Function of hpTlyA as 2'-O-methyltransfrase of ribosomal RNA

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Certificate of Examination

This is to certify that the dissertation titled **"Function of hpTlyA as 2'-O-methyltransferase of ribosomal RNA"** submitted by **Mr. Anoop Kumar** (Reg. No. MS10071) 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.

Dr. Arunika Mukhopadhaya Dr.Samarjit Bhattacharayya Dr.KausikChattophadhyay

(Supervisor)

Dated: April 24, 2015

Declaration

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

Anoop Kumar
MS10071
Dated : April 24,2015

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 Chattopadyay
(Supervisor)

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Notations

PFTs	Pore-forming toxins
hpTlyA	*Helicobacter pylori* associated TlyA-like protein
SDS-PAGE	Sodium dodecyl sulphate Polyacrylamide Gel Electrophoresis
rRNA	Ribosomal RNA
Cap	Capreomycin
CD	Circular Dichroism
PCR	Polymerase Chain Reactions

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Abstract

Pore-forming toxins are the most common virulence factors of bacterial system to invade the host immune system. The gram negative, *Helicobater pylori* is also one such bacterial system that harbours different classes of pore-forming toxins. hpTlyA is one of the recently identified pore-forming toxin of *Helicobater pylori* toxins which has been found to be playing role in hemolytic as well as cytotoxic activity. This toxin is also suspected to do methyltransferase activity. In our lab we try to address this functionality of hpTlyA and observed that mutation at K-D-K-E tetrad of this toxin can perturb the methyltransferase activity which can be monitored in the presence of capreomycin. Recombinant *E.coli*. cells expressing all different constructs of hpTlyA show normal growth of cells in the presence of methylated ribosomal targeting drug, capreomycin, which is the opposite response of methylated ribosome containing cells, indicating that variants of hpTlyA unable to properly methylate the ribosomal RNA and lead to normal growth of the cell. Thus, we can say hpTlyA can have methyltransferase activity.

Chapter-1
INTRODUCTION

1.0 Pore-forming toxins

Most of the diseases in human and animal species are due to infection caused by bacteria and virus species. Lack of the knowledge of exact mechanisms of bacterial and virus infection lead to deaths of millions of people every year worldwide. For any infection in the host, bacterial and virus species require some virulence factors. The very first way to invade the host system is to mediate-proper adhesion followed by subsequent weakening of their immune system. The mode of infection of bacteria and virus species involve some molecules that are secreted either in the extracellular or intracellular environment of host cells and lead to the perturbation of host immune system (Gonzalez et.al., 2008, Ichovahe et.al., 2008). In case of bacteria, it is found that bacterial species secrete water soluble pore-forming toxins in the extracellular environment in their inactive form and assemble in the lipid bilayer of host cells to form active form of toxin. Activated form of toxin either directly interact with host intracellular environment or help the extracellular contents to pass through lipid bilayer which trigger the imbalance in the concentration of ions across the lipid bilayers that lead to death of host cell (Rai and chattopadhyay, 2013). Evolution of such toxins in many species indicates their functional role to remain alive in the harsh environments.

Bacterial species like *Clostriudium septicum, Streptococcus aureus, Streptococcus pnemoniae, Clostridium perfrigens Bacillus thurgiensis,* and *Vibrio Cholerae* are the typical pore-forming toxins producing organisms. Pore-forming toxins (PFTs) produced by these bacteria can be classified based on their pore sizes, receptors and their mode of actions (Gonzalez et.al., 2008). Basically pore-forming toxins are classified in two broad categories as β-PFTs and α-PFT. β-PFTs are named so because of having high percentage of β-structure. β-PFTs vary greatly in their size from 2 nm-50 nm in diameter. β-PFTs are generally secreted as water soluble monomers and at high concentration they multimerize and form circular structure. One or more β-hairpin structures form amphipathic β-barrel exhibits hydrophilic cavity and hydrophobic outer surface and allow insertion in lipid bilayer of host cells and make irregular pores (Gonzalez

et.al.,2008, Los et.al 2013, Iocovache et.al., 2008). Further, β-PFTs are sub-classified as Cholesterol dependent PFTs (CDC), Binary PFTs and Small PFTs. CDCs category of β-PFTs include streptolysin, listeriolysin, pneumolysin and perfringolysin. Binary PFTs include anthrax toxin. α-toxin of *Streptococcus aureus* and *V*ibrio cholerae cytolysin of *Vibrio cholerae* come in small PFT category. Another class of PFTs is α-PFT which consists of α-helices predominantly. Schematic diagram of classification of bacterial pore-forming toxin can be given as follow

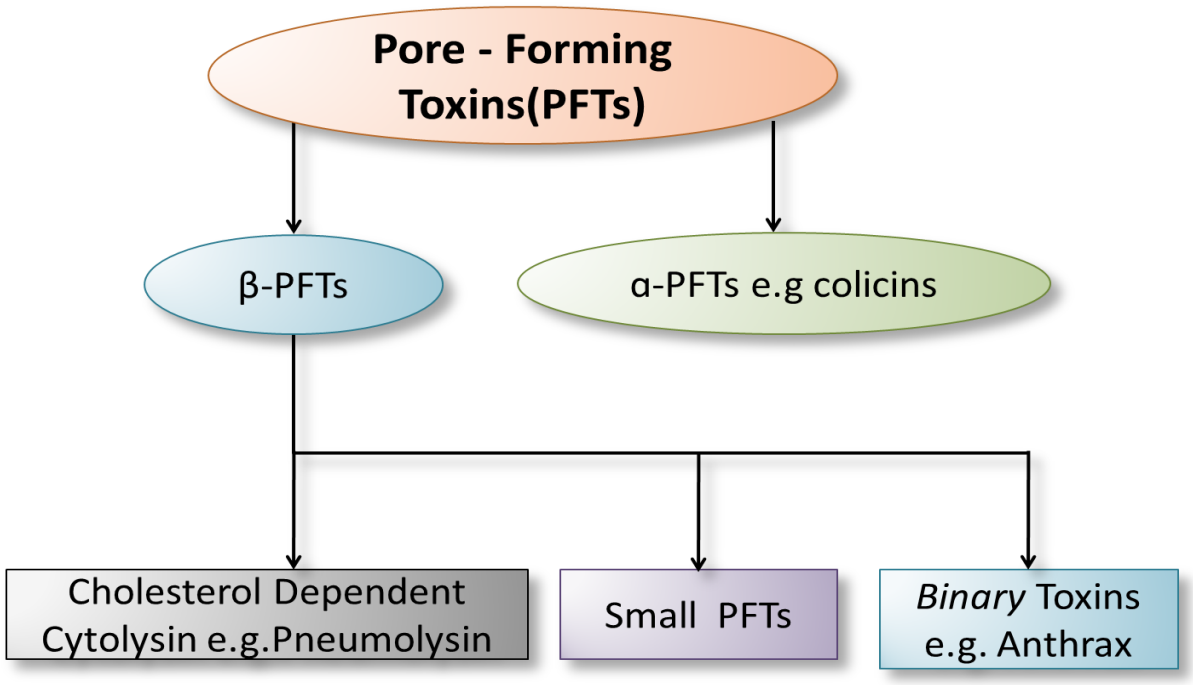


Figure 1.0 General classification of pore-forming toxins

1.1General mode of action of PFTs

Most of the β-PFTs are dimorphic in nature. These toxins are secreted as water-soluble monomeric form and then assemble in circular form to create pore in lipid bilayers of cells, for example *Vibrio cholerae* cytolysin and α-hemolysin. The monomeric units of these toxins multimerize into mushroom like structure whose stalk insert into the lipid bilayer of cell and render the cell permeable which lead to osmosis and bursting of the cell (Gonzalez et.al.,2008, Rai and Chattpadhyay , 2013).

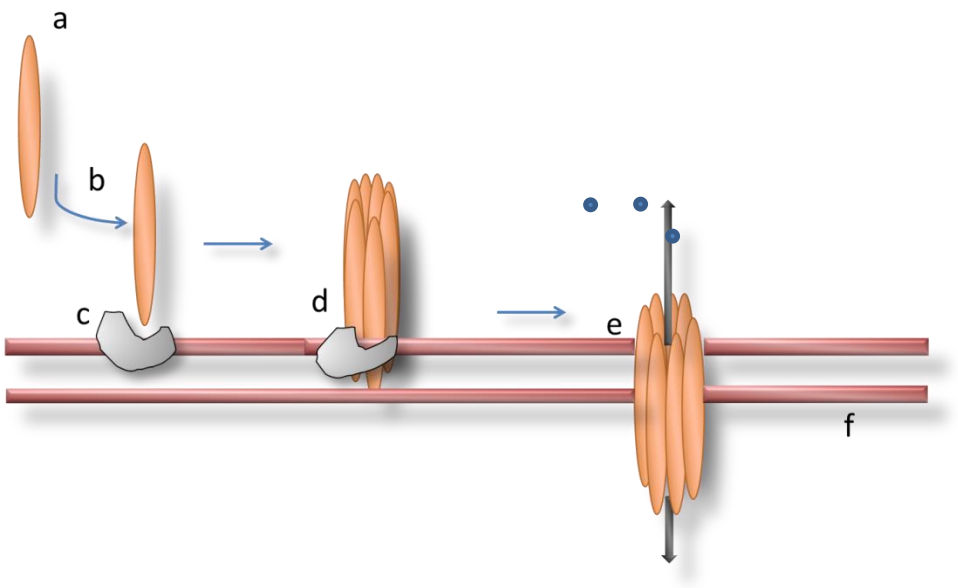


Figure 1.1The generalized mode of action of β-PFTs – (a) Water soluble monomeric state of PFT (b) Interaction of monomeric unit to their receptor present at the surface of lipid bilayer of host cell (c) Receptor present on the bilayer (d) Multi-merization of monomeric units to form circular pore like structure (e) Functional pore formation show leakage of contents, (f) the lipid bilayer

Some of the PFTs consist of two component 'A' and 'B'. Therefore they are termed as AB type/Binary toxins. During their mode of action, 'B' component facilitate the entrance of the enzymatic payload into the cell and by forming pore, and then 'A' component enter the cell and lead to the alteration of normal activities of cell (Gonzalez et.al.,2008, Geny and popoff et.al., 2006).

