Probing into membrane interaction mechanism of *Helicobacter pylori* TlyA

Anjali Gupta

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BS-MS dual degree in Science

Department of Biological Sciences

Indian Institute of Science Education and Research Mohali

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Certificate of Examination

This is to certify that the dissertation titled **"Probing into membrane interaction mechanism of *Helicobacter pylori* TlyA"** submitted by **Ms. Anjali Gupta** (Reg. No. MS09016) for the partial fulfillment of BS-MS dual degree programme of the Institute, has been examined by the thesis committee duly appointed by the Institute. The committee finds the work done by the candidate satisfactory and recommends that the report be accepted.

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(Supervisor)

Dated: April 25, 2014

Declaration

The work presented in this dissertation has been carried out by me under the guidance of Dr. Kausik Chattopadhyay at the Indian Institute of Science Education and Research Mohali. This work has not been submitted in part or in full for a degree, a diploma, or a fellowship to any other university or institute. Whenever contributions of others are involved, every effort is made to indicate this clearly, with due acknowledgement of collaborative research and discussions. This thesis is a bonafide record of original work done by me and all sources listed within have been detailed in the bibliography.

Anjali Gupta

(Candidate)

Dated: April 25, 2014

In my capacity as the supervisor of the candidate's project work, I certify that the above statements by the candidate are true to the best of my knowledge.

Dr. Kausik Chattopadhyay

(Supervisor)

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Firstly I wish to thank god for bringing courage and patience in me.

I would like to express my heartfelt gratitude to Dr. Kausik Chattopadhyay, my supervisor for his useful advice, pertinent guidance and constant encouragement without which this project would not have been a success. I am thankful for the invaluable opportunity to have worked under him.

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I am grateful to the faculty, staff and the research students at IISER Mohali for maintaining a healthy research environment. A special thanks to the Library, IISER Mohali for providing me access to the research articles needed for my work.

At the end I will not forget to thank my family for providing all necessary support and unconditional help.

Anjali Gupta

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A**bbreviations**

- PFTs: Pore forming toxins
- WT: Wildtype
- LB: Luria Bertani
- RBCs: Red Blood Cells
- RMSD: Root mean square deviation
- IPTG: isopropyl-β-d-thiogalactopyranoside
- PBS: Phosphate buffer saline
- PCR: Polymerase chain reaction
- HpTlyA: *Helicobacter pylori* TlyA
- Trp: Tryptophan
- Bp: Base pairs
- Aa-amino acid

CONTENTS

INTRODUCTION

Infections caused by bacteria constitute the major type of illnesses among living individuals. Bacterial infections are the leading causes of morbidity and mortality worldwide, and bacteria can cause infections in nearly all host tissues. To cause damage to the host, bacteria colonize into the host and release certain virulence factors. Of all these bacterial virulence factors one third i.e. about 25 to 30% of cytotoxic bacterial proteins are pore-forming toxins (PFTs) (Los et al., 2013). They are frequently cytotoxic (i.e., they kill cells) by creating unregulated pores in the membrane of targeted cells. In addition to pore formation there are other mechanisms also by which these toxins cause damage into the host cell. These pore-forming proteins have been identified in a wide range of organisms including bacteria, plants, fungi, and animals, and in some cases show mechanistic and structural homologies across vast evolutionary distances. Pore-forming toxins (PFTs) are protein toxins, typically, (but not exclusively) produced by bacteria, such as *Clostridium septicum* and *Staphylococcus aureus* and certain other organisms such as cnidarians, mushrooms and plants. In fact, in mammals perforins are an important part of innate immune defense (Gonzalez et al., 2008).

PFTs function to punch a hole in membranes of host cells, predominantly the plasma membrane but in some cases intracellular organelle membranes also. It has been classically hypothesized that it does so to kill target cells directly, for intracellular delivery of other bacterial or external factors, to release nutrients, or for phagosomal escape in the case of intracellularly acting PFTs (Los et al., 2013). Loss of PFTs generally causes pathogenic bacteria to be less virulent or completely avirulent. Conversely, transgenic expression of a PFT can turn an otherwise harmless bacterium into a parasite or a pathogen (Kho et al., 2011). Generally, PFTs are secreted as water-soluble molecules. Then they diffuse towards their specific target and recognize their specific

receptor. On recognition, because of high affinity they bind to their specific receptors and associate with the target membrane, form multimers, and undergo a conformational change, leading to the formation of an aqueous pore in the membrane. Intracellularly acting toxins bound to their receptor are trapped into endocytic vesicles and follow various intracellular pathways until they deliver their enzymatic domain into the cytosol (Geny and Popoff, 2006).

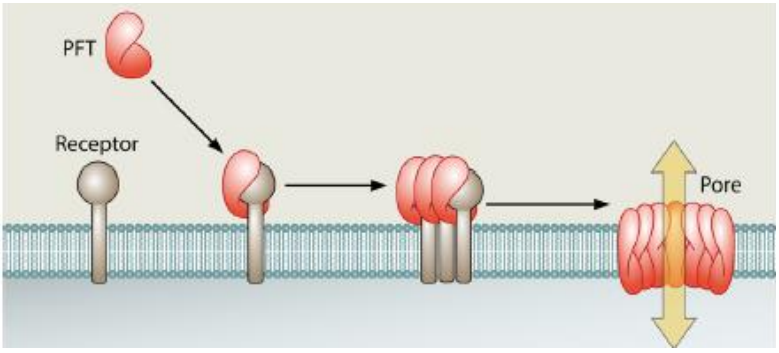


Figure1: Generalized mechanism of pore-formation by PFTs

On the basis of structural and mechanistic features bacterial toxins which form pores can be broadly classified into two groups, those that interact with membrane through α-helices and those that interact through β-structures. Interestingly, if PFTs are sorted based on the presence or absence of hydrophobic stretches in their primary sequence then also same categories are formed. α-PFTs are characterized by stretches of hydrophobicity that are predicted to be helical and are able to span lipid bilayers. In contrast, ß-PFTs are not predicted to be transmembrane as revealed by the analysis based on hydrophobicity. They however contain pairs of amphipathic ß-strands, which when combined in multi-protein structures generate a sufficiently hydrophobic surface for membrane insertion to occur (Iacovache et al., 2008). The divisions and subdivisions are shown as below-

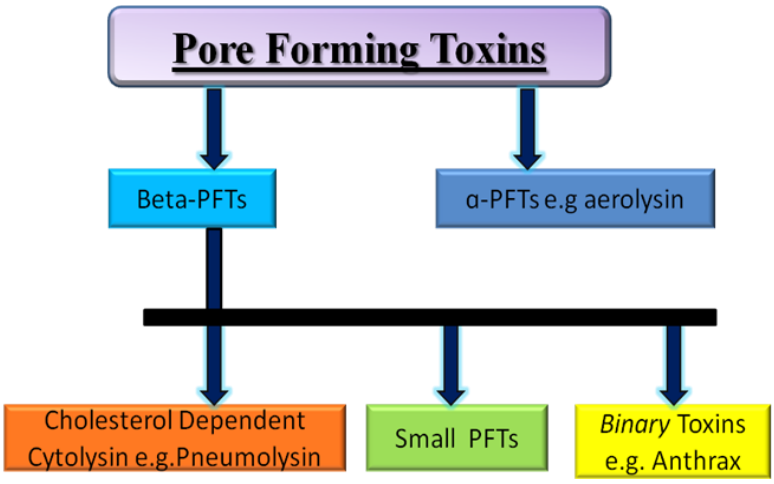


Figure 2: Classification of Pore-forming toxins

PFTs use two main mechanisms to form pores in the cell membrane, according to the structure of the toxin domain building the channel: insertion of amphipathic α-helices or insertion of amphipathic β hairpins organized in a β-barrel.

Some of the examples of toxins that interact through α-helices are AB5 family of toxins, the bacterial colicin and *Bacillus thuringiensis* δ endotoxins. Examples of AB5 toxins are the heat-labile enterotoxins of several pathogenic bacteria such as *Vibrio cholerae* (the causative agent of cholera), *Shigella dysenteriae* (dysentery), *Bordetulla pertussis* (whooping cough), and enterotoxigenic *Escherichia coli*. The *B. thuringiensis* δ endotoxins are agriculturally important insecticidal agents which are thought to act through oligomerization. Colicins are agents of bacterial warfare which kill sensitive strains of *E. coli*; their mechanism is a matter of controversy (Iacovache et al., 2008).

Among PFTs, the term hemolysins has been used to refer those bacterial toxins that lyse erythrocytes by cell wall disruption and are often more correctly referred to as cytolysins. Hemolysins have been demonstrated in a number of pathogens, including streptococcal and staphylococcal species, *E. coli*, *Serpulina hyodysenteriae*, *Mycobacterium tuberculosis*, *Trypanosoma cruzi*, and *Listeria monocytogenes* (Andrews and Portnoy,

1994, Bhakdi et al., 1996, Morgan et al., 1996), and some of these have been shown to be important virulence factors (Braun and Focareta, 1991). Despite the facts that *Helicobacter pylori* is hemolytic when grown on unlysed blood agar plates, and hemolytic activity is increased under iron-limiting conditions, the availability of its genome sequence, and at least half of the world's human population is known to be infected with *H. pylori* (Martino et al., 2001); lesser attention has been paid to characterize *H. pylori* hemolysins.

H. pylori is a microaerophilic spiral shaped Gram-negative bacterium. It is a human specific gastric pathogen that colonizes largely within the gastric mucus layer in the stomach. In 2005, Barry Marshall and Robin Warren were awarded Nobel prize in physiology for the discovery of this pathogen and describing its role in pathogenesis. It was initially named *Campylobacter pylori* but because of its distinct characteristics it was given a separate genus and was named *H. pylori* (Kusters et al., 2006). Infection with *H. pylori* is associated with the development of duodenal and gastric ulcers, gastric adenocarcinoma, and mucosa-associated lymphoid tissue lymphoma (Kuipers, 1997). It is the first identified bacterial carcinogen and under WHO classification it is kept in category of class I carcinogens (Kusters et al., 2006). It has been found that at least half of world's human population is infected with *H. pylori* but only 10-20 % of them develop gastric disorders. Complex interaction between environmental influences and bacterial virulence factors determines the clinical outcome of *H. pylori* infection (Hohenberger and Gretschel, 2003). It has also been found that polymorphism in host cytokines genes varies the response of an individual towards *H. pylori* infection as these genes are responsible for quantitative cytokine production (Basso and Plebani, 2004).

