

GENERATION AND CHARACTERIZATION OF RANDOM AND SITE-  
DIRECTED MUTANTS OF SHIGA-LIKE TOXIN 1A BY *Escherichia*  
*Coli* O157:H7 IN *Saccharomyces Cerevisiae*

By

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Written under the direction of

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## ABSTRACT OF THE THESIS

Generation and characterization of random and site-directed mutants of  
Shiga-like toxin 1A by *Escherichia coli* O157:H7 in *Saccharomyces*  
*cerevisiae*

By VARSHA SHETE

Thesis Director: Dr. Nilgun Tumer

Stx1 produced by enterohemorrhagic *Escherichia coli* is essentially similar to Shiga toxin produced by *Shigella dysenteriae*. Food-borne illnesses are mainly associated with Shiga-like toxins (Stx1 and Stx2) produced by various serotypes of enterohemorrhagic *Escherichia coli* (EHEC). These serotypes are collectively known as STEC (Stx-producing *E. coli*). One of these serotypes *E. coli* O157:H7 has been the major cause of food-borne illnesses recently in US, Canada and Japan. The clinical manifestations of EHEC infections range from watery diarrhea, severe bloody diarrhea, abdominal cramps and hemorrhagic colitis (HC), to the most severe outcome, life-threatening hemolytic uremic syndrome (HUS) resulting in kidney failure.

The mechanism by which Shiga-like toxin 1 exhibits its cytotoxicity in cells is unknown. This study aims at identifying intra-molecular regions of Shiga-like toxins and studying its protein structure-function relationships. Shiga-like toxin 1A gene was cloned and transformed into *Saccharomyces cerevisiae* system. Random and site-directed mutants were generated and characterized for protein expression, ribosome depurination and loss of cytotoxicity. The results provide an understanding of various regions in the Shiga-like toxin 1 that are crucial for its cytotoxicity to the cells. This data may be an important tool in developing treatments against the diseases caused by Shiga-like toxin 1.

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# **Generation and characterization of random and site-directed mutants of Shiga-like toxin 1A by *Escherichia coli* O157:H7 in *Saccharomyces cerevisiae***

## **INTRODUCTION**

### **Shiga-like toxins and outbreaks**

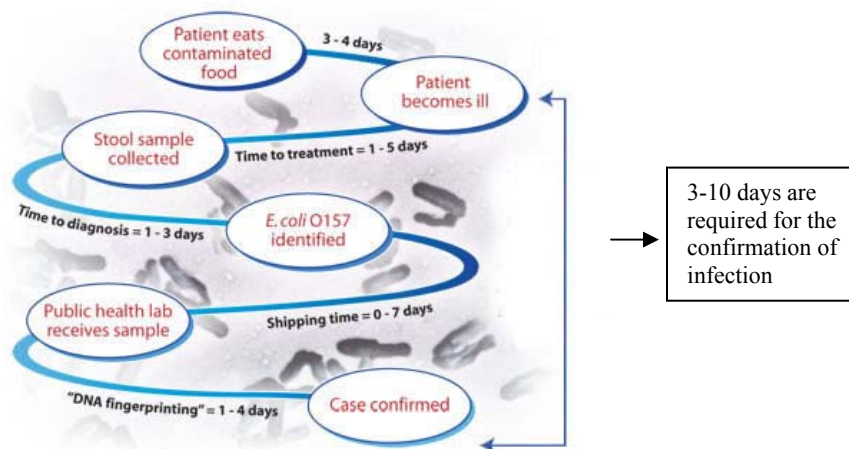
Ribosome inactivating proteins (RIPs) have gained a lot of attention recently due to their RNA-N-glycosidase activity and their ability to depurinate the ribosomal subunit. The bacterial toxins Shiga toxin, Shiga-like toxins and the plant toxin ricin are type II ribosome inactivating proteins. Although these toxins are found in different plants and bacteria, they are known to exhibit several common characteristics and the mechanism of action. These toxins are known to inactivate ribosome and inhibit protein synthesis by removing a specific adenine residue from the highly conserved  $\alpha$ -sarcin ricin loop of the 28S eukaryotic rRNA.

The Shiga toxin family includes Shiga toxin from *Shigella dysenteriae* and Shiga-like toxins-1 and -2 (Stx1 and Stx2). They all are highly related to each other in structure and function (Johannes *et al.*, 2002). Shiga-like toxins are also produced by *Citrobacter freundii*, *Aeromonas hydrophila*, *Aeromonas caviae* and *Enterobacter cloacae* (Paton *et al.*, 1998). Shiga-like toxin 1 (Stx1) is more homogenous as compared to Shiga-like toxin 2 (Stx2) as far as the structural details are concerned. Stx2 has five subtypes, namely, Stx2, Stx2c, Stx2d, Stx2e and Stx2f.

Stx1 produced by *E.coli* is essentially similar to Shiga toxin produced by *Shigella dysenteriae*. Food-borne illnesses are mainly associated with Shiga-like toxins (Stx1 and

Stx2) produced by various serotypes of enterohemorrhagic *Escherichia coli* (EHEC). These serotypes are collectively known as STEC (Stx-producing *E. coli*). One of these serotypes *E. coli* O157:H7 has been the major cause of food-borne illnesses recently in US, Canada and Japan. The clinical manifestations of EHEC infections range from watery diarrhea, severe bloody diarrhea, abdominal cramps and hemorrhagic colitis (HC), to the most severe outcome, life-threatening hemolytic uremic syndrome (HUS) resulting in kidney failure (Fraser *et al.*, 2004).

Typically it takes about 6-10 days to confirm whether an outbreak showing symptoms of Stx infection is indeed caused due to STEC. The typical timeline for the reporting of the case of infection caused by *E. coli* O157:H7 contamination can be schematically represented as following:



**Figure 1 Timeline for the onset of the infection caused due to the consumption of food contaminated with *E. coli* O157:H7, as indicated by the Center for Disease Control and Prevention on [www.cdc.gov](http://www.cdc.gov).**

As the timeline in Fig 1 indicates, when a person consumes food contaminated with *E. coli* O157:H7 of the amount sufficient to cause infection, it takes about 3-4 days

by the time the symptoms like bloody diarrhea exhibit themselves. Further it takes approximately 7-10 days to confirm the case as an infectious outbreak caused by STEC.

In the United States alone, several cases of outbreaks of *E. coli* O157:H7 infection have been reported. Between July 20, 2007 and October 10, 2007 at least 8 people were hospitalized and 4 people developed HUS resulting in kidney failure due to infection by *E. coli* O157:H7 in Illinois, Kentucky, Missouri, New York, Ohio, Pennsylvania, South Dakota, Tennessee, Virginia and Wisconsin. This multistate outbreak was believed to be caused due to consumption of frozen pepperoni pizza which was contaminated with STEC. This pizza was produced by General Mills under the brand name Totino's and Jeno's ([www.cdc.gov](http://www.cdc.gov)).

An outbreak of STEC infection associated with consumption of iceberg lettuce from Taco Bell restaurants in Northeastern states was reported between November 20 and December 2, 2006. Totally 71 cases were reported where 53 persons were hospitalized and 8 cases developed HUS. Similarly, about 199 cases of *E. coli* O157:H7 infection were reported in 26 states between August 19 and September 5, 2006. These were caused by the consumption of bagged spinach and products containing spinach which was produced in Californian counties. This was at least 26<sup>th</sup> outbreak of *E. coli* O157:H7 infection reported since 1993 that was associated with green leafy vegetables. Since 1982, more than 400 outbreaks were reported which were caused by enterohemorrhagic *E. coli* O157:H7 infection (Dennis *et al.*, 2006).

On September 10, 2007, 21.7 million pounds of ground beef patties were recalled due to a STEC outbreak caused due to consumption of Topp's brand frozen beef patties.



Totally 40 cases of infection were reported where 21 people were hospitalized and 2 of them produced HUS. All these outbreaks indicate that shiga-like toxin produced by *E. coli* O157:H7 has been a major cause of more than 73,000 cases of food-borne illnesses per year in the United States ([www.cdc.gov](http://www.cdc.gov)).

