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Multiple deletions reveal the essentiality of the DedA membrane protein family in *Escherichia coli*

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The DedA family is a highly conserved, ancient family of membrane proteins with representatives in most sequenced genomes. A characteristic of prokaryotic DedA family genes is extensive gene duplication, with most bacterial genomes carrying two or more homologues. The *Escherichia coli* genome carries eight DedA genes, each individually nonessential. We previously described an *E. coli* mutant (BC202; Δ*yghB*::*kan*^{R}, Δ*yqjA*::*tet*^{R}) with in-frame deletions of two DedA genes encoding proteins with 61 % amino acid identity. BC202 fails to complete cell division or grow at elevated temperatures. Here, we report that restoration of normal growth and cell division of BC202 is possible by overexpression of a subset of the eight *E. coli* DedA genes (*yabI*, *yohD*, *yqjA and yghB*) but not others (*dedA*, *ydjX*, *ydjZ* and *yqaA*), suggesting the existence of two functional groups within the family. We have constructed individual *E. coli* strains in which all eight DedA genes are deleted in a nonpolar manner, and growth is supported by a single DedA family gene under control of an inducible promoter. Strain BAL801 (with growth supported by cloned *dedA*) and BAL802 (with growth supported by cloned *yqjA*) exhibit slow growth that is absolutely dependent upon the presence of the arabinose inducer. Growth in the presence of glucose results in cell death. These results indicate that while not individually essential, the *E. coli* DedA family proteins are collectively essential. These observations suggest important functions for the *E. coli* DedA protein family.

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 in the NCBI genome database (Khafizov *et al.*, 2010). The number of DedA homologues present within an individual bacterial genome can vary from one, as in *Borrelia burgdorferi* (Fraser *et al.*, 1997), to as many as eight, as in *Escherichia coli* (Blattner *et al.*, 1997). These eight *E. coli* DedA family genes are annotated *yqjA*, *yghB*, *yabI*, *yohD*, *yqaA*, *ydjX*, *ydjZ* and *dedA* (the *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 *et al.*, 2006). Our previous work on the DedA family in *E. coli* suggests a role in maintaining envelope integrity (Sikdar & Doerrler, 2010; Thompkins *et al.*, 2008). BC202 (W3110; Δ*yqjA*::*tet*^{R}, Δ*yghB*::*kan*^{R}) 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 *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) pathway, leading to an accumulation of these proteins in the cytoplasm (Sikdar & Doerrler, 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 Δ*tat* and Δ*ami* mutants in several respects. Δ*tat* (Ize *et al.*, 2003; Stanley *et al.*, 2001) and Δ*ami* (Heidrich *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, 2006; Thompkins *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, as-yet-unknown roles of DedA family proteins in cellular physiology.

Insight into the DedA family came from studies involving the Lyme disease pathogen *B. burgdorferi* (Liang *et al.*, 2010). Unlike many bacteria, the borrelia genome harbours only a single *dedA* family gene, annotated *bb0250* (Fraser *et al.*, 1997). *bb0250* encodes a membrane protein that

Abbreviation: DIC, differential interference contrast.

Five supplementary figures and two supplementary tables are available with the online version of this paper.

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 Δ*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 *et al.*, 2010). Additionally, the phenotypes seen in DXL-01 are independent of any role that the DedA proteins may play in the Tat pathway, since the *B. burgdorferi* genome does not encode homologues of TatABC or any proteins with a predicted Tat-dependent signal peptide (Dilks *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 homologue (BCG2664) that confers resistance to the antibiotic halicyclamine A, when expressed in *Mycobacterium smegmatis* (Arai *et al.*, 2011). BCG2664 shares significant sequence identity with five of the eight *E. coli* DedA proteins (EcDedA, YohD, YqjA, YabI and YghB), all with BLAST E-values $<10^{-8}$ (Sayers *et al.*, 2012). Halicyclamine A is an alkaloid isolated from an Indonesian marine sponge as an anti-dormant mycobacterial substance (Arai *et al.*, 2008; Jaspars *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 *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 that 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, harbouring pBAD_EcdedA) and BAL802 (harbouring 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 have also allowed us to begin to functionally dissect the roles of the *E. coli* DedA protein family in regard to cell division, physiology and cell viability.

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; 5 g yeast extract l^{-1}, 10 g tryptone l^{-1}, 10 g NaCl l^{-1}, pH 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 S1. PCR products and pBADHisA vector (Invitrogen) were purified with a QIAquick PCR purification kit (Qiagen) and digested with appropriate restriction enzymes (Table S1). Digested pBADHisA was subsequently treated with Antarctic phosphatase (NEB). PCR product and vector were ligated using T4 DNA ligase. Ligation reactions were transformed into chemically competent XL1-Blue cells, ampicillin-resistant colonies were selected and plasmid DNA was isolated using a QIAquick Spin Miniprep kit (Qiagen). DNA sequencing (primers listed in Table S2) was conducted at the LSU College of Basic Science Genomics Facility and confirmed the sequences of all cloned PCR products.

