Knockout of OmpF and OmpC does not affect kanamycin susceptibility in two different strains of *Escherichia coli* K-12

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SUMMARY OmpF and OmpC are key outer membrane diffusion porins in *Escherichia coli*. Previous studies have tested OmpF and OmpC mutants for resistance to the antibiotic kanamycin, with conflicting results. We set out to determine if the inconsistent results were due to the different *E. coli* K-12 strains employed by each study. Side by side minimum inhibitory concentration (MIC) assays were conducted to test kanamycin resistance of OmpF and OmpC mutants from 2 different *E. coli* genetic backgrounds. We observed no change in kanamycin resistance in the OmpF and OmpC mutants in either genetic backgrounds tested. Based on these results, we conclude that OmpF and OmpC play no role in kanamycin resistance in *E. coli* K-12. The genetic basis of the *ompF* deletion in *E. coli* strain JF700 had yet to be characterized in literature. We uncovered a 1195 bp IS5 transposon insertion within the *ompF* gene using Sanger sequencing. This insertion explains the lack of OmpF protein expression in this mutant.

INTRODUCTION

OmpC and OmpF are major outer membrane diffusion porins in Gram-negative bacteria (1). These porins consist of homotrimeric beta barrels with charged cores for facilitating diffusion of small hydrophilic molecules across the outer membrane (2). Different antibiotics such as ampicillin have been to shown to utilize the Omp porins for transport into bacterial cells (3).

Kanamycin is an aminoglycoside antibiotic, which are some of the most frequently used broad-spectrum antibiotics in clinical settings (4). This class of antibiotics works by inhibiting bacterial protein synthesis through binding of prokaryotic ribosomes, and therefore must transported into bacterial cells in order to exert its antimicrobial function (4). The small hydrophilic nature of kanamycin makes the Omp porins a prime target for outer membrane transport. Understanding how aminoglycosides such as kanamycin enter bacterial cells, is important to combating antibiotic resistance, as well as developing new antimicrobial therapies. Previous studies have looked into whether kanamycin enters *Escherichia coli* (*E. coli*) cells via the Omp porins, but have shown inconsistent results (5,6). A 1991 study by Hancock *et al.* determined that neither OmpF nor OmpC were involved in kanamycin resistance in *E. coli* (5). This study was followed up in 2018 by Chang *et al.*, who observed increased kanamycin resistance in Δ*ompF* mutants, leading them to conclude OmpF was indeed involved in kanamycin resistance (6). While the two studies were performed under identical growth and media conditions, the key difference was the strains of *E. coli* used.

Hancock *et al.* utilized strains generated by Foulds and Chai, arising from spontaneous mutations screened by phage and antimicrobial resistance (7). Chang *et al*. generated their strains by removing the kanamycin resistance cassette from single gene knockouts from the Keio strain collection (8). This difference in strain generation resulted in the two studies being conducted on different genetic backgrounds. Analysis of the CGSC database showed genetic variation across the two parent strains in a minimum of 17 genes (Table S1)(9).

Based on the variation in the genetic background between the strains used in each study, we hypothesize that the observed inconsistencies in kanamycin resistance in the Δ*ompF* mutants was due to strain specific effects. In this study we tested the minimum inhibitory concentration (MIC) of kanamycin required to inhibit growth in the Hancock *et al*. strains and the Chang *et al.* strains when tested side by side.

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METHODS AND MATERIALS

Strains and Media. *E.coli* K-12 BW25113 (CGSC#: 7636) and JF699 (CGSC#: 6043) served as WT controls. JW0912-2 (6) derived from JW0912-1 (CGSC #8925), and JF700 (CGSC#: 6044) served as Δ*ompF* strains. JW2203-2 (6) derived from JW2203-1 (CGSC #9781), and JF733 (CGSC#: 6044) served as Δ*ompC* strains. BW25113, JW0912-2 and JW0912-1 were acquired from Chang *et al.* and JF699, JF700, JF733 were purchased from the Yale Coli Genetic Stock Center. All strains were first streaked on low salt LB agar (1% peptone 0.5% yeast extract, 0.5% NaCl, and 1.2% agar), isolated colonies were then cultured in low salt LB broth (1% peptone 0.5% yeast extract and 0.5% NaCl).

Strains					
Strains used by Hancock *et al.*			Strains used by Chang *et al.*		
JF699 (WT)	JF700 *(ΔompF)*	JF733 (*ΔompC*)	BW25113 (WT)	JW0912 *(ΔompF)*	JW2203 *(ΔompC)*
CGSC#: 6043	CGSC#: 6044	CGSC#: 6044	CGSC#: 7636	CGSC #8925	CGSC #9781

Colony PCR of JW0912, JW2203 and BW25113. Colony PCR was performed with primers flanking *ompC* and *ompF*. *ompC* and *ompF* from BM25113 wildtype was also amplified as a positive control. In order to lyse bacteria prior to PCR, colonies were added to a PCR tube with 20uM NaOH, and placed in the thermocycler at 100^{o}C for 8 min. After the lysis step, Invitrogen Platinum *Taq* DNA (Catalog number: 10966018), manufacturer provided buffer, dNTPs and 10uM of the primers shown in Table 1 were added to the PCR tubes. Cycle conditions complied with manufacturer recommended temperatures. Initial 94^{o}C 30 sec denaturation step was followed by an annealing temperature of 52.5^{o}C for *ompC* primers and 55.3^{o}C for *ompF* primers for 30 seconds. Extension time was done for 1.5 minutes at 72^{o}C. The cycle was repeated 35 times followed by a final extension of 72^{o}C for 5 minutes.

Colony PCR of JF699, JF700 and JF733. Primers were made to amplify the region 240 bp upstream of *ompC* gene up to the end of *ompC*. As there is no annotated genome for JF700, *ompF* primers were made based of MG1655, starting 201bp upstream of *ompF* and ending 289 bases downstream of *ompF*. The colony PCR protocol above was repeated with annealing temperatures changed to 52^{o}C.

Gel Electrophoresis. After the PCR, amplicons were confirmed for both PCRs on a 1.5% agarose gel, in 0.5% TBE buffer with Invitrogen SYBR Safe DNA. The gels were then ran with a ThermoFischer TrackIt™ 1 Kb Plus DNA Ladder (Catalog number: 10488085) at 100 V for 45-55 minutes and photographed under UV light.

TABLE 1 *ompF* and *ompC* primers for Hancock *et al*. Chang *et al*. strains for PCR amplification and Sanger sequencing.

Target	Orientation	Sequence
ompC- Chang *et al.* strain	Forward	5'-GCAGGCCCTTTGTTCGATATCAATC-3'
	Reverse	5'-ATCAGTATGCAGTGGCATAAAAAAGC-3'
ompF- Chang *et al.* strain	Forward	5'-CGGCATTTAACAAAGAGGTGTGC-3'
	Reverse	5'-ACGGCAGTGGCAGGTGTC-3'
ompC- Hancock *et al.* strain	Forward	5'-GCCTTTTATCGTCTTGTTTATA-3'
	Reverse	5'-TTACGATCATTTAAACCAACTCCA-3'
ompF- Hancock *et al.* strain	Forward	5'-TTTGCCCGTCTCATCTTTATA-3'
	Reverse	5'-CACAAAGTTCCTTAAATTTTACTTTT-3'

Growth Curves of JF699, JF700, JF733, JW0912, JW2203 and BW25113. Overnight cultures of each strain were grown in LB and standardized by diluting to an OD_{600} of 0.05 using a spectrophotometer. 200ul was plated in quadruplicate in a 96 well plate. Plate was incubated in a BioTek automatic plate reader at 37°C for 16 hours with OD_{600} measured every 10 mins in order to generate a growth curve.

Minimum Inhibition Assays. Minimum inhibition assays were performed in triplicates in 96 well plates. Overnight cultures of each strain were prepared in LB broth and subsequently normalised to an OD_{600} of 0.08 on the day of experiment. 1:1 serial dilutions of stock kanamycin (100mg/mL) in LB broth yielded a range of 128 ug/mL to 1ug/mL kanamycin LB broth. 50uL of broth containing a bacterial strain and 50uL containing kanamycin were added to each well, totalling 100uL. Each well had a final concentration of kanamycin ranging from 64ug/mL to 0.5ug/mL. Plates were sealed and placed on the shaker and incubated at 37°C and 180rpm for 16-20 hours. Readings of the plates were performed by eye and via the BioTek Plate Reader**.** The MIC was determined to be the lowest kanamycin concentration with no growth in at least two of the three replicates.

RESULTS

Growth yield and growth rate were similar across all strains. We set out to determine the growth rate and yield of all 6 strains in our experiment, to assess if there were any intrinsic growth differences that could impact our MIC assay incubation times or results. After performing a 16-hour growth curve at 37°C, we found that JW9012, JW2203, and BW25113 grew to a higher optical density compared to strains JF699, JF700, and JF733, but the growth rates of all the strains were similar during log phase (Fig. 1). From this we determined we could use equal incubation times for our MIC assays of each strain for consistency, without compromising validity of our data.

PCR Genotyping of the JW0912, JW2203 and BW25113 strains used by Chang *et al.* To confirm the identity of our isolated WT, Δ*ompF,* and Δ*ompC* strains, we performed PCR to detect the presence or absence of the *omp* genes. Primers flanking the *ompF* or *ompC* genes were utilized to amplify the intervening gene segment (Fig. 2). A no template

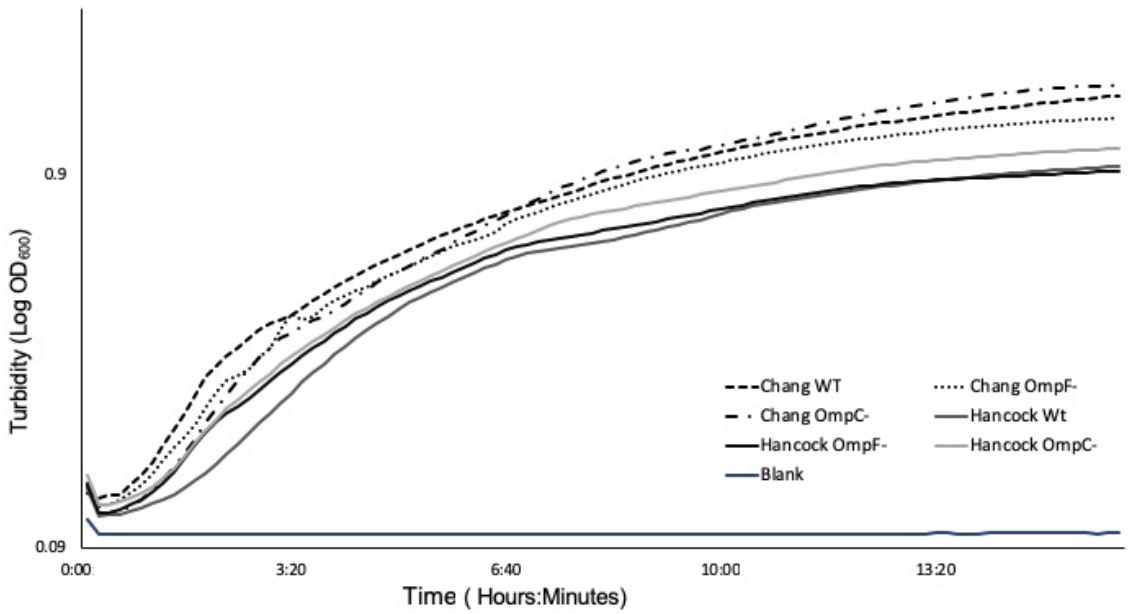

FIG. 1 Growth curve comparisons of Chang *et al*. Δ*ompC*, Δ*ompF, and* WT strains compared to Hancock *et al*. Δ*ompC*, Δ*ompF* and WT strains. Bacterial growth of all studied strains were measure for 16 hours with OD_{600} readings every 10 mins. (n=1).

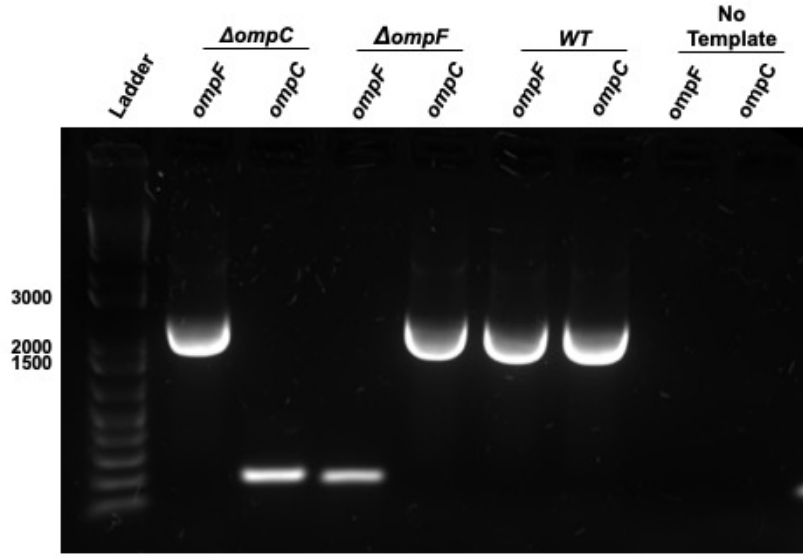

FIG. 2 PCR and Gel analysis confirming *ΔompF* and *ΔompC* mutants for JW0912, JW2203, BW25113. Using colony PCR, ompF and ompC primers amplified their respective genes in the *ΔompC, ΔompF,* and WT *E. coli* K-12 keio strains. Amplicons were then run on 1.5% agarose gel for 50 minutes at 100V. Each set of primers were used on all 3 strains along with a no template control and PCR control. OmpC and OmpF sequences correspond to bands between 1500 and 200 bp. Scar sequences correspond to 300 bp.

negative control detected no nonspecific primer amplification. The ΔompC mutant showed full length *ompF* and the scar sequence replacing its *ompC* gene. The scar sequence is a small, known nucleotide fragment that was used by Chang *et al.* to replace the kanamycin cassette previously present in the Keio strains. The Δ*ompF* mutant had a full length *ompC* gene and a deleted *ompF* gene. The WT had full length *ompF* and *ompC* genes. These data matched with the methods Chang *et al.* used to create their strains, where the mutant gene of interest had been replaced with a short scar sequence, while the WT genes were left untouched. As our data matched the desired genotypes, we deemed our characterization successful at confirming the desired identity of our strains.

Sanger sequencing of the JF699, JF700 and JF733 strains used by Hancock *et al.* and novel characterization of the JF700 *ΔompF* mutant. In order to confirm the identity of the strains used by Hancock et al., we used PCR to amplify their *ompF* and *ompC* genes and then performed Sanger sequencing on the purified products. It is known that JF733 has a point mutation in the promoter sequence of its *ompC* gene that is believed to prevent transcription and subsequently lead to lack of OmpC protein (10). This mutation is located 90 bp upstream of the *ompC* gene (10). We sequenced this region of the genome and found 100% sequence similarity with the previously characterized JF700 genome (Fig. 3) (Supp. sequencing data JF733). The *ΔompF* mutant is known to have developed from a spontaneous mutation, but had yet to be genetically characterized in literature (7). We observed the presence of a larger *ompF* gene in the *ΔompF* mutant relative to the WT (Fig. S1). Further sequencing showed a 1195bp IS5 transposon insertion 283 bp from the end of the *ompF* gene (Fig. 3) (Supp. Sequencing data JF700). From these data, we are able to confirm the JF733 *ompC* mutant and characterization of an insertion within the *ompF* gene of the *ompF* mutant JF700.

No difference in kanamycin susceptibility across strains or *omp* mutants. Once our strains were characterized, we aimed to perform MIC assays to compare kanamycin susceptibility of our strains. We used a 96 well liquid MIC assay with serial dilutions of kanamycin and observed growth inhibition using a BioTek plate reader. We found there was no difference between the Hancock *et al. ompF* and *ompC* mutants and WT within each replicate in terms of kanamycin susceptibility (Table 2). There was also no difference in kanamycin susceptibility between the Chang *et al. ompF* and *ompC* mutants and WT (Table 2). In terms of absolute MIC, there was no difference between the strains, as both strains

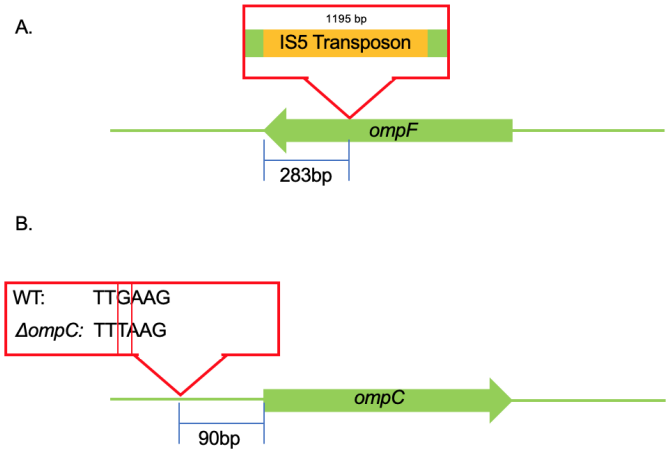

FIG. 3 Loss of function mutations in JF700 and JF733: Using specific primers, *ompC* and *ompF* were amplified using colony PCR and sent for Sanger sequencing at GENEWIZ. Sequences were searched for alignments to sequences in the NCBI GenBank non-redundant database using BLAST. *ompC* was aligned against the previously annotated *ompC* sequence. Transposon insertions found in *ompF* were confirmed with the IS finder BLASTN tool, and searched against the ISfinder_Nucl database.

consistently showed an MIC of 16 ug/ml. A single Hancock *et al.* replicate did show a reduced MIC of 8 ug/mL, but within that replicate there was no MIC difference between any of the mutants and WT (Table 2).

DISCUSSION

Previous studies done by Chang *et al.* and Hancock *et al.* testing kanamycin resistance in Δ*ompC* and Δ*ompF* strains with different genetic backgrounds, yielded conflicting results (5,6). In this study, we observed no differences in kanamycin resistance across any mutant or any strain (Table 1). This finding supports the previous work by Hancock *et al.*, who saw no effect of OmpC or OmpF deletion on kanamycin resistance (5). After repeated attempts, we were unable to replicate the observed increase in kanamycin resistance in the Δ*ompF* mutants observed by Chang *et al.* When performing our MIC assays, we found the Chang *et al.* strains to be much more inconsistent in growth patterns, and had to repeat the experiment many times in order to get consistent and reproducible results. Within each trial, our technical replicates often did not align with one another and higher kanamycin concentration occasionally showed increased levels of growth than lower concentrations. This inconsistency was also reported by Chang *et al.*, and the lack of replicates they performed may have lead them to report a difference in resistance that was not truly representative (6). The limited number of biological replicates performed by Chang *et al.* is our best explanation for their reported results, and we feel if they had repeated their assays numerous times they would have seen data in line with Hancock *et al.* and our study. No such variations were observed when testing the strains used by Hancock *et al.*

In order to better understand the growth differences amongst the strains, a 16 hour growth curve was done (Fig. 1). Hancock *et al.* and Chang *et al.* strains all had similar growth rates between 3 and 6 hours of incubation. However, the strains from Chang *et al.* had a slightly higher final growth yield than those used by Hancock *et al.*. This increase in growth was minimal and consequently, we did not deem it an important consideration for our MIC assays.

We next sought to confirm the mutations of the Hancock *et al.* strains by Sanger sequencing. Previous studies show that the JF733 *ompC* promoter region lies 110-80 bp upstream from the start codon (11). Consequently, it has been shown that JF 733 bears an T to G substitution mutation 90 bp upstream from the start codon that is thought to be sufficient in preventing transcription of the *ompC* gene (10). Our sequenced JF733 ompC was compared to the previously annotated JF733 genome (10). Sequence alignments revealed 100% identification with the JF733 reference sequence, including the -90bp

TABLE 2 Minimum Inhibitory Concentration Assays. LB inoculated with *E. coli* standardized to OD_{600} of 0.08 were added to serial dilutions of kanamycin. OD_{600} readings from BioTek plate reader were used to determine minimum concentration of kanamycin required to inhibit bacterial growth. 3 technical replicates were performed per assay (n=5 Hancock *et al*., n=4 Chang *et al*.).

	MIC (ug/mL Kanamycin)					
	Strains used by Hancock *et al.*			Strains used by Chang *et al.*		
Trial #	699 (WT)	700 (*ΔompF)*	733 (*ΔompC*)	25113 (WT)	0912 (*ΔompF)*	2203 (*ΔompC)*
1	16	16	16	16	16	16
2	8	8	8	16	16	16
3	16	16	16	16	16	16
4	16	16	16	16	16	16
5	16	16	16	-	-	-

substitution mutation. Although the appropriate mutation was found, whether OmpC is being transcribed and translated remains to be tested. Past reports have showed that JF733 does not produce OmpC however many of these studies are dated, and newer confirmation may be beneficial (7).

Strain JF700 had never before been sequenced in the literature, and upon analysis of the *ompF* and *ompC* gene regions, a 1195 bp transposon insertion was discovered in the *ompF* gene (Supp. sequencing data JF700). Previously the loss of OmpF in JF700 was attributed to an unknown spontaneous mutation (7). Using BLAST sequence alignment, this insertion mapped to a IS5 transposon that inserted 283bp downstream of the *ompF* start codon. As the IS5 transposon inserted directly into *ompF,* this insertion is almost certainly the cause of the gene loss of function.

Once all the strains were confirmed for their appropriate mutations, we proceeded to perform MIC assays. Throughout our MIC assay replicates, the strains used by Chang *et al.* did not always show the almost perfect consistency and reproducibility of the strains used by Hancock *et al*. Many trials were necessary for establishing a reliable kanamycin MIC for the strains, since the technical replicates often displayed variance. Although we were able to establish consistency after extensive technical practice, further experiments into growth kinetics of the strains used by Chang *et al.* might help explain the variation in uniform growth and susceptibility.

Our MIC assays reveal that there were no difference in kanamycin MIC between the Hancock *et al.* strains and the Chang *et al.* Strains (Table 1). This finding is in accord with the previous observations of Hancock *et al.* while investigating the roles of OmpC and OmpF in aminoglycoside resistance (5). Nevertheless, the absolute kanamycin MICs that we observed were double those reported by Hancock *et al.* (5). This difference may be explained by the different ways that MIC be assessed, as Hancock *et al.* used visual observation while we used a spectrophotometer. Furthermore, the drop off in turbidity as the MIC of kanamycin was reached were different between the Hancock *et al.* and Chang *et al.* strains. The growth of the Chang *et al.* strains dropped steadily with increasing kanamycin concentration. In contrast, the Hancock *et al.* strains had a distinct sharp drop in turbidity at their MIC, when measured on the plate reader. This may suggest a difference between the strains in membrane permeability in the context of kanamycin uptake.

Regardless of the difference in MIC between our study and the Hancock study, the MIC between Omp knockouts and WT were identical and consistent amongst replicates. We initially hypothesized that differences in genetic background between the Hancock *et al.* and Chang *et al.* strains was the source of their conflicting findings on kanamycin resistance. However, since we found no observable difference in kanamycin resistance

between the Hancock *et al.* and Chang *et al.* strains, and between OmpF and OmpC mutants, we conclude our initial hypothesis was incorrect, and the differences observed by Chang *et al.* are due to a different cause. The lack of kanamycin resistance amongst Omp knockouts was not restricted to the strains from Hancock *et al.,* but was also true for the Chang *et al.* strains.

Future Directions Based on the confidence of our findings, and the previous work by Hancock *et al.,* we do not feel further studies into kanamycin susceptibility in Δ*ompC* and Δ*ompF E. coli* is worthwhile. Since OmpF and OmpC are very similar porins it is possible there is some redundancy in their function. It may be interesting to study if a Δ*ompC*Δ*ompF* double knockout mutant in the same genetic background used by Chang *et al.* displays any variation in kanamycin susceptibility. This has previously been tested by Hancock *et al.* who saw increased kanamycin susceptibility in Δ*ompC*Δ*ompF,* but using a different genetic background may provide further clues about kanamycin transport across the outer membrane (5).

In order to supplement the characterisation of JF733 and JF700, western blots could be performed on colonies probing for OmpC and OmpF respectively to confirm lack of protein. A functional analysis of the Omp porins could also be analysed by testing ampicillin resistance on the mutants, since Omp porins are known to have a role in ampicillin transfer (3).

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CONTRIBUTIONS

All wet lab work was evenly distributed with each member performing each task at various time points.

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