Mutagenesis Of Beta-Barrel Membrane Proteins To Evaluate The Role Of Sequence And Structural Motifs

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LIST OF ABBREVIATIONS

β-barrel	Beta barrel
α-helical	Alpha helical
KDa	Kilo Dalton
OmpF	Outer membrane protein F
OmpC	Outer membrane protein C
OmpA	Outer membrane protein A
OmpT	Outer membrane protein T
OmpR	Outer membrane protein R
λ- Red plasmid	Lamda Red plasmid
PCR	polymerase chain reaction
SDS-PAGE	sodium dodecyl sulfate polyacrylamide gel electrophoresis
octyl-POE	n-Octyl-oligo-oxyethylene
PLB	Planar Lipid Bilayer

SUMMARY

Proteins are a major component of any living cell, playing an important role in nutrient uptake, metabolism, transport and many other functions. The structure of the protein affects the way it functions and with the advances in NMR and X-ray crystallography extensive research is being done on hundreds of proteins on the structural level. Membrane proteins are coded by 25% of the genes in an organism's genome and act as channels, pumps, sensors and receptors. In spite of having such vital functions, membrane proteins have been left out of the crystallographer's repertoire probably due to the difficulty in crystallizing them in the critical environmental requirements in which they function.

Previous studies suggest a series of models on the structural prediction of the *ß*-barrel transmembrane proteins [7, 8]. In those models they were able to identify sequence and spatial patterns such as alanine- tyrosine motifs, antimotifs, chaperone binding motifs, aromatic rescue of glycine and amino acid regional propensities for specific locations that are statistically important but biological significance has not been determined. To elucidate which amino acids or patterns of amino acids are important for *in vivo* folding and which influence thermodynamic stability of *ß*-barrel membrane proteins they proposed a series of experiments with outer membrane protein F (OmpF).

The *ompF* gene was deleted from MG1655 *E.coli* strain and was replaced with a kanamycin resistant gene using homologous gene recombination. Site directed mutagenesis was done on pBAD24F plasmid to produce different mutants of *ompF* gene. Proteins expressed by wild type and mutant plasmids in the omp8 strain (*ompF* null) were checked on SDS-PAGE for expression and thermal stability.

1. INTRODUCTION

(A) *ß*-barrel membrane proteins

ß-barrel membrane proteins are one of two main classes of membrane protein. These are found in the outer membrane of Gram-negative bacteria, mitochondria and chloroplasts. Approximately 2-3% of the Gram-negative bacterial genes in their genomes encode for *ß*-barrels [1]. α-helical membrane proteins (Fig.1A) are another main category of membrane proteins.

Advancements in the fields of genomics, proteomics, genetics and structural biology lead to the discovery of numerous *ß*-barrel membrane proteins. Unfortunately, only about 25 non homologous *ß*-barrel membrane protein three-dimensional structures are known. These proteins perform different roles in different organisms such as facilitated, nonspecific, energy-dependent transport and as receptors [1]. These proteins also act as lipases, proteases, pore-forming toxins and protein secretion pores [1].

ß-barrel trans-membrane proteins fold into a cylinder-like structure across the lipid bilayer. The *ß*-strands are connected into a closed barrel giving a *ß*-meander topology with alternating loops and turns [1]. Typically, one end of the *ß*-barrel has tight turns and the other side has loops as shown in Fig. 1B. The interstrand side chain hydrogen bonds and the nonlocal backbone hydrogen bond interactions stabilize and rigidify the barrel. *ß*-barrels are very stable and not readily unfolded in the membrane because of these hydrogen bonds [3]. Residues exposed to the lipid bilayer have nonpolar side chains and the residues facing the barrel interior have polar side chains [2]. The lipid-exposed surface of the *ß*-barrel mainly contains of tyrosine, tryptophan, phenylalanine, valine and leucine. Aromatic amino acids are abundant at the bilayer interfaces. The interior of the *ß*-barrel is mainly occupied with small and polar amino acids such as glycine, threonine, serine, asparagine and glutamine.

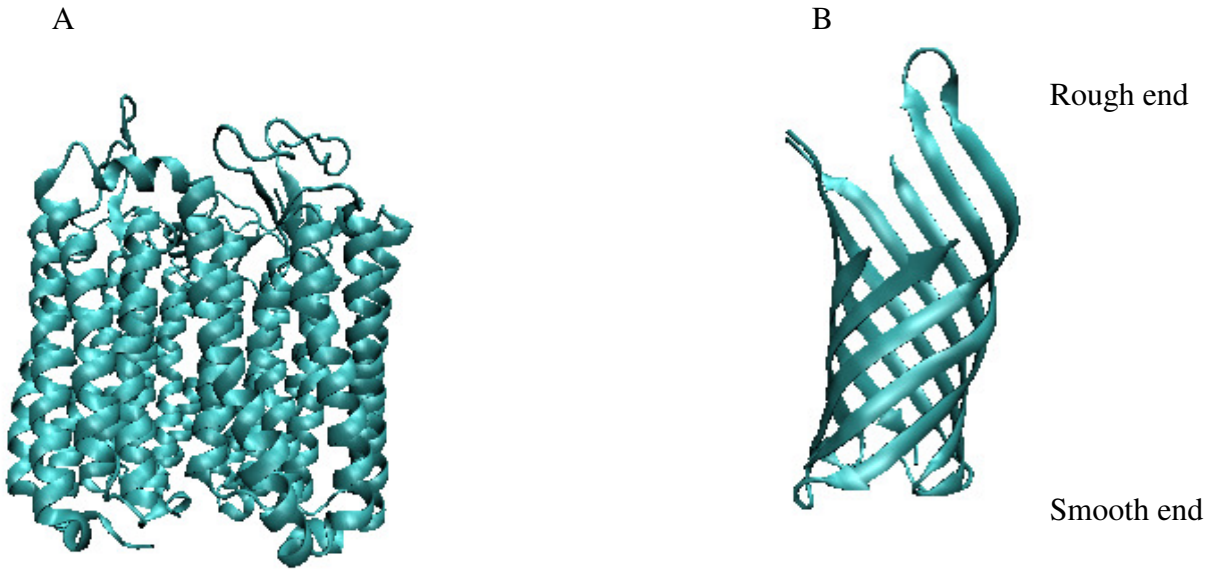

Figure 1. Ribbon diagram representing the topology of

A. α-helical membrane protein, Halorhodopsin (HR) from *Natronomonas pharaonis*

B. *ß*-barrel membrane protein, OmpA from *Escherichia coli*

(B) Bacterial cell wall

The bacterial cell wall defines the shape of the microorganism, controls the entry and exit of nutrients/ waste material and play a role as receptors. Most of the bacteria are divided into two major groups, Gram-positive and Gram-negative. Gram-positive bacterial cell wall contains a single membrane and a thick peptidoglycan layer, whereas Gram-negative bacteria contain two membrane layers separated by the periplasmic space [25].

Phospholipids are the main constituent of bacterial membranes. Gram-negative bacteria have a thin peptidoglycan layer sandwiched between two membranes in the periplasmic space. Gram-positive bacteria have an inner membrane covered with a very thick peptidoglycan layer. The inner membranes of both Gram-positive bacteria and the Gram-negative bacteria consist of the phospholipids. In contrast, the outer membrane of Gram-negative bacteria is asymmetric in terms of the phospholipid arrangement. The inner leaflet of the outer membrane contains phospholipids and the outer leaflet contains some phospholipids and lipopolysaccharides as shown in Fig.2 [26].

The inner membrane of Gram-negative bacteria contains α-helical membrane proteins and the outer membrane contains β-barrel membrane proteins such as OmpF, OmpC, OmpA, OmpT, LamB and α-Hemolysin [3, 7]. Having an extra bilayer around the Gram-negative bacteria gives them significant protection from external environmental threats and it also acts as a barrier for the transport of nutrients and waste material [3]. β-barrel membrane proteins that are present in the outer membrane facilitate the entry and exit of nutrients/waste from the bacteria [3].

The increased resistance to antibiotics that Gram-negative bacteria possess because of the outer membrane can be a problem when it comes to killing them. This problem can be solved by

studying the β-barrel membrane proteins which are facilitating transport across the outer membrane [8].

(C) Challenges associated with the study of integral membrane proteins

Membrane proteins are suitable targets for pharmaceutical drugs because they are involved in many vital functions. In spite of the fact that membrane proteins are crucial, our knowledge of membrane proteins is very limited. This disparity is best exemplified by the fact that, as of December 2010, only 263 unique high-resolution structures of membrane proteins have been deposited in the Protein Data Bank [25], whereas the structures of about seventy thousand water-soluble proteins are known as of September 06th, 2011.

The lack of information of membrane proteins is due to:

a. Membrane proteins are often cytotoxic when over expressed in cell culture; hence it is extremely difficult to obtain sufficient purified quantities for biophysical, biochemical and structural studies [5].

b. Membrane proteins are hydrophobic and usually require the presence of detergents or lipids during the purification process and often with experiments using the purified protein. This makes membrane proteins difficult to handle and unsuitable for many techniques [5].

In addition to the difficulties in purification of membrane proteins, many of them have biological activities only in specific environments such as complex membranes or membrane-mimetic environments. Some membrane proteins are naturally abundant, so their purification and crystallization is relatively easy. But, in some cases they are not present in sufficient quantities, in that case we have to overexpress the protein in bacterial inclusion bodies and refold it *in vitro* [5].

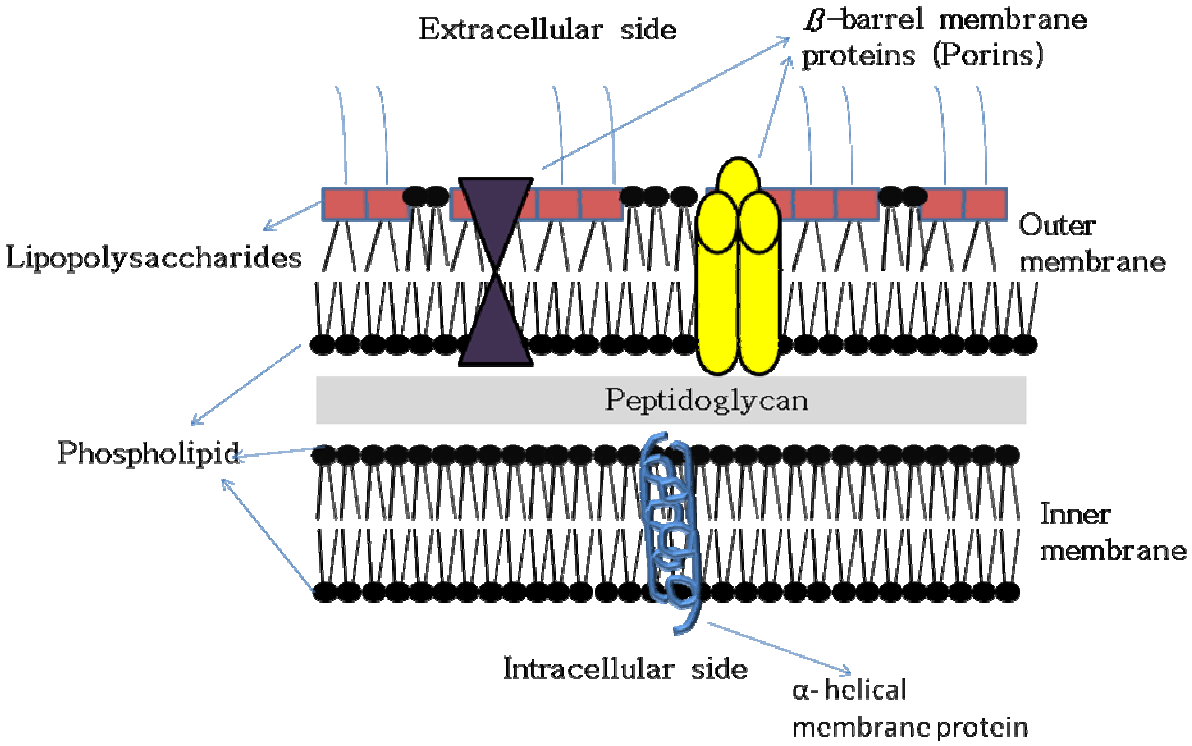

Figure 2. Diagram of a Gram-negative bacterial cell wall [26]. The inner membrane is a phospholipid bilayer and the outer membrane is an asymmetric bilayer of phospholipid and Lipopolysaccharide. α-helical membrane protein and β-barrel membrane proteins are represented in inner and outer membranes respectively. A thin peptidoglycan layer is sandwiched between the two bilayers.

(D) Outermembrane protein F (OmpF) structure

OmpF is a general diffusion pore with a small net charge. It has a solute exclusion size of ~600 Da and growth conditions determine its expression. OmpF protein is composed of 340 amino acids that are organized into16 beta sheets in anti-parallel fashion (Fig. 3), with a shear number 20 [7]. It forms a pseudo-cyclic structure by a salt bridge within the 16^{th} beta strand that links the amino and carboxy termini. The turns/ loops that join the beta strands on the periplasmic end are of similar length, as shown in Fig.3, and are defined as the smooth end. The tightly packed extracellular loops have different lengths and extend towards the barrel axis. This is considered the rough end, shown in Fig. 3. Loop L3, one of the long loops, folds into the barrel leaving a gap in the wall between 4th and 7th beta strands.

OmpF forms a very stable trimer (Fig. 4) due to the strong hydrophobic interactions between the monomers. Loop L2 contributes to the integrity of the trimer by numerous hydrophilic interactions and extends from one monomer to another forming a "latching loop". The combination of the hydrophobic and hydrophilic interactions involves 35% of the surface of each isolated monomer, the porin cannot be dissociated even under high concentrations of chaotropic agents or organic solvents other than phenol [7].

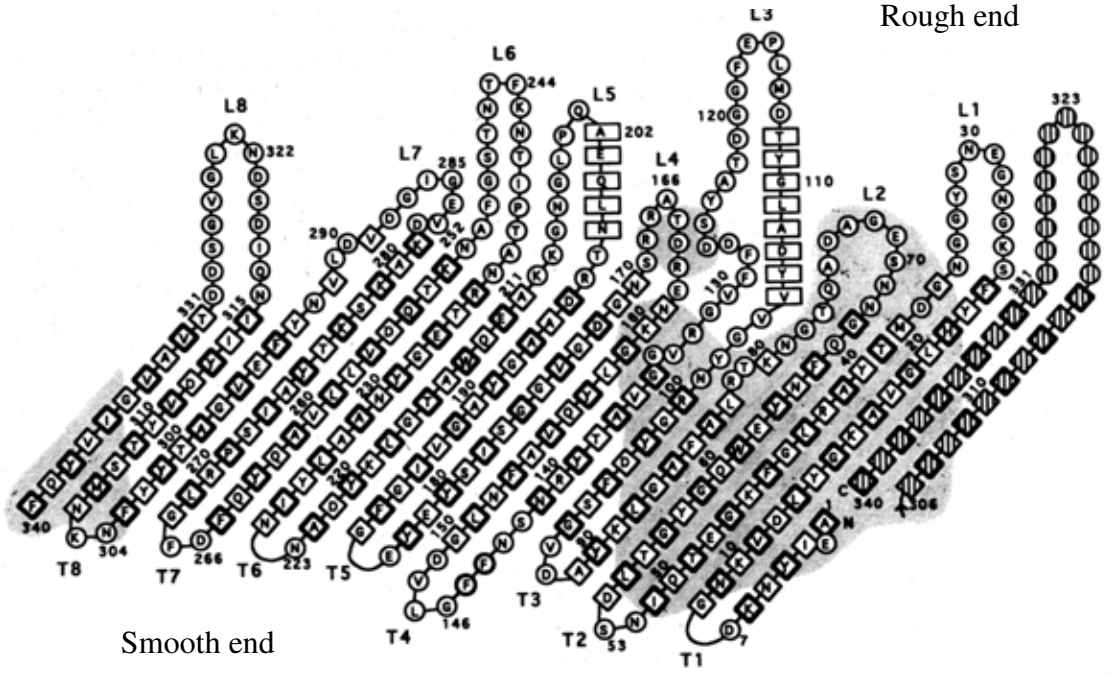

Figure 3. View of an unrolled 16- stranded β-barrel membrane protein OmpF. The amino acid sequence of the OmpF protein is showed in one letter code [7].

