Roles of members of the conserved DedA/Tvp38 membrane protein family in Escherichia coli drug resistance and alkaline pH tolerance

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ROLES OF MEMBERS OF THE CONSERVED DedA/Typ38 MEMBRANE PROTEIN FAMILY IN *ESCHERICHIA COLI* DRUG RESISTANCE AND ALKALINE pH TOLERANCE

A Dissertation

Submitted to the Graduate Faculty of the Louisiana State University and Agricultural and Mechanical College in partial fulfillment of the requirements for the degree of Doctor of Philosophy in

The Department of Biological Sciences

by

Sujeet Kumar
B.Tech, Vellore Institute of Technology, 2011
August 2016
ACKNOWLEDGEMENTS

The accomplishment of this doctoral dissertation would not have been possible without the dynamic support, continuous backing and nonstop inspiration from numerous individuals. I would like to convey my heartfelt appreciation and genuine gratitude to all of them.

To start with, I am extremely grateful to my research advisor, Dr. William Doerrler, for providing me the opportunity to work in his lab. It has been an honor to learn under his guidance and I appreciate all his contributions of time, ideas, and consistent encouragement to make my graduate studies fruitful and exciting. His insightful advice, great knowledge, constant motivation, prolific discussions, extreme enthusiasm, and immense experience in research, make him a great mentor. Thank you, Sir, for all your help and backing.

I would also extend my deepest gratitude to my committee members, Dr. Marcia Newcomer and Dr. Anne Grove for their unmatched support, time and thoughtful criticism on my work, and attention to my progress. They have been some of the best educators who intensely influenced and affected my graduate life at LSU. I also thank my dean’s representative Dr. Charles Lee for his supportive analysis of my research work and important suggestions. I also thank my Biochemistry instructors Dr. Erin Hawkins and Dr. Johnna Roose for their spontaneous discussions on my work, and their guidance for teaching Biochemistry Lab, and allowing me to explore the hidden teacher inside me.

The present and past members of the Dr. Doerrler lab have contributed enormously to my personal and professional time at LSU. First of all, I want to thank Dr. Rakesh Sikdar and Dr. Lisa Boughner for their huge help and training me in the lab during the early years
of graduate life at LSU. I am also grateful to Megan Justice and Pradip Panta for their cooperation and providing an exceptional atmosphere at work. These two lab mates formed the core of my research time. I would also like to acknowledge all undergraduate students who have contributed to the progress of our research. I am thankful to all my friends here at LSU and back in India who have been there for me at all times regardless of locations or conditions. My special thanks are to Ms. Priscilla Milligan and Ms. Chimene Boyd for keeping me updated with the deadline of the Graduate school and helping with all the necessary documents.

Lastly, I would like to dedicate this dissertation and extend my heartfelt appreciation to the most important persons of my life- my father Shri Phoolchandra Mishra, my mother- Mrs. Kiran Mishra, my brother- Mr. Ajeet Mishra and my sister in law- Mrs. Anamika Mishra. I thank them, for their absolute love, continuous encouragement, and offering me the extreme freedom to chase my dream of pursuing research as my career. My sincere gratitude is also to my family, both from paternal and maternal side for their caring attitude and continuous blessings through all these years.
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<table>
<thead>
<tr>
<th>Abbreviation</th>
<th>Full Form</th>
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<tbody>
<tr>
<td>ABC</td>
<td>ATP binding cassette</td>
</tr>
<tr>
<td>BC</td>
<td>Benzalkonium Chloride</td>
</tr>
<tr>
<td>CL</td>
<td>Cardiolipin</td>
</tr>
<tr>
<td>CPA</td>
<td>Cation Proton Antiporter</td>
</tr>
<tr>
<td>CTAB</td>
<td>Cetyltrimethyammonium Bromide</td>
</tr>
<tr>
<td>IM</td>
<td>Inner membrane</td>
</tr>
<tr>
<td>LOL</td>
<td>Localization of lipoproteins</td>
</tr>
<tr>
<td>LPS</td>
<td>Lipopolysaccharide</td>
</tr>
<tr>
<td>LPT</td>
<td>Lipopolysaccharide transport</td>
</tr>
<tr>
<td>MDR</td>
<td>Multidrug Resistance</td>
</tr>
<tr>
<td>MFS</td>
<td>Major Facilitator Superfamily</td>
</tr>
<tr>
<td>MFP</td>
<td>Membrane Fusion Protein</td>
</tr>
<tr>
<td>MATE</td>
<td>Multidrug and Toxic compounds Extrusion</td>
</tr>
<tr>
<td>MV</td>
<td>Methyl Viologen</td>
</tr>
<tr>
<td>NCBI</td>
<td>National Center for Biotechnology Information</td>
</tr>
<tr>
<td>OM</td>
<td>Outer membrane</td>
</tr>
<tr>
<td>OMF</td>
<td>Outer Membrane Factor</td>
</tr>
<tr>
<td>OMPs</td>
<td>Outer membrane proteins</td>
</tr>
<tr>
<td>PDB</td>
<td>Protein Data Bank</td>
</tr>
<tr>
<td>PE</td>
<td>Phosphatidylethanolamine</td>
</tr>
<tr>
<td>PG</td>
<td>Phosphatidylglycerol</td>
</tr>
<tr>
<td>PL</td>
<td>Phospholipids</td>
</tr>
<tr>
<td>Acronym</td>
<td>Description</td>
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<tr>
<td>---------</td>
<td>--------------------------------------------</td>
</tr>
<tr>
<td>PMF</td>
<td>Proton motive force</td>
</tr>
<tr>
<td>PSMR</td>
<td>Paired Small Multidrug Resistance</td>
</tr>
<tr>
<td>QACs</td>
<td>Quaternary Ammonium Compounds</td>
</tr>
<tr>
<td>RND</td>
<td>Resistance Nodulation Cell Division</td>
</tr>
<tr>
<td>SMP</td>
<td>Small Multidrug Pumps</td>
</tr>
<tr>
<td>SMR</td>
<td>Small Multidrug Resistance</td>
</tr>
<tr>
<td>SUG</td>
<td>Suppressor of groEL mutation Protein</td>
</tr>
<tr>
<td>TAT</td>
<td>Twin-arginine translocation</td>
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<tr>
<td>TMD</td>
<td>Transmembrane Domain</td>
</tr>
<tr>
<td>TMH</td>
<td>Transmembrane Helices</td>
</tr>
<tr>
<td>TMSs</td>
<td>Transmembrane Segments</td>
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<tr>
<td>TPP</td>
<td>Tetraphenylphosphonium</td>
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ABSTRACT

The objective of this dissertation is to understand the functions of *Escherichia coli* YqjA and YghB, which are members of the conserved DedA/Tvp38 membrane protein family. YqjA and YghB are inner membrane (IM) proteins with multiple predicted membrane-spanning domain, sharing 62% amino acid identity and partly overlapping functions. Simultaneous in-frame deletions of these two genes in a strain named BC202 results in various phenotypes including cell division defects, temperature sensitivity, and sensitivity to drugs and alkaline pH. The cell division defect of BC202 is due to the inefficient secretion of periplasmic amidases by the twin arginine transport (Tat) pathway into the periplasm and drug sensitivity is due to the inefficient function of various drug efflux pumps. Both these phenotypes are related to the loss of proton motive force (PMF) in BC202. Overexpression of MdfA, a Na⁺-K⁺/H⁺ antiporter or growth in acidic media has the ability to rescue all the phenotypes of BC202. In addition, the ΔyqjA mutant (but not the ΔyghB mutant) is alkaline sensitive and overexpression of yqjA can restore growth at alkaline pH only when more than 100mM of sodium or potassium ions is present in the growth medium. Osmotic pressure augments the YqjA mediated growth at alkaline pH. Furthermore, charged amino acids are also essential for YqjA and YghB function that were previously shown important for various secondary transporters. Additionally, yqjA expression is higher at alkaline pH and increased expression of yqjA also required sodium/potassium salts above pH 9.0. The transcriptional regulator CpxR is required for the expression of yqjA at alkaline pH in the presence of Na⁺/K⁺. Based on these results, we suggest YqjA and YghB are osmosensing proton-dependent transporters required for *E. coli* drug resistance and alkaline pH tolerance.
CHAPTER 1
GENERAL INTRODUCTION

1.1 The structure and composition of Gram-negative bacteria cell envelope

Bacteria are the oldest form of the living organism, as ancestors of present bacteria appeared on earth approximately four billion years ago. Over the extraordinary period of time, the random mutations, shifting environments and natural selection gave rise to two different cell wall structures in bacteria; the Gram negative and Gram positive cell envelope varieties. The designation of Gram positive and Gram negative bacteria are based on the different reaction of the cell envelope toward Gram staining due to their structural and chemical composition. The cell envelope offers bacteria their shape, provide a site for energy generation used for growth and division, and promotes pathogenesis and confers evasion from host immune responses. Importantly, the cell envelope continues to be a well-known target in the search for novel drugs to Figure the rise in multidrug resistance (Kleanthous and Armitage 2015). A key feature of Gram-negative bacteria like *Escherichia coli* is their cell envelope as shown in Figure 1.1. The Gram-negative cell envelope is formed by three layers; the outer membrane (OM), the periplasm which contains the peptidoglycan layer, and the inner membrane (IM).

The outer membrane (OM): The Gram-negative bacteria can be distinguished from Gram-positive bacteria due to the presence of the outer membrane. It is an asymmetric lipid bilayer with an inner leaflet made of phospholipids and outer leaflet composed of lipopolysaccharide (LPS). LPS is an amphipathic molecule, made up of lipid A, a core oligosaccharide and an O-antigen polysaccharide. It is responsible for the endotoxic shock linked with the septicemia (Raetz and Whitfield 2002; Nikaido 2003). LPS blocks the diffusion of numerous hydrophobic molecules, which can simply diffuse across
phospholipid bilayers (Nikaido 2003). LPS assembly on the OM occurs through the Lpt (lipopolysaccharide transport) pathway (Okuda, Sherman et al. 2016). The hydrophobicity of LPS makes it very challenging to treat Gram-negative pathogens as most of the known drugs are relatively hydrophobic in nature (Okuda, Sherman et al. 2016). Apart from lipids, OM also contains two distinct types of protein namely lipoproteins and β-barrel proteins.

Figure 1.1. Structure and composition of the *E. coli* cell envelope. The outer membrane (OM) is an asymmetric bilayer with phospholipids (PL) on the inner leaflet and lipopolysaccharide (LPS) on the outer leaflet. It also contains lipoproteins anchored to the inner leaflet and β-barrel protein (porins). The periplasm is an aqueous cellular compartment sandwiched between OM and IM and contains a thin peptidoglycan layer. The inner membrane (IM) is made of phospholipids on both inner and outer leaflet. This Figure was replicated with authorization from Nature Publishing Group with slight modifications: Nature Reviews Microbiology (Ruiz, Kahne et al. 2009).

The lipoproteins are assembled by Lol (localization of lipoproteins) pathway to the OM (Silhavy, Kahne et al. 2010). Lipoproteins possess N-terminal lipid moieties which help them in associating with the inner leaflet of OM. There are around 100 OM lipoproteins present in *E. coli*, however, the functions of most of them are still unknown (Miyadai, Tanaka-Masuda et al. 2004). The β-barrel proteins which (also known as outer membrane proteins (OMPs)) are also part of OM usually forms channels and are essential
for nutrient uptake but may also be enzymes. The β-barrel channels only permit the diffusion of small hydrophilic molecules through it but not larger molecules (Silhavy, Kahne et al. 2010). The OMPs are gathered to the OM by the Bam (β-barrel assembly machinery) complex (Ruiz, Kahne et al. 2006). The OM also serves as a protective barrier shielding Gram negative bacteria from various toxic chemicals and therefore it is an essential organelle (Ruiz, Kahne et al. 2006; Silhavy, Kahne et al. 2010). The Mla (maintenance of lipid asymmetry) is an ABC transport system that helps in conserving the OM lipid asymmetry. There are at least six different Mla proteins present in all the compartments of cell envelope and that assist retrograde transport of phospholipid from OM to the inner membrane. The Mla pathway is thought to be a bacterial intermembrane phospholipid trafficking system (Malinverni and Silhavy 2009; Sutterlin, Shi et al. 2016).

The Periplasm: The periplasm is the aqueous compartment sandwiched between the outer membrane and inner membrane (Ruiz, Kahne et al. 2006). It is an extremely viscous layer of the cell envelope packed densely with soluble proteins and the peptidoglycan layer. The periplasm occupies around 40% of the total cell volume of Gram-negative bacteria. The peptidoglycan layer consists of repeating units of N-acetyl glucosamine and N-acetyl muramic acid and crosslinked pentapeptide side chains that contribute to the cell shape and blocking cell lysis that can occur as a result of the high turgor pressure in the cytoplasm (Vollmer, Blanot et al. 2008; Silhavy, Kahne et al. 2010). Moreover, it is covalently linked to the outer membrane by Braun’s lipoprotein (Ruiz, Kahne et al. 2006). The periplasm represents an evolutionary ancestor of the lysosomes of eukaryotic cells due to the presence of degrading enzymes like alkaline phosphatase or
RNAse (De Duve and Wattiaux 1966). Additionally, it has an oxidizing environment and lacks the presence of any energy source such as ATP.

The inner membrane (IM): The inner membrane or the cytoplasmic membrane borders the cytoplasm and is essential for cell integrity. It consists of both proteins and phospholipids. The content of phospholipids varies between bacterial species; for example in *E. coli* phosphatidylethanolamine (PE) is 70-80%, phosphatidylglycerol (PG) is 15-20% and cardiolipin (CL) is 5% or less (Kanemasa, Akamatsu et al. 1967). There are two predominant forms of IM proteins; integral inner membrane proteins and lipoproteins. Integral IM proteins possess α-helical transmembrane domains that are inserted perpendicular to the plane of the membrane, and lipoproteins are attached to the outer leaflet of the IM by lipid modification of an amino terminal cysteine residue (Tokuda and Matsuyama 2004; du Plessis, Nouwen et al. 2011). The IM is a semipermeable barrier that assists in the transport of molecules into and out of the cell. The IM serves as a site for many cellular processes like ATP synthesis, protein translocation, and lipid biosynthesis. Some IM proteins act as sensors of certain chemicals, stresses or physical stimuli and help in transducing the signal to the cytoplasm (Ruiz, Kahne et al. 2009). Approximately 25-30% of the genes in the sequenced genomes are predicted to encode integral IM proteins (Daley, Rapp et al. 2005); however, to date only relatively few structures of IM proteins are present in the Protein Data Bank (PDB) because they are highly hydrophobic in nature and resistant to form 3D crystals for structural analysis (Engel and Gaub 2008; White 2009; Rose, Beran et al. 2011).
1.2 Genetics of the *E. coli* DedA/Tvp38 membrane protein family

There are eight genes (*yqjA, yghB, yabI, yohD, dedA, yqaA, ydjX* and *ydjZ*) in the *E. coli* genome that encode members of the DedA/Tvp38 protein family (Doerrler, Sikdar et al. 2013). Presently, YqjA and YghB are the best-characterized members of this protein family. A number of years ago, a unique *E. coli* mutant was isolated in the Doerrler laboratory named Lud135 (Thompkins, Chattopadhyay et al. 2008). It was found that Lud135 had mutations in two non-essential genes- a nonsense mutation (W92TGA) in *yqjA* and a missense mutation (G203D) in *yghB*. Thereafter, an *E. coli* strain (named BC202) with simultaneous deletion of *yqjA* and *yghB* was created in the parent strain W3110, which showed various phenotypes identical to Lud135 (Thompkins, Chattopadhyay et al. 2008). YqjA and YghB are proteins of 219 and 220 amino acids, respectively, and share 62% amino acid identity with each other. The other six proteins of the *E. coli* DedA family display 25 to 30% amino acid identity with each other and YqjA/YghB. BC202 exhibits numerous phenotypes that provide a tool to study the roles of the DedA protein family. The distinguishing phenotypes of BC202 (∆yqjA ∆yghB) and ∆yqjA are described as follows:

The cell division defect: Bacterial cells divide into two identical daughter cells through a process of binary fission (Haeusser and Margolin 2016). The process of cell splitting (cytokinesis) occurs through a complex cooperating network of cytoplasmic, periplasmic and membrane bound cell division proteins that get recruited to the division site. If the process of cytokinesis is inactivated through any means, the bacterial cells grow as cylindrical rods and bacterial chromosomes continue to replicate and segregate, resulting in filamentous cells with numerous nucleoids (Haeusser and Margolin 2016). BC202 displays a cell division defect phenotype with distinctive cell chaining at all growth
temperatures. An exponential phase culture of BC202 growing at 30°C consists of a heterogeneous mixture of cells bearing wild-type shape as well as cells with membrane bulges and irregular shapes unlike parent W3110 when seen under the microscope (Figure 1.2, A) (Thompkins, Chattopadhyay et al. 2008). Our lab has demonstrated that the inefficient export of amidase (AmiA and AmiC) into the periplasm by the twin-arginine transport (TAT) pathway is responsible for the cell division defect of BC202 (Sikdar and Doerrler 2010). The twin-arginine translocation (Tat) protein export system is a PMF dependent protein export pathway present in the cytoplasmic membranes of most bacteria and archaea and helps in transporting fully folded proteins to the periplasm (Palmer and Berks 2012; Berks 2015).

Figure 1.2. The cell division defect and temperature sensitivity of BC202. (A) The wild type W3110 cells divide normally but BC202 displays a cell division defect under similar growth conditions. (B) BC202 grows at 30°C but not at 42°C while wild type W3110 grow fine at all temperatures (not shown). This Figure was modified and replicated with authorization from American Society for Microbiology: Journal of Bacteriology (Thompkins, Chattopadhyay et al. 2008).

Temperature sensitivity: BC202 shows similar growth as wild type W3110 at 30°C but fails to grow at a temperature above 42°C (Figure 1.2, B) (Thompkins, Chattopadhyay et al. 2008). The temperature sensitivity of BC202 is a phenotype independent of cell
division. The exact reason behind the temperature sensitive phenotype still remains unclear.

Altered membrane phospholipid composition: Membrane lipid homeostasis is very critical for the bacterial cell physiology. Many bacteria have the ability to change their membrane phospholipids that help them to survive in various environmental conditions. The phospholipids used in the biosynthesis of the membranes components often get recycled and they are essential for the stability of the cell envelope in the dividing cells (Zhang and Rock 2008). However, BC202 exhibits an altered membrane phospholipid composition with increased amounts of acidic phospholipids PG and CL and reduced amount of zwitterionic phospholipid PE at all growth temperature (Thompkins, Chattopadhyay et al. 2008). The cause behind and the significances of the altered membrane composition in BC202 remain unclear.

Activation of envelope stress response pathways: Envelope stress responses (ESRs) pathways in Gram-negative bacteria have mainly been defined in relation to their role in the biogenesis and maintenance of the bacterial envelope. Moreover, the ESRs also control resistance to antimicrobial agents through proteolysis of unfolded proteins, changes in proton motive force (PMF), and the expression of multidrug resistant genes (Guest and Raivio 2016). Six different stress response systems are characterized in E. coli- $\sigma^{32}$ (RpoH) cytoplasmic stress pathway, $\sigma^{E}$ (RpoE) periplasmic stress response pathway and the Cpx, Psp, Rcs and Bae extracytoplasmic stress response pathways. These stress response systems allow bacteria to survive in hostile environmental conditions like extreme temperature, pH, and osmolarity. Out of these stress response pathways, extracytoplasmic stress response pathways- Cpx, Psp, Rcs and Bae are highly activated in BC202 at
permissive growth condition supporting the role of YqjA and YghB in envelope maintenance (Sikdar, Simmons et al. 2013).

Loss of membrane potential ($\psi$): The proton motive force (PMF) in *E. coli* is composed of two components - the transmembrane electrical potential difference, $\Delta \psi$ ($\psi_{in} - \psi_{out}$), with $\psi_{in}$ (electrical potential inside) more negative than $\psi_{out}$ (electrical potential outside), and the transmembrane pH difference, $\Delta \text{pH}$ ($\text{pH}_{in} - \text{pH}_{out}$) with $\text{pH}_{in}$ (intracellular pH) being more alkaline than $\text{pH}_{out}$ (extracellular pH) under standard growth conditions. BC202 exhibits a significant loss of membrane potential as compared to wild type W3110 strain suggesting the requirement of YqjA and YghB for PMF homeostasis as shown in Figure 1.3 (Sikdar, Simmons et al. 2013).

Drug sensitivity: Resistance to various antibiotic and biocides is often provided by various secondary membrane transporters, which utilize PMF as a source of energy. BC202 was found to be sensitive to numerous antibiotics and biocides, which are effluxed in a
PMF-dependent manner (Kumar and Doerrler 2014). The details of the drug sensitivity phenotype are discussed later in this dissertation (Chapter 3).

Motility and biofilm formation defect: Motility in bacteria is generally connected with the flagella with certain exceptions. This feature helps them to escape adverse environments. BC202 is non-motile unlike the parent strain W3110 (unpublished data). Additionally, BC202 is also not able to form biofilms when compared to wild type W3110 strain (unpublished data), a phenotype that might be due to loss of motility (Pratt and Kolter 1998).

Alkaline pH sensitivity: Neutrophilic bacteria, like *E. coli* have the ability to survive in a wide range of pH between 5.5 and 9.0. However, the Δ*yqjA* mutant is sensitive to alkaline pH at 37⁰C (Kumar and Doerrler 2015). The specifics and cause of this phenotype are discussed in later part of this dissertation (Chapter 4).

The distinguishing phenotypes of BC202 (Δ*yqjA ΔyghB*) and Δ*yqjA* are summarized in (Figure 1.4):

![Figure 1.4. The Summary of phenotypes of *E. coli* DedA family mutants.](image-url)

Figure 1.4. The Summary of phenotypes of *E. coli* DedA family mutants.
The eight genes from *E. coli* are further functionally and evolutionary grouped into a complementing group (*yqjA, yghB, yabI*, and *yohD*) and non-complementing group (*dedA, yqaA, ydjX*, and *ydjZ*) depending on their ability to complement the cell division and temperature sensitivity phenotypes of DedA family mutant (Boughner and Doerrler 2012). These results suggested that two groups of proteins have coevolved in *E. coli*. Our lab has pioneered in the characterization of the DedA/Tvp38 protein family using BC202 as a primary mutant and discovered various suppressors (Figure 1.5) that helped in deciphering the functions of these proteins. Based on a series of publications from our lab, we have proposed that members of conserved DedA/Tvp38 protein family are likely to be a novel family of transporters (Thompkins, Chattopadhyay et al. 2008; Liang, Xu et al. 2010; Sikdar and Doerrler 2010; Boughner and Doerrler 2012; Doerrler, Sikdar et al. 2013; Sikdar, Simmons et al. 2013; Kumar and Doerrler 2014; Kumar and Doerrler 2015).

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<tbody>
<tr>
<td>YqjA/YghB/YohD/ YabI overexpression</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes/partially</td>
</tr>
<tr>
<td>10 mM Mg(^{2+}) or 400 mM Na(^+) in growth media</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>ND</td>
</tr>
<tr>
<td><em>B. Burgdorferi</em> DedA homolog (BB0250) overexpressed</td>
<td>Yes</td>
<td>Yes</td>
<td>No</td>
<td>Yes</td>
<td>ND</td>
</tr>
<tr>
<td>Low external pH (pH 6.0)</td>
<td>Yes</td>
<td>Yes</td>
<td>ND</td>
<td>Yes</td>
<td>ND</td>
</tr>
<tr>
<td>MdfA overexpression</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Yes</td>
<td>Partially</td>
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</tbody>
</table>
1.3 The DedA/Tvp38 membrane protein family: *E. coli* and beyond

The DedA/Tvp38 protein family is an ancient and highly conserved family of inner membrane proteins, present in most sequenced genomes, including bacteria, archaea, and eukarya (Doerrler, Sikdar et al. 2013). The DedA family got its name from a 1987 publication when a group of scientists titled specific DNA region found between the *hisT* and *purF* genes (downstream [of *hisT*] *E. coli* DNA gene A) as DedA (Nonet, Marvel et al. 1987). Tvp38, a eukaryotic homolog of this family present in *Saccharomyces cerevisiae*, was isolated in proteomic analysis of Golgi subcompartments. Tvp38 was involved in a cooperative network that consists of late Golgi proteins and helps in the effective maintenance/function of the late Golgi/endosomal compartments (Inadome, Noda et al. 2007). In general, the members of DedA/Tvp38 family have 4-6 predicted transmembrane domains and contain 200-250 amino acids. The DedA domain is highly conserved and includes both a cytoplasmic and a transmembrane region, as well as an amphipathic helix. All the proteins containing a DedA domain are assembled in NCBI Clusters of Orthologous groups (COG0586) (Doerrler, Sikdar et al. 2013). They do not show any similarity to known enzymes, channels, transporters or signaling proteins. In short, the function of this protein family remains obscure. However, a novel software AlignMe has shown that the bacterial DedA family proteins may share structural motifs with the LeuT protein superfamily based on the hydrophobicity profile (Khafizov, Staritzbichler et al. 2010). LeuT is a bacterial homolog of the neurotransmitter sodium symporter, and contain two sets of five transmembrane domains having a pseudo two-fold axis of symmetry along the plane of the membrane (Faham, Watanabe et al. 2008).
Thousands of prokaryotic homologs of DedA proteins are present in the NCBI protein database, and many bacteria have several members of this family in their genome (Figure 1.6.) For example, there are eight predicted DedA homologs in the *E. coli* genome: *yqjA*, *yghB*, *yohD*, *yabI*, *dedA*, *yqaA*, *ydjX*, and *ydjZ*. Individually, each of these genes is nonessential as single knock-outs have been created and are present in the Keio collection (Baba, Ara et al. 2006). However, collectively the DedA family is essential in *E. coli* as a strain with all eight genes deleted is non-viable and requires expression of a DedA family member *in trans* from a plasmid for survival (Boughner and Doerrler 2012). A multiple sequence alignment using ClustalW of DedA homologs is shown in Figure 1.7.

<table>
<thead>
<tr>
<th>Bacterial Strains</th>
<th>Number of DedA family homologs</th>
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</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em> K-12 str. W3110</td>
<td>8*</td>
</tr>
<tr>
<td><em>Salmonella typhimurium</em> LT2</td>
<td>6</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em> PAO1</td>
<td>5</td>
</tr>
<tr>
<td><em>Helicobacter pylori</em> 399</td>
<td>2</td>
</tr>
<tr>
<td><em>Borrelia burgdorferi</em> B31</td>
<td>1**</td>
</tr>
<tr>
<td><em>Caulobacter crescentus</em> CB15</td>
<td>3</td>
</tr>
<tr>
<td><em>Vibrio cholerae</em> ElTor N16961</td>
<td>3</td>
</tr>
<tr>
<td><em>Neisseria meningitides</em> Z2491</td>
<td>3</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em> st. 168</td>
<td>6</td>
</tr>
<tr>
<td><em>Bacillus anthracis</em> str. Ames</td>
<td>8</td>
</tr>
<tr>
<td><em>Mycobacterium smegmatis</em> MC2</td>
<td>1</td>
</tr>
<tr>
<td><em>Chlamydia trachomatis</em> D/UW-3/CX</td>
<td>0</td>
</tr>
<tr>
<td><em>Mycobacterium tuberculosis</em> H37Rv</td>
<td>4</td>
</tr>
<tr>
<td><em>Synechocystis</em> sp. strain PCC6803</td>
<td>3</td>
</tr>
<tr>
<td><em>Halobacterium salinarum</em> NRC-1</td>
<td>1</td>
</tr>
</tbody>
</table>

Figure 1.6. Numbers of DedA family homologs in several bacterial species. This table lists the number of DedA family homologs present in sequenced genomes of representative eubacterial and archaeal species with (protein BLASTp E-value < 0.02) of *E. coli* *yqjA*, *yghB*, *dedA*, *yohD*, *yabI*, *ydjX*, *ydjZ*, and *yqaA* were included in the numbers of proteins displayed in the second column. As shown, various bacterial species contain multiple DedA homologs but some obligate intracellular pathogenic bacterial species like *Chlamydia* sp. do not have any significant DedA homologs.* The DedA family homologs have been shown to be collectively essential in *E. coli* (Boughner and Doerrler 2012). ** The single DedA homolog of *B. burgdorferi* has also been shown to be essential (Liang, Xu et al. 2010).
Figure 1.7. Multiple sequence alignments of DedA homologs using ClustalW 2.1. The multiple sequence alignment using eight DedA homologs of *E. coli* K-12 (protein name preceded by Ec_), BCG2664 of *Mycobacterium bovis*, BB0250 from *Borrelia burgdorferi* and HP1162 from *Helicobacter pylori*, are aligned with the help of ClustalW 2.1 (www.ebi.ac.uk/Tools/msa/clustalw2/). The boxed region signifies the DedA domain (COG0586) and predicted transmembrane domains are shown in green and the blue. The transmembrane regions are predicted using TMHMM software (Sonnhammer, von Heijne et al. 1998). The only conserved Glycine (G) amino acid is shown in bold and is either in or near the predicted amphipathic helix for all the aligned homologs. This Figure was modified and replicated with authorization from American Society for Microbiology: Journal of Bacteriology (Doerrler, Sikdar et al. 2012).
The DedA family is also essential in *Borrelia burgdorferi*, the causative agent of Lyme disease, as deletion of its only gene (*bb0250*) of this organism results in death (Liang, Xu et al. 2010). Moreover, using hyper-saturated transposon mutagenesis coupled with high-throughput sequencing, an essential dedA genes in *Caulobacter* was discovered (Christen, Abeliuk et al. 2011). In that study, the *dedA* genes are also reported essential in *Caulobacter crescentus* (Christen, Abeliuk et al. 2011). Apart from these, the occurrence of DedA homologs in various reduced genomes, including bacterial and archaeal species, extends support to the necessity of the DedA protein family for species survival (Doerrler, Sikdar et al. 2013). Recently, a phylogenetic study has evaluated three DedA homologs in cyanobacterium *Synechocystis* sp. PCC6803 named Slr0232, Slr0509 and Slr0305 (Keller and Schneider 2013; Keller, Schleppi et al. 2015). In that study, the investigators found that Slr0232 and Slr0509 are more closely related to *E. coli* YqiA and Slr0305 are more closely related to *E. coli* YdjX (Keller and Schneider 2013).

