Structure and function of a neuraminidase from *Streptococcus pneumoniae*

Submitted for the degree of PhD

May 2000

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STATEMENT OF ORIGINALITY

The accompanying thesis submitted for the degree of PhD entitled

Structure and function of a neuraminidase from *Streptococcus pneumoniae.*

is based on work conducted by the author in the Department of Microbiology and Immunology at the University of Leicester mainly during the period between October 1994 to March 1998.

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

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

Signed..... Date....9/10/98

Abstract

Streptococcus pneumoniae is a major cause of diseases such as pneumonia and meningitis especially in the developing countries, the elderly and infants. Treatment with antibiotics is becoming less effective due to increasing resistance and the current vaccine has many limitations, highlighting the need for an improved vaccine. Previously the virulence and pathogenicity was attributed to the capsule, now other factors have been implicated, these include neuraminidase, pneumolysin, and autolysin. The aim of this project was to characterise a neuraminidase from *Streptococcus pneumoniae* by relating its amino acid sequence to the enzymatic activity of the protein, leading to the production of mutated neuraminidases that could be tested as protective immunogens.

The sequence of the cloned neuraminidase gene (*nan A*), was compared to other bacterial neuraminidases to identify conserved residues, and also utilising crystallography data, predictions were made of the residues likely to be important in catalysis. Three residues, glutamic acid (E) 647, arginine (R) 663 and tyrosine (Y) 752 were chosen for further study. To assess the importance of these residues in catalysis, conservative substitutions of these residues (E647>Q, R663>H and Y752>F) were made and the subsequent effect on enzyme activity measured. The wild-type and mutated neuraminidase genes were cloned into the expression vector pQE30 and purified by Ni-NTA affinity chromatography. The purified neuraminidases were assayed for enzyme activity using a colorimetric assay. The E647>Q and Y752>F mutations resulted in reduction in specific activity to a level below the detection range of the assay ($<0.012\mu mol\ min^{-1}\ ml^{-1}$). The R663>H mutation resulted in a decrease in activty to 2% of that of the wild type. The protective effects of the mutated neuraminidases in mice was investigated by immunization followed by challenge with virulent *Streptococcus pneumoniae*. Mice immunized with heat-inactivated wild-type neuraminidase survived for 90 hours after challenge, mice immunized with E647>Q, Y752>F or R663>H survived for 97, 191 or 140 hours respectively. The antibody levels in the sera of the mice immunized with wild type or E647>Q neuraminidases were measured before and after immunization, the antibody levels were increased following immunization, however no relationship was detected between antibody levels and the survival time of the individual mice.

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Abbreviations

A	Absorbance unit
Amp	Ampicillin
AMPS	Ammonium persulphate
BAB	Blood agar base
BHI	Brain heart infusion
bp	base pair
BSA	Bovine serum albumin
C-terminal	Carboxyl terminal
CD	Circular dichroism
CIAP	Calf intestinal alkaline phosphatase
CRM_{197}	Cross reacting molecule 197
CsCl	Caesium chloride
CSF	Cerebrospinal fluid
DANA	2-deoxy-2,3-dehydro-N-acetylneuraminic acid
dATP	Deoxyadenosine triphosphate
dCTP	Deoxycytidine triphosphate
dGTP	Deoxyguanosine triphosphate
dNTP	Deoxynucleoside triphosphate
dTTP	Deoxythymidine triphosphate
DNA	Deoxyribonucleic acid
DNAse	Deoxyribonuclease
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
ELISA	Enzyme linked immunosorbant assay
EmM	Millimolar coefficient
FCS	Foetal calf serum

GlcNAc	N-acetyl-glucosamine
GMT	Geometric mean titres
GST	Glutathione-S-transferase
Hib	Haemophilus influenzae type b
IPTG	β-D-isopropyl-thiogalactopyranoside
Kan	Kanamycin
kb	kilo base
kD	Kilodaltons
K_i	Inhibition constant
K_m	Michaelis constant
LA	Luria-Bertani agar
LB	Luria-Bertani broth
MIC	Mean inhibitory concentration
MUAN	2'-(4-methylumbelliferyl)α-D-N-acetyl-neuramininc acid
MW	Molecular weight
MWCO	Molecular weight cut off
N-terminal	amino terminal
NaCl	Sodium chloride
NANA	N-acetylneuraminic acid
NaOAc	Sodium acetate
NaH_2PO_4	Sodium dihydrogenphosphate
Na_2HPO_4	Disodium hydrogenphosphate
NCCLS	National committee for clinical laboratory standards
Ni-NTA	Nickel nitrilo-tri-acetic acid resin
OD	Optical density
OPD	o-phenylenediamine dihydrochloride

ORF	Open reading frame
PAF	Platelet activating factor
PAGE	Polyacrylamide gel electrophoresis
PBP	Penicillin binding protein
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PdB	Pneumolysin toxoid
PMNLs	Polymorphonuclear leukocytes
PNP-NANA	2-O-(p-nitrophenyl)-α-D-N-acetylneuramininc acid
PRP-D	Phosphate-diptheria toxoid
RNA	Ribonucleic acid
RNAse	Ribonuclease
rpm	Revolutions per minute
SDS	Sodium dodecyl sulphate
TAE	Tris-acetate-EDTA buffer
TBE	Tris-borate-EDTA buffer
TE	Tris-EDTA buffer
TI	Thymus independent
TE	Tris-EDTA
TEMED	N,N,N',N'-tetramethylethylenediamine
Tm	Melting temperature
TMP-SMX	trimethoprim-sulfamethoxazole
TRIS	Tris-(hydroxymethyl)-aminomethane
Vmax	Maximal velocity
v/v	Volume for volume equivalent
w/v	Weight for volume equivalent
X-gal	5-Bromo-4-chloro-3-indoyl-β galactopyranoside

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Dedicated to the loving memory of my parents,
Vina and Raj.

Chapter 1: Introduction

1.1 General characteristics of Streptococcus pneumoniae

Streptococcus pneumoniae is a major pathogen of humans worldwide. It is responsible for pneumonia, meningitis and less serious diseases such as otitis media (Mitchell *et al* ., 1997). Individuals at the extremes of age and those with immunological deficiencies are at an increased risk of invasive disease (Gillespie, 1989).

Streptococci are Gram positive cocci that divide in one plane to form chains or pairs (Colman, 1990). Taxonomically they belong to the family *Streptococcaceae*, genus *Streptococcus* (Austrian, 1984). The term *Streptococcus* was applied by Billroth in 1874 to a chain-forming coccus he saw in infected wounds (Colman, 1990). It was later isolated by Sternberg (1880) in the U.S.A, who named it *Micrococcus pasteuri* and by Pasteur (1881) who named it *Microbes septicemique du salive*. (Austrian, 1984). The term pneumococcus was in general use by 1880s due to its regular association with lobar pneumonia. In 1926 the name *Diplococcus* was applied because of the organism's appearance in Gram stained sputum. In 1974 its morphology in liquid growth media led to the now generally used *Streptococcus pneumoniae* designation (Musher, 1995).

Pneumococci are normally ovoid or spherical in shape and range in size from 0.5-1.25µm. They usually exist in pairs but are sometimes found in chains or singly. The distal ends of each pair of organisms tend to be pointed or lance shaped (Rotta, 1986). They are catalase negative, facultative anaerobes with a growth temperature range between 25 - 42 °C (Rotta, 1986). Their metabolism is fermentative with the production mainly of L(+)lactic acid from sugars, and no formation of carbon dioxide (Colman, 1990).

Pneumococci are usually sensitive to optochin (ethylhydrocupreine) and this characteristic is often used in their identification (Colman, 1990). All capsulated pneumococci, but not all non-capsulated variants, are lysed by bile (Colman, 1990). Bile activates N-acetylmuramyl - L - alanine amidase which cleaves the bond between alanine and muramic acid in peptidoglycan (Colman, 1990).

Colonies of capsulated pneumococci on blood agar are usually raised, circular and about 1mm in diameter; those of type 3 are usually larger and have a mucoid appearance (Colman, 1990). Pneumococci exhibit alpha-haemolysis when incubated aerobically and beta-haemolysis when incubated anaerobically (Rotta, 1986). Alpha-haemolysis is characterized by a zone of green agar surrounding the the pneumococcal growth which results from the breakdown of haemoglobin by α-haemolysin (Musher, 1995). Beta-haemolysin is characterized by a complete zone of clearing around the colony (Colman, 1990) and is caused by the action of pneumolysin O (Rotta, 1986).

Pneumococci are typed on the basis of their polysaccharide capsule. Virtually all clinical isolates of *S. pneumoniae* possess a capsule made of repeating oligosaccharides polymerised by transferases. This capsule enables them to resist phagocytosis (van Dam *et al* ., 1990). Occasionally unencapsulated isolates have been implicated as the causative agents of disease such as conjunctivitis (Pease *et al* ., 1986). There are 90 distinct serotypes (Henrichsen, 1995) of *S. pneumoniae* based on antigenic differences in the polysaccharide and these are classified by two systems. The American system assigns serotype numbers in order of first isolation (from 1-90) (Eddy, 1944). The Danish system serotypes on the basis of antigenic similarities; cross reactive serotypes are placed in groups designated by numbers and types within groups by a number preceeding a capital letter (Lund and Henrichsen, 1978). For example the Danish system places serotypes 6A and 6B which differ only in the rhamnosyl-ribitol bond in their linkage (1-3 in 6A and 1-4 in 6B) in the same group, whereas their equivalent American nomenclature is 6 and 26 respectively (Gillespie, 1989; Austrian, 1984). The Danish system of *S. pneumoniae* classification is more widely adopted (Musher, 1992).

1.2 Pneumococcal carriage and serotype distribution

The pneumococcus is a constituent of the typical nasopharyngeal microflora (Austrian, 1984). There is a high probability that every individual is a carrier of *S.pneumoniae* at some stage, and in certain populations carriage may be greater than 70% (Riley and Douglas, 1981). Colonization occurs soon after birth and reaches a level similar to that of the mother by 12 weeks (Riley and Douglas, 1981). Generally in adults, carriage lasts for approximately 6 weeks, but some individuals may be carriers for more than a year (Musher, 1992). Disease is more likely to result from colonization by a new strain, rather than

following long-term carriage of a particular strain (Boulnois, 1992; Gray *et al* ., 1980).

In healthy individuals carriage rates of pneumococci are inversely related to age, being lowest in adults and greatest in pre-school children (Hendley, 1975). This relationship correlates with anticapsular antibody levels in the respective age groups. The immune status of the host is therefore an important determinant of the prevalence and longevity of carriage (Gwaltney, 1975). Carriage in adults is also dependent on exposure to children. A study of 11 families with at least one preschool child in the home showed a carriage rate of 25% compared with 2% in families whose children were at junior high school (Hendley, 1975).

Simultaneous carriage of more than one pneumococcal serotype can occur. In a study by Gundel and Okuru (1933) of 95 school children aged between 12 and 14 years, simultaneous carriage of two, three and even four pneumococcal types was found in 54, 14 and 2 children respectively. In another study five pneumococcal types have been detected in one nasopharyngeal secretion (Hodges *et al* ., 1946). Concominant infections with more than one serotype are rare, nonetheless simultaneous infection with two types of pneumococci has been shown in 1% of pneumococcal otitis media patients (Austrian, 1977). Colonization with one serotype does not necessarily confer resistence to re-colonization by another strain of the same serotype. Generally <50% of carriage results in resistence to re-challenge, for example 42% for serotype 3 and 32% for seotype 19. There is an increase in the number of individuals resistant to colonization by a particular strain correlating with increasing age, presumably due to previous colonization events (Dowling *et al* ., 1971).

Riley and Douglas (1981) have suggested (but no data were provided) that the rate of disease within a population is determined by the frequency of carriage of invasive serotypes. However some serotypes are rarely carried in the population but are frequently isolated from patients. These isolates are considered particularly virulent with the carrier state being merely transient. The converse is also true; serotypes are carried at a high population frequency but are rarely associated with disease (Boulnois, 1992).

The spread of pneumococci is proportional to the frequency and intimacy of contact between people. Closed communities and cramped accomodation such

as military barracks therefore provide optimal conditions for the transmission of pneumococcal diseases (Gillespie, 1989). In the gold mines of South Africa, amongst S. African gold miners there is a high rate of carriage of invasive serotypes due to the constant influx of new non-immune subjects. The new subjects are at an increased risk of pneumonia for 6 months after their arrival in a compound; thereafter the risk falls dramatically (Gillespie, 1989).

Studies have shown that pneumococcal colonization is greatest in the Autumn, Winter and early Spring in adults in closed communities and in the population at large (Smillie, 1936; Hodges *et al* ., 1946; Gray *et al* ., 1982). This association between season and carriage may be mediated through seasonal incidence of viral illness. In a community surveillance program in Houston, USA the greatest number of respiratory viruses were isolated in Autumn, Winter and Spring and the increase in viral disease preceded the onset of invasive pneumococcal disease (Kim, 1996). *In vitro* observations suggest that pneumococci adhere more readily to virus-infected cells than uninfected cells (Fainstein *et al* ., 1980; Hakansson *et al* ., 1994). For example, prior influenza virus infection leads to improved adherence of pneumococci to tracheal epithelial cells, mediated by the viral neuraminidase, exposing receptors for pneumococcal attachment (Plotkowski *et al* ., 1986). Viral infections predispose to pulmonary infection by causing bronchoconstriction, increased mucus production (Berendt *et al* ., 1975; Nickerson and Jakab, 1990), decreased ciliary action (Carson *et al* ., 1985), damage to mucosal cells and leukocyte dysfunction (Berendt *et al* ., 1975).

Of the 90 recognised serotypes, only a small percentage are responsible for the majority of pneumococcal diseases (Musher, 1992). The distribution of serotypes isolated varies between adults and children and also with geographic location and time (Austrian, 1984). For example, in the elderly type 3 pneumococci are a common cause of infection whereas in adolescents infections caused by type 1 pneumococci predominate (Austrian, 1984). The geographical variation in serotypes is shown by the distribution of serotypes 45 and 46. These are a common cause of infection in Southern Africa (Austrian *et al* ., 1976) and Papua New Guinea (Austrian, 1982) but have rarely been isolated in USA (Greenwood *et al* ., 1980). Another example is the frequency of serotypes 2 and 25 to cause disease in South Africa but rarely in USA (Austrian, 1981). The frequency of isolation and clinical importance of a serotype also changes slowly with time. For example, serotypes 2 and 5 frequently caused

disease in North America and Northern Europe in the early part of this century, but since World War II they have almost disappeared in these areas but remain important in other areas (Austrian, 1984).

1.3 Predisposition to pneumococcal disease

In the nasopharynx *S. pneumoniae* has a commensal relationship with the host. However when it spreads to other areas of the respiratory tract or the systemic circulation it causes disease (Austrian, 1986). Usually the host is protected by a variety of specific and non specific defence mechanisms, however when these are compromised, replication of pneumococci occur leading to disease.

Non-specific defences include filtration, cough reflexes, secretions and mucociliary transport (Busse, 1991). These defences can be compromised by events such as sleep, alcohol/drug abuse and anaesthesia. Viral infections of the respiratory tract can also interfere with the non-specific defences of the lungs and cause predisposition to pneumococcal infections. The cellular and humoral components of host defence are also important in preventing infection of the respiratory tract (Busse, 1991). Antibody and complement components in the alveolar lining fluid act as opsonins to facilitate phagocytosis. Initially the resident alveolar macrophage is the key cell in phagocytosis, but later complement cleavage products, bacterial products and macrophages also recruit polymorphonuclear leucocytes (PMNLs). Following ingestion by the phagocytic cells, pneumococci are rapidly killed (Johnston, 1991).

There are many factors which interfere with the functioning of the host immune system leading to increased susceptibility to pneumococcal infection, some of these are listed in Table 1.1

Acquired or congential defects in antibody formation lead to an increased susceptibility to pneumococcal infections. The impact of pneumococcal infections in congenital agammaglobulinemia (Bruton, 1952; Lederman and Winkelstein 1985), and acquired agammaglobulinemia (Hausser *et al* ., 1983; Cunningham-Rundles, 1989) are well documented. Individuals with multiple myelomas and lymphomas which are associated with reduced IgG are particularly susceptible to pneumococcal infection. However after

chemotherapy and hospitalization Gram negative infections tend to predominate in these patients (Twomey, 1973; Savage *et al* ., 1982).

Conditions predisposing to pneumococcal infection
Immunodeficiency syndromes
Splenectomy/ Sickle cell disease
Acute alcoholism/liver cirrhosis
Chronic obstructive pulmonary disease
Congestive heart failure
Malignancy
Diabetes mellitus
Steroid treatment
Prior respiratory infection e.g. Influenza
Excess chance of exposure, e.g. military training camps, day care centres
Cold exposure
Stress/Fatigue
Infancy and aging

Table 1.1: Conditions predisposing to pneumococcal infection (Adapted from Watson and Musher 1995; Musher, 1995)

People with HIV seem to be particularly susceptible to pneumococcal diseases such as bacteremia and pneumonia. In a San Francisco study (Redd *et al* ., 1990) the incidence of pneumococcal bacteremia was 9.4 cases per 1000 AIDS patients per year, an incidence more than a 100 - fold greater than for an age matched population. Also, another study showed that the incidence of pneumococcal pneumonia in AIDS patients was 5.5 - fold and 17.5 - fold higher than for the general population in Boston and New York respectively (Reviewed by Janoff *et al* ., 1992). In spite of the increased susceptibility to pneumococcal infections in HIV patients, no parallel increase in mortality has been observed (Redd *et al* ., 1990).

Defects in the complement system which interfere with C3b production are also associated with pneumococcal infection, whereas defects in C6, C7, C8 and C9 are not. A small increase in susceptibility is associated with C1, C2 and C4 deficiency (Figueroa and Densen, 1991). The spleen is thought to be important in pneumococcal clearance from the blood (Wara, 1981; Styrt, 1990),

complement activation and antibody syntheisis. Its importance in pneumococcal infections is reflected in the high incidence of pneumococcal diseases in splenectomised individuals (Wara, 1982). Children with sickle cell disease have a 100 - fold increase in incidence of pneumococcal bacteremia and meningitis (Wara, 1981; Zarkowsky *et al* ., 1986; Wong *et al* ., 1992). The combined effects of spleen dysfunction and low levels of circulating antibody are contributive factors for the observed high mortality rates from pneumococcal disease in sickle cell patients (Pearson, 1996).

Individuals at the extremes of age are also at increased risk from pneumococcal disease (Gillespie, 1989; Austrian, 1984). Young children respond poorly to carbohydrate antigens and a peak of incidence is found in this age group. The peak incidence of meningitis and otitis media are in infants aged between three and five months (Klein and Marcy 1976), and six to twelve months respectively (Teele *et al* ., 1980). The incidence of pneumococcal disease remains low in adult life, but peaks again in old age (Gillespie, 1989). In the elderly, senescence of the immune system, decreased physical activity, weakening of the gag reflex, malnutrition and other diseases may play an important part in increased susceptibility (Musher, 1995).

There is no conclusive evidence linking liver disease, alcoholism and diabetes melitus with increased pneumococcal infection (Bruyn and van Furth, 1991). However there is poor prognosis for invasive pneumococcal disease in such patients (Bruyn and van Furth, 1991). High infection levels also occur in malnourished individuals, particularly those who are also alcoholics (Musher, 1992).

The importance of cold exposure, fatigue and stress in the development of pneumococcal pneumonia has been described by Heffron (1979).

1.4 Diseases caused by *S. pneumoniae*

Despite the advent of antibiotic therapy, *S. pneumoniae* remains an important pathogen of man worldwide (Mitchell *et al* ., 1997). It is accountable for the majority of cases of community-acquired pneumonia (Roberts, 1995). It is also a common cause of bacterial meningitis in adults and children (Musher, 1992). The pneumococcus is also an important cause of bacteremia and the most frequent cause of otitis media (Roberts, 1995).

1.4.1 Pneumonia

Pneumonia is defined as inflammation and consolidation of lung tissue due to an infectious agent (Marrie, 1994). In the United States it is the main cause of death from infection (Campbell, 1994), where there are between 2 and 4 million cases per year (Garibaldi, 1985). It has the greatest incidence at the extremes of age. In the USA the overall attack rate is 10-12 cases per 1000 adults per year (Marrie, 1994). Studies from United States and Finland show incidence rates of 12 - 18 and 35 per 1000 population per year respectively in infants (Foy *et al* ., 1979; Jokinen *et al* ., 1993). In patients over 75 years of age, in Seattle, USA, rates greater than 30 cases per 1000 population per year were observed. Also in the same study the incidence rates were 1-5 per 1000 people aged between the ages of 5 and 60 (Foy *et al* ., 1979). Pneumococcal pneumonia is the leading cause of death in children in developing countries, where it kills 10% of all children under 5 years of age (The Jordan report, NIH, 1998).

The mortality varies depending on the source of pneumonia. Community aquired pneumonia has a mortality ranging from <5% in mild cases to 50% in more severe cases. In nosocomial pneumonia, the mortality from early onset cases is <10% increasing to 30% for late onset cases. In immunocompromised individuals the mortality rate can be as high as 50% (Woodhead, 1995). In England and Wales 5% of all registered deaths are due to pneumonia (Collee and Watt 1990).

As mentioned previously in Section 1.3 there are many conditions that pre dispose to the development of pneumonia. Many host defence mechanisms exist to resist bacterial infection but when these are compromised by other conditions then bacterial infection may follow. Pneumococcal pneumonia occurs from aspiration of pneumococci into the lower respiratory tract that has previously been damaged by viruses or physical/chemical agents. In the injured respiratory epithelium the clearance mechanism has lower efficiency and allows bacterial replication and the development of pneumonitis (Austrian, 1984). Pneumococcal pneumonia is often preceeded by viral respiratory infections (Austrian, 1984). Many animal and human studies have shown that disruption of the epithelium, excessive mucus production and dampened ciliary function which follow a common cold attack can predispose to pneumococcal pneumonia (Loosli, 1940; Douglas and Riley 1979; Gray and Dillon 1989).

The main symptoms of pneumonia are cough and sputum production which are due to bacterial proliferation and resulting inflammatory response in the alveoli, and fever resulting from the systemic and local release of cytokines and other pyrogens (Musher, 1995). 10-30% of pneumonia patients also have headaches, nausea, vomiting, abdominal pain, diarrohea, myalgia, and arthralgia (Marrie, 1994).

Two forms of pneumococcal pneumonia are recognised, primary lobar pneumonia and bronchopneumonia. Primary lobar pneumonia is a severe illness characterised by sudden onset, high fever and consolidation of the lung. If left untreated, patients may die during the acute phase of the illness or make a recovery after 7-10 days (Collee and Watt, 1990). There are four overlapping stages in lobar pneumonia: engorgement, red hepatization, gray hepatization and resolution (Johnston, 1991). The first stage is known as engorgement and is characterized by the presence of pneumococci and serum in the alveoli (Tuomanen *et al* ., 1995). The fluid is thought to provides nutrients for the bacteria and aid their dissemination in the lung (Harford and Hara, 1950). Red hepatization occurs as a result of passage of erythrocytes, neutrophils and macrophages from capillaries into the alveoli (Loosli and Baker, 1962; Sorokin, 1977). Later when the capillaries are compressed, the erythrocyte content is reduced and the leukocyte migration increases, leading to gray hepatization (Johnston, 1991). The pneumococci are phagocytosed by the leukocytes (Boulnois, 1992). Resolution is achieved by the appearance of macrophages which remove cell debris, (Austrian, 1981). On recovery there is no necrosis of alveoli or interstitium despite the inflammatory events, and the lung returns to normal (Loosli and Baker, 1962; Johnston, 1991). The occurrence of lysis-by-crisis associated with lobar pneumonia was common before the use of antibiotics. The fever would decrease, the pneumococci would be cleared from the blood and the patient would go from a critical state to safety. Crisis is frequently seen during the resolution stage and is accompanied by the presence of anticapsular antibodies (Johnston, 1991).

Bronchopneumonia refers to the patchy consolidation of the lung (Collee and Watt, 1990). This is different to the lobar or segmental consolidation seen in lobar pneumonia. Inflammation may affect the bronchi/ole walls leading to interstitial pneumonia. This leads to incomplete resolution and fibrosis as opposed to the complete resolution and restoration of the lung to its normal

state that is observed in lobar pneumonia (Collee and Watt, 1990). Bronchopneumonia may be primary (as in infants) but more often it is secondary to other illnesses such as bronchitis, gastroenteritis, diabetes, nephritis, anaemia and carcinoma. It may be preceeded by surgery, particularly to the abdomen or by aspiration of food or vomit into the trachea (Collee and Watt, 1990). Cases may occur in outbreaks as seen during an influenza epidemic or they may be sporadic. The prevalence of predisposing factors, immunological status of individuals and nature of invading organism all determine the frequency of bronchopneumonia (Collee and Watt, 1990).

There is still no clear explanation of why death occurs in pneumococcal pneumonia. Individuals treated with antibiotics, in whom pneumococci are cleared have still died (Austrian and Gold, 1961). Many substances are thought to be involved in the toxic nature of pneumococcal infection, including pneumolysin, cell wall constituents and neuraminidase. However there is no clear evidence implicating a specific substance (MacLeod, 1970), although pneumolysin (a thiol-activated toxin) has many attributes which can contribute to inflammation and systemic effects. For example, it can activate complement thorough the classical pathway (Paton *et al* ., 1984), bind and lyse neutrophils, and stimulate granulocyte migration and release of granule enzymes (Johnson *et al* ., 1981).

1.4.2 Meningitis

Despite the advent of antibiotics meningitis still remains an important cause of mortality and morbidity (Quagliarello and Scheld, 1992). Meningitis refers to inflammation of the leptomeninges, which are the membranes that surround the brain and spinal column. Meningitis arises when bacteria enter the cerebro spinal fluid (CSF) which fills the space between the two membranes. *Streptococcus pneumoniae* is a major cause of meningitis in all age groups but particularly in the elderly and children. It is the most common cause of bacerial meningitis in adults and children (Schuchat, 1997). In England and Wales it accounts for 20% of all bacterial meningitis (Noah, 1987) and in the USA for 13% (Schlech and Ward, 1985). The incidence of disease reaches a peak in the winter months. Mortality from all bacterial meningitis remains high in spite of antibiotic and steroid therapy. Between 25-50% of neonates (Mulder and Zanen, 1984; Bell *et al* ., 1989) and 3-10% of children affected after the neonatal period die (Goldacre, 1976; De Louvois *et al* ., 1991). Survivors have a

high incidence of neurologic sequelae (Bohr *et al* ., 1984; Dodge *et al* ., 1984). For example 6-12% of meningitis survivors have hearing difficulties (Fortnum, 1992).

Factors which predispose to pneumococcal meningitis include pneumonia, sinusitis, infective endocarditis, head trauma, CSF leak, splenectomy, sickle cell anaemia, bone marrow transplantation and alcoholism (McGee and Baringer, 1990). Pneumococcal meningitis may occur as a complication of pneumonia and bacteremia. Observations in adults (Austrian, 1979) and children (Belsey, 1967) have shown that pneumococci may gain access to the systemic circulation in the absence of respiratory tract infection with meningitis developing as a sequel to bacteremia. Meningitis may also result from extension of infection of the middle ear or paranasal sinuses to involve the bony structure of the skull and ultimately the meninges. Following a skull fracture and tearing of the meninges the organisms colonizing the upper respiratory tract may extend to the subarachnoid space and cause meningitis (Austrian, 1984).

The most common symptoms in affected individuals under 6 months of age are fits, irritability, diarrhoea, and bulging fontanelles. Vomiting, drowsiness and poor feeding are common in individuals between 6 months and 2 years whereas neck stiffness, focal neurology, headache and vomiting are commonest in those over 10 years old. Fever and foci of infections outside the CSF were common in all age groups, with chest infections being significantly associated with mortality (Kirkpatrick, 1994).

The combined effects of bacteria and their products and the host inflammatory response contribute to the brain damage associated with meningitis. The inflammatory response in the CSF results in production of chemotactic factors (Nolan *et al* ., 1975), immunoglobulins (Ganrot-Norlin, 1978;), polymorphonuclear leukocytes (PMNLs), and complement mediated opsonization (Simberkoff *et al* ., 1980). The inflammatory process would help to control bacterial multiplication in the course of infection. However Tuomanen *et al* ., (1985) have found that inflammatory products like the cell wall released by drug induced autolysis could cause further damage to host tissue and even mortality despite the sterilization of CSF.

Bacterial virulence factors cause inflammation and increased blood-brain barrier permeability which results in brain edema, increase in intracranial pressure and cerebral blood flow changes (Tunkel *et al* ., 1990). Mediators of these changes include cytokines, cyclo-oxygenase metabolites and PAF (Kornelisse *et al* ., 1995).

To limit the neurologic sequelae and death in meningitis patients, treatment strategies are being aimed at at regulating the damaging host response to invading bacteria (Kornelisse *et al* ., 1995). These include intervention with anti-inflammatory agents or monoclonal antibodies in combination with antibiotics (Tunkel *et al* ., 1990).

1.4.3 Bacteremia

Before the advent of antibiotics most cases of bacteremia were caused by pneumococci, *Streptococcus pyogenes* and *Staphylococcus aureus*. Since the use of antibiotics there has been a decrease in the number of bacteremias caused by streptococci and pneumococci and an increase in the bacteremias caused by *Enterobacteriaceae* and other Gram negative bacilli (Phillips and Eykyn, 1990). The pneumococcus still sometimes causes bacteremia in healthy adults but it is now more often the cause of disease in individuals with pre-existing disorders such as bronchopulmonary disease and alcoholism (Phillips and Eykyn, 1990).

The incidence of bacteremia is thought to be around 10 per 100,000 persons per annum (Filice *et al* ., 1980, Broome *et al* ., 1980). Higher attack rates are seen in infancy and later life. The rate of pneumococcal bacteremia is approximately 21 per 100,000 in patient over 60 years (Austrian, 1984). In infants 61-78% of bacteremias are caused by the pneumococcus (Hamrick and Murphy, 1978)

Pneumococcal bacteremia occurs in a variety of ways. Bacteremia can occur as a continuation of pneumonia when pneumococci travel from the lung to hilar lymph nodes and then enter the thoracic duct and the systemic circulation via the left subclavian vein (Austrian, 1981). It can also be linked with meningitis caused by head injury when pneumococci enter the systemic circulation after infected CSF passes from subarachnoid spaces to venous sinuses (Austrain, 1964). More recently it has been recognised that pneumococci can enter the systemic circulation without an apparent source of

infection (Austrian, 1981). For example children aged 6-24 months developed bacteremia without an obvious site of infection in the respiratory tract (Belsey, 1967). Many recovered spontaneously but some developed meningitis. A similar event occured in adult South African gold miners who developed fever, malaise and headaches in the absence of any respiratory infection. They all had pneumococci in their blood but most recovered without antibiotic therapy. One individual developed pneumonia (Austrian, 1981), which suggested that pneumococci in these infections probably originated in the lungs.

The liver, and more importantly the spleen, function to clear pneumococci from the blood. Therefore splenectomised individuals and those with functional asplenia, such as sickle cell disease, are at a greater risk from bacteremia (Austrian, 1981). The spleen is important in antibody synthesis, bacterial clearance from the non - immune host, and in complement activation (Wara, 1982).

1.4.4 Otitis media

Otitis media is defined as an inflammation of the middle ear cavity (Juhn, 1984). It has been estimated that 75% of all children will have at least one attack of otitis media by the time they reach 6 years of age. (Howie, 1975). *Streptococcus pneumoniae* is the main cause of bacterial otitis media and accounts for 30% of all cases of this disease (Giebank, 1989). Antimicrobial therapy has reduced the complications of otitis media such as meningitis and mastoiditis (Giebank, 1976) but long term effects of otitis media such as language difficulties and school learning impairments (Zincus *et al* ., 1978) still persist. Of the 90 pneumococcal serotypes that exist, types 6A, 6B, 14, 19F and 23F are responsible for 60% of episodes of acute otitis media (Austrian *et al* ., 1977). It has been suggested that the enhanced adherence and colonization capabilities of these strains may be the reason for their involvement in otitis media (Kamme *et al* ., 1970).

Much has been learnt about the pathogenesis of otitis media by the use of animal models. Some of the models used include gerbil, guinea pig, rat and chinchilla (Giebank, 1987). Of these, the chinchilla is widely used because it has a similar auditory physiology and anatomy to humans (Miller, 1970) and is not commonly susceptible to middle ear infections (Ward and Duvall, 1971).

Many factors are involved in the pathogenesis of otitis media, including Eustachian tube malfunction and viral infection (Juhn, 1984). Studies on the chinchilla have shown that nasopharyngeal colonization in the absence of negative pressure in the middle ear rarely leads to otitis media. However a decrease in pressure of the Eustachian tube brought about by Influenza A infection does lead to otitis media (Giebank, 1981). Studies on the chinchilla have highlighted many probable events that occur during otitis media. Inoculation of *S.pneumoniae* in this animal model leads to changes in the cell-surface carbohydrate structure along the roof and neck of the Eustachian tube which is paralleled by the ascension of Eustachian tube by *S.pneumoniae* leading to the exposure of N-acetyl-glucosamine GlcNAc (a component of the *S.pneumoniae* adherence receptor) (Linder *et al* ., 1992). The exposure of this receptor may facilitate colonization of the nasopharynx by *S.pneumoniae* and promote invasion of the middle ear and induction of otitis media (Linder *et al* ., 1994).

Other studies using the rhesus monkey model showed that the nature and length of otitis media was dependent on the severity and duration of impairment in Eustachian tube function (Doyle, 1984). In children the muscular opening function of the Eustachian tube is poorer than in adults, therefore they frequently suffer from negative pressure which predisposes to obstruction of ET and inflammation of the middle ear (Portman, 1984). The poorly developed mucosal immune system of the middle ear is also considered to be one of the factors which render the middle ear susceptible to infections (Mogi, 1984).

1.5 Antibiotic resistance

1.5.1 Introduction

Pneumococcal infections are treated mainly with antibiotics (Watson *et al* ., 1995). The most popular antibiotic for over 50 years has been penicillin (Watson *et al* ., 1993) but now various cephalosporins are also used successfully

(Musher, 1992). Over the past two decades there has been an increase in the resistance of *S.pneumoniae* to various antibiotics, including penicillin, (Appelbaum, 1992) which has lead to increased use of vancomycin (Musher, 1992). However there is now concern that overuse of this antibiotic will lead to the emergence of vancomycin - resistant strains (Grimwood *et al* ., 1997). Recently strains of *S. pneumoniae* that are tolerant to vancomycin have been identified (Novak *et al* ., 1999). As tolerance (the ability of a bacteria to survive but not replicate in the presence of the antibiotic) is a precursor phenotype to resistance (Tomasz *et al* ., 1970), it is only a matter of time before vancomycin resistant strains emerge. The issue of increasing antibiotic resistance is now recognised as a major health problem and novel therapeutic strategies are under investigation.

Since the 1960s pneumococci resistant to penicillin have emerged and spread rapidly in Australia and New Guinea, through South Africa in the 1970s, and throughout countries in Africa, Asia and Europe during the 1980s (Jacobs *et al* ., 1978; Appelbaum, 1992; Caputo *et al* ., 1993; Jernigan *et al* ., 1996). More worrisome is the emergence of strains that are also resistant to other unrelated antibiotics such as erythromycin, trimethoprim/sulfamethoxazole (TMP-SMX), tetracyclines and chloramphenicol (Grimwood *et al* ., 1997). The outcome is that pneumococcal infections are harder to treat (Grimwood *et al* ., 1997). Particularly in the case of meningitis, previously recommended therapy can no longer be used with confidence (John, 1994).

1.5.2 Resistance

A strain of *S.pneumoniae* is defined as susceptible to penicillin if the mean inhibitory concentration (MIC) is less than 0.1μg/mL; intermediate if 0.1-1.0 μg/mL; and highly resistant if the MIC is equal to or greater than 2.0 μg/ml as defined by the United States National Committee for Clinical Laboratory Standards (NCCLS). The NCCLS recommendations for cefotaxime or ceftriaxone indicate susceptibility if the MIC is less than 0.5μg/mL, intermediate if 0.5-1.0μg/mL and highly resistant when MIC is equal to or greater than 2.0μg/mL. The maximum MICs reported worldwide for penicillin and third generations cephalosporins are 16 and 32 μg/mL respectively (NCCLS 1993).

Multidrug resistance is defined as resistance to three or more antibiotics of different classes (those with different mechanisms of action (Estrada and Steele, 1995).

1.5.3 Mechanism of pneumococcal antibiotic resistance

In pneumococci resistance to antibiotics is chromosomally mediated, probably due to exchange of genetic material with other pneumococci and other bacterial genera (Klugman, 1990). Resistance to penicillin is due to alterations in the penicillin-binding proteins (PBP). The PBP are involved in peptidoglycan synthesis and are the binding sites for penicillin (Estrada and Steele, 1995). Penicillin kills pneumococci by binding to penicillin binding proteins which then interfere with cell wall synthesis and activate amidase leading to autolysis. Penicillin resistant pneumococci have altered PBPs that have a decreased affinity for penicillin which renders the antibiotic much less effective. (Grimwood *et al* ., 1997). Many changes in the PBPs have now been found and account for the variation in the resistance of different pneumococcal strains to β-lactam antibiotics (Schreiber and Jacobs, 1995).

The spread of penicillin resistant pneumococci may occur by clonal spread or gene transfer. An example of clonal spread occurred in Iceland. Before 1989 there were few antibiotic resistant isolates of *S. pneumoniae* in Iceland (Soares *et al* ., 1993). In 1989 a multiple drug-resistant capsular type 6B *S. pneumoniae* emerged in Reykjavik and spread rapidly. Multiple drug-resistant capsular type 6B *S. pneumoniae* were also found in Spain, which was a popular holiday destination for the Icelandic. The capsular type 6B isolates from Iceland were indistinguishable from a subgroup of multiply resistant type 6B from Spain. From this it was postulated that the multi drug-resistant type 6B was brought to Iceland from Spain by holidaymakers (Soares *et al* ., 1993). Another mechanism for the spread of penicillin resistance is by the transfer of resistance genes into penicillin susceptible pneumococci. The resistance genes may originate in other pneumococci or other bacterial species. Diverse resistance genes were found in 68 clinical isolates of serogroups 6 or 19 studied in South Africa (Simth *et al* ., 1993). Nucleotide analysis suggested that DNA from different species was transferred into *S. pneumoniae* on many different occasions (Lonks and Medeiros, 1995).

Many highly resistant pneumococci have also acquired different mechanisms of resistance to other unrelated antibotics, which are transferred by plasmids and transposons (Klugman, 1990). Resistance to chloramphenicol is by an inducible chloramphenicol acetyltranferase enzyme. Alterations in the ribosomal target proteins is the basis of resistance to tetracyclines and macrolides, whereas resistance to TMP-SMX is mediated by an altered dihydrofolate reductase (Grimwood *et al* ., 1997). Finally erythromycin resistance is due to the *erm*-AM gene encoding adenine dimethylase, whose action on the 23s ribosomal RNA causes reduced affinity of erythromycin for binding to the ribosome (Schreiber and Jacobs, 1995).

1.5.4 Treatment of infections caused by antibiotic resistant pneumococci

The increase in antibiotic resistant pneumococci has had a big influence on the therapy of serious infections caused by the pneumococcus (Estrada and Steele, 1995). The treatment is usually empiric and anecdotal because of the lack of prospective antimicrobial therapy trials (Schreiber and Jacobs, 1995). Factors which influence the choice of therapy are: site and seriousness of the infection, immune status of host, existence of multiply-resistant strains in the community and resistance to other antibiotics. The treatment options of penicillin resistant pneumococci are summarized in Table 1.2.

Infection site	Empiric therapy	Intermediate resistance (0.1-1.0μg/mL)	High-level resistance (≥ 2.0 μg/mL)
Pneumonia/ Bacteremia	Penicillin	Penicillin	Vancomycin and /or Cefotaxime/ Ceftriaxone
Meningitis	Cefotaxime/ Ceftriaxone	Cefotaxime/ Ceftriaxone	Vancomycin and Ceftriaxone
Otitis media	Amoxycillin	Amoxycillin	Amoxycillin or IM ceftriaxone

Table 1.2. Treatment of infections caused by antibiotic resistant pneumococci. (Adapted from Schreiber and Jacobs, 1995; Grimwood *et al* ., 1997).

1.5.5 Prevention

The sensible use of antibiotics and the introduction of conjugate vaccines will help reduce the number of antibiotic resistant pneumococci (Klugman, 1996). For example antibiotics should not be used for long-term prophylaxis of otitis media, tonsillitis or for probable viral infections but for specific treatments only (Grimwood *et al* ., 1997). It is hoped the the major impact on the reduction of antibiotic resistant strains will come from the introduction of conjugate vaccines. It is known that many multiply resistant pneumococci belong to serogroups 6, 9, 14, 19, and 23. The inclusion of these in a conjugate vaccine will hopefully lead to their decreased carriage and reduce the burden of antibiotic resistant pneumococcal disease (Klugman, 1996). However recent work has found that pneumococci are able to switch their capsular types by acquisition of DNA from other resistant pneumococci. During an outbreak of multiresistant pneumococci amid AIDS patients in New York, an unusual event occurred, a multidrug-resistant strain of pneumococcus that usually expresses capsular type 23F, acquired capsular type 3. This change increased its virulence by more than a millionfold and susceptibility testing showed it to be resistant to all antibiotics except vancomycin. The widespread use of vaccines may cause selective pressure for this kind of capsular switching among pneumococci which means that increased surveillance for resistant pneumococci is required (The Jordan report, NIH, 1998).

In the United States a working group was formed in 1994 to develop strategies for monitoring, investigating, and controlling infections due to drug-resistant *S. pneumoniae* (DRSP). The main aims of the group include the establishment of an electronic laboratory-based surveillance system (ELBS) for reporting DRSP and providing advice on the sensible use of antibiotics. It is hoped that the strategy will reduce complications of DRSP infections like long-term sequelae, health care costs, morbidity and mortality (Jernigan *et al* ., 1996).

1.6 Vaccination

1.6.1 Introduction

Despite the susceptibility of pneumococci to many antibiotics, the mortality rate from pneumococcal disease still remains high. For example pneumococcal bacteremia has a fatality rate of 25-30%, (Gillespie, 1989). Also pneumococcal

diseases are becoming increasingly difficult to treat because of the increase in penicillin-resistant and multi-drug resistant pneumococci (Section 1.5) (Linares *et al* ., 1992). The use of antibiotics in developing countries is further exacerbated by financial and transportation considerations. Pneumococcal diseases are a frequent cause of expensive hospitalization and the burden on resources will increase due to the rise in elderly population. For example, 30-50% of pneumonia requiring hospitalization are caused by *S. pneumoniae* (Fedson and Musher, 1994). Furthermore, there is an increased risk of death with increasing age and presence of co-existing disease (Gillespie, 1989). The high mortality and morbidity of pneumococcal disease, combined with the problems in treatment, suggest a need for alternatives to chemotherapy. Vaccination is considered to be an efficient and inexpensive solution (Andrew *et al* ., 1994).

1.6.2 History of pneumococcal vaccination

The first attempts at pneumococcal vaccination were made by Almroth Wright in 1911. He vaccinated 50,000 South African gold miners (a group with high risk of pneumococcal pneumonia) with whole heat-killed pneumococci. (Bruyn and van Furth, 1991). The vaccine was ineffective, probably because not enough organisms were used in its formulation and no consideration was given to the serotype (Gillespie, 1989).

F. Spencer Lister continued the work of Wright and increased the understanding of pneumococcal type-specificity (Lister, 1913), immunity and dose requirements for stimulation of immunity (Lister, 1916). However his immunization trials using a pentavalent vaccine (Heidelberger *et al* ., 1946) in South African miners were inconclusive because of epidemiological considerations and the lack of supporting bacteriological data (Austrian, 1981).

During World War I, vaccination of military populations with whole pneumococcal cells produced results which suggested that the vaccine was effective. However the results were deemed inconclusive because of experimental design considerations (Reviewed by Cecil, 1925 ; Heffron, 1979).

In 1930 pneumococcal polysaccharide was shown to be immunogenic in man (Francis and Tillett, 1930), and this observation led to vaccine trials based on the polysaccharide rather than the whole organism. Trials of a bivalent

polysaccharide vaccine in Civilian Conservation Corps in the 1930s failed to give conclusive evidence on the efficacy of vaccination for reasons similar to those affecting African trials (Ekwurzel *et al* ., 1938). MacLeod *et al* ., (1945), however conclusively showed the efficacy of vaccination using a tetravalent vaccine containing polysaccharide types 1, 2, 5 and 7 in a group of USA army personnel. This crude vaccine was effective in protecting vaccinees against disease from serotypes contained within the vaccine but was largely ineffective against other serotypes not included in the vaccine. Later it was shown that six polysaccharides would make an effective vaccine (Heidelberg *et al* ., 1949). In the 1940s two hexavalent polysaccharide vaccines were licensed for clinical use but were later withdrawn because of the increasing preference for antibiotics (Gillespie, 1989).

The persistence of a high mortality rate from pneumococcal disease (Gillespie, 1989), despite the use of antibiotics, combined with the emergence of antibiotic resistant strains (Appelbaum, 1992), refocused attention on vaccination. A 14-valent and a 23-valent vaccine were licensed for use in the USA in 1977 and 1983 respectively. The 23-valent vaccine, containing 25µg of each polysaccharide, was licensed for use in the U.K in 1989 (Sharpiro, 1991). There are two commercial preparations of this pneumococcal vaccine: Pnu-Immune 23 from Lederle labortories and Pneumovax 23 from Merck Sharp and Dohme. The following polysaccharides are included in the vaccine: 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F and 33F. They represent the serotypes most often associated with disease in USA, Europe and Western Africa (Mitchell and Andrew, 1995). The vaccine induces type specific antibodies for five years following vaccination (Mufson *et al* ., 1983) and is recommended for individuals at a high risk of pneumococcal infection such as the elderly, immunocompromised and those with chronic diseases (Bruyn and van Furth, 1991). However the vaccine is not recommended for children under 2 years of age because an appropriate antibody response is not evoked in this age group (Bruyn and van Furth, 1991).

1.6.3 Drawbacks of the 23 valent polysaccharide vaccine

The 23 valent vaccine currently in use has two major disadvantages; it is poorly immunogenic in infants and protects only against the 23 serotypes present in the vaccine and a few other serotypes that are closely related to those present in the vaccine (such as types 6A and 6B which are highly cross-reactive

(Robbins *et al* ., 1979). Children under 2 years of age (Infants) respond poorly to the vaccine (Fedson and Musher, 1994). Some infants vaccinated at six months of age can mount a reasonable immune response to types 3 and 18C and to other serotypes at one year age. However revaccination at the age of one year leads to minimal or no increase in antibodies (Fedson and Musher, 1994). The poor response in infants is attributed to the fact that polysaccharides are thymus-independent (TI) antigens. Generally thymus-independent antigens induce a predominant IgM response and poor memory (Feldmann and Male, 1991). The high molecular weight and repeating structure of polysaccharides enables them to activate B-cells directly without help from T-cells, by cross-linking surface antigen receptors (Mitchell and Andrew, 1995). Thymus-independent antigens are classified as Type 1 TI antigens (TI-1) or type 2 TI antigens (TI-2) based on their ability to induce antibody responses in CBA/N mice (Armsbaugh *et al* ., 1972). These mice carry an X-linked immunodeficiency and their B-cells are similar to immature B-lymphocytes, so the genetic defect can be thought of as a maturation defect. Type 1 TI antigens (TI-1), such as lipopolysaccharide, can activate immature neonatal B-cells and induce an immune response in CBA/N mice. Type 2 TI antigens (TI-2), such as polysaccharide, can only activate mature B-cells and therefore can not induce a response in CBA/N mice which lack mature B-cells. Infants lack mature B-cells, therefore anti-TI-2 antibody responses are not generally observed and no protective antibody is generated to polysaccharide vaccines (Mitchell and Andrew, 1995).

Another disadvantage of the vaccine is that it only provides serotype specific protection. The serotypes included in the vaccine represent those most frequently isolated in North America and Europe (Robbins *et al* ., 1983). However due to the geographic variation in serotypes distribution, serotypes frequently associated with disease in other parts of the world are not well represented in the vaccine. For example the 23 valent vaccine covers 90% of isolates in North America but only 62.9% in Taiwan and 79.9% in China (Lee, 1987). The serotypes isolated also vary with age of individuals (Nielsen and Henrichsen, 1992) and with time (Finland and Barnes, 1977) so the vaccine is aimed at an ever changing target.

In addition to the serotypic variation, there is also evidence of variation of antibody response to pneumococcal polysaccharide with geography and age. For example Gambian infants respond better than Finnish infants to type 1

polysaccharides but less well to type 19F (Temple *et al* ., 1991). Type 3 polysaccharides are good immunogens at all ages but types 6 and 19 are not good over a long age range (Douglas *et al* ., 1983).

1.6.4 Efficacy of polysaccharide pneumococcal vaccines

The efficacy of a polysaccharide pneumococcal vaccine is dependent on a number of factors including the age and immunological status of the individual (Mufson, 1981; Bruyn and van Furth, 1991). A number of trials of the vaccine have been performed in institutionalised adults, urban elderly, young adults and children (Austrian, 1984). The vaccine is very efficient in young, immunocompetent individuals (Schwartz, 1982), however efficacy is less clear in infants, elderly and immunocompromised individuals.

The efficacy of the pneumococcal polysaccharide vaccine in adults has been demonstrated by several trials (MacLeod *et al* ., 1945; Austrian *et al* ., 1976; Smit *et al* ., 1977; Sharpiro *et al* ., 1991). Early evidence of the efficacy of the pneumococcal vaccine came from randomised controlled trials of the 13-valent vaccine conducted in South African gold miners in the 1970s. It was found that the vaccine reduced the incidence of pneumonia and bacteremia caused by serotypes present in the vaccine by 78% and 82% respectively (Austrian *et al* ., 1976). A more recent study by Shapiro *et al* (1991) has provided further evidence of the efficacy of the pneumococcal polysaccharide vaccine. In this prospective case-controlled study of 1054 patients with invasive pneumococcal disease (862 immunocompetent and 191 immunocompromised) it was found that the efficacy of the vaccine was related to the immune status and age of the individual and the time since vaccination. The vaccine was more effective in immunocompetent patients compared to those that were immunocompromised. If only the immunocompetent patients (862 patients) of the group were considered than the protective efficacy of the vaccine was 61% ($P < 0.00001$). If, however the immunocompromised patients (191patients) were considered than the efficacy fell to 21% and was not statistically significant. The efficacy of the vaccine decreased with increasing age of the patient. For example in immunocompetent patients aged under 55 years who had been vaccinated within the previous three years the efficacy was 93%. However in patients over 85 years of age the efficacy was only 46%. The efficacy of the vaccine also decreased with increasing time since vaccination. For example in those aged

under 55 years vaccinated within the previous three years the efficacy was 93% but this declined to 85% if five years or more had elapsed since vaccination (Shapiro *et al* ., 1991).

There are contrasting data on the efficacy of the vaccine in children. Infant trials in Australia (Douglas and Miles, 1984) gave disappointing results but trials in Papua New Guinea, where acute respiratory infections are common showed significant differences in the mortality rates of vaccinated and unvaccinated children. The efficiency of vaccination in reducing death from lower respiratory tract infections was estimated at 59% in children under 5 years old and 50% in children under 2 years of age (Riley *et al* ., 1986). Trials of the 23-valent vaccine in Gambian children also had variable results. All the Gambian children who were given the vaccine at 2, 4, 6 or 9 months of age developed moderate IgG responses to type 1, 3 and 5 but only older children developed antibodies to type 19F and 23F and very few children from any age group responded to type 6A (Temple *et al* ., 1991). Other trials in Finland focussing on the prevention of otitis media and bacteremia in children by the polysaccharide vaccine have given poor support for the use of the vaccine in children (Makela *et al* ., 1981). These trials have showed the following: (a) variable degrees of protection against infection was afforded by the serotypes to which the vaccinees responded at time of vaccination; (b) there was no immune response against serotype 6A and subsequently no reduction in illness caused by serotype 6A; (c) protection against type specific infection was apparent but it was for less than 6 months; (d) vaccination did not result in reducing the incidence of otitis media (Makela *et al* ., 1981). In view of the conflicting evidence, the vaccine is not currently recommended for use in children under 2 years of age in the USA and UK (Anon, 1990).

Elderly individuals have a decreased immune response (Fedson and Musher, 1994), but they still respond well to the polysaccharide vaccine. Many studies have shown that the antibody response following vaccination in healthy elderly individuals is similar to that in younger adults (Hilleman *et al* ., 1981; Ruben and Uhrin, 1985). The polysaccharide vaccine is considered to be effective in the elderly, but vaccine trials in elderly subjects have shown conflicting results. Two double-blind randomized controlled trials on the efficacy of the 12-valent in the elderly were done in the United States of America. One trial involving psychiatric patients at the Dorothea Dix hospital in North Carolina (Austrian, 1981) found no reduction in pneumonic illness

of vaccinees compared to unvaccinated controls. Also there was no bacteremia in either group. A difference in seroconversion between the two groups was observed. Vaccinees who had a two-fold or greater rise in serum antibodies against pneumococcal capsular polysaccharide types in the vaccine had no cases of radiological pneumonia, whereas the control group had 16 cases ($p<0.01$). The result of these findings were considered dubious without a concomitant pneumococcal isolate, because it is uncertain whether the seroconversion occurred from pneumococcal infection or from infection with a cross-reacting species (Austrian, 1981). Also seroconversion may not even occur from pneumococcal infection because its known that pneumococcal polysaccharides do not give rise to a secondary response (Bruyn and van Furth, 1991). The other trial at the Kaiser Permanente Medical Center in California (Austrian, 1981), also showed no reduction in pneumonic illness of vaccinated individuals. A lower incidence of bacteremia was observed between the vaccinated and unvaccinated individuals but this was not statistically significant. As in the previous trial there was a reduction (80%) in the incidence of radiologically confirmed pneumonia in vaccinated individuals who showed seroconversion (Austrian, 1981).

Another study by Forrester *et al .*, (1987) using 89 veterans found no difference in the bacteraemic rates in vaccinated individuals compared to control subjects. Moreover 65% of the blood isolates from nonvaccinated bacteremic patients were included in the vaccine compared with 69% of those in the vaccinated group. These studies cast doubt on the efficacy of the polysaccharide vaccine in the elderly but there have been other studies which shed a positive light on vaccination in the elderly. A study by Gaillat *et al .*, (1985) found that elderly French individuals vaccinated with the 14-valent vaccine had a 77.1% ($P < 0.0001$) reduction in the incidence of pneumonia. However this study was weakened by the lack of serotyping and microbiological data. Another study by Shapiro and Clemens (Shapiro and Clemens, 1984) comparing the frequency of antecedent immunization in groups with and without pneumococcal disease found that the efficacy of vaccination was 70% for healthy elderly patients over 55 years of age. Yet another study by Sims *et al .*, (1988) examined *Streptococcus pneumoniae* cultures from otherwise sterile body fluids in immunocompetent elderly. They found that 8% and 20.8% of the patients and controls respectively had been vaccinated indicating a vaccine efficacy of 70%. Despite the conflicting efficacy trial results the vaccine is currently recommended for all adults over 65 years of age however some experts suggest vaccination at 55 years of age

when the immune response may be better (Long and Kyllonen, 1997). Patients need to be revaccinated a few years later because the efficacy of vaccination decreases with increasing age and with increasing interval since vaccination (Shapiro *et al* ., 1991). For example Shapiro *et al* ., (1991) found that 3-5 years after vaccination the efficacy of vaccination was 89% in those under 55 years of age but decreased to 82% and and 71% in the 55-64 years and 65-74 years age groups respectively. The efficacy of vaccination in the 55-64 years age group was 93% during the 3 years after vaccination but decreased to 85% after 5 years following vaccination.

In other high risk individuals vaccination has varying protection. Amman *et al* ., (1977) have demonstrated the efficacy of vaccination in children or young adults with sickle cell disease or splenectomy. However most pneumococcal infections in this group occur before the age of two years when the antibody response to pneumococcal antigens is poor. Therefore penicillin prophylaxis between 6 months and 5 years (Gaston *et al* ., 1986) and vaccination at two years of age is recommended (Bruyn and van Furth, 1991). Individuals with chronic obstructive pulmonary disease have been reported to respond normally to vaccination (Musher *et al* ., 1986). Patients with Systemic Lupus Erythematosus (Klippel *et al* ., 1979) and Sjogren's syndrome (Karsh *et al* ., 1980) responded normally to the vaccine, however individuals with Hodgkins disease, multiple myeloma and bone marrow transplants exhibited a poor response to vaccination (Bruyn and van Furth, 1991).

In HIV infected patients the efficacy of the vaccine varied with the disease status of the patient. Patients with fewer than 500 CD4 lymphocytes per mm^3 produced less antibody to the vaccine than normal controls. However antibody levels in patients with greater than 500 CD4 lymphocytes per mm^3 were similar to those in normal controls (Rodriguez-Barradas *et al* ., 1992). Despite the variable efficacy of the vaccine, it is still recommended by the Centers for Disease Control for all patients with HIV infection regardless of disease stage.

1.6.5 Recommendations for pneumococcal vaccination

The groups for which the pneumococcal vaccine is currently recommended are shown in Table 1.3.

Immunocompetent individuals • Individuals over 50 years of age. • Patients with chronic illnesses in which there is an increased risk of pneumococcal disease. These include cardiovascular, obstructive pulmonary, renal and hepatic diseases, diabetes melitus and chronic cerebrospinal fluid leakage.
Immunodeficient individuals • Patients with asplenia and sickle cell disease. • Individuals with immunoglobulin and classical complement pathway deficiency. • Asymptomatic or symptomatic HIV patients. • Patients with Hodgkin disease.
High risk individuals in communities • Individuals in nursing homes, residential homes and other institutions. • Patients at high risk of influenza complications especially pneumonia. • Populations epidemiologically at risk from a generalised outbreak in the population due to a single pneumococcal type included in the vaccine.

Table 1.3. Groups for whom the pneumococcal vacine is recommended. (Adapted from Bruyn and van Furth, 1991; Pneumovax 23, Pasteur Merieux MSD).

1.6.6 Improvements to the pneumococcal vaccine

Due to the poor efficacy of the current 23 valent vaccine in infants and other groups at high risk from pneumococcal diseases, improvements to the vaccine are necessary. Bruyn and van Furth (1991) suggest that the new vaccine must meet the following requirements:

1) The vaccine should be very immunogenic, inducing at least a two fold increase in antibody levels against all polysaccharide antigens.

2) The antibody levels should persist for several years.

3) The new vaccine must be safe.

4) Efficacy should be demonstrated in a randomized double-blind placebo-controlled study.

Many approaches are being used to improve the current vaccine, these include conjugating polysacchrides to proteins (Anderson and Betts, 1989; Schneerson *et al* ., 1986; Peeters *et al* ., 1991), addition of adjuvants to improve antigenic presentation (Zigterman *et al* ., 1988) and incorporating semisynthetic serotypes in liposomes (Snippe *et al* ., 1983). Of these the strategy of conjugating

pneumococcal polysaccharides to proteins appears to be most promising (Bruyn and van Furth, 1991) and is therefore discussed in detail in Section 1.6.7.

Either as an alternative to or as a complement to the existing polysaccharide vaccine, the use of pneumococcal proteins as immunogens is also being investigated. These are discussed in Section 1.6.7.6.

1.6.7 Conjugate vaccines

1.6.7.1 Introduction

The inclusion of protein antigens in the vaccine on their own or in conjugation with polysaccharides may improve the immunogenicity of the vaccine.

Unlike polysaccharides, proteins are thymus dependent antigens and immune response to them requires the cooperation between B and T lymphocytes and cytokine mediated events (Noelle and Snow, 1991). Thymus dependent antigens are immunogenic in children, for example healthy infants colonized/infected with *S.pneumoniae* produce an antibody response to pneumococcal proteins which is similar to that observed in adults (Alonsodevelasco *et al* ., 1995). Thymus dependent antigens also induce memory and a booster effect is observed upon repeated immunizations (Alonsodevelasco *et al* ., 1995). They also induce a larger ratio of IgG to IgM (Mitchell and Andrew, 1995).

Many studies (Goebel and Avery, 1929; Paul *et al* ., 1971) show that conjuagation of polysaccharide to protein leads to an enhanced immune response to the polysaccharide and results in protective immunity against *S.pneumoniae* (AlonsoDeVelasco et al ., 1993; Snippe *et al* ., 1983). This enhanced immunogenicity is attributed to the conversion of the polysaccharide from a thymus independent antigen to a thymus dependent antigen (Schneerson *et al* ., 1980). The benefits of conjugating protein to polysaccharides were further confirmed by the success of *Haemophilus influenzae* type b (Hib) conjugated vaccines. *Haemophilus influenzae* type b capsular polysaccharide-diphtheria toxoid conjugate vaccine (polyribosylribitol phosphate-diphtheria toxoid [PRP-D]) was shown to be 94% effective in

protecting Finnish infants against *H. influenzae* type b infections (Eskola *et al* ., 1990).

1.6.7.2 Examples of conjugate vaccines

There are many reports of conjugating pneumococcal capsular polysaccharides to a variety of carrier molecules. Some of these are shown in Table 1.4. A few of these are discussed in greater detail below.

A pentavalent vaccine containing capsular polysaccharide of serotypes 6B, 9V, 14, 18C and 23F conjugated to a nontoxic variant of diphtheria toxin: cross reacting molecule 197(CRM_{197}) was shown to be safe and immunogenic when given as a three dose series to infants at 2, 4, and 6 months of age (Åhman *et al* ., 1996; Leach et al ., 1996). Furthermore vaccination with a booster dose of the licensed polysaccharide vaccine results in an increase in titres of antibodies to serotypes in the pentavalent vaccine (Käyhty *et al* ., 1996). However it was found that if serotypes 4 and 19F were added to the pentavalent vaccine then the potential to prevent infections would increase from 65% to 85% (Butler *et al* ., 1995).

Name	Polysaccharides	Protein	Reference
PNCRM5	6B, 9V, 14, 18C, 23F	Diphtheria toxin cross reactive molecule 197 (CRM_{197})	Åhman *et al* ., 1996.
PNCRM7	4, 6B, 9V, 14, 18C, 19F, 23F	CRM_{197}	Rennels *et al* ., 1998.
PncD	6B, 14, 19F, 23F	Diphtheria toxoid	Åhman *et al* ., 1988.
PncT	6B, 14, 19F, 23F	Tetanus toxoid	Nieminen *et al* ., 1998
_	6B, 14, 19F, 23F	Meningococcal outer membrane protein (MOMP)	Vella *et al* ., 1992

Table 1.4. Examples of some pneumococcal conjugate vaccines

To this end Rennels *et al.* (1998) conjugated polysaccharide of serotypes 4, 6B, 9V, 14, 18C, 19F and 23F to CRM_{197} (PNCRM7) and vaccinated infants at 2, 4, and 6 months of age. A booster dose of the same vaccine was administered at 12 and 15 months of age. Results from this study showed that vaccination with PNCRM7 was safe and led to significant increases in antibody to all 7 serotypes. Also comparison of antibody concentrations after the three dose primary series with those achieved after the PNCRM5 vaccinations (Daum *et al* ., 1997) suggested that the addition of serotypes 9V and 4 to the pentavalent vaccine did not lead to decreased immunogenicity. Another important observation from this study was that there was no increase in rate or severity of local reactions with repeated doses of the vaccine. This had been a concern because revaccination with the polysaccharide vaccine within a few years of the first immunization resulted in local erythema, swelling, and pain attributed to formation of antibody-antigen complexes at injection site (Borgono *et al* ., 1978). The different reactogenicities may be explained by the lower amount of polysaccharide in the PNCRM7 vaccine (16μg) compared to that in the 23-valent polysaccharide vaccine (573μg) (Rennels *et al* ., 1998).

To determine the efficacy of the PNCRM7 vaccine against otitis media and invasive disease two prospective controlled trials are underway in Finland and North California (Rennels *et al* ., 1998).

The Californian trial is at Kaiser Permanente, a prepaid health maintenance organization. The first phase of the trial involving 300 infants has been completed. It evaluated the safety and immunogenicity of the conjugate. Overall systemic and local reactions occurred less frequently with the pneumococcal vaccine than with the diphtheria toxoid-tetanus toxoid-whole-cell pertussis vaccine or the *H. influenzae* type b (DTP/HbOC) vaccine. No severe allergic or neurologic reactions were noted. The geometric mean titres (GMT) after the third dose were between 0.98 and 3.42 μg/mL and fell to between 0.24 and 1.95 μg/ml before the fourth dose. After the booster dose all GMTs were above 1.0μg/ml. The efficacy portion of the study is still in progress. It involves 35,000 infants and the primary and secondary end points are the determination of the vaccines effectiveness against invasive pneumococcal disease and otitis media respectively (Shinfield, 1998).

Another conjugate vaccine currently under investigation is the nine-valent pneumococcal vaccine conjugated to CRM197. The pneumococal disease

research unit in Johannesburg, South Africa conducted a double-blind, randomised, prospective clinical trial in 1997 to assess the immunogenicity, safety and impact on carriage of this vaccine. The results showed the presence of high levels of antibody four weeks following the third dose of the vaccine. Significant antibody responses were obtained to all nine antigens included in the vaccine and the nasopharyngeal carriage of all serotypes was reduced. There was an increase in carriage of nonvaccine serotypes which the authors suggested was caused by the unmasking of previously hidden subdominant strains or from the acquisition of extra strains. A phase III, double-blind, prospective vaccine trial is currently underway in Soweto, South Africa. The primary end points of this study are protection against serotype-specific invasive pneumococcal disease and protection against radiologically confirmed pneumonia. Secondary end points include protection against invasive disease and pneumonia among HIV-infected children (Klugman, 1998).

1.6.7.3 Future of conjugate vaccines

Given the promising results of efficacy trials, the conjugate vaccines may be in use by the end of this century. Their immunogenicity in children will enable them to be included in routine childhood immunization schedules and reduce the impact of pneumococcal disease. A remaining challenge will be the reduction of manufacturing costs of such vaccines so that they are available to all the children in the world (Siber, 1994).

The use of a conjugate vaccine may reduce high childhood mortality rates in developing countries, however the geographic and temporal variation in pneumococcal strains isolated from sick children suggests that a species-wide, protein based vaccine may provide more widespread protection (Sniadack *et al* ., 1995). A protein that would be useful for inclusion in a pneumococcal vaccine is pneumolysin (Section 1.6.7.6.1). Pneumolysin is produced by all clinical isolates (Paton *et al* ., 1993) and there is little variation in its primary structure (Mitchell *et al* ., 1990). Although the native protein is toxic and therefore unsuitable for inclusion in a human vaccine, genetically detoxified derivatives have been developed. One such toxoid (PdB) containing a Trp>Phe mutation at position 433 which reduces its haemolytic activity by 99% has been shown to protect mice immunized with it against challenge with pneumococci of nine different serotypes (Alexander *et al* ., 1994). Moreover immunization of

mice with a single dose of a conjugate of PdB and 19F polysaccharide (a polysaccharide which is poorly immnogenic in young humans and mice) has been shown to induce high titres of antibody against the polysaccharide and the pneumolysin compared to mice immunized with the polyaccharide alone (Paton *et al* ., 1991). A significant boost in antibody levels after one or two additional doses was also observed. Additionally the immunized mice survived longer than control mice following challenge with virulent pneumococci (Lee *et al* ., 1994). These results point to the potential of the pneumolysoid-polysaccharide conjugate as human vaccine components.

1.6.7.4 Factors affecting conjugate vaccine immunogenicity

The immunogenicity of conjugate vaccines is influenced by many factors including the length and terminal structure of the saccharide and the type of coupling chemistry used (AlonsoDeVelasco *et al* ., 1995). Another important consideration is the nature of the carrier protein (Van de Wijgert *et al* ., 1991). Until recently the carrier proteins were chosen on the basis of their T-cell activating capacities (AlonsoDeVelasco *et al* ., 1995). Therefore many commonly used childhood vaccines had the same carrier protein, such as diphtheria (Seppälä *et al* ., 1988) or tetanus toxoids. Using the same carrier proteins in a pneumococcal vaccine would avoid the need to introduce new agents and may provide a booster effect (Andrew *et al* ., 1994). However the repeated exposure to the same carrier protein may lead to non-specific suppression of the response to the carried epitopes. This phenomenon is called carrier-induced epitope suppression (Peeters *et al* ., 1991) has been described for synthetic peptides coupled to tetanus toxoid (Gaur *et al* ., 1990). The carrier molecule for a pneumococcal vaccine will have to be chosen carefully as the pneumococcal vaccines will contain multiple doses of the carrier protein (one for each type of polysaccharide). Also the repeated use of a particular carrier may cause hypersensitivity reactions at the site of injection due to the deposition of immune complexes.

1.6.7.5 Limitation of conjugate vaccines

Polysaccharide-protein conjugate vaccines have overcome many of the limitations of the polysaccharide vaccines but they still have some disadvantages. Firstly pneumococcal conjugate vaccines induce an immunity that may be short lived, particularly in infants (Sell *et al* ., 1981). To overcome

this, frequent vaccinations would be required during the first few years of life which would be expensive in developed and developing countries. Secondly the regional variation in predominant infecting serotypes means that custom made vaccines suited to the regional epidemiology are required (Giebink, 1994). These are both expensive and technologically difficult. For example, a vaccine designed to prevent invasive disease in the USA may be unsuitable for use in other regions of the world because of the difference in serotype prevalence in various locales (Butler, 1997). Furthermore the serotype prevalence in many countries may need to be monitored continuously to ascertain whether major changes in serotype prevalence are occuring. For example a study by Finland and Barnes (1977), found that serotypes prevalent in Boston, MA, altered considerably over forty years. Thirdly pneumococci are able to take up heterologous DNA (Tomasz and Hotchkiss, 1964) and change their capsular serotype which means that protection from anticapsular antibodies may be short lived as vaccine types change their surface polysaccharide in response to mucosal antibodies (The Jordan Report, NIH, 1998). Fourthly, because of economic and immunologic considerations (Rennels *et al* ., 1998) fewer serotypes can be included in the conjugate vaccine giving a narrower spectrum of coverage (Moore *et al* ., 1998). The inclusion of a large number of serotypes would be expensive and the presence of multiple doses of carrier protein (one for each serotype) would increase the chance of carrier-induced suppression. Therefore the development of a 23-valent conjugate pneumococcal vaccine is unlikely.

To address these problems research has focused on developing a common antigen vaccine. In particular pneumococcal proteins generating cross serotype protection are being investigated to act as carriers in a conjugate vaccine or as antigens in their own right (Andrew *et al* ., 1994). These proteins are discussed in Section 1.6.7.6.

1.6.7.6 Pneumococcal proteins in vaccination

As previously discussed the current polysaccharide vaccine has two major limitations. Firstly it is poorly immunogenic in certain age groups such as the elderly and infants. Secondly the protection is serotype specific and of the 90 different serotypes that exist only 23 are covered by the vaccine. Polysaccharide-protein conjugate vaccines currently under clinical trials have addressed the problem of poor immunogenicity in young children. However because of

economic and practical considerations the number of capsular polysaccharides included in the conjugate formulations is likely to be less than 23, therefore the problem of serotype coverage persists (Paton, 1998). An octavalent conjugate vaccine is more likely providing 60-70% coverage in most areas. To provide protection against the remaining 30-40% of pneumococcal isolates, much attention has been focused on pneumococcal protein antigens that are common to all serotypes (Andrew *et al* ., 1994). Pneumococcal proteins would provide cross-serotype protection and be highly immunogenic and induce immunological memory because of their T-cell dependent nature (Paton, 1998). There are many pneumococcal proteins that may be involved in virulence and have potential as protective immunogens. These include, autolysin, pneumolysin, pneumococcal surface protein A (PspA) and neuraminidase (Andrew *et al* ., 1994). The latter three proteins will be discussed in greater detail.

1.6.7.6.1 Pneumolysin

Pneumolysin is a 53-kDa protein produced by all clinical isolates of *S.pneumoniae* (Paton *et al* ., 1993). It is a member of the thiol-activated cytolysins family of toxins which are produced by many Gram positive bacteria (Paton *et al* ., 1993). These toxins undergo irreversible inactivation on treatment with cholesterol and are susceptible to inactivation by oxidation that can be reversed by thiol-reducing agents (Rubins and Janoff, 1997). They also share a common two-step mode of action. The first stage involves binding of the toxin to the membrane cholesterol followed by temperature-dependent insertion of pneumolysin into the lipid bilayer. The next stage involves the lateral diffusion and oligomerization of 20-80 toxin molecules to form pores 30-35nm in diameter which are visible by electron microscopy as arc or ring structures. (Bhakdi and Tranum-Jensen, 1986). These pores cause cytolysis by leakage of cytosolic proteins (Bhakdi and Tranum-Jensen, 1986). Pneumolysin is not actively secreted but is located within the cytoplasm (Johnson, 1977), it is released by the action of pneumococcal autolysin on the cell wall (Boulnois, 1992).

At sublytic concentrations pneumolysin has many effects which may contribute to the pathogenesis of disease. Certain activities such as the bactericidal action of phagocytes, neutrophil migration (Paton and Ferrante, 1983), lymphocyte proliferation and antibody synthesis are inhibited by

pneumolysin (Ferrante *et al* ., 1984). However other activities such as production of inflammatory cytokines like tumor necrosis factor alpha and interleukin-1ß by human monocytes are stimulated by pneumolysin (Houldsworth *et al* ., 1994). Another important activity of pneumolysin is its ability to activate the classical complement pathway in the absence of anti-toxin antibody (Paton *et al* ., 1993). Also pneumolysin has effects in other biological systems. For example pneumolysin inhibits ciliary beating of human respiratory tract epithelium, disrupts cultured epithelial cell monolayers from the upper respiratory tract and from alveoli (Feldman *et al* ., 1990). It causes physiological and anatomical changes in guinea-pig cochlea in a model of toxin-induced deafness (Comis *et al* ., 1993). Therefore pneumolysin may enhance inflammation by its ability to activate complement, disrupt respiratory tract epithelium and stimulate production of cytokines (Alonsodevelasco *et al* ., 1995).

There is considerable evidence to implicate pneumolysin in disease. Firstly the increase in anti-pneumolysin antibody titres during infection suggest that it is produced during infection (Jalonen *et al* ., 1987). Secondly mice immunized with native pneumolysin are protected to a certain degree against challenge with virulent pneumococci (Paton et al ., 1983). Also in the same murine model a pneumolysin-negative mutant showed reduced virulence and faster clearance from the bloodstream compared to wild-type parents (Berry et al ., 1989).

The importance of pneumolysin in pneumococcal pathogenesis and its immunogenicity in man makes it ideal for inclusion in conjugate vaccines (Rubins and Janoff, 1998). Native pneumolysin can not be used because of its toxicity but pneumolysin derivatives with reduced toxicity can be used (Boulnois *et al* ., 1991). Structure-function studies on pneumolysin (Boulnois *et al* ., 1991) have led to the production of pneumolysin derivatives lacking anti-cellular and/or complement-activating activity (Andrew *et al* ., 1994). In experimental animals the mutated pneumolysins were well tolerated and were as immunogenic as wild type toxin (Paton *et al* ., 1991). Studies have shown that mice immunised with either of two mutant toxoids in Freund's adjuvant survived longer than unimmunised mice upon challenge with type 2 pneumococci (Paton *et al* ., 1991).

Further studies on the Trp433>Phe toxoid show the potential of pneumolysin derivatives. The Trp433>Phe toxoid has a single amino acid change and less than 1% of the cytolytic activity of wild-type toxin but complete complement-activating activity (Andrew *et al* ., 1994). Mice were immunized with the Trp433>Phe toxoid and then challenged either intraperitoneally or intranasally with pneumococci covering nine different serotypes. It was found mice immunized with the Trp433>Phe toxoid produced significantly higher levels of anti-pneumolysin antibodies and survived longer than non immunized mice when challenged with nine strains of pneumococci (Alexander *et al* ., 1994). However the degree of protection varied according to the strain used and and the route of challenge. For example immunization with the Trp433>Phe toxoid did not protect against intranasal challenge with type 3 strain GB05, but did protect against intranasal challenge with another type 3 strain. Also immunization with Trp433>Phe toxoid protected against intraperitoneal challenge with type 3 strain GB05 but not against intranasal challenge with the same strain. The absence of protection afforded by Trp433>Phe toxoid against intranasal challenge with type 3 strain GB05 suggested that other pneumococcal immunogens should be explored in addition to pneumolysin (Alexander *et al* ., 1994).

The benefits of conjugating a bacterial polysaccharide to a protein carrier have been highlighted by the recent success of *H. influenzae* conjugate vaccines (Eskola *et al* ., 1990). Conjugate vaccines induce high levels of polysaccharide specific antibodies and booster responses in infants and children (Rubins and Janoff, 1998). A conjugate vaccine linking pneumolysin to pneumococcal polysaccharide would also have the benefit of inducing protective neutralizing antibodies to pneumolysin that may prevent pneumolysin induced injury and also protect against serotypes whose polysaccharides are not covered in the vaccine (Rubins and Janoff, 1998). A pneumococcal conjugate was produced by linking the Trp433>Phe toxoid to type 19F pneumococcal polysaccharide (Paton *et al* ., 1991). When mice were immunized with this toxoid they produced higher levels of anti-polysaccharide antibodies compared to mice given the 19F polysaccharide alone. Moreover a booster effect was observed following one or two additional doses (Paton *et al* ., 1991). Pneumolysin stimulates mucosal immunity in murine models so pneumococcal-polysaccharide conjugate vaccines can potentially induce local immunity to protect against mucosal injury caused by pneumolysin during respiratory infections (Rubins and Janoff, 1998).

1.6.7.6.2 Pneumococcal surface protein A (PspA)

Another promising immunogen is pneumococcal surface protein A (PspA). McDaniels *et al* ., (1981) demonstrated the presence of this protein in pneumococcal cell walls by the use of monoclonal antibodies. The surface location of PspA means that antibodies produced against it are likely to be opsonic and lead to bacterial clearance. This is in contrast to pneumolysin antibodies which most probably act by neutralizing the toxin (Mitchell *et al* ., 1997).

PspA is highly variable in both molecular weight and immunologically but is produced by all pneumococci (Crain *et al* ., 1990) and is necessary for full virulence (McDaniel *et al* ., 1987). There is a lot of evidence suggesting that PspA should be considered as a protective immunogen. For example passive immunization with polyclonal and monoclonal antibodies against PspA provides protection against pneumococcal infection in mice (McDaniel *et al* ., 1984; McDaniel *et al* ., 1991). Normally immunization of mice with rough pneumococci protects against challenge by virulent pneumococci, however if the PspA is inactivated prior to immunization then no protection is observed. Moreover it has been demonstrated that in the first hour following intravenous challenge of mice, PspA-deficient pneumococci were cleared faster than isogenic wild-type pneumococci. This finding correlates with the longer median survival time of the PspA challenge group (McDaniel *et al* ., 1987).

Cloning of the coding sequence from PspA of a capsular serotype 2 strain has allowed predictions to be made on the probable structure of PspA (Yother and Briles, 1992). There are four structural domains consisting of an amino terminal highly charged α-helical coiled coil, a proline rich area of 82 amino acids, a carboxy terminal comprising 10 highly conserved 20-amino acid repeats followed by a 17 amino acid tail. The carboxy terminus of PspA is involved in attachment of the protein to the cell (Paton *et al* ., 1993). Antigenic and size variations in the PspA from different *S.pneumoniae* strains (Paton, 1998) is accounted for by variations in the amino terminal region.

There are at least 30 dfferent serotypes of PspA, and these are widely distributed through the capsular serotypes (Crain *et al* ., 1990). This high serological variation could prove to be limiting in vaccine development (Andrew *et al* ., 1994). However there is evidence to support cross capsular serotype protection

(Andrew *et al* ., 1994). Talkington *et al* ., (1991) have purified the 43-KDa amino terminal half of PspA from type 2 pneumococal culture and used it to immunise mice. The mice were protected upon subsequent challenge with pneumococci from two different capsular serotypes but not from challenge with a type 2 strain (Talkington *et al* ., 1991). Another study by McDaniel *et al* ., (1991) showed that recombinant PspA cloned from D39 (type 2 strain) was able to elicit cross-protection against three pneumococcal isolates of two different capsular serotypes (McDaniel *et al* ., 1991). These results indicate that unlike the polysaccharide vaccine, immunization with a single PspA will protect against strains of more than one capsular type (McDaniel *et al* ., 1991).

It has been found that in PspA from a Rx1 strain (Capsular serotype 2), the epitopes eliciting protection are located between amino acids 192 and 260 in the α-helical region of the amino terminus (McDaniel *et al* ., 1994). PCR analysis of this region has shown that it is very conserved (McDaniel *et al* ., 1994). Therefore proteins or peptide containing this region may be useful as vaccine components.

1.6.7.6.3 Neuraminidase

Neuraminidase is the main topic of this study and is discussed in detail in Section 1.7.

1.7 Neuraminidase

1.7.1 Introduction

Sialidases (neuraminidases, N-acylneuraminosyl glycohydrolases EC 3.2.1.18) are enzymes that catalyze the hydrolytic removal of terminal sialic acid residues from oligosaccharides, gangliosides and glycoproteins (Sweeley, 1993). Sialidases are found principally in higher eukaryotes, in some viruses, bacteria and protozoans (Vimr, 1994). The various aspects of sialidases including their occurence, biological function and purification are discussed below.

1.7.2 Historical background

The initial work implicating the existence of a sialidase was done by Hirst in 1941. He suggested that an enzyme was responsible for the release of influenza virus from the surface of chicken erythrocytes that had been agglutinated by the virus (Hirst, 1941). The enzyme was termed "receptor destroying enzyme" (RDE) when it was discovered that extracts of *Vibrio cholerae* and *Clostridium perfringens* also destroyed the receptor sites for influenza virus on the surface of human erythrocytes (Burnet *et al* ., 1946). Further studies demonstrated that the RDE removed sialic acids from glycoproteins (Gottschalk and Lind, 1949). The name "sialidase" was proposed when it was found that pneumococcal extracts caused the release of sialic acid from salivary mucins (Heimer and Meyer, 1956). Consequently the name neuraminidase was proposed (Gottschalk, 1957) and both names have been used interchangeably ever since.

1.7.3 Occurence of neuraminidases

Neuraminidases are widely distributed in nature (Schauer, 1982). They have been found in viruses, bacteria (Drzeniek, 1972; Rosenberg and Schengrund, 1976; Schauer, 1982; Corfield, 1992), in protozoa such as *Trypanosoma cruzi* (Pereira, 1983) and in animals including human tissue (Rosenberg and Schengrund, 1976). Examples of some neuraminidase producing organisms are given in Table 1.5.

In viruses, neuraminidases are found in the orthomyxoviruses (e.g. influenza virus), in paramyxoviruses (e.g. Newcastle disease virus) and also in metamyxoviruses (e.g. respiratory syncytial viruses). Viral neuraminidases are localized on the outer surface of the virion (Drzeniek, 1973). They are linked to the virus by non-covalent bonds (Drzeniek, 1973). The neuraminidases from influenza viruses have been well characterized. The X-ray structures of many influenza subtypes have been determined (Varghese *et al* ., 1983; Burmeister *et al* ., 1992), and these are being used to design inhibitors of the virus (von Itzstein *et al* ., 1993).

In bacteria the majority of neuraminidase producing organisms belong to two taxonomic orders, the *Eubacteriales* and *Pseudomonadales* (Rosenberg and Schengrund, 1976). The ability to produce neuraminidase can vary between closely related species (Roggentin *et al* ., 1993) or even within strains of the

Organism	Reference
Viruses	
Influenza A	Colman, 1994
Influenza B	Burmeister *et al* ., 1994
Newcastle Disease Virus	Iorio *et al* ., 1989
Bacteria	
Actinomyces viscosus	Henningsen *et al* ., 1991
Bacteroides fragilis	Tanaka *et al* ., 1992
Bacteroides loeschii	Takeshita*et al* ., 1991
Clostridium chauvoei	Heuermann *et al* ., 1991
Clostridium perfringens	Roggentin *et al* ., 1988
Clostridium septicum	Rothe *et al* ., 1991
Corynebacterium aquaticum	Sondag-Thull *et al* ., 1989
Klebsiella aerogenes	Pardoe, 1970
Micromonospora viridifaciens	Sakurada *et al* ., 1992
Pasteurella haemolytica	Straus *et al* ., 1993
Pseudomonas aeruginosa	Leprate and Michel-Briand, 1980
Salmonella typhimurium	Hoyer *et al* ., 1991
Streptococci Group B	Milligan *et al* ., 1980
Streptococcus pneumoniae	Hughes and Jeanloz, 1964
Streptococcus sanguis	Varki and Diaz, 1983
Vibrio cholerae	Ada *et al* ., 1961
Protozoa	
Acanthamoeba castellanii	Pellegrin *et al* ., 1991
Endotrypanum Sp	Medina-Acosta*et al* ., 1994
Trypanosoma cruzi	Schenkman *et al* ., 1994
Mammals	
Chicken (embryo tissue)	Cook and Ada, 1963
Human (intestinal mucosa)	Ghosh *et al* ., 1968
Rabbit (kidney)	Mahadevan *et al* ., 1967
Sheep (urinary tract)	Candiotti *et al* ., 1972

Table 1.5. Neuraminidase producing organisms

same species (Hoyer *et al* 1992). Interestingly many neuraminidase producing bacteria have a close association with animals as commensals or as pathogens (Roggentin *et al* ., 1993). Although some of these bacterial neuraminidases have been implicated in disease (Corfield 1992), it is thought that their main function is nutritional (Section 1.7.7.4.3) (Hoyer et al ., 1992; Taylor *et al* ., 1992). The neuraminidase may be cell bound or secreted. The cell-bound enzyme may have a purpose in the periplasmic space or it may be in a stored form prior to release (Corfield, 1992). Examples of cell associated neuraminidases include the enzymes from *Pasteurella multicocida* (Scharmann *et al* ., 1970) and *Klebsiella aerogenes* (Pardoe, 1970). Secreted neuraminidases include those of *Vibrio cholerae* (Burnet and Stone, 1947) and *Clostridium perfringens* (McCrea, 1947). The enzyme activity can be induced by sialic acids, sialo-glycoconjugates and related compounds (Corfield, 1992). It has been demonstrated that the production of pneumococcal neuraminidase can be induced by adding sialic acid to the culture medium (Kelly *et al* ., 1966). Similarly the production of *Klebsiella aerogenes* neuraminidase can be induced by the addition of sialoglycocongugates to the culture medium (Pardoe, 1970).

