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Phosphate deficiency restores SDS-EDTA resistance in an *Escherichia coli* K12 *ompC* knockout mutant

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SUMMARY The asymmetric outer membrane (OM) of Gram-negative bacteria protects against external insults, such as antibiotics and detergents. OmpC is a general diffusion porin found in the OM that plays a role in maintaining OM asymmetry. Previous studies have shown that *E. coli ompC* knockout strains are sensitive to treatment with sodium dodecyl sulfate (SDS) and EDTA compared to wild type. PhoE is another general diffusion porin that is similar in structure and function to OmpC, but normally expressed only under phosphate-deficient conditions. We hypothesized that phosphate deficiency would restore SDS-EDTA resistance in *ΔompC* mutants by inducing PhoE expression, which could compensate for the lack of OmpC. To test this, we performed growth assays of wild type and *ΔompC* mutant strains grown in phosphate-sufficient or phosphate-deficient media with increasing SDS-EDTA concentrations. Our study found that the *ΔompC* mutant strain is resistant to SDS-EDTA when grown in phosphate-deficient media compared to phosphate-sufficient media. SDS-PAGE was used to investigate the expression of PhoE in phosphate-deficient media. Our results suggest that phosphate deficiency results in changes in cellular protein expression and restores SDS-EDTA resistance in *E. coli ΔompC* strains.

INTRODUCTION

Gram-negative bacteria, such as *Escherichia coli*, have an asymmetric outer membrane (OM) with lipopolysaccharides (LPS) in the outer leaflet and phospholipids in the inner leaflet (1). The OM is highly hydrophobic and has low fluidity due to lateral interactions between the LPS molecules in the outer leaflet, conferring protection against membrane-disrupting compounds (2). Studies have shown that as OM asymmetry is disrupted and phospholipids accumulate in the outer leaflet, bilayer patches start to form which become portals of entry for small lipophilic molecules (3). The cells then become more sensitive to external insults including detergents, bile salts and antibiotics, and demonstrate decreased rates of survival (2,4).

The Mla pathway is an ATP-binding cassette transport system that maintains OM asymmetry in *E. coli* by mediating retrograde transport of misplaced phospholipids in the outer leaflet (3,4). The Mla pathway is the only system that maintains OM asymmetry via direct extraction of misplaced phospholipids, and it has therefore been identified as an important virulence factor in several types of Gram-negative bacteria (3). OmpC is a major general diffusion porin spanning the OM that forms an amphipathic trimeric ß-barrel which serves as a size-selective channel for the diffusion of small hydrophilic molecules (5). OmpC forms a stable complex with MlaA, a protein in the Mla pathway, and plays a role in OM resistance against membrane-disrupting compounds such as ethylenediaminetetraacetic acid (EDTA) and sodium dodecyl sulphate (SDS) (1,3,6).

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EDTA is a chelating agent that displaces divalent cations in the OM, resulting in increased repulsion between negatively-charged LPS molecules (4). The increased repulsion causes migration of phospholipids into the affected areas of the outer leaflet (4). The newly migrated phospholipids then disrupt packing interactions between LPS and form areas with increased permeability to detergents, such as SDS, that cause protein denaturation (4). Thus, cells that lose OM asymmetry become susceptible to SDS-EDTA treatment. *ΔMla* knockout mutants in *E. coli* have been shown to cause increased SDS-EDTA sensitivity (4). Previous studies have also shown that *E. coli ΔompC* mutants are more susceptible to SDS-EDTA treatment compared to wild type due to loss of membrane asymmetry (6,7). Altogether these results demonstrate the necessity of the Mla system and OmpC in maintaining OM asymmetry to resist external chemical insults.

PhoE is another trimeric general diffusion porin that is induced only under phosphate-deficient conditions by the Pho regulon, which responds to changes in phosphate concentration (8). There are 61% identical amino acid residues between OmpC and PhoE (8). The major difference in gene sequence between the two porins exists in the 5' and 3' noncoding regions, resulting in different gene expression patterns (8). OmpC and PhoE form diffusion pores with similar diameters and have molecular weights of 40.3 kDa and 38.9 kDa, respectively (8). Like OmpC, PhoE is capable of transporting small hydrophilic molecules, such as phosphate and phosphorylated molecules (8). It remains unknown whether PhoE interacts with components in the Mla pathway in order to maintain membrane asymmetry. However, the purpose of our study is not to investigate the involvement of PhoE in the Mla pathway, but rather to determine the effect of PhoE expression in *ΔompC* mutants.

OmpF is another general diffusion porin similar to OmpC (9). A previous study has suggested that OmpF decreases membrane stability and therefore has an opposing effect to OmpC (9). As such, despite similarities between OmpC and OmpF, this study does not examine the role of OmpF in terms of rescuing membrane asymmetry in *ΔompC* mutants.

We hypothesized that phosphate deficiency can restore SDS-EDTA resistance in *E. coli* K12 *ΔompC* mutants due to compensation by PhoE. To test this, we developed and performed SDS-EDTA growth assays on wild type and *ΔompC* strains. We grew both strains in either phosphate-sufficient or phosphate-deficient media in the presence of increasing concentrations of SDS-EDTA and assessed optical density for 16 hours. We also investigated protein expression patterns in our *ΔompC* mutant grown in phosphate-deficient media using SDS-PAGE.

TABLE 1. Primer sequences for PCR genotype validation.

Primer	Nucleotide sequence (5' to 3')
ompC forward	G AGA ATG GAC TTG CCG ACT GAT TAA TGA G
ompC reverse	C ACG CCA GAA GGT ACC CAT AGT TTT G
phoE forward	GA TAT CAA ACG AAC GTT TTA GCA GGA CTG TCG TCG GTT G
phoE reverse	GA GCT GGA AGC GCA GGA ATC CCG TTT TAC
Control forward	GCA AAT AAA GGC ATA TAA CAG AGG GTT AAT AAC ATG
Control reverse	C AGG CCC TTT GTT CGA TAT CAA TCG AGA TTA

METHODS AND MATERIALS

Bacterial strains. *E. coli* K12 strain BW25113 (hereafter referred to as wild type), JW2203-1 (*ΔompC*), and JW0231-2 (*ΔphoE*) from the CGSC Keio collection were used (10). The wild type strain was grown on Lysogeny Broth (LB) Agar plates while the mutant strains were grown on LB Agar plates supplemented with kanamycin at 100 µg/mL. All strains were grown at 37°C.