Bacterial virulence factors are the ones which distinguish a pathogenic bacterium from a non-pathogenic one. In *H. pylori* it is poorly understood why some of its strains are

pathogenic and some are not (Atherton, 1997). Factors involved in *H. pylori* virulence, includes urease, motility, the VacA cytotoxin, CagA, the neutrophil-activating protein NapA, adhesins, iron acquisition, and lipopolysaccharide. Out of these, VacA and CagA have been studied in molecular detail. VacA genotype is shown to play important role in cytotoxin activity, and signal sequence type correlates closely with peptic ulceration. Infection with strains possessing CagA (cytotoxin associated gene A) is more common among people with peptic ulceration or gastric adenocarcinoma than without CagA strain. CagA is a marker for the cag pathogenicity island, which includes genes necessary for the enhanced inflammation induced by pathogenic strains. *H. pylori* genome consists of approximately 1590 coding genes analysis of which indicates presence of additional virulence factors that are yet to be characterized in molecular detail.

Apart from these virulence factors *H. pylori tlyA* gene which has been annotated to encode a protein with sequence similarity to the TlyA hemolysin like proteins of several pathogenic bacteria has not been extensively characterized. *H. pylori* genome harbors *tlyA* gene of 708 Bp (HP1086 in the genome of *H. pylori*, strain 26695) (Tomb et al., 1997) which encodes for a protein consisting of 235 amino acid which does not include any tryptophan residue in it and its blast analysis shows that TlyA homologs are present in many prokaryotes and some eukaryotes. TlyA amino acid sequence alignment demonstrated nearly 35% homology with *M. tuberculosis* and *S. hyodysenteriae.*

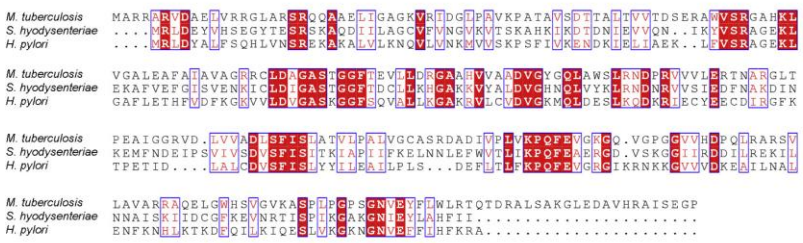


Figure 3: Amino acid sequence alignment of *H. pylori* TlyA with the TlyA-like proteins from *M. tuberculosis* and *S. hyodysenteriae*.

It has been reported that *H. pylori* adherence to AGS cells was reduced in the absence of *tlyA* gene indicating that TlyA is important for adherence of *H. pylori* with host cells (Zhang et al., 2002). It has also been demonstrated that *in-vitro* hemolytic activity is decreased in *tlyA* deficient mutant of *H. pylori*. Moreover, when wild type and *tlyA* deficient mutant *H. pylori* cells were coincubated with dextran 5000 the hemolytic activity was significantly reduced in the mutant strain. These two evidences suggest that the expression of TlyA is a prerequisite for the colonization of *H. pylori* in the mouse model, and also TlyA is an important virulence determinant, and its mode of action is by pore formation (Martino et al., 2001). *H. pylori* TlyA (HpTlyA) recombinant protein was purified in our lab for the first time and its characterization revealed certain interesting features. The results suggest that TlyA purified protein kills human erythrocytes as well as human gastric cancer cells (AGS cells) (Lata et al., 2014). Purified recombinant TlyA protein interacts with various eukaryotic cell membranes like human RBC and AGS cells. It was also shown that TlyA exhibits hemolytic activity which is facilitated by membrane pore formation in the target cells. Cytotoxicity studies like MTT assay showed cytotoxic effects of TlyA on AGS cells (Lata et al., 2014). These studies strongly suggest that TlyA of *H. pylori* is a hemolytic, pore-forming and membrane-damaging cytotoxin. However, there is very little or no information in the scientific literature about what biophysical changes occur while TlyA interacts with cell membrane and cause cytotoxicity.

Considering the fact that HpTlyA is a tryptophan-less protein and in view of the above mentioned information the present study was designed to understand the mode of action of this novel virulence factor TlyA using Trp point mutations to probe into cell membrane-protein interaction. To conduct the study a number of single Trp variants of Hp*tlyA* were created along the sequence of *tlyA* gene to directly monitor binding-induced folding changes as well as residue specific localization of the polypeptide chain on the

cell membrane. As the crystal structure of this toxin is not yet solved the sites of mutation were decided based on preliminary bioinformatics analysis of TlyA. Results obtained from characterization of TlyA tryptophan incorporated mutants have provided a new understanding of how this toxin is interacting with the membrane and the residues which can be important to maintain the functionality of this protein. However, more detailed analysis is required to have a complete understanding of mechanism. For this purpose, we are performing alanine scanning mutagenesis which will enable us to understand the importance of a specific residue or a stretch of residues in context of stability and maintaining function of protein.

MATERIALS AND METHODS

1. Strains, culture conditions, plasmids and chemicals

Strains and plasmids used in this study are listed in table 1. *E. coli* strains like Top10 (Novagen), Origami B (Novagen) were grown in LB (Luria Bertani) medium at 37° C in the presence of appropriate antibiotics wherever required. *E. coli* Top10 was used as plasmid host and *E. coli* Origami B was used as expression system. All chemicals used for growing bacteria were procured from Hi-media (Mumbai, India), chemicals used in enzymatic assays were purchased from Sigma, USA.

Table1: Plasmids used in the present study

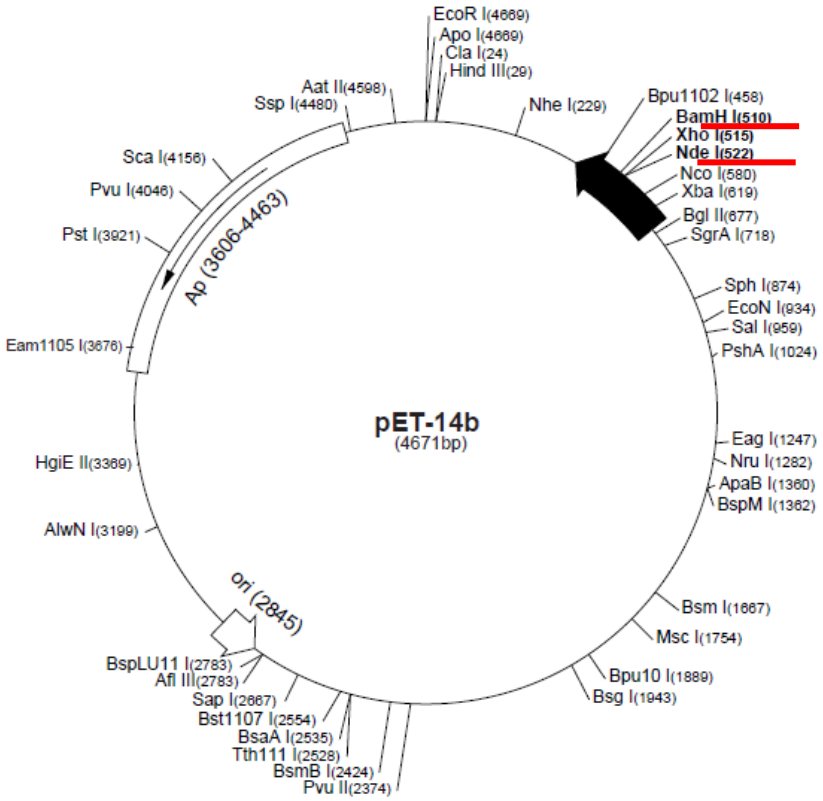
Plasmid name	**Type**
pET-14b	*E. coli* expression vector
pCR®2.1, linearized	*TA cloning vector*

Table2: Details of primers

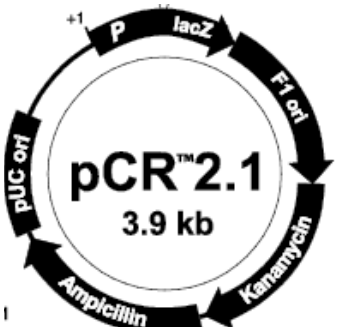
Primer	**Sequence (5'-3')**
WT TlyA Forward primer	AATGTACATATGATGCGCTTA
WT TlyA Reverse primer	ACAAGTGGATCCTTAGGCTCG
A6W TlyA Forward primer	AATGTAcatatgATGCGCTTAGATTACtggTTATTCAGTCAGCA
G224W TlyA Reverse primer	ACAAGTggatccTTAGGCTCGCTTGAAATGGATAAAAAATTCA ACATTccaGTTTTTCCCTTTCAC
Y153W TlyA Forward primer	GTGAGCTTTATTTCTTTATATtggATTTTAGAAGCGATT
Y153W TlyA Reverse primer	AGGCAAAATCGCTTCTAAAATccaATATAAAGAAATAAA
F148W TlyA Forward primer	TTAGCGCTTTGCGATGTGAGCtggATTTCTTTATATTAT
F148W TlyA Reverse primer	TAAAATATAATATAAAGAAATccaGCTCACATCGCAAAG

Primers were designed using DNAstar software. In order to make protein purification convenient 6X His tag was utilized from pET-14b plasmid. Restriction sites *Nde*I and *Bam*HI were also inserted in constructed primers.

Vector map of pET-14b is shown below:



Vector map of TA cloning vector pCR 2.1 is shown below:



2. TlyA protein and site-directed mutagenesis:

TlyA: The nucleotide region 1145032-1145739 of *H. pylori* is 708 base pairs long *tlyA* gene which encodes TlyA protein of 235 amino acid residues. The molecular weight of translated protein is 26.6 kDa and ~28 kDa with 6X His tag.

