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# Regulation of Osmotic Stress by OmpC but not OmpF Increases Kanamycin Resistance in *Escherichia coli*

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**Changing the ratio of outer membrane proteins, OmpF and OmpC, is a mechanism by which *Escherichia coli* are able to develop antibiotic resistance. Osmotic stress is known to affect the outer membrane protein ratio by increasing OmpC and decreasing OmpF expression. Our study assessed whether kanamycin resistance under high osmolarity was mediated by an increase in the ratio of OmpC to OmpF. Minimum inhibitory concentration assay for kanamycin and an SDS-PAGE analysis of the outer membrane proteins were performed for wild-type, TALT12W-1 (Δ*omp*C), and TALT12W-2 (*omp*F$^+$) bacteria grown in media containing varying NaCl concentrations.TALT12W-2 was initially thought to be Δ*omp*F, but our PCR showed the presence of the *ompF* gene and the SDS-PAGE analysis also showed OmpF protein expression in the strain. Our results showed an increase in kanamycin resistance with increasing osmotic stress; however, we also saw an increase in resistance comparable to the wild-type cells in the Δ*omp*C mutants at higher NaCl concentrations. Although we observed an increase in OmpC protein levels with increasing NaCl concentrations in the wild-type and *ompF*$^+$ cells, we did not see a corresponding decrease in OmpF. Taken together, these results suggested that the ratio of OmpC to OmpF plays a role in gaining resistance to antibiotics with the increase in OmpC expression, but may not be the only mechanism conferring resistance.**

*Escherichia coli* have been shown to develop resistance to antibiotics through a number of mechanisms, such as increasing the ratio of OmpC to OmpF in their outer membranes (1, 2, 3). This resistance may be the result of the smaller pore diameter of OmpC compared to OmpF, which are 38 and 37 kDa, respectively (4, 5). Kanamycin, an aminoglycoside, is one example of an antibiotic that could diffuse into the cell at a lowered rate due to an increase in the OmpC to OmpF protein ratio.

*E. coli* has a number of mechanisms for regulating the relative expression of OmpF and OmpC such as the EnvZ and OmpR regulatory system (3). In the system, EnvZ within the cytoplasmic membrane senses an increase in the surrounding osmolarity, undergoes autophosphorylation, and then phosphorylates the OmpR regulatory protein. The phosphorylated OmpR will then repress the transcription of the *ompF* gene, and increase the transcription of the *ompC* gene (3).

This study aimed to determine the relationship between OmpC and OmpF expression following osmotic shock and the effects of this relationship on kanamycin susceptibility. Our results demonstrated that the osmotic stress-induced regulation of OmpC, but not OmpF, led to an increase in kanamycin resistance.

## MATERIALS AND METHODS

**Bacterial strains and growth media**. *E. coli* K-12 from the Keio collection (6) housed at the Coli Genetic Stock Centre (CGSC) were obtained through the MICB 421 culture collection of the Microbiology and Immunology Department at the University of British Columbia. Strains BW25113 (wild-type, CGSC#7636), JW0912 (Δ*omp*F, CGSC #8925), and JW2203 (Δ*omp*C, CGSC #9781) were cultured using Luria-Bertani (LB) liquid media made using the Keio collection specifications (6) at a concentration of 0.085, 0.2, 0.4, or 0.6 M of NaCl (Fisher #102039). Solid LB was prepared by adding 1.5% bacto-agar (Invitrogen #30391-023).

**Preparation of competent cells.** Electrocompetent JW2203 and JW0912 cells were prepared from methods previously described by Sidhu *et al.* (7). 1.5 ml of each overnight culture was centrifuged and washed twice with cold, sterile distilled water, followed by one wash with 100 µl ice-cold 10% glycerol. The final pellets were resuspended in 50 µl of 10% glycerol.