### **Modes of transmission and entry into the food chain**

The concentrations of *E. coli* O157:H7 in the food responsible for many of the outbreaks were found to be low indicating low doses of the organism are sufficient to cause the infection. For example, the multistate outbreak caused to due consumption of hamburger patties in 1993 had a dose of less than 700 organisms per pound before cooking, which was believed to have been further lowered during the consumption of food. This suggests that since this bacterium can induce disease symptoms at lower concentrations, it is necessary to detect it at lower concentrations in the food in order to avoid consumption and subsequent infection.

*E. coli* O157:H7 outbreaks have been linked to consumption of ground beef in many cases. The first STEC outbreak in 1982 was believed to be due to consumption of contaminated ground beef (Armstrong *et al.*, 1996). The presence of STEC was found in both healthy and ill cattle, younger animals showing the highest prevalence rate of the bacterium. Ground beef is the main mode of transmission for the largest portion (58%) of the illnesses, as shown in the Table 1. Other food products showing presence of STEC are meat products from sheep and chickens. Raw milk had been tested positively for the presence of this bacterium. Other foods that have been linked with the outbreaks due to

STEC infection are unrefrigerated sandwiches, potatoes, apple cider, mayonnaise, cantaloupe, alfalfa seeds, radish sprouts, iceberg lettuce, spinach and green onions. In Scotland and South Africa, drinking water, which was probably contaminated with bovine feces, has been linked with the outbreaks.

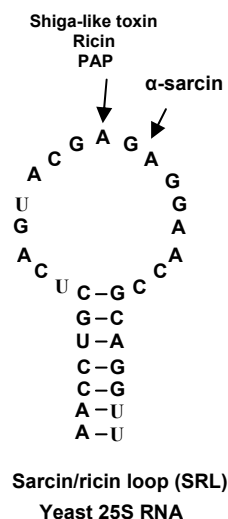
<b>Vehicle</b>	<b>No. of outbreaks</b>	<b>No. of individuals involved</b>
<b>All foods</b>	<b>38</b>	<b>1,541</b>
<b>Ground beef</b>	<b>22</b>	<b>1,137</b>
<b>Beef products and milk</b>	<b>26</b>	<b>1,278</b>
<b>Drinking water</b>	<b>3</b>	<b>276</b>

**Table 1 Outbreaks of *E. coli* O157:H7 as reported by the Center for Disease Control and Prevention 1982-1994 data. The above data indicates the mode of infection involved and number of cases reported (Armstrong *et al.*, 1996).**

The benefit of toxin production to the survival of the bacterium *E. coli* O157:H7 has been a subject of interest. It was showed that the toxin produced during infection helps in the adherence of the bacterium to enterocyte surface, thus promoting its intestinal colonization (Robinson *et al.*, 2006).

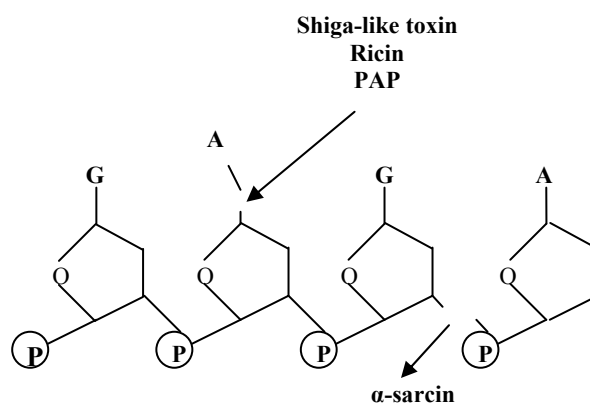
### **Ribosome inactivating proteins (RIPs)**

Ribosomes are complexes of protein and RNA that are found in all cells and they play a major role in the process of translation through which protein synthesis occurs in the cells. They use messenger RNA as a template to form a protein assembly with amino acids in order to initiate the process of translation. Ribosome inactivating proteins (RIPs) are known to remove specific adenine residue (A4324) from a universally conserved  $\alpha$ -sarcin ricin loop in the 28S subunit of the eukaryotic rRNA. When this loop structure is altered it no longer binds to the elongation factors. This interferes with the translocation which leads to inhibition of translation and subsequent cell death. Thus the main function of RIPs is believed to be in the defense mechanism of the host cell (Park *et al.*, 2004). Initially it was believed that adenine is the only target for the action of RIPs. Recent studies have revealed that some RIPs like Saporin can release adenine by attacking other substrates as mRNA, tRNA, rRNA and DNA (Barbieri *et al.*, 1992).



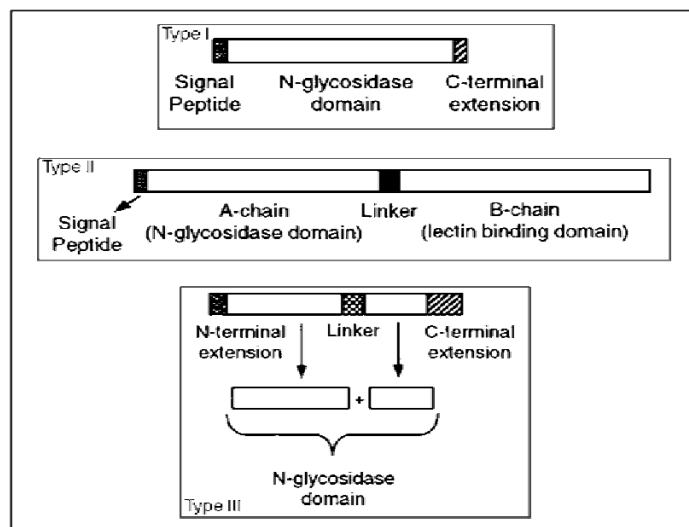
**Figure 2** Schematic presentation of sarcin/ricin loop (SRL) of yeast 25S RNA. The arrows indicate the site of target for ribosome inactivating proteins and ribotoxin  $\alpha$ -sarcin.

Ribosomal sequence in the loop shown in Fig 2 above is highly conserved. Ricin is a plant toxin found in castor beans while  $\alpha$ -sarcin is an endoribonuclease produced by *Aspergillus giganteus*. The loop sequence found in the principal RNA of large ribosomal subunits is the site of attack for these two toxins  $\alpha$ -sarcin and ricin, thereby giving it the name  $\alpha$ -sarcin ricin loop (SRL).



**Figure 3 The sites of attack for Ribosome Inactivating proteins and  $\alpha$ -sarcin from GAGA sequence in the sarcin/ricin loop.**

As shown in the Fig 3, ribosome inactivating proteins attack the adenine site while  $\alpha$ -sarcin hydrolyzes the ribosome by cleaving the bond between adjacent guanine and adenine residues. Thus plant toxins ricin, PAP and bacterial toxin Shiga-toxin differ from ribotoxin  $\alpha$ -sarcin in the mechanism through which they inactivate ribosomes though all these toxins contribute to RIP class of proteins.