***E. coli* chromosome engineering.** λ Red recombination was used for gene replacement during strain generation (Yu *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. cam^R cells were isolated and the gene deletion was confirmed by PCR using flanking primers. A P_1 phage lysate prepared from the cam^R strain was subsequently used to transduce W3110, resulting in the generation of BAL202 (Table 1). PCR and DNA sequencing, using the primers listed in Table S2, verified gene deletions in all mutant strains. For each gene in *E. coli*, there is a single-deletion, kanamycin-resistant (kan^R) strain available from the Keio collection (Baba *et al.*, 2006) (Table 1). 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 shown in Fig. 4. 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 2), 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 & Wackernagel, 1995; Datsenko & Wanner, 2000). In place of the kan^R cassette, an 81 bp scar sequence remained encoding a 27 residue internal peptide (referred to herein as a scar peptide). Deletion of *yghB* was carried out 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 *et al.* (1990). Certain mutants, especially those of the BAL700 series and BAL420,

Table 1. Genotypic descriptions of strains and mutants used in this study

Strain	Description	Source or reference
W3110	Wild-type; F^-, λ^-, *IN*(*rrnD-rrnE*)*1*, *rph-1*	*E. coli* Genetic Stock Center, Yale University
W3110A	*aroA*::Tn*10* (Tet^r $P1_{vir}$ transductant of W3110; LCB273 donor)	Doerrler *et al.* (2004)
BC202	W3110, Δ*yqjA*::*tet*^{R}, Δ*yghB*::*kan*^{R}	Thompkins *et al.* (2008)
JW5005	F^-, Δ(*araD-araB*)*567*, Δ*yabI772*::*kan*, Δ*lacZ4787*(::rrnB-3), λ^-, *rph-1*, Δ(*rhaD-rhaB*)*568*, *hsdR514*	Baba *et al.* (2006)
JW2314	F^-, Δ(*araD-araB*)*567*, Δ*lacZ4787*(::rrnB-3), λ^-, Δ*EcdedA726*::*kan*, *rph-1*, Δ(*rhaD-rhaB*)*568*, *hsdR514*	Baba *et al.* (2006)
JW2124	F^-, Δ(*araD-araB*)*567*, Δ*lacZ4787*(::rrnB-3), λ^-, Δ*yohD762*::*kan*, *rph-1*, Δ(*rhaD-rhaB*)*568*, *hsdR514*	Baba *et al.* (2006)
JW2976	F^-, Δ(*araD-araB*)*567*, Δ*lacZ4787*(::rrnB-3), λ^-, Δ*yghB781*::*kan*, *rph-1*, Δ(*rhaD-rhaB*)*568*, *hsdR514*	Baba *et al.* (2006)
JW3066	F^-, Δ(*araD-araB*)*567*, Δ*lacZ4787*(::rrnB-3), λ^-, Δ*yqjA785*::*kan*, *rph-1*, Δ(*rhaD-rhaB*)*568*, *hsdR514*	Baba *et al.* (2006)
JW2664	F^-, Δ(*araD-araB*)*567*, Δ*lacZ4787*(::rrnB-3), λ^-, Δ*yqaA770*::*kan*, *rph-1*, Δ(*rhaD-rhaB*)*568*, *hsdR514*	Baba *et al.* (2006)
DY330	W3110, Δ*lacU169*, *gal490*, *λcI857* Δ(*cro-bioA*)	Yu *et al.* (2000)
BAL202	W3110, Δ*ydjXYZ*::*cam*	This work
BAL300	W3110, Δ*ydjXYZ*::*cam*, Δ*yabI772*::*kan*	This work
BAL301	W3110, Δ*ydjXYZ*::*cam*, Δ*yabI772*	This work
BAL400	W3110, Δ*ydjXYZ*::*cam*, Δ*yabI772*, Δ*EcdedA726*::*kan*	This work
BAL401	W3110, Δ*ydjXYZ*::*cam*, Δ*yabI772*, Δ*EcdedA726*	This work
BAL500	W3110, Δ*ydjXYZ*::*cam*, Δ*yabI772*, Δ*EcdedA726*, Δ*yohD762*::*kan*	This work
BAL501	W3110, Δ*ydjXYZ*::*cam*, Δ*yabI772*, Δ*EcdedA726*, Δ*yohD762*	This work
BAL60A	W3110, Δ*ydjXYZ*::*cam*, Δ*yabI772*, Δ*EcdedA726*, Δ*yohD762*, Δ*yqjA785*::*kan*	This work
BAL601	W3110, Δ*ydjXYZ*::*cam*, Δ*yabI772*, Δ*EcdedA726*, Δ*yohD762*, Δ*yqjA785*	This work
BAL60B	W3110, Δ*ydjXYZ*::*cam*, Δ*yabI772*, Δ*EcdedA726*, Δ*yohD762*, Δ*yghB781*::*kan*	This work
BAL602	W3110, Δ*ydjXYZ*::*cam*, Δ*yabI772*, Δ*EcdedA726*, Δ*yohD762*, Δ*yghB781*	This work
BAL700	W3110, Δ*ydjXYZ*::*cam*, Δ*yabI772*, Δ*EcdedA726*, Δ*yohD762*, Δ*yqjA785*, Δ*yqaA770*::*kan*	This work
BAL701	W3110, Δ*ydjXYZ*::*cam*, Δ*yabI772*, Δ*EcdedA726*, Δ*yohD762*, Δ*yqjA785*, Δ*yqaA770*	This work
BAL702	W3110, Δ*ydjXYZ*::*cam*, Δ*yabI772*, Δ*EcdedA726*, Δ*yohD762*, Δ*yghB781*, Δ*yqaA770*::*kan*	This work
BAL801	W3110, Δ*ydjXYZ*::*cam*, Δ*yabI772*, Δ*EcdedA726*, Δ*yohD762*, Δ*yqjA785*, Δ*yqaA770*, Δ*yghB781*::*kan*, pBAD_EcdedA	This work
BAL802	W3110, Δ*ydjXYZ*::*cam*, Δ*yabI772*, Δ*EcdedA726*, Δ*yohD762*, Δ*yqjA785*, Δ*yqaA770*, Δ*yghB781*::*kan*, pBAD_yqjA	This work
BAL803	W3110, Δ*ydjXYZ*::*cam*, Δ*yabI772*, Δ*EcdedA726*, Δ*yohD762*, Δ*yqjA785*, Δ*yqaA770*, Δ*yghB781*::*kan*, pBAD_yohD	This work
BAL804	W3110, Δ*ydjXYZ*::*cam*, Δ*yabI772*, Δ*EcdedA726*, Δ*yohD762*, Δ*yqjA785*, Δ*yqaA770*, Δ*yghB781*::*kan*, pBAD_yabI	This work
BAL420	W3110, Δ*yqjA*::*tet*^{R}, Δ*yabI772*, Δ*yohD762*, Δ*yghB781*::*kan*	This work

