Characterization of the OmpU mutants affecting pore size in inducing apoptosis in mammalian cell line

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Certificate of Examination

This is to certify that the dissertation titled "Characterization of the OmpU mutants affecting pore size in inducing apoptosis in mammalian cell line" submitted by Mrs. Priyanka Jeeth (Reg. No. MS10049) 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.Samarjit Bhattacharya (Supervisor) Dr. Kaushik Chattopadhyay Dr. Arunika Mukhopadhaya

Dated: April 24,2015

Declaration

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

Priyanka Jeeth D P

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. Arunika Mukhopadhaya
(Supervisor)

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List of abbreviations

OmpU	Outer Membrane Protein U
PCR	Polymerase Chain Reaction
SDS PAGE	Sodium Dodecyl Sulfate Poly-Acrylamide Gel Electrophoresis
PBS	Phosphate Buffer Saline
Wt	Wild Type
DNA	Deoxy ribose Nucleic Acid
AIF	Apoptosis Inducing Factor
PI	Propidium Iodide
JC-1	5,5′,6,6′-tetrachloro-1,1′,3,3′-tetraethylbenzimidazolylcarbocyanine iodide
MMPT	Mitochondrial Membrane Permeability Transition
PCD	Programmed cell death

ABSTRACT

It has been demonstrated in versatile modes, the role of mitochondria in determining the life and death strategy of the cell [1, 2, 3, 4].The permeability transition property of mitochondria is one such key event in deciding the death of cell upon certain stimuli [1, 2].Considering, the permeability pores of mitochondria, there have been contrasting reports of the ensuant response of certain porins on interaction with the mitochondrial membrane. Porins are transmembrane beta barrel proteins that can literally form pores on the cell membrane. The amino acids lining the constriction zone or the eyelet region of the porin is claimed to determine the size of the pore and thereby the channel property of the protein. Mutations of the amino acids present in this region can have drastic impact on the pore size. In the present study, we check whether any alteration in the channel property of the porin OmpU has an effect on the magnitude of apoptosis induced in the target cell lines.

1. INTRODUCTION

While we still debate about the ‘death’ of unicellular organisms, incontestably, there exists a well-choreographed yet sophisticated execution mechanism in all multicellular forms. More than a philosophical concept, ’death’ is a crucial phenomenon that defines the strategy of the cell and is universal in some sense in all existing cellular machineries. At times of stress, cellular system tends to sacrifice some among them hoping the benefit of the whole, neglecting the individual cells [16]. There is an exponential increase in the perceptual structure of these execution mechanisms over the decades in linear to the technological advancements. The constant decision making of the cell in this context determines the fate of the beings as it is.

It is difficult to draw a clear boundary between different modes of cell death. The general criteria for the classification includes the morphological appearance of the cell, involvement of proteases such as caspases, functional aspects such as programmed or accidental as well as the immunological features [21]. It would be unfair to categorize a particular mode of cell death on the basis of certain limited features as the increasing evidence suggests the mechanistic overlap between the various execution machineries [20]. Apart from the programmed way of cell death, cell death can be accidental as well. Energy level of the cell may be the predominating factor here in deciding the programmed as well as the accidental mode of cell death [22, 23]. Sufficient energy is required to execute the death in a well programmed manner. If the cell fails to meet the minimum energy requirement, it may end up in necrosis, where cell contents leak out and may cause damage to the neighboring cells [22, 23].

In the present study we focus on one among the well characterized form of programmed cell death known as apoptosis. Apoptosis, as the name suggests is the fall off of cell. Various parameters characterize the apoptotic form of cell death including nuclear and cytoplasmic condensation, blebbing of plasma membrane which in fact influenced by the cytoskeletal rearrangement, fragmentation of DNA and most noticeably, formation of the apoptotic bodies [29]. Still today, apoptosis takes the centre stage as the prime mode of execution mechanism that subsists from embryonic stage until senescence [5].Initially,

caspases were claimed as the ‘central apoptosis executing molecules [6, 7, 16], but the increasing evidence suggests the independent existence of apoptosis irrespective of caspases [8, 9].

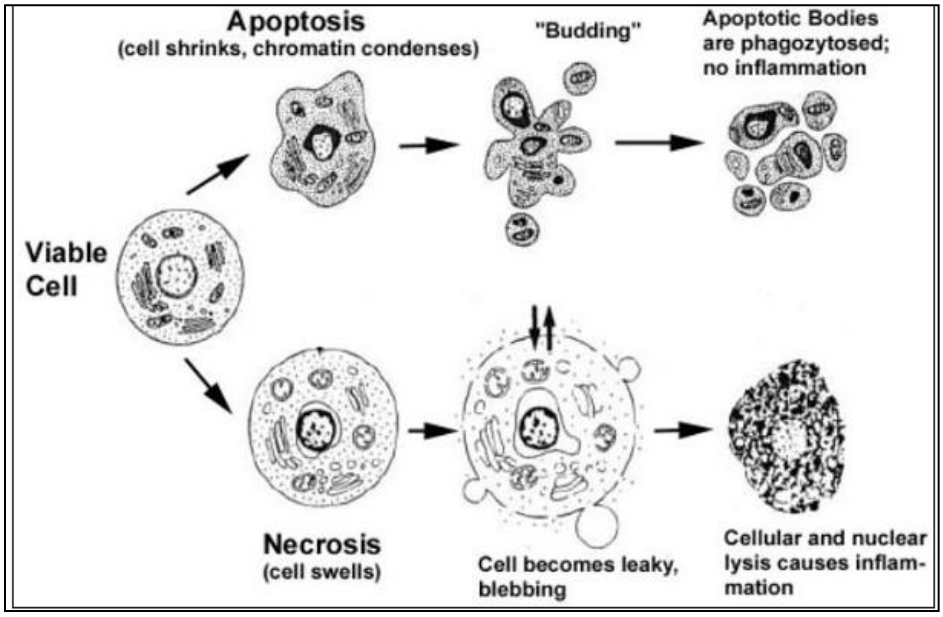

Fig 1: Representation of apoptotic and necrotic mode of cell death [25]

There are two established pathways for apoptosis, as of now, extrinsic and intrinsic. This study focuses on the intrinsic pathway, in which mitochondria is the sole player in determining the mode of death. The electrochemical potential gradient across the mitochondrial membrane is an indispensable factor for its membrane integrity [10]. There is a significant number of data that suggests the importance of the membrane integrity of mitochondria for its functional existence. Besides, once the MMPT happens, there seems to be an irreversible commitment towards the cell death [10, 11, 12]. Demise of the cell can be of two reasons, because of the release of AIF from mitochondria that can directly chop up the DNA [10]; secondly, since mitochondria is the limiting factor of the energy supply of the cell, cell may experience the shortage of energy with time and can end up in necrosis[22,23].

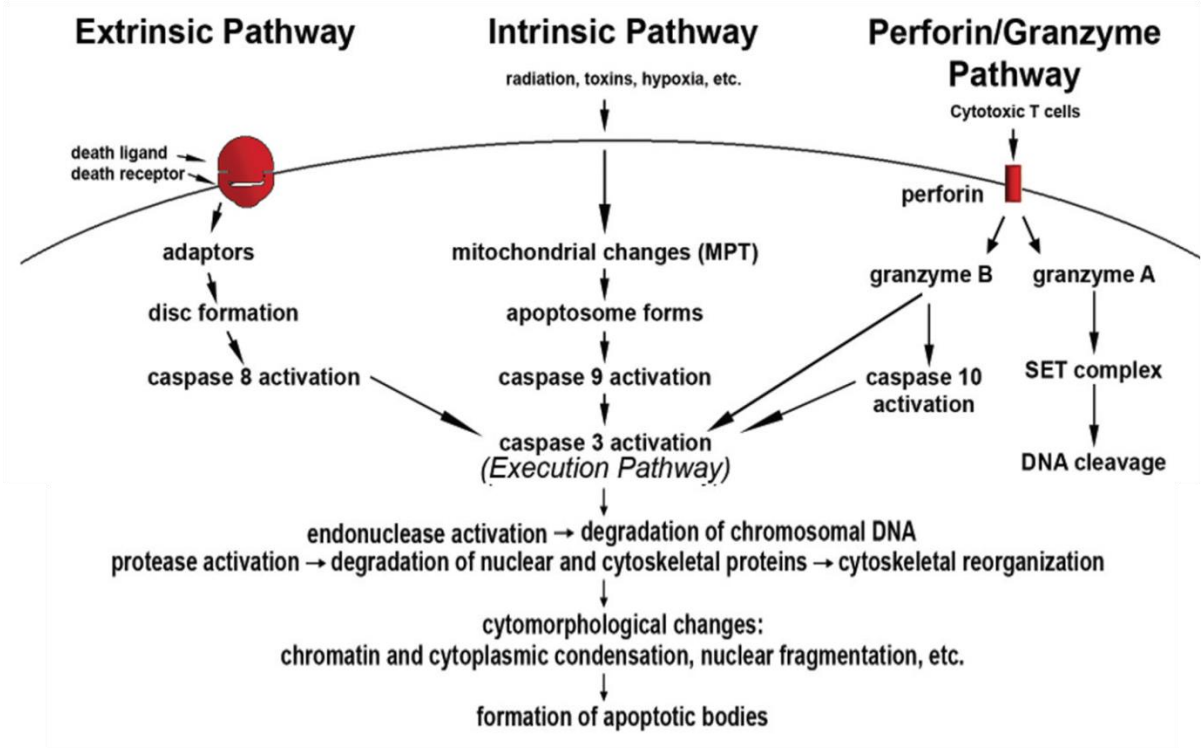

Fig 2: Schematic representation of the apoptotic events [24]

