by bouyant density centrifugation through CsCl as described for the production of plasmid DNA. All procedures were carried out gently to reduce mechanical shearing of the DNA.

2.24 Preparation of *in vitro* Packaging Extracts

The method of packaging extract preparation was based on the method of Priefer *et al* (1984). The two strains used in the production of packaging extracts, *E coli* BHB2688 and *E. coli* BHB2690 were routinely checked for temperature sensitivity, prior to use. An aliqot from the glycerol stock of each strain was streaked onto two L-agar plates, one of which was incubated at 30°C and the other at 42°C. If no growth was observed at 42°C, a single colony from each strain grown at 30°C was picked into 50 ml of L-broth and incubated overnight at 30°C with vigorous shaking. Five ml of each overnight culture was added to 200 ml of L-broth, prewarmed to 30°C, and

incubated at 30°C with vigourous shaking until an OD_{650} of 0.3-0.4 was reached. To each culture was added 200 ml of L-broth, prewarmed to 65°C, and the cultures incubated at 45°C for a further 25 min with occasional gentle shaking. The cultures were then transferred to 37°C, and incubated, with vigorous shaking, for a further 2-3 hr. After this incubation, 1 ml of each culture was added to 600 µl of chloroform and gently mixed, if lysis occurred, the bulk cultures were chilled on ice/water for 5 min and mixed together. The cell debris was collected by centrifugation (GSA rotor, 5 K for 10 min at 0°C). Each pellet was resuspended in 10 ml of ice cold complementation buffer and transferred to sterile plastic universals (Nunc) and recentrifuged (Heraeus Christ, 5 K for 10 min at 0°C). The pellet was resuspended in 5 ml of ice cold complementation buffer/ 5mM ATP and centrifuged as before. Each pellet was finally resuspended in 2.5 ml of ice cold complementation buffer/5mM ATP. Forty µl aliquots of the cells were transferred into precooled sterile 1.5 ml microfuge tubes and immediately frozen in liquid nitrogen. The packaging extracts were stored at -70°C until required.

Complementation buffer

50 ml	80 mM Tris-HCl pH 8.0, 10 mM $MgCl_2$
0.1 ml	β-mercaptoethanol
7 ml	DMSO
225 mg	Spermidine
161 mg	Putrescine
	SDW to 100 ml and store at -20°C.

Complementation buffer/ 5mM ATP

100 ml Complementation buffer

300 mg ATP

2.25 *In Vitro* Packaging

The packaging efficiency of each batch of packaging extracts was determined as follows. To a thawing packaging extract, kept in an ice water bath, was added 1 μl of λ DNA (BRL) made up to 5 μl with λ diluent. The contents were mixed thoroughly and incubated at room temperature for 60 min. One ml of L-broth and 50 μl of chloroform were added to the packaging extract and mixed gently by inversion until the solution was homogeneous. The contents were centrifuged for 5 min to pellet bacterial debris. Serial dilutions of the supernatant from 10^{-1} to 10^{-5} were prepared in SM buffer. Each dilution (100 μl) was added to 100 μl of indicator cells (*E. coli* LE392 grown to mid-log phase) and incubated at 37°C for 20 min. To this was added 2.5 ml of top agar, and the mixture poured over dry BBL agar plates (Tryptone 10 g/L, NaCl 5 g/L, Agar 15 g/L). All plates were incubated at 37°C overnight in an inverted position. After incubation the number of plaques formed at each dilution was counted and the packaging efficiency per μg of λ DNA determined.

Packaging extracts yielding less than 10^6 plaque forming units per μg of λ DNA were discarded, as were any packaging extracts that were found to contain contaminating phage.

The above protocol was used when producing libraries of pneumococcal sequences cloned into bacteriophage or cosmid vectors. In these

cases the supernatant was stored at 4°C until required and centrifuged for 5 min prior to replating. For λgt10 recombinants *E. coli* BNN102 was used as the indicator cells.

2.26 Liquid Lysate Preparation of Recombinant 'Phage DNA

Two plaques of recombinant λgt10 phage were picked from a fresh plate and added to 1 ml of *E.coli* BNN102, (grown overnight, washed and resuspended in an equal volume of λ diluent) and incubated at 37°C for 30 min. This culture was added to 500 ml of L-broth and incubated with vigourous shaking at 37°C overnight. To the overnight culture was added 10 ml of chloroform and the incubation continued for a further 30 min. If lysis was observed then DNase and RNase, to a final concentration of 1 μg/ml, was added and the lysate allowed to stand at room temperature for 30 min. PEG 6000 (50 g) and NaCl (29.2 g) was added to the lysate and stirred slowly until completely dissolved. The 'phage were precipitated by storing on ice for 2 hr and collected by centrifugation (GSA rotor, 7 K for 10 min). The supernatant was discarded and the bacteriophage pellets resuspended in 2 ml of SM buffer. The bacteriophage were then purified as described for λgt10 and recombinant DNA isolated by phenol extraction as described previously (section 2.22).

2.27 DNA Sequence Determination

2.27 i. Preparation of random fragments for sequence determination.

The DNA fragment to be sequenced was purified after elution from agarose gels. The fragment was self ligated, in a final volume of 50

μl, to give recircularised and concatermerised molecules. The ligated fragments were fragmented by sonication (Perry sonicating water bath) for 40 sec. The extent of fragmentation was determined by agarose gel electrophoresis. Where necessary the sonication was repeated for intervals of 5 sec, until DNA fragments up to 1 kb in length were produced. The fragments were end repaired, and fragments from between 500 and 800 bp in length were purified as described. These fragments were ligated into M13.mp18 digested with *Hinc*II and dephosphorylated. The products of the ligation were transfected into *E. coli* JM101 and recombinant plaques isolated as described.

2.27 ii. Production of single stranded template DNA.

To 25 ml of L-broth was added 50 μl of a fresh overnight culture of *E.coli* JM101. Two ml aliquots were transferred into sterile glass test tubes (6"x 1"). Each tube was inoculated with a single recombinant plaque and incubated for 6 hr at 37°C with vigorous shaking. After incubation the contents of each tube were decanted into a 1.5 ml microfuge tube and the bacteria pelleted by centrifugation in a microfuge for 5 min. The supernatant (800 μl) was removed into a fresh microfuge tube and 200 μl of 20 % PEG, 2.5 M NaCl, was added and the contents mixed well. The tubes were left to stand at room temperature for 30 min. The samples were centrifuged for 5 min and the supernatant carefully removed. The precipitated 'phage were resuspended in 100 μl of 1.1 M Sodium acetate pH 7.0. To each tube was added 100 μl of PCI and the contents vortexed for 10 sec. The phases were separated by centrifugation for 2 min, and the top aqueous layer carefully removed into a 500 μl microfuge tube. Chloroform (60 μl) was added, the two phases were mixed by vortexing,

and separated by centrifugation for 2 min. The top aqueous layer was carefully removed and tranferred to a separate 1.5 ml microfuge tube. Ice cold ethanol (250 µl) was added and the mixture stored overnight at -20°C. The DNA was collected by centrifugation for 10 min, the supernatant was discarded and the pellet washed in 500 µl 70 % ethanol in water. The supernatant was again carefully removed and the DNA pellets dried under vacuum. The DNA was finally resuspended in 50 µl of TE buffer.

2.27 iii. Preparation of gradient gels

Two electrophoresis plates (200 mm x 500 mm), one of which had been previously siliconised were assembled, separated by 0.4 mm spacers (BRL), and the edges and bottom of the plates sealed with tape. To 40 ml of 0.5x TBE acrylamide and 10 ml of 2.5x TBE acrylamide was added 300 µl and 90 µl of 10 % APS respectively. The solutions were degassed under vacuum for a few minutes. To each of the acrylamide solutions was added 20 µl of TEMED. Eight ml of the 0.5x TBE acrylamide was taken up in a 25 ml pipette followed by 8 ml of 2.5x TBE acrylamide. The acrylamide solution was gently poured between the two glass plates. The remainder of the gel space was filled with 0.5x TBE acrylamide and the straight edge of a sharkstooth comb (BRL) was inserted into the top of the gel to a depth of 2-3 mm. The gel was laid horizontally and allowed to set. When set, the gel was placed into a vertical gel apparatus, 0.5x TBE was poured into the top reservoir and lx TBE added to the bottom reservoir. Before the samples are loaded the surface of the acrylamide was washed to remove excess acrylamide and urea. The sharkstooth comb was inserted into the acrylamide so that the teeth penetrated the surface to a depth of

0.5-1 mm. The samples were loaded between the teeth of the sharktooth comb. The gel was run at a constant 40 watts until the bromophenol blue dye front reached the bottom of the gel.

After electrophoresis, the plates were gently separated leaving the gel attached to the non siliconised plate. The gel was then fixed in 10 % methanol:10 % acetic acid in water for 5 min. Whatman 3MM paper pre-wetted with water was laid over the gel and excess water removed. The gel, attached to the 3MM paper was lifted from the plate, covered with saranwrap and dried under vacuum at 80°C for 45 min. The dried gel was placed against X-ray film at room temperature for at least 24 hr.

2.27 iv. DNA sequence determination.

The method of DNA sequence determination was based on the dideoxy chain termination method (Sanger *et al* 1977). An annealing reaction consisting of 2.5 µl of template DNA solution and 2.5 µl of primer mix was set up for each sequece determination. The primer was annealed to the template DNA by incubation at 65°C for 30 min. Klenow mix (4 µl) was then added to each reaction and mixed by gentle pipetting. For each reaction, 2 µl of A, T, C and G mixes were aliquoted into 4 separate microfuge tubes. Two µl of the klenow /template reaction mix was added to each of the four tubes and the reaction initiated by centrifugation, followed by incubation at 37°C for 15 min. Second strands not terminated by the incorporation of dideoxynucleotides were chased out by the addition of 1 µl of 2.5 mM dNTP mix followed by a further incubation for 15 min at 37°C. To each reaction tube was added 1 µl of formamide dye and the contents

denatured by boiling for two min. The fragments are loaded onto a 6% acrylamide gradient gel and electrophoresed as described.

The insert present in M13 recombinants could be rapidly verified by carrying out a single dideoxy termination reaction on each template DNA, a screening usually referred to as T tracking. For each reaction, 1 μl of template DNA was added to 1 μl of primer mix and annealed as before. To this annealing reaction was added 2 μl of T track mix. The reactions are treated the same way as for sequence determinations.

Sequencing solutions

40 % Acrylamide solution per 100 ml

38 g Acrylamide

2 g Methylene Bis acrylamide

The solution was deionised with amberlite for 10 min, filtered, degassed under vacuum and stored in the dark at 4°C

2.5x TBE acrylamide per 500 ml

215 g Urea

125 ml 10x TBE buffer

75 ml 40 % Acrylamide

25 g Sucrose

25 mg Bromophenol Blue

0.5x TBE acrylamide per 500 ml

215 g Urea

25 ml 10x TBE buffer

75 ml 40 % Acrylamide

Primer mix per template

0.5 µl Universal primer at 2 ng/µl (BRL)

0.5 µl TM buffer (100 mM Tris-HCl pH 7.5, 50 mM $MgCl_2$)

1.5 µl SDW

Klenow mix per template

1 µl ^{35}S dATP >1000 Ci/mM (Amersham)

0.25 µl DNA polymerase Klenow fragment (1 unit)

2.8 µl SDW

Deoxy / Dideoxy reaction mixes

	A mix	T mix	C mix	G mix
0.5 mM dTTP	20 µl	1 µl	40 µl	20 µl
0.5 mM dCTP	20 µl	20 µl	2 µl	20 µl
0.5 mM dGTP	20 µl	20 µl	40 µl	1 µl
1 mM ddATP	16 µl	-	-	-
10 mM ddTTP	-	6 µl	-	-
1 mM ddCTP	-	-	5 µl	-
1 mM ddGTP	-	-	-	6 µl
TE Buffer	84 µl	75 µl	155 µl	75 µl

T track reaction mix per 10 templates

10 μl T mix

1 μl ^{35}S dATP > 1000 Ci/mM

0.5 μl Klenow fragment DNA polymerase 2 units

8.5 μl SDW

dNTP chase mix

5 μl 50 mM dATP, dGTP, dTTP, dCTP

980 μl SDW

Formamide dye

10 ml De-ionised formamide

10 mg Xylene cyanol FF

10 mg Bromophenol blue

200 μl 500 mM EDTA

2.28 Detection of Proteins by ELISA

The method used was adapted from that described by Redmond *et al* 1985 Rabbit anti-pneumolysin serum (100 μl of a 1/100 dilution in coating buffer) was added to the wells of a microtitre dish (Nunc immunoplate type II) which was incubated for 2 hr at 37°C. To each well was added 20 μl of a 5 % BSA solution in coating buffer, and the incubation continued for a further 30 min at 37°C. The wells were washed four times in PBS-T, and excess wash solution removed. Serial 1/2 dilutions in PBS-T of the test antigen and a purified pneumolysin solution, of known concentration, were carried out and added to the wells of the microtitre plate. The plate was incubated for 2 hr at 37°C, and then washed in PBS-T as before. To every well

was added 100 µl of rabbit anti-pneumolysin IgG conjugated to horseradish peroxidase (diluted 1/500 in PBS-T) and incubated at 37°C for 30 min. The wells were again washed as before. The presence of bound conjugate was identified by the addition of 100 µl ofsubstrate solution to each well and the plate incubated in the dark at 37°C for 30 min. The reaction was stopped by the addition of 20 µl of 12 % sulphuric acid to each well. The absorbance of each well at 495nm was determined using a non-conjugate control as a blank.

The dilution at which an arbitary end-point for the standard pneumolysin was noted and the number of ELISA units (EU) present were calculated as the reciprocal of this dilution. From the amount of pneumolysin used in the control, the number of EU that correspond to 1 µg of antigen can be calculated. The dilution of the test samples, corresponding to the end point determined for the control sample was noted, and the number of EU/ml of test solution determined and converted to µg/ml of antigen.

ELISA Solutions

Coating Buffer. (0.05 M carbonate pH 9.6)

1.59 g/L Na_2CO_3

2.93 g/L $NaHCO_3$

PBS-T (Phosphate buffered Tween pH 7.4)

8.0 g/L NaCl

0.2 g/L KH_2PO_4

1.12g/L Na_2HPO_4 (anhydrous)

0.2 g/L KCl

0.5 ml/L Tween 20

Substrate Buffer (0.15 M citrate/phosphate pH 5.0)

2.1 g/ 100 ml Citric acid (0.1 M)

3.56 g/ 100 ml Na_2HPO_4

Substrate solution.

49 ml 0.1 M citric acid

51 ml 0.2 M Na_2HPO_4

34 mg O-phenylenediamine

40 µl 30 % H_2O_2

2.29 Western Blotting

The procedure used was adapted from the method of Towbin *et al* (1979). Proteins were separated by SDS-PAGE. Nitrocellulose filter and Whatman 3MM paper, cut to the size of the gel, were soaked in transfer buffer for 30 min. The nitrocellulose filter was placed over the gel, avoiding air bubbles, and both were then placed between Whatman 3MM papers. The sandwich was placed in the plastic frame of the transfer apparatus (Biorad) with the nitrocellulose filter towards the anode. Sufficient transfer buffer was added to cover the gel. The proteins were transferred to the nitrocellulose for 1.5 hr at 0.6 Amp. After transfer the nitrocellulose filter was washed briefly in Tris NaCl (10 mM Tris-HCl pH 7.4, 150 mM NaCl) and non-specific binding of antibody blocked by incubation of the nitrocellulose filter in a 3 % solution of dried milk (Marvel) in Tris NaCl for 2 hr at 37°C. The filter was washed three times in Tris NaCl. Anti-pneumolysin antibody conjugated to horseradish peroxidase and diluted 1/500 in Tris NaCl was added to the filter and incubated at 37°C for 2 hr. The filter was washed 3 times in

Tris-NaCl and placed in a fresh solution of 4-chloro-1-naphthol(30 mg in 6 ml methanol, plus 50 ml Tris NaCl). The bound antibody was visualised by the appearance of a purple colour.

Transfer buffer	per litre
	3.03 g Tris base
	14.4 g Glycine
	200 ml Methanol (Analytical grade)
	Water to 1 litre

2.30 Oligonucleotide Site Directed Mutagenesis

The method used to generate site directed mutants was based on the single primer method of Zoller and Smith (1983).

Oligonucleotides were phosphorylated as described. Oligonucleotide (2 µl / 5pm) was added to 10 µl of single stranded recombinant DNA carrying the sequence to be mutated, together with 1 µl of 10x polB buffer and 1 µl 500 mM NaCl. The oligonucleotides were annealed to the template DNA by incubation at 65°C for 30 min. To each annealing reaction was added 10 µl of extension-ligation mix, and the mixture incubated at 16°C overnight. Four microlitres of the extension ligation reaction were analysed by agarose gel electrophoresis, to check for the production of RF DNA. If the extension ligation reaction was successful, then 10 µl of the reaction mix was transfected into *E. coli* TG1, and plated out onto minimal media. Plaques were picked in duplicate onto L-agar plates and minimal agar plates, and incubated overnight at 37°C. Colonies grown on the L-agar plates were screened for appearance of mutants by overlaying with a blood agarose overlay. Colonies associated with a reduced

haemolysis or those devoid of haemolysis were noted. Colonies were picked from the minimal medium plate and single stranded DNA prepared. Changes in the DNA sequence were determined by an appropriate dideoxynucleotide screen.

10x polB buffer

200 mM Tris-HCl pH 7.5

100 mM $MgCl_2$

10 mM DTT

Extension ligation mix

1 µl 10x polB buffer

4 µl 2 mM dNTP

1 µl 10 mM ATP

1 µl T_4 ligase (Pharmacia)

0.7 µl Klenow fragment DNA polymerase

2.3 µl SDW

2.31 *E.coli* Cell-Free *in vitro* Transcription Translation of Plasmid DNA

The method used was that described by Pratt *et al* 1981 and Zubay 1973. The cell free extracts and solutions were kindly provided by Ms R. L. Allen.

The cell free extract was thawed on ice for one hr. In a separate microfuge tube, 0.5 µg of plasmid DNA , in a volume of 5 µl, was added to 1 µl ^{35}S methionine (1360 Ci/mM) [Amersham], 8.5 µl LMM (Collins 1979), 2.5 µl 0.1M magnesium acetate and made up to 25 µl

with SDW treated with DEPC. The mixture was incubated at 37°C for 4 min. To this was added 5 µl of the cell free extract and the mixture incubated for a further 30 min at 37°C. Methionine (5 µl at 44 mg/ml) was added, to chase the synthesis reactions to completion, and the incubation continued for a further 5 min at 37°C. To the reaction mix was added an equal volume of SDS loading buffer and the mixture boiled for 5 min prior to loading. Each sample (20 µl) was loaded into the wells of a SDS-polyacrylamide gel, together with 10 µl of ^{14}C labelled protein markers, and the proteins separated at 200 V until the bromophenol blue dye front reached the bottom of the gel. The proteins were fixed by soaking the gel in 10 % acetic acid for 15 min, after which the gel was rinsed briefly in water and soaked in 'Amplify' (Amersham) for 15 min. The gel was dried under vacuum at 80°C and placed against X-ray film with an intensifying screen at -70°C.

2.32 Purification of Modified Pneumolysin

The protocol used was amended from that developed for pneumolysin by Dr. T. J. Mitchell using a high performance liquid chromatography system (LKB). A crude cell extract (5 ml) containing modified toxin was loaded onto a hydrophobic interaction column (TSK phenyl-5PW), and subjected to chromatography in TBS at a flow rate of 0.5 ml/min. Fractions (2.5 ml) were collected and analysed for haemolytic activity. Those fractions exhibiting haemolytic activity were pooled and re-applied to the hydrophobic interaction column. Two column volumes of TBS were run through the column and absorbed protein eluted from the column with water. Fractions corresponding to the single protein peak were collected and assayed for haemolytic

activity. The purity of each sample was determined by SDS-polyacrylamide gel electrophoresis.

TBS buffer 10 mM Tris-HCl pH 7.2
250 mM NaCl

2.33 Estimation of Protein in Solution

The concentration of protein in solution was determined using the method of Bradford (1976). Test solution (100 µl) was added to 5 ml of protein assay stock solution (Bio-Rad) diluted 1 in 5 with water. The samples were kept at room temperature for at least 5 min. The absorbance was read at 495 nm, and the concentration of protein estimated by comparison against a protein standard curve. An absorbance of 0.1 equated to 100 µg of protein / ml of solution.

TABLE 3. Bacterial strains
(*E. coli* unless stated)

STRAIN	GENOTYPE	REFERENCE or SOURCE
BHB2688	N205 *recA* (λ *imm*^{434} *cI ts* b_2 *red*$^-$ *Eam Sam*)	Hohn 1979
BHB2690	N205 *recA* (λ *imm*^{434} *cI ts* b_2 *red*$^-$ *Dam Sam*)	Hohn 1979
BNN 93	*hsdR hsdM*$^+$ *supE thr leu thi lacY*1 *fhuA*21	Young and Davis 1983
BNN 102	*hsdR hsdM*$^+$ *supE thr leu thi lacY*1 *fhuA*21 *hflA*150[chr::Tn*10*]	Young and Davis 1983
JM83	*ara* Δ(*lac proA,B*) *rpsL* Φ80 *lacZ*Δ*M*15	Vieira and Messing 1982
JM101	*SupE thi* Δ(*lac proA,B*)/*F' traD*36 *proA,B lacI*^{q}*Z M*15	Yanisch-Perron *et al* 1985
LE392	*F*$^-$ *hsdR*514 (R_K^-,M_K^-) *SupE*44 *SupF*58 *lacY*1 *galK*2 *galT*22 *metB*1 *trpR*55 λ^-	Maniatis *et al* 1982
MC1061	*araD* 194 Δ(*ara-leu*)7697 Δ*lacX*74 *galU galK hsdR hsdM*$^+$ *rpsL*	Casadaban and Cohen 1980
TG1	K 12 Δ(*lac-pro*) *SupE thi hsd*D5/*F' traD*36 *proA,B lacI*^{q} *lacZ*M15	Amersham
NCTC 7465	*Streptococcus pneumoniae* serotype 1	N.C.T.C.
NCTC 7466	*Streptococcus pneumoniae* serotype 2	N.C.T.C.
NCTC 10319	*Streptococcus pneumoniae* serotype 47	N.C.T.C.
GJB194	*Streptococcus pneumoniae*	Clinical isolate LRI

Table 4. Plasmid and Bacteriophage Vectors.

Vector	genotype	relevant phenotype	reference
λgt10	λimm434, b527		Huynh *et al* 1985
pOU71		Amp^r, low copy	Larsen *et al* 1984
pUC9		Amp^r, high copy	Vieira and Messing 1982
pUC18 pUC19		Amp^r, high copy	Norrander *et al* 1983
M13mp18 M13mp19			Yanisch-Perron *et al* 1985

CHAPTER 3

CLONING AND CHARACTERISATION OF THE PNEUMOLYSIN GENE, SUBCLONING INTO PLASMID VECTORS.

3.i CLONING OF THE PNEUMOLYSIN GENE

In initial attempts to construct representative libraries of pneumococcal DNA sequences, commonly available cloning vectors, such as bacteriophage λ L47, cosmid vector pEMBL Cos4 and the plasmid pBR322 were used. Despite analysis of each step in the cloning procedure, to ensure that each stage was working optimally, no recombinants were obtained with any of the above vectors. One common factor in all these initial experiments was the pneumococcal chromosomal DNA. It had been observed during the cloning of *slo* that the production of representative libraries of streptococcal sequences required chromosomal DNA that had been purified by bouyant density centrifugation through CsCl (M Kehoe, personal communication). With this knowledge, further attempts to construct libraries of pneumococcal sequences used chromosomal DNA that had been purified by centrifugation through CsCl.

The bacteriophage vector, λgt10, was used in all further attempts at constructing gene libraries of pneumococcal DNA. Bacteriophage λgt10 has a single *Eco*RI cloning site within the *c*I repressor gene (Fig 1). Non recombinant, $c\mathrm{I}^{+}$ 'phage, can form lysogens and therefore produce turbid plaques when grown on *E. coli* (Murray *et al* 1977). Insertion of DNA fragments into the *Eco*RI site result in $c\mathrm{I}^{-}$ phage, which are committed to the lytic cycle, and produce clear plaques when grown on *E. coli*. Recombinant λgt10 bacteriophage can also be selected by growth on *E. coli* carrying the high frequency lysogeny mutation, *HflA* 150. The product of the Hfl gene in wild type strains of *E. coli* represses lysogeny (Hoyt *et al* 1982). In *E.coli* (*HflA*) strains, non recombinant $c\mathrm{I}^{+}$ phage are committed to lysogeny so efficiently they do not form plaques. However, recombinant $c\mathrm{I}^{-}$ phage, committed to

the lytic cycle form plaques on *E. coli* (*HflA*) strains with no loss of plating efficiency. The upper limit of the size of DNA that can be inserted into λgt10 is only 7.6 kb.

The construction of representative libraries of pneumococcal DNA fragments in λgt10 involve many manipulations of the insert DNA fragments (Huynh *et al* 1985). In order to test the suitability of the pneumococcal DNA for cloning, a pilot experiment was undertaken as outlined below (Fig 1).

Chromosomal DNA from *S. pneumoniae* serotype II was partially digested with *Eco*RI. DNA fragments were separated by preparative agarose gel electrophoresis, and fragments from 4-6 kb and 6-9 kb isolated from the gel. Vector DNA of λgt10 was digested to completion with *Eco*RI. The pneumococcal DNA fragments and λgt10 vector DNA were analysed by agarose gel electrophoresis (Fig 2) and the concentration of DNA present in each sample was estimated empirically.

Vector and chromosomal DNA fragments were mixed together in various ratios (Table 5), ligated together, and the products of the reaction analysed by agarose gel electrophoresis. The appearance of higher molecular weight fragments, with the concomitant disappearance of the pneumococcal fragments and λgt10 arms observed in Fig. 2, was taken to indicate successful ligation of the DNA (results not shown).

High molecular weight DNA fragments in each ligation reaction were packaged into 'phage heads *in vitro* and an aliquot of the packaged 'phage plated out onto *E. coli* BNN102. After overnight incubation the number of plaques were counted (Table 5). The results indicated that the smaller fragments (4 - 6 kb) produced approximately 10 times

more recombinants than the equivalent amount of the larger (6 - 9 kb) fragments. About 6000 recombinant phage (Ligation A, B and C) containing pneumococcal fragments from 4-6 kb in length, were plated out onto *E. coli* BNN102. The resulting plaques were screened for haemolytic activity by overlaying the plaques with 10% sheep erythrocytes in agarose. After three hours incubation at 37°C faint haemolysis was observed around two recombinant plaques. 'Phage from within these plaques were subjected to several rounds of plaque purification until all single plaques exhibited haemolytic activity. The zones of haemolysis surrounding plaques of purified recombinants are shown in Fig 3A. One of the recombinants, λPLY003, was selected for further analysis.

3.ii CHARACTERISATION OF THE HAEMOLYTIC PRODUCT PRODUCED BY λPLY003

If the haemolytic product produced by λPLY003 was pneumolysin, it was anticipated the activity of this product would be inhibited by cholesterol. *E. coli* BNN102 was infected with λPLY003 and plated out on L- agar containing various concentrations of cholesterol. The plates were incubated overnight at 37°C and the resulting plaques were overlaid with blood-agarose. The degree of haemolysis around plaques grown at each concentration of cholesterol was noted (Fig 3). The extent of haemolysis decreased with increasing concentrations of cholesterol in the media (Fig 3). The inclusion of cholesterol in the media appeared to have no detrimental effect on the growth of *E. coli* BNN102 nor on the efficiency of plating of λPLY003. The inhibition of haemolysis by cholesterol was the first indication that the haemolytic agent was pneumolysin.

Further characterisation of the haemolytic product was carried out on cell-free lysates produced by λPLY003. The haemolytic activity of such lysates was of the order of 1 x 10^4 HU/ml of lysate. One HU being defined as the amount of toxin causing 50 % lysis of a 0.85 % suspension of sheep erythrocytes at 37°C in 30 min. This activity gradually fell during storage of the lysates at 4°C (Table 6, Fig 4). The haemolytic activity of the lysates stored at 4°C could be recovered when DTT (1mM) was added to the lysates prior to use in the haemolytic assay. The haemolytic activity of DTT-activated lysates was reduced after incubation with cholesterol and anti-SLO antibody (Table 6, Fig 4). The loss of haemolytic activity on storage and its recovery by thiol agents, together with the inhibitory activity of cholesterol and neutralisation by antibodies raised against the related toxin SLO, were further proof that the haemolytic agent produced by λPLY003 was pneumolysin.

3.iii ANALYSIS OF λPLY003

Since the haemolytic product from λPLY003 had been shown to be pneumolysin, an analysis of the insert present in the recombinant 'phage DNA was undertaken.

DNA from λPLY003 was isolated and digested to completion with *Eco*RI and the fragments separated by agarose gel electrophoresis. The size of the insert fragments was determined after comparison of their mobilities against λ DNA fragments digested with *Hind*III (Fig 5). The insert present in λPLY003 was 5.6 kb in length and comprised of two *Eco*RI fragments of approximately 3.3 and 2.3 kb.

DNA from λPLY003 was partially digested with *Eco*RI and the 5.6 kb, 3.3 kb and 2.3 kb insert fragments were purified after separation on a preparative agarose gel. The three fragments were ligated into λgt10 cut with *Eco*RI. The products of the ligation were packaged *in-vitro* and were plated out onto *E. coli* BNN102. The resulting recombinant plaques were screened for haemolytic activity. Haemolytic activity was only observed around those plaques resulting from the ligation reaction which included the 5.6 kb *Eco*RI fragment (results not shown). The above observation indicated the requirement for both fragments for the production of pneumolysin, and implied that either the structural gene for pneumolysin or regulatory sequences for pneumolysin gene expression spanned the internal *Eco*RI site.

3.iv SUBCLONING OF THE INSERT FROM λPLY003 INTO pOU71

Manipulation of the insert in bacteriophage vectors is made laborious by the large size of the vector. In addition, the lack of other suitable cloning sites in λgt10, precluded the use of λPLY003 in further analysis of the pneumolysin gene. Therefore the subcloning of the insert from λPLY003 into plasmid vectors was a priority.

Initial attempts to clone the 5.6 Kb *Eco*RI fragment into pBR328 were not successful (results not shown). This failure may be explained by the instability of large pneumococcal DNA fragments at high copy number in *E. coli* (Chen and Morrison 1987). Further attempts at producing subclones in plasmid vectors used the RI plasmid-based vector pOU71 (Larsen *et al* 1984). This vector has a copy number of 1-2 at 30°C, which is controlled by the λ repressor gene product.

