Investigations on the essentiality of the Escherichia coli DedA membrane protein family

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INVESTIGATIONS ON THE ESSENTIALITY OF THE *ESCHERICHIA COLI* DedA MEMBRANE PROTEIN FAMILY

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Dedication

In memory of my Aunt

Marie E. Rocheleau

June 27, 1927 – August 10, 2010
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Abstract

The DedA protein family is a highly conserved family of membrane proteins, with members present throughout all domains of life: Bacteria, Archaea, and Eukarya. Investigation of the DedA membrane protein family began with the isolation of BC202, an *Escherichia coli* mutant with in-frame deletions of two DedA proteins of unknown function (YqjA and YghB) that share 61% amino acid identity. BC202 demonstrates temperature sensitivity, inefficient cell division, an altered phospholipid composition, increased expression of extracytoplasmic stress response pathways, as well as an inability to maintain the cell membrane proton motive force (PMF). Additionally, *Borrelia burgdorferi* has a single DedA homolog (Bb0250), deletion of this gene was only possible in the presence of a cloned and inducible copy of the gene. The essentiality of the DedA membrane protein family in *B. burgdorferi*, instigated the investigation into the essentiality of the family in *E. coli*, which has eight individually non-essential DedA genes. Discussed herein is the generation of mutants in which all eight *E. coli* DedA genes are deleted, which again, was only possible in the presence of a cloned and arabinose inducible DedA protein. One mutant was further characterized, BAL801 (cloned EcDedA), identifying that the essential function of the *E. coli* DedA membrane protein family may be to play a vital role in the proper maintenance and segregation of the bacterial nucleoid. Also, complementation of BC202’s temperature sensitivity and cell division defects are complemented by only four of the eight *E. coli* DedA proteins (C-group; *yqjA, yghB, yohD*, and *yabI*), the remaining four were subsequently classified as non-complementing (NC-group; *ydjX, ydjZ, yqaA*, and *dedA*). The identification of the C- and NC-groups within *E. coli*'s DedA proteins, lead to the generation of another
mutant series in which the NC-group and the C-group proteins were independently deleted, to aide in the continued understanding of this essential and redundant membrane protein family.
I. Introduction

Life consists of three domains: Archaea, Bacteria, and Eukarya. A major distinguishing characteristic of the bacterial domain lies within the cell envelope – whether the organism is Gram-positive or Gram-negative, distinguished by a thick or thin peptidoglycan layer, respectively. The cell envelope of a Gram-negative cell is composed of an inner membrane (IM), periplasmic space (with a thin peptidoglycan layer), as well as a thicker and more complex outer membrane (OM) [Figure I-1 and (Ruiz, N, Kahne, D et al., 2006)]. *Escherichia coli*, is one of many Gram negative organisms and the model organism used in this work. The inner membrane of *E. coli* consists of mainly phospholipids [typically 70-80% phosphatidylethanolamine (PE), 15-20% phosphatidylglycerol (PG), and less than 5% cardiolipin (CL)], as well as integral membrane proteins (α-helical regions that cross the IM), and lipoproteins (present in the periplasmic space, but anchored to the outer leaflet of the IM). The highly fluid periplasmic space of *E. coli*’s cell envelope has, in addition to the peptidoglycan layer mentioned above, other soluble proteins. The more complex outer membrane in *E. coli* is asymmetric, such that the inner leaflet is composed of phospholipids, and lipopolysaccharides (LPS) in the outer leaflet.

I.1. Cell Division

*E. coli*, like most single celled organisms, undergoes a process of reproduction referred to as binary fission, or cytokinesis. The rod/bacillus shaped *E. coli* cell will elongate, while the genome is being replicated and subsequently cytokinesis will split the cell in half through the accumulation of multiple proteins (Dajkovic, A & Lutkenhaus, J, 2006) to the well-defined and monitored midpoint of the cell.
(Goehring, NW & Beckwith, J, 2005). The FtsZ ring (first step of cytokinesis) forms once the two daughter chromosomes have been segregated into the soon to be daughter cells (Figure I-2). The formation of the FtsZ ring begins the stepwise organization of multiple cell division proteins at a point midway (midcell) between the two poles of the elongating mother cell. Polymerization of the FtsZ ring instigates the formation of the cytokinesis apparatus, consisting of FtsA, followed by FtsEX, FtsK, FtsQ, FtsL and FtsB, FtsW, FtsI, FtsN, and AmiC (one of the amidases responsible for cleaving the peptidoglycan layer) [Figure I-3 and (Dajkovic, A & Lutkenhaus, J, 2006)]. The accumulation of some proteins involved in cytokinesis were found to depend on the proton-motive force of the inner membrane of the bacterial cell (Strahl, H & Hamoen, LW, 2010).

![Figure I-1](image)

**Figure I-1:** The cell envelope of *Escherichia coli*. The cell envelope of *E. coli* consists of an inner membrane (IM), periplasmic space (containing the peptidoglycan layer) and a more complex outer membrane (OM). The inner membrane is composed of phospholipids (PL), as well as integral membrane proteins (α-helical regions that cross the IM), and lipoproteins (proteins in periplasmic space, anchored to outer leaflet of the IM). The periplasmic space contains the peptidoglycan layer and other soluble proteins. The more complex outer membrane consists of phospholipids in the inner leaflet, and lipopolysaccharides (LPS) in the outer leaflet, where proteins are β-barrel (rather than α-helical). Figure reproduced with permission (Appendix I: Reproduction permissions, Chapter I) from Nature Reviews Microbiology, (Ruiz, N, Kahne, D et al., 2006).
Figure I-2: Cytokinesis in *Escherichia coli*.
Division of the *E. coli* cell is a highly complex and regulated process involving many different systems (see text and cited references for more detail). Figure demonstrates the general process by which the *E. coli* mother cell is split into two daughter cells (i.e. cytokinesis). Figure reproduced with permission (Appendix I: Reproduction permissions, Chapter I) from Current Biology, Elsevier (Goehring, NW & Beckwith, J, 2005).

Figure I-3: Proteins of the Cytokinesis apparatus.
Following the formation of the FtsZ ring at midcell, begins the stepwise assembly of the cytokinesis apparatus. Depicted, are the many proteins involved in splitting the bacterial cell. Figure reproduced with permission (Appendix I: Reproduction permissions, Chapter I) from Journal of Molecular Microbiology and Biotechnology (Dajkovic, A & Lutkenhaus, J, 2006).
I.2. The Bacterial Nucleoid

The *E. coli* genome consists of a single circular chromosome of approximately 4.6 megabases, a physical length of $1.6 \times 10^3 \mu m$, which is compacted into a 1 $\mu m$ region inside the cell, i.e. the nucleoid (Holmes, VF & Cozzarelli, NR, 2000). Therefore, it comes as no surprise, that the bacterial nucleoid has been found to be a highly ordered and organized structure, involving many different protein groups (Boeneman, K & Crooke, E, 2005; Niki, H, Jaffe, A et al., 1991; Petrushenko, ZM, She, WF et al., 2011; Strunnikov, AV, 2006; Wang, X, Llopis, PM et al., 2013). The initiator of DNA replication is DnaA-ATP, binding of this protein to the DnaA boxes in and around the origin of replication (*oriC*), results in DnaA-ADP. The regeneration of DnaA-ATP is a highly regulated process involving many mechanisms: regulatory inactivation of DnaA (RIDA), *datA* locus titration of DnaA (Camara, JE, Breier, AM et al., 2005; Kaguni, JM, 2006; Kasho, K & Katayama, T, 2013). As well as the recently identified *datA*-dependent DnaA-ATP hydrolysis pathway (DDAH), in which DnaA-ATP hydrolysis occurs in a *datA* locus- and IHF binding-dependent manner (Kasho, K & Katayama, T, 2013). The newly synthesized hemi-methylated DNA is bound by SeqA which sequesters the daughter chromosomal origins to the membrane, aiding in the limitation of chromosomal replication initiation to once per round of cell division. Another process is referred to as nucleoid occlusion, whereby the *E. coli* FtsZ ring is inhibited from forming (by SlmA and the Min proteins) until the two daughter chromosomes have been segregated into the soon to be daughter cells [Figure I-4 and (Wu, LJ & Errington, J, 2011)].
Nucleoid occlusion is the process by which the formation of the FtsZ ring is inhibited until the two newly replicated daughter chromosomes have been segregated to the soon to be new daughter cells. Process involves the Min proteins, and SlimA. Figure reproduced with permission (Appendix I: Reproduction permissions, Chapter I) from Nature Reviews (Wu, LJ & Errington, J, 2011).
There are many models currently under debate as to how the bacterial cell both maintains the genomic content within the cell, while still being able to efficiently transcribe required genes and replicate the genome. Some of those models consist of entropic forces (Jun, S & Mulder, B, 2006; Jun, S & Wright, A, 2010), in which the topological arrangement of the nucleoid, i.e. the twisting of the genome, results in the necessary compaction and organization of the genome, which was found to be plausible under slow growth conditions (low temperature and/or minimal media); however, the entropic force model was found insufficient under an increased growth rate (optimal temperature and rich media) (Hadizadeh Yazdi, N, Guet, CC et al., 2012). Another model is the co-transcriptional transertion (protein translocation) model (Woldringh, CL, 2002), in which transcription and subsequent translation of mRNA both occur while the newly synthesized membrane proteins are simultaneously inserted into the membrane. Discussed below is another newly proposed model by which the *E. coli* chromosome is maintained within the cell. In actuality, the nucleoid is likely organized through a collaborative effort including all proposed models.

I.3. DedA membrane protein family

The DedA membrane protein family is a group of proteins that is just beginning to be understood, all are predicted to locate to the inner membrane, and DedA proteins are found throughout all domains of life (Archaea, Bacteria, and Eukarya). In fact, greater than 90% of sequenced bacterial genomes contain a significant (BLAST E-value ≤ 10^{-4}) homolog of the DedA membrane protein family [discussed more below; (Doerrler, WT, Sikdar, R et al., 2013)]. The number of DedA homologs present within
an organism can vary from as many as eight [like in *E. coli* (Blattner, FR, Plunkett, G et al., 1997)] to as few as one [as in *Borrelia burgdorferi* (Fraser, CM, Casjens, S et al., 1997)]. The eight DedA genes in *E. coli* are individually non-essential (Baba, T, Ara, T et al., 2006), and consist of *yqjA, yghB, yohD, yabI, ydjX, ydjZ, yqaA*, and *dedA*; herein the *E. coli* *dedA* gene/protein is referred to as Ec*dedA*/EcDedA to avoid confusion with the DedA membrane protein family.

I.3.1. Two *Escherichia coli* DedA proteins, YqjA and YghB, are together required for normal cell division and growth at elevated temperatures

The first significant DedA mutant to be isolated and characterized was BC202 – an *Escherichia coli* strain with deletions of two DedA genes (Δ*yqjA::tet*R, Δ*yghB::kan*R) that share 61% amino acid identity. The initially identified characteristics of BC202 consisted of temperature sensitivity, inefficient cell division, and an inner membrane with increased acidic phospholipids (phosphatidylglycerol and cardiolipin) (Thompkins, K, Chattopadhyay, B et al., 2008). The cell division defect of BC202 was later found to be due to inefficient export of amidase proteins (AmiA and AmiC) by the twin arginine transport (TAT) pathway (Sikdar, R & Doerrler, WT, 2010). It was recently identified that BC202 also has increased expression of extracytoplasmic stress response pathways (Bae, Cpx, Rcs, and Psp) as well as the inability to properly maintain the proton motive force (PMF) (Sikdar, R, Simmons, AR et al., 2013).
I.3.2. The single *Borrelia burgdorferi* DedA family protein, Bb0250, is essential

While the *E. coli* genome contains eight individually non-essential DedA genes (Baba, T, Ara, T *et al.*, 2006; Blattner, FR, Plunkett, G *et al.*, 1997), *Borrelia burgdorferi* contains only one DedA gene, annotated as *bb0250* (Fraser, CM, Casjens, S *et al.*, 1997). In order to investigate the essentiality of the DedA family and to expand our knowledge of DedA family function, a mutant of *B. burgdorferi* was created, in which *bb0250* was deleted [strain DXL-01; (Liang, FT, Xu, QL *et al.*, 2010)]. *B. Borrelia Δbb0250* could only be generated in the presence of a cloned and inducible copy of Bb0250 (Liang, FT, Xu, QL *et al.*, 2010). Under non-inducing conditions, DXL-01 exhibits inefficient cell division and membrane deformities, all preceding cell death (Liang, FT, Xu, QL *et al.*, 2010). In other words, the borrelia DedA mutant phenotypes resemble those of BC202, except that DXL-01 requires *bb0250* at all temperatures for viability. In addition, cloned *bb0250* can fully complement the growth and cell division phenotypes of BC202, even though Bb0250 displays only ~19% amino acid identity to *E. coli*’s YqjA (Liang, FT, Xu, QL *et al.*, 2010). These results demonstrate a high degree of functional conservation for DedA family proteins among two widely distributed Gram-negative organisms.

I.4. The DedA Domain

The DedA membrane protein family is widely conserved, with members present throughout all domains of life (Archaea, Bacteria and Eukarya). The polypeptides belonging to the DedA family typically contain 4-6 predicted transmembrane

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domains, between 150 and 250 amino acids and a conserved domain. This DedA Domain contains both transmembrane and cytoplasmic domains, as well as a likely amphipathic helix. A strictly conserved amino acid sequence is not present across the entire domain; however, there is at least one universally conserved glycine residue which occurs in or near the potential amphipathic helix (Figure I-5a), and is found in all defined DedA proteins (NCBI Clusters of Orthologous Groups, COG0586; The DedA Domain).

I.5. Phylogenetic Distribution of the DedA membrane protein family

To investigate the distribution of the DedA protein family, we scanned a total of 350 sequenced bacterial genomes (Wu, DY, Hugenholtz, P et al., 2009) and 100 sequenced archaeal genomes (Brochier-Armanet, C, Forterre, P et al., 2011) found in the NCBI database with Protein BLAST using amino acid sequences of all eight E. coli DedA proteins. Using a very conservative Protein BLAST score (E-value ≤10^{-4}) as significant, we found that 33 (9.2%) of bacterial species and 27 (27%) of archaeal species lack a significant DedA homolog (Figure I-5b, c). The largest proportion of bacterial species that lack a significant DedA homolog can be found in the phylum Tenericutes (with 13/16 species lacking a clear DedA homolog), followed by the Thermotogae (5/5) and Alphaproteobacteria (4/46). As for the Archaeal domain, the largest proportion of species lacking a significant DedA homolog is in the Euryarchaeota (14/70) and Crenarchaeota (12/24) phyla. However it is important to note that the majority of sequenced Archaeal species fall within the Euryarchaeota phylum (Brochier-Armanet, C, Forterre, P et al., 2011).
Figure I-5: Phylogenetic analysis of the DedA Protein Family.
(a) Alignment of *E. coli* DedA proteins, and homologs found in *Borrelia burgdorferi*, *Mycobacterium bovis*, and *Helicobacter pylori* from the NCBI database to illustrate the DedA Domain, COG0586 (boxed in region). Predicted Transmembrane (TM) domains are highlighted in Green, and partial TM regions, possibly amphipathic helices, are highlighted in Blue. The singularly conserved amino acid residue of the DedA Domain (glycine) is in bold. Of interest, the only conserved glycine residue is in or near the amphipathic helix for all aligned members. TM prediction software used was TMHMM (Krogh, A, Larsson, BÈ et al., 2001). (b) Bacterial and (c) Archaeal Domain representative trees. Numbers in red demonstrate the proportion of species lacking a significant DedA homolog (Protein BLAST E-value ≤10^{-4}) in each bacterial (b) or archaeal (c) phylum. Otherwise, all species of the phylum contain at least one significant DedA homolog (numbers in blue). Phylogenetic trees were constructed with MEGA (Tamura, K, Peterson, D et al., 2011), using a single 16S rRNA sequence from a representative species of each phylum. Previously published phylogenetic trees for both Bacteria (350 species) (Wu, DY, Hugenholtz, P et al., 2009) and Archaea (100 species) (Brochier-Armanet, C, Forterre, P et al., 2011), were used as a basis for phylogenetic analyses, though additional species were investigated, presented values are solely from published trees. Significant DedA homologs are found within the *Thermotogae* phylum although not in the five completed genomes analyzed.
Figure I-5
The presence of a significant DedA homolog is not consistent among organisms of similar habitats; for example, a significant DedA homolog is present within *Neisseria* spp., *Mycoplasma synoviae/fermentans*, *Anaplasma phagocytophilum* and *Ehrlichia chaffeensis*, but absent in *Chlamydia*, *Ureaplasma*, and *Neorickettsia sennetsu* (Detels, R, Green, AM et al., 2011; Hotopp, JCD, Lin, MQ et al., 2006; Larsen, B & Hwang, J, 2010). In fact, a DedA member is not present among any of the sequenced *Chlamydia/Chlamydophilia* spp. or *Ureaplasma* spp.; however, *Neisseria* spp. have multiple, and some very significant (E-value <10^{-100}) DedA homologs. Interestingly, the majority of *Mycoplasma* spp. and *Rickettsia* spp. do not have any significant DedA members, but DedA homologs are present within the Genera, ex. *Mycoplasma synoviae/fermentans*, and *Rickettsia felis/bellii* (Kowalczewska, M, Vellaiswamy, M et al., 2012; Sprong, H, Wielinga, PR et al., 2009). The majority of *Clostridium* spp. (including *C. botulinum*) do have a DedA homolog, whereas *Clostridium thermocellum* does not (Nerandzic, MM, Cadnum, JL et al., 2012; Shao, XJ, Raman, B et al., 2011; Siddiqui, AR & Bernstein, JM, 2010; Zhang, W, Ma, J et al., 2011). There are many additional genera in which the presence of a DedA homolog is variable, for example *Psychrobacter*, *Bartonella*, and *Mycobacterium* (Liu, QY, Eremeeva, ME et al., 2012; Wirth, SE, Ayala-Del-Rio, HL et al., 2012). The significance of the observed distribution of the DedA protein family is as yet unclear.

Another inconsistency among the distribution of DedA homologs is among the reduced genome symbionts and obligate symbionts of various organisms. The DedA family is found in the genomes of several symbionts including *Wigglesworthia*
glossinidia (Akman, L, Yamashita, A et al., 2002) and Buchnera spp. (Shigenobu, S, Watanabe, H et al., 2000). Some symbionts that lack a DedA homolog are “Candidatus Sulcia muelleri”, “Candidatus Amoebobhilus asiaticus”, “Candidatus Phytoplasma mali”, “Candidatus Zinderia insecticola”, “Candidatus Carsonella ruddii”, “Candidatus Hodgkinia cicadicola”, and “Candidatus Tremblaya princeps” [reviewed in (Mccutcheon, JP & Moran, NA, 2011)]. The possibility exists that this variability of the DedA distribution is related to the genetic makeup and/or physiology of the symbionts’ host species (Mccutcheon, JP & Moran, NA, 2010; Moran, NA, Tran, P et al., 2005; Penz, T, Horn, M et al., 2010).