Mammalian neuraminidases have been less well characterized by comparison to bacterial and viral neuraminidases. This is because of difficulties in their purification (Schauer, 1982). Neuraminidase activity has been detected in many mammalian organs and may be an integral component of organs containing sialyl compounds. Examples of organs which have sialidase activity include the brains of humans and pigs, liver and kidney of rats and ram testis (Rosenberg and Schengrund, 1976).

1.7.4 Action of neuraminidase

Neuraminidases catalyze the hydrolytic removal of α-glycosidically linked sialic acids from sialylated glycoconjugates (Figure 1.1) (Sweeley, 1993). The result is the formation of the free sialic acid and the desialylated glycoconjugate. The importance of this reaction is best understood by an appreciation of the importance of sialic acids in biological systems. Therefore the structure and function of these molecules in biological systems is discussed below.

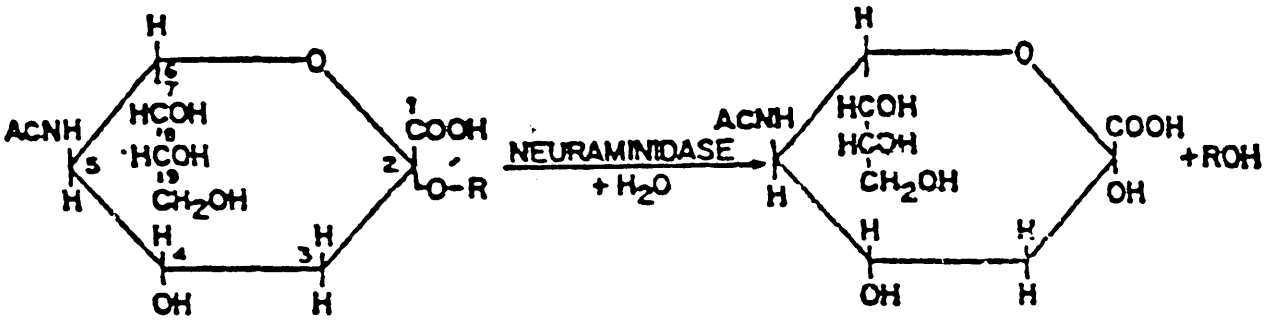


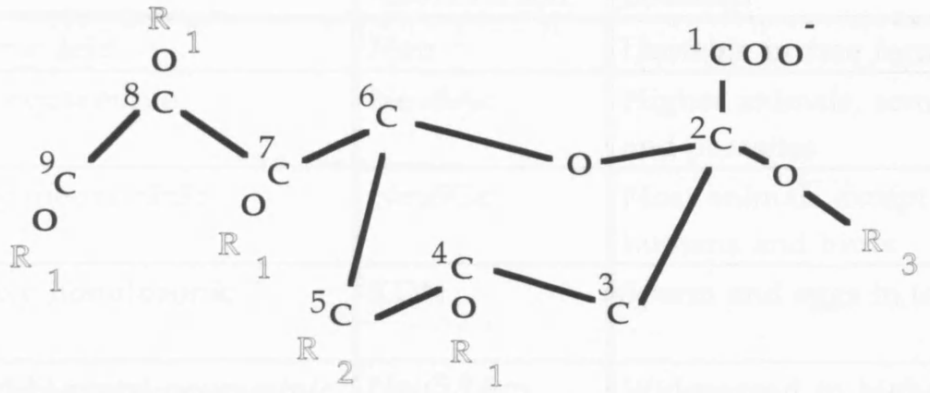
Figure 1.1 : (Taken from Drzeniek, 1972).
The release of N-acetyl neuraminic acid from N-acetyl neuraminic acid α-ketoside by the action of neuraminidase. R = Monosaccharides, oligosaccharide, aromatic or aliphatic alcohols, glycoproteins, glycolipids. Ac = acetyl or glycolyl in naturally occurring substances. The carbon atoms are numbered from 1 - 9.

1.7.5 Sialic acids

1.7.5.1 Structure

The sialic acids are a group of 9-carbon carboxylated sugars generally found as terminal monosaccharides of animal oligosaccharides (Varki, 1992). The general structure of sialic acid is shown in Figure 1.2. The term sialic acid was contrived by Blix, Gottschalk and Klenk in 1957 and covers all N- and O- acyl derivatives of neuraminic acid (Neu) isolated from natural materials (Schauer, 1982).

The commonest sialic acid, N-acetylneuraminic acid (Neu5Ac) (Pilatte *et al* ., 1993) was first isolated by Gottschalk (1951) following the action of influenza viruses on urinary mucins. N-acetylneuraminic acid is believed to be the precursor for all other sialic acids (Pilatte *et al* ., 1993). Modifications of Neu5Ac are extensive, and result in the high diversity of sialic acids (reviewed by Varki, 1992). For example the hydroxylation of the N-acetyl group leads to the formation of N-glycolyl-neuraminic acid (Neu5Gc) (Varki, 1992). The replacement of the 5-amino group by a hydroxyl group leads to the formation of 2-keto-3-deoxy-nonulosonic acid (KDN) (Varki, 1992). The substitution of the hydroxyl groups of Neu5Ac, Neu5Gc or KDN with acetyl, methyl, lactyl, phosphate or sulphate groups gives rise to most other sialic acids (Varki, 1992.



R_1 = H, Acetyl (4,7,8,9), Lactyl (9), Methyl (8), Sulphate (8), Phosphate (9), Anhydro (4,8 or 2,7), Sialic Acid (8,9), Fucose (4), Glucose (8), or Galactose (4)

R_2 = N-Acetyl, N-Glycolyl, Amino, Hydroxyl

R_3 = Gal (3/4/6), GalNAc (6), GlcNAc (4/6) or Sialic Acid (8/9) (Absent in 2,6 and 3,7 anhydro compounds)

The numbers in brackets refer to the carbon atom where the substitutions indicated have been found.

Figure 1.2. The general structure of sialic acids (Adapted from Varki, 1992)
The 9-carbon backbone prevalent in all sialic acids is displayed in the chair conformation. Natural substitutions described to date at positions R_4, R_5, R_7, R_8 and R_9 are shown. Greater diversity is obtained by different types of glycosidic linkages at R_2, by generation of lactones at R_1, by dehydro forms (eliminating R_2) and anhydro forms.

The diversity of the sialic acids is further increased by unsaturated and dehydro-forms and by different linkages to the sugar chain (Varki, 1992). More than 30 sialic acids differing in the nature of the N-substituent of neuraminic acid and in the position, number or combination of O- substituents have been identified to date and some of these are listed in Table 1.6. The biosynthetic pathways leading to modifications of Neu5Ac have been reviewed by Varki (1992).

Name	Abbreviation	Location
Neuraminic acid	Neu	Unstable in free form.
N-acetyl-neuraminic acid	Neu5Ac	Higher animals, some bacteria and parasites
N-glycolyl-neuraminic acid	Neu5Gc	Most animals except adult humans and birds
Keto-deoxy-nonulosonic acid	KDN	Sperm and eggs in teleost fish
9-O-acetyl-N-acetyl-neuraminic acid	$Neu5,9Ac_2$	Widespread in higher animals and certain bacteria
9-O-lactyl-N-acetyl-neuraminic acid	Neu5Ac9Lt	Found in higher animals in bound and free forms
8-O-methyl-N-acetyl-neuraminic acid	Neu5Ac8Me	Found in echinoderms
9-O-lactyl-2,3 didehydro 2,6 anhydro-N-acetyl-neuraminic acid	Neu2en5Ac9Lt	Porcine submandibular gland

Table 1.6. Some commonly found sialic acids, (Adapted from Varki, 1992).

1.7.5.2 Occurrence of sialic acids

In nature, sialic acids are found in most higher animals and in some bacteria, viruses and protozoa (Schauer, 1982). In bacteria the sialic acids always contain N-acetylneuraminic acid occasionally with side chain O-acetylation. Recently the presence of 4-O-acetylated Neu5Ac in Salmonella has been reported (Reuter and Gabius, 1996). The relationship between the presence of sialic acid and pathogenicity is unclear since both the highly pathogenic Neisseria and non-pathogenic Rhodobacter (Reuter and Gabius, 1996) contain sialic acid. In meningococci and gonococci, the sialylation of the lipopolysaccharide has been shown to affect pathogenicity (Smith *et al* ., 1995), however in other bacteria such as *Haemophilus influenzae* and *Campylobacter jejuni* the influence of sialylation on pathogenicity has not been assessed (Smith *et al* ., 1995).

In viruses, sialic acids have a role in their adhesion to cell membranes (Reuter and Gabius, 1996). Sialic acids are also present in the surface glycoprotein of

many viruses including Epstein-Barr virus and human immunodeficiency virus. A definite correlation between the presence of sialic acid and pathogenicity for viruses has not been documented (Reuter and Gabius, 1996).

Generally sialic acids are not found in plants (the exception being buckwheat) and lower invertebrates (Rosenberg and Schengrund, 1976). The only report of the presence of sialic acid in insect has been in *Drosophila melanogaster*, where N-acetylneuraminic acid has been detected during the embryo stage (Reuter and Gabius, 1996).

Usually sialic acids occur in low concentrations as free molecules in biological materials, in human urine and serum (Schauer, 1982), in tissues having an active sialic acid metabolism (for example , bovine submandibular glands , and human saliva (Schauer, 1982). Higher concentrations of free exogenous sialic acids have been found in digestive glands of lobsters , fish eggs and in bodily fluids of human diseases such as sialuria (Schauer, 1982).

Sialic acids are components of oligosaccharides, polysaccharides and glycoconjugates (Ng and Dain, 1976). Sialic acids are usually bound to other sialic acids, galactose (Gal), 2-acetamido-2-deoxy-D-galactose (GalNAc), or 2-acetamido-2-deoxy-D-glucose (GlcNAc) (Schauer, 1982). Linkage of sialic acid molecules to each other usually occurs via a α–(2–8) bonds. The repetition of this bonding leads to formation of oligosialyl groups found in gangliosides and glycoproteins (Schauer, 1982; Ng and Dain, 1976). In complex carbohydrates, sialic acids are often linked to Gal by α–(2–3) or α–(2–6) linkages. Binding at GalNAc residues usually occurs at O-6.

Sialic acids are often found at terminal positions of oligosaccharide chains. In glycoproteins, gangliosides and oligosaccharide sialic acids may form a branch by binding to the side position of an oligosaccharide chain. GlcNAc, Gal or GalNAc bind to sialic acids in the aforementioned manner (Schauer, 1982).

1.7.5.3 Distribution of sialic acids

Current opinion indicates that distribution of modified sialic acids is species specific and depends on the cell type and its developmental stage (Varki, 1992; Schauer *et al* ., 1995).

Some sialic acid modifications are even molecule specific, and are found only on certain types of glycoconjugates in a given cell type (Varki, 1992). The modification may be specific for a certain sialic acid residue in a particular group of molecules. For example 9-O-acetylation in the ganglio series gangliosides is found only on a specific terminal α2-8-linked sialic acid residue (Varki, 1992). These findings suggest a very specific role for these modifications in tissue development and/or organization (Varki, 1992).

1.7.5.4 Functions of sialic acids

Due to their wide distribution in exposed positions of many biological compounds, their strong electronegativity (pK value around pH 2) and the existence of many tissue-specific forms, sialic acids affect many functions (Schauer, 1985). These functions can be divided into three groups.

1.7.5.4.1 Functions due to negative charge of sialic acids

Sialic acids are responsible for the negative charge present on the surface membrane of many cells (Schauer, 1982). The negative charge prevents the aggregration of certain cell types such as erythrocytes (Born and Palinsky, 1989) but facilitates the aggregration of other cell types, for example chick embryonic muscle cells (Kemp, 1970).

The repulsive electrostatic forces of the sialic acid are also responsible for the rigidity of cell surfaces. For example enzymic removal of sialic acids from the zona pellucida of rabbit ovum decreases the rigidity of the cell and makes it impenetrable to spermatozoa (Soupart and Clewe, 1965).

At cell surfaces, sialic acids are involved in the binding and transport of ions and amino acids. For example, the uptake of 2-amino-2-methylpropanoic acid by HeLa cells is decreased after treatment with sialidase (Brown and Michael, 1969).

The binding of cationic compounds to macromolecules is facilitated by sialic acids. For example the presence of sialic acids on the surface of L1210 mouse-leukemia cells influence the transport of potassium ions through the cell membrane (Schauer, 1982). In muscle cells sialic acids are important Ca^{2+} binding sites (Harding and Halliday, 1980). Reports on the influence of

sialidase treatment on muscle contractibility are conflicting. The removal of sialic acid from cardiac sarcolemma caused no change in the strength of contraction of stimulated guinea-pig atrial muscle cells (Harding and Halliday, 1980). In contrast the injection of sialidase into the spinal-cord segment of the frog caused increased neurone activity (Romer and Rahmann, 1979). These changes in activity are thought to be related to the degree of sialylation of gangliosides, which have been shown to bind Ca^{2+} ions (Schauer, 1982).

1.7.5.4.2 Effect of sialic acid on macromolecular structure

Sialic acids affect the conformation of glycoproteins (Kelm and Schauer, 1997). This has an impact on the glycoprotein arrangement in the cell membranes, on the activity of glycosylated enzymes and the resistance of glycoproteins to proteases (Schauer, 1985).

Sialic acids are frequently found on enzymes and their removal has an effect on the activity of the enzyme. For example, sialidase treatment of monoamine oxidase A (EC 1.4.3.4) destroys its activity (Houslay and Marchmont, 1980).

The involvement of sialic acids in the resistance of glycoproteins to proteases has been well documented. For example, the proteolytic resistance of the intrinsic factor and its concomitant binding capacity for vitamin B12 are lost after the release of sialic acid (Schauer, 1982). Likewise the sialoglycoproteins of the jelly coat of the frog's egg are resistant to proteolysis (Schauer, 1982). Sialic acids contribute to the high viscosity of many mucus secretions such as those from the respiratory, digestive and urogenital tracts (Schauer, 1982). These secretions act as defensive agents in bodily cavities exposed to the environment (Reid *et al* ., 1978). The high viscosity of the mucous is an important component of immune protection (Lamont, 1992), therefore the removal of the sialic acids which leads to a decrease in the viscosity of these secretions can lead to reduced immune protection at these areas.

1.7.5.4.3 Sialic acids as regulators of cellular and molecular interaction

Sialic acids play a dual role in the regulation of cellular and molecular interactions (Varki, 1992). They can either act as recognition determinants or they can mask recognition sites, such as subterminal carbohydrate structures of

glycoprotein glycans (Schauer *et al* ., 1997). These effects are briefly discussed below.

a) Receptor functions of sialic acids

The exposed position of sialic acids on cell surfaces allows them to act as recognition markers in multicellular organisms, and this may be exploited as a receptor for invading microorganisms (Kelm and Schauer, 1997). Many proteins of plant, animal and microbial origin (Sialic acid binding lectins) that recognise sialic acids have been described. An excellent table listing the various lectins can be found in Varki, 1997. A few selected examples are discussed below, however they have been exhaustively reviewed elsewhere (Mandal and Mandal, 1990; Varki 1992; Varki 97; Kelm and Schauer, 1997; Sharon and Lis, 1995)

Lectins are prevalent on surfaces of bacteria, protozoa and viruses (Sharon and Lis, 1995). The influenza virus haemagglutinin, the model viral lectin, is a surface glycoprotein specific for the most common sialic acid, N-acetyl-neuraminic acid (NeuAc). The lectin mediates the attachment of the virus to the cell by combining with disaccharide NeuAc(α2-6)Gal which is found mainly on human cells. Fusion of the viral and cellular membranes follows allowing release and subsequent replication of the virus. Treatment of cell membranes with sialidase (to remove sialic acid), eliminates binding and prevents infection (Wiley and Skehel, 1987).

An important role of bacterial lectins is to mediate the adhesion of bacteria to host cells (Ofek and Sharon, 1990). Bacterial lectins are often in the form of fimbriae (pilli). Some strains of *E. coli* express fimbrial lectins specific for glycoconjugates containing sialic acid.

Many plant lectins have been characterized but their function remains unknown. Some suggestions are that lectins are involved in protecting plants against pathogens and insects or that lectins mediate the binding of symbiotic rhizobia to the roots of leguminous plants (Sharon and Lis, 1995).

The primary structure of about 100 animal lectins is known. Despite variations in the overall structure the carbohydrate binding activity has been associated to a certain polypeptide region of each lectin, called the carbohydrate recognition domain (CRD). Two main types of CRDs are the C and S types. Lectins

containing these domains are called the C-type lectins and galectins respectively. The galectins bind exclusively to β-galactosides such as N-acetyl-lactosamine [Gal(β1-4)GlcNAc] and do not require metal ions for their activity (Barondes, *et al* ., 1994). The C-type lectins require a Ca^{2+} ion for their activity and have a broader binding specificity: some bind galactose and N-acetylgalactosamine and others bind oligosaccharides. On the basis of their structure and function the C-type lectins are further divided into three subgroups: endocytic lectins, collectins and selectins (Sharon and Lis, 1995).

The selectins provide the best example of carbohydrate-lectin interaction in biological recognition. The selectins (E-, P-, L-selectin) present on activated endothelial cells, platelets and endothelial cells and circulating leukocytes respectively, (Reuter and Gabius, 1996) are membrane bound proteins that bind specifically to the trisaccharide NeuAc(α2-3) Gal(β1-4) [Fuc(α1-3)GlcNAc (sialyl $Lewis^x$) (Sharon and Lis, 1995). They play an important part in directing leucocytes to regions of inflammation and in the migration of lymphocytes to specific lymphoid organs (Sharon and Lis, 1995). Selectins may also be involved in the spread of cancer cells from the main tumour to other parts of the body (Sharon and Lis, 1995). More recently another family of sialic acid binding lectins, I-type lectins have been recognized (Kelm *et al* ., 1996). It includes other immunoglobulin superfamily members like sialoadhesins found on specific subsets of macrophages, CD22, a B-cell specific protein, CD33, a molecule expressed by myeloid progenitor cells, myelin-associated glycoprotein (MAG) (Kelm and Schauer, 1997). Studies have shown that all these proteins are able to mediate cell adhesion via recognition of sialylated cell surface glycans (Kelm and Schauer, 1997). Sialoadhesin may mediate macrophage interactions with developing myeloid precursors (Varki, 1997) and MAG may interact with gangliosides on neuronal cells to maintain myelin integrity and function (Varki, 1997).

Beside the selectins and I-type lectins, other proteins that recognise sialic acid have been found but as yet not classified. These include the lectins that bind complement factor H and human placental lectin (Reuter and Gabius, 1996; Varki, 1997).

Sialic acids, principally as components of gangliosides are also involved in the binding of many toxins. For example cholera toxin and diptheria toxin (Schauer, 1982).

b) Anti-recognition effects of sialic acids

One of the most important functions of sialic acids is their ability to act as biological masks. Through steric hindrance and/or electrostatic repulsion they are able to stop or decrease the accessibility of recognition sites in many biological systems, including the immune system (Schauer *et al* ., 1984). The anti-recognition effect was initially demonstrated by Ashwell and Morell (1974). They found that sialic acids mask the D-galactosyl residues of serum glycoproteins and prevents their breakdown. Removal of sialic acid by a sialidase exposes the galactose residues. The desialylated molecules are then bound by a hepatocyte lectin and cleared from the circulation. Sialic acids also regulate the life time of blood cells. In man the enzymic removal of sialic acids (10-20%) from erythrocytes causes their rapid clearance from the bloodstream (Schauer, 1982). The life-time of erythrocytes decreases from 120 days to 2 hours following sialidase tretment (Schauer, 1982).

Sialic acids act as biological masks in many other systems. For example they decrease the reactivities of IgG with the Fc receptor of human T lymphocytes, killer cells and some receptors for drugs or hormones (Schauer *et al* ., 1984)

Another example of the masking of antigenic sites by sialic acids is found in the reproductive system. A sialic acid rich glycoprotein layer exists on the surface of trophoblast cells. This acts as an immunobarrier between the mother and foetus and prevents the formation of anti-foetus antibodies by the mother (Schauer, 1982).

Antigenic supression by sialic acids is a common property of cells and molecules and the loss of these sugars has been proposed to account for some autoimmune diseases (Schauer, 1982). The high degree of sialylation observed on the surface of tumours may be responsible in protecting them from the immune system (Schauer, 1985).

c) Sialaic acid and the complement system

Sialic acids also play a very important role in regulating the activation of the complement pathways (Pilatte *et al* ., 1993). The complement system consists of a number of inactive blood proteins that are activated sequentially in an amplifying series of reactions which are ultimately able to bring about cell lysis (Frank and Fries, 1989). There are two complement activation pathways, the classical and the alternative. Activation of the classical pathway requires the

formation of antigen-antibody complexes, whereas activation of the alternative pathway does not require antibody fixation to target cells. Although the initial stages of the two pathways are different, they both lead to the deposition of C3b (a fragment of the third complement component) on to the cell membrane. This event then leads to binding of complement components C5, C6, C7, C8 and C9 which form the membrane attack complex, producing cell lysis (Frank and Fries, 1989). In order to prevent autoimmune reactions, the activation of the complement system has to be tightly regulated. The presence of sialic acid on cell surfaces is important in descrimination of activating (foreign) and non-activating (host) surfaces and the activation of the two complement pathways.

With regards to the classical pathway, it has been found that treatment of human red cells and lymphocytes (Lambre *et al* ., 1985; Johannsen *et al* ., 1979) with bacterial sialidase results in the production of antibodies to these cell types. These antibodies have been found to activate classical complement and cause lysis of the red cells (Lambre *et al* ., 1985) and lymphocytes (Johannsen *et al* ., 1979).

Sialic acids play an important role in the self/non-self discrimination of the alternative pathway. The alternative pathway is able to discriminate between self and non-self because it can lyse bacteria, parasites and tumour cells without harming normal cells (Frank and Fries, 1989). This discriminatory ability of the alternative pathway was shown by Fearon (1978) using sheep red cells. He found that sialylated sheep red cells were unable to activate the alternative complement pathway, but red cells that had been desialylated by treatment with sialidase were able to activate the human alternative pathway. There is partial understanding of the molecular mechanism of this activation. The first step in the activation of the alternative complement pathway is binding of C3b to the cell surface. This occurs spontaneously on foreign particles and host cells. C3b can then be either inactivated or it can promote the formation of a C3 convertase (C3bBb) which then leads to the formation of the membrane attack complex. The fate of C3b is controlled by a regulatory protein, factor H. Factor H can prevent the formation of the C3 convertase or promote its dissociation. However the activity of factor H depends on the cell surface that C3b is bound to. Factor H has lower affinity for C3b bound to an activator of the alternative pathway but a higher affinity for C3b that is located on host cell surfaces (Pilatte *et al* ., 1993). Sialic acids located on cell surfaces regulate the

activity of factor H, by increasing its affinity for C3b. The increased affinity of factor H for C3b prevents the formation of the C3 convertase and consequently the membrane attack complex (Pilatte *et al* ., 1993). In summary, cell surface sialic acids prevent the activation of the alternative complement pathway. Many microorganisms use this system to their advantage and express sialic acid on their outer membrane or capsule (e.g. *Neisseria meningitidis* B and *E. coli* K1) to avoid alternative complement attack (Frank, 1992).

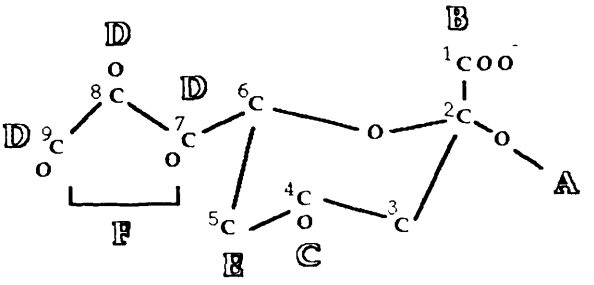
1.7.6 The substrate specificity of neuraminidases

Neuraminidase catalyses the hydrolytic removal of α-glycosidically linked sialic acid residues from sialylated glycoconjugates at an acidic pH (Sweeley, 1993) as described in Section1.7.4. The rate of the reaction is affected by many factors including the type of linkage between the sialic acid and the glycoconjugate and the nature of substituents on the sialic acid. These issues have been extensively reviewed by Drzeniek (1973), Rosenberg and Schengrund (1976) and Corfield (1992). The principal characteristics of neuraminidase substrate specificity are shown in Figure 1.3 .

Two of these characteristics are very important with relation to infectious diseases. These are the nature and linkage of the glycoconjugate and the substitution of the hydroxyl groups at C_{7-9} (Corfield 1992). In macromolecular compounds (glycoconjugates or complex carbohydrates) sialic acids are joined to sugars (such as galactose or N-acetylgalactosamine) or to other sialic acid molecules by α-glycosidic linkages (Schauer, 1982). (Natural β-glycosidic linkages of sialic acids are only found in CMP glycosyl esters (Ng and Dain, 1976). Most frequently sialic acids are linked to sugars by α-(2–> 3) or α-(2–> 6) linkages (Schauer, 1982) and to other sialic acid molecules by α-(2–> 8) linkages (Drzeniek 1973). Neuraminidases can only cleave α-glycosidic linkages (Schauer, 1982) and generally α-(2–> 3) linkages are hydrolysed faster than α-(2–> 6) or α-(2–> 8) linkages (Sweeley, 1993). An exception is the neuraminidase of *Arthrobacter ureafaciens* which cleaves α-(2–> 6) bonds more efficiently than α-(2–> 3) or α-(2–> 8) (Schauer, 1982).

Moiety	Effect on neuraminidase action
A-aglycone	Activity related to: -Nature of glycosidic linkage -Nature of linkage monosaccharide -Nature of glycoconjugate - Packing/organization of oligosaccharide
B-carboxyl	Activity is dependent on an intact, unblocked carboxyl group
C-hydroxyl C4	Substitution with acyl group inhibits enzyme activity
D-hydroxyl C7-9	Substitution with acyl groups results in decreased activity
E-N-acyl	Activity usually N-acetyl> N-glycolyl. Also depends on type of glycoconjugate
F-C7-9 side chain	Shortening of side chain may reduce activity

Figure 1.3a. Neuraminidase substrate specificity. (Adapted from Corfield, 1992)



Substitutions of the core sialic acid structure which can affect neuraminidase specificity are indicated by letters A - F as described in Figure 1.3 a.

Figure 1.3b. Diagram of sialic acid. (Adapted from Varki, 1992)

The second important feature with respect to infectious diseases is the substitution of hydroxyl groups at C7-9. Increased O-acetylation of these hydroxyl groups reduces the activity of the neuraminidase. In humans the most common site of O-acetylation are the saliva and colon (Corfield 1992).

Other important factors which affect the activity of the neuraminidase include the substitution of the C-4 by O-acetyl groups which reduces or blocks the action of the enzyme (Sweeley, 1993). However this substitution is rare and not normally found in humans (Corfield, 1992). The carboxyl group (Figure 1.3) is essential for neuraminidase activity and its blockage prevents sialic acid release (Schauer, 1982).

Other substituents have a limited effect on sialidase activity. The N-glycolyl group (E, Figure 1.3) is not normally found in man (Varki, 1992 and does not greatly affect the action of bacterial neuraminidases (Corfield *et al* ., 1981).

1.7.7 Biological role of neuraminidases

Sialic acid, the substrate of neuraminidase, has a wide occurence and function in nature. Therefore it is not surprising that the action of neuraminidases also has a wide range of consequences. These are discussed below, with the major emphasis being on the bacterial neuraminidases.

1.7.7.1 Viral neuraminidases

Many roles have been proposed for viral neuraminidases. These include the viral penetration into host cells, provision of metabolites for host metabolism, release of new virus from host cells, and viral binding to host cell. However there is no consensus view on the role of viral neuraminidases because of the presence of conflicting lines of evidence for each hypothesis (Rosenberg and Schengrund, 1976).

Studies on the best characterised neuraminidases of Influenza A virus have shown that the neuraminidase does not play a role in viral entry, assembly or replication. Mutant viruses, deficient in neuraminidase production were still able to assemble, replicate and infect new cells (Liu *et al* ., 1995). The more important role of the viral neuraminidase is thought to be in the release of progeny viruses from cells (Liu *et al* ., 1995). It is postulated that interactions between the glycoconjugates on the surface of the viral (particularly the haemaglutinin) and host cells causes the newly synthesised virions to aggregate on the cell surface and consequently prevents further replication. The neuraminidase facilitates infection by cleaving the sialic acids from glycoconjugates thereby releasing the virions and allowing them to infect new cells (Liu *et al* ., 1995).

The importance attached to the involvement of neuraminidase in viral disease is highlighted by the considerable efforts that are expended in the search for neuraminidase inhibitors (von Itzstein and Thomson, 1997). Recently the anti-influenza compound called Zanamivir (GG167) has been developed on the basis of the influenza neuraminidase structure. Its main

target is the active site of the enzyme. Early clinical trials have shown that the drug is safe and effective at preventing and treating influenza in humans (Hayden *et al* ., 1996). Phase three clinical trials of this drug are currently underway.

1.7.7.2 Mammalian neuraminidases

Mammalian neuraminidases have not been well characterized (Taylor *et al* ., 1992) but are thought to play a part in many areas. These include reproduction (Yaginuma 1972,), change in the half life of circulating sialo-compounds (Schauer, 1982), formation of blood clots (Vermylen et al ., 1974), hormone interactions (Cuatrecasas and Illiano 1971), neurotransmission (Rosenberg and Schengrund, 1976), cell-cell interactions and cellular transformations (Schauer 1982; Varki, 1992).

1.7.7.3 Protozoan neuraminidases

In protozoa sialic acids and neuraminidases are also involved in interactions between the parasite and the host (Schenkman *et al* ., 1994). In the genus *Trypanosoma* a neuraminidase termed *trans*-sialidase has been described (Pereira *et al* ., 1991). It is responsible for shifting sialic acids from host glycoconjugates to acceptor molecules on the plasma membrane of the parasite. There is considerable evidence suggesting the participation of this reaction in the invasion of host cells by parasites (for a review see Schenkman *et al* ., 1994) For example, it has been shown that *T. cruzi* trypomastigotes carry a stage specific sialylated epitope, Ssp-3 which is involved in attachment and penetration of host cells (Andrews *et al* ., 1987). *T. cruzi* does not synthesise sialic acid (Schauer *et al* ., 1983) and trypomastigotes just released from host cells do not express the Ssp-3 epitope (Frevert *et al* ., 1992). However emerging trypomastigotes rapidly acquire sialic acid by the action of the *trans*-sialidase. The *trans*-sialidase transfers sialic acid from host glycoconjugates to acceptors on the trypomastigote surface, thereby generating the Ssp-3 epitope (Schenkman *et al* ., 1992). The presence of sialic acid on the trypomatigote surface (as a result of *trans*-sialidase activity) also contributes to the evasion of the parasite from complement attack. The parasite membrane associated sialic acid prevents efficient assembly of the central complement cascade-amplifying enzyme, C3 convertase C3bBb (Joiner *et al* ., 1984) (Section 1.7.5.4.3. c) and consequently the activation of complement.

1.7.7.4 Bacterial neuraminidases

1.7.7.4.1 Introduction

Bacterial neuraminidases are divided into two families on the basis of their molecular weight (Hoyer *et al* ., 1992). The "small family" have molecular weights of about 42 kDa (Hoyer *et al* ., 1992), do not require a divalent metal ion for maximal activity (Crennell *et al* ., 1993) and preferentially cleave substrates with α(2-3)-linked sialic acids (Roggentin *et al* ., 1993). The "large family" have molecular weights greater than 60 kDa (Roggentin *et al* ., 1993), require a divalent metal ion for maximal activity (Crennell *et al* ., 1993) and have a broader substrate range (Roggentin *et al* ., 1993). Examples of neuraminidases belonging to the two families are shown in Table 1.7. The tertiary structures of neuraminidases from the large (*V. cholerae*) (Crennell *et al* ., 1994) and small (*S. typhimurium*) (Crennell *et al* ., 1993) families have been solved and has provided further insight into the catalytic mechanism and function of the neuraminidases. These neuraminidases are discussed in Section 1.7.9.2.

Family and organism	Neuraminidase size (kDa)	Reference
Small family		
Arthrobacter ureafaciens	39 and 51 kDa	Uchida *et al* ., 1979
Clostridium perfringens	42 kDa	Kruse *et al* ., 1996
Clostridium sordellii	44 kDa	Rothe *et al* ., 1989
Micromonospora viridifaciens	41 kDa	Gaskell *et al* ., 1995
Salmonella typhimurium	42 kDa	Hoyer *et al* ., 1991
Large family		
Bacteroides fragilis	165 kDa	Tanaka *et al* ., 1992
Bacteroides loescheii	87 kDa	Takagaki *et al* ., 1991
Clostridium perfringens	73 kDa	Nees *et al* ., 1975
Clostridium septicum	110 kDa	Rothe *et al* ., 1991
Micromonospora viridifaciens	68 kDa	Gaskell *et al* ., 1995
Vibrio cholerae	83 kDa	Taylor *et al* ., 1992

Table 1.7. The two families of bacterial neuraminidases

1.7.7.4.2 Role of bacterial neuraminidases in disease

The exact role of bacterial neuraminidases is unknown but until recently they have been linked mainly with pathogenicity. Now, however a nutritional function has also been attributed to bacterial neuraminidases (Corfield, 1992). These two very different functions of neuraminidases are briefly discussed below, followed by a more detailed account of the role of the pneumococcal neuraminidase.

a) General features

Neuraminidase activity can be localised to specific areas or be widespread, depending on the disease. For example, in peridontal disease and glomerulonephritis the neuraminidase activity is localized, whereas in peritonitis or gas gangrene the neuraminidase activity is spread throughout the circulation (Corfield, 1992).

Bacterial neuraminidases are thought to be virulence factors which facilitate the invasion and spread of the organism within the host (Muller, 1974). The action of neuraminidase on glycoconjugates throughout the host results in many changes which are summarized in Table 1.8.

Many bacteria that are involved in disease secrete large amounts of neuraminidase (Corfield, 1992). Moreover the neuraminidase activity is often inducible, by free sialic acid or a variety of glycoconjugates. This has been demonstrated by isolating bacteria from a host followed by culturing in media deficient in sialic acid. It was found that these cultures lacked neuraminidase activity (Muller, 1974).

b) Diseases

Many pathogenic bacteria produce neuraminidase and this enzyme has been implicated in the process of disease due to these pathogens. Examples include *Clostridium* (Fraser, 1978), *Bacteroides* (Guzmán *et al* ., 1990), *Vibrio cholerae* (Galen *et al* ., 1992) and the pneumococcus (Vishniakova and Reztsova, 1991).

Mucosal surfaces Mucus viscoelasticity and defensive properties are lost Bacterial binding sites are created on epithelial cells which lead to invasion Unmasking of crypt antigens
Vascular cells Loss of negative charge on surface of endothelial cells Loss of cell surface receptor specificity for enzymes, hormones and cells Unmasking of crypt antigens
Blood cells Panagglutination of erythrocytes Haemolytic anaemia Decrease in circulating half life of erythrocytes and leukocytes Thrombocytopenia Unmasking of crypt antigens
Soluble blood glycoconjugates Increased blood viscosity Decreased half-life of circulating glycoconjugates Increased formation of immune complexes Increased titre of autoantibodies Unmasking of crypt antigens

Table 1.8. Events that occur as a result of neuraminidase action in bacterial infection. (Adapted from Corfield, 1992).

One of the best characterized neuraminidases with respect to disease is the neuraminidase of *V. cholerae*. This neuraminidase is responsible for cleaving higher order gangliosides to GM1 (the receptor for cholera enterotoxin). It is thought that this action results in increased local concentrations of GM1 which enhances the binding and uptake of cholera toxin. Evidence supporting the involvement of neuraminidase in the binding of cholera toxin comes mainly from *in vitro* results using purified neuraminidase and cholera toxin. For example, it has been shown that isolated rabbit small-intestine brush borders treated with neuraminidase bound double the amount of cholera toxin (compared to untreated tissue) (Griffiths *et al* ., 1986). Also the exposure of cultured mouse neuroblastoma cells to neuraminidase caused the binding of cholera toxin to increase by five to seven fold (Miller-Podraza *et al* ., 1982).

Neuraminidase may also have an indirect involvement in pathogenicity by potentiating the action of IgA1 protease. The latter enzyme cleaves IgA1 in the hinge region thus separating the antigen binding and effector functions of the molecule. IgA1 protease is produced by many microbes including oral streptococci, *Neisseria meningitidis*, *S.pneumoniae* and urogenital pathogens (Kilian *et al* ., 1996). IgA has many and varied functions which include inhibition of microbial and viral adherence to surfaces, neutralizing bacterial toxins and enzymes, elimination of immune complexes and antibody-dependent cellular cytotoxicity (Kilian *et al* ., 1996). Therefore the cleavage of the IgA will have a direct effect on these activities.

It has also been suggested that the extensive cleavage of IgA1 observed *in vivo* at mucosal membranes may result in IgA1 deficiency that in turn would facilitate the colonization of the protease producer and other microbes (Kilian *et al* ., 1996).

Experiments show that the neuraminidase activity of *S. oralis* and *S.miti* may increase the formation of plaque by enhancing the degradation of IgA1 by IgA1 protease (Reinholdt *et al* ., 1990)

1.7.7.4.3 Nutritional role of bacterial sialidases

Neuraminidases also are produced by non-pathogenic bacteria. Here they have a nutritional function in scavenging host cell sialic acid to use as carbon and energy source (Gaskell *et al* ., 1995) Some of these bacteria are located at mucosal surfaces such as the human oral cavity and large intestine and function in symbiotic relationships (Corfield, 1992). Examples include the mucin degrading bacteria (*Bifidobacterium* and *Ruminococcus* species) found in the human large intestine. These bacteria are capable of completely degrading glycoproteins present in mucus of mucosal surfaces. The neuraminidase production in these bacteria is constitutive and acts on gangliosides and sialo-glycoproteins (Corfield, 1992). It has been shown that the degradation of mucin by neuraminidases provides a substantial proportion of the bacterial energy supply (Corfield, 1992).

A nutritional role for neuraminidase has also been demonstrated in *S. typhimurium* using the substrate sialyl α(2-3)-lactose (Corfield, 1992). *Salmonella* strains are unable to use lactose as a carbon source, therefore the

sialic acid has to be released prior to its uptake and metabolism. It was found that mutants deficient in neuraminidase were unable to grow on sialyl α(2-3)-lactose because they were incapable of releasing the sialic acid. In contrast the wild type bacteria were able to cleave the sialic acid and consequently grew on the sialyl α(2-3)-lactose medium.

Some neuraminidases are involved both in pathogenesis and nutrition. An example is the *V.cholerae* neuraminidase which plays a role in pathogenesis by unmasking the receptor sites for the binding of cholera toxin and also releases sialic acids for use as a carbon source (Gaskell *et al* ., 1995).

1.7.7.4.4 Role of pneumococcal neuraminidases

The exact role of the pneumococcal neuraminidase is unknown. However there are many hypotheses about the part played by the neuraminidase. Scanlon *et al* (1989) suggested that the neuraminidase could help establish infection in two ways. Neuraminidase could decrease the viscosity of the mucus and as a consequence interfere with its function of trapping and eliminating invading organisms. Alternatively neuraminidase may aid in infection by exposing sites for the attachment of the pneumococcus. It has been shown by Andersson *et al* ., (1983) that glycoconjugates containing the disaccharide unit GlcNAcß1—3Galß were the receptors for pneumococcal binding to human pharyngeal epithelial cells. Neuraminidase could expose this receptor by removing the terminal sialic acid. Another carbohydrate receptor containing GalNAcß1—4Galß1 has been found in glycolipids and is recognized by *S. pneumoniae* (Krivan *et al* . , 1988). Moreover, Linder *et al* ., (1994) have shown that intranasal inoculation of pneumococci into chinchillas causes changes in surface carbohydrates of middle ear mucosa and Eustachian tube, which lead to the unmasking of pneumococcal receptors. Neuraminidase may be a major contributor to this phenomena. However, Feldman *et al* (1992) have used organ culture of human nasal turbinate tissue to show that *S.pneumoniae* initially persists and multiplies in the mucous layer alone. Therefore in terms of colonization the attachment of pneumococcus to epithelial cells may not be as important as the interaction with the epithelial-derived secretions (Feldman *et al* ., 1992).

There is considerable circumstantial evidence implicating pneumococcal neuraminidase in disease. Two studies which examined 112 fresh clinical

isolates of *S. pneumoniae* (representing a heterogenous population of capsular types) found that all strains produced neuraminidase (Kelly *et al* ., 1967; O'Toole *et al* ., 1971). The first study by Kelly *et al.*, (1967) examined 77 clinically significant pneumococci isolated from sputum, blood, cerebrospinal fluid (CSF), throat, conjunctiva and external auditory canal. They found that all the isolates had neuraminidase activity. In contrast only 7 out of 15 laboratory adapted *S.pneumoniae* had neuraminidase activity. A later study by O'Toole *et al* ., (1971) examined the CSF of patients infected with *H. influenzae, N. meningitidis* and *S. pneumoniae*. They found that all *S. pneumoniae* isolates produced neuraminidase but no neuraminidase activity was detectable from the meningococci or *H. influenzae*. Moreover when the level of free sialic acid in the CSF of patients with pneumococcal meningitis was measured, it was found that about 50% of the patients had elevated levels of free sialic acid (1.42mg/100ml compared to 0.37mg/100ml in the control group). This increased level of sialic acid was associated with the development of coma and bacteremia and led to suggestions that neuraminidase was involved in the pathogenesis of meningitis (O'Toole *et al* 1971).

Further evidence for the involvement of neuraminidase comes from an examination of middle ear effusion (MEE) from children with chronic otitis media. It was discovered that *S.pneumoniae* was one of the organisms most commonly found in MEE. Moreover 96% of MEEs containing *S.pneumoniae* had neuraminidase activity compared with only 21-45% of MEEs containing other organisms. As the middle ear epithelium is a rich source of sialic acid containing compounds (gangliosides, glycoproteins and mucins), it has been suggested that neuraminidase may play an important role in disease by removing these terminal sialic acids and exposing receptors for pathogens or toxins (Lamarco *et al* ., 1984).

Additional evidence for the involvement of neuraminidase in disease came from experiments with purified neuraminidase. Kelly *et al* ., (1970) found that intraperitoneal inoculation of mice with partially purified neuraminidase caused death. Furthermore histochemical analysis of the liver and kidney showed a decreased content of sialic acid compared to controls. Surprisingly the intrathecal inoculation of dogs with the purified neuraminidase resulted in no symptoms (O'Toole and Stahl, 1975). Further evidence for the involvement of neuraminidase in disease was provided by the immunization experiments of Lock *et al* ., (1988a). They immunized mice with

neuraminidase purified from *S.pneumoniae* and then challenged the mice with virulent pneumococci. It was found that the immunized mice had a significantly increased ($P < 0.05$) survival time compared to control mice given PBS.

Two genes, *nanA* (Camara *et al* ., 1991) and *nanB* (Berry *et al* ., 1996) coding for neuraminidase activity have been cloned (Section 1.7.8.2). A *nanA*- negative mutant created in our laboratory by insertion-duplication mutagenesis provides direct evidence for the involvement of neuraminidase in disease (J. Hill, unpublished data). In the murine model of pneumonia this mutant had reduced virulence than the wild-type parent. In the blood the mutant pneumococci grew less well than the wild-type and were beginning to decrease after 48 hours, whereas mice infected with the wild type pneumococci had died by 48 hours. The number of mutant pneumococci in the lungs of intranasally infected mice remained unchanged over a 3 day period. In contrast, the number of wild-type pneumococci rose by 4 log over 2 days after which time the mice died. This suggested that neuraminidase enabled the pneumococci to survive and multiply in the lungs.

1.7.8 Cloning of neuraminidase genes

1.7.8.1 Introduction

Several neuraminidase genes have been cloned and some of these are listed in Table 1.9. The cloning of the pneumococcal neuraminidase genes are discussed in greater detail.

Organism	Reference
Vibrio cholerae	Vimr *et al* ., (1988)
Clostridium perfringens	Roggentin *et al* ., (1988)
Clostridium sordellii	Rothe *et al* ., (1989)
Clostridium septicum	Rothe *et al* ., (1991)
Bacteroides fragilis	Russo *et al* ., (1990)
Actinomyces viscous	Yeung and Fernandez (1991)
Salmonella typhimurium	Hoyer *et al* ., (1992)
Micromonospora viridifaciens	Sakurada *et al* ., (1992)

Table 1.9. Cloned neuraminidase genes

1.7.8.2 Cloning of the pneumococcal neuraminidase genes

There has been controversy regarding the number and size of neuraminidases produced by the pneumococcus. Tanenbaum *et al* ., (1970 and 1971) had described the existence of several pneumococcal neuraminidase isoenzymes of approximately 70 kDa. Stahl and O'Toole (1972) also reported the isolation of a neuraminidase with a molecular weight of 69.8 kDa . Lock *et al* ., (1988b) on the other hand suggested the existence of a single neuraminidase and postulated that the previously reported isoenzymes were a consequence of proteolytic degradation of a single neuraminidase. In the presence of protease inhibitors, they were able to purify a neuraminidase with a molecular weight of 107 kDa from *S. pneumoniae* lysates. However in the absence of protease inhibitors they isolated many active forms of neuraminidase with molecular weights as low as 86 kDa (Lock *et al* ., 1988b). Lock *et al* ., (1988b) suggested that the 86 kDa neuraminidase was one of the more stable products in the degradation process but not the end-product. Later Scanlon *et al* ., (1989) purified a neuraminidase with a molecular weight of 65 kDa in the absence of protease inhibitors. These studies suggested that there was a single pneumococcal neuraminidase of about 107 kDa which underwent proteolytic degradation and resulted in the production of the observed smaller molecular weight neuraminidases.

The cloning of the pneumococcal neuraminidase genes in *E. coli* has provided a molecular explanation for the conflicting data regarding the size and number of pneumococcal neuraminidases. There have been three reports of the cloning of pneumococcal genes in *E. coli* (Berry *et al* .., 1988; Camara *et al* ., 1991; Berry *et al* ., 1996). These are discussed below in detail, because of their relevance to the work of this thesis.

The initial attempts at cloning were performed by Berry *et al* ., (1988). They constructed a gene bank of pneumococcal DNA fragments in *E. coli* K-12. About 1500 clones were produced and screened for neuraminidase activity. One clone was able to cleave 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUAN), and harboured a plasmid termed pJCP301 which contained 3kb of pneumococcal DNA. Neuraminidase expression was obtained from this clone but it was not stable. The neuraminidase encoded by pJCP301 was subjected to western blot analysis. *E. coli* [pJCP301] lysates contained one protein that reacted with anti-neuraminidase. This protein was 98 kDa and not present in lysates of *E.coli* harbouring the cosmid vector

pHC79. The neuraminidase produced by pJCP301 was smaller than the native neuraminidase produced by *S. pneumoniae*. Two explanations were given to explain this observation. One possibility was that the neuraminidase underwent proteolytic processing resulting in smaller forms of the protein. However a more likely explanation was that the complete neuraminidase gene was not cloned because the 3.0kb pneumococcal DNA insert in pJCP301 was scarcely sufficient to encode a 107 kDa protein.

In order to isolate more stable neuraminidase-producing clones and to clone the whole neuraminidase gene, the vector pJDC9 (Chen and Morrison, 1987) was utilized. The use of this vector had been shown to improve the stability of cloned pneumococcal DNA fragments in *E. coli* (Chen and Morrison, 1987). The chromosomal DNA from *S. pneumoniae* was digested with various enzymes in single or double digests. The digested DNA was electrophoresed and Southern blots were hybridized with pJCP301 DNA (3.6 kb *Hind* III - *Acc* I fragment). The Southern blot analysis showed that the sequence encoding the neuraminidase was situated on a 3.3 kb *Hind* III fragment. To isolate the whole neuraminidase gene, the DNA was cleaved with *Hind* III and 3.3 kb fragments were cloned into pJDC 9. Colony blots of the resultant clones were probed with a 900bp *Pst* I - *Kpn* I fragment from pJCP301. One clone hybridized strongly with the probe and contained a recombinant plasmid (termed pJCP302) with a 3.3 kb pneumococcal DNA insert. However no neuraminidase activity was obtained from this recombinant. It was thought that a mutation which abolished neuraminidase activity occurred in the 3.3 kb chromosomal DNA fragment.

In summary the cloning by Berry *et al* ., (1988) resulted in the production of a unstable neuraminidase-producing clone which could not be further characterized.

The second cloning attempt was performed by Camara *et al* ., (1991) and resulted in the cloning of a stable neuraminidase encoding gene termed *nan* A. This work is described in detail below as it formed the starting point for this current study. Previous efforts by Berry *et al* ., (1988) to clone the neuraminidase gene were hampered by the instability of the cosmid vectors used. To overcome this Camara *et al* ., (1991) constructed a genomic library of pneumococcal DNA from the strain R36A (NCTC10319) in a LamdaEMBL vector (Lathe *et al* ., 1987). The R36A strain used for the construction of the

genomic library was a nonencapsulated, non-pathogenic derivative of D39, a type II strain highly pathogenic for mice (Camara, 1992). The genomic library was screened for the presence of functional neuraminidase enzyme by the use of the fluorogenic substrate 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUAN; Sigma). One recombinant clone expressing neuraminidase activity was isolated. It contained an insert of 18.5 kb. and was designated EMBL301 - neu 1.

DNA from the lambda recombinant EMBL301 - neu 1 was sub cloned into plasmid pJDC9 and transformed into *E. coli* JM101. The resulting clones were screened for neuraminidase activity. The smallest plasmid selected (pMC2150) contained a 4.3 kb insert. A restriction map of this plasmid showed no similarity to the restriction map of pJCP301 cloned by Berry *et al* ., (1988). DNA hybridization studies were performed to determine whether there was sequence homology between the neuraminidase gene cloned by Berry *et al* ., (1988) and carried on pJCP301 and that present in pMC2150. The result of the hybridization studies showed that the two genes present in pJCP301 and pMC2150 were different (Camara *et al* ., 1991). Furthermore the genome of five pneumococcal isolates were analysed by Southern blotting and found to contain both genes (Camara *et al* ., 1991).

Sequencing of plasmid pMC2150 indicated the presence of three ORF's. However the 3' end of the largest one (ORF1) was missing from pMC2150 (Figure 1.4). In order to determine the entire sequence of ORF1, a DNA fragment was subcloned from the recombinant phage lambda EMBL301-Neu1. On the basis of neuraminidase production and its restriction pattern another recombinant containing a 7.0 kb insert was selected. This clone designated pMC4170 included the complete ORF1. ORF1 (3.1 kb) was proposed to represent the neuraminidase structural gene (*nanA*), because the predicted amino acid sequence from ORF1 contained the aspartic box SXDXGXTW motif that is found in other bacterial neuraminidases (Roggentin *et al* ., 1989).

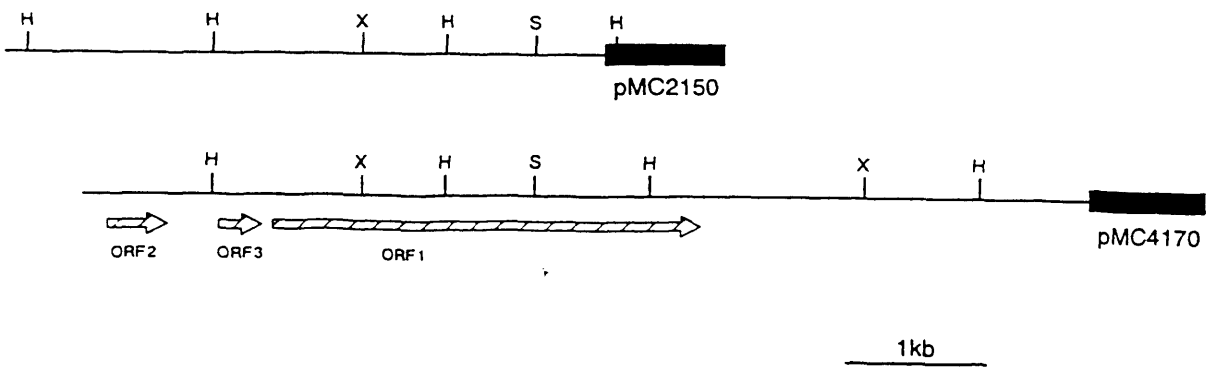


Figure 1.4 . (Taken from Camara *et al* ., 1994).
Restriction map of cloned pneumococcal DNA in pMC2150 and pMC4150. Vector sequence is represented by solid lines. Orientation and location of ORFs is shown by arrows. Restriction endonuclease sites are as follows: H, *Hind* III; S, *Sph* I; X, *Xba* I.

In order to confirm that the neuraminidase activity from pMC4170 was associated with ORF1, the entire ORF1 was cloned into the expression vector pQE30 (Qiagen, see purification Section 4.2.5 for more details) (Camara *et al* ., 1994). The recombinant was designated pQMC1 (Section 3.1.2, Figure 3.1) and was transformed into *E. coli* SG13009::pREP4. The correct cloning of ORF1 was ascertained by sequencing the ends of the inserted pneumococcal DNA in pQMC1. Neuraminidase activity was present in the sonicates of bacteria harbouring pQMC1, which confirmed the prediction that neuraminidase is encoded by ORF1 in pMC4170.

nan A has three potential start codons but only two of them are associated with a putative Shine-Dalgarno sequence (Figure 1.5). Initiation of translation from these would give proteins of 112 and 110 kDa including a signal peptide at the N terminus. Removal of the signal peptide would give a neuraminidase of about 108 kDa (Camara *et al* ., 1994; Berry *et al* ., 1996). This is very similar to the 107kDa neuraminidase reported by Lock *et al* ., 1988b) . Furthermore N-terminal sequencing of the 107 kDa neuraminidase has shown that the first 10 amino acids are identical to the sequence predicted for nanA after cleavage of the signal peptide (Berry *et al* ., 1996).

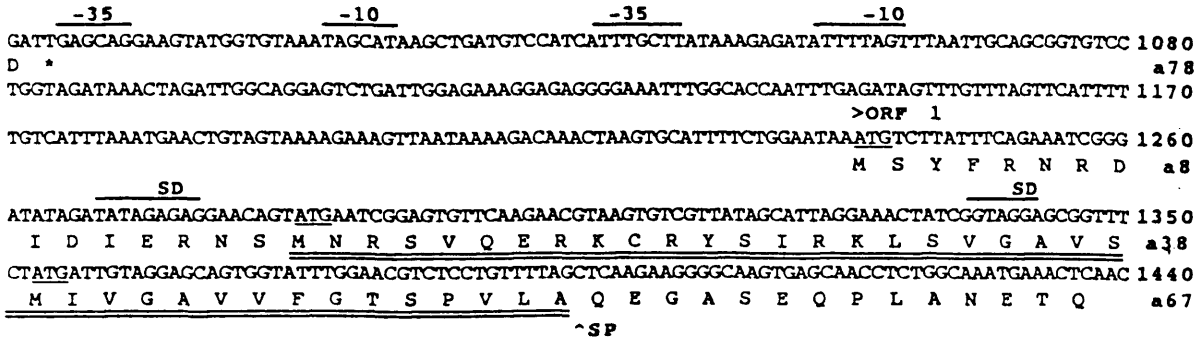


Figure 1.5. (Taken from Camara *et al* ., 1994).
Figure showing the 5'sequence of the *nan* A gene. The nucleotide sequence (5' to 3') is shown on the upper line and the deduced amino acid sequence (of Nan A) is shown underneath.
The figures to the right of the sequence indicate the nucleotide and amino acid numbers.
The three potential start codons are underlined and the two putative ribosome binding sites (SD) are indicated. The two putative -35 and -10 promoter sequences are shown upstream of ORF1. A putative signal peptide (double underlined) and signal paptidase (arrowhead) are also shown.

More recently, work by Berry *et al* ., (1996) led to the cloning and characterisation of another neuraminidase gene designated *nan* B. They constructed a cosmid gene bank of pneumococcal DNA in *E. coli* K - 12 strain DH1 (Hanahan, 1983) using a low copy number vector pOU61cos. The library consisting of 600 clones was screened with mouse anti-NanA serum. One cosmid clone was isolated and lysates of it were able to cleave MUAN. The recombinant cosmid from this clone was termed pJCP309. Purification and restriction analyses of pJCP309 showed that it contained a 32 kb pneumococcal DNA insert. In order to isolate smaller DNA fragments which coded for the pneumocccal neuraminidase, pJCP309 was cleaved with *Pst* I and fragments were cloned into pBluescript SK. The resulting plasmids were transformed into *E. coli* DH5α and recombinants were screened for the neuraminidase activity. One subclone designated pJCP310 was able to cleave MUAN and contained 5.2 kb of pneumococcal DNA. DNA from pJCP310 failed to hybridize to a *nan A* specific probe. However the *nan A* probe hybridized strongly to pJCP309 DNA which suggested that pJCP309 encoded two different neuraminidase genes. The position of the two putative neuraminidase coding regions in pJCP309 were mapped (Figure 1.6).

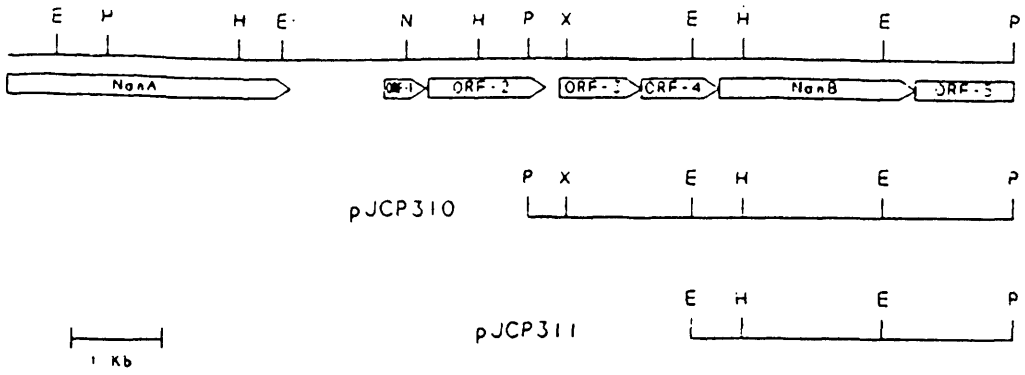


Figure 1.6. (Taken from Berry *et al* ., 1996)
Restriction map of a section of the pneumococcal DNA insert in recombinant cosmid pJCP309. The horizontal lines represent *S. pneumoniae* DNA cloned in pJCP309 and subclones thereof cloned in pBluescript (pJCP310 and pJCP311). ORFs are shown below the map. Restriction sites are as follows: E, *Eco* RI; H, *Hind* III; N, *Nco* I, P,; *Pst* I; X, *Xho* I.

The gene encoded by the insert in pJCP310 was termed *nan B*. The complete sequence of pJCP10 was obtained to enable further characterisation of the *nan B* gene. The entire sequence between *nan A* and *nan B* also was determined. It was found that *nan B* was located about 4.5kb downstream of *nan A* and on a separate operon. The predicted size of Nan B after cleavage of the signal peptide was 74.5 kDa. However SDS-PAGE analysis of NanB purified from recombinant *E. coli* showed it to be a 65 kDa protein. The discrepency between the predicted and actual weight was attributed to C-terminal processing or anamalous behaviour on SDS-PAGE (Berry*et al* ., 1996).

It is apparent that *S. pneumoniae* produces more than one neuraminidase and that the different molecular weights previously observed probably were not a consequence of proteolytic cleavage of a single neuraminidase.

1.7.9 Structure of bacterial neuraminidases

1.7.9.1 Primary sequence

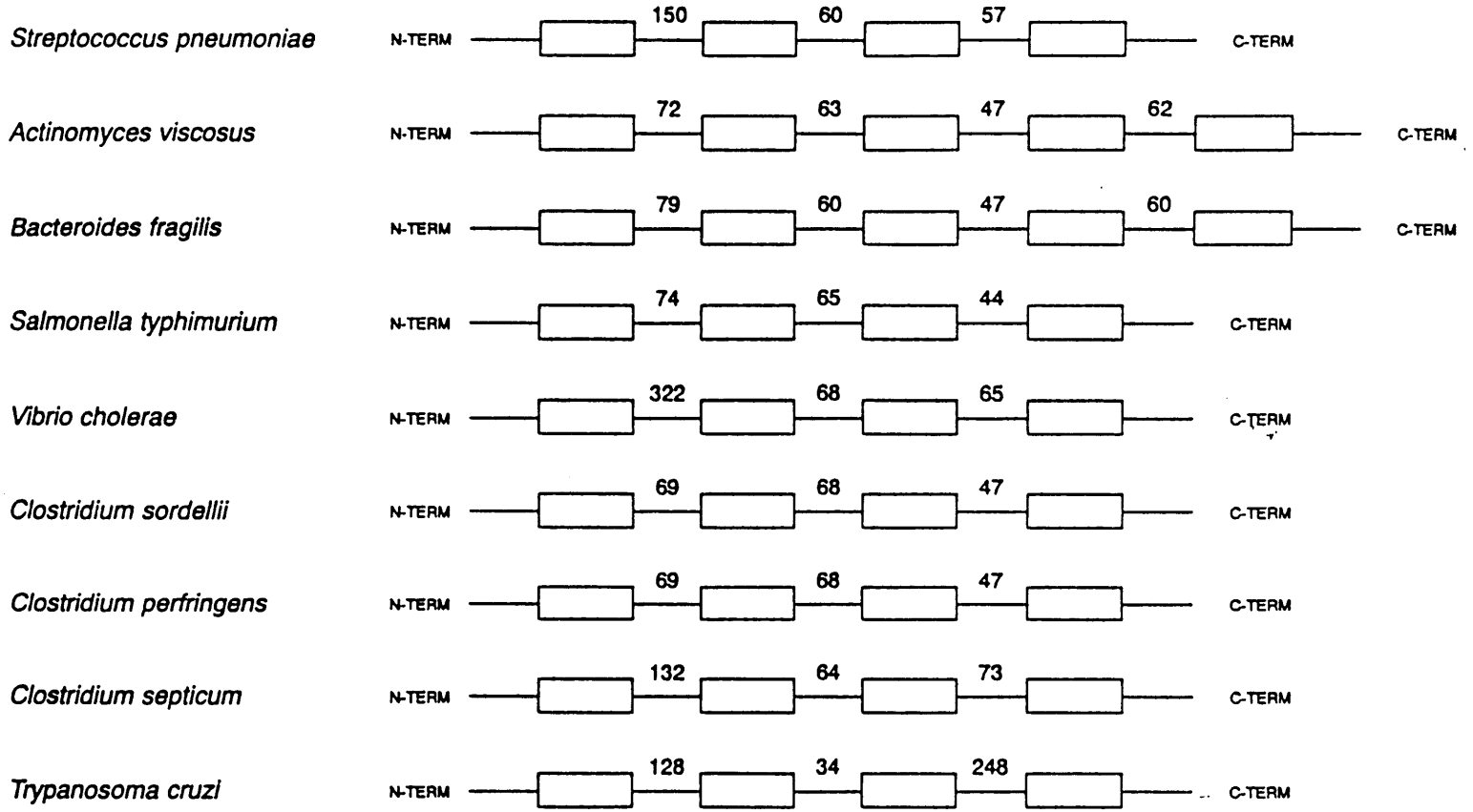
The first bacterial neuraminidase gene (designated *nanH*) to be cloned was that of *V. cholerae* in 1988 (Vimr *et al* ., 1988). Subsequently many other bacterial, protozoan and mammalian neuraminidase genes have been cloned and sequenced (Vimr , 1994). Most members of the neuraminidase superfamily

have amino acid sequence identity of between 20-30%, increasing to 50% if conservative amino acid substitutions were also considered (Hoyer *et al* ., 1992; Roggentin *et al* ., 1993).

A distinguishing characteristic of all neuraminidases was the presence of a short sequence of 12 amino acids termed the "Asp-box" which was repeated four to five times in the protein (Roggentin *et al* ., 1989) (Figure 1.7). Examination of the primary sequence of the neuraminidase from *Clostridium sordellii* G12, *C. perfringens* A99, *Salmonella typhimurium* LT-2 and *V. cholerae* 395 showed that five of the 12 residues were highly conserved at fixed positions: Ser-X-Asp-X-Gly-X-Thr-Trp. Moreover the distance separating the "Asp-boxes" was also highly conserved and was usually between 45 - 71 residues (Roggentin *et al* ., 1989). The Asp-boxes have also been detected in viral and protozoal neuraminidases (Roggentin *et al* ., 1989; Pereira *et al* ., 1991). The precise function of the Asp boxes is unknown but it is thought that they are not involved in catalysis because of their distance from the substrate-binding site (Vimr, 1994) (Section 1.7.9.2). Hoyer *et al* ., (1992) have predicted that Asp-boxes were located at ß- turns and so were exposed at the sialidase surface and may be important for protein folding. Asp-boxes have also been detected in many sugar-binding proteins and had led to suggestions that they are involved in sugar binding. Additionally the detection of Asp-boxes in the yeast vacuolar-protein-sorting-peptide Vps has led to suggestions that they may have a more general function in protein folding and/or interactions with other proteins (Vimr, 1994).

At the N-terminus of many bacterial and protozoal neuraminidases another group of conserved amino acids, RXP, is found. The amino acids, Arg (R) and Pro (P) were present in all seven bacterial and two protozoan neuraminidases examined (Roggentin *et al* ., 1993). It has been suggested that the Arg residue in this region is involved in binding substrate (Roggentin *et al* ., 1993). Evidence for this hypothesis comes from experiments on the neuraminidase of *C. perfringens* . It has been shown that site-specific mutation of the Arg>Lys in the *C. perfringens* neuraminidase RXP region caused a significant change in the catalytic properties of the mutant enzyme. The mutant enzyme had an increased K_m (determined using the substrate MUAN) by comparison to the wild type enzyme and was resistant to competitive inhibition by 2, 3 didehydro 2,6 anhydro-n-acetyl neuraminic acid (DANA) (Roggentin *et al* ., 1992).

Figure 1.7. Diagram showing the Aspartic box motif found in bacterial neuraminidases. (Taken fom Camara *et al* ., 1994)
The aspartic box consensus motif (SXDXGXTW) is depicted as an open box. The number of amino acids seperating the asp boxes is indicated.



1.7.9.2 Crystal structure

1.7.9.2.1 Crystal structure of the neuraminidase from *S. typhimurium*

The first bacterial neuraminidase to be crystallized was that of *S. typhimurium*, using the method of multiple isomorphous replacement (MIR). The neuraminidase is a 42 kDa monomer. The structure of the *S. typhimurium* and influenza virus neuraminidases are shown schematically in Figure 1.8. Comparison of the crystal structure of the *S. typhimurium* and influenza neuraminidases revealed that the overall folding of the enzyme and the location of specific amino acids in the active site were very similar in both enzymes. However some differences in the folding of the two enzymes were also observed (Crennell *et al* ., 1994).

The neuraminidase is composed of predominantly ß-sheets, two small α-helical segments and a shallow active site crevice which is opposite a deep cleft which extends 15Å into the structure (Crennell *et al* ., 1993). The structure of the *S. typhimurium* neuraminidase (STNA) is described by Crennell *et al* (1993) as a canonical ß-propeller. This is because the six four-stranded antiparallel ß-sheets are arranged like propeller blades around an axis which crosses through the active site as shown in Figure 1.8 (Crennell *et al* ., 1993).

The Asp-boxes are situated on the bend between the third and fourth ß-sheets of the first four strands. The bend is stabilized by aromatic residues which arrange into a hydrophobic centre and the aspartic residues which point out into the solvent (Crennell *et al* ., 1996). The same fold with a rms value of 0.13-0.40 Å between the Cα atoms of the Asp boxes was found at each bend. Due to their surface location and position far from the active site, it has been postulated that the ASP boxes may be involved in protein secretion or folding. (Crennell *et al* ., 1993). In contrast to the influenza neuraminidase which has eight disulphide bridges, the *S.typhimurium* neuraminidase only has one disulphide bridge (Crennell *et al* ., 1996). No disulphide bridges were found in the other two bacterial neuraminidases whose structure was known (*V. cholerae* and *M. viridifaciens*, (Crennell *et al* ., 1994; Gaskell *et al* ., 1995 respectively), therefore Crennell *et al* ., (1996) concluded that the presence of the disulphide bridge in *S.typhimurium* neuraminidase was not functionally important. In *S.typhimurium* the disulphide bridge occurs between the first and second sheets, whereas in influenza the disulphide bridges occur mostly

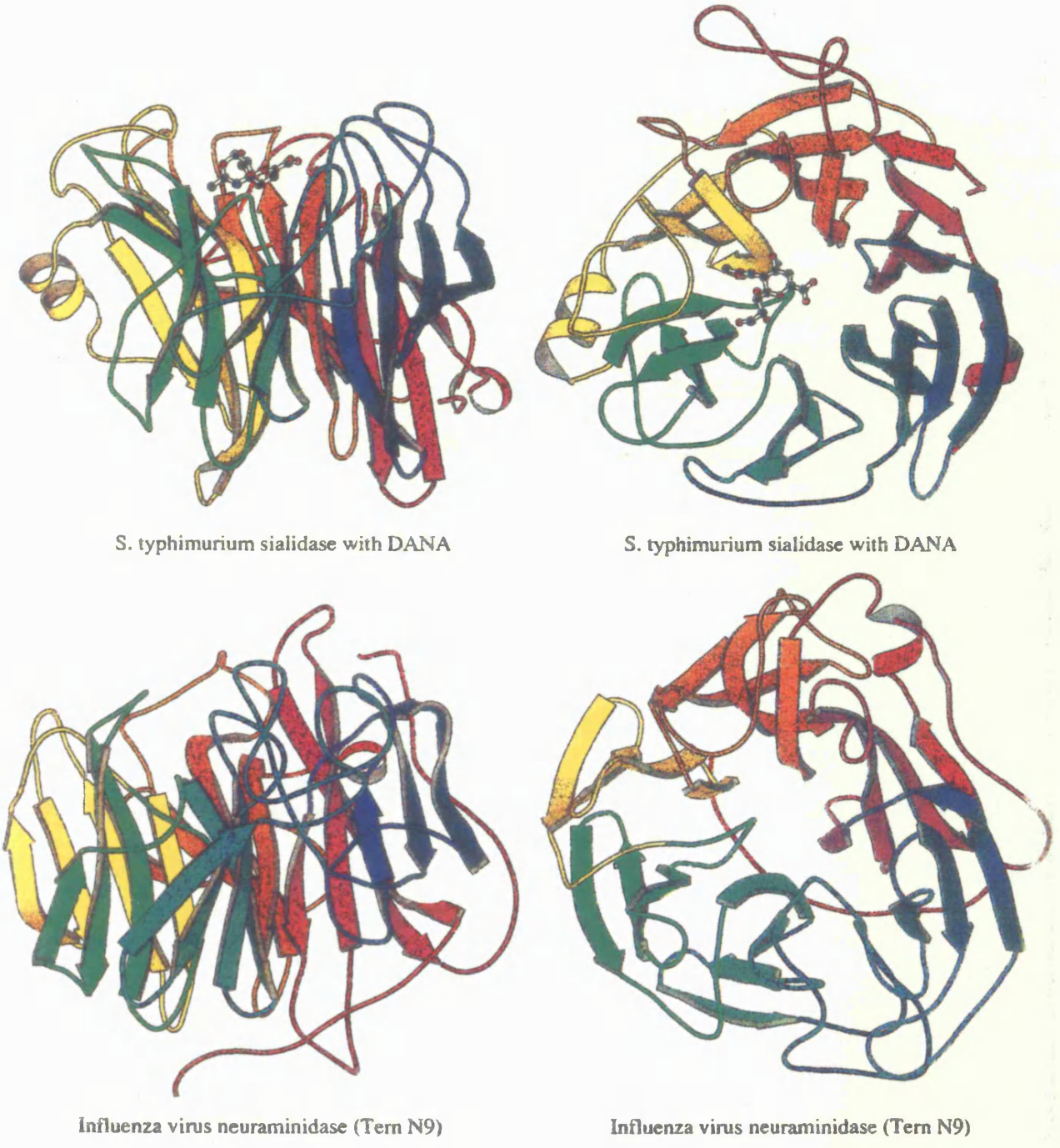


Figure 1.8. (Taken from Crennell *et al*., 1990).
Schematic ribbon diagram of the neuraminidase from *S. typhimurium* bound to the inhibitor DANA (upper) and the neuraminidase from tern influenza virus subtype N9 (lower) generated by the MOLSCRIPT program. The side view is shown on the left hand side and the view into the active site from above is shown on the right hand side. The N9 structure is shown following optimal alignment with the bacterial structure using the SHP program. The N and C termini are shown in red and purple respectively. The chains are coloured from red at the N terminus to violet at the C terminus.

inside the sheets. Although the disulphide bridges in the influenza neuraminidase do not lead to tighter packing of the strands within the sheets, they may cause the sheets near the base of the molecule to pack together more closely and consequently close the cleft which is observed in the *S.typhimurium* neuraminidase (Crennell *et al* ., 1996).

The inhibitor 2-deoxy-2,3-dehydro-N-acetylneuraminic acid (DANA) or the product N-acetylneuraminic acid (NANA) have been used to study the active site of the *S.typhimurium* and influenza neuraminidases (Crennell *et al* ., 1993; Crennell *et al* ., 1996). There was a great similarity in the overall folding and arrangement of the catalytic residues in the two enzymes. However some differences in the active site were also observed. These are discussed below. The active site of the two neuraminidases are shown in Figure 1.9.

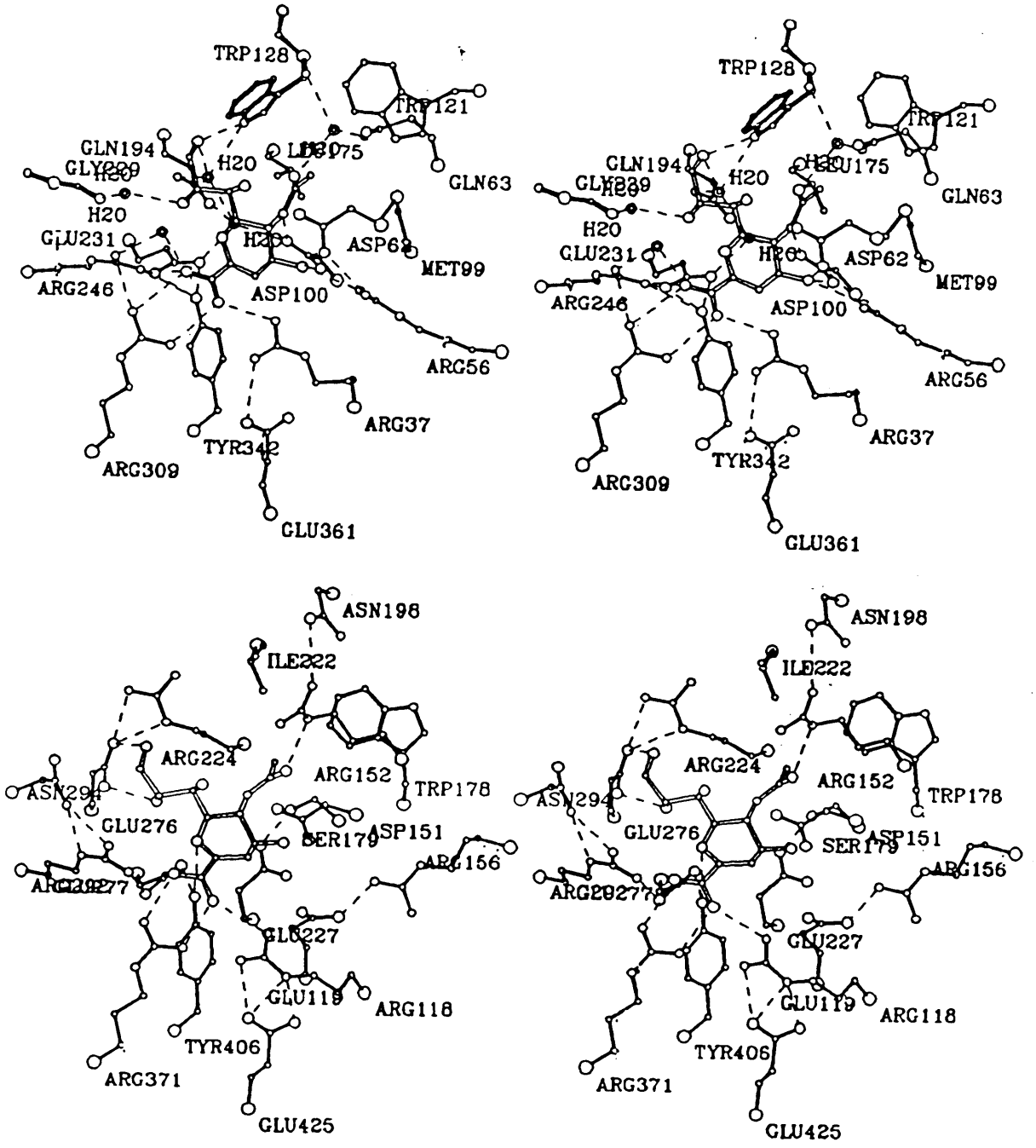


Figure 1.9. (Taken from Crennell *et al* ., 1996). The inhibitor DANA bound to the active sites of the *S. typhimurium* (upper) and Tern influenza virus type N9 (lower) neuraminidases. Both sites are viewed from the same direction looking into the active site. Water molecules and hydrogen bonds are shown by concentric circles and broken lines respectively.

A comparison of the active site of the *S. typhimurium* and influenza neuraminidases (using DANA) showed that the active site of the two enzymes share some common features. These are shown in Table 1.10.

Features of the *S. typhimurium* neuraminidase active site that are similar to the influenza neuraminidase active site.
The carboxylic acid group present in all sialic acid derivatives is stabilized by three arginine residues (37, 246, 309).
One of the triad arginines is stabilized by a glutamic acid (361.)
The hydroxyl group of the tyrosine (342) is very close (3Å) to the C1 and C2 carbons of the inhibitor DANA. This led to suggestions of its involvement in stabilizing the carbonium ion transition state intermediate (Burmeister *et al* ., 1993).
Residues Met-99, Trp-121, Trp-128 and Leu-175 form a hydrophobic pocket which houses the N-acetyl group of the sialic acid.
A proton donating glutamate (231) residue at the base of the active site is thought to be involved in the catalytic mechanism (Burmeister *et al* ., 1993).
An aspartic acid residue (62) which could either donate a proton to the glycosidic oxygen, or be involed in stabilizing a proton-donating water molecule.

Table 1.10. (Adapted from Crennell *et al* ., 1994). Features shared by the active sites of the influenza and *S. typhimurium* neuraminidases. The numbers in brackets refer to the residues in *S. typhimurium* neuraminidase.

Some differences were also observed between the catalytic site of the *S. typhimurium* and influenza neuraminidases. These were mainly in the interaction of the active site with the glycerol and O4 groups of the sialic acid (Crennell *et al* ., 1993).

1) *S.typhimurium* neuraminidase has a fragile bond between Trp 128 and O9 of the sialic acid DANA, whereas in influenza neuraminidase two strong hydrogen bonds exist between a glutamic residue and O8 and O9 of glycerol

side group of DANA and NANA (Burmeister *et al* ., 1992; Varghese *et al* ., 1992). This difference may explain the different kinetics of the two enzymes. *S.typhimurium* neuraminidase has a turnover of $2700s^{-1}$ (Hoyer *et al* ., 1991), while for the influenza neuraminidase it is $10s^{-1}$ (Crennell *et al* ., 1993).

2) The other main difference is in the interactions with the O4 of sialic acid. In the *S. typhimurium* neuraminidase the O4 of DANA is linked to Arg 56 and Asp 100 by strong hydrogen bonds and to Asp 62 by weak hydrogen bonds. In contrast the influenza neuraminidase only has a weak bond between Asp 62 and O4 of DANA. This difference reflects the different substrate preferences of the two enzymes. For example the influenza neuraminidase is able to hydrolyse N-acetyl-4-O-acetylneuraminyl-(2-3)-lactose, but the *S. typhimurium* neuraminidase cannot (Messer, 1974).

1.7.9.2.2 Crystal structure of the *Vibrio cholerae* neuraminidase

A year after the crystallization of the small bacterial neuraminidase of *S. typhimurium* another neuraminidase, belonging to the large family of bacterial neuraminidases, was crystallized. This was the neuraminidase of *V. cholerae* which has a molecular weight of 83 kDa. It has 16-24% sequence similarity with other neuraminidases and requires a Ca^{2+} ion for activity (Crennell *et al* ., 1994).

The structure of the *V.cholerae* neuraminidase (VCNA) is shown schematically in Figure 1.10. The neuraminidase consists of 54 ß-strands and 5 α-helices arranged into three domains. This central domain has the ß-propeller canonical fold and is flanked by two domains (Wings) which have identical topology to a legume lectin (Crennell *et al* ., 1994). One flanking domain is at the N-terminus and the other is located between the second and third ß-sheets of the propeller (Crennell *et al* ., 1994).

The topology of the VCNA is shown in Figure 1.11. Residues 1 - 24 encode a signal peptide which is cleaved prior to release of the enzyme into the extracellular medium. Residues 25 - 216 of the polypeptide forms the first lectin-like domain. Next the polypeptide forms the outer strand of the sixth ß-sheet of the neuraminidase domain (as previously seen in the *S.typhimurium* (Crennell *et al* ., 1993) and influenza (Varghese *et al* ., 1983) neuraminidases). The first two ß-sheets of the central domain are then formed. Next the

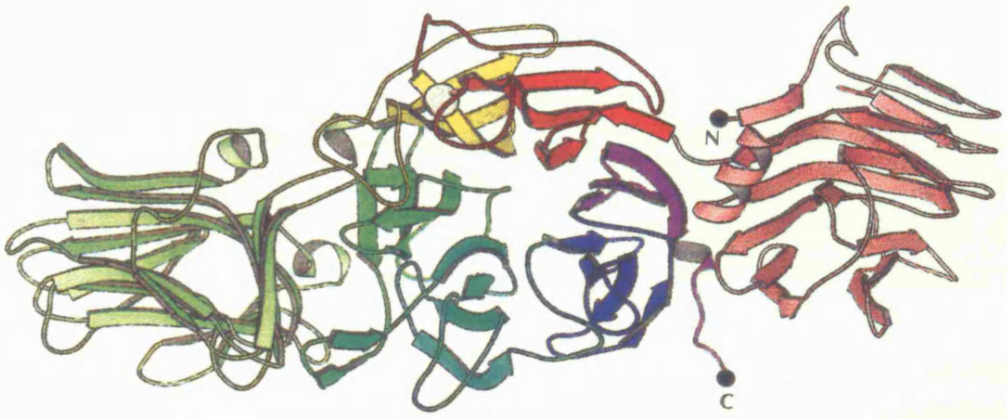


Figure 1.10. (Taken from Crennell *et al* ., 1994).
Schematic diagram of the *V. cholerae* neuraminidase where the protein domains are coloured as follows: pink - amino-terminal wing-1, red to purple - the canonical neuraminidase domain, pale green - wing-2 . The grey sphere represents the calcium ion which is required for activity.

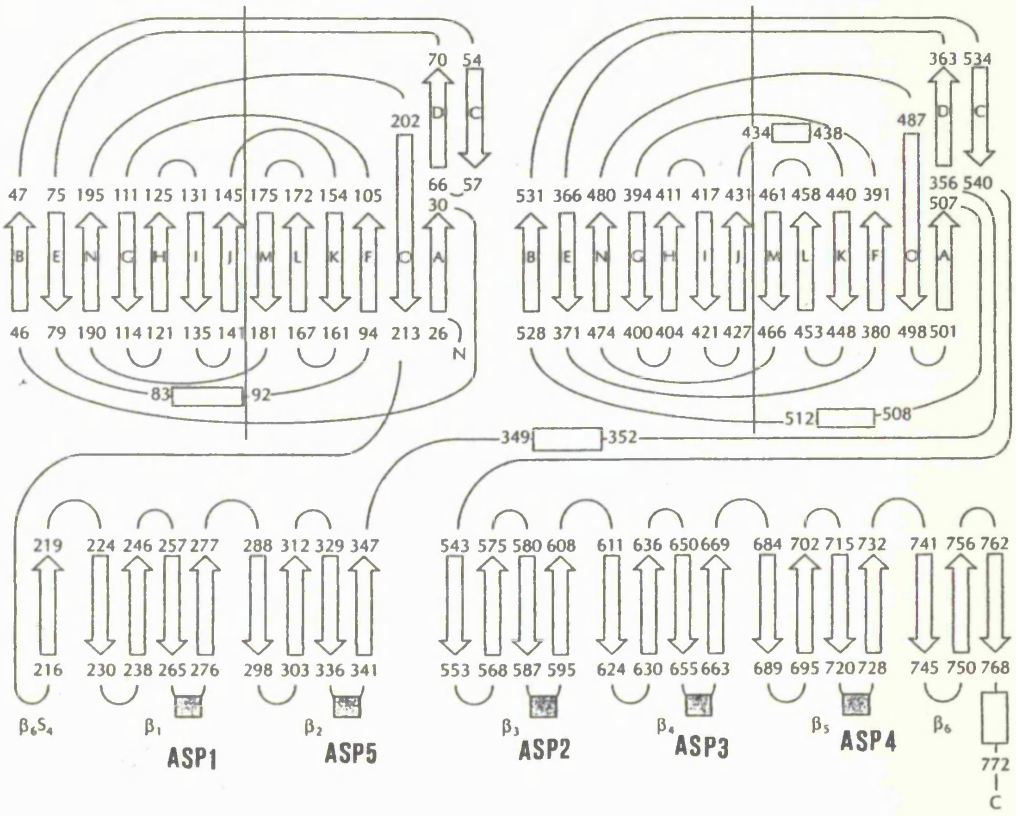


Figure 1.11. Topology of *Vibrio cholerae* neuraminidase (Taken from Crennell *et al* ., 1994). The polypeptide chain is numbered from 25 - 781. Residues 1 - 24 encode a leader peptide which is cleaved prior to release into the extracellular medium. The arrows represent the ß-strands and the open and shaded boxes represent the α-helices and Asp-boxes respectively. The four Asp boxes (labelled Asp1, Asp2, Asp3 and Asp4) are at topologically equivalent position to those found in *Salmonella typhimurium* neuraminidase and have the S-X-D-X-G-X-T/N-W consensus sequence. The existence of a fifth Asp box (labelled Asp 5) is proposed and has a primary sequence (Y-D-V-A-S-G-N-W) that is different from the consensus. The ß sandwich of the two wings is formed by closing the two sheets of ß-strands around the vertical lines that is shown running through both wings.

polypeptide forms the second lectin-like domain before completing the other four ß sheets of the central domain. Finally as previously observed in the *S. typhimurium* neuraminidase (Crennell *et al* ., 1993), the polypeptide ends in a small α-helix. No cysteine residues were present in the protein. Five Asp boxes are present and are located at topologically equivalent position to those in *S.typhimurium* neuraminidase (Crennell *et al* ., 1994).

