Pneumolysin as a vaccine and study of structure and function

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by

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Abstracts

Streptococcus pneumoniae is a Gram-positive bacterium capable of causing the life threatening invasive diseases pneumonia, septicemia and meningitis. It can also cause the highly prevalent, but less serious diseases, acute otisis media (AOM) and sinusitis.

Pneumolysin, a 52Kda protein toxin, is not only an important virulence factor of *Streptococcus pneumoniae* but toxoid versions can be protective immunogens, although they suffer from residual activity. The toxin is produced by all serotypes and has very limited structural variation. Structurally pneumolysin consists of four domains. The mechanism of action of pneumolysin has been proposed to involve binding to the cell surface via its domain 4, then the toxin monomers oligomerize on the cell surface and finally structural changes in domain 3 results alpha helices being converted into beta hairpins that insert into the membrane to form a pore. To understand how the structure explains this function of pneumolysin, two sets of mutants were constructed to investigate the role of individual amino acids in these events. In one set, cystine bridges were introduced to lock different domains; C262+C278 was found to have locked TMH2. In the second set, mutants were made to test hypotheses on which amino acids took part in oligomerization, a double mutant 18A: 84A was found to be inactive.

Currently the available pneumococcal vaccines are polysacchride based (Pneumovax) and a protein conjugate vaccine (Prevenar). The problem with Pneumovax is that it does not elicit protection in people at high risk and it covers only 23 serotypes. Prevenar is expensive and has even more limited serotype coverage. An ideal vaccine solution would be a molecule with little or no variation among different serotypes and capable of inducing a T-cell dependent response.

The functionally important C-terminal domain of pneumolysin (D4) was investigated for its immunogenicity as a protective antigen and as an adjuvant. Domain 4 (D4) of pneumolysin elicits antibody response and boost it as an adjuvant but didn't provide protection on its own. Domain 4 mixed in equimolar amounts with domains 1-3 was non-toxic and it provided protection against a lethal dose of *S. pneumoniae*.

(1) Introduction:

(1.1) *Streptococcus pneumoniae*: *Streptococcus pneumoniae* is a classic extracellular Gram positive bacterium. It is a round or oval shaped non spore forming immotile bacterium usually present in pairs or short chains. Sensitivity of Pneumococcus to optochin and alpha hemolysis on blood agar plates are characteristics extensively used for isolation and identification. Pneumococcus is a catalase negative bacterium, first identified by Louis Pasteur and George Sternberg independently in 1881. Louis Pasteur isolated the bacterium from saliva of rabies patient while Sternberg isolated it from his own saliva. (Sternberg 1881; Helen *et al.,* 2006; Weiser *et al.,* 1994).

It was named as *Diplococcus pneumoniae* by Weichselbaum in 1886 (Austrian 1960) and was known as *Diplococcus pneumoniae* until 1974 and *Streptococcus pneumoniae* afterwards as a result of the observation that it can grow as chains in liquid media. *Streptococcus pneumoniae* is a commensal of the human nasopharyngeal cavity (Cartwright 2002).

The carriage rate for this bacterium is variable and varies among the 91 different serotypes, but nasopharyngeal carriage rates may exceed 70% in certain populations (Riley and Douglas 1981).

After gaining access to the sterile parts of the respiratory tract it can cause some life threatening diseases like pneumonia, bacteraemia and meningitis if it breaches the blood brain barrier. Despite the availability of effective chemotherapy, the pathogen is responsible for around one million deaths worldwide. The main reason for this mortality is the emergence of antibiotic resistance strains. What is even more worrying that these

drug resistant strains belong to the serotypes isolated from children. Pneumococcus is also a major agent responsible for commonly prevalent but less serious infections like acute otitis media (AOM) and sinusitis (Paton 1996, Marchese *et al.,* 1995, Linares *et al.,* 1992, Tai 2006). Like most other pathogens, pneumococcal antibiotic resistant strains are on the rise, Penicillin as a drug of choice against pneumococcal infection is now history. What is even more worrying that resistant strains to newer antibiotics, such as vancomycin and cephalosporins, are on the rise, which makes the treatment even more difficult (Minton and Macfarlane 1996; Novak *et al.,* 1999).

People at risk to the pneumococcal infections include children, elderly, immunocompromised and cigarette smokers. Young children are particularly susceptible to this pathogen. The problem of child mortality due to pneumonia is more pronounced in the developing countries as compared to developed countries where pneumococcus is the agent involved in at least one third of the cases (Greenwood, 1999).

According to a systemic literature review, total episodes of pneumococcal disease in children aged 1-59 months worldwide are estimated to be about 14.5 million of which 826,000 children die annually. Over 61% of the mortality is associated with 10 African and Asian countries. The underlying problem may be due to the financial burden, which is hard on part of these countries, furthermore, despite the availability of antibiotics in the developed world the mortality rates due to pneumonia are still high i.e mortality rates of 5-22% have been reported elsewhere while it is 14% in the UK (Macfarlane et al., 1982, Lim et al., 2001, Mufson et al., 1982, O'Brien et al., 2009) .

Annually in the United States 3,000 cases of meningitis and 50,000 cases of bacteraemia are recorded (Butler *et al.,* 1999). In adults 60-87% of bacteraemia caused by pneumococcus are linked with pneumonia (Afessa *et al.,* 1995). In the United States pneumococcus caused 40,000 deaths, which are more than deaths caused by any other bacterial pathogen (Alexander *et al.,* 1994). In a review and analysis of fourteen studies conducted about lung aspirates of children diagnosed with pneumonia one of the single major agent involved for causing the disease was found to be *Streptococcus pneumoniae* (Berman, 1991).

In the UK 76% of cases of community acquired pneumonia were attributed to the pneumococcus (Macfarlane et al., 1982). The risk of death increases with coexistence of another disease and age is also an important determining factor as with increasing age no difference was found in the mortality rate between those with and without underlying conditions. Adults with AIDS are 100% more susceptible while adults with sickle cell anemia are 30-100% more prone to the disease as compared to the healthy population (Jetté and Lamothe, 1989). In the case of acute otitis media (AOM) estimated 75% of children suffer at least one episode of this disease by the time they reach 6 years of age and 67% of children of the same age group has to go through this painful ordeal in the United States (Austrian, 1984b).

It is estimated that annually 5 million children having age less than 5 years die of pneumoniae, with the pneumococcus being the most common agent involved (Lancet 1985). Lee *et al.,* 2010 isolated *Streptococcus pneumoniae* from patients with respiratory tract infections. They found out that resistance to erythromycin, clindamycin and tetracycline were high and 56% of isolates were multidrug resistant (MDR) to three or

more of the tested antibiotics. The MDR strains of serotypes 19A, 3, 6A and 11Aa were of particular interest as they are the non PCV-7 serotypes. Furthermore, antibiotics that are bacteriolytic in nature may result in release of the bacterial toxin and due to the detrimental effect of the toxin, the patient can be left with organ deficits (Spreer *et al.,* 2003, Hirst *et al.,* 2004).

A schematic diagram of the virulence factors of *S. pneumoniae* is given in Fig.1.1 As can be seen in the figure, the virulence factors of *S. pneumoniae* include the pneumococcal capsule, pneumococcal surface protein A, hyaluronate lyase, choline binding protein A, autolysin, neuraminidase, pneumococcal surface antigen A, pneumococcal surface protein C and pneumolysin.

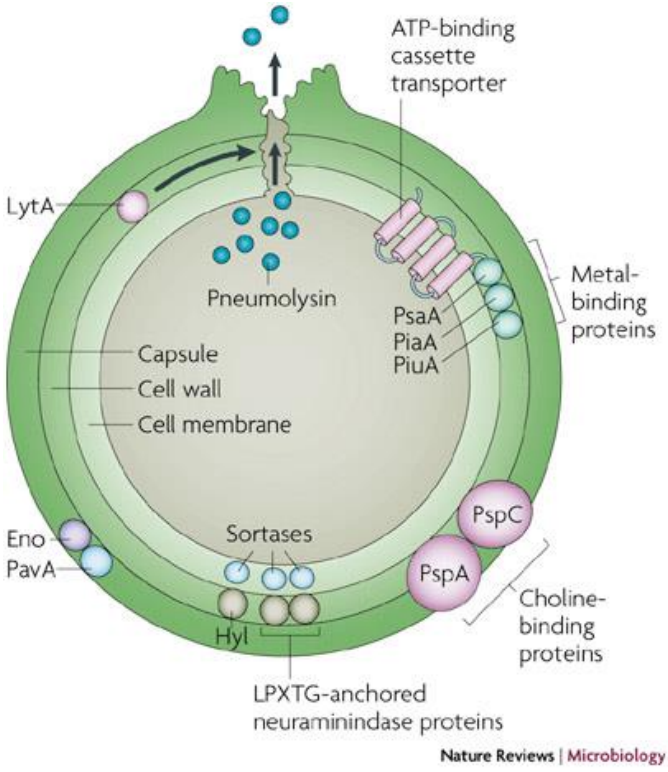


Fig 1.1: Some of the imporatnt virulence factors of *Streptococcus pneumoniae* include: The capsule, the cell wall, PspA, PspC (Choline binding proteins), autolysin,

Pneumolysin (PLY), enolase (eno), LPXTG anchored neuraminindase proteins, metal binding proteins (PsaA, PiaA, PiuA). Some of which are described in detail. (Kadioglu et al., 2008)

1.2 Some important virulence factors of the pneumococcus

1.2.1 The pneumococcal Capsule:

The outer most 200-400nm thick layer of the pneumococcal cell is the capsular polysaccharide (CPS) which is perhaps the most important virulence factor (Sorensen *et al.,* 1988; Kadioglu *et al.,* 2008). Based on the difference in structure of capsular polysaccharide, a total of 90 serologically different serotypes have been described so far (Henrichsen 1995). Regardless of differences in the capsular polysaccharide they all function to reduce opsonophagocytosis. The degree of inhibition of phagocytosis varies among different serotypes depending on the nature of capsular polysaccharides (Jonsson *et al.,* 1985; Melin *et al.,* 2010).

The pneumococcal capsule is an important virulence factor in invasive pneumococcal disease. Watson and Musher 1990 infected two groups of mice intraperitoneally with serotype 3 pneumococci having functional and non-functional capsule, respectively. They found that pneumococci with non-functional capsules were less virulent. Morona *et al.,* 2007 used serotype 2 pneumococci having functional and non-functional capsule and infected mice intranasally. They found significantly lower number of bacteria present in the lungs and blood of mice infected with pneumococci having a non-functional capsule

as compared to mice infected with pneumococci having a functional capsule. Antibodies against cell wall constituents including, teichoic acids and cell surface proteins were found to bind complement (Winkelstein *et al.,* 1981; Musher, 1992).

Another factor responsible for reduced phagocytosis is the net negative charge of the capsule at physiological pH, which electrostatically repels bacteria from phagocytic cells (Lee *et al.,* 1991; Magnusson, 1989).

The capsule also adds to the virulence of *S. pneumoniae* due to its adhesive function (López & García, 2004). The capsule is essential for colonisation and helps the bacterium escape mechanical removal by mucus (Nelson *et al.,* 2007). It also limits autolysis and reduces exposure to antibiotics (Van der Poll and Opal 2009). The virulence of *S. pneumoniae* differs significantly between capsular serotypes (Austrian, 1997). One factor contributing to the variation in capsular polysaccharide is the accessibility of bacterial surface adhesions, which interact with host ligands, affecting the virulence of bacterial cells (Carlos *et al.,* 2011).

The ability of particular strain or serotype to cause disease often depends on the presence of the capsule. Non-capsulated strains are less virulent as compared to encapsulated strains (MacLEOD & Kraus, 1950; Hostetter *et al.,* 1986). However, the capsule itself is non-inflammatory (Mitchell *et al.,* 1997). Virulent clinical isolates are encapsulated. Spontaneous non-encapsulated derivatives of these strains have been found to be almost completely avirulent (Lee *et al.,* 1991; Briles *et al.,* 1992). However, some non-encapsulated strains have been found associated with superficial infections like conjunctivitis (Martin *et al.,* 2003).

The degree of complement deposition has demonstrated to be correlated with the capsular type. The capsule impairs C3 deposition on the bacterial surface and conversion of surface bound C3b to iC3b (Abeyta *et al.,* 2003; Hyams *et al.,* 2010; Melin *et al.,* 2010b).

1.2.2 Pneumococcal surface protein A (PspA)

Pneumococcal surface protein A (PspA) is a cell surface exposed choline binding protein present in all strains of *Streptococcus pneumoniae* discovered so far (Jedrzejas, 2006; McDaniel *et al.,* 1984; Crain *et al.,* 1990). The molecular weight of PspA is variable between different strains and ranges between 67-99 KDa. PspA consists of three structural domains: The N-terminal domain, considered to be the functional part of the protein, mainly consists of repeated α-helices. These α-helices extend from the cell wall and protrude outside of the capsule. It is believed that this domain interacts with antibodies (Jedrzejas *et al.,* 2001 and 2000).

The outer domain of PspA is highly electronegative and believed to have anti-complement properties thus helps in preventing *S. pneumoniae* from host complement system (Jedrzejas *et al.,* 2000; Briles *et al.,* 1997; Ren *et al.,* 2004). However, studies on the role of PspA in complement inhibition were conflicting and not conclusive. Some studies have shown that PspA protects the bacteria from complement-mediated phagocytosis by interfering with complement C3 activation on pneumococcal cell surface (Tu *et al*., 1999; Ren *et al*., 2003; Ren *et al.,* 2004). Other studies show that inhibition of complement is due to PspA interfering with C4 deposition on the surface of *S. pneumoniae* (Li *et al.,* 2007; Li *et al.,* 2009).

PspA is considered for alternative vaccine approaches as it is present in all serotypes and elicits an antibody response (Nabors *et al.,* 2000; Jedrzejas *et al.,* 2001). PspA, an essential virulence factor, is highly immunogenic eliciting an immune response against *S. pneumoniae* (McDaniel *et al.,* 1987; Crain *et al.,* 1990; Jedrzejas *et al.,* 2001). Within different strains PspA is not only antigenically heterogenic but also elicits cross protection against strains of different capsular type (Kolberg *et al.,* 2001; McDaniel, 2007; Moreno *et al.,* 2010). A study found promising prospects about PspA when administered as an intranasal vaccine. Specific IgGs has a protective role against secondary pneumonia after influenza (Ezoe *et al.,* 2011).

Immunization with recombinantly fused PspA to a flagellin from *Vibrio vulnificus* (FlaB an agonist of TLR5) was shown to elicit a more efficient protective mucosal immune response against pneumococcal infection in mice than immunization with PspA alone (Nguyen *et al.,* 2011).

1.2.3 Pneumococcal surface protein C (PspC)

Pneumococcal surface protein C (PspC) also known as choline binding protein A (CbpA) is another pneumococcal surface protein and the only molecule homologous to PspA in its structure and also similar in function (Mook-Kanamori *et al.,* 2011; Briles *et al.,* 1997). PspC being a surface exposed protein is attached to the cell wall through specific choline binding motifs (Jedrzejas 2001).

PspC is one of the important virulence factors of *Streptococcus pneumoniae* as it has a crucial role in adherence to the host cells, colonisation and therefore immunogenicity to *S. pneumoniae.* Rosenow *et al.,* (1997) demonstrated that a PspC mutant shows reduced

nasopharyngeal colonistaion as compared to the wild type strain because of the compromised *in vitro* binding to epithelial cells and silaic acid residues of epithelial cells. The contribution of PspC to virulence in lung infection and during systemic infection is strain dependent and high genetic variability is found among PspC from different pneumococcal strains.

PspC mutants of serotype 2 and 3 were found to be less virulent in a sepsis model (Ogunniyi *et al.,* 2007; Kerr *et al.,* 2006, Iannelli *et al.,* 2004). PspC evades the host complement system by binding to the negative regulator of the alternative pathway which is factor H, thus preventing the *S. pneumoniae* from the action of alternative pathway (Duthy *et al.,* 2002**;** Dave *et al.,* 2001; Hammerschmidt *et al.,* 1997**)**. The three potential mechanism described for this binding are; 1. Dissociation of factor B from C3 convertase of the alternative pathway which may reduce C3b deposition on the surface of S. pneumoniae. 2. Factor H acts as a cofactor and degrade C3b through factor I-dependent cleavage of C3b bound to the bacterial surface to iC3b. 3. C3 convertase formation on the bacterial surface might be inhibited due to the binding of factor H to C3b. This in turn might dissociate C3b from factor B and decay the C3 convertase (Dave *et al.,* 2004). PspC can also block the activation of classical pathway of complement by binding to the fluid phase complement inhibitor C4b binding protein (Dieudonne-Vatran *et al.,* 2009).

1.3 Pneumococcal infection and host defense

A series of mechanisms from the host innate immune system and cooperation from the adaptive immune system is required for a successful defence against pneumococcal infection (Andersen & Feldman 2011). First in the line to attack *S. pneumoniae* are

mucosal antibodies of which secretory IgA are important, however, IgG antibodies are directed against the pneumococcal capsule and provides type specific immunity against the bacterium by inhibiting its binding to the nasopharyngeal mucosa. Binding of these antibodies to the Pneumococcus may facilitate phagocytosis through antigen presenting cells and neutrophils (Kurono *et al.,* 1991; Bogaert *et al.,* 2004). Antibodies against certain pneumococcal proteins like anti-PspC, anti-PspA and anti pneumolysin antibodies may also be present in this region but are less efficient in protection as compared to secretary IgG (Ogunniyi *et al.,* 2007).

Lactoferrin, a bactericidal iron scavenger found in body fluids like saliva, nasal secretions and also carried by neutrophils has direct activity on the cell wall of *S. pneumoniae*. Unbound lactoferrin can induce lysis of the bacterium by disrupting its cell wall by an unknown mechanism (Esposito *et al.,* 1990, Raphael *et al.,* 1989; Arnold *et al.,* 1980; Senkovich *et al.,* 2007).

The complement system is also an important component of mucosal immunity. Activation of the complement cascade results in subsequent cleavage of several complement factors, each having and leading to a different biological activity like opsonisation, phagocystosis, leukocyte recruitments and formation of the membrane attack complex. Neutrophils of the host kill the pathogen by opsonophagocytosis; a process, requiring opsonisation of the bacterium by the complement system (Bogaert *et al.,* 2010; Lysenko *et al.,* 2007). Complement activation also results in deposition of C3 on the surface of the bacterium, which in turn promotes interaction with the complement receptors and thus facilitates phagocytosis (Dalia and Weiser 2011; Lambris *et al.,* 2008).

Another mechanism of the innate immune response is mediated through C-reactive protein. C-reactive protein (CRP) is produced by the liver during the acute phase of infection and is also expressed in respiratory epithelial cells. CRP can bind to several bacteria including *S. pneumoniae* and activates the classical pathway of complement through C1q or may promote phagocytosis by binding to Fcγ receptor on macrophages and dentritic cells to induce and promote cytokine production (Gould & Weiser 2001; Winkelstein & Tomasz 1977; Suresh *et al.,* 2006; Mold & Du 2006; Thomas-Rudolph *et al.,* 2007).

The pneumococcal toxin (Pneumolysin) is known to augment bacterial virulence, however at sublytic concentrations it may benefit the host by eliciting a protective immune response. Pneumolysin in sublytic concentrations binds to the host epithelial cells producing sub lethal pores which may lead to Ca^{++} influx. This influx of calcium ions may result in the activation of several transcription factors and thus activating pro inflammatory cytokines like IL-8 which promote recruitment of neutrophils. This whole process is helpful in controlling early colonisation (Ratner *et al.,* 2006; Koga *et al.,* 2008; Mathias *et al.,* 2008).

The pattern recognition molecules known as toll-like receptors, and which functions as part of the innate immune system, interact with pneumolysin. Toll-like receptors also link adaptive immunity to innate immunity by up regulation of co-stimulatory molecules on antigen presenting cells and through the secretion of pro-inflammatory cytokines. Pneumolysin, after binding to TLR-4 initiates a cascade of signals inducing and promoting the release of proinflammatory cytokines IL-8, IL-6 and IL-1b and TNFa. These proinflammatory cytokines helps in the chemoattraction and recruitment of

neutrophils (Malley *et al.,* 2003; MacLeod *et al.,* 2007). Protection against *S. pneumoniae* is provided by these proinflammatory responses. However, hyper activation of these responses can result in overproduction of the cytokines, which can cause disruption of the branchoalveolar epithelial barrier due to inflammation. The disruption of the epithelial barrier may lead to extra pulmonary dissemination of the pathogen (Garcia-Suarez Mdel *et al.,* 2007; Martner *et al.,* 2008).

1.4 Pathogenesis of pneumococcal infection

Streptococcus pneumoniae, normally a commensal of the human nasopharyngeal tract can transfer from one individual to other directly via air borne transmission. Under specific conditions the host may become more susceptible to infections and the commensal is transformed into a pathogen causing disease. The disease may be local or invasive and may disseminate to other organs of the body through the blood stream as shown in Fig. 1.2 (Bogaert *et al.,* 2004).

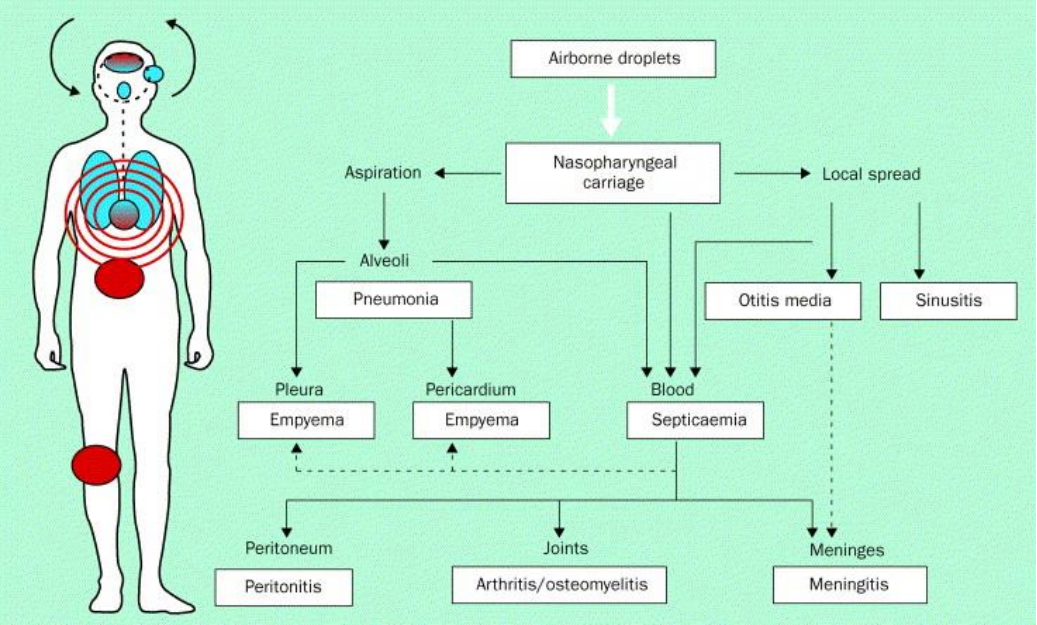


Figure 1.2: Different routes followed during the course of infection by *Streptococcus pneumoniae* starts from the nasopharynx through airborne droplets and subsequent spreading to other organs of the body. Organs infected through airborne showed in blue, and organs infected through blood showed in red (Bogaert *et al.,* 2004).

1.4.1 Colonisation

S. pneumoniae colonises the mucosal surface of the nasopharynx in the upper respiratory tract of the host as a commensal under normal circumstances. Colonisation of *S. pneumoniae* is asymptomatic and disease can occur only when the bacterium gains access to the sterile part of the body i.e. lungs, the middle ear and the blood stream. Colonisation with multiple strains of *S. pneumoniae* in humans starts with birth and carriage is throughout childhood. Carriage frequency in childhood changes with age i.e. it may be as high as 50% in the beginning and may gradually decline to 10% at the age of 5 years (Bogaert *et al.,* 2004, Musher, 2003).

Transfer of the bacterium from one individual to another is through coughing and sneezing. Successful colonisation is dependent on adherence, nutrition and replication of the bacterium. To fulfil these requirements, the bacteria have to overcome the host's natural barrier including the one formed by the respiratory mucosa, immune system of the host. They also have to compete for the niche with other colonising pathogens. Mucus secretions being the first barrier on respiratory mucosal surface entrap *S. pneumoniae* but the pneumococcal capsule helps the bacterium to escape. The pneumococcal capsule is negative charged which repulses the negatively charged sialic acid residues in the mucus (Coonrod *et al.,* 1991, Nelson *et al.,* 2007).

The pneumococcus expresss certain enzymes like neuraminidase A (Nan A), β-galactosidase A (BgA), β–N-acetylglucosaminidase (StrH) and neuraminidase B (NanB). These exoglycosidases help to remove the terminal sialic acids, galactose and N-acetylglucosamine. They help to reveal receptors for adherence, affect the function of host glycosylated molecules, modify the surface of cohabiting competing bacteria. BgA and Nan A are also known to contribute to adherence of pneumococcal cells to the host epithelial cells (Davis *et al.,* 2008, King *et al.,* 2006, Tong *et al.,* 2000).

Another factor that may help in the escape of the Pneumococcus from entrapment by the mucus is the pneumococcal toxin pneumolysin. Pneumolysin is known to inhibit cilliary beating of the epithelial cells thus aids in the binding of the bacterium to epithelial cells without any interference from the mucus (Feldman *et al.,* 2002).

The presence of lysozyme on the mucosal cell surfaces is another challenge faced by *S. pneumoniae*, lysozyme like muramidase, catalyse the hydrolysis of the peptidoglycan; a

major constituent of the pneumococcal cell wall. Secretory IgA-mediated opsonisation in the nasopharynx is limited by the pneumococcal capsule (Fasching *et al.,* 2007).

IgA1 protease of the Pneumococcus cleaves secretory IgA at the hinge region and further reduces phagocytosis. The remaining portion of IgA (Fab) helps in adhesion of the pneumococcus to the epithelial cells by binding to pneumococcal cell wall and exposes choline-binding protein (CBP). The exposure of CBP confers greater electronegative surface charge and facilitate adherence to host cells through nonspecific physiochemical interactions (Swiatlo et al., 2002, Weiser *et al.,* 2003, Sabharwal *et al.,* 2009). Apolactoferrin (ALF) is an important component of the mucosal innate immune system. ALF has lytic effects against a wide range of microorganisms due to its iron chelating ability. PspA inhibits bactericidal activity of human apolactoferrin by binding to its active site in the nasopharynx helping the bacteria to thrive (Shaper *et al.,* 2004).

1.4.2 Binding to the epithelium

The phenomenon of phase variation in the Pneumococcus is very important as the bacteria may exist as an opaque (with thick capsule) variant or transparent (with thin capsule) variant. The thick capsule in its opaque form is helpful in evading mucus entrapment and prevents phagocytosis, but once the mucosal barrier is breached transparent variants of the bacterium starts to dominate the population. In the transparent variant (with thin capsule) adhesion molecules for binding to the host epithelium becomes exposed *(Cundell et al., 1995, Weiser et al.,* 1994, Li-Korotky *et al.,* 2008).

By the use of SpxB, ami, msrA, *and* plpA *S. pneumoniae* binds to the glycoconjugates present on the surface of epithelial cells. This binding may be direct or indirectly by upregulating the binding molecules on the epithelial cell lining (Fig. 1.3)(Cundell *et al.,* 1995, Cundell and Tuomanen, 1994, Wizemann *et al.,* 1996). A non-protein virulence factor Phosphorylcholine which is bound as a hydrophilic polar head group to some of the phospholipid and abbreviated as ChoP mediates the initial binding by interacting with platelet activating factor (PAF) receptor on the epithelium (Preston and Dockrell, 2008, Kadioglu *et al.,* 2008).

Pneumococcal surface protein A and pneumococcal surface protein C interact with epithelial polymeric immunoglobulin receptors and helps in adhesion of the bacterium by acting as adhesins (Hammerschmidt *et al.,* 1997; Rajam *et al.,* 2008).

Pilus-like structures found in some serotypes of *S. pneumoniae* also act as adhesins to unknown receptors on the human buccal cells in the human nasopharynx (Barocchi *et al.,* 2006; Bagnoli *et al.,* 2008). Papasergi *et al.,* (2010) described plasminogen and fibronectin binding proteins to have enhanced the binding of *S. pneumoniae* to the host epithelial cells. Nan A, Nan B and Nan C cleaves the terminal sialic acids from the glycan chains on the epithelial cells thus exposing the potential binding sites for the bacterial adhesins and enhancing biofilm formation due to the reduced size of the bacterium at this stage (Soong *et al.,* 2006; Trappetti *et al.,* 2009).

A multitude of mechanisms are utilized by *S. pneumoniae* after establishment of its binding to the host epithelial cells and colonisation in order to fight the host's innate and adaptive immune systems which includes enzymatic modification of the peptidoglycan of the cell wall in order to establish resistance to lysozymes (Davis *et al.,* 2008), biofilm

formation (Hall-Stoodley & Stoodley, 2009) and curtailing the effect of complement activation pathways by PspA, PspC and pneumolysin (McCool & Weiser 2005; Lu *et al.,* 2008). While additional protection to the pneumococcus is provided by zinc metalloproteinase which cleaves the secretary IgA (Wani *et al.,* 1996; Kadioglu *et al.,* 2008).

1.4.3 Biofilm formation

Biofilm formation is necessary for successful colonisation of *S. pneumoniae*. It not only provides insulation against the host defense mechanisms i.e cellular and humoral, but also against other anti-bacterial compounds. Bacteria in a biofilm remain sequestered and are surrounded by a hydrated and self-generated extracellular polymeric substance matrix. The bacteria usually re-emerge from its biofilm to cause a secondary infection or invasive disease when the host becomes immunocompromised (Hall-Stoodley & Stoodley, 2009; Grau *et al.,* 2009; Murdoch *et al.,* 2009).

A recent study demonstrated the role of pneumolysin in biofilm formation where the pneumolysin knockout mutants produced less biofilm mass than the wild type. Transmission electron microscopy also confirmed the presence of pneumolysin on the bacterial cell surfaces and in the biofilm matrix. The role of pneumolysin in biofilm formation and disease increases the importance of it as a vaccine (Shak et al., 2013)

1.4.4 Invasive disease

The likelihood of invasive pneumococcal disease depends on the colonising strain (especially when the host has no adaptive immunity against it), its virulence factors and efficiency of the host defence system (Bogaert *et al.,* 2004; Feldman & Anderson, 2009).

The mechanisms for epithelial transmigration of the Pneumococcus are described as under;

1. Pneumococcal phosphorylcholine (ChoP) binds to platelet activating factor (PAF) on activated epithelial and endothelial cells of the host and follows the PAF receptor recycling pathway as in Fig 1.3. As a result, the bacterium is carried to the basement membrane of epithelial cells, which leads to invasive disease (Cundell *et al.,* 1995; Rijneveld *et al.,* 2004; Radin *et al.,* 2005; Mook-Kanamori *et al.,* 2011).
2. The second mechanism is analogous to the PAF receptor recycling pathway and involves PspC binding to pIgR, an Fc receptor (secretary component) expressed on the epithelial cells. As a result, the bacteria are transferred to the basal membrane of the epithelial cells (Elm *et al.,* 2004; Zhang *et al.,* 2000).
3. To cross the endothelial or endothelial layers of the host intercellular or pericellular layers of the respiratory barrier *S. pneumoniae* binds to plasminogen; which bind to specific receptors i.e enolase, GlyPh and Cbpe present on the bacterial surface and can enhance the adhesion of pneumococcus to the epithelial and endothelial cells leading to the cleavage of intercellular cadherin junctions. The cleavage of these junctions by plasmin allows the intercellular migration of pneumococci. (Bergmann *et al.,* 2001; Attali *et al.,* 2008; (Shenoy and Orihuela, 2016). Furthermore, the damage induced as a result of Pneumolysin activity to the tissue barrier also helps in pneumococcal entry into the systemic circulation (Rayner *et al.,* 1995). Hyluran is degraded in the extracellular matrix by hyaluronidase to facilitate the invasive disease (Jedrzejas *et al.,* 2007).

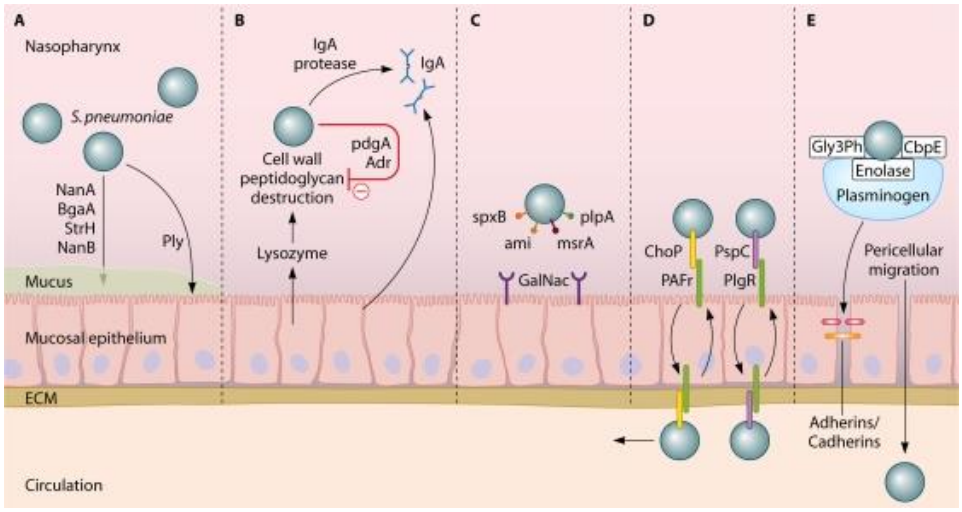


Fig 1.3. Colonization of *S. pneumoniae.* (A) Enzymes (NanA, NanB, BgaA and StrH) degrades mucus to facilitate colonization in the nasopharynx while the pneumococcal toxin (Pneumolysin) greatly reduce the ciliary beating of the epithelial cells thus enhancing the bacterial adherence. (B) Evasion of proteolytic enzymes i.e lysozyme to destroy cell wall peptidoglycans of the pneumococcus by is inhibited by pneumococcal cell surface peptidoglycan molecules like PdgA and Adr. (C) For binding to GalNac of host epithelial cells the pneumococcus uses SpxB, Smi, MsrA, and PlpA. (D) The binding of pIgR with PspC (or PAF receptor [PAFr] with ChoP), pneumococci utilize the pIgR or PAF receptor recycling pathway and transport through the epithelial cell layer. (E)The binding of Gly3Ph, CbpE, and enolase to plasminogen enhances epithelial cell binding and degrades interepithelial adherens junctions to allow pericellular migration (Mook-Kanamori *et al.,* 2011).

1.5 Pneumolysin (PLY):

Pneumolysin was identified in 1942 and its physical properties described (Cohen *et al.,* 1942, Kreger and Bernheimer, 1969). PLY belongs to a group of toxins called cholesterol-binding toxins (CBT's) or cholesterol dependent cytolysins (CDC's), which is a more appropriate name to the group than the original "Thiol activated toxins", or Thiol activated cytolysins. This misnomer arose from the fact that the biological activity of the toxins was suppressed by SH group blocking agents and can be restored by reducing agents. Later on this hypothesis was disapproved by mutagenesis. Three mutants of PLY were created where Cys428 was mutated to Alanine, Ser and Gly respectively. It was

found that the mutant Cys428Ala behaved exactly the same to the wild type toxin proving that the cysteine residue at position 428 has nothing to do with binding or insertion of PLY in the host cell membrane (Saunders *et al.,* 1989).

Toxins of this family are secreted by 23 different species of five Gram-positive genra. The five gram-positive genra producing CDC's are *Streptococcus*, *Clostridium*, *Listeria, Archanobacterium* and *Bacillus*. It includes perfringolysin (PFO) from *Clostridium perfringes,* Alveolysin (ALV) from *Bacillus alvei* Pyolysin (PLO) from *Archanobacterium pyogenes* and Listeriolysin (LLO) from *Listeria monocytogenes.* Some if not all of these toxins are proven to be important virulence factors i.e Listeriolysin, pneumolysin, perfringolysin and alveolysin (Alouf, 2000, Gilbert et al., 1998).

Pneumolysin is the shortest member of cholesterol dependent cytolysin (CDC) family (Gonzalez *et al.,* 2008). Cholesterol dependent cytolysins generally can be found in the following forms; (i) a water-soluble form in which the toxin exists as a monomer or dimer (ii) bound to cholesterol where the toxin is probably in the dimer form and (iii) bound to membrane where large oligomers are formed while embedded in the lipid bilayer of the target cell membrane. (Kelly 2000; Rubins 1998).

Pneumolysin (PLY) one of the essential virulence factors of pneumococcus, is a toxin 471 amino acids long and has a MW of 53KDa (Walker *et al.,* 1987). Pneumolysin is an asymmetrical 4-domain protein, which is slightly curved rather than globular (Morgan 1994). Its production by all serotypes of the clinical isolates makes it a strong vaccine candidate (Mitchell 1999).

The thiol group is not essential to cholesterol binding or plasma membrane insertion of PLY, hence the description of these toxins as a thiol activated cytolysins is a misnomer at least for purified toxin *in vitro* (Marriott *et al.,* 2008).

Attachment of the toxin to cell membrane is cholesterol-dependent, rapid, pH dependent but temperature independent (Johnson *et al.,* 1980). Pneumolysin is not only haemolytic but can also activate the classical pathway of complement. Pneumolysin also has the capability to inhibit ciliary beating, chemotaxis of neutrophils and microbial killing (Helen *et al.,* 2006 and 2008).

Pneumolysin action disrupts the alveolar capillary and thus may end pneumococcal invasion finally into the blood stream. Being cytotoxic it can inhibit phagocytosis and the function of immune cells which may suppress the host inflammatory and immune responses (Hirst *et al*., 2004).

PLY is also capable of inducing apoptosis in neurons, brain micro vascular endothelial cells, tissue macrophages, respiratory epithelial cells and can induce necrosis in lung epithelial cells and neutrophils. (Zysk *et al.,* 2001, Braun *et al.,* 2002, Bermpohl *et al.,* 2005, Marriott *et al.,* 2004, Srivastava *et al.,* 2005, Schmeck *et al.,* 2004). The production of nitric oxide (NO) during inflammation is protective but can contribute to the host induced tissue damage and PLY is capable of inducing the production of NO from macrophages (Braun *et al.,* 1999). PLY also inhibits the oxygen dependent phagocyte respiratory burst, induces cytokine synthesis and activates CD4+T cells (Kadioglu *et al.,* 2004, Nandoskar *et al.,* 1986).