As for the Archaeal domain, the distribution of DedA members is quite unpredictable; one note of interest is that no sequenced genomes within the Halobacteriales or Sulfolobales orders lack a significant DedA homolog. Also, the reduced genomes of archaeal species, “Candidatus Parvarchaeum spp.” and “Candidatus Micrarchaeum acidiphilum”, all contain a significant DedA homolog (Baker, BJ, Comolli, LR et al., 2010). The presence of DedA homologs within several reduced genomes, both bacterial and archaeal, further supports the essentiality of the DedA protein family for species viability. Published phylogenetic trees of the Bacterial and Archaeal domain were used as a guide for investigating the distribution of DedA members (Brochier-Armanet, C, Forerre, P et al., 2011; Wu, DY, Hugenholtz, P et al., 2009).

In regards to the distribution of the DedA protein family, there may be subtle differences between organisms that lack a DedA homolog, which has allowed for a select few to counteract the necessity of DedA proteins. For example, it is possible
that other proteins have taken over their role, or symbiotic relationships enable this selective genotypic evolution. Regardless, these species have found a way to exist without the DedA protein family. It is important to note however, that the identification of significant DedA homologs using a strict BLAST score cutoff (E-value $\leq 10^{-4}$) may have overlooked DedA family members with lower sequence identity to the *E. coli* DedA proteins.

I.6. Investigations of the *Escherichia coli* DedA membrane protein family

The beginnings with BC202 instigated investigations into the DedA membrane protein family, leading to the identification that this family is functionally redundant and collectively essential with intriguing prospects for future understandings of organismal physiology across all domains of life. Discussed herein is the identification of functional, and possibly evolutionarily related groups within the *E. coli* DedA membrane protein family, as four out of the eight *E. coli* DedA proteins are able to complement (C-group; *yqjA, yghB, yabI*, and *yohD*) BC202’s temperature sensitivity and cell division inefficiency [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012)]. The remaining four *E. coli* DedA proteins were grouped into the non-complementing group (NC-group; *ydjX, ydjZ, yqaA*, and *Ec*dedA*) since they could not restore normal growth to BC202 [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012)]. The *E. coli* DedA membrane protein family was also identified as being a collectively essential group of proteins, where generation of a mutant deleted for all eight *E. coli* DedA proteins was only possible in the presence of a cloned and inducible DedA protein (namely EcDedA) [Chapter II, pages 16-44;
(Boughner, LA & Doerrler, WT, 2012)]. The essential function of the *E. coli* DedA membrane protein family, shared among all DedA members, was proposed to play a critical role in the maintenance and organization of the bacterial nucleoid [Chapter III, pages 45-74; (Boughner, LA, Kumar, S et al.)]. Finally, utilizing the identification of the *E. coli* functional DedA protein C- and NC-groups, a subset of mutants were generated in order to help further characterize the non-essential functions of each *E. coli* DedA membrane protein (Chapter IV, pages 75-93; Manuscript in preparation). Also investigated, was the potential for mutant generation in other organisms to further investigate and identify potentially similar functional groups (C- and NC-groups) as was found in *E. coli* (Chapter IV, pages 75-93; Manuscript in preparation).
II. Multiple Deletions Reveal The Essentiality of The DedA Protein Family in *Escherichia coli*

II.1. Introduction

The DedA protein family is a highly conserved family of inner membrane proteins, present throughout all Domains of life. At present, there are more than 3000 members of the DedA family found in the NCBI genome database (Khafizov, K, Staritzbichler, R *et al.*, 2010). The number of DedA homologs present within an individual bacterial genome can vary from one, as in *Borrelia burgdorferi* (Fraser, CM, Casjens, S *et al.*, 1997) to as many as eight as in *E. coli* (Blattner, FR, Plunkett, G *et al.*, 1997). These eight *E. coli* DedA family genes are annotated yqjA, yghB, yabI, yohD, yqaA, ydjX, ydjZ and dedA (*dedA* gene is referred to herein as *EcdedA* to avoid confusion). Deletion of any single DedA family gene in *E. coli* results in a strain without a growth phenotype, indicating that these genes are individually nonessential (Baba, T, Ara, T *et al.*, 2006). Our previous work on the DedA family in *Escherichia coli* suggests a role in maintaining envelope integrity (Sikdar, R & Doerrler, WT, 2010; Thompkins, K, Chattopadhyay, B *et al.*, 2008). BC202 (W3110; ΔyqjA::tetR, ΔyghB::kanR) is an *E. coli* mutant with deletions of two of the DedA family genes, yqjA and yghB, encoding inner membrane proteins with 61% amino acid identity. We found that BC202 is not viable at 42 °C and displays defects in cell division at all growth temperatures (Thompkins, K, Chattopadhyay, B *et al.*, 2008).

The cell division deficiency of BC202 is known to be caused by inefficient export of periplasmic amidases AmiC and AmiA by the twin arginine transport (TAT)

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2 Reprint permission granted from the Society of General Microbiology (Boughner, LA & Doerrler, WT, 2012). See Appendix II: Reprint Permissions Chapter II
pathway, leading to an accumulation of these proteins in the cytoplasm (Liang, FT, Xu, QL et al., 2010). Growth and cell division defects of BC202 can be corrected by overexpression of the TatABC operon from a plasmid; however, BC202 is unlike both $\Delta$tat and $\Delta$ami mutants in several respects. $\Delta$tat (Ize, B, Stanley, NR et al., 2003; Stanley, NR, Findlay, K et al., 2001) and $\Delta$ami (Heidrich, C, Ursinus, A et al., 2002) mutants grow at elevated temperatures and display pleiotropic outer membrane defects unlike BC202. BC202 is not sensitive to detergents or antibiotics, indicative of an intact outer membrane (Doerrler, WT, 2006; Thompkins, K, Chattopadhyay, B et al., 2008). Therefore, while inefficiency of the TAT pathway in BC202 is responsible for the cell division defect, this is likely a secondary effect of additional, yet unknown, roles of DedA family proteins in cellular physiology.

Insight into the DedA family came from studies involving the Lyme disease pathogen Borrelia burgdorferi (Liang, FT, Xu, QL et al., 2010). Unlike many bacteria, the borrelia genome harbors only a single DedA family gene, annotated bb0250 (Fraser, CM, Casjens, S et al., 1997). bb0250 encodes a membrane protein that displays only 19% amino acid identity to E. coli YqjA. In spite of this low level of sequence identity, expression of Bb0250 can restore growth and normal cell division to E. coli mutant BC202. This suggests a high degree of functional conservation between these proteins of two evolutionarily distinct species, B. burgdorferi and E. coli. A borrelia $\Delta$bb0250 mutant (DXL-01) expressing an IPTG-inducible copy of bb0250 displays cell division defects when grown in the absence of IPTG prior to cell death and lysis (Liang, FT, Xu, QL et al., 2010). Additionally, the phenotypes seen in DXL-01 are independent of any role the DedA proteins may play in the TAT pathway,
since the *B. burgdorferi* genome does not encode homologs of TatABC or any proteins with a predicted TAT dependent signal peptide (Dilks, K, Rose, RW *et al.*, 2003).

Additional evidence that the DedA protein family carries out essential functions in bacteria came from the identification of a *Mycobacterium bovis* DedA homolog (Bcg2664) that confers resistance to the antibiotic halicyclamine A, when expressed in *Mycobacterium smegmatis* (Arai, M, Liu, L *et al.*, 2011). Bcg2664 shares significant sequence identity with five of the eight *E. coli* DedA proteins (EcDedA, YohD, YqjA, Yabl, and YghB); all with BLAST E-values <10^-8 (Sayers, EW, Barrett, T *et al.*, 2012). Halicyclamine A is an alkaloid isolated from an Indonesian marine sponge as an antidormant mycobacterial substance (Arai, M, Sobou, M *et al.*, 2008; Jaspars, M, Pasupathy, V *et al.*, 1994). The ability of the overexpressed *bcg2664* gene to confer resistance to this substance suggests that it may encode the target of this antimycobacterial compound (Arai, M, Liu, L *et al.*, 2011). Further studies are needed to define the target of this drug in mycobacteria but the finding that a DedA protein may encode a drug target is consistent with our studies suggesting the general essentiality of the DedA family.

In order to extend our knowledge of the DedA family, we describe here an *E. coli* strain with deletions of all eight DedA family genes (*ydjX, ydjZ, yabI, EcdedA, yohD, yqjA, yqaA* and *yghB*). We found this was possible only if one family member was expressed from an inducible promoter and the strain was grown in the presence of inducer (arabinose). The strains we describe here are termed BAL801 (*E. coli* mutant deleted for all eight DedA family genes, harboring pBAD_EcdedA) and
BAL802 (harboring pBAD_yqjA). Growth in the presence of glucose, to repress gene expression, causes each of these strains to undergo lysis and die. The essentiality of the DedA family was also found to be independent of a DedA family member’s ability to complement BC202 for temperature sensitivity and cell division defects. We discovered two functional groups in the E. coli DedA protein family, those that complement BC202 are in the C-group (Complementing group; YqjA, YghB, YabI, and YohD), and those that do not complement BC202 belong to the NC-group (Non-Complementing group; EcDedA, YdjX, YdjZ and YqaA). These results demonstrate that in spite of extensive gene redundancy in this well characterized model organism, the DedA protein family is essential in E. coli. These studies also have allowed us to begin to functionally dissect the roles of the E. coli DedA protein family in regards to cell division, physiology and cell viability.

II.2. Materials and Methods

Materials: All materials were purchased from VWR International, Sigma-Aldrich, New England Biolabs or Qiagen.

Microbiological growth conditions: All bacterial cultures were grown in Luria-Bertani medium (LB: yeast extract 5 g L⁻¹, tryptone 10 g L⁻¹, NaCl 10 g L⁻¹ and a pH of 7) with respective antibiotics 10 μg chloramphenicol ml⁻¹, 30 μg kanamycin ml⁻¹, 100 μg ampicillin ml⁻¹, and where indicated supplemented with 0.2% glucose (w/v) or 0.1% arabinose (w/v) (unless otherwise noted). All cultures were grown at 30 °C unless otherwise stated.

Plasmid construction: E. coli genes ydjX, ydjZ, yabI, yohD, EcdedA, yqaA, yqjA, and yghB were amplified from genomic DNA prepared from strain W3110A using the
primers listed in Table II-1. PCR products and pBADHisA vector (Invitrogen) were purified on a QIAquick PCR purification kit (Qiagen, Valencia CA), and digested with appropriate restriction enzymes (Table II-1). Digested pBADHisA was subsequently treated with Antarctic phosphatase. PCR product and vector were ligated using T4 DNA ligase. Ligation reactions were transformed into chemically competent XL1blue cells, ampicillin resistant colonies were selected and plasmid DNA was isolated using QIAquick Spin Miniprep kit (Qiagen). DNA sequencing (primers listed in Table II-2) was conducted at the LSU College of Basic Science Genomics Facility and confirmed the sequences of all cloned PCR products.

Table II-1: Primers used to amplify DedA genes and clone into pBADHisA. Restriction sites are underlined.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence 5’ to 3’</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>XFRE_ydjX</td>
<td>GCGGCCTCAGATGAACGCTGACGGTAAATTTC (<strong>XhoI</strong>)</td>
<td>This Study</td>
</tr>
<tr>
<td>XRRE_ydjX</td>
<td>GCGACGATTCCTATCTCCATTTTTTGAGGTAGT (<strong>HindIII</strong>)</td>
<td>This Study</td>
</tr>
<tr>
<td>XFRE2_ydjZ</td>
<td>GGCACCCCTCAGATGATGATGATGACATCGGAAA (<strong>XhoI</strong>)</td>
<td>This Study</td>
</tr>
<tr>
<td>XRRE2_ydjZ</td>
<td>CGGCAGCATGCTCTGAGGCGTTC (<strong>HindIII</strong>)</td>
<td>This Study</td>
</tr>
<tr>
<td>FRE_yabl</td>
<td>GCGACGCTCAGATGACCGATTGCTGGAACAC (<strong>XhoI</strong>)</td>
<td>This Study</td>
</tr>
<tr>
<td>RRE_yabl</td>
<td>CGACTAAGCTTCAACACACCACTTTACGC (<strong>HindIII</strong>)</td>
<td>This Study</td>
</tr>
<tr>
<td>FRE_yqiA</td>
<td>GCGAGCCTCAGATGAGCTTTGGACACTTGTGACCACATGCT (<strong>XhoI</strong>)</td>
<td>This Study</td>
</tr>
<tr>
<td>RRE_yqiA</td>
<td>CGGCAGCATGCTCTGAGGCACCACTTTACGC (<strong>HindIII</strong>)</td>
<td>This Study</td>
</tr>
<tr>
<td>FRE_yqaA</td>
<td>GCGACGCTCAGATGACCGATTGCTGGAACAC (<strong>XhoI</strong>)</td>
<td>This Study</td>
</tr>
<tr>
<td>RRE_yqaA</td>
<td>CGACTAAGCTTCAACACACCACTTTACGC (<strong>HindIII</strong>)</td>
<td>This Study</td>
</tr>
<tr>
<td>FRE_yghB</td>
<td>GCGACGCTCAGATGACCGATTGCTGGAACAC (<strong>XhoI</strong>)</td>
<td>This Study</td>
</tr>
<tr>
<td>RRE_yghB</td>
<td>CGACTAAGCTTCAACACACCACTTTACGC (<strong>HindIII</strong>)</td>
<td>This Study</td>
</tr>
<tr>
<td>FRE_EcdedA</td>
<td>GCCCATGGATGCCACCATGACCATGCC (<strong>NcoI</strong>)</td>
<td>This Study</td>
</tr>
<tr>
<td>RRE_EcdedA</td>
<td>GCAAGCTTGAACCAGGGACTTATTTATTCATC (<strong>NcoI</strong>)</td>
<td>This Study</td>
</tr>
<tr>
<td>FRE_yohD</td>
<td>GGCATGGATCTCAATACACCTTATTCAC (<strong>NcoI</strong>)</td>
<td>This Study</td>
</tr>
<tr>
<td>RRE_yohD</td>
<td>GCAAGCTTGAACCAGGGACTTATTTATTCATC (<strong>HindIII</strong>)</td>
<td>This Study</td>
</tr>
</tbody>
</table>
Table II-2: PCR Primers used to confirm genotypic composition of mutants.

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequence 5’ to 3’</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>FfP_ydjX</td>
<td>CGCTAATTTAGCAGCTCTCCTG</td>
<td>This Study</td>
</tr>
<tr>
<td>RfP_ydjX</td>
<td>CGTATGGTTATTGGCATCGACA</td>
<td>This Study</td>
</tr>
<tr>
<td>FfP_ydjZ</td>
<td>GCAGCAGAAGAGAAGAAAACGGGT</td>
<td>This Study</td>
</tr>
<tr>
<td>RfP_ydjZ</td>
<td>GCTGACAAGCGATCGTACTACCG</td>
<td>This Study</td>
</tr>
<tr>
<td>FfP_Yabl</td>
<td>TGACGATCAACTCTATTCTGCG</td>
<td>This Study</td>
</tr>
<tr>
<td>RfP_Yabl</td>
<td>GCCTGGCAAGGGATGACCA</td>
<td>This Study</td>
</tr>
<tr>
<td>FXfP_EcededA</td>
<td>GCCGTGCAATGCCCAGTCCCAGA</td>
<td>This Study</td>
</tr>
<tr>
<td>RXfP_EcededA</td>
<td>ACATCTTTCGGCTCAAGCTCGC</td>
<td>This Study</td>
</tr>
<tr>
<td>FfP_YohD</td>
<td>GATGGGAAAAACAGTCCCCAGAC</td>
<td>This Study</td>
</tr>
<tr>
<td>RfP_YohD</td>
<td>ACACCACGGGCAGTCGTTG</td>
<td>This Study</td>
</tr>
<tr>
<td>FfP_YqjA</td>
<td>GAACAACGTTGACCTTTTGTTAC</td>
<td>This Study</td>
</tr>
<tr>
<td>RfP_YqjA</td>
<td>AAAGGAATGCCATGAGCGTC</td>
<td>This Study</td>
</tr>
<tr>
<td>FfP_yqaA</td>
<td>GGATTACGCCATTATTTTGACG</td>
<td>This Study</td>
</tr>
<tr>
<td>RfP_yqaA</td>
<td>ACCTAATGCTTCAGGATGACC</td>
<td>This Study</td>
</tr>
<tr>
<td>FfP_YghB</td>
<td>TCTGGAAGATGTCGACGATCTG</td>
<td>This Study</td>
</tr>
<tr>
<td>RfP_YghB</td>
<td>AGGCCGGATGATGATCATCC</td>
<td>This Study</td>
</tr>
<tr>
<td>FP_pBAD</td>
<td>ATGCCATAGCATTAGTTTATCC</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>RP_pBAD</td>
<td>GATTTAATCTGTATCC</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

E. coli chromosome engineering: λ. Red recombination was used for gene replacement during strain generation (Yu, DG, Ellis, HM et al., 2000). Briefly, primers were designed with 50 bp flanking ydjXYZ plus 20 bp for amplifying the chloramphenicol cassette. Amplified ydjXYZ::cam product was electroporated into DY330. CamR cells were isolated and the gene deletion was confirmed with PCR using flanking primers. A P1 phage lysate prepared from the camR strain was subsequently used to transduce W3110, resulting in the generation of BAL202 (Table
II-3). PCR and DNA sequencing, using the primers listed in Table II-2, verified gene deletions in all mutant strains. For each gene in *E. coli*, there is an available single deletion, kanamycin resistant (kan\(^R\)) strain from the Keio collection (Baba, T, Ara, T *et al.*, 2006) (Table II-3). A P\(_1\) phage lysate generated from each of the single DedA deletion strains was then used to generate each of the successive deletions. The order of gene deletion is described in Figure II-1 (page 26). Each kan\(^R\) cassette is flanked by FLP recombinase recognition target (FRT) sites so the same selectable marker was used for each successive deletion, following removal of the prior kan\(^R\) cassette. By transforming each new strain with the temperature sensitive pCP20 plasmid expressing FLP recombinase (Table II-4), the FRT flanked kan\(^R\) cassette was excised during the first overnight growth and the plasmid cured by a second overnight growth at 42 °C (Cherepanov, PP & Wackernagel, W, 1995; Datsenko, KA & Wanner, BL, 2000). In place of the kan\(^R\) cassette remains an 81 bp scar sequence encoding a 27 residue internal peptide (referred to herein as a scar peptide). Deletion of *yghB* was reserved for last since this step generated the first temperature sensitive strain precluding the ability to cure pCP20.