Site-directed Mutagenesis in Hp*tlyA* gene: Hp*tlyA* gene was PCR amplified using primers (table 2) from the provided pET14b vector harboring WT *tlyA* gene. The primers were designed to perform specific mutation at desired site and then mutagenesis is performed by primer extension method. The amplified product was gel purified (Qiagen, USA) and digested with *Nde*I and *BamH*I restriction enzymes

and was subsequently ligated with the similarly digested vector pET-14b, where the cloned product was expressed under the control of the endogenous T7 promoter. *E. coli* Top10 cells, were subsequently transformed with the ligation mix and the cells were grown in LB Broth (Himedia, Mumbai) containing ampicillin (50µg/mL) at 37°C. Clones obtained were confirmed by restriction digestion and sequencing.

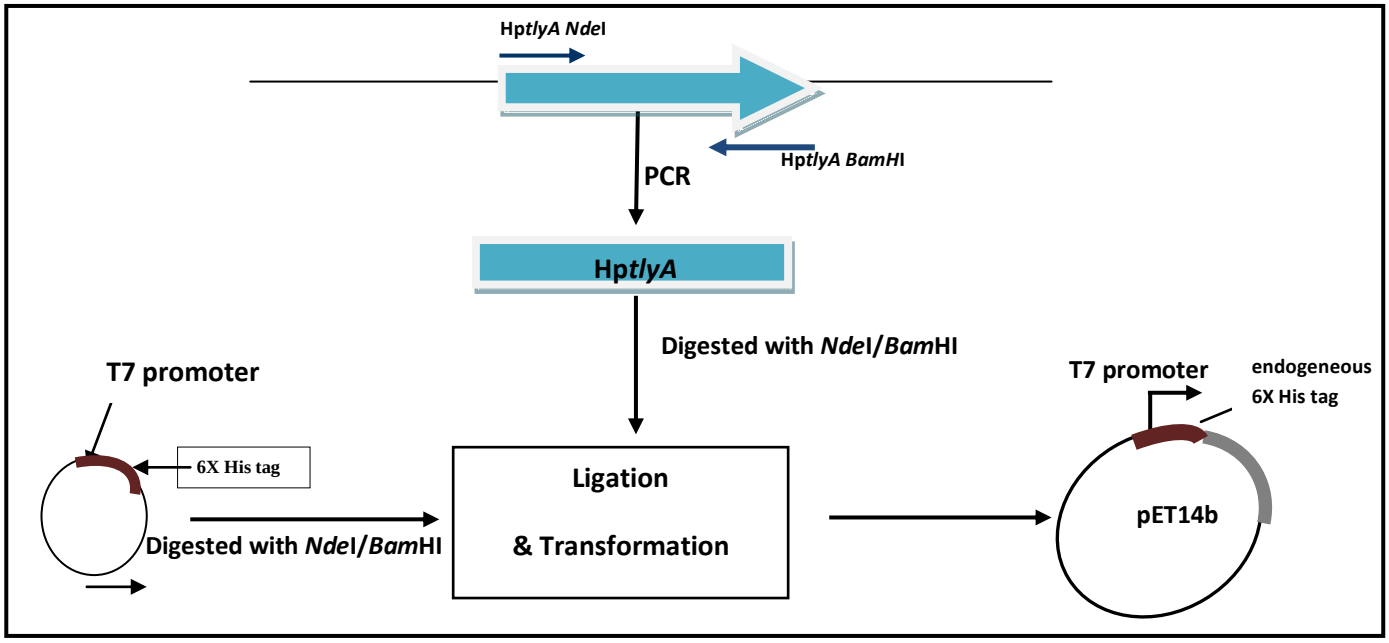


Figure 4. Schematic representation of site-directed mutagenesis of Hp*tlyA* gene under T7 promoter in pET-14b.

3. Homology-search of *H. pylori* TlyA

Sequence homology analysis results revealed that this gene harbored in *H. pylori* genome has been annotated to encode a protein with sequence similarity to the TlyA proteins of several pathogenic bacteria including *M. tuberculosis* and *S. thermophilus*. TlyA of *H. pylori* shows 58% homology with TlyA of both *S. thermophilus* (34% identity) and *M. tuberculosis* (32% identity).

The homologs of *H. pylori* TlyA are known to exhibit many activities such as of a toxin, methyltransferase and RNA-binding. Sequence based domain prediction showed presence of two major domains:

- **S4 domain:** It is a small domain consisting of 60-65 amino acid residues. It was detected in the bacterial ribosomal protein S4, eukaryotic ribosomal S9 etc. and a number of uncharacterized, small proteins that may be involved in translation

regulation (Davis et al., 1998). This domain is known to mediate RNA binding (Aravind et al., 1999).

- **FtsJ-like methytransferase domain:** The source of FtsJ is from various bacterial and archaeal species where FtsJ plays the role of a methyltransferase, but in reality has no effect on cell division. Crystal structure of FtsJ in complex with its cofactor S-adenosylmethionine suggests that FtsJ has a methyltransferase fold. This family also includes the N terminus of flaviviral NS5 protein which is hypothesised to be a methyltransferase involved in viral RNA capping (Koonin EV, 1993).

Source	Domain	Start	End
Pfam A	S4	1	48
Pfam A	FtsJ	56	234

Figure 5: Sequence based domain prediction (software used Pfam)

TM Pred analysis of HpTlyA predicted the presence of a **putative transmembrane region** from residue 146 to 168.

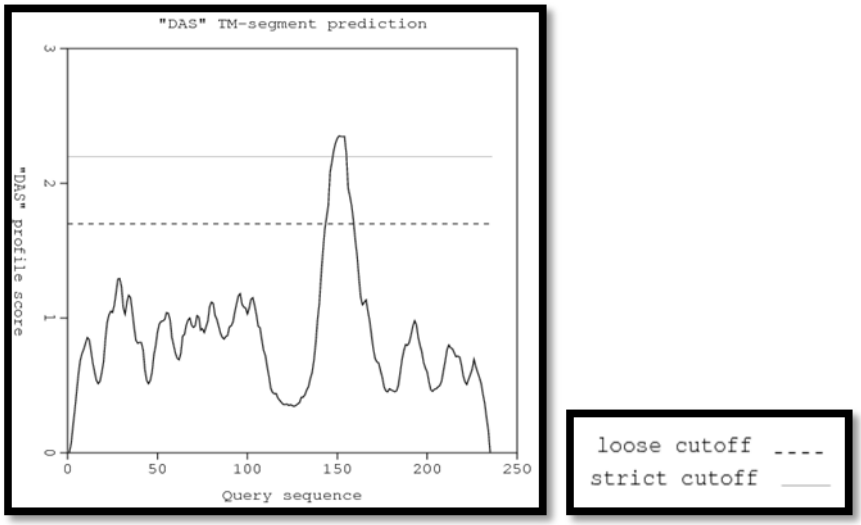


Figure 6: Transmembrane region analysis of HpTlyA sequence (software: DAS Transmembrane prediction server).

4. **Mutation scheme performed to map the cell membrane binding region**

The parameters obtained by homology modeling suggested that the structure of hemolysin from *Streptococcus thermophilus* was a suitable template for HpTlyA.

- Identity (37.2%,RMSD 0.44 °A, E-value 4.7e-26)
- Domain architecture was similar to TlyA protein

Structural coordinates were obtained from the Protein Data Bank (PDB) (entry 3hp7 for *Streptococcus thermophilus* TlyA). Visualisation of structural models was done using PyMOL.

Figure 7: Homology modeling based structural model of HpTlyA using online Swiss PDB viewer program

In order to figure out how HpTlyA interacts with the cell membrane a number of mutants of TlyA were created. Tryptophan was incorporated at various sites in the protein with an intention to exploit intrinsic fluorescence in an otherwise tryptophan-less protein. The basic idea was to use this introduced intrinsic fluorescence in monitoring how various parts of protein interact with cell membrane and how important role do they play towards the function of protein.

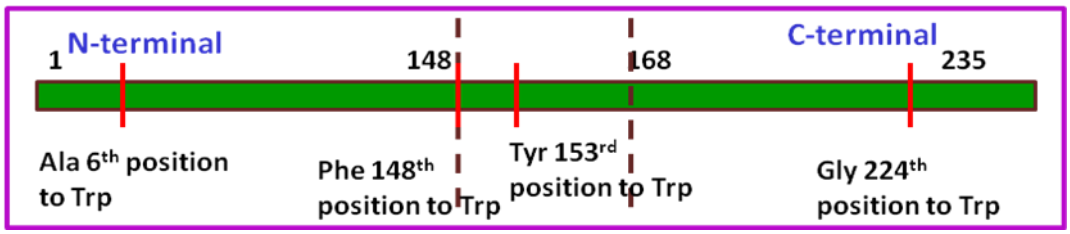


Figure 8: Regions of protein alongwith labeled sites of mutation. Trp positions are indicated using a red mark.