Comparison of the central domain with the *S.typhimurium* and influenza neuraminidases shows a greater homology with the former (Crennell *et al* ., 1994). The central domain is strongly associated with the two outer domains (wings). The first wing is linked by 18 hydrogen bonds, whereas the second wing is linked by predominantly hydrophobic interactions. Despite the low sequence identity (23%) between the wings they are structurally very similar as shown by a root mean square (r.m.s.) fit of 1.8Å for 146Cα atoms (Crennell *et al* ., 1994). The wings are structurally very similar to the legume lectins. However the wings do not have any metal binding loops which are present in other lectins (Crennell *et al* ., 1994). It is not clear which ligands are recognized by the wings, and whether the wings share a common binding site. Nonetheless it is known that the carbohydrate binding site of other lectins is located on the concave fold (Crennell *et al* ., 1994). As the concave surfaces of the wings point away from the central domain and are exposed to solvents, it is very probable that the wings also recognize some ligands.

The inhibitor DANA was used to characterize the catalytic site of the VCNA. The active site was found to be similar to the active site of the *S. typhimurium* and influenza neuraminidases. All the common features described in Table 1.10 were also present in the VCNA. The active sites of the VCNA, STNA and influenza neuraminidases are shown in Figure 1.12 and the residues important for activity are indicated. Some differences were also observed between the catalytic sites of the VCNA, *S. typhimurium* and influenza neuraminidases. These were mainly in the interaction of the active site with the glycerol and O4 groups of the sialic acid. In the influenza neuraminidase a strong hydrogen bond exists between the glutamate residue (residue 276 in neuraminidase from tern influenza virus subtype N9) and O8 and O9 of the glycerol group of the sialic acid (Bossart-Whitaker *et al* ., 1993). This glutamate residue is absent in the VCNA and STNA, so consequently there is virtually no interaction between the glycerol group of the sialic acid and the active site of these neuraminidases (Crennell *et al* ., 1994.

Active site of the cholera, salmonella and influenza neuraminidases.

Vibrio cholerae
neuraminidase

Salmonella typhimurium
neuraminidase

Tern influenza (subtype N9)
neuraminidase

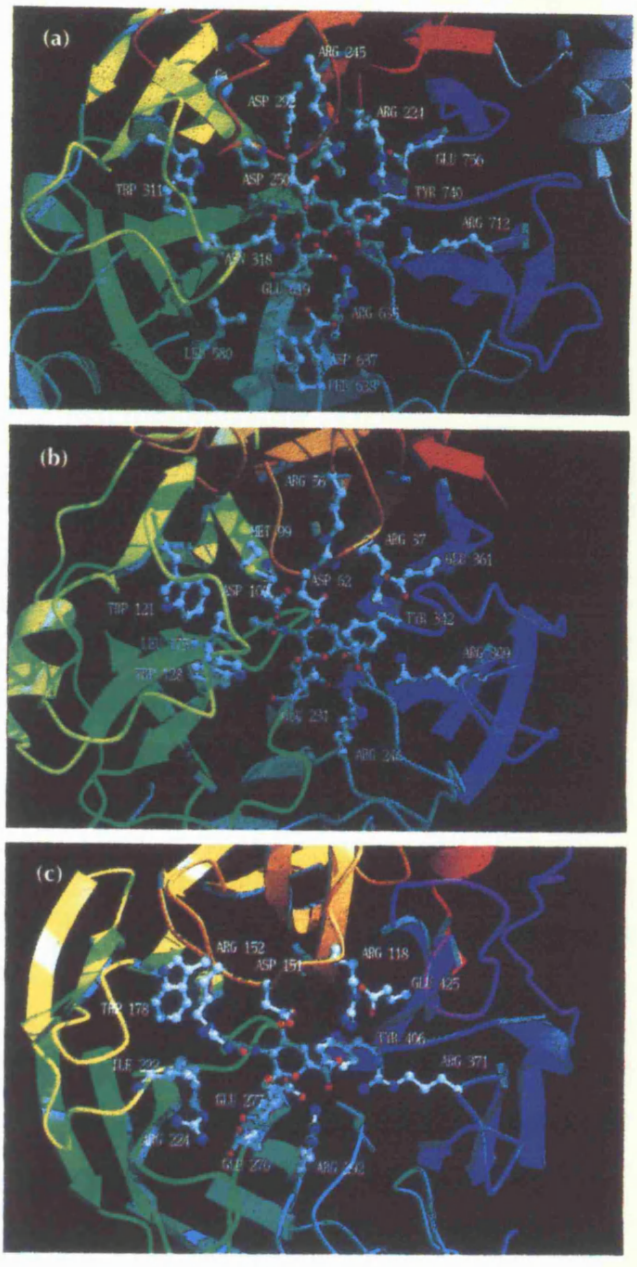


Figure 1.12. (Taken from Crennell *et al* ., 1994).
Schematic diagram of the active sites of cholera, salmonella and influenza neuraminidases showing the amino acids that interact with the inhibitor DANA.

The other main difference between the three neuraminidases is in the interaction of the active site with the O4 of the sialic acid. In the VCNA, residues Arg 245 and Asp 292 form strong hydrogen bonds with the O4 of sialic acids. Similarly in the STNA residues Arg 56 and Asp 100 are linked to the O4 of sialic acids by hydrogen bonds (Crennell *et al* ., 1994). In contrast these bonds are not present in the influenza neuraminidase. The resulting cavity in the influenza neuraminidase active site has been used to design inhibitors specific for this enzyme (von Itzstein *et al* ., 1993).

The VCNA is dependent on Ca^{2+} ion for activity. This cation is situated on the surface of the catalytic domain and functions to stabilize the Arg245, Asp250, Trp311 and Asn318 residues which are critical for enzyme activity (Crennell *et al* ., 1994).

1.7.9.2.3 Crystal structure of the *Micromonospora viridifaciens* neuraminidase

The crystal structure of one other bacterial neuraminidase (from *Micromonospora viridifaciens,* a non-pathogenic soil bacterium) has also been solved (Gaskell *et al* ., 1995) and shows many similarities to the structure of the STNA and VCNA. The structure of the *M. viridifaciens* neuraminidase has been described in detail (Gaskell *et al* ., 1995) and the essential features are briefly described below.

The *M. viridifaciens* neuraminidase was initially purified from bacterial cultural filtrates (Aisaka and Uwajima, 1987). It was found that a 68kDa enzyme was secreted when milk casein was used for induction, but a 41kDa enzyme was secreted if the inducer was colominic acid (Aisaka *et al* ., 1991). Cloning and sequencing of the neuraminidase gene followed by expression in *Streptomyces lividans* showed that the neuraminidase was 68kDa. Sakurada *et al* ., (1992) suggested that the 68kDa protein was cleaved by the action of the *S. lividans* protease to produce the 51 and 41 kDa forms that were both enzymically active. The 41, 51 and 68 kDa forms had similar catalytic properties which suggested that the catalytic domain resides in the 41 kDa form (Sakurada *et al* ., 1992)). The 68 and 41kDa forms of the enzyme have have been crystallized by Gaskell *et al* ., (1995).

The 41kDa form of the neuraminidase exists as a ß-propeller structure similar to that observed in the *S.typhimurium* and *V.cholerae* neuraminidases

(Figure 1. 13). The five Asp boxes were located between the third and fourth strand of each sheet. The active site contained all the motifs found in the other neuraminidases described so far (Table 1.10).

The 68kDa form of the neuraminidase is organized into three domains: a 41kDa ß-propeller domain, a ß-sandwich linker domain and a galactose binding domain (Figure 1.14). The ß-propeller domain is structurally similar to the domain found in the 41kDa form. The ß-sandwich linker domain is similar to the immunoglobulin constant domains (Bork *et al* ., 1994) and consists of four antiparallel strands over three antiparallel strands. This domain functions as a linker between the ß-propeller and galactose binding domains. The galactose binding domain is a ß-sandwich made up of five antiparallel ß-strands arranged over a three stranded antiparallel ß-sheet. This domain is structurally very similar to the galactose binding domain of a galactose oxidase from *Dactylium dendroides* (Ito *et al* ., 1994). It is thought that the galactose binding domain is involved in binding specific substrates or in cell adhesion (Gaskell *et al* ., 1995).

1.7.10 Characteristics of pneumococcal neuraminidases

There is little information available regarding the structure of the pneumococcal neuraminidases. However the cloning and sequencing of the two pneumococcal neuraminidase genes (*nanA* and *nanB*) in *E.coli* has revealed certain features. These are discussed below.

1.7.10.1 *nan* A

The cloning of the pneumococcal neuraminidase gene into *E.coli* has been described in Section 1.7.8.2. Analysis of the predicted protein sequence from *nan A* by Kyte-Doolittle analysis revealed that *nanA* was a hydrophillic protein with hydrophobic domains at the N- and C- termini (Camara *et al* ., 1994).

The amino acid sequence at the N-terminus was typical of the signal sequences found in other Gram positive bacteria (Austen and Westwood, 1991). The characteristics of the sequence are a hydrophobic domain (amino acids 32-49), which is followed by a stretch of basic and neutral amino acids, a proline at position 50 and the signal peptidase cleavage site (VLA) at position 51-53.

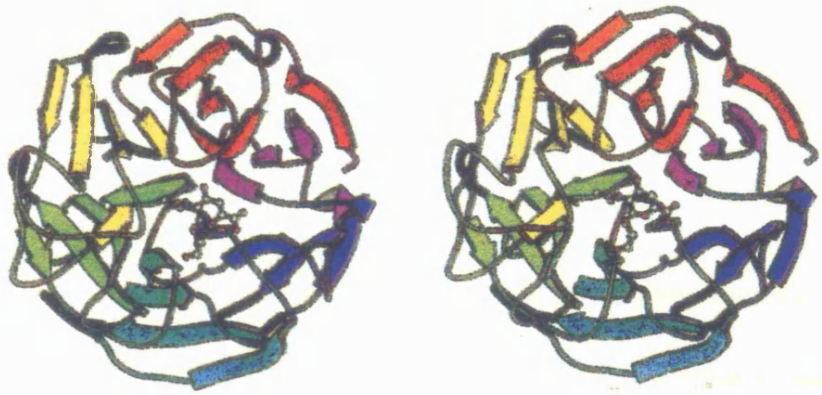


Figure 1.13. (Taken from Gaskell *et al* ., 1995).
Diagram of the 41 kDa form of the *M. viridifaciens* neuraminidase bound to the inhibitor DANA. The thick black turns indicate the position of the Asp boxes.

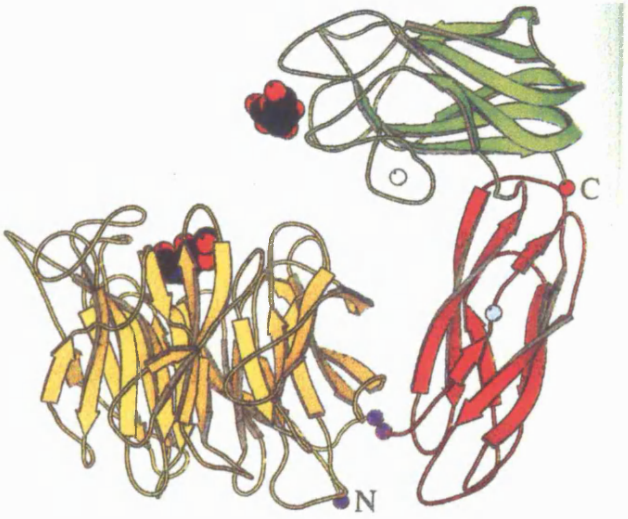


Figure 1.14. (Taken from Gaskell *et al* ., 1995).
Drawing of the 68 kDa form of *M. viridifaciens* neuraminidase. The protein domains are coloured as follows: yellow - 41 kDa domain, red - the linker domain and green - galactose-binding domain. The galactose and DANA molecules are drawn in space-filling mode. The grey sphere represents the monovalent cation (possibly Na^+), the purple spheres represent the two glycines (Gly 402 and 403) which may form the hinge and the light blue sphere represents the proline residue in the linker domain which causes the first strand to dogleg between the sheets.

The presence of this sequence suggested that the protein was exported from the cell. A signal peptidase cleavage site was also present (Camara *et al* ., 1994).

The C-terminus amino acid sequence resembled the sequences present at the C-terminus of surface proteins from other Gram positive bacteria (Schneewind *et al* ., 1993). This anchor motif consists of 50-125 residues (a large proportion of which are proline/glycine and threonine/serine residues) a LPETGN sequence, and a hydrophobic domain which is followed by five charged amino acids (Schneewind *et al* ., 1993). The anchor motif sequences have been shown to play a part in fixing proteins to the cell wall of *Staphylococcus aureus* (Schneewind *et al* ., 1992; Schneewind *et al* ., 1993). Camara *et al* ., (1994) have suggested that the C-terminal amino acids may play a similar role in anchoring the neuraminidase onto the surface of the pneumococcus.

In common with other bacterial neuraminidases, four copies of the conserved sequence SXDXGXTW (Asp box) was found in ORF1 (Camara *et al* ., 1994) (Figure 1.7). Furthermore it was found that the number of amino acids separating the boxes was similar to the spacing of the Asp boxes in other neuraminidases (Roggentin *et al* ., 1989). The function of the Asp boxes is unknown, however it has been suggested by Crennell *et al* ., (1993) that they may be involved in stabilsing the protein structure.

Located 108 bases upstream of the first Asp box (at position 347) is another sequence called R-I-P which is the "RXP" motif which is highly conserved in bacterial neuraminidases (Roggentin *et al* ., 1989). As discussed above, structural studies on the neuraminidases from *V. cholerae* (Crennell *et al* ., 1994) and *S. typhimurium* (Crennell *et al* ., 1993) have shown that the arginine of the "RXP" motif interacts with the carboxylate group of the sialic acid and is therefore important for the activity of the enzyme. The "RXP" arginine may play a similar role in the pneumococcal neuraminidase.

Little is known about the active site of the pneumococcal neuraminidase. However, as discussed above, structural studies have indicated that certain amino acids are important for the activity of the neuraminidase from *S. typhimurium* (Crennell *et al* ., 1993), *V. cholerae* (Crennell *et al* ., 1994) and influenza A (Varghese *et al* ., 1983). Comparison of the primary sequence of the pneumococcal enzyme with those from *S. typhimurium, Vibrio cholerae* and influenza neuraminidases in conjunction with the crystallography data

enabled us to make predictions of the amino acids in the active site of the pneumococcal neuraminidase. These are shown in Table 1.11.

Proposed function	Neuraminidase			
	VC NA	ST NA	Influ. A NA	SP NA
Three arginines which stabilize the carboxylic acid group present in all sialic acids	R224	R37	R118	R347
	R635	R246	R292	R663
	R712	R309	R371	Likely equivalent residues are R721 or R730
Glutamic acid which stabilizes the first arginine of the triad	E756	E361	E425	E990
A tyrosine whose hydroxyl group is close to C1 and C2 of sialic acid	Y740	Y342	Y406	Likely equivalent residue is Y777
A glutamate thought to donate a proton and implicated in viral neuraminidase mechanism	E619	E231	E277	E647
A conserved tryptophan present in the hydrophobic pocket which houses the N-acetyl group of sialic acid	W311	W121	W178	No equivalent amino acid identified
An aspartic acid that is exposed to the solvent and may function as a proton donor or stabilize a proton donating water molecule	D250	D62	D151	D372

Table 1.11. Amino acids considered important for the activity of *V. cholerae, S. typhimurium* , influenza and *S. pneumoniae* neuraminidases.

1.7.10.2 *nan B*

The cloning of the second neuraminidase gene designated *nanB* has been described in Section 1.7.8.2. *nanB* is 2094 bp in length and located 4.5kb downstream of *nanA* on the pneumococcal chromosome (Berry *et al* ., 1996). *nanB* encodes a protein (NanB) of 698 amino acids (Berry *et al* ., 1996). Analysis of the deduced amino acid sequence revealed the presence of a putative signal peptide of 29 residues at the N-terminus (Berry *et al* ., 1996). The predicted size

of NanB after cleavage of the signal peptide was 74.5 kDa. Sequence comparisons with other bacterial neuraminidases showed limited homology to NanA but 30% homology to the neuraminidase of *Clostridium septicum*. (Berry *et al* ., 1996). The region of greatest homology was the R-I-P sequence (located 111 bases upstream of the first Asp box) which is the RXP motif found in the active site of bacterial neuraminidases (Roggentin *et al* ., 1989). In common with other bacterial neuraminidases NanB contained three Asp boxes (Berry *et al* ., 1996).

1.7.11 Project aims

The aim of this project was to characterise a neuraminidase from *S. pneumoniae* by relating particular amino acids to the enzyme activity of the protein. At the start of this project the gene for a pneumococcal neuraminidase (*nanA*) had already been cloned (Camara, 1992). Comparison of the primary sequence of the pneumococcal neuraminidase with those of other bacterial neuraminidases (See appendix II), and crystallography data of the *S. typhimurium* neuraminidase, enabled predictions to be made about the amino acids likely to be important for the activity of the pneumococcal neuraminidase. From a total of 17 amino acids predicted to be important for the activity of the pneumococcal enzyme (Table 1.11), three were selected for mutagenesis. These were E647, R663 and Y752. Residue E647 was equivalent to residues E231 and E277 in the *S. typhimurium* and influenza neuraminidases respectively. The glutamate is thought to be a proton donor and has been implicated in the influenza neuraminidase mechanism. Residue R633 is equivalent to residues R246 and R292 in the *S. typhimurium* and influenza neuraminidases respectively. In these neuraminidases the arginine stabilizes the carboxylic acid group of sialic acids. Residue 752 was selected on the basis of information provided by Peter Roggentin (Biochemisches Institut, Kiel, Germany, personal communication) who had found an equivalent residue to be important for the activity of the *C. perfringens* neuraminidase. To understand the role of these amino acids in the activity of the enzyme, site directed mutagenesis followed by purification and enzymology of the resultant protein was undertaken. This approach was proposed to lead to the production of inactivated neuraminidases that could be tested as protective immunogens against pneumococcal disease in mice.

Chapter 2: Materials and Methods

2.1 Bacteria, Plasmids, Oligonucleotides, Media and Antibiotics

2.1.1 Bacterial Strains and Plasmids

The bacterial strains and plasmids used in this study are listed in Tables 2.1 and 2.2 respectively. The oligonucleotide primers used in this work are listed in Table 2.3.

Bacterial strain	Genotype/characteristic	Reference/source
Escherichia coli		
DH5α	*sup* E44 *hsd* R17 *rec* A1 Δ*lac* U169 (ø80 *lac* ZΔM15) *end* A1 *gyr* A96 *thi*- 1 *rel* A1	Hanahan, (1983)
JM109	*rec* A1 *sup* E44 *end* A1 *hsd* R17 ($^{r}k^{-}$ $^{m}k^{+}$) Δ(*lac* -*pro* AB) *thi gyr* A96 (Nal^{r}) F'*tra* D36 *lacIq* Δ (*lac* Z) M15 *pro* $A^{+}B^{+}/e14^{-}$ (Mcr A^{-})	Yanisch-Perron *et al.*, (1985)
SG13009 [pREP4]	*Nal* ^{s} *Str*^{s} *rif*^{s} *lac* $^{-}$ *ara* $^{-}$ *gal* $^{-}$ *mtl* $^{-}$*F* $^{-}$ *rec* A^{+} *uvr*$^{+}$	Gottesman *et al.*, (1981)
Streptococcus pneumoniae		
D39	Virulent type 2 strain	NCTC 7466

Table 2.1 Bacterial strains

Plasmid	Comment	Source or Reference
pUC 18	High copy number plasmid cloning vector	Yanisch - Perron (1985)
pCR-Script™SK (+)	High copy number vector derived from pBluescript® II SK (+) phagemid. Exhibits ampicillin resistance and blue-white selection.	Stratagene (Short *et al.*, 1988)
pQE 30	High level expression vector derived from pDS56/RBSII.	Qiagen (Stuber *et al.*, 1990)
pQMC1	Neuraminidase structural gene (*nan A*) cloned into the expression vector pQE 30	Camara *et al* ., (1994)
pMC4170	Neuraminidase producing clone isolated from a gene bank of pneumococcal DNA from the nonencapsulated strain R36A (NCTC 10319)	Camara, (1992)
pSS2A	pUC 18 containing middle *Hind* III fragment of *nan A*	This study
pSSEQscript	pCR-Script™SK (+) containing 1.5kb fragment from pSS2A amplified by primers M13 Reverse and M13 Forward, then mutated E>Q at position 647	This study
pSS3A	As pQMC1 but lacking 466 bp from the 3' end of *nan A* , and lacking 14 bp from the MCS of pQE30 (between *Sal* I and *Hind* III sites)	This study
pSSEQvec	1.4kb E>Q mutated *Hind* III/pQMC1 fragment of *nan A* from pSSEQscript ligated into pSS3A	This study
pSSWTvec	As pSSEQvec but without the mutation	This study

Table 2.2 Plasmids

Plasmid	Comment	Source or Reference
pSSGT	*nan A* lacking 114 bases from the 5' end and 705 bases from the 3' terminus cloned into pQE 30	This study
pSSRHscript	pCR-Script™SK (+) containing 1.5kb fragment from pSS2A amplified by primers M13 Reverse and M13 Forward, then mutated R>H at position 663	This study
pSSRHvec	1.4Kb R>H mutated *Hind* III fragment of *nan A* from pSSRHscript ligated into pSS3A	This study
pDAYFscript	pCR-Script™SK (+) containing 1.5kb fragment from pSS2A amplified by primers M13 Reverse and M13 Forward, then mutatedY>F at position 752	This study
pSSYFvec	1.4kbY>F mutated *Hind* III/pQMC1 fragment of *nan A* from pDAYFscript ligated into pSS3A	This study

Table 2.2 Plasmids (continued)

Primer	Sequence (5′ - 3′)	Function	Origin
M13 Reverse	AGCGGATAACAATTTCA CACAGGA	Sequencing pSS2A and mutagenesis	This study
M13 Forward	GTAAAACGACGGCCAGT	Sequencing pSS2A and mutagenesis	This study
NeuE 647.1	TGCGCAAAATACACAAT CAACGGTGGTACA	For mutating E>Q at position 647 in pSS2A	This study
NeuE 647.2	TGTACCACCGTTGATTGT GTATTTTGCGCA	For mutating E>Q at position 647 in pSS2A	This study
Y752F T1	AGAGTTTGCCTTTAATTC GCTCC	For mutating Y>F at position 752 in pSS2A	This study
Y752F T2	GGAGCGAATTAAAGGC AAACTCTC C	For mutating Y>F at position 752 in pSS2A	This study
A663H 1	CTCTTTATGCATGGTTTG AC	For mutating R>H at position 663 in pSS2A	This study
A663H 2	GTCAAACCATGCATAAA GAG	For mutating R>H at position 663 in pSS2A	This study
SOM1	CAACGATAACCGTCAGG TAG	For sequencing pSSEQvec,PSSRHvec and pSSYFvec	This study
SOM2	GCAACCTGAAGATCTCC AGT	For sequencing pSSEQvec,PSSRHvec and pSSYFvec	This study
SOM3	GGCACGTGTCGAAGAAA ATG	For sequencing pSSEQvec,PSSRHvec and pSSYFvec	This study
SOM4	CAGTATGTTCATACAAG ATGCC	For sequencing pSSEQvec,PSSRHvec and pSSYFvec	This study
SOM5	CTACGATGAACAATAGA CG	For sequencing pSSEQvec,PSSRHvec and pSSYFvec	This study

Table 2.3 Oligonucleotides used in this study

Primer	Sequence (5' - 3')	Function	Origin
SOM6	CCTTCTCCCAAGTCACTC C	For sequencing pSSEQvec,PSSRHvec and pSSYFvec	This study
EQV1F	ATCGGTATGGTCATCAG A CG	For sequencing pSSEQvec,PSSRHvec and pSSYFvec	This study
EQV2F	ACAGGTTTGGCAGAAGG TGC	For sequencing pSSEQvec,pSSRHvec and pSSYFvec	This study
EQV2R	CCAGATGGAGTTCTGAG GTC	For sequencing pSSEQvec,pSSRHvec and pSSYFvec	This study
EQV3R	CTTGAACCAACACCATA TCG	For sequencing pSSEQvec,pSSRHvec and pSSYFvec	This study
SSNA1	TCAGGATCCCAAGAAGG GGCAA	For amplifying *nan A* gene lacking 114 bases from the 5' end and 705 bases from the 3' end from pMC4170	This study
SSNA2	CTAGTCGACCTATTATTT CGCTTCGGT	For amplifying *nan A* gene lacking 114 bases from the 5' end and 705 bases from the 3' end from pMC4170	This study

Table 2.3 Oligonucleotides used in this study (Continued).

2.1.2 Chemicals

All the chemicals were obtained from Fisons and Sigma unless otherwise stated. All bacteriological growth media were purchased from Oxoid Unipath Ltd.

2.1.3 Media and conditions for bacterial growth

All of the *E. coli* strains were propagated on Luria agar or in Luria Broth at 37°C. The *S. pneumoniae* strains were grown on blood agar base (Oxoid) containing 5% (v/v) defibrinated horse blood and incubated in a gas jar containing a lit candle to remove the oxygen. Liquid cultures of *S. pneumoniae* were incubated statically in brain heart infusion broth (BHI, Oxoid). Appropriate amounts of antibiotics were added to the media for selection of recombinant clones.

For long term storage *E. coli* strains were kept in Luria broth with 20% (v/v) glycerol as a cryoprotectant and stored at -70°C. *S. pneumoniae* strains were kept in BHI broth with 15% (v/v) glycerol as a cryoprotectant and stored at -70°C.

All media was made with distilled water and sterilized by autoclaving at 15 psi (pounds per square inch), 121°C for 15 minutes.

Media composition

Luria-Bertani broth (LB) (Roth, 1970)

Peptone/Tryptone	10g
Yeast extract	5g
NaCl	10g

Distilled water was added to give a final volume of 1 litre, followed by autoclaving.

Luria-Bertani Agar (LA)

As for Luria-Bertani broth but with addition of 1.5% (w/v) agar before autoclaving.

Blood Agar Base (BAB)

40g of BAB was dissolved in distilled water to a final volume of 1 litre and then autoclaved and cooled to 45°C. Defibrinated horse blood (TCS Microbiology) was added to a final concentration of 5% (v/v).

Brain Heart Infusion (BHI)

BHI was prepared by dissolving 37g of BHI in distilled water to a final volume of 1 litre followed by autoclaving.

2.1.4 Antibiotics

The antibiotics used in this study, their preparation and working concentrations are listed in Table 2.4. All antibiotics were filter sterilised prior to use.

Antibiotic	Solvent	Stock concentration	Working concentration	Storage
Ampicillin	Distilled water	100mg/ml	100µg/ml	-20°C
Kanamycin	Distilled water	50mg/ml	25µg/ml	-20°C

Table 2.4 Antibiotics used in this study

2.2 DNA techniques

2.2.1 Agarose gel electrophoresis of DNA

DNA fragments were separated by gel electrophoresis using the method of Sambrook *et al* ., (1989). Agarose gels from 0.7 - 2.0% (w/v) were prepared as appropriate for the expected size of DNA fragments. Agarose (Seakem HGT) was mixed with 1 x TAE buffer and dissolved by heating in a microwave oven. 0.5µg/ml ethidium bromide was added to the molten agarose and the mixture was maintained molten until required. Gels were cast in a perspex gel tray and wells were formed using a perspex comb. The DNA sample was mixed with loading buffer prior to electrophoresis and run alongside molecular weight markers (1Kb ladder, Gibco/BRL) The gel was submerged

in 1 x TAE buffer and run at a voltage of 30-100V. The DNA fragments were visualised on a ultraviolet transilluminator.

1 x TAE

Tris-Acetate	40mM
EDTA (pH 8.0)	1mM

Final pH adjusted to pH 7.8 with glacial acetic acid

Loading Buffer

Bromophenol Blue	0.25% (w/v)
Xylene Cyanol	0.25% (w/v)
Ficoll(40,000MW, Pharmacia)	15% (w/v)

made up in nanopure water

2.2.2 Extraction of DNA from agarose gels

Sephaglass bandprep (Pharmacia) and Qiaquick gel extraction (Qiagen) kits were used for purifying DNA fragments from agarose gels.

2.2.2.1 Sephaglass Bandprep (Pharmacia)

The DNA fragment of interest was excised from the gel with a clean scalpel blade and dissolved in gel solubiliser buffer by heating at 60°C for 10 minutes. Sephaglass BP suspension (5µl/µg of DNA) was added and incubated for 5 minutes at room temperature with gentle mixing at every minute. The DNA and resin were pelleted by centrifugation for 15 seconds at 13,000 rpm (≥ 10,000 x g) in a Sanyo MSE Micro Centaur benchtop microfuge and the supernatant discarded. The DNA was washed three times in wash buffer, air dried and finally eluted by incubation with elution buffer for 5 minutes at room temperature. The DNA was briefly spun at 13,000 rpm (≥ 10,000 x g) to remove any sephaglass residue and stored at -20°C until required.

2.2.2.2 QIAquick gel extraction (Qiagen)

All centrifugation steps were performed at 13,000rpm (≥ 10,000 x g), at room temperature in a microfuge. The DNA fragment of interest was excised from the gel with a clean scalpel and weighed in a 1.5ml tube. Three volumes of buffer QX1 were added to one volume of gel. The gel was dissolved by incubation at 50°C for 10 minutes. After the gel had dissolved if the pH of the mixture was ≥ 7.5 as indicated by orange or violet colouration then 10µl of 3M sodium acetate (pH 5.0) was added to turn the mixture yellow and return pH to ≤ 7.5. One gel volume of isopropanol was added and mixed. DNA binding was achieved by applying the mixture to a QIAquick column and spinning for 1 minute. The eluate was discarded and 0.5ml of buffer QX1 was applied to the column to remove traces of agarose. The DNA was washed by addition of 0.75ml of buffer PE to QIAquick column and centrifugation for 1 minute. After discarding the eluate the column was spun for a further minute and placed in a clean 1.5ml microfuge tube. The DNA was eluted by adding 30µl of 10mM Tris-HCl pH 8.5 into the centre of the QIAquick column and centrifuging for 1 minute. The DNA was stored at -20°C until required.

2.2.3 Quantitation of DNA

The concentration of DNA was measured by the method of Sambrook *et al* ., (1989). The quantity and purity of DNA was ascertained by measuring the optical density (OD) of the DNA at wavelengths 260 and 280nm in an Ultrospec 3000, UV/Visible Spectrophotometer (Pharmacia Biotech). An OD_{260} of 1 is equal to 50µg/ml of double stranded DNA, 40µg/ml of single stranded DNA or RNA and 20µg/ml of single stranded oligonucleotide (Sambrook *et al* ., 1989). The DNA was diluted in nanopure water and the OD_{260} and OD_{280} measured. The following calculation was applied.

$$OD_{260} \times \text{Dilution Factor} \times 50 = [\text{DNA}]\ \mu g/ml$$

The ratio of OD_{260} to OD_{280} is indicative of the purity of DNA. A ratio of 1.8 is obtained with pure DNA, whereas a lower ratio indicates the presence of contaminating protein or phenol. A ratio greater than 1.8 is indicative of contaminating nucleotides (Sambrook *et al* ., 1989).

2.2.4 Phenol/Phenol Chloroform extraction of DNA

Unwanted proteins were removed from nucleic acid solutions by the method of Sambrook *et al* ., (1989). The DNA was mixed with an equal volume of phenol equilibrated with 0.1M Tris pH 8.0, or phenol:chloroform:isoamyl alcohol (25:24:1). The mixture was vortexed for 15 seconds and then centrifuged for 2 minutes at 13,000rpm (≥ 10,000 x g) in a microfuge. The upper aqueous phase was transferred to a fresh tube and the process repeated. Finally an equal volume of chloroform:isoamyl alcohol was added and the centrifugation repeated. The DNA in the aqueous phase was then precipitated with sodium acetate and ethanol as described in section 2.2.5.

2.2.5 Ethanol precipitation of DNA

Nucleic acids were concentrated by the ethanol precipitation method of Sambrook *et al* ., (1989). DNA was precipitated from solution by the addition of 0.1 sample volume of 3M sodium acetate (pH 5.2) and 2.5 sample volumes of cold 100% ethanol followed by incubation on dry ice for 30 minutes. The DNA was spun at 13,000rpm (≥ 10,000 x g) for 15 minutes in a microfuge and the supernatant discarded. 70% (v/v) ethanol was applied to the DNA and the centrifugation repeated for 5 minutes. After discarding the ethanol the tube was inverted on a paper towel and the DNA allowed to air dry for 15-30 minutes. The DNA was resuspended in nanopure water or TE buffer and quantitated by UV spectroscopy (Section 2.2.3).

2.2.6 Restriction endonuclease cleavage of DNA

Digestion of DNA with restriction endonucleases was performed as described by Sambrook *et al* ., (1989). Restriction digestion of DNA was performed using enzymes from Gibco BRL, according to the manufacturers's instructions. Typically reactions were performed using 1 unit of enzyme per μg of DNA in the reaction buffer supplied and the reaction allowed to proceed for at least 1-2 hours at the recommended temperature. To optimise the reaction, the glycerol concentrations were kept to below 5% (v/v) of the final reaction volume. Finally the reaction was terminated by heating at 65°C for 10 minutes. If the DNA was to be analysed directly on a gel then loading buffer was added to the digest (section 2.2.1). If the DNA was to be purified

then it was extracted twice with phenol/chloroform (2.2.4) followed by ethanol precipitation (2.2.5). The restriction enzymes and their buffers are shown in Tables 2.5 and 2.6 respectively.

Enzyme	Cleavage site 5′ - 3′	Incubation temperature	REact Buffer
Bam H I	G/GATCC	37°C	3
Bst E II	G/GTNAC	60°C	2
Eco R I	G/AATTC	37°C	3
Hind III	A/AGCTT	37°C	2
Nde I	CA/TATG	37°C	2
Not I	GC/GGCCGC	37°C	3
Pst I	CTGCA/G	37°C	2
Pvu I	CGAT/CG	37°C	7
Sal I	G/TCGAC	37°C	6
Sph I	GCATG/C	37°C	6
Xba I	T/CTAGA	37°C	2

Table 2.5 Restriction Endonucleases used in this study

Buffer	Tris-HCl (mM)	pH	$MgCl_2$ (mM)	NaCl (mM)	KCl (mM)
REACT 2	50	8.0	10	50	-
REACT 3	50	8.0	10	100	-
REACT 6	50	7.4	6	50	50
REACT 7	50	8.0	10	50	50

Table 2.6 Concentration of 1 x REACT buffers

2.2.7 Dephosphorylation of DNA

To prevent recirculization of the vector DNA, the 5′ phosphates were removed from linear DNA with calf intestinal alkaline phosphatase (CIAP) (Seeburg *et al.*, 1977; Ullrich *et al.*, 1977). The vector DNA was dephosphorylated prior to ligation by the method of Sambrook *et al* ., (1989).

calf intestinal alkaline phosphatase (Gibco/BRL) was used according to manufacturer's instructions. The DNA to be dephosphorylated was digested fully with the restriction enzyme and purified from the agarose gel. It was then subjected to 0.01 units of CIAP per pmol of DNA 5'-protruding ends at 37°C for 30 minutes in 1 x dephosphorylation buffer. For blunt DNA ends 1 unit CIAP per pmol DNA ends was incubated in 1 x dephosphorylation buffer for 1 hour at 50°C. A second addition of CIAP was made and the reaction continued for another 30 minutes. The CIAP was inactivated by heating at 75°C for 10 minutes in the presence of 5mM EDTA (pH 8.0) followed by 2 x phenol/chlorofom extraction (Section 2.2.4) and ethanol precipitation (Section 2.2.5).

1 x Dephosphorylation Buffer	
Tris-HCl (pH8.5)	50mM
EDTA	0.1mM

2.2.8 DNA ligation

Ligations were performed using T4 DNA ligase and buffer from Gibco BRL. The recommendations of Sambrook *et al* ., (1989) were followed for optimal vector:insert ratios. Typically reactions were set up with 50ng of vector in a final volume of 20µl with 1 x ligase buffer. A 3:1 molar ratio of insert:vector was included with 1 unit of T4 DNA ligase. The mixture was incubated at 4°C overnight, then precipitated with ethanol and the pellet resuspended in 4µl of nanopure water. 1-2µl of this DNA was transformed into 40µl of electrocompetent *E. coli*. For blunt ended cloning 0.1 - 1.0µg of DNA was used with 1 unit of DNA ligase followed by incubation at 14°C for 24 hours. The following ligation controls were included: 1) phosphorylated vector alone, 2) dephosphorylated vector alone and 3) insert DNA alone.

5 x DNA Ligase Reaction Buffer	
Tris-HCl (pH 7.6)	250mM
$MgCl_2$	50mM
ATP	5mM
DTT	5mM
PEG-8000	25% (w/v)

2.2.9 Transformation of *E.coli*

Transformation of *E. coli* was achieved by the electroporation method of Dower *et al* ., 1988. This method was chosen because of its high efficiency. With this method transformation efficiencies of 10^9 - 10^{10} transformants/µg of DNA have been achieved by optimizing various parameters, such as the strength of the electric field, the length of the electric pulse and the concentration of DNA.

2.2.9.1 Preparation of electrocompetent cells

Electrocompetent cells were prepared by the method of Dower *et al* ., (1988). One litre of LB was inoculated with 10ml of an overnight culture of *E. coli* and grown to an OD_{600} of 0.6 - 0.75. Cells were harvested by centrifugation at 5,000rpm (4225 x g), 4°C for 15 minutes in a Sorval RC-5B centrifuge, GS-3 rotor. The supernatant was removed and the cells were resuspended in 10ml of ice cold distilled water. More ice cold distilled water was added to bring the final volume to 1 litre. The centrifugation step was repeated and the cells were resuspended in 20ml of 10% (v/v) ice cold glycerol. Following a final centrifugation step at 5000rpm (4225 x g), 4°C for 10 minutes the cells were resuspended in 2 ml of 10% (v/v) ice cold glycerol. Cell suspensions were divided into 40µl aliquots and stored at -70°C.

2.2.9.2 Transformation by electroporation

Competent cells were placed on ice until thawed (10 minutes), then placed in chilled electroporation cuvettes (Bio Rad Gene Pulser Cuvette, 0.2cm electrode gap). The electroporator (Bio-Rad Gene Pulser) was set at a capacitance of 25µFD, resistance of 200Ω and a voltage of 2.5KV (Dower *et al* ., 1988). 10 - 20ng of DNA was added to the competent cells and the cuvette tappped gently to allow mixing of DNA and cells. The cuvette was left on ice for a further 5 minutes, then the outside was dried thoroughly before placing in electroporation chamber. Immediately following delivery of the pulse, 1ml of SOC medium was added to the cuvette. The cuvette contents were transferred to a sterile 1.5ml tube and incubated with shaking at 37°C for 60 minutes. 50 - 200µl of cells were plated onto LA plates containing the appropriate antibiotics and incubated at 37°C overnight.

SOC medium

Bactotryptone	2.0% (w/v)
Yeast Extract	0.5% (w/v)
NaCl	10mM
KCl	25mM
$MgCl_2$	10mM
$MgSO_4$	10mM
Glucose	20mM

Distilled water was added to final volume followed by autoclaving.

2.2.10 Procedures for DNA Extraction

The following methods were used to prepare *E. coli* plasmid DNA.

2.2.10.1 Small scale extraction of plasmid DNA (Miniprep)

This method was adapted from Holmes and Quigley (1981). 1.5ml of an overnight culture was centrifuged for 2 minutes at 13,000rpm (≥ 10,000 x g) in a microfuge and the pellet resuspended in 200µl of STET buffer. The cells were placed in a boiling water bath for 2 minutes then immediately placed on ice for 10 minutes. Following centrifugation at 13,000rpm (≥ 10,000 x g) for 10 minutes the pellet was removed with a toothpick. The DNA was precipitated by the addition of 20µl 10M ammonium acetate and 400µl cold ethanol. Following incubation on ice for 10 minutes the DNA was centrifuged at 13,000rpm (≥ 10,000 x g) for 15 minutes. The DNA was then washed with 70% (v/v) ethanol and redissolved in 20µl TE (10mM Tris - HCl pH 8.0, 1mM EDTA) containing 50µg/ml boiled RNase A (BDH). 5 - 10µl of DNA was analysed by restriction endonuclease digestion.

STET buffer

Sucrose	8% (w/v)
EDTA, pH 8.0	50mM
Tris-HCl pH 8.0	50mM
Triton X-100	0.5% (v/v)
Lysozyme	1mg/ml (BDH)

Tris - HCl/EDTA buffer (TE)

Tris-HCl (pH 8.0)	10mM
EDTA	1mM

2.2.10.2 Large scale extraction of plasmid DNA (Maxiprep)

In the early stage of this study DNA extraction was performed by equilibrium centrifugation in caesium chloride - ethidium bromide gradients. During the later stage of the study Qiagen plasmid purification kits were used. Generally less DNA was obtained from the latter method but the process was quicker. The quality of DNA obtained from both methods was comparable as judged by the OD 260nm to OD 280nm ratio (Section 2.2.3).

2.2.10.2.1 Caesium Chloride plasmid preparation

This method was adapted from Sambrook *et al* ., (1989). A single colony was used to inoculate 10ml of LB containing the appropriate antibiotics. This culture was grown for 6-8 hours at 37°C, with shaking. 1ml of this was used to inoculate 500ml LB plus antibiotics. This was grown for 14-16 hours with shaking at 37°C then harvested by centrifugation at 5,000rpm (4225 x g), 4°C for 10 minutes in a Sorvall RC-5B centrifuge, GS-3 rotor. The pellet was resuspended in 2.5ml of ST buffer, and then 1ml of ST buffer containing 20mg/ml lysozyme was added and incubated on ice for 10 minutes. 1.5ml of 0.5M EDTA (pH8.0) was added and incubated as above. 2.5ml of Triton lysis mix was added and incubated on ice for 10 -15 minutes or until lysis was apparent by the clearing of the solution. The solution was centrifuged at 20,000rpm (47,807 x g), 4°C for 45 minutes in a Sorvall SS34 rotor. The supernatant was carefully decanted and placed in a 15ml graduated plastic tube. For every 1ml of supernatant present 1g of CsCl (Optical grade) (Gibco - BRL) was added and the mixture incubated at 37°C for 10 minutes or until the CsCl had dissolved. The mixture was centrifuged at 2,500rpm (1398 x g) in a MSE Mistral 3000i centrifuge, 20°C for 5 minutes. The DNA/CsCl was removed carefully using a 10ml syringe and 16 gauge needle avoiding any crud material and placed in Beckman heat seal tubes which had been previously filled with 50µl of 10mg/ml ethidium bromide solution. The tubes were heat sealed and centrifuged in an BeckmanOptima TL ultra centrifuge (TLN 100 rotor) at 100,000rpm, 20°C for 4-5 Hours or at 80,000rpm, 20°C overnight. Following centrifugation, two bands of DNA were visible in daylight. The top band consisted of nicked circular plasmid DNA and linear bacterial DNA (chromosomal DNA). The bottom band consisted of closed circular plasmid DNA. The latter was harvested as follows. A 20 gauge

needle was inserted into the top of the tube to act as vent and then a second 20 gauge needle attached to a 2ml syringe was inserted at the site immediately below the lower band and the DNA was collected in this second syringe. The ethidium bromide was removed by mixing the DNA with an equal amount of CsCl saturated isopropanol and centrifuging for 5 minutes at 2,500 rpm (1398 x g) in a MSE Mistral 3000i centrifuge at 20°C. The bottom aqueous phase was removed into a clean tube and the process repeated 3-4 times until no more pink colour was visible in the aqueous phase. The DNA was then precipitated by the addition of 3 volumes TE and 2 volumes of ethanol. The DNA was recovered by centrifugation at 2,500rpm (1398 x g) in a MSE Mistral 3000i centrifuge, 4°C for 30 minutes. The pellet was washed in 70% (v/v) ethanol, dried and resuspended in TE.

ST buffer	
Sucrose	25% (w/v)
Tris-HCl pH 8.0	50mM

Triton lysis mix	
Triton X 100	1.5% (v/v)
Tris-HCl pH 8.0	50mM
EDTA pH 8.0	50mM

2.2.10.2.2 Qiagen purification of plasmid DNA (using Qiagen-tip 500)

The Qiagen plasmid purification technique was based on the alkaline lysis method of Birnboim and Doly (1979). A single colony from an overnight growth on selective LA plate was used to inoculate 5ml of LB containing the appropriate antibiotics. The culture was incubated for 6 - 8 hours at 37°C with shaking at 200rpm. The starter culture was diluted 1:500 into LB containing the appropriate antibiotics. For high copy plasmids 100ml of LB was used and for low copy plasmids 250ml of LB was used. After 12 - 16 hours incubation at 37°C with shaking the cells were harvested by centrifugation at 5,000rpm (4225 x g), 4°C for 15 minutes in a Sorvall GS - 3 rotor and the pellet was resuspended in 10ml of buffer P1 containing 100µg/ml RNase A. 10ml of buffer P2 was mixed by gentle inversions of the tube and incubated at room temperature for exactly 5 minutes during which the screw cap was attached

to the outlet nozzle of the QIAfilter cartridge. 10ml of chilled buffer P3 was added to the lysate and mixed by tube inversion. The lysate was immeditely poured into the QIAfilter cartridge barrel and incubated for 10 minutes. The QIAGENn-tip 500 was equilibrated by allowing passage of 10ml of buffer QBT by gravity flow. The cell lysate was filtered into the previously equilibrated QIAGEN-tip by removal of the cap from the QIAfilter outer nozzle and insertion of the plunger into the QIAfilter maxi cartridge. After the cleared lysate had entered the resin by gravity flow, the QIAGEN-tip was washed twice with 30ml of buffer QC. The DNA was eluted with 15ml of buffer QF and precipitated by addition of 10.5ml of isopropanol at room temperature. Centrifugation at 11,000rpm (14,461 x g), 4°C for 30 minutes in a Sorvall SS34 rotor followed and the supernatant was carefully removed. The DNA pellet was washed with 70% (v/v) ethanol at room temperature, then air dried before being resuspended in a suitable volume of TE buffer. The DNA yield was determined as described in section 2.2.3

Buffer P1	
Tris-HCl (pH 8.0)	50 mM
EDTA	10 mM
RNase A	100 µg/ml

Buffer P2	
NaOH	200 mM
SDS	1% (w/v)

Buffer P3	
Potassium acetate (pH 5.5)	3 M

Buffer QBT	
NaCl	750 mM
MOPS (pH 7.0)	50 mM
Ethanol	15% (v/v)
Triton X-100	0.15% (v/v)

Buffer QC	
NaCl	1 M
MOPS (pH 7.0)	50 mM
Ethanol	15% (v/v)

Buffer QF	
NaCl	1.25 M
Tris-HCl (pH 8.5)	50 mM
Ethanol	10% (v/v)

2.2.11 DNA sequencing

DNA sequencing was performed manually using Sequenase version 2.0 (Amersham Life Sciences, USB), in the early stages of this study. In the later

stage of the project automated sequencing using the ABI Prism dye terminator cycle sequencing ready reaction kit was performed.

2.2.11.1 Manual DNA sequencing

The sequencing technique was based upon the chain termination dideoxy method (Sanger *et al* ., 1977). The kit (Sequenase version 2.0) was supplied by Amersham Life Sciences, USB, and contained all the necessary reagents and the enzyme sequenase version 2.0. All reactions were performed according to the DNA sequencing protocol, 9th edition supplied by Amersham Life Sciences, USB.

2.2.11.1.1 Alkaline denaturation

3-5µg of double stranded DNA was made single stranded by alkaline lysis. DNA denaturation was achieved by adding 0.1 volume of 2M NaOH, 2mM EDTA (pH 7.5) and incubating at 37°C for 30 minutes. The DNA was precipitated by adding 0.1 volume of 3M sodium acetate (pH 5.2) and 2.5 volumes of 100% (v/v) ethanol. Following a 15 minute incubation at -70°C, the DNA was centrifuged at 13,000rpm (≥ 10,000 x g) for 15 minutes in a microfuge then washed in 70% (v/v) ethanol. The DNA was air dried for 20 - 30 minutes then resuspended in 7µl of nanopure water.

2.2.11.1.2 Annealing and labelling reaction

To 6µl of the denatured DNA, 2µl sequenase buffer and 2µl of primer (1.25pmoles/µl) were added. The reaction was mixed and incubated at 65°C for 2 minutes then allowed to cool slowly (30 - 60 minutes) to room temperature. To the annealed DNA mixture was added 1µl of 0.1M DTT, labelling mix (2µl), [α^{35}S] dATP (0.5µl) (Amersham International PLC) and diluted sequenase polymerase (2µl). After mixing, the reaction was incubated at room temperature for 5 minutes. The sequenase polymerase was diluted 1:8 in ice cold dilution buffer (10mM Tris-HCl, pH 7.5, 5mM DTT, 0.5mg/ml BSA) immediately prior to use. The labelling mix (stock concentrations: 1.5µM dITP, 7.5µM dCTP, 7.5µM dTTP) was diluted 1: 4 before use.

2.2.11.1.3 Termination reaction

3.5µl of the labelling reaction was transferred to one of four tubes containing 2.5µl of termination mixture (ddGTP, ddATP, ddTTP, ddCTP). The reactions were mixed, incubated at 37°C for 5 minutes then stopped by the addition of 5µl of stop solution (95% Formamide (v/v), 20mM EDTA, 0.05% bromophenol blue (w/v), 0.05 % xylene cyanol FF (w/v). The reactions were analysed by gel electrophoresis.

2.2.11.1.4 Sequencing gel preparation

Glass plates (20cm x 50cm) were cleaned, separated with 0.4mm spacers and placed in an Anachem gel pouring apparatus. 32ml of solution A and 14ml of solution B were measured out. 64µl of 25% (w/v) ammonium persulphate (APS) and 64µl of TEMED were added to solution A and 28µl of 25% (w/v) APS and 28µl of TEMED were added to solution B. 25 ml of solution A was drawn into a 25ml pipette followed by 7 ml of solution B. Several air bubbles were drawn into the pipette to allow TBE gradient formation. The mixture was poured between the plates and topped up with solution A to cover the length of the plate. A teflon comb was inserted and the gel allowed to polymerise for an hour. The comb was removed and the wells were washed thoroughly with running buffer prior to sample loading. The gels were run with 0.5 x TBE in the top chamber and 1 x TBE in the bottom chamber. The gels were pre-run at 30W for 15 - 20 minutes. The gels were run at a constant power of 37W for 1.5 - 3 hours depending on where the sequence of interest lay. Following electrophoresis the plates were separated and the gel was fixed by immersion in 10% (v/v) methanol, 10% (v/v) acetic acid for 10 minutes. The gel was rinsed in distilled water, then transferred to 3MM chromatography paper (Whatman) and dried under vacuum at 80°C for 1 hour using a Bio-Rad gel drier. The gel was transferred to an autoradiograph cassette and exposed to blue sensitive X-ray film (Genetic Research Instrumentation Ltd) overnight. The films were developed in an Agfa-Geveart automatic film processor.

40% (w/v) Acrylamide stock

Acrylamide	380g
Bis-acrylamide	20g
distilled water to final volume	1 litre

Solution A

40% stock acrylamide (w/v)	150ml
10 x TBE	50ml
Urea	460g
distilled water to final volume	1 litre

Solution B

40% stock acrylamide (w/v)	75ml
10 x TBE	125ml
Urea	230g
Bromophenol blue	0.02g
distilled water to final volume	0.5 litre

10 x TBE

Tris Base	109g
Boric acid	55g
EDTA	9.3g
distilled water to final volume	1 litre

2.2.11.2 Automated DNA sequencing

The ABI PRISM Dye Terminator cycle sequencing kit (Perkin-Elmer) was used in association with an ABI model 377 DNA sequencer.
Automated sequencing was perfomed using the Perkin-Elmer/Applied Biosystems Division dRhodamine terminator ready reaction mix or Bigdye terminator ready reaction mix. The pre-prepared reaction mix contains the dye terminators, deoxynucleoside triphosphates, enzymes and buffer. The sequencing enzyme used was AmpliTaq® DNA Polymerase, FS. which is derived from *Thermus aquaticus* DNA polymerase and contains a point mutation in the active site. This mutation reduces discrimination against dideoxynucleotides leading to more even peak intensity patterns. AmpliTaq® DNA Polymerase, FS also has another mutation in the amino terminal domain which almost eliminates the 5' > 3' nuclease activity. The ready reaction premix contained optimum amounts of dye-labeled dideoxynucleotides and deoxynucleotides to give a balanced distribution of signal between base 10 and base 700+. The dNTP mix contained dITP in place of dGTP, to minimise band compressions. Essentially the reaction

consisted of mixing terminator mix (8µl), DNA (400 -700ng), primer (3.2pmoles) and nanopure water to a final volume of 20µl in a 0.5ml tube on ice, followed by thermal cycling.

Terminator Premix

A-Dye Terminator labelled with dichloro[R6G], C-Dye Terminator labelled with dichloro[TAMRA], G-Dye Terminator labelled with dichloro [R110], T-Dye Terminator labelled with dichloro [ROX], dITP, dATP, dCTP, dTTP, Tris-HCl (pH 9.0). $MgCl_2$, thermal stable pyrophosphatase and AmpliTaq® DNA Polymerase, FS. The concentration of these reagents was not disclosed by the manufacturer (Perkin-Elmer).

The mixture was overlaid with 20µl of mineral oil and placed in a thermal cycler preheated to 96°C. The cycling parameters were 96°C for 30 seconds, 50°C for 15 seconds and 60°C for 4 minutes for 25 cycles. The reactions were held at 4°C until required.

The sequencing mix was removed from beneath the mineral oil and precipitated on ice for 15 minutes with 2µl 3M sodium acetate (pH5.2) and 50µl of 100% (v/v) cold ethanol. The DNA was centrifuged at 13,000rpm (≥ 10,000 x g) for 15 minutes in a microfuge then washed in 70% (v/v) cold ethanol. After air drying for 30 - 40 minutes the samples were analysed on a 377 DNA sequencer by Mr. Stuart Bayliss at the Protein and Nucleic Acid Sequencing Laboratory, University of Leicester.

2.2.12 Polymerase Chain Reaction (PCR)

2.2.12.1 Precautions to limit PCR contamination

The sensitive nature of the PCR can result in problems with reagent or sample contamination. This can occur when products from a previous reaction or material from an exogenous source contaminates the reaction mixture giving rise to false-positive signals (Erlich, 1989). A number of precautions were taken to minimise contamination. These included the physical separation of the reaction preparation and analysis areas, use of positive displacement pipettes or aerosol resistant pipette tips and pre-aliquoting of reagents. A separate stock of pipettes, pipette tips, Eppendorf

tubes and reagents were kept specifically for PCR. Distilled water, Eppendorfs and pipette tips were autoclaved prior to use.
A negative control containing distilled water instead of template was always included to highlight any contamination.

2.2.12.2 Oligonucleotide primers

2.2.12.2.1 Source of oligonucleotide primers

The primers used and their sequences are shown in Table 2.3. During the early stages of the project primers were obtained from the Protein and Nucleic Acid Sequencing Laboratory, University of Leicester at a 40 nM scale, stored under ammonia. Primers were recovered by ethanol precipitation (Section 2.2.5) and the concentration determined spectrophotometrically by the optical density at 260nm (2.2.3). During the later stages of the project primers were purchased from Gibco/BRL in a lyophilysed form (50 nM scale) and resuspended in 300µl of TE buffer prior to quantitation and use.

2.2.12.2.2 Calculation of primer concentration

The primer was diluted in nanopure water so that it gave readings in the range 0.1 - 1.0 for the OD_{260}. The concentration of the primer was ascertained by calculating a value for the millimolar coefficient (EmM) for each primer. The EmM depends on the base composition of the oligonucleotide and was calculated by summing the contribution of each base.

Base	Contribution to EmM ($mM^{-1} cm^{-1}$)
A	15.4
C	7.3
G	11.7
T	8.8

The oligonucleotide concentration was calculated from the following equation

$$\text{Concentration of oligonucleotide (mM)} = \frac{OD_{260}}{\text{EmM}}$$

Example:

primer - SOM1. Sequence 5′ CAA CGA TAA CCG TCA GGT AG 3′

OD_{260} (100 fold dilution) = 0.194

A	7 x 15.4 =	107.8
C	5 x 7.3 =	36.5
G	5 x 11.7 =	58.5
T	3 x 8.8 =	26.4
		Σ229.2

0.194 x 100 (dilution factor) = 19.4

$\frac{19.4}{229.2} \times 1000 = 85\text{pmoles}/\mu\text{l}$ (0.085mM)

2.2.12.2.3 Calculation of the melting temperature (T_m) of an oligonucleotide

The following formula was used to calculate the melting temperature of a primer.

$$T_m\ (°C) = \Sigma\ [\ \{\ 2 \times (^{\#}A + {}^{\#}T)\ \} + \{\ 4 \times (^{\#}C + {}^{\#}G)\ \}\ ]$$

represents the total number of each base (A, T, C, G) in the primer

2.2.12.3 Polymerase Chain Reaction (PCR)

The polymerase chain reaction (Saiki *et al.*, 1988) was used to amplify target DNA between 5′ and 3′ primers, for site directed mutagenesis and also for colony screening following a cloning procedure. *Vent* DNA polymerase (New England Biolabs) was used because of its high fidelity (lower rate of nucleotide misincorporation). The standard reaction (Sambrook *et al* ., 1989) worked for all the templates and primers used in this study. A standard reaction is shown below.

Standard PCR reaction

10 x Vent buffer	10µl
A mix of 4 dNTPs, each at a final concentration of 1.25mM	16µl
Primer 1	100pmol
Primer 2	100pmol
DNA template	1-20ng
Vent DNA polymerase	1µl
Nanopure water to final volume of 100 µl.	

1 x Vent buffer:

KCl	10mM
Tris-HCl (pH 8.8)	20mM
$(NH_4)_2SO_4$	10mM
$MgSO_4$	2mM
Triton X-100	0.1% (v/v)

The reaction was set up on ice and the enzyme added last. The mixture was overlaid with 50µl of mineral oil to prevent evaporation and incubated in a thermal cycler. The standard incubation cycle was:

"Standard" PCR incubation cycle

DNA denaturation	94°C for 1 minute
DNA annealing	58°C for 1 minute
DNA polymerisation	72°C for 1 minute/kb of amplified product

The 3 stages were repeated for 35 cycles then the reaction held at 72°C for 10 minutes. On completion of the cycles, the PCR mixture was removed from below the oil and analysed on an agarose gel. For the mutagenesis it was necessary to purify the amplified band from the agarose.

2.2.13 PCR colony screening

PCR screening (Gussow and Clackson, 1989) was used as a rapid method for identifying the presence of a particular sequence in a number of clones. A colony was removed with a sterile toothpick and swirled in an 1.5ml tube containg 100µl of nanopure water. This was boiled for 5 minutes and cooled on ice. 2µl of the solution was added to 8µl of the PCR master mix and 0.5µl *Vent* polymerase and incubated as follows: 1 cycle at 94°C for 5 minutes, 35 cycles at 94°C for 1 minute, 58°C for 2 minutes, 72°C for 2 minutes. Finally the reaction was held at 72°C for 10 minutes.

PCR master mix	
10 x Vent buffer	20µl
A mix of 4 dNTPs, each at a final concentration of 1.25mM	32µl
Primer 1	200pmol
Primer 2	200pmol
Nanopure water to	200µl

2.2.14 PCR mutagenesis

2.2.14.1 Introduction

PCR provides a faster and easier method for modifying DNA compared to other recombinant DNA techniques. The reason for this is that sequence changes can be made more easily in oligonucleotide primers chemically than by manipulating DNA fragments with restriction and ligation enzymes. Also PCR products can readily accept such sequences like convenient restriction sites (Higuchi, 1989).

Mutagenesis of PCR fragments via mismatched primer or 5' "add-on" sequences is restricted to the ends of the fragments by the primer lengths that can be achieved by chemical DNA synthesis (Scharf, 1990). However Higuchi *et al* ., (1988) have described a method which combines primer-introduced sequence modification with PCR fragment joining to introduce sequence alterations at any position in the PCR fragment.

The principle of mismatch PCR mutagenesis is illustrated in Figure 2.1. The mismatch PCR mutagenesis protocol used during this project is an adaptation of the original method by Higuchi *et al* ., (1988) and is described below. Two "inside" primers which anneal to opposite strands of the same segment of target DNA are designed to mismatch the target sequence at a single base. Another two primers with no mismatches are designed to anneal some distance from the inside primers. Initially two separate primary PCR reactions are performed under standard PCR conditions. The "left" PCR is performed with outside primer (A) and inside primer (D) and the "right" PCR is performed with outside primer (B) and inside primer (C). Due to the mismatch primers the same alteration occurs in the two primary PCR products. The primary PCR products are separated from excess primer, denatured and allowed to anneal together at a lower temperature resulting in the formation of two heteroduplexe products. The heteroduplexes with recessed 3' ends can be extended by *Taq* DNA polymerase to produce a fragment that is the sum of the two overlapping PCR products. A subsequent reamplification of this fragment with the outside primers (A and B) leads to the production of a full-length secondary product that has a specific base change far from its ends.

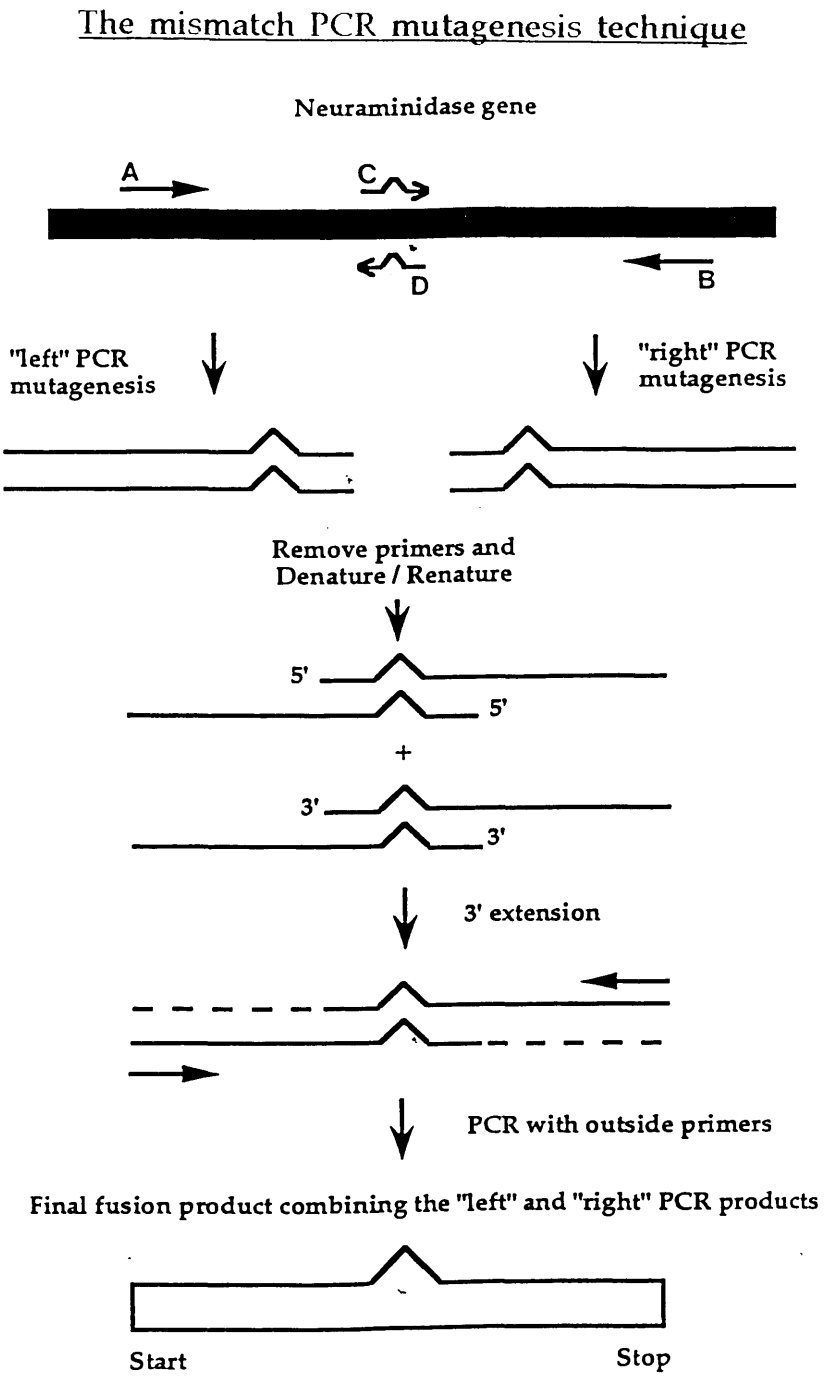


Figure 2.1 Diagrammatic representation of the strategy used for introduction of site specific mutations into pSS2A using the mismatch PCR technique.
Oligonucleotide primers are represented by arrows adjacent to their annealing sites in the target sequence. The two "inside" primers (C and D) anneal to the same segment of target DNA but on opposite strands, are shown mismatched to the target sequence at a single base. The mismatch produce the same sequence change in both of the primary PCR products. The two primary PCRs (PCR1 and PCR2) are performed separately. The primary products are denatured and allowed to reanneal together, producing two possible heteroduplex products. DNA extension of heteroduplexes with recessed 3' ends leads to a fragment that is the sum of the two overlapping products. A subsequent reamplification of this fragment with the "outside" primers (A and B) results in a full length product with the muation in the middle.

2.2.14.2 Design of primers for mutagenesis

The 1433 bp fragment of the neuraminidase gene termed *Hind* III/pQMC1 in plasmid pSS2A (Section 3.3) was the target DNA for mutagenesis. The outside primers were universal sequencing primers (M13 Reverse and M13 Forward) which annealed to the pUC18 DNA flanking the neuraminidase gene fragment *Hind* III/pQMC1 (Figure 2.2).

Two pairs of inside primers (about 30 nucleotides long) were designed for each base change and checked for binding specificity using a PCR application program (Amplify, version 1.2., Engels, 1992). They were mismatched to the target sequence at the substituted base (Figure 2.3). Primers were designed with a similar G+C content to the target sequence, minimal secondary structure and low complementarity to each other especially in the 3' region (Taylor, 1991).

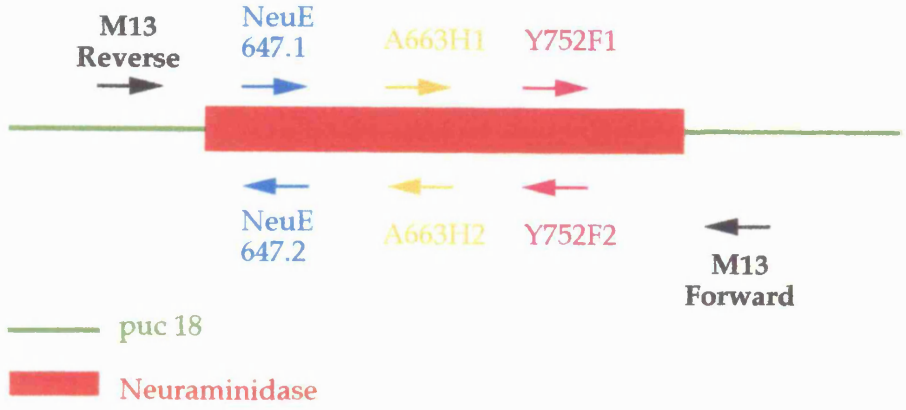


Figure 2.2 Position of "outside" primers relative to the "inside" primers in pSS2A.
M13 Forward and M13 Reverse are the "outside" primers and anneal to pUC18.
NeuE647.1 and NeuE647.2 are the mismatched primers for causing the base change (G>C) in the *Hind* III/pQMC1 fragment at position 647.
A663H1 and A663H2 are the mismatched primers for causing the base change (G>A) in the *Hind* III/pQMC1 fragment at position 663.
Y752F1 and Y752F2 are the mismatched primers for causing the base change (A>T) in the *Hind* III/pQMC1 fragment at position 752.

A) Mutation of glutamic acid (GAA) to glutamine (CAA) at position 647 in pSS2A

Primer Neu E 647.1	5'	TGCGCAAAATACA**CAA**TCAACGGTGGTACA 3'
Target sequence (pSS2A)	5'	TGCGCAAAATACA**GAA**TCAACGGTGGTACA 3'
	3'	ACGCGTTTTATGT**CTT**AGTTGCCACCATGT 5'
Primer Neu E 647.2	3'	ACGCGTTTTATGT**GTT**AGTTGCCACCATGT 5'

B) Mutation of arginine (CGT) to histidine (CAT) at position 663 in pSS2A

Primer A663H1	5'	TAAACTCTTTATG**CAT**GGTTTGACTGGAGA 3'
Target sequence pSS2A	5'	TAAACTCTTTATG**CGT**GGTTTGACTGGAGA 3'
	3'	ATTTGAGAAATAC**GCA**CCAAACTGACCTCT 5'
Primer A663H2	3'	ATTTGAGAAATAC**GTA**CCAAACTGACCTCT 5'

C) Mutation of tyrosine (TAT) to phenylalanine (TTT) at position 752 in pSS2A

Primer Y752F1	5'	AGGAGAGTTTGCC**TTT**AATTCGCTCCAAGA 3'
Target sequence pSS2A	5'	AGGAGAGTTTGCC**TAT**AATTCGCTCCAAGA 3'
	3'	TCCTCTCAAACGG**ATA**TTAAGCGAGGTTCT 5'
Primer Y752F2	3'	TCCTCTCAAACGG**AAA**TTAAGCGAGGTTCT 5'

Figure 2.3 Sequence of the mismatch primers used in mutagenesis and the corresponding target sequence

2.2.14.3 PCR mutagenesis conditions

2.2.14.3.1 Primary PCR

Primary PCR reactions were carried out with the primer combinations shown in Table 2.7. The position of the primers relative to each other is shown in Figure 2.2. The constituents of the reaction were the same as that of a standard PCR (Section 2.2.12.3) and the cycling parameters were the "standard" PCR incubation cycle. (Section 2.2.12.3).

Mutation	Primary PCR	"Inside" primer	"Outside" primer
E>Q 647	Left	NeuE647.2	M13 Reverse
E>Q 647	Right	NeuE647.1	M13 Forward
R>H 663	Left	A663H2	M13 Reverse
R>H 663	Right	A663H1	M13 Forward
Y>F 752	Left	Y752F2	M13 Reverse
Y>F 752	Right	Y752F1	M13 Forward

Table 2.7 Primer combinations used during the primary PCRs

The products of the "left" and "right" PCR were visualised on a 0.7% agarose gel and purified from the agarose with the bandprep kit (2.2.2.1). The purification of the PCR product from the gel had two advantages. Firstly any unmutagenized template was removed and secondly the inside primers were removed, which favoured the production of the full-length product during the secondary PCR.

2.2.14.3.2 Secondary (Recombinant) PCR

The next stage involved amplification of the full length mutagnised product with the outside primers M13 Reverse and M13 Forward. The gel purified products of the "left" and "right" primary PCRs (6µl of each) were used as the template in a secondary PCR where the annealing temperature was reduced to 40°C for the first two cycles to favour the formation of recombinant heteroduplex molecules. The PCR cycling parameters were: 1 cycle of 5 min at 94°C followed by 2 cycles of 1' at 94°C, 2' at 40°C, 3' at 72°C followed by 35 cycles of 1' at 94°C , 2' at 58°C, 2' at 72°C. The final cycle was 1'

at 94°C, 2' at 58°C and 10' at 72°C. The product of the secondary PCR was analysed on a 0.7% agarose gel, then gel purified using the bandprep kit. The next stage involved the cloning of the mutagenised PCR product into the vector pCR-Script™ SK (+) (2.2.15).

2.2.15 Cloning of the mutagenized recombinat PCR product into pCR-Script™ SK (+)

2.2.15.1 The pCR-Script™ SK (+) cloning kit

The pCR-Script™ SK (+) cloning kit (Stratagene) was chosen for the cloning of the mutagenized PCR product because it gives efficient cloning of PCR fragments with a high yield and a low rate of false positives (pCR-Script™ SK(+) Cloning Kit Instruction Manual, Stratagene, 1994).

PCR products are incubated with predigested pCR-Script™ SK (+) cloning vector in the presence of *Srf* I and T4 DNA ligase (Figure 2.4). The use of the restriction enzyme in the ligation reaction maintains a high steady state concentration of digested vector DNA and allows the use of non-phosphorylated, unmodified PCR primers (Bauer *et al* ., 1992). The ligation efficiency of blunt-ended DNA fragments is increased by the simultaneous, opposite reactions of *Srf* I restriction enzyme and T4 DNA ligase on nonrecombinant, religated vector DNA (Liu and Schwartz, 1992). *Srf* I is a rare-cleavage restriction enzyme which recognizes the sequence 5' - GCCC/GGGC- 3'.

The pCR-Script cloning vector is derived from the pBluescript® II SK (+) phagemid. It includes an ampicillin-resistance gene, a *lac* promoter for gene expression, T3 and T7 RNA polymerase-binding sites, an f1 intergenic region for single-stranded DNA rescue and the SK multiple cloning site (MCS), which is modified to include the *Srf* I restriction-endonuclease target sequence (Figure 2.5), (pCR-Script™ SK(+) Cloning Kit Instruction Manual, Stratagene, 1994).

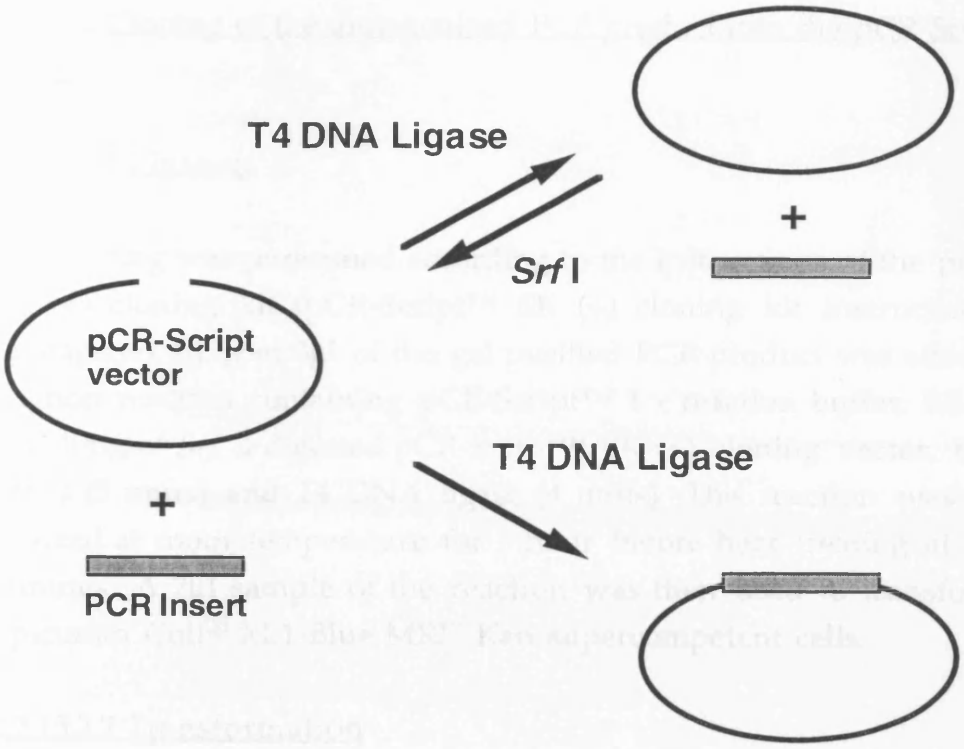


Figure 2.4. The principle of the pCR-Script™ SK (+) cloning method (adapted from pCR-Script™ SK (+) cloning kit instruction manual, Stratagene).
The ligation reaction was set up using an aliquot of the PCR product along with reaction buffer, rATP and *Srf* I digested pCR-Script SK (+) cloning vector. Following the addition of *Srf* I and T4 DNA ligase, the reaction was incubated at 65° C for 10 minutes. Next an aliquot of the reaction was transformed into Epicurian Coli® XL1-Blue MRF' Kan supercompetent cells (Stratagene).

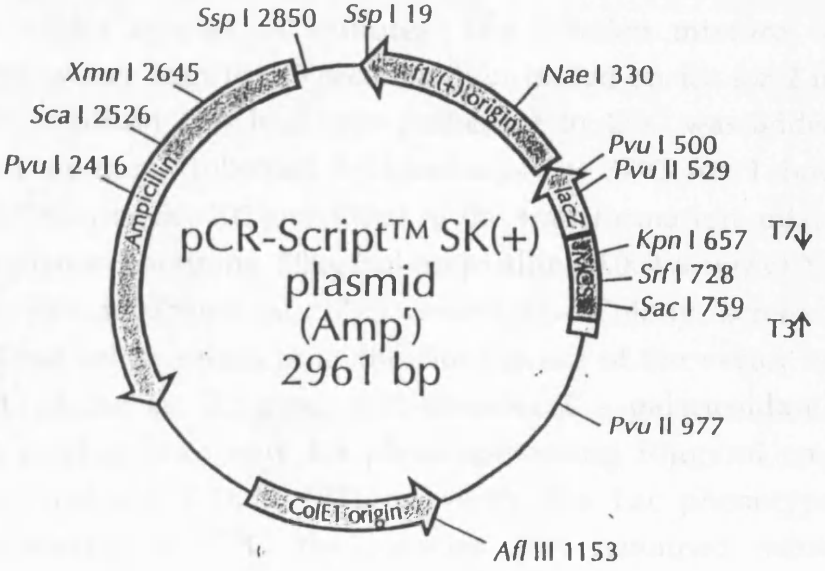


Figure 2.5 Diagram of the pCR-Script™ SK (+) plasmid. (Taken from pCR-Script™ SK (+) cloning kit instruction manual, Stratagene). It was derived by incorporating a *Srf* I site into the pBluecript SK (+) phagemid. The orientation of the polylinker is such that transcription proceeds from *Sac* I to *Kpn* I as shown by the SK designation.

2.2.15.2 Cloning of the mutagenized PCR product into the pCR-Script cloning vector

2.2.15.2.1 Ligation

The cloning was performed according to the instructions of the pCR-Script™ SK (+) cloning kit (pCR-Script™ SK (+) cloning kit instruction manual, Stratagene). In brief 3µl of the gel purified PCR product was added to a 10µl ligation reaction containing pCR-Script™ 1 x reaction buffer, 0.5 mM rATP and 10ng of *Srf* I digested pCR-Script™ SK (+) cloning vector, the enzyme *Srf* I (5 units) and T4 DNA ligase (4 units). The reaction was allowed to proceed at room temperature for 1 hour before heat treating at 65°C for 10 minutes. A 2µl sample of the reaction was then used to transform 40µl of Epicurian Coli® XL1-Blue MRF' Kan supercompetent cells.

2.2.15.2.2 Transformation

40µl of Epicurian Coli® XL1-Blue MRF' Kan supercompetent cells were placed into a prechilled 15ml Falcon polypropylene tube. 0.7µl of ß-mercaptoethanol (1.44M) was added to the cells to give a final concentration of 25mM. The cells were incubated on ice for a further 10 minutes. 2µl of the DNA from the ligation (Section 2.2.15.2.1) was added to the cells and incubated on ice for another 30 minutes. The reaction mixture was heat pulsed in a 42°C water bath for 45 seconds then placed on ice for 2 minutes. 0.45 ml of SOC medium that had been preheated to 42°C was added to the transformation mixture, followed by incubation at 37°C for 1 hour with shaking at 225-250 rpm. 50, 100 and 190µl of the transformation mixture was plated on LA plates containing 50µg/ml ampicillin, 0.004% (w/v) X-gal and 0.1mM IPTG and incubated at 37°C overnight. Colony screening was facilitated by blue/white selection as the cloning site of the vector is located in the middle of the *lac Z* gene, that encodes ß - galactosidase. White colonies were patched onto new LA plates containing 50µg/ml ampicillin, 0.004% (w/v) X-gal and 0.1mM IPTG to verify the Lac phenotype. After overnight incubation at 37°C, the colonies that remained white were screened by colony PCR (Section 2.2.13) using the primers M13 Reverse and M13 Forward. Plasmid DNA was prepared from colonies that produced an approximately 1.5 kb product in the PCR screening procedure and subjected to restriction enzyme analysis.

SOC medium	
Tryptone	20.0g
Yeast extract	5.0g
NaCl	0.5g
Distilled water	970ml
Autoclaved then added	
1M $MgCl_2$	10ml
1M $MgSO_4$	10ml
2M glucose solution	10ml
Filter sterilize	

2.3 Protein Techniques

2.3.1 SDS Polyacrylamide gel electrophoresis (SDS - PAGE)

Protein fractions were analysed by the Laemmli (1970) system of discontinuous polyacrylamide gel electrophoreis (PAGE). A mini protein 2 cell modular mini electrophoresis system (Bio-Rad) was used according to manufacturer's instructions. The components for the gels are listed below. Following the addition of TEMED and ammonium persulphate (APS) to the resolving solution, the mixture was poured between the plates. It was overlaid with water saturated iso-butanol. After polymerisation, the iso-butanol was removed and the gel surface was washed with distilled water. TEMED and APS were added to the stacking solution which was then poured above the resolving gel. A Teflon comb was inserted to create wells. 27µl of protein solution was added to 3µl of 10 x loading buffer and boiled for 5 minutes prior to loading on to the gel alongside high molecular weight markers. Proteins were run through the stacking gel at 100V, then at 200V through the resolving gel until the blue dye reached the bottom. The gels were stained in Coomassie blue (Sigma) stain for 30 minutes then destained for 2 - 3 hours in several changes of destain solution. The gels were dried on gel drying film (Bio - Rad).

SDS - PAGE solutions

Resolving gel (8%)

Distilled water	2.3ml
30% (w/v) stock acrylamide	1.3ml
1.5M Tris - HCl (pH 8.8)	1.3ml
10% SDS (w/v)	0.05ml
10% APS (w/v)	0.05ml
TEMED	0.003ml

Stacking gel (5%)

Distilled water	2.1ml
30% (w/v) stock acrylamide	0.5ml
1.5M Tris-HCl (pH6.8)	0.38ml
10% SDS (w/v)	0.03ml
10% APS (w/v)	0.03ml
TEMED	0.003ml

SDS - PAGE electrophoresis buffer

Tris base	25mM
Glycine (pH 8.3) (electrophoresis grade)	250mM
SDS	0.1% (w/v)

SDS - PAGE loading buffer

Tris-HCl (pH6.8)	100mM
Dithiothreitol	200mM
SDS	4% (w/v)
Bromophenol blue	0.2% (w/v)
Glycerol	20% (v/v)

SDS - PAGE destain solution

Distilled water	800ml
Methanol	800ml
Acetic acid	160ml

SDS - PAGE stain solution

Coomassie brilliant blue R250 stain	1.1g
made up in destain solution	200ml

2.3.3 Purification of histidine tagged proteins by affinity chromatography

2.3.3.1 The protein expression and purification system

The QIAexpress system (Qiagen) was selected for the expression and purification of proteins. The system consists of pQE expression vectors and Nickel nitrilo-tri-acetic acid resin (Ni-NTA,Qiagen). The pQE expression vectors provide high-level expression in *E. coli* of proteins or peptides that contain a tag of 6 consecutive histidines (6xHis). The Ni-NTA resin has a high affinity for proteins or peptides that contain a 6xHis affinity tag at either their N- or C- terminus (Hochuli *et al* ., 1988). The combined advantages of a high-level bacterial expression system and the high affinity of the Ni-NTA resin allows for a one-step purification of recombinant proteins under either native or denaturing conditions.

2.3.3.2 Outline of the protein expression procedure

The protein expression system is described in brief below and in greater detail in sections 2.3.3.3 - 2.3.3.6 inclusive. The cloning, expression and purification of neuraminidase was done according to the manufacturer's instructions ("The QIAexpressionist", Qiagen, 1992).
The neuraminidase structural gene was subcloned into the pQE30 expression vector (Camara *et al* ., 1994) in the same reading frame as the 6xHis affinity tag. The pQE expression construct was transformed into SG13009 [pREP4] host strain carrying the pREP4 repressor plasmid. Transformants were selected on plates containing ampicillin and kanamycin. Cultures were grown in the presence of ampicillin and kanamycin until they reached an OD_{600} of 0.7 - 0.9, then induced with IPTG. The cells were harvested after they had expressed the recombinant protein for 2 hours. The cells were lysed and the cleared lysate was loaded onto Ni-NTA cloumn. The pure protein was eluted after a number of washing steps.

2.3.3.3 Preparation of expression constructs

The neuraminidase structural gene (*nan A*) was cloned into the *Bam* H I/*Sal* I cloning site of the expression vector pQE30 (Camara *et al* ., 1994). The resulting construct had the 6xHis tag 5' to the polylinker (N-terminus of the protein). The primers for the amplification of *nan A* were designed so as not

to include the ATG start codon, because internal starts from control sequences provided by the inserted fragment would result in proteins that do not contain the 6xHis tag and hence cannot be purified by Ni-NTA. The recombinant was transformed into *E. coli* SG13009:pREP4. Tight regulation of protein expression was achieved by the *lac* repressor encoded by the *lacI* gene (Farabaugh, 1978) carried by the pREP4 plasmid. Expression from pQE vectors was induced by IPTG which inactivated the repressor and cleared the promoter.

