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Development of Pneumolysin as a vaccine candidate

A thesis submitted to the University of Glasgow for the degree of

Doctor of Philosophy

By

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Submitted January 2014

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Author's Declaration

This thesis is the original work of the author unless stated otherwise.

Muhammad Kashif Mughal

January 2014

Abstract

More than 90 serotypes of *Streptococcus pneumoniae* are responsible for causing a number of invasive and non-invasive diseases in humans. To combat this pathogen there are two types of vaccines available namely pneumococcal polysaccharide vaccine (PPV) and pneumococcal conjugate vaccine (PCV). Although these vaccines have reduced the incidence and prevalence of pneumococcal diseases yet these vaccines are unable to curb the disease and have some potential drawbacks. The problems associated with PPV is inability to mount appropriate immune response in young children (<2 years) of age and with PCV, disadvantages are serotype replacement and affordability. These disadvantages have shifted the focus of attention on developing vaccines that can provide serotype independent protection and is cost effective and can be used in developing and under-developing countries as well.

Pneumolysin (PLY) is one of the members of cholesterol dependent cytolysin (CDC) family and is one of the key virulence factors of the bacterium. PLY has a wide variety of functions but the two most important functions are the ability to lyse the host cell by forming pores and activating the complement pathway. Studies have shown recombinant PLY to be a good vaccine candidate; however PLY is toxic and cannot be used as a vaccine. A number of toxoid versions have been made targeting residues responsible for toxin's lytic activity and tested *in-vivo* models. $\Delta 6$ PLY is a toxoid made by deleting two amino acid residues and seems to be a potential candidate to be included in next generation pneumococcal vaccines. $\Delta 6$ PLY is immunogenic and act as an adjuvant as well,

however $\Delta 6$ PLY causes the aggregation of red blood cells and therefore cannot be used in the vaccine.

The present study is carried out to identify the residue/s responsible for causing this agglutination behaviour. A number of molecular biology techniques were used in this study for making mutants in different backgrounds and were tested for their lytic and agglutination activity. The study identified the single residue in domain 4 causing aggregation of the red blood cells. The agglutination negative mutant was then tested at different concentrations through a series of experiments including hemagglutination assays, fluorescence microscopy and fluorescence activated cell sorter (FACS).

The study also highlighted some important regions of the toxins responsible for maintaining structure of the toxin. This new toxoid seems to have the potential to be used in future pneumococcal vaccines alone, in combination with other pneumococcal proteins and/or polysaccharides.

Table of Contents

Acknowledgement	2
Abstract	5
List of Tables.....	10
List of Figures.....	11
Abbreviations	14
1 Introduction.....	19
1.1 Disease burden.....	21
1.2 Pneumococcal carriage dynamics	23
1.3 Pneumococcal diseases	24
1.3.1 Pneumonia	24
1.3.2 Meningitis.....	25
1.3.3 Acute otitis media.....	26
1.3.4 Other diseases	26
1.4 Pneumococcal virulence factors (VF)	27
1.4.1 Capsule	28
1.4.2 Pili.....	29
1.4.3 Cell wall	29
1.4.4 Pneumococcal cell surface proteins.....	30
1.4.4.1 Choline binding proteins.....	31
1.4.4.2 Lipoproteins	31
1.4.4.3 LPXTG anchored proteins.....	31
1.5 Pneumococcal vaccines.....	35
1.5.1 Pneumococcal polysaccharide vaccine (PPV)	35
1.5.2 Pneumococcal conjugate vaccines.....	36
1.5.3 Other potential vaccine candidates	37
1.6 Cholesterol dependent cytolysin (CDCs)	38
1.7 PLY.....	40
1.7.1 Release of PLY	40
1.7.2 Structure of PLY.....	41
1.7.3 Biological effects of PLY	42
1.8 Pore formation.....	52
1.8.1 Binding.....	53
1.8.1.1 Undecapeptide and hydrophobic loops (L1-3)	55
1.8.2 Oligomerisation.....	56
1.8.3 Formation of β barrel pore	57
1.8.4 PLY as a vaccine candidate	58
1.8.5 $\Delta 6$ PLY a potential vaccine candidate.....	60
Aims of the study.....	62
2 Materials and Methods.....	63
2.1 Materials	63
2.1.1 Chemicals.....	63
2.1.2 Enzymes	63
2.1.3 Proteins kits and supplies	63
2.1.4 Antibiotics.....	64
2.2 Bacterial strains	64
2.3 Vectors	64
2.3.1 pET33b and pPAL7	64
2.4 General molecular biology techniques	65
2.4.1 PCR.....	65
2.4.2 Agarose gel electrophoresis	65

2.4.3	dNTPs	66
2.4.4	Plasmid purification.....	66
2.4.5	Bacterial transformation	66
2.4.6	Sequencing	67
2.4.7	Oligonucleotides.....	67
2.5	Construction of plasmids by site directed mutagenesis (SDM)	70
2.5.1	Construction of pET33bD4PLY ^(T459G.L460G) and pET33beGFPD4PLY ^(T459G.L460G)	71
2.5.2	Construction of pPAL7Δ6 PLY	71
2.5.3	Construction of pET33beGFPΔ6 PLY ^(L363A) , pET33beGFPΔ6 PLY ^(T459G.L460G) and pET33beGFPΔ6 PLY ^(L363A. T459G.L460G)	71
2.6	Mutations made by random mutagenesis (RM)	72
2.6.1	Random mutagenesis in WT PLY and Δ6 PLY background	75
2.7	Library of mutants from DNA 2.0	81
2.8	Expression and purification of the recombinant proteins	82
2.8.1	Isopropyl β-D-thiogalactopyranoside (IPTG).....	82
2.8.2	Protein expression	83
2.8.3	Cell lysis	83
2.8.4	Protein purification	84
2.8.5	Nickel affinity chromatography	84
2.8.6	Anion exchange chromatography	85
2.8.7	Profinity eXact TM protein purification system	86
2.8.8	Buffer exchange of protein samples.....	88
2.8.9	Storage of protein samples	89
2.8.10	Concentration of the purified protein.....	89
2.9	Characteristics of the purified proteins	90
2.9.1	SDS-PAGE	90
2.9.2	Western blotting	90
2.9.3	Red blood cells assays	92
2.9.3.1	Haemolytic assay.....	92
2.9.3.2	Hemagglutination assay.....	94
2.9.4	Fluorescence microscopy.....	95
2.9.5	Cell binding assay by fluorescent activated cell sorter (FACS)	96
2.10	Statistical analysis.....	97
3	Construction, purification and characterisation of cholesterol binding PLY mutants	98
3.1	Summary	98
3.2	Construction of D4PLY and eGFPD4PLY (459,460 TL>GG) by site directed mutagenesis	99
3.2.1	Purification of D4PLY ^(T459G.L460G)	100
3.2.2	Purification of eGFPD4PLY ^(T459G.L460G)	102
3.2.3	Purification of eGFPD4PLY and D4PLY.	105
3.3	Concentration, yield and LPS levels in the purified proteins	106
3.4	Western blot of the purified proteins	106
3.5	Red cell assay of the purified proteins	107
3.6	Fluorescence microscopy	111
3.7	Cell binding by fluorescence activated cell sorting (FACS).....	114
3.8	Discussion	122
4	Purification and characterisation of mutants made by random mutagenesis 126	
4.1	Summary	126
4.2	Random mutagenesis in pET33bD4PLY	127

4.2.1	Problems in random mutagenesis with pET33bD4PLY.....	133
4.3	Random mutagenesis in pPAL7 Δ 6 PLY and WT PLY	135
4.3.1	NotI site removal in pPAL7VECTOR	135
4.3.2	Generation of pPAL7 Δ 6 PLY	135
4.3.3	PLY amplification with TagfreePLY primers.....	136
4.3.4	Construction of random mutants in pPAL7 Δ 6 PLY and WT PLY ...	137
4.4	Protein expression and purification.....	141
4.5	Western blot of the purified proteins	143
4.6	Characterisation of the mutant proteins.....	144
4.6.1	Lytic activity of the mutant proteins.....	144
4.6.2	Hemagglutination activity of the mutant proteins	147
4.7	Mapping of mutants	149
4.8	Issues with Random Mutagenesis using Tn insertion.....	151
4.8.1	Purification of the mutant in pET33D4PLY	151
4.8.2	Purification of insoluble mutants in pPAL-7 vector	152
4.9	Single amino acid addition and substitution in PLY using SDM kit.....	154
4.9.1	Purification and characterization.....	155
4.10	Discussion	157
5	Screening, characterisation and purification of PLY mutants	161
5.1	Summary	161
5.2	Library from DNA 2.0.....	162
5.3	Screening of mutants for haemolytic activity	164
5.3.1	Haemolytic assays titres measured as end points	167
5.4	Screening of library for hemagglutination negative mutants	175
5.5	Characterisation and purification of the agglutination negative mutant Δ 6PLY ^(L363A)	177
5.5.1	Purification of mutant by NAC and AEC.....	177
5.5.2	Hemagglutination assay of the purified protein	178
5.6	Purification and characterisation of eGFP tagged mutants in pET33bvector.....	180
5.6.1	Purification of eGFP-linked PLY mutants	180
5.6.2	SDS-PAGE and western blot of the purified proteins.....	180
5.6.3	Concentration of the purified proteins	181
5.7	Cell binding of mutant protein by FACS	182
5.8	Fluorescence microscopy	188
5.9	Other Cholesterol dependent cytolysins (CDCs).....	189
5.10	Discussion:	191
6	Concluding remarks and future perspectives:	195
6.1	Future work.....	200
Appendices		202
7	Purification and characterisation of Δ 6 PFO	202
7.1	Protein purification	202
7.2	Hemagglutination assay of the purified protein.....	203
Publication, conference participation and presentations		205
List of References		206

List of Tables

Table 1-1 Summary of important virulence factors (VF) of <i>S.pneumoniae</i> and their functions	32
Table 1-2 List of currently known Cholesterol dependent cytolysins (CDCs)	39
Table 1-3 Biological effects of PLY on cells and tissues	51
Table 2-1 Conditions used for PCR Reactions	65
Table 2-2 List of oligonucleotides used in the study	68
Table 2-3 Reagent composition of SDM Reaction	70
Table 2-4 Cycling parameters of a typical SDM reaction	71
Table 2-5 Reaction protocol for the <i>in-vitro</i> transposition reaction.....	74
Table 2-6 List of plasmids generated in the study.....	76
Table 2-7 Nickel affinity chromatography (NAC) buffers	85
Table 2-8 Anion exchange buffers (AEC)	86
Table 2-9 Profinity exact buffers	87
Table 2-10 SDS-PAGE and western blotting reagents	91
Table 3-1 Concentration, yield and LPS levels in the purified proteins.....	106
Table 3-2 Specific haemolytic activity of the purified proteins	110
Table 4-1 List of mutants generated by transposon mediated random mutagenesis	140
Table 4-2 Concentration of the purified mutant proteins.....	143
Table 4-3 Specific haemolytic activity and some additional information of the mutant proteins.....	147
Table 4-4 Haemagglutination results of the mutant proteins.....	149
Table 5-1 Library of mutants from DNA 2.0	166
Table 5-2 Haemolytic titre end points (HU/ml) of wells	167
Table 5-3 Concentration of the purified protein.....	181

List of Figures

Figure 1-1 Pie chart showing cause-specific mortality worldwide in children less than 5 years old.....	22
Figure 1-2 Diagrammatic representation of main pneumococcal virulence factors	30
Figure 1-3 Crystal structure of PFO showing the 4 domains.....	41
Figure 1-4 Schematic presentation of proposed models of pore formation by CDCs	53
Figure 1-5 Undecapeptide region and three hydrophobic loops in domain 4 of CDCs	56
Figure 1-6 Different stages of pore formation of PLY	58
Figure 1-7 Protein alignment of CDCs showing highlighted residues alanine and arginine rendering CDCs non-lytic	60
Figure 2-1 Different stages of <i>in-vitro</i> transposition reaction using mutation generation system TM	73
Figure 2-2 Random15 basepair insertions in the target DNA. Figure modified from the online MGS TM instruction manual F-701	73
Figure 2-3 Translation of 15 base pair insertions in the three reading frames... ..	75
Figure 2-4 Profinity eXact purification system	88
Figure 2-5 Phenotypes observed on a red cell assay	94
Figure 3-1 His tag column output of D4PLY ^(T459G.L460G) AKTAprime TM	100
Figure 3-2 SDS-PAGE of D4PLY ^(T459G.L460G) after NAC Purification	101
Figure 3-3 Anion exchange chromatography output and SDS-PAGE of D4PLY ^(T459G.L460G) AKTA after AEC Purification	102
Figure 3-4 SDS-PAGE of eGFPD4PLY ^(T459G.L460G) after NAC Purification	103
Figure 3-5 SDS-PAGE of eGFPD4PLY ^(T459G.L460G) and D4PLY ^(T459G.L460G) after AEC Purification	104
Figure 3-6 SDS-PAGE of eGFPD4PLY and D4PLY	105
Figure 3-7 Western blotting results of the purified proteins.....	107
Figure 3-8 Red cell assay of the purified proteins.....	108
Figure 3-9 Red cell assay showing non-agglutinating and clearing well effect phenotypes.	109
Figure 3-10 Haemolytic activity of the recombinant proteins	110
Figure 3-11 Fluorescence microscopy images showing eGFP tagged recombinant proteins binding	112
Figure 3-12 FL1-ve and FL1+ve gates drawn on negative control (Red cells with PBS).....	115
Figure 3-13 FACS results showing proteins binding with the red blood cells	116
Figure 3-14 FACS results showing eGFPD4PLY ^(T459G.L460G) binding with horse red blood cells.....	117
Figure 3-15 FACS results showing the protein binding with the horse red blood cells in forward scatter channel (FSC)	118
Figure 3-16 Graph showing unpaired t-tests between the control eGFP only and the eGFPD4PLY ^(T459G.L460G)	120
Figure 3-17 Graph showing the comparison of fluorescence index of three different concentrations of eGFPD4PLY ^(T459G.L460G)	121
Figure 3-18 Model showing PLY binding to red blood cells	124
Figure 4-1 Vector pET33bD4PLY showing different restriction sites.....	128
Figure 4-2 Restriction enzyme digestion of pET33bD4PLY.....	129
Figure 4-3 PCR reactions with <i>NotI</i> mini primers	130
Figure 4-4 DNA gel pictures of transposon insertion and PCR with <i>NotI</i> mini primer	131

Figure 4-5 Transposon insertions in different regions of the pET33bD4PLY	132
Figure 4-6 Sequencing results showing two amino acids deletion in WT PLY....	136
Figure 4-7 DNA gel picture showing the PLY amplification with new set of primers	136
Figure 4-8 DNA gel showing transposon insertion in PLY gene	137
Figure 4-9 DNA gel picture showing the amplification of $\Delta 6$ PLY and transposon with PLY and transposon specific primers	138
Figure 4-10 Sequencing results showing addition of 15 base pairs (highlighted in yellow) in $\Delta 6$ PLY background using Vector NTI software programme.....	139
Figure 4-11 SDS-PAGE of $\Delta 6$ PLY, Mutant29.5 $\Delta 6$ PLY, Mutant1 WT PLY and Mutant2 WT PLY	141
Figure 4-12 SDS-PAGE showing purified WT PLY and $\Delta 6$ PLY mutants	142
Figure 4-13 Western blot of the purified mutant proteins	144
Figure 4-14 Haemolytic activity of the mutant proteins.....	145
Figure 4-15 Hemagglutination assay of the mutant proteins.....	148
Figure 4-16 Structural model of PLY generated using computational software for structure prediction (Phyre2) and 5 amino acids mutation mapped using PYMOL	150
Figure 4-17 Western blot showing PLY in insoluble fraction	153
Figure 4-18 SDS PAGE and the western blot of the purified mutant proteins ...	155
Figure 4-19 Red cell assay of the purified mutant proteins.....	156
Figure 5-1 Diagrammatic representation of DNA 2.0 library	163
Figure 5-2 Haemolytic assay of Cell lysate from DNA 2.0 library.....	168
Figure 5-3 The graph showing screening results of cell lysate from DNA 2.0 mutant Library	170
Figure 5-4 Lytic activity of WT PLY mutants form DNA 2.0 Library.X-axis on all the graphs showing mutant's name and Y axis is% lytic activity compared to the WT PLY. All important regions are highlighted. Loop1, 2, 3 and undecapeptide region are highlighted as pink, blue, green and brown respectively. Residue 363 is highlighted as red.....	171
Figure 5-5 Haemolytic activity comparisons of mutant proteins with single alanines substitutions with WT PLY	172
Figure 5-6 Screening of DNA 2.0 library lysates ($\Delta 6$ PLY background) for hemagglutination activity	176
Figure 5-7 SDS-PAGE of $\Delta 6$ PLY(L363A) after AEC Purification	178
Figure 5-8 Hemagglutination assay of the purified mutant protein $\Delta 6$ PLY ^(L363A)	179
Figure 5-9 SDS PAGE and western blot of eGFP-PLY linked proteins.....	181
Figure 5-10 FACS showing binding of mutant proteins with horse red blood cells	183
Figure 5-11 Fluorescent index (FI) of the mutant proteins.....	185
Figure 5-12 FACS results showing red blood cell population (single/multiples) on forward scatter channel.....	187
Figure 5-13 Fluorescence microscopy images showing eGFP tagged recombinant proteins binding	188
Figure 5-14 Sequence alignment of member of CDCs using CLC genomic software	189
Figure 5-15 Structural model of PLY generated using Phyre2 and highlighting residue responsible for aggregation along with loops and undecapeptide region	190
Figure 6-1 Showing orientation and distance of residues L363 and L460 (one of the cholesterol binding motifs) in PLY	196

Figure 7-1 Purified fraction of recombinant $\Delta 6$ PFO on a pre cast gel after size exclusion chromatography (SEC)	202
Figure 7-2 Hemagglutination of the purified $\Delta 6$ PFO	203

Abbreviations

Å	Angstrom
°C	Degrees Celsius
Δ	Deletion
-/-	Deficient
%	Percent
μl	Micro litre
μM	micro molar
nM	Nano molar
A	Absorbance
A.A	Amino acid
Ab	Antibody
AEC	Anion Exchange Chromatography
AOM	Acute Otitis Media
ANOVA	One way analysis of variance
ASM	Alanine Scanning Mutagenesis
BBB	Blood Brain Barrier
C	Complement
Cbp	Choline Binding Protein
CD	Circular dichroism

CDC	Cholesterol-dependent Cytolysin
CNS	Central Nervous System
CPS	Capsule Polysaccharide
CSF	Cerebrospinal Fluid
CV	Column Volumes
dH ₂ O	Distilled Water
DNA	Deoxyribonucleic acid
dNTP	Deoxyribonucleotide triphosphate
DTT	Dithiothreitol
<i>E. coli</i>	<i>Escherichia coli</i>
EDTA	Ethylenediaminetetraacetic acid
eGFP	Enhanced Green Fluorescent Protein
ELISA	Enzyme Linked Immunosorbent Assay
ETOH	Ethanol
EU	Endotoxin Units
FACS	Fluorescence activated cell sorter
g	Gram
H ₂ O ₂	Hydrogen peroxide
His-Tag	Histidine Affinity tag
HRP	Horseradish peroxidase
HU	Haemolytic Units
Hyl	Hyaluronidase

H ₂ O ₂	Hydrogen peroxide
Ig	Immunoglobulin
IL	Interleukin
ILY	Intermedilysin
IMAC	Immobilised metal affinity chromatography
IPTG	Isopropyl-β-D-Thiogalactopyranoside
Kb	Kilobase
kDa	Kilodalton
L	Litre
LB	Luria Broth
LDC	Ligation Dependent Cloning
LPS	Lipopolysaccharide
LytA	Autolysin A
M	Molar
mAb	Monoclonal Antibody
MAPK	Mitogen activated protein kinase
mg	Milligram(s)
μg	microgram
MGS	Mutation Generation System MGS™
ml	Millilitre
MLST	Multi-Locus Sequence Typing
mM	Millimolar

Mol.wt	Molecular weight
MyD88	Myeloid differentiation factor 88
NAC	Nickel Affinity Chromatography
Nan	Neuraminidase
NO	Nitric Oxide
NOD	NOD-like receptor
OD	Optical Density
PBS	Phosphate Buffered Saline
PCR	Polymerase Chain Reaction
PHYRE	Protein Homology/Analogy Recognition Engine
PCV	Pneumococcal Conjugate Vaccine
PdB	Pneumolysin carrying W433F substitution
PdT	Pneumolysin carrying triple mutations: D385N, C428G, W433F
PFO	Perfringolysin O
Pht	Pneumococcal histidine triad protein
Pho	Phosphorylcholine
Pia	Pneumococcal iron acquisition protein
Piu	Pneumococcal iron uptake protein
PLY	Pneumolysin
PLYD1	Toxoid made by substituting three residues (65TC, G293C, C428A)
PPR	Pattern recognition receptors
PPV	Pneumococcal Polysaccharide Vaccine

PS	Polysaccharide
Psa	Pneumococcal surface adhesion protein
Psp	Pneumococcal surface protein
RM	Random mutagenesis
rpm	Revolutions per minute
SP	<i>Streptococcus pneumoniae</i>
SDM	Site-directed mutagenesis
SDS-PAGE	Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
SEM	Standard Error of the Mean
SLO	Streptolysin O
ST	Sequence Type
SLY	Suilysin
TAT	Thiol Activated Toxins
TL	Threonine and Leucine
TLR	Toll-like Receptor
TMH	Transmembrane Helices
TNF	Tumour Necrosis Factor
U	Units
VF	Virulence factors
V	Volts
v/v	Volume/volume
WT	Wild type

1 Introduction

Streptococcus pneumoniae, also known as pneumococcus, is a gram positive facultative anaerobic organism involved in an array of human diseases ranging from non-invasive otitis media and sinusitis to invasive life threatening bacterial pneumonia, meningitis and sepsis (O'Brien *et al.*, 2009). The bacterium is able to cause such diseases in all age groups, but infants, elderly and the immunocompromised are more vulnerable (Tai, 2006).

The bacterium grows in chains or pairs, hence the name diplococcus, and was simultaneously isolated by two scientists named Pasteur and Steinberg in 1881 (Sternberg, 1881, Pasteur, 1881). Pneumococcus is classified according to the immunochemistry of the polysaccharide capsule that surrounds them by the Quellung reaction (swelling reaction) which was discovered by Neufeld in 1902 (Neufeld, 1902). The reaction is visualised as capsular swelling/bacterial agglutination with anti sera raised against the homologous capsular polysaccharide. More than 90 different capsular types (serotypes) of the organism have been identified (Henrichsen, 1995, Park *et al.*, 2007). The distribution of these different serotypes vary geographically and the different subtypes within a serotype can cross react with each other (Austrian, 1981, Calix & Nahm, 2010). This cross reactivity led to the reclassification of different serotypes from chronological order of discovery to the Danish nomenclature; which places them in different serogroups (Dochez & Gillespie, 1913). Quellung reaction is quite a useful tool in pneumococcal characterisation and is still used in laboratories but has certain limitations. The anti sera are expensive and mistyping of certain sera occurs due to cross reactivity. Moreover, there are some strains which do not express capsules (un-encapsulated) or have down

regulated their capsular expression genes so cannot be typed (non-serotypables) (Pai *et al.*, 2006, Smart, 1986).

The advancement in the molecular biology tools has led to more accurate and specific typing. One of these is Multi locus Sequencing Technique (MLST) which is simpler, reliable and based on the sequencing of different isolates for the seven housekeeping genes which are glucose kinase (*gki*), shikimate dehydrogenase (*aroE*), glucose-6-phosphate dehydrogenase (*gdh*), signal peptidase I (*spi*), transketolase (*recP*), D-alanine-D-alanine ligase (*ddl*) and xanthine phosphoribosyltransferase (*xpt*). The sequencing results of each allele is matched on the MLST website (<http://spneumoniae.mlst.net/>) and based on the results is assigned a number. This number is compared with other isolates already present on the database and based on the allelic variation of genes, isolates are assigned specific sequence type (ST) (Enright & Spratt, 1998). A number of STs can exist within a serotype and also some STs can be found in more than one serotypes thus exhibiting capsular switching. Capsular switching enables pneumococcus to escape from vaccines targeted against capsular polysaccharide (Coffey *et al.*, 1998, Jacobs *et al.*, 2009, Jefferies *et al.*, 2004, Pai *et al.*, 2006). MLST is a useful online tool and is helpful in maintaining the same standard in majority of laboratories across the globe. It enables researchers and clinicians to monitor spread of any virulent isolate in a specific geographical area or emergence of any antibiotic resistant strains and thus provide useful information about the epidemiology and genetic variation of the organism (Enright & Spratt, 1999, McGee *et al.*, 2001).

The microarray analysis of clinical isolates has pointed out certain limitation of MLST by exhibiting genetic variations in isolates that were found to be identical

by serotyping and MLST (Silva *et al.*, 2006, Dagerhamn *et al.*, 2008). However, the cost of microarrays is too expensive to be used routinely for identification in diagnostic labs. The next generation genome sequencing is by far the most accurate method of identification of the clinical isolates but the high cost and affordability, especially in the developing and under developed countries remains the major issue with these new techniques. There is a dire need of quicker, reliable and cheaper technique for identification and diagnosis of pneumococcal infections in the developing and under developed countries where the disease burden is highest with limited resources.

1.1 Disease burden

S.pneumoniae is among the leading bacterial pathogens causing respiratory tract infections cumulating 14.5 million pneumococcal cases worldwide with 11% mortality (excluding pneumococcal deaths in HIV positive) in children below 5 years of age (O'Brien *et al.*, 2009). Children under two years of age are more susceptible because of insufficient development of immune system and passive immunity acquired in the form of maternal antibodies (IgG) from placenta, which diminishes approximately after 3 months. After 3 months the immune system of healthy babies starts producing their own antibodies. Overall, Asia and Africa account for 66% of pneumococcal cases worldwide; and the top four out of five countries with highest number of cases are present in Asia (India 27%, China 12%, Nigeria 5%, Pakistan 5% and Bangladesh 4%) (O'Brien *et al.*, 2009).

According to World Health Organization (WHO) statistics over 2 million deaths in children occur due to pneumonia each year and it accounts for more deaths in children than measles, malaria and AIDS combined. One out of every five children worldwide dies before reaching the fifth birthday due to this deadly

disease (figure 1)

http://whqlibdoc.who.int/publications/2006/9280640489_eng.pdf.

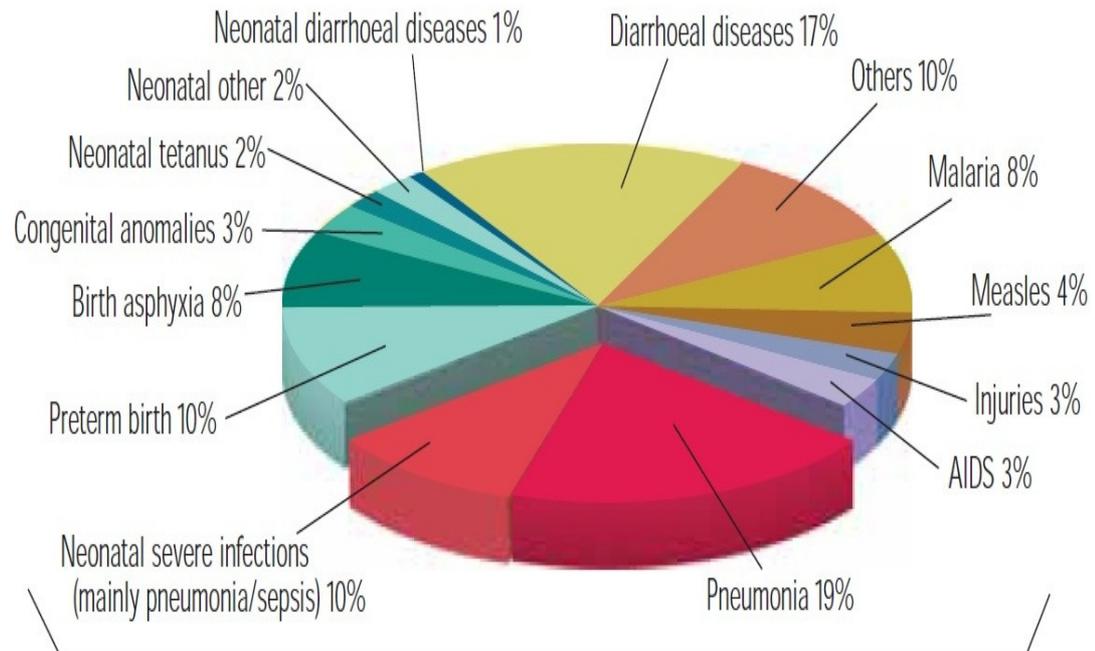


Figure 1-1 Pie chart showing cause-specific mortality worldwide in children less than 5 years old

Figure adapted from http://whqlibdoc.who.int/publications/2006/9280640489_eng.pdf

Figure 1.1 showing that pneumonia leads the list of causes of mortality worldwide in children less than 5 years of age. The old age group people are also susceptible to pneumococcal disease mainly due to decrease in immunity as antibody body titres goes down with increasing age (Simell *et al.*, 2008). Other risk factors predisposing pneumococcal infections include alcoholism, heart and lung diseases and patients receiving immunosuppressive therapies (Koivula *et al.*, 1994).

In the USA alone nearly 50,000 people died due to pneumonia in the year 2009 and 1.1 million people required hospitalization

(<http://www.cdc.gov/Features/Pneumonia/>). These figures reflect the magnitude of this disease in a developed country and the numbers are far higher in developing and under developed countries where access to health facilities are limited, the diagnostic facilities poor, the data recording is not accurate, the resources spent on health of people are sparse and, and less than 20% of children receive the recommended antibiotic treatment (http://whqlibdoc.who.int/publications/2006/9280640489_eng.pdf).

The mortality associated with pneumococcal bacteremia is very high. A study done in a developing African country (Kenya) showed a high incidence rate of disease 597 per 100 000 children under five years of age with a mortality rate of 16% (Brent *et al.*, 2006). The disease can be treated with antibiotics but less than 1/3 of the children in the world receive proper antibiotic treatment <http://www.who.int/mediacentre/factsheets/fs331/en/index.html>.

1.2 Pneumococcal carriage dynamics

Pneumococcus resides in the upper respiratory tract (nasopharynx) as a harmless coloniser along with the other bacterial species like *Moraxella catarrhalis*, *Haemophilus influenzae*, *Neisseria meningitidis*, *Staphylococcus* and other streptococcus species. Every individual experiences colonisation with one of these organisms at least once in a life time. This colonisation is the first step in route of the development towards invasive disease if the organism manages to break the immunological barriers (Bogaert *et al.*, 2004). Since colonisation is an important step the organism has evolved certain mechanisms to combat its survival. Pneumococcus produces hydrogen peroxide (H₂O₂), neuraminidase and bacteriocin which can inhibit the growth of other microorganisms (e.g. *H.influenzae*, *N.meningitidis*) in the nasopharynx which minimises the chances

of these organisms of causing invasive diseases (Pericone *et al.*, 2000, Shakhnovich *et al.*, 2002, Dawid *et al.*, 2009).

Colonisation by pneumococcus starts at a very early age and in the developing world 95% of healthy children (under 3 years of age) and 40% of adults are carriers of up to four serotypes (Obaro & Adegbola, 2002, Gray *et al.*, 1980). The colonisation declines with increasing age as the immune system matures and pneumococcal carriage drops to less than 5% in adults. (IgG production starts three months after birth and reaches adult levels at 2-7 years thus resulting in this decrease in colonization) (Flamaing *et al.*, 2010). Other risk factors can also increase the chances of pneumococcal carriage and hence disease which includes overcrowding, smoking, alcoholism, asthma, ethnicity and the use of antibiotics (Kurtti *et al.*, 1998, Bogaert *et al.*, 2004, Greenberg *et al.*, 2006, Hoge *et al.*, 1994).

1.3 Pneumococcal diseases

1.3.1 Pneumonia

S.pneumoniae is also called pneumococcus because of the ability to cause pneumonia. It is one of the most important causative agents of community acquired pneumonia. Pneumonia is defined as inflammatory response of the lung tissues as a consequence of an infection resulting in the consolidation and exudation of alveolar spaces and thereby hindering the gaseous perfusion between the blood stream and alveoli.

In the year 2000, pneumonia accounted for more than 95% of all the pneumococcal cases and had a global incidence of 13.8 million cases in children

under the age of 5, out of which 0.74 million lost their lives due to this disease (O'Brien *et al.*, 2009). In Pakistan alone, one in four deaths in children under 5 years of age is due to this disease (Owais *et al.*, 2010).

A number of other factors are also responsible for predisposing individuals to pneumonia which includes old age, cigarette smoking, chronic obstructive pulmonary disease (COPD), alcohol abuse, liver disease, congestive heart failure, HIV infection and previous history of pneumonia (Musher *et al.*, 2000).

Pneumonia may lead to bacteremia which is a state where bacteria are found in the normal sterile blood stream, which can lead to sepsis and death (Laterre *et al.*, 2005).

1.3.2 Meningitis

Pneumococcus can cause meningitis, a disease characterised by the inflammation of the fluid filled thin membranous covering surrounding the brain and the spinal cord. The disease is normally preceded by an infection at a site other than Central Nervous System (CNS). It has been shown that 30% of patients with pneumococcal meningitis had focus of infection in the ear followed by 18% and 8% in the lungs and sinus (Ostergaard *et al.*, 2005). However, it has been shown in a murine model that organism can invade and infect the CNS directly through olfactory neurons (van Ginkel *et al.*, 2003).

In the year 2000, there were more than a hundred thousand pneumococcal meningitis cases worldwide with a mortality rate of nearly 60% (O'Brien *et al.*, 2009). The symptoms of the disease are neck stiffening, severe headaches, fever, seizures and coma which may lead to death. The case fatality of meningitis is 21% even in industrialised nations and up to 30% of the surviving

cases face the devastating long-term sequelae of hearing loss and mental impairment (Schuchat *et al.*, 1997, Koedel *et al.*, 2002).

The mechanism through which pneumococcus crosses the blood brain barrier (BBB) and gains entry into the CNS requires further investigation. The toxin Pneumolysin (PLY) plays a role in damaging cerebral endothelial cells and allowing the bacterium to cross the BBB and gaining access to the cerebral compartment (Zysk *et al.*, 2001). The role of neuraminidase (NanA), a pneumococcal cell surface protein as an important mediator of the pathogen entry into the CNS has also been reported (Uchiyama *et al.*, 2009).

1.3.3 Acute otitis media

Acute otitis media (AOM) is a relatively benign but a very common middle ear infection in children. In the United States, it is the one of the leading causes for physicians visit and ranks first among all the infectious disease for which antibiotics are prescribed. The disease has a huge economic burden and in the US alone, it costs more than 5 billion dollars (Klein, 2000). The magnitude of the disease can be determined by the fact that more than 80% of children have more than or equal to one episode and 46% have greater than or equal to 3 episodes of the disease during their first 3 years of life (Teele *et al.*, 1989). Although this disease is rarely fatal, but it can cause morbid conditions including hearing loss and developmental delays (Klein, 2000).

1.3.4 Other diseases

Pneumococcus is also responsible for some relatively less common infections which are described in the subsequent paragraphs.

Pneumococcus can cause conjunctivitis which is the redness and the inflammation of the thin membrane that covers the front of the eye (conjunctiva). The disease is usually caused by the unencapsulated (non-typable) strains in disease outbreaks although sporadic cases of conjunctivitis have also been found (Ertugrul *et al.*, 1997, Barker *et al.*, 1999). The current vaccines target pneumococcal capsule and therefore have no effect on this non-typable strains.

Pneumococcus can also cause endocarditis (inflammation of the inner most layer of tissue the lining heart chamber) and cerebral abscesses (collection of pus in brain). Although these diseases are very uncommon but are associated with a significant morbidity and mortality (Eastham *et al.*, 2004, Bruyn *et al.*, 1990, Carpenter *et al.*, 2007).

1.4 Pneumococcal virulence factors (VF)

The bacterium includes the following most important virulence factors (VF) namely, the capsule, pneumolysin, cell surface proteins and various enzymes. Virulence factors of bacterium aids in establishing disease in the host of particular species and increases its potential to cause disease. In pneumococcus, VF are responsible for colonisation of the organism and/or causing disease and aids pneumococcus in evading the host defences. A number of other putative virulence factors have been identified by full genome sequencing and signature tagged mutagenesis (Frolet *et al.*, 2010, Hava & Camilli, 2002, Hiller *et al.*, 2007, Lau *et al.*, 2001, Polissi *et al.*, 1998, Tettelin *et al.*, 2001). The function of a number of these putative VF remain unknown but research is ongoing in this field and studies are being conducted and published to ascertain their functions

(Frolet *et al.*, 2010). The well studied VF and a summary of their functions are summarized in the table 1.1.

1.4.1 Capsule

The capsule is one of the foremost important virulence factors of the bacterium as it evades the host immune response by its anti-phagocytic property. (Hyams *et al.*, 2010, Melin *et al.*, 2010) Capsular Polysaccharide (CPS) importance can be ascertained by the fact that all clinical isolates that cause invasive disease are encapsulated and loss of capsule through genetic mutation resulted in decreased virulence in animal models of infection (Watson & Musher, 1990). Un-encapsulated strains rarely cause invasive disease and are isolated from cases of sporadic and epidemic pneumococcal conjunctivitis which is a non invasive disease causing inflammation of conjunctiva (Barker *et al.*, 1999, Martin *et al.*, 2003).

The thickness of capsule determines the colony morphology. In the nasopharynx, the transparent colonies (having thin layer of capsule) enables cell surface proteins and pili to adhere to the nasal mucosa and hence associated with colonisation (Weiser, 1998). The opaque colonies are usually recovered from blood in the invasive disease and have a thick capsule enabling the organism to escape from antibody and/or complement mediated phagocytosis (Serrano *et al.*, 2006, Hyams *et al.*, 2010). However, these colony morphologies were found to be reversible and aided in transition of bacterium from carriage to invasive disease (Hammerschmidt *et al.*, 2005).

Pneumococcus has the ability to switch its capsular serotype through horizontal gene transfer and hence escape the vaccines which are directed against the

specific serotypes (Coffey *et al.*, 1998). This has resulted in the evolution of specific pneumococcal isolates of particular STs within different serotypes (Coffey *et al.*, 1999, Pai *et al.*, 2006). This serotype replacement has shifted the attention of scientists on other virulence factors for designing vaccines other than the CPS.

1.4.2 Pili

It was initially thought that capsule is the first point of contact with the host cells before pilus was discovered and was shown to extend beyond the polysaccharide capsule and responsible for mediating the attachment of the bacterium to the mucosal surfaces. There are two pilus islet in the pneumococcus which encodes for the pilus, PI-1 and PI-2 (Hava & Camilli, 2002, Bagnoli *et al.*, 2008).

A study done by Barocchi *et al.*, 2006 showed introduction of PI-1 to a pilus negative encapsulated strain increased the attachment of the bacterium to the lung epithelial cells and provided competitive advantage *in-vivo* when infected simultaneously with the pilus negative strain. Furthermore, pilus expressing isolates were more virulent in a murine model of pneumonia and bacteremia than the non piliated deletion mutants (Barocchi *et al.*, 2006).

1.4.3 Cell wall

The cell wall of pneumococcus is made up of peptidoglycan backbone covalently linked with the teichoic acid and lipoteichoic acid which is attached to the plasma membrane. Phosphorylcholine (Pho), a component of both teichoic and lipoteichoic acid provides the site of attachment for the choline binding proteins (Mosser & Tomasz, 1970, Yother & White, 1994). The cell wall components can

induce the production of inflammatory mediators and challenges with the cell wall components in animal models can reproduce symptoms of pneumonia, otitis media and meningitis (Giebink *et al.*, 1988, Hoffmann *et al.*, 2007, Riesenfeldorn *et al.*, 1989, Tuomanen *et al.*, 1987).

1.4.4 Pneumococcal cell surface proteins

Pneumococcal cell surface proteins are divided into three groups based on their attachment with cell wall. These three families of proteins along with the other important virulence factors are shown in the figure 1.2. The surface cell surface proteins are classified as choline-binding proteins (nearly 16 members), Lipoproteins (around 50 members), and LPXTG anchored proteins (up to 18 members) (Perez-Dorado *et al.*, 2012).

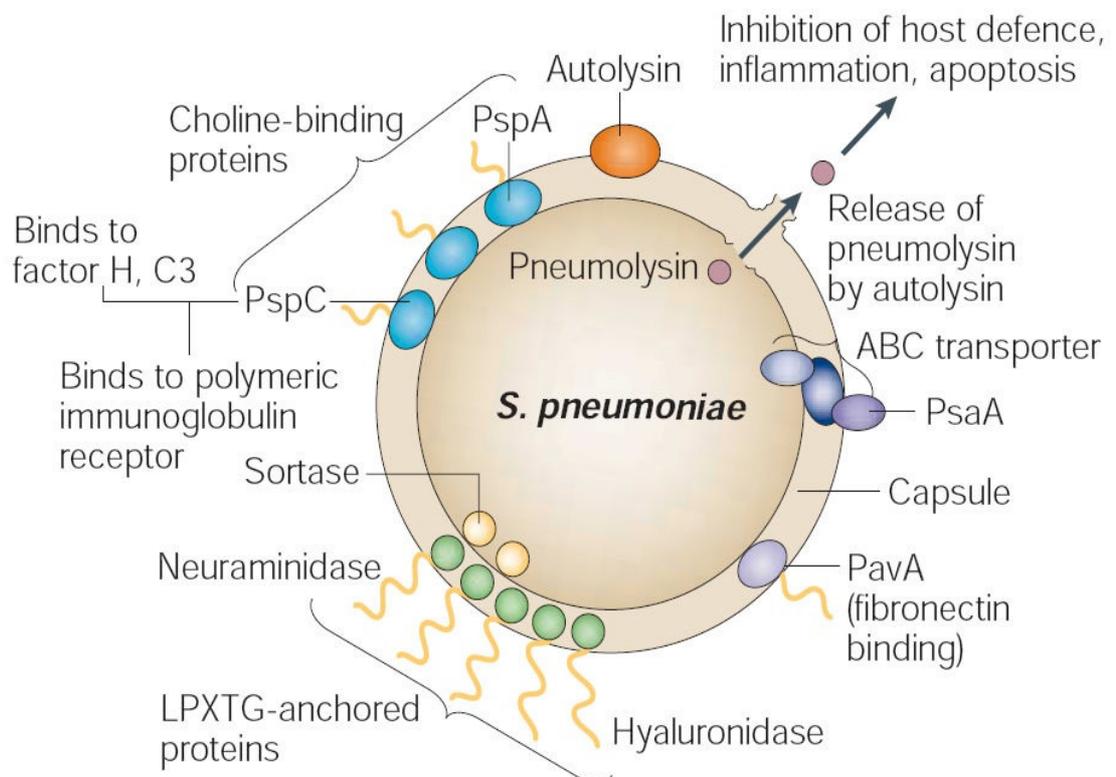


Figure 1-2 Diagrammatic representation of main pneumococcal virulence factors

The three families of cell surface proteins are choline binding proteins, lipoproteins and LPXTG-anchored proteins PavA, pneumococcal adhesion and virulence A; PsaA, pneumococcal surface antigen A; PspA, pneumococcal surface protein A; PspC, pneumococcal surface protein C. Figure taken from (Mitchell, 2003).

1.4.4.1 Choline binding proteins

CBPs possess carboxy terminal repeat regions that non-covalently anchor them to the Pho of the cell wall. The important proteins included in this family are autolysin (LytA), pneumococcal surface protein A (PspA) and pneumococcal surface protein C (PspC) also known as Choline binding protein A (CbpA) (Mitchell, 2003). All these proteins have important roles in virulence and a brief summary of their specific functions are mentioned in Table 1.1.

1.4.4.2 Lipoproteins

This family of cell surface proteins are linked to the cell membrane through an enzyme called diacylglyceryl transferase (Lgt) (Chimalapati *et al.*, 2012). These proteins have diverse functions and the majority of them form a component of the ATP-binding cassette-type (ABC) binding proteins responsible for uptake of Mn^{+2} , Zn^{+2} ions and other ions (Bergmann & Hammerschmidt, 2006, Lawrence *et al.*, 1998). The members of this family of proteins include pneumococcal cell adhesin (PsaA), pneumococcal iron uptake protein A (PiuA) and pneumococcal iron acquisition protein A (PiaA) (Brown *et al.*, 2001).

1.4.4.3 LPXTG anchored proteins

This family of proteins are anchored covalently to the cell wall via LPXTG motif (Leucine, proline, any amino acid, threonine and glycine) and is located at the carboxy-terminal end. This motif is recognised by the sortase enzyme that links the protein via a threonine residue to the peptidoglycan of the cell wall. Two important members of this family include hyaluronidase and neuraminidase (Mitchell, 2003, Tettelin *et al.*, 2001).