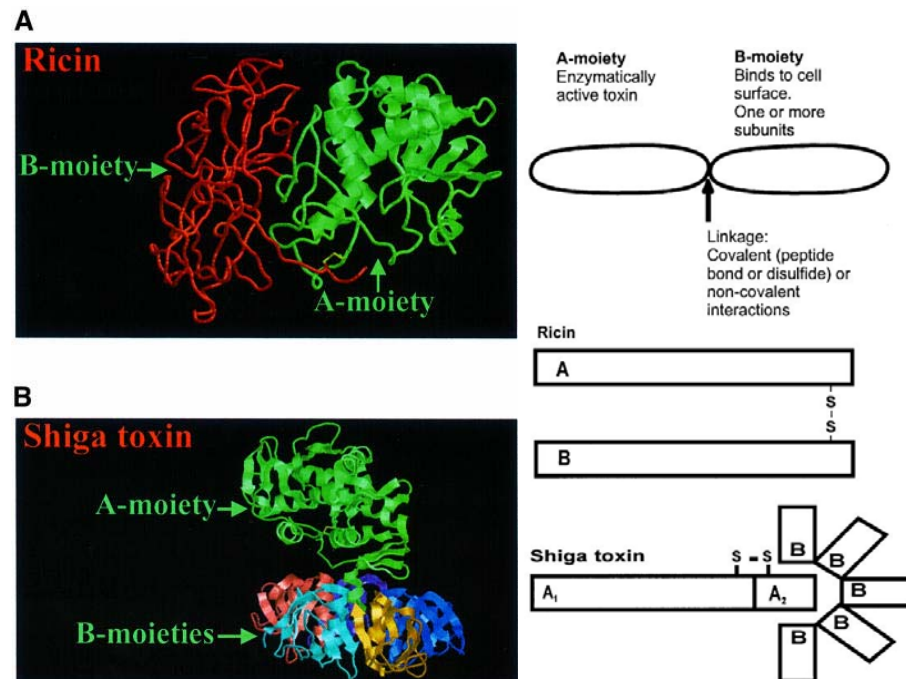
**Figure 4 Schematic representation of three types of Ribosome Inactivating Proteins (Park *et al.*, 2004)**

As shown in the schematic representation of RIPs in Fig 4, type I RIPs such as PAP and Saporin consist of single peptide chain. Whereas type II RIPs, such as ricin and Shiga-like toxin, consist of single peptide A chain which is covalently bonded to B chain which helps in binding of the protein for the entry into the cells. Type I RIPs lacking the presence of lectin binding B chain, do not exhibit optimal binding to the cell surface and thus are considerably less toxic than type I RIPs. Type III RIPs, which are found in maize and barley, consist of single chain proteins. These type of RIPs need to undergo proteolysis in order to be active and exhibit their toxicity.

## Structure of Shiga-like toxins

Structure of Shiga-like toxins 1 and 2 consists of one A subunit and five B subunits as shown in the schematic representation in Fig 5. A subunit is enzymatically active while B subunits help in binding to the cell surface (Park *et al.*, 2004). The A subunit to which the B subunits are non-covalently bonded, a trypsin sensitive site underlying a disulfide bridge. A1 and A2 subunits are further generated by proteolytic processing of the loop area and reduction of the disulfide bonds. The A1 fragment inhibits protein synthesis after release in the cytosol by removing one adenine from the 28S RNA of the 60S ribosomal subunit. The binding moiety B subunit consists of five identical subunits. All the B subunits can bind to the glycolipid receptors, the globotriaosylceramide (Gb<sub>3</sub>) at the cell surface. One variant of the Shiga-like toxin 2, Stx2e, binds to the Gb<sub>4</sub> receptor. B subunits have peptide extensions, which help in binding of the Gb<sub>3</sub> receptor (Hagnerelle *et al.*, 2002). B subunit of molecular weight 7.7kDa is a monopentamer which forms a ring like structure. This ring is formed by peripheral anti-parallel  $\beta$  sheets and a central pore made of five  $\alpha$ -helix structures, each representing one of the five subunit chains. At the level of the central pore, A subunit makes a contact with the B subunit ring-like structure to form covalent bonds.

The molecular weight of the intact toxin is about 70kDa, with the A subunit of about 32kDa and each B subunit of 7.7kDa as shown in Fig 6. Stx1A-A fragment has 315 amino acids, whereas Stx2-A has 318 amino acids.



**Figure 5** Ribbon structures of Ricin and Shiga toxin are showed on left. A chain and B chains are indicated in order to show their position in 3D structure of protein. Schematic representation on the right shows A chain and B chain of ricin and A chain and five B subunits of Shiga toxin bonded by disulfide bonds (Sandvig *et al.*, 2000)

All the B subunits have 89 amino acids. X-ray crystallographic analysis demonstrated that the B-subunit pentamers form a doughnut shaped structure with the carboxy-terminus of the A-subunit inserted into the central pore of B-subunits.

A primary mechanism of Stx-mediated damage is direct Stx cytotoxicity for vascular endothelial and renal epithelial cells. The epithelial cells of the kidneys are rich in Gb<sub>3</sub> receptors. B subunits of Stx bind to these Gb<sub>3</sub> receptors and facilitate the cell surface binding of the toxin.

It is very important to understand the mechanism of action of STEC on a molecular level in order to find efficient treatment for the diseases caused by STEC that

enter water and food chain. When the toxin affects humans, causing disease symptoms like bloody diarrhea and HUS in more severe cases, there is no effective and immediate cure available at the present date. While there is indirect evidence that human vaccination against STEC may be effective in preventing illness, at present, there are no vaccines or therapeutics for human STEC infections. A successful human vaccine would need to elicit antibodies either aimed at preventing STEC colonization in the intestinal tract or at neutralizing Stx to prevent the development of HUS. Although STEC strains are generally susceptible to a variety of antibiotics, studies have shown that the use of antibiotics negatively alters the outcome of STEC infections causing increased incidence of HUS (Park *et al.*, 2004). This is likely as the lysis of bacteria by some antibiotics leads to increased release of toxin as well as to increased toxin synthesis during the induction of lysogenic toxin-producing bacteriophage. Non-toxic recombinant vaccines need to be developed for the prevention and treatment of diseases caused by Shiga-like toxin. This study makes an attempt towards understanding the mechanism of action of Shiga-like toxins at the molecular level, which may provide an important step towards developing an effective treatment against the diseases caused by STEC.



## MATERIALS AND METHODS

### Random Mutagenesis of NT890 (Stx1A in pYES vector)

The Stx1A plasmid which was cloned into the yeast expression vector pYES2.1/V5-His-Topo (*Invitrogen*) was incubated with 7% hydroxylamine for 20 hr at 37°C. Hydroxylamine is known to act as a mutagen producing several point mutants in the genes. The plasmid treated with hydroxylamine was then precipitated and transformed into yeast. Yeast cells were plated onto Uracil dropout media supplemented with 2% glucose. These colonies were then individually plated onto Uracil dropout media (SD-Ura) containing 2% galactose. The colonies that were observed to be growing on galactose containing medium were separately replicated from the original SD-Ura plates containing 2% glucose. The Stx1A plasmid was isolated by Smash and Grab method from the yeast colonies that were able to grow on galactose. For Smash and Grab method, 0.3 g of glass beads and 0.2 ml of lysis buffer (10 mM Tris, pH 8.0, 1 mM EDTA, 100 mM NaCl, 1% SDS, 2% Triton X-100) and 0.2 ml of 1:1 mix of phenol and chloroform were added to the cell pellet. After vortexing the tube, 0.2ml of TE (10 mM Tris, 1 mM EDTA, pH 8.0) were added. After centrifuging the tubes at high speed, only the aqueous layer was collected and mixed with 100% ethanol and centrifuged again. The pellet is washed with 70% ethanol and after removing the supernatant the pellet was air-dried and resuspended in sterile H<sub>2</sub>O. These plasmids were then transformed in DH5 $\alpha$  bacterial cells by electroporation transformation. Electroporation provides a method of transforming *E. coli* to efficiencies greater than those with the best chemical methods. The survival and

transformation of cells are of much higher efficiencies by electroporation. To prepare competent bacterial cells for transformation by electroporation, fresh cells were treated with ice cold water and 10% glycerol. These cells were then transferred to sterile cuvettes along with the DNA plasmid obtained from the yeast colonies as stated above. Gene pulser apparatus set at 25 $\mu$ F sends pulse through the mixture, which is then plated onto plates containing lactose broth medium with ampicillin. The bacterial colonies were grown to isolate plasmids which were subsequently retransformed into yeast to confirm that the resistance was due to the plasmid. Plasmids isolated from colonies expressing Stx1A were characterized by sequence analysis.