2.3.3.4 Preparation of expression cultures

The antibiotic selection for expression clones based on the pQE30 vector was 100µg/ml ampicillin and 25µg/ml kanamycin.
A sample from the glycerol stock culture of the expression clone was streaked onto LA plates containing antibiotics and incubated overnight at 37°C. A single colony was used to inoculate 20ml of LB containing antibiotics and incubated at 37°C with shaking at 200 rpm. The 20ml overnight culture was used to inoculate 1 litre LB containing antibiotics and grown to OD_{600} = 0.6 - 0.9. The culture was induced with IPTG (Boehringer) at a final concentration of 2mM for 2 hours and the cells harvested by centrifugation at 5,000rpm (4225 x g), 4°C for 15 minutes in a GS-3 rotor. The pellet was resuspended on ice in 8 ml of equilibration buffer then stored at -70°C or sonicated immediately.

The sonicator probe was pre-chilled in ice prior to use and the sonication sample was kept on an ice slurry at all times. Sonication was performed in ten 15 second pulses at an amplitude of 7.5 microns with a Soniprep 150 ultrasonic disintegrator (Sanyo - Gallenkamp). There was a 45 second cooling period between pulses. The sonicate was centrifuged at 20,000rpm (47,807 x g), 4°C for 30 minutes in a Sorvall SS34 rotor and the supernatant containing the soluble proteins was filtered through a 0.45µm acrodisk. It was then either immediately purified or stored at -20°C until required.

Equilibration buffer

Sodium phosphate (pH8.0)	50mM
NaCl	300mM

Sodium phosphate buffer

Sodium dihydrogen orthophosphate	0.5M
Disodium hydrogen orthophosphate	0.5M

Varying amounts of the above two solutions were mixed to achieve sodium phosphate buffer of the desired pH.

2.3.3.5 Preparation of Ni-NTA columns

All protein purification procedures were done at 4°C to minimise enzyme inactivation and proteolysis. A 10ml XK column (0.9cm diameter x 15cm, 9ml bed volume, Pharmacia) was filled with 5ml of water to check for leaks. Approximately 4 ml of water was drained off by opening the bottom cap of the column, leaving 1ml in the column. The Ni-NTA was supplied pre-swollen and charged with Ni^{2+} as an aqueous suspension (50% w/v) containing 30% (v/v) ethanol as preservative. The Ni-NTA resin was thoroughly resuspended by vigorous shaking and 10ml transferred to a sterile tube. The resin was centrifuged at 550rpm (67 x g), 4°C for 13 minutes in a MSE Mistral 3000i centrifuge. The supernatant was discarded and the resin was mixed with 5ml of water. The slurry was poured into the column and allowed to settle for 2-3 hours. The excess water was removed, leaving about 1cm of water above the resin. The resin was packed by inserting an upper frit into the column and pushing the frit until it was about 0.25cm above the resin. A peristaltic pump was attached to the column and nanopure water pumped through at 3-4 column volumes per hour. Simultaneously the bottom cap was opened. The resin was washed with 5 column volumes of water and equilibrated with 10 column volumes of equilibration buffer. The Ni-NTA resin was re-used no more than 3 - 5 times for purifying the same recombinant protein.

2.3.3.6 Ni-NTA affinity column chromatography

At the start of this project protein purification was attempted by the "original protocol" devised by M. Camara. Throughout the course of the project this was modified to give the "new purification protocol". Both of these protocols are described below.

2.3.3.6.1 The "original purification protocol"

A flow rate of 6-8 column volumes per hour was used throughout the purification. The filtered crude lysate (CRUDE) was applied to the equilibrated column and the unbound material collected (OUTFLOW). Equilibration buffer was applied for 20 minutes and the eluate collected (EQUILIBRATION). The column was washed with wash buffer (pH 8.0) for 12 - 14 hours and the protein eluted with a 0 - 0.5M gradient of imidazole in wash buffer. The gradient was formed by placing wash buffer in chamber A and 0.5M imidazole dissolved in wash buffer in chamber B of a gradient former (Bio-Rad model 395). Chamber A was attached to the pump and gradual mixing of solution from chamber B occured. 5 minute fractions were collected over a period of 4 - 6 hours and analysed by SDS - PAGE.

Wash buffer	
Sodium phosphate (pH 8.0)	50mM
NaCl	500mM
Glycerol	10% (v/v)
Tween - 20	0.5% (v/v)

Gradient buffer	
Imidazole.HCl (pH 8.0)	0.5M
made up in wash buffer	

2.3.3.6.2 The "new purification protocol"

As the "original purification protocol" but with the following changes. The pH of the wash buffer was reduced to 6.0, the wash length was reduced to 2.5 hours and the flow rate was reduced to 3-4 column volumes per hour.

Wash buffer	
Sodium phosphate (pH 6.0)	50mM
NaCl	500mM
Glycerol	10% (v/v)
Tween - 20	0.5% (v/v)

Gradient buffer	
Imidazole.HCl (pH 6.0)	0.5M
made up in wash buffer	

2.3.4 Buffer exchange and concentration of protein

Ultrafiltration was used to remove the imidazole from the protein prior to vaccination of mice. Several rounds of ultrafiltration were employed to progressively purify macromolecules from contaminating solutes. Initially Amicon stirred cells with YF membranes were used but later in the study Vivaspin centrifugal concentrators were the method of choice.

2.3.4.1 Amicon stirred cell diafiltration

The purest fractions of protein were pooled and filtrated against 0.5 x phosphate-buffered saline (PBS). 6ml of protein was mixed with 44ml of 0.5 x PBS and placed in an Amicon stirred cell containing a pre-rinsed YF 10K membrane. The protein was filtered under pressure from nitogen until 5ml of liquid remained in the cell. More 0.5 x PBS was added to bring the volume to 50ml and the process repeated for a total of 3 times. During the final run, filtration was continued until approximately 1ml of protein solution remained in the bottom cell. The protein solution was removed and its concentration was measured as described in sections 2.3.5.

1 x Phosphate-buffered saline (PBS)

NaCl	8g
KCl	0.2g
Na_2HPO_4	1.44g
KH_2PO_4	0.24g
Distilled water to	1l

and pH adjusted to 7.4 with HCl

2.3.4.2 Vivaspin diafiltration

Vivaspin cetrifugal filter units were purchased from Vivascience and used according to manufacturer's instructions. The unit consisted of a thin channel filtration chamber with a vertical polysulfone membrane housed in a polypropylene collection tube. Vivaspin 15 with a 50,000 molecular weight cut off (MWCO) was used. In brief, 10ml of the pooled protein fractions were added to 190ml of 0.5 x PBS. 50ml of this mixture was poured into the vivaspin unit and centrifuged at 2,500rpm (1398 x g) in a MSE mistral 3000i centrifuge at 4°C until about 1 - 2 ml remained in the upper chamber. More

PBS/protein mix was added and filtered. When the last of the PBS/protein mix was added, ultrafiltration was continued until about 0.75-1ml remained in the concentration chamber, this was removed with a Gilson pipette and the protein concentration measured (Section 2.3.5).

2.3.5 Measurement of protein concentration

The protein concentration was measured by the absorbance at 260nm and 280nm or by the Bradford assay.

2.3.5.1 Absorbance at 260nm and 280nm

The method of Warburg and Christian (1941) involves measuring the absorbance (A) of an appropriately diluted protein solution at 260nm and 280nm in a 1cm path length cell. The ratio of A_{280} and A_{260} is used to find the factor from Table 2.8 and the protein concentration is given by:

$$\text{Protein concentration (mg/ml)} = A_{280} \times \text{Factor}$$

The values in Table 2.8 and the equation were calculated from the absorbance values of crystalline yeast enolase (for a concentration of 1mg/ml, A_{260} 0.512, A_{280} 0.894) and purified yeast nucleic acid for (1mg/ml, A_{260} 22.1, A_{280} 10.8). The method is strictly accurate only for a mixture of proteins and nucleic acids falling between these absorbance values. A_{280} values for most proteins at a concentration of 1mg/ml fall in the range of 0.5 to 2.0.

2.3.5.2 Bradford assay

The Bradford micro assay (Bradford, 1976) for measuring proteins in the range (0 - 10ug/ml) was performed using reagents and instructions from Bio-Rad. Dilutions of protein in nanopure water (total volume 800µl) were placed in tubes and 200µl dye reagent added sequentially at 30 second intervals to each tube. 20 minutes after the addition of reagent to the first tube the A_{595} of each sample was measured at 30 second intervals. Protein concentrations were estimated by reference to a standard curve of the A_{595} of bovine serum albumin at concentrations of 0 - 10µg/ml.

A280/A260	Nucleic acid % (*)	Factor
1.75	0	1.118
1.60	0.30	1.078
1.50	0.56	1.047
1.40	0.87	1.011
1.30	1.26	0.969
1.25	1.49	0.946
1.20	1.75	0.921
1.15	2.05	0.893
1.10	2.4	0.863
1.05	2.8	0.831
1.00	3.3	0.794
0.96	3.7	0.763
0.92	4.3	0.728
0.90	4.6	0.710
0.88	4.9	0.691
0.86	5.2	0.671
0.84	5.6	0.650
0.82	6.1	0.628
0.80	6.6	0.605
0.78	7.1	0.581
0.76	7.8	0.555
0.74	8.5	0.528
0.72	9.3	0.500
0.70	10.3	0.470
0.68	11.4	0.438
0.66	12.8	0.404
0.64	14.5	0.368
0.62	16.6	0.330
0.60	19.2	0.289

Table 2.8 Factor for calculation of protein concentration (adapted from Warburg and Christian, 1941)

* Nucleic acid is expressed as a percentage of the total (protein + nucleic acid).

2.3.6 Assays for neuraminidase activity

A qualitative assay utilising the fluorogenic substrate 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUAN, Sigma) was routinely used to test for the presence of neuraminidase activity. For quantitative measurements the substrate 2-O-(O-nitrophenyl)-α-D-N-Acetylneuraminic acid (PNP-Nana, Sigma) was used.

2.3.6.1 Qualitative MUAN assay

The method was a variation of that used by Lock *et al* ., (1988). Neuraminidase cleaves the MUAN to release 4-methylumbelliferone which can be visualised under ultraviolet light. 1-5µl of the protein sample was incubated with 1µl of MUAN (10mg/ml in nanopure water) and 25µl of 50mM citric acid/Na_2HPO_4 pH 6.5 for 10 minutes at 37°C. The reaction was stopped by the addition of 100µl of 0.5M Na_2CO_3, pH 9.6 and the fluoresence visualised under a ultraviolet trans illuminator.

2.3.6.2 Quantitative PNP - NANA assay

This method was a personal communication from Dr. V. Clarke (Cambridge University, U.K.). PNP - Nana is cleaved by neuraminidase with the release of free PNP which can be measured in a spectrophotometer at 400nm. 5µl of neat and diluted protein samples were placed in clean test tubes on ice. The tubes were placed in a 37°C water bath and 50µl of 0.3mM PNP - Nana (dissolved in dilution buffer) added sequentially at 10 second intervals. 10 minutes after the start of the first reaction, 100µl of 0.5M Na_2CO_3 pH 9.6 was added to the tubes at 10 second intervals, in the same sequence, to stop the reaction. The tubes were removed from the water bath and the absorbance at 400nm measured against a baseline of nanopure water. A reaction blank containing 5µl of enzyme dilution buffer in place of enzyme was included. Each sample was measured in duplicate. Dilutions of enzyme were made such that the absorbances produced were within the linear range of the assay from 0.2 to 0.8 (after subtraction of blank).

Dilution buffer

50mM Citric acid/Na_2HPO_4 (pH 6.5)	1920µl
Nanopure water	1776µl
BSA (25mg/ml)	154µl
Azide (10% w/v)	8µl

Citric acid/Na_2HPO_4 buffer (pH6.5)

0.1M Citric acid	2.9mls
0.2M Na_2HPO_4	7.1mls

Substrate dilution

5mg of PNP - NANA was dissolved in 3.85ml of dilution buffer giving a stock substrate of 3mM. This was divided into 110µl aliquots and stored at -20°C. When required, an aliquot was thawed on ice and diluted 10 - fold in dilution buffer to give 0.3mM substrate.

Calculation of enzyme activity

The activity of the neuraminidase enzyme was calculated using the equation derived by Dr. V. Clarke (Cambridge University, U.K.).

$$\text{Enzyme activity } (\mu\text{mol min}^{-1}\text{ ml}^{-1}) = \frac{\text{Absorbance at 400nm} \times \text{Dilution factor} \times \text{Volume of reaction (l)} \times 1 \times 10^6}{\text{Extinction coefficient of PNP-anion (17,700)} \times \text{Time of reaction (min)} \times \text{Volume of enzyme in assay (ml)}}$$

2.4 Techniques used for the *in vivo* investigation of the effects of neuraminidase toxoids in mice

2.4.1 General details of work on animals

Female MF1 outbred mice weighing 30 - 35g were obtained from Harlan Olac Ltd. (Shaw's Farm, Bicester, Oxon., GB). After arrival they were kept in stock cages with free access to food and water, in groups of 10 for a minimum of 2 weeks prior to use. Mice $\geq$ 9 weeks old but $\leq$ 15 weeks old were used.

2.4.2 Strain validation

Bacteria were streaked to single colonies from original strain stocks on BAB containing 5% (v/v) aerated horse blood. Optochin sensitivity was confirmed by placing an optochin antibiotic disc on the site of the first streak. Plates were incubated in a CO_2 gas jar at 37°C overnight. 4 - 5 colonies were inoculated into BHI and incubated statically overnight at 37°C. The cells were harvested by centrifugation at 3000rpm (1000 x g) for 15 minutes in a Heraeus-Christ centrifuge. The pellet was resuspended in 1ml freshly made serum broth (BHI plus 20%(v/v) irradiated, heat inactivated, filter sterilised foetal calf serum) (Gibco). An appropriate amount of culture suspension was added to 3ml of fresh serum broth to give an OD_{500} of 0.7. The culture was incubated at 37°C for 5 hours then divided into aliquots and stored at -70°C. After 24 hours the strain was checked for contamination by streaking out to single colonies on BAB + 5% (v/v) horse blood plates. Tests for Optochin sensitivity and the Quellung reaction (Section 2.4.3) were also performed.

2.4.3 Quellung reaction

The Quellung reaction (Merrill *et al* ., 1973) was done to check for the presence of type - specific polysaccharide capsule. A loop of overnight broth culture was smeared onto a microscope slide and allowed to air dry. 10ml of anti-type capsule antiserum (Statens Serum Institut, Copenhagen, Denmark) and 1% (w/v) methylene blue in water were mixed on a coverslip. The coverslip was placed fluid side down on the culture smear and examined by oil immersion microscopy at x 1000 magnification. Comparisons were made with a control slide prepared with non-immune serum (heat inactivated foetal calf serum). Bacteria were counted as positive if the capsule was distinctly outlined around the blue stained cells.

2.4.4 Viable counting

The sample to be tested was thawed at room temperature. 20µl of the sample was serially diluted in a microtitre plate containing 180µl of sterile nanopure water. The well contents were mixed by repeated drawing and expulsion of the solution into the tip of a Gilson pipette. Serial dilutions were performed to 10^{-6} with a tip change after each dilution. Dried BAB + 5% (v/v) horse

blood plates were marked into six sectors and 3 x 20µl drops from each dilution were plated onto one sector. The plating was performed in duplicate for each dilution. The spots were allowed to dry into the agar and the plates incubated in a CO_2 gas jar at 37°C overnight. Colonies were counted on sectors where 100 - 200 colonies were visible on the lowest dilution sector.

2.4.5 Passage of *S. pneumoniae* through mice

Streptococcus pneumoniae was streaked to single colonies on BAB + 5% (v/v) aerated horse blood and incubated in an anaerobic gas jar overnight at 37°C. 4 - 5 colonies were inoculated into 10ml of BHI broth (+ antibiotics if appropriate) and incubated statically at 37°C overnight. Bacteria were harvested by centrifugation at 3000rpm (1000 x g) in a Heraeus-Christ centrifuge at room temperature for 15 minutes. The pellet was resuspended in 10ml of sterile PBS and kept on ice until required. 200µl of the suspension was injected intraperitoneally into each of two MF1 mice, using a 0.5ml fine insulin syringe. After 24 hours, the animals were anaesthetised with 5% (v/v) fluothane in 1L O_2/min. The effectiveness of anaesthesia was confirmed by pinching joints and observing for no reflex reaction from the animal. Exsanguination was achieved by cardiac puncture using a 23 gauge needle attached to a syringe (Performed by P.W.Andrew). The mouse was killed by dislocation of the neck. Blood from each mouse was processed separately and kept on ice until required. 50µl of blood or serum was inoculated into 10ml of BHI (with antibiotics if appropriate) and incubated statically at 37°C overnight. Optochin sensitivity was confirmed by streaking to single colonies on BAB + 5% (v/v) aerated horse blood as described in section 2.4.2. Antibiotic sensitivity was confirmed by streaking to single colonies on BAB + blood plates with and without the antibiotic.

2.4.6 Preparation of standard inoculum

The pneumococci prepared in section 2.4.5 were centrifuged at 3000 rpm(1000 x g) at room temperature for 15 minutes in a Heraeus-Christ centrifuge and resuspended in 1ml freshly prepared serum broth (BHI broth and 20% (v/v) irradiated, heat inactivated, filter sterilised foetal calf serum) (Gibco). After resuspension the pellet was diluted in serum broth to give an OD_{500} of 0.7. The culture was incubated statically at 37°C for 5 - 8 hours until the OD_{500} reached 1.6 and 0.5ml aliquots of this culture were stored at -70°C until

required. It was observed that under these conditions pneumococci could be stored for 1 month without a significant reduction in viability. Viable counting of thawed aliquots was performed in triplicate on BAB + 5% (v/v) horse blood (+ antibiotics if appropriate). The sample was thawed at room temperature and the bacteria harvested by centrifugation at 13,000rpm (≥ 10,000 x g) for 1 minute in a microfuge. Appropriate dilutions were made in sterile PBS prior to challenge of mice.

2.4.7 Virulence testing of standard inoculum

An aliquot of the standard inoculum was thawed at room temperature and 400µl transferred to a sterile tube. The bacteria were harvested by centrifugation at 13,000rpm (≥ 10,000 x g) for 1 minute in a microfuge, and the pellet resuspended in 400µl sterile PBS. Based on the viable count data (Section 2.4.4) further dilution of cells were made in PBS to achieve a CFU of 2×10^7/ml of PBS. The cells were kept on ice until challenge of mice. Two MF1 mice (≥ 9 weeks old) were anaesthetised with 2.5% v/v fluothane in 1L O_2/min, administered using a calibrated vaporiser in a fume hood. Anaesthesia was confirmed by lack of reflex upon pinching joints. The animals were scruffed with the nose upright and the pneumococcal dose was administered by placing 50µl of culture onto the nostrils with a Gilson pipette. A series of small droplets were placed onto the nostrils, allowing inhalation of one droplet before introduction of the next. After inoculation the animal was laid on its back until recovery and placed in an isolator. Observation and recording of the disease symptoms of the animals were made for 72 hours or until they became moribund (Section 2.4.9). The time when the animals became moribund was recorded and the animals then culled by dislocation of the neck. Death was confirmed by lack of joint and eye reflexes.

2.4.8 Intranasal challenge of mice with pneumococcal samples of known virulence

An aliquot of standard inoculum from a batch that had been previously tested for virulence (2.4.7) was thawed and harvested as described in section 2.4.6. From the viable count data the CFU/ml was known. Further dilutions in PBS were made to achieve a CFU in 50µl of PBS equal to the 100% effective dose (ED_{100}) i.e. the dose which results in 100% of the group

injected becoming moribund within 3 days. The ED_{100} for MF1 outbred mice challenged with D39 was found to be 1 x 10^6. The dose was kept on ice until required and administered intranasally as described in section 2.4.7. Viable counting of the dose was performed as described in section 2.4.4.

2.4.9 Monitoring system for disease symptoms

After infection with *Streptococcus pneumoniae* , mice were monitored 3-4 times a day between the hours of 9am and 4pm for symptoms of disease. The mice were assessed on the following criteria:
1) condition of coat (starry or smooth)
2) posture (hunched or normal)
3) lethargy
4) moribund
5) found dead
Mild symptoms were given a score of 1+, severe symptoms were given a score of 2+. Personal judgement was used in each case to limit the suffering of mice but mice that showed a 2+ score for coat condition, posture, lethargy and were moribund were humanely culled by dislocation of the neck.

2.4.10 Collection of blood from mice

Mice were warmed in a 37°C incubator for 20 - 30 minutes prior to removal of blood. The mice were individually placed in a restrainer which enclosed the mouse body but allowed sampling of the blood from a tail vein. A 0.5ml fine insulin syringe (Becton-Dickinson) was used to remove 30 - 50µl of blood which was placed in a clean tube. When all the mice had been processed, the bacterial numbers in the blood were enumerated by viable counting (Section 2.4.4), or the blood samples were stored at -20°C. When serum was required the samples were thawed at room temperature and centrifuged at 6000rpm (2000 x g) for 2 minutes in a microfuge. The upper layer of serum was removed for measurement of circulating antibody levels by the enzyme linked immunosorbent assay (ELISA) (Section 2.4.13).

2.4.11 Enumeration of bacteria in the blood and lungs of mice

At predetermined time points following infection, preselected mice were deeply anaesthatised with 5% (v/v) fluothane in 1L O_2/min. Anaesthesia

was confirmed by observing no reflex movement on pinching the joints. The mice were exsanguinated by cardiac puncture using a 23 gauge needle and the blood was placed in a pre-weighed 1.5 ml tube. The mouse was culled by dislocation of the neck before recovery from the anaesthesia and the fur was drenched in ethanol or hibitane. With the aid of scissors and forceps pre-sterilised in ethanol, the fur was removed from the chest and abdomen, care was taken not to penetrate the chest cavity and peritoneum. The instruments were resterilised in ethanol and the chest and peritoneum opened whilst avoiding the internal organs. The required organs were aseptically removed and placed in pre-weighed tubes containing 10ml of sterile distilled water and kept on ice. The weight of organs and blood was determined. Within one hour of removal the tissues were aseptically transfered to to sterile stomacher bags and homogenised (90 seconds for lungs) in a Stomacher-Lab blender (Seward Medical). The contents were transferred back to the original tube and the bacterial numbers determined by the viable count procedure (Section 2.4.4).

2.4.12 Vaccination of mice

The protein antigen was administered in combination with the adjuvant $AlPO_4$ (Alum) (RIVM, National Institute of Public Health and the Environment, The Netherlands). The protein to be tested was mixed with alum in a ratio of protein:alum 1:75 and kept on ice until required. Blood samples were taken from the mice prior to immunization as described in section 2.4.10. The mice were scruffed and held at an angle of about 45° with the abdominal area facing upwards. The abdominal area was swabbed with ethanol and the antigen to be tested was administered into the intraperitoneal area with a 0.5ml insulin syringe. The control mice were immunized with 200µl of filter sterilised PBS. The mice were monitored for signs of distress, twice daily during the immunization period. Two booster doses of the same antigen were given at 14 day intervals. One month after the third immunization the mice were anesthetised and challenged intranasally with *Streptococcus pneumoniae* (Section 2.4.8). Blood samples were taken before each vaccination and prior to challenge with *S. pneumoniae* and the sera were tested for the presence of antibodies by enzyme linked immunosorbent assay (ELISA, section 2.4.13). After challenge the mice were kept in an isolator and monitored for visible clinical symptoms for 14 days, at which point the experiment was ended. Mice that

had survived this period were classified as survivors. Mice that became moribund during the 14 day period were judged to have reached the end point of the experiment. The time at which they became moribund was recorded and they were humanely sacrificed by dislocation of the neck.

2.4.13 Measurements of circulating antibody

Antibody levels in the blood samples from the mice (2.4.10) were measured using an enzyme linked immunosorbent assay (ELISA) according to Jalonen *et al* ., (1989). A Nunc-Immuno Maxisorp plate (Gibco) was coated with 200µl/well of coating buffer containing 0.05µg/µl antigen. A control well was coated with 200µl of coating buffer alone. The plate was incubated at 4°C overnight then washed 3 times in PBS-Tween (PBS containing 0.01% (v/v) Tween 20), then blocked with 200µl/well of coating buffer containing 1% (w/v) caesin and 3% (v/v) foetal calf serum. The plate was incubated for 2 hours at 37°C followed by 3 washes in PBS-Tween. The plate was incubated with 200µl/well of a dilution of the primary antibody (usually 1:500 for serum) in 1% (w/v) BSA in PBS-Tween for 2 hours. Three washes in PBS-Tween were followed by a 1 hour incubation at 37°C with 200µl/well of a 1:2000 dilution of Anti-mouse IgG (whole molecule) peroxidase conjugate raised in goat (Sigma A4416) in coating buffer. The plate was washed three times in PBS-Tween, then 200µl/well of substrate solution was added and incubated for 30 minutes at 37°C. The reaction was stopped by the addition of 50µl/well of 12% (v/v) sulphuric acid. The absorbance at 490nm was measured against a reference of 650nm in an ELISA plate reader (Dynatech).

Coating buffer

Sodium Carbonate	1.6g
Sodium Hydrogen Carbonate	2.9g

Distilled water to 1 litre

The pH was adjusted to pH 9.6 prior to use.

20 x PBS (phosphate buffered saline)

Sodium Chloride	320g
Potassium dihydrogen orthophosphate	13.6g
Dipotassium hydrogen orthophosphate	48.4g

Distilled water to 2 litres

Diluted 20 x for use with a final pH of 7.2 after dilution

Substrate solutions

Solution A

Citric acid	10.5g
Distilled water to 500ml	

Solution B

Disodium hydrogen orthophosphate	14.2g
Distilled water to 500ml	

Substrate solution was prepared by dissolving 15mg of O-phenylenediamine dihydrochloride (OPD) in a mixture of 11ml of solution A, 14ml of solution B and 25ml of distilled water. 6µl of hydrogen peroxide (30% (v/v) stock) was added immediately prior to use.

2.4.14 Statistical analysis

The INSTAT programme on the Macintosh was used for statistical analysis. Data were analysed by the Mann-Whitney U-test (Jones, 1973), for median survival times and by the Fisher's exact test for survival rates. Antibody data were analysed by analysis of variance followed by the Tukey-Kramer multiple comparison test.

Chapter 3: Cloning and mutagenesis of the neuraminidase gene

3.1 Cloning of the neuraminidase gene.

3.1.1 Introduction

Previous efforts by Berry *et al* ., (1988) to clone the neuraminidase gene were hampered by the instability of the cosmid vectors used. To overcome this Camara *et al* ., (1991) constructed a genomic library of pneumococcal DNA from the strain R36A (NCTC10319) in a LamdaEMBL vector (Lathe *et al* ., 1987; Camara, 1992). The R36A strain used for the construction of the genomic library was a nonencapsulated, non-pathogenic derivative of D39, a type II strain highly pathogenic for mice (Camara, 1992).

The genomic library was screened for the presence of functional neuraminidase enzyme by the use of the fluorogenic substrate 2'-(4-methylumbelliferyl)-α-D-N-acetylneuraminic acid (MUAN; Sigma). One recombinant clone expressing neuraminidase activity was isolated. It contained an insert of 18.5 kb and was designated EMBL301 - neu 1.

DNA from the lambda recombinant EMBL301 - neu1 was subcloned into plasmid pJDC (Chen and Morrison, 1987) and transformed into *Escherichia coli* JM101. The resulting clones were screened for neuraminidase activity. One positive recombinant containing a 7.3kb insert was selected for further analysis. The plasmid pMC4170 contained four open reading frames. ORF1 (3.1 kb) was proposed to represent the neuraminidase structural gene (*nanA*) (See Section 1.7.8.2, Figure 1.4).

In order to confirm that the neuraminidase activity from pMC4170 was associated with ORF1, the entire ORF1 was cloned into the expression vector pQE30 (Qiagen, Section 4.2.5 for more details) (Camara *et al* ., 1994). The recombinant was designated pQMC1 and was transformed into *E. coli* SG13009::pREP4. The correct cloning of ORF1 was ascertained by sequencing the ends of the inserted pneumococcal DNA in pQMC1. Neuraminidase activity

was present in the sonicates of bacteria harbouring pQMC1, which confirmed the prediction that neuraminidase was encoded by ORF1 in pMC4170.

All of the above work was carried out by Miguel Camara at Leicester University (Camara *et al* ., 1991; Camara, 1992; Camara *et al* ., 1994) and is described here to establish the background to the project.

3.1.2 Requirement of a small target for PCR mutagenesis

The neuraminidase gene was obtained from a plasmid expression vector (pQMC1) made previously in our laboratory by Miguel Camara (Camara *et al* ., 1994). One of the aims of this project was to create specific mutations in the neuraminidase gene and to assess their effect on the function of the resulting protein. The mutagenesis technique chosen necessitated the sub-cloning of the mutagenesis target region of *nan A* from pQMC1 into pUC18.

The mismatch PCR mutagenesis technique (Section 2.2.14) was selected for the introduction of point mutations. Although this method of introducing mutations into DNA is relatively straightforward, occasionally unwanted sequence alterations can be introduced (Higuchi, 1989). In a mutation reversion assay, *Taq* DNA polymerase has been estimated to incorporate an incorrect nucleotide once every 9000 nucleotides incorporated and produce frameshift errors once every 41,000 nucleotides (Tindall and Kunkel, 1988). If the PCR product is to be used collectively then the fact that a small proportion of molecules have an incorrect base is not very significant (Krawczak *et al* ., 1989). However, if the PCR product is to be cloned (as in present case) then the fact that one molecule cloned has a sequence alteration, other than at the desired site, may be significant (Higuchi, 1989).

Cloned constructs generated by the PCR methodology need to be sequenced to verify that no unwanted mutations have occurred. In order to reduce the likelihood of such mutations and to minimise the sequencing, it was desirable to keep the construct size to a minimum. To achieve this it was necessary to subclone and mutate a small portion of DNA from pQMC1. From the restriction map of pQMC1 it was predicted that cleavage with *Hind* III would result in the production of three fragments of sizes 4588, 1433 and 480 bp (Figure 3.1). Since the 1.4 kb fragment contained the bases to be mutated, it was decided to subclone this fragment into pUC18 for the mutagenesis. The 1.4 kb

fragment will be referred to as the *Hind* III/pQMC1 fragment throughout the text. The overall cloning and mutagenesis strategy is shown in Figure 3.2.

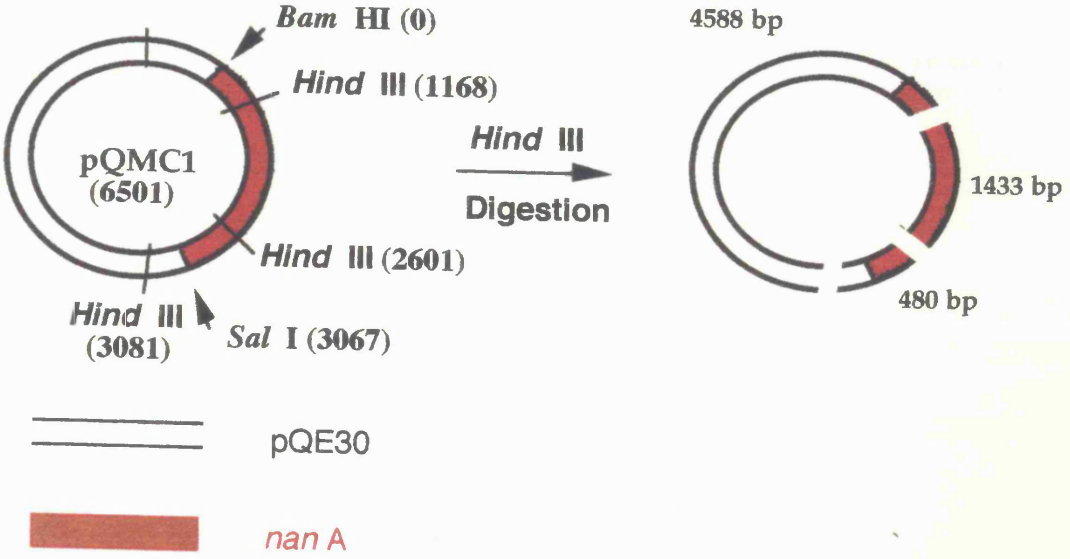


Figure 3.1 Restriction map of pQMC1.
Digestion of pQMC1 with *Hind* III should produce three fragments of approximately 0.48, 1.4 and 4.6 kb.

3.1.3 Cloning the 1433 bp *Hind* III/pQMC1 fragment from pQMC1 into pUC18

3.1.3.1 Cleavage of pQMC1 with *Hind* III to produce the 1433 bp *Hind* III/pQMC1 fragment

A large scale preparation of pQMC1 DNA was obtained using the CsCl method of plasmid isolation (Section 2.2.10.2.1). Plasmid pQMC1 (37µg) was digested with an excess of *Hind* III (Section 2.2.6) and the resulting fragments analysed by agarose gel electrophoresis (Figure 3.3). Three fragments of approximate sizes 0.48, 1.4 and 4.6 kb were distinguishable (Figure 3.3, lanes 2 and 3). These were purified using a commercial purification kit (Bandprep kit, Section 2.2.2.1) and stored at -20°C until required. To confirm recovery of the DNA, a small sample of the Bandprep product was re-analysed by gel electrophoresis.

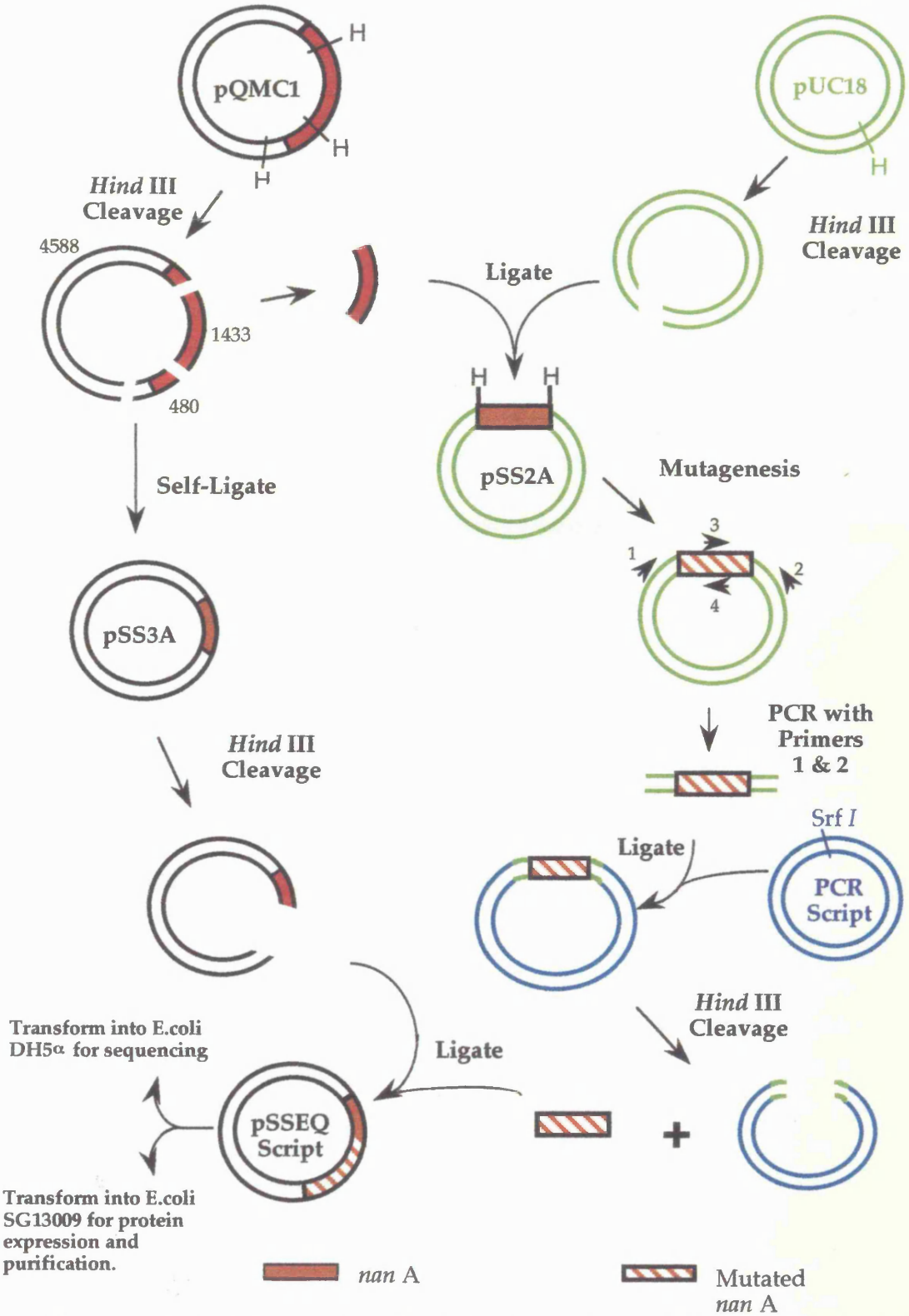


Figure 3.2 Summary of the strategy used to subclone the *Hind* III fragment from pQMC1 into pUC 18, followed by mutagenesis and cloning into pCR Script™ SK (+) vector and subsequent cloning into pSS3A.
The plasmid pQMC1 was digested with *Hind* III and the resulting 1433 bp fragment was ligated into the *Hind* III site of pUC18. Mutagenesis was performed on the *Hind* III/pQMC1 fragment using mutagenic primers (3 and 4) and flanking primers (1 and 2). The 4588 bp fragment from the cleavage of pQMC1 with *Hind* III was self-ligated and then introduced into E. coli strain JM109 to produce the plasmid pSS3A. The mutated *Hind* III/pQMC1 fragment with flanking pUC18 DNA was cloned into the pCR Script™ SK (+) vector, followed by cloning of the *Hind* III/pQMC1 fragment into pSS3A to generate the plasmid pSSEQscript. The plasmid pSSEQscript was introduced into *E. coli* strains DH5a and SG13009 for DNA sequencing and protein expression, respectively.

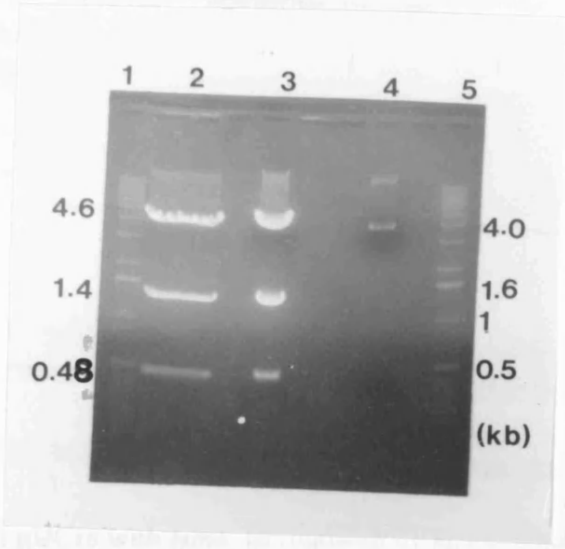


Figure 3.3 Cleavage of pQMC1 with *Hind* III followed by agarose gel electrophoresis.
Lanes 1 and 5: 1kb DNA ladder. Lanes 2 and 3: *Hind* III digested pQMC1 producing fragments of 0.48, 1.4 and 4.6 kb. Lane 4: undigested pQMC1

3.1.3.2 Cleavage of pUC 18 with *Hind* III

To sub clone the *Hind* III/pQMC1 fragment, pUC18 was digested with *Hind* III and the resulting fragments analysed by electrophoresis (Figure 3.4). The digestion produced some linearized plasmid of approximately 2.7 kb (Figure 3.4, lane 3). This fragment was purified from the agarose gel as described in section 2.2.2.1 and dephosphorylated with calf intestinal alkaline phosphatase. The success of the alkaline phosphatase reaction was checked by a religation reaction of the dephosphorylated vector followed by transformation into electrocompetent *E.coli* JM109 cells. Typically less than 5 colonies were obtained which was an acceptable level of background. Electroporation was the method of choice for DNA transformations because of its high efficiency (Dower *et al.*, 1988).

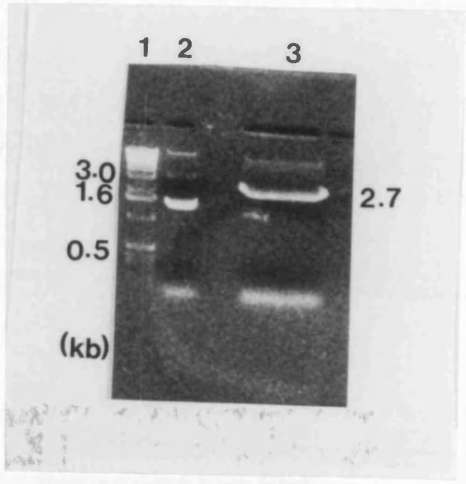


Figure 3.4 Cleavage of pUC18 with *Hind* III followed by agarose gel electrophoresis.
Lane 1: 1kb DNA ladder. Lane 2: undigested pUC18. Lane 3: *Hind* III digested pUC18 producing a single band of 2.7 kb.

3.1.3.3 Ligation of *Hind* III/pQMC1 fragment into the pUC18 vector, producing plasmid pSS2A.

Ligation reactions were set up between the *Hind* III/pQMC1 fragment released from the digestion of pQMC1 with *Hind* III (Section 3.1.3.1), and dephosphorylated *Hind* III cleaved pUC18 vector (Section 3.1.3.2). The ratios of insert to vector used were: 1.5:1, 3:1, and 6:1.

A total of 23 colonies were obtained on the 3 plates originating from the 3 different ratios of insert to vector used. The most (14 colonies) originated from the 6:1 ratio of insert to vector. To determine the outcome of the cloning, small scale plasmid preparations (minipreps) were done for each of the 23 colonies and digested with *Hind* III. The resulting fragments were analysed by agarose gel electrophoresis. Restriction digestion of minipreps 1 - 12 is shown in Figure 3.5.

Restriction digestion of miniprep DNA with *Hind* III was anticipated to release the insert from the vector and this would be evidenced by two bands of 1.4 and 2.7 kb. Restriction digestion of miniprep DNAs 4, 6, 7, 8, 10, 11 and 12 (Figure 3.5, lanes 5, 7, 8, 9, 11, 12 and 13 respectively) produced bands of approximately 1.4 and 2.7 kb which corresponded to the expected size of the insert released from vector. Miniprep DNAs 13 - 23 also produce two fragments

of about 1.4 and 2.7 kb. (Results not shown). Restriction digestion of clones 5, and 9 produced a single band around 2.7kb (Figure 3.5, lanes 6 and 10 respectively) which was probably *Hind* III cut pUC 18 vector. Miniprep DNAs 1, 2 and 3 (Figure 3.5, lanes 2, 3 and 4 respectively) did not produce the required fragments and were ignored. The DNA isolated from the 23 clones was also linearised with *EcoR* I, and with the exception of clones 3, 5, 9, 10, 11 and 12 produced a single band of approximately 4.1 kb (Results not shown). This confirmed that a single *Hind* III/pQMC1 fragment had ligated into pUC 18.

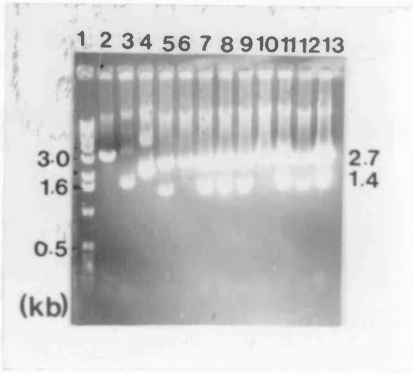


Figure 3.5 The *Hind* III/pQMC1 fragment was cloned into *Hind* III digested pUC18 vector. Miniprep DNA was obtained from twelve clones and digested with *Hind* III. The desired clones produced two bands of 1.4 and 2.7 kb, lanes 5, 7, 8, 9, 11, 12 and 13. Minipreps in lanes 2, 3, 4, 6 and 10 did not produced the 1.4 kb band and were ignored.
Lane 1: 1kb DNA ladder. Lanes 2 - 13: *Hind* III digested miniprep DNAs 1 - 12.

Based on the results of the *Hind* III and *Eco R* I digestions, clones 4, 6, 7 and 8 were selected for further analysis. Preparative DNA purifications were performed using the CsCl method of plasmid isolation (Section 2.2.10.2.1). The DNA from each preparation was digested with *Hind* III and *Eco R* I in separate reactions and analysed by agarose gel electrophoresis (Results not shown). The electrophoresis patterns from the restriction digests of the maxiprep DNA was identical to that observed in the miniprep digests, confirming the successful cloning of the *Hind* III/pQMC1 fragment into pUC 18.

The 1.4 kb fragment isolated from pQMC1 could ligate into pUC18 in one of two orientations (Figure 3.6). The direction of cloning could be elucidated by digestion with *Sph* I because there is a single *Sph* I site present in the vector and insert. Therefore if the insert had ligated in the correct orientation then fragments of about 3.5 and 0.6 kb would be produced. If however the insert had ligated in the opposite direction then fragments of about 3.3 and 0.8 kb would be produced.

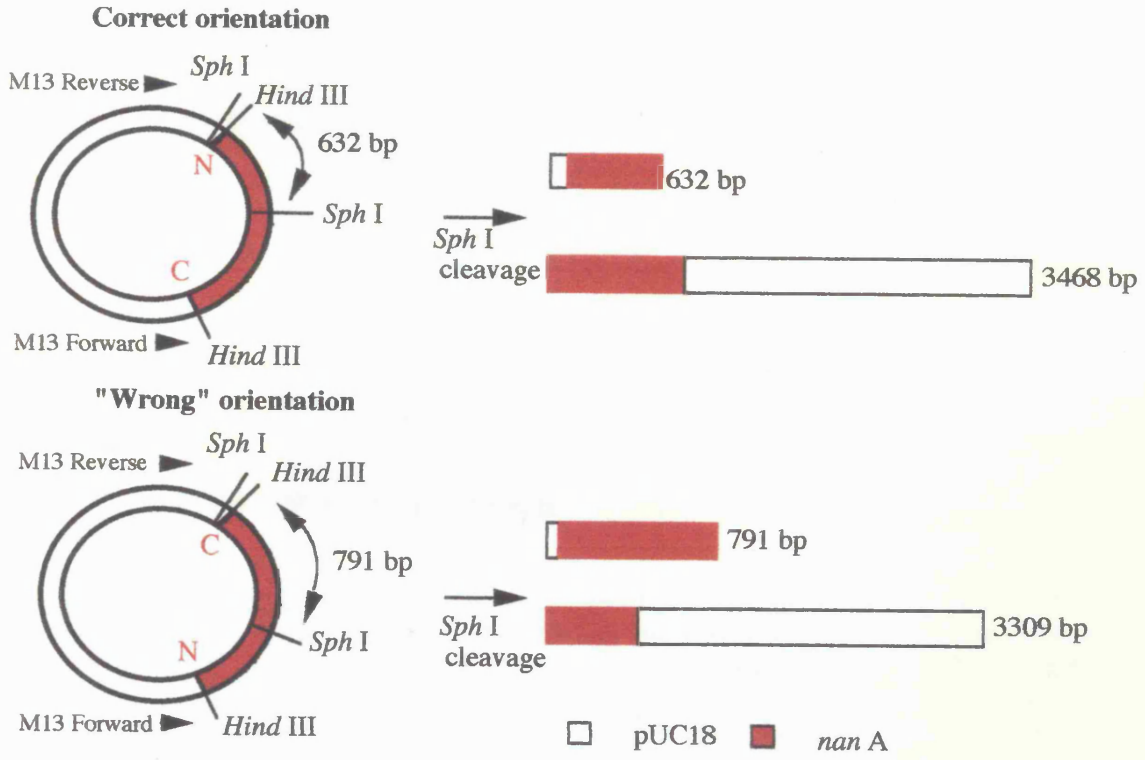


Figure 3.6 The two potential orientations of the *nan* A pQMC1/*Hind* III fragment cloned in pUC18.
If the pQMC1/*Hind* III fragment had ligated into pUC18 in the correct orientation then digestion of the maxiprep DNA with *Sph* I would result in two fragments of 632 and 3468 bp. If however the insert had been cloned in the opposite direction then cleavage with *Sph* I would result in two fragments of 791 and 3309 bp.

The orientation of the insert was confirmed by cleavage of the maxiprep DNA with *Sph I* (Figure 3.7). Digestion of DNA from clones 6 and 7 (Figure 3.7, lanes 2 and 3 respectively) produced bands of about 3.5 and 0.6 kb which were consistent with the ligation of the insert in the correct orientation. Digestion of DNA isolated from clones 4 and 8 (Figure 3.7, lanes 1 and 4 respectively) produced bands of about 3.3 and 0.8 kb which were consistent with the ligation of the insert in the incorrect orientation.

Clone 6 representing the 1.4 kb *Hind* III/pQMC1 fragment ligated into pUC18 in the correct orientation was selected for future work and termed pSS2A (Figure 3.2). Plasmid pSS2A formed the starting material for the PCR mutagenesis (Section 3.2).

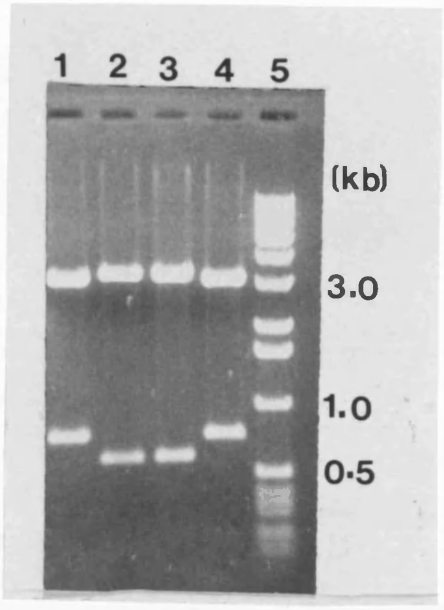


Figure 3.7. The *Hind* III/pQMC1 fragment was cloned into the *Hind* III digested pUC18 vector. Maxiprep DNA was obtained from clones 4, 6, 7 and 8 and digested with *Sph* I. The correct clones produced two bands of 3.5 and 0.6 kb (lanes 2 and 3). Clones 4 and 8 prduced bands of 3.3 and 0.8 kb (lanes 1 and 4) which was consistent with the ligation of the insert in the incorrect orientation. Lanes 1 - 4: *Sph* I digested maxiprep DNAs 4, 6, 7 and 8 respectively. Lane 5: 1kb DNA ladder.

3.2 PCR mutagenesis

3.2.1 Introduction

The aim of this project was to characterise a neuraminidase from *Streptococcus pneumoniae* by relating its amino acid sequence to its enzyme activity. There is no published data for which amino acids are required for activity in the pneumococcal neuraminidase. However primary sequence information and crystallography data from other bacterial neuraminidases enabled predictions to be made about the residues likely to be important in catalysis (Section 1.7.10.1). Three residues thought to be involved in catalysis were glutamic acid (E) 647, arginine (R) 663 and tyrosine (Y) 752. To assess the importance of these residues in catalysis, conservative substitutions of these residues (E_{647}>Q, R_{663}>H and Y_{752}>F) were made and the subsequent effect on enzyme activity measured. The mutations were introduced using the mismatch PCR mutagenesis protocol (Section 2.2.14). The outcome of the mutagenesis is described below.

3.2.2 Mutagenesis of Glutamic acid (GAA) to Glutamine (CAA) at position 647 in pSS2A.

3.2.2.1 Primary PCR

Primary PCR reactions were performed with the primer combinations M13 Reverse/NeuE647.2 and M13 Forward/NeuE647.1. The "Standard" PCR incubation cycle (Section 2.2.12.3) was used.

3.2.2.1.1 Left primer mutagenesis

A fragment of about 744 bp was amplified from plasmid pSS2A using the primers M13 Reverse and NeuE647.2, using standard PCR conditions with *Vent* polymerase (New England Biolabs). *Vent* polymerase was chosen because of its high fidelity which is derived partly from the 3'>5' proofreading exonuclease activity (Mattila *et al* ., 1991; Kong *et al* ., 1993). The primer NeuE647.2 was mismatched from the target sequence at position 647 (G>C base change). The "left" mutated PCR product was visualised on a 0.7% agarose gel (Figure 3.8, lane 4) and extracted from the gel using the Bandprep kit. The purification of the PCR product from the gel removes both unmutated template and the "inside primers" which favours the production of the full-length product during the secondary PCR (Higuchi, 1989). As anticipated, a band of approximately 0.7 kb was visible in lane 4 of Figure 3.8. A "blank" reaction containing no template DNA was included to detect contamination. This negative control reaction gave no bands (Figure 3.8, lane 2) thus confirming that the amplification of the "left" portion of the mutated *Hind* III/pQMC1 fragment was successful.

3.2.2.1.2 Right primer mutagenesis

An 843 bp fragment was amplified from pSS2A using the primers M13 Forward and NeuE647.1 under standard PCR conditions (Sambrook *et al* ., 1989) with *Vent* polymerase. The primer NeuE647.1 was mismatched from the target sequence at position 647 (C>G). The "Right" mutated PCR product was visualised on a 0.7% agarose gel (Figure 3.9) and extracted from the gel using the Bandprep kit. A band corresponding to the expected 843 bp was present in

lane 3 of Figure 3.9. A "blank" reaction containing no template DNA was included to detect contamination. This negative control reaction gave no bands (Figure 3.9, lane 1) thus confirming that the amplification of the "right" portion of the mutated *Hind* III/pQMC1 fragment was successful.

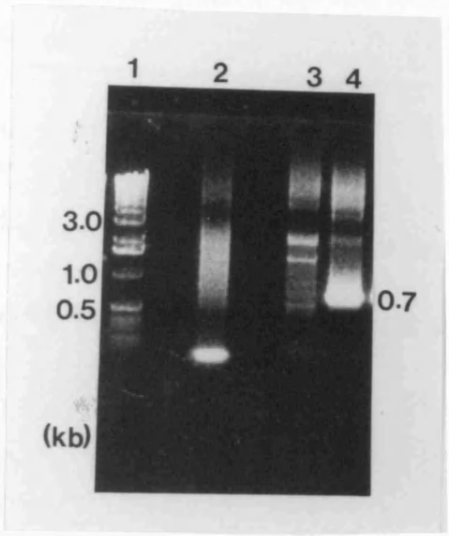


Figure 3.8 Left primer mutagenesis of plasmid pSS2A.
Plasmid pSS2A was amplified using primers NeuE647.2 and M13 Reverse to produce the "left" primary PCR product. Lane 1: 1kb DNA ladder. Lane 2: negative PCR control. Lane 3: Product of a separate experiment which is not relevant here. Lane 4: 744 bp "left" PCR product.

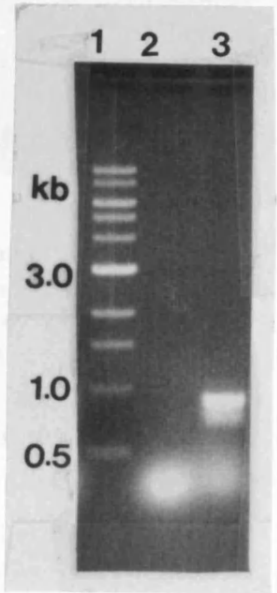


Figure 3.9 Right primer mutagenesis of plasmid pSS2A.
Amplification of pSS2A using primers NeuE647.1 and M13 Forward producing the "right" primary PCR product. Lane 1: 1 kb DNA ladder, Lane 2: negative PCR control. Lane 3: 843 bp "right" PCR product.

3.2.2.2 Secondary (Recombinant) PCR.

The next stage involved amplification of the full length mutated PCR product with the outside primers M13 Reverse and M13 Forward. The gel purified products of the "left" and "right" primary PCRs (6µl of each) were used as the template in a secondary PCR. The annealing temperature in these reactions was reduced to 40°C for the first two cycles in order to favour the formation of recombinant heteroduplex molecules. The PCR cycling parameters were: 1 cycle of 5 min at 94°C followed by 2 cycles of 1 min at 94°C , 2 min at 40°C, 3 min at 72°C followed by 35 cycles of 1 min at 94°C , 2 min at 58°C, 2 min at 72°C. The final cycle was 1 min at 94°C, 2 min at 58°C and 10 min at 72°C. The product of the secondary PCR was analysed on a 0.7% agarose gel (Figure 3.10), then isolated from the gel using the bandprep kit. A band of about 1.5 kb in lane 3 of Figure 3.10 suggested the successful amplification of the full length mutated *Hind* III/pQMC1 fragment. The mutated PCR product was then cloned into the pCR-Script™ SK (+) vector.

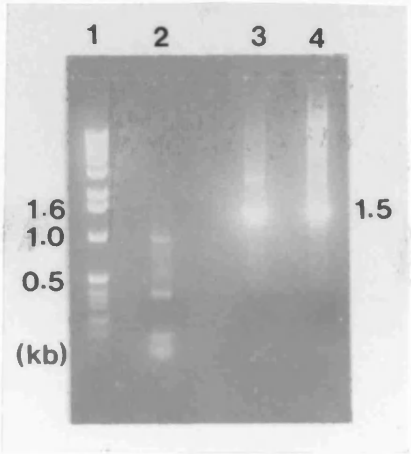


Figure 3.10 Analysis of the recombinant PCR product (E_{647}>Q). The recombinant PCR product obtained from the "left" and "right" primary PCR reactions with primers M13 Forward and M13 Reverse was analysed by agarose gel electrophoresis. Lane 1: 1Kb DNA ladder. Lane 2: negative control. Lane 3: 1555 bp full length recombinant PCR product. Lane 4: Positive control

3.2.2.3 Cloning of the mutated *nan A* gene PCR product into pCR-Script™ SK (+) vector.

A commercial pCR-Script™ SK (+) kit (Stratagene) was chosen for the cloning of the mutated *nan A* PCR product because it combines efficient high yield cloning of PCR fragments with a low rate of false positives (Section 2.2.15).

The ligation was performed according to the instructions of the pCR-Script™ SK (+) cloning kit (Stratagene), followed by transformation into 40µl of Epicurian Coli® XL1 - Blue MRF' Kan supercompetent cells (Section 2.2.15). Transformants were selected on LA plates containing 50µg/ml ampicillin, 0.004% (w/v) X-gal, and 0.1mM IPTG. Colony screening was facilitated by blue/white selection since the cloning site of the vector is located in the middle of the *lac Z* gene. A total of 36 white colonies were patched onto new LA plates containing 50µg/ml ampicillin, 0.004% (w/v) X-gal, and 0.1mM IPTG to verify the Lac phenotype. After overnight incubation at 37°C, 10 colonies remained white. These were screened by colony PCR using the primers M13 Reverse and M13 Forward. The DNA from colonies 7, 9, 12, 13 and 18 produced the expected amplified PCR product of approximately 1.5 kb. Plasmid DNA from these colonies was prepared and subjected to restriction analysis. The DNA from these clones was initially digested with *Not I* then extracted twice with phenol/chloroform followed by ethanol precipitation and digestion with *BstE* II. The resulting fragments were analysed by gel electrophoresis and the results are shown in Figure 3.11.

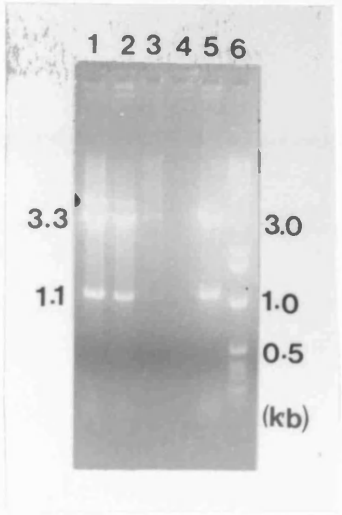


Figure 3.11 Cleavage of miniprep DNAs 7, 9, 12, 13 and 18 with *Not* I and *Bst* E II.
The recombinant PCR product (E_{647}>Q) was cloned into the pCR-Script™ SK (+) vector. Small scale DNA preparations were performed from clones 7, 9, 12, 13 and 18 and digested with *Not* I and *Bst* E II (Lanes 1 - 5 respectively). Lane 6: 1 kb DNA ladder.

The mutated PCR product could have been cloned into the pCR-Script™ SK (+) vector in two potential orientations. This is shown diagramatically in Figure 3.12, (however here either orientation of the insert can be used in subsequent cloning). Also shown is the size of fragments that would be produced upon restriction digestion with *Pvu* I.

If the insert had ligated into the pCR-Script™ SK (+) cloning vector in "orientation A" then DNA fragments of 4085 and 431 bp would be expected upon digestion with *Not* I and *BstE* II. However, if the insert had ligated into the vector in "orientation B" then bands of 3364 and 1152 bp would be observed. Clones 7, 9, 12 and 18 were found to contain the insert in "orientation B" as confirmed by the presence of two bands of approximately 3.3 and 1.1 kb (Figure 3.11, lanes 1,2,3 and 5 respectively). Clone 13 was not digested and therefore excluded from further analysis. Large scale DNA was prepared from clones 12 and 18 and digested with *Not* I and *BstE* II. Electrophoresis of the cleaved DNA revealed two fragments of approximately 3.3 and 1.1 kb (Results not shown). This was identical to the restriction pattern of the miniprep DNA and confirmed the cloning of the 1.5 kb PCR product into pCR-Script™ SK (+) in "orientation B".

One clone, (clone 18) was chosen for further use and designated pSSEQscript. It consisted of the 1433 bp *Hind* III/pQMC1 fragment containing a glutamic acid to glutamine mutation at position 647 (protein sequence), flanked by short stretches of pUC18 DNA ligated into the pCR-Script™ SK (+) vector in "orientation B".

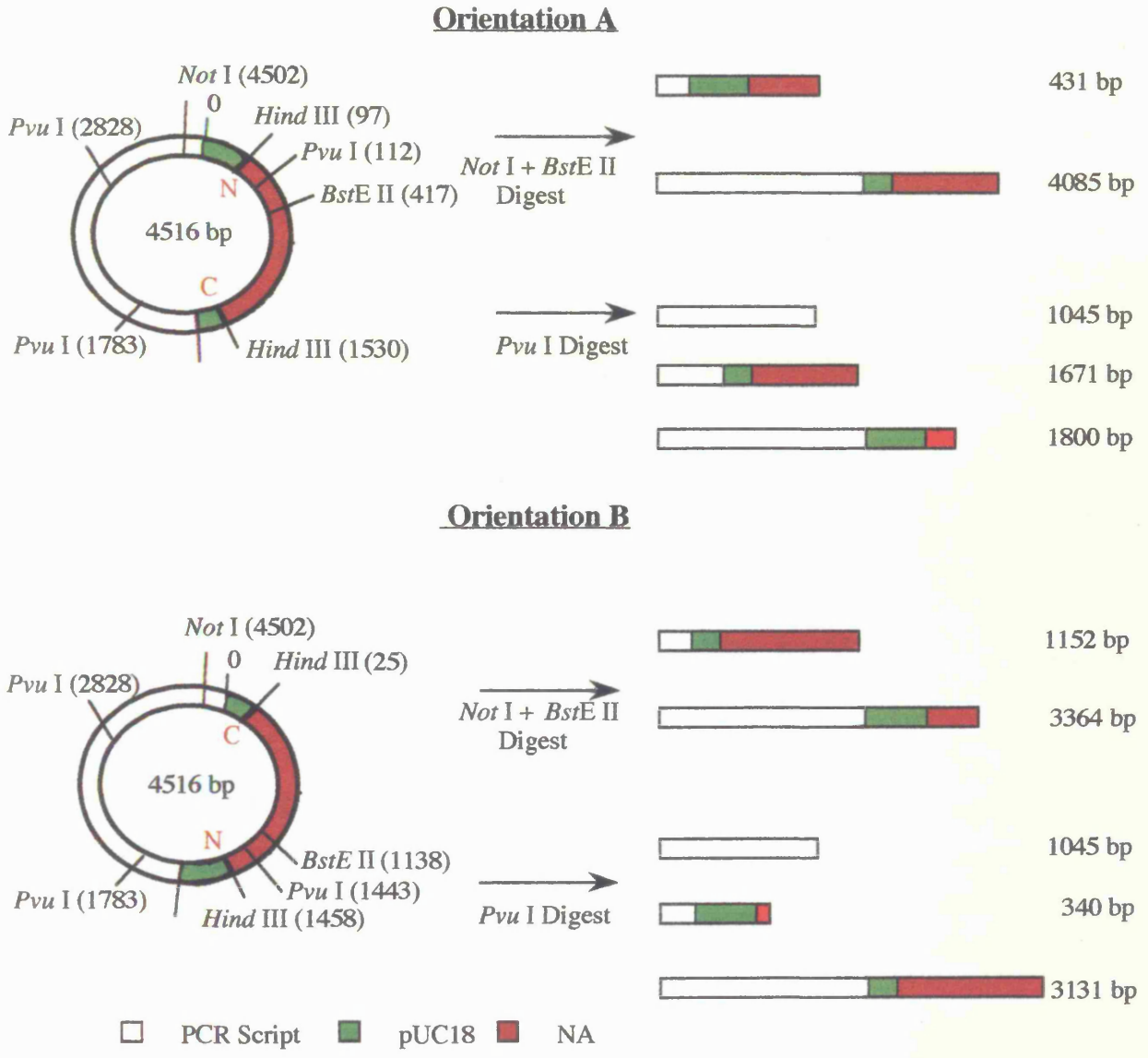


Figure 3.12 Possible outcomes of the cloning of the PCR product into the pCR-Script™ SK (+) vector.
The cloning of the PCR product in "orientation A" would give bands of 4085 and 431 bp upon digestion with *Not* I and *Bst*E II. Cleavage with *Pvu* I would result in fragments of 1045, 1671 and 1800 bp Cloning in "orientation B" would result in bands of 3364 and 1152 bp upon digestion with *Not* I and *BstE* II. Cleavage with *Pvu* I would produce fragments of 1045, 340 and 3131 bp.

3.2.2.4 Cloning of the *Hind* III/pQMC1 fragment from pSSEQscript containing the (E>Q) mutation at position 647 back into the expression vector pQE30.

In section 3.1.3.1 cleavage of the plasmid pQMC1 with *Hind* III was described. This resulted in the production of three fragments of sizes 0.48 , 1.4 and 4.6 kb. The 1.4 kb*Hind* III/pQMC1 fragment was mutated as described in Section 3.2.2 then cloned into the pCR-Script™ SK (+) vector to give pSSEQscript. The next stage of the project required the expression and purification of the mutated neuraminidase protein, in order to assess the effect of the mutation on the activity of the neuraminidase. To achieve this it was necessary to ligate the mutated *Hind* III/pQMC1 fragment from pSSEQscript with the 4.6 kb fragment which contains the 5' end of the neuraminidase gene in the expression vector pQE30 (Figure 3.2). The first step in this process was the self-ligation of the 4.6 kb fragment and is described below.

3.2.2.4.1 Self ligation of the 4.6 kb fragment

The 4.6 kb fragment of DNA (purified from an agarose gel in Section 3.1.3.1) was self ligated with T4 DNA ligase and transformed into electrocompetent JM109 as described in Section 2.2.9.2. DNA was prepared from 12 transformants and subjected to restriction analysis. All clones produced the expected size fragments of approximately 1.6 and 3 kb upon restriction digestion with *Xba* I (Figure 3.13, lanes 1 -12). Four clones 1, 2, 3 and 4 were selected for large scale DNA preparation (Caesium Chloride method, section 2.2.10.2.1). Restriction digestion with *Xba* I, of the maxiprep DNA from clones 1-4 also produced the expected fragments of approximately 1.6 and 3 kb (Results not shown). Clone 4 which represents the self ligated 4.6 kb fragment from pQMC1, was designated pSS3A.

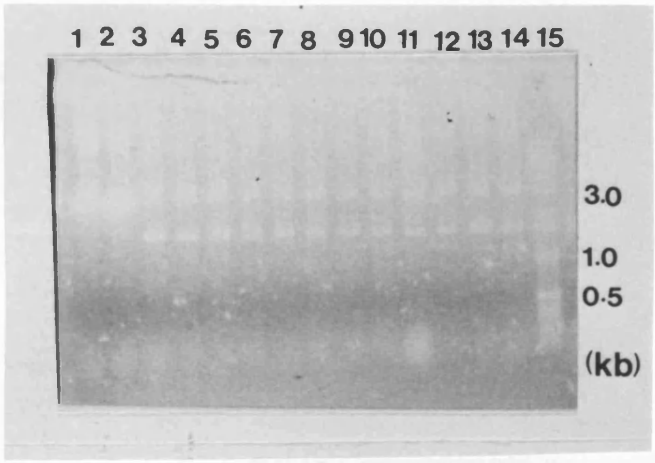


Figure 3.13 Cleavage of minipreps 1 - 12 with *Xba* I.
The 4.6 kb fragment produced by the digestion of pQMC1 with *Hind* III (Section 3.1.3.1) was self-ligated. Small scale DNA was prepared from 12 clones, digested with *Xba* I and the resulting fragments analysed by agarose gel electrophoresis. Lanes 1 - 12: cleavage of DNAs 1 - 12 with *Xba* I. Lane 13: 1 kb DNA ladder.

3.2.2.4.2 Cloning of the *Hind* III/pQMC1 fragment from pSSEQscript containing the (E>Q) mutation at position 647 into the *Hind* III site of pSS3A.

Plasmid pSSEQscript (20µg) was digested with *Hind* III and the 1.4kb mutated *Hind* III/pQMC1 fragment isolated using the Qiaquick gel extraction kit (Figure 3.14, lane 3).

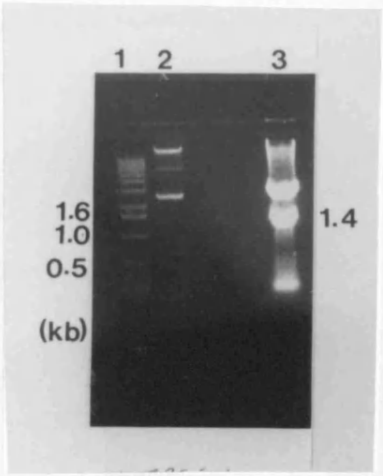


Figure 3.14 *Hind* III digestion of pSSEQscript.
Plasmid pSSEQscript was digested with *Hind* III and the resulting fragments analysed by gel electrophoresis. Lane 1: 1 kb DNA ladder. Lane 2: undigested pSSEQscript. Lane 3: *Hind* III digested pSSEQscript.

The plasmid pSS3A (30µg) was also linearized with *Hind* III, followed by gel purification of the 4.6 kb band (Figure 3.15, lane 1) with the Qiaquick gel extraction kit.

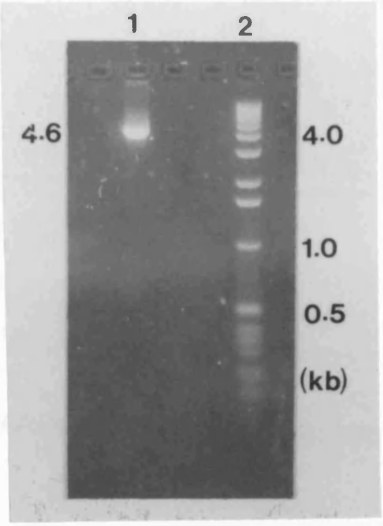


Figure 3.15 *Hind* III digestion of pSS3A.
The plasmid pSS3A was cleaved with *Hind* III and the resulting 4.6 kb fragment was gel purified and analysed by gel electrophoresis. Lane 1: pSS3A cleaved with *Hind* III. Lane 2: 1 kb DNA ladder.

The pSS3A vector was then dephosphorylated (Section 2.2.7) and ligated in a ratio of 3:1, insert:vector to the 1.4kb *Hind* III/pQMC1 fragment isolated from pSSEQscript. The DNA from the ligation reactions was transformed into JM109 cells by electroporation. The transformants were selected on LA plates containing 100µg/ml ampicillin. DNA from 12 transformants was prepared (Section 2.2.10.1) and digested with *Hind* III and *Pvu* I in separate reactions.

Upon digestion with *Hind* III, all clones produced the expected bands of 1.4 and 4.6 kb (Figure 3.16, lanes 1 - 12) representing the 1.4 kb insert and the 4.6 kb vector.

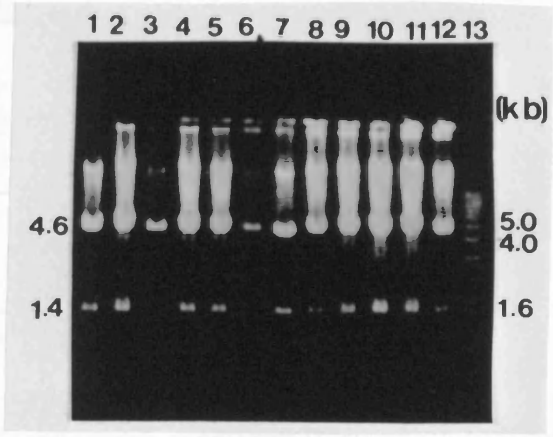


Figure 3.16 *Hind* III digestion of 12 miniprep DNAs of pSSEQvec.
Lanes 1 - 12: *Hind* III digestion of minipreps 1 - 12. Lane 13: 1 kb DNA ladder.

The *Hind* III/pQMC1 fragment isolated from pSSEQscript could ligate into pSS3A in one of two orientations (Figure 3.17). The direction of cloning could be elucidated by digestion with *Pvu* I because there is a single *Pvu* I site present in the vector and insert. Therefore if the insert had ligated in the correct orientation then fragments of 2.0 and 4.0 kb approximately would be produced. If however the insert had ligated in the opposite direction then bands of about 2.7 and 3.3 kb would be produced. Digestion of miniprep DNA from clones 5, 9, 11 and 12 with *Pvu* I (Figure 3.18, lanes 6, 10, 12 and 13 respectively) produced bands of about 2 kb and 4 kb which were consistent with the ligation of the insert in the correct orientation. Digestion of miniprep DNA isolated from the other clones (Figure 3.18, lanes 2, 3, 4, 5, 7, 8, 9, and 11) did not produce the required bands and were abandoned.

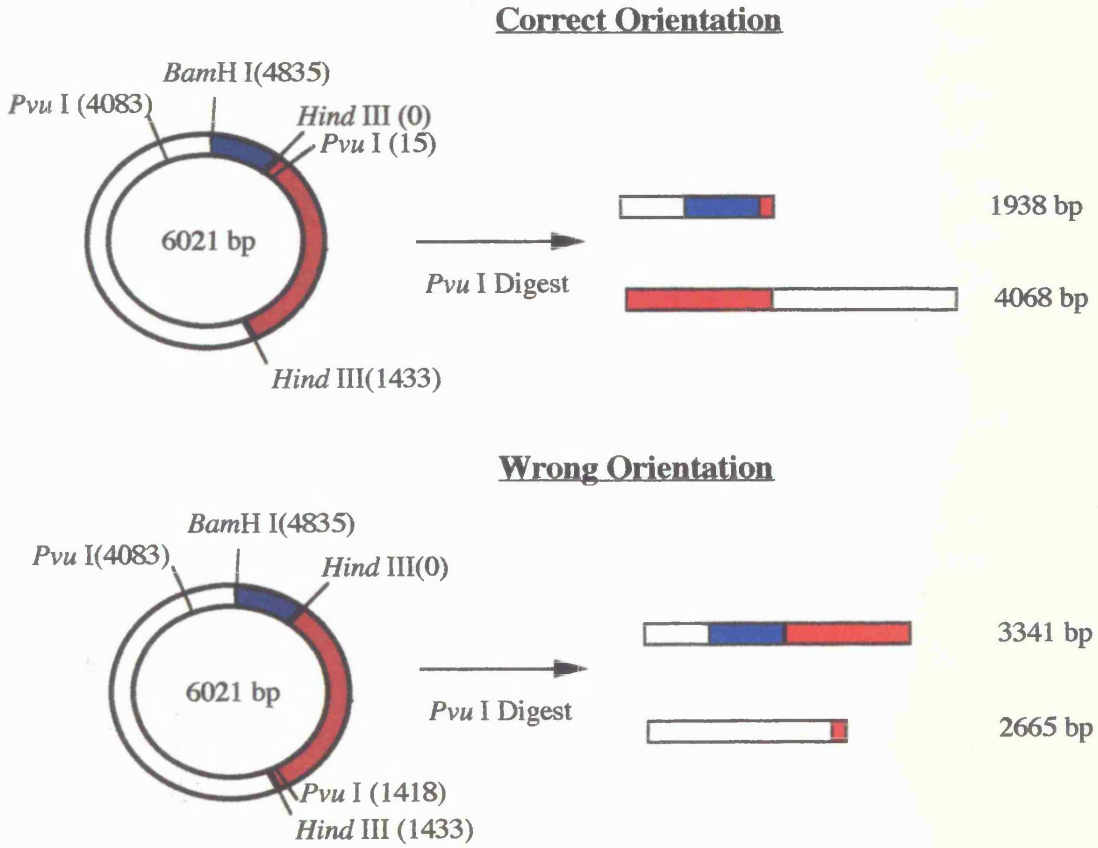


Figure 3.17 Potential outcome of cloning the *Hind* III fragment from pSSEQscript into pSS3A. The *Hind* III fragment isolated from pSSEQscript could ligate into pSS3A in one of two orientations. Cleavage of the transformants with *Pvu* I revealed the orientation of the insert. An insert cloned in the correct direction would yield DNA fragments of 1938 and 4068 bp. Cloning of the insert in the incorrect orientation would give fragments of 2665 and 3341 bp.

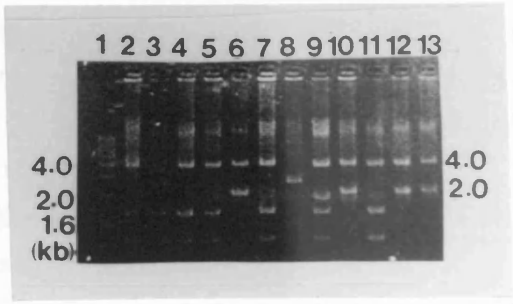


Figure 3.18 *Pvu* I digestion of 12 miniprep DNAs of pSSEQvec.
Lane 1: 1 kb DNA ladder. Lanes 2 - 13: minipreps 1 - 12 digested with *Pvu* I.

Large amounts of DNA were prepared from clones 11 and 12 by the caesium chloride method (Section 2.2.10.2.1) and cleaved with *Hind* III and *Pvu* I (in separate reactions) to confirm the identity of the constructs. The restriction pattern (Results not shown) was identical to that observed with the digestion of miniprep DNAs. The *Hind* III digestion of all clones released the insert from the vector and produced two bands of approximately 1.4 and 4.6 kb. Digstion of clones 11 and 12 with *Pvu* I resulted in two bands of about 2.0 and 4.0 kb, which confirmed the correct orientation of the insert. Clone 11 was termed pSSEQvec and selected for further work.

Plasmid pSSEQvec is the DNA encoding the neuraminidase structural gene containing a GAA > CAA mutation (glutamic acid to glutamine) at position 647 (protein sequence), within the expression vector pQE30. It is worth noting that the cloning process leading from the initial digestion of pQMC1 (Section 3.1.3.1) to the formation of pSSEQvec had resulted in the loss of the 466 bases from the 3' end of the neuraminidase structural gene and 14 bases from the vector pQE30 (between *Sal* I and *Hind* III in the multiple cloning site of pQE30) (Figure 3.2).

The plasmid pSSEQvec was transformed into electrocompetent DH5α cells to facilitate DNA sequencing (Section 3.3) and into electrocompetent SG13009:pREP 4 for protein expression and subsequent purification studies (Chapter 4).

3.2.3 Construction of pSSWTvec

In order assess the effects of the mutations created in the neuraminidase gene, it was necessary to compare the construct pSSEQvec with an identical construct which did not have any mutations. The creation of this construct, designated pSSWTvec is described below. It essentially consisted of the ligation of the wild type *Hind* III/pQMC1 fragment (Section 3.1.3.1) with pSS3A (Section 3.2.2.4.1) as shown in Figure 3.19.

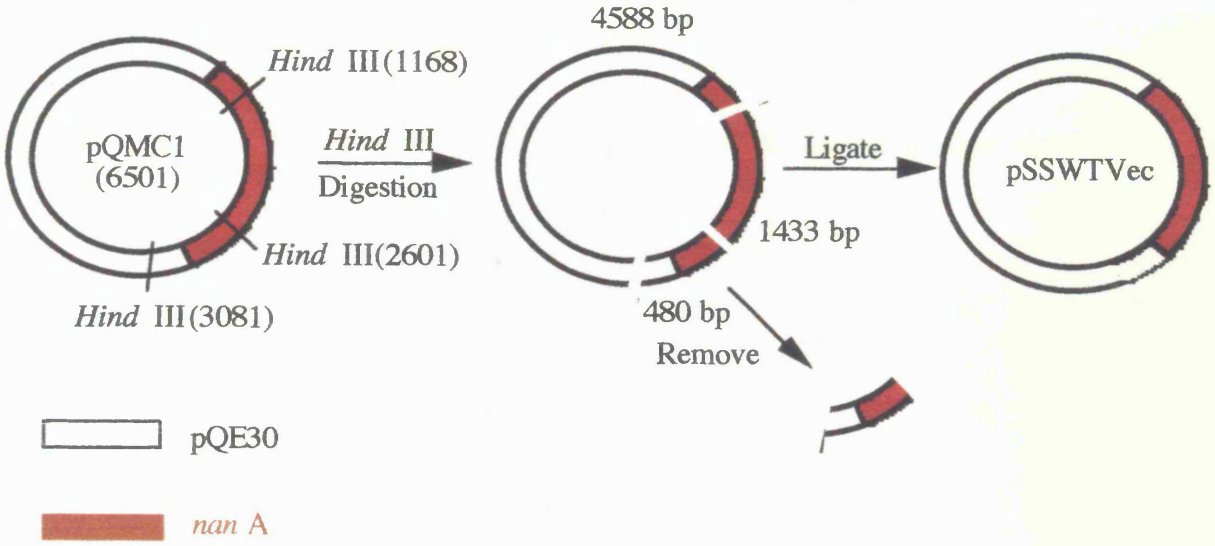


Figure 3.19 Diagrammatic representation of the construction of plasmid pSSWTvec. Cleavage of plasmid pQMC1 with *Hind* III resulted in three fragments of sizes 4588, 1433 and 480 bp. Religation of the two larger fragments resulted in the formation of pSSWTvec.

3.2.3.1 Cloning of the *Hind* III/pQMC1 fragment into pSS3A.

Cleavage of pQMC1 to produce the *Hind* III/pQMC1 fragment and the construction of pSS3A were described in sections 3.1.3.1 and 3.2.2.4.1 respectively. Approximately 5µg of pSS3A was digested with *Hind* III, dephosphorylated and ligated with the wild type *Hind* III/pQMC1 fragment in a ratio of insert: vector, 3:1 and 6:1. The resulting DNA was electroporated into electrocompetent JM109. Transformants were selected with ampicillin. The DNA from 12 colonies was prepared and digested with *BstE* II to discover the orientation of the insert. The possible outcomes of the cloning were identical to that shown in Figure 3.17, except that here the enzyme used was *BstE* II. If the insert had ligated in the correct orientation then bands of approximately 0.4 and 5.6 kb would be observed. However if the insert had ligated in the incorrect orientation then bands of 1.1 and 4.9 kb would be observed. Analysis of the fragments by electrophoresis showed the presence of a single band of

approximately 4.6 kb which corresponded to the size of the pSS3A vector (Figure 3.20, lanes 2 - 13). This suggested that the cloning had been unsuccessful. Miniprep DNA was prepared from a further 12 colonies and digested with *BstE* II but once again only a 4.6 kb band was present (Result not shown) which suggested that the cloning had been unsuccessful.

New stocks of vector and insert were prepared and the cloning was repeated. DNA was prepared from 24 clones and digested with *Bst* E II. Once more the presence of a single 4.6 kb band representing the vector alone showed that the cloning had not been successful (Result not shown).

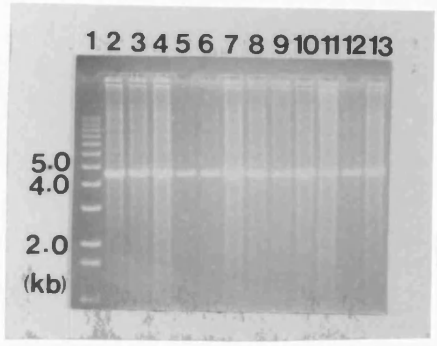


Figure 3.20 *Bst* E II digestion of 12 miniprep DNAs of pSSWTvec
Lanes 1: 1kb DNA ladder. Lanes 2 - 13: minipreps 1 - 12 digested with *Bst* E II.

3.2.3.2 Partial Digestion of pQMC1

Because there seemed to be no obvious explanation why the cloning was unsuccessful, another approach was undertaken; that of partial digestions. The rationale was that if the pQMC1 DNA was digested over a 30 minute period with *Hind* III then at some time during this 30 minutes the DNA will only be digested in one or two of the three *Hind* III sites. This would lead to the formation of fragments with varying sizes (Figure 3.21). If it proved possible to isolate a 6021 bp fragment (which would represent the sum of the 4.6 kb pSS3A and the 1.4 kb *Hind* III/pQMC1 fragments), a self-ligation of this DNA would yield pSSWTvec.

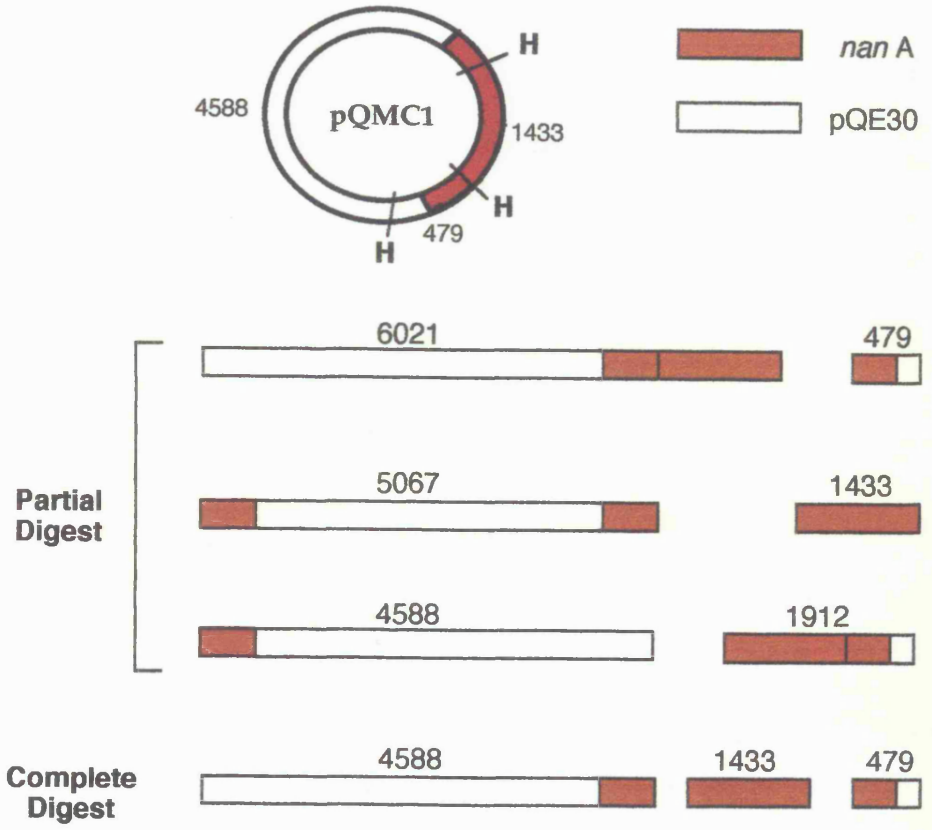


Figure 3.21 Diagrammatic representation of the DNA fragments that could result from the incomplete digestion of pQMC1 with *Hind* III.