Table 1-1 Summary of important virulence factors (VF) of *S.pneumoniae* and their functions

<u>Virulence Factor</u>	<u>Location on bacterium</u>	<u>Function</u>
Capsular Polysaccharide (CPS)	Cell surface	<p>Resists complement activity and protects bacterium from being phagocytosed (Anti-phagocytic).</p> <p>Hides cell surface antigens from the host immune system and altering capsular expression during disease (Hammerschmidt <i>et al.</i>, 2005).</p>
Cell Wall	Beneath the capsule	<p>Surface proteins anchored to the cell wall.</p> <p>Cell wall components initiate inflammatory response (Tuomanen <i>et al.</i>, 1985).</p> <p>Pho binds to PAF (Platelet activating factor). PAF is up regulated in viral infection which might explain the secondary pneumococcal infection (Mitchell, 2003).</p>
Pili	Cell surface	<p>Mediates initial attachment of bacterium with the host.</p> <p>Second pilus discovered with its involvement in host cell adhesion (Bagnoli <i>et al.</i>, 2008).</p>
Autolysin (LytA)	Cell wall; choline-binding	<p>Degrades cell wall and hence release of other virulent factor e.g. PLY (Howard & Gooder, 1974).</p> <p>Has an important role in the pathogenesis of pneumococcal meningitis (Hirst <i>et al.</i>, 2008).</p> <p>LytA deficient mutants have decrease ability of replication in the lungs (Orihuela <i>et al.</i>, 2004).</p>
LytB and LytC		<p>These autolysins have a role in nasopharyngeal colonisation (Gosink <i>et al.</i>, 2000).</p>

<p>Pneumococcal surface protein (PspC) also called Choline Binding protein (CbpA)</p> <p>Cbp D,E,F,I,L and G</p>	<p>Cell wall; choline-binding</p>	<p>Has a major role in adherence of bacterium to the nasopharynx (Rosenow <i>et al.</i>, 1997).</p> <p>Binds Factor H (Dave <i>et al.</i>, 2001) which is the regulator of the complement activation and exploit it for bacterium survival (Herbert <i>et al.</i>, 2012).</p> <p>Mutants show reduced virulence in both bacteremia and pneumonia models possibly due to less effective nasopharyngeal colonisation (Kerr <i>et al.</i>, 2006, Rosenow <i>et al.</i>, 1997).</p> <p>CbpD,E and G have role in nasopharyngeal colonisation (Gosink <i>et al.</i>, 2000).</p> <p>CbpF,I and L binds to elastin, a major component of blood and lung vessels (Frolet <i>et al.</i>, 2010).</p>
<p>Pneumococcal Surface Protein A (PspA)</p>	<p>Cell wall; choline-binding</p>	<p>Highly variable protein expressed by all clinically important serotypes (Crain <i>et al.</i>, 1990).</p> <p>Inhibit deposition of the complement C3 and thus reducing complement mediated clearance (Mukerji <i>et al.</i>, 2012).</p> <p>Binds to human lactoferrin, an iron binding protein thus involved in iron acquisition at the site of infection (Hakansson <i>et al.</i>, 2001).</p>
<p>Pneumococcal cell adhesion (PsaA)</p>	<p>Cell surface; lipoprotein</p>	<p>Part of ABC transport system for uptake of Mn^{+2} and Zn^{+2} into bacterial cytoplasm (Lawrence <i>et al.</i>, 1998).</p> <p>Has a role in oxidative stress (Tseng <i>et al.</i>, 2002).</p>
<p>Pneumococcal iron acquisition(Pia)</p>	<p>Cell surface; lipoprotein</p>	<p>Part of pneumococcal iron ABC transport system and was in mouse model required for full virulence (Brown <i>et al.</i>, 2001).</p>

and Pneumococcal iron uptake (Piu)		Antibodies protection is via opsonophagocytosis rather than iron transport inhibition (Jomaa <i>et al.</i> , 2005).
Hyaluronidase (Hyl)	Cell wall; LPXTG anchored. also secreted	Breaks down hyaluronic-acid in the mammalian connective and extracellular matrix and enhances pneumolysin-mediated epithelial cell damage (Feldman <i>et al.</i> , 2007, Humphrey, 1948).
Neuraminidase (NanA)	Cell wall; LPXTG anchored. also secreted	Cleaves sialic acid from host glycolipids, oligosaccharide and lipoproteins revealing receptors for pneumococcal binding (King <i>et al.</i> , 2006). Mediates entry of bacterium in the CNS (Uchiyama <i>et al.</i> , 2009). Role in nasopharyngeal colonisation in chinchilla model (Tong <i>et al.</i> , 2005). Desialylates competitor colonizing species (Shakhnovich <i>et al.</i> , 2002).
Pneumococcal Histidine Triad (Pht) proteins	Cell surface	Inhibit complement deposition on pneumococcus through recruiting of human Factor H (Ogunniyi <i>et al.</i> , 2009). Antibodies against PhtD and E prevent pneumococcal adherence to airway epithelial cells (Khan & Pichichero, 2012).
Pneumococcal adhesion and virulence A (PavA)	Cell surface	Binds to fibronectin and inactivation of PavA gene resulted in markedly reduced virulence (Holmes <i>et al.</i> , 2001).
Hydrogen Peroxide (H ₂ O ₂)	Cell surface	Produced during aerobic growth and inhibits/kills other colonisers of the upper respiratory tract (Pericone <i>et al.</i> , 2000).

PLY	Cytoplasm	Has two major activities; lysis of the cells and activation of the complement. The role of PLY is explained in detail as the toxin is the major focus of this study (Mitchell, 2003).
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1.5 Pneumococcal vaccines

The history of pneumococcal vaccine dates back to early 1900, when for the first time whole killed pneumococci as vaccine was used with some efficacy against the bacterium (Chien *et al.*, 2010). The discovery of antibiotics resulted in the loss of interest for vaccines but the focus shifted again on developing vaccines due to the emergence of antibiotic resistant strains (Tomasz, 1997).

Currently used vaccines involve the use of CPS and are known as Pneumococcal Capsular Polysaccharide (PPV) and Pneumococcal Conjugate Vaccine (PCV).

1.5.1 *Pneumococcal polysaccharide vaccine (PPV)*

PPV contains capsular polysaccharide which acts as active immunogen and produce sero-type specific antibody protection promoting opsonisation and complement mediated phagocytosis (Malley & Anderson, 2012). A PPV vaccine with a mixture of 14 most prevalent capsular polysaccharide antigens were introduced in the United States in the year 1977 and the numbers increased to 23 in 1983 (Robbins *et al.*, 1983). The vaccine contain CP of the following 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 and covers 85 to 90% of IPDs in the developed world (Butler *et al.*, 1999).

PPV induces a T-cell independent response and the resulting response is short lived and lacks T-memory cells (Weintraub, 2003). Another major limitation in the use of PPV in young children (< 2 years of age) is its inability to generate an effective response against the vaccine due to immature immune system (Deficiency in maturation of distinct B lymphocyte sub populations) (Barrett, 1985). Since this age group (<2 years) is highly affected by pneumococcal disease, an alternative vaccine for the protection of this group was required.

1.5.2 Pneumococcal conjugate vaccines

PCV was designed by conjugating the selected capsular polysaccharides to a protein carrier resulting in the T-cell dependent response. The response generated is long lived and has a memory T-cell response, unlike PPV which engages B cell and hence lacks memory response. PCV7 contains the purified capsules of the following serotypes 4, 6B, 9V, 14, 18C, 19F, and 23F combined with detoxified diphtheria toxin (CRM147). This vaccine is processed as a separate protein antigen rather than entering the polysaccharide pathway.

In 2009-10 two more PCVs were introduced; a 13 valent PCV containing capsular polysaccharides from serotypes 1, 3, 4, 5, 6A, 6B, 7F, 9V, 14, 18C, 19A, 19F, 23F and a 10 valent vaccine (Synflorix) using *H.influenzae* carrier protein containing CPS from serotypes 1, 4, 5, 6B, 7F, 9V, 14, 18C, 19F and 23F (Prymula & Schuerman, 2009).

The introduction of PCVs has resulted in significant reduction of both non-invasive and invasive pneumococcal diseases in children and infants (Principi & Esposito, 2012, Fitzwater *et al.*, 2012). However, there are certain disadvantages with PCVs as limited serotypes can be included with this vaccine

and strain replacement/capsular switching (serotype replacement) by pneumococcus may result in long term vaccine failure (Coffey *et al.*, 1998). The vaccine escape isolates of serotype 19A associated with PCV-7 has already emerged in the USA (Croucher *et al.*, 2011).

The other major concern with PCV is its high cost which is unaffordable in developing countries. There is a desperate need to develop cheaper, affordable and sero-type independent vaccines for use in the developing world.

1.5.3 Other potential vaccine candidates

Whole-cell killed vaccines have been tested in mice and have shown some promising results by inducing both the humoral and cell mediated immunity. This type of approach uses non-capsular antigens common to all serotypes (Malley & Anderson, 2012).

Another approach to overcome the issue of serotype specificity is the use of conserved pneumococcal cell surface proteins as a vaccine. These recombinant protein based vaccines are easy to manufacture, cheap, robust and globally accessible. The interaction of these protein antigens with the immune system will result in the generation of antibodies resulting in host protection. However, since these proteins are surface exposed they are under constant selective pressure of the host immune response and exhibit some variability across serotypes. Cytoplasmic proteins will be under less selective pressure since they will be protected from such assault (PLY as vaccine candidate discussed in section 1.8.4 and 1.8.5).

Recently, a study conducted showed that mice immunised with PLY toxoids fused with PspA fragments provided protection against pneumococcal infection (Goulart *et al.*, 2013). Other studies have also shown the protective effect of PspA and Ply toxoid ((PdB) discussed later)) in murine model of pneumonia and sepsis (Briles *et al.*, 2003, Ogunniyi *et al.*, 2007). Furthermore, a study showed that chemically detoxified PLY given in combination with PhtD confers protection against *S. pneumoniae* induced pneumonia in rhesus macques (Denoel *et al.*, 2011). Immunisation with iron-uptake ABC transporter proteins piaA and piuA protected mice with I.N challenge with *S.pneumoniae* (Jomaa *et al.*, 2005).

1.6 Cholesterol dependent cytolysin (CDCs)

CDCs also known in the past as thiol-activated toxins (TATs), are a family of β -barrel pore-forming toxins, secreted mainly by more than 20 species of Gram positive bacteria. PLY belongs to this family and recently two CDC Desulfolysin (DLY) and Enterolysin (ELY) from Gram negative bacteria have also been identified and characterised (Alouf *et al.*, 2006, Heuck *et al.*, 2010, Hotze *et al.*, 2013). The current known members of this family are listed in the table below (Heuck *et al.*, 2010).

Table 1-2 List of currently known Cholesterol dependent cytolysins (CDCs)

Species	Name of CDC	Abbreviation
<i>Arcanobacterium haemolyticum</i>	Arcanolysin	ALN
<i>Arcanobacterium pyogenes</i>	Pyolysin	PLO
<i>Bacillus anthracis</i>	Anthrolysin O	ALO
<i>Bacillus cereus</i>	Cereolysin O	CLO
<i>Bacillus thuringiensis</i>	Thuringiensilysin O	TLO
<i>Bacillus weihenstephanensis</i>	Weihenstephanensilysin	WLO
<i>Brevibacillus brevis</i>	Brevilysin	BVL
<i>Brevibacillus laterosporus</i>	Laterosporolysin	LSL
<i>Clostridium bifermentans</i>	Bifermentolysin	BFL
<i>Clostridium botulinum</i> B	Botulinolysin B	BLYb
<i>Clostridium botulinum</i> C	Botulinolysin C	BLYc
<i>Clostridium botulinum</i> E3	Botulinolysin E3	BLYe
<i>Clostridium butyricum</i>	Butyriculysin	BRY
<i>Clostridium chauvoei</i>	Chauveolysin	CVL
<i>Clostridium histolyticum</i>	Histolyticolysin	HLO
<i>Clostridium novyi</i>	Novylilysin	NVL
<i>Clostridium tetani</i>	Tetanolysin O	TLY
<i>Clostridium perfringens</i>	Perfringolysin O	PFO
<i>Clostridium septicum</i>	Septicolysin O	SPL
<i>Desulfobulbus propionicus</i>	Desulfolysin	DLY
<i>Enterobacter lignolyticus</i>	Enterolysin	ELY
<i>Gardnella vaginalis</i>	Vaginolysin	VLY
<i>Lactobacillus iners</i>	Inerolysin	INY
<i>Listeria ivanovii</i>	Ivanolysin	ILO
<i>Listeria monocytogenes</i>	Listeriolysin O	LLO
<i>Listeria seeligeri</i>	Seeligeriolysin O	LSO
<i>Lysinibacillus sphaericus</i>	Sphaericolysin	SPH
<i>Paenibacillus alvei</i>	Alveolysin	ALV
<i>Streptococcus canis</i>	Streptolysin O c	SLOc
<i>Streptococcus dysgalactiae</i> *	Streptolysin O e	SLOe
<i>Streptococcus intermedius</i>	Intermedilysin	ILY
<i>Streptococcus mitis</i> (Lectinolysin)	Lectiniolysin	LLY
<i>Streptococcus mitis</i>	Mitilysin	MLY
<i>Streptococcus pneumoniae</i>	Pneumolysin	PLY
<i>Streptococcus pseudomononiae</i>	Pseudopneumolysin	PSY
<i>Streptococcus pyogenes</i>	Streptolysin O	SLO
<i>Streptococcus suis</i>	Suilysin	SLY

Adapted mainly from (Heuck *et al.*, 2010) with additional information taken from (Billington *et al.*, 2000, Rampersaud *et al.*, 2011, Jost *et al.*, 2011, Hotze *et al.*, 2013). *subsp. *Equisimilis*.

The toxins were historically named as TATs due to the observation that modification of an important cysteine residue renders it inactive. However, later studies showed the thiol group not to be contributing in toxin binding and or membrane insertion and hence TAT description for these toxins is a misnomer (Marriott *et al.*, 2008, Saunders *et al.*, 1989). The toxins are referred as CDCs because of the absolute requirement of cholesterol in the host target membrane for binding and pore formation (Heuck *et al.*, 2010).

1.7 PLY

PLY belongs to the family of cholesterol dependent cytolysin (CDCs) family. PLY is regarded as one of the main virulence factors of *S.pneumoniae*. Despite the fact that the toxin was recognised more than 80 years back several fundamental mechanisms regarding its release and its localisation remain ill defined (Rubins & Janoff, 1998).

1.7.1 Release of PLY

All the members of the CDC family (except *S.pneumoniae*, *S.mitis* and *S.pseudoneumoniae*) have a cleavable signal sequence and are secreted extracellularly. PLY is different from other CDCs as it lack a N-terminal secretion signal and its released is dependent on autolysin (Jedrzejjas, 2001). However, its release is still unclear with some studies showing PLY release independent of LytA (Balachandran *et al.*, 2001) and others predicting its domain 2 dependent export (Price *et al.*, 2012). The localisation of PLY is also debatable with some studies suggesting its localisation in cell wall compartment (Price & Camilli, 2009).

1.7.2 Structure of PLY

The crystal structure of PLY is yet to be elucidated but the crystal structures of PFO (Rossjohn *et al.*, 1997), ILY (Polekhina *et al.*, 2005), SLY and ALO (Bourdeau *et al.*, 2009, Xu *et al.*, 2011) have been determined. Figure 1.3 shows the crystal structure of PFO, a CDC produced by *Clostridium perfringens*. The crystal structure shows that the protein is rich in β sheets (Rossjohn *et al.*, 1997). The structure and/or function studies of PLY are based on the model built on the basis of PFO. PFO has 48% sequence identity and 60% similarity with PLY (Gilbert *et al.*, 1999)

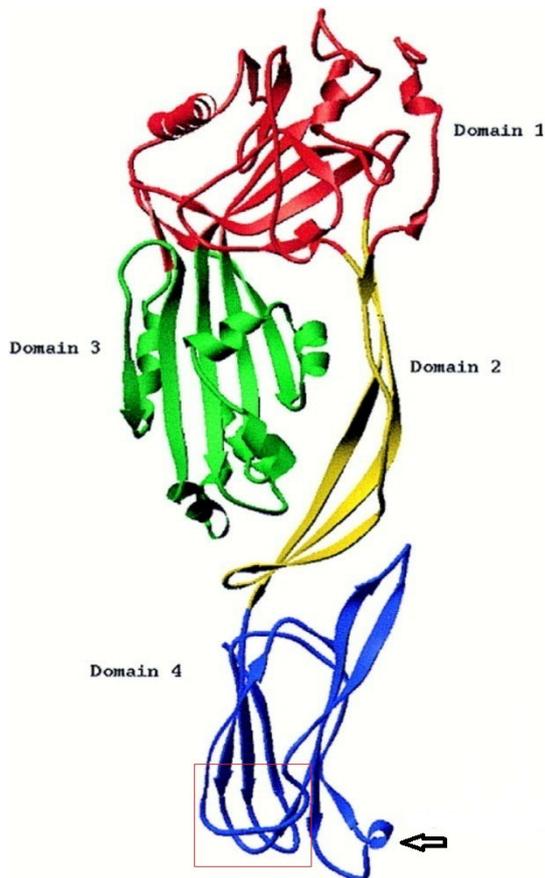


Figure 1-3 Crystal structure of PFO showing the 4 domains

The black arrow indicates Trp-rich motif in undecapeptide region of domain 4 of the toxin. The red box indicates the three hydrophobic loops (L1-3). Adapted from (Rossjohn *et al.*, 1997). Domains 1, 2, 3 and 4 are represented by red, yellow, green and blue.

PLY is a 53kDa protein with 471 amino acids (Walker *et al.*, 1987) and like other CDCs it has 4 domains (Gilbert *et al.*, 1999); Domain 1 (residues 6-21, 58-147, 198-243, 319-342), domain 2 (residues 22-57, 343-359) and domain 3 (residues 148-197, 244-318). Domains (1-3) form the N-terminal part while domain 4 (360-469) forms the important C-terminal part of the protein (Rossjohn *et al.*, 1998). Domains 1-3 are connected to domain 4 via domain 2 which is considered as the “neck” of the molecule (Rossjohn *et al.*, 1997).

Domain 4 is a compact β -sandwich shaped domain and has a Trp-rich motif (black arrow in figure 1.3) at its end. This motif resides in an important region of the toxin known as undecapeptide region (427-437) which is highly conserved among CDCs (Rossjohn *et al.*, 1998). In addition to the undecapeptide region, domain 4 also houses three short hydrophobic loops (Loops1, 2 and 3; shown in a red square in figure 1.3) and their roles will be discussed in detail in the subsequent headings (Soltani *et al.*, 2007).

1.7.3 Biological effects of PLY

PLY is a multifunctional toxin and is present virtually in all clinical isolates (Kancierski & Mollby, 1987). The biological properties of the toxin have been thoroughly studied both *in-vitro* and *in-vivo* models. The toxin interaction with the host cells (host pathogen interaction) has also been studied in detail with both PLY deficient and PLY proficient strains. It has a range of functions and its importance can be determined from the fact that in a murine model of pneumonia, mutants lacking PLY genes are less virulent as compared to isogenic toxin-producing strains. PLY can induce many pro-inflammatory and physiological mediators and the biological effects of toxin on different cells and tissues are given in the table 1.3. PLY gene is highly conserved across all serotypes with

little variation (3.3%) over time and geographical location (Mitchell, 2006, Marriott *et al.*, 2008). However, some sequence variations e.g. in (Serotypes 1, ST306) have some important consequences and are discussed later. The two main important functions of toxin is the ability to form pores and activating the complement pathway. The pore forming mechanism of the toxin is discussed in section 1.8.

PLY pore forming ability makes it toxic to cells containing cholesterol and can damage eukaryotic pulmonary epithelial, endothelial cells (Steinfort *et al.*, 1989, Rubins *et al.*, 1992) and neuronal cells (Reiss *et al.*, 2011, Zysk *et al.*, 2001). PLY was found in the CSF from meningitis cases (Wall *et al.*, 2012), in the lung tissue from mice pneumonia model (Witzenrath *et al.*, 2006), in sputum from pneumonia patients (Wheeler *et al.*, 1999) and in the middle ear cavity from otitis media (Palmu *et al.*, 2004).

The cytotoxic effect of PLY on the respiratory epithelial causes cytoplasmic blebbing, mitochondrial swelling and cell death (Steinfort *et al.*, 1989). PLY helps pneumococcus to move from alveoli to interstitium and into the blood stream. This is achieved by breaking the epithelial tight junctions, increasing alveolar permeability, inflammation and thus aiding its spread into the blood (bacteremia) (Rubins *et al.*, 1995, Orihuela *et al.*, 2004, Kadioglu *et al.*, 2002). In addition to this, PLY also impedes ciliary bronchial epithelial cell clearance and helps in propagation of organism in the lower respiratory tract. This effect is dependent on haemolytic activity as a mutant lacking haemolytic activity (W433F) showed reduced effect. Thus PLY perturbs host defences in respiratory tract which helps in proliferation and invasion of the bacterium (Feldman *et al.*, 1990, Steinfort *et al.*, 1989, Rayner *et al.*, 1995).

PLY causes neuronal cell death by increase in the calcium influx mediated through its pore forming ability and also induces mitochondrial damage (Braun *et al.*, 2007, Stringaris *et al.*, 2002). PLY also causes ependymal ciliary stasis, sloughing and cytoplasmic extrusion as it is toxic to ependymal cells (Mohammed *et al.*, 1999). The toxin helps the organism in crossing the (BBB) by damaging brain's microvascular endothelial cells (Zysk *et al.*, 2001). The importance of PLY in pathogenesis of pneumococcal meningitis disease is evident from the fact that mutant lacking PLY had reduced virulence when compared with PLY proficient strains in a rat model of meningitis (Hirst *et al.*, 2008).

The role of PLY in AOM is not well known and pathogenesis of AOM might be due to cell wall components, cell surface proteins, inflammation in the middle ear and a minor contribution from the toxin (Sato *et al.*, 1996, Stol *et al.*, 2009). Anti-PLY antibodies have been found from the middle ear fluid from the children with AOM and direct instillation of PLY induced damage on the cochlear hair cells of guinea pigs suggesting that PLY may contribute to deafness associated with adverse effects of AOM and meningitis (Rapola *et al.*, 2000, Comis *et al.*, 1993). A study conducted by Franco-Vidal *et al.*, 2008 on rat cochlear hair cells found that debilitating effects of toxin on cells can be minimised by zinc supplementation. Zinc supplementation possibly blocks the influx of calcium ions in the membrane by interfering with the toxin binding and subsequent pore forming ability (Franco-Vidal *et al.*, 2008).

PLY plays a role in eye infections and in a rabbit model the pathogenesis of endophthalmitis was directly related to the toxin's pore forming lytic activity. The study carried by Green *et al.*, 2008 showed that antibodies against PLY

confer protection against *S.pneumoniae* keratitis (Green *et al.*, 2008, Sanders *et al.*, 2008).

Pore forming ability of the toxin was directly responsible for neuronal cell death (Stringaris *et al.*, 2002), retinal layers damage (Sanders *et al.*, 2008), damage to the phagocytes and immune cell response (Jedrzejewski, 2001) and can induce apoptosis in dendritic cells (Littmann *et al.*, 2009). PLY was thought to be lytic in all strains and its lytic property was assumed to be important for the pneumococcal disease progression until the identification of the non-haemolytic version of the toxin in serotype 1, ST306 causing invasive disease outbreak in Scotland. The protein alignment of ST306 with the D39 strain revealed 4 amino substitutions (Y150H, T172I, K224R and A265S) and 2 amino acid deletions (Δ V270K271). This implies that lytic activity of the toxin is not required by some serotypes like ST306 to cause invasive disease. This also suggests that there is less selective pressure on these strains for conserving the nucleotide sequence encoding lytic activity and hence resulted in mutations described in this study (Kirkham *et al.*, 2006b).

Some serotypes (7F and 8) with reduced haemolytic than WT PLY were also identified and this reduction was attributed to the amino acid substitution at position 172 (Threonine-Isoleucine) (Lock *et al.*, 1996). To date, there are 19 distinct PLY alleles and recently a study done by Harvey *et al.*, 2011 has shown that serotype 1 PLY variant (PLY04496) having reduced haemolytic activity have a higher bacterial count in the first 15 hours post challenge compared to WT strain. This implies that these alleles have a growth advantage in early stages of bacteremia which could be important for invasive serotypes. However, PLY

immunisation provided protection against all the strains irrespective of their haemolytic activity (Harvey *et al.*, 2011, Jefferies *et al.*, 2010).

The functions of PLY extend beyond its lytic activity and complement activation is another major biological effect of the toxin. This immunomodulatory effect of toxin is independent of its pore forming ability. PLY has sequence homology to acute-phase protein C-reactive protein and can interact directly with C1q or indirectly through Fc portion of the immunoglobulin IgG, thereby activating the complement. The complement activation can however occur in the absence of anti-PLY antibody as well. The complement activation was shown to be mediated by the residue Aspartate 385 (D385), residing in domain 4 of the toxin and this region is shown to be homologous to the C-reactive protein. A mutation made in this region of the toxin (D385N) abolished complement binding (Berry *et al.*, 1995, Mitchell *et al.*, 1991, Paton *et al.*, 1984). PLY's complement activation decreased C3 deposition and thereby reduces opsonophagocytosis. A study conducted showed that *in-vitro* PLY deficient strains have increased complement deposition compared to isogenic PLY proficient strains (Yuste *et al.*, 2005). The importance of this pathway in pneumococcal infection was further strengthened by a study performed in mice with genetic deficiencies of different complement. Their results suggested that a loss of activation of the complement pathway resulted in severe septicaemia (Brown *et al.*, 2002). The highly important role of complement in humans has also been established by many studies and it has been shown that patients having complement deficiencies (C2) have increased susceptibility to pneumococcal infections (Yuste *et al.*, 2008, Skattum *et al.*, 2011).

The importance of complement inhibition in disease pathogenesis can be observed by the fact that complement-activation makes specific contribution in the pneumococcal pneumonia by a different mechanism and at separate stage of infection from the haemolytic activity of the toxin (Rubins *et al.*, 1995, Rubins *et al.*, 1996). In addition, it has been demonstrated in previous studies that both properties of the toxin are required for virulence of the organism in the model of bronchopneumonia and lobar pneumonia (Rubins *et al.*, 1996, Alexander *et al.*, 1998). However, mutants lacking both the complement and lytic activity were still virulent compared to the PLY negative strains suggesting other important functions of the toxin contributing to its virulence (Benton *et al.*, 1997).

A study done has shown that in the host lung tissues T-cell recruitment response was influenced by the complement activating activity of the toxin and the neutrophil recruitment was mediated by the pore forming activity of the toxin (Jounblat *et al.*, 2003). Neutrophils are first cells to respond during pneumococcal infection and are responsible for clearing bacteria from the lungs (Jones *et al.*, 2005). The neutrophil recruitment from the vascular spaces to the nasopharynx has also been observed during pneumococcal colonisation (van Rossum *et al.*, 2005). Neutrophils play a key role in clearing infection as impaired recruitment resulted in severe pneumococcal pneumonia in mice (Sun *et al.*, 2007, Nakasone *et al.*, 2007).

Resident alveolar macrophages are another group of phagocytic cells that provide immunity by engulfing the bacterium and releasing the inflammatory mediators. The loss of their function significantly alters the inflammatory response and release of cytokines (Jonsson *et al.*, 1985, Marriott *et al.*, 2008). The effect of the toxin, on the host immune response, results in the

persistence or clearance of the bacterium hence implying that a fine balance must be maintained. This manipulation of neutrophilic response in pneumococcal pneumonia is extremely crucial since a subset of individuals die mainly due to dysregulated inflammatory response induced despite the clearance of the organism. This response results due to excessive secretion of pro inflammatory cytokines by neutrophils and hence switching off these pro inflammatory cytokines at appropriate time is beneficial for host survival (Dockrell *et al.*, 2012).

Toll like receptors (TLRs) also known as pattern recognition receptors (PRRs), a component of innate immune system recognises microbial invasion and elicits a range of host defence mechanisms. There are other PRRs which include cytosolic NOD-like receptors (NLRs) and DNA sensors, e.g. DAI (DNA-dependent activator of interferon (IFN)-regulatory factors). TLR activation generates signal transduction pathways resulting in the formation of gene products which trigger inflammatory responses (recruitment of neutrophils and macrophages) and also leads to the activation of antigen specific adaptive immunity. The TLR signalling can also cause over activation of immune response and as a consequence fatal immune disorders can occur, so a negative feedback mechanism is required to prevent hyperactivation (Akira & Takeda, 2004, Takeda *et al.*, 2003, Takeda & Akira, 2005, Koppe *et al.*, 2012).

Toll receptor was first discovered as transmembrane receptor for establishing dorso-ventral polarity in *Drosophila* during embryogenesis (Hashimoto *et al.*, 1988). It showed remarkable similarity with the mammalian Interleukin-1 (IL-1) pathway, which leads to the activation of transcription factor NF- κ B, responsible for innate and inflammatory response (Belvin & Anderson, 1996).

So far, 12 mammalian TLRs (10 in humans and 12 in mice) have been discovered that are controlled by a family of regulator proteins adapters such as Myeloid differentiation factor (MyD88) that provide specificity to the response by each of these TLRs (Takeda & Akira, 2005, Koblansky *et al.*, 2013).

PLY has been shown to interact with TLR-4 and defined mutation in TLR-4 renders mice more susceptible to colonisation and invasive disease when intranasally challenged with a PLY producing pneumococcus compared to the WT mice (Malley *et al.*, 2003). An *in-vitro* study has shown TLR-4 dependent production of cytokines, chemokines (IL-1 β , IL-6 and KC) and neutrophil influx after high dose of PLY (Dessing *et al.*, 2009).

The role of other TLRs ((TLR-2 (van Rossum *et al.*, 2005) and TLR-9 (Albiger *et al.*, 2007)) in pneumococcal infections have also been studied *in-vivo* and some conflicting results have been reported. Pneumococcal cell wall components e.g. lipoteichoic acids (LTA) have shown to activate TLR-2 and TLR-2 dependent neutrophil influx (Dessing *et al.*, 2008). Neutrophil recruitment in a TLR-4 dependent fashion has also been found in another study after PLY instillation. These differences might be in part due to the redundancies that exist between different TLRs (Dessing *et al.*, 2009, Koppe *et al.*, 2012).

A study has shown that PLY interacts with other PPR; (Nod)-like receptor (NLR) P3 inflammasome independent of TLR-4. This interaction results in maturation of dendritic cells and production of IL-17A and IFN- γ by T cells. The study showed NLRP3^{-/-} deficient mice were more susceptible to infection compared to the WT group. The study also identified the NLRP3 activation is dependent on the pore forming ability of the toxin and activation of lysosomal Cathepsin B for IL1- β

release. IL-1 β has an important role in IL-17A activation and provides an important role against pneumococcal infection. IL-17 helps recruiting neutrophils and lymphocytes at the site of infection (Kafka *et al.*, 2008, McNeela *et al.*, 2010). The pore forming ability required to activate NLRP3 inflammasome was also shown by a study which demonstrated impaired IL-1 β production by strains with reduced haemolytic activity; for instance, ST 306 (Serotype 1). This might also explain that some strain (PLY004496) mentioned above have survival advantages in the blood within the first 15 hours of infection post infection since they do not engage the NLRP3 inflammasome thus facilitating the spread of bacterium in the blood. The full lytic activity of the toxin may hinder the early bacterial proliferation in blood and hence non-lytic strains show early growth advantage (Witzenrath *et al.*, 2011, Harvey *et al.*, 2011).

PLY induces damage in a variety of host cells and exerts its pathogenic effect by another mechanism known as programme cell death or apoptosis. The apoptotic response is usually observed in CNS following pneumococcal meningitis (Berpohl *et al.*, 2005, Braun *et al.*, 2002). This pathway involves the activation of p38-mitogen-activated protein kinase (MAPK) activation, calcium influx of calcium (Ca⁺²) mediated by pore forming ability of the toxin, reactive oxygen species (ROS) production and direct mitochondrial damage and release of apoptosis-inducing factor. PLY induced neuronal apoptosis is independent of caspases (1, 3 and 8) activation (Stringaris *et al.*, 2002, Braun *et al.*, 2007). In addition to neuronal cells, PLY can induce apoptosis in neutrophils, macrophages, dendritic and endothelial cells involving caspases activation (Dockrell *et al.*, 2003, Littmann *et al.*, 2009, Zhou *et al.*, 2012, Colino & Snapper, 2003, Zysk *et al.*, 2000).

Overall the toxin acts as a “double edge sword” during host-pathogen interaction whereby on one hand toxin stimulates excessive inflammatory response by releasing pro inflammatory mediators as described above and on the other hand toxin limits critical host immune responses (e.g. complement activation, inhibition of lymphocyte proliferation and antibody response) and thus exacerbating injury to the host (Marriott *et al.*, 2008).

Table 1-3 Biological effects of PLY on cells and tissues

Cells/Tissues Types	Effects
Alveolar and Bronchial epithelial cells	Disrupts cells and slows ciliary beating which in turn facilitates spread of the organism and infection in the surroundings (Rubins <i>et al.</i> , 1993, Steinfors <i>et al.</i> , 1989, Feldman <i>et al.</i> , 1990).
Pulmonary endothelial cells	Disrupts pulmonary endothelial barrier and thus possibly helps in disseminating bacterium from lung to blood stream (Bacteremia). Also activates phospholipase A contributing to inflammation and tissue damage (Rubins <i>et al.</i> , 1994, Rubins <i>et al.</i> , 1992).
Neutrophils	<p>Inhibition of respiratory burst, chemotaxis and migration of neutrophils and thus facilitating the spread of the organism (Paton & Ferrante, 1983).</p> <p>Dysregulated release of IL-8 which is a neutrophil chemo attractant, resulting in excessive inflammation and damage to the respiratory epithelium. This favours microbial spread by subverting host immune response (Cockeran <i>et al.</i>, 2002).</p>
Monocytes and Macrophages	<p>Release of IL-1β and TNF-α from monocytes resulting in inflammation and tissue damage (Houldsworth <i>et al.</i>, 1994).</p> <p>Stimulates Nitric oxide (NO), TNF-α and IL-6 production and resulting in excessive inflammation and injury (Malley <i>et al.</i>, 2003, Braun <i>et al.</i>, 1999).</p>

Erythrocytes	Cause the lysis of red blood cells hence lytic activity must be abolished before using as vaccine (Mitchell <i>et al.</i> , 1989).
Lymphocytes and B cells	Inhibits lymphocytes proliferation and antibody production thereby altering the adaptive immune response (Ferrante <i>et al.</i> , 1984).
Dendritic cells	PLY inhibits DC maturation, release of pro inflammatory cytokines and induces caspase 1 dependent apoptosis. This helps pneumococcus to evade DC mediated host immune response (Littmann <i>et al.</i> , 2009).
Spleen	Induce IFN- γ production independent of its pore forming ability which in turns stimulates NO production (Baba <i>et al.</i> , 2002).

1.8 Pore formation

The pore formation mechanism of CDCs still remains controversial. There are two proposed theories by which CDCs, including PLY, forms pores in the cholesterol containing membranes (Figure 1.4). One model suggests that 1-2 toxins monomers bind to the membrane and forms a small pore and other monomers bind to the pre-existing pore followed by oligomerisation and this eventually results in the formation of a large pore. This model was based on studies done on Streptolysin O (SLO) (Palmer *et al.*, 1998). The second model was based on studies done on PFO and suggests that toxin monomers binds to the membrane and forms a prepore first by oligomerising on the membrane and then the entire oligomer inserts in the phospholipid layer to form a large pore (Hotze *et al.*, 2002, Shepard *et al.*, 2000). However, a study done by Heuck *et al.*, 2003 has demonstrated that SLO can also form a prepore state (Heuck *et al.*, 2003).

The prepore model is the most acceptable model based on studies done and in case of PLY, the cryo-EM study also confirms that it does not form pores as suggested by Palmer *et al.* 1998, rather a prepore is formed followed by a large oligomeric 260Å pore (Tilley *et al.*, 2005, Heuck *et al.*, 2010).

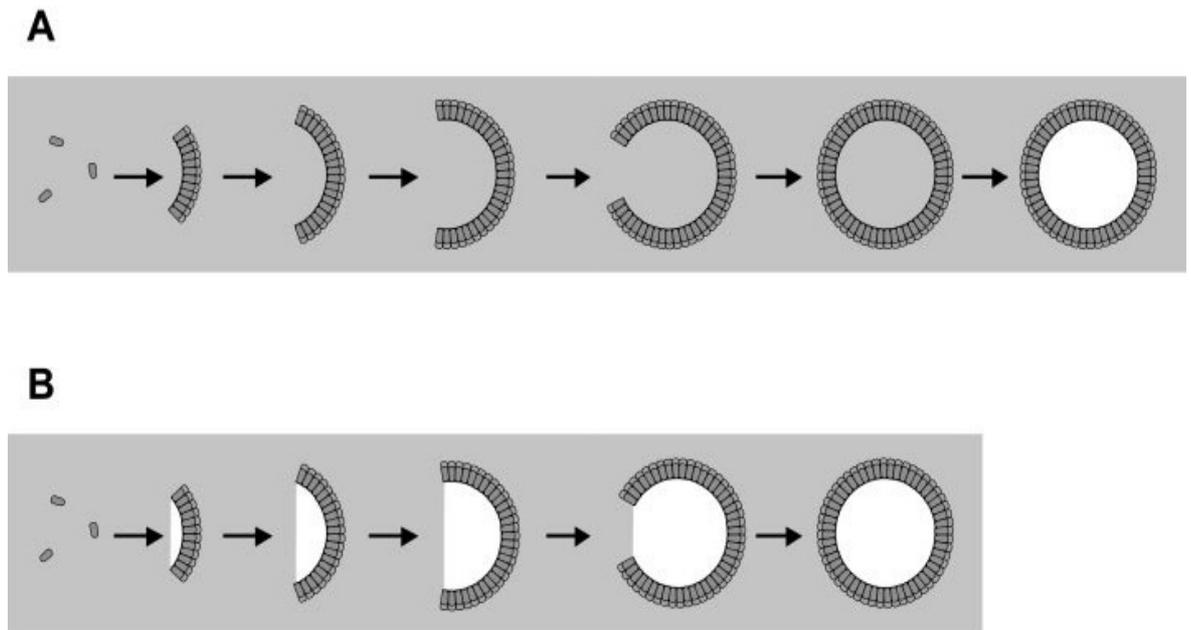


Figure 1-4 Schematic presentation of proposed models of pore formation by CDCs

A) Individual monomers bind to the membrane to form a prepore complex first which is followed by large transmembrane pore. B) Individual monomers insert and form a small pore followed by monomers joining the pre-existing oligomer, until the pore reached its desired size. The membrane is shown as light gray from above and the transmembrane pore is shown as white (Heuck *et al.*, 2003).

The 3 steps in pore formation include binding of the toxin, oligomerisation to form a prepore state and finally the formation of a large pore.

1.8.1 Binding

The initial step in pore formation is the binding to the toxin to the target membrane (Tweten, 2005, Flanagan *et al.*, 2009). It has been long proposed that toxin uses cholesterol as direct receptors since pre incubating the toxin with

free cholesterol blocks binding probably due to the saturation of the receptors on the toxin (Nollmann *et al.*, 2004).

The observations drawn from studies that CDCs use cholesterol as receptors got confounded with the discovery of Intermedilysin (ILY) a human specific CDC which interact via non sterol receptor human (hu) CD59 (Giddings *et al.*, 2004). However, in ILY initial binding does not require cholesterol but subsequent prepore and pore formation requires toxin interaction with cholesterol. This was shown by a study conducted by Giddings *et al.*, 2003 whereby depleting membrane cholesterol resulted in the failure of toxins (PFO, ILY and SLO) to form pores in the membrane (Giddings *et al.*, 2003). Thus cholesterol does not function only as receptor for binding but its functions extend beyond binding. Besides binding, cholesterol promotes oligomerisation step, triggers pore formation and stabilises pore (Rossjohn *et al.*, 1997).

D4 of the toxin initially interacts with the cholesterol containing membrane and CD59 in case of ILY (Giddings *et al.*, 2004, Baba *et al.*, 2001). A study has shown that recombinant D4 can bind to the cell membranes and immobilised cholesterol. Moreover, addition of purified D4 to cholesterol blocks the binding for the full length toxin and also the lytic activity. Hence D4 of the toxin was responsible for mediating initial contact of the toxin with the membranes and subsequent lytic activity (Baba *et al.*, 2001). A study conducted has shown that in Listeriolysin (LLO), one of the members of the CDCs, domains (1-3) can also bind to the cell membranes along with D4. The purified domains LLO-d1-3 and LLO-d4 can also reassemble into a hemolytically active form and slight mutations made in this linker renders protein inactive (Dubail *et al.*, 2001).

1.8.1.1 Undecapeptide and hydrophobic loops (L1-3)

Domain 4 of all CDCs has 11 amino acid conserved Trp rich loop (ECTGLAWEWWR) known as undecapeptide region (residues 427-437 in PLY). This region is well conserved among all CDCs with slight variation in some members (Figure 1.5). A number of mutations have been made in the undecapeptide region of the toxin to evaluate its contribution in binding and subsequent lytic activity (SekinoSuzuki *et al.*, 1996, Nakamura *et al.*, 1998). The insertion and extension of the toxin into cholesterol containing membranes was thought to be mediated by Trp-rich region which forms a loop like structure acting as a “hydrophobic dagger” (Rossjohn *et al.*, 1997). The importance of this region can be identified from the fact that the first tryptophan residue of the undecapeptide in PLY when substituted to phenylalanine (W433F) resulted in the significant reduction in the lytic activity of the toxin (Paton *et al.*, 1991).

Domain 4 also houses three short hydrophobic loops (L1-3) and these loops along with the tip of the undecapeptide region are in contact with the lipid bilayer while the rest of the domain 4 is surrounded by water (Ramachandran *et al.*, 2002). This led to the search for residues responsible for the initial interaction of D4 with membrane and a study showed that D4 loops (1-3) and not the undecapeptide are involved in the initial recognition of the toxin with the membrane (Soltani *et al.*, 2007). Further research to track down the amino acids was carried by performing mutational analysis of all residues present in the three loops. The study found that two amino acids (threonine, leucine) in Loop 1 PLY^(T459G.L460G) was responsible for mediating this initial interaction with the cholesterol containing membranes and was present in all CDCs (Figure1.5). In ILY, the toxin initially interacts with CD59 but the cholesterol recognition motif is responsible for subsequent steps since substitution of this motif TL>GG in ILY

toxin binds to the membrane resulting in the conformational change dissociating $\beta 5$ subunit and exposing $\beta 4$ which then interacts with $\beta 1$ strand of the neighbouring monomer which is always exposed (Ramachandran *et al.*, 2004, Tweten, 2005).

1.8.3 Formation of β barrel pore

The prepore oligomer upon reaching the size of complete ring structure is followed by the transition to pore complex. The 3α helices in D3 form 2 amphipathic transmembrane β hair pin loops (TMH). The pore formation commences upon insertion of these TMH per monomer from 35 to 50 monomers in the lipid bilayer eventually forming a large transmembrane β barrel pore. The vertical collapse of D2 of about 40 Å brings these TMH within the striking distance of the lipid bilayer and the β -barrel pore has a diameter of about 250 to 300 Å (Tweten, 2005, Czajkowsky *et al.*, 2004).

In summary, D4 first binds with the membrane via cholesterol recognition motif followed by insertion of the other loops and undecapeptide which strengthens the interaction (Farrand *et al.*, 2009, Ramachandran *et al.*, 2002). This is followed by oligomerisation where monomers bind and form a prepore complex. After oligomerisation, domain 2 begins to dissociate from domain 4 which brings the domain 3 closer to the membrane. Domain 3 unfolds and forms beta(β) hairpins loops which traverses into the membrane to form pores as shown in figure 1.6 below (Tilley *et al.*, 2005).

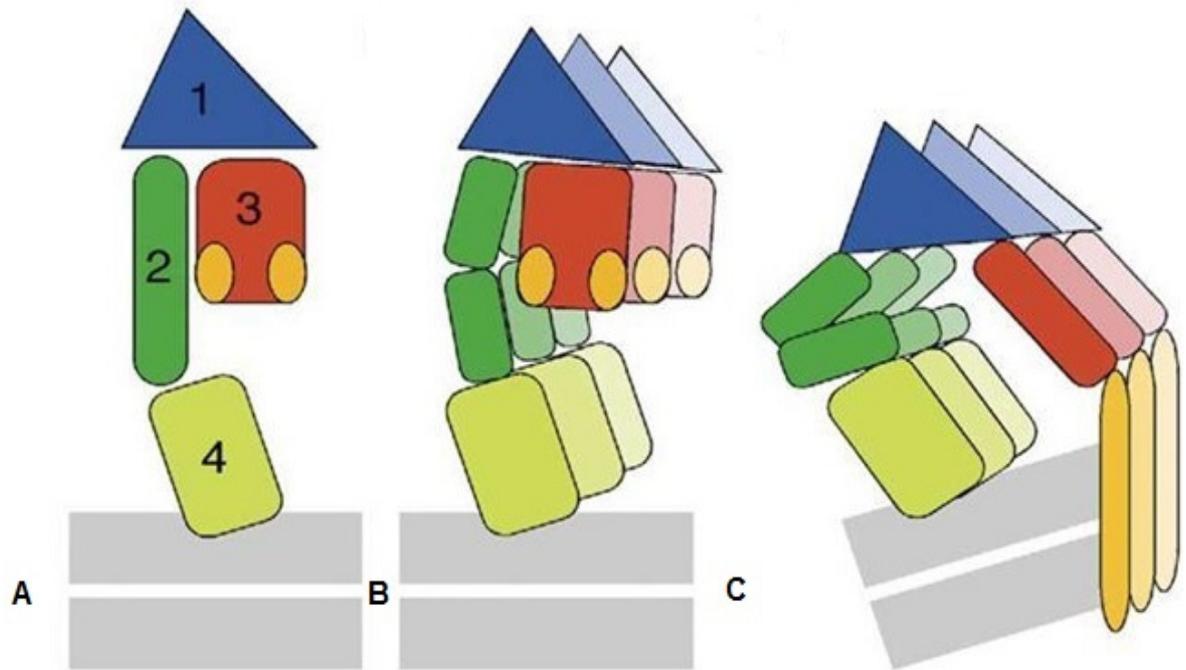


Figure 1-6 Different stages of pore formation of PLY

The domains are numbered (1-4) and the lipid bilayer is coloured grey. **A)** Monomer binding with the membrane. **B)** Prepore oligomer formed by 3 subunits. **C)** Dissociation of domain 2 and insertion of TMH forming β -barrel pore (Tilley *et al.*, 2005).

1.8.4 PLY as a vaccine candidate

PLY is one of the potential candidates to be used as vaccine since it is produced by nearly all serotypes with little variation over the time. PLY has been shown to provide protection when used alone (Kirkham *et al.*, 2006a), as a protein carrier with current PCV (Kuo *et al.*, 1995, Lee *et al.*, 2001) and as a mucosal adjuvant (Douce *et al.*, 2010). Anti-PLY antibodies given to mice also provide protection against the pneumococcal pneumonia (Garcia-Suarez *et al.*, 2004, Salha *et al.*, 2012). However, PLY is toxic to be used as a vaccine ((forms large pores on host cell membranes) (Mitchell, 2006)) so a number of mutants were devised to reduce its toxicity yet maintaining its immunogenicity (Paton *et al.*, 1984).

A number of mutants with reduced cytotoxic activity have been devised by site directed and random mutagenesis approach and tested as vaccines in animal models (Hill *et al.*, 1994, Berry *et al.*, 1995). One of the well characterised mutants is PdB with a single amino acid substitution of phenylalanine instead of tryptophan at position 433 in the undecapeptide region. PdB toxoid has shown to be protective in animal models when used alone and in combination with CbpA (Goulart *et al.*, 2013, Paton *et al.*, 1991, Neill *et al.*, 2013). However, this mutant has unacceptable levels of toxicity (0.1 to 1% residual haemolytic activity compared to the WT) and therefore cannot be used as a vaccine (Paton, 1996, Korchev *et al.*, 1998).

Recently, a toxoid version of the vaccine (PLYD1) has been made by making three substitutions (T65C, G293C and C428A) and has been shown to provide protection against pneumococcal lung injury and infection. This toxoid is designed based on the structure of the toxin and apart of its haemolytic activity, no other *in-vitro* experiments regarding its biological activity (binding assays, immune parameters) were mentioned in the study (Oloo *et al.*, 2011). The result of this study and a previous study has shown that substituting C428A has no effect on the lytic activity of the toxin (Saunders *et al.*, 1989).

Another non-lytic PLY version (L460D) is constructed by mutating one of its cholesterol recognition motif and has shown to provide protection against pneumococcal colonisation in mice. However, the results of the excipient assays showed that toxoid was exhibiting aggregation phenotype without adding sugars (trehalose, sucrose and sorbitol; which exert stabilising effects) and was not conformationally stable when adsorbed on the surface of alhydrogel

(Alhydrogel is one of the currently licensed adjuvant to be used in human vaccines) (Hu *et al.*, 2013).

