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Phosphate deficiency confers transient SDS-EDTA resistance in *Escherichia coli* K12 *ompC* knockout mutant

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SUMMARY OmpC is a general diffusion protein located in the outer membrane (OM) of gram-negative bacteria. It is known to associate with components of the Mla pathway to facilitate OM lipid asymmetry, which protects against harmful external stressors such as antibiotics and detergents. It has been demonstrated that *Escherichia coli* Δ*ompC* mutants are more sensitive to sodium dodecyl sulfate-ethylenediaminetetraacetic acid (SDS-EDTA) treatment than the wild type strain. PhoE is another general diffusion protein that shares structural similarity with OmpC. While *ompC* is constitutively expressed, *phoE i*s thought to be upregulated in phosphate limiting conditions. Phosphate deficiency in minimal media has been shown to restore SDS-EDTA resistance in Δ*ompC* strains over a period of 16 hours and is thought to be attributed to the upregulation of *phoE.* However, it has also been found that phosphate deficiency can hamper growth, and that passaging cells in phosphate limiting media helps mitigate this issue. This study investigated changes to the resistance of *E. coli* Δ*ompC* mutants to SDS-EDTA under phosphate sufficient and deficient conditions when grown until the end of stationary phase, as well as the effects of serially passaging wild type and Δ*ompC* mutants in phosphate deficient and sufficient media. We hypothesized that SDS-EDTA resistance would be observed in Δ*ompC* mutants. We also hypothesized that adaptation of cells to the phosphate limiting minimal media would decrease the length of the lag phase. To test this, we serially passaged wild type and Δ*ompC* strains in phosphate sufficient and phosphate deficient media, and then performed SDS-EDTA growth assays. We found that serially passaging the strains a minimum of 4 times in phosphate sufficient or deficient media resulted in a decreased lag phase, higher growth rates, and higher growth yields for both Δ*ompC* mutants and wild type cells compared to non-passaged cells. Additionally, we replicated the previously established findings that Δ*ompC* mutants grow better in SDS-EDTA during exponential phase than the wild type, but this resistant phenotype was not observed in the transition to stationary phase. On the basis of our results and those of other studies, it is possible that *phoE* may be upregulated upon primary exposure to SDS-EDTA in *E. coli* cells lacking *ompC,* after adaptation to phosphate deficient media. However, SDS-EDTA resistance appear to be transient and there may be several other components influencing the resistant phenotype.

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INTRODUCTION

The outer membrane (OM) is integral to the survival of certain gram-negative bacteria, such as *Escherichia coli*, because it provides a protective barrier against harmful external insults such as antibiotics, detergents and other toxic compounds (1). The OM is an asymmetric lipid bilayer composed of lipopolysaccharides (LPS) on the outer leaflet and phospholipids (PLs) on the inner leaflet (2). The Mla pathway is important in maintaining lipid asymmetry, and studies have shown that the lipoprotein MlaA associates with the

osmoporin OmpC at the OM in order to remove outer leaflet PLs from the OM (3). It has been found that Δ*mlaA E. coli* mutants are sensitive to sodium dodecyl sulfate-ethylenediaminetetraacetic (SDS-EDTA) treatment, and Δ*ompC* mutants may show a similar phenotype (4). In one proposed mechanism, EDTA chelates divalent cations, which neutralize the repulsive negative forces between phosphate residues in LPS. Phosphate repulsions in LPS destabilize the membrane, allowing PLs to enter the outer leaflet (4). In wild type *E. coli*, EDTA-mediated membrane perturbation is thought to be reversed by OmpC and the Mla pathway (4). Membrane packing of LPS may be disrupted to such an extent that sodium dodecyl sulfate (SDS) can enter the cell via gaps in the lipid bilayer, although these two phenomena are not found to be directly correlated (4). SDS is an amphipathic surfactant that denatures proteins by unfolding the tertiary structure (5). The interactions between the hydrophobic tail of SDS and the interior regions of the protein normally buried away disrupt the hydrophobic effect and lead to denaturation and cell death (5). It is suggested that Δ*ompC E. coli* mutants may not be able to maintain membrane asymmetry, which could render them more sensitive to treatment with SDS and EDTA (4).

There are many other osmoporins in the OM, one of which is PhoE, another general diffusion protein with a similar structure to OmpC (6). However, unlike OmpC, PhoE is supposedly upregulated under phosphate limiting conditions or when exposed to osmotic shock, where it has been shown to fold into a hexagonal lattice and associate with LPS molecules in the OM (6). Some groups have further suggested that in cells lacking OmpC, the presence of PhoE may be able to compensate for its function in maintaining OM asymmetry (7).

Phosphate limiting conditions can also delay, and perhaps hinder the growth of *E. coli* cells. In another study, when cells were incubated in media deprived of phosphate for 15 hours, they were found unable to grow normally when inoculated into fresh, defined media (8). It has been suggested that phosphate deficiency may downregulate certain components of the cell machinery necessary for division (8). Studies have also shown that *E. coli* can be adapted to minimal media by serial passaging, a method which can increase growth rates and decrease lag phases of the cells (9, 10).

Boen *et al.* have demonstrated resistance to SDS-EDTA in Δ*ompC E. coli* cells grown in phosphate deficient media (11). They suggested that this resistant phenotype is due to the upregulation of *phoE* (11). However, one limitation of the study was that they were only able to incubate cells in SDS-EDTA until the exponential/early stationary phase (after 16 hours), to which they have noted may not provide a complete picture of the growth patterns of this strain (11). Boen *et al.* also noted delayed growth responses of both the wildtype and Δ*ompC* mutants in both phosphate sufficient and deficient media, but have not yet attempted adaptation experiments to try to resolve this.

We hypothesized that SDS-EDTA resistance would be maintained over the course of 22 hours in Δ*ompC* mutants in phosphate-deficient media, and that serial passaging of this strain in phosphate limiting media would shorten the lag phase of the bacterial growth curve. To test this, we modified the SDS-EDTA growth assay of Boen *et al.* to include adapted strains that had been serially passaged a minimum of 4 times in either phosphate deficient or sufficient media, and those that had not been adapted. Cells were then grown in their respective media in the presence of SDS with or without EDTA for 22 hours, while recording optical density (OD_{600}) and interpreting this as a measure of growth. We compared growth rates and yields of both our wild type and Δ*ompC* mutant *E. coli* strains, which showed novel changes in relative growth yield upon longer incubation with SDS-EDTA than what has been previously reported.