To achieve this goal, trial partial digestions were set up to estimate the time required to produce various sized fragments. Several 1µg samples of pQMC1 were digested with *Hind* III for varying periods of time (1, 2, 3, 4, 5, 10, 15, 20 or 30 minutes), after which the cleavage reaction was stopped by the addition of phenol/chloroform. The DNA was ethanol precipitated and analysed on a 0.7% (w/v) agarose gel (Figure 3.22). The required 6021bp fragment was clearly visible after the 30, 20,15 and 10 minute reactions (Figure 3.22 lanes 1, 2, 4 and 5).

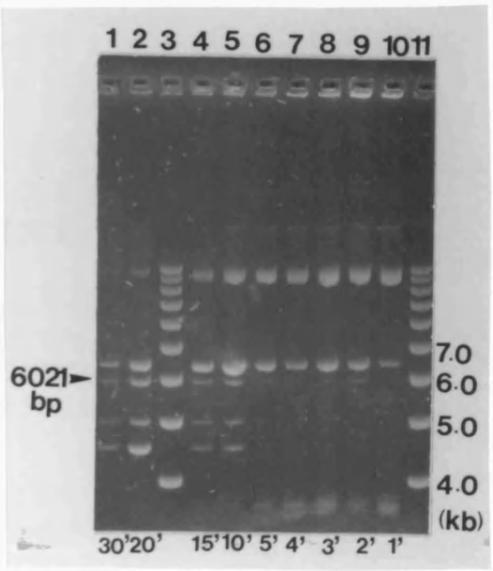


Figure 3.22 Trial partial digestion of pQMC1 with *Hind* III.
Lanes 1 and 2: 30 minutes and 20 minutes digestions of pQMC1 with *Hind* III. Lanes 3 and 11: 1kb DNA ladder. Lanes 4 - 10: Digestion of pQMC1 with *Hind* III for 15, 10, 5, 4, 3, 2 and 1 minutes.

Analysis of the efficiency of the trial digestions suggested that the 20 minute incubation was the most efficient. Therefore two samples of pQMC1 DNA (2.5µg and 5µg) were digested with *Hind* III for 18, 20 and 22 minutes. The resulting fragments are shown in Figure 3.23. The 6021 bp band (very feint) was visible in lanes 7, 9, and 11. The 6021bp bands from lanes 7, 9 and 11 were pooled and gel purified with the Qiaquick gel extraction kit. A self-ligation reaction was set up with this DNA and the ligation mix was transformed into electrocompetent JM109. The ligation and transformations were repeated twice but yielded no colonies.

Due to the inconsistent results of the partial digestion approach and the failure of the self ligation reactions this strategy was abandoned and the initial approach of cloning the *Hind* III/pQMC1 fragment into pSS3A was reattempted.

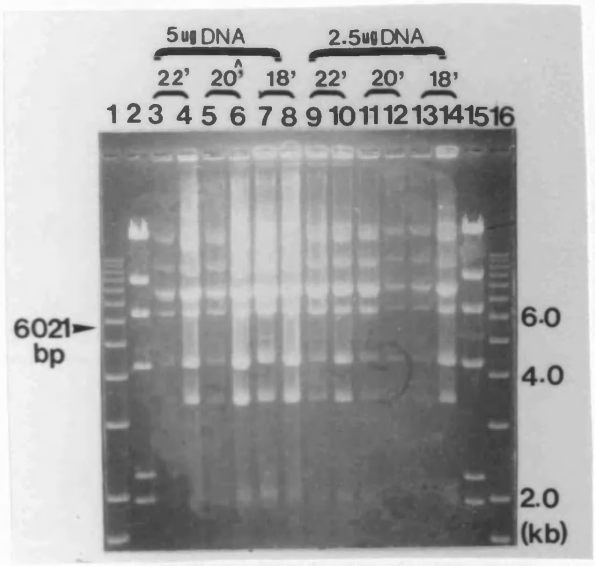


Figure 3.23 Partial digestion of pQMC1 (2.5 and 5µg) with *Hind* III for 18, 20 and 22 minutes. Lanes 3 - 8 contained 5 µg pQMC1 DNA. Lanes 9 - 14 contained 2.5µg of pQMC1 DNA. Lanes 1 and 16: 1 kb DNA ladder. Lanes 2 and 15: λ Hind III marker. Lanes 3, 4, 9 and 10: 22 minutes digestion of pQMC1 with *Hind* III. Lanes 5, 6, 11 and 12: 20 minutes digestion of pQMC1 with *Hind* III. Lanes 7, 8, 13 and 14: 18 minutes digestion of pQMC1 with *Hind* III.

3.2.3.3 Cloning of the *Hind* III/pQMC1 fragment into pSS3A (Second attempt).

The failure of previous attempts at cloning the 1.4 kb wild type *Hind* III/pQMC1 fragment into pSS3A (Section 3.2.3.1) may have been linked to the use of the Qiaquick gel extraction kit. Other workers in the laboratory had communicated difficulties in cloning DNA that had been purified using the Qiaquick gel extraction kit. It was therefore decided to digest the pSS3A DNA (17µg) with *Hind* III and dephosphorylate it without an intermediate Qiaquick gel purification stage. Ligation reactions were set up between vector prepared in this way and the *Hind* III/pQMC1 prepared as in section 3.1.3.1. The ligation reactions were transformed into JM109 cells. DNA was prepared from 12 colonies and cleaved in separate reactions with *Hind* III and *Pvu* I (Figures 3.24 and 3.25). Analysis of the *Hind* III cleavage products revealed the presence of approximately 1.4 and 4.6 kb bands (Figure 3.24, lanes 1 - 12, not 4) which represented the insert and vector respectively, indicating that the cloning was successful. Clone 4 was an exception and did not produce the anticipated bands (Figure 3.24, lane 4). Restriction analysis with *Pvu* I would reveal the orientation of the insert as shown previously in Figure 3.17 . Clones 2, 3, 6 and 9 -11 had the insert in the correct orientation and produced bands of 2.0 and 4.0 kb (Figure 3.25, lanes 2, 3, 6 and 9 -11 respectively). The ligation of the insert in the incorrect orientation resulted in bands of about 2.7 and 3.3 kb as evidenced by clones 1, 5, 7, 8 and 12 (Figure 3.25 lanes 1, 5, 7, 8 and 12 respectively). Clone number 3 was chosen for future work and designated pSSWTvec, it consisted of the *Hind* III/pQMC1 fragment ligated into pSS3A in the correct orientation.

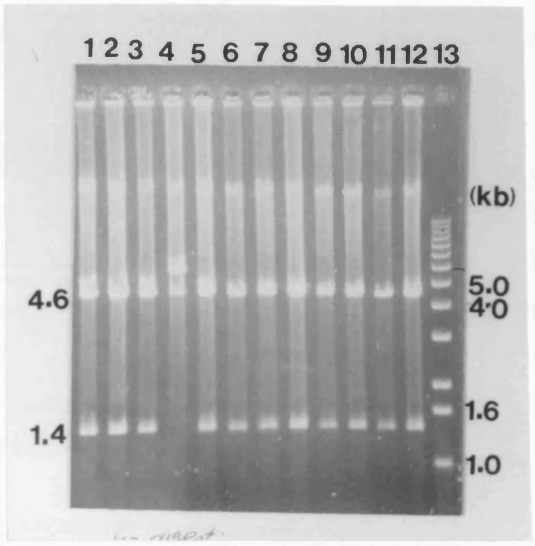


Figure 3.24 Cleavage of miniprep DNAs of pSSWTvec with *Hind* III.
The *Hind* III/pQMC1 fragment was cloned into pSS3A. Small scale DNA was prepared from 12 transformants, cleaved with *Hind* III and the resulting fragments analysed by electrophoresis.
Lanes 1 - 12: minipreps 1 - 12 cleaved with *Hind* III. Lane 13: 1kb DNA ladder.

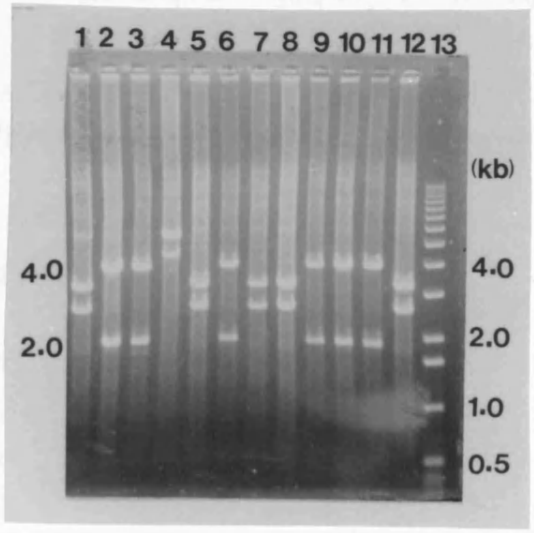


Figure 3.25 Cleavage of miniprep DNAs of pSSWTvec with *Pvu* I.
The *Hind* III/pQMC1 fragment was cloned into pSS3A. Small scale DNA was prepared from 12 transformants, cleaved with *Pvu* I and the resulting fragments analysed by electrophoresis.
Lanes 1 - 12: minipreps 1 - 12 cleaved with *Pvu* I. Lane 13: 1kb DNA ladder

3.2.4 Mutagenesis of Arginine (CGT) to Histidine (CAT) at position 663 in pSS2A.

The mutagenesis of arginine (CGT) to histidine (CAT) at position 663 (protein sequence) in pSS2A, followed by cloning of the mutated *Hind* III/pQMC1 fragment, first into pCR-Script™ SK (+) vector and then into the pQE30 expression vector, was performed in exactly the same manner as described in sections 3.2.2, 3.2.2.3 and 3.2.2.4 respectively. The relevant details of the construction of the arginine, position 663 to histidine neuraminidase mutant are summarised below.

3.2.4.1 Primary PCR

3.2.4.1.1 Left primer mutagenesis

The primers M13 Reverse and A663H2 (Section 2.2.14.2) were used to amplify a fragment of approximately 885 bp from pSS2A. The primer A663H2 was mismatched from the target sequence at position 663 (G>A). The 885 bp "left" mutated PCR product was visualised on a 0.7% (w/v) agarose gel (Figure 3.26, lanes 7,8 and 9) and gel purified using the Bandprep kit. No bands were present in the "left" PCR negative control (Figure 3.26 lanes 2 and 3) confirming the success of the PCR.

3.2.4.1.2 Right primer mutagenesis

An approximately 700 bp fragment of DNA was amplified using the primers M13 Forward and A663H1. The primer A663H1 was mismatched from the target sequence at position 663 (C>T). The "Right" mutated PCR product was visualised on a 0.7% (w/v) agarose gel (Figure 3.26, lanes 10, and 12) and gel purified using the Bandprep kit. No bands were evident in the "right" PCR negative control (Figure 3.26 lanes 4 and 5).

3.2.4.2 Secondary (Recombinant) PCR.

The gel purified products of the "left" and "right" primary PCRs (6µl of each) were used as the template in a secondary PCR to produce the full length mutated product. The primers used were M13 Reverse and M13 Forward. The product of the secondary PCR (approximately 1.5 kb) was analysed on a 0.7%

(w/v) agarose gel (Figure 3.27, lane 4), gel purified using the bandprep kit, then cloned into the pCR-Script™ SK (+) cloning vector.

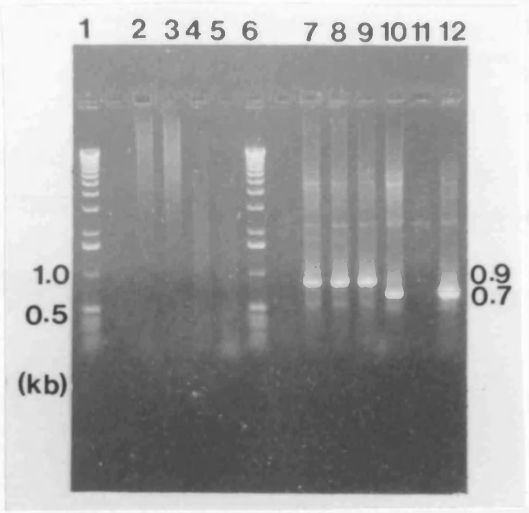


Figure 3.26 Products of "left" and "right" primary PCRs of pSS2A.
Plasmid pSS2A was amplified using primers A663H2 and M13 Reverse to produce the "left" PCR product and with primers A663H1 and M13 Forward to produce the "right" PCR poduct.
Lanes 1 and 6: 1kb DNA ladder. Lanes 2 and 3: "left" PCR negative control. Lanes 4 and 5: "right" PCR negative control. Lanes 7 - 9: 885 bp "left" PCR product (A663H2 and M13 Reverse). Lanes 10 - 12: 700 bp "right" PCR product (A663H1 and M13 Forward).

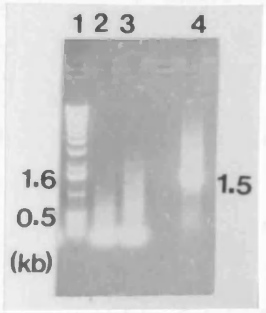


Figure 3.27 Analysis of the R_{663}>H recombinant PCR product.
The product of the primary PCRs were purified from the agarose gel, combined and amplified with the "outside" primers M13 Forward and M13 Reverse.
Lane1: 1kb DNA ladder. Lanes 2 and 3: negative control. Lane 4: 1555 bp full length recombinant PCR product (Primers M13 Forward and M13 Reverse).

3.2.4.3 Cloning of the mutated PCR product into the pCR-Script cloning vector.

As described in section 3.2.2.3, 3µl of the gel purified mutated PCR product was cloned into pCR-Script™ SK (+) cloning vector.

50 white colonies were screened by colony PCR using the primers M13 Reverse and M13 Forward on the standard amplification program. The DNA from 21 colonies produced a PCR product of approximately 1.5 kb. Plasmid DNA from 12 of these colonies was prepared and subjected to restriction analysis. The DNA was digested with *Pvu* I and the resulting fragments analysed by gel electrophoresis (Figure 3.28).

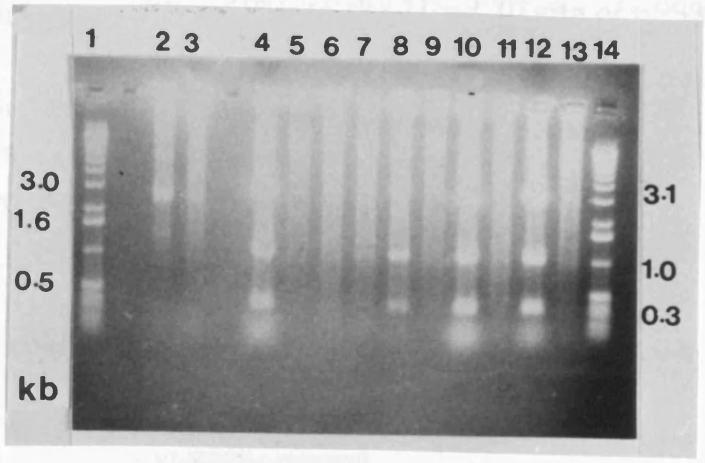


Figure 3.28 *Pvu* I digestion of 12 minipreps of pSSRHscript.
The recombinant PCR product (R_{663}>H) was cloned into the pCR-Script™ SK (+) vector. Small scale DNA preparations were done from 12 clones, digested with *Pvu* I and the resulting fragments analysed by gel electrophoresis.
Lanes 1 and 14: 1kb DNA ladder. Lanes 2 - 13: minipreps 1 - 12 cleaved with *Pvu* I.

The possible orientations of the insert were the same as those shown previously in Figure 3.12. However in this case the cleavage was done with *Pvu* I This would produce fragments of 1.7, 1.8 and 1.0 kb for an insert cloned in the "orientation A" and fragments of 3.1 and 1.0 and 0.3, kb for an insert cloned in "orientation B". The insert cloned in either direction is suitable at this stage.

Four out of twelve clones (clones 3, 7, 9 and 11 produced bands of about 3.1, 1.0 and 0.3 kb (Figure 3.28, lanes 4, 8, 10 and 12) indicating that the insert was in "orientation B". The other eight clones (1, 2, 4, 5, 6, 8, 10 and 12) produced very

feint or no bands at all, (Figure 3.28, lanes 2, 3, 5, 6, 7, 9, 11 and 13 respectively) and were excluded from further study.

Clone 9 representing the 1.4 kb *Hind* III/pQMC1 fragment mutated at position 663 (R>H), flanked by short stretches of pUC18 DNA ligated into the pCR-Script™ SK (+) vector was designated pSSRHscript. Large amounts of pSSRHscript DNA were purified by the caesium chloride method and the presence and orientation of the insert was confirmed by restriction digestion with *Pvu* I (Results not shown). As observed with the miniprep digestions, the large scale DNA also produced the expected fragments of 3.1, 1.0 and 0.3 kb upon cleavage with *Pvu* I.

3.2.4.4 Cloning of the *Hind* III/pQMC1 fragment from pSSRHscript containing the (R>H) mutation at position 663 into the *Hind* III site of pSS3A.

Plasmid pSSRHscript (30µg) was digested with *Hind* III (Figure 3.29). The resulting 1.4 kb mutated *Hind* III/pQMC1 fragment was purified from the gel using the bandprep gel extraction kit (Section 2.2.2.1).

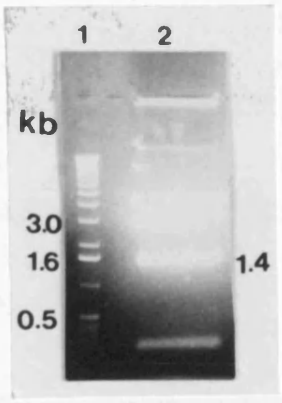


Figure 3.29 *Hind* III cleavage of pSSRHscript.
pSSRHscript was digested with *Hind* III and the 1.4 kb fragment was gel purified.
Lane 1: 1 kb DNA ladder, Lane 2: *Hind* III cleaved pSSRHscript.

The plasmid pSS3A was previously cleaved with *Hind* III and dephosphorylated as described in section 3.2.3.3. Ligation reactions were set up between the 1.4 kb mutated *Hind* III/pQMC1 fragment and the *Hind* III cleaved pSS3A.

Miniprep DNA was prepared from 12 transformants and digested with *Hind* III to check the efficiency of the cloning (Figure 3.30) and with *Pvu* I to identify the orientation of the insert (Figure 3.31).

Digestion with *Hind* III produced bands of 1.4 and 4.6 kb from all clones confirming the success of the cloning (Figure 3.30, lanes 2 - 13).

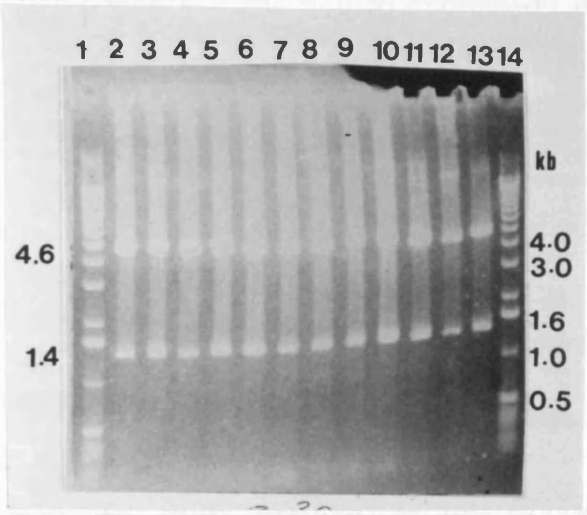


Figure 3.30 Cleavage of 12 minipreps of pSSRHvec with *Hind* III.
The *Hind* III/pQMC1 fragment was isolated from pSSRHscript and cloned into pSS3A. Small scale DNA was prepared from 12 clones, digested with *Hind* III and the resulting fragments analysed by gel electrophoresis.
Lanes 1 and 14: 1 kb DNA ladder. Lanes 2 - 13: minipreps 1 - 12 cleaved with *Hind* III.

Digestion with *Pvu* I revealed the orientation of the insert. The two potential orientations of the insert were identical to that shown in Figure 3.17. The *Pvu* I digestion produced fragments of 2.0 and 4.0 kb for clones that contained the insert in the correct orientation clones 2 and 6 (Figure 3.31, lanes 3 and 7 respectively). Clones containing the insert in the incorrect orientation produced fragments of 2.7 and 3.3 kb. These were clones 1, 3 - 5, 7 - 12 (Figure 3.31, lanes 2, 4 - 6, 8 - 13 repectively).

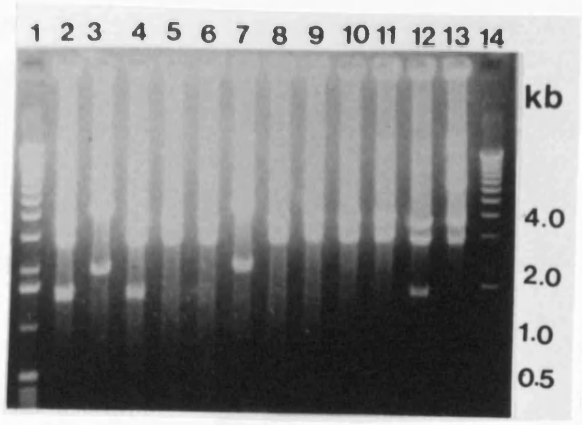


Figure 3.31 Digestion of 12 minipreps of pSSRHvec with *Pvu* I.
The *Hind* III/pQMC1 fragment isolated from pSSRHscript was cloned into pSS3A. Small scale DNA was prepared from 12 clones and digested with *Pvu* I. Analysis of the resulting fragments by gel electrophoresis revealed the orientation of the insert.
Lanes 1 and 14: 1kb DNA ladder. Lanes 2 - 13: minipreps 1 - 12 digested with *Pvu* I.

Clone 2 was designated pSSRHvec. It represents the the *Hind* III/pQMC1 fragment of *nan* A mutated at position 663 (R>H) and then cloned into the plasmid pSS3A which consists of the pQE30 expression vector and 5' end of the neuraminidase structural gene. This plasmid pSSRHvec was transformed into DH5α cells to facilitate DNA sequencing (Section 3.3) and into SG13009:pREP 4 for protein expression and subsequent purification studies (Chapter 4).

3.2.5 Mutagenesis of tyrosine (TAT) to phenylalanine (TTT) at position 752 in pSS2A.

The mutagenesis of tyrosine (TAT) to phenylalanine (CAT) at position 752 in pSS2A, followed by cloning of the mutated *Hind* III/pQMC1 fragment into pCR-Script™ SK (+) vector then into the expression vector pQE30 was performed in exactly the same manner as the previous glutamic acid to glutamine mutation at position 647 (Sections 3.2.2, 3.2.2.3 and 3.2.2.4 respectively). The relevant results for the mutation of tyrosine 752 to phenylalanine and the subsequent cloning steps are shown below.

The work in sections 3.2.5.1, 3.2.5.2 and 3.2.5.3 was performed by Denise Atkinson, a final year undergraduate student, under my supervision.

3.2.5.1 Primary PCR

3.2.5.1.1 Left primer mutagenesis

A fragment of approximately 1.1 kb was amplified from pSS2A using the primers M13 Reverse and Y752F2, the latter was mismatched from the target sequence at position 752 (A>T). The "left" mutated PCR product was visualised on a 0.7% (w/v) agarose gel (Figure 3.32, lane 3) and gel purified using the Bandprep kit.

3.2.5.1.2 Right primer mutagenesis

The primers M13 Forward and Y752F1 were used with *Vent* polymerase under standard PCR conditions to amplify a fragment of approximately 0.4 kb from pSS2A. The primer Y752F1 was mismatched from the target sequence at position 752 (T>A). The "Right" mutated PCR product was visualised on a 0.7% agarose gel (Figure 3.32, lane 2) and gel purified as described previously (2.2.2.1).

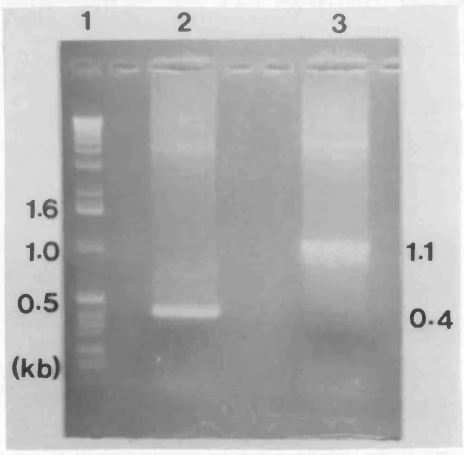


Figure 3.32 Products of "left" and "right" primary PCRs of plasmid pSS2A.
The plasmid pSS2A was amplified using primers Y752F1 and M13 Forward to produce the "right" PCR product, and with primers Y752F2 and M13 Reverse to produce the "left" PCR product. Lane 1: 1 kb DNA ladder . Lane 2: 426 bp "right" PCR product (Y752F1 and M13 Forward). Lane 3: 1154 bp "left" PCR product (Y752F2 and M13 Reverse).

3.2.5.2 Secondary (Recombinant) PCR.

The gel purified products of the "left" and "right" primary PCRs (6µl of each) were used as the template in a secondary PCR to produce the full length mutated product (Figure 3.33). The band of approximately 1.5 kb (Figure 3.33, lane 3) was extracted from the gel by the bandprep kit and then cloned into the pCR-Script™ SK (+) cloning vector. The negative control (Figure 3.33, lane 2) produced no bands.

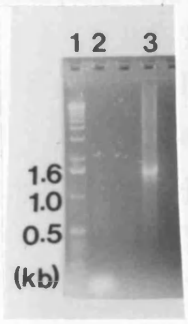


Figure 3.33 Analysis of the Y_{752}>F recombinant PCR product.
The primary PCR products were gel purified, combined and amplified using the primers M13 Forward and M13 Reverse to produce the full length recombinant PCR product. Lane 1: 1kb DNA ladder. Lane 2: negative control. Lane 3: 1555 bp full length recombinant PCR product (Primers M13 Forward and M13 Reverse).

3.2.5.3 Cloning of the mutated PCR product into the pCR-Script cloning vector.

Cloning into the pCR-Script™ SK (+) cloning vector was performed according to the manufacturer's instructions. The gel purified PCR product (3µl) was ligated with 10ng of *Srf* I-digested pCR-Script™ SK (+). Seven transformants were screened by colony PCR (Section 2.2.13) using the primers M13 Reverse and M13 Forward on the standard amplification program. Three transformants (termed 1,2 and 3) produced a PCR product of approximately 1.5 kb. Plasmid DNA was prepared from these colonies and linearised with *Bst E* II. All clones produced a single band of about 4.5 kb (Figure 3.34, lanes 5, 6 and 7) confirming that a single fragment had ligated into the pCR-Script™ SK (+) cloning vector.

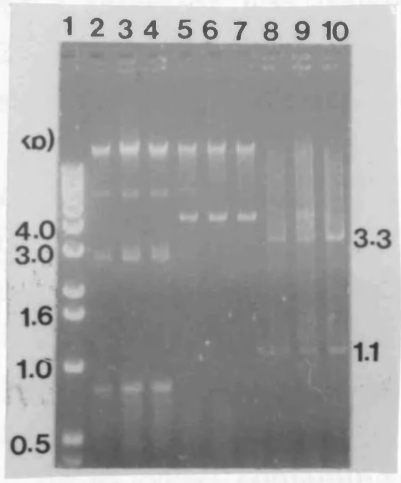


Figure 3.34 Cleavage of minipreps 1, 2 and 3 with *Not* I and *BstE* II Lane 1: 1kb DNA ladder. Lanes 2 - 4: undigested DNAs 1, 2 and 3 respectively. Lanes 5 - 7: *BstE* II cleaved DNAs 1, 2 and 3 respectively. Lanes 8 - 10: *Not* I and *BstE* II cleavage of miniprep DNAs 1 - 3 respectively.

To ascertain the direction of cloning the DNA was digested with *Not* I and *BstE* II. The possible orientations of the insert were the same as those shown previously in Figure 3.12 for the E_{647}>Q PCR product. If the insert had ligated into the pCR-Script™ SK (+) cloning vector in "orientation A" then DNA fragments of 4085 and 431 bp would be expected upon digestion with *Not* I and *BstE* II. However, if the insert had ligated into the vector in "orientation B" then bands of 3364 and 1152 would be observed. Either orientation of cloning is acceptable at this stage.

The cleavage of DNA from all three transformants produced bands of approximately 3.3 and 1.1 kb (Figure 3.34, lanes 8,9 and 10) which was consistent with the insert being in "orientation B".

Clone 1 was designated pDAYFscript. It represented the 1433 bp *Hind* III/pQMC1 fragment mutated at position 752 (A>T), flanked by short stretches of pUC18 DNA ligated into the pCR-Script™ SK (+) vector in "orienation B". Large quantities of pDAYFscript DNA was purified by the caesium chloride method (Section 2.2.10.2.1) and checked by restriction digestion with *Not* I and *BstE* II (Results not shown) prior to cloning into pSS3A. Cleavage with *Not* I and *BstE* II produced the expected fragments of 3364 and 1152 bp.

3.2.5.4 Cloning of the *Hind* III/pQMC1 fragment from pDAYFscript containing the (Y>F) mutation at position 752 into the *Hind* III site of pSS3A.

Plasmid pDAYFscript was digested with *Hind* III and the resulting 1.4 kb mutated *Hind* III/pQMC1 fragment was isolated using the bandprep gel extraction kit (Section 2.2.2.1). The mutated *Hind* III/pQMC1 fragment was ligated with *Hind* III cleaved pSS3A prepared in section 3.2.3.3.

Plasmid DNA was prepared from 12 transformants and digested with *Pvu* I to reveal the orientation of the insert. The two potential orientations of the insert were identical to that shown in Figure 3.17. The *Pvu* I digestion produced fragments of 2.0 and 4.0 kb for clones 1, 4, 5, 10 and 12 (Figure 3.35, lanes 2, 5, 6, 11 and 13 respectively) that contained the insert in the correct orientation. Bands of 2.7 and 3.3 were produced from clones 2, 3, 6 - 9 and 11 (Figure 3.35, lanes 3, 4, 7 - 10, and 12 respectively) containing the insert in the incorrect orientation.

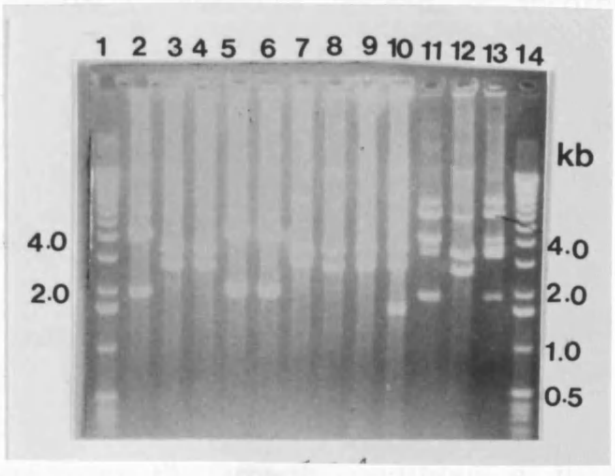


Figure 3.35 Digestion of 12 miniprep DNAs of pSSYFvec with *Pvu* I.
The *Hind* III/pQMC1 fragment isolated from pDAYFscript was cloned into pSS3A. Small scale DNA was prepared from 12 clones and digested with *Pvu* I. Analysis of the resulting fragments by gel electrophoresis revealed the orientation of the insert.
Lanes 1 and 14 : 1kb DNA ladder. Lanes 2 - 13: minipreps 1 - 12 digested with *Pvu* I.

Clone 10 was designated pSSYFvec. It represented the DNA encoding the neuraminidase structural gene containing a TAT > TTT mutation (phenylalanine to tyrosine) at position 752 (protein sequence) in the expression vector pQE30.

The plasmid pSSYFvec was transformed into DH5α cells to facilitate DNA sequencing (Section 3.3) and into SG13009:pREP 4 for protein expression and subsequent purification studies (Chapter 4).

3.3 DNA sequence analysis of mutated neuraminidases

3.3.1 Introduction

In order to confirm the presence of the mutations introduced into pSSEQvec, pSSRHvec and pSSYFvec by mismatch PCR in sections 3.2.2, 3.2.4 and 3.2.5, it was necessary to sequence these constructs. The 1.4 kb *Hind* III/pQMC1 fragment of neuraminidase DNA in these constructs was sequenced because that was the region that underwent PCR amplification during site-directed mutagenesis.

DNA sequence analysis of the mutated neuraminidase genes was done using the ABI PRISM dRhodamine Terminator Cycle Sequencing Ready Reaction Kit from Perkin Elmer as described in section 2.2.11.2. Automated sequencing is based on the same principle as the chain termination DNA sequencing method (Sanger *et al* ., 1977). However instead of incorporating radiolabelled nucleotides for visualization of the labeled chains by autoradiography, automated sequencing utilizes fluorescene labelled dye terminators which are then resolved by an ABI PRISM 377 DNA Sequencer. The benefits of this method include single-tube reactions for all terminators and a greater reproducibility of results.

The purity of the DNA template is critical for automated sequencing (Dusterhöft, 1998). An important initial factor in plasmid template purification is the quality of the culture from which the DNA is extracted. Two areas of particular concern were the growth conditions of the culture and the host strain (Düsterhöft, 1998). Bacterial cultures for plasmid preparation were started from fresh colonies and grown on selective media to a cell density of approximately 1.5 - 4.0 x 10^9 cell/ml (OD_{600} 1.4 - 4 units/ml). The medium of choice for bacterial growth was LB. *E. coli* grown in LB medium reaches the

stationary phase after 10-12 hours and continues to grow 4-5 hours before cells begin to lyse. However with an enriched medium such as Terrific broth (TB), stationary phase is reached earlier (8 hours) and ends sooner. Therfore an overnight culture grown in LB will contain fewer lysed cells than a comparable TB culture (Düsterhöft, 1998). Lysed cells release degraded genomic and plasmid DNA and yield plasmid DNA of a lower quality (Düsterhöft, 1998). The host strain for propagation of plasmids was changed from JM109 to DH5α because the former strain contains large amounts of carbohydrates which are released during lysis. JM109 also has an intact endA locus and produces large amounts of nucleases (Sambrook *et al* ., 1989). The bacterial strain for propagation was changed by transforming DNA from constructs pSSEQvec, pSSRHvec and pSSYFvec into DH5α competent cells as described in section 2.2.9.2.

Highly pure DNA for sequencing was obtained using the Qiagen maxiprep kits (Section 2.2.10.2.2). The use of the Qiagen anion exchange resin enabled the removal of impurities such as RNA, protein, carbohydrates and small metabolites (Düsterhöft, 1998).

The sequencing reactions were performed according to the instructions of the kit manufacturer (Perkin-Elmer). Each construct was sequenced in both the forward and reverse directions. The mutated region in each construct was sequenced twice in both directions. The primers used in sequencing and their positions relative to the mutations are shown in Figure 3.36.

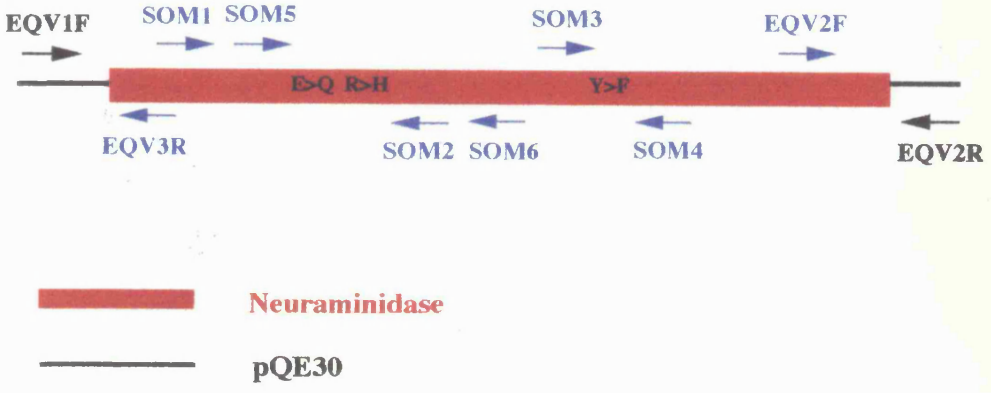


Figure 3.36 Position of sequencing primers relative to the mutations in pSSEQvec, pSSRHvec and pSSYFvec plasmids.

The results obtained from the Leicester University seqencing service were in the form of a chromatogram of the fluorescent output and a sequence based on this chromatogram. Any ambiguities in the sequence were designated by an "N". This sequence was aligned with the reference seqence using the SEQUENCE NAVIGATOR software package (Applied Bioystems). Any areas of sequence mismatch could usually be resolved by examination of the corresponding chromatogram.

3.3.2 Sequencing of plasmid pSSEQvec

The presence of the E>Q mutation, a change from GAA to CAA at position 647 (protein), was confirmed by sequencing with primers SOM1 in the forward direction and by SOM2 in the reverse direction. The chromatogram depicting this change is shown in Figure 3.37 alongside a chromatogram showing the corresponding wild-type sequence in pSSWTvec. No other changes were detected in the remainder of the*Hind* III/pQMC1 region. Therefore the pSSEQvec construct contained only the desired mutation of E>Q at position 647.

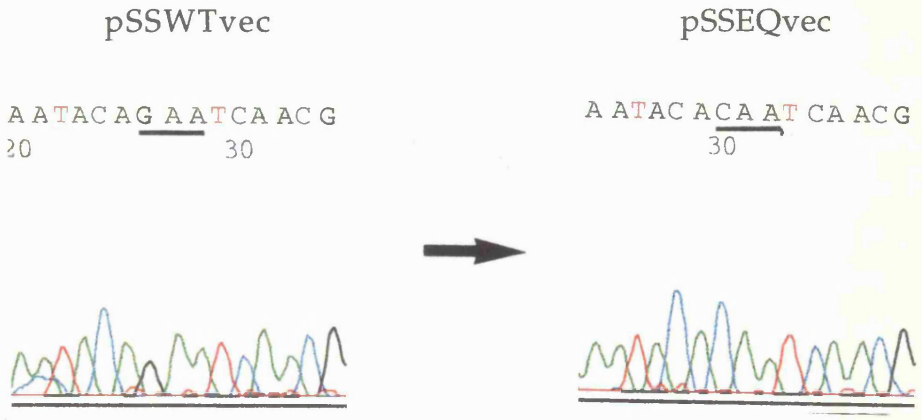


Figure 3.37 Chromatograms of pSSWTvec and pSSEQvec.

3.3.3 Sequencing of plasmid pSSRHvec

The presence of the R>H mutation, a change from CGT to CAT at position 663 (protein), was confirmed by sequencing with primers SOM5 in the forward direction and by EQV2R in the reverse direction. A chromatogram showing this change alongside the corresponding unmutated sequence is ahown in Figure 3.38. One other change at position 449 (protein), 642 bases upstream

from the R>H mutation was observed when sequencing with primers EQV1F and SOM6. The change of bases AAA to AGA resulted in a change of lysine to aginine. Both of these amino acids are basic and have a similar structure. Therefore the effect of the R>H mutation on the function of the neuraminidase cannot be considered in isolation. The lysine to arginine secondary change may also have a role.

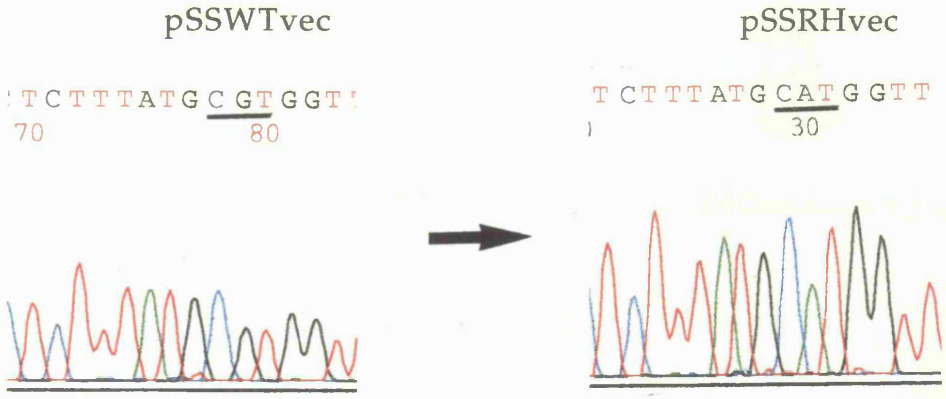


Figure 3.38 Chromatograms of pSSWTvec and pSSRHvec.

3.3.4 Sequencing of plasmid pSSYFvec

The presence of the expected Y>F mutation (TAT to TTT) at position 752 (protein), was confirmed by sequencing with primers SOM3 and EQV2R in the forward and reverse directions respectively. A chromatogram showing this change alongside the wild-type sequence in pSSWTvec is shown in Figure 3.39. Additionally another unexpected sequence change was observed at position 812 (protein) when sequencing with primers SOM5 and EQV2R. This was a non-coding change of ATT (isoleucine) to ATA (isoleucine). Therefore only the desired mutation of Y>F (protein position 752) was present in the construct pSSYFvec.

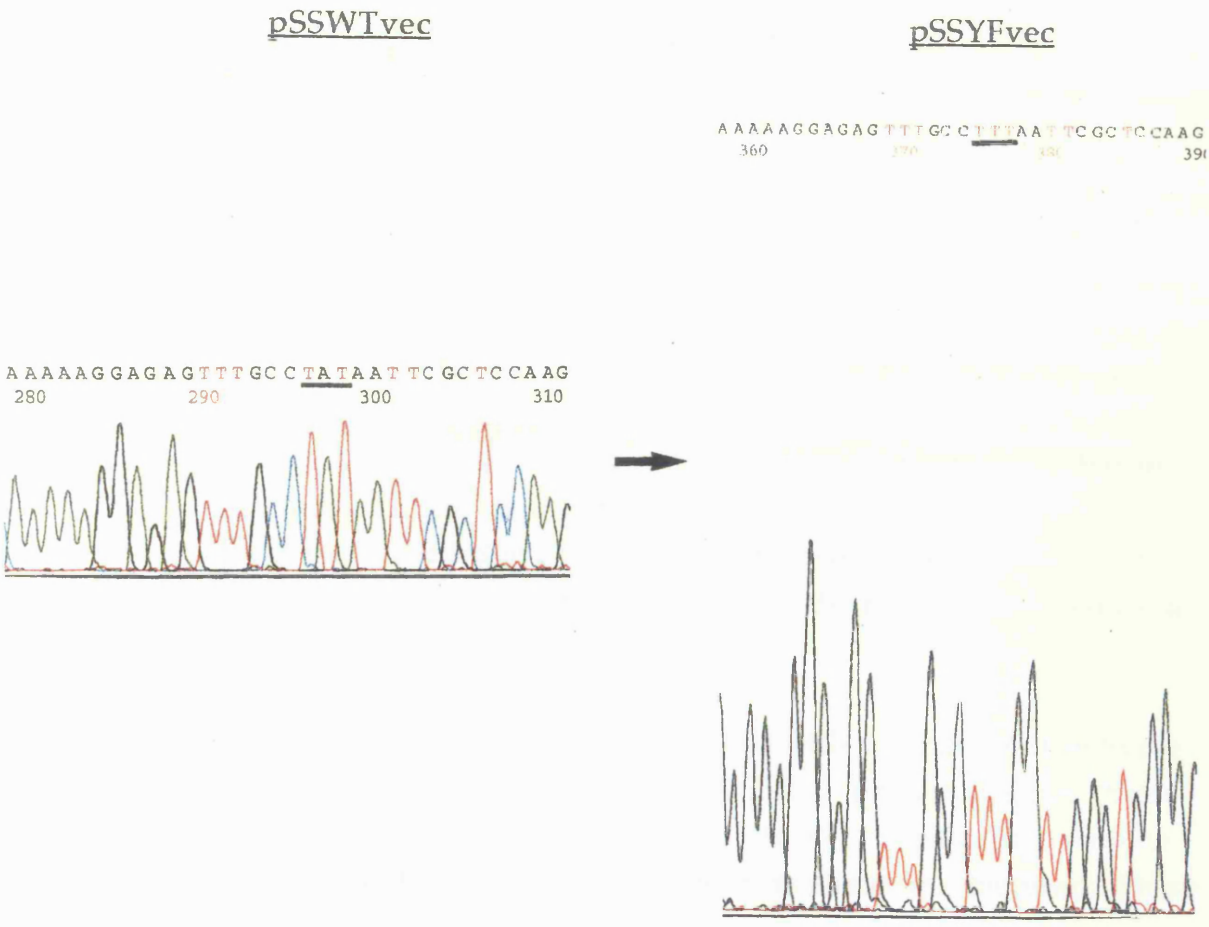


Figure 3.39. Chromatograms of pSSWTvec and pSSYFvec

Chapter 4: Protein expression and purification

4.1 Introduction

In order to study the structure and function of neuraminidase and to allow examination of the *in vivo* protection effects of mutated forms of the protein, purification of the enzyme was necessary. Previous methods of neuraminidase isolation have involved numerous chromatography steps, such as ion exchange and gel permeation (Stahl and O'Toole, 1972; Lock *et al* ., 1988). In addition to being time consuming, a disadvantage of having multiple steps in a purification scheme is the effect of loss at each stage on the final yield of the end product. The effects of recovery at each stage on the final yield are cumulative, so it is desirable to keep the number of separation steps to a minimum (Harris, 1995).

Previous attempts to purify neuraminidase had little success, and enzyme preparations were contaminated by many different protein species (Stahl and O'Toole, 1972; Tanenbaum, 1970). A more efficient purification scheme, with fewer steps than the existing protocols, was therefore required. This was attempted by use of a commercial protein expression and purification system; QIAexpress (Qiagen, Germany). The QIAexpress system consists of a high-level expression vector (pQE vector) and a nickel nitrilo-tri-acetic acid resin (Ni-NTA, Qiagen). The pQE expression vectors provide high-level expression in *E. coli* of proteins possessing a tag of 6 consecutive histidines (6xHis). The Ni-NTA resin has a high affinity for proteins or peptides that contain a 6xHis affinity tag at either their N- or C- terminus (Hochuli *et al* ., 1988). The combined benefits of a high-level bacterial expression system and the high affinity Ni-NTA resin enables single-step purification of recombinant proteins under either native or denaturing conditions.

Accordingly the *nanA* gene was cloned into the pQE30 expression vector. This step attaches a 6xHis tag on to the N-terminus of the protein and enables purification by affinity chromatography on Ni-NTA resin. The expression and purification system are described in greater detail below.

4.2 Protein expression and purification system

4.2.1 The QIAexpress pQE30 expression vector

Plasmid pQE30 belongs to the pDS family of plasmids (Bujard *et al.*, 1987) and were derived from plasmids pDS56/RBSII and pDS781/RBII-DHFRS (Stüber *et al* ., 1990). The map and characteristics of plasmid pQE30 is shown in Figure 4.1.

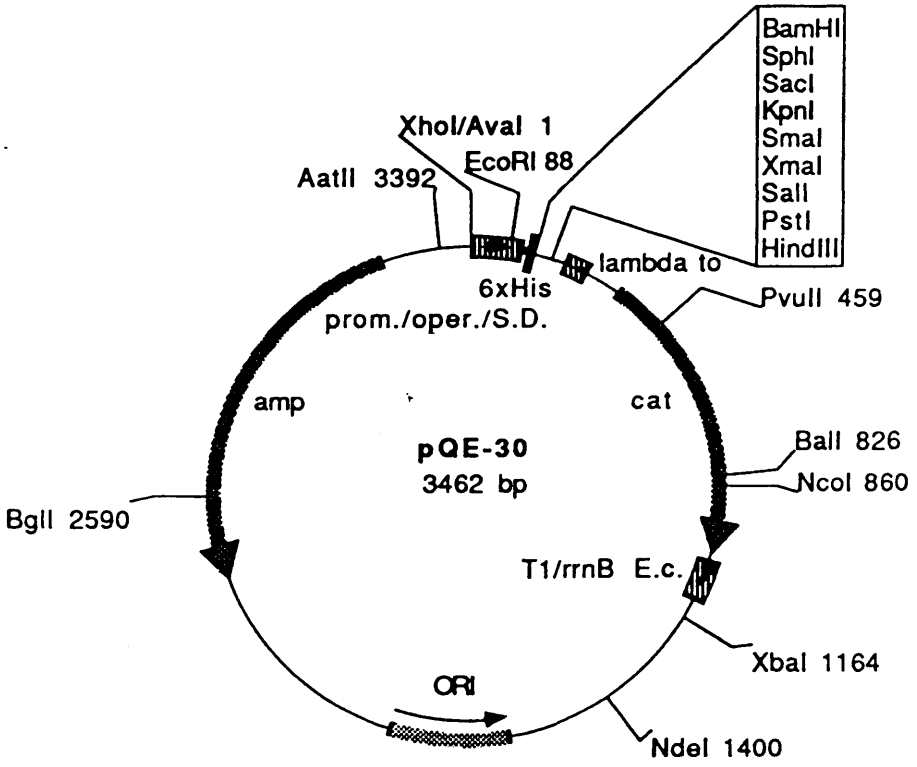
4.2.2 Regulation of expression

The *E.coli* host cells in the QIAexpress system contain multiple copies of the plasmid pREP4, which carries the *lac I* gene encoding the lac repressor (Farabaugh, 1978). Expression from the promoter/operator region (which contains two *lac* operator sequences and the phage T5 promoter) is highly efficient and can only be prevented by the presence of high levels of *lac* repressor. The presence of multiple copies of the pREP4 plasmid ensure high levels of *lac* repressor and tight regulation of protein expression. Under these conditions, expression from the pQE30 vector requires induction by the addition of IPTG ("The QIAexpressionist", Qiagen, 1992).

4.2.3 The Ni-NTA resin

Metal chelate affinity chromatography has become a widely used protein purification technique. The NTA resin possesses four chelating sites which can interact with metal ions. When the NTA resin is charged with Ni^{2+}, the NTA occupies four of the six ligand binding sites in the coordination sphere of the Ni^{2+} ion, and two sites are left free to interact with the 6xHis tag. This enables the NTA resin to bind metal ions more stably than other chelating resins (Hochuli, 1989). The high affinity of the Ni-NTA resin for the 6xHis tag allows the purification of proteins from less than 1% originally to greater than 95% homogeneity (Janknecht *et al* ., 1991). Elution of affinity - tagged proteins from the resin can be achieved by lowering the pH to protonate histidine residues, or by the addition of imidazole, which competes with the tagged proteins for binding sites on the Ni-NTA resin. The latter method requires milder elution conditions and is more suited to proteins susceptible to denaturation by low pH.

Restriction map of plasmid pQE 30



Features of the expression vector

- A regulable promoter/operator element containing the *E. coli* phage T5 promoter and two *lac* operator sequences.
- A synthetic ribosome binding site (RBS II).
- Coding sequence for 6xHis affinity tag.
- A multiple cloning site.
- Translation stop codon.
- The transcriptional terminator "t_o" from phage lambda (Schwarz *et al* ., 1987).
- Replication region and ß - lactamase gene from plasmid pBR322 (Sutcliffe, 1979).

Figure 4.1 Restriction map and characteristics of plasmid pQE30 (Adapted form "The QIAexpressionist, Qiagen, 1992)".
The *nan* A gene from *Streptococcus pneumoniae* was cloned into the *Bam* HI/ *Sal* I site of pQE30.

4.2.4 The 6xHis affinity tag

A number of affinity tags exist which can be fused to the ends of expressed proteins, for example glutathione-S-transferase (Smith and Johnson, 1988), maltose-binding protein (Maina *et al* ., 1988) and thioredoxin (LaVallie *et al* ., 1983). However due to their large size, these tags have the potential to interfere with the function of the protein of interest (LaVallie, 1995). Proteolytic removal of the tags is therefore a necessary step in the purification of recombinant proteins of this type. This is both time-consuming, and often inefficient. In contrast, the affinity tag that binds the Ni-NTA resin consists of only six additional residues and therefore it is less likely to affect protein activity ("The QIAexpressionist", Qiagen, 1992). The 6xHis affinity tag is poorly immunogenic in all species tested except some monkeys. It is uncharged at physiological pH and generally does not affect the secretion, compartmentalization, or folding of the attached protein ("The QIAexpressionist", Qiagen, 1992). Studies at Hoffmann-La Roche have shown that the 6xHis tag does not interfere with the structure and function of various purified proteins, including enzymes (Döbeli *et al* ., 1990) and transcription factors (Janknecht *et al* ., 1991). It was therefore not deemed necessary to remove the 6xHis tag from the recombinant neuraminidase after Ni-NTA resin purification.

4.2.5 Cloning of *nanA* into the pQE 30 expression vector

The work described below was done by M.Camara at Leicester University (Camara *et al* ., 1991; Camara, 1992; Camara *et al* ., 1994) and is described here to establish a background to the project.

The construction of the plasmid pMC4170 which contained the *nanA* structural gene was described in Section 3.1.1. The full length, predicted neuraminidase structural gene (*nanA*) was amplified by PCR from pMC4170. The amplified DNA was cloned into the *Bam* H I/*Sal* I cloning site of the expression vector pQE30 (Camara *et al* ., 1994). This resulted in the attachment of a 6xHis affinity tag to the N-terminus of the protein (Camara *et al* ., 1994). The resulting construct was designated pQMC1. The N-terminus was chosen in preference to the C-terminus for attachment of the 6 x His affinity tag, because amino terminally tagged proteins are expressed 2-4 times more efficiently than proteins with a C-terminal affinity tag ("The

QIAexpressionist", Qiagen, 1992). The *nanA* gene was subcloned in the same reading frame as the 6 x His affinity tag.

The pQE expression construct, pQMC1 was transformed into *E. coli* SG13009 [pREP4] which carried the pREP4 repressor plasmid for control of expression.

4.2.6 An overview of the expression and purification of the neuraminidase from pQE constructs

For *in vivo* expression of neuraminidase the pQE expression construct was transformed into the *E.coli* SG13009:pREP4 and grown overnight in LB medium containing 100μg/ml ampicillin and 25μg/ml kanamycin, in order to select for the presence of the expression plasmid pQE and the repressor plasmid pREP4 respectively. 20 ml of the uninduced overnight culture was used to inoculate 1L of LB containing 100μg/ml ampicillin and 25μg/ml kanamycin. The culture was grown at 37°C with shaking until the cells reached an A_{600} of 0.6 - 0.9. IPTG to a final concentration of 2mM, was added to induce expression of the neuraminidase. The cells were harvested two hours after induction as previously described (Section 2.3.3.4). The cells were then lysed by sonication, and the soluble fraction separated from the insoluble fraction by centrifugation. The soluble fraction containing the recombinant neuraminidase was applied to the Ni-NTA resin. After washing, the protein was eluted by the application of a gradient of increasing imidazole (0 - 0.5M). The imidazole competes with the 6xHis tag for Ni^{2+} binding sites on the Ni-NTA resin and consequently displaces the tagged protein. An overview of the purification scheme is shown in Figure 4.2.

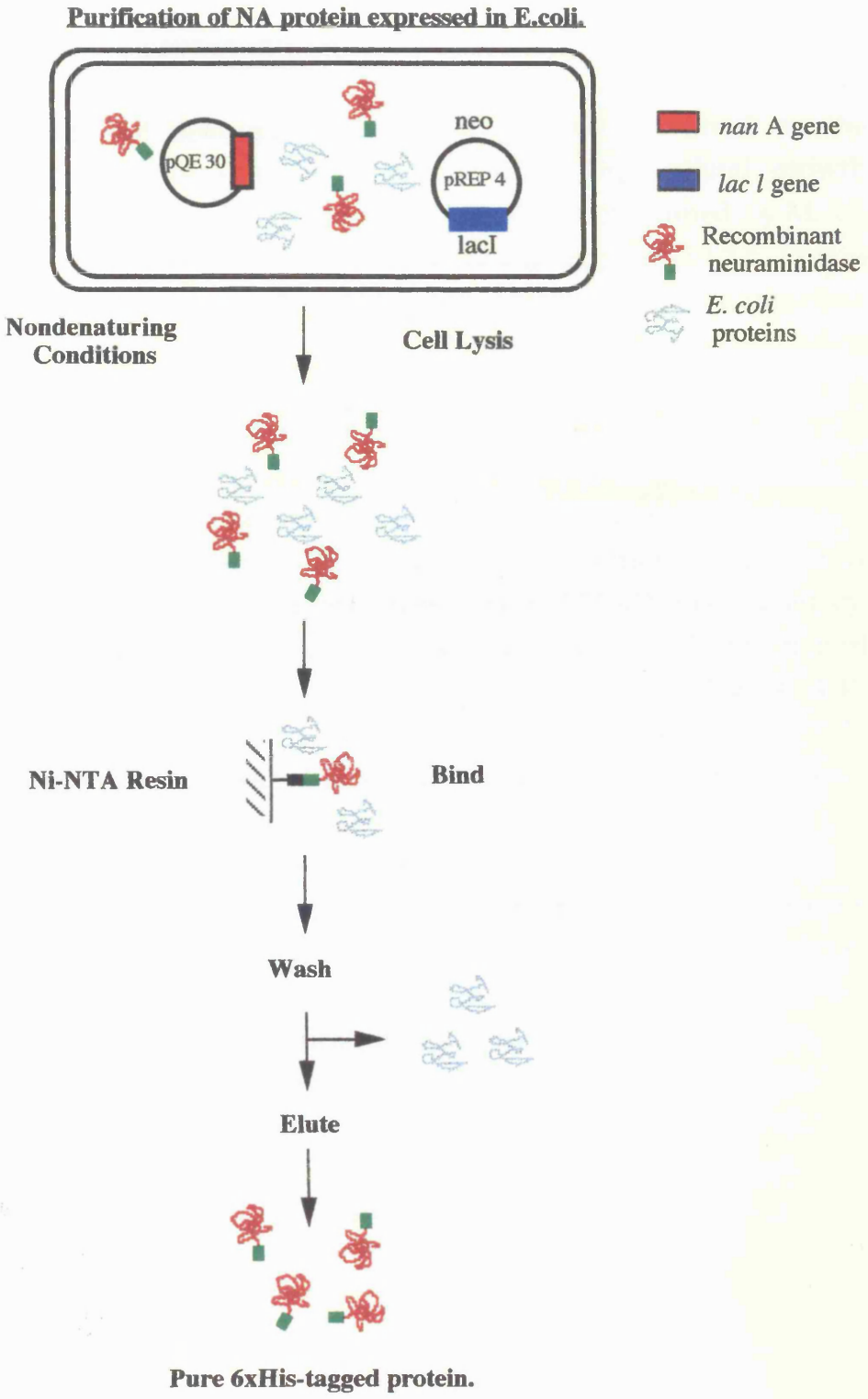


Figure 4.2. Principle of the Ni-NTA affinity purification procedure. (Adapted from 'The QIAexpressionist, Qiagen, 1997).The *E. coli* host strain containing the expression (pQE30) and repressor (pREP4) plasmid was grown in the presence of ampicillin and kanamycin until the OD_{600} was between 0.6 - 0.9. The cells were induced by the addition of IPTG and allowed to express the protein for two hours. The cells were then harvested, lysed and the cleared lysate was loaded onto the Ni-NTA column. The column was washed to remove contaminating proteins and the pure protein was eluted.

4.3 Purification of neuraminidase

Initial attempts at neuraminidase purification were centred on the full length protein expressed from pQMC1 (3.4.4.1). The optimal growth and induction conditions for the neuraminidase were determined by M. Camara (personal communication) and are described in Section 2.3.3.4. Details of a purification scheme devised by M. Camara (personal communication) are referred to as "The original protocol", and are described in Section 2.3.3.6.1.

4.3.1 Initial attempts at puification of neuraminidase

Initially attempts at neuraminidase purification were made using the "The original protocol" (Section 2.3.3.6.1). Upon elution with imidazole occasionally high purity neuraminidase (about 170 kD) (as judged by SDS-PAGE) was obtained (Figure 4.3). However more frequently the majority of neuraminidase co-purified with many other proteins (Figure 4.4). The neuraminidase had an apparent molecular mass of approximately 170 kDa, which is higher than expected from the deduced amino acid sequence (114 kDa). This discrepancy may be attributed to anomalous behaviour on SDS-PAGE. The irreproducibility of the results obtained using this method meant that improvements were necessary. Two potential areas for improvement were increasing the expression of the neuraminidase and thereby increasing the proportion of neuraminidase that was present and improving the affinity chromatography purification procedure. The overexpression of the protein had already been achieved (M.Camara, unpublished results), and so offered little scope for further improvement. However the Ni-NTA affinity chromatography parameters had not been studied in detail before and so became the basis for further investigations.

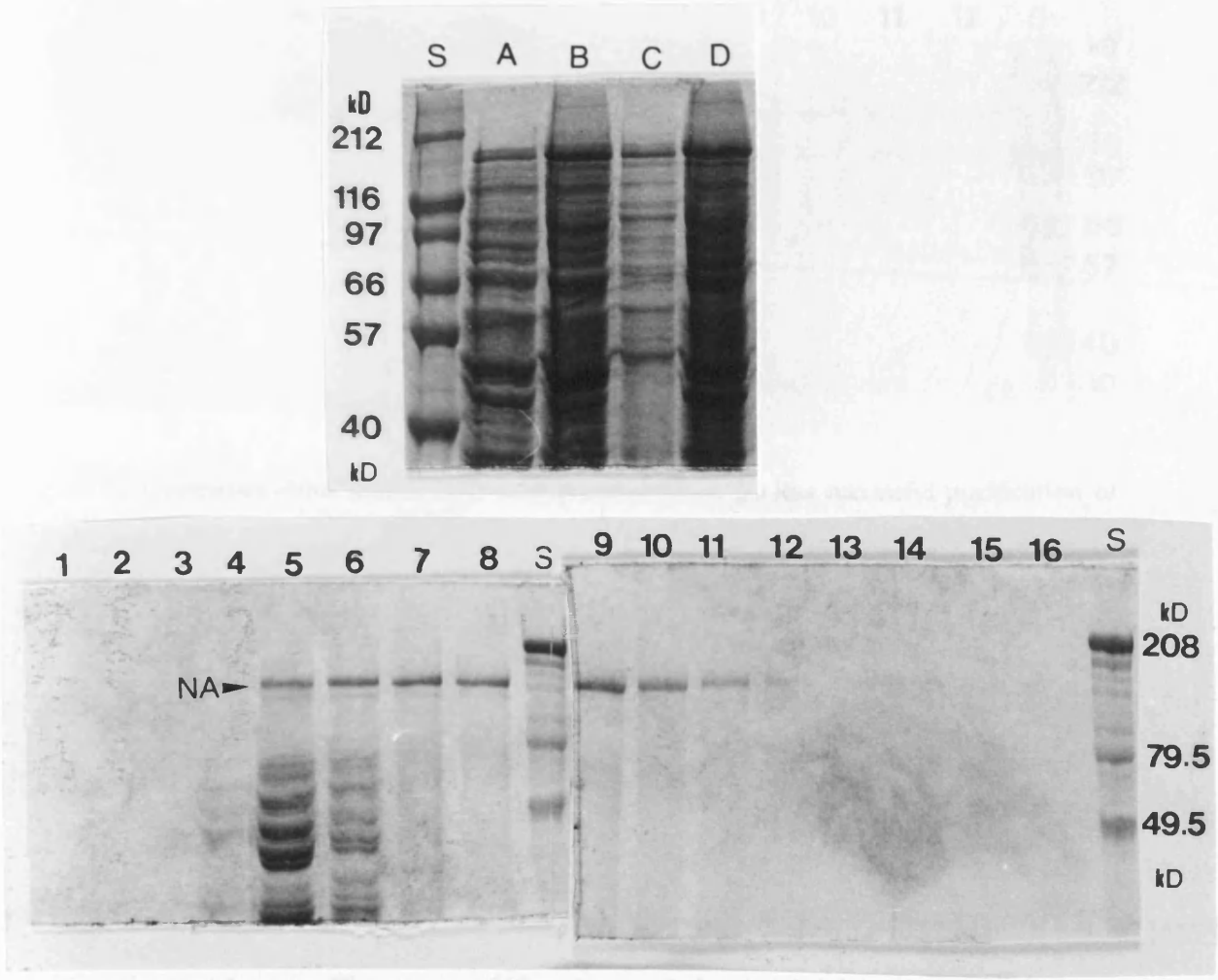


Figure 4.3. Coomassie - blue stained SDS-PAGE showing the purification of neuraminidase expressed from plasmid pQMC1.
The crude lysate was prepared as described in section 2.3.3.4. After filtering through a 0.45µm acrodisk, 8 mls of the lysate was loaded at a flow rate of 6 - 8 column volumes per hour onto a Ni-NTA column (0.9cm diameter x 15cm, 9ml bed volume, Pharmacia), that had been previously equilibrated with equilibration buffer. The flow through was collected (OUTFLOW). The column was washed with equilibration buffer for 20 mins and the eluate collected in two fractions (EQUILIBRATION). After washing the column for 12 - 14 hours with wash buffer the neuraminidase was eluted with a 0 - 0.5M gradient of imidazole in wash buffer. The eluted proteins were collected in 5 minute fractions. The column flow-through and eluted products were analysed by SDS-PAGE. Equal volumes (27µl) of each fraction were electrophoresed through an 8% gel.
Lane S - Standard molecular weight markers
Lane A - CRUDE
Lane B - OUTFLOW
Lanes C and D - EQUILIBRATION
Lanes 1 - 16: Eluted protein fractions 1 - 16.

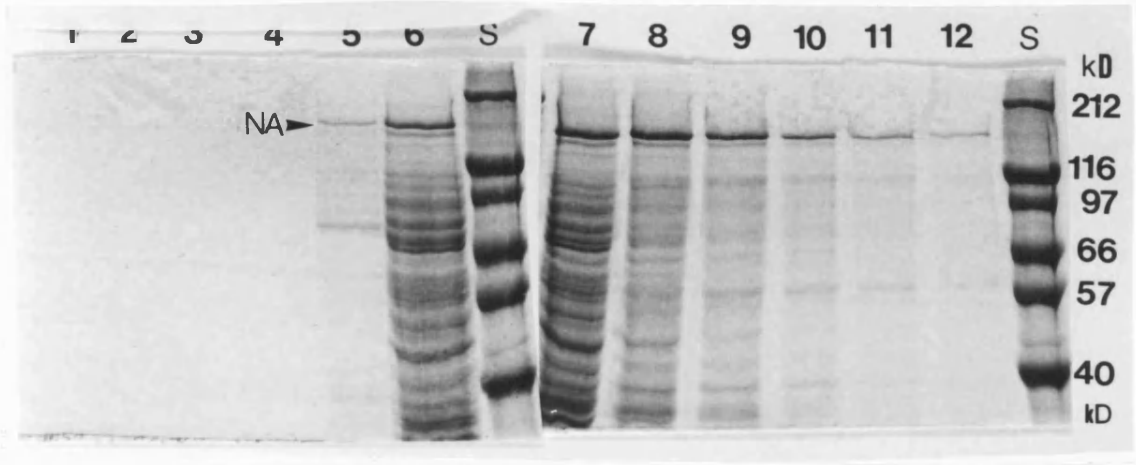


Figure 4.4 Coomassie - blue stained SDS - PAGE demonstrating a less successful purification of neuraminidase expressed from pQMC1.
The Ni-NTA cloumn was loaded, washed and eluted as described in the previous figure legend. The flow-through and eluted product were analysed by SDS-PAGE. Only the eluted protein fractions are shown (Lanes 1 - 12, fractions 1 - 12). The CRUDE, OUTFLOW and EQUILIBRATION fractions were identical to those described in Figure 4.3. Lane S - Standard molecular weight markers

4.3.2 Modification of the purification procedure

Improving the purity of the Ni-NTA resin purified neuraminidase could be achieved in two ways, either in the first instance by preventing the binding of unwanted proteins or by removing bound contaminants more effectively during the wash step. Drastic modifications of the initial binding conditions to minimise co-binding of contaminating proteins was felt to be detrimental to the attachment of the neuraminidase to the resin. Therefore, it was decided to retain the initial binding conditions, and to concentrate instead on more effective removal of contaminating proteins during the post-binding wash step.

Experiments aimed at maximising the efficiency of the wash step in removing contaminating host proteins whilst maintaining the efficient binding of the neuraminidase are discussed below.

4.3.2.1 Maximising the efficiency of the wash step

Background contamination can arise from: proteins that contain neighboring histidines; proteins linked to the 6xHis-tagged protein by disulfide bonds; proteins that associate non-specifically with the tagged protein and nucleic acids that associate with the tagged protein. These

contaminants can be removed by washing the resin under various conditions ("QIAexpressionist", Qiagen, 1992).

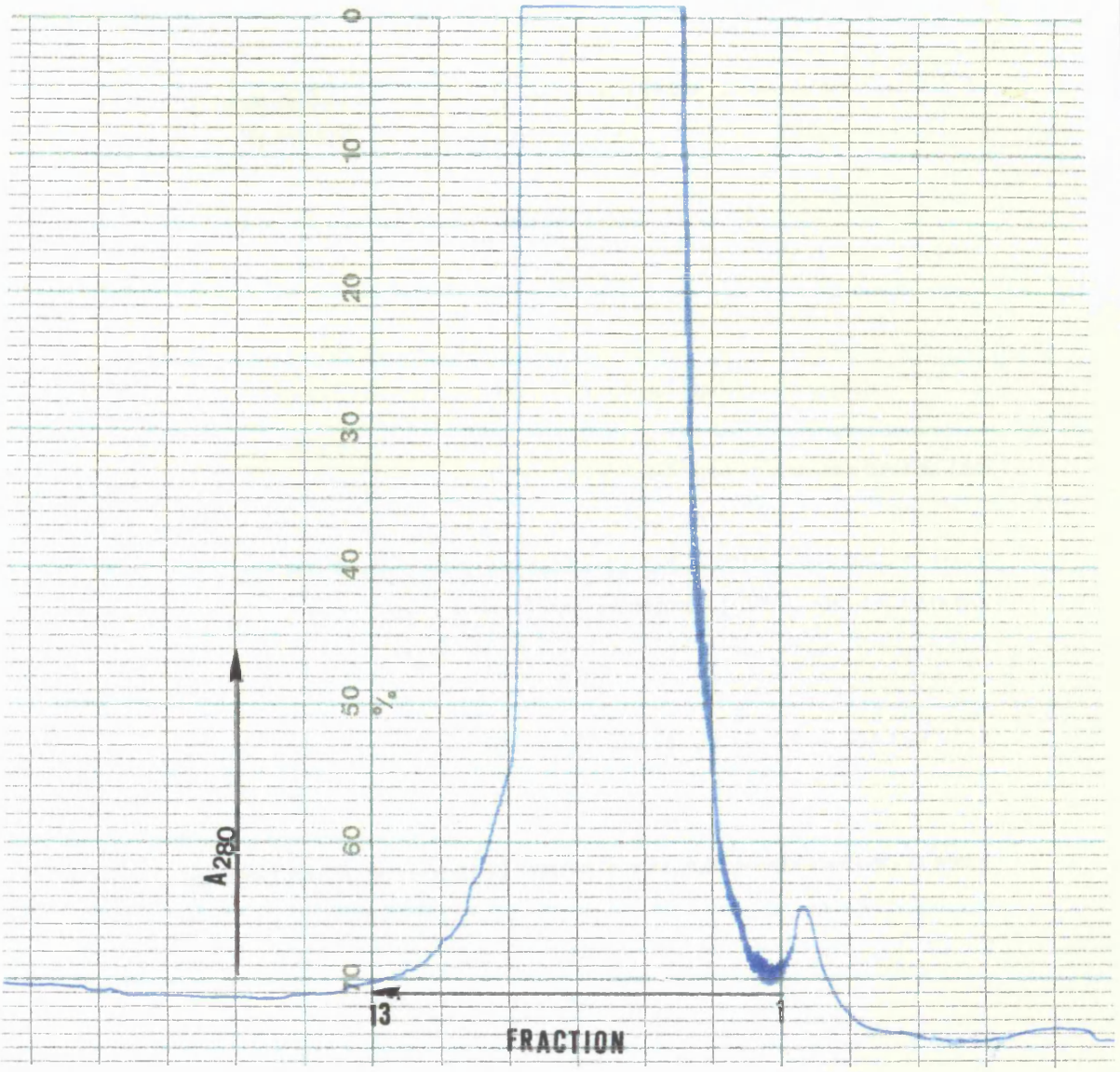
One method of removing the background proteins is by altering the pH of the wash buffer. Since the interactions between proteins and the resin will be predominantly ionic (charge mediated), a decrease in the pH of the column wash buffer will therefore protonate the histidine residues and give non-specific elution of bound material.

4.3.2.1.1 Binding and elution of *E.coli* SG13009 proteins from the Ni-NTA resin with a pH 8.0 wash buffer

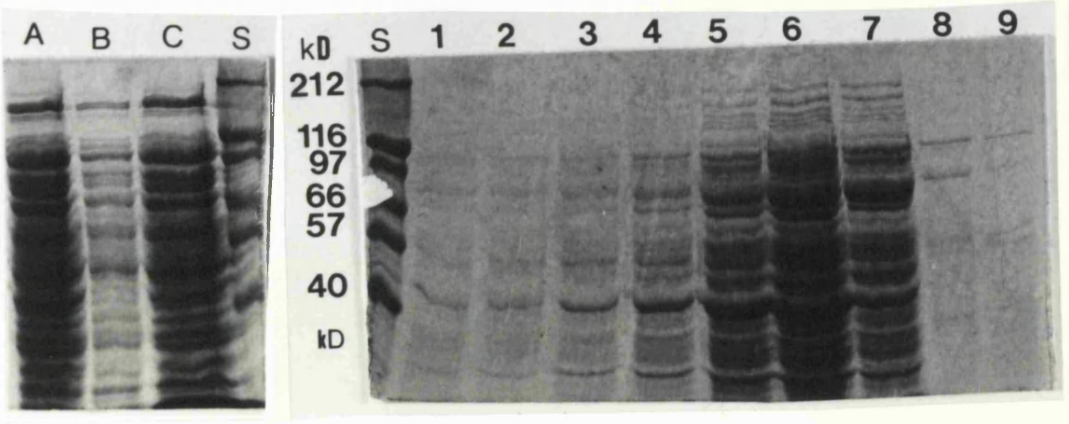
The contaminating proteins that co-eluted with the neuraminidase (Section 4.3.1, Figure 4.4) were the host cytoplasmic proteins. To obtain pure neuraminidase it was necessary to remove these proteins from the column during the wash step. To this end crude lysates of *E.coli* SG13009 were prepared in the same manner as described in Section 2.3.3.4 except for the induction step which was omitted. IPTG was not added to *E.coli* cultures because it did not contain the pQMC1 plasmid and host strains that do not carry *lac* inducible plasmids do not exhibit a change in protein expression upon addition of IPTG as judged by SDS-PAGE of cytoplasmic and membrane extracts (Dr. P. Freestone, Leicester University, personal communication). The crude lysate (containing approximately 100mg of protein) was applied to the Ni-NTA resin and the washing and elution steps were performed according to the original protocol (Section 2.3.3.6.1). The elution profile is shown in Figure 4.5, panel a. A large peak with an area in excess of 46 cm^2 was observed in fractions 10 - 20, which corresponded to an imidazole range of 62 - 125 mM. Further analysis of these fractions by SDS-PAGE (Figure 4.5, panel b) showed the presence of many proteins. The fact that these *E.coli* proteins eluted at the same imidazole concentration as the neuraminidase suggested that they were responsible for the contamination observed in the earlier purifications (Section 4.3.1).

The removal of the *E.coli* proteins from the resin during the wash step was required. To this end the ability of a range of wash buffers (differing in their pH) to remove the *E.coli* proteins from the resin was tested. Approximately the same amount of protein (100mg) was loaded on the column in each case.

a


0
10
20
30
40
50
%
60
70
A280
13
FRACTION

b


A
B
C
S
kD
S
1
2
3
4
5
6
7
8
9
212
116
97
66
57
40
kD

4.3.2.1.2 Binding and elution of *E.coli* SG13009 proteins from the Ni-NTA resin with a pH 6.0 wash buffer

Crude lysates of *E.coli* SG13009 were applied to the Ni-NTA resin as described previously. However in this experiment the pH of the wash buffer was reduced to 6.0. The elution profile is shown in Figure 4.6, panel a. In comparison to the profile obtained at pH 8.0 (Section 4.3.2.1.1), the new conditions resulted in a smaller peak with an area of approximately 3 cm^2. The eluted proteins were analysed by SDS-PAGE (Figure 4.6, panels b and c). From Figure 4.6 it was apparent that fewer contaminating host proteins (as judged by visual inspection of the SDS-PAGE gel) were eluted from the column washed with pH 6.0 buffer as compared to a column washed with a pH 8.0 buffer. Therefore a majority of the host proteins had been removed from the resin during the wash stage.

4.3.2.1.3 Binding and elution of *E.coli* SG13009 proteins from the Ni-NTA resin with a pH 5.0 wash buffer

When the above procedure was repeated using a pH 5.0 wash buffer, a very small protein peak with an area of about 0.4 cm^2 was observed on elution corresponding to fractions 9 - 10 Figure 4.7, panel a. Analysis of these fractions by SDS-PAGE showed the presence of very few proteins (as judged by visual inspection of the coomassie stained gel) (Figure 4.7, panel b). This result suggested that most of the contaminating *E. coli* proteins were removed from the resin by washing with pH 5.0 buffer. Therefore it was decided to examine the effect of the pH 5.0 wash buffer on purification of neuraminidase.

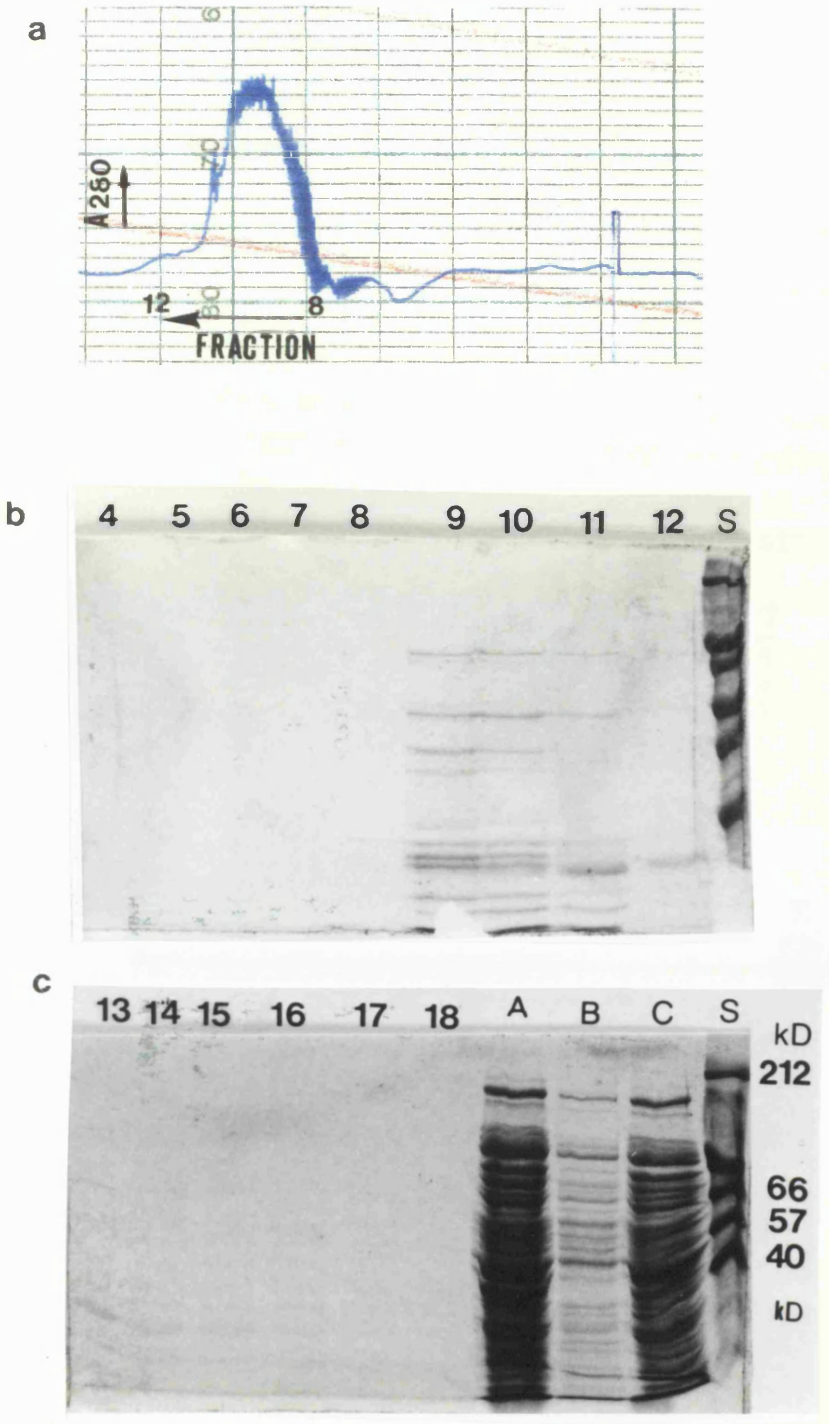
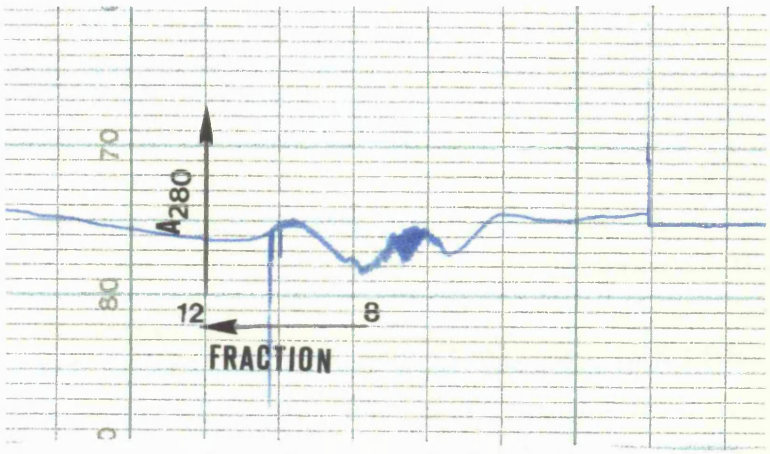


Figure 4.6 Analysis of the behaviour of *E. coli* proteins on Ni-NTA resin.
Crude lysates of *E. coli* SG13009 were prepared as described in Section 2.3.3.4. 10 ml of crude lysate was loaded on to a pre-equilibrated Ni-NTA column at a flow rate of 6 - 8 column volumes per hour. The washing and elution steps were performed as previously described in Section 2.3.3.6.1, except that the pH of the wash buffer was reduced to pH 6. The elution profile (Panel a) and the SDS - PAGE analysis of the flow-through and eluted fractions (Panels b and c) is shown. Equal volumes (27µl) of fractions from the affinity column were analysed by SDS-PAGE (10%) and visualized by coomassie blue staining.

Lane 4 - 18 : Elution fractions 4 - 18 respectively
Lane A - CRUDE
Lane B - OUTFLOW
Lane C - EQUILIBRATION
Lane S - Standard molecular weight markers

a



b

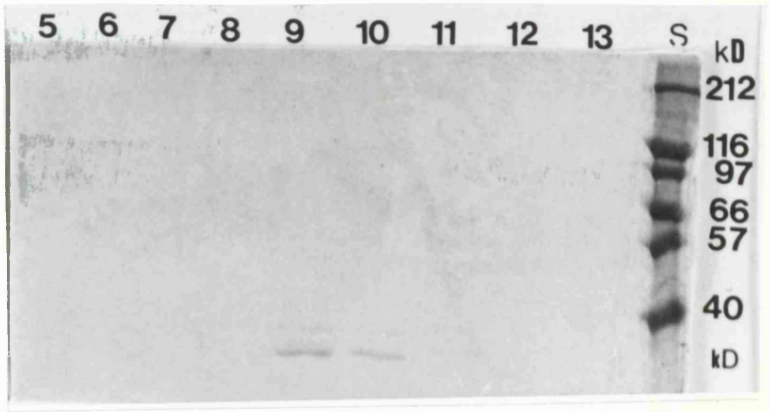
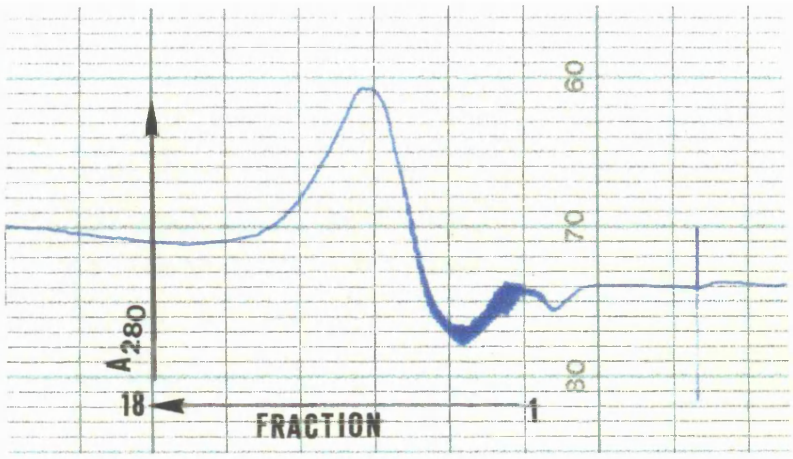


Figure 4.7 Analysis of the behaviour of *E. coli* proteins on Ni-NTA resin.
Crude lysates of *E. coli* SG13009 were prepared as described in Section 2.3.3.4. 10 ml of crude lysate was loaded on a pre-equilibrated Ni-NTA column at a flow rate of 6 - 8 column volumes per hour. The washing and elution steps were performed as previously described in Section 2.3.3.6.1, except that the pH of the wash buffer was reduced to pH 5. The elution profile of the eluted proteins is shown above (Panel a). The SDS-PAGE analysis of just the eluted protein fractions is shown (Panel b). The CRUDE, OUTFLOW and EQUILIBRATION fractions were identical to those described in Figures 4.5 and 4.6. Equal volumes (27µl) of each fraction were electrophoresed.
Lanes 5 - 13 : Fractions 5 - 13 respectively
Lane S - Standard molecular weight marker

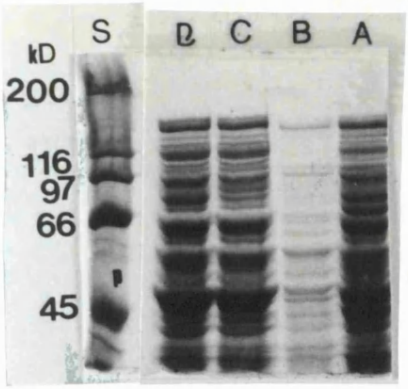
4.3.2.1.4 Purification of neuraminidase with a pH 5.0 wash buffer

Purification of the neuraminidase encoded by pQMC1 was repeated using the original method (Section 2.3.3.6.1), except that the wash buffer pH was reduced to 5.0. The elution profile and SDS - PAGE analysis of the eluted proteins are shown in Figure 4.8, panels a - d. The neuraminidase (approximately 170 kD) eluted in fractions 1 - 18 with trace amounts of contaminating host proteins (Panel d, lanes 1 - 18). An analysis of the wash fractions (Figure 4.8, Panel c, lanes A - I) shows the presence of the vast majority of the host derived proteins. Therefore washing the resin with pH 5.0 wash buffer resulted in better purification of the neuraminidase. This experiment was repeated twice more and gave consistent results (not shown). As judged by visual inspection of the SDS-PAGE, the purity of the neuraminidase was increased but the yield of the protein was decreased. However at this stage the loss was considered acceptable in view of the overall reduction in contaminating protein evident in Figure 4.8.