**Isolation of pCP20 plasmid.** PureLink® HiPure Plasmid Filter Midiprep Kit (Invitrogen #K2100-15) was used to isolate pCP20 plasmids from *E. coli*BT340 (CGSC #7629). Concentration and purity of the isolated plasmid were assessed via 1.2% agarose gel electrophoresis and a UV-visible NanoDrop (Thermo Scientific, model 2000c).

**Removal of kanamycin resistance gene from JW2203 and JW0912.** To remove the kanamycin resistance genes inserted into the JW2203 and JW0912 genomes, cells were transformed with pCP20 plasmid, which carries FLP recombinase and ampicillin resistance. An adapted protocol from Sidhu *et al.* (7) was used. Electrocompetent cells were transformed using a 0.2 cm GenePulser® Cuvette (BioRad #165-2082) in a BioRad MicroPulser® following the Ec2 setting. The 1 hr outgrowth at 30°C was performed using 1 ml of SOC medium (Invitrogen #15544-034). Following outgrowth, transformants were spread plated onto LB-ampicillin (100 µg/ml) and incubated overnight at 30°C. Subsequently, a colony for each strain was re-streaked to a fresh LB-ampicillin plate and incubated overnight at 30°C. Ampicillin resistant colonies were re-streaked onto LB plates and incubated overnight at 42°C. This step was repeated by re-streaking colonies onto LB plates and incubating overnight at 42°C. Following the final incubation, colonies were grid plated onto LB-ampicillin (100 µg/ml), LB-kanamycin (50 µg/ml), and LB, then grown overnight at 30°C. A doubly-sensitive colony for each strain was selected, with the JW2203 transformant renamed as TALT12W-1, and the JW0912 transformant renamed as TALT12W-2. These strains were used in all subsequent assays.

**Determination of the minimum inhibitory concentration of kanamycin.** The protocol from Wiegand *et al.* (8) was used for the minimum inhibitory concentration (MIC) assay. Overnight cultures of BW25113, TALT12W-1, and TALT12W-2 were diluted to an $OD_{600}$ of 0.15 in LB with 0.085, 0.2, 0.4, or 0.6 M of NaCl and grown at 37°C to an $OD_{600}$ of 0.6. Osmotically stressed

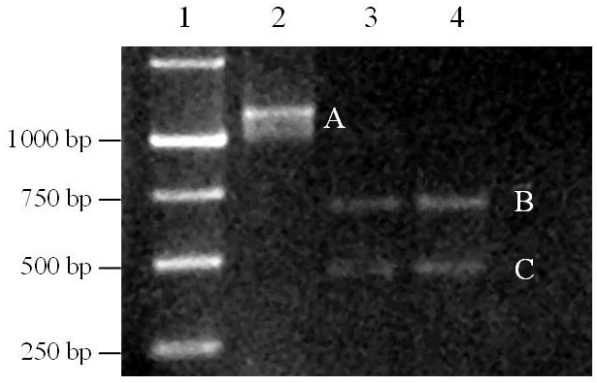


**FIG 1** Presence of *ompF* in TALT12W-2 confirmed by PCR and restriction enzyme analysis. Lane 1 molecular weight ladder; lane 2 undigested PCR products of *ompF* gene; lane 3 PvuII digest for TALT12W-2 (transformant; *ompF*$^+$); lane 4 PvuII digest for BW25113 (wild-type). Bands A, B, and C refer to 1139, 693, and 446 bp fragments, respectively.

cells were diluted to an $OD_{600}$ of 0.005 and 50 µl cells was added to 50 µl 2X kanamycin stocks in a 96-well plate (Becton-Dickinson #30115). Plates were incubated for 24 hours at 37°C, after which the plates were visually interpreted. The MIC was estimated as the lowest concentration of kanamycin where there was no visible growth.