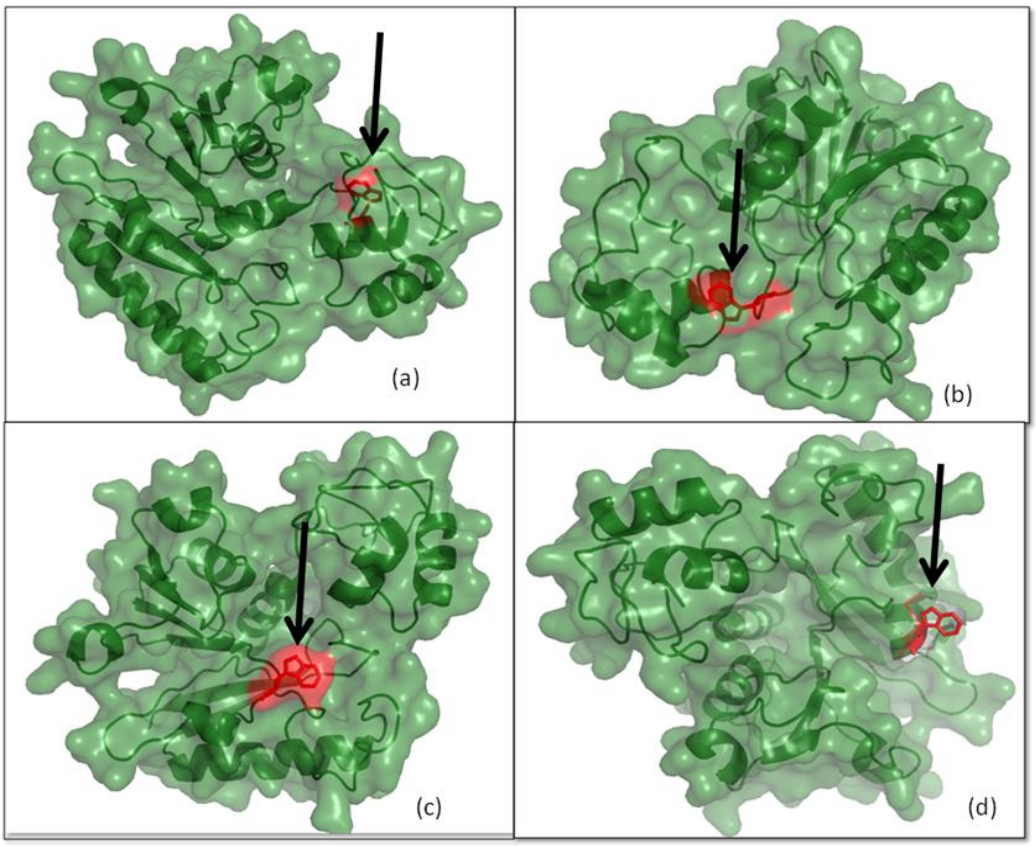


Figure 9: Structural models depicting the sites of mutation (tryptophan incorporation) in sequence based homology modeling generated HpTlyA model where (a), (b), (c), (d) are A6W, G224W, F148W and Y153W TlyA mutants respectively (Software used: PyMOL)

Alanine scanning mutagenesis

It is a method to figure out the importance of a particular residue or a stretch of residues in maintaining stability and functionality of protein.

To characterize the role of putative transmembrane region in maintaining the structure and function of protein various single point variants of TlyA are created in which some residues in the region of focus are replaced with alanine.

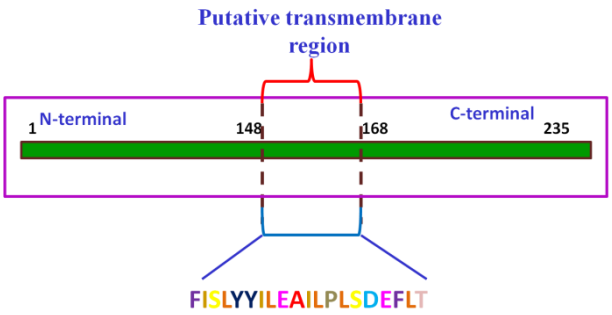


Figure 10: Sequence targeted for alanine scanning mutagenesis.

Mutagenesis, Expression and Purification of WT TlyA and TlyA mutants

5. Preparation of LB-Agar plates (500 mL)

1) 12.5g LB medium powder and 7.5g agar was dissolved in 500 mL distilled water and autoclaved at 121° C for 15 min at 15lb pressure.
2) The autoclaved medium was cooled and poured into Petri dishes.

6. PCR Reaction conditions

Primers used for PCR are listed in table2.

PCR reaction mixture (33µL) consisted of the following components:

Component	**Stock concentration**	**Final concentration**	**Volume added in a reaction (µL)**
Invitrogen Platinum Super mix	1X	1X	30
Forward primer	10µM	0.3µM	1.0
Reverse primer	10µM	0.3µM	1.0
DNA template (100ng/µl)			1.0
PCR grade water			0.0
		Total	**33 µL**

PCR reaction was carried out in BIO-RAD's thermocycler using the following conditions.

Program used for PCR:

Segment	**Number of cycles**	**Temperature**	**Duration**
1	1	94°C	5min
2	30	94°C	30 sec
		56°C	30 sec
		72°C	1min
3	1	72°C	7 min
4	1	4° C	∞

Concept of TA cloning*:* It uses specifically *Taq* polymerase for PCR amplification. *Taq* polymerase exhibits a nontemplate-dependent activity that adds a single deoxyadenosine (A) to the 3´ ends of PCR products. It uses linearized vector pCR2.1 which carries single 3´ deoxythymidine (T) residues. This allows PCR inserts to ligate efficiently with the

vector. This method of cloning does not involve restriction digestion by restriction enzymes.

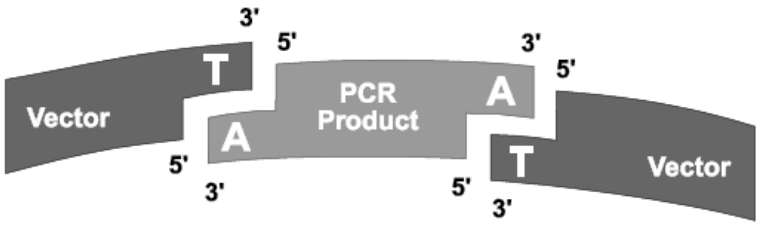


Figure 11: Schematic of TA cloning method

Agarose gel electrophoresis

PCR products/ plasmid were routinely checked on 1% agarose gel electrophoresis and visualized using UV- transilluminator.

Purification of PCR product

Purification was done either by using Gel Extraction Kit or PCR purification Kit (Qiagen, USA).

7. Restriction digestion

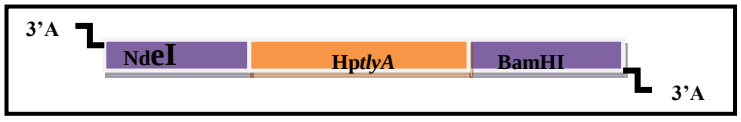


Figure 12 :*Taq* polymerase PCR amplified fragment of Hp*tlyA*

A. **Double digestion of pET-14b, mutated Hp*tlyA* fragment:** Here *Taq* polymerase amplified fragments which have an A overhangs at 3' positions are cleaved as they are after restriction sites.
B. Samples were then incubated at 37°C for 3.5 hours. Then products were purified by PCR purification kit (Qiagen, USA). Purified products were checked on agarose gel.

The restriction digestion mixture (10 µl) consisted of the following components:

Component	**Stock concentration**	**Final concentration**	**Volume added in a 10µl reaction**
Restriction digestion buffer	10X	1X	1 µl
pET-14b/Inserts	300µg	4 µg	7 µl
Restriction Enzyme (*Nde*I+*BamH*I) (NEB)		1 Unit each	1 µl each

C. TA cloning does not require restriction digestion as it is not based on sticky-end ligation concept.

8. Ligation Reaction

A. Ligation reaction to ligate digested pCR2.1 with Taq polymerase amplified insert (Hp*tlyA*).

The following components were added in the ligation mixture (10 µl).

Component	**Negative Control**	**Test Sample**
Digested vector pCR2.1 (provided in invitrogen kit)	2 µl (100 ng)	1 µl (100 ng)
Taq polymerase amplified Hp*tlyA*	NIL	4 µl (135 ng)
T4 DNA Ligase	1 µl	1 µl
MilliQ water	3µl	3µl

B. Ligation reaction to ligate digested pET-14b with double digested (*Nde*I/*Bam*HI) insert (mutated Hp*tlyA* fragment).

Component	**Negative Control**	**Test Sample**
Digested vector pET-14b	1 µl (100 ng)	1 µl (100 ng)
Double digested PCR product (Hp*tlyA*)	NIL	7 µl (135 ng)
T4 DNA Ligase (NEB)	1 µl	1 µl
10X T4 DNA ligase buffer (NEB)	1 µl	1 µl

Conditions:

Samples were incubated at 25° C for ~15 minutes.

9. Competent cell preparation:

Competent *E. coli* Top10 cells were prepared by $CaCl_2$ method and used for cloning. *E. coli* Origami cells were also prepared by $CaCl_2$ method and used for expression studies.

Protocol followed is as follows:

i) Seed culture was prepared by inoculating a loop full of the culture into 10 mL LB broth and was incubated at 37° C with shaking for 16 hours.

ii) 200µL of overnight grown culture was added into fresh 10 mL LB and was incubated at 37° C till O.D. reaches 0.6.

iii) Culture was centrifuged at 4000 rpm for 15 minutes at 4° C.

iv) Supernatant was decanted and 10 mL of 0.1 M $CaCl_2$ (Sigma) was added to the pellet and mixed well.

v) The suspension was kept on ice for 5 minutes.

vi) Cells were pelleted down at 4000 rpm for 5 minutes and supernatant was decanted.

vii) Pellet was carefully mixed with 5 mL of 50 mM $CaCl_2$ and was kept on ice for 45 minutes.

viii) Cells were centrifuged at 4000 rpm for 5 minutes.

ix) Discard the supernatant and to the pellet 1 mL of 85% 0.1M $CaCl_2$ with 15% glycerol was added.

x) Aliquots of 100 µL were made and stored in -80°C.

10. Transformation of *E. coli*

Chemically prepared *E. coli* strain Top10 (Novagen) and Origami (Novagen) was transformed with the desired ligated product using the following protocol:

i) 10µL ligated product (2µL of plasmid) was added to 100 µl competent cells kept on ice and was mixed well by tapping.

ii) Cells with ligated product were incubated in ice for 10-15 minutes.

iii) Then it was incubated at 42° C for 1 minute.

iv) Then it was kept in ice for 5 minutes.

v) 1 mL of LB was added and was mixed well by tapping.

vi) Then cells were incubated at 37° C for 1 hour.

vii) Cells were then centrifuged at 5000 rpm for 1 minute.

viii) Supernatant was discarded and pellet was resuspended in 100µL LB.

ix) Then the cells with ligated product were plated onto LB-agar plates containing ampicillin (50µg/mL).

11.

