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Genetic characterization and investigation of kanamycin susceptibility of *ompC* and *ompF* single gene deletion mutants of *Escherichia coli* K-12

Chuan Chuan Chang, Ching Wing Yan, Regina Onal, and Yue Zhang

Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada

SUMMARY OmpF and OmpC are two highly similar proteins that form porins on the outer membranes of of Gram-negative bacteria. Though the two porins have been shown to transport a variety of antibiotics, much remains unknown about aminoglycoside transport through OmpF and OmpC porins as previous studies relating OmpF to kanamycin resistance have shown inconsistent observations. We first performed genetic characterization of kanamycin sensitive *Escherichia coli* K-12 *ompC* and *ompF* single gene deletion mutants, which were generated from the removal of a kanamycin resistance cassette through FLP-FRT recombination of the original Keio collection Δ*ompC and* Δ*ompF* mutants. We then performed minimum inhibitory concentration assays to determine the lowest inhibitory kanamycin concentration of the mutants. We have shown the gene deletion mutations are accurate and precise in our Keio mutants, and we have preliminary results showing that the *ompF* deletion mutant has a greater resistance to kanamycin in comparison to the wildtype and *ompC* deletion mutant. This finding implicates that *ompF* may play a greater role in kanamycin susceptibility.

INTRODUCTION

OmpF and OmpC are proteins that form trimeric general diffusion porins on the outer membranes (OM) of Gram-negative bacteria. The two porins are highly similar; both porins are made of 16-stranded beta barrels and contain a central hydrophilic core made of clusters of acidic and basic residues (1). Negatively-charged residues are found on the L3 loop of the Omp proteins, while positively-charged residues are clustered on the opposite barrel wall. Together with the opposite barrel wall, the L3 loop forms the constriction zone of these porins (2). The OmpF and OmpC porins have been shown to transport a variety of antibiotics. And thus, loss of OmpF and OmpC expression has been implicated in antibiotic resistance (3).

With respect to the aminoglycoside kanamycin, much remains unclear about its transport through OmpF and OmpC porins since experiments have focused mostly on OmpF and the results have been inconsistent (4). Moreover, several experiments have yielded inconsistent observations. The *Escherichia coli* CO strain shows kanamycin resistance as well as no expression of OmpC, suggesting the loss of OmpC may be involved in the development of kanamycin resistance (5). However, in other bacterial species, lack of OmpF, but not OmpC, increased antibiotic minimum inhibitory concentration (MIC) values (6).

Our prediction for the effect of *ompC* and *ompF* gene deletion on kanamycin resistance in *E. coli* was based on evidence that supports the channel-specific nature of drug translocation through the Omp porins and the chemical structure of our antibiotic of interest. We expected that the expression of *ompF* or *ompC* would play a role in translocation of kanamycin into the cell.

Computational modelling by Im and colleagues predicted that cations are drawn to Omp porin L3 loops, where negatively charged residues are clustered. Overall, their experiments suggest that cation permeation through the porins does not follow simple diffusion (7). Experiments by Nestorovich and colleagues demonstrated that zwitterionic ampicillin and amoxicillin act as transient open channel blockers for the OmpF porin; ampicillin and amoxicillin also showed the highest diffusion rates through the OmpF porin (8). The blocking is hypothesized to be due to complementary charge distribution of the drugs to that of the L3

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loop and opposing barrel wall. These findings further support the idea that antibiotic permeation through the OmpF porin may be channel-specific and that the channel-specific binding of antibiotics to the Omp porins can facilitate drug translocation into *E. coli* cells (8).

Since the OmpC and OmpF porins are highly similar in terms of size, geometry, and arrangement of charged residues in their constriction zones (9-11), we expected to see channel-specific drug translocation in the OmpC porin that is mechanistically similar to that of OmpF. The difference is that OmpC was found to have greater preference for cations, possibly due to its more negatively-charged pore lumen. Kanamycin is a polycationic species because its amine moieties are mostly protonated at biological pH (12). We predicted that the complete deletion of *ompC* would confer greater kanamycin resistance compared to the loss of OmpF porin in *E. coli*, since OmpC prefers cations which will encourage the permeation of cationic kanamycin into *E. coli*. Our hypothesis, however, was disproved following minimum inhibitory concentration (MIC) assays experiments using the Keio *ΔompF* and *ΔompC* single gene deletion *E. coli* mutants.