**Transformation:** Chemically competent cells were prepared and transformations were carried out according to Inoue (Inoue, H, Nojima, H *et al.*, 1990). Certain mutants, especially those of the BAL700 series and BAL420 were not efficiently transformed using the Inoue method and were instead subjected to electroporation. Electroporation was carried out in a MicroPulser electroporator (Bio-Rad) according to the manufacturer’s instructions.
Table II-3: Genotypic descriptions of strains and mutants used in this study.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>W3110</td>
<td>Wild type; F-, Δ, ΔIN(rrnD-rrnE)1, rph-1</td>
<td>E. coli genetic stock center, Yale University</td>
</tr>
<tr>
<td>W3110A</td>
<td>aroA::Tn10 (Te1 P1,wv transductant of W3110; LCB273 donor)</td>
<td>Doerrler, WT, Gibbons, HS et al., 2004</td>
</tr>
<tr>
<td>BC202</td>
<td>W3110, ΔyqjA::tcr, ΔyghB::kanR</td>
<td>Thompkins, K, Chattopadhyay, B et al., 2008</td>
</tr>
<tr>
<td>JW5005</td>
<td>F-, Δ(araD-araB)567, Δyabl772::kan, ΔlacZ4787::rrnB-3, Δ, rph-1, Δ(rhaD-rhaB)568, hsdR514</td>
<td>Baba, T, Ara, T et al., 2006</td>
</tr>
<tr>
<td>JW2314</td>
<td>F-, Δ(araD-araB)567, ΔlacZ4787::rrnB-3, Δ, ΔEcdedA726::kan, rph-1, Δ(rhaD-rhaB)568, hsdR514</td>
<td>Baba, T, Ara, T et al., 2006</td>
</tr>
<tr>
<td>JW2124</td>
<td>F-, Δ(araD-araB)567, ΔlacZ4787::rrnB-3, Δ, ΔyohD762::kan, rph-1, Δ(rhaD-rhaB)568, hsdR514</td>
<td>Baba, T, Ara, T et al., 2006</td>
</tr>
<tr>
<td>JW2976</td>
<td>F-, Δ(araD-araB)567, ΔlacZ4787::rrnB-3, Δ, Δyabl772::kan, rph-1, Δ(rhaD-rhaB)568, hsdR514</td>
<td>Baba, T, Ara, T et al., 2006</td>
</tr>
<tr>
<td>JW3066</td>
<td>F-, Δ(araD-araB)567, ΔlacZ4787::rrnB-3, Δ, ΔyajjA785::kan, rph-1, Δ(rhaD-rhaB)568, hsdR514</td>
<td>Baba, T, Ara, T et al., 2006</td>
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<tr>
<td>JW2664</td>
<td>F-, Δ(araD-araB)567, ΔlacZ4787::rrnB-3, Δ, Δyqja770::kan, rph-1, Δ(rhaD-rhaB)568, hsdR514</td>
<td>Baba, T, Ara, T et al., 2006</td>
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<td>DW330</td>
<td>W3110, ΔlacU169, gal490, Δc1857 Δ(cro-bioA)</td>
<td>Yu, DG, Ellis, HM et al., 2000</td>
</tr>
<tr>
<td>BAL202</td>
<td>W3110, ΔydjXYZ::cam</td>
<td>This work</td>
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<tr>
<td>BAL300</td>
<td>W3110, ΔydjXYZ::cam, Δyabl772::kan</td>
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<td>BAL301</td>
<td>W3110, ΔydjXYZ::cam, Δyabl772</td>
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<td>BAL400</td>
<td>W3110, ΔydjXYZ::cam, Δyabl772, ΔEcdedA726::kan</td>
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<td>BAL401</td>
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<td>BAL500</td>
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<td>BAL501</td>
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<td>BAL60A</td>
<td>W3110, ΔydjXYZ::cam, Δyabl772, ΔEcdedA726, ΔyohD762, Δyqja785::kan</td>
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<td>BAL602</td>
<td>W3110, ΔydjXYZ::cam, Δyabl772, ΔEcdedA726, ΔyohD762, Δygb781</td>
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<td>BAL700</td>
<td>W3110, ΔydjXYZ::cam, Δyabl772, ΔEcdedA726, ΔyohD762, Δyqja785::kan</td>
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<td>BAL701</td>
<td>W3110, ΔydjXYZ::cam, Δyabl772, ΔEcdedA726, ΔyohD762, Δyqja785, Δyqja770</td>
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(Table II-3 continued)
Table II-3 continued

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<th>Strain</th>
<th>Description</th>
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<tr>
<td>BAL702</td>
<td>W3110, ΔydiXYZ::cam, Δyabl772, ΔEcdedA726, ΔyghB781::kan</td>
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<td>BAL801</td>
<td>W3110, ΔydiXYZ::cam, Δyabl772, ΔEcdedA726, ΔyqjA785, ΔyqA770, ΔyghB781::kan, pBAD_EcdedA</td>
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<td>BAL802</td>
<td>W3110, ΔydiXYZ::cam, Δyabl772, ΔEcdedA726, ΔyqjA785, ΔyqA770, ΔyghB781::kan, pBAD_yqjA</td>
<td>This work</td>
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<tr>
<td>BAL803</td>
<td>W3110, ΔydiXYZ::cam, Δyabl772, ΔEcdedA726, ΔyqjA785, ΔyqA770, ΔyghB781::kan, pBAD_yohD</td>
<td>This work</td>
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<td>BAL804</td>
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<td>This work</td>
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<tr>
<td>BAL 420</td>
<td>W3110, ΔyqjA::tetR, Δyabl772, ΔyohD762, ΔyghB781::kan</td>
<td>This work</td>
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</table>

**P₁ transduction:** P₁ lysates were prepared and P₁ transductions were carried out as described (Silhavy, TJ, Berman, ML et al., 1984). 1 ml of overnight cultures were centrifuged at 10,000 rpm for 30 seconds, and cells were resuspended in 0.5 ml of 0.1 M MgSO₄ and 0.01 M CaCl₂ and incubated with P₁ phage lysate prepared from a donor strain. Once mixed, reactions were incubated at 30 °C for 40 minutes after which 1 ml LB containing 0.1 M Na-citrate was added and cells were shaken at 225 rpm for 2 hours at 30 °C. Reactions were centrifuged and all cells were spread on LB plates containing appropriate antibiotics plus 4 mM Na-citrate and incubated overnight at 30 °C.

**Measurement of growth and β-galactosidase assay:** Overnight cultures were diluted to a starting OD₆₀₀ of ~0.01 in fresh media with no sugars, grown to an OD₆₀₀ of ~0.3 and diluted 1:10 in pre-warmed media with glucose/arabinose as indicated, and appropriate antibiotics (centrifugation of cultures before initial dilution removed any traces of arabinose in glucose culture). All absorbances were read using a SmartSpec Plus Spectrophotometer from Bio-Rad with a 1 cm path length cuvette.
The absorbance was read at 30-minute intervals and cell free media was saved to assay for activity of released β-galactosidase as a sign of cell lysis. Assays for β-galactosidase activity were carried out according to Miller (Miller, JH, 1972). Briefly, 0.1 ml of media was added to 0.9 ml of pre-warmed Z buffer (60 mM Na₂HPO₄·7H₂O, 40 mM NaH₂PO₄·H₂O, 10 mM KCl, 1 mM MgSO₄·7H₂O, 50 mM β-mercaptoethanol), and incubated at 30 °C for 5 minutes. To start the assay 0.1 ml of 8 mg ml⁻¹ 2-Nitrophenyl β-D-galactopyranoside (ONPG) was added to reaction, allowed to sit in the dark at 30 °C until yellow color was visible, after which 0.25 ml of 2 M Na₂CO₃ was added to stop the reaction. Assayed reactions were centrifuged and A₄₂₀ read. For each time point, activity was measured in triplicate.

### Table II-4: Vectors used in study.

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBADHisA</td>
<td>Expression vector, Amp’, N-terminal polyhistidine tag and Xpress epitope, with the araBAD promoter.</td>
<td>Invitrogen</td>
</tr>
<tr>
<td>pBAD_ydjX</td>
<td>pBAD expressing N-terminal His tagged ydjX; Amp’</td>
<td>This Study</td>
</tr>
<tr>
<td>pBAD_ydjZ</td>
<td>pBAD expressing N-terminal His tagged ydjZ; Amp’</td>
<td>This Study</td>
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<td>pBAD_yabl</td>
<td>pBAD expressing N-terminal His tagged yabl; Amp’</td>
<td>This Study</td>
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<tr>
<td>pBAD_yqjA</td>
<td>pBAD expressing N-terminal His tagged yqjA; Amp’</td>
<td>This Study</td>
</tr>
<tr>
<td>pBAD_yqaA</td>
<td>pBAD expressing N-terminal His tagged yqaA; Amp’</td>
<td>This Study</td>
</tr>
<tr>
<td>pBAD_yghB</td>
<td>pBAD expressing N-terminal His tagged yghB; Amp’</td>
<td>This Study</td>
</tr>
<tr>
<td>pBAD_yohD</td>
<td>pBAD expressing yohD; Amp’</td>
<td>This Study</td>
</tr>
<tr>
<td>pBAD_EcdedA</td>
<td>pBAD expressing EcdedA; Amp’</td>
<td>This Study</td>
</tr>
<tr>
<td>pCP20</td>
<td>FLP’, λ.cI857*, λ.Rep&lt;sup&gt;ts&lt;/sup&gt; promoter, Amp’, Cam&lt;sup&gt;R&lt;/sup&gt;</td>
<td>E. coli Genetic Stock Center; (Cherepanov, PP &amp; Wackernagel, W, 1995)</td>
</tr>
<tr>
<td>pBAD-TatABC</td>
<td>pBADHisA expressing TatABC operon; Amp’</td>
<td>(Sikdar, R &amp; Doerrler, WT, 2010)</td>
</tr>
</tbody>
</table>
**Figure II-1**: Order of sequential deletion of all DedA family genes within *E. coli*. A DIC image is next to each generated strain (samples prepared as described in Methods and images captured with a Leica DM RXA2 deconvolution microscope); all images are of equal scale. No visible phenotypes were observed unless both yghB and yqiA were deleted (Figure II-8, page 36). See Table II-3 for genotypic descriptions.

**Microscopy**: Overnight cultures of strains were diluted 1:100 and grown to an OD$_{600}$ of approximately 0.5. Cells were resuspended in fresh LB to make an OD$_{600}$ of 1.0 and 10 μl was then added to agar coated slides. All DIC images were captured using Leica DM RXA2 deconvolution microscope (LSU Socolofsky Microscopy Center).

**Phospholipid extraction**: Overnight cultures were diluted to a starting OD$_{600}$ of ~0.1 in fresh media with respective antibiotics and glucose/arabinose as indicated. Cultures were centrifuged and overnight media removed to eliminate any traces of arabinose in glucose cultures. Absorbance was read every 30 minutes and cells were collected for lipid extraction (Bligh, EG & Dyer, WJ, 1959) at one-hour intervals. $^{32}$P$_i$ was added to 10 μCi ml$^{-1}$ (3.7 x $10^5$ Bq ml$^{-1}$) and growth was continued for 10 min.
0.2 ml of growing culture was used for lipid analysis. Chloroform and methanol were added to cells to a final ratio of chloroform: methanol: water::1:2:0.8. The extraction mixture was allowed to incubate for 1 hr at room temperature with occasional mixing. Insoluble material was removed by centrifugation for 10 min at 13,000 rpm. The supernatant was transferred to a new tube and chloroform and water were added to adjust the ratio of chloroform, methanol and water to 1:1:0.8, resulting in a two-phase mixture. The aqueous upper phase was discarded and the lower phase was washed with fresh pre-equilibrated upper phase. Pellets were dried in vacufuge for >30 minutes and lipid species were resolved by TLC on silica gel 60 plates (Merck) using the solvent chloroform: methanol: acetic acid (65:25:10) and analyzed using a Phosphorimager equipped with IQMac software.

II.3. Results

**Growth and cell division defects of BC202 are restored by a subset of DedA family proteins:** To begin our analysis of the DedA family, all eight *E. coli* DedA family genes were amplified and cloned into a vector to allow for arabinose dependent expression (Table II-4). BC202 does not grow at the non-permissive temperature of 42 °C, and forms chains resembling amidase mutants (Heidrich, C, Ursinus, A et al., 2002) because of an inefficiency in the TAT pathway (Sikdar, R & Doerrler, WT, 2010). Each cloned gene was individually tested for its ability to restore cell division and temperature sensitive growth to BC202. Due to problems with toxicity certain genes were expressed using low concentrations of both ampicillin and arabinose (Figure II-2). Cloned *yqiA*, *yghB*, *yabI*, and *yohD* each restored growth (Figure II-2) and normal cell division (Figure II-3) to BC202, while cloned *ydjX*, *ydjZ*,
yqaA, and EcdedA failed to restore growth and normal cell division to BC202 (Figure II-2 and Figure II-3). Therefore each *E. coli* gene able to complement BC202 for temperature sensitivity also complemented cell division and, conversely, each gene that did not complement BC202’s temperature sensitivity also failed to restore cell division. This complementation analysis suggests that the eight genes can be separated into two functional groups, one group that functions similarly to YqjA/YghB and one group that does not. We will hereforth refer to these groups as the C (complementing) and NC (non-complementing) groups, respectively.

**Figure II-2:** Complementation of growth at 42 °C of BC202 with cloned DedA family genes. BC202 (W3110; ΔyqjA::tetR, ΔyghB::kanR) was transformed with (a, b) pBAD, pBAD_yqaA, pBAD_yabl, (c, d) pBAD_yghB, pBAD_ydjZ, pBAD_EcdedA, (e, f) pBAD_yohD, pBAD_yqjA, pBAD_ydjZ (Table II-4), and grown on LB/amp plates supplemented with 0.1% arabinose (a, b) or 0.002% arabinose (c, d, e, f) and 50 μg amp ml⁻¹ at both 30 °C and 42 °C.

Figure II-4 shows how the *E. coli* DedA proteins may fall within evolutionary groups. Using the Maximum Parsimony Algorithm, all the NC proteins appear to branch together separately from the C proteins on the tree (see also Table II-5, page
While this is just one of many algorithms available for phylogenetic analysis of proteins, the results are striking, and perhaps reflect a functional grouping of this family. In addition, this analysis does not reflect upon the essentiality of the DedA protein family. Intriguingly, as discussed below, both yqiA (C-group) and EcdedA (NC-group) are each capable of supporting viability of a BAL800 series mutant, suggesting that shared among all DedA family proteins is an unknown essential function that is required for a cell’s viability.

Figure II-3: Complementation of BC202 cell division defects with cloned DedA family genes.
BC202 was transformed with (a) plasmid pBAD (Table II-4, page 25) or pBAD with cloned (b) pBAD_yqiA, (c) pBAD_yghB, (d) pBAD_yabl, (e) pBAD_yohD, (f) pBAD_EcdedA, (g) pBAD_yqaA, (h) pBAD_ydjX, or (i) pBAD_ydjZ and were grown in LB/amp supplemented with 0.1 % arabinose at 30 °C. Cells were harvested at an OD$_{600}$ of ~0.5 and visualized using a Leica DM RXA2 deconvolution microscope.
Figure II-4: Proposed Evolutionary relationship of *E. coli* DedA proteins. Alignments were generated using nucleotide sequences of *E. coli* DedA family proteins and analyzed with MEGA, using the Maximum Parsimony algorithm (Tamura, K, Peterson, D et al., 2011). Tree was rooted with the 16S RNA gene, *rrsH*. Proteins with an asterisk (*) restore both normal growth and cell division when expressed in BC202 (see Table II-5, page 32; Figure II-2, page 28; Figure II-3, page 29). The branch lengths (vertical and horizontal) were drawn to scale with units signifying the number of changes over whole sequence.

**Generation of mutants with deletions of eight DedA family genes:** The approach for generating BAL800s, with deletions of all eight DedA family genes is shown in Figure II-1 (page 26). Initially, *ydjXYZ* was deleted with a single chloramphenicol cassette because the two DedA genes (*ydjX* and *ydjZ*) are expressed from a single operon. Simultaneous deletion also avoids polar effects due to individual deletion of the two genes. *ydjX* and *ydjZ* encode DedA family membrane proteins while *ydjY* encodes a predicted subunit of an ABC transporter. The *ydjY* gene is not essential,
as a $\Delta ydjY$ mutant was isolated (Baba, T, Ara, T et al., 2006) and has no apparent phenotypes. Also, deletion of $ydjY$ during the mutant generation process resulted in no observable phenotypes and it was only after deletion of all members of the DedA protein family that the strain(s) became non-viable (see Figure II-1, page 26). While we have not formally ruled out a role for loss of $ydjY$ in our observable phenotypes, we felt removal of $ydjY$ was necessary to avoid unintended polar effects. This initial $ydjXYZ$ deletion was followed by subsequent individual deletions of DedA family members (Figure II-1, page 26) using P$_1$ lysates prepared from the corresponding Keio mutants (Baba, T, Ara, T et al., 2006). Kan$^R$ cassettes were removed using FLP recombinase and deletions confirmed with PCR (Figure II-5; Table II-2, page 21) prior to proceeding to the next deletion. During mutant generation, no temperature sensitivity or cell division defects (Figure II-1, page 26) were observed until both $yqjA$ and $yghB$ were absent. Either $yghB$ or $yqjA$ was the final gene deleted during mutant construction because of the known inability of BC202 to grow at 42 °C, the temperature required to cure the plasmid used to remove the kan$^R$ cassette. A pBAD cloned DedA family gene plus the presence of arabinose was required to isolate every BAL800 series mutant (see below).

During sequencing of BAL801, a single base pair deletion within the $\Delta yabl$ scar peptide was observed. In the *E. coli* genome, *yabl* is flanked by *araC* as well as *thiQ*. *araC* of course has its own promoter sequence (Hirsh, J & Schleif, R, 1977), and according to RegulonDB, *yabl* is also known to have its own promoter (Gama-Castro, S, Salgado, H et al., 2011). As for *thiQ*, it is in the opposite orientation of *yabl* and is part of the *sgrR-sroA-tbpA-thiPQ* operon. Therefore, the observed single base
pair deletion of BAL801 within the Δyabi scar peptide, though a polar deletion, is ~100 bp away from surrounding genes, and is unlikely to impact the transcription or translation of genes in the vicinity of Δyabi in BAL801. Other than this, DNA sequencing confirmed that all gene deletions of BAL801 were nonpolar.

Table II-5: The *E. coli* DedA family.

<table>
<thead>
<tr>
<th>Group</th>
<th>Gene Name</th>
<th>Position on Chromosome</th>
<th>Complements BC202*</th>
<th>BAL800 Series Mutant</th>
</tr>
</thead>
<tbody>
<tr>
<td>C group</td>
<td>yqjA</td>
<td>69.96</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>yghB</td>
<td>67.93</td>
<td>Yes</td>
<td>Non-culturability**</td>
</tr>
<tr>
<td></td>
<td>yohD</td>
<td>47.93</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>yabI</td>
<td>1.54</td>
<td>Yes</td>
<td>Yes</td>
</tr>
<tr>
<td>NC group</td>
<td>yqaA</td>
<td>60.66</td>
<td>No</td>
<td>Non-culturability**</td>
</tr>
<tr>
<td></td>
<td>EcdedA</td>
<td>52.42</td>
<td>No</td>
<td>Yes</td>
</tr>
<tr>
<td></td>
<td>ydjZ</td>
<td>39.50</td>
<td>No</td>
<td>Yes; unexpected genotype***</td>
</tr>
<tr>
<td></td>
<td>ydjX</td>
<td>39.47</td>
<td>No</td>
<td>Yes; unexpected genotype***</td>
</tr>
</tbody>
</table>

C – complementing and NC – for non-complementing.
*Complementation defined as ability to restore both growth at 42 °C and normal cell division at 30 °C to BC202 (Table II-3, page 23).
**Small colonies were observed following P1 transduction but could not be grown on plates or in liquid media.
***Denoting a duplication of the yghB gene regions during the generation of BAL800s.