1.2 Role of bacterial PFTs

It is found that bacteria invest large amount of their energy to form PFTs which can constitute to 10-15 % of the dry mass of bacteria at sporulation. This indicates that these PFTs indeed have some functional role within bacteria. Some of the following functions can be exhibited by PFTs

- Bacteria can utilized PFTs for invading the host cell by evading the immune system by preventing phagocytosis

- Bacteria can utilize PFTS for by making structural and functional changes in some proteins during translation and transcription stages to resist the antimicrobial drug.
- Bacteria can utilize PFTs to fulfil the requirement of food resources by killing host cell
- Some bacteria grow in the anaerobic condition that can be easily attained if the bacteria get the entry in the mammalian host cells because immune system of mammals create the anaerobic condition for metabolism.

1.3 *Helicobacter pylori (H. pylori)* and its toxins

For many years, peptic ulcer and stomach related diseases were thought to be related with stress and hyperacid production, but discovery of *Helicobacter pylori* in 2005 by Barry Marshall and Robin Warren found the real cause. For this discovery they were honoured with Nobel Prize (Tomb et.al., 1997).

H. pylori is a gram negative microaerophilic bacterium which has spiral morphology and flagellated structure for mobility. Initially *H. Pylori* was known as *Campylobacter pylori* but due to its distinct features it was classified in a new genus and named as *Helicobacter pylori* (Kusters et.al.2006). Unique feature of the bacteria is that it can reside in low pH of stomach and colonize on epithelial cells successfully. Recently people have found that *H. pylori* is a hemolytic bacteria which can lyse human erythrocytes (Marntino et.al., 2001, Boren et.al., 2001).

Association of the bacteria in various stomach related diseases like gastric adenocarcinoma and peptic ulcer attracted people to understand the pathogenesis of this bacteria. *H. pylori* strain 26695 genome consists of circular chromosome having 1,666,867 bp in size which includes various cytotoxic pathogenicity islands (Tomb et.al., 1997). Various kinds of toxins and proteins are secreted by the bacteria to properly adhere with epithelial cells. Urease secreted by the pathogen is a potent multi-functional protein which can neutralize the acidic environment of stomach and help the bacteria to colonize in host epithelial cells (Tomb et.al., 1997). As the bacteria colonize, various kind of cytotoxic factors are produced by the bacteria which lead to acute to chronic infections. Most predominant cytotoxic factors of *H. Pylori* are cytotoxin associated protein (CagA) and vacuolating cytotoxin (VacA). VacA helps in the formation of acidic vacuoles in host cells and cause ulcer related diseases (Tomb et.al., 1997). People also

found that *H. pylori* utilize various kinds of adhesion molecules to adhere with epithelial cells. Most commonly known adhesins of *H. pylori* are proteins of Hop Family (outer membrane protein) which include AlpA, AlpB (help in adherence with gastric epithelial cells) and BabA which mediates attachment to blood group antigen Lewis^{b} (Martino et.al., 2001, Zhang et al., 1993).

1.4 TlyA and TlyA-like proteins

H. pylori also contain a gene which has sequence similarity with TlyA protein of many bacteria like *Mycobacterium tuberculosis* and *Serpulina hyodyseneiriae*, *Thermus. thermophiles* and *Mycobacterium smegmatis*. HP1086 gene of *H. pylori* strain 26695 shows sequence similarity with TlyA encoding gene of all the above mentioned bacteria. HP1086 gene consists of 708 bp which encode for a hemolysin of 235 amino acid residues, known as hpTlyA (Tomb et.al 1997). Based on the length of N and C termini of sequences of TlyA or TlyA- like proteins, people have classified them in two groups. Group I TlyA known as "TlyA^{I}" and group II TlyA known as "TlyA^{II}". Group I TlyA proteins are short at their 'N' terminal while group II TlyA protein have at least four additional amino acid residues and their 'C' termini also extended up to fifty amino acid residues. In group II TlyA, extension of 'N' termini is thought to be as a functional motif for interaction with SAM (S-adenosylmetheonine) (Monshupanee et.al.,2012)

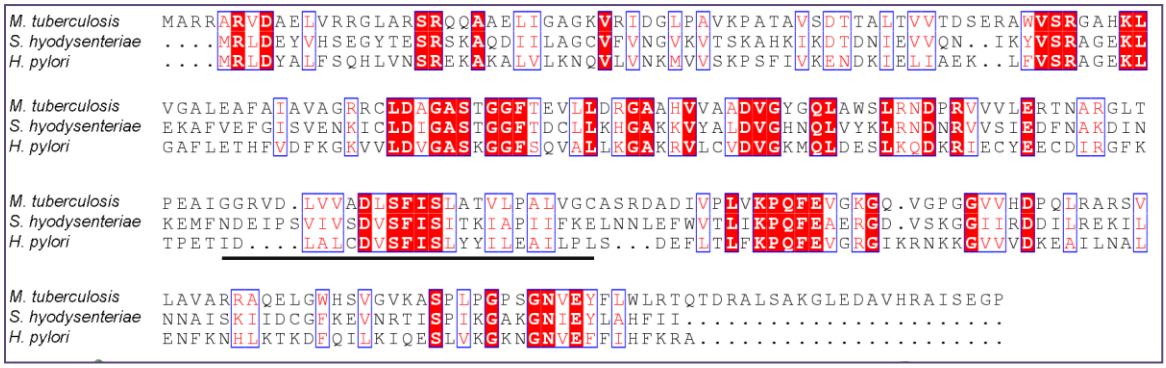


Figure 1.2 Sequence alignment results of TlyA protein of *Mycobacterium tuberculosis* and *Serpulina hyodyseneiriae*

Sequence alignment of hpTlyA with other two bacterial TlyA showed 35% sequence homology. Based on the sequence alignment we can say that hpTlyA belongs to the group I category of TlyA proteins ($TlyA^{I}$) and also the hpTlyA has short 'N' and 'C' termini.

It has been found that in the absence of hpTlyA, the adherence property of *H. pylori* reduces as compared to the wild type strain (Zhang at.al, 2002). Reduced adherence of mutant strain indicates that hpTlyA has role in adherence of the bacteria. It is reported that hpTlyA can exhibit hemolytic activity and show cytotoxic effect on human specific gastric cancer cells (AGS cells) as studied by MTT assay (Lata et.al., 2014). Since this toxin is recently reported, therefore very little is known about its functions and mechanisms of action.

TlyA proteins of *M. tuberculosis* and *S. hyodyseneiriae* are also reported as methyltransferase of ribosomal RNA (Rehman et.al.2010). Methylation of rRNA by enzyme present in bacteria is necessary for the joining of two ribosomal subunits for protein translation.

It is also reported that expression of tlyA recombinant *E.coli* increases in susceptibility to the capreomycin (Monshupanee et.al.,2012). Capreomcin is antimicrobial drug which interact with methylated ribosome in bacteria. It is not clearly known that how it target the ribosome but it is reported that capreomycin binding sites lie at the inter bridge B2a between helix 44 of 16S rRNA and helix 69 of 23SrRNA (Monshupanee et. al., 2012). Interaction of capreomycin with methylated ribosome inhibits the growth of bacteria by stabilizing the tRNA in the A site during the pre- translation state that lead to inhibition of protein synthesis (Monshupanee et. al., 2012. People also found out that mutation in TlyA confer the capreomycin resistance in *M. tuberculosis* which indicate that TlyA protein has capability to methylate the rRNA. It is also reported that Group I TlyA protein only methylate 23S rRNA nucleotide at C1920 position in 50S ribosomal unit while group II TlyA protein can target both ribosomal subunit at 16S rRNA of 30S ribosomal unit at C1409 position and 23S rRNA nucleotide at C1920 position in 50S ribosomal unit (Monsupanee et.al.,2012).

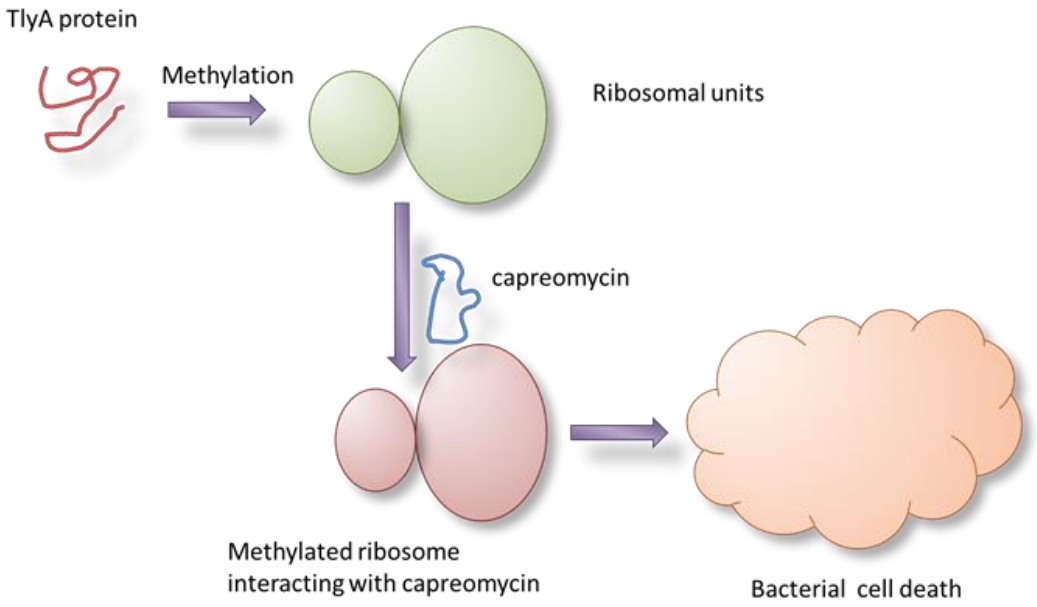


Figure 1.3 Schematic diagram of methylation of ribosomal RNA in ribosomal units and subsequently capreomycin effect on bacterial cell containing TlyA methylated ribosome.