1.8.5 $\Delta 6$ PLY a potential vaccine candidate

$\Delta 6$ PLY, a toxoid created by deleting the two amino acids at alanine and arginine positions 146 and 147 is one of the potential candidates to be used as a vaccine (Kirkham *et al.*, 2006a). Protein alignment has also shown the presence of these residues in other CDCs (Figure 1.7).

The deletion of two amino acids resulted in the loss of oligomerisation and subsequent pore formation but the toxin still binds to the membranes. This mutant unlike PdB, was non-lytic even at concentrations $>1\text{mg/ml}$ and was immunogenic and confer protection to mice challenged with *S.pneumoniae* (Kirkham *et al.*, 2006a).

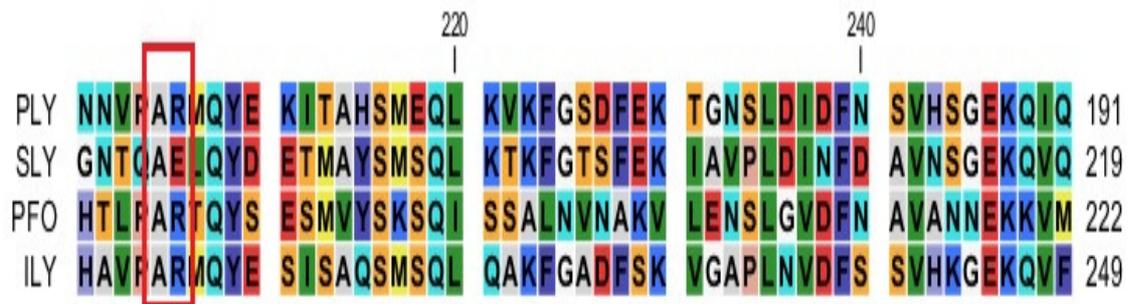


Figure 1-7 Protein alignment of CDCs showing highlighted residues alanine and arginine rendering CDCs non-lytic

The protein sequence of all CDCs was obtained from NCBI and protein alignment was done using CLC Genomics Workbench3.

$\Delta 6$ PLY fused with a protein antigen given to mice has proven to be a strong mucosal adjuvant eliciting a strong antibody response to the antigen fused (Douce *et al.*, 2010). This antigen response is independent to the fused antigen

and various other pneumococcal surface proteins (PspA, PspC and PhtD) fused with $\Delta 6$ PLY have been tested for their adjuvanticity in the TJM group laboratory (T.J.Mitchell, personal communication, 2011).

However, purified $\Delta 6$ PLY was shown to cause red blood cells to aggregate. The fluorescence microscopy images and FACS experiments also showed the red blood cells aggregation with $\Delta 6$ PLY (This work and T. J. Mitchell, personal communication, 2009)

This aggregation phenomenon makes the toxin unsuitable for using as a vaccine and therefore requires further investigation as to which residue/s are responsible for causing this aggregation behaviour. The identification of this residue/s will lead to the non-aggregation, non-lytic and immunogenic variant which can be used in vaccines alone, in combination with other pneumococcal protein antigens and/or conjugated to the capsular polysaccharide vaccines.

Aims of the study

The aim of this study was to identify the toxin residue/s responsible for causing the aggregation/agglutination phenotype of the red blood cells.

A number of molecular biology techniques (Site directed mutagenesis/Random mutagenesis/Alanine scanning mutagenesis) were used to track down the residue/s responsible for this phenotype. A number of mutants were designed and were tested in a 96 well round bottom plate.

As the study progressed we were also able to identify the importance of residues in terms of maintaining the toxin's structure and their contribution in the lytic activity.

ASM (Alanine scanning mutagenesis) was done for each individual residue of D4PLY of the toxin and its lytic activity was measured.

2 Materials and Methods

2.1 Materials

2.1.1 Chemicals

All chemicals used in this study were purchased from Sigma Aldrich, Fisher Scientific, Loughborough, UK, unless otherwise stated.

2.1.2 Enzymes

The oligonucleotides used in this study were purchased from Sigma Aldrich, UK. The restriction enzymes and GoTaq DNA used was purchased from Promega, UK.

2.1.3 Proteins kits and supplies

The Quick Change® II XL site directed-mutagenesis kit used in this study, for constructing site directed mutants, was purchased from (Stratagene,UK).The random mutants constructed using Mutation Generation System™ Kit (MGS™ Kit) was purchased from (Finnzymes, UK). The prepacked HisTrap™ HP affinity columns for Immobilized Metal Ion Affinity Chromatography (IMAC), Hitrap™ Capto Q™ for anion exchange and PD10-Desalting columns for buffer exchange were obtained from GE Healthcare Buckinghamshire, UK. The Bio-scale™ Mini Profinity eXact™ 1ml cartridges for tagfree purification was purchased from Bio-Rad,UK.

2.1.4 Antibiotics

The antibiotics used routinely were ampicillin, kanamycin and chloramphenicol. The stock solutions of these antibiotics were made in respective solvent i.e. (dH₂O or ETOH), filter sterilised using 0.2µm filter and stored at -20°C until required. The working concentration of antibiotic was calculated and added to the sterile broth media and agar after cooling them.

2.2 Bacterial strains

E.coli strains were grown overnight from a single colony with appropriate antibiotics in Luria Broth (L.B) at 37°C degree shaking at 180 rpm. 1 ml aliquots were made and were stored with 15% Glycerol at -80°C. The XL-1 blues (Stratagene,UK) were used for cloning and for storage of plasmids. The BL-21 (DE3) (Stratagene,UK) chemical competent cells were used for protein expression.

2.3 Vectors

2.3.1 pET33b and pPAL7

The plasmids used in this study are listed in table 2.6. Plasmid pET33b(+) (Novagen,UK) containing a kanamycin resistance gene along with pPAL7 containing ampicillin resistance gene (patented vector from Biorad) were used in this study. The former was used for making site directed mutants and latter was also used for generating random mutations in PLY gene.

2.4 General molecular biology techniques

2.4.1 PCR

PCR was performed using 200 μ l PCR tubes from (Starlabs, Milton Keynes, UK) using Techgene thermal cycler (Bibbi Scientific, Staffordshire UK). The annealing temperature varied accordingly for different reactions depending on the melting temperature (T_m) of the primer used. A typical PCR reaction is outlined below.

Table 2-1 Conditions used for PCR Reactions

Reaction Step	Temperature ($^{\circ}$ C)	Time (Seconds)
1X Initial Denaturation	95	60
30X		
Melting	95	45
Annealing	50	45
Extension	72	60 per kb length
1X Final Extension	72	600

2.4.2 Agarose gel electrophoresis

The agarose gels 0.8% w/v in tris-acetate-EDTA (TAE) buffer were used for analysis of plasmid DNA and PCR products and restriction digests. 100ml of the gel was prepared and for staining and visualization of products, 5 μ l of SyberSafe was added after cooling. 5 μ l of the products and 0.4 μ g of 1Kbp+ DNA standard ladder (Invitrogen, Paisley UK) were loaded and gel was run with 1 X TAE buffer at 100 Volts for 20 minutes. The UVpro-gel documentation system (UVtech) was used for gel visualisation and images.

2.4.3 dNTPs

For PCR reaction mixes, a set of dNTPs (dATP, dCTP, dGTP, dTTP) from Invitrogen was used. 10 μ M final concentration was made in a ratio of 1:1:1:1 in DNase/RNase ultra pure H₂O and store at -20°C until required.

2.4.4 Plasmid purification

Plasmid DNA was purified using QIAspin Miniprep Kit (Qiagen) according to the manufacturer's protocol.

2.4.5 Bacterial transformation

All plasmid DNA generated (listed in table 2.6) were transformed in chemical competent XL-1 Blues and BL-21(DE3). 100ng of the expression vector was added to 50 μ l of *E. coli* (either XL-1 Blues or BL-21 (DE3)) in pre chilled 1.5ml ependorfs. After 20 minutes of incubation on ice, cells were heat-shocked for 30sec at 42°C. The cells were kept again on ice for 2 minutes and 0.5ml of L.B broth was added and the reaction was incubated for 1 hour at 37°C. After incubation, 200 μ l of the culture was plated on L.B agar with appropriate antibiotics (kanamycin or ampicillin) at 50 μ g/ml. In random mutagenesis reactions, L.B agar was supplemented with 10 μ g/ml chloramphenicol (since transposon has chloramphenicol resistance gene) and 50 μ g/ml ampicillin (target plasmid). The next day colonies were picked and colony PCR reaction was done for successful transformation using primers flanking to the gene inserted.

2.4.6 Sequencing

All plasmid DNA purified from the positive colonies (listed in table 2.6) were sent for sequencing to the Sequencing Service, University of Dundee using primers 7F and 7G listed in the list of oligonucleotides (Table 2.2).

2.4.7 Oligonucleotides

All oligonucleotides used in the study were reconstituted to 100mM concentration by addition of DNase/RNase free water. The 100mM was further diluted to 10mM working concentration by the addition of DNase/RNase free water. Both the stock and working concentration were kept at -20°C until required.

Table 2-2 List of oligonucleotides used in the study

Primer Reference	Primer Name	Sequence (5' to 3')	Primer Type (Sequencing/SDM/PCR)
7F	T7 old Fwd	TAATACGACTCACTATAGGG	Sequencing and PCR
7G	T7 old Rev	GCTAGTTATTGCTCAGCGGTG	Sequencing and PCR
23B	Delta6 forwards	GGTCAATAATGTCCCAATGCAGTATGAAAAATAACGGCTC	SDM
23C	Delta6 reverse	GAGCCGTTATTTTTTCATACTGCATTGGGACATTATTGACC	SDM
SDMD4	Forward(459,460)	CCTCTACCTGAGGATAGCCGCCTGTTCCCAAATAGAAATCGTCC	SDM
SDMD4	Reverse(459,460)	GGACGATTTCTATTTGGGGAACAGGCGGCTATCCTCAGGTAGAGG	SDM
<i>NotI</i>	Forward	GAATTCCTCGAGGCGCGGCATAAGCCCGAAAGG	SDM
<i>NotI</i>	Reverse	CCTTTCGGGCTTATGCCCGCGCCTCGAGGAATTC	SDM
PCRTagfree	Forward	GTAGAAGAAGACAAGCTCTTCAAAGCTTTG	PCR
PCRTagfree	Reverse	CCGCGCCTCGAGGAATTC	PCR

Delta6PLY363L>A	Forward	CAGCTTACAGAAACGGAGATGCACTGCTGGATCATAGTGG	SDM
Delta6PLY363L>A	Reverse	CCACTATGATCCAGCAGTGCATCTCCGTTTCTGTAAGCTG	SDM
Delta6PLYW>F	Forward	GTACCGGGCTTGCCTTTGAATGGTGGCGTACG	SDM
Delta6PLYW>F	Reverse	CGTACGCCACCATTCAAAGGCAAGCCCGGTAC	SDM

2.5 Construction of plasmids by site directed mutagenesis (SDM)

The Quick Change® II XL site directed-mutagenesis kit can successfully make point mutations, replacing amino acids and deleting or inserting single or multiple adjacent amino acids. The mutagenic oligonucleotide primers (25-45 base pair length) are designed which complement each other and to the opposite strand except in the middle where the desired mutations (deletions or insertions) have to be made. The PCR was set up with 100ng of the plasmid according to the conditions in table 2.3. The high-fidelity pfuUltra II DNA polymerase reads the entire plasmid on each cycle and plasmids with desired mutations are created. The resultant PCR product was incubated with 1µl of restriction enzyme *DpnI* at 37°C for 2 hours in order to digest the parental methylated plasmid. The nicked unmethylated plasmid was chemically transformed in *E.coli* (XL-1 blues) for nicked repair.

Table 2-3 Reagent composition of SDM Reaction

Reagent	Concentration and Volume µl
Forward Primer	15pmol, 15µl
Reverse Primer	15pmol, 15µl
dNTP	10mM, 10µl
Template DNA	100ng
10X Reaction Buffer	5µl
pfu Ultra II DNA polymerase	1µl

Table 2-4 Cycling parameters of a typical SDM reaction

Reaction Step	Temperature (°C)	Time (Seconds)
1X Initial Denaturation	95	120
18X		
Melting	95	60
Annealing	60	60
Extension	68	120sec/kb length
1X Final Extension	68	600

2.5.1 Construction of pET33bD4PLY^(T459G.L460G) and pET33beGFPD4PLY^(T459G.L460G)

The plasmids pET33bD4PLY and pET33beGFPD4PLY was used to make the desired mutation at position 459 and 460 (TL>GG) using site directed mutagenesis protocol mentioned in section 2.5. The primers used in this reaction are given in the list of oligonucleotides. The plasmids from successful colonies were purified and were sent for sequencing.

2.5.2 Construction of pPAL7Δ6 PLY

The pPAL7 containing PLY gene was taken and Δ6 PLY was made by deleting the two amino acids using mutagenic primers 23B and 23C using the site directed mutagenesis kit following the same protocol described in section 2.5.

2.5.3 Construction of pET33beGFPΔ6 PLY^(L363A), pET33beGFPΔ6 PLY^(T459G.L460G) and pET33beGFPΔ6 PLY^(L363A. T459G.L460G)

The mutation 363 (lysine>alanine) was introduced in pET33beGFPΔ6PLY using SDM Kit with SDM L363A primers and following the same protocol outlined in

section 2.5. The pET33beGFP Δ 6PLY was also used to make double mutation T459G, L460G using the same set of primers as used before for making this mutation in pET33bD4PLY. Finally, a triple mutant was made pET33beGFP Δ 6PLY (L363A, T459G, L460G) by using pET33beGFP Δ 6PLY L363A and introducing the double amino acid substitution (TL>GG) using the same set of primers and protocol as explained in section 2.5.

2.6 Mutations made by random mutagenesis (RM)

A panel of mutants was generated in pPAL7 WTPLY and pPAL7 Δ 6PLY by random transposon-based insertion of five amino acids using Mutation Generation SystemTM Kit (MGSTM Kit) (Taira *et al.*, 1999, Haapa *et al.*, 1999). The system involves a bacteriophage Mu that inserts a transposon (Entranceposon) into any target plasmid at random sites. This *in-vitro* transposition reaction is catalysed by an enzyme MuA Transposase.

The insertion clones generated as a result of this transposition reaction are digested by restriction enzyme *NotI* which removes the body of the Entranceposon. This results in the in-frame fifteen base pair insertion in the target DNA after the closure of *NotI* digestion clones by self ligation. Figures 2.1 and 2.2 showing how random 5 amino acid insertions are made using this kit.

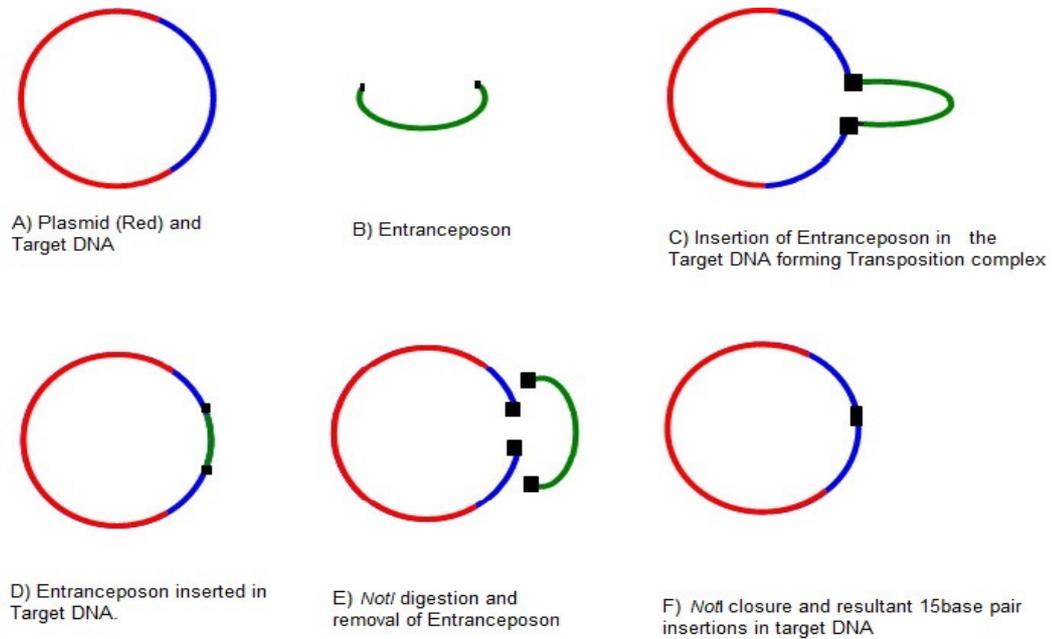


Figure 2-1 Different stages of *in-vitro* transposition reaction using mutation generation system™

A and B Target plasmid (Red), Target gene (Blue) and entranceposon (Green) (C and D) Entranceposon insertion in target DNA followed by *NotI* digestion (E and F) to remove the body of entranceposon and re-ligation with T4 DNA ligase resulting in 15 basepair insertion in target DNA. Figure made using Microsoft word 2007.

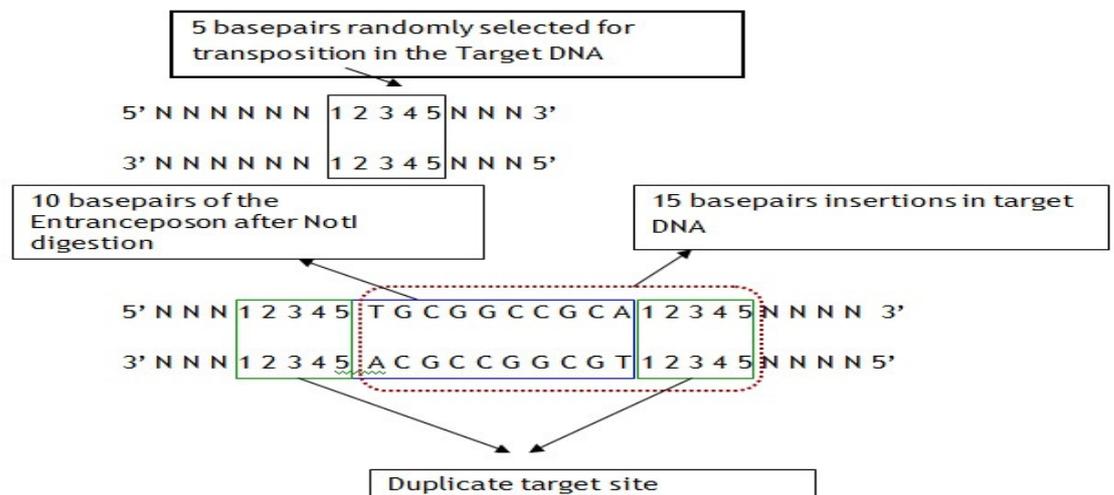


Figure 2-2 Random 15 basepair insertions in the target DNA. Figure modified from the online MGS™ instruction manual F-701

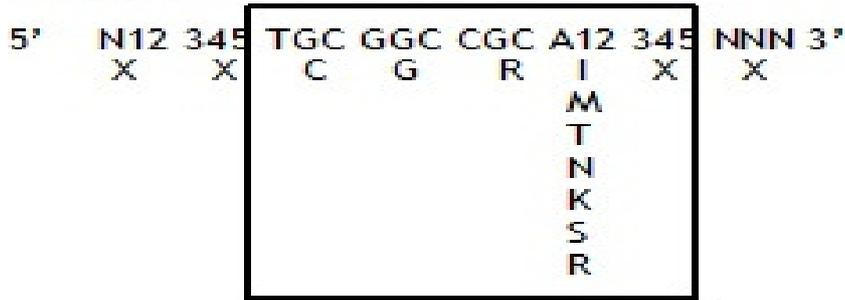
Table 2-5 Reaction protocol for the *in-vitro* transposition reaction

Reagent	Volume μl
Target DNA	size of target Plasmid(kb)x40ng (3 μl)
Reaction Buffer	4 μl
Entranceposon (M1-Cam ^R)	100ng (1 μl)
MuA Transposase	1 μl
Deionized water	11 μl

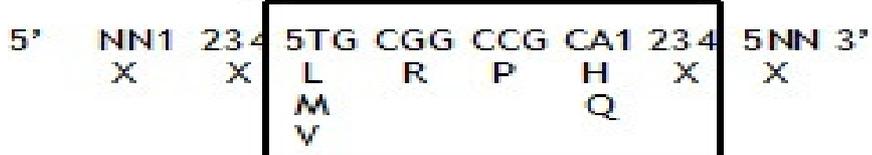
The reaction outlined in table 2.5 was allowed to proceed for one hour at 30°C and then MuA Transposase was deactivated by incubating at 75°C for 10 minutes. This plasmid with transposon was then chemically transformed into the XL-1 blues according to the protocol mentioned in section 2.4.5. The next day all the individual colonies were picked and colony PCR was performed with the PLY primers. The colonies with transposon were treated with *NotI* primer to remove the body of the transposon and were religated with T4 DNA Ligase (Figure 2.1). The plasmid was again chemically transformed again into the XL-1 blues and purified with Qiaspin Miniprep kit.

The purified plasmid was then sent for sequencing and a PCR reaction was also set up with *NotI* miniprimers supplied with the kit to check the site of five amino acid addition. The sequencing results were analysed using Vector NTi software programme (Invitrogen, UK) and insertion of 5 amino acids were confirmed. The translation of 15 base pair insertions were done using figure 2.3.

Frame 1



Frame 2



Frame 3



X = Any amino acid

X* = Any amino acid except Q, E, K, M, W

Figure 2-3 Translation of 15 base pair insertions in the three reading frames

Figure modified from the online MGS™ instruction manual F-701.

2.6.1 Random mutagenesis in WT PLY and Δ6 PLY background

The transposon based five amino acid random mutagenesis was made in both pPAL7 WT PLY and pPAL7 Δ6 PLY vector according to the protocol outlined in table 2.5.

Table 2-6 List of plasmids generated in the study

Plasmid	Description	SDM/RM	Origin
pET33bD4PLY	Contains domain4 PLY gene attached to N-terminal His ₆ tag.		Dr Jiang Tao Ma
pET33beGFPD4PLY	Contains eGFP- D4PLY fusion attached to N-terminal His ₆ tag. The GFP was amplified from plasmid pNF320 and mutated to F64L and S65T using primers 24W and 24X and generating enhanced (e)GFP.		Dr Graeme Cowan and Dr Jiang Tao Ma
pET33bD4PLY ^(T459G.L460G)	Contains D4PLY gene with mutations at sites T459G.L460G attached to N-terminal His ₆ tag.	SDM	This work
pET33beGFPD4PLY ^(T459G.L460G)	Contains eGFP-D4PLY fusion with same mutations in D4PLY	SDM	This work

pPAL7 PLY	Contains the PLY gene without any tag attached. The gene encodes from residue M1 to D471.		Dr Jiang Tao Ma
pPAL7 Δ 6 PLY	Contains the PLY gene with two amino acids deletions at 146 and 147(AR) made by using SDM primers 23B and 23C.	SDM	This work
pPAL7 Δ 6 PLY ^(W433F)	Contains the PLY gene with two amino acids deletions at position (146,147) (AR) and one amino acid substitution at position W433F by using primers mentioned in the oligonucleotide list (Table 2.2).	SDM	This work
pET33beGFP Δ 6PLY	Contains eGFP- Δ 6 PLY fusion attached to N-terminal His ₆ tag. The Δ 6 version is made by deleting 2 residues	SDM	Dr Jiang Tao Ma

	(146,147) (AR) by using primers 23B and 23C.		
pET33beGFPΔ6PLY ^(L363A)	Contains eGFP-Δ6 PLY ^(L363A) fusion attached to N-terminal His ₆ tag. This mutant contains additional substitution at residue L363A using mutagenic primers.	SDM	This work
pET33beGFPΔ6PLY ^(T459G.L460G)	Contains eGFP-Δ6 PLY ^(T459G.L460G) fusion attached to N-terminal His ₆ tag. This mutant contains additional substitutions at residues T459G.L460G using mutagenic primers.	SDM	This work
pET33beGFPΔ6PLY ^(L363A.T459G.L460G)	Contains eGFP-Δ6 PLY ^(L363A.T459G.L460G) fusion attached to N-terminal His ₆ tag. This mutant contains additional substitutions at residues	SDM	This work

	T459G.L460G and L363A using mutagenic primers.		
pPAL7 PLY-1	Contains PLY gene with 5 additional residues after residue T410.	RM	This work
pPAL7 PLY-2	Contains PLY gene with 5 additional residues after residue Q24.	RM	This work
pPAL7 PLY-6	Contains PLY gene with 5 additional residues after residue K389.	RM	This work
pPAL7 PLY-9	Contains PLY gene with 5 additional residues after residue D312.	RM	This work
pPAL7 PLY-12	Contains PLY gene with 5 additional residues after residue F112.	RM	This work
pPAL7 PLY-13	Contains PLY gene with 5 additional residues after residue G305.	RM	This work

pPAL7 PLY-19	Contains PLY gene with 5 additional residues after residue E441.	RM	This work
pPAL7 PLY-21	Contains PLY gene with 5 additional residues after residue W433.	RM	This work
pPAL7 Δ 6 PLY-1	Contains Δ 6 PLY gene with 5 additional residues after residue Q136.	RM	This work
pPAL7 Δ 6 PLY-5	Contains Δ 6 PLY gene with 5 additional residues after residue G326.	RM	This work
pPAL7 Δ 6 PLY-13	Contains Δ 6 PLY gene with 5 additional residues after residue S318.	RM	This work
pPAL7 Δ 6 PLY-27.10	Contains Δ 6 PLY gene with 5 additional residues after residue R449.	RM	This work
pPAL7 Δ 6 PLY-29.4	Contains Δ 6 PLY gene with 5 additional residues	RM	This work

	after residue N7.		
pPAL7 Δ 6 PLY-29.5	Contains Δ 6 PLY gene with 5 additional residues after residue Q215.	RM	This work
pPAL7 Δ 6 PLY-35	Contains Δ 6 PLY gene with 5 additional residues after residue L365.	RM	This work
pPAL7 Δ 6 PLY-44.1	Contains Δ 6 PLY gene with 5 additional residues after residue N420.	RM	This work
pPAL7 Δ 6 PLY-44.2	Contains Δ 6 PLY gene with 5 additional residues after residue T410.	RM	This work

2.7 Library of mutants from DNA 2.0

A library of mutants was designed and purchased commercially from DNA2.0 (USA). The library was devised in such a way that every single residue in D4PLY was replaced by alanines and where alanines were present in the gene, they were replaced by glycines. The mutations were made both in WT PLY background and Δ 6 PLY background. In total 112 mutants were made each in WT

PLY and $\Delta 6$ PLY background. The mutants were constructed in vector pJexpress 401 with a six His tag attached on the N-terminal of the gene and an upstream T5 promoter which is IPTG inducible.

The mutants were received from DNA 2.0 (CA, USA) stabbed in 96 well plates in L.B agar. Once the mutants were received, they were re grown in 5ml LB supplemented with 50 μ g/ml Kanamycin. All mutants were grown and glycerol stocks for all mutants were made in duplicates and were stored at -80° C.

2.8 Expression and purification of the recombinant proteins

This section elaborates the method deployed for the expression and the purification of the different proteins. Specific features of the proteins are discussed in the subsequent chapters.

All proteins used in this project were purified by the author except for the purified eGFP only. eGFP was purified by one of the previous members of TJM group, Dr Jiang Tao Ma from expression vector pET33beGFP. This protein was used as control in fluorescent activated cell sorter experiments (FACS).

2.8.1 Isopropyl β -D-thiogalactopyranoside (IPTG)

IPTG used in this study for protein expression was purchased from (Melford, UK). 1M stock was prepared in dH₂O; filter sterilised with a 0.22 μ m filter syringe and stored at -20°C until required.

2.8.2 Protein expression

The BL-21(DE3) cells containing expression vectors were grown in 2 X 1 Luria Broth (L.B) with 50µg/ml appropriate antibiotic. The flasks were incubated at 37°C with shaking at 180rpm. Once the O.D of the culture reached to 0.8-1.0, 1mM Isopropyl-β-D-thio-galactopyranoside (IPTG) at a final concentration was added into the culture to induce expression. After inducing with IPTG, cells were further grown for 6 hours in L.B at 30° C with shaking at 180rpm. After that the cells were centrifuged at 4000rpm at 4°C for 35 minutes using a Beckman J-6B large volume centrifuge. The pellet was saved and the supernatant was discarded.

Each individual mutant of the library was grown in 10ml overnight culture with 100µg/ml kanamycin. 1ml of this overnight culture was then grown in 250ml conical flask having 50ml Luria Broth (L.B) with 50 µg/ml Kanamycin. The expression was further carried in the same way as the described in 2.8.2.

2.8.3 Cell lysis

Cell pellet was thawed on ice and was re-suspended in pre-chilled 15ml/litre with phosphate saline buffer (PBS) (Oxoid,UK). 300 units of DNase I and 10mg of proteinase inhibitor benzamidine (Sigma-Aldrich Dorset,UK) was added per litre of cell culture. The cell suspension was sonicated 5 times for 30 seconds with a minute break between each cycle at 100 % by cell sonicator (Constant systems,Ltd, Warwick,UK). The cell lysate was then centrifuged at 14000g for 30 min at 4°C in a 4K15 centrifuge (Sigma-Aldrich-UK). The supernatant were filtered by syringe using 0.22µm filter and the pellet discarded.

The cell lysis of the individual mutants of the library was done the same way as all other mutants except 1ml of pre-chilled phosphate saline buffer (PBS) was added and no proteinase inhibitors or DNase were added. The individual cell suspension was sonicated 5 times for 10 seconds with a 30 seconds break between each cycle at 10 Amplitude microns by cell sonicator. (Soniprep 150 MSE Ltd,UK). The cell lysate was then centrifuged at 13000g for 5 minutes in a table top centrifuge. The supernatant was saved and the pellet discarded.

2.8.4 Protein purification

The two methods were used for the purification of recombinant proteins during the study. The proteins with a His tag attached on the N-terminal were purified in a two step purification system. The proteins without a tag were purified in a single step purification following Profinity eXact™ protein purification system protocol as explained in section 2.8.7.

2.8.5 Nickel affinity chromatography

The pET33b vector allows addition of six histidines (His tag) attached to N-terminal sequence of the required protein so all the recombinant proteins having His tag were purified by immobilized metal affinity chromatography first since His tag can bound tightly to the metal. The buffers used in the purification steps are outlined in the table 2.7. 1ml Histrap FF crude column (GE-Healthcare) was equilibrated 5X times with the wash buffer. The bacterial supernatant was passed through the column using a bench-top peristaltic pump (Master flex pump C/L) at a rate of 2ml/minute. The His tagged proteins bound to the column while other proteins and contaminants flowed through. The column was again

washed 5X with the wash buffer to remove any unbound proteins and then the column was attached to the ÄKTAPrime™ Plus Purification system (GE Health Care) on which the Nickel Affinity Programme was run. The trapped proteins were eluted with a continuous gradient of 0-500mM of imidazole in PBS. The purified protein fractions were run on a sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) gels as described in section 2.9.1.

Table 2-7 Nickel affinity chromatography (NAC) buffers

Name	Composition
Nickel Affinity Wash Buffer	20mM imidazole in 1 X PBS. Filter sterilised with 0.22µM filter
Nickel Affinity Elution Buffer	500mM imidazole in 1 X PBS. Filter sterilised with 0.22µM filter
Phosphate Buffered Saline (PBS)	NaCl 8g/L KCL 0.2g/L Na ₂ HPO ₄ 1.44g/L KH ₂ PO ₄ 0.24g/L pH adjusted to 7.4 and filter sterilised with 0.22µM filter.

2.8.6 Anion exchange chromatography

The selected fractions from Nickel Affinity Chromatography were buffer exchanged into Anion Exchange Wash buffer to remove contaminating proteins, lipopolysaccharides (LPS) and DNA. 1ml Hi Trap Cpto Q column (GE Health Care)

was washed 5X with anion exchange wash buffer at a rate of 2ml/minute. After washing the proteins were then loaded on the column at a rate of 1ml/minute to allow for maximum binding, washed again 5X times with the Anion exchange wash buffer before attaching to the AKTAPrime™ Plus Purification system (GE Health Care) on which the anion exchange programme was run. The proteins were eluted using a linear continuous gradient of anion exchange elution buffer. The collected fractions were run on SDS-PAGE gels.

Table 2-8 Anion exchange buffers (AEC)

Anion Exchange Wash Buffer	20mM tris-HCL pH 8.0. Filter sterilised with 0.2µM filter
Anion Exchange Elution Buffer	20mM tris-HCL and 500mM NaCl at pH 8.0. Filter sterilised with 0.2µM filter

2.8.7 Profinity eXact™ protein purification system

The pPAL7 uses the exact affinity cleavage technology by utilising an immobilized, extensively engineered protease, subtilisin S189 which binds to a small N-terminal co-expressed affinity tag in the protein fusion. The protease performs a specific, controlled cleavage after the addition of elution buffer; resulting in the removal of tag from the fusion protein directly on the column. During the elution step, the cleaved tag remain tightly bound to the column's resin resulting in the release of highly purified recombinant protein without any tag attached to the N-terminus.

The buffers used in the tagfree purification are listed in the table 2.9. 1ml of Bio-Scale Mini Cartridge was washed with 10ml of wash buffer at a rate of 3ml/minute using a bench-top peristaltic pump (Master flex pump C/L). The cell lysate was loaded on the column at a rate of 1ml/minute to allow maximum binding of the tag to the column resin. The elution buffer was then run through the cartridge at rate of 2ml/minute so as to saturate the cartridge with the elution buffer. The peristaltic pump was then stopped and the cartridge was incubated with elution buffer for 30 minutes at room temperature. The elution buffer was again run at a rate of 3ml/minute and the purified tagfree protein was eluted from the column leaving the tag attached to the resin. The protein was run on SDS-PAGE gel to confirm the presence of proteins.

Table 2-9 Profinity exact buffers

Name	Composition
Wash Buffer	100mM sodium phosphate in dH ₂ O.pH 7.2 adjusted with acetic acid. Filter sterilised with 0.22µM filter.
Elution Buffer	100mM sodium phosphate and 100mM sodium fluoride in dH ₂ O.pH 7.2 adjusted with acetic acid. Filter sterilised.

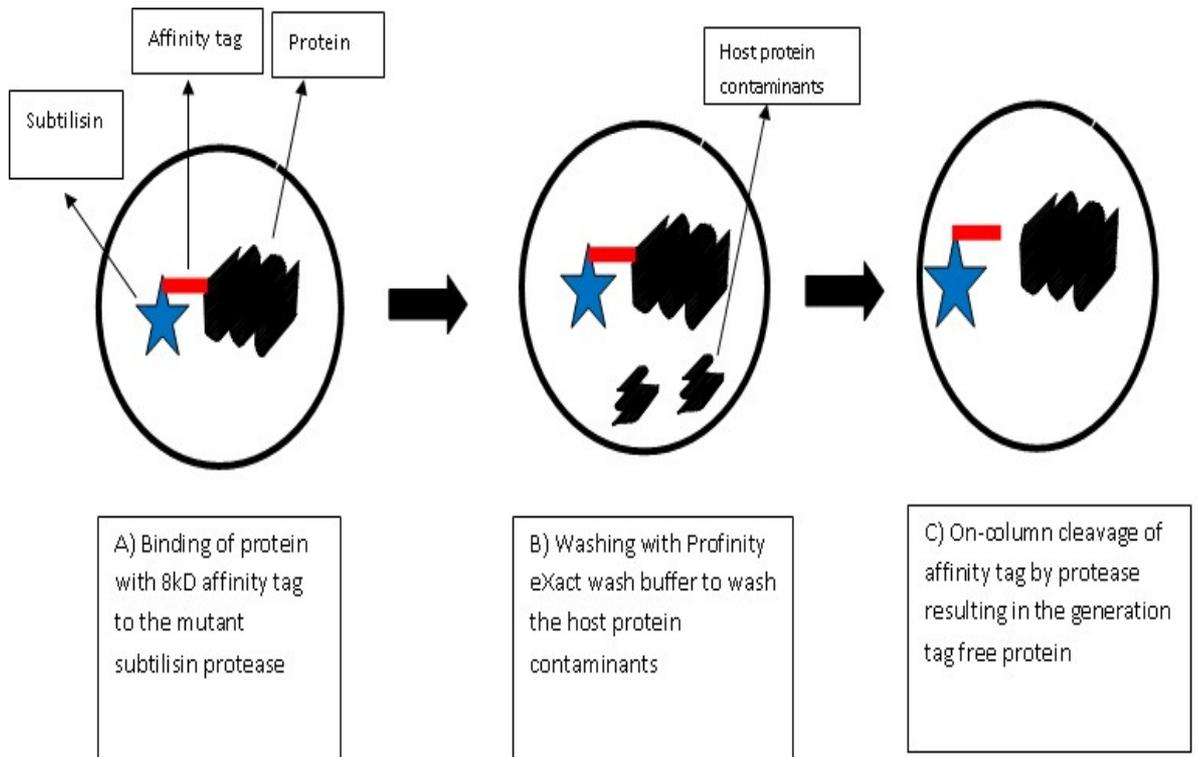


Figure 2-4 Profinity eXact purification system

The figure showing stages of Tagfree purification system. On column binding of protein followed by washing with wash buffer to remove contaminants and then cleavage of tagfree protein using elution buffer shown in A, B and C respectively. Figure drawn using Microsoft word 2007.

2.8.8 Buffer exchange of protein samples

The protein samples of 15ml or above were buffer exchanged in a dialysis tubing of 8kDa molecular weight cut off (Medicell international Ltd, London, UK). The proteins were dialysed against a greater than 50 fold volume of the buffer to be exchanged at 4° C for 30 hours. The proteins samples of less than 15ml were dialysed using PD-10 desalting columns (GE Healthcare) according to the manufacturer's protocol.

2.8.9 Storage of protein samples

All recombinant proteins were buffer exchanged to PBS using the protocol mentioned in 2.8.8. Before storage, purified proteins were concentrated by using Amicon Ultra Centrifugal Concentrators 50, 30 or 10kDa (Millipore, Watford UK) cut off depending on the size of the protein sample. The sample was centrifuged at 4000g, 4°C for 2 minutes in a 4K15 centrifuge (Sigma Aldrich). This step is repeated until desired concentration of protein is reached. This additional step further removes any contaminants from the purified protein. The proteins were aliquoted into 0.25ml volumes at -80°C for long time storage and at -20°C and for short term storage.

2.8.10 Concentration of the purified protein

The concentration of the purified proteins was measured by using a derivation of Beer-Lambert law.

$$A_{\lambda} = \epsilon c L$$

Where A = Absorbance at 280nm (A₂₈₀)

ϵ = extinction coefficient of the protein sample

L = Light path length in centimetres

$$\epsilon = A_{\lambda} / c L \text{ (Since } L = 1\text{cm)}$$

$$\epsilon = A_{\lambda} / c$$

The absorbance reading at 280nm (A280) was measured using a NanoDrop ND-1000 (Fisher Scientific, Loughborough UK). The Vector NTi software programme (Invitrogen, UK) was used to calculate extinction coefficient of the sample from amino acid sequence of the protein.

2.9 Characteristics of the purified proteins

2.9.1 SDS-PAGE

The SDS-PAGE gels used in this study were pre-made NuPage 4-12% bistris gels 15 wells from (Invitrogen UK). Additionally some gels were made following the TJM lab manual. The self-made gels (SDS 10% W/V) were poured using 10% resolving gel and 4% stacking gel and were run on Bio-Rad mini-protein apparatus. The protein marker SeeBlue® Plus 2 Pre-stained (Invitrogen UK) was used for indicating the size of the protein. 2-12µl of the protein was loaded with NuPage 4X loading buffer supplemented with 10mM dithiothreitol (DTT) and denatured for 10 minutes at 70°C in a heat block. The proteins along with the 5µl protein marker were loaded on the gel and the gel was run at 180 volts for 40 minutes. The SDS-PAGE was then stained with Coomassie® R250 stain for several hours and then destained with several changes of destain and dH₂O.

2.9.2 Western blotting

The proteins were further analysed by Western blotting. For Western blotting proteins were first run on the SDS-PAGE following the same protocol described in section 2.9.1. The proteins were then transferred to a Hybond™ -P PolyVinylidene Fluoride (PVDF) membrane (Amersham Biosciences GE Health

Care) using a Xcell II blotting module (Invitrogen) for 90 minutes at 30 volts. The membrane was blocked overnight in 3% skimmed milk in tris NaCl (pH 7.4) at 4°C. The membrane was blotted next day with anti-PLY primary antibody (1:1000 dilutions in 3% skimmed milk) for 2 hours at 37°C in the shaking incubator. The membrane was then washed five times with tris-NaCl (pH 7.4) and then blotted with horseradish hydrogen peroxide-linked secondary antibody (1:2000 dilution in 3% skimmed milk) at 37°C for 1 hour in the shaking incubator. The membrane was again washed five times with tris-NaCl (pH 7.4) before developing in the dark using 4-Chloro-1-naphthol (Sigma Aldrich UK) as developer. The reaction was stopped by addition of dH₂O.

Table 2-10 SDS-PAGE and western blotting reagents

1X Nu Page MES Running	Proprietary formulation	Diluted from 25X stock concentration with dH ₂ O
10x tricine running Buffer	30g tris, 144g glycine, 10g SDS	Diluted from 10x concentrated stock with dH ₂ O
Destain	50% dH ₂ O, 40% methanol and 10% glacial acetic acid	
Stain	0.5g Coomassie R250 stain in 0.5L Destain	

Tris NaCl (pH 7.4)	tris base 1.2g, NaCl 8.7g make up to 1L with dH ₂ O. pH with HCL	
Developer	30mg 4chloro-1-naphthol, 10ml methanol added to 40ml tris NaCl(pH 7.4). 30µl H ₂ O ₂ added in the end.	Make just before use

2.9.3 Red blood cells assays

The haemolytic assays were run to measure the haemolytic activity in WT PLY mutants and hemagglutination assays were run to observe the aggregation phenotype of $\Delta 6$ PLY mutants and D4 PLY mutants.

2.9.3.1 Haemolytic assay

The haemolytic assay was analysed by using 96 well round and flat bottomed wells (Costar, UK) by using a haemolytic assay described previously (Walker *et al.*, 1987), with slight modifications. 1ml horse blood (E&O Laboratories, Scotland) was taken and washed 5 times with PBS to remove the lysed cells and antibodies. 2% horse blood was prepared by adding 200µl of washed horse blood in 10ml of PBS. 50µl of PBS was added in all wells of R-plate using multi-channel pipette. Purified WT PLY of known concentration was diluted (1:1000) and added in the first well of row 1 as positive control and 50µl of PBS only was added in

the first well of second row as negative control. The 50 μ l of purified protein of known concentration or 10 μ l cell lysate and 40 μ l of PBS (lysate in case of DNA 2.0 library) were placed in duplicates in first wells of subsequent rows 3 to 8. Serial dilutions were made from of each row of by removing 50 μ l from previous well into the next well and repeating until 50 μ l is discarded from last well of all the row. 50 μ l of 10 μ M dithiothreitol (DTT) was placed in all the wells as reducing agent to oxidize any reduced CDC protein. 50 μ l of 2% of washed horse blood was added in all the wells and incubated for 15 minutes at 37°C. 50 μ l of PBS was added again in all wells and R-plate was centrifuged at 1000g for 1 minute in a 4K15 centrifuge (Sigma Aldrich, UK) to pellet the intact cells. 100 μ l supernatant was transferred from each well into the flat bottomed plate and absorbance was measured at 540nm by FLUOstar Optima plate-reader (BMG Labtech, UK) to measure the levels of haemoglobin released in each well.

From these results, the percentage (%) lysis in each well was calculated by using mean negative controls from PBS wells where 0% lysis has occurred, as 0% lysis value and the mean value from positive wells (Purified PLY) where 100% lysis has occurred, as 100% value. A curve of % lysis was generated against each well for each purified toxin. A typical sigmoid curve was then made by using GraphPad prism 5 software (GraphPad software, USA).

For cell lysates from DNA 2.0 library in WT PLY background haemolytic activity was measured by measuring the endpoint. The reciprocal of the dilution at which the endpoint is reached gives the Haemolytic Units (HU) per ml of sample.

2.9.3.2 Hemagglutination assay

The hemagglutination assay was carried out in the same way as the haemolytic assay described in section 2.9.3 with some modifications. The positive control in hemagglutination assays was $\Delta 6$ PLY and $\Delta 6$ PLY lysate as compared to WT PLY which was used in haemolytic assays. $\Delta 6$ PLY form red blood cells aggregates which can easily be appreciated in R-Plate after centrifugation. The hemagglutination assay was therefore used to screen the $\Delta 6$ PLY mutants which were not forming the typical red cell aggregates as formed by $\Delta 6$ PLY.

The different phenotypes observed during the hemagglutination experiments are shown in figure 2.5.

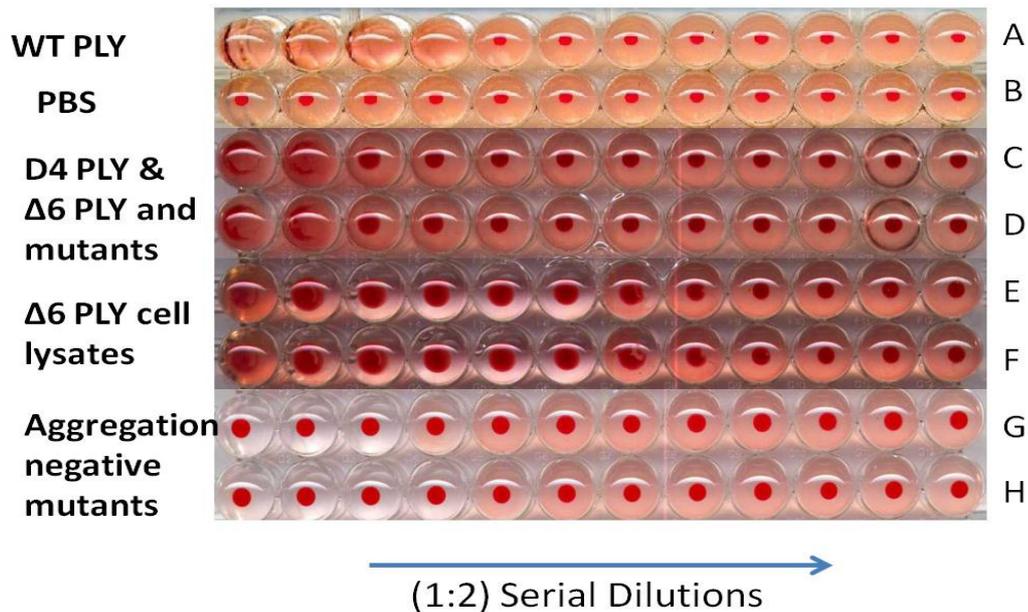


Figure 2-5 Phenotypes observed on a red cell assay

Figure 2.5 showing different phenotypes on a 96 well plate. A) WTPLY used as positive control causes complete lysis of sheep red blood cells and a pinkish tinge in wells is observed due to the released haemoglobin. B) PBS used a negative control shows no lysis and red blood cells as pellets are seen after the final centrifugation step. C&D) Purified D4, $\Delta 6$ PLY and their variants causing red blood cell aggregation and red cells appear to form clumps around the edges of the wells. This phenotype is marked in the first few wells and decreases as the concentration of protein drops in subsequent wells. E&F) $\Delta 6$ PLY mutant lysates of DNA 2.0 library also showing aggregation phenotype slightly different from purified proteins and G& H) aggregation negative mutants were not causing red blood cell aggregation and a red cell pellet was observed after the final step. The aggregation negative mutants showed a clearing effect (white background instead of pink as seen in PBS wells) in the first few wells, the cause of which is unknown.