A. Transformation of *E. coli* Top10 cells prepared by Chemical $CaCl_2$ method with pET14b-Hp*tlyA*

Plated LB-Amp plates were incubated at 37° C overnight. Colonies were observed the very next day (~14-16 hours).

B. Transformation of *E. coli* Top10 cells prepared by Chemical $CaCl_2$ method with pCR 2.1-Hp*tlyA*

In TA cloning to check whether clone is positive or not insertional inactivation is employed. There is lacZ gene in pCR2.1 plasmid which gets disrupted when gene of interest is inserted. As a result of which positive transformants when exposed to X-gal substrate produce white colonies. In case of no transformation lacZ gene is not disrupted and synthesizes β-galactosidase which reacts with X-gal substrate to produce chromogenic product (blue colony).

Protocol:

i) 20mg/mL stock of X-gal is prepared in dimethylformamide (DMF).

ii) 40µL of X-gal stock is spread on LB-Ampicillin plate. Plate was allowed to soak X-gal.

iii) Transformed cells were then plated onto the plates.

iv) Plated LB-Amp plates were incubated at 37° C overnight. Colonies were observed the very next day.

12. Colony PCR to check if there is any positive colony among obtained colonies.

Component	**Stock concentration**	**Final concentration**	**Volume added in a 23µl reaction (µL)**
Genei PCR master mix	2X	1X	10
Forward primer	10µM	0.3µM	1.0
Reverse primer	10µM	0.3µM	1.0
DNA template(colony)			Using tip
PCR grade water			10.0

13. **Sequencing of cloned plasmid:** For further clone confirmation, the plasmids were given for sequencing (Eurofins Genomics India Pvt Ltd, Bangalore).
14. **Transformation of cloned pET14b-Hp*tlyA* plasmid in Origami cells.**

    For protein expression Origami competent cells (Novagen) (prepared by Calcium chloride method) were transformed with the recombinant plasmid pET14b-Hp*tlyA*. Transformation of Origami cells (Novagen) prepared by Calcium Chloride method was done using the protocol mentioned in section 10.

15. **Expression studies of HpTlyA**

    Small scale expression:

    Clones transformed in Origami cells were inoculated and were incubated overnight at 37° C. Overnight grown culture was reinoculated in 10 mL LB-Amp media. Culture was set on vigorous shaking and was incubated till it attained OD ~ 0.4-0.6. Half of the culture was given 1mM IPTG induction and rest half was kept uninduced. Both the cultures were again incubated for 3 hrs at 37° C.

    Then cells were spinned down at 5,000 rpm for 5 min. Supernatant was removed and the cells were resuspended in 50 µL loading dye. Sample was then boiled at 99° C for 10 minutes. 20 µL of both induced and uninduced cell lysates were loaded on SDS PAGE gel and compared to check the induction.

16. **Large scale expression and Protein Purification of HpTlyA mutants and wildtype protein :**

    After checking the small scale expression of recombinant proteins they were now produced in large scale. Transformed *E. coli* Origami B cells were grown in LB media containing ampicillin (50 µg/mL) at 37°C under constant shaking at 190 rpm. It was allowed to reach upto $O.D._{600}$ of 1.0, at which cells were induced with 0.5 mM isopropyl-β-d-thiogalactopyranoside (IPTG) for 3 h at 30°C. Cells were harvested by pelleting them down at 3800 rpm for 30 minutes and re-suspended in 1X PBS (20 mM sodium phosphate, 150 mM NaCl (pH 7.0)). Bacterial protease inhibitor cocktail (Sigma) was then added to cell suspension and it was stored at -80°C if not to be processed at that time.

    After obtaining desired induction large scale protein purification was done.

- Large scale protein purification:

  Composition of buffers:

  Lysis buffer:

- 1X Phosphate buffer saline pH 7.4
- 20 mM Imidazole (pH 8.0)

  Wash Buffer:

  First washing

- 1X Phosphate buffer saline pH 7.4
- 20 mM Imidazole (pH 8.0)

  Second washing:

- 1X Phosphate buffer saline pH 7.4
- 50mM Imidazole (pH 8.0)

  Elution Buffer:

  - 1X PBS
  - 200 mM Imidazole

Protocol:

1. The pellet in lysis buffer (excluding 20 mM imidazole) stored at -80° C was thawed.
2. Lysate was sonicated at amplitude 20 with 30sec ON/10sec OFF pulses for about 15 minutes.
3. Centrifuged at 13,000 rpm 4° C 30 min in table top centrifuge and clear supernatant was collected discarding the obtained pellet.
4. Ni-NTA (Qiagen) column was washed with 1X PBS.
5. 20 mM Imidazole was added to obtain supernatant.
6. Supernatant obtained after centrifugation (in step 3) was loaded onto the Ni-NTA (Qiagen) beads slowly (2 mL in one go).
7. Column was washed with 50 mL 1X PBS.
8. Column was then washed with around 40 mL of two wash buffers each.
9. Bound protein was eluted with elution buffer. Fractions each of 5 mL were obtained.
10. Fractions were checked constantly at each step using Bradford reagent.

11. Fractions were also checked by SDS-PAGE.

Ion Exchange chromatography:

Protein obtained after affinity chromatography contained some undesirable bands of non-specific proteins because of which ion exchange chromatography was performed to get rid of those bands and to get the pure recombinantly expressed protein.

pI of HpTlyA protein = 9.11

Cation exchanger:

Column used- SP Sepharose (Sigma) anionic 6 mL columns.

Buffers used:

Wash buffer- 50 mM Phosphate buffer pH 7.4

Wash buffer- 50 mM Phosphate buffer pH 7.4 + 100 mM NaCl

Elution buffer- 50 mM Phosphate buffer pH 7.4 + 400 mM NaCl

Structural and functional characterization of TlyA mutants

17. Hemagglutination assay:

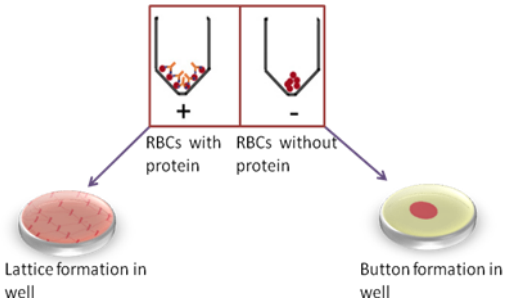


Figure 13: Schematic representation of hemagglutination assay

Characterization of WT HpTlyA has revealed that the primary activity of this protein is hemagglutination i.e. one protein molecule binds to several erythrocytes clumping them together. This assay is a quick indicator of activity of protein (Lata et al., 2014 under review).

Two-fold serial dilutions of protein (WT TlyA and TlyA mutants) were prepared, mixed with a specific amount of human erythrocytes, and added to the wells of V-bottom 96 well plate.

OBSERVATION:

- Erythrocytes not bound by protein: Sink to the bottom of the well and form a button shaped clump.
- Erythrocytes attached to protein: Form a lattice that coats the well.

18. Circular dichroism to figure out if there are any changes in secondary structure content in TlyA mutants compared to WT TlyA

To study the regular secondary structural features of TlyA mutant proteins and compare them with the secondary structure content of WT, CD spectra of all mutants along with WT were collected in far-UV range (190nm-260nm).

Secondary structure conformation changes induced by single point mutations in WT TlyA were monitored by means of far-UV CD spectrum of WT TlyA and its mutant proteins in 10 mM Phosphate buffer (pH 7.4). The spectra were acquired with a Chirascan spectropolarimeter (Applied Photophysics, Leatherhead, UK) equipped with a Peltier-based temperature controller, using 5mm pathlength quartz cuvette. Far-UV CD spectrum of the protein was corrected for the baseline by subtracting the buffer spectrum. Ellipticities are reported as milli degrees. Data points were collected with a band width of 1 nm. Each spectrum was an average of three consecutive scans. Buffer (10mM Phosphate buffer) scans were accumulated under the same conditions and subtracted from the protein spectra before further analysis. Estimation of secondary structure content was done using CDNN software.

19. Preparation of Asolectin cholesterol liposomes

For preparation of Asolectin-cholesterol liposomes, appropriate amounts of lipids were first dissolved in chloroform (sigma) in a round-bottomed flask. In this study cholesterol (Sigma Aldrich) and asolectin (Sigma Aldrich) were used in ratio 1:1. Immediately chloroform was stirred rapidly so that it forms a thin layered film in flask. The solvent was evaporated at room temperature and the lipid film was dried under vacuum for 3.5 h, followed by resuspension in 1X PBS for 4 h at 37 °C with constant stirring at 200rpm.

20. Steady state fluorescence spectroscopy

(a) To study environment of incorporated tryptophan residues

(b) To study the effect of liposome binding on intrinsic fluorescence in different TlyA mutants

(c) To study the change in environment of tryptophan on amyloid formation

Intrinsic tryptophan fluorescence spectra of TlyA mutants were monitored using Fluoromax-4 (Horiba Scientific, Edison, NJ, USA) spectrofluorimeter equipped with a Peltier-based temperature controller, using a 1-cm cuvette. Tryptophan fluorescence was monitored upon excitation at 290 nm, with slit widths of 1 and 5 nm for excitation and emission, respectively. The emission spectra were background subtracted. In liposome binding experiments liposome used was asolectin-cholesterol liposome prepared in 1:1 ratio. To study the change in intrinsic fluorescence in amyloid state, protein was heated upto 50°C at which it is known that WT TlyA protein forms amyloids and was then allowed to cool down till 25°C. Spectra were then collected at 25°C.

The concentrations of proteins used were in the range of 3-4 μM for all the studies.

21. Asolectin-cholesterol liposome-protein pull down assay to study the binding efficiency of TlyA mutant proteins as compared to WT TlyA protein qualitatively

In liposome-protein pull down assay 5 μM protein was incubated at room temperature with asolectin-cholesterol liposome (1mg/mL) for 1 hour. Then boiled and unboiled samples of incubated protein-liposome samples were run on SDS-PAGE in denaturing conditions (with β-mercaptoethanol). As a control, boiled and unboiled protein without liposome in same concentration was also run on SDS-PAGE.