**Outer membrane protein isolation.** The isolation of outer membrane proteins was performed following the procedure described by Carson *et al.* (9). Overnight cultures of BW25113, TALT12W-1, and TALT12W-2were diluted and grown to 0.6 $OD_{600}$with 0.085, 0.2, 0.4, or 0.6 M of NaCl. 200 ml of the cultures were pelleted by centrifuging at 7,000 x g for 10 min in the Sorvall RC-5B centrifuge using the SLA-1500 rotor. The pellets were resuspended in 5 ml L1 lysis buffer (9) and lysed at 1,500 psi using a French pressure cell for 2 cycles. The lysate was centrifuged at 2500 x g for 25 min at 10°C in a Beckman Coulter Avanti J30-I centrifuge using a JA-20 rotor. The supernatant was then centrifuged at 35,000 x g for 40 min at 10°C using a JA-30.5 rotor. The supernatant was discarded; the pellet was resuspended in 10 ml of W2 wash buffer (9) and then centrifuged at 35,000 x g for 40 min at 10°C using a JA-30.5 rotor once more. To solubilize the inner membrane, the pellets were resuspended in 4 ml of 2% Triton X-100 (BioRad #1610407) in 10 mM Tris-HCl pH 8, incubated for 30 min at room temperature, and then stored at -80°C prior to buffer exchange.

**Buffer exchange for outer membrane proteins.** To solubilize the outer membranes for SDS-PAGE analysis, the buffer was exchanged to 2% SDS (BioRad #161-0302) in 8 mM Tris-HCl pH 8. Buffer exchange was performed by centrifuging the isolated outer membrane proteins in an Amicon Ultra-0.5 with 30 kDa cut off (Millipore #UFC503024) at 14,000 rpm for 10 min in a microcentrifuge (Eppendorf, Model 5415D), and then subsequently washing with 2% SDS buffer twice. Final recovered volume was 250 µl, which was stored at -20°C prior to SDS-PAGE analysis.

**Determining concentration of outer membrane proteins.** Protein concentration was determined using the bicinchoninic acid assay (BCA). The Micro BCA Protein Assay Kit (Thermo Scientific #23235), substituting cupric sulfate (Sigma #C-2284) for Reagent C, was used. Bovine serum albumin (Sigma #A-2153) was used to create a standard curve and all samples were read in a Beckman Coulter 8 mm HIGH cuvette (#523540) in a Biochrom Ultraspec 3000.

**SDS-PAGE of outer membrane proteins.** The procedure described by Carson *et al.* (9) was followed, with a4% stacking and 12% separating gel used. The gel was loaded with 10 µg protein in 5X loading buffer and a high standard protein ladder (Sigma), then run at 100 V for 3 hrs.

**Colony PCR and restriction enzyme analysis.** The PCR reaction mixture consisted of primers for the *ompF* gene based off of sequences previously determined in the Keio collection (6), (forward primer: 5'-GAGGTGTGCTATTAGAACTGGTAAACGATACC-3' and reverse primer: 5'-GACGGCAGTGGCAGGTGTCAT-3') and *Taq* DNA polymerase (Invitrogen) following the reaction composition suggested by the manufacturer. To the 50 µl reaction mixture, a small amount of BW25113 or TALT12W-2 colony was added. The first cycle consisted of extended initial denaturation of 12 min at 94°C, followed by 35 cycles of 94°C for 1 min, 56°C for 0.5 min, and 72°C for 1.5 min. Following PCR, 10 µl of product was digested with PvuII (Fermentas) for 1 hr at 37°C. The expected *ompF* PCR product was 1139 bp, and the PvuII enzyme was expected to cut *ompF* once to yield a 693 and 446 bp fragment.

## RESULTS

**PCR suggested TALT12W-2 as having *ompF*.** Previous results from Kuzhiyil *et al.* (10) suggested that OmpF may be expressed, therefore implying the presence of *ompF* in the JW0912 (Δ*ompF*) strain. PCR performed using primers flanking the *ompF* gene showed that the TALT12W-2 strain (derived from JW0912) and wild-type BW25113 strain shared an approximate 1 kb product (lane 2) in the same position (Fig. 1). Restriction digestion with PvuII further identified the band to be *ompF*, thus confirming TALT12W-2 and JW0912 to be *ompF*$^+$.