There are no known studies which characterize the Keio *ompC* and *ompF* single gene deletion mutants. Furthermore, Angeles and colleagues found a band corresponding to the *ompF* DNA fragment when the products of a PCR reaction using primers flanking the *ompF* gene deletion region of a supposed Keio *ΔompF* mutant were run on a 1% agarose gel (13). The presence of the assumed *ompF* band suggests that the Keio *ΔompF* mutant is incorrect and may still contain the *ompF* gene. We thus first performed genetic characterization of the Keio *ompC* and *ompF* single gene deletion mutants before using these mutants to test for any differences in kanamycin susceptibility.

METHODS AND MATERIALS

Bacterial strains, media, and growth conditions. *E. coli* K-12 BW25113 (wildtype, CGSC #7636), JW0912-1 (Δ*ompF*, CGSC #8925), and JW2203-1 (Δ*ompC*, CGSC #9781) were ordered from the Yale Coli Genetic Stock Centre. BT340 strain was cultured for the isolation of pCP20 plasmid used for FLP-FRT recombination. Low salt LB Lennox (1% Bacto-Tryptone, 0.5% yeast extract, 0.5% NaCl) was used as described for the culturing of Keio strains, with the addition of 1.5% agar for solid media (14). BW25113 was cultured on media as described, with no antibiotics. JW0912-1 and JW2203-1 were cultured on media with the addition of kanamycin to a final concentration of 30 μg/mL. LB broth and agar (1% Bacto-Tryptone, 0.5% yeast extract, 1% NaCl) with a 100 μg/mL final concentration of ampicillin were used to culture BT340. All strains were cultured aerobically, and all strains except for BT340 were cultured at 37℃. BT340 was cultured at 30℃.

Preparation of electrocompetent JW0912-1 and JW2203-1 cells. Electrocompetent JW0912-1 and JW2203-1 cells were prepared using methods described by the Barrick lab (15). Strains were grown overnight in culture conditions as described previously. Strains were sub-cultured by adding 200 μL of the overnight cultures into 20 mL of LB-Kan, and were grown under the same conditions until an OD_{600} of around 0.6 was reached. Subcultures were centrifuged to pellet the cells and dispose of the supernatant. Cells were washed four times with cold, sterile 10% glycerol by resuspension and centrifugation. The pellets were resuspended in 200 μL of 10% glycerol and divided into 30 μL aliquots, which were stored at -80ºC.

Isolation and and purification of pCP20 plasmid. PureLink Quick Plasmid MiniPrep Kit (Invitrogen K210011) was used to isolate and purify pCP20 plasmids from *E. coli* BT340. Plasmids were eluted in TE buffer, provided by the kit manufacturer. The concentration and purity of isolated pCP20 plasmids were assessed with a UV-visible NanoDrop spectrophotometer.

Removal of kanamycin resistance cassette gene from JW0912-1 and JW2203-1. FLP-FRT recombination was conducted to remove the kanamycin resistance cassette gene in JW0912-1 and JW2203-1 strains, with a protocol adapted from the Barrick lab (15). Purified pCP20 (3 μL of 62.5 ng/μL DNA) was added to 30 μL aliquot of competent cells, and electroporated with 0.2 cm GenePulser Cuvette in a BioRad MicroPulser, under the Ec2

setting. Immediately after electroporation, 1 mL of LB broth was added to the cuvette, and the entire mixture was transferred to a 1.5 mL microcentrifuge tube for a 1 hour outgrowth at 30°C on a shaking incubator. Transformants were selected by plating on LB-Amp agar plate and incubated at 30°C for at least 20 hours. A colony was selected from the LB-Amp plate for each strain and inoculated into sterile LB broth without antibiotic. The inoculated cultures grew at 43°C overnight. We made 10^{-6} dilutions of the overnight cultures and plated 50 µL onto LB agar plates. The plates were incubated at 30℃ overnight. A colony for each strain was selected and patched onto LB-Kan, LB-Amp and LB plates to screen for candidates that had lost kanamycin resistance and were ampicillin sensitive. The LB-Kan and LB plates were incubated at 37°C overnight whereas the LB-Amp plates were incubated at 30°C overnight.

