

Deletion of OmpF is Not Sufficient to Confer Sensitivity to Ethylenediaminetetraacetic Acid in *Escherichia coli* Strain K-12

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The MlaA pathway maintains lipid asymmetry in the outer membrane of Gram negative bacteria. OmpF and OmpC proteins both interact directly with MlaA. Previous studies have demonstrated that $\Delta ompC$ mutants of *Escherichia coli* are more susceptible to ethylenediaminetetraacetic acid, a membrane disrupting agent, than wildtype cells due to disruption of the MlaA pathway. However, double $\Delta ompF/\Delta ompC$ mutants exhibit resistance to membrane disrupting agents, comparable to that of wildtype cells. To gain more insight into this apparent reversion in phenotype, we tested the sensitivity of $\Delta ompF$ mutants to ethylenediaminetetraacetic acid using a broth dilution minimal inhibitory concentration assay adapted from Hartstein *et al.*. This allowed for the effect of knocking out each gene in the $\Delta ompF/\Delta ompC$ double knockout mutants to be individually tested. Since OmpC and OmpF share similar topology, we expected $\Delta ompF$ mutants to behave similarly to $\Delta ompC$ mutants. The results of our adapted minimum inhibitory assay quantitatively demonstrated resistant wildtype and $\Delta ompC/\Delta ompF$ phenotypes, and a sensitive $\Delta ompC$ phenotype, both of which are in accordance with previous work. However, we found $\Delta ompF$ cells to exhibit increased resistance as compared to the $\Delta ompC$ strain, which was unexpected. Our results may suggest the presence of redundant mechanisms replacing OmpF and OmpC's functions, and that OmpF might disrupt the function of the MlaA lipoprotein in the absence of OmpC.

Asymmetric lipid distribution of the outer membrane (OM), with the accumulation of lipopolysaccharides (LPS) in the outer leaflet and phospholipids (PL) in the inner leaflet, is necessary for barrier function in *Escherichia coli*. In the presence of ethylenediaminetetraacetic acid (EDTA), a divalent chelator that disrupts the OM, this asymmetric distribution of membrane lipids is disrupted (1). Loss of divalent cations in the OM induces repulsion between negatively charged acyl chains of LPS resulting in the shearing of the outer leaflet (2). PL in the inner leaflet displace the sheared LPS in the outer leaflet, disrupting membrane asymmetry (2). The MlaA pathway is an ATP binding cassette (ABC) transport system that maintains OM asymmetry (1), and *E. coli* is more sensitive to EDTA when the *mlaA* gene is knocked out (3). Studies have shown that MlaA, a major protein in the MlaA pathway, interacts with the osmoporins OmpC and OmpF (3). In their study, Chong *et al.* suggested that OmpC facilitates

MlaA to maintain OM asymmetry during stationary growth while OmpF does not seem to be involved in maintaining OM asymmetry (3).

OmpC and OmpF are barrel-shaped osmoporins in the outer membrane of *E. coli* that facilitate the transport of small hydrophilic molecules in response to changes in environmental osmolarity. Increasing evidence suggests that they play a role in maintaining outer membrane structural stability: they are abundant in the OM and form trimers while associated with peptidoglycans (4). Yamada and Mizushima found that OmpC and OmpF both form hexagonal latticed arrays and act as constituents of a protective basal framework of the OM when treated with the membrane-disrupting detergent sodium dodecyl sulfate (SDS) (5). It is therefore possible that this crystal structure formed by the osmoporins plays a role in conferring resistance to EDTA.

Many studies have found that *ompC* single knockout mutants ($\Delta ompC$) are more sensitive to EDTA than the wildtype (WT) (3, 4). However, Hartstein *et al.* found *ompC/ompF* double knockout mutants ($\Delta ompC/\Delta ompF$) to be resistant to EDTA (6). The phenotype of *ompF* single

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knockout mutants ($\Delta ompF$) to EDTA has yet to be tested. Due to the structural similarities between the osmoporins, we expected that the absence of either OmpC or OmpF would weaken the OM structure integrity and have comparable phenotypes when treated with EDTA regardless of OmpC's involvement with the Mla pathway.

MATERIALS AND METHODS

Bacterial strains. The *E. coli* strains studied are listed in Table 1. All strains were grown in Luria Bertani broth at 37 °C with or without antibiotics. Strains JW2203, JW0912 were grown in 50 mg/mL Kanamycin (Kan); strain KJ740 was grown in 100 mg/mL Streptomycin; strain BW25113 does not require antibiotic selection (7-9). The strains are all derivatives of K-12, with the parent strain of JW2203 and JW0912 being BW25113 (7, 8). KJ740 is derived from AW06 (9).

Minimum inhibitory concentration broth assay. The minimum inhibitory concentration (MIC) broth assay was adapted from Hartstein *et al.* (6). Four different *E. coli* strains (BW25113, JW0912, JW2203, KJ740) were tested in a MIC broth assay experiment. In each experiment, 4×10^4 cells were seeded into each well of a 96-well polypropylene plate containing LB, EDTA and SDS (10). In each well, the final concentration of SDS was kept constant at 0.0125%, as suggested by Hartstein *et al.*, to further resolve the effects of EDTA on the cells (6). EDTA concentrations in each well ranged from 10 mM to 60 mM (10 mM, 15 mM, 20 mM, 25 mM, 30 mM, 35 mM, 40 mM, 45 mM, 50 mM, 55 mM, 60 mM). The final volume in each well was 300 μ L. The plates were then sealed with sterile aluminum foil and incubated at 37 °C for 20 hours. Following incubation, the wells were mixed thoroughly with a micropipette and 175 μ L of the culture from each well was transferred onto a transparent 96-well polystyrene plate. The polystyrene plate was measured for optical density (O.D.) at 660 nm. Each plate contained triplicates of each test and two lanes of negative controls. Outliers were removed based on the Grubb's outlier test ($p = 0.01$), and instances of known human error. The growth curves were standardized according to the O.D. of the 0 mM EDTA wells for each strain, after taking into account background signal derived from the negative controls of each 96-well plate. Error bars for the standardized bar graphs (Fig. 1B) were calculated via error propagation of the standard errors of the mean of each triplicate. Two-tailed T-Tests ($p = 0.05$) were performed between different strains to test for statistical significance at each tested EDTA concentration. The exact p-value was not calculated for comparisons between each EDTA concentration for simplicity and clarity. The T-stat was simply compared with the critical t-value for $p = 0.05$ to give either a pass or fail for each null hypothesis.

RESULTS

***E. coli ompF* deletion mutants are resistant to EDTA in the presence of SDS.** To investigate the role of OmpF and

OmpC in conferring resistance to EDTA in the presence of 0.0125% SDS, we performed the MIC broth assay on $\Delta ompF$, $\Delta ompC$ and $\Delta ompC/\Delta ompF$ knockouts. BW25113 (WT), the parental strain to both JW0912 ($\Delta ompF$) and JW2203 ($\Delta ompC$), expresses both OmpC and OmpF, and served as the positive control. JW2203 ($\Delta ompC$) was included as an additional control for comparison. Though we measured the resistance of the strains against varying EDTA concentrations, our experiment aimed to build upon the research performed by Hartstein *et al.*. In order to reproduce results and compare them, SDS was added alongside EDTA to replicate the same experimental conditions as that in the study by Hartstein *et al.*. Figure 1A shows the O.D. of each strain at each EDTA concentration with 0.0125% SDS. The differences in O.D. between strains are more pronounced at higher EDTA concentrations (between 25 mM and 50 mM), and then converges sharply for wells containing 55 mM and 60 mM EDTA. We noticed that the strains grow at slightly different rates under the same conditions. The difference in growth rate is not related to EDTA as this was observed in the absence of EDTA (specifically the 0 mM EDTA/0% SDS treatment). Since the focus of our experiment was on strain-resistance of EDTA, we standardized the data based on the number of cells present at the 0 mM EDTA/0% SDS treatment (set as 100%). In Figure 1B (the standardized data), the cell viability of JW2203 ($\Delta ompC$) after standardization was significantly lower compared to BW25113 (WT), JW0912 ($\Delta ompF$), and KJ740 ($\Delta ompC/\Delta ompF$) when subjected to EDTA concentrations from 25 mM to 50 mM ($p < 0.05$). The cell viability at EDTA concentrations ranging from 10 mM to 20 mM was not statistically different ($p > 0.05$). The cell viability of JW0912 ($\Delta ompF$) did not show significant disparity at EDTA concentrations of 35 mM, 40 mM, and 45 mM. Despite the T-Test between JW0912 ($\Delta ompF$) and WT passing for the other concentrations of EDTA ($p < 0.05$), the magnitude of the differences between these two strains was relatively small, and has no distinguishable correlation with increasing EDTA concentration across all concentrations, making the rejection of the null hypothesis difficult without more replicates. The minimum inhibitory concentration for BW25113 (WT), JW0912 ($\Delta ompF$) and KJ740 ($\Delta ompC/\Delta ompF$) were all at 55 mM EDTA/0.0125% SDS (Fig. 1B). At 40 mM EDTA, the differences between strains were the most pronounced. Figure 1C highlights this significant difference in viability between JW2203 ($\Delta ompC$) and other strains at 40 mM EDTA. JW0912 ($\Delta ompF$) did not show significant difference in cell viability compared to BW25113 (WT). JW2203 ($\Delta ompC$) was close to its minimum inhibitory concentration at the 40 mM EDTA treatment. Its cell viability was significantly lower than the other strains. Results shown in Figure 1 were from a single experiment containing triplicates of each strain. In short, the data suggests that JW2203 ($\Delta ompC$) is more sensitive to

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

| Strain | Mutation | Reference/Source |
|---------|---------------------------|------------------|
| BW25113 | WT | 7 |
| JW2203 | $\Delta ompC$ | 8 |
| JW0912 | $\Delta ompF$ | 8 |
| KJ740 | $\Delta ompC/\Delta ompF$ | 9 |

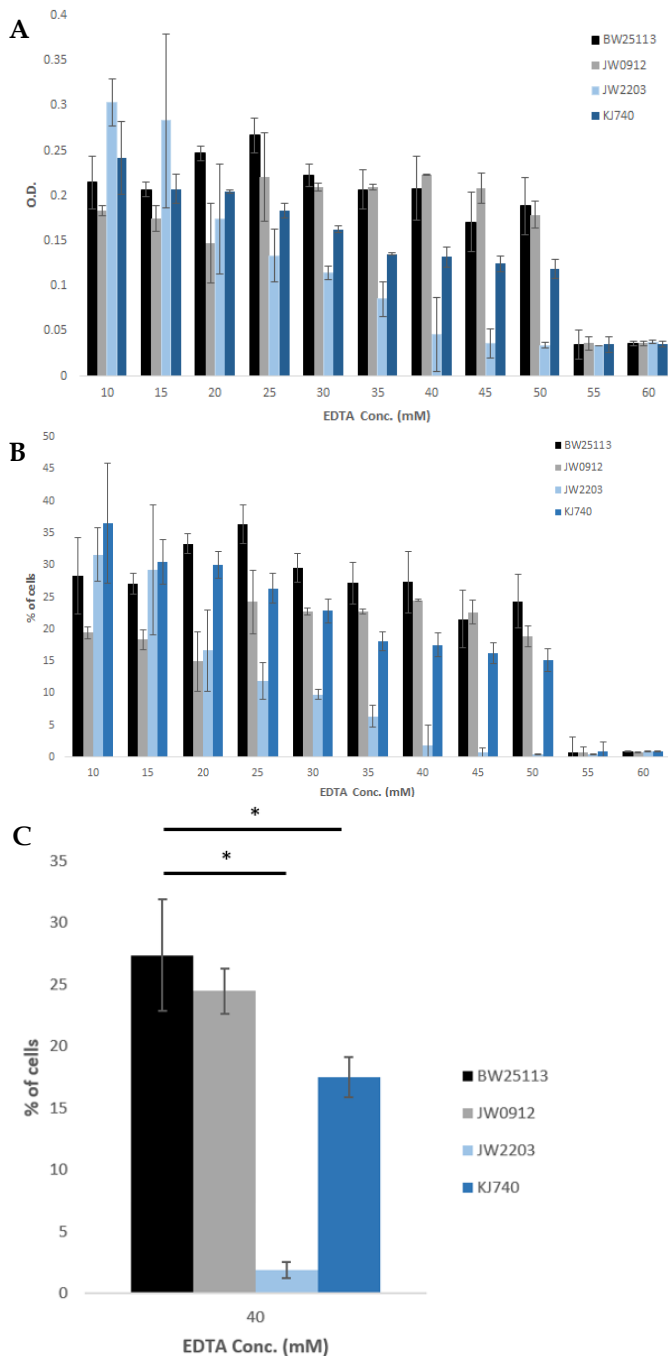


FIG. 1 (A) Effect of varying EDTA concentrations (10 mM to 60 mM) on growth of *E. coli* strains. O.D. measurements for the 4 strains: BW25113 (Wildtype), JW0912 ($\Delta ompF$), JW2203 ($\Delta ompC$), and KJ740 ($\Delta ompF/\Delta ompC$). SDS Concentration = 0.0125%. Plates were incubated at 37 °C for 20 hours. **(B)** Effect of varying EDTA concentrations on standardized cell growth of *E. coli* strains. Standardized each well to the average O.D. of the LB only treatment (as 100%, not shown) of each strain. Error bars are calculated via error propagation of the standard deviations of the triplicates of each strain. **(C)** % of cells relative to respective controls for strains BW25113, JW0912, JW2203 and KJ740 at an EDTA concentration of 40 mM. Asterisks symbolize positive T-Test between the two indicated strains with $p < 0.05$.

EDTA compared to BW25113 (WT). Moreover, JW0912 ($\Delta ompF$) and KJ740 ($\Delta ompC/\Delta ompF$) did not exhibit conclusive differences to EDTA sensitivity relative to BW25113 (WT).

DISCUSSION

OmpF and OmpC are traditionally discussed in the context of their porin function, but increasing evidence and their abundance in the OM suggests that they are important for maintaining OM structural stability as well. Studies have previously used qualitative broth dilution assays to measure the MIC of EDTA and demonstrated that $\Delta ompC$ mutants are susceptible to EDTA (6, 10). We further developed Hartstein *et al.*'s qualitative broth dilution assay into a quantitative microplate-based growth assay wherein the growths (O.D.s) of strains are measured using spectrophotometry with a microtiter plate reader at a wavelength of 660 nm to determine the MIC of EDTA of *E. coli* strains (6). Our adapted, quantitative approach allows insight into the response of the various tested strains to EDTA at various time points after treatment, as opposed to the simple end-point analysis performed by Hartstein *et al.*

Consistent with previous studies (1, 6), we found that $\Delta ompC$ *E. coli* are more sensitive to EDTA as compared to isogenic WT strains. To our knowledge, the sensitivity of $\Delta ompF$ mutants to EDTA has not been tested before (3, 4, 6). It was thought that due to its functional, biochemical and physiochemical similarities to OmpC, $\Delta ompF$ would be sensitive in these conditions (4, 5). However, we show here that $\Delta ompF$ is resistant to EDTA stress. Additionally, we confirmed that $\Delta ompC/\Delta ompF$ strain is resistant to EDTA (6). Despite the fact that KJ740 ($\Delta ompC/\Delta ompF$) was not isogenic to the rest of the strains tested, and the comparison is therefore not wholly valid, Hartstein *et al.* have described multiple other OmpF- and OmpC- lacking *E. coli* strains that display a similar resistant phenotype to EDTA as BW25113 (WT) (6).

The total inhibitory concentration of EDTA was observed to be 55 mM for three of our tested strains exhibiting resistant EDTA phenotype. This included the wildtype (BW25113), $\Delta ompF$ mutant (JW0912), and $\Delta ompC/\Delta ompF$ mutant (KJ740). The decline of O.D. at 55 mM EDTA after 20 hours of incubation in all trials of the experiment is sharp. This uniform decrease in cell viability at 55 mM across all tested strains was rather unexpected, but did not conflict with the results of previous papers (3, 6). This phenomenon may either be the result of human error during reagent preparation, or hint at a potential drastic change induced by a critical

threshold of EDTA concentration in *E. coli*. On the other hand, the total inhibitory concentration of EDTA was observed to be between 35 and 40 mM for JW2203 ($\Delta ompC$). This range was in accordance with the MIC results of Hartstein *et al.* It is worth noting that O.D. of JW2203 ($\Delta ompC$) decreased gradually with increased EDTA concentration up until the total inhibitory concentration, contrary to the rather sharp decrease in O.D. seen at 55 mM for the resistant strains, potentially underlining the existence of interplay of multiple mechanisms for determining cell viability of *E. coli* in the presence of EDTA.

The MIC broth assay could be improved by including an additional column of wells containing 0.0125% SDS but no EDTA. The implementation of this treatment may serve to better reflect on the difference in O.D. due to increments of EDTA, and potentially explain the large decrease in O.D. seen globally across replicates when going from 0 mM EDTA/0% SDS to 10 mM EDTA/0.0125% SDS.

At present, there is no explanation for the resistant phenotype associated with $\Delta ompF$ and $\Delta ompC/\Delta ompF$ mutants. Although the strains used in this study were validated via PCR by Hartstein *et al.*, it is known that $\Delta ompC/\Delta ompF$ cells tend to evolve into revertants or pseudorevertants (expressing OmpF) at a high rate (6, 11). It has also been shown the absence of OmpF in the membrane results in the upregulation of other OM porins like PhoE, to potentially compensate for OmpC and OmpF's roles in maintaining outer membrane integrity (11). These osmoporins share extensive sequence homology and several biochemical and physiochemical characteristics with OmpC and OmpF, and carry out similar functions (12). Mutants devoid of OmpF and OmpC constitutively produce PhoE, which is normally upregulated only when phosphate is limited and has been shown to assemble into a hexagonal lattice with LPS under conditions of osmotic shock (11). This evidence suggests that PhoE is capable of compensating for the structural defect in strains lacking OmpC and OmpF.

Furthermore, it is possible that the presence of OmpF in the OM inhibits the MlaA protein function in maintaining membrane asymmetry thereby rendering the cells sensitive to EDTA. Chong *et al.* have shown that both OmpC and OmpF physically associate with MlaA and that OmpC facilitates MlaA function (3). However, they observed that membrane asymmetry is disrupted only in cells lacking OmpC and not in cells lacking OmpF (3). In $\Delta ompF$, the absence of OmpF may allow MlaA to associate with the resident OmpC and keep the

membrane stable. However, in $\Delta ompC$ the presence of OmpF in conjunction with the absence of OmpC might leave the cells sensitive to EDTA. This model suggests that in WT cells, OmpF and OmpC cells regulate MlaA function in a dynamic equilibrium. Additionally, in the absence of both OmpC and OmpF, MlaA may localize to the OM via its lipid anchor and perform its function. In conclusion, we have re-appropriated Hartstein *et al.*'s qualitative assay into a growth-based MIC assay to determine the MIC of EDTA of *E. coli* K-12 strains. We established that phenotypic differences between $\Delta ompC$ and the WT *E. coli* arise at 40 mM EDTA at 0.0125% SDS. Unexpectedly, we observed that *E. coli ompF* deletion mutants are as resistant to EDTA as the WT and $\Delta ompC/\Delta ompF$ mutants which might point toward a complex relationship between OmpC, OmpF, and MlaA in the OM.

FUTURE DIRECTIONS

OmpC and OmpF are both implicated to be important in maintaining OM stability of *E. coli* in conjunction with the MlaA pathway (3, 6). Understanding the molecular mechanism through which OmpC facilitates the establishment of an asymmetric OM is a key point that remains to be elucidated. Testing mutants with defective OmpC porins in the assay would determine if the activity of the channel is required for the resistance to EDTA. Lou *et al.* have constructed a plasmid carrying an OmpC mutant with amino acid substitutions within the porin channel that restrict passage of solutes could be used (13). Screening for *E. coli* mutants in which OmpF and MlaA do not bind to each other and determining their EDTA resistance relative to WT cells would determine if OmpF indeed plays a vital or negative role in the MlaA pathway.

Additionally, Nogami *et al.* suggested that PhoE maintains the surface structure of *E. coli* in the absence of OmpC and OmpF (11). In their study, Tris-HCl and osmotic shock treatment had no effect on OmpF/OmpC or PhoE expressing cells but was lethal to cells lacking those proteins (11). It would be prudent to confirm the expression of PhoE in the $\Delta ompC/\Delta ompF$ strains used in our study, which can be accomplished using qPCR or SDS-PAGE (with commercially available antibodies) after purification of the protein from the cell envelope.

It is also possible that OmpF plays an inhibitory role in the expression of compensatory proteins like PhoE. Click *et al.* demonstrated that the export of other outer membrane proteins can be regulated by *ompC* overexpression (14). Similarly, *ompF* expression in the $\Delta ompC$ strains could potentially down-regulate the expression of compensatory proteins, resulting in susceptibility to EDTA. Expressing OmpF in a low copy plasmid and transcomplementing them into

$\Delta ompC/\Delta ompF$ may be an interesting experiment that could elucidate the mechanism behind $\Delta ompF$ resistance and $\Delta ompC$ sensitivity to EDTA.

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