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The Role of *Escherichia coli* Porins OmpC and OmpF in Antibiotic Cross Resistance Induced by Sub-inhibitory Concentrations of Kanamycin

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Previous studies have shown that pretreatment of *E. coli* with low doses of kanamycin have generated an increase in the resistance to high doses of kanamycin as well as induced cross resistance to a broad range of unrelated antibiotics, and that multi-antibiotic-resistant *E. coli* had lower levels of OmpF porin. In this study, the role of OmpC and OmpF porins in the development of cross resistance was investigated by comparing the levels of these porins in kanamycin untreated and pretreated *E. coli* K-12. To demonstrate the difference in protein levels, outer membrane protein fractions from wild type, Δ*omp*C, and Δ*omp*F strains were isolated by French press and analyzed by SDS-PAGE. Minimal Inhibitory Concentration (MIC) assays were used to confirm the development of the cross resistance due to exposure to low doses of kanamycin and related to the levels of porins visualized using SDS-PAGE. Outer membrane protein profile of the wild type showed lower levels of OmpC after pretreatment with kanamycin. Kanamycin pretreatment did not seem to alter the outer membrane protein profiles of both mutant strains. Surprisingly, the *Δomp*F strain had outer membrane protein profile identical to that of the wild type strain. Resistance to kanamycin of both mutants was 32-fold higher than that of the wild type. Upon kanamycin pretreatment, only the wild type showed a slight but reproducible increase in MIC. *E. coli* K-12 mutant strains *Δomp*C and *Δomp*F did not display any reproducible changes in MIC in response to kanamycin pretreatment. Our results indicated that OmpC porin may have a role in the development of resistance to kanamycin in the wild type *E. coli* K-12, but it is an unlikely factor in development of cross resistance to other tested antibiotics. The role of OmpF in the cross resistance could not be clearly identified.

The growing number of antibiotic-resistant bacterial strains requires an in-depth understanding of the mechanisms that confer antibiotic resistance. Although initially it was believed that sub-inhibitory doses of antibiotics were responsible for selecting for bacterial cells with increased resistance to inhibitory doses of the related antibiotics (13, 14), further studies revealed that growth of bacteria in the presence of sub-lethal doses of certain antibiotics protected the bacterial cells against a broad range of other unrelated antibiotics (4) as well as organic solvents and biocides (8). These observations suggest that the mechanism for this cross resistance must be general rather than specific. A number of plausible general mechanisms for the development of cross-resistance have been suggested. One proposed non-specific resistance mechanism involves cell envelope modifications which serve to prevent entry of solutes into the cell such as formation of capsule or regulation (6, 7) and expression of outer membrane porins (3, 5).

Cross resistance to various unrelated antibiotics had previously been reported in *Escherichia coli* K-12 following exposure to a sub-lethal dose of kanamycin (4). In this study we have attempted to relate this observation to the levels of expression of OmpC and OmpF outer membrane porins. Since OmpC and OmpF are major porins in the *E. coli* outer membrane involved in non-specific solute transport, we reasoned that they are likely to be involved in the establishment of cross resistance to unrelated classes of antibiotics. Multiple-antibiotic resistant clinical isolates of *E. coli* have been shown to have lower levels of OmpF porin (5). Hence, we hypothesized that a change in OmpF and OmpC protein expression levels has an important role in the establishment of cross resistance to a broad range of antibiotics which resulted from pretreatment of *E. coli* K-12 with sub-lethal doses of kanamycin. Given the reciprocal relationship between the levels of OmpC and OmpF (10), the expected decrease in OmpF due to exposure to kanamycin was expected to result in an increase in the amount of OmpC produced by the cell.

In order to demonstrate this effect, we isolated the outer membrane proteins from *E. coli* K-12 wild type, Δ*omp*C mutant, and Δ*omp*F mutant strains that were cultured either in the presence or the absence of a sub-lethal concentration of kanamycin. We then visualized the outer membrane protein expression levels by SDS-PAGE with subsequent staining and compared the levels of OmpC and OmpF in the control cultures and the cultures pretreated with kanamycin. Concurrently, we have tested the samples of the same cultures for the development of cross resistance by using MIC assays. Based on our results, OmpC appeared to be unimportant for cross resistance induced by kanamycin and the role of OmpF still remains unclear.

