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Deletion of *ompF* and *ompC* in *Escherichia coli* K12 does not affect kanamycin resistance

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SUMMARY OmpC and OmpF are major *Escherichia coli* outer membrane general diffusion porins that are involved in the influx of small cationic hydrophilic molecules. It has been suggested that these two porins are involved in resistance to antibiotics such as kanamycin. OmpC and OmpF are reciprocally regulated by growth medium osmolarity. At low osmolarity, OmpF is preferentially expressed and at high osmolarity, OmpC is preferentially expressed. Previously reported results for kanamycin susceptibility of *ΔompC* and *ΔompF E. coli* K12 mutants are inconsistent. Furthermore, these previous studies were all done under low salt conditions which does not account for the differential expression of OmpC and OmpF under different osmolarities. This study aims to address the discrepancy between previously reported kanamycin resistance of *ΔompC* and *ΔompF* mutants by repeating the experiments. The differential expression of the two porins will also be addressed by determining resistance of *ΔompC* and *ΔompF* mutants cultured under different medium osmolarities. Minimum inhibitory concentration assays were conducted on *E. coli* K12 wild type, *ΔompC* and *ΔompF* mutants grown under three different salt condition (0.5, 1.0 and 1.5% NaCl) LB media. Results show that deletion of *ompC* and *ompF* does not affect kanamycin resistance. However, high salt growth conditions did increase kanamycin resistance in all three strains.

INTRODUCTION

The outer membrane of gram-negative bacteria is an impermeable asymmetric lipid bilayer that protects the bacteria from harmful environmental factors and changes in osmotic pressure (1, 2). Influx of nutrients and disposal of waste products across the outer membrane are facilitated by specific protein translocators and general diffusion porins (1, 2). OmpF and OmpC are major general diffusion porins found in the outer membrane of *Escherichia coli* (1). These porins are involved in the uptake of small hydrophilic molecules including antibiotics (2). It has been suggested that loss or mutation of OmpC and OmpF may increase resistance to antibiotics (3, 4, 5).

The structures of OmpC and OmpF are transmembrane homologous trimers of 16-stranded antiparallel β-barrels (2, 3). Within the lumen of each monomer is a conserved L3 loop that forms the intracellular constriction zone (3, 6). Acidic residues on the L3 loop are found directly opposite basic residues on the barrel wall which creates a strong electric field that facilitates the diffusion of molecules into the cell (2, 6). OmpC and OmpF are both cationic selective with OmpC having a greater preference for cations due to its more negatively charged lumen (1, 7). Kanamycin is a small polycationic aminoglycoside bactericidal antibiotic that targets the 30S ribosomal subunit and inhibits protein synthesis (8). It has been suggested that OmpC and OmpF contain a binding site for polycations that allows the influx of kanamycin into the bacteria (4, 9).

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OmpC and OmpF are reciprocally regulated by medium osmolarity through the EnvZ-OmpR two-component His-Asp phosphorelay signal transduction system (10, 11, 12). EnvZ is a histidine kinase that is the transmembrane osmosensor and OmpR is a transcription factor that is the response regulator (12). EnvZ has both kinase and phosphatase activity and it regulates the level of phosphorylated OmpR in the cell (12). At low growth medium osmolarity, the phosphatase activity of EnvZ is greater than its kinase activity and so the level of phosphorylated OmpR is lower (11). In this situation the lower level of phosphorylated OmpR is sufficient to activate *ompF* expression but not *ompC* (11). At high growth medium osmolarity, the EnvZ kinase activity is greater than its phosphatase activity and so the level of phosphorylated OmpR is increased (11). When the level of phosphorylated OmpR is high, expression of *ompF* is repressed and *ompC* is upregulated (11). Therefore, at low osmolarity OmpF is preferentially expressed, and at high osmolarity OmpC is dominant (10, 11).