Table 2. Vectors used in study

Plasmid	Description	Source or reference
pBADHisA	Expression vector, Amp^r, N-terminal polyhistidine tag and Xpress epitope, with the *araBAD* promoter	Invitrogen
pBAD_ydjX	pBAD expressing N-terminal His-tagged *ydjX*; Amp^r	This study
pBAD_ydjZ	pBAD expressing N-terminal His-tagged *ydjZ*; Amp^r	This study
pBAD_yabI	pBAD expressing N-terminal His-tagged *yabI*; Amp^r	This study
pBAD_yqjA	pBAD expressing N-terminal His-tagged *yqjA*; Amp^r	This study
pBAD_yqaA	pBAD expressing N-terminal His-tagged *yqaA*; Amp^r	This study
pBAD_yghB	pBAD expressing N-terminal His-tagged *yghB*; Amp^r	This study
pBAD_yohD	pBAD expressing *yohD*; Amp^r	This study
pBAD_EcdedA	pBAD expressing *EcdedA*; Amp^r	This study
pCP20	FLP^+, λcI857$^+$, λRep^{ts} promoter, Amp^r, Cam^R	*E. coli* Genetic Stock Center; Cherepanov & Wackernagel (1995)
pBAD-TatABC	pBADHisA expressing TatABC operon; Amp^r	Sikdar & Doerrler (2010)

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.

P_1 transduction. P_1 lysates were prepared and P_1 transductions were carried out as described by Silhavy *et al.* (1984). One millilitre volumes of overnight cultures were centrifuged at 10 000 r.p.m. for 30 s, and cells were resuspended in 0.5 ml of 0.1 M $MgSO_4$ and 0.01 M $CaCl_2$, and incubated with P_1 phage lysate prepared from a donor strain. Once mixed, reactions were incubated at 30 °C for 40 min, after which 1 ml LB containing 0.1 M sodium citrate was added and cells were shaken at 225 r.p.m. for 2 h at 30 °C. Reactions were centrifuged, and all cells were spread on LB plates containing appropriate antibiotics plus 4 mM sodium citrate and incubated overnight at 30 °C.