The crystal structure of Perfringolysin O (PFO) of *Clostridium perfringens* (a sister toxin to PLY) was used as a base to present a homology model soluble monomeric and oligomeric forms of PLY (Rossjohn *et al.,* 1998). PFO shares 48% sequence identity and 60% sequence similarity with Pneumolysin which reflects the probability of resemblance in structure and function ((Solovyova et al., 2004)Rossjohn *et al.,* 1997).

Studies have shown that PLY plays direct role in the virulence of pneumococcal bacteria and is immunogenic as immunization of mice with PdB (a defective version of the toxin) provided significant protection in mice against virulent pneumococci (Paton *et al.,* 1991). Pneumolysin is a focal point of consideration for inclusion in a possible vaccine because of the role it plays in the pathogenicity and virulence of *S. pneumoniae* (Helen *et al.,* 2008). Immunisation studies with PLY will be discussed later in this chapter and in the discussion chapter.

Pneumolysin is unique among other cholesterol binding toxins (CBT's), it was believed previously that PLY is released as a result of autolysis of the bacterium. Although, PLY has no signal peptide for secretion but interestingly it was found in at least 18 clinical isolates that PLY is localised to the cell wall before autolysis of the bacterium. In the absence of any cell wall anchorage motif a novel non-covalent mechanism of binding to the cell wall was suggested which is yet to be determined. The mechanism underlying the release of cell wall localised PLY is unknown, however, as determined by its hemolytic assay the cell wall localised pneumolysin is active. In early exponential phase PLY is localised in the protoplast fraction but starts appearing in the cell wall fractions at later stages during the growth of the bacterium (Price and Camilli, 2009).

The PLY released on autolysis was found to be unable to re-associate with intact cells. This behaviour of the released PLY suggests a possible export mechanism that is somehow related to the cell wall localization of the protein. Experimentally it was demonstrated that *B.subtilis* can export PLY which augments the possibility of a conserved pathway for the export of the protein. Finally, through domain swapping and truncation analyses it was shown that export is dependent on domain 2 of PLY (Price *et al.,* 2012).

The complement is activated via three distinct pathways; The classical pathway of complement (CP), the alternative pathway of complement (AP) and the lectin pathway of complement (LP). The classical pathway is activated when C1q binds to antigen-antibody complexes or to charged clusters thus it may be due to the presence of non-specific IgM or IgG or due to the presence of specific immunoglobulins like IgM, IgG1, IgG2 or IgG3 (Ali *et al.,* 2012).

The activation of lectin pathway is dependent on MBL, ficolins and collectin 11 (CL-11). The alternative pathway of complement actually works as an amplification loop for the CP and AP which can activate via antibody dependent or antibody independent route (Ali *et al.,* 2013). The classical pathway of complement has been found to be the dominant pathway for immunity to Pneumococcus in mice (Brown *et al*., 2002) and PLY has been shown to activate complement independent of anti-pneumolysin anti bodies ((Paton *et al.,* 1984, Mitchell *et al.,* 1991). It was found that 1µg/ml of PLY is sufficient to activate the complement because of its sequence homology (two regions of limited sequence similarity) with a contiguous sequence with in acute phase protein including human C-Reactive protein. Inappropriate activation of the complement by PLY may subvert

normal complement activation by depletion of complement components (Mitchell *et al* 1991). Complement activation by PLY facilitates bacterial growth in animal models (Rubins *et al.,* 1995). Reduction in both the cytotoxic activity and complement activating activity decrease the virulence of pneumococci and these functions are attributed to different parts of the toxin molecule. It is evident from isogenic mutants of a type 2 pneumococcal strain carrying point mutations in regions effecting either the cytotoxic or complement activating activity or mutants carrying mutations to effect both properties at the same time (Alexander *et al.,* 1998).

Mice were challenged via the intraperitoneal route with D39 wild type strain and its mutant strains produced as a result of insertion duplicated mutagenesis. No significant difference was found in the survival time for mice challenged with wild type D39 strain and a mutant D39 strain carrying Asp-385-Asn in the PLY gene. The mutation Asp-385-Asn in the PLY gene abolishes its ability to activate the complement. On the other hand, mutant strains of D39 that were having mutations to effect the cytotoxic property of the toxin i.e. His-367-Arg, Trp-433-Phe and Cys-428-Gly had significantly higher median survival times as compared to wild type hence it was proved that virulence of PLY is largely attributed to its cytotoxic properties rather than its ability to the activate the complement(Berry *et al.,* 1995).

Complement activation is independent of haemolytic activity of the toxin as greater C3 conversion (26.6% as compared to 17%) was observed in serum treated with Pneumolysin that had been inactivated by prior exposure to cholesterol in equimolar amount (Paton *et al*., 1984). The regions in CRP (thought to be similar in sequence to regions of PLY) are close to each other while in Pneumolysin they are not close by as its

region 1 (PLY residues 256-296) is located in domain 3 while region 2 (PLY residues 368-397) is located in the domain 4 of pneumolysin. Site directed mutagenesis studies of PLY suggested that region 2 (Residues 368-397) is involved in complement activation. A single point mutation D385N in the domain 4 of pneumolysin renders the toxin unable to activate the complement. Although domain 4 and Fc have no detectable sequence similarity but both adopt β-sandwich structures in aggregated states, which might be the possible underlying mechanism of complement activation (Rossjohn *et al.,* 1998).

Giddings *et.al.*, 2003 studied the effect of depletion of cholesterol from membrane of human erythrocytes and found that cholesterol depletion prevents insertion of the transmembrane β- barrel and traps the CDC in the prepore complex, they found an 11000-fold increase in the haemolytic dose (HD50) of PFO and 10-fold decrease in binding to hRBC's, a 4400-fold increase in the HD50 for SLO and 3000-fold increase in the HD50 for ILY. SLO and ILY binding to human red blood cells were largely unaffected, Interestingly, in all cases the haemolytic activity was reduced to 0.1% of its original activity confirming the dependence of these toxins on cholesterol.

Purified CD+ T cells migration to the Pneumococcus *in vitro* is dependent on T cell activation and occurs only in response to *in vivo* grown bacteria, Pneumolysin might play an important role in this migration as Pneumolysin deficient pneumococci stimulate less migration. It is unclear as how this stimulation is done but Toll like receptor4 (TLR4) expression of T cells may be of relevance (Komai-koma *et al* 2004).

Malley *et. al.,* 2003 have found that the *in vitro* proinflammatory effect of Pneumolysin is TLR4 dependent. Pneumolysin has been shown to directly interact with TLR4 and as a

result can induce apoptosis in the host cell *in vitro* and *in vivo*. Apoptosis as a result of pneumolysin-TLR4 signalling is a protective host response. The PLY-TLR4 interaction and the resultant response is significant only in the host air way surfaces as the absence of it makes mice more susceptible and has no effect on the survival rate and blood bacterial counts post intravenous infection (Srivastava *et al.,* 2005, Benton *et al.,* 1997).

Korchev *et al*., 1998 studied the effect of mutagenesis on activity of the toxin with in the undecapeptide (a well conserved tryptophan rich loop) of the toxin, they found a great loss in activity of the toxin as compared to wild type toxin when Cys428 was mutated to Gly or Ser but no change in activity when Cys428 was mutated to Ala.

Similarly, they changed Trp433 to Phe and the toxin lost 99% of its haemolytic activity, Glu434 to Gln had 80% loss in activity, Glu434 to Asp had 50% loss in activity, Trp435 to Phe had 80% loss and Trp436 to Phe had 50% loss in haemolytic activity. They demonstrated all the three-tryptophan residues in the conserved region to be important for haemolytic activity but the effect of Trp433 was more pronounced as it formed more of large than small channels in planer lipid bilayer the opposite of which is true for wild type pneumolysin. Hill *et al.,* 1994 studied the effects of mutation R31C located in domain 2 and H156Y located in domain 3 and found the haemolysis to be reduced by 25% and 98% respectively. Residue 428 of domain 4 in pneumolysin is necessary for its most favourable haemolytic activity and is important to the general structure of the toxin (Hill *et al*., 1994).

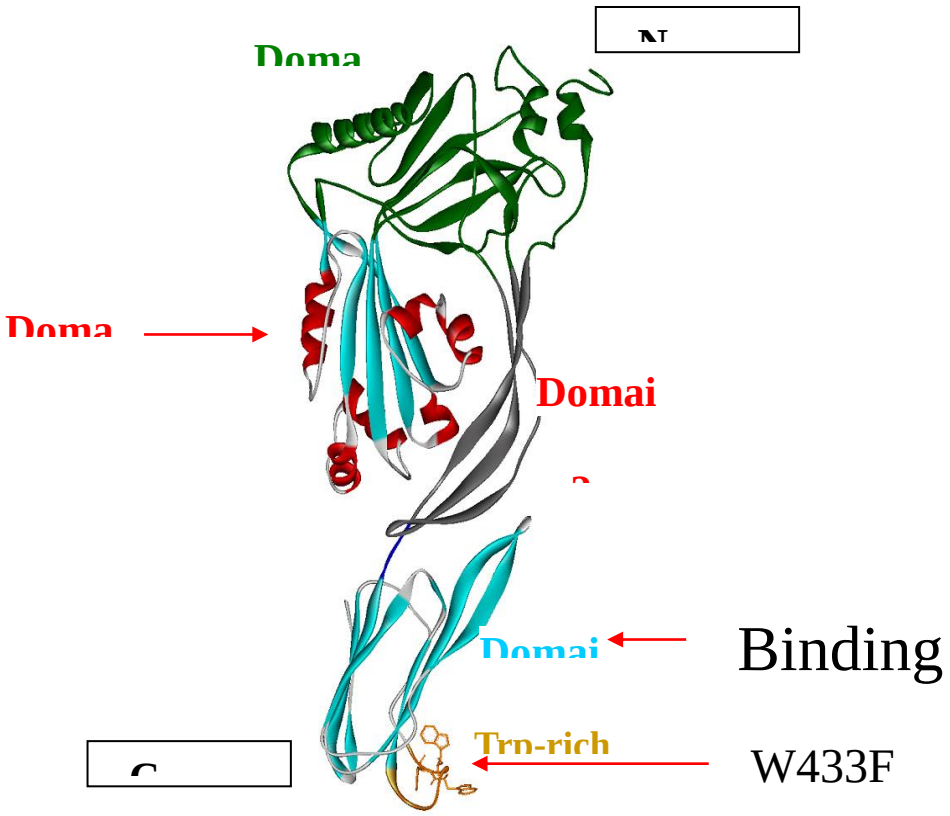


Figure 1.4 The monomeric form of Pneumolysin (Rossjohn *et.al 1998*). The four domains are numbered. The Trp-rich loop involved in membrane binding is at the bottom right. The three small helices (red) in domain 3, adjacent to domain 2, have been proposed to refold to a β hairpin structure and enter the membrane during pore formation (Gilbert et al 1999).

Braun et al 2001&2007 found that live pneumococci cause neuronal cell death by inducing rapid increase in the levels of intracellular reactive oxygen species (ROS) and Ca^{+2} which is then followed by early mitochondrial damage and release of the mitochondrial apoptosis inducing factor (AIF) which shows that it causes caspases

independent cell death. They used Fluo-4 fluorescence and detected increase in intra cellular Ca+2 level as early as 30 minutes after the addition of Pneumolysin to neural cells, for ROS detection they used DHR123 as uncharged non fluorescent DHR123 is oxidized intracellularly to the cationic fluorescent Rhodamine 123 derivative in the presence of ROS. They found massive swellings of the mitochondria and Pneumolysin bound to the mitochondrial membrane detected by anti Pneumolysin antibody and concluded that it is a central feature of pneumolysin's toxicity and release of bioactive Pneumolysin as a result of lysis of the bacterium by antibiotics needs to be neutralized as a potential strategy to reduce cell demage in invasive pneumococcal infections.

Pneumolysin is able to self-associate in solution to form the same oligomeric structure as on membranes, this is done through a weak interaction manifested at low concentrations in a dimeric toxin form. The inhibition of toxin self-interaction by derivitization of the single cysteine residue in Pneumolysin with the thiol active agent dithio (bis) nitro benzoic acid indicates that self-interaction is mediated by the fourth domain of the protein, which has a fold similar to other self-associated proteins (Gilbert *et al.,* 1998).

A panel of monoclonal antibodies were raised by de los toyos et al (2003). Three of the monoclonal antibodies (PLY 4, PLY 5 and PLY7) have anti haemolytic properties, of which PLY5 and PLY 7 prevents binding of PLY to erythrocytes and PLY 4 seem to interfere with oligomerization process of the toxin. Residues 40-98 (fragment B), 199-248 (fragment E), 299-353 (fragment G), 352-414 (fragment H) and 415-471 (fragment I) were found as immunogenic and antigenic. All these stretches include well accessible and surface exposed sites of the toxin with fragment B and E mostly with in domain 1, residues 299-318 of fragment G are part of domain 3 while 319-353 are part of control

loop on the convex face of domain 1. Fragment H corresponds to first 4 β-strands of the β-sandwich of domain 4 while fragment I corresponds to the last 4 β-strands of domain 4. These results indicate the immunological significance of domain 1 and domain 4 (Suarez-Alvarez *et al.,* 2003).

Also it was found that one of the monoclonal antibody PLY-5 recognizes an epitope in all thiol-activated cytolysins tested so far. Epitope mapping revealed that this antibody reacts with the conserved Trp rich motif as pre incubation of toxin with PLY-5 prevented binding to membrane and the epitope was no longer accessible after membrane binding of the toxin. This suggested that the Trp rich motif is responsible for binding by inserting into the host cell membrane (Jacobs *et al.,* 1999).

Sonnen *et al.,* 2008 studied the interaction of Pneumolysin with cholesterol crystals and found the toxin binding to edges and faults of the cholesterol crystals. They suggested higher affinity of Pneumolysin for molecules having their hydrophobic nucleus more accessible. They found only arcs visible on the cholesterol crystals and no complete rings about which they assumed to because of limited mobility of the toxin on crystals which indicated the importance of migration with in the fluid mosaic of membrane in forming PLY rings.

The homology model of PLY is based on the crystal structure of PFO (Rossjohn *et al.,* 1997).

The model of PLY has dimensions of 110A^{o}X 55A^{o} X 30A^{o} and is rod shaped consisting of 4 domains. Domain 1 consists of residues 6-21, 58-147, 198-243, 319-342, domain 2 consists of residues 22-57, 343-359, domain 3 consists of 148-197, 244-318 and domain

4 consisting of residues 360-469. The model is comprised of 41% sheet and 21% helix structure (Rossjohn *et al.,* 1998).

Domain 1 of PLY is negatively charged, structurally associated with domain 3 and contributes to the orientation of oligomers in the membrane (Owen *et al.,* 1994).

Domain 1 is having two faces, a concave and a convex face. The concave face of domain 1 comprises of five strands of anti-parallel β-sheets whilst the convex face is comprised of longest helix in PLY and a few smaller helices and loops. The sheet of domain 1 extends into domain 3 and a marked curvature is observed at in the sheet at the domain 1/ domain 3 junction and the residues 197-202, 240-244 and 328-331 found over there are well conserved. (Rossjohn *et al.,* 1998).

Domain 3 sheet has two faces and both are packed against α-helices. One face is packed against three small α-helices (residues 160-186) which are in turn loosely packed against domain 2, the other face of the sheet is shielded from the solvent by packing against two α-helices namely α-12 (residues 307-316) and α-10 (259-268).(Rossjohn et al., 1998).

The interface between domain 2/domain 3 is polar and have 5 polar interactions and one aromatic/ aromatic interaction.

Domain 2 is a linking domain connecting domain 1 with domain 4, the elongated loop at the top of domain 4 consisting of residues 380-392 near domain 2/domain 4 interface are well conserved. Domain 4 is mainly composed of β-sheets. Domain 1 and domain 4 provides most of the oligomerization contacts, the concave face of domain 1 interacts with the convex face of the neighbouring monomer as it is supported by work with monoclonal antibody directed towards residues 142 and 143 of Pneumolysin rendered the

ability of the toxin to form pores although it was able to bind to erythrocytes. (Rossjohn et al., 1998 & Toyos et al., 1996).

Domain 4 is critical for binding to the membrane of the host cells its truncation led to defect in the binding activity of PLY as was shown by Toyos *et al.,* (1996). Direct binding of PLY to membrane cholesterol through domain 4 (residues 360-470) is one part of it to exert the lytic activity. It has been shown that domain 4 has inhibitory effect on hemolysis and binding of full length PLY to sheep erythrocytes by Baba *et.al* ., 2001.

Two mechanisms were proposed for pore formation by CDC's, studies with streptolysin O led to the proposal that monomers bind to the cell membrane and insert before oligomerization takes place in the membrane (Palmer et al., 1998). Heuck *et al.,* 2003 demonstrated that streptolysin O can form a prepore which is in accordance with studies carried out with perfringolysin and support the idea that members of this family share a common mechanism of pore formation.

The mechanistic scheme of pore formation was described by Tilley et al., 2005. As can be seen in Fig 1C domain 1 provides a stable platform for the other domains, binding of the toxin to the membrane surface is free of any movement although there might be some local changes occurring in the tryptophan rich loop as it penetrates the upper leaflet of the bilayer (Rossjohn et al., 1997).

In the tryptophan rich motif W433 is wedged in a hydrophobic pocket formed by the long side chains of Tyr376, Lys424, Arg426 and Gln374. Conformation of the loop is maintained by W433 as mutations of this particular residue effects the stability of the region. This hydrophobic pocket is the point of contact for PLY with cholesterol, as a

result the W433 is displaced and the Trp rich loop starts to flip out forming a hydrophobic dagger (Rossjohn et al., 1998). The tryptophan rich loop may form an unusual elongated loop that folds back on the sheet of domain 4 (Tilley et al., 2005).

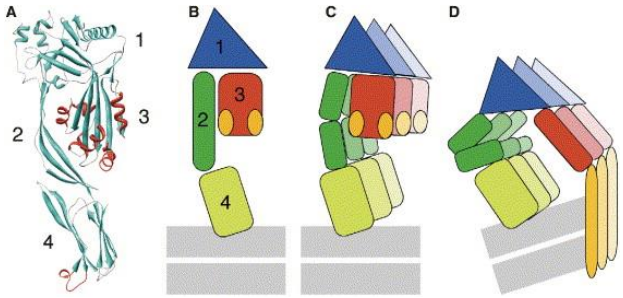


Fig 1C. A is a pneumolysin monomer based on homology model of PFO. B, C and D is explaining the mechanism of binding to membrane and pore formation (Tilley *et al.,* 2005).

Membrane binding of the toxin promotes oligomerization which results in the interface change between domain 2 and domain 4, which also disrupts the bracing action of the loop at the top of domain 4 that supports the long thin domain 2. Domain 2 starts to bend losing its hydrophilic interface with domain 3, domain 3 also bends slightly. These changes together with oligomerization forms the prepore state of the toxin while transition from prepore to pore state requires complete dissociation of the interface between domain 2/domain 3, in the process domain 2 buckles fully forming β-sandwich between two halves of the domain.

The collapse of domain 2 brings domain 3 close to the membrane and provides it with the opportunity to insert its helices into the membrane. The movement of domain 2 also tilt the top of domain 4 away from the oligomers axis imposing a sharp bend on the membrane and ultimately breaking the bilayer to form the 230-260A° diameter hole. (Tilley et al., 2005)

1.6 History of pneumococcal vaccines

The history of pneumococcal vaccine can be traced back to the investigative study in South African gold miners performed by Almorth Wright in 1914. Wright for the first time injected heat killed whole pneumococci into South African gold miners as his test subject, although his study was not conclusive but it provided a platform for further studies by Lister and his colleagues in the 1930s. These studies were not successful in terms of the efficacy of vaccination but gave an idea of the type specific immunity and epidemiology of serotypes with in the subject community. In the same year Francis and Tillet showed that pneumococcal polysaccharides when injected via the intradermal route are immunogenic and produce antibodies.

This observation paved the way for pneumococcal polysaccharides to be used as a vaccine rather than the heat killed whole pneumococci. Macleod *et al.,* 1945 used a vaccine formulation containing 4 polysaccharides from serotype 1, 2, 5 and 7 in US army personnel and was shown to be efficient as the carriage rate and colonisation was reduced for the types included in the vaccine. In this study 8586 vaccinated adult males were compared to 8449 adult non-vaccinated males for pneumococcal attack rates. They found 4 cases of pneumonia in the vaccinated and 26 cases in the non-vaccinated adults.

The incidence of disease for the types not included in the vaccine was the same for both vaccinated and placebo groups. Heidelberger *et al.,* 1949 formulated a six valent vaccine, these two formulations were licensed, however, due to the overoptimistic view about the efficacy of antibiotics, these vaccines were withdrawn in the 1950s. The observation of high mortality rates despite treatment of pneumonia with available antibiotics shifted the interest to the polysaccharide vaccine. The 14 valent and 23 valent polysaccharide vaccines in the US were licensed in 1977 and 1983 respectively. In the UK this vaccine was licensed in 1989. The commercial version of this vaccine available from different companies contains 25ug of each of the polysaccharide. It consists of polysaccharides from serotypes 1, 2, 3, 4, 5, 6B, 7F, 8, 9N, 9V, 10A, 11A, 12F, 14, 15B, 17F, 18C, 19F, 19A, 20, 22F, 23F and 33F covering the most prevailing disease causing serotypes in the USA, Europe and parts of western Africa (Andrew *et al.,* 1994).

This vaccine can induce type specific antibody response in certain populations, 37 healthy volunteers were vaccinated with capsular polysaccharides of type 6, 8, 9 or 13 and were tested 4, 5 or 6 years post vaccination for type specific pneumococcal antibody in their sera. Some of the individuals received 8 valent or tridecavalent vaccine combined with bivalent influenza virus vaccine in a single syringe. It was found that 4 years after immunisation the mean circulating type specific antibody level was 90% while after 5 years it was 76% in comparison to that of the antibody level achieved four weeks after immunisation.

Based on these findings they suggested the interval between repeated doses to be at least 5 years. Antibody response and adverse reaction to the pneumococcal polysaccharide vaccine were accessed by vaccinating healthy individuals with either 2 or 3 doses of the

vaccine. Local and systemic reactions were found to be low and mild and no fever was observed in the 12 individuals vaccinated. This study also revealed that primary immunisation with pneumococcal vaccines induced significant antibody rise while a second dose did not further increase antibody levels of to the same antigens (Mufson *et al.,* 1983, Mufson *et al.,* 1984). To see the adverse effects of vaccination and revaccination in elderly people 15 individuals aged 57-79 were vaccinated with 14 valent polysaccharide vaccine and 6 years later revaccinated with 23 valent vaccine. No adverse effects were found with the first vaccination although 5 individuals had mild tenderness at the site of injection after the booster dose. The antibody level dropped to one third and then was raised to about half the levels after primary vaccination by the booster dose (Mufson *et al.,* 1991).

The license for the 14 valent vaccine was preceded by trials of 6 and 12 valent vaccines among south African gold miners. In this study 1523 individuals received pneumococcal vaccines and 3171 were included as controls. The trials being successful showed a 76% decrease in disease episodes by 6 valent and 92% decrease in the incidence of the disease was observed for the 12 valent vaccine (Smit *et al.,* 1977).

A case control study of US adults between 1984-1990 demonstrated the effectiveness of the polyvalent vaccine and it was concluded from this study that both the 14 valent and 23 valent vaccines are protective in immunocompetent patients. This study also highlighted a major drawback associated with the polysaccharide vaccines, which is; it does not offer protection against those serotypes that are not included in the vaccine formulation. (Shapiro and Clemens, 1984, Shapiro *et al.,* 1991).

There are about 90 serotypes of *S. pneumoniae* some of which are more invasive than others and the choice of a serotype to be included in the vaccine formulation is solely based on the most commonly prevalent serotypes, which do vary geographically. For example the 23 valent polysaccharide vaccine covers about 90% of the serotypes prevalent in the United States, Canada and a mere 62.9% in Taiwan. The 468 pneumococcal isolates in a study by Jette and Lamothe belonged to 30 different serogroups. They found out that the most common serotypes in adults belonged to serotype 4, 3, 9, 6, 8 and 19 while the most common in young individuals were serotype 14, 19, 18, 6, 9 and 4. The coverage of the 23 valent polysaccharide vaccine for serotypes of adults was 91% and it was 98% for the serotypes most prevalent in young individuals. The mortality rate was found to be 12.8% and most of them (52 out of 60) had received appropriate antibiotic treatment (Jetté and Lamothe, 1989, Lee, 1987). Serotypes 1 and 5 are common in Israel and rare in Finland and for serotype 19 it is the other way around (Dagan *et al.,* 1992, Eskola *et al.,* 1992). Furthermore, the distribution of serotypes causing invasive disease may vary with time, geography and age, so that serotypes can be classified as related to adults or pediatric. For example serotype 19 and 23 are relatively more common in Europe and North America while serotype 1 and 5 are more common in South America. As regards distribution of serotypes in relation to age, the risk of disease decreases and follow different gradients with serogroup 6, 14, 18, 19 and 23.

The reduction in risk is most abrupt for type 14 and most gradual for serogroup 18. Interestingly, the risk increases for serotypes 8 and 3, although serotype 3 polysaccharide even elicits high antibody titre among children under 2 years of age. Serotype 23 and 7 are usually associated with the third decade of life (Scott *et al.,* 1996, Austrian, 1984a,

Pedersen *et al.,* 2004). Because of this unusual distribution among different age groups it is very difficult to aim the polysaccharide vaccine at a specific target.

Another major drawback of the polysaccharide vaccine is related to its immunological property, polysaccharides are known to be poorly immunogenic type 2 thymus independent antigens in infants induce poor memory (Borgoño *et al.,* 1978). Polysaccharides have high molecular weight with repeating structures and can directly cross link to antigen receptors and activate B cells (Andrew *et al.,* 1994). Thymus independent antigens can be further subdivided into two groups; thymus independent type 1 and thymus independent type 2, based on their ability to induce antibody response in CBA/N mice having an X-linked immune defect. The B cells in these mice have properties in common with immature B cells so that it can be considered as a maturation defect. Thymus independent type 2 antigens can only activate more mature B cells which in the case of CBA/N mice are absent (Amsbaugh *et al.,* 1972).

Similarly in individuals under the age of 2 years, anti-thymus independent type 2 antibodies are not generally detected and, as a result, no protective antibody is generated. Since the start of vaccination an overall decrease in invasive pneumococcal disease is observed in a serotype-specific manner but at the cost of carrying a disadvantage of not being protective in children under the age of two years and the elderly (Huss *et al.,* 2009). Immunocompromised individuals also have a poor response to polysaccharides which are T cell independent antigens (Ho and Lin 2005). About the 23 valent polysaccharide vaccine it can be said safely that it is most effective in young healthy individuals. The disadvantages carried by this vaccine warrants the need for an alternative vaccine approach which should be protective in young, elderly and immunocompromised.

Taking into account the disadvantages associated with the 23 valent polysaccharide vaccine a new vaccine was developed, This new vaccine (Prevnar) is widely available and is a protein conjugate vaccine covering 7 of the 90 serotypes of *S. pneumoniae*. The serotypes included in PCV-7 are 4, 6B, 9V, 14, 18C, 19F and 23F each conjugated separately to diptheria toxin and is very effective in children under the age of 5 years (Black *et al.,* 2000) and immunocompromised individuals, due to the induction of this vaccine in the immunisation programme in 2000, the rate of invasive pneumococcal disease has fallen by 63-74% in the United States (Lynch and Zhanel 2010). Currently, a 9 valent conjugated vaccine is used in Gambia to combat pneumococcal disease and is proving to be effective (Cutts *et al.,* 2005). The 9 valent conjugate vaccine also elicits a protective response in HIV infected individuals (Klugman *et al.,* 2003).

A 10 valent conjugate pneumococcal vaccine hit the market in the year 2010 (Lynch and Zhanel 2010). There are some disadvantages associated with the pneumococcal conjugate vaccines, when one considers the highly prevalent otitis media the protection by pneumococcal polysaccharide conjugate vaccine is very low (6% overall and 36% in culture confirmed episodes) specifically in young children under the age of three.

The number of episodes of acute otitis media related to serotypes included in the vaccine was reduced by 57 percent but an increase of 33 percent was observed for serotypes not included in the vaccine (Taylor *et al.,* 2012, Eskola *et al.,* 2001) also its costly production making it inaccessible to the poor countries in south east Asia and Africa (Mu *et al.,* 2008). Singleton et al., 2007 reported serotype shift in native America and Alaskan population. A non-vaccine serotype 19A was found to be involved in 70% of the invasive

pneumococcal disease in the year 2003-2004 showing that the number of cases due to this serotype doubled in the post-vaccination era (Pai *et al.,* 2005).

In such a scenario, and taking into consideration the burden of pneumococcal disease, there is need for the creation of a vaccine which can provide protection in a serotype-independent manner and less expensive than Prevnar.

1.7 New approaches to tackle the pneumococcal problem:

To tackle the pneumococcal vaccine problem many new approaches are under consideration. One of such approach is to use well-conserved pneumococcal proteins. In this regard several pneumococcal proteins have been tested for its immunogenicity and protective efficacy against *S. pneumonaie* (Bogaert *et al.,* 2004). This can be helpful to prevent pneumococcal serotype shift, will not be expensive and furthermore pneumococcal proteins are easy to produce taking into consideration recombinant DNA technology (Paton *et al.,* 1991).

There are many pneumococcal proteins tested so far for their protective efficacy. One such vaccine candidate is Pneumolysin, the toxin produced by *S. pneumoniae.* Pneumolysin is well characterized in immunogenicity studies and is known to be a protective antigen, however, the problem lies in its toxicity and ideally a non-toxic version of pneumolysin is required to be used in a vaccine. The non-toxic version of PLY to be used as a vaccine should retain the ability to produce functional antibodies against the wild type toxin.

Paton *et al.,* (1983) used native pneumolysin for the first time in an immunisation study. They purified native pneumolysin from *S. pneumoniae* and mixed it with Freunds

complete adjuvant. Mice were immunised via the intraperitoneal route 3 times, 10 days apart. Finally, they challenged mice with a lethal dose of *S. pneumonaie* D39 a serogroup 2 strain. The results demonstrated that immunised mice had a significantly longer survival time as compared to the non-immunised mice. Furthermore, regarding the immunogenicity they found 40 times more antibody levels in the serum of immunised mice as compared to the non-immmunised mice (Paton *et al.,* 1983).

Paton *et al.,* (1991) used the toxoids PdB and PdA which had 0.1% and 0.001% hemolytic activity respectively to immunise Balb/C mice three times, 14 days apart. Mice were challenged 14 days after the last immunisation with strain D39 intranasally or intraperitoneally. The results demonstrated immunised mice had significantly longer survival times than the non-immunised mice and there was no significant difference between the PLY, PdB or PdA immunised mice. When challenged intraperitoneally the PdB toxoid increased the survival time as compared to PLY and PdA.

Alexander *et al.,* (1994) immunised mice with PdB and their results demonstrated that the toxoid is protective against 9 serotypes of *S. pneumoniae*. The PdB immunised mice after the challenge survived for 14 days as compared to 3.2 days on average for the non-immunised mice. Elisa results for the serum antibody collected throughout the course of the experiment showed IgG1 and IgG2a in high levels. IgG subclass response is important as it is elicited in a thymus dependent reaction. This response is also long lasting as compared to the short-term memory response elicited by IgM antibodies.

Ogunniyi *et al.,* (2000) adapted a different approach to the above-described studies. They used a combination of different pneumococcal virulence factors with pneumolysin. In

this study the virulence factors were shown to confer protection against pneumococcal disease. Interestingly, during this study, PdB did not elicit a protective immune response in Balb/C mice when challenged intraperitoneally (Ogunniyi *et al.,* 2000). This is in contrast to the study performed by Paton *et al.,* (1991). The only noticeable difference between these two studies is the use of a different adjuvant. The combination of pneumococcal proteins (PdB, PspA and PspC) as antigens provided a better protective response as mice immunised with the combination of these pneumococcal virulence factors had significantly higher survival times compared to the non-immunised control group.

Basset *et al.,* (2007) used pneumococcal surface protein C, pneumococcal surface protein A and PdT to immunise C57BL/6J mice. In this study cholera toxin was used as an adjuvant. The authors demonstrated that $CD4^{+}$T cells also have a role in the protective response and that the protection is not always antibody mediated. In this experiment one group of mice was depleted of $CD4^{+}$T cells before challenging them with *S. pneumoniae*. It was found that colonisation in the normal immunised mice was greatly reduced as compared to the non-immunised mice. However, this protective response was completely lost in the $CD4^{+}$T cells depleted mice (Basset *et al.,* 2007).

Wu *et al.,* (2010) constructed and used delta-146Ply to immunise mice. This single amino acid mutation makes the toxin inactive when incubated in microgram amounts with RBC's. Mice were immunised 3 times10-18 days apart and were challenged intraperitoneally 4 weeks after the last immunisation, the immunised mice survived significantly longer than the non-immunised mice.

Other new approaches to pneumococcal immunisation include the efforts to control nasopharyngeal colonisation. If pneumococcus is successfully stopped from nasopharyngeal colonization it may help to prevent all the invasive pneumococcal disease. The prevention of nasopharyngeal colonization will largely depend mucosal immunity due to vaccination. Villena *et al.,* (2010) showed that a pneumococcal protein expressed in *Lactococcus lactis* is able to induce mucosal immunity and is protective. However, the potential risk it carries is that the niche might be occupied by a pathogenic bacterium instead of the commensal pneumococci due to mucosal immunity.

Another idea to prevent pneumococcal disease is the use of heat-killed whole cell bacteria as a vaccine (Malley *et al.,* 2001). Animal studies have shown that immunisation with an uncapsulated autolysin deficient strain (RX1) could be protective against colonisation and invasive disease when administered with cholera toxin. Cholera toxin is a known mucosal immunogen as well as an adjuvant which can enhance mucosal and systemic antibody response to co-delivered antigens. The problem with this method or vaccine approach is that the bacteria will not always produce the same proteins in the correct conformation required for protection (Maley *et al.,* 2001). Lu *et al.,* (2010) carried the inactivation of unencapsulated bacteria. This type of vaccine will not produce immunity against one of the important virulence factor, namely the capsule.

All of the above approaches are actually an effort to produce a new, more widely and readily available vaccine. Most of the approaches for production of vaccines these days are taking into consideration the issue of serotype replacement. Highly conserved protein vaccines are one possible solution to this problem. Pneumolysin is one of such a vaccine candidate.

Aims and objectives:

To obtain domain 4 of pneumolysin by applying different strategies.

To check immunogenicity of domain 4 as a protective antigen.

To check domain 4 of pneumolysin as an adjuvant.

Mutants of pneumolysin to study the structure to function relationship in pneumolysin.

Using site-directed mutagenesis study for point mutations to study the important residues that might be involved in oligomerization.

To check if D1-3+D4 of pneumolysin when used as an antigen provides protection against *Streptococcus pneumoniae.*

CHAPTER 2

(2) METHODS:

(2.1) Preparation of ampicillin stocks

Ampicillin was purchased from Sigma as ampicillin sodium salt. 1g of ampicillin was dissolved in 10ml of dH_2O in a universal tube to give a final concentration of 100mg/ml. The dissolved ampicillin was filter sterilized through a syringe filter (0.2um from Nalgene) with the help of a 10ml sterile syringe (from BD) into a new universal tube. Using a 1ml Gilson pipette 500ul each were dispensed into separate eppendorf tubes to make aliquots and stored at -20°C until used.

(2.2) Culture of *E. coli* harbouring the recombinant plasmids

E. coli carrying the constructs were streaked onto Luria agar (LA) plates containing ampicillin at a final concentration of 100ug/ml. The stock of ampicillin had a concentration of 100mg/ml. and incubated overnight at 37°C in a static incubator. A sterile pipette tip was used to pick bacteria from the overnight plate and a fresh LA plate was inoculated as was 10ml Luria broth (LB) containing ampicillin (100µg/ml). The LA plates were incubated overnight at 37°C in a static incubator while the LB culture was incubated overnight at 37°C in a shaking incubator set at 220RPM.

(2.3) Extraction of plasmid pKK233-2*:ply*

Plasmid pKK233-2*:ply* was extracted using a mini prep plasmid extraction kit (from Qiagen) by following the protocol provided by the supplier. 3ml of the overnight culture was centrifuged in a tabletop centrifuge at 13000 RPM (17900Xg) to form a pellet.

Complete resuspension of the pellet was ensured by adding 250µl of buffer P1 (RNaseA added at a final concentration of 0.1mg/ml, provided as 10mg/ml solution, in a total volume of 200ml, Qiagen) and then vortexing it so that no cell clump remained. 250µl of buffer P2 was added and mixed thoroughly by inverting the tube 4-6 times until the solution became clear and viscous. 350µl of buffer N3 was added and mixed immediately by inverting the tube gently until the solution became cloudy and there were no traces of blue left. The eppendorf tube was centrifuged at 17900g for 10 minutes and the supernatant was applied to a Qiagen spin column. The spin column was centrifuged for 30- 60seconds and the eluate was discarded. The spin column was washed by adding 0.75ml buffer PE and centrifuged for 30 seconds at 17900g. After completion, the eluate was discarded and the column centrifuged for additional 1 minute to remove any residual wash buffer. Finally, the column was placed in a sterile 1.5ml microcentrifuge tube and 50µl of elution buffer (EB) was added (Tris-HCl, pH8.5). The tube was left to stand for 1 minute and then centrifuged for 1 minute. The DNA was in the eluate.

(2.4) Determination of DNA concentration 1µl of DNA was diluted in 69ml of autoclaved nanopure water and its A260 and A280 were determined. The concentration of DNA was calculated as A260 X 50 X 70, where 70 is the dilution factor. The purity of DNA was assessed from the A260: A280 ratio. Or in most of the cases nanodrop was used to determine the DNA concentration. Briefly the nanodrop was cleaned and two microliters of nH2O was used to start the apparatus and then equilibrated with 2ul of EB for a blank. Finally, the DNA sample (2ul) was put and its A280 determined. It gives the concentration of DNA in ng/ul and also the A260:A280 ratio for determination of DNA purity.

(2.5) Digestion of DNA

Normally 1 unit of enzyme was used for digestion of 1µg of DNA. The enzyme was not allowed to exceed 5% v/v of the total volume of reaction (1µl for 20µl of reaction). For digestion 5µl of template DNA (250-300ng), 12µl of nanopure water (from Bioline), 2µl of 10X reaction buffer (supplied with enzyme by the New England Biolabs) and lastly 1µl of restriction enzyme (New England Biolabs) was added and a quick pulse in the centrifuge was given to ensure that all the reaction components were mixed properly. The reaction was incubated in a water bath at 37°C for 2 hours. After completion of the reaction the DNA was analyzed via agarose gel electrophoresis.