This product is inactivated at high temperature, so that maintenance of the host at 42°C, leads to derepression of λP_r and overexpression of repA genes, resulting in a high copy number (up to 1000 copies / cell) and a consequent increase in expression of inserted gene sequences.

λPLY003 DNA was digested with *Eco*RI and the 5.6, 3.3 and 2.3 kb *Eco*RI fragments were purified. The fragments were ligated separately into *Eco*RI cut and dephosphorylated pOU71. The products of each ligation were transformed into *E.coli* MC1061 and cells plated out on L-agar containing 100 μg/ml ampicillin. Colonies were overlaid with blood agarose, and those colonies exhibiting haemolytic activity identified. Haemolytic activity was only observed around colonies resulting from the ligation reaction containing the 5.6 kb *Eco*RI fragment. Plasmid DNA was prepared from haemolytic transformants and transformants resulting from the ligation reaction which involved the 3.3 kb and the 2.3 kb fragments. The plasmid DNA was digested with *Eco*RI, and the products of the digestion analysed by agarose gel electrophoresis (results not shown). Only those transformants with both *Eco*RI fragments exhibited haemolytic activity. This confirmed the previous observation that both *Eco*RI fragments are required for the production of pneumolysin.

The orientation of the insert present in the recombinants encoding haemolytic activity was investigated. Plasmid DNA from four haemolytic recombinants, identified above, were digested with *Pst*I and *Cla*I and the fragments separated by agarose gel electrophoresis (Fig 6). The digests indicated the presence of two recombinants (designated pJW510 and pJW520) differing only in the orientation of the insert fragment with respect to the λP_r promoter (Fig 7).

A 5 kb fragment was observed in both pJW510 and pJW520, after digestion with *Cla*I, that was wholly within the insert present in λPLY003. The 5 kb *Cla*I fragment was isolated from pJW510 and ligated into the *Cla*I site of pOU71. The products of this ligation were transformed into *E. coli* MC1061 and colonies checked for haemolysis by overlaying with blood agarose. Several haemolytic colonies were identified. Plasmid DNA was prepared from transformants and digested with *Cla*I. The fragments were separated by agarose gel electrophoresis. The sizes of the fragments observed were consistant with the construction outlined in Fig 7. Plasmid pJW451 (Fig 7) was used in further analysis

Attempts to achieve high levels of pneumolysin production from pJW451 via runaway replication (Nicaud *et al* 1985) were unsuccessful. The reasons for this are unclear.

3.v SUBCLONING INTO pUC VECTORS

The construction of stable recombinants based on pOU71 vectors had been achieved, but the lack of suitable cloning sites in pOU71 restricted further manipulation of the pneumococcal insert. The greater number of potential cloning sites in the pUC family of vectors, together with the possibility of a higher level of expression of the pneumolysin gene made these vectors a convenient choice for the construction of more useful subclones.

The construction of pUC based subclones, described below, were carried out after the location of the pneumolysin gene, within the insert present in pJW451, had already been determined (Chapter 4).

This approach enabled the construction of recombinants with defined inserts, which ensured that each subclone carried the complete coding sequence of the pneumolysin gene.

From the DNA sequence, a 2.5 kb *Tth*III 1 fragment was the smallest that could be extracted from pJW451, with a single digest, that still carried the complete coding sequence of the pneumolysin gene. Plasmid DNA of pJW451 was digested to completion with *Tth*III 1 and the fragments separated by agarose gel electrophoresis. The 2.5 kb fragment was isolated and the ends of the fragment repaired with T_4 DNA polymerase. The repaired *Tth*III 1 fragment was ligated into pUC9 cut with *Hinc*II and dephosphorylated. The products of the ligation were introduced into *E. coli* JM83 by transformation, and plated out onto L-agar containing 100 μg/ml ampicillin plus X-gal. *E. coli* JM83 transformed by recombinant molecules produce white colonies on this media, whilst transformants produced by vector molecules produce blue colonies. White colonies arising from the transformation of *E. coli* JM83, were picked onto fresh plates of L-agar containing 100 μg/ml ampicillin and incubated overnight at 37°C. Colonies exhibiting haemolytic activity were identified by a blood agarose overlay. Plasmid DNA was prepared from these haemolytic transformants and preliminary digests with *Eco*RI indicated the presence of two different constructs. The structure of these two recombinants were verified by restriction digest of the DNA. The two recombinants, designated pJW252 and pJW254, differed only in the orientation of the *Tth*III 1 fragment with respect to the *lac* promoter (Fig 8). Plasmid DNA of pJW252 or pJW254 was transformed into *E. coli* MC1061 for all further analysis.

The haemolytic activities of crude cell free extracts of *E. coli* MC1061 harbouring the two recombinants pJW252 and pJW254 were similar (Table 7), indicating that expression of the pneumolysin gene was not dependent on expression from the *lac* promoter. It was noted, however, that the numbers of colonies lacking haemolytic activity increased with serial subculture of cells transformed with either plasmid. This loss of haemolytic activity implied that the constructs pJW252 and pJW254 were unstable.

Due to the instability of pJW254 and pJW252 further subclones, involving smaller fragments carrying the pneumolysin gene, were constucted in pUC vectors. The choice of fragments was made on the basis of the DNA sequence and is briefly oulined below. A 1.9 kb *Sma*I-*Eco*RV fragment was isolated from pJW254 and ligated into *Hinc*II digested pUC18. The products of the ligation were transformed into *E. coli* MC1061 and cells plated out on blood agar containing 100μg/ml ampicillin. DNA from colonies exhibiting haemolytic activity was prepared and digested with *Eco*RI, the products of the digest separated by agarose gel electrophoresis. The sizes of fragments obtained, indicated that the 1.9 kb *Sma*I-*Eco*RV fragment was present in both orientations with respect to the *lac* promoter. These subclones were designated pJW191 and pJW192 (Fig 8). A further set of subclones was constructed using a 2.2 kb *Xho*II fragment from pJW252 which was cloned into the *Bam*HI site of pUC18. These constructs, designated pJW208 and pJW209, had the *Xho*II fragment in opposite orientations (Fig 8). A 1.7 kb *Kpn*I- *Eco*RV fragment isolated from pJW208 was inserted into pUC18 digested with *Kpn*I and *Hinc*II (Fig 8) the resulting transformant, designated pJW158, was used for the mutagenesis of the pneumolysin gene (see Chapter 5).

The levels of pneumolysin in cell free extracts produced by each subclone were determined (Table 7) and indicated that pJW252, pJW254, pJW191 produced less pneumolysin than the other subclones. It was also noted that these subclones were less stable, as observed by the loss of haemolytic activity upon subculture. The reasons for this instability were not investigated. The production of pneumolysin by each construct and its stability did not appear related to the orientation of the insert with respect to *lac* expression or the size of insert (Table 7).

3.vi ANALYSIS OF THE ORGANISATION OF THE PNEUMOLYSIN GENE

The organisation of the pneumolysin gene in other serotypes of the pneumococcus was investigated by Southern blotting. Chromosomal DNA from *S. pneumoniae* serotypes 1, 2, 47 and from an untyped clinical isolate, designated GJB194, were digested to completion with *Eco*RI and *Cla*I. The fragments were separated by agarose gel electrophoresis, transferred to nitrocellulose and probed with the 2.5 kb *Tth*III 1 fragment carrying the pneumolysin gene. After hybridisation the filters were washed under stringent conditions, and bound probe detected by autoradiography (Fig 9). Homology was detected between the probe and *Eco*RI fragments of 3.3 and 2.3 kb in all isolates, and a 5 kb *Cla*I fragment in serotypes 1, 2 and 47. This was consistant with the sizes of fragments cloned in λPLY003 and indicate that the organisation of the pneumolysin gene and flanking sequence is probably very similar in different serotypes of the pneumococcus. The presence of a restriction site polymorphism could be the cause of the increase in size of the homologous *Cla*I fragment in GJB194.

3.vii DISCUSSION

The reasons for the failure to produce representative libraries of pneumococcal sequences in cosmid and bacteriophage vectors were not extensively investigated once the successful cloning of the pneumolysin gene in λgt10 had been achieved. However, considering the reported cloning of other pneumococcal genes, and the benefit of hindsight, factors that may have contributed to these failures can be considered. Pneumococcal sequences have been found to be difficult to clone and express in *E. coli* vectors (Chen and Morrison 1987), probably due to the presence of more promoter-like sequences, resulting from the high AT content of pneumococcal DNA, recognised by the more permissive *E.coli* RNA polymerase. This may result in higher expression of cloned sequences leading to the instability of recombinants plasmids, though the exact mechanism of this instability remains unclear.

The size of the insert DNA, therefore, appears to be one parameter of some importance in determining the stability of cloned pneumococcal sequences. Thus the larger sizes of pneumococcal insert DNA required for cloning into λ or cosmids may have resulted in reduced cloning efficiency. The comparatively smaller fragment sizes required for cloning into λgt10 may have contributed to a greater stability of recombinants.

The quality of the DNA used to prepare fragments is another factor assumed to have some effect on the efficiency of cloning. The successful cloning of the SLO gene from *S. pyogenes* was achieved only after purification of the DNA by bouyant density centrifugation through CsCl (M. Kehoe personal communication). Whether the

successful cloning of the pneumolysin gene was due to this means of purification of the DNA and/or the use of λgt10 was not investigated.

The DNA insert from λPLY003 was pneumococcal in origin as shown by DNA hybridisation experiments (results not shown). The properties of the haemolytic product produced by λPLY003 were consistent with the product being pneumolysin. Although there have been no reports of the production of more than one haemolysin by the pneumococcus (Johnson and Aultman 1977, Lorian *et al* 1973), there have been occurences when recombinant expressing a haemolytic product have erroneously been thought to encode a sulphydryl-activated cytolysin, for example listeriolysin (Vicente *et al* 1985) and cereolysin (Kreft *et al* 1983).

Additional evidence that the haemolytic product expressed by the cloned pneumococcal sequence was pneumolysin was obtained from *in-vitro* transcription translation of the plasmids pJW510 and pJW252 (Walker *et al* 1986, 1987). In an *E.coli* cell free *in-vitro* transcription translation system both clones directed the production of a polypeptide of 53 kD, which was similar in size to the reported size of native pneumolysin (Paton *et al* 1983). The 53 kD polypeptide was precipitated by serum raised against either SLO (Geoffroy and Alouf 1984) or alveolysin (J. E. Alouf, personal communication) and indicated that the insert carried a full length copy of the pneumolysin gene (Walker *et al* 1986, 1987).

Hybridisation between the cloned pneumolysin gene and DNA from several hundred clinical isolates of the pneumococcus, obtained from the Leicester Royal Infirmary (S. Pratt, personal communication), indicated the presence of the gene in all isolates tested. This is

consistent with the observation that pneumolysin is produced by nearly all clinical isolates of the pneumococcus (Kanclerski and Mollby 1987, Lorian *et al* 1973). The organisation of sequences flanking the pneumolysin gene appeared to be highly conserved in those serotypes tested, though restriction site differences between the insert in λPLY003 and the pneumolysin gene isolated by Paton *et al* (1986) are apparent. Whether these differences reflect single base changes in the DNA sequence or significant changes to flanking DNA has not been established.

Explanations for the instability of certain pUC sub-clones carrying the pneumolysin gene and levels of pneumolysin produced by these sub-clones are less than staightforward. Though size of insert appears to be of little importance, the direction of expression of the pneumolysin gene with respect to *lac* expression and the presence of flanking DNA either side of the pneumolysin gene are perhaps more significant. The contribution of either of these factors remains to be clarified. It is possible that high levels of transcription of the pneumolysin gene and/or efficient translation of the message, could lead to high levels of intracellular pneumolysin. This may stress the bacterium, leading to the loss of the plasmid carrying the pneumolysin gene, so that a lower yield of pneumolysin is observed. It may be concluded therefore, that the levels of pneumolysin produced by pJW208, for example, represent the maximal levels of pneumolysin, consistent with stability, that the cells can tolerate. Clearly the synthesis of pneumolysin in *E. coli* and its relationship with the stability of recombinants carrying the gene is complex and requires further study to elucidate those parameters that affect expression and stability.

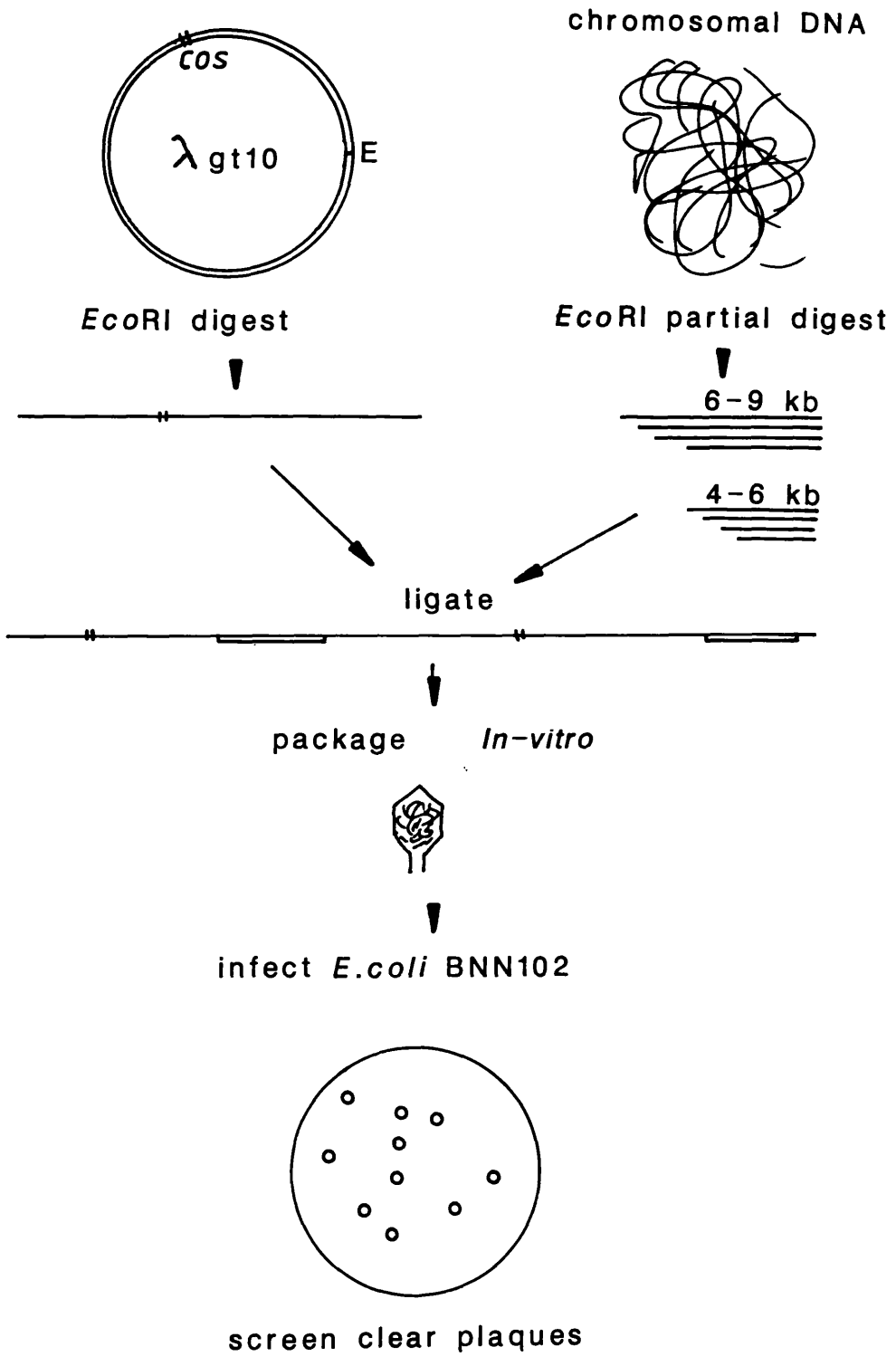

FIGURE 1. Cloning strategy employed in the isolation of recombinants from chromosomal DNA of *S. pneumoniae* serotype II.

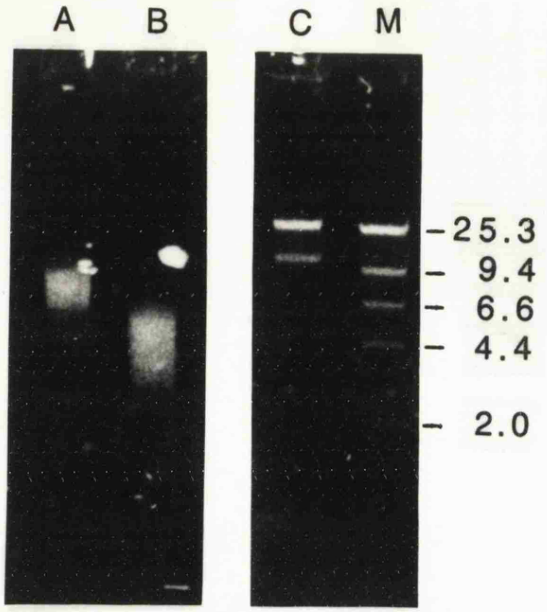

FIGURE 2. Restriction endonuclease *Eco*RI digests of DNA from *S.pneumoniae* serotype II and λgt10 separated by agarose gel electrophoresis.

A. DNA fragments 6-9 kb in length obtained from a partial *Eco*RI digest of *S.pneumoniae* serotype II.

B. DNA fragments 4-6 kb in length obtained from a partial *Eco*RI digest of *S.pneumoniae* serotype II.

C. DNA of λgt10, digested to completion with *Eco*RI

M. λ DNA digested to completion with *Hind*III. Sizes are in kb

TABLE 5

Numbers of recombinants obtained from ligations of pneumococcal sequences in λgt10 during the construction of gene libraries.

LIGATION	RATIO [a]	PLAQUES OBTAINED [b]	
		4-6 kb	6-9 kb [c]
A	10 : 1	64	3
B	5 : 1	108	11
C	2 : 1	126	15
D	1 : 1	150	8
E	1 : 2	7	9

a Ratio of λgt10 vector DNA to pneumococcal DNA in each ligation.

b Number of plaques obtained from 50μl of packaged phage supernatant

c Range of size of pneumococcal DNA fragments ligated into λgt10

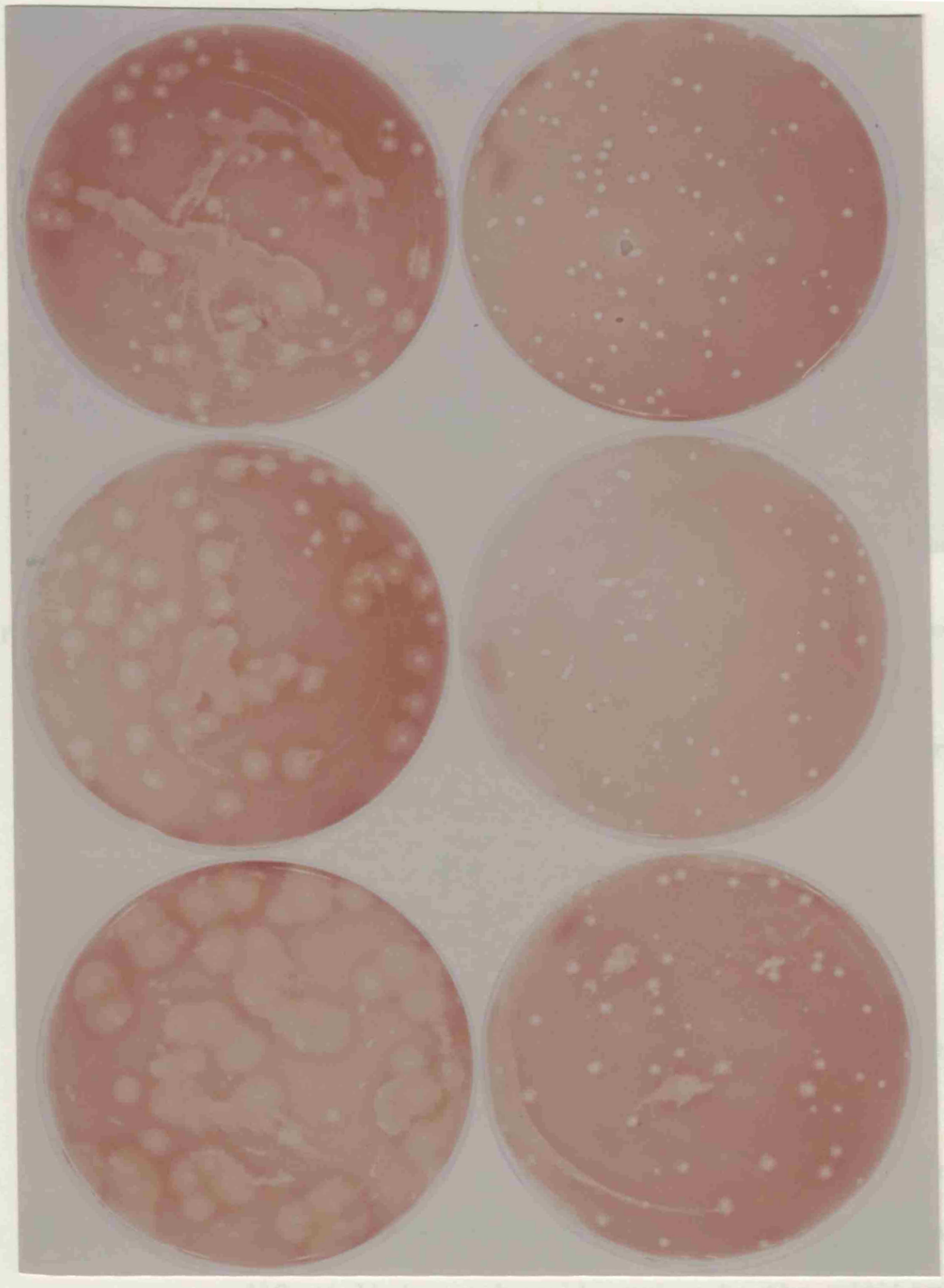

FIGURE 3. Zones of haemolysis, observed around plaques of recombinant λPLY003, following growth on media containing increasing concentrations of cholesterol in the media.

A. No cholesterol B. 8 μg/ml of cholesterol per plate.

C. 20 μg/ml of cholesterol D. 40 μg/ml of cholesterol.

E. 80 μg/ml of cholesterol F. 120 μg/ml of cholesterol

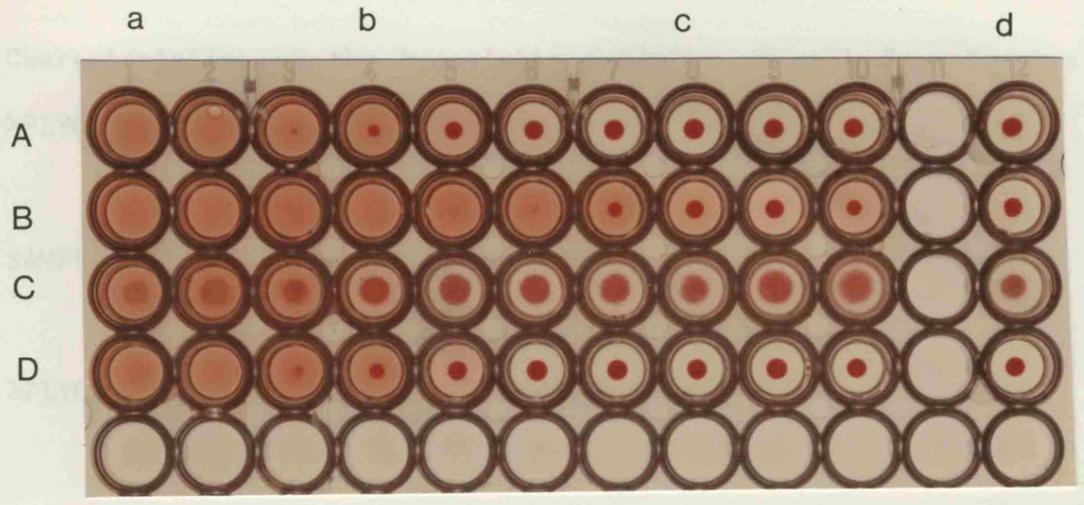

FIGURE 4. Characterisation of the haemolytic activity of cell free lysates of λPLY003.

Lysate (50μl) was added to the first well and serial 1/2 dilutions in PBS carried out to well 10. The end points, corresponding to approximately 50% lysis of the erythrocyte suspension are indicated.

A. Supernatant from liquid lysates of λPLY003 stored at 4°C for two weeks.

B. As A, but lysate pretreated with 1mM DTT prior to use.

C. As B, but after the addition of 5μl of cholesterol (10mg/ml) to each well, prior to the addition of erythrocytes.

D. As B, but after the addition of 5μl of serum containing anti-SLO antibodies, prior to the addition of erythrocytes.

a Fifty microlitres of supernatant in well 1, serially diluted 1/2 to well 10.

b End point of reactions A, C and D.

c End point of reaction B.

d Control samples without the addition of lysate.

TABLE 6

Characteristics of the haemolytic activity of cell free lysates of λPLY003

SAMPLE	HAEMOLYTIC ACTIVITY (HU/ml)[a]
λPLY003 (untreated)[b]	1.6×10^3
λPLY003 (+ DTT)[c]	1.2×10^4
λPLY003 (+ DTT and cholesterol)[d]	1.6×10^3
λPLY003 (+ DTT and anti-SLO)[e]	8×10^2

a Haemolytic units are expressed as the reciprocal of the dilution of the lysate giving 50 % lysis of a 1.7 % sheep erythrocyte suspension

b Lysate stored at 4°C for two weeks prior to dilution, no DTT added

c Lysate as *b*, incubated with 1 mM DTT prior to the addition of erythrocytes

d Lysate treated as in *c* but incubated with 5 μl of cholesterol prior to the addition of erythrocytes

e Lysate treated as in *c* but incubated with 5 μl of anti-SLO antibodies prior to the addition of erythrocytes

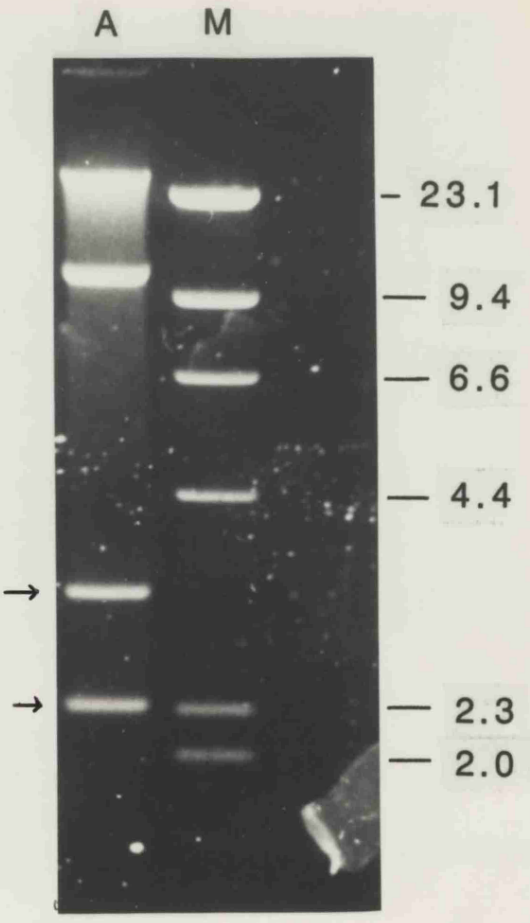

FIGURE 5. Agarose gel electrophoresis of an *Eco*RI restriction digest of λPLY003

A. λPLY003 digested to completion by *Eco*RI, the insert fragments of 2.3 kb and 3.3 kb are indicated by arrows.

M. λ DNA digested with *Hind*III. Sizes of fragments are shown in kilobases

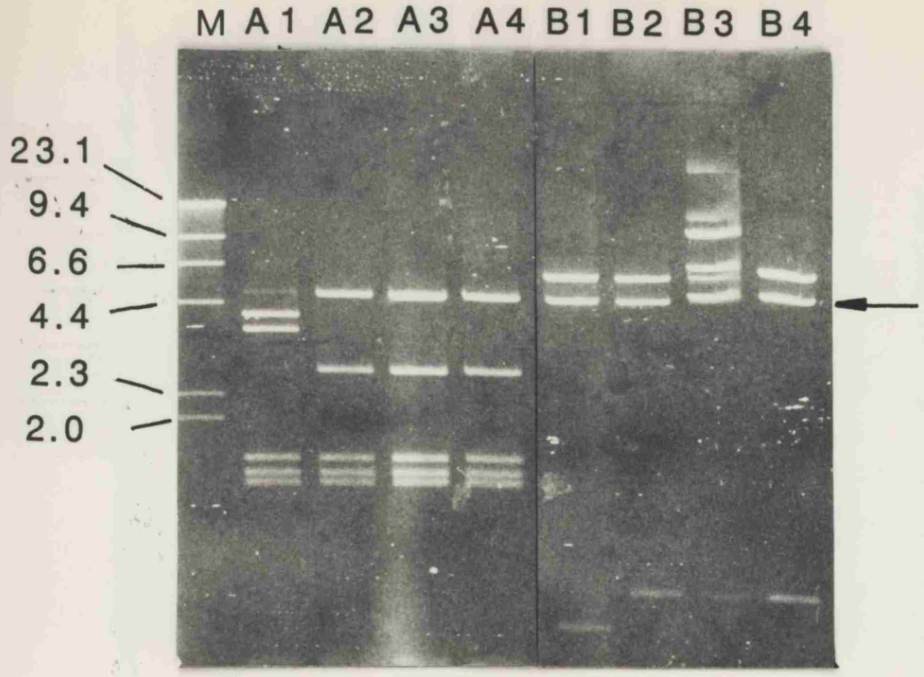

FIGURE 6. Agarose gel electrophoresis of restriction fragments from plasmid DNA obtained from four recombinants, expressing haemolytic activity.

A1–A4 Plasmid DNA from each recombinant, 1–4, digested with *Pst*I

B1–B4 Plasmid DNA from each recombinant, 1–4, digested with *Cla*I,

M λ DNA digested to completion with *Hind*III. Sizes are in kilobases.

The presence of a *Cla*I fragment wholly within the insert of pneumococcal DNA, in all recombinants, 1–4, is indicated by an arrow.