### **Site-directed mutagenesis of NT890 (Stx1A in pYES vector)**

Sites for making the mutations were determined using the position of the active site and the nucleotides that are potentially critical for the activity and retrotranslocation of the toxin. Mutations in the Stx1A plasmid were introduced using the Stratagene QuikChange™ site-directed mutagenesis kit, a technique followed by Dr. Rong Di from our laboratory. A set of oligomeric primers were used for PCR amplifications of the template plasmid with wild-type Stx1A. After PCR amplification, the template plasmid was removed by *DpnI* digestion. The mutated plasmids were transformed into *E.coli* DH5 $\alpha$ . Mutations were confirmed by sequencing the plasmids. The mutant plasmids were retransformed in yeast for confirmation of the mutation and further characterization.

### **Analysis of growth rate**

Yeast cells were grown in SD-Ura containing 2% glucose to an  $A_{600}$  of 0.3 and were then transferred to SD-Ura containing 2% galactose. Aliquots were taken every 2 h, and the  $A_{600}$  was recorded. Doubling times were calculated based on exponential growth between 6 and 12 h post induction.

### **Cell viability assay**

Yeast cells expressing Stx1A mutants were grown on SD-Ura containing 2% glucose to an  $A_{600}$  of 0.3 and then transferred to SD-Ura medium containing 2% galactose to induce Stx1A protein expression. A serial dilution of cells was plated on SD-Leu plates containing 2% glucose at 0, 4, 6 and 10h post induction. Plates were incubated at 30°C for approximately 48 h. The cell growth was recorded by scanning the plates.

### **Protein extraction from yeast cells**

Yeast cells showing growth on galactose were grown in SD-Ura medium with 2% glucose. They were pelleted and resuspended in SD-Ura medium with 2% galactose. At the 6<sup>th</sup> hour of induction, these cells were centrifuged at 2000 g for 2 minutes. Cells were resuspended in 1x low-salt buffer (20 mM HEPES-KOH, pH 7.6, 0.1 M potassium

acetate, 5 mM magnesium acetate, 1 mM EDTA, 2 mM dithiothreitol, and 0.1 mM phenylmethylsulfonyl fluoride) and lysed using glass beads. Samples were centrifuged briefly at 2300 rpm to remove cell debris and glass beads. The supernatant was transferred to a new tube and centrifuged at 15,000 rpm for 15 min to separate cell membranes and cytosolic fraction. After removing the cytosolic fraction, the membrane pellet is resuspended in 10  $\mu$ l of 1x low salt buffer.

### **rRNA depurination assay**

Shiga-like toxin family of toxins is known to depurinate ribosomes by removing specific adenine residue from highly conserved  $\alpha$ -sarcin ricin loop from the large subunit of the rRNA as previously described. Dual primer extension analysis was conducted to quantify rRNA depurination. 2  $\mu$ g of total yeast RNA from cells expressing Stx1A was hybridized with  $10^6$  cpm of end-labeled depurination primer (5'-AGCGGATGGTGCTTCGCGGCAATG-3'). The second primer hybridized upstream of the depurination site close to the 5' end of the 25S rRNA. For accurate quantification, the labeled 25S control primer was diluted 1:4 with unlabeled 25S control primer. Superscript II reverse transcriptase was used in the primer extension assay. Extension primers for the control and depurination fragments (100 nt and 73 nt, respectively) were separated by running on a 7 M urea-5% polyacrylamide denaturing gel and quantified as the percentage of the depurination caused due to wild type Stx1A (Parikh *et al.*, 2002).

## RESULTS

### Random and Site-directed Mutagenesis

Stx1A plasmid was treated with 7% hydroxylamine for generating mutants as described. The colonies that were observed to be growing on SD-Ura medium with 2% glucose after the plasmid was treated with 7% hydroxylamine were replicated on SD-Ura medium with 2% galactose. The colonies that were growing on galactose containing medium are selected for further characterization and confirmation of loss of cytotoxicity. This is because the Stx1A gene was previously cloned into the yeast expression vector containing galactose inducible *GALI* promoter. The growth on galactose indicates that the cells are not killed due to toxin production and thus the mutant that grows on galactose containing medium is regarded as a non-toxic mutant. Out of all the colonies that were replicated on the galactose containing medium, 131 colonies were able to show considerable amount of growth. These mutants were selected for further characterization. The colonies were grown and plasmids were isolated from yeast cells by Smash and Grab method. These plasmids were further transformed into bacterial DH5 $\alpha$  cells and then sequenced in order to find out the site where the mutation has taken place. These plasmids were retransformed into yeast cells in order to confirm the presence of the mutation. Site-directed mutants were created as described before at active sites and nucleotides that are potentially critical for the activity and retrotranslocation of the protein. N75A, Y77A and E167A are single point mutants created by site-directed mutagenesis. E167 and R170 residues contribute to the active site of the toxin. C terminal

mutants, C242\*, N241\*, L240\* and V236\* were obtained by targeting the specific nucleotides by site-directed mutagenesis method. Double mutant, E167A/R170A which disrupts both active sites of the toxin was also obtained by site-directed mutagenesis.

### Group I

Amino acid change	Toxicity	Doubling time (hr)	Depurination (%)
pYES (vector control)	N	2.96	-
Stx1A (WT)	Y	12.1	Y(100)
R21H	Y	12.9	Y (96.7)
G25D	N	7.3	Y (100)
G25R	N	5.7	Y
R63W	Y	11.3	Y (21.3)
N75A (S.D.)	N	6.3	Y (92.5)
Y77A (S.D.)	N	6.0	Y (23.2)
G80R	N	5.8	Y (100)
S96Y	N	8.7	Y (34.8)
R119C	Y	13.2	Y (28.7)
T137I	Y	15.2	Y (34.3)
A155R	N	6.5	Y (34.7)
R160W	Y	14.7	Y (21.9)
E167K	N	7.8	Y (33.2)
E167A (S.D.)	N	6.5	Y (21.1)
R170C	Y	18.0	Y (20.8)
R172W	Y	10.3	Y (36.5)
R172Q	Y	13.1	Y (41.0)
R179H	Y	14.7	Y (100)
G234E	Y	19.2	Y (98.3)

**Table 2** Group I contains random mutants with mutations at single amino acid site. These mutants were characterized for cytotoxicity, doubling time and depurination, the comparison is as shown in the table 2. (S.D. denotes mutants generated by site-directed mutagenesis).

Analysis of nucleotide sequence indicated presence of 38 different mutations. These mutants showed loss of toxicity by growth on galactose. The loss of cytotoxicity was confirmed by viability assay. Many of the mutations were obtained multiple times

indicating the saturation of mutation screening by hydroxylamine. The mutants were divided into three groups. Group I contained mutations at single amino acid. 20 such mutants were obtained which were characterized for loss of cytotoxicity, expression of proteins and depurination.

### Group II

Amino acid change	Toxicity	Doubling time (hr)	Depurination (%)
Q173*	N	11.8	N
Q175*	N	7.3	N
W203*	N	9.6	Y (11)
Q216*	N	4.9	N (10)
G227*	N	9.4	Y (88)
V236*(S.D.)	N	12.6	Y (73)
L240*(S.D.)	N	8.8	Y (83)
N241*(S.D.)	N	12.3	Y
C242*(S.D.)	Y	14.7	Y (49)

### Group III

Amino acid change	Toxicity	Doubling time (hr)	Depurination (%)
D58N/G177R	N	8.7	Y (51)
N75A/R119T	N	6.7	Y (33)
V78M/N83D	N	11.8	Y (68)
A166T/A250V	N	8.9	Y (100)
R119C/R156K	N	4.3	Y (96.5)
R119C/R289K	N	6.5	Y (100)
R120H/L201F	N	12.9	Y (95)
S134L/A284G	N	12.7	Y (93)
E167A/R170A(S.D.)	N	5.8	Y (68)

Table 3 Group II contains random and site-directed mutants with amino acid changed to a stop codon. Group III contains random mutants with mutations at two amino acid sites. These mutants were characterized for cytotoxicity, depurination and doubling time as indicated above.

Group II contained 9 different mutations with a premature stop codon. This resulted in truncation of the Stx1A gene and thus truncation of the protein produced. Group III contained 9 different mutants with mutations at more than one amino acid. All of these mutants were retransformed into yeast cells.