Considering all the fact of TlyA and TlyA like proteins, our present study focused on to address the question whether hpTlyA can act as methyltransferase or not, and if yes, then whether this characteristic is related to its cytotoxic activities. In order to conduct this study, different variants of hpTlyA were generated to monitor the methyltransferase activity. Due to lack of crystal structure availability, mutation sites responsible for methyltrasferase activity were determined only by bio-information based studies of hpTlyA and capreomycin effects on E.coli cell expressing all constructs were analysed. Results of this study not only provide the insight into functional aspects of it but also give a clue to mechanistic way of drug target. Knowledge of exact mechanism of drug target sites can help people to design drug properly.

Chapter -2
Materials and Methods

This chapter includes the information about all materials and methods that were utilized during the dissertation work. Material section includes information about all those chemical regents that were used to prove the methyltransferase activity of protein and to get the desired protein starting from cloning. Description of protocol and procedure followed during all the experiments is given in the methods section

2.1 Molecular Biology methods of clone generation

2.1.1 Generation of hpTlyA variants

Recombinant hpTlyA variants (K63A, D145A, K170A, E227A) harbouring point mutations were generated by Polymerase Chain Reaction (PCR) based method. PCR method utilizes a thermostable DNA polymerase enzyme to amplify a gene of interest. The gene containing point mutation was PCR amplified by using gene specific primers. Primers were designed based on wild type sequence of hpTlyA. Forward and reverse primers also include the restriction sites for Nde1 and BamH1.

K63A _TlY _FW	AGGGCTGGGGAAgccTTAGGGGCTTTTTTAGAAACCCATT
K63A _TlY_RC	AAAAGCCCCTAAggcTTCCCCAGCCCTGCTAACGAAAAGT
D145A_ Tly_FW	TTAGCGCTTTGCgccGTGAGCTTTATTTCTTTATATTATA
D145A_ Tly_RC	AATAAAGCTCACggcGCAAAGCGCTAAATCAATTGTTTCT
K170A _Tly _FW	TTAACACTTTTCgccCCGCAATTTGAAGTGGGCAGAGGAA
K170A _Tly _RC	TTCAAATTGCGGggcGAAAAGTGTTAAAAATTCATCGCTT
E227A_ Tly_RC	ACAAGTggatccTTAGGCTCGCTTGAAATGGATAAAAAAggcAACA

Table 1 – Details of primers

A standard PCR was performed in final reaction volume of 30 microliter (µL) containing given composition of reaction mixture in Table 2.

Reaction mixture A	**Reaction mixture B**
PCR mastermix = 25 µL	PCR mastermix = 25 µL

Wt - hpTlyA forward primer = 1.5 µL	Wt - hpTlyA revrse primer = 1.5 µL
K63A – TlyA reverse Primer = 1.5 µL	K63A - TlyA forward primer = 1.5 µL
Wt – hpTlyA gene template = 1 µL	Wt – hpTlyA gene template = 1 µL

Table 2 Reaction mixture composition

PCR of both reaction mixtures was carried out under the following program made in PCR machine.

Temperature	Purpose	Time
95˚C	Heating the lid and DNA	5:00 min
95˚C	Denaturing DNA	0:30 min
56˚C	Annealing	0:30 min
72˚C	Polymerase Binding and extension	1:00 min
Go To 30 X (repeat the above program for 30 cycles)	Multiplication of template	
72˚C	Extension	5:00 min
4˚C	Storing	Depend on the requirement

Table 3- Details PCR protocol

2.1.2 Agarose gel electrophoresis

To analyse the amplified DNA agarose gel electrophoresis was used. Analysis of amplified DNA was done based on molecular weight. DNA fragment of higher molecular weight will move slowly while DNA fragments of lower molecular move faster. To analyse plasmids and PCR products, we mixed 1 unit volume of 6X loading dye and run in 1% agarose gel. We utilized the 1X TAE buffer (40 mM Tris, 1% acetic acid (v/v), 1 mM EDTA, pH 8.0) for agarose gel preparation. Agarose gel was run on 90 V. DNA ladder (1kb size) (Invitrogen) was also loaded in agarose gel to estimate the size of DNA in applied sample. Band sizes of different DNA fragments were visualized by Gel Doc^{TM} EZ Imager (Bio-Rad).

2.1.3 DNA Extraction and Elution

For the extraction the DNA fragment from agarose gel, we utilized the clean sharp scalpel and cut the desired DNA band from agarose gel under UV light and extracted by Qiagen gel extraction kit and eluted in 50 µL autoclaved water. Eluted first round PCR products were utilized in second round PCR. Second round contain the following composition of reaction mixture.

Reaction Mixture
PCR mastermix = 30 µL
Wt-TlyA forward primer = 1.5 µL
Wt-TlyA reverse primer = 1.5 µL
Round - 1 PCR product A = 1 µL
Round - 1 PCR product B = 1 µL

Both rounds of PCR also include the positive and negative control parallel to applied sample in 1 % agarose gel. Second round PCR products were also extracted and eluted by above written method (2.1.3).

2.1.4 Restriction and ligation of desired DNA into pET14b vector

Desired DNA fragments and pET14b vector were digested by restriction enzymes at their restriction sites to efficiently ligate in next step. To digest the desired DNA and pET14B vector appropriate amount of restriction enzymes (Nde1 and Bamh1) was used. For proper digestion to take place, PCR products and undigested pET14b vector were incubated for 3 hrs at 37˚C. Further estimation of digested products is done by running all samples in 1 % agarose gel and all desired DNA fragments were extracted and eluted by gel extraction kit (Qiagen).

To ligate the digested DNA fragment and digested pET14b, T4 DNA ligase was used (New England Biolab). Sample containing ligation mixture (containing digested pET14B vector, digested gene (K63A-TlyA), ligation buffer and T4 DNA ligase) were incubated at 25˚C temperature for 1 hour.

2.1.5 Transformation of chemically competent *E.coli* Top10 Cells

In order to make competent cells, a tip containing a few cells were inoculated in 5 mL autoclaved media and incubated overnight at 37˚C. Next morning 2.5 % of overnight grown culture was inoculated in 10 mL autoclaved LB media. At OD-0.5 culture was cooled on ice for 10 min. and centrifuged at 5000 rpm at 4˚ C for 15 min. Pellet was re-suspended in ice cold 10 mL of 100mM $CaCl_2$ and left on ice for 5min. After 5 min. suspension was further centrifuged at 5000 rpm at 4˚C temperature. Supernatant was thrown and pellet was re-suspended with 5 mL of 50 mM $CaCl_2$ and left for 45 min at ice cold condition. After 45 min. re-suspended were again centrifuged at above given conditions. Supernatant was thrown and pellet was re-suspended in 85% of 100 mM $CaCl_2$ and 15 % glycerol. Finally aliquots of 100 µL volume were prepared and stored at -80˚ C temperature for further use.

After ligation step, ligated products are transformed in competent *Escherichia coli* Top10 (Novagen) cells and spread on LB-Agar ampicillin plates. Only transformants *E.Coli* Top 1o cell will grow on ampicillin containing LB-Agar plates because of ampicillin resistant gene in pET14b vector.

Competent E.coli cells were transformed by adding 10 uL of ligated products in freshly prepared 100 µL aliquot of competent cells and left on ice for 15 min at 42 ˚C. After 15 min cells were subjected to cold shock for 1 min. and inoculated with 1 mL LB media and incubated at 37˚C temperatures for 1 hour. After 1 hour cells were centrifuged at 5000 rpm and suspensions was spread on ampicillin containing LB-Agar plates. To check the successfully transformed cells, different colonies were picked up from plates and their colony PCR. In each screen we utilized pET14b primers (T7 fw and rc). Sometime gene specific were also utilized for screening. Efficiency of successfully transformed colonies is verified in the 1% agarose gel. Small scale cultures were also prepared inoculating rubbed tips (containing colonies which are subjected to colony PCR) in 5 mL LB media and incubated overnight to get the plasmid. Plasmid was isolated by DNA extraction mini-prep kit (Qiagen) and sequencing of plasmid containg desired gene was done.

2.1.6 Checking of the expression of variants of hpTlyA

In order to check the expression of various constructs, competent origami B cells of *E.coli* strain cells were transformed with plasmid of each construct and spread on LB - Agar plate containing ampicillin (50 ug/mL). Single colony from each plate is selected for small scale culture preparation. Small scale culture was prepared by picking up single colony by tip and inoculating the tip containing few cells in 5 mL LB Media. Induction check of half of small scale culture was done at OD_{650} - 0.5 by adding 1mM IPTG to increase the expression of desired protein and other half of culture was remain un-induced. After addition of IPTG, cultures were incubated at 37˚C for 3 Hours. Finally, 1mL induced and un-induced cells from each culture were centrifuged at 5000 rpm for 5 min. Pellet of each culture is boiled at 99˚ C with 40 µL 5X SDS loading dye and run in 12.5% SDS PAGE with all controls (including un-induced samples).

2.1.7 Preparation of large scale culture of recombinant hpTlyA and hpTlyA variant expessing *E.coli* cells .

Large scale cultures for wild type hpTlyA and all mutants were prepared after successful expression check of small scale culture. Seed cultures for all mutants were prepared by inoculating ampicillin (50 µL/mL), glucose (500mg/mL) and Origami B cells containing plasmid of different constructs into autoclaved LB media. Inoculated LB media was incubated for overnight at 37˚C temperature. Very next day large scale cultures were prepared by adding 1 mL ampicillin (50 µg/ml working concentration), and 20ml of seed culture into 1L autoclaved LB media. Inoculated media was incubated at 37˚C temperature under constant shaking condition at 180 rpm till OD reaches to 0.8-0.9 and then shaker temperature is subjected to lowered down to 30˚C for minimum 15 min. After 15 min, 0.5mM IPTG (119mg/L) was added to it so that leaky expression of another proteins inhibit and expression of desired proteins increase. Induced cultures were maintained under constant shaking condition at 30˚C temperatures for 3 hours at 180rpm. Induced Cultures were centrifuged at 4000 rpm at 4˚C temperature for 30 min and then re-suspended in 25mL 1X PBS (20mM sodium phosphate and 150 mM NaCl, pH 7.0). To inhibit the activities of protease, 1X protease inhibitor cocktail was added in suspended cells and suspended cells were stored at -80˚C temperature for purification steps. After induction of large scale cultures of different constructs, stored pellets of each constructs were subjected to purification process. Purification of wild type hpTlyA and all mutants involve following composition of different buffers.