22. Asolectin-cholesterol liposome-protein binding study quantitatively by ELISA based assay:

1. Liposome stock of concentration (1mg/mL) was prepared.
2. Working concentration of liposome used was (10 μg/mL) (9.9 mL PBS + 100 μl liposome).
3. ELISA plate was coated with 100 μl of diluted liposome and was incubated at 4°C overnight.

4. Next day coated liposome was discarded from the plate followed by washing 3 times with 1X PBS with 0.05% tween20 (PBST), 200µl each well.

5. Plate was blocked with 3% milk in PBS, 200µl each well for 1 hr.

6. Protein dilutions were prepared meanwhile, in serial dilution for triplicate set. Dilution was prepared for 100µl in each well.

7. The plate was washed 3 times with PBST 200 µl each well.

8. 100 µl protein dilution was added in each well and was incubated for 2 hrs at room temperature.

9. Washing was done thrice with 200 µl PBST.

10. Primary antibody dilution (1:5000) was prepared in 1X PBS, 100 µl in each well and incubated at RT.

11. Washing was done thrice with 200 µl PBST.

12. The secondary antibody dilution was prepared in 1X PBS (1:5000), incubated for 1 hr at RT.

13. Washing was done thrice with 200 µl PBST.

14. The substrate (10 mL of sodium citrate buffer + 10 mg of OPD+ 20 µl of H_2O_2, pH 4.5) was prepared and added 100 µl in each well.

15. Immediately 100 µl of 2N H_2SO_4 is added to terminate the reaction.

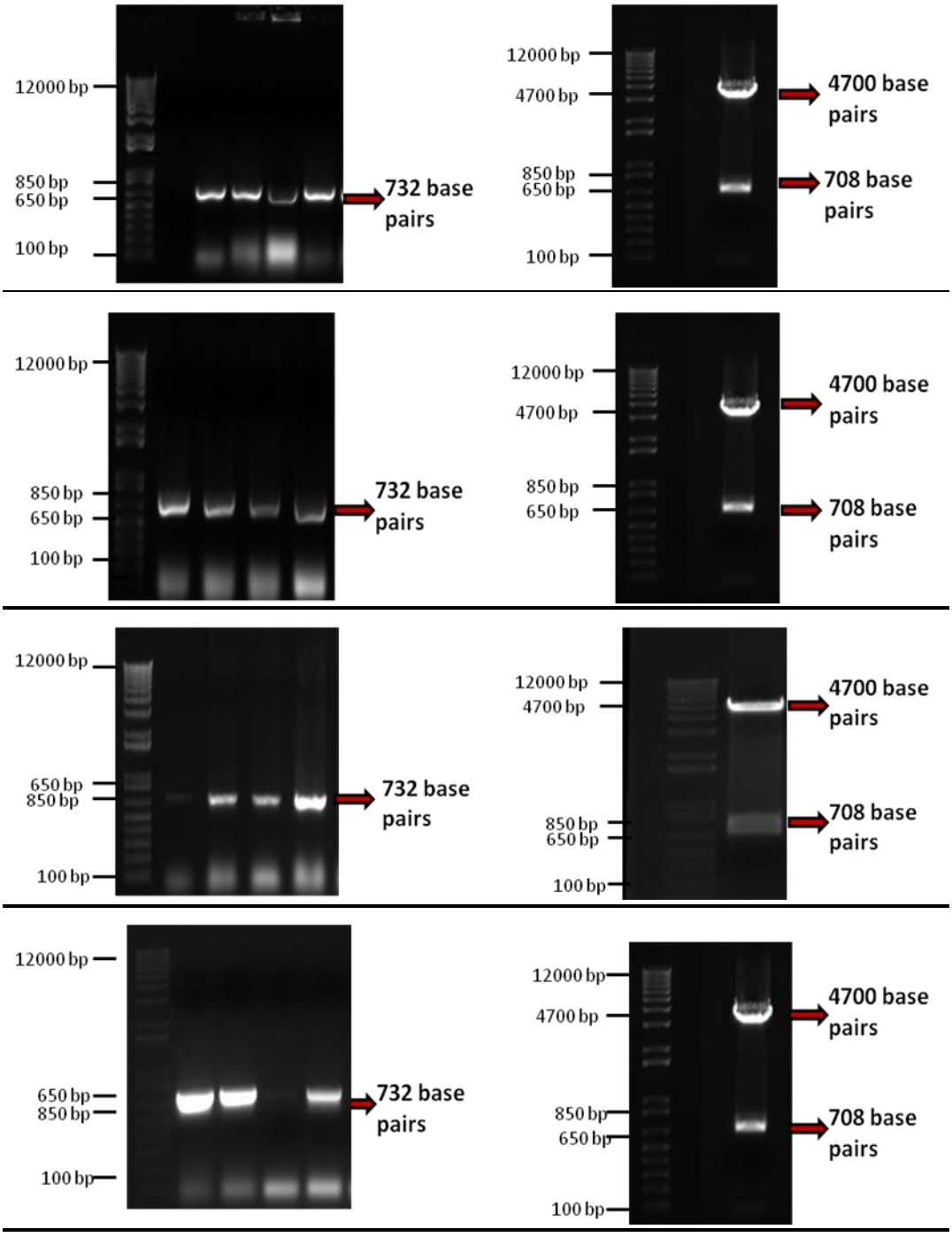
16. Absorbance of the plate was taken at 490 nm using multiwell spectrometer.

23. Protein induced liposome aggregation monitoring by measuring the changes in turbidity of liposome dispersions at 500 nm

Asolectin-cholesterol liposomes (molar ratio of 1:1) were added to a cuvette in such a concentration that optical density at 500 nm becomes 0.25. Immediately after the addition of protein (WT TlyA and TlyA mutant proteins), protein-induced aggregation of liposomes was monitored spectrophotometrically at 500nm by measuring the increase in optical density of the liposome dispersion over a period of 5-6 min at room temperature.

RESULTS AND DISCUSSION

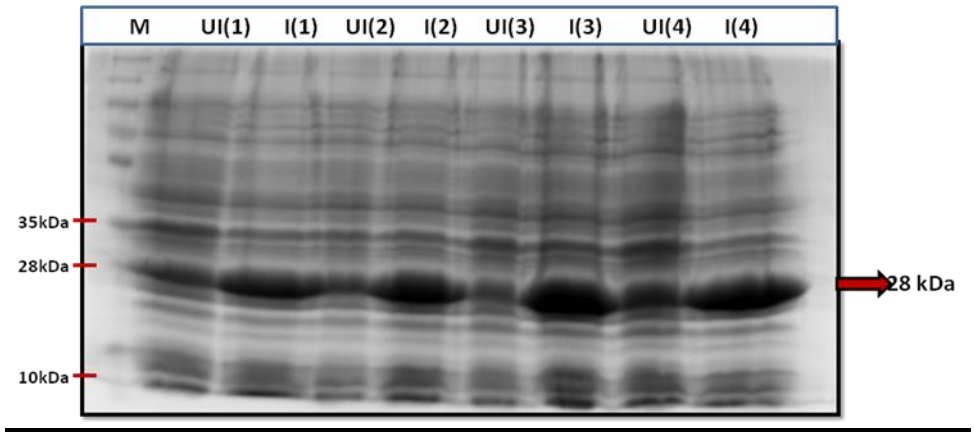
1. Site-directed mutagenesis of Hp*tlyA*



Using site-directed mutagenesis a number of single tryptophan variants of HpTlyA were created. They were verified by colony PCR and restriction digestion. After colony PCR the amplified product was visualized on agarose gel was of 732 base pairs. After digestion of cloned plasmid a fragment of 708 base pairs was released. All the recombinant constructs were verified by DNA sequencing.

Protein purification and expression:

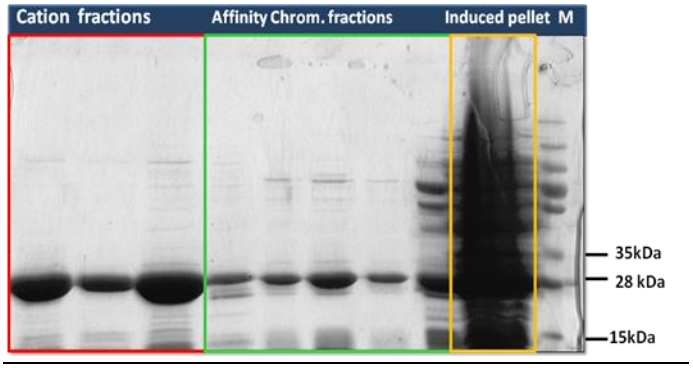
2. Overexpression of WT TlyA and TlyA mutants

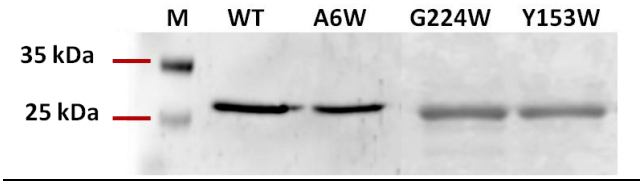


1: pET14b-WT TlyA 2: pET14b-G224W TlyA
3: pET14b-Y153W TlyA 4: pET14b-A6W TlyA
UI: Uninduced Sample I: Induced Sample

In this study, *H. pylori* TlyA wild type protein and TlyA mutants with N-terminal hexahistidine-tag transformed in *E. coli* Origami cells were recombinantly over-expressed on IPTG induction. F148W TlyA was not overexpressed in *E. coli*.