**NaCl-induced osmotic stress increased the MIC of kanamycin.** To determine the effects of osmotic stress on kanamycin resistance, an MIC for all three strains grown in varying concentrations of NaCl (Table 1) was performed, which showed an increase in resistance to kanamycin as a function of increasing NaCl concentrations.

The MIC value of 13 µg/ml kanamycin measured for the wild-type *E. coli* at 0.085 M NaCl was also consistent with the value from the same strain used by Sidhu *et al.* (7).The MICs for the wild-type and *ompF*$^+$ were highest in 0.4 M NaCl, where the observed MIC showed an approximate two-fold increase in value compared to 0.2 M NaCl. In contrast, the Δ*ompC* strain exhibited an approximate three-fold increase in MIC when cultured in 0.085 M to 0.2 M NaCl and showed less than a two-fold increase in 0.2 to 0.4 M NaCl. Δ*moo*ched a decreased ability to improve its resistance to kanamycin as the concentration of NaCl increased, although it achieved resistance comparable to

**TABLE 1** MIC values of kanamycin for *E. coli* strains at varying NaCl concentrations.

| [NaCl] (M) | MIC of Kanamycin (µg/ml) | | |
|---|---|---|---|
| | wild-type (BW25113) | Δ*ompC* (TALT12W1) | *ompF*$^+$ (TALT12W2) |
| 0.085 | 13 | 6 | 13 |
| 0.2 | 15 | 20 | 20 |

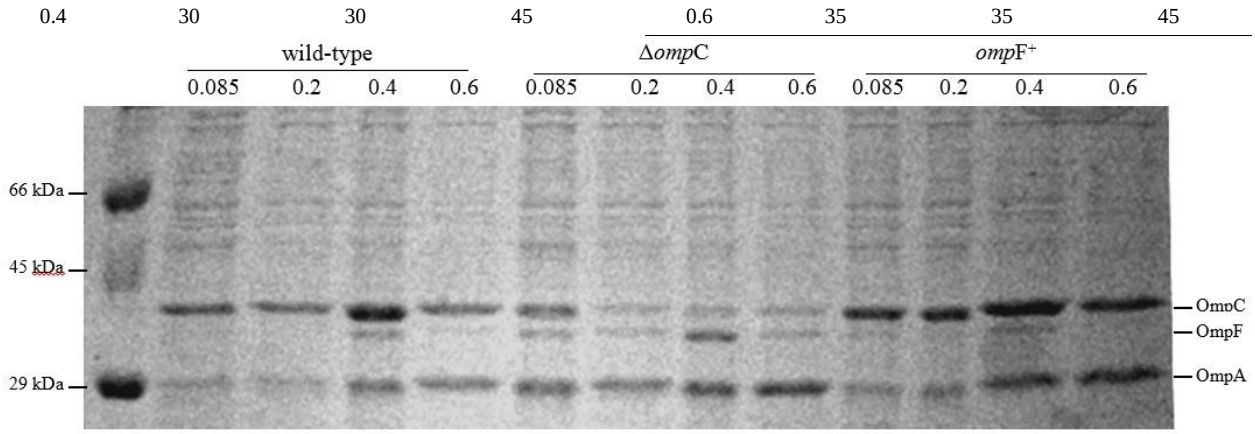


**FIG 2** Growth of *E. coli* in varying concentrations of NaCl affects the expression of OmpC and OmpF. First lane corresponds to protein standard; numbers above each lane refers to concentration (M) of NaCl added to growth medium.

wild-type. In contrast to the wild-type cells, higher MIC values were observed for *omp*F$^+$ at 0.4 and 0.6 M NaCl, which were not expected since *omp*F$^+$ should be similar to wild-type.