Measurement of growth and β-galactosidase assay. Overnight cultures were diluted to a starting OD_{600} of ~0.01 in fresh medium with no sugars, grown to OD_{600} ~0.3, and diluted 1 : 10 in pre-warmed medium 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 min intervals, and cell-free medium 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 (1972). Briefly, 0.1 ml medium was added to 0.9 ml pre-warmed Z buffer (60 mM $Na_2HPO_4.7H_2O$, 40 mM $NaH_2PO_4.H_2O$, 10 mM KCl, 1 mM $MgSO_4.7H_2O$, 50 mM β-mercaptoethanol), and incubated at 30 °C for 5 min. To start the assay, 0.1 ml of 8 mg ONPG ml^{-1} was added to the reaction, and allowed to sit in the dark at 30 °C until yellow colour was visible, after which 0.25 ml 2 M Na_2CO_3 was added to stop the reaction. Assayed reactions were centrifuged and A_{420} read. For each time point, activity was measured in triplicate.

Microscopy. Overnight cultures of strains were diluted 1 : 100 and grown to OD_{600} ~0.5. Cells were resuspended in fresh LB to OD_{600} 1.0, and 10 µl was then added to agar-coated slides. All differential interference contrast (DIC) images were captured using a 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 medium with respective antibiotics and glucose/arabinose, as indicated. Cultures were centrifuged and overnight medium removed to eliminate any traces of arabinose in glucose cultures. Absorbance was read every 30 min and cells were collected for lipid extraction (Bligh & Dyer, 1959) at 1 h intervals. $^{32}P_i$ was added to 10 µCi ml^{-1} (3.7×10^5 Bq ml^{-1}) and growth was continued for 10 min. A 0.2 ml volume of growing culture was used for lipid analysis. Chloroform and methanol were added to cells to a final chloroform/methanol/water ratio of 1 : 2 : 0.8. The extraction mixture was allowed to incubate for 1 h at room temperature with occasional mixing. Insoluble material was removed by centrifugation for 10 min at 13 000 r.p.m. (16 000 ***g***). 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 a Vacufuge (Eppendorf) for >30 min, and lipid species were resolved by TLC on Silica gel 60 plates (Merck) using the solvent chloroform/methanol/acetic acid (65 : 25 : 10) and analysed using a Phosphorimager equipped with IQMac software.

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 2). BC202 does not grow at the non-permissive temperature of 42 °C, and forms chains resembling those of amidase mutants (Heidrich *et al.*, 2002) because of an inefficiency in the Tat pathway (Sikdar & Doerrler, 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 (Fig. 1). Cloned *yqjA*, *yghB*, *yabI* and *yohD* each restored growth (Fig. 1) and normal cell division (Fig. 2) to BC202, while cloned *ydjX*, *ydjZ*, *yqaA* and *EcdedA* failed to restore growth and normal cell division to BC202 (Figs 1 and 2). Therefore, each *E. coli* gene able to complement BC202 for temperature sensitivity also complemented cell division and, conversely, each gene that did not complement the temperature sensitivity of BC202 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 henceforth refer to these groups as the C (complementing) and NC (non-complementing) groups, respectively.

Fig. 3 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 3). 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 *yqjA* (C group) and *EcdedA* (NC group) are capable of supporting the viability of a BAL800 series mutant, suggesting that shared among all DedA family proteins is an unknown essential function that is required for cell viability.

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 Fig. 4. 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 Δ*ydjY* mutant has been isolated (Baba *et al.*, 2006) and has no apparent

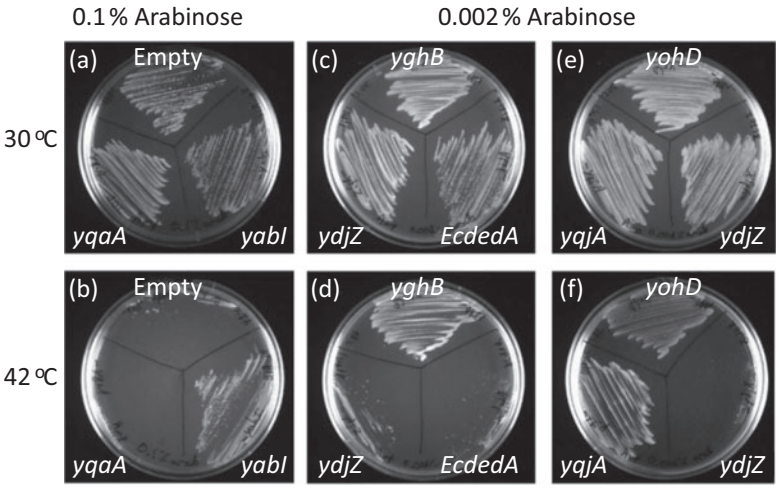

Fig. 1. Complementation of growth at 42 °C of BC202 with cloned DedA family genes. BC202 (W3110; Δ*yqjA*::*tet*^{R}, Δ*yghB*::*kan*^{R}) was transformed with (a, b) pBAD, pBAD_yqaA, pBAD_yabI, (c, d) pBAD_yghB, pBAD_ydjZ, pBAD_EcdedA, (e, f) pBAD_yohD, pBAD_yqjA, pBAD_ydjZ (Table 2), and grown on LB/ampicillin plates supplemented with 0.1 % arabinose (a, b) or 0.002 % arabinose (c–f) and 50 μg ampicillin ml^{-1} at both 30 and 42 °C.