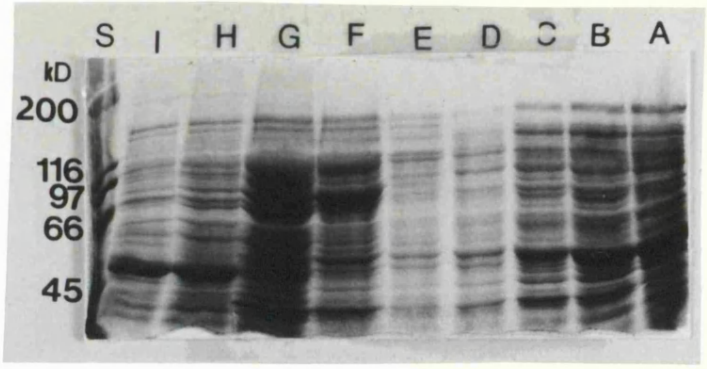
a


60
70
80
A_{280}
18
FRACTION
1

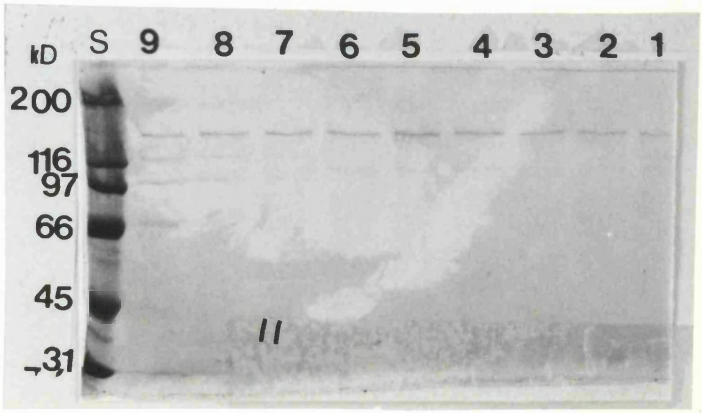
b

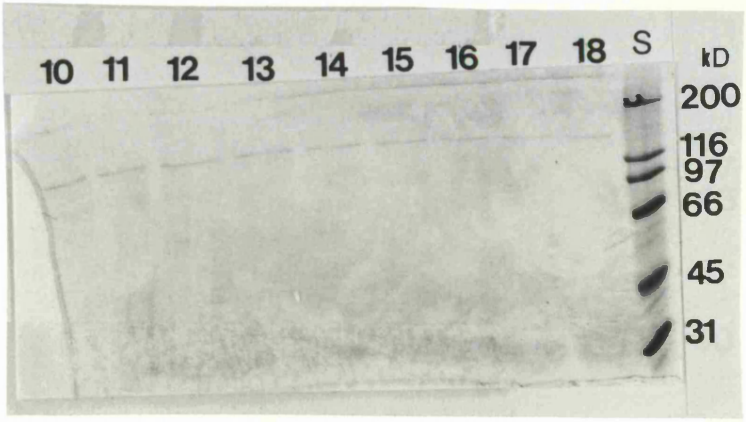

kD
S
D
C
B
A
200
116
97
66
45

c


S
I
H
G
F
E
D
C
B
A
kD
200
116
97
66
45

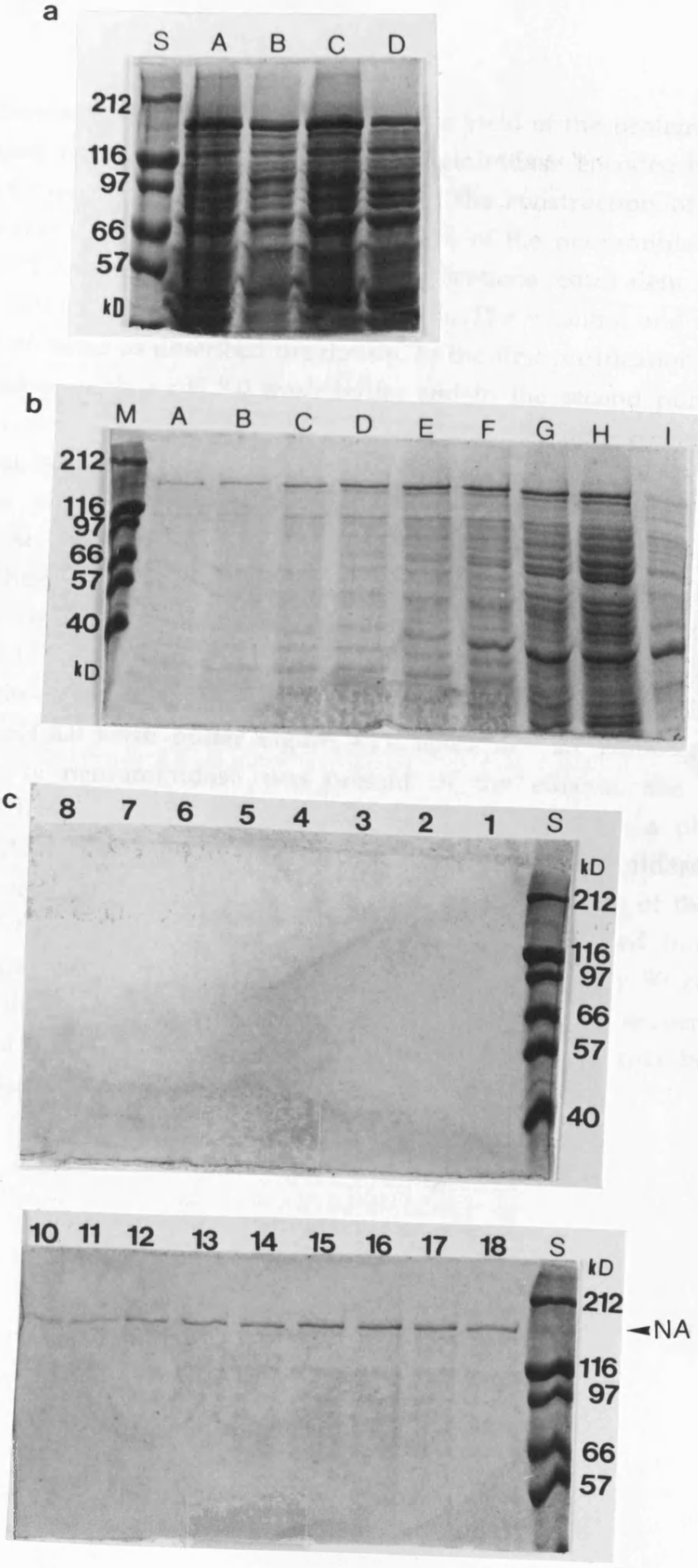
d


kD
S
9
8
7
6
5
4
3
2
1
200
116
97
66
45
31


10
11
12
13
14
15
16
17
18
S
kD
200
116
97
66
45
31

Several batches of neuraminidase were purified using a pH 5.0 wash buffer and it became apparent that the yield from this purification scheme was not sufficient to meet the requirements for procedures such as X-ray crystallography which require large quantities of protein (Martin, 1996). In any purification procedure there is a link between the purity of the protein and its overall yield. In order to improve the yield of the neuraminidase, it maybe necessary to reach a compromise with purity and vice versa.

In order to improve the yield of the neuraminidase, a compromise between purity and yield was reached by performing the purification with a pH 6.0 wash buffer. Analysis of the eluted proteins by SDS-PAGE (Figure 4.9) show that, an acceptable decrease in purity occured but the yield of the neuraminidase was significantly improved (as judged by SDS-PAGE of the eluted proteins. It was decided to perform future purifications using a pH 6.0 wash buffer.


a
S A B C D
212
116
97
66
57
kD
b
M A B C D E F G H I
212
116
97
66
57
40
kD
c
8 7 6 5 4 3 2 1 S
kD
212
116
97
66
57
40
10 11 12 13 14 15 16 17 18 S
kD
212
NA
116
97
66
57

The effect of the pH 6.0 wash buffer on the yield of the protein was further illustrated during purification of the neuraminidase encoded by the pSSGT plasmid (See Section 4.3.6.1, for details on the construction of the plasmid and its features). Two separate purifications of the neuraminidase encoded by pSSGT were conducted. In both purifications equivalent amounts of protein (100mg) were loaded on the column. The washing and elution steps were performed as described previously. In the first purification the column was washed with a pH 5.0 wash buffer and in the second purification the column was washed with a pH 6.0 wash buffer. The eluted proteins were analysed by SDS-PAGE and are shown in Figures 4.10 and 4.11. The purity and the yield of the eluted proteins from the two purifications were compared by visual analysis of the SDS-PAGE. In the first purification, where the column was washed with a pH 5.0 wash buffer a small amount of purified neuraminidase (about 90 kD) was visible in eluted fractions 11 - 18, lanes 11 - 18 (Figure 4.10). By comparison a greater amount of neuraminidase was present in the fractions eluted from the coulmn washed with a pH 6.0 wash buffer Figure 4.11, lanes 15 - 23. Although a greater amount of neuraminidase was present in the eluants, the purity was reduced (compared to the neuraminidase purified using a pH 5.0 wash buffer). A few other proteins co-purified with the neuraminidase. However this reduction in purity was considered acceptable because of the increased yield of the neuraminidase. The neuraminidase expressed from plasmid pSSGT had an apparent molecular mass of approximately 90 kDa. This is higher than expected from the deduced amino acid sequence, which predicted a size of approximately 83 kDa. This discrepancy may be attributed to anomalous behaviour on SDS-PAGE.

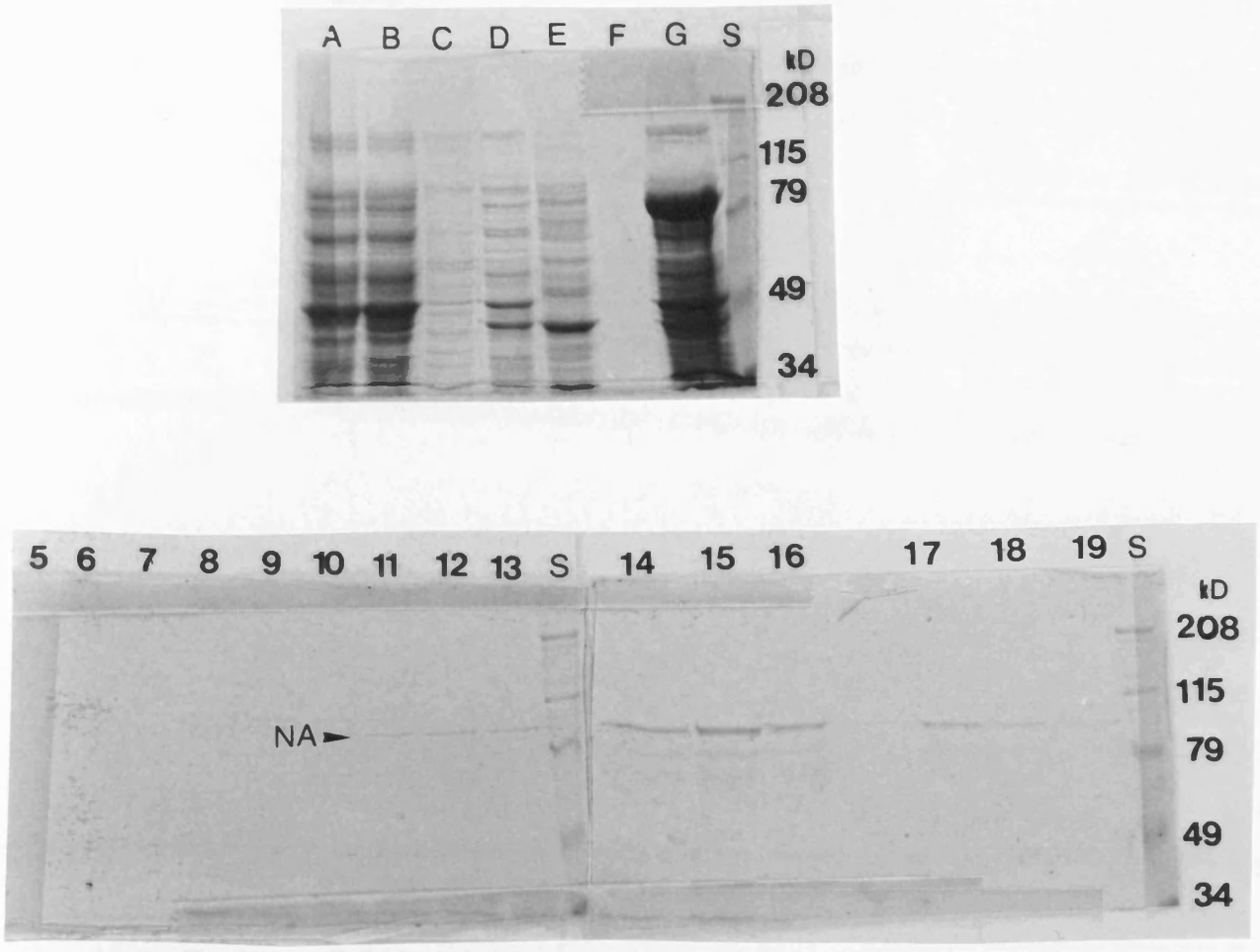


Figure 4.10 Coomassie - blue stained SDS - PAGE gels showing the purification of neuraminidase expressed from plasmid pSSGT, using a pH 5.0 wash buffer.
Crude lysates were prepared as described in Section 2.3.3.4. 10 ml of crude lysate was loaded at a flow rate of 6 - 8 column volumes per hour on a Ni-NTA column pre-equilibrated with equilibration buffer. The washing and elution steps were performed as previously described in Section 2.3.3.6.1, with the exception that the pH of the wash buffer was reduced to pH 5.0. The flow - through and eluted proteins were analysed by SDS - PAGE. Equal volumes (27µl) of each fraction were electrophoresed.

Lanes A and B - EQUILIBRATION
Lane C - OUTFLOW
Lane D - Wash fraction 1
Lane E - Wash fraction 4
Lane F - last wash fraction
Lane G - CRUDE.
Lanes 5 - 19: Eluted fractions 5 -19
Lane S - Standard molecular weight marker

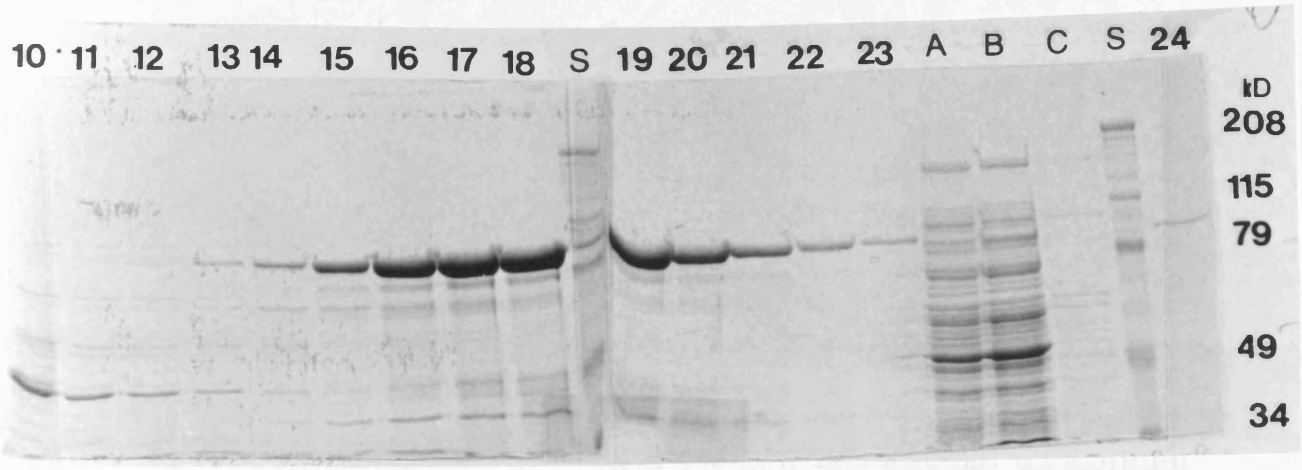


Figure 4.11 Coomassie - blue stained SDS - PAGE gels showing the purification of neuraminidase expressed from plasmid pSSGT, using a pH 6.0 wash buffer.
Crude lysates were prepared as described in Section 2.3.3.4. 10 ml of crude lysate was loaded at a flow rate of 6 - 8 column volumes per hour on a Ni-NTA column pre-equilibrated with equilibration buffer. The washing and elution steps were performed as previously described in Section 2.3.3.6.1, with the exception that the pH of the wash buffer was reduced to pH 6.0. The flow - through and eluted proteins were analysed by SDS - PAGE. Equal volumes (27µl) of each fraction were electrophoresed.

Lanes 10 - 24: eluted fractions 10 - 24
Lanes A and B - EQUILIBRATION
Lane C - OUTFLOW
Lane S - Standard molecular weight marker
The SDS - PAGE analysis of the CRUDE is not shown but was identical to that shown in Figure 4.10.

4.3.2.1.5 Alterations in the length of the wash step

Having identified the optimum pH of the wash buffer, it was decided to examine the effect of changing the length of the wash step and changing the flow rate compared to the previous purifications.

In the original purification protocol (Section 2.3.3.6.1) the resin was washed for 12 - 14 hours following application of the crude lysate, however the resin manufacturer recommended a wash period of 6 - 7 hours. A long period of washing would result in slow leaching of proteins from the resin. To test the effects of a reduced wash step on the purity and yield of the neuraminidase, two purifications were performed on the neuraminidase expressed from plasmid pSSWTvec. In one purification the resin was washed with pH 6.0 wash buffer for 7 hours and in another the wash period was reduced to 2.5 hours. The reduced wash period appeared to make no difference to the purity or yield of the purified protein (as judged by SDS-PAGE gels of the purified protein). The neuraminidase eluted from a column washed for either 7 hours or 2.5 hours is shown in Figures 4.12 and 4.13 respectively. In view of these reults it was decided to reduce the wash step to 2.5 hours in future purifications.

In addition to the change in wash length, the flow rate through the column was decreased to 3 -4 column volumes per hour. This rate was recommended by the manufacturers (Qiagen) producing an increased number of fractions collected, and was considered to be optimal.

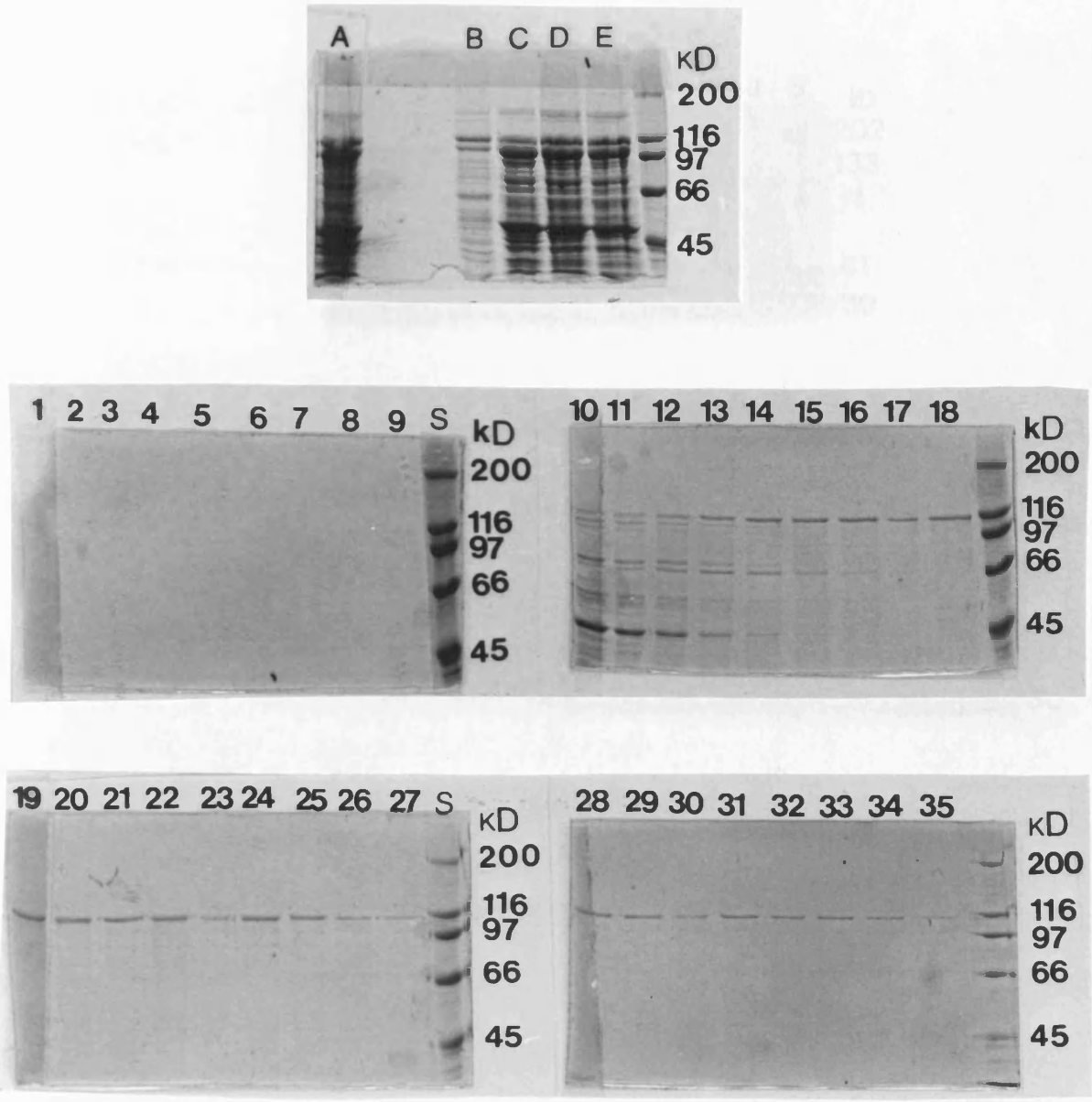


Figure 4.12 Coomassie - blue stained SDS - PAGE gels showing the purification of neuraminidase expressed from plasmid pSSWTvec, using a pH 6.0 wash buffer, a 6 -7 hour wash length and a flow rate of 3 - 4 column volumes per hour.
Crude lysates were prepared as described in Section 2.3.3.4. 10 ml of crude lysate was loaded at a flow rate of 3 - 4 column volumes per hour on to a Ni-NTA column pre-equilibrated with equilibration buffer. The washing and elution steps were performed as previously described in Section 2.3.3.6.1, with the exception that the pH of the wash buffer was reduced to pH 6.0 and the wash period was reduced to 6 - 7 hours. The flow - through and eluted proteins were analysed by SDS - PAGE. Equal volumes (27µl) of each fraction were electrophoresed.

Lanes A - CRUDE
Lane B - OUTFLOW
Lanes C , D and E - EQUILIBRATION
Lanes 1 - 35 show eluted fractions 1 - 35 respectively
Lane S - Standard molecular weight marker

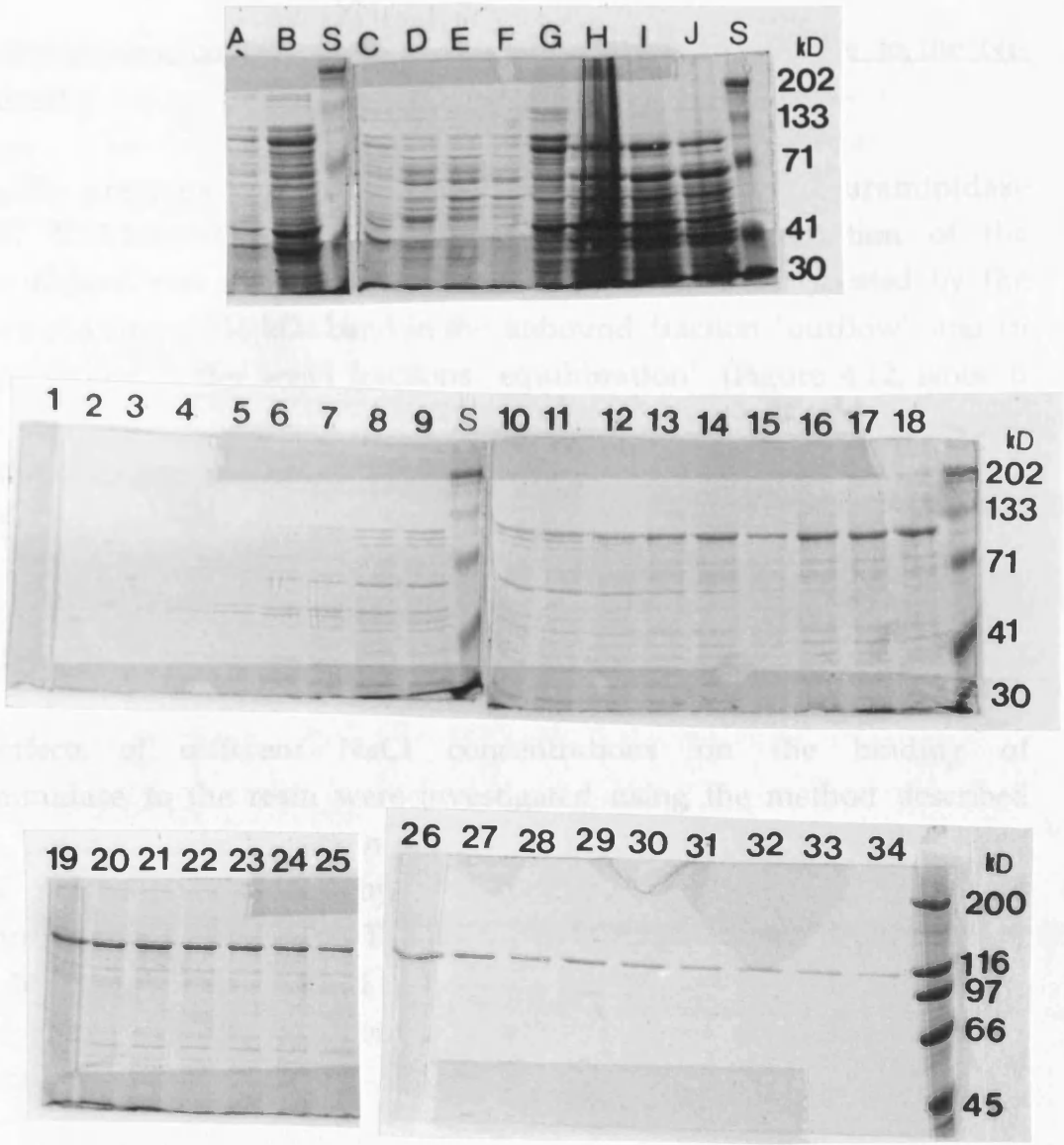


Figure 4.13 Coomassie - blue stained SDS - PAGE gels showing the purification of neuraminidase expressed from plasmid pSSWTvec, using a pH 6.0 wash buffer, a 2.5 hour wash length and a flow rate of 3 - 4 column volumes per hour.
Crude lysates were prepared as described in Section 2.3.3.4. 10 ml of crude lysate was loaded at a flow rate of 3 - 4 column volumes per hour on to a Ni-NTA column pre-equilibrated with equilibration buffer. The washing and elution steps were performed as previously described in Section 2.3.3.6.1, with the exception that the pH of the wash buffer was reduced to pH 6.0 and the wash period was reduced to 2.5 hours. The flow - through and eluted proteins were analysed by SDS - PAGE. Equal volumes (27µl) of each fraction were electrophoresed.

Lanes 1 - 34: eluted fractions 1 - 34 respectively
Lanes A - F : Wash fractions 1 - 6 respectively
Lane G - OUTFLOW
Lanes H and J - CRUDE
Lane I - EQUILIBRATION
Lane S - Standard molecular weight marker

4.3.2.2 Optimizing conditions for binding of the neuraminidase to the Ni-NTA resin

During the previous experiments on the purification of the neuraminidase (Section 4.3.2.1.5), it became apparent that a large proportion of the neuraminidase was not binding to the resin. This was suggested by the presence of a strong 116 kDa band in the unbound fraction "outflow" and in the equilibrium buffer wash fractions "equilibration" (Figure 4.12, lanes B and C respectively). Therefore to obtain an increased yield of the end product, it was necessary to improve the binding of the neuraminidase to the resin.

4.3.2.2.1 Determination of the effect of varying the NaCl concentration in the crude lysate on the binding of neuraminidase to the Ni-NTA resin.

The effects of different NaCl concentrations on the binding of neuraminidase to the resin were investigated using the method described below. The NaCl concentration of crude lysate (prepared as described in Section 2.3.3.4) was altered by the addition of 225µl of each of the equilibration buffers at pH 8.0. This resulted in the final NaCl concentrations of 120, 165, 210, 255 and 300mM for each crude lysate. The lysates were then applied to the resin to monitor the effect of the different NaCl concentrations on binding.

4.3.2.2.1.1 Binding protocol

The following protocol was adapted from the "The QIAexpressionist", (Qiagen, 1992) and is shown schematically in Figure 4.14.
All centrifugations were performed at 13,000rpm (≥ 10,000 x g) in a microfuge and mixing was performed in a rotary wheel. In brief 25µl of the crude lysate prepared as described in Section 2.3.3.4 was mixed with 225µl of equilibration buffer that was being tested and then added to 50µl of Ni-NTA resin that had been equilibrated in the same buffer. After gently agitating for 10 minutes the mixture was centrifuged for 2 minutes. The supernatant (SN1) was removed to a clean 1.5 ml centrifuge tube and kept on ice. 500µl of wash buffer (50mM Na-phosphate, 300mM NaCl, 10% (v/v) Glycerol, 0.5% (v/v) Tween, pH 6.0) was added to the resin and the mixing and centrifugation steps were repeated. The supernatant (SN2) was removed to a

clean tube and placed on ice. The wash step was repeated and the supernatant (SN3) was collected and kept on ice. The protein that had bound to the resin was eluted by adding 50µl of 100mM EDTA (pH8.0) to the resin followed by gentle agitation for 10 minutes. The eluted protein was collected by centrifugation for 2 minutes, the supernantant (SN4) and the resin were placed on ice. When the above procedure had been repeated using each of the equilibration buffers, all the supernatants were analysed by SDS-PAGE. The results are shown in Figure 4.15.

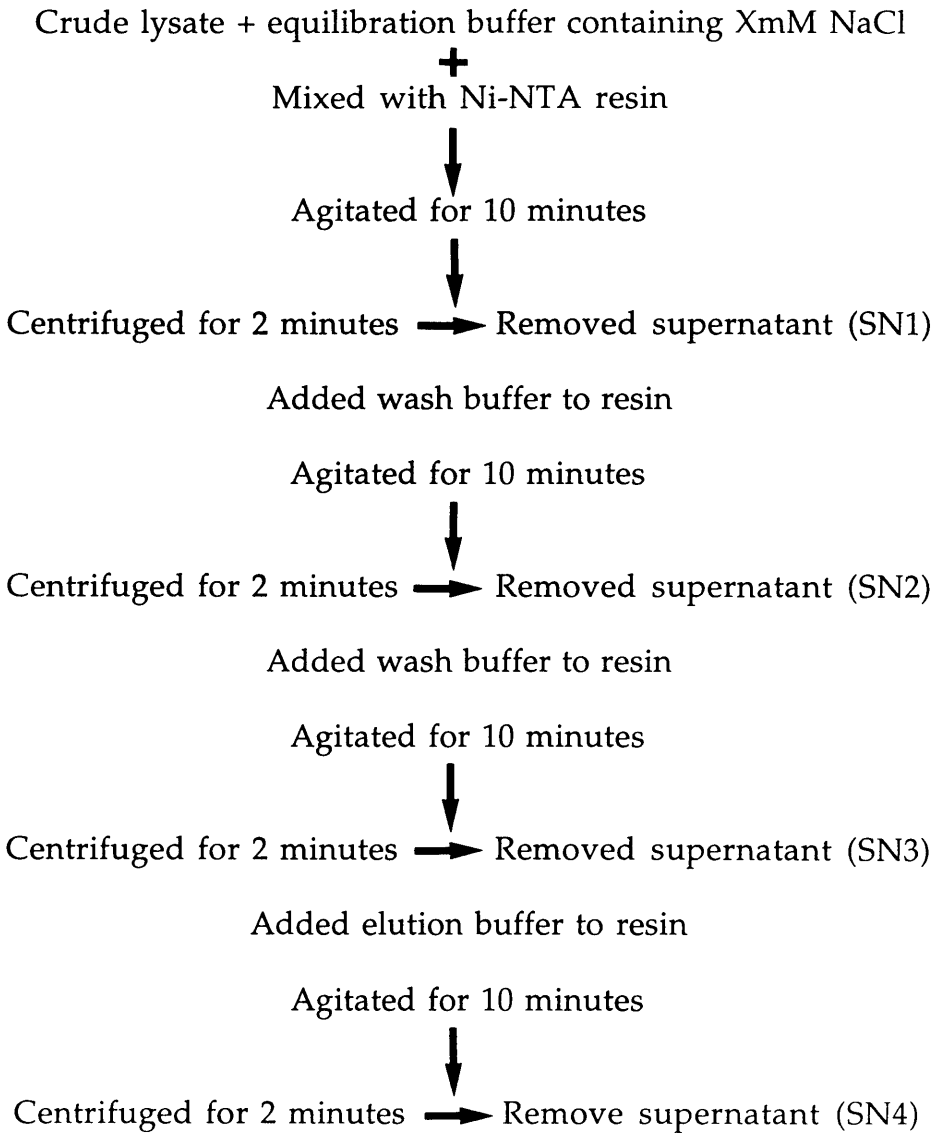


Figure 4.14. Schematic representation of the procedure used to measure the effect of different NaCl concentrations in the equilibration buffer on binding of neuraminidase to the Ni-NTA resin (See Section 4.3.2.2.1.1 for more detail)

Supernatant 1 (SN1) represented the proteins that did not bind to the resin. Supernatant 2 and 3 (SN2 and SN3) were the proteins that were removed

from the resin during the wash steps. Supernatant 4 represented the protein that remained bound to the resin after the wash steps. As can be seen from Figure 4.15, the same pattern of binding and elution was observed with all the equilibration buffers used. The different salt concentrations tested did not alter the binding of the neuraminidase to the resin. Therefore it was decided to continue using the equilibration buffer containing 300mM NaCl at pH 8.0.

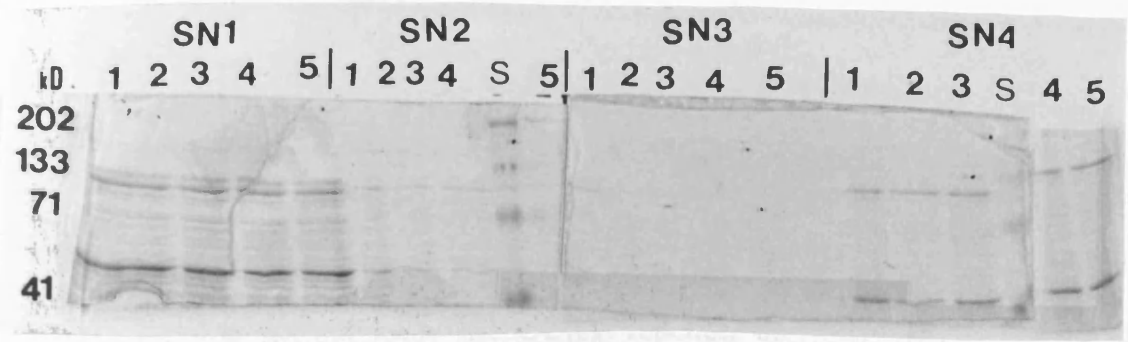


Figure 4.15. SDS-PAGE analysis of the supernatants that resulted from the experiment conducted to measure the effect of different NaCl concentrations on the binding of neuraminidase to Ni-NTA resin (See Section 4.3.2.2.1.1 for more detail).
Neuraminidase was expressed from plasmid pSSWTvec in *E. coli* . In brief crude lysate containing a certain concentration of NaCl was mixed with Ni-NTA resin. The unbound material was collecetd and termed SN1. The resin was washed twice with wash buffer and the outflow samples collected and termed SN2 and SN3. Finally the bound proteins (SN4) were eluted by the application of EDTA .
27µl of each supernatant (SN1 - SN4) was analysed by SDS-PAGE followed by staining with coomassie blue.

Lanes numbered 1 - 5 refer to increasing NaCl concentration of the crude lysate that was applied to the Ni-NTA resin.

Lane 1 - 120mM
Lane 2 - 165mM
Lane 3 - 210mM
Lane 4 - 255mM
Lane 5 - 300mM
Lane S - Standard molecular weight marker

4.3.2.2.2 Trial purification under denaturing conditions

Another reason for the poor binding of the neuraminidase to the resin could be due to the 6 x His tag being "hidden" inside the protein and consequently not being available for binding to the resin. To improve accessibility of the 6 x His tag to the resin protein purification was attempted under denaturing conditions.

4.3.2.2.2.1 Denaturing purification protocol

The following protocol was adapted from the "The QIAexpressionist" (Qiagen, 1992) and is shown schematically in Figure 4.16. A single colony of *E.coli* SG13009: [pREP4] [pSSWT] was used to inoculate 1.5 ml of LB containing 100µg/ml ampicillin and 25µg/ml kanamycin and the cultures was grown overnight at 37°C. 500µl of the overnight cultures were used to inoculate 1.5 ml of prewarmed LB (100µg/ml ampicillin and 25µg/ml kanamycin) and grown until the OD_{600} reached 0.7 - 0.9. Expression was induced by the addition of IPTG to a final concentration of 2 mM. After 2 hours, 1ml of the culture was transferred to a microcentrifuge tube and the cells harvested by centrifugation at 13,000rpm (≥ 10,000 x g) in a microfuge for 30 seconds. The cells were resuspended in 200µl of buffer B (8M urea, 0.1 M Na-phosphate, 0.01 M Tris-HCl pH 8.0), and lysed by gentle vortexing. The lysate was centrifuged for 12 minutes at 13,000rpm (≥ 10,000 x g) in a microfuge and the supernatant was transferred to a clean microfuge tube. 50µl of a 50% (w/v) slurry of Ni-NTA resin was added to the supernatant and mixed gently for 30 minutes at room temperature. The resin was pelleted by centrifugation at 13,000rpm (≥ 10,000 x g) for 10 seconds. The supernatant (SN1) was transferred to a clean tube and stored on ice. The resin was washed 2 x with 1 ml of buffer C (8 M urea, 0.1 M Na-phosphate, 0.01 M Tris-HCl pH 6.3) and the supernatants collected and stored on ice (SN2, SN3). 20µl of buffer C containing 100 mM EDTA was added to the resin and incubated at room temperature for 2 minutes with gentle mixing. After centrifugation at 13,000rpm (≥ 10,000 x g) the supernatant (SN4) was removed to a clean tube. All the supernatants were analysed by SDS-PAGE.

Purification of NA protein expressed in E.coli. under denaturing conditions

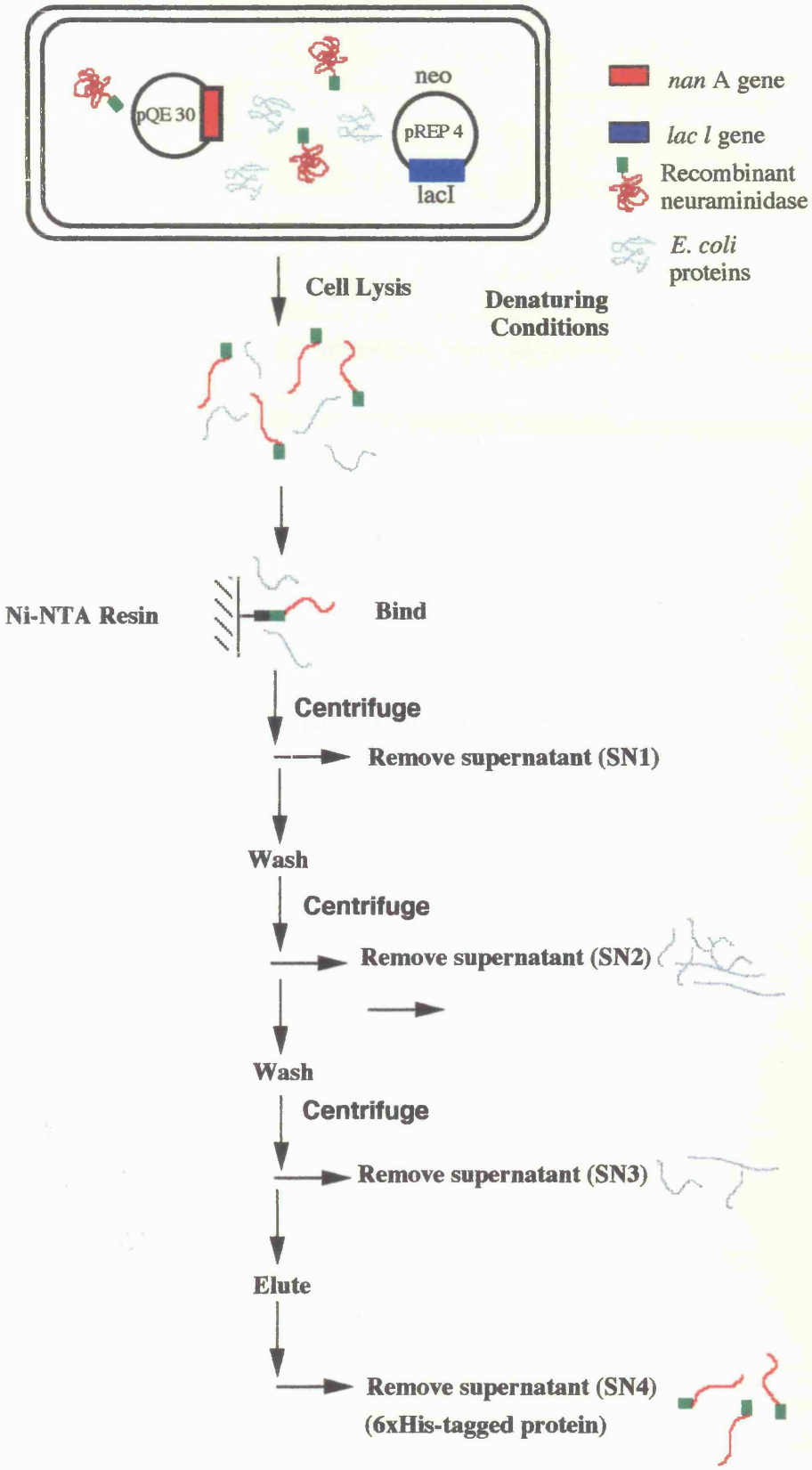


Figure 4.16.Schematic representation of the procedure used to perform a trial purification of neuraminidase under denaturing conditions (See Section 4.3.2.2.2. 1 for more detail). (Adapted from "The QIAexpressionist, Qiagen, 1997).

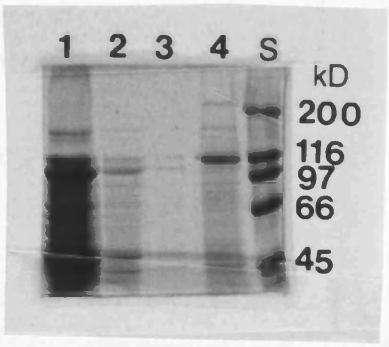


Figure 4.17. SDS-PAGE analysis of the supernatants that resulted from a trial purification of neuraminidase under denaturing conditions. (See Section 4.3.2.2.2.1 for more detail). In brief, neuraminidase was expressed from plasmid pSSWTvec in *E. coli* cultures which were pelleted and resuspended in dentauring buffer. Following cell lysis (by vortexing) the crude lysate was mixed with Ni-NTA resin. The unbound material was collected and termed SN1. The resin was washed twice with wash buffer and the outflow samples collected and termed SN2 and SN3. Finally the bound proteins (SN4) were eluted by the application of EDTA .
27µl of each supernatant was analysed by SDS-PAGE followed by staining with coomassie blue.

Lane 1 - SN1 (Unbound material)
Lane 2 - SN2 (Wash 1)
Lane 3 - SN3 (Wash 2)
Lane 4 - SN4 (Eluate)
Lane S - Standard molecular weight marker

As can be seen from Figure 4.17, even under denaturing conditions a large proportion of the neuraminidase did not bind to the resin as evidenced by the presence of large amounts of this protein in the outflow fraction (SN1) (Figure 4.17, Lane 1).

It was decided not to proceed with the denaturation protocol because there was no improvement in the binding of the neuraminidase. Furthermore prior to functional studies the protein would need to be correctly refolded, which would not be without its own problems and would likely be time consuming.

4.3.3 Removal of imidazole from the protein fractions

One of the principal intended uses of the purified neuraminidase was in an immunization study in mice (Chapter 5). Purification of the neuraminidase requires elution from the Ni-NTA affinity resin by competition with imidazole. Since the imidazole was considered to be potentially toxic to mice, it was necessary to remove this compound from neuraminidase preparations. The final stage of the purification procedure involved the removal of the imidazole from the protein.

In the early stages of the study imidazole removal was achieved by concentration, dilution and re-concentration in Amicon stirred cells (Section 2.3.4). Later this was replaced by Vivaspin centrifugal filter units because they were more convenient and faster to use.

4.3.4 Summary of binding and washing conditions

After consideration of the results from various binding and washing conditions, a final protocol (described in Section 2.3.3.6.2) was devised for the purification of neuraminidase. Essentially the protocol differed from the original protocol (Section 2.3.3.6.1) in the length of the wash step, the flow rate and the pH of the wash buffer. Both wild type and mutated forms of neuraminidases used for vaccination of mice and enzymatic analysis were purified using this method. The results of a typical purification for each form of the neuraminidase are described below.

4.3.5 Purification and activity of wild type and mutated neuraminidases

A representative purification of each neuraminidase is described below. All samples were kept at 4°C and assayed for enzyme activity and protein content within 48 hours of purification.

4.3.5.1 Purification of wild type neuraminidase

The results of a typical wild type neuraminidase purification are summarized in Table 4.1 and SDS-PAGE analysis is shown in Figure 4.18. The volume, enzyme activity and protein content of all fractions were

measured. These allowed estimations to be made of the yield and degree of purification.

After harvesting 1 litre of bacterial cells a "crude" lysate (9.5ml) containing 98mg of total protein with a total neuraminidase activity of 2260 μmol min^{-1} was obtained (Section 2.3.3.4). Analysis of this sample by SDS-PAGE shows the presence of several major protein bands. One of the most abundent protein was of approximately 116 kDa and was considered to be the neuraminidase (Figure 4.18, Lane A). The "crude" lysate was applied to a Ni-NTA column as described in Section 2.3.3.6.2. The unbound material termed "outflow" (8.7ml) was collected. The "outflow" fraction consisted of 11.9mg of total protein with a neuraminidase activity of 396μmol min^{-1} . Analysis of the "outflow" by SDS-PAGE (Figure 4.18, Lane B) revealed the presence of the same protein bands that were previously seen in the "crude" fraction but at a lower intensity. Once again one of the most prominent bands had a molecular weight of 116 kDa. By comparison of the SDS-PAGE profile of the "crude" and "outflow" it was concluded that a proportion of the neuraminidase was not binding to the Ni-NTA resin.

In the next stage the resin was washed with equilibration buffer for 20 minutes and 5.8ml of the eluate termed "equilibration" was collected. The greatest loss of enzyme activity occurred at this stage. The "equilibration" fraction had an enzyme activity of 1122 μmol min^{-1} (50 % of loaded). The specific activity of this pool had decreased from 32.5 to 25.4 μmol min^{-1} mg^{-1} of protein. Approximately 50% of the total protein, which is greater than expected was also present in this fraction. Analysis of this fraction by SDS-PAGE (Figure 4.18, Lane C) revealed an almost identical pattern and intensity of proteins to that observed in the "crude" fraction. The most intense protein band (as judged by visual analysis of the SDS-PAGE) corresponded to a protein of 116 kDa, which was thought to be the neuraminidase. This suggested that a substantial proportion of the neuraminidase had not bound to the resin.

Next the Ni-NTA column was washed with wash buffer for 2.5 hours to remove proteins that were associated with the neuraminidase or resin due to non-specific interactions. The material that came off the column during this period was collected and termed the "wash" fraction (45ml). The greatest removal of protein occurred at this stage. The "wash" contained 60.8mg of

protein with an enzyme activity of 898 μmol min^{-1}. The specific activity had correspondingly decreased to 14.7 μmol min^{-1} mg^{-1}. Analysis of the "wash" fraction by SDS-PAGE showed that a majority of the contaminants were removed from the column in the initial stage of the wash (Fractions 1- 8 corresponding to Figure 4.18, Lanes D - K respectively).

Finally the neuraminidase was eluted from the column with a 0 - 0.5M gradient of imidazole in wash buffer. The eluted fractions were analysed by SDS - PAGE (Figure 4.18). The neuraminidase eluted mainly in fractions 15-42 and had an apparent molecular mass of approximately 116 kDa. This is higher than expected from the deduced amino acid sequence, which predicted a size of approximately 96 kDa. This discrepancy may be attributed to anomalous behaviour on SDS-PAGE. The peak of neuraminidase activity eluted in the 56 - 86 mM imidazole range, corresponding to fractions 15 - 23 (Figure 4.18, Lanes 15 - 23). Several other co-purified proteins were also present in these fractions. A reduced amount of neuraminidase (as judged by visual analysis of SDS-PAGE gels) was also found in fractions 24 - 33 (Figure 4.18, Lanes 24 - 33) (imidazole concentration of 90 - 124 mM). Considerably lower amounts of co-purifying proteins were observed in these fractions. The purest neuraminidase (as judged by SDS - PAGE) was observed in fractions 34 - 42 (Figure 4.18, Lanes 34 - 42) (imidazole concentration of 127 - 158mM). Although the amount of neuraminidase present in this fraction was low, its purity was higher because no contaminaing proteins were visible in the SDS-PAGE gels of this preparation. These fractions were then pooled and the mixture was termed "pool".

The "pool" fraction contained 4.6mg of protein with an enzyme activity of 16 μmol min^{-1}. 10ml of the "pool" was added to 190ml 0.5 x PBS. Buffer exchange and concentration of the protein were achieved by diafiltration of the "pool"/0.5 x PBS mixture in Vivaspin cetrifugal filter units (Section 2.3.4.2). Ultrafiltration was continued until 1.6ml of protein/PBS remained in the concentration chamber. This protein sample was termed "concentrate A". The specific activity of this the most highly purified neuraminidase sample was 2.9 μmol min^{-1} mg^{-1}.

4.3.5.1.1 Summary of the purification of wild-type neuraminidase

Overall the degree of purification of neuraminidase is not reflected in terms of increasing specific activity. This is because of extensive losses of both protein and activity during the binding and washing steps. Inspite of this sufficient quantities of purified neuraminidase were obtained for kinetic studies and for immunization studies in mice (Chapter 5). The overall purification of neuraminidase from the crude lysate was 0.12 fold with a yield of 0.2%. The values for the yield and purification fold are based on comparison of the starting and final activities, therefore the extensive losses of activity and protein that occur during the purification procedure lead to the low values for yield and purification fold. Finally, a total amount of about 1.6mg of purified neuraminidase was obtained from 1 litre of bacterial culture.

Table 4.1. A representative scheme for the purification of wild-type pneumococcal neuraminidase.

Fraction	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Enzyme activity (µmol/ min/ml)	Total activity (µmol/ min)	Specific activity (µmol/ min/mg)	Purification (fold)	Yield (%)
Crude	9.5	10.4	98	238	2260	22.9	-	100
Outflow	8.7	1.4	11.9	46	396	32.5	1.4	18
Equilibration	5.8	7.6	44.3	193	1122	25.4	1.1	49
Wash	45.0	1.4	60.8	20	898	14.7	0.6	39
Pool	13.8	0.3	4.6	1.2	16	3.5	0.15	0.7
Concentrate A	1.6	1.0	1.6	2.9	4.6	2.9	0.12	0.2

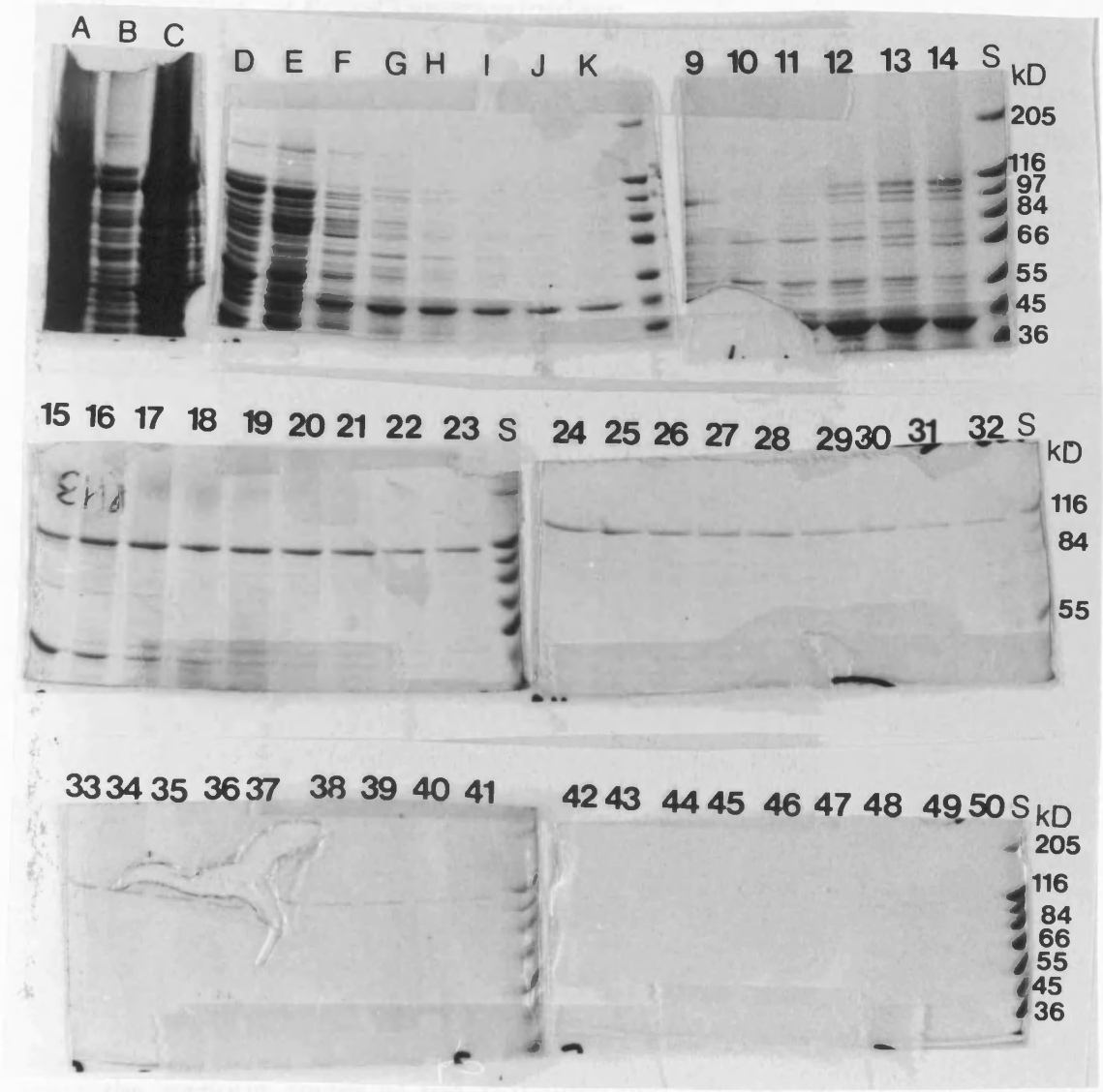


Figure 4.18. Coomassie - blue stained SDS-PAGE gels showing the purification of neuraminidase expressed from plasmid pSSWTvec.
The crude lysate was prepared as described in section 2.3.3.4. 9.5 mls of the lysate was loaded at a flow rate of 3 - 4 column volumes per hour onto a Ni-NTA column (0.9cm diameter x 15cm, 9ml bed volume, Pharmacia), that had been previously equilibrated with equilibration buffer. The flow through was collected (OUTFLOW). The column was washed with equilibration buffer for 20 mins and the eluate collected (EQUILIBRATION). The column was washed for 2.5 hours with wash buffer (pH 6.0) and the outflow collected (WASH FRACTION). Finally the neuraminidase was eluted with a 0 - 0.5M gradient of imidazole in wash buffer (ELUTED FRACTIONS). The column flow-through and eluted products were analysed by SDS-PAGE. Equal volumes (27µl) of each fraction were electrophoresed.

Lane A - CRUDE LYSATE.
Lane B - OUTFLOW
Lane C - EQUILIBRATION
Lanes D - K: WASH FRACTIONS 1 - 8 respectively.
Lanes 9 - 50: ELUTED FRACTIONS 9 - 50 respectively
Lane S - Standard molecular weight markers.

4.3.5.2 Purification of E_{647}>Q neuraminidase

The E_{647}>Q neuraminidase was purified in an identical manner to the wild type neuraminidase. Measurements were made of the volume, protein content and enzymic activity of all fractions. The results of a typical E_{647}>Q neuraminidase purification are summarized in Table 4.2 and SDS-PAGE analysis of the various fractions is shown in Figure 4.19.

Table 4.2. A representative scheme for the purification of E_{647}>Q neuraminidase

Fraction	Total volume (ml)	Protein (mg/ml)	Total protein (mg)
CRUDE	8.5	11.2	95
OUTFLOW	8.3	0.7	5.6
EQUILIBRATION	6.4	7.0	44.8
WASH	47	1.4	65.8
POOL	12.2	0.3	3.2
CONCENTRATE A	1.5	0.9	1.4
CONCENTRATE B	0.9	1.4	1.3
CONCENTRATE C	0.2	4.3	0.7

None of the E_{647}>Q neuraminidase samples showed enzyme activity when assayed using the PNP-NANA method of estimation. Therefore it was not possible to determine values for the degree of purification and the yield. Nonetheless it was still possible to follow the progression of the protein during the various stages of the purifications scheme by reference to the SDS-PAGE gels and by comparisons with the wild type neuraminidase purification.

The purification of the E_{647}>Q neuraminidase was similar to that of the wild type neuraminidase in terms of yield and purity. Crude lysates contining similar amounts of protein (95-98mg) were applied to the Ni-NTA resin in each case. Analysis of the E_{647}>Q neuraminidase "crude" fraction (Figure 4.19, Lane A) shows the presence of many protein bands, including a predominant band of 116 kDa, which was thought to represent the neuraminidase.

The "outflow" fraction contained 5.6 mg of protein and had a similar SDS-PAGE profile to the "crude" but of a reduced intensity (Figure 4.19, Lane B). Again the 116 kDa protein band was prominent. The "equilibrium" fraction

of the E_{647}>Q and wild type neuraminidases contained a similar amount of protein (44.3 and 44.8 mgs respectively). The SDS-PAGE analysis of the former (Figure 4.19, Lane C) showed a protein profile that was similar to the "crude", including a predominant protein band of 116 kDa. This suggested that a proportion of the neuraminidase did not bind the resin and was removed by the equilibration wash.

Similar amounts of protein (61 - 66mgs) were present in the wash fractions of the E_{647}>Q and wild type neuraminidase purifications. The SDS-PAGE analysis of the first nine E_{647}>Q "wash" fractions (Figure 4.19, Lanes D - L) revealed the presence of many protein bands, including a prominent 116 kDa band. This suggested that the washing of the resin removed not only the contaminating proteins but also a high proportion of neuraminidase.

The E_{647}>Q neuraminidase eluted at a similar range of imidazole to the wild type neuraminidase. The peak of E_{647}>Q neuraminidase activity eluted in the 56-86mM imidazole range, corresponding to fractions 15-23 (Figure 4.19, Lanes 15 - 23). A reduced amount of neuraminidase was also present in fractions 24-28 (Imidazole concentration 90 - 105mM) (Figure 4.19, Lanes 24 - 28). Notably fewer contaminating proteins were present in these fractions. The purest neuraminidase (as judged by SDS-PAGE) was present in fractions 29-36 (imidazole concentration 108 - 135mM). Despite the low amount of neuraminidase in these fractions, its purity was higher (as judged by SDS-PAGE) (Figure 4.19, Lanes 29 - 36). These fractions were then pooled. In the first instance the pool (12.2ml) containing E_{647}>Q neuraminidase at a concentration of 0.27mg/ml was concentrated to 0.9mg/ml (1.5ml). This sample was termed "Concentrate A" and contained 1.4 mg of protein. Approximately 1.8 mg of protein (56%) had been lost during the ultrafiltration. The enzyme activity of "concentrate A" was measured using the PNP-NANA method of estimation. Even when the maximum practical assay volume (15µl) was used, absorbance readings similar to that of the blank reaction were obtained. This indicated that the activity of "Concentrate A" was below the detection range of the PNP - NANA assay.

Next the remainder of "Concentrate A" was further concentrated in Vivaspin concentrators to 1.4mg/ml. This sample, termed "Concentrate B" was removed and assayed for protein concentration and enzymic activity. The protein loss from the second round of ultrafiltration was much lower

compared to the first round and 1.3mg of protein was recovered. "Concentrate B"also failed to show enzymic activity even when the maximum assay volume of 15µl was used in the PNP-NANA assay. Finally 0.8ml of "Concentrate B" was diafiltrated until 0.17ml remained in the concentration chamber. This sample was termed "Concentrate C" and contained 0.7mg of protein. Assays for enzyme activity were once again negative. Therefore it was concluded that the E_{647}>Q mutation abolished the activity of the neuraminidase to a level that cannot be measured by the PNP - NANA assay.

4.3.5.2.1 Summary of the purification of E_{647}>Q neuraminidase

Overall the purification of E_{647}>Q neuraminidase was similar to that of the wild type in terms of elution and final yield. A major difference was observed in the amount of protein that did not bind to the Ni-NTA resin and was present in the outflow fraction. The E_{647}>Q neuraminidase "outflow" contained 50% less protein than its wild type equivalent. This suggested an improved binding of proteins to the resin in the E_{647}>Q neuraminidase purification. "The "equilibration" fraction contained approximately the same amount of protein in both the wild type and E_{647}>Q neuraminidase purifications. The "wash" fraction from the E_{647}>Q neuraminidase and wild type purification also contained comparable amounts of total protein (66 and 61mg respectively). The neuraminidase eluted at approximately the same range of imidazole in the two purifications. The total yield of the E_{647}>Q neuraminidase (from 1 litre of bacterial culture) after the initial vivaspin diafiltration was 1.4 mg which is comparable to the 1.6mg obtained from the purification of the wild type neuraminidase.

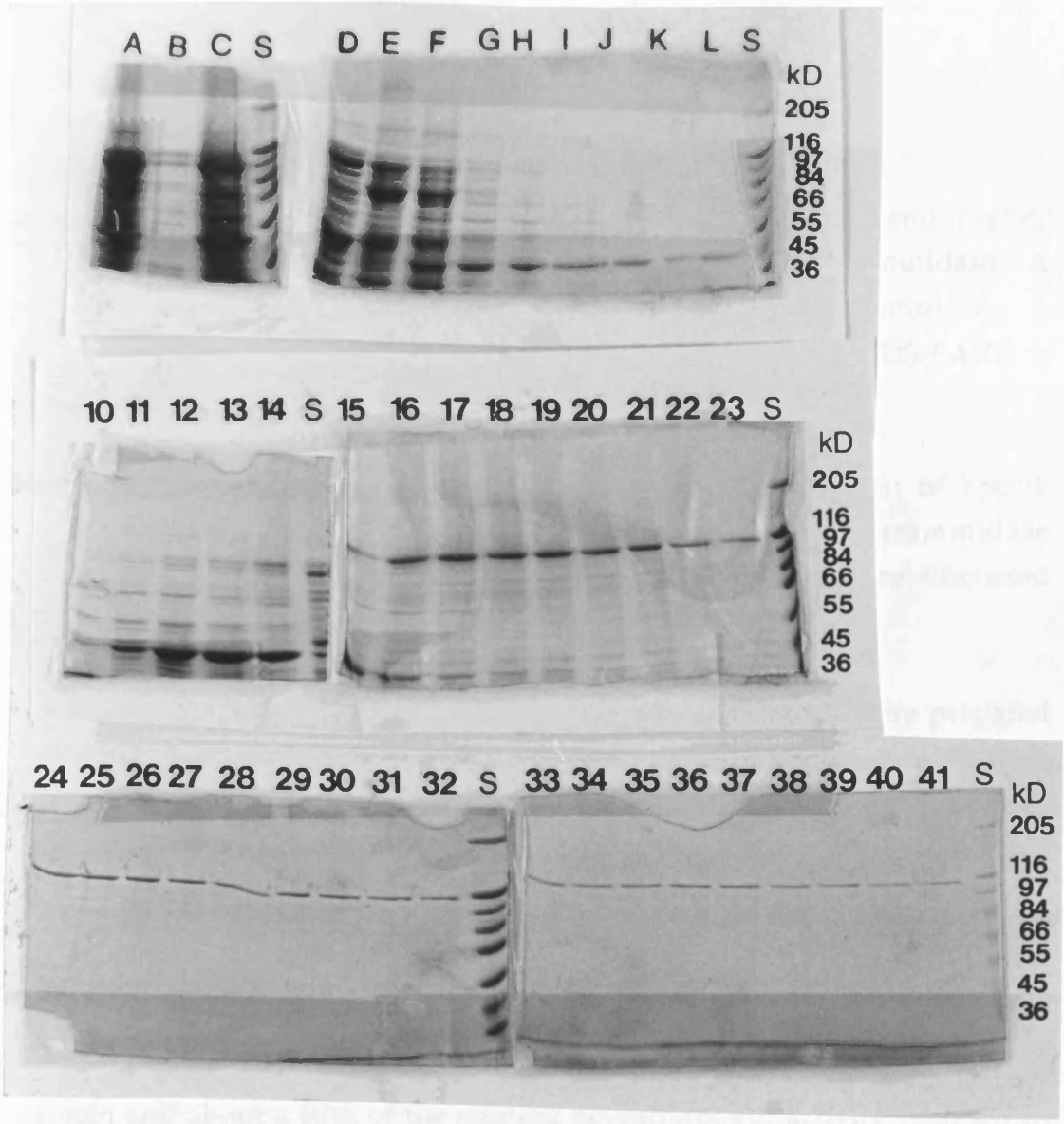


Figure 4.19. Coomassie - blue stained SDS-PAGE gels showing the purification of neuraminidase expressed from plasmid pSSEQvec.
The crude lysate was prepared as described in section 2.3.3.4. 8.5 mls of the lysate was loaded at a flow rate of 3 - 4 column volumes per hour onto a Ni-NTA column (0.9cm diameter x 15cm, 9ml bed volume, Pharmacia), that had been previously equilibrated with equilibration buffer. The flow through was collected (OUTFLOW). The column was washed with equilibration buffer for 20 mins and the eluate collected (EQUILIBRATION). The column was washed for 2.5 hours with wash buffer (pH 6.0) and the outflow collected (WASH FRACTION). Finally the neuraminidase was eluted with a 0 - 0.5M gradient of imidazole in wash buffer (ELUTED FRACTIONS). The column flow-through and eluted products were analysed by SDS-PAGE. Equal volumes (27µl) of each fraction were electrophoresed.

Lane A - CRUDE LYSATE
Lane B - OUTFLOW
Lane C - EQUILIBRATION
Lanes D - L: WASH FRACTIONS 1 - 9 respectively.
Lanes 10 - 41: ELUTED FRACTIONS 10 - 41 respectively
Lane S - Standard molecular weight marker

4.3.5.3 Purification of Y_{752}>F neuraminidase

The procedure utilized for the purification of the wild type and E_{647}>Q neuraminidases was also used for purifying the Y_{752}>F neuraminidase. A detailed analysis of an example purification of Y_{752}>F neuraminidase is shown in Table 4.3. The analysis of the various fractions by SDS-PAGE is shown in Figure 4.20.

The various binding and washing steps used in the purification of Y_{752}>F were identical to those of the wild type and E_{647}>Q neuraminidase purifications. The salient features of the Y_{752}>F purification are discussed below.

Crude lysates of *E.coli* SG13009:pREP4 harbouring pSSYFvec were prepared as described previously (Section 2.3.3.4). Analysis of this sample by SDS-PAGE (Figure 4.20, Lane A) shows the presence of several intensely stained protein bands. The most intense band was of approximately 116 kDa and was thought to be the neuraminidase. 8ml of this crude lysate containing 80mg of total protein with a neuraminidase activity of 1.1 μmol min^{-1} was applied to the Ni-NTA resin.

The unbound material (outflow) was collected and found to contain 10.8 mg of protein and about a fifth of the starting neuraminidase activity (0.21 μmol min^{-1}). The specific activity of the neuraminidase in the "outflow" fraction was 0.02 μmol min^{-1} mg^{-1}. The SDS-PAGE profile of the "outflow" was identical to that of the "crude" but of a lower intensity (Figure 4.20, Lane B). Once again the most prominant protein band had a molecular weight of 116 kDa which suggested that a large proportion of the neuraminidase (116 kDa protein) had not bound to the Ni-NTA resin.

Next 3ml of "equilibrium" with a total protein content of 20.7mg was collected. A preliminary assay using the maximum practical volume of "equilibrium" in the assay (15μl) gave absorbance readings which were above the linear range of the assay. Unfortunately more accurate measurements using less enzyme were not made. Therefore it was concluded that the "equilibration" fraction had neuraminidase activity but its level was unknown. However it can be stated that the enzyme activity of the "equilibrium" was at least 0.012 μmol min^{-1} ml^{-1} which is the

minimum value that can be measured by the assay. The SDS-PAGE profile of the "equilibrium" showed a similar intensity and distribution of protein bands to the "crude" fraction (Figure 4.20, Lanes C). Once again the 116 kDa protein band predominated, suggesting the removal of a large proportion of neuraminidase from the resin by the equilibrium wash.

A total of 36ml of "wash" was collected. The major loss of protein and enzyme activity were observed at this stage. The "wash" contained 81mg of protein with an enzyme activity of 1.1 μmol min^{-1}. The specific activity had decreased to 0.012 μmol min^{-1} mg^{-1}. Analysis of the pooled "wash" fractions by SDS-PAGE revealed the presence of many protein bands (Figure 4.20, Lane D). The 116 kDa neuraminidase band predominated which suggested that a significant proportion of neuraminidase was removed from the column during the wash step.

The elution of the Y_{752}>F neuraminidase followed a similar pattern to the wild type and E_{647}>Q neuraminidases. A large proportion of the neuraminidase eluted in fractions 15 - 23 (45 - 69 mM imidazole) (Figure 4.20, Lanes 15 - 23) along with several other co-purified proteins. A reduced amount of neuraminidase (as judged by visual analysis of SDS-PAGE gels) eluted in fractions 24 - 33 (72 - 99 mM imidazole) (Figure 4.20, Lanes 24 - 33). Considerably lower amounts of co-purifying proteins were observed in these fractions. The purest neuraminidase (as judged by SDS - PAGE) was observed in fractions 34 - 42 (102 - 126 mM imidazole) (Figure 4.20, Lanes 34 - 42). Although the amount of protein present was low, its purity was higher because no contaminaing bands were evident in the preparation. These fractions were pooled and the mixture was termed "pool". The "pool" contained 3.6mg of protein. No enzyme activity was detectable in the "pool" using the PNP-NANA assay.

The "pool" was concentrated to 4.4mg/ml (using Vivaspin cetrifugal filter units as described in Section 2.3.4.2) and a final volume of 0.28ml. This sample was termed "concentrate A" and consisted of 1.23 mg of neuraminidase. No enzyme activity was detectable in "concentrate A" even when the maximum allowable volume (15μl) of enzyme was assayed. In comparison the wild type "concentrate A" had a specific activity of 2.9 μmol min^{-1} mg^{-1}. Therefore it was concluded that the Y_{752}>F mutation reduced

the activity of the neuraminidase to a level that was not detectable by the PNP-NANA assay.

4.3.5.3.1 Summary of the purification of Y_{752}>F neuraminidase

Overall the purification of Y_{752}>F neuraminidase was similar to that of the wild type in terms of binding, elution and final yield. From 1 litre of culture of *E. coli* harbouring pSSYFvec a total of 80mg of total protein was obtained, this was comparable to the 98mg of total protein obtained from a 1 litre culture of pSSWTvec. By comparison of the SDS-PAGE of the crude lysates its apparent that the neuraminidase content of both is similar. A difference was observed in the volume and protein content of the "equilibration" and "wash" fractions compared to those obtained during the purification of the wild type neuraminidase. This was most likely a consequence of technical malfunctioning of the pump which caused a reduced flow of the equilibrium and wash buffers onto the column. The reduced amount of protein present in the equilibrium fraction is compensated by an increased amount of protein in the wash fraction of the Y_{752}>F neuraminidase purification. Therefore in terms of the amount of protein coming off the column, there was no difference between the wild type and Y_{752}>F neuraminidase purifications. The neuraminidase eluted at approximately the same range of imidazole in the two purifications. The total yield of the Y_{752}>F neuraminidase (from 1 litre of bacterial culture) after the initial vivaspin diafiltration was 1.2 mg which is comparable to the 1.6mg obtained from the purification of the wild type neuraminidase.

Table 4.3. A representative scheme for the purification of Y752>F neuraminidase

Fraction	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Enzyme activity (µmol/ min/ml)	Total activity (µmol/ min	Specific activity (µmol/ min/mg)	Purification (fold)	Yield (%)
Crude	8	10	80	0.14	1.12	0.014	-	100
Outflow	7.7	1.4	10.8	0.03	0.21	0.02	1.4	18.75
Equilibration	3	6.9	20.7					
Wash	36	2.25	81	0.03	1.08	0.012	0.8	96.4
Pool	11	0.33	3.63					
Concentrate A	0.28	4.4	1.23					

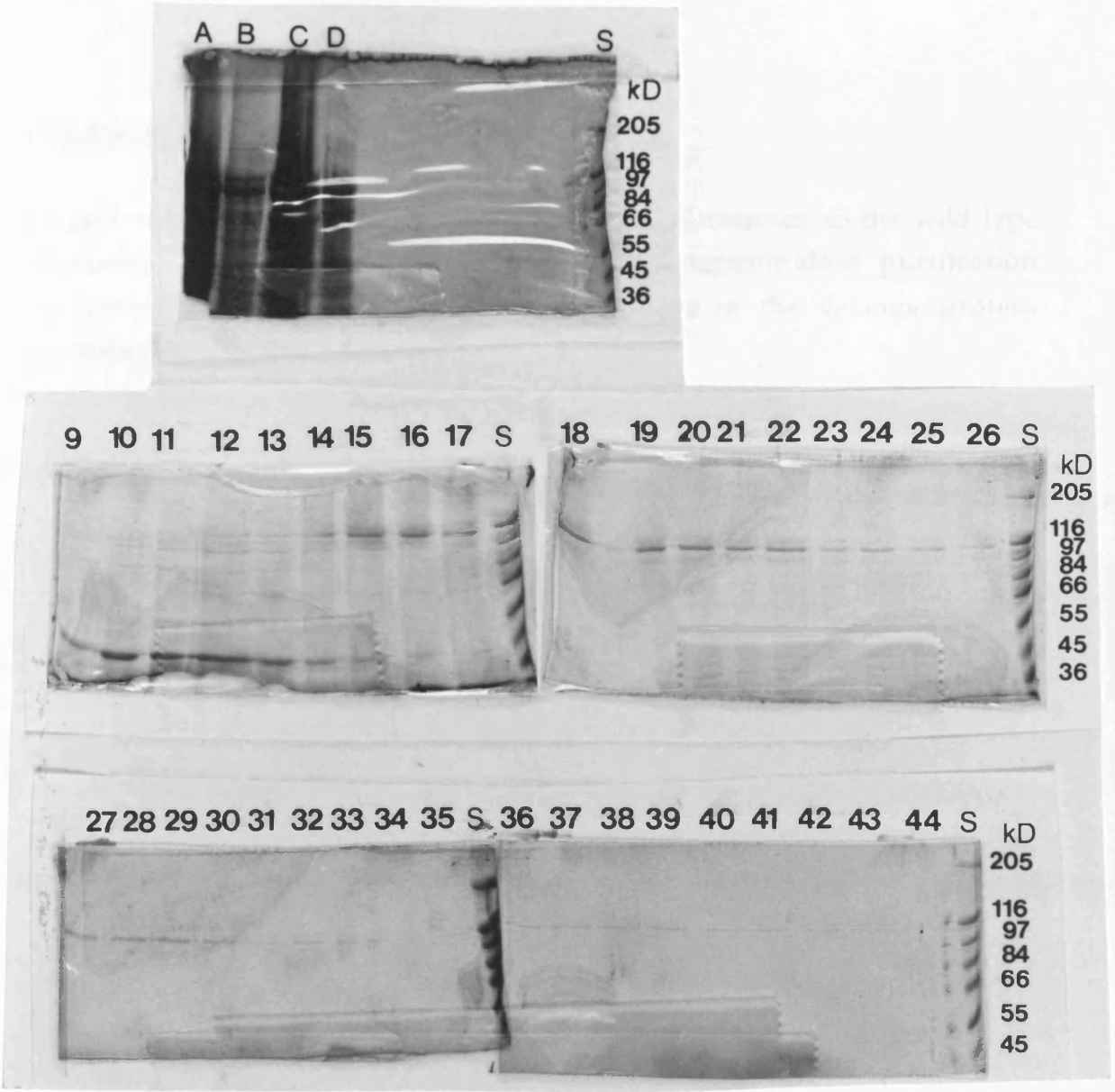


Figure 4.20. Coomassie - blue stained SDS-PAGE gels showing the purification of neuraminidase expressed from plasmid pSSYFvec.
The crude lysate was prepared as described in section 2.3.3.4. 8 mls of the lysate was loaded at a flow rate of 3 - 4 column volumes per hour onto a Ni-NTA column (0.9cm diameter x 15cm, 9ml bed volume, Pharmacia), that had been previously equilibrated with equilibration buffer. The flow through was collected (OUTFLOW). The column was washed with equilibration buffer for 20 mins and the eluate collected (EQUILIBRATION). The column was washed for 2.5 hours with wash buffer (pH 6.0) and the outflow collected (WASH FRACTION). Finally the neuraminidase was eluted with a 0 - 0.5M gradient of imidazole in wash buffer at a flow rate of 3 column volumes per hour (ELUTED FRACTIONS). The column flow-through and eluted products were analysed by SDS-PAGE. Equal volumes (27µl) of each fraction were electrophoresed.

Lane A - CRUDE LYSATE.
Lane B - OUTFLOW
Lane C - EQUILIBRATION
Lanes D : Pooled WASH FRACTIONS.
Lanes 9 - 44: ELUTED FRACTIONS 9 - 44 respectively
Lane S - Standard molecular weight markers.

4.3.5.4 Purification of R_{663}>H neuraminidase

R_{663}>H neuraminidase was purified in an identical manner to the wild type neuraminidase. The results of a typical R_{663}>H neuraminidase purification are shown in Table 4.4. The SDS-PAGE analysis of the various protein fractions is shown in Figure 4.21.

Crude lysate was prepared from a 1 litre culture of *E. coli* harbouring pSSRHvec. 8.5 ml of the crude lysate containing 78mg of total protein with a total neuraminidase activity of 33.6 µmol min^{-1} was applied to the Ni-NTA resin. The unbound material, "outflow" contained 4.4mg of protein with a total neuraminidase activity of 1.7 µmol min^{-1}. Analysis of the "crude" and "outflow" fractions (Figure 4.21, Lanes A and B respectively) revealed a similar protein banding pattern. The protein bands from the "outflow" fraction were of a reduced intensity. In both fractions the 116 kDa neuraminidase predominated.

The "equilibration" fraction contained 23.9mg of protein with a total neuraminidase activity of 14.6 µmol min^{-1}. This represented 30% of the total protein and 50% of the neuraminidase activity that was present in the crude lysate. The SDS-PAGE analysis of the "equilibration" fraction (Figure 4.21, Lane C) was very similar to the "crude lysate". Many protein bands were present with a prominent 116 kDa neuraminidase band. This suggested that the proteins which did not bind the resin with a high affinity were eluted in the "equilibrium" fraction.

40ml of "wash" was collected and found to contain 68mg of total protein and 27 µmol min^{-1} of activity. This greatest loss of protein and enzyme activity occured at this stage. Analysis of the pooled wash fractions by SDS-PAGE showed the presence of many protein bands including a prominent neuraminidase band at about 116 kDa (Figure 4.21, Lane D).

The elution profile of the R_{663}>H neuraminidase was similar to that of the wild type neuraminidase. The neuraminidase started eluting at about 60mM imidazole which corresponded to fraction 24. In fractions 24 - 32 (imidazole range 60 - 80mM), an intense neuraminidase band of 116 kDa was observed accompanied by several contaminating protein bands of a lower intensity (Figure 4.21, Lanes 24 - 32). The majority of the neuraminidase eluted in fractions 33 - 41 (82 - 102mM imidazole). This neuraminidase was also

accompanied by co-purifying proteins (Figure 4.21, Lanes 33 - 41). The purest neuraminidase eluted in fractions 42 - 50 (105 - 125mM imidazole) (Figure 4.21, Lanes 42 - 50). These fractions were pooled and the mixture was designated the "pool". The "pool" contained 2.7 mg of protein with a total activity of 0.6 μmol min^{-1}. At this stage a 0.48 fold purification of the neuraminidase with a specific activity of 0.2 μmol min^{-1} mg^{-1} was achieved.

9.15ml of "pool" was reduced to a final volume of 0.330ml in Vivaspin centrifugal filter units (Section 2.3.4.2). This protein sample was termed "concentrate A" and consisted of 1.5 mg of neuraminidase. The protein concentration had increased from 0.3mg/ml in the "pool" to 4.5mg/ml in "concentrate A". Approximately 50% of the protein was lost during the ultrafiltration procedure, and the total activity had decreased from 0.6 to 0.1 μmol min^{-1}. The specific activity of the most highly purified neuraminidase was 0.06 μmol min^{-1} mg^{-1}. By comparison the wild type "concentrate A" had a specific activity of 2.9μmol min^{-1} mg^{-1}. The purification of neuraminidase from the pool was 0.14 fold with a yield of 0.29%. Once again the extensive losses of activity and protein that occur during the purification procedure are reflected in the low values for yield and purification fold.

4.3.5.4.1 Summary of the purification of R_{663}>H neuraminidase

The purification of R_{663}>H neuraminidase was similar to that of wild type neuraminidase in terms of binding, washing and elution of the protein. A total amount of 1.5 mg of purified neuraminidase was obtained from 1 litre of bacterial culture, this was comparable to the amounts of wild type, E_{647}>Q and Y_{752}>F neuraminidases that were obtained.

Table 4.4 A representative scheme for the purification of R663>H neuraminidase

Fraction	Total volume (ml)	Protein (mg/ml)	Total protein (mg)	Enzyme activity (µmol/ min/ml)	Total activity (µmol/ min)	Specific activity (µmol/ min/mg	Purification (fold)	Yield (%)
Crude	8.5	9.2	78	3.9	33.64	0.42	-	100
Outflow	8	0.55	4.4	0.2	1.7	0.38	0.9	5
Equilibration	4.5	5.3	23.9	3.2	14.6	0.61	1.4	43
Wash	40	1.7	68	0.7	27	0.40	0.9	80
Pool	9.15	0.3	2.7	0.06	0.6	0.2	0.48	1.8
Concentrate	0.330	4.5	1.5	0.29	0.1	0.06	0.14	0.29

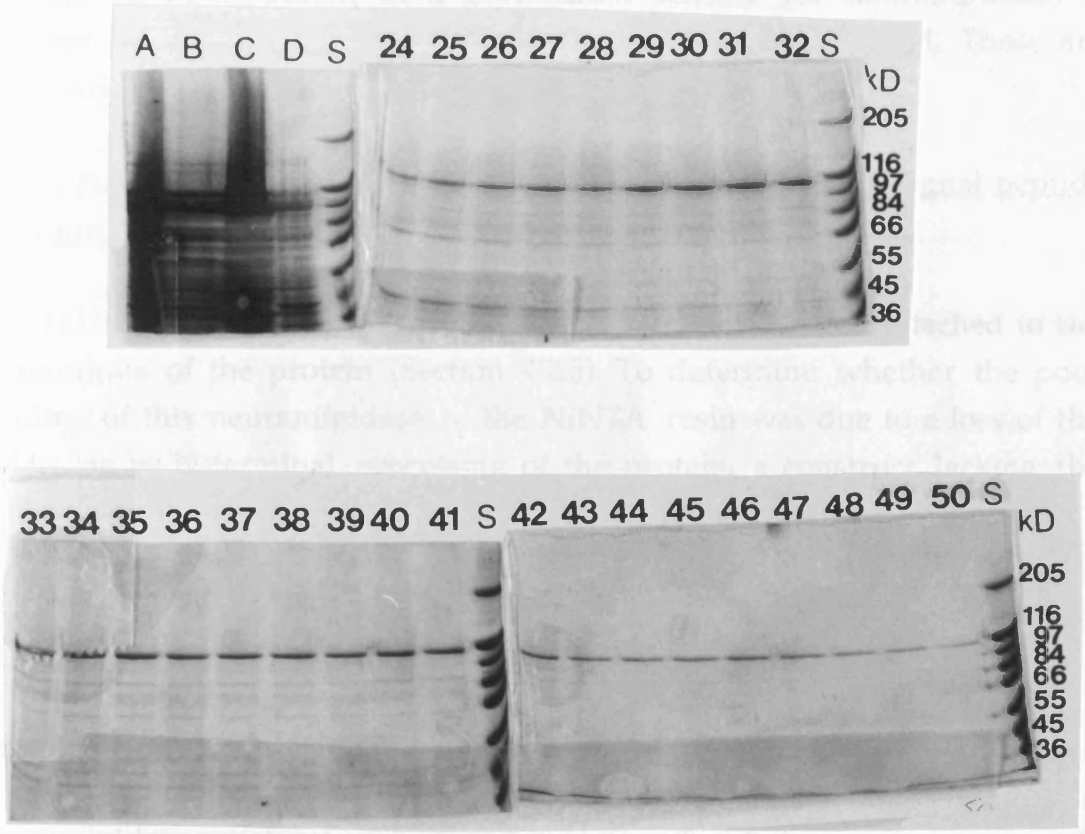


Figure 4.21. Coomassie - blue stained SDS-PAGE gels showing the purification of neuraminidase expressed from plasmid pSSRHvec.
The crude lysate was prepared as described in section 2.3.3.4. 8.5 mls of the lysate was loaded at a flow rate of 3 - 4 column volumes per hour onto a Ni-NTA column (0.9cm diameter x 15cm, 9ml bed volume, Pharmacia), that had been previously equilibrated with equilibration buffer. The flow through was collected (OUTFLOW). The column was washed with equilibration buffer for 20 mins and the eluate collected (EQUILIBRATION). The column was washed for 2.5 hours with wash buffer (pH 6.0) and the outflow collected (WASH FRACTION). Finally the neuraminidase was eluted with a 0 - 0.5M gradient of imidazole in wash buffer at a flow rate of 2 - 3 column volumes per hour (ELUTED FRACTIONS). The column flow-through and eluted products were analysed by SDS-PAGE. Equal volumes (27µl) of each fraction were electrophoresed.

Lane A - CRUDE LYSATE.
Lane B - OUTFLOW
Lane C - EQUILIBRATION
Lane D : Pooled WASH FRACTIONS
Lanes 24 - 50: ELUTED FRACTIONS 24 - 50 respectively
Lane S - Standard molecular weight markers.

4.3.6 Alternative purification schemes

During the development of a purification scheme for neuraminidase a number of additional purification techniques were investigated. These are summarized below.

4.3.6.1 Purification of a neuraminidase lacking the N-terminal signal peptide (38 amino acids) and the C-terminal anchor motif.

The 6xHis tag used for purification of the neuraminidase was attached to the N-terminus of the protein (Section 4.2.5). To determine whether the poor binding of this neuraminidase to the NiNTA resin was due to a loss of the 6xHis tag by N-terminal processing of the protein, a construct lacking the signal peptide was created. This truncated neuraminidase protein was missing the first 38 amino acids (114 bp) containing the putative signal peptide domain, and also had a deletion of 235 amino acids (705 bp) from the C-terminus. It was predicted that the loss of the 235 amino acids from the C-terminus would not result in loss of enzyme activity because a similar deletion of the *nanA* gene has been shown to produce active neuraminidase (Camara *et al* ., 1994). The production of this truncated neuraminidase construct termed pSSGT is described below.

4.3.6.1.1 Amplification and cloning of the neuraminidase gene lacking 114 bp from the 5' - terminus and 705 bp from the 3' - terminus.

The plasmid pMC4170 (Section 3.1.1) containing the ORF1 (which represented the *nan A* structural gene) was the starting point for the cloning. A 2248 bp fragment of the neuraminidase structural gene was amplified from plasmid pMC4170 using the primers SSNA1 and SSNA2, under standard PCR conditions with *Vent* polymerase (New England Biolabs). The primer SSNA1 annealed at bases 1389 to 1410 in pMC4170. A *Bam* HI site was incorporated into SSNA1 to facilitate cloning into the expression vector pQE30. The primer SSNA2 annealed at position 3653 to 3627 and had a *Sal* I site incorporated into it.

SSNA1 5' TCAGGATCCCAAGAAGGGGCAA 3' (*Bam* HI site underlined)
SSNA2 5'CTAGTCGACCTATTATTTCGCTTCGGT 3' (*Sal* I site underlined)

The PCR product was visualised on a 0.7% (w/v) agarose gel (Figure 4.22). As anticipated, a band of approximately 2.2 kb was visible in lane 4 of Figure 4.22. A "blank" reaction containing no template DNA was included to detect contamination. This negative control reaction gave no bands (Figure 4.22, lanes 2 and 3) thus confirming the successful amplification of the truncated neuraminidase gene.

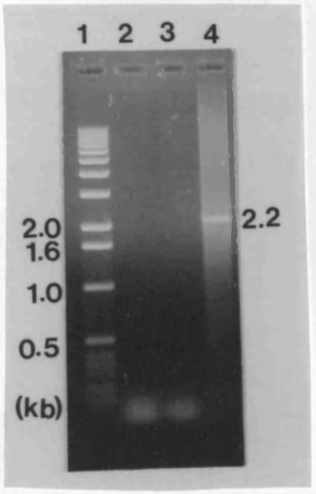


Figure 4.22. PCR amplification of the neuraminidase gene lacking 114 and 705 bps from the N and C termini respectively.
Lane 1: 1kb DNA ladder. Lanes 2 and 3: Negative PCR control (PCR reaction minus template DNA): Lane 4: 2.2 kb PCR product.

4.3.6.1.2 Cloning the truncated neuraminidase gene into pQE 30

The presence of the *Bam* HI site was confirmed by cleaving the PCR product with *Bam* HI and *Eco* RI which resulted in three fragments of 3506 and 1756 bps. The 506 and 1756 bp fragments were visible on a 0.7% (w/v) agarose gel (Figure 4.23, lane 2). Confirmation of the presence of the *Sal* I site was made by cleaving the PCR product with *Sal* I and *Eco* RI which resulted in fragments of 9509 and 1747 bps. The 509 and 1747 bp fragments were visible on a 0.7% (w/v) agarose gel (Figure 4.23, lane 3).

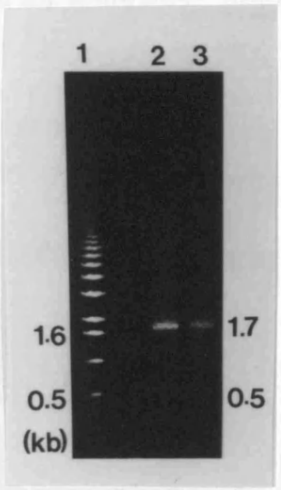


Figure 4.23. Cleavage of the truncated PCR product by *Bam* HI and *Eco* RI or by *Sal* I and *Eco* RI.
Lane 1: 1 kb DNA ladder. Lane 2: PCR product cleaved with *Bam* HI and *Eco* RI. Lane 3: PCR product cleaved with *Sal* I and *Eco* RI.

The PCR product (15µg) (Insert) and the expression vector pQE30 (15µg) were digested with *Bam* H I and *Sal* I. The vector and insert were then gel-purified (Section 2.2.2.1) prior to insert ligation in order to remove residual nicked and supercoiled plasmid.

Ligation reactions were set up between the *Bam* H I and *Sal* I cleaved PCR product and the *Bam* H I and *Sal* I cleaved pQE30 vector. The ratios of insert to vector used were: 3:1, 1.5:1 and 6:1. The ligation reactions were transformed into electrocompetent JM109 cells. To determine the outcome of the cloning, small scale DNA was prepared from 12 colonies and digested with *Bam* H I and *Sal* I. The resulting fragments were analysed by agarose gel electrophoresis (Figure 4.24).

Restriction digestion of miniprep DNA with *Bam* H I and *Sal* I was anticipated to release the insert from the vector and this would be evidenced by two bands of 3462 and 2248 bps. Restriction digestion of miniprep DNA 2 (Figure 4.24, lane 2) produced bands of approximately 3.4 and 2.2 kb which corresponded to the expected size of the insert released from vector. Cleavage of the other miniprep DNAs produced a restriction pattern that

was not consistent with the expectation and were excluded from further analysis.

Clone 2 representing the 2.2 kb truncated neuraminidase gene ligated into pQE30 was selected for future work and termed pSSGT. This plasmid pSSGT was transformed into SG13009:pREP 4 for protein expression and subsequent purification studies.

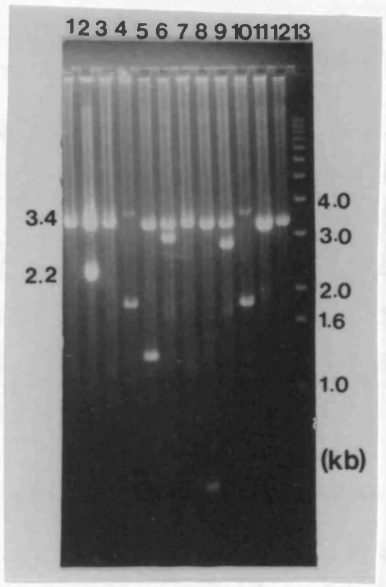


Figure 4.24 Cleavage of minipreps 1 - 12 with *Bam* H I and *Sal* I.
The 2.2 kb truncated neuraminidase gene was cloned into the pQE30 vector. DNA was prepared on a small scale from 12 transformants, cleaved with *Bam* H I and *Sal* I and the resulting fragments analysed by electrophoresis.
Lanes 1 - 12: minipreps 1 - 12 cleaved with *Bam* H I and *Sal* I. Lane 13: 1kb DNA ladder.

Crude protein extracts prepared from this construct were purified by the original purification protocol (Section 2.3.3.6.1). The only change to the protocol was that a pH 6.0 wash buffer was used. The crude (Figures 4.10), unbound and eluted fractions (Figure 4.11) were analysed by SDS-PAGE and are shown in Figures 4.10 and 4.11. An intense band of about 90 kDa was visible in the crude fraction (Figure 4.10, lane G) and was thought to represent the neuraminidase. The molecular mass of the neuraminidase was higher than expected from the deduced amino acid sequence , which predicted a size of approximately 83 kDa. This discrepancy may be attributed to anomalous behaviour on SDS-PAGE. In the unbound fractions (Figure 4.11, lanes A, B and C) several protein bands were visible but the (90 kDa) band corresponding to the neuraminidase was very feint. This suggested

that the majority of the neuraminidase had bound to the resin. Analysis of the eluted fractions (Figure 4.11, lanes 10 - 23) demonstrated that the majority of the neuraminidase co-eluted with contaminating proteins.

The neuraminidase encoded by pSSGT appeared to have improved binding to the resin because less of it was present in the unbound fractions. However the purity of the eluted protein was reduced. In an effort to improve the purity of the eluted neuraminidase the above experiment was repeated using pH 5.5 wash buffer. The crude, unbound and eluted fractions were analysed by SDS-PAGE (Figure 4.10). The improved binding of the neuraminidase was again observed. The neuraminidase was also undetectable in the wash fractions (Figure 4.10, lanes D, E and F) which suggested that the neuraminidase remained bound to the resin under stringent wash conditions. However the yield of the eluted neuraminidase was now so diminished that it was difficult to determine if the reduction in pH of the wash buffer resulted in increased purity of the neuraminidase. In view of these findings no further work was done on the pSSGT derived protein.

To summarize the removal of 114 and 705 bases from the 5' and 3' ends of the *nan* A gene respectively resulted in improved binding of the encoded protein to the Ni-NTA resin. However the purity of the eluted neuraminidase was decreased compared to the wild type neuraminidase. It would have been worthwhile experimenting with purification conditions which improved the purity of the neuraminidase but did not significantly compromise the yield of the protein. However time constraints made further studies impractical. Furthermore by this stage mutagenised constructs containing the signal peptide had already been made. Therefore it was decided to persevere with purification of the original pSSEQvec, pSSYFvec and pSSRHvec constructs. Furthermore the purified neuraminidase encoded by pSSGT was enzymically active as judged by the presence of fluoresence on incubation with MUAN. Quantitative assays of enzyme activity with PNP-NANA were not performed.

Chapter 5: Investigations of the protective effects of the mutated neuraminidases.

5.1 Introduction

Lock and co-workers (1988a) found that native neuraminidase was not a protective immunogen in mice. Their explanation for this finding was that the toxicity of the enzyme outweighed its protective ability. This hypothesis was supported by experiments which showed that treatment of native neuraminidase with formaldehyde prior to immunization led to a significant increase in the survival time of mice subsequently challenged with virulent *S. pneumoniae*. (Lock *et al* ., 1988a). This method of enzyme inactivation removed the toxicity of the enzyme, but potentially at the expense of immunogenicity. Formalization may alter potentially protective epitopes thereby reducing the protective potential of the neuraminidase (Lock *et al* ., 1988a).

A better approach would be to target the active site of the neuraminidase by site-directed mutagenesis of the neuraminidase gene. This approach would have two advantages, firstly protein engineering would create a product whose toxicity was reduced but whose immunogenicity was unaltered. Secondly the reduction in toxicity would not be dependent on the effectiveness of the formalization procedure and so the product would be safer and more amenable to quality control. This proposition was supported by studies on the pneumococcal toxin pneumolysin. A pneumolysin with a W>F mutation was a superior immunogen compared to a chemically inactivated wild-type version (Mitchell *et al* ., 1992).

To this end we set about testing the genetically altered neuraminidases that had been made as part of a structural study on neuraminidase (Section 3.2). To test whether the mutated neuraminidases were immunogenic in mice a series of immunization experiments were conducted. The first mutant constructed was E_{647}>Q, and this was tested in parallel with wild-type neuraminidase. Whilst this test was in progress, mutants R_{663}>H and Y_{752}>F were created. These genetically altered neuraminidase toxoids were then

tested. Finally the basis of the protection afforded by the E_{647}>Q neuraminidase toxoid was examined.

5.2 Inactivation of wild-type neuraminidase

5.2.1 Introduction

The original plan was to compare the protective ability of the genetically altered neuraminidases, with wild type neuraminidase inactivated by treatment with formaldehyde. Difficulties arose with the formaldehyde treatment that made this goal unfeasable (Section 5.2.2). Therefore the genetically altered neuraminidases were compared with wild type neuraminidase that had been inactivated by heat treatment (Section 5.2.3). These experiments could not test the original hypothesis that enzyme inactivation by genetic means was superior to enzyme inactivation by treatment with formaldehyde, but it could give valuable information about the immunogenicity of genetically manipulated neuraminidases.

5.2.2 Inactivation of wild type neuraminidase by treatment with formaldehyde.

Lock and co-workers investigated the optimal conditions (maximum inactivation with minimal reduction in immunogenicity) for formaldehyde treatment of neuraminidase (Lock *et al* ., 1988a). They found that exposure of neuraminidase to formaldehyde at a concentration of 3.4% (v/v) for 18 hours at 23°C, did not significantly reduce its immunoreactivity but decreased its activity against the artificial substrate MUAN by 60%. Immunization of mice with neuraminidase treated in this way increased the survival time of mice subsequently challenged with *S. pneumoniae*.

After considering these results, it was decided to treat the wild type neuraminidase with 3.4% (v/v) formaldehyde for 18 hours at 23°C. It was then necessary to remove the formaldehyde due to its toxicity in mice. This was achieved by dialysis against 0.5 x PBS. When the formaldehyde-treated dialysed protein sample was analysed by SDS-PAGE, no bands were visible. The absence of protein bands on the gel was attributed to the low concentration of protein in the dialysed sample. To verify this assumption,

the dialysed protein sample was concentrated by the use of Amicon stirred cells and analysed by SDS-PAGE. Again no protein was visible on the gels.

To ascertain if the protein loss resulted from the formaldehyde exposure, dialysis or amicon concentration, it was necessary to analyse the effect of each treatment individually. Initially the effect of formaldehyde treatment on neuraminidase was examined. Equal amounts of purified neuraminidase were placed in two tubes. To one tube 3.4% (v/v) formaldehyde was added, and no additions were made to the control tube. Both tubes were incubated at 23°C for 18 hours and then the protein samples were analysed by SDS-PAGE. A protein band was present from the untreated sample but absent from the formaldehyde treated sample (Figure 5.1). This result suggested that the formaldehyde treatment was causing destruction of the neuraminidase.

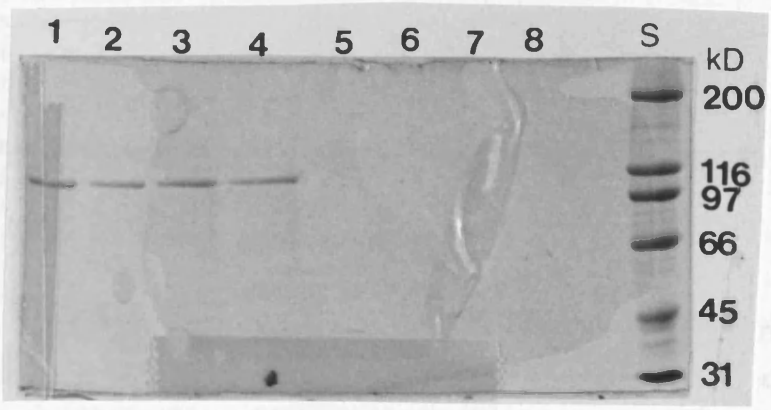


Figure 5.1 Formaldehyde treatment of neuraminidase.
100µl aliquots of neuraminidase were incubated either in the presence or absence of 3.4 % (v/v) formaldehyde for 18 hours at 23°C. 27µl samples were then analysed by SDS-PAGE.
Lanes 1 - 4: SDS-PAGE analysis of purified neuraminidase incubated at 23°C for 18 hours.
Lanes 5 - 8: SDS-PAGE analysis of purified neuraminidase incubated in the presence of 3.4 % (v/v) formaldehyde at 23°C for 18 hours.
Lane S: Protein molecular weight standards.

Contrary to the findings of Lock *et al* ., (1988a), our experiments have shown that treatment of neuraminidase with 3.4% (v/v) formaldehyde was inappropriate. This led to investigation of the effects of different formaldehyde concentrations and incubation periods on neuraminidase. Neuraminidase samples were exposed to the following formaldehyde concentrations: 0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.4% (v/v) for 18 hours at 23°C. The samples were then analysed by SDS-PAGE. No protein bands were discernible from any of the samples which had formaldehyde added to them (irrespective of the formaldehyde concentration). The neuraminidase

sample that had no fomaldehyde added to it was clearly visible on the SDS-PAGE gel (Figure 5.2, lane 1). This suggested that in addition to the formaldehyde concentration, other factors such as the length and temperature of incubation may have been important.

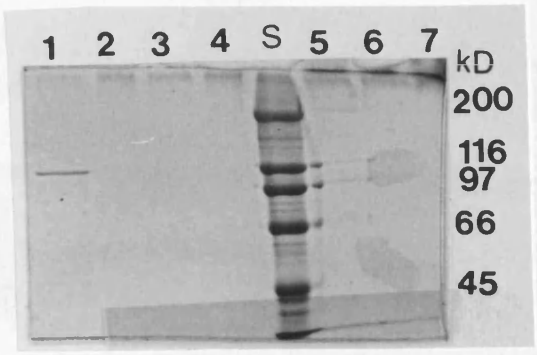


Figure 5.2. Effect of various formaldehyde concentrations on neuraminidase.
100µl samples of purified neuraminidase were exposed to the following formaldehyde concentrations: 0, 0.5, 1.0, 1.5, 2.0, 2.5 and 3.4% (v/v) for 18 hours at 23°C. 27µl samples were then analysed by SDS-PAGE.
Lane 1: Untreated neuraminidase.
Lane 2: Neuraminidase exposed to 0.5 % formaldehyde.
Lane 3: Neuraminidase exposed to 1.0 % formaldehyde.
Lane 4: Neuraminidase exposed to 1.5 % formaldehyde.
Lane S: Protein molecular weight standards.
Lane 5: Neuraminidase exposed to 2.0 % formaldehyde.
Lane 6: Neuraminidase exposed to 2.5 % formaldehyde.
Lane 7: Neuraminidase exposed to 3.4 % formaldehyde.

The next step was to examine the effects of different periods of formaldehyde exposure. Equal amounts of purified neuraminidase were placed in two clean tubes. To one tube formaldehyde was added to a final concentration of 3.7% (v/v) and to the control tube no additions were made. Then immediately 100µl samples were removed from both tubes and frozen at -70°C (0 hrs). Both tubes were then incubated at 23°C. At 15 minute intervals 100µl of the protein was removed from each tube and frozen at -70°C. These procedures were continued for 5.5 hours. The frozen samples were thawed and analysed by SDS-PAGE. Protein bands were visible from all the control samples not exposed to formaldehyde but not from any of the formaldehyde-treated samples. Even when the protein was analysed immediately after the addition of formaldehyde (0 hrs), no bands were visible (Figure 5.3, lane 2). An explanation for this observation was that the formaldehyde was causing the protein to precipitate out of solution.

This use of formaldehyde as a method of enzyme inactivation was discarded due to the inability to recover the protein. An alternative method of enzyme inactivation by heat treatment was investigated (Section 5.2.3).

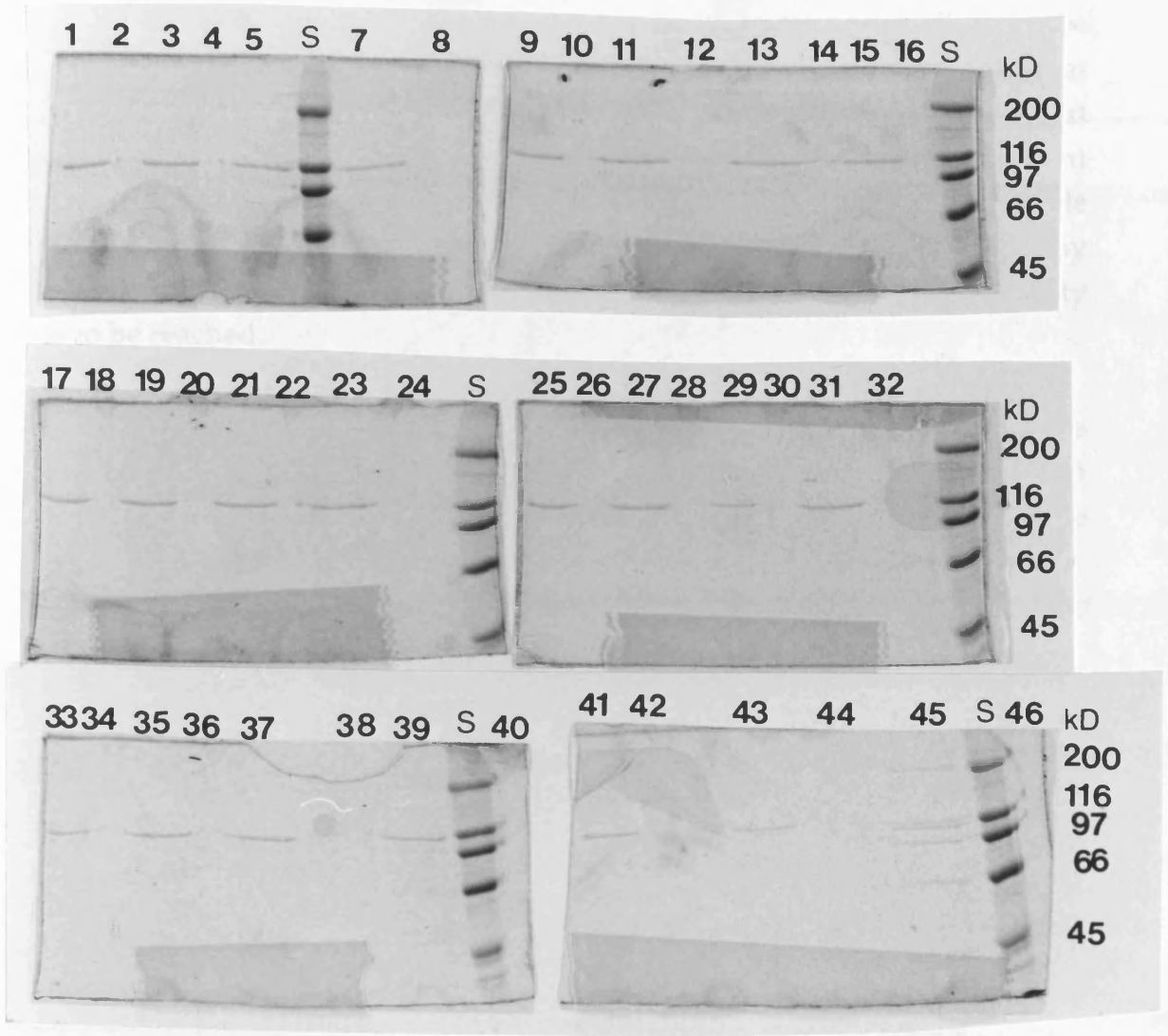


Figure 5.3. Coomassie stained SDS-PAGE showing the the effect of increasing periods of formaldehyde exposure on neuraminidase.
Equal amounts of purified neuraminidase were placed in two tubes. Formaldehyde at a final concentration of 3.7% (v/v) was added to one tube and the other was left untreated. Both tubes were incubated at 23°C. Immediately following the addition of formaldehyde at time point 0 and at 15 minute intervals subsequently (for 5.5 hours), 100µl of the protein was removed from each tube and frozen at -70°C. 27µl of the protein sample at each time point was analysed by SDS-PAGE. Odd and even numbered lanes show untreated and formaldehyde - treated neuraminidases respectively. Lanes 1 and 2 show samples taken at time 0. Lanes 3 and 4 show samples taken at 15 minutes. Subsequent samples were taken at 15 minute intervals until 5.5 hours (lanes 45 and 46). The formaldehyde-treated sample at 30 minutes (sample 6) was not analysed.

5.2.3 Inactivation of neuraminidase by heat treatment

Heat treatment is a fast and simple method of denaturing proteins, but it has disadvantages. In order for a protein to act as an immunogen it must be soluble and maintain a suitable conformation for epitope presentation. Heat treatment of proteins may cause the protein to aggregate and precipitate out of solution or cause conformational changes that result in loss of important epitopes. Also the effects of heat denaturation are not always reproducible and heating may not necessarily result in loss of activity. Therefore in any inactivation protocol, a balance between inactivation and immunogenicity has to be reached.

Despite these potential drawbacks this method of enzyme inactivation was used because problems were encountered in the formaldehyde inactivation method. Also limitations in time made it impractical to investigate alternative methods of inactivation.

5.2.3.1 Determination of optimal conditions for heat inactivation

To determine the period of heating which resulted in a significant reduction in enzyme activity, a time-course of heat inactivation was conducted on purified wild type neuraminidase. In brief 100µl samples of purified enzyme (100µg/ml) were exposed to 56°C for 30, 60, 90 and 120 minutes. After this period they were assayed immediately for enzyme activity. The results are shown in Table 5.1 and in Figure 5.4

Period of heating (Minutes)	Enzyme activity (µmol/min/ml)	Enzyme activity expressed as a % of unheated NA
0	1.36	100
30	0.235	17.3
60	0.145	10.7
90	0.120	8.9
120	0.105	7.7

Table 5.1 Effect of heat treatment (56°C) on the activity of pneumococcal neuraminidase.

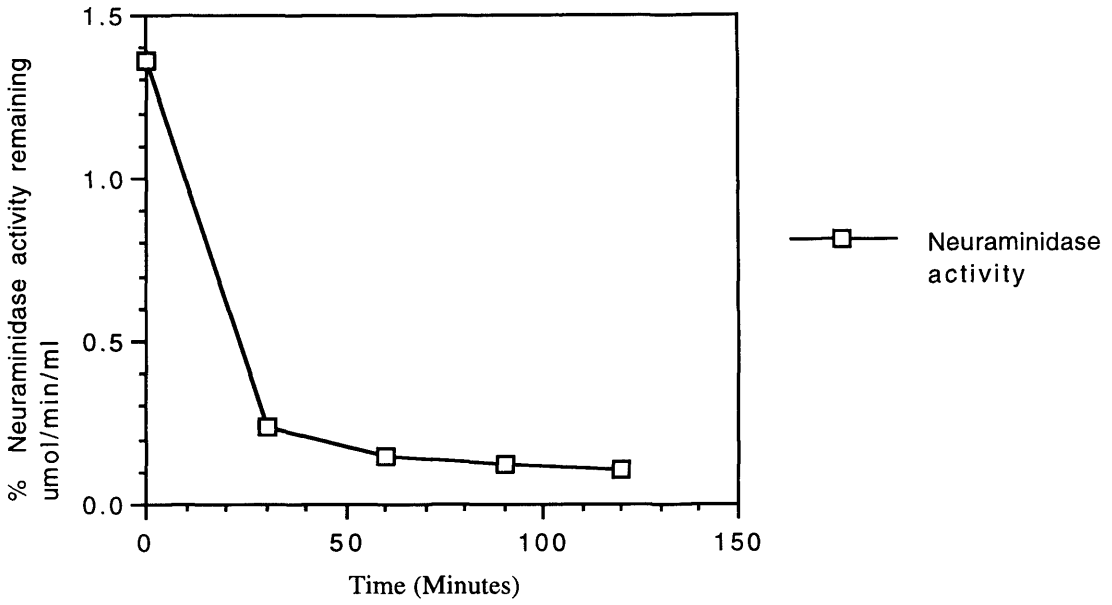


Figure 5.4 The effect of heat treatment (56°C) on the activity of pneumococcal neuraminidase.

From Table 5.1 and Figure 5.4 it was apparent that a dramatic drop in activity occured by 30 minutes of exposure to 56°C, a reduction in activity from 100% to 17.3%. After 60 minutes the effects of heating were no longer notable. For example heating for another 60 minutes minutes caused the activity to decrease by only another 3%. This experiment was repeated using a neuraminidase sample that was more concentrated (226µg/ml) and had a higher starting activity (6.04 µmol/min/ml). This resulted in a similar pattern of decrease in activity and is shown in Table 5.2 and Figure 5.5.

Period of heating (Minutes)	Enzyme activity (µmol/min/ml)	Enzyme activity expressed as a % of unheated NA
0	6.0	100
30	0.4	7.3
60	0.2	3.7
120	0.2	2.6

Table 5.2 Effect of heat treatment (56°C) on the activity of pneumococcal neuraminidase

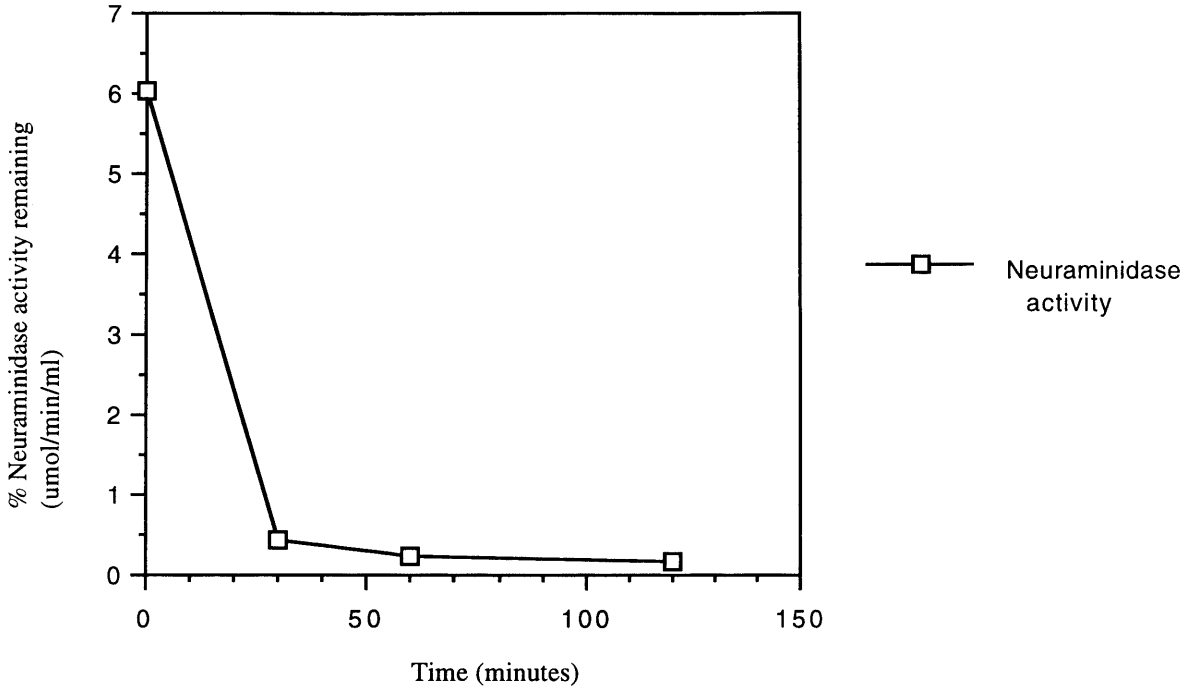


Figure 5.5 The effect of heat treatment (56°C) on the activity of pneumococcal neuraminidase.

The dramatic decrease in enzyme activity was observed after exposure to 56°C for 30 minutes, a reduction from 100% to 7.3%. Heating for a further 90 minutes caused a decrease of only another 4 - 7%.

After consideration of the results of the heat inactivation experiments it was decided to heat treat the neuraminidase for 60 minutes prior to the vaccination of mice. This period of heating was chosen because it reduced the activity of the neuraminidase considerably, and a longer heating period would have contributed little to the overall decrease in activity of the enzyme but would have the potential drawback of destroying the protein.

5.3 Investigation of the effects of immunization of mice with E_{647}>Q and wild type neuraminidases

5.3.1 Immunization of mice with E_{647}>Q and wild type neuraminidases

The following groups of mice were immunized intraperitoneally.

Group 1 - PBS (negative control), 10 mice.
Group 2 - Pneumolysin toxoid-PdB (positive control), 9 mice.
Group 3 - Wild type neuraminidase heated at 56°C for 60 minutes, 15 mice.
Group 4 - E_{647}>Q neuraminidase, 20 mice.

The neuraminidases were purified as described in section 2.3.3.6.1 and the imidazole was removed using Amicon stirred cells as described in section 2.3.4.1. The pneumolysin toxoid used in this study as a positive control (PdB) was kindly provided by Dr. James Paton (Women's and Children's Hospital, North Adelaide, Australia). The PdB toxoid had a W_{433}>F mutation caused by oligonucleotide-directed mutagenesis of the pneumolysin gene cloned from the type 2 strain NCTC 7466. This mutation reduced its haemolytic activity to 1% that of the wild-type toxin (Boulnois *et al* ., 1991). Immunization with PdB has been shown to protect mice against subsequent challenge with *S. pneumoniae* (Alexander *et al* ., 1994).

The immunizations were conducted as described in section 2.4.12. The interval between the three vaccinations was approximately 20 days. After each vaccination the mice were monitored daily for adverse reactions. No such reactions were observed in any of the mice which showed that the antigens were not toxic at the levels used. Mice were challenged intranasally 39 days after the third vaccination with *S. pneumoniae* strain D39 as described in section 2.4.8. Mice were monitored for visible clinical symptoms for 14 days (Section 2.4.9), at which point the experiment was ended. Mice that had survived this period were classified as survivors. Mice that became moribund during the 14 day period were judged to have reached the end point of the experiment. The time at which they became moribund was recorded as their survival time and they were humanely sacrificed.

5.3.2 Survival times of E_{647}>Q and wild type neuraminidase immunized mice

The survival times of mice challenged intranasally are shown in Figure 5.6, and the median survival time of mice, the percentage survival and statistical analysis for each group of mice is shown in Table 5.3

Group	Median survival time (hours)	P[a]	% Survival	P[b]
PBS	42.5	N/A	10	N/A
PdB	66.0	<0.05	11	> 0.1(NS)
Wild type NA	90.0	<0.01	20	> 0.1(NS)
E_{647}>Q NA	97.0	<0.01	40	> 0.1(NS)

Table 5.3 Protection against intranasal challenge with *S. pneumoniae* elicited by immunization of mice with E_{647}>Q neuraminidase, wild type neuraminidase and the pneumolysin toxoid PdB.

[a] Significance of difference in median survival time compared with that of group given PBS; Mann-Whitney U test.

[b] Significance of difference in percentage survival time compared with that of group immunized with PBS; Fisher's Exact test. NS = not significant.
Challenge dose (CFU) = 1.3×10^5

The pneumolysin toxoid PdB has been shown to be protective in previous *in vivo* studies (Paton *et al* ., 1991; Alexander *et al* ., 1994) and was the reason why it was selected to act as a positive control in this experiment. In this trial, mice immunized with PdB survived significantly longer ($P < 0.05$) than mice that were given PBS, confirming the results of previous studies.

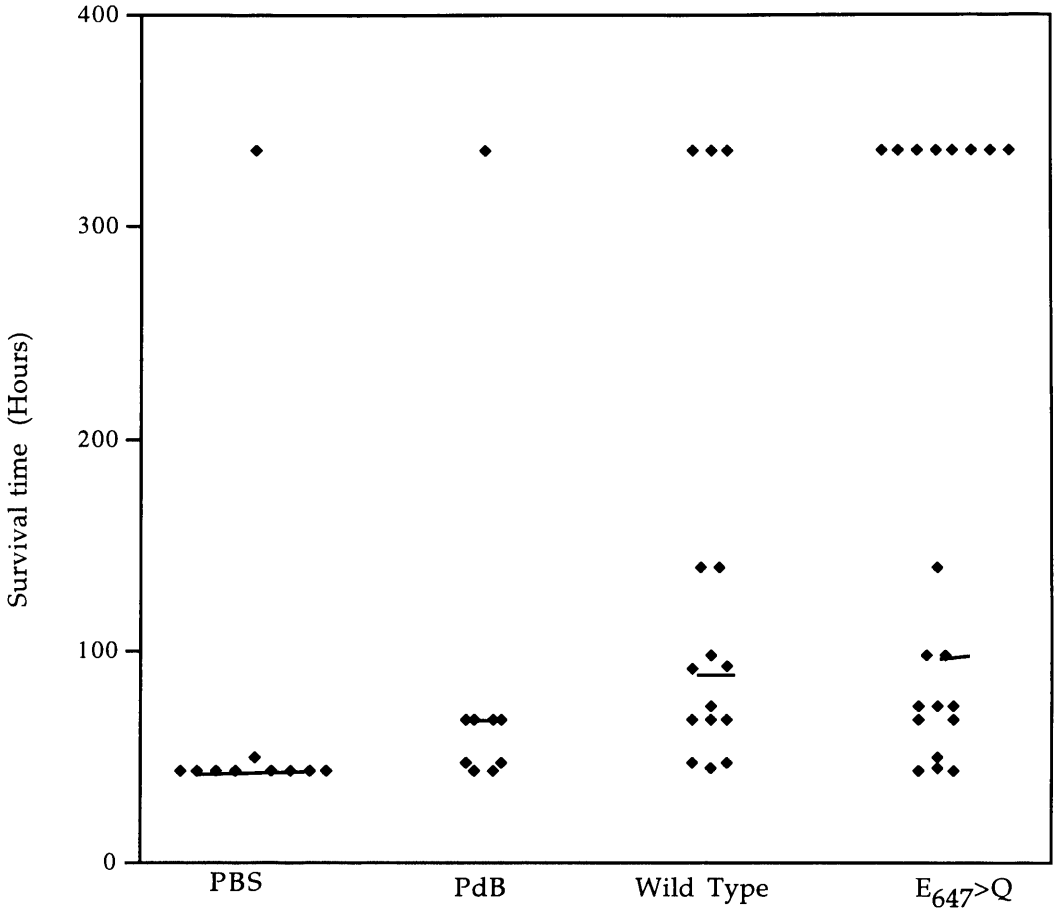


Fig 5.6 Survival time of mice challenged with D39. Groups of mice were immunized with 3 doses of 20ug of the antigens shown as described in section 5.3.1 then challenged intranasally with virulent *S.pneumoniae* (Section 2.4.8) . The time taken by each mouse to reach the moribund state is represented by a dot and the median survival time of each group is shown by a horizontal line. The mice that were alive at 336 hours were considered to have survived the challenge. PdB is pneumolysin toxoid, E_{647}>Q is neuraminidase toxoid.

There was a highly significant difference ($P < 0.01$) in the median survival times of mice challenged with heat treated wild type neuraminidase (90 hours) compared to mice given PBS (42.5 hours). Also there was a highly significant difference ($P < 0.01$) in the median survival times of mice immunized with E_{647}>Q neuraminidase (97 hours) compared to PBS immunized mice (42.5 hours). This suggests that the mice immunized with the neuraminidase are being protected to a certain degree against challenge by virulent pneumococci. However the difference in median survival time of mice immunized with wild type (90 hours) and E_{647}>Q mutated neuraminidase (97 hours) was not significant ($P > 0.05$) which suggests that both toxoids were equally immunogenic. Immunization of mice with either the wild type or E_{647}>Q neuraminidases resulted in longer survival times

(90 and 97 hours respectively) than after immunization with the pneumolysin toxoid PdB (66 hours). The difference between the median survival times of the PdB toxoid and E_{647}>Q neuraminidase immunized mice was statistically significant ($P < 0.05$) but the difference in the median survival time of the PdB toxoid and wild type neuraminidase immunized mice did not reach statistical significance ($P > 0.05$).

5.3.3 Analysis of serum response to immunization

Serum samples were taken from mice prior to each immunizing dose and before challenge, as described in section 2.4.10. Due to limited quantities of purified neuraminidase antigen, only a small fraction of the sera were assayed for circulating antibody levels by ELISA as described in section 2.4.13.

5.3.3.1 Comparison of the antibody levels in sera of mice following two immunizations with PdB or PBS

In the first instance the levels of circulating antibodies against pneumolysin in mice immunized with PdB were measured by antigen capture ELISA (Section 2.4.13). The sera from mice given PBS were used as a negative control. Sera were taken from five mice in each group prior to the first vaccination and after two vaccinations. The results and the statistical analysis of the data is shown in Table 5.4

Antigen	OD490 values pre-immunization ± SD [a]	OD490 values post-immunization ± SD [a]	P[b]
PBS	0.08 ± 0.03	0.15 ± 0.05	>0.05 (NS)
PdB	0.14 ± 0.05	1.31 ± 0.26	<0.001
P[c]	>0.05 (NS)	<0.001	-

Table 5.4 Antibody levels prior to and after immunization with the PdB toxoid or PBS and their significance of difference (p values)
The results of the ELISA are given as OD490 values

[a] Values shown are mean OD490 of 5 mice ± Standard deviation

[b] Significance of difference in OD490 values before and after immunization, Analysis of variance followed by Tukey-Kramer multiple comparisons test

[c] Significance of difference in OD490 values of PBS and PdB treated mice, Analysis of variance followed by Tukey-Kramer multiple comparisons test

The results of the ELISA are given as OD490 values. The OD490 values can be considered to reflect the circulating antibody levels in the blood of immunized mice. Therefore an increase in the OD490 levels reflect an increase in antibody levels. The results show a significant difference ($P < 0.001$) in the OD490 values from sera of mice before (0.14) and after (1.31) immunization with PdB. This indicates anti-pneumolysin antibody levels were significantly increased following immunization. In comparison the difference in OD490 values observed for control mice given PBS was not significant ($P > 0.05$). It was not expected that mice given PBS would generate an immune response to pneumolysin. As expected the OD490 levels before immunization in the PBS and PdB groups were not significantly different ($P > 0.05$).

5.3.3.2 Determination of the relationship between OD490 levels and survival time of mice immunized with PdB or PBS.

Next it was decided to ascertain the relationship (if any) between the survival time of the mice and the OD490 levels of the sera. The survival times of mice immunized with either PBS or PdB and the OD490 levels of the sera are shown in Figure 5.7

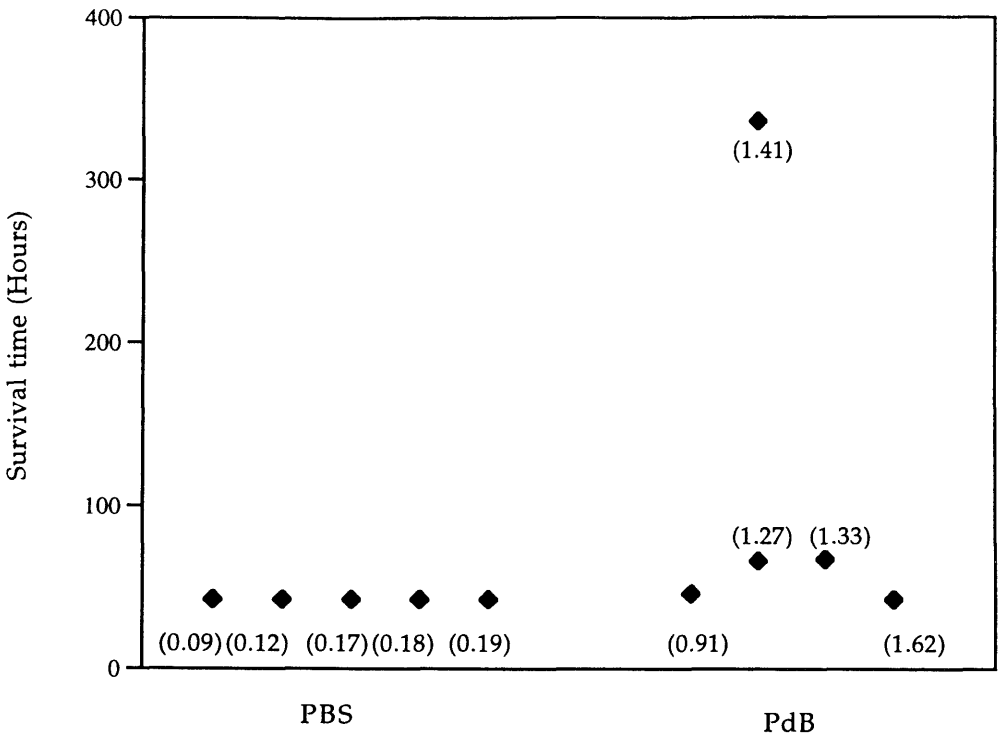


Figure 5.7 Survival time of mice immunized with either PBS or PdB. The OD 490 levels of the individual serum samples after ELISA for antibody are shown in bracket.

From Figure 5.7 it is apparent that there is no correlation between the survival time and the OD490 values. For example the serum from the PdB immunized mice with the longest survival time (336 hours) gave an OD490 reading of 1.41. However the sera from two mice with the shortest survival time gave OD490 values of 0.91 and 1.62. The OD490 values of the sera from the PBS treated mice ranged from 0.09 - 0.19.

The absence of a link between the survival time of the mice and the OD490 levels of the corresponding serum is further illustrated by Figures 5.8 (A) and 5.8 (B) which show the survival time of the PBS and PdB treated mice versus the corresponding OD490 levels of the sera.

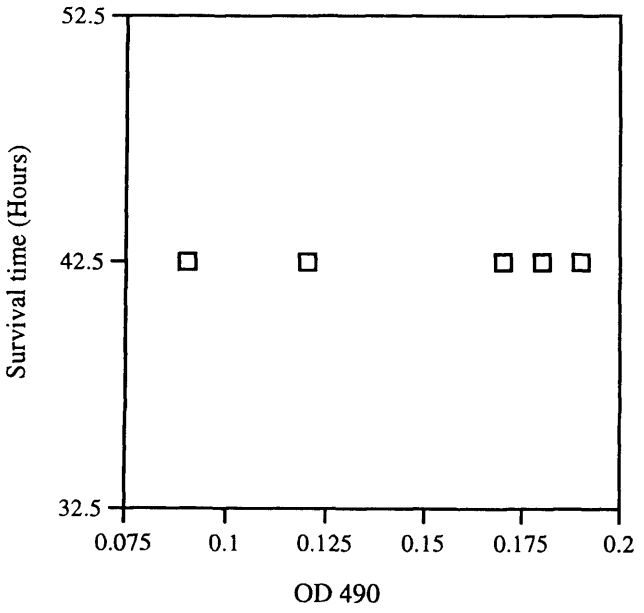


Figure 5.8 (A) Survival times of PBS treated mice versus the OD 490 levels of the individual serum samples after ELISA for antibody.

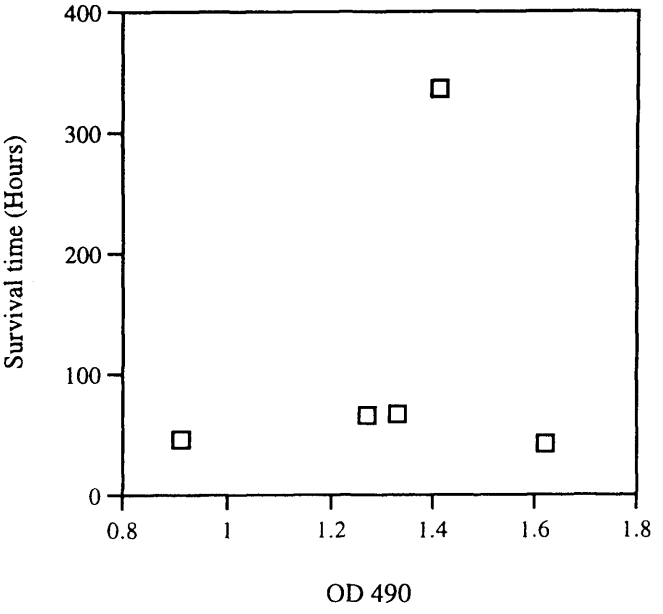


Figure 5.8 (B) Survival time of PdB immunized mice versus OD 490 levels of the individual serum samples after ELISA for antibody.

5.3.3.3 Comparison of the antibody levels in sera of mice following two immunizations with PdB, wild type neuraminidase or E_{647}>Q toxoid.

The circulating antibody levels in the sera of mice immunized with two doses of PBS, PdB, wild type and E_{647}>Q neuraminidases were determined. Unfortunately due to insufficient quantities of purified protein required in the ELISA, the circulating antibody levels in sera of mice prior to immunization with wild type and E_{647}>Q neuraminidase were not measured. However the circulating antibody levels in the sera of mice given PBS may still be compared to the circulating antibody levels in the sera from mice immunized with neuraminidase or pneumolysin. The circulating antibody levels after two immunizations with PBS, PdB, wild type or E_{647}>Q neuraminidases, and statistical analysis of the data is shown in Table 5.5.

Antigen	Mean OD490 [a]	P[b]
PBS	0.15 ± 0.05	-
PdB	1.31 ± 0.26	<0.05
Wild type NA	2.39 ± 1.10	<0.001
E_{647}>Q NA	2.97 ± 0.03	<0.001

Table 5.5 Antibody levels following immunization with the PdB, wild type or E_{647}>Q neuraminidase toxoids and their significance of difference (p values)

[a] Values shown are mean OD490 of 5 mice ± Standard deviation

[b] Significance of difference in OD490 value compared to that from PBS treated mice. Analysis of variance followed by Tukey-Kramer multiple comparisons test

An absorbance of 0.15 was obtained for the PBS treated mice. In comparison to this, the absorbance, and therefore the circulating antibody levels, in the PdB, wild type and E_{647}>Q neuraminidase immunized mice were significantly greater. There was not a statistically significant difference between the wild type and E_{647}>Q neuraminidase-immunized mice either in their circulating antibody levels or median survival times, 90 hours compared to 97 hours respectively ($P > 0.05$) (Section 5.3.2).

The high standard deviation of the mean OD490 obtained from the sera of mice immunized with wild-type neuraminidase was a consequence of the serum from one mouse having a low OD490 value of 0.47, compared to the sera from 4 other mice in the group whose average OD490 value was 2.88.

Interestingly the mouse with the serum OD490 value of 0.47 had one of the lowest survival times of 46 hours. (See Section 5.3.3.4 for more detail)

In Table 5.5 the OD490 values from the sera of mice immunized with either PdB, wild-type or E_{647}>Q neuraminidase were compared to those of mice given PBS. The statistical analysis showed that the sera of all the toxoids had significantly ($P < 0.01$) greater antibody levels than PBS treated mice. Next to determine if any one toxoid provided better protection than the others, the OD490 levels from the sera of mice immunized with the PdB, wild-type or E_{647}>Q neuraminidase toxoids were compared against each other. The results of the statistical analysis are shown in Table 5.6

	PBS	PdB	Wild type NA	E_{647}>Q NA
PBS	-	<0.05	<0.001	<0.001
PdB	-	-	<0.05	<0.001
Wild type NA	-	-	-	>0.05 (NS)
E_{647}>Q NA	-	-	-	-

Table 5.6. The significance of difference (p values) between the post immunization OD490 values of sera obtained from mice immunized with the different toxoids.

Significance of difference. Analysis of variance followed by Tukey-Kramer multiple comparisons test
NS - not significant

The statistical analysis of the data indicates that the OD490 values obtained from the sera of mice immunized with PdB, wild type and E_{647}>Q neuraminidases were statistically different to those of control mice given PBS. The OD490 values of sera from mice immunized with wild type neuraminidase were not significantly different ($P > 0.05$) from values obtained by immunization with E_{647}>Q toxoid. However the difference in the OD490 values of sera from the mice immunized with wild type neuraminidase and PdB was statistically significant ($P < 0.05$).

The OD490 values from sera of mice immunized with E_{647}>Q toxoid were not significantly different to those of wild type neuraminidase immunized mice but were significantly different ($P < 0.001$) to those of PdB immunized

mice. This is reflected by the similar survival times of E_{647}>Q (97 hours) and WT NA (90 hours) immunized mice ($P > 0.05$), and the significantly different ($P < 0.05$) survival times of E_{647}>Q (97 hours) and PdB (66 hours) immunized mice (Section 5.3.2).

5.3.3.4 Determination of the relationship between OD490 levels and survival time of mice immunized with either wild type or E_{647}>Q neuraminidase toxoids.

It was decided to ascertain the relationship (if any) between the survival time of the mice and the OD490 levels of the sera. A graph showing the survival time of the mice immunized with either wild type or E_{647}>Q neuraminidase toxoids and the corresponding OD490 levels of the sera is shown in Figure 5.9.

From Figure 5.9 it is apparent that there is no correlation between the survival time and the OD490 values. For example the serum from the wild type immunized mice with the longest survival time (336 hours) gave an OD490 reading of 2.63 However the sera from another mouse gave a higher OD490 reading of 2.87, yet survived for a shorter time of 90 hours.

Similarly two of the mice immunized with the E_{647}>Q neuraminidase toxoid survived for 336 hours and had sera OD490 levels of 2.97. However another mouse survived for only 49 hours but had serum OD490 levels of 2.93.

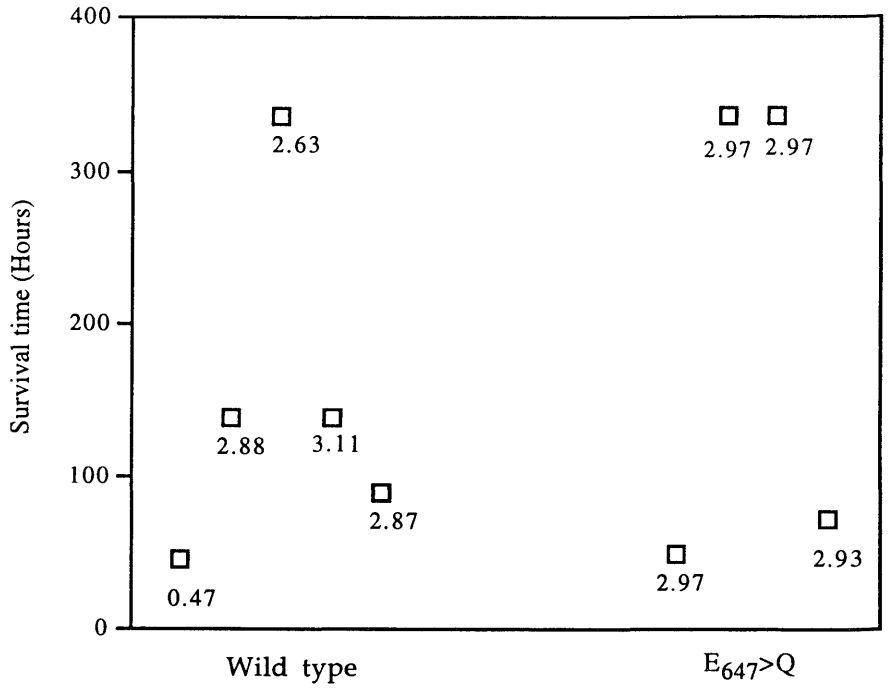


Figure 5.9 Survival time of mice immunized with either wild type or E_{647}>Q neuraminidase toxoid. The OD 490 levels of the individual serum samples after ELISA for antibody are shown.

The absence of a link between the survival time of the mice and the OD490 levels of the corresponding serum is further illustrated by Figures 5.10 (A) and 5.10 (B) which show the survival time of the wild type and E_{647}>Q neuraminidase toxoid immunized mice and the corresponding OD490 levels of the sera.

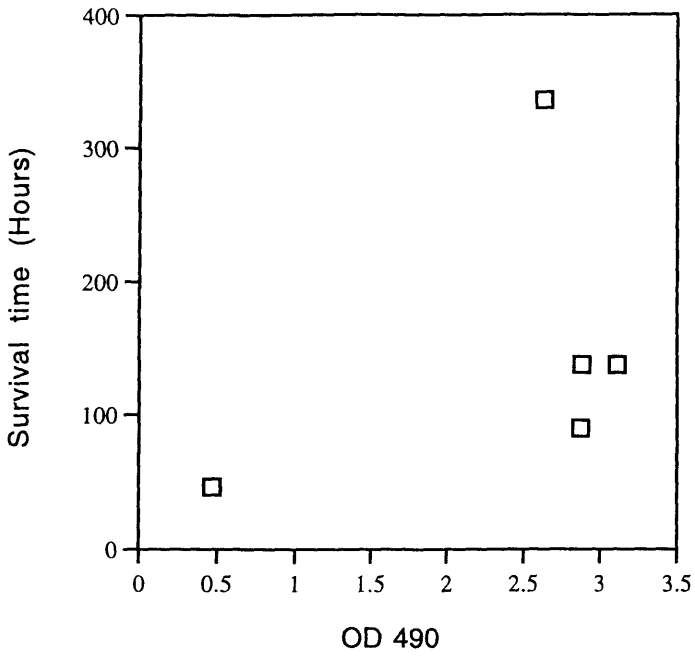


Figure 5.10 (A) Survival time of wild type neuraminidase immunized mice versus OD490 levels of the individual serum samples after ELISA for antibody.

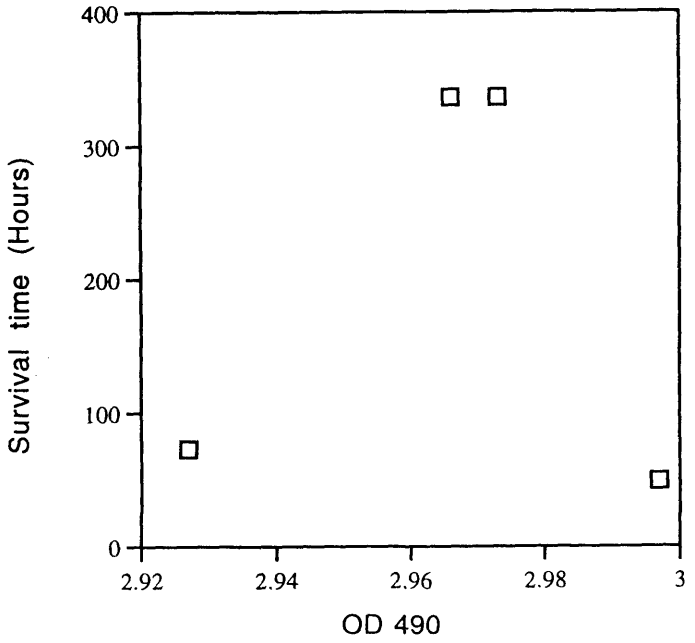


Figure 5.10 (B) Survival time of E_{647}>Q neuraminidase immunized mice versus OD490 levels of the individual serum samples afterELISA for antibody

5.4 Investigation of the effects of immunization of mice with Y_{752}>F and R_{663}>H neuraminidases.

5.4.1 Immunization of MF1 outbred mice with Y_{752}>F and R_{663}>H neuraminidases.

To extend the structure-function analysis of neuraminidase, two additional mutants were created; Y_{752}>F and R_{663}>H. The effect of these mutations on the immunogenicity of the neuraminidase was determined in mice in a similar way to the E_{647}>Q mutant. A new method of imidazole removal was used (Vivaspin Cells, Section 2.3.4.2), which resulted in higher protein recovery as judged by the A260 and A280 ratio (Section 2.3.5.1) compared to the Amicon stirred cells procedure (Section 2.3.4.1). In addition a new batch of mice was obtained, and given three consecutive immunizations of PdB, Y_{752}>F or R_{663}>H as described earlier (Section 5.3.1). The PdB used in these immunizations came from the same batch as that used in the E_{647}>Q immunizations (Section 5.3.1). The mice were monitored after each vaccination and showed no signs of distress for all the toxoids used. A fresh preparation of the standard inoculum was prepared (Section 2.4.6). Groups of immunized and control mice were challenged intranasally with *S. pneumoniae* (Section 2.4.8) and monitored for symptoms of disease for 14 days at which point the experiment was terminated.

The survival times of mice are shown in Figure 5.11, the median survival time of mice, the percentage survival and statistical analysis for each group of mice is shown in Table 5.7. A significant increase ($P < 0.01$) in the median survival time of immunized mice compared to control mice was seen in each case, except for mice immunized with PdB ($P > 0.05$).

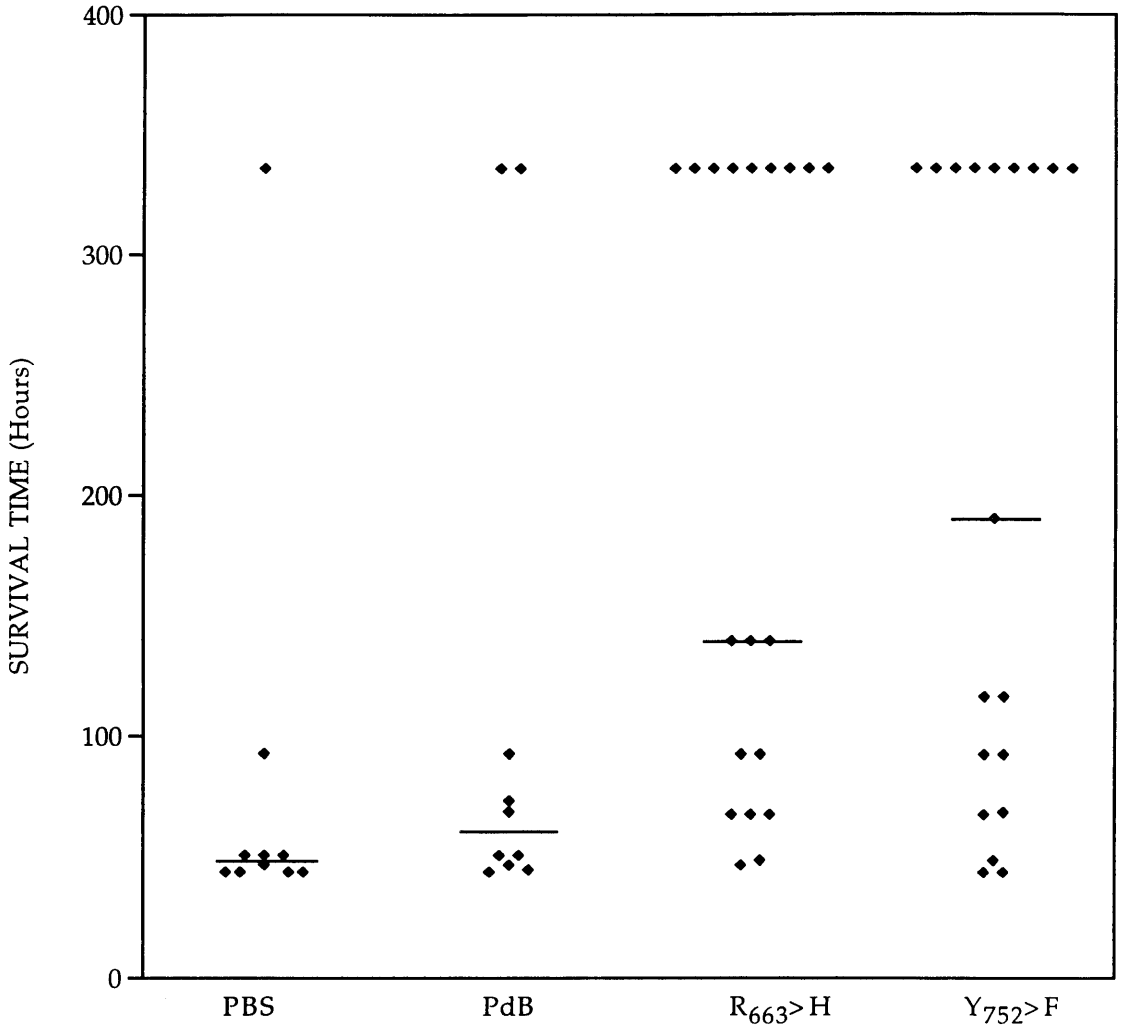


Figure 5.11 Survival time of mice challenged with D39. Groups of mice were immunized with 3 doses of 20ug of the antigens shown as described in section 5.4.1 then challenged intranasally with virulent *S.pneumoniae* (Section 2.4.8) . The time taken by each mouse to reach the moribund state is represented by a dot and the median survival time of each group is shown by a horizontal line. The mice that were alive at 336 hours were considered to have survived the challenge. PdB is pneumolysin toxoid, R_{663}>H and Y_{752}>F are neuraminidase toxoids.

Group	Median survival time (hours)	Pa	% survival	Pb
PBS	49	-	10	-
PdB	60	>0.05 (NS)	20	>0.05 (NS)
Y_{752}>F	191	<0.01	47	>0.05 (NS)
R_{663}>H	140	<0.005	47	>0.05 (NS)

Table 5.7. Protection against intranasal challenge with *S. pneumoniae* elicited by immunization of mice with Y_{752}>F and R_{663}>H neuraminidase toxoids and the pneumolysin toxoid PdB.

[a] Significance of difference in median survival time compared with that of group given PBS; Mann-Whitney U test.

[b] Significance of difference in percentage survival time compared with that of group immunized with PBS; Fisher's Exact test. NS= not significant.

Challenge dose (CFU) = 1×10^6

An unexpected finding was that immunization with PdB did not impart protection to mice. Mice immunized with PdB did not have a significantly different ($P > 0.05$) median survival time, or percentage survival ($P > 0.05$) compared to mice given PBS. This was surprising because PdB had shown protection in the preceding experiment (5.3), and in previous studies by Paton and co-workers (Paton *et al* ., 1991). Immunization of mice with either the Y_{752}>F or R_{663}>H neuraminidases resulted in longer survival times (191 and 140 hours respectively) than after immunization with the pneumolysin toxoid PdB (60 hours). The difference between the median survival times of the PdB toxoid and R_{663}>H neuraminidase immunized mice was statistically significant ($P < 0.05$) but the difference in the median survival time of the PdB toxoid and Y_{752}>F neuraminidase immunized mice did not reach statistical significance ($P > 0.05$).

Mice immunized with the Y_{752}>F and R_{663}>H neuraminidase toxoids survived substantially longer than mice that received PBS. The difference in median survival times of mice immunized with the Y_{752}>F toxoid (191 hours) and PBS control mice (49 hours) was statistically significant ($P < 0.01$). A similar observation was made in mice immunized with the R_{663}>H toxoid. Here the median survival time of R_{663}>H immunized mice (140 hours) was highly significantly different ($P < 0.005$) to that of PBS immunized mice. This suggests that immunization of mice with the neuraminidase toxoids leads to the production of anti-neuraminidase antibodies. However the Y_{752}>F toxoid was not more protective than the R663>H toxoid. The difference in median survival times between groups of mice immunized with Y_{752}>F (191 hours) and those receiving R_{663}>H (140 hours) was not statistically significant ($P > 0.1$).

5.4.2 Comparison of the effectiveness of the E_{647}>Q, Y_{752}>F and R_{663}>H toxoids in protecting mice against pneumococcal infection

The effectiveness of the neuraminidase toxoids (wild type neuraminidase, E_{647}>Q, Y_{752}>F and R_{663}>H) in protecting mice against disease from *S. pneumoniae* were compared. The results from wild type and E_{647}>Q neuraminidase immunized mice (Section 5.3.2) were compared with the Y_{752}>F and R_{663}>H groups from the second experiment (Section 5.4.1). The statistical analysis of the median survival time of mice immunized with these neuraminidase toxoids is shown in Table 5.8.

	Wild type NA (90hrs)	E_{647}>Q (97hrs)	Y752>F (191hrs)	R_{663}>H (140hrs)
Wild-type NA (90hrs)*	-	-	-	-
E_{647}>Q (97hrs)	> 0.05 (NS)	-	-	-
Y_{752}>F (191hrs)	> 0.05 (NS)	> 0.05 (NS)	-	-
R_{663}>H (140hrs)	< 0.05 (S)	> 0.05 (NS)	> 0.05 (NS)	-

Table 5.8. The significance of difference (p values) in median survival time of the different neuraminidase toxoids.

Significance of difference in median survival time; Mann-Whitney U test
* Figure in bracket indicates median survival time.
NS - not significant
S - significant

The comparison of median survival times of the mice given the toxoids was influenced by a difference in the median survival times observed for the PBS treated control mice. The mice given PBS in section 5.3.1 had a median survival time of 42.5 hours which was significantly different ($P < 0.01$) from those in section 5.4.1 at 49.0 hours. This observation is important and influences any comparisons that are made in the survival times of the mice from the two groups. For example the longer survival time of the PBS immunized mice in Section 5.4.1 may lead to underestimations of the survival and hence the effectiveness of the R_{663}>H and Y_{752}>F toxoids as protective antigens. The reason for the difference in the survival times of the two groups of PBS treated mice could be attributed to many factors such as slight variation in their housing and differences in their levels of anaesthetization.

The results of the Mann-Whitney U test (Table 5.8) indicate that two of the mutated neuraminidases (E_{647}>Q and Y_{752}>F) were no better than the wild type heat inactivated neuraminidase in protecting mice against challenge with virulent pneumococci. For the R_{663}>H toxoid, protection was just significantly better ($P < 0.05$) than wild type heat inactivated neuraminidase. However because of the P value obtained, a cautious interpretation would be that the two treatments were equally effective in protecting mice from virulent pneumococci.

5.4.3 Analysis of serum response to immunization with neuraminidase and pneumolyin toxoids

To ascertain whether the increased survival times of the mice immunized with Y_{752}>F and R_{663}>H toxoids reflected an increase in circulating antibody levels, serum samples were collected from mice, before and after each immunization. The serum samples obtained prior to and after the second immunization were analysed by ELISA and compared for circulating antibody levels. The statistical analysis of the ELISA results are shown in Table 5.9.

Toxoid	Mean OD490 ± SD [a] (Pre-immune)	Mean OD490 ± SD (Post-immune)	p[b]
PBS	0.28 ± 0.05	0.33 ± 0.04	> 0.05 (NS)
PdB	0.32 ± 0.29	0.63 ± 0.17	> 0.05 (NS)
Y_{752}>F	0.33 ± 0.07	2.92 ± 0.16	< 0.001
R_{663}>H	0.23 ± 0.04	2.47 ± 0.27	< 0.001

Table 5.9 Antibody levels prior to and after immunization with the pnemolysin PdB toxoid and Y_{752}>F, R_{663}>H mutated neuraminidase toxoids and their significance of difference (p values).

[a] Values shown are mean OD490 of 5 mice ± standard deviation (SD)

[b] Significance of difference between pre- and post immunization OD490 levels; Analysis of variance followed by Tukey-Kramer multiple comparisons test
NS - Not significant

The sera from mice immunized with Y_{752}>F and R_{663}>H neuraminidases exhibited an increase in the ELISA OD490 values after immunization, whereas the sera from mice given either PdB or PBS showed no such increase. In both the Y_{752}>F and R_{663}>H groups of immunized mice the OD490 values before and after immunization were significantly different ($P < 0.001$).

5.4.4 Determination of the relationship between antibody levels and survival time.

It was hypothesised that the survival times of the mice were related to their antibody levels. For example the long median survival times of the Y_{752}>F (191 hours) and R_{663}>H (140 hours) neuraminidase immunized mice (compared to control PBS treated mice, 49 hours) may be a consequence of their sera having high levels of antibody. To determine the relationship between the antibody level and the survival time of the mice a plot of the survival time of the mice immunized with either PBS, PdB or Y_{752}>F and R_{663}>H neuraminidase toxoids versus their post immunization OD490 levels was made (Figure 5.12).

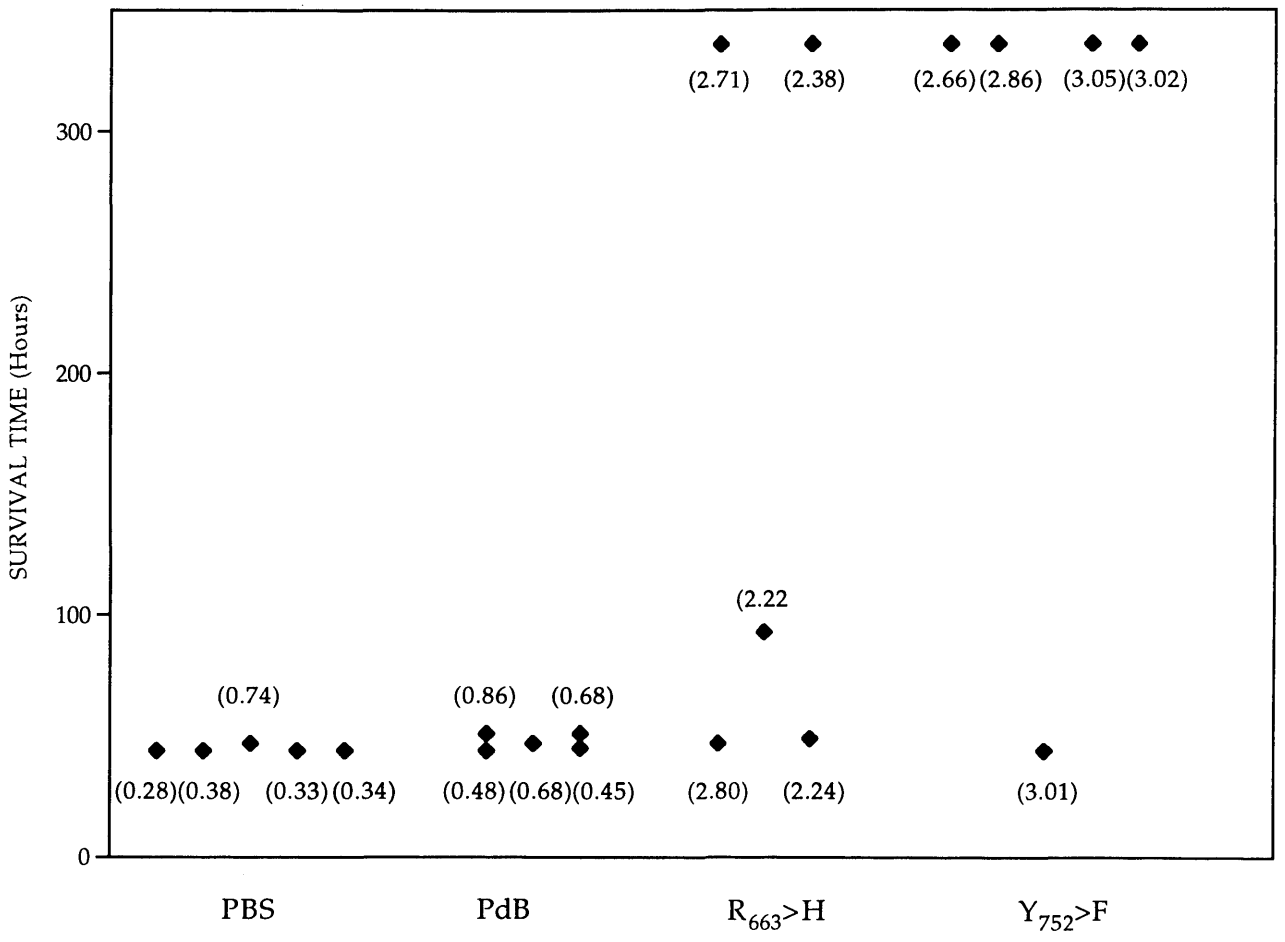


Figure 5.12 Survival time of mice treated with either PBS or immunized with PdB toxid, R663>H or Y752>F neuraminidase toxoid and the OD 490 levels of the sera.

From Figure 5.12, it is apparent that there was no link between the survival time of the mice and the OD490 of their sera. For example four of the mice immunized with the Y_{752}>F toxoid survived for 336 hours and their OD490 levels ranged between 2.66 and 3.02. However one mouse imunized with the Y_{752}>F neuraminidase toxoid had an OD490 of 3.01, yet it survived for only 44 hours following infection with *S. pneumoniae*. Similarly two of the mice immunized with the R_{663}>H neuraminidase toxoid survived for 336 hours and their OD490 values were 2.71 and 2.38. However another mouse immunized with the same toxoid survived for 47 hours yet had a higher OD490 level of 2.80.

The survival times of mice immunized with PBS, PdB, R_{663}>H or Y_{752}>F neuraminidase toxoids and their corresponding OD490 levels are shown in Figures 5.13 A - D respectively. This further highlights the lack of correlation between the survival time and the OD490 values of sera from immunized mice.

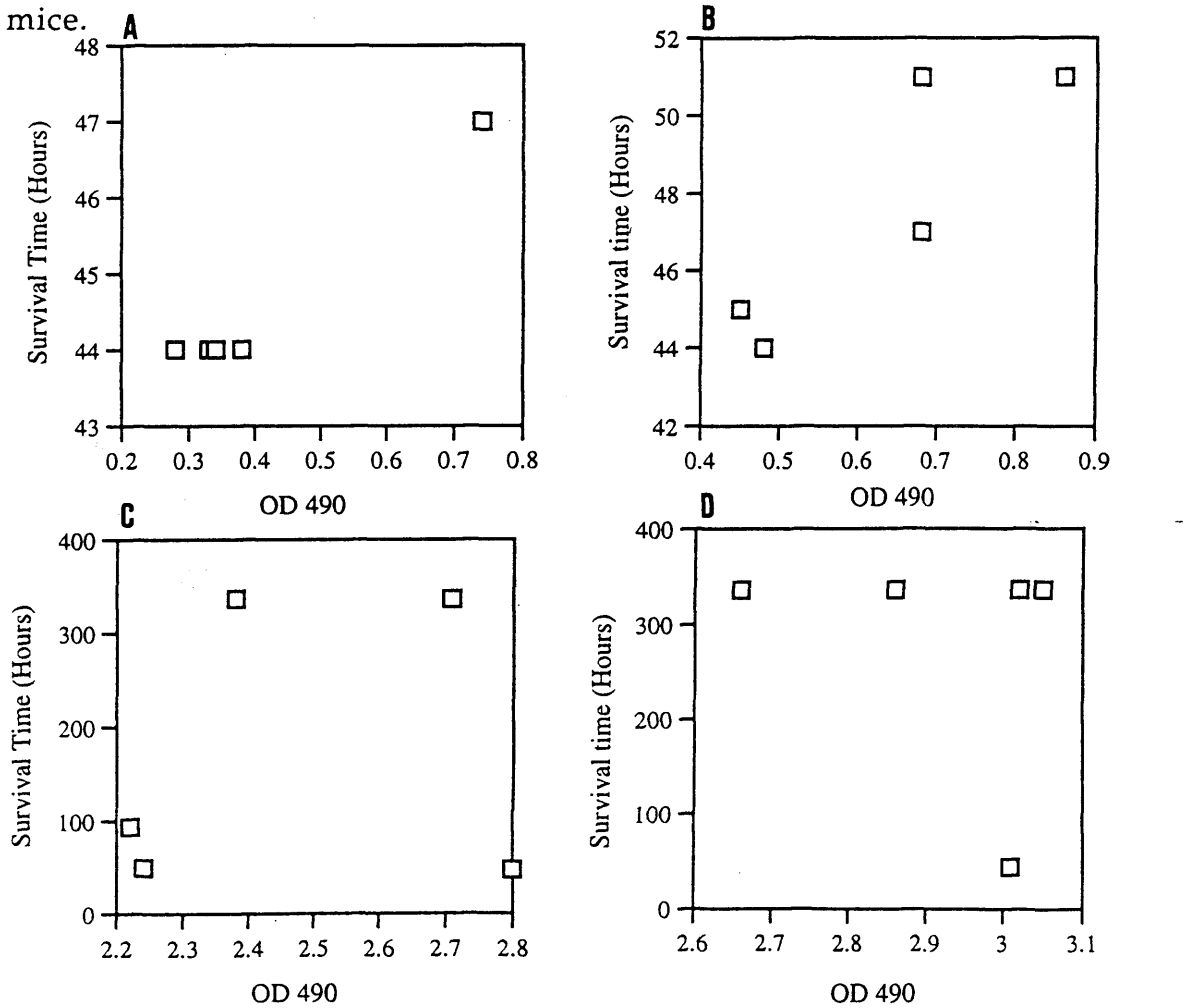


Figure 5.13. Survival time of (A) PBS, (B) PdB, (C) R_{663}>H and (D) Y_{752}>F immunized mice versus the OD 490 levels of the individual serum samples after ELISA for antibody.

5.5 Investigation of the protective effects of E_{647}>Q mutated neuraminidase in mice.

5.5.1 Introduction

Immunization with the E_{647}>Q toxoid was shown to protect mice against challenge with *S. pneumoniae* (Section 5.3.1). It was hypothesised that the protection was due to the retardation of bacterial growth in the lungs and blood of mice by the E_{647}>Q toxoid.

To test this hypothesis the growth of bacteria in the lungs and blood of mice immunized with the E_{647}>Q toxoid or PBS were determined. Thirty mice were immunized intraperitoneally with 20μg of purified E_{647}>Q neuraminidase in Alum at a ratio of protein:alum of 1:75 as previously described (Section 2.4.12). The mice were challenged intranasally 37 days after the last vaccination as described in Section 2.4.8. At pre-chosen intervals following challenge, blood samples were collected from the tail vein (Section 2.4.10) of pre-selected groups of mice. The mice were then sacrificed and the lungs were removed and homogenized and viable counts in homogenates and blood were determined as described in section 2.4.11. The number of viable bacteria in the lungs and blood of mice is shown in Figures 5.14 and 5.15 respectively .

The original plan was to sacrifice five E_{647}>Q immunized mice at time points 0, 24, 36, 72, 96 and 120 hours post challenge. Five mice per time point were sacrificed at times 0, 24 and 36 hours post challenge. By 72 hours however, there were only a total of five mice surviving and these were destined to be culled at 72 hours (2 mice), 96 hours (1 mouse) and 120 hours (2 mice) time points. In order to obtain results amenable to statistical analysis, it was decided to sacrifice all these mice for the 72 hour time point and terminate the experiment.

5.5.2 Bacterial numbers in the lungs of mice immunized with E_{647}>Q neuraminidase or PBS

The number of viable bacteria at timepoints between 0 and 72 hours post challenge in the lungs of mice immunized with E_{647}>Q mutated neuraminidase or PBS are shown in Figure 5.14.

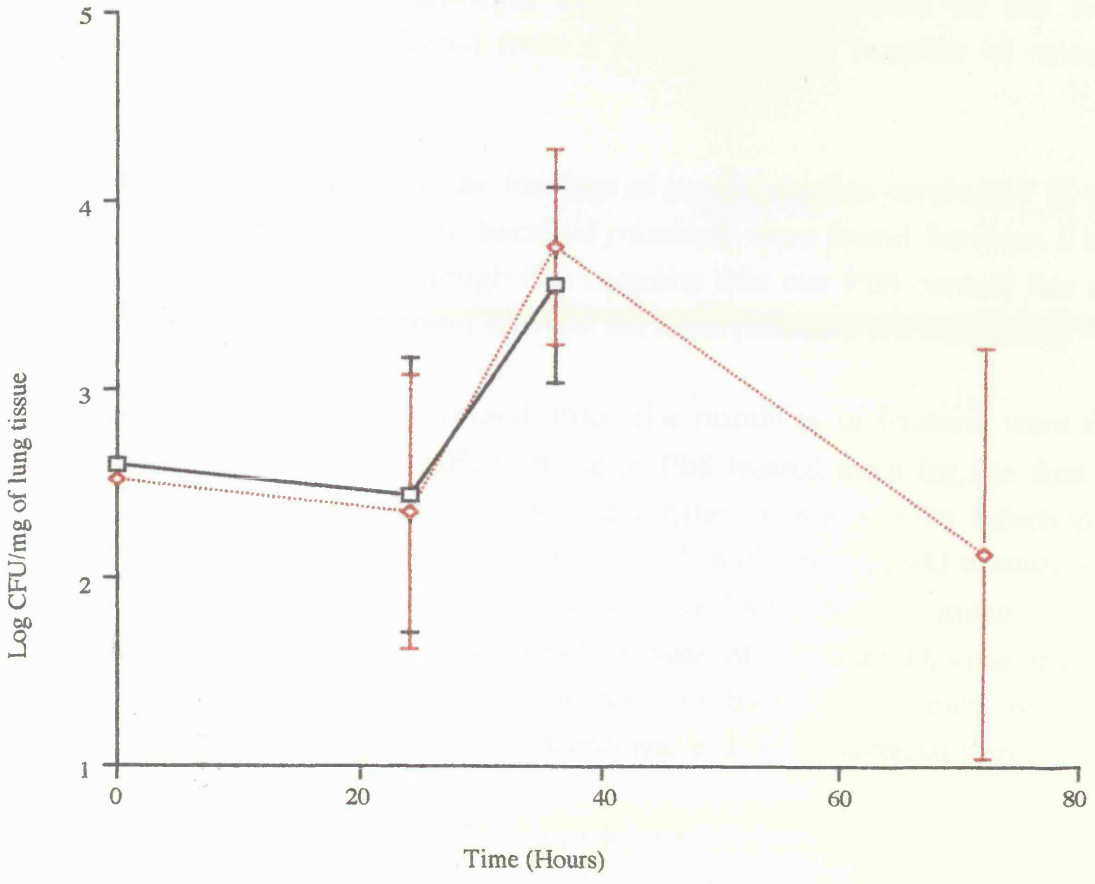


Figure 5.14. Number of viable *S. pneumoniae* in lungs of mice over 72 hours after infection with 8 x 10^5CFU of bacteria. Each point represents the mean of five mice ± standard error of the mean. □ PBS, ◇ E_{647}>Q toxoid

At 0 hours post-infection, a mean 2.6 log CFU/mg of tissue of bacteria were detected in the lungs of mice treated with PBS. By 24 hours post-infection

there was no significant difference ($P > 0.05$) in bacterial numbers compared to 0 hours. At 36 hours post infection the number of bacteria appeared to have increased to a mean of 3.56 log CFU/mg of tissue but the difference in bacterial numbers at 24 and 36 hours was not statistically significant ($P > 0.05$). After this time no further animals were tested because it was known from previous studies that mice succumb to infection with *Streptococcus pneumoniae* and die between 40 - 50 hours. Statistical analysis of the bacterial numbers at times 0, 24 and 36 hrs post-infection showed that the variation among the mean CFU/mg of lung tissue at different times after infection was not significantly greater than expected by chance ($P > 0.05$). The absence of statistically significant data could be attributed to the wide variation in the data collected from a relatively small number of mice at each time-point.