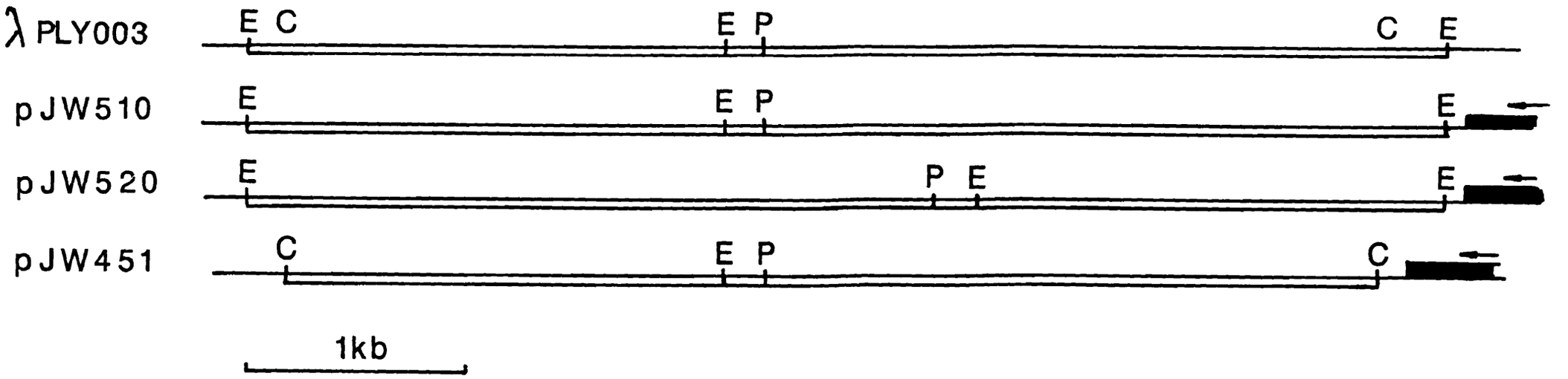

FIGURE 7. Restriction digest map of pOU71 subclones.

E = *Eco*RI, C = *Cla*I, P = *Pst*I, ■ = cI_{857} gene,

← = direction of transcription from λP_R promoter.

— = pOU71 vector DNA. Open boxes represent DNA of pneumococcal origin.

FIGURE 8. Restriction maps of recombinant plasmids carrying the pneumolysin gene.

pneumolysin gene

flanking pneumococcal DNA

lac Z gene in pUC vector.

direction of expression of the *lac* promoter

T= *Tth*III 1, X= *Xho*II, V= *Eco*RV.

The 5' end of the pneumolysin gene is to the left in all cases

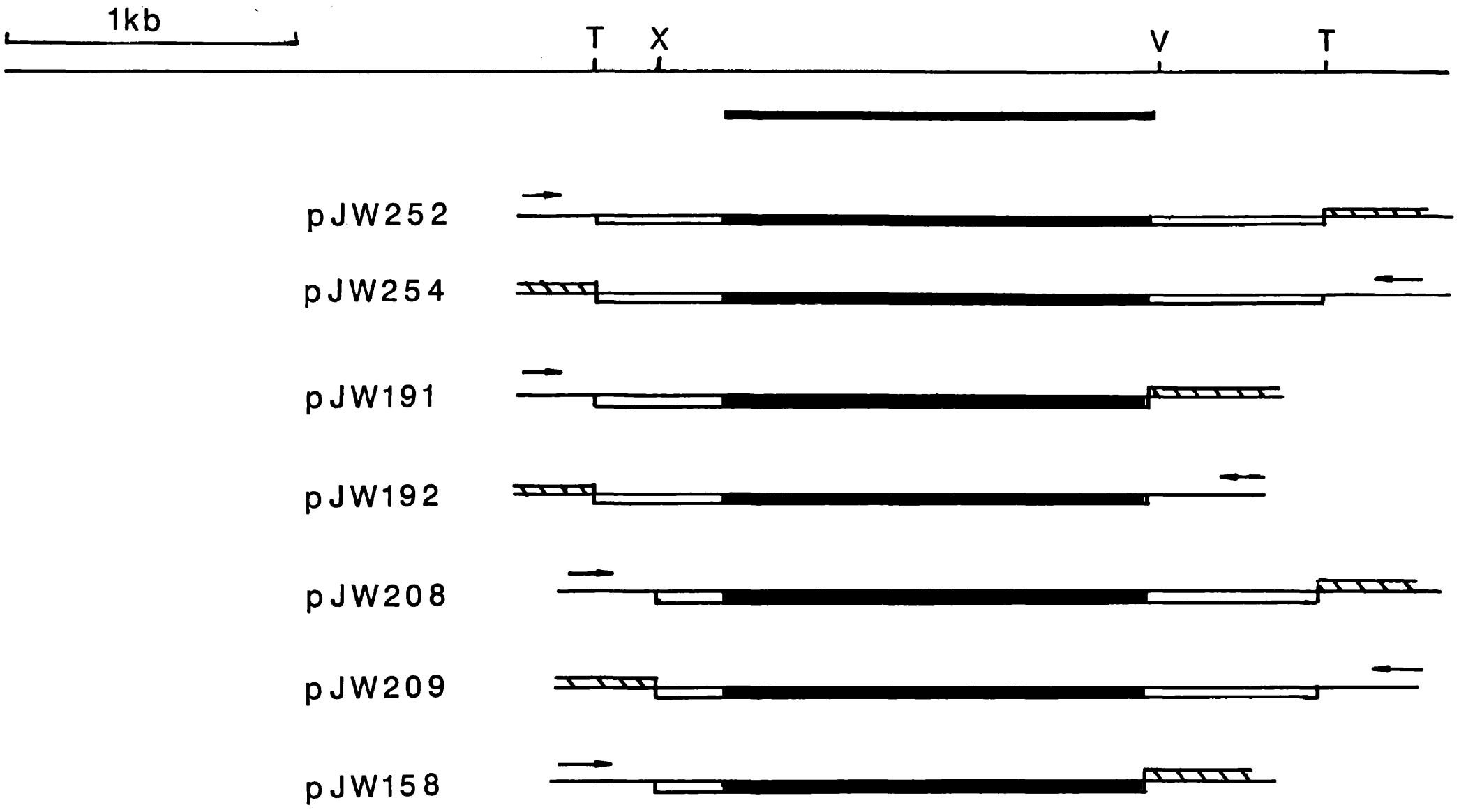
1kb
T
X
V
T
pJW252
pJW254
pJW191
pJW192
pJW208
pJW209
pJW158

TABLE 7

Haemolytic activity in cell free supernatants of bacteria harbouring various recombinant plasmids.

PLASMID	HAEMOLYTIC ACTIVITY [a]	INSERT SIZE [b]	STABILITY [c]	EXPRESSION [d]
pJW252	6.3×10^3	2.5 kb	-	→
pJW254	6.3×10^3	2.5 kb	-	←
pJW191	$<4.0 \times 10^2$	1.9 kb	-	→
pJW192	2.0×10^5	1.9 kb	+	←
pJW208	4.0×10^5	2.2 kb	+	→
pJW209	4.0×10^5	2.2 kb	+	←

[a] Haemolytic activity expressed in HU/ml of crude cell extract

[b] Size of insert DNA carrying the pneumolysin gene

[c] The stability of the recombinant was determined empirically from observations of the number of colonies expressing haemolysis after subculture. +, Stable on subculture, no loss of haemolytic activity. -, unstable, reduction in the numbers of colonies exhibiting haemolysis after subculture.

[d] Orientation of the expression of the pneumolysin gene with respect to the expression of the *lac* gene in pUC. → in the same direction as *lac*. ← in the opposite direction to *lac*. (see Fig. 8)

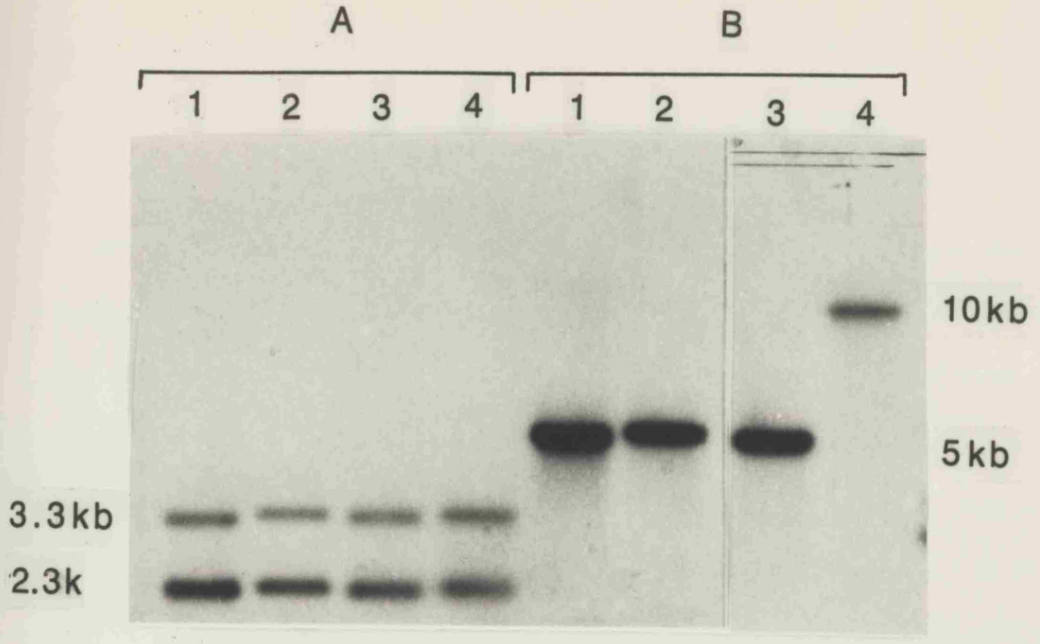

FIGURE 9. Southern Blot of pneumococcal chromosomal DNA probed with pneumolysin sequences.

A. Chromosomal DNA digested to completion with *Eco*RI

B. Chromosomal DNA digested to completion with *Cla*I

Tracks 1. Chromosomal DNA from *S. pneumoniae* serotype I

Tracks 2. Chromosomal DNA from *S. pneumoniae* serotype II

Tracks 3. Chromosomal DNA from *S. pneumoniae* serotype 47

Tracks 4. Chromosomal DNA from *S. pneumoniae* GJB 194

The filters were probed with a 2.5 kb *Tth*III 1 fragment, washed under stringent conditions (0.1x SSC), and bound probe detected by autoradiography.

CHAPTER 4

DNA SEQUENCE DETERMINATION OF SULPHYDRYL-ACTIVATED CYTOLYSIN GENES

4.i INTRODUCTION

Investigations into the mode of action of pneumolysin have been greatly inhibited by the lack of information on the structure of the protein. The isolation of the pneumolysin gene, and its stable maintenance in *E. coli*, permits the determination of the nucleotide sequence of the pneumolysin gene, from which the primary sequence of the toxin can be predicted. The elucidation of the primary protein sequence will facilitate the identification of those domains within the protein which may be important in the biological activity of pneumolysin. Knowledge of these domains will allow a directed investigation into the structure function relationships of pneumolysin. This information will also help in the identification of sequences common to other sulphydryl-activated cytolysins.

4.ii PNEUMOLYSIN GENE SEQUENCE

The complete DNA sequence of the insert present in pJW451 was determined using a shotgun cloning strategy (Deininger 1983) and nucleotide sequencing by the dideoxy chain termination method (Sanger *et al* 1977).

The 5Kb *ClaI* fragment carrying the pneumococcal insert was purified from pJW451 and circularised, the resulting DNA molecules were fragmented by sonication. The fragments produced by sonication were end repaired to allow ligation of these randomised fragments into *Hinc*II digested M13mp18. Fragments of between 500 and 800 bp in length were isolated from a preparative agarose gel and ligated into *Hinc*II digested and dephosphorylated M13mp18. The products of these

ligations were transfected into *E. coli* JM101 and recombinants identified on minimal media containing X-gal and IPTG, on which recombinant plaques are clear and vector only plaques are blue. Single stranded 'phage DNA from 150 recombinant phage was prepared. To determine the randomness of the inserts present in these recombinant 'phage the inserts were screened using a single 'T' sequencing reaction, known as T tracking. Twenty of the 150 recombinants were screened in this way and found to contain unique sequences of insert DNA. The DNA sequence of all remaining template DNAs was determined without prior screening.

The DNA sequence of nearly 100 random clones was determined and the presence of overlapping sequences determined by computer analysis (Staden 1980). Overlapping sequences were joined together until a contiguous sequence, 4,860 bp in length, was obtained (Fig 10). The length of this sequence was very similar in size to that of the *Cla*I fragment from pJW451, as determined from agarose gel electrophoresis. A predicted restriction map of this sequence was determined by a computer program (Staden 1980) which revealed the presence of a single *Cla*I site (Fig 10). The presence of a *Cla*I site in the sequence, together with the observation that the ends of the sequence did not join to form a circle, indicated that the DNA sequence of the entire insert had not been determined. The DNA sequence of the missing region was obtained by cloning specific restriction fragments from pJW451 into M13. A 500 bp *Ssp*I fragment, predicted to contain the undetermined sequence, was cloned into M13mp18. The sequence of this fragment was determined, and so the complete nucleotide sequence of the insert in pJW451 was determined (Fig 10).

The final nucleotide sequence was 4995 bp in length with a GC content of 38% (Fig 11). The individual sequences used to assemble the 4995 bp nucleotide sequence are shown (Fig 10). A complete restriction digest map was predicted using programs written for the VAX mainframe computer (Deveraux *et al* 1984). A restriction map of selected sites is shown in Fig 15. Although not all the sites, predicted by computer analysis of the nucleotide sequence, have been checked by restriction digest analysis of the cloned DNA, there have been no inconsistencies between the predicted restriction sites and those determined experimentally.

Analysis of the DNA sequence revealed only one open reading frame (ORF) that was large enough to code for the polypeptide observed in *in vitro* transcription and translation analysis (Walker *et al* 1987) (Fig 12). This ORF was 1413 bp in length, which would code for a protein of 471 amino acids. Translation of this ORF into amino acid sequence gave a predicted protein with a molecular weight of 52,860 (Fig 13). Sequencing gels of the 5' and 3' ends of the coding sequence, are shown in Fig 14 (see also Fig 10). The N-terminal amino acid sequence of this translated sequence matched exactly that determined for the native toxin (Walker *et al* 1987). This was the final proof that the insert cloned carried the full coding sequence of the pneumolysin gene.

4.iii SEQUENCE DETERMINATION OF THE ALVEOLYSIN GENE

The amino acid composition of native alveolysin as determined from the native protein (Geoffroy and Alouf 1983) appears very different to that determined for pneumolysin, significantly in the presence of

four cysteine residues in alveolysin. It has been suggested that the mode of action of alveolysin may be different to that hypothesised for SLO, the type protein of the sulphydryl-activated cytolysins (Thelestam *et al* 1981). At this time however there is no primary protein sequence data available for alveolysin, or any other of the sulphydryl-activated cytolysins of the Bacillaceae. Comparison of pneumolysin and SLO have highlighted sequences that may be important in the structure and function of these toxins. The recent cloning of sequences from *B. alvei* that direct the production of alveolysin (Geoffroy and Alouf personal communication) provides an opportunity for the determination of the primary structure of alveolysin.

A recombinant plasmid that expressed alveolysin, (Geoffroy and Alouf personal communication) was kindly made available for the determination of the DNA structure of the alveolysin gene. The gene, directing the production of alveolysin was carried on a 3 kb *Hind*III fragment cloned into pBR322. A detailed restriction map of the insert was constructed (Fig 16b) which was in agreement with that constructed by Geoffroy and Alouf (Fig 16a). The recombinant (pALVI, my notation) directed the production of a polypeptide with a molecular weight of 63 kD (Geoffroy and Alouf, personal communication), which is comparable to that of native alveolysin (Geoffroy and Alouf 1983). This recombinant protein possessed all the basic biochemical and immunological characteristics of sulphydryl-activated cytolysins (Geoffroy and Alouf, personal communication).

The procedure used for the determination of the alveolysin sequence was similar to that used for pneumolysin. The 3 kb *Hind*III fragment was purified, circularised, and fragmented by sonication. The single

stranded ends of the fragments produced by sonication were repaired. Fragments of insert DNA between 500 - 800 bp in length were cloned into M13mp18 that had been cut with *Hinc*II and dephosphorylated. The products of the ligation were transfected into *E. coli* JM101 and cells plated out onto minimal media plates containing IPTG and X-gal. About 30 random clones were isolated and their DNA sequences determined. In addition to random clones specific restriction fragments were inserted into M13mp18 and mp19 to facilitate the assembly of the contiguous nucleotide sequence of the insert in pALV1 (Fig 17).

The nucleotide sequence of the insert in pALV1 was 2910 bp in length (Fig 18). The restriction map predicted from this sequence (Fig 16c), was reasonably consistent with that determined by restriction digest analysis (Fig 16a,b), considering the rudimentary nature of the restriction maps determined experimentally.

To locate the alveolysin gene within the insert in pALVI the nucleotide sequence of the insert was screened for ORFs (Fig 19). No ORF large enough to encode a protein of 63 kD was observed. The nucleotide sequences of both strands was translated into amino acid sequence in all three frames. The amino acid sequences in all six translations were screened with the N-terminal amino acid sequence determined from native alveolysin. No homology was observed in any of the six translations. A re-evaluation of the sequence of existing M13 clones, and the determination of sequence data from newly isolated clones, failed to substantially alter the sequence of the insert (Fig 18).

The failure to locate an ORF within the nucleotide sequence of the insert in pALV1 is inconsistent with the observation of the production of a 63 kD haemolytic polypeptide by pALVI. The most logical explanations are that the DNA sequence of the insert in pALV1 contains many errors or has been incorrectly compiled. At this point no further work was carried out on the alveolysin gene sequence.

4.iv DISCUSSION

4.iv a Pneumolysin gene sequence

The pneumolysin coding sequence was 1,413 bp in length, and encoded a protein of 471 amino acids. The predicted amino acid composition (Table 8) gave a protein with a molecular weight of 52.8 kD. This is in very close agreement with the size of purified native pneumolysin (Paton *et al* 1983) and that of the recombinant pneumolysin seen in the *E. coli* cell free *in-vitro* transcription translation of plasmids carrying the pneumolysin gene (Walker *et al* 1987).

Pneumolysin, unlike other sulphydryl-activated cytolysins, is not secreted from its host organism and is located in the pneumococcal cytoplasm (Johnson 1977). Examination of the predicted N-terminal amino acid sequence of pneumolysin revealed no consensus signal sequences which are usually found in secreted proteins (Oliver 1985, Sarvas 1986). The N-terminal amino acid sequence predicted from the nucleotide sequence is identical to that of native toxin (Walker *et al* 1987) and the recombinant toxin purified from *E. coli* (Mitchell *et al*, in preparation), indicating that no post-translational processing, other than the removal of f-methionine, occurs.

Analysis of the amino acid composition of pneumolysin revealed a single cysteine residue at position 428 in the predicted amino acid sequence, 42 amino acids from the C terminus of the protein. The presence of a single cysteine in perfringolysin has also been reported (Yamakawa *et al* 1977). Using chemical analysis of perfringolysin the single cysteine has been located to within 5 kD of the C terminus (Iwomato *et al* 1987), and is therefore at a similar position to the location of the cysteine in pneumolysin as predicted from translation of the nucleotide sequence.

It is currently accepted that sulphydryl groups play an important role in the activity of pneumolysin. Oxidation and reduction of the protein reflecting the formation and breakage of intramolecular disulphide bonds (reviewed by Alouf 1980), the subsequent conformational changes induced in the protein resulting in inactive or active toxin respectively. Clearly, however, the presence of a single cysteine in pneumolysin precludes the formation of intramolecular disulphide bridges. The formation of intermolecular disulphide bridges with other proteins in crude extracts, which may result in inactive toxin, cannot be discounted.

Analysis of sequences 5' to the ATG start codon of the pneumolysin gene (position 2508) revealed a typical ribosome binding site or Shine-Delgarno sequence of GGAGG at the optimum distance of six bp upstream of the translation initiation codon. The presence of putative promoter sequences upstream of the initiation codon have also been identified (Fig 13). These sequences are similar to the consensus sequences for *E. coli* promoters (Rosenberg and Court 1979). The distance between these putative promoter sequences and the initiation codon is relatively long compared to many of the promoter

sequences determined for other pneumococcal genes (Ferretti and Curtiss 1987). Whether these sequences are the ones involved in the expression of pneumolysin in the pneumococcus or in *E. coli* has yet to be investigated. The production of high levels of pneumolysin by pUC based recombinants, where the pneumolysin gene is expressed in the opposite orientation to the expression of the *lac* gene (Table 7), supports the hypothesis that the pneumolysin gene is expressed from a promoter present on the insert.

The stability of pneumococcal sequences in *E. coli* have been shown to depend on efficient termination of transcription (Chen and Morrison 1987), however, there were no consensus *E. coli* transcription terminator signals observed in the sequence downstream from the stop codon of the pneumolysin gene.

A significant observation of the pneumolysin gene sequence was the presence of an amber translation termination codon (TAG) at the end of the coding sequence. The termination of translation by amber stop codons in *E.coli* can be suppressed in the presence of various mutations, notably *SupE*. In strains carrying *SupE* mutations, glutamine residues are sometimes inserted into the polypeptide at TAG codons, resulting in elongation of the transcript, until termination occurs at the next in frame transcription termination codon. *E.coli* LE392 harbouring a pneumolysin clone, (pJCP191), constructed by Paton *et al* (1986), produces two immunologically related polypeptides of 52 kD and 54 kD. The higher molecular weight polypeptide is not seen when the cloned pneumolysin gene was expressed in the Sup^0 strains, *E. coli* DS410 (Paton *et al* 1986) or *E. coli* MC1061 (T. Mitchell, personal communication). The 2 kD difference can be accounted for if suppression of the stop codon at 3923 occurs, with termination at the

next in frame stop codon (Fig 13). Whether the production of two related polypeptides reduces the yield of full length recombinant pneumolysin in *SupE* strains of *E. coli* has not been thoroughly investigated, though cell-free extracts of *E. coli* MC1061 (pJCP 191) had a higher haemolytic activity than those of *E. coli* LE392(pJCP 191) (T. Mitchell, personal communication). In view of the production of aberrant polypeptides related to pneumolysin, with unknown activity, in *SupE* strains of *E. coli*, the use of Sup^0 strains such as *E. coli* MC1061, routinely used for the propogation of all subclones described in this thesis, is essential.

4.iv b Comparison of pneumolysin and SLO

The determination of the structure of the gene for SLO (Kehoe *et al* 1987), subsequent to the determination of the sequence of the pneumolysin gene allows, for the first time, the direct comparison of primary amino acid sequences of two sulphydryl-activated cytolysins.

The DNA sequence of the SLO gene was 1713 bp in length and coded for a protein of 571 amino acids, with a predicted molecular weight of 63.6 kD (Kehoe *et al* 1987). A consensus secretion signal sequence (Sarvas 1986) was observed at the N-terminus of the protein, which if removed, would generate a protein of approximately 538 amino acids long, with a predicted molecular weight of 60 kD. It has been observed that native SLO exists in two haemolytically active forms, of 69 kD and 57 kD in size (Bhakdi *et al* 1985), the recombinant SLO also existed in two forms of 68 kD and 61 kD (Kehoe and Timmis 1984). It has been suggested that the lower molecular weight form represented a cleavage product of the 69 kD protein (Bkakdi *et al* 1985). The N-terminal amino acid sequence of the lower molecular

weight fragment of the native SLO had been tentatively determined as SER-ASP-GLU-ASP. A similar sequence of SER-GLU-GLU-ASP was identified at amino acids 101-104 in the predicted SLO sequence. This sequence, preceded by a lysine residue, suggests a protease sensitive site. The loss of the 67 amino acids between the end of the secretion signal sequence and amino acid 101 could account for the 7 kD difference in size between the high and low molecular weight forms of recombinant SLO observed in minicells (Kehoe and Timmis 1984). It is interesting to note that removal of 100 amino acids from the full length SLO sequence gives a low molecular weight form of SLO containing 470 amino acids, almost identical in size to that predicted for pneumolysin. Also of great interest was the observation of a single cysteine in the predicted SLO sequence at a similar position to the C-terminus of the protein as determined for pneumolysin.

The nucleotide sequences of the pneumolysin and SLO genes were compared using a dot-plot algorithm (Maizel and Lenk 1981). This algorithm produces a dot matrix in which matches of a particular stringency are scored. At a stringency of 66% (14 matches within a sequence window of 21 bases) a line of homology was detected between the two sequences (Fig 20). The resulting plot aligned the pneumolysin gene with a SLO sequence 300 bp in from the start of the gene. The similarity of the two nucleotide sequences throughout the length of the coding region was very low, about 21 %, and accounts for the failure to detect homology between the SLO gene and pneumococcal chromosomal DNA in Southern blots (Kehoe and Timmis 1984).

When the pneumolysin amino acid sequence was aligned with the primary transcipt of SLO using a best fit algorithm (Smith and Waterman 1981), the sequences were aligned at the single cysteine in the two proteins (Fig 21a). This alignment was achieved with the inclusion of one gap, of a single amino acid, in the SLO sequence and resulted in 42% of residues being identical in both sequences. The similarity of the two sequences was considered significant, since randomised sequences of SLO and pneumolysin only shared 15 % homology when compared by best fit programs (Smith and Waterman 1981). The significance of this alignment is that not only is it consistent with that observed in the DNA alignment, as expected, but that it aligns the C-terminus of pneumolysin with the predicted C-terminus of the proposed low molecular weight form of SLO.

The similarity of the pneumolysin and SLO sequences was even higher when the protein sequences were compared after the amino acids had been simplified into six groups based on the properties of each amino acid. The groups were neutral, acidic, basic, hydrophobic, aromatic and cysteine (Fig 21b). Comparison of the simplified sequences resulted in 62% similarity in both sequences, with the same alignment observed with the comparison of identical amino acids.

The comparison of pneumolysin and SLO protein sequences using identical and similar amino acids aligned the single cysteine in both sequences at similar positions with respect to the C-Termini of both toxins. The longest sequence of identical amino acids in both toxins consisted of 12 amino acids surrounding the cysteine (Fig 22), a similar sequence (11 out of 12 amino acids) has also been found in the related toxin listeriolysin O (Mengaud *et al* 1988). The presence of regions of identical amino acids surrounding the cysteine in

pneumolysin, SLO and listeriolysin-O implies that such a sequence may define a functionally important domain within the protein. This hypothesis is based on the observation that cysteine residues appear essential in the biological activities of this group of proteins. The actual involvement of such a domain in the activity of sulphydryl activated cytolysins remains to be determined.

The hydropathic indexes of pneumolysin and the low molecular weight form of SLO, as measured by the algorithm of Kyte and Doolittle (1982), are very similar, especially toward the C-terminus of both proteins (Fig 23). This reinforces the hypothesis that the two proteins are structurally very similar and probably have similar if not identical modes of action.

The comparison of the amino acid composition of pneumolysin (my data), with perfringolysin (Yamakawa *et al* 1977) and the low molecular weight form of SLO (Kehoe *et al* 1987) (Table 8) shows little overall conservation in numbers of individual amino acids. However, there is a similarity in the percentage of each group of amino acids, with similar properties (Fig 21 b). The overall composition of amino acids is similar to that determined for prokaryotic proteins (Doolittle 1986), and may even contain a slightly higher proportion of charged amino acids. Clearly, therefore, the hydrophobic nature of pneumolysin and SLO observed experimentally (Johnson *et al* 1982), cannot be due to a disproportionately high number of hydrophobic amino acids and must result from the secondary structure of the protein. The presence of hydrophobic domains throughout the amino acid sequence of both pneumolysin and SLO (Fig 23) is consistant with the hypothesis that folding of the protein is responsible for its hydrophobic character.

FIGURE 10. The individual gel sequences that were used in compiling the complete nucleotide sequence of the insert present in pJW451.

A. Initial 4860 bp sequence compiled from random clones.

B. Final 4995 bp sequence after determination of the missing sequence, not present in A.

Solid lines represent sequence determined from random clones. Broken lines indicate the sequence determined from specific restriction fragments.

C= *Cla*I, H= *Hpa*II, S= *Ssp*I, V= *Eco*RV.