The generation of BAL800 series mutants was successfully completed only when EcdedA, yqjA, yohD or yabI was expressed from an arabinose inducible plasmid (BAL801, BAL802, BAL803, and BAL804, respectively; Table II-3, page 23; Table II-4, page 25) and only when arabinose was included during the selection of transductants. Note that the ability of a gene to complement growth and cell division of BC202 appears to be independent of its ability to support growth of BAL800s, as EcdedA can support this strain but is unable to complement either phenotype of
BC202 (Figure II-2, page 28; Figure II-3, page 29). BAL801 and BAL802 were subsequently further characterized more fully.

![Amplified region](image)

**Figure II-5:** PCR confirmation of BAL801 gene deletions. Flanking primers for each gene deletion were used to confirm genotypic composition of strains. Primer sites are approximately 200 bp up/downstream each of the DedA genes, except for EcdedA which are ~400 bp up/downstream. Since yghB was the last gene deleted, the kan\(^\text{R}\) cassette is still present at this site in BAL801, resulting in the increased size of this PCR product (yghB region, strain B lane). **W:** W3110A, and **B:** BAL801.

**Growth of mutants in liquid culture:** Working with BAL800 series mutants requires great consideration. While these strains grow reproducibly on LB plates, we found liquid cultures of BAL800s grew best when flasks were inoculated directly from -80°C freezer stocks, otherwise growth rates were inconsistent. Also, BAL800s were sensitive to centrifugation and dilution, completing these before beginning growth curve analysis removed some of the observed growth inconsistency. Cells grown in the presence of glucose began to enter growth arrest after 308 ± 51 minutes of growth. We observed some day-to-day variation as far as the specific timing of growth arrest. The presence of \(\beta\)-galactosidase was not detectable in the media until approximately 270 minutes after the shift of BAL801 to glucose media, indicating that
the mutant cells had lysed releasing the cytoplasmic contents to the culture media (Figure II-6). A similar growth dependency upon arabinose was observed when yqjA was expressed from the inducible plasmid (BAL802; Figure II-7) with the exception that cultures grew much slower when arabinose was present in the growth media, possibly reflecting toxic effects of the overexpressed protein. This data strongly suggests that the DedA family is essential in *E. coli*.

**Figure II-6**: Growth of BAL801 in liquid culture. Growth of *E. coli* with in frame deletions of all eight DedA family genes (ΔyqjA, ΔyghB, Δyabl, ΔyohD, ΔEcdedA, ΔydiXYZ, ΔyqaA) with the plasmid pBAD_EcdedA (strain BAL801) is dependent upon induction with arabinose. Cultures were diluted at 180 minutes into LB supplemented with 0.1% arabinose (blue triangles) or 0.2% glucose (red stars) and diluted ten-fold whenever the OD$_{600}$ reached 0.3-0.5. The plotted OD$_{600}$ is the cumulative growth yield. Aliquots were removed from growing cultures to assay cell-free media for β-galactosidase activity as a measure of cell lysis (at 120, 270 and 450 minutes). Cells were found to lyse around minute 450 when grown in the presence of glucose (red bar).
Microscopic analysis of mutants: BAL801 grown in the presence of arabinose (Figure II-8a) displays similar morphological deformities and cell division defects as BC202 as expected due to the inability of Ec*dedA* to complement BC202 (Figure II-3, page 29). BAL801 also exhibited increased cell clumping when grown in the presence of arabinose (Figure II-9). When expression of cloned Ec*dedA* is repressed, the deformities of BAL801 increase until the cells lyse and die (Figure II-8b).

Figure II-7: Growth curve of BAL802. BAL802 contains deletions of eight DedA family genes with yqjA expressed from an arabinose inducible promoter (pBAD_yqjA; Table II-4, page 25). Overnight cultures were diluted to a starting OD*600* of ~0.1 in pre-warmed LB/antibiotics, grown to ~0.3 and diluted into fresh media with antibiotics and sugars 0.1% arabinose (blue triangles) or 0.2% glucose (red stars). Cultures were re-diluted 10 fold into fresh pre-warmed media to maintain logarithmic growth. The plotted OD*600* is the cumulative growth yield. BAL802 with glucose initially grows at an increased rate, until approximately 360 minutes when growth begins to slow.
BAL802 does not share the same deformities and appears almost as wild type when grown in the presence of arabinose (Figure II-8c) due to the ability of \textit{yqjA}\ to restore normal morphology and cell division to BC202 (Figure II-3, page 29). However, in the absence of \textit{yqjA}\ expression when cells are grown in the presence of glucose, the cell morphological deformities return and the cells lyse and die (while at the same time failing to form chains since cells are entering growth arrest) (Figure II-8d). All images are representative of cells or chaining cells. Therefore either \textit{EcedA}\ or \textit{yqjA}\ can support growth of a mutant lacking all eight DedA family genes, regardless of their ability to restore normal growth and cell division to BC202.

\textbf{Figure II-8:}\ Microscopic analysis of BAL801 and BAL802. Overnight cultures of BAL801 (a, b) and BAL802 (c, d) were diluted 1:100 into LB plus antibiotics and grown to an OD$_{600}$ of ~0.5 in the presence of either 0.1% arabinose (a, c) or 0.2% glucose (b, d). Most BAL802 cells were in the process of undergoing lysis under these conditions during growth with glucose and what are likely representative cell ghosts are shown (panel d). All cultures were grown at 30 °C. Cells were harvested and visualized with a Leica DM RXA2 deconvolution microscope.
Membrane phospholipid composition: We previously reported that BC202 grows under permissive conditions with an altered membrane phospholipid composition (Thompkins, K, Chattopadhyay, B et al., 2008). We therefore measured phospholipid composition in BAL801 when grown under both inducing and repressing conditions. The phospholipid composition of W3110 consists of 73.5% PE (phosphatidylethanolamine), 24.6% PG (phosphatidylglycerol) and 1.9% CL (cardiolipin), and for BC202 membranes contain 58.4% PE, 35.3% PG and 6.3% CL (Figure II-10). When BAL801 is grown with EcdedA expressed, the phospholipid composition is approximately the same as in BC202. Also, the phospholipid composition of BAL801 remains relatively constant whether the EcdedA gene is induced or not, even after the cells begin to die (Figure II-11). This data indicates that along with inability of EcdedA to rescue cell division and growth defects of BC202 (Figure II-2, page 28; Figure II-3, page 29), EcdedA is also unable to restore wild type phospholipid composition (Figure II-10). This altered phospholipid composition is independent of BAL801 growth arrest.

Effect of TatABC overexpression on DedA C-group deletion mutant: We previously reported that the cell division defect observed with strain BC202 (ΔyghB::kanR, ΔyqjA::tetR) was due to inefficient transport of periplasmic amidases AmiA and AmiC across the plasma membrane by the TAT pathway (Sikdar, R & Doerrler, WT, 2010). Overexpression of the TatABC operon from an inducible plasmid rescued the cell division defect and, surprisingly, the temperature sensitivity of BC202. We were interested in determining whether overexpression of TatABC was capable of restoring growth to mutants with additional DedA family mutations.
We found that deletion of the four C-group genes (*yqjA, yghB, yohD*, and *yabl*; BAL420, Table II-3, page 23) resulted in a strain with similar growth and cell division defects as BC202 but these could no longer be corrected by overexpression of TatABC (Figure II-12, page 42). Therefore, the presence of *yabl* and/or *yohD* is required for TatABC overexpression to correct cell division and temperature sensitivity of DedA family mutants. In light of this result, the technically more challenging experiment of overexpressing TatABC in a BAL800 mutant was not attempted.

![Figure II-9: Clumping of BAL801.](image)
Growth of BAL801 in the presence of arabinose at 30 °C leads to an increase of cell aggregation. The cause of this is currently unclear.

II.4. Discussion

In *Escherichia coli*, as in most organisms, it is predicted that roughly 20-25% of transcribed genes encode integral membrane proteins (Elolsson, A & Von Heijne, G, 2007). One estimate states that more than half of all drugs currently produced are directed against membrane proteins, mostly G-protein receptors (Klabunde, T & Hessler, G, 2002). In contrast, less than 1% of all known protein structures in the
Protein Data Bank are membrane proteins (Rose, PW, Beran, B et al., 2011) and the functions of many membrane proteins are only poorly understood. The DedA protein family has now been demonstrated to be essential in two species of Gram-negative bacteria: B. burgdorferi, possessing one essential DedA family member (Fraser, CM, Casjens, S et al., 1997; Liang, FT, Xu, QL et al., 2010), and E. coli, possessing eight (Blattner, FR, Plunkett, G et al., 1997) collectively essential members.

**Figure II-10**: Comparative phospholipid composition of wild type W3110, BC202, and BAL801.

Overnight cultures of W3110, BC202 were diluted 1:100 in LB and grown to an OD<sub>600</sub> of ~0.5 for lipid analysis. BAL801 overnight culture was diluted to a starting OD<sub>600</sub> of ~0.1 in fresh LB/antibiotics supplemented with 0.1% arabinose or 0.2% glucose as indicated. BAL801 grown in both arabinose and glucose was maintained in logarithmic growth and monitored every 30 minutes. After 360 minutes, growth of BAL801 in glucose halted (see Figure II-11), shown here are the lipids extracted at this time point. Aliquots were removed for phospholipid analysis as described in methods. Following quantification of individual lipid species, lipid composition for each species was plotted at % total phospholipid signal for each individual species. Shown is representative experiment of two different determinations. Abbreviations: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin.
In the case of *B. burgdorferi*, all the DedA functions are maintained by a single protein (Bb0250). The DedA protein in *B. burgdorferi* was found to be essential and did complement BC202 for cell division and temperature sensitivity in spite of displaying 19% or less amino acid identity to its *E. coli* counterparts (Liang, FT, Xu, QL *et al.*, 2010). Depletion of Bb0250 protein in a Δbb0250 *B. burgdorferi* background results in rapid cell death preceded by striking cell division defects (Liang, FT, Xu, QL *et al.*, 2010). While this degree of functional conservation may not be observed across all species it certainly points to some level of conserved functionality, and suggests that the DedA family may be essential in many other species as well, bacterial or otherwise.

A recent report suggests that the DedA family may represent a potential drug target. The genomes of all *Mycobacterium* species encode multiple DedA proteins, and one DedA homolog (Bcg2664) from *M. bovis* is possibly the target for the antibiotic halicyclamine A, as *bcg2664* confers resistance to this drug when overexpressed in *M. smegmatis* (Arai, M, Liu, L *et al.*, 2011). Halicyclamine A was first isolated from a marine sponge *Haliclona sp.* and was initially thought to target inosine monophosphate dehydrogenase (IMPDH) (Arai, M, Sobou, M *et al.*, 2008; Jaspars, M, Pasupathy, V *et al.*, 1994). There exists a possibility that halicyclamine A and/or derivatives of this drug may act as general inhibitors of the widely distributed and highly conserved DedA protein family.
**Figure II-11**: Phospholipid composition of BAL801. 

(a) Growth curve of cultures used for phospholipid extraction. Overnight cultures were diluted to starting OD$_{600}$ ~0.1 in fresh LB with either 0.1% arabinose (blue triangles) or 0.2% glucose (red stars). Circled time points signify points where cells were removed and labeled for lipid analysis. 

(b) BAL801 phospholipid composition (%) when grown in 0.1% arabinose. 

(c) BAL801 phospholipid composition (%) when grown in 0.2% glucose. Cells were labeled with $^{32}$P$_{i}$ as described in Materials and Methods and lipids were extracted, resolved by TLC and individual lipid species were quantified using a Phosphorimager. Relative amounts of phosphatidylethanolamine (PE; blue diamonds), phosphatidylglycerol (PG; red squares) and cardiolipin (CL; green triangles) were expressed for each time point as a percentage of total labeled phospholipid. BAL801 cells begin dying at 240 minutes, but phospholipid composition does not change, signifying cell death is not related to a changing phospholipid composition.
Figure II-12: Overexpression of TatABC does not restore normal cell division or growth at elevated temperatures to BAL420. BAL420, a mutant with deletions of all C-group (complementing) genes (yqjA, yghB, yabl and yohD), was transformed with pBAD-TatABC and grown in the presence of 0 % (a) or 0.1 % (b) arabinose to mid-log density and imaged using a Leica DM RXA2 deconvolution microscope. The same strain was grown on LB/agar plates containing 0.1 % arabinose and incubated at 30 °C or 42 °C (panels c and d). These same conditions were found to correct cell division and growth defects of BC202 in previous work (Sikdar, R & Doerrier, WT, 2010).

Complementation analysis of BC202 indicates that in E. coli there are at least two functional groups of DedA family genes (Table II-5, page 32): we categorize those that can rescue BC202 as belonging to the C-group (yqjA, yghB, yabl, yohD), and those that do not rescue belong to the NC-group (EcdedA, yqaA, ydjX and ydjZ) (Figure II-2, page 28; Figure II-3, page 29). In spite of this disparity of function, at least one member from either the C- or NC-groups can support the generation and growth of BAL800 series mutants with chromosomal deletions of each DedA family gene. These results suggest that retained among E. coli DedA proteins is an unknown essential function that is likely independent of the growth and cell division defects displayed by BC202. Given the high degree of conservation of DedA proteins across all Domains of life, including humans, it is not surprising that in E.
coli, the DedA proteins are collectively essential. Though not all DedA family proteins are able to complement the phenotypes displayed by BC202, and many functions are shared, there is at least one essential function shared by many DedA family members.

Additionally, any role the DedA proteins may play in the efficiency of the TAT pathway is independent of the essentiality of the DedA proteins in E. coli. BAL420, a mutant with deletions of all C-group DedA proteins (Table II-3, page 23), is similar to BC202 in that it is also sensitive to growth at high temperatures and has cell division defects. However, unlike BC202, where growth and cell division abnormalities are restored by overexpression of the TatABC operon (Sikdar, R & Doerrler, WT, 2010), overexpression of TatABC does not restore normal cell morphology and growth to BAL420 (Figure II-12), further indicating that the essential role of DedA proteins is independent of temperature sensitivity and cell division defects. The ability of overexpressed TAT pathway proteins to rescue BC202 but not BAL420 is attributed to the availability of yabI and yohD still present in BC202. It is possible that the expression levels of yabI and/or yohD are increased in BC202 enabling the recovery of this strain, and not BAL420.

While the phenotypes of BAL801 described here do not point to a clear function for the DedA family, it is clear that these mutants lyse and are likely experiencing high levels of envelope stress. The stability of these mutants is quite variable, possibly due to toxic effects associated with the individual DedA genes being expressed, or that the shared essential function is not individually retained among all members of the family, such that some members may require a complex of
DedA proteins. This is likely why some DedA members did not allow for the isolation of a BAL800 series mutant (i.e. \textit{ydx}, \textit{ydz}, \textit{yqaA}, and \textit{yghB}). For example, the time it takes for growth arrest in the absence of an expressed DedA family member varies from strain to strain. Furthermore, certain DedA family mutants in our hands have displayed intriguing properties that include the apparent duplication of a gene region, abnormal accumulation of replicating chromosomal DNA, loss of chromosomal organization within a mutant cell, and large scale genomic inversions between remnant chromosomal scar peptides, likely caused by homologous recombination [Chapter III, pages 45-74; (Boughner, LA, Kumar, S \textit{et al.})]. It is conceivable that the DedA family may play an as-of-yet undefined role in regulating the replication and/or organization of genomic DNA during various phases of the cell cycle. We are currently exploring this possibility.

The DedA protein family present in \textit{E. coli} has now been demonstrated to be collectively essential for cellular viability. Regardless of consistent functional homology among DedA proteins, there does seem to be a highly conserved essential function. These data support a role for this family that is independent of the growth and cell division phenotypes displayed by BC202. Future studies will focus on elucidating the role DedA proteins play in the viability of a cell and its genome, and possibly provide new targets for therapeutic interventions for bacterial diseases.

\[^{1}\text{After multiple attempts, generation of a BAL800 series mutant containing a cloned pBAD copy of } yghB \text{ was possible, though the mutant is extremely slow growing. The drastically reduced growth rate is likely why isolation of this mutant was so challenging.}\]
III. The *Escherichia coli* DedA protein family may be required for proper organization and segregation of the bacterial nucleoid.

III.1. Introduction

The DedA membrane protein family is highly conserved and found within all domains of life (Bacteria, Archaea, and Eukarya) [Chapter I, pages 1-15; (Doerrler, WT, Sikdar, R et al., 2013)]. However, their functions are still poorly understood. Genes encoding DedA protein family members are found at varying frequencies within different sequenced genomes (Doerrler, WT, Sikdar, R et al., 2013; Liang, FT, Xu, QL et al., 2010). For example, the *Escherichia coli* genome possesses eight DedA family genes (*yqjA, yghB, yabl, yohD, ydjX, ydjZ, yqaA*, and *dedA*) encoding integral membrane proteins with 25-61 % amino acid identity; herein, the *E. coli* DedA protein/gene is referred to as EcDedA/Ec*dedA* respectively. *Borrelia burgdorferi*, in contrast has a single DedA gene (*bb0250*). We have shown that the DedA family is essential in both of these organisms [Chapter III, pages 45-74; (Boughner, LA & Doerrler, WT, 2012; Liang, FT, Xu, QL et al., 2010)]. Other sequenced bacterial genomes often contain multiple DedA family homologs and, by our estimation, >90% of sequenced bacterial genomes possess at least one DedA family member [Chapter I, pages 1-15; (Doerrler, WT, Sikdar, R et al., 2013)].

Our previous work suggests that the DedA protein family is involved in maintaining the integrity of the inner membrane (Liang, FT, Xu, QL et al., 2010; Sikdar, R & Doerrler, WT, 2010; Sikdar, R, Simmons, AR et al., 2013; Thompkins, K, Chattopadhyay, B et al., 2008) and may be a new class of membrane transporters (Doerrler, WT, Sikdar, R et al., 2013; Sikdar, R, Simmons, AR et al., 2013). For
example, BC202—an *E. coli* mutant with deletions of two DedA genes (*yghB* and *yqjA*), exhibits defects in cell division (Sikdar, R & Doerrler, WT, 2010), fails to grow at 42 °C (Thompkins, K, Chattopadhyay, B *et al*., 2008), has increased activation of multiple envelope stress response pathways and fails to maintain the membrane proton motive force (PMF) (Sikdar, R, Simmons, AR *et al*., 2013). The cell division defect is caused by inefficient export of periplasmic amidases through the twin arginine transport (TAT) pathway (Sikdar, R & Doerrler, WT, 2010). YghB and YqjA are two of the inner membrane DedA proteins that share 61% amino acid identity; they carry out redundant functions, since the individual deletion mutants have no, or only mild, phenotypes (Baba, T, Ara, T *et al*., 2006; Price, NL & Raivio, TL, 2009; Thompkins, K, Chattopadhyay, B *et al*., 2008) and overexpression of either gene in BC202 corrects the cell division and growth phenotypes [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012; Thompkins, K, Chattopadhyay, B *et al*., 2008)].