These results are contrary to the findings of similar studies conducted in our laboratory, where increases in bacterial numbers were found between 0 and 36 hours post infection. Although this suggests that our PBS control has not worked ideally, there is a trend that the bacterial numbers are increasing.

In the lungs of E_{647}>Q immunized mice the numbers of bacteria were not significantly different ($P > 0.05$) to those in PBS treated mice for the first 36 hours after infection with *S.pneumoniae*. After 36 hours post infection a different picture emerges in mice immunized with the E_{647}>Q toxoid. The bacterial numbers in the lungs appear to be declining from a mean of 3.76 log CFU/mg of lung tissue at 36 hours to a mean of 2.13 log CFU/mg of lung tissue at 72 hours. Although this decrease in bacterial numbers was not statistically significant ($P > 0.05$), the trend was a drop in bacterial numbers.

The variation in the mean CFU/mg of lung tissue at times 0, 24, 36 and 72 was not statistically significant ($P > 0.05$). The relatively small numbers of animals in the study and the wide variation in the numbers of bacteria isolated from animals at a given sampling point may be responsible for this observation.

5.5.3 Bacterial numbers in the blood of E_{647}>Q and PBS immunized mice.

The number of viable bacteria in the blood of mice immunized with E_{647}>Q or treated with PBS as a control at various time points after challenge is shown in Figure 5.15.

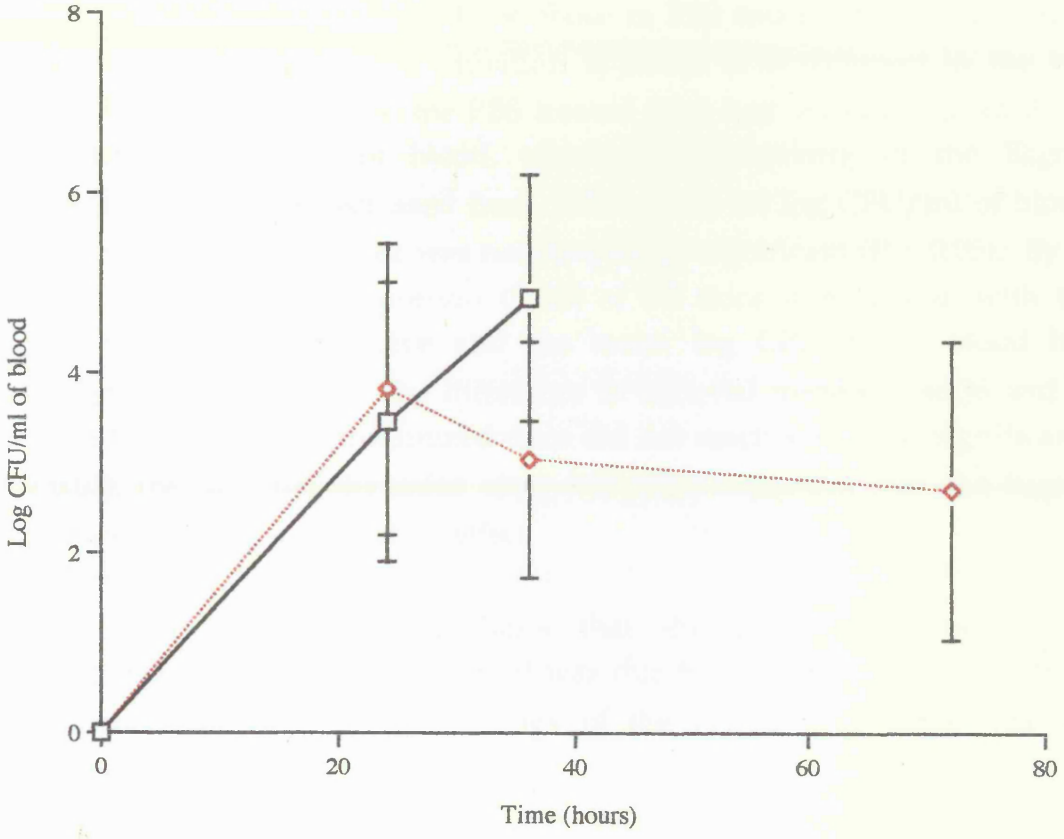


Figure 5.15. Number of viable *S.pneumoniae* in blood of mice over 72 hours after infection with 8×10^5 CFU of bacteria. Each point represents the mean of five mice ± standard error of mean.

PBS E647>Q Toxoid

Immediately after infection no bacteria were detected in the blood of mice treated with PBS. At 24 hours post-infection a mean of 3.5 log CFU/ml of blood of bacteria were detected in the blood of these mice, but this increase was not statistically significant compared to the bacterial numbers at 0 hours ($P > 0.05$). 36 hours post-infection the bacterial numbers appeared to have increased to mean 4.8 log CFU/ml, but the difference in bacterial numbers at 24 and 36 hours did not reach statistical significance ($P > 0.05$). Also the difference in bacterial numbers between 0 and 36 hours post infection did

not reach significance ($P > 0.05$). After 36 hours following infection the mice treated with PBS would have died, so after this time no sampling of mice was planned.

In mice immunized with the E_{647}>Q toxoid no bacteria were detected in the blood immediately following infection. By 24 hours post-infection the bacterial numbers had increased to mean 3.8 log CFU/ml of blood but were not significantly different ($P > 0.05$) to those in PBS treated mice at 24 hours. By 36 hours post infection the situation appeared to be different in the two groups. Bacterial numbers in the PBS treated mice had increased from 3.5 to mean 4.8 log CFU/ml of blood, whereas the numbers in the E_{647}>Q immunized mice had decreased from mean 3.8 to 3.0 log CFU/ml of blood. However again the difference was not statistically significant ($P > 0.05$). By 72 hours post-infection a proportion (5/15) of the mice immunized with the E_{647}>Q toxoid were still alive and the mean log CFU/ml of blood had decreased to 2.7. Although the difference in bacterial numbers at 36 and 72 hours in E_{647}>Q toxoid immunized mice did not reach statistical significance ($P > 0.05$), the fact that the mice were still alive suggested that the E_{647}>Q toxoid was exerting a protective effect.

In summary, the initial hypothesis that the protection observed by immunization with the E_{647}>Q toxoid was due to its effect on the growth of pneumococci in the blood and lungs of the immunized mice was not proven to be correct.

Chapter 6 - Discussion

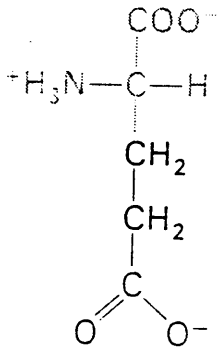
6.1 Mutagenesis

6.1.1 Introduction

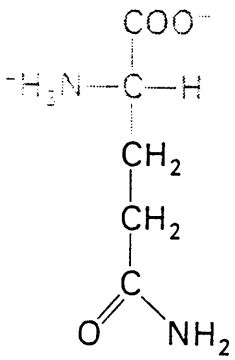
The principal aim of this study was to characterise a neuraminidase (Nan A) from *Streptococcus pneumoniae* by relating its amino acid sequence to its enzymic activity. The amino acid residues involved in the catalytic mechanism of the pneumococcal neuraminidases are unknown. However comparison of the primary amino acid sequences of bacterial and protozoan neuraminidases (Roggentin *et al* ., 1993) in conjunction with the crystallography data (Crennell *et al* ., 1993) had suggested that all neuraminidases have a similar catalytic site. Moreover, comparison of the primary sequence of one of the pneumococcal neuraminidases (Nan A) with the primary sequence of other bacterial neuraminidases helped in the prediction of certain amino acids likely to be involved in the active site. In particular, information from the primary sequence and crystal structure of the *S. typhimurium* neuraminidase led us to hypothesize that glutamic acid (E) 647, arginine (R) 663 and tyrosine (Y) 752 are important residues for catalytic activity. To test this hypothesis we substituted glutamic acid 647 with glutamine, arginine 663 with histidine and tyrosine 752 with phenylalanine by site directed mutagenesis. The mutated and wild type neuraminidase genes were expressed in *E. coli* , purified and the activity of the purified enzymes was determined. For each amino acid substitution, the outcome of the mutagenesis and its effect on enzyme activity are discussed below.

6.1.2 Selection of amino acids for mutagenesis

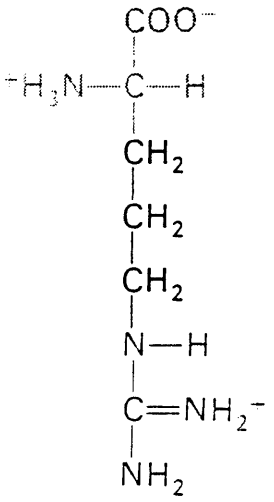
In order to minimise alterations in the secondary structure of the protein, conservative amino acid substitutions were made. The structures of the amino acids in the wild type and mutated neuraminidases are shown in Figure 6.1. The choice of amino acids to be mutated was based on the primary sequence and crystal structure information obtained from the neuraminidases of influenza virus and *Salmonella typhimurium* (Section 1.7.9.2). Residues E647, R663 and Y752 were chosen for mutagenesis because of their potential involvement in the catalytic mechanism. As discussed in greater detail in



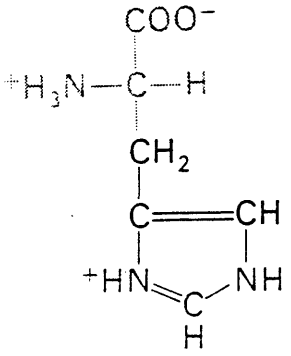
Glutamate
(Glu, E)



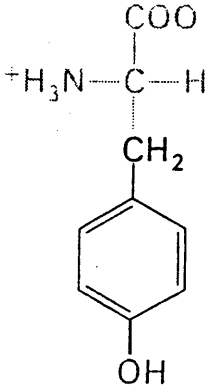
Glutamine
(Gln, Q)



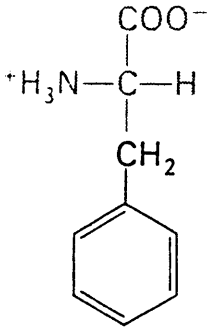
Arginine
(Arg, R)



Histidine
(His, H)



Tyrosine
(Tyr, Y)



Phenylalanine
(Phe, F)

Figure 6.1. The structures of the amino acids present in the wild type (left column) and mutated neuraminidases (right column). Adapted from Stryer, 1988.

sections 1.7.10.1 and 1.7.11, residues E647, R663 were equivalent to residues E231 and R246 in *S. typhimurium* . In *S. typhimurium* these residues are considered to play an important role in the active site of the neuraminidase (Crennell *et al* ., 1993; Crennell *et al* ., 1996). To determine if these residues (E647 and R663) are involved in the active site of the pneumococcal neuraminidase, they were substituted by conservative amino acids and the subsequent effect of these changes on enzyme activity studied. Residue Y752 is also highly conserved among bacterial and protozoan neuraminidases and was chosen for mutagenesis on the basis of unpublished results provided by Dr. Peter Roggentin (Biochemisches Institut, Kiel, Germany). He found that a residue equivalent to the Y752 residue of the pneumococcal enzyme was important for the enzyme activity of the *C. perfringens* enzyme (Unpublished results). Based on these results it was postulated that Y752 may be important for the catalytic activity of the pneumococcal neuraminidase. To investigate this possibility the Y752 was substituted with phenylalanine and the effect on enzyme activity was examined.

6.1.3 Mutagenesis

The starting point for the mutagenesis was the neuraminidase gene, *nan A* that had been previously cloned by Camara *et al* ., (1991). The neuraminidase gene was obtained from a plasmid expression vector (pQMC1). To facilitate mutagenesis, a small portion of the *nan A* gene (*Hind* III fragment of pQMC1) was subcloned into pUC18 and the resulting construct termed pSS2A (Chapter 3, section 3.1.3). Plasmid pSS2A formed the starting material for the PCR mutagenesis (Section 3.2). After mutagenesis the *Hind* III-pQMC1 fragment was ligated with the 4.6 kb fragment which contained the 5' end of the neuraminidase gene and the expression vector sequence. The new constructs containing the mutated neuraminidase gene was called pSSXXvec, where the XX represented the amino acid substitution. For example the substitution of glutamic acid (E) to glutamine (Q) at position 647 resulted in the construct pSSEQvec. Similarly the replacement of tyrosine (Y) with phenylalanine (F) at position 752 resulted in the construct pSSYFvec, and the mutation of arginine (R) to histidine (H) at position 663 resulted in the construct pSSRHvec.

6.1.4 Mutagenesis of E>Q at position 647

The first amino acid to be changed was the glutamic acid to glutamine at position 647. Sequencing of the pSSEQvec construct confirmed the presence of the E>Q mutation, a change from GAA to CAA. No other changes were detected in the remainder of the *Hind* III/pQMC1 region of pSSEQvec. Therefore the site directed mutagenesis of the pSSEQvec construct had resulted in the desired mutation.

Next the neuraminidase was expressed in *E. coli* and purified by affinity chromatography. Measurements of the enzyme activity of the crude and purified neuraminidase revealed the importance of the E647 for biological activity. Replacement of this residue with glutamine resulted in total loss of detectable enzyme activity. By comparison purified wild type neuraminidase had a specific activity of 2.9 μmol min^{-1} mg^{-1}. This result lends support to our original hypothesis that the glutamine at position 647 has an important role in the active site of the neuraminidase.

There is considerable evidence from other bacterial and viral neuraminidases about the importance of the corresponding glutamic acid residue in catalysis. The glutamic acid is highly conserved in viral and bacterial neuraminidases (Roggentin *et al* ., 1993; Crennell *et al* ., 1996). In the case of the *S.typhimurium* neuraminidase, complexes of the enzyme with the inhibitor DANA have shown that the corresponding residue E231 is hydrogen bonded to one of the triad arginines (R246) (Chapter 1, Figure 1.9) (Crennell *et al* ., 1996). The arginine triad stabilizes the carboxylic group present in all sialic acids. Therefore the residue E231 functions in maintaining an environment suitable for catalysis (Crennell *et al* ., 1993). To determine if similar interactions occur in the pneumococcal enzyme, the crystal structure of the DANA/enzyme complex could be examined.

Studies on the influenza neuraminidase have also helped to illustrate the importance of the equivalent glutamic acid residue in catalysis. The crystal structure of the neuraminidase from many influenza viruses have been solved (Varghese *et al* ., 1983; Varghese and Colman, 1991). Also the structure of the viral neuraminidase complexed with the substrate and inhibitors has also been elucidated (Burmeister *et al* ., 1992; Varghese *et al* ., 1992; Bossart-Whitaker *et al* ., 1993). These studies have shown that despite an amino acid sequence identity

of only 30% between influenza A and B, the amino acids and their arrangement in the active site are highly conserved (Burmeister *et al* ., 1992). The active site is composed of two shells of amino acids (Burmeister *et al* ., 1992). The first shell comprises nine fully conserved residues which interact directly with the sialic acid. The second shell is composed of ten amino acids (eight of which are highly conserved) which function to stabilize the first shell. Despite the wealth of structural information known about the influenza neuraminidase there is still no agreement about the mechanism of the enzyme. Amongst the many mechanisms put forward (Lentz *et al* ., 1987; Chong *et al* ., 1992) a popular mechanism is the one which involves the formation of a sialosyl cation transition-state intermediate (Chong *et al* ., 1992). To determine the role of the active site residues in catalysis many groups have performed site directed mutagenesis on conserved amino acids and studied the effects on enzyme structure and function (Lentz *et al* ., 1987; Wei *et al* ., 1987, Ghate and Air, 1998). The earlier studies of Lentz *et al* ., (1987) and the more recent studies of Ghate and Air (1998) have the most relevance to our mutagenesis studies and are therefore discussed further.

Lentz *et al* ., (1987) mutated a total of 14 residues (12 of which were conserved) in the active site of the influenza A/Tokyo/3/67 neuraminidase. The amino acid substitutions were 146 Asn>Ser, 152 Arg>Ile, 152 Arg>Lys, 178 Trp>Leu, 198 Asp>Asn, 222 Ile>Val, 274 His>Tyr, 274 His>Asn, 276 Glu>Gln, 276 Glu>Asp, 277 Glu>Asp, 346 Thr>Asn, 371 Arg>Lys, 406 Tyr>Phe. The mutated proteins were analysed for expression, transport, three-dimensional structure and enzymic activity. They found that with the exception of 146 Asn>Ser and 406 Tyr>Phe, all the mutated proteins had the correct three dimensional structure and were transported to the cell surface in a similar manner to the wild type. Two of these retained their enzymic activity, but seven exhibited a complete loss of activity (152 Arg>Ile, 152 Arg>Lys, 178 Trp>Leu, 198 Asp>Asn, 276 Glu>Gln, 276 Glu>Asp, 277 Glu>Asp).

Based on these results the following mechanism was proposed (Lentz *et al* ., 1987) for neuraminidase action (Figure 6.2). Following binding of substrate into the active site a proton is donated by His274 to Glu276. The proton is used by Glu276 to cleave the glycosidic bond resulting in the liberation of the polysaccharide minus its terminal sialic acid. A carboxonium ion intermediate may form and be stabilized by one of the conserved amino acids (probably Glu277). Finally a water molecule reprotonates the His274 and donates a

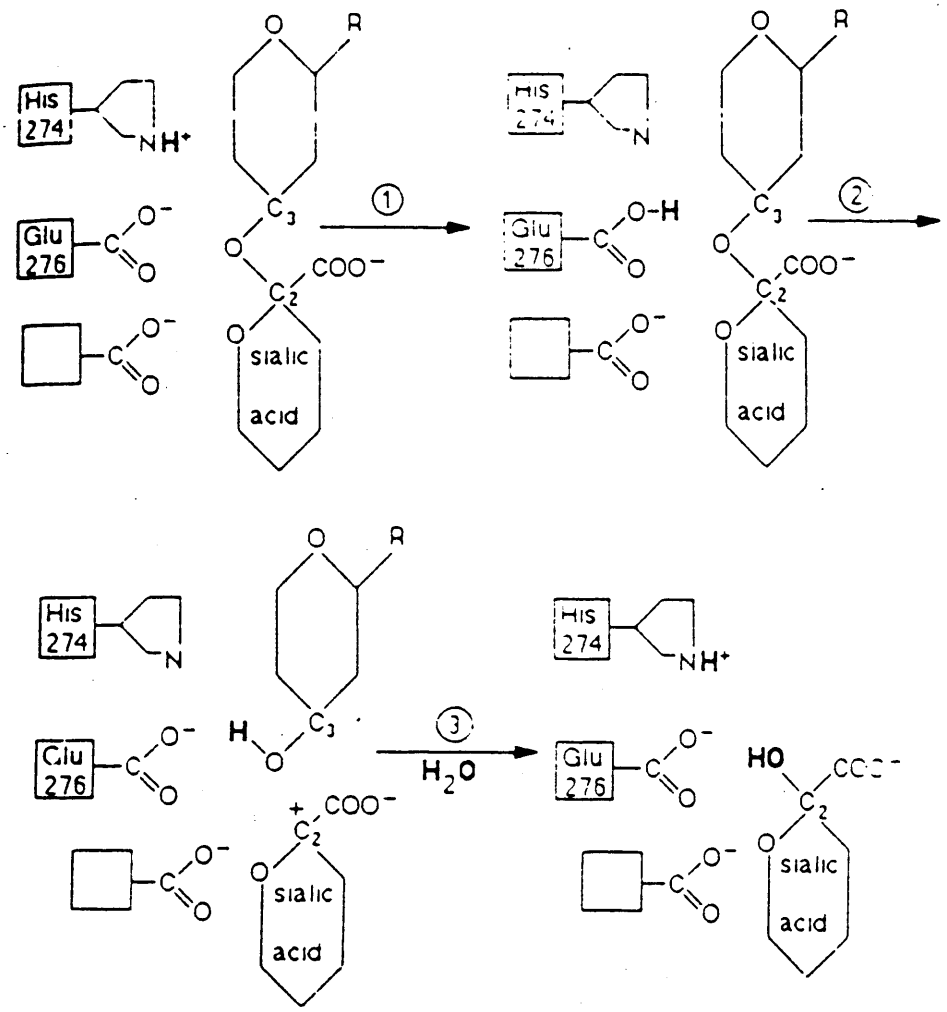


Figure 6.2. Diagram showing the proposed catalytic mechanism of influenza A neuraminidase. (Taken from Lentz *et al* ., 1987).

The sialic acid in the substrate is linked by an α (2,3) linkage to a polysaccharide chain (R).
Step 1: Following binding of the substrate into the active site, a proton is donated by His 274 to the ionized side chain of Glu 276.
Step 2: The proton is used by Glu 276 to cleave the glycosidic bond which results in the liberation of the polysaccharide minus its terminal sialic acid. A carboxonium ion intermediate may form and be stabilized by one of the conserved amino acids (probably Glu277).
Step 3: A water molecule reprotonates the His 274 and donates a hydroxyl group to the transition-state intermediate resulting in release of free sialic acid (Lentz *et al* ., 1987).

hydroxyl group to the transition state intermediate resulting in release of free sialic acid (Lentz *et al* ., 1987). One of the seven mutated neuraminidases made by Lentz *et al* ., (1987) with total loss of activity was the Glu277Asp mutant. This mutant is of particular interest because residue Glu277 is equivalent to the Glu647 of the pneumococcal enzyme and so may give some clues about the role of the glutamate in catalysis. The mutation of Glu277 to Asp resulted in no alteration in the charge (as both are negatively charged at pH6.0), however there was a decrease in the length of the side chains. It was suggested that the decreased length of the side chain in Asp may help to explain the loss of enzyme activity in the mutant. It was proposed that the Glu277 forms ionic interactions with the substrate in the wild type enzyme but these interactions are not possible in the mutant enzyme because of the decreased length of the side chain. Another role proposed for Glu277 was its involvement in stabilizing the transition-state intermediate (Lentz *et al* ., 1987).

The Glu647 residue could play a similar role in the pneumococcal neuraminidase. Although the change from E>Q does not result in alteration of the length of the side chain, it does result in change in net charge (From negatively charged to uncharged). This alteration of charge of an active site residue may cause changes in the interaction of the active site with the substrate which in turn could be responsible for the observed loss of enzyme activity of the E647Q mutant.

A more recent study by Ghate and Air (1998) showed the importance of the corresponding glutamate residue (Glu275) in the neuraminidase from influenza type B (B/Lee/40). They mutated seven conserved active site residues and measured the enzyme activity of the altered proteins. They found a reduction in the catalytic constant, K_{cat} (turnover number) of all the mutants. The K_{cat} is defined as V_{max} / E_t, where E_t = total amount of folded protein. Furthermore variations in K_i were also observed with the transition-state analogue inhibitor (2-deoxy-2,3-didehydro-N-acetylneuraminic acid). The results of the inhibitor studies enabled interpretation of the effect of mutations in terms of transition-state binding and product release. This is because the K_i is a reflection of the affinity of the enzyme for an inhibitor. If the inhibitor is a transition-state analogue then it can be assumed that the affinity of the enzyme with the transition state is reflected by the K_i. Therefore a high K_i reflects weaker binding to the inhibitor and can be seen as a destabilization of the transition state in the mutant. Conversely a low K_i is a reflection of stronger

binding of the enzyme to the inhibitor and reflects increased stabilization of the transition state in the mutant by comparison to the wild type (Ghate and Air, 1998)

From the studies mentioned above, it was concluded Asp149, Arg223, Glu275, Arg374 and Tyr409 were important for the activity of the influenza neuraminidase (Ghate and Air, 1998). Mutation of glutamate at position 275 to aspartate resulted in a decrease in the K_m and K_{cat} to approximately 2% of the wild type, but the catalytic efficiency (K_{cat}/K_m) was maintained. Modelling studies suggested that movement in the aspartate side chain oxygens resulted in loss of interaction with Arg223. However, a decreased K_i with (2-deoxy-2,3-didehydro-N-acetylneuraminic acid) was observed which led the authors to propose that the shorter length of the Asp side chain was better able to accomodate the transition state. Therefore it was suggested that the reduced K_{cat} was due to the tighter binding of the oxocarbonium ion intermediate (of the sialic acid) to the mutant neuraminidase which resulted in slowing of the product formation step (Ghate and Air, 1998). From these studies a slightly different mechanism of catalysis was proposed. It was suggested that catalysis was mediated by the binding and distortion of the substrate, facilitated by stabilization of the oxycarbonium ion intermediate. The presence of the reaction intermediate in the active site led to the suggestion that upon binding of the substrate to the active site, the sialyl group of the substrate undergoes a ring distortion. This is brought about by the interactions between the substrate carboxylate and and the guanidinium group of arginines 116, 292 and 374. The acetyl group is bound to the hydrophobic pocket on the other side of the active site. The C2 adopts a planar conformation and the glycosidic oxygen is protonated by the solvent (Ghate and Air, 1998).

The role of the equivalent glutamate residue (Glu230) in the *Clostridium perfringens* neuraminidase has also been investigated (Chien *et al* ., 1996). Glu230 was one of eight residues mutated in the active site of the *C. perfringens* neuraminidase. The mutation of Glu230 to Valine resulted in a 1000 fold reduction in the K_{cat}/K_m value (using the substrate α-(2,3) sialyllactose) by comparison to the wild type (Chien *et al* ., 1996). Similar to the findings of the influenza A neuraminidase mutants, it was suggested that in the *C. perfringens* neuraminidase (wild type) the negative charge of the Glu230 side chain may be involved in stabilizing the positively charged carbonium ion intermediate. The substitution of Glu with the uncharged Val resulted in loss of this interaction

and so could explain the reduced enzyme activity of the mutant (Chien *et al* ., 1996). Correspondingly in the pneumococcal neuraminidase the mutation of E>Q at position 647 results in loss of charge, and loss of enzyme activity. By analogy with the *C. perfringens* and influenza A enzymes, the loss of enzyme activity of the pneumococcal neuraminidase may be explained by the inability of the uncharged glutamine to stabilize the positively charged carbonium ion intermediate of the sialic acid.

From the above discussion it is apparent that the glutamine residue is important for the catalytic actvity of neuraminidases from many organisms. Due to lack of structural information about the pneumococcal neuraminidase, it is not possible to state the precise role of the E647 residue. However the active sites of bacterial neuraminidases studied to date appear to have a similar topology, therefore we can predict that the E647 reisidue is involved in stabilising the carbonium ion transition state intermediate.

Kinetic studies were not performed on the wild type and mutated pneumococcal neuraminidases because of the lack of sufficient quantities of the purified enzymes.

6.1.5 Mutation of arginine to histidine at position 663

The second amino acid to be changed was the arginine to histidine at position 663. Sequencing of the pSSRHvec construct confirmed the presence of the R>H mutation, a change from CGT to CAT at position 663 (protein). In addition one other undesirable (but conservative) change at position 449 (protein), 642 bases upstream from the R>H mutation was also observed. This was a PCR artefact. The change of bases AAA to AGA, at position 449 resulted in a change of lysine to arginine. Both of these amino acids are basic and have a similar structure.

The presence of the unwanted mutation at position 449 meant that the effect of the R>H mutation on the function of the neuraminidase cannot be considered in isolation. The lysine to arginine change may also have a role. It is unknown how conserved this residue is in other neuraminidases because our sequence alignment showed no homology in this region. The elucidation of the crystal structure of the pneumococcal neuraminidase would help to establish the role of the Lys449. However until such information is available we have to assume that the reduction in activity of the R663>H mutant could be a result of the

R663>H or K449>R mutation or a combination of the two. We had planned to re-attempt the mutagenesis in the hope of obtaining a neuraminidase with just a single mutation at R663>H, however time constraints made this unfeasible.

The R663>H neuraminidase was purified from sonicates of *E. coli* harbouring pSSRHvec and assayed for enzyme activity by using the substrate PNP-NANA. The purified neuraminidase had a specific activity of 0.06 μmol min^{-1} mg^{-1}. This was 48 fold lower than the activity of the wild-type neuraminidase (2.9 μmol min^{-1} mg^{-1}). By comparison to the other two mutants this was the most active neuraminidase. It is likely that the predominant effect on activity was from the R663>H mutation because this residue is part of the triarginyl cluster that been found in all neuraminidases to date (Crennell *et al* ., 1994). The function of the three arginines is to stabilise the carboxylic acid group present in all sialic acids (Crennell *et al* ., 1993). Therefore a mutation of one of these key argines is likely to lead to instability of the carboxylic acid group and hence a decrease in enzyme activity. The conversion of the R663>H results in no alteration in charge but a change in the side chain. A shorter but heterocyclic arrangement of the side chain may preclude interactions with the carboxylic group of the sialic acid. The consequence of this would be an unstabilised carboxylic group leading to reduced enzyme activity.

The importance of the equivalent arginine residue is demonstrated by studies in other neuraminidases. For influenza virus (A) neuraminidase, variants which have an R292>K mutation (R292 corresponds to the R663 of pneumococcal neuraminidase) have been found to have a specific activity that was 20% of the wild type. Moreover the mutant neuraminidase exhibited reduced sensitivity to all neuraminidase inhibitors (Gubareva *et al* ., 1997). Similarly in the *C. perfringens* neuraminidase mutation of the arginine at position 245 to isoleucine resulted in a decreased enzyme activity of the mutant compared to the wild type. The K_{cat}/K_m value for the R245>I mutation was ten fold less than the value for the wild type (Chien *et al* ., 1996).

As described earlier R663 was predicted to be part of the triarginyl cluster conserved in many neuraminidases. Our results show that mutation of R>H in pneumococcal neuraminidase results in decreased activity. It is likely that the R663 plays a part in stabilizing the carboxylic group of the sialic acid.

6.1.6 Mutagenesis of Tyrosine to Phenylalanine at position 752

The final amino acid substitution was a change of tyrosine at position 752 to phenylalanine. DNA sequence analysis of the mutated neuraminidase (Y752>F) confirmed the presence of this mutation. Another non-coding change of ATT (isoleucine) to ATA (isoleucine) at position 812 (protein) was also observed.

The Y752>F neuraminidase was purified from sonicates of *E. coli* harbouring pSSYFvec and assayed for enzyme activity by using the substrate PNP-NANA. The purified neuraminidase had no measurable activity as judged by the assay with PNP-NANA. In comparison, the wild type neuraminidase had a specific activity of 2.9 μmol min^{-1} mg^{-1}. Enzyme activity was detectable in the "crude", "outflow" and "wash" fraction of the Y752>F neuraminidase. In comparison to the wild-type neuraminidase present in the crude homogenates of *E. coli* the Y752>F neuraminidase "crude" was 1636 times less active. Similarly the Y752>F neuraminidase "outflow" and "wash" fractions were 1625 times and 1225 times less active than the wild type "outflow" and "wash" fractions respectively.

The presence of Y752>F neuraminidase activity during the early stages of the purification ("crude", "outflow" and "wash") and its absence from the final product could be attributed to the huge losses in protein and enzyme activity that occur during the purification procedure. For example in the purification of the wild type neuraminidase we started with an activity of 238 μmol min^{-1} ml^{-1}, but only 2.9 μmol min^{-1} ml^{-1} remains in the final purified product. This represents a 82 fold loss of activity. The Y752>F neuraminidase was purified in an identical manner to the wild type, therefore, if a similar loss of activity had occurred during the purification of the Y752>F neuraminidase then we would expect to see 1/82 of 0.14 μmol min^{-1} ml^{-1} (the activity present in the crude), which is 0.0017μmol/min/ml. Such a low level of activity is below the detection range of the PNP-NANA assay (minimum detection range is 0.012 μmol min^{-1} ml^{-1}) and therefore could not be measured.

In contrast to the E647>Q mutation which resulted in complete loss of enzyme activity (as judged by the PNP-NANA assay), the mutation of Y>F does not completely inactivate the neuraminidase. Although the mutation does substantially decrease the activity of the enzyme, some activity was present in the "crude", "outflow" and "wash fractions. This suggests that in contrast to

residue Glu647 which appears to be essential for activity, residue Y752 has a significant although not absolutely essential role in catalysis.

The results of this study show that replacement of tyrosine with phenylalanine at position 752 reduces the activity of the neuraminidase to a level that cannot be detected by the assay with PNP-NANA. This result lends support to our original hypothesis that this residue is important for the catalytic activity of the neuraminidase. The corresponding tyrosine residue has been shown to be important for the catalytic activity of the neuraminidase from *C. perfringens*. In a study of the *C. perfringens* neuraminidase the technique of chemically induced dynamic nuclear polarization (CIDNP) was used to assess the effect of amino acid substitutions on the positioning of specific amino acid side chains (Tyr, Trp and His) (Siebert *et al* ., 1996). Amongst the three mutants made was a Tyr>Phe substitution at position 336. This was equivalent to the tyrosine at position 752 and 331 in neuraminidases from *S. pneumoniae* and *S. typhimurium* respectively. Kinetic analysis of the Y336>F mutant with the substrate methyl-umbelliferyl-N-acetylneuraminic acid showed a decreased V_{max} in comparison to the wild type (11 % of the wild type). The CIDNP analysis revealed that substituting Phe for Tyr at position 336 had caused a change in positioning of many amino acid side chains, i.e. those of Tyr82, Tyr204, Tyr267, Tyr310, Tyr375 and Trp124. The alteration in the positioning of many widely separated residues in conjunction with the kinetic data suggested that the Y336>F mutation had an important impact on the structure of the neuraminidase. However no suggestions were made about the precise role played by the Y336 in the activity of the Clostridial neuraminidase (Siebert *et al* ., 1996).

We can conclude that the tyrosine 752 does play an important role in the catalytic activity of the pneumococcal neuraminidase. However due to lack of structural data on the enzyme it is not possible to comment on its precise role.

In summary it has been shown that point mutations in conserved regions of the *nanA* gene drastically diminishes its neuraminidase activity. At present we cannot distinguish the precise functions of residues E647, R663 and Y752, however the elucidation of the crystal structure of the pneumococcal neuraminidase will lead to clarification about their precise function. Work is currently in progress to solve the crystal structure of the enzyme.

6.1.7 Future work

There are three main areas for future work. The first involves the continued introduction of site directed mutations in other residues predicted to be important for the activity of the enzyme and an analysis of their effect on enzyme activity. The second involves an analysis of the substrate preferences of the mutated neuraminidase. The third approach involves an examination of the secondary and tertiary structure of the wild type and mutated neuraminidases

There are several residues in the pneumococcal enzyme which are worthy of further study. The first would be the other arginines in the tri-arginyl cluster. In *S. typhimurium* the arginine triad comprises residues R37, R246 and R309. The equivalent of the first two arginines have been found in the pneumococcal enzyme, these are R347 and R663. The equivalent residue of R309 is likely to be either R721 or R730. The R663 has been mutated in this study and found to be fully or partly responsible for the reduction in activity of the neuraminidase. It would be interesting to see if mutation of R347 resulted in a reduction in enzyme activity. In *Clostridium perfringens* the arginine triad is likely to be formed by residues R37, R245 and R312. Mutation of arginine 37 to lysine (Roggentin *et al* ., 1992) or isoleucine (Chien *et al* ., 1996) has resulted in drastic reductions in activity of the neuraminidase. From these results a role for R37 (as part of the arginine traid) in substrate binding has been proposed (Roggentin

et al ., 1992). A similar role may be played by the R347 of the pneumococcal enzyme.

A hydrophobic pocket containing a conserved tryptophan has been found in the active site of *Salmonella typhimurium, Vibrio cholerae* and influenza neuraminidases (Crennell *et al* ., 1994). The pocket houses the N-acetyl group of sialic acids and is considered to be important for activity of the enzyme. As the hydrophobic pocket is part of the active site of the three neuraminidases, it is highly likely that such a pocket exists in the pneumococcal enzyme. Moreover as tryptophans are conserved in this structure in the bacterial and viral neuraminidases, it is reasonable to predict that tryptophans will be present in the pneumococcal hydrophobic pocket. In order to identify such a pocket, the tryptophans should be mutated and their effects on enzyme activity analysed. Since the pneumococcal neuraminidase only has eleven tryptophans, it would be a practical to mutate each one. The first candidate for mutagenesis should be W220. The reason being that W220 is equivalent to residue W128, a constituent of the hydrophobic pocket in *S. typhimurium* neuraminidase (Crennell *et al* ., 1993).

Another target for mutagenesis would be the "Asp" boxes (conserved sequence SXDXGXTW) that are present in all bacterial neuraminidases. Four to five "Asp" boxes are found in all bacterial neuraminidases and the number of amino acids separating the central boxes is always between 45 - 71 (Roggentin *et al* ., 1989). The conservation in the sequence, number and spacing of the "Asp" boxes suggests that they have an important role in enzyme activity. It has been proposed that they are involved in maintenance of protein structure and/or secretion (Crennell *et al* ., 1993). The role of the "Asp" boxes can be clarified by mutagenesis. Since tryptophans are present in all four "Asp" boxes, mutagenesis of tryptophans would result in targeting the "Asp" boxes. If the "Asp" boxes are important for protein folding then mutagenesis of the tryptophan would result in altered folding of the protein and consequently a reduction in enzyme activity.

Finally the hydroxyl group of a tyrosine residue in *S. typhimurium* (Y342), influenza A (Y406), influenza B (Y408) and *V. cholerae* (Y740) has been found in close proximity to the C1 and C2 carbons of sialic acid (Crennell *et al* ., 1994; Crennell *et al* ., 1996). This observation has led to proposals of a role for it in stabilizing the carbonium ion transition state intermediate (Burmeister *et al* .,

1993). Mutation of this tyrosine in influenza B neuraminidase to phenylalanine resulted in complete loss of activity of the influenza neuraminidase (Ghate and Air, 1998). It was proposed that the loss of activity was due to disruption of the interaction of the enzyme with the substrate. In the wild type enzyme the negative charge on the hydroxyl group of the tyrosine may help to stabilize the positive charge of the carbonium ion intermediate. However in the mutant enzyme the hydroxyl group is missing, the side chain of phenol is non-polar and cannot participate in hydrogen bonding (Ghate and Air, 1998). In another study on the *C. perfringens* neuraminidase the mutation of Y347>F resulted in decreased V_{max} by comaprison to the wild type (0.009 % compared to 100% for the wild type) and an alteration in the positioning of many amino acid side chains (Siebert *et al* ., 1996). An equivalent tyrosine has not been identified in the pneumococcal enzyme, however a tyrosine is present at position 777 in the pneumococcal enzyme. This may represent the key tyrosine because it is only one residue downstream from the equivalent positions (347 and 342) in the *C. perfringens* and *S. typhimurium* neuraminidases respectively. Therefore it would be interesting to change Y777>F and examine the effect on activity.

In addition to the amino acids discussed above for which have experimental evidence for their involvement in the activity of a small neuraminidase exists, more than twelve other amino acids have been found to be conserved between the small and large neuraminidases (Roggentin *et al* ., 1993). The function of these residues in the pneumococcal enzyme can be tested by site directed mutagenesis. For example towards the N-terminus is the presence of the RIP sequence which is highly conserved in neuraminidases. The arginine is the previously discussed R347 of the arginine triad. However the function of the isoleucine and proline are unknown and so can be the basis for future mutagenesis studies.

Another area worth investigating is the substrate preference of the wild type and mutated neuraminidases. This may give some indication of the relationship between structure of the enzyme and its substrate specificity. Also the substrate specificty of the neuraminidase may give indications of the primary site of action and physiological function (Corfield, 1992). The small family of neuraminidases hydrolyse a small range of substrates with a preference for α2-3 linked sialic acids, while the larger family have a wider substrate range (Roggentin *et al* ., 1993). On the basis of molecular weight we presume that the neuraminidase belongs to the large family, so it would be

interesting to see if it utilizes a wide range of substrates. Previous studies by Scanlon *et al* ., (1989) had shown that purified pneumococcal neuraminidase was able to hydrolyse many natural and synthetic substrates. However the relative preference for α2-3 or α2-6 linkages was only indirectly explored and the activity against α2-8 linkages and the effect of O-acetylation were not investigated. Future studies could make a more detailed analysis of the substrate prefence of the wild-type and mutated neuraminidases. Sialyl α2-3 lactose, sialyl α2-6 lactose and colominic acid (poly α2-8 NANA) can be used to investigate linkage preference. The activity against sialoproteins can be tested using fetuin, transferrin and α1 acid glycoproteins (Corfield 1992, Scanlon *et al* ., 1989). These substrates will also provide indirect but confirmatory evidence of linkage preference since they have different proportions of α2-3 and α2-6 linkages. Activity against sialo glycolipids can be investigated using a mixture of brain gangliosides. The effect of O-acetylation on activity can be investigated by comparisons of the activity on native and saponified bovine submaxillary mucin (BSM). Native BSM exists in various O-acetylated forms. The acetyl groups are removed by treatment with sodium carbonate (Gibbons, 1963).

Future experiments could examine the secondary structure of the protein using circular dichroism spectroscopy. This would show whether the introduced mutations have resulted in alterations of the folding of the protein. This would be especially valuable when examining the "Asp" box mutants.

Finally the elucidation of the crystal structure of the pneumococcal neuraminidase would provide invaluable information about the active site. For example for the influenza neuraminidase, the catalytic site was identified crystallographically by the binding of sialic acid (Burmeister *et al* ., 1992; Varghese *et al* ., 1992). Similar studies on the interaction of the pneumococcal neuraminidase with inhibitors such as NANA and DANA would help to identify the catalytic residues. It would be interesting to see if the crystallography studies confirm our predictions about the residues required for activity. In the present study it was not possible to carry out any crystallography studies due to the lack of sufficient quantities of the purified protein. However future improvements to the purification procedure should overcome this problem. Moreover initial attempts to crystallise the pneumococcal neuraminidase are being made by Crennell *et al* ., (Bath University) using the pSSGT construct.

6.2. Protein purification.

6.2.1 Introduction

In order to study the structure and function of neuraminidase and to allow examination of the *in vivo* protection effects of mutated forms of the protein, purification of the enzyme was required. Previous methods of neuraminidase isolation have involved numerous chromatography steps, such as ion exchange and gel permeation (Stahl and O'Toole, 1972; Lock *et al* ., 1988b). These schemes were time consuming and led to enzyme preparations contaminated by many different protein species (Stahl and O'Toole, 1972; Tanenbaum *et al* ., 1970).

A more efficient purification scheme with fewer steps than the existing protocols was therefore desirable. In an attempt to achieve this the neuraminidase structural gene was fused to a 6 x His tag and a single stage purification by affinity chromatography attempted. The use of this scheme enabled the production of purified neuraminidase. Although the quantities of pure neuraminidase obtained by this method were sufficient to allow basic enzymatic characterization and investigation of the vaccine potential (Chapters 4 and 5 respectively), they were insufficient to allow detailed structural and kinetic characterization. Two of the factors which may have contributed to the low yield were the initial inefficient binding of the neuraminidase to the Ni-NTA resin and the large losses of the protein that did bind during the wash stage. During the course of this study these factors were investigated and are discussed below.

6.2.2. Binding of the neuraminidase to the Ni-NTA resin

One of the major problems encountered during the purification of neuraminidase was the poor binding of the 6 x His tagged protein to the affinity resin. There were several possible explanations for this: the 6 x His tag may have been absent from the expression construct; the 6 x His tag may have been inaccessible to the resin; the 6 x His tag had been degraded or the conditions for binding to the Ni-NTA resin were sub-optimal (The QIAexpressionist, Qiagen, 1992). Several of these possibilities were investigated and are discussed below.

6.2.2.1 Investigation of the effect of NaCl concentration on the binding of the 6xHis tagged protein to the neuraminidase

The manufacturers of Ni-NTA resin recommended a concentration of 300mM NaCl in the binding buffer (The QIAexpressionist, Qiagen, 1992). However inclusion of this concentration of NaCl resulted in poor binding of the neuraminidase to the resin. It was possible that the binding of the neuraminidase to the Ni-NTA resin involved charge based interactions. If this was the case then a high concentration of NaCl in the binding buffer may interfere with the interaction between the resin and neuraminidase leading to the inefficient binding. To test this idea it was therefore decided to reduce the NaCl concentration in the binding buffer. To this end the NaCl concentration of the binding buffer was altered to 120mM, 165mM, 210mM, 255mM and 300mM and its effect on binding examined (Section 4.3.2.2.1). From the SDS-PAGE analysis of the unbound fractions it was apparent that the use of the different NaCl concentrations produced no difference in the binding of the neuraminidase. This result suggested that ionic interactions resulting from high NaCl were not responsible for reducing the affinity of the 6 x His neuraminidase to the Ni-NTA resin. Accordingly the concentration of NaCl recommended by the manufacturers (Qiagen) was used in the purification process

6.2.2.2. Determination of the accessibility of the His tag

Another possible explanation for the poor binding of the His tagged neuraminidase to the Ni-NTA resin may have been the inaccessibility of the His tag residues. During folding the His tag may have become "wrapped" or "buried" by the neuraminidase protein chain and become inaccesible to the resin (The QIAexpressionist, Qiagen, 1992). If this were the case then denaturation of the protein prior to purification might make the His tag accessible to the resin, and consequently improve the binding of the neuraminidase. To determine if the poor binding of the neuraminidase was a consequence of the His tag being hidden, the purification was performed under denaturing conditions (Section 4.3.2.2.2). The results of this experiment showed that even under denaturing conditions the majority of the 6 x His neuraminidase did not bind to the resin. It was therefore concluded that factors other than inaccessibilty of the His tag were responsible for the poor affinity of the neuraminidase for the Ni-NTA resin.

6.2.2.3. Experiments to determine if the poor binding is a consequence of the His tag being removed

Another explanation for the poor binding of the neuraminidase was that the His tag was attached to a portion of the neuraminidase that was processed after translation (The QIAexpressionist, Qiagen, 1992). In the neuraminidase expression construct the His tag is followed by a signal peptide. It is therefore possible that cleavage of the signal peptide could also result in loss of the His tag. Accordingly another neuraminidase His-tagged expression construct (pSSGT) was created which lacked the signal peptide. This was purified in an identical manner to the full length neurminidase. The truncated neuraminidase exhibited improved binding to the resin as judged by the absence of large quantities of neuraminidase in the SDS-PAGE analysis of the unbound fractions (Figure 4.10). However the purity of the neuraminidase was reduced as judged by SDS-PAGE analysis (Figure 4.11). In view of these results it was possible that cleavage of the signal peptide and consequent loss of the His tag was responsible in part or fully for the poor binding of the neuraminidase to the affinity resin. N-terminal sequencing of the purified protein would have answered this question, however attempts at N-terminal sequencing were unsuccessful because of extreme difficulty in obtaining sufficient quantities of the purified protein. It may have been profitable to investigate further optimisation of purification of the truncated neuraminidase, however time constraints made this impractical. Furthermore by this stage the mutagenised neuraminidases containing the signal peptide had already been constructed. Therefore it was decided to on advice persevere with the purification of the neuraminidase containing the signal peptide.

Another reason for the poor binding of the neuraminidase could be due to the presence of *nan A* start codons downstream of the His tag. Initiation of translation from these codons would result in production of a full-length neuraminidase without a His tag. Once again N-terminal sequencing would have resolved this possibility. In future constructs the His tag could be placed at the carboxyl terminus to ensure that only full-length protein will bind to the Ni-NTA resin. However it should be noted that a histidine tail at the carboxyl terminal is more likely to be inaccessible to the resin (Petty, 1995) and carboxyl terminal tagged proteins are less well expressed than amino tagged proteins (The QIAexpressionist, Qiagen, 1992). Alternatively the His tag can be left at the

N-terminus but the signal peptide sequence and *nan A* start codons are excluded.

6.2.3. Alterations in the wash conditions

Another major problem which affected recovery of the neuraminidase was the loss of large amounts of enzyme during the pre-elution wash stage. The wash stage was necessary to remove the background contamination that could have originated from many sources (Section 4.3.2.1).

There are several ways of removing the contaminating proteins. One method investigated was the alteration of the wash buffer pH. Proteins co-purifying with the neuraminidase would have originated from the host *E. coli* strain. Removal of these proteins from the resin prior to elution of neuraminidase should therefore result in a more purified preparation of the enzyme. To investigate this possibility the ability of wash buffers with pH values ranging from 5.0 to 8.0 to remove *E. coli* proteins from the resin were tested. It was found that pH 5.0 wash buffer was most effective at removing contaminating *E. coli* proteins. However when a pH 5.0 wash buffer was used for the purification of neuraminidase, it was found that although the levels of contaminating *E. coli* proteins were much reduced the yield of the purified neuraminidase was also correspondingly reduced. As a compromise between purity and yield a wash buffer of pH 6.0 was chosen for the neuraminidase purification scheme. The use of a pH 6.0 wash buffer for the purification of neuraminidase resulted in quantities and purity of neuraminidase suitable for basic enzymology and *in vivo* protection studies (Chapters 4 and 5 respectively).

If time had been available, other methods of reducing background contamination would have been investigated. These would have included the addition of low concentrations of imidazole in the binding and wash buffers to prevent contaminating proteins from binding to the Ni-NTA resin in the first instance. Also the addition of ß-mercaptoethanol (ß-ME) to the sonication buffer to reduce the formation of disulphide bonds between contaminating proteins and 6-His tagged proteins (The QIAexpressionist, Qiagen, 1992) could have been investigated . Another parameter worthy of investigation was the determination of the optimal ratio of Ni-NTA resin to the tagged protein. It has been suggested that the total binding capacity of the resin should be

matched to the amount of 6 x His protein to be purified (Schmitt *et al* ., 1993). This is because 6 x His tagged proteins have a greater affinity for the Ni-NTA resin than do background proteins. Consequently if the tagged protein fills the majority of binding sites then very few background proteins will be retained on the resin. If, however, too much resin is used then other proteins may bind nonspecifically to unoccupied sites and elute as contaminants (The QIAexpressionist, Qiagen, 1997).

An investigation into the effect of the length of the wash period on the purity and yield of the neuraminidase was also made. The original neuraminidase purification protocol (Section 2.3.3.6.1) included a wash period of 12 - 14 hours prior to elution of the protein from the resin. It was felt that this may have resulted in slow leaching of the protein from the resin and contributed to the final low yield of the purified neuraminidase. To investigate this possibility the wash length was reduced to 7.0 and 2.5 hours in two separate purifications and the effect on yield and purity of the neuraminidase examined. The reduced wash periods made no difference to the purity and yield of the neuraminidase (as judged by SDS-PAGE analysis of the purified neuraminidase), therefore the shorter wash length of 2.5 hours was included in the final purification procedure. Furthermore it was desirable that the neuraminidase was adsorbed to the resin for the minimum length of time in order to minimise spontaneous denaturation and inactivation.

After extensive attempts at optimisation of the binding and wash conditions a purification scheme for the *S. pneumoniae* neuraminidase was devised (Section 2.3.3.6.2). Although the final yield of the purified neuraminidase was not as high as anticipated the purification scheme did provide neuraminidase of sufficient quantity and purity for animal protection studies and enzyme assays.

6.2.4. Future developments

In view of the problems encountered with the single step purification of neuraminidase, future developments would examine alternative methods. Two main areas worthy of consideration are the development of a multi-stage purification scheme and/or the use of a different affinity tag. These options are discussed below.

6.2.4.1. Multi-step purification scheme

In order to avoid the problems associated with traditional multi-stage purification schemes, the technique of affinity chromatography was chosen to purify neuraminidase. In theory the technique is capable of separating the target protein from a total protein population in a single step (Österlund and Janson, 1997). However our experiences with the neuraminidase purification suggest that a single stage method may not be the best course. The affinity chromatography may be more appropriate as a final stage after the bulk of the contaminants had been removed by the use of other lower resolution techniques.

A suitable strategy for neuraminidase purification would involve an initial precipitation stage using ammonium sulphate to remove the large portions of bulk proteins and concentrate the target protein. The next logical step would be hydrophobic interaction chromatography (HIC) which is able to deal with the high ionic strength sample obtained from the previous salt precipitation stage. The adsorbed proteins should be removed from HIC by elution with a decreasing salt concentration gradient, making the subsequent fractions suitable for application to an ion exchange column. Finally affinity chromatography can be used to remove trace impurities.

6.2.4.2. Alternative tags

Another alternative would be to use a different affinity tag such as glutathione-S-transferase (GST) (Smith and Johnson, 1988) or thioredoxin (TRX) (LaVallie *et al* ., 1993). As the cloned neuraminidase is already available the construction of another affinity tagged protein would be relatively straight forward.

A new affinity protein expression and purification system sold by Stratagene claims to provide an alternative to the 6 x His tag system (Felts *et al* ., 1998, Stratagene). It consists of a calmodulin-binding peptide tag (CBP tag) and calmodulin affinity resin. The CBP tagged proteins can be purified to near homogeneity in a single step using calmodulin affinity resin. The CBP tag binds the calmodulin resin in the presence of low concentration of Ca^{2+} (0.2 - 2.0 mM $CaCl_2$) and the bound proteins are eluted by the application of 2mM EGTA at neutral pH. These are milder elution conditions compared to 6 x His tagged proteins which require a low pH or high concentrations of imidazole for

elution. In contrast to the 26 kDa GST and 40 kDa maltose binding protein (MBP) tags the CBP tag is small (4 kDa) and therfore less likely to affect the biological function of the protein of interest. The features of the various tags are shown in Table 6.1.

	6xHis tag	GST	CBP
Small tag	Yes	No	Yes
Mild elution	No	Yes	Yes
One-column purification	Yes	Yes	Yes

Table 6.1. The features of the various affinity tags used for protein purification.

6.3. Investigation of the protective effects of the mutated neuraminidases.

6.3.1. Introduction

The currently available pneumococcal vaccine has many limitations (Section 1.6.3), one of the main ones being that that it imparts only serotype specific protection. In an attempt to address this problem, several investigators have examined the potential use of pneumococcal proteins to provide more extensive protection. Some of the pneumococcal proteins investigated as vaccine targets include PspA and pneumolysin (Section 1.6.7.6). This study has examined the protective effects of one of the pneumococcal neuraminidases.

Previous studies on pneumococcal neuraminidase have been complicated by the presence of apparent multiple forms of the enzyme (Stahl and O'Toole, 1972; Tanenbaum *et al* ., 1970; Tanenbaum and Sun, 1971). However, it is now clear that there are at least two distinct neuraminidases, NanA and NanB (Section 1.7.8). To date, only the role of NanA in virulence has been examined (Lock *et al* ., 1988a; J. Hill, University of Leicester, unpublished results). Lock *et al* purified NanA from clinical isolates of *S.pneumoniae* and examined its efficacy as a protective immunogen in mice. Mice were immunized with purified neuraminidase and then challenged intranasally with virulent pneumococci. A significant ($P < 0.05$) increase in survival time of immunized mice compared to non-immunized controls was observed. However this protection was only observed if the enzyme was inactivated with 3.4% (v/v) formaldehyde prior to immunization. The requirement for inactivation

suggested that the lack of protection of the native protein was due to its toxicity. Although formaldehyde treatment reduced the toxicity of the neuraminidase it may also have impaired the immunogenicity of the enzyme by altering important epitopes. A more selective method of enzyme inactivation, such as mutation of the gene may produce an enzyme that is reduced in toxicity but still possesses the antigenic structure of the native molecule. The genetically altered enzyme may also be a safer vaccine component. To investigate this possibility the neuraminidases were altered by site directed mutagenesis (Section 3.2), then tested for their potential to protect mice from pneumococcal disease.

6.3.2 Protective effects of E_{647}>Q, Y_{752}>F and R_{663}>H neuraminidase toxoids.

Three neuraminidases, lacking significant levels of enyme activity (E_{647}>Q, Y_{752}>F and R_{663}>H) were created by site directed mutagenesis. These neuraminidase toxoids were purified and used to immunize mice. To enable comparisons to be made with the wild-type enzyme, a group of mice were also immunized with heat inactivated wild-type neuraminidase. Although the original intention was to compare the immunogenicity of the genetically altered neuraminidases with that of formaldehyde inactivated neuraminidase as used by Lock *et al* ., (1988a), difficulties in re-solubilising the protein after 3.4% formaldehyde treatment made this impractical. This further highlights the problems of using formaldehyde to inactivate the neuraminidase. As a positive control, one group of mice were immunized with a pneumolysin toxoid, PdB, which has been shown to impart protection against pneumococcal challenge (Alexander *et al* ., 1994). As a negative control, one group of mice were sham-immunized with PBS. Following immunization all groups of mice were challenged intranasally with virulent pneumococci, and their symptoms and survival times monitored. A number of interesting observations arose from the immunization studies and these are discussed below.

None of the mice showed any adverse reactions following immunization with any of the neuraminidase toxoids which suggested that the toxoids were well tolerated and not toxic at the levels given. It was particularly interesting to note that even the two neuraminidase toxoids (Y_{752}>F and R_{663}>H) which retained some enzymatic activity produced no discernible toxic effects in the immunized mice. From these observations it can be concluded that the

mutagenesis procedure had been successful in eliminating the *in vivo* toxicity of the neuraminidase at the dose given.

All of the neuraminidase toxoids provided protection against challenge with virulent pneumococci. Measurement of the survival time of the immunized mice following pneumococcal challenge showed that all the neuraminidase toxoids significantly ($P < 0.01$) increased the survival time of the immunized mice compared to PBS treated controls (Sections 5.3.2 and 5.4.1). Moreover with the exception of the R_{663}>H toxoid all the other neuraminidase toxoids were equally protective against pneumococcal infection (Section 5.4.2). Statistical analysis of the survival data (Table 5.8) showed that the difference in protection from R_{663}>H toxoid compared to the wild-type, just reached significance ($P < 0.05$). However this result needs to be viewed with caution as the P value obtained is on the threshold of statistical significance.

There was no correlation between the degree of protection elicited by a particular toxoid and its enzyme activity. This suggests that for the neuraminidase to be protective its activity only needs to be reduced to a threshold level. Further decreases beyond this do not result in increased protection or lack of toxicity.

In summary it can be concluded that all the neuraminidase toxoids were safe and effective in protecting mice against challenge with virulent pneumococci and therefore would be suitable for inclusion into a trial of pneumococcal vaccine in humans. Although the genetically altered and heat inactivated neuraminidases were equally effective in protecting mice from pneumococcal challenge, from a vaccine development perspective the former method would be preferable. As discussed previously in Section 3.6.2.2, using heat to inactivate proteins has many drawbacks including variations in the level of inactivation achieved and difficulties in recovering the protein. Neuraminidase inactivation at the genetic level would be much more amenable to quality control. It may even be prudent to engineer a genetically-altered neuraminidase which contained more than one mutation in order to decrease the likelihood of toxic revertants being formed during vaccine production. Since there was no relationship between the enzymic activity of the neuraminidase toxoid and the protection it elicited, it may be better to have a toxoid with some residual activity so that its purification could be monitored by assay of activity.

Another observation worth noting was that the median survival time of the PBS control mice in the first challenge trial (with toxoids PdB, E_{647}>Q and wild type neuraminidase, Section 5.3) was significantly different ($P < 0.01$) to that in the second trial (with toxoids PdB, Y_{752}>F and R_{663}>H, Section 5.4). This is surprising since the challenge dose in the second trial was ten-fold higher than in the first. Therefore the survival time of the PBS treated mice would if anything, be expected to decrease rather than increase. This suggests that the longer survival time of the PBS immunized mice in the second trial may have led to underestimation of the protection afforded by the PdB, Y_{752}>F and R_{663}>H toxoids in the second immunization trial. For example in the first challenge trial immunization with PdB significantly ($P < 0.05$) increased the survival time of the mice compared to PBS controls. In contrast, in the second challenge trial the survival of the PdB immunized mice was not significantly different ($P > 0.05$) from PBS controls.

Previous studies by Lock *et al* ., (1988a) have shown that immunization with pneumolysin confers greater protection than formaldehyde-inactivated neuraminidase. Mice immunized with formaldehyde-inactivated neuraminidase survived significantly longer ($P < 0.05$) than non-immunized mice. However the median extension in survival time was significantly less ($P < 0.01$) than that of mice immunized with pneumolysin. From these results Lock *et al* ., (1988a) suggested that pneumolysin may be a better candidate than neuraminidase for supplementing the existing polysaccharide vaccine. The results from the present study (Sections 5.3 and 5.4) are contrary to these findings. It was found that mice immunized with either of the neuraminidase toxoids (heat inactivated and genetically inactivated) survived significantly longer than PdB treated mice. Immunization of mice with either the E_{647}>Q (Section 5.3.2) or R_{663}>H (Section 5.4.1) neuraminidase toxoids resulted in significantly ($P < 0.05$) increased survival times compared to PdB treated mice. Mice immunized with heat treated wild-type (Section 5.3.2) or Y_{752}>F (Section 5.4.1) neuraminidase survived longer than PdB immunized mice, however the difference in their median survival times did not reach statistical significance ($P > 0.05$). These results suggest that neuraminidase may be a superior immunogen than pneumolysin. Moreover, neuraminidase has the advantage that it is a surface protein and may induce antibodies with opsonic value, unlike pneumolysin, which is a cytoplasmic protein.

In conclusion, this study has shown that immunization with either of the neuraminidase toxoids confers protection against *S. pneumoniae*. Neuraminidase toxoids should therefore be considered as promising candidates for inclusion in improved pneumococcal vaccines. Future studies investigating vaccine potential could be directed at conjugating the neuraminidase toxoids to pneumococcal polysaccharide and determining the immunogenicity of the conjugate. Similar experiments have been conducted with pneumolysin toxoid (PdB) conjugated to type 19F polysaccharide (a polysaccharide which is poorly immunogenic in infants (Paton *et al* ., 1991)). Mice immunized with this conjugate had significantly higher levels of anti-polysaccharide antibodies than mice immunized with 19F alone. Significant anti-pneumolysin titres were also obtained. Moreover, conjugation converted the 19F polysaccharide into an antigen capable of inducing a booster effect.

Another aspect worth investigating are the protective effects of PdB and neuraminidase in combination. Previous studies by Lock *et al* ., (1988a) have shown that pneumolysin provides more protection than formaldehyde treated neuraminidase. However the protection afforded by immunization with a combination of pneumolysin and formaldehyde inactivated neuraminidase was not significantly greater than that from immunization with pneumolysin alone. Therefore the protective effects of pneumolysin and neuraminidase were not additive. It would be interesting to see if immunization with a combination of PdB and the neuraminidase toxoids made in this study resulted in increased protection compared to mice immunized with PdB or neuraminidase alone.

Immunization with any one of the neuraminidase toxoids produced in this study provided protection against pneumococcal infection. The protection is likely to be mediated by the production of antibodies. This idea is supported by ELISA experiments which measured the serum levels of circulating antibodies following immunization with the various neuraminidase toxoids (Sections 5.3.3. and 5.4.3). In the case of the wild type and E_{647}>Q neuraminidase toxoids the serum antibody levels after two immunizations were measured and found to be significantly higher ($P < 0.001$) than the levels obtained from the sera of mice given two doses of PBS (Section 5.3.3.3). In the case of the Y_{752}>F and R_{663}>H toxoids comparison of the antibody levels prior to immunization and following the second immunization revealed a significant increase ($P < 0.001$)

(Section 5.4.3). Therefore an increase in specific antibody levels does occur following immunization with neuraminidase.

Interestingly there was no statistical difference ($P > 0.05$) in the post immunization antibody levels induced by either the wild type and E_{647}>Q neuraminidase toxoids (Section 5.3.3.3, Table 5.6). This suggests that both the neuraminidase toxoids were equally immunogenic. Further support for this hypothesis comes from the statistical comparison of the median survival times of the mice immunized with the various toxoids (Section 5.4.2, Table 5.8), which showed that the survival times of the mice immunized with the wild type, E_{647}>Q and Y_{752}>F neuraminidase toxoids were not significantly different ($P > 0.05$). The median survival time of mice immunized with the R_{663}>H toxoid compared to the wild-type neuraminidase were statistically different ($P < 0.05$). However the statistical value obtained is on the threshold of significance therefore needs to be treated with caution.

One limitation of this type of *in vivo* study is that it only provides partial information about the protective effects of the neuraminidase toxoids. Although there was an increase in antibody levels following immunization with the neuraminidase toxoids, there appeared to be no correlation between antibody levels in a particular mouse and its length of survival (Sections 5.3.3.4 and 5.4.4). This study gives no information about the function of the antibody. It is possible that a threshold antibody level is required for survival and after that level is reached further increases do not influence the survival time. Therefore knowing that antibodies are elicited after immunization is only partially informative, one also needs to know the function of the antibody. Future experiments directed at determining the opsonic and neutralizing titres of the antibodies would help clarify the situation.

6.3.3. Investigation of the protective effects of E_{647}>Q mutated neuraminidase in mice.

It was hypothesised that neuraminidase may be exerting its protective effect by reducing the number of pneumococci in the infected animals. To test this hypothesis and also to gain some idea of when and how protection was working, a "time course" of infection was done. Groups of mice were immunized with either the E_{647}>Q neuraminidase toxoid or PBS and then challenged with virulent pneumococci. The growth of bacteria in the lungs

and blood of mice was measured at pre-determined time points up to 72 hours following infection (Section 5.5).

In the lungs (Section 5.5.2) the statistical analysis of bacterial numbers suggested that there was no difference in the growth of bacteria in the PBS or E_{647}>Q immunized mice over the first 36 hours. Between 36 and 72 hours the numbers of bacteria appeared to have decreased in the lungs of the E_{647}>Q immunized mice but this drop was not statistically significant ($P > 0.05$) from 0 hours. No PBS treated mice were sampled after 36 hours.

A similar scenario was observed in the blood (Section 5.5.3). During the first 24 hours following infection, the growth of bacteria in the PBS and E_{647}>Q immunized mice was identical. Between 24 and 36 hours the bacterial numbers in the PBS immunized mice appeared to be increasing while those in the E_{647}>Q immunized mice appeared to have decreased. The decrease in bacterial numbers in E_{647}>Q immunized mice continued until 72 hours. However all the apparent changes in bacterial numbers for both the lungs and blood proved to be not statistically significant ($P > 0.05$).

From these results it appears that immunization with the E_{647}>Q toxoid does not affect the bacterial numbers in the lungs and blood of immunized mice. The results obtained from this experiment were less satisfactory than expected, possibly due to several factors. Firstly the number of bacteria in the PBS treated mice did not grow as expected. From the statistical analysis it appears that there is no increase in bacterial numbers between 0 and 36 hours in either the lungs or the blood of mice treated with PBS. This is in contrast to the results obtained by other workers in our laboratory and also by Alexander *et al* ., (1994). Secondly the number of bacteria in two to three of the five mice at any given time point (with the exception of the 0 hrs point) were very different from the others at that time point. This had the effect of skewing the mean values for each time point. Consequently the difference in bacterial numbers at the different time points became statistically insignificant. To compensate for the high variability in the bacterial numbers of two to three mice, statistical analysis of log transformed data was performed. However this did not alter the conclusions.

Therefore in view of the variation, if this experiment was to be repeated more than five mice at each time point, and also a greater number of time points (such as 48, 96, 120 and 144 hours post infection for the E_{647}>Q immunized mice), should be used. This would lead to a better representation of the growth profile during the experiment. In this study it was not possible to include a greater number of mice at each time point because of the lack of sufficient quantities of the purified protein for immunization, and lack of time.

It was hoped that this experiment would provide some indication of the nature of the antibody response. However given the results obtained it is not possible to draw such conclusions.

It has been proposed that neuraminidase may have a nutritional role in bacteria (Section 1.7.7.4.3). It has been suggested that bacterial neuraminidases may play a part in nutrition by degrading sialo-glycoconjugates and releasing carbohydrates as a bacterial energy source. If this were the case in the pneumococcal neuraminidase then a reduction in the growth rate of the bacteria in neuraminidase immunized mice might be expected. However from the results obtained here it is not possible to comment on this putative nutritional role.

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Appendix I

The Amino Acid Sequence of NanA.

```
        10        20        30        40        50        60        70
MSYFRNRDIDIERNSMNRSVQERKCRYSIRKLSVGAVSMIVGAVVFGTSPVLAQEGASEQPLANETQLSG 70
ESSTLTDTEKSQPSSETELSGNKQEQERKDKQEEKIPRDYYARDLENVETVIEKEDVETNASNGQRVDLS 140
SELDKLKKLENATVHMEFKPDAKAPAFYNLFSVSSATKKDEYFTMAVYNNTATLEGRGSDGKQFYNNYND 210
APLKVKPGQWNSVTFTVEKPTAELPKGRVRLYVNGVLSRTSLRSGNFIKDMPDVTHVQIGATKRANNTVW 280
GSNLQIRNLTVYNRALTPEEVQKRSQLFKRSDLEKKLPEGAALTEKTDIFESGRNGKPNKDGIKSYRIPA 350
        360       370       380       390       400       410       420
LLKTDKGTLIAGADERRLHSSDWGDIGMVIRRSEDNGKTWGDRVTITNLRDNPKASDPSIGSPVNIDMVL 420
VQDPETKRIFSIYDMFPEGKGIFGMSSQKEEAYKKIDGKTYQILYREGEKGAYTIRENGTVYTPDGKATD 490
YRVVVDPVKPAYSDKGDLYKGNQLLGNIYFTTNKTSPFRIAKDSYLWMSYSDDDGKTWSAPQDITPMVKA 560
DWMKFLGVGPGTGIVLRNGPHKGRILIPVYTTNNVSHLNGSQSSRIIYSDDHGKTWHAGEAVNDNRQVDG 630
QKIHSSTMNNRRAQNTESTVVQLNNGDVKLFMRGLTGDLQVATSKDGGVTWEKDIKRYPQVKDVYVQMSA 700
        710       720       730       740       750       760       770
IHTMHEGKEYIILSNAGGPKRENGMVHLARVEENGELTWLKHNPIQKGEFAYNSLQELGNGEYGILYEHT 770
EKGQNAYTLSFRKFNWDFLSKDLISPTEAKVKRTREMGKGVIGLEFDSEVLVNKAPTLQLANGKTARFMT 840
QYDTKTLLFTVDSEDMGQKVTGLAEGAIESMHNLPVSVAGTKLSNGMNGSEAAVHEVPEYTGPLGTSGEE 910
PAPTVEKPEYTGPLGTSGEEPAPTVEKPEYTGPLGTAGEEAAPTVEKPEFTGGVNGTEPAVHEIAEYKGS 980
DSLVTLTTKEDYTYKAPLAQQALPETGNKESDLLASLGLTAFFLGLFTLGKKREQ 1035
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APPENDIX II

Amino acid sequence alignment of bacterial neuraminidases.

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SP:MSYFRNRDIDIERNSMNRSVQERKCRYSIRKLSVGAVSMIVGAVVFGTSPVLAQEGASEQP---------
VC:MRF---KNVK--KTALMLAMFGMATSSNAALFDYNATGDTEFDSPAKQGWMQDNTNNGSGV---------
CP:MC----NKNNTFEKN-------------LD--ISHKPEPLILFNKDNNIWNSKYFRIPNIQ---------
ST:MTV---EKSVVFKAE-------------GEHFTDQKGNTIVGSGSGGT---TKYFRIPAMC---------
AV:M-----TSHSPFSRRRLPALLGSLPLAATGLIAAAPPAHAVPTSDGLADVTITQVNAPADG---------

SP:--------------------------LANETQLSGESSTLTDTEKSQPSSETELSGNKQE----------
VC:--------------------------LTNADGMPAW--LVQGIG-GRAQWTYSLSTNQHA----------
CP:--------------------------LLNDGTILTFSDIRYNGP-DD-HAYIDIASARST----------
ST:--------------------------TTSKGTIVVFADARHNTA-SD-QSFIDTAAARST----------
AV:--------------------------LYSVGDVMTFNITLTNTS-GEAHSYAPASTNLSG----------

SP:---QERKDKQEEKIP--RDYYARDLENVETVIEKEDVETN--------------ASNGQRVDLSSELDKL
VC:---QASSFGWR-M----TTEMKVLSGGMITNYYANGTQRV-----------------LPIISLDSSGNLV
CP:---DFGK-TWS-------------------------------------------------YNI--AMK
ST:---DGGK-TWN-KK-----------------------------------------------I--AIY
AV:---NVSKCRWR-NVP--AGTTKTDCTGLATHTVTAEDLKA--------------GGFTPQIAYEVKAVEY

SP:KKLENATVHMEFKPDAKAPAF--YNLFSVSSATKKDEYFTMAVYNNTATLEG------RGSDGKQFYNNY
VC:VEFEGQTGRTVLATGTAATEYHKFELVFLPGSNPSASFYFDGKL---IRDNI------QPTASKQNMIV-
CP:NNRIDSTYSRVMDSTTVIT--------------------------------N---------TGRIILIAG
ST:NDRVNSKLSRVMDPTCIVA--------------------------------N------IQGRETILVMVG
AV:AGKALSTPETIKGATSPVKA-NSLRVESITPSSSQENYKLGDTVSYTVRVRS------VSDKTINVAATE

SP:NDAPLKVKPGQWNSVTFTVEKPTAELPKGRVRLYVNGVLSRTSLRSGNFIKDMPDVTHVQIGATKRANNT
VC:-----------WGNG--SSNTDGVAAYRDIKFEIQGDVIFRGPDRIPSIVASSVTPGVVTAFAEKRVGGG
CP:SWNT----NGNW-----------------------------------A----------------------
ST:KWNN---NDKTW-----------------------------------GA----------------------
AV:SSFDDLGRQCHWGGL--KPGKGAVYNCKPLTHTITQADV--DAGRWTPSITLTATGTDGATLQTLTATGN
```
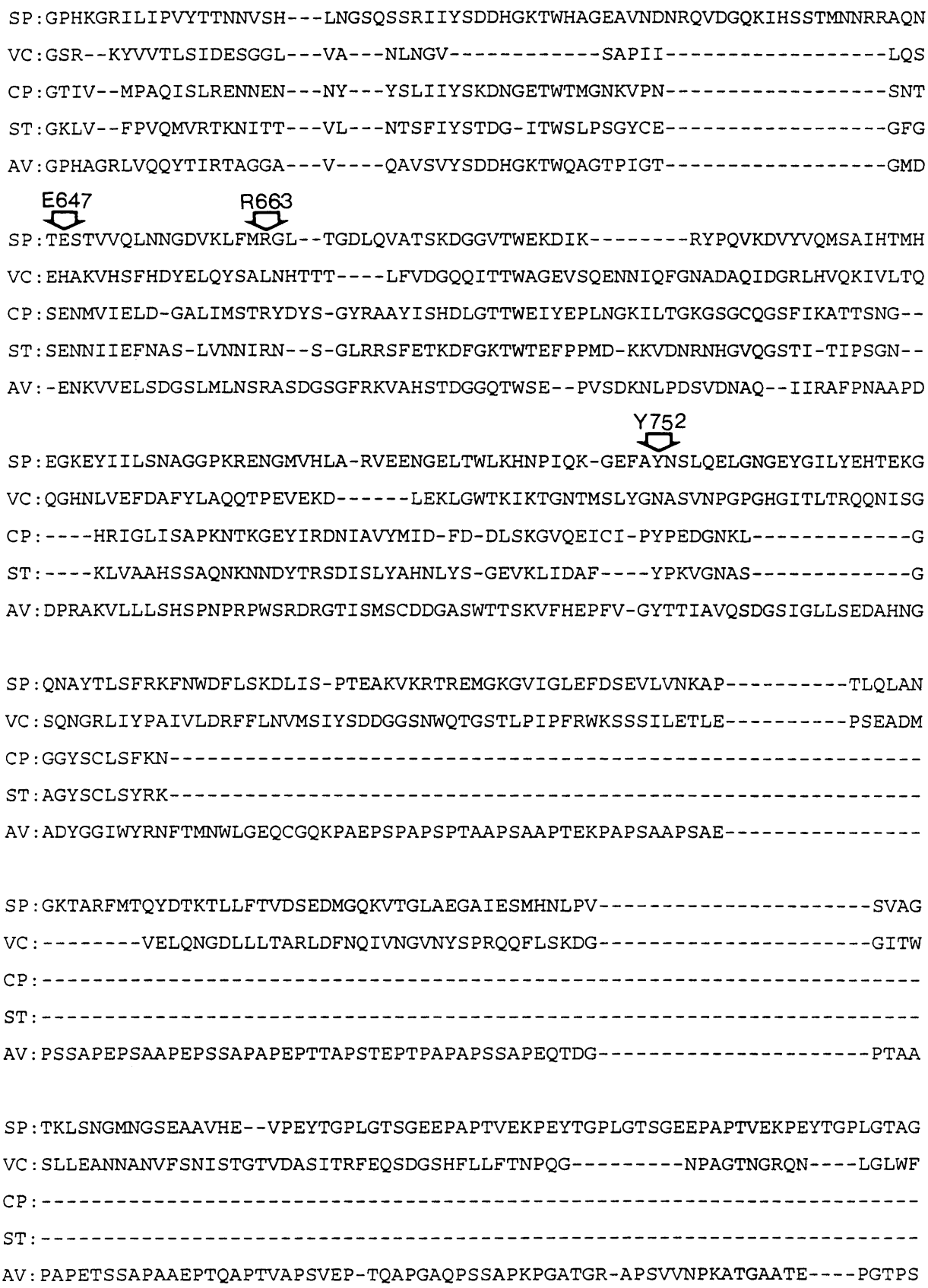

```
SP:VWGSN---------LQIRNLT----------VY-NRALTPEEVQKRSQL--------------FKRSDL
VC:-------------------------------------DPGALSNTNDI--------------ITRTSR
CP:------------------------------------------------------------------
ST:------------------------------------------------------------------
AV:-------------------------------------PINVVGDHPQA--------------TPAPAP

SP:EKKLPEGAALTEKTDIFESGRNGKPNKDGIKSYRIPALLKTDKGTLIAGADER------------RLHS
VC:DGGITWDTELNLTEQI--------NVSDEFDFSDPRPIYDPSSNTVLVSYARW------------PTDA
CP:------------------------------------------------------------------
ST:------------------------------------------------------------------
AV:DASTELPASMDQAQHL--------AANTATDNYRIPAITTAPNGDLLISYDER------------PKDN

SP:SDWGDI-----GMVIRRSEDNGKTW---------GDRVTITNLRDNPKASDPSIGSPVNIDMV-------
VC:AQNG---------------DRIKPW----------MPNGIFY--SVYDVASGNWQAPIDV----------
CP:-MTTSTRRSDWSVQMIYSDDNGLTW---------SNKIDLTK--DSSKVKN----QPSNT----------
ST:-YRDKAPDTDWDLVLYKSTDDGVTF---------SKVETNIH--DIVT-KN----GTISA----------
AV:GNGGSDAPNPNHIVQRRSTDGGKTW---------SAPTYIHQ--GTETGKKVGYSDPSYV----------

SP:-----------LVQDPETKRIFSIYDMFPEGKG-------IFGMSSQKEEAYKKIDGKTYQILYREGEKG
VC:-------------TDQVKERSF-------------------------------QI------AG-WGGS--
CP:-------------I--------------------------------------------------G-W-----
ST:-------------M------------------------------------------------------
AV:-------------VDHQTGTIF-------------------------------NFHVKSYDQG-WGGSRG

SP:AYTI----RENGTVYT-------------PDGKATDYRVV------VDPVKPAYSDKGDLYKGNQLLGNI
VC:-------------------------------------EL------YRRNTSLNSQQD------------
CP:------------------------------------------------------------------
ST:------------------------------------------------------------------
AC:--------------------------------GTDPENRGI------IQAEVSTSTDNG------------

SP:YFTTNKTSPFRIAKDSYLW---------MSYSDDDGKTWSAPQDITPMVKAD-WMKFLGVGPGTGIVLRN
VC:------------------W------------------QSNAKIRIVD-------------GAANQIQVAD
CP:-----------------------------------------------------------LG-GVGSGIVMDD
ST:-----------------------------------------------------------LG-GVGSGLQLND
AV:------------------W------------------TWTHRTITADITKDKPWTARFA-ASGQGIQIQH
```


SP:GPHKGRILIPVYTTNNVSH---LNGSQSSRIIYSDDHGKTWHAGEAVNDNRQVDGQKIHSSTMNNRRAQN
VC:GSR--KYVVTLSIDESGGL---VA---NLNGV----------SAPII----------LQS
CP:GTIV--MPAQISLRENNEN---NY---YSLIIYSKDNGETWTMGNKVPN----------SNT
ST:GKLV--FPVQMVRTKNITT---VL---NTSFIYSTDG-ITWSLPSGYCE----------GFG
AV:GPHAGRLVQQYTIRTAGGA---V----QAVSVYSDDHGKTWQAGTPIGT----------GMD
E647
R663
SP:TESTVVQLNNGDVKLFMRGL--TGDLQVATSKDGGVTWEKDIK--------RYPQVKDVYVQMSAIHTMH
VC:EHAKVHSFHDYELQYSALNHTTT----LFVDGQQITTWAGEVSQENNIQFGNADAQIDGRLHVQKIVLTQ
CP:SENMVIELD-GALIMSTRYDYS-GYRAAYISHDLGTTWEIYEPLNGKILTGKGSGCQGSFIKATTSNG--
ST:SENNIIEFNAS-LVNNIRN--S-GLRRSFETKDFGKTWTEFPPMD-KKVDNRNHGVQGSTI-TIPSGN--
AV:-ENKVVELSDGSLMLNSRASDGSGFRKVAHSTDGGQTWSE--PVSDKNLPDSVDNAQ--IIRAFPNAAPD
Y752
SP:EGKEYIILSNAGGPKRENGMVHLA-RVEENGELTWLKHNPIQK-GEFAYNSLQELGNGEYGILYEHTEKG
VC:QGHNLVEFDAFYLAQQTPEVEKD------LEKLGWTKIKTGNTMSLYGNASVNPGPGHGITLTRQQNISG
CP:----HRIGLISAPKNTKGEYIRDNIAVYMID-FD-DLSKGVQEICI-PYPEDGNKL----------G
ST:----KLVAAHSSAQNKNNDYTRSDISLYAHNLYS-GEVKLIDAF----YPKVGNAS----------G
AV:DPRAKVLLLSHSPNPRPWSRDRGTISMSCDDGASWTTSKVFHEPFV-GYTTIAVQSDGSIGLLSEDAHNG
SP:QNAYTLSFRKFNWDFLSKDLIS-PTEAKVKRTREMGKGVIGLEFDSEVLVNKAP----------TLQLAN
VC:SQNGRLIYPAIVLDRFFLNVMSIYSDDGGSNWQTGSTLPIPFRWKSSSILETLE----------PSEADM
CP:GGYSCLSFKN----------
ST:AGYSCLSYRK----------
AV:ADYGGIWYRNFTMNWLGEQCGQKPAEPSPAPSPTAAPSAAPTEKPAPSAAPSAE----------
SP:GKTARFMTQYDTKTLLFTVDSEDMGQKVTGLAEGAIESMHNLPV----------SVAG
VC:--------VELQNGDLLLTARLDFNQIVNGVNYSPRQQFLSKDG----------GITW
CP:----------
ST:----------
AV:PSSAPEPSAAPEPSSAPAPEPTTAPSTEPTPAPAPSSAPEQTDG----------PTAA
SP:TKLSNGMNGSEAAVHE--VPEYTGPLGTSGEEPAPTVEKPEYTGPLGTSGEEPAPTVEKPEYTGPLGTAG
VC:SLLEANNANVFSNISTGTVDASITRFEQSDGSHFLLFTNPQG---------NPAGTNGRQN----LGLWF
CP:----------
ST:----------
AV:PAPETSSAPAAEPTQAPTVAPSVEP-TQAPGAQPSSAPKPGATGR-APSVVNPKATGAATE----PGTPS

```
SP:EEAAPTVEKPEFTGGVNGTEPA-VHEIAEYKGSDSLV----------TLTTKEDYTYKAPLAQQALPET
VC:SFDEGVTWKGP----------IQLVNGASAYSDIY----------QLDSENAIVIVE---------T
CP:----------------------N----HLGIVYEANG----------NIEYQDLTPY----------
ST:----------------------NVDKKHCMLSMKPME----------VLSSRTLAVI----------
AV:SSASPAPSRNAAPTPKPGMEPDEIDRPSDGTMAQPTG----------GASAPSAAPTQAAKAGSRLSRT

SP:GNKESDLLASLGLTAFFL-GLFT----------L--GK-KREQ
VC:DNSN---MRILRMPITLLKQKLT----------L------SQN
CP:--------------------YS----------LI-----NKQ
ST:--------------------Y--------------------Q
AV:GTNA---LLILGLAGVAVVGGYL----------LLRAR-RSKN
```

Amino acid sequence alignment of bacterial neuraminidases using Clustal method with PAM250 residue weight table.
Codes represent neuraminidases from: SP (*S. pneumoniae*, nan A); VC (*V. cholerae*); CP (*C. perfringens*); ST (*S. typhimurium*); AV (*A. viscosus*).
The residues mutated in this study in the *S. pneumoniae* neuraminidase are indicated.