Our study focuses on the apoptotic role of the Outer membrane porinU (OmpU) of the cholera causing organism, *Vibrio cholerae.* Porins are transmembrane ß barrel proteins that forms triple barrel structures across the surface of the cell membrane[13,14].Their presence has been identified in gram negative bacteria as well as in mitochondria. Their primary role is reported to be in the transportation of hydrophilic molecules. The high expression rate of porins (10^4-10^6 copies per cell)[13]in the bacterial cell signifies their importance in the bacterial system .Depending on the environmental constraints, porins can adopt either a trimeric or a monomeric conformation[14].On the cellular membrane they often forms a triple barrel channel ,in which each channel consists of an individual pore. At times of stress, bacteria tend to modulate the expression level [13, 15] as well as the structural organization of the porins, owing to the high genetic flexibility.

The role of porins on cell membrane is in the transportation of hydrophilic molecules [27, 28]. This channel property of the porins is actually determined by the construction zone or the eyelet region. Aminoacids lining the construction zone are crucial in determining the channel size of the porin. The structural stability of the porin is defined by the electrostatic

interaction between the negatively charged residues of the L3 loop and the positively charged aminoacids lying opposite to them [17, 18, 19].The high structural dynamicity of the pore region depends on the charge as well as the side chain length of the aminoacids present on the eyelet region of the porin[17,19]. Hence mutations affects the aminoacids lining the constriction zone of the porin can have drastic effects on the channel property of the porin.

It has been observed that the *V.cholerae* OmpU can translocate to the mitochondrial membrane. Hence we are interested to evaluate whether the mutants of *V.cholerae* OmpU have any effect on translocation to mitochondria. Besides, we would like to look at the role played by the mutants of OmpU of *V.cholerae* in inducing apoptosis in mammalian cell line through the mitochondrial pathway.

2. METHODOLOGY

2.1 Cloning of R46A and R86A mutant of *V. cholerae* OmpU

Bacterial strains and plasmids: *V. cholerae* El Tor O1 strain (MTCC Code 3905) was used for the experimental purposes. For cloning and expression of the mutants, *E. coli* Top10 (Invitrogen) and Origami cells (Novagen) were used respectively. Wild type, as well as, mutant-genes was cloned into the *Nde*1 (New England Biolabs (NEB) and *Bam*H1 (NEB) sites of pET 14b vector (Novagen) for transformation in *E. coli* Top10 cells. After confirming the mutations by sequencing, clones were retransformed into Origami expression cells.

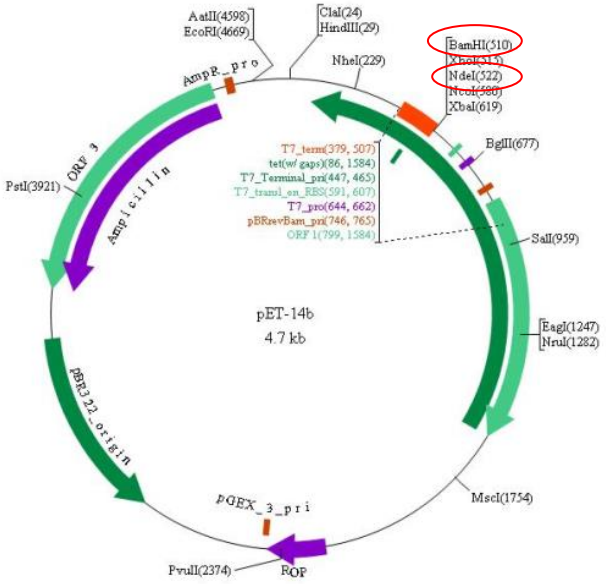

Fig 3: Schematic representation of pET 14b expression vector [26]

Construction of mutants and cloning in expression vector: Mutation sites were chosen according to the reported data [19] which shows the increase in the pore size upon mutation at sites 46 and 86, replacing the arginine by alanine[R46A & R86A] in the *Vibrio cholera*e genome.

MDNKLGLNKMNKTLIALAVSAAAVATGAYADGINQSGDKAGSTVYSAKGTSLEVGGRAEARLSLKDGKAQDNSR
VRLNFLGKAEINDSLYGVGFYEGEFTTNDQGKNASNNSLDNRYTYAGIGGTYGEVTYGKNDGALGVITDFTDIMSY
HGNTAAEKIAVADRVDNMLAYKGQFGDLGVKASYRFADRNAVDAMGNVVTETNAAKYSDNGEDGYSLSAIYTFG
DTGFNVGAGYADQDDQNEYMLAASYRMENLYFAGLFTDGELAKDVDYTGYELAAGYKLGQAAFTATYNNAETAK
ETSADNFAIDATYYFKPNFRSYISYQFNLLDSDKVGKVASEDELAIGLRYDF

Fig 4. *V.cholerae* OmpU amino acid sequence

For the introduction of the mutation at the desired sites, the primers were designed according to the available *V.cholerae* OmpU gene sequence (http://blast.ncbi.nlm.nih.gov) Aminoacid codon for the mutation sites were chosen on the basis of the most frequently occurring genetic code for alanine.

Primer Sequence

- VC OmpU_FW:5'-AAT GTT CAT ATG GAC GGA ATC AAC CAA AGC GGT GAC A-3'
- VC OmpU_RC:5'-ATT CAA GGA TCC TTA GAA GTC GTA ACG TAG ACC ATA GCC -3'
- R46A_RC:5'-CAA GAA GTT TAG AGC TAC GCG AGA GTT GTC TTG TGC CTT A -3'
- R46A_FW:5'-AAC TCT CGC GTA GCT CTA AAC TTC TTG GGT AAA GCA GAA A-3'
- R86A_FW:5'-AGC CTA GAC AAC GCT TAT ACC TAC GCT GGT ATC GGT GGC A-3'
- R86A_RC:5'-AGC GTA GGT ATA AGC GTT GTC TAG GCT GTT GTT AGA CGC G-3'

2.1.1 Cloning of R46A mutant

PCR amplified product containing the desired mutation (R46A) was constructed using wild type *Vibrio cholerae* OmpU gene sequence as the template. After confirming the size of the mutants by agarose gel electrophoresis, it was cloned into pET14b vector, transformed *E.coli* Top10 cells to get sufficient copy number and into *E.coli* Origami cells for the expression of proteins. The detailed description of the procedure is given below.

2.1.1A. Polymerase Chain Reaction (PCR)

Mutation at the desired sites for both the mutants was generated using site-directed mutagenesis. Steps followed for the construction of the mutated sequences for both R46A and R86A mutants is given below.

2.1.1B. Construction of mutated template using VCOmpU forward (VC OmpU_FW) &46 reverse (R46A_RC) primers

To generate the above mentioned template using PCR, the following reaction mixture was used.

Table 1: PCR components

VC OmpU template (1:100 of 100ng/µl)	0.3µl
R46A _RC (1µM)	2 µl
VC OmpU_FW (.3µM)	0.5 µl
Invitrogen Platinum PCR mix (1x)	22.2 µl
Reaction volume	25 µl

Table 2: PCR conditions

Segment	Temperature	Time
1	95 ^{o}C	5m
2	95 ^{o}C	30s
3	56 ^{o}C	30s
4	72 ^{o}C	30s
5	34x	
6	72 ^{o}C	10m
7	4 ^{o}C	∞

2.1.1C. Construction of mutated template using 46 forward (R46A_FW)& VC OmpU reverse (VC OmpU_RC) primers

Table 3: PCR components

VC OmpU template (1:100 of 100ng/µl)	1µl
R46A _FW (1µM)	1 µl
VC OmpU_RC (.3µM)	1 µl
Invitrogen Platinum PCR mix (1x)	37 µl
Reaction volume	40 µl

Table 4: PCR programme

95 ^{o}C	5m
95 ^{o}C	30s
56^{o}C	30s
72 ^{o}C	1m
34x	
72 ^{o}C	10m
4 ^{o}C	∞

2.1.1D. Construction of mutant gene [r46a] using the above generated templates

Table 5: PCR components

Template- 46F-VC R(23.68ng/µl) 46R-VC F(1:10 of 18.21ng/µl)	2µl each
VC OmpU_FW (.3µM)	1 µl
VC OmpU_RC (.3µM)	1 µl
Invitrogen Platinum PCR mix (1x)	44 µl
Reaction volume	50 µl

Table 6: PCR conditions

95 ^{o}C	5m
95 ^{o}C	30s
56^{o}C	30s
72 ^{o}C	1m
34x	
72 ^{o}C	10m
4 ^{o}C	∞

The PCR product was analyzed by agarose gel electrophoresis. After confirming the size of the gene, PCR product was extracted from the agarose gel (Gel extraction kit-Qiagen). Concentration of the DNA (15.86ng/ µl) was measured using nanodrop (Jenway).