*Borrelia burgdorferi* possesses a single DedA gene (annotated *bb0250*). A *B. burgdorferi Δbb0250* mutant (DXL-01) requires a plasmid copy of this DedA gene and inducing agent (IPTG) for viability. Growth of DXL-01 in the absence of IPTG results in cell death preceded by striking defects in cell division and cellular morphology (Liang, FT, Xu, QL *et al*., 2010). This study was the first to demonstrate the essentiality of a DedA family gene (Liang, FT, Xu, QL *et al*., 2010). 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 (Liang, FT, Xu, QL *et al*., 2010). These results demonstrate a strong conservation of function for the DedA family among these distantly related species of
Gram-negative bacteria. Intriguingly, the *B. burgdorferi* genome does not encode a functional twin arginine pathway or any predicted TAT substrates (Dilks, K, Rose, RW *et al.*, 2003), indicating that the DedA family is involved in functions independent of the TAT pathway.

While these phenotypes represent a significant advancement towards identifying the functions of DedA proteins, many questions still remain unanswered. For example, ΔTAT mutants exhibit cell division defects similar to BC202 (since BC202 inefficiently operates the TAT pathway), but ΔTAT mutants (unlike BC202) are not temperature sensitive (Sikdar, R & Doerrler, WT, 2010). BC202 is also extremely sensitive to growth in alkaline pH and cannot maintain the membrane PMF (Sikdar, R, Simmons, AR *et al.*, 2013). MdfA is a member of the major facilitator superfamily involved in drug efflux, a Na⁺-K⁺/H⁺ antiporter (Krulwich, TA, Lewinson, O *et al.*, 2005). Overexpression of *mdfA* from a plasmid corrects the growth and cell division defects of BC202 (Sikdar, R, Simmons, AR *et al.*, 2013) and therefore, the DedA family may represent a new family of membrane transporters (Doerrler, WT, Sikdar, R *et al.*, 2013; Sikdar, R, Simmons, AR *et al.*, 2013). Under permissive growth conditions, BC202 has a number of constitutively activated extracytoplasmic stress response pathways, including the Cpx, Rsc, Psp and Bae pathways (Sikdar, R, Simmons, AR *et al.*, 2013). However, activation of these pathways, by themselves, are not responsible for the observed temperature sensitivity of BC202, or for that matter, the cell division defects (Sikdar, R, Simmons, AR *et al.*, 2013). Growth and cell division defects found in BC202 can be restored by expression of some, but not all *E. coli* DedA family genes. Individual expression of four out of the eight *E. coli*
DedA genes [yqjA, yghB, yohD and yabI: a.k.a the C (complementing) group], restores normal growth and cell division to BC202; conversely, the NC (non-complementing) group consists of EcdedA, yqaA, ydjX and ydjZ [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012)].

In an effort to better understand the functions of the DedA family, we previously created an E. coli strain with in-frame deletions of all eight DedA family genes [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012)]. Isolation of such a mutant was only possible if a cloned member of the DedA protein family was present and the strain was grown in the presence of inducer (i.e. arabinose). Strikingly, we were able to create such a DedA family mutant just as readily if an NC-group protein (EcDedA; BAL801) was expressed as when a C-group protein was expressed (YqjA; BAL802) [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012)]. Growth of BAL801 or BAL802 in the presence of repressor (glucose) results in cell lysis. Under permissive growth conditions (with arabinose at 30 °C) BAL801, expressing an NC-group protein, displays cell division defects and is temperature sensitive for growth (due to a lack of YqjA and YghB) but are viable. BAL802, expressing a C-group protein, displays no temperature sensitivity or cell division defects, but is dependent upon arabinose for viability [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012)]. Thus, the DedA protein family is collectively essential in E. coli [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012)], just as it is in B. burgdorferi (Liang, FT, Xu, QL et al., 2010). We conclude from these observations that the E. coli DedA family carries out an essential function that may be independent of a putative transporter function.
Here we further characterize the mutant with nonpolar deletions of all DedA family members. BAL801 (pBAD_EcdedA) exhibits severe morphological deformities, is deficient for cell division, sensitive to growth at 42 °C and in the presence of repressor (glucose), these deformities increase before cells lyse and die [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012)]. During the generation of BAL801 some very anomalous, and consistent, observations were made regarding the chromosome; specifically, the duplication of genomic regions, and double crossover events of large portions (~16 minutes) of the chromosome. In addition, we report here that the deletion (and depletion) of all eight DedA protein members in E. coli results in a significant increase of genomic content, followed by cell anucleation (cells devoid of nucleoid), before the cells lyse and die. Here we aim to understand the essential function of the E. coli DedA protein family by examining the genomic anomalies of BAL800 generational family mutants, and specifically BAL801.

III.2. Materials and Methods

**Materials:** All materials were purchased from VWR International, Sigma-Aldrich, New England Biolabs, Qiagen, Amersham - GE Healthcare Life Sciences or Bio-Rad.

**Bacterial Growth conditions:** All cultures were grown in Luria-Bertani medium (LB: yeast extract 5 g L⁻¹, tryptone 10 g L⁻¹, NaCl 10 g L⁻¹ and a pH 7), with respective antibiotics (10 μg chloramphenicol ml⁻¹, 30 μg kanamycin ml⁻¹, 100 μg ampicillin ml⁻¹, and/or 100 μg streptomycin ml⁻¹) and where indicated supplemented with either 0.1% w/v arabinose or 0.2% w/v glucose. Unless otherwise stated, cultures were grown in
10 ml and 50 ml volumes (in 50 ml and 250 ml flasks, respectively) at 30 °C in a (225 rpm) shaking incubator.

**Table III-1**: Primer Sequences for mutant genotype confirmation and cloning (restriction sites are underlined)

<table>
<thead>
<tr>
<th>Primer</th>
<th>Sequence</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>FfP_ydjX</td>
<td>CGCTAATTTAGCAGCTCCTCCTG</td>
<td>(Boughner, LA &amp; Doerrler, 2012)</td>
</tr>
<tr>
<td>RfP_ydjZ</td>
<td>GCTGACAAGCGATCGTACTACCG</td>
<td>(Boughner, LA &amp; Doerrler, 2012)</td>
</tr>
<tr>
<td>FfP_YabI</td>
<td>TGACGATCAACTCTATTTTCGCG</td>
<td>(Boughner, LA &amp; Doerrler, 2012)</td>
</tr>
<tr>
<td>RfP_YabI</td>
<td>GCCTGGCAGGGTATGACCAA</td>
<td>(Boughner, LA &amp; Doerrler, 2012)</td>
</tr>
<tr>
<td>FXfP_EcdedA</td>
<td>GCGGTGCAGTGCAGCTCCTG</td>
<td>(Boughner, LA &amp; Doerrler, 2012)</td>
</tr>
<tr>
<td>RXfP_EcdedA</td>
<td>ACATCTTTCGGCTAAGCTC</td>
<td>(Boughner, LA &amp; Doerrler, 2012)</td>
</tr>
<tr>
<td>FfP_YohD</td>
<td>GATGGGAAAACAGTCCCCAGAC</td>
<td>(Boughner, LA &amp; Doerrler, 2012)</td>
</tr>
<tr>
<td>RfP_YohD</td>
<td>ACACCACCGGCGAGTCTGTG</td>
<td>(Boughner, LA &amp; Doerrler, 2012)</td>
</tr>
<tr>
<td>FfP_YqjA</td>
<td>GAACACGTTGAACCTTTGTAC</td>
<td>(Boughner, LA &amp; Doerrler, 2012)</td>
</tr>
<tr>
<td>RfP_YqjA</td>
<td>AAAGGAATGCGATGACGTC</td>
<td>(Boughner, LA &amp; Doerrler, 2012)</td>
</tr>
<tr>
<td>Ffp_yqaA</td>
<td>GGATTACGCCATTATTTTGACG</td>
<td>(Boughner, LA &amp; Doerrler, 2012)</td>
</tr>
<tr>
<td>RfP_yqaA</td>
<td>ACCTAATGCTTACGAGATGACC</td>
<td>(Boughner, LA &amp; Doerrler, 2012)</td>
</tr>
<tr>
<td>FfP_YghB (i.e. FP yghB)</td>
<td>TCTGGAAGATGTCGACGATCTG</td>
<td>(Boughner, LA &amp; Doerrler, 2012)</td>
</tr>
<tr>
<td>RfP_YghB (i.e. RP yghB)</td>
<td>AGGCGCGAGATGATGATCATCC</td>
<td>(Boughner, LA &amp; Doerrler, 2012)</td>
</tr>
<tr>
<td>FP_topA</td>
<td>AGTCCATATCGGTAACCTCGTTGC</td>
<td>This Study</td>
</tr>
<tr>
<td>RP_topA</td>
<td>TCCACTTAGCACGCGATCGAAAC</td>
<td>This Study</td>
</tr>
</tbody>
</table>
DNA sequencing: Sequencing of all PCR amplified DNA was performed with listed primer pairs (Table III-1) using an ABI 3130XL DNA Sequencer in the LSU Department of Biological Sciences Genomics Facility.

Transformations: Transformations were carried out using chemically competent (Inoue, H, Nojima, H et al., 1990), or electrocompetent cells, as previously published [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012)].

Mutant generation: Generation of mutants was completed as previously described [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012)]. In order to select for the topA10 allele present in strain RS2, a P1vir lysate was generated from the Keio collection (Baba, T, Ara, T et al., 2006) strain JW1253-1 (ΔtrpB::kanR, ~0.3 minutes from topA) and transduced into RS2 (giving rise to RS2_B). A lysate of RS2_B was then used to introduce the topA10 allele into BAL701 (giving rise to BAL703) followed by the curing of the kanamycin cassette from trpB::kanR, with temperature sensitive plasmid pCP20 (BAL704; Table III-2) [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012; Cherepanov, PP & Wackernagel, W, 1995; Datsenko, KA & Wanner, BL, 2000)]. After which, deletion of the final DedA gene, yghB, was attempted. All transduction reactions were carried out as previously described [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012; Silhavy, TJ, Berman, ML et al., 1984)]. All mutant genotypes were confirmed by PCR band analysis.

DNA extraction experiment: An overnight culture of BAL801 was diluted to a starting OD600 of ~0.03 in fresh LB, ampicillin and either 0.1% arabinose or 0.2% glucose. All DNA extractions were completed in triplicate following the manufacturer’s instructions at each indicated time point, from the same cell density (OD600 = 0.2).
Extractions were completed using Invitrogen's EasyDNA kit with the addition of allowing the DNA to precipitate in 100% ethanol at -20 °C overnight to maximize the extracted/precipitated DNA. DNA was resuspended in an equal volume of TE buffer, treated with RNase and concentration of DNA was measured using a Nanodrop Spectrophotometer.

**Table III-2: Vector Description**

<table>
<thead>
<tr>
<th>Vector</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>pBAD_EcEdA</td>
<td>pBAD expressing EcEdA; Amp&lt;sup&gt;+&lt;/sup&gt;</td>
<td>(Boughner, LA &amp; Doerrler, WT, 2012)</td>
</tr>
<tr>
<td>pCP20</td>
<td>FLP&lt;sup&gt;+&lt;/sup&gt;, λcl857&lt;sup&gt;+&lt;/sup&gt;, λRep&lt;sup&gt;+&lt;/sup&gt; promoter, Amp&lt;sup&gt;+&lt;/sup&gt;, Cam&lt;sup&gt;R&lt;/sup&gt;</td>
<td>E. coli Genetic Stock Center; (Cherepanov, PP &amp; Wackernagel, W, 1995)</td>
</tr>
<tr>
<td>pBADHisA</td>
<td>Expression vector, Amp&lt;sup&gt;+&lt;/sup&gt;, N-terminal polyhistidine tag and Xpress epitope, with the araBAD promoter</td>
<td>Invitrogen</td>
</tr>
</tbody>
</table>

**Fluorescence microscopy:** An overnight culture of BAL801 was diluted to a starting OD<sub>600</sub> of ~0.03 in LB/ampicillin and either 0.1% arabinose or 0.2% glucose, and grown until repressed culture reached desired growth phase (either entering growth arrest or death), and cells were resuspended in fresh LB to an OD<sub>600</sub> of 2.0. For staining, 20 μl of cells were mixed with 2 μl of DAPI (4',6-diamidino-2-phenylindole; 100 μg ml<sup>-1</sup>), and 10 μl of this mixture was placed on an agarose coated slide. Differential interference contrast (DIC) and all fluorescent images were captured with a Leica DM RXA2 deconvolution microscope (LSU Socolofsky Microscopy Center). Examination of the chromosome from wild type cells exposed to a proton ionophore, carbonyl cyanide p-chlorophenylhydrazone (CCCP) (Jovanovic, G, Lloyd, LJ et al.,
2006), was completed on strains grown and treated as recently published (Sikdar, R, Simmons, AR et al., 2013). Briefly, an overnight culture of W3110/pBADHisA was diluted 1:100, grown to OD$_{600}$~1.0, then diluted 1:3 into LB/amp with and without 50 μM CCCP and grown for an additional 60 minutes.

III.3. Results

**Genomic anomalies of DedA deletion mutants**: The genome of *E. coli* contains eight members of the DedA family distributed throughout the chromosome (Figure III-1). The strategy for the generation of BAL801 has been described in detail [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012)]. Briefly, the genomic *ydhXYZ* region was deleted by homologous recombination of a generated PCR product (*ydhXYZ::cam*)$^R$. Subsequent transductions were completed using lysates of the single DedA family gene deletions obtained from the Keio collection (Baba, T, Ara, T et al., 2006), followed by removal of the kan$^R$ cassette (Cherepanov, PP & Wackernagel, W, 1995; Datsenko, KA & Wanner, BL, 2000). Resulting in a series of mutants leading to BAL701 harboring only a chromosomal copy of *yghB* [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012)]. Final deletion of *yghB* from BAL701 to create BAL801 required prior transformation with a plasmid (pBAD) encoding Ec*dedA*. Growth of BAL801 was entirely dependent upon the presence of arabinose in the growth media; growth in the presence of glucose resulted in cell lysis [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012)].
Figure III-1: The DedA family and the *E. coli* chromosome. Relative chromosomal positions of the eight *E. coli* DedA genes within the major structural domains of the *E. coli* MG1655 bacterial nucleoid as previously described (Mercier, R, Petit, MA et al., 2008). (Depicted domains redesigned/recreated after Figure S7 of Mercier et al. 2008.) NSR/NSL: non-structure right/left.

We attempted to generate BAL800 mutants using a number of different cloned DedA family genes expressed from the pBAD plasmid. In some cases, unexpected anomalies occurred. Independent and repeated attempts to generate a BAL800 mutant with cloned *ydjX* or *ydjZ*, consistently resulted in a genomic duplication of the *yghB* gene region, where wild type *yghB* and *yghB::kan^R* were both present (Figure
Also, the process for mutant generation was pursued through two pathways, differing only in whether \textit{yqjA} or \textit{yghB} was the final DedA gene deleted [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012)]. The BAL800s were successful generated with BAL701 as the preceding mutant; however during the generation of BAL702 (where only chromosomal \textit{yqjA} remains after deletion of \textit{yqaA}; Table III-3, page 59) a consistent result was the apparent inability to generate a PCR product using respective flanking primers for two DedA genes, Ec\textit{dedA} and \textit{yghB} (Figure III-3a, page 58). To investigate the possibility of a double crossover event between ΔEc\textit{dedA} and Δ\textit{yghB} [located at 52.42’ and 67.93’, respectively (Figure III-1)], a PCR reaction using flip-flopped forward and reverse primers of Ec\textit{dedA} and \textit{yghB} gene regions, surprisingly, resulted in PCR products recovering the ‘missing’ genes (Figure III-3b, page 58). Sequencing this ‘flip-flopped’ PCR product confirmed that a crossover event occurred within the scar peptide (81 bp sequence that remains following kan\textsuperscript{R} curing [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012) and references therein]) in place of both remnant DedA genes (Ec\textit{dedA} and \textit{yghB}).

As can be seen in Figure III-2 (page 58) and Figure III-3 (page 58), the sequential removal of particular \textit{E. coli} DedA protein family members consistently resulted in chromosomal abnormalities. Since no such recombination occurred prior to these penultimate DedA mutant generation stages, we conclude that loss of multiple DedA family proteins results in inappropriate DNA recombination, possibly due to a loss of nucleoid organization and/or maintenance.

**Genomic content within BAL801:** The observed genomic anomalies lead to the question: what is happening to the genome and how is it affected by the loss of this
family of inner membrane proteins? Since BAL801 dies when expression of EcDedA is repressed, and given the results depicted in Figure III-2 and Figure III-3, the possibility exists that the essentiality of the DedA family lies in the maintenance of the genome. To begin to examine this hypothesis, DNA was extracted from cells of an equal OD$_{600}$ at various time points of induced and repressed BAL801 growth (i.e. logarithmic, growth arrest, and death phase) and its concentration was measured. We repeatedly observed that the genomic DNA concentration of repressed BAL801 cells significantly increased as the cells entered growth arrest, and ultimately died (Figure III-4a, page 60; Figure III-5a, page 62). The genomic content of BAL801 cells grown under inducing conditions showed no differences when compared at either phase (Figure III-4a, page 60) or to the DNA extracted from wild type cells, W3110A (Figure III-4, page 60). Additional controls consisted of completing the same experiment with two different temperature sensitive strains, one that enters growth arrest [WD2 (Doerrler, WT, Reedy, MC et al., 2001)] and a second that undergoes lysis [SM101 (Galloway, SM & Raetz, CR, 1990)] when grown at 42 °C. Also examined was the impact of growth at elevated temperatures on DNA concentration in wild type cells (W3110A). In all cases, the DNA concentration either was not significantly different (SM101; data not shown) or decreased (WD2 and W3110A. Figure III-4b, page 60) when grown at 42 °C. We conclude that the observed significant increase in DNA concentration is specific for a mutant devoid of all DedA proteins.

**Imaging the Genome**: Because of the genomic anomalies, we used the nucleotide stain 4’,6-diamidino-2-phenylindole (DAPI) to visualize DNA *in vivo*, as the cellular
organization of the nucleoid may not have been normal upon depletion of EcDedA in repressed BAL801. We found that when EcDedA was depleted and cells entered the death phase, the chromosome content within a cell appeared to be either drastically increased or missing entirely (cell anucleation) (Figure III-5, page 62). The number of anucleated cells observed is comparable to the approximate 15% observed using this staining procedure with ΔmukB mutants (Niki, H, Jaffe, A et al., 1991), where MukB is a known E. coli protein involved in the structural maintenance of the chromosome (SMC). The genomic organization of BC202, missing only two DedA genes, yghB and yqjA, is comparable to wild type cells [Figure III-6, page 64; (Thompkins, K, Chattopadhyay, B et al., 2008)]. Since significant anucleation only appears once BAL801 begins to die, and not simply when cells enter growth arrest, this suggests that the nucleoid is not properly organized or segregated once BAL801 is depleted of all DedA proteins.