MATERIALS AND METHODS

Bacterials strains and growth conditions. *Escherichia coli* K-12 strains used in this study were obtained from the Keio collection (Coli Genetic Stock Centre) (1): BW25113 (wild type, CGSC #7636), JW0912 (Δ*omp*F, CGSC #8925), and JW2203 (Δ*omp*C, CGSC #9781). All the strains were cultured in Luria Bertani (LB) broth (1% tryptone (Becton Dickinson (BD) #211701), 0.5% yeast extract (BD #212750), 1% NaCl (Fisher #S271-3), pH adjusted to 7.0 with 5N NaOH). All inoculated cultures were incubated at 37°C, on a shaking platform at 200 rpm, and ml of a 1:10 diluted overnight culture, was used to innoculate cultures for MIC assays and protein isolaton.

Preparation of antibiotic stock solutions. Concentrated stocks of the following antibiotics dissolved in distilled water (except the tetracycline in 70% ethanol) were used to make working stocks: 20 mg/ml kanamycin monosulfate (Sigma #K4000) solution, 25 mg/ml tetracycline hydrochloride (Sigma #T-3383) solution, 25 mg/ml streptomycin sulphate (Sigma #S6501-50G), and 100 mg/ml ampicillin sodium (Sigma #A-9518) solution. Piperacillin working stock was prepared from piperacillin sodium salt (Sigma #P-8396). All antibiotics used in this study were prepared to a final concentration of 512 ug/ml with sterile LB broth before use in subsequent assays. Prepared working stocks were filter-sterilized by 0.45 um milipore filter (Millipore #HAWP01300) and stored at -20°C.

Minimal inhibitory concentration (MIC) assays. MIC assays were carried out in 96 well flat bottom plates (Sarstedt #82.1581.001), with each well filled with 100 ul LB broth. In two duplicate wells, 100 ul of each antibiotic at 512 ug/ml was loaded and mixed in the first column. 100 ul of the mixed antibiotics and LB was then transferred to the adjacent well, producing ½ dilution of the prior antibiotic concentration. The concentrations of the antibiotics in each column were 256, 128, 64, 32, 16, 8, 4, 2, 1, 0.5 and 0.25 ug/ml. To this 100 ul mixture of antibiotic and LB, 5 ul of 10^4 cells/ml strain samples were added to each well, except for the negative controls. Wells containing only antibiotic and LB, or only LB and bacterial cells, were used as negative and positive controls respectively. All plates were incubated at 37°C for 20 h. The MIC for each test was the wells containing the lowest concentration of antibiotic in which no bacterial growth was visible.

Determining the sub-lethal concentration of kanamycin. The MIC of kanamycin in both deletion strains was 128 µg/ml and the wild type strain had 3 ug/ml (data not shown). Sub-lethal dose of kanamycin were defined as a ¼ the kanamycin MIC. Therefore, kanamycin pretreatment of the knock out strains was at a final concentration of 32 µg/ml, whereas the wild type strain was treated with 0.75 ug/ml of kanamycin. After 1 h incubation with sub-lethal dose of kanamycin, additional kanamycin was added to the untreated conditions in order to equalize final kanamycin concentration prior to performing subsequent MIC experiments. Growth was monitored at OD_{600} and it was determined that 2.5 h incubation was sufficient to allow bacterial growth to the density required for MIC assays and outer membrane protein extraction. Cultures were subsequently pretreated with kanamycin for 1 h prior to harvest for outer membrane isolation and MIC assays.

Outer membrane protein isolation. Six 500 ml cultures were prepared for all three strains, with or without kanamycin pretreatment, and grown as described above. Cells were harvested and outer membrane proteins were isolated as described by Carson *et al*. (2). Solubilized membrane protein samples were aliquoted in 1 ml and stored at -20°C.

Buffer replacement of the outer membrane protein samples. Prior to running the outer membrane protein samples on SDS-PAGE, Triton-X100 in 500 ul sample was displaced with SDS buffer as described by Carson *et al* (2).

Estimation of the total protein concentration in membrane preparation samples. Protein quantification was carried out using a bicinchoninic acid (BCA) assay. To prepare for the BCA working solution, 4% w/v cupric sulphate (Sigma #C-7631) solution was added in 1:50 ratio to BCA assay solution (Sigma #B-9643). To each well in a 96-well flat bottomed plate (Sarstedt #82.1581.001), a 25 ul of ½ diluted samples was mixed with 200 ul of the BCA working. The plate was incubated for 30 minutes at 37°C, followed by 5 min cooling at room temperature. The concentration of outer membrane protein samples was determined using a standard curve of BSA of known concentrations at A_{595}.