(2.6) Agarose gel electrophoresis

(2.6a) Agarose gel preparation

Agarose gel was prepared according to the method described in sambrook *et al.,* Briefly 0.8% w/v agarose (DNase and RNase free from Bioline) in 1X TAE buffer (50X TAE buffer: 242g Tris base, 57.1ml glacial acetic acid, 37.2g Na2EDTA.2H2O and DH2O to final volume of 1000ml, pH 8.5) was dissolved by heating in a microwave oven at full power for 3 minutes. When cooled to hand-hot, 2µl of ethidium bromide (from stock having concentration of 10mg/ml) was added and mixed by gentle shaking. Then the agarose was poured into a prepared mould with a comb in place. The gel was allowed to solidify, the comb was removed gently and then transferred to a gel tank containing 1X TAE buffer (running buffer).

(2.6b) Preparation and loading of samples

(2.6b.1) Preparation of 1 kb DNA ladder

1µl of 1Kb DNA ladder (New England Biolabs), 1µl of 6X blue loading dye (provided with the ladder) and 4µl of nanopure water (from Bioline) were added and mixed in an eppendorf tube to make a 1kb ladder for the gel electrophoresis.

(2.6b.2) Preparation of samples

20µl DNA samples (Digested) and 4µl of 6X blue loading dye were mixed in an eppendorf tube. Similarly, 5µl of DNA (uncut) and 1µl of 6X blue loading dye were mixed in a separate tube to serve as a control sample. The samples and 1kb ladder were gently transferred to separate wells of an agarose gel with the help of a Gilson pipette. Electrophoresis was done at 70-80 volts for 50 minutes. When electrophoresis was complete the gel was visualized under the UV light of a gel doc system and the image captured with an attached camera.

(2.7) Small-scale protein expression

E. coli BL21 DE3 Gold: pKK233-2*:ply* was inoculated into10ml of LB containing ampicillin (100µg/ml) and was incubated overnight at 37^{o}C in a shaking incubator. A negative control (LB containing ampicillin) was also incubated overnight. 1:10 dilutions of the overnight culture were made in fresh LB with ampicillin (100µg/ml) and incubated at 37^{o}C in a shaking incubator set at 220RPM. After approximately 1 hour its optical density OD600nm was confirmed to be 0.5-0.6 and 1M IPTG was added to the culture at

a final concentration of 1mM (1ul/ml from the 1M stock) and incubated again until an OD600nm of 1.5-1.6 was attained. The culture was then centrifuged in a precooled Beckman Coulter AllegraTm X-22R centrifuge at maximum speed for 15minutes, the supernatant was discarded and the pellet re-suspended in 300µl of PBS. EDTA was added to to give a final concentration of 1mM and mixed gently.

The bacterial cells were lysed by sonication. The amplitude used for sonication was 6microns and the cycle 15 seconds of sonication followed by 45 seconds of rest. The cycle was repeated 9-12 times. Cell lysis was confirmed by change of the suspension colour from white to yellowish by naked eye. The cell lysates were centrifuged (in the same precooled centrifuge) at maximum speed for 15minutes. The pellet was retained at -20^{o}C and the supernatant (Crude extracts) was transferred to a sterile eppendorf tube and assayed for its hemolytic activity as described by Owen *et., al* 1994.

(2.8) Haemolytic assay

Haemolytic activity was assayed (Owen et al., 1994) to determine the activity of the toxin. For this assay 10ml of PBS and 10ml of sheep defebrinated blood (from Oxoid) were put into separate universal tubes and centrifuged in a Beckman Coulter Allegra X-22R centrifuge at 3000RPM (4250g) at 4^{o}C for 15 minutes. After completion the supernatant was discarded and the red blood cells pellet was retained.

To get a 4% w/v RBC suspension, 400µl of the blood cell pellet was taken carefully with a Gilson pipette and mixed well with 9600µl of PBS. A 96-well round bottom microtitre plate (from Nunc) was taken and 50µl of PBS was added to each well in row A and row B with a Gilson pipette. Then 50µl of the crude extracts Containing pneumolysin was

added to the Ist well of row B. It was mixed and two-fold serial dilution was carried across the entire row and mixed well after each addition until reached the last well from which 50µl was discarded.

After this, 50µl of 4%w/v red blood cells suspension in PBS was added to each well in row A (served as a control) and row B, the lid was placed on the microtitre plate and the contents mixed by gently taping the plate on the sides with finger. Finally, the plate was incubated at 37^{o}C for 30 minutes in a static incubator. Hemolysis was observed by eye and the hemolytic units/ml determined by counting the two fold serial dilutions (20, 40, 80, 160 and so on) across the row until the well having 50% hemolysis compared to control (PBS+sRBC's) in row 1. The concentrations of protein samples were determined by using a Nanodrop system and the specific activity for each sample was determined.

(2.9) Polymerase Chain Reaction (PCR) for amplification of ply gene from plasmid pKK233-2*:ply*.

Polymerase chain reaction was performed as described by Sambrook *et al.*, 1989. Before setting up the reaction, the bench space was wiped well with IMS (industrial methylated spirit) and microsol (1% v/v from Anachem). This was also done with the Gilson pipettes. The Gilson pipettes were treated with UV light so as to avoid any DNA contamination in the reaction. The reaction mixture was prepared in 50µl per tube (thin walled PCR tube) by adding 5µl of 10X accubuffer (from New England Biolabs), 1.5µl of 50mM MgCl2 to give a final concentration of 1.5mM in the reaction mixture, 1µl of 10mM dNTP's mixture to give a final concentration of 0.2mM of each in the reaction mixture, 2µl of both forward and reverse primers (Table 1) from the 10mM stock and

38.5µl of nanopure water (from Bioline) was added to each tube (total 4 tubes). 1µl (20ng) of template DNA was added to 3 of the tubes while the fourth tube served as a negative control. Finally, 1µl (1U) of accuenzyme DNA polymerase was added to each tube and mixed gently. The tubes were immediately put in a thermocycler (Biometra from Thistle Scientific) with a pre-set programme for the reaction given as under;

A. 95^{o}C for 1 minute (Initial denaturation)

B. 94^{o}C for 30 seconds (Denaturation),

65^{o}C for 30 seconds (Annealing) and

72^{o}C for 2 minutes (Extension). Step B was repeated for 30 cycles.

C. 72^{o}C for 10minutes (Final extension)

D. The reaction was held at 4^{o}C. After completion the amplified DNA was run on 1% agarose gel and then visualized with gel doc system and analysed.

(2.10) Mutagenesis and Transformation with Quick-change lightning multisite directed mutagenesis kit (from Stratagene).

(2.10a) Mutagenesis reaction

The Mutagenesis reaction (25µl in volume) was done according to the protocol provided by the manufacturer with the Quick-change lightning multisite directed mutagenesis kit (from Stratagene). 2.5µl of 10X Quick-change lightning multi reaction buffer (from Stratagene), 16.5µl of nanopure water (from Bioline), 1µl of Quick solution (from Stratagene), 100ng of template DNA (plasmid pKK233-2: ply), 1µl of dNTPs mixture

(from Stratagene), 1µl of primer (forward or reverse for each reaction) and finally 1µl of Quick-change multi enzyme blend was added to a thin walled PCR tube and mixed gently with a Gilson pipette or tapped the tube with finger. Similarly, in another tube all the reaction components were mixed, except primer, and this served as a control. The reaction was transferred to a thermocycler (Biometra from Thistle Scientific) as soon as possible using the cycling parameters recommended by the manufacturer of the kit (Stratagene);

A: 95^{o}C for 2 minutes (initial denaturation)

B: 95^{o}C for 20 seconds (Denaturation)

55^{o}C for 30 seconds (Annealing) and

65^{o}C for 3 minutes (Extension). Step B was repeated for 30 cycles.

C: 65^{o}C for 5 minutes (Final Extension).

In some cases, the annealing temperature to 62^{o}C and the extension and final extension temperature was 68^{o}C, the extension time was also increased from 30sec/Kb to 1 minute/Kb for optimisation purpose.

After completion the reaction was immediately placed on ice to cool to 37^{o}C and then 1µl of Dpn1 restriction enzyme was added, mixed with a Gilson or spun in a micro centrifuge for 10 seconds, followed by immediate incubation at 37^{o}C for 5 minutes or 1 hour. In some cases, the digestion reaction was carried out overnight at 4^{o}C. After completion of the digestion reaction the DNA was immediately placed on ice.

Table 1. Primers used for creating the TEV protease cleavage site in pneumolysin

S.No	Name	5'-3' sequence
1	1AF3	5'-CAGACTATGTTGAGACTAAGGAGAACCTGTACAGAAACGGAGATTTACTG-3'
2	1AR3	5'-CAGTAAATCTCCGTTTCTGTACAGGTTCTCCTTAGTCTCAACATAGTCTG-3'
3	2AF2	5'-GAGACTAAGGAGAACCTGTACTTTCAAGGAGATTTACTGCTGGATCAT-3'
4	2AR2	5'-ATGATCCAGCAGTAAATCTCCTTGAAAGTACAGGTTCTCCTTAGTCTC-3'
5	FT356-N356	5'-GTTGAGACTAAGGTTAACGCTTACAGAAACGGA-3'
6	RT356-N356	5'-TCCGTTTCTGTAAGCGTTAACCTTAGTCTCAAC-3'
7	FAR5759-LF5759	5'-GAGACTAAGGTTACACTGTACTTCAACGGAGATTTACTG-3'
8	RAR5759-LF5759	5'-CAGTAAATCTCCGTTGAAGTACAGTGTAACCTTAGTCTC-3'
9	NQ360 Forward	5'-GTTACACTGTACTTCCAAGGAGATTTACTGCTG-3'
10	NQ360 Reverse	5'-CAGCAGTAAATCTCCTTGGAAGTACAGTGTAAC-3'
11	FTN5660-NQ5660	5'-GTTGAGACTAAGGTTAACCTGTACTTCCAAGGAGATTTACTGCTG-3'
12	RTN5660-NQ5660	5'-CAGCAGTAAATCTCCTTGGAAGTACAGGTTAACCTTAGTCTCAAC-3'
13	E355 FOR	5'-GACTATGTTGAGACTAAGGAGAACCTGTACTTCCAAGGAGAT-3'
14	E355 REV	5'-ATCTCCTTGGAAGTACAGGTTCTCCTTAGTCTCAACATAGTC-3'

Table 2. Primers used for creating the CYS mutants of pneumolysin

S.No	Name	5'-3' sequence
1	G388-C388 FOR	5'-TCCTATGATCATCAATGCAAGGAAGTCTTGACTC-3'
2	G388-C388 REV	5'-GAGTCAAGACTTCCTTGCATTGATGATCATAGGA-3'
3	W278-C278 FOR	5'-GCTCCTCAGACAGAGTGCAAGCAGAATTTGGAC-3'
4	W278-C278 REV	5'-GTCCAAATTCTGCTTGCACTCTGTCTGAGGAGC-3'
5	E35-C35 FOR	5'-GAAAATCGTTTCATCAAATGTGGTAATCAGCTACCC-3'
6	E35-C35 REV	5'-GGGTAGCTGATTACCACATTTGATGAAACGATTTTC-3'
7	K34-C34 FOR	5'-GAAAATCGTTTCATCTGTGAGGGTAATCAGCTA-3'
8	K34-C34 REV	5'-TAGCTGATTACCCTCACAGATGAAACGATTTTC-3'
9	V163-C163 FOR	5'-ATGGAACAACTCAAGTGCAAGTTTGGTTCTGAC-3'
10	V163-C163 REV	5'-GTCAGAACCAAACTTGCACTTGAGTTGTTCCAT-3'
11	A262-C262 FOR	5'-AGTGATGAAGTAGAGGCTTGCTTTGAAGCTTTGATAAAAGGA-3'
12	A262-C262 REV	5'-TCCTTTTATCAAAGCTTCAAAGCAAGCCTCTACTTCATCACT-3'
13	T55-C55 FOR	5'-AATATCACTTGTATTACACGACAAGCTCCGCTT-3'
14	T55-C55 REV	5'-AAGCGGAGCTTGTCGTGTAATACAAGTGATATT-3'

Table 3. Primers for creating PLY W433F, primers for pneumolysin gene and primers for cloning of PLY D4 into pEcoli Nterm 6XHN vector from clontech.

S.NO	Name	5'-3' sequence
1	W433F FOR	5'-CTTGCCTGGGAATGGGCGCGTACGGTTTATGAA-3'
2	W433F REV	5'-TTCATAAACCGTACGCGCCCATTCCCAGGCAAG-3'
3	Ply Forward	5'-ATGGCAAATAAAGCAGTAAATGAC-3'
4	Ply Reverse	5'- CTAGTCATTTTCTACCTTATCCTC -3'
5	Infusion Forward	5'-TAAGGCCTCTGTCGAGACTATGTTGAGACTAAGG-3'
6	Infusion Reverse	5'CAGAATTCGCAAGCTCTAGTCATTTTCTACCTTATCCTC-3'

(2.10b) Transformation reaction

XL10 Gold ultra-competent cells (from Stratagene) were thawed on ice and 45µl of the cells were added to each prechilled 14ml BD Falcon polypropylene round bottom tube. Then 2µl of β-ME mix (from Stratagene) was added to each tube and incubated on ice for 10minutes with gentle swirling every 2 minutes. After completion, 1.5µl of Dpn1 treated DNA from each mutagenesis reaction was added to each tube and incubated on ice for 30minutes with gentle swirling every two minutes. NZY+ broth (4g NZ amine (enzymatic casein hydrolysate), 2g yeast extract, 2g NaCl and DH2O to a final volume of

400ml) was preheated by putting in a water bath at 42°C. After completion of incubation on ice, the reaction in the round bottom tubes was heat pulsed in a water bath at 42°C for 30 seconds and was immediately placed on ice for 2 minutes.

After completion, 0.5ml of preheated NZY+ broth was added to each reaction and incubated at 37°C for one hour in a shaking incubator (220- 225RPM). Appropriate volume of each reaction was plated on LA-ampicillin plates with a Gilson pipette and spread with a sterile spreader. The plates were incubated overnight at 37°C in a static incubator.

(2.11) SDS PAGE: Sodium Dodecyl Sulphate Polyacrylamide Gel Electrophoresis

(2.11a) Gel preparation. SDS PAGE gels were prepared as described by Sambrook et al. (1989) and consisted of two gels, the resolving and the stacking gels. The gel mould was washed and assembled carefully ensuring no leakage of gel. The resolving gel was then prepared in a 20 ml universal tube by adding 6.9 ml H2O, 4 ml of 10% w/v polyacrylamide mix (Protogel), 3.8 ml 1.5 M Tris-HCl (pH 8.8), 150µl 10% w/v SDS, 150µl 10% w/v ammonium persulphate (0.1g into 1 ml of DH_2O) and 9ul TEMED respectively and finally vortexed.

The gel was then poured carefully using a Gilson pipette into the slot between the two gel plates and some water was added on top of the gel. The stacking gel was prepared by mixing in a 20 ml universal tube, 5.5 ml H2O, 1.3 ml of 30% acrylamide, 1 ml of 1 M Tris- HCl (pH 6.8), 80µl of 10% w/v SDS, 80µl 10% w/v ammonium persulphate and 8µl TEMED respectively and finally vortexed. The water film on top of the resolving gel was removed and the stacking gel was poured on top of the resolving gel. Finally, a comb

was placed carefully in the stacking gel, which was left to set before loading the samples.

(2.11b) Sample preparation and loading

Some distilled water was put in a beaker and placed on hot plate to boil, in the meantime samples were prepared. To each pellet sample, 8µl of PBS and 3µl of SDS loading buffer (NuPAGE LDS sample buffer 4X from Invitrogen or 10X SDS loading buffer: 6.25 ml of 0.5M Tris-HCl (pH 6.8), 1g SDS, 10 ml glycerol, 10mM DTT, 50mg bromophenol blue and 6.25ml H2O) was added to an eppendorf tube. 4µl of SDS loading buffer was added to 20µl of crude extract in an eppendorf tube.

The lid of each eppendorf tube was pierced with a needle and the samples were put in a float in the boiling water. Pellet samples were boiled for 10 minutes while crude extracts were boiled for 5 minutes. The gel mould was placed in the SDS gel tank (from BIO RAD) with the thin glass facing inwards and 1X SDS running buffer added. Running buffer was 15.1g Tris 94 glycine, 50ml 10% w/v SDS and 1000ml distilled H2O). The comb was removed carefully and the samples (20µl) were loaded onto the wells with 10ul of protein molecular weight marker added to one well (from Precision plus). The current was set to 20mA (milliamperes) and was increased to 30mA when the protein samples entered the resolving gel.

Electrophoresis was stopped when the dye front neared the bottom of the gel. The gel was carefully removed from the mould with a wedge and transferred immediately to the staining solution (0.1% w/v commasie brilliant blue R250 (0.4g), 40%v/v methanol (160ml), 10%v/v acetic acid (40ml) and DH2O give volume of 200ml). The gel was left in the staining solution on a shaker overnight or for 3-4 hours then the staining solution

was discarded and the gel rinsed in tap water thoroughly. The gel was placed in destaining solution (25% v/v isopropanol (100ml), 10% v/v acetic acid (40ml) and 260ml DH_2O) for 3-4 hours with shaking. After destaining the gel was wrapped in a cling film and was scanned with a computer scanner.

(2.12) Western blotting

Western blotting was performed as in Sambrok et al., (1989), the monoclonal antibody (PLY-4 & PLY-7 provided by delos Toyos et.al., 1996) to pneumolysin was used. The sponges and filter papers were soaked in transfer buffer (5.9g Tris, 2.9g Glycine, 100ml methanol, 3ml 10% w/v SDS, 900ml DH_2O, pH8.3).

After electrophoresis, the gel and nitrocellulose membrane (cut according to the size of the gel) were placed in the transfer buffer and a gel sandwich was constructed (Soaked sponge, soaked filter paper, gel, nitrocellulose membrane, soaked filter paper and soaked sponge) inside the blotting cassette. The cassette was closed and placed in the blotter tank filled with ice cold transfer buffer with the membrane placed towards the positive terminal and the gel towards the negative terminal of the blotter.

The blotting was performed at 230mA for 1 hour and after completion the membrane was blocked with blocking buffer (2.5g (5% w/v) skimmed milk (from BD) in 50ml of PBS) for 3 hours at room temperature in a large Petri dish. The membrane was washed 4 times (10minutes each wash) with PBS-Tween20 (100ml PBS, 500ul Tween) on a shaking platform. The membrane was then incubated for 2-2.5 hours with diluted primary antibody (10ul of Pneumolysin monoclonal antibody (1mg/ml PLY-4) in 10ml of 1% w/v skimmed milk in PBS) on a shaker. The membrane washing was repeated with PBS-

Tween and incubated with diluted (2ul anti mouse IgG alkaline phosphatase in 12ml of 1% w/v skimmed milk in PBS) secondary antibody with shaking for 2-3 hours. The washing with PBS-Tween was repeated and 5ml of 5-Bromo-4-Chloro-Indolyl-Phosphatase (BCIP) solution was added to develop the membrane by gentle shaking for 5-10 minutes. The membrane was wrapped in a cling film and was scanned for a picture.

2.13 Expression of D4 fused to MBP:

The vector pLEICS-10 has an N-terminal Maltose binding protein (MBP) tag, D4 amplified with primers specifically designed for pLEICS-10 was cloned and then transformed into BL21 DE3 *plyss* chemical competent cells. The MBP tags after expression of recombinant protein can be cleaved from the recombinant protein by Tobacco etch virus (TEV) protease.

The cells were grown in 10ml of LB having ampicillin overnight in Falcon tubes at 37°C and 220RPM, next day the overnight culture was added to 1L of LB having ampicillin and incubated at 37°C and 220RPM until the OD_{600} reached 0.8 approx after 3.5 hours. The culture was then induced with 1mM IPTG and incubated overnight at 30°C.

Next day the cells were harvested at 3000RPM, 4°C for 30 minutes in Beckman coulter Avanti J-E centrifuge to make a pellet. The cells were re-suspended in 100ml of Sterile PBS and separated into two Falcon tubes. This time they were centrifuged in Allegra at 3500RPM and 4°C for 20 minutes. The supernatant was discarded and the pellets were stored at -80°C overnight. Next day the pellets were re-suspended in 1ml of 10X Bugbuster (from Novagen) and 20ml of buffer (50mM Tris pH 7.5, 150mM NaCl and 0.5 Protease inhibitor cocktail (PIC) tablet was added.

The falcon tubes were left on rockers for 30-45 minutes at maximum speed and room temperature. The samples were sonicated 5 times while keeping it on ice to avoid overheating (10 seconds sonication and 50 seconds rest each time) at amplitude of 6micron. The sonicated samples (Lysed cells) were centrifuged at 20,000 RPM for 30 minutes at 4°C in Beckman coulter Avanti J-E centrifuge to make a pellet. The supernatant (Crude extract) was transferred to a fresh Falcon tube and filtered through a 0.45um Acrodisc filter.

2.14 Purification of D4 fused to MBP:

D4 fused to MBP was purified via Amylose affinity chromatography. 1ml of amylose high flow resin (from New England Biolabs) was applied to Poly-Prep Chromatography column (from Bio-Rad) using a Gilson and left to set. The column was washed with 10ml of 50mMTris, 150mM NaCl (pH7.5). The supernatant (Filtered Crude extract) were passed through the column 3-4 times in order to ensure good binding of MBP fused D4 to the amylose resin.

The column was washed thoroughly with 30ml of 50mMTris, 150mM NaCl (pH7.5). The protein was finally eluted in four 1ml fractions by washing the column with 50mMTris, 150mM NaCl and 10mM Maltose (pH7.5).

The elutes were checked by running on 12% SDS gel and fractions containing MBP-D4 were then subjected to cleavage with TEV protease (having stock concentration of 7.5units/ul) to remove the N-terminal MBP tag.

20ul of TEV protease from the stock was added to each ml of elutes and incubated overnight (19hours in this case) at 4°C. The cleaved samples were run on 15% SDS gel to confirm the cleavage and check the efficiency of the protease.

Cleaved samples were loaded onto a Superdex 200 16/60 gel filtration column and eluted using 50mMTris, 150mM NaCl (pH7.5) at 1ml/min. The protein was concentrated using 3,000 MWCO centrifugal filter unit (from GE Health care) and its concentration was determined by Bradford assay. The concentration of D4 was then set to 400ug/ml to be used in immunisation studies.

2.15 Blood Agar Base (BAB) culture plates with 5% v/v horse blood (Blood Agar Plates):

16 g of Blood Agar base medium was put in 400ml of dH_2O and autoclaved at 15psi (103kPA) for 20 minutes. The medium was then allowed to cool to 56°C before 20ml of horse defebrinated blood was added making sure that the blood was evenly distributed with stirring gently to prevent bubble formation. The medium (BAB+5% v/v horse blood) was poured into sterile Petri dishes (approx 20 ml to each plate) and allowed to solidify before inverting the plates. The plates were half opened near a lit Bunsen burner for about 30 minutes to dry. The plates were stored at 4°C.

2.16 Determination of CFU/ml for the passaged stock:

Passaged stock of Streptococcus pneumoniae Strain D39 from lab stock kept at -80°C was thawed at room temperature and then centrifuged at 13000rpm for 2 minutes in a table top centrifuge. The supernatant was discarded aseptically by inverting the tube in a

small Petri dish and the bottom of the tube was tapped with finger gently in order to make sure no residual medium of the stock is left.

The pellet was re-suspended in 400ul of sterile PBS with a Gilson pipette. PBS (180ul) was added to 6 wells in row A (A1 to A6) and row C (C1 to C6) of a 96 well Nunc plate aseptically near Bunsen flame. 20ul each from the re-suspended stock was added to wells A1 and C1 with the help of a Gilson pipette and each row was serially diluted while changing tips each time and mixing well before each transfer. Two blood agar plates (pre incubated at 37°C) were divided into 6 sections on the backside with a permanent marker representing 10^{-1}, 10^{-2}, 10^{-3}, 10^{-4}, 10^{-5} and 10^{-6}. The 180ul PBS +20ul bacteria from the stock serially diluted in the 96 well plate was mixed well with a Gilson pipette and 3 drops (each 20ul) from each dilution were plated on the blood agar plate in their respective halves starting from the higher dilution. The drops were allowed to dry on the agar plate; the plates were inverted in CO_2 jar and incubated overnight in a static incubator at 37°C. Next day the colonies were counted in 10^{-5} section (colonies were counted in the section where the number of colonies were 30-300) and the CFU/ml calculated as follows;

CFU/ml = Number of Colonies in section x dilution factor x 1000/60.

2.17 Preparation of Dose (1 x 10^6 CFU/50ul) from the stock:

A vial from the already Passaged stock containing 500ul of D39 was thawed at room temperature and centrifuged at 13000 rpm for 2 minutes in a table top centrifuge. The

supernatant was discarded carefully and the pellet was re-suspended in 400ul of sterile PBS with the help of a Gilson. The calculations were done as follows;

$$\frac{\text{Required CFU/50ul}}{\text{Stock CFU/1000ul}} \times 2300\text{ul} = X$$

Where X was the required amount to be taken from the stock and put in sterile PBS to make a final volume of 2300ul containing 1.5 x 10^6 CFU/50ul. The required amount of dose was 2000ul.

The prepared dose was administered with in 30 minutes of its preparation intra nasally to MF1 female mice (from Harlan UK or from CR). Each mouse before administration of bacterial dose was anesthetized with 5% v/v Isoflorhane (1.6-1.8L O_2/min) in an anaesthetic box and then administered with 50ul of bacteria intra nasally using a Gilson pipette while holding the mice in vertical position. Care was taken that the mice do not sneeze the dose out. The mice were then laid on their back in the cage and after regaining their conscious the cage was closed properly and put in the infected section of cages. Mark sheets were made to record the health of mice at different time points.

The inoculated dose was plated on blood agar plates for checking the viability. The dose was serially diluted in sterile PBS using a 96 well plate from Nunc. The pre incubated blood agar plate was divided into 6 sections on the backside with a permanent marker representing the dilution factors. Three drops of 20ul from each dilution factor was put on

the plate and waited to dry into the blood agar plate. The plate was inverted and incubated in CO_2 jar at 37°C overnight. The numbers of colonies were counted (where it was between 30-300) and the CFU/ml was calculated.

2.18 Purification of PCR product by using Qiagen PCR Purification Kit

5 volumes of buffer PBI was added to 1 volume of the PCR sample, mixed and the resultant colour of the mixture was ensured to be yellow. The QIAquick Spin column was placed in the collection tube provided with the kit, to bind the DNA the sample was applied to it and centrifuged at 17,900 x g (13000 rpm) for 1 minute in a table top micro-centrifuge at room temperature. The flow through was discarded and the QIAquick column was placed back into the same tube. The sample was washed by adding 0.75 ml of buffer PE to the sample and centrifuging for 1 minute at 17900 x g in a table top micro-centrifuge at room temperature, the flow-through was discarded and centrifugation repeated to remove residual ethanol from buffer PE. To elute DNA, the QIAquick column was placed in a clean 1.5 ml micro-centrifuge tube and 50ul of buffer EB (10mM Tris-Cl, pH 8.5) or water was added carefully to the centre of the QIAquick membrane and centrifuged for 1 minute. Finally, the purified DNA was analysed on 0.8% agarose gel by mixing 5ul of DNA with 1ul of loading dye and its concentration was determined by Nanodrop.

2.19 Purification of PCR product by using Qiagen gel Extraction Kit

The DNA fragment was excised from the agarose gel with a clear and sharp scalpel carefully. Removal of as much extra agarose as possible was ensured to minimized the size of the gel slice. The gel Slice was weighed in a micro-centrifuge tube and 3 volumes of buffer QG was added per 1 volume of gel (for 100mg = 300ul was added) and incubated at 50°C until the gel slice completely dissolved. It was made sure that the colour of the mixture was yellow (similar to buffer QG) indicating optimum pH of 7.5 for DNA binding to the membrane.

To increase the yield of DNA Isopropanol was added to the sample (1ul/mg) and mixed. The QIAquick spin column was placed in the 2ml tube provided with the kit. To bind DNA to the column the sample up to 800ul was applied and then centrifuged at 17900x g (13000 rpm) for 1 minute at room temperature in a micro-centrifuge. The flow-through was discarded and the QIAquick column was placed back in the same collection tube. In order to remove all traces of agarose 0.5ml of buffer QG was applied to QIAquick column and centrifuged again at 17,900 X g (13000 rpm) in a micro-centrifuge at room temperature. Washed the DNA by adding 0.75ml of buffer PE to QIAquick column, left to stand for 3 minutes and finally centrifuged.

The flow-through was discarded and the QIAquick column was centrifuged for an additional 1 minute to get rid of the residual ethanol from buffer PE. The QIAquick column was placed into a clean 1.5ml micro-centrifuge tube and 50ul of buffer EB (10mM Tris-Cl, pH 8.5) was carefully added to the centre of the QIAquick membrane and centrifuged for 1 minute at 17900 X g (13000 rpm) in a microcentrifuge. Finally, the

purified DNA was visualized on 0.8% agarose gel by mixing 5ul of DNA with 1ul of loading dye and its concentration determined via Nanodrop.

2.20 Cloning of D4 of pneumolysin:

D4 of pneumolysin was amplified by using primers Infusion1 and Infusion2 (Table 3 of primers) specifically designed for the p*E.coli* N-term 6XHN vector (from Clontech). The primers were obtained from MWG operon and were kept at a stock concentration of 100pmol/ul. The PCR reaction (master mix) contained 40ul of 10X NH4 buffer, 16ul of 50mM MgCl2, 8ul of 25mM dNTP's Mix, 6ul of template pKK233-3:*ply* (50ng/ul), Primers 1ul each (100pmol/ul stock) added lastly after removing 1 reaction for negative control, 8ul of *pfu* polymerase and 317ul nH_2O (from Bioline). The master mix was dispensed into seven thin walled PCR tubes.

The cycling parameters for amplification were as follows;

1. Initial denaturation at 95°C for 1 minute
2. Denaturation at 94°C for 30 seconds,

   Annealing at 48-60°C for 30 seconds

   Extension at 72°C for 2 minutes

   Step 2 was 30 times
3. Final extension at 70°C for 10 minutes
4. Hold at 4°C

Although the PCR was set up to gradient but we used the slots for 48°C, 49.2°C, 51.8°C, 54.8°C, 57.6°C, 59.7°C and 60°C.

The amplified DNA for D4 of pneumolysin was visualized on 1% Agarose gel. The PCR product from the 54.8°C and 57.6°C was pooled and cleaned with PCR purification kit from Qiagen. Its concentration was determined to be 50ng/ul using Nanodrop system. Ligation was performed according to the protocol of In-Fusion^{TM} Dry-Down PCR cloning Kit.

Shortly the purified PCR Fragment and p*E.coli* N-term6XHN Vector were mixed in 2:1 molar ration in 10ul of deionized water. The aluminum seal from the tube was peeled off carefully and 10ul of the mixture (Vector, Fragment and H2O) was added and mixed properly with a Gilson pipette. The reaction was incubated at 37°C for for 15 minutes followed by incubation at 50°C for 15 minutes. Finally, the tube was placed on ice now ready for transformation.

The *pE.coli* N-term6XHN vector is linear and only become circularized after successful ligation. The Ligation reaction was transformed into XL10 gold Chemical competent cells from Stratagene. Next day a few colonies were selected and the plasmid DNA extracted by using Qiagen Mini prep kit. The extracted plasmid DNA was sequenced and then transformed into *E.coli* BL21 DE3 *plyss* chemical competent cells for expression purpose.

2.21 Two stage PCR for site directed Mutagenesis:

A set of primers (forward and reverse) was designed carrying three amino acid substitutions. The primers (1AF and 1AR) were obtained from MWG Operon. Two PCR tubes with master mix and template were set up. PCR water from Bioline was added to make the final volume of 50ul. Single primer (forward or reverse) was added to each tube

for amplification of mutagenic *ss*DNA. The cycling parameters for the first 10 cycles of amplification were;

1. Initial denaturation at 95°C for 1 minute

2. Denaturation at 95°C for 1 minute,

   Annealing at 57°C for 1 minute

   Extension at 70°C for 6 minutes (1Kb/minute).

After the completion of the Ist stage/round of PCR the tubes were removed from the thermocycler and 25ul of reaction from each of the amplified *ss*DNA i.e amplified with forward or reverse primers was mixed immediately into a fresh PCR tube in order to perform the secondary PCR. O.5ul of *Pfu* polymerase (from Stratagene) was added lastly to the tube before putting it in the thermocycler. The same cycling parameters as above were applied for another 18 cycles, finally the reaction was held at 4°C. After completion, the reaction and parental *ds*DNA (As a control for the efficiency of Dpn1) were digested overnight with restriction enzyme Dpn1 at 37°C. The digestion reaction contained the template (PCR product), 5ul of 10X buffer no 4 from New England Biolabs and 1ul of Dpn1 (200 units/ul). The template after digestion was visualized on 0.8% agarose (DNase and RNase free from Bioline) gel via agarose gel electrophoresis.

2.22 Mutagenesis by two stage PCR reaction using two sets of primers:

Two primers D1-3R Tm primer and D4 Tm primer were deigned which carried the TEV protease cleavage site. The primers were obtained from MWG Operon. The template DNA used was pKK233-3:*ply*. The PCR master mix contained 15ul of 10X NH4 buffer

from NEB, 6ul of 50mM MgCl2, 2.5ul of 25mM dNTP's mix, 2ul of template (100ng), 117.5ul of DNase and RNase free water from Bioline, 3ul of Biotaq polymerase from Bioline. The master mix was dispensed into three separate thin walled PCR tubes (48.66 into each tube) and finally 4ul of each primer (40pmol in total) were added. To tube1 primers ply F and ply R were added (which served as a positive control), to tube 2 primers D4 Tm primer and ply R and to tube 3 D1-3R and ply F were added. The cycling parameters were;

1.Initial denaturation at 95°C for 30 seconds.

2. Denaturation at 30°C for 30 seconds,

annealing at 58°C for 1 minute and

Extension at 70°C for 6minutes.

Step 2 was repeated for 30 cycles and then the reaction was held at 4°C. The resultant products were visualized on 0.8% agarose gel (from Bioline) via agarose gel electrophoresis. The size of the resultant products was consistent with D1-3, D4 and whole length pneumolysin (positive control). Both the PCR products i.e D1-3 with overhang having TEV protease cleavage site at the C-terminus and D4 with overhang having TEV protease cleavage site at the N-terminus were cleaned with PCR purification kit from Qiagen. Their concentration was determined to be 124ng/ul and 35ng/ul respectively using Nanodrop system.

For the secondary PCR 4ul of D4 and 1.2ul of D1-3 DNA was mixed to be used as a template, The PCR master mix was prepared (as above) for 3 50ul reactions and ply F

and ply R primers were used to join and amplify full length pneumolysin. A gradient PCR was set and the slots for 54.8°C, 57.6°C and 60°C were used. The PCR product was visualized on 0.8% agarose gel, the product was then PCR purified using Qiagen PCR purification kit. Finally, the product was gel purified using Qiagen gel purification kit and sent for sequencing to PNACL Leicester.

2.23 Mutagenesis with Quick change XL Site Directed Mutagenesis Kit:

The Mutagenesis reaction (50µl in volume) was done according to the protocol provided by the manufacturer with the Quick-change XL site directed mutagenesis kit (from Stratagene). Each of the reaction contained 5µl of 10X-reaction buffer

1 µl (30-50 ng) of dsDNA template, 1 µl (125 ng) of forward primer, 1µl (125 ng) of reverse primer, 1 µl of dNTP mix, 3 µl of Quick Solution, ddH_2O to a final volume of 50 µl (from Bioline) and 1ul of *pfu* turbo DNA polymerase from Stratagene.

The cycling parameters used for each reaction were;

1. Initial denaturation at 95°C for 1 minute,
2. Denaturation at 95°C for 50 seconds,

   Annealing at 60°C for 50 seconds and

   Extension at 68°C for 6minutes (1Kb per minute).

   Final extension at 68°C for 7 minutes.

The reaction was transferred to ice and incubated for a minute or two to cool it down and then 1µl of *Dpn1* restriction enzyme was added, mixed with a Gilson or spun in a micro-

centrifuge for 10 seconds, followed by immediate incubation at 37°C for 5 minutes. After completion of the digestion reaction the DNA was placed on ice.

The mutagenesis reaction was followed by transformation and then extraction of plasmid DNA. The extracted DNA was sent for sequencing and after confirmation of mutations the DNA was transformed into *E.coli* BL21 DE3 gold *plyss* chemical competent cells from Stratagene. The Starting template for the mutagenesis reactions was pKK233-3:*ply* in each reaction. The following aminoacids mutations in pneumolysin were performed with this method, all the primers used for these mutations were obtained from MWG Operon.

1. N360 to Q360
2. G388 to C388
3. V163 to C163
4. W278 to C278
5. T55 to C55
6. K34 to C34
7. E35 to C35
8. V355 to E355
9. T357R359 to L357F359
10. V355T356 to E355N356
11. W433 to F433

The mutant ply where G388 was mutated to C388 was used a template for K34 to C34 and E35 to C35 thus giving rise to two double mutants C34C388 and C35C388. Similarly, the mutants where the residue W278 was mutated to C278 and the mutant

where the residue V163 was mutated to C163 were used a template for mutating residues A262 to C262 and T55 to C55 respectively.

Each of the mutagenesis reaction was transformed into XL10 Gold chemical competent cells, its DNA was extracted and sent to PNACL for sequencing. The mutants were then amplified from pKK233-3;*ply* with primers designed for pLEICS-05. The amplified DNA was visualized on 0.8% agarose via agarose gel electrophoresis, PCR purified by using Qiagen PCR purification kit and then gel purified by Qiagen gel purification kit. Sending the purified product to PROTEX for cloning into pLEICS-05. The constructs were sequenced and then transformed into *E.coli* BL21 DE3 gold *plyss* chemical competent cells for expression.

2.24 ELISA

The concentration of protein was adjusted to 10ug/ml in coating buffer (100mM carbonate/bicarbonate pH 9.6). To coat the microtitre plate 100ul of it (1ug of protein) per well was added to the microtitre plate overnight at 4°C. The microtitre plate was covered with cling film to prevent evaporation and any possible contamination.

The liquid then was removed and the plate washed six (6) times with 200ul of washing buffer (PBS+0.05%Tween20) per well.

The plate was then blocked with 5% $_{W/V}$ skimmed milk (200ul/well) by incubating at 37°C in a static incubator for 1 hour 30 minutes duration during which time the plate was covered with cling film to prevent evaporation. The liquid then was removed and the plate washed 6 times with washing buffer as described earlier.