Genotype validation of *E. coli* wild type, *ΔompC*, and *ΔphoE* strains. Primers for *ompC* and *phoE* PCR amplification were designed at least 100 base pairs away from each gene on both sides using the *E. coli* BW25113 genomic sequence obtained from NCBI as template.

Wild type, *ΔompC* and *ΔphoE* genomic DNA was isolated using Invitrogen™ PureLink™ Genomic DNA Mini Kit (Cat#1977688). The *ompC* and *phoE* primers shown in Table 1 were used to amplify the *ompC* and *phoE* genes in wild type while the same primers were utilized to amplify the kanamycin resistance cassette inserted into *ΔompC* and *ΔphoE* mutants. In each PCR tube, 50 ng genomic DNA, 0.5 µM of each primer (forward and reverse), 0.2 mM dNTP mixture, 1X buffer, 1.5 mM $MgCl_2$, and 2.5 units/reaction Platinum Taq polymerase (ThermoFisher Scientific, Cat #10966026) were added to a final volume of 50 µL. The *ompC* PCR reaction was performed accordingly: 94°C for 3 minutes; followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 56°C for 30 seconds, and extension at 72°C for 3 minutes; then a final extension of 72°C for 10 minutes. The *phoE* and positive control reactions used the same parameters, except with respective 62°C and 54°C annealing temperatures. The positive control extension time was set for 2 minutes instead due to a shorter sequence.

A 0.8% agarose gel was made using a final concentration of 1X TAE buffer with 1X SYBR™ Safe DNA gel stain (ThermoFisher Scientific, Cat #S33102). 10 µL of amplified DNA was loaded with 6X DNA loading dye and run with the 1 Kb Plus DNA ladder from ThermoFisher Scientific (Cat #10787018). The gel was run at 100 V for 45 minutes in 1X TAE buffer, and then visualized using AlphaImager® EC (Alpha Innotech Corporation) under UV exposure.

Each PCR reaction was cleaned up using the Wizard® SV Gel and PCR Clean-up System (Promega, Cat #A9282). The DNA concentration and purity were assessed on the NanoDrop 2000c Spectrophotometer (ThermoFisher Scientific), then sent for sequencing through Genewiz. The *ompC* primer pair was used to sequence the wild type and *ΔompC* mutant strains while the *phoE* primer pair was used to sequence the wild type and *ΔphoE* mutant strains. Resulting sequences were aligned against their respective insert sequence at the flanking regions using the NCBI nucleotide BLAST tool. Upon confirmation of the correct sequences, the ExPASy translate tool was used to determine the OmpC, PhoE, and kanamycin resistance cassette protein sequences. The protein sequences were further aligned against their respective insert protein sequence using the NCBI protein BLAST tool for confirmation.

Preparation of phosphate-sufficient and phosphate-deficient media. The protocol was adapted from Tommassen *et al.* (11). The minimal media contained final concentrations of 0.12 M HEPES, 0.08 M NaCl, 0.02 M KCl, 0.02 M NH_4Cl, 3 mM Na_2SO_4, 1 mM $MgCl_2 \cdot 6H_2O$, 0.2 mM $CaCl_2 \cdot 2H_2O$, 3.3 µM $FeCl_3$, 0.1 M glucose, and 3.8 µM thiamine. Phosphate-sufficient media contained 660 µM K_2HPO_4 while phosphate-deficient media contained 42 µM K_2HPO_4. HEPES buffer was prepared using HEPES sodium salt and adjusted to pH 7.42 using 1 M HCl. Salts were dissolved in autoclaved distilled water. All components were filter-sterilized through a 0.45 µm filter and then combined aseptically. Media was covered in aluminum foil and stored at 4°C.

SDS-EDTA growth assay. A solution of 5 mM EDTA was prepared at pH 8.0 to dissolve EDTA, and filter sterilized through a 0.45 µm filter. A 1% w/v stock of SDS was prepared and also filter sterilized. *E. coli* wild type and *ΔompC* strains were grown in phosphate-sufficient and phosphate-deficient media for 48 hours in a shaking incubator at 37°C. The media for the *ΔompC* mutant strain was supplemented with 100 µg/mL of kanamycin. $1x10^5$ cells from each culture were added to each well in a 96-well plate containing a 0.05% w/v SDS and final concentrations of either 0 mM, 0.05 mM, 0.30 mM, or 0.60 mM EDTA in a

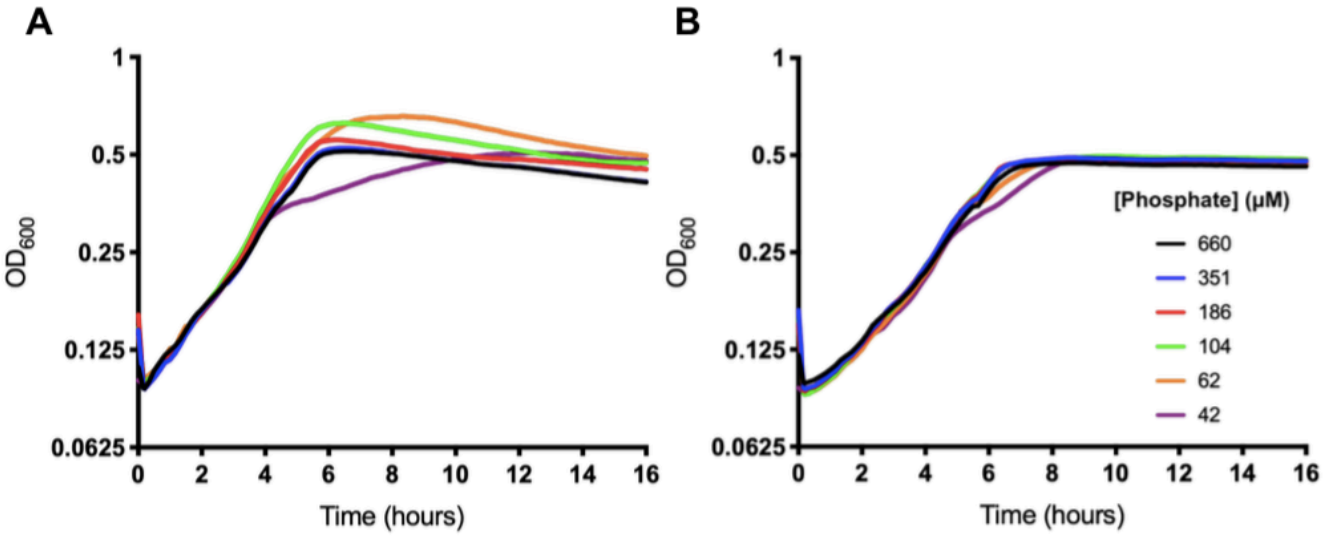

FIG. 1 Growth curve of *E. coli* wild type and *ΔompC* strain in minimal media of varying phosphate concentrations (A) *E. coli* wild type growth curve (B) *ΔompC* growth curve. 1:10 dilutions of overnight LB cultures were transferred to a 96-well plate and grown in minimal media with different concentrations of phosphate at 37°C for 16 hours. Growth was measured at OD_{600} every 10 minutes (n=3).

final volume of 250 µL. The 96-well plate was incubated at 37°C in a BioTek Plate Reader that recorded OD_{600} readings every 10 minutes for 16 hours, shaking for 3 seconds before each reading.