2.1.1E. Digestion, ligation and transformation of the mutants

The amplified PCR product was double digested using *Bam*H1 and *Nde*1 restriction enzymes along with cut smart buffer .The reaction mixture was as follows

Table 7: Restriction Digestion

Template(15.89ng/ µl)	25 µl	pET 14b(12ng)	7 µl
*Bam*H1	1 µl	*Bam*H1	1.5µl
*Nde*1	1µl	*Nde*1	1.5µl
Cut smart buffer(1x)	3µl	Cut smart buffer(1x)	1.5µl
Reaction volume	30µl	Reaction volume	15µl

The reaction mixture was incubated for 3h at 37^{o}Cafter thorough mixing. The digestion was confirmed using agarose gel electrophoresis. The digested product (4.5ng/µl) was then ligated together after eluting from the gel. The ligation mixture was as follows

Table 8: Components of ligation reaction

	Test Sample	Negative Control
Vector	3 µl (12ng)	3µl(12ng)
PCR amplified R46A	5 µl (52ng)	nil
T4 DNA ligase	1 µl	1 µl
Ligation buffer	1 µl	1 µl

For a reaction volume of 10µl.The reaction mixture was incubated for 1h at room temperature. The ligated product was transformed into *E.coli* Top10 competent cells and kept overnight at 37^{o}C and confirmed the transformation efficiency by colony PCR.

2.1.1F. Competent cell preparation and transformation

Competent cells for the uptake of the external DNA was prepared as follows.

Protocol followed for the competent cell preparation

- LB media (10ml) was inoculated with 2% seed culture in falcon tube (50ml)
- The culture was kept in the shaking incubator at 37^{o}Ctill O.D reaches 0.4-0.5
- The culture was subjected to centrifugation (4000rpm,4^{o}C,15m)
- Decanted the supernatant and 0.1M $CaCl_2$(10ml) was added

- The solution was kept in ice for about 5m after through mixing
- Centrifuged the solution(4000rpm,4^{o}C ,15m)
- Suspended the pellet in 50mM $CaCl_2$
- The mixture was kept in ice for about 45m and subjected to centrifugation at the same conditions
- The supernatant was discarded and the pellet was suspended in 1ml of 85% $CaCl_2$ and 15% glycerol
- Distributed in 100µl aliquots and kept at -80^{o}C unless used right away

2.1.1G. Transformation of the ligated product (DNA fragment + pET14(b)) into competent E.coli Top10 cells

Transformation of the ligated product into the competent cells was done by the following procedure

- Competent cells(100µl) were kept at 4^{o}C.
- 5µl of ligated product was added to it.
- The solution was mixed well by tapping from outside.
- Kept in ice for about 15m.
- Heat shock (for 1m at 42^{o}C in the waterbath)(do not exceed time) was given.
- Mixture was kept in ice for 5m.
- LB media (1ml) was added to this, by tapping from outside (in laminarhood).
- Kept at 37^{o}C for 45m for about an hour.
- Spin (5000rpm, 2) and discarded the supernatant in a single shot.
- The pellet was resuspended in the remaining LB.
- 100µl from it was spreaded on LB-Amp plate using sterile glass spreader.
- Plate was kept overnight invertably at 37^{o}C.
- Colonies were screened using colony PCR.

Control: - *E.coli* Top10 cells pET(14)b without insert was spreaded into the LB-amp plate as the negative control

2.1.2 Cloning of R86A mutant

2.1.2A. Construction of mutated template using VC OmpU_FW –R86A_RC primers

To generate the mutant template using the above mentioned primers, the following reaction mixture was used.

Table 9: PCR components

VC OmpU template(1:100 of 100ng/µl)	0.4 µl
R86A_RC (1µM)	1 µl
VC OmpU_FW (0.3µM)	2 µl
Invitrogen Platinum PCR mix (1x)	22 µl
Water	
Reaction volume	25 µl

Table 10: PCR Conditions

95 ^{o}C	5m
95 ^{o}C	30s
58.5 ^{o}C	30s
72 ^{o}C	30s
34x	
72 ^{o}C	10m
4 ^{o}C	∞

2.1.2B. Construction of mutated template using R86A_FW -VC OmpU_RC primers

Table 11: PCR components

VCOmpUtemplate(1:100 of 100ng/µl)	2 µl
R86A_FW (1µM)	1 µl
VC OmpU_RC (1µM)	1 µl
Platinum PCR mix (1x)	21 µl
Reaction volume	25 µl

Table 12: PCR Conditions

95 ^{o}C	5m
95 ^{o}C	30s
60 ^{o}C	30s
72 ^{o}C	1m
30x	
72 ^{o}C	10m
4 ^{o}C	∞

2.1.2C. Construction of mutant gene [r86a] using the above generated templates

Table 13: PCR components

R86_FW-VC OmpU_RC(1:10 of 12.84ng/µl	2µl
R86A_RC-VC OmpU_FW(1:10 of 18.21ng/µl)	2 µl
VC OmpU_FW (0.3µM)	1.6 µl
VC OmpU_RC (0.3µM)	1.6 µl
Platinum PCR mix (1x)	53 µl
Reaction volume	60 µl

Table 14: PCR Conditions

95 ^{o}C	5m
95 ^{o}C	30s
56 ^{o}C	30s
72 ^{o}C	1m
34x	
72 ^{o}C	10m
4 ^{o}C	∞

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The PCR product was analyzed by the agarose gel. Concentration was found to be 10ng/µl.

2.1.2D. Digestion, Ligation and Transformation of the mutants

The amplified PCR product was then double digested using *Bam*H1 and *Nde*1 restriction enzymes along with cut smart buffer .The reaction mixture is given below

Table 15: Components of restriction digestion

Template(10.29ng/ µl)	25 µl	pET 14b(8ng)	7 µl
*Bam*H1	1 µl	*Bam*H1	1.5µl
*Nde*1	1µl	*Nde*1	1.5µl
Cut smart buffer(1x)	3µl	Cut smart buffer(1x)	1.5µl
Reaction volume	30µl	Reaction volume	15µl

The reaction mixture was incubated for 3h at 37^{o}C after thorough mixing. The digestion was confirmed by analyzing the PCR product on agarose gel. Concentration of the digested product and the plasmid was found to be 5.37ng/µl and 7.48ng/µl respectively. The product was then ligated after eluting from the gel. The ligation mixture is as follows.

Table 16: Components of ligation reaction

	Test Sample	Negative Control
Vector	3 µl	3µl
PCR product	4 µl	nil
T4 DNA ligase	0.5 µl	0.5 µl
Ligation buffer	1 µl	1 µl

For a reaction volume of 10µl.The reaction mixture was incubated for 1h at room temperature. The ligated product was transformed into *E.coli* Top10 competent cells and spreaded on LB-Amp plate to check for the positive clones. Transformation of the mutants was confirmed by colony PCR.

After cloning, nucleotide sequence encoding the desired point mutated regions for both the mutants [R46A and R86A] were confirmed by DNA sequencing [using the DNA sequencing services of the Amnion Biosciences Pvt. Ltd, Sequencing Dept, Bangalore-560091, Karnataka, India]

2.2 Over-expression and Purification of mutant proteins

Mutants were purified as reported by khan *et al*. Bacterial culture was grown in Luria Bertani Broth (pH 7.5) (Merck) (contained 1% casein enzymic hydrolysate, 0.5% yeast extract, 1% NaCl) in the presence of 50µg/ml of ampicillin at 37^{o}C in the shaking incubator. Protein expression was enhanced by induction with 1mM isopropylthiogalactoside(IPTG) when the optical density of the bacterial culture reached 0.5-0.6 at 600nm.After induction, the culture was incubated under similar conditions. After induction, cells (OD>1.05) were harvested at 4000 rpm, 30m at 4^{o}C. Pelleted cells were then dissolved in PBS (1x) and subjected to sonication. Bacterial protease inhibitor cocktail (Sigma) was added prior to sonication to inhibit the protease activity that may degrade the proteins when cells were lysed (20 A ,20s pulse on followed by 10s pulse off). Lysed cells were then pelleted by centrifuging at 11,000xg at 4^{o}C. The supernatant and the pellet were analyzed by SDS-PAGE to check the presence of protein.

A thick band corresponding to the molecular weight of OmpU was observed in the pellet indicating the presence of bulk amount of protein in inclusion bodies. Hence the pellet was processed further for the purification of the protein. Pellet was washed thrice using 1xPBS + 100mM NaCl and resuspended in 8M Urea prepared in 1xPBS. Resuspended protein was incubated in urea for 1 h at room temperature prior to centrifugation (11,000xg for 30 min) to remove all undissolved debris. The supernatant obtained was collected in a fresh falcon. Unless used right away, the supernatant was stored at -20^{o}C.