3. Purification of WT TlyA and TlyA mutant proteins





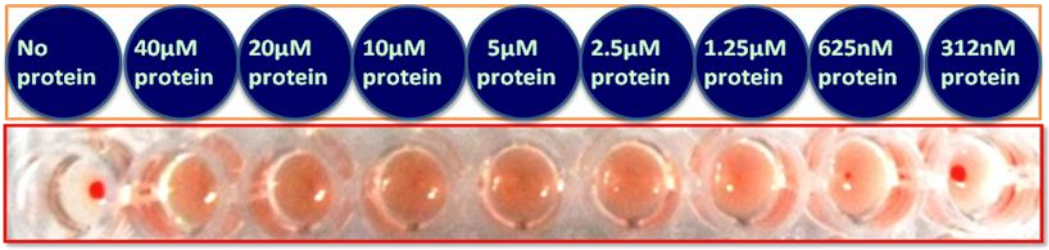
6X His-tagged TlyA WT and mutant proteins were expressed in the cytosolic fraction of the *E. coli* cells were first subjected to Ni–NTA agarose affinity chromatography for first round of purification, and as some non-specific proteins

were present they were subsequently purified via SP-Sepharose cation-exchange chromatography. Cation–exchange chromatography was effective here as the pI of the protein was 9.1 and purification was done at the pH of 7.0.

Recombinantly produced TlyA WT and mutant TlyA proteins were visualized on SDS-PAGE/Coomassie staining under reducing condition. All of them displayed a molecular mass of ~28kDa.

Structural and Functional characterization of Tryptophan incorporated TlyA mutants

4. Hemagglutination activity

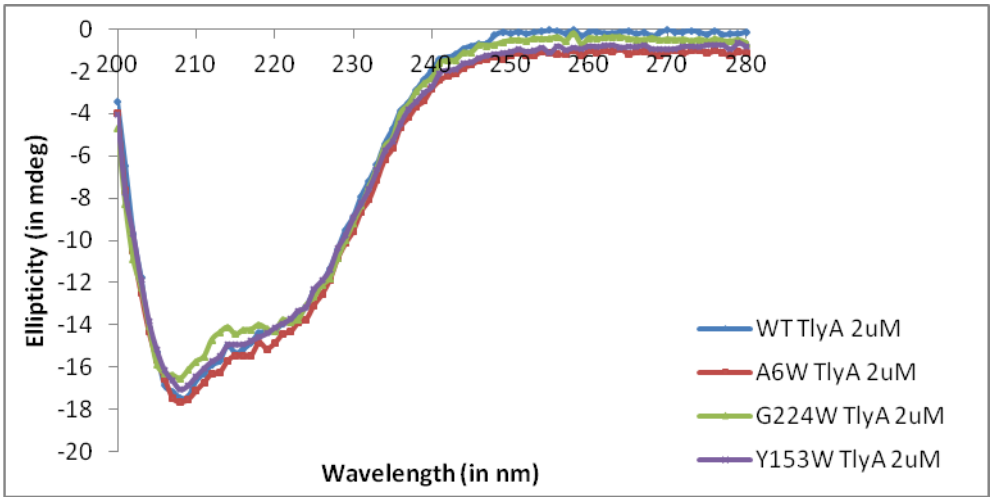


It has been found in previous studies that the primary activity of WT TlyA is hemolysis (Lata et al. 2014) and hemagglutination (Lata et al. Unpublished data). To test the effects of the mutations on the functional activities of TlyA, in this work hemagglutination activity was studied for purified TlyA mutant proteins, and such activities were also compared with that of WT TlyA protein. It was found that the minimum concentration required for the hemagglutinating activity of WT protein was ~1µM.

G224W TlyA exhibits haemagglutination activity comparable to. A6W TlyA appears to exhibit slightly less activity. Y153W TlyA appears to exhibit almost no agglutinating activity. This suggests that mutation in putative transmembrane region critically compromised hemagglutination activity of protein.

5. Far-UV CD spectra of WT HpTlyA and HpTlyA mutants

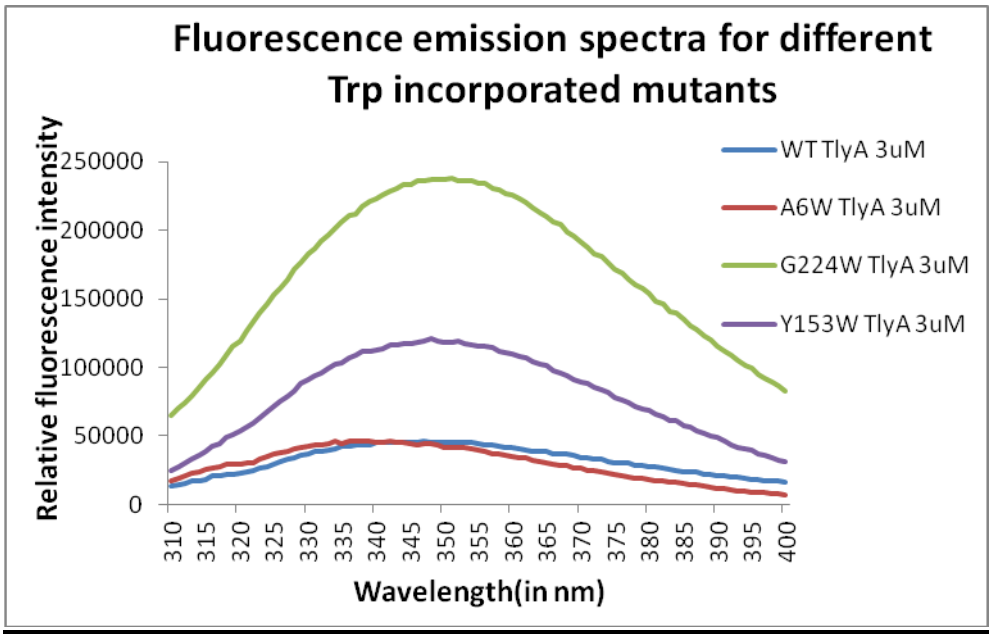
To study if the point mutations have induced any major secondary structure deviations from that of the WT protein, far-UV CD spectra of TlyA mutants and WT TlyA were monitored. CDNN analysis of the far-UV CD profile estimated the presence of 17% helix, and 34% β-secondary structures in the protein and about 47% of random coils. There was no significant difference in secondary structure contents of TlyA mutants as compared to WT TlyA.



6. Steady state fluorescence spectra of WT HpTlyA and HpTlyA mutants

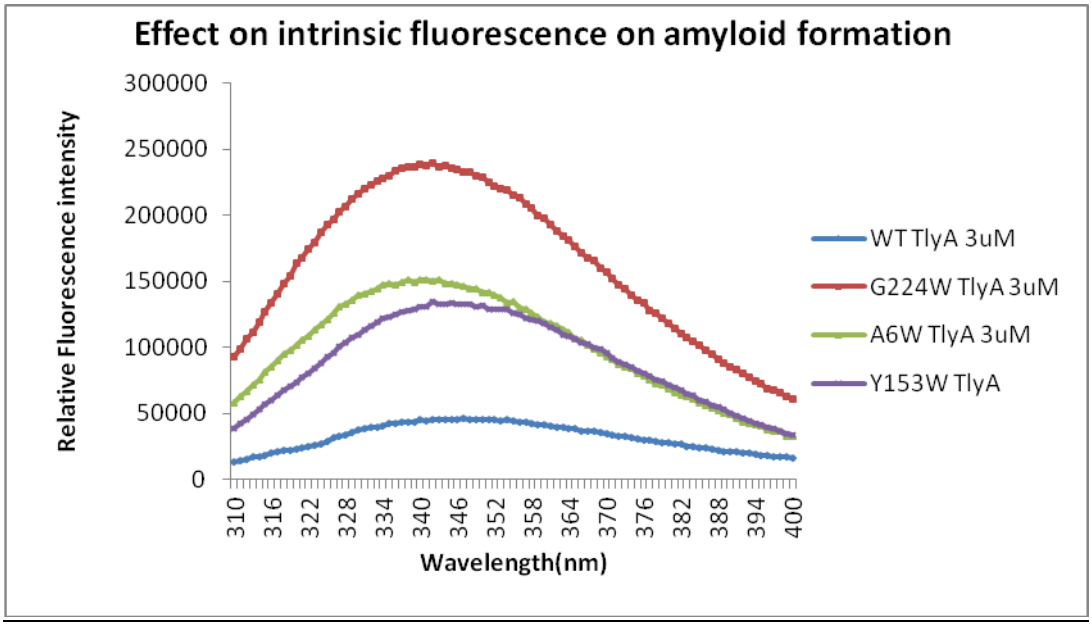
WT TlyA does not contain any Trp residue. So, it is not possible to study the structural characteristic of the protein by intrinsic tryptophan fluorescence spectroscopy. However, the recombinant mutants of TlyA generated in the present study contain Trp residues at distinct sites across its primary structure. Intrinsic tryptophan fluorescence spectra of these mutants would therefore provide valuable insights regarding the environments of the introduced Trp residues, and hence the local structural features. The spectra were collected on exciting at 290 nm and collecting the emission in the range of 310 nm-400 nm.

It was found out that the maxima for A6W TlyA was obtained at 337 nm which indicates that the tryptophan introduced in this mutant is completely buried in hydrophobic environment. For G224W TlyA peak maxima was obtained at 351 nm which shows that the tryptophan residue incorporated in this mutant is completely solvent exposed. The peak maxima for Y153W TlyA was obtained at 348 nm which means tryptophan incorporated in this mutant is partially buried.



7. Effect of amyloid formation on intrinsic fluorescence

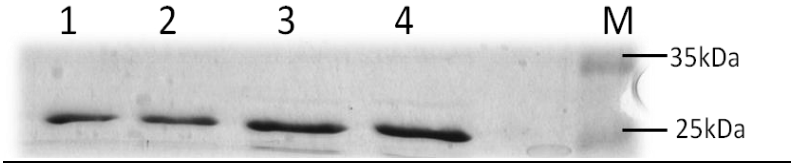
As revealed by the molecular characterization of WT TlyA, TlyA protein can exist in multiple activity states. This protein has a tendency to form amyloids also (Lata et al. unpublished data). The functional implication of this amyloid formation is still not well understood. In this study we monitored the effect of amyloid formation on intrinsic tryptophan fluorescence. Interestingly, it was observed that in case of G224W TlyA in which tryptophan has been incorporated at the C-terminus, peak maxima was observed at 342 nm. This means that there is a blue shift of about 10 nm indicating that this tryptophan which was completely solvent exposed in native conformation gets completely buried on amyloid formation. A6W TlyA which was buried in hydrophobic core of protein undergoes no significant difference in terms of environment of incorporated tryptophan. Y153W TlyA also undergoes a blue shift of about 6 nm and becomes buried. These fluorescence measurements suggest that these mutants can be used as probes to understand the process of amyloid formation in TlyA protein.