A previous study conducted by Chang *et al*. determined the kanamycin MIC of *ΔompC* and *ΔompF* mutants (13). It was observed that the *ΔompF* mutant resulted in a slower growth rate and two-fold higher kanamycin MIC compared to the wild type (WT) and *ΔompC* mutant (13). Based on this result, Chang *et al*. suggested that OmpF may be the dominant porin in the transportation of kanamycin into the bacterial cell (13). However, this study was conducted solely under low salt conditions (Lysogeny Broth medium with 0.5% NaCl). At low osmolarity, OmpF expression would be elevated and OmpC expression downregulated, which means that for the *ΔompF* mutant, these conditions may have essentially created a double *ΔompFΔompC* mutant phenotype. Therefore, results obtained by Chang *et al.* may have been a result of the low salt condition of the growth media used in the experiments. It was also noted that results obtained by Chang *et al.* are inconsistent with a previous study conducted by Hancock *et al*. which also used *ΔompC* and *ΔompF* mutants to determine kanamycin MIC under low salt conditions (9, 13). Both papers reported using the mirodilution method for their MIC assays, however, Hancock *et al*. reported that deletion of OmpC and OmpF had no effect on kanamycin susceptibility (9, 13).

In order to resolve these discrepancies, we conducted another study to confirm whether OmpC and OmpF play a role in conferring kanamycin susceptibility. We also aimed to determine whether the results observed by Chang *et al.* were due to the low salt conditions used in the experiments. In high salt conditions, OmpC is the dominantly expressed porin so for the *ΔompF* mutant it would be able to express the dominant porin while *ΔompC* mutant would not be able to. In this study, we adjusted medium osmolarity by varying the amount of NaCl in Lysogeny Broth (LB) media. Based on research conducted by Omotoyinbo and Omotoyinbo, growth rate of *E. coli* is similar when grown in LB media with 0.5 and 1.0% NaCl and decreases when grown in 3.0% NaCl LB media (14). Abdulkarim *et al*. also conducted a similar growth curve analysis on *E. coli* and found that growth remains constant between 0.5 and 1.0% NaCl concentrations and then decreases when grown in 1.5% NaCl (15). Therefore, low, normal and high salt conditions for this study were defined as 0.5, 1.0 and 1.5% NaCl, respectively. We hypothesized that OmpC and OmpF are equally involved in conferring kanamycin susceptibility. Therefore, under high salt conditions the *ΔompC* mutant will show increased resistance to kanamycin compared to *ΔompF* and wild type *E. coli* and under low salt conditions the *ΔompF* mutant will show increased resistance.

METHODS AND MATERIALS

Bacterial strains, media, reagents and growth conditions. *E. coli* K12 BW25113 (wild type; CGSC #7636), JW0912-1 (*ΔompF* Kan^R; CGSC #8925) and JW2203-1 (*ΔompC* Kan^R; CGSC #9781) were obtained from the Keio Collection (16). Kanamycin resistant strains were used as positive genotyping controls. Chang *et al*. made kanamycin sensitive strains, JW0912-K2 (*ΔompF* Kan^S) and JW2203-K5 (*ΔompC* Kan^S), by removing the kanamycin resistance cassette through FLP-FRT recombination (13). These strains were obtained from a strain collection in the Department of Microbiology and Immunology in the University of British Columbia and used in subsequent experiments in order to test kanamycin resistance. Osmolarity of the LB growth medium (1% Tryptone, 0.5% Yeast Extract) was adjusted with different concentrations of NaCl. Low, normal and high salt medium contained 0.5, 1.0 and 1.5% NaCl, respectively. Stock solution of kanamycin sulfate (50 mg/ml) was prepared in sterile water and filter-sterilized through a 0.2 µm PES syringe filter. JW0912-1 and JW2203-

1 were cultured on LB agar plates (1% Tryptone, 0.5% Yeast Extract, 1.0% NaCl, 1.5% agar) containing kanamycin sulfate (50 µg/ml). BW25113, JW0912-K2 and JW2203-K5 were cultured on LB agar plates without kanamycin sulfate. All strains were cultured aerobically at 37ºC prior to experiments.