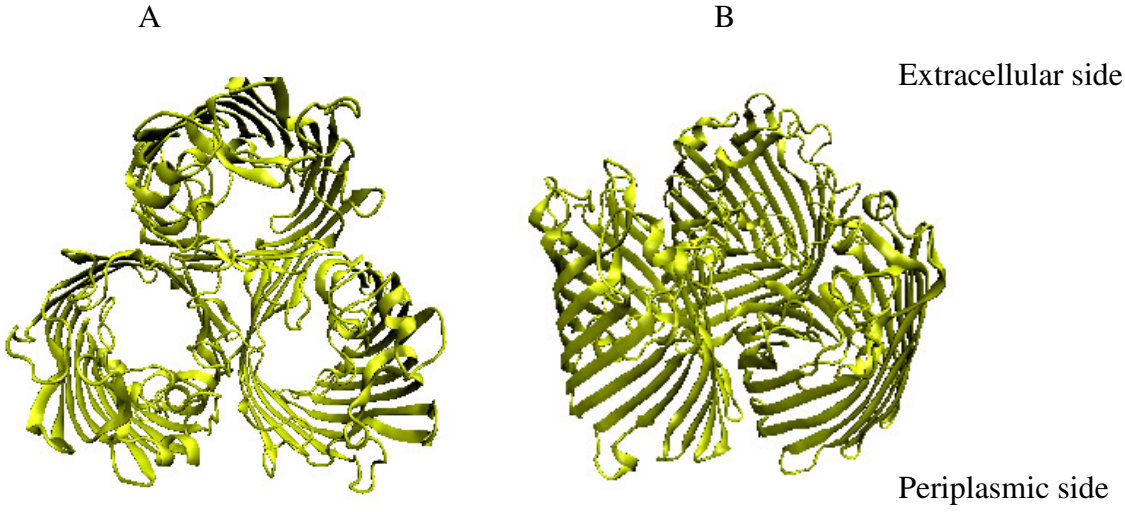

Figure 4. OmpF trimer images drawn using Visual Molecular Dynamics (VMD) software.

(E) Regulation of Outermembrane protein F

OmpF protein expression is dependent on the osmolarity of the external environment. EnvZ, a histidine kinase in inner membranes of *E. coli*, responds to the changes in the osmolarity of the growth medium by regulating the phosphorylation state of a transcription factor OmpR [6, 9]. Osmolarity in the surrounding environment is detected by EnvZ, which transfers a phosphoryl group on to an aspartic acid residue in the N –terminal receiver domain of OmpR. Phosphorylated OmpR binds to the promoter regions of either *ompF* or *ompC* and alters the expression of the porin [6]. High and low osmolarity in the environment triggers the expression of OmpC and OmpF proteins, respectively. This in turn controls the expression of OmpF and OmpC [6].

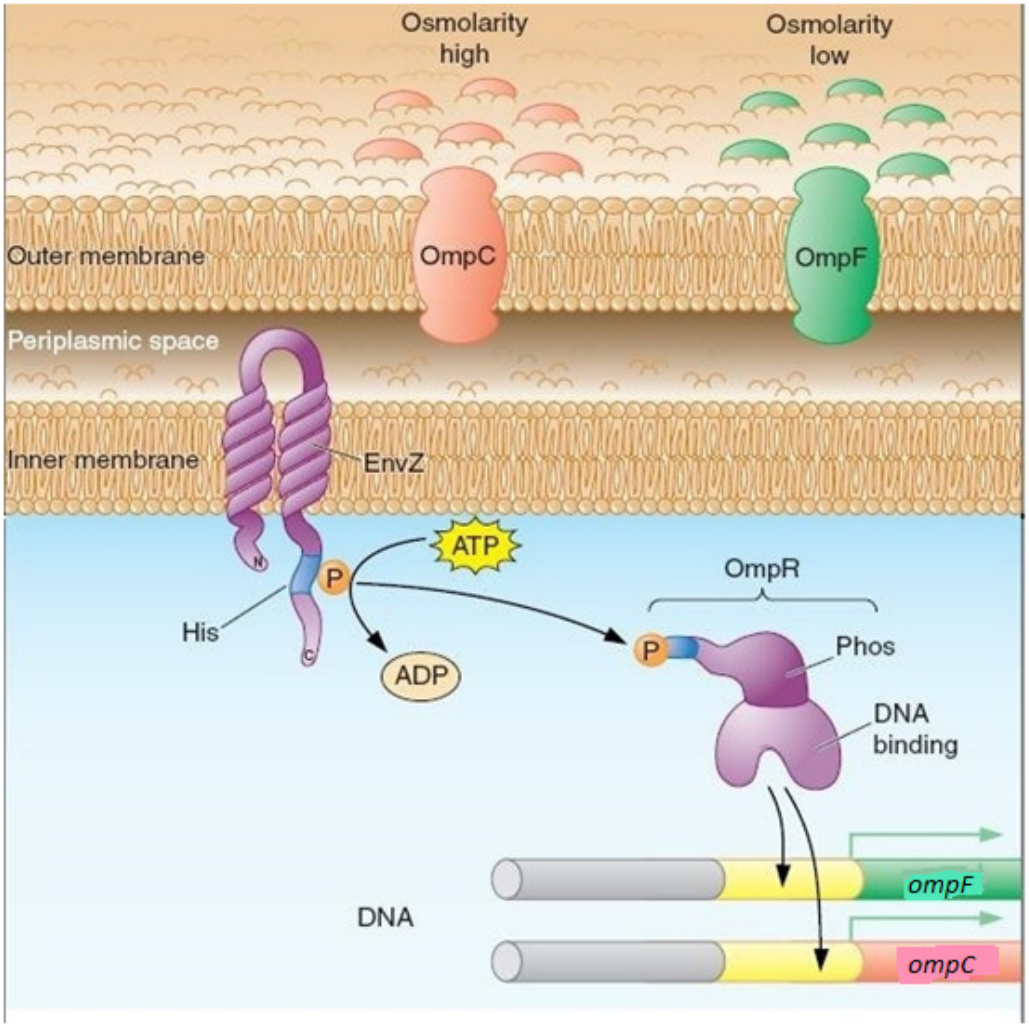

Figure 5. EnvZ/ OmpR regulate the expression of outer membrane protein F and outer member protein C. EnvZ, membrane bound kinase detects the osmolarity conditions outside the cell and OmpR gets phosphorylated upon the transfer of phosphoryl group from EnvZ. Phospho-OmpR binds to promoter regions of either *OmpF* or *OmpC* and alters the expression of porins [6].

(F) Statistical analysis of *β*-barrel membrane proteins and the discoveries

Jackups *et al.* investigated the sequence motifs of residue pairs separated by an arbitrary number on *β*-strands from the genome of 78 Gram-negative organisms using a rigorous statistical model. This study identified characteristic sequence motifs and antimotifs in transmembrane *β*-strands and noted that the tyrosine plays an important role in several such motifs [10]. This study also found that tyrosine linked with aliphatic amino acids to form favorable motifs (Aliphatic-Try) and unfavorable antimotifs (Try-Aliphatic). Based on his results, an experimentally testable hypothesis was proposed to test the significance of the motifs for *in vivo* sorting of *β*- barrel membrane proteins. To study the importance of the motif on the folding behavior, several mutants were proposed. For example, an AY2 motif mutated to a YA2 antimotif reveals the impact of the AY2 motif on the folding properties and stability of the membrane protein. Another type of experiment involved mutating the motifs that were interacting spatially on neighboring strands [10].

Jackups *et al.* developed a probabilistic model that calculates the p-values and propensity values of residues for different spatial locations, using the reference state of exhaustive permutation of residues within the same *β*-strand in a data set of 19 non-redundant *β*-barrel membrane proteins (See Table 1) [11]. Calculating exact p-values predicts the patterns that are statistically significant. This study revealed that there are characteristic preferences to the residues for different membrane locations. Residues such as arginine and lysine that are positively charged are highly favored in the extracellular cap region of the barrel and disfavored in the periplasmic cap region of the barrel. This study also found that a residue pair such as Gly-Aromatic prefers strong backbone H-bonded interstrand pairing and an Aromatic-Aromatic residue pair prefers non H-bond pairing. Another finding was that tyrosine and phenylalanine

contributes to aromatic rescue by shielding glycine from the polar environment. Results of this study were used to predict the hydrogen bond patterns (registration of strand pairs) and the structure with 44% accuracy. Based on these interesting observations, experiments were suggested to elucidate the mechanism of *in vivo* folding and sorting of *β*-barrel membrane proteins [11]. For example, substitution of residues in the periplasmic cap region with arginine or lysine was proposed to investigate the sorting behavior of a mutant in order to verify the positive outside rule in *β*- barrel membrane proteins. Substitution of tyrosine that shields the glycine residue (aromatic rescue of glycine) was proposed to understand the aromatic rescue of glycine in *β*- barrel membrane proteins.

A recent model established by Naveed *et al.* on predicting weakly stable regions, oligomerization state and protein-protein interfaces in *β*-barrel membrane proteins was able to predict whether the *β*-barrel was a monomer or an oligomer with 91% accuracy, and the protein-protein interaction interface with 82% accuracy [16]. This model predicts the unstable strands of a *β*-barrel membrane protein and the residues that contribute to the instability of the strand. Experimental studies were proposed to evaluate the efficiency of this model.

An unpublished evolutionary analysis of *β*-barrel membrane proteins identified residues that are highly conserved in *β*-barrel membrane proteins by Jimenez-Morales *et al*. [27]. Based on these discoveries, an experimental study was proposed to understand the role of glycine residues that are highly conserved at the trimer interface of the OmpF porin protein.

Protein	Organism	Architecture	Strands	PDB ID
OmpA	*E. coli*	Monomer	8	1BXW[46]
OmpX	*E. coli*	Monomer	8	1QJ8[47]
NspA	*N. meningitidis*	Monomer	8	1P4T[48]
OpcA	*N. meningitidis*	Monomer	10	1K24[49]
OmpT	*E. coli*	Monomer	10	1I78[50]
OMPLA	*E. coli*	Dimer	12	1QD6[51]
NalP	*N. meningitidis*	Monomer	12	1UYN[52]
Porin	*R. capsulatus*	Trimer	16	2POR[53]
Porin	*R. blastica*	Trimer	16	1PRN[54]
OmpF	*E. coli*	Trimer	16	2OMF[55]
Omp32	*C. acidovorans*	Trimer	16	1E54[56]
LamB	*S. typhimurium*	Trimer	18	2MPR[57]
ScrY	*S. typhimurium*	Trimer	18	1A0S[58]
FepA	*E. coli*	Monomer	22	1FEP[59]
FhuA	*E. coli*	Monomer	22	2FCP[39]
FecA	*E. coli*	Monomer	22	1KMO[60]
BtuB	*E. coli*	Monomer	22	1NQE[61]
TolC	*E. coli*	Trimer	4	1EK9[62]
α-Hemolysin	*S. aureus*	Heptamer	2	7AHL[4]

All proteins share no more than 26% pairwise sequence identity. Crystal structures have a resolution of 2.6 Å or less. Three identical chains of TolC and seven of α-hemolysin form a single barrel; all other proteins listed form whole barrels with a single peptide chain.

Table 1. Dataset of the β-barrel membrane proteins used in the study by Jackups *et.al.* [11].

(G) Porin protein patterns of OmpF and proposed experiments

OmpF porin protein was chosen as a representative *β*-barrel membrane protein to identify and analyze the patterns found in the statistical analysis [10, 11]. OmpF porin was selected because of the known crystal structure and the wide variety of experimental information. The trimerization properties of OmpF were successfully used to determine the effect of amino acid ssubstitution in the sequence and structural motifs.

(a) Sequence motifs, antimotifs and neighboring motifs

Among the sequence patterns discovered in the statistical analysis of *β*-barrel membrane proteins, highly favored Ala-Try motifs and disfavored Try-Ala antimotifs were very interesting. There were number of other motifs discovered and tyrosine was involved in many of these motifs [10]. These statistically significant motifs were predicted to be important for the protein folding and sorting, so substitution in these regions would likely have an effect on protein stability or flexibility.

The Ala-Tyr motifs and Try-Ala antimotifs on the OmpF monomer are shown in Fig.6. There are 5 motif and 2 antimotif pairs found in an OmpF monomer, motif pairs A137-Y139, A261-Y263, A229-Y231,A299-Y301 and A1-Y3 and antimotif pairs are Y191-A193 and Y220-A222.

The motif interactions on two different *β*-sheets on an OmpF monomer are shown in Fig 7. Motifs V155-Y157 and A137-Y139 interacts with each other on neighboring strands.

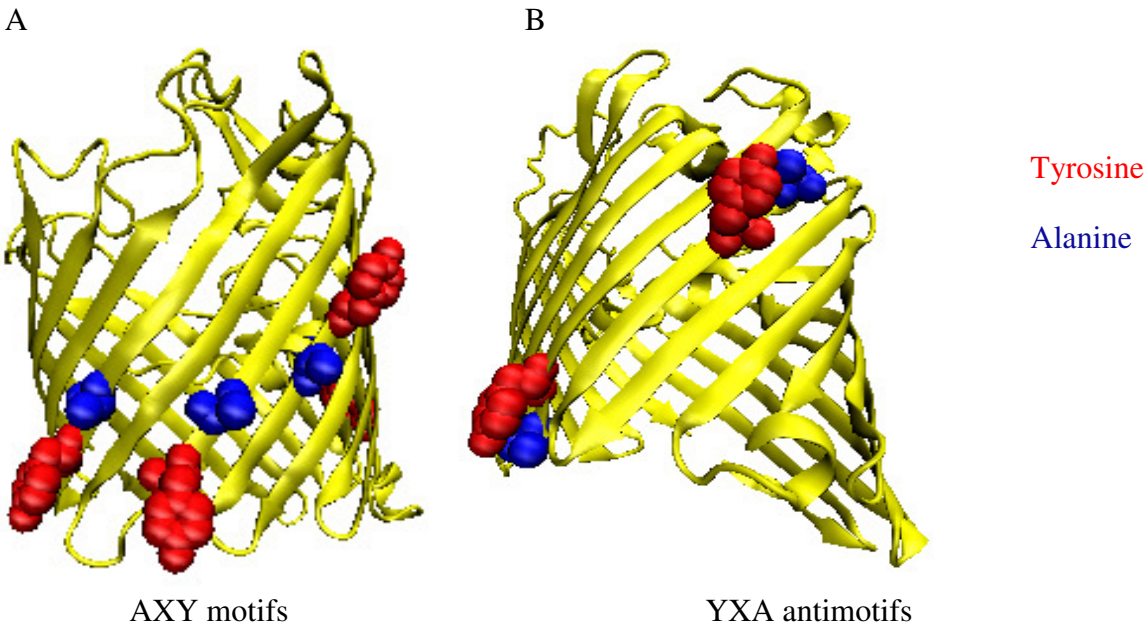

Figure 6. A.The Ala-Tyr motifs and B. the Tyr-Ala antimotifs on an OmpF monomer are shown.