Whole cell lysate SDS-PAGE. 30 µL of β-mercaptoethanol was added to 570 µL of 2x Laemmli sample buffer (65.8 mM Tris-HCl, pH 6.8, 26.3% (w/v) glycerol, 2.1% SDS, 0.01% bromophenol blue), as per the Bio-Rad™ Laemmli sample buffer protocol, to obtain a final working solution of Laemmli with 355 mM β-mercaptoethanol. *E. coli* wild type, *ΔompC,* and *ΔphoE* strains were grown in phosphate-sufficient and phosphate-deficient media for 48 hours in a shaking incubator at 37°C. The media for the *ΔompC* and *ΔphoE* mutant strains was supplemented with 100 µg/mL of kanamycin. To normalize the number of cells from which lysates were collected, 1.92 x 10^8 cells were pelleted at 13,000 rpm for 10 minutes. To prepare the whole cell lysates, each cell pellet was resuspended in 100 µL of sample buffer. The cells were subsequently heated at 95°C for 10 minutes, spun down, and the cell lysate separated on a 4-20% Bio-Rad™ Mini-PROTEAN® TGX Stain-Free™ precast protein gel at 200 V for 30 minutes. The gel was run in 1X Tris/Glycine/SDS running buffer (25 mM Tris-HCl, 192 mM glycine, 0.1% SDS, pH 8.3). Samples were run with the Bio-Rad™ Precision Plus Protein™ All Blue Prestained Protein Standards ladder and visualized with the BioRad™ ChemiDoc MP Imaging System with a 45-second exposure time.

RESULTS

Genotype validation of *E. coli* wild type, *ΔompC*, and *ΔphoE* strains. *E. coli* strains JW2203-1 (*ΔompC*) and JW0231-2 (*ΔphoE*) bear kanamycin resistance cassette insertions within the *ompC* and *phoE* genes, respectively (10). To confirm the genotypes of these strains, we designed primer pairs flanking the *ompC* and *phoE* coding regions (Table 1). Wild type strain BW25113 served as a control for both *ΔompC* and *ΔphoE* mutants since it is expected to contain a functional copy of both gene sequences. A band of the correct size in the positive control (1165 bps) indicates a successful PCR experiment.

Based on the lengths of the kanamycin resistance cassette (1303 bps), the *ompC* gene (1104 bps) and the *phoE* gene (1062 bps), PCR product lengths of *ΔompC* (1671 bps) and *ΔphoE* (1850 bps) as well as wild type *ompC* (1448 bps) and *phoE* (1579 bps) would be expected. All PCR gene products had one distinct bright band, indicating high primer

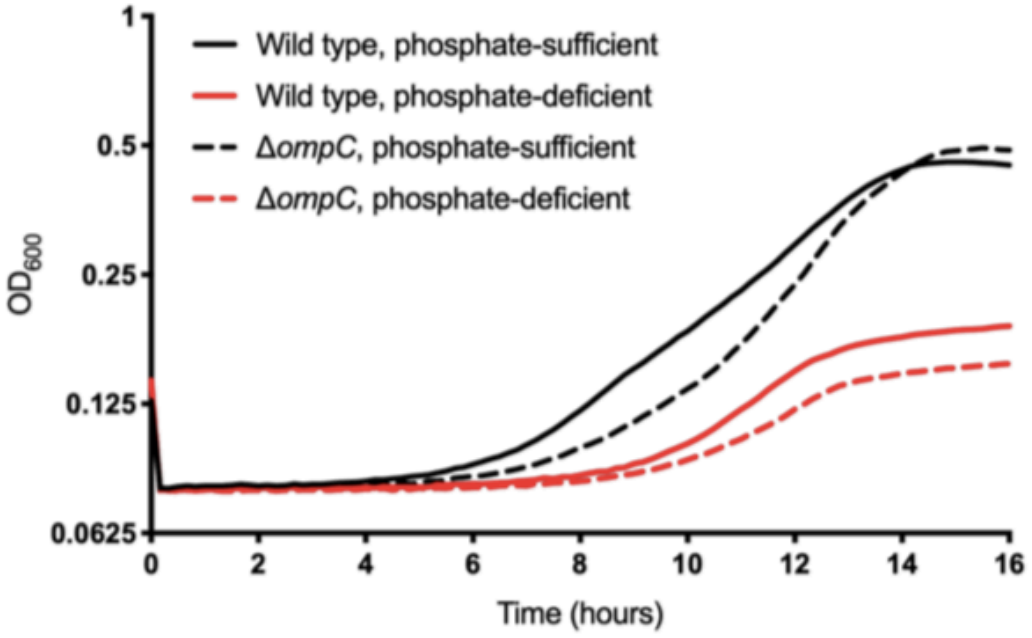

FIG. 2 Growth curve of *E. coli* wild type and *ΔompC* strain in phosphate-sufficient and phosphate-deficient media. $1x10^5$ cells from 48-hour overnight cultures were transferred to a 96-well plate and incubated at 37°C for 16 hours. Growth was measured at OD_{600} every 10 minutes (n=6).

specificity to anneal to their respective regions (Fig. S1). In addition, all the bands present correspond to their expected sizes. The identity of each amplicon was successfully confirmed by sequencing.

Wild type and *ΔompC* mutant strains are viable in minimal media containing phosphate concentrations between 42 µM - 660 µM. Viability of the wild type and *ΔompC* strains in a range of phosphate concentrations was determined by performing a 16 hour growth curve assay with serial dilutions of minimal media. As shown in Figure 1, both strains are viable in all ranges of phosphate concentrations. Both strains reached similar OD_{600} values of 0.50 at 16 hours. Growth of both strains in 42 µM phosphate minimal media slowed down around 4 hours, but caught up by 10 and 8 hours for wild type and *ΔompC*, respectively. With the exception of the 42 µM phosphate condition, all wild type strains peaked around 6 hours at an approximate OD_{600} of 0.65 and decreased to OD_{600} values around 0.45 at 16 hours. In contrast, with the exception of the 42 µM phosphate condition, all *ΔompC* strains peaked at 6 hours and remained stable. 660 µM and 42 µM of phosphate were used in subsequent assays as phosphate-sufficient and phosphate-deficient media, respectively, as adapted from Tommassen *et al.* (11).