Figure 2.5 showing different phenotypes on a 96 well plate. The WT and the mutant proteins displayed these phenotypes during the red cell assay experiments. The different phenotypes include complete lysis in the wells shown by WT PLY, no lysis by PBS, red cell aggregation phenotypes by D4, $\Delta 6$ PLY and $\Delta 6$ PLY lysates of the DNA 2.0 mutant library and clearing well effect. The clearing effect means that the usual pinkish tinge in wells after the last centrifugation step is not observed as seen in PBS treated wells, rather a white background seen is in aggregation negative mutants. The reason for this clearing of wells is unknown and probable reasons are discussed in section 3.8.

2.9.4 Fluorescence microscopy

The fluorescent microscopy images were taken to show the mutant protein binding to horse erythrocytes. The horse red blood cells were prepared in water and PBS. 1ml of horse blood was taken in 2 eppendorfs and centrifuged at 13k rpm for 1 minute. The supernatant was removed and each pellet was resuspended in PBS and water. The cell suspension was centrifuged again and supernatant removed. The above procedure was repeated three times. 100 μ l of 2% horse blood and 140nM of the purified proteins were added to eppendorfs. For controls 100 μ l of horse blood only and 100 μ l of 2% horse blood with 140nM of eGFP only was taken. The eppendorfs were incubated for 15 minutes at 37°C and then centrifuged at 1500g for 5 minutes. The supernatant was discarded and the pellet was again resuspended in PBS. 200 μ l of the sample with 200 μ l of 4% paraformaldehyde (w/v) and incubated at room temperature for 20 minutes to fix cells. After incubation, the cells were again washed with PBS to remove any unbound proteins. 20 μ l of sample was put on glass slide and was air dried. A

drop of fluorescence mounting media (Dako UL Ltd, UK) was put directly on the spot and the cover slip placed directly over it. The slides were either kept on dark or observed under the microscope with ultraviolet (UV) light. The images were generated using the software velocity Image analysis software (PerkinElmer, USA).

2.9.5 Cell binding assay by fluorescent activated cell sorter (FACS)

The protein binding was assessed using fluorescent activated cell sorter (FACS). 4% of horse blood was made after washing the horse blood with PBS as mentioned in 2.9.5. The proteins were diluted to 14nM/L, 140nM/L and 1400 nM/L. Proteins samples were added to the washed horse blood in FACS tubes and incubated at 37° C for 15 minutes. For controls eGFP only at three concentrations (14, 140 and 1400nM) and RBCs only were taken. The numbers of events noted for each reading was 50,000 and each sample was run in quadruplet. The proteins were transferred to the FACS tubes and readings were noted using the Cell Sorter.

The results were analysed using the Flowjo Software. Red blood cells suspended in PBS have a known amount of cell auto-fluorescence. To minimise the background cell auto-fluorescence gates FL1+ and FL1-ve gates were drawn based on the controls.

2.10 Statistical analysis

The GraphPad® InStat (GraphPad® InStat Software Inc., San Diego, CA) was used for FACS data analysis. In the study unpaired t-test was used to compare the mean and the standard error mean (SEM) between two groups. For comparison between the three groups, one way analysis of variance (ANOVA) was conducted using Dunnet's method to adjust the p-value for multiple comparisons. In all experiments, $p < 0.05$ was considered significant.

3 Construction, purification and characterisation of cholesterol binding PLY mutants

3.1 Summary

As described in section 1.8.1.1, the mutant version of PLY (T459G.L460G), mentioned by (Farrand *et al.*, 2009), shows that residues threonine and leucine (TL) in D4 are responsible for initial binding of the toxin to the cholesterol containing membranes. D4 of the toxin is responsible for initial binding with the cholesterol containing membrane followed by the cell lysis (Baba *et al.*, 2001). A study conducted recently has shown that D4PLY binds to human erythrocytes whereas D123 exhibited no binding and was required for the subsequent lytic process (Lim *et al.*, 2013). Similar results were found in this study when purified D4 and purified D123 were compared in a haemolytic assay. Domains 123 was not binding to red blood cells (showing a similar phenotype as PBS treated wells) where as purified D4 was not causing lysis of the red cells, but rather binding and causing red cells to aggregate (Figure 2.5) ((This work and (Ma, 2011))). However, Listeriolysin (LLO) secreted by *Listeria monocytogenes*, both domain 123 and domain 4 bind to the cell membranes and can reassemble into a haemolytically active form when secreted simultaneously, by the bacterium (Dubail *et al.*, 2001). Recently binding of PLY (domains 1-3) with the bone marrow derived macrophages has also been reported by one of the members in the laboratory (Dr Catherine Dalziel, unpublished data). This binding may be due to different receptors on the surface of bone marrow derived macrophages not found on the surface of red blood cells.

The phenomenon of red blood cell aggregation was also observed using $\Delta 6$ PLY and similar sort of aggregation was observed with purified D4PLY ((This study

and(Ma, 2011)). This suggests that residues responsible for this phenotype reside in the D4 of the toxin.

The project was started by making the same mutations at residues (T459G.L460G) in the domain 4 of the toxin. For the ease of understanding and writing D4PLY^(T459G.L460G) is referred at some places in this chapter and or figures in the thesis as TL mutant, since these two residues threonine and leucine were replaced by glycines.

The eGFP version of the mutant toxin was also made so that the binding with red blood cells could be analysed on the fluorescent activated cell sorter and by fluorescence microscopy. The eGFP was fused to the D4PLY by ligation dependent cloning and the same construct was used as a template for mutation of residues (T459G.L460G) using SDM kit.

Wild type D4, eGFP tagged WT domain 4 of the toxin and eGFP only proteins were purified for experimental controls.

3.2 Construction of D4PLY and eGFPD4PLY (459,460 TL>GG) by site directed mutagenesis

The double amino acid substitutions were successfully made in the D4 and eGFPD4 of PLY using Quick Change ® II XL Site Directed-Mutagenesis kit following the protocol described in section 2.5.

To analyse protein binding to the cholesterol containing membranes constructs encoding WT and the mutant versions; (pET33bD4PLY, pET33beGFPD4PLY,

pET33bD4PLY^(T459G.L460G) and pET33b eGFPD4PLY^(T459G.L460G) were transformed in the BL21 (DE3) cells for protein expression.

All four proteins were subjected to a two step purification process, first by immobilized metal affinity chromatography and then further purified by anion exchange chromatography (AEC).

3.2.1 Purification of D4PLY^(T459G.L460G)

D4PLY^(T459G.L460G) was purified first by loading the cell lysate onto a His Trap® column using a peristaltic pump. The column was then attached to the ÄKTAprime™ Plus (GE Healthcare) purification system on which the Nickel Affinity Chromatography programme was run. (Figure 3.1)

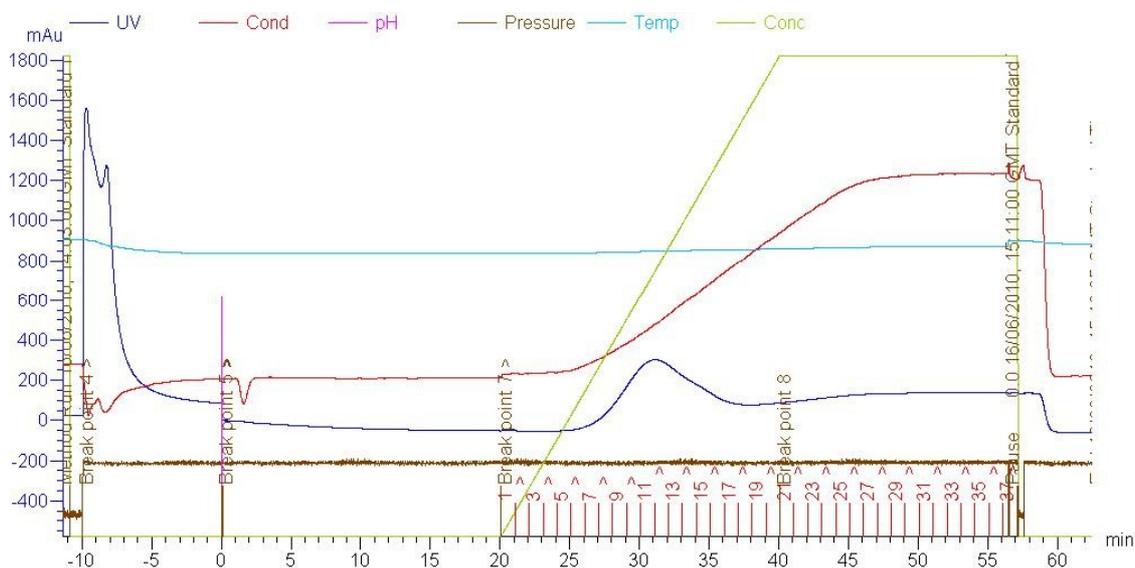


Figure 3-1 His tag column output of D4PLY^(T459G.L460G) AKTAprime™ Nickel Affinity Column (NAC) output of D4PLY^(T459G.L460G) from AKTA showing protein elution and fractions numbers. Fractions 7-20 were run on an SDS-PAGE figure 3.2 below. The blue line (UV) is A₂₈₀nm protein reading, which measures protein content. The green line represents imidazole concentration (0-0.5M).

The purified protein D4PLY (T459G.L460G) Histag₆ has a predicted molecular weight of 18kDa. This was confirmed by running the eluted fractions on an SDS-PAGE with a known molecular weight protein marker.

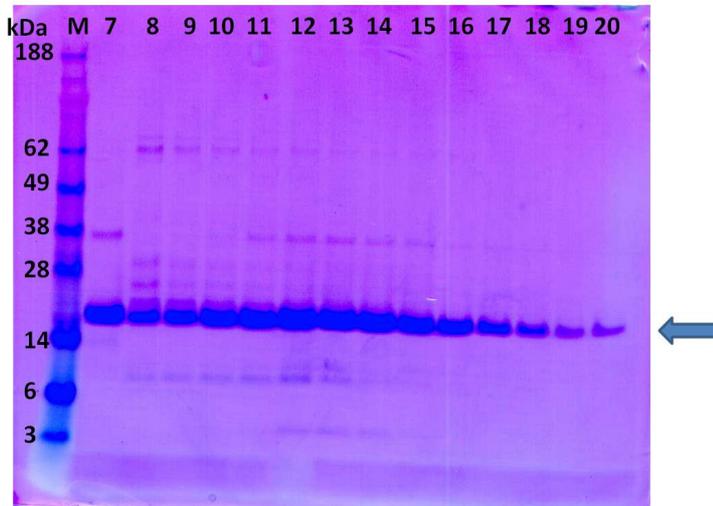


Figure 3-2 SDS-PAGE of D4PLY^(T459G.L460G) after NAC Purification
Mol.wt of D4PLY^(T459G.L460G) is 18kDa. 10 μ l of the recombinant protein was loaded on the gel. Lane M contains 5 μ l of the mol. wt. marker SeeBlue®Plus2, followed by Histag column purification fractions 7 to 20 from AKTA. The arrow showing the recombinant protein D4PLY^(T459G.L460G) corresponding to the right molecular weight compared with known molecular weight markers. There were other bands seen on the gel (contaminants) which were removed subsequently after the second AEC purification.

The D4PLY^(T459G.L460G) after NAC chromatography had some contaminants including LPS so it was further purified by using a 1ml CptoQ Anion Exchange columns. The protein binds to the negatively charged column while the contaminating Lipopolysaccharides (LPS) being negatively charged will not bind to the column, this will be collected in the flow through. The protein was eluted from the column using a linear gradient of 0- 0.5M NaCl. The eluted fractions were then analysed by SDS-PAGE (Figure 3.3B). The selected AEC fractions were dialysed at 4°C with 2 litres pre-chilled PBS buffer.

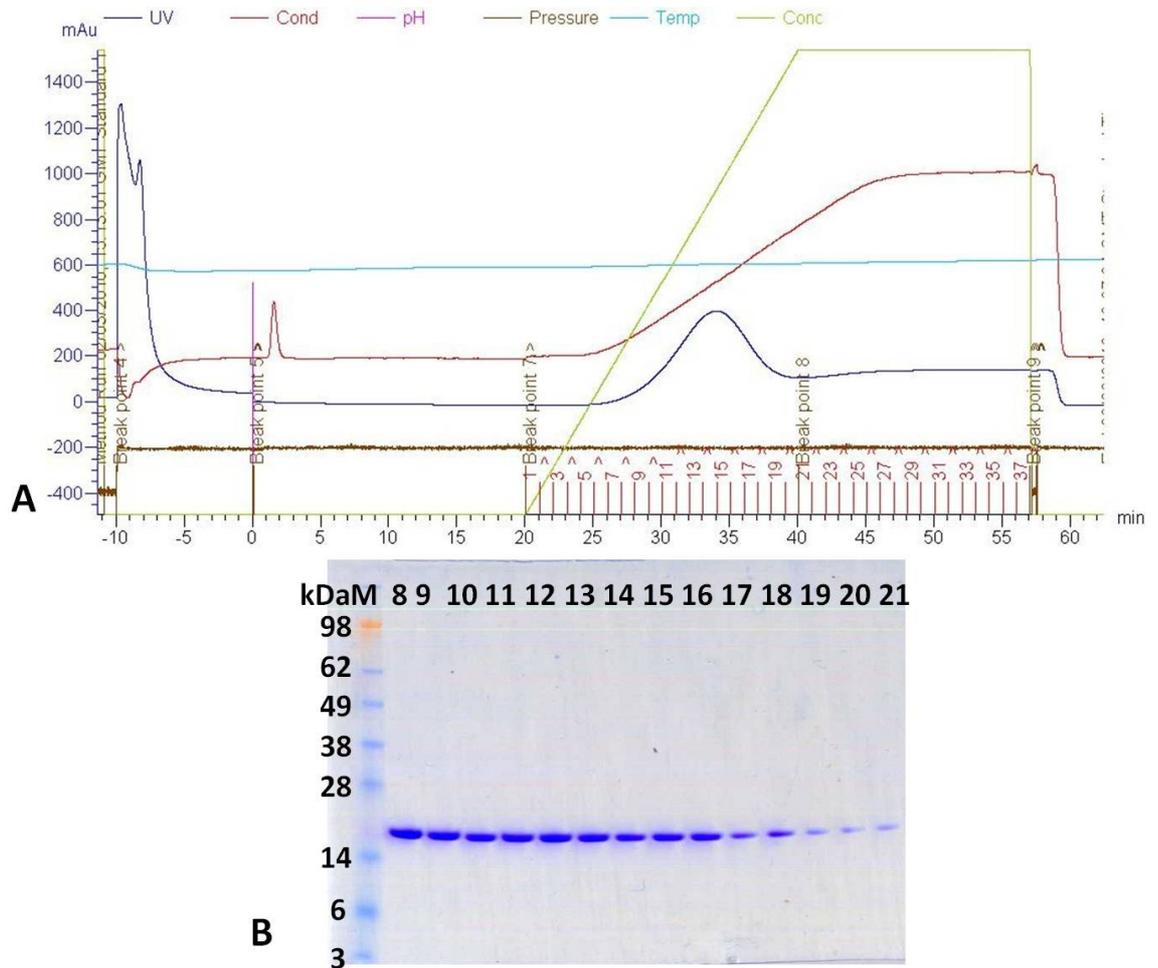


Figure 3-3 Anion exchange chromatography output and SDS-PAGE of D4PLY^(T459G.L460G) AKTA after AEC Purification
A) AEC output of D4PLY^(T459G.L460G) from AKTA showing protein elution and fraction numbers. B) SDS-PAGE of D4PLY^(T459G.L460G) on which 10 μ l of the recombinant protein from fractions 8-21 were loaded. Lane M showing 5 μ l of the mol. wt. marker (SeeBlue®Plus2).

Figure 3.3 shows that the second purification step yield a high quality protein which corresponds to the right size with the protein marker run on the gel. The fractions were pooled together and buffer exchanged into PBS before storing them at -80°C .

3.2.2 Purification of eGFPD4PLY^(T459G.L460G)

The recombinant protein eGFPD4PLY^(T459G.L460G) was purified in a similar manner to the D4PLY^(T459G.L460G).

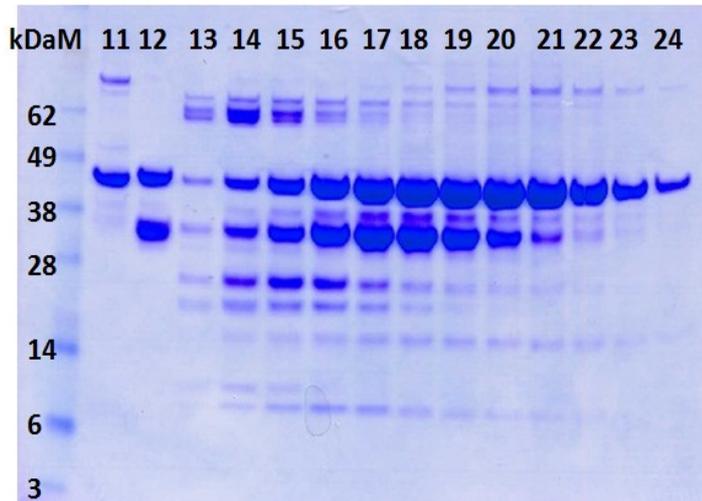


Figure 3-4 SDS-PAGE of eGFPD4PLY^(T459G.L460G) after NAC Purification
 10 μ l of the recombinant protein was loaded. Mol.wt of eGFPD4PLY^(T459G.L60G) is 44kDa. Lane M contains 5 μ l of the mol. wt. marker SeeBlue®Plus2 followed by His tag column purification fractions from 11-21. The fractions 12-21 show degradation, the reason for which is unknown. These fractions also showing many contaminants and therefore were discarded. Fractions 11 and 22-24 were saved as there was no degradation and have few contaminants as compared to other fractions. These fractions were saved for subsequent purification.

The recombinant eGFPD4PLY^(T459G.L460G) has a molecular weight of 44kDa. After the nickel purification a single peak was produced but some of the purified fractions (14 to 21) were degraded/cleaved when the fractions were analysed by SDS-PAGE. The two fragments appeared to be of 44kDa and 34kDa. This suggests that protein is not cleaved from the linker linking eGFP to the D4PLY as D4 is 18kDa and eGFP is 26kDa. A similar sort of degradation was observed by previous laboratory member while purifying one of the protein (eGFP SLY) of cholesterol dependent cytolysin family (McInally, 2012). The reason of this degradation is unknown and could be due to errors in protein folding. There were some fractions (22-24) at the end of the gel without any degradation which corresponds to the right protein size to the molecular weight markers on the SDS-PAGE. These fractions were pooled together and run through the anion exchange purification step (Figure 3.5).

The rest of the fractions were collected and they were separately analysed through the anion exchange purification step. The purified protein fractions again were degraded/cleaved so were not saved and discarded (Lane 1 of Figure 3.5 (A) SDS-PAGE gel).

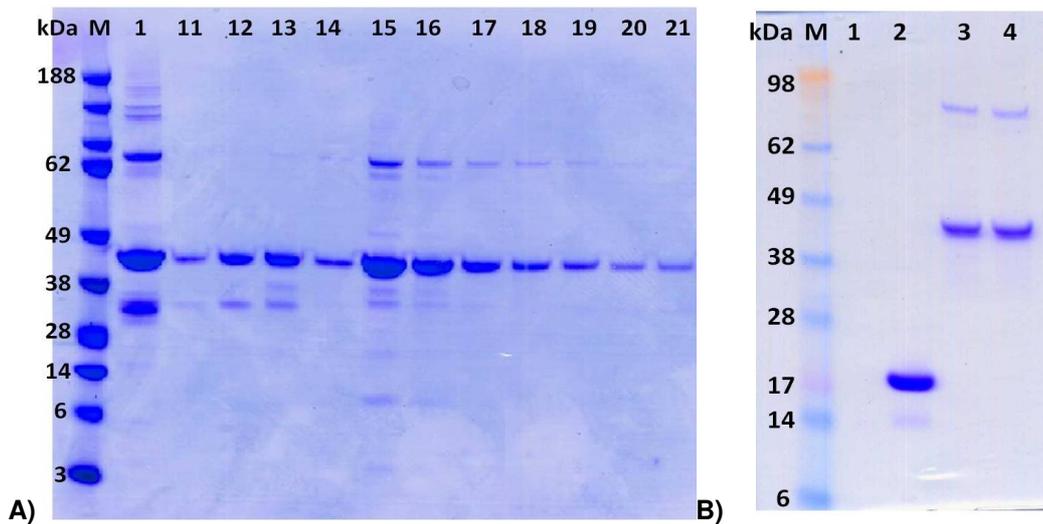


Figure 3-5 SDS-PAGE of eGFPD4PLY^(T459G.L460G) and D4PLY^(T459G.L460G) after AEC Purification

A) 10 μ l of the recombinant protein was loaded. Lane M contains 5 μ l of the mol.wt. marker SeeBlue®Plus2. Fraction 1 is the pooled degraded fractions from NAC run separately on AEC and one of fraction was loaded on the gel. Fractions 11-21 are fractions eluted from AEC from previously saved non degraded NAC fractions. A few bands (contaminants) were also seen and were removed by the using Amicon Ultra Centrifugal concentrators as mentioned under heading 2.8.9. **B)** Lane M contains 5 μ l of the mol. wt. Marker, Lane 1 Blank, Lane 2 Pooled D4PLY^(T459G.L460G) fractions in PBS after AEC and Lane 3&4 Pooled eGFPD4PLY^(T459G.L460G) fractions in PBS after AEC. Two small bands were also seen around 88kDa which might suggest dimer formation.

The fractions obtained after AEC were analysed on an SDS-PAGE with a known molecular weight marker and the fractions corresponding to the right molecular weight were pooled together and buffer exchanged into PBS. The degraded fractions were also run separately on AEC and the one of the eluted fractions was run on the SDS-PAGE but that fraction was degraded as shown in lane 1 of figure 3.5A. The proteins with one single band, corresponding to the right molecular weight was further concentrated using Amicon Ultra-15 30kDa cut off centrifugal filters which further removed any contaminants less than 30kDa. The purified proteins D4PLY^(T459G.L460G) and eGFPD4PLY^(T459G.L460G) after AEC were

pooled together and were buffer exchanged to PBS and was again analysed on the SDS-PAGE (Figure 3.5 B)

3.2.3 Purification of eGFPD4PLY and D4PLY.

The two proteins (D4PLY eGFPD4PLY) were used as controls in the experiments including red cell assays, fluorescence microscopy and fluorescence activated cell sorter (FACS) along with the eGFP. The two proteins were purified in a similar manner to the TL mutants.

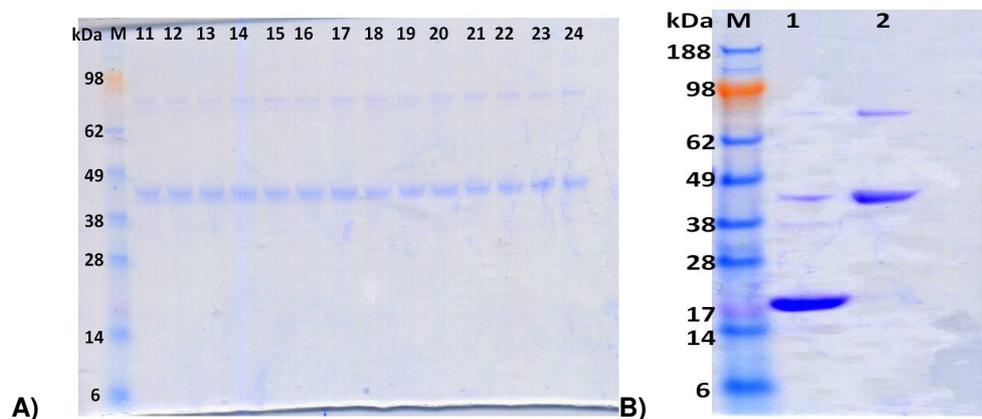


Figure 3-6 SDS-PAGE of eGFPD4PLY and D4PLY

10 μ l of the recombinant protein eGFPD4PLY was loaded. A) Lane M contains 5 μ l of the mol. wt. marker SeeBlue®Plus2. Fractions 11-24 are eGFPD4PLY fractions after AEC. There are some bands around 88kDa which might suggest dimer formation as seen in figure 3.5B B) Lane M is 5 μ l of the mol. wt. Marker, Lane 1 is 10 μ l of recombinant D4PLY after NAC and AEC and Lane 2 is pooled eGFPD4PLY fractions in PBS after the two step purification. There is one additional band (apart from D4PLY band) seen in lane 1 which might suggest overflow from lane 2 and the small band around 88kDa in lane 2 suggesting dimer formation.

D4PLY and eGFPD4PLY have a predicted molecular weight of 18 and 44kDa respectively and SDS-PAGE confirmed its size compared to the protein marker. The dimer formation could be checked by running protein on size exclusion chromatography (gel filtration) and comparing the height of the eluted fraction with the controls.

3.3 Concentration, yield and LPS levels in the purified proteins

The concentration of the purified proteins was measured as described in Materials and Methods section 2.8.10. The LPS Levels were measured to ensure that LPS are low (less than 5 International Unit (IU)/dose) and will not stimulate the immune system non-specifically if they were to be used in *in-vivo* experiments (Douce *et al.*, 2010). The LPS levels was measured using Endosafe.-PTS™ hand held spectrophotometer using the FDA-licensed disposable cartridges from Charles River following manufacturer's instructions.

Table 3-1 Concentration, yield and LPS levels in the purified proteins

Name of the Protein	Molecular weight	mg	LPS Levels IU
WT D4PLY	18	2.74	0.266
WT eGFPD4 PLY	44	2.07	0.0402
D4 PLY (TL>GG) (459,460)	18	2.128	0.198
eGFPD4 PLY (TL>GG)(459,460)	44	1.182	0.102

3.4 Western blot of the purified proteins

The purified recombinant proteins were further analysed by Western Blotting as outlined in section 2.9.2. Briefly, 1µg of the purified protein was loaded on the gel along with molecular weight protein marker SeeBlue®Plus2. The primary antibody used was rabbit anti PLY and the secondary antibody used in all of the samples was horseradish hydrogen peroxide-linked secondary antibody. The membrane was washed five times after adding both the antibodies and developed in the developer.

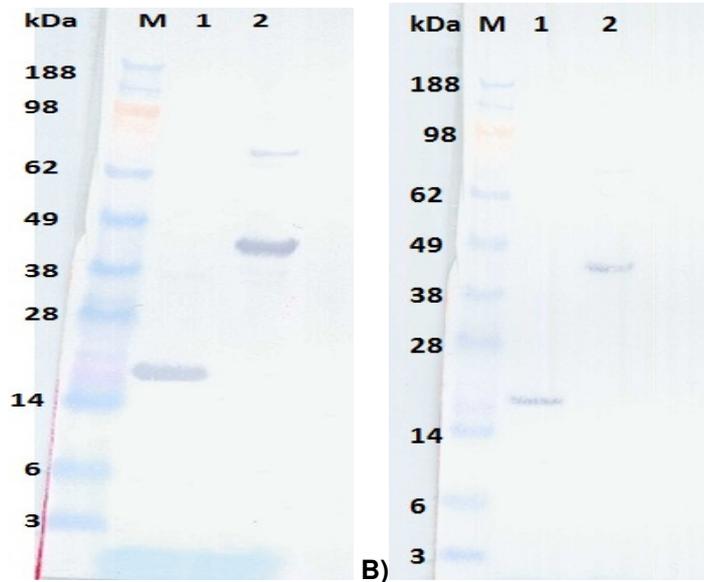


Figure 3-7 Western blotting results of the purified proteins

A) Lane M is the protein molecular weight marker followed by 1 μ g of the purified proteins. Lane 1 is WT D4PLY and Lane 2 is WT eGFPD4PLY. (B) Lane M is the protein molecular weight marker. Lane 1 is D4PLY^(T459G.L460G) and Lane 2 is eGFPD4PLY^(T459G.L460G). There was a band seen in figure 3.7 A&B around 88kDa in lane 2 of both gels possibly showing dimer formation.

The western blot results (Figure 3.7A and B) further confirmed the size and identity of all the four purified proteins.

3.5 Red cell assay of the purified proteins

CDCs cause the lysis of red blood cells so to ensure that the purified recombinant proteins were still functionally active, red cell assay using sheep red blood cells were run for all of the purified proteins. The cell lysis was measured indirectly by the amount of haemoglobin released. As expected none of the purified D4 recombinant proteins were lytic. The full length toxin was lytic even at amounts as low as 1ng of the purified protein. The WT PLY used as positive control was diluted in (1:1000) in PBS and then 50 μ l was added into the first well of 96 well round bottom plate and serial dilutions were made as explained in the section 2.9.3. In negative controls wells PBS was added. For all D4 and eGFP versions 1 μ g of the neat protein was added in the first well and serial dilutions were made thereafter.

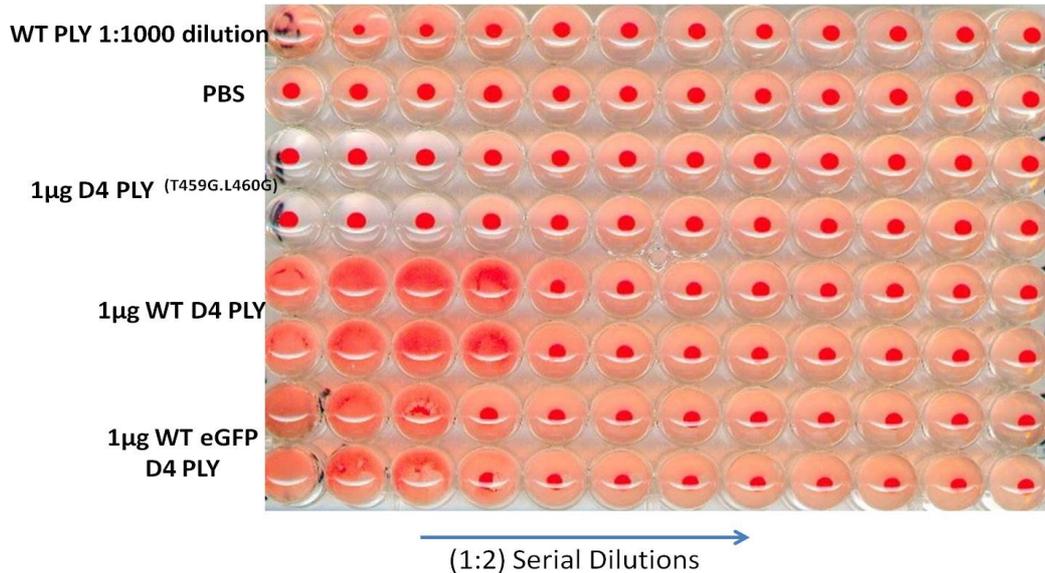


Figure 3-8 Red cell assay of the purified proteins

1µg of D4PLY, eGFPD4PLY and D4PLY^(T459G.L460G) was added in the first well of a 96 well plate and serial dilutions (1:2) were made thereafter. WT PLY (1:1000 dilution) and 50µl PBS was used as positive and negative controls. WTPLY causing red blood cell lysis and a pinkish tinge observed in WTPLY treated wells due to released haemoglobin. PBS only wells, causing no lysis and a red cell pellet is seen in centre after the last centrifugation step. 1µg of D4PLY, eGFPD4PLY cause no lysis but aggregation of red blood cells are seen in first few wells. D4PLY^(T459G.L460G) treated wells showing no visible aggregation and lysis but a clearing effect (white background as compared to pink in PBS wells) is seen in first 3 wells. These phenotypes are explained in figure 2.5.

WTPLY in figure 3.8 used as positive control cause lysis of red blood cells (no intact pellet seen in first two wells) where as PBS used as negative control showing no lysis (all red cells pellet are intact). 1µg of WTD4PLY and WT eGFPD4PLY cause no lysis but rather aggregates red blood cells and this is consistent with finding of previous studies showing the same phenotype of these proteins on a 96 well plate (Ma, 2011).

Δ6PLY also form the same aggregation phenotype as D4PLY on a 96 well plate suggesting the site/s responsible for this behaviour resides in the D4 region of the toxin. Interestingly, this aggregation phenotype was not observed with TL mutants. There is a clearing effect in the wells treated with D4PLY mutant and a pinkish tinge in the wells (first three wells in both lanes of mutant protein) as seen with negative control (PBS) is not found. A similar effect was seen when

red blood cells were treated with purified D123 and eGFPD4PLY^(T459G.L460G)

(Figure 3.9). To further confirm this clearing effect other non-lytic streptococcal proteins PspC and PspA and non- pneumococcal protein e.g. BSA (Bovine Serum Albumin), were tested and the same clearing effect was seen suggesting non specific binding of the proteins.

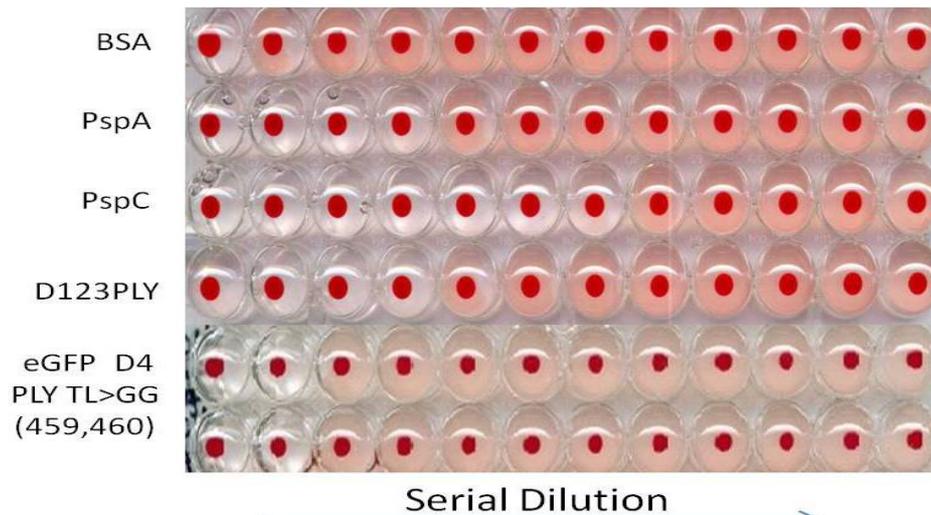


Figure 3-9 Red cell assay showing non-agglutinating and clearing well effect phenotypes.

10 μ l of the above proteins were added in the first well of the 96 well plate and serial dilutions were made. The non-lytic pneumococcal proteins (PspA and PspC) and non-pneumococcal protein (BSA) are also showing the same clearing well effect phenotype as eGFPD4PLY^(T459G.L460G). D123 also showing the same background white colour in the first 4 wells as the other proteins. All the above proteins are non-lytic as there is no lysis and the red cell pellet is intact after the last step.

The specific haemolytic activity of the proteins (D4PLY, eGFPD4PLY, D4PLY^(T459G.L460G) and eGFPD4PLY^(T459G.L460G)) were measured by calculating the percent lysis of the red blood cells at a known protein concentration. As expected D4PLY, eGFPD4PLY and the mutant versions were found to be non-lytic.

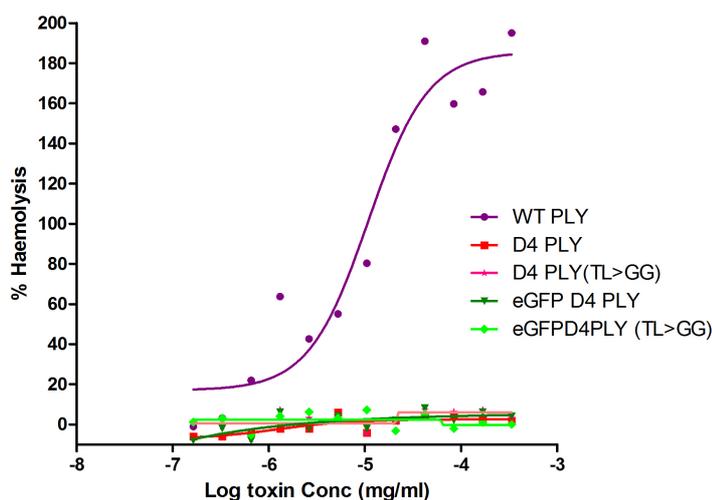


Figure 3-10 Haemolytic activity of the recombinant proteins

A sigmoid dose response curve of 50 % lysis was generated against each well for each purified toxin by using mean negative controls from PBS wells where 0% lysis has occurred and the mean value from positive wells (Purified PLY of known concentration) where 100% lysis has occurred. X-axis is log toxin concentration in mg/ml and Y-axis denotes % haemolysis. WT PLY (purple), D4PLY (red), D4PLY^(T459G.L460G) (pink), eGFPD4PLY (dark green), eGFPD4PLY^(T459G.L460G) (light green) were all tested under the same conditions in duplicates.

The specific activity of the toxin was then calculated as Haemolytic units (HU). 1 haemolytic unit is defined as the amount of toxin that causes 50% lysis of red blood cells. Thus the specific activity in HU/mg was then calculated and stated in the table 3.2.

Table 3-2 Specific haemolytic activity of the purified proteins

Name of the Protein	Haemolytic Activity HU/mg
PLY	2.63×10^5
D4PLY	0
eGFP D4 PLY	0

Figure 3.8 shows that D4PLY^(T459G.L460G) was not giving the same appreciable aggregation phenotype on the 96 well plate as seen with WTD4 and eGFPD4PLY. To further evaluate the binding of these mutant versions of the toxin with the red blood cells fluorescence microscopy and flow cytometry experiments were done.

3.6 Fluorescence microscopy

The fluorescence microscopy was done with eGFP tagged versions of the proteins to assess the protein binding with the red blood cells. The binding was seen both with intact cells and red cell membranes.

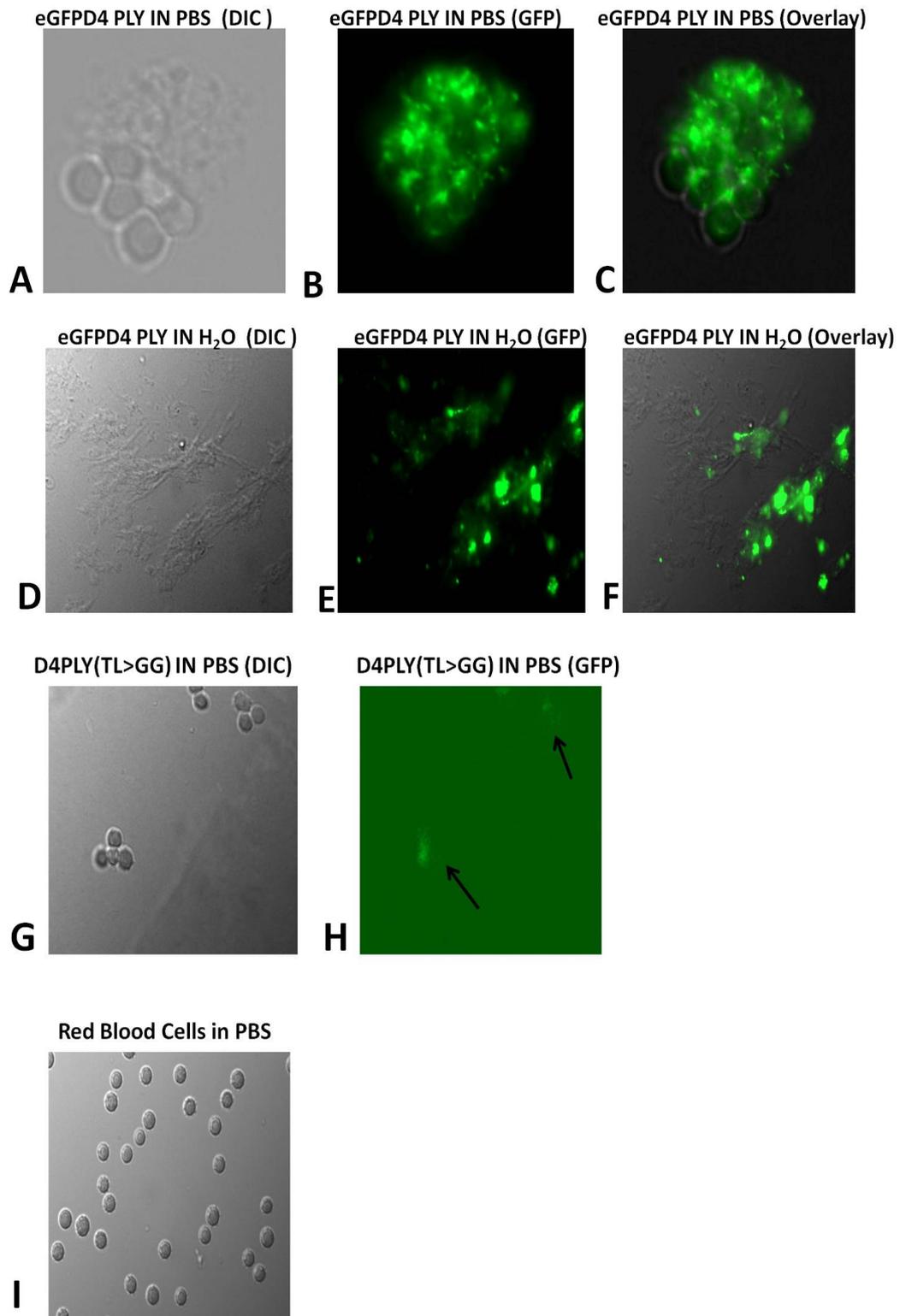


Figure 3-11 Fluorescence microscopy images showing eGFP tagged recombinant proteins binding

The protein binding was observed using horse red blood cells. 140nM of the purified proteins were incubated with horse red blood cells at 37°C for 15minutes. (A, B & C) eGFPD4PLY showing binding and causing aggregation of horse red blood cells in DIC, GFP and overlay. (D, E & F) eGFPD4PLY showing binding and causing aggregation of horse red blood cells membranes in DIC, GFP and overlay. G&H) eGFPD4PLY^(T459G.L460G) in DIC and GFP bound to the red blood cells and causing aggregation (small group of red cells shown in Figure 10G) which is comparatively less as compared to WTeGFPD4PLY (A,B and C). I) Red cells controls only in PBS showing single cells in DIC. Red blood cells binding with GFP (140nM) only used as one of the controls are shown in figure 5.13.

In Figure 3.11, the results of eGFPD4PLY binding with red cells are similar to ones observed in previous studies (Ma, 2011). D4PLY forms large clumps of red blood cells which are visible in the 96 well plate as well (3.11 A, B & C). To further confirm the binding, red cell membranes were made by lysing the cells with dH₂O and washing the membranes thrice with PBS. The membranes were incubated with 140nM of eGFPD4PLY at 37°C and binding was observed under the microscope (Figure 3.11 D, E & F).

As expected at 140nM concentration of eGFPD4PLY^(T459G.L460G), the binding of toxin was greatly reduced as compared to the eGFPD4PLY. Since threonine and leucine residues at position 459 and 460 of the PLY are the responsible for initial binding with the cholesterol containing membranes, it was expected that the toxin will not bind to the red blood cells at all. However, some binding of the toxin to the red cells was visible as judged by clumping of red blood cells in figure 3.11 G&H. This binding was also picked up by the GFP mode (black arrows in Figure 3.11 H). However, the signals were low and were not picked on the overlay. The red blood cells were also forming small aggregates of red blood cells with eGFPD4PLY^(T459G.L460G) (Figure 3.11G). These small aggregates were not as profound as with the WT eGFPD4PLY. Red blood cells only in PBS observed under the microscope show no aggregation of the red blood cells (Figure 3.11I).

The eGFP only protein used as one of the controls was neither binding nor causing the aggregation of the red blood cells and results were similar to red blood cells (Figure 5.13 A & B).

3.7 Cell binding by fluorescence activated cell sorting (FACS)

The cell binding was further confirmed and quantitatively measured by FACS. The proteins were tagged with eGFP in order to assess the protein binding with red blood cell by the fluorescence channel (FL1) of the cytometer. The forward scatter channel (FSC) was also used to distinguish if there are single cells or more than single cells. The experiment was set up as explained in the section 2.9.5. The FACS experiments were done on three different occasions to ensure consistency.

In order to measure the intensity of protein binding with the red blood cells arbitrary units such as fluorescence Index (FI) was used. FI was calculated by multiplying the percentage of fluorescent cells with the mean geometrical fluorescence (GEM). This method has been used before to assess the bacterial binding with the complement (Hyams *et al.*, 2010).

Red blood cells only in PBS have a minimum amount of background auto-fluorescence. This back-ground cell auto-fluorescence was minimised by drawing gates FL1+ and FL1-ve based on the controls (Figure 3.12).

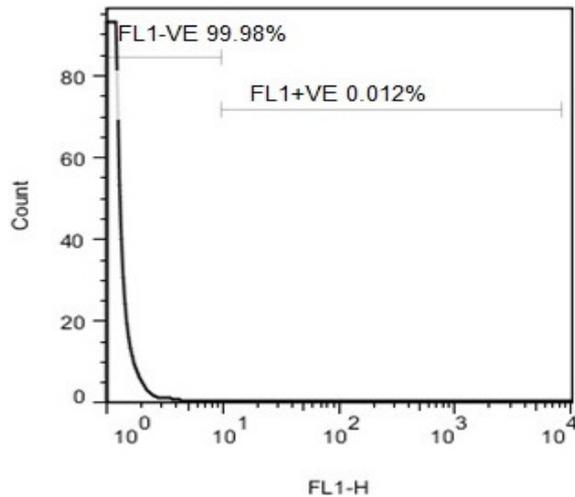


Figure 3-12 FL1-ve and FL1+ve gates drawn on negative control (Red cells with PBS)

X-axis showing FL1+ve gate to measure background auto-fluorescence and Y axis showing number of events which in this case is 50,000.

Figure 3.12 shows that the red blood cells with PBS only have negligible percentage of fluorescent cells (0.012%) and FL1-ve gates (no fluorescence) in all the samples were drawn keeping the FL1-ve gate at 10^1 on the X-axis (FL1-H channel). The cells which lie after 10^1 on the X axis were labelled as fluorescent cells and gated as FL1+ve. The percentage of FL1+ve cells for all controls was very small (less than 0.1%) as it is shown in the above figure. The samples with eGFPD4 at 1400nM concentrations had the highest number of fluorescent cells and the eGFP only controls had the minimum fluorescence (Figure 3.13).