Analysis of the outer membrane protein preparation. Outer membrane preparation of the *E.coli* K-12 strains were resolved by sodium dodecyl sulfate polyacrylamide agarose gel electrophoresis (SDS-PAGE). All protein gels presented in this study used 11% running gel and 5% separating gel, electrophoresed in running buffer (25 mM Tris base pH 6.8, 192 mM glycine, 1% SDS) at 120V for 90 minutes. Following protein concentration normalized as described above, 10 ul of the diluted protein samples were mixed with 10 ul of 2X loading buffer (50 mM Tris pH 6.8, 100 mM dithiothreitol (DTT) (Sigma # 3483-12-3), 2% SDS, 0.1% bromophenol blue (Sigma #161-0404**),** 10% glycerol (Fisher # BP229-1)), with one lane reserved for a protein ladder (Fermentas, #SM0441 or #SM0431). The final protein concentration in each lane, in conclusion, was equalized to 7 ug. The polyacrylamide gels were stained for 30 minutes with Coomassie staining solution (0.05% Coomassie Brilliant Blue R-250 (Thermo Scientific #20278), 25% v/v isopropanol (Sigma #190764), 10% glacial acetic acid (Fisher #95685)), followed by de-staining with 7% acetic acid solution overnight.

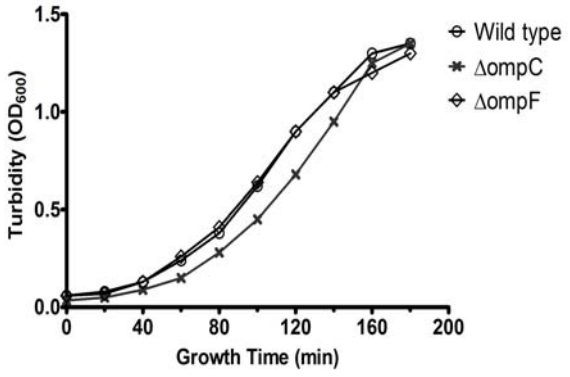

FIG. 1. Comparative growth curve of the three strains of *E.coli* K-12.

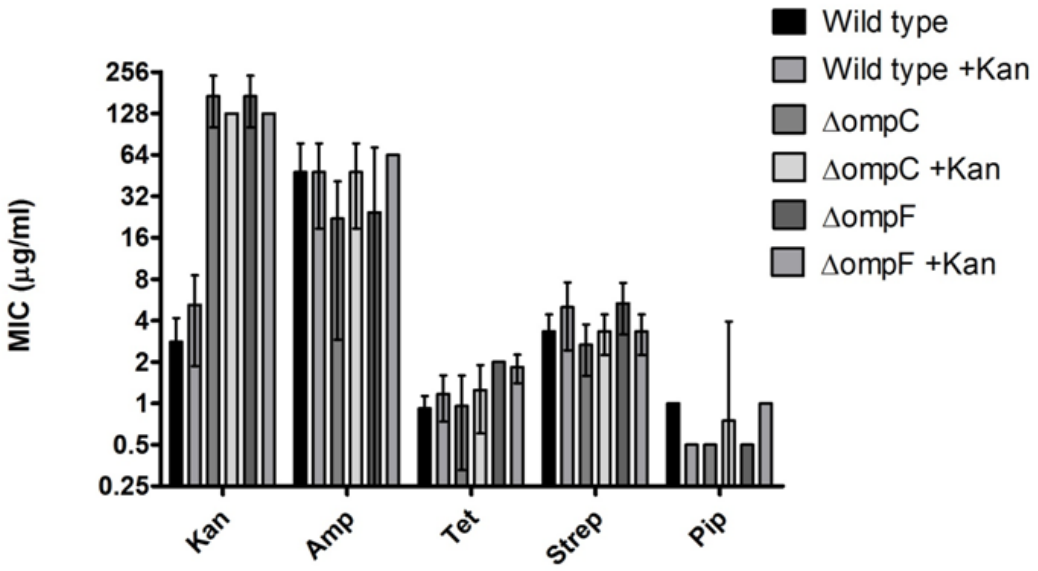

FIG. 2. The effect of sub-inhibitory kanamycin pretreatment on the MIC of five antibiotics on wild type, *ΔompC* and *ΔompF* strains of *E.coli* K-12. Plotted data shows the mean MIC +/- 95% confidence intervals based on three different MIC experiments for kanamycin, ampicillin, tetracycline, and streptomycin. Piperacillin MIC was determined in a single experiment. All values were measured in duplicates. Kan, kanamycin; Amp, ampicillin; Tet, tetracycline; Strep, streptomycin; Pip, piperacillin.

RESULTS

Determination of growth phenotype. In order to assess the effect that deficiency in OmpC or OmpF had on the growth rate of *E.coli* K-12, the growth of the wild type, and both mutant strains was assessed over a period of 180 minutes (Fig. 1). When compared to wild type and Δ*omp*F, the growth of Δ*omp*C lagged approximately 20 minutes (Fig. 1). These growth curves were consistent with the expectation that removal of a key outer membrane porin would have a slight effect on the ratre of growth. It was also observed that all strains appear to be within the log phase of growth between 80 and 140 min past the start of incubation (Fig. 1). As can be seen in Figure 1, all strains reached a plateau turbidity of approximately 1.33 OD_{600} at 180 min following inoculation of the cultures. These results indicated that growth rates were similar enough that all strains could be inoculated at the same time, and harvested during log phase growth after two hours incubation time.

Determination of the minimal inhibitory concentration (MIC) of tested antibiotics in *E.coli* K-12 wild type and Δ*omp*C, Δ*omp*F mutants, with and without pretreatment with kanamycin. The average MIC was determined for each of the five antibiotics used over the course of all experiments: ampicillin, kanamycin, tetracycline, streptomycin and piperacillin (Fig. 2). The standard error indicates the mutant strains did not see a significant increase in MIC after pretreatment with kanamycin. The wild type strain did not see a significant increase in ampicillin MIC, or piperacillin after kanamycin pretreatment. The 95% confidence intervals for each average MIC were found to overlap between the kanamycin treated and untreated conditions for all strains, with the exception of piperacillin in Δ*omp*F (Fig. 2). It should be noted that increased MIC of piperacillin observed after treatment with kanamycin in Δ*omp*F was only observed in one experiment; the experiment was not reproduced due to time constraints. Strong resistance to kanamycin was observed in Δ*omp*C and Δ*omp*F knock out strains (approximately a 32-fold increase in MIC when compared to wild type), indicating a successful crossover event, and the insertion of the kanamycin resistance gene during the creation of both knock out strains (1). While the effect of kanamycin pretreatment in average MIC for the wild type was not observed to be significant in a 95% confidence interval, the increase in MIC was observed reproducibly in each of three assays (data not shown). An increase in MIC between kanamycin treated and untreated conditions was seen repeatedly with tetracycline (2 of 3 assays) and streptomycin (2 of 3 assays), but was less prominent (data not shown). All strains were observed to possess strong resistance to ampicillin, clinically defined as an MIC greater than 16 ug/ml, however this level resistance was not observed for piperacillin (9).

Purification of membrane protein from *E.coli* K-12 wild type and Δ*omp*C, Δ*omp*F mutants. Significant differences were observed between the 95°C boiled samples (Fig. 3) and the 37°C incubated samples

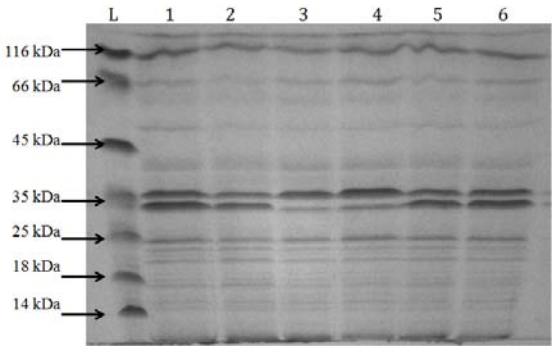

Fig 3. Effect of pre-treatment with sub-inhibitory kanamycin on the outer membrane protein profile of *E.coli* K12. SDS-PAGE of the membrane protein extracts of strains pretreated with kanamycin (+Kan) or untreated as follows: lane 1, WT; lane 2, WT+Kan; lane 3, *Δomp*C; lane 4, *Δomp*C+Kan; lane 5, *Δomp*F; lane 6, *Δomp*F+Kan; lane L, a prestained protein molecular marker. Samples were denatured at 95^0C for 5 minutes.

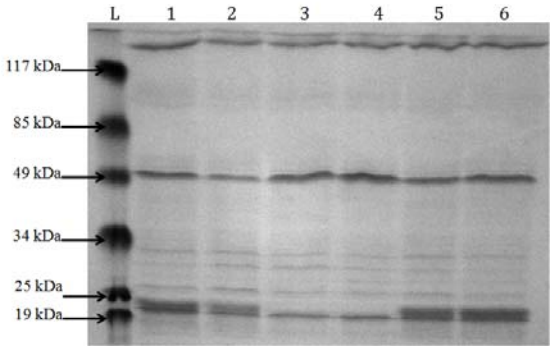

Fig 4. Effect of pretreatment with sub-inhibitory kanamycin on the outer membrane protein profile of *E.coli* K12. SDS-PAGE of the membrane protein extracts of strains pretreated with kanamycin (+Kan) or untreated as follows: lane 1, WT; lane 2, WT+Kan; lane 3, *Δomp*C; lane 4, *Δomp*C+Kan; lane 5, *Δomp*F; lane 6, *Δomp*F+Kan; lane L, an unstained protein molecular marker. Samples were incubated at 37^0C for 30 minutes prior to electrophoresis.

(Fig. 4). The faint band seen in figure 3, identified at approximately 49 kDa, was observed to be the same position as the strong band identified at the 49 kDa position seen in figure 4. An intense doublet of bands was seen on the gel containing the boiled sample in figure 3 at the 34 kDa and 36 kDa level. The wild type strain displayed a clear membrane phenotype difference between the kanamycin treated and untreated conditions; kanamycin treated wild type showed less intense banding at gel positions corresponding to 34, 36 (Fig. 3), and 49 kDa (Fig. 4). The Δ*omp*C mutant displayed a clear membrane phenotype, seen as a sharp reduction in intensity of the bands located at approximately 32 kDa (Fig. 3) and approximately 23 kDa (Fig. 4), as well as a slight reduction in intensity at 49 kDa (Fig. 3) for both the untreated and treated conditions when compared to the wild type kanamycin untreated condition. A slight increase in band intensity was observed gel position correlating to 27 kDa in size (Fig. 4) when the Δ*omp*C kanamycin treated condition was compared to the Δ*omp*C untreated. The kanamycin treated and untreated conditions of the Δ*omp*F mutant were observed to display the same phenotype as the wild type untreated condition (Fig. 3 and Fig. 4). However, specific identification of OmpC and OmpF was difficult.

DISCUSSION

Pretreatment of *E. coli* K-12 with a sub-lethal dose of kanamycin resulted in an increase in the minimum inhibitory concentration (MIC) of the wild type (WT) strain but did not appear to confer cross resistance to ampicillin, tetracycline, streptomycin, or piperacillin, as the MIC of these antibiotics were virtually unchanged (Fig. 2). Chen *et al* (4) similarly observed that kanamycin pretreatment did not confer cross resistance to tetracycline and ampicillin, but they did report a six-fold increase in the MIC of pretreated *E. coli* to streptomycin—a finding that was not observed in our experiment. This was a surprising result given that these two aminoglycosides would likely use the same pathway of entering the cell given their similar chemical structures.

Intrinsic resistance to the β-lactam ampicillin was suspected as all strains under both conditions showed universally high MIC levels (Fig. 2). To investigate this phenomenon further we decided to do an additional MIC assay with a second β-lactam: piperacillin. The *E. coli* K-12 mutant and WT strains had very low MICs against piperacillin (Fig. 2), leading us to conclude that specific mechanisms were at work rather that general mechanisms, such as the presence of a β-lactamase. According to the MIC breakpoint definitions established by Macgowan *et al* (9), the strains are resistant to ampicillin and are susceptible to piperacillin; additionally, since these are laboratory strains and not clinical isolates, it's likely that the strains contain an ampicillin resistance cassette. The nearly 32-fold increase in the MIC of kanamycin against the Δ*omp*F and Δ*omp*C *E. coli* K-12 strains is due to the resistance gene that replaced the *omp* genes when the strains were constructed (Fig. 2). The MIC results for the only other aminoglycoside we tested, streptomycin, did not show a dramatic increase and was very similar to the wild type MIC against both aminoglycosides because of the limited nature of the resistance gene used in the strains. The sub-lethal doses of kanamycin used for pretreatment of our strains were consistant proportions of the strain's MIC. This method was

done in an attempt to optimize the sub-lethal dose for each strain. However, the differences between our results and those of Chen *et al.* (4) may be due to the differences in the amount of kanamycin used for pretreatment. Similar studies have optimized the sub-lethal concentrations by obtaining growth curves for strains treated with a range of MIC proportions.