Recently we published that the mutant BC202 cannot maintain the cytoplasmic membrane PMF (Sikdar, R, Simmons, AR et al., 2013), and to account for the possible involvement of the PMF on the nucleoid organization of repressed BAL801, parent strain W3110/pBADHisA (Table III-2, page 52) was grown in the presence of the proton ionophore CCCP [carbonyl cyanide p-chlorophenylhydrazone; known to deplete cellular PMF (Jovanovic, G, Lloyd, LJ et al., 2006)] for 60 minutes, and stained with DAPI to visualize the nucleoid. Though such cells grown in the presence of CCCP were beginning to die, the organization of the nucleoid within each cell appeared normal under these conditions (Figure III-7, page 64). We conclude that loss of PMF by itself does not independently affect chromosomal organization.
Figure III-2: Incomplete generation of certain *E. coli* DedA family BAL800 series deletion mutants. Genomic DNA from BAL810 (lanes 1 and 2), BAL820 (lanes 3 and 4), BAL803 (lane 5), and W3110A (lane 6) was the template in PCR reactions using respective primer pairs flanking specified gene regions. BAL810 harbors pBAD-cloned *ydjX*, BAL820 (cloned *ydjZ*), and BAL803 (cloned *yohD*) (see Table III-3 for complete genotype descriptions). With BAL810 and BAL820, the amplification of the chromosomal *yghB* gene region results in two bands, wt *yghB* and *yghB::kan*<sup>R</sup>. BAL803 is an example of a successful mutant generation [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012)]. All primers listed in Table III-1 (page 50).

Figure III-3: PCR confirmation of DedA gene deletions and evidence of double crossover event. (a) PCR amplification of BAL702 isolates 1-4, and wild type W3110A DNA. Through multiple and independent mutant generation attempts, it was not possible to amplify the DedA gene regions of ΔEc*dedA* and Δ*yghB*. Ec*dedA* and *yghB* are located at 52.42' and 67.93', respectively, on the *E. coli* chromosome (see Figure III-1, page 54). (b) PCR amplification of ‘missing’ gene regions ΔEc*dedA* and Δ*yghB* of BAL702 isolates 1-3, using flip-flopped primer pairs (FP_*yghB* with RP_Ec*dedA*; and RP_*yghB* with FP_Ec*dedA*). All primers are listed in Table III-1 (page 50).
<table>
<thead>
<tr>
<th>Strain</th>
<th>Description</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>W3110</td>
<td>Wild-type; F, λ, IN(rrnD-rrnE)1, rph-1</td>
<td>The Coli Genetic Stock Center</td>
</tr>
<tr>
<td>W3110A</td>
<td>aroA::Tn10 (Tetr P1vir transductant of W3110; LCB273 donor)</td>
<td>(Doerrler, WT, Gibbons, HS et al., 2004)</td>
</tr>
<tr>
<td>BAL801</td>
<td>W3110, ΔydjXYZ::cam, Δyabl772, ΔEcdedA726, ΔyohD762, ΔyqjA785, ΔyqaA770, ΔyghB781::kan, pBAD_EcededA</td>
<td>(Boughner, LA &amp; Doerrler, WT, 2012)</td>
</tr>
<tr>
<td>BAL810</td>
<td>W3110, ΔydjXYZ::cam, Δyabl772, ΔEcdedA726, ΔyohD762, ΔyqjA785, ΔyqaA770, ΔyghB781::kan, pBAD_ydjX</td>
<td>This Study</td>
</tr>
<tr>
<td>BAL820</td>
<td>W3110, ΔydjXYZ::cam, Δyabl772, ΔEcdedA726, ΔyohD762, ΔyqjA785, ΔyqaA770, ΔyghB781::kan, pBAD_ydjZ</td>
<td>This Study</td>
</tr>
<tr>
<td>BAL803</td>
<td>W3110, ΔydjXYZ::cam, Δyabl772, ΔEcdedA726, ΔyohD762, ΔyqjA785, ΔyqaA770, ΔyghB781::kan, pBAD_yohD</td>
<td>(Boughner, LA &amp; Doerrler, WT, 2012)</td>
</tr>
<tr>
<td>RS2</td>
<td>F-, gal-25, λ-, topA10, pyrF287, fnr-1, rpsL195(strR), iclR7(const), trpR72(Am)</td>
<td>The Coli Genetic Stock Center; (Sternglanz, R, Dinardo, S et al., 1981)</td>
</tr>
<tr>
<td>JW1253-1</td>
<td>Δ(araD-araB)567, ΔlacZ4787::rrnB-3, λ-, ΔtrpB769::kan, rph-1, Δ(rhaD-rhaB)568, hsdR514</td>
<td>The Coli Genetic Stock Center; (Baba, T, Ara, T et al., 2006)</td>
</tr>
<tr>
<td>RS2_B</td>
<td>RS2, P1vir ΔtrpB769::kan</td>
<td>This Study</td>
</tr>
<tr>
<td>BAL701</td>
<td>W3110, ΔydjXYZ::cam, Δyabl772, ΔEcdedA726, ΔyohD762, ΔyqjA785, ΔyqaA770</td>
<td>(Boughner, LA &amp; Doerrler, WT, 2012)</td>
</tr>
<tr>
<td>BAL702</td>
<td>W3110, ΔydjXYZ::cam, Δyabl772, ΔEcdedA726, ΔyohD762, ΔyghB781, ΔyqaA770::kan</td>
<td>(Boughner, LA &amp; Doerrler, WT, 2012)</td>
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<tr>
<td>BAL703</td>
<td>W3110, ΔydjXYZ::cam, Δyabl772, ΔEcdedA726, ΔyohD762, ΔyqjA785, ΔyqaA770::kan, topA10, ΔtrpB769::kan</td>
<td>This Study</td>
</tr>
<tr>
<td>BAL704</td>
<td>W3110, ΔydjXYZ::cam, Δyabl772, ΔEcdedA726, ΔyohD762, ΔyqjA785, ΔyqaA770, topA10, ΔtrpB769</td>
<td>This Study</td>
</tr>
<tr>
<td>BAL840</td>
<td>W3110, ΔydjXYZ::cam, Δyabl772, ΔEcdedA726, ΔyohD762, ΔyqjA785, ΔyqaA770, topA10, ΔtrpB769, ΔyghB781::kan</td>
<td>This Study</td>
</tr>
<tr>
<td>BAL420</td>
<td>W3110, ΔyqjA::tetR, Δyabl772, ΔyohD762, ΔyghB781::kan</td>
<td>(Boughner, LA &amp; Doerrler, WT, 2012)</td>
</tr>
<tr>
<td>WD2</td>
<td>W3110, aroA::Tn10, msbA2</td>
<td>(Doerrler, WT, Reedy, MC et al., 2001)</td>
</tr>
</tbody>
</table>
Figure III-4: Measured DNA concentrations of *E. coli* strains. 
(a) BAL801 was grown at 30 °C in presence of inducer (arabinose) and repressor (glucose) until cell death was observed for repressed BAL801. DNA extractions were carried out (in triplicate) at the times indicated and concentration of DNA was measured. (b) W3110A and WD2 [a temperature sensitive strain (Doerrler, WT, Reedy, MC et al., 2001)], were grown at 30 °C and 42 °C, DNA was extracted when WD2 grown @ 42 °C entered growth arrest. In all cases, DNA concentration was measured with a Nanodrop spectrophotometer. *P*-value (*) < 0.01 and (**) < 0.02 and (***)) < 0.005 using two-tailed Student’s t-test.
Lack of suppression by known SMC mutant suppressor, topA10: Known SMC proteins, including *E. coli* MukB, are cytoplasmic and all have a very specific three dimensional structure (see discussion) (Gruber, S, 2011; Hirano, T, 2005). *E. coli* DedA proteins are inner membrane proteins, unlike known SMCs. DedA proteins are not predicted to have ATPase activity, which is necessary to initiate MukB’s condensin activity (Carter, SD & Sjogren, C, 2012). topA10 is an allele of DNA topoisomerase I (*topA*) that is known to suppress ΔmukB phenotypes due to its increased negative supercoiling activity (Sawitzke, JA & Austin, S, 2000). Due to the observed phenotypic similarities between ΔmukB and repressed BAL801, we attempted to isolate a BAL800 series mutant with the addition of topA10 without a cloned DedA protein. In other words, can *E. coli* exist without the DedA protein family in the presence of the topA10 allele? However, successful isolation of a BAL800 series mutant (i.e. true deletion of DedA family) with topA10 was not possible, as the genome became very unstable. All isolates had evidence of non-amplifiable genes (DedA gene regions with scar peptides; Δyabl, ΔEcdedA, ΔyohD, ΔyqjA, and/or ΔyqaA) and either genomic duplication of the yghB region (Figure III-8a) or the double transduction of ΔyghB::kanR with wild type yqjA (Figure III-8b and c). Some of the non-amplifiable genes were confirmed to be double crossover events, but it was not possible to confirm all, due to the large number of potential recombinations. The inability of topA10 to suppress the genomic instabilities of a mutant devoid of the DedA family, suggests that the family may be necessary for proper topological and locational arrangement of the nucleoid *in vivo*, and that nucleoid changes observed in repressed BAL801 are different from those observed in ΔmukB cells.
**Figure III-5:** Imaging nucleoid of BAL801 grown under inducing and repressing conditions. Growth curves were continued until repressed BAL801 entered growth arrest (a), and cell death (f). Mutant was grown in the presence of 0.1% arabinose (inducing conditions) or 0.2% glucose (repressing conditions). DIC and DAPI images were captured at the indicated times for each growth curve (b-e when entering growth arrest and g-j when repressed BAL801 cells were beginning to die). DNA concentration was measured as cells were entering growth arrest (panel a). (b-e and g-j) DIC and fluorescence images of DAPI treated BAL801 grown in the presence of arabinose (b/c) and (g/h); and grown in the presence of glucose (d/e) and (i/j). Cells for DNA extraction in (a) were placed on ice until DAPI images were captured using a Leica DM RXA2 deconvolution microscope. *P*-value (*) < 0.01 using two-tailed Student’s t-test.
Figure III-5
**Figure III-6**: Microscopic images of DAPI treated BC202 (ΔyqiA and ΔyghB) (Thompkins, K, Chattopadhyay, B et al., 2008). (a) Differential interference contrast (DIC) and fluorescent DAPI overlay, (b) DAPI only. All images were captured using a Leica DM RXA2 deconvolution microscope.

**Figure III-7**: Disruption of PMF using proton ionophore CCCP does not affect nucleoid organization. W3110/pBADHisA grown in the absence (a/b) and presence (c/d) of 50 μM CCCP (carbonyl cyanide p-chlorophenylhydrazone). All differential interference contrast (DIC) (a/c) and DAPI (b/d) images were captured using a Leica DM RXA2 deconvolution microscope. There were no apparent differences between the nucleoids of either W3110/pBADHisA with or without CCCP, indicating that a deficient PMF alone does not impact the organization of *E. coli*'s nucleoid under these conditions.
Figure III-8: Incomplete generation of a mutant devoid of all DedA members (chromosomal or cloned) in the presence of DNA topoisomerase I allele topA10. TopA10 increases negative supercoiling of the chromosome, suppressing the cell anucleation phenotype of ΔmukB mutants (Sawitzke, JA & Austin, S, 2000). (a) PCR amplification of six isolates (BAL840; Table III-3, page 59) generated in the attempt to create a BAL800 mutant with topA10 (no cloned DedA). In all cases (multiple and independent transduction reactions), there are instances of non-amplifiable gene regions and either duplication of the yghB genomic region or double transduction of yghB::kanR and the wild type yqjA gene. (b) and (c) Microscopic images of the single isolate that resulted in the double transduction of yghB::kanR and the wild type yqjA gene, as well as non-amplifiable gene regions; differential interference contrast (DIC) (b) and DAPI (c) images were captured using a Leica DM RXA2 deconvolution microscope. The cell morphology and genomic organization of the double transduction BAL840 mutant are as wild type (Figure III-7).

III.4. Discussion

The DedA protein family is highly conserved across all domains of life, suggesting that they play a significant physiological role. Whether that role is the same for all DedA homologs present within the same species, or across species is unknown. The significance of the DedA family has been demonstrated in two different organisms in which the family is essential, B. burgdorferi (Liang, FT, Xu, QL et al., 2010) and E. coli [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012)]. In
addition, it has been reported that the *Mycobacterium bovis* DedA homolog (Bcg2664) is a possible drug target (Arai, M, Liu, L et al., 2011). As such, the identification of the essential function of the *E. coli* DedA protein family would be a dramatic and significant step towards understanding this widely distributed family.

The generation of BAL800 series mutants resulted in some interesting anomalous observations regarding the genome. The duplication of the *yghB* region, as well as the inability to PCR amplify two DedA gene remnants due to a crossover event (Figure III-2, page 58; Figure III-3, page 58), suggests that if certain DedA protein members are the only remaining member in *E. coli* (i.e. *ydjX* or *ydjZ; yqjA*), genomic instability ensues. Also, the double crossover event of BAL702 consistently occurred within the scar peptides of \( \Delta EcdedA \) and \( \Delta yghB \), and not in any other combination of the total four scar peptides (\( \Delta EcdedA, \Delta yghB, \Delta yohD, \) and \( \Delta yabI \)). The genomic regions surrounding the *EcdedA* and *yghB* genes may be located physically near each other in the cell. In this scenario, homologous recombination between these particular scar peptides (as opposed to \( \Delta yohD \) and \( \Delta yabI \)) occurs out of locational convenience (Figure III-1, page 54). These instabilities were consistently observed during multiple and independent mutant generation attempts, and may be due to the function of the remaining DedA proteins going unchecked from a lack of (or improper localization of) regulating member(s). The *topA10* allele, capable of suppressing the anucleation phenotypes of \( \Delta mukB \) mutants, had no effect upon the genomic instabilities of a BAL800 mutant, in fact, the genomic instabilities increased. This is consistent with an altered nucleoid topological structure which cannot be corrected by the increased negative supercoiling activity of TopA10.
Upon further investigation, it was discovered that the genome of repressed BAL801 displays a significant increase in DNA concentration, a loss of genomic organization and cell anucleation, all preceding cell death. Detailed reviews regarding the replication and segregation of the bacterial genome have been published and as such will only briefly be covered herein (Boeneman, K & Crooke, E, 2005; Bravo, A, Serrano-Heras, G et al., 2005; Britton, RA, Lin, DCH et al., 1998; Carter, SD & Sjogren, C, 2012; Cobbe, N & Heck, MMS, 2000; Dame, RT & Dorman, CJ, 2010; Dillon, SC & Dorman, CJ, 2010; Hirano, T, 1999; Holmes, VF & Cozzarelli, NR, 2000; Kaguni, JM, 2006, 2011; Le Chat, L & Espéli, O, 2012; Mott, ML & Berger, JM, 2007; Strunnikov, AV, 2006). Regulation of genomic replication is mediated in part by both DnaA and SeqA. DnaA-ATP binds the DnaA boxes in and around the origin of replication (oriC) leading to initiation of replication, and newly synthesized hemi-methylated origins are sequestered near the membrane by SeqA, thereby limiting replication initiation to once per cell division cycle (Bahloul, A, Meury, J et al., 1996; Charbon, G, Riber, L et al., 2011; Slater, S, Wold, S et al., 1995). DnaA-ATP is also regenerated through titration of DnaA molecules with the datA locus and regulatory inactivation of DnaA (RIDA) (Camara, JE, Breier, AM et al., 2005; Kaguni, JM, 2006; Kasho, K & Katayama, T, 2013). Recently identified was a new mechanism by which DnaA-ATP hydrolysis occurs in a datA locus- and IHF binding-dependent manner, the datA-dependent DnaA-ATP hydrolysis pathway (DDAH) (Kasho, K & Katayama, T, 2013).

The increased genomic content within repressed BAL801 which occurs prior to cell anucleation (Figure III-4a, page 60; Figure III-5a, page 62), suggests that
chromosomal replication may no longer be properly regulated, followed by the improper segregation of the chromosome (anucleation) (Figure III-5, page 62), ultimately leading to cell death. The cause of death in repressed BAL801 may be due to double strand breaks that result from over-initiation of chromosomal replication, as this is known to be toxic (Simmons, LA, Breier, AM et al., 2004). Also, eukaryotic cohesion and E. coli HU proteins are known to play a role in repairing double strand breaks (Carter, SD & Sjogren, C, 2012; Hashimoto, M, Imhoff, B et al., 2003; Lindroos, HB, Strom, L et al., 2006; Strom, L, Lindroos, HB et al., 2004; Unal, E, Arbel-Eden, A et al., 2004), as well as the role nucleoid compaction is suspected to play in the survivability of radiation-resistant organisms (Toueille, M, Mirabella, B et al., 2012). The loss of chromosomal replication regulation may lead to the cell anucleation, or the DedA proteins themselves may possibly play a direct role in the maintenance and segregation of the chromosome, as EcDedA has been found to bind DNA (Kumar S. and Doerrler WT, manuscript in preparation). The identification of a new method by which DNA replication is regulated through DDAH (Kasho, K & Katayama, T, 2013), indicates that genomic regulation occurs through multiple (and possibly not yet identified) pathways/mechanisms.
Figure III-9: Predicted Topology of *E. coli*’s DedA proteins and potential significance of the cytoplasmic loop.

(a) Predicted topology of DedA proteins, based on TMHMM v.2.0 Predictive transmembrane domain software (http://www.cbs.dtu.dk/services/TMHMM/), the experimental topological investigation of *E. coli*’s YqjA (Thompkins, KS, 2010), and the experimental determination that all *E. coli* DedA proteins (excluding YqaA) have their C-terminus in the cytoplasm (Daley, DO, Rapp, M et al., 2005). (b) Alignment of amino acid sequences of the cytoplasmic loop regions from *E. coli* DedA family proteins. The identified Glycine residue (indicated with *), is the single conserved residue in the DedA domain [Chapter I, pages 1-15; (Doerrler, WT, Sikdar, R et al., 2013)]. Residues were highlighted to indicate predicted preference for either aqueous or hydrophobic environments (Kyte, J & Doolittle, RF, 1982). Yellow (positively charged R group), blue (polar uncharged) or green (negatively charged) indicates hydrophilic residues (<3.5 hydropathy index), and those in purple are hydrophobic residues (>3.5 hydropathy index). Basic residues are R,H,K.

All *E. coli* DedA family proteins are predicted to possess a cytoplasmic loop located between transmembrane helices 2 and 3 containing a number of positively charged amino acids (EcDedA amino acids 98-156; loop pI = 10.44), seemingly
regularly spaced (Figure III-9). The predicted topology of *E. coli* DedA proteins stems from multiple sources: the topological predictive software TMHMM v.2.0, experimental evidence of YqjA’s topology (Thompkins, KS, 2010), comparative analysis of DedA protein alignments (Doerrler, WT, Sikdar, R et al., 2013), as well as the determination that all *E. coli* DedA proteins (excluding YqaA) have a cytoplasmic C-terminus (Daley, DO, Rapp, M et al., 2005). This cytoplasmic loop is predicted to fold into a secondary structure, rich in $\alpha$-helices, using the prediction software Phyre2 [Protein Homology/analogy Recognition Engine V 2.0 (Kelley, LA & Sternberg, MJE, 2009)] and is thought the interacting region between EcDedA and DNA (Kumar S. and Doerrler WT, manuscript in preparation). It is unusual, but not unprecedented for a membrane protein to bind DNA (Ulbert, S, Antonin, W et al., 2006; Ulbert, S, Platani, M et al., 2006).