***ΔompC* mutant strain has a similar growth pattern compared to wild type strain in both phosphate-sufficient and phosphate-deficient media.** Growth curves conducted in the absence of selective agents (i.e. SDS, EDTA, or kanamycin) were measured for the wild type and *ΔompC* strains in phosphate-sufficient and phosphate-deficient media to assess possible growth differences between the strains. As shown in Figure 2, growth patterns between the strains are comparable to each other in both media over 16 hours. Both strains grew to similar OD_{600} values at 16 hours in their respective media. In phosphate-sufficient media, growth began around the 5 hour time point and both strains reached similar densities around OD_{600} 0.50 at the 16 hour end point. In phosphate-deficient media, growth started approximately 8 hours after inception of the assay and reached densities around OD_{600} 0.175. Of note, growth of both strains in phosphate-sufficient media occurred at a faster rate and reached a higher

optical density at 16 hours than growth in phosphate-deficient media. Additionally, the *ΔompC* mutants grew at a slower rate compared to the wild type strain in both media.

***ΔompC* mutant strain is sensitive to SDS-EDTA in phosphate-sufficient media.** SDS-EDTA growth assays were conducted for the wild type and *ΔompC* strains in phosphate-sufficient media to confirm results previously observed by Hartstein *et al.* (7). The wild type strain in phosphate-sufficient media grew comparably for all conditions, irrespective of the concentration of SDS-EDTA (Fig. 3A). Growth entered exponential phase for all conditions around the 6 hour mark, steadily increased to 14 hours, then plateaued around OD_{600} 0.40. The *ΔompC* strain without SDS-EDTA treatment (0 mM) grew similar to wild type, with growth starting around 6 hours, followed by a steady increase and plateau at 14 hours around OD_{600} 0.45 (Fig. 3B). In contrast, growth yield of the *ΔompC* strain notably decreased when treated with any concentration of SDS-EDTA (Fig. 3B). Growth started around the 6 hour mark for all conditions in the presence of SDS-EDTA. The *ΔompC* strain in the presence of 0.05 mM EDTA increased up to 14 hours, followed by a decrease. Similarly, growth of the *ΔompC* strain in the presence of 0.30 mM EDTA increased up to 15 hours, and also followed by a decrease. *ΔompC* growth in the presence of 0.60 mM EDTA was notably slower and increased up to the end of the assay at 16 hours. The final OD_{600} values were approximately 0.25 for all SDS-EDTA treated conditions. None of the treated *ΔompC* strains reached OD_{600} values higher than 0.30. Based on the observed growth patterns, the *ΔompC* mutant strain is sensitive to SDS-EDTA in phosphate-sufficient media. In contrast, the wild type strain is resistant to SDS-EDTA treatment in phosphate-sufficient media.

It should be noted that although typical SDS concentrations used to examine OM integrity are 0.50% SDS, our results do indicate growth inhibition using merely 0.05% SDS (6). Our methods were adapted from Hartstein *et al.* and Fung *et al.*, who both used 0.0125% SDS to disrupt OM integrity (7,9).

Phosphate deficiency restores SDS-EDTA resistance in *ΔompC* mutant strain. SDS-EDTA growth assays were conducted for the wild type and *ΔompC* strains in phosphate-deficient media to determine if phosphate deficiency can restore resistance to SDS-EDTA in a *ΔompC* mutant. The wild type strain in phosphate-deficient media was able to grow in the presence of 0.05% SDS with 0.05 mM, 0.30 mM, and 0.60 mM EDTA (Fig. 4A). Consistent

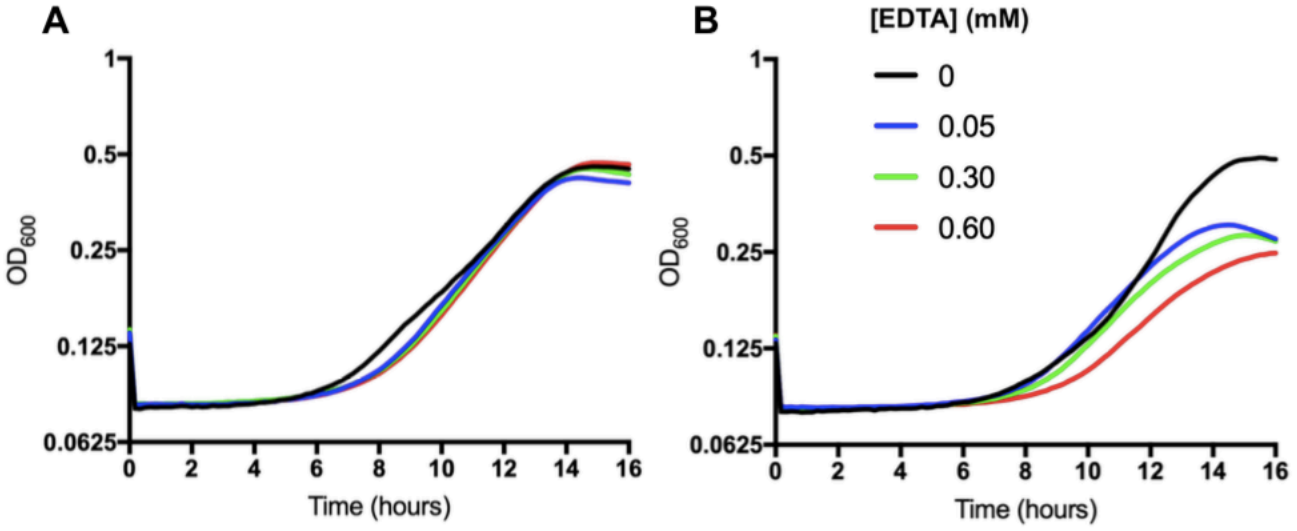

FIG. 3 SDS-EDTA growth assay of *E. coli* (A) wild type and (B) *ΔompC* strains in phosphate-sufficient media with 0.05% SDS and varying concentrations of EDTA. 1x10⁵ cells from 48-hour overnight cultures were transferred to a 96-well plate and incubated at 37°C for 16 hours. The 0 mM EDTA condition contained neither SDS nor EDTA. Growth was measured at OD_{600} every 10 minutes (n=6).

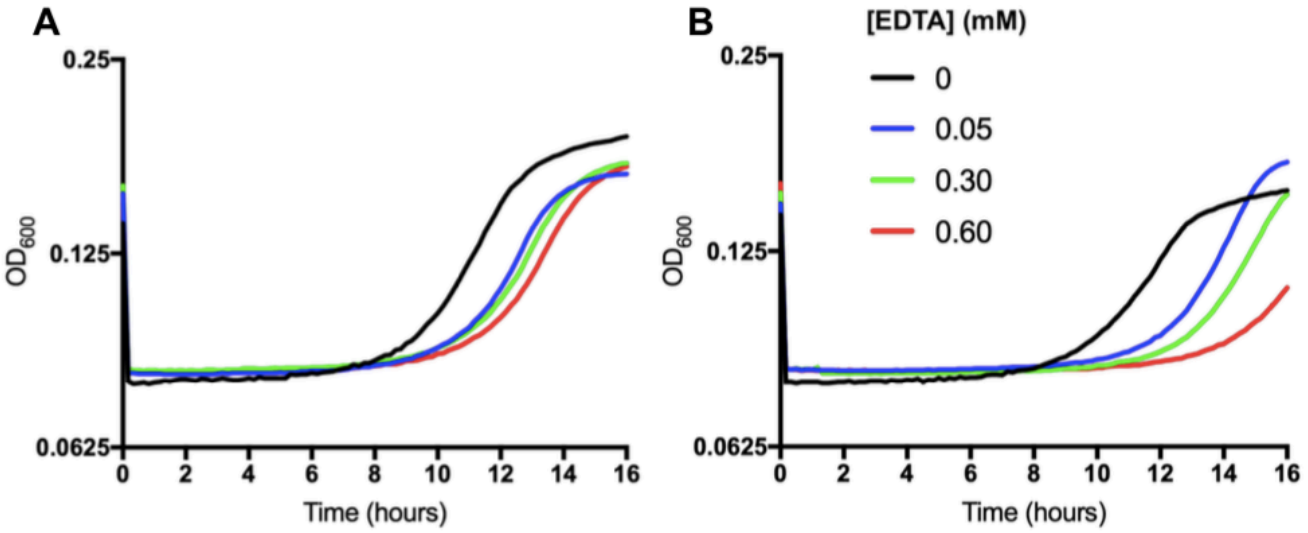

FIG. 4 SDS-EDTA growth assay of *E. coli* (A) wild type and (B) *ΔompC* strains in phosphate-deficient media with 0.05% SDS and varying concentrations of EDTA. 1x10^5 cells from 48-hour overnight cultures were transferred to a 96-well plate and incubated at 37°C for 16 hours. The 0 mM EDTA condition contained neither SDS nor EDTA. Growth was measured at OD_{600} every 10 minutes (n=6).

with the pattern observed in phosphate-sufficient media (Fig. 3A), growth began around the 8 hour mark, steadily increased to 14 hours, and began to plateau by 16 hours. The *ΔompC* strain grown in phosphate-deficient media was able to grow in the presence of 0.05 mM and 0.30 mM EDTA, reaching similar growth densities at 16 hours as the no SDS-EDTA control (Fig. 4B). As shown in Fig. 4B, SDS-EDTA treatment delayed, but did not completely inhibit, the growth of the *ΔompC* strain compared to the no SDS-EDTA control.

In order to assess the SDS-EDTA sensitive or resistant phenotype of the strains in phosphate-sufficient compared to phosphate-deficient media, OD_{600} values at the 16 hour time point were used as measures of growth yield. The 16-hour time point was used to examine the effect as cells had the most time to acclimate to their conditions at this time point in our study. For each condition, the OD_{600} at 16 hours of each SDS-EDTA concentration was divided by the no SDS-EDTA control to determine the relative growth yield (Fig. 5). An unpaired, two-tailed t-test was used to compare the relative growth yields at 16 hours between phosphate-sufficient and phosphate-deficient media, at each concentration of SDS-EDTA. For the wild type strain, there was no difference in relative growth yield in 0.05 mM and 0.30 mM EDTA in phosphate-sufficient compared to phosphate-deficient media. At the highest EDTA concentration of 0.60 mM, growth yield was significantly higher in phosphate-sufficient media. For the *ΔompC* strain, there was a significant difference in relative growth yield between phosphate-sufficient and phosphate-deficient media, with the latter showing a higher relative growth yield. This observation was significant at all three EDTA concentrations tested and indicates that the *ΔompC* strain, which is sensitive to SDS-EDTA treatment under normal phosphate-sufficient conditions becomes resistant when grown in phosphate-deficient media. The difference in relative growth yields between the media decreases with increasing EDTA concentration, due to decreasing growth yield. Taken together, these data suggest that phosphate deficiency restores SDS-EDTA resistance in the *ΔompC* strain.

Phosphate deficiency induces changes in protein expression in wild type, *ΔompC*, and *ΔphoE* strains. SDS-PAGE was performed on whole cell lysates for wild type, *ΔompC*, and *ΔphoE* strains grown in both phosphate-sufficient and phosphate-deficient media in order to analyze differences in protein expression. The objectives were to confirm that OmpC is not detected in the *ΔompC* mutant and to determine if PhoE is induced by growth in phosphate-deficient media. PhoE and OmpC are 38.9 kDa and 40.3 kDa, respectively, and the region of interest on the gel was between the 37-50 kDa markers of the ladder (red box). A distinct