The decreased OmpC band intensity observed by SDS-PAGE following sub-inhibitory kanamycin pretreatment of wild type *E. coli* (Fig. 3) suggests that this porin may in fact play a role in uptake of this antibiotic. Note that in Figure 3 the putative OmpF band appeared as a doublet (Fig. 3). This could be due to incomplete denaturation during the boiling step or it could represent OmpF a pre-OmpF protein that still has its amino acid signal sequence. Surprisingly, the Δ*omp*F strain also appeared to express OmpF protein. This finding casts doubt on not only the validity of our Δ*omp*F strain, but also on the identification of the OmpF protein in our gels. However, this strain differed greatly from the wild type, as indicated by its altered antibiotic susceptibility, as determined by MIC assays (Fig. 2).

Variable SDS-PAGE migration is a well documented phenomenon for membrane proteins and may explain the drastically different migration rates of the putative OmpF and OmpC bands in our two gels. Investigations by Rath *et al.* (12) have shown that the main determinants of PAGE migration are SDS binding and protein conformation; further, they have shown that membrane proteins, with their many hydrophobic regions, tend to aggregate SDS molecules and thereby bind them at ratios several times that of globular proteins. The result is that these membrane proteins often migrate at unpredictable rates and do not correlate well with the globular proteins of the molecular weight standards—a process termed "gel shifting" (12). Given that the boiled, denatured membrane proteins would likely have vastly different conformations than their unboiled counterparts, our results indicating differing migration rates is not unexpected and may be a result of variable SDS binding capacities. An issue that may improved by optimizing the SDS concentration used to treat the proteins.

The separation of the outer membrane fraction via SDS-PAGE revealed slight differences when comparing WT and Δ*omp*C strains with kanamycin treated and untreated conditions when denatured at 95 °C (Fig. 3), but failed to reveal any marked differences between these conditions for the Δ*omp*F strain. The decrease in band intensity seen in the WT between kanamycin untreated and treated conditions for the putative OmpC band suggests that the OmpC protein expression is affected by kanamycin pretreatment, and may play a role in the development of resistance to kanamycin, as implied by the observed MIC results (Fig. 2). This conclusion is in agreement with many studies that implicate altered porin expression profiles in the antibiotic resistance mechanims of *E. coli* as well as many other microbial species (11).

Based on the protein banding observed by SDS-PAGE for Δ*omp*C and Δ*omp*F strains, OmpC expression appears to be down-regulated after pretreatment with sub-inhibitory kanamycin, the Δ*omp*F strain shows no evidence of any change in expression of both ompF and ompC proteins after pretreatment. The marked increase in intensity of the OmpF doublet for the Δ*omp*C strain (Fig. 3) may suggest that OmpF was over-expressed after kanamycin pretreatment compared to the untreated band. It should be noted, however, that this upregulation did not translate into any detectable increase in MIC for any of the five antibiotics tested in this experiment (Fig. 2).

Our data suggests that OmpC may not be involved in resistance mechanisms to streptomycin, ampicillin, tetracycline, and piperacillin; however we cannot rule out its role in upregulating resistance mechanisms to kanamycin. Conversely, implications for OmpF are inconclusive since the Δ*omp*F phenotype appeared to be missing from that strain. Additional research will be required to determine the involvement of these outer membrane proteins in antibiotic resistance mechanisms and further explore the role played by porins in regulating the development of antibiotic cross-resistance in *E. coli* following treatment with sub-inhibitory concentrations of kanamycin.

FUTURE DIRECTIONS

Further research should focus on the anomalous behaviour of the Δ*omp*F strain. Since the Δ*omp*F mutant did not display the corresponding phenotype, an alternative deletion mutant of *omp*F gene can be considered for identifying the OmpF band in SDS-PAGE. In addition, the kanamycin resistance gene in the Keio mutant strains could be eliminated by targeting the FRT sequence flanking the gene with an FLP expression plasmid which could remove the resistance gene via homologous recombination so that the strains should have similar MIC and can be tested by pre-exposure to equivalent amounts of kanamycin.

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