Two successful recombinants for each gene deletion mutant were selected and renamed. JW2203-K2, JW2203-K5 were the two *ΔompC* mutants after kanamycin cassette removal, while JW0912-K2, JW0912-K5 were the two *ΔompF* mutants after kanamycin cassette removal. The -K5 strains were taken as backup and were not used in further experiments beyond colony PCR and gel electrophoresis for confirmation of their mutations.

Colony PCR and agarose gel electrophoresis of strains to observe presence or absence of *ompC* and *ompF*. Colony PCR was performed with two sets of primers: one set flanking *ompC* and one set flanking *ompF*. Primer sequences are shown in Table 1. Strains BW25113 (wildtype), JW0912-1 (original Keio *ΔompF* mutant), JW2203-1 (original Keio *ΔompC* mutant), as well as the four post-kanamycin resistance removal mutants were used in colony PCR twice, once with each primer set. We used Invitrogen Platinum *Taq* DNA Polymerase and used the PCR mix and cycle settings recommended by the manufacturer for the polymerase, with modifications. The initial denaturation at 94℃ was carried out for 2 min. Each cycle has denaturation of 30 seconds at 94℃. Annealing temperature used was 52.5℃ for *ompC* primers and 55.3℃ for *ompF* primers. Annealing time was 30 seconds, and extension time was 1.5 min at 72℃. We ran the PCR for 35 cycles.

All PCR products were resolved using 1.5% agarose gel electrophoresis in 0.5X TBE buffer. SYBR Safe DNA gel stain in 0.5X TBE (Invitrogen) was used to make the gel for visualization of the DNA bands. Gels were run at 100V for 45 min.

PCR product purification and Sanger sequencing of mutated regions. PCR product purification was carried out using the PureLink PCR Purification Kit (Invitrogen), using protocol provided by the manufacturer. Only the PCR products that were suspected to have amplified kanamycin resistance gene and scar sequences were sent for sequencing: JW2203-1 before and after kanamycin cassette removal, amplified by *ompC* primers, and JW0912-1 before and after kanamycin cassette removal, amplified by *ompF* primers. Purity of PCR products were assessed using the NanoDrop spectrophotometer. Sanger sequencing was conducted by GENEWIZ. Samples were prepared as per instructions for Sanger sequencing of purified PCR products. The same primers used for amplification of the respective PCR products were used for sequencing. Scar sequences from our sequencing results were compared to that described by literature (14).

Table 1 - Primers used for colony PCR and Sanger sequencing of gene deletion regions

Gene targeted	Primer orientation	Sequence
ompC	forward	5’-GCAGGCCCTTTGTTCGATATCAATC-3’
ompC	reverse	5’-ATCAGTATGCAGTGGCATAAAAAAGC-3’
ompF	forward	5’-CGGCATTTAACAAAGAGGTGTGC-3’
ompF	reverse	5’-ACGGCAGTGGCAGGTGTC-3’

Establishment of growth curves for BW25113, JW0912-K2 and JW2203-K2. 500 µL overnight cultures of BW25113, JW0912-K2 and JW2203-K2 were introduced to 50 mL of fresh, sterile LB broth in separate flasks. The cultures were incubated at 37°C on a shaking incubator at 200 rpm. The optical density at 625 nm of each culture was measured every 20 minutes using a Beckman spectrophotometer, over a course of 8 hours.