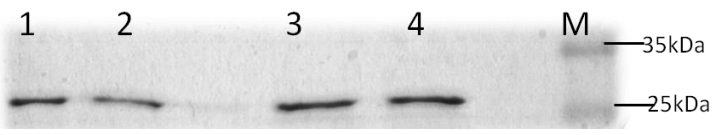
Protein-liposome binding studies

8. Liposome pull-down assay to study the binding of WT TlyA and TlyA mutants with Asolectin-cholesterol liposomes

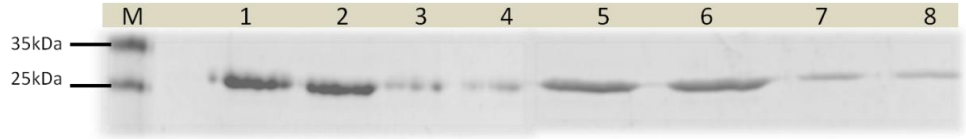
In the liposome pull-down assays it was observed that the binding ability of G224W TlyA toward Asolectin-cholesterol liposome is comparable with that of WT TlyA. In case of A6W TlyA it was found to be slightly reduced but in case of Y153W TlyA the binding efficacy was reduced significantly as compared to WT TlyA.



M: Marker; **1:** WT TlyA protein-liposome unboiled; **2:** WT TlyA protein-liposome boiled; **3:** WT TlyA protein unboiled; **4:** WT TlyA protein boiled



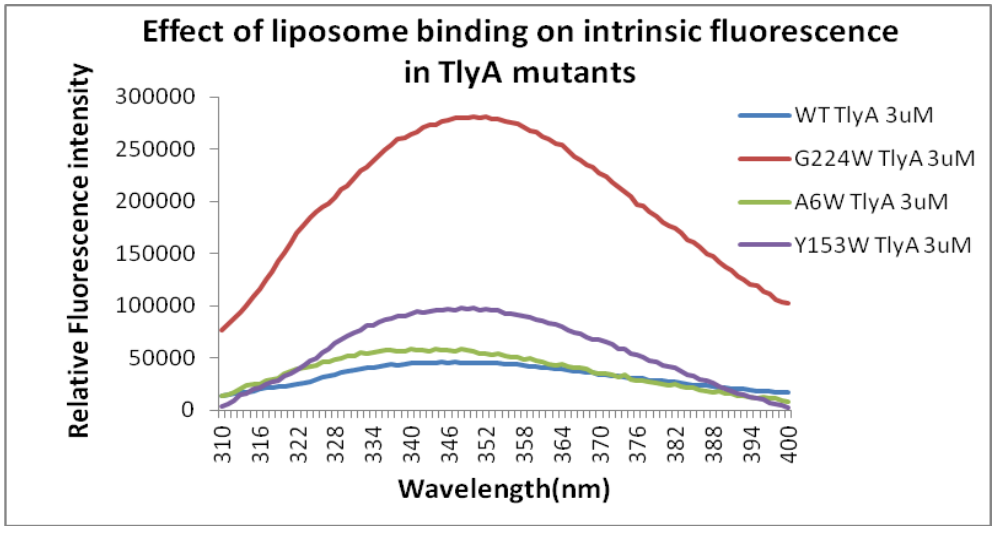
M: Marker; **1:** G224W TlyA protein-liposome unboiled ; **2:**G224W TlyA protein liposome boiled ; **3:** G224W TlyA protein unboiled ; **4:** G224W TlyA protein boiled



M: Marker; **1:** A6W TlyA protein unboiled; **2:**A6W TlyA protein boiled; **3:** A6W TlyA liposome unboiled; **4:** A6W TlyA-liposome boiled; **5:** Y153W TlyA protein unboiled **6:** Y153W TlyA protein boiled; **7:** Y153W TlyA-liposome unboiled; **8:** Y153W TlyA-liposome boiled

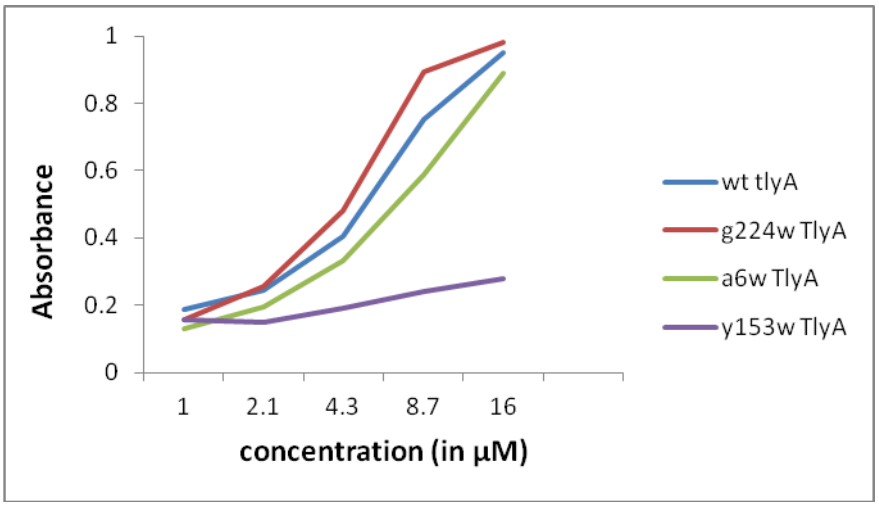
9. Effect of liposome binding on intrinsic fluorescence of Trp-incorporated TlyA mutants

In order to monitor if liposome binding causes any significant change in the environment of incorporated tryptophan, fluorescence spectra were collected for protein incubated with liposome for ~10 minutes. Excitation wavelength was 290 nm and emission was observed in the range of 310 nm - 400 nm. The peak maxima for A6W TlyA, G224W TlyA and Y153W TlyA were 348 nm, 350 nm and 348 nm respectively. This showed binding of TlyA mutants with Asolectin-cholesterol liposome does not cause significant effect on intrinsic fluorescence which indicated the environment of tryptophan is not altered.



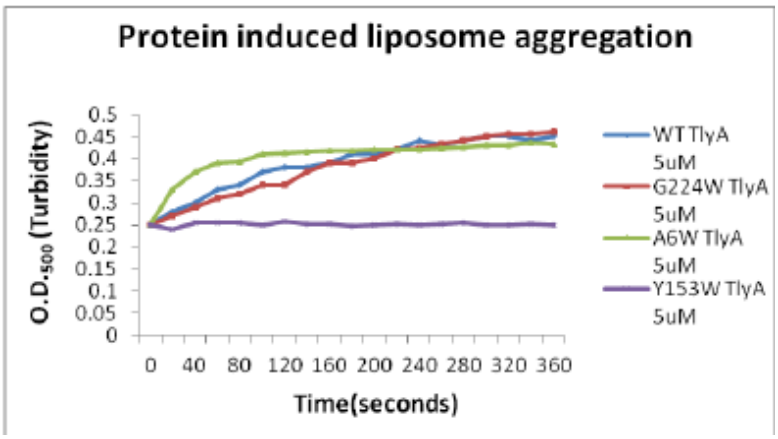
10. Quantitative analysis of Asolectin-cholesterol liposome-protein binding of WT TlyA and TlyA mutants by performing ELISA-based binding assay

Binding of WT TlyA and TlyA mutants was studied by performing ELISA based binding assay. It displayed quantitatively that asolectin-cholesterol liposome binding was significantly reduced in Y153W TlyA mutant whereas in A6W TlyA and G224W TlyA binding tendency was almost comparable with WT TlyA with some subtle differences in the minimum inhibitory concentration.



11. Protein induced liposome aggregation monitoring by measuring the changes in turbidity of liposome dispersions at 500 nm

Liposome aggregation and fusion induced by WT TlyA and TlyA mutants was studied by measuring the changes in turbidity of liposome dispersions at 500 nm. It was observed that in case of WT TlyA, G224W TlyA and A6W TlyA turbidity increased with time and became constant after a timepoint indicating saturation of the system. Whereas in Y153W TlyA turbidity was constant throughout as there was increase in $O.D._{500}$ on addition of protein which suggests that asolectin-cholesterol liposome aggregation tendency was significantly reduced in Y153W TlyA mutant.



CONCLUSION AND FUTURE DIRECTIONS:

In this study, site-directed mutagenesis performed at various sites in the WT *tlyA* sequence proved to be very helpful to initiate mechanistic studies of this novel bacterial toxin as it provided a superficial idea about the dynamics and the functional motifs of this protein which laid down a platform for many future studies which will be in similar direction.

Incorporation of tryptophan in an otherwise tryptophan-less protein enabled us to monitor the dynamics of this protein in various activity states such as in amyloid conformation and liposome bound state. The structural and functional properties of G224W TlyA were unperturbed as compared to WT TlyA and displayed a blue shift of ~10 nm on amyloid formation from peak maxima obtained in fluorescence read out of native structure. This mutant can be used as a probe to study amyloid formation in TlyA.

It was found that Y153W TlyA lost its activity completely but there was no significant change in the secondary structure when compared with WT TlyA. F148W TlyA was not overexpressed probably because the protein synthesis was critically disrupted. These two observations are interesting as both these residues lie in the bioinformatically derived putative transmembrane region. These results indicate when residues in putative transmembrane region were mutated they critically compromised the activity of TlyA protein and posed problems in its expression. These results suggest that perhaps residues in the putative transmembrane region are critical for maintaining function of the protein. This has provided a hunch which motivated us to conduct a study focusing on this region specifically so that the crucial region of HpTlyA protein for cell-membrane binding ability is studied providing information at residue level. In this direction we are performing alanine scanning mutagenesis specifically in this region in order to delineate the importance of residues in maintaining function and stability of this protein.

With this work we intend to get some valuable information about the membrane-interaction mechanism of HpTlyA protein.

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