Buffer Name	Compositions	pH	Purpose
IX Phosphate Buffer Saline		7.4	1 Washing of Ni-NTA column, 2 maintaining cells, & maintaining Ni-NTA purified fraction of desired proteins.
Imidazole (20mM,200mM)	For 1M imidazole, add 6.808g solid imidazole in 70mL milliQ water and adjust the pH 7.0-7.2 by adding conc.HCl. Add water to make up the volume	7.0-7.2	1 Equilibrate the Ni-NTA column, 2 washing of non- specific protein (by 20mM prepared in 1X PBS) and eluting desired protein(by 200mM prepared in 1X PBS)
50 mM Sodium Phosphate Buffer	Add 6.8895g of Na_2HPO_4 and 1.55827g of NaH_2PO_4 in 800mL water to make its pH 7.2-7.4.Check the pH and adjust the volume to 1000mL	7.0-7.4	1 Washing the SP-Sepharose column, 2 For removal non-specific protein 100mM NaCl solution of Sodium Phosphate buffer was used. 3 For elution of desired proteins 400 mMNaCl solution of Sodium Phosphate buffer was used

Table 4- Washing and Elution Buffers compositions

2.1.8 Protein purification from *E.coli* origami B cells

2.1.8a Nickel – NTA affinity chromatography

Purification of desired proteins (Wt. hpTlyA, K63A-TlyA, D145A-TlyA, K170A TlyA, and E227A-TlyA) involve following steps:-

1. First of all, stored pellet at -80˚C was thawed.
2. Thawed lysate/pellet was sonicated at amplitude =20, process time =15 min, pulse on time = 30 seconds and pulse off time =10 seconds.
3. Sonicated pellet was centrifuged at 12000 rpm for 30 min at 4˚ C temperature and cleared supernatant was collected in separate falcon tube.
4. In mean time Ni-NTA column was washed with 1X PBS.
5. After washing of column, column was equilibrated with 20mM imidazole.
6. 20 mM of imidazole was also added to collected supernatant according to collected volume before loading to column.
7. Column containing Ni-NTA beads, was loaded with around 2 – 3 mL volume of supernatant in single go.
8. Column was again washed with 30-45 mL 1X PBS.
9. In order to remove bound non-specific proteins column was washed with 20mM imidazole prepared in 1X PBS.
10. To get desired protein, column was eluted with 200 mMimidazole prepared in 1X PBS and collected in around 2.5 mL 50 mM sodium phosphate buffer in 15 mL falcon tube.
11. Collected fractions was checked by taking 5µLfrom each collected fraction and added to 20 µL Bradford regent.

    Note- All purification steps are carried at ice cold condition

2.1.8b Ion Exchange chromatography

Sometime purified proteins from Ni-NTA affinity give unwanted band in SDS-PAGE due to elution of non-specific protein with higher concentration of imidazole. To get rid of this problem it is necessary to process the eluted fractions by ion exchange chromatography. This method relies on the pI of protein and charge on the protein. Therefore column utilize the charged resins which can bind only charged protein. Our protein Ni-NTA fractions were processed by SP-

Sepharose cation exchanger column. The pI of Wt. hpTlyA is 9.11.Purification of protein after loading to column involve following steps.

1. All the eluted fractions from Ni-NTA column were diluted up to 4 fold in 50 mM sodium phosphate buffer.
2. Before loading the diluted fractions column was washed with 15-20 mL 50 mM sodium phosphate buffer.
3. After washing, column was loaded with diluted fractions of protein.
4. Further column was washed with 10 mL of 50 mM sodium phosphate buffer.
5. To remove the non-specific protein from resins column was washed with 100 mMNaCl prepared in sodium phosphate buffer.
6. To get the desired protein, column was loaded with elution buffer (400 mMNaCl prepared in sodium phosphate buffer) and collected in 15 mL falcon tubes.
7. Eluted fractions were checked with Bradford regent.

Note- All purification steps carried out under ice cold conditions

In Similar manner all the mutants were purified.

Purity of all mutants was checked by 12.5 % SDS-PAGE coomassies staining.

2.1.9 Measuring the concentration of different variants of hpTlyA

After checking the purity of all mutants of hpTlyA in SDS –PAGE, concentrations of all mutants and Wt. hpTlyA protein were measured using spectrometer at 280 nm in quartz cuvette having path length 1 cm. The extinction coefficient of Wt hpTlyA is 0.220 (1 mg/ml). For better quality of protein the ratio of 260/280 should be around 0.6 - 0.9.

2.1.9 Checking of secondary structure of different variants of hpTlyA

Understandings of the secondary structures of proteins are crucial aspects to know their functional features. Study of secondary structure show the percentage contents of alpha and beta strands arrangement.

One of the best methods to study secondary structure of the protein samples is done by circular dichroism (CD) technique discovered by Jean-Baptiste Biot, Augustin Fresnel, and Aimé Cotton. This technique use circular polarized light. CD spectra of alpha, beta and random coil have distinct shape and absorption value. Therefore this information can be utilized to get percentage content of alpha, beta in any protein sample.

Mutation in protein sequence at certain position can alter the secondary structure feature of protein. Therefore it becomes necessary to study the CD spectra of mutated protein.

CD spectra of Wt. hpTlyA and all mutants were recorded in the Far-UV range. Samples of all proteins having concentration 2-4 µM were prepared in 1mM Tris solution (pH 7.2). CD spectra for all the proteins were acquired using Chirascanspectroplorimeter (Applied Photo-physics Leatherhead, UK) which is equipped with Peltier based temperature controller. Quartz Cuvette having path length 5mm was used for sample insertion in machine. Each spectrum was collected by taking average of three consecutive scan. Baseline correction was done by subtracting buffer spectrum from each protein's Far- UV spectra and plot of each spectrum was made.

2.1.10 Checking of the hem-agglutination activity of various recombinants of hpTlyA

Many bacterial and virus species contains envelop or surface proteins which have characteristic feature to bind with red blood cells. This property of binding of surface protein was used by American virologist George Hirst to know the presence of viral titer in blood. Viral and bacterial proteins stick with red blood cells and make clumps. This clumping of red blood cells is known as hem-agglutination. During agglutination process protein molecule can stick more than one red blood cell that lead to the formation of lattice film on the wall of the 96 wells microtiter 'V' bottom plate. The efficiency of making lattice film or extent to doing the hem-agglutination activity is determined by the last dilution.

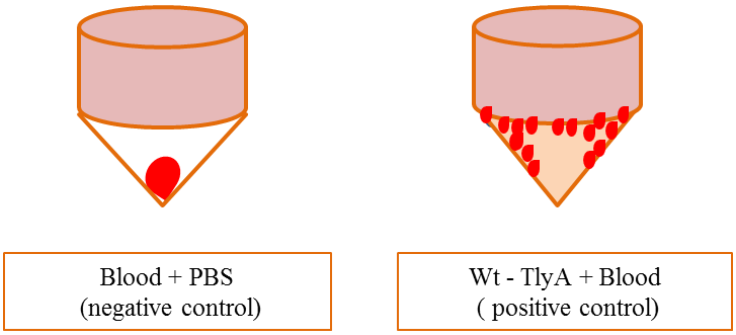


Figure 2.0- Schematic diagram of hem-agglutination

Hem-agglutination activity was performed by treating fresh blood (OD = 0.4) with Wt. hpTlyA and all mutants in 96 wells microtiter 'V' bottom plate. Following steps were taken during hem-agglutination assay:-

1. OD of fresh blood was adjusted to 0.4.
2. First of all, in microtiter 'V' bottom plate, 50 µL of PBS was added into Blank label well, and all the wells which are going to be undergone dilutions with protein load excluding well containing neat protein.
3. Well of neat load was loaded with 50 µL of highest available concentration of protein and 50 µL blood while blank label well contain only blood and PBS.
4. Well of first dilution of protein load contain half of the concentration of neat protein.
5. Serial dilution from well of first dilution was carried out for minimum 7-8 dilutions.
6. Reaction mixture of 100 µL volume was prepared by adding blood in each well. Mixture was properly mixed without forming any bubble.
7. Finally plate was covered with aluminium foil and incubated at room temperature without any disturbance for 2-3 hours.
8. After 2 - 3 hours of incubation, hem-agglutination was checked.

2.1.11 Checking of Methyltransferase activity of hpTlyA and their mutants

2.1.11a Performing the growth kinetic of Origami B cells containing only pET14b and pet14b with hpTlyA gene in presence of capreomycin.