As expected with eGFPD4PLY (1400nM), binding was significantly higher as compared to the mutant version (Figure 3.13). Similar results were found on fluorescence microscope where large clumps of cells were seen at this protein concentration. Interestingly, the mutant version eGFPD4PLY^(T459G.L460G) showed some residual binding (Figure 3.14). This binding was greatly reduced compared to the WT D4PLY. This suggests that there may be another binding site apart of this TL site which is contributing to this residual finding since TL substitution

does not completely remove the binding of the red blood cells. To exclude the fact that this is not because of eGFP only, controls were used at the same concentration as the proteins (eGFP only used at 14, 140 and 1400nM).

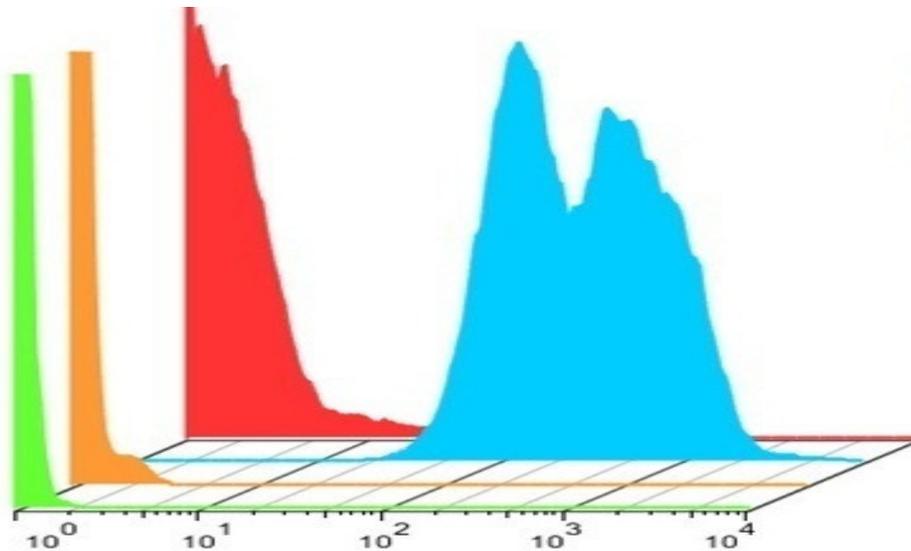


Figure 3-13 FACS results showing proteins binding with the red blood cells

X axis is FL1+ve channel which was set on log scale at 400 volts. Y axis is the number of events measured for every reading and in this case is 50,000. The protein binding with the red blood cells is seen by shifting of the graph to the right. Light green represents PBS with red blood cells, yellow shows eGFP at 1400nM, red shows eGFPD4PLY^(T459G.L460G) at 1400nM and light blue represents eGFPD4PLY at 1400nM concentration. There were two peaks seen with eGFPD4PLY suggesting two or more cells. Figure was drawn using Flowjo software.

In the case of eGFPD4PLY the graph has clearly shifted to the right suggesting more binding as compared to the mutant protein. Some binding of the TL mutant with the red blood cells was observed as compared to the controls. With eGFPD4PLY more than one prominent peak was seen as compared to other proteins. Similar peaks (more than one) were also observed previously with this protein at high concentrations with red blood cells (Ma, 2011). These double peaks may be the doublets, triplets or multiple cells giving different fluorescence signal with eGFPD4PLY.

The binding of mutant protein with red blood cells was concentration dependent as it is increased with the increasing concentration of the protein. The three

concentrations of the TL mutant were noted for the binding along with the controls. The binding was minimal with the 14nM and was highest for the highest concentration 1400nM.

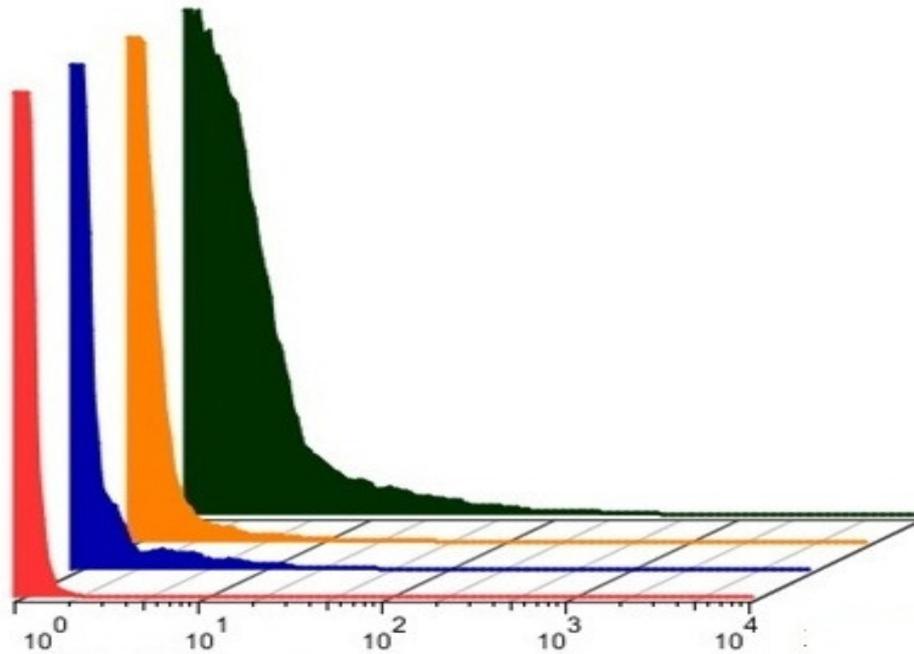


Figure 3-14 FACS results showing eGFPD4PLY^(T459G.L460G) binding with horse red blood cells Three different concentrations (14,140 and 1400nM) of eGFPD4PLY^(T459G.L460G) were taken. X axis is FL1+ve channel which was set on log scale. Y axis is the number of events measured and is 50,000. The pink colour represents the control eGFP only (14nM), blue, yellow and green colour represents 14, 140 and 140nM of eGFPD4PLY^(T459G.L460G).

The results in figure 3.14 shows binding of protein with the red blood cells increased with the increase in the concentration suggesting that TL are not the only two residues responsible for binding to red blood cells and there might be some more residue/s contributing for this binding.

The forward scatter channel (FSC) was used to observe any changes in the size of the cells that might have happened due to binding of the proteins.

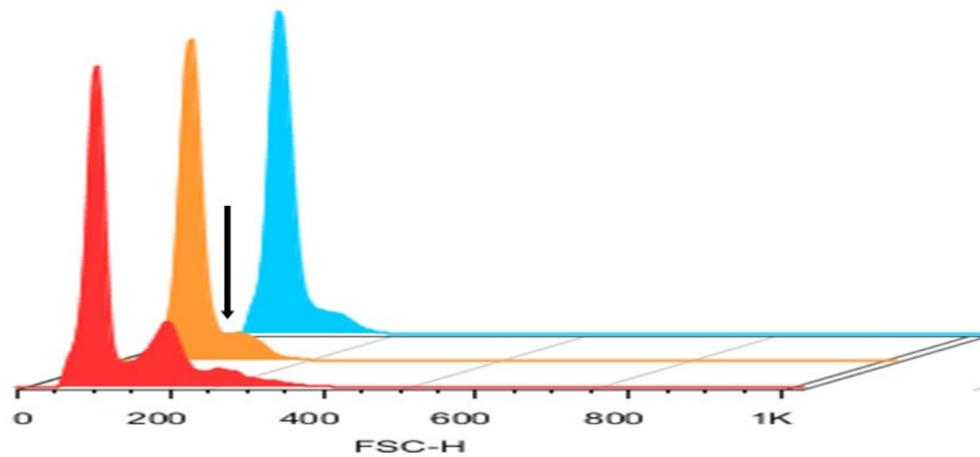


Figure 3-15 FACS results showing the protein binding with the horse red blood cells in forward scatter channel (FSC)

X axis is the forward scatter channel (FSC) which was set on linear scale at 350 volts. The Y axis shows the number of events captured during each reading and in this case is 50,000. The red graph is eGFPD4PLY (1400nM), yellow is eGFPD4PLY^(T459G.L460G) (1400nM) and blue is eGFP only at (1400nM). The black arrow indicating the second peak observed with TL mutant.

The eGFPD4PLY appears to be binding with more than one cell as more than two peaks are observed with it in Forward scatter channel (FSC). The first peak is the single cells and the second and the small third in eGFPD4PLY seems to be more than one cell. The two peaks were also seen with eGFPD4PLY in fluorescence channel (FL-1) which might explain that eGFPD4PLY form more than one clumps of cells and these may give different fluorescence signals and hence more peaks, when seen with FL-1 channel. The results show that with eGFP only control no second peak was observed where as with eGFPD4PLY^(T459G.L460G) a small second peak was seen. The aggregation results of mutant proteins were evaluated as percentage (% of single and multiple cells) in section 5.7 (Figure 5.12).

This data presented in here is in line with microscopy results which shows aggregation of two or three cells with the mutant protein where as the cell aggregation is more with eGFPD4PLY (more than 15-20), which might suggest that there is still some residual binding with TL (459,460) residues even after substituting them with glycines.

The FI (fluorescence index) of the different protein concentrations was calculated and their results were compared. The FI was calculated by multiplying the total number of fluorescence cells by mean geometrical fluorescence. The shifting of peak to far right in case of eGFPD4PLY in FL-1 channel (Figure 3.13) also resulted in high FI (fluorescence index) of eGFPD4PLY in comparison to both the TL mutant and the eGFP only, where the readings were not as high. The FI readings of eGFPD4PLY at all concentrations (14,140 and 1400nM) differ significantly when compared with both TL mutant and eGFP only (data not shown).

The FI of TL mutant at three different concentrations (14,140 and 1400nM) was compared with the eGFP only at the same concentration. The different protein concentration of TL mutants and eGFP only were compared by using unpaired t-test.

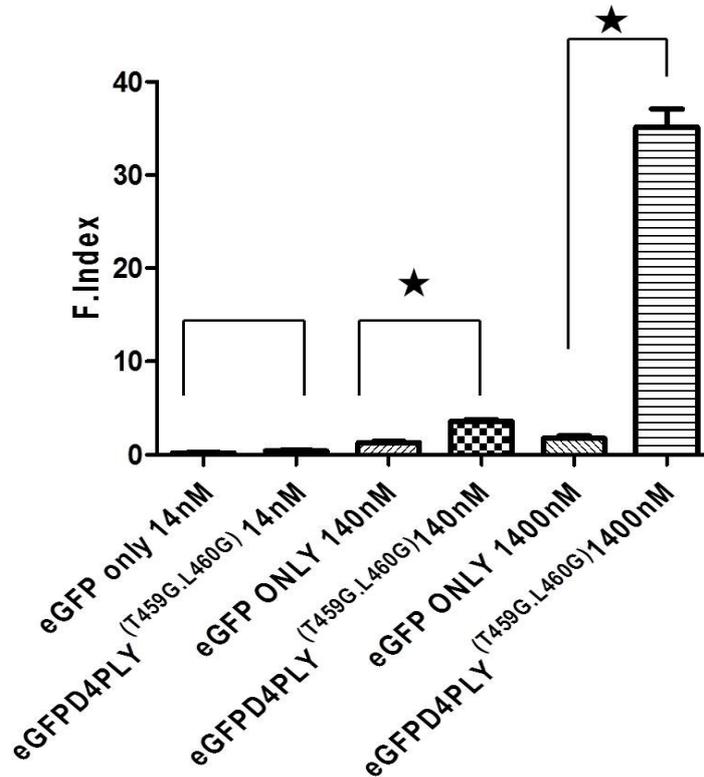


Figure 3-16 Graph showing unpaired t-tests between the control eGFP only and the eGFPD4PLY^(T459G.L460G)

The graph represents different concentrations of the TL mutant (14,140 and 1400nM) with corresponding eGFP only concentrations using unpaired t-tests. The P value <0.05 was considered to be significant. The * symbol shows that P value is significant (* = p<0.05).

Figure 3.16 shows that there is no significant difference between 14nM of TL mutant and 14nM of eGFP only. However, significant differences were observed at higher concentrations 140M and 1400nM when compared with their corresponding controls (eGFP only 140nM and 1400nM). This result suggests a second site/s apart from this TL site responsible for binding with the red blood cells or there could be some residual binding with glycines in the mutant protein.

The data for all three different concentrations of the TL mutant 14nM, 140nM and 1400nM was plotted and compared by using again One-way Anova to see if

they differ significantly. The values were compared with the 14nM of eGFPD4PLY^(T459G.L460G).

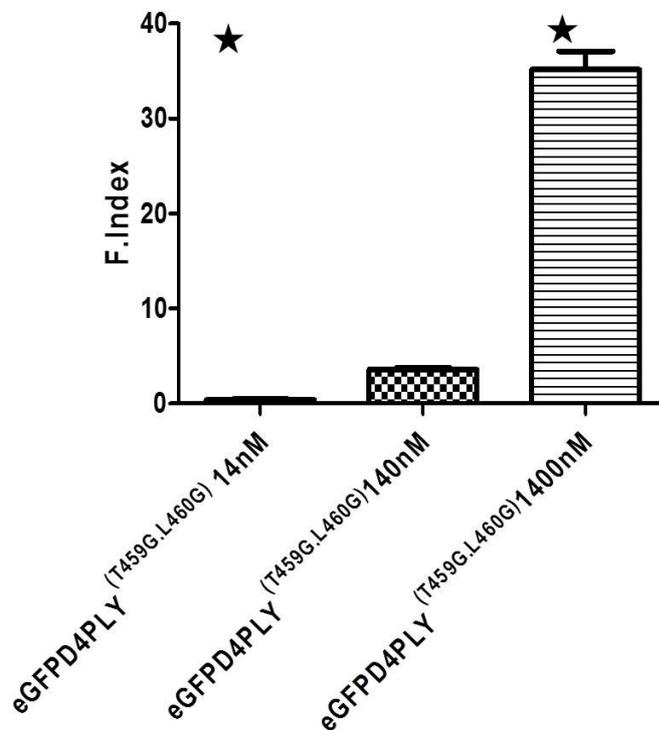


Figure 3-17 Graph showing the comparison of fluorescence index of three different concentrations of eGFPD4PLY^(T459G.L460G)

The graph shows the comparison of three different concentrations of the TL mutant (14,140 and 1400nM) using analysis of variance Anova. The P value <0.05 was considered to be significant. The * symbol shows that P value is significant (* = p<0.05)

Figure 3.17 shows that as the concentration of the TL mutant is increased from 14nM to 1400nM, there is an increase in the F.I and this increase is significant (P value <0.05) when compared between concentration 14nM and 1400nM. This result confirms our previous finding of FACS results (figure 3.14) which is also showing the increase in binding of the toxin to red blood cells with the increased concentration.

3.8 Discussion

The plasmid pET 33bD4PLY was constructed by ligation dependent cloning (LDC). The sequence encoding D4 of PLY was ligated in pET33b+. The plasmid pET33b eGFPD4PLY was also constructed by LDC by cloning D4 first in pET 33b+ and inserting eGFP gene later in the vector (Ma, 2011). These constructs were used by the author to construct TL versions of the mutant proteins by using the site directed mutagenesis kit. Since this traditional ligation dependent technique leaves some undesirable amino acids encoded by restriction sites ligating the two segments of DNA together (in this case eGFP with D4 of PLY) which might explain the degradation/cleaving of recombinant eGFPD4PLY.

The red cell assays showed the aggregation of red blood cells with D4PLY and eGFPD4PLY. The same aggregation pattern was also observed when examined under the fluorescence microscope which might suggest that fusing eGFP to the N- terminal D4PLY does not interfere with this aggregation phenotype. This also suggests that there might be a site other than the TL site that is responsible for this aggregation. This site must be in the D4 of the toxin since the purified D123 does not give this aggregation phenotype (Figure 3.9).

The TL mutant showed no visible aggregation on the plate but clearing effect (results figure 3.8) of wells was observed on the red cell assay plate. A similar effect was also observed with D123 of PLY (Figure 3.9) and a non-haemolytic pneumolysin variant ST-306 (Data not shown) (Kirkham *et al.*, 2006b). It is assumed that this clearing effect might be non-specific interaction of the protein with either DTT and/or PBS in the red cell assay plate since it was also seen with non-lytic Pneumococcal proteins like PspC and PspA and other non

pneumococcal proteins like BSA (Figure 3.9). This clearing effect is not seen until the last centrifugation step of the red cell assay experiment. It was noted that if the plate was left on bench without this last centrifugation step, this clearing effect phenomenon is not observed suggesting the centrifugation step causes the protein to bind non-specifically with PBS and or DTT.

The FACS results of D4PLY binding with horse red blood cells were consistent with the previous results (Ma, 2011). Interestingly, the TL mutant did not demonstrate the same aggregation phenotype as of D4PLY on the red cell assay plate but showed some residual binding, which increased as the concentration of the toxin was increased from 14 to 1400nM. This may suggest that binding of protein with the red cell membrane may be concentration dependent. This residual binding of TL mutant might suggest that TL is not only the site responsible for binding to the cholesterol membrane and other sites in D4 may contribute to this binding. This also suggests that substituting TL residues with glycines does not completely abolish binding and there is some binding with the glycines since the microscopy results show small cell aggregates (2-3) as compared to the WT protein (Figure 3.11). The Farrand *et al.*, 2009 study showed in PFO when TL residues were individually substituted with either alanines or glycines, there was binding observed with red blood cells and when both the substitutions were made there was no binding and the %lytic activity was less than 0.2% (Farrand *et al.*, 2009).

However, contrary to Farrand *et al.*, 2009, significant difference was found in fluorescence index (FI) value of the mutant protein when compared with 140nM and 1400nM of eGFP only.

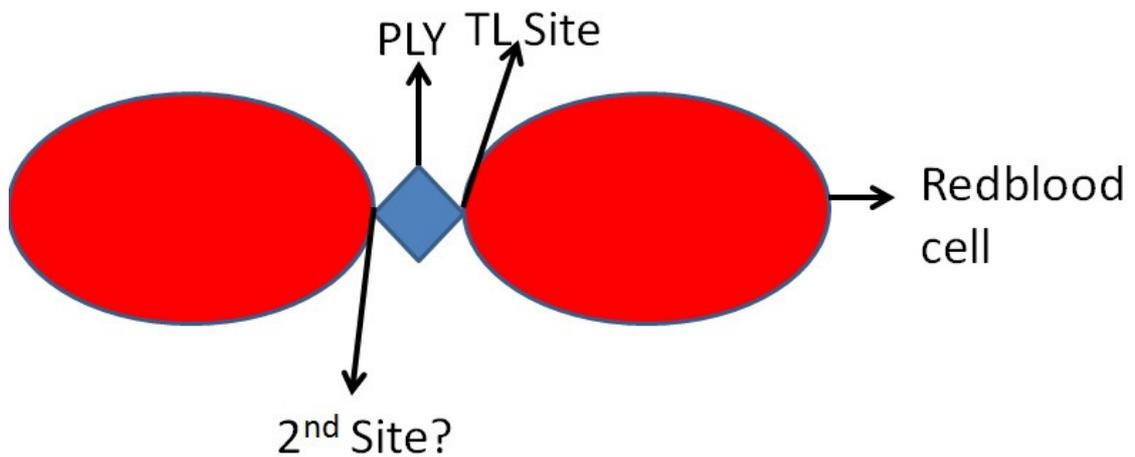


Figure 3-18 Model showing PLY binding to red blood cells

The above figure showing two sites, one is TL site and a possible second binding site.

Based on the above results a model was hypothesised which might explain the binding of the toxin with the cholesterol membranes. PLY may have two binding sites; one is the TL site and there may be another binding site other than the TL site which is responsible for this residual binding with the red blood cells.

Another possible explanation may be that once the TL site and this new site binds to the cholesterol membrane there may be conformational change in the structure of the protein that exposes the D4 loops (L1, L2 and L3) residues and the undecapeptide which results in the insertion of the tryptophan residues and then coupled with the insertion of D3 transmembrane β -hair pin loops (Heuck *et al.*, 2000). Another possibility might be that this second site is binding with other red blood cells which may explain the aggregation phenomenon observed if the lytic activity is abolished by making $\Delta 6$ version or using purified D4 only.

TL residues initially bind to the cholesterol and then this interaction is strengthened by the other L1-3 residues. TL mutant does not perturb the structure of the protein so these changes are subtle and well tolerated (Farrand *et al.*, 2009). The results of this study suggest that TL with the other binding site

interacts at the same time or TL binds first resulting in a conformational change which might result in the interaction of this new site with cholesterol and this phenomenon disappears when we block the TL site but we still get some residual binding with the new site.

It will be interesting if the other binding site, as described in the model, is discovered (Figure 3.18) and binding phenomenon with the red blood cells is observed only by substituting this new site and leaving the TL site intact and also by substituting both the TL and new site.

One potential problem might be that the recombinant proteins used in the above experiments were His-tagged protein and His-tag attached to the N-terminal of the protein might be responsible for this residual binding of the protein since the tag was also attached to eGFP version of the mutants. To overcome this issue a tagfree version of the proteins can be used in the experiments which will exclude any doubt of the His-tag binding with the red blood cells. This will be discussed in detail in the next chapter of the thesis.

4 Purification and characterisation of mutants made by random mutagenesis

4.1 Summary

The results of previous chapter showed that mutant version of PLY^(T459G.L460) was showing residual binding/aggregation with the red blood cells suggesting that there might be another site other than the TL site interacting with the red blood cells. The exact position of the site is not known but the red cell assay results demonstrate that the site is present in D4 of the toxin. Therefore random mutations were made in the D4 of the toxin using Mutation Generation system (MGSTM).

The random mutants were initially made using the pET33bD4PLY vector but some issues in generation and purification of the mutants were observed. The reasons are described in detail in this chapter. As discussed in chapter 3, pET33bD4PLY has a His tag attached to its N-terminal which might be contributing to this residual binding. Due to these potential problems the tag free purification system was used.

The tagfree WT PLY construct was made using Profinity eXact Purification System. The WT PLY is lytic even at very low concentrations and one of the aims of this study is to identify the sites which are responsible for causing the binding/aggregation of the red blood cells. It would be difficult to identify these sites in the WT PLY background because of its lytic activity so $\Delta 6$ version of the toxin was made (Kirkham *et al.*, 2006b). This would not only abolish the lytic

activity the toxin but also at the same time will be useful in identifying the sites responsible for this residual binding/aggregation.

The random transposon based insertion was made both in the WT PLY and the $\Delta 6$ PLY background. The random mutations in the WT PLY background were made to identify any other residues in addition to the already identified 146 and 147 residues (alanine and arginine) responsible for the lytic activity of the toxin (Kirkham *et al.*, 2006a). The random mutants generated in the WT PLY background will also be useful to identify sites responsible for any other biological activity of the toxin. The random mutants made in the tagfree $\Delta 6$ PLY background will eventually solve the potential issue of His tag interference in binding with the red blood cells.

A number of mutants were generated by using the method described in 2.8.7. These mutant proteins were then purified using Profinity eXactTM protein purification system. These purified mutant proteins were then screened for lytic activity in case of tag free WT PLY mutants, and for aggregation phenotype in case of $\Delta 6$ PLY mutants. Based on these results further SDM mutagenesis was also carried out in interesting mutants to see whether an addition of single amino acid addition/substitution results in phenotypic change.

4.2 Random mutagenesis in pET33bD4PLY

The residual binding site(s) was observed by making random mutations in the pET33bD4PLY. The 15 base pair random mutations were made by inserting transposon named as Entranceposon in the target DNA at any site. The body of the transposon can be removed later by using a *NotI* restriction enzyme that

specifically cleaves the body of the transposon leaving 15 additional base pairs in the target DNA at any site. For this specific reason the vector should not have any *NotI* recognition site. The pET33b vector has a *NotI* recognition site (Figure 4.1) so it was removed before inserting the transposon. The two possible ways to remove this site were to either do a restriction digest with *XhoI* and *SacI* restriction enzyme and religating it or to do a site directed mutagenesis.

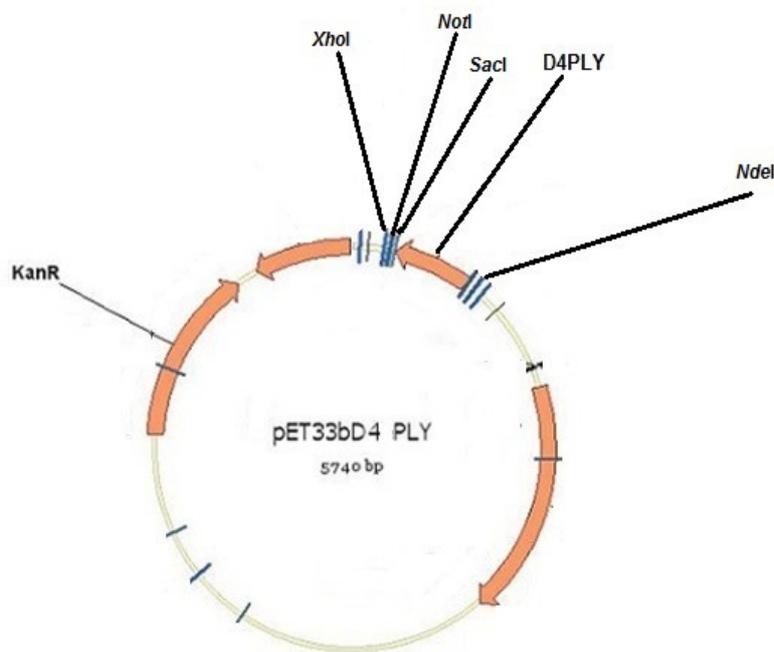


Figure 4-1 Vector pET33bD4PLY showing different restriction sites

The diagram was generated using Vector NTI software and showing D4PLY gene along with restriction sites *NotI*, *SacI*, *XhoI* and *NdeI* respectively.

The pET33bD4PLY *NotI* restriction site was removed by doing a restriction enzyme digestion with *XhoI* and *SacI*. After digestion, plasmid was ligated with T4 DNA ligase overnight and was chemically transformed in XL-1 blues. To confirm that the *NotI* site has been removed the *NotI* digested pET33b and the parent pET33b was treated with *NotI* and *NdeI*.

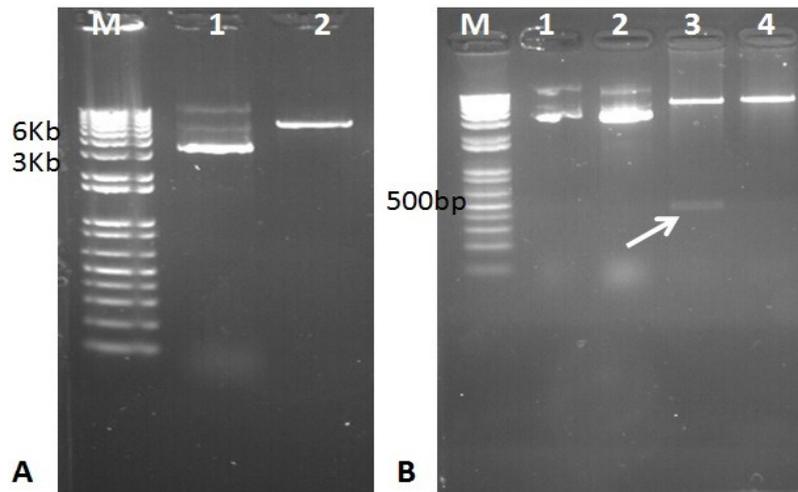


Figure 4-2 Restriction enzyme digestion of pET33bD4PLY

A) DNA Gel showing lane M= DNA marker followed by lane 1= Uncut vector and lane 2= vector cut sequentially first with *XhoI* and *SacI* enzyme.

B) DNA gel showing lane M=DNA marker followed by lane 1= parent vector, lane 2 = vector with *NotI* site removed, lane 3= parent vector cut with *NdeI* and *NotI* and lane 4= Vector with *NotI* site removed cut with *NotI* and *NdeI*. The small arrow showing the D4 gene with the parent vector.

Figure 4.2 shows that when the *NotI* site was removed and same plasmid treated with *NotI* and *NdeI* along with the parent plasmid, the parent plasmid yielded a small fragment of about 500 base pairs (small white arrow in figure 4.2B) whereas the vector from which the *NotI* site was removed did not give any fragment. The removal of *NotI* site from the vector was further confirmed by sequencing results.

The next step was to treat the *NotI* cut plasmid with the MGS kit as explained in Materials and Methods section 2.5. The plasmid was treated with transposon for one hour. The plasmid has a kanamycin resistance gene and the transposon has a chloramphenicol resistance gene.

The next day the plate was checked and colonies which had appropriate antibiotic selection were screened using a PCR reaction. All positive colonies were picked and colony PCR was performed using the primers (7F and 7G) flanking the PLY gene. The positive colonies where the transposon insertion has

occurred in PLY gene were 1254 base pairs bigger (size of Tn is 1254bp; mentioned in MGS™ protocol) than WT PLY. The plasmid DNA was purified from the positive colonies and was then treated with *NotI* enzyme to remove the body of the transposon, re-ligated with T4 DNA ligase and was chemically transformed in XL-1 blues.

The plasmid DNA was purified from positive XL-1 blue colonies and was confirmed by sequencing. The 15 base pair insertions have a *NotI* site recognition site (GC GGCC GC) (Figure 4.10). The MGS kit also has a palindromic *NotI* mini primer supplied which can anneal to both the strands of the target DNA.

In order to roughly evaluate the position of the transposon insertion a PCR reaction was set up using *NotI* mini primer with 7F and *NotI* mini primer with 7G.

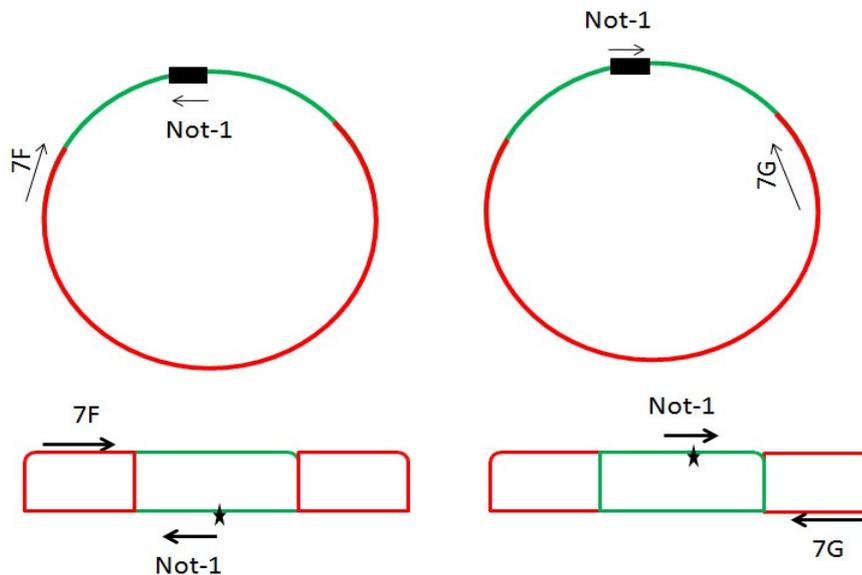


Figure 4-3 PCR reactions with *NotI* mini primers

The figure showing primer annealing and amplification of D4PLY gene (green line), pET33b plasmid (red line) with primers 7F and *NotI* and 7G and *NotI*. The small black indicates transposon with *NotI* site. Two separate PCR reactions were set up; one with *NotI* miniprimer and 7F and the other with *NotI* and 7G.

Figure 4.3 shows the amplification with a combination of *NotI* mini primer and the vector-binding forward and vector-binding reverse primer. For each individual positive colony two reactions were set up, one with the vector-binding forward primer and *NotI* mini primer and the second with the vector binding reverse primer and *NotI* mini primer.

The PCR products were analysed on a gel and the size of the PCR product will roughly determine where the 15 base pair insertion has gone in relation to the two vector-binding forward and reverse primers. The exact location of the 15 base pair insertions (5 amino acid residues) was determined by sequencing results.

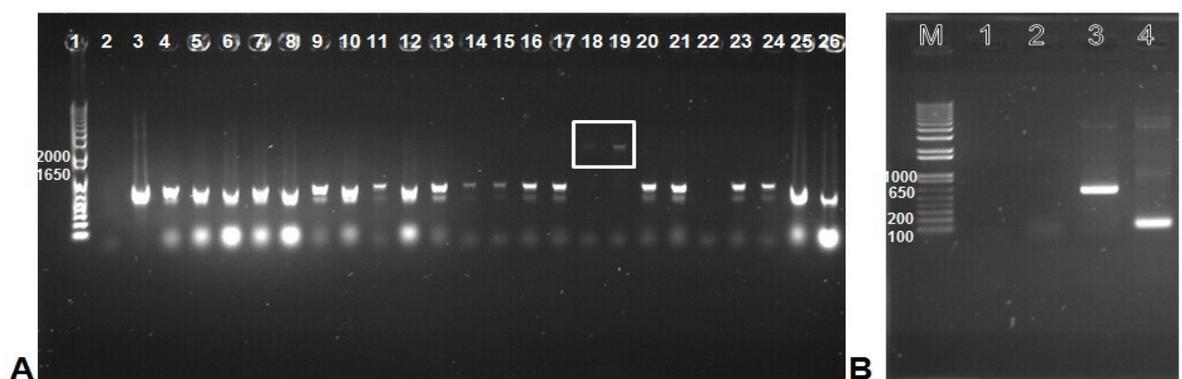


Figure 4-4 DNA gel pictures of transposon insertion and PCR with *NotI* mini primer

4A) Showing the results of colony PCR with 7F and 7G primers. The white box indicates the two positive colonies (lanes 18 and 19) with transposon insertion. In all other lanes transposon was not inserted in D4PLY gene since all the bands are of the same size as positive control (D4PLY amplified with 7F and 7G primers) in lane 3. 4B) Lane M is the DNA marker followed by Lane 1 negative control (No plasmid with 7F and *NotI* mini primer), Lane 2 Negative control again (colony where Tn has not inserted in the PLY gene) with 7F and *NotI* mini primer, Lane 3 (positive colony where Tn has inserted in the PLY gene) with 7F and *NotI* mini primer and Lane 4 same positive colony with 7G and *NotI* miniprimer.

Figure 4.4 A shows the two plasmids highlighted with a white box in lanes 18 and 19 appeared to have transposon inserted in them. They were treated with *NotI* to remove the body of the transposon, religated and plasmid DNA purified and

was sent for sequencing. A colony PCR was also done with *NotI* mini primer in figure 4.4 B.

The result 4.4 B shows that with the positive colony (Tn insertion), a band of approximately 800 base pairs were present with 7F and *NotI* mini primer and a small band of approximately 200 base pairs present with 7G with *NotI* mini primer. There are few other bands present which might suggest non specific amplification because of the small size of the *NotI* mini primer (mentioned in the MGSTM protocol).

The sequencing results for the three positive colonies were checked to see where exactly the five amino acids insertions have gone in the D4PLY.

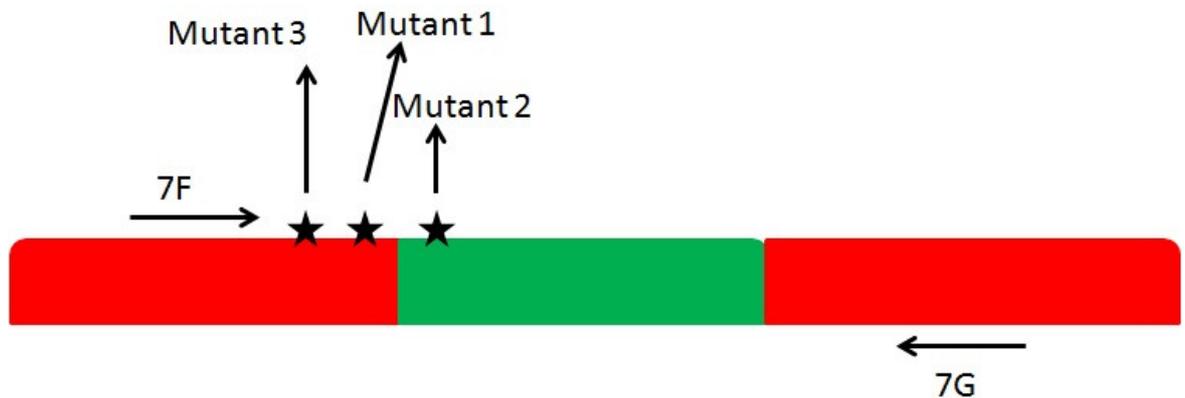


Figure 4-5 Transposon insertions in different regions of the pET33bD4PLY

The above figure showing places of three positive transposon insertions (black stars) in D4PLY gene (green rectangular box), 7F and 7G primers annealing to the plasmid (red rectangular boxes). In Mutant 1 and 3, Tn has gone in the vector backbone and Mutant 2 having Tn inserted in D4PLY gene.

Figure 4.5 showing the two mutants (Mutant 1 and Mutant 3) were not actually in the D4PLY gene and therefore were discarded. The Mutant 2 was the only successful mutant where the transposon had actually gone into the D4PLY.

This plasmid containing the mutant DNA (five amino acid insertion) was then chemically transformed into BL-21 cells for expression. The protein was expressed according to the same protocol as mentioned in section 2.8. This new mutant could not be purified due to formation of inclusion bodies. Several attempts were made to improve expression including change of induction temperature (37 °C to 20°C), concentration of IPTG (2mM to 0.5mM), use of auto induction media and time post induction (3 hours to 12 hours) but still the protein could not be expressed in the soluble form.

One of the mutants generated in the WT PLY background (tag free system) discussed later in this chapter has the same 5 amino insertions (after residue 389) and that mutant protein was soluble so further purification attempts of this mutant in pET33bD4PLY background were not carried out (Table 4.1).

4.2.1 Problems in random mutagenesis with pET33bD4PLY

There were several issues with transposon insertion in the pET33bD4PLY. Firstly there were not many positive colonies with transposon insertion in the actual D4PLY gene even after several attempts. The large number of colonies that grew on the plate after treating with the transposon showed that transposon insertion had occurred in the plasmid since the reaction was plated on the agar supplemented with both kanamycin and chloramphenicol. The colony PCR was done to pick the positive colonies in the D4 of the gene. The colony PCR results showed very few (only three) colonies with the transposon insertion. The possible explanation for not getting transposon insertion in the D4 gene itself could be that the D4PLY is relatively small and is roughly $\frac{1}{4}$ of the full length of the PLY gene and most of the transposon insertion was going in the vector back

bone rather than the actual gene. The transposon itself has no preference for any specific target sequence (MGSTM kit manual).

Secondly, the primers (7F and 7G) used to amplify D4PLY gene were few hundred base pairs away from the gene and was amplifying some portion of the vector along with the D4PLY (figure 4.5). Two of the three positive colonies (black asterisk in figure 4.5) went in the vector instead of going in the actual gene. This was confirmed by the sequencing results of the three mutants.

The next challenge was the purification of the mutant protein. As stated earlier several attempts were made to get the protein in the soluble fraction but were unsuccessful. The presence of the mutant protein was confirmed in the insoluble fraction by doing a western blot with anti-PLY antibody. The 5 amino acid insertion at the same place in full length PLY (WT PLY background) was soluble showing that 5 amino acid insertions are not tolerated by D4PLY alone (Table 4.1).

To address these issues, full length PLY in a tagfree system was used as a target so that a larger sequence region was provided for the transposon insertion; also any possible interference from His tag is removed to allow observation of residual binding/aggregation with the red blood cells. The new primers were also designed that were flanking PLY gene only (Figure 4.7).

The tagfree vector (pPAL7) having the WT PLY gene was used. The WT PLY causes the lysis of the red blood cells and since the aim of the project is to identify sites that were responsible for causing the aggregation/binding of the red blood cells, so a pPAL-7 Δ 6 PLY was constructed by deleting the two amino

acids at positions (146,147) using the SDM kit. The primers used were 23B and 23C as mentioned in the list of oligonucleotides (table 2.2). The random mutants were made both in the WT and $\Delta 6$ PLY background. Previous experiments showed no differences in the lytic activity between the His tag purified PLY and the tagfree PLY (T.J.Mitchell, personal communication). $\Delta 6$ PLY tagfree protein was compared with the His tag purified $\Delta 6$ PLY on a hemagglutination assay to observe if there was still the same aggregation phenotype.

4.3 Random mutagenesis in pPAL7 $\Delta 6$ PLY and WT PLY

4.3.1 NotI site removal in pPAL7VECTOR

The pPAL7 vector has a *NotI* site and the *NotI* site was removed by a SDM reaction using the SDM kit following the same protocol as described in the materials and methods section 2.5. The SDM reaction was transformed in XL-1 blues and plasmid DNA purified and sent for sequencing. The sequencing reaction confirmed that the *NotI* site was successfully removed from the vector.

4.3.2 Generation of pPAL7 $\Delta 6$ PLY

The pPAL7 $\Delta 6$ PLY was constructed from pPAL-7 WT PLY according to the same protocol as described above and in materials and methods section 2.5. The resultant reaction was transformed in XL-1 blues and plasmid DNA was purified and sent for sequencing. The sequencing results confirmed that the two amino acids at position 146 and 147 (alanine and arginine) were successfully deleted.

		720	730	740	750	760	770	780	790	800
» tagfreejiang	713	AAGTGGCATCAAGATTATGGTCAGGTC	CAATAATGTCCCA	GCTAG	ATGCAGTATGAAAAAATAACGGCTC	CACAGCATGGAACAACTCAAGGTC				
» tagfreewitho...	713	AAGTGGCATCAAGATTATGGTCAGGTC	CAATAATGTCCCA	GCTAG	ATGCAGTATGAAAAAATAACGGCTC	CACAGCATGGAACAACTCAAGGTC				
» Delta6tagfree...	713	AAGTGGCATCAAGATTATGGTCAGGTC	CAATAATGTCCCA	-----	ATGCAGTATGAAAAAATAACGGCTC	CACAGCATGGAACAACTCAAGGTC				
« 34cutr	1				A-----	ATGCAGTATGAAAAAATAACGGCTC	CACAGCATGGAACAACTCAAGGTC			
» 33cutf	659	AAGTGGCATCAAGATTATGGTCAGGTC	CAATAATGTCCCA	-----	ATGCAGTATGAAAAAATAACGGCTC	CACAGCATGGAACAACTCAAGGTC				

Figure 4-6 Sequencing results showing two amino acids deletion in WT PLY

The above diagram shows the two amino acids (alanine and arginine) deletions at position 146 and 147. The sequencing results (34 cut and 33 cut) were aligned with the parent (tagfree jiang, tag free with out *NotI*) and the new (Delta6 TagfreePLY) sequence using Vector NTI software.

4.3.3 PLY amplification with TagfreePLY primers

The 7F and 7G primers amplified the D4PLY gene along with few hundred bases on either side of vector. This led to transposon insertion in the vector region in two out of the three mutant generated in pET33bD4PLY (Figure 4.5). To overcome this issue, new primers were designed to amplify the PLY gene only. With the same PCR conditions the new primers were tried along with the 7F and 7G and PLY was successfully amplified with both the primer pairs.

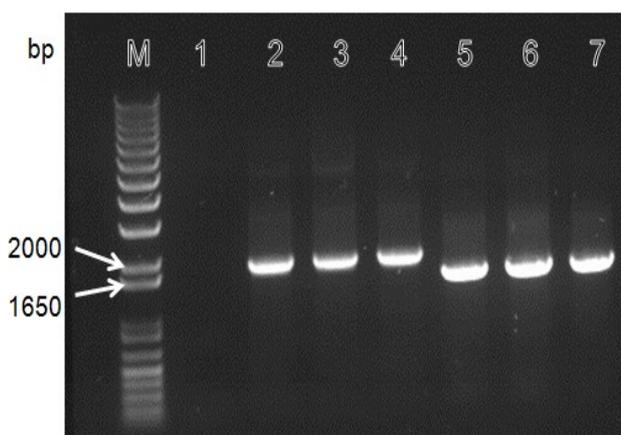


Figure 4-7 DNA gel picture showing the PLY amplification with new set of primers

Lane M showing DNA ladder followed by lane 1, negative control, Lane 2-4 PLY amplification with 7F and 7G primers and Lane 5-7 PLY amplification with new primers. The two bands of 1650 and 2000 base pairs are indicated by the small white arrows.

Figure 4.7 showing PLY amplification with the new primers. The product amplified with the new primers was few hundred base pairs smaller since only the PLY gene was amplified.

All positive colonies screened with these new primers will reduce any chance of false positive colonies (transposon insertion in the vector back bone) as seen with the 7F and 7G primers.

4.3.4 Construction of random mutants in pPAL7 Δ 6 PLY and WT

PLY

A number of mutants were generated in pPAL7 WT and Δ 6 PLY background. The mutants were generated by using the protocol as described in the materials and methods section 2.6.



Figure 4-8 DNA gel showing transposon insertion in PLY gene

Lane M showing DNA ladder, Lane 1 is the negative control, Lane 2 is the positive control PLY gene. Lane (3-9) and 17 is the PLY gene with transposon insertion. Lane (10-16) and (18-25) showing colonies without transposon insertion.

Figure 4.8 showing the initial screening of the positive colonies after the *in-vitro* transposition reaction. A colony PCR was set up with new primers and colonies with transposon insertion in Δ 6 PLY gene are marked in boxes (White boxes in

above figure). The colonies with transposon insertion were bigger since transposon was 1254bp in size and PLY gene was 1408bp. The PCR was set up again with the PLY primers and transposon specific primers (M1 Cam^R) to see the presence of both the PLY gene and the transposon.

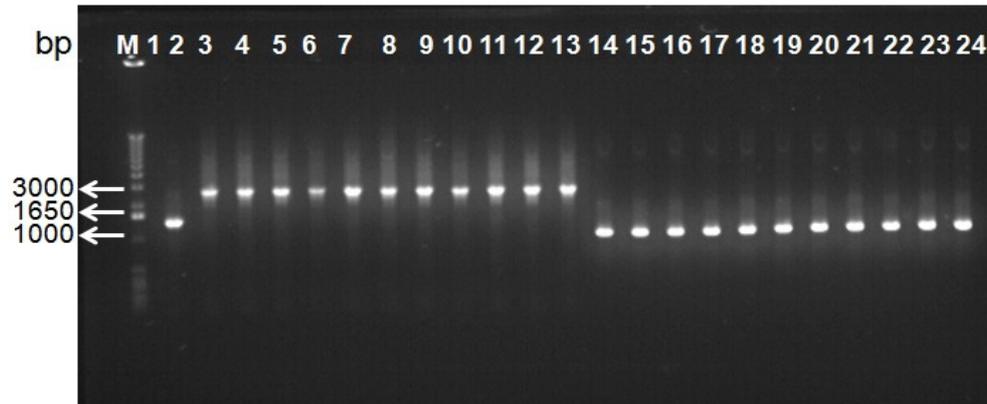


Figure 4-9 DNA gel picture showing the amplification of $\Delta 6$ PLY and transposon with PLY and transposon specific primers

The Lane M shows 1Kb+ Ladder followed by Lane 1 negative control, Lane 2 positive control WT PLY, Lane 3-13 colonies amplified with new PLY primers where transposon insertion have occurred in the PLY gene and Lane 14-24 are the same colonies amplified with transposon specific primers to confirm the presence of transposon.