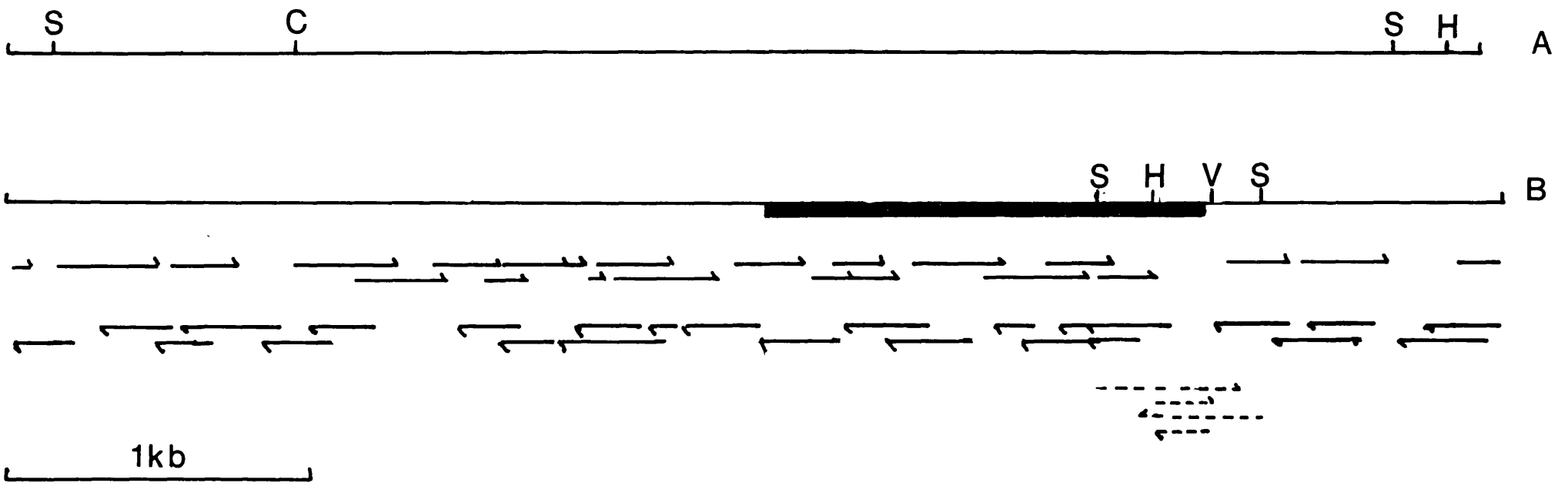
S
C
S
H
A
S
H
V
S
B
1kb

FIGURE 11. Complete nucleotide sequence of the 4995 bp *Cla*I fragment present in pJW451. The numbers refer to the position of nucleotides within the sequence.

```
   1  GATTTCTAAC AATGTTTTAG AAGCAGAAGT GTACTATTCT AGTTTCAATC    50
      CTAAAGATTG TTACAAAATC TTCGTCTTCA CATGATAAGA TCAAAGTTAG

  51  TACTATAGTT AAATCTGCGG TCAAGTCTAC TGGTGAATCT ATGATTGTAA   100
      ATGATATCAA TTTAGACGCC AGTTCAGATG ACCACTTAGA TACTAACATT

 101  TACTCTTCCA AAATCTCATC AACCACGTCA GTCTTGCCTT GCAGTCTGTA   150
      ATGAGAAGGT TTTAGAGTAG TTGGTGCAGT CAGAACGGAA CGTCAGACAT

 151  TCTTACTGAC CAAGCTAGTG ATGGACTTAG AATAGGTGAT TTGGAGCGTC   200
      AGAATGACTG GTTCGATCAC TACCTGAATC TTATCCACTA AACCTCGCAG

 201  CTATTGGCTA GGAAATGCTG ATCATAGTCC TTTGCTGAGG CTAGGGTGTT   250
      GATAACCGAT CCTTTACGAC TAGTATCAGG AAACGACTCC GATCCCACAA

 251  TCAACATTCA ACACTCAACT GGTTGATCTA GTTGATAGGA AGGGAATTAC   300
      AGTTGTAAGT TGTGAGTTGA CCAACTAGAT CAACTATCCT TCCCTTAATG

 301  TATAAAATAC TCAGTCTTCC ATCATATTTT TTGAAACGAT TGTGTAATCA   350
      ATATTTTATG AGTCAGAAGG TAGTATAAAA AACTTTGCTA ACACATTAGT

 351  AAATGTACCA ATTATTGTAG TATTGGTACA GAAGATGTTG TGAATGGATA   400
      TTTACATGGT TAATAACATC ATAACCATGT CTTCTACAAC ACTTACCTAT

 401  AATATATCAT AACTGCTGTC TCTCTATGTA AAAAGAAGGC TGTATCAAAA   450
      TTATATAGTA TTGACGACAG AGAGATACAT TTTTCTTCCG ACATAGTTTT

 451  AGATTTCATA TGTCTGTGCA TATATATAAT AGACTTCCTG CAAAACTAGA   500
      TCTAAAGTAT ACAGACACGT ATATATATTA TCTGAAGGAC GTTTTGATCT

 501  ATCCTAGTTC ATGATTGATA ATACCAGCAC TCAAATTCAT TCGTAATCCG   550
      TAGGATCAAG TACTAACTAT TATGGTCGTG AGTTTAAGTA AGCATTAGGC

 551  AAGCGTTTAC GATGATTTCG ACAGGTTGTT GAAAATTTTA AACGTTTTTA   600
      TTCGCAAATG CTACTAAAGC TGTCCAACAA CTTTTAAAAT TTGCAAAAAT

 601  CTTTGGCAAA GATGTTCTCA ACCTTGCTTC TCTCCTTAGA TAGCGCATGG   650
      GAAACCGTTT CTACAAGAGT TGGAACGAAG AGAGGAATCT ATCGCGTACC

 651  TTATAGACTT TATCTTCAAC TGTTAGTGGC TTGAGTTTGC TGGATTTACG   700
      AATATCTGAA ATAGAAGTTG ACAATCACCG AACTCAAACG ACCTAAATGC

 701  TGGAGTTTGT GCTTGAGGAT ATATCTTCAT GAGCCCTTGA TAATTACTGT   750
      ACCTCAAACA CGAACTCCTA TATAGAAGTA CTCGGGAACT ATTAATGACA

 751  CAGCCAAGAT TTTACCAGCT TGTCCGACTT TTTTGATAAG CTGTTTTTAA   800
      GTCGGTTCTA AAATGGTCGA ACAGGCTGAA AAAACTATTC GACAAAAATT

 801  TACAGCTAAC ATCTCTTCAA AAGTGGTACG CTGAACACCA ACAAGACGCT   850
      ATGTCGATTG TAGAGAAGTT TTCACCATGC GACTTGTGGT TGTTCTGCGA

 851  TAAATCGTGT ATCAGTTAAT TGTTTACTTG CTTCATCATT CATAGAACTA   900
      ATTTAGCACA TAGTCAATTA ACAAATGAAC GAAGTAGTAA GTATCTTGAT

 901  CTATACCATA TTTTGTTTCG CAGGGAGTCT AATAAGTATA GGCCATTTTT   950
      GATATGGTAT AAAACAAAGC GTCCCTCAGA TTATTCATAT CCGGTAAAAA

 951  ACTATACTTT GATATTCAAA TATGTTATAC TATGGTTATC AAAGTAAATG  1000
      TGATATGAAA CTATAAGTTT ATACAATATG ATACCAATAG TTTCATTTAC

1001  AAAGGAGTTA CGAATATGAC GCCAGAAGAA ATGTACCTGA CAGAGCGATT  1050
      TTTCCTCAAT GCTTATACTG CGGTCTTCTT TACATGGACT GTCTCGCTAA

1051  AGACGTACAG ATAGCTCATT TTTTAAAGAA AAGCGTTCAA CATCGTAGGC  1100
```

```
      TCTGCATGTC TATCGAGTAA AAAATTTCTT TTCGCAAGTT GTAGCATCCG

1101  GCTATAAGGT ATTAAAAATA ACAGAAATCG TGGCAGGTTT CCTCATAGCT  1150
      CGATATTCCA TAATTTTTAT TGTCTTTAGC ACCGTCCAAA GGAGTATCGA

1151  GTCTTTTGTG CTATTCCTAT GCCAGGTGAT CGCTACCGTT TGATTTCGGT  1200
      CAGAAAACAC GATAAGGATA CGGTCCACTA GCGATGGCAA ACTAAAGCCA

1201  TGCCTTATCC AGTCTCGGCT TGCTGTGTGA GGGGATTATC AATTTGTATA  1250
      ACGGAATAGG TCAGAGCCGA ACGACACACT CCCCTAATAG TTAAACATAT

1251  ATGCAAAGGA AAATTGGATT TCTTACCAAA AAACTGCGCA ACTCCTGGAG  1300
      TACGTTTCCT TTTAACCTAA AGAATGGTTT TTTGACGCGT TGAGGACCTC

1301  AAAGAAAAAT TCCTCTATCA ACGCCAAACG GAGAAATATG CAGGAAAGAC  1350
      TTTCTTTTTA AGGAGATAGT TGCGGTTTGC CTCTTTATAC GTCCTTTCTG

1351  CAAGGCTTTT GCCCTATTTG TCAAGACATG CGAAGGTCTT ATCTCAGAGG  1400
      GTTCCGAAAA CGGGATAAAC AGTTCTGTAC GCTTCCAGAA TAGAGTCTCC

1401  AGATTAACCA GTGGGAAAGT ATCCAGTCAA AAGAAGTGGC AGCTAGTGCA  1450
      TCTAATTGGT CACCCTTTCA TAGGTCAGTT TTCTTCACCG TCGATCACGT

1451  GATGCTCCAG TTAAAAAAGA GTAGGAGGTA GAGGAAATGT CTCAATCCAG  1500
      CTACGAGGTC AATTTTTTCT CATCCTCCAT CTCCTTTACA GAGTTAGGTC

1501  TTACCTGTCG CCCTTGCTCT GGTTAAAAAA AGAAGCCGAT AAGGAAAAGA  1550
      AATGGACAGC GGGAACGAGA CCAATTTTTT TCTTCGGCTA TTCCTTTTCT

1551  TGAGCGCGAC CCAGTGCCAG ATATTTTTCT TTTACTATCA AATGTTTGAG  1600
      ACTCGCGCTG GGTCACGGTC TATAAAAAGA AAATGATAGT TTACAAACTC

1601  CTCTTATTTG CTAGAGAAAG CGACATGAAA GACTTATGTC TGGGAACGAA  1650
      GAGAATAAAC GATCTCTTTC GCTGTACTTT CTGAATACAG ACCCTTGCTT

1651  AGGTTTTTAT TTCTCGCAGT TAGAGAAAAA TTTGCTTTCT GGAGTTTCCC  1700
      TCCAAAAATA AAGAGCGTCA ATCTCTTTTT AAACGAAAGA CCTCAAAGGG

1701  GATTTCTAAA AAACTTGGAG GGGAAAGTAA CTCTCAAGGC TAACCAAGAA  1750
      CTAAAGATTT TTTGAACCTC CCCTTTCATT GAGAGTTCCG ATTGGTTCTT

1751  GTATCAGCTC GCAAAGCCCT TTTTCTAGCC TTGACAACTA GCCAATCAGA  1800
      CATAGTCGAG CGTTTCGGGA AAAAGATCGG AACTGTTGAT CGGTTAGTCT

1801  TTGGCAGGAG TTAGCTCCTG TTTTTGATTT TTATCAGACT ATCGGGAGGC  1850
      AACCGTCCTC AATCGAGGAC AAAAACTAAA AATAGTCTGA TAGCCCTCCG

1851  TTGAAAATCC TTCTCTCTTG AGTTCTCAGG ACAGACAACA TCTGATGTGG  1900
      AACTTTTAGG AAGAGAGAAC TCAAGAGTCC TGTCTGTTGT AGACTACACC

1901  ATTTACCAGT CAGCTTTGGA GAAGGATTAT ATTGTCAAGG TTATTGGCGA  1950
      TAAATGGTCA GTCGAAACCT CTTCCTAATA TAACAGTTCC AATAACCGCT

1951  CAAGCATTTT GTATTGAAGA GACAAGATGC TACTAAATTG ACAGCGCGCC  2000
      GTTCGTAAAA CATAACTTCT CTGTTCTACG ATGATTTAAC TGTCGCGCGG

2001  AAACTCAAAC TTTGGAAATT CTGAGTCAAT CAGAAGACTT GGTCAATCCT  2050
      TTTGAGTTTG AAACCTTTAA GACTCAGTTA GTCTTCTGAA CCAGTTAGGA

2051  GTCTATGTTA CATTAGGAGA AAAGGGGGTG CTCTTGCTTG ATTAAGAGAG  2100
      CAGATACAAT GTAATCCTCT TTTCCCCCAC GAGAACGAAC TAATTCTCTC

2101  GAGATGTTGT AGCTCTTTAT TTGCCTTTTC CGACTATTAG TAGCGATTTG  2150
      CTCTACAACA TCGAGAAATA AACGGAAAAG GCTGATAATC ATCGCTAAAC

2151  GCTGTGAAGA ATCATATGTA TATCTGTATT GACAACAGCA TGACTAAAAA  2200
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      CGACACTTCT TAGTATACAT ATAGACATAA CTGTTGTCGT ACTGATTTTT

2201  CAAAGAGTTG GTTAAAAATC AGACCTTCAA ACCAGCTCTC TTGACCAGAC  2250
      GTTTCTCAAC CAATTTTTAG TCTGGAAGTT TGGTCGAGAG AACTGGTCTG

2251  GTTTGGTCAA GAACTTTATG ATAGAAGAGC CGGATCTAGC TCGTAATCCT  2300
      CAAACCAGTT CTTGAAATAC TATCTTCTCG GCCTAGATCG AGCATTAGGA

2301  TTACAAGACC AACCTTGATT GACTTAGATA AGGTATTTAT GTTGGATAAT  2350
      AATGTTCTGG TTGGAACTAA CTGAATCTAT TCCATAAATA CAACCTATTA

2351  ACGGTTATTC CGACTTCTTA TCTAGCCAGA CGGCGACGCA ATGTCTCAGA  2400
      TGCCAATAAG GCTGAAGAAT AGATCGGTCT GCCGCTGCGT TACAGAGTCT

2401  AGAATTGTAC GAGGAAATTT TGGATCACTT AGTCCAACCA CGGCTGATTT  2450
      TCTTAACATG CTCCTTTAAA ACCTAGTGAA TCAGGTTGGT GCCGACTAAA

2451  CGCTGAACAA GTCTGAGTTT ATGCAACTCA ATCCAGGAAC TTATTAGGAG  2500
      GCGACTTGTT CAGACTCAAA TACGTTGAGT TAGGTCCTTG AATAATCCTC

2501  GTAGAAGATG GCAAATAAAG CAGTAAATGA CTTTATACTA GCTATGAATT  2550
      CATCTTCTAC CGTTTATTTC GTCATTTACT GAAATATGAT CGATACTTAA

2551  ACGATAAAAA GAAACTCTTG ACCCATCAGG GAGAAAGTAT TGAAAATCGT  2600
      TGCTATTTTT CTTTGAGAAC TGGGTAGTCC CTCTTTCATA ACTTTTAGCA

2601  TTCATCAAAG AGGGTAATCA GCTACCCGAT GAGTTTGTTG TTATCGAAAG  2650
      AAGTAGTTTC TCCCATTAGT CGATGGGCTA CTCAAACAAC AATAGCTTTC

2651  AAAGAAGCGG AGCTTGTCGA CAAATACAAG TGATATTTCT GTAACAGCTA  2700
      TTTCTTCGCC TCGAACAGCT GTTTATGTTC ACTATAAAGA CATTGTCGAT

2701  CCAACGACAG TCGCCTCTAT CCTGGAGCAC TTCTCGTAGT GGATGAGACC  2750
      GGTTGCTGTC AGCGGAGATA GGACCTCGTG AAGAGCATCA CCTACTCTGG

2751  TTGTTAGAGA ATAATCCCAC TCTTCTTGCG GTTGATCGTG CTCCGATGAC  2800
      AACAATCTCT TATTAGGGTG AGAAGAACGC CAACTAGCAC GAGGCTACTG

2801  TTATAGTATT GATTTGCCTG GTTTGGCAAG TAGCGATAGC TTTCTCCAAG  2850
      AATATCATAA CTAAACGGAC CAAACCGTTC ATCGCTATCG AAAGAGGTTC

2851  TGGAAGACCC CAGCAATTCA AGTGTTCGCG GAGCGGTAAA CGATTTGTTG  2900
      ACCTTCTGGG GTCGTTAAGT TCACAAGCGC CTCGCCATTT GCTAAACAAC

2901  GCTAAGTGGC ATCAAGATTA TGGTCAGGTC AATAATGTCC CAGCTAGAAT  2950
      CGATTCACCG TAGTTCTAAT ACCAGTCCAG TTATTACAGG GTCGATCTTA

2951  GCAGTATGAA AAAATAACGG CTCACAGCAT GGAACAACTC AAGGTCAAGT  3000
      CGTCATACTT TTTTATTGCC GAGTGTCGTA CCTTGTTGAG TTCCAGTTCA

3001  TTGGTTCTGA CTTTGAAAAG ACAGGGAATT CTCTTGATAT TGATTTTAAC  3050
      AACCAAGACT GAAACTTTTC TGTCCCTTAA GAGAACTATA ACTAAAATTG

3051  TCTGTCCATT CAGGTGAAAA GCAGATTCAG ATTGTTAATT TTAAGCAGAT  3100
      AGACAGGTAA GTCCACTTTT CGTCTAAGTC TAACAATTAA AATTCGTCTA

3101  TTATTATACA GTCAGCGTAG ACGCTGTTAA AAATCCAGGA GATGTGTTTC  3150
      AATAATATGT CAGTCGCATC TGCGACAATT TTTAGGTCCT CTACACAAAG

3151  AAGATACTGT AACGGTAGAG GATTTAAAAC AGAGAGGAAT TTCTGCAGAG  3200
      TTCTATGACA TTGCCATCTC CTAAATTTTG TCTCTCCTTA AAGACGTCTC

3201  CGTCCTTTGG TCTATATTTC GAGTGTTGCT TATGGGCGCC AAGTCTATCT  3250
      GCAGGAAACC AGATATAAAG CTCACAACGA ATACCCGCGG TTCAGATAGA
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3251  CAAGTTGGAA ACCACGAGTA AGAGTGATGA AGTAGAGGCT GCTTTTGAAG   3300
      GTTCAACCTT TGGTGCTCAT TCTCACTACT TCATCTCCGA CGAAAACTTC

3301  CTTTGATAAA AGGAGTCAAG GTAGCTCCTC AGACAGAGTG GAAGCAGATT   3350
      GAAACTATTT TCCTCAGTTC CATCGAGGAG TCTGTCTCAC CTTCGTCTAA

3351  TTGGACAATA CAGAAGTGAA GGCGGTTATT TTAGGGGGCG ACCCAAGTTC   3400
      AACCTGTTAT GTCTTCACTT CCGCCAATAA AATCCCCCGC TGGGTTCAAG

3401  GGGTGCCCGA GTTGTAACAG GCAAGGTGGA TATGGTAGAG GACTTGATTC   3450
      CCCACGGGCT CAACATTGTC CGTTCCACCT ATACCATCTC CTGAACTAAG

3451  AAGAAGGCAG TCGCTTTACA GCAGATCATC CAGGCTTGCC GATTTCCTAT   3500
      TTCTTCCGTC AGCGAAATGT CGTCTAGTAG GTCCGAACGG CTAAAGGATA

3501  ACAACTTCTT TTTTACGTGA CAATGTAGTT GCGACCTTTC AAAACAGTAC   3550
      TGTTGAAGAA AAAATGCACT GTTACATCAA CGCTGGAAAG TTTTGTCATG

3551  AGACTATGTT GAGACTAAGG TTACAGCTTA CAGAAACGGA GATTTACTGC   3600
      TCTGATACAA CTCTGATTCC AATGTCGAAT GTCTTTGCCT CTAAATGACG

3601  TGGATCATAG TGGTGCCTAT GTTGCCCAAT ATTATATTAC TTGGGATGAA   3650
      ACCTAGTATC ACCACGGATA CAACGGGTTA TAATATAATG AACCCTACTT

3651  TTATCCTATG ATCATCAAGG TAAGGAAGTC TTGACTCCTA AGGCTTGGGA   3700
      AATAGGATAC TAGTAGTTCC ATTCCTTCAG AACTGAGGAT TCCGAACCCT

3701  CAGAAATGGG CAGGATTTGA CGGCTCACTT TACCACTAGT ATTCCTTTAA   3750
      GTCTTTACCC GTCCTAAACT GCCGAGTGAA ATGGTGATCA TAAGGAAATT

3751  AAGGGAATGT TCGTAATCTC TCTGTCAAAA TTAGAGAGTG TACCGGGCTT   3800
      TTCCCTTACA AGCATTAGAG AGACAGTTTT AATCTCTCAC ATGGCCCGAA

3801  GCCTGGGAAT GGTGGCGTAC GGTTTATGAA AAAACCGATT TGCCACTAGT   3850
      CGGACCCTTA CCACCGCATG CCAAATACTT TTTTGGCTAA ACGGTGATCA

3851  GCGTAAGCGG ACGATTTCTA TTTGGGGAAC AACTCTCTAT CCTCAGGTAG   3900
      CGCATTCGCC TGCTAAAGAT AAACCCCTTG TTGAGAGATA GGAGTCCATC

3901  AGGATAAGGT AGAAAATGAC TAGGAGAGGA GAATGCTTGC GACAAAAAGA   3950
      TCCTATTCCA TCTTTTACTG ATCCTCTCCT CTTACGAACG CTGTTTTTCT

3951  GGCGATGATC TCTCTGCGGA TATCTGAAAG TTTATCTCTT GCCTAGCGAT   4000
      CCGCTACTAG AGAGACGCCT ATAGACTTTC AAATAGAGAA CGGATCGCTA

4001  TTCCATTGGA GTATGCTCGT GATGTTAAAA TGAGTCTATC AAACAGGATG   4050
      AAGGTAACCT CATACGAGCA CTACAATTTT ACTCAGATAG TTTGTCCTAC

4051  TGTAAAAAAG TAAAAAGGGT AGCCAATTCC TTGATATACA GGGGATTGAT   4100
      ACATTTTTTC ATTTTTCCCA TCGGTTAAGG AACTATATGT CCCCTAACTA

4101  TATCCTTTAC TTGTTTTAGA TGATTGGAAA TTCAAGAATG ACACGTATCG   4150
      ATAGGAAATG AACAAAATCT ACTAACCTTT AAGTTCTTAC TGTGCATAGC

4151  GAAATATTTT GCTGTTTATA TCTAATACCC ATTTATGGAA AAAATGAAAT   4200
      CTTTATAAAA CGACAAATAT AGATTATGGG TAAATACCTT TTTTACTTTA

4201  TAAATATGAA CAAATCAATT GCTAGCAATG GTTTAGAAGT GCCAGTGTAC   4250
      ATTTATACTT GTTTAGTTAA CGATCGTTAC CAAATCTTCA CGGTCACATG

4251  TAGTCTAACT TCTCCATCTT AGGAACACGT AAATTATACT CTTAGAAAAT   4300
      ATCAGATTGA AGAGGTAGAA TCCTTGTGCA TTTAATATGA GAATCTTTTA
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4301  CTCTTCAAAC CATGTCAGCC CTATCCGCAA CCTCAAAACA GTGTTTTGAG   4350
      GAGAAGTTTG GTACAGTCGG GATAGGCGTT GGAGTTTTGT CACAAAACTC

4351  CAACCTGCGG CTAGCTTTCT AGTTTGCTCT TTGATTTTTA TTGAGTATTA   4400
      GTTGGACGCC GATCGAAAGA TCAAACGAGA AACTAAAAAT AACTCATAAT

4401  GAGGTTTGCT TAGAAACTCT TCTATTGATT TCTTAGAATA CAATAGCGCC   4450
      CTCCAAACGA ATCTTTGAGA AGATAACTAA AGAATCTTAT GTTATCGCGG

4451  TATAAGAACT GTTGAATTTC AAAGTATTTA CTTGAACTAT GATATGTCTG   4500
      ATATTCTTGA CAACTTAAAG TTTCATAAAT GAACTTGATA CTATACAGAC

4501  GAGTAGACTG TCAACAAAAG GTGCAGTATG ATTGTTTTTG TCGCTTATAT   4550
      CTCATCTGAC AGTTGTTTTC CACGTCATAC TAACAAAAAC AGCGAATATA

4551  CTACAAACTT CAAACAGTAA TTTGAGCTGA CTTTGTCAAA CTTGGCTGAA   4600
      GATGTTTGAA GTTTGTCATT AAACTCGACT GAAACAGTTT GAACCGACTT

4601  ACTTTCTATT TGCAGATAAT GGTCTAGTTT GTTTTTTGAA TGTGGATGAG   4650
      TGAAAGATAA ACGTCTATTA CCAGATCAAA CAAAAAACTT ACACCTACTC

4651  TGTCTGTTGA TAAAGATAGA AAGGCAAGAC CATCTTAGCA GGTAAAGTGC   4700
      ACAGACAACT ATTTCTATCT TTCCGTTCTG GTAGAATCGT CCATTTCACG

4701  TATCCTAGCT TGATTTGCTA ATAAAATCCA TTTCCTCTAG TGAAAATCAA   4750
      ATAGGATCGA ACTAAACGAT TATTTTAGGT AAAGGAGATC ACTTTTAGTT

4751  AAAAACTTGT GTTATAATAA GAAAGATTAA AATGTGAAAA AAGGAGATTC   4800
      TTTTTGAACA CAATATTATT CTTTCTAATT TTACACTTTT TTCCTCTAAG

4801  CTAATGGGAC GTAAATGGGC CAATATCGTA GCCAAGAAAA CGGCTAAAGA   4850
      GATTACCCTG CATTTACCCG GTTATAGCAT CGGTTCTTTT GCCGATTTCT

4851  TGGAGCCAAC TCTAAAGTAT ATGCAAAATT TGGTGTAGAA ATCTATGTAG   4900
      ACCTCGGTTG AGATTTCATA TACGTTTTAA ACCACATCTT TAGATACATC

4901  CAGCTAAAAA AGGTGATCCA GATCCAGAAT CAAACTCAGC TTTGAAATTC   4950
      GTCGATTTTT TCCACTAGGT CTAGGTCTTA GTTTGAGTCG AAACTTTAAG

4951  GTTATCGACC GTGCTAAACA AGCCCAAGTG CCAAAACACA TTATC  4995
      CAATAGCTGG CACGATTTGT TCGGGTTCAC GGTTTTGTGT AATAG
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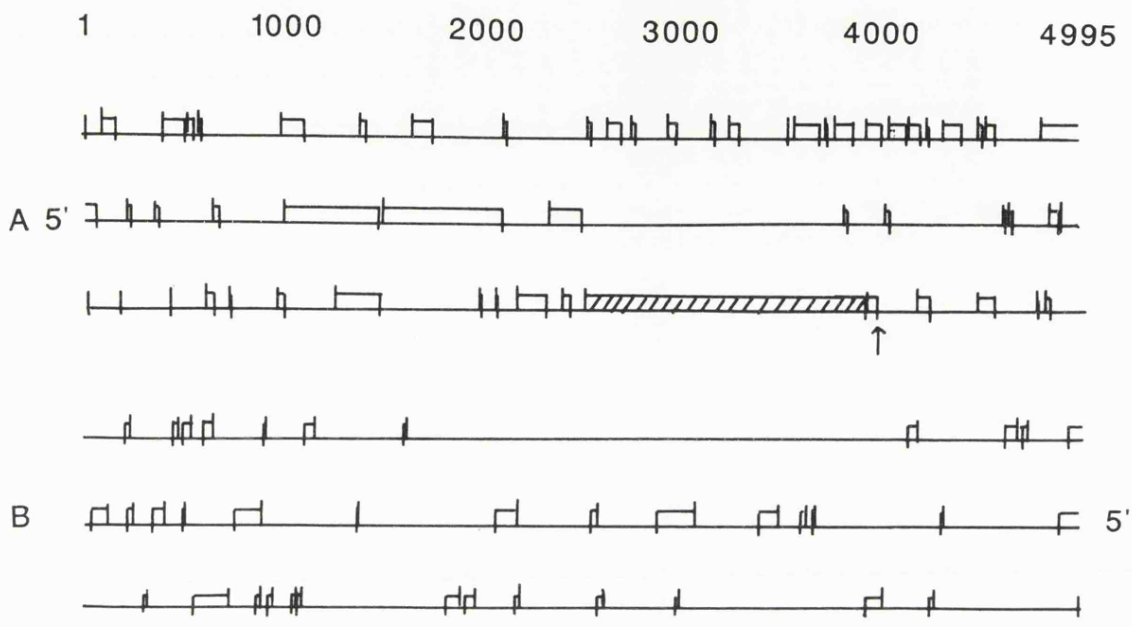


FIGURE 12. Open reading frames present in the DNA sequence of the insert in pJW451.

A. Top strand B. Bottom strand reading in the opposite direction. The 5' end of each strand is indicated. Vertical lines indicate start codons (⊥) or stop codons (⊣). Open boxes represent open reading frames. The numbers at the top of the figure refer to the position of nucleotides in the sequence shown in Fig. 11

The shaded box represents the pneumolysin coding region. The arrow indicates the position of a second in frame stop codon that may be used in *Sup*E strains of *E. coli*.

FIGURE 13. Nucleotide sequence of the pneumolysin gene showing the translated amino acid sequence in single letter code. Potential promoter sequences in the flanking DNA 5' of the pneumolysin coding sequence are underlined. The boxed sequence of GGAGG represents the putative Shine-Delgarno sequence or ribosome binding site. A second in frame stop codon in the 3' flanking sequence of the pneumolysin gene is underlined.