Determination of the minimum inhibitory kanamycin concentration for BW25113, JW0912-K2 and JW2203-K2. Minimum inhibitory concentration assays for kanamycin were performed using 96-well, flat-bottom polyethylene plates by LB broth dilution methods as per Wiegand and colleagues (16). BW25113, JW0912-K2 and JW2203-K2 strains were diluted to OD_{625} of 0.08 with sterile LB broth and incubated at 37°C while shaking at 200 rpm. Triplicates of kanamycin concentrations serially diluted from 64 µg/mL to 0.05 µg/mL were prepared, along with negative and positive controls. 50 µL of each diluted working strain was introduced to appropriate wells to make a total volume of 100 µL. The plate was incubated at 37°C for 16-20 hours. The negative control was 100 µL of sterile LB broth, whereas the positive control was 50 µL of respective bacterial strain in 50 µL of sterile LB broth. The lowest minimum inhibitory kanamycin concentration for each working strain was determined by the first well with no visible turbidity. The assay was repeated twice, each with 3 technical replicates.

RESULTS

Colony PCR and gel electrophoresis of mutant strains using primers flanking *ompC* and *ompF* gene regions show correct gene deletions. Analysis of the colony PCR products of the *ompC* and *ompF* gene regions of the mutant strains before and after removal of kanamycin resistance show that the gene deletions were correct. As shown in Figure 1A, primers that flank the *ompC* gene on JW2203-1 before kanamycin cassette removal amplified a region of around 1.5 kb. This region should be the kanamycin cassette that replaced the *ompC* and *ompF* genes in their respective mutants. After kanamycin cassette removal, the amplicon drops to around 300 bp for JW2203-K2 and JW2203-K5. Since *ompC* is much longer than