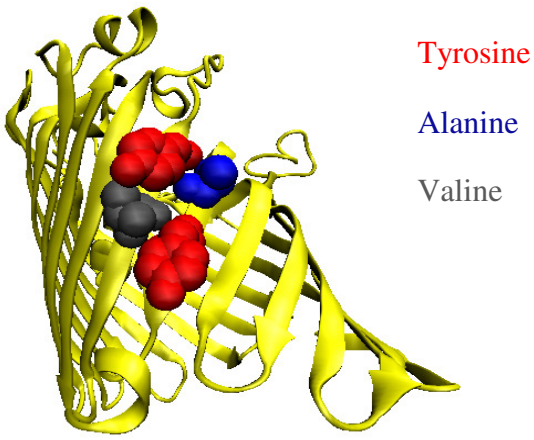

Figure 7. The Interactions of neighboring motifs (Val-Try and Ala-Try) on an OmpF monomer are shown.

(b) Aromatic rescue of Glycine

An interstrand spatial interaction between Gly- Tyr was interesting because normally tyrosine is found outside of the barrel. In the case of Gly- Tyr interactions, tyrosine adopts an unusual conformation and stays inside of the barrel. The analysis on 39 Gly-Try interactions in the data set showed that 32 interactions have tyrosine on the inside of the barrel. This unusual behavior of the tyrosine serves to protect the exposure of glycine from the polar environment and at the same time minimizes the area of aromatic ring exposed to solvent. It was predicted that substitution of the tyrosine residue may have an effect on the stability of the OmpF monomer. This observation in β-barrel membrane proteins was similar to aromatic rescue of glycine in soluble β-sheets [11]. Fig. 8 illustrates the Gly-Tyr interstrand interactions of an OmpF monomer.

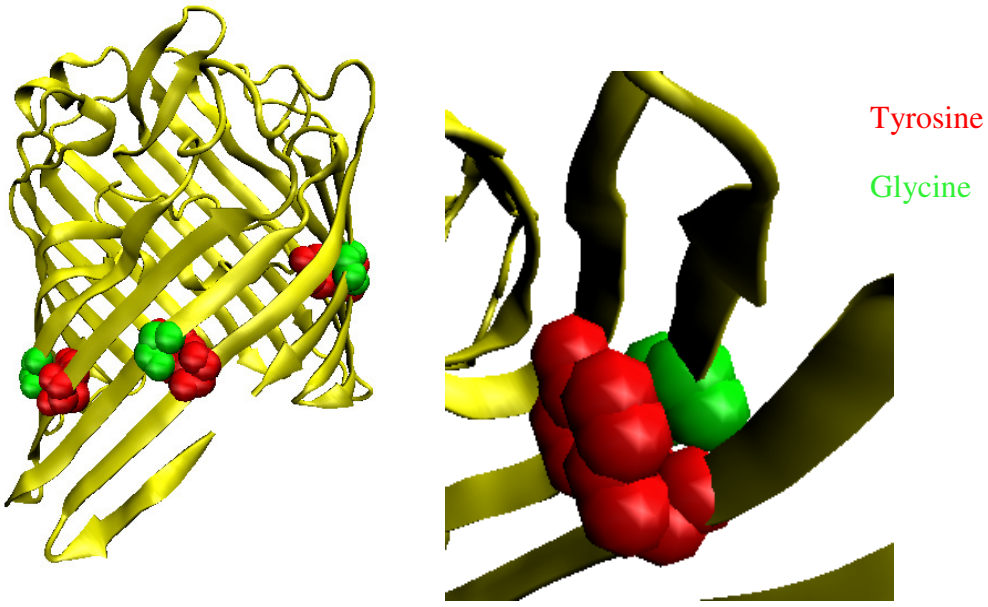

Figure 8. Gly-Tyr interstrand spatial interactions on an OmpF monomer. Tyrosine is in an unusual conformation bends into the barrel and protects the Glycine from polar environment.

(c) Trimer interface interactions

The probabilistic model developed to quantify the residue propensity for interstrand pairwise contact interactions was used to identify statistically significant interactions [11]. A strong propensity was always observed between a small residue and an aromatic residue with a strong backbone H-bond interaction such as Gly-Tyr and Gly-Phe. The interstrand spatial interaction between glycine and phenylalanine is shown in Fig. 9 on each monomer of the OmpF trimer. Substitution to disturb this contact affected the trimer stability. Substitutions were proposed in this region in order to determine the effectiveness of the potential function, which calculated the statistical significance of the Gly-Phe interaction. Using the potential function, leucine residues at the trimer interface of an OmpF porin were predicted to be statistically significant. Fig.10 shows leucine residues at the trimer interface interacting with each other. If this interaction is biologically important, substitution of leucine would be expected to reduce the trimer stability significantly.

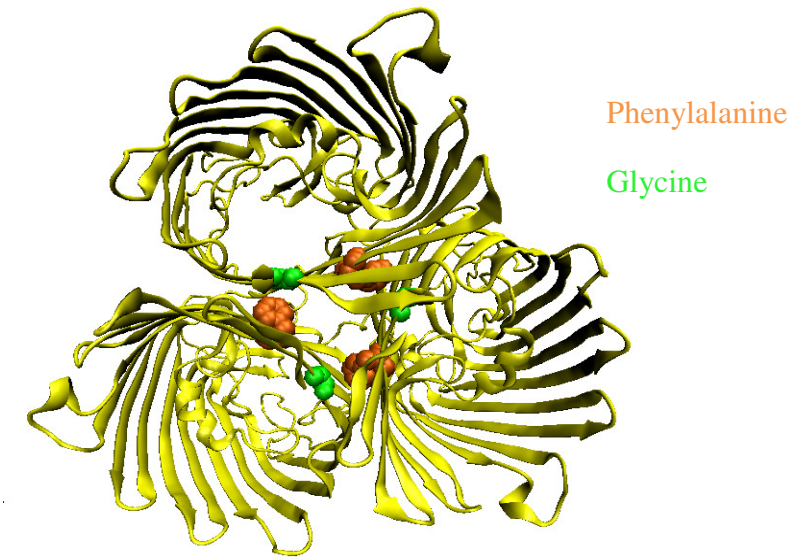

Figure 9. Interaction of glycine and phenylalanine at the trimer interface of an OmpF porin.

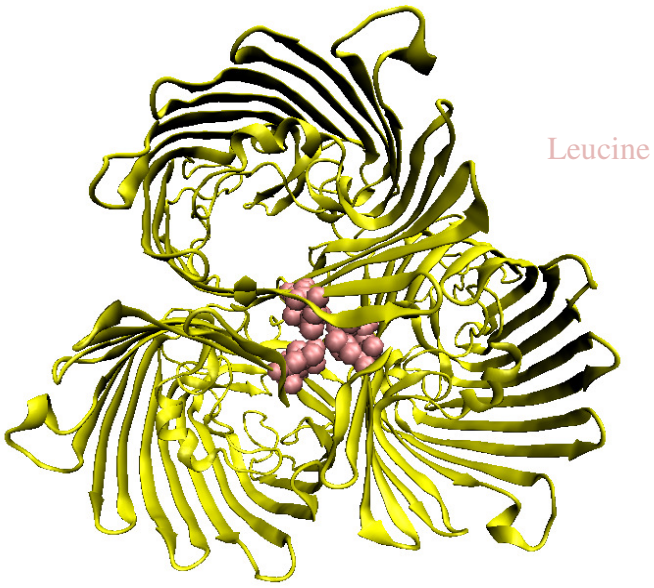

Figure 10. Interaction of leucine residues at the trimer core of an OmpF porin.

(d) Positive outside rule

Using the probabilistic models developed, the significance was calculated for the location preferences of different types of residues in *ß*- barrel membrane proteins [11]. One of the interesting observations was that basic residues such as arginine and lysine have a high preference to stay in the extracellular cap region. This observation is similar to the positive-inside rule of α-helical membrane proteins. This property of *ß*- barrel membrane proteins is called the positive outside rule and this propensity may play a role in insertion of the protein into the membrane [11]. Fig. 11 shows that arginine residues in an OmpF monomer prefer occupying the extracellular end of an OmpF porin.

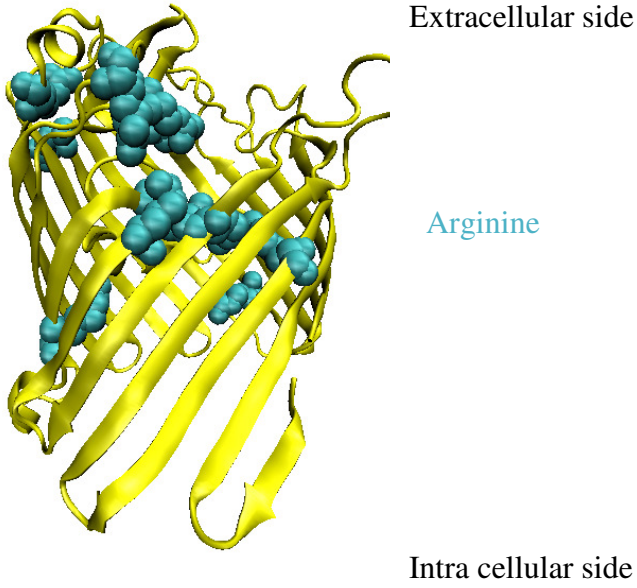

Figure 11. Arginine residues on an OmpF monomer located at the extracellular side of the protein.

(e) Glycine residues that are highly conserved on an OmpF trimer

Evolutionary analysis of the *β*-barrel membrane proteins suggested that the glycines at the protein-protein interaction interface are extremely conserved [27]. Fig. 12 shows the glycines at the trimer interface of an OmpF trimer.

(f) Highly unstable residues of an OmpF porin

Investigation of weakly stable regions in *β*-barrel membrane proteins provided information on the strands that have the highest overall energy in the protein and the residues that are responsible for the strand instability [16]. Residues that contribute to strand instability were predicted by the energy profile of the protein. G135, G19 and R100 are the residues on an OmpF monomer that are predicted to have high energy. G135W, G19W and R100V substitution studies were suggested on an OmpF porin protein. Fig. 13 shows the G135 and R100 residues on an OmpF trimer.

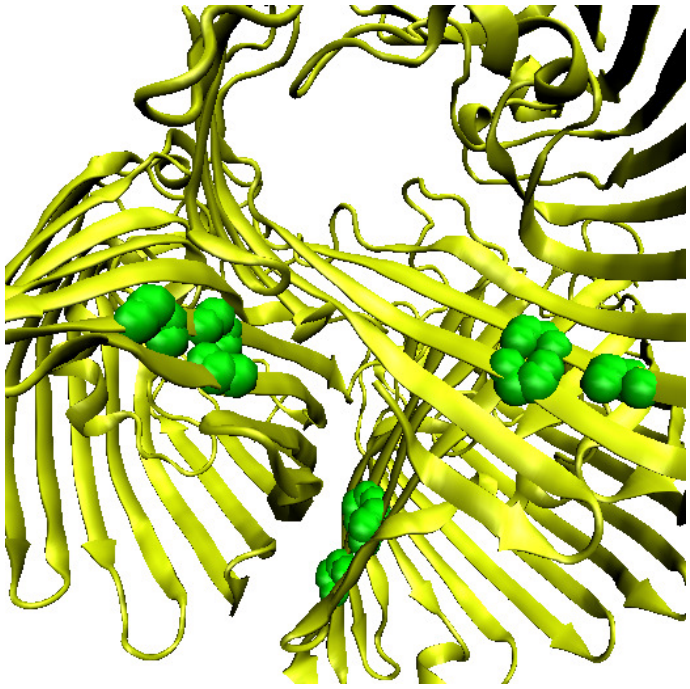

Figure 12. Glycines residues at an OmpF trimer interface. Glycine residues at the 47, 57 and 59th positions are shown here.

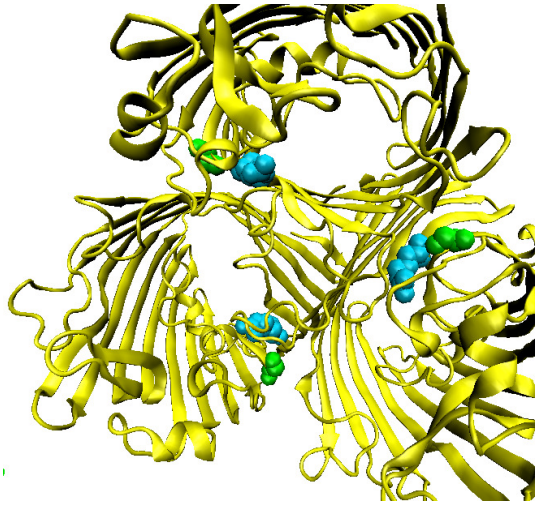

Figure 13. Glycine on the sixth strand at 135th position and an arginine on fifth strand at the 100th position of an OmpF trimer are shown here.

2. RATIONALE OF THIS STUDY

ß-barrel membrane proteins are widely expressed. These proteins are involved in major cellular functions such as nutrients/waste transport and cell adhesion. Some of these proteins act as lipases, proteases and pore forming toxins. With the advancements in the fields of genomics and proteomics, we were able to identify large numbers of them. Very few 3-dimensional structures are known to date because of the difficulties associated in purification and crystallization of membrane proteins.

Sequence and structural details of *ß*-barrel membrane proteins has been conducted in Dr. Liang's laboratory [10, 11, 16, 27]. Their published papers suggest that there are several sequence and structural motifs that are statistically important. Thus, this thesis work was focused on investigating the role of the sequence and structural motifs of the *ß*-barrel membrane protein stability and function. In particular, OmpF proteins with different sequence and structural motif substitutions were examined for thermal stability.

3. METHODS

(A) Deletion of the *ompF* gene

To delete the *ompF* gene from the wild type MG1655 strain, I used a one step inactivation of a chromosomal gene in *Escherichia coli* K-12 by homologous gene recombination of the *ompF* gene with a Kanamycin resistance gene [17]. A template plasmid, pKD13, with a Kanamycin resistance gene was used to produce a PCR product with the Kanamycin resistance gene flanked by *ompF* homologous ends. The primers contained 62 base pairs with either 41 or 42 nucleotide extensions that were homologous to the *ompF* gene ends (Fig. 14). Recombination of the target gene with the PCR product requires the λ- Red recombinase. The pKD46 λ- Red containing plasmid was transformed into MG1655. Wild type cells with pKD46 λ- Red were made electrocompetent and the PCR Kanamycin gene product was transformed into the cells by electroporation. The cells were plated on kanamycin (50 μg/ml) plates to select for recombination.