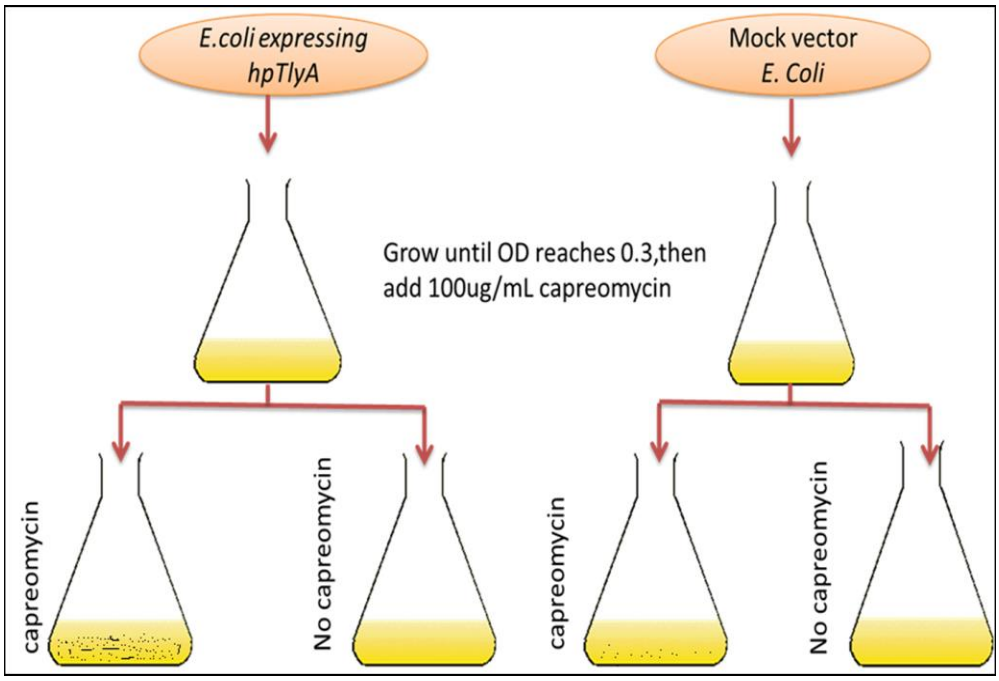


Figure 2.1- Experimental setup for growth kinetics

1. First of all, seed cultures of Origami B cells containing mock vector (pET14b only) and hpTlyA were grown overnight at 37 C temperature.
2. Growth of each culture was observed on next day morning by using spectrometer.
3. Each secondary culture was prepared in 50 ml freshly autoclaved LB-media and intial OD was set to 0.02.

Formula used
X = required OD of secondary culture (0.02) * 50 ml LB media / OD of seed culture

Where 'X' is volume taken from each culture to make 0.02 OD of secondary culture

4. Add 50 uL of ampicillin in each four equally autoclaved LB media flasks.
5. Flasks were divided into two pairs and labelled as ‘pET14B’, ‘pET14B with capreomycin’, ‘hpTlyA’ and ‘hpTlyA with capreomycin’.
6. After addition of ampicillin ‘X’ volume from both cultures was added to four flasks.
7. Initial OD of each culture was noted to ensure whether it is 0.02 or not.
8. At OD = 0.3, 50 µL capreomycin (100 µg/mL) was added.
9. Subsequently OD of every culture was noted after every hour.
10. Relative growth curves wereplotted .

2.1.11b Performing the growth kinetic of Origami B cells containing hpTlyA, K63A-TlyA, D145A-TlyA, K170A-TlyA and E227A-TlyA in pET14B vector in presence of capreomycin

In similar manner growth curves were analysed for every mutant. Here comparison was made only between ‘Wt.-hpTlyA with capreomycin’ and ‘mutant with capreomycin’. Wt.-hpTlyA without capreomycin was considered as standard to compare the relative inhibition in growth.

CHAPTER 3
RESULTS

3.0 Identification of the critical amino acid residues involved in methyltransferse activity

Prediction of critical amino acid residues responsible for methyltransferaseactivity, was carried out by sequence alignment of Wt. hpTlyA of *H. pylori* with TlyA protein sequence of *Mycobacterium tuberculosis* and *Serpulina hyodysenteriae.* Sequence alignment results show that *M.tuberculosis and S.hyodysenteriae* have 35 % sequence similarity with *H.pylori* and also reveal the presence of K-D-K-E tetrad. TlyA gene in *M. tuberculosis and S.hyodysenteriae*, encode a rRNA methyltransferase which methylate 2'-hydroxyl group of ribose moiety of ribosomal RNA. K-D-K-E tetrad position in hpTlyA protein sequence can be shown at 63-145-170-227 respectively. Presence of such tetrad also confirmed by 3D structure, generated by Swiss-model and Phyre2 (online software).

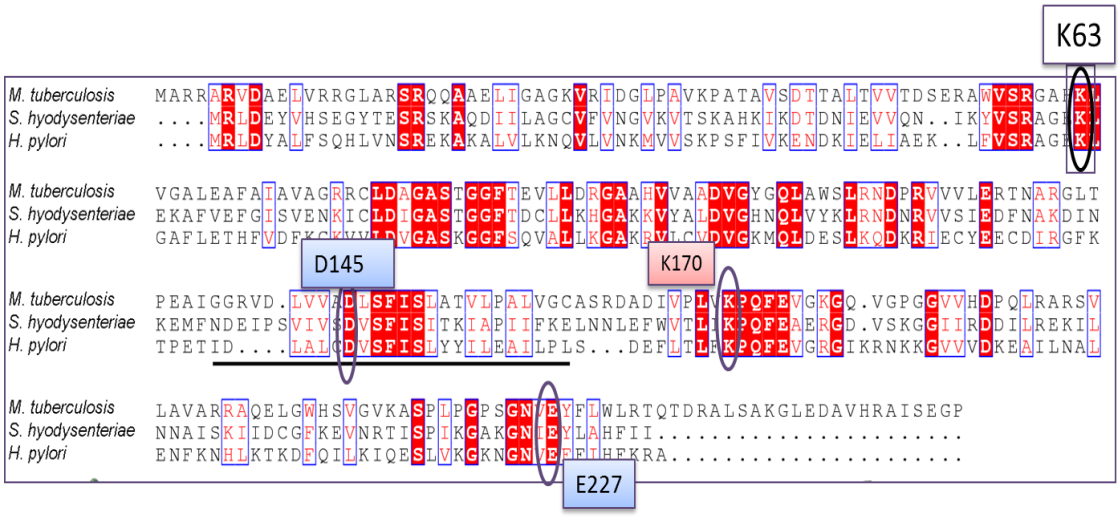


Figure 3.0 Sequence alignment of TlyA protein of *Mycobacterium tuberculosis* and *Serpulinahyo dyseneiriae* with critical amino acid residues positions of tetrad

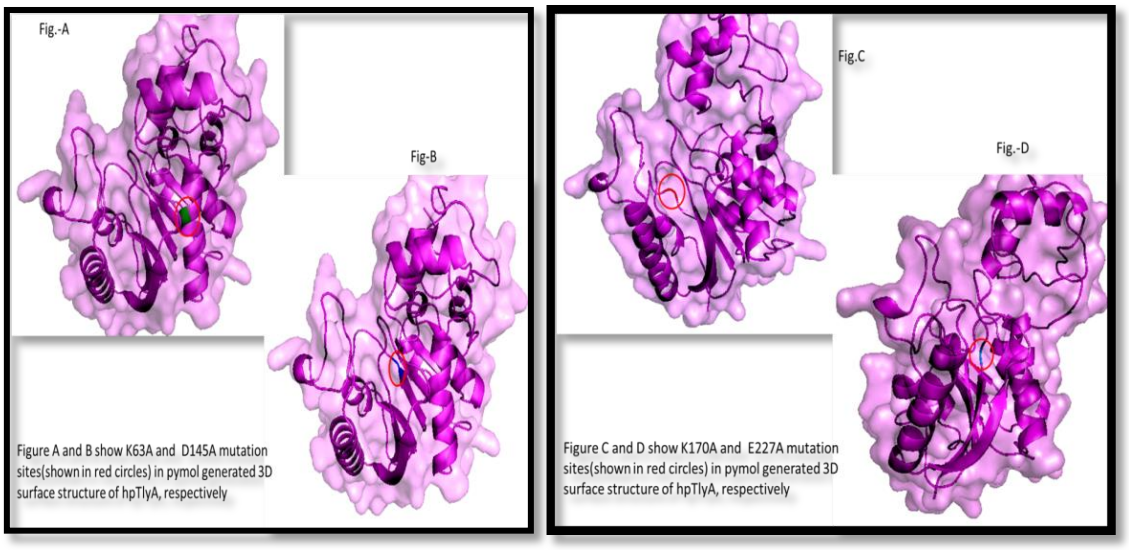
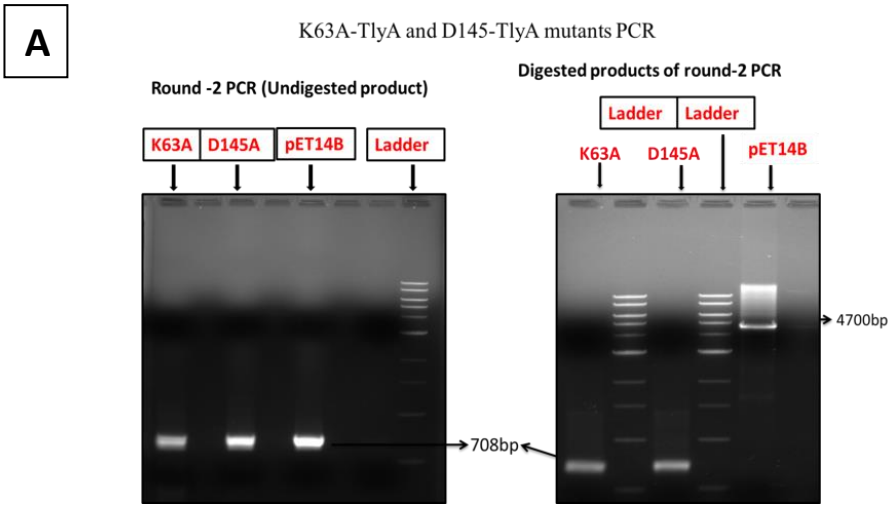


Figure 3.1 Left panel show K63 (Fig A) and D45 (Fig B)(red circles) position in 3D structure of hpTlyA and right panel show K170 (Fig C) and E227 (Fig D) (red circles) positions.