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 Fig. 4). While we have not formally ruled out a role for loss of *ydjY* in our observable phenotypes, we felt that removal of *ydjY* was necessary to avoid unintended polar effects. This initial *ydjXYZ* deletion was followed by subsequent individual deletions of DedA family members (Fig. 4) using P_1 lysates prepared from the corresponding Keio mutants (Baba *et al.*, 2006). kan^R cassettes were removed using FLP recombinase and deletions confirmed by PCR (Fig. S1, Table S2) prior to proceeding to the next deletion. During mutant generation, no temperature sensitivity or cell division defects (Fig. 4) 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 Δ*yabI* scar peptide was observed. In the *E. coli* genome, *yabI* is flanked by *araC* as well as *thiQ*. *araC* of

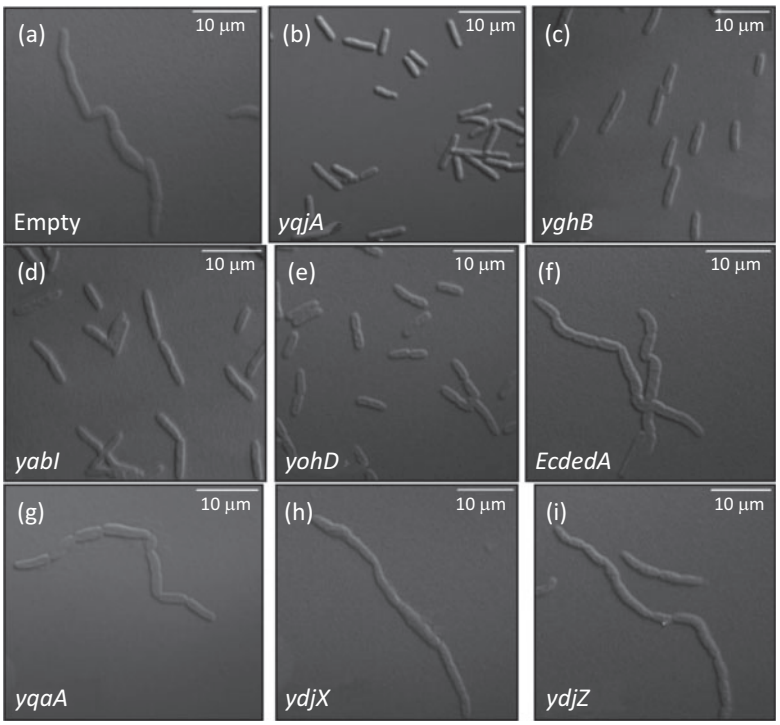

Fig. 2. Complementation of BC202 cell division defects with cloned DedA family genes. BC202 was transformed with (a) plasmid pBAD (Table 2) or pBAD with cloned (b) pBAD_yqjA, (c) pBAD_yghB, (d) pBAD_yabI, (e) pBAD_yohD, (f) pBAD_EcdedA, (g) pBAD_yqaA, (h) pBAD_ydjX or (i) pBAD_ydjZ, and grown in LB/ampicillin medium supplemented with 0.1 % arabinose at 30 °C. Cells were harvested at OD_{600} ~0.5 and visualized using a Leica DM RXA2 deconvolution microscope.

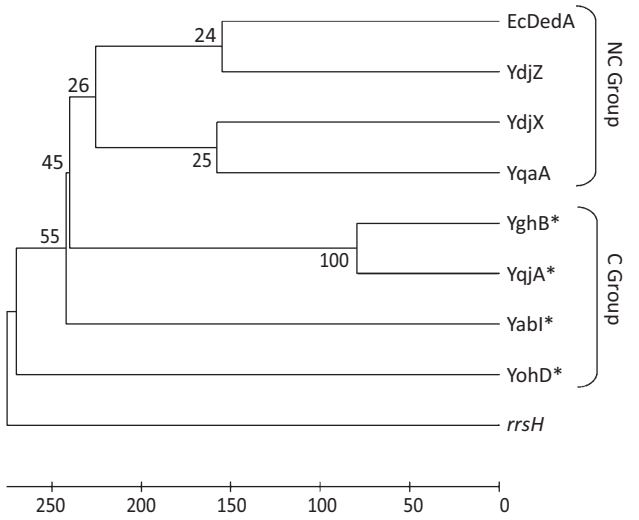

Fig. 3. Evolutionary relationship of *E. coli* DedA proteins. Alignments were generated using nucleotide sequences of *E. coli* DedA family proteins and analysed with MEGA, using the maximum-parsimony algorithm (Tamura *et al.*, 2011). The tree was rooted with the 16S RNA gene *rrsH*. Proteins with an asterisk restore both normal growth and cell division when expressed in BC202 (see Table 3, Figs 1 and 2). The branch lengths (vertical and horizontal) are drawn to scale with units signifying the number of changes over the whole sequence.