PCR and agarose gel electrophoresis to verify deletion of *ompC* and *ompF* and removal of kanamycin cassette. Genomic DNA was isolated and purified from overnight cultures of JW0912-1, JW2203-1, JW0912-K2 and JW2203-K5 using PureLink Genomic DNA mini kit (Invitrogen Kit K182002). The protocols outlined by the manufacturer for preparing gram negative bacterial cell lysate and purifying genomic DNA using spin columns were followed (17). DNA was eluted with 100 µL of Genomic Elution Buffer. Concentration and quality of isolated DNA were assessed with a UV-visible NanoDrop spectrophotometer. PCR was performed with 10 ng of purified genomic DNA using Platinum *Taq* DNA Polymerase (Invitrogen) and *ompC* and *ompF* primers (Integrated DNA Technologies) (Supplementary Table 1). The PCR mix and protocol given by the manufacturer was followed exactly (18). Annealing temperatures were 52.5ºC for *ompC* and 55.3ºC for *ompF* reactions. Extension time was 2 minutes at 72ºC and PCR was run for 35 cycles.

PCR products were resolved on a 1.5% agarose gel prepared with SYBR Safe DNA gel stain in 0.5X TBE (Invitrogen) to verify the deletion of *ompC* and *ompF*, in addition to the removal of the kanamycin resistance cassette in the mutantconsts ructed by Chang *et al*.

Establishment of growth curves for wild type (BW25113), *ΔompC* (JW2203-K5) and *ΔompF* (JW0912-K2) strains. Growth curves were generated to determine if gene deletions or different salt concentrations had a notable effect on growth rate of a particular strain. Overnight cultures of wild type, *ΔompC* and *ΔompF* strains were grown in normal salt LB medium. Overnight cultures were then diluted 1/100 into the 3 different salt condition LB media and 200 µL of each diluted culture was plated per well into a 96-well plate. An automated plate reader (BioTek Synergy H1 Microplate Reader) was used to measure OD_{600} every 10 minutes for 16 hours. Growth curves were generated with 3 technical replicates and negative control wells with just 200 µL of the three different salt condition LB media was included as well.

Pilot MIC assay in the low salt condition. A pilot minimum inhibitory concentration (MIC) assay was done in the low salt condition. The protocol for MIC assay described by Chang *et al*. was followed (13). Overnight cultures of wild type, *ΔompC* and *ΔompF* strains were grown in low salt LB Medium. Cultures were diluted 1/10 and then grown to an OD_{600} of 0.08. 50 µL of bacterial cells per well were inoculated into a 96-well plate containing 50 µL of 2-fold kanamycin serial dilutions to give final kanamycin concentrations of 128 to 0.5 µg/ml. Positive control wells with no antibiotic were included to ensure normal bacterial growth and negative control wells with no bacteria were included to test sterility. Plates were incubated at 37ºC for 16-20 hours before determining MIC with an absorbance plate reader (BioTek) at OD_{600}. This assay was done once with 2 technical replicates.

Determination of MIC for wild type (BW25113), *ΔompC* (JW2203-K5) and *ΔompF* (JW0912-K2) strains at different salt conditions. Overnight cultures of wild type, *ΔompC* and *ΔompF* strains were grown in normal salt LB Medium. Overnight cultures were then diluted 1/100 into the 3 different salt condition LB media and allowed to grow for 2 hours. OD_{600} was measured using a spectrophotometer and cells were diluted to 1 x 10^5 cells/ml in the respective salt condition LB media. This calculation is based on the assumption that a culture OD_{600} of 1.0 is equal to an average of 1 x 10^8 cells/ml. Separate sets of kanamycin serial dilutions were prepared with the different salt condition LB media separately in Eppendorf tubes and then plated into the 96-well plates. 50 µL of diluted cells were then inoculated into the 96-well plate containing 50 µL of the 2-fold kanamycin serial dilutions to give final kanamycin concentrations from 128 to 0.5 µg/ml. Positive and negative control wells were included as above. Plates were incubated at 37ºC for 16 hours before determining MIC with an absorbance plate reader (BioTek) at OD_{600}. Each assay was repeated 3 times with 3 technical replicates. Absorbance values between the triplicates were averaged and the

negative control absorbance value was subtracted. Corrected absorbance values less than 0.04, representing double the average of the negative control wells, was not considered growth for the purposes of determining the kanamycin MIC. The kanamycin MIC for each strain was determined as the first well with what is considered to be no growth based on the corrected absorbance value.