Figure 4.9 showing amplification of positive colonies (lanes 14-24) with transposon specific primers and a band of approximately 1200bp since the transposon was 1254bp in length. The same colonies (lanes 3-13) when amplified with PLY primers showed bands of around 2.8 kb since PLY was 1408bp and transposon was 1254bp. This reaction confirmed the presence of transposon in the PLY gene in these colonies. New PLY primers used were only amplifying the gene and not any portion of the plasmid.

The plasmid DNA was purified for these positive $\Delta 6$ PLY colonies and was treated with *NotI* enzyme to remove the body of the transposon, religated with T4 DNA Ligase and was again transformed in XL-1 Blues cells. The plasmid DNA was purified from the XL-1 Blues cells and was sent for sequencing to identify the

exact place of 5 amino acids additions. The same colonies were also treated with *NotI* mini primer as it was done with the one positive colony of pET33bD4PLY to identify the position of 5 amino acids insertion in the PLY gene (Figure 4.4b).

		920	930	940	950	960	970	980	990	1000	1010	1020
>Delta6tagfree...	914	TATACAGTCAGCGTAGACGCTGTTAAAAATCCAGGAGATGTGTTCAA						-----GATACTGTAACGGTAGAGGATTTAAAAACAGAGAGGAATTTCTGCAGAGCGT				
tagfreewithou...	914	TATACAGTCAGCGTAGACGCTGTTAAAAATCCAGGAGATGTGTTCAA						-----GATACTGTAACGGTAGAGGATTTAAAAACAGAGAGGAATTTCTGCAGAGCGT				
<< 29_V_2_REV	274	TATACAGTCAGCGTAGACGCTGTTAAAAATCCAGGAGATGTGTTCAA				TGCGGCCGCATTCAA		GATACTGTAACGGTAGAGGATTTAAAAACAGAGAGGAATTTCTGCAGAGCGT				
>> 29_V_2_FWD	795	TATACAGTCAGCGTAGACGCTGTTAAAAATCCAGGAGATGTGTTCAA				TGCGGCCGCATTCAA		GATACTGTAACGGTAGAGGATTTAAAAACAGAGAGGAATTTCTGCAGAGCGT				

Figure 4-10 Sequencing results showing addition of 15 base pairs (highlighted in yellow) in $\Delta 6$ PLY background using Vector NTI software programme

Figure showing sequencing reaction of 15 base pair additions (highlighted yellow) from of the positive colonies aligned to the parent sequence using Vector NTI software.

Figure 4.10 shows the sequencing results of one of the mutants 29.5 (Table 4.1) generated in $\Delta 6$ PLY background. The results of the forward and reverse primers were aligned to the parent vectors $\Delta 6$ PLY without *NotI* site and Tag free PLY with *NotI* site. The results show the fifteen base pair addition (5 amino acids) in the plasmid. The 15 base pair addition is highlighted in yellow and is (TGCGGCCGCATTCAA). *NotI* sequence is GCGGCCGC which is present in the 15 base pair addition (Figure 4.10) and *NotI* mini primer at this site along with 7F and 7G anneals and amplifies the gene in a PCR reaction and roughly identifies the 5 amino acid insertions as explained in figure 4.4.

The mutants in WT PLY back ground were made exactly the same way as for the $\Delta 6$ PLY back ground. A number of mutants were generated using the transposon mediated random mutagenesis in both WT PLY and $\Delta 6$ PLY back ground are listed in the table 4.1 below. The insertion place and translation of five amino acids residues are also mentioned in the table 4.1.

Table 4-1 List of mutants generated by transposon mediated random mutagenesis

S.No	Name of the Mutants	Background	Insertion Place in the PLY gene	Amino acid sequence	PLY domain 1,2 & 3 or Domain 4	Vector
		WT/ Δ 6/D4				
1	Mutant 1	WT PLY	After amino acid 410	SAAAT	Domain 4	pPAL7
2	Mutant 2	WT PLY	After amino acid 24	VRPHQ	Domain 1,2 and 3	pPAL7
3	Mutant 6	WT PLY	After amino acid 389	CGRRG	Domain 4	pPAL7
4	Mutant 9	WT PLY	After amino acid 312	CGRRG	Domain 1, 2 and 3	pPAL7
5	Mutant 12	WT PLY	After amino acid 112	CGRSF	Domain 1, 2 and 3	pPAL7
6	Mutant 13	WT PLY	After amino acid 305	GAAAT	Domain 1, 2 and 3	pPAL7
7	Mutant 19	WT PLY	After amino acid 441	CGRNE	Domain 4	pPAL7
8	Mutant 21	WT PLY	After amino acid 433	CGRTW	Domain 4	pPAL7
9	Mutant 1	Δ 6 PLY	After amino acid 136	CGRNQ	Domain 1, 2 and 3	pPAL7
10	Mutant 5	Δ 6 PLY	After amino acid 326	CGRTR	Domain 1, 2 and 3	pPAL7
11	Mutant 13	Δ 6 PLY	After amino acid 318	CGRSS	Domain 1, 2 and 3	pPAL7
12	Mutant 27.10	Δ 6 PLY	After amino acid 449	MRPQR	Domain 4	pPAL7
13	Mutant 29.4	Δ 6 PLY	After amino acid 7	CGRSL	Domain 1, 2 and 3	pPAL7
14	Mutant 29.5	Δ 6 PLY	After amino acid 215	CGRIQ	Domain 1, 2 and 3	pPAL7
15	Mutant 35	Δ 6 PLY	After amino acid 365	CGRNC	Domain 4	pPAL7
16	Mutant 44.1	Δ 6 PLY	After amino acid 420	CGRIT	Domain 4	pPAL7
17	Mutant 44.2	Δ 6 PLY	After amino acid 410	CGRTT	Domain 4	pPAL7
18	Mutant 2	D4 PLY	After amino acid 389	SAAAV	Domain 4	pET33b

The table 4.1 explains the list of random mutants generated in WT PLY, Δ 6 PLY and D4PLY background. It also shows exactly where the transposon has inserted, which new amino acid residues have been added to the protein and in which domains the insertions have gone.

Table 4.1 also shows that the insertion place (after amino acid 389) of the only mutant generated in pET33bD4PLY. This mutant could not be purified due to problems mentioned in this chapter under the heading 4.2.1.

4.4 Protein expression and purification

$\Delta 6$ PLY was purified first by the Profinity eXact purification protocol. In total 8 mutants were generated in WT PLY and 9 mutants were generated in $\Delta 6$ PLY background. All plasmids containing the mutant DNA were chemically transformed in BL-21 (DE3) for protein expression and were purified by Profinity eXact™ protein purification system as described in the materials and methods section 2.8.7.

The purified proteins were analysed by SDS-PAGE gels.

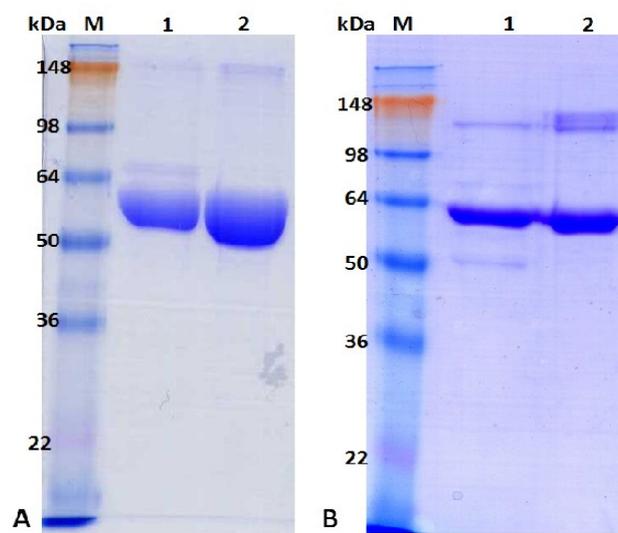


Figure 4-11 SDS-PAGE of $\Delta 6$ PLY, Mutant29.5 $\Delta 6$ PLY, Mutant1 WT PLY and Mutant2 WT PLY
A) Mol.wt of $\Delta 6$ PLY and M29.5 is 53kDa. Lane M contains 5 μ l of the mol. wt.marker SeeBlue® Plus 2 followed by 5 μ l recombinant protein $\Delta 6$ PLY and M29.5 $\Delta 6$ PLY (Lane 1 and 2). **B)** Mol.wt of M 1 WT PLY and M6 WT PLY is 53kDa. Lane M contains 5 μ l of the mol. wt .marker SeeBlue®Plus2, followed by 5 μ l purified protein M1 WT PLY and M6 WT PLY (Lane 1 and 2). A few bands were seen (contaminants) in both the gels (A&B) and were removed by using Amicon Ultra Centrifugal concentrators as explained in section 2.8.9.

Figure 4.11 showing purified proteins after one step purification and the protein size also correspond to the molecular weight markers. The purified proteins were passed through Amicon Ultra-15 50K centrifugal filter removing any contaminates less than 50kDa molecular weight. Unlike the TL mutants these random mutants were purified from 0.5 litre of the culture and screening was

done for aggregation negative mutants in case of $\Delta 6$ PLY and non-lytic in case of WT PLY background. All purified mutants were then run on as SDS-PAGE.

All mutants in WT and $\Delta 6$ PLY back ground were purified in a similar way and purity was checked by running the SDS-PAGE (Figure 4.12). The proteins were then buffer exchanged into PBS and were stored at -80°C .

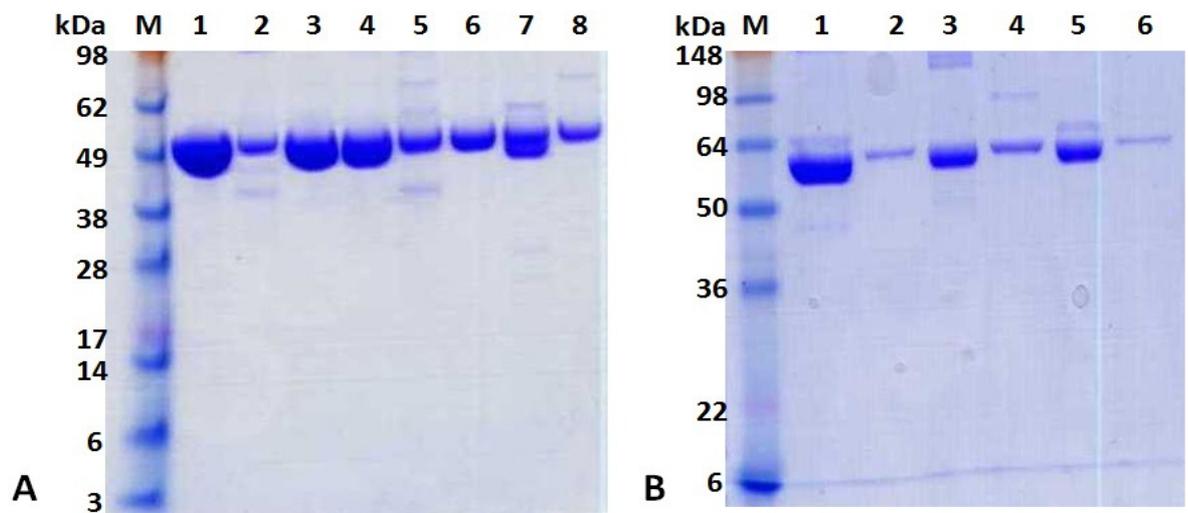


Figure 4-12 SDS-PAGE showing purified WT PLY and $\Delta 6$ PLY mutants

A) All purified $\Delta 6$ PLY mutants on a precast gel. Lane M is the 5 μl mol.wt marker SeeBlue® Plus 2 followed by 5 μl of M1, M35, M13, M27.10, M29.4, M5, M44.1 and M44.2 $\Delta 6$ PLY in Lanes 1-8 respectively. All mutant proteins have expected size of 53kDa. **B)** All purified WT PLY mutants on a self poured gel. Lane M is the 5 μl mol.wt marker SeeBlue® Plus 2 followed by 5 μl of M2, M9, M12, M13, M21 and M19 WT PLY in Lanes 1-6 respectively. The above proteins in gels A and B were purified by single step purification and hence some contaminants are present in the gels (small bands below and above 53kDa size). The proteins were passed through ultra centrifugal filters to remove contaminants before storage.

All the purified proteins correspond to the expected size as the molecular weight marker. There were some contaminants and were removed using Ultra centrifugal filters before storage as explained in 2.8.9.

The concentration of the all purified proteins were measured using Nano Drop and following the same method as explained in the materials and methods

section 2.8.10. The table 4.2 shows the concentration of the purified proteins from 0.5 litre of bacterial culture.

Table 4-2 Concentration of the purified mutant proteins

S.No	Name of the Mutants	Background WT or $\Delta 6$	mg
1	Mutant 1	WT PLY	2.13
2	Mutant 2	WT PLY	5.616
3	Mutant 6	WT PLY	2.28
4	Mutant 9	WT PLY	1.35
5	Mutant 12	WT PLY	5.6
6	Mutant 13	WT PLY	0.69
7	Mutant 19	WT PLY	0.5
8	Mutant 21	WT PLY	3.39
9	Mutant 1	$\Delta 6$ PLY	2.1
10	Mutant 5	$\Delta 6$ PLY	8
11	Mutant 13	$\Delta 6$ PLY	5.64
12	Mutant 27.10	$\Delta 6$ PLY	1.65
13	Mutant 29.4	$\Delta 6$ PLY	1.008
14	Mutant 29.5	$\Delta 6$ PLY	3.12
15	Mutant 35	$\Delta 6$ PLY	0.496
16	Mutant 44.1	$\Delta 6$ PLY	2.25
17	Mutant 44.2	$\Delta 6$ PLY	1.6

4.5 Western blot of the purified proteins

All purified mutant proteins were further analysed by western blotting. The western blotting was done according to the same protocol as mentioned in the materials and methods section 2.9.2.

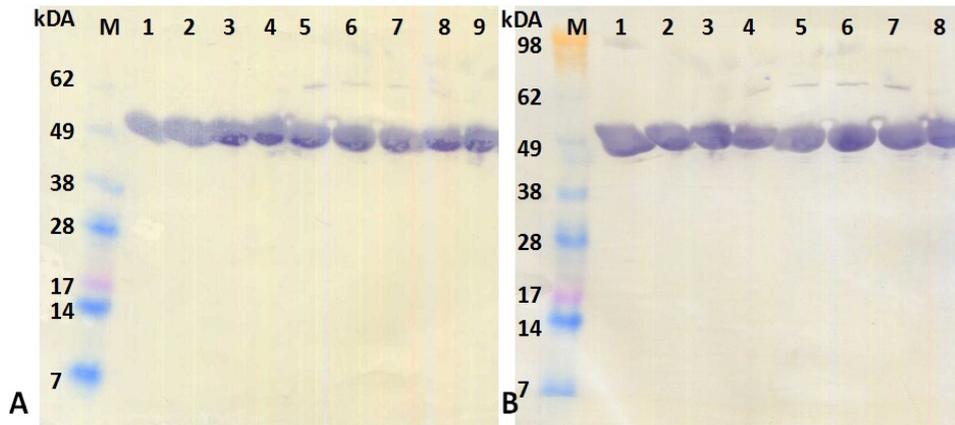


Figure 4-13 Western blot of the purified mutant proteins

A) Western blots of $\Delta 6$ PLY mutants. 1 μ g each of the purified proteins was loaded on the gel. Lane M is the mol wt marker followed by M1, M5, M13, M27.10, M29.4, M29.5, M35, M44.1 and M44.2 in Lanes 1-9 respectively. B) Western blots of WT PLY mutants. 1 μ g each of the purified proteins was loaded on the gel. Lane M is the mol wt marker followed by M1, M2, M6, M9, M12, M13, M12, and M21 in Lanes 1-8 respectively. There was a faint band above some of the purified proteins (Lanes 5-7 on both blots) which might be some non-specific antibody binding.

4.6 Characterisation of the mutant proteins

The lytic activity WT PLY mutants were analysed on the red cell assay plate. The $\Delta 6$ PLY mutants were assessed for red blood cell aggregation on a hemagglutination assay.

4.6.1 Lytic activity of the mutant proteins

All 8 random mutants created in WT PLY background were assessed for their lytic activity. Lytic activity of each of individual mutant is plotted on the graphs (Figure 4.14).

These experiments were set up to identify any other residues apart for the two residues ($\Delta 146A\Delta 147R$) identified by (Kirkham *et al.*, 2006a) that may be responsible for the lytic activity.

In WT PLY background; four mutants (1, 6, 19 and 21), the 5 amino acid insertions had gone into the domain 4 whereas the other four mutants (2, 9, 12 and 13), the insertions had gone into the domains 1-3 of the protein. All the 5 amino insertion mutants in domain 4 were plotted in the same graph (Figure 4.14A) and rest of the mutants were plotted in the other graph (Figure 4.14B).

The lytic activity of these 5 amino acid random mutants for erythrocytes was assessed by indirect measurement of released haemoglobin. The specific haemolytic activities of the toxins were then calculated in HU/mg and are stated in table 4.3.

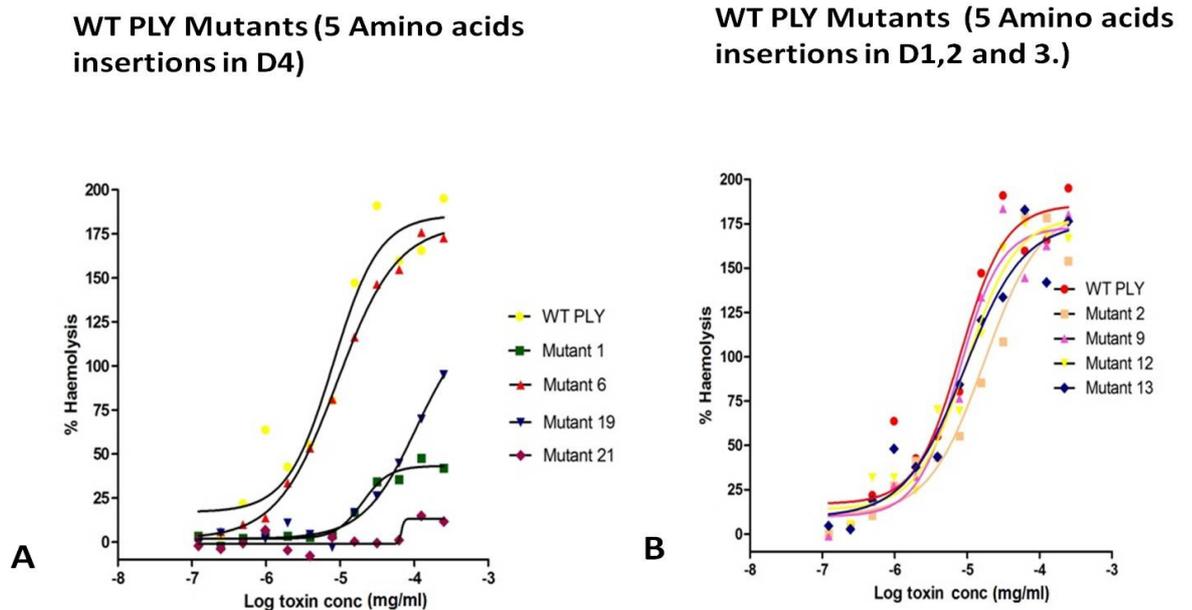


Figure 4-14 Haemolytic activity of the mutant proteins

% lytic activity of individual mutant (Y-axis) plotted on the graphs against log toxin concentration (X-axis) of each mutant.

Figure 4.14A showing WT PLY mutants with 5 amino acid insertions in D4 of the toxin resulted in a decrease in lytic activity of the all of them apart from Mutant 6, where the lytic activity is almost comparable to the WT PLY. The 5 amino acid

(a.a) insertions in Mutant 6 WT PLY were exactly at the same place (after 389 amino acids) where the five a.a insertions in pET33bD4PLY had gone (Table 4-1). In case of D4PLY, this 5 amino acid random insertions made the mutant insoluble but in case of full length PLY it was soluble. The 5 amino acids residues inserted in case of Mutant 6 were (CGRRG) where as in D4 background it was (SAAAV) which may have made former soluble and the latter insoluble.

The 5 amino acid insertions in three other mutants in D4PLY have all gone in important positions. In Mutant 21, the 5 amino acid insertion has gone in the undecapeptide region after first tryptophan residues and has made this mutant non-lytic. In Mutant 19, the 5 amino acid insertion has gone very near to the undecapeptide region exactly after 4 amino acids after the undecapeptide region and in Mutant 1; the 5 residues were inserted after the 3rd hydrophobic loop (L-3) of domain 4 PLY (Figure 4.16A).

The results of this study were comparable with the findings of the previous studies (Korchev *et al.*, 1998, Berry *et al.*, 1995) which showed that undecapeptide region (residues 427-437) is an important region of the toxin and mutation made in this region at one of the first three tryptophan residue 433 (W>F) drastically reduced the lytic activity of the toxin to 1%. Although in this study single amino acids were not substituted, but 5 amino acids were inserted at the same position.

Table 4-3 Specific haemolytic activity and some additional information of the mutant proteins

Name of Protein	Haemolytic activity HU/mg	Additional Information	Lytic/Non-Lytic
WT PLY	3.38×10^5		
Mutant 1	0	3 Amino acids after 3rd small hydrophobic loop of D4 PLY. Same insertion place as of Mutant 44.2 in $\Delta 6$ PLY background.	Non-Lytic
Mutant 2	1.72×10^5		Lytic
Mutant 6	2.76×10^5		Lytic
Mutant 9	2.75×10^5		Lytic
Mutant 12	2.77×10^5		Lytic
Mutant 13	3.18×10^5		Lytic
Mutant 19	1.38×10^4	Inserted after 4 amino acids of undecapeptide	Lytic
Mutant 21	0	Insertion in undecapeptide after first tryptophan residue.	Non-Lytic

The lytic activity of the other mutants where the 5 amino acid insertions were in the domains 1, 2 and 3 (Mutant 2, 9, 12 and 13) were similar to the WT PLY suggesting that addition of 5 amino acid residues in other domains does not have an effect on the lytic activity of the toxin. These random mutants had not gone in any well studied regions of the protein. None of these mutants had gone near the two residues ($\Delta 146\Delta 147R$) which are responsible for abolishing the lytic activity of the toxin (Figure 4.16A).

4.6.2 Hemagglutination activity of the mutant proteins

The random mutants generated in the $\Delta 6$ PLY background were checked for their ability to aggregate/agglutinate red blood cells. In total 9 mutants were generated; four mutants (Mutant 27.10, 35, 44.1 and 44.2) had 5 amino acids residues inserted in domain 4 and five mutants (Mutant 1, 5, 13, 29.4 and 29.5) had insertions in domains 1, 2 and 3 of the toxin. The assay was done according to the same protocol as mentioned in the materials and methods section 2.9.3.

The tagfree was also causing the same aggregation/ agglutination phenotype as His-tagged purified $\Delta 6$ PLY. $\Delta 6$ PLY is non-lytic so the specific lytic activity for

these mutants was not calculated and they were only screened for their aggregation activity.

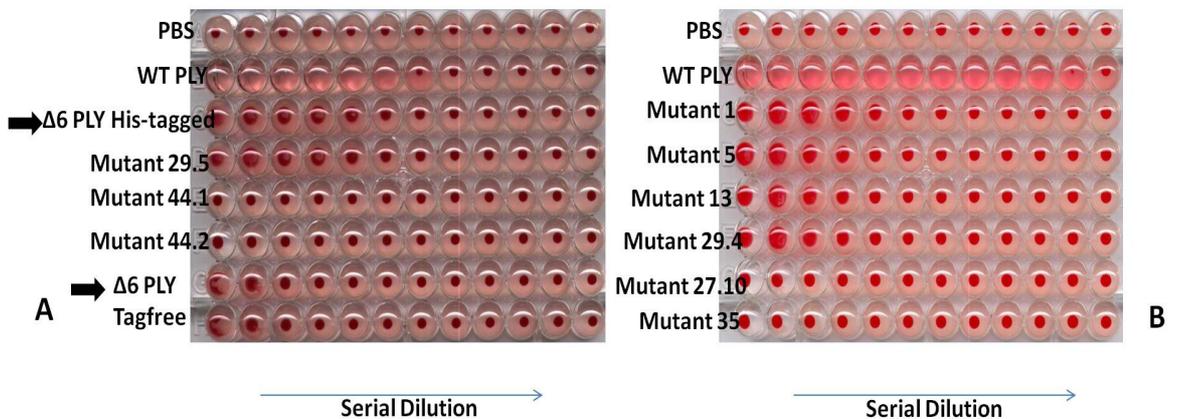


Figure 4-15 Hemagglutination assay of the mutant proteins

1 μ g of mutant proteins was added in the first well the plate and serial dilutions were made (1:2) across the 96 well plate. The plates were observed for the agglutination of the red blood cells as seen with the $\Delta 6$ PLY. 1 ng of WT PLY and 50 μ l PBS were used as positive and negative controls. The small arrows showing the Histagged and tagfree version of $\Delta 6$ PLY.

Figure 4-15 showing the different phenotypes as explained in figure 2.5. PLY used as positive control was causing lysis and thus the pinkish tinge observed in the wells. PBS was not causing any effect and hence a red blood cell pellet was seen. The result shows that both the His-tagged and the tagfree PLY (black arrows) were showing aggregation phenotype. Therefore the His-tag seems not to be causing this aggregation phenotype. Mutants 1, 5, 13, 29.4, 29.5 was causing the same aggregation phenotype as cell lysates of the DNA 2.0 Library and Mutants 27.10, 35, 44.1 and 44.2 showing the same aggregation negative phenotype and clearing well effect as seen with TL mutants in figure 3.8.

The results suggest that Mutants 27.10, 35, 44.1 and 44.2, where 5 residues were inserted in the domain 4 of the toxin were aggregation negative and Mutants 1, 5, 13, 29.4 and 29.5 where amino acids were inserted in domains 1, 2 and 3 were agglutination/aggregation positive.

In these hemagglutination assays the same clearing effect of the wells was observed in Mutant 27.10, Mutant 44.2 and Mutant 35 as seen with the TL mutants. The clearing effect however, was less pronounced in Mutant 44.1.

These mutations were mapped on the structure and figure 4.16B showing that all the aggregation negative phenotype mutants were near or in the well studied regions of the toxin whereas the aggregation positive phenotype mutants were in domains 1, 2 and 3 (all mutants highlighted in blue).

Table 4.4 states the aggregation/agglutination type, domains of PLY where these 5 amino acid insertions had gone and any important information of all the purified mutants generated in $\Delta 6$ PLY background.

Table 4-4 Haemagglutination results of the mutant proteins

S.No	Name of the Mutant	PLY domain 1,2 & 3 or Domain 4	Additional Information	Aggregation Phenotype
1	Mutant 1	Domain1,2 and 3		Positive
2	Mutant 5	Domain 1, 2 and 3		Positive
3	Mutant 13	Domain 1, 2 and 3		Positive
4	Mutant 27.10	Domain 4	11 amino acids before Cholesterol binding site TL (459,460)	Negative
5	Mutant 29.4	Domain 1, 2 and 3		Positive
6	Mutant 29.5	Domain 1, 2 and 3		Positive
7	Mutant 35	Domain 4	One amino acid before the 2 nd small hydrophobic loop of D4PLY	Negative
8	Mutant 44.1	Domain 4	6 amino acids before undecapeptide	Negative
9	Mutant 44.2	Domain 4	3 Amino acid after the 3 rd small hydrophobic loop of D4 PLY.Same as M1 WT PLY	Negative

4.7 Mapping of mutants

The crystal structure of PLY is not solved and the structure prediction was made by using one of the computational tools for structure prediction, named the protein homology/analogy recognition engine (Phyre) (Kelley & Sternberg, 2009). PLY sequence was submitted to the website and the resulting PLY structure was then used to map the 5 amino acid mutations using PYMOL.

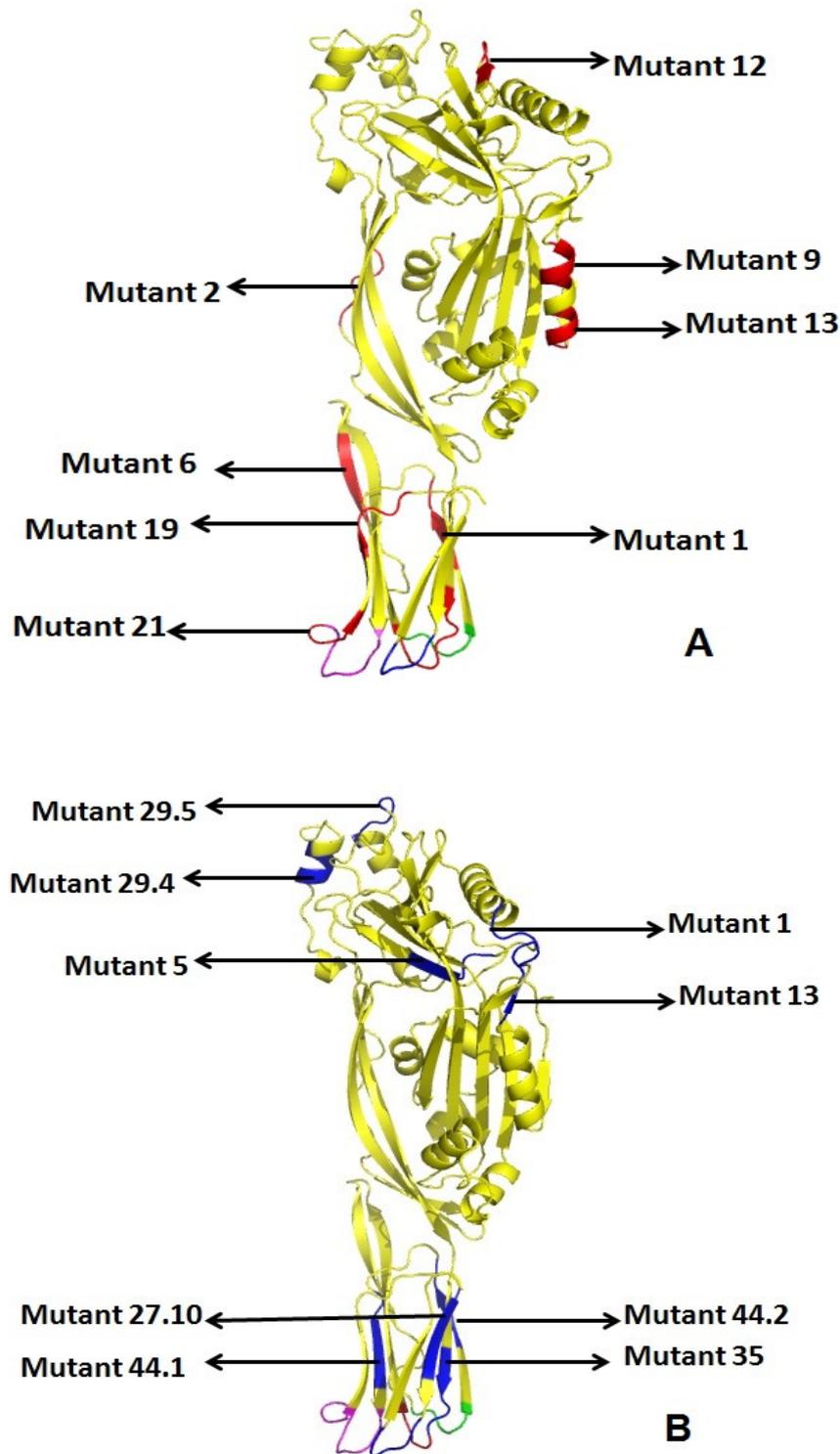


Figure 4-16 Structural model of PLY generated using computational software for structure prediction (Phyre2) and 5 amino acids mutation mapped using PYMOL

PLY sequence was submitted to the website

<http://www.sbg.bio.ic.ac.uk/phyre2/html/page.cgi?id=index>, which predicts the structure of

the protein based on the closely related solved protein structures. A) In WT PLY background, 5 amino acid insertions in mutants 1, 6, 19 and 21 are in D4 whereas in mutant 2, 9, 12 & 13 insertions are in D1, 2 & 3 of PLY. B) In $\Delta 6$ PLY background 5 a.a insertion in mutants 27.10, 35, 44.1 and 44.2 are in D4 whereas Mutant 1, 5, 29.4 and 29.5 insertions are in D1, 2 & 3 of PLY. Undecapeptide, D4 loops 1, 2 and 3 are highlighted in magenta, blue, red and green.

In Figure 4.16A, insertions in M.1 and M.19 have gone in β - sheets of D4 and possibly interfering the subsequent pre-pore and pore formation after initial binding, thereby exhibiting decrease in lytic activity (table 4.3) where as in M.21 the insertions are in the undecapeptide region and therefore this mutant may not have accomplished complete binding and thus showing no lytic activity at all. Mutant 6 lytic activity is similar compared to WT PLY since the mutation is neither in any loops (L1-3) nor the well conserved undecapeptide region and therefore might not be interfering with the binding and subsequent pore formation.

In $\Delta 6$ PLY background (Figure 4.16B) 5 amino acid insertions in M27.10. 35, 44.1 and 44.2 are in β -sheets of D4PLY near the undecapeptide region and near the loops (L1-3) and presumably hindering the perpendicular orientation of the domain 4 loops and undecapeptide thereby showing the agglutination negative phenotype.

4.8 Issues with Random Mutagenesis using Tn insertion

4.8.1 Purification of the mutant in pET33D4PLY

The only mutant (Mutant 2) generated in pET33bD4PLY vector backbone was insoluble and due to time constraints, purification of this mutant from inclusion bodies was not performed. It was assumed that 5 amino acid insertions in this region of the protein might have made this mutant insoluble. However, mutation at the same position in full length WT PLY (Mutant 6) was soluble and almost 2mg of the recombinant mutant protein was purified (Table 4.1 and 4.2). This suggests that five amino acids insertion effect is different in D4PLY gene only compared to the full length toxin, which due to its larger structure may have

stabilised the mutant protein compared to the smaller domain 4 of the toxin, which renders it insoluble. The other reason might be the different amino acids insertions in these two mutants. In case of D4 PLY mutant, the 5 amino acids were SAAAV and in case of WTPLY, it was CGRRG.

In both WT PLY and $\Delta 6$ PLY in pPAL-7 vector backbone, almost 50% (5 out of 9 in $\Delta 6$ PLY and 4 out 8 in WTPLY) of the transposon insertion went in D4 region of the gene. D4 is just a quarter ($\frac{1}{4}$) the size of the full length toxin and the reason for this high percentage of Tn insertion in D4 is unclear as transposon has no specificity for any particular residues. In most instances the transposon inserted in the vector back bone as well.

4.8.2 Purification of insoluble mutants in pPAL-7 vector

Unlike D4PLY, mostly all the mutants in pPAL-7 vector were soluble although 3 mutants were insoluble. In these three mutants (Mutant 27.3 in $\Delta 6$ PLY background and Mutant 25 and 42 in WT PLY background), the transposon was inserted between residues 190-198. In Mutant 27.3, the insertion was after residue 198, in Mutant 25 it was after 193 and in Mutant 42 after amino acid residue 190. Several things were tried (Induction time, amount of IPTG, temperature and auto induction media) but it was still not possible to purify these mutants. The most likely explanation could be that insertion at this site (190-198) might have made these mutants insoluble. These proteins were expressed in BL21 (DE3) and after induction both the pellet and supernatant was checked for protein expression. The pellet was again resuspended in the wash buffer and sonicated as explained in materials and methods section 2.8.3. The

supernatant and pellet was checked for presence of PLY by blotting with anti PLY antibody.

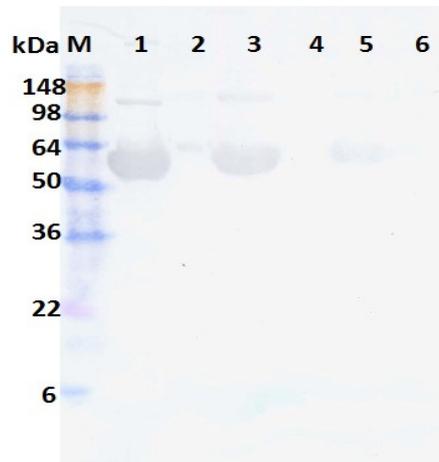


Figure 4-17 Western blot showing PLY in insoluble fraction

5 μ l each of the sample (pellet and supernatant) was loaded on the gel. Lane M is the mol wt marker followed by M 27.3 Δ 6 PLY insoluble fraction (pellet) in lane 1 and soluble fraction (supernatant) in lane 2 followed by M 25 insoluble fraction (pellet) and soluble fraction (supernatant) in lanes 3 and 4 and M 42 WT PLY insoluble fraction (pellet) and soluble fraction (supernatant) in lanes 5 and 6 respectively. There were a band seen in lane 1 and lane 3 probably suggesting a dimer formation.

Figure 4.17 shows that all these mutants were expressed but were not folding properly and hence were not included in further experiments. These three mutants could be co expressed with a protein chaperone (GroES / GroEL) that could minimise the chances of protein mis-folding. However due to time constraints this was not tried.

Region (190-198) mapped on the structure showed that these residues were on one of the β sheet of toxin and insertions at this place might result in improper folding since this sheet is near to the 3 α helices (α helices undergo transition to form β hair pin loops after prepore state(1.8.3)).

The random transposon mediated mutagenesis identified the regions where mutations can be made. The results of this study also suggest that up to 5 amino

acids insertions can be made in various regions of the gene. Previously all the studies were either deleting or substituting one to two amino acid residues.

4.9 Single amino acid addition and substitution in PLY using SDM kit

Mutant 27.10 in $\Delta 6$ PLY background has the 5 amino acid insertions between the actual cholesterol binding site (459,460) and the undecapeptide (residues 427-437) and was showing aggregation negative phenotype on a hemagglutination assay plate (Table 4.4 & Figure 4.15B). In order to test the sensitivity of this region of the toxin to amino acid alterations, a single amino acid (Methionine) addition was made rather than five (MRPQR) using the site directed mutagenesis kit and its effect was compared with the 5 amino acid insertions (Figure 4.19).

A single amino acid substitution was also made in $\Delta 6$ PLY background in at position W433F to observe its effect on the red blood aggregation. This is one of the three tryptophan residues in the undecapeptide region of the toxin. It is already known from the previous studies in WT PLY that mutation made in this residue results in the reduction of the lytic activity of the toxin but its aggregation phenotype was not checked (Paton *et al.*, 1991). In a study done in PFO, all the tryptophan residues in the undecapeptide region were replaced with phenylalanines and resulted in decreased binding and hence reduced lytic activity and the same study also suggested Trp436 is responsible for maintaining the toxin structure (SekinoSuzuki *et al.*, 1996). The results described here in this study also show that insertion of five amino acids at this place in the WT PLY back ground also resulted in reduction of the lytic activity of the toxin (Figure 4.14A).

The above mentioned mutations were made successfully in pPAL-7 $\Delta 6$ PLY tagfree system using the site directed mutagenesis kit and following the same protocol as mentioned in section 2.5.

4.9.1 Purification and characterization

After the sequencing results were proved to be correct the mutants were chemically transformed into BL-21 (DE3) for expression. The proteins were expressed and purified following the same protocol as mentioned in section 2.8.7. The proteins were successfully purified and after purification, proteins were analysed on an SDS-PAGE and a Western blot. The proteins were also run on a hemagglutination assay plate to observe the aggregation phenotype.

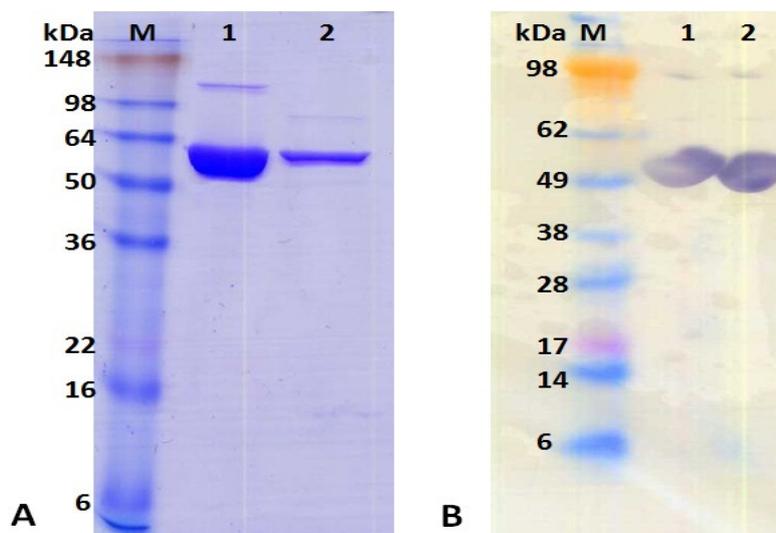


Figure 4-18 SDS PAGE and the western blot of the purified mutant proteins

A) 10 μ l of the purified proteins were loaded on the gel. Lane M contains the 5 μ l protein mol. wt marker followed by purified M.27.10 (methionine added after residue 449) followed by purified protein $\Delta 6$ PLY^(W433F) in lanes 1 and 2 respectively. There was a band present in lane 1 and 2 possibly suggesting some contaminants which were removed using Amicon Ultra centrifugal filters.

B) Western blots of mutant proteins. 1 μ g each of the purified proteins was loaded on the gel. Lane M is the mol wt marker followed by M.27.10 $\Delta 6$ PLY (methionine added after residue 449) and $\Delta 6$ PLY^(W433F) in Lanes 1 and 2 respectively. A faint band is seen which might suggest non-specific antibody binding.

The purified mutant proteins were pure and both were recognised by the anti-PLY antibody. The protein concentration was also measured using nano drop and following the same protocol as stated in section 2.8.10. The concentrations of purified proteins were 1.38mg for $\Delta 6$ PLY (433 W>F) and 0.5mg for M.27.10 (Methionine added after residue 449).

The red cell assay for both of these mutant proteins was performed to check their agglutination phenotype. The assay was performed according to the same protocol mentioned in section 2.9.3.

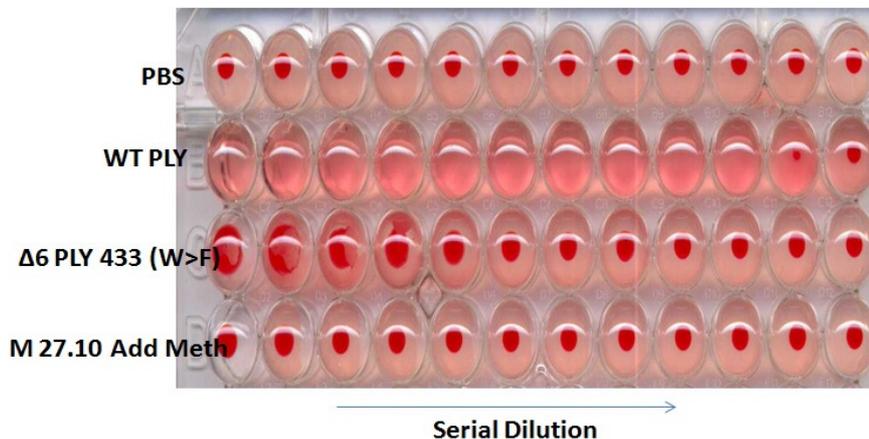


Figure 4-19 Red cell assay of the purified mutant proteins

1 μ g of mutant proteins for $\Delta 6$ PLY (433 W>F) and M.27.10 (methionine added after residue 449) was added in the first well the plate and serial dilutions were made (1:2) across the 96 well plate. The plates were observed for the agglutination of the red blood cells as seen with the $\Delta 6$ PLY. 1ng of WT PLY and 50 μ l PBS were used as positive and negative controls.

Figure 4-19 shows that the single amino acid substitution W433F in the undecapeptide region results in the aggregation of the red blood cells as seen with the $\Delta 6$ PLY. This suggests substitution or addition at this place results in the reduction of the lytic activity but does not eliminate the aggregation phenotype from this mutant; if the amino acid is substituted at this place. This also predicts

that reduction of lytic activity does not eliminate the aggregation activity from the toxin and this is done by site other than the binding site of the toxin.

On the other hand addition of 1 amino acid instead of 5 at the same place in Mutant 27.10 gives the same non-aggregation phenotype showing that addition of even a single amino acid in domain 4 is not tolerable for aggregation phenotype. As mentioned earlier, addition of 5 amino acid residues in Mutant 27.10 were between the cholesterol binding site(s) and the undecapeptide region and even a single addition of amino acid residue may perturb the structure of the toxin, resulting in non-aggregation phenotype. One more possibility could be that presence of second binding site in this region of the toxin which may have resulted in this non-aggregation phenotype. It would be interesting to compare the results by substituting one single amino acid at this place.

4.10 Discussion

The five amino acid insertions were successfully added for the first time using this technique in D4, $\Delta 6$ and WT PLY. A total of 21 mutants were generated through this transposon based random mutagenesis. All of them were created in pPAL-7 vector with full length PLY except one (Mutant 2), which was made in pET33b D4PLY (Table 4.1). The aim of these experiments was to generate a large library of mutants to screen for the lytic activity and aggregation activity but this technique was unable to generate a large number of mutants mainly because a lot of transposon insertion went into the backbone of the plasmid rather than the gene. Transposon insertion is non-specific and it should insert at any site in target plasmid.

Previously, a study was carried out in which random single amino acid substitutions were made in the PLY gene and found an interesting region (H156) in the N-terminal region of toxin. This residue was shown to reduce the lytic activity of the toxin compared to the WTPLY (Hill *et al.*, 1994). The results of this study are comparable with the previous studies (Korchev *et al.*, 1998, Berry *et al.*, 1995, SekinoSuzuki *et al.*, 1996) and one of the mutants generated in WT PLY background (Mutant 21) has 5 amino acids (CGRTW) insertions in the well studied undecapeptide region. This mutant has reduced lytic activity as shown in figure 4.14. All the mutants listed in table 4.2 were successfully purified by using the Profinity eXact protein purification system using the 1 ml Bio-Scale Mini cartridge as described in 2.8.7.

The lytic activity of mutants in WT PLY background (Figure 4.14 A and B) where 5 amino acid insertions were in the domains 1, 2 and 3 of the toxin were comparable with the WT PLY toxin where as the insertions in D4 of the region resulted in a decrease in the lytic activity of the toxin apart from one mutant (Mutant 6). One probable reason for this might be that Mutant 6 is not inserted in any of important well studied region of D4, three small hydrophobic loops (L1, L2 and L3) and undecapeptide.

Similar results were observed with mutants made in $\Delta 6$ PLY background where all mutants were found to be aggregation/agglutination negative with 5 amino acids insertion in D4 whereas all mutants were agglutination/aggregation positive with insertions in domains 1, 2 and 3 (Figure 4.15). This suggests that D4 of the toxin is more sensitive to amino acid insertions compared to domains 1, 2 and 3.

The random single base substitution described by (Hill *et al.*, 1994) also found the lytic activity was not reduced much when mutations were made in domains 1,2 and 3 apart from one mutant (H156Y) which resulted in a 98% reduction in lytic activity compared to WTPLY. This results of this study has shown no reduction in lytic activity in WT PLY mutants where 5 a.a insertions have gone in domains 1-3.

The result of single amino acid substitution at (W433F) in $\Delta 6$ PLY background gives the same aggregation phenotype as $\Delta 6$ PLY where as single amino acid insertion is not tolerable in D4 and gives aggregation negative phenotype in Mutant 27.10. If time permitted, it would be very interesting to see how the toxin behaves by addition of one single amino acid at position 433 in $\Delta 6$ PLY background. The result of this will give a clue that whether toxin can tolerate single amino acid residue at important regions (Undecapeptide, Loops 1-3).

The other experiments in future work would be doing the Circular dichroism (CD) spectroscopy of the mutant proteins and observing any significant structural changes by addition or substitution of the amino acids. Although it is already known from the previous work that substituting the cholesterol binding site with the glycines or alanines does not result in significant secondary structural changes when compared with the WT PFO (Farrand *et al.*, 2009). CD experiments will reveal whether PLY mutants made by site directed mutagenesis and random mutagenesis result in the structural changes of the toxin.

One of the mutants in $\Delta 6$ PLY with a non aggregation phenotype could also be tagged by eGFP and binding observed with the horse blood cells under the fluorescent microscope. Since tagging each individual mutant with gfp is labour

intensive, alternative approaches could be used including the use of secondary labelled fluorescent antibody which can bind to the toxin already bound with red cells. The proteins can also be directly labelled with Alexa Flour dye® and then treating them with cells and assessing binding by fluorescence microscopy.

Similarly, FACS experiments could also be done with the eGFP tagged protein or secondary labelled fluorescent antibody, to observe the mutants' binding with the red blood cells. The FACS experiments not only can determine the binding but can also calculate the extent of binding of different mutant versions of the toxins with the red blood cells.

The toxins can also be used *in-vivo* experiments to determine their effectiveness in eliciting an immune response and compare the response of different versions of the toxins.

5 Screening, characterisation and purification of PLY mutants

5.1 Summary

The random mutations made by addition of amino acids residues in of PLY did not give the clue as to which amino acid/s are responsible for the residual binding/aggregation of the red blood cells. However, a mutant with a single amino acid substitution made in one of the tryptophan residues in the undcapeptide region was still agglutinating red blood cells. This tryptophan residue was responsible for the lytic activity of the toxin as described in the results of the previous chapter. This result suggested that single amino acid substitution in D4PLY of the gene is tolerable as it gives the positive agglutination phenotype with W433F Δ 6PLY mutant and every single amino acid substitution in D4PLY of the gene will eventually track the amino acid/s responsible for this residual binding/agglutination.

In order to identify site/s responsible for this residual binding site, subtle changes were made in the D4 of the toxin. A library of mutants was designed and purchased commercially from DNA 2.0 (CA, USA). The details of the library are explained in this chapter under the heading 5.1.2. To date, no study has carried out scanning mutagenesis of every single residue across the whole length of D4PLY.

The library was screened for the lytic activity in WT PLY background and for the agglutination activity in the $\Delta 6$ PLY background. The lytic activities of the mutants were compared with the studies done previously.

The screening activity for the aggregation negative mutants done in the $\Delta 6$ PLY background on a haemagglutination plate revealed an interesting mutant that was not causing the aggregation of the red blood cells. This mutant was purified and was tagged with eGFP and then again checked on a haemagglutination plate with different known protein concentrations. The aggregation/binding activity with the red blood cells was further confirmed by fluorescence microscopy and fluorescence activated cell sorting (FACS) experiments.

The cholesterol binding region (TL) in full length toxin was mutated to glycines and was tagged with eGFP and an eGFP tagged triple mutant PLY (363,459,460 LTL > AGG) was also constructed.

This residue L363A was also checked in the other cholesterol dependent cytolysins (CDCs) by doing the protein aligning using the CLC Genomics software.

5.2 Library from DNA 2.0

As mentioned earlier, the addition of 5 amino acid transposon based random mutations did not reveal the site/s responsible for agglutination of red blood cells in $\Delta 6$ PLY background. Therefore, subtle mutations by substituting one single amino acid were made across the whole domain 4 of the toxin. The library was again made both in WT PLY and $\Delta 6$ PLY background.

In this library every single amino acid residue in D4 of the toxin was replaced by alanine and where alanines were present, they were changed to glycines. D4 of toxin extends from residues 360 to 471. All these individual residues (360-471) were replaced by alanines or glycines and then each individual mutant was either re-ligated in $\Delta 6$ or WT PLY background.

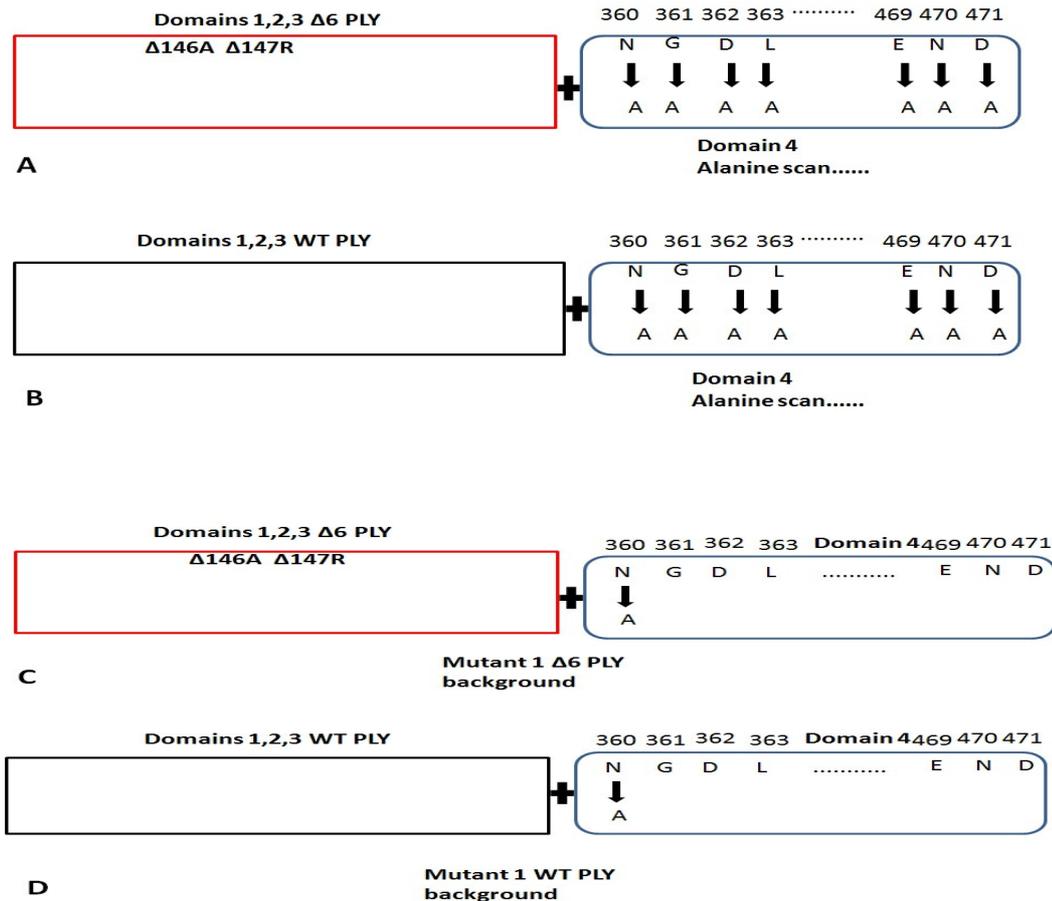


Figure 5-1 Diagrammatic representation of DNA 2.0 library

A&B) Mutants generated in both $\Delta 6$ PLY and WT PLY background. **C&D)** Mutant 1 of the library shown in both WT and $\Delta 6$ PLY background.

In total 112 mutants were generated in WT PLY background and the same number of mutants were generated in $\Delta 6$ PLY background. WT PLY mutants were checked for the lytic activity and the $\Delta 6$ PLY were checked for the aggregation/agglutination activity since the background lysis were removed from them. Mutant 1 of the library both WT and $\Delta 6$ PLY represents the first amino acid residue of D4, asparagine which in the library is replaced by alanine,

mutant 2 of the library represents 2 amino acid residue of D4, glycine replaced by alanine until the last amino acid residue of D4 which is aspartic acid, replaced by alanine.

The mutants were made in pJexpress 401 vector and a His tag attached to the N-terminal of every mutant. The results of experiments described in previous chapters show that His tag has no effect on the residual binding/aggregation of the red blood cells. The promoter attached to the vector is T5 which is IPTG inducible and can work in any *E.coli* host strain. All mutants received were stabbed in a 96 well plates with cultures grown in *E.coli* strain (DH10 β) with 15% glycerol.

5.3 Screening of mutants for haemolytic activity

All WT PLY mutants of library were screened for the haemolytic activity. The assay was set up according to the protocol as stated in materials and methods section 2.9.3.

Due to time constraints 112 WT PLY mutants were not purified instead the lytic activities of the individual mutants were screened by using the cell lysate (standardised *E.coli* cell lysate with recombinant PLY). The concentration of PLY in all the cell lysates of the library was assumed to be equal as all the experiments were carried using same technique. If time permitted, concentration of recombinant proteins in cell lysate and hence specific lytic activity in mg/ml could be measured by setting up an ELISA and/or doing a western blot. However, the lytic activity (HU/ml) calculated in this study from the cell lysates were comparable with the results of the studies done previously

with purified recombinant mutant proteins. Cell lysate of WT PLY from library was used as a positive control and PBS only was used as a negative control.

Unlike the purified proteins where specific haemolytic activity was calculated and expressed as percentage lysis, the lytic activity of the individual mutant was measured using the end point and every mutant of the library was compared to the end point from the lysate of the WT PLY.

The detail of the every single mutant in the library and their haemolytic activities are given in the table below. There are five alanine residues at positions (337, 370, 396, 406 and 432) in domain 4 of the toxin so all of them were replaced by glycines.

Table 5-1 Library of mutants from DNA 2.0

PLY Residue Number	Haemolytic Activity HU/ml	Mutant Name	PLY Residue Change	PLY Residue Number	Haemolytic Activity HU/ml	Mutant Name	PLY Residue Change	PLY Residue Number	Haemolytic Activity HU/ml	Mutant Name	PLY Residue Change	PLY Residue Number	Haemolytic Activity HU/ml
360	102400	29	G>A	388	51200	57	G>A	416	51200	85	D>A	444	25600
361	102400	30	K>A	389	102400	58	N>A	417	51200	86	L>A	445	12800
362	51200	31	E>A	390	25600	59	V>A	418	51200	87	P>A	446	51200
363	12800	32	V>A	391	51200	60	R>A	419	51200	88	L>A	447	51200
364	102400	33	L>A	392	51200	61	N>A	420	102400	89	V>A	448	51200
365	12800	34	T>A	393	51200	62	L>A	421	51200	90	R>A	449	25600
366	102400	35	P>A	394	102400	63	S>A	422	102400	91	K>A	450	12800
367	6400	36	K>A	395	51200	64	V>A	423	102400	92	R>A	451	6400
368	51200	37	A>G	396	102400	65	K>A	424	51200	93	T>A	452	25600
369	51200	38	W>A	397	51200	66	I>A	425	102400	94	I>A	453	3200
370	102400	39	D>A	398	51200	67	R>A	426	51200	95	S>A	454	51200
371	3200	40	R>A	399	102400	68	E>A	427	12800	96	I>A	455	25600
372	25600	41	N>A	400	102400	69	C>A	428	51200	97	W>A	456	12800
373	51200	42	G>A	401	102400	70	T>A	429	3200	98	G>A	457	12800
374	25600	43	Q>A	402	102400	71	G>A	430	12800	99	T>A	458	12800
375	25600	44	D>A	403	102400	72	L>A	431	3200	100	T>A	459	6400
376	51200	45	L>A	404	102400	73	A>G	432	12800	101	L>A	460	1600
377	51200	46	T>A	405	3200	74	W>A	433	800	102	Y>A	461	12800
378	51200	47	A>G	406	25600	75	E>A	434	12800	103	P>A	462	25600
379	25600	48	H>A	407	6400	76	W>A	435	800	104	Q>A	463	51200
380	51200	49	F>A	408	51200	77	W>A	436	800	105	V>A	464	51200
381	25600	50	T>A	409	102400	78	R>A	437	12800	106	E>A	465	51200
382	102400	51	T>A	410	102400	79	T>A	438	51200	107	D>A	466	51200
383	51200	52	S>A	411	102400	80	V>A	439	12800	108	K>A	467	51200
384	51200	53	I>A	412	51200	81	Y>A	440	3200	109	V>A	468	51200
385	102400	54	P>A	413	102400	82	E>A	441	25600	110	E>A	469	51200
386	102400	55	L>A	414	51200	83	K>A	442	12800	111	N>A	470	51200
387	102400	56	K>A	415	51200	84	T>A	443	12800	112	D>A	471	12800

5.3.1 Haemolytic assays titres measured as end points

The end point is measured in Haemolytic units (HU) per ml (HU/ml) of the sample. The reciprocal of the dilution at which end point is reached is calculated as e.g. the first well has 10 μ l of the sample and 90 μ l of PBS which is a 10 fold dilution, from this well 50 μ l is taken and added in the subsequent well and 50 μ l DTT is added to the first well resulting in a 20 fold dilution.

50 μ l of red blood cells was again added and finally 50 μ l of PBS was added before centrifuging the plate which makes the final dilution in the first well to 80 fold.

This dilution is only in a total volume of 200 μ l and since the dilution is to be calculated in HU/ml, so we multiplied it by 5 to make the dilution per ml, which is 1/400 for the first well. This means well 2 represents 1/800 dilution, well 3 will be 1/1600 dilution and well 4 will be 1/3200 dilution and so on. Therefore if the end point was well 4 the haemolytic activity would be 3200 HU per ml of the sample as shown in the table below.

Table 5-2 Haemolytic titre end points (HU/ml) of wells

Well No	Titre (HU/ml)	Well No	Titre (HU/ml)
1	400	7	25600
2	800	8	51200
3	1600	9	102400
4	3200	10	204800
5	6400	11	409600
6	12800	12	819200

The end point calculated for WT PLY lysate was well no 9 and therefore the lytic activity was calculated to be 102400 (HU/ml) (Figure 1). All mutants in the

library are compared with this value. The haemolytic assay plates for screening the undecapeptide region of WT PLY library are shown below.

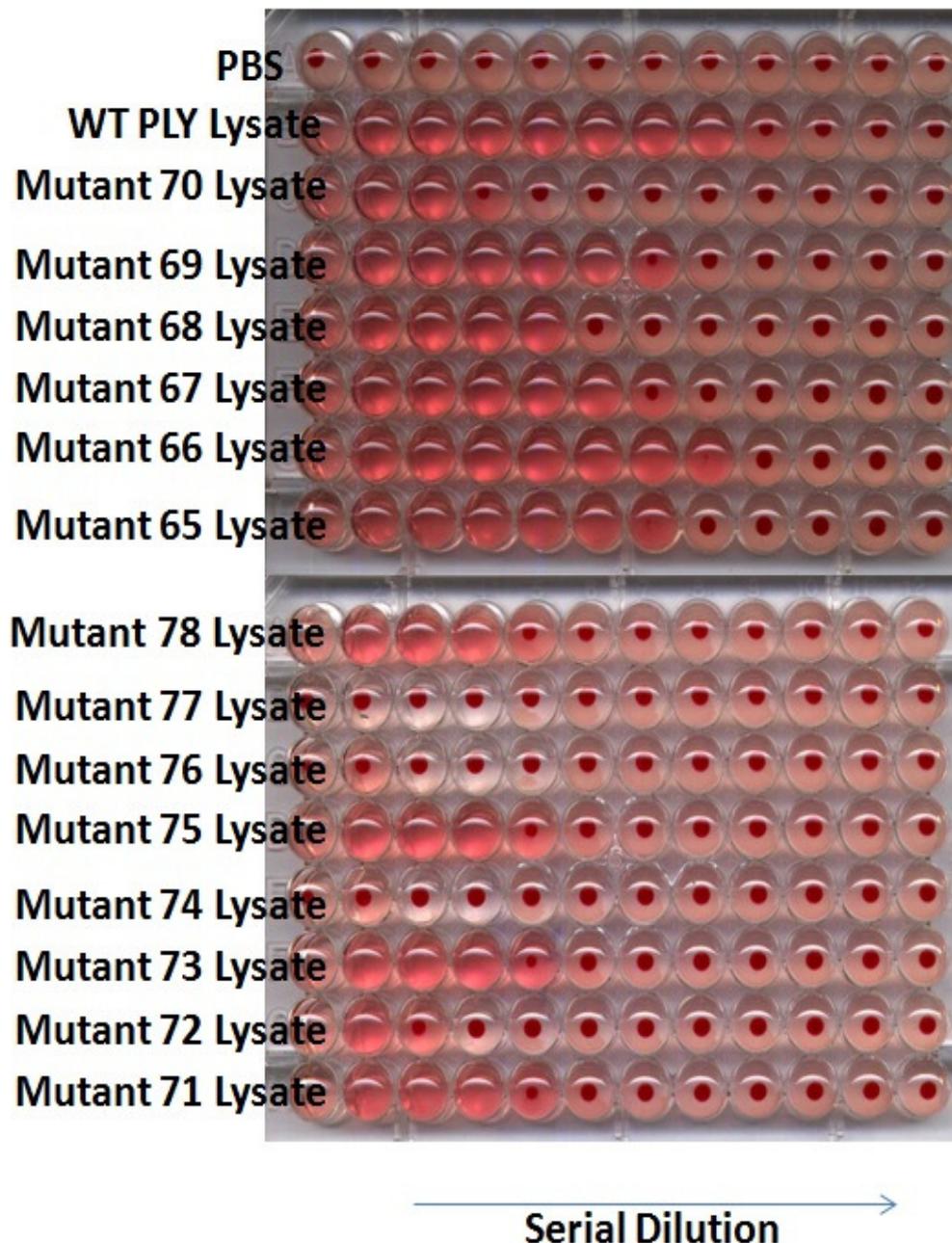


Figure 5-2 Haemolytic assay of Cell lysate from DNA 2.0 library

10 μ l of cell lysate of mutant proteins was added in the first well the plate and serial dilutions were made (1:2) across the 96 well plate. The plates were observed for the lysis of the red blood cells. 10 μ l cell lysate of WT PLY and 50 μ l PBS were used as positive and negative controls. WT PLY showing lysis of red blood cells and PBS showing no lysis.

Figure 5.2 result showing end point (well 9) of WT PLY lysate in the haemolytic assay plate and hence the lytic activity calculated as 102400 HU/ml. In other 112

mutants of the library the lytic activity was either at end point 9 or less and none of the mutants had 0% lytic activity. All residues of the undecapeptide regions are represented by mutant numbers (68-78) of the library. Only three mutants in the library were found to have end points at well 2 on the haemolytic plate and these were the three tryptophan residues 433, 435 and 436 (Mutant 74, 76 and 77 of DNA 2.0 library) of the undecapeptide region (Figure 5.2). None of cell lysates of the other mutants in the library had lytic activity equal to less than these mutants. Mutant 74, 76 and 77 are the three tryptophan residues in the undecapeptide region and have been known to reduce the lytic activity of the PLY in studies done previously (Korchev *et al.*, 1998).

All residues in the undecapeptide region are less lytic as compared to WT PLY lysate. The undecapeptide region in PLY starts from residue 427 and end at residue 437. The mutant 68 in the library represents the first residue Glutamine of undecapeptide region and mutant 78 of the library represents last residue Arginine of the undecapeptide region.

The lytic activity measured as end points for all the mutants in the library are collectively shown in the figure 5.3 below. The % lytic activity of each individual mutant compared to the WT PLY of the library is shown in the figure 5.4.

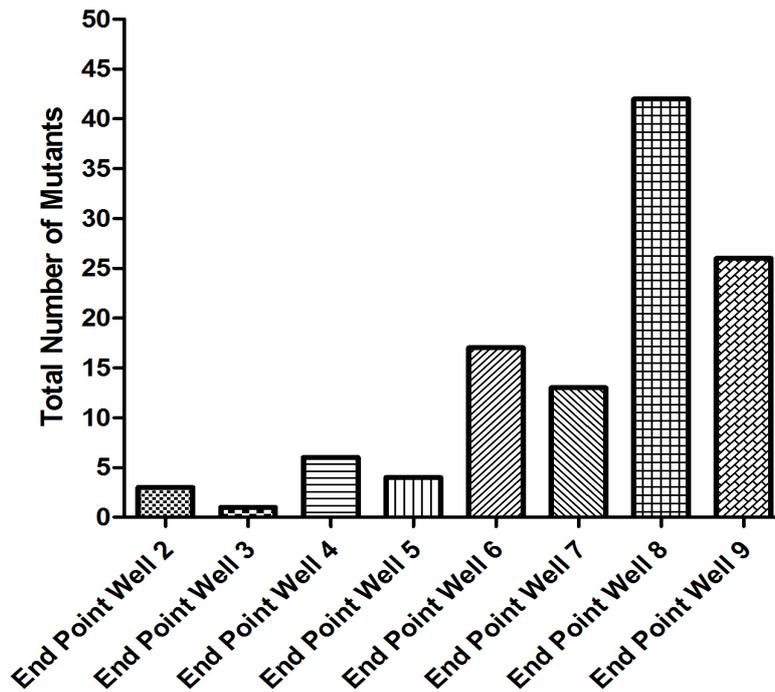


Figure 5-3 The graph showing screening results of cell lysate from DNA 2.0 mutant Library. The X axis on the graph showing the end points wells of the Haemolytic assay plate and Y axis showing the number of mutants for each end point.

The screening results show that the end point was well 8 for 42 mutants (37.5%) out of 112 where as 26 mutants (23.2%) have same end point (Well No 9) as WT PLY. This means that more than 60% of the mutants in D4PLY have lytic activity similar to or 50% in comparison to WT PLY when single alanines substitutions are made. The total number of mutants having end points at well 5 or below was 14 and represents less than 12.5% of the total number of mutants in the library. The total number of mutants having end point well 6 and 7 were 30 and represents 26.6% of the total mutants.

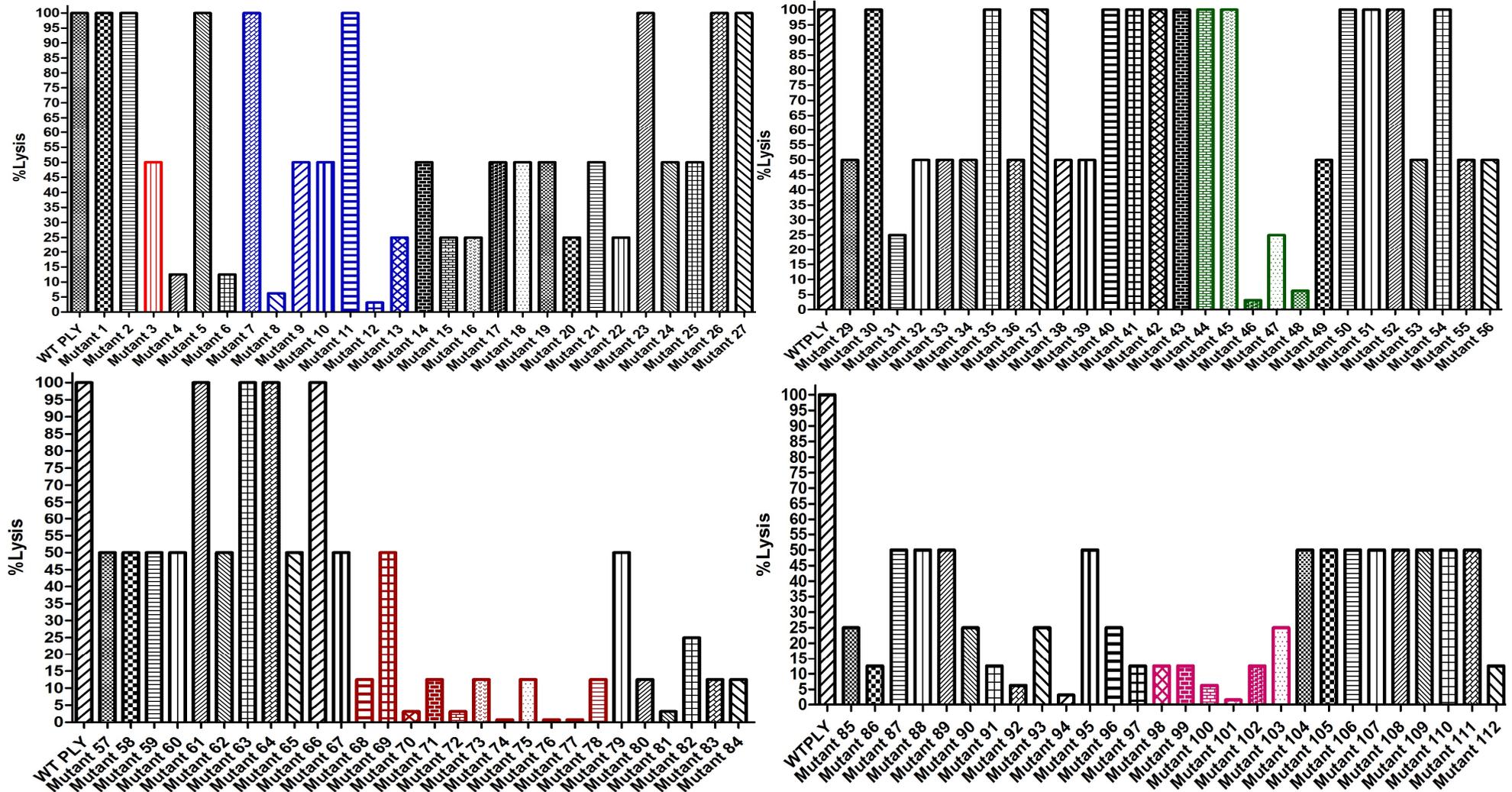


Figure 5-4 Lytic activity of WT PLY mutants form DNA 2.0 Library.X-axis on all the graphs showing mutant's name and Y axis is% lytic activity compared to the WT PLY. All important regions are highlighted. Loop1, 2, 3 and undecapeptide region are highlighted as pink, blue, green and brown respectively. Residue 363 is highlighted as red.

As expected figure 5.4 shows that all well studied important regions of the toxin have a significant drop of % lytic activity as compared to the WT PLY. These important regions are highlighted with different colours. The individual lytic activities of the highlighted important regions (Undecapeptide region and three hydrophobic loops L1, L2 and L3) of the toxin when replaced by alanines are represented in the figure 5.5 below.

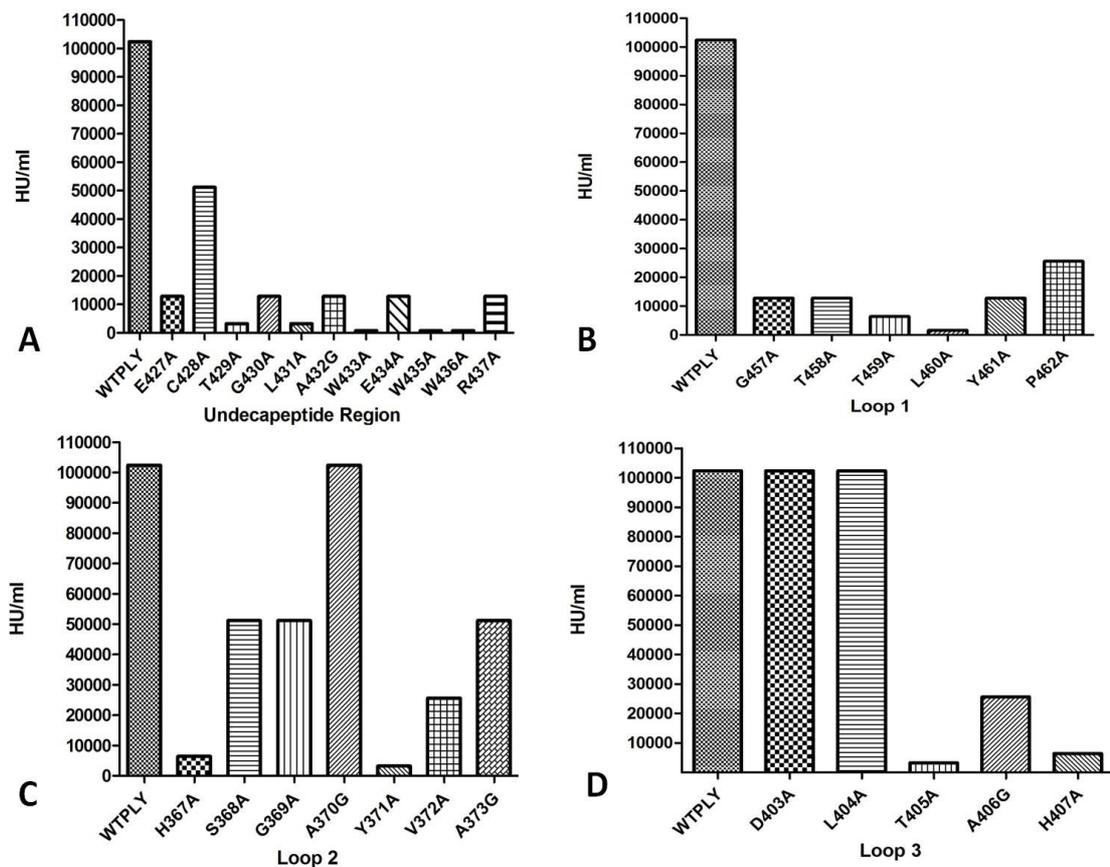


Figure 5-5 Haemolytic activity comparisons of mutant proteins with single alanine substitutions with WT PLY

The X-axis showing the amino acid residues and Y axis represents the lytic activity in haemolytic unit per mg (HU/ml). A) The lytic activity of cell lysate of undecapeptide region (427-437) with WT PLY lysate. B) Comparison of lytic activity of cell lysate of loop 1, C) Loop 2 and D) Loop 3 of D4PLY with WT PLY lysate.

Figure 5.5 shows that the two amino acid residues (TL) or Cholesterol binding motif identified by Farrand *et al.*, 2009 in loop 1, responsible for initial binding with the membrane cholesterol mentioned were represented by Mutant 100 and

101 of the DNA 2.0 library. The end point calculated was well No 5 and 3 and the haemolytic activity in (HU/ml) for these mutants were 6400 and 1600 respectively. This activity is less than 7% and 2% when compared with WT PLY lysate. The lytic activity of cell lysate of the cholesterol binding motif 459(T>A) and 460(L>A) was more than the tryptophan residues of the undecapeptide.

A study conducted has shown that substituting L460D removes toxin's cytolytic activity whereas above results shows partial lytic activity when substituted with alanine (Hu *et al.*, 2013). Farrand *et al.*, 2009 substituted both these residues with glycines and found the lytic activity <0.02% compared to WT PLY. In this study every single amino acid in D4 of the toxin was substituted with alanines and lytic activity compared with WT PLY lysate. A study done recently has shown that PLY^(L460E) and PLY^(L460G) have different effects on oligomerisation activity of the toxin where the former renders toxin unable to oligomerise and latter has no effect. However, both the mutant versions have decreased lytic activity as compared to the WT PLY (PLY^(L460E) is non-lytic even at 480nM where as PLY^(L460G) is lytic at this concentration and non-lytic at concentrations below 8nM) (Taylor *et al.*, 2013). The above results suggests that substituting different amino acid at same position gives different lytic and oligomerisation activity which might explain that difference in lytic activity in our results compared to previous studies (Farrand *et al.*, 2009, Hu *et al.*, 2013). It will be interesting to make double substitutions with alanine and/or any other amino acids in both the cholesterol recognition motifs and measuring the lytic activity of the toxin.

The hydrophobic loop1 consists of residues GTTLYP from position 457-462 in PLY and in the library represented by mutants (98-103). All the residues in loop 1 have shown a reduction in lytic activity up to 12.5 apart from proline where the

reduction is 25%. This is in line with a study done previously which showed proline (462) substitution with serine resulted in 27% (Owen *et al.*, 1994).

Loop 2 consists of residues HSGAYVA from position (367-373) and represented by mutants (8-14) of the library. These results show a huge reduction in lytic activity in residues 367 and 371 (histidine and tyrosine). In a previous study carried by Berry *et al.*, 1995 showed that histidine when substituted with arginine resulted in a 0.02% reduction in lytic activity and in this study when substituted with alanine resulted in 6.25% reduction in lytic activity (Berry *et al.*, 1995). The lytic assay of residue PLY^(A370G) in loop 2 showed no reduction in lytic activity as compared to WT PLY which is in line with a study done recently and showing recombinant PLY^(A370G) has same lytic activity as compared to WT PLY (Taylor *et al.*, 2013).

Loop 3 consists of DLTAH at positions (403-407) and represented (Mutant 44-48) of DNA 2.0 library. These results show a decrease in lytic activity in the last three residues with the highest reduction observed in threonine at position 405.

The lytic results in the undecapeptide region show that apart from cysteine residue at 428, all other mutants showed a decrease in lytic activity up to 85% or more when compared with the WT PLY lysate. The highest lytic activity observed was for cysteine which was 51200HU/ml and lowest was for all tryptophan residues of the undecapeptide at 800HU/ml.

The results of the previous experiments also suggest that cysteine has no involvement in binding or insertion of the toxin into the membrane and when it

was substituted with alanine retained its haemolytic activity (Saunders *et al.*, 1989).

The above results also show that some of the residues in loop 2 and 3 (370, 403 and 404) when substituted to alanines seems to have no effect on the lytic activity of the toxin. The cell lysate of these residues have same lytic activity as the WT PLY lysate. None of the mutants with substitution in the undecapeptide or loop 1 have lytic activity equal to WT PLY.

5.4 Screening of library for hemagglutination negative mutants

The 112 mutants in $\Delta 6$ PLY background were screened for hemagglutination negative mutants. The 112 mutants were screened on a 96 well round bottom plate as described in the materials and methods section 2.9.3 and were observed for hemagglutination activity.

Due to time constraints it was not possible to purify the 112 mutants of the library so the cell lysates (standardised *E. coli* cell lysate with recombinant PLY) were checked for hemagglutination activity. Purified $\Delta 6$ PLY and $\Delta 6$ PLY lysate was used as positive control whereas PBS only was used as negative controls. The results of hemagglutination assays were noted as positive or negative and were not quantitatively measured since the aim of these experiments were to identify agglutination negative mutants.

50 μ l of the cell lysate of the mutants was used to measure agglutination activity in the plate. A typical hemagglutination assay plate is shown below in figure 5.6.

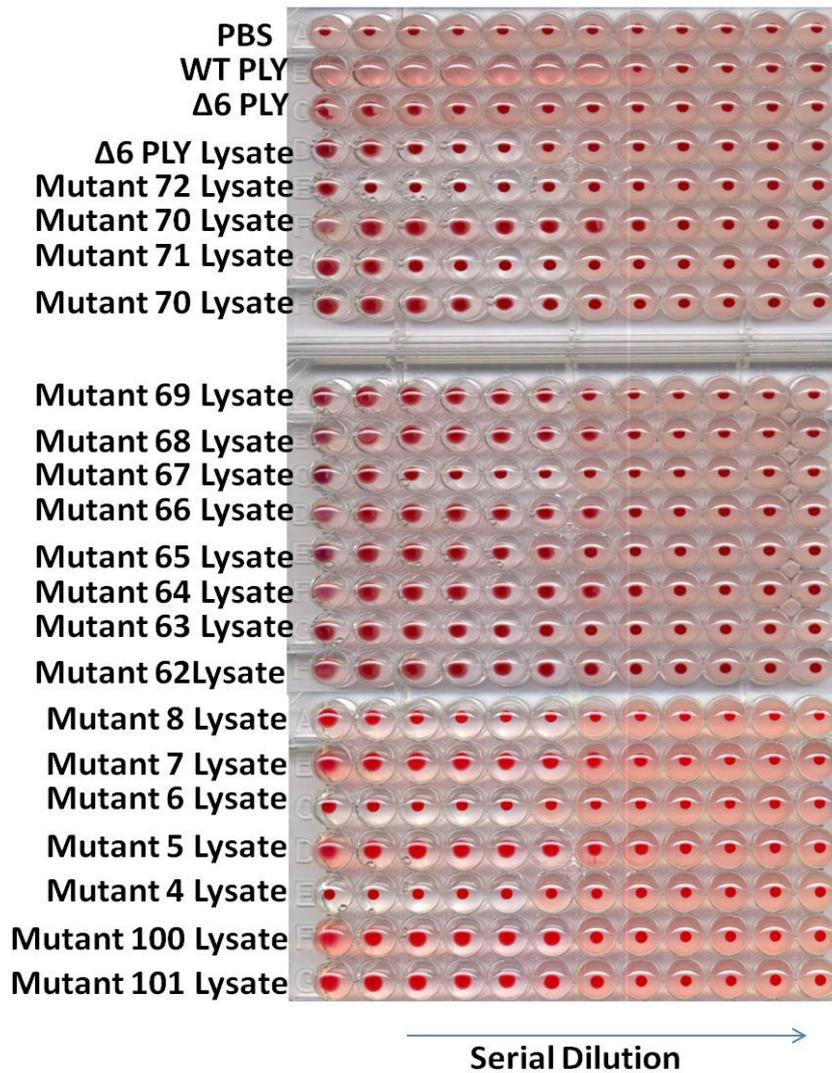


Figure 5-6 Screening of DNA 2.0 library lysates ($\Delta 6$ PLY background) for hemagglutination activity

50 μ l of cell lysate of mutant proteins from DNA 2.0 library was added in the first well of the 96 well round bottom plate and serial dilutions were made (1:2) across the 96 well plate. The plates were observed for the haemagglutination of the red blood cells. 1 μ g $\Delta 6$ PLY, 50 μ l cell lysate of $\Delta 6$ PLY was used as positive control and 50 μ l PBS was used as negative controls. All different phenotypes are explained in figure 2.5

All mutants in the library apart from Mutant 4 caused the agglutination of the red blood cells. The same mutant L363A (Mutant 4) has a reduced lytic activity (12.5%) in WT PLY background (Figure 5.4).

The cholesterol binding motif when replaced individually with alanines (T459A and L460A) was agglutination positive suggesting that these residues do not account for the agglutination property of the toxin but substituting both residues

with glycines do not result in appreciable agglutination on 96 well plate. However, some aggregation of red cells was seen with the fluorescence microscopy (Figure 3.11). It will be interesting to compare the agglutination behaviour on a hemagglutination plate by substituting both these TL residues with alanines.

The only agglutination negative mutant, Lysine at position 363 when changed to alanine PLY (L363A) did not cause red blood cells to aggregate. This mutant was further purified and the purified protein was again checked for agglutination activity on a 96 well round bottom plate. This mutant (L363A) was giving the same clearing effect as observed with cholesterol binding motif in figure 3.8.

In WT PLY background, the same amino acid substitution (L363A) resulted in 87.5% reduction in lytic activity compared to WTPLY lysate (Figure 5.4 highlighted in red) which may suggest its role in binding to red blood cells.

5.5 Characterisation and purification of the agglutination negative mutant $\Delta 6\text{PLY}^{(\text{L363A})}$

5.5.1 Purification of mutant by NAC and AEC

The agglutination negative mutant $\text{PLY}^{(\text{L363A})}$ was subjected to a two step purification process, first by immobilized affinity chromatography and then further purified by anion exchange chromatography (AEC). The collected fractions after AEC were analysed on an SDS-PAGE with a known protein molecular weight marker (Figure 5.7).

The purified proteins concentration was measured as described in materials and methods section 2.8.10. The concentration of the purified protein was measured as explained in section 2.8.10 and is found to be 0.375mg. The purified protein after AEC was dialysed at 4°C with 2 litres pre-chilled PBS buffer and saved at -80°C.

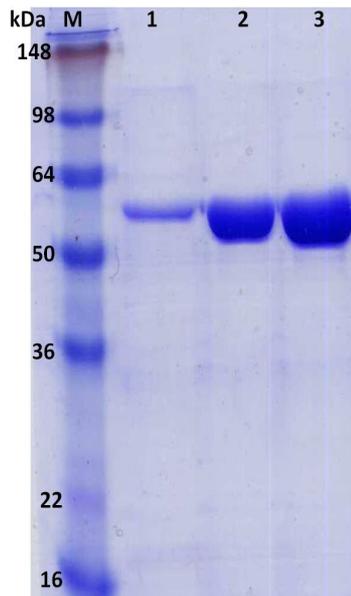


Figure 5-7 SDS-PAGE of $\Delta 6$ PLY(L363A) after AEC Purification

Lane M contains 5 μ l of the mol. wt .marker SeeBlue® Plus2, followed by 10 μ l recombinant $\Delta 6$ PLY (L363A) after AEC purification in lanes 1-3.

The purified protein was further checked for its agglutination ability on a 96 well round bottom plate.

5.5.2 Hemagglutination assay of the purified protein

The purified protein mixed with red blood cells at concentration up to 5 μ g was analysed on a 96 well plate to observe whether it still agglutinates the red blood cells.

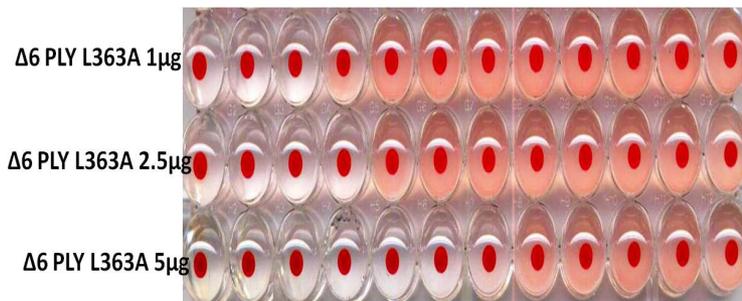


Figure 5-8 Hemagglutination assay of the purified mutant protein $\Delta 6\text{PLY}^{\text{(L363A)}}$

1, 2.5 and 5 μg of the mutant protein was added into the first well of rows and serial dilutions were made (1:2) across the 96 well plate. The plates were observed for the haemagglutination of the red blood cells.

Figure 5.8 showing hemagglutination was not observed at adding 1, 2 and 5 μg of the purified protein directly into the first well of the corresponding rows. This result confirmed our previous results done with the cell lysate of the mutant which was agglutination negative. This suggests that this residue might be responsible for causing the agglutination when the lytic activity was toxin is abolished by deleting the residues at position 146 and 147 (alanine and arginine).

The results with the purified protein suggests that cell lysate and purified protein have similar agglutination effects when run on a 96 well round bottom plate. The clearing effect of wells was also observed in the assay and increased with the increase in the concentration of the toxin.

To further confirm the results by fluorescence microscopy and fluorescence activated cell sorting, eGFP version of the mutant protein was required so that the binding and the agglutination can be observed.

The pET33beGFP $\Delta 6\text{PLY}$ construct was used as template to make mutant versions by site directed mutagenesis using the SDM kit following the protocol mentioned

in section 2.5.3. For negative control eGFP only was used while purified eGFP $\Delta 6$ PLY was used as positive control.

5.6 Purification and characterisation of eGFP tagged mutants in pET33bvector

The site directed mutagenesis was done using the SDM kit and the sequencing results of all the mutants were checked before expression and purification. All recombinant proteins have a six histidine tag attached to it and were subjected to a two step purification process, first by immobilized metal affinity chromatography then by anion exchange chromatography.

5.6.1 Purification of eGFP-linked PLY mutants

The fractions of the purified proteins from AKTA PrimeTM were run on an SDS-PAGE gel (Figure 5.9) and their predicted molecular weights were confirmed with the known molecular weight protein marker on the gels. The predicted molecular weight of all the proteins is 83kDa.

5.6.2 SDS-PAGE and western blot of the purified proteins

The purified recombinant proteins were further analysed by Western Blotting. 1 μ g of the purified protein was loaded on the gel against the known molecular weight protein marker SeeBlue[®] Plus2.

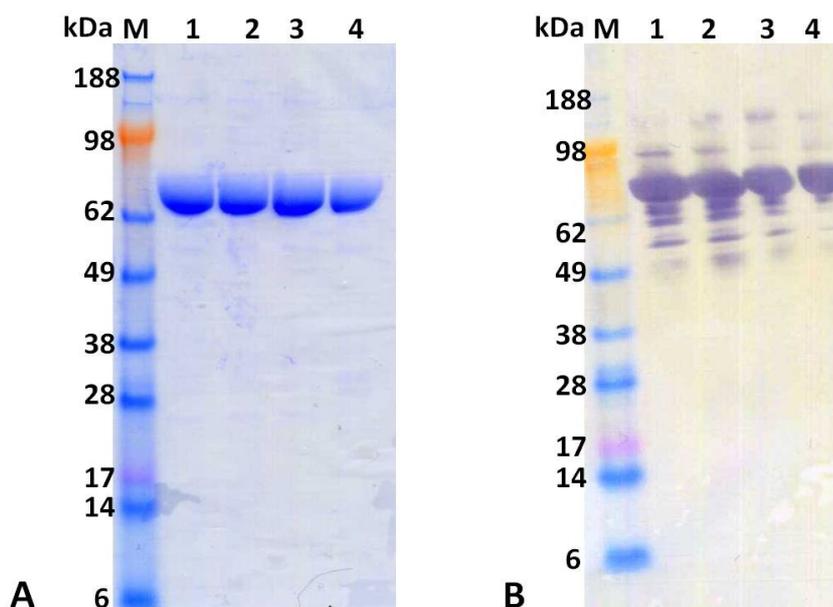


Figure 5-9 SDS PAGE and western blot of eGFP-PLY linked proteins

A and B Lane M contains 5 μ l of the mol. wt .marker SeeBlue® Plus2, followed by 10 μ l recombinant proteins eGFP Δ 6PLY, eGFP Δ 6PLY^(L363A), eGFP Δ 6PLY^(T459G L460G) and eGFP Δ 6PLY^(L363A T459G L460G) in lanes 1-4. There were some bands seen on western blot (5.9B) showing some background and were not appreciable on the SDS-PAGE.

Figure 5.9 showing some background on the western blot which is not appreciated on the SDS-PAGE.

5.6.3 Concentration of the purified proteins

The concentrations of the purified proteins were measured and are stated in the table 5.3.

Table 5-3 Concentration of the purified protein

S.No	Name of the Mutants	mg
1	eGFP Δ 6PLY	5.72
2	eGFP Δ 6PLY L363A	1.005
3	eGFP Δ 6PLY T459G L460G	2.2
4	eGFP Δ 6PLY L363A T459G L460G	0.7

5.7 Cell binding of mutant protein by FACS

The cell binding of the mutant proteins with red blood cells were measured by using fluorescence activated cell sorter (FACS). The experiment was carried out following the same protocol as explained in the materials and methods section 2.9.5. The gates FL1+ and FL-ve were drawn based on the controls (eGFP with red blood cells and red blood cells in PBS) to minimise the back ground auto fluorescence.

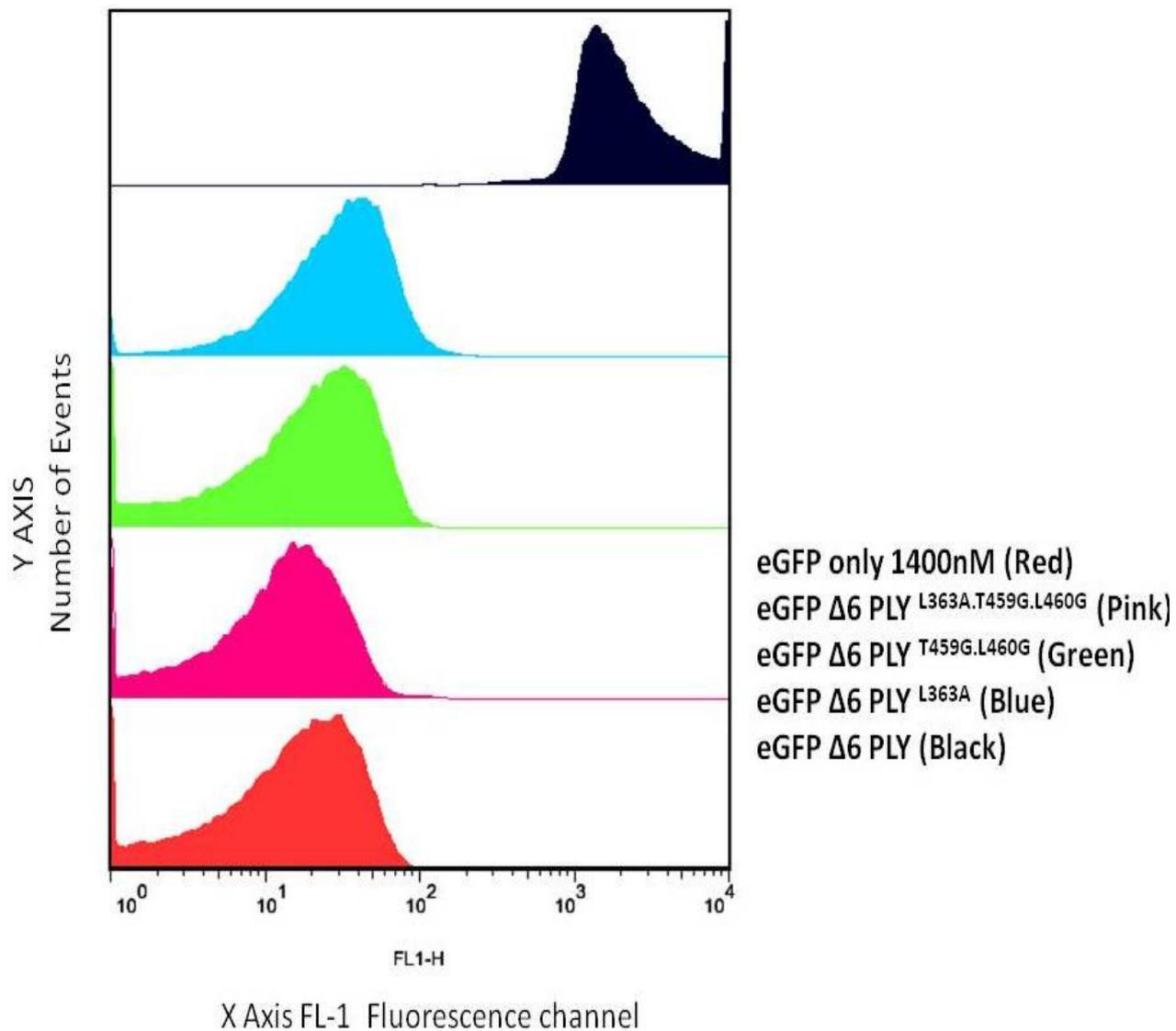


Figure 5-10 FACS showing binding of mutant proteins with horse red blood cells

X-axis is the fluorescent channel (FL1) set on log scale and Y axis is the number of events measured which in this case is 50,000. 1400nM of the eGFP only (red) eGFP $\Delta 6$ PLY^(L363A.T459G.L460) (pink), eGFP $\Delta 6$ PLY^(T459G.L460) (green), eGFP $\Delta 6$ PLY^(L363A) (blue) and eGFP $\Delta 6$ PLY (black) were analysed for binding with red cells based on shifting of the graph to the right side, in case of positive binding. The figure was drawn using the flow jo software.

As expected there is a clear shift in the graph on the right in case of eGFP $\Delta 6$ PLY showing maximal binding. A second peak can also be seen with eGFP $\Delta 6$ PLY at 10^4 on the X-axis similar to eGFPD4PLY (Figure 3.13 first results chapter) but in this case it is off scale.

The cell binding has reduced considerably in all the mutant proteins when compared to eGFP $\Delta 6$ PLY. However, protein binding to red blood cells triple mutant eGFP $\Delta 6$ PLY^(L363A.T459G.L460) is similar to the control eGFP only 1400nM whereas there is slight shift on the right side for both the mutant proteins eGFP $\Delta 6$ PLY^(L363A) and eGFP $\Delta 6$ PLY^(T459G.L460) suggesting some residual binding of cells with the mutant proteins. These results also suggest that residue 363, leucine when replaced with alanine can reduce the binding of PLY to red blood cells comparable to the TL mutant. This is evident from the results where the shifting of peak to the right side on the X-axis in case of L363A is similar to TL mutant.

On a haemolytic assay plate the same mutant (L363A) resulted in a lytic activity reduction of more than 85% compared to WT PLY suggesting its role in binding along with the cholesterol binding mutant.

The results were further confirmed by quantitatively measuring the binding of proteins with the red blood cells using the fluorescent Index (FI). The FI was measured as explained in the first results chapter under section 3.7. The FI of all the mutant proteins concentrations were compared with corresponding controls.

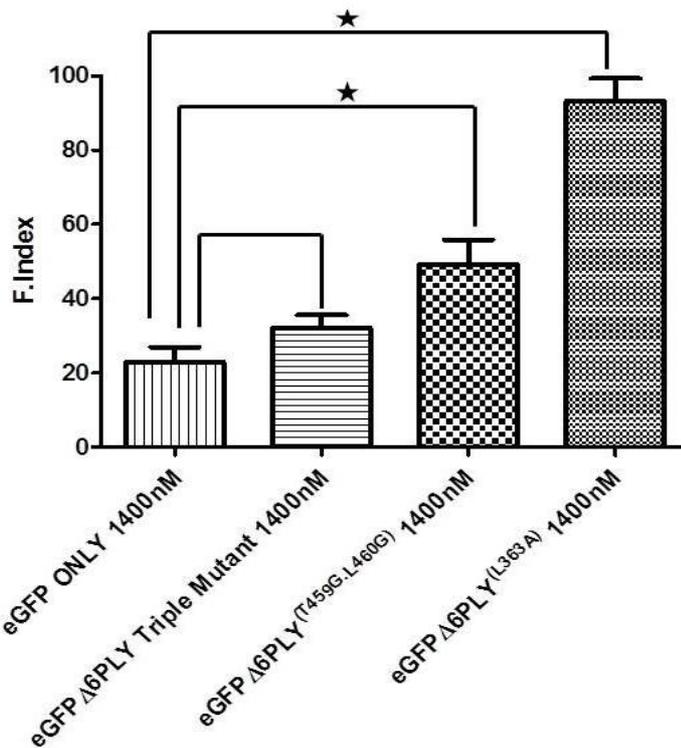


Figure 5-11 Fluorescent index (FI) of the mutant proteins

The graph showing the comparison of 1400nM of the three mutant proteins compared with control. Statistical significance was determined by using One way anova using Dunnet's post test to determine the significance between different groups compared with the control. The P value <0.05 was considered to be significant. The * symbol shows that P value is significant (* = $p < 0.05$). FI is calculated multiplying the % of fluorescent cells with the mean geometrical fluorescence.

Figure 5.11 illustrates that there is no significant difference between the eGFP only and eGFP Δ 6 PLY^(L363A.T459G.L460) at 1400nM of the protein concentration. The other two mutant Δ 6 PLY^(T459G.L460) and Δ 6 PLY^(L363A) differ significantly when compared with the controls. This suggests that the residue 363 was responsible for this residual binding seen with the TL mutant and mutating this residue resulted in the complete loss of binding of protein with the red blood cells.

L363A mutation causes a significant decrease in the binding of protein with the red blood cells compared to eGFP Δ 6 PLY (not shown in figure 5.11) which might explain the reduced lytic activity of the mutant L363A (12.5%) in the WT PLY background compared to the WT PLY.

The t-test was used to compare the other two concentrations (14 and 140nM) of the mutant proteins with the corresponding controls. There was no significant difference at 14nM of all mutant proteins when compared with 14nM of eGFP only whereas significant differences were observed at 140nM of the protein concentration except the triple mutant eGFP $\Delta 6$ PLY^(L363A.T459G.L460) where no significant difference was observed when compared with the controls (data not shown). These results are in line with the previous results where no significant difference was observed between TL mutant at 14nM where as at 140 and 1400nM the binding differ significantly when compared with the controls at the same concentration.

The mutants were further analysed for their agglutination activity on forward scatter (FS Linear) and forward scatter area (FS Area). Unlike the previous results chapter where the cell population was identified on the peaks this time a more quantitative approach was taken by drawing a gate (black line) around the control eGFP only 1400nM. The same gate was applied on all the observations and cell population within and outside the gates recorded. The cell population lying within the gate was marked as single cell and in case of negative control was 98.3%.

As already shown in figure 5.10 that the increase in binding resulted in the shifting of the graph towards the right and two peaks indicating more than single cells where as in case of forward channel (linear vs. Area), the cells well spread out on Y axis (FS-area) were labelled as more than single cells. The single cells were present in drawn polygonal gate or area surrounding it.

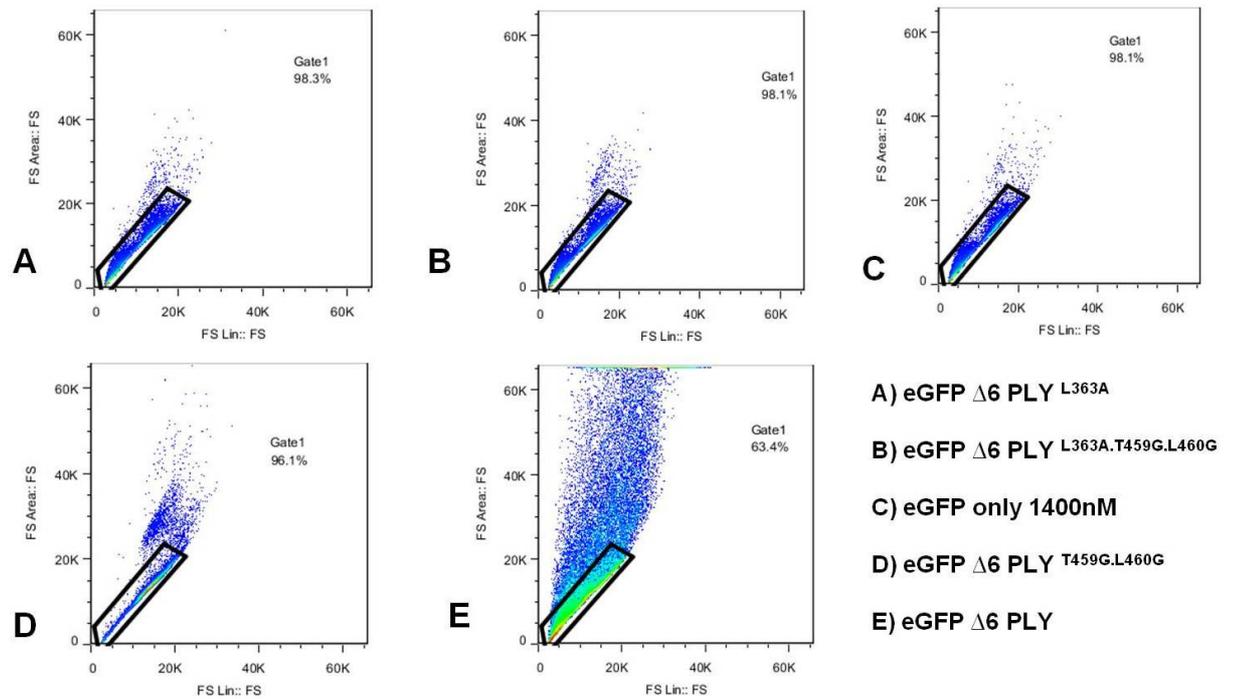


Figure 5-12 FACS results showing red blood cell population (single/multiples) on forward scatter channel

X axis the forward scatter channel (Linear) set on the linear scale and Y axis is the Forward scatter channel (area) also set on linear scale. 1400nM of the protein concentration was used. eGFP only and eGFP Δ6 PLY were used as negative and positive controls. Black line represents polygonal gate drawn around the single cells. The percentage of single cells are given for each individual proteins.

Figure 5.12 showing that in negative control (eGFP only) more than 98% of the cell population are in the gate (single cells) where as in the positive control eGFP Δ6 PLY than 36.6% of the population are more than single cells. The cells have well spread around the Y axis (FS area) in case of positive control. The results of eGFP Δ6 PLY^(L363A) and eGFP Δ6 PLY^(L363A.T459G.L460) (A&B) were similar to the control eGFP 1400nM (C) and the same cell population 98% was seen inside the drawn gate. In case of eGFP Δ6 PLY^(T459G.L460), there was more population of cells (blue dots) outside the drawn gate compared to the A & B. The single cell population inside the gate was 96% which is 2% less as compared to the controls and the other two mutants.

5.8 Fluorescence microscopy

The fluorescence microscopy was done to assess the binding of proteins with the red blood cells.

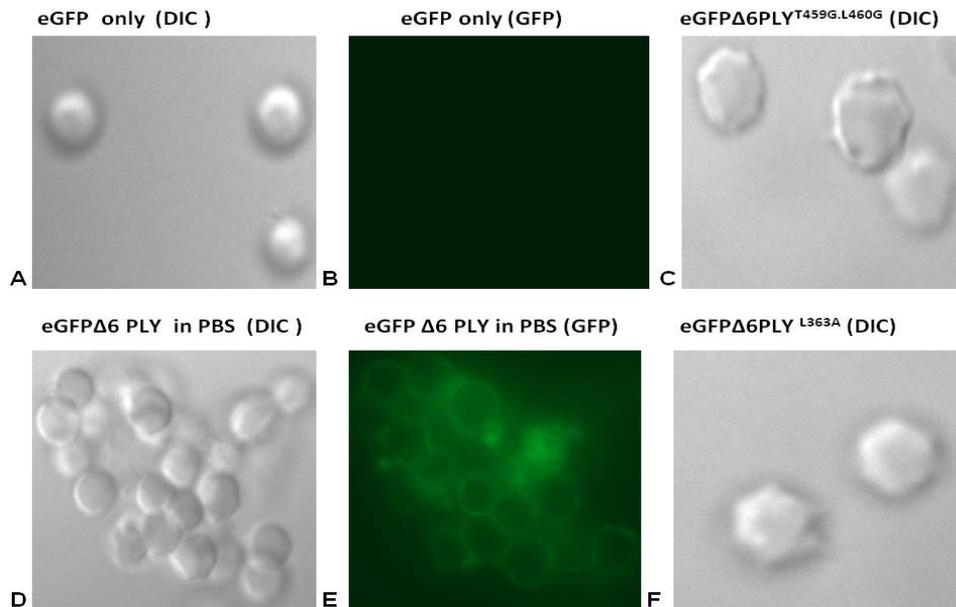


Figure 5-13 Fluorescence microscopy images showing eGFP tagged recombinant proteins binding

The binding of different proteins versions with red blood cells was analysed. (A,B) eGFP only with red blood cells in PBS in DIC and GFP. (C) eGFPΔ6PLY^(T459G.L460G) binding with red blood cells in DIC. (D&E) eGFPΔ6PLY binds and forming clumps with red blood cells as seen DIC and GFP. (F) eGFP Δ6 PLY^(L363A) binding in DIC. GFP mode of mutant proteins are not shown as the binding was not picked up by this channel. GFP only and eGFPΔ6PLY were used as negative and positive controls.

These results are in line with the previous findings with eGFP Δ6 PLY forming large clumps of red blood cells and aggregation pattern is similar to the pattern observed in purified eGFPD4PLY as seen in the chapter 3 (Figure 3.11).

From the above and previous results (Figure 3.11) it is observed that binding of mutant proteins with red blood cells is not readily picked up the green fluorescence (gfp) channel of the microscope. The result of mutant proteins under gfp channel of the microscope is similar to eGFP only. It might be that binding is significantly reduced and not picked up by the gfp channel. The FACS

analysis also predicts a small difference (2%) between eGFP and TL mutant as shown in figure 5.12.

5.9 Other Cholesterol dependent cytolysins (CDCs)

The above results show that residue 363 (leucine) in domain 4 is an important residue in PLY responsible for significant residual binding of the proteins with the red blood cells. The presence of this residue was checked by doing a protein alignment across other members of CDCs using CLC GenomicWorkbench3 software.

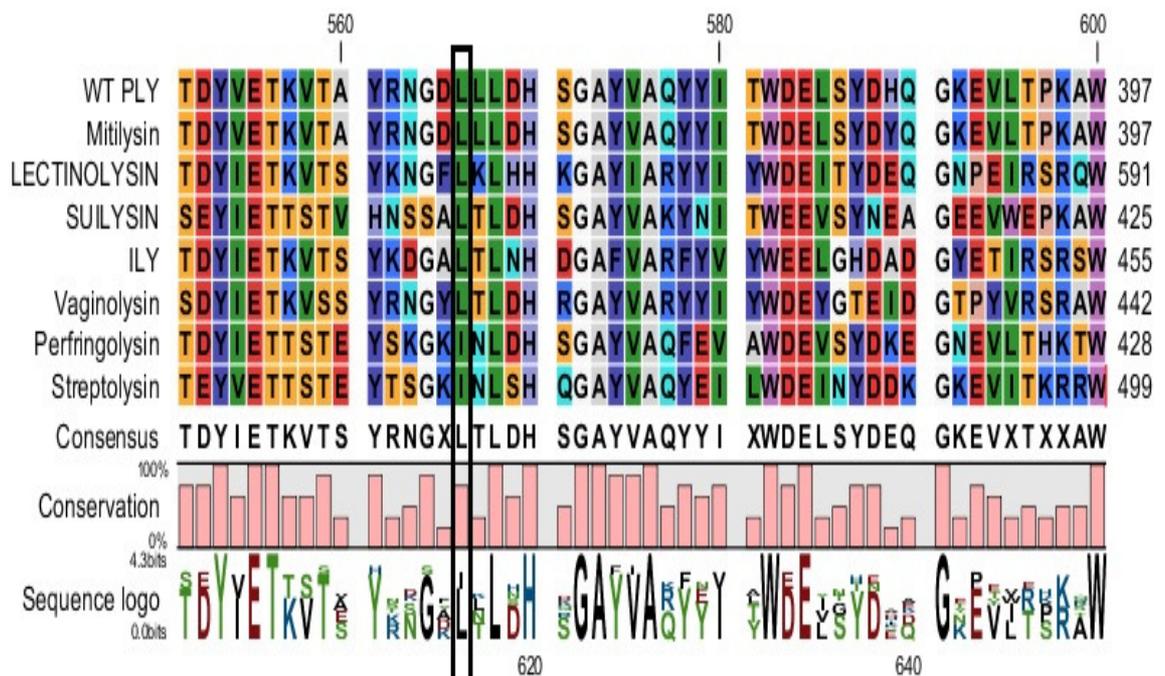


Figure 5-14 Sequence alignment of member of CDCs using CLC genomic software

The sequences were downloaded from the NCBI website were aligned by using the CLC genomics workbench 3.7.1

The above alignment shows the residue leucine highlighted with black line, responsible for residual binding and or/aggregation of red blood cells is present in almost all members of CDCs aligned, apart from Perfringolysin (PFO) and Streptolysin O (SLO) which have isoleucine at this same position.

$\Delta 6$ Sulysin (SLY) has leucine at the same position and gives the same aggregation phenotype as $\Delta 6$ PLY (Dr Carol McNally, Personal communication, 2012). PFO and SLO naturally have isoleucine instead of leucine but PFO is lytic so this aggregation pattern cannot be observed in WT PFO. However, the effect could be observed in these two toxins by abolishing their lytic activity.

The L363A mutation was also mapped using PYMOL software and is shown in the figure 5.15.

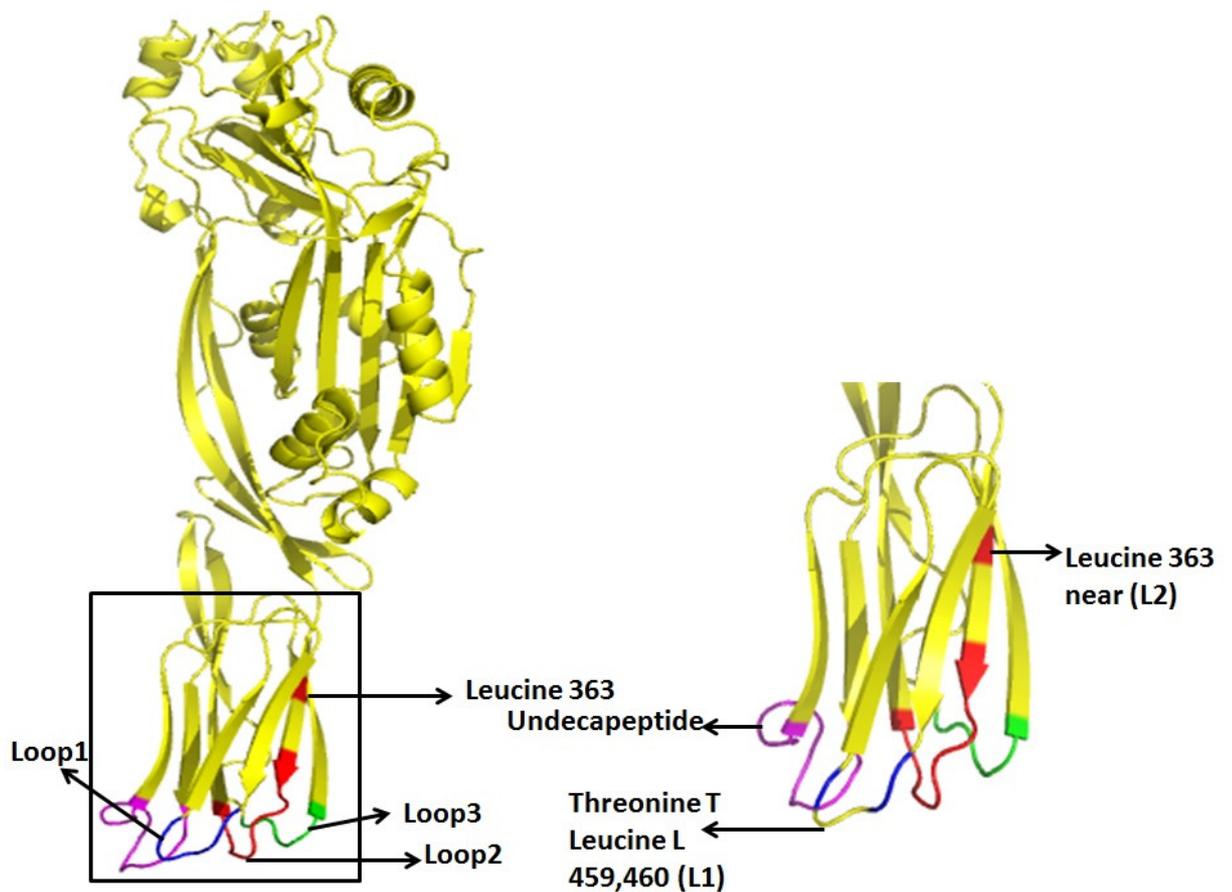


Figure 5-15 Structural model of PLY generated using Phyre2 and highlighting residue responsible for aggregation along with loops and undecapeptide region

PLY structure is predicted based on the solved crystal structures of the proteins already present in the protein data bank using computational software programme Phyre2. Residue 363 (leucine) causing agglutination is highlighted as red along with the 3 loops and undecapeptide region. The above diagrams were drawn using PYMOL software. The region having mutation L363A was zoomed again with the three loops (L1, L2, L3) and the undecapeptide region which are highlighted with blue, red, green and magenta. The TL residues in loop1 (zoomed picture) are highlighted in yellow.

Figure 5.15 shows that L363A mutation is on the first β sheet of D4 and close to the hydrophobic loop 2 of D4PLY. Mutating L363A might interfere with the subsequent binding after the initial attachment of the cholesterol recognition motif (TL) with the membrane.

The undecapeptide and loops (1-3) are assumed to anchor the toxin in the perpendicular orientation after binding so mutation in this residue close to loop 2 might have been affected and resulted in either the abolishment of prepore oligomerisation and/or pore formation (Ramachandran *et al.*, 2002). One possible explanation is that L363A mutation might be interfering with the cholesterol docking after the initial binding and hence reduction in lytic activity.

5.10 Discussion:

A total of 112 mutants were screened for the lytic activity in WT PLY background through this alanine scanning mutagenesis approach. To our knowledge, no previous study has scanned the whole D4 of PLY but instead has substituted important residues in D4PLY (L1-3 loops and undecapeptide). In this study cell lysates were assessed for lytic activity and although this technique is not optimal but still gives us some clues as to which mutants might be important for lytic activity of the toxin. This alanine scanning mutagenesis has been done in a study done recently in LLO showing *in-vivo* effect of single substitution on the virulence of the organism. Their study was able to identify a number of 39 novel residues in LLO previously uncharacterised (Melton-Witt *et al.*, 2012). The results of this study has also identified some mutants (4, 6, 80, 83, 84, 86, 91 and 97) and (81, 92 and 94) not residing in L1-L3 and undecapeptide region and reducing the lytic activity by 87% and more than 90%. However, further

experiments needs to be conducted with these mutants and lytic activity measured using purified recombinant proteins.

The haemolytic assay results in the study are comparable with the studies done previously. The haemolytic activity of tryptophan residues in the undecapeptide region in our study is less than 1% of the WT PLY and the reduction in lytic activity has also been reported in the studies done previously (Korchev *et al.*, 1998, SekinoSuzuki *et al.*, 1996). In the same study, residue 428 cysteine when replaced with glycine and serine resulted in the reduction of haemolytic activity to 3 and 25% of the WT PLY. However, in this study the activity was reduced to 50% and this is supported by studies done by Soltani *et al.*, 2007 which suggests that modification at this residue does not affect the binding in PFO but rather blocks the subsequent tryptophan residues insertion (Soltani *et al.*, 2007). Cysteine 428 replaced with alanine had no effects on the lytic activity and binding with the red blood cells was also shown by a study carried by Saunders *et al.*, 1989 which showed similar effects as WT PLY (Saunders *et al.*, 1989). The same mutation C428A made by another study also found no difference in lytic activity compared to the WT PLY (Oloo *et al.*, 2011).

The residue 385 (aspartic acid) known to be involved in activating the complement without affecting the haemolytic activity was shown by study done by Alexander *et al.*, 1998 and the results of this study also showed no difference in the lytic activity when this residue is replaced with alanine (Alexander *et al.*, 1998).

The % binding activities in D4PLY loops 1, 2 and 3 in PFO when replaced with alanines were assessed by Farrand *et al.*, 2009 and found that in PFO, residues

(T489A, Y492A) in loop 1, (S399A, V403A) in loop 2 and (D434A) in loop 3 have 50% or more binding when compared with WT PFO and the results of this study also suggests the same residues have haemolytic activities ranging from 12.5 to 100% (Farrand *et al.*, 2009). Their study also reported 100% or more binding with residue A401G in loop 2 and this study also found the lytic activity of the same residue to be 100%.

The histidine substitution with arginine in loop 2 resulted in reduction of lytic activity by 0.02% in a study done by Berry *et al.*, 1995 whereas this study has shown reduction by 6.25% when replaced with alanine which might be due to the different nature of residues; arginine being polar basic and alanine non-polar hydrophobic (Berry *et al.*, 1995).

The role of the C-terminus in binding was also observed which showed that truncated versions with 6 and 11 amino acid deletions in the C-terminal resulted in the loss of more than 99% of the haemolytic activity and a reduction in 98% binding compared to the WT PLY. The results of this study also shows a reduction of 12.5 to 50% in the lytic activity when these 11 residues were individually substituted with alanines (Owen *et al.*, 1994).

Recently, a study conducted to evaluate cholesterol-dependent binding of different mutants of PFO (L491C, A401C, V403C and A437C). PFO^(L491A) (one of the cholesterol binding motifs in PFO) and PFO^(V403) (in loop 2) requires more cholesterol concentration to trigger binding. Their results depicts that D4 residues located around the conserved cysteine in the undecapeptide region modulates the cholesterol threshold content required for toxin membrane

interaction. They also suggested that residues other than TL residues might also be involved in binding and requires further investigation. (Johnson *et al.*, 2012).

The hemagglutination assays of the 112 mutants in the library revealed residue 363 responsible for the residual binding/agglutination of the red blood cells and this residue lies in close proximity of loop 2 of the D4 of the toxin. This substitution might have several consequences including structural perturbation of the loop-1 resulting in loss of binding. Another possible explanation could be that this mutation is affecting the conformational change required after the initial binding of the TL residues since mutation at this residue only resulted in the reduction of the lytic activity of the toxin by 87.5%. The involvement of residues (in and/or near loop 2) in binding with the membranes has also been suggested by a recent study done in LLO (Melton-Witt *et al.*, 2012).

Further evaluation of this residue for binding can be done by running cholesterol binding assays and substituting residue 363 with any other charged residue and/or glycine and by comparing the aggregation and lytic effect of the toxin with alanine substitution.

6 Concluding remarks and future perspectives:

The primary aim of the project was to locate site/s in $\Delta 6$ PLY responsible for aggregating/agglutinating red blood cells in order to further improve $\Delta 6$ PLY as a vaccine candidate.

The flow chart below explains how the above explained aims were achieved during the progress of this study.

Mutant generated in D4PLY by substituting cholesterol binding motifs (Farrand *et al.*, 2009) TL>GG by SDM (pET33bD4PLY^(TL>GG)) (Chapter No 3).



Mutants made in full length toxin (WT & $\Delta 6$ PLY) backgrounds by RM. The mutants generated in WT PLY were checked for their lytic activity and $\Delta 6$ PLY were analysed for their aggregation/agglutination behaviour (Chapter 4).



ASM approach was taken and mutants were devised again by mutating every single residue in D4PLY by alanines and/or glycines in both the (WT & $\Delta 6$ PLY) backgrounds (Chapter 5).

Mutants generated in $\Delta 6$ PLY background were tested and eventually the amino acid (L363A) responsible for this activity was located. L363A substitution completely abolished the aggregation activity associated with $\Delta 6$ PLY at concentrations up to 5 μ g.

The crystal structure of PLY has not yet been solved and identification of the residue was done by screening the library of all individual residues present in the D4 of the molecule. The 363 residue in PLY responsible for agglutination of red blood cells was mapped on PYMOL software and its position, orientation of side chain and distance (363 and TL) was measured.

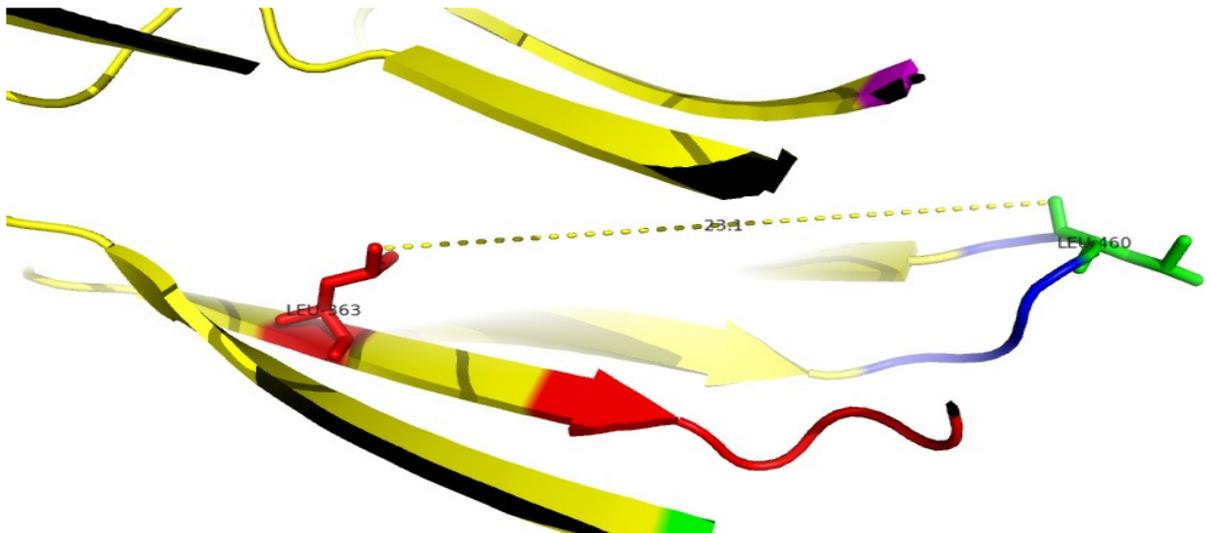


Figure 6-1 Showing orientation and distance of residues L363 and L460 (one of the cholesterol binding motifs) in PLY

PLY model was made using computational software for protein prediction PHYRE2. The side chain distances between residues 363 and leucine 460 on loop 1 is measured. L363 and L460 are highlighted as red and green The figure was generated by PYMOL software.

Figure 6.1 show that 363 residue lies near the C-terminal on one of the first β strands (near loop 2,) of domain 4 PLY. 363 and TL residues are not right next to each other and distance of side chains between residues ((363L and 460L, (one of the cholesterol binding motif)) measured was found to be 23.1 Å. The distance between the residues and the position of side chain suggest that it may not be interacting with the red blood cells at the same time. Although, the side chain of L363 is pointing inwards, it could be that this residue is not directly accessible and thus not involved in initial binding but conformational change of molecule after initial binding with TL residues could result in the subsequent

binding with other red blood cells hence causing agglutination by this second site as hypothesised in the model (Figure 3.18). In WT PLY background, L363A substitution resulted in 87.5% reduction of lytic activity compared to the WT PLY. Another explanation might be that this residue is second binding site as suggested in the model (Figure 3-18) and is interacting with mannose rather than cholesterol since a study done recently have shown that isolated D4 binds to mannose (Lim *et al.*, 2013). However, this requires further investigation.

A study conducted in LLO suggests residues flanking loop2 may have some underappreciated role in binding with the membranes (Melton-Witt *et al.*, 2012). Circular dichroism (CD) experiments can be done to evaluate any difference in secondary and tertiary structure of the mutant compared to the WT PLY. Studies done previously have shown that up to double amino acid substitutions in PLY gene is well-tolerated and does not result in any perturbation of the toxin molecule (Farrand *et al.*, 2009).

In this study for the first time in PLY gene, 5 amino acid insertions were made by RM and it was well-tolerated. These mutations gave a clue as to which regions are important in terms of structure and biological activity of the toxin. The haemolytic activity of the purified proteins was similar when amino acids were substituted/added and is comparable to the previous studies. However, a single amino acid addition in C-terminal region of D4 region of PLY is intolerable probably suggesting that this region is not suitable for conjugating with other proteins and/or polysaccharides. Another region (190-198) of the toxin in domains (1-3) was also identified which renders it insoluble.

In terms of $\Delta 6$ PLY as a vaccine molecule, unlike W433F, it is non-toxic at high protein concentrations (mg/ml) and addition of L363A substitution seems to make it ideal to be used as a vaccine molecule alone. $\Delta 6$ PLY has also been shown to act as strong adjuvant and its adjuvant activity is independent of the carried antigen fused (Douce *et al.*, 2010). The mechanism of $\Delta 6$ PLY^(L363A) interacting with cells is interesting and the experiment conducted in this study shows that substituting L363A blocks agglutination activity of the molecule. The comparison of this new toxin with $\Delta 6$ PLY warrants further investigation by setting up experiments to check the adjuvant activity of this new mutant. The reasons of Δ PLY adjuvant activity are still unclear. It could be that the aggregation of red blood cells is responsible for $\Delta 6$ PLY's adjuvanticity.

$\Delta 6$ PLY^(L363A) presumably has some biological activities which appears to be “non-toxic” to the host and can be retained? One of them is TLR-4 activation which in turns results in the activation of the host immune response and thus suggesting its immunogenic potential. A few preliminary *in-vitro* experiments were run as an off-shoot of this project and suggest that the TLR-4 binding sites are located in regions around residues 138-142 (YGQVN>AAAAA). The results of experiment show that a PLY mutant with 5 amino acid substitution at this place has no effect on the lytic activity compared to the WT PLY but IL-1 β production is significantly reduced. This implies that ideal vaccine should be interacting with immune cells and therefore isolated domains (D1-3 and D4) fused with eGFP were unable to mount an appropriate immune response when given in mice (Ma, 2011).

The study succeeded in making a toxoid which is non-toxic, binds to cells and does not aggregate but whether it is beneficial in terms of immunity requires further follow up experiments. The next stage probably is set up some *in-vivo* experiments with or with-out any added adjuvants (Alum, Aluminium hydroxide) and to check this version ($\Delta 6$ PLY^{L363A}) with a version that does not bind to the cells at all (PLY^{L363A, T459G, L460G}), a toxoid that binds to cells and cause aggregation of cells ($\Delta 6$ PLY), W433F, PLYD1 and comparing immune parameters (antibody production, Th-17 response, effect on colonisation) of these tested toxoids.

The search of an ideal PLY vaccine candidate has been going on for the last few decades. The protein is toxic so designing a molecule which is non-toxic, immunogenic, safe, cheap, and efficacious and provides broad serotype-independent protection is the ultimate goal. So far, only one toxoid PLYD1 has reached phase 1 clinical trials and results published recently results show it has fewer side effects in adults yet the safety has not been checked in the main target population (infants and kids) (Kamtchoua *et al.*, 2012). The success of $\Delta 6$ PLY as adjuvant described by the study done by Douce *et al.*, 2010 is promising since it provides a generic platform and different pneumococcal protein/s can be fused and higher concentration of toxin can be given to achieve maximal response. However, the study also showed that membrane binding of protein was essential for adjuvant activity.

The success of Synflorix and PCV-13 is promising and instead of conjugating *H. Influenza* carrier protein and detoxified Diphtheria toxin, to capsular polysaccharides, $\Delta 6$ PLY and or $\Delta 6$ PLY^(L363A) can be used which can provide non-serotype specific protection. The studies for the search of next generation

pneumococcal vaccines are ongoing and it is likely that a combination of pneumococcal proteins as vaccines can also be used in future. The present study has identified the residue responsible for agglutination of the red blood cells and hence enhanced the safety potential of $\Delta 6$ PLY to be used in the next generation of pneumococcal vaccines

6.1 Future work

Data generated from this project identified the residue (L363) in PLY responsible for causing agglutination of red blood cells. However, the exact mechanism how it stops agglutination is unclear. It will be interesting to observe the behaviour of this residue among other CDCs. Future experiments supporting the proposed model and leading to the eventual goal of suitable PLY toxoid to be used in future pneumococcal vaccine should include:

- Cholesterol binding assays, whereby pre incubating $\Delta 6$ PLY with water soluble cholesterol might result in no agglutination activity on the hemagglutination assay plate due to saturation of TL site which fits in the hypothesised model (Figure 3-18). The second binding site could be binding to mannose (Lim *et al.*, 2013). This binding of toxin to mannose can also be calculated by setting up experiments and comparing the % mannose binding with $\Delta 6$ PLY, Δ PLY^(L363A) and $\Delta 6$ PLY^(T459G.L460G).
- The residue should be checked in other CDCs and mutating the corresponding 363 residue of PLY in other CDC members to check if it blocks the agglutination activity. $\Delta 6$ SLY causes agglutination of red blood cells and has same residue leucine as PLY, therefore changing $\Delta 6$ SLY

corresponding residue to alanine and or other residue to check it stops agglutinating red cells. PFO has isoleucine at the same place and $\Delta 6$ PFO was made but the agglutination could not be observed since $\Delta 6$ PFO was lytic (Section 7.1 and 7.2). The residue 363 in PLY could also be change to other residues e.g. to isoleucine to check if agglutination activity is due to iso-group. The residue can also be substituted with other basic (e.g. lysine), acidic (aspartic acid) or residue with no side chain (glycine) and observing the effect. As mentioned earlier CD experiments should be done with these mutants to observe any change in secondary or tertiary structure.

- Mutant 363 had reduce lytic activity compared to WT PLY so purification of recombinant PLY^(L363A) in WT background and observe binding and pore formation of this toxin with cells by electron microscopy (EM) and comparing results with WT and $\Delta 6$ PLY. The % haemolytic activity in HU/mg of purified recombinant protein can also be calculated and compared with WT PLY.
- *In-vivo* experiments and measuring antibody response in mice and checking its adjuvant activity as explained in the above paragraph.

Appendices

7 Purification and characterisation of $\Delta 6$ PFO

7.1 Protein purification

The recombinant protein $\Delta 6$ PFO was purified in the same way as the other mutant proteins except it was not passed through the AEC column. After the initial purification through the nickel affinity chromatography the protein was further purified by size exclusion chromatography (SEC) column following the protocol as explained below.

$\Delta 6$ PFO after NAC was further purified using Hi Load 16/60 Superdex 200 pg column from GE Health Care. The column was washed overnight with PBS at 0.5ml/minute. $\Delta 6$ PFO was first buffer exchanged into PBS and then loaded onto the column at 0.1ml/minute. The column was attached to the AKTAPrime™ plus purification system (GE Health Care) and the SEC Programme was run.

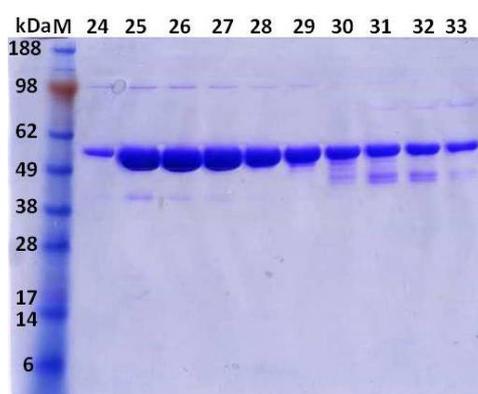


Figure 7-1 Purified fraction of recombinant $\Delta 6$ PFO on a pre cast gel after size exclusion chromatography (SEC)

Lane M contains 5 μ l of the mol. wt. marker SeeBlue® Plus 2 followed by 5 μ l recombinant protein $\Delta 6$ PFO fractions after SEC in lanes 24 to 33. There were few bands (contaminants) which were removed before storing proteins at -80°C.

Purified $\Delta 6$ PFO has a predicted molecular weight of 54kDa. The purified $\Delta 6$ PFO fractions loaded on the SDS-PAGE correspond to the same molecular weight when compared to molecular weight markers. The purified fractions were aliquoted and stored at -80°C .

7.2 Hemagglutination assay of the purified protein

$\Delta 6$ PFO was run on a 96 well hemagglutination assay plate according to the same protocol as followed for the other hemagglutination assays.

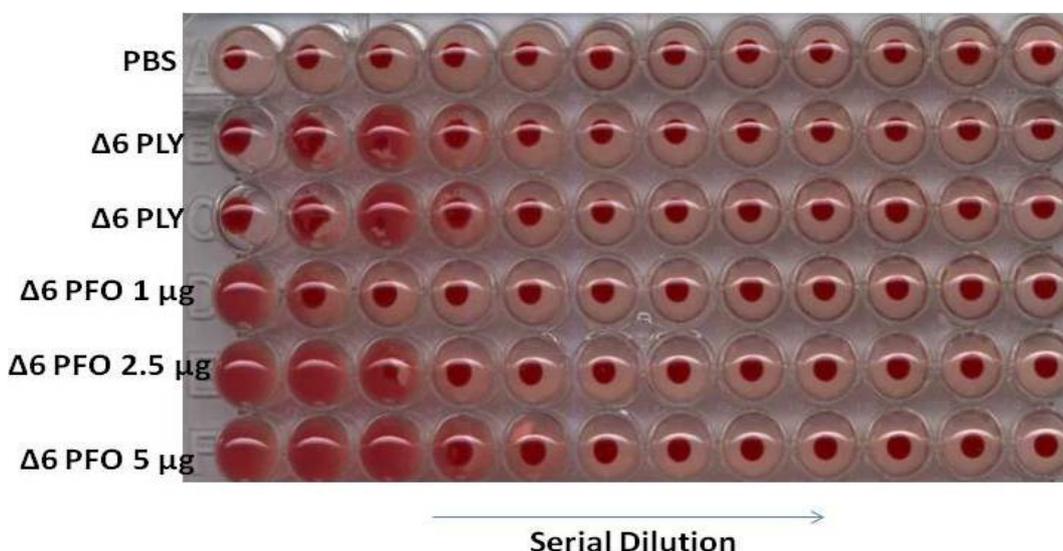


Figure 7-2 Hemagglutination of the purified $\Delta 6$ PFO

1, 2 and 5 μg of purified $\Delta 6$ PFO was placed in the first well of the corresponding rows and then serially diluted across the whole plate. PBS only and $\Delta 6$ PLY were used as negative and positive controls.

The toxin was checked with 3 different concentrations of 1, 2.5 and 5 μg . The toxin has naturally isoleucine instead of leucine at position 363 so it was suspected that the $\Delta 6$ PFO will be agglutination negative but results showed that even after deleting the two residues alanine and arginine the toxin is still lytic

even at 1 μ g. Therefore we were unable to ascertain the aggregation phenomenon with $\Delta 6$ PFO.

Publication, conference participation and presentations

Activation of memory Th17 cells by domain 4 pneumolysin in human nasopharynx-associated lymphoid tissue and its association with pneumococcal carriage.

Gray C, Ahmed MS, Mubarak A, Kasbekar AV, Derbyshire S, McCormick MS, Mughal MK, McNamara PS, Mitchell T, Zhang Q.

Pdf version of the paper attached after the list of references.

Poster presentations (Presenting author underlined)

M.K.Mughal and T.J.Mitchell. (September 2012) “Development of Pneumolysin as a vaccine candidate.” Society of General Microbiology (SGM), Birmingham, UK.

M.K.Mughal and T.J.Mitchell. (November 2010) “Development of Pneumolysin as a vaccine candidate.” World Pneumonia Day: perspectives on pneumonia and pneumococcal disease. Glasgow, UK.

M.K.Mughal and T.J.Mitchell. (June 2010 and 2011) “Structure and function studies of Pneumolysin.” Internal poster presentations at the University of Glasgow, UK.

Oral presentation

M.K.Mughal and T.J.Mitchell. (May 2012) “Development of Pneumolysin as a vaccine candidate.” Internal seminar at the Institute of Infection, Immunity and Inflammation, Glasgow, UK.

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