### **Indication of loss of cytotoxicity of the mutants by cell viability assay**

The mutants generated by random and site-directed mutagenesis were further characterized using viability assay, immunoblot analysis and ribosome depurination analysis. Viability assay involved induction of cells with galactose containing medium, this induced the protein expression which is lethal to the cells. Wild type Stx1A showed high level of cytotoxicity by viability assay. By 10 hr after induction, it reduced viability of the cells to a minimum as seen in Fig. 7 A. Whereas, the mutants exhibited higher viability of the yeast cells even after induction with galactose for 10 hr. Mutants G25D, G25R, N75A, Y77A, G80R, S96Y, A155R, E167K, E167A from group I (Fig. 7 A&B) showed growth comparable to the empty vector used as a control, indicating loss of cytotoxicity. Random mutants from group II, Q173\*, Q175\*, W203\*, Q216\*, G227\* showed viability of cells, which indicates loss of cytotoxicity after 10 hr induction. Site-directed mutants V236\*, L240\*, N241\* showed loss of cytotoxicity but C242\* showed reduction in viability of cells after 10 hr induction indicating the mutant is in fact cytotoxic to the cells. All random two point mutants from group III, D58N/G177R, N75A/R119T, V78M/N83D, A166T/A250V, R119C/R156K, R119C/R289K, R120H/L201F, S134L/A284G and the site-directed mutant E167A/R170A showed loss of cytotoxicity upon 10 hr induction (Fig. 8 and Fig. 9).



A)

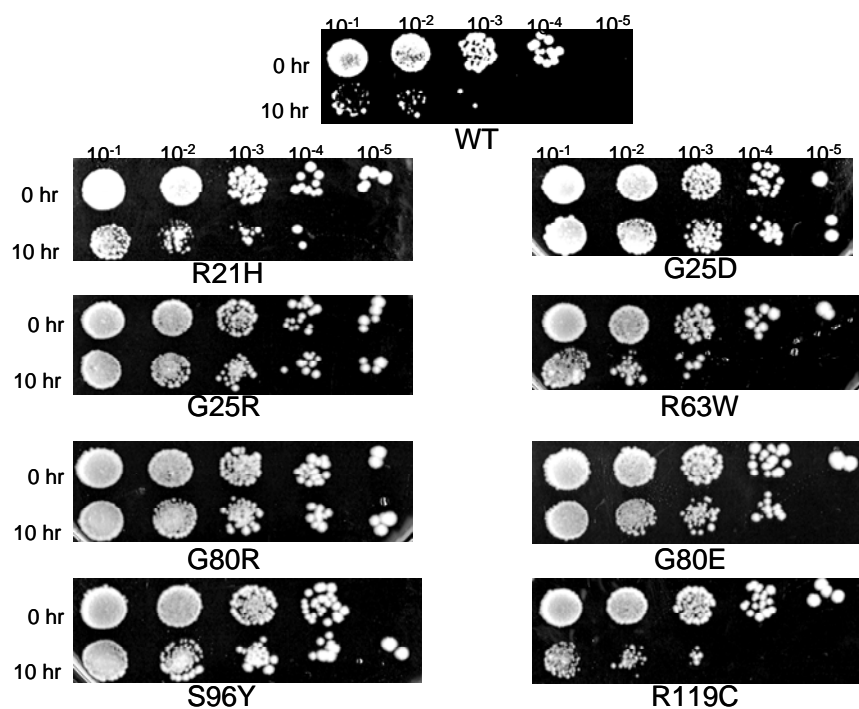


Figure 7 A) Viability assay for the random mutants from Group I

B)

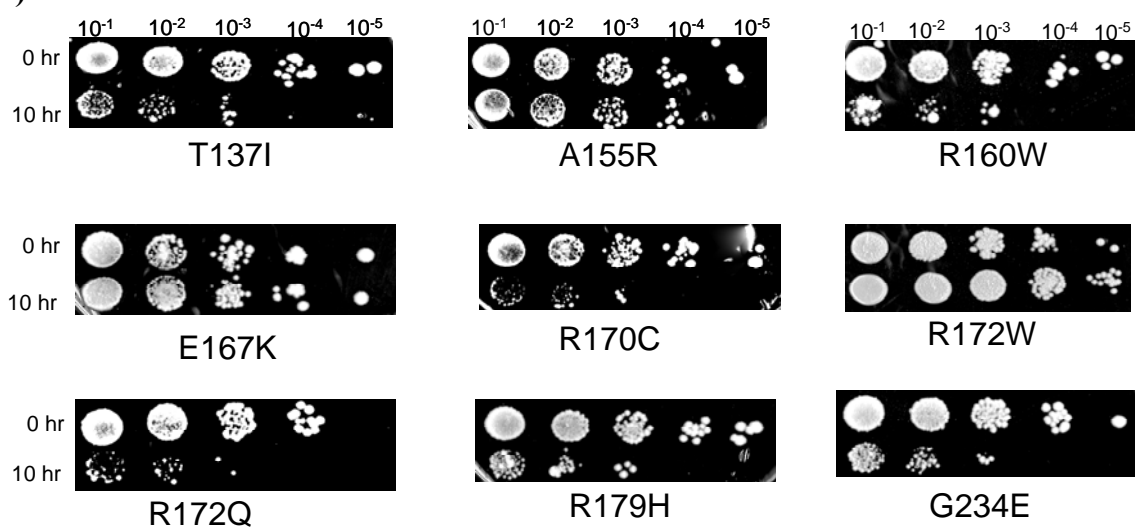
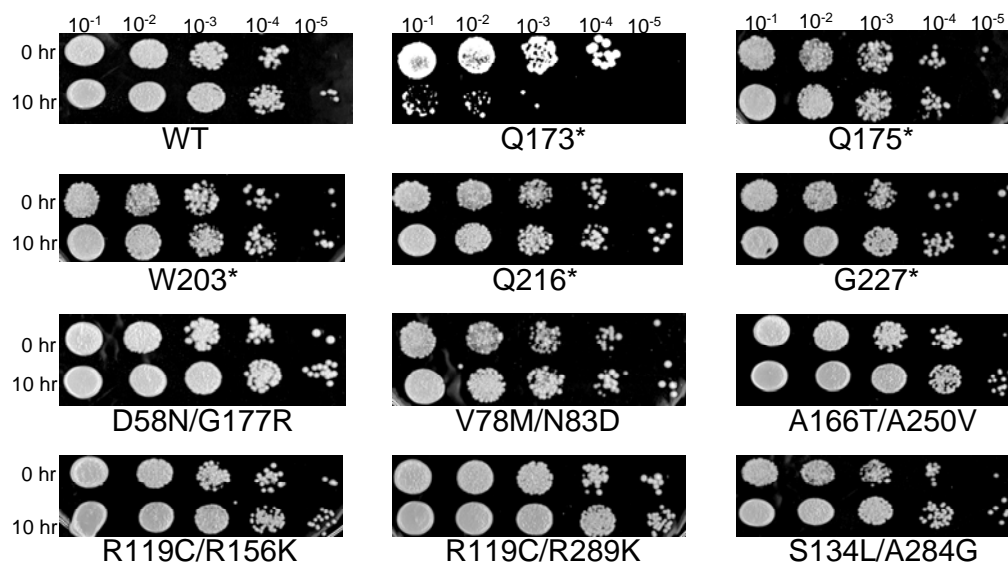
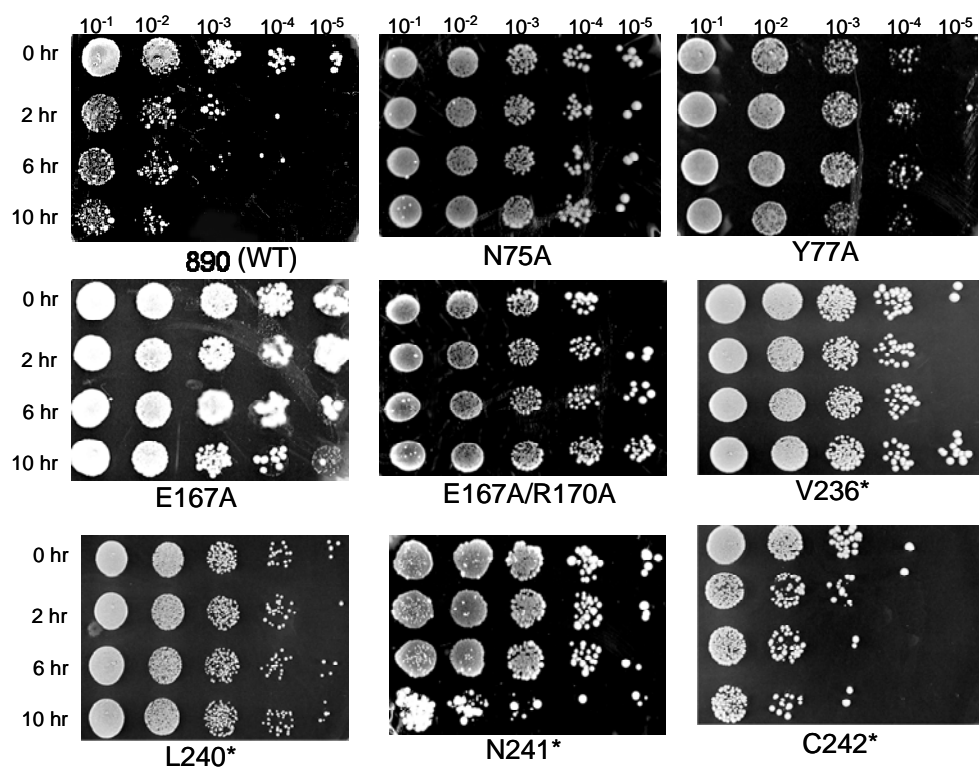


Figure 7 A) and B) Viability assay for all the random mutants from Group I is shown above. The growth at 10hr post induction is directly compared against that of wild type Stx1A in order to determine whether loss of cytotoxicity is observed in the mutants.



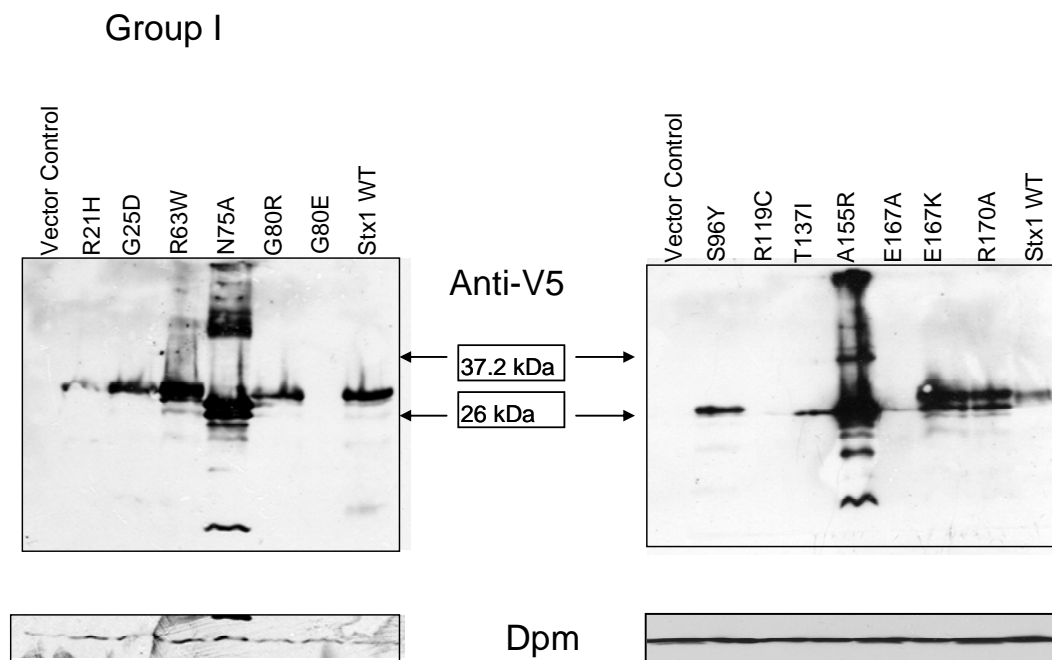
**Figure 8** Viability assay for random mutants with stop codons and double mutants from Group II and III respectively is shown above. Growth at 10 hr post induction is compared with that of the wild type Stx1A in order to determine whether the mutation is toxic or non-toxic.



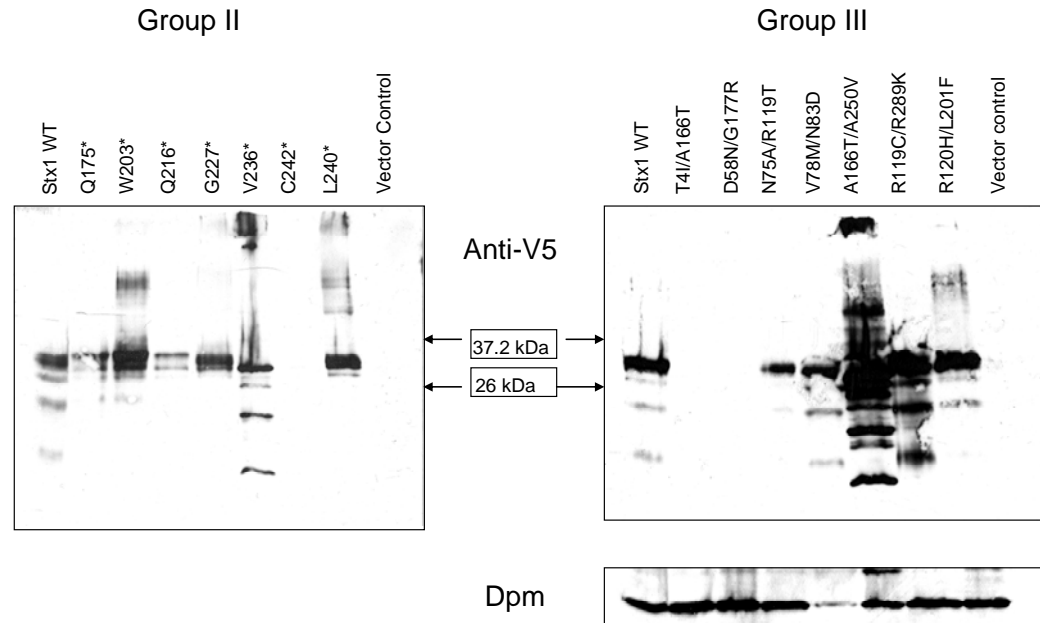
**Figure 9 Viability assays for site-directed mutants (mutants with stop codons, single point mutation and double mutations) at 10 hr post induction is compared with that of the wild type Stx1A.**

### **Wild-type Stx1A and nontoxic mutants expressed in yeast**

All the mutants were induced for 6hr with galactose containing SD-Ura medium in order to examine the protein expression. Cells containing wild-type Stx1A consistently showed a single protein band of size 32kDa on the Western blot. The vector in which Stx1A gene was cloned is V5 tagged, thus anti-V5 antibodies were used for examination of protein expression. ER membrane fractions of the wild-type Stx1A as well as the mutants expressed the protein, indicating that the presence of protein is mainly associated with the ER membrane and not the cytosolic fractions.