Forward
ATGATGAAGCGCAATATTCTGGCAGTGATCGTCCCTGCTCTGTGTAGGCTGGAGCTG
CTTCG
Reverse
TTAGAACTGGTAAACGATACCCACAGCAACGGTGTCGTCTGTTCCGGGGATCCGTCG
ACCTG

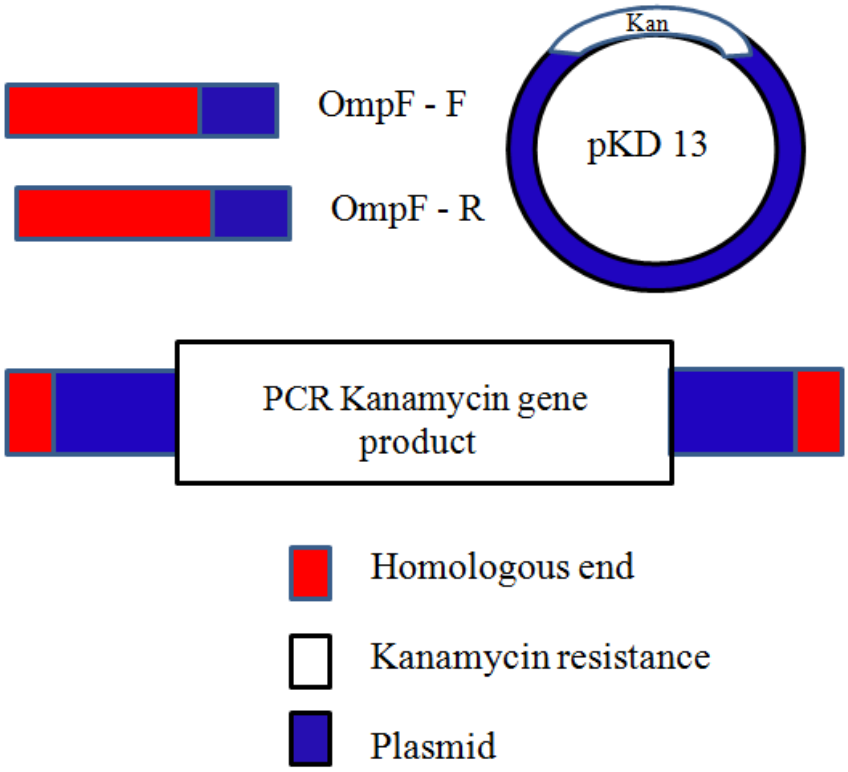

Figure 14. Amplification of the Kanamycin resistance gene with *ompF* gene homologous ends. OmpF-F and OmpF-R primers were used in the PCR to amplify the Kanamycin gene on the pKD13 plasmids. The PCR product, Kanamycin resistance gene flanked by *ompF* homologous ends was obtained.

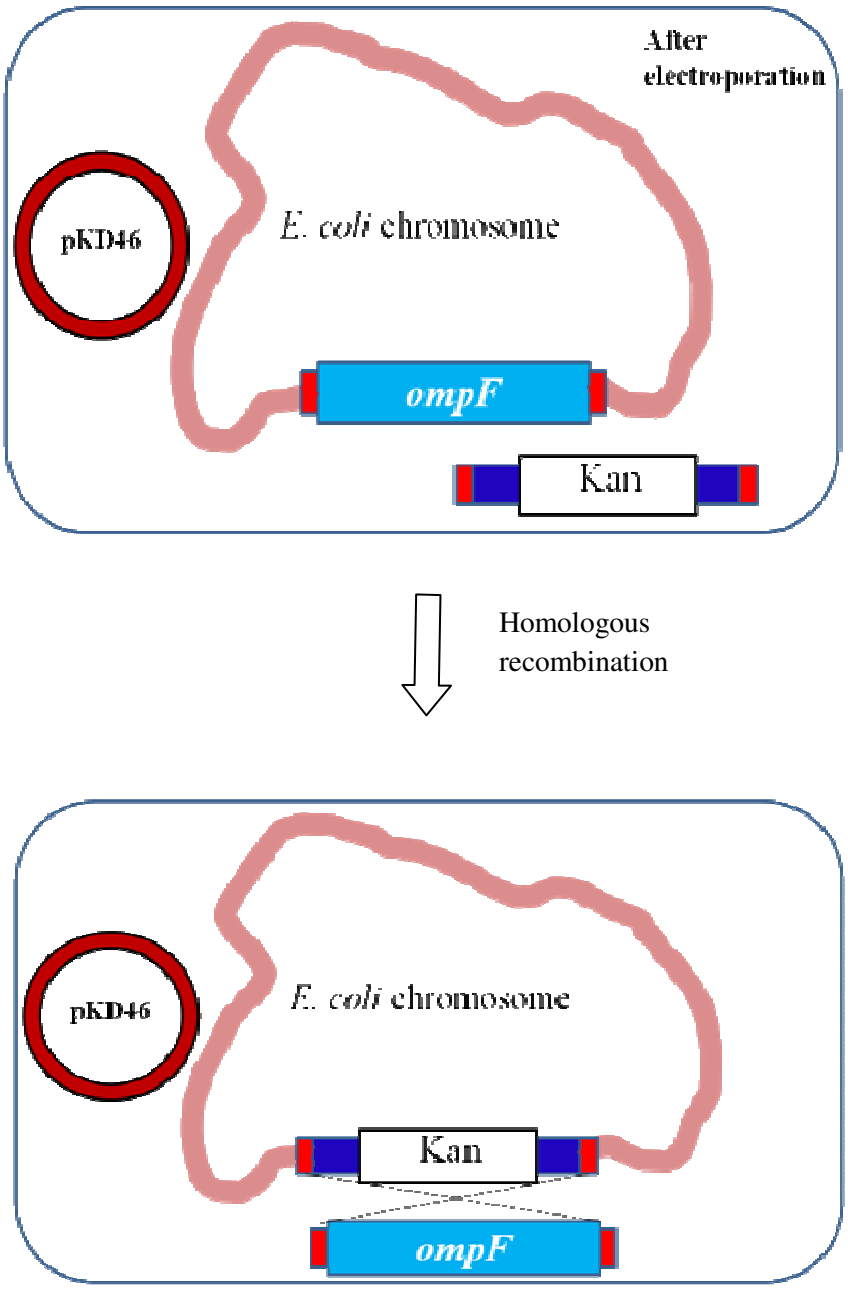

Figure 15. Electroporation and recombination of kanamycin resistance gene with *ompF* homologous ends.

(B) Cloning the *ompF* gene

The *ompF* gene was amplified using primers (CCGGATCCATGATGAAGCGCAATAT TCTGGC and GGCTCGAGGAACTGGTAAACGATACCC) with flanking restriction sites (Nco1 and Xho1) and ligated into vector TOPO 2.1 [24]. The ligation product was transformed into DH5α cells and screened on a kanamycin (50µg/ml) and X-gal (40µg/ml) plate for kanamycin resistance and *β-gal* gene activity. Colonies which did not show *β-gal* gene activity (white in color) were used to isolate plasmid DNA and the *ompF* insert sequence was confirmed by sequencing. The *ompF* gene insert in TOPO2.1 vector was digested with Ncol1 and Xho1 restriction enzymes and separated on a 1.5% agarose gel followed by gel purification with a Millipore DNA gel extraction kit. The pET28 vector was also digested with Ncol1 and Xho1 and the fragments were separated and the large linear fragment of DNA ~5Kb was gel purified. A ligation reaction was performed at 16°C overnight between the *ompF* gene fragment (~1.9Kb) and the pET28 vector (~5Kb). The next day, 4µl of the reaction mixture was transformed into DH5α competent cells and plated on a kanamycin plate (50µg/ml). Colonies resistant to kanamycin were selected and used for the isolation of plasmid DNA. The construct was confirmed by sequencing.

(C) Outer membrane profile

The outer membrane profile was examined to ensure the expression of OmpF porin in the outermembrane [15]. Overnight cultures of the *ompF* null stain with pBAD24F or mutants of pBAD24F were grown in LB medium containing 50 µg/ml of ampicillin and kanamycin. The next day, they were subcultured into 10 ml of minimal media A (7 g Nutrient broth powder, 1 g Yeast extract, 2 g glycerol, 3.7 g K_2HPO_4, 1.3 g KH_2PO_4 per liter) containing 50 µg/ml of

ampicillin and kanamycin. The osmolarity of the medium was adjusted by adding water or 30% sucrose. Cells were induced with 0.2% arabinose at the time of subculturing and grown until OD_{600} of ~0.6 at 37 °C. Cells were harvested by centrifugation at 3000×g for 10 minutes. The pellet was collected and air dried for 15 minutes and then kept on ice for 10 minutes. The pellet was resuspended by vortexing in 200 µl of a buffer containing 20% sucrose and 30 mM Tris-HCl at pH 8.8, then 50 µl lysis buffer was added. Lysis buffer contains equal parts of 10 mg/ml of lysozyme and 0.2 M EDTA pH 8.0. Test tubes were kept on ice for 30 minutes followed by addition of 5 ml of 30 µM EDTA. Complete cell lysis was assured by sonication for 1 minute at setting 7 on a Fisher Scientific model 100 Sonic Dismembranator. The cell debris and unbroken cells were separated by centrifugation at 3000×g for 10 minutes at 4°C in an Avanti J26XP centrifuge. The supernatant collected from this step was centrifuged at 21,000× g for 1 hour at 4°C. The membrane fraction was collected in the pellet. The pellets were dissolved in Laemmli buffer and heated at 95°C for 10 minutes and separated on a Urea denaturing SDS PAGE gel.

(a) <u>Urea denaturing SDS-PAGE gel:</u>

The resolving gel contained 3.6 g urea, 3.5 ml of 1M Tris pH 8.8, 4.0 ml of 30% acrylamide, 0.8% bisacrylamide, 0.2 ml 10% SDS,100 µl 10% APS and 10 µl TEMED

The stacking gel contained 1.25 ml of Tris pH 6.8, 1.0 ml of 30% acrylamide, 0.8% bisacrylamide, 0.2 ml 10% SDS,7 ml H_2O, 200 µl 10% APS and 20 µl TEMED.

(D) <u>Site directed mutagenesis</u>

To create mutants of the *ompF* gene, site directed mutagenesis [19] was performed on plasmid DNA with wild type *ompF* (pBAD24F). Primers with the desired mutation (See Table 2) were used to amplify pBAD24F. The PCR product contains both the original template plasmid

and amplified DNA with the mutation. The mixture of DNA was treated with Dpn1 restriction enzyme to digest the original template DNA. 2 µl of this mixture was transformed into 50 µl of DH5α competent cells by heat shock at 42 °C for 40 seconds and then kept on ice briefly. 500 µl of LB media was added to the cells and placed in a shaker at 37°C for 30 minutes. Cells were plated on an ampicillin plate and screened for ampicillin resistance. A few colonies were selected randomly for isolation of plasmid DNA and the mutation was confirmed by sequencing.

(E) Plasmid extraction

The Quiagen plasmid mini prep kit [20] was used to prepare all of the plasmid DNA.

(F) Plasmid sequencing

Plasmids were sequenced at the Research Resources Center (RRC), UIC [21] using plasmid specific primers. The forward primer used was CTGTTTCTCCATACCCGTT and the reverse primer used was CTCATCCGCCAAAACAG.

Mutant	Forward primer	Reverse primer
Sequence motifs		
Y139A	CGTGTTGGCGGCGTTGCTACCGCGCGTAACTCCAACTTCTTTGG	CCAAAGAAGTTGGAGTTACGCGCGGTAGCAACGCCGCCAACACG
A137Y-Y139A	CGTGTTGGCGGCGTTTATACCGCGCGTAACTCC	GGAGTTACGCGCGGTATAAACGCCGCCAACACG
Y231A	CCTGGCAGCGAACGCGGGTGAAACCCG	CGGGTTTCACCCGCGTTCGCTGCCAGG
A229Y-Y231A	CATCTACCTGGCATATAACGCGGGTGAAACC	GGTTTCACCCGCGTTATATGCCAGGTAGATG
Antimotifs		
Y191A	TGGTATCGTTGGTGCTGCTGGTGCAGCTGACCGTACC	GGTACGGTCAGCTGCACCAGCAGCACCAACGATACCA
Y191A-A193Y	GGTGCTGCTGGTTATGCTGACCGTACC	GGTACGGTCAGCATAACCAGCAGCACC
Neighboring sequence motifs		
V155A-Y157A+ A137-Y139A	CCTGAACTTCGCTGCTCAGGCCCTGGGTAAAAACG	CGTTTTTACCCAGGGCCTGAGCAGCGAAGTTCAGG
V155Y-Y157A+ A137Y- Y139A	CCTGAACTTCGCTTATCAGGCCCTGGGTAAAAACG	CGTTTTTACCCAGGGCCTGATAAGCGAAGTTCAGG
Aromatic rescue		
Y14A-G44	GTAGATCTGGCCGGTAAAGCTGTTGG	CCAACAGCTTTACCGGCCAGATCTAC
Trimer interface		
L13A	GGCAACAAAGTAGATGCGTACGGTAAAGC	GCTTTACCGTACGCATCTACTTTGTTGCC
F45A - G15	GCCCGTCTTGGTGCGAAAGGGGAAACTC	GAGTTTCCCCTTTCGCACCAAGACGGGC
Positive outside rule		
D92R	GGGTCTTAAATACGCTCGCGTTGGTTCTTTCG	CGAAAGAACCAACGCGAGCGTATTTAAGACCC
Evolutionary analysis mutants		
G47I	CCGTCTTGGTTTTAAAATTGAAACTCAAATCAATTC	GGAATTGATTTGAGTTTCAATTTTAAAACCAAGACGG
G47I-G57I	CCGATCTGACCATCTATGGTCAGTG	CACTGACCATAGATGGTCAGATCGG
G57I-G59L	CCGATCTGACCATCTATCTTCAGTGGG	CCCACTGAAGATAGATGGTCAGATCGG
Energy function mutants		
G19W	CGGTAAAGCTGTTTGGCTGCATTA	TAATGCAGCCAAACAGCTTTACCG
G135W	GGTCGTGTTGGCTGGGTTGC	GCAACCCAGCCAACACGACC
G135W-R100V	CTTTCGATTACGGCGTGAACTACGG	CCGTAGTTCACGCCGTAATCGAAAG
G135W-R100L	CGATTACGGCCTGAACTACGG	CCGTAGTTCAGGCCGTAATCG

Table 2. List of primers used for site-directed mutagenesis.

(G) **Purification of OmpF protein from outer membranes**

Either pBAD24F or pBAD24F (with the mutation(s)) was transformed into strain *omp8* (BL21 (DE3), *ΔlamB ompF*: :Tn5 *ΔompA ΔompC)* [18] and ampicillin and kanamycin resistant-colonies were selected. The cells were grown in LB containing 50 µg/ml of ampicillin and kanamycin at 37 °C. Overnight cultures were subcultured into 100 ml of LB with 50 µg/ml of ampicillin and kanamycin and at OD_{600} ~0.6 the cells were induced with 0.2% arabinose. After 3 hours of growth, the cells were harvested and porins were purified by the Rummel and Rosenbush method [9].

(a) Procedure

The pellet was resuspend in lysis buffer containing 2% SDS in 20 mM Tris-HCl, pH 8.0. The cell were incubated at 60°C for 1 hour to ensure all were broken and sonicated on ice by using 3 pulses for 40 seconds with a 3 minute cool down between pulses. (Sonication was done at power setting 5 on a Fisher Scientific Model 100 sonic dismembranator). And the envelope fraction was collected the by centrifugation at 100,000×g for 1 hour at 4°C in a Beckman L8 70M class H ultra centrifuge. The pellet was washed with 20 mM sodium phosphate buffer, pH 7.3 to remove the residual SDS and then resuspended and extracted with 0.125% octyl-POE in 20 mM phosphate buffer at 37°C for 1 hour to remove the majority of the contaminants. The sample was subjected to centrifugation at 100,000×g for 1 hour at 4°C and the supernatant was discarded. The pellet was resuspended and extracted in 3% octyl-POE in 20 mM phosphate buffer for 1 hour at 37°C. Then the sample was subjected to centrifugation at 100,000×g for 1 hour at 25°C. The supernatant with porins was collected and dialyzed against 1% octyl-POE in 20 mM phosphate buffer overnight. The porin samples were separated on 12% SDS- PAGE gel to confirm the presence of the OmpF porin.