DnaA, or more specifically DnaA-ATP, is responsible for initiating the replication of *E. coli’s* genome (Aranovich, A, Gdalevsky, GY et al., 2006; Crooke, E, 2001). DnaA localizes to the membrane (Newman, G & Crooke, E, 2000), and regeneration of DnaA-ATP is stimulated by acidic phospholipids (Aranovich, A, Gdalevsky, GY et al., 2006; Crooke, E, 2001). Though BAL801 demonstrates an increase in acidic phospholipids (cardiolipin and phosphatidylglycerol) similar to BC202 [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012)], the genomic content within BC202 is not affected (Figure III-6, page 64). The possibility remains that the *E. coli* DedA proteins may aid in the localization of DnaA to the membrane where regeneration of DnaA-ATP takes place, since it is only after EcDedA protein depletion that the genomic content of repressed BAL801 increases, regardless of
lipid composition. Although the PMF in BC202 was recently found to be deficient (Sikdar, R, Simmons, AR et al., 2013), this deficiency does not affect the organization of the genome, as the chromosome of W3110/pBADHisA cells grown in the presence of CCCP [known to impair the PMF (Jovanovic, G, Lloyd, LJ et al., 2006)] appear very similar to cells grown without CCCP (Figure III-7, page 64). The idea that membrane associated protein(s) may be involved in regulating genomic replication has been proposed (Boeneman, K & Crooke, E, 2005); however to our knowledge, no protein has been identified as essential for this function in E. coli, or genomic maintenance in general.

Other mutants have demonstrated similar phenotypes, specifically those proteins involved in the structural maintenance of the chromosome (SMC). Most of the $\Delta$SMC mutants identified thus far are viable and display similar phenotypes (discussed below); and an SMC protein in E. coli has yet to be demonstrated as essential for survival (Britton, RA, Lin, DCH et al., 1998; Niki, H, Jaffe, A et al., 1991). Known SMC proteins all have a similar ‘V’ like structure, with the ability to hydrolyze ATP, bind DNA and potentially other proteins required for functionality (Britton, RA, Lin, DCH et al., 1998). The ATP hydrolysis of MukB (an E. coli SMC protein) is only required to initiate the condensin activity (Carter, SD & Sjogren, C, 2012). In most cases, deletion of SMC proteins results in ~15% cell anucleation, temperature sensitivity, reduced ability to form colonies, as well as chaining of cells (Britton, RA, Lin, DCH et al., 1998; Niki, H, Jaffe, A et al., 1991). The sequence homology for known SMC proteins is not highly significant; for example, MukB does possess the expected ‘V’-like structure, however it does not share significant sequence homology.
with other SMC proteins [ex. NCBI BLAST alignment (Sayers, EW, Barrett, T et al., 2012), querying MukB against Bacillus subtilis Smc, results in E-value = 0.005]. Conversely, DedA proteins are predicted inner membrane proteins, for which sequence analysis does not suggest any functional similarity to known proteins (transporters, enzymes, channels, or two-component signal transducers), though there has been speculation of certain DedA family proteins functioning as transporters (Doerrler, WT, Sikdar, R et al., 2013; Khafizov, K, Staritzbichler, R et al., 2010; Sikdar, R, Simmons, AR et al., 2013). Interestingly, a BLAST analysis of the B. subtilis Smc protein against the E. coli genome most significantly results in transporters and enzymes, followed by MukB with a lower significance [BLAST (Sayers, EW, Barrett, T et al., 2012) E-value = 4.3].

Of particular interest is the species Deinococcus radiodurans, since SMC mutants (Δsmc) do not demonstrate any significant phenotypes (merely an increased sensitivity to gyrase inhibitors); the D. radiodurans genome contains only four of the twelve nucleoid associated proteins (NAPs) thus far identified in E. coli (Bouthier De La Tour, C, Toueille, M et al., 2009). However, the NAP HU protein was found to be essential in D. radiodurans and the null mutant displayed similar phenotypes to other ΔSMC mutants (i.e. E. coli ΔmukB) discussed above (Nguyen, HH, La Tour, D et al., 2009). A protein BLAST analysis of all eight E. coli DedA homologs against the D. radiodurans R1 genome indicates three significant (E-value ≤ 10⁻⁴) DedA homologs. We believe that the genomic anomalies observed during the generation of BAL800s, as well as the phenotypes of repressed BAL801, occurred due to the essential role E. coli DedA protein members play in the maintenance and organization of the nucleoid.
(Figure III-10). In the absence of any DedA member, the chromosome content within a cell either increases significantly, or disappears entirely (cell anucleation) (Figure III-4, page 60; Figure III-5, page 62).

We propose here that the essential function of the *E. coli* DedA protein family is to play a crucial, but as yet unidentified, role in the maintenance and organization of the bacterial nucleoid (see model in Figure III-10). However, each DedA member may play a slightly different role, such that each DedA protein may have a specific and independent part to play in maintaining the nucleoid. As an *E. coli* cell devoid of all DedA proteins is incapable of properly maintaining and organizing its chromosome (Figure III-4, page 60; Figure III-5, page 62), ultimately leading to cell death; and the singular presence of some DedA proteins (YdjX, YdjZ, and YqjA), leads to anomalous genomic instabilities (Figure III-2, page 58; Figure III-3, page 58). In vivo, DedA proteins may function as homo- or hetero-dimers, and likely function collaboratively with other nucleoid associated proteins (NAPs) in maintaining the nucleoid (Figure III-10).

Recently, a new condensin bacterial family was identified, MksBEF (*MukB*-like SMC proteins) (Petrushenko, ZM, She, WF *et al.*, 2011). The Mks proteins are highly conserved, occurring in organisms in conjunction with either of the well characterized SMC protein groups (MukBEF or SMC-ScpAB), and as suggested by the authors, the organization of the bacterial nucleoid is more complex than was previously thought, involving multiple SMC protein groups (Petrushenko, ZM, She, WF *et al.*, 2011). Therefore, the involvement of the DedA membrane protein family with nucleoid organization and maintenance is highly possible, especially considering the high
degree of speculation for inner membrane, or membrane associated protein(s) playing a role in chromosome maintenance. The DedA protein family was already known to be essential for cell viability in two species, *E. coli* [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012)] and *B. burgdorferi* (Liang, FT, Xu, QL *et al.*, 2010), however this work demonstrates for the first time, that the essentiality of this inner membrane protein family, in *E. coli*, is directly tied to maintenance of the bacterial nucleoid. Future work will aim to determine the specific mechanism by which these essential membrane proteins maintain *E. coli*’s chromosome.

**Figure III-10:** Model proposing the most probable essential role of the *E. coli* DedA protein family. In the absence of any DedA protein member, or the singular presence of particular members, the chromosome becomes highly disordered and unstable. The proposed essential role of the *E. coli* DedA protein family is to play a role in maintaining and organizing the bacterial nucleoid, along with other SMC and NAP proteins. DedA family proteins may function as homo- or hetero-dimers. See text for further discussion.
IV. Preliminary Investigations into the C (complementing) and NC (non-complementing) functional groups of the DedA membrane protein family as defined in *Escherichia coli*

IV.1. Introduction

The DedA membrane protein family has been demonstrated as a collectively essential group of proteins in two distinct Gram negative organisms, *Escherichia coli* [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012)], and *Borrelia burgdorferi* (Liang, FT, Xu, QL et al., 2010). The DedA membrane protein family is widely distributed, with members found across all domains of life (Bacteria, Archaea, and Eukarya). The number of members within each organism varies from organism to organism (Liang, FT, Xu, QL et al., 2010), from the single DedA homolog in *B. burgdorferi* (Fraser, CM, Casjens, S et al., 1997), to as many as eight DedA proteins in *E. coli* (Blattner, FR, Plunkett, G et al., 1997). The *E. coli* DedA protein family consists of *yqjA, yghB, yabI, yohD, ydjX, ydjZ, yqaA* and *dedA* (to avoid confusion with the DedA protein family, hereafter the *E. coli* dedA/DedA gene/protein is referred to as EcdedA or EcDedA, respectively). The DedA membrane protein family was found to be collectively essential in both *B. burgdorferi* (Liang, FT, Xu, QL et al., 2010) and *E. coli* [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012)].

The functions of this family are just beginning to come to light; with specific members likely playing a role in membrane transport, as well as the general maintenance of the inner membrane’s integrity, including the proton-motive force (PMF) (Doerrler, WT, Sikdar, R et al., 2013; Sikdar, R & Doerrler, WT, 2010; Sikdar, R, Simmons, AR et al., 2013; Thompkins, K, Chattopadhyay, B et al., 2008). Thus
far, most characterizations of the DedA protein family have been based on investigations of the *E. coli* mutant BC202 (Δ*yqjA::tet*, Δ*yghB::kan*). The phenotypes of BC202 consist of temperature sensitivity, inefficient cell division, an altered phospholipid composition, as well as a decreased membrane potential. Many of these phenotypes can be complemented by a number of factors, including overexpression of other proteins (DedA family C-group, TatABC, or H⁺ transporters), or growth of the mutant in acidic media (pH 6). Based on complementation analysis of the varying *E. coli* DedA proteins in BC202, it was determined that there are two functional groups: those that are able to restore normal cell division and growth to BC202 at high temperatures (YqjA, YghB, YabI, and YohD; the Complementing, C-group) and those that cannot (YdjX, YdjZ, YqaA, and EcDedA; the Non-Complementing, NC-group) [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012)].

Given the redundancy within the *E. coli* DedA family, the identification of the two functional groups was seen as an opportunity to further characterize the *E. coli* DedA membrane protein family. Beginning with the generation of a new series of DedA mutants, in which combinations of the two functional groups were deleted to aid in defining the role each DedA member actually plays. Here we report the results of complementation analyses of these new C-group (complementing) and NC-group (non-complementing) mutants of the DedA membrane protein family, in which unexpected phenotypic discrepancies were observed.
IV.2. Methods and Materials

Materials: All materials were purchased from VWR, Sigma-Aldrich, BioRad, Qiagen, or New England Biolabs.

Microbial Growth Conditions: All strains were grown at 30 °C in Luria-Bertani medium (LB: yeast extract 5 g L⁻¹, tryptone 10 g L⁻¹, NaCl 10 g L⁻¹ and pH 7), (unless otherwise noted) and with respective antibiotics: 10 μg chloramphenicol ml⁻¹, 30 μg kanamycin ml⁻¹, 12.5 μg tetracycline ml⁻¹, or 100 μg ampicillin ml⁻¹.

Mutant Generation: Mutant strains were generated following the protocol already described [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012)]. Briefly, a mutant deleted for all NC-group proteins began with strain BAL202 (Table IV-1). BAL202 was created by transducing W3110 with a lysate from a strain (DY330) that was capable of undergoing homologous recombination with a ydjXZY::camR PCR product [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012)]. Subsequently, the individual lysates of single DedA gene deletion mutants from the Keio collection (Baba, T, Ara, T et al., 2006), were used to transduce the preceding mutants and the kanR cassette was removed using the temperature sensitive plasmid pCP20 [FLP⁺, λcl857⁺, λRepⅨ promoter, AmpR, CamR (Cherepanov, PP & Wackernagel, W, 1995; Datsenko, KA & Wanner, BL, 2000)]. Generation of a mutant deleted for all C-group proteins followed a similar process but began with strain BC203 [yqjA::tetR, (Thompkins, K, Chattopadhyay, B et al., 2008)]. The order in which the DedA genes were deleted from ΔNC-group and ΔC-group strains, are depicted in Figure IV-1. See Table IV-1 for a full genotypic description of all strains.
Figure IV-1: Depiction of the strategy utilized to generate each of the two functional group mutant series. (a) Depicts the series of mutants generated for the DedA proteins that cannot complement BC202, ΔNC-group. (b) Generated mutant series in which the DedA proteins that are able to complement BC202 are deleted, ΔC-group.

Microscopy: Overnight cultures of strains were diluted 1:100 into fresh LB with respective antibiotics and grown to an OD$_{600}$ of ~0.6. 1 ml of cells was centrifuged and resuspended to an OD$_{600}$ of 1.0. 20 μl of cells was mixed with 2 μl of DAPI (4',6-diamidino-2-phenylindole; 100 μg ml$^{-1}$), and 10 μl of DAPI stained cells was added to an agarose coated slide. All microscopic images (DIC and fluorescence) were captured using a Leica DM RXA2 deconvolution microscope (LSU Socolofsky Microscopy Center).

Growth of ΔC- and ΔNC-group mutants at 42 °C with and without magnesium: Strains BC202, BAL310, BAL320, BAL410 and BAL420 were streaked across kanamycin plates at 42 °C and 30 °C to determine each strain’s ability to grow at elevated temperatures (for full genotypic descriptions, see Table IV-1). Upon confirmation of temperature sensitivity in all ΔC-group mutants, BC202, BAL310, BAL320, and BAL410 were streaked across kanamycin plates with both 10 mM and
Table IV-1: Strain descriptions.

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<th>Strain</th>
<th>Genotype</th>
<th>Source</th>
</tr>
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<td>Wild type; F-,  ( \lambda ), ( \Delta \text{rrnD-rrnE}1 ), rph-1</td>
<td>E. coli genetic stock center, Yale University</td>
</tr>
<tr>
<td>W3110A</td>
<td>( aroA::\text{Tn10} ) (Tet' P1\text{vir} transductant of W3110; LCB273 donor)</td>
<td>(Doerrler, WT, Gibbons, HS et al., 2004)</td>
</tr>
<tr>
<td>BC202</td>
<td>W3110, ( \Delta \text{yqjA::tet}^R ), ( \Delta \text{yghB::kan}^R )</td>
<td>(Thompkins, K, Chattopadhyay, B et al., 2008)</td>
</tr>
<tr>
<td>BAL202</td>
<td>W3110, ( \Delta \text{ydjXYZ::cam}^R )</td>
<td>(Boughner, LA &amp; Doerrler, WT, 2012)</td>
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<tr>
<td>BC203</td>
<td>W3110, ( \Delta \text{yqjA::tet}^R )</td>
<td>(Thompkins, K, Chattopadhyay, B et al., 2008)</td>
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<tr>
<td>JW5005</td>
<td>F-, ( \Delta \text{(araD-araB)}567 ), ( \Delta \text{yabI772::kan} ), ( \Delta \text{lacZ4787::rrnB-3} ), ( \lambda ), rph-1, ( \Delta \text{(rhaD-rhaB)}568 ), ( \text{hsdR514} )</td>
<td>(Baba, T, Ara, T et al., 2006)</td>
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<td>JW2314</td>
<td>F-, ( \Delta \text{(araD-araB)}567 ), ( \Delta \text{lacZ4787::rrnB-3} ), ( \lambda ), ( \Delta \text{Ec}d\text{edA726::kan, rph-1, } \Delta \text{(rhaD-rhaB)}568 ), ( \text{hsdR514} )</td>
<td>(Baba, T, Ara, T et al., 2006)</td>
</tr>
<tr>
<td>JW2124</td>
<td>F-, ( \Delta \text{(araD-araB)}567 ), ( \Delta \text{lacZ4787::rrnB-3} ), ( \lambda ), ( \Delta \text{yohD762::kan, rph-1, } \Delta \text{(rhaD-rhaB)}568 ), ( \text{hsdR514} )</td>
<td>(Baba, T, Ara, T et al., 2006)</td>
</tr>
<tr>
<td>JW2976</td>
<td>F-, ( \Delta \text{(araD-araB)}567 ), ( \Delta \text{lacZ4787::rrnB-3} ), ( \lambda ), ( \Delta \text{yghB781::kan, rph-1, } \Delta \text{(rhaD-rhaB)}568 ), ( \text{hsdR514} )</td>
<td>(Baba, T, Ara, T et al., 2006)</td>
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<tr>
<td>JW3066</td>
<td>F-, ( \Delta \text{(araD-araB)}567 ), ( \Delta \text{lacZ4787::rrnB-3} ), ( \lambda ), ( \Delta \text{yqjA785::kan, rph-1, } \Delta \text{(rhaD-rhaB)}568 ), ( \text{hsdR514} )</td>
<td>(Baba, T, Ara, T et al., 2006)</td>
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<tr>
<td>JW2664</td>
<td>F-, ( \Delta \text{(araD-araB)}567 ), ( \Delta \text{lacZ4787::rrnB-3} ), ( \lambda ), ( \Delta \text{yqjA785::kan, rph-1, } \Delta \text{(rhaD-rhaB)}568 ), ( \text{hsdR514} )</td>
<td>(Baba, T, Ara, T et al., 2006)</td>
</tr>
<tr>
<td>BAL310</td>
<td>W3110, ( \Delta \text{yqjA::tet}^R ), ( \Delta \text{yabI772, yghB781::kan} )</td>
<td>This work</td>
</tr>
<tr>
<td>BAL320</td>
<td>W3110, ( \Delta \text{yqjA::tet}^R ), ( \Delta \text{yghD762, yghB781::kan} )</td>
<td>This work</td>
</tr>
<tr>
<td>BAL410</td>
<td>W3110, ( \Delta \text{ydjXYZ::cam}^R ), ( \Delta \text{Ec}d\text{edA726, yqaA770::kan}^R )</td>
<td>This work</td>
</tr>
<tr>
<td>BAL420</td>
<td>W3110, ( \Delta \text{yqjA::tet}^R ), ( \Delta \text{yabI772, yohD762, yghB781::kan} )</td>
<td>(Boughner, LA &amp; Doerrler, WT, 2012)</td>
</tr>
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60 mM MgCl$_2$, and grown at 42 °C and 30 °C. BAL410 was included during magnesium complementation analysis to determine if successful growth at elevated temperature was affected by magnesium. Due to the observed discrepancies between BAL310 and BAL320, the complementation of BAL420 at 42 °C with magnesium was investigated in liquid culture. Overnight cultures of BAL420 and BC202 were diluted 1:10 and 1:100 respectively (dilution difference between strains...
due to BAL420’s reduced growth rate) in fresh LB/kanamycin, grown at 30 °C to an OD$_{600}$ ~0.5 and shifted to pre-warmed media with 20 mM MgCl$_2$ at 42 °C.

**Generation of phylogenetic trees:** DedA homologs were identified within differing organisms through a BLAST (Sayers, EW, Barrett, T et al., 2012) analysis of all *E. coli* DedA protein sequences against organisms of interest. Proteins with a BLAST E-value ≤ 10^{-4} were considered to be likely members of the DedA protein family and used in comparative phylogenetic tree analysis. Tree generation was completed using the alignment program MEGA (Tamura, K, Peterson, D et al., 2011), with amino acid sequences of proteins of interest and rooted with an *E. coli* 16S rRNA gene.

**IV.3. Results**

**Phenotypes of *Escherichia coli* ΔC-group and ΔNC-group mutants:** As expected, any strain in which both *yqjA* and *yghB* were deleted resulted in temperature sensitivity (Figure IV-2), inefficient cell division (Figure IV-3) and a reduced growth rate (Figure IV-4, page 86). The deletion of *yqjA* and *yghB* in addition to only one other C-group protein (either *yabl* or *yohD*, BAL310 or BAL320 respectively) results in a strain with phenotypes similar to BC202. However, when *yohD* is the only remaining chromosomal C-group DedA gene (BAL310; Δ*yqjA*, Δ*yabl*, Δ*yghB*), the strain completely loses the ability to be complemented for growth at 42 °C in the presence of magnesium, even at a concentration of 60 mM MgCl$_2$ (Figure IV-5, page 87). Interestingly, when all C-group proteins (*yqjA*, *yghB*, *yohD* and *yabl*) are deleted (BAL420; Table IV-1), the resulting strain demonstrates increased growth sensitivity,
slower growth rate compared to BC202, and regains the ability to be complemented for growth at high temperatures in the presence of magnesium at 42 °C (Figure IV-6, page 88).