METHODS AND MATERIALS

Bacterial strains. *E. coli* K12 strain BW25113 (hereafter referred to as wild type), strain JW2203-1 (ΔompC), and strain JW2203-2 (ΔphoE) were obtained from the CGSC Keio strain collection (11). For storage purposes, the wildtype, Δ*ompC*, and Δ*phoE* strains were propagated on Lysogeny Broth (LB) agar plates at 37°C, and media for the latter two strains was supplemented with 100 µg/mL of kanamycin. Growth data of the Δ*phoE* strain was not considered in our experiments.

Validation of wildtype, ΔompC, and ΔphoE strains. Polymerase chain reaction (PCR) was performed on *E. coli* K-12 wild type, and mutant strains Δ*ompC* and Δ*phoE* strains. Genomic DNA was extracted from each strain using the Invitrogen™ PureLinkTM Genomic DNA Mini Kit (Cat no. K1820-01). Primers at least 100 bp outside of the *phoE* and *ompC* genes were designed by Boen *et al.* (11) (Table S1). The primers were used to amplify the wildtype gene, as well as the kanamycin resistance cassettes in the mutant strains. Amplification of the pUC19 plasmid was used as a positive control. In each reaction tube, 50 ng of genomic DNA, 0.2 µM of the forward and reverse primers, 0.2 µM dNTP mixture, 1X PCR Buffer-Mg, 1.5 mM $MgCl_2$, and 2 units per reaction Invitrogen™ Platinum™ Taq DNA Polymerase were combined with autoclaved dH_20 in a total volume of 50 µL. The *ompC* PCR protocol was performed as follows: initial denaturation at 94°C for 3 min, followed by 35 cycles of denaturation at 94°C for 45 seconds, annealing at 56 °C for 30 seconds, extension at 72°C for 3 minutes, and a final extension of 72°C for 10 min. The *phoE* PCR protocol followed the same parameters as *ompC*, with the exception of a 62°C annealing temperature for *phoE*, and a 56°C annealing temperature for the positive control reaction.

All PCR products combined with 6X DNA loading dye, and a 1 kB ladder Plus DNA ladder from Thermo Fisher Scientific, were run on a 1% (w/v) agarose gel containing SYBR™ Safe in 0.5X Tris/Borate/EDTA buffer for 60 minutes at 110V. The gel was visualized via UV exposure using the AlphaImager® EC (Alpha Innotech 105 Corporation). All PCR products were purified using the PureLink® PCR Purification Kit and sent to GeneWiz for Sanger sequencing. The resulting sequences were analyzed using the 4Peaks and SerialCloner (2.6.1) software and the NCBI BLAST®n search tool to confirm the presence of the wild type genes and mutant kanamycin resistance cassettes.

Preparation of phosphate defined media. Phosphate defined media was prepared using the protocol defined by Boen *et al.* (11). The following components were combined and dissolved in 1 L of distilled water to prepare the minimal media base with final concentrations of: 0.12 M HEPES, 0.08 M NaCl, 0.02 M KCl, 0.02 M NH4Cl, 3 mM Na2SO4, 1 mM MgCl2 • 6H2O, 0.2 mM CaCl2 • 2H2O, 3.3 µM FeCl3, and 0.1 M glucose. HEPES buffer was adjusted to pH 7.42 following the protocol outlined by Boen *et al.* (11). The base media was filter-sterilized through a 0.22 µm filter using a vacuum filter apparatus and stored at 4°C. A 1000x stock solution of thiamine in distilled water was also filter-sterilized using a 0.22 µm filter and stored in the dark at 4°C, and was added to the base media immediately prior to inoculation for a final concentration of 3.8 µM. A sterile 10x solution of K_2HPO_4 was also prepared by filter-sterilization in a similar manner and added to the base media for a final concentration of 660 µM for phosphate sufficient media, and 42 µM for phosphate deficient media.

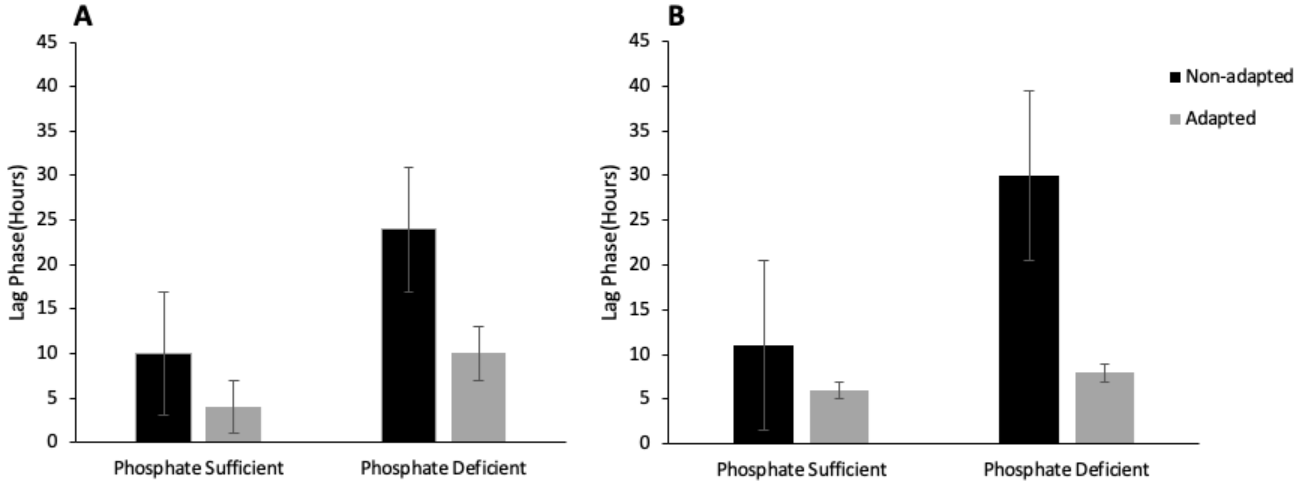

FIG. 1 Lag phase comparison between non-adapted and adapted *E.coli* (A) wild type and (B) *ΔompC* mutants in phosphate sufficient and deficient media. 10^6 cells, either adapted or 10^5 cells from 48-hour starter cultures (non-adapted), were grown in the presence of 0.05% SDS at 37°C in a 96-well plate. Data represent lag phase time in hours with standard error bars (n=3).