Sera collected from immunized and non-immunized were used as a source of primary antibody and is referred to as test serum here while Mab PLY-7 (from De los Toyos) was used as a positive control.

3ul of test sera were diluted in 660ul of 1%$_{W/V}$ Skimmed milk and 0.4ul of Mab PLY-7 also was prepared in the same amount of 1%$_{W/V}$ Skimmed milk (to be used a positive control).

Serial dilutions of the test sera and Mab PLY-7 were prepared in a separate microtitre plate. The dilution was then transferred to the ELISA plate and incubated at 37°C for 1 hour 30 minutes. The liquid was removed from the microtitre plate and washed 6times with washing buffer. Anti-mouse IgG alkaline phosphatase (from Sigma) was used as a secondary antibody (1:2000 dilutions in 1%$_{W/V}$ Skimmed milk in PBS) 100ul was applied to each well before incubation at 37°C for 1 hour 30 minutes. After incubation the liquid was removed from the plate and it was washed 6 times with 200ul per well of PBS+0.05% Tween20.

100ul of alkaline phosphatase substrate solution was added to each well and it was incubated at room temperature for 5-8 minutes until the colour started to develop in the test wells.

By adding 3M NaOH the reaction was stopped and the A_{405} was measured with a Bio-Rad plate reader and the data exported to Excel.

2.25 Preparation of formalin fixed *S. pneumoniae* to be used as a positive control in complement assays

Formalin fixed bacteria were prepared to be used in C3 deposition assays as follows; *S. pneumoniae* were cultured overnight in 10ml of brain heart infusion (BHI, Oxoid) at 37°C in a static incubator. Next day the overnight culture was centrifuged at 3000Xg for 10 minutes. The supernatant was discarded and the bacterial pellet washed thrice with phosphate buffer saline (PBS) and suspended in 0.5% formalin (Sigma Aldrich) in PBS for 1-3 hours at room temperature. After formalin fixation the bacteria were centrifuged again and washed twice with PBS and finally re-suspended in coating buffer (15mM Na_2CO_3, 35mM $NaHCO_3$, pH 9.6) and the OD_{550} was adjusted to 0.6 (Lynch *et al.,* 2004).

2.26 C3 deposition assay

To measure C3 deposition on the surface of *S. pneumoniae* Nunc Maxisorb microtitre Elisa plate was coated overnight with 100ul/well of already prepared formalin fixed bacteria in triplicates, 100 ml of 10ug/ml of PLY-D4 in coating buffer, 100 ul of 10ug/ml of mannan (Sigma Aldrich) and 100 ul of 10ug/ml of PLY. The plate was incubated overnight at 4°C and sealed with cling film to prevent evaporation. Residual protein binding sites were blocked next day with 1% BSA in TBS buffer.

The plate was washed thoroughly 3-5 times with washing buffer. Serum was diluted twofold in BBS (4mM barbital, 145mM NaCl, 2mM $CaCl_2$, 1mM $MgCl_2$ and pH 7.4) starting from 1/80. Plates with serum were incubated at 37°C for 90 minutes and washed again. Rabbit anti-C3c (Dako) was diluted 1:5000 in washing buffer and 100ul of it was added per well. The plate was then incubated for 90 minutes at 37°C and then washed 3 times. Alkaline phosphatase conjugated goat anti-rabbit (Sigma Aldrich) was diluted

1:10000 and 100ul of it per well was added to the plate followed by 90 minutes of incubation at room temperature.

The plate was washed at least three times before adding 100ul per well of colorimetric substrate p-nitrophenyle phosphate (pNPP from Sigma Aldrich). The plate was left at room temperature until the colour started to develop and the absorbance was measured at A_{405} with a Bio-Rad microtitre plate reader.

2.27 Structure of Pneumolysin based on homology model of PFO:

Structure of pneumolysin was obtained by using the pdb file for pneumolysin based on the homology of PFO by using the software MAC pymol. Site directed mutagenesis was performed Insilco to calculate the distance in A° between the sulphur atoms of target residues.

2.28 Blast

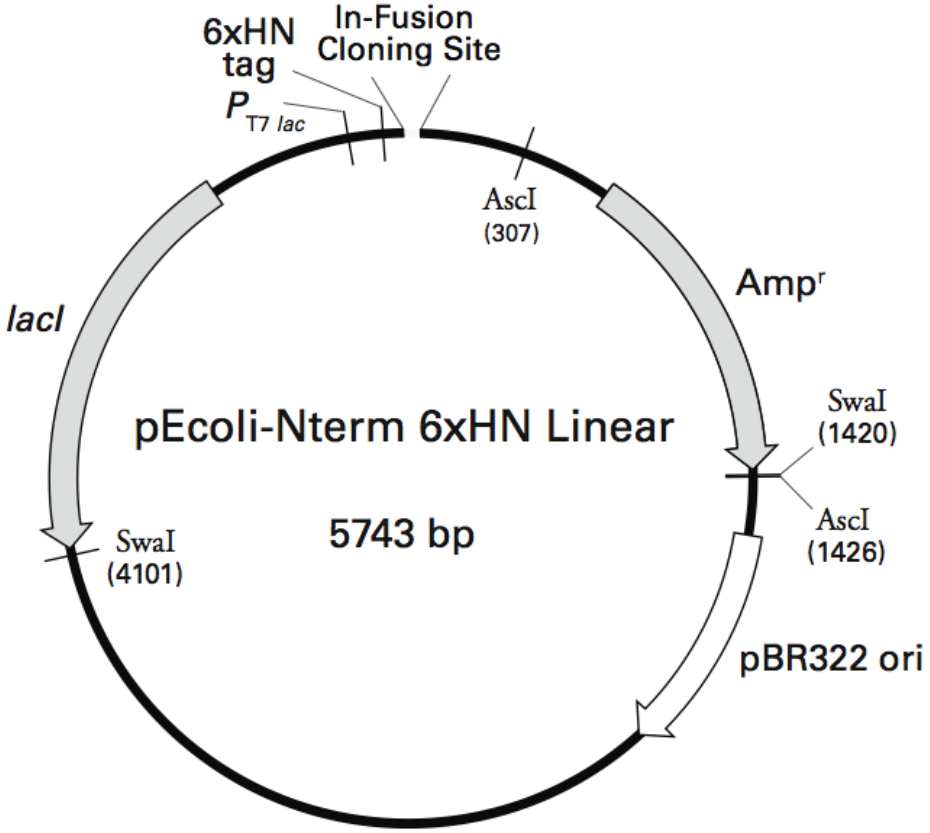
For analysis of the sequences and reading of the sequencing files a software for Mac called 4 peaks was used. For confirmation of mutagenesis NCBI nucleotide blast or alignment tool was used.

2.29 Protein

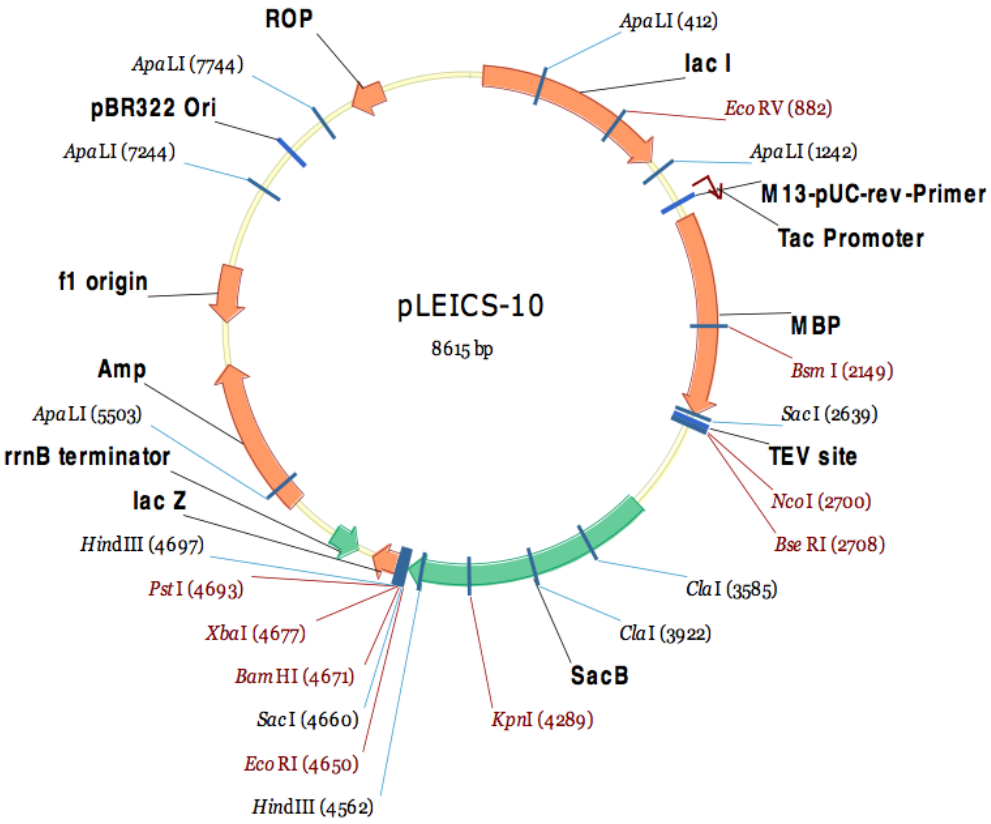
For translation of the nucleotide sequence EXPASY translate was used.

2.30 Vectors Used in this project

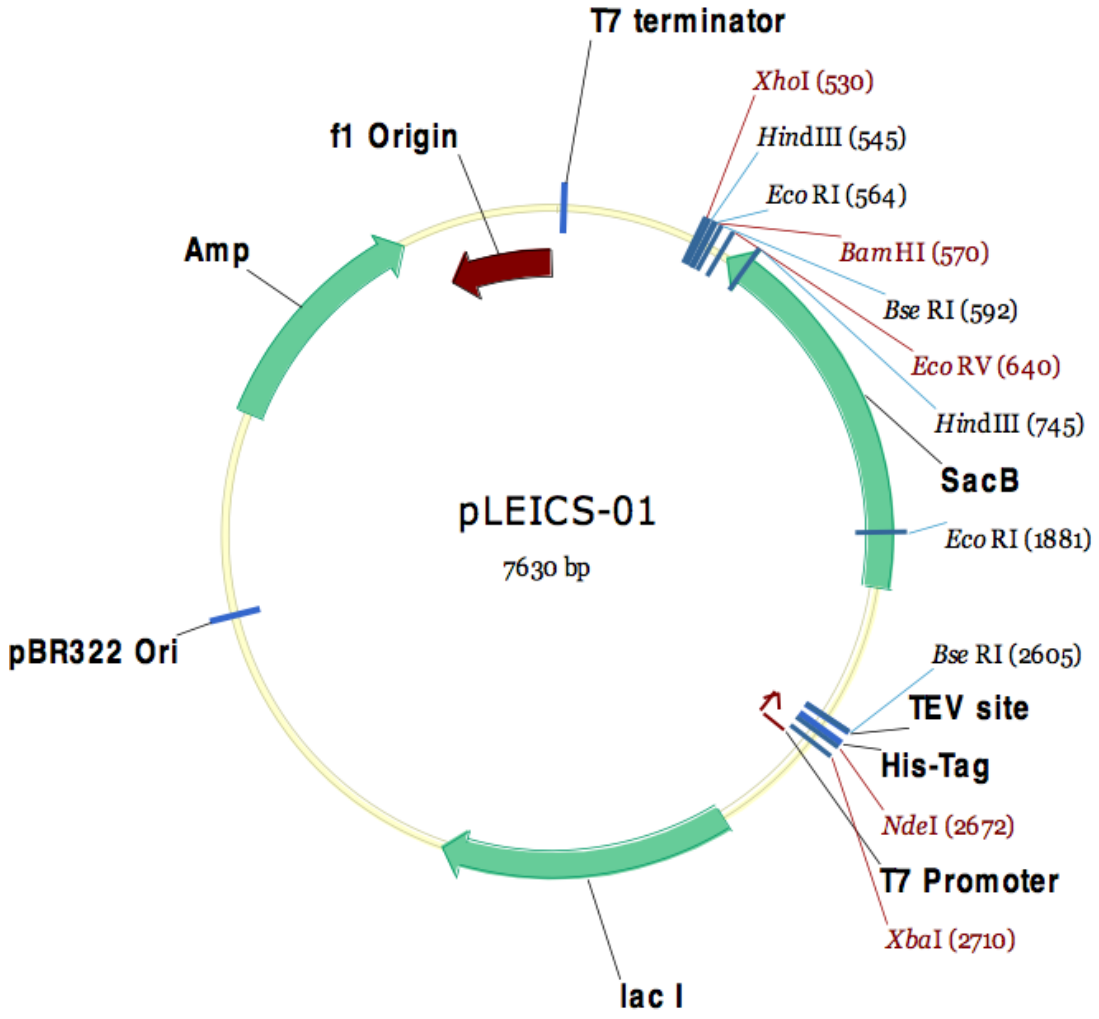
The various vectors used in this project included pEcoli-Nterm 6XHN vector from clontech technologies, pLEICS-10, pLEICS-01, pLEICS-03 (from PROTEX Leicester) and pHISTEV (from Rana lonen). The maps of these vectors are given as under;



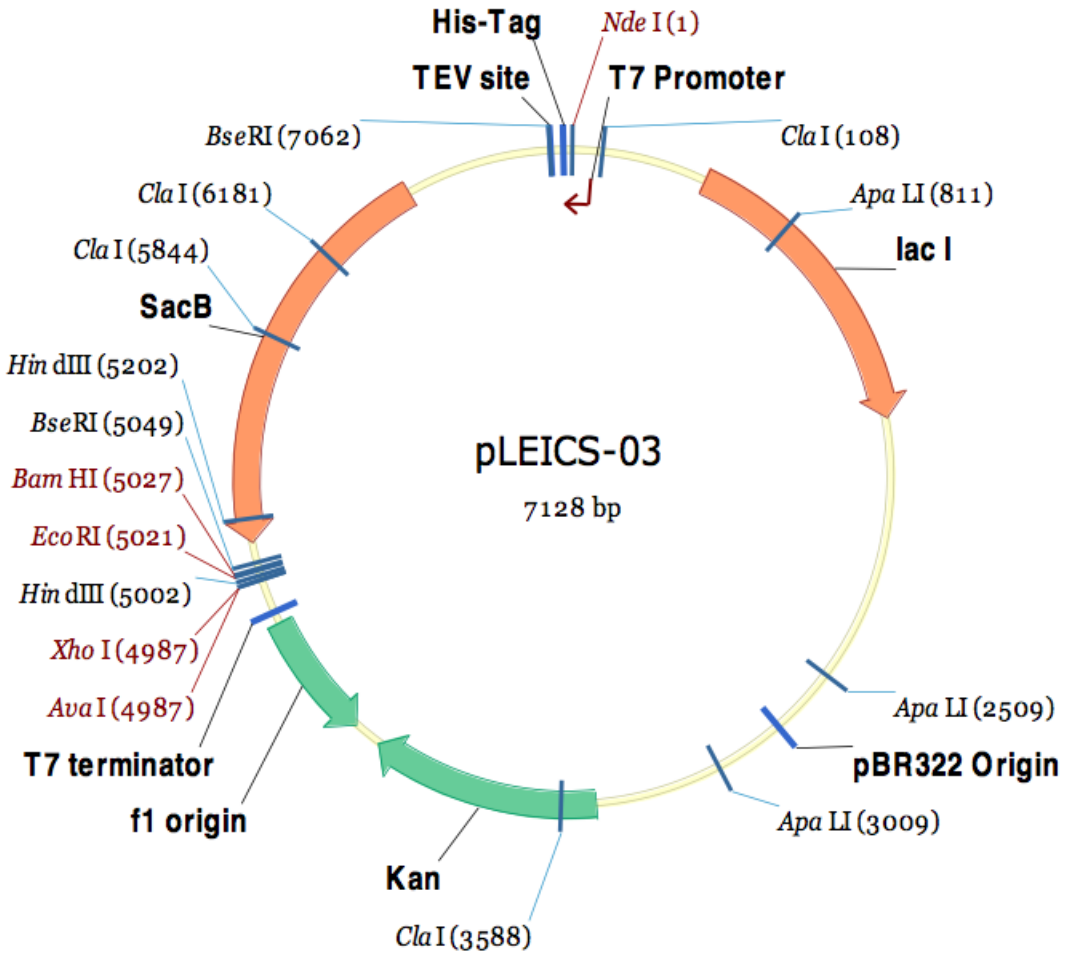
Map for pEcoli-Nterm 6xHN vector used for cloning of domain 4 (D4) of pneumolysin (From clontech technologies)



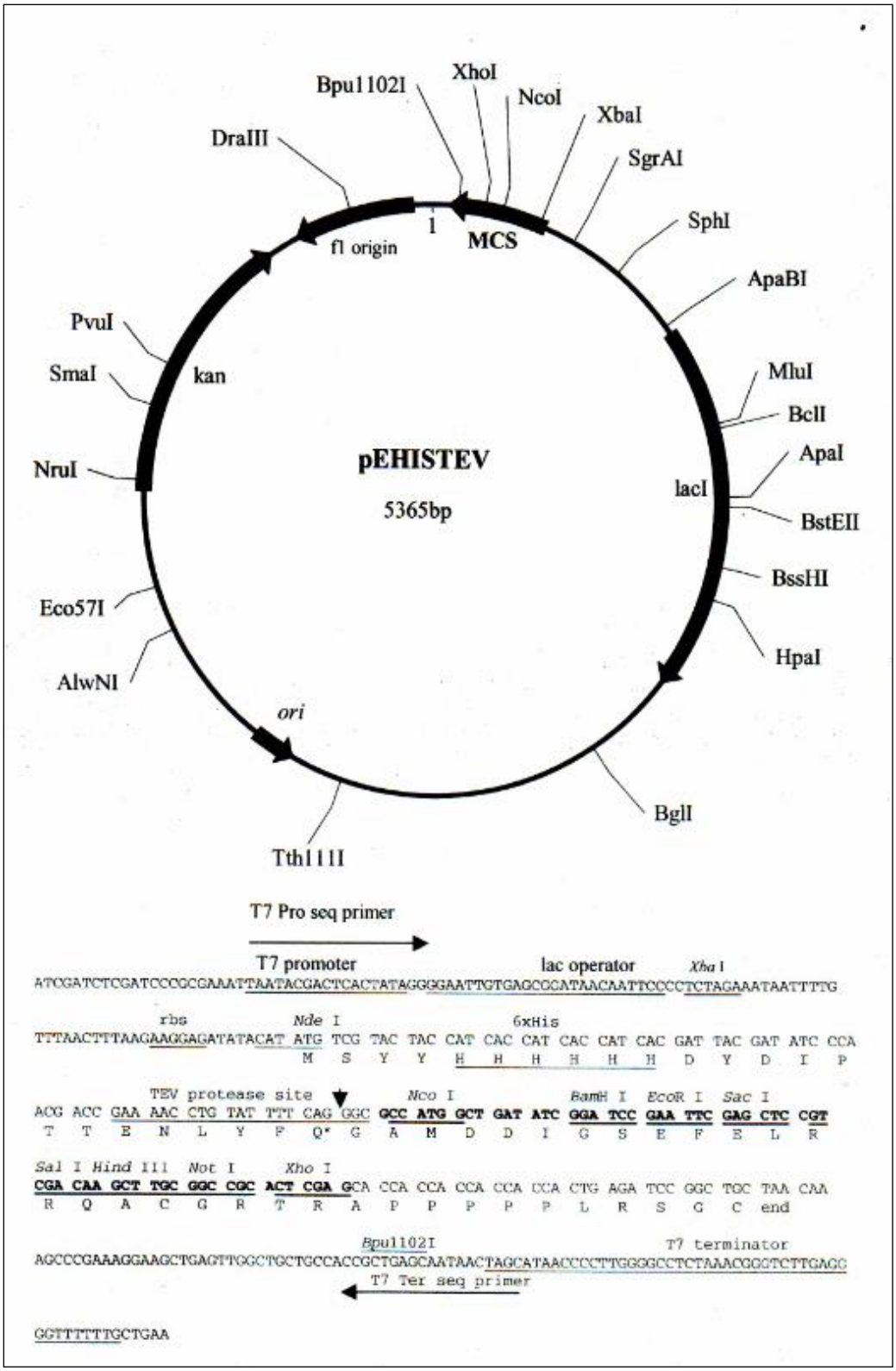
Map for pLEICS-10: Used for expression of MBP-D4 and MBP-D1-3Chis (from PROTEX Leicester)



Map for pLEICS-01 used for expression of CYS mutants of pneumolysin (From PROTEX Leicester)



Map for pLEICS-03 used for expression of CYS mutants of pneumolysin, TEV mutant of pneumolysin, pneumolysin (PLY) mutants 17A, PLY 18A, PLY 80A, PLY 84A, PLY 79Q and PLY double mutant 18A84A.



Map of pEHISTEV vector used for expression of pneumolysin and mutant PLY W433F.

CHAPTER 3:

RESULTS

3.1 Confirmation of pneumolysin gene in pKK233-2:*ply*: The available plasmid in the lab (pKK233-2:*ply*) harbouring pneumolysin gene was extracted from *E.coli* XL10 gold as described in methods and was used a template in PCR to confirm the presence of pneumolysin gene. Primers Ply forward and reverse (as in table) were used to amplify pneumolysin. As can be seen in fig x a 1410 bp fragment of DNA was amplified which is in accordance to the size of pneumolysin gene. To further confirm the amplified product was gel purified and sequenced by sending it to PNACL. The sequencing results made sure that pKK233-2:*ply* contains the gene of interest.

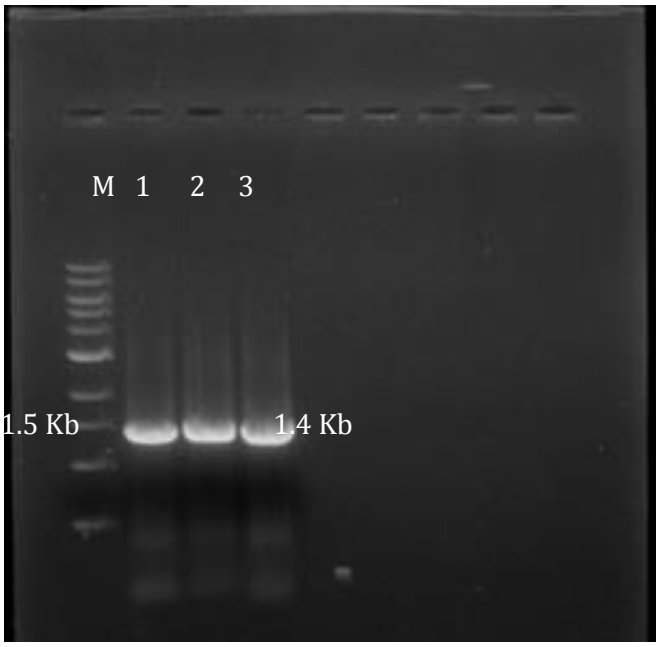


Fig 3.1 PCR for confirmation of ply in the construct pKK233-2:*ply* The amplified DNA for pneumolysin gene was visualized on 1% W/V agarose gel. Lane 1 contains 1Kb DNA ladder marker (from New England biolabs), lane 2, 3 and 4 contains amplified pneumolysin (size 1410bp).

3.2 Cloning of pneumolysin gene into pLEICS vectors: The plasmid pKK233-2:*ply* carrying pneumolysin gene had no tag to aid in purification of the toxin after its expression. It was used as template for amplification of pneumolysin gene with primers specifically designed for pLEICS vectors so that resultant PCR product can be readily ligated. pLEICS vectors as can be seen in their maps in methods carry an N-terminal or C-terminal 6XHis tag.

The 6XHis tag aids in purification of the recombinant protein by binding tightly/strongly to a Ni column via the immidazole ring of the Histidine. The bound protein to the Ni column can then be eluted/stripped off with immidazole and the tag removed by cleaving it from the purified protein by utilizing the cleavage site for TEV protease between the recombinant protein and the tag. As can be seen in figure 3.2 pneumolysin gene was amplified and gel purified before sending it to PROTEX (a commercial service at university of Leicester) for ligation. The received positive clones from PROTEX were verified by colony PCR using primers Ply forward and reverse (sequence shown in table of primers) and then for further confirmation and analysis (to make sure the insert is inframe) the extracted plasmid from *E. coli* XL10 Gold competent cells was sequenced using the service of PNACL at the University of Leicester.

The sequence confirmed plasmid was then transformed into *E. coli* BL21 DE3 plyss competent cells for expression and purification purposes as described earlier.

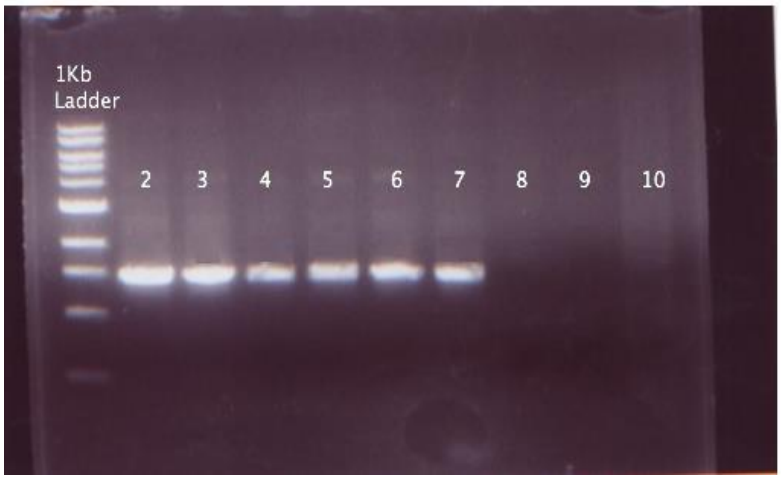


Fig 3.2 Amplification of ply gene for cloning into pLEICS vectors. The amplified DNA for pneumolysin gene was visualized on 1% W/V agarose gel. Lane 1 is 1 Kb DNA ladder (from New England Biolabs) and lane 2-6 contains DNA (1410 bp) for pneumolysin gene amplified with specifically designed primers ready to be cloned into pLEICS-01 and pLEICS-03 vectors. Lane 7 contains positive control (ply gene) while lane 8-10 are negative controls.

3.3 Expression and purification of pneumolysin (PLY): Pneumolysin was expressed and then purified as described in methods. The AKTA purifier and Ni affinity chromatography was used to purify the recombinant pneumolysin. The affinity columns used for this purpose were His Trap HP (5ml) prepacked columns from GE health care. With these columns having the Ni sepharose highly crosslinked agarose beads with an immobilised chealating group made sure strong binding of the his tag recombinant protein. The protein was eluted with 300mM of immidazole in the binding buffer. The purified fractions were run on a 15% SDS gel to check for size and for analysis of the

purified fractions of protein. As can be seen in Fig 3.3 the recombinant protein was about 53 Kda in size which corresponds to the molecular weight of pneumolysin and was about 70-85% pure. The final yeild for one litre of culture in LB was about 35mg of recombinant pneumolysin.

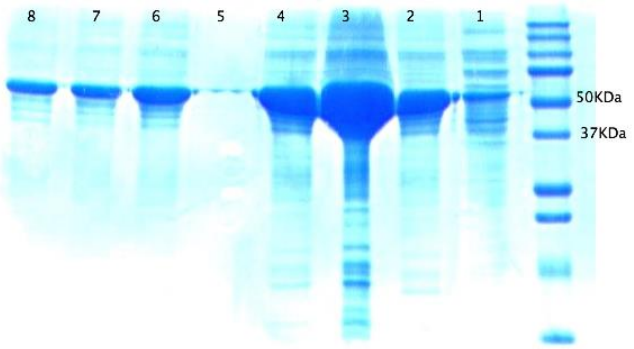


Figure 3.3a SDS PAGE Gel for the purified C-his pneumolysin: The purified fractions of pneumolysin were run on a 15% SDS PAGE gel for analysis. Lanes contain protein standard from Bio-rad, lane 1 contains column wash, lane 2,3, 4, and 6,7, 8 contains different eluates of the his tag purified recombinant pneumolysin.

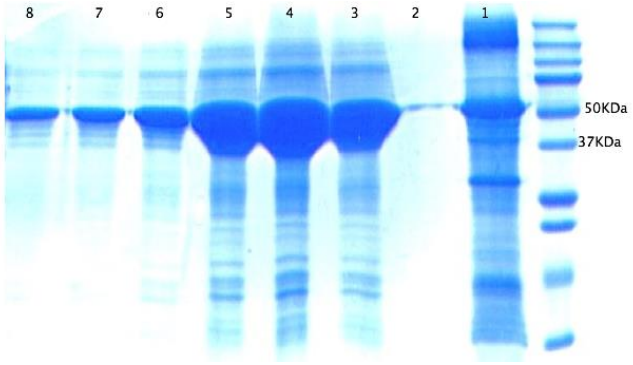


Figure 3.3b SDS PAGE Gel for the purified N-his pneumolysin: The purified fractions of pneumolysin were run on a 15% SDS PAGE gel for analysis. Lane 1 (from right of the viewer) contains protein standard from Bio-rad, lane 2 contains column wash, lane 4, 5, 6, 7, 8 and 9 contains different eluates of the his tag purified recombinant pneumolysin.

3.4 Pneumolysin mutants with predicted disulphide bonds: The technique Site directed mutagenesis was used for making the point mutations as described in methods. The template used for the mutagenesis reaction was pKK233-2:*ply*. The primers carrying the mutations were designed according to the guidelines provided with the mutagenesis kit and are listed in the primer table in methods. The aim of these experiments was to make four sets of mutations in the pneumolysin gene. Specific nucleotides coding for amino acid residues 34, 388, 55, 163, 262 and 278 were mutated to the sequence coding for cystine residue. The resultant double mutants produced were;

1. pKK233-2:*ply* K34G388-C34C388 or (PLY K34G388-C)
2. pKK233-2:*ply* E35G388-C35C388 or (PLY E35G388-C)
3. pKK233-2:*ply* T55V163-C55C163 or (PLY T55V163-C)
4. pKK233-2:*ply* A262W278-C262C278 or (PLY A262W278-C)

For this purpose, first the nucleotide sequence coding for the amino acid G388 in the wild type gene was mutated to a sequence coding for C388. The reason it was selected to be attempted first was that it can serve as a template for two other mutagenesis reactions. Attempts with the newer version of mutagenesis kit called "Lightning quick Multi site directed mutagenesis kit" from Stratagene were not fruitful. Therefore, the older and basic version of the kit from the same manufacturer called "XL-site directed mutagenesis kit" was used ultimately to get the desired mutations. The mutagenesis reaction is based on two complimentary primers having the desired mutation in the middle and 10-15 base pairs on both sides identical to the wild type gene. The primers for the mutagenesis reactions are listed in table for primers in methods.

The mutations were successfully attained and were sequence verified by sending the template to PNACL. All the point mutations were done with the "XL Site directed mutagenesis kit or with site directed mutagenesis as described in methods.

3.5 Cloning of CYS mutants into pLEICS vectors:

All the four double mutants were amplified from pKK 233-2:*ply* with primers specific of pLEICS vectors (from PROTEX Leicester) as described earlier for wild type pneumolysin and cloned. The clones were sequence verified before attempts to express and purify the mutant proteins.

3.6 Expression and purification of the mutant PLY C55+C163

The mutant PLY E55V163-C was expressed and purified as described in section 3.3. The final yield of the mutant protein was 4mg/ml with some co contaminants (*E. coli* proteins). The rocombinant protein bound to the column was eluted with 250 mM of immidazole in the eluting buffer. The purified fractions were run on a 15% SDS gel to check for size and for analysis of the purified fractions of protein. As can be seen in Fig 3.4 the recombinant protein was about 52 Kda in size which corresponds to the molecular weight of pneumolysin and was about 70% pure. As can be seen in the SDS gel. The co contaminants (*E. coli* proteins) could have been separated easily by gel filtration chromatography.

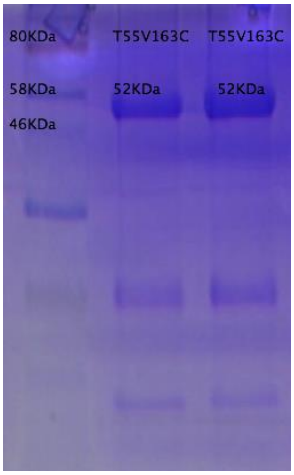


Figure 3.4 SDS PAGE Gel for the purified N-his PLY C55+C163: The purified fractions of pneumolysin were run on a 15% SDS PAGE gel for analysis. Lane 1 contains protein standard from Bio-rad, lane 2 and 3 contains his tag purified fractions of the mutant PLY T55V163-C.

3.7 Expression and purification of the mutant PLY K34G388-C

The double mutant PLY K34G388-C was expressed and purified as described in section 3.3. The final yield of the mutant protein was 7mg/ml with some co contaminants. The protein was eluted with 250 mM of immidazole in the eluting buffer. The purified fractions were run on a 15% SDS gel to check for size and for analysis of the purified fractions of protein. As can be seen in Fig 3.5 the recombinant mutant protein was about 53 Kda in size which corresponds to the molecular weight of pneumolysin and was about 65% pure. As can be seen in the SDS gel.

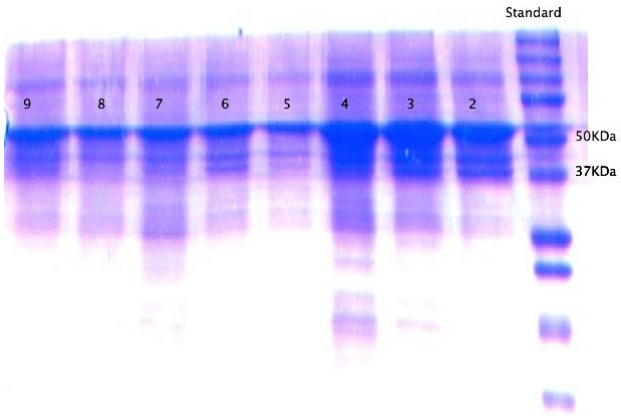


Figure 3.5 SDS Gel for the purified N-his PLY K34G388-C : The purified fractions of PLY K34G388-C were run on a 15% SDS gel for confirmation and analysis. Lanes contain (From the right of the viewer) protein standard from Bio-rad, and lanes having numbers from 2-9 contain different fractions of the eluted recombinant protien stripped off the column with 250mM of immidazole in the eluting buffer.

3.8 Expression and purification of the mutant PLY E35G388-C

The mutant PLY E35G388-C was expressed and purified as described in section 3.3. The final yield of the mutant protein was 7mg/ml with some co contaminants. The protein was eluted with 250 mM of immidazole in the binding buffer. The purified fractions were run on a 15% SDS gel to check for size and for analysis of the purified fractions of protein. As can be seen in Fig 3.6 the recombinant protein was about 53 Kda in size which corresponds to the molecular weight of pneumolysin and was about 80% pure.

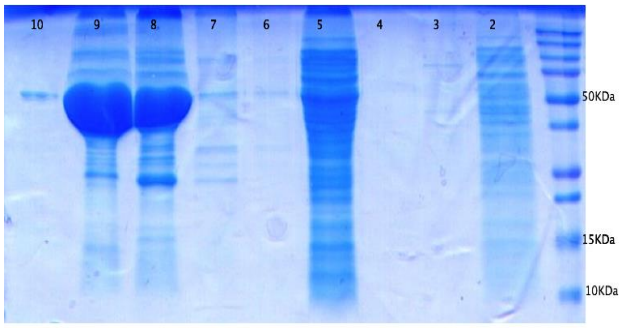


Figure 3.6 SDS PAGE Gel for the purified N-his PLY E35G388-C: The purified fractions of PLY E35G388-C were run on a 15% SDS PAGE gel for analysis. Lanes contains (From the right of the viewer) protein standard from Bio-rad, lane numbered 2 in the picture contains column wash, 3 and 4 are empty, lane numbered 5 contains flow through of the column, lane numbered 6-10 contains different fractions of the his tag purified mutant protein.

3.9 Expression and purification of the mutant PLY A262W278-C

The mutant PLY A262W278-C was expressed and purified as described in section 3.3. The final yield of the mutant protein was 1mg/ml with *E. coli* proteins as contaminants. The protein was eluted with a concentration of 250 mM of immidazole in the eluting buffer. The purified fractions were run on a 15% SDS gel to check for the size and for analysis of the purified fractions of the mutant recombinant protein. As can be seen in Fig 3.7 the recombinant protein was about 53 Kda in size which corresponds to the molecular weight of the pneumococcal toxin (pneumolysin) and was about 35-40 % pure considering the contaminants seen in given picture for its SDS PAGE picture.

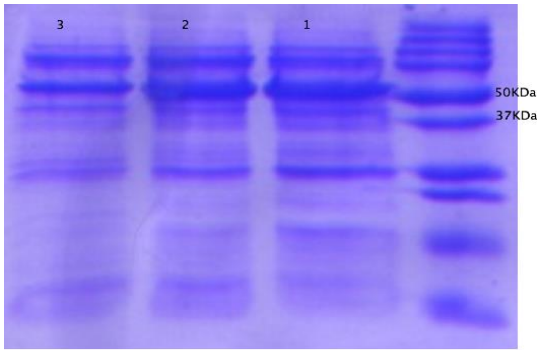


Figure 3.7: SDS PAGE Gel picture for the His tag purified PLY A262W278-C: The purified fractions of the mutant PLY A262W278-C were run on a 15% SDS gel for analysis. Lanes (from the right of the viewer) contain protein standard from Bio-rad, lane numbered as 1,2 and 3 contains different fractions of the eluted protein. As can be seen the purified/eluted protein is not pure to the required standards possibly due to its low yeild and therefore it contains a lot of contaminats (*E. coli* proteins). The low purity and yeild warrants further optimisation (Change of vector) so that the protein can be produced in high quantities and pure form for further characterization.

3.10 Western blot for confirmation pneumolysin and its mutants: The purified fractions of pneumolysin were loaded to a 12% SDS gel and transferred to a nitrocellulose membrane as described in methods. The monoclonal antibody PLY-7 (from de los toyos) was used as a primary antibody in the western blot for confirmation of pneumolysin. As can be seen in figure 3.8 the binding of monoclonal antibody PLY-7 to the recombinant proteins confirmed the size and identity of the expressed protein to be pneumolysin and its mutants.