course has its own promoter sequence (Hirsh & Schleif, 1977), and according to RegulonDB, *yabI* is also known to have its own promoter (Gama-Castro *et al.*, 2011). As for *thiQ*, it is in the opposite orientation to *yabI* and is part of the *sgrR-sroA-tbpA-thiPQ* operon. Therefore, the observed single base-pair deletion of BAL801 within the Δ*yabI* 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 Δ*yabI* in BAL801. Other than this, DNA sequencing confirmed that all gene deletions of BAL801 were nonpolar.

The generation of BAL800 series mutants (Table 2) was successfully completed only when *EcdedA*, *yqjA*, *yohD* or *yabI* was expressed from an arabinose-inducible plasmid (BAL801, BAL802, BAL803 or BAL804, respectively; Table 1) 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 (Figs 1 and 2). BAL801 and BAL802 were subsequently characterized more fully.

Growth of mutants in liquid culture

Working with BAL800 series mutants requires great consideration. While these strains grow reproducibly on LB plates, we found that 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 min of growth. We observed some day-to-day variation with respect to the specific timing of growth arrest. The presence of β-galactosidase was not detectable in the medium until approximately 270 min after the shift of BAL801 to glucose medium, indicating that the mutant cells had lysed, releasing the cytoplasmic contents to the culture medium (Fig. 5). A similar growth dependency upon arabinose was observed when *yqjA* was expressed from the inducible plasmid (BAL802; Fig. S2), with the exception that cultures grew

Table 3. The *E. coli* DedA family

C, complementing; NC, non-complementing.

Group	Gene name	Position on chromosome (minutes)	Complements BC202*	BAL800 series mutant
C group	*yqjA*	69.96	Yes	Yes
	yghB	67.93	Yes	Non-culturable†
	yohD	47.93	Yes	Yes
	yabI	1.54	Yes	Yes
NC group	*yqaA*	60.66	No	Non-culturable†
	EcdedA	52.42	No	Yes
	ydjZ	39.50	No	Yes; unexpected genotype‡
	ydjX	39.47	No	Yes; unexpected genotype‡

*Complementation defined as ability to restore both growth at 42 °C and normal cell division at 30 °C to BC202 (Table 1).
†Small colonies were observed following P_1 transduction but could not be grown on plates or in liquid medium.
‡Denoting a duplication of the *yghB* gene regions during the generation of BAL800s.

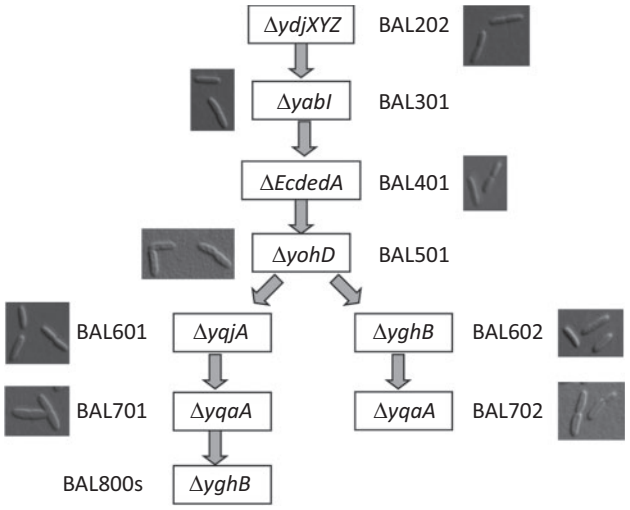

Fig. 4. Order of sequential deletion of all DedA family genes within *E. coli*. A DIC image is next to each generated strain (samples prepared as described in Methods and images captured with a Leica DM RXA2 deconvolution microscope); all images are of equal scale. No visible phenotypes were observed unless both *yghB* and *yqjA* were deleted (Fig. 6). See Table 1 for genotypic descriptions.

much more slowly when arabinose was present in the growth medium, possibly reflecting toxic effects of the over-expressed protein. These data strongly suggest that the DedA family is essential in *E. coli*.

Microscopic analysis of mutants

BAL801 grown in the presence of arabinose (Fig. 6a) displayed similar morphological deformities and cell division defects as BC202, as expected, due to the inability of *EcdedA* to complement BC202 (Fig. 2). BAL801 also exhibited increased cell clumping when grown in the presence of arabinose (Fig. S3). When expression of cloned *EcdedA* was repressed, the deformities of BAL801 increased until the cells lysed and died (Fig. 6b).