```
2310  CAACCTTGATTGACTTAGATAAGGTATTTATGTTGGATAATACGGTTATTCCGACTTCTT  2369

2370  ATCTAGCCAGACGGCGACGCAATGTCTCAGAAGAATTGTACGAGGAAATTTTGGATCACT  2429

2430  TAGTCCAACCACGGCTGATTTCGCTGAACAAGTCTGAGTTTATGCAACTCAATCCAGGAA  2489

2490  CTTATTAGGAGGTAGAAGATGGCAAATAAAGCAGTAAATGACTTTATACTAGCTATGAAT  2549
                          M  A  N  K  A  V  N  D  F  I  L  A  M  N

2550  TACGATAAAAAGAAACTCTTGACCCATCAGGGAGAAAGTATTGAAAATCGTTTCATCAAA  2609
      Y  D  K  K  K  L  L  T  H  Q  G  E  S  I  E  N  R  F  I  K

2610  GAGGGTAATCAGCTACCCGATGAGTTTGTTGTTATCGAAAGAAAGAAGCGGAGCTTGTCG  2669
      E  G  N  Q  L  P  D  E  F  V  V  I  E  R  K  K  R  S  L  S

2670  ACAAATACAAGTGATATTTCTGTAACAGCTACCAACGACAGTCGCCTCTATCCTGGAGCA  2729
      T  N  T  S  D  I  S  V  T  A  T  N  D  S  R  L  Y  P  G  A

2730  CTTCTCGTAGTGGATGAGACCTTGTTAGAGAATAATCCCACTCTTCTTGCGGTTGATCGT  2789
      L  L  V  V  D  E  T  L  L  E  N  N  P  T  L  L  A  V  D  R

2790  GCTCCGATGACTTATAGTATTGATTTGCCTGGTTTGGCAAGTAGCGATAGCTTTCTCCAA  2849
      A  P  M  T  Y  S  I  D  L  P  G  L  A  S  S  D  S  F  L  Q

2850  GTGGAAGACCCCAGCAATTCAAGTGTTCGCGGAGCGGTAAACGATTTGTTGGCTAAGTGG  2909
      V  E  D  P  S  N  S  S  V  R  G  A  V  N  D  L  L  A  K  W

2910  CATCAAGATTATGGTCAGGTCAATAATGTCCCAGCTAGAATGCAGTATGAAAAAATAACG  2969
      H  Q  D  Y  G  Q  V  N  N  V  P  A  R  M  Q  Y  E  K  I  T

2970  GCTCACAGCATGGAACAACTCAAGGTCAAGTTTGGTTCTGACTTTGAAAAGACAGGGAAT  3029
      A  H  S  M  E  Q  L  K  V  K  F  G  S  D  F  E  K  T  G  N

3030  TCTCTTGATATTGATTTTAACTCTGTCCATTCAGGTGAAAAGCAGATTCAGATTGTTAAT  3089
      S  L  D  I  D  F  N  S  V  H  S  G  E  K  Q  I  Q  I  V  N

3090  TTTAAGCAGATTTATTATACAGTCAGCGTAGACGCTGTTAAAAATCCAGGAGATGTGTTT  3149
      F  K  Q  I  Y  Y  T  V  S  V  D  A  V  K  N  P  G  D  V  F

3150  CAAGATACTGTAACGGTAGAGGATTTAAAACAGAGAGGAATTTCTGCAGAGCGTCCTTTG  3209
      Q  D  T  V  T  V  E  D  L  K  Q  R  G  I  S  A  E  R  P  L

3210  GTCTATATTTCGAGTGTTGCTTATGGGCGCCAAGTCTATCTCAAGTTGGAAACCACGAGT  3269
      V  Y  I  S  S  V  A  Y  G  R  Q  V  Y  L  K  L  E  T  T  S

3270  AAGAGTGATGAAGTAGAGGCTGCTTTTGAAGCTTTGATAAAAGGAGTCAAGGTAGCTCCT  3329
      K  S  D  E  V  E  A  A  F  E  A  L  I  K  G  V  K  V  A  P

3330  CAGACAGAGTGGAAGCAGATTTTGGACAATACAGAAGTGAAGGCGGTTATTTTAGGGGGC  3389
      Q  T  E  W  K  Q  I  L  D  N  T  E  V  K  A  V  I  L  G  G

3390  GACCCAAGTTCGGGTGCCCGAGTTGTAACAGGCAAGGTGGATATGGTAGAGGACTTGATT  3449
      D  P  S  S  G  A  R  V  V  T  G  K  V  D  M  V  E  D  L  I

3450  CAAGAAGGCAGTCGCTTTACAGCAGATCATCCAGGCTTGCCGATTTCCTATACAACTTCT  3509
      Q  E  G  S  R  F  T  A  D  H  P  G  L  P  I  S  Y  T  T  S

3510  TTTTTACGTGACAATGTAGTTGCGACCTTTCAAAACAGTACAGACTATGTTGAGACTAAG  3569
      F  L  R  D  N  V  V  A  T  F  Q  N  S  T  D  Y  V  E  T  K

3570  GTTACAGCTTACAGAAACGGAGATTTACTGCTGGATCATAGTGGTGCCTATGTTGCCCAA  3629
      V  T  A  Y  R  N  G  D  L  L  L  D  H  S  G  A  Y  V  A  Q

3630  TATTATATTACTTGGGATGAATTATCCTATGATCATCAAGGTAAGGAAGTCTTGACTCCT  3689
      Y  Y  I  T  W  D  E  L  S  Y  D  H  Q  G  K  E  V  L  T  P

3690  AAGGCTTGGGACAGAAATGGGCAGGATTTGACGGCTCACTTTACCACTAGTATTCCTTTA  3749
      K  A  W  D  R  N  G  Q  D  L  T  A  H  F  T  T  S  I  P  L

3750  AAAGGGAATGTTCGTAATCTCTCTGTCAAAATTAGAGAGTGTACCGGGCTTGCCTGGGAA  3809
      K  G  N  V  R  N  L  S  V  K  I  R  E  C  T  G  L  A  W  E

3810  TGGTGGCGTACGGTTTATGAAAAAACCGATTTGCCACTAGTGCGTAAGCGGACGATTTCT  3869
      W  W  R  T  V  Y  E  K  T  D  L  P  L  V  R  K  R  T  I  S

3870  ATTTGGGGAACAACTCTCTATCCTCAGGTAGAGGATAAGGTAGAAAATGACTAGGAGAGG  3929
      I  W  G  T  T  L  Y  P  Q  V  E  D  K  V  E  N  D  *

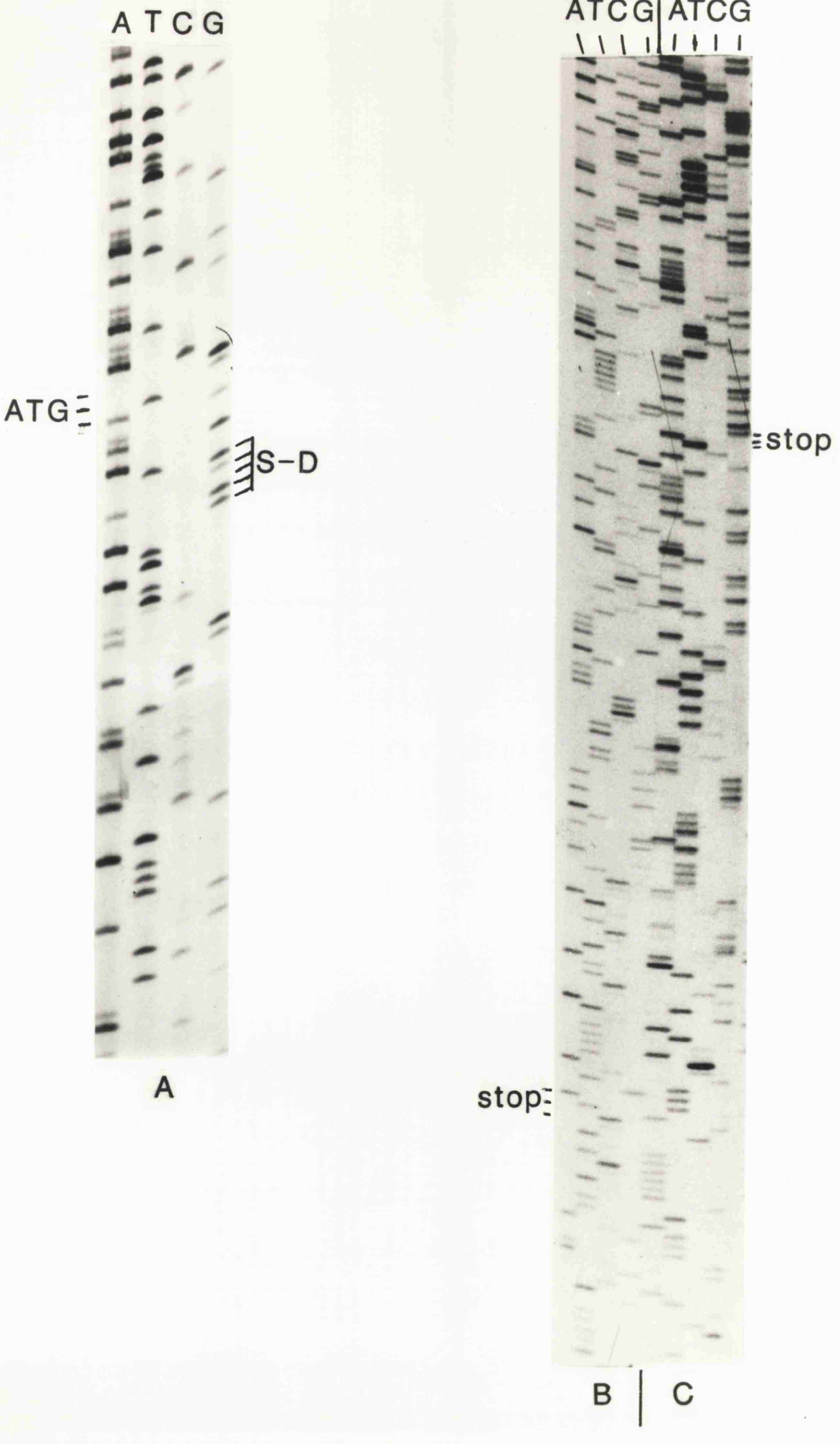
3930  AGAATGCTTGCGACAAAAAGAGGCGATGATCTCTCTGCGGATATCTGAAAGTTTATCTCT  3989
```

FIGURE 14. Autoradiographs of DNA sequences from the coding region of the pneumolysin gene.

A. Sequence of a randomly generated fragment showing the 5' end of the pneumolysin gene. The initiation codon ATG and the putative Shine-Delgarno sequence GGAGG are indicated.

B. Sequence of the 170 bp *Hpa*II-*Eco*RV restriction fragment that spans the 3' end of the coding region, reading from left to right on the bottom strand of the sequence, Fig 11.

C. The same fragment as in B, but reading from right to left on the top strand of the sequence, Fig 11. The stop codon, TAG, at position 3923 in the sequence, Fig. 11 is marked in both sequences.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S-D
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stop
B
C

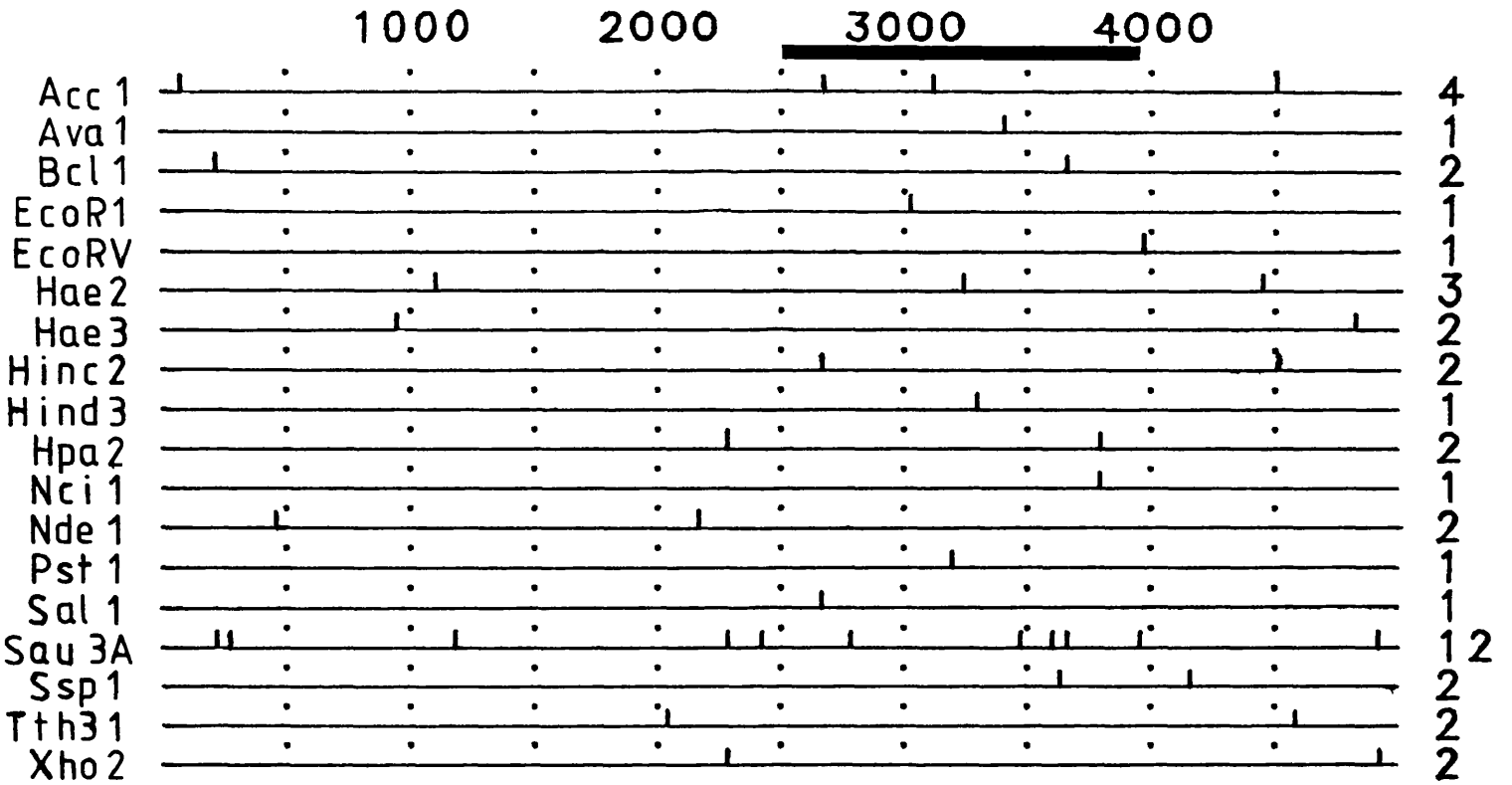

FIGURE 15. Restriction map of the insert present in pJW451.

Only selected restriction sites predicted from the sequence are shown.

The bar refers to the position of the pneumolysin coding sequence within the insert.

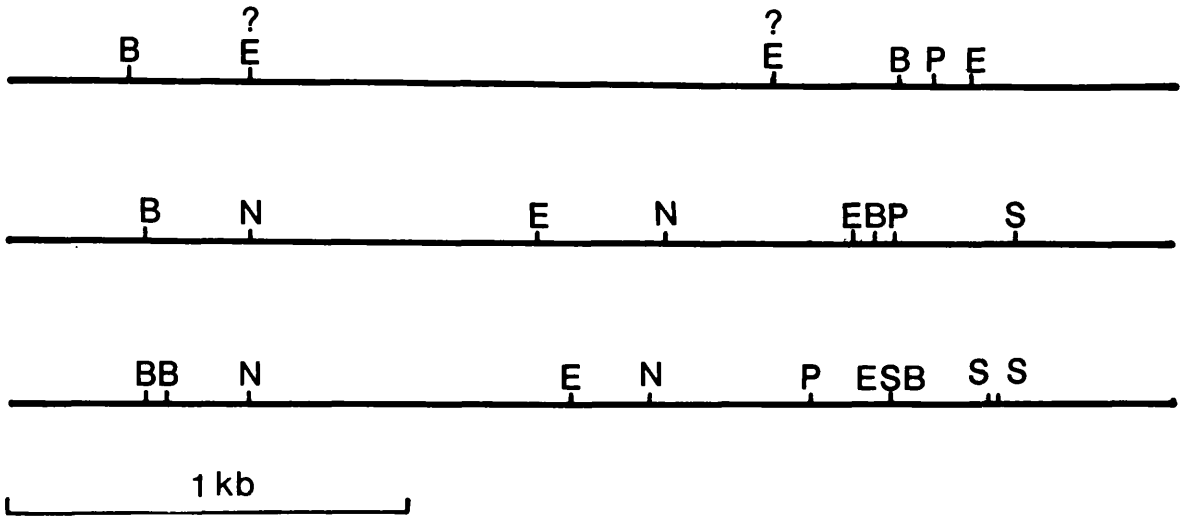

FIGURE 16. Restriction digest maps of the 3 kb *Hind*III insert present in pALV1.

A. Restriction map (Geoffroy and Alouf, personal communication). E (?) represent probable *Eco*RI sites.

B. Restriction map determined by restriction digests (this thesis)

C. Restriction map derived from the nucleotide sequence of the insert present in pALV1.

E = *Eco*RI, P = *Pst*I, B = *Bam*HI, N = *Nde*I, S = *Sph*I

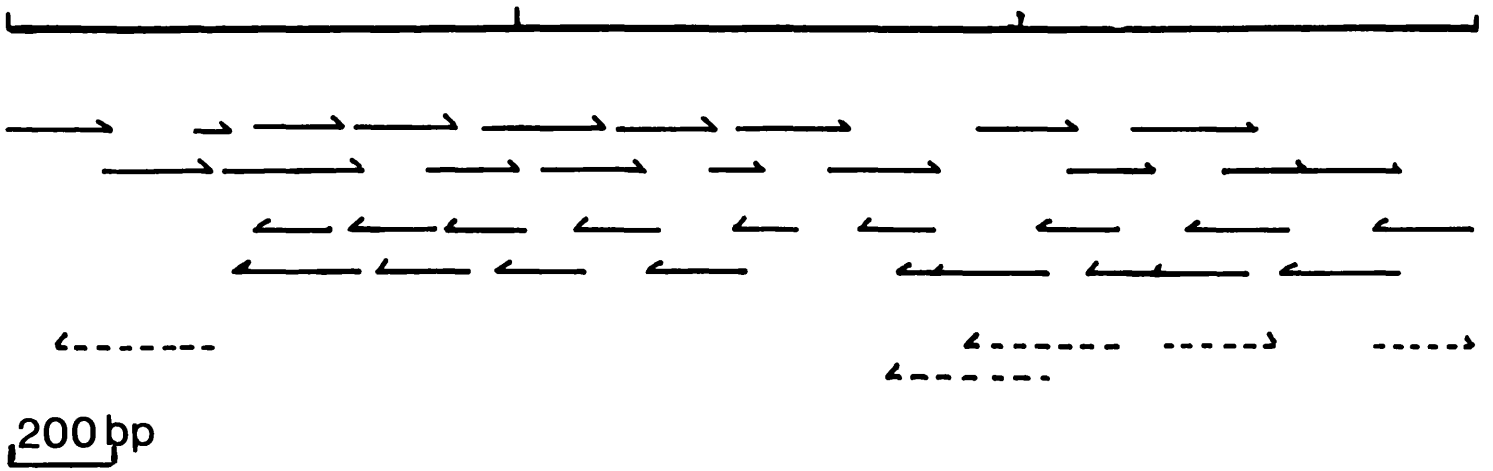

FIGURE 17. Compilation of the nucleotide sequence of the insert in pALV1.

A. 2910 bp sequence constructed from individual clones, solid lines indicate the sequence obtained from randomly generated clones and broken lines that sequence obtained from specific restriction fragments.

FIGURE 18. Complete DNA sequence of the 2910 bp insert present in pALV1. The numbers refer to the nucleotide positions.

```
   1  GGTATATATG GCAATCGTTC ATGGTCGTCC CCCACAGAAC AGCGATGCAT    50
      CCATATATAC CGTTAGCAAG TACCAGCAGG GGGTGTCTTG TCGCTACGTA

  51  TAACGCATCA TTTGCTGAAG GATGTGCGTA CGAACACAGT TCGCGCCGTT   100
      ATTGCGTAGT AAACGACTTC CTACACGCAT GCTTGTGTCA AGCGCGGCAA

 101  GCTCCGAATA CCCCTGGAGC CAAAGAAGCG CGATTGTCTT ATGAAGTAGC   150
      CGAGGCTTAT GGGGACCTCG GTTTCTTCGC GCTAACAGAA TACTTCATCG

 151  TGGTATGAGC GGAGATTTGA CGCTTGTACG AATACAACTT CATACCGGAC   200
      ACCATACTCG CCTCTAAACT GCGAACATGC TTATGTTGAA GTATGGCCTG

 201  GGCCACATCA AATACGGGTT CAGATGAAGA CAATCGGTTG TCCGTTGTAC   250
      CCGGTGTAGT TTATGCCCAA GTCTACTTCT GTTAGCCAAC AGGCAACATG

 251  GGAGATCAAA AGTACGGCGC CGAATGCAAT AAGCCTGGAC AGCAGATCGC   300
      CCTCTAGTTT TCATGCCGCG GCTTACGTTA TTCGGACCTG TCGTCTAGCG

 301  ACTATGGTCT GTCCGGCTGT CGTTTCCTCA TCCGACTGCG AAGGAAGTCA   350
      TGATACCAGA CAGGCCGACA GCAAAGGAGT AGGCTGACGC TTCCTTCAGT

 351  TGGACTTCTA TTCGAGACCG CCCGAGGAAT ATCCGTGGAA CTTATGGGAT   400
      ACCTGAAGAT AAGCTCTGGC GGGCTCCTTA TAGGCACCTT GAATACCCTA

 401  CCGTCTATCT ATGCATCCTG TTAATGTACA GGATCCCTGC CTATCCACCA   450
      GGCAGATAGA TACGTAGGAC AATTACATGT CCTAGGGACG GATAGGTGGT

 451  TGAAGTATTG TGATCACCTT ACTGGCTAGT AATCAGTCAT ATCACACAGC   500
      ACTTCATAAC ACTAGTGGAA TGACCGATCA TTAGTCAGTA TAGTGTGTCG

 501  ATCCTGTTTG TACGATCGCA TTGCGGAGGA ACTTACTTGC ATCTCTTCAA   550
      TAGGACAAAC ATGCTAGCGT AACGCCTCCT TGAATGAACG TAGAGAAGTT

 551  TGCGATTGTA CATAGGTGTA CGCCAATAAG CTCAGGTAAC GATTAAGCAT   600
      ACGCTAACAT GTATCCACAT GCGGTTATTC GAGTCCATTG CTAATTCGTA

 601  ATGTGCATAT CGAACTTGTT TTGAGCTTGA AAGGAGGGGA ATATGGAGAT   650
      TACACGTATA GCTTGAACAA AACTCGAACT TTCCTCCCCT TATACCTCTA

 651  GAGCAACAGA CGTTATGTAT CAATATGGAT GCTGATAATC GTAGTTATTC   700
      CTCGTTGTCT GCAATACATA GTTATACCTA CGACTATTAG CATCAATAAG

 701  TTTTACTCTC ATCCACCGCT TGTGCAATTG GATTCGCCAT TGCGTTCAGC   750
      AAAATGAGAG TAGGTGGCGA ACACGTTAAC CTAAGCGGTA ACGCAAGTCG

 751  GGCTCAGGAT CATTTCAATA CAGAGCCTGC AACCAACGAC AACCAACGGA   800
      CCGAGTCCTA GTAAAGTTAT GTCTCGGACG TTGGTTGCTG TTGGTTGCCT

 801  CCAAATCTCC GAAATCGAAT AATTCGATGC CTAATAGCAG CTCTCATTCA   850
      GGTTTAGAGG CTTTAGCTTA TTAAGCTACG GATTATCGTC GAGAGTAAGT

 851  ACATCTGCTA CCGATCATTC CATCTCTACA GCCGACATCC CGGCAAGCAC   900
      TGTAGACGAT GGCTAGTAAG GTAGAGATGT CGGCTGTAGG GCCGTTCGTG

 901  CACCGTTCCA AGCAATGAAA AAATAAAGCC CTATACCTAT CCAGCAAAAT   950
      GTGGCAAGGT TCGTTACTTT TTTATTTCGG GATATGGATA GGTCGTTTTA

 951  TCTTGCTGTA GGAGATATTA TGATGCACTC TCCTCAATTC CCTGCTTATC  1000
      AGAACGACAT CCTCTATAAT ACTACGTGAG AGGAGTTAAG GGACGAATAG

1001  TGAATAAGAA GACAGGCACC TACAATTTTA ACAACTATTT CACAAAGGTT  1050
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      ACTTATTCTT CTGTCCGTGG ATGTTAAAAT TGTTGATAAA GTGTTTCCAA

1051  AAGCCTATTC TCGAAGATGC AGATTGGTGT TGGGCAAATC TAGAGACTCC   1100
      TTCGGATAAG AGCTTCTACG TCTAACCACA ACCCGTTTAG ATCTCTGAGG

1101  ATTGCTTGGC GGAGAGAAGG TATACACTGG GTATCCGATG TCAATGCCCC   1150
      TAACGAACCG CCTCTCTTCC ATATGTGACC CATAGGCTAC AGTTACGGGG

1151  CCCAGAACTA GCAGATGCAC TGAAGTACGC CGGCTTCAAT ATCGTTACGA   1200
      GGGTCTTGAT CGTCTACGTG ACTTCATGCG GCCGAAGTTA TAGCAATGCT

1201  CTGCAAACAA TCATTCCATG GATCGATACG AAGCTGGAGC ATTGCGCACA   1250
      GACGTTTGTT AGTAAGGTAC CTAGCTATGC TTCGACCTCG TAACGCGTGT

1251  AGAGAAATGC TGAAGAAAAA AGGATTGGTG ACGAAGGGCA TCTCAGCTTC   1300
      TCTCTTTACG ACTTCTTTTT TCCTAACCAC TGCTTCCCGT AGAGTCGAAG

1301  ATTATGGGAG TCCAATCAAC CAACCTTATT TGAGAAAAAC GGAATACAAT   1350
      TAATACCCTC AGGTTAGTTG GTTGGAATAA ACTCTTTTTG CCTTATGTTA

1351  TAGGGATTCT TGCTTATACG TACGGTACAA ACGGAATTCC ACTTCCGAAG   1400
      ATCCCTAAGA ACGAATATGC ATGCCATGTT TGCCTTAAGG TGAAGGCTTC

1401  CAAAAACCGT ATCTCGTATC TCTCATTGAT GAGAAGCGTA TGATTCAGGA   1450
      GTTTTTGGCA TAGAGCATAG AGAGTAACTA CTCTTCGCAT ACTAAGTCCT

1451  TATTCAAAAA ACGAAAAAGG CTGGTGCAGA TGTTGTAGCG ATTGCATTGC   1500
      ATAAGTTTTT TGCTTTTTCC GACCACGTCT ACAACATCGC TAACGTAACG

1501  ACTTCGGTAC AGAATATGAG CATACACCGA ATCAAGATCA GATCCGACTA   1550
      TGAAGCCATG TCTTATACTC GTATGTGGCT TAGTTCTAGT CTAGGCTGAT

1551  GCGCAGATCT TATCCAGTCT GGAGCAGATA TTATATTAGG CTCTCATCCA   1600
      CGCGTCTAGA ATAGGTCAGA CCTCGTCTAT AATATAATCC GAGAGTAGGT

1601  CATGTCATCC AGCCATATGA GCGTATTACT GTAAGAGAGC CCGACGGCTC   1650
      GTACAGTAGG TCGGTATACT CGCATAATGA CATTCTCTCG GGCTGCCGAG

1651  CAAGCGTGAT GGGCTAATTA TCTATTCTAT GGGGAACTTT ATATCCAATC   1700
      GTTCGCACTA CCCGATTAAT AGATAAGATA CCCCTTGAAA TATAGGTTAG

1701  AGACAGGCAA TGGCACGAAT ATAGGAGTAA TCTTCGGTGT GGGAAATTAA   1750
      TCTGTCCGTT ACCGTGCTTA TATCCTCATT AGAAGCCACA CCCTTTAATT

1751  CAAACACATG CCXGAAGGTA CAATCGAGCT AAAAAAATAA CAACAATTCC   1800
      GTTTGTGTAC GGXCTTCCAT GTTAGCTCGA TTTTTTTATT GTTGTTAAGG

1801  AACATGGGTG CATATTGACG GTGCTCCGAC AAGCGCAAAT ATCGCGTACT   1850
      TTGTACCCAC GTATAACTGC CACGAGGCTG TTCGCGTTTA TAGCGCATGA

1851  CCCATTGGAG CAAACAATTA AAGCCCGTTC TGACAAACGG TAAGCACAAA   1900
      GGGTAACCTC GTTTGTTAAT TTCGGGCAAG ACTGTTTGCC ATTCGTGTTT

1901  GCAGTATCAG GCTATGAAAA AGATGCTCAA CAGCACATAC AGCCATCTGG   1950
      CGTCATAGTC CGATACTTTT TCTACGAGTT GTCGTGTATG TCGGTAGACC

1951  CATCAAGAGC GGCTGTTCCC GTTATTCTCC CTGCCCCAGT CTCACCTTAG   2000
      GTAGTTCTCG CCGACAAGGG CAATAAGAGG GACGGGGTCA GAGTGGAATC

2001  CTATTGCCCA GCACGTACAG GCTCTGACGC TGCAGCCGTC TCTTTCTCAA   2050
      GATAACGGGT CGTGCATGTC CGAGACTGCG ACGTCGGCAG AGAAAGAGTT
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2051  CAGCAGACGG TTGCGGCGAT TGAGTCAGCT TGCCAAGCAA GAGAATAAGT   2100
      GTCGTCTGCC AACGCCGCTA ACTCAGTCGA ACGGTTCGTT CTCTTATTCA

2101  AGCTGCTGAT TGTGTGTAGA GATACGATGC AAGATGCCAT TCCAATAATT   2150
      TCGACGACTA ACACACATCT CTATGCTACG TTCTACGGTA AGGTTATTAA

2151  CTCGCGAATC GTTACTCCGC GGCGAATTTC CTGCATGCCG AATTCTGTAG   2200
      GAGCGCTTAG CAATGAGGCG CCGCTTAAAG GACGTACGGC TTAAGACATC

2201  CTTGGATCCA CACGATTCGA CGATCATCCT GATCCCGCTC GCGAATAATT   2250
      GAACCTAGGT GTGCTAAGCT GCTAGTAGGA CTAGGGCGAG CGCTTATTAA

2251  AGTGCATTCT TCTCCATGCG ATCCATCAAT GTTGTAATTG CGGCCGGGGT   2300
      TCACGTAAGA AGAGGTACGC TAGGTAGTTA CAACATTAAC GCCGGCCCCA

2301  CGTAGCTAGA TGAGGCAATA AATCAGAGGG CTTGACCTTT CCATATTCCG   2350
      GCATCGATCT ACTCCGTTAT TTAGTCTCCC GAACTGGAAA GGTATAAGGC

2351  CAATTCGCTC CAACACGGTT AACTGACTCT CTGTCAGACT TGGCGAAAGG   2400
      GTTAAGCGAG GTTGTGCCAA TTGACTGAGA GACAGTCTGA ACCGCTTTCC

2401  GCTTCGTCCA TCATCAACTT ATAATCCTTG GATAATTTCG ACCATAGCTT   2450
      CGAAGCAGGT AGTAGTTGAA TATTAGGAAC CTATTAAAGC TGGTATCGAA

2451  GGCAAATTCG GCTGCATGCA TGCAAGCGAC TCCCCTTTAT TCCCATGTCG   2500
      CCGTTTAAGC CGACGTACGT ACGTTCGCTG AGGGGAAATA AGGGTACAGC

2501  ATCTACAAAC AACTAAATTA TACCACATTT TTCTAGTCGG GATCATCCCT   2550
      TAGATGTTTG TTGATTTAAT ATGGTGTAAA AAGATCAGCC CTAGTAGGGA

2551  CTTCTGAATG TAGTTTCATC CCAAACTCAT ATCGCTTCTT GTTACGATTT   2600
      GAAGACTTAC ATCAAAGTAG GGTTTGAGTA TAGCGAAGAA CAATGCTAAA

2601  GGAACCGTGC AATCATTTCC CACATATAAT AACATATAGG CATTTGCCAT   2650
      CCTTGGCACG TTAGTAAAGG GTGTATATTA TTGTATATCC GTAAACGGTA

2651  ACATTCTCTA ACATTTACAT TGCTTCATAC TGCGAGGAGG GAACACAATG   2700
      TGTAAGAGAT TGTAAATGTA ACGAAGTATG ACGCTCCTCC CTTGTGTTAC

2701  GATCAGGCCA AAGCCGACAT TTTTCAAAAA GCAGTGAAAA TCGGGATCAT   2750
      CTAGTCCGGT TTCGGCTGTA AAAAGTTTTT CGTCACTTTT AGCCCTAGTA

2751  AAAAGACCCA CAGTGGGCAG AGCGGCTCGA TGAGCCCGTC CCATTGTGGG   2800
      TTTTCTGGGT GTCACCCGTC TCGCCGAGCT ACTCGGGCAG GGTAACACCC

2801  TCCTATTAGA AATTGTCATT ACCCTGCACG ATGTGCTGAA TCCACCGCAC   2850
      AGGATAATCT TTAACAGTAA TGGGACGTGC TACACGACTT AGGTGGCGTG

2851  AAGCCCTTCG ATTAAGAAGA TGGCGGTTAT TTACAATTGT ATCAACCTAT   2900
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2901  AATGACTGTA   2910
      TTACTGACAT
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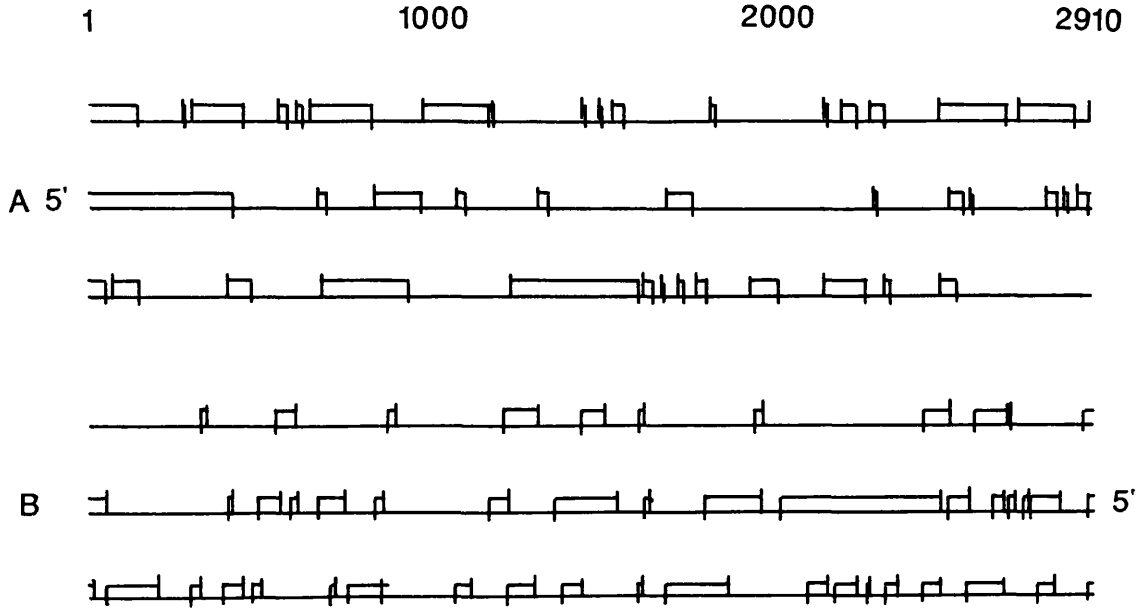


FIGURE 19. Open reading frames present in the DNA sequence of the insert in pALV1.