3.1 Cloning and preparation of variants of Wt-hpTlyA by Site–directed mutagenesis

To explore the metyltranferase activity of hpTlyA, we generated variants of hpTlyA in the K-D-K-E tetrad. All variants of hpTlyA (K63A-TlyA, D145A-TlyA, K170A and E227A-TlyA) are generated by site directed mutagenesis. PCR of all variants were done and quality of all PCR products was checked with 1% agarose gel. All variants show similar 708 bp size amplification (Figures 1a, 1b, 1c, 2a, 2b, 2c). Finally mutation sites in each construct were confirmed by sequence analysis**.**



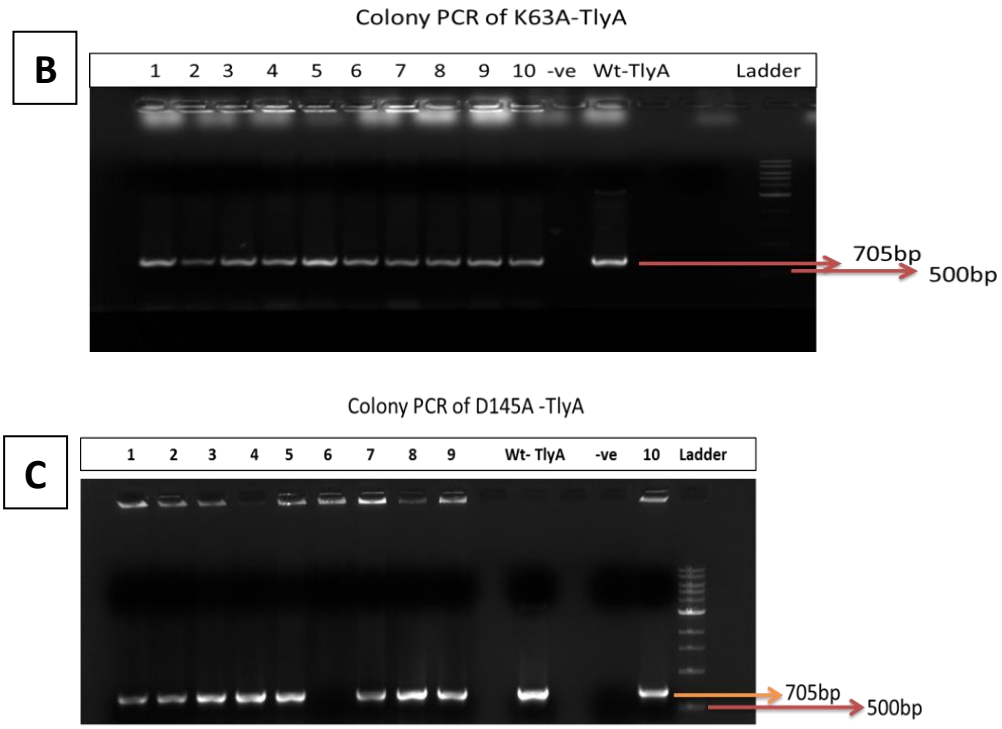


Figure 3.2(A) Undigested PCR products of K63A-TlyA and D145A-TlyA in left paneland digested PCR products of both products in right panel. (B) and (C) show colony PCR of 9-10randomlselectecoloniesof transformed E.coli with both mutated gene of Wt-hpTly

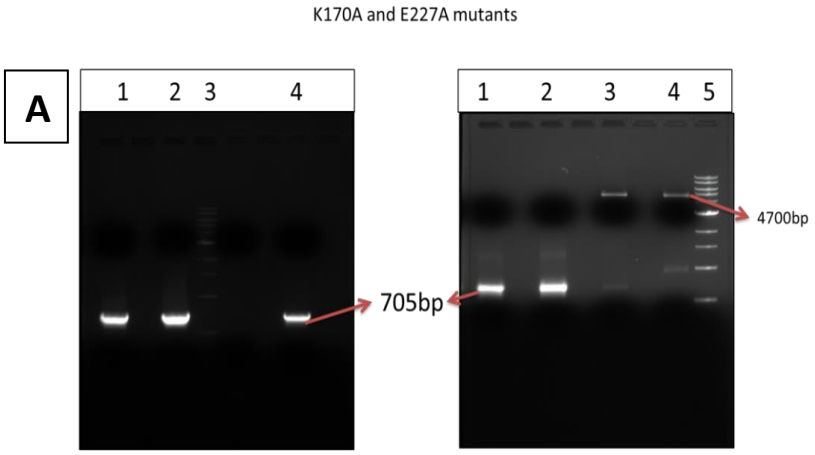


Figure A show undigested products of K170A and E227A in lane 1 and 2 respectively. Lane 3 and 4 show DNA ladder and wt-TlyA respectively

Figure-B-Lane1 and2 show digested products of K170A, E227A TlyA respectively. Lane 3 ,4 show digested pET14B and lane 5 show DNA ladder

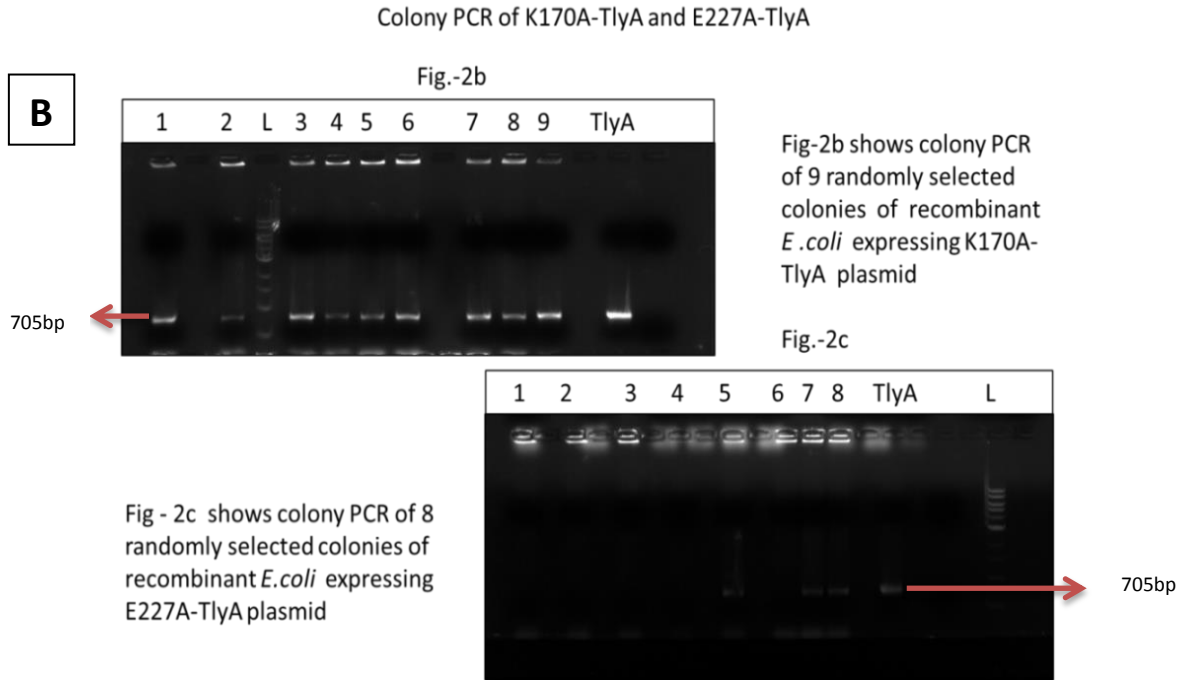
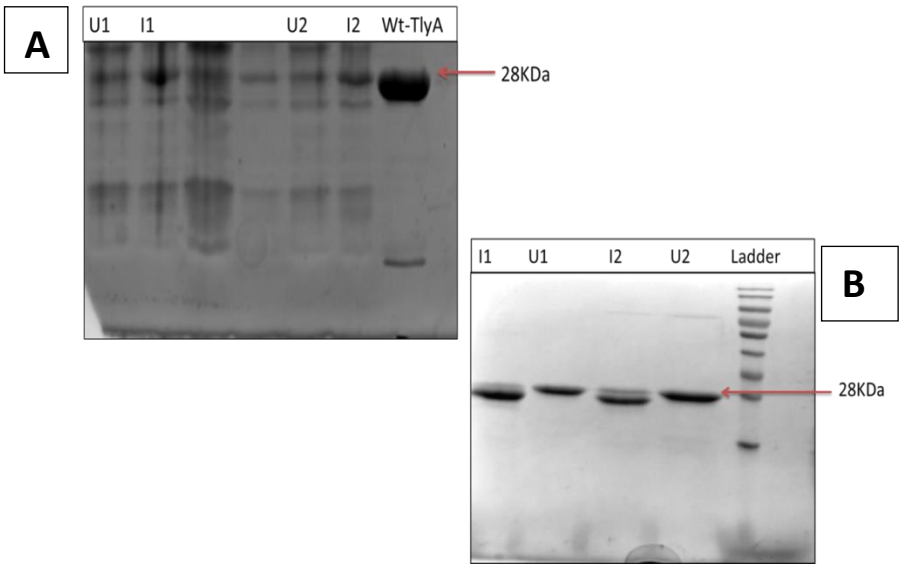


Figure 3.3 (A) Undigested PCR products of K170A-TlyA and E227A TlyA constructs in left panel and right panel show digested products of both mutant constructs of Wt-hpTlyA. (B) and (C) colony PCR of 9-10 randomly selected colonies of transformed *E.coli* with both mutated gene of Wt-hpTlyA

3.2 Purification of the variants of hpTlyA

To check the effects of mutagenesis in hpTlyA methyltranfearse, firstly variants of hpTlyA were expressed in *E.coli* origami B cells. The different constructs of hpTlyA were induced with IPTG and their expression level was checked on 12.5% SDS PAGE by coomassie gel staining (Figures 3a and 3b).His-tagged variants of hpTlyA expressed in the cytosol of origami B cells were first purified with Ni-NTA affinity chromatography and subsequently Ni-NTA purified fractions were processed by SP-sepharose column. Purified variants were run on the 12.5 % SDS coomassie staining gel (Fig. 4). In order to check any change in secondary structure of the variants, Far- UV spectra of all variants was monitored (Fig. 5). Analysis of CD spectra of all variants show structural similarity with wild type hpTlyA**.**



Figures 3.4 (A) and (3) show induction check of all variants of Wt-hpTlyA in 12.5 % SDS PAGE coomassie staining. (A) U1 and I1 show expression of protein in un-induced and induced culture of K170-TlyA mutant while U2 and I2 show expression of protein in un-induced and induced culture of E227A-TlyA mutant with Wt-hpTlyA as positive control at 28KDa respectively. (B) U1 and I1 show expression of protein in un-induced and induced culture of K63-TlyA mutant while U2 and I2 show expression of protein in un-induced and induced culture of D145A-TlyA at 28KDa respectively.