Serial passaging of wild type and ΔompC strains (adaptation growth experiment). Wild type and Δ*ompC* colonies from LB plates were inoculated in 3 mL cultures of phosphate sufficient and deficient media in duplicate and incubated at 37°C and 200 rpm. Once the cultures reached a minimum of OD_{600} of 0.6 within a 48-hour time period, 45 µL of the previous phosphate sufficient and 30 µL of the phosphate deficient cultures were seeded into 3 mL of fresh media with the corresponding amounts of phosphate. Adapted strains were defined as having been serially passaged a minimum of 4 times. As a control, non-adapted cells were inoculated into starter cultures with 3 mL of phosphate sufficient and deficient media from colonies on LB agar plates incubated for 48 hours at 37°C and 200 rpm. 10^6 cells of adapted and 10^5 cells of non-adapted cells were then inoculated into a 96-well plate containing either phosphate deficient or phosphate sufficient media and 0.05% SDS in triplicate, in a total volume of 250 µL. The 96-well plate was incubated at 37°C in a BioTek Plate Reader and OD_{600} readings were taken every 10 minutes for 22 hours. All media for the mutant strain Δ*ompC* was supplemented with 100 µg/mL of kanamycin.

SDS-EDTA growth assay. A 5 mM EDTA stock solution (pH adjusted to 8.0) and 1 % w/v stock solution of SDS were prepared separately in distilled water. Both solutions were filter-sterilized using a 0.22 µm filter and stored at room temperature. Wild type and Δ*ompC* strains were serially passaged a minimum of 4 times in phosphate sufficient and deficient media as described above prior to the SDS-EDTA growth assay. Approximately 10^6 cells from phosphate sufficient and deficient starter cultures were inoculated in triplicate into wells containing their respective media as well as 0.05 % SDS and either 0 mM or 0.3 mM EDTA in a final volume of 250 µL. Inoculum sizes were determined by converting OD_{600} readings to the expected number of cells present based on the assumption that a measurement of 1 OD corresponds to 10^8 bacterial cells. Growth in media without EDTA was used as a control. All media for the Δ*ompC* strain was supplemented with 100 µg/mL of kanamycin. The 96-well plate was incubated in a BioTek Plate reader at 37°C for 22 hours and OD_{600} readings were taken every 10 minutes. We performed an additional SDS-EDTA growth assay using cells that were not passaged in phosphate sufficient and deficient media (Fig. S2). $1x10^5$ cells from starter cultures grown for 48 hours prior to the growth assay were inoculated in a 96-well plate containing media as described earlier and incubated for 48 hours at 37°C to investigate the possibility of sustained resistance to SDS-EDTA during stationary phase. OD_{600} readings were taken every 10 minutes for 48 hours (n=3).

Statistical tests and data analysis. Unpaired two-tailed t-tests were used to calculate statistical significance. This is pertinent for comparing OD_{600} values at 22 hours between adapted and non-adapted cells in both phosphate sufficient and phosphate deficient media for wild type and Δ*ompC* mutants (Fig. 2), as well as for growth yield analysis between growth in phosphate sufficient and deficient media for both strains at different time points (Fig. 5). In addition, the length of the lag phase in hours was compared quantitatively with a bar graph (Fig. 1) of the non-serially passaged and serially passaged cells, because this phase differed nearly 3-fold when analyzing the growth curves qualitatively. Growth rate constants of the adapted and non-adapted strains were calculated when grown in both phosphate sufficient and phosphate deficient media (Table 1). Growth rate constants represent the rate of growth during exponential phase. They were calculated by subtracting the initial OD_{600} from the final OD_{600} over the time duration of the exponential phase.

RESULTS

Genotype validation of wild type, Δ*ompC*, and Δ*phoE* strains. The Δ*ompC* and Δ*phoE* strains, obtained from the *E. coli* Keio collection, have kanamycin resistance cassettes in place of *ompC* and *phoE*, respectively (12). The PCR product lengths of the mutant strains were expected to be greater than the wild type as the kanamycin resistance cassette is longer (1300 bp) compared to the *ompC* (1104 bp) and *phoE* (1062 bp) genes. PCR product lengths of Δ*ompC* (1671 bp), Δ*phoE* (1850 bp), and wildtype *ompC* (1448 bp) and *phoE* (1579 bp) were all expected. The expected band sizes for all reactions was observed using gel electrophoresis

TABLE 1. Growth rate constants of non-adapted and adapted *E.coli* wild type and Δ*ompC* cells in phosphate sufficient and deficient media.

		Wild type	Δ*ompC*
Condition	**Type**	**Rate (Hour^{-1})**	**Rate (Hour^{-1})**
Phosphate Sufficient	Non-adapted	0.11	0.10
	Adapted	0.17	0.16
Phosphate Deficient	Non-adapted	0.14	0.19
	Adapted	0.18	0.21

analysis (Fig. S1). The negative control, lacking template, resulted in no amplification, suggesting no DNA contamination occurred (Fig. S1). In addition, all PCR products produced one distinct band, indicating an absence of any non-specific amplification or primer dimers.

The identity of each amplicon was confirmed using Sanger sequencing and BLAST® analysis. The wild type *ompC* and *phoE* amplicons aligned to their respective genes. Both PCR products of the mutant strains aligned with the pKD13 plasmid containing the kanamycin resistance cassette. Overall, our gel electrophoresis and sequencing results confirmed the presence of the kanamycin resistance cassettes in the Δ*ompC* and Δ*phoE* strains, and the presence of the *ompC* and *phoE* genes within the wild type strain.