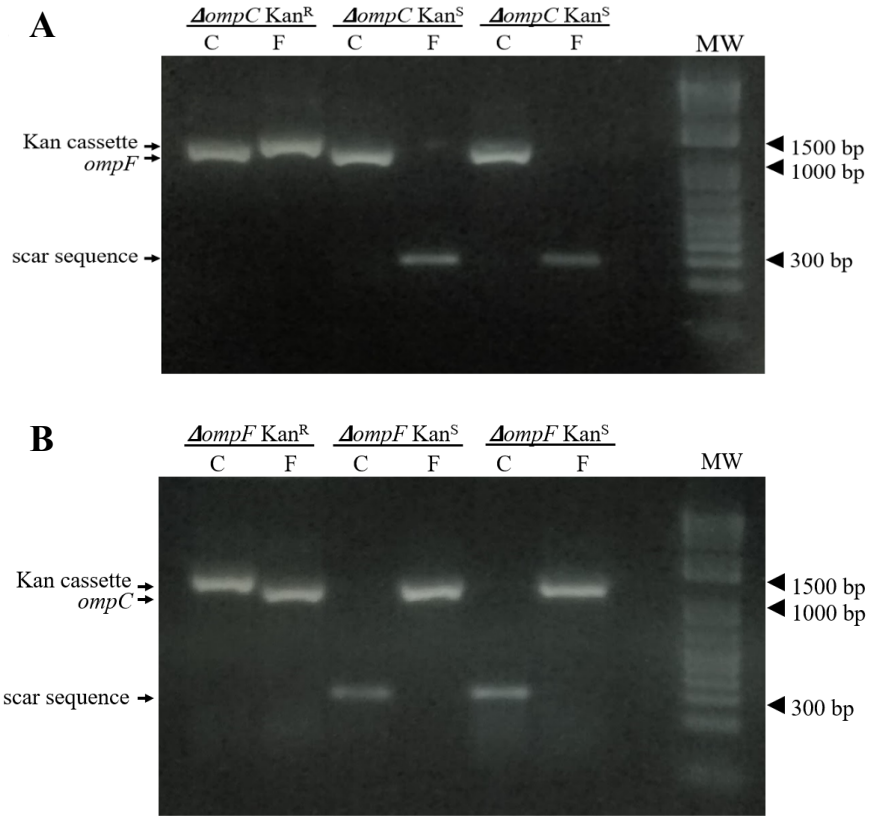

FIG. 1 PCR analysis confirm *ompC* and *ompF* deletions in *E. coli* strain K-12. Keio strains before and after removal of kanamycin cassette were analyzed with primers specific for both *ompC* and *ompF*. PCR products were resolved on 1.5% agarose gel for 45 min at 100 V. The primer set used to get the product for each lane is indicated by 'C' for *ompC* and 'F' for *ompF*. (A) Colony PCR of *ΔompC* mutants before and after kanamycin cassette removal to compare the gene deletion regions amplified by *ompC* primers. Lanes with *ompF* primers show the presence of wildtype *ompF*. (B) Colony PCR of *ΔompF* mutants before and after kanamycin cassette removal, comparing the gene deletion regions amplified by primers for *ompF*. Lanes with *ompC* primers show the presence of wildtype *ompC*.

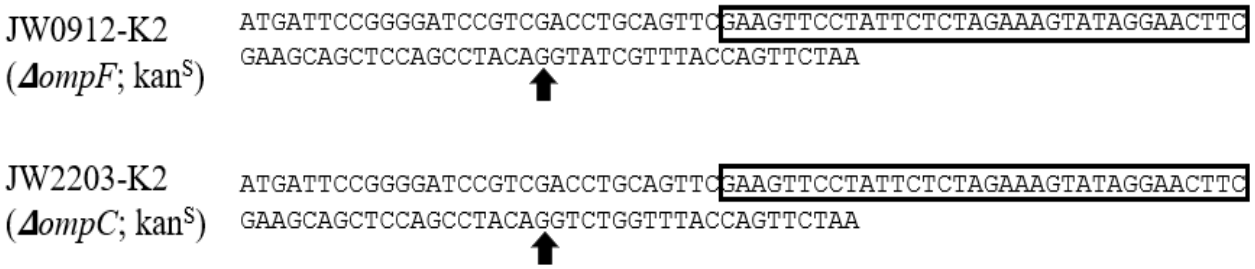

FIG. 2 Sequences for the gene deletion scars of *ΔompF* and *ΔompF* mutants after kanamycin cassette removal. The scar sequences for both JW0912-K2 and JW2203-K2 are identical, except for the last 21 bp (starting after the arrow), which are the last 21 bp of the *ompF* and *ompC* deleted genes, respectively. The boxed sequences show the FRT sites that are left over from the FLP recombination.

300 bp, we can be sure that kanamycin cassette was removed from the correct location in the chromosome, and that *ompC* is deleted. The primers flanking *ompF* in contrast, amplified the *ompF* gene that is still present in the Δ*ompC* mutant, producing an expected band of around 1.1 kb. The same is shown in Figure 1B for *ΔompF* mutants before and after FLP-FRT recombination. The regions of around 1.5 kb of JW0912 before the kanamycin cassette removal, and around 300 bp after the removal were amplified by the *ompF* primers instead. Primers flanking *ompC* amplified the wildtype *ompC* gene still present in these mutants.

Scar sequences of Keio mutant strains after kanamycin cassette removal are exactly as expected. Sequencing results from forward and reverse primers were compiled for each sample. The annotated scar sequences of JW0912-K2 and JW2203-K2 are shown in Figure 2, and contain the same elements as described by Baba and colleagues (14). One FRT site was identified in each scar sequence, which is the expected result after conducting FLP recombination (14). The scar sequence is an open reading frame consisting of 102 bp. The last 21 bp of each scar sequence are the last 21 bp of the deleted gene.

Much longer sequences of around 1300 bases were obtained for the mutated regions of JW0912-1 and JW2203-1 that do not match up to the original *ompF* and *ompC* genes, respectively (see supplementary Figures S1 and S2). We expect to, and do, see two FRT sites in each kanamycin cassette insertion sequence; FRT sites are required for the FLP recombination. Our primers were unable to sequence the entire length of the kanamycin cassette insertion, as bases from the wildtype BW25113 genome were unable to be identified within the sequencing results.