RESULTS

Confirmation of mutant genotypes. PCR amplification was done to confirm the correct gene deletions in the kanamycin resistant and susceptible strains. Primers flanking *ompC* and *ompF* were used to amplify the respective genes in *ΔompF* and *ΔompC* mutants. The kanamycin resistance cassette that replaced the genes in the single deletion mutants has an expected size of approximately 1.5 kb. Wild type *ompC* and *ompF* genes have an expected size of about 1.0 kb. After removal of the kanamycin resistance cassette it is expected that the amplified region will drop to approximately 300 bp representing the remaining scar sequence. As seen in Figure S1 (Appendix), the kanamycin resistance cassette and expected wild type bands were amplified in the kanamycin resistant strains. In kanamycin susceptible strains, the scar sequence and expected wild type bands were amplified, confirming the removal of the kanamycin cassette and the deletion of *ompF* and *ompC* genes in JW0912-K2 (*ΔompF* mutant) and JW2203-K5 (*ΔompC* mutant) respectively.

Wild type, *ΔompC* (JW2203-K5) and *ΔompF* (JW0912-K2) mutants showed similar growth rates at low, normal and high salt LB medium. Growth curves were generated for all 3 strains in each salt condition to determine if gene deletions and salt conditions affect growth rates. This is important in determining whether the growth rates will affect the subsequent MIC assays. As illustrated in Figure 1, the growth rates and final growth yields of the wild type, *ΔompF* and *ΔompC* strains at the three different salt conditions are all

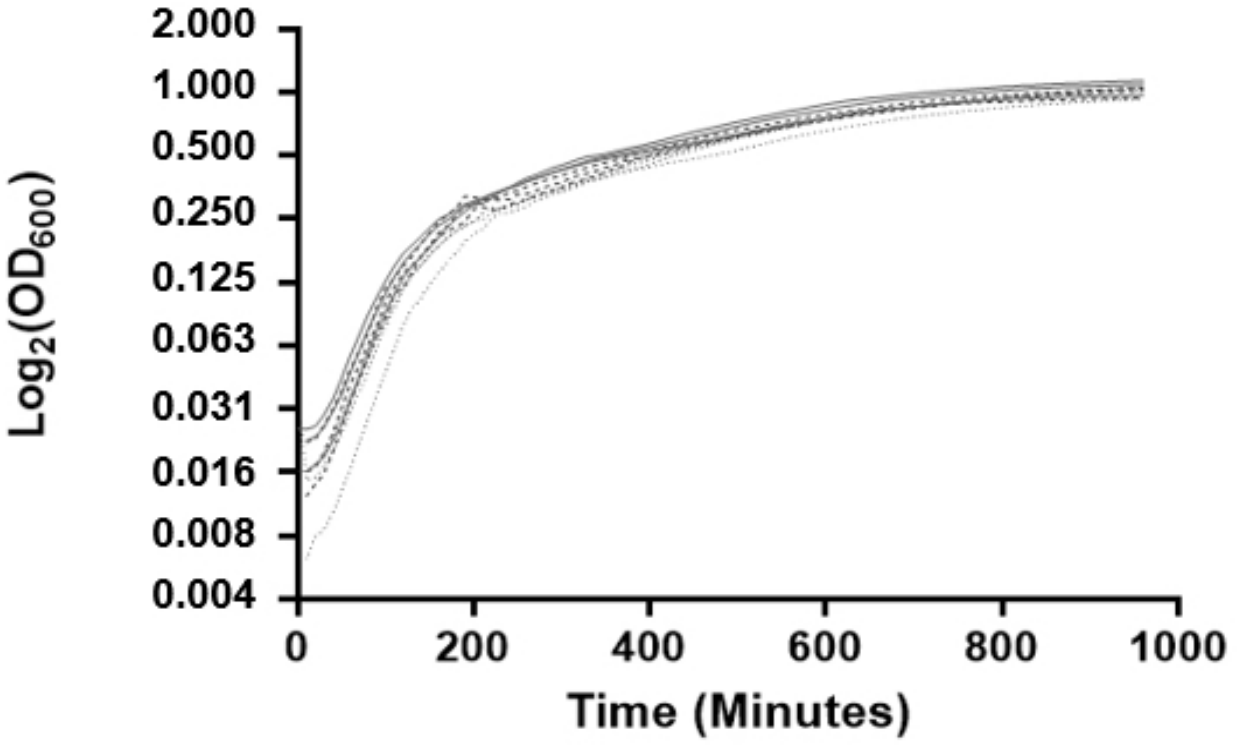

FIG. 1 Log_2 transformed growth rate curves of wild type, *ompC* and *ompF* deletion mutants under growth media of different osmolarities. Absorbance was measured every 10 minutes for 16 hours. Absorbance (OD_{600}) values are averaged between triplicates and the absorbance value of the negative control was subtracted. Overlapping curves indicate similar growth rates at each salt condition. Solid lines represent wild type strain, dashed lines represent *ΔompF* mutant and dotted lines represent *ΔompC* mutant.

relatively similar. These results show that gene deletions and different salt conditions do not affect growth rate under the conditions used in our assay; therefore, no normalization or considerations regarding growth were required when analyzing MIC assay results.

Pilot MIC assay showed no difference in kanamycin resistance between strains at low salt condition. All 3 strains were cultured in low salt media and then plated in low salt media containing 2-fold kanamycin serial dilutions from 128 to 0.5 µg/ml. In our pilot experiment, the MIC was consistently 8 µg/ml for wild type, *ΔompF* and *ΔompC* mutant strains indicating no difference in kanamycin resistance between the three strains.

Kanamycin resistance was not affected by deletion of *ompF* or *ompC* at any salt concentration. To test whether deletion of *ompC* and *ompF* will affect kanamycin resistance at different salt concentrations, MIC assays with 2-fold dilutions of kanamycin from 128 to 0.5 µg/ml were repeated in the three salt conditions. As shown in Table 1, the MIC values between strains at the three different salt conditions are relatively consistent between replicates. There is no notable difference in kanamycin resistance between wild type, *ΔompF* and *ΔompC* mutants at any of the salt conditions used in this experiment.

Although deletion of *ompC* and *ompF* did not seem to affect kanamycin resistance, salt conditions did appear to have an effect. Table 1 shows that at high salt conditions a consensus MIC value of 16 µg/mL was obtained for the three strains. At low and normal salt conditions, kanamycin MIC was generally 8 µg/mL across the three strains. At high salt conditions, there is greater kanamycin resistance and at low and normal salt conditions there is lower kanamycin resistance.

DISCUSSION

Our study investigated the roles of OmpC and OmpF porins in conferring kanamycin resistance while taking into account the effect of osmolarity on expression of both porins. *ompC* and *ompF* expression is upregulated at high and low osmolarity respectively. We hypothesized that kanamycin resistance would increase in the *ΔompC* mutant at high salt condition due to downregulation of OmpF. We also expected to see increased resistance in the *ΔompF* mutant at low salt condition due to downregulation of OmpC. To test this, we performed growth curve analysis and MIC assays for wild type, *ΔompF* mutant and *ΔompC* mutant at low, normal and high salt conditions. Growth curve analysis shows that different conditions do not notably affect the growth rates of wild type, *ΔompC,* and *ΔompF* mutants.

***E. coli ΔompC* mutant does not show increased kanamycin resistance at high salt condition.** Under low salt conditions, Chang *et al*. saw a two-fold difference in MICs for *ΔompF* mutant compared to the wild type strain and *ΔompC* mutant. However, a study done by Hancock *et al.* showed no difference in kanamycin resistance between wild type, *ΔompC* and *ΔompF* mutants at low salt conditions as all three strains had a MIC of 4 µg/ml (9). Our MIC results were inconsistent with our hypothesis and kanamycin resistance was not affected by OmpC and OmpF at the same salt concentrations. This result is consistent with the conclusion made by Hancock *et al*. which is strong evidence that OmpC and OmpF do not play a significant role in kanamycin resistance (9).

TABLE 1 Minimum inhibitory kanamycin concentration (MIC) of BW25113 (wild type), JW0912-K2 (*ΔompF*) and JW2203-K5 (*ΔompC*) *E. coli* K12 grown under different medium osmolarities.

NaCl Concentration in LB Medium	Minimum Kanamycin Inhibitory Concentrations (µg/mL)								
	BW25113 (WT)			JW0912-K2 (*ΔompF*)			JW2203-K5 (*ΔompC*)		
	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3	Trial 1	Trial 2	Trial 3
High (1.5%)	16	16	16	8	16	16	8	16	16
Normal (1.0%)	16	8	8	8	8	8	8	8	8
Low (0.5%)	4	8	8	8	8	8	8	8	8

High salt growth conditions increase kanamycin resistance in *E. coli.* Our results show that kanamycin resistance increased in high salt concentration. Since our study suggests that *ompC* and *ompF* deletions do not affect resistance, then another possible explanation for this observation could be an increase in efflux activity. AcrAB-TolC complex is a multidrug efflux pump system encoded by genes *acrA, acrB and tolC* (19). A study done by Belmans *et al.* found that kanamycin susceptibility increased in *ΔacrA* mutants compared to wild type strain (20). Another study done by Manlu and Xiongfeng investigated the relationship between AcrAB-TolC complex and antibiotic resistance in *E. coli* at high salt condition (19). They showed upregulated expression of *acrA*, *acrB* and *tolC* under high salt conditions (19). They also found that *acrB* and *tolC* single gene deletion mutants were more susceptible to tetracycline and chloramphenicol than wild type growing in high salt conditions (19). Together, both studies suggest that high salt conditions could cross-protect *E. coli* from kanamycin through upregulated expression of AcrAB-TolC. It is important to note that Manlu and Xiongfeng used 0.1M (0.6% NaCl) and 0.4M NaCl (2.3% NaCl) in glucose minimal medium as normal and high salt conditions respectively (19).

Conclusions Our results showed no difference in kanamycin resistance between *E. coli* K12 wild type, *ΔompF* and *ΔompC* mutants at each salt condition, suggesting that OmpC and OmpF do not play a role in kanamycin resistance. This conclusion contradicts our hypothesis but supports the findings of Hancock *et al.* (9). Additionally, we observed increased kanamycin resistance for all strains at high salt conditions.

Future Directions As expression of OmpF and OmpC are regulated by medium osmolarity, protein expression levels at the different salt conditions should be quantified using Western Blot analysis. Alternatively, qRT-PCR could also be conducted to quantify the expression of OmpF and OmpC at the transcriptional level. This would make our result more conclusive as it would verify whether OmpC and OmpF expression were significantly affected by the salt conditions we selected. Also, an *ΔompFΔompC* double deletion mutant could be included in the study as Hancock *et al.* observed a 2-fold decrease in kanamycin resistance for the double mutant strain. It would also be worth including a mutant that overexpresses OmpF or OmpC to clearly determine their role in kanamycin influx. Our study revealed no role for OmpF and OmpC in kanamycin resistance, but did uncover a relationship between media salt concentration and kanamycin resistance. Future studies could further examine the upregulated efflux activity of AcrAB-TolC in response to high NaCl concentrations.

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