Although most of the studies about DedA family are done on prokaryotes, a bit of information is also available about DedA proteins from the eukaryotic organisms. Using *Mos1* transposon mutagenesis, a DedA family member, Bus-19 (bacterially unwollen-19; having similarity with YdjX of *E. coli*) was found in a screen for *Caenorhabditis elegans* mutants which displayed altered the response to the bacterial pathogen *Microbacterium nematophilum* (Yook and Hodgkin 2007). The bacteria were no longer able to colonize the rectum of ∆bus-19 mutants, possibly due to loss of cell surface receptor in nematode (Yook and Hodgkin 2007). This study consistent with the idea that DedA family proteins may play a role in the function of the eukaryotic secretory pathway. Moreover, a membrane-associated protein, Tvp38 (Tlg-2 compartment Vesicle protein of 38kD), was found in the
Tlg2-containing membrane by proteomic analysis of immunoisolated Golgi subcompartments of *Saccharomyces cerevisiae* (Inadome, Noda et al. 2007). Tvp38 was involved in an interactive network that consists of the late Golgi/endosomal proteins. Apart from nematodes and yeast, DedA homologs are also present in the *Mus musculus* and *Homo sapiens* genomes. Tmem41A is a protein present in *Mus musculus* and *Homo sapiens* that shares a 25% amino acid similarity with *E. coli* YdjX (Doerrler, Sikdar et al. 2013).

DedA proteins have been reported to play a role in the virulence of some species and may represent a possible drug target. A DedA homolog (BCG2664) from *Mycobacterium bovis* provides resistance to the antibiotic halicyclamine A when overexpressed in *M. smegmatis* (Arai, Liu et al. 2011). Furthermore, DedA proteins are also necessary for resistance to cationic peptides in both *Salmonella enterica* (Shi, Cromie et al. 2004) and *Neisseria meningitidis* (Tzeng, Ambrose et al. 2005). In *Salmonella*, YqjA provides resistance to protamine and cationic peptide magainin 2. Generally, covalent modifications of lipid A are required for resistance to cationic peptides (Zhou, Ribeiro et al. 2001), but the *Salmonella ΔyqjA* mutant displays wild-type lipid A profile. Therefore, the role of YqjA in providing resistance to cationic peptide remains unknown in *Salmonella* (Shi, Cromie et al. 2004). Likewise, *Neisseria NMB1052 (dedA)* mutant was found susceptible to polymyxin. For resistance to cationic peptides, *Neisseria* utilizes lipopolysaccharide modification, efflux pumps, and type IV secretion (Tzeng, Ambrose et al. 2005). Many Gram-negative pathogens use Type III secretion system to transfer effector proteins into the host cells. In *Yersinia pestis*, the DedA family gene *ctgA* (similar to *E. coli* YabI) was found to play a role in the secretion of Yops, as a mutant named CHI 1345
having insertion mutation in ctgA had compromised Yops secretion and attenuated virulence in mouse infection model (Houppert, Kwiatkowski et al. 2012).

This dissertation will mainly focus on the roles of YqjA and YghB in the drug resistance and alkaline pH tolerance in E. coli. I provide evidence to support the hypothesis that these proteins function as proton dependent transporters.

1.4 References


CHAPTER 2
PROTON DEPENDENT MULTIDRUG TRANSPORTERS AND ALKALINE pH HOMEOSTASIS IN BACTERIA

2.1 Introduction

Bacterial cells possess a group of cytoplasmic membrane transport systems, which participate in the vital uptake of nutrients, efflux of toxic substances, energy generation, and maintenance of cellular homeostasis. In the past two decades, the advancement in cloning and sequencing technologies led to the identification of many such transport systems (Paulsen, Brown et al. 1996). Based on the direction of transport of substance, membrane transporters can be classified into three types: uniporter, symporter, and antiporters (Figure 2.1). A uniporter transports one kind of substance down its concentration gradient, for example, ion channels. For symporters, movement of one molecule down its concentration gradient allow the co-transport of another molecule against its concentration gradient in the same direction like in the case of lactose permease, which co-transport s a proton and lactose. Finally, an antiporter allows the movement of one molecule down its concentration gradient and another molecule against its concentration gradient in the opposite direction such as MdfA which catalyzes Na⁺/H⁺ antiport.

Figure 2.1. Three classes of transporter (Uniporter, symporter, Antiporter).
The development of numerous antibiotics for controlling pathogenic organisms has been one of the most significant achievements in the past century. However, the extensive use of antibiotics has led to the rise of bacterial strains which can survive in the presence of multiple antibiotics (Putman, van Veen et al. 2000). This acquisition of multidrug resistance (MDR) has become a severe threat to modern health care due to the increasing problem in attaining effective clinical treatments (Kumar, He et al. 2016). The resistance to antibiotics or other drugs may arise through various mechanisms, including enzymatic inactivation of antibiotics, target alteration, decreased membrane permeability, and drug metabolism (Figure 2.2) (Putman, van Veen et al. 2000; Higgins 2007). However, in most cases efflux systems provide resistance to the antibiotics or biocides (Van Bambeke, Balzi et al. 2000). Multidrug transporters play a major role in providing MDR by exporting a large number of structurally distinct compounds. The substrates for MDR transporters mainly consist of different types of antibiotics, biocides, dyes, detergents, cationic peptides, fatty acids and bile salts. The MDR transporters are of great clinical significance due to their multispecific nature that provides a severe hindrance to chemotherapy of infectious disease (Nishino and Yamaguchi 2001).

Many species of bacteria have multiple MDR transporter genes in their genome (for example 39 putative genes are reported in E. coli) (Nishino and Yamaguchi 2001; Sulavik, Houseweart et al. 2001). As a group, they form a coordinated system of transporters with overlapping specificities, which provide a robust survival strategy (Tal and Schuldiner 2009). Previously, it was believed that multispecificity of MDR transporters occurs by an indirect mechanism in which there is not a direct contact between the transporters and its substrate. However, recent studies including the recent crystal structures of MDR
transporters suggested the direct interaction of substrates to transporters and the broad substrate range is due to their multiple substrate binding capacities (Higgins 2007). Multidrug transporters have large hydrophobic binding pockets in their transmembrane region, mainly consisting of aromatic amino acids which help in nonspecific hydrophobic interactions, and also a few charged residues that interact with oppositely charged substrates (Zheleznova, Markham et al. 2000; Murakami, Nakashima et al. 2006). In certain cases, the binding pocket can also accommodate two different substrates at the same time (Lewinson and Bibi 2001).

Figure 2.2. Mechanisms used by bacteria for drug resistance. They have numerous strategies to protect themselves from the toxic effect of antimicrobial compounds including (a) altering the drug target, (b) enzymatic degradation of drugs, (c) adjusting the cell membrane permeability, (d) use of multidrug efflux pump. In this Figure the antimicrobial agents are represented by yellow color and red color arrow show the direction of movement of drugs. This Figure was replicated with authorization from Hindawi Publishing Corporation (Kumar, Mukherjee et al. 2013).
2.2 Families of multidrug transporters

MDR transporters are well categorized on the basis of sequence homology and are extensively documented (Saier and Paulsen 2001; Paulsen 2003). Depending on the structural difference and bioenergetics used, multidrug transporters can be classified into five superfamilies which are involved in the extrusion of toxic compounds to the outside of the cell (Figure 2.3) (Paulsen, Brown et al. 1996; Du, van Veen et al. 2015).

Figure 2.3. The schematic representation of multidrug efflux transporters. Based on the phylogeny, bioenergetics used and transport mechanism, multidrug resistance transporters are classified into five superfamilies of efflux pumps. ABC transporters (left), MFS, SMR, RND and MATE families. This Figure is self-drawn based on idea of (Delmar, Su et al. 2014).

Members of these families are highly conserved and are present in all the domains of life (Yen, Chen et al. 2010). The ATP-binding cassette (ABC) superfamilies have the distinct feature of containing the ATP-binding cassette (ABC), and utilize ATP hydrolysis as an energy source to pump the drug out of the cells. Example are human P-glycoprotein
and SAV1866 from *S. aureus* (Dawson and Locher 2007). Since they use ATP hydrolysis as their energy source, they are also called as “primary active transporters” (Murakami 2008). All other multidrug transporters fall into the category of secondary transporters as they transport protons or sodium ions down their electrochemical gradients to extrude the drugs outside the cells (Putman, van Veen et al. 2000). The secondary transporters are further classified into four different groups: the major facilitator superfamily (MFS) (Griffith, Baker et al. 1992; Marger and Saier 1993; Yin, He et al. 2006), resistance nodulation cell division (RND) (Saier, Tam et al. 1994; Seeger, Schiefner et al. 2006), small multidrug resistance (SMR) (Paulsen, Skurray et al. 1996; Chen, Pornillos et al. 2007), and multidrug and toxic compounds extrusion (MATE) families (Brown, Paulsen et al. 1999; Tanaka, Hipolito et al. 2013) as shown in (Figure 2.3). Generally, these transporters vary in size, with 400-450 amino acids in MFS family and MATE transporters, around 1000 amino acids in the RND transporters and approximately 100 amino acids in SMR family transporters (McKeegan, Borges-Walmsley et al. 2003). ABC transporters are less common in bacteria and provide resistance to specific drugs unlike the secondary multidrug transporter (Marquez 2005). The details of proton-dependent superfamilies are discussed as follows.

**2.2.1 The major facilitator superfamily (MFS):** The MFS is ancient, well characterized, universal, and the largest known superfamily of secondary transporters and passive solute transporters, comprising more than 15000 sequenced members from all kingdoms of life (Hoglund, Nordstrom et al. 2011; Saier, Reddy et al. 2014; Yan 2015; Kumar, He et al. 2016). It was discovered by Henderson and coworkers (Henderson and Maiden 1990) but the term “major facilitator superfamily” was coined by Saier et al
in order to phylogenetically classify the solute permease and drug-resistance protein (Yan 2013; Yan 2015). The MFS participates in all types of transport including uniport, symport, and antiport of numerous substances (Marger and Saier 1993; Paulsen, Brown et al. 1996). Based on the transport process involved, the members of MFS were initially divided into five different clusters which take part in (a) drug resistance (b) sugar uptake (c) uptake of Krebs cycle intermediates (d) phosphate ester/phosphate antiport, and (e) oligosaccharide uptake (Marger and Saier 1993). Currently, this superfamily has more than 74 families in it, each of which are involved in transport of broad spectrum of substrates including monosaccharide, oligosaccharides, amino acids, lipids, organic ions, vitamins, enzyme cofactors, chromophores, and nucleobases (Saier and Paulsen 2001; Yen, Chen et al. 2010; Reddy, Shlykov et al. 2012).

Most members of this superfamily have 12-14 transmembrane helices (TMH) and contain 400-600 amino acids (Law, Almqvist et al. 2008; Du, van Veen et al. 2015). In spite of low sequence similarity, dissimilar substrate specificities and coupling mechanism, these transporters share an extraordinary degree of structural similarity. The basic structural design involves two domains, each grouped into two pseudo symmetrical 6-TM bundles in the membrane plane and connected by a long cytoplasmic loop or two transmembrane helices (Sun, Zeng et al. 2012; Deng, Xu et al. 2014; Wisedchaisri, Park et al. 2014; Du, van Veen et al. 2015). The substrate binding pocket is mainly formed by the membrane-embedded cavity present in-between the two domains (Heng, Zhao et al. 2015). They also have a conserved hallmark sequence, DRXXRR, at identical positions on the N- and C-terminal part of the proteins (Law, Almqvist et al. 2008). Based on the sequencing studies, it was suggested that the C-terminal regions of these proteins participate mainly in
substrate recognition and the N-terminal halves are involved in proton translocation (Paulsen, Brown et al. 1996). These proteins operate by an alternating access mechanism, in which the substrate binding cavity is accessible from either sides of the membrane at some point in the transport cycle. A rocker-switch-type movement leads to conformational changes between inward open, occluded, and outward open states during transport of substrate in exchange of protons. However, the types of transport coupling may vary between the family members. (Bapna, Federici et al. 2007; Schaedler and van Veen 2010; Du, van Veen et al. 2015). In *E. coli*, MdfA is one of the most studied and best characterized bacterial MFS family antiporter (Edgar and Bibi 1997; Sigal, Cohen-Karni et al. 2006).

*MdfA, a model for secondary MDR transport from MFS family and a known suppressor of BC202*

MdfA is one of the suppressors of the BC202 phenotypes since overexpression of MdfA from a plasmid can rescue the cell division and temperature sensitivity phenotypes (Sikdar, Simmons et al. 2013). It also corrects the drug sensitivity of BC202 and partially corrects the alkaline pH sensitivity of ΔyqjA mutant (Kumar and Doerrler 2014)(Kumar and Doerrler 2015)). MdfA is a cytoplasmic membrane protein with 12 transmembrane helices (Figure 2.4 A) and containing 410 amino acids. MdfA was previously called Cmr because it was thought to be a chloramphenicol specific transporter (Nilsen, Bakke et al. 1996; Edgar and Bibi 1997; Adler and Bibi 2002). However, overexpression of MdfA from a multicopy plasmid provides resistance to a wide range of structurally discrete compounds including neutral, zwitterionic, and lipophilic monovalent cations, such as chloramphenicol, ethidium bromide, tetraphenylphosphonium (TPP), benzalkonium chloride, some aminoglycosides, and fluoroquinolones in *E. coli* (Edgar and Bibi 1997; Bibi, Adler et al. 2001). Homologs of MdfA are found in many pathogenic bacteria.
including *Shigella flexneri* (99% identity) (Jin, Yuan et al. 2002), and *Salmonella typhimurium* (90% identity) (Parkhill, Dougan et al. 2001).

Figure 2.4. The secondary structure of MdfA and mechanism of drug/H\(^+\) transport by MdfA. (A) Based on hydropathy profile and gene fusion analysis MdfA has 12 transmembrane helices in it. This part of the Figure is replicated with authorization from American society for microbiology: journal of Bacteriology (Adler and Bibi 2002). (B) The model shows the mechanism in which the transport of drug/H\(^+\) takes place. This part of the Figure is replicated with authorization from Elsevier: Molecular cell (Fluman, Ryan et al. 2012).
Remarkably, MdfA is promiscuous not only in its substrate profile but also in the use of PMF components to drive electroneutral or electrogenic transport. The transport of cationic substrates is electroneutral in nature in which no net movement of charge occurs, whereas the neutral substrate is transported in an electrogenic manner resulting in net movement of electric charges (Lewinson, Adler et al. 2003). The electrogenic transport is governed by both transmembrane potential (Δψ) and a transmembrane pH (ΔpH) component of the PMF (ΔµH), whereas electroneutral transport only requires ΔpH component (Bibi, Adler et al. 2001). The stoichiometry of drug/proton determines the electrogenic or electroneutral transport of drugs. Generally, MdfA has a stoichiometry of 1: monovalent cationic drugs, signifying movement of one proton per antiport cycle. Because of this, the transport of divalent cationic drugs is energetically unfavorable by MdfA. However, providing an additional acidic residue either by random mutagenesis or by rational design in the predicted substrate binding pocket may allow MdfA to transport divalent cationic compounds as well (Tirosh, Sigal et al. 2012).

Biochemical and structural studies have shown the complexity of multidrug recognition and transport mechanism of MdfA. Mutational analysis suggested no irreplaceable acidic amino acid for the transport activity of MdfA (Sigal, Molshanski-Mor et al. 2006). Moreover, the amino acids cysteine (C21), glutamate (D26), glycine (39), valine (V54), and threonine (T56), alanine (A128, A147, and A191) and valine (V335) were proposed to form the drug binding site for MdfA and permit the recognition of various drugs (Edgar and Bibi 1997; Adler and Bibi 2004). The membrane-embedded negatively charged residues glutamic acid (E26) and aspartic acid (D34), and the positively charged arginine (R112) are proposed to participate in the transport function (Edgar and Bibi 1999;
Recently, a model for MdfA transport activity was proposed in which aspartate (D34) is shown to be the proton binding site and it was suggested that substrate and protons compete for binding with each other but they bind at a different location within the protein during the transport cycle (Fluman, Ryan et al. 2012). The detailed mechanism of MdfA mediated drug/H⁺ transport, explaining the remarkable way of indirect competition, is shown in (Figure 2.4 B). In the first step, the proton is released from aspartate (D34) to the cytoplasm, which also requires the help of glutamate (E26). The drugs represented by (S) (Figure 2.4 B) cannot bind to the protein in the proton bound state, which is shown by dashed line. In the second step, the drug (S) bind to the protein upon deprotonation at a site other than D34. Once the drug is bound to the protein, there is a conformational switch that allows the drug bound pocket to open towards the periplasm. The drug is released in the periplasm. Thereafter, the release of the drug will allow a proton to bind D34 from the periplasmic side. The binding of proton again brings back MdfA to inward-facing conformation.

As an MFS protein, MdfA is expected to have the ‘rocker switch’-type mechanism that enables alternating access of the binding pocket to either side of the membrane. According to this model, the MdfA mediated drug transport cycle starts with the proton release from aspartate (D34) to the cytoplasm which also requires the help of glutamate (E26). The deprotonation of protein causes drugs to bind at a site other than D34. Once the drug is bound to the protein, there is a conformational switch that allows the drug bound pocket to open towards the periplasm. Next, the drug is released into the periplasm and release of drug allow proton to bind D34 from the periplasmic side. The binding of proton
again brings back MdfA to inward-facing conformation (Fluman, Ryan et al. 2012). MdfA forms a monomer in detergent solution and in proteoliposomes (Sigal, Lewinson et al. 2007). The complementary gene fusion analysis showed that the C-terminal region of MdfA is not essential for the transport function (Adler and Bibi 2002). Three crystal structures of MdfA-ligand complexes are resolved all in inward facing conformation and has supported the previous biochemical evidence of importance of charged amino acids in transport cycle (Heng, Zhao et al. 2015). Apart from drug/proton transport, MdfA also catalyzes a low-affinity Na⁺-K⁺/H⁺ antiport activity and thus provides alkaline pH tolerance to *E. coli* (Lewinson, Padan et al. 2004).

2.2.2 The Resistance-Nodulation-Division (RND) Superfamily: Based on the sequence homology of functionally related membrane proteins, the RND protein superfamily was first described in 1994 (Saier, Tam et al. 1994). The unique designation of resistance/nodulation/division came from the identification of homologs involved in the nodulation of legumes by *rhizobia* (NolGHI) (Gottfert 1993), cell division protein (EnvD) (Klein, Henrich et al. 1991), and drug resistance. The RND superfamily can be further classified into seven protein families depending on their participation in the transport of transition metals, organic substances, drugs, and polypeptides (Tseng, Gratwick et al. 1999; Kim, Nies et al. 2011). These proteins are omnipresent in all the domains of life including bacteria, archaea, and eukaryotes (Tseng, Gratwick et al. 1999). Out of all MDR efflux pumps, the RND superfamily has the broadest range of antimicrobial substrates (Nikaido 1998) and is present in most pathogenic bacteria including, *Burkholderia pseudomallei*, *Escherichia coli O157:H7*, *Haemophilus influenzae*, *Neisseria gonorrhoeae*, *Pseudomonas aeruginosa*, *Salmonella typhimurium*, and *Vibrio cholerae* (Helms, Vastrup
et al. 2002; Livermore 2003; Poole 2003). In Gram-negative bacteria, the efflux pumps belonging to the RND superfamily, such as AcrAB of *E. coli* and MexAB of *Pseudomonas aeruginosa* are the major players for providing multidrug resistance, since mutants lacking these genes are susceptible to most of the tested drugs (Kim, Nies et al. 2011). RND efflux pumps are a hindrance to the development of new potential antibiotics or drugs because of their specificity for a broad variety of substrates (Li, Zolli-Juran et al. 2004; Caughlan, Jones et al. 2012). RND efflux pumps also play a major role in virulence of pathogenic bacteria by helping them in colonization, evading phagocytosis, and biofilm formation (Paulsen, Brown et al. 1996; Nikaido and Takatsuka 2009).

RND proteins usually contain around 700-1300 amino acids, and their predicted structure consists of 12 transmembrane segments (TMS) with two large extracytoplasmic loops between TMS 1 and TMS 2, and TMS 7 and TMS 8 (Saier, Tam et al. 1994; Paulsen, Brown et al. 1996). Comparative sequence investigation have suggested that the N-terminal halves have sequence similarity with the C-terminal halves, indicating the tandem intragenic duplication of these transporters during evolution, similar to the MFS transporters (Saier, Tam et al. 1994; Paulsen, Brown et al. 1996). The RND family proteins are usually functional as a tripartite system (Figure 2.5) with the RND pump protein (AcrB) in the cytoplasmic membrane, a periplasmic adaptor protein of the MFP (membrane fusion protein) family (AcrA) (Zgurskaya and Nikaido 2000) and an outer membrane channel such as TolC of *E. coli* from the outer membrane factor (OMF) family (Fralick 1996) to extrude substrates out of cells (Nikaido 2011). Two different types of efflux occur through this tripartite system (i) transenvelope efflux and (ii) periplasmic efflux, using the proton gradient as an energy source (Figure 2.5).
In transenvelope efflux, the substrate is directly effluxed to the external environment from the cytoplasm using an RND protein in the inner membrane and an outer membrane protein. However, in periplasmic efflux the substrate is extruded out from the periplasm (Kim, Nies et al. 2011). Recently, the crystal structure of all the three components of the RND efflux pump (AcrAB) were published detailing the mechanism of the assembly and transport process (Koronakis, Sharff et al. 2000; Murakami, Nakashima et al. 2002; Akama, Kanemaki et al. 2004; Akama, Matsuura et al. 2004). AcrA-AcrB-TolC of *E. coli* and MexA-MexB-OprM of *Pseudomonas aeruginosa* are two best-characterized examples of RND family proteins.
**AcrAB-TolC multidrug efflux system from RND family**

The *E. coli* AcrAB-TolC multidrug efflux pump is thought to be the best-studied efflux system and primarily responsible for the resistance to a wide variety of biocides and antibiotics. The *acrAB* operon contains two genes *acrA* and *acrB* which encode AcrA (member of MFP family) and AcrB (member of RND family) respectively (Ma, Cook et al. 1995) while the gene for TolC is present at a different position on the chromosome (Fralick 1996). All together they form a tripartite efflux system in which AcrB is at the cytoplasmic membrane, AcrA is periplasmic protein and TolC is a channel spanning the outer membrane. The schematic representation of the *E. coli* AcrAB-TolC efflux system is shown in Figure 2.6. Each component of the tripartite system is explained in details as follows.

![Schematic representation of E. coli AcrAB-TolC efflux system](image)

**Figure 2.6.** Schematic representation of *E. coli* AcrAB-TolC efflux system: Three components (i) AcrB are present in cytoplasmic membrane (ii) a periplasmic adapter protein AcrA and (iii) the outer membrane channel TolC. The Figure is replicated with authorization from Elsevier publisher: Current opinion in microbiology journal (Blair and Piddock 2009).

AcrB : the inner membrane component : AcrB is an inner membrane protein that utilizes proton motive force to extrude different substances including antibiotics like penicillin, cephalosporin, fluoroquinolones, macrolides, tetracycline and rifampicin,
cationic dyes such as acriflavine, crystal violet and ethidium bromide, and detergents such as Triton X-100, bile acids and organic solvents out of the cytoplasm (Ma, Cook et al. 1995; Yu, Aires et al. 2003; Nikaido and Takatsuka 2009). Crystallographic studies have shown that AcrB is a homotrimer complex of three identical protomers in the inner membrane, which form a “jellyfish” like shape as shown in Figure 2.7 (Murakami, Nakashima et al. 2002; Du, van Veen et al. 2015). Each protomer has 1049 amino acids and is divided into three domains (i) the transmembrane domain (TMD), (ii) periplasmic domain (PD), (iii) a TolC-docking domain in the periplasm (Figure 2.7) (Murakami, Nakashima et al. 2006; Tornroth-Horsefield, Gourdon et al. 2007; Nikaido 2011).

Figure 2.7. The Structural arrangement of E. coli AcrAB-TolC efflux system: This system consists of the membrane fusion protein (MFP) AcrA and two membrane proteins: TolC, a channel in the outer membrane, and AcrB, a proton-dependent drug antiporter in the inner membrane. AcrB consists of the transmembrane domain, the porter (pore) domain, and the TolC docking domain. TolC is present on the outer membrane (OM) with its β-barrel domain and forms a long channel which tapers to a closed entrance at the proximal end in the periplasm by its α-helical domain. AcrA is separated into the membrane proximal domain, β-barrel domain, the lipoyl domain, and the α-helical hairpin. AcrA is linked with the inner membrane via an N-terminally attached lipid anchor. The Figure is replicated with authorization from Elsevier publisher: Chemistry and biology (Oswald and Pos 2011).
In vivo site-directed disulfide crosslinking experiments showed that AcrB opens like a funnel into the TolC-docking domain (Tamura, Murakami et al. 2005), and loops from the TolC docking domain help in holding all three protomers together (Sennhauser, Amstutz et al. 2007). AcrB has 12 transmembrane helices out of which the 4th and 10th transmembrane domains have a triplet of charged amino acids, Asp 407, Asp 408, and Lys 940, all of which are important for proton translocation and are located distally from the substrate binding site (Murakami, Nakashima et al. 2006; Su, Li et al. 2006; Seeger, von Ballmoos et al. 2009). The structure of AcrB is asymmetric in nature as each protomer are organized differently and various conformations of each monomer are necessary for the rotating mechanism (also called “peristaltic” transport mechanism) in which the substrates go through different modes of binding that include access, binding, and extrusion and back to access mode in the transport cycle as shown in (Figure 2.8) (Murakami, Nakashima et al. 2006; Sennhauser, Amstutz et al. 2007; Takatsuka and Nikaido 2009; Du, van Veen et al. 2015). Recently, novel pyrano-pyridine based inhibitors of AcrB were developed having more powerful inhibition capacity than previously known inhibitors that will help in combating MDR gram-negative pathogens (Sjuts, Vargiu et al. 2016).

AcrA: The periplasmic component: AcrA is a periplasmic protein necessary for the transport activity of AcrAB-TolC tripartite system. It belongs to the membrane fusion protein family and is coexpressed with AcrB. AcrA is crucial for the multi-drug resistance phenotype along with AcrB (Ma, Cook et al. 1995; Paulsen, Brown et al. 1996). AcrA is categorized as a lipoprotein based on sequence similarity and it is bound to the inner membrane through N-terminal lipid moiety (Ma, Cook et al. 1995).
Figure 2.8. Schematic representation of “peristaltic” transport mechanism of *E. coli* AcrB. The three protomers of AcrB adopt different conformations individually upon substrate binding, namely Loose (L), Tight (T) and Open (O). The top panel shows a cross section (two protomers of the AcrB/TolC trimers or AcrA hexamer). TolC is at the top and AcrA is contacting both AcrB and TolC. The middle panel is the view of AcrB trimer with respect to the periplasmic domain of the pump. The lower panel shows the movement of the substrate between the binding pockets in protomers. The various geometric representation like a triangle (low), rectangle (high), and circle (no) show the binding affinity for the substrates. In the first state of the cycle (from left to right), a monomer binds a substrate (acridine) in its transmembrane domain (L conformation), subsequently transports the substrate from the transmembrane domain to the hydrophobic binding pocket (conversion to T conformation) and finally releases the substrate in the funnel toward TolC (O conformation). Peristaltic transport of drugs through the AcrB tunnels and through TolC would account for a strict unidirectional movement towards the outside of the cell. The conversion from the T monomer to the O monomer conformation is suggested to be the major energy-requiring (proton motive force-dependent) step in this functional rotation cycle and requires the binding of a proton to the proton translocation site (D407, D408, and K940) from the periplasm. The release of a proton from the proton translocation site to the cytoplasm might occur during conversion from the O monomer to the L monomer (as depicted) or from the latter to the T monomer. AcrA is expected to participate in the transduction of the conformational changes from AcrB to TolC, which results in the movement of the proximal part of TolC and the facilitation of drug extrusion to the outside of the cell. The Figure is replicated with authorization from Elsevier publisher: Current opinion in Structural biology journal (Du, van Veen et al. 2015).
The structure of AcrA has four domains (a) the C-terminal domain which is conserved in the membrane-fusion proteins (b) the β-barrel domain (c) the lipoyl domain, which is suggested to function as swinging arm and participating in carrying substrate to their destination and (d) a helical domain which interacts with TolC as shown in (Figure 2.7) (Johnson and Church 1999; Zgurskaya and Nikaido 1999; Mikolosko, Bobyk et al. 2006; Ge, Yamada et al. 2009).