Our sequencing results are further evidence that JW2203-K2 and JW0912-K2 have the correct gene deletion mutation and no longer have the kanamycin gene insertion.

Table 2 Kanamycin susceptibility of wildtype, *ΔompC* and *ΔompF E. coli* K-12 determined using broth dilution method.

Strain	Genotype	Minimum inhibitory kanamycin concentration (μg/-ml)	
		Trial 1	Trial 2
BW25113	wildtype	16	32
JW0912-K2	*ΔompC*	16	32
JW2203-K2	*ΔompF*	32	64

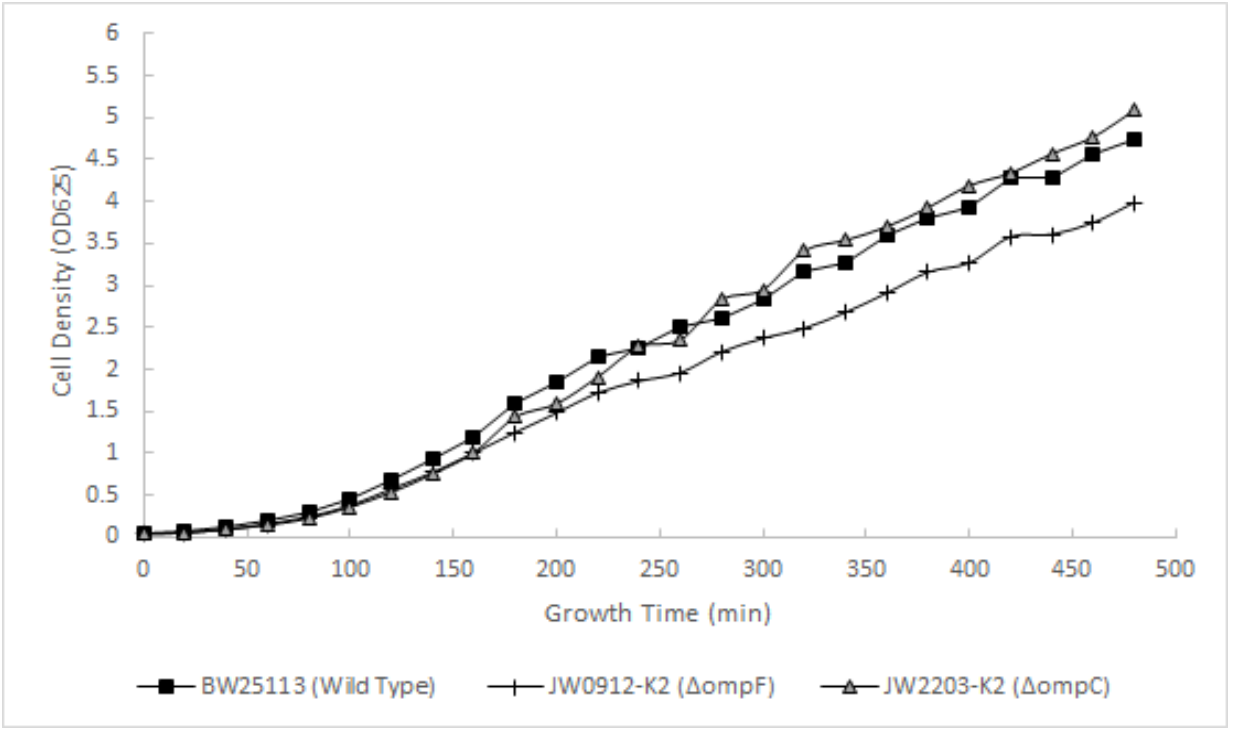

FIG. 3 Characterization and growth curve comparison of the wildtype strain BW25113, *ΔompF* mutant strain JW0912-K2 and the *ΔompC* mutant strain JW2203-K2. The bacterial growth curves were monitored, starting from the introduction of 500 μL respective overnight cultures into separated, sterile 50 mL of LB broth (t=0). 1 mL of inoculated bacterial culture was drawn and transferred to a clean cuvette. The optical density at 625 nm was used to measure bacterial growth every 20 minutes.