Serial passaging decreases lag phase and increases growth rates and yields. After performing the SDS-EDTA growth assay on wild type and Δ*ompC* cells grown for only 48 hours in minimal media prior to the assay, we observed long lag phases and low final OD_{600} values (Fig. S2). Compared to the 6 and 8-hour lag phases observed by Boen *et al.* (11) in phosphate sufficient and deficient media respectively, our lag phases were longer, lasting for approximately 12 and 24 hours respectively. Moreover, their cells were able to reach an OD_{600} of 0.45 in phosphate sufficient media (11) whereas we observed a maximum OD_{600} of ~0.35 (Fig. S2). We therefore decided to investigate whether adding a higher inoculum size would decrease lag phase times. We initially performed growth curve analysis using a total of 10^9

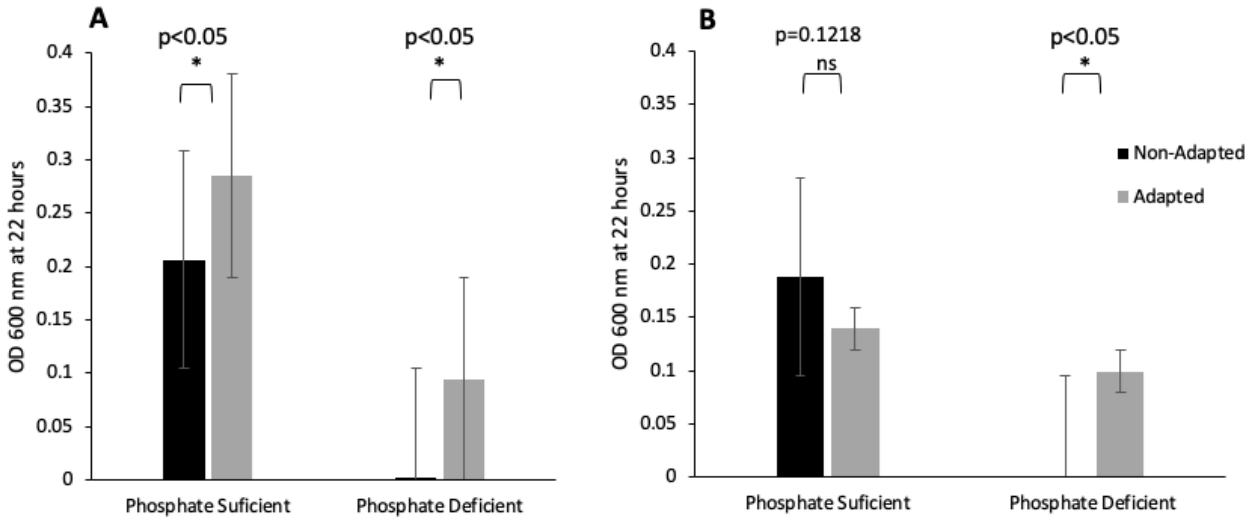

FIG. 2 Growth yield comparison between non-adapted and adapted *E.coli* (A) wild type and (B) *ΔompC* mutant in phosphate sufficient and deficient media at 22 hours. 10^9 cells, either adapted or 10^9 cells from 48 hour starter cultures (non-adapted), were grown in the presence of 0.05% SDS at 37°C in a 96-well plate. Data represents OD_{600} at 22 hours with statistical differences calculated using unpaired two-tailed t-test.

cells and observed similarly long lag phases of ~12- 24 hours, (data not shown) where we seeded 10^9 cells into test tubes containing 3 mL phosphate sufficient and deficient media. OD_{600} values were measured every 12 hours until an OD_{600} of ~0.6. Next, we decided to investigate whether serially passaging the cells in phosphate sufficient and deficient media prior to performing the growth assay would allow the strains to adapt to the minimal media conditions and decrease lag phases. Accordingly, we began by serially passaging the wild type and Δ*ompC* cells in phosphate sufficient and deficient media. Based on the experiments performed by LaCroix *et al*. (9), as well as the time allowances of our study, we chose to passage cells at least 4 times over the course of 2 weeks, and then performed the growth curve analysis again. Using inoculum sizes of 10^6 and 10^5 cells for adapted and non-adapted strains, respectively, we measured differences in lag phase, growth rates, and growth yields of adapted versus non-adapted cells grown in the absence of EDTA. We defined these serially passaged cells as adapted cells. We observed that adapted cells needed to be incubated for ~30 hours to grow to an OD_{600} of ~0.6 prior to the SDS-EDTA growth assay compared to the 48 hours required for the non-adapted cells (data not shown).

Figure 1 depicts the lag phase duration of non-adapted and adapted wild type and Δ*ompC* cells when grown under phosphate sufficient and deficient conditions. Lag phase duration of the wild type strain passaged in phosphate sufficient and deficient media decreased by 6 and 14 hours respectively, compared to the non-adapted wild type. Similarly, serially passaging of Δ*ompC* cells resulted in a 5-hour decrease in lag phase time under phosphate sufficient conditions. The difference in lag phase time was more pronounced under phosphate deficient conditions, as it decreased by 22 hours after passaging. These results indicate that serially passaging decreases lag phase.

Table 1 depicts growth rate constants (μ) of adapted and non-adapted wild type and Δ*ompC* mutants when grown in phosphate sufficient and deficient media. The growth rate constants are represented by the slope of the exponential phase for each strain grown under each condition. We observed that adapted cells grew to a greater cell concentration per hour compared to non-adapted cells under both phosphate conditions. Our results indicate that adapted wild type and Δ*ompC* cells grow faster than non-adapted cells.

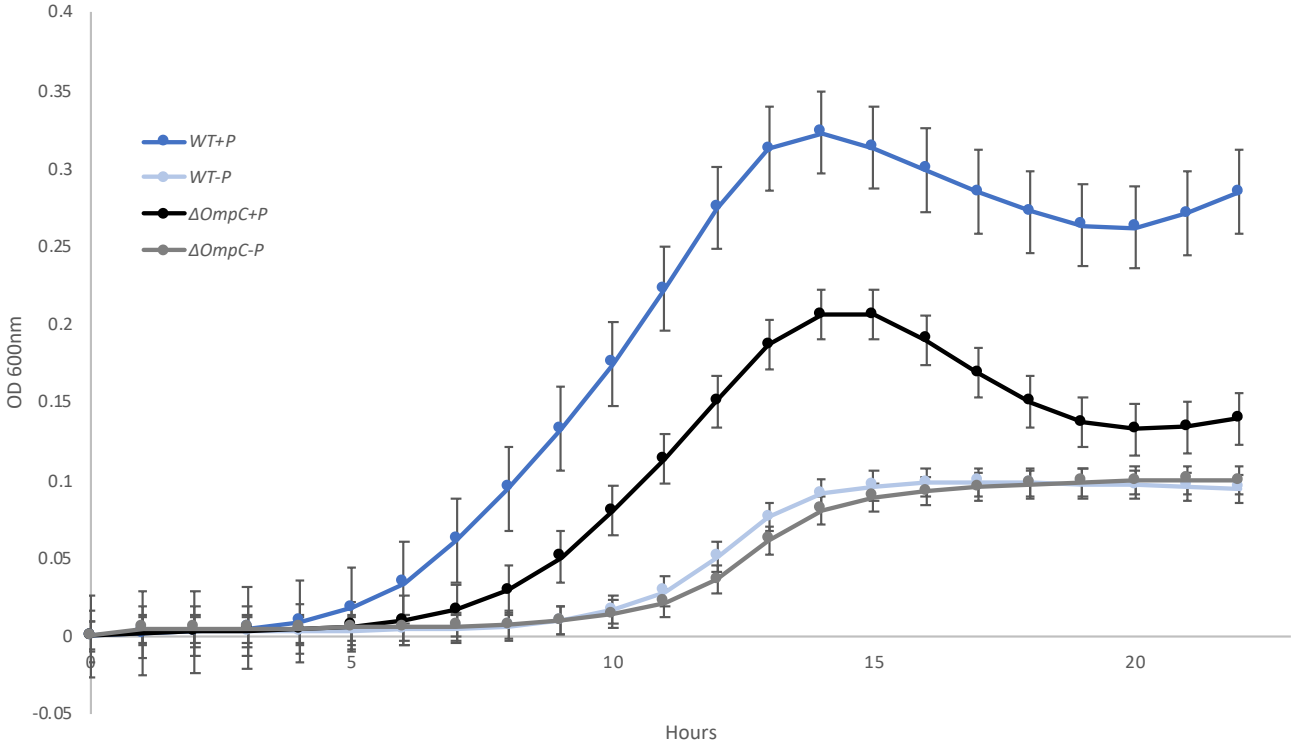

FIG. 3 Growth curve of wild type (WT) and *ΔompC* cells in phosphate sufficient (+P) and deficient (-P) conditions. 10^6 cells from starter cultures grown for 30 hours were seeded in a 96 well plate and incubated at 37°C for 22 hours. OD_{600} readings were taken every 10 minutes to generate the curves. Error bars represent standard error of technical replicates (n=3).

Figure 2 compares the OD_{600} values at 22 hours between adapted and non-adapted cells in phosphate sufficient and deficient media. For wild type cells, we observed a 1.4-fold and 45-fold increase in optical density when grown in phosphate sufficient and deficient conditions, respectively. Similarly, we observed a significant 71-fold increase in optical density after adaptation of Δ*ompC* cells were grown in phosphate deficient conditions. In contrast, non-adapted Δ*ompC* cells grew to a higher optical density compared to the adapted cells in phosphate sufficient media. Taken together, our results indicate that adapted cells grow to higher growth yields compared to non-adapted when inoculum sizes of 10^6 are used.

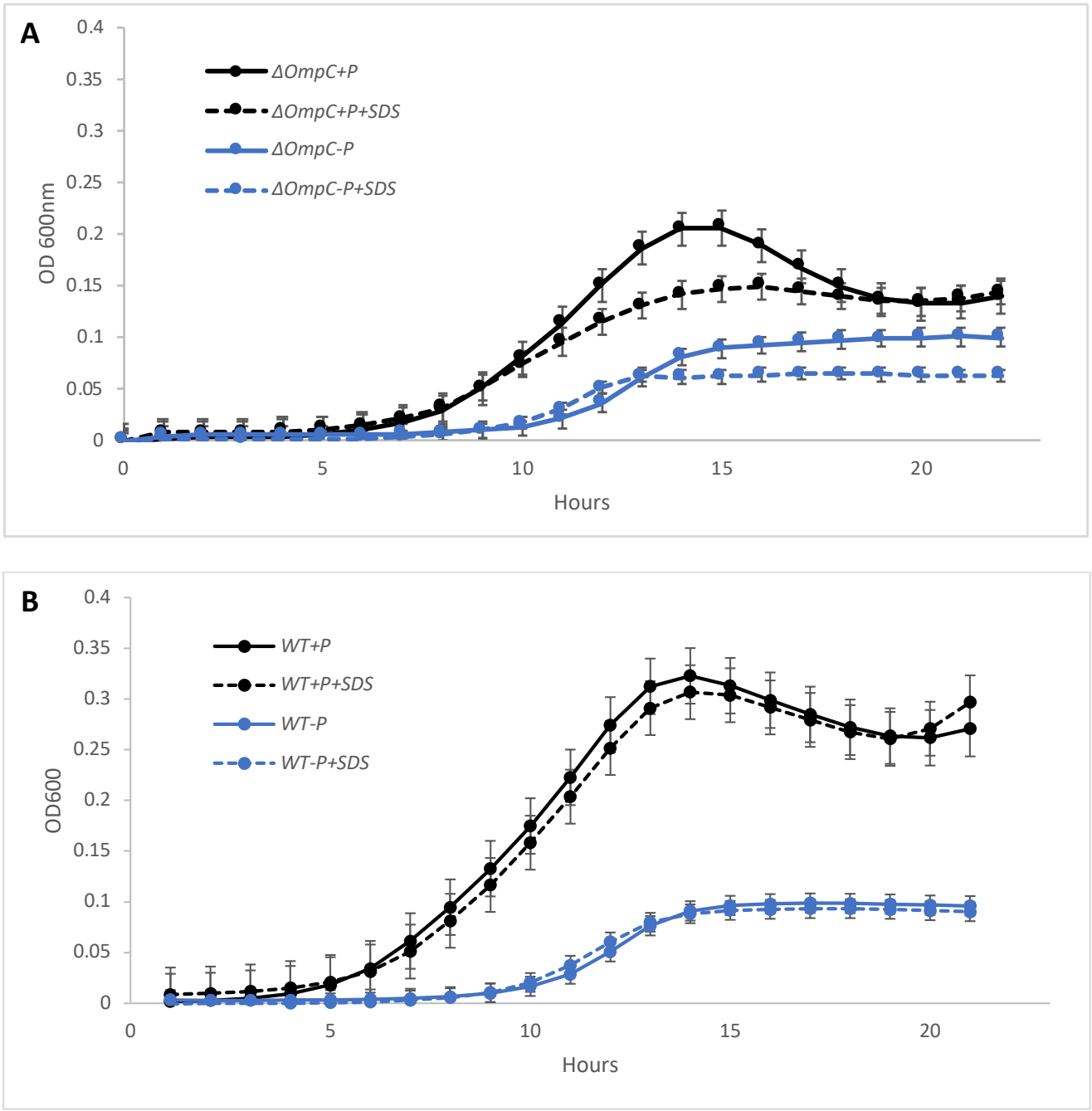

FIG. 4 SDS-EDTA growth assay for (A) wild type (WT) and (B) *ΔompC* cells in phosphate sufficient (+P) and deficient (-P) conditions. 10^6 cells from 30-hour starter cultures were seeded into a 96 well plate and incubated at 37°C for 22 hours. 0.05 % SDS and 0.3 mM EDTA were added to the test conditions and no EDTA was added to the control conditions. Growth was measured every 10 minutes to generate the curves. Error bars represent standard error of technical replicates (n=3).

Δ*ompC* grew similar to wild type in phosphate deficient media but not in phosphate sufficient media. In order to look at the baseline growth of the wild type and Δ*ompC* strains in both phosphate sufficient and phosphate deficient conditions prior to subjecting the cells to EDTA, we measured growth by optical density readings. Figure 3 shows that both strains grew with shorter lag phases and had a higher final growth yield in phosphate sufficient conditions compared to phosphate deficient conditions, which was expected. Relative to the wild type strain, the Δ*ompC* mutant strain had a greater lag phase and lower maximum optical density value in phosphate sufficient media, but the overall trend of growth is similar to the wild type strain. In contrast, in phosphate-deficient conditions, we see similar growth patterns in Δ*ompC* compared to wild type cells. However, as the Δ*ompC* mutants were grown in sublethal levels of kanamycin, we cannot conclude that growth patterns observed are directly attributed to *ompC* expression. These results suggest the absence of *ompC* may affect growth in phosphate sufficient conditions, but not in phosphate deficient conditions.

Phosphate deficiency transiently restores SDS-EDTA resistance in Δ*ompC* mutant *E. coli*. To test whether SDS-EDTA impacts cell growth due to a gene deletion of *ompC*, optical density measurements were taken. The control wild type strain in Figure 4A shows that the addition of SDS-EDTA in both phosphate sufficient and deficient conditions resulted in similar growth patterns. We conclude that the wild type strain is not sensitive to SDS-EDTA, at the concentrations tested in this study. The mutant Δ*ompC* in SDS-EDTA in phosphate sufficient conditions grew to a similar final optical density compared to the untreated (i.e. no EDTA) control (Fig. 4B). In contrast, the Δ*ompC* strain did not sustain resistance in phosphate deficient conditions in the presence of EDTA after 13 hours. The Δ*ompC* mutant also grew to a lower final OD_{600} value in phosphate deficient conditions with SDS-EDTA compared to the untreated (i.e. no EDTA) control, indicating sensitivity to EDTA. These results indicate that Δ*ompC* growth is affected by the addition of EDTA in phosphate sufficient and deficient conditions, when SDS is present.

In order to test whether phosphate deficient conditions restore SDS-EDTA resistance in Δ*ompC* cells over an extended period of time, we measured the relative growth yield of cells at different phases of bacterial growth across a 22-hour incubation period. Relative growth yields for cells in phosphate sufficient and deficient conditions were calculated by dividing the optical density value obtained with the SDS-EDTA culture by the optical density value for the corresponding untreated (i.e. no EDTA) culture at the same time-point. Optical density readings were taken during exponential phase, immediately after exponential phase, and at 2 time points during stationary phase (Fig. 5). An unpaired, two-tailed t-test was used to compare the relative growth yields at each of the time points between phosphate sufficient and phosphate deficient SDS-EDTA cultures for each strain.

In all phases of growth, the wild type strain was not sensitive to SDS-EDTA, as indicated by the relative growth yield values ($p>0.05$) (Fig. 5A-D). In contrast, the Δ*ompC* strain showed varied relative growth yields depending on growth phase. The Δ*ompC* relative growth yield in phosphate deficient media compared to phosphate sufficient media was significantly higher ($p<0.05$) during exponential phase (Fig. 5A), indicating that phosphate deficiency may confer SDS-EDTA resistance after 12 hours of growth. There was no significant difference in relative growth yields after 13 hours (Fig. 5B). However, the relative growth yields of Δ*ompC* are similar ($p>0.05$) in both phosphate sufficient and deficient media in stationary phase at 14.5 hours (Fig. 5C). Furthermore, at the second point of stationary phase taken at 19.5 hours, we observed a slightly greater ($p>0.05$) relative growth yield in phosphate sufficient conditions as compared to phosphate deficient conditions in the first two time points (Fig. 5D). This data suggests that phosphate deficiency was able to confer SDS-EDTA resistance in Δ*ompC* during exponential phase, but resistance was not sustained throughout stationary phase.

DISCUSSION

The osmoporin OmpC is known to interact with MlaA and maintain OM asymmetry by facilitating the removal of PLs from the outer leaflet of the OM in gram-negative bacteria (Fig. S3A) (1, 4). Due to its structural similarity to OmpC, it has been suggested that the

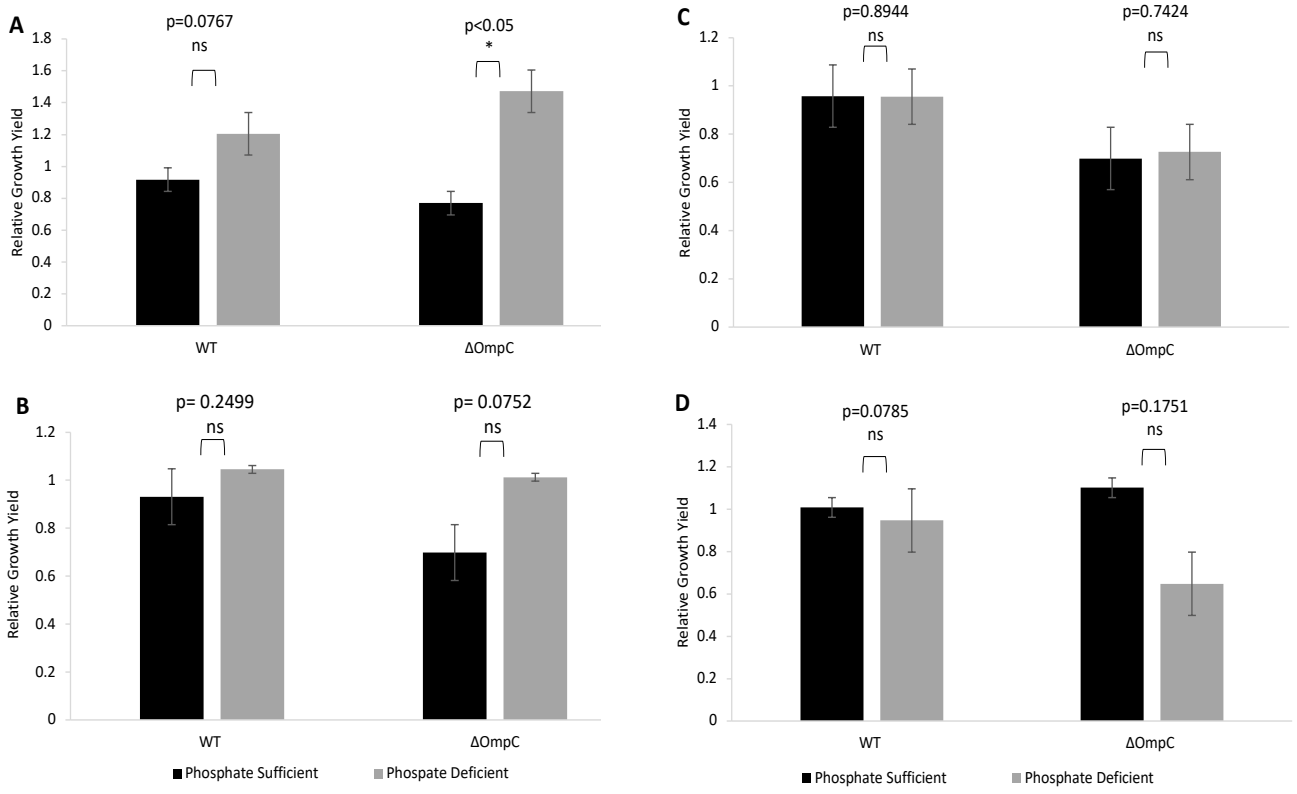

FIG. 5 Relative growth yields at (A) 12 hours (B) 13 hours (C) 14.5 hours (D) 19.5 hours for wild type (WT) and Δ*ompC* strains in phosphate sufficient compared to phosphate deficient conditions with SDS-EDTA. Relative growth yields were calculated by dividing the OD_{600} value at each time point for the addition of EDTA by the no-EDTA control. Statistical significance was calculated between the phosphate sufficient and deficient conditions using an unpaired, two tailed, t-test (n=3).

osmoporin PhoE may play an analogous role in *E. coli* (7, 11). PhoE is upregulated in phosphate limiting conditions and has been shown to associate with LPS in the OM (6). Some groups have hypothesized that PhoE may be able to compensate for OmpC in *E. coli* when *ompC* has been deleted in phosphate limiting conditions to maintain membrane asymmetry (7, 11). Additionally, phosphate limiting conditions alter global protein expression in *E. coli* (13), as well as their growth patterns (8). Our study aimed to determine whether Δ*ompC* cells exhibit sustained resistance to SDS-EDTA in phosphate limiting conditions, and whether serially passaging cells in phosphate limiting media would influence their growth patterns.

Phosphate deficiency transiently restores SDS-EDTA resistance in Δ*ompC* strains. To determine the effects of phosphate deficiency on SDS-EDTA resistance in Δ*ompC* strains over the entire growth period until the lag phase, we constructed growth curves of adapted wild type and Δ*ompC* strains in phosphate deficient and sufficient media without EDTA. As expected, wild type cells in both phosphate deficient and sufficient media showed an almost identical growth pattern in conditions with and without EDTA, indicating that the cells were not affected by the addition of EDTA (Fig. 4A). This is consistent with previous findings (11), and likely due to *ompC* expression, which has been shown to maintain OM asymmetry and thus offer protection against EDTA entry into the cells (4). The decreased growth yield and longer lag phase of the wild type cells in phosphate deficient media relative to phosphate sufficient media was expected, as phosphate limitation is a stressor and nutrient depletion likely occurs more quickly in these conditions (8). In contrast, we did not observe the expected growth patterns between SDS-EDTA and untreated (EDTA-free) conditions for the Δ*ompC* strain (Fig. 4B). Although Δ*ompC* in phosphate sufficient conditions without EDTA reached a higher maximal optical density, the final growth yield of the cells with and without EDTA appeared to be similar. This result was compelling, as Δ*ompC* in phosphate sufficient

conditions has previously only been shown to be susceptible to SDS-EDTA up until exponential phase (11). Our results indicate that Δ*ompC* does not exhibit susceptibility to SDS-EDTA after exponential phase.

Interestingly, Δ*ompC* mutants showed a higher growth yield during the exponential phase relative to wild type in phosphate deficient conditions. This was also observed in non-adapted cells (Fig. S2). To examine this observation further, we calculated relative growth yields at multiple time points. The Δ*ompC* strain had a significantly higher relative growth yield in phosphate deficient media during exponential phase (after 12 hours), indicating resistance to SDS-EDTA (Fig. 5A). These findings were consistent with previous findings (11). However, this resistant phenotype was not sustained after the exponential phase (12 hours) between the growth curves of the Δ*ompC* strain grown in phosphate deficient conditions, when comparing the SDS-EDTA treated cells with cells treated with only SDS (Fig. 5B-D). This was also not previously measured.