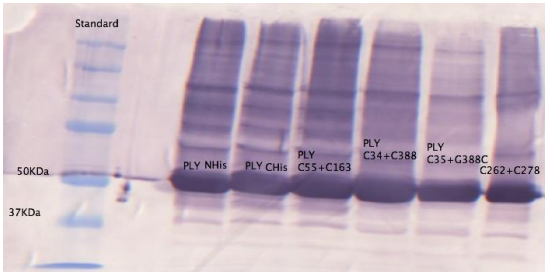


Figure 3.8: western blot for pneumolysin and PLY CYS mutants. As can be seen in the picture for western blot lane 1 (from left of the viewer) contains protein standard from

Bio-rad, lane 2 is empty, lane 3 contains PLY Nhis, lane 4 contains PLY Chis, lane 5 contains PLY T55V163-C, lane 6 contains PLY K34G388-C, lane 7 contains PLY E35G388-C and lane 8 contains A262W278-C.

3.11 Expression and purification of pneumolysin mutant having TEV cleavage site at the junction of domain 3 and domain 4: In order to obtain domain 4 of pneumolysin one of the strategies adopted was to mutate pneumolysin gene at the junction of the two domains (domain 3 and domain 4) in such a way so that after cleavage of the recombinant protein domain 4 of pneumolysin is separated from the rest of the molecule.

The rationale behind this strategy was that domain 4 of pneumolysin will be obtained in pure form with correct conformation and folding. However, doing mutations at the highly conserved region made the mutant protein insoluble as most of the expressed protein was found in the insoluble fraction (Pellet).

The soluble protein as a result of expression was very low in yield and that too with huge amounts of contaminants (*E. coli* proteins). Attempts to increase the yield and enhance the solubility of the recombinant protein were to no avail. Finally, PLY TEV mutant was purified from inclusion bodies. The amount of recombinant protein was very high and pure but when checked for its hemolytic activity it was completely inactive.

The inactivity of the PLY TEV mutant was attributed to its improper folding and conformation. Furthermore, when the PLY TEV mutant purified from inclusion bodies was subjected to cleavage with TEV protease the cleavage was not efficient which further raised questions about the conformation and folding of the recombinant mutant protein. The mutations at the conserved hinge region in

pneumolysin might have been a bit overambitious and this strategy for obtaining domain 4 of pneumolysin was abandoned.

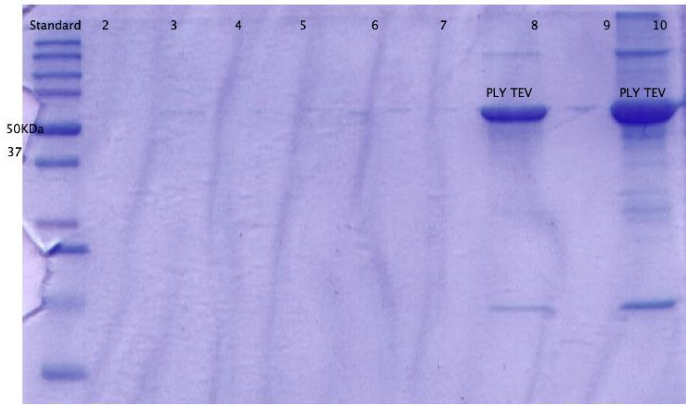


Figure 3.9 SDS PAGE gel picture for PLY TEV mutant purified from inclusion bodies: As can be seen in the figure (from left of the viewer to the right) the first lane contains protein standard (from Bio-rad), lane numbered from 2-7 and lane labelled as 9 are empty while lanes 8 and 10 contains fractions of PLY TEV mutant purified from inclusion bodies and cleaved with TEV protease. The size of the purified protein is 52KDa, which confirms it to be pneumolysin while the lower faint bands in lane 8 and 10 are representing domain 4 of pneumolysin. The yield of domain 4 via this strategy was not what was required and considering the labour and issues with the recombinant protein obtained via this method it was abandoned.

3.12 Expression and purification of domain 4 of pneumolysin expressed as a fusion partner to Maltose binding protein (MBP): The DNA coding for domain 4 of pneumolysin was cloned into pLEICS-10 vector and after sequence confirmation was transformed into *E. coli* BL21 DE3 *plyss* cells for expression. The expressed protein (domain 4 fused to MBP) was termed MBP-D4. The MBP tag not only helps in purification but also work as a solubility enhancer. MBP was later separated by utilizing the cleavage site for TEV protease in the construct, shortly, the expressed protein was purified by gravity columns containing amylose resin (from NEB) and the purified fractions were cleaved by incubating overnight with TEV protease (from PROTEX) as described in methods. The cleaved product was loaded to a gel filtration column for size

exclusion chromatography so as to separate MBP and domain 4 of pneumolysin. Domain 4 of pneumolysin obtained via this method was almost 95% pure and the final yield per litre was 8mg of the recombinant protein which makes it the method of choice. The MBP tag expressed and separated after gel filtration chromatography was also utilized in immunization experiments which are described in the next chapter.

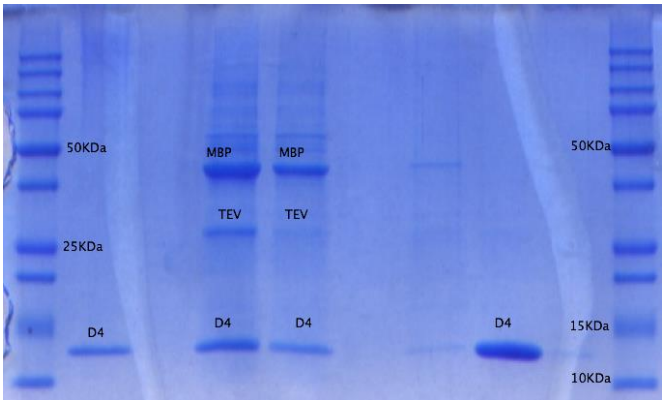


Figure 3.10 SDS PAGE gel picture for the expressed MBP-D4 cleavage with TEV protease isolation of domain 4 of pneumolysin to homogeneity: As can be seen in the figure from left of the viewer to the right lane 1 contains protein standard (from Bio-rad), Lane 2 contains purified domain four of pneumolysin, lane 3 is empty, lane 4 and 5 contains the digestion reactions where MBP-D4 was incubated with TEV protease for separation of MBP from domain 4 of pneumolysin. The enzymatic reaction was very efficient as the amount of fused MBP-D4 (the product of expression) is very small when compared to the cleaved product (MBP and D4 separated). Lane 6 and 7 are empty, lane 8 contains domain four of pneumolysin after gel filtration. The purified recombinant protein was about >95-98% pure as can be seen and had the correct size of about 13KDa corresponding to D4 of pneumolysin.

3.13 Comparison of His tag and MBP tag for the production of domain 4 of pneumolysin: As described earlier in methods domain four of pneumolysin was cloned into pE.coli 6XHN vector (from Clontech) failed to express domain 4 of pneumolysin, pLEICS-03 vector having a his tag that aids in purification but most of it was in the insoluble fraction despite many attempts to optimise the expression. Finally, cloned into

pLEICS-10 vector (from PROTEX Leicester) carrying MBP tag, that not only aids in purification but also enhances solubility of its fusion partner. The constructs were transformed into expression cells and the protein expressed to compare and assess the efficacy and quantity of the production of the recombinant protein. Comparing the final yield and stability of the final product the production of MBP-D4 method was the preferred method for obtaining domain 4 of pneumolysin, as domain 4 of pneumolysin when produced with his tag (at least with pLEICS-03 vector) is prone to precipitation when kept at room temperature besides its low solubility. Domain 4 of pneumolysin produced with its fusion partner was very stable and highly soluble when compared to domain 4 produced with a conventional His tag.

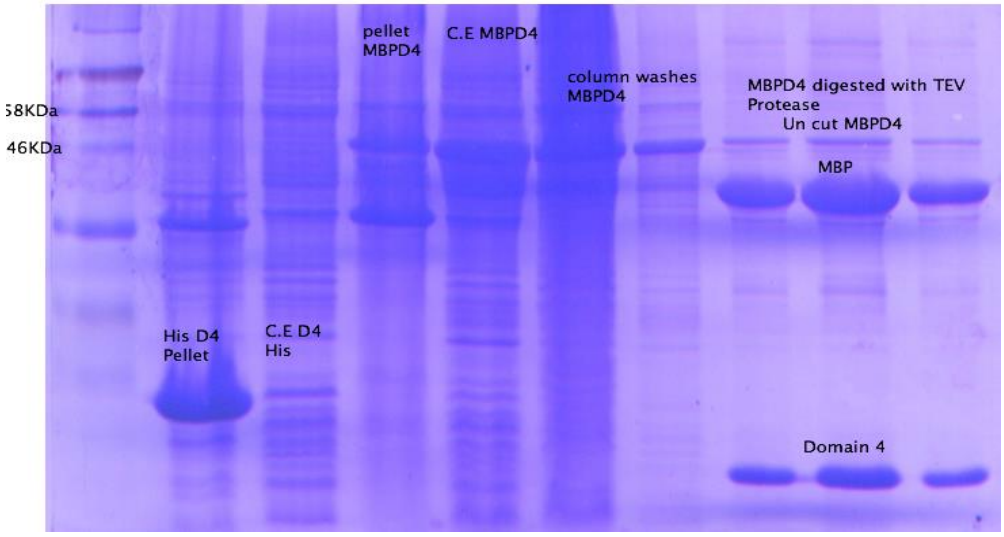


Figure 3.11 SDS PAGE gel picture for the comparison of the production and solubility of domain 4 of pneumolysin between His tag and MBP tag: As can be seen in the picture production of domain 4 of pneumolysin with His tag, most of the recombinant protein is in the insoluble fraction (Lane 2 from left of the viewer), Lane 3 contains crude extracts for domain 4 of pneumolysin which has very small amount of the recombinant protein. Comparing lane 4 and 5 for MBP-D4 production the amount of protein in the soluble fraction is considerably higher than in the insoluble fraction. Lane 6

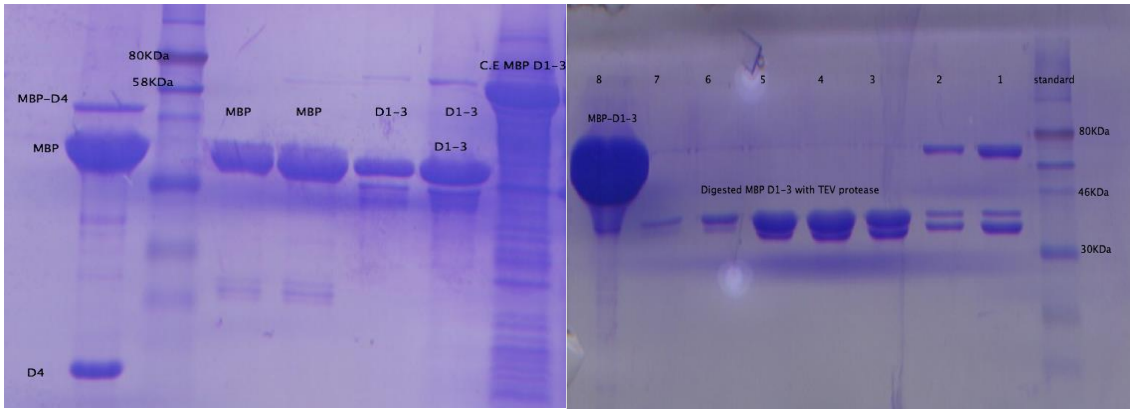
and 7 contain column washes while lane 8,9 and 10 has the purified protein MBP-D4 cleaved with TEV protease.

3.14 Expression and purification of the first 3 domains (D1-3) of pneumolysin fused to MBP: The DNA for domain 1-3 of pneumolysin was cloned into pLEICS-10 vector to be expressed as a fusion protein with MBP, which not only aids in purification but is a solubility enhancer. The decision to express it with MBP was due to the reason as evident from section 3.13. Theoretically, it was straight forward that after expression and purification, the purified fractions will be desalted to get rid of maltose (from the elution buffer). The dialysed purified protein was supposed to be passed through the column after cleavage with TEV protease where the MBP will bind to the column while domain 1-3 was supposed to be collected in the flow through.

This strategy was devised as the size of MBP and domain 1-3 of pneumolysin was almost identical and separation through gel filtration chromatography was not possible. Looking into P.I values of both the proteins it was also similar so it was difficult to isolate through hydrophobic interaction and ion exchange chromatography. However, when the dialysed cleaved protein was passed through the column interestingly MBP did not bound even to newly prepared amylose column for some unknown reasons.

The on column cleavage digestion strategy also failed to for obtaining domain 1-3 of pneumolysin in highly purified form. To solve the problem an alternative strategy was adopted which was to put an extra tag (His tag in this case) at the C-terminus of the recombinant protein so that after cleavage the digested products will be passed through a nickel column where the D1-3 of pneumolysin will bind through its C-terminal his tag while the MBP alone will be pass through without binding to the column. This strategy

proved successful and domain 1-3 of pneumolysin was obtained in pure from to be used in immunization experiments described in the next chapter.



3.12a and 3.12b SDS PAGE gel picture for domain 1-3 of pneumolysin expressed as a fused partner with MBP: The recombinant protein expressed as a fusion partner with MBP was termed as MBP D1-3. As can be seen in the above picture (Left of the viewer) lane 1 contains MBP-D4 cleaved with TEV protease, Lane 2 contains protein standard (from Bio-rad), lane 3 and 4 contains maltose binding protein (MBP), lane 5 and 6 contains purified fractions of domain 1-3 Chis of pneumolysin and lane 7 contains the crude extracts (soluble fraction) for the expressed recombinant protein (MBP D1-3). In the second picture from right to left of the viewer the first lane contains protein standard (from Bio-rad), lane labelled as 1 and 2 contains MBP D1-3 digested with TEV protease and lanes 3,4, 5 and 6 contains the flow through of the digested products after passing through the amylose coumn. lane numbered as 8 contains the purified recombinant protein MBP domain 1-3 of pneumolysin.

3.15 Haemolytic activity for the mutant PLY E35G388-C to check the formation of disulphide bond:

Hemolytic activity for PLY C35+C388 was performed as described in methods, briefly 4% sheep red blood cells were incubated with PLY C35+C388 for 30 minutes in a static incubator and the hemolysis was determined by eye. As these mutants were intended to have a disulphide bond which ideally will stop or reduce hemolysis to a great extent.

To confirm the formation of disulphide bond one row is added with a reducing agent to see any difference in the activity of the toxin. This experiment confirms that no disulphide bond is formed between the intended residues based on the level of hemolysis in both native and reducing states of the mutant toxin.

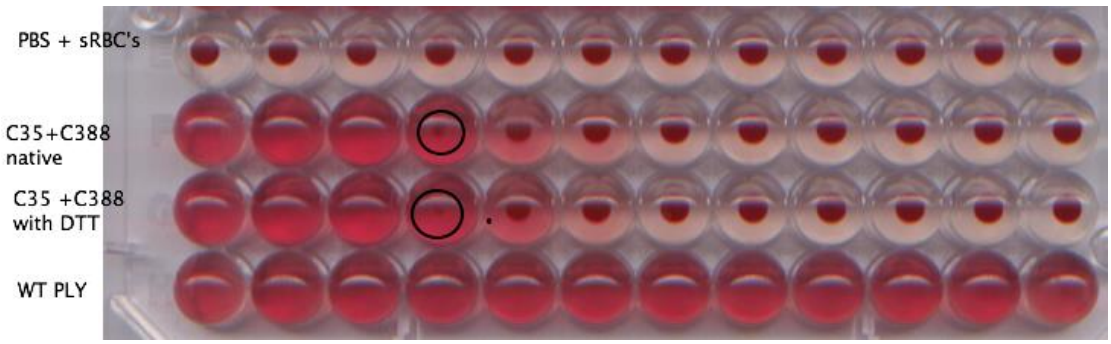


Fig 3.13 Hemolytic assay for PLY E35G388-C: Row one as labelled has PBS+ 4% sRBC's which serves as a negative control (no hemolysis) to the test wells. The second row has the mutant PLY E35G388-C incubated with 4% sRBC's in PBS under native conditions, row 3 has its PLY E35G388-C preincubated with DTT (a reducing agent) for 25 minutes at 37°C for reduction of the disulphide bond if any. The last row contains WT pneumolysin incubated with sRBC's for 30 minutes at 37°C. The haemolytic activity of the mutant is greatly reduced as compared to the wild type toxin (the mutant having 160HU while the wild type is showing activity beyond 40960HU). Both the mutant and the wild type had a concentration of 9ug in the first well and then serially diluted across the row but the mutant apart from containing contaminants was unstable and was prone to precipitation and aggregation which might have been the cause of showing the reduction in activity.

3.16 Haemolytic activity for the mutants PLY K34G388-C, PLY T55V163-C and PLY A262W278-C:

Hemolytic activity for the rest of the three mutants e.g. PLY K34G388-C, PLY T55V163-C and PLY A262W278-C was performed as described in 3.11 and in methods.

Fig 3.14: Hemolytic assay for PLYK34G388-C, PLY T55V163-C and A262W278-C: Row one as labelled has PBS+sRBC's which serves as a negative control for the test wells. Row two has WT PLY incubated with 4% sRBC's in PBS, row 3 has PLY K34G388-C incubated with sRBC's (native conditions), Row 4 has PLY K34G388-C (reducing conditions) pre-incubated with DTT for 25 minutes at 37°C to reduce the disulphide bond. row 5 contains PLY T55V163-C in native while row 6 contains the same under reducing conditions, row 7 contains PLY A262W278-C in native while row 8 contains the same under reducing conditions. The haemolytic activity of the mutants as seen in the picture shows that PLY K34G388-C has the same activity to to that of the wild type toxin (25160 HU). PLY T55V163-C has activity of 640 HU while PLY A262W278-C has a reduced activity of 80HU only and of all the 4 mutants designed to have a disulphide bond it is the only mutant which confirmed formation of the disulphide bond when checked for its hemolytic activity under native and reducing conditions. The significant reduction in its activity under native conditions and then regaining its activity under reducing conditions proves the rationale and the sound approach for design of the experiment.

3.17 Mutagenesis in domain 1 of pneumolysin : This set of mutaions were intended to break the contacts of the individual toxin monomer to its neighboring monomer during

the process of oligomerization. The target residues to be mutated were based on the crytal structure of pneumolysin. The identified residues were mutated by Site directed mutagenesis as described in methods. The template used for these muations were pHIS TEV and pLEICS-03. Both of these vactors carry a his tag which adds in purification. The target amino acids residues and finally the resultant mutants were; PLY K17A, PLY K18A, PLY D79Q, PLY E80A, PLY E84A and a double mutant PLY 18A84A.

All of the above mutants were sucessfully created as confirmed by its sequencing. The mutants were expressed and purified as described before in section 3.3. The his tag mutants were first purified by IMAC and for furtehr purification to homogeneity via size exclusion chhromatography it was loaded on to a gel filtration column and finally the eluates were concentrated and run on a gel. As can be seen in the gel picture the proteins were very pure almost 95% so to determine its exact activity and if these residues have role in oligomerization when the toxin comes into contact with the hsot cell membrane. The purified proteins were run on a 15% SDS gel.

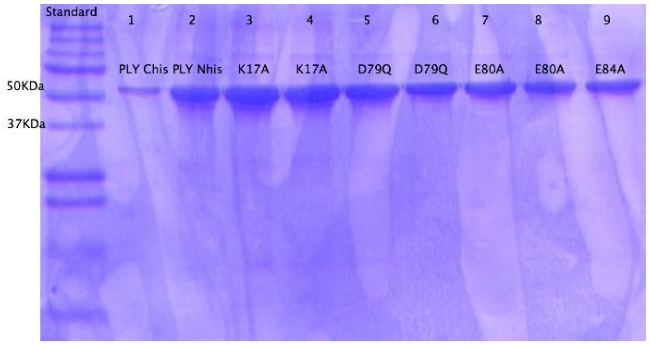


Figure 3.15 SDS PAGE Gel picture for the highly purified pneumolysin muatnts: The purified PLY mutants after gel filtration chromatoghraphy and subsequent concentration were assayed with SDS PAGE to check for purity and homogeneity. As can be seen in the image for SDS PAGE (from left of the viewer) the first lane contains protein standard from Bio-rad, the next lane numbered as 1 has PLY Chis, 2 has PLY Nhis, 3 has PLY K17A, 4 has PLY K18A, lane numbered as 5 and 6 has PLY D79Q, lane

7 and 8 has PLY E80A and lane 9 has PLY E84A. The purity of the protein as seen in the picture of the gel is estimated to be 90-95%.

3.18: Hemolytic activity of the mutants: Hemolytic assay to determine the activity of the muatnat toxins was performed according to the protocol as described in methods. Breifly the mutant PLY toxins were serialy diluted across the rows of the round bottom nunc plate and were incubated with 4% RBC's for 30 minutes at 37°C. HU were detrmined by eye and in the figure the 50% hemolysis is marked with a circle for each mutant toxin.

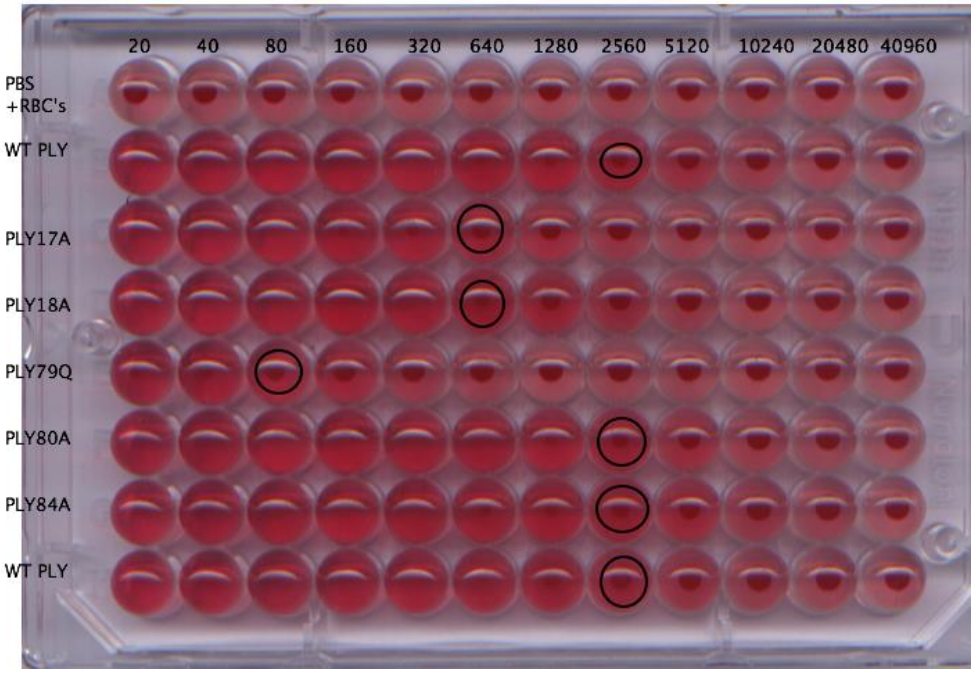
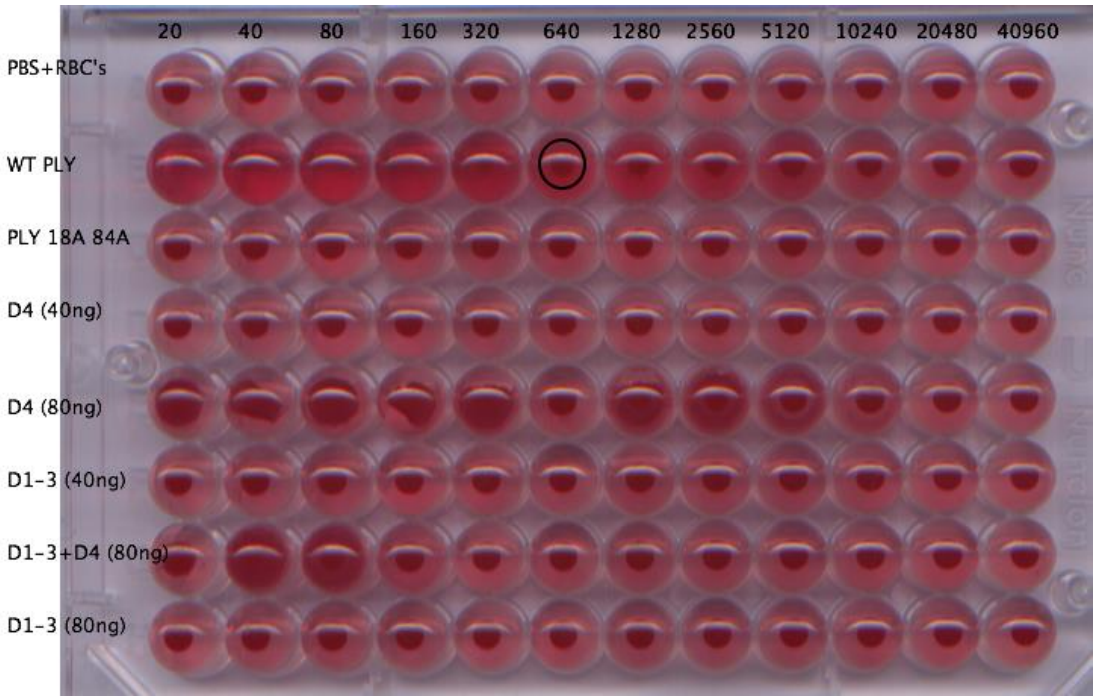


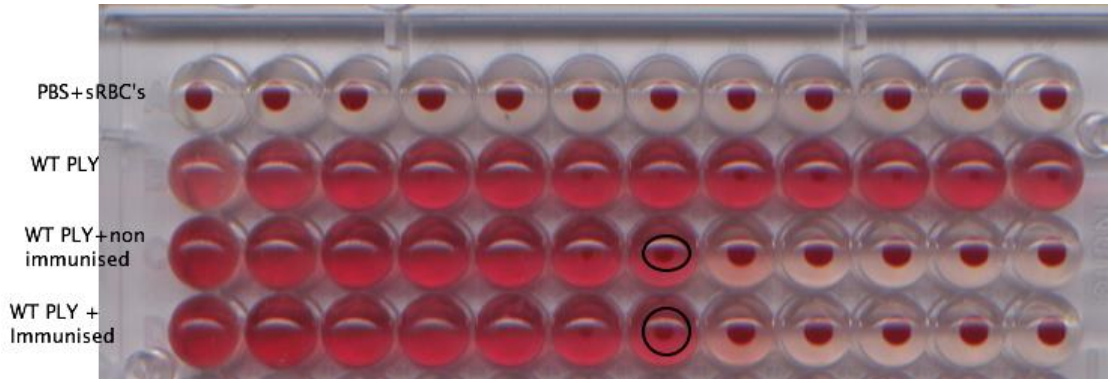
Fig 3.16 Hemolytic assay for domain 1 mutants of pneumolysin: Row one as labelled has PBS+sRBC's which serves as a negative control and to compare the level of hemolysis in the test wells. Row two has WT PLY incubated with 4% sRBC's in PBS, row 3 has the mutant PLY K17A (640HU) incubated with sRBC's, Row 4 has PLY K18A (640HU), row 5 contains PLY D79Q (80HU), row 6 contains PLY E80A (2560HU), row 7 contains PLY E84A (2560HU) and row 8 contains WT PLY (2560HU). These results show that PLY 17A, PLY 18A and PLY 79A have reduced activity and is not fully attenuated.

3.19 Hemolytic activity for the double mutant PLY 18A84A, domain 4 of pneumolysin, domain 1-3 of pneumolysin and domain 1-3 + D4 of pneumolysin in combination: The hemolytic activity for the pneumolysin double mutant PLY 18A84A and domains of pneumolysin was determined according to the protocol as described in 3.18. Briefly, the proteins were serially diluted across the rows in the round-bottom nunc plate and incubated for half an hour at 37 degrees centigrade. The hemolytic units were determined by eye.

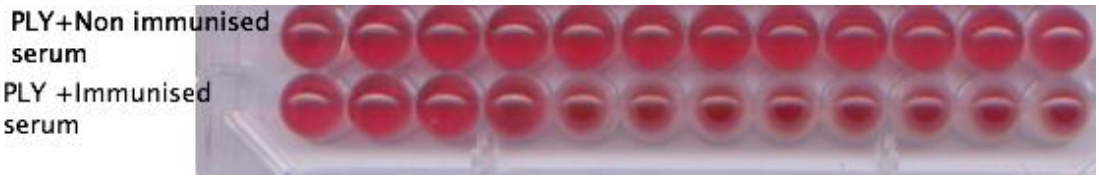


3.17 Hemolytic assay for PLY double mutant and PLY domains: As can be seen in the picture for hemolytic assay row one contains the negative control (PBS+RBC's), row 2 of has wild type pneumolysin (640 HU), row 3 of the plate has the double mutant designed to break the contacts between the individual monomers and as a result stop oligomerization of the toxin on the host cell surface. Row 4 and 5 contains domain 4 of pneumolysin at concentration of 40ng and 80ng respectively, the result for domain 4 of pneumolysin is interesting as it is forming a mesh like structure or making the cells to aggregate instead of doing hemolysis. This aggregation is speculated to be due to the multiple binding sites in domain 4 of pneumolysin to the host cell. No hemolysis or activity is seen for domain 1-3 of pneumolysin.

3.20 Hemolytic assay to check the inhibition of hemolysis with serum from mice immunized with PLY-D4 and mice immunized with PLY D1-3+ PLY D4 in combination: Serum from a group of mice immunized with domain 4 of pneumolysin was pooled in one tube and the serum from the non-immunized mice (negative control) was pooled in another tube. The purpose of this experiment was to check the inhibitory property of the antibodies that were generated in mice as a result of immunization. Briefly, 9ug of wild type toxin was serially diluted across two rows of a round bottom nunc plate, 2ul of the pooled serum (immunized to one row and non-immunized to the other) was added to each well and then incubated for 30 minutes. Finally, 4% RBC's were added to each well and incubated again for half an hour. Hemolysis was observed by eye.



3.18 Hemolytic inhibition assay with serum of mice immunized with PLY-D4: As can be seen in the figure, the serum of mice immunized with domain of pneumolysin is unable to inhibit the activity of the toxin. Row 1 is the negative control containing PBS and RBC'S, row 2 contains wild type pneumolysin incubated with RBC'S, row 3 has pneumolysin preincubated with pooled serum from non-immunized mice while row 4 has pneumolysin pre-incubated with pooled serum from mice immunized with domain 4 of pneumolysin. No difference between the inhibitory activity of serum from immunized and non-immunized mice was detected which correlates with the survival curve and hence lack of protection with domain 4 of pneumolysin when used as a vaccine.



3.19 Hemolytic inhibition assay with serum of mice immunized with PLY D1-3+D4: As can be seen in the figure when a similar assay (as in 3.18) was performed for the pooled serum of mice immunized with domain 1-3 of pneumolysin and domain 4 of pneumolysin in equimolar ratios the resulting antibodies were able to inhibit the activity of the toxin. The inhibition of the toxin in this assay is in correlation with the survival curve (described in the next chapter) where immunized mice had significant protection against a lethal dose of *Streptococcus pneumoniae*. Row 1 in the picture contain pneumolysin pre-incubated with pooled non-immunised serum showing no sign of inhibition of hemolysis. Row 2 contain serially diluted pneumolysin (starting from 9ug) pre-incubated with 2ul of pooled immunised serum. Inhibition of the toxin is one way to know that the mixture of domains of pneumolysin has great potential to be used as a vaccine on its own or can be included in any vaccine formulations.

3.21 Creation of W433F to be used in immunization experiments: The purpose of creation of PLY W433F was to obtain the mutant protein (pdB toxin) known for its reduced hemolytic activity. In some previous studies like that of Paton et al., 1991 when used as an immunogen it provided protection and was considered as a strong case for a potential vaccine. However, recently when used in our department (PhD thesis of Sarah Smeaton) it failed to give protection against a lethal dose of the pneumococcus.

The purpose of its production was to get the protein and use fresh batch of it for immunisation purpose rather than relying on other sources. If it provides protection will serve as a positive control or at least its protective efficacy will be evaluated once more. PLY W433F was created using site directed mutagenesis; the template used for creating this mutant was pHIS TEV. The protein PLY W433F was expressed and purified as

described in methods. The yield of the protein per litre was 15mg. The purity of W433F as can be seen in the gel pictures is about 80-85%.

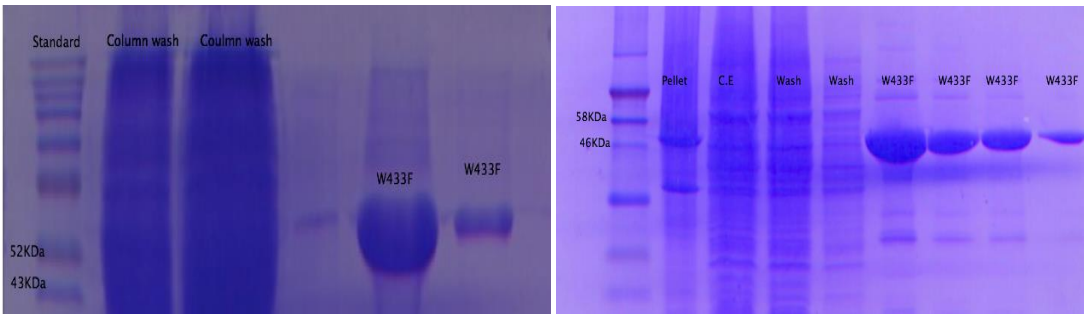


Figure 3.20: SDS PAGE Gel picture for the purified PLY W433F: (Left) The purified fractions of PLY W433F were run on an SDS gel, as can be seen in the SDS PAGE picture from left of the viewer to the right lanes contain protein standard from Bio-rad, the next two lanes contain column wash with binding buffer, and lanes marked with W433F contain the purified protein. The second picture (to the right of the viewer) Lane 1 is standard, lane 2 contain pellet after induction, lane 3 contain crude extracts, lane four and five contain column wash, and lanes 6-9 conatin the purified PLY W433F. The purified protein was about 80-85% pure and the yeild per litre was 15mg of the recombinant protein.

CHAPTER 4:

IMMUNIZATIONS

D4 as an adjuvant:

The structurally and functionally important C-terminal domain of pneumolysin, also known as domain 4 of pneumolysin (PLY-D4) is responsible for binding of the toxin to the host cell. Domain 4 alone is capable of binding to host cells. Also it can inhibit hemolysis when incubated with human red blood cells (hRBC's) presumably by competing the binding sites for the toxin (Baba *et al.,* 2001)).

Pneumolysin is capable of activating the human complement system (Paton *et al.,* 1984) and site-directed mutagenesis studies of pneumolysin showed that a single mutation (D385N) in its domain 4 can abolish the ability of toxin to activate the complement system (Mitchell *et al.,* 1991). Domain 4 is also essential for toxicity of pneumolysin. A classic example is that of a point mutation (W433F) in the well conserved tryptophan rich loop of domain 4 of pneumolysin. The mutation W433F in pneumolysin effects the toxicity and the mutant toxin is left only with residual toxicity.

Domain 4 of pneumolysin is not hemolytic and from our previous immunisation experiments it was known that it is immunogenic although it did not provide protection against a lethal dose of *Streptococcus pneumoniae*. Here the hypothesis that domain 4 of pneumolysin boosts the antibody level, i.e acts as an adjuvant was investigated. For this purpose mice were immunised three times and serum collected after each immunisation as described in the materials and methods section. Antibody levels in the serum of mice

immunised with maltose binding protein (MBP) and mice immunized with maltose binding protein fused to domain 4 of pneumolysin (MBP-D4) were compared by Elisa as described in the materials and methods section.

As can be seen in figure 4.1 anti-MBP antibody levels in mice serum after first immunization were significantly different between the immunized groups ($p < 0.0001$). The mean A405 reading for anti-MBP antibody in mice serum immunised with maltose binding protein was 0.1826 while it was 0.6404 in mice immunized with MBP-D4.

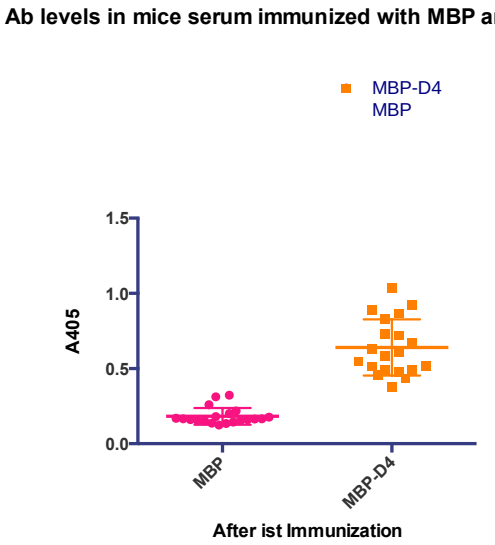


Fig 4.1 Antibody levels in mice serum immunised with MBP and MBP-D4 The figure shows antibody levels after ist immunization in the sera of two groups of MF1 mice immunised with maltose binding protein (MBP) and MBP fused to domain 4 of pneumolysin (MBP-D4). Serum samples were used in triplicate and the mean of three A405 readings for individual mice sera were plotted ($p<0.0001$).

Anti-MBP antibody levels in mice sera after 2nd immunisation was compared by an Elisa as can be seen in the figure the levels of anti-MBP antibody in mice immunised with

MBP-D4 was significantly higher ($p>0.0001$) to those immunised with MBP alone. After first immunization the antibody titre in the group of mice immunised with MBP alone was not high with a mean A405 reading of 0.1826, whereas after the second immunisation it was 0.4384. The antibody titre in mice immunised with MBP alone and mice immunised with MBP-D4 were significantly different with the sum of means 8.769 and 15.68 respectively ($p<0.0001$). The mean A405 reading for serum samples of mice immunised with MBP and MBP-D4 were 0.4384 and 0.7841 respectively.

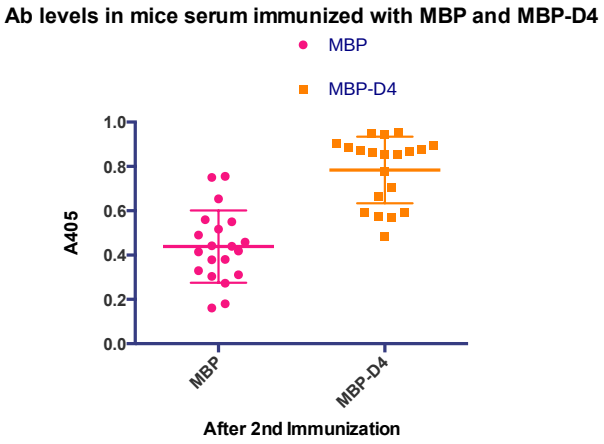


Fig 4.2 Antibody levels in mice serum immunised with MBP and MBP-D4 The figure shows antibody levels after 2nd immunization in the sera of two groups of MF1 mice immunised with maltose binding protein (MBP) and MBP fused to domain 4 of pneumolysin (MBP-D4). Serum samples were used in triplicate on the Elisa plate and the mean of three A405 readings for individual mice sera were plotted ($p<0.0001$).

Similarly, anti-MBP antibody levels in mice sera of both groups of mice i.e Immunised with MBP alone and Immunised with MBP-D4 after 3rd immunisation was compared by an Elisa as described earlier. As can be seen in the figure the levels of anti-MBP antibody in the mice immunised with MBP-D4 was significantly higher ($p>0.0002$) to those

immunised with MBP alone. The level of antibody in the sera of mice immunized with MBP alone was on the increase as compared to its first immunisation, here after third immunisation though the difference in the antibody level between the two groups i.e mice immunised with MBP alone and mice immunised with MBP-D4 was still significant ($p<0.0002$) but we did not see significant increase in the antibody levels of mice immunised with MBP-D4 when compared to the antibody levels after second immunisation. The sum of means for mice immunized with MBP and MBP-D4 was 13.14 and 15.98 respectively. The mean A405 reading for serum samples of mice immunised with MBP and MBP-D4 were 0.657 and 0.799 respectively ($p<0.0002$).

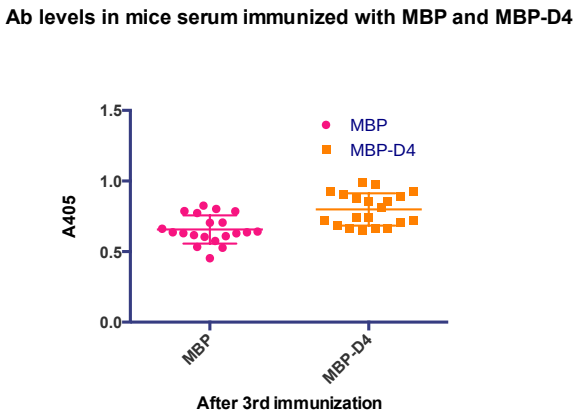


Fig 4.3 Antibody levels in mice serum immunised with MBP and MBP-D4 The figure shows antibody levels after 3rd immunization in the sera of two groups of MF1 mice immunised with maltose binding protein (MBP) and MBP fused to domain 4 of pneumolysin (MBP-D4). Serum samples were used in triplicate on the Elisa plate and the mean of three A405 readings for individual mice sera were plotted ($p<0.0002$).

A bar chart to was constructed to summarize the antibody levels in both groups after each immunisation. The means of anti-MBP antibody levels where each bar as labelled represents the means of antibody levels after first, second and third immunisation in both

the immunised groups of mice i.e mice immunised with MBP alone and mice immunised with MBP-D4. As can be seen in the figure the level of anti-MBP antibody is significantly different between the two immunised groups after each immunisation. The boosting effect of anti-MBP antibody can be clearly seen after first and 2nd immunisation in the group of mice immunised with MBP-D4 as compared to the group of mice immunised with MBP alone. In the case of serum antibody levels to MBP-D4 between second and third immunisation the difference is not significant with the sum of means 15.68 and 15.98 respectively. Elisa for anti-MBP antibodies in the sera for all the three immunisations was performed simultaneously to observe the increase in the antibody titre.

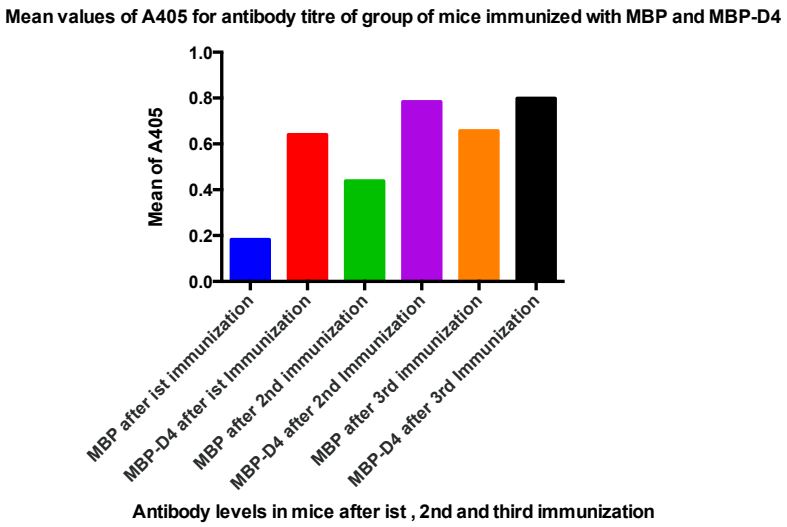


Fig 4.4 Antibody levels in mice serum immunised with MBP and MBP-D4 The figure compares antibody levels in the serum of both groups of MF1 mice immunized with maltose binding protein (MBP) and MBP fused to domain 4 of pneumolysin (MBP-D4). Each bar in the figure is representing the sum of mean of A405 readings for a group of 20 mice after each immunisation.

Domain of of pneumolysin as a vaccine:

Protective efficacy of domain 4 of pneumolysin when administered intraperitoneally: The purpose of this experiment was to investigate if domain 4 of pneumolysin confers protection against a challenge dose of *Streptococcus pneumoniae* that is known to be lethal in non-immunised mice. For this purpose, one group of mice were immunised intraperitoneally with domain 4 of pneumolysin in alum and the second group with alum alone, as described in methods section.

One month after the final immunisation mice were challenged with a lethal dose of *Streptococcus pneumoniae*. The disease signs were monitored closely, and the end point when a mice became severely lethargic was recorded as its survival time. As can be seen in the figure 4.5 no significant difference (P 0.05) was observed in the survival time of the immunised and non-immunised groups of mice. The experiment revealed that domain 4 of pneumolysin on its own can't provide protection against *streptococcus pneumoniae* when administered intraperitoneally.

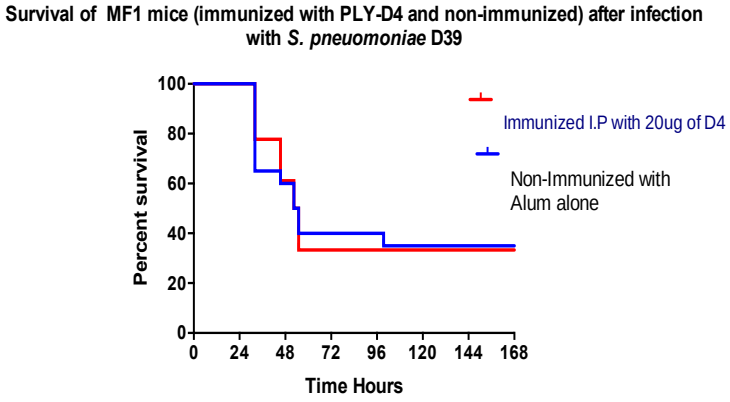


Fig 4.5 Survival curve for groups of MF1 mice after immunised with PLY-D4 and the negative control non-immunised group (alum alone) after infection with *S. pneumoniae* D39. 20 female MF1 mice were immunised three times (ten days apart) with 20ug of domain 4 of pneumolysin in alum (---) and another group of 20 female MF1 mice administered with alum alone (-). The immunisation was followed by intranasal challenge of both groups with 2 X 10^6 *S. pneumoniae* D39. No significant difference between the survival curves was observed ($P > 0.05$).

Protective efficacy of domain 4 of pneumolysin when administered via the intranasal route: The purpose of this experiment was to investigate can domain 4 of pneumolysin when administered via the intranasal route of pneumolysin confers protection against a lethal dose of *Streptococcus pneumoniae*? For this purpose, one group of mice were immunised via the intranasal route with domain 4 of pneumolysin in PBS and the second group of mice (Negative control) with PBS alone.

Three doses 10 days apart were administered to individual mouse; each dose was 30ug of the recombinant pneumolysin domain 4 for the immunised group while to the non-immunised group only PBS was administered. One month after the final immunisation mice were challenged with a challenge dose of *Streptococcus pneumoniae*. The disease signs were monitored closely and the end point for each mouse was considered and recorded when it became severely lethargic.

As can be seen in figure 4.6 no significant difference (P 0.05) in the survival time between the two groups of mice (immunised and non-immunised) was observed. The experiment revealed that domain 4 of pneumolysin on its own can't provide protection against a lethal dose of *streptococcus pneumoniae* when used as a vaccine.

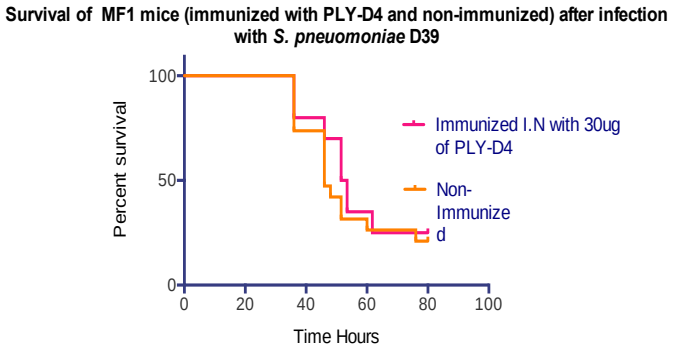


Fig 4.6 Survival curve for the groups of MF1 mice immunised with PLY-D4 and the non-immunised group after infection with *S. pneumoniae* D39. 20 female MF1 mice were immunised three times with 30ug of domain 4 of pneumolysin in PBS (---) and 20 female MF1 mice (non immunised) administered with PBS alone (-) were challenged via the intranasal route with 2 X 10^6 *S. pneumoniae* D39. No significant difference between the survival curves (P > 0.05) was observed.

Protective efficacy of domain 4 of pneumolysin when administered via the intraperitoneal route:

The purpose of this experiment was to investigate if domain 4 of pneumolysin confers protection against a lethal dose of *Streptococcus pneumoniae* when administered via the intraperitoneal route. For this purpose, one group of mice were immunised intraperitoneally with 30ug of recombinant domain 4 of pneumolysin in alum, a second group of mice was immunised with MBP-D4 in alum and the third group of mice (the non-immunised negative control group) with alum alone. Three doses ten days apart were

administered and one month after the final immunisation mice were challenged with *Streptococcus pneumoniae* via the intranasal route. The disease signs were monitored closely and severely lethargic was considered as the end point for each mouse. The end point was recorded and considered as its survival time. As can be seen in figure 4.7 there was no significant difference ($P > 0.05$) in the survival time between the immunised and non-immunised groups. The experiment revealed that domain 4 of pneumolysin alone or infusion with maltose binding protein (MBP-D4) when administered intraperitoneally can't provide protection against a standard intranasal dose of *Streptococcus pneumoniae*.

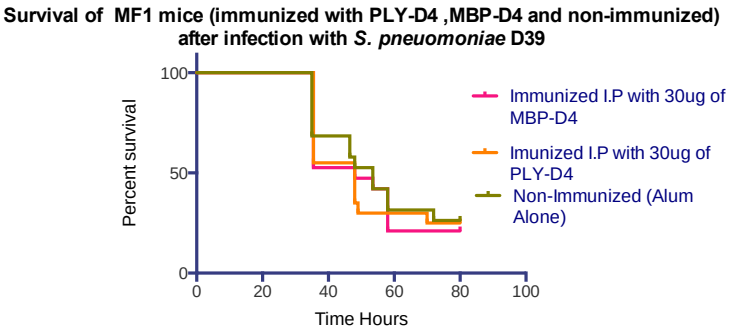


Fig 4.7. **Survival curve for the groups of MF1 mice after immunisation with PLY-D4, MBP-D4 and non-immunised (Negative control) group challenged with *S. pneumoniae* D39.** 20 female MF1 mice were immunised three times with 30ug of domain 4 of pneumolysin, 20 female MF1 mice with MBP-D4 in alum (---) and the third group of 20 mice were administred with alum alone (-) before intranasal challenge with 2 X 10^6 *S. pneumoniae* D39. There was no significant difference between the the survival curves ($P > 0.05$).

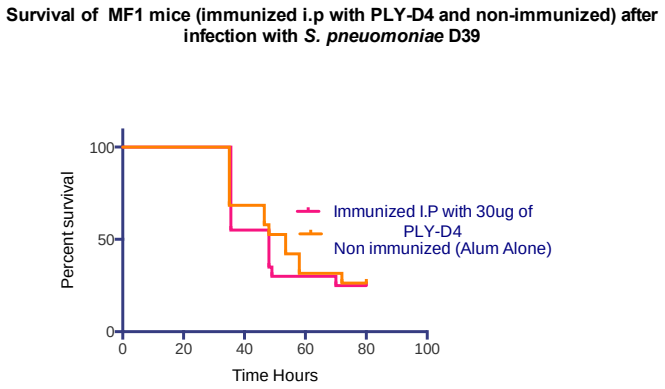


Fig4.8 Survival curve for MF1 mice immunised with 30ug of PLY-D4 in alum and the non- immunised group after challenge with *S. pneumoniae* D39. 20 female MF1 mice were immunised three times ten days apart with 30ug of domain 4 of pneumolysin in alum (---) and similarly 20 mice were administered 3 doses of alum alone (-) before intranasal challenge with 2 X 10^6 *S. pneumoniae* D39. There was no significant difference between the survival curves for the immunised and non-immunised groups ($P > 0.05$).

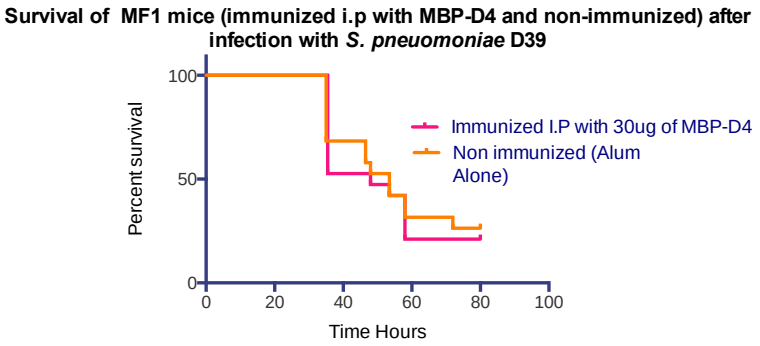


Fig4.9 Survival curve for MF1 mice immunised with MBP-D4 in alum and the non-immunised group after challenge with *S. pneumoniae* D39. 20 female MF1 mice were immunised three times with 30ug of MBP-D4 in alum (---) and 20 were administered with alum alone (-). Finally, one month after the last immunisation both groups were challenged with 2 X 10^6 *S. pneumoniae* D39. There was no significant difference between the the survival curves for the immunised and non-immunised group ($P > 0.05$).

Combination of PLY D1-3 + PLY-D4 as a vaccine: Pneumolysin a known immunogen cannot be used a vaccine due to its cytotoxicity which is mainly derived from its hemolytic property. The mutants produced in pursuit to get rid of its activity do carry some residual activity i.e PLY toxoid W433F (Pdb toxin). The ideal mutant candidate of pneumolysin must have no activity and should be immunogenic to the level of the wild type pneumolysin. Taking into consideration these above problems and facts associated with pneumolysin as a vaccine a hypothesis developed which was that the mixture of domains of pneumolysin (domain 1-3 +D4) will not only be inactive but will also represent and act like the wild type toxin when it comes to protective efficacy and immunogenicity.

To check this hypothesis of the mixture of domains of pneumolysin as a vaccine, domain 1-3 and domain 4 of pneumolysin was expressed and purified as described in results. The purified recombinant PLY D1-3 protein when checked for activity was completely inactive alone and also in combination with domain 4 of pneumolysin. Immunisation experiments with the mixture of domains were performed and its immunogenicity and protective efficacy was investigated as can be seen in the following results.

Comparison of serum antibody levels between the immunised (with the mixture of domain 1-3+ domain 4) and the non-immunised groups:

The purpose of this experiment was to check the difference in antibody levels in the serum of immunised mice and non-immunised (control group) mice after first immunization via the intranasal route. For this purpose, an Elisa was performed as described in methods. As can be seen in the figure the levels of antibodies in mice

immunized with PLY D1-3+D4 in PBS was significantly higher (P value 0.0021) to the mice administered with PBS alone. The sum of means for mice immunised with PLY D1-3+D4 and non-immunised (PBS alone) was 2.382 and 1.864 respectively. The mean A405 reading for PLY antibody in mice serum immunised with PLY D1-3+D4 was 0.1191 while it was 0.0932 in the non- immunised mice with PBS alone ($p<0.0021$).

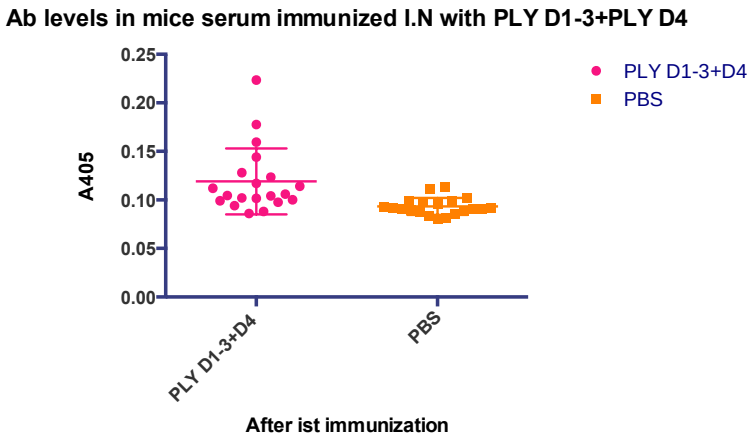


Fig 4.10 Antibody levels in mice serum immunised with PLY D1-3+D4 in PBS and PBS alone The figure shows antibody levels after first immunization in the sera of two groups of MF1 mice where one group was immunised with PLY D1-3+D4 in PBS and the other administered with PBS alone (non-immunised group). Serum samples for individual mice sera were used in triplicate on the Elisa plate and the mean of three A405 readings for individual mice sera were plotted ($p<0.0021$).

Similarly, the antibody levels in the serum of mice after second immunization in both the immunised and non-immunised groups of mice were compared. For this purpose, an Elisa was performed where each mice serum was tested in triplicates and then the mean reading was taken. As can be seen in the figure 4.10 the levels of PLY antibodies in the mice immunised with PLY D1-3+D4 was significantly higher (P value 0.0001) to that of

the non-immunized group treated with PBS alone. The sum of means for mice immunised with PLY D1-3+D4 and non-immunised (PBS alone) was 15.88 and 2.250 respectively. The mean A405 reading for PLY antibody in mice serum immunised with PLY D1-3+D4 was 0.7940 while it was 0.1125 in the non-immunised mice treated with PBS alone ($p<0.0001$).

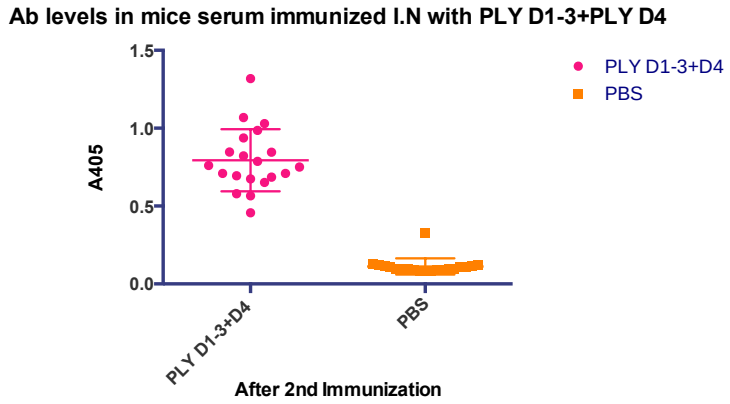


Fig 4.11. Antibody levels in mice serum immunised with PLY D1-3+D4 in PBS and PBS alone The figure shows antibody levels after second immunisation in the sera of two groups of MF1 mice where one group was immunised with PLY D1-3+D4 in PBS and the other (non- immunised) treated with PBS alone. Serum samples were used in triplicate on the Elisa plate and the mean of three A405 readings for individual mice sera were plotted ($p<0.0001$).

PLY antibodies level in mice sera for both groups of mice i.e Immunised with PLY D1-3+D4 in PBS and non-immunised with PBS alone after 3rd immunisation was compared by an Elisa. As can be seen in the figure 4.12 the levels of PLY antibodies in mice sera immunised with PLY D1-3+D4 was significantly higher ($p>0.0001$) to that of the non-immunised group treated with PBS alone. The sum of means for mice immunised with PLY D1-3+D4 and non-immunised (PBS alone) was 29.65 and 5.07 respectively. The

mean A405 reading for PLY antibody in mice serum immunised with PLY D1-3+D4 was 1.482 while it was 0.25 in the non-immunised group of mice treated with PBS alone ($p<0.0001$).

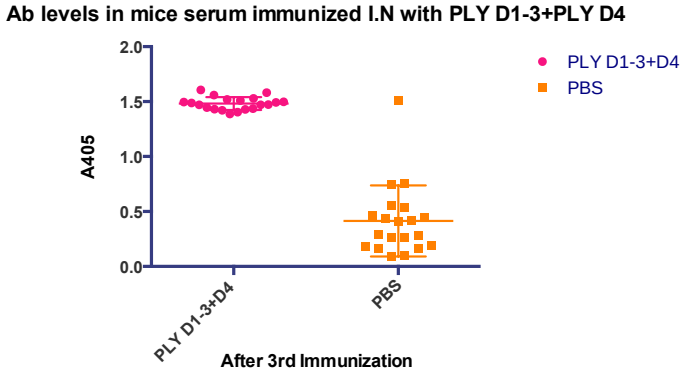


Fig 4.12 Antibody levels in mice serum immunised with PLY D1-3+D4 in PBS and the non- immunised control group: The figure depicts the pneumolysin antibody levels in the sera of mice after third immunisation. The sera belonged to two groups of MF1 mice where one group was immunised with PLY D1-3+D4 in PBS and the other non-immunised treated with PBS alone. Serum samples for individual mice were used in triplicate and the mean of three A405 readings sera were plotted for analysis ($p<0.0001$).

To get a complete picture for the antibody levels and its comparison between the immunised and non-immunised groups a bar chart was constructed. The sum of means of PLY antibody levels in both groups after each immunisation are represented as a single bar as labelled in figure 4.13 for mice immunised with PLYD1-3+D4 and non-immunised treated with PBS alone. As can be seen in the figure the level of PLY antibody is significantly different between the two groups after each immunisation. The difference in

the level of antibody in mice sera is more pronounced after the second and third immunisation. The sum of means for mice immunised with PLY D1-3+D4 after first, second and third immunisation were 2.382, 15.88 and 29.65 respectively which shows increase in antibody level in the serum with each additional treatment. Elisa for PLY antibodies in the sera for all the three immunisations were performed simultaneously to observe the increase in the antibody titre.

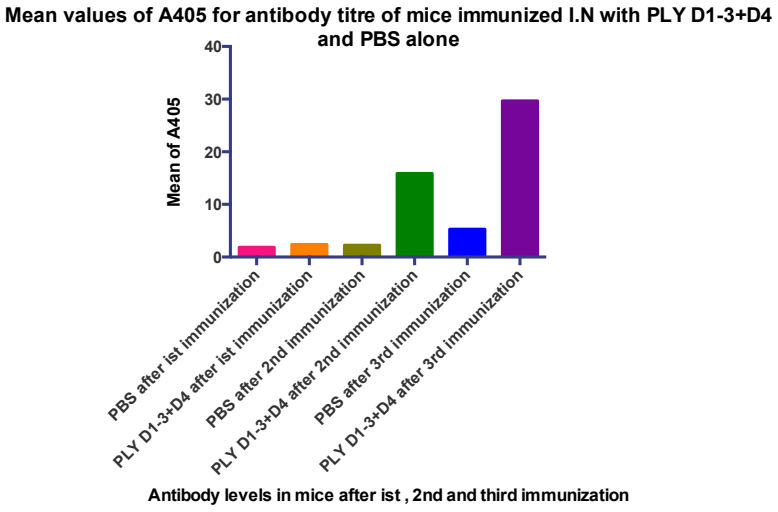


Fig 4.13 Difference of antibody levels in mice serum of two groups immunised with domain 1-3+ domain 4 of pneumolysin and treated with PBS: The figure compares antibody levels in the serum of both groups of MF1 mice immunised with PLY D1-3+D4 and the non-immunised treated with PBS alone. Each bar in the figure is representing the sum of mean of A405 readings for a group of 20 mice after first, second and third immunisation.

The difference in serum antibody levels for mice immunised with the mixture (domain 1-3 of pneumolysin + domain 4 of pneumolysin) and the non-immunised mice treated with alum alone via the intraperitoneal route was determined.

An Elisa was performed for this purpose. As can be seen in the figure the levels of PLY antibodies in the group of mice immunized with PLY D1-3+D4 in alum was significantly higher (P value 0.0001) to that of the non-immunised group treated with alum alone.

The sum of means for mice immunised with PLY D1-3+D4 and non-immunised (alum alone) was 2.649 and 2.045 respectively. The mean A405 reading for PLY antibody in mice serum immunised with PLY D1-3+D4 was 0.132 while it was 0.1023 for the non-immunised mice treated with alum alone ($p<0.0001$).

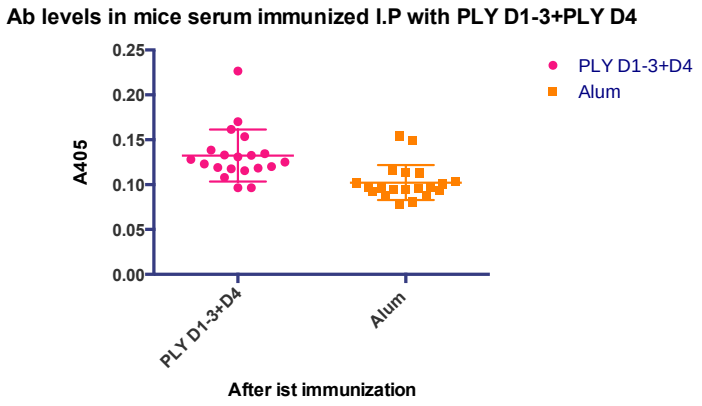


Fig 4.14 Antibody levels in mice serum immunised with PLY D1-3+D4 in alum and alum alone: The figure shows antibody levels after first immunization in the sera of two groups of MF1 mice where one group was immunised with PLY D1-3+D4 in alum and the other administered with alum alone (non-immunised group). Serum samples for individual mice sera were used in triplicate on the Elisa plate and the mean of three A405 readings for individual mice sera were plotted ($p<0.0001$).

The antibody levels in the serum of mice after second immunization in both the immunised and non-immunised groups of mice were compared. For this purpose, an Elisa was performed. As can be seen in the figure the levels of PLY antibodies in the group of mice immunised with PLY D1-3+D4 was significantly higher (P value 0.0001) to that of the non-immunized group treated with alum alone. The sum of means for mice immunised with PLY D1-3+D4 and non-immunised (PBS alone) was 14.52 and 2.43

respectively. The mean A405 reading for PLY antibody in mice serum immunised with PLY D1-3+D4 was 0.8695 while it was 0.2855 for the non-immunised mice treated with alum alone ($p<0.0001$).

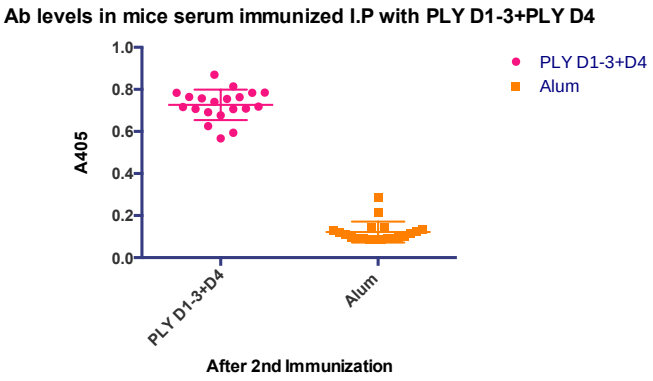


Fig4.15 Antibody levels in mice serum immunised with PLY D1-3+D4 in alum and non-immunised treated with alum alone: The figure shows antibody levels after the second immunization in the sera of two groups of MF1 mice where one group was treated with PLY D1-3+D4 in alum and the other non-immunised treated with alum alone. Serum samples were used in triplicate on the Elisa plate and the mean of three A405 readings for individual mice sera were plotted ($p<0.0001$).

Similarly, the antibody levels in the serum of mice after third immunization in both the immunised and non-immunised groups of mice were compared. For this purpose, an Elisa was performed. As can be seen in the figure 4.16 the levels of PLY antibodies in mice immunised with PLY D1-3+D4 was significantly higher (P value 0.0001) to that of the non-immunized group of mice treated with alum alone. The sum of means for mice immunised with PLY D1-3+D4 and non-immunised (PBS alone) was found to be 27.26 and 5.211 respectively. The mean A405 reading for PLY antibody in mice serum

immunised with PLY D1-3+D4 was 1.363 while it was 0.2605 for the non-immunised group of mice treated with alum alone ($p<0.0001$).

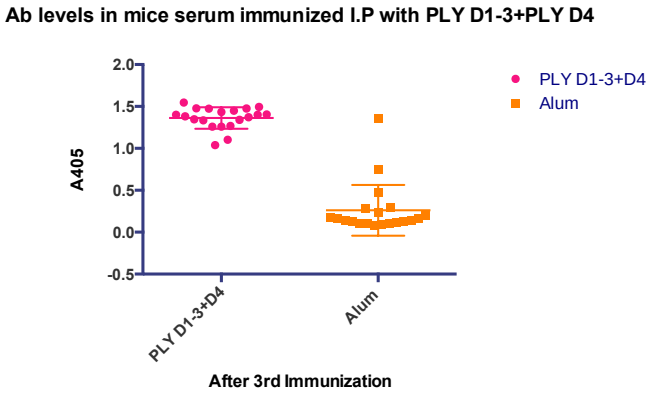


Fig 4.16 Antibody levels in mice serum immunised with PLY D1-3+D4 in alum and non-immunised group treated with alum alone: The figure shows antibody levels after third immunisation in the sera of two groups of MF1 mice where one group was immunised with PLY D1-3+D4 in alum and the other non-immunised treated with alum alone. Serum samples were used in triplicate on the Elisa plate and the mean of three A405 readings for individual mice sera were plotted ($p<0.0001$).

As described in 4.13 a bar chart was constructed. As can be seen in the figure 4.17 the level of PLY antibody is significantly different between the immunised and non-immunised group after each immunisation. The difference in the level of antibody developing in mice sera is more pronounced after second and third immunisations. The sum of means for mice immunised with PLY D1-3+D4 after first, second and third immunisation were 2.649, 14.52 and 27.26 respectively which shows increase in antibody level in the serum with each immunisation. Elisa for PLY antibodies in the sera for all the

three immunisations were performed simultaneously to observe the increase in the antibody titre.

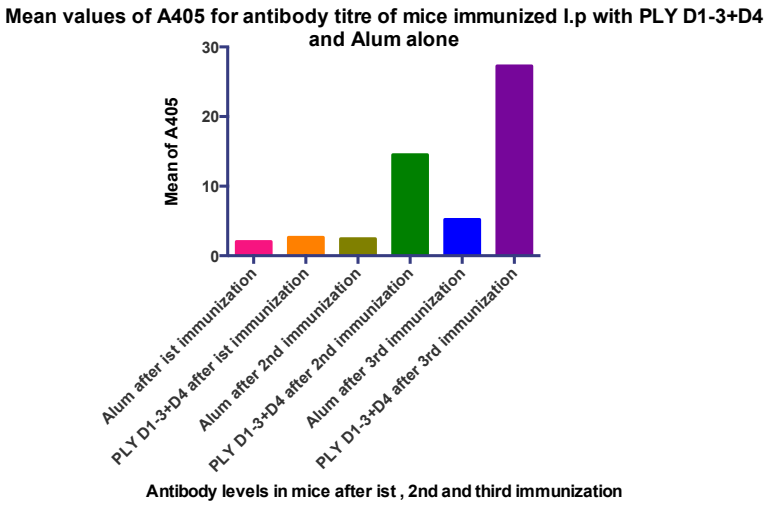


Fig 4.17 Antibody levels in mice serum immunised with PLY D1-3+D4 (in alum) and non-immunised treated with alum alone: The figure compares antibody levels in the serum of both groups of MF1 mice immunised with PLY D1-3+D4 and non-immunised with alum alone. Each bar in the figure is representing the sum of mean of A405 readings for a group of 20 mice after each immunisation.

Protective efficacy of Pneumolysin domain 1-3+ D4 as a mixture

The purpose of this experiment was to investigate if a mixture of D1-3 + domain 4 of pneumolysin confers protection against a standard lethal dose of *Streptococcus pneumoniae*. For this purpose, one group of mice were immunised via the intranasal route three times ten days apart with D1-3 +domain 4 of pneumolysin in PBS and the second group (Non Immunised) of mice treated with PBS alone. One month after the final immunisation mice were challenged with *Streptococcus pneumoniae*. The disease signs were monitored and the end point for each mouse was recorded as its survival time. As can be seen in figure 4.14 there was significant difference ($P > 0.05$) in the survival time of the immunised and non-immunised mice. The experiment proved the hypothesis about the mixture of domains of pneumolysin to be used as a vaccine as it provided protection against a standard dose of *streptococcus pneumoniae*.

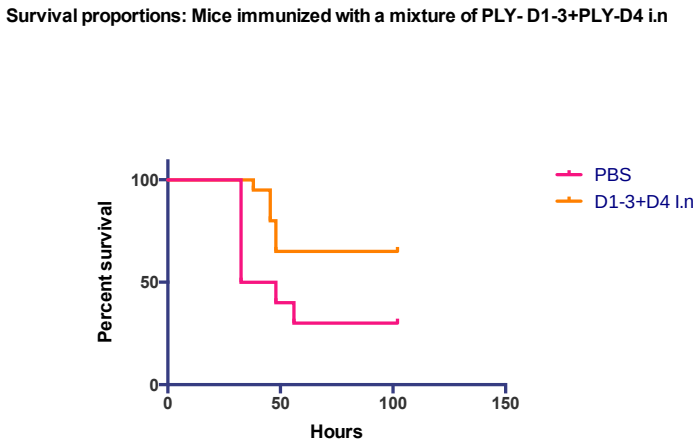


Fig 4.14 Survival curve for immunised and non-immunised mice challenged with a lethal dose of *S. pneumoniae* D39. 20 female MF1 mice were immunised three times with a mixture of PLY D1-3+domain 4 in PBS (---) and 20 female MF1 mice (Non immunised) treated with PBS alone (-) and finally one month after the third immunisation were challenged via the intranasal route with a dose of 2 X 10^6 *S. pneumoniae* D39. Significant difference between the survival times was observed as evident from the figure ($P > 0.05$).

Comparison of serum antibody levels between the immunised (with domain 4) and the non-immunised groups of mice: The purpose of this experiment was to check the antibody levels in the serum of mice. For this purpose, an Elisa was performed, as can be seen in the figure 4.15 the levels of antibodies to domain 4 in the serum of mice (immunised with PLY D4 in PBS) was significantly higher (P value 0.0001) to that of the control group (non immunised) treated with PBS alone. The sum of means for mice immunised with PLY D4 and non-immunised (PBS alone) was 7.36 and 1.64 respectively. The mean A405 reading for PLY D4 antibody in mice serum immunised with PLY D4 was 0.3683 while it was 0.0843 in non- immunised mice treated with PBS alone ($p<0.0001$).

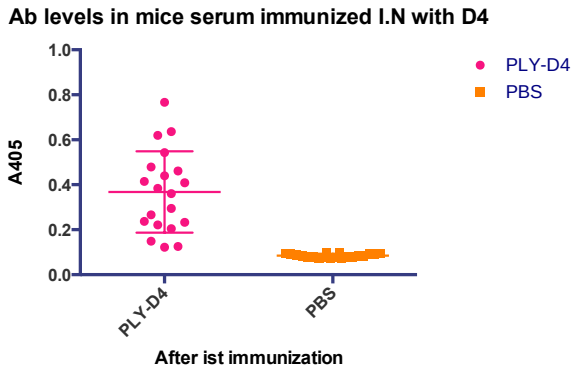


Fig4.15 Antibody levels in mice serum immunised with PLY D4 in PBS and PBS alone The figure shows antibody levels after first immunization in the sera of two groups of MF1 mice where one group was immunised with PLY D4 in PBS and the other non-immunised with PBS alone. Serum samples were used in triplicate on the Elisa plate and the mean of three A405 readings for individual mice sera were plotted ($p<0.0001$).

The antibody levels in the sera of mice after second immunization for both groups were compared. As can be seen in the figure 4.16 the levels of PLY D4 antibodies in mice

immunised with PLY D4 was significantly higher (P value 0.0001) to that of the non-immunized group treated with PBS alone. The sum of means for mice immunised with PLY D4 and non-immunised (PBS alone) was 22.31 and 3.5 respectively. The mean A405 reading for PLY D4 antibody in mice serum immunised with PLY D4 was 1.15 while it was 0.1780 for the non-immunised mice treated with PBS alone ($p<0.0001$).

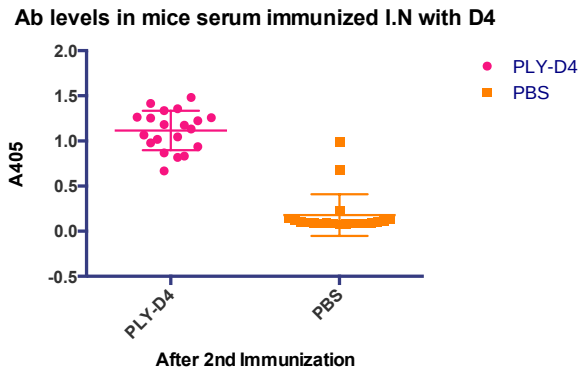


Fig4.16 Antibody levels in mice serum immunised with PLY D4 in PBS and PBS alone: The figure shows antibody levels after second immunization in the sera of two groups of MF1 mice where one group was immunised with PLY D4 in PBS and the other non-immunised treated with PBS alone. Serum samples were used in triplicate on the Elisa plate and the mean of three A405 readings for individual mice sera were plotted ($p<0.0001$).

Similarly, PLY-D4 antibodies level in mice sera of both groups i.e Immunised with PLY-D4 in PBS and non-immunised treated with PBS alone after 3rd immunisation was

compared. As can be seen in the figure 4.17 the levels of PLY D4 antibodies in mice sera immunised with PLY D4 was significantly higher ($p>0.0001$) to that of the non-immunised treated with PBS alone. The sum of means for mice immunised with PLY D4 and non-immunised (PBS alone) was 25.88 and 8.64 respectively. The mean A405 reading for PLY-D4 antibody in mice serum immunised with PLY D4 was 1.29 while it was 0.432 for the non-immunised mice treated with PBS alone ($p<0.0001$).

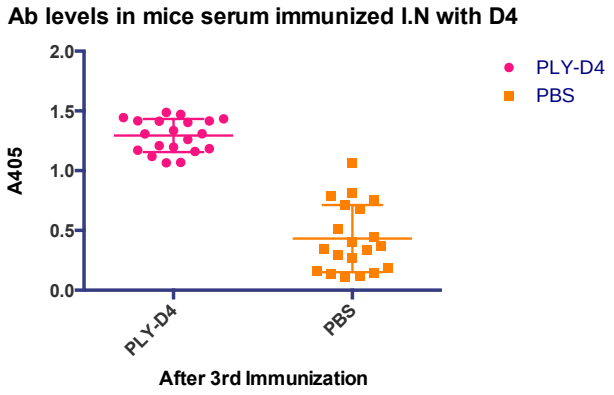


Fig 4.17 Antibody levels in mice serum immunised with PLY D4 in PBS and mice treated with PBS alone: The figure shows antibody levels after third immunization in the sera of two groups of MF1 mice where one group was immunised with PLY D4 in PBS and the other non-immunised treated with PBS alone. Serum samples were used in triplicate on the Elisa plate and the mean of three A405 readings for individual mice sera were plotted ($p<0.0001$).

A bar chart to was constructed to summarize the antibody levels in both groups after each immunisation. The sum of means of PLY-D4 antibody levels in both groups after each immunisation are represented as a single bar as labelled. As can be seen in the figure 4.18

the level of PLY-D4 antibody is significantly different between the immunised and non-immunised group after each immunisation. The difference in the level of antibody in mice sera is more pronounced after second and third immunisations. The sum of means for mice immunised with PLY D4 after first, second and third immunisation were 7.36, 22.31 and 25.88 respectively, which shows increase a boosting effect in antibody level in the serum with each immunisation. Elisa for PLY-D4 antibodies in the sera for all the three immunisations was performed simultaneously to observe the increase in the antibody titre.

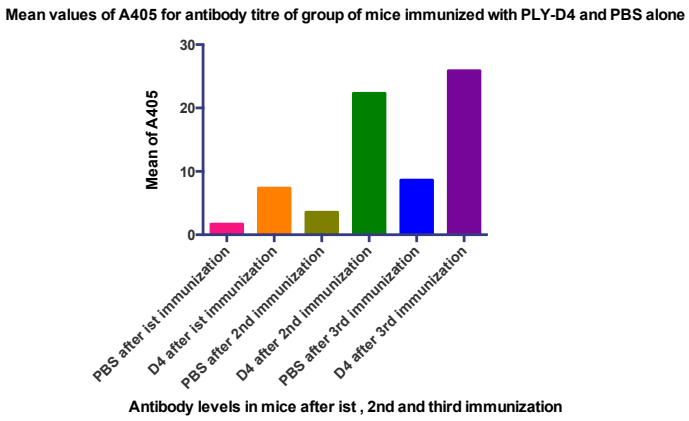


Fig 4.18 Antibody levels in mice serum immunised with MBP and MBP-D4 The figure compares antibody levels in the serum of both groups of MF1 mice immunised with PLY D4 and non-immunised treated with PBS alone. Each bar in the figure is representing the sum of means of A405 readings for a group of 20 mice after each immunisation.

Protective efficacy of domain 4 of pneumolysin

The purpose of this experiment was to investigate if domain 4 of pneumolysin confers protection against a challenge dose of *Streptococcus pneumoniae*. For this purpose, one

group of mice were immunised intranasally with domain 4 of pneumolysin in PBS and the second group (Non immunised) with PBS alone, as described in methods section. One month after the final immunisation mice were challenged with *Streptococcus pneumoniae*. The disease signs were monitored closely and the end point for each mouse was recorded as its survival time when it became severely lethargic. As can be seen in figure 4.19 there was no significant difference ($P > 0.05$) in the survival time between the immunised and non-immunised groups of mice. The median survival time for immunised group was 48 hours and the median survival time for non-immunised group was 40 hours. The experiment revealed that domain 4 of pneumolysin on its own can't provide protection against *streptococcus pneumoniae*.

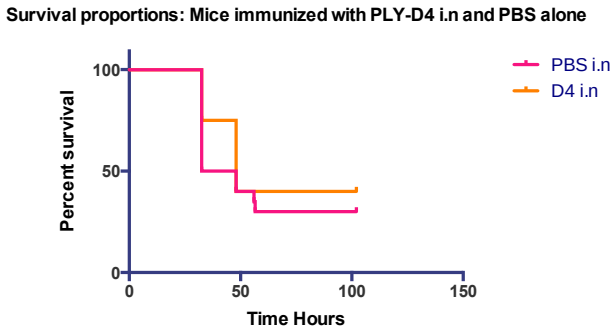


Fig 4.19 Survival curve of MF1 mice immunised with D4 and non-immunised (control group) challenged with *S. pneumoniae* D39. 20 female MF1 mice were immunised three times with PLY D4 in PBS (---) and 20 MF1 mice served as a control and were given PBS alone (-) before intranasal challenge with 2 X 10^6 *S. pneumoniae* D39. No significant difference between the survival curves was observed ($P > 0.05$).

Pneumolysin mutant W433F, its protective efficacy as a vaccine:

The purpose of this experiment was to investigate PdB toxin (PLY W433F) as a vaccine. The mutant pneumolysin W433F was generated via site directed mutagenesis, expressed and purified to homogeneity. It was then used to immunise mice three times ten days apart via the intraperitoneal route. The antibody levels in the serum of mice after each immunisation in both groups of mice i.e immunized and non-immunised mice were compared by Elisa. As can be seen in the figure 4.20 the levels of PLY antibodies in the serum of mice immunised with PLY W433F in alum and in the serum of non-immunised mice after first immunisation is not significantly different (P value 0.3789). The sum of means for mice immunised with PLY W433F and non-immunised (alum alone) was 2.43 and 2.29 respectively. The mean A405 reading for PLY antibody in mice serum immunised with PLY W433F was 0.1216 while it was 0.1149 for the non- immunised mice treated with alum alone ($p<0.3789$).

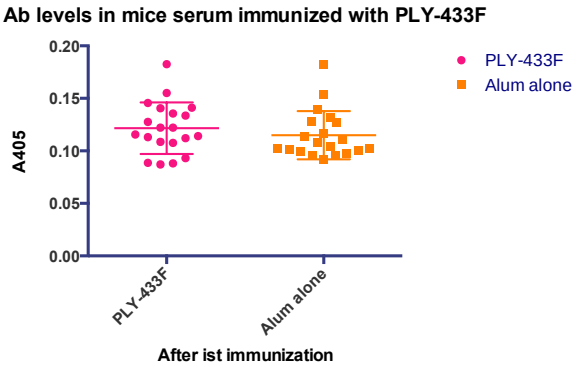


Fig 4.20 Antibody levels in mice serum immunised with PLY W433F in alum and non-immunised treated with alum alone The figure shows antibody levels after first immunisation in the sera of two groups of MF1 mice where one group was immunised intraperitoneally with PLY W433F in alum and the other non-immunised with alum alone. Serum samples were used in triplicate on the Elisa plate and the mean of three A405 readings for individual mice sera were plotted ($p<0.3789$).

To check the antibody levels in the serum for both groups of mice immunised (with PLY W433F) and non-immunised (alum alone) after second immunisation an Elisa was performed. As can be seen in the figure 4.21 the levels of PLY antibodies in the serum of mice immunised with PLY W433F in alum and in the serum of non-immunised mice is not significantly different (P value 0.7421). The sum of means for mice immunised with PLY W433F and non-immunised (alum alone) was 4.172 and 3.802 respectively. The mean A405 reading for PLY antibody in mice serum immunised with PLY W433F was 0.2086 while it was 0.1902 for the non-immunised mice with treated with alum alone ($p<0.7421$).

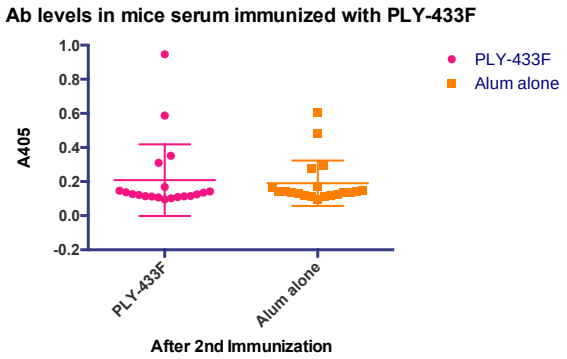


Fig 4.21 Antibody levels in mice serum immunised with PLY W433F in alum and non-immunised treated with alum alone: The figure shows antibody levels after second immunisation in the sera of two groups of MF1 mice where one group was immunised intraperitoneally with PLY W433F in alum and the other non-immunised treated with alum alone. Serum samples were used in triplicate on the Elisa plate and the mean of three A405 readings for individual mice sera were plotted ($p<0.3789$).

Similarly, to check the antibody levels in the serum of both groups of mice after third immunisation an Elisa was performed as described earlier and in methods. As can be seen in the figure 4.22 the levels of PLY antibodies in the serum of mice immunised with PLY W433F (in alum) and in the serum of non-immunised mice is not significantly different (P value 0.0887). The sum of means for mice immunised with PLY W433F and non-immunised (alum alone) was 5.83 and 3.72 respectively. The mean A405 reading for PLY antibody in mice serum immunised with PLY W433F was 0.2919 while it was 0.1861 for the non-immunised mice treated with alum alone ($p<0.0887$).

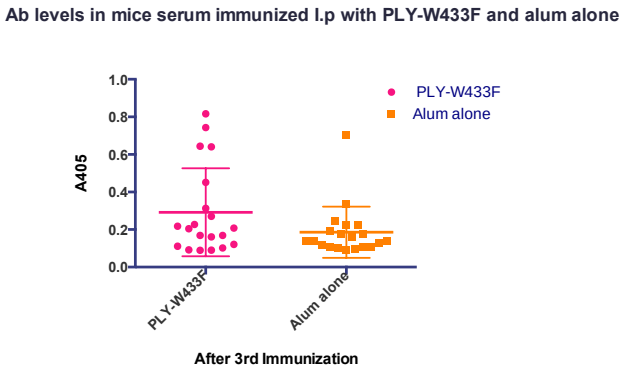


Fig4.23 Antibody levels in mice serum immunised with PLY W433F in alum and non-immunised treated with alum alone: The figure shows antibody levels after third immunisation in the sera of two groups of MF1 mice where one group was immunised intraperitoneally with PLY W433F (in alum) and the other non-immunised with alum alone. Serum samples were used in triplicate on the Elisa plate and the mean of three A405 readings for individual mice sera were plotted ($p<0.3789$).

A bar chart was constructed to summarize the antibody levels in both groups after each immunisation i.e first immunisation, second immunisation and third immunisation. The sums of means of PLY antibody levels in both groups after each immunisation are represented as a single bar and can be seen in the figure as labelled. As can be seen in the figure 4.24 the level of PLY antibody generated in mice is not significantly different between the immunised and non-immunised group after each immunisation. The level of antibody in mice sera is not increasing, in contrast to the expected even after second and third immunisation. The sum of means for mice immunised with PLY W433F after first, second and third immunisation were 2.43, 4.172 and 5.83 respectively. Elisa for PLY antibodies in the sera for all the three immunisations was performed simultaneously to observe the increase in the antibody titre.

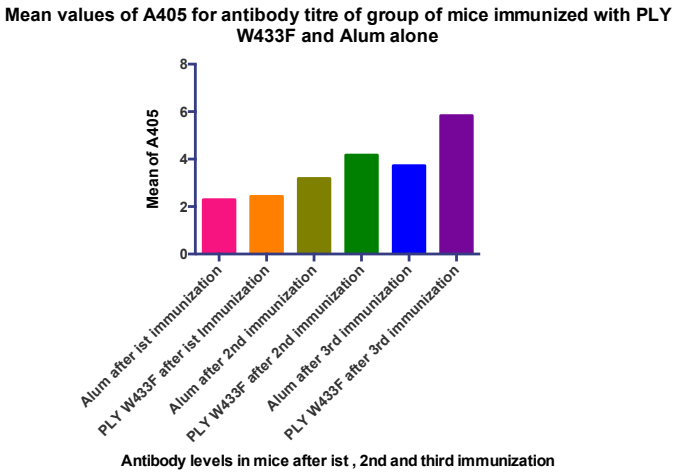


Fig 4.24 Antibody levels in mice serum immunised with PLY W433F (in alum) and non-immunised treated with alum alone: The figure compares antibody levels in the serum of both groups of MF1 mice immunised with PLY W433F and non-immunised with alum alone. Each bar in the figure is representing the sum of means of A405 readings for a group of 20 mice after each immunisation.

Protective efficacy of PLY W433F

The purpose of this experiment was to investigate and check PLY W433F as a positive control in immunisation experiment as it was proven to be immunogenic and protective

in mice when challenged with a dose of *Streptococcus pneumoniae* that is lethal to the non-immunised animals. For this purpose, one group of mice were immunised intraperitoneally with PLY W433F in alum and the second group (non immunised) with alum alone. One month after the final immunisation mice were challenged with *Streptococcus pneumoniae*. The disease signs were monitored closely for 4 days and the end point for each mouse was recorded as its survival time when it became severely lethargic. As can be seen in figure 4.25 there was no significant difference ($P > 0.05$) in the survival time between the immunised and non-immunised groups. The experiment did not work properly as shown by elisas for antiPLY antibodies. With such low levels of antibodies PLY W433F failed to protect mice against a lethal dose of *streptococcus pneumoniae*.

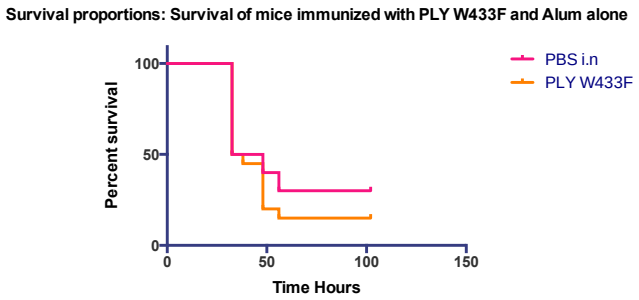


Fig4.25 Survival curve of MF1 mice after immunisation with PLY W433F and non immunised with alum alone after infection with *S. pneumoniae* D39. 20 female MF1 mice were immunised three times with PLY W433F in alum (---) or non immunised with alum alone (-) before intranasal challenge with 2 X 10^6 *S. pneumoniae* D39. There was no significant difference between the survival curves ($P > 0.05$).

CHAPTER 5

Discussion of structure to function studies

The use of mutagenesis of the pneumolysin gene to study the protein is decades old, e.g. it was with site directed mutagenesis that Saunders *et al.,* (1989) provided the evidence that the only Cys at position 428 in pneumolysin (PLY) is not responsible for the function of the toxin and therefore the term thiol activated cytolysin is a misnomer. Hill *et al.,* (1994) used random mutagenesis in order to identify amino acids crucial for the hemolytic activity of the toxin. By the use of insertion duplication mutagenesis in Pneumolysin it was found that the contribution of PLY to virulence is largely dependent on its hemolytic activity and not on its complement activating ability (Berry *et al.,* 1995). Paton *et al.,* (1991) used site directed mutagenesis to create the toxoids PdA and PdB (Paton *et al.,* 1991). There are other techniques available in the literature used for mutagenesis, such as a modification to the original mutagenesis primer design protocol where the non-overlapping sequence of the primer is extended at the 3' ends in both the forward and reverse primers to increase the Tm of the primer by 5 to 10^{o}C as a result of which the yield and efficiency of PCR and mutagenesis is greatly increased (Liu and Naismith, 2008), Ligation during amplification (LDA) in ciruclar DNA using sequence specific primer is a useful technique for site directed mutagenesis (Chen and Ruffner, 1998), a one tube site directed mutagenenesis using extending primers (Chapnik et al., 2008), inverse PCR based method where partially complimentary primers are used for deletion, insertion and replacement in the selected gene (Qi and Scholthof, 2008), Overlap extension PCR where overlaping 3' ends products are produced by internal primers and are then joined by flanking primers in subsequent PCR (Heckman and Pease, 2007) and site directed ligase independent mutagenesis (Chiu et al., 2004).

In this project site directed mutagenesis was applied to create mutants of pneumolysin for different purposes. The first purpose was to create a mutant of pneumolysin with a TEV protease cleavage site at the junction of domain 4 to the rest of the molecule so that after purification domain 4 of pneumolysin is isolated from the rest of the molecule. The isolation of domain 4 of pneumolysin was intended for immunisation studies. For this purpose, mutagenic primers carrying the desired mutations were designed. The sequence of the wild type pneumolysin (the presence of ply was first verified by PCR and then sequencing) from the plasmid pKK233-2:*ply* was used for designing the primers. using the sequence of pneumolysin gene from NCBI that carried the translation for TEV protease cleavage site (ENLYFQ/G) in the middle with 15bp of nucleotides on each side homologous to pneumolysin gene. Quick-change lightning site directed mutagenesis kit was used for this purpose as this method not only seemed simple and robust but multiple mutations could be inserted at a time. Briefly it required a PCR with the mutant complimentary primers carrying the desired mutations, followed by digestion with the Dpn1 enzyme to digest the parental methylated and hemi methylated DNA for 5 minutes to an hour at 37°C and finally transforming the mutagenesis reaction into chemical competent cells provided with the kit. Multiple attempts with primers carrying the sequence for the desired mutations for mutagenesis of pneumolysin gene in pKK 233-2:*ply* using this kit were not fruitful. To trouble shoot the problem the competence of the cells and efficiency of the Dpn1 enzyme was checked by transforming wild type plasmid, the transformation reaction was successful and the Dpn1 was efficient as no colony was observed after transforming the digested reaction into chemical competent cells. The apparent draw back of the method is that the DNA after its amplification is not visible on

agarose gel, let alone the digested PCR reaction with Dpn1. The manual of the kit advises to run 20ul of the PCR reaction on agarose gel and continues to say it may or may not be visible but nevertheless to proceed to the next step.

In the first few attempts the primers used were carrying all the desired 5 mutations (ENLFQ) as the amino acids Y and G were already present in pneumolysin at the desired place to complete the sequence for the protease cleavage site. The primers used with the kit had the optimal length of 42 and 39.5% GC content (kit requires 40%) but its melting temperature was 72°C while the kit advised 78°C. To design mutagenic primers according to the requirements of the mutagenesis kit having the desired Tm would require a 96 bp long primer for the intended region for mutagenesis. The idea of getting a new mutagenic primer having all the desired five mutations was abandoned as it would have potentially caused other problems like self-annealing, strong dimer and hair pin formations within a 96-mer oligo. Instead two new sets of mutagenic primers were designed, carrying 3 and 2 of the desired mutations respectively, in accordance with the requirement of the kit. Attempts with both of these new sets of primers also failed to produce the mutants. The increase of extension time from 30sec/Kb to 1 minute /kb for amplification and increasing the annealing temperature, as advised in the kit for trouble shooting, made no difference. The reasons for these failures are unclear but rather than seeking solutions it was decided that another approach should be taken.

The mutagenesis kit was changed to "XL site directed mutagenesis kit" from the same manufacturer was previously used in the lab for successful mutagenesis. New primers were designed for this kit carrying one mutation at a time. The protease cleavage site was constructed with site directed mutagenesis in 5 steps by introducing one mutation at a

time. The stepwise mutagenic primers are given in Table 1 of the methods. An alternative strategy to create the mutant by extension PCR was also applied. Briefly, the sequence for domain 1-3 and domain 4 were amplified with two sets of primers wherein the internal primers had some homologous sequences (namely, the overhangs carrying the sequence for the protease cleavage site) and finally the two PCR products were joined together with the external primers (which in this case were ply forward and ply reverse) as described before (Heckman and Pease, 2007). The final product was gel purified and cloned into the pLEICS-01 vector, sequencing results confirmed that both the approaches were successful in creating the desired mutant. Thus although the approach of introducing the five mutations one at a time was successful, the method of overlap extension proved to be more acceptable because of the saving of time.

Unfortunately, in spite of the successful mutagenesis to insert the cleavage sequence, the pneumolysin TEV mutant was abandoned after several attempts of expression failed because the solubility of the protein was very poor, most probably due to improper folding.

In retrospect, the introduction of 5 mutations at an important region for the structure and function may have been considered ambitious, given how important this region could be for the overall structure, function and conformity of the toxin. The final yield of the purified protein was low and when checked in a hemolytic assay for its activity, it was 90 times lower than the wild type toxin. The exact hemolytic activity was not determined as the purified toxin despite the low yield had co contaminants. The presence of co contaminants in the purified fractions can be explained by the high binding capacity of

the Ni column. Other strategies were applied to obtain domain 4 of pneumolysin and these are discussed in a later discussion section.

The second set of mutants were produced that contained pairs of CYS residues at specific sites in pneumolysin. This was done in order to lock different parts of the pneumolysin molecule with the intention of preventing them moving apart during pore formation. This was done to arrest the pore formation process at intermediate stages. The amino acids that were replaced by CYS in pneumolysin were K34, E35, T55, V163, A262, W278 and G388. Four mutants of pneumolysin (each carrying two mutations) were created by site directed mutagenesis. The four sets of mutants included; T55C + V163C (intended to lock domain 2 to TMH1 of domain 3 of pneumolysin), K34C + G388C, E35 + G388C (intended to create a disulphide bridge between domain 2 and domain 4) and A262 + W278C (intended to lock TMH2). The prediction of these residues was based on the homology model of pneumolysin (plyB homology model) (Rossjohn et al., 1998) as no structure was available for pneumolysin itself. Rossjohn et al., (1998) presented the homology model for pneumolysin in its monomeric and oligomeric form. Based on this model and a pdb file for plyB, pneumolysin was mutated *in silico* using the software macpymol to estimate the distance, in angstroms, between the respective CYS residues. The predicted structures for the intended mutations are shown below (Fig 5.2 -5.4).

Distance between T55C + V163C

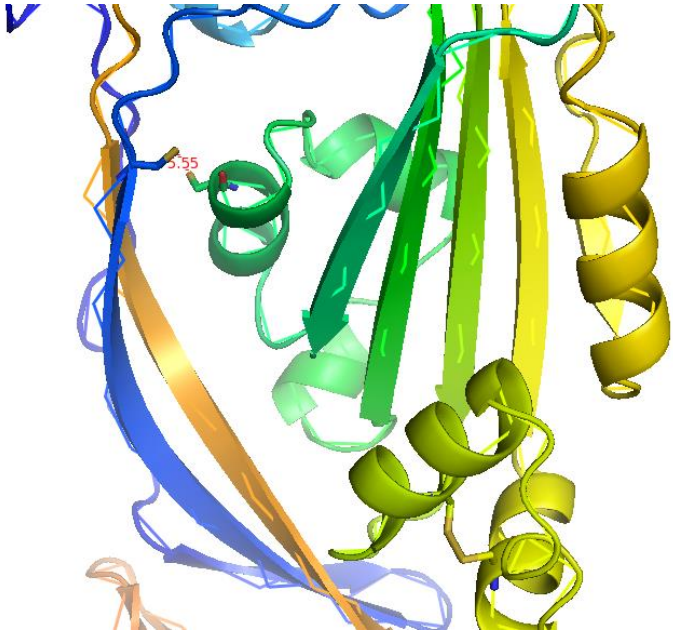


Fig 5.1 Distance between T55C: V163C : The distance between the Sulphers of C55 and C163 is 5.5A° as calculated on the basis of the homology model of PLY.

Distance between E35C : G388C

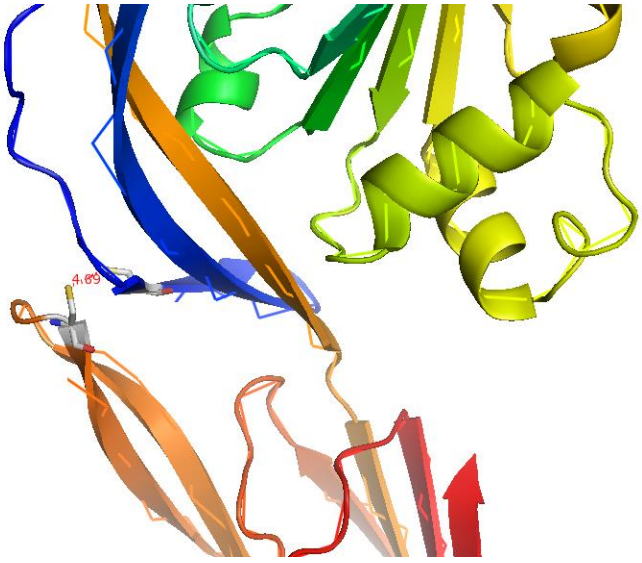


Fig 5.2

Distance between E35C : G388C : The distance between the Sulphers of C35 and C388 is 4.89A° as calculated on the basis of the homology model of PLY.

Distance between K34C : G388C

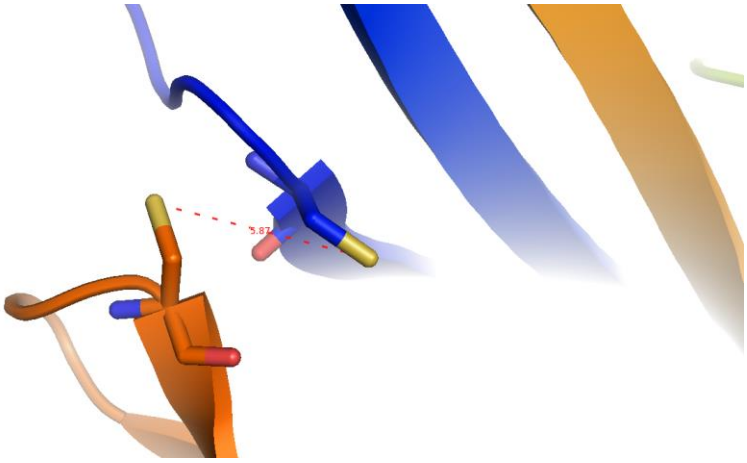


Fig 5.3 Distance between K34C : G388C : The distance between the Sulphers of C34 and C388 is 5.89A° as calculated on the basis of homology model of PLY .

Distance between A262C : W278C

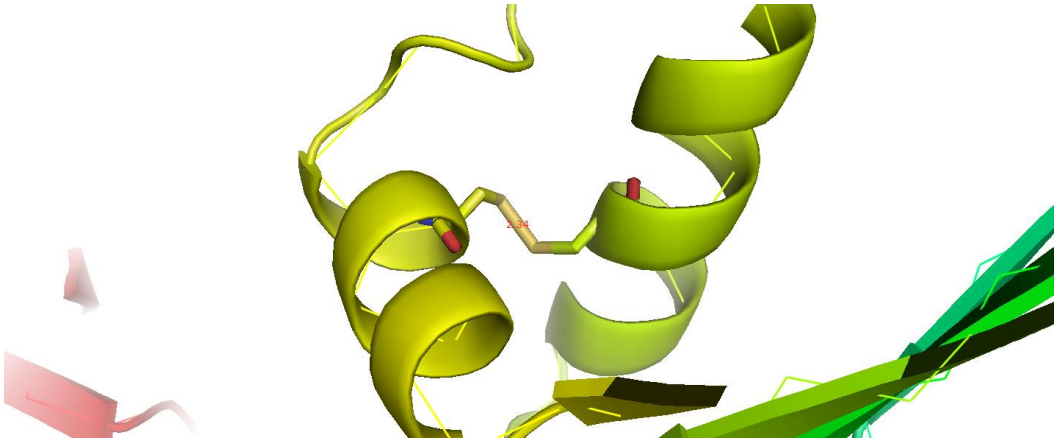


Fig 5.4. The distance between A262C : W278C : The distance between the sulphers of C262 and C278 is 2.34A° as calculated on the basis of homology model of PLY.

The purpose of creating these mutants was to understand the mechanism of action of pneumolysin as described in a paper by (Tilley et al., 2005). To elucidate it further the mechanism of action of pneumolysin is briefly explained. The first step is contact of pneumolysin with the host cell. Pneumolysin comes into contact with the host cell via its domain 4, which contains the important Tryptophan residues thought to be involved in contact with the host cell membrane. It is believed that part of domain 4 inserts into the host cell membrane and is involved with contacts to the neighbouring molecules, thus helping in the process of oligomerization. However, a study with perfringolysin demonstrated that domain 4 has limited contact only at its tip with the host cell membrane while the rest of the domain lies away (Shepard et al., 1998, Ramachandran et al., 2002). The role of domain 4 of pneumolysin might be that it anchors the molecule into the host cell membrane and acts as a fulcrum to the rest of the changing molecule. Upon contact, the monomers starts interacting and oligomerize to form a Prepore

assembly (Shepard et al., 2000). Pneumolysin in the prepore state may contain 38-44 monomers. Oligomerization is followed by some astonishing changes to convert the prepore form of the bound toxin to puncture the host cell membrane. This involves an overall change in the conformation of the toxin; the trigger for these changes is not understood. These changes include the bending of domain 2 into half bringing domain 1 and 4 closer. Two of our mutants (K34C+G388C, E35C+G388C) were intended to lock domain 2 to domain 4 via a disulphide bond so that the toxin can be trapped in this state before any conformation changes occur. Releasing the toxin then would have been possible by reducing the disulphide bond by adding DTT. The next change that occurs during the conversion of prepore form to the pore form is the radical change in the helices on each side of domain 3 into extended beta hair pins structure that finally traverse and goes into the host cell membrane to punch a hole. The pore formed by pneumolysin may contain up to 176 □ strands, four from each monomer (Gilbert et al., 2000, Heuck et al., 2000, Gilbert et al., 1999, Tilley et al., 2005).

Another of the created mutant (T55C+V163C) was intended to lock TMH1 of domain 3 to domain 2 of pneumolysin, while the other mutant (A262C+W278C) was intended to lock TMH2. The hypothesis behind the predicted disulphide bond between these residues was that if successful it would trap the toxin and help to delineate the mechanism of pore formation. The inactivity of the toxin will be attributed to the disulphide bond once it became active after reduction with DTT or other reducing agents.

The mutants were successfully created and purified. The first test to check the mutants was to determine their hemolytic activities under reducing and native conditions to see if there is any difference and thus confirm the formation of the predicted disulphide bond.

The first mutant (PLY C34C388) intended to create a disulphide bond between domain 2 and domain 4 was fully active both in natural and reducing conditions. This might be due to two reason either the intended to be locked domains did not happen or another possible explanation and reason could be that the distances between the residues and their relative positions were based on the homology model of PFO which in reality might be different. The mutant PLY C55C163 had lost 50 percent of activity as compared to the wild type toxin in the reduced and oxidized form. The formation of disulphide bond could not be confirmed as there was no difference between the hemolytic activities of the toxin in native condition and after DTT was added. The fourth mutant, intended to block the beta hairpin insertion into the membrane, had greatly reduced activity in the oxidized state (activity of 6.25% of the wild type toxin). It was the only of our four mutants where we could confirm the formation of the disulphide bond with a hemolytic assay. When DTT was added to reduce the disulphide bond the mutant fully regained its hemolytic activity. This result confirmed that the experimental approach is sound. This mutant confirms that during the pore forming mechanism the alpha helices in the domain 3 (TMH2) undergo movement, consistent with the model that they unfold and insert into the host cell membrane as beta hairpins. The 6% residual activity of this toxin could be due to the insertion of the TMH1 alone.

The third set of mutants were intended to study the role of different amino acids in the oligomerization of pneumolysin on the host cell membrane. A structural model of pneumolysin helped to identify some residues in domain 1 of pneumolysin, which were thought to be involved in contacts between two monomers during oligomerization of the toxin (Jamie Marshall, personal communication). These candidate residues were K17,

K18, D79, E80 and E84. Considering two PLY molecules in an oligomer and given the position of these residues there seemed every chance that they might interact, especially K18 with E84. Theoretically, the mutation of these residues will disrupt the contact to the adjacent monomer and will render the toxin inactive. The 1st mutant produced for this purpose was K17A. The purified protein was checked for the level of its hemolytic activity because the hemolytic activity of the toxin is a good way to check oligomerization as it is the step preceding pore formation. If oligomerization is inhibited it should result in no hemolytic activity or reduced hemolytic activity. The mutant D79Q had a reduced reduction in activity and was about 80HU as compared to 25160 HU of the wild type toxin. However, looking into the crystal structure of the toxin D79 is not the ideal candidate to form contact with residues in the neighboring monomers. The structure suggests two residues K18 and E84 to have more chance of contacts, therefore a double mutant K18A+E84A was created which is inactive when checked for hemolysis however one cannot be sure as further studies are warranted to establish that the reasons for inactivity of this mutant is the loss of oligomerization. One method to confirm if these two residues are involved in oligomerization would be to mutate both of these to CYS, if they were to come in contact the two CYS would form a disulphide bond and the toxin will be active but once some reducing agent like DTT is added the disulphide will break thus releasing the monomers and hence the toxin will become inactive.

CHAPTER 6

Discussion of immunisation studies

Pneumolysin (PLY), the major protein toxin by *Streptococcus pneumoniae* is not only cytotoxic but also is capable of activating the complement system. The genetic variability of PLY genes across different serotypes of pneumococcus is very limited. This protein is considered as a potential vaccine candidate. For inclusion in a vaccine, a nontoxic form of pneumolysin is required which conserves the protective immunity of the wild type toxin. Alexander *et al.*, (1994) made single and double mutants of PLY (PdB and PdBD) with reduced toxicity using site directed mutagenesis. They made a point mutation in the tryptophan rich loop of domain 4 of PLY derived from serotype 2 pneumococci and termed the mutant toxin as PdB. They purified the PdB toxoid and found that the mutation W433F has significantly compromised the hemolytic activity of the toxin. They immunised 6-8 week old female MF1 mice with the purified PdB toxoid and finally challenged the mice with *S. pneumoniae* D39 at a dose of $1X10^6$ in 50ul. Immunisation with PdB was protective and 72% of the mice immunised with PdB survived in contrast to 6% of the control non-immunised mice following acute challenge. The antibody titres in the PdB immunised mice were significantly higher to those of the non-immunised mice after two immunisations. These antibodies were functional and were able to block PLY haemolytic activity *in vitro*. Alexander *et al.*, (1994) showed that the antibodies as a result of PdB immunisation were able to protect against the tested 9 different serotypes of pneumococci in mice. PdB immunised mice were also protected from intraperitoneal

challenge against 9 different serotypes of pneumococci. The protection offered by PdB against different serotypes may be because of the low genetic variation (3.3%) of pneumolysin in pneumococcal strains tested so far (Marriot *et al.*, 2008).

Berry *et al.,* (1995) used insertion duplication mutagenesis to incorporate mutations in the chromosome of strain D39, the introduced point mutations were targeted to effect the cytoxicity of pneumolysin, the complement activating ability of pneumolysin or both at the same time. The mutants created were with single mutation of Asp385Asn, His367Arg, Trp433Phe, a mutant with double mutations (Trp433Phe Cys428Gly) and a mutant carrying three mutations (Trp433Phe, Cys428Gly and Asp385Asn) in the pneumolysin gene. Mice were challenged to compare the virulence of these mutant strains in an intra peritoneal challenge model. No significant difference between the median survival time or overall survival rate was found between mice challenged with D39 expressing wild type pneumolysin and mutant D39 with the point mutation Asp385Asn in the pneumolysin gene. The mutation Asp385Asn abolishes the complement activating ability of pneumolysin. However, the mutant strains wherein the mutations were aimed at reducing the cytotoxicity of pneumolysin had significantly greater median survival time and overall survival rate than mice challenged with strain D39 expressing wild type pneumolysin. In the same study they challenged mice with the D39 mutant having three point mutations (Trp433Phe, Cys428Gly and Asp385Asn) and another mutant having two point mutations (Trp433Phe and Cys428Gly) in the pneumolysin gene and found no difference in their virulence. They concluded that the contribution of pneumolysin to the virulence is associated with its hemolytic activity (cytotoxicity) and not with its complement activating ability.

Berry *et al.*, (1999) deleted a portion of pneumolysin gene encoding for aminoacids 55-437 in type 2 *S. pneumoniae* and compared the virulence of this strain to strain D39 expressing wild type pneumolysin, PLN-A (an insertion duplication mutant) and another mutant strain (carrying PdT) with triple mutations in its pneumolysin gene. They found intermediate virulence for the strain carrying PdT and concluded that the toxin must have some additional properties that are not abolished by the point mutations, which reduces the cytotoxicity and complement activation to virtually undetectable levels. PdT is less toxic than the wild type pneumolysin but it carries residual toxicity, the intermediate virulence level found for this derivative strain might have been due the residual toxicity of PdT. Dorton *et al.,* (2000) conducted a study to determine and compare the toxic profiles of PdB, PdBD and pneumolysin itself in a single and repeated dose studies. In their single dose studies they administered pneumolysin via the intravenous (IV) route to a group of 4 male rats and found that LD50 is 30-45ug/Kg of bodyweight for WT pneumolysin while 300ug/Kg for both the mutant toxins. Additional detoxification of the mutants with formaldehyde increased the LD50 to 900ug/Kg of body weight. Interestingly they found no difference in the suspected primary targets of pneumolysin, namely blood cells and endothelium, between animals treated with WT pneumolysin and animals treated with mutants with reduced toxicity after a single dose administration. In a repeated dose toxicity study where male and female animals were treated once a week for five consecutive weeks, it was found that pneumolysin had no effects on haemotology, clinical chemistry, organ weight, bone marrow cell counts and immunological assays. Increased IgM levels in the serum of only female mice was found after high dose of sub cutaneous treatment. Increase in the number of B-cells in the spleen was found by FACS

analysis in animals which were sub cutaneously treated and relative and absolute decrease in T-cells counts after both intravenous and sub cutaneous treatment of animals with pneumolyin. Pneumolysin was found to have caused major damage to the kidney i.e glomerular as well as tubular. Female rats were found to be more susceptible to kidney damage as compared to male rats. The *in vivo* toxicity of PdB as determined by Dorton *et al.,* (2000) was 10% of the wild type toxin, which is not agreement with the *in vitro* toxic profile of PdB where it is 1% of the wild type pneumolysin. From this study they concluded that the toxic effects of pneumolysin and the mutant toxins may be due to membrane toxicity of erythrocytes and blood vessels and advised that if any derivative of pneumolysin is used in a vaccine it should be non-toxic and preserve the immunogenicity of the wild type toxin.

Basset *et a*l., (2007) showed PdT to be a protective antigen against nasopharyngeal colonization. They immunised inbred C57BL/6J mice (susceptible to pneumococcal infection) intranasally with purified PdT in combination with PspA and PspC followed by challenge with a colonisation dose of serotype 6B. The results of this study indicated a significant decrease in the number of pnueumococci recovered from the nasopharynx in mice immunised with all the three proteins. They also demonstrated the important role played by $CD4^{+}$T cells in protection from nasopharyngeal colonisation. $CD4^{+}$T cells antibody was administered before the challenge in mice to both PdB immunised and non-immunised groups. The results of this study indicated that mice with depleted $CD4^{+}$T cells lost the protection previously seen with $CD4^{+}$T cells as similar numbers of pneumococci were recovered from the nasopharynges. The antibodies produced as a result of immunisation with PdB were functional as they were capable of inhibiting

hemolytic activity of pneumolysin showing the importance of $CD4^+$T cells (Basset *et a*l., 2007).

Immunisations with PdB elicits antibody response that is capable of decreasing the number of pneumococci in the nasopharynx, the specific anti PdB antibodies are also capable of neutralising the pore forming activity of the toxin *in vitro*. Inhibition of the hemolytic activity of the toxin is very important as the specific antibodies will bind to any toxin released on autolysis of *Streptococcus pneumoniae* (as previously believed) and also it will bind to any pneumolysin that might be present on the surface of the bacterium as found by Price and Camilli (2009). This binding of antibody to pneumolysin may prevent many immunomodualtory actions. It may even prevent pneumolysin from halting of the cilia which in turn may effect colonisation of the bacterium i.e movement of the epithelium may make it difficult for the bacteria to adhere to the surface. The anti pneumolysin antibodies may also stop disruption of the cell tight junctions which in turn will affect the invasion of epithelial cell layers. These antibodies can inhibit neutrophil respiratory burst which is an important factor if immunity against *Streptococcus pneumoniae* is mediated by $CD4^+$T cells as the response depends upon the presence of neutrophils.

The production of pneumolysin by different strains of *Streptococcus pneumoniae* is not constant and varies among different serotypes. Benton *et al*., (1997) showed that strain A66 produces 5 times less cytoplasmic pneumolysin while 8 times more extracellular pneumolysin as compared to strain D39. In the pneumococcal murine model of infection, it has been demonstrated that production of different amounts of pneumolysin is not of particular importance as the pneumococcal strains producing less amounts of

pneumolysin have the same growth characteristics and generate the same inflammatory response by the host. The role played by pneumolysin in colonisation is controversial as some studies suggest that pneumolysin has no role in colonisation of the upper respiratory tract.

Rubins *et al.,* (1998) showed that a serotype 14 strain deficient in pneumolysin has the same ability to colonise the nasopharynx as that of the wild type strain. Kiadioglu *et al*., (2002) demonstrated that serotype 3, strain A66 deficient in pneumolysin, colonised the nasopharynx in a similar fashion to that of the wild type strain, which was expressing pneumolysin. However, PLN-A, a serotype 2 D39 strain (deficient in pneumolysin) when tested failed to colonise the nasopharynx. The differences in these results of colonisation ability of different serotypes may infer to the fact that they possess different capsular polysaccharides. The role of pneumolysin in colonisation may be dependent on the type of capsular polysaccharide or it may imply the importance of pneumolysin for colonisation in some serotypes while not in others. There is not enough data available regarding the importance of pneumolysin in colonisation in different serotypes of *Streptococcus pneumoniae* to conclude about it.

Briles *et al*., (2003) subcutaneously immunised 6-8 weeks old CBA/N mice with 20ug of PdB three times fourteen days apart. Mice were challenged 14 days after the last immunisation intravenously with D39. The median survival time recorded was 72 hours however mice when challenged with serotype 6B the median survival time for the PdB immunised mice increased to 8 days. These results showed that PdB was unable to prevent sepsis and death when challenged with D39 but is able to increase the survival time when serotype 6B is used as a challenge dose. In this study they also demonstrated

that PspA is more effective in preventing sepsis of mice following intravenous challenge than does PdB (Briles *et al.,* 2003). Musher *et al.,* (2001) showed that mice immunised with anti-PLY antibodies had protection against intravenous challenge of serotype 4 pneumococci. The authors of the study went on to suggest that the anti pneumolysin antibodies are protective for as long as the host start producing anti capsular antibodies and proposed that both protein specific and capsular specific antibodies are important for protection against pneumococcal sepsis. Until 2001 it was commonly believed that pneumolysin, having no signal peptide, is released on autolysis of the cell but Price and Camilli in 2009 showed pneumolysin to be present attached to the cell surface (Price and Camilli, 2009). Pneumolysin on the cell surface may facilitate opsonophagocytosis and it might have been the reason of the protective response as seen by Musher and colleagues (2001) in their study (Musher *et al.,* 2001).

Zhang *et al.,* (2002) showed that PdB is not efficient as a vaccine to stimulate mucosal immunity in humans. PdB was able to stimulate the release of secretory IgA in human adenoid B cells in only 2 out of eight patients. On the other hand Simell *et al.,* (2001) found anti PLY antibodies and secretary IgA in the saliva of children after acute otitis media (AOM) infection (Simell *et al.,* 2001).

The objective of our study was to use a nontoxic derivative of pneumolysin (as an immunogen) which is an important virulence factor of *S. pneumoniae* and a known protective antigen when used for immunisation in animal models. As discussed earlier other derivatives of pneumolysin, although less toxic than the wild type pneumolysin still

carried some residual toxicity. This residual toxicity makes them unsuitable to be used in a vaccine. Looking into the structure and function of pneumolysin and the available literature, which highlights the importance of domain 4 of pneumolysin i.e domain 4 is responsible for binding of the toxin to the host cell membrane and its role in the complement activation, it was used as an immunogen in our immunisation studies to see if domain 4 of pneumolysin can work as a protective antigen against a lethal dose of *Streptococcus pneumoniae* as theoretically anti domain 4 antibodies should stop pneumolysin from binding to the host cell and therefore stop it from the toxic effects it can have on the host.

As described in the methods, and later can be seen in the results, to obtain domain 4 of pneumolysin different strategies were applied. The first strategy was to put a protease cleavage site at the junction where domain 4 is attached to the rest of the molecule with a single exposed polypeptide. Looking into the available literature, different options of putting a protease cleavage site were available. It could have been a cleavage site for one of a common protease, for example Thrombin, Factor Xa and Enterokinase, but none of these options were employed. The reasons for not utilizing one of these options were that these proteases are not accurate in cleaving, digests may be incomplete, high concentration of the protease is required and are expensive (Jenny *et al.*, 2003, Blommel and Fox, 2005). The other available options for putting a protease cleavage site were for Tobacco ETCH virus protease (TEV protease) and Tobacco vein molting virus protease (TVMV protease). These two viral proteases are closely related and are very efficient in cleaving its target sequence. Out of these two options, TEV protease was selected as it is

specific in cleavage, is efficient, active in various buffers, active at low temperatures and has no P2 specificity except it cannot be Pro (Kapust *et al.,* 2002).

Although, TVMV protease is better in terms of the properties of not auto inactivating and it does not self-cleave at higher concentrations to which the wild type TEV protease is prone but a mutant TEV protease does not auto inactivate or self-cleave (Kapust *et al.*, 2001). The advantage of TEV protease is that it has best results if a protease cleavage site is placed between two domains of a protein as in this project (Nallamsetty *et al.,* 2004, Fang *et al.,* 2007). Furthermore, TEV protease was readily available at low cost from PROTEX Leicester. The TEV protease site ENLYFQ/G was constructed as desired by mutating amino acids at the junction of domain 4 to the rest of the molecule in pneumolysin by site directed mutagenesis, as described in the methods. The mutant pneumolysin (PLY TEV mutant) was cloned into pLEICS-01 and pLEICS-03 vectors carrying N-his and C-his terminal tags for purification. The choice of His tag for purification was based on; its wide use for the purification of proteins since its first description by Hochuli et al., 1987, His tag is relatively smaller in size and charge ensures that the protein activity is least effected. Another reason was the simple method of IMAC purification as His interacts with an immobilized metal ion on (Cu^{2+}, Co^{2+}, Ni^{2+} or Zinc) matrices via its imidazole ring. The option of utilizing both terminal tags was based on the fact that the optimal placement of the tag is protein specific and there are no current rules to decide the end of putting the tag (Terpe, 2003). The mutations at the hinge region (at the junction of domain 4 to the rest of the molecule) effected the solubility and may have effected the proper folding of the toxin as the final yield per litre of culture was only 200ug of PLY TEV mutant.

The concern it raised was about the final yield of domain 4 of pneumolysin after cleavage of PLY TEV mutant with TEV protease. The purified TEV mutant when checked for its hemolytic activity showed reduction in activity as compared to the wild type toxin. Theoretically, under ideal conditions the final yield of domain 4 of pneumolysin, taking into consideration its molecular weight, would have been about 50ug/litre. Practically it was not feasible to get the required amounts of domain 4 of pneumolysin as the protease cannot have 100% efficiency and furthermore, passing it through the gel filtration column for its separation would have resulted in some loss of protein. To purify PLY TEV mutant from inclusion bodies will be discussed later in this chapter.

The second strategy was to clone domain 4 of pneumolysin into a suitable vector for expression and purification. First D4 of pneumolysin was cloned in frame with the HA coding sequence of pE.coli-Nterm 6xHN vector from Clontech. This vector is based on the pET system and the expression of protein is tightly regulated. The vector comes in a linear form and gets circularised after successful ligation of the insert. It carries an N-terminal 6HisAsn tag, which can be subsequently removed after purification by utilizing the enterokinase cleavage site. Several attempts to express and purify domain 4 of pneumolysin were not fruitful with this construct therefore we had to change the vector. Domain 4 part of pneumolysin gene was cloned into pLEICS-01 vector from PROTEX Leicester with a C-terminal His tags. The choice of pLEICS-01 vectors was made because it was not only cost effective but also gave us the option to put and check if the C-terminal his tag helps in the expression of domain 4 of pneumolysin.

It did help to an extent by expressing domain 4 of pneumolysin but the yield per litre was very low and the purified protein contained a lot of co-contaminants. Furthermore, domain 4 of pneumolysin was highly unstable and was prone to precipitation. First this problem was thought be associated with the presence of imidazole in the purified fractions. To rule out this possibility and provide a possible solution the expression and purification was repeated, and by exchanging the buffer several times the protein was desalted. The problem still persisted, looking into the available literature, domain four of pneumolysin was previously expressed and purified by Baba (Baba *et al.*, 2001). They were contacted about the construct and asked for any possible solution. Their information was that they had faced the same problem of instability but rather than solve this problem they did the required assays as soon after its purification as possible (Personnel communication).

The problem of proteins expressed in *E.coli* as inclusion bodies is not new, although it's not desirable. Proteins expressed as inclusion bodies are not bioactive molecules, however these bodies have certain advantages where the activity of the protein is not required; expression in high level, easy isolation, resistant to proteases of the host, less contaminants and a straight forward purification (Clark, 1998).

Available literature (Tao *et al.*, 2010, Singh and Panda, 2005, Vallejo and Rinas, 2004) suggests different methods to purify proteins from inclusion bodies and to make them soluble and refold but all of these methods are tedious, involve denaturation with high concentration of urea which may already have or can produce cyanate, guanidinium chloride, addition of reducing agents like DTT or beta merceptoethanol, time consuming and addition of certain detergents like sarkosyl, triton X100 and chaps. The chances of

getting correctly folded proteins from inclusion bodies might be very low (Tao *et al.*, 2010, Singh and Panda, 2005, Vallejo and Rinas, 2004). While the purification of inclusion bodies is simple and robust, the refolding part is challenging and demanding, it is not straight forward and at the end the purified proteins may aggregate and not fold properly (Vallejo and Rinas, 2004). To tackle this problem there are other robust ways to make the protein soluble. One of such strategy is to express the protein fused to a highly soluble protein, which can work as a solubility enhancer. One such high solubility enhancer is the N-terminal fragment of translation initiation factor (IF2), which is 17KDa in size and after expression can be purified via nickel affinity chromatography (Sørensen *et al.*, 2003). Maltose binding protein (MBP) and NusA (N utilizing substance A) are one of the most powerful solubility enhancers to date. MBP (40KDa) and Nus A (54.8KDa) are suitable for proteins that tend to form inclusion bodies. Maltose binding protein (MBP) is a highly soluble *E.coli* protein and is used as a solubility enhancer (Fox and Waugh, 2003, Kapust and Waugh, 1999). The mechanism by which MBP acts as a solubility enhancer is not understood but it may act as a chaperone (Kapust and Waugh, 1999). The disadvantage with MBP is that it does not always bind efficiently to the amylose resin but by including an extra His tag at the C-terminus of the passenger protein can solve this problem (Waugh, 2005). Among these solubility enhancers MBP was selected for fusion with domain 4 of pneumolysin as it is well characterized and is neither too large nor too small. To fuse domain 4 of pneumolysin to an N-terminal MBP it was cloned into the vector pLEICS-10. Given the size of domain 4 of pneumolysin (13KDa) it was the ideal candidate to be expressed as a fusion partner with MBP.

Expression of domain 4 of pneumolysin fused to MBP turned out to be the method of choice and after purification with amylose resin (from NEB) domain 4 of pneumolysin was cleaved from its fusion partner efficiently with TEV protease utilizing the cleavage site between MBP and domain 4 of pneumolysin. The purified protein was desalted to remove maltose. The final product was run on an SDS gel for size confirmation and checking of purity. The purified protein was assessed to be of >95% purity and was stable. The stability of domain 4 produced in fusion with MBP, in contrast to the His tagged domain 4, might be due to the solubility enhancing effect and chaperone-like function of MBP (Nallamsetty and Waugh, 2007).

The idea of using the structurally and functionally important C-terminal domain of pneumolysin (PLY-D4) as a protective antigen was based on, and supported by, the literature (Paton *et al.*, 1984, Mitchell *et al.*, 1991). To recapitulate, the two main functions of the important pneumococcal virulent factor pneumolysin are its cytotoxicity and its ability to activate the complement system in the absence of specific antibody (Paton *et al.*, 1984, Mitchell *et al.*, 1991). The importance of domain 4 as the functional domain of pneumolysin was established with the creation of the PdB toxin. This toxoid has reduced hemolytic activity because of one point mutation (W433F) in the well-conserved tryptophan rich loop located in the domain 4 of pneumolysin (Peeters *et al.*, 1994).

The only cysteine residue (C428) lies in domain 4 which was thought to be very important for the overall structure and function of the toxin and due to this misconception the misnomer “Thiol activated cytolysins” arose which was later proved to be wrong (Saunders *et al.*, 1989). A single amino acid mutation in the C-terminal part of domain 4

(D385N) renders the toxin unable to activate the complement system (Mitchell *et al.*, 1991). Looking into the mechanism of pore formation the first event is binding to the host cell and it is domain 4 that first comes in contact with the host cell.

Domain 4 alone can bind to the host cells and mutagenesis studies in pneumolysin and for its sister toxin Perfringolysin O (PFO) it has been demonstrated that two amino acids at the tip of the C-terminal domain of pneumolysin (in domain 4) are responsible for binding to cholesterol (Yukiko *et al.*, 2002, Baba *et al.*, 2001, Farrand *et al.*, 2010). Western blot analysis by using anti-PLY rabbit IgG and polyclonal mice serum recognized the antigenic and immunogenic regions in pneumolysin. The recognized stretches were, 40-98, 199-248, 325-414 and 415-471 emphasizing the importance of domain 1 and domain 4 of pneumolysin (Suárez-Alvarez *et al.*, 2003).

A C3 deposition assay revealed that domain 4 of pneumolysin could activate the complement system in both human and mice serum. Domain 4 of pneumolysin was shown to be non-toxic in terms of its hemolytic activity, when tested at low concentrations (20 ng/ml) but in micrograms amounts (starting from 5ug and then serially diluted in a microtiter plate) it does not cause lysis but it causes the red blood cells to form a sheet-like structure or matrix-like structure, rather than a pellet. The mechanism for this phenomenon is unknown and warrants further investigation. It is therefore speculated that domain 4 might have some toxicity at the sub cellular level.

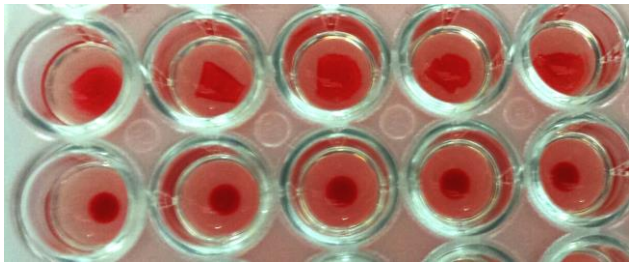


Fig 6.1. Hemolytic assay for domain 4 of pneumolysin: The top row contains the sheet/matrix like structure as a result of domain 4 incubation with hRBC's, the bottom row having the compact pellet contains PBS+hRBC's.

To check the hypothesis of domain 4 as a protective antigen, mice were immunised three times, 10 days apart, via the intraperitoneal route, in alum and sera collected as described in the methods. The sera collected were checked for anti-domain 4 antibodies by Elisa (El-Rachkidy *et al.*, 2007). It was found that serum antibody levels increased significantly after the second immunisation as compared to the sham immunised mice (Briles *et al.*, 2003, Paton *et al.*, 1983), however no significant increase was noticed for antibody levels in the serum of immunised mice between the second and third immunisations. Mice were challenged via the intranasal route with a tested virulent strain of pneumococcus (D39) at the end of the experiment to see if domain 4 of pneumolysin confers protection but no difference was observed in the survival times (Median survival for both groups 53.75) for immunised and non-immunised mice. Although the challenge dose of *S. pneumoniae* (D39) was 2 x 10^6 in 50ul, some of the mice in both the groups (immunised and non-immunised) survived to the end of the experiment i.e 6 out of 20 in the non-immunised and 7 out of 20 in the immunised group. This was surprising because in independent experiments this batch of pneumococci was 100% lethal. To further confirm the outcome of the experiment the antibody level (Elisa reading for serum

antibody) of each mice was checked for correlation with its survival times, however no correlation was found as can be seen in figure 6.2.

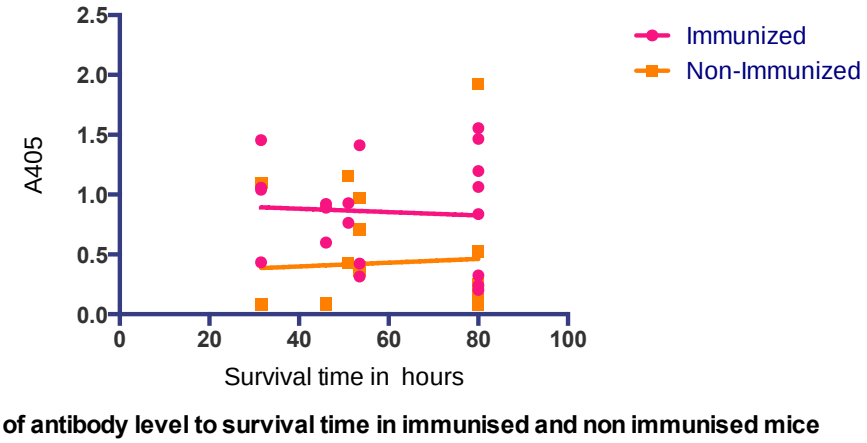


Fig 6.2. Lack of correlation between serum antibody level and survival time of immunised and non-immunised mice: Serum Elisa readings for the antibody in immunised and non-immunised mice were plotted against the survival time to see the correlation by using the software prism6. There was no correlation between the survival time and antibody (P>0.05).

An important *in vitro* test to check the functionality of antibodies produced is to see if it can inhibit the toxin of its hemolytic activity (Alexander *et.al*., 1994). Inhibiting the toxin pneumolysin of its hemolytic activity can potentially stop many of its detrimental effects to the host i.e monoclonal antibodies raised against pneumolysin completely abolish the binding of the toxin to the membrane (de los Toyos *et al.*, 1996). These include but not limited to prevent many immunomodulatory actions. Pneumolysin is known to effect beating of the cilia of the host epithelium, which in the presence of a functional antibody may make life difficult for the bacterium to adhere to the surface of epithelial cells. The

antibodies produced as a result of immunisation with domain 4 of pneumolysin in this study were not able to inhibit the toxin. With these results in hand we could not conclude about the protective response of domain 4 of pneumolysin as there might have been some technical issues with the experiment and also it is a well-known fact that the results of immunisation experiments is effected by certain factors like the virulence and serotype of the challenge strain, the route of immunisation, the route of challenge, dose, selection of adjuvant, the strain of mice and the batch of the purified proteins (Ogunniyi *et al.*, 2000, Alexander *et al.*, 1994, Paton *et al.*, 1991).

Later on it was found that the mice used for our experiment was from a genetically contaminated colony which might have effected the outcome of our result, particularly the failure to achieve 100%lethality in the non-immunised group. Having done the pilot study for immunisation with domain 4 of pneumolysin and with these questionable results in hand we could not conclude about the protective effect of domain 4 of pneumolysin as an antigen. The immunisation experiment was repeated with certain modifications. These modifications included the change of mice provider from Harlan UK to CHARLES RIVER UK, increase of immunisation dose from 20ug to 30ug of recombinant protein as a possible reason of the dose in the previous experiment not enough to elicit a protective response, inclusion of MBP-D4 to rule out any possibility of effect on the folding of domain 4 after cleavage from its partner, inclusion of intranasal route for immunisation as pneumolysin may directly interact with TLR4 (Malley *et al.*, 2003). TLR4 signalling can induce host cell apoptosis, a protective response, as mice with inhibited TLR4 were more susceptible to pneumococcal challenge (Srivastava *et al.*, 2005). The significance of TLR4 in pneumococcal infection is restricted to the airway

surfaces as the absence or presence of TLR4 makes no difference to the survival rates and blood bacterial counts after intravenous infection of mice (Srivastava *et al.,* 2005 Benton *et al.,* 1997).

Other concerns about the first immunisation experiment were, it turned out later that the colony of mice obtained from Harlan UK had issues of genetic contamination and it was thought that this might have affected the results. Also domain 4 after cleavage might not be properly folded, as it would have been in the fused form before cleavage from its fusion partner. With these concerns about the first immunisation experiment any solid conclusion about domain 4 of pneumolysin could not be drawn and therefore in our next immunisation experiment 30ug of domain 4 or domain four fused to MBP (MBP-d4) were administered via the intraperitoneal route and intranasal route to mice (from Charles River). The negative controls (sham immunised) used in this immunisation study were mice with PBS alone (for the intranasal route) and Alum alone for the intraperitoneal route. The same protocol was followed for the rest of the immunisation experiment and at the end one month after the final immunisations mice were challenged with a previously tested but fresh stock of a virulent strain of *S. pneumoniae* (D39), the antibody titres were checked by doing Elisa's as described earlier for serum collected after each immunisation.

The Elisa results were consistent with our last experiment and the antibody levels in the immunised mice were significantly different after the second immunisation. We did not find any significant difference between the survival times of immunised and non-immunised mice when challenged via the intranasal route with a lethal dose of *S. pneumoniae.* The mean survival time for mice immunised via the intranasal route was

52.5 hours as compared to the sham immunised mice where the mean survival time was 46 hours. In the mice immunised via the intraperitoneal route the mean survival time was lower (48 hours) than that of the sham immunised mice (53.5 hours) with PBS and Alum alone. The antibodies produced as a result of immunisations of mice with D4 of pneumolysin and MBP-D4 were lacking in the ability to inhibit pneumolysin.

After the second immunisation experiment we reached the conclusion that domain 4 of pneumolysin on its own cannot provide protection against a lethal dose of *S. pneumoniae*, however, it was noticed that the intranasal route for immunisation was more promising in the pneumococcal challenge model. Immunisation with domain 4 of pneumolysin I.N did not increase the survival time significantly but at least it increased the mean survival time by 5.5 hours than the sham immunised mice as compared to intraperitoneal immunisation of mice.

From this experiment the conclusion drawn was that in order to have protection against *S. pneumonaie* at least another domain of pneumolysin was required for the antibodies to have synergetic effect. Monoclonal antibodies PLY-5 and PLY-7 recognize epitopes in domain 4 of pneumolyisn. PLY-5 binds to the highly conserved stretch of 11 aminoacids (undecapeptide) or the tryptophan rich loop while PLY-7 recognizes one of the loop at the bottom of domain 4 while PLY-4 recognize a stretch in domain 1 thought to be involved in oligomerization of the toxin on the host cell membrane (García-Suárez *et al.*, 2004, Suárez-Alvarez *et al.*, 2003).

The next hypothesis was that pneumolysin when used in two parts i.e in two parts D1-3 of pneumolysin and Domain 4 of pneumolysin mixed in equimolar amounts can provide

protection as it will not only be non-toxic but also will provide all the four domains of wild type pneumolysin required for production of functional antibodies.

We already had domain 4 of pneumolysin so for this new immunisation experiment we cloned PLY d1-3 into pLEICS-01 vector for expression. This vector contains a T7 promoter and the resultant His tag protein expressed can then be purified with Ni affinity chromatography. PLY d1-3 was expressed, mostly insoluble (in the pellet).

The soluble protein from the crude extracts was purified as described in methods containing co contaminants and hence not suitable for immunisation experiments, we required highly purified protein at least 95% pure. To address and solve this problem we cloned it into pLEICS-10 vector with an N-terminal MBP to get a soluble protein, although there was a concern about its separation as the size of MBP is almost the same to that of PLY-d1-3. The theoretical PI value for both the protein were exactly the same but we thought we can separate MBP from its fusion partner after cleavage with TEV protease by passing it through amylose column. The idea was that MBP will bind to amylose resin as it always does and we will collect PLY d1-3 in the flow through. The recombinant PLY d1-3 fused to MBP was expressed and purified as described in methods. The purified protein was run on SDS gel for checking the size and assessing the purity, further purified and desalted with gel filtration chromatography and later on cleaved with TEV protease. The cleaved product was passed through an amylose column but MBP did not bind. Interestingly neither MBP nor PLY d1-3 bind to the amylose column and both were collected in the flow through. The recombinant protein was re-expressed and this time after purification an on-column cleavage was performed to obtain PLY d1-3 but to no avail as it contained co contaminants, as well as some uncleaved

protein. A C-terminal his tag was included with PLY d1-3 and then it was fused to MBP in pLEICS-10 in order to aid the purification. The recombinant protein was expressed and then the crude extracts were passed through a Ni-column After binding, the column was washed thoroughly and protein eluted with imidazole. The His-tag proved very successful with huge amounts of the recombinant proteins being purified, probably because of the binding capacity of the column. About 40mg of protein was purified from one litre of culture. The purified protein was desalted and were passed through the same Ni column for rebinding and the column was rewashed with binding buffer before an on-column cleavage with TEV protease was performed overnight. The cleavage was very efficient and the cleaved MBP did not bind to the Ni column. After extensive washing d1-3 was eluted with imidazole.

The purity of the purified protein was assessed with an SDS gel and found to be 95% pure. The purified PLY d1-3 was checked for hemolysis by doing a hemolytic assay and PLY d1-3 was found to be non-hemolytic. Both PLY d4 and PLY d 1-3 when mixed in equimolar amounts show no sign of hemolysis, thus it can be concluded that it is non-toxic, although it is possible that some toxicity may occur at sub cellular levels and this will require some further investigation. PLY d1-3 and PLY d4 were mixed in equimolar amounts in PBS for intranasal administration to mice and with alum for intraperitoneal administration as described in the methods. The sera collected after each of the three immunisations were analysed for antibody and as can be seen in results there was significant difference of antibody titre between the immunised and non-immunised mice. The antibody in the serum after second immunisation was able to inhibit the toxin when pneumolysin was preincubated with the serum for 30 minutes at 37°C. One month after

the last immunisation mice were challenged with a lethal dose of *S. pneumoniae* and were monitored carefully for signs of disease and the survival time was recorded. The immunised mice survived significantly longer than the non-immunised mice as can be seen in the Results. The survival among the immunised mice was 65% as compared to 25 % in the mice immunised with PBS alone.

To check the terminal domain of PLY as an adjuvant mice were immunized with 20ug of MBP-D4 or MBP alone in PBS via the intranasal route three times ten days apart. The serum collected was checked for anti MBP antibody from both groups. The results show that the level of anti MBP antibody in the serum was significantly higher in the group of mice immunised with MBP-D4 than the one immunised with MBP alone even after 1st immunisation. The anti MBP antibody level only starts increasing after the second immunisation in mice immunised with MBP alone. The antibody levels in the serum of both immunised groups (Immunised with MBP or MBP-D4) is still significantly different as can be seen in results. Our results show that D4 of pneumolysin can work as an adjuvant by enhancing the antibody response of its fused partner.

CHAPTER 7

Conclusion 1

Pneumolysin as a vaccine

The pneumococcal toxin "Pneumolysin" has different functions to facilitate the bacterium's survival inside the host. Such functions of the toxin include but is not limited to the activation of the complement system away from the bacterium, lysis of red blood cells, activation of cytokines, disrupting the host epithelial cell lining, disrupting the tight junctions. Looking into the structure and function of Pneumolysin, its C-terminal (4^{th} domain) is the most important domain.

Certain point mutations in the C-terminal part of this domains renders the toxins ability to bind to the host cell, mutations in the Tryptophan rich region results in loss of hemolytic activity and another mutation D385N renders the toxin unable to activate the complement system. Looking into the literature made us believe that if we use D4 of Pneumolysin as a vaccine it might provide protection, for this purpose we devised certain strategies to isolate domain 4 of Pneumolysin in highly purified form so that it can be used in immunological studies.

One of such strategies was to put a protease cleavage site (TEV cleavage site) at the junction of domain 3 and domain 4 of Pneumolysin. Site directed mutagenesis was used for this purpose and the protease cleavage site was successfully achieved at the desired position in Pneumolysin, however, making mutations at such important region led the protein to be insoluble after expression in a suitable host.

Another strategy for obtaining domain 4 of Pneumolysin was to clone the sequence coding for it in a suitable vector, express and purify it on its own. This strategy proved to be of no help as the obtained D4 was in the insoluble friction. Strategies to solubilize the insoluble protein were abandoned as the process doesn't always lead to the correct conformation of a protein.

Here in this case the protein was required in its proper conformation and highly soluble form so it was expressed as a fusion partner to maltose binding protein. Maltose binding protein (MBP) is a well-known solubility enhancer and in this case is the ideal candidate. Domain four expressed in fusion with the solubility enhancer (MBP) had a protease cleavage site (TEV protease site) in between them and after expression and purification it was cleaved off its fusion partner and separated with gel extraction chromatography.

The conclusion about expression and purification of domain 4 of Pneumolysin is that it is best to express it with a solubility enhancer when one looks to the final yield and the percentage of soluble protein. It was accepted as the most feasible and cost effective way to produce domain 4 of Pneumolysin for further immunological studies and its interaction with the complement system.

Using highly purified domain 4 of Pneumolysin, mice were immunized via intra-peritoneal and intra-nasal route to check if it can provide protection against a lethal challenge dose of *Streptococcus pneumoniae*. Shortly mice were immunized three times 10 days apart and then waited on for another month and finally challenged. The survival times were noted carefully, looking into the data we conclude that although domain 4 of Pneumolysin is immunogenic as it can elicit an antibody response and the antibody

response increases with booster doses but is not capable to provide protection against a lethal dose of *Streptococcus pneumoniae*.

Looking into this data it was hypothesized that domain 4 of Pneumolysin (due to its binding ability) may boost antibody response as an adjuvant. In the next trail domain 4 of Pneumolysin was used as an adjuvant with MBP, the antibody response (as can be seen in results) for MBP alone is significantly lower than the antibody response to MBP-D4. on the basis of these results in hand we can conclude that domain 4 of Pneumolysin can be used as an adjuvant.

Based on these results it was concluded that domain 4 of Pneumolysin is unable to provide protection on its own therefore a toxoid version of whole Pneumolysin was required. The goal was to get a toxoid version of Pneumolysin which must be nontoxic or least less toxic to all the predecessor versions of toxoids tried so far. On the other hand, loosing cytotoxicity must not compromise the immunogenicity of the toxin, for this purpose the best strategy seemed was to use domain 4 of Pneumolysin in combination with the rest of the toxin molecule (D1-3) in equimolar concentrations to mimic for the wild type Pneumolysin.

Domain 4 of Pneumolysin was already in hand so DNA coding for the rest of the 3 domains was cloned into suitable vector for its expression and strategies were adopted to get it in highly soluble and pure form. The two recombinant proteins domain 1-3 and domain 4 of Pneumolysin (constituting the whole Pneumolysin) were mixed and assayed for its hemolytic activity in order to find out its cytotoxicity. The mixture of the domains proved to be the most inactive toxoid we have encountered so far, this was used to

immunize mice 3 times ten days apart and were finally challenged with a lethal dose of *Streptococcus pneumoniae*.

As can be seen in the results and the accompanying Elisa data it can be safely concluded that immunization with the mixture of domains is one fine strategy for getting protection against *Streptococcus pneumoniae* and can be used as a vaccine. Our data suggests that the mixture of domains of Pneumolysin is not only protective on its own but can be used in fusion with the current available pneumococcal vaccines, it is suggested that it will not only increase the efficacy of the current available vaccines but also will get rid of the pneumococcal serotype shift phenomenon because pneumolysin is one of the most conserved proteins of pneumococci.

Chapter 8

Conclusion 2

Mutagenesis in pneumolysin

To recapitulate pneumolysin is hemolytic in nature and is known to activate the complement system away from the bacterium thus helping and giving a chance for the bacteria to thrive inside the host. Looking into the mechanism of action of pneumolysin it is well understood that domain 4 of pneumolysin (PLY-D4) is responsible for contact with the host cell membrane as certain mutations with in the domain 4 of Pneumolysin i.e. W433F makes the toxin less toxic. The tip of PLY-D4 comes in contact with the host cell membrane while the rest of the molecule lies away.

PLY-D4 anchors pneumolysin into the host cell membrane and acts as a fulcrum to the rest of the transforming molecule. Upon contact the monomers starts interacting probably through residues of Domain 1 of pneumolysin and oligomerise to form a prepore assembly. PLY in the prepore assembly may contain 38-44 monomers, oligomerization is followed by some astonishing changes to convert the prepore form of the bound toxin to puncture the host cell membrane resulting in lysis of the host cell.

The conversion from prepore to pore form involves an overall change in the conformation of the toxin, the trigger force to bring these changes is not known and is unresolved. However, this radical transformation includes the bending of domain 2 of pneumolysin into half as a result it brings domain 1 of pneumolysin closer to PLY-D4. The next change that occurs during the conversion of prepore form to the pore form is the

radical changes in the helices on each side of domain 3 of pneumolysin. This radical change is the transformation of the helices into extended beta hairpin structure that finally traverse and goes into the host cell membrane punching a hole.

To confirm the mechanism of action of pneumolysin certain residues in pneumolysin were mutated in pneumolysin with the intention to lock different parts of the molecule preventing them moving apart during pore formation. Two of these double mutants were created with the intention to lock domain 2 of pneumolysin to PLY-D4 via a disulphide bond so that the toxin can be trapped before any conformation changes takes place.

The residues mutated for this purpose were K34, E35 and G388. The two resultant double mutants created were PLY C34C388 and PLY C35C388, both of whom were active in the reduced and native condition. The possible explanation for the failure is that these residues were selected on the basis of *insilico* work and the actual distance for the Sulphur atom might be different than calculated on the basis of homology model.

The criteria for bond formation was checked and confirmed by observing difference in the hemolytic activity of the said mutants in native and reduced conditions. No difference in the activity of the mutant toxins was observed on the basis of which it can be concluded that the disulphide bond was not formed as intended.

Another such mutant PLY C55V163 was created with the intention to lock domain 2 of pneumolysin to TMH1 of domain 3 to stop the beta hair pin formation and its insertion into the host cell membrane. On the basis of results for its hemolytic activity it is concluded that the intended bond was not formed.

The fourth mutant of this series was PLY C262C278, which was intended to lock TMH2 and block the insertion of the beta hairpin into the host cell membrane proved to be successful. The disulphide bond was formed as intended and when checked for the activity in native conditions it had greatly reduced activity in comparison to the wild type toxin. The retained residual activity of the the mutant toxin is supposed to be due to TMH1 which was free to transform. The formation of disulphide bond in TMH2 was confirmed by the difference of activity of the toxin in native and reduced conditions, once reduced with DTT the mutant toxin regained the activity level of the wild type toxin.

This explains the soundness of approach and design in creating these mutants for understanding the mechanism of action of pneumolysin, further work is warranted for the failed mutants to look into the now available structure of pneumolysin and search for residues that could lock via a disulphide bridge and arrest pneumolysin at different stages of pore formation.

The studies done with these mutants is not complete and could be carried to the next step by properly confirming the bond formation with probes and binding the mutants to host cell and and observing the behaviour of the mutants in comparison to the the wild type toxin.

As mentioned earlier in introduction chapter the toxin after establishing contact with the host cell starts to form oligomers which then give rise to the prepore complex. Considering the prepore complex and the available structure for pneumolysin it becomes evident that certain residues in pneumolysin are very close and there is a great possibility

that oligomerization is the result of those interaction between the neighbouring pneumolysin molecules.

The third set of mutants were solely intended to study the role of different amino acids in the oligomerization of pneumolysin on the host cell membrane. The candidate residues selected were K17, K18, D79, E80 and E84 in pneumolysin. Looking into the available structure there seemed every possibility that these residues may interact considering two PLY molecules in an oligomer.

Theoretically, the mutation of these residues were disrupting the contact between the adjacent monomers which in turn was rendering the toxin inactive. The first mutant produced for this purpose was PLY 17A, the purified protein when checked for its hemolytic activity was as active as the wild type toxin itself. Checking hemolytic activity of the toxin is a good way to check oligomerization as it is the step preceding pore formation. If oligomerization is inhibited it should result in no hemolytic activity or reduced hemolytic activity.

The mutant PLY 18A had the same results when checked for its hemolytic activity while the mutant PLY 79Q had reduction in activity and was about 80HU as compared to 25160 HU of the wild type toxin. However, looking into the crystal structure of the toxin D79 is not the ideal candidate to form contact with residues in the neighbouring monomers.

The crystal structure of PLY suggested two residues K18 and E84 to have more chance of contacts, therefore a double mutant PLY 18A84A was created. The double mutant PLY 18A84A when checked for its activity was inactive.

The results give a glimpse of the blocking of the contact of residues that are essential for oligomerization of the toxin on the host cell membrane. However, one cannot be sure as further studies are warranted to establish that the reasons for inactivity of this mutant is the loss of oligomerization. One method to confirm if these two residues are involved in oligomerization would be to mutate both of these to CYS, if they were to come in contact the two CYS would form a disulphide bond and the toxin will be active but once some reducing agent like DTT is added the disulphide will break thus releasing the monomers and hence the toxin will become inactive.

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