BAL802 did not share the same deformities and appeared almost as the wild-type when grown in the presence of arabinose (Fig. 6c), due to the ability of *yqjA* to restore normal morphology and cell division to BC202 (Fig. 2). However, in the absence of *yqjA* expression when cells were grown in the presence of glucose, the cell morphological deformities returned and the cells lysed and died (while at the same time failing to form chains, since cells were entering growth arrest) (Fig. 6d). All images are representative of cells or chaining cells. Therefore, either *EcdedA* or *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.

Membrane phospholipid composition

We previously reported that BC202 grows under permissive conditions with an altered membrane phospholipid composition (Thompkins *et al.*, 2008). We therefore measured phospholipid composition in BAL801 when grown under both inducing and repressing conditions. The phospholipid composition of W3110 consisted of 73.5 % phosphatidylethanolamine (PE), 24.6 % phosphatidylglycerol (PG) and 1.9 % cardiolipin (CL), and for BC202 membranes contained 58.4 % PE, 35.3 % PG and 6.3 % CL (Fig. 7). When BAL801 was grown with *EcdedA* expressed, the phospholipid composition was approximately the same as in BC202. Also, the phospholipid composition of BAL801 remained relatively constant whether the *EcdedA* gene was induced or not, even after the cells

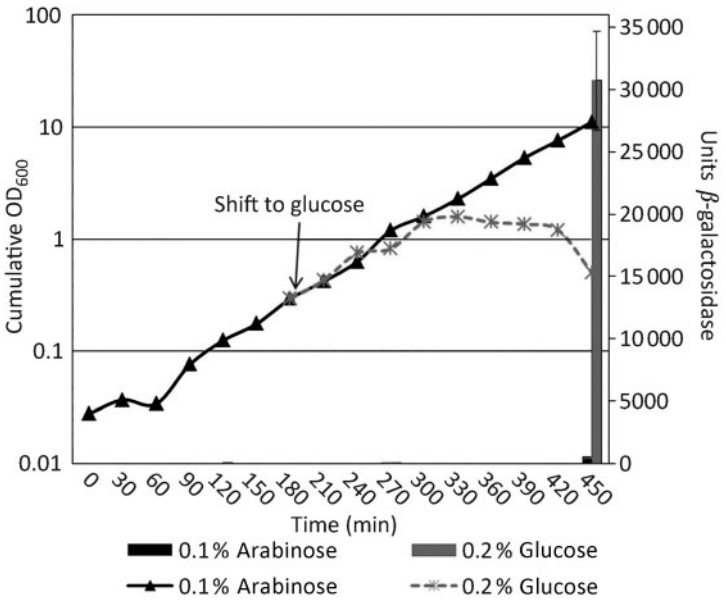

Fig. 5. Growth of BAL801 in liquid culture. Growth of *E. coli* with in-frame deletions of all eight DedA family genes (Δ*yqjA*, Δ*yghB*, Δ*yabI*, Δ*yohD*, Δ*EcdedA*, Δ*ydjXYZ*, Δ*yqaA*) with the plasmid pBAD_EcdedA (strain BAL801) is dependent upon induction with arabinose. Cultures were diluted at 180 min into LB supplemented with 0.1 % arabinose (triangles) or 0.2 % glucose (stars) and diluted 10-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 medium for β-galactosidase activity as a measure of cell lysis (at 120, 270 and 450 min). Cells were found to lyse at around minute 450 when grown in the presence of glucose (grey bar).

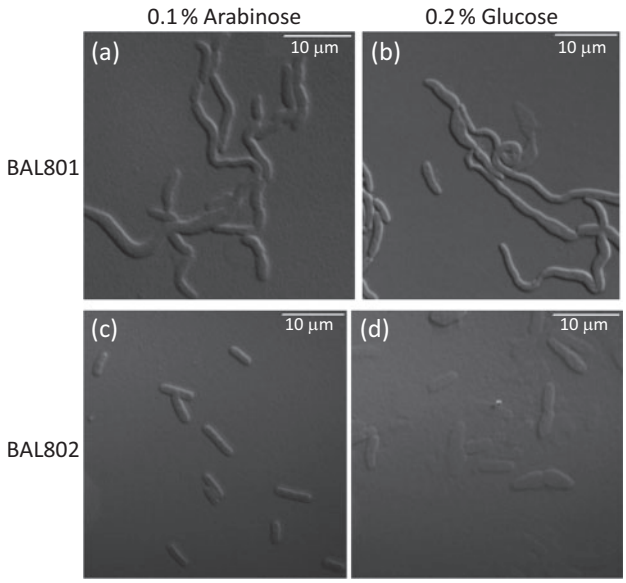

Fig. 6. Microscopic analysis of BAL801 and BAL802. Overnight cultures of BAL801 (a, b) and BAL802 (c, d) were diluted 1 : 100 into LB plus antibiotics and grown to OD_{600} ~0.5 in the presence of either 0.1 % arabinose (a, c) or 0.2 % glucose (b, d). Most BAL802 cells were in the process of undergoing lysis under these conditions during growth with glucose, and what are likely representative cell ghosts are shown (d). All cultures were grown at 30 °C. Cells were harvested, and visualized with a Leica DM RXA2 deconvolution microscope.