It has been shown that the accumulation of PLs in the OM occurs more frequently in Δ*ompC* cells in stationary phase (4), thus contributing to increased OM asymmetry. This could explain why SDS-EDTA may have been able to enter the disrupted membrane and thus, resistance was not sustained in stationary phase in our experiment. Additionally, phosphate starvation has been shown to alter global protein expression in *E. coli* cells (13). VanBogelen *et al.* found that only 10% of the protein-expressing genes in the genome are responsive to growth under phosphate deficiency (13). Moreover, initial adaptation results largely in an upregulation of gene expression, while later adaptation in stationary phase primarily constitutes repression of gene expression (13). As mentioned earlier, PhoE is similar in protein structure to OmpC and as such, PhoE may contribute to SDS-EDTA resistance in phosphate deficient conditions, when it is hypothesized to be upregulated by (6, 7). A downregulation of genes in stationary phase may account for the decrease in resistance we observed in the stationary phase. However, much further testing, such as an investigation of the turnover rate of PhoE in the outer membrane, should be conducted to determine its abundance in the membrane in phosphate limiting conditions during stationary phase.

Serially passaging cells increases growth yield and rate and decreases lag phase. We demonstrated that serial passaging of the wild type strain resulted in a 2.4-fold decrease in lag phase time in phosphate sufficient and deficient media. Similarly, there was a 2-fold and 4-fold decrease in phosphate sufficient and deficient conditions, respectively for the Δ*ompC* strain (Fig. 1). In addition, we observed significant increases in growth yields at 22 hours post-adaptation, as there was a 45-fold and 71-fold increase in wild type and Δ*ompC* growth in phosphate deficient media, respectively (Fig. 2). Our results are in accordance with previous studies of *E. coli* in minimal media, that have shown cells can be adapted to minimal media by serial passaging, which increases growth rates and decrease lag phases (9, 10).

We also observed an overall increase in final growth yields of all strains when comparing adapted and non-adapted cells. However, the Δ*ompC* strain grew to a similar growth yield compared to wild type in phosphate deficient conditions, but not in phosphate sufficient conditions (Fig. 3). Previous studies have shown that *E. coli* cells that have been adapted to various types of minimal media are known to sustain mutations to the *rpoB* gene, which encodes the beta subunit of the RNA polymerase (9, 14). Mutations in this gene are thought to influence transcription on a genome-wide scale (9). Though it is unclear whether our strains sustained genetic mutations in the adaptation process, studies have shown that serially passaging cells may result in various mutations and differential transcriptional states of cells may occur in respective cultures. However, adapted cultures will exhibit a convergent phenotype of increased fitness in minimal media (15). Depending on the transcriptional state of cells in a culture, certain populations may exhibit a maximum threshold for growth yield, which does not change regardless of additional passaging steps (15). Our observations indicate that the Δ*ompC* strain that was adapted to phosphate sufficient minimal media reached a maximum threshold of growth lower than the wild type strain adapted to phosphate sufficient media. This suggests that the Δ*ompC* strain may have entered an adapted transcriptional state limiting growth. Further testing is needed to determine whether these differences in growth are due to divergent paths of adaptive mutations, or mere phenotypic changes that are not sustained when transferred to rich media and subsequently grown on

limiting media again. Additionally, our strains may have acquired variant mutations while serially passaged in phosphate sufficient and deficient media, leading to non-isogenic strains.

In conclusion, we determined that phosphate limiting conditions did not confer lasting SDS-EDTA resistance in Δ*ompC* cells, however, resistance is observed during exponential phase. Additionally, we found that adapting wild type and Δ*ompC E. coli* K-12 in phosphate-defined minimal media and using inoculum sizes of 10^6 cells effectively decreases lag phases and increases growth yields and rates. Our results may be of use in future experiments involving phosphate-defined limiting media.

Limitations This study was able to determine that phosphate deficiency only transiently restores SDS-EDTA resistance in Δ*ompC* cells, but we were unable to determine the mechanism by which this occurs. As previously mentioned, PhoE may play a role in maintaining OM asymmetry; however, we attempted, but were unable to successfully optimize RT-qPCR experiments to implicate its involvement, and it remains unknown whether *phoE* is upregulated to a greater extent during exponential phase than stationary phase. We were also unable to confirm the underlying adaptive mechanisms that resulted in decreased lag phase times and increased growth yields in our strains, as we only observed these changes by conducting growth curve experiments. Additionally, due to the evolving direction of our project and time constraints, we were only able to conduct one biological replicate of this experiment and we did not rigorously control for the effect of inoculum size on decreasing lag phase times in phosphate sufficient and deficient media for adapted and non-adapted cells.

Future Directions Our data suggests that phosphate deficiency only transiently confers resistance to SDS-EDTA in the Δ*ompC* mutant. However, in this experiment, we were unable to determine the mechanism involved in conferring transient resistance to SDS-EDTA under phosphate limiting conditions. In order to further investigate the involvement of other osmoporins such as *phoE* in this mechanism, we recommend analyzing expression levels using a promoter trap assay or RT-qPCR in exponential versus stationary phase. If *phoE* is found to be upregulated in these conditions, one can further validate its role in conferring SDS-EDTA resistance by using a gain of function approach and complimenting a Δ*ompC*Δ*phoE* strain with *phoE* under phosphate deficient conditions.

With respect to our adaptation of *E. coli* wild type and Δ*ompC* strains, we did not determine the effects of serial passaging on the morphology of the OM of our cells, which may be done using electron microscopy experiments. In addition, we could not verify whether serial passaging resulted in genotypic changes. Further study of the effects of adaptation on *rpoB* mutations in phosphate limiting conditions may help elucidate the underlying mechanisms of the phenotypic changes we observed in our cells. Additionally, to observe whether the initial adapted phenotype is sustained or is reversible, adapted cells may be transferred to LB medium, and subsequently to phosphate defined minimal media again.

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CONTRIBUTIONS

PK: Result Figures, Results, Material and methods, Supplementary Figures. **PS:** Results, Result Tables and Figures, Future Directions, Supplementary Figures. **CS:** Abstract, Introduction, Supplementary Figures, References. **AH:** Discussion, Materials and Methods, Future Directions, Supplementary Figures

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