(H) Thermal stability analysis

The trimer dissociation temperature of the wild type and various mutant porins was determined using a thermal stability assay. Protein samples were incubated for 10 minutes at various temperatures (55-85 °C, in increments of 5 °C) along with SDS and *ß*-mercaptoethanol and separated by 15% SDS-PAGE. The porin protein band shifts from ~94KDa to ~37KDa as the trimer dissociates into monomers [13]. The temperature at which the trimer begins to form monomers (Td) and complete dissociation of the trimer (Tc) were noted. The Td values of the mutants were compared to the wild type value to determine the effect of the substitution on trimer stability.

(I) Planer Lipid Bilayer assay

Planar Lipid Bilayer (PLB) experiments to measure the conductance of OmpF porin were performed by Janhavi Giri in the laboratory of Dr. Robert Eisenberg, Rush University [14]. The experimental set up contains a cuvette that was divided into two compartments named *cis* and *trans* compartments. A small aperture of 150 µm diameter between the *cis* and the *trans* compartments was present. The *cis* and the *trans* compartments were connected to headstage and ground, respectively, by the means of 3M KCl/2% agar salt bridges. This set up was established in a Faraday cage to minimize the responses from unwanted external voltages (See Fig. 16).

The *cis* and the *trans* sides of the cuvette were filled with 1 M KCl. The lipid bilayer, phosphatidylethanolamine and phosphatidylcholine (4:1) dissolved in n-decane (10 mg/ml), was painted across the 150 µm diameter aperture. Once a good lipid bilayer was formed, OmpF protein was introduced into the *cis* side of the cuvette and stirred gently to reconstitute OmpF

porin. At this point, voltage was applied on the *trans* side and several response plots were obtained.

The data was sampled digitally at 5 KHz and filtered digitally at 300 Hz using a low-pass 8-pole filter to screen out the background noise. Conductance of the OmpF porin was calculated by measuring the slope of the conductance of the fully opened trimer with equal concentrations of ions on both the *cis* and the *trans* side [14].

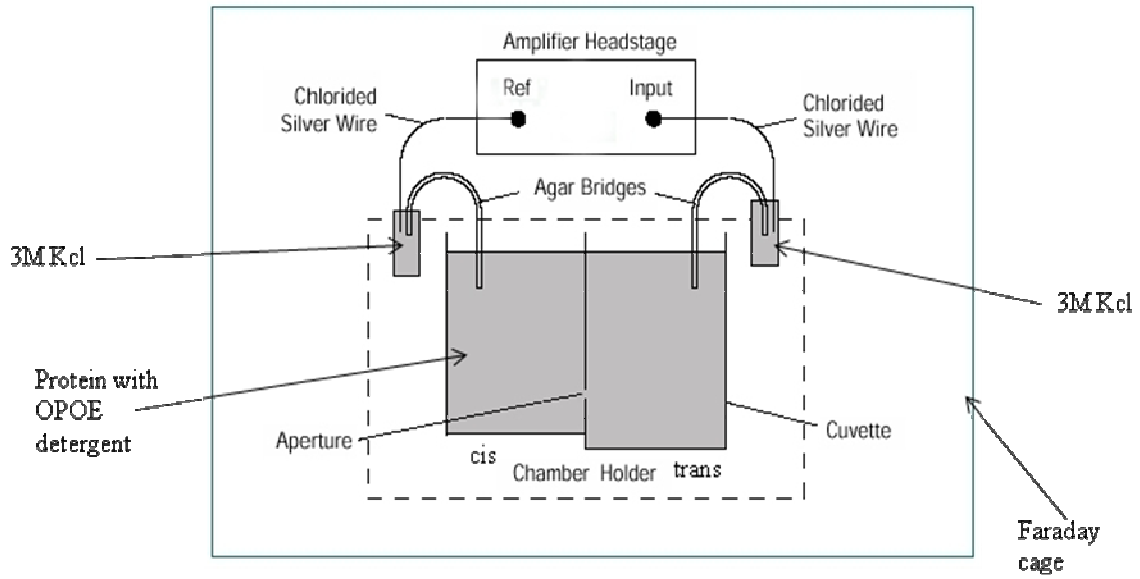

Figure 16. Experimental setup for the planar lipid bilayer assay. The porin sample that is introduced into the cis side of the cuvette inserts into the 150 µm slit. Voltage is applied on the trans side and the voltage responses are recorded at the amplifier headstage.

4. RESULTS

(A) Constructing and verification of an *ompF* null strain

In order to express the OmpF wild type and mutant proteins from the plasmid, a strain without the chromosomal *ompF* gene is required. To construct a strain with no *ompF* gene, the *ompF* gene deletion on the chromosome of wild type MG1655 and the deletion was verified.

Using homologous gene recombination with the λ-Red recombinase system, the *ompF* gene was successfully deleted from the wild type MG1655 and replaced with a kanamycin resistance gene (Kan^R) as described in methods section A. *ompF* null cells were selected by kanamycin resistance on a LB Agar plate containing 50 ug/ml of kanamycin. The *ompF* gene deletion was also confirmed by checking the expression of the OmpF porin in the outer membrane as described in methods section C. Wild type MG1655 expressed OmpF porin protein in low osmolarity media (Figure 17, lane 2) but not in high osmolarity media (lane 3). As expected, in the *ompF* null strain OmpF porin was not expressed in low osmolarity media (lane 4), confirming the deletion of the *ompF* gene. The *ompF* gene on the chromosome of the wild type MG1655 was deleted. This strain was named as *ompF* null (*ΔompF*) strain. The *ompF* null strain does not express OmpF porin protein in either high (not shown) and low osmolarity media.

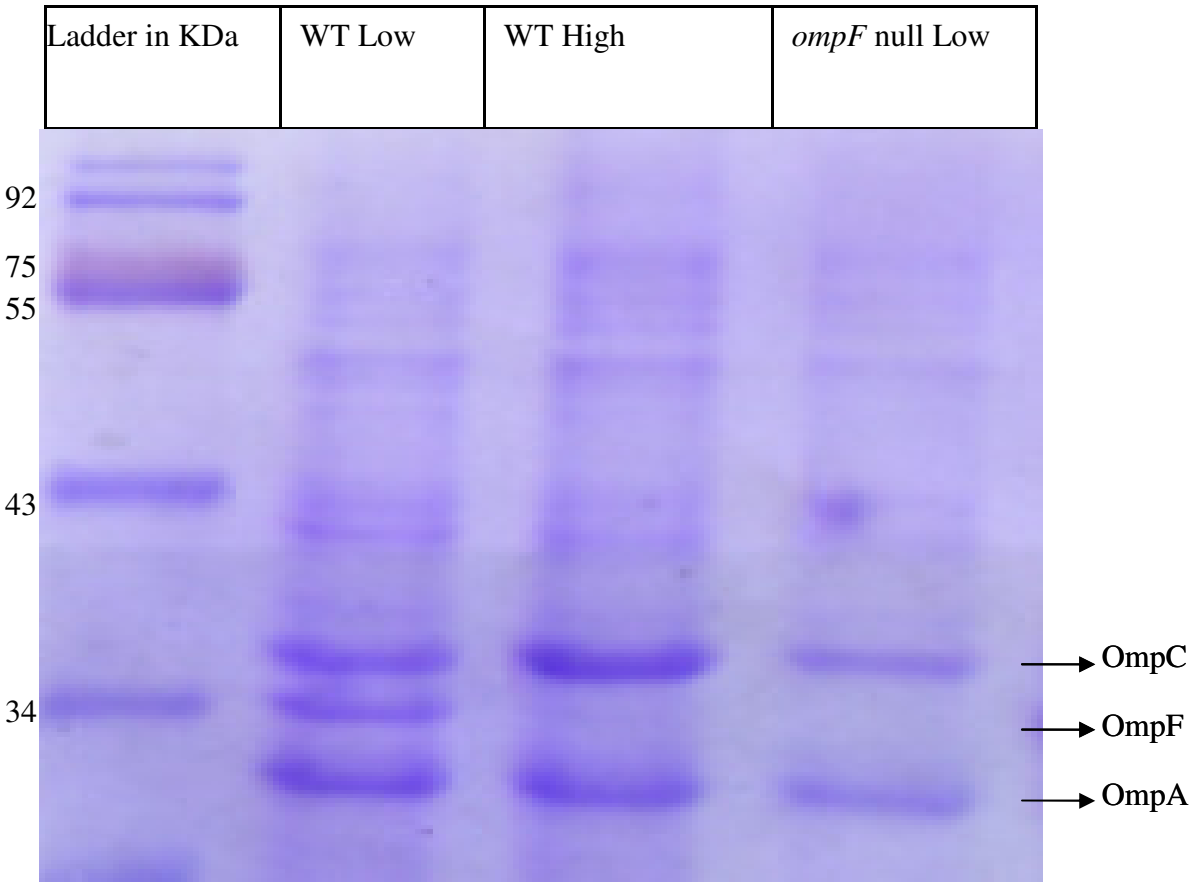

Figure 17. Expression of outer membrane proteins in wild type MG1655 at low and high osmolarity and the isogenic *ompF* null strain at low osmolarity. Low indicates 0% sucrose and high indicates 15% sucrose added to growth medium A.

(B) Constructing OmpF mutants and examination of their expression

Statistical analysis of *β*-barrel membrane proteins identified several sequence and structural motifs [10, 11, 16, 27]. To understand which motifs are statistically important, different motif substitutions were made on the plasmid pBAD24F and then the expression of the mutant OmpF proteins in the *ompF* null strain was examined. Site-directed mutagenesis was performed using plasmid pBAD24F and 19 different mutations were constructed as described in methods section D. Expression of the mutant protein was confirmed by examining the outer membrane profile of each mutant strain as described in methods section E. After extracting the outer membranes, extracts were separated by SDS-PAGE. All of the mutants expressed OmpF porin protein in low osmolarity media except the following: G47I-G57I, G57I-G59L, G135W-R100V and G135W-R100L. The outer membrane expression profiles of the *ompF* null strain with pBAD24F, Y139A, A137Y-Y139A, Y231A or A229Y-Y231A mutations are shown in Figure18, lanes 3-7. Mutant OmpF porin proteins (lanes 4-7) ran identically to the wild type OmpF protein expressed in the *ompF* null strain (lane 3).

Ladder KDa	Wild type	*ompF* null strain + pBAD24F				
		pBAD24F	Y139A	A137Y- Y139A	Y231A	A229Y- Y231A

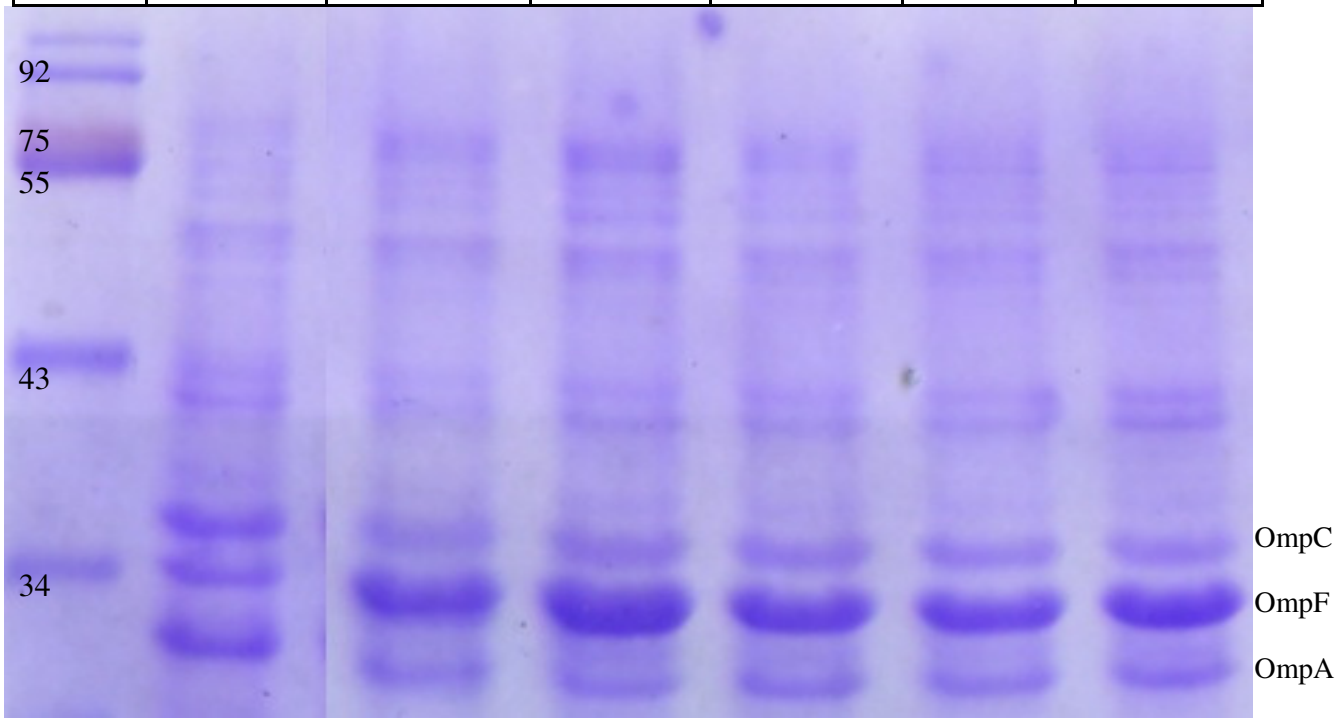

Figure 18. Outer membrane profiles of wild type MG1655 and *ompF* null strains under low osmolarity conditions. The *ompF* null strain contains either pBAD24F or pBAD24F with the indicated mutation.

(C) Purification of OmpF from outer membranes

OmpF porin protein is required in purified form to study the thermal stability and to conduct biophysical tests. Thus, the OmpF protein (wild type and mutant) from outer membranes of *E. coli* was purified. Purification of the OmpF protein can be achieved either by overexpressing the OmpF proteins in inclusion bodies followed by denaturing them and refolding the denatured protein or by purifying the OmpF trimers from outer membranes. I have purified the OmpF protein from the outer membranes of *E.coli* using Octyl-POE detergent as described in methods section G. The OmpF porins are held in the outer membrane by strong hydrophobic interactions, hence the porins cannot dissolve in aqueous solutions. To dissolve the membrane porins in the membrane, the presence of detergent is required. The detergent Octyl-POE was used to extract and purify the OmpF wild type and mutant porin proteins from the *omp8* strain with plasmid pBAD24F (or mutation(s)) [12]. Porins were collected after 3% octyl-POE extraction and separated on SDS-PAGE with or without dialysis against 1% octyl-POE in 20 mM sodium phosphate buffer.

The wild type OmpF and 15 mutant porins were recovered by Octyl-POE extraction and stored in 1% Octyl-POE at 4°C. The mutations G47I-G57I, G57I-G59L, G135W-R100V and G135W-R100L were not recovered. Porins collected in this manner are stable even after prolonged storage. We observed multiple bands on SDS-PAGE (Figure 19, lane 7-9) in some of the porin extracts. This could indicate a possible modification of the OmpF porins, which we plan to investigate further by mass spectrometry.