**Growth in NaCl altered the expression profile of OmpC and OmpF in the outer membrane.** To determine if the observed increase in resistance to kanamycin was mediated by the relative expression of OmpC and OmpF in response to osmotic stress, an SDS-PAGE of the isolated outer membrane proteins (Fig. 2) was performed which showed a mixed expression profile of OmpF and OmpC. In each lane, the lowest band at about 29 kDa corresponds to OmpA, above it is OmpF (37 kDa), and then OmpC at the highest (38 kDa). We attempted to normalize the band intensities by observing the relative band intensity of OmpC and OmpF compared to OmpA. Therefore, the decreased band intensity seen with 0.6M compared to 0.4 M NaCl wild-type condition would actually appear darker, if the same amount of protein had been loaded on the gel. Interestingly, for the wild-type, we expected to see strong OmpF bands in 0.085 and 0.2 M NaCl conditions, yet we observed no bands. Additionally, the gel indicated that *omp*F$^+$ had a band corresponding to the OmpF molecular weight region in all salt concentrations. This would indicate that either it was expression of a protein with similar molecular weight, or more likely that the strain expresses OmpF, as we found that the strain still contains the *omp*F gene. Overall, the expression of OmpF in TALT12W-2was similar in intensity to the bands seen in the wild-type strain. Furthermore, the gel indicated that the Δ*omp*C mutant expressed OmpC, most apparent at 0.085 M NaCl. The band intensity was lower than what was observed in the other two strains at this concentration, which could be indicative of a knockdown in expression, as opposed to a knockout. We observed that the highest expression of OmpC was seen in *omp*F$^+$ at 0.4 and 0.6 M NaCl which, when combined with the MIC results, suggested that OmpC contributed to the increased kanamycin resistance. The wild-type strain showed a similar trend, where the highest expression of OmpC in 0.4 and 0.6 M NaCl led to a higher MIC value, although lower than what was seen for *omp*F$^+$. However, where the wild-type strain had maximum OmpC expression at 0.4 and 0.6 M NaCl, Δ*omp*C showed minimal expression, yet yielded the same MIC value.

## DISCUSSION

Inducing osmotic stress in *E. coli* by increasing the NaCl concentration has shown to confer an increase in the resistance of bacteria to kanamycin, which involved OmpC regulation. When subjected to osmotic stress, wild-type *E. coli* showed an increased resistance to kanamycin. The increased resistance correlated to the levels of OmpC and OmpF in the outer membranes, as seen in the SDS-PAGE, for the wild-type strain (Fig. 2). The increase in OmpC levels in wild-type cells grown in increasing salt concentrations confirmed the results by Pratt *et al.* (3). These observations also confirmed previous studies that have shown that at a higher osmolarity, the EnvZ/OmpR system regulates the activation of *omp*C transcription, leading to increased OmpC expression (11).This increase in OmpC is necessary for resistance because OmpC, having a smaller pore diameter than OmpF, will effectively slow the diffusion rate of solutes into the cell (3).Therefore, the increase in OmpC protein observed on the gel may explain the increased resistance to kanamycin observed in the MIC assays.

The Δ*omp*C mutant should lack expression of OmpC and have only OmpF in the outer membrane. Having only OmpF proteins to regulate cellular osmolarity would suggest that the Δ*omp*C strain should show increased susceptibility to kanamycin relative to the wild-type. However, the wild-type and Δ*omp*C strain did not support this theory as they showed similar MIC values. Additionally, it was unexpected that the Δ*omp*C strain would have bands corresponding to OmpC proteins on the gel, which appeared to have a trend of decreasing levels of OmpC as a function of increasing salt concentrations. Because the *ompC* gene was deleted, we expected only basal levels of OmpC expression. Any basal level of OmpC protein that appeared on the gel should increase with increasing salt concentrations, which was opposite to

what was observed. Also, the OmpF protein for the Δ*omp*C strain appeared to have the same unexpected increasing trend with increasing salt concentrations as the wild-type, up to 0.4 M NaCl. Because little correlation exists between the observed kanamycin resistance and the relative Omp proteins for the Δ*omp*C strain, it is likely that regulating the ratio OmpC and OmpF is not the only mechanism contributing to the increased resistance. It has been shown by Kuzhiyil *et al.* that as the concentration of salt increased, the thickness of the resulting *E. coli* capsule also increased, which conferred kanamycin resistance (10).It is therefore possible that capsule formation may play a larger role in the associated increase in kanamycin resistance than does the OmpC and OmpF proteins.