2.3 Purification of denatured protein using Ni-NTA affinity chromatography

The initial purification of the protein was done by Ni-NTA agarose affinity chromatography (Qiagen).The pET 14b plasmid which was used for the cloning purpose was tagged with 6x His tags. Hence proteins can bind to the nickel raisins in the column because of the affinity between histidine and nickel raisins in the column. Before loading the protein, the column was equilibrated by passing the mixture of 1xPBS + 8M Urea through the column, since the protein suspension was in the same mixture. After equilibration, the

column was incubated with protein for about 1 h. The flow through was collected prior to the washing of column by 8M Urea in PBS+ 20mM immidazole to wash off all feeble bounded proteins. Finally, the protein was eluted out using 8M Urea in PBS+300mM immidazole. Individual fractions were analyzed by SDS-PAGE and the ones with desired concentrations were proceeded for refolding. All the above mentioned steps were done at the room temperature since urea tends to crystallize at low temperatures.

2.4 Refolding of the denatured protein

Proteins present in the inclusion body may not be properly folded. Hence they have to be refolded back. The refolding was done by slowly adding the eluted nickel purified protein into the refolding buffer (1xPBS+10%, glycerol+0.5%LDAO) with constant shaking, maintaining the 4^{o}C. Protein was incubated overnight at 4^{o}C for proper refolding. Refolded protein was then subjected to centrifugation at 11000xg for 1h at 4^{o}C to remove aggregates. The protein was concentrated using 10kDa cut off Millipore membrane.

2.5 Purification of the refolded protein by size exclusion chromatography

The concentrated refolded protein was passed through the sephacryl S-200 column to exchange buffer and to eliminate the impurities of immidazole. The column was equilibrated with the elution buffer (10mM Tris+10mM Nacl+0.5%LDAO) prior to the loading of the protein. Protein was loaded on the column at a flow rate of 1ml/m. Purified protein fractions were collected from the column at the same flow rate. Samples of individual fractions were analyzed by SDS-PAGE to check the degradation status of the protein and pooled the sufficiently concentrated intact fractions together.

2.6 Mammalian cell culture

All the experiments were carried out in THP-1, human monocytic cell line. Cell line was maintained in RPMI media supplemented with 10% fetal bovine serum and 1% penicillin-streptomycin, in the incubator conditions (5% CO_2, 37^{o}C).

2.7 AnnexinV-FITC staining

The flipping of phosphatidyl serine to the outer leaflet of the cell membrane is one of the hallmarks of early apoptotic cells. Hence to detect the early apoptotic cells, we applied annexin V-FITC/PI staining procedure. For that, cells were conditioned in 2% FBS and kept for 12h prior to the experiment. THP-1cells were counted (Hemacytometer) and plated at a density of 10^6cells/ml in a 6-well plate. After plating, cells were kept for 2h and treated with wt OmpU as well as the mutant proteins (10µg/ml each) and incubated for a time period of 24h.Cells were then harvested (2500rpm,4 ^oC,5m) and washed twice with 1xPBS (1ml each).After washing, the pellet was resuspended in 1x binding buffer(750µl each). 100µl of cells from each vial was then transferred to FACS tubes and stained with Annexin V-FITC (2.5µleach) and PI (2.5µl each).Cells were then incubated in dark for about 15m at RT. After incubation, FACS tubes were placed in ice and 1x binding buffer (400µl each) was added to each tube and mixed well. Cells were analyzed using flow cytometry. For the assay we used the Annexin V-FITC kit from BD Pharmingen(556547).

2.8 JC-1 assay

Cells were counted and plated at a density of 10^6 cells/ml in 2%FBS in a 6-well plate and kept for 12h prior to the experiment. After plating, cells were treated with wt OmpU and the mutant proteins (10µg/ml) and incubated for 24h.Treated cells were stained using JC-1 dye (1x staining buffer was prepared from 5x JC-1 stock solution) and kept for 20m in the incubator (37^oC, 5% CO_2). After incubation, cells were harvested at 3000rpm for 5m at 4 ^oC. Harvested cells were washed twice using 1xPBS (3000rpm, 5m, 4 ^oC).The pellet obtained was dissolved in 1xPBS (500µl) and transferred to FACS tubes and the readings were taken using flow cytometry. The JC-1 assay was carried out using the mitochondria staining kit from Sigma (CS0390-1KT).

2.9 Preparation of mitochondrial fraction

To detect the translocation efficiency of the mutant proteins, THP-1 cells were treated with wt OmpU and the mutant proteins (10µg/ml) for a period of 1h.After incubation, mitochondrial fraction was isolated and checked for the presence of the protein. For

mitochondrial isolation, we followed the manufacture's protocol (mitochondrial isolation kit-Sigma).After treatment cells were harvested (600xg, 5m, 4 ^{o}C) and washed twice with ice cold PBS(1x).Mitochondrial fraction was isolated using detergent lysis method, that employs lyses of cells using detergent. Cells were resuspended in lysis solution and incubated on ice for 5m (do not exceed time).Extraction buffer was added to it and subjected to centrifugation (600xg, 10m, 4 ^{o}C). The supernatant obtained was transferred to a fresh tube and centrifuged (11,000 xg, 10m, 4 ^{o}C).The supernatant was carefully removed and the pellet enriched with the mitochondrial fraction was suspended in a suitable buffer.

2.10 Western Blotting

Western blotting was employed to analyze the translocation efficiency of the mutant proteins to the mitochondrial membrane. For that THP-1 cells were treated with wt OmpU and the mutant proteins (10µg/ml each) and the mitochondria were isolated from the cells. Equal concentration of the mitochondria was loaded on SDS-PAGE after checking the concentration using Bradford's method. The proteins were then transferred to poly vinylidene fluoride membrane (PVDF,Merck,Millipore).After transferring, the membrane was blocked using 5% BSA in TBST buffer and kept for 1h at RT . After blocking, membrane was treated with rabbit anti-OmpU antibody (Sigma Aldrich) diluted in TBST (1:1000) and kept for 4h at RT. Membrane was then washed twice using TBST and kept for 10m at RT. Finally, the membrane was treated with the secondary antibody, anti-rabbit IgG coupled with horseradish peroxidase (1:5000, Sigma Aldrich) and kept for 1h at RT. The membrane was then washed 4 times using TBST and the blot was developed. TIM 23 was used as the mitochondrial marker as well as the loading control.

2.11 Homology modeling

To compare the structural homology of the proteins, we did homology modeling [Swiss-Prot]. We compared the differences in the eyelet region of the mutant proteins as well as the wt OmpU. We tried to analyze the results using I-TASSER as well, and the results appeared to be the same.

3 RESULTS AND DISCUSSION

3.1 Cloning, expression and purification of the mutant proteins

3.1.1 Cloning of R46A mutant of *V. cholerae* OmpU

The mutated sequence of R46A was generated by the PCR amplification of the wt OmpU gene sequence using site directed mutagenesis. The mutation was done in two steps. Firstly, generated the mutated sequences using R46A_RC and VC OmpU_FW as well as using R46A_FW and VC OmpU_RC primers. After optimizing the ratio between these two sequences, the whole length R46A mutant was generated using VC OmpU_FW and VC OmpU_RC primers. The PCR product was ran on the agarose gel. Results obtained are shown below.

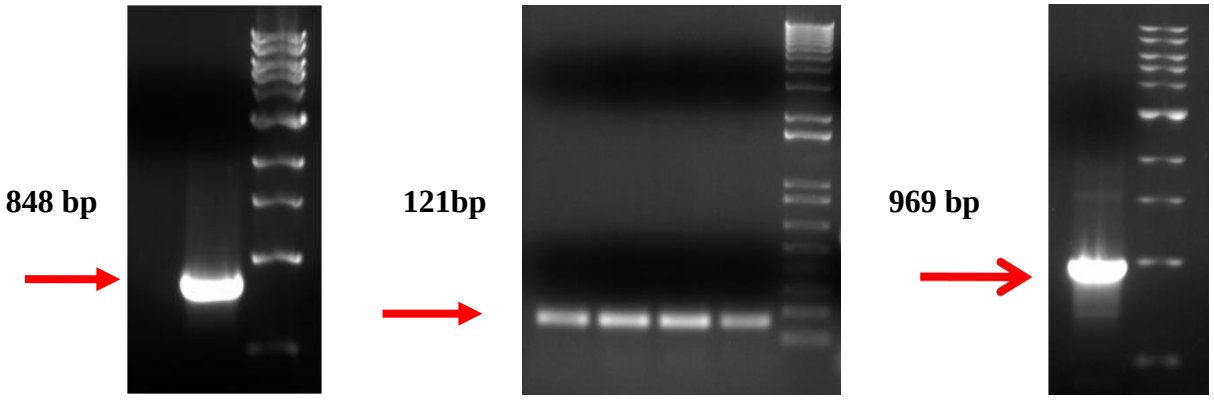


Figure 4A **Figure 4B** **Figure 4C**

Figure 4A: PCR amplification of the mutated gene by R46A_FW and VC OmpU_RC

Figure 4B: PCR amplification of the mutated gene by VC OmpU_FW and R46A_RC

Figure 4C: PCR amplification of R46A mutant by VC OmpU_FW and VC OmpU_RC

After generating the individual fragments, we tried to figure out the optimum ratio between them. After several trials, we could generate the R46A mutant gene sequence following the ratio optimization of the above generated templates.

3.1.2 Cloning of R86A mutant of *V. cholerae* OmpU

The mutated gene sequence of R86A was generated the similar way as that of R46A.Mutation at the desired site was done in two steps. Firstly, generated the mutated sequences using R86A_FW and VC OmpU_RC as well as VC OmpU_FW and R86A_RC primers.R86A mutant gene sequence was generated using VC OmpU_FW and VC OmpU_RC primers upon ratio optimization of the above mentioned templates.

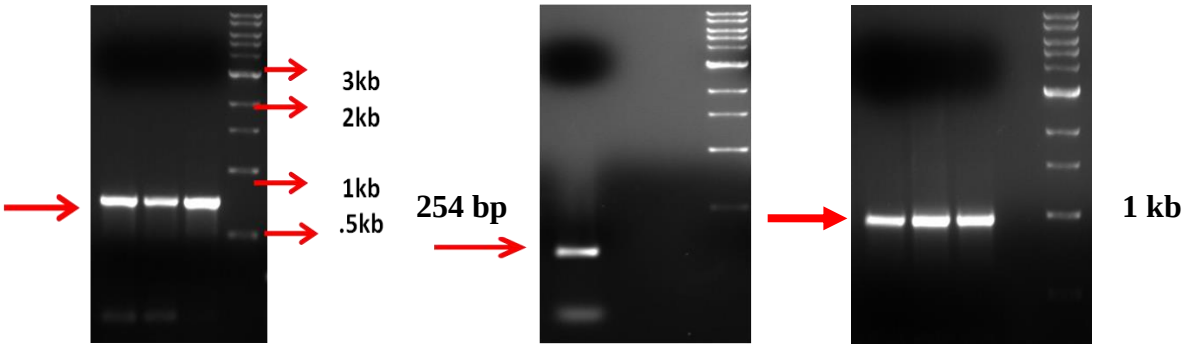


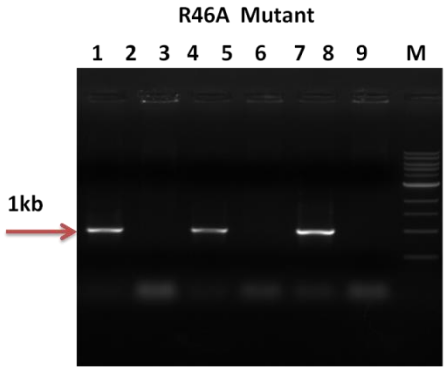
Figure 5A **Figure 5B** **Figure 5C**

Figure 5A: PCR amplification of the mutated gene using VC OmpU_RC & R86A_FW primers
Figure 5B: PCR amplification of the mutated gene using VC OmpU_FW & R86A_RC primers
Figure 5C: PCR amplification of the mutated gene using VC OmpU_RC & VC OmpU_FW primers

Standardization of the template concentration and the ratio optimization of the template sequences were the main aspects needed to be optimized in the cloning procedure. The initial concentration of the template DNA used for the PCR reaction was100ng/µl. But we couldn't get an intact band using this concentration, hence different dilutions were tested for the template [**1:10^2, 1:10^3, 1:10^4**] and checked for the optimum concentration. The template which was diluted 1:100 times seems to work fine [1:100 of 100ng/µl] (confirmed by agarose gel electrophoresis). Hence throughout the cloning procedure, we preferred to use the above mentioned template concentration.

3.1.3 Screening of the transformed colonies by colony-PCR

The mutated gene sequences for both the mutants were cloned into *E.coli* Top10 competent cells to achieve high copy number. The transformed colonies were spreaded on LB-Amp plate and screened using colony PCR.



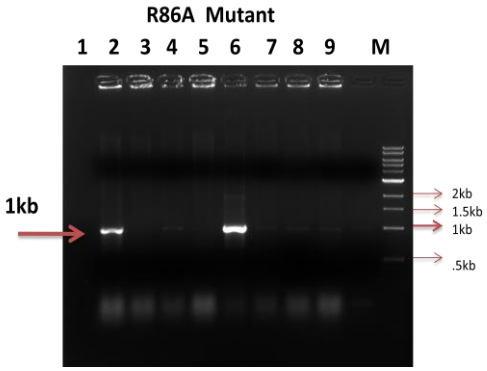
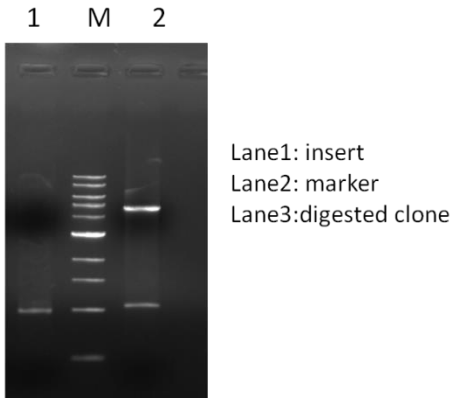


Figure 6A **Figure 6B**

Figure 6A & Figure 6B: Verification of the mutants [R46A and R86A] created using site-directed mutagenesis by colony PCR.

3.1.4 Confirmation of the clones by double enzyme digestion



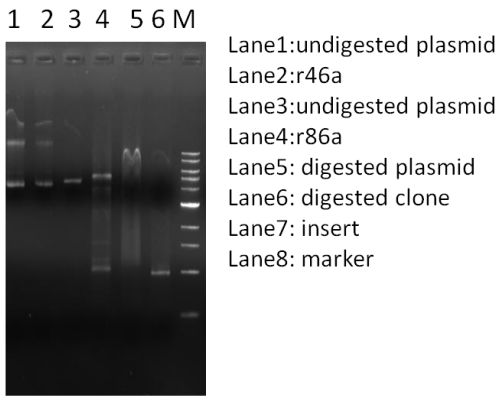


Figure 7A **Figure 7B**

Figure 7A: Nde1/BamH1 digested **pET-R46A** clone
Figure 7B: Nde1/BamH1 digested **pET-R86A** clone

Positive clones obtained by colony PCR was subjected to restriction digestion. Transformations of the clones were confirmed by agarose gel electrophoresis.

3.1.5 Purification of mutant proteins using Ni-NTA affinity chromatography

The bacterial culture was grown in bulk amount for the purification of the protein. Initial purification of the protein was carried out using Ni-NTA affinity chromatography. The transformed plasmid contains the 6x His tags which can bind to the nickel raisins in the column with high affinity. Bounded protein was finally eluted out from the column using immidazole.

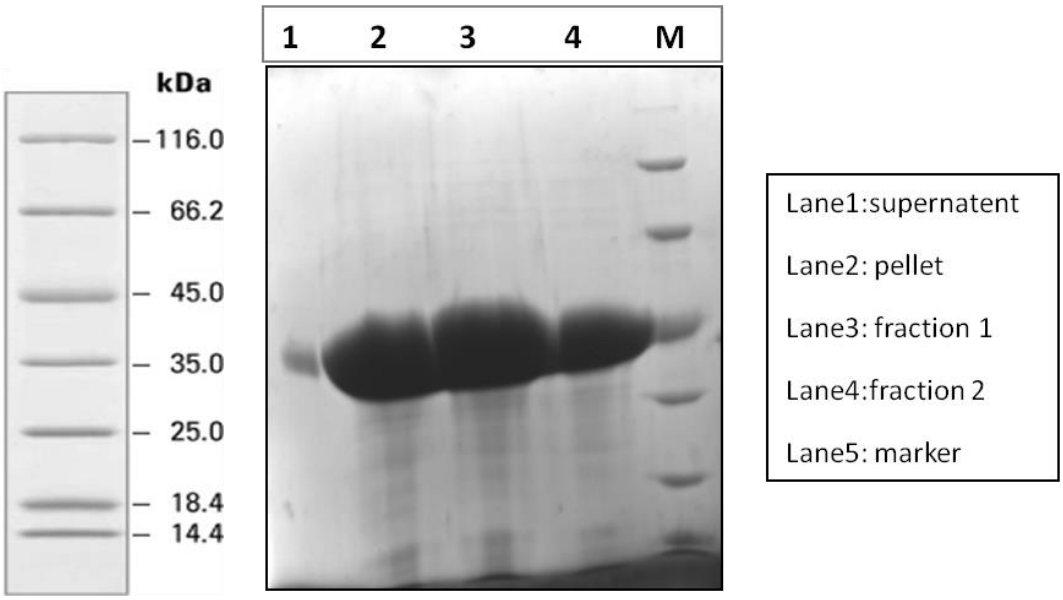


Figure 8: SDS-PAGE shows the purification of protein using Ni-NTA affinity chromatography

While preparing the bacterial culture, we observed that the growth of the mutants in the luria broth media was in a slow pace (>4h to reach O D-0.45 at 600nm) compared to the wt OmpU(2.5-3h to reach O D-0.5 at 600nm).Mutant proteins were initially tried to express in BL21 cells, but failed to get any expression upon induction with IPTG. Besides, we observed that this cell line looses plasmid (might be because of its recombination property), which was observed several times by the decrease in the optical density of the bacterial culture after reaching a certain threshold.