TolC: The outer membrane component: TolC is present in the OM and accountable for extrusion of hydrophobic and amphipathic drugs out of the cell. The crystallographic studies have shown that TolC is made of three identical protomers, consisting of 428 amino acids that form a “channel-tunnel like” structure. Each protomer consists of a β-barrel domain (channel domain) attached to the outer membrane and a helical domain (tunnel domain) projecting into the periplasm as shown in (Figure 2.9) (Koronakis, Li et al. 1997; Koronakis, Eswaran et al. 2004).

Figure 2.9. The complete structure of TolC. The three protomers of TolC are colored in red, yellow and blue. The beta barrel representing the channel domain is at the top and α-helical structure showing the tunnel domain is at the bottom. The Figure is replicated with authorization from nature publishing group: nature with slight modifications (Koronakis, Sharff et al. 2000).
Apart from AcrAB efflux pump, many other multidrug transporter including AcrEF (Kobayashi, Tsukagoshi et al. 2001), YhiUV (Nishino and Yamaguchi 2002), EmrAB (Borges-Walmsley, Beauchamp et al. 2003), MdtABC (Nagakubo, Nishino et al. 2002), and the MacAB (Kobayashi, Nishino et al. 2001) are dependent on TolC for expulsion of drugs outside the cell. TolC is also responsible for hemolysin secretion (Wandersman and Delepelaire 1990).

2.2.3 The small multidrug resistance (SMR) superfamily: Members of SMR superfamily are usually small integral membrane proteins having 100 to 140 amino acids and participate in the efflux of lipophilic cationic drugs and numerous quaternary ammonium compounds (QACs) (Paulsen, Skurray et al. 1996; Bay, Rommens et al. 2008). The well-known substrates for SMR are methyl viologen (MV), tetraphenylphosphonium (TPP), benzalkonium chloride, cetyltrimethylammonium bromide (CTAB), ethidium bromide, and acriflavine (Yerushalmi, Lebendiker et al. 1995; Chung and Saier 2001). Phylogenetic studies of the SMR family suggested that all the members of this superfamily are not drug transporters and they are further classified into three types: (i) small multidrug pumps (SMP), which provide multidrug resistance to bacteria as well as archaea (for example EmrE from *E. coli*), (ii) suppressor of GroEL mutation proteins (SUG) which depend on the ability to phenotypically suppress the mutation in *groE* (Greener, Govezensky et al. 1993), and (iii) the paired SMR (PSMR) which need coexpression of two separate SMR gene products providing resistance in the bacteria (for example YdgE and YdgF from *E. coli*) (Bay, Rommens et al. 2008).

Typically all classes of the SMR superfamily have four predicted transmembrane domains. Multiple sequence alignment and membrane topology studies have shown the
presence of conserved amino acid motifs in each of four predicted transmembrane domains (Figure 2.10). However, the knowledge of topology and orientation of these proteins are often the subject of controversial debate. Both uni-directional orientation and the bi-directional concept of the dual topology of a homodimer have been proposed in the literature (Pornillos and Chang 2006; von Heijne 2006). EmrE is a model for studying the evolution and mechanism of SMR family (Schuldiner 2009).

Figure 2.10. The representation of three classes of small multidrug resistance protein (SMR) topology. (A) *E. coli* EmrE belong to SMP class (B) SugE represent the SUG class (C) YdgE and (D)YdgF from *E. coli* belong to PSMR class. SMR protein consists of four transmembrane helices. The amino acids of each protein are depicted by circles. Site-directed mutagenesis studies have suggested that the amino acids highlighted in red affect the drug resistance activity. The conserved motif for all SMR proteins is shown by blue color and conserved residue specific to SMP are shown by green, SUG is shown by yellow and PSMR is shown by violet. The Figure is replicated with authorization from Elsevier publication: Biochimica et Biophysica Acta with slight modifications (Bay, Rommens et al. 2008).
EmrE: model for studying mechanism of transport of SMR family

EmrE is a small (110 amino acids) SMR transporter that provides resistance to multiple hydrophobic positively charged drugs, including ethidium bromide, proflavine, methyl viologen, erythromycin and tetracycline in *E. coli* (Higgins 2007; Schuldiner 2009). EmrE forms the basis of a unique experimental model system to study the coupling mechanism of secondary transporter not only due to its small size and stability, but also because the detergent-solubilized proteins is able to show the antiport of a proton and substrate, replicating catalytic activity in the membrane (Schuldiner 2007). Based on hydropathy analysis, EmrE has four predicted transmembrane domains (Figure 2.10). Transport experiments have shown that the energy requisite for activity depends on the nature of the substrate. For example, monovalent cationic compounds like TPP⁺ are transported in an electrogenic manner whereas transport of divalent cations like methyl viologen (MV) are electroneutral in nature (Rotem and Schuldiner 2004).

Altogether, there are eight charged amino acids in EmrE, of which only glutamate (E14) is located in the transmembrane region and is highly conserved in hundreds of homologs in bacteria and archaea (Schuldiner 2009; Schuldiner 2014). Site-directed mutagenesis and pH dependent studies have shown that E14 is indispensable for both the substrate and proton binding, out of which only one can bind at one time (Schuldiner 2009). Moreover, biochemical and structural data have suggested that EmrE functions as a homodimer, but whether it functions as parallel or antiparallel dimers has been highly controversial (Schuldiner 2009). The parallel dimer is symmetric in nature in which both subunits are in the same orientation, whereas anti-parallel dimers are asymmetric having
both subunits in opposite membrane orientation (dual topology) (Figure 2.11) (Schuldiner 2009) (Sanders 2015).

![Parallel homodimers](image1)

**Figure 2.11.** The schematic representation of the topology of *E. coli* EmrE. Theoretically, two subunits of EmrE can either acquire a parallel or anti-parallel orientation. (A) A parallel orientation is when the N- and C-termini of both subunits in the dimer are on the same side of the membrane. (B) In contrast, for an anti-parallel orientation, the N- and C-termini of each subunit are on opposite sides of the membrane and as a result, obtain a dual topology, where the dimers are a combination of both C_{in} and C_{out}. The Figure is drawn based on the idea from (Rapp, Granseth et al. 2006; Schuldiner 2007; Schuldiner 2009; Woodall, Yin et al. 2015).

The structural data from Cryo-EM, X-ray crystallography (Chen, Pornillos et al. 2007; Lloris-Garcera, Slusky et al. 2013) and NMR dynamics experiment (Morrison, DeKoster et al. 2012), along with the biochemical evidence (Lloris-Garcera, Bianchi et al. 2012) support the anti-parallel orientation of EmrE (Figure 2.12, b). Moreover, the membrane topology sequence determinants, arginine and lysine residues present in EmrE loops (K_{R} bias) further support the dual topology orientation (Rapp, Granseth et al. 2006). The anti-parallel dimer adopts the dual topology in a cotranslational manner by the ribosome and translocon (Woodall, Yin et al. 2015). On the other hand, there are various
biochemical data backing the parallel orientation including the design of functional EmrE constructs in which the monomers are associated with short polar loops making the constructs energetically unfavorable to cross the membrane (Steiner-Mordoch, Soskine et al. 2008).

Figure 2.12. The model of single-site alternating access mechanism of EmrE transport. (A) EmrE translocates one cationic substrate in exchange for two protons. In this model, EmrE undergoes a cycle of alternatingly inward and outward facing binding pocket on one side of the membrane during the transport cycle. The change in conformation only takes place when either two protons or cationic substrate compete for binding to a single glutamate residue (E14). (B) The cartoon structure of conformational interconversion of anti-parallel EmrE in substrate bound form is shown. This Figure is replicated with authorization from Nature publishing group: Nature (Morrison, DeKoster et al. 2012).
Additionally, crosslinking of amino acids present in opposite sides of the membrane in the anti-parallel model is possible without affecting the transport activity supporting the parallel orientation (Soskine, Mark et al. 2006). Furthermore, electron paramagnetic resonance (EPR) studies of spin-labelled EmrE in TM3 showed restricted constraint, further backing the parallel orientation concept (McHaourab, Mishra et al. 2008).

As far as the transport mechanism is concerned, the single site alternate access mechanism has been suggested for anti-parallel EmrE in which EmrE switches the inward and outward-facing conformations to transport the substrate across the membrane. In this transport process, the proton and substrate interact with a single binding site (E14) leading to a conformational change (Figure 2.12) (Morrison, DeKoster et al. 2012). E14 acts as the carrier for the two protons that are released into the cytoplasm to energetically initiate the EmrE antiport cycle (Schuldiner 2009). Moreover, the pH gradient across the E. coli membrane may help in altering the equilibrium of EmrE in support of an inward-open resting conformation important for substrate binding (Gayen, Leninger et al. 2016).

2.2.4 The Multidrug and Toxic Compound Extrusion (MATE) Family: The transporters of the MATE family help in the extrusion of cationic, lipophilic compounds and xenobiotics and provide multidrug resistance to pathogenic bacteria and cancer cells (Omote, Hiasa et al. 2006; Kuroda and Tsuchiya 2009). They are functionally flexible in using H⁺ or Na⁺ motive force for their transport activity (Jin, Nair et al. 2014). Around 900 MATE transporters have been reported so far based on the amino acid similarity and classified into the NorM, DinF (DNA-damage-inducible protein F), and eukaryotic subfamilies (Brown, Paulsen et al. 1999; Lu 2015). Out of these subfamilies, NorM and DinF have the ability to utilize either the proton or sodium gradient to energize transport
while eukaryotic subfamilies are mainly proton-dependent transporters (Morita, Kataoka et al. 2000; Su, Chen et al. 2005; Masuda, Terada et al. 2006; Lu 2015). Typically, MATE family proteins have 400-700 amino acids and contain 12 predicted transmembrane domains. Biochemical and structural data have suggested that these transporters also follow a rocker-switch mechanism similar to the MFS transporters (Du, van Veen et al. 2015).

2.2.5 Coordination between the multidrug transporters: a strong tool for survival:
Genomic studies have revealed that most bacteria have a vast number of drug transporter genes. Various methods have been employed to study the roles of these drug transporters. Sulavik et al. created knock out strains of various efflux pumps and studied the effect of 35 different compounds including antibiotics, dyes, and detergents on these strains (Sulavik, Houseweart et al. 2001). In another study, overexpression of a complete library of *E. coli* putative drug transporters from a plasmid showed that around 20 genes provide resistance to at least one of the 26 biocides tested (Nishino and Yamaguchi 2001). These studies supported the idea that deletion of efflux pumps other than AcrAB-TolC has minimal effect on the drug sensitivity in *E. coli*. The minimal effect of gene deletion of other transporters except AcrAB-TolC can be explained on the basis of overlapping function of one transporter to shield the loss of other transporters (Tal and Schuldiner 2009). The model in (Figure 2.13) was proposed explaining the coordinated network of transporters with overlapping capacity in providing a strong survival strategy against toxic environments. According to this model, there is a two stage functional organization of membrane transporters in which MFS (MdfA) and SMR (EmrE) like transporters associated with the cytoplasmic membrane transport the toxic substrates from the
cytoplasm to periplasm and AcrAB-TolC removes the toxic compounds from periplasm out of the cell (Tal and Schuldiner 2009).

Figure 2.13 Model of functional co-ordination among AcrAB-TolC, EmrE, and MdfA in E. coli. The EmrE and MdfA are single component multidrug transporters present in the cytoplasmic membrane. These transporters remove their respective substrates from the cytoplasm into the periplasm. Thereafter, AcrAB-TolC captures those substrates and extrudes them out of the cells. This Figure is replicated with authorization from PNAS (Tal and Schuldiner 2009).
2.3 Alkaline pH homeostasis in *E. coli*

The proton motive force (PMF) is an electrochemical gradient of protons across the cell membrane established by active proton pumping and moderated by secondary ion movement (Mitchell 1961; Krulwich, Sachs et al. 2011). Usually, the bacterial PMF has two components: a transmembrane electrical potential ($\Delta \psi$), which is negative inside as compared to outside and a transmembrane pH gradient ($\Delta p$H), which is usually alkaline inside relative to the outside (Rottenberg 1979; Kashket 1985). $\Delta \psi$ is generated by electrically unbalanced movement of ions across the membrane and defined as the potential difference across the membrane when measured with two identical electrodes (Rottenberg 1979). $\Delta \text{pH}$ is the difference between the cytoplasmic pH ($\text{pH}_{\text{in}}$) and outside pH of the environment ($\text{pH}_{\text{out}}$) (Padan and Schuldiner 1987). The PMF of a bacterial cell is represented by the equation: \text{PMF (mV)} = \Delta \psi - (2.303RT/F)*\Delta \text{pH}, where $R$ is the gas constant, $T$ is the absolute temperature, and $F$ is the Faraday constant (Krulwich, Sachs et al. 2011). Primary proton pumps including the respiratory or other redox potential-driver pumps facilitate electrogenic transport of protons outward and help in generation of PMF across the membrane (Padan and Schuldiner 1987). Secondary transporter and rotary nanomachines like the ATP synthase and the flagellar motor can utilize the PMF to energize active transport and mechanical processes, ultimately contributing to the pH homeostasis of specific bacteria (Krulwich, Sachs et al. 2011). Additionally, the proton concentration across the cell membrane plays a crucial role in bioenergetics of the bacterial cell (Mitchell 1966).

Bacteria are classified into three categories based on the pH requirement: acidophile, neutrophile and alkaliphiles. Acidophiles like *Lactobacillus* can grow in pH <
5, neutrophiles like *Escherichia coli* can survive at external pH values of 5.5-9.0 and alkaliphiles like *Bacillus alcalophilus* can live at pH 10-13.0 range. Depending on the demands of pH homeostasis, bacterial species can regulate the relative level of both PMF components (Krulwich, Sachs et al. 2011). The environmental pH partially controls the cytoplasmic pH, or intracellular pH, which ultimately affects protein stability, enzymatic functions, nucleic acid structure, and other important biological molecules (Slonczewski, Fujisawa et al. 2009). Therefore, pH homeostasis is essential for the existence of bacterial cells at unfavorable pHs. There have been extensive studies and reports about the acidic pH homeostasis of bacteria (Matin 1999; Foster 2004), but here I will mainly concentrate on describing the bacterial alkaline pH homeostasis which is an important aspect of many pathogen life styles.

There are many natural habitats with elevated pH, including sea water, and bacteria are able to reside in such places. For instance, enteric bacteria inhabiting the human gut have to survive in pH > 10 in the pancreatic duct below the pylorus (Evans, Pye et al. 1988). Additionally, neutrophilic enterobacteria *E. coli* and *Vibrio cholerae* can survive in alkaline marine environments over a period of time, where they are a risk to public health (Hood and Ness 1982). The ability of bacteria to persist and grow at alkaline pH is of great importance for physiology, ecology, and pathogenesis, and also for remediation and industrial applications (Padan, Bibi et al. 2005). Although neutrophiles like *E. coli* can grow in a broad range of external pH, from 5.5 – 9.0, they have to retain their cytoplasmic pH to a narrow range of pH 7.4-7.8 for their survival (Krulwich, Sachs et al. 2011). At alkaline pH, alkaliphiles and neutrophiles have an inverted ΔpH (acid inside) which gets deducted from PMF resulting in constant cytoplasmic pH inside and only the Δψ
component of PMF plays the major role in the maintenance of cytoplasmic pH (Slonczewski, Fujisawa et al. 2009). Additionally, the surface-associated proton concentration as relative to cytoplasmic proton concentration could establish a ΔpH, which is chemiosmotically suitable than the bulk ΔpH during the growth at alkaline pH (Krulwich, Guffanti et al. 1990).

The ability to survive in alkaline environments is critical for many bacterial species. Studies aimed at understanding alkaline tolerance have applications in biotechnology, bioremediation and medicine (Horikoshi 1999; Margesin and Schinner 2001). Alkaline tolerance is important for the ability of the microbiome and bacterial pathogens to adapt to the alkaline environment of the gut (Krulwich, Sachs et al. 2011), for the bioprecipitation of uranium under alkaline conditions by *Deinococcus radiodurans* (Kulkarni, Ballal et al. 2013) and it is even relevant to the origin of life, which is believed to have occurred in alkaline hydrothermal vents (Martin, Baross et al. 2008).

Transcriptome and proteome analysis, along with genetic studies, suggested various adaptive mechanisms are employed by bacteria for alkaline pH homeostasis. These include: (a) upregulation of ATP synthase that brings in protons during ATP generation (Krulwich, Sachs et al. 2011) (b) metabolic switching for acid production (Stancik, Stancik et al. 2002), (c) increased synthesis of acidic metabolite by amino acid deaminases like TnaA tryptophanase (Blankenhorn, Phillips et al. 1999), (d) cell surface modifications such as increased synthesis of anionic phospholipids or specific neutral lipids that aid in cytoplasmic proton preservation (Clejan, Krulwich et al. 1986), and (e) increased expression and activity of monovalent cation/proton antiporters (Padan, Bibi et al. 2005). Amongst these strategies, cation/proton antiporters play a dominant role in the survival of
bacteria to challenges of alkaline pH tolerance, salt, temperature or osmolarity (Padan and Schuldiner 1994; Padan, Gerchman et al. 1999). The different strategies employed by *E. coli* on exposure to alkali challenge are shown in Figure 2.14.

Figure 2.14. Mechanisms of alkaline pH adaptation by neutrophilic bacteria like *E. coli*. In order to survive alkaline environments, *E. coli* must maintain its cytoplasmic pH to a narrow range and therefore the genes such as TnaA (tryptophan deaminase), ATP synthase, Non-proton-pumping Cyd and Na⁺/H⁺ antiporters get highly activated and on the other hand, proton pumping Nuo proteins get down regulated mainly to keep the cytoplasmic pH in a range of 7.4 to 4.8. Cyd, cytochrome *bd*; NhaA, Na⁺/H⁺ antiporters; Nuo, NADH–ubiquinone oxidoreductase; TnaA, tryptophan deaminase (also known as tryptophanase). The Figure was reproduced with the permission from nature publishing group: Nature review Microbiology (Krulwich, Sachs et al. 2011).

Genomic studies have shown the existence of five to nine dissimilar genes encoding transporters that can act as cation/proton antiporters in most sequenced bacterial genomes (Krulwich, Hicks et al. 2009). To date, five cation/proton antiporters (NhaA, NhaB, ChaA, MdfA, and MdtM) have been reported to play a role in alkaline pH tolerance in *E. coli* (Padan and Landau 2016). A comprehensive phylogenetic analysis of both eukaryotic and prokaryotic genomes have grouped the putative antiporter genes into three different monovalent cation proton antiporter superfamilies (CPA) (Chang, Lin et al. 2004).
CPA1 family mainly consists of the eukaryotic sodium-hydrogen exchanger (NHE) Na\(^+\)/H\(^+\) antiporters along with a few prokaryotic orthologs like Mj-NhaP1 (Chang, Lin et al. 2004). The CPA2 family includes NhaA as its prokaryotic member and also has orthologs in some eukaryotic genomes including the human genome. Lastly, the CPA3 family consists of Mrp-type antiporters from alkaliphilic Bacillus (Swartz, Ikewada et al. 2005). Mrp-type antiporters involves six to seven membrane proteins forming a hetero-oligomeric complex for its transport activity, unlike other cation/proton antiporters which only need one membrane protein for their activity (Morino, Suzuki et al. 2014).

Na\(^+\) and H\(^+\) are the most predominant ions in the cell that has an important roles in cell physiology and bioenergetics. A balanced concentration of these ions is critical for protein functions inside the cell and any fluctuation in their concentration results in cell stress (Padan, Venturi et al. 2001). The Na\(^+\)/H\(^+\) antiporters utilize these ions for regulation of the intracellular pH and also balance the Na\(^+\) ion content. These Na\(^+\)/H\(^+\) antiporters were first discovered by Mitchell and his colleagues in 1974 (West and Mitchell 1974). They proposed that Na\(^+\)/H\(^+\) antiporters have a vital role in maintaining a balanced concentration of Na\(^+\) and H\(^+\) ions in the cells. Since then, Na\(^+\)/H\(^+\) antiporters have been identified in the membranes of most cells including microorganisms, plants and animals (Orlowski and Grinstein 2007; Fliegel 2008). Importantly, a number of these antiporters are also recognized as potential drug targets in humans (Karmazyn 2013). The Na\(^+\)/H\(^+\) antiporters usually show the presence of a sensor and transducer region, which helps them to sense the ion concentration and react and control activity through transducer regions to maintain homeostasis (Padan, Venturi et al. 2001). Multiple cation/proton antiporters are present in bacteria, which maintain the Na\(^+\) and H\(^+\) cycle, not all of them participate in the alkaline
pH homeostasis. The schematic representation of Na\(^+\) and H\(^+\) cycle and different cation/proton antiporter systems in *E. coli* is shown in Figure 2.15.

Each antiporter has distinct properties that including stoichiometry and rate, expression profile, affinity for substrate and transport mechanism and structure-functional relationship (Padan, Bibi et al. 2005). Usually, in alkaline environments (pH > 7.6), to maintain cytoplasmic pH around 7.6, protons should accumulate in the cytoplasm. Therefore, Na\(^+\)/H\(^+\) antiporters should be electrogenic in nature rather than electroneutral allowing \(\Delta\psi\) to drive the net accumulation of cytoplasmic proton concentration (Macnab and Castle 1987; Krulwich, Ito et al. 1998). For example, *E. coli* NhaA has a coupling stoichiometry of 2H\(^+\)/Na\(^+\) and NhaB has a stoichiometry of 3H\(^+\)/2Na\(^+\) (Taglicht, Padan et
al. 1993; Pinner, Padan et al. 1994). Some antiporters other than Na\(^+\)/H\(^+\) antiporter may also help in maintaining alkaline tolerance by utilizing another substrate. For example, in *Bacillus subtilis* an electroneutral Bs-MleN, a parologue of the Na\(^+\)/H\(^+\) antiporter Bs-NhaC, catalyzes 2H\(^+\) - malate \(^{-2}\) / Na\(^+\) - lactate \(^{-1}\) transport and helps in net cytoplasmic accumulation of H\(^+\) (Wei, Guffanti et al. 2000). Moreover, multifunctional Bs-Tet(L) and Bs-Mrp antiporters utilize K\(^+\), along with PMF to catalyze Na\(^+\) (K\(^+\))/ H\(^+\) exchange (Cheng, Baldwin et al. 1996; Ito, Guffanti et al. 1999). Additionally, many bacteria also use K\(^+\)/H\(^+\) antiporters for alkaline pH homeostasis as implicated in many studies (Plack and Rosen 1980; Kakinuma and Igarashi 1995). Recently, MdtM was found to be a low-affinity antiporter that catalyzes an electrogenic exchange of Na\(^+\), K\(^+\), Rb\(^+\) or Li\(^+\) for H\(^+\) (Holdsworth and Law 2013). Here, I will discuss the role of five different cation/proton antiporters (NhaA, NhaB, ChaA, MdfA, MdtM) in providing alkaline pH tolerance in *E. coli* as shown in Figure 2.16.

![Figure 2.16](image_url)

Figure 2.16. Cation/proton antiporters in *E. coli*. NhaA, NhaB, ChaA, MdfA, MdtM are the cation/proton antiporters reported to play a role in alkaline pH tolerance. These antiporters have the ability to use different types of cations in exchange of proton for their activity at alkaline pH as shown. The Figure is self-drawn.
2.3.1 NhaA, the main Na\(^+\)/H\(^+\) antiporter of *E. coli*, serves as a prototype for Na\(^+\)/H\(^+\) antiporter: *E. coli* NhaA is a model for the prokaryotic Na\(^+\)/H\(^+\) antiporter that utilizes the proton electrochemical gradient to exchange sodium ions for protons in the cytoplasm and helps in adaptation to alkaline pH in the presence of sodium ions. Apart from Na\(^+\), NhaA also has the ability to transport Li\(^+\), thereby helping in lithium detoxification (Inaba, Kuroda et al. 1994). Topological analysis studies have suggested a 12-transmembrane helical structure for NhaA with N and C termini located in the cytoplasm as shown in Figure 2.17 (Rothman, Padan et al. 1996).

![Figure 2.17](image.png)

**Figure 2.17.** Model for the secondary structure of *E. coli* NhaA (Na\(^+\)/H\(^+\) antiporter) containing 12-transmembrane domains. The roman numbers represent the number of transmembrane segments. The amino acids required for the function of NhaA in response to pH are represented. The amino acids involved in the transport activity is shown in a rectangle, residues important for pH response are shown in hexagon and circle represent the amino acid found in suppression mutation screening that upset pH response. Trypsin cleavage occurs at the K249 as shown by the arrow. The Figure was reproduced with the permission from Elsevier publishing group: Biochimica et Biophysica acta (Padan, Venturi et al. 2001).
NhaA contains 362 amino acids with a molecular weight of 42 kDa and forms a homodimer in which two monomeric units are attached by a beta-hairpin at the periplasmic side of the membrane (Rimon, Tzubery et al. 2007). However, monomers are the functional unit for NhaA, and dimers are essential only in stress conditions because of better stability in a harsh environment (Rimon, Tzubery et al. 2007). Biochemical studies have revealed numerous properties of NhaA, which suggest its role in alkaline pH homeostasis. Most importantly, it is an electrogenic antiporter having a stoichiometry of $2H^+/Na^+$ that facilitates the maintenance of cytoplasmic pH in alkaline conditions (Taglicht, Padan et al. 1993). Additionally, the turnover rate of its transport activity is very high ($10^5$ exchanges per minute). Furthermore, the NhaA activity is strongly pH dependent, showing an increase in activity by three orders of magnitude between pH 6.5 and 8.5 (Taglicht, Padan et al. 1993; Krulwich, Sachs et al. 2011). The predicted secondary structure of NhaA suggested six charged residues in putative transmembrane helices including four conserved aspartates (D65, D133, D163, and D164) towards the N-terminal and two lysines (K300 and K362) in the C-terminal region. Most of these residues are conserved in members of all NhaA antiporter family members except K362 (Rothman, Padan et al. 1996) (Padan, Venturi et al. 2001). Mutagenesis studies showed the importance of D133, D163 and D164 for cation binding or transport activity of NhaA and H225 and G338 a part of pH sensor region in NhaA (Inoue, Noumi et al. 1995) (Gerchman, Olami et al. 1993).

NhaA stays in its active conformation at pH > 6.5 and in downregulated conformation at pH below 6.5. The crystal structure in its downregulated conformation at pH 4 aided in understanding the antiporter’s mechanism and pH regulation. Additionally, molecular dynamics simulations elucidated the residues necessary for ion exchange in
NhaA. The protonation and deprotonation of aspartate residues (D164 and D163) help in the completion of transport cycle. Protonation of D164 leads to the release of Na\(^+\) and deprotonation cause Na\(^+\) to bind D164 of NhaA. In addition to that, the protonation of D163 causes a conformational change in NhaA allowing Na\(^+\) to get released into the periplasm. However, if D163 is not protonated, Na\(^+\) is released back into the cytoplasm (Arkin, Xu et al. 2007) (Hunte, Screpanti et al. 2005).

NhaA with its 12 transmembrane segments (TMSs) forms a cytoplasmic funnel and periplasmic funnel that are separated by a barrier made of hydrophobic amino acids as shown in Figure 2.18, A. A negatively charged funnel consisting of TMSs II, IX, IV and V forms the cytoplasmic funnel and the periplasmic funnel is made by TMSs II, VIII and XI (Hunte, Screpanti et al. 2005). The ionizable residues forming the pH sensor region is located at the entry site of the cytoplasmic funnel and when these residues are mutated the pH profile but not the Na\(^+\)/H\(^+\) antiporter activity is affected. However, amino acids at the bottom of cytoplasmic funnel forms the active site and affect the ion translocation (Gerchman, Rimon et al. 1999; Venturi, Rimon et al. 2000; Tzubery, Rimon et al. 2004). The amino acid residues playing a role in pH sensing and ion translocation are shown in (Figure. 2.18, B). The monomeric units of NhaA are organized in a unique inverted topology repeats as shown in (Figure. 2.18, (C)) containing three TMSs (TMSs III, IV, V and TMSs X, XI, XII). Remarkably, one of the TMs in each repeat is interrupted by an extended chain in the middle of the membrane leaving two short segments oriented to periplasm and cytoplasm respectively (Hunte, Screpanti et al. 2005).

The antiporter follows the classical alternating access mechanism. Cryo-electron microscopy of NhaA 2D crystals has found a two-step conformational change in response
to pH or substrate ions. As the pH rise from 6 to 7, the first transition occurs from inactive to pH-activated state. The pH activation causes the enlargement of the NhaA monomer and slight rearrangement of the N-terminus. The second conformational change occurs in the response to substrate (Na\(^+\) or Li\(^+\)) at pH above 7 and causes movement of transmembrane IV leading to a charge imbalance at the ion binding site and prompting the release of substrate ion and open periplasmic funnel (Appel, Hizlan et al. 2009). The pH and substrate-induced conformational changes are shown in Figure 2.18, D.