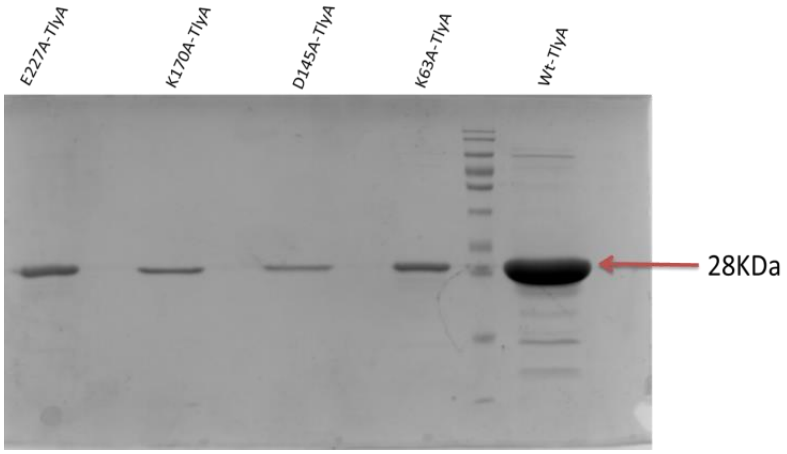


Fig.3. 5 show SDS PAGE coomassie staining of all mutants in 12.5% SDS gel with Wt.TlyA protein as control at 28KDa

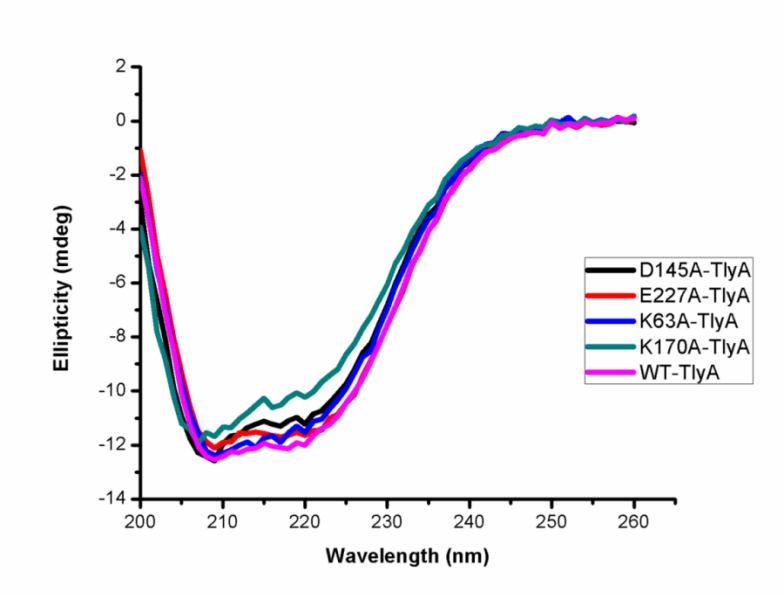
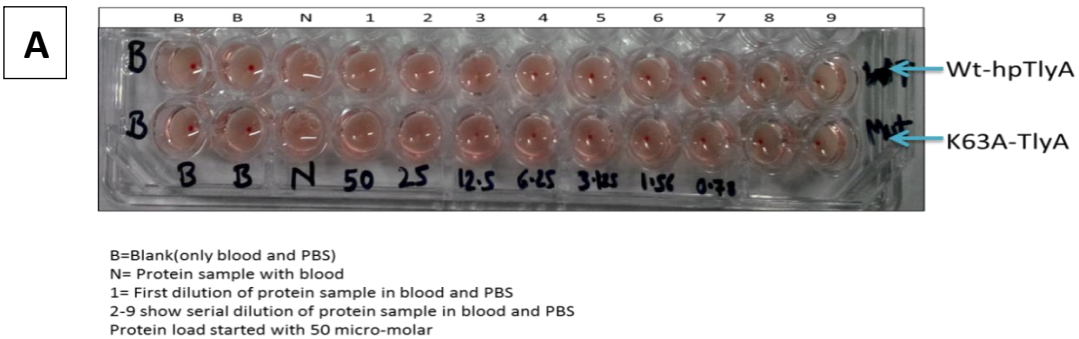


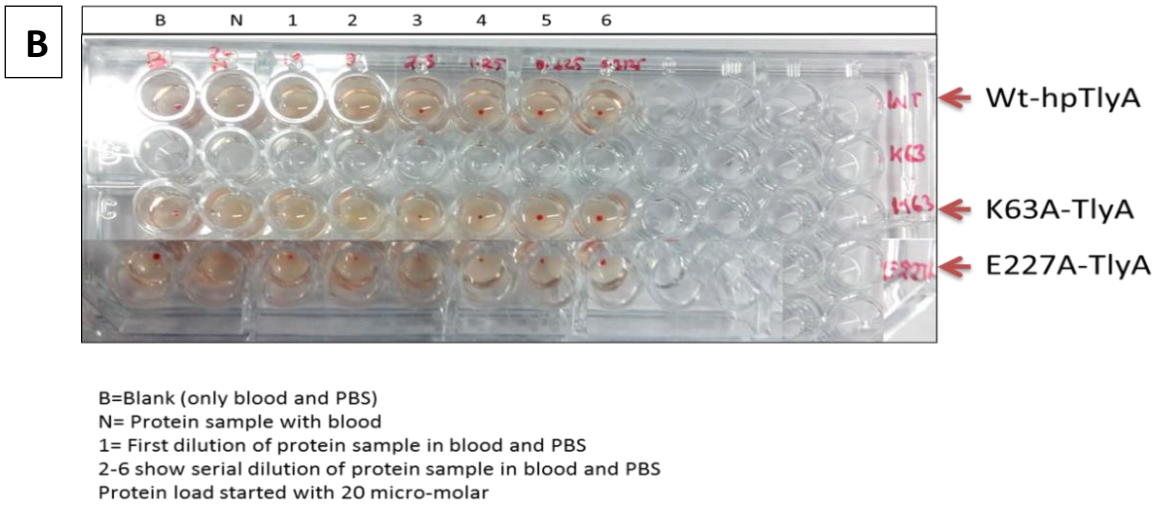
Figure 3.6 CD spectra of proteins of all constructs. All variants show resemblance with Wt-hpTlyA protein which indicate that mutations at different positions do not cause any change in secondary structural. CD spectra of all variants also show presence of combination of alpha helices and beta sheets in their secondary structure like Wt-hpTlyA.

3.5 Cytotoxic effects of variants of hpTlyA on human erythrocytes

Wt-hpTlyA is a pore forming toxin and its cytotoxicity is determined by hemagglutination assay. It has been reported that Wt-hpTlyA can exhibits pore formation by performing hem-agglutination first and then subsequently do hemolysis of human erythrocytes. In order to check the functional activity of hpTlyA variants, we compared the hem-agglutination ability of the variants with the Wt-hpTlyA. It was observed that the variants showed similar level of hem-agglutination as the wt-hpTlyA(Figures 6a and 6b). This result indicates that the mutation in the K-D-K-E tetrad has no effect on hem-agglutination ability and all the variants prepared are functionally active



B=Blank(only blood and PBS)
N= Protein sample with blood
1= First dilution of protein sample in blood and PBS
2-9 show serial dilution of protein sample in blood and PBS
Protein load started with 50 micro-molar



Figures 3.7 (A) and (B) Hem-agglutination activity by K63A-TlyA and E227A-TlyA. Wt-hpTlyA protein is also used to compare the extent of hem-agglutination by both mutants. Comparisons of hem-agglutination activity by both mutants with Wt-hpTlyA do not show any significant difference.

3.6 Methyltransferase activity of hpTlyA

In order to check the methyltransferase activity, growth kinetics of origami B cells expressing Wt-hpTly, was monitored in the presence of antimicrobial drug capreomycin and relative growth kinetics of origami B cells expressing pET14B and plasmid of all variants including Wt-hpTlyA were analysed. First of all, growth kinetics of Origami B cells expressing pET14B and hpTlyA , were monitored in the presence of capreomyicn (Figure 7) and then all variants were monitored. For relative growth curve plot, 'pET14B without capreomycin' was taken as reference (pET14B 100%) while in case variant, growth line of 'hpTlyA without capreomycin' (TlyA 100 %) was taken as reference line (Figure 8). Relative growth kinetics of all variants clearly show growth of Wt-hpTlyA retard up to 70-80 % in presence of capreomycin while variants show only 20-32 % retardation in their growth which indicate that mutations at K-D-K-E tetrad can perturb the growth kinetics of Wt-hpTlyA because un-methylated sites in RNA were not recognized by capreomycin which indicate that Wt-hpTlyA is a methyltransferase and its functionality is dependent on K-D-K-E tetrad.