The Δ*omp*F strain should lack the expression of OmpF, and have only OmpC available to regulate cellular osmolarity. A true Δ*omp*F strain should exhibit greater resistance to kanamycin relative to the wild-type and the Δ*omp*C strain due to a slower diffusion rate of kanamycin into the cell. Because the expression level of OmpF seen in the *omp*F$^+$ strain seemed comparable to the level seen in the wild-type strain, the supposed *omp*F deletion mutant used may have had an intact *omp*F gene. This was confirmed by PCR and a subsequent restriction digest, suggesting that the Δ*omp*F strain obtained from the Keio collection may not have the said deletion and is genotypically similar to the wild-type strain for the *omp*F gene. However, a higher expression of OmpC was observed in this strain compared to wild-type, which corresponds to the higher MIC values. Although the strain was not a true *omp*F mutant, the MIC assay indicated that it did not appear to be the same as the wild-type either. The fact that the strain grew when first plated with kanamycin suggests that a gene deletion may have occurred elsewhere in the genome besides the *omp*F gene, which would explain why the strain was still *omp*F$^+$ like wild-type, but had a different MIC value. These accounts may be attributed to the construction of the deletion mutants in the Keio collection.

The Keio collection was generated by replacing the gene to be deleted with a kanamycin cassette through homologous recombination, followed by PCR analysis to verify deletion (6). Because the *omp*C (JW2203) and *omp*F (JW0912) deletion mutants grew in the presence of kanamycin while the wild-type strain did not, the resistance cassette must have been successfully inserted. However, it is possible that when incorporated into the genome, the kanamycin cassette recombined elsewhere, which removed a gene segment other than the desired *omp* gene. Considering OmpC and OmpF are factors essential to the normal regulation of cell osmolarity, the growth of the *omp*F and *omp*C deletion mutants may be slower than mutants with random inserts elsewhere in the genome. Thus, over time, it is possible that those mutants with intact *omp*F or *omp*C may outgrow the original mutants. This could explain why the strains were resistant to kanamycin but still expressed either OmpC or OmpF proteins, and in the case of the Δ*omp*F mutant, still carried the *omp*F gene. It is important to note that the PCR confirmation of the gene deletion was done only for the *omp*F and not the *omp*C deletion mutant.

Our results confirmed that osmotic stress led to resistance to kanamycin in wild-type *E. coli*. The increased MIC values were correlated to higher levels of OmpC in the outer membrane, suggesting the importance of OmpC in conveying resistance to kanamycin. However, due to issues with the *omp*F$^+$ and Δ*omp*C strains, the importance of the role that the outer membrane proteins play in the resistance could not be conclusively determined. More research is needed to better understand the deletion strains and the functional importance of the OmpF and OmpC protein resistance mechanism compared to other resistance mechanisms, such as capsule formation.

## FUTURE DIRECTIONS

It was stated in the paper by Baba *et al.* (6) that *omp*F deletion in JW0912 was confirmed via PCR using a combination of locus- and kanamycin-specific primers. However, results from our PCR indicated the presence of *omp*F gene despite having gained resistance to kanamycin. Further work is therefore needed to understand the discrepancy between the data. A verified *omp*F deletion mutant should be used as a positive control for *ompF* deletion rather than working with the JW0912 strain. A new batch of JW0912strain should also be requested in the event that there was a mix-up between the strains. In addition, because our SDS-PAGE showed bands with sizes corresponding to OmpC, the *omp*C gene may still be present in the TALT12W-1strain. Thus, PCR confirmation of *omp*C deletion should also be performed. Additionally, other approaches to control the outer membrane proteins such as using conditional knockdown mutations should be considered if complete *omp*C and *omp*F gene deletions are lethal and mutants cannot be stably generated.

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