Kanamycin-sensitive *ΔompF* mutant showed slower growth rate than the wildtype and kanamycin-sensitive *ΔompC* mutant. In order to characterize the effect of the loss of OmpC or OmpF porin on the growth rate of *E.coli* K-12 strains, growth curve analysis was performed over a period of eight hours comparing the mutants to the wildtype (Fig. 3). As shown in Figure 3, the JW0912-K2 has a slower growth rate starting at the 4-hour time point. These growth patterns are inconsistent with the results shown by Agafitei and colleagues which saw that lack of *ompC* caused a 20 min lag in growth compared to the wildtype and the *ΔompF* mutant. Our results may be more reliable as we have confirmed the correct genotypes of our mutants, whereas they suspected that they did not have a true *ΔompF* mutant (17). Furthermore, the growth curve experiment was conducted for a longer period of time (17).

Kanamycin-sensitive *ΔompF* mutant shows greater resistance to kanamycin than the wildtype and kanamycin-sensitive *ΔompC* mutant. We hypothesized that the lack of *ompC* would incur a greater resistance to kanamycin, as compared to the wildtype and *ΔompF* mutant. To validate this, minimum inhibitory concentration assays was performed using the broth dilution method as described previously. Our trials exhibit consistent results in terms of the MIC values for each strain. As shown in Table 2, minimum inhibitory kanamycin concentrations for BW25113, JW2203-K2 and JW0912-K2 mutant are 16, 16 and 32 µg/mL, respectively for one trial, and 32, 32, and 64 µg/mL, respectively for the second trial. Despite the inconsistent values, the takeaway is the *ΔompF* mutant consistently displayed a two-fold increase in kanamycin resistance, as compared to the wildtype and *ΔompC* mutant.

DISCUSSION

Our study performed FLP recombination on and genetically characterized the *ompC* and *ompF* single gene deletion mutants from the Keio collection in order to determine any differences in kanamycin susceptibility between these mutants. In doing so, we hoped to investigate the degree to which OmpF or OmpC porins were involved the bactericidal activity of kanamycin. Through colony PCR, gel electrophoresis, and sequencing, we have genetically

characterized the *ompC* and *ompF* single gene deletion mutants from the Keio collection. We confirmed that the JW2203-1 and JW0912-1 Keio strains have had the correct genes, *ompC* and *ompF* respectively, replaced by the kanamycin cassette. We have also created new strains from the Keio mutants, called JW2203-K2 (*ΔompC*, Kan^S) and JW0912-K2 (*ΔompF*, Kan^S), which are the Keio single gene deletion mutants that have had the kanamycin resistance gene recombined out through FLP recombination. Using these new mutant strains, we have characterized their laboratory growth patterns compared to BW25113. JW0912-K2 has been shown to lag slightly in growth. Lastly, we have gathered preliminary data suggesting that the JW0912-K2 is more resistant to kanamycin than JW2203-K2 and BW25113.

Single gene deletion strains JW0912-1 and JW2203-1 from the Keio collection have the correct genotype. Kanamycin sensitive *ΔompC* and *ΔompF* Keio mutants were generated from kanamycin resistant Keio mutants. Polymerase chain reaction (PCR) of the kanamycin sensitive mutants using primers designed to bind outside the porin gene deletion regions was performed, followed by Sanger sequencing of the PCR products. Growth curve analyses were also performed to determine growth patterns and characteristics of our kanamycin sensitive wildtype and mutants. We have results to show that the Keio *ΔompC* and *ΔompF* single gene deletion mutants are accurate and precise, and that there is a change in growth pattern for the *ΔompF* mutant.

Previous studies have suggested that *ompF* may still be present in JW0912-1, based on the results of PCR and cell wall extraction experiments (13, 17). Our results contradict their findings and show that *ompF* is not present in JW0912-1. Since we ordered new JW0912-1 and JW2203-1 from the CGSC for our project, previous papers working with the older stock of JW0912-1 may have encountered contamination of the stock with wildtype or another strain that still contains *ompF*, from which they cultured bacteria with the unexpected genotype.

The new strains created from the original Keio knockouts, JW2203-K2 and JW0912-K2, were shown to be sensitive to kanamycin. Using colony PCR, we have confirmed the precise deletion of *ompC* and *ompF*, respectively, leaving behind a characterized scar sequence (Figure 2). These new mutants can be used to investigate the role of *ompC* and *ompF* in *E. coli* K-12.

***E. coli ompF* mutant shows reduced growth rate compared to wildtype *E. coli*.** Previous studies by Agafitei suggested that lack of *ompC* in *E. coli* K-12 had slight effect on growth rate (17). The study showed that the growth of the *ΔompC* mutant lagged behind the growth of the wildtype and *ΔompF* mutant, but the data may be unreliable since their genetic analysis suggested that they did not have an actual *ΔompF* mutant (17). All three strains did not reach their stationary phase within the timeframe of our growth curve analysis, but the deviation of growth curve of JW0912-K2 from JW2203-K2 and BW25113 is evident, as seen in Figure 3. The slower growth rate of *ΔompF* strain may be an indicative of the importance of OmpF porin in the influx of nutrients during bacterial growth, especially in low salt concentration, which was the condition of our cultures (6). The growth curve established for BW25113, JW0912-K2 and JW2203-K2 should be repeated to validify this finding.

***E. coli ompF* mutants are more sensitive to kanamycin than *ompC* mutants at low salt concentration.** Due to the polycationic nature of kanamycin, we hypothesized that it would have a greater interaction with the more negatively charged OmpC porins than the less negatively charged OmpF porins. As a result, we speculated that the deletion of *ompC* gene should result in a greater resistance to kanamycin, as compared to wildtype and *ΔompF* mutant. Our result suggests the opposite, and deletion of *ompF* resulted in a two-fold increase in kanamycin resistance. The actual MIC values could not be determined from our results because of inconsistent values obtained between trials. More repetitions of the MIC assay would be necessary to support our claim, but the two-fold difference of the kanamycin MIC values of JW0912-K2 compared to those of BW25113 and JW2203-K2 were consistent.

OmpC porin has generally much lower permeability than OmpF, especially at low osmolarity, even though the two porins have very similar channel size (18). Since our strains were grown in low salt LB, the higher permeability of OmpF could be more involved in

passive diffusion of kanamycin across the outer membrane. This could also be linked to the level of expression of OmpF compared to OmpC porins at different osmolarities. OmpC and OmpF porin expressions are regulated through the EnvZ/OmpR two-component signal transduction system, which upregulates the expression of OmpF at lower osmolarities and induces expression of OmpC at higher osmolarities (19). Since we conducted our experiments at low salt concentrations, OmpF would have been the dominant porin that would be expressed. The wildtype and *ΔompC* mutant would be able to express the dominant porin, while the *ΔompF* mutant would be unable to do so, perhaps almost creating the phenotype of a double *ΔompCΔompF* mutant. If there are less porins on the outer membrane of the *ΔompF E. coli* available for kanamycin entry, it may also pose an explanation for the higher kanamycin resistance that was seen.

Future Directions The *ΔompC* and *ΔompF* Keio mutants we have characterized can be used in a variety of future studies. Repeats of the MIC assay would be necessary to confirm the observation of higher kanamycin resistance for the *ΔompF* mutant. Additionally, repeated growth curve analysis with regards to characterizing the lack of OmpF porin would determine the significance of OmpF porin in nutrient uptake. Furthermore, it may be interesting to test the sensitivity to other polycationic antibiotics such as polymyxin with the obtained mutant strains. This would determine if the cation selective property of porins is a specific mechanism for internalizing polycationic antibiotics. Lastly, MIC assays using media of increasing salt concentrations may be performed to see how porin expression levels are affected by osmolarity, which in turn may affect kanamycin susceptibility phenotypes.

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