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Escherichia coli OmpC Mutants Are Sensitive to Ethylenediaminetetraacetic acid and Sodium Dodecyl Sulfate Treatment Whereas Double OmpC and OmpF Mutants Are Not

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The Mla pathway is hypothesized to be an ATP binding cassette transport system in *Escherichia coli* that maintains outer membrane asymmetry. Outer membrane proteins C (OmpC) and F (OmpF) have been shown to interact with MlaA, a key lipoprotein in the Mla pathway. *Escherichia coli ompC* mutants in strain MC4100 have previously been shown to be more sensitive to treatment with ethylenediaminetetraacetic acid (EDTA) and sodium dodecyl sulfate (SDS) which disrupt lipids stability and solubilizes the outer membrane. Sensitivity to these agents has been used as an indicator for maintenance of outer membrane asymmetry. In this study we sought to test additional strains of *E. coli* bearing *ompC* mutations to further characterize this phenotype. We acquired several strains of *E. coli* bearing mutations in either *ompC* or both *ompC* and *ompF*. We adapted a broth dilution minimal inhibitory concentration assay to compare resistance of *E. coli* as a proxy for membrane asymmetry. Our data are consistent with previous reports showing EDTA/SDS sensitivity in *ompC* mutants to treatment with EDTA / SDS. Surprisingly, *ompC* and *ompF* double mutants showed a similar level of sensitivity to EDTA/SDS as the isogenic wild type strain of *E. coli*. This observation may suggest a relationship between OmpC, OmpF, and MlaA in the Mla pathway.

An asymmetric distribution of lipids in the outer membrane (OM) of Gram-negative organisms is necessary for barrier function (1). The lipid distribution of the OM is described as asymmetric because lipopolysaccharide accumulates on the outer leaflet of the OM while phospholipids accumulate on the inner leaflet. This distribution can be disrupted by the addition of chelating agents which bind divalent cations in the outer membrane and induce repulsion between negatively charged acyl chains of lipopolysaccharide causing shearing of the outer leaflet (1). Lipid asymmetry is disrupted as phospholipids from the inner leaflet replace the regions of sheared lipopolysaccharide in the outer leaflet (1). First described by Maliverni and Silhavy (2009), the Mla pathway is an ATP binding cassette (ABC) transport system that is hypothesized to traffic phospholipids in retrograde: from the outer leaflet of the OM to the inner membrane (1). The Mla pathway is composed of several proteins including MlaA, MlaB, MlaC, MlaD, MlaE, and MlaF and is proposed to be involved in maintaining OM lipid asymmetry (1). *Escherichia coli* (*E. coli*) becomes more susceptible to OM destabilization by the divalent cation chelating agent ethylenediaminetetraacetic acid (EDTA) and membrane solubilizing agent, sodium dodecyl sulfate (SDS), when *mlaA* is knocked out. These data imply that MlaA is required for maintenance of membrane stability (1). Maliverni and Silhavy (2009) also linked this phenotype to increased abundance of phospholipids in the outer leaflet of the outer membrane (1). Studies performed by Chong *et al*. have shown that MlaA interacts with the osmoporins OmpC and OmpF (2). This study suggested that the OmpC functions in conjunction with MlaA to maintain OM asymmetry during stationary growth. Although similar in structure to OmpC, the OmpF porin was not found to be involved in maintaining membrane asymmetry. Both of these studies were conducted using *E. coli* strain MC4100 and used spot assays on solid media to assay growth in the presence of different concentration of EDTA and SDS.

In addition to functioning as porins, OmpC and OmpF play key structural roles in the outer membrane. As such, mutation of the genes encoding for OmpC or OmpF can

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perturb the structural integrity of the cell and result in phenotypic changes (e.g. genetic reversion, upregulation of other membrane proteins) to compensate. We therefore decided to examine the EDTA / SDS sensitive phenotype previously observed in strain MC4100 in other strains of *E. coli* lacking OmpC or OmpF. We have acquired five different mutants and tested them side by side in a broth dilution assay using SDS and EDTA as selective agents. Our results are consistent with previous studies which showed a more SDS / EDTA sensitive phenotype in *E. coli* strains lacking OmpC. However, we observed a similar level of sensitivity to SDS / EDTA in wild type *E. coli* strains and OmpC and OmpF double knock out strains.

