

Role of OmpF and OmpC in Kanamycin-induced Resistance to Kanamycin and Transient Cross-Resistance to Ampicillin in *Escherichia coli* K12

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The expression of porins may have a role in the induced resistance and transiently induced cross-resistance that *E. coli* B23 exhibits following pre-treatment with sublethal levels of kanamycin. This study aimed to ascertain whether OmpF plays a role in protecting cells from the full effects of subsequent inhibitory levels of kanamycin treatment and lethal levels of ampicillin treatment. Minimal inhibitory concentration assays were used to determine subinhibitory levels of kanamycin, which were then used in a pre-treatment of wildtype (C149) *E. coli* K12 as well as OmpF- (C150), OmpC- (C157), and OmpC- OmpF- (C160) *E. coli* K12 mutants. The sublethal treatment was followed by treatment with inhibitory levels of either kanamycin or ampicillin. By comparing pre-treated cultures with untreated cultures and by comparing the resistance in the mutant strains with wildtype K12 we were able to analyse potential effects OmpF and OmpC have on induced resistance and cross-resistance. Results indicated that OmpF and OmpC proteins were not the sole contributors to the induced resistance phenomenon and may only play minor roles.

Low antibiotic dosing and prescription of unnecessary antibiotic treatments have contributed to increasing resistance in bacteria (15). Consequently, there is high demand for new antibiotics. Previous endeavours in the development of novel antibiotics have maintained the gross structure of an earlier antibiotic while modifying only a small reactive location. An example of such intricate manipulation is ampicillin, which is a modification of penicillin, but has been formulated into a zwitterion (5, 18). In order to create drugs that can have a useful lifetime as antimicrobial agents in prophylaxis, there must be a complete understanding of current resistance mechanisms.

The mechanism of action of aminoglycosides has been studied extensively and relies on the ability of the antibiotics to cross the outer membrane (OM) of Gram negative bacteria. Although there is a wealth of information regarding specific cellular processes that aminoglycosides target, there is little knowledge as to the method in which they cross the OM. Previous studies have shown that *Escherichia coli* cells subjected to sublethal levels of the aminoglycoside kanamycin demonstrate an increase in antibiotic resistance upon exposure to subsequent lethal levels of exposure to the same antibiotic (1, 3, 16). Suggested mechanisms of resistance include increased capsular production, inner membrane restructuring, and decreased porin

production (1, 2). It is already known that many antibiotics enter Gram negative bacteria through either the OmpF or OmpC porins in the OM of *E. coli* and the level of expression of porins may regulate the rate of entry of antibiotics into the cell (1, 3, 4, 17, 8). It has been observed that exposure of *E. coli* to sublethal levels of kanamycin induces transient cross-resistance to structurally and functionally unrelated antibiotics (1). Since this same cross-resistance is not seen in the long term, it has been suggested that the bacteria are modulating the permeability of their outer membranes in response to kanamycin, thus inducing transient resistance (1, 23).

The porin regulon of *E. coli* is a two-component signal transduction system that functions to regulate the osmolarity inside the cell in correspondence with the surrounding environment. The regulon consists of the EnvZ sensor, the OmpR regulator, the OmpF OM pore of large diameter, and the OmpC OM pore of smaller diameter (17). Both OM pores allow small hydrophilic molecules to passively diffuse through the OM and into the cell (13, 17). The total amount of OmpF and OmpC pores remains constant in the *E. coli* membrane with only the relative levels of each varying in response to changes in environment (4, 17). When osmolarity is low, OmpF is expressed to a higher degree than OmpC (4, 13, 17). If osmolarity is high, then OmpC expression is increased and OmpF decreased (4, 13,

17). This is because the rate of diffusion of molecules less than 600 Da is lower through OmpC than through OmpF (12, 13). Due to the large size of kanamycin, entry into the cell is most likely accomplished through the larger OmpF porin (9). If kanamycin enters the cell through OmpF then it follows that the *E. coli* cells would lower their expression of OmpF as a means to limit the amount of antibiotic that passes the OM. This may not be evident during the first exposure of a cell to kanamycin due to the decrease in rates of transcription caused by the antibiotic (6, 7), but could possibly be evident upon a second exposure.