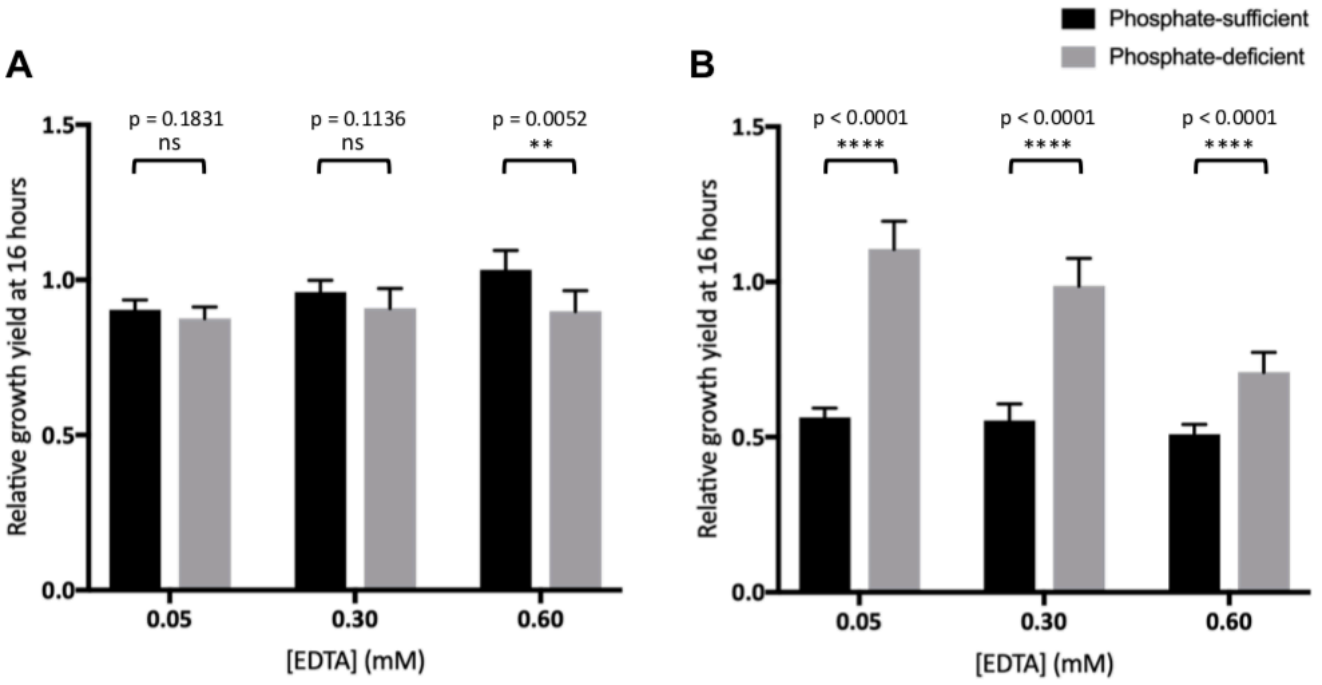

FIG. 5 Relative growth yield at 16 hours of *E. coli* (A) wild type and (B) *ΔompC* strains in phosphate-sufficient media compared to phosphate-deficient media. Relative growth yield was calculated by dividing the OD_{600} at 16 hours of each SDS-EDTA condition by the no SDS-EDTA control. Statistical significance was calculated between phosphate-sufficient and phosphate-deficient media using unpaired, two-tailed t-test (n=6).

band of approximately 40 kDa was observed in both the wild type (lanes 1-2) and *ΔphoE* strains (lanes 5-6) under both phosphate-sufficient and phosphate-deficient conditions, but was absent in *ΔompC* mutants (lanes 3-4) (Fig. 6). This band was consistent with the size of OmpC, and the band pattern across the conditions was consistent with expected OmpC expression. The data show that OmpC is not expressed in the *ΔompC* mutant under both phosphate-sufficient and phosphate-deficient conditions, confirming a knockout phenotype.

Differences in protein expression between the *ΔompC* and*ΔphoE* mutant strains grown under phosphate-deficient conditions were indistinguishable in the same 37 to 50 kDa size range (Fig. 6). However, clear differences were observed in protein expression between phosphate-sufficient and phosphate-deficient media for all three strains (Fig. 6). These results suggest that phosphate deficiency induces changes in global protein expression, which may be involved in maintaining membrane asymmetry and resistance to SDS-EDTA in the absence of OmpC.

DISCUSSION

The outer membrane porin OmpC interacts with the protein MlaA in the Mla pathway to maintain OM asymmetry in Gram-negative bacteria, conferring resistance to SDS-EDTA treatment (5). Studies have demonstrated that *ΔompC* mutants show increased sensitivity to SDS-EDTA compared to wild type counterparts (5). PhoE is another general diffusion porin that is similar in structure and function to OmpC, but is only expressed under phosphate-deficient conditions (7). Based on these observations, we hypothesized that phosphate deficiency would restore SDS-EDTA resistance in *ΔompC* mutants due to the induced expression of PhoE (Fig. 7). We expected that the wild type strain would be resistant to SDS-EDTA treatment in both phosphate-sufficient and phosphate-deficient media (Fig. 7A and 7C). Furthermore, we expected that the *ΔompC* mutant would be sensitive to SDS-EDTA in phosphate-sufficient conditions due to loss of OM integrity (Fig. 7B). We hypothesized that phosphate deficiency would restore resistance due to the induction of *phoE* expression (Fig.

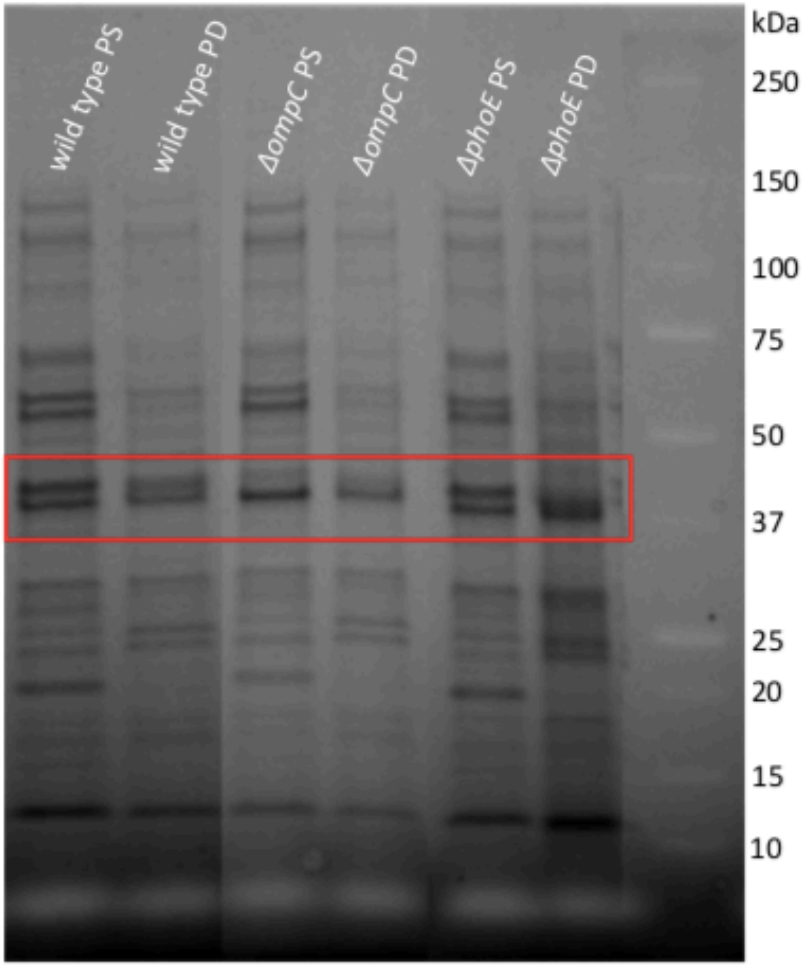

FIG. 6 Whole cell lysates of Wild Type, *ΔompC*, and *ΔphoE* strains grown in phosphate-sufficient (PS) media and phosphate-deficient (PD) media. After 48 hours of growth in respective phosphate media, cell numbers were normalized and cell lysates were subjected to 4-20% SDS-PAGE at 200 V for 30 minutes.

7D). The mechanism of this potential effect and whether the Mla pathway plays a role was not examined in this study.

After confirming viability of the wild type and *ΔompC* strains in minimal media containing various phosphate concentrations, we conducted growth curves to examine the growth patterns. Both wild type and *ΔompC* grew at a faster rate and to a higher density in phosphate-sufficient media compared to phosphate-deficient media (Fig. 2). The delay in growth could be due to the cells acclimating to both the nutrient-limited environment and the cold media that was used to dilute the cells before the assay. Nutrient depletion also occurs earlier in the phosphate-deficient media, accounting for the lower growth yields observed. Growth patterns are comparable between strains in the same media, suggesting that growth behavior does not account for the differences in relative growth yield observed in the SDS-EDTA growth assays.

We confirmed resistance and sensitivity to SDS-EDTA in the wild type and *ΔompC* strains, respectively, in phosphate-sufficient media, as previously shown by Hartstein *et al.* (7). The OD_{600} values at 16 hours for the *ΔompC* strain in all three concentrations of SDS-EDTA reached only half that of the control of no SDS-EDTA (Fig. 3). For the *ΔompC* mutant, we observed a dose-dependent decrease in growth yield that is not observed for the wild type strain, indicating that growth of the *ΔompC* strain in the presence of SDS-EDTA is inhibited compared to the control, while the wild type strain is unaffected.

In phosphate-deficient media, the wild type strain is resistant to SDS-EDTA (Fig. 4). Although growth in SDS-EDTA is delayed compared to the control, the rate of growth and final growth yield at 16 hours is comparable for both strains. Similar to wild type, the growth of the *ΔompC* strain in SDS-EDTA is delayed but reached the same final density at 16 hours. Growth in 0.60 mM EDTA is the most delayed and only starts to increase at the 16-hour cut-

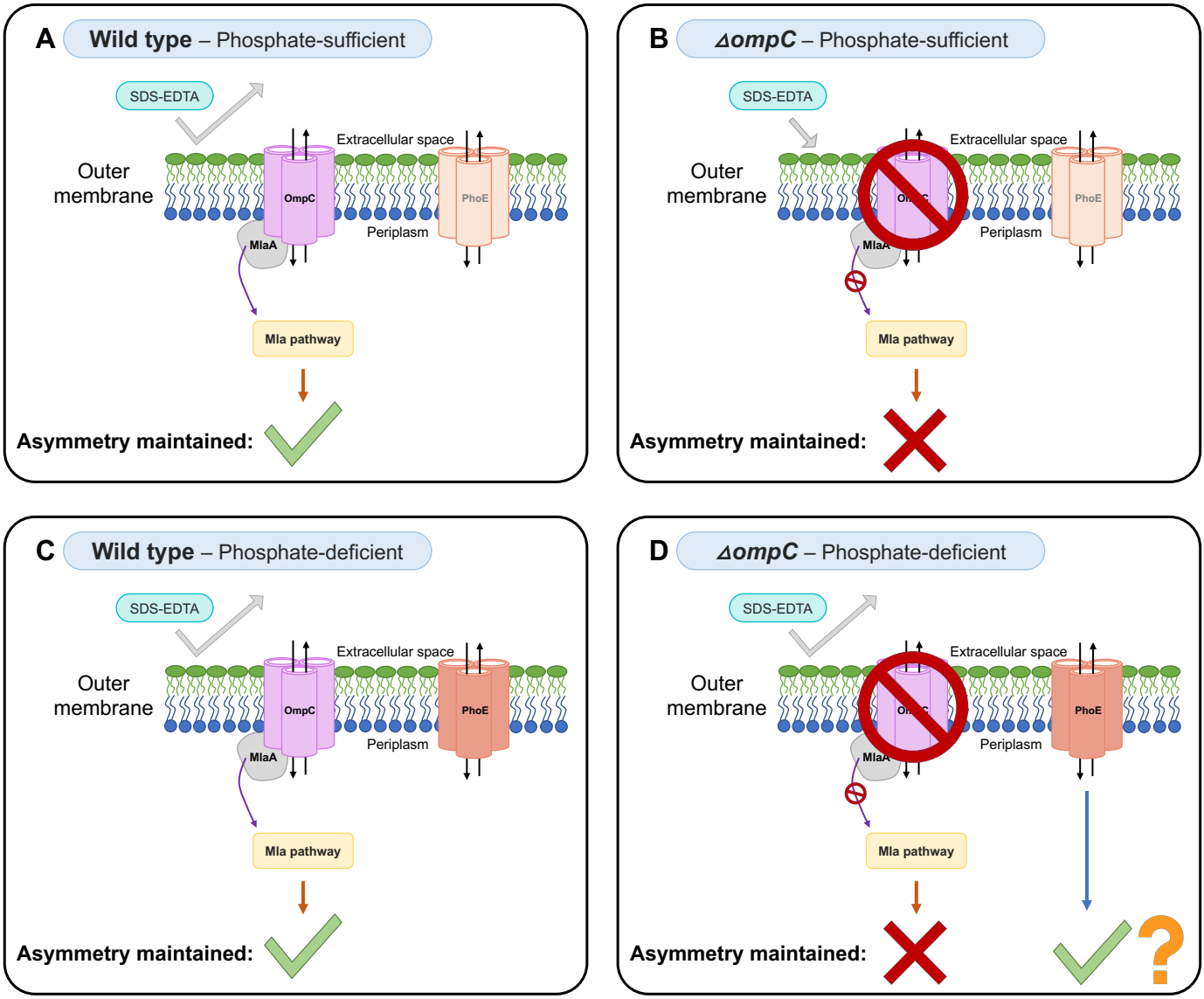

FIG. 7 Proposed mechanism of action of OmpC and PhoE on *E. coli* K-12 membrane asymmetry in phosphate-sufficient and phosphate-deficient conditions. In phosphate- sufficient media, PhoE is not expressed. In phosphate-deficient media, PhoE is expressed. (A and C) OmpC maintains membrane asymmetry in response to external insults through the Mla pathway. (B) Lack of OmpC disrupts membrane asymmetry in response to harmful agents. (D) In the absence of OmpC, PhoE expression restores maintenance of membrane asymmetry through currently unknown methods.

off. This may be due to the combination of a nutrient-limited environment and a high concentration of disrupting agents causing delayed growth.

We compared the relative growth yields at 16 hours by normalizing the growth in SDS-EDTA to the no SDS-EDTA control, thereby accounting for the delayed growth and differences in final optical density between the media (Fig. 5). Figure 5A shows that there is no statistical difference between the growth in phosphate-sufficient compared to phosphate-deficient media for the wild type strain in 0.05 mM and 0.30 mM EDTA. At the highest EDTA concentration, a higher relative growth yield in phosphate-sufficient media is observed. This difference is likely due to the combination of a nutrient-limiting environment and high concentration of EDTA in the media. Relative growth yields for the wild type control are close to 1 in both phosphate-sufficient and phosphate-deficient media, suggesting resistance to SDS-EDTA treatment. In contrast, Figure 5B shows that relative growth yields for the *ΔompC* strain are close to 0.5 in phosphate-sufficient media, indicating sensitivity to

SDS-EDTA treatment. Relative growth yields for *ΔompC* in phosphate-deficient media ranges between 0.7 and 1.2. At each EDTA concentration, there is a significant difference between relative growth yields for the *ΔompC* strain in phosphate-sufficient compared to phosphate-deficient media at 16 hours. Therefore, we concluded that in phosphate-deficient media, the *ΔompC* mutant is resistant and able to grow in the presence SDS-EDTA, compared to inhibited growth in phosphate-sufficient media. A biological replicate of the SDS-EDTA growth assay was conducted and we observed the same trend (Fig. S2).

The results from our SDS-EDTA growth assays do not indicate the responsible factor for the restored resistance under low phosphate conditions. As we hypothesized the involvement of PhoE, we investigated its expression in our *ΔompC* mutant using SDS-PAGE. Using SDS-PAGE, we were able to confirm the lack of OmpC expression in our *ΔompC* mutant (Fig. 6). However, under phosphate-deficient conditions, we were unable to distinguish differences in protein expression between the *ΔompC* and *ΔphoE* mutants (Fig. 6). Distinguishing small differences to determine PhoE expression was challenging as the entire cell lysate was used, thereby displaying the presence of numerous other proteins. As such, the involvement of PhoE in SDS-EDTA resistance in *ΔompC* can neither be confirmed nor denied through this SDS-PAGE. However, the gel does demonstrate clear differences in protein expression for all three strains between phosphate-sufficient and phosphate-deficient media (Fig. 6). These observations suggest a change in cellular protein expression in phosphate-deficient media, which may be involved in restoring SDS-EDTA resistance in the absence of OmpC.

In conclusion, our SDS-EDTA growth assays of *E. coli* K12 wild type and *ΔompC* strains grown in phosphate-sufficient and phosphate-deficient media indicate that phosphate deficiency restores SDS-EDTA resistance in *ΔompC* mutants.

Limitations Due to time and resource constraints, we were unable to confirm the expression of PhoE in phosphate-deficient media using whole cell lysate SDS-PAGE. Whole cell lysates contain many other proteins, making it difficult to compare particular protein expression changes, especially since PhoE and OmpC are similar in size. Additionally, our SDS-EDTA growth assay could not control for other changes in protein expression in phosphate-deficient media, preventing us from identifying the affected components.

Future Directions While our data suggests that phosphate deficiency restores SDS-EDTA resistance in *ΔompC* mutants, the exact mechanism involved remains unknown. We believe that the PhoE porin may be involved. Although an SDS-PAGE experiment of the *E. coli* wild type, *ΔompC,* and *ΔphoE* whole cell lysates grown in both phosphate-sufficient and phosphate-deficient media could not confirm the presence or absence of PhoE, it was demonstrated that phosphate deficiency induces changes in protein expression. These changes in protein expression as a result of phosphate deficiency may be the reason for recovery of SDS-EDTA resistance in *ΔompC* mutants, but we are unable to deduce the exact protein(s) involved. To examine the potential role of PhoE in our model, SDS-PAGE may be repeated while samples are prepared through enrichment of outer membrane proteins. Alternatively, *phoE* expression could be confirmed through more sensitive methods such as RT-qPCR or western blot, provided the availability of an antibody.

Furthermore, due to the delayed growth of wild type and *ΔompC* in both phosphate-sufficient and phosphate-deficient minimal media, the 16 hour incubation time in our SDS-EDTA growth assays provided an incomplete picture of the growth patterns. To acquire a further understanding of growth behavior of the two strains, we suggest repeating the SDS-EDTA growth assays with an incubation time of 30 hours.

One potential process for the restoration of SDS-EDTA resistance in *ΔompC* mutants is that the PhoE porin may be involved by interacting with the Mla pathway in order to maintain membrane asymmetry in the absence of OmpC. PhoE expressed under phosphate limiting conditions could be interacting with MlaA of the Mla pathway analogous to OmpC (8). In wild type *E. coli*, OmpC interacts with MlaA to form a complex that anchors MlaA to the OM (6). It has been proposed that the OmpC-MlaA complex may maintain membrane asymmetry by removing phospholipids from the outer leaflet, implicating a function for OmpC in the transport of lipids (6). Due to similar primary amino acid sequences and protein structures between OmpC and PhoE, one potential model is that PhoE can also interact with

MlaA and anchor it to the OM (8). PhoE can then restore resistance to SDS-EDTA by maintaining OM asymmetry under phosphate-deficient conditions in *ΔompC* mutants. One study by Nogami *et al.* demonstrated that *ΔompCΔompF* double mutants are sensitive to lysis by Tris-HCl treatment, whereas strains expressing PhoE are as stable as wild type, suggesting a possible role for PhoE in membrane integrity (12). It should be noted that our results do not demonstrate that PhoE is involved in the Mla pathway. To test its potential role, *E. coli ΔompC* mutants in phosphate-deficient media should be exposed to concentrations of 0.80-1.0 mM EDTA since *ΔmlaA* mutants are not sensitive to EDTA below 0.80 mM (6).

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