A. Top strand B. Bottom strand reading in the opposite direction. The 5' end of each strand is indicated. Vertical lines indicate start codons (⊥) or stop codons (⊥). Boxes represent open reading frames. The numbers at the top of the figure refer to the position of nucleotides in the sequence shown in Fig. 18.

No open reading frames large enough to encode a protein of 63 kD were observed.

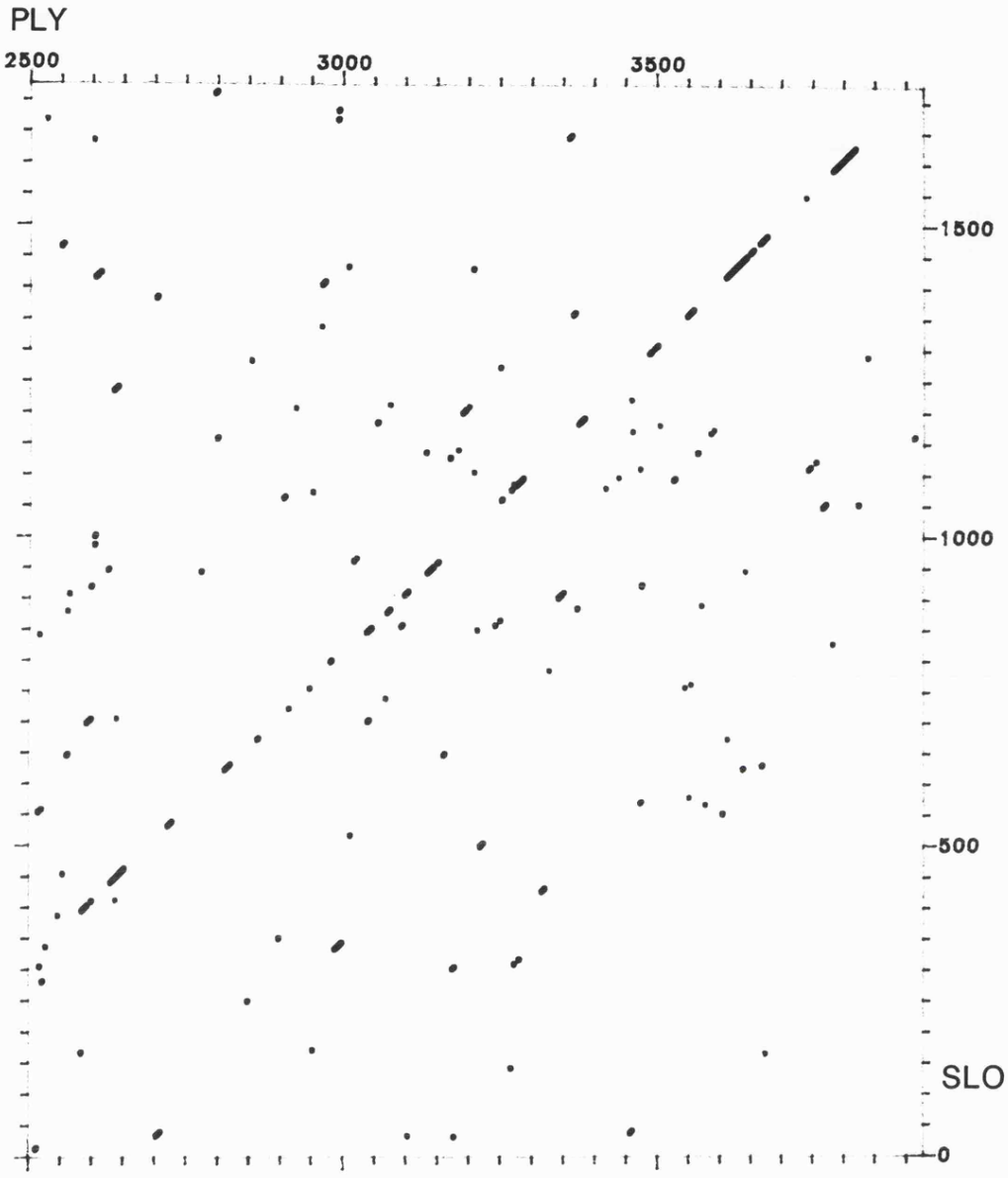


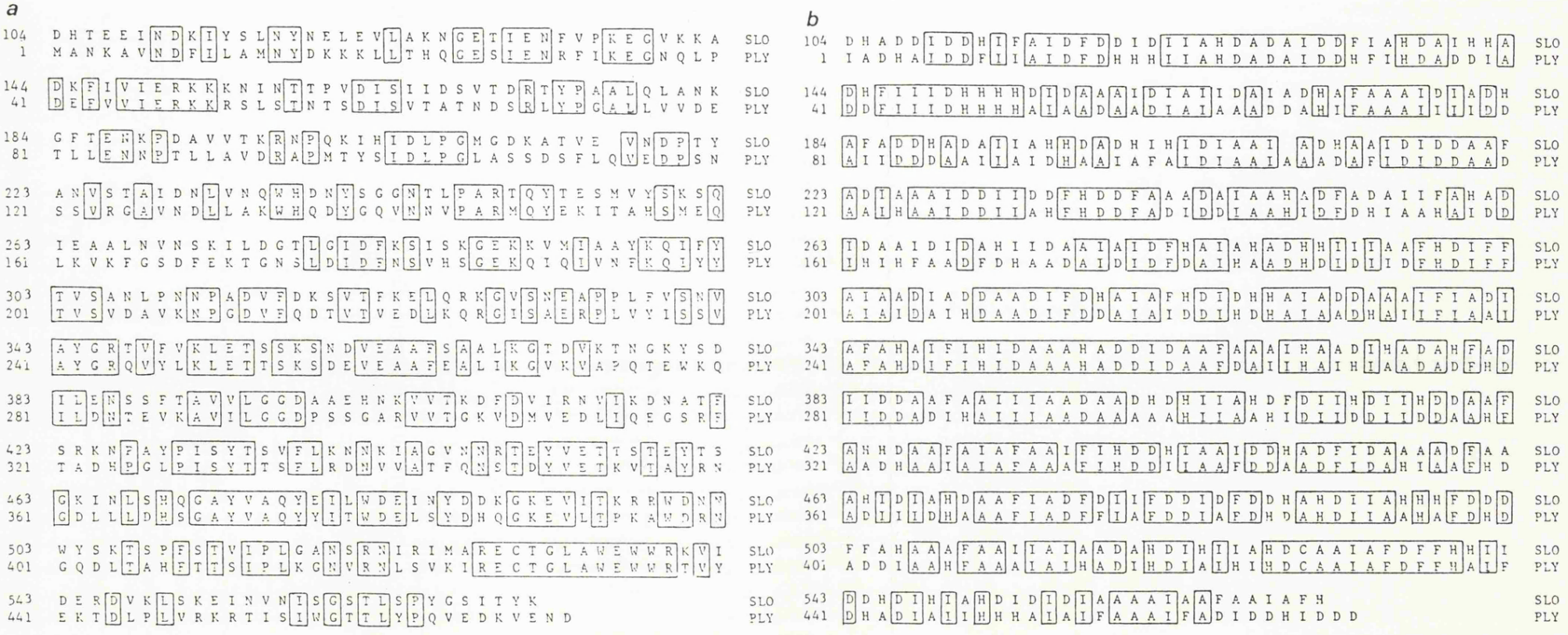
FIGURE 20. Dot matrix comparison of the nucleotide sequences of the pneumolysin, (Y axis), and SLO gene, (X axis). The match was carried out at a stringency of 66% (14 matches in a window of 21 nucleotides).

FIGURE 21. Comparison of the predicted amino acid sequences of pneumolysin and SLO.

The sequences were aligned by a best-fit program (Smith and Waterman 1981).

a alignment of identical residues, represented by single letter code. The alignment, which includes a gap of a single base in the sequence of SLO, shows 42% homology.

b Alignment of similar residues; A= neutral, weakly hydrophobic (P,A,G,S,T); D= hydrophilic, acid amine (Q,N,E,D); H= hydrophilic, basic (H,K,R); I= hydrophobic (L,I,V,M); F= hydrophobic, aromatic (F,Y,W); C= cysteine. Homology is 62%.


a
b

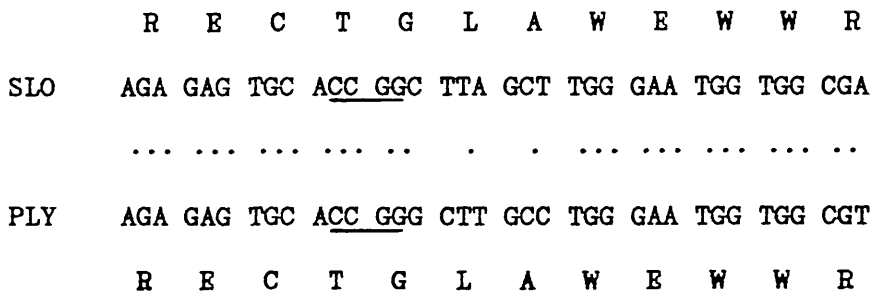


FIGURE 22. DNA and amino acid sequence of the region surrounding the single cysteine in pneumolysin (PLY) and SLO. The *Hpa*II site conserved in both DNA sequences is shown underlined. Nucleotides common to both sequences are marked with a dot.

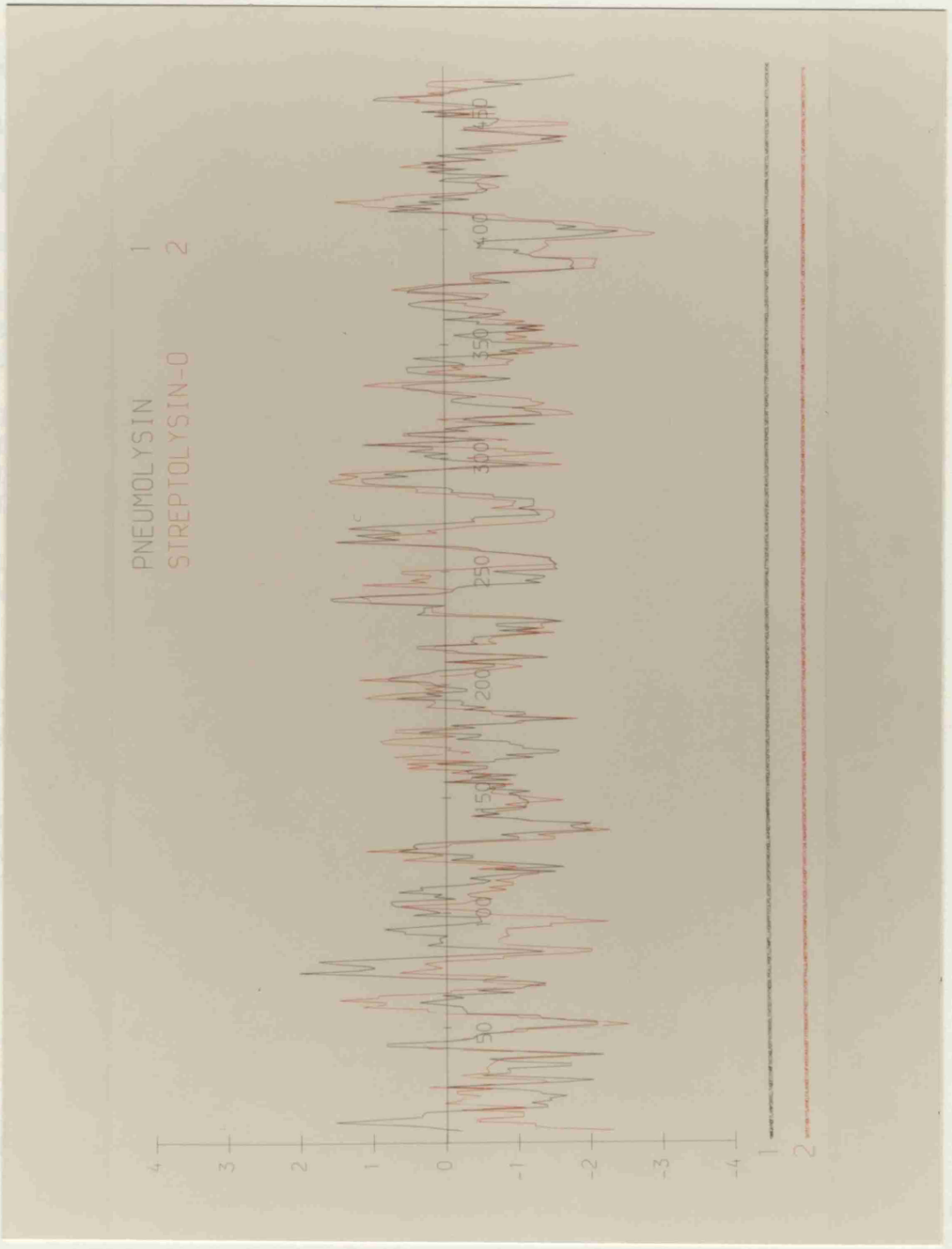


FIGURE 23. Hydropathic indexes of pneumolysin and the low molecular weight form of SLO.

Positive values indicate hydrophobic domains. The black trace represents pneumolysin and the red trace SLO.

TABLE 8

Amino acid compositions of pneumolysin, SLO and perfingolysin.

AMINO ACID	PLY[a]		SLO[b]		PER[c]		PRO[d]
	no.	%	no.	%	no.	%	%
CYSTEINE (C)	1	0.2	1	0.2	1	0.2	
PROLINE (P)	17	3.6	17	3.6	22	4.7	4.1
SERINE (S)	35	7.4	38	8.0	43	9.2	6.0
THREONINE (T)	36	7.6	36	7.6	33	7.2	5.9
ALANINE (A)	29	6.2	33	7.0	32	6.9	9.5
GLYCINE (G)	26	5.5	24	5.1	25	5.45	8.0
ASPARAGINE (Q)	25	5.3	9	1.9	76	16.42	9.9
ASPARTIC A.(N)	36	7.6	42	8.9	"	" "	"
GLUTAMINE (E)	30	6.4	31	6.6	38	8.4	10.0
GLUTAMIC A.(D)	21	4.5	29	6.2	"	" "	"
HISTIDINE (H)	9	1.9	5	1.0	6	1.3	2.0
ARGININE (R)	20	4.2	16	3.4	11	2.5	4.1
LYSINE (K)	31	6.6	25	5.3	43	9.2	5.7
METHIONINE (M)	6	1.3	4	0.8	5	1.0	2.5
ISOLEUCINE (I)	23	4.9	34	7.2	31	6.7	5.7
LEUCINE (L)	40	8.5	25	5.3	26	5.5	8.9
VALINE (V)	45	9.6	40	8.5	38	8.3	7.1
PHENYLALANINE (F)	14	3.0	15	3.2	13	2.8	3.8
TYROSINE (Y)	19	4.0	21	4.5	22	4.6	3.3
TRYPTOPHAN (W)	8	1.7	7	1.5	-	-	1.3

a The pneumolysin sequence was determined from the translation of the nucleotide sequence determined in this thesis

b The SLO sequence was determined from the translation of the nucleotide sequence (Kehoe *et al* 1987).

c The perfringolysin sequence was determined from chemical analysis of the purified protein (Yamakawa *et al* 1977).

d Prokaryotic consensus amino acid composition (Doolittle 1986).

CHAPTER 5

MUTAGENESIS OF THE PNEUMOLYSIN GENE

5.i INTRODUCTION

As discussed previously, the accumulated knowledge of the biological properties of sulphydryl-activated cytolysins has so far failed to resolve their mechanism of action. The similarity of pneumolysin and SLO amino acid sequences has focused attention on domains common to each protein, which may be important in their biological activity. The most significant region being the 12 amino acid sequence, surrounding the single cysteine, present in both proteins. To investigate the relative importance of the cysteine residue and these sequences in the activity of pneumolysin two separate experiments, involving mutagenesis of the pneumolysin gene, were undertaken. The first attempted to determine the extent of functional similarity between the sequences of pneumolysin and SLO, and the second was to determine the importance of the cysteine residue in the biological activity of the toxin

5.ii CONSTRUCTION OF A HYBRID PROTEIN OF PNEUMOLYSIN AND SLO

It has been assumed, from many observations of the biological activities and immunological cross reactivities of sulphydryl-activated cytolysins, that these toxins possess significant structural similarities. The high level of homology observed between the amino acid sequences of pneumolysin and SLO has therefore indicated the possibility that chimeric proteins may possess biological properties similar to those of the parent proteins. To test this hypothesis, a fusion of the pneumolysin and SLO genes was constructed which would direct the production of a chimeric protein of 469 amino acids in length, containing the first 428 amino acids from pneumolysin with the last 41 amino acids from SLO.

5.ii a Construction of the Hybrid Gene

The scheme for the production of a pneumolysin-SLO fusion is outlined in Fig 24, and made use of the conserved *Hpa*II restriction site in each gene which lies adjacent to the cysteine codon. The required fragment from the pneumolysin gene was isolated as follows. Plasmid pJW208 was digested to completion with *Kpn*I and *Sph*I, and a 2.2 kb fragment carrying the pneumolysin gene was isolated from a preparative agarose gel. This 2.2 kb fragment was digested with *Hpa*II to generate a 1.5 kb *Kpn*I-*Hpa*II fragment carrying the coding sequence sequence of the first 428 amino acids of pneumolysin.

The SLO derived fragment was isolated from pMK205 as described below. Plasmid pMK205 was digested with *Hae*III and a 700bp fragment carrying the 3' end of the SLO gene was purified. This 700 bp *Hae*III fragment was digested with *Hpa*II and a 250 bp fragment carrying the coding sequence of the last 41 amino acids of SLO was isolated from a polyacrylamide gel.

The pneumolysin and SLO DNA fragments were ligated together and the products of the ligation analysed by agarose gel electrophoresis. A population of ligated fragments were observed, which were digested with *Kpn*I and *Hae*III to obtain the required fusion of pneumolysin and SLO sequences. Since there was insufficient DNA to purify from an agarose gel, the population of restriction fragments were ligated into pUC18 that had been digested with *Kpn*I and *Hinc*II. The products of the ligation were transformed into *E. coli* JM83 and cells plated on Blood-agar containing 100 μg/ml ampicillin and X-gal. Transformants producing white colonies were examined for haemolytic activity. After overnight growth no zones of haemolysis were

observed around white colonies. After a further eight hours incubation at 37°C, faint zones of haemolysis were observed around most white colonies.

5.ii b Analysis of Recombinants

The low haemolytic activity of the recombinants indicated that either the hybrid protein was less active than pneumolysin or SLO, or that the incorrect construct had been produced. The integrity of the construct was investigated as follows.

Plasmid DNA from a pUC18 recombinant possessing weak haemolytic activity was prepared and digested with *Sph*I and *Sst*I, *Eco*RI, *Hind*III, *Nde*I or *Hinc*II and the fragments separated by agarose gel electrophoresis. The sizes of the fragments obtained indicated that the construct, designated pCHI8, had the correct restriction digest pattern predicted for the gene fusion (Fig 25). The sequence of the fusion site was determined by DNA sequence analysis of the insert in pCHI8. A 1.7 kb *Sph*I-*Sst*I fragment carrying the insert from pCHI8 was isolated and ligated into M13mp18 digested with *Sph*I and *Sst*I. Recombinant 'phage DNA was prepared from white plaques and the sequence of the insert was determined from the universal primer site. The sequence (Fig 26), indicated that the gene fusion outlined in Fig 24. had been correctly constructed.

5.ii c Analysis of the Haemolytic Product of pCHI8

The nature of the haemolytic polypeptide encoded by the gene fusion was investigated. Plasmid DNA of pCHI8, and a similar plasmid, designated pCHI9, which differed only in the orientation of the

insert, were purified by bouyant density centrifugation through CsCl. Polypeptides encoded by the two plasmids were analysed in an *E. coli* cell free *in-vitro* transcription translation system. The products of the reaction were separated by SDS-PAGE and polypeptides visualised by fluorography (Fig 27). In both pCHI8 and pCHI9 polypeptides of approximately 53 kD were synthesised in addition to those encoded by the vector. These polypeptides were of a similar size to those produced by pJW159 and pJW208 which carry the pneumolysin gene and implies that these are the fusion proteins of pneumolysin and SLO. The greater production, *in vitro*, of the 53 kD protein from pCHI9 is consistent with the larger zones of haemolysis observed around colonies of *E. coli* MC1061(pCHI9) when compared to *E. coli* MC1061 (pCHI8).

The production of fusion proteins *in vivo* was examined by western blotting of crude cell free extracts of *E. coli* MC1061 harbouring pCHI8 and pCHI9. Proteins present in crude cell free extracts were separated by SDS-PAGE and transferred to nitrocellulose. Bound proteins were probed with anti-pneumolysin IgG conjugated to horseradish peroxidase. The presence of proteins cross-reactive with anti-pneumolysin IgG were visualised by the addition of the chromogen 4-chloro-1-naphthol. Proteins of about 53 kD, that were cross-reactive with anti-pneumolysin antibodies, were identified in both extracts pCHI8 and pCHI9 (Fig 28).

5.ii d Specific Activity of the Chimeric Protein

Cell-free extracts of *E. coli* MC1061(pCHI9) were prepared and were found to contain a haemolytic activity of only 40 HU/ml of cell-free extract. This low activity precluded analysis of the properties of

the fusion protein as far as neutralisation by specific antibody or the inhibition of the haemolytic activity by cholesterol was concerned. The low activity also complicated the purification of the chimeric protein, since it was not possible to screen each fraction for the presence of haemolytic activity. It was necessary therefore to determine the amount of chimeric protein in crude extracts using a capture ELISA. The amount of antigen in crude extracts of *E. coli* MC1061 (pCHI9) was calculated to be 50 μg/ml. This calculation assumes that the affinity of the anti-pneumolysin IgG is the same for the fusion protein as for the native toxin. The specific activity of the chimeric protein was calculated to be approximately 1x 10^3 HU/mg of protein, some three orders of magnitude lower than native or recombinant pneumolysin.

5.iii SITE DIRECTED MUTAGENESIS OF THE PNEUMOLYSIN GENE

The importance of cysteine residues in the activity of sulphydryl-activated cytolysins has been recognised for many years, though the role of the residue remains to be clarified. The single cysteine in pneumolysin could therefore be regarded as essential in the activity of this toxin. To explore the role of the cysteine residue in the activity of pneumolysin, a series of modified toxins were produced, by oligonucleotide-mediated site-directed mutagenesis, in which the cysteine residue had been substituted by an amino acid of not too dissimilar structure. The advantage of this technique is that it produces a homologous population of modified toxin containing a defined change and circumvents many of the problems encountered with chemical modifications of the cysteine residue.

The method used for the production of oligonucleotide site directed mutations of the pneumolysin gene was the single primer method (Zoller and Smith 1982). The gene to be mutated was cloned into the single stranded bacteriophage vector M13. The required codon change was included in a synthetic oligonucleotide complementary to the sequence present in the M13 recombinant. The oligonucleotide was annealed to single stranded recombinant DNA and the second strand synthesised *in-vitro*. Duplex DNA is then transfected into *E. coli* and recombinant plaques isolated. DNA from recombinant 'phage were screened to identify the presence of mutant sequences.

5.iii a Synthesis of Mutant Oligonucleotides

The scheme for the production of the oligonucleotide site directed mutants is shown in Fig 29. Oligonucleotides that would direct the changes from a cysteine to serine and from cysteine to glycine were identified. Using computer based comparison programs (Staden 1980, Deveraux *et al* 1984), the only homology observed between these two oligonucleotides and the insert in pJW158 was with the complementary sequence surrounding the cysteine codon. Oligonucleotides were synthesised by John Keyte, Department of Biochemistry and purified as described. The M13 recombinant, designated M13.JW158, was constructed by inserting a 1.7 kb *Sph*I-*Sst*I fragment carrying the pneumolysin gene, from pJW158, into M13 mp18 cut with *Sph*I-*Sst*I.

5.iii b Oligonucleotide Site-Directed Mutagenesis of the Pneumolysin Gene

The synthetic oligonucleotides were phosphorylated and annealed to single stranded DNA of M13.JW158. As a control, a similar reaction was set up containing no primer. The synthesis of the second strand was carried out *in-vitro* and the products of the extension ligation reaction were analysed by agarose gel electrophoresis for the production of RF DNA (Fig 30). The results indicated that some RF DNA was produced by all extension ligation reactions, but in insufficient amounts to purify from the gel. Therefore, the whole reaction mix was tranfected into *E. coli* TG1 and plaques isolated on minimal media. *E.coli* TG1 was used since it does not contain the repair system for hemi-methylated DNA, thus enriching the recovery of mutant M13 genomes. The number of plaques obtained using mutant primers was only slightly higher than the number obtained in the no primer control, which indicated that many of the plaques arising from the primed extention-ligation reactions were due to wild type phage. Despite this, plaques were analysed for haemolytic activity, presuming that mutant genomes would direct the production of modified pneumolysin with reduced haemolytic activity. From each transfection 250 clear plaques were picked onto both minimal agar and L-agar. Six plaques from the no primer control were also picked, as positive controls. All plates were incubated at 37°C overnight. The minimal plates were stored at 4°C. Colonies growing on the L-agar plates were screened by blood overlay for haemolytic activity. The plates were incubated at 37°C and examined for haemolytic activity every 5 minutes. Those colonies exhibiting a lower haemolytic activity, or devoid of haemolysis, were noted. The majority of colonies, as expected, exhibited full haemolytic activity.

Single stranded DNA was prepared from those colonies identified as causing a decreased haemolysis. The DNA from ten potential cysteine to glycine mutants and twenty potential cysteine to serine mutants were screened using DNA sequence reactions. Potential mutants were identified using either a single dideoxy A or T reaction. In the A track cysteine to glycine mutants would be identified by the loss of a band in the autoradiographs at a position corresponding to the cysteine codon, whilst serine mutants would be identified by the appearance of a band in the T track at the same position.

One mutant from the glycine substitution reaction and 11 mutants from the serine substitution reaction were originally identified (Fig 31). A mutant phage carrying the cysteine to glycine change, designated M13.GLY428 and one of the 'phage carrying the cysteine to serine change, designated M13.SER428 were selected for further analysis and subjected to two rounds of plaque purification (Fig 32). The complete DNA sequence of the inserts present in M13.SER428 and M13.GLY428 were determined using oligonucleotides that were complementary to sequences, approximately 250 bp apart, present on the insert in M13.JW158. The sequence of inserts in M13.GLY428 and M13.SER428 were identical to that determined for the wild type pneumolysin gene, with the exception of the base change introduced at position 3789. The DNA sequence of the region surrounding the base change in both mutant genes is shown (Fig 33).

5.iii c Haemolytic Activity of Modified Toxins

The changes in the activity of the modified proteins, designated S^{428} pneumolysin or G^{428} pneumolysin, brought about by substitution of the cysteine residue by serine or glycine respectively were

assessed. Haemolytic activities of crude cell extracts of *E. coli* TG1(M13.SER428) and *E. coli* TG1(M13.GLY428) was determined. The cell extract of *E. coli* TG1(M13.SER428) contained 1.2x 10^4 HU/ml, which was approximately 5 % the activity of cell extracts of *E. coli* TG1 (M13.JW158) (Table 9). In contrast, the haemolytic activity in cell extracts of *E. coli* TG1(M13.GLY428) was 800 HU/ml, which represented about 0.3% of the activity of the cell exracts containing wild type pneumolysin (Table 9). Crude cell extracts of *E.coli* TG1 producing either ^{S428} pneumolysin or ^{G428} pneumolysin lost no activity upon storage at 4°C, nor was the haemolytic activity raised by the addition of DTT (Table 9). The haemolytic activity of both modified toxins was neutralised by anti-pneumolysin antibodies and inhibited by cholesterol (Table 9).

The observation that both ^{S428} pneumolysin and ^{G428} pneumolysin, appear to possess a reduced haemolytic activity must be considered with some caution when assigning actual values to the activities obtained from crude cell extracts. At this time there was no information available concerning the expression of the mutant genes or the stability of the modified pneumolysin proteins. To assess the actual changes in the activity of modified toxins brought about by the substitution of the cysteine residue it was necessary to purify both the modified toxins.

5.iii d Purification and Specific Activity of Modified Toxins

To facilitate purification of the two modified toxins, the inserts present in M13.SER428 and M13.GLY428 were sub-cloned into pUC18. A 1.7 kb fragment was isolated from RF DNA of M13.SER428 and M13.GLY428 by a *Sph*I-*Sst*I double digest. 1.7 kb fragments carrying either

mutant gene were ligated separately into pUC18 previously digested with *Sph*I and *Sst*I. The ligation products were transformed into *E. coli* MC1061 and cells plated out on Blood agar containing 100 μg/ml ampicillin. Haemolytic recombinants from each transformation were identified. Plasmid DNA was prepared from these transformants, recombinants carrying either the glycine mutation or the serine mutation were designated pGLY008 and pSER008 respectively.