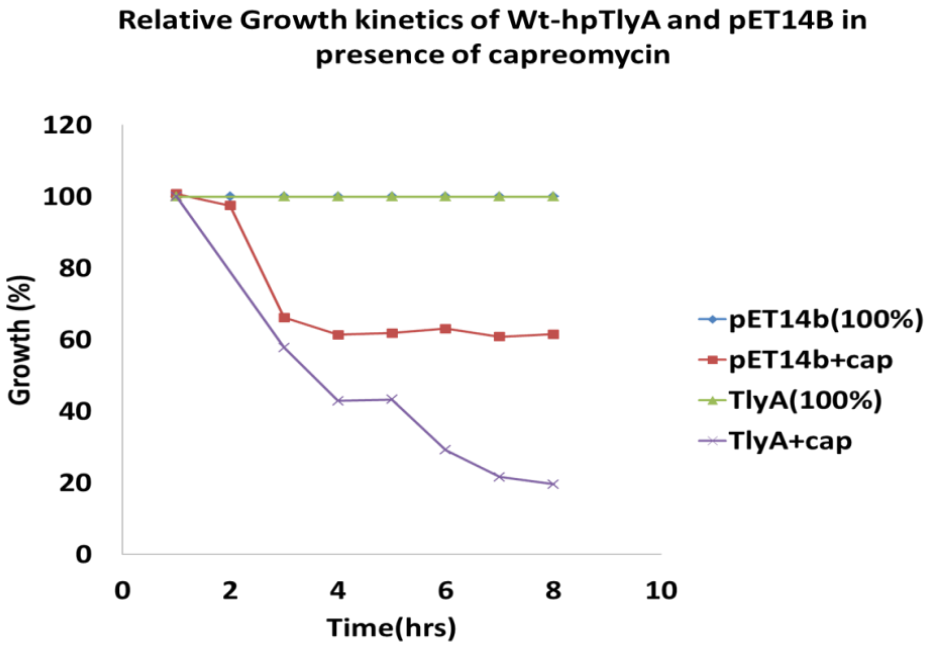


Figure 3.8 Relative growth kinetics of recombinant E.coli containing Wt-hpTlyA and pET14B in presence of capreomycin (100 µg/ mL). Relative growth plot were made by considering pET14B (without capreomycin) as reference line. Plots show up-to 80 % growth inhibition in Wt-hpTlyA as compared to pET14B. pET14B with capreomycin show up-to 20-30 % growth inhibition which indicating the possibility of having methyltransferase activity by Wt-hpTlyA protein.

Comparisons of growth kinetic of Origami B cells containing hpTlyA, K63A-TlyA, D145A-TlyA, K170A-TlyA and E227A-TlyA in pET14B vector in presence of capreomycin

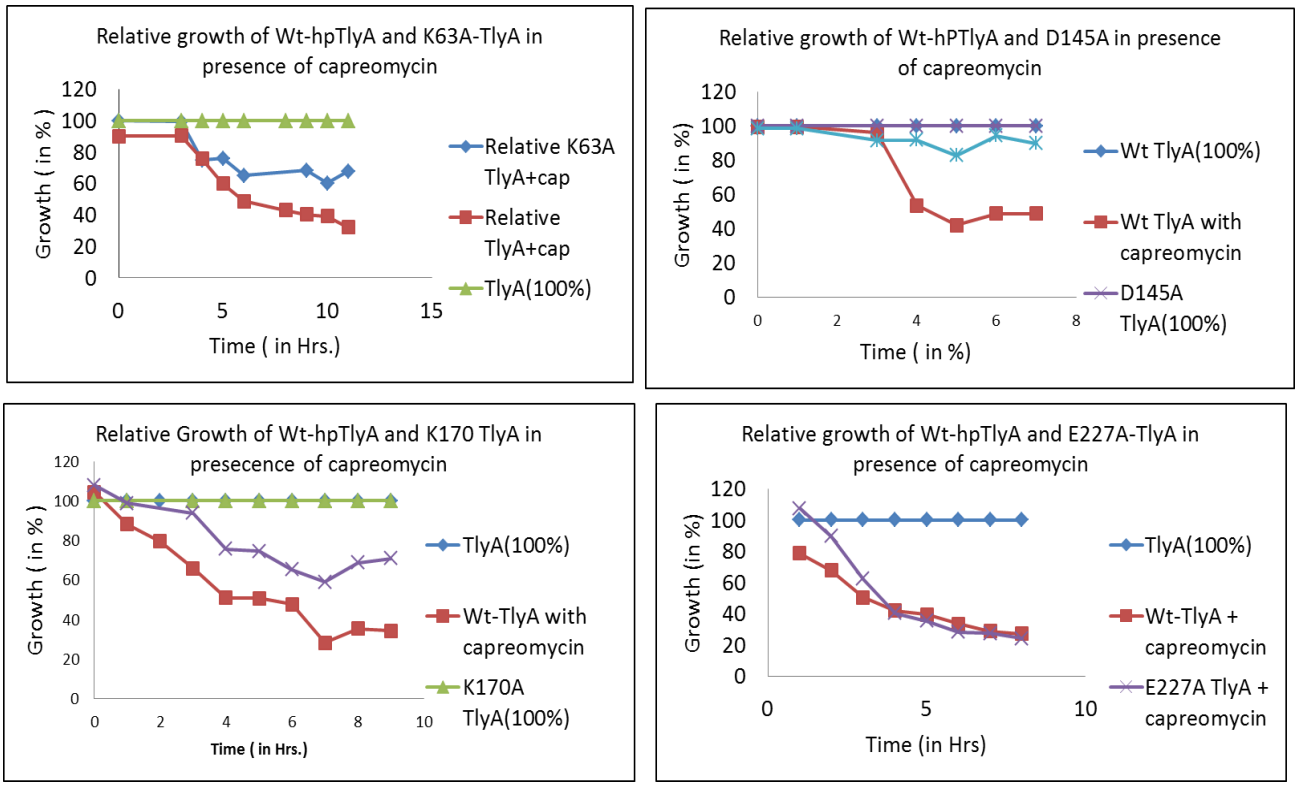


Figure 3.9 Relative growth kinetics of all variants (K63A.D145A,K170A,E227A-TlyA) of Wt-hpTlyA in presence of capreomycin (100µg/mL). Wt-hpTlyA (without capreomycin) growth line was taken as reference line to make relative growth of all variants in presence of capreomycin. In all cases Wt-hpTlyA show 70-80 % growth inhibition in presence of capreomycin while K63A,D145A,K170A and E227A-TlyA show 25-35%, 20-30%, 25-35% and 60-70% growth inhibition respectively. Survival ship of*E.coli* cells, expressing variants of Wt-hpTlyA except E227A-TlyA, in presence of capreomycin,indicating the loss of methyltransferase activity that is why capreomycin is unable to recognize the binding sites.

Discussion

Site directed mutagenesis at various sites of Wt-hpTlyA sequence provide insight to understand the underlying mechanism of various structural and functional aspects of toxin.

Substitution of the four amino acid residues (K63-D145-K170-E227 tetrad) in the Wt-hpTlyA sequence by alanine, make us enable to monitor the methyltransferase property of toxin. Experimental sequence analysis of all four constructs after whole cloning procedure show the right substitution of critical amino acid residues by alanine, at their respective positions which indicate that all constructs contain desired mutations.

Protein purification from Origami B cells, expressing variants of Wt-hpTlyA and then subsequently their bands on SDS-PAGE coomassie gel staining show exactly same size like Wt-hpTlyA. This indicates that purified proteins were desired products.

CD spectra of all variants of Wt-hpTlyA show similar secondary structure features like Wt-hpTlyA. There was no significant difference found in secondary structure of all variants which clearly indicates that mutations do not cause any disturbance in structural stabilities of all variants.

It was also possible that mutations can prevent the hem-agglutination activity by this toxin. In order to check this possibility, hem-agglutination assay was performed and it was observed that K63A and E227A-TlyA perform this activity in same extent like Wt-hpTlyA.

Methylation of rRNA is quite crucial for the protein synthesis and subsequently the regulation of various events in bacteria for their survival. Loss of methylation can prevent the synthesis of protein and ultimately lead to death of bacteria. Capreomycin is a peptide antibiotic drug which target methylated ribosome and inhibits the protein synthesis machinery and lead to growth inhibition of bacteria. We utilize this property to show our toxin as a methyltransferase of rRNA. In order to show this, first of all we compare the growth kinetic of Origami B cells containing only pET14B and pet14B with Wt-hpTlyA gene in presence of capreomycin. It was observed that Wt-hpTlyA show 70-80 % growth inhibition in presence of capreomycin while pET14B

show only 20-30 % growth inhibition which indicating that Wt-hpTlyA might be a methyltrasferase which methylating the rRNA. Further, to prove this, growth kinetic of origami cell expressing all variants of Wt-hpTlyA was monitored and comparisons were made for all variants. It was observed that all variants show normal growth like pET14B in presence of capreomycin except E227A-TlyA mutant. Normal growth of all variants except E227A-TlyA indicates that capreomycin has not recognized the target sites in ribosome due to loss of methylation of rRNA. Normal growth of all variants also be possible due to presence of other methyltranferase in bacteria that methylate rRNA at different position irrespective of capreomycin target sites.

Thus in short, we can that K63-D145-K170-E227 tetrad is necessary for methylation property of Wt-hpTlyA. Methyltransferase and ham-agglutination activity are independent features of this toxin giving place to it in multi-functional proteins

Conclusions and future directions

Residing of *H. pylori* bacteria in low pH of human stomach and then colonization of the bacteria to epithelial cells is one of its unique features. The ability of bacteria to survive in such a harsh environment requires strong system to adapt with multifunctional proteins which can regulate metabolic and protein synthesis machinery. To understand the functions of hpTlyA toxin is a little attempt to work in this direction.

Having ability of hpTlyA to act as methyltransferase of rRNA in *H.pylori* can be a crucial function which can modulate the metabolic and protein synthesis pathways. Primary methods clearly show that hpTlyA can do methylation of rRNA and help in protein synthesis. Further advanced methods like inhibition of in vitro translation of S30 extract of recombinant *E.coli* in presence of capreomycin (require alpha hemolysin reporter gene) and reverse primer extension of rRNA (involve radiolabel H for methyl group of SAM or P for nucleotide), can be employed to robust the results.

Lack of knowledge of exact mechanism of bacterial infection and cytotoxicity of their toxin is always crucial in drug designing. Therefore, knowledge of finding of such activity is not necessary only to explore the function aspect of toxin but it also gives a clue for drug designing.

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