**Figure 9 Group I random mutants were characterized for protein expression by Immunoblotting**

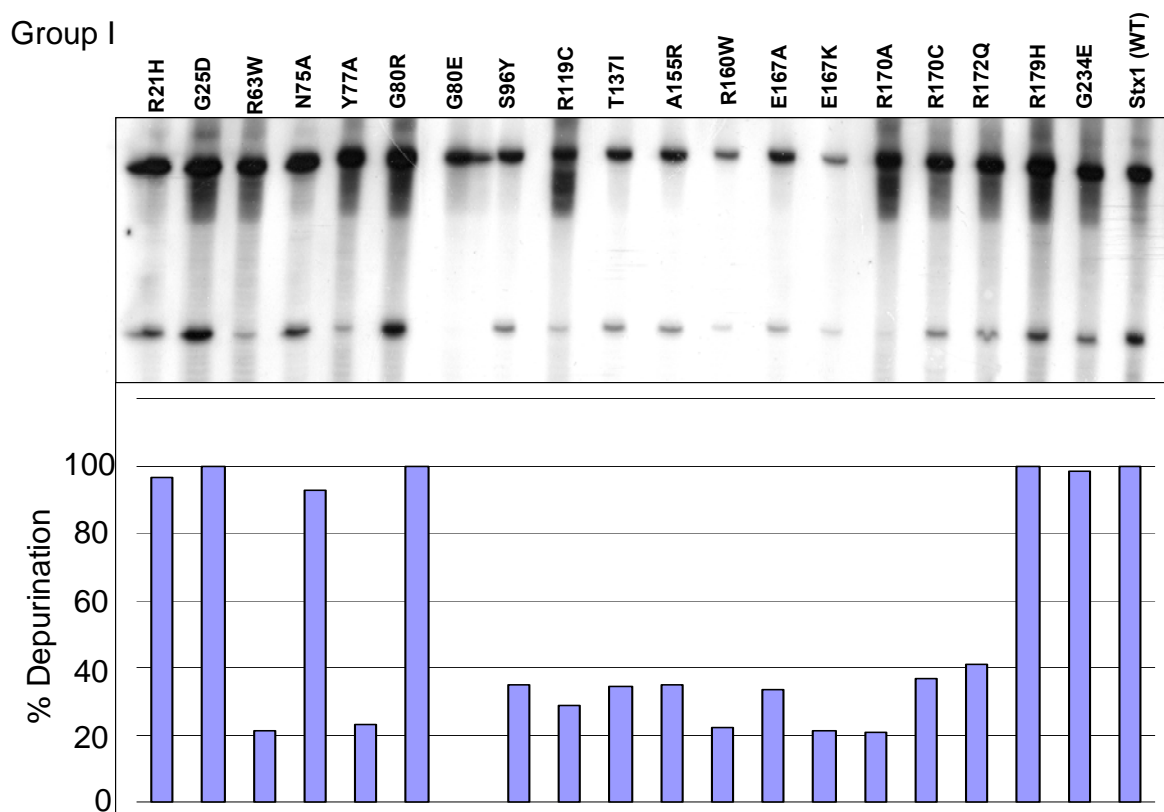


**Figure 10 Group II and Group III mutants were characterized for protein expression by Western Blotting using anti-V5 antibody**

As indicated in Fig. 9 and Fig. 10, twenty two mutants showed expression of protein by immunoblot analysis. Dpm1p protein antibody was used for hybridization of the blots in order to ensure equal loading of the protein. Yeast cells carrying non-toxic form of Stx1A expressed comparable levels of protein as in case of wild-type Stx1A. Most of the mutants showed the expression of protein which had a size comparable to that of the wild-type Stx1A. Some mutants like N75A, A155R, V236\* and A166T/A250V showed multiple bands indicating that possibly the protein was not stable and it might be undergoing processing or degradation. Apart from this, mutants R119C, C242\*, D58N/G177R did not express protein while R119C and C242\* are toxic to the cells and D58N/G177R is non-toxic. A possible explanation could be that the mutation at that particular site makes the protein unstable and thus the mutant fails to express the protein on the immunoblot.

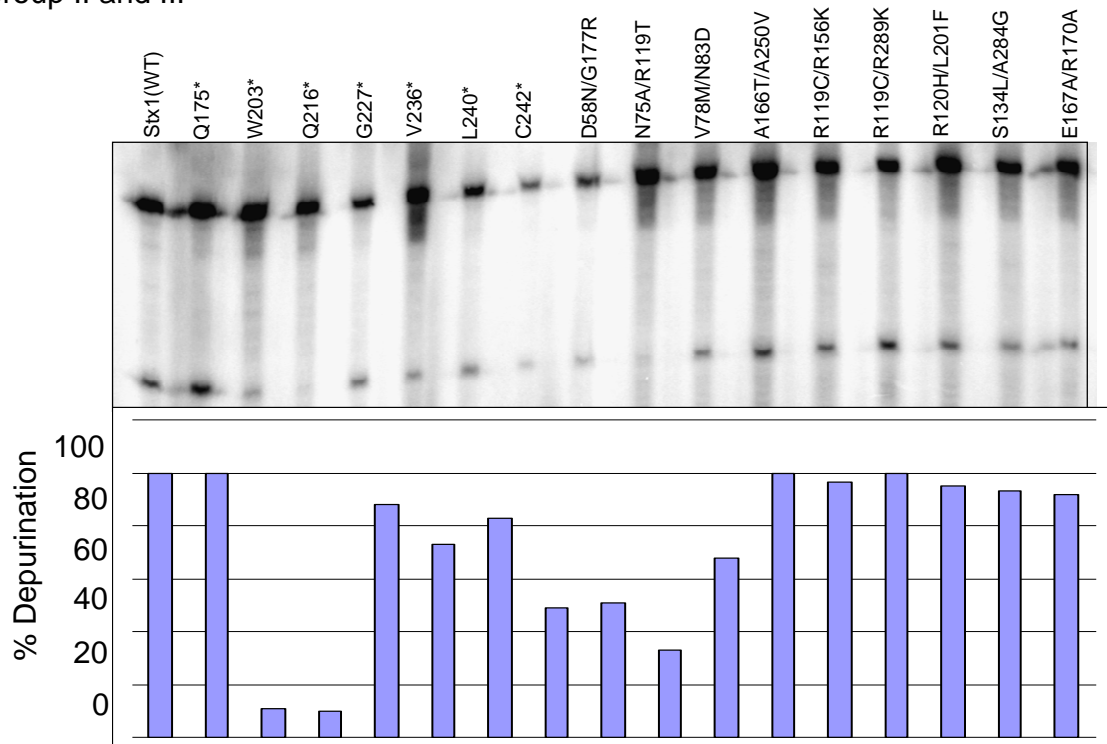
### Nontoxic mutants depurinate the rRNA

The random and site-directed mutants were examined for depurination of rRNA by dual primer extension analysis. Total RNA was isolated from the yeast cells and primer extension reaction was carried out. Analysis of the depurination assay showed that mutants expressing Stx1A protein, R21H, G25D, N75A, G80R, G227\*, V236\*, L240\*, C242\*, V78M, A166T/A250V, R119C/R156K, R120H/L201F showed ribosome depurination (Fig. 11 & Fig. 12).



**Figure 11** Group I random mutants were characterized for ribosome depurination by primer extension assay. The levels of depurination were quantified and plotted as a bar graph

## Group II and III



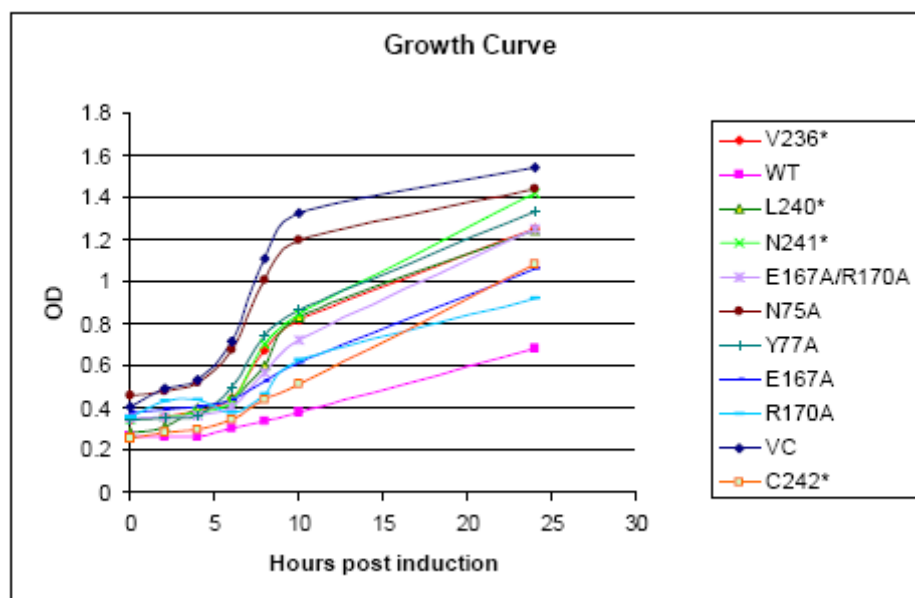
**Figure 12** Group II and III random mutants were characterized for ribosome depurination by primer extension assay and the quantification of depurination was plotted as a bar chart below \*(This Fig. is a representative gel for all the group II and III mutants)

G80R showed depurination which was comparable to the cells expressing wild-type Stx1A. Mutants R179H and G234E showed cytotoxicity as indicated by viability assay and also showed level of depurination similar to that of wild type Stx1A. Most of the mutants showed depurination of ribosomes less than the wild type Stx1A and they had showed reduction in toxicity by viability assay. Mutants like G80R, N75A showed high levels (100% and 92.5%) of ribosome depurination which was comparable to the wild type Stx1A, though they have showed reduction in cytotoxicity as indicated by the

viability assays. This suggests that ribosome depurination alone is not sufficient for cytotoxicity.

### **Doubling time of the cells of nontoxic mutants does not correlate with ribosome depurination**

Cell growth rate was examined by measuring the doubling time of the mutants. Yeast cells expressing wild type Stx1A showed a doubling time of 12.1 hr while the empty vector showed a doubling time of 2.96 hr. Doubling time of most of the mutants was between the doubling times for the empty vector and the wild-type Stx1A cells as shown in Tables 2 and 3.



**Figure 13** Optical density of the cells at  $A_{600}$  was measured at 0, 2, 4, 6, 8, 10 and 24 hr post induction. The growth curve of the mutants is compared with wild type Stx1A and vector control

The doubling time of the single point mutants R21H, R119C, T137I, R160W, R170C, R172Q, R179H and G234E as well as mutants with stop codons V236\*, N241\* and C242\* showed higher doubling time than the cells expressing wild-type Stx1A, thereby suggesting slower growth rate. Though all these mutants showed depurination of the ribosomes, they also exhibited loss of cytotoxicity by viability assay. These results demonstrate that the doubling time of the mutants was not related to the ribosome depurination. This shows that cytotoxicity exhibited by the cells is not due to ribosome depurination alone.



## DISCUSSION

A large scale mutagenesis was carried out using 7% hydroxylamine on Stx1A wild type plasmid DNA. *Saccharomyces cerevisiae*, commonly known as yeast, is used as a simple system as it is tractable to classical genetic techniques and functions in yeast have been studied in great detail by biochemical approaches. As expected for hydroxylamine mutagenesis, most of the mutants showed C-to-T and G-to-A base pair changes. This shows exchange of one purine to another purine (A-to-G or G-to-A) and exchange of one pyrimidine to another pyrimidine (C-to-T or T-to-C), which is classified as transition mutation. In case where a purine is exchanged for a pyrimidine or vice versa, the mutation is classified as transversion. A transition is caused by mutagens like nitrous acid and hydroxylamine, the later was used to produce point mutations in our laboratory.

Though hydroxylamine is known to produce mutations predominantly at a single amino acid, out of total 38 random mutants, 7 mutants showed change of existing amino acid to a stop codon. Whereas 9 mutants showed changes at two different amino acids producing double mutants. Many of these mutants were isolated twice or thrice indicating the saturation of mutation screen using hydroxylamine. Such a large scale mutagenesis was carried out in order to isolate nontoxic Stx1A mutants from yeast. Each mutant was characterized in order to determine the precise effects of the mutation on cytotoxicity, ribosome depurination and translation inhibition and it suggests that ribosome depurination is not solely responsible for the cytotoxicity of Shiga-like toxin 1 (Stx1A). Mutations that were produced in first 21 amino acids with stop codons were not isolated, since a change to stop codon would result in a short peptide chain which will not be

detected by immunoblot analysis. Mutations in the N-terminal extension encoding first 21 amino acids may act in the same way as pre-RTA (A chain of ricin) mutants in the N-terminal region, in a way that these mutants may disrupt the ability of the protein to translocate through the endoplasmic reticulum membrane with little or no change in its cytotoxicity.

Glutamic acid at amino acid position 167 and Arginine at amino acid position 170 constitute to the active site of the Shiga-like toxin 1. Site directed mutations were produced at individual amino acid as well as double mutant containing changes at both the active site residues. Loss of cytotoxicity was observed in single active site mutants (E167A and R170A) as well as the double mutant (E167A/R170A) as observed by the growth on galactose containing medium as well as viability assays. This indicates that both residues at the active site 167 and 170 amino acid positions are critical for exhibiting the cytotoxicity of Stx1A. These mutants were also characterized for ribosome depurination. Single point mutants at both active sites (E167A and R170A) exhibited some (~30%) depurination of the ribosome. The double mutant E167A/R170A where both the active sites are changed to Alanine exhibited increased amount (68%) of ribosome depurination when compared with ribosome depurination due to wild type Stx1A as 100%. These results indicate loss of toxicity does not necessarily cause total loss of the ability of the mutants to depurinate ribosomes.

Mutants obtained by random mutagenesis of Glycine at amino acid 25 changed to Aspartic acid, Glycine at amino acid 80 changed to Arginine exhibited total loss of toxicity with doubling times of 7.3 hr and 5.8 hr as opposed to 12.1 hr doubling time of

the wild type Stx1A. These two mutants showed ribosome depurination as high as caused due to wild type Stx1A, emphasizing that ribosome depurination alone is not sufficient for cytotoxicity of the Stx in yeast cells.

At the same time, Arginine at 179 amino acid position changed to Histidine did not indicate loss of toxicity with a doubling time of 14.7 hr but it also showed ribosome depurination as high as the wild type Stx1A as showed by the non toxic mutants. This suggests that ability to depurinate ribosomes is not correlated with cytotoxicity of the mutants.

The random mutant Q175\* showed depurination as comparable to the wild type initially. This assay was repeated after retransformation of the plasmid into the yeast by Eric Kyu from our laboratory and this particular mutation did not show any depurination. This can be explained as an error in the assay carried out previously and this error was confirmed and eliminated by repeating the assay.

C-terminal region has been known to be critical for transport of the protein into the cytosol as observed in case of RIPs ricin and PAP (Sandvig *et al.*, 2000). Site-directed mutations were produced at this region with each amino acid changed to stop codon starting at the amino acid Valine at 236 to Cysteine at 242 amino acid position. All these site-directed mutants showed loss of cytotoxicity except Cysteine at amino acid 242. V236\*, L240\* mutants with stop codons showed depurination of ribosomes of the order of 73% and 83%. Whereas, the non toxic stop codon mutant C242\* showed reduced depurination of (49%).

Different methods of mutagenesis are used to produce random as well as site-directed mutants of the RIPs. X ray crystallographic studies are done to indicate the critical residues in the structure as well as in order to study the binding of antidote inhibitors which are known to disrupt the activity of this class of enzymes (RIPs). So far to our knowledge, this is the first attempt at studying the correlation of cytotoxicity and ribosome depurination property of Shiga-like toxin 1 by production and characterization of its non-toxic mutants. The cytotoxicity and depurination studies reveal that ribosome depurination is not sufficient for the cytotoxicity of Shiga-like toxin 1 (Stx1A).

## **FUTURE DIRECTIONS**

Generation and characterization of random and site-directed mutants in an *in vitro* system like *Saccharomyces cerevisiae* is important for identifying intra-molecular regions of Shiga-like toxins and for studying its protein structure-function relationships. Further studies would include the investigation of regions of Stx1A that are critical for translocation and the activity of the enzyme. Targeting these specific regions might provide insights into production of vaccines and other treatments to treat the illnesses caused in humans due to Shiga-like toxins produced by *E. coli* O157:H7.

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