Purification of ^{S428} pneumolysin and ^{G428} pneumolysin from crude cell-extracts was undertaken using a protocol developed for the the purification of pneumolysin using HPLC (Mitchell *et al*, in preparation). Each crude cell-extract was subjected to hydrophobic interaction column chromatography and the collected fractions analysed for haemolytic activity. Those fractions exhibiting haemolytic activity were pooled and subjected to a second hydrophobic interaction column chromatography. The modified toxins were eluted from the column with water. The fraction corresponding to the protein peak was collected, purified ^{G428} pneumolysin was eluted in a single fraction, whilst ^{S428} pneumolysin was collected in two fractions, (f.1) and (f.2). The haemolytic activity and protein concentration of each fraction was determined (Table 10). The specific activity of ^{G428} pneumolysin was 9.2×10^3 HU/mg of protein. The activity of ^{S428} pneumolysin (f.1) and (f.2) was 1.9×10^4 HU/mg and 1.1×10^4 HU/mg of protein respectively. The lower specific activity of ^{S428} pneumolysin in f.2 may be due to the presence of contaminating proteins (see Figure 34). These results indicate that both the modified toxins are of a similar specific activity, at around 1% the specific activity of wild type pneumolysin.

5.iii e Western Blot analysis of Modified Toxins

Crude cell extracts of *E. coli* MC1061(pSER008) and *E. coli* MC1061 (pGLY008), purified ^{G428} pneumolysin, ^{S428} pneumolysin (f.1 and f.2) and wild type recombinant pneumolysin were subjected to SDS-PAGE. When stained, the gel (Fig 34 *A*) showed that the purified modified toxins were of the same size as wild type recombinant pneumolysin. No other proteins were observed in the ^{G428} pneumolysin or ^{S428} pneumolysin sample (f.1), whilst ^{S428} pneumolysin (f.2) was contaminated with other proteins. This implies that the specific activity of ^{S428} pneumolysin (f.1) is probably the more accurate. The proteins from a similar gel to that seen in Fig 34 *A* were transferred to nitrocellulose and probed with anti-pneumolysin IgG, the resulting blot (Fig 34 *B*) showed the cross reactivity of purified modified toxins with anti-pneumolysin IgG.

5.iii f Estimation of the Specific Activity of Modified Toxins in Crude Cell-Extracts.

It was observed, from western blots, that high concentrations of modified toxins were present in crude cell extracts of *E.coli* MC1061 (pSER008) and (pGLY008). However, the haemolytic activity of the crude cell extracts appeared low, and inconsistent with the specific activity of purified modified toxins. In an attempt to determine the specific activity of modified toxins in crude cell extracts, the amount of antigen present, as measured by ELISA, was determined (Table 10). The concentration of ^{S428} pneumolysin in crude cell extracts was calculated to be 25 mg of antigen /ml of cell extract, which gave a specific activity of 1.6×10^3 HU/mg of antigen. Whilst the concentration of ^{G428} pneumolysin in similar extracts was

determined at 12.5 mg of antigen /ml of extract, giving a specific activity of 400 HU/mg of antigen (Table 10). These specific activities are very much lower than those determined for purified proteins. This may be due to the presence of a large amount of inactive toxin in crude cell extracts. Though the cause of this inactivity is unknown, it is unlikely to be due to degradation of the toxin, since western blotting of crude cell extracts of modified toxin indicated that the majority of the modified toxin had a relative molecular weight of around 53 kD (Fig 34b).

5.iv DISCUSSION

The observation that crude extracts of modified toxin may contain up to 90% haemolytically inactive toxin, reflects the situation observed for wild type recombinant pneumolysin (T. Mitchell, personal communication). The inactive toxin is not degraded since it appears full size as determined by western blotting. It is known that high expression of foreign genes in *E. coli* can lead to the formation of inclusion bodies composed of aggregated protein, but whether these are formed in *E.coli* harbouring plasmids expressing either wild type pneumolysin or the modified toxins requires further investigation.

5.iv a Chimeric Protein

The construction of a gene fusion was intended to confirm experimentally the hypothesis that similar activities of pneumolysin and SLO were due to extensive structural similarity, as deduced from their amino acid sequences.

A fusion of pneumolysin and SLO genes, constructed to encode for the 428 N-terminal amino acids of pneumolysin and 41 amino acids from the C-terminus of SLO, was predicted to direct the production of a chimeric protein 469 amino acids in length. The integrity of the construct, pCHI8, carrying the gene fusion, was determined by restriction digests and some DNA sequence analysis. The results indicated that the construct ought to direct the production of the expected chimeric protein. In agreement with this, the polypeptide produced both *in vivo* and *in vitro*, by pCHI8 and pCHI9, was indistinguishable in size from wild type pneumolysin produced in the same strain of *E. coli*. The protein was also cross reactive with anti pneumolysin antibodies in both western blotting and ELISA. However, the chimeric protein had a very low specific activity. This low haemolytic activity of the protein was not a consequence of an incorrect assembly of the gene fusion or translation of the mutant gene as determined by analysis of the construct pCHI8.

The low haemolytic activity of crude cell extracts, together with the low yield of the chimeric protein made purification of this protein difficult, thus preventing a more precise analysis of its biochemical properties.

The construction described above was based on the assumption that the amino acid sequences of pneumolysin and SLO, being very similar throughout the protein, would confer a similar secondary structure on these toxins. However, relative to the remainder of the sequence, the C-terminal 41 amino acids of both proteins are quite diverse. This may suggest that the C-terminal portion of pneumolysin and SLO may have significantly different properties. The C-terminus of SLO is clearly important for activity since truncated proteins, lacking

only a few amino acids, are inactive (M. Kehoe, personal communication). Whether the C-terminal portion of the SLO molecule is directly involved in the lytic process or simply required for the overall conformation of the protein remains to be determined.

5.iv b Site directed mutagenesis of the pneumolysin gene

The role of the cysteine residue in the activity of sulphydryl-activated cytolysins has been the subject of intense investigation using a variety of sulphydryl modifying agents (see Chapter 1, Smyth and Duncan 1978, Geoffroy and Alouf 1982, Iwamoto *et al* 1987). Such an approach, though useful in defining the importance of the cysteine in the activity of these toxins has limitations since it is not known whether or not the changes induced by chemical modification only affect the cysteine residue or induce a conformational change in the protein. The substitution of the cysteine residue by other amino acids mediated by oligonucleotide site-directed mutagenesis circumvents many of these problems.

The specific activity of both the modified toxins, ^{S428} pneumolysin and ^{G428} pneumolysin, were similar at about 1% of wild type pneumolysin. It is interesting to note that this level of activity is similar to that of perfringolysin after the cysteine had been modified chemically by NBS (Iwomato *et al* 1987). The chemically-modified perfringolysin had a lower binding capacity to erythrocytes as well as reduced lytic activity. Whether or not the reduced activity of ^{S428} and ^{G428} pneumolysin is a consequence of their lower binding to erythrocytes or the subsequent lytic activity remains to be determined. The slightly higher specific activity of ^{S428} pneumolysin may reflect the greater similarity of the serine

($R=CH_2OH$) and cysteine ($R=CH_2SH$) side chains, though again this requires further investigation.

It would appear from the reduced activities of both of the modified proteins, that the cysteine residue plays an important role in the biological activities of these proteins. However, it has been found that substitution of the cysteine residue with alanine, designated ^{A428} pneumolysin, results in a modified toxin that has a similar specific activity to wild type pneumolysin (T. Mitchell, personal communication). This observation would imply that cysteine residues are not directly involved in the mechanisms of binding or subsequent lysis, though it is likely that they contribute to the overall conformation of a functional domain. The precise function of cysteine residues cannot be determined from the rudimentary characterisation of the modified proteins descibed in this chapter. Further substitutions of the cysteine residue are required to determine the exact role of this residue in the biological activity of pneumolysin.

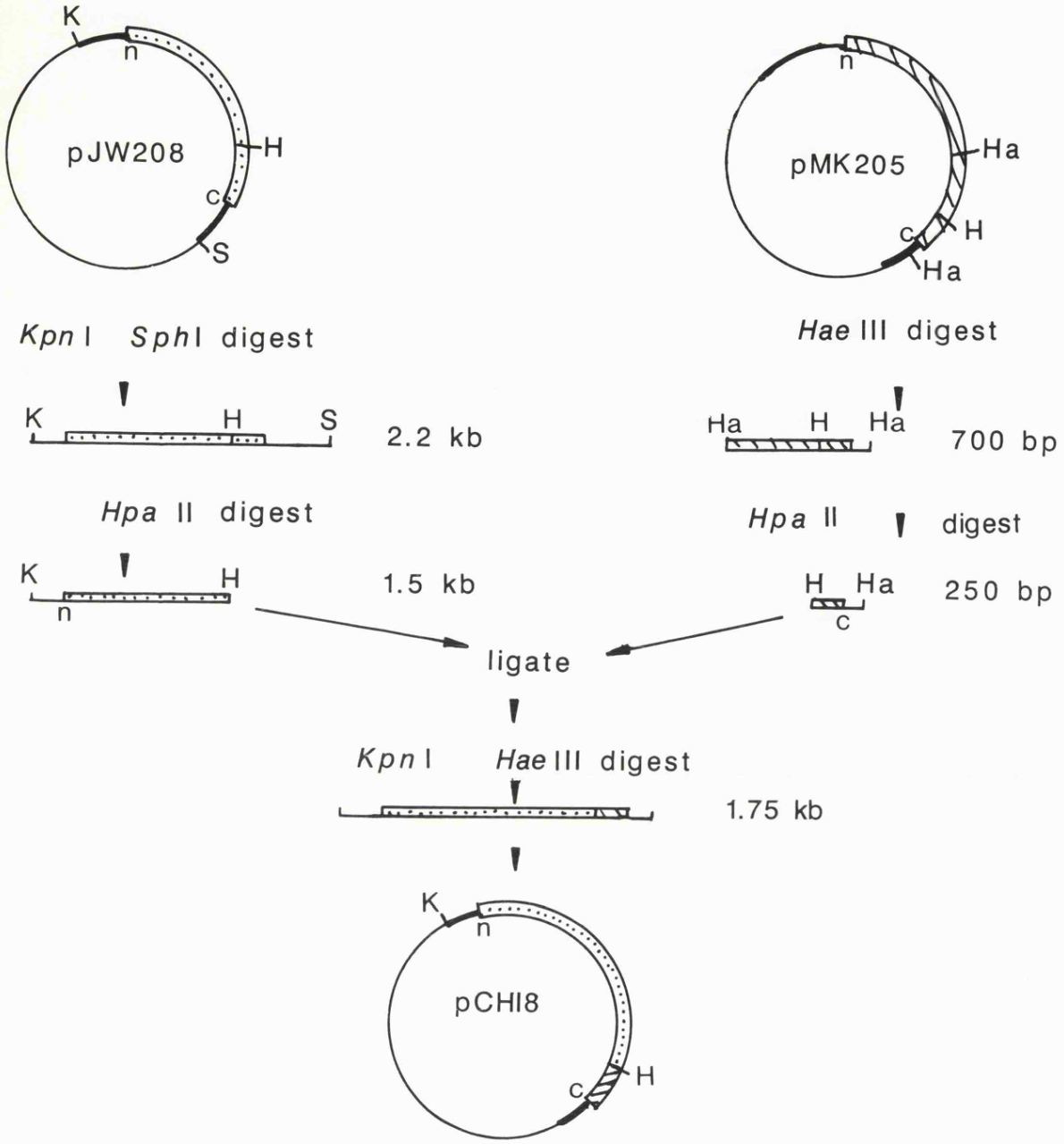


FIGURE 24. Scheme for the generation of a fusion of the pneumolysin and SLO genes.

represents the pneumolysin gene and the SLO gene. n = The start of each coding region.

c = end of each coding region.

— Vector DNA ▬ DNA flanking each toxin gene

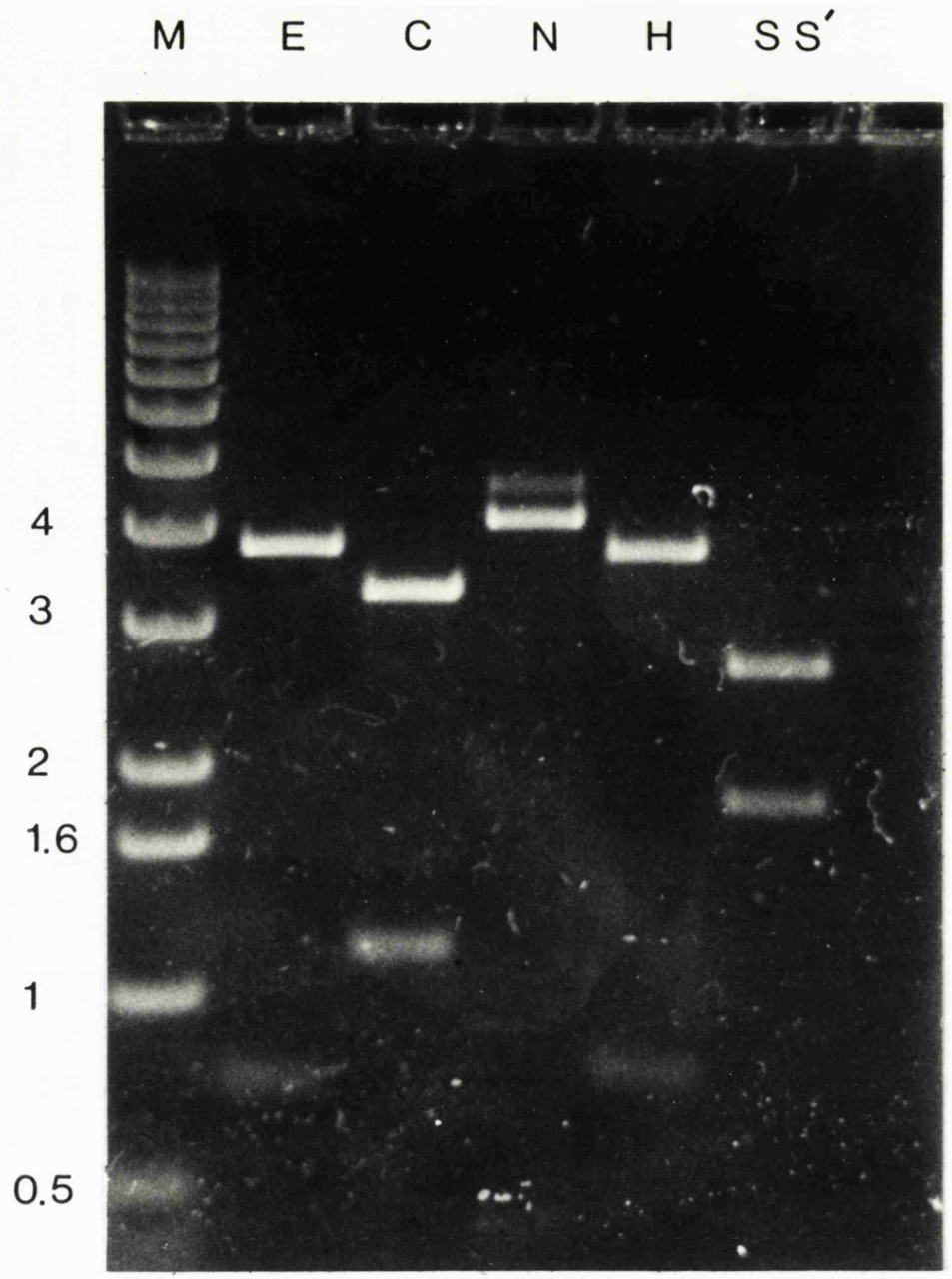
K = *Kpn*I, Ha = *Hae*III, H = *Hpa*II, S = *Sph*I

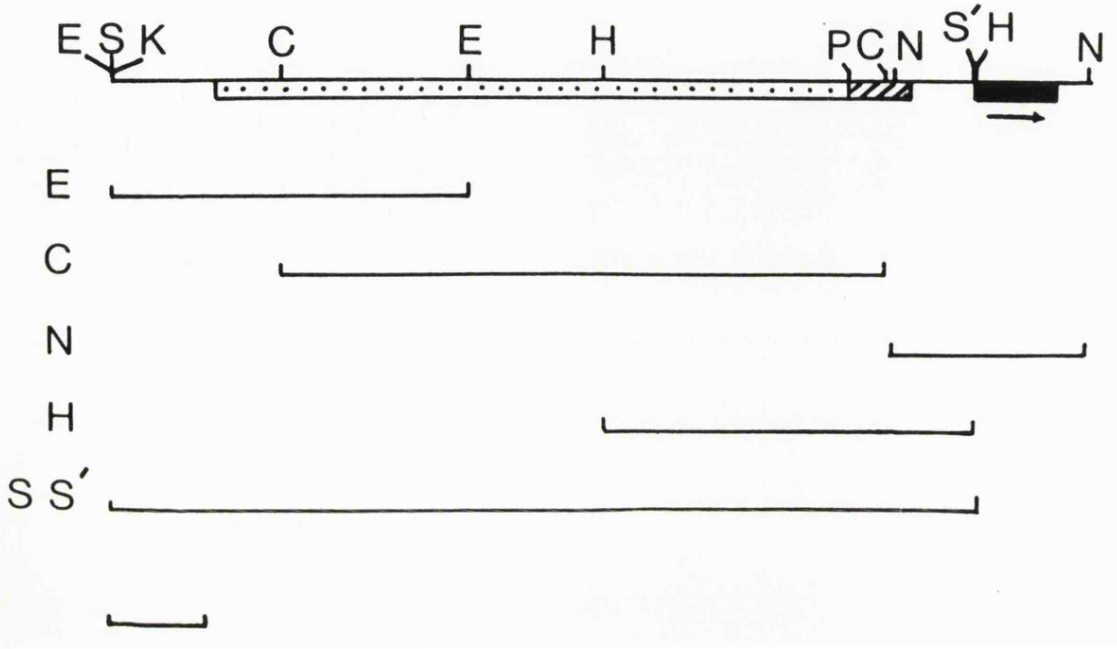
FIGURE 25. Agarose gel electrophoresis of fragments of pCHI8 generated by various restriction enzymes and below it the restriction map of the insert constructed from these digests.

E= *Eco*RI, C= *Hinc*II, N= *Nde*I, H= *Hind*III, S S'= *Sph*I-*Sst*I P= *Hpa*II, M= 1 kb ladder (sizes of fragments are in increments of 1kb unless indicated).

 represents the pneumolysin gene sequence and  the SLO gene sequence. The *lac* gene is represented by the solid box. The arrow indicates the direction of expression of both the *lac* gene and the hybrid gene

 = 200 bp


M
E
C
N
H
S S′
4
3
2
1.6
1
0.5


E S K
C
E
H
P C N
S′ H
N
E
C
N
H
S S′

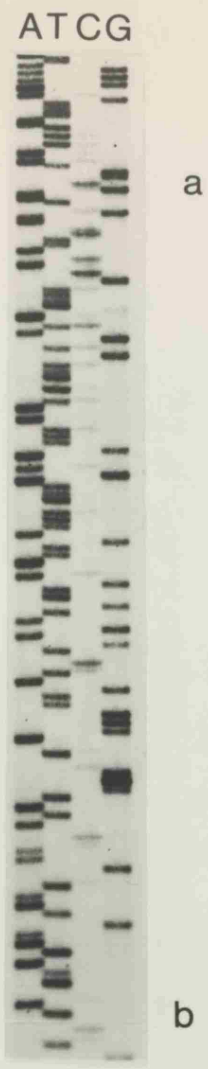


FIGURE 26. DNA sequence of the 3'end of the hybrid gene construction.

The insert from pCHI8 was cloned into M13mp18 and the sequence of the end of the fragment determined from the universal primer.

a. The *Hpa*II restriction site forming the boundary of the fusion between pneumolysin and SLO b. The end of the SLO coding sequence.

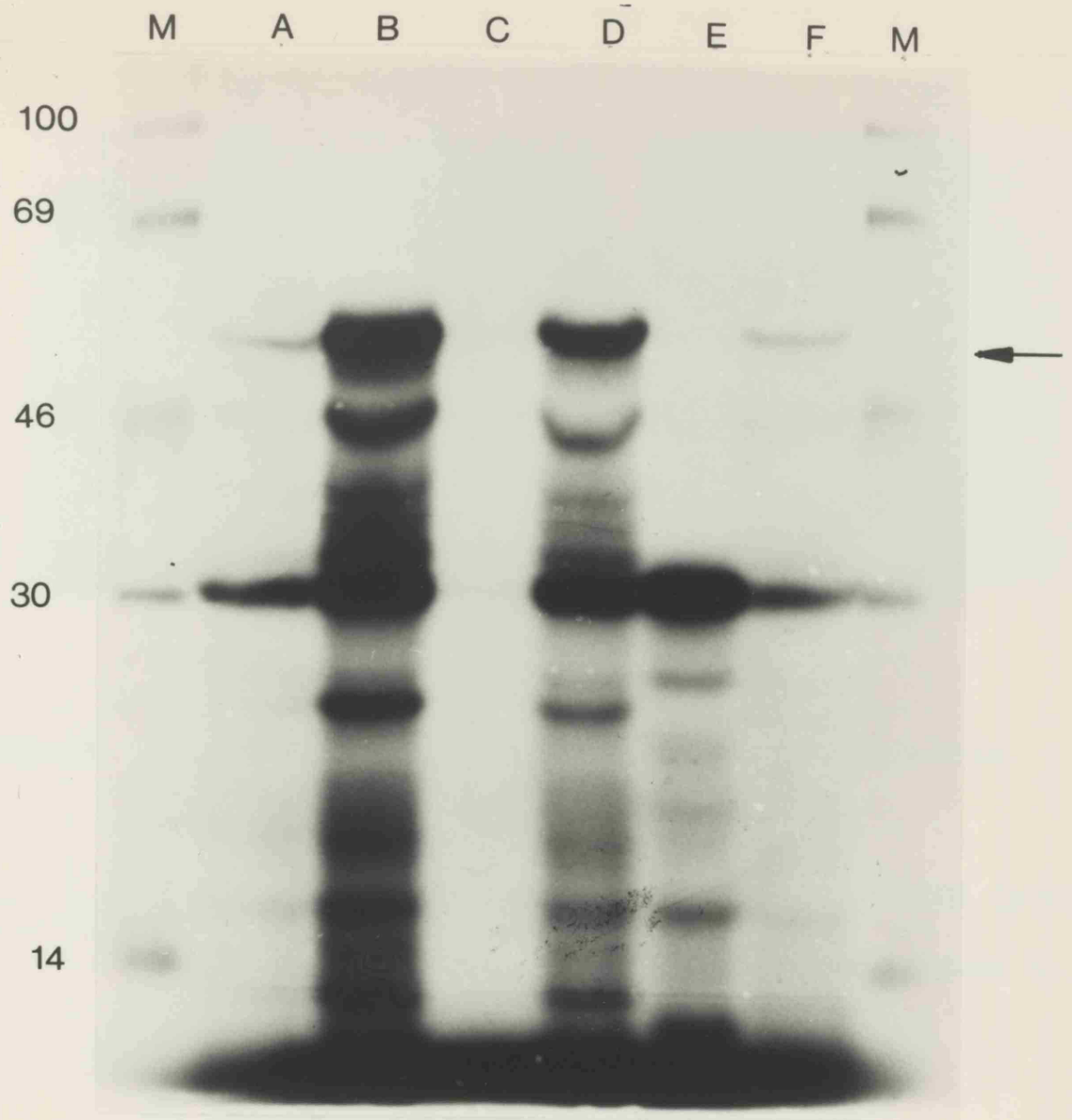


FIGURE 27. Autoradiograph of labelled proteins produced by a cell free *E. coli in vitro* transcription translation system.

A= pCHI8, B= pCHI9, C= no plasmid control, D= pJW209, E= pUC18, F= pJW158, M= C^{14} labelled protein markers, sizes shown in kD. The protein corresponding to pneumolysin is indicated by an arrow.

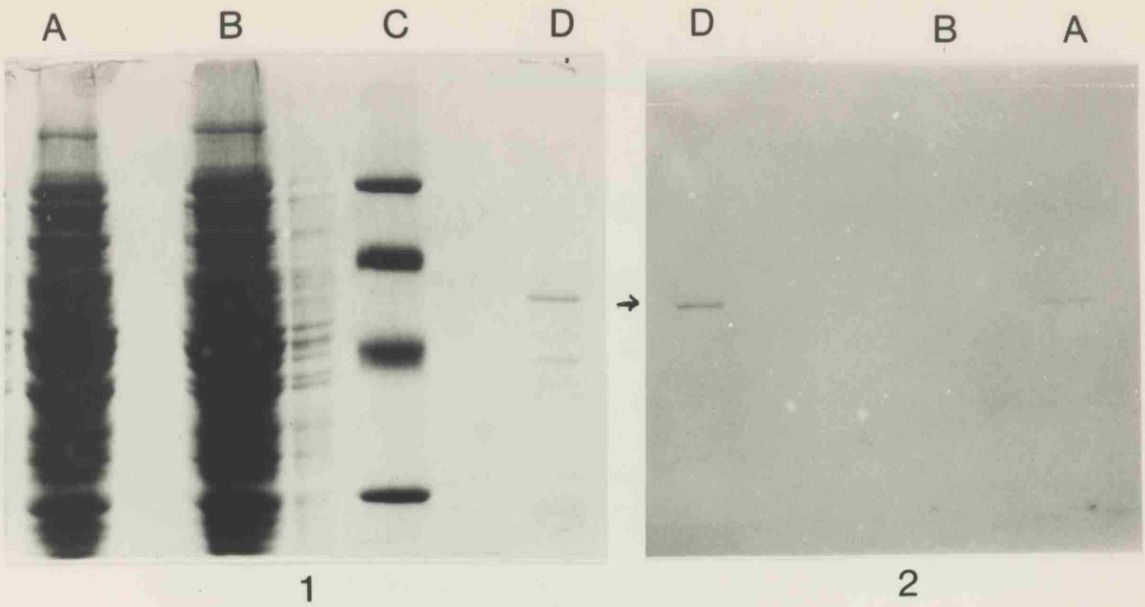


FIGURE 28 1. SDS-PAGE of crude cell extracts stained with coomassie blue.

A = *E.coli* MC1061 (pCHI9), B = *E. coli* MC1061 (pCHI8)

C = Standard protein markers (molecular weights in kD from the top 100, 69, 46, 30)

D, partially purified pneumolysin (10μg).

2. Western blot of a similar gel to that shown in 1. The proteins were probed with polyclonal IgG antibodies raised against pneumolysin. Samples as in 1. The presence of proteins reactive with the antibody is indicated by the arrow.

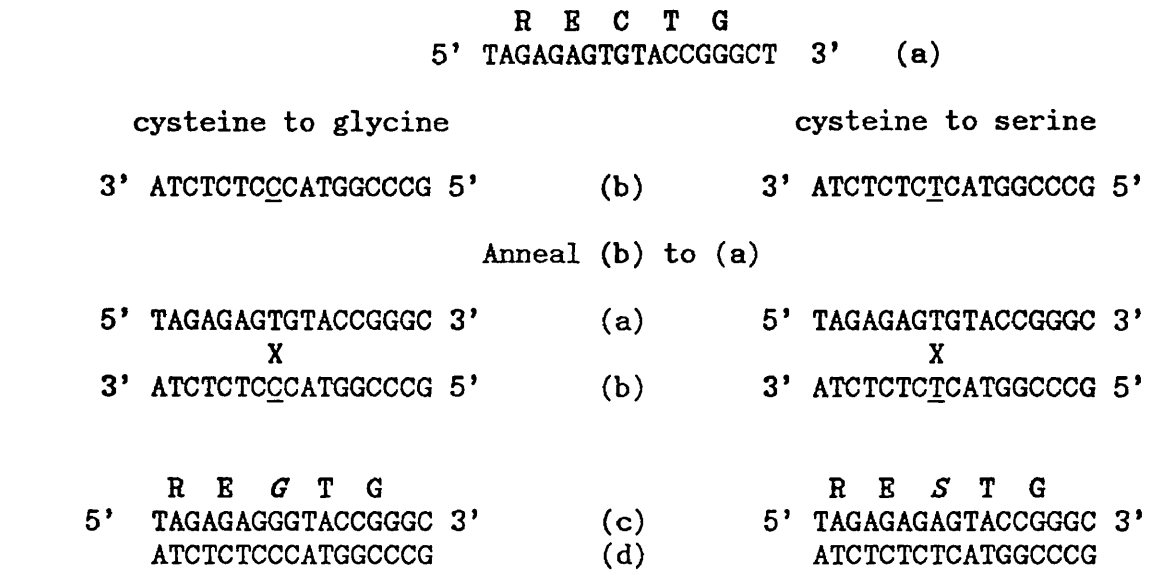


FIGURE 29 Scheme for the production of site directed mutants of the pneumolysin gene.

a= strand cloned in M13.JW158

b= synthetic oligonucleotide carrying the required base change (shown underlined)

c= changes introduced into the + strand of M13.JW158. The original and altered amino acid sequences are in bold type, the altered amino acids are shown in italics.

d= predicted DNA sequence determined from mutant M13 (see also Fig 33)

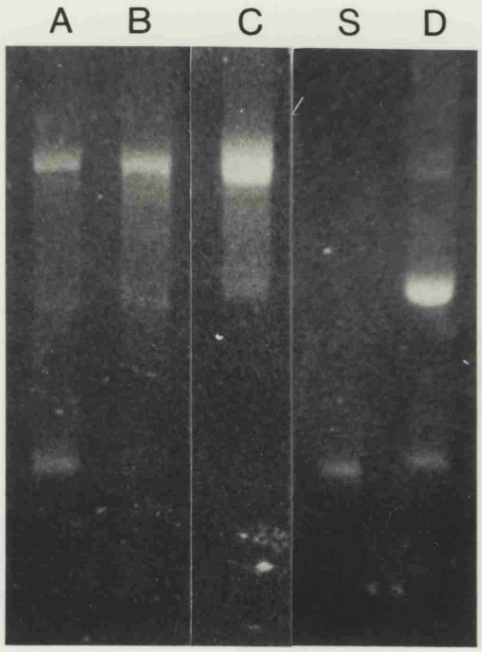


FIGURE 30. Agarose gel electrophoresis of extension-ligation reactions of M13.JW158 and mutant oligonucleotides.

A= No primer control, B= M13.JW158 annealed with glycine substituion oligonucleotide. C= M13.JW158 annealed with serine substitution oligonucleotide. S= Single stranded DNA of M13.JW158, D= RF of M13.JW158.