3.1.6 Purification of the refolded protein using gel filtration

The denatured nickel purified protein was refolded back using refolding buffer. The protein was concentrated using 10kDa cut off millipore membrane prior passing through the sephacryl column. After purifying the protein using the sephacryl column,it was ran on SDS-PAGE to check the degradative status.Individual fractions with intact bands were aliquoted and stored at-20 ^{o}C.

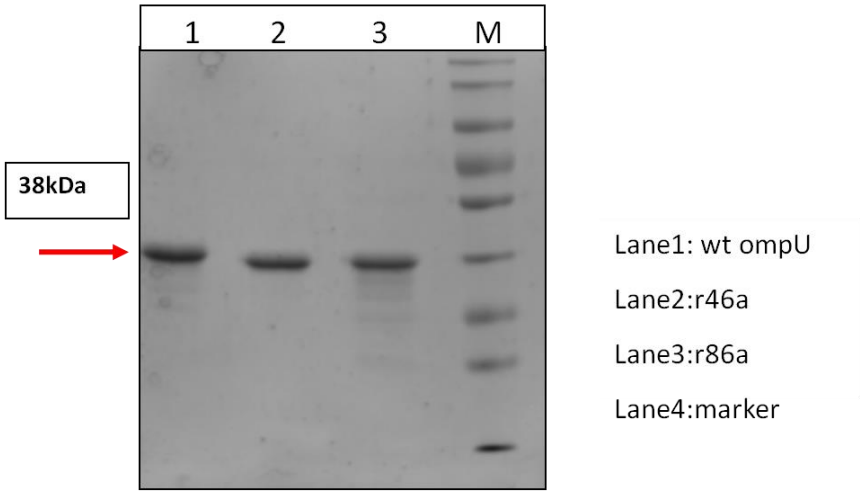
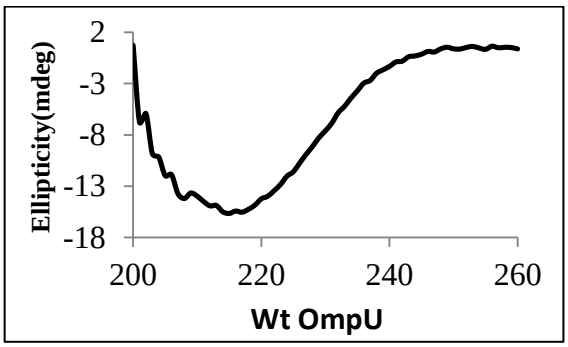


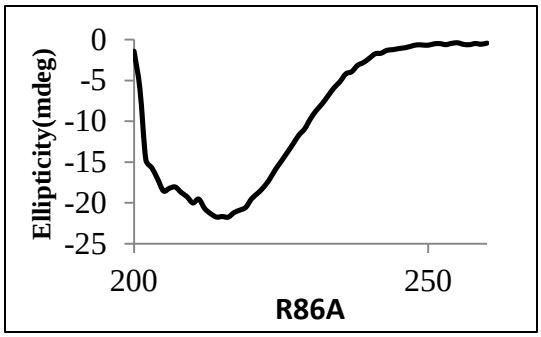
Figure 9: SDS-PAGE:The refolded protein after sephacryl purification.

.3.2 Secondary Structure analysis by circular dichroism

We analyze the secondary structural features of the mutant proteins, by circular dichroism (Chirascan spectropolarimeter). We scanned the protein regions by wavelength ranging from 200nm-260nm.Each graph is an average of three consecutive scans. Buffer (10mMTris, 10mM Nacl, 0.5%LDAO) value obtained under the same conditions was subtracted from the protein spectra prior to analysis.

Figure 10A





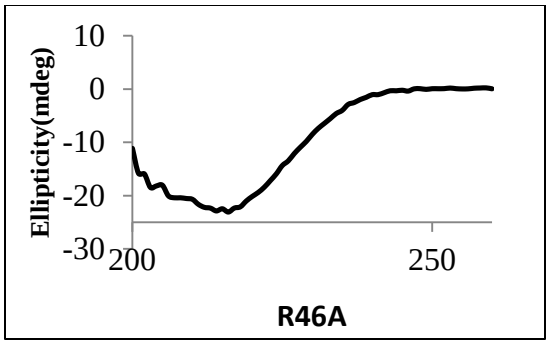


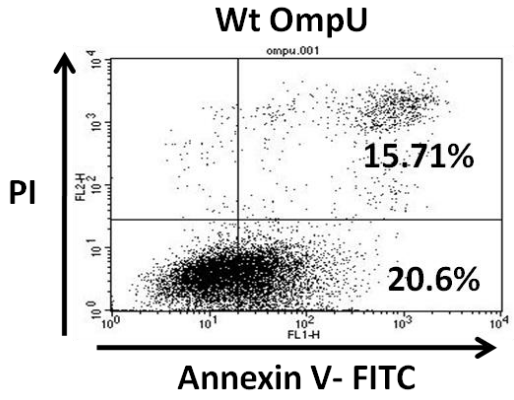
Figure 10 B **Figure 10 C**

Figure 10A,10B & 10C: Secondary structure analysis of the corresponding proteins using circular dichroism

Negative dip in the beta region (218nm) suggests that the structure is rich in ß strands. In the case of mutants, random coils are also present (dip in the region of 200nm-220nm).The data suggests that the mutation doesn't affect the secondary structure conformation of the protein.

3.3 Detection of apoptosis using Annexin V-FITC staining

After confirming the secondary structure of the mutant proteins, we tried to look at their effect on human monocytic cell line (THP-1).For that we treated cells with wt OmpU as well as the mutant proteins and checked for apoptosis. Asymmetry of the cell membrane due to the flipping of the phosphatidyl serine to the outer leaflet of the cell is considered as a defined feature of the cells in the early apoptotic stage. Hence we analyzed the cells using annexin V-FITC staining and the data was analyzed using flow cytometry.



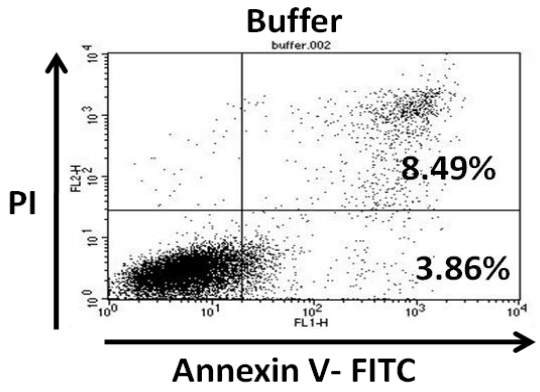
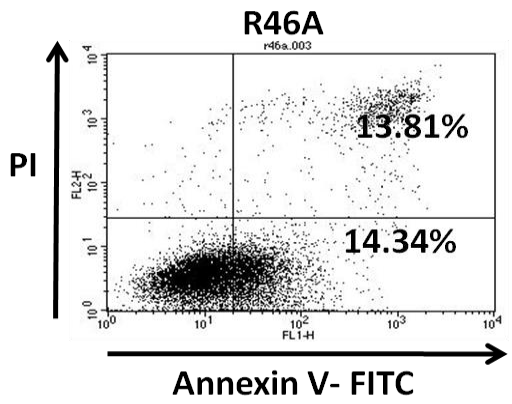


Figure 11 A **Figure 11 B**



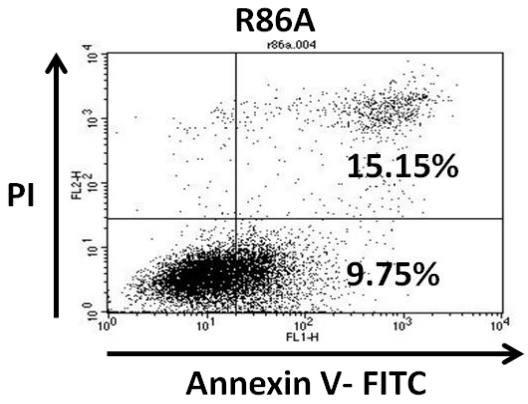


Figure: 11 C **Figure 11 D**

Figure 11 A-11 D. Percentage of apoptotic cells stained positive for Annexin V-FITC staining

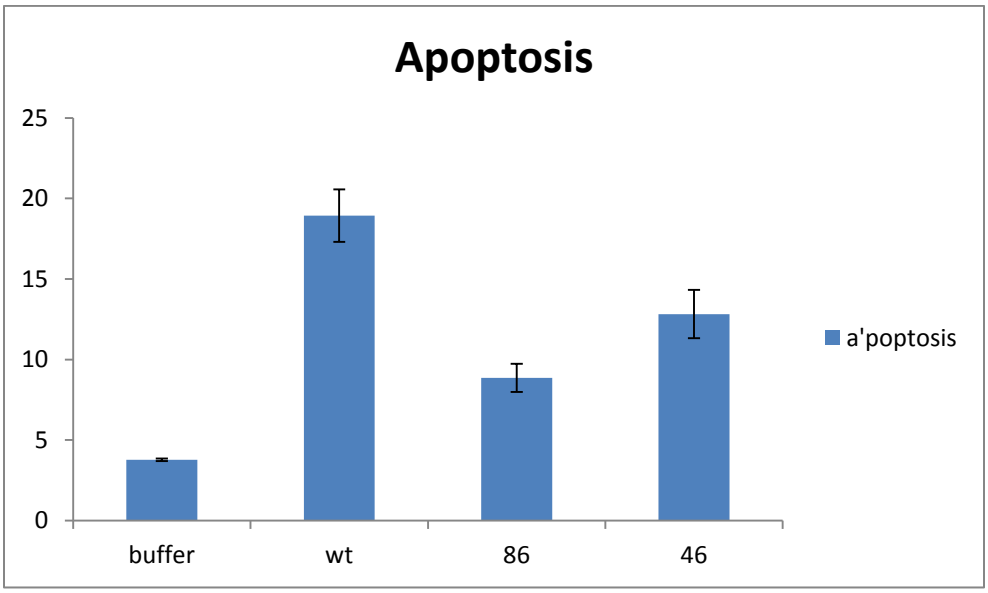


Figure 11 E: Apoptotic cells stained positive for Annexin V-FITC staining (%)

Population of cells in the early apoptotic stage will stain positive for Annexin V-FITC,because of the flipping of PS to the outer membrane in apoptotic cells. From the graph we can understand that the mutant proteins are showing less apoptosis (almost 50% less) than wt OmpU.

3.4 Mitochondrial translocation of the mutant proteins

Since we observed the reduction in apoptosis in the cells treated with the mutant proteins, we were interested in looking at the translocation efficiency of the mutant proteins to the mitochondrial membrane. For that, we treated THP-1 cells with the wt OmpU as well as the mutant proteins (10µg/ml) for a time period of 1h.Mitochondrial translocation of the mutant proteins were assessed by western blotting using anti-OmpU antibody. Time period was chosen as per the date for the wt OmpU (S.Gupta et al-manuscript submitted), where the maximum translocation of the protein was observed at 60m.After treatment, mitochondria was isolated from the cells and performed the western blotting. Isolation of mitochondria was done using detergent lysis method followed by low (600xg) and high speed (11,000xg) centrifugation (mitochondria isolation kit-sigma).

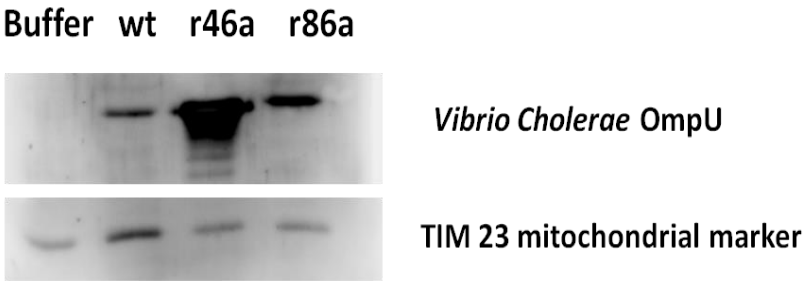


Figure 12: Western Blot:Mitochondrial translocation of the mutant proteins

Western blot represents the translocation efficiency of the proteins to the mitochondrial membrane. Interestingly, the flux of the mutant proteins is higher compared to the wt OmpU, particularly in R46A where the translocation seems to be two fold or more than that compared to R86A as well as the wt OmpU. Besides, there seems to be considerable degradation in the R46A mutant protein. The substantial increase in the translocation efficiency of the mutant protein suggests the involvement of the mutated residues in translocation to the mitochondrial membrane.

3.5 Detection of mitochondrial membrane permeability transition using JC-1 assay

The detonation of mitochondrial membrane potential gradient is considered as a marked event during the early phase of apoptosis. Since we found that the translocation efficiency of the mutant proteins is higher compared to the wt OmpU, we looked at the effect of the mutant proteins in mitochondrial membrane permeability transition (MMPT). To study the impact of mutants on the permeability transition of mitochondria, THP-1 monocytes were treated with wt OmpU and mutants (10µg/ml) for a time period of 24h.Cells were treated with buffer as the negative control and valinomycin (1:200) as the positive control. Following incubation, cells were harvested (3000 rpm) and stained with JC-1 dye (25µl/5ml) and the data was analyzed using flow cytometry.

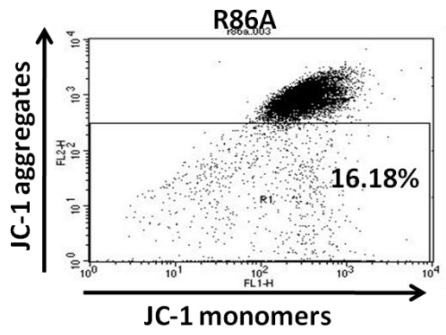


Figure 13A

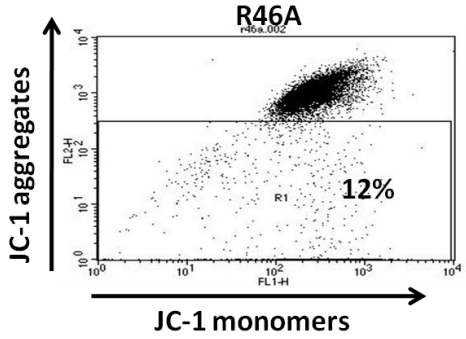


Figure 13B

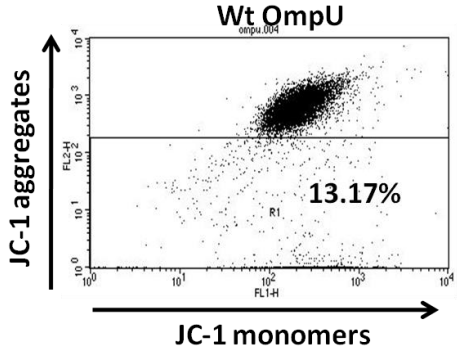


Figure 13C

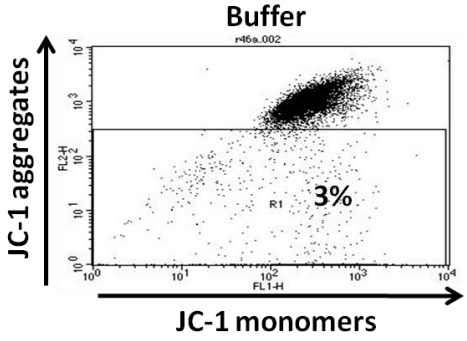


Figure 13D

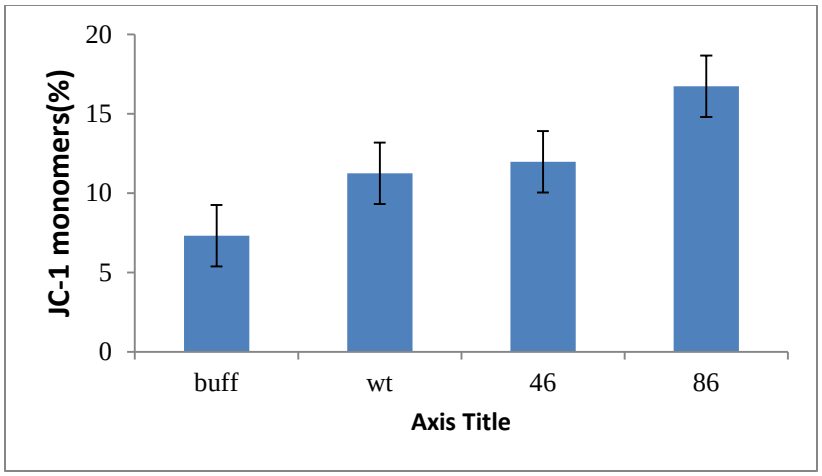
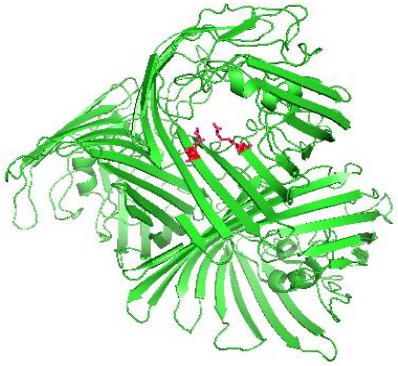


Figure 13 E.: Percentage of mitochondrial membrane permeability transition

Red fluorescent aggregates of JC-1 represents the mitochondria with intact membrane potential, where JC-1 can form aggregates in the mitochondrial matrix. Whereas in the mitochondria with disrupted membrane potential, JC-1 will be dispersed throughout the cell as monomers which can be detected by the green fluorescence. Valinomycin, which can permeabilize the membrane for k^+ ions was treated as the positive control. From the graph it is clear that the mutant proteins hardly have any effect on mitochondrial membrane permeability transition.

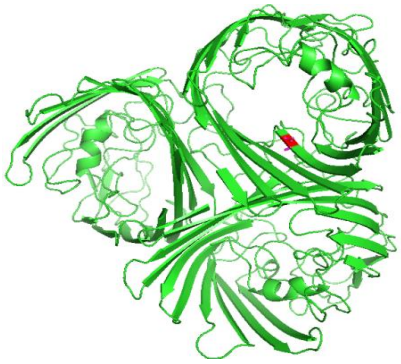
3.6. Homology modeling

To understand the participatory role of the mutated residues in the pore formation, we did homology modeling. Modelling was done using Swiss-Prot. The structure was confirmed using I-TASSER as well, after doing threading.



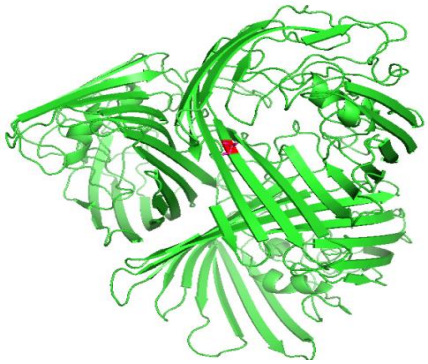
WtOmpU: Projection of arginines lining the barrel wall opposite to the L3 loop, towards the pore lumen in the eyelet region

Figure 14A: wt OmpU



R86A: Projection of alanine (86th position in the aminoacid chain) towards the pore lumen. Present in the 5th antiparallel ß strand in the eyelet region.

Figure 14B:R86A



R46A: Projection of alanine(46th position in the aminoacid chain) towards the pore lumen. Present in the 3rd antiparallel ß strand in the eyelet region.

Figure 14C:R46A

The mutated residues were found to line the eyelet region of the pore, which suggests the involvement of the residues in determining the pore size of the porin protein. I-TASSER

results show that the residues are not exposed to the surface; rather they are buried inside the eyelet region.

4. CONCLUSION

Mitochondrial membrane permeability transition (MMPT) is determined by a number of variables, and it is not as straight as simple diffusion through a channel. The present study suggests the same. The Annexin V-FITC staining assay suggests the reduction in apoptotic population by the mutant proteins compared to the wt OmpU. The JC-1 assay suggests that the increase in the pore size of the OmpU protein has hardly any effect on the MMPT. These data suggests that, it can be the charge, the chain length of the aminoacid in the insertion loop or the combined effect of both or some other characteristics of the aminoacid lining the membrane that actually determines the MMPT rather than the channel size of the porin. And it is clear that the mutant proteins have complete access to the mitochondria which can be deduced from the western blot data. The visible change in the translocation efficiency of the mutant proteins suggests the involvement of mutated residues in translocation to the mitochondrial membrane.

5. REFERENCES

1. Chipuk JE, Green DR. 2008. How do BCL-2 proteins induce mitochondrial outer membrane permeabilization?Trends Cell Biol.18:157–64

2. Kroemer G, Galluzzi L, Brenner C. 2007. Mitochondrial membrane permeabilization in cell death.Physiol. Rev.87:99–163

3. Suen DF, Norris KL, Youle RJ. 2008. Mitochondrial dynamics and apoptosis.Genes Dev.22:1577–90

4. Youle RJ, Strasser A. 2008. The BCL-2 protein family: opposing activities that mediate cell death.Nat.Rev. Mol. Cell Biol.9:47–59

5. Takei N, Endo Y. Ca^{2+}ionophore-induced apoptosis on cultured embryonic rat cortical neurons.Brain Res1994;652:65–70.

6. Muzio, M., Stockwell, B.R., Stennicke, H.R., Salvesen, G.S. & Dixit, V.M. An induced

proximity model for caspase-8 activation. J. Biol. Chem.273, 2926–2930 (1998)

7.Yang QH, Church-Hajduk R, Ren J, Newton ML, Du C. 2003. Omi/HtrA2 catalytic cleavage of inhibitorof apoptosis (IAP) irreversibly inactivates IAPs and facilitates caspase activity in apoptosis.Genes Dev.17:1487–96

8.Marzo I, P´ erez-Gal´ an P, Giraldo P, Rubio-F´ elix D, AnelA,Naval J. Cladribine induces apoptosis in human leukaemia cells by caspase-dependent and -independent pathways acting on mitochondria.Biochem J2001;359: 537–546

9.Bortner CD, Cidlowski JA. Caspase independent/dependentregulation of K+, cell shrinkage, and mitochondrial membrane potential during lymphocyte apoptosis.JBiolChem 1999;274: 21953–21962

10 J. D. Ly, D. R. Grubb∗and A. Lawen The mitochondrial membrane potential ($\Delta\psi m$)

in apoptosis; an update Department of Biochemistry and Molecular Biology, School of Biomedical Sciences, Monash University, Building 13D,Melbourne 3800, Australia

11.Gorka Basan˜ez1*, Lucian Soane2, J. Marie Hardwick 2 A New View of the Lethal Apoptotic Pore Biophysics Unit, Spanish Science Research Council (CSIC) and University of the Basque Country (UPV/EHU), Bilbao, Spain,2Department of Molecular Microbiology and Immunology, Johns Hopkins School of Public Health, Baltimore, Maryland, United States of America

12.Tait SW, Green DR (2010) Mitochondria and cell death: outer membrane permeabilization and beyond. Nat Rev Mol Cell Biol 11: 621–632

13.Koebnik, R., Locher, K.P. and Van Gelder, P. (2000) Structure and function of bacterial outer membrane proteins: barrels in a nutshell.Mol. Microbiol. 37, 239^253.

14.Nikaido, H. (1996) Outer membrane. In: Escherichia coliandSalmonella, Cellular and Molecular Biology (Neidhardt, F.C., Ed.), pp. 29^47. ASM Press, Washington, DC

15.Pratt, L.A., Hsing, W., Gibson, K.E. and Silhavy, T.J. (1996) Fromacids to osmZ: multiple factors in£uence synthesis of OmpF and OmpCporinsinEscherichia coli. Mol. Microbiol. 20, 911^917.

16.Muller, A., Gu «nther, D., Du«x, F., Naumann, M.MeyerT.F. andRudel, T. (1999) Neisserialporin (PorB) causes rapid calcium in£ux in target cells and induces apoptosis by the activation of cysteine proteases. EMBO J. 18, 339^352.

17.Cowan, S.W., Schirmer, T., Rummel, G., Steiert, M., Ghosh, R.,Pauptit, R.A., Jansonius, J.A. and Rosenbusch, J.P. (1992) Crystal structures explain functional properties of twoE. coliporins. Nature 358, 727^733

18.Weiss, M.S. and Schulz, G.E. (1992) Protein, Structure of porin re-¢ned at 1.8 Aî resolution. J. Mol. Biol. 227, 493^509

19.Melissa Pagel, Vale´rieSimonet, Jie Li,#MathildeLallemand,§ Brian Lauman, and Anne H. Delcour* Phenotypic Characterization of Pore Mutants of the *Vibrio cholerae* PorinOmpU Department of Biology and Biochemistry, University of Houston, 369 Science and Research Building II, Houston, Texas 77204-5001

20. R.A. Lockshin, Z. Zahra, Int. J. Biochem. Cell Biol. 36 (2004) 2405–2419.

21.G.Kroemer *et al*,classification of cell death,PMC,2009 Jan;16(1):3-11

22. Yutaka Eguchi, Shigeomi Shimizu, and Yoshihide Tsujimoto[2] Intracellular ATP Levels Determine Cell Death Fate by Apoptosis or Necrosis[1], Cancer Res May 15, 1997.

23.TsujimotoY, Apoptosis and necrosis: intracellular ATP level as a determinant for cell death modes,Europe Pubmed Central,. [1997, 4(6):429-434]

24. Apoptosis: A Review of Programmed Cell Death,Susan Elmore,Toxicol Pathol. Author manuscript; available in PMC 2007 December 6

25. www.celldeath.de

26. www.biovisualtech.com

27.Nikaido, H. (1996) Outer membrane. In: Escherichia coliandSalmonella, Cellular and Molecular Biology (Neidhardt, F.C., Ed.), pp. 29 47. ASM Press, Washington, DC.

28. Koebnik, R., Locher, K.P. and Van Gelder, P. (2000) Structure and function of bacterial outer membrane proteins: barrels in a nutshell.Mol. Microbiol. 37, 239^253

29.Kerr *et al*.,1972, Apoptosis: a basic biological phenomenon with wide-ranging implications in tissue kinetics, J.cancer, 1972 Aug;26(4):239-57.

30.Khan *et al.*2012. Refolding and functional assembly of the Vibrio cholera porin OmpU

recombinantly expressed in the cytoplasm of *Escherichia coli*.Elsevier.85:204-210