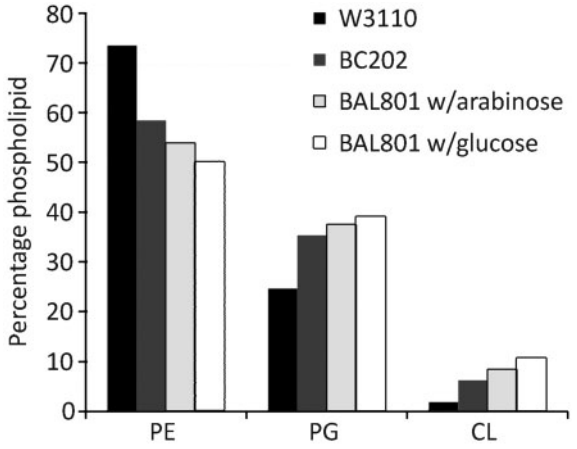

Fig. 7. Comparative phospholipid composition of wild-type W3110, BC202 and BAL801. Overnight cultures of W3110 and BC202 were diluted 1 : 100 in LB and grown to OD_{600} ~0.5 for lipid analysis. BAL801 overnight culture was diluted to a starting OD_{600} of ~0.1 in fresh LB/antibiotics supplemented with 0.1 % arabinose or 0.2 % glucose, as indicated. BAL801 grown in both arabinose and glucose was maintained in exponential growth and monitored every 30 min. After 360 min, growth of BAL801 in glucose halted (see Fig. S4); 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 as the percentage total phospholipid signal for each individual species. Shown is a representative experiment of two different determinations. Abbreviations: PE, phosphatidylethanolamine; PG, phosphatidylglycerol; CL, cardiolipin.

began to die (Fig. S4). These data indicate that along with the inability of *EcdedA* to rescue cell division and growth defects of BC202 (Figs 1 and 2), *EcdedA* is also unable to restore wild-type phospholipid composition (Fig. 7). This altered phospholipid composition is independent of BAL801 growth arrest.

Effect of TatABC overexpression on a DedA C-group deletion mutant

We previously reported that the cell division defect observed with strain BC202 ($\Delta yghB::kan^R$, $\Delta yqjA::tet^R$) was due to inefficient transport of periplasmic amidases AmiA and AmiC across the plasma membrane by the Tat pathway (Sikdar & Doerrler, 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 *yabI*; BAL420; Table 1) resulted in a strain with similar growth and cell division defects to those of BC202, although these could no longer be corrected by overexpression of TatABC (Fig. S5). Therefore, the presence of *yabI* and/or *yohD* is required for TatABC overexpression to correct the cell division defects 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.

DISCUSSION

In *E. coli*, as in most organisms, it is predicted that roughly 20–25 % of transcribed genes encode integral membrane proteins (Elofsson & von Heijne, 2007). One estimate states that more than half of all drugs currently produced are directed against membrane proteins, mostly G-protein receptors (Klabunde & Hessler, 2002). In contrast, fewer than 1 % of all known protein structures in the Protein Data Bank are membrane proteins (Rose *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 *et al.*, 1997; Liang *et al.*, 2010); and *E. coli*, possessing eight (Blattner *et al.*, 1997) collectively essential members.

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 *et al.*, 2010). Depletion of BB0250 protein in a Δ*bb0250 B. burgdorferi* background results in rapid cell death preceded by striking cell division defects (Liang *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 homologue (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 *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 *et al.*, 2008; Jaspars *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.

Complementation analysis of BC202 indicates that in *E. coli* there are at least two functional groups of DedA family genes (Table 3): we categorize those that can rescue BC202 as belonging to the C group (*yqjA*, *yghB*, *yabI*, *yohD*) and those that do not rescue BC202 belong to the NC group (*EcdedA*, *yqaA*, *ydjX*, *ydjZ*) (Figs 1 and 2). 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 that 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 1), 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 & Doerrler, 2010), overexpression of TatABC does not restore normal cell morphology and growth to BAL420 (Fig. S5), 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 due to the fact 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. *ydjX*, *ydjZ*, *yqaA* and *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 (L. A. Boughner & W. T. Doerrler, unpublished observations). It is conceivable that the DedA family may play an as-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 *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 that DedA proteins play in the viability of a cell and its genome, and possibly provide new targets for therapeutic interventions for bacterial diseases.

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