Figure 2.18. Overall structural and functional organization of \textit{E. coli} NhaA (Na\(^+\)/H\(^+\) antiporter). (A) A ribbon representation of the overall architecture of NhaA having 12-transmembrane domains is shown as viewed parallel to the membrane. The funnels (black ellipses), novel fold showing the interrupted helices (green) and parts of transmembrane II and IX lining the cytoplasmic funnel (magenta) are represented. (B) The site-directed mutagenesis of amino acids shown in magenta result in failure in the pH response, yellow color represents the amino acids affecting both the pH response and ion translocation (yellow) and black color residues only affect the translocation process. (C) The inverted repeat formed by the transmembranes (TMSs) III, IV, and V and TMSs X, XI and XII is shown. (D) The conformational switch occurring due to pH change and ions transport is shown. The Figure was reproduced with the permission from nature publishing group: Nature review Microbiology (Krulwich, Sachs et al. 2011).
NhaA also has the ability to use lithium (Li+) as its substrate for its transport activity. In general, Li+ binding to NhaA causes the displacement of two proton from the binding site. Mutational analysis showed that Li+ binding, H+ release, and antiporter activity get affected to similar magnitude when Li+ binding site residues (D163E, D163N, D164N, and D164E) are mutated. However, mutation of D133C causes the stoichiometry of Li+/H+ antiport to change to 1:4. Recently, the isothermal titration calorimetry studies have shown that the Li+/H+ binding to NhaA is antagonistically coupled. However, the antagonistic binding of NhaA can be changed to synergistic binding by the mutation of A167P, which has so far been only reported in the Cl-/H+ antiporter (Dwivedi, Sukenik et al. 2016). Expression analysis studies utilizing a LacZ fusion have shown that nhaA expression is induced by the presence of Na+ and Li+ ions in the medium thereby reflecting its necessity in adaptation to high salt and alkaline pH in E. coli (Karpel, Alon et al. 1991). Furthermore, Na+ induced nhaA expression is positively regulated by NhaR, a homolog of bacterial regulatory protein LysR, and has also affected the expression of nhaA (Dover, Higgins et al. 1996).

2.3.2 NhaB, second Na+/H+ antiporter of E. coli: NhaB-like antiporters belong to a second class of Na+/H+ antiporters and were recognized in bacteria more than 20 years ago. Although nhaB-like gene sequences have been found in many different bacterial genomes, only a few of them have been functionally characterized till now. Previous studies have identified a few pH-sensitive and -insensitive NhaB-like antiporters, but the mechanisms that regulate their pH responses still remain unknown (Kiriyama, Honma et al. 2013). In E. coli, NhaB is an IM protein having 504 amino acids that also works as cation/proton antiporter. Although NhaA and NhaB do not show noticeable sequence similarity, they
have similar putative secondary structures of twelve transmembrane segments linked with hydrophilic loops (Pinner, Padan et al. 1992). Overexpression of NhaB from a plasmid can complement the ΔnhaA strain (NM81) to the toxic effects of Na⁺ and Li⁺, and show increased Na⁺/H⁺ antiporter activity. The deletion of the nhaB gene in strain EP431 revealed that nhaB is the housekeeping gene, which becomes essential only in the absence of nhaA (Pinner, Padan et al. 1992). EP431 lacks the Na⁺/H⁺ antiporter activity up to pH 8, where other antiporters like NhaA and ChaA become active, although NhaA supports the viability through its activity in the background for all ranges of pH and Na⁺ concentration (Thelen, Tsuchiya et al. 1991; Pinner, Kotler et al. 1993). The mutant named EP432 lacking both nhaA and nhaB is the most Na⁺ sensitive strain and was used for cloning the E. coli third antiporter chaA which has Na⁺ (Ca⁺²)/H⁺ activity (Ivey, Guffanti et al. 1993; Pinner, Kotler et al. 1993). The two triple knock out strains (named KNabc and TO114) having a deletion of nhaA, nhaB and chaA have been used to identify novel Na⁺(K⁺)/H⁺ antiporter genes from various bacteria (Ohyama, Igarashi et al. 1994; Padan and Schuldiner 1994; Nozaki, Inaba et al. 1996). NhaB displays very low pH-dependent antiporter activity, and thus it is necessary for Na⁺/H⁺ activity in the usual physiological pH range (Pinner, Padan et al. 1992). Kinetic and thermodynamic studies showed NhaB transport activity is electrogenic in nature with the stoichiometry of 2Na⁺/3H⁺ and thereby facilitates in cytoplasmic pH regulation in alkaline conditions (Pinner, Padan et al. 1994). Additionally, NhaB also helps in Li⁺ extrusion and protects the cell from lithium toxicity, however, to a lesser extent than NhaA (Inaba, Kuroda et al. 1994).
2.3.3 ChaA, third Na\(^+\)(Ca\(^{2+}\))/H\(^+\) antiporter of *E. coli*: ChaA is a cytoplasmic protein having 11 TMSs. ChaA was recognized as Na\(^+\)/H\(^+\) antiporter on the basis of its ability to complement the ΔnhaA ΔnhaB strain (EP432) when overexpressed from a multicopy plasmid (Ivey, Guffanti et al. 1993). Based on the biochemical analysis of everted membrane vesicles, ChaA also possesses Ca\(^{2+}\)/H\(^+\) antiporter activity and thus participates in calcium ion circulation at alkaline pH (Ohyama, Igarashi et al. 1994). Moreover, ChaA has pH dependent Na\(^+\)/H\(^+\) activity which increases as the pH becomes more alkaline (similar to NhaA on this note). Additionally, ChaA extrudes sodium ions primarily at an alkaline pH above 8 (Sakuma, Yamada et al. 1998). Mutational analysis has shown glutamate (E85) is important for the Na\(^+\)/H\(^+\) activity of ChaA (Fukaya, Tanaka et al. 2010). Promoter activity assays using a LacZ fusion have suggested that expression of chaA is upregulated by pH and osmolarity of the medium (Shijuku, Yamashino et al. 2002). ChaA can also act as a K\(^+\)/H\(^+\) antiporter and help *E. coli* in adaptation to K\(^+\) stress at alkaline pH and maintain K\(^+\) homeostasis (Radchenko, Tanaka et al. 2006).

2.3.4 MdfA as Na\(^+\)(K\(^+\))/H\(^+\) antiporter of *E. coli*: Apart from acting as a multidrug transporter, MdfA also provides alkaline pH tolerance to *E. coli* through its Na\(^+\)(K\(^+\))/H\(^+\) activity. The ΔmdfA mutant is sensitive to alkaline pH and overexpression of mdfA rescues the alkaline sensitivity phenotype (Lewinson, Padan et al. 2004). Biochemical studies using inverted vesicles showed that MdfA act as Na\(^+\)(K\(^+\))/H\(^+\) antiporter at alkaline pH. It was suggested that MdfA provides alkaline tolerance at pH> 9 at which NhaA does not function and MdfA utilizes K\(^+\) as the main cation for its activity (Lewinson, Padan et al. 2004).

2.3.5 MdtM as Na\(^+\)(K\(^+\))/H\(^+\) antiporter of *E. coli*: MdtM is the most recently reported cation/proton antiporter playing a role in *E. coli* alkaline pH homeostasis. It is a
homolog of MdfA having 12-transmembrane segments and also belongs to the major facilitator superfamily. Through its drug/H\(^+\) activity MdtM provides intrinsic resistance to multiple drugs including ethidium bromide, chloramphenicol and quaternary compounds in \textit{E. coli} (Holdsworth and Law 2012; Holdsworth and Law 2013). The deletion of \textit{mdtM} causes alkaline pH sensitivity and millimolar concentrations of Na\(^+\) or K\(^+\) is required for MdtM mediated alkaline pH tolerance. Moreover, biochemical studies using inverted vesicles showed MdtM is a low-affinity antiporter that catalyzes electrogenic transport of Na\(^+\), K\(^+\), Rb\(^+\) or Li\(^+\) in exchange for H\(^+\) (Holdsworth and Law 2013).

2.4 References


3.1 Introduction

Bacterial multidrug resistance to biocides and antibiotics is increasing at a worrying speed and is a serious threat to the human health; therefore, there is a critical requirement to discover ways for battling the bacterial multidrug resistance. Mechanisms used by bacteria for multidrug resistance include antibiotic inactivation, biofilm formation, target alteration, membrane modification, and efflux by drug pumps (Khameneh, Diab et al. 2016). Resistance is often conferred by expression of efflux pumps that can accommodate numerous unrelated drugs (Krulwich, Lewinson et al. 2005; Piddock 2006). There are a plethora of efflux pumps present in Gram-negative bacteria that transport a wide range of structurally different compounds including antibiotics, dyes, out of the bacterial cell thereby allowing bacteria to survive at higher drug concentration (Blair, Richmond et al. 2014). Efflux pumps belonging to the major facilitator superfamily (MFS), small multidrug resistance (SMR) family and ABC transporter families are found widespread throughout evolution. The study of these efflux pumps is of significant importance, not only for understanding the function of efflux pumps, and its role in drug resistance but also as targets for the development of the novel drugs (Mahmood, Jamshidi et al. 2016). While the functions of these protein families have been studied in detail, genome sequencing

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projects have revealed the existence of many families of membrane proteins whose functions are not well understood (Bernsel and Daley 2009).

DedA is a large superfamily of membrane proteins found within all three domains of life and is present in nearly all sequenced bacterial genomes (Doerrler, Sikdar et al. 2013). Protein databases do not classify the DedA family as membrane transporters. They bear no amino acid similarity to the MFS, SMR family, ABC transporters, ion channels or other types of transporters. They bear limited similarity to the LeuT family of transporters when analyzed using a novel type of evolutionary analysis called AlignMe (Khafizov, Staritzbichler et al. 2010). The *Escherichia coli* genome encodes eight members of the DedA family displaying ~25-60% amino acid identity (annotated as *dedA*, *yqjA*, *yghB*, *yabI*, *yohD*, *yqaA*, *ydjX*, and *ydjZ*) (Blattner, Plunkett et al. 1997). *YqjA* and *YghB* are DedA family proteins of 220 and 219 amino acids, respectively, with 61% amino acid identity and partially redundant functions. Simultaneous in-frame deletion of these two DedA family genes in strain BC202 (W3110; Δ*yghB, ΔyqjA*) results in numerous phenotypes including temperature sensitivity (Thompkins, Chattopadhyay et al. 2008), cell division defects due to inefficient export of periplasmic amidases by the twin arginine pathway (Sikdar and Doerrler 2010), activation of envelope stress response pathways and compromised membrane protonmotive force (PMF) (Sikdar, Simmons et al. 2013). Viability at elevated temperatures and normal cell division can be restored to BC202 by growth at pH 6.0 (Sikdar, Simmons et al. 2013), overexpression of a subset of DedA family genes (Thompkins, Chattopadhyay et al. 2008; Boughner and Doerrler 2012) or *mdfA* (Sikdar, Simmons et al. 2013), encoding a Na\(^+\)-K\(^+\)/H\(^+\) antiporter belonging to the MFS (Kruilwich, Sachs et al. 2011). These results suggest roles for *YqjA/YghB* in the
maintenance of the pH gradient (ΔpH) component of the protonmotive force (PMF), composed of both the electrical potential (ΔΨ) and the ΔpH (Krulwich, Sachs et al. 2011). The ΔyqjA mutant is sensitive to alkaline pH (Price and Raivio 2009) and yqjA expression is repressed at low pH (Maurer, Yohannes et al. 2005), suggesting that one function of YqjA may be to promote alkaline tolerance. Similar functions have been shown for E. coli MdfA and Bacillus subtilis Tet(L) (Cheng, Guffanti et al. 1996; Lewinson, Padan et al. 2004; Krulwich, Lewinson et al. 2005). A role for YqjA/YghB in PMF maintenance is also supported by our own direct measurements of ΔΨ in BC202 using the JC-1 dye-based assay (Sikdar, Simmons et al. 2013).

In addition to functioning as an ion transporter, MdfA exports numerous cationic and zwitterionic lipophilic compounds (Lewinson, Adler et al. 2003). Overexpression of MdfA increases resistance of E. coli to a wide variety of structurally diverse drugs and biocides (Edgar and Bibi 1997) and ΔmdfA mutants are sensitive to exposure to such compounds (Tal and Schuldiner 2009). Given the ability of MdfA to correct the observed phenotypes of BC202, we measured the growth of BC202 and its parent strain W3110 in the presence of biocidal drugs and dyes belonging to different classes. We found that BC202 is hypersensitive to a number of structurally diverse compounds. Resistance can be restored to BC202 by expression of DedA family genes yghB or yqjA. Mutation of membrane-embedded acidic amino acids compromised the ability of YghB and YqiA to restore resistance to BC202 suggesting that these E. coli membrane proteins are possibly a new class of membrane proton-dependent transporters.
3.2 Materials and Methods

Materials: All chemicals were reagent grade and purchased from Sigma-Aldrich or VWR. Restriction enzymes, DNA polymerases, and T4 DNA ligase were purchased from New England BioLabs. The following compounds were used for the susceptibility testing and their classes/mode of action and sources are represented in parenthesis: chloramphenicol (Sigma-Aldrich), erythromycin (macrolide; Sigma-Aldrich), ciprofloxacin (fluoroquinolone; Sigma-Aldrich), norfloxacin (fluoroquinolone; Sigma-Aldrich), nalidixic acid (quinolone precursor; Sigma-Aldrich), sodium dodecyl sulfate (detergent; BioRad), acriflavine hydrochloride (intercalator; Acros Organics), ethidium bromide (intercalator; Amresco), cetyltrimethyl-ammonium bromide (quaternary ammonium compound; Amresco), benzalkonium chloride (quaternary amino compound; Alfa Aesar), methyl viologen (redox cycling drug, Acros Organics), Oxacillin (penicillin, Sigma), Cloxacillin (Penicillin, sigma), Piperacillin (Penicillin, TCI America).

Table 3.1: E. coli Strains and plasmids in this study:

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Plasmids

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</tr>
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<td>BB0250(E39A)-GFP expression, lac promoter. Amp&lt;sup&gt;R&lt;/sup&gt;</td>
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Table 3.2 Primers used in the study

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<tr>
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Bacterial growth conditions: Bacterial cultures were grown in Luria-Bertani broth (LB) (1% tryptone, 0.5% yeast extract and 1% NaCl) unless otherwise stated. LB media of pH 6.0 was buffered with 100 mM 2-(N-morpholino)-ethanesulfonic acid (MES) (Sikdar, Simmons et al. 2013). Solid media were obtained by addition of 1.5 % agar (w/v). When required, the media was supplemented with ampicillin (Amp) 100 µg/ml, kanamycin (Kan) 30 µg/ml, tetracycline (Tet) 12.5 µg/ml, chloramphenicol (Cam) 30 µg/ml, arabinose (0.002% or 0.1%, w/v) or isopropyl-1-thio-β-D-galactopyranoside (IPTG; 0.5 mM). All cultures were grown at 30 °C unless otherwise indicated.

Strain construction: The multiple-deletion mutants were generated in E. coli W3110 and correct configuration was verified through PCR with primers flanking the appropriate gene. In order to eliminate the kanamycin-resistance (kanR) gene from BC202, plasmid pCP20 expressing FLP recombinase was used as described previously (Cherepanov and Wackernagel 1995). Since this protocol requires overnight incubation at 42 °C to cure pCP20 (nonpermissive for BC202), the yghB::kanR strain was first made markerless (W3110 background). YqjA::tetR (Thompkins, Chattopadhyay et al. 2008) was then introduced by P1 transduction. Additional mutations (i.e. ΔemrE::kanR, ΔmdfA::kanR, or ΔacrB::kanR) were introduced by P1 transduction with P1vir lysate prepared from the indicated E. coli strains (Table 3.1) obtained from the Keio collection (Baba, Ara et al. 2006). P1 transductions were carried out as described (Silhavy, Berman et al. 1984). All strains and genotypes are listed in Table 3.1.

Microscopy: Overnight cultures of E. coli strains were diluted 1:100 in fresh LB media with suitable antibiotics and additives, and grown to OD600 ~ 0.6 at 30 °C in a shaking incubator. Cells were resuspended to final OD600 1.0 in LB and 10 µL of cells were
applied to a 1% agarose coated glass slide for imaging. A Leica DM-RXA2 deconvolution microscope was used for all the Differential Interference Contrast (DIC) micrographs. Observations were made by a 100X, 1.30-numerical-aperture oil immersion objective lens. The images were captured through a DIC filter by a cooled Cooke SensiCamQE 12 bit, 1, 3-megapixel, charge-coupled device digital camera and recorded using Slidebook software (Intelligent Imaging Innovation, Denver, CO).

Susceptibility to toxic compounds: For testing the susceptibility on solid medium, overnight cultures of *E. coli* strains were freshly diluted 1:100 in LB media with appropriate antibiotics and additives, and grown to OD\(_{600}\) ~ 0.6 at 30°C in a shaking incubator. 5 μl of serially log\(_{10}-\)diluted cells were spotted on LB agar plates containing various concentrations of corresponding biocides. Growth was analyzed after incubation for 20 to 24 hours at 30 °C. All experiments were repeated at least three times.

Minimal inhibitory concentration (MIC) determination: The MICs of biocides were carried out using a two-fold dilution technique in either 1.5 x 15 cm glass tube or in 96-well microtiter plates in liquid medium (Wiegand, Hilpert et al. 2008). Overnight cultures were freshly diluted 1:100 into LB medium with suitable antibiotics and additives. Exponentially growing cultures at OD\(_{600}\) ~1.0 were inoculated at a density of 10\(^5\) cells per ml into LB medium supplemented with a series of two-fold dilutions of indicated biocide. Cell growth was determined visually after incubation at 30°C for 24 hours. All experiments were repeated at least three times.

Site-directed mutagenesis: Site-specific mutants were created by using previously published protocol (Zheng, Baumann et al. 2004). The primers carrying the site-specific mutations (Table 3.2) were used in a PCR reaction to amplify a vector containing the
specified wild type gene. The PCR product was then digested with \textit{DpnI} and used to transform competent XL1-blue cells. Colonies obtained after transformation were screened by colony PCR using gene-specific primers. The mutations confirmed by DNA sequencing conducted at the LSU College of Science Genomics Facility.

Membrane preparation and Western blotting: Cell membranes was prepared from exponential phase cultures of BC202 containing appropriate plasmid DNA grown under inducing condition (arabinose or IPTG) using a previously published protocol (Doerrler and Raetz 2002). Equal amounts of protein were resolved by 12% SDS-PAGE and transfer to PVDF. Western blotting was done using penta-His (Qiagen) or GFP (JL-8; Clontech) primary antibody at 1:5000 dilution and goat-anti-mouse horseradish peroxidase (HRP) conjugated secondary antibody (Pierce) at 1:5000. Detection was carried out using the ImmunStar HRP kit (Bio-Rad).

3.3 Results

\textit{A DedA family mutant is highly sensitive to a number of biocides.} Ethidium bromide (EthBr) efflux is a hallmark of several bacterial organic cation transporters including MdfA (Lewinson, Adler et al. 2003; Lewinson, Padan et al. 2004), EmrE (Purewal 1991) and QacA (Brown and Skurray 2001). We tested for sensitivity to EthBr using a standard growth assay and found that BC202 is significantly more sensitive than parent strain W3110 to this cationic dye (Figure.3.1A). We also observed increased sensitivity of BC202 to biocides known to be exported by other drug efflux proteins (Sulavik, Houseweart et al. 2001; Tal and Schuldiner 2009; Lim and Nikaido 2010) including methyl viologen (resistance conferred by EmrE), Benzalkonium chloride (mainly MdfA), cetyltrimethyl ammonium bromide (mainly AcrAB), nalidixic acid (AcrAB),
acriflavine (EmrE, AcrAB) and β-lactam antibiotics (AcrAB) on solid (Figure 3.1, B, C) and/or liquid media (Figure 3.2).

Figure 3.1. Sensitivity of BC202, multidrug resistance mutants and combination mutants to select biocides. BC202 (ΔygHB, ΔyqJA) is sensitive to (A) ethidium bromide (EthBr), (B) methyl viologen (MV) and (C) acriflavine (Acr) while the corresponding multidrug resistance mutants display sensitivity approximately equal or less than BC202. MdfA promotes EthBr resistance, EmrE promotes MV resistance, and AcrB is required for acriflavine resistance (Tal and Schuldiner 2009). Combination mutants (multidrug resistance gene deletions in the BC202 genetic background) display additive or synergistic sensitivity (see also Table 1). (D) BC202 is not appreciably sensitive to SDS, chloramphenicol (CAM) or ciprofloxacin (Cipro), norfloxacin (Norflo) or erythromycin (Erythro) on solid LB plates. Dilutions of log phase cells of the indicated genotype were spotted onto LB plates containing the indicated concentrations of biocide. All strains grew equally well on LB plates with no added biocide.
Figure 3.2. Minimal inhibitory concentration (MIC) values for *Escherichia coli* strains. W3110 (parent), BC202, and efflux pump mutations in either the W3110 (ΔmdfA, ΔemrE and ΔacrB) or BC202 background strains (BC202M, BC202E and BC202A) were grown in liquid culture in the presence of a series of dilutions of the indicated compounds. In strain BC202, increased sensitivity to the indicated compounds is shown in bold. In strains BC202M, BC202E and BC202A additive or synergistic effects are shown in bold. Abbreviations (units are µg/ml unless indicated in parentheses): SDS, sodium dodecyl sulfate (%); CAM, chloramphenicol; BC, benzalkonium chloride; NA, nalidixic acid; CTAB, cetyl trimethyl ammonium bromide; MV, methyl viologen; Acr, acriflavine hydrochloride; EthBr, ethidium bromide; Erythro, erythromycin; Cipro, ciprofloxacin (ng/ml); Norflox, norfloxacin (ng/ml); Ox, Oxacillin; Clox, cloxacillin; Pip, piperacillin. *BC202 was not sensitive to erythromycin, ciprofloxacin and norfloxacin on plates (Figure 3.1) but was slightly sensitive in liquid culture.

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We have tested BC202 for sensitivity to many antibiotics and other compounds (Thompkins, Chattopadhyay et al. 2008) and found BC202 is not generally hypersensitive to all biocides. For example, BC202 is not sensitive to SDS indicating that the outer membrane is intact in this strain or, intriguingly, chloramphenicol- a known substrate of the MdfA efflux pump (Figure. 3.1, D, Figure 3.2). Remarkably, BC202 is sensitive to concentrations of EthBr that are roughly ten-fold lower than what is needed to kill an ΔmdfA mutant (Figure 3.1, A and Figure 3.2). Expression of either yqjA or yghB individually was capable of restoring resistance to BC202 indicating these proteins have redundant functions (Figure. 3.3). These results indicate that YqjA and YghB are either directly involved in drug efflux or are required for proper function of other membrane transporters.

Figure 3.3. Expression of yqjA and/or yghB restores drug resistance to BC202. BC202 was transformed with plasmids pACYC184, pBADHisA, pACYC-yghB or pBAD-yqjA in all combinations and spotted on LB plates containing (A) no additives (B) CTAB, cetyl trimethyl ammonium bromide, 25 µg/ml (C) acriflavine, 25 µg/ml (D) MV, methyl viologen 20 µg/ml or (E) BC, benzalkonium chloride 20 µg/ml. Plates were incubated overnight at 30 °C.
Deletion of known efflux pumps causes an increase in BC202 sensitivity. Introducing deletions of known transporters into BC202 consistently resulted in a marked increase in drug sensitivity on both solid and liquid media (Figure 3.1, A-C; Figure 3.2). MdfA and EmrE have been proposed to be the major contributors to EthBr resistance of E. coli (Nishino and Yamaguchi 2001). The presence of either the ΔmdfA or ΔemrE mutation in the BC202 background strain results in additional sensitivity, suggesting independent resistance mechanisms by these protein families. We see the same pattern with other biocides and the mutants of the corresponding export proteins involved in their efflux including EthBr, acriflavine and methyl viologen (Figure 3.1, A-C; Figure 3.2). Strikingly, the presence of the ΔacrB deletion in BC202 results in a nearly synergistic increase in sensitivity to a number of compounds including SDS, many-fold higher than what is observed in either background individually (Figure 3.2). These results suggest that the effect on drug sensitivity of these DedA family deletions is independent of other known drug transporters.

Membrane embedded acidic amino acids are essential for function of DedA family members. In many multi-drug resistance (MDR) transporters, including MdfA, TetL, and EmrE, it has been demonstrated that membrane embedded acidic residues such as glutamic acid (E) and aspartic acid (D) play a role in PMF-dependent drug transport (Table 3.4) (Edgar and Bibi 1999; Soskine, Adam et al. 2004; Seeger, von Ballmoos et al. 2009; Sigal, Fluman et al. 2009; Fluman, Ryan et al. 2012). E26 and D34 both play roles in proton translocation in exchange of substrate by MdfA (Fluman, Ryan et al. 2012). Many DedA proteins contain a predicted membrane-embedded or membrane-proximal glutamic acid
and/or aspartic acid in the first transmembrane (TM) spanning region (E39 and D51 in YqjA and YghB).

<table>
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<td>DedA</td>
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<td>NP417482</td>
<td>DedA</td>
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<td>VFFSLILAGLNPSEDAILVM**</td>
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Figure 3.4. Membrane-embedded acidic amino acids in TM1 of DedA family proteins and select drug transport proteins. Conserved acidic amino acids are shown in red. Database sequences were analyzed for predicted membrane domains using SOSUI (Hirokawa, Boon-Chieng et al. 1998). Table is for illustration purposes only and is not a complete list of transport proteins. Abbreviations: TM1, transmembrane region 1; MFS, major facilitator superfamily; SMR, small multidrug resistance. *YghB D51 is predicted to lie just outside TM1, but is also required for function. **BB0250 is an essential DedA family protein of *Borrelia burgdorferi* (Liang, Xu et al. 2010). E39 and D40 are each required for BB0250 to complement growth and cell division defects of BC202. ***E26 and D34 both play roles in transport by MdfA (Fluman, Ryan et al. 2012)

We performed site-directed mutagenesis on these residues and found the YqjA point mutants (YqjA-E39A, YqjA-D51A) no longer restore growth at 42 °C (Figure. 3.5, A), cell division (Figure. 3.5, B) or drug resistance (Figure. 3.5, C) when expressed in BC202. Protein expression levels were equivalent to wild type levels in purified membranes (Figure. 3.5, D) suggesting that the mutant proteins were correctly folded. Wild type yqjA completely restored growth and cell division (Figure. 3.5, A and B), in agreement with previous reports (Thompkins, Chattopadhyay et al. 2008; Boughner and Doerrler
2012), as well as drug resistance to BC202 (Figure 3.5, C). To further support this data, we created isosteric substitutions at positions E39 and D51 in YqjA, converting these residues to glutamine and asparagine, respectively.

Figure 3.5. Mutation of YqjA E39 or D51 abolishes ability to restore growth, cell division or drug resistance to BC202. (A) BC202 transformed with pBAD, or pBAD-yqjA, pBAD-yqjA(E39A), or pBAD-yqjA(D51A) was grown at 30 °C (left) or 42 °C (center and right). Wild type yqjA but not point mutants restores growth to BC202 at 42 °C. BC202/pBAD-yqjA(D51A) also grew at 30 °C (not shown). (B) Micrographs of BC202 transformed with pBAD, or pBAD-yqjA, pBAD-yqjA(E39A), or pBAD-yqjA(D51A) grown at 30 °C. Wild type yqjA but not point mutants restores normal cell division to BC202. Bar = 10 μm. (C) Sensitivity of BC202 transformed with control vector, cloned yqjA, yqjA(E39A), or yqjA(D51A) to EthBr (75 μg/ml), acriflavine (25 μg/ml), nalidixic acid (0.5 μg/ml) and methyl viologen (25 μg/ml). All strains grew on LB plates without biocides at 30 °C. Wild type yqjA but not point mutants restores drug resistance to BC202 at 30 °C. (D) Expression of yqjA, yqjA(E39A) and yqjA(D51A) in membrane fractions as determined by Western blotting with anti-hexahistidine antibody. All growth with pBAD-yqjA was carried out in LB/amp/0.002% arabinose.
YqjA-E39Q and YqjA-D51N were unable to restore drug resistance (Figure 3.6, A), growth at 42 °C or normal cell division (Figure 3.6, C,D) in spite of membrane expression levels similar to wild-type (Figure 3.6, B).