Figure IV-2: Temperature sensitivity of C- and NC-group DedA protein mutants. BC202, BAL310, BAL320, BAL420, and BAL410 were grown at 30 °C (left) and 42 °C (right) on kanamycin plates. Only BAL410 (ΔNC-group mutant) was able to grow at 42 °C. See Table IV-1 for strain descriptions.

Since the essential function of the *E. coli* DedA membrane family may be to play a role in the maintenance and organization of the bacterial nucleoid [Chapter III, pages 45-74; (Boughner, LA, Kumar, S et al.)], a logical question became whether complete, and independent, deletion of the two functional groups had an impact on the bacterial nucleoid. In all cases, whether the C-group or NC-group was deleted, the nucleoid segregates to each new daughter cell and the content within each cell appears as normal (Figure IV-3). Microscopic images of the *E. coli* DedA functional group deletion mutants, indicates that it is only in the complete absence of the entire DedA family that the nucleoid’s organization and maintenance is affected [repressed
Figure IV-3: Cell division of C- and NC- group mutants.
Microscopic images of wild type (W3110A; a/b), BC202 (ΔyqiA, ΔyghB; c/d), BAL310 (ΔyqiA, Δyabl, ΔyghB; e/f), BAL320 (ΔyqiA, ΔyohD, ΔyghB; g/h), BAL420 (ΔC-group: ΔyqiA, Δyabl, ΔyohD, ΔyghB; i/j), and BAL410 (ΔNC-group: ΔydiXYZ, ΔEceddA, ΔyqaA; k/l). Cell division inefficiency is observed in any strain deleted for both yqiA and yghB. DIC/DAPI overlay (left), DAPI alone (right). All images were captured using a Leica DM RXA2 deconvolution microscope.
(Figure V-3 continued)
(Figure V-3 continued)
As of yet, no phenotypes are apparent for a mutant deleted for all NC-group proteins (BAL410; Table IV-1, page 79) of the *E. coli* DedA membrane protein family.

**Comparative DedA Family Phylogenetic Trees for Different organisms:** The presence of DedA members within particular organisms can vary from a single homolog (*B. burgdorferi*), to as many as eight (*E. coli*), see Table 1 in reference (Liang, FT, Xu, QL *et al.*, 2010). In the interest of understanding the evolutionary development of the DedA family, phylogenetic trees were generated of the *E. coli* DedA family with the multiple DedA homologs identified within investigated organisms, to potentially identify C- and NC-groups of those organisms. The potential identification of DedA functional groups within other organisms, could possibly provide a basis for new mutant generation analysis using an organism other than *E. coli*, i.e. is there another “BC202” type strain possible among other organisms, due to the absence of particular DedA members? An organism quite similar to *E. coli*, *Salmonella enterica*, has six identified DedA homologs (NCBI BLAST E-value ≤ 10^-4); a phylogenetic tree comparing the DedA families for both organisms suggests that a *S. enterica* strain deleted for *stm3226* and *stm3162*, may result in a mutant demonstrating phenotypes similar to BC202 (Figure IV-7, page 89), i.e. temperature sensitivity, inefficient cell division, inability to maintain PMF, and/or increased activation of multiple extracytoplasmic stress response pathways (Sikdar, R & Doerrler, WT, 2010; Sikdar, R, Simmons, AR *et al.*, 2013; Thompkins, K, Chattopadhyay, B *et al.*, 2008). Whether this hypothetical *S. enterica* strain (Δ*stm3226*, Δ*stm3162*) would result in a mutant with phenotypes similar to BC202 is
unknown; however, investigations into the degree of similarity of the DedA families across multiple organisms could provide valuable information regarding the physiological functions of this highly conserved protein family.

Other similar trees were generated with organisms containing multiple identified DedA family members, however, none so promising as with *S. enterica*. For example *Pseudomonas aeruginosa* (five DedA homologs, NCBI BLAST E-value ≤ 10⁻⁴), pa4029 groups with *E. coli* C-group DedA family proteins *yqjA, yghB* as well as *Ec*dedA, suggesting deletion of this single gene from *P. aeruginosa*, may result in a strain with interesting phenotypes (Figure IV-8, page 90). Also, a tree of the *Bacillus anthracis* (eight DedA homologs, NCBI BLAST E-value ≤ 10⁻⁴) and *E. coli* DedA families, suggests that the phylogenetic relationship of *B. anthracis* DedA proteins may be very different than that of the *E. coli* DedA family (Figure IV-9, page 91).

![Figure IV-4: Growth of ΔC- and ΔNC-group DedA mutants in liquid media at 30 °C.](image)

BC202 (Δ*yqjA, ΔyghB*), BAL310 (Δ*yqjA, Δ*yabl, Δ*yghB*), BAL320 (Δ*yqjA, Δ*yohD, Δ*yghB*), BAL420 (ΔC-group: Δ*yqjA, Δ*yabl, Δ*yohD, Δ*yghB*), and BAL410 (ΔNC-group: Δ*ydjXYZ, Δ*Ec*dedA, Δ*yqaA).
Figure IV-5: Complementation of ΔC-group mutants in the presence of Magnesium. Mutant strains were streaked on magnesium plates (10 mM MgCl$_2$ a/b; 60 mM MgCl$_2$ c/d) and grown overnight at 30 °C (a/c) and 42 °C (b/d). Any strain in which both *yghB* and *yqjA* are deleted will not grow above 42 °C (Figure IV-2, page 81), but millimolar concentrations of magnesium will restore BC202’s (Δ*yqjA*, Δ*yghB*) ability to grow at elevated temperatures (Thompkins, K, Chattopadhyay, B et al., 2008). BAL320’s (Δ*yqjA*, Δ*yohD*, Δ*yghB*) temperature sensitivity is restored by growth in the presence of magnesium. Surprisingly, BAL310’s (Δ*yqjA*, Δ*yahl*, Δ*yghB*) temperature sensitivity is not complemented by growth in the presence of magnesium, at neither 10 mM nor 60 mM MgCl$_2$ (b/d). BAL410 (ΔNC-group) is unaffected by both high temperatures and growth in the presence of magnesium.
Figure IV-6: Complementation abilities of magnesium for growth of BC202 and BAL420 at 42 °C. BC202 ($\Delta$yqjA, $\Delta$yghB) and BAL420 ($\Delta$C-group: $\Delta$yqjA, $\Delta$yabI, $\Delta$yohD, $\Delta$yghB) were grown in liquid media at 30 °C and shifted to media pre-warmed to 42 °C in the presence of 20 mM MgCl$_2$. Growth of BAL420 at 42 °C is partially restored with magnesium, unlike BAL310 (Figure IV-5).
Figure IV-7: Escherichia coli vs. Salmonella enterica DedA families. Phylogenetic tree generated using the E. coli DedA membrane protein family, along with the six identified homologs present within S. enterica (STM2367, STM0105, STM2170, STM3162, STM3226, and STM2819). Depicted tree used as an aid to potentially identify the specific homologs of the E. coli DedA membrane proteins within S. enterica (see text for more description). Branch node values (%) represent the number of times that node was replicated out of the 500 trees generated in bootstrap test. Node values of 100 suggest that the grouped proteins are very closely related, and as such, some nodes with <50% bootstrapping were not collapsed to better demonstrate the potential evolutionary relationships across the two DedA families. Tree was constructed using the Maximum Parsimony method with MEGA5 (Tamura, K, Peterson, D et al., 2011) and rooted with a 16S rRNA sequence from E. coli (Ec_rrsH).
**Figure IV-8: Escherichia coli vs. Pseudomonas aeruginosa DedA families.**
Phylogenetic tree generated using the *E. coli* DedA membrane protein family, along with the five identified homologs present within *P. aeruginosa* (PA4029, PA2752, PA4011, PA5244, and PA1209). Depicted tree used as an aid to potentially identify the specific homologs of the *E. coli* DedA membrane proteins within *P. aeruginosa* (see text for more description). Branch node values (%) represent the number of times that node was replicated out of the 500 trees generated in bootstrap test. Node values of >90% suggest that the grouped proteins are very closely related, and as such, some nodes with <50% bootstrapping were not collapsed to better demonstrate the potential evolutionary relationships across the two DedA families. Tree was constructed using the Maximum Parsimony method with MEGA5 (Tamura, K, Peterson, D *et al.*, 2011) and rooted with a 16S rRNA sequence from *E. coli* (Ec_rrsH).
Figure IV-9: *Escherichia coli* vs. *Bacillus anthracis* DedA families
Phylogenetic tree generated using the *E. coli* DedA membrane protein family, along with the eight identified homologs present within *B. anthracis* (BA_5082, BA_1139, BA_0600, BA_0810, BA_2842, BA_2048, BA_5272, and BA_0415). Depicted tree used as an aid to potentially identify the specific homologs of the *E. coli* DedA membrane proteins within *B. anthracis* (see text for more description). Branch node values (%) represent the number of times that node was replicated out of the 500 trees generated in bootstrap test. Node values of >90% suggest that the grouped proteins are very closely related, and as such, some nodes with <50% bootstrapping were not collapsed to better demonstrate potential evolutionary relationships across, and within, the two DedA families. Tree was constructed using the Maximum Parsimony method with MEGA5 (Tamura, K, Peterson, D *et al.*, 2011) and rooted with a 16S rRNA sequence from *E. coli* (Ec_rrsH).
IV.4. Discussion

Homologs of the DedA membrane protein family have been identified throughout all domains of life. Investigations into the *E. coli* DedA protein family led to the identification of functional groups (C- and NC-groups), and possibly even evolutionary groups [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012)]. Mutant analysis of the DedA family in *E. coli* further implicates the suggested functional and evolutionary relationship of the *E. coli* DedA membrane family, as a mutant deleted for the entire C-group (BAL420) is viable but demonstrates increased growth sensitivities. But interestingly, BAL420 demonstrates reduced abilities for complementation (as compared to BC202) in the presence of magnesium (Figure IV-6, page 88) and over expression of the twin arginine transport (TAT) pathway [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012)]. As opposed to BAL410 (∆NC-group) which as of yet, demonstrates no apparent phenotypes.

Astonishingly, a mutant in which YohD is the only remaining DedA family C-group protein (BAL310; ∆yqjA, ∆yabl, and ∆yghB; Table IV-1, page 79) cannot be complemented for growth at 42 °C in the presence of magnesium, even at the drastic concentration of 60 mM MgCl₂ (Figure IV-5, page 87). In this background (BAL310), the presence of YohD is not sufficient to confer growth at high temperatures in the presence of magnesium. This discrepancy between BAL310 and BAL320 is most likely due to both the chromosomal presence of yohD and absence of yabl. Expression analysis of the DedA proteins mRNA, particularly yohD and yabl in the C-group mutant series, would address whether there is an expression difference of these two proteins under particular growth conditions.
The accumulated analyses into the functional and evolutionary groups of the DedA family are highly indicative of functional C- and NC-groups, and likely evolutionary groups of this family. There remains much work to be done in characterizing each member of the *E. coli* DedA family, and the mutants reported herein will likely provide the background in which the function of each DedA member can be determined. Particularly for the C-group proteins, as discrepancies have already been observed between the two generated C-group triple mutants (BAL310 and BAL320) (Figure IV-5, page 87), as well as between BC202 and BAL420 [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012; Sikdar, R & Doerrler, WT, 2010)]. Evolutionary analyses between the DedA families of *E. coli* and other organisms suggest many divergent pathways along which this protein family has evolved. Furthermore, mutant analysis of the DedA family within other organisms, particularly *S. enterica* (*Δ*stm3226 and *Δ*stm3162; Figure IV-7, page 89) would greatly aid in the understanding of this most physiologically significant protein family.
V. Conclusion

The DedA membrane protein family is a widely distributed group of proteins, with homologs present across all domains of life (Archaea, Bacteria, and Eukarya). In fact, we estimate that greater than 90% of the sequenced bacterial genomes contain a significant (BLAST E-value ≤10^{-4}) DedA homolog [Chapter I, pages 1-15; (Doerrler, WT, Sikdar, R et al., 2013)]. Thus far, the DedA family has been identified as collectively essential in two organisms, Borrelia burgdorferi (Liang, FT, Xu, QL et al., 2010) and Escherichia coli [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012)], indicating that the DedA membrane protein family plays a significant and important physiological role. E. coli DedA members (YqjA and YghB) play a role in maintaining membrane phospholipid composition, growth at high temperatures (Thompkins, K, Chattopadhyay, B et al., 2008); cell division (as division defect is due to an inefficient export of amidase proteins through the TAT pathway) (Sikdar, R & Doerrler, WT, 2010). YqjA/YghB also play a role in maintaining the proton motive force and normal physiological levels of extracytoplasmic stress response pathways, under permissive growth conditions (Sikdar, R, Simmons, AR et al., 2013). Additionally, other organismal DedA homologs (Mycobacterium bovis, Bcg2664) have been indicated to play a possible role in drug targeting (Arai, M, Liu, L et al., 2011).

The E. coli DedA membrane protein family was found to contain two functional groups, containing four members in each group: the C-group – which are able to complement BC202 for growth at high temperatures and restore normal cell division (YqjA, YghB, YabI, and YohD); whereas the NC-group – are incapable of restoring both temperature sensitive growth and normal cell division to BC202 [Chapter II,
Interestingly, mutant analysis of the identified functional groups within *E. coli*, suggests that there is likely an inter-regulatory network at play involving the DedA membrane proteins; considering that mutants of the C-group have varying abilities to be complemented by physical/chemical components. For example, BC202’s temperature sensitivity and inefficient cell division can be restored by growth in the presence of magnesium (Thompkins, K, Chattopadhyay, B *et al.*, 2008) and overexpression of the TAT pathway proteins [TatABC; (Sikdar, R & Doerrler, WT, 2010)]; however, different ΔC-group mutants are viable, but unable to be similarly complemented. BAL420 will not grow at elevated temperatures in the presence of overexpressed TatABC [Chapter II, pages 16-44; Figure II-12, page 42; (Boughner, LA & Doerrler, WT, 2012)]. Nor will BAL310 grow at elevated temperatures in the presence of magnesium, even when the concentration is drastically increased to 60 mM MgCl₂ (Chapter IV, pages 75-93, Figure IV-5, page 87); however most strikingly, BAL420’s ability to grow at an elevated temperature in the presence of magnesium is restored, though at a reduced growth rate compared to BC202 at the same Mg²⁺ concentration (Chapter IV, pages 75-93, Figure IV-6, page 88). Expression analysis of the DedA proteins from various mutants under differing conditions, would address whether there is in fact an inter-regulatory network at play.

The DedA protein family being collectively essential in two separate organisms, is a most intriguing discovery; *Borrelia burgdorferi* (Liang, FT, Xu, QL *et al.*, 2010) and *E. coli* [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012)]. The essential function of the *E. coli* DedA membrane protein family is hypothesized to play an
integral role in the maintenance and segregation of the bacterial nucleoid [Chapter III, pages 45-74; (Boughner, LA, Kumar, S et al.)]. Strain BAL801, a mutant devoid of all chromosomal DedA proteins, is only viable because of an inducible plasmid encoding EcDedA. When expression of EcDedA is induced, BAL801 exhibits cell membrane deformities, but the strain is viable [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012)]. However, when EcDedA is repressed, the cell membrane deformities increase, as well as demonstrating a significant increase in DNA concentration, followed by cellular anucleation, all preceding cell death [Chapter III, pages 45-74; (Boughner, LA, Kumar, S et al.)]. The involvement of membrane proteins in maintenance of the bacterial nucleoid is a most unexpected discovery but not unheard of, as nuclear membrane proteins do play a role in the maintenance of eukaryotic DNA (Ulbert, S, Antonin, W et al., 2006; Ulbert, S, Platani, M et al., 2006). The proposed essential function for the *E. coli* DedA membrane protein family (to play a vital role in maintaining the bacterial nucleoid) likely occurs in collaboration with other models of bacterial nucleoid organization and segregation [entropic forces, (Jun, S & Mulder, B, 2006; Jun, S & Wright, A, 2010); co-transcriptional transertion, (Woldringh, CL, 2002)]. Future work will continue to pursue the potential involvement of the DedA membrane protein family in maintaining the nucleoid, particularly in regulating chromosomal replication, as it is possible that in the absence of DedA proteins, the bacterial cell dies due to double strand breaks that can result from overinitiation of replication.

Understanding the DedA protein family and its members is just beginning, but the physiological importance of this mostly uncharacterized family is becoming quite
clear. With members playing significant roles in maintaining the integrity of the cell membrane (Sikdar, R & Doerrler, WT, 2010; Sikdar, R, Simmons, AR et al., 2013; Thompkins, K, Chattopadhyay, B et al., 2008), as well as the essentiality of the DedA family being identified in two separate organisms [Chapter II, pages 16-44; (Boughner, LA & Doerrler, WT, 2012; Liang, FT, Xu, QL et al., 2010)], and most intriguingly, the hypothesized essential function for the E. coli DedA membrane protein family to be an integral player in the maintenance of the bacterial nucleoid [Chapter III, pages 45-74; (Boughner, LA, Kumar, S et al.)].

Investigations into the DedA membrane protein family has proven to be a most intriguing journey. Future investigations of the DedA membrane protein family will consist of determining the specific role DedA members play in organizing, maintaining and segregating the bacterial nucleoid. Do DedA members play a collaborative effort with other proteins, if so, which proteins? Is replication of the genome in repressed BAL801 being overinitiated? If so, is this overinitiation (likely resulting in DNA double-strand breaks) what ultimately leads to death of repressed BAL801? Do DedA proteins bind DNA, at specific or non-specific binding sites? If so, what role does the binding of DedA proteins to DNA play in a genotypically normal cell? Are DedA proteins part of an inter-regulatory network, playing a role in gene expression, and/or does expression of particular DedA proteins vary in different mutant backgrounds under differing conditions? Do DedA proteins within all organisms, single and multicellular alike, share a common physiological role? If so, is the shared role related to maintaining the nucleoid or as membrane transporters?
These are just a few of the directions in which a continued pursuit of the DedA family will proceed.
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Lisa A. Boughner graduated from the University of Windsor (Winsor Ontario, Canada) with a Bachelor of Science, Honours Biological Sciences with thesis, in the year 2006. Afterwards, she worked for a couple of years at the Greenhouse and processing crops research center in Harrow Ontario, Canada. Subsequently, she joined the graduate students at Louisiana State University, to continue her pursuit of a higher education. During her time at Louisiana State University, she has had many diverse experiences, all of which have led her to the conclusion that academia is not a job, it’s a passion. Upon obtaining a Doctorate in Biology, she plans to continue in academia with a postdoctoral position at Michigan State University with Dr. Jon M. Kaguni; aspiring to obtain a professorial position at a university that values both teaching and research equally.