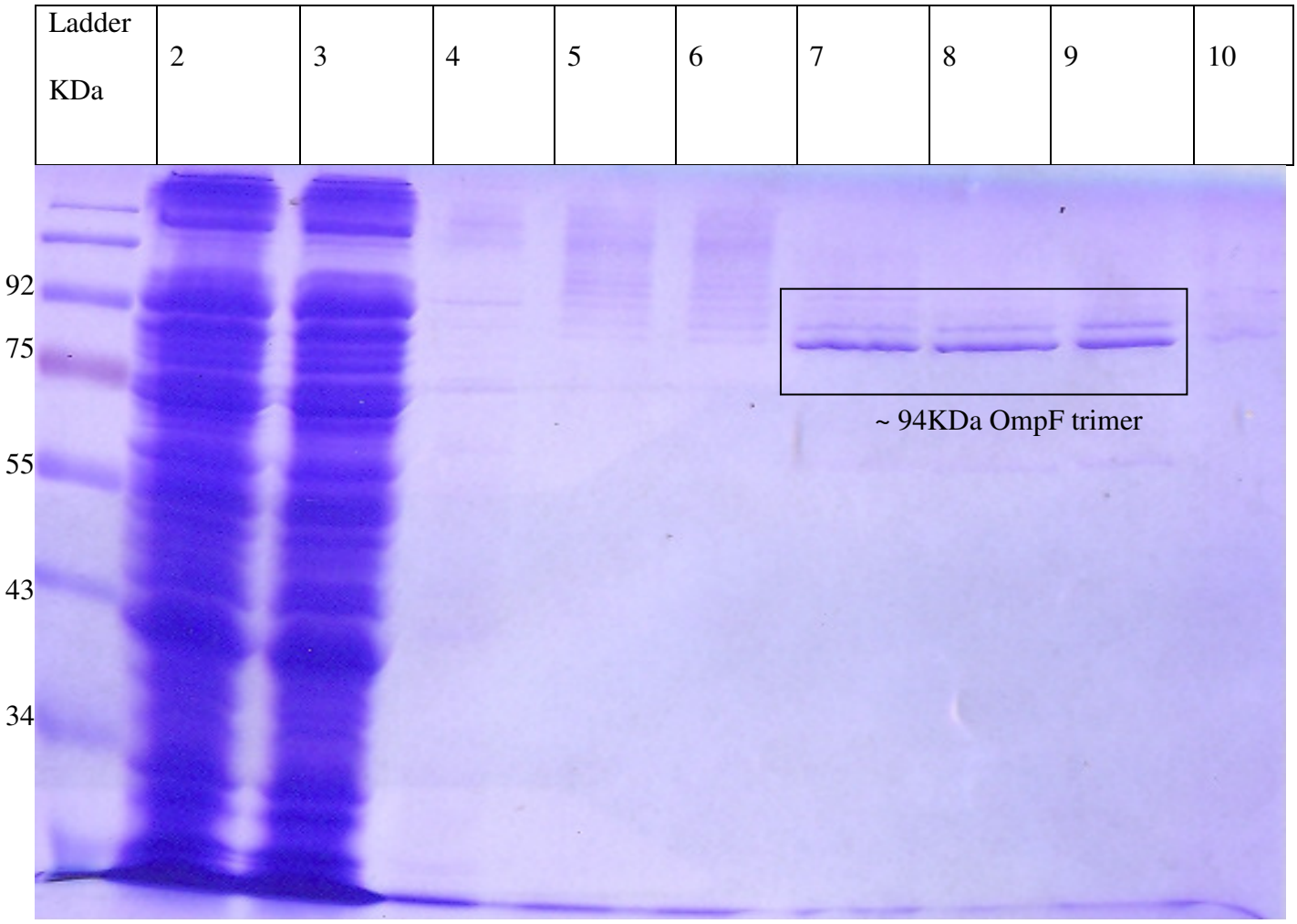

Figure 19. Samples from each step of the porin extraction process were separated on a 12% SDS-PAGE. The whole cell lysate is in lane 2 and the supernatant after ultracentrifugation is in lane 3. The pellet was washed with 20 mM sodium phosphate buffer (lane 4), and then extracted with 0.125% octyl-POE (lane 5). After ultracentrifugation, the supernatant (lane 6) was discarded and the pellet was further extracted in 3% octyl-POE (lane 7). After additional ultracentrifugation, the supernatant containing the porins was observed as a band around 94 KDa (lane 8). The porin extract from lane 8 was dialyzed against 1% octyl-POE (lane 9) and stored at 4°C. The pellet remaining in the final centrifugation step was separated on the gel and shows little porin protein (lane10).

(D) **Thermal stability of the wild type and the mutants**

The OmpF porin protein forms a very stable trimer by the hydrophobic interactions of 3 monomers. A thermal stability assay with wild type OmpF protein and the mutant OmpF proteins was performed to measure the temperature at which the OmpF trimer dissociates into monomers. By comparing the dissociation temperatures of the wild type and the mutants, the effect of the substitution on the trimer stability was estimated. The wild type and mutant OmpF porin proteins that were purified from the outer membranes of the *omp8*strain were used for thermal stability studies. The temperature at which the trimer dissociates into monomers was determined by heating the stable trimer protein at different temperatures ranging 55 °C to 85 °C, in increments of 5 °C (See method H).

(a) Wild type OmpF porin

Wild type OmpF porin ~ 94 KDa was stable between 55 °C to 75 °C, but dissociated into monomers of ~34 KDa at 80 °C. As seen in Fig. 20, between temperatures 55 °C and 75°C, OmpF is observed around 94 KDa. At 80 °C, a band around 94 KDa and a band around 34 KDa were observed. Hence, the monomerization start point was ~80 °C. At 85 °C, the band around 94 KDa completely disappears, indicating complete monomerization of OmpF porin.

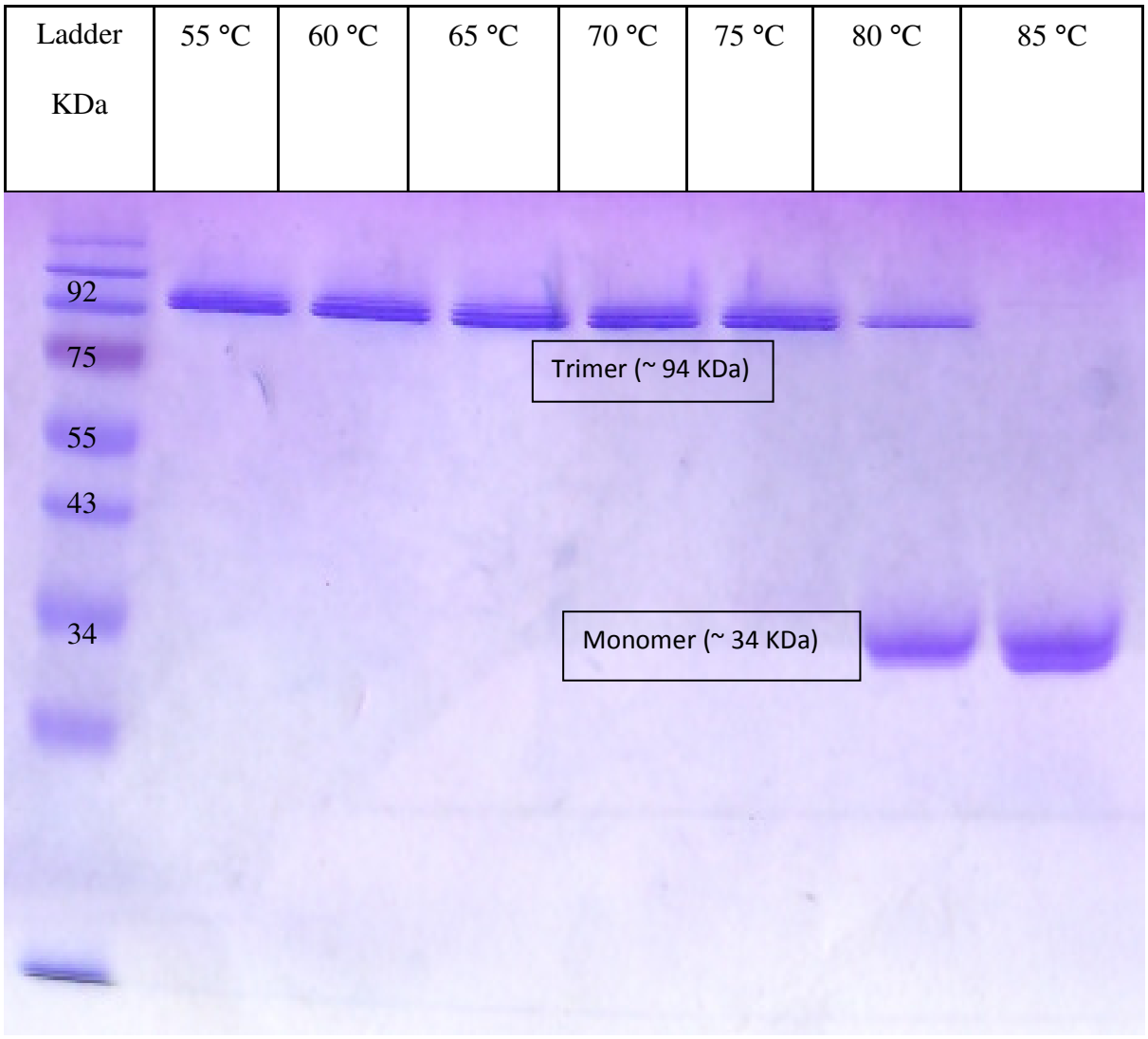

Figure 20. Thermal stability analysis of the wild type OmpF porin separated by SDS-PAGE. The OmpF trimers were observed at around 94 KDa. At 80 °C, OmpF trimers started to form monomers and the monomer band was obsevered at around 34 KDa. Trimers were completely dissocicated into monomers at 85 °C.

(b) Sequence motif mutants

Two sets of sequence motif mutants were constructed to identify the role of the sequence motifs on protein stability and function. They were Y139A, A137Y-Y139A and Y231A, A229Y-Y231A. The sequence motif mutants A137Y-Y139A and A229Y-Y231A were created using a two step process. For example, in the first step A137-Y139 has the tyrosine substituted to A137- Y139**A** (referred to as Y139A) and then the plasmid DNA from that mutation was used to substitute alanine 137 with tyrosine. This results in the double mutant A137**Y**- Y139**A**. Mutant OmpF porin proteins were purified by Octyl-POE extraction and analyzed for thermal stability.

Both Y139A and A137Y-Y139A mutant porins start forming monomers at 75°C and complete monomerization occurred at 80°C. Hence, the mutation in the Y137-A139 sequence motif reduces the thermal stability of the OmpF porin by ~5°C. In the case of the A229-Y231 motif, the intermediate mutant Y231A mutant has a trimer dissociation temperature of 80°C (Y231A, lane7), whereas A229Y-Y231A has a similar thermal dissociation temperature as the wild type and dissociates at 85°C (A229Y-Y231A, lane 8).

Y139A

A137Y-Y139A

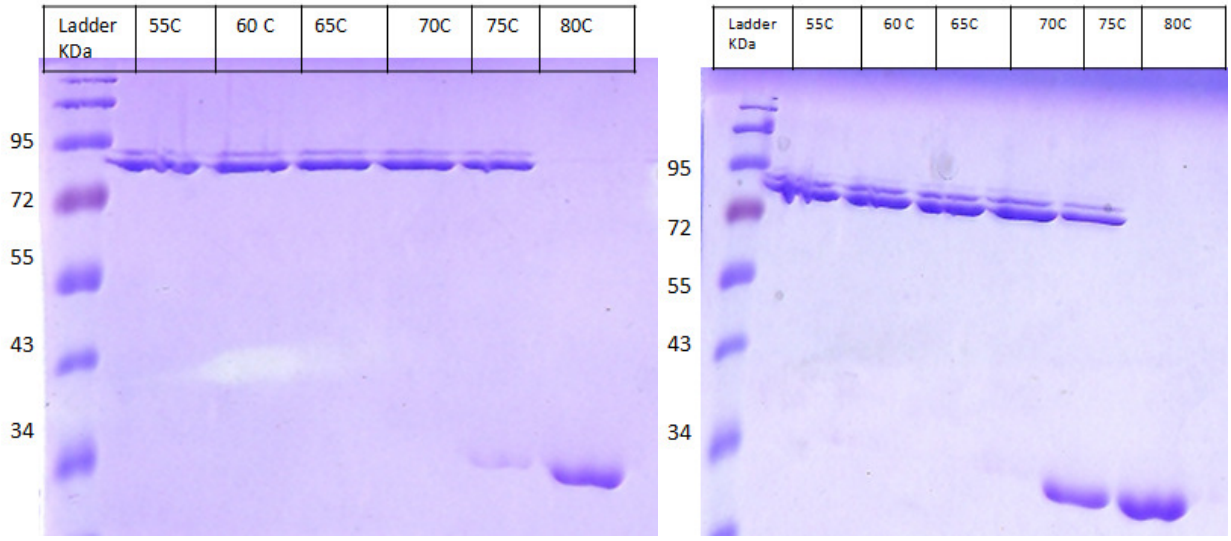

Figure 21. SDS-PAGE analysis for the thermal stability of the Y139A and the A137Y-Y139A mutant porin proteins.

Y231A

A229Y- Y231A

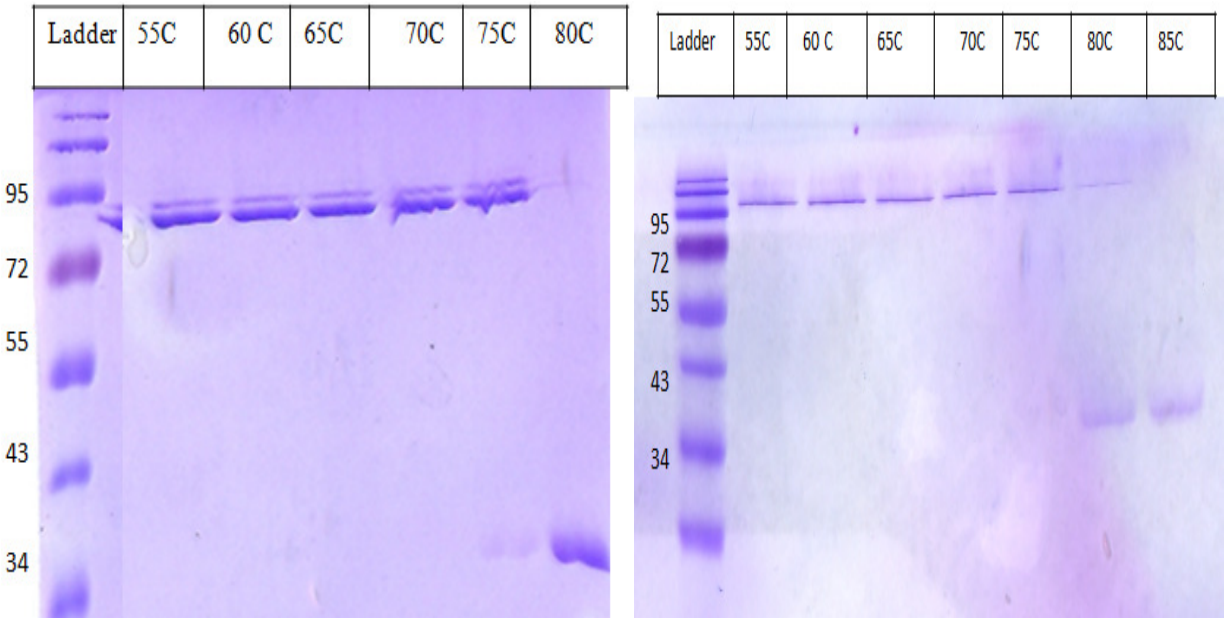

Figure 22. SDS-PAGE analysis for the thermal stability of the Y231A and the A229Y-Y231A mutant porin proteins.

(c) Antimotif mutants

OmpF porin proteins with substitutions(s) in the amino acid sequence were examined to understand the role of the antimotif in protein stability and function. With the two step process Y191- A193 antimotif was mutated to Y191**A**-A193 (referred as Y191A) and Y191**A**-A193**Y**. The mutant OmpF porin proteins were purified by the Octyl-POE method and examined for thermal stability. Niether of the antimotif mutants differed from the wild type thermal stability profile. Trimers began to dissociate into monomers at 80°C (Y191A and Y191A-A193Y, lane 7) and dissociated completely at 85°C (Y191A and Y191A-A193Y, lane 8), similar to the wild type OmpF porin protein (Fig. 23).

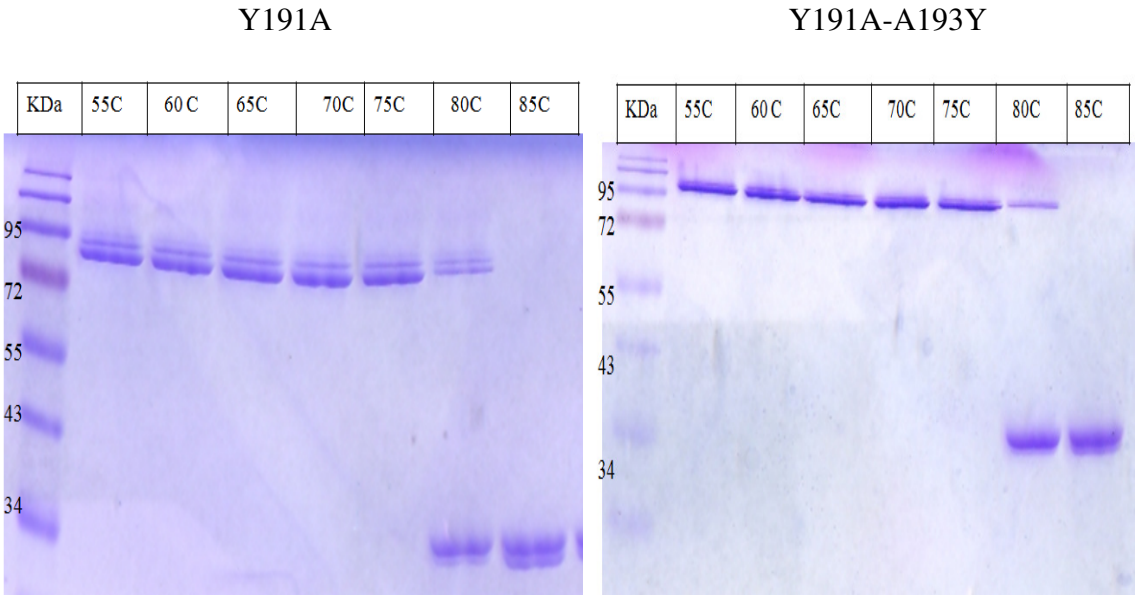

Figure 23. SDS-PAGE analysis for thermal stability of the Y191A and theY191A-A193Y mutant porin proteins.

(d) Neighboring motif mutants

Neighboring motifs are the sequence motifs that interact spatially. OmpF porin proteins with substitution(s) in these regions were analyzed to understand the importance of the spatial interaction motifs.

The neighboring motif pair V155-Y157 + A137-Y139 was mutated to either V155A-Y157A + A137- Y139A or V155Y-Y157A+ A137Y-Y139A. The mutant OmpF porin proteins were purified using Octyl-POE extraction and analyzed by thermal stability. When compared to wild type, the thermal stability of the trimer was slightly decreased when all the residues in the neighboring motifs were mutated to alanine (V155A-Y157A+A137-Y139A). However, substitution of motifs (VY2 and AY2) with YA2 antimotifs did not affect the trimer stability. V155A-Y157A+A137Y-Y139A mutant OmpF porin begins to dissociate into monomers at 75°C (V155A-Y157A+A137Y-Y139A, lane 6), whereas V155Y-Y157A+A137Y-Y139A starts to dissociate at 80°C (V155Y-Y157A+A137Y-Y139A, lane 7). With both mutants, complete formation of monomers occurred at 85°C (V155A-Y157A+A137Y-Y139A and V155Y-Y157A+A137Y-Y139A, lane 8), which is same as the wild type OmpF porin protein.

V155A-Y157A+A137Y-Y139A V155Y-Y157A+A137Y-Y139A

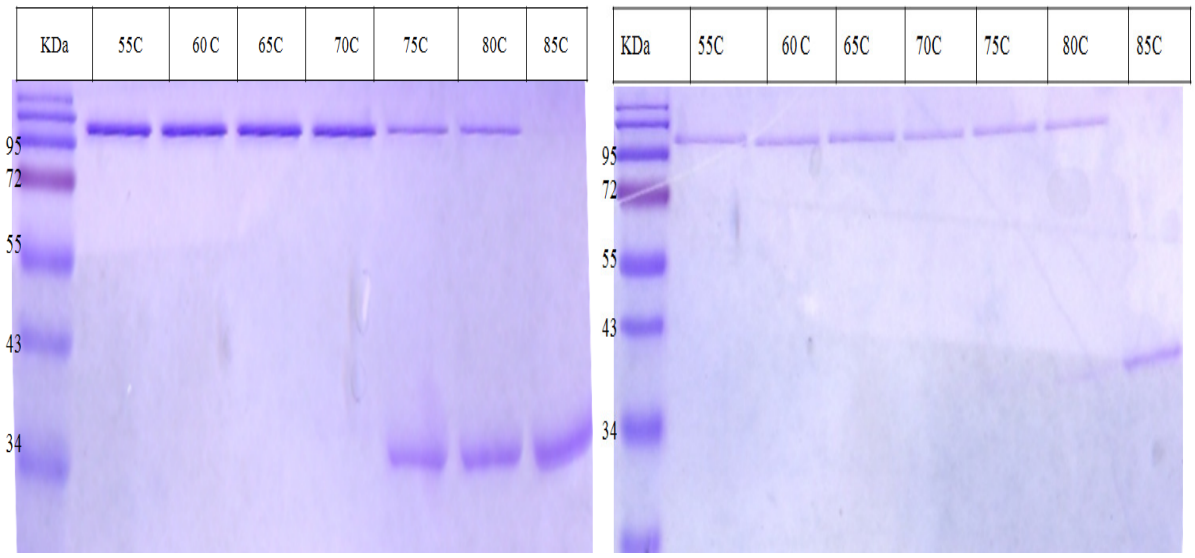

Figure 24. SDS-PAGE analysis for thermal stability of the V155A-Y157A + A137- Y139A and the V155Y-Y157A+ A137Y-Y139A mutant porin protein.

(e) Aromatic rescue mutant

The property of tyrosine residues that occupy an unusual conformation to protect glycine residues from a polar environment was termed aromatic rescue. A tyrosine residue that appeared to participate in such a phenomenon was mutated and the resulting mutant protein was analyzed to understand the role of aromatic rescue. To test the aromatic rescue of glycine in OmpF porin protein Y14A mutant was constructed. OmpF Y14A mutant protein was purified by Octyl-POE extraction and analyzed by thermal stability (See methods section H).

When compared to the wild type OmpF porin protein, Y14A mutant porin did not exhibit any change in trimer stability. This was anticipated, because there was no change in the back bone structure that affects trimer stability. Y14A mutant OmpF porin protein begins to form monomers at 80°C and complete monomerization occurred at 85°C.

Y14A

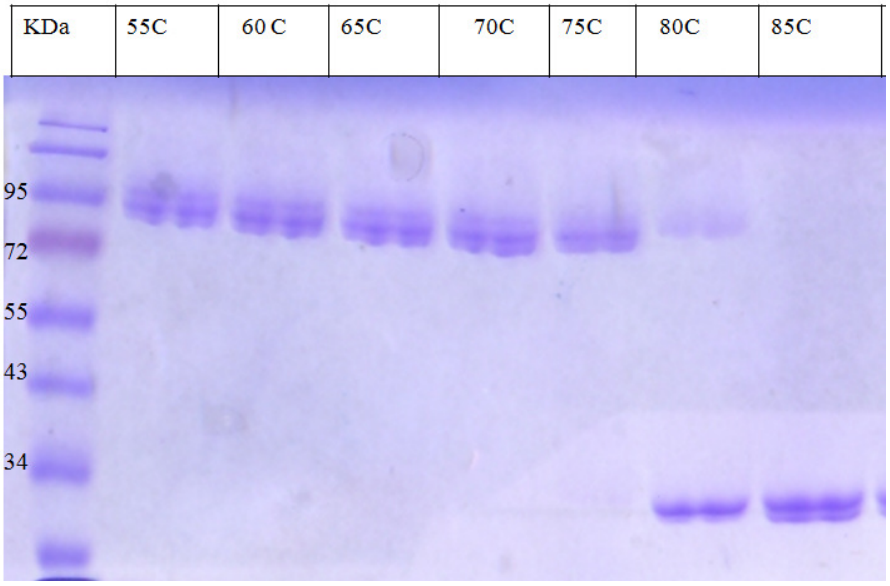

Figure 25. SDS-PAGE analysis for thermal stability of the Y14A mutant porin protein.

(f) Trimer interface mutants

Analysis of the trimer interface and the residue propensity at the trimer interface using the potential function proposed by Jackups *et al.* suggested interesting amino acid interactions on the OmpF protein. A leucine residue projecting its long side chain to the core of the timer and the phenylalanine and Glycine resides interacting at the trimer interface were found to be statistically significant. L13A and F45A mutant OmpF porin proteins were analyzed to understand the role of these interactions on the trimer stability. L13A and F45A mutants were created in plasmid pBADF and the mutant proteins were expressed. Mutant porin proteins were purified by the Octyl-POE extraction method and analyzed by the thermal stability assay (See methods section H). When compared to wild type OmpF porin protein, the thermal stability of the F45A mutant porin trimer stability was slightly decreased. However, the L13A mutation did not affect trimer stability. F45A mutant OmpF porin begins to dissociate into monomers at 75°C, whereas L13A mutant porin starts to dissociate at 80°C.

L13A F45A

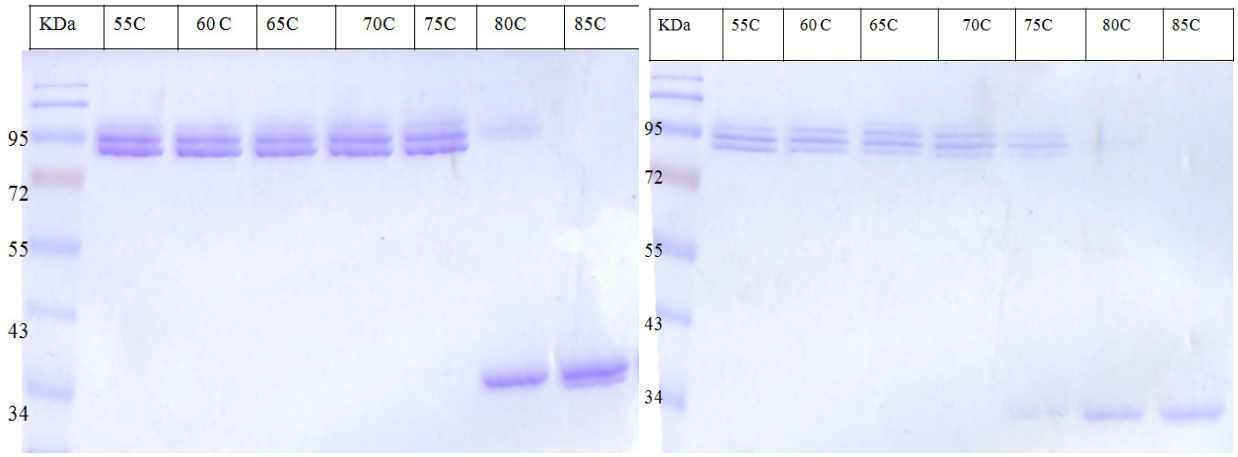

Figure 26. SDS-PAGE analysis for thermal stability of the L13A and the F45A mutant porin protein.

(g) Positive outside rule mutant

The observation that β-barrel membrane proteins have basic residues such as lysine and arginine at the extracellular side of the barrel was termed the positive outside rule. To understand the role of the basic residues at the extracellular side of the barrel, it was proposed to introduce basic residues on the periplasmic side and analyze protein stability. Thus, a D92R mutant was created and D92R mutant protein was analyzed for thermal stability. The D92R mutation was created in plasmid pBAD24F and the protein expressed and purified by the Octyl-POE extraction method. The D92R mutant protein was analyzed for thermal stability (See methods section H).

This substitution did not effect the thermal stability of the D92R OmpF porin protein when compared to the wild type OmpF porin. The D92R mutant OmpF porin begins to dissociate into monomers at 80°C and complete monomerization occurred at 85°C.

D92R

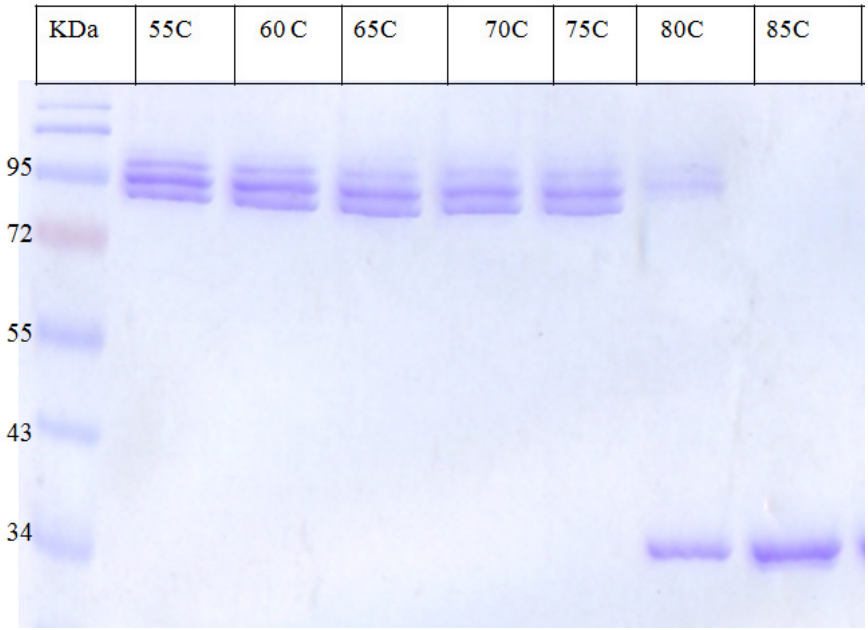

Figure 27. SDS-PAGE analysis for thermal stability of the D92R mutant porin protein.

(h) Evolutionary analysis mutants

Based on an evolutionary analysis, glycine residues that are highly conserved in β-barrel membrane proteins were substituted and the mutant proteins were examined for thermal stability. G47I, G47I-G57I and G57I-G59L mutants were created in plasmid pBAD24F and mutant proteins were purified by the Octyl-POE extraction method. Thermal stability of the mutant protein was examined by a thermal stability assay as described in methods section H. We were able to purify G47I mutant porin protein but G47I-G57I and G57I-G59L mutant porin proteins was not recovered. This could be because, substitution of glycines with a bulky amino acid effects trimerization. The G47I mutant protein was examined for thermal stability. The wild type OmpF porin protein begins monomer formation at 80°C, whereas the G47I mutant porin protein forms monomers at 70°C. Even though the G47I mutant porin protein starts to form monomers at lower temperature when compared to the wild type OmpF protein, both the wild type and G47I mutant proteins formed complete monomers at 85°C.

G47I

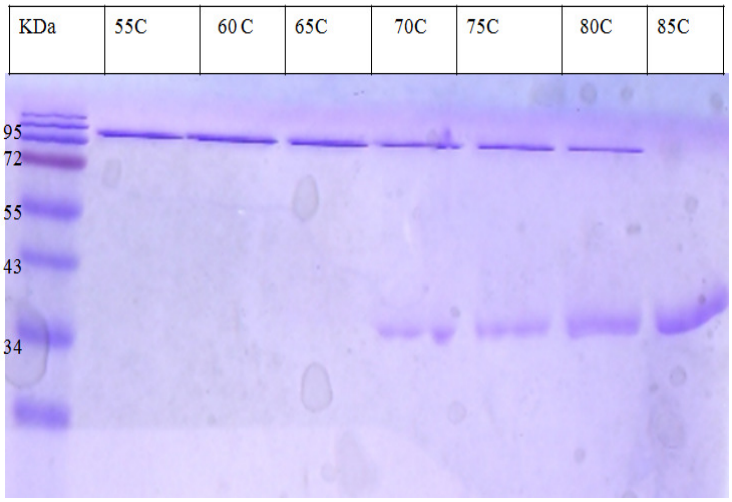

Figure 28. SDS-PAGE analysis for the thermal stability of the G47I mutant porin protein.

(i) Energy function mutants

Mutants that were proposed by the analysis of the highly unstable regions of β- barrel membrane proteins were analyzed by a thermal stability assay. G19W, G135W, G135W-R100V and G135W-R100L were predicted to have an effect on trimer stability, using the energy profile of the weakly stable regions of OmpF protein.

G19W, G135W, G135W-R100V and G135W-R100L mutations were created in plasmid pBAD24F and used to express the mutant protein. The G135W and the G19W mutant OmpF porins were purified and a thermal stability assay was performed on them. The G135W-R100V and the G135W-R100L mutant proteins were not purified by the Octyl- POE extraction process. This suggested that either stable trimers were not formed or there was a problem with insertion of the trimer into the outermembrane. The G135W and the G19W mutant porin proteins had reduced thermal stability when compared with wild type OmpF porin. G135W forms the monomers at 65 °C (G135W, lane 5), whereas G19W begins to form monomers at 60°C (G19W, lane 3) and complete monomers were formed at 65 °C (G19W, lane 4). Both the G135W and G19W mutant proteins form monomers from trimers at 65 °C, which is 20 °C lower when compared with the wild type OmpF protein thermal stability profile.

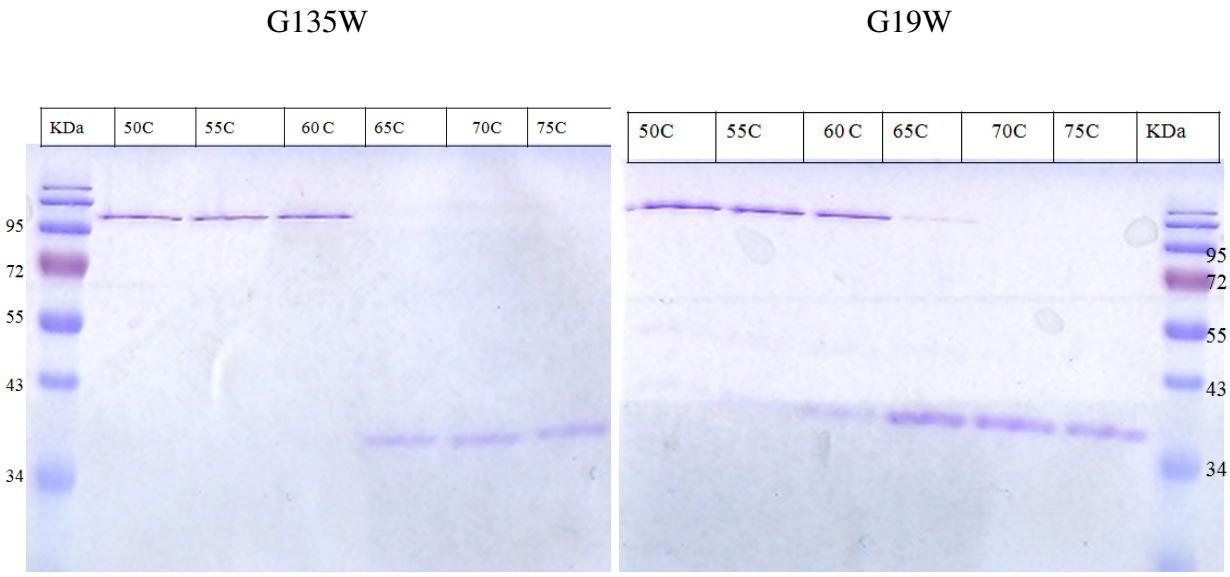

Figure 29. SDS-PAGE analysis for the thermal stability of the G135W and the G19W mutant porin proteins.

(j) Summary of thermal stability assay

Mutation	Td	Tc	Mutation	Td	Tc
WT	80	85	**Aromatic rescue**		
Sequence motifs			Y14A-G44	80	85
A137Y-Y139A	75	80	**Trimer interface**		
Y139A	75	80	L13A	80	80
A229Y-Y231A	80	85	F45A - G15	75	80
y231A	75	80	**Positive outside rule**		
Antimotifs			D92R	80	85
Y191A	80	85	**Evolutionary analysis**		
Y191A-A193Y	80	85	G47I	70	85
Neighboring sequence motifs			**Energy function**		
V155A-Y157A+ A137-Y139A	75	85	G19W	60	65
V155Y-Y157A + A137Y- Y139A	80	85	G135W	65	65

Table 3. Table summarizes the temperatures at which the wild type and mutant proteins formed monomers. The temperature at which the trimer begins to dissociate (Td) and the temperature at which complete monomerizatoin occurs (Tc) of the mutants (in °C) is given.

(E) Wild type OmpF porin conductance

In order to determine the conductance of the OmpF trimer and to confirm the properties of the OmpF trimer purified by the Octyl-POE method, planar lipid bilayer experiments were performed. OmpF porin was inserted into a planar lipid bilayer with 1M KCl on both sides as described in the methods section I. Upon a voltage trigger, the behavior of OmpF in the lipid bilyaer was monitored and the conductance was calculated by the slope of the conductance of a fully open trimer OmpF porin. The conductance of the wild type OmpF porin was 3.8 nS. The conductance of 3.8 nS is in the range of OmpF porin protein conductance. Different states such as fully open, fully closed, 1 monomer open were observed in OmpF reconstituted (Fig. 30). Hence, the porin extraction procedure resulted in an active, functional protein and was used for the extraction process of mutant porin proteins.

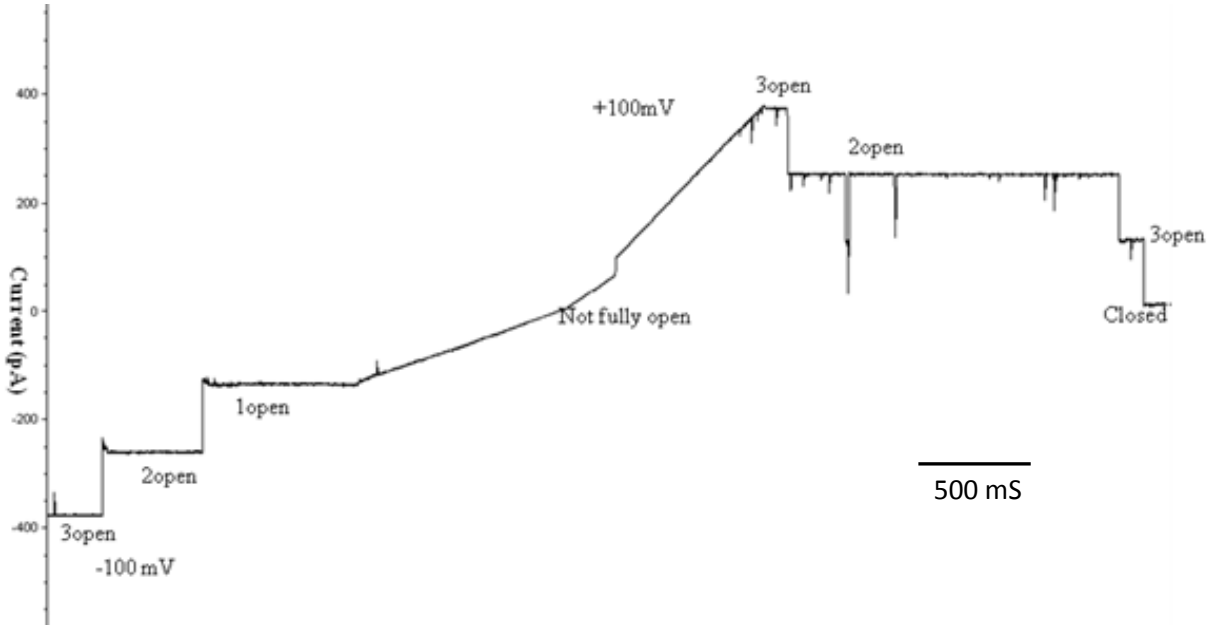

Figure 30.The conductance states of the wild type OmpF trimer inserted into a planar lipid bilayer. A ramp voltage (0V to -100mV to +100mV) protocol was used to obtain the plot of the OmpF porin protein conductance states [14].

5. Discussion

The goal of this study was to determine if statistically important sequence and structural motifs of OmpF porin proteins are important in vivo. To do this, OmpF mutants were created based on the proposals from statistical studies and an evolutionary study performed in Dr. Liang's laboratory [10, 11, 16, 27]. We examined 19 OmpF porin mutants for the expression of protein in the outer membrane and observed their thermal stability. Through this evaluation several interesting conclusions can be made regarding the role of sequence and structure motifs of the *β*-barrel membrane proteins, in particular the OmpF porin protein.

There are only about 25 *β*-barrel membrane proteins with known 3 dimensional structures. These proteins are hard to purify and crystallize [4, 5]. Hence, it is very beneficial to understand structure using computational methods. OmpF is a stable trimer of about 97 KDa [3] and is a general outer membrane porin of *E. coli,* expressed during low osmolarity [15]. Several mutant studies were previously done on expression, trimerization and thermal stability of OmpF protein [13, 14, 18]. In this thesis work, we examined 19 OmpF mutants that were proposed by Jackup *et al*., Naveed *et al*. and Jimenez-Morales *et al*. to determine the significance of these predictions [10, 11, 16, 27].

Outer membrane protein properties were examined for 19 OmpF mutants (See Table 2, column 1). The expression profile of the wild type OmpF protein was consistent with the results published by other groups [13, 15]. All the mutants that were suggested based on the motif analysis were expressed in the outermembrane and showed similar thermal stability when compared to the wild type OmpF protein. Conductivity of wild type OmpF protein was examined

by inserting it into an artificial planar lipid bilayer. A conductivity value of 3.8 nS was observed and this value is consistent with other published results [14].

We examined three main categories of OmpF mutants: 1. sequence and structural motif mutants which includes motifs, antimotifs, trimer interface mutants, positive outside rule mutants and aromatic rescue mutants. 2. Energy function mutants, which includes substitutions at weakly stable regions on OmpF. 3. An evolutionary analysis mutants, which includes substitutions at conserved regions of OmpF.

Mutating a motif AY2 (A137-Y139 and A229-Y231) to an antimotif YA2 (A137Y-Y139A and A229Y-Y231A) did not change the stability of OmpF porin protein. This might be because the total aromatic contents of the porin did not change. For perhaps the same reason, neighboring motifs did not show any effect on thermal stability when mutated to neighboring antimotifs. It would be interesting to see whether multiple motif mutations would effect protein stability. The mutants that were examined for aromatic rescue, trimer interface interaction and positive outside rules also did not show a significant effect on the thermal stability of the porin, except for F45A. The slight decrease (~5°C) in the thermal stability of the F45A mutant (trimer interface mutant) may be due to the reduced aromatic content of the porin [Fig. 9, Fig 26 F45A].

Naveed *et al.* proposed an energy function that predicts weakly stable regions of the *ß*-barrel membrane proteins [16]. Based on this study, he predicted specific amino acid interactions with high energy in OmpF protein and suggested substitution of these amino acids would result in significant change of both expression and thermal stability of the protein. I examined G19W, G135W, G135W-R100V and G135W-R100L mutants. Both G19W and G135W were expressed in the outermembrane and extracted by Octyl-POE extraction method. These point mutants have

a lower thermal stability (~20-25 °C) less than the wild type OmpF. G135W-R100V and G135W-R100L mutants were not expressed in the outermembrane. The lack of OmpF double mutants in the outermembrane could be because stable trimers were not formed. It would be interesting to examine whether a double mutant OmpF forms a stable monomer.

Based on an evolutionary analysis of the *β*-barrel membrane proteins, substitutions at conserved regions of OmpF showed a significant change in expression and thermal stability of the OmpF protein. Point mutant G47I decreased thermal stability of the porin significantly by 15 °C, whereas double mutants (G47I-G57I and G47I-G59L) were not expressed in the outer membrane. This could be because the mutant OmpF monomer was unstable or because of a trimerization or insertion problem.

Statistical studies of the *β*-barrel membrane proteins gave us a tremendous amount of information about motifs, conserved regions and highly unstable regions of these proteins. But the experimental studies conducted based on Jackups *et al.* statistical analysis showed that many of these predictions that we examined did not have a significant role in thermal stability and the mutants proposed using energy function and an evolutionary analysis showed a significant effect in vivo. This study helped to understand the significance of the statistical predictions in vivo and suggests the improvement of the statistical methods.

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Research Experience

Voluntary work

Mammalian cell culture, Maintaining the human embryonic kidney 293T cell line.
Purification of OmpR protein using FPLC, Purified outer membrane protein R with a His tag using Ni-NTA column on AKTA FPLC system and analyzed the protein yield on SDS PAGE.
Imidazol that was present in the purified OmpR protein solution was removed by running a Heparin column on AKTA FPLC system.
Mice handling,Genotyping of mice and simple surgery to collect tissue.
Westernblot of homogenized heart sample to detect the expression of Angiotensin receptor.

Master's Thesis

Mutagenesis of OmpF protein to evaluate the stability contribution of different sequence and structural motifs in *ß*- barrel membrane proteins
The *ompF* gene was deleted by homologous recombination using λ-Red recombinase from *E.coli* MG1655.
P1*vir* was used to achieve the *ompF* gene deletion form *E.coli* MG1655 by generalized transduction.
Cloned the *ompF* gene into pBAD24 and pET28 vectors.
OmpF mutants were created using site-directed mutagenesis.
S35-Methionine labeling of OmpF protein was used to observe the trimerization phenomenon.
The wild type and mutant proteins of OmpF were purified and analyzed for the thermal stability and conductance.

Teaching Experience

Teaching Assistant, Bio chemistry course (BioS 454), University of Illinois at Chicago 01/2008 to 05/2008.

Bachelor's project

Cloning and sequencing of protein phosphatase 2C (*pp2C*) gene from *sorghum bicolor* (l.) Moench,RNA was extracted from sorghum plant grown in high salinity conditions and used for cDNA synthesis.

The *pp2C* gene was amplified and cloned into pTZ vector and construct was confirmed by sequencing.

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