Figure 3.6. Isosteric mutations of YqjA E39 or D51 abolish ability to restore drug resistance, growth and cell division to BC202. (A) Sensitivity of BC202 transformed with control vector pBAD, cloned yqjA, yqjA(E39Q), or yqjA(D51N) to Benzalkonium chloride (20 µg/ml), acriflavine (25 µg/ml), CTAB (100 µg/ml), EthBr (75 µg/ml) and methyl viologen (25 µg/ml). All strains grew on LB plates without biocides at 30 °C. Wild type yqjA but not point mutants restores drug resistance to BC202 at 30 °C. (B) Expression of yqjA, yqjA(E39Q) and yqjA(D51N) in membrane fractions as determined by Western blotting with anti-hexahistidine antibody. All growth with pBAD-yqjA was carried out in LB/amp/0.002% arabinose. (C) BC202 transformed with pBAD, or pBAD-yqjA, pBAD-yqjA(E39Q), or pBAD-yqjA(D51N) was grown at 30 °C (left) or 42 °C (right). Wild type YqjA but not point mutants restores growth to BC202 at 42 °C. (D) Micrographs of BC202 transformed with pBAD, or pBAD-yqjA, pBAD-yqjA(E39Q), or pBAD-yqjA(D51N) grown at 30 °C. Wild type yqjA but not point mutants restores normal cell division to BC202. Bar = 10 µm.
Similar observations were made with YghB and its corresponding point mutants YghB-E39A and YghB-D51A (Figure. 3.7). The presence of membrane embedded acidic amino acids that are essential for activity strongly supports the notion that YqjA and YghB are proton dependent transporters.

Figure 3.7. Inability of YghB E39A or D51A point mutants to restore growth, cell division or drug resistance to BC202. (A) BC202 was transformed with empty vector pBADHisA, pBAD-yqjA, pBAD-yghB(E39A) or pBAD-yghB(D51A) and grown at 30 or 42 °C. BC202/pBAD-yghB(D51A) also grew at 30 °C (not shown). (B) BC202 was transformed with empty vector, pBAD-yqjA, pBAD-yghB(E39A) or pBAD-yghB(D51A) and grown at 30 °C in liquid media. Cells were visualized using a with a Leica DM-RXA2 deconvolution microscope. Wild type yghB, included as a positive control, could restore both growth and cell division to BC202, in agreement with our previously published results (Thompkins, Chattopadhyay et al. 2008; Boughner and Doerrler 2012). Bar = 10 μm. (C) Sensitivity of BC202 with control vector, cloned yghB, yghB(E39A), yghB(D51A) or mdfA to EthBr (75 μg/ml), acriflavine (25 μg/ml), cetyltrimethyl-ammonium bromide (CTAB) (100 μg/ml) and methyl viologen (25 μg/ml). All strains grew on LB plates without biocides at 30 °C. Wild type yghB or mdfA but not yghB point mutants restores drug resistance to BC202 at 30 °C. (D) Expression of yghB, yghB(E39A) and yghB(D51A) in membrane fractions as determined by Western blotting with anti-hexahistidine antibody. All growth with pBAD-yghB was carried out in LB/amp/0.002% arabinose. This lower concentration of inducer was used due to toxicity observed when overexpressing yghB in 0.1% arabinose.
We were interested in determining whether these properties applied to DedA family members found in other bacterial species. BB0250 is the sole DedA family protein of the Lyme disease pathogen *Borrelia burgdorferi* and is essential for viability (Liang, Xu et al. 2010). Depletion of BB0250 in *B. burgdorferi* results in cell death preceded by defects in cell division. In other words, the borrelia mutant phenotypes resemble those of BC202, with the exception being that *bb0250* is essential at all temperatures.

![Figure 3.8](image_url)

Figure 3.8. Inability of *Borrelia burgdorferi* BB0250 E39A and D40A point mutants to restore growth, cell division or drug resistance to BC202. (A) BC202 was transformed with empty vector pBB0, pBB0250-GFP, pBB0250 (E39A)-GFP or pBB0250 (D40A)-GFP and grown at 30 or 42 °C. BC202/pBB0250(D40A)-GFP also grew at 30 °C (not shown). (B) BC202 was transformed with empty vector, pBB0250-GFP, pBB0250 (E39A)-GFP or pBB0250 (D40A)-GFP and grown at 30 °C in liquid media. Cells were visualized using a with a Leica DM-RXA2 deconvolution microscope. Bar = 10 µm. Wild type *bb0250*, included as a positive control, could restore both growth and cell division to BC202, in agreement with our previously published results (Liang, Xu et al. 2010). (C) Sensitivity of BC202 transformed with control vector, pBB0250-GFP, pBB0250 (E39A)-GFP or pBB0250(D40A) to EthBr (50 µg/ml), acriflavine (25 µg/ml), cetyltrimethyl-ammonium bromide (CTAB) (100 µg/ml) and methyl viologen (30 µg/ml). All strains grew on LB plates without biocides at 30 °C. Wild type *bb0250* but not *bb0250* point mutants restores drug resistance to BC202 at 30 °C. (D) Expression of *bb0250*, *bb0250*(E39A) and *bb0250*(D40A) in membrane fractions as determined by Western blotting with anti-GFP antibody. All growth was carried out in LB/amp/0.5 mM IPTG.
In addition, cloned bb0250 can fully complement the growth and cell division phenotypes of *E. coli* BC202 even though BB0250 displays only ~19% amino acid identity to *E. coli* YqjA. BB0250 also possesses acidic amino acids within its first predicted transmembrane domain (Figure 3.4). We performed site-directed mutagenesis on residues E39 and D40 in TM1 of BB0250 and show that each BB0250 point mutant is unable to restore cell division, temperature sensitivity and drug resistance to BC202 (Figure. 3.8, A-C) while also being expressed at wild type levels (Figure. 3.8, D). This data collectively suggests that members of the larger DedA family are likely proton-dependent membrane transporters necessary for resistance to a number of structurally diverse compounds in *E. coli* and likely other species of bacteria.

*Artificially increasing PMF restores drug resistance to BC202.* One potential explanation for these results is that YqjA/YghB are themselves drug efflux pumps. Alternatively, these DedA family proteins may indirectly affect the ability of other efflux pumps to function by compromising the proton motive force (PMF). A role for YqjA/YghB in PMF maintenance is supported by our own published data showing correction of BC202 growth and cell division by growth at acidic pH or by overexpression of the Na\(^+\)-K\(^+\)/H\(^+\) antiporter MdfA (Sikdar, Simmons et al. 2013). PMF is also required for proper functioning of the twin arginine transport pathway (Palmer and Berks 2012), which functions inefficiently in BC202 leading to the observed cell division defect (Sikdar and Doerrler 2010). To directly test this question, we expressed *mdfA* from a plasmid in BC202 and tested its sensitivity to the collection of biocides to which it is sensitive. If *mdfA* overexpression corrects sensitivity to all drugs, this would support a role for YqjA/YghB in PMF maintenance, which is indirectly affecting the drug export mechanism. We found
that expression of *mdfA* corrects sensitivity of BC202 to all drugs, including those that are not transported directly by MdfA (i.e. acrifavine, methyl viologen and nalidixic acid) (Figure. 3.9, A). Growth at pH 6.0 (increasing the ΔpH component of the PMF) also restores drug resistance to BC202 (Figure. 3.9, B and Figure 3.10) indicating the sensitivity is likely due to loss of PMF.

Figure 3.9. *MdfA* overexpression or Growth at pH 6.0 restores drug resistance to BC202. (A) BC202 harboring vector alone or pBAD-MdfA was spotted on plates containing LB (Amp, 0.1% arabinose) alone or this media supplemented with 20 μg/ml benzalkonium chloride (BC; exported by MdfA), 1 μg/ml nalidixic acid (NA, exported by AcrAB), 30 μg/ml methyl viologen (MV, exported by EmrE), 25 μg/ml acriflavine (Acr, exported by AcrAB), 100 μg/ml cetyl trimethyl ammonium bromide (CTAB, mainly exported by AcrAB) or 50 μg/ml ethidium bromide (EthBr, exported by MdfA, EmrE and others). (B) BC202 and parent W3110 was spotted on LB plates containing buffered to pH 6.0 or 7.0 containing no additive, 10 μg/ml BC, 1μg/ml NA, 25 μg/ml MV, 25 μg/ml Acr, 50 μg/ml CTAB or 100 μg/ml EthBr. LB media was buffered at pH 6.0 or 7.0 as described (Sikdar, Simmons et al. 2013). Nalidixic acid was toxic to both strains at pH 6.0 for reasons unknown.
Figure 3.10. Minimal inhibitory concentration (MIC) values of W3110 and BC202 in LB media at pH 6.0. The pH of LB media was adjusted to pH 6 as described in materials and methods. Abbreviations are as for Table 1 and all units are μg/ml. BC, Benzalkonium chloride; NA, nalidixic acid; CTAB, Cetyl trimethyl ammonium bromide; MV, methyl viologen; Acr, acriflavine hydrochloride; EthBr, ethidium bromide.

Growth in the presence of 400 mM NaCl or 10 mM Mg^{++}, both conditions that restore growth and cell division to BC202 (Thompkins, Chattopadhyay et al. 2008; Sikdar, Simmons et al. 2013), also restores BC202 drug resistance to most compounds tested (Figure 3.11). Each of these conditions that restore resistance (mdfA expression, growth at pH 6.0 or in the presence of 400 mM NaCl or 10 mM Mg^{++}) likely assists in restoring pH homeostasis to BC202 (see discussion and (Sikdar, Simmons et al. 2013)).

3.4 Discussion

Members of the DedA family of membrane proteins are found in the vast majority of sequenced bacterial genomes (Doerrler, Sikdar et al. 2013) and there are virtually thousands of DedA members in the protein databases. While it has been reported that members of this family are evolutionarily related to the LeuT superfamily of transporters (Khafizov, Staritzbichler et al. 2010), they have never been defined as true membrane transporters. Deletion of functionally redundant genes yqjA and yghB in strain BC202 causes a number of pleiotropic effects on *E. coli.*
Figure 3.11. Growth on LB media containing high salt or 10 mM MgCl$_2$ restores drug resistance to BC202. BC202 and parent W3110 was spotted on plates containing LB (normally containing 170 mM NaCl), LB containing a total of 400 mM NaCl, or LB with 170 mM NaCl and 10 mM MgCl$_2$. Each of these growth media was used alone or additionally supplemented with 10 µg/ml benzalkonium chloride (BC), 1 µg/ml nalidixic acid (NA), 25 µg/ml methyl viologen (MV), 25 µg/ml acriflavine (Acr), 50 µg/ml cetyl trimethyl ammonium bromide (CTAB) or 100 µg/ml ethidium bromide (EthBr). The control plates grown on LB in the left column are identical to the controls shown in Figure 3B from the main article. 400 mM NaCl did not restore resistance to 50 µg/ml CTAB for reasons that are not clear. Plates were incubated at 30 ºC overnight.
BC202 is unable to grow at elevated temperatures or complete cell division (Thompkins, Chattopadhyay et al. 2008; Sikdar and Doerrler 2010) and activates numerous envelope stress responses (Sikdar, Simmons et al. 2013). In this work, we show that BC202 is highly sensitive to several cationic biocides that are known substrates of PMF dependent efflux pumps. It appears that loss of YqjA and YghB may indirectly cause massive drug sensitivity due to impairment of the PMF in BC202. This is likely the reason that Salmonella ΔyqjA mutants are sensitive to magainin (Shi, Cromie et al. 2004), a cationic antimicrobial peptide that likely disrupts the membrane PMF (Westerhoff, Juretic et al. 1989). Moreover, recently it is shown that ΔyqjA is also sensitive to a model cationic antimicrobial peptide (CAMP), protamine (Weatherspoon-Griffin, Yang et al. 2014). In addition, PMF disrupting lipopeptides have recently been reported to sensitize E. coli to a number of antibiotics (Goldberg, Sarig et al. 2013). Overexpression of DedA family genes in BC202 does not by itself increase resistance to any of the biocides tested here beyond the level of resistance seen in parent strain W3110. However, the essentiality of membrane embedded amino acids strongly suggests that DedA proteins themselves are proton dependent transporters as similar acidic amino acids are found in many other classes of such transporters. In the case of MdfA, it is has been shown that acidic amino acids E26 and D34 play roles in proton translocation during substrate transport (Fluman, Ryan et al. 2012). It is possible that such amino acids serve a similar function for DedA family proteins. Whether this family also functions as antiporters and the nature of the transported substrate(s) is currently unknown.

Growth at 42 °C, normal cell division and wild type drug resistance can be restored to BC202 by over expression of mdfA (Figure. 3.9, A) growth at pH 6.0 (Figure. 3.9, B) or
in the presence of 400 mM NaCl or 10 mM Mg$^{++}$ (Figure 3.11). (400 mM NaCl did not restore resistance to 50 μg/ml CTAB for reasons that are unclear.) It is likely that each of these conditions helps to restore an altered cytoplasmic pH. For example, MdfA is an antiporter that can provide proton influx in exchange for Na$^+$ or K$^+$ (Lewinson, Padan et al. 2004). In a similar manner, media buffered to pH 6.0 results in an increased ΔpH component of the PMF. An increase in extracellular Mg$^{++}$ can enhance membrane integrity and can also inhibit the activity of the F$_0$/F$_1$ proton extruding ATPase (Bulygin and Vinogradov 1991) which is upregulated in response to alkaline growth conditions (Krulwich, Sachs et al. 2011). Finally, high levels of monovalent cations such as Na$^+$ or K$^+$ can stimulate a number of proton dependent antiporters such as NhaA and MdfA which play critical roles in cellular pH homeostasis (Lewinson, Padan et al. 2004; Mager, Rimon et al. 2011). Interestingly, the ability of 400 mM Na$^+$ or 10 mM Mg$^{++}$ to restore growth and cell division to BC202 is dependent upon an intact Cpx envelope stress response pathway suggesting that some Cpx-induced gene may be required for this effect (Sikdar, Simmons et al. 2013).

One of the more interesting properties of the DedA family that sets it apart from many membrane transporter families is its apparent essentiality. This is supported by several lines of evidence. First, the DedA family is collectively essential in E. coli (Boughner and Doerrler 2012) and essential DedA genes are found in Borrelia burgdorferi (BB0250) (Liang, Xu et al. 2010) and Caulobacter crescentus (CCNA_01607) (Christen, Abeliuk et al. 2011). Furthermore, expression of a DedA family gene from Mycobacterium bovis BCG (BCG2664) confers resistance to and may be the target of the anti-tuberculosis drug halicyclamine A (Arai, Liu et al. 2011). In this work, we show that members of the
DedA family are likely membrane transporters required for the normal function of several families of drug efflux pumps possibly due to their requirement for PMF maintenance. DedA family proteins are thus an intriguing class of membrane proteins and potential targets for anti-bacterial drug design.

3.5 References


CHAPTER 4
ESCHERICHIA COLI YqjA, A MEMBER OF THE CONSERVED DedA/Tvp38 MEMBRANE PROTEIN FAMILY, IS A PUTATIVE OSMOSENSING TRANSPORTER REQUIRED FOR GROWTH AT ALKALINE pH*

4.1 Introduction

Bacterial alkaline pH tolerance is a key feature of pathogenic, ecological and industrially important bacteria. Microbes have many naturally occurring alkaline habitats including the human body (Krulwich, Sachs et al. 2011). Neutralophilic bacteria including *Escherichia coli* and *Vibrio cholerae* can stay viable in alkaline marine environments and can cause threats to public health (Herz, Vimont et al. 2003). They have the ability to maintain their intracellular pH in range of 7.5-7.7 when grown within a wide range of pH values between 5.5 and 9.0. Cytoplasmic pH maintenance is vital for structural integrity and function of essential proteins needed for growth (Padan, Bibi et al. 2005; Krulwich, Sachs et al. 2011). In order to survive at alkaline pH, bacteria employ various strategies including increased expression and activity of cation/proton antiporters, which play a crucial role in alkaline pH homeostasis (Padan, Bibi et al. 2005). To date, five such antiporters (NhaA, NhaB, ChaA, MdfA, and MdtM) have been reported to function in alkaline pH homeostasis in *E. coli* (Pinner, Kotler et al. 1993; Lewinson, Padan et al. 2004; Padan, Bibi et al. 2005; Radchenko, Tanaka et al. 2006; Holdsworth and Law 2013). Inward active transport of protons by these antiporters is coupled to the export of cations such as Na⁺, K⁺ or Ca++.  

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The DedA/Tvp38 family is a highly conserved membrane protein family with the corresponding genes present in most sequenced genomes (Doerrler, Sikdar et al. 2013). However, the functions of these proteins are not well understood. YqjA and YghB are members of the DedA family sharing 62% amino acid identity and partially redundant functions. We have previously shown that an E. coli strain lacking these two genes (named BC202) is defective in twin arginine transport (Tat) pathway protein export and cell division (Thompkins, Chattopadhyay et al. 2008; Sikdar and Doerrler 2010), maintenance of the proton motive force (PMF) (Sikdar, Simmons et al. 2013), and is sensitive to a number of antibiotics and biocides normally subject to efflux in a PMF-dependent manner (Kumar and Doerrler 2014). Interestingly, all BC202 phenotypes appear to be corrected if the strain is grown in slightly acidified growth medium of pH 6.0 (Sikdar, Simmons et al. 2013; Kumar and Doerrler 2014).

Many DedA proteins contain membrane-embedded acidic amino acids in the first predicted transmembrane spanning region (Glu39 and Asp51 in both YqjA and YghB as predicted using SOSUI (Hirokawa, Boon-Chieng et al. 1998)). Such membrane acidic amino acids are found in many proton dependent transporters belonging to several different families (Edgar and Bibi 1999; Soskine, Adam et al. 2004; Seeger, von Ballmoos et al. 2009; Sigal, Fluman et al. 2009; Fluman, Ryan et al. 2012). We have also shown that DedA proteins require these acidic amino acids for their ability to complement the phenotypes of BC202 described above, suggesting they may represent a new family of proton dependent transporters (Kumar and Doerrler 2014).

In this study, we show that the E. coli ΔyqjA mutant (but not the ΔyghB mutant) is unable to grow at an external pH range of 8.5-9.5; unlike the parent E. coli strain.
Overexpression of \( yqjA \) from a plasmid restores growth at alkaline pH, but only if monoivalent cation sodium or potassium is supplied in the growth media. Moreover, acidic amino acids within the first transmembrane domain are functionally important for YqjA to support growth at pH>9. We also observed that YqjA could rescue growth of the mutant at elevated pH in the absence of any salt if grown in the presence of higher osmotic pressure provided by sucrose, mannitol or sorbitol, raising the possibility that YqjA may possess an osmosensing capability. Based upon these data, we suggest that YqjA is a newly identified proton-dependent transporter playing a significant role in alkaline pH homeostasis in \( E. coli \).

4.2 Materials and Methods

Bacterial growth conditions: Bacteria cultures were grown in LB medium (1% tryptone, 0.5% yeast extract and 1% NaCl) with antibiotics (ampicillin (Amp), 100 µg/ml, kanamycin (Kan) 30 µg/ml, tetracycline (Tet) 12.5 µg/ml where specified. In certain experiments growth media was additionally supplemented with 0.002% or 0.02% arabinose (w/v). Media were buffered with 70 mM BTP (Bis-Tris propane) and pH was adjusted as required with HCl. Cultures were grown at 37 °C in a shaking incubator unless otherwise indicated.

Strains and molecular techniques: All strains and plasmids are listed in Table 1. Mutations from Keio collection strains (Baba, Ara et al. 2006) were typically introduced into \( E. coli \) W3110 by P1 transduction (Silhavy, Berman et al. 1984) and correct configuration was verified through PCR and DNA sequencing with primers flanking the appropriate gene. DNA sequencing was conducted at the LSU College of Science Genomics Facility.
Alkaline pH sensitivity growth assay on solid medium: For testing the alkaline pH sensitivity on solid medium, overnight cultures of *E. coli* were freshly diluted 1:100 in LB media with appropriate antibiotics and additives, and grown to OD$_{600}$ ~ 0.6 at 37°C in a shaking incubator. 5 μl of serially log$_{10}$-diluted cells were spotted on LB agar plates of various pH’s with appropriate antibiotics and additives. For testing the effect of Na$^+$ and K$^+$ ions at alkaline pH on growth of bacteria salt free media (1% tryptone (w/v), 0.5% yeast extract (w/v)) was supplemented with different concentrations of salts as indicated. Although we refer to this media as “salt-free”, residual amounts (1-10mM) of both sodium and potassium are likely present (Ohyama, Igarashi et al. 1994; Radchenko, Tanaka et al. 2006). The bacterial cells were washed at least two times in salt free media before making dilutions and plating. Growth was analyzed after incubation for 20 to 24 hours at 37°C. All the experiments were repeated at least three times.

Table 4.1: Strain and plasmid in this study:

<table>
<thead>
<tr>
<th>Strains</th>
<th>Description</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>W3110</td>
<td>Wild Type, F$^{-}$λ-IN(rrnD-rrnE)1,rph-1</td>
<td><em>E. coli</em> genetic stock center, Yale University</td>
</tr>
<tr>
<td>BC203</td>
<td>W3110,ΔyqjA::TetR</td>
<td>Thompkins et al.(2008)</td>
</tr>
<tr>
<td>XL1 Blue</td>
<td>recA1 endA1 gyrA96 thi-1 hsdR17 supE44 relA1 lac [F’ proAB lacIqZAM15 Tn10 (TetR)]</td>
<td>Stratagene</td>
</tr>
</tbody>
</table>

Plasmids

| pBADHisA | Expression Vector; *araBAD* promoter, Amp$^R$ | Invitrogen. |
| pBAD-YqjA | H$_6$.yqjA expression vector; *araBAD* promoter,Amp$^f$ | Sikdar et al.(2013) |
| pBAD-MdfA | H$_6$.mdfA expression vector; *araBAD* promoter,Amp$^R$ | Sikdar et al.(2013) |
| pBAD-YqjA E39A | H$_6$.yqjA E39A expression vector; *araBAD* promoter,Amp$^f$ | Kumar et al.(2014) |
| pBAD-YqjA D51A | H$_6$.yqjA D51A expression vector; *araBAD* promoter,Amp$^f$ | Kumar et al.(2014) |
Alkaline pH sensitivity growth assay in liquid medium: Overnight cultures from a single colony were freshly diluted to 1:100 in LB media with appropriate antibiotics and additives, and grown to OD \(_{600} \approx 1.0\) at 37°C in a shaking incubator. 1ml of culture was further inoculated into 20ml of prewarmed fresh LB medium buffered to indicated pH and containing appropriate antibiotics and additives. During growth at pH 7, 0.002% arabinose was used when inducing expression of \(yqjA\) due to toxicity issues. At higher pH, 0.02% arabinose was used. The cells were further grown aerobically at 37°C in a shaking incubator and OD \(_{600}\) was measured each hour for 8 hours. In order to see the dependency of sodium and potassium ions at alkaline pH, the cells were first washed in salt-free media twice before growth was initiated in LB media containing additives at the indicated concentrations.

4.3 Results

*E. coli* lacking \(yqjA\) is unable to grow at alkaline pH. In order to study the physiological roles of YqjA, we tested the growth of wild type *E. coli* and a \(\Delta yqjA\) mutant at different alkaline pH’s in liquid media. At pH 7 and pH 8, both the strains grew well at 37°C (Figure. 4.1, A). However, as the pH increased above pH 8.25 to pH 9.5 the growth of \(\Delta yqjA\) mutant was diminished compared to the wild type strain. Similar results were observed when dilutions of these strains were spotted and grown on solid growth media (Figure. 4.1, B). Above pH 9.5 both wild type and the \(\Delta yqjA\) mutant did not show any or only marginal growth on either solid or in liquid media (data not shown). Our results are in agreement with the alkaline sensitivity of a \(\Delta yqjA\) mutant observed previously (Price and Raivio 2009). These observations show that YqjA plays an important role in providing alkaline tolerance to *E. coli*. While YghB displays 62 % amino acid identity to YqjA, and
can substitute for YqjA in many processes (Doerrler, Sikdar et al. 2013), the ∆yghB mutant did not display sensitivity to growth under alkaline conditions (Figure 4.2).

Figure 4.1. An E. coli ∆yqjA mutant fails to grow at elevated pH. (A) Parent E. coli W3110 (filled squares) and ∆yqjA (open triangles) were grown to mid log phase at pH 7, and inoculated into liquid LB medium at pH 7.0, 8.0, 8.25, 8.5, 8.75, 9.0, 9.25, 9.5 or 9.75. Growth at 37 °C was monitored hourly. (B) Parent strain W3110 and ∆yqjA were grown to mid log phase at pH 7.0, then 5 µl of serially diluted cells were spotted onto LB medium plates at pH 7.0, 8.5, 8.75, 9.25 and 9.5 and the plates were incubated at 37 °C for 20-24 hours.
Figure 4.2. YqjA, but not YghB, is required for growth at elevated pH. ΔyqjA, ΔyghB and BC202 (ΔyqjA, ΔyghB) were grown to mid log phase at pH 7.0, and 5 μl of serially diluted cells were spotted onto LB-Amp plates at pH 8.5, 8.75, 9.0, 9.25 and 9.5. The plates were subsequently incubated at 37 °C for 20-24 hours. BC202, but not the single deletion strains, grows slowly at 37 °C, perhaps explaining its increased sensitivity at pH 8.5 and pH 8.75 compared to that of the ΔyqjA strain.

Overexpression of yqjA from a multicopy plasmid corrects the alkaline sensitive phenotype. We next examined the ability of overexpression of yqjA from an inducible multicopy plasmid to rescue the alkaline pH sensitivity phenotype. In liquid media at pH 7.0, the ΔyqjA mutant harboring either empty vector or vector expressing yqjA grew similarly (Figure 4.3, A). As the pH increased into the range of 8.5-9.5, the mutant harboring the control vector grew more and more poorly while plasmid expression yqjA was capable of supporting growth of the ΔyqjA mutant to wild type levels under these conditions. On solid plates, ΔyqjA cells containing vector or expressing wild type yqjA grew well until about pH 8.5. However, as the pH was increased above 8.5 the cells overexpressing yqjA were able to survive as compared to cells containing control vector (Figure 4.3, B). Taken together, these results suggest that expression of functional YqjA is
necessary for a ΔyqjA mutant to survive at alkaline pH. Expression of yghB can restore normal growth and cell division to BC202 (ΔyqjA, ΔyghB), suggesting it possesses functions in common with YqjA (Thompkins, Chattopadhyay et al. 2008; Boughner and Doerrler 2012). We tested the ability of plasmid expression of yghB to facilitate growth of ΔyqjA at elevated pH. At pH 8.75 and 9.0, yghB expression could enhance growth of the ΔyqjA mutant compared to the vector control, but only yqjA expression could restore growth at the higher pH of 9.25 (Figure 4.4). This result suggests a partial but not complete overlap of functions of these DedA family proteins.

Figure 4.3. Expression of yqjA or mdfA display differing capacities to support growth of the ΔyqjA mutant at elevated pH. (A) ΔyqjA harboring control vector pBAD (open triangles) or pBADyqjA (filled squares) were grown to mid log phase at pH 7.0, and inoculated into liquid LB-Amp medium at pH 7.0, 8.5, 8.75, 9.0, 9.25 or 9.5. Growth was monitored hourly by measuring OD₆₀₀. (B) ΔyqjA harboring control vector pBAD, pBADyqjA or pBADmdfA was grown to mid log phase at pH 7.0, and 5 μl of serially diluted cells were spotted onto LB-Amp plates at pH 8.75, 9.0 and 9.25. The plates were subsequently incubated at 37 °C for 20-24 hours. In both experiments, growth at pH 7.0 was carried out in the presence of 0.002% arabinose while growth at higher pH was in the presence of 0.02% arabinose due to toxicity issues with yqjA expression at pH 7.0.
Figure 4.4. YghB can partially support growth of ΔyqjA up to pH 9.0 but no higher. (A) ΔyqjA harboring control vector pBAD, pBADyqjA or pBADyghB was grown to mid log phase at pH 7.0, and 5 µl of serially diluted cells were spotted onto LB-Amp plates containing 0.02% arabinose at pH 8.75, 9.0, and 9.25. The plates were subsequently incubated at 37 °C for 20-24 hours. (B) ΔyqjA harboring control vector pBAD (open triangles) or pBADyghB (filled squares) were grown to mid log phase at pH 7.0, and inoculated into liquid LB-Amp medium at pH 8.5, 8.75, 9.0, or 9.25. Growth was monitored hourly by measuring OD_{600}.

Overexpression of MdfA partially complements the alkaline pH sensitivity of the ΔyqjA mutant. We reported previously that overexpression of mdfA could rescue growth, cell division, and drug sensitivity of BC202 (ΔyqjA, ΔyghB) (Sikdar, Simmons et al. 2013; Kumar and Doerrler 2014). MdfA is a Na^+-K^+/H^+ antiporter belonging to the major facilitator superfamily and is involved in both alkaline tolerance (Lewinson, Padan et al. 2004) and drug resistance (Edgar and Bibi 1997; Lewinson, Adler et al. 2003) in *E. coli*. Therefore, we tested whether mdfA overexpression can correct the alkaline pH sensitivity of the ΔyqjA mutant. We found that MdfA can partially complement the alkaline sensitivity
of ΔyqjA up to about pH 9.0, but not at higher pH’s on both solid media (Figure 4.3, B) and when grown in liquid media (Figure 4.5), suggesting that YqjA plays a significant role in survival of *E. coli* at extreme alkaline pH.

Figure 4.5. MdfA can partially restore growth to ΔyqjA up to pH 8.75 but not at higher pH in liquid media. ΔyqjA harboring control vector pBAD (open triangles) or pBADmdfA (filled squares) were grown to mid log phase at pH 7.0, and inoculated into liquid LB-Amp medium at pH 8.5, 8.75, or 9.0. Growth was monitored hourly by measuring OD<sub>600</sub>.

Figure 4.6. ΔyqjA and ΔnhaA mutants but not ΔmdfA and ΔmdtM mutants are sensitive to alkaline pH. Parent W3110, ΔyqjA, ΔmdfA, as well as ΔmdfA, ΔmdtM, ΔnhaA and ΔnhaB (in the Keio parent strain BW25113) were grown to mid log phase at pH 7.0, and 5 µl of serially diluted cells were spotted onto LB-Amp plates at pH 8.75, 9.0 and 9.25. The plates were subsequently incubated at 37 °C for 20-24 hours.

We could not perform the reverse experiment, expressing yqjA in a ΔmdfA mutant or, for that matter, a ΔmdtM mutant, because both mutants (obtained from the Keio collection, Table 4.1) grew well at all pH’s in our hands (Figure 4.6). These observations
are not in agreement with previous reports (Lewinson, Padan et al. 2004; Holdsworth and Law 2013). In contrast, the $\Delta$nhaA mutant does display pH sensitivity in our hands, consistent with previous reports (Padan, Maisler et al. 1989). The reasons for these differences are unclear and may be related to differences in the genetic backgrounds of the strains. We have reported that the $\Delta$mdfA mutant is sensitive to drugs and biocides in our hands (Kumar and Doerrler 2014), consistent with previous reports (Tal and Schuldiner 2009), and the genetic identity of all Keio strains was confirmed by PCR with primers flanking each gene and DNA sequencing. Plasmid overexpression of $yqjA$ did not reverse the pH sensitivity of $\Delta$nhaA (data not shown).

Transmembrane acidic amino acids are required for YqjA dependent alkaline tolerance. In many proton-dependent transporters including those involved in drug efflux, it has been demonstrated that membrane embedded acidic residues play a role in proton transport and recognition of cationic substrates (Edgar and Bibi 1999; Soskine, Adam et al. 2004; Seeger, von Ballmoos et al. 2009; Sigal, Fluman et al. 2009; Fluman, Ryan et al. 2012). Many DedA proteins contain membrane-embedded glutamic acids and/or aspartic acids in the first predicted transmembrane spanning region (E39 and D51 in both YqjA and YghB). We have shown that these residues are required for YqjA to restore growth, cell division and drug resistance to BC202 (Kumar and Doerrler 2014). Similar acidic residues are found in the $B. burgdorferi$ DedA protein BB0250 (E39 and D40) that are essential for its ability to complement the growth, cell division and drug sensitivity phenotypes of BC202 (Kumar and Doerrler 2014). We tested whether YqjA residues E39 and D51 are individually required for YqjA to provide alkaline tolerance to $E. coli$. In both liquid and on solid media buffered at pH 9.0 and above, each acidic amino acid is required
However at pH 8.75, E39 appears to be dispensable for this activity, as growth was observed for the yqjA mutant expressing YqjA (E39A) at this pH (Figure 4.7, A, B). This suggests that residue D51 plays a major role in the ability of YqjA to provide alkaline tolerance while E39 is only required at the more extreme alkaline pH’s above 9.0.

Figure 4.7. YqjA acidic amino acids E39 and D51 are required for YqjA to support growth of E. coli at elevated pH. (A) ΔyqjA harboring either control vector pBAD (open triangles), pBADyqjA (filled squares), pBADyqjA-E39A (filled triangles), or pBADyqjA-D51A (filled circles) were grown to mid log phase at pH 7.0, and inoculated into liquid LB-Amp medium at pH 8.75, 9.0 and 9.25. (B) ΔyqjA harboring control vector pBAD, pBADyqjA, pBADyqjA-E39A, or pBADyqjA-D51A was grown to mid log phase at pH 7.0, and 5 μl of serially diluted cells were spotted onto LB-Amp medium plates at pH 8.75, 9.0 and 9.25, and 9.5. The plates were subsequently incubated at 37 °C for 20-24 hours.

Sodium or potassium is required for YqjA to support growth under alkaline conditions. Many of the proteins that have been reported to be necessary for alkaline tolerance (NhaA, NhaB, ChaA, MdtM and MdfA) in bacteria are antiporters that exchange protons for other cations such as sodium, potassium, lithium, or calcium (Pinner, Kotler et
Figure 4.8. Sodium or potassium is required for YqjA to support growth at alkaline pH. (A, B) ΔyqjA harboring pBADyqjA was grown to mid log phase at pH 7.0, then inoculated into liquid LB-Amp medium at pH 9.25 containing 0 mM (open triangles), 25 mM (filled circles), 50 mM (filled triangles) or 100 mM (filled squares) sodium chloride (A) or sodium gluconate (B) and growth was monitored. (C, D) ΔyqjA harboring either control vector pBAD or pBADyqjA were grown to mid log phase at pH 7.0, and 5 μl of serially diluted cells were spotted onto LB-Amp medium plates at pH 9.25 containing 75, 100 or 150 mM NaCl or sodium gluconate (C) and KCl or potassium gluconate (D). The plates were incubated at 37 °C for 20-24 hours. For reasons that are not understood, slight differences were observed in optimal salt concentrations needed for growth when strains were grown on solid or in liquid media.
We tested the cation requirements for YqjA to support alkaline tolerance by growing strains at elevated pH in the presence of varying concentrations of extracellular salts. Either sodium or potassium was found to be necessary for this YqjA activity. While no growth was seen in the \textit{yqjA}-complemented \textit{ΔyqjA} mutant at pH’s above 8.75 in the absence of extracellular salts (Figure. 4.8A, Figure.4.9 and 4.10), YqjA could support growth of the \textit{ΔyqjA} mutant at pH 9.25 if 100 mM sodium was included in the growth media (Figure. 4.8 A-C). Sodium could be provided in the form of either sodium chloride or sodium gluconate thereby demonstrating that the chloride anion is not necessary. Similarly, potassium, supplied as potassium chloride or potassium gluconate at 100 mM could support growth at pH 9.25 under these conditions (Figure. 4.8 D and Figure.4.9). Choline chloride could not support the ability of YqjA to provide alkaline tolerance at any concentration up to 150 mM (Figure. 4.11, D), further supporting no role for the chloride anion.

![Figure 4.9. Growth of ΔyqjA::pBADyqjA in liquid media at pH 9.25 containing potassium salts. (A, B) ΔyqjA harboring pBADyqjA was grown to mid log phase at pH 7.0, then inoculated into liquid LB-Amp medium at pH 9.25 containing 0 mM (open triangles), 25 mM (filled circles), 50 mM (filled triangles) or 100 mM (filled squares) potassium chloride (A) or potassium gluconate (B) and growth was monitored hourly by measuring OD$_{600}$.](image)
Figure 4.10. Growth of ΔyqjA in media containing no salt at alkaline pH’s. ΔyqjA was grown to mid log phase at pH 7.0, and inoculated into salt-free liquid LB-Amp medium at pH 8.5 (filled squares), 8.75 (filled triangles), 9.0 (open triangles), 9.25 (open circles) or 9.5 (open diamonds). Growth was monitored hourly by measuring OD$\text{600}$. 

_Raising osmotic pressure can enhance YqjA mediated alkaline tolerance._ While no growth was seen when the ΔyqjA mutant expressing _yqjA_ was grown at pH 9.25 in the absence of extracellular salt (Figure 4.10), we were surprised to find that sucrose, mannitol or sorbitol could enhance the growth of this strain at this elevated pH (Figure 4.11). While the growth rate in the presence of increased osmotic pressure was only ~25 % of that seen in the presence of 150 mM NaCl or KCl (Figure.4.11, B), it was reproducible and significantly better than the undetectable growth rate seen in the absence of salt (although trace levels of both sodium and potassium are present in the broth; see materials and methods). This growth rate enhancement was seen with 100-300 mM sucrose (Figure.4.11, C), as well as with mannitol or sorbitol (Figure. 4.11, D) but was not seen with a molar equivalent amount of choline chloride (Figure. 4.11, D).
Figure 4.11. Increasing the osmotic pressure can partially substitute for cations in supporting the ability of YqjA to permit growth at elevated pH. (A) ΔyqjA harboring pBADyqjA was grown to mid log phase at pH 7.0, then inoculated into salt-free liquid LB-Amp medium at pH 9.25 containing 0 mM (open triangles), 100 mM (filled circles), 200 mM (filled triangles) or 300 mM sucrose (filled squares) and growth was monitored. (B) Comparison of growth rates between ΔyqjA harboring pBADyqjA in salt-free LB-Amp medium (open triangles), salt-free LB-Amp medium containing 300 mM sucrose (closed circles) and LB-Amp medium containing 150 mM NaCl (filled squares). ΔyqjA harboring control vector pBAD or pBADyqjA was grown to mid log phase at pH 7.0, then 5 µl of serially diluted cells were spotted onto LB-Amp medium plates at pH 9.25 containing 100, 200 or 300 mM sucrose (C), 300 mM mannitol, 300 mM sorbitol or 150 mM choline chloride (D). The plates were incubated at 37 °C for 20-24 hours.
Figure 4.12. Sucrose enhances the ability of YqjA to provide alkaline tolerance in the presence of a suboptimal concentration of sodium. (A) ΔyqjA harboring pBADyqjA was grown to mid log phase at pH 7.0, then inoculated into salt-free liquid LB-Amp medium at pH 9.25 containing 0 mM sucrose, 0 mM NaCl (open triangles), 300 mM sucrose, 0 mM NaCl (open circles), 0 mM sucrose, 50 mM NaCl (filled circles), 300 mM sucrose, 50 mM NaCl (filled triangles) or 0 mM sucrose, 100 mM NaCl (filled squares) and growth was monitored. (B) ΔyqjA harboring control vector pBAD or pBADyqjA was grown to mid log phase at pH 7.0, then 5 μl of serially diluted cells were spotted onto LB-Amp medium plates at pH 9.25 containing 0 mM NaCl, 50 mM NaCl, 100 mM sucrose, 50 mM NaCl + 100 mM sucrose, or 100 mM NaCl. The plates were incubated at 37 °C for 20-24 hours.
Sodium and potassium salts or sucrose can support growth of W3110 but not ΔyqjA at elevated pH. Parent W3110 and ΔyqjA were grown to mid log phase at pH 7.0, and 5 µl of serially diluted cells were spotted onto salt-free LB plates containing the additional salts or sucrose at pH 7.0, 9.0 and 9.25. The plates were subsequently incubated at 37 °C for 20-24 hours.

We further tested if increased osmotic pressure could enhance YqjA function by growing the ΔyqjA mutant expressing yqjA at pH 9.25 in the presence of a sub-optimal concentration of sodium chloride (50 mM; see Figure 4.8) with or without 300 mM sucrose. On both plates and in liquid medium, sucrose could enhance the growth of this strain in 50 mM NaCl to levels close to what is seen at 100 mM NaCl in the absence of additional osmotic pressure (Figure 4.12). No growth was seen under these conditions with the ΔyqjA mutant harboring a control vector. As expected, the wild type parent strain W3110 also grows poorly at high pH in the absence of added salt, but growth can be restored by the inclusion of NaCl, KCl or sucrose in the growth media (Figure 4.13). To our knowledge, this is the first time it has been demonstrated that sucrose can aid growth of *E. coli* at elevated pH in the presence of limiting salts and our data suggests that YqjA alone is

<table>
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<tr>
<td>W3110</td>
<td>W3110</td>
<td>W3110</td>
</tr>
<tr>
<td>ΔyqjA</td>
<td>ΔyqjA</td>
<td>ΔyqjA</td>
</tr>
<tr>
<td>no salt</td>
<td>no salt</td>
<td>no salt</td>
</tr>
<tr>
<td></td>
<td>150 mM NaCl</td>
<td>150 mM KCl</td>
</tr>
<tr>
<td></td>
<td>no salt, 300 mM Sucrose</td>
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</table>

Figure 4.13. Sodium and potassium salts or sucrose can support growth of W3110 but not ΔyqjA at elevated pH. Parent W3110 and ΔyqjA were grown to mid log phase at pH 7.0, and 5 µl of serially diluted cells were spotted onto salt-free LB plates containing the additional salts or sucrose at pH 7.0, 9.0 and 9.25. The plates were subsequently incubated at 37 °C for 20-24 hours.
responsible for this. This data collectively supports the hypothesis that the transport activity of YqjA required for growth at elevated pH and is stimulated in the presence of increased osmotic pressure.

4.4 Discussion

Adaptation to alkaline conditions by neutralophiles remains a poorly understood process. A key process necessary is maintenance of an acidic cytoplasm relative to the pH of the surrounding environment. This can be accomplished in a number of ways, but import of protons from the outside of the cell is one of the most common. This is usually accomplished by the activity of high affinity proton dependent antiporters. To control cytoplasmic pH, the cell by definition must control its internal proton concentration, doing this by dictating the relative magnitudes of the transmembrane proton gradient (ΔpH) and the membrane electrical potential (ΔΨ), the two components of the protonmotive force (PMF). Under conditions of alkaline stress, neutralophiles like *E. coli* cannot use the ΔpH to drive proton accumulation inside the cell because the cytoplasm is already more acidic than the outside. To overcome this, antiporters use ΔΨ to promote electrogenic Na⁺/H⁺ and/or K⁺/H⁺ exchange to acidify the cytoplasm (Krulwich, Sachs et al. 2011). In *E. coli*, there are five known membrane transporters that play a role in adaptation to alkaline conditions: MdfA, NhaA, NhaB, ChaA and MdtM (Pinner, Kotler et al. 1993; Lewinson, Padan et al. 2004; Padan, Bibi et al. 2005; Radchenko, Tanaka et al. 2006; Holdsworth and Law 2013). All are proton dependent antiporters that function under specific environmental conditions. Mutants lacking these genes are often sensitive to alkaline pH (Krulwich, Sachs et al. 2011). We asked whether *E. coli* DedA family mutants can grow at elevated pH. Surprisingly, we found that YqjA, but not YghB, is required for growth in the range of pH
8.5-9.5, in support of previous observations (Price and Raivio 2009). A ΔyqjA mutant cannot grow above pH 9.0 (Figure. 4.1) and expression of yqjA from a plasmid can restore growth to the ΔyqjA mutant, but only if sufficient extracellular sodium or potassium is supplied (> ~100 mM) (Figures. 4.3 and 4.8). Mutation of YqjA acidic amino acids E39 or D51 abolishes this activity (Figure.4.7), in spite of the expression and membrane association of the mutant proteins at levels equivalent to the wild type proteins (Kumar and Doerrler 2014).

We also observed that increasing the osmotic pressure by the addition of sucrose, mannitol, and sorbitol at concentrations of 100-300 mM could enhance the ability of YqjA to provide adaptation to alkaline growth conditions (Figure.4.11 and 4.12). Each of these agents is capable of imposing increased osmotic pressure on bacteria (Verkhovskaya, Barquera et al. 1998; van den Bogaart, Hermans et al. 2007; Kitko, Wilks et al. 2010). One interpretation of this surprising result is that YqjA may additionally possess osmosensing capabilities. A number of osmosensing transporters are known and belong to different membrane transporter families. These include ProP of E. coli (Major facilitator superfamily), OpuA of Lactococcus lactis (ABC transporter superfamily) and BetP of Corynebacterium glutamicum (betaine-carnitine-choline transporter family (Wood 2011). Interestingly, BetP is a homotrimer of 12 transmembrane helix subunits (Ressl, Terwisscha van Scheltinga et al. 2009) possessing the LeuT fold (Forrest, Kramer et al. 2011). Members of the DedA are predicted to be evolutionarily related to LeuT and are predicted to also possess LeuT fold transmembrane helices (Khafizov, Staritzbichler et al. 2010).

The yqjA gene is found in an operon with yqjB, also known as MzrA. MzrA was reported to modulate the activity of the EnvZ/OmpR two component system which
responds to osmotic stress (Gerken, Charlson et al. 2009). MzrA interacts with inner membrane sensors kinases EnvZ and CpxA and promotes enhanced phosphorylation of these proteins perhaps coordinating the output of the osmolarity sensing EnvZ and the stress sensing CpxA. Therefore, it is quite intriguing that we find an osmotic pressure stimulated activity of YqjA in promoting adaptation to alkaline growth conditions. EnvZ is not necessary for YqjA to promote adaptation to alkaline growth conditions (Kumar S and Doerrler WT, unpublished observations), consistent with the previous determination that the EnvZ regulator MzrA functions independently of YqjA (Gerken, Charlson et al. 2009).

YqjA is also a member of the Cpx regulon (Price and Raivio 2009), which responds to periplasmic stress caused by alkaline conditions and overexpression of certain envelope proteins among other stressors (Raivio and Silhavy 1999). These stresses cause the activation of sensor kinase CpxA which in turn results in the activation of response regulator CpxR. CpxR activation results in the upregulation a number of periplasmic chaperones and proteases. YqjA is therefore one member of the Cpx regulon required for adaptation to alkaline conditions (Price and Raivio 2009), suggesting a specific environmental niche for YqjA activity.

The DedA/Tvp38 family of membrane proteins are highly conserved and found within all domains of life (Doerrler, Sikdar et al. 2013). Nearly all sequenced bacterial genomes possess at least one DedA family member. Our genetic approaches support roles for YqjA and its close homologue YghB in maintenance of the protonmotive force, possibly by acting as proton-dependent transporters (Sikdar, Simmons et al. 2013; Kumar and Doerrler 2014). Our findings reported herein support a critical role for YqjA in
adaptation to alkaline conditions by the neutralophile *Escherichia coli*. Furthermore, YqjA appears to possess characteristics similar to a number of reported osmosensing transporters (Wood 2011) although additional work, including in vitro studies, are required to prove this. While other transporters have been reported to counteract alkaline stress (Pinner, Kotler et al. 1993; Lewinson, Padan et al. 2004; Padan, Bibi et al. 2005; Radchenko, Tanaka et al. 2006; Holdsworth and Law 2013), we believe YqjA and possibly other DedA family members are candidates to serve major roles in adaptation to alkaline and possibly other stressful environments.

4.5 References


5.1 Introduction

The DedA/Tvp38 family is a highly conserved and poorly understood family of membrane proteins present in most bacteria as well as many archaea and eukaryotes (Doerrler, Sikdar et al. 2013). Computational analysis has suggested DedA family proteins share an evolutionary relationship and structural fold with LeuT, belonging to the neurotransmitter: sodium symporter family (Khafizov, Staritzbichler et al. 2010; Keller, Ziegler et al. 2014). Our laboratory has pioneered characterization of the DedA/Tvp38 membrane protein family in E. coli. (Thompkins, Chattopadhyay et al. 2008; Liang, Xu et al. 2010; Sikdar and Doerrler 2010; Boughner and Doerrler 2012; Sikdar, Simmons et al. 2013; Kumar and Doerrler 2014; Kumar and Doerrler 2015).

YqjA and YghB of E. coli are the best characterized members of the DedA family. YqjA and YghB possess 220 and 219 amino acids, respectively, along with multiple predicted membrane-spanning domains, sharing 62 % amino acid identity and partially redundant functions (Thompkins, Chattopadhyay et al. 2008). Simultaneous in-frame deletions of these two genes in strain BC202 results in numerous phenotypes including temperature sensitivity (Thompkins, Chattopadhyay et al. 2008), cell division defects due to inefficient export of periplasmic amidases by the twin arginine pathway (Sikdar and Doerrler 2013), and sensitivity to various antibiotics and biocides which are normally

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in a PMF-dependent manner from the cytoplasm (Kumar and Doerrler 2014). Phenotypes of BC202 can be rescued by growth at pH 6.0 or overexpression of either \textit{yqjA}, \textit{yghB} or \textit{mdfA} (Sikdar, Simmons et al. 2013; Kumar and Doerrler 2014). MdfA is an unrelated Na\textsuperscript{+}-K\textsuperscript{+}/H\textsuperscript{+} antiporter and drug efflux pump belonging to the major facilitator superfamily (Lewinson, Adler et al. 2003). The \textit{ΔyqjA} mutant (but not the \textit{ΔyghB} mutant) is unable to survive above pH 8.5 and sodium or potassium is required for YqjA-mediated alkaline tolerance (Kumar and Doerrler 2015). Cation/proton antiporters play a major role in alkaline pH homeostasis in \textit{E. coli} (Pinner, Kotler et al. 1993; Lewinson, Padan et al. 2004; Padan, Bibi et al. 2005; Holdsworth and Law 2013). Based on these results, we suggested YqjA/YghB are proton-dependent membrane transporters (Sikdar, Simmons et al. 2013; Kumar and Doerrler 2014; Kumar and Doerrler 2015).

Charged amino acids are known to play essential roles in various secondary transporters including MdfA, NhaA, MdtM and LacY (Gerchman, Olami et al. 1993; Noumi, Inoue et al. 1997; Abramson, Iwata et al. 2004; Adler and Bibi 2004; Sigal, Vardy et al. 2005; Fluman, Ryan et al. 2012; Holdsworth and Law 2012). Membrane embedded acidic amino acids were shown to be important for the function of YqjA and YghB of \textit{E. coli} (E39 and D51 of both proteins) (Kumar and Doerrler 2014). Furthermore, the significance of membrane embedded basic amino acids like arginine are well documented in the literature and shown to be crucial in several biological processes including regulation of redox potential (Cutler, Davies et al. 1989; Winn, Ludemann et al. 2002), voltage detection across a lipid bilayer (Jiang, Ruta et al. 2003; Long, Campbell et al. 2005; Tao, Lee et al. 2010), and proton transport (Cain and Simoni 1989; Hellmer, Teubner et al. 2003; Sigal, Vardy et al. 2005). Here, we analyze the effect of point mutations of conserved basic
amino acids on the function of YqjA and YghB and show that conserved arginines at positions 130 and 136 of YqjA and YghB are essential for the function of both proteins. Furthermore, mutation of R130 to lysine results in a functional protein indicating a charge dependence at this position. In contrast, the R136K mutation remains nonfunctional indicating an important role played by arginine at this position. This study provides mechanistic insight into YqjA and YghB and supports the hypothesis that they function as membrane transporters.

5.2 Materials and Methods

Materials: All chemicals were reagent grade and purchased from Sigma-Aldrich, VWR, New England Biolabs or Qiagen.

Bacterial growth conditions: Bacteria cultures were grown in LB medium (1% tryptone, 0.5% yeast extract and 1% NaCl) with antibiotics ampicillin (Amp) at 100 µg/ml, kanamycin (Kan) at 30 µg/ml and tetracycline (Tet) at 12.5 µg/ml where specified. In certain experiments growth media was additionally supplemented with 0.002% or 0.02% arabinose (w/v). For pH experiments, the growth media were buffered with 70mM BTP (Bis-Tris propane) and pH was adjusted as required with HCl.

Site-directed mutagenesis: Point mutants were created according to a previously published protocol (Kumar and Doerrler 2014). The point mutation was introduced using primers carrying the site-specific mutations (Table S2) in a PCR reaction to amplify a vector having the indicated wild type gene. The DpnI-digested PCR products were used to transform competent XL1 blue cells. Amp resistant colonies were screened by colony PCR by means of gene-specific primers. Mutations were confirmed by DNA sequencing conducted at the LSU College of Science Genomics Facility.
Table 5.1 Strains and plasmids used in this study:

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Plasmids

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Table 5.2: Primers used in the study

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<tr>
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<tr>
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<tr>
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<td>CATTACCTGAGCTTCATGCGTTAATGACGTAATGACGTTCGATGATCTTGCCATTCATATCATT AAGCC</td>
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Microscopy: Microscopy was performed as previously described (Kumar and Doerrler 2014). Overnight cultures of specified strains were diluted 1:100 in LB media with appropriate antibiotics and additives, and grown shaking to OD$_{600}$ ~ 0.6 at 30 °C. The cells were resuspended to a final OD$_{600}$ of 1.0 and 10 µl of cells were used for imaging on a 1% agarose coated glass slide. Differential interference contrast (DIC) micrographs were obtained using a Leica DM-RXA2 deconvolution microscope (LSU Shared Instrument Facility).
Drug resistance measurements: Growth was carried out on solid media as described (Kumar and Doerrler 2014). Overnight cultures of *E. coli* were diluted 1:100 into LB media containing Amp and 0.1 % arabinose and grown to OD$_{600}$ ~ 0.6 at 30°C. 5 μl of serially log$_{10}$-diluted cells were spotted on LB agar plates containing 0.002 % arabinose and the indicated drugs/biocides. Plates were incubated at 30 °C for 20 to 24 hours.

Alkaline pH sensitivity: Alkaline pH sensitivity was tested as previously described (Kumar and Doerrler 2015). Overnight cultures of *E. coli* were diluted 1:100 in LB media with appropriate antibiotics and additives, and grown to OD$_{600}$ ~ 0.6 at 37°C. 5 μl of serially log$_{10}$-diluted cells were spotted on LB agar plates at the indicated pH with appropriate antibiotics and additives. Plates were incubated at 37°C for 20 to 24 hours. All experiments were repeated three times.

Membrane Preparation and Western Blotting: Isolation of membranes and Western blotting was performed as previously described (Kumar and Doerrler 2014). Cell membranes were prepared from the log phase cultures of BC202 expressing the indicated proteins. Protein samples were incubated with 2X SDS PAGE buffer and heated at 95 °C for 5 minutes before 12 % SDS-PAGE and transfer to PVDF. Western blotting was done using Penta-His primary antibody (Qiagen) at 1:5000 dilution and goat-anti-mouse IgG horseradish peroxidase secondary antibody (Pierce) at 1:10,000 and detection with the ImmunStar HRP kit (Bio-Rad).

5.3 Results

*Identification of amino acids required for YajA and YghB function:* Numerous studies have confirmed the role of charged amino acids in the transport mechanism of membrane transporters (Gerchman, Olami et al. 1993; Wiebe, Dibattista et al. 2001; Adler
and Bibi 2004; Sigal, Vardy et al. 2005; Holdsworth and Law 2012). Therefore, we investigated the significance of conserved, charged residues in YqjA and YghB by site directed mutagenesis. Previously, we reported the importance of membrane embedded acidic amino acids E39 and D51 in the function of both proteins (Kumar and Doerrler 2014). In addition, a mutational analysis of YqjA identified numerous non-essential residues (Keller, Schleppi et al. 2015). Here, we studied the effect of mutations of conserved and charged amino acids of YqjA and YghB, mutating amino acids that were found at identical positions in both YqjA and YghB (Figure 5.1). Mutations were introduced into YqjA at 6 different positions (D15, H121, R130, R136, E185 or D186) and YghB at four positions (R130, R136, E185 and D186). All genes were cloned into vector pBAD in-frame with an N-terminal His₆ tag which allows detection of expression and membrane association of proteins using Western blot. The vector with the desired mutation in yqjA or yghB was transformed into BC202 or ΔyqjA to study its ability to complement certain mutant phenotypes.

Rescue of temperature sensitivity of BC202 by point mutants: We first tested the ability of the mutant proteins to complement temperature sensitivity of BC202. Of the six YqjA mutants, YqjA-D15A, -H121A and -D186A were functional; able to support growth of BC202 at 42 ºC (Figure 5.2, A, top panel). Of the four YghB mutants, only YghB-D186A supported growth at 42 ºC (Figure 5.2, C, top panel). All mutants are well expressed in membranes indicating proper folding of the expressed mutant proteins with the exception of YqjA-D15A (Figure 5.2, B), in spite of its ability to complement BC202 at 42 ºC (Figure 5.2, A). While the reasons for poor expression are unclear, residue D15 appears to be dispensable for YqjA function from our data.
Figure 5.1. Amino acid alignment of YqjA and YghB and summary of mutation data. (A). Amino acid alignment of YqjA and YghB. ClustalW (Larkin, Blackshields et al. 2007) was used to create an alignment between the two protein sequences. The charged amino acids which are conserved between both the proteins were chosen for site directed mutagenesis study. Essential acidic (blue) and basic (red) residues are highlighted. (B) Predicted membrane topology model of YqjA/YghB based on the TMHMM program (Sonnhammer, von Heijne et al. 1998). The amino acids E39 and D51 (white type, black background) were previously shown important for the function of YqjA/YghB (Kumar and Doerrler 2014). YqjA/YghB residues mutated in this study are represented as ovals. Mutation of YqjA/YghB R130, R136 or E185 results in loss of function (yellow type, black background). Mutation of YqjA D15, H121, and YqjA/YghB D186 results in proteins with wild type activity (black type, white background). *Mutation of YqjA/YghB E185 results in a partial loss of function in certain assays (See Figure 2 and 3). All other amino acids shown in the model are non-essential for the function for YqjA (Keller, Schleppi et al. 2015). (C) A hydrophobicity profile of YqjA and YghB. The profiles were generated by using the AlignMe program (Khafizov, Staritzbichler et al. 2010; Stamm, Staritzbichler et al. 2013; Stamm, Staritzbichler et al. 2014) along with the scale from Hessa, White & van Heinje (HWvH) with a threshold for gap penalty of -0.05. The green and red plots represent the hydrophobicity profile of YghB and YqjA, respectively.
Since the R130A and R136A mutants of YqjA and YghB failed to rescue growth of BC202, we constructed R130K and R136K mutants replacing the positively charged arginine with positively charged lysine to distinguish between the effect of size or charge in their loss of function. Notably, the R130K but not the R136K mutant of both YqjA and YghB regain their ability to support growth of BC202 at 42 °C (Figure. 5.2A, C bottom). This suggests that amino acid charge at position 130 is critical for the function of both these proteins. However, arginine is absolutely required at position R136 as lysine cannot
substitute. YqjA-E185A and YghB-E185A also fail to restore growth to BC202 at 42 ºC (Figure. 5.2, A, C). These mutants gave contradictory results in some of our assays and are discussed in more detail below.

Rescue of BC202 cell division by point mutants: We proceeded to measure the ability of each mutant to support proper cell division of BC202. These results largely agree with the data from Figure 5.2. BC202 transformed with control vector forms long chains of incompletely constricted cells (Figure 5.3, A, B) as previously reported (Thompkins, Chattopadhyay et al. 2008). BC202 transformed with pBAD-yqjA or pBAD-yghB displays proper cell division and there is no sign of elongated cells. YqjA-D15A, -H121A and -D186A were functional in that they were each capable of restoring cell division to BC202 while YqjA-R130A, –R136A and E185A were nonfunctional in that they failed to restore cell division to BC202. When we made arginine-to-lysine substitutions at positions 130 and 136 of YqjA, we again found that YqjA-R130K regained function but not YqjA-R136K, suggesting an absolute dependence upon arginine at position 136 of YqjA (Figure 5.3, A). Similar observations were made in the corresponding YghB mutants with the exception of YghB-E185A, which was functional in that it did restore normal cell division to BC202 (Figure. 5.3, B).

Rescue of drug sensitivity of BC02 by point mutants: BC202 is also sensitive to several biocides and antibiotics which are normally effluxed from the cell via PMF-dependent pumps including EmrE, MdfA and AcrB (Kumar and Doerrler 2014). BC202 transformed with control vector displays sensitivity to acriflavine, benzalkonium-Cl, ethidium bromide, and methyl viologen (Figure 5.4, A and B). Resistance to these compounds is normally conferred by AcrB, MdfA/AcrB, MdfA/EmrE/AcrB and EmrE,
respectively (Sulavik, Houseweart et al. 2001; Tal and Schuldiner 2009). BC202 expressing wild type \( yqjA \) or \( yghB \) display normal resistance to these compounds as do cells expressing \( YqjA-D15A, -H121A, \) and \( -D186A \) or \( YghB-E185A \) and \( D186A \). Once again, \( YqjA \) and \( YghB-R130A \), \( R136A \) and \( E185A \) mutants were nonfunctional in that they failed to restore resistance to BC202. Lysine could substitute for arginine at position 130 but not position 136 in each of the two proteins.

Figure 5.3. Correction of cell division defects of BC202 by \( YqjA \), \( YghB \) and point mutants. BC202 transformed with vector, \( pBAD-yqjA \) and \( YqjA \) point mutants (A) or vector, \( pBAD-yghB \) and \( YghB \) point mutants (B) grown at 30°C in liquid media having 0.1 % arabinose were visualized with a Leica DM-RXA2 deconvolution microscope. Bar = 10 μm.
Figure 5.4. Correction of drug sensitivity of BC202 by YqjA, YghB and point mutants. BC202 transformed with vector, pBAD-yqjA, and YqjA point mutants (A) and vector, pBAD-yghB, and YghB point mutants (B) was tested for sensitivity to acriflavine (25 μg/ml), Benzalkonium chloride (40 μg/ml), ethidium bromide (100 μg/ml) and methyl viologen (30 μg/ml). All strains grew well on LB plates without biocides at 30 °C.

Rescue of alkaline sensitivity of ΔyqjA by point mutants: The E. coli ΔyqjA mutant (but not the ΔyghB mutant) cannot grow at a pH exceeding 8.5 (Kumar and Doerrler 2015). Plasmid copies of yqjA or yghB rescue growth of ΔyqjA up to pH 9.5 or 9.0, respectively. We therefore tested each point mutant for their ability to rescue growth of the ΔyqjA mutant at elevated pH (Figure 5.5). Our results largely agree with the results for BC202 in Figures 5.2-4 in that only YqjA and YghB -R130A and -R136A mutants are nonfunctional in that
they fail to restore growth to the $\Delta$yqjA mutant at alkaline pH. Again, lysine can substitute for arginine at position 130 but not 136 in each protein (Figure 5.5, A and B).

Figure 5.5. Correction of alkaline sensitivity of $\Delta$yqjA by YqjA, YghB and point mutants. The $\Delta$yqjA mutant transformed with vector, yqjA and YqjA point mutants (A) and vector, pBAD-yghB, and YghB point mutants (B) was grown to mid log phase at pH 7.0 and 5 μl of serially diluted cells were spotted onto LB plates at pH 7.0, 9.0 and 9.25 (A) or pH 7.0, 8.75 and 9.0 (B). The plates were incubated at 37 °C for 20-24 hours. Growth at pH 7.0 was carried out with 0.002% arabinose and 0.02% arabinose was used at pH 9 and 9.25.

To our surprise, we found a discrepancy in the ability of YghB-E185A to rescue BC202 compared to the YqjA-E185A mutant. While YqjA-E185A rescues drug (Figure 5.3, A) and pH sensitivity (Figure 5.4, A), it fails to rescue temperature sensitivity (Figure 5.1, A) and cell division (Figure 5.2, A). In contrast, YghB-E185A corrects the drug
sensitivity (Figure. 5.3, B) and cell division defect (Figure. 5.2, B) of BC202 and the pH sensitivity of ΔyqjA (Figure. 5.4, B), while not correcting the temperature sensitivity of BC202 (Figure. 5.1, C). All mutants proteins are expressed well in the membrane fraction, indicating proper folding and targeting (Figure 5.1, B, D). The reasons for the differing ability of E185A mutants of YqjA and YghB to rescue cell division are at present unclear but both mutants are classified here as partially functional.

### 5.4 Discussion

YqjA and YghB belong to DedA/Tvp38 membrane protein family of *E. coli* and genes encoding homologs are present in most sequenced bacterial genomes (Doerrler, Sikdar et al. 2013). Previously, we characterized the importance of YqjA/YghB in growth at elevated temperatures, cell division, drug resistance, and alkaline pH tolerance in *E. coli* (Thompkins, Chattopadhyay et al. 2008; Sikdar and Doerrler 2010; Doerrler, Sikdar et al. 2013; Kumar and Doerrler 2014; Kumar and Doerrler 2015). Our observations suggest DedA/Tvp38 is a new family of membrane transporters (Sikdar, Simmons et al. 2013; Kumar and Doerrler 2014; Kumar and Doerrler 2015). In spite of our genetic and physiological evidence, mechanistic and structural details of this protein family remain unclear. A structural model of one DedA family member, Slr0305 of *Synechocystis* sp. PCC6803, has been published based upon a predicted similar fold to LeuT (Keller, Ziegler et al. 2014).

To provide mechanistic insight into DedA family proteins, we introduced mutations of conserved and charged amino acids of YqjA and YghB and measured the effect of the mutant proteins on numerous phenotypes of BC202 and ΔyqjA. We observed that R130A and R136A mutations abolish the ability of YqjA and YghB to rescue BC202 and ΔyqjA
suggesting an essential role of these amino acid in their function. In order to distinguish whether the effect was due to changes in size or charge we created the R130K and R136K variants of YqjA and YghB. We found that YqjA-130K and YghB-R130K regain full function suggesting that charge at this position is important for the function of both proteins. However, in spite of being well expressed, the R136K mutant of both proteins were not able to complement the BC202 and ΔyqjA phenotypes implying that arginine at this position is essential for their function.

The importance of membrane embedded arginines has been observed in a number of different families of membrane transporters including GlpT (Law, Almqvist et al. 2008), UhpT (Fann, Davies et al. 1998; Law, Almqvist et al. 2008), F0F1 ATPase (Cain and Simoni 1989), MjNhaP1 (Hellmer, Teubner et al. 2003), TetA (Kimura, Nakatani et al. 1998), MdtM (Holdsworth and Law 2012) and MdfA (Fann, Davies et al. 1998; Sigal, Vardy et al. 2005; Law, Almqvist et al. 2008; Holdsworth and Law 2012). In these examples, arginine is thought to be directly involved in substrate binding and faces the translocation channel.

MdfA appears to share some functional similarity to YqjA. Both the ΔyqjA and the ΔmdfA E. coli strains display alkaline sensitivity (Lewinson, Padan et al. 2004; Kumar and Doerrler 2015). Overexpression of MdfA is sufficient to suppress each mutant phenotype of BC202 and to partially restore pH resistance to ΔyqjA (Sikdar, Simmons et al. 2013; Kumar and Doerrler 2014; Kumar and Doerrler 2015). Therefore the importance of charged residues in the membrane domains of both proteins is potentially significant. Acidic residues residing in the first transmembrane domain of both proteins (MdfA E26/D34 and YqjA/YghB E39/D51) have been shown to be functionally important (Fluman, Ryan et al.
Substitutions resulting in a loss of the positive charge at amino acid R112 in MdfA resulted in sensitivity to antibiotics (Sigal, Vardy et al. 2005). It was suggested that R112 of MdfA could function in proton recognition (Sigal, Vardy et al. 2005) similar to R302 of LacY (Abramson, Iwata et al. 2004). Recently, the inward facing substrate bound structure of *E. coli* MdfA was published and confirmed the importance of membrane embedded R112 for transport activity (Heng, Zhao et al. 2015).

LeuT, a member of the neurotransmitter: sodium symporter family appears to share structural features with DedA based upon computational analysis (Khafizov, Staritzbichler et al. 2010; Keller, Ziegler et al. 2014). In this transporter, a water mediated salt bridge formed between amino acids R30 and D404 is thought to form the extracellular gate (Noskov 2008; Singh, Piscitelli et al. 2008; Krishnamurthy, Piscitelli et al. 2009). We propose, based on these studies that R130 and R136 of both YghB and YqjA are either exposed to the substrate translocation pore or play a role in substrate binding. An alternate possibility is that these residues form salt bridges with negatively charged amino acids helping to maintain protein conformation during the transport cycle (Law, Almqvist et al. 2008). Their involvement in the specific transport mechanism may differ from those examples cited above and clarification awaits future structural studies. In conclusion, we have demonstrated that R130 and R136 are critical for the function of YqjA and YghB. These results will aid in elucidating the mechanism of transport of DedA/Tvp38 family members.

### 5.5 References


CHAPTER 6
EXPRESSION ANALYSIS OF yqjA

6.1 Introduction

Alkaline pH tolerance is a vital feature of pathogenic, ecological and industrially important bacteria. Neutrophilic bacteria like *E. coli* have the capability to retain their intracellular pH in the range of 7.5-7.7 when grown under alkaline pH conditions. Recent physiological and genetic studies have shown several adaptations which contribute towards alkaline pH homeostasis including higher expression of transporters and enzymes which stimulate proton transport into the cell (for example the ATP synthase and monovalent cation/proton antiporters), metabolic changes which lead to up-regulation of amino acids deaminase such as tryptophan deaminase, or catabolic pathways that yield organic acids (Padan, Bibi et al. 2005). Na\(^+\) and H\(^+\) are the predominant ions necessary for bioenergetics inside the cell. A correct balance of these ions is critical for protein function and any fluctuation in the concentration causes stress to the cell (Padan, Venturi et al. 2001). Cation proton antiporters have been found in most prokaryotes, and some species have multiple antiporters. For instance, five cation/proton antiporters (NhaA, NhaB, ChaA, MdfA, and MdtM) are known to function in *E. coli* alkaline pH homeostasis (Padan and Landau 2016). Inward active transport of protons by these antiporters is coupled to the export of cations such as Na\(^+\), K\(^+\), Li\(^+\) or Ca\(^{2+}\).

The presence of multiple sodium ion extrusion systems in bacteria can be explained based on the use of different systems under different environmental conditions (Kobayashi, Saito et al. 1999; Kobayashi, Saito et al. 2000; Shijuku, Yamashino et al. 2002). For example, ChaA extrudes sodium ions primarily at alkaline pH above 8, and NhaB functions at pH below 8, whereas NhaA is mainly responsible for adaptation to Li\(^+\) toxicity and
growth at alkaline pH in the presence of Na\textsuperscript{+} ions (Karpel, Alon et al. 1991) (Sakuma, Yamada et al. 1998). \textit{nhaB} does not play a role in adaptation to high salinity at alkaline pH but becomes essential in the absence of \textit{nhaA} activity (Ohyama, Imaizumi et al. 1992; Pinner, Padan et al. 1994). The gene expression of \textit{nhaA} is most studied and has been well documented (Karpel, Alon et al. 1991; Dover, Higgins et al. 1996; Dover and Padan 2001; Shijuku, Saito et al. 2001). A study using \textit{nhaA'-lacZ'} fusion showed the expression of \textit{nhaA} is specifically regulated by intracellular Na\textsuperscript{+} and Li\textsuperscript{+} ions and not by ionic strength or osmolarity (Karpel, Alon et al. 1991; Dover, Higgins et al. 1996). Additionally, the transcription of \textit{nhaA} in response to the high intracellular Na\textsuperscript{+} ions is positively regulated by NhaR (Rahav-Manor, Carmel et al. 1992), a member of LysR- OxyR family of regulatory proteins (Schell 1993; Storz and Altuvia 1994). The increase in pH by itself does not alter \textit{nhaA} expression but does affect the sensitivity of expression towards Na\textsuperscript{+} or Li\textsuperscript{+} ions (Karpel, Alon et al. 1991). Two promoters, P1, and P2 were identified by primer extension analysis for \textit{nhaA}, of which only P1 is Na\textsuperscript{+} and NhaR –dependent (Dover and Padan 2001). Furthermore, the expression of \textit{chaA} is dependent on osmolarity and pH of the medium (Shijuku, Yamashino et al. 2002). Additionally, experiments with a \textit{ΔchaA} mutant have shown that ChaA is necessary for survival in a high concentration of K\textsuperscript{+} at alkaline pH (Radchenko, Tanaka et al. 2006).

The DedA/Tvp38 family is a highly conserved but poorly understood family of membrane proteins and is present in most sequenced genomes including bacteria, archaea, and eukaryotes (Doerrler, Sikdar et al. 2013). Our lab has pioneered the characterization of the DedA/Tvp38 membrane protein family in \textit{E. coli} (Thompkins, Chattopadhyay et al. 2008; Liang, Xu et al. 2010; Sikdar and Doerrler 2010; Boughner and Doerrler 2012;
Doerrler, Sikdar et al. 2013; Sikdar, Simmons et al. 2013; Kumar and Doerrler 2014; Kumar and Doerrler 2015). YqjA and YghB are the best-characterized members of the DedA family and share 62% amino acid identity and partially redundant functions (Thompkins, Chattopadhyay et al. 2008). Simultaneous deletion of these two genes in a strain named BC202 (W3110 ΔyqjA, ΔyghB) results in various phenotypes such as temperature sensitivity (Thompkins, Chattopadhyay et al. 2008), cell division defects due to inefficient export of amidases by the twin-arginine transport pathway (Sikdar and Doerrler 2010), and drug sensitivity due inefficient function of PMF-dependent efflux pumps (Kumar and Doerrler 2014). These phenotypes of BC202 are rescued by overexpression of mdfA or by growth at pH 6.0 suggesting a role for YqjA/YghB in the maintenance of proton motive force (PMF) (Sikdar, Simmons et al. 2013). Furthermore, membrane-embedded charged amino acids are essential for the function of YqjA and YghB (Kumar and Doerrler 2014; Kumar and Doerrler 2015; Kumar, Bradley et al. 2016), that were previously shown to be important for the function of numerous secondary proton-dependent transporters including MdfA, NhaA, MdtM and LacY (Abramson, Kaback et al. 2004) (Sigal, Vardy et al. 2005; Fluman, Ryan et al. 2012; Holdsworth and Law 2012). The ΔyqjA mutant (but not the ΔyghB mutant) is sensitive to alkaline pH and the presence of Na+ or K+ is also necessary for the function of YqjA at alkaline pH (Kumar and Doerrler 2015). Based on these observations, we have recently shown YqjA is a putative osmosensing transporter required for the growth at alkaline pH (Kumar and Doerrler 2015).

yqjA gene expression is under the regulation of both Cpx and σE stress response pathways (Dartigalongue, Missiakas et al. 2001; Price and Raivio 2009). The Cpx (conjugative pilus expression) stress response pathway is mediated by a two-component
system comprising of an inner membrane sensor histidine kinase (CpxA) and a cytoplasmic response regulator (CpxR) (Vogt and Raivio 2012). CpxR becomes activated in response to the overexpression of envelope proteins such as NlpE or pilus subunits, elevated pH, altered membrane composition, high osmolarity, adhesion, indole, and metal ions (Cu^{2+}, Zn^{2+}) (Raivio 2014). CpxR controls the expression of genes that participate in numerous cellular processes including degradation of misfolded proteins in the periplasm (proteases like DegP), protein secretion, motility, multidrug efflux, regulation of two component systems, cell division, biogenesis and modifications of phospholipids and lipopolysaccharide and other uncharacterized physiological processes (Vogt and Raivio 2012; Raivio 2014; Guest and Raivio 2016). The $\sigma^E$ stress response pathway is facilitated by the essential sigma factor $\sigma^E$ (RpoE), which senses stress that compromises the envelope integrity like misfolded polypeptides and is involved in regulating extracytoplasmic and extracellular functions (Danese and Silhavy 1997; Dartigalongue, Missiakas et al. 2001). Cpx and $\sigma^E$ have partially overlapping regulon (Raivio and Silhavy 1999; Vogt and Raivio 2012).

In order to distinguish the uses of various transport systems and their physiological roles, it is important to study the effects of environmental stimuli on the expression of these genes (Shijuku, Yamashino et al. 2002). Therefore, in this study, we have analyzed the expression of $yqjA$ using a reporter gene fusion of the $yqjA$ promoter to lacZ. We found that $yqjA$ expression was higher at alkaline pH than at acidic and neutral pH. Moreover, increased expression of $yqjA$ at elevated pH also required sodium or potassium salts. We observed that the transcriptional regulator CpxR is responsible for the expression of $yqjA$ at alkaline pH and presence of Na$^+$ or K$^+$ ions at alkaline pH. These results further support
our previous observations and help in better understanding of the role of this newly identified family of membrane transporters in *E. coli*.

6.2 Materials and Methods

**Materials:** All chemicals were reagent grade and purchased from Sigma-Aldrich, VWR, New England Biolabs or Qiagen.

**Bacterial growth conditions:** Bacteria cultures were grown in LB medium (1% tryptone, 0.5% yeast extract and 1% NaCl) with antibiotics ampicillin (Amp) at 100 µg/ml, kanamycin (Kan) at 30 µg/ml, tetracycline (Tet) at 12.5 µg/ml, and chloramphenicol (Cam) at 30 µg/ml where indicated. For studies at alkaline pH, the growth media were buffered with 70mM BTP (Bis-Tris propane) and pH was adjusted as required with HCl. For acidic conditions (pH 5 and pH 6) the growth media were buffered with 100mM 2-(N-morpholino)-ethane sulfonic acid (MES) as previously described (Sikdar, Simmons et al. 2013). For some experiments growth media was additionally supplemented with 0.002% or 0.02% arabinose (w/v). Cells were grown at 37°C.

**Alkaline pH sensitivity on solid plate media:** Alkaline pH sensitivity was tested as previously described (Kumar and Doerrler 2015). Overnight cultures of *E. coli* were diluted 1:100 in LB media with appropriate antibiotics and additives, and grown to OD$_{600} \sim 0.6$ at 37°C. 5µl of serially log$_{10}$-diluted cells were spotted on LB agar plates at the indicated pH with appropriate antibiotics and additives. During growth at pH 7, 0.002% arabinose was used when inducing expression of *yqjA* and *cpxR* due to toxicity issues. At higher pH, 0.02% arabinose was used. Plates were incubated at 37°C for 20 to 24 hours. All experiments were repeated three times.
Alkaline pH sensitivity growth assay in liquid medium: Alkaline pH sensitivity in liquid growth media was carried out as previously described (Kumar and Doerrler 2015). Overnight cultures from a single colony were freshly diluted to 1:100 in LB media with appropriate antibiotics, and grown to OD$_{600}$ ~ 1.0 at 37°C in a shaking incubator. 1ml of culture was further inoculated into 20ml of prewarmed fresh LB medium at required pH and containing appropriate antibiotics. The cells were grown aerobically at 37°C in a shaking incubator and OD$_{600}$ was measured each hour for 8 hours. All growth curves were repeated three times.

Strain construction: All strains used are listed in Table 6.1. Strain TB28 (Bernhardt and de Boer 2003) was used to provide a ΔlacZYA background for certain strains. Mutations from Keio collection strains (Baba, Ara et al. 2006) were typically introduced into E. coli W3110 or TB28 background by P1 transduction (Silhavy, Berman et al. 1984) and correct configuration was verified by PCR and DNA sequencing with primers flanking the appropriate gene. DNA sequencing was conducted at the LSU College of Science Genomics Facility.

Construction of plasmids (pSK1, pSK2, pSK3) for β-galactosidase assay: All PCR primers are listed in table 6.2. The E. coli lacZ gene was amplified from W3110 genomic DNA. The PCR product obtained was purified using QIAquick PCR purification kit (Qiagen, Valencia CA), digested with SphI and BamHI, and cloned into a likewise treated and dephosphorylated pACYC184 vector (NEB). The upstream region containing the promoter of the particular genes (yqjA, alx, nhaA) in the study was amplified using W3110 genomic DNA and cloned into pACYC184 plasmid at SalI and SphI sites such that the promoter of that gene lies upstream of lacZ on the plasmid and controls the expression of
the lacZ gene. The plasmids generated are listed in table 6.1. Sequencing of all constructs was performed at the LSU College of Science Genomics Facility.

β-Galactosidase Assay: The β-galactosidase (β-gal) assays were performed by using the method of Miller (Miller 1972). Overnight cultures of indicated strains were diluted 1:100 in fresh LB supplemented with appropriate antibiotics and grown shaking at 37°C to an OD600 of ~1.0. 1ml of culture was then diluted into 5ml of fresh growth media at desired pH and salt concentrations and grown for an additional 1 hour. To analyze the effect of Na+ and K+ ions on the expression of genes, salt-free media (1% tryptone (w/v), 0.5% yeast extract (w/v)) was supplemented with different concentrations of salts as specified. Although we refer to this media as “salt-free”, residual amounts (1-10mM) of both sodium and potassium are likely present (Ohyama, Igarashi et al. 1994; Radchenko, Tanaka et al. 2006). Cells were washed at least two times in salt-free media before adding the fresh media. After growing the cells in the experimental conditions, 0.1ml of cells were then added to Z buffer (Miller 1972) to a total volume of 1 ml and the cells were lysed by the addition of 50 µl chloroform and 25 µl 0.1% SDS and vortexing. The reaction was started by addition of 0.1 ml of 8 mg/ml (w/v) 2-nitrophenyl β-D-galactopyranoside (ONPG) and the tubes were incubated at 30°C till sufficient yellow color developed (reaction time varied from 20 minutes to 6 hrs). The reaction was stopped by adding 0.4 ml of 1.25 M Na2CO3 to the reaction mix and vortexing. The mixture was centrifuged at 13,000 rpm for 5 min to remove cell debris and absorbance was read at 420 nm. Cells harboring the empty vector gave a reading at 420 nm that was not above background. The β-galactosidase units were corrected for the culture density and correspond to 1nmole ONPG hydrolyzed per minute at 30°C.
### Table 6.1. *E. coli* strains and plasmids used in this study

<table>
<thead>
<tr>
<th>Strains</th>
<th>Relevant Genotype/Genetic Markers</th>
<th>Source or Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>W3110</td>
<td>Wild-type, F⁻ λ⁻</td>
<td><em>E. coli</em> genetic stock center, Yale University</td>
</tr>
<tr>
<td>BC203</td>
<td>W3110, ΔyqjA::Tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Thompkins, Chattopadhyay et al. 2008)</td>
</tr>
<tr>
<td>BC202</td>
<td>W3110, ΔyqjA::Tet&lt;sup&gt;R&lt;/sup&gt; ΔyghB:: Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Thompkins, Chattopadhyay et al. 2008)</td>
</tr>
<tr>
<td>BC203C</td>
<td>W3110, ΔyqjA::Tet&lt;sup&gt;R&lt;/sup&gt; ΔcpxR772:: Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>TB28</td>
<td>MG1655, lacIZYA&lt;&gt;frt</td>
<td>(Bernhardt and de Boer 2003)</td>
</tr>
<tr>
<td>RS28A</td>
<td>TB28, ΔyqjA::Tet&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Sikdar, Simmons et al. 2013)</td>
</tr>
<tr>
<td>RS28C</td>
<td>TB28, ΔcpxR772:: Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Sikdar, Simmons et al. 2013)</td>
</tr>
<tr>
<td>SK28AC</td>
<td>TB28, ΔyqjA::Tet&lt;sup&gt;R&lt;/sup&gt;, ΔcpxR772:: Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work</td>
</tr>
<tr>
<td>Plasmids</td>
<td></td>
<td></td>
</tr>
<tr>
<td>pBADHisA</td>
<td>Expression Vector; araBAD promoter, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>Invitrogen.</td>
</tr>
<tr>
<td>pACYC184</td>
<td>Low Copy Expression Vector; Tet&lt;sup&gt;R&lt;/sup&gt;, Kan&lt;sup&gt;R&lt;/sup&gt;</td>
<td>New England Biolabs.</td>
</tr>
<tr>
<td>pSK1</td>
<td>yqjA promoter responsive vector; Cam&lt;sup&gt;R&lt;/sup&gt;, pACYC184 (yqjA promoter – lacZ)</td>
<td>This work.</td>
</tr>
<tr>
<td>pSK2</td>
<td>alx responsive vector; Cam&lt;sup&gt;R&lt;/sup&gt;, pACYC184 (alx promoter – lacZ)</td>
<td>This work.</td>
</tr>
<tr>
<td>pSK3</td>
<td>nhaA responsive vector; Cam&lt;sup&gt;R&lt;/sup&gt;, pACYC184 (nhaA promoter – lacZ)</td>
<td>This work.</td>
</tr>
<tr>
<td>pBAD-YqjA</td>
<td>H&lt;sub&gt;6&lt;/sub&gt;-yqjA expression vector; araBAD promoter, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>(Boughner and Doerrler 2012)</td>
</tr>
<tr>
<td>pBAD-CpxR</td>
<td>H&lt;sub&gt;6&lt;/sub&gt;-cpxR expression vector; araBAD promoter, Amp&lt;sup&gt;R&lt;/sup&gt;</td>
<td>This work.</td>
</tr>
</tbody>
</table>
Table 6.2 PCR primers used in this study.

<table>
<thead>
<tr>
<th>Name</th>
<th>Sequence (5’-&gt;3’) [Restriction Sites Underlined]</th>
</tr>
</thead>
<tbody>
<tr>
<td>CpxR_FP (XhoI)</td>
<td>CGGCGCCTCGAGATGAATAAAAATCCTGTTAGTTG</td>
</tr>
<tr>
<td>CpxR_RP (HindIII)</td>
<td>GCCGGGCAAGCTTTTTCATGAAGCAGAAAACCATCAGATAGCCG</td>
</tr>
<tr>
<td>LacZ_FP (SphI)</td>
<td>CGCGGCATGCACCATGATTACGGATTCACCTGGCC</td>
</tr>
<tr>
<td>LacZ_RP (BamHI)</td>
<td>CGCGGGATCCCTTATTGAGCACCAGACCAACTG</td>
</tr>
<tr>
<td>YqjA_PR_FP (SalI)</td>
<td>CGGGGTCGACAAATCTGTTGGACAGGGCGCTGCAAATGCG</td>
</tr>
<tr>
<td>YqjA_PR_RP (SalI)</td>
<td>CGGGGCATGCATTCTTGGTAATCGGTTAACGCC</td>
</tr>
<tr>
<td>Alx_PR_FP (SalI)</td>
<td>CGGGGTCGACATTGCCAATTACGGATTACGGATTACGGATATGTAAC</td>
</tr>
<tr>
<td>Alx_PR_RP (SalI)</td>
<td>GCCGGGCATGCCATAGAAGTTTTACACATAAAAAAACG</td>
</tr>
<tr>
<td>NhaA_PR_FP (SalI)</td>
<td>CGGGTACCGTCTATGCGGATCAATGCGCTGAGA</td>
</tr>
<tr>
<td>NhaA_PR_RP (SalI)</td>
<td>CGGGGATGCACCATTGTATTATTTTCTCTTTCAGGTGAATAGATCG</td>
</tr>
</tbody>
</table>

6.3 Results

*Effect of pH on the expression of yqjA:* We have previously shown that the ΔyqjA mutant is alkaline sensitive (Kumar and Doerrler 2015). In order to further explore the physiological roles of yqjA, we analyzed the expression of yqjA at different pH’s. We used a ΔlacZYA parent strain, TB28 (Bernhardt and de Boer 2003) for our expression analysis study. In agreement with our previous observation, the RS28A (TB28ΔyqjA) (Table 6.1)
was also alkaline sensitive and was used similarly as BC203 (Table 6.1) depending upon requirements of the specific experiments. We constructed plasmids pSK1, pSK2, and pSK3 (Table 6.1), in which expression of lacZ was under the control of the yqjA, alx (ygjT), and nhaA promoter respectively (Figure. 6.1, A) and measured β-galactosidase activity (Miller units). We tested the expression of yqjA in the range of pH from 5 to 9.25. We found that expression of yqjA increased as the pH of the media was increased and decreased in the acidic pH range (Figure. 6.1, B) consistent with a previously published observation (Maurer, Yohannes et al. 2005). Previously, a microarray study has reported that yqjA expression is repressed at acidic pH (Maurer, Yohannes et al. 2005). Additionally, the alx (ygjT) gene appeared in the same acid low cluster as yqjA, showing its lowest expression at pH 5, higher expression at pH 7 and highest at pH 9 and served as a control for our experiment (Maurer, Yohannes et al. 2005). Alx (YgjT) is a predicted membrane bound redox regulator (Stancik, Stancik et al. 2002). We also observed the expression of alx was low at pH 5 and highest at pH 9 (Figure. 6.1, C) in agreement with the previous observation (Maurer, Yohannes et al. 2005). Furthermore, the expression of nhaA was strongly induced by the presence of Na+ ion at alkaline pH (Karpel, Alon et al. 1991; Dover and Padan 2001). We also observed the similar expression profile for nhaA in our experiment (Figure. 6.1, D). These observations suggest yqjA is induced at alkaline pH.