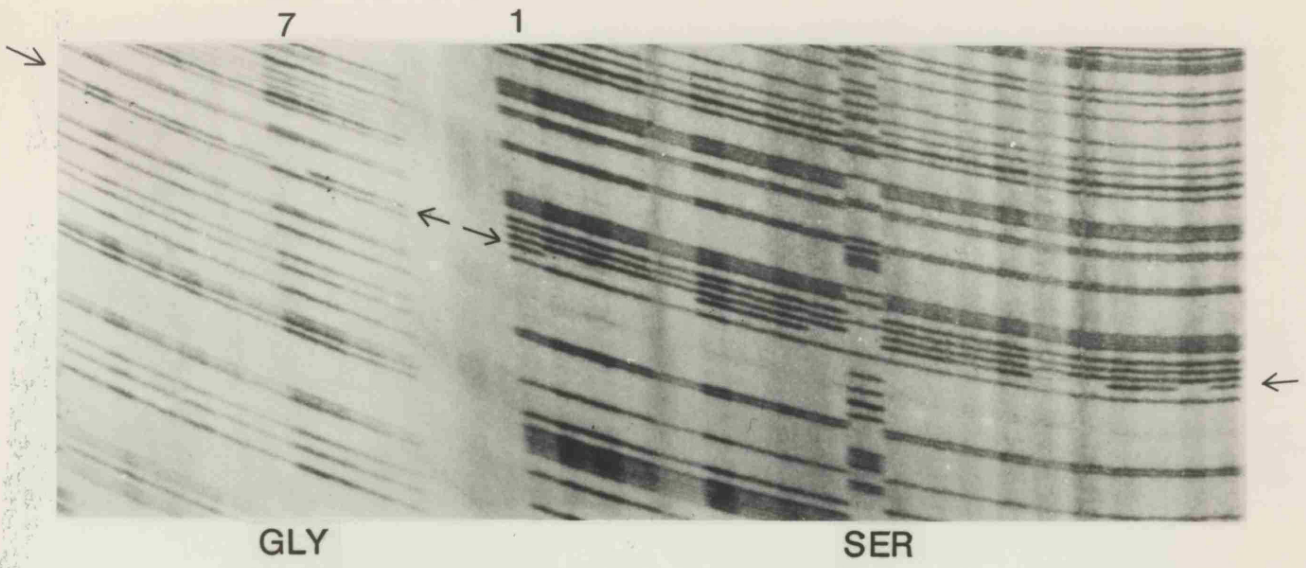


FIGURE 31. Gel showing A or T screening of potential oligonucleotide site directed mutants of the pneumolysin gene.

GLY= 'A' Screens of single stranded DNA from potential cysteine to glycine mutants. The arrows indicate the position of the mutated base. Track 7 represents the mutant, designated M13.GLY428, used in further analysis of the mutant.

SER= 'T' Screens of single stranded DNA from potential cysteine to serine mutants. The position of mutated bases is indicated by arrows. Track 1 represents a mutant, designated M13.SER428, used in further analysis.

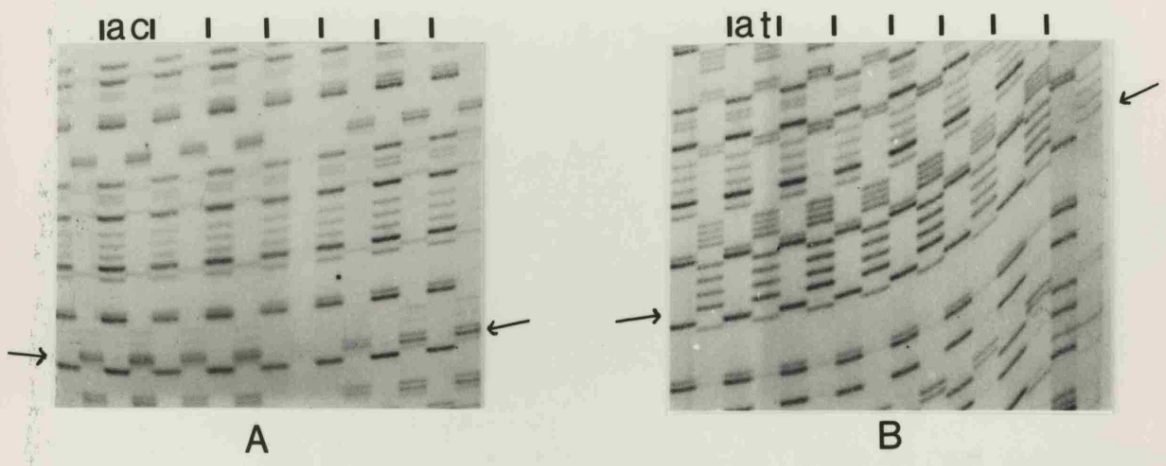


FIGURE 32. Samples of plaque purified M13.SER428 and M13.GLY428

A= M13.GLY428, screened by A and C reactions (ac)

B= M13.SER428, screened by A and T reactions (at)

Absence of wild type sequences in all samples was indicated by the lack of a band in the A track at the locations marked by the arrows

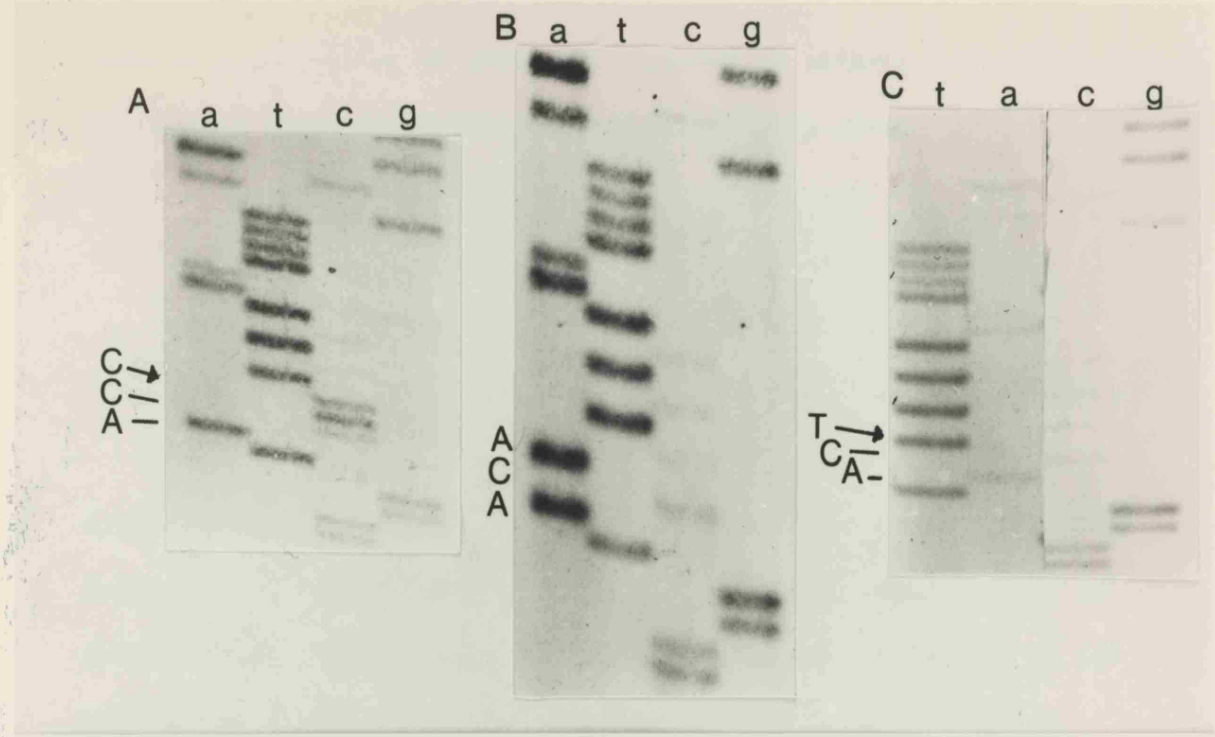


FIGURE 33. DNA sequence of recombinant and both mutant pneumolysin genes.

A= M13.GLY428 (3' ATCTCTCCCATGGCCCG 5')

B= M13.JW158 (3' ATCTCTCACATGGCCCG 5')

C= M13.SER428 (3' ATCTCTCTCATGGCCCG 5')

The sequences read 5'-3', from the bottom of the gels, the underlined bases are indicated next to the relevant sequence. The base altered in both the serine and glycine substitutions is marked by an arrow. a t c and g refer to the order of loading of each sequence reaction. The sequences shown above are consistent with those predicted in Fig 29.

TABLE 9

Haemolytic activities in crude cell lysates of bacteria harbouring recombinant plasmids encoding mutant pneumolysin genes.

SAMPLE [a]	HAEMOLYTIC ACTIVITY [b]			
	no DTT [c]	DTT [d]	Sterol [e]	IgG [f]
E.coli MC1061 (M13.JW158)	6.4×10^4	2.5×10^5	8×10^1	8×10^1
E.coli MC1061 (M13.SER428)	1.2×10^4	1.2×10^4	4×10^1	4×10^1
E.coli MC1061 (M13.GLY428)	8×10^2	8×10^2	8×10^1	4×10^1

a Cell extracts from 1 ml of overnight culture, lysed by sonication

b Haemolytic activity in HU/ml of supernatant.

c Haemolytic activity of the supernatant after storage for 1 week at 4°C, without the addition of DTT to the assay

d As c, but after the addition of 1 mM DTT prior to the addition of erythrocytes

e As d, but after the addition of 5 µl of cholesterol (1 mg/ml)

f As d, but after the addition of 1 µl of anti-pneumolysin IgG antibodies.

TABLE 10

Specific activities of modified toxins compared with pneumolysin, as determined on pure samples of toxin and in crude cell extracts.

A = Specific activities of purified modified toxin

B = Specific activities of modified toxin in crude cell extracts and purified samples, as determined by ELISA

a = Purified samples of modified toxin

b = Crude cell extracts of *E.coli* harbouring mutant plasmids

c = Haemolytic activity in HU/ml of solution

d = Concentration of protein in samples of modified toxin

e = Specific activity of modified toxin in HU/mg of protein

f = Percentage activity compared to wild type pneumolysin

g = Number of EU/ml of solution

h = Protein concentration per ml of solution, calculated on the basis of 3.3 EU/μg of antigen

j = Specific activity of modified toxins, calculated from the protein concentration in *h*

TABLE 10

A	SAMPLE		*c* HAEMOLYTIC ACTIVITY	*d* PROTEIN CONC.	*e* SPECIFIC ACTIVITY	*f* PERCENT
G428	Pneumolysin	*a*	6.4x 10^2	70μg/ml	9.2x 10^3	1.5 %
S428	Pneumolysin (f.1)	*a*	2.6x 10^3	130μg/ml	1.9x 10^4	3.2 %
S428	Pneumolysin (f.2)	*a*	2.6x 10^3	220μg/ml	N.D.	N.D.
Wild type	pneumolysin	*a*	8x 10^4	120μg/ml	6x 10^5	100 %

B	SAMPLE		*c* HAEMOLYTIC ACTIVITY	*g* ELISA	*h* PROTEIN CONC.	*j* SPECIFIC ACTIVITY
E.coli	MC1061(pGLY008)	*b*	5x 10^3	4x 10^4	12.5 mg	4x 10^2
E.coli	MC1061(pSER008)	*b*	4x 10^4	8x 10^4	25 mg	1.6x 10^3
G428	Pneumolysin	*a*	6.4x 10^2	1.6x 10^2	48 μg	1.3x 10^4
S428	Pneumolysin (f.1)	*a*	2.6x 10^3	3.2x 10^2	97 μg	2.6x 10^4

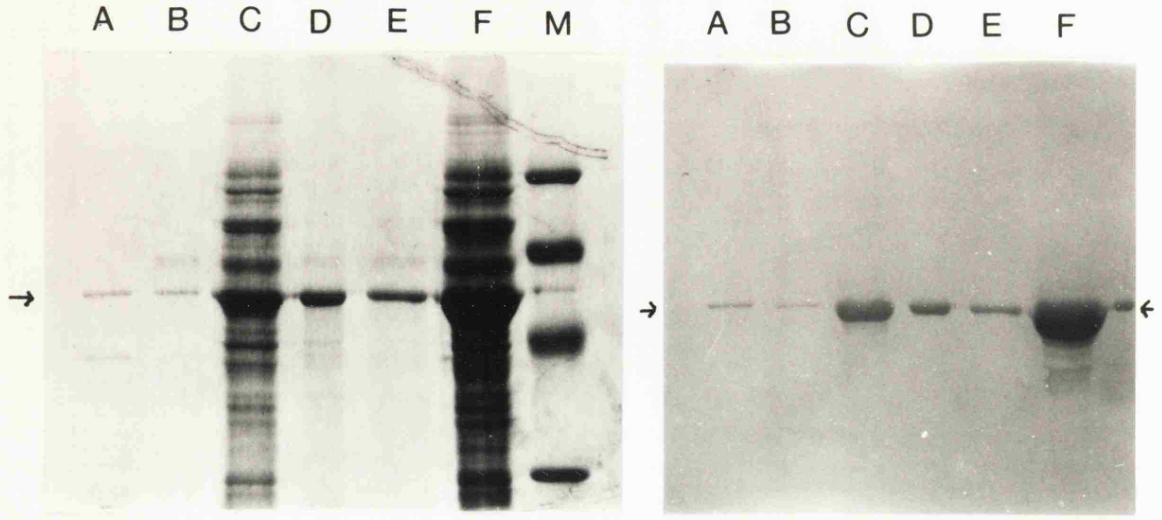


FIGURE 34. 1 SDS-PAGE of crude cell extracts and purified samples of modified pneumolysin, ^{S428} pneumolysin and ^{G428} pneumolysin

A= Partially purified wild type pneumolysin

B= Purified modified toxin, ^{G428} pneumolysin

C= Crude extract of *E.coli* MC1061(pGLY.008)

D= Purified modified toxin, ^{S428} pneumolysin (f.2)

E= Purified modified toxin, ^{S428} pneumolysin (f.1)

F= Crude extract of *E.coli* MC1061(pSER.008)

M= Standard protein markers (sizes in kD, from the top of the gel are 100, 69, 46, 30)

→ = Position of wild type pneumolysin

2 Western blot of a similar gel to that shown in 1. The proteins were probed with polyclonal IgG antibodies raised against pneumolysin. Samples as in 1. The presence of proteins reactive with the antibody is indicated by the arrow.

CHAPTER 6

DISCUSSION

Pneumolysin, the sulphydryl-activated cytolysin of the pneumococcus is of interest because of its potential role in the virulence of the pneumococcus. Such a role is implied by its ability to damage eukaryotic cell membranes, leading to either cell death or significant changes in cell physiology. Despite the spectrum of cytotoxic and sublytic effects mediated by pneumolysin, the mechanisms by which the protein exerts these effects remains unknown. This lack of definitive data has lead to many assumptions concerning the mode of action of pneumolysin and other sulphydryl-activated toxins. For example, it is generally assumed that cholesterol is the receptor in cell membranes for these toxins, despite a lack of direct evidence in support of this hypothesis. Similarly, the presence of cysteine residues in these proteins is thought to be essential for their cell damaging activities, yet it is not clear what is the function of this amino acid. Perhaps most significantly, is the general assumption that the sulphydryl-activated cytolysins exert their effects by similar mechanisms, so that data obtained for one toxin can be applied to all other toxins of the group.

Studies aimed at elucidating the mechanisms by which pneumolysin exerts its biological effects have been hampered by two major obstacles. Firstly, much of the original experimental work was carried out using protein preparations that, at best, were only partially pure. Secondly, that there is no structural data available on the protein. Such data, and the use of pure toxin, must be regarded as essential in investigations intended to clarify the mechanism of toxin binding to a variety of cell membranes and the subsequent effects exerted by pneumolysin.

In the light of these obstacles, the determination of the primary sequence of pneumolysin was considered a priority. The use of recombinant DNA technology provides the most rapid route to this goal (see chapter 4). In addition, this methodology enables the construction of precisely defined mutations as a means of probing structure function relationships (see chapter 5).

Perhaps the most significant observation from primary sequence of pneumolysin was the presence of a single cysteine close to the C-terminus of the toxin (see chapter 4.iv.a). Previous reports (Alouf 1980) had predicted the presence of two cysteine residues in pneumolysin, which supported the theory that the breakage and formation of intramolecular disulphide bridges resulted in the production of active or inactive toxin respectively. The presence of a single cysteine proscribes this mode of thiol-activation. Previous studies had shown that cysteines were essential for the activity of sulphydryl-activated cytolysins. The importance of cysteine residues in activity of these toxins was further emphasised by the discovery of a single cysteine in SLO (Kehoe *et al* 1987). Of interest was the observation that the longest sequence of identical amino acids in pneumolysin and SLO consisted of a 12 amino acid sequence surrounding the single cysteine (Kehoe *et al* 1987). It may not be coincidental that the longest sequence of identical amino acids should surround a residue thought essential to the activity of these toxins. This observation raised the question of the role of this sequence in the activity of the toxins. A possible role for this domain and the cysteine residue in the activity of pneumolysin will be discussed later.

The essential nature of the cysteine residue in pneumolysin was investigated by its substitution with either a serine or glycine using oligonucleotide-mediated site-directed mutagenesis (Chapter 5.iii.b). A rudimentary characterisation of the haemolytic activity of both of these modified toxins indicated, surprisingly, that they possessed haemolytic activity, albeit at a lower level than wild type pneumolysin. The relative specific activities of modified toxin and wild type pneumolysin was reflected in their activity towards PMNL. The amount of modified toxin required to reduce the H_2O_2 production of PMNL by 50 % was 20 times higher for the serine mutant and 50 times higher for the glycine mutant than the amount required of wild type pneumolysin (K. Saunders, personal communication).

The currently accepted mechanism for cytolytic activity of SLO, and therefore by inference pneumolysin, separates the lytic process into two distinct phases. An initial binding of the toxin to cholesterol in the erythrocyte membrane is followed by an aggregation of toxin molecules in the lipid bilayer, which results in the production of transmembrane channels and results in lysis (Bhakdi *et al* 1985). The lower specific haemolytic activity of the modified toxins might result from either a reduction in the ability of the toxins to bind to erythrocyte membranes, a failure to aggregate in the lipid bilayer, the formation of non-functional pores or a combination of all of these. Some of these possibilities have been investigated (T. Mitchell, personal communication).

Investigation of the binding process indicated that the kinetics of binding of both the serine- and glycine-modified toxins to erythrocyte membranes at 0°C appeared similar to that of wild type pneumolysin. This experiment, which involved detecting bound toxin

by haemagglutination of erythrocytes after the addition of anti-pneumolysin IgG, showed that both modified toxins and wild type pneumolysin bound rapidly to erythrocytes even at 0°C.

The ability of the modified toxins to form high molecular weight aggregates in erythrocyte membranes was also investigated. Membranes of erythrocytes lysed by either of the modified toxins were solubilised by DOC, and membrane constituents separated by centrifugation through sucrose gradients. Fractions of decreasing buoyant density were collected and proteins present in each fraction analysed by SDS-PAGE. Modified toxins were detected in fractions of high buoyant density. The formation of high molecular weight aggregates in membranes by each of the modified toxins indicates that these toxins form structures similar to those produced by wild type pneumolysin. The amount or size of the high molecular weight aggregates was not quantifiable, so the extent of aggregation can not be determined.

These results indicate that there is little difference in the abilities of either of the modified toxins to bind to erythrocyte membranes or, once bound, to aggregate. Whether or not the reduced activity of the modified toxins reflects an inability to generate functional pores remains unclear. The determination of the exact cause of this reduced activity will require more sensitive and quantitative experimentation than has been possible to date. The results, however, do appear to be consistent with the hypothesis that the cysteine residue is important for the activity of pneumolysin. This view was dramatically challenged when the properties of another modified toxin, in which the cysteine had been substituted by an alanine residue, were analysed (T. Mitchell, personal communication).

The alanine-modified toxin possessed a specific haemolytic activity, and activity towards PMNL, almost indistinguishable from that of wild type pneumolysin (T. Mitchell and K. Saunders, personal communication). Although further analysis of the properties of the three modified pneumolysin toxins is required to determine the specific differences between them, a radical reassessment of the importance of cysteine residues in the mode of action of pneumolysin, and by inference other sulphydryl-activated cytolysins is now required.

As described previously, it is currently accepted that the lytic mechanism of these toxins involves two distinct stages. Toxin, containing a reactive sulphydryl group, initially binds to cholesterol in the cell membrane and once bound it undergoes a conformational change. This change reveals a lytic site in the toxin, the accessibility of which promotes aggregation which results in the formation of trans-membrane pores which lead to lysis.

The presence of a highly conserved dodecapeptide sequence containing the single cysteine in pneumolysin, SLO (Kehoe *et al* 1987) and listeriolysin O (Mengaud *et al* 1988), suggests this sequence has an important role in the activity of these toxins. Though it is not possible to determine the role of this domain just from the sequence data currently available, it may be postulated that this sequence represents the receptor binding domain of pneumolysin. This hypothesis is supported by two lines of evidence. Firstly, it is the most highly conserved sequence present in pneumolysin, SLO and listeriolysin O and it surrounds a residue that appears essential to the activity of wild type toxin. Since the interaction with cholesterol, the proposed cell membrane receptor is so specific, the

conservation of a domain capable of recognising membrane cholesterol specifically would appear highly plausible. Secondly, if we assume that most of the biological activities depend at least on the ability of toxin to bind to cell membranes, and the dodecapeptide is involved in this process, then the simultaneous alteration of both the cytolytic and sub-lytic activities following modification of the cysteine residue would be inevitable.

The nature of the lytic domain remains unclear. Since the homology between pneumolysin, SLO and listeriolysin O is spread throughout the molecule, it may be that the overall folding of the polypeptide confers on the protein the ability to insert into, and be maintained within the lipid bilayer. A conformational change in the protein subsequent to binding may not be necessary to reveal the lytic site, since toxin has been observed to aggregate in solution in the absence of cholesterol or membranes containing cholesterol. However the nature of these aggregates remains to be determined.

Hydropathic indexes of the amino acid sequence of pneumolysin and SLO reveal them to be basically hydrophilic proteins with hydrophobic domains separated along the length of the protein (Fig 23). A folding of the protein may bring these hydrophobic sequences into close proximity, resulting in a sufficiently large hydrophobic surface to allow integration of the toxin into the lipid bilayer. Analysis of other membrane damaging proteins, such as staphylococcal α-toxin (Gray and Kehoe 1984) and aerolysin (Howard *et al* 1987) have revealed hydrophobic domains throughout their sequences rather than a single highly hydrophobic region.

Insertion of pneumolysin into the lipid bilayer may bring hydrophilic domains of the protein into contact with lipids forcing the bilayer aside and resulting in the formation of arc like structures and the generation of a pore large enough to allow the passage of small ions and water. This is thought to occur in *E. coli* haemolysin (Bhakdi and Tranum-Jensen 1986). The sublytic effects of pneumolysin on cells may result from the insertion of single protein molecules or the aggregation of small numbers of protein monomers with a concommitant change in cell permeability. Such a mechanism has been suggested for the activity of staphylococcal α-toxin (Suttorp *et al* 1985). However, the ability of pneumolysin to aggregate in the bilayer may allow the more extensive forcing aside of lipid resulting in the formation of large functional lesions, assumed to be important in the lysis of erythrocytes.

If the above hypothesis represents a more accurate explanation of lytic and sub-lytic events, there still remains the question of the role of the cysteine in these processes. The production of a modified toxin, lacking a cysteine residue, that has a similar activity to wild type pneumolysin implies that the function of the cysteine may be simply to contribute to the overall folding and tertiary structure of the putative receptor binding domain. The amino acids that contribute to the "active site" of the protein being elsewhere within this dodecapeptide sequence. Other residues, such as the three tryptophans at positions 433, 435 and 436 (Fig 13), may be of importance in the overall conformation of this sequence, and have been targeted for further study.

There is some indirect evidence that the C-terminal region of the sulphydryl-activated toxin is important for activity. This portion

is more highly conserved than the N-terminus in all three sequences of sulphydryl-activated cytolysins so far determined (Kehoe *et al* 1987, Mengaud *et al* 1988). A preliminary investigation of the role of this portion of the molecule in biological activity has been undertaken. Truncated mutants of SLO were made lacking the 5 C-terminal amino acids (M. Kehoe, personal communication). The truncated protein had a 200 fold reduction in haemolytic activity compared to wild type SLO. What stage of the haemolytic process was affected by this truncation was not determined. The importance of the C-terminal portion of pneumolysin was emphasised following the construction of a chimeric protein containing pneumolysin and SLO sequences. Substitution of the C-terminal portion of pneumolysin by the C-terminal portion of SLO resulted in a chimeric protein with nearly a 1000 fold reduction in haemolytic activity compared to wild type parents. It was noted that the portion of SLO replacing the pneumolysin sequence (Fig 21) was significantly different. This difference may be sufficient to interfere with the folding of the protein and therefore its biological activity. It would appear, therefore, that the C-terminus of pneumolysin has a very definite function in the activity of this toxin.

Pneumolysin and SLO have been observed to activate the classical complement pathway, apparently in the absence of specific antibody (Paton *et al* 1984, Bhakdi and Tranum-Jensen 1985). This raised the question as to whether activation involved a specific domain on pneumolysin or SLO. A sequence in pneumolysin, from amino acid residues 379 to 397, has a high degree of homology with a region of CRP (D. Burton, personal communication). The ability of CRP to activate the classical pathway of complement (Mold and Gewurz 1981), has focused our attention on this region as to whether it is involved

in the activation of complement. Mutations in the gene which code for this region have been made (T. Mitchell, personal communication), and the resulting modified toxins have a reduced ability to activate complement. These modified toxins have specific haemolytic activities indistiguishable from wild type pneumolysin. This indicates that mutations in this region do not affect the overall tertiary structure of the toxin. Whether this means that pneumolysin can activate the classical complement pathway, in the absence of anti-pneumolysin antibody, via the binding of IgG to a specific region of the toxin remains to be determined.

All three modified proteins produced by the substitution of the cysteine residue activated complement, as measured by C3 conversion, to the same degree as wild type toxin. Thus the regions responsible for the activation of complement and cytolytic activity reside in different parts of the protein. This suggests that there are at least two domains in pneumolysin. One domain is defined by the dodecapeptide surrounding the cysteine and is important in cytolytic activity and a second domain, amino acid residues 379-397, that appears concerned with the activation of complement. These two sequences are now being subjected to further investigation.

The immunological relatedness of the sulphydryl-activated cytolysins together with their similar biological activities had indicated that they possessed structural similarities. From the analysis of the sequence this appears to be the case. However, significant differences in the activities of pneumolysin, SLO and listeriolysin O have been apparent for some time. These differences include the intracellular nature of pneumolysin, the lower optimal pH required for listeriolysin O activity, and the presence of two active forms of

SLO. It is likely that there are other subtle differences between these toxins that are so far undetected. The differences between these toxins may have arisen as a result of the differing physiology of each bacteria. Whether these different activities imply different roles for the toxins in the disease processes of the producing bacterium remains to be determined.

It has been assumed that pneumolysin, SLO and Listeriolysin O genes have diverged from a common ancestor (Kehoe *et al* 1987, Mengaud *et al* 1988). This would be consistent with the observation that the protein sequences are more similar than the corresponding nucleotide sequences and that they are from either the same or closely related genera. The question is raised therefore, whether other toxins of this group, for example from Bacillus or Clostridia are structurally similar to the pneumolysin prototype. This may not be the case. The number of cysteine residues has been found to vary in other toxins, four being identified in alveolysin (Geoffroy and Alouf 1983), two in cereolysin (Cowell *et al* 1976), though a single cysteine residue has been determined in perfringolysin (Yamakawa *et al* 1977). Whether the differences in cysteine content of toxins from the Bacillaceae are significant in terms of their structure and function remains unclear, but this should be clarified when their primary sequence is determined.

A key unanswered question is the role of pneumolysin in pneumococcal infections. The determination of the nucleotide sequence of the pneumolysin gene enabled the construction, *in-vitro,* of deletion mutations in the gene (results not shown). Transformation of pneumococci with the deleted gene sequence, followed by homologous integration of the sequence into the pneumococcal chromosome ought to

produce a specific pneumolysin negative pneumococcus. This approach, attempted during the period of the work described in this thesis was not completed. A similar approach however (J. Paton, personal communication), succeeded in producing a pneumolysin negative pneumococcus of serotype 1. The mutant had a lower capacity to cause disease in mice than its isogenic parent and was an indication that pneumolysin has a role to play in pneumococcal disease, although the precise role for pneumolysin was not defined.

One possible function of pneumolysin that has not received much attention is its role in the normal physiology of the pneumococcus. A pneumolysin negative mutant isolated after treatment with EMS (Johnson *et al* 1982) was shown to produce more H_2O_2 than did its pneumolysin producing parent (Johnson *et al* 1984). Since the pneumococcus does not produce catalase or other peroxide detoxifying enzymes, the suggestion was that pneumolysin functions to "mop up" peroxide, allowing the pneumococcus to grow at higher oxygen tensions. This hypothesis is extremely controversial, especially considering the possibilities that multiple mutations, affecting other important cell functions, could have been introduced during the EMS treatment. However, the intracellular location of the toxin does not preclude a function for pneumolysin in normal bacterial physiology. The use of defined pneumolysin-negative mutants will allow further testing of this hypothesis.

The sulphydryl-activated toxins are only one example of membrane damaging toxins produced by bacteria from a variety of Gram-positive and Gram-negative genera. These include *E. coli* haemolysin, staphylococcus α-toxin, aerolysin from *Aeromonas hydrophila* and *Pseudomonas aeruginosa* cytotoxin. The sulphydryl-activated

cytolysins are unique compared with these other toxins in that they appear to bind to a specific receptor in the cell membrane, and form much larger functional pores (Bhakdi and Tranum-Jensen 1986).

Comparison of the primary structures of *E. coli* haemolysin (Felmlee *et al* 1985), Staphylococcal α-toxin (Gray and Kehoe 1984), aerolysin (Howard *et al* 1987) and pneumolysin has failed to establish any extensive similarity between them. An understanding of the mechanism of membrane binding, pore formation and subsequent membrane damage of these toxins may be invaluable to our understanding of the sulphydryl-activated cytolysins.

From the work described in this thesis it should now be possible to initiate many new avenues of investigation to probe the structure and function of pneumolysin. Monoclonal antibodies raised against pneumolysin will help in the identification of binding sites, sites concerned with aggregation of the toxin (lytic sites) and epitopes shared with other proteins that damage cell membranes. Such a study would be aided in no small measure by the determination of the structure of pneumolysin by X-ray crystallography. The use of site directed mutants can then be extended to establish a more definitive role for certain amino acids in functional domains. Wider aspects of the relationship between functional domains of these proteins can also be investigated by the construction of a variety of chimeric proteins. Finally, a precisely defined pneumolysin negative mutant of the pneumococcus will be invaluable in the study of the contribution of pneumolysin to the physiology and virulence of the pneumococcus.

CHAPTER 7

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