MATERIALS AND METHODS

Bacterial strains. *E. coli* strains used are listed in Table 1. Strains were grown at 37°C in Lyogeny Broth (LB) in the presence of either 50 ug/mL kanamycin (strain JW2203, LMM-PDS1) or 100 ug/mL streptomycin (strain KJ740). Strains BW25113, MG1655, C157, and C161 were grown in the absence of antibiotic selection.

Colony PCR amplification of *ompC*. A small portion of one colony was suspended in 20 uL of sterile deionized water (distilled H_2O) in a PCR tube. The suspension was heated at 95°C for 5 minutes to lyse the cells. The lysate was centrifuged at high speed for 30 seconds to pellet debris. The supernatant was collected for use in a PCR reaction. The PCR reaction used primers specific for *omp*C (Forward: 5′-GCA TTT ACA TTT TGA AAC ATC TAT AGC G-3′) (Reverse: 5′-GGG TTG TGG TTT TTG ATC GC-3′) and *rpo*S primers as a positive control (Forward: 5′-TCG CTT GAG ACT GGC CTT TCT G 3′) (Reverse: 5′-CGG AAC CAG GCT TTT GCT TGA ATG-3′). Per 50 uL PCR reaction mix, 1X Buffer, 200 uM dNTP mixture, 200 uM primers (10uM), 1.5 mM $MgCl_2$, 2 units/reaction Taq polymerase, and 1 uL DNA-containing supernatant were used. Each reaction mix was filled to 50 uL with DIH_2O. PCR reaction was held at 94°C for 12 minutes, followed by 35 cycles of 94°C for 1 minute, 48°C for 0.5 minute, and 72°C for 0.5 minutes. 1% agarose gel was made using 0.5X TBE buffer containing SYBR Safe nucleic acid staining dye. 10 uL of each sample and 2 uL of 6X Loading Buffer (Thermo Fischer Scientific) were loaded in each lane and 10 uL of 1 kb plus DNA ladder (Thermo Fischer Scientific) was used for determining the size of gel products. The gel was run at 100 V for 45 minutes in 1X TBE buffer.

DNA sequencing. PCR products for JW2203, LMM-PDS1, and the wild type DH5α (WT DH5α) were purified using PureLink® PCR Purification Kit by Invitrogen™ (CAT# K3100-1). The purified products were sent for sequencing at the NAPS Unit located at the University of British Columbia. Forward and reverse sequencing reactions were performed on each sample, using the same *ompC* primers as in colony PCR.

Minimum inhibitory concentration assay. The concentrations of SDS and EDTA to be used in the MIC assay were first determined using a serial dilution assay of EDTA and SDS on JW2203 and MG1655 in a 96-well polystyrene plate. The first well contained 0.2% SDS and 0.1 mM EDTA, as this was the lowest concentration of EDTA used by Malinverni and Silhavy (1). Subsequent wells were diluted by one half and each well was topped up with LB such that the final volume of each well was 300 µl after seeding with 4 x 10^4 cells. The plates were incubated at 37°C for 24 hours. Following the incubation, growth was visually assessed to determine the minimum inhibitory concentrations of SDS and EDTA. MIC assays were conducted in 96 well polystyrene plates with 0.0125% SDS and varying concentrations of EDTA at 0.0 mM, 0.02 mM, 0.05 mM, 0.1 mM, 0.15 mM, 0.20 mM, 0.25 mM, 0.3 mM, 0.35 mM, 0.40 mM and 0.45 mM were performed on the 7 strains used in this study, C157, C161, KJ740, JW2203, LMM-PDS1, MG1655, and BW25113. Each well was filled with LB then seeded with 4 x 10^4 cells. The plates were incubated at 37°C for 24 hours. Growth was visually assessed to determine the minimum inhibitory concentrations of SDS and EDTA.

TABLE 1 *Escherichia coli* strains and plasmid used in the study.

Strain	Mutation	Reference / Source
BW25113	WT	(7)
MG1655	WT	(8)
JW2203	Δ*ompC*	(3)
KJ740	Δ*ompC*, Δ*ompF*	(5)
C157	Δ*ompC*	Gift from R.E.W. Hancock
C161	Δ*ompC*, Δ*ompF*	Gift from R.E.W. Hancock
LMM-PDS1	Δ*ompC*	(6)

RESULTS

Genetic characterization of *E. coli ompC* mutant strains. We sought to further characterize the EDTA / SDS sensitive phenotype linked to *ompC* (1). To do this we acquired three different *E. coli* strains bearing deletions of *omp*C and two *E. coli* strains bearing a deletion of *omp*C and *omp*F (Table 1). Strains JW2203 and LMM-PD21 bear kanamycin resistance cassette insertions within the *omp*C gene and were generated using parent strain BW25113 via lambda red recombination (3). Strain MG1655 is an isogenic parent to BW25113; these two strains were used as wild type controls (4). Strain KJ740 was generated by bacteriophage transduction to disrupt *ompC*/*ompF* (5). Based on the absence of PCR products, C157 and C161 were presumptively generated by bacteriophage as the region flanked by the PCR primers were too large to successfully amplify the region. Isogenic parent strains were not available for KJ740, C157, or C161.

To confirm that the *E. coli* strains were indeed *omp*C mutants we performed PCR followed by DNA sequencing of the amplicon using primers flanking the *ompC* coding region (Fig. 1A). Based on the lengths of the kanamycin resistance cassettes (795 bp) and the flanking sequences (6), a PCR product of 1.7 kb was expected from amplification in JW2203 and 1.9 kb product was expected for LMM-PDS1. Based on the length of *ompC*, the amplicon for the WT DH5α was expected to be 1.3 kb. *E. coli* strain DH5□ was used as a positive control since it is expected to contain a functional copy of the *omp*C gene sequence. As shown in Figure 1B, colony PCR of *E. coli* strain DH5□ using *omp*C

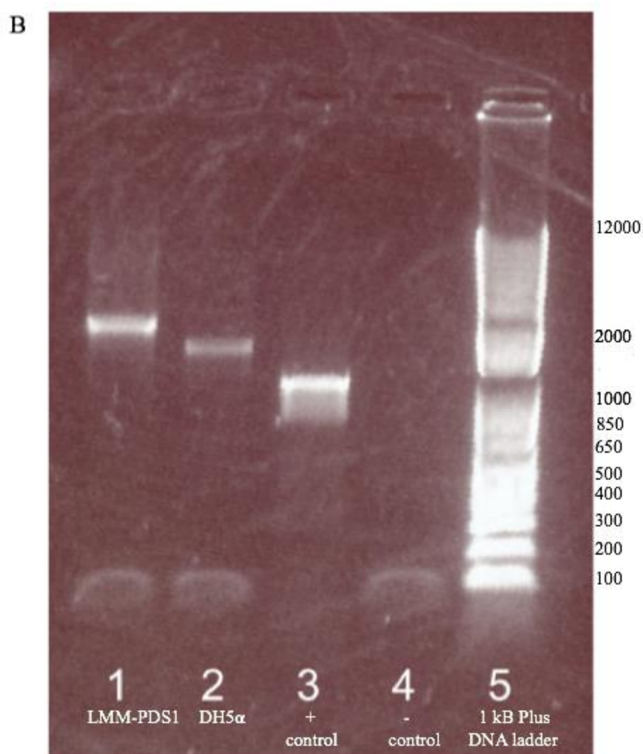

FIG. 1 Agarose gel electrophoresis of PCR products from *E. coli* strains. Lane labelled (+) control was a PCR using primers to rpoS. Lane labelled (–) control did not contain template DNA.

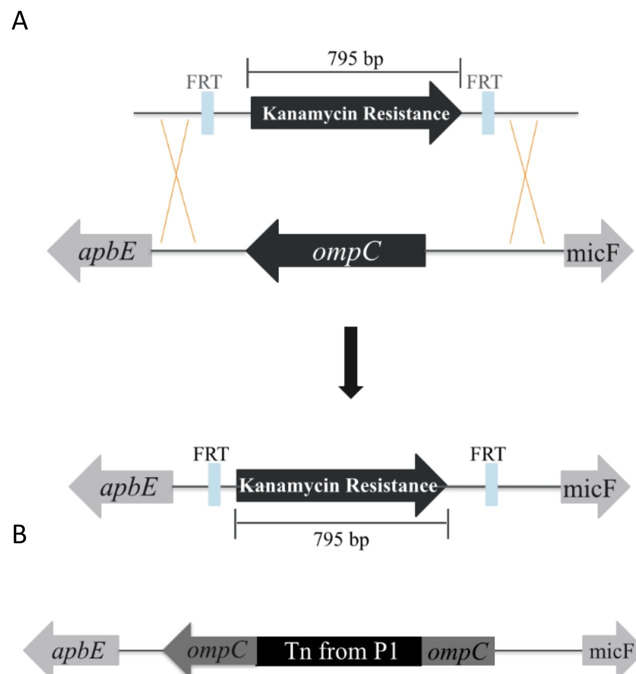

FIG. 2 Illustration of the *E. coli* Δ*ompC* constructs. **(A)** JW2203 and LMM-PDS1: PCR product containing a kanamycin resistance cassette flanked by FLP recognition target (FRT) and homologous sequences to the chromosome sequences adjacent to *ompC* (3, 6). **(B)** C157, C161, and KJ740: *ompC* deletion and nearby transposon (Tn) in bacteriophage P1 was inserted in the *E. coli* chromosome to delete *ompC* (5).

primers resulted in a 1.3 kBp amplicon. Sequencing of this 1.3 kBp PCR product confirmed its identity as *omp*C (Fig. 2A). PCR amplification of *E. coli* strains JW2203 and LMM-PDS1 resulted in amplicons of 1.7 kBp and 1.9 kBp, respectively. PCR products of this size were expected as the gene was replaced with a kanamycin resistance cassette (Fig. 1A). DNA sequencing results for JW2203 and LMM-PDS1 confirmed that *ompC* was indeed replaced by a kanamycin resistance cassette (Fig. 2B, 2C). Colony PCR of *E. coli* strains C157, C161, and KJ740 did not result in a PCR product. Since these strains were generated by the disruption of *ompC* with bacteriophage (Fig. 1A) we postulate that the fragments could not be amplified due to the large size of the inserted sequence (Figure 1B). These data show that *E. coli* strains JW2203 and LMM-PDS1 are *ompC* knockouts.

In broth dilutions assays *omp*C mutant strains of are more sensitive to SDS and EDTA whereas *omp*C and *omp*F double mutants show sensitivity to SDS and EDTA compared to wild type *E. coli* strains. The role of OmpC and OmpF in resistance to treatment with SDS and EDTA was tested in broth dilution assays. Each strain was seeded into wells containing 0.0125% SDS and incrementally decreasing concentrations of EDTA ranging from 0.45 mM to 0 mM (Table 2). After 24 hours of incubation, the uninoculated negative control wells showed no signs of turbidity while the positive control wells containing just LB medium were turbid as expected (Table 2). Both of the wild type strains (MG1655 and BW25113) showed growth up to concentrations of 0.45 mM EDTA whereas the Δ*ompC* mutant strains, JW2203, LMM-PDS1, and C157 showed turbidity in concentrations of EDTA up to 0.20 mM. It is

TABLE 2 Effect of varying concentrations of EDTA on growth of *Escherichia coli* strains.

Strain	SDS (%)	0	0.0125									
	EDTA (mM)	**0.0**	**0.02**	**0.05**	**0.1**	**0.15**	**0.20**	**0.25**	**0.30**	**0.35**	**0.40**	**0.45**
BW25113		+	+	+	+	+	+	+	+	+	[+]	[+]
MG1655		+	+	+	+	+	+	[+]	[+]	[+]	[+]	[+]
JW2203		+	+	+	+	+	[+]	+/-	-	[-]	-	-
LMM-PDS1		+	+	+	+	+	+	+/-	-	-	-	-
KJ740		+	+	+	+	+	+	[+]	[+]	[+]	[+]	[+]
C161		+	+	+	+	+	+	[+]	[+]	[+]	[+]	[+]
C157		+	+	+	+	+	[+]	+/-	[-]	[-]	[-]	[-]

+ indicates growth in all 4 replicates, [+] for growth in ¾ replicates, +/- for 2/4 replicates, [-] for growth in 1/4 replicates and – for no growth in any replicate.

important to note that *omp*C mutant strains JW2203 and LMM-PDS1 are isogenic mutant strains of parent strain BW25113. The Δ*ompC* Δ*ompF* double mutant strains, KJ740 and C161, showed turbidity in concentrations of EDTA up to 0.45 mM, similar to the wild type control strains. Taken together these data suggest that compared to wild type strains of *E. coli,* strains bearing a deletion of *omp*C are more sensitive to treatment with SDS/EDTA while strains bearing a double deletion of both *omp*C and *omp*F show a similar phenotype. Growth curves conducted in the absence of selective agents (e.g. SDS or EDTA) were measured for each strain to assess possible physiological differences associated with each strain. As shown in Figure 4, growth rates of the strains were similar, although the C161 (□*omp*C and □*omp*F) and C157 (□*omp*C) grew at a slightly slower rate. These data suggest that the relative growth rates of each strain do not account for the differences observed between strains in the broth dilution assays.

DISCUSSION

Lipid distribution in biological membranes is a fundamental aspect of cell biology. The MlaA pathway represents a strategy that Gram negative bacteria have evolved to maintain an asymmetric distribution of lipids in the OM (1). Chong *et al*. showed that OmpC interacts with MlaA and that it plays a key role in this process as evidenced by increased sensitivity to SDS and EDTA in growth assays performed on solid media (2). In this study, we sought to cross examine this EDTA /SDS sensitive phenotype in several other OmpC mutant strains of *E. coli*. To facilitate side by side comparison we developed a broth dilution assay to measure the minimum inhibitory concentration of SDS and EDTA. Consistent with the work of Maliverni and Silhavy (2009), our data show that *E. coli* strains bearing deletions of *omp*C are more sensitive to SDS/EDTA compared to isogenic wild type strains (1). It is worth noting that strain *omp*C mutant C157 (for which we had no wild type control) showed a similar phenotype to *omp*C mutants JW2203 and LMM-PDS1 which were generated more recently using parent strain BW25113 (3, 6).

Surprisingly, strains C161 and KJ740 bearing deletions of both *omp*C and *omp*F showed similar levels of SDS / EDTA resistance to the wild type control strains. Although we don't have an isogenic wild type strain for C161 and KJ740 to draw a direct comparison, this result is compelling since these two *omp*C/*omp*F mutants (C161 and KJ740) were generated independently by different research groups. Why the *omp*C and *omp*F double mutant survival is similar to wild type *E. coli* in the presence of SDS /EDTA is unknown. It is possible that the presence of OmpF somehow destabilizes MlaA in the absence of OmpC thereby rendering the cells sensitive to SDS / EDTA. This model would suggest that in the absence of both OmpC and OmpF, MlaA can localize to the OM and perform its function, perhaps via its lipid anchor.

In conclusion, we have developed a broth dilution assay to compare the MIC of SDS/EDTA of various *E. coli* strains. Consistent with previous studies with *E. coli* strain MC4100, we have shown here that other *E. coli* strains bearing a deletion of *ompC* are more sensitive to treatment with SDS / EDTA. These data are consistent with the observation that *ompC* mutants are more sensitive to SDS and EDTA (1, 2) and show that the phenotype is not limited to only *E. coli* strain MC4100. These additional *ompC* mutant strains expand the toolbox which will enable deeper and broader research into the mechanism by which *ompC* and *mlaA* regulate membrane asymmetry. Unexpectedly, we observed that deletion of both *ompC* and *ompF* restores an SDS /

EDTA resistance phenotype which may point toward a complex relationship between OmpC, OmpF and MlaA in the OM.

FUTURE DIRECTIONS

Understanding how OmpC facilitates the maintenance of membrane asymmetry as part of the MlaA pathway is a key question that remains to be elucidated. Chong *et al*. have proposed that lipids may somehow traffic through the channel formed by the OmpC porin (2). In this regard, it would be interesting to complement the *omp*C mutants in our collection with OmpC variants bearing mutations within the channel. Lou *et al.* (4) have constructed a plasmid (pOmpC33, Dr. J.H. Naismith, Centre for Biomolecular Sciences, University of St. Andrews) which expresses a mutant form of OmpC with amino acid substitutions within the channel which restrict the passage of antibiotics across the OM. Whether or not the activity of the channel is required for resistance to EDTA/SDS could be tested. Additionally, the role of OmpF in maintaining membrane asymmetry remain unknown. The data presented here suggest that it may impact the function of the MlaA pathway in the absence of OmpC. Testing a mutant with a single mutation of OmpF may be an interesting avenue for future research. Finally, it is important to note that the several of the strains tested in the study did not have isogenic control strains for side by side comparison. However, the different strains showed trends that corresponded with the expected phenotype (e.g. all *omp*C mutants showed similar level of resistance and the two *ompC/ompF* mutants showed similar levels of resistance) supporting our conclusions. Nonetheless, we recommend tempering conclusions until follow up experiments can be performed using isogenic mutant and wild type strains.

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