Effect of sodium or potassium on the expression of yqjA. We have also shown previously that Na+ and K+ are required for YqjA mediated growth at alkaline pH (Kumar and Doerrler 2015). We measured the expression profile of yqjA in response to changes in sodium or potassium in the medium at various pH. At pH 7, the expression of yqjA is not
Figure 6.1. Expression of the yqjA-promoter-lacZ fusion gene at different pH. (A). Schematic representation of the construct yqjA-promoter-lacZ fusion used to carry out expression analysis. TB28 containing plasmid pSK1 (B), pSK2 (C), or pSK3 (D) were cultured in LB(NaCl) media at 37°C at the desired pH and assayed for β-galactosidase activity. The alx(C) and nhaA(D) construct acted as control for the experiment and showed expression pattern as previously reported (Maurer, Yohannes et al. 2005) (Karpel, Alon et al. 1991). Each bar represents the average and standard deviation of three replicates of a particular experiment. The β-galactosidase activity is in miller units.

dependent on the presence of sodium or potassium ion (Figure 6.2, A), however as the pH of media (above pH > 8.5) was increased the expression of yqjA was increased with the increase in concentration of sodium or potassium (Figure. 6.2, B). We observed a similar pattern of expression with both chloride and gluconate form of sodium and potassium salts (Figure. 6.2, B). The effect of Na⁺ and K⁺ on expression was not connected to the change in osmolarity caused by ions since the addition of sucrose does not increase the expression of yqjA (Figure. 6.2, B). NhaA served as a control in our experiment since it is specifically regulated by Na⁺ or Li⁺ and not by ionic strength or osmolarity (Karpel, Alon et al. 1991).
In agreement with the previous study (Karpel, Alon et al. 1991), we also observed that the expression of *nhaA* was related specifically to Na\(^+\) ions, and not associated with a change in osmolarity caused by ions (Figure. 6.2, C and 6.2, D). These observations suggest that *yqjA* expression is induced in the presence of Na\(^+\) and K\(^+\) ion at alkaline pH.

![Graph of yqjA expression](image)

**Figure 6.2.** Effect of Na\(^+\) and K\(^+\) ions on the expression of the *yqjA* at pH 7 and pH 9. TB28 containing plasmid pSK1(A, B) or pSK3 (C, D) were cultured in LB media at 37\(^\circ\)C at the desired pH containing increasing concentrations (0mM, 100mM, 200mM) of either NaCl, KCl, Sucrose, Na-Gluconate, K-Gluconate and assayed for β-galactosidase activity. The *nhaA* construct acted as control for the experiment and showed an expression pattern as previously reported (Karpel, Alon et al. 1991). Each bar represents the average and standard deviation of three replicates of a particular experiment. The β-galactosidase activity is in miller units.

*Deletion of cpxR causes an increase in ΔyqjA mutant alkaline pH sensitivity:* It has been previously shown that the *E. coli* Δ*yqjA* mutant displays an induction of the Cpx stress response and is alkaline sensitive (Price and Raivio 2009; Sikdar, Simmons et al. 2013; Kumar and Doerrler 2015). CpxR is the cytoplasmic response regulator of the two-
component systems of Cpx stress response pathway and controls the expression of a plethora of genes in response to a variety of perturbations (e.g., pH changes, and change in membrane composition) related to the bacterial cell envelope (Raivio and Silhavy 2001). Additionally, the ΔcpxR mutant was shown to be alkaline sensitive signifying its critical role in the adaptation to alkaline pH stress (Danese and Silhavy 1998; Price and Raivio 2009). yqjA transcription is regulated by both CpxR (Price and Raivio 2009) as shown in Figure 6.3, (A) and σE (RpoE) (Dartigalongue, Missiakas et al. 2001). In the previous study of genes under the control of CpxR responsible for the growth at alkaline pH only yqjA mutant showed an alkaline sensitive phenotype (Price and Raivio 2009).

We wanted to know whether yqjA is the only gene under the control of CpxR responsible for alkaline pH tolerance or there are additional genes under CpxR regulation necessary for alkaline pH tolerance. The introduction of a ΔcpxR mutation into a ΔyqjA mutant resulted in increased alkaline pH sensitivity on both solid and liquid media (Figure. 6.3, B and 6.3, C) suggesting that there might be other genes apart from yqjA under the control of CpxR regulation required for alkaline tolerance. The overexpression of cpxR from a plasmid does not correct the alkaline pH sensitivity of ΔyqjA mutant (Figure. 6.3, D). Similarly, overexpression of yqjA does not provide alkaline pH tolerance to ΔcpxR mutant to wild-type level (Figure. 6.3, D). Furthermore, as expected deletion of cpxR reduces the expression of yqjA at alkaline pH (Figure. 6.3, E). Altogether, these results suggest that other genes in the CpxR regulon apart from yqjA are important for alkaline pH tolerance in E. coli.
Figure 6.3. Effect of ΔcpxR deletion in ΔyqjA mutant background. (A). The schematic representation of yqjA regulation by CpxR. (B). Parent E. coli W3110, ΔyqjA, ΔcpxR, BC203C (ΔyqjA, ΔcpxR) mutants were grown to mid-log phase till OD_{600}≈0.6 at pH 7 and then 5µl of serially diluted cells was spotted onto LB media plates at pH 7.0, 8.25, 8.5, 8.75, 9, and 9.25 and the plates were incubated at 37^\circ C for 20 to 24 hours. (C). All strains were grown till OD≈ 1.0 and inoculated into fresh LB media at pH 7, 8.25, 8.5, 8.75, 9.0. The growth of the cells at 37^\circ C were measured hourly. The error bars indicate standard deviations of three independent measurements. (D). The ΔyqjA, ΔcpxR, BC203C (ΔyqjA, ΔcpxR) mutants harboring the control vector pBAD or pBAD-yqjA, or pBAD-cpxR were grown to mid-log phase till OD_{600}≈ 0.6 at pH 7, and 5µl of serially diluted cells were spotted onto LB-Amp plates at pH 7, 8.5, 8.75, 9. The plates were then incubated at 37^\circ C for 20 to 24 hours. The growth at pH 7.0 was carried out in the presence of 0.002% arabinose, while growth at higher pH was in the presence of 0.02% arabinose (E). The expression of the yqjA at pH 7, 8, 8.5 in TB28 and TB28ΔcpxR strains. Each bar represents the average and standard deviation of three replicates of a particular experiment.
6.4 Discussion

For neutralophiles like *E. coli*, the maintenance of acidic cytoplasm relative to the surrounding environment is critical for the survival at alkaline pH. The proton motive force (PMF) consists of two components: the transmembrane proton gradient (ΔpH) and the membrane electrical potential (Δψ) (Krulwich, Sachs et al. 2011). The relative magnitudes of these two components help in controlling the cytoplasmic pH. Although there are numerous strategies for alkaline pH homeostasis, the cation/proton antiporters play a vital and dominant role (Padan, Bibi et al. 2005). In *E. coli*, there are five known cation/proton transporters that help in adaptation to alkaline conditions: NhaA, NhaB, ChaA, MdfA, MdtM (Padan and Landau 2016). *E. coli* utilizes these antiporters under different environmental conditions.

We have previously shown that the ΔyqjA mutant is sensitive to alkaline pH and that Na⁺ and K⁺ are required for YqjA mediated growth at alkaline pH (Kumar and Doerrler 2015). If any gene product is required for survival at alkaline pH then its expression should increase under alkaline conditions (Padan, Bibi et al. 2005). Therefore, we measured the expression of *yqjA* at various pH’s and salt concentrations. The expression of *yqjA* was higher at alkaline pH as compared to neutral pH (Figure 6.1, B) and expression was low at acidic pH (Figure 6.1, B) consistent with previous observations (Maurer, Yohannes et al. 2005). The expression profile of *alx* (Figure 6.1, C) and *nhaA* (Figure 6.1, D) was also in agreement with previous reports and served as controls in these experiments (Maurer, Yohannes et al. 2005) (Karpel, Alon et al. 1991).

We also observed that increasing the Na⁺ and K⁺ concentration in the medium does not have much effect on *yqjA* expression at pH 7 (Figure 6.2, A) but increases the
expression of \textit{yqjA} at alkaline pH (Figure. 6.2, B). NhaA, the main Na\textsuperscript{+}-K\textsuperscript{+}/H\textsuperscript{+} antiporter of \textit{E. coli} was suggested to function primarily at alkaline pH containing a high level of sodium (Sakuma, Yamada et al. 1998; Padan, Venturi et al. 2001; Shijuku, Saito et al. 2001). We observed using \textit{nhaA}-promoter-\textit{lacZ} gene fusion that the expression of \textit{nhaA} is induced specifically by Na\textsuperscript{+} and not by K\textsuperscript{+} or medium osmolarity (Figure. 6.2, C and 6.2, D) in agreement with the previous reports (Karpel, Alon et al. 1991; Dover and Padan 2001). YqjA mediated growth at alkaline pH required the presence of Na\textsuperscript{+} or K\textsuperscript{+} in the medium (Kumar and Doerrler 2015). Our present results suggest that \textit{yqjA} expression at high pH is specific to the presence of Na\textsuperscript{+} and K\textsuperscript{+} ions but independent of osmolarity of the medium. The presence of multiple systems for one function can be explained on the basis of different usage of multiple systems under different environmental conditions (Kobayashi, Saito et al. 2000) (Kobayashi, Saito et al. 1999; Shijuku, Yamashino et al. 2002). For example, the transcription of \textit{nhaA} is induced by Na\textsuperscript{+} or Li\textsuperscript{+} ions, however varying the pH by itself does not affect the expression but increases the sensitivity of expression towards Na\textsuperscript{+} or Li\textsuperscript{+} (Karpel, Alon et al. 1991) (Dover and Padan 2001). NhaB functions at neutral and acidic pH whereas ChaA extrude sodium ions at alkaline pH (Sakuma, Yamada et al. 1998; Shijuku, Yamashino et al. 2002). Moreover, \textit{chaA} expression is regulated by osmolarity and pH of the medium (Shijuku, Yamashino et al. 2002).

\textit{YqjA} is a member of Cpx regulon (Price and Raivio 2009) that gets activated in response to disturbances to the cell envelope and to periplasmic stress caused by alkaline conditions (Raivio and Silhavy 1999). The Cpx stress response pathway is controlled by a two-component system consisting of the sensor kinase CpxA and the response regulator CpxR (Raivio, Leblanc et al. 2013). CpxR activation results in upregulation of many
periplasmic proteases and chaperones. An important role in adaptation to alkaline pH was proposed for YqjA based on alkaline pH sensitivity of both ΔcpxR and ΔyqjA mutants (Price and Raivio 2009) which was further confirmed by us previously (Kumar and Doerrler 2015). The deletion of ΔcpxR in the ΔyqjA mutant background resulted in additional alkaline pH sensitivity suggesting the presence of another gene responsible for alkaline pH tolerance under CpxR regulation (Figure 6.3, A and 6.3, B). However, the expression of yqjA at alkaline pH is mainly under the CpxR control as deletion of cpxR abolishes induction of yqjA (Figure 6.3, E). Autokinase and phosphotransfer activities of the reconstituted CpxAR system were stimulated by KCl, NaCl whereas osmotic active solutes as glycine betaine, sucrose, and proline had no effect (Fleischer 2006).

YqjA and YghB are the best-studied members of the DedA/Tvp38 membrane protein family. Previously, we characterized the importance of YqjA/YghB in PMF-dependent processes in E. coli (Thompkins, Chattopadhyay et al. 2008; Sikdar and Doerrler 2010; Sikdar, Simmons et al. 2013; Kumar and Doerrler 2014; Kumar and Doerrler 2015). MdfA, a well-known Na⁺-K⁺/H⁺ antiporter, is a suppressor of the BC202 phenotypes and also partially rescues ΔyqjA mutant growth at alkaline pH (Sikdar, Simmons et al. 2013; Kumar and Doerrler 2014; Kumar and Doerrler 2015). Moreover, DedA proteins appear to share evolutionary structural fold similarity to LeuT, a member of neurotransmitter : sodium symporter family based on computational analysis (Khafizov, Staritzbichler et al. 2010). These observations suggest that YqjA and YghB may function as proton dependent membrane transporters (Sikdar, Simmons et al. 2013; Kumar and Doerrler 2014; Kumar and Doerrler 2015). The results of the present study further supports our previous observations and helps in better understanding the physiological role of yqjA in E. coli.
6.5 References


CHAPTER 7
CONCLUSIONS AND FUTURE DIRECTIONS

In the final chapter of this dissertation, the important findings from this research will be summarized in a point by point manner. Based on the present knowledge and experimental data, a model will be presented for YqjA/YghB proteins. Moreover, new experimental data that are not incorporated in the major chapters will also be presented. At the end, future experiments will be proposed as the extension of present findings to elucidate the function of these proteins.

7.1 Important Conclusions

YqjA and YghB of *E. coli* are the two best-characterized members of the conserved DedA/Tvp38 protein family. However, the actual function of these proteins is not known yet. In order to decipher the function of these proteins, we investigated the phenotypes of the Δ*yqjA* mutant and double mutant named BC202 (Δ*yqjA* Δ*yghB*). BC202 displays multiple phenotypes including cell division defects, drug sensitivity, and alkaline pH sensitivity, induction of Cpx and Psp stress responses, weak biofilm formation and motility defects. The research presented here mainly focuses on the alkaline pH sensitivity of Δ*yqjA* and drug sensitivity of BC202.

BC202 is highly sensitive to numerous compounds like ethidium bromide, benzalkonium chloride, methyl viologen, and acriflavine as compared to wild type W3110. However, BC202 is not hypersensitive to all biocides. Deletion of known efflux pumps causes additional drug sensitivity in BC202 suggesting the drug sensitivity phenotype is independent of other drug transporters. Overexpression of either YqjA or YghB corrects the drug sensitivity phenotypes, indication of an overlapping function of these proteins. Artificially increasing PMF either by overexpression of the Na⁺-K⁺/H⁺ antiporter MdfA or
growing BC202 in acidic media of pH 6 restores the drug sensitivity to most drugs signifying the indirect role of YqjA and YghB in *E. coli* drug resistance.

The Δ*yqjA* mutant is not able to grow at alkaline pH, unlike wild-type W3110. While YqjA and YghB show 62% amino acid similarity and redundant function, the Δ*yghB* mutant does not display alkaline sensitivity likely due to the presence of *yqjA* on the chromosome. However, the double mutant BC202 shows a more pronounced alkaline pH sensitivity. Overexpression of *yqjA* can complement the alkaline sensitivity of the Δ*yqjA* mutant, but only in the presence of Na⁺ or K⁺. Additionally, overexpression of *mdfA* or *yghB* partially rescues the growth of the Δ*yqjA* mutant at alkaline pH. Moreover, increasing the osmotic pressure also boosts the YqjA-mediated alkaline tolerance.

We found that membrane-embedded acidic amino acids, a hallmark of the proton-dependent transporters, are important for the function of YqjA and YghB. We also identified conserved arginines (Figure 7.1) which are present at the same position in YqjA and YghB and found that they are essential for the function of these proteins. Charged amino acids are known to play important roles in numerous secondary transporters.

The expression of *yqjA* is highly induced at alkaline pH as compared to pH 7. Moreover, the expression of *yqjA* is increased with increasing concentration of Na⁺ or K⁺ salt at alkaline pH, but not at pH 7. The osmolarity of the medium does not control the expression of *yqjA* since increasing the concentration of sucrose does not have much effect. Lastly, deletion of *cpxR* in Δ*yqjA* causes increase in alkaline pH sensitivity, suggesting that there may be other genes controlled by CpxR that play a role in alkaline pH tolerance.
7.2 Putative transporter function of YqjA and YghB in *E. coli*

Based on the genetic and physiological evidence, we propose that YqjA and YghB are novel membrane transporters linked to roles in the stabilization of membrane and quality control, and helping in the maintenance of PMF. Although the structural and mechanistic details of these proteins remain unclear, we propose a model (Figure 7.1) supported by knowledge gathered by us and other labs over the past several years in support of YqjA and YghB as putative membrane transporters and PMF maintenance. The evidence is summarized below:

The deletion of both genes in the same strain (BC202) causes cell division defects due to the inefficient export of periplasmic amidases by the twin-arginine transport pathways (Sikdar and Doerrler 2010) and drug sensitivity due to inefficient function of drug efflux pumps (Kumar and Doerrler 2014). The common factor for these phenotypes is the dependence on the PMF and we have shown using a JC-1 dye-based assay that BC202 has significant loss of PMF (Sikdar, Simmons et al. 2013).

A known transporter, MdfA, has the ability to complement the phenotypes of BC202 and ∆yqjA (Sikdar, Simmons et al. 2013; Kumar and Doerrler 2014; Kumar and Doerrler 2015). To date, two functions of MdfA have been reported that include efflux of drugs from the cytoplasm using the PMF and a role in alkaline pH homeostasis as a Na⁺-K⁺/H⁺ dependent transporter (Lewinson, Padan et al. 2004).

YqjA is required for the growth of *E. coli* at alkaline pH and Na⁺ or K⁺ is required for YqjA mediated growth at alkaline pH (Kumar and Doerrler 2015). Generally, cation/proton antiporters play a major role in alkaline pH homeostasis in *E. coli* (Padan,
Bibi et al. 2005). Moreover, expression of \textit{yqjA} increases at alkaline pH and also with the presence of Na\textsuperscript{+} and K\textsuperscript{+} alkaline pH (manuscript in preparation).

The importance of charged amino acids for the function of \textit{YqjA} and \textit{YghB}. Membrane-embedded acidic amino acids, a hallmark of proton-dependent transporters were shown to be essential for the function of \textit{YqjA} and \textit{YghB} (Figure 7.1) (Kumar and Doerrler 2014; Kumar and Doerrler 2015). Moreover, membrane-embedded basic amino acids like arginine are also critical for the function of \textit{YqjA} and \textit{YghB} (Figure 7.1) (Kumar, Bradley et al. 2016). Arginines have been shown to be important for membrane proteins involved in various biological processes like regulation of redox potential (Cutler, Davies et al. 1989), voltage detection (Tao, Xie et al. 2010) and proton transport (Hellmer, Teubner et al. 2003; Sigal, Vardy et al. 2005).

AlignMe, a novel program, showed the evolutionary similarity of DedA proteins to LeuT family of transporters based on hydropathy profile (Khafizov, Staritzbichler et al. 2010). The LeuT family transporters exist as a dimer of two 5-TM helices with a pseudo 2-fold axis symmetry and it was hypothesized that DedA proteins may adopt dual topology and form dimers as shown in Figure 7.2 (Faham, Watanabe et al. 2008; Khafizov, Staritzbichler et al. 2010). It was recently confirmed that \textit{YqjA} actually forms dimers in the membrane (Keller, Schleppi et al. 2015). A model of the structure of \textit{YqjA} based on the LeuT structure is shown in Figure 7.1.

The DedA homolog plays a role in selenite uptake in \textit{Ralstonia metallidurans} (Ledgham, Quest et al. 2005) and likely iron uptake in \textit{Clostridium} (Zhang, Ma et al. 2011). Apart from these, DedA domains appear in secondary transporters of the Tripartite ATP- independent periplasmic transporter (TRAP- T) superfamily.
Figure 7.1 Structural model of DedA/Tvp38 family member YqjA of *Escherichia coli* showing locations of cytoplasmic N- and C-termini and critical amino acids E39, D51, R130 and R136 performed using the (PS) 2-v2 Protein Structure Prediction Server and the structure of the repeat domain of LeuT (PDB: 2a65_A) as a template. The Figure was reproduced with permission from FEMS Microbiology Letters journal (Kumar, Bradley et al. 2016).

Figure 7.2 Proposed mechanistic model of YqjA and YghB representing the likely transporter function of YqjA and YghB. A model suggesting the role of YqjA and YghB in the PMF maintenance as a proton transporter. Previously, DedA proteins were hypothesized to form dimers in the membrane based on fold similarity to LeuT transporters (Khafizov, Staritzbichler et al. 2010) and take part in proton transport into the cytoplasm, in exchange of symport or antiport of yet to be identified substrate. Using a crosslinking approach it is now confirmed that YqjA forms homodimers (Keller, Schleppi et al. 2015). Moreover, we propose YqjA and YghB likely act as Na⁺-K⁺/H⁺ antiporter similarly to MdfA during PMF homeostasis (Lewinson, Padan et al. 2004).
Based on these observations, we proposed that YqjA and YghB form homodimers and act as proton transporters similarly to MdfA as shown in Figure 7.2. However, unless proved through biochemical assay, both symport or antiport of yet unknown substrate is a possibility and is something for future consideration. Therefore, potential future experiments are required to answer our hypothesis that YqjA and YghB are actually proton dependent transporters.

7.3 Additional interesting observations

Previously, our lab had shown that BC202 has impaired motility and produces outer membrane vesicles with increased amounts of flagellin subunit FliC (unpublished observation). These phenotypes suggest inefficient functioning of type III secretion system (T3SS), which might be due to loss of PMF in BC202. Moreover, B202 is unable to form biofilms (unpublished observations), a phenotype which might be linked to a motility defect (Pratt and Kolter 1998). Quorum sensing is a method in bacteria to control gene expression by recognizing the cell density through small signaling molecule such as autoinducer 2 (AI-2) that are secreted into the environment (Bassler 1999). AI-2 is known to stimulate biofilm formation in E. coli (Gonzalez Barrios, Zuo et al. 2006). DNA microarray studies in E. coli showed that AI-2 controls the expression of numerous genes including those for chemotaxis, motility, flagellar synthesis and virulence factor (DeLisa, Wu et al. 2001; Ren, Bedzyk et al. 2004). It is important to note that expression of yghB is induced by AI-2 quorum signaling and might play a role in biofilm formation (DeLisa, Wu et al. 2001). The occurrence of “C- group” DedA homologs- YabI and YohD as a multicopy suppressor for BC202 phenotypes make it difficult to analyze the function of YqjA and YghB (Thompkins, Chattopadhyay et al. 2008; Boughner and Doerrler 2012).
YabI also has the ability to rescue the ΔyqjA mutant growth at alkaline pH when overexpressed from a plasmid (unpublished observation). Alternatively, there is also a possibility of different roles for these homologs in contrast to YqjA and YghB, since they become important only in the absence of yqjA and yghB in *E. coli*. Using a transposon-directed insertion sequencing (TraDIS) screening approach, it was found that an *E. coli* ΔyabI mutant has modest IR survival rate as compared to wild-type cells (Byrne, Chen et al. 2014) suggesting a link between yabI and IR survival in *E. coli*. IR causes double-stranded breaks (DSBs) and other DNA damage. Therefore, genes involved in IR survival are suggested to play an essential role in bacteria.

**7.4 Future directions**

In spite of our genetic and physiological observations, mechanistic and structural information of DedA/Tvp38 family remains unclear. However, using LeuT as a template a structural model of a DedA homolog, Slr0305 of *Synechocystis* sp. PCC6803, was recently published (Keller, Ziegler et al. 2014). We know *E. coli* DedA family mutants (ΔyqjA and BC202) are growth-compromised at alkaline pH. Moreover, overexpression of yqjA can correct alkaline pH sensitivity only if adequate extracellular sodium or potassium is supplied (> ~100 mM). Additionally, the activity of the yqjA promoter also increases at elevated pH, but, again, only if extracellular cations are present. All the evidence points to Na\(^+\) or K\(^+\) as potential substrates and therefore symporter or antiporter assays using membrane vesicles are needed to determine the direction of transport. We also need to measure cytoplasmic pH under different extracellular pH’s to address the role of YqiA in alkaline adaptation and measure ΔΨ to address the energy requirements of transport. We think that the cytoplasmic pH of ΔyqjA and BC202 is likely to be more alkaline than
normal. Intracellular pH can be measured in live *E. coli* cells at several external pH’s by ratiometric fluorescence measurements of the pH sensitive, cell permeable fluorescent probe 2,7-bis-(2-carboxyethyl)-5-carboxyfluorescein (Holdsworth and Law 2013). Alternatively, the use of $^{14}$C-methylamine, which gathers in the cell in a ΔpH dependent manner is also used in some cases (Lewinson, Padan et al. 2004). Additionally, to know the stoichiometry of transport cycle or measure the ΔΨ we need to determine if YqjA-mediated transport is electrogenic using the ΔΨ-sensitive fluorophore [bis-(3-phenyl-5-oxoisooxazol-4-yl) pentamethine oxonol] (Holdsworth and Law 2013).

Furthermore, our observations show the expression of *yqjA* is highly induced at alkaline pH and with increasing concentration of Na$^+$ or K$^+$ salt. To further support our reporter gene assay results of *yqjA* expression and other DedA genes we will carry out quantitative PCR to determine the mRNA levels at the alkaline conditions.

Antibiotics are one of the most important discoveries of science, used by the healthcare units for reducing mortality and morbidity in severe bacterial infections (Leibovici, Paul et al. 2016). However, the extensive use of antibiotics has led to the rise of bacterial strains which can survive in the presence of multiple antibiotics (Putman, van Veen et al. 2000). The rise in multidrug resistant bacterial pathogens represents a global public health concern due to difficulty in finding efficient treatments (Kumar, He et al. 2016). There are numerous studies that suggest DedA proteins may represent potential drug targets and may be important for virulence in some species. A DedA homolog (BCG2664) from *Mycobacterium bovis* confers resistance to the antibiotic halicyclamine A, when overexpressed in *Mycobacterium smegmatis*, suggesting a probable target for this antibiotic (Arai, Liu et al. 2011). Additionally, DedA proteins seem to be necessary for
resistance to cationic peptides in both *Salmonella enterica* and *Neisseria meningitidis*. In *Salmonella*, the ΔyqjA mutant is susceptible to protamine and α-helical cationic peptide, including magainin 2 and melittin (Shi, Cromie et al. 2004). Covalent modifications to lipid A often provides resistance to cationic peptides (Raetz, Reynolds et al. 2007), however *Salmonella ΔyqjA* exhibits wild-type lipid A profile (Shi, Cromie et al. 2004). Therefore, the role of YqjA in providing resistance to cationic peptides in *Salmonella* is not yet clear. Additionally, a *Neisseria* NMB1052 (dedA) mutant was found hypersensitive to polymyxin (Tzeng, Ambrose et al. 2005). A combination of lipopolysaccharide (LPS) modification, efflux pumps and type IV pilin secretion is used by *Neisseria* to get resistance against cationic peptides (Tzeng, Ambrose et al. 2005; Lewis, Choudhury et al. 2009). The roles of DedA genes in *Neisseria* is also unclear, however, the similarity of the *Neisseria* and *Salmonella* DedA mutants further suggests these genes to be a potential drug targets. Many other pathogenic bacteria like *Pseudomonas, Burkholderia, Vibrio* and *Klebsiella* have either one or multiple DedA family genes in their genomes. Therefore, it will be interesting to study the roles of DedA family genes in the drug resistance of these pathogenic organisms. In short, studies of the DedA/Tvp38 family promise to fundamentally change our understanding of bioenergetics and physiology in bacteria.

### 7.5 References


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Figure 1.2
Figure 1.3

Title: Multiple Envelope Stress Response Pathways Are Activated in an Escherichia coli Strain with Mutations in Two Members of the DesA Membrane Protein Family

Author: Rakesh Siddar, Angelica R. Simmons, William T. Doerfler et al.

Publication: Journal of Bacteriology

Publisher: American Society for Microbiology

Date: Jan 1, 2013

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Title: Escherichia coli YqfA, a Member of the Conserved DdeA/Tvp38 Membrane Protein Family, Is a Putative Osmosensing Transporter Required for Growth at Alkaline pH

Author: Sujeeet Kumar, William T. Doerrier

Publication: Journal of Bacteriology
Publisher: American Society for Microbiology
Date: Jul 15, 2015
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