Although structurally and functionally unrelated, ampicillin, like kanamycin, is a hydrophilic molecule less than 600 Da and so can diffuse through *E. coli* porins (9). Moreover, there is evidence that *E. coli* mutants with porin deficiencies have higher minimal inhibitory concentrations (MIC) to ampicillin than wildtype strains (10, 11, 22). This experiment tested whether OmpF-, OmpC-, or OmpF- OmpC- mutant strains showed an increase in resistance to kanamycin or ampicillin after exposure to sublethal levels of kanamycin.

MATERIALS AND METHODS

Bacterial strains. Isogenic *E. coli* K12 strains C149 (wildtype), C150 (*ompF*-254), C157 (*ompC*-264), and C160 (*ompF*-254, *ompC*-263), were obtained from the MICB 421 culture collection in the Microbiology and Immunology Department of the University of British Columbia (24).

Culture methods. Overnight cultures were prepared by inoculating 5 ml of Luria-Bertani (LB) broth (0.5% w/v yeast extract, 1% w/v tryptone, and 0.5% w/v NaCl, pH 7.0) with a loopful of bacteria. The inoculated culture was placed overnight on a shaking platform at 170 rpm in 37°C.

Minimal inhibitory concentration assay. MICs of kanamycin monosulfate (Sigma #K4000-5G) and ampicillin sodium salt (Sigma #A-9518) were performed in 96-well flat bottom polypropylene plates (Falcon 3915). Cultures were started with a 1/100th dilution of an overnight culture in 5 ml of LB and then incubated at 37°C overnight. The lowest concentration that showed no bacterial growth was considered the MIC.

Sublethal antibiotic treatment. Overnight cultures were diluted 1-in-10 into 4.5 ml of LB and were incubated on a shaking platform at 170 rpm in 37°C until an optical density at 595 nm (OD₅₉₅) of 1.0 was reached. Cultures were diluted with LB to make all the OD₅₉₅ values ~ 1.0 and then were placed on ice. Turbidity readings were taken with a Biochrom Ultrospec 3000 spectrophotometer. Kanamycin was added to four 16 x 125 mm glass test tubes containing 4.5 ml of LB to create sublethal concentrations of 12 µg/ml. Each tube was then inoculated with 0.5 ml of one culture strain that was placed on ice. The tubes were incubated on a shaking platform at 170 rpm in 37°C for 90 min. Untreated control cultures were made by inoculating 0.5 ml of each culture that was placed on ice into 4.5 ml of LB without antibiotic and then incubated for 90 min on a shaking platform at 170 rpm in 37°C.

Lethal antibiotic treatments. Chilled pre-treated and untreated cultures were diluted to an OD₅₉₅ of 0.4 in LB and exposed to kanamycin final concentrations at 0, 12.5, 25, 50, and 100 µg/ml or to ampicillin final concentrations at 0, 6, 12, 24, and 100 µg/ml. Empty wells were filled with distilled sterile water to prevent desiccation. Plates were incubated at 37°C and turbidity readings were taken

every 20 min for a total of 160 min. Turbidity readings were taken using a BioRad 680XR microplate reader.

RESULTS

Selection of strains. Previous studies which looked into the phenomenon of kanamycin-induced short term cross-resistance to antibiotics have used *E. coli* B23 strains, whereas we used *E. coli* K12 strains C149 (wt), C150 (*OmpF*-), C157 (*OmpC*-), and C160 (*OmpF*-*OmpC*-) (1, 16). B23 and K12 strains have numerous differences, but the most important difference which could affect our experiment is the presence of both the OmpF porin and the OmpC porin in K12, whereas B23 strains lack the OmpC porin (24).

MIC determination. To quantify the antimicrobial activity of kanamycin and ampicillin, MICs for all four strains were determined (Table 1). MIC values were higher than expected, with 50 µg/ml of kanamycin required to inhibit growth for wildtype and *OmpF*-. The *OmpC*- and *OmpF*- *OmpC*- strains had lower kanamycin MICs of 20 and 8 µg/ml, respectively. When subjected to ampicillin, the wildtype, *OmpF*-, and *OmpF*- *OmpC*- strains all showed the same MIC value of 8 µg/ml, whereas *OmpC*- had an MIC of only 2 µg/ml. This is surprising since *OmpF*- and *OmpF*-*OmpC*- are *OmpF* deficient and we expected that they would have higher ampicillin MICs than wildtype, owing to the fact that ampicillin enters the cell primarily through the OmpF porin (5, 10, 11, 17).

Determination of sub-inhibitory kanamycin concentration. For kanamycin, we obtained MIC values which were in great excess to previously reported MIC values of 4 µg/ml (1, 9). Additionally, we obtained ampicillin MIC values which were also several fold higher than previously reported values (5, 10). We initially thought this could be due to the polystyrene plastic of the 96-well plates. Polystyrene can bind charged molecules including antibiotics, thereby lowering the effective antibiotic concentrations (14). However, we performed a second MIC using polypropylene 96-well plates, which do not bind antibiotics, and obtained similar MIC values. This would seem to indicate an intrinsic resistance to kanamycin and ampicillin in the strains we used compared to B23 strains. There was no difference in

TABLE 1. MIC values for *E. coli* strains used in this study.

Strain	Kanamycin (µg/ml)
C149 (wildtype)	50
C150 (<i>OmpF</i> -)	50
C157 (<i>OmpC</i> -)	20
C160 (<i>OmpF</i> -, <i>OmpC</i> -)	8

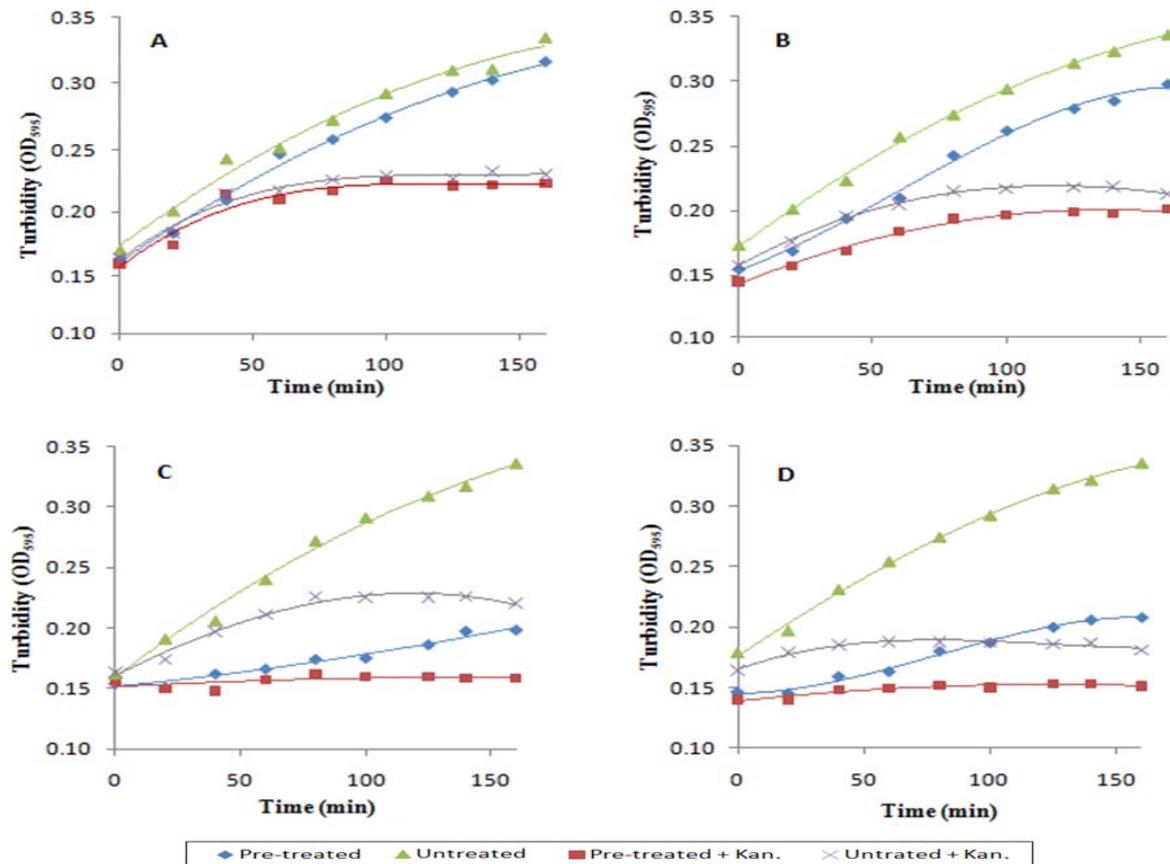


FIG. 1. Effect of 100 µg/ml kanamycin on the growth of untreated and pre-treated wildtype (A), *OmpF*⁻ (B), *OmpC*⁻ (C), and *OmpF*⁻, *OmpC*⁻ (D) *E. coli* K12 strains. The pre-treated cells were prepared by exposing them to kanamycin at 12 µg/ml for 90 min on a shaking platform at 170 rpm in 37° C and then subjected to 100 µg/ml of kanamycin.

MIC values between wildtype and *OmpF*⁻ cultures with regards to ampicillin, which was fascinating and unpredicted considering ampicillin enters through *OmpF*. Also, previous reports suggest the absence of *OmpF* will induce an observable resistance to ampicillin (21). This made determining the antibiotic concentrations to use in our experiment difficult since the values were quite high and had a degree of variance. A kanamycin concentration of 12.0 µg/ml was chosen for pre-treatment as we wanted to maximize the effect for strains with high MICs closer to 50 µg/ml, but minimize the inhibitory effect on the strains with lower MICs near 8 µg/ml.

Determination of lethal antibiotic concentration. A range of concentrations were tested for ampicillin and kanamycin lethal values. The greatest differences in both lysis and growth rates between the pre-treated and untreated cultures were observed at 100 µg/ml ampicillin and 100 µg/ml kanamycin.

Inhibitory kanamycin treatment. The growth rates of pre-treated and untreated cultures were compared in LB without kanamycin and LB with 100 µg/ml kanamycin. When subjected to 100 µg/ml kanamycin for 90 min, the wildtype and *OmpF*⁻ cultures had a 60 min period of growth before inhibition was observed (Fig. 1A and Fig. 1B). There was no significant advantage observed for the cultures pre-treated with kanamycin since they did not show a longer growth period and were inhibited at 60 min. In addition, the treated cells did not show a faster growth rate before reaching the inhibition point and grew at the same rate as the untreated cells.

However, in Fig. 1C, pre-treated *OmpC*⁻ was inhibited almost immediately in the 100 µg/ml kanamycin, but the untreated *OmpC*⁻ grew for 60 min. This indicates that the *OmpC*⁻ mutants somehow got sensitized to kanamycin after the pre-treatment. This sensitization is consistent with Fig. 1C and Fig. 1D which shows pre-treated *OmpC*⁻ and *OmpF*⁻ *OmpC*⁻

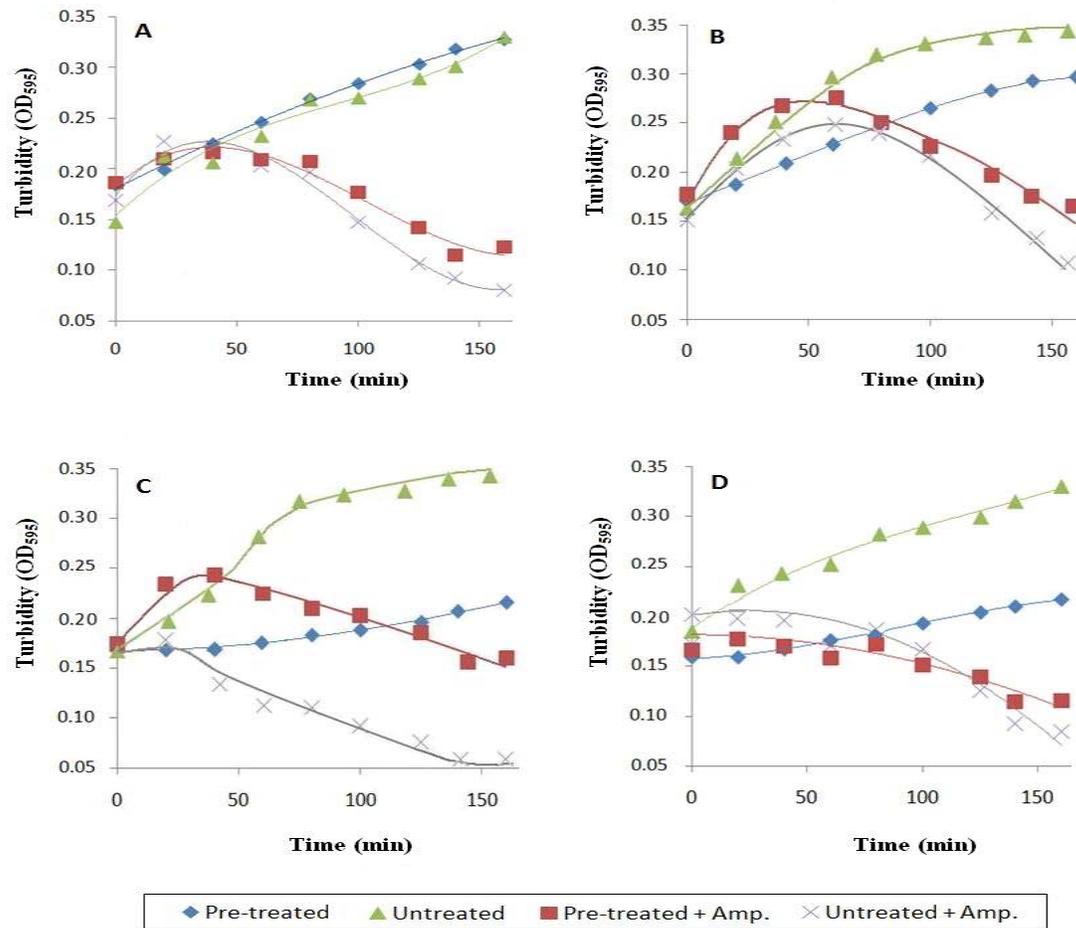


FIG. 2. Effect of 100 µg/ml ampicillin on the growth of untreated and pre-treated wildtype (A), *OmpF*- (B), *OmpC*- (C), and *OmpF*-, *OmpC*- (D) *E. coli* K12 strains. The pre-treated cells were prepared by exposing them to kanamycin at 12 µg/ml for 90 min on a shaking platform at 170 rpm in 37° C and then subjected to 100 µg/ml of ampicillin.

cells grew at a slower rate compared to untreated *OmpC*- and *OmpF*- when not subjected to further antibiotic treatment. It appears the *OmpC*- and *OmpF*- *OmpC*- cells could not protect themselves from the sub-inhibitory kanamycin treatment.

Inhibitory ampicillin treatment. For the ampicillin treatment, differences were observed between pre-treated and untreated cultures. We assessed the difference in growth rate by evaluating variation in the average instantaneous lysis rates at 60 and 120 min. The lysis curves are estimated to be linear between the 60 and 120 min time points (Fig. 2).

The rate of lysis for the pre-treated cultures in 100 µg/ml of ampicillin was lower in each strain compared to the untreated cultures (Fig. 2). Surprisingly, the *OmpF*- strain had the greatest decrease in lysis rate, which can be equated with the greatest survival compared to the untreated culture upon treatment with

ampicillin. The *OmpF*- culture demonstrated a 72% relative lysis rate (pre-treated divided by untreated). Pre-treated wildtype had a 75% relative lysis rate based on the 60 and 120 min time points. *OmpC*- and *OmpF*- *OmpC*- mutants had an 80% relative lysis rate of pre-treated culture compared with untreated culture. Despite *OmpF*- showing the greatest decrease in average lysis rate after pre-treatment, it is not significantly different from the decreases observed in the other strains (Fig. 3).

DISCUSSION

Changes in growth rates and lysis rates were used to determine changes in resistance. The 60 and 120 min time points were chosen as the sole data to calculate the lysis rate slopes in ampicillin because they occur after the antibiotic has taken effect and before the transient

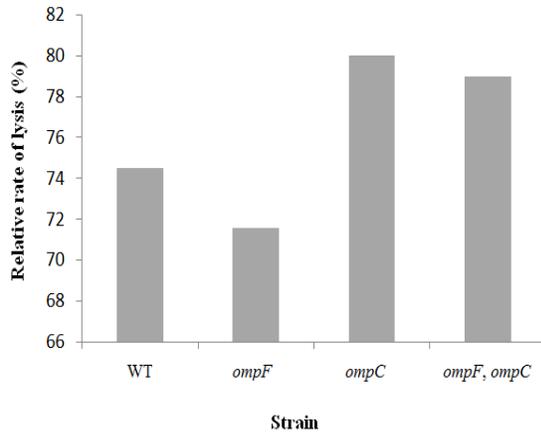


FIG. 3. Comparison of the rate of lysis between pre-treated and untreated strains when subjected to ampicillin.

resistance has faded away. We are able to do this estimation because the ampicillin lysis curves are almost linear between 60 and 120 min (Fig. 2). We used antibiotic concentrations of 100 $\mu\text{g/ml}$ for our treatments since this is inhibitory for all four strains, but we did not want to use a concentration high enough to override any protective benefits pre-treatment would have provided. Moreover, this concentration of antibiotic showed the greatest difference in resistance between pre-treated and untreated cultures.

It is demonstrated in Fig. 1 that the wildtype and OmpF- strains showed no change in their growth rates in 100 $\mu\text{g/ml}$ kanamycin after the 90 min sub-inhibitory kanamycin pre-treatment. The strains that showed a difference, namely OmpC- and OmpF- OmpC- did not show any increased resistance. These two strains demonstrated an increased susceptibility to 100 $\mu\text{g/ml}$ kanamycin. The above findings are in contrast with previous studies (1). Although no induced resistance to kanamycin was seen, Fig. 2 shows that all of the strains demonstrated a slight cross-resistance to ampicillin after the sublethal kanamycin treatment. In lower ampicillin concentrations, we saw almost no effect with regards to growth rates. In higher and lethal ampicillin concentrations, we saw relative lysis rates (pre-treated divided by untreated) between 72-80% after pre-treatment with little variance between strains. The OmpF- mutant strain exhibits a 28% increase in resistance (72% relative lysis) after the kanamycin pre-treatment. Interestingly, the increase is higher than the cross-resistance seen in the wildtype strain. This might be due to the higher intrinsic resistance of the wild type strain to ampicillin, as seen in our MIC results. Based on the data, we cannot reject the null hypothesis that OmpF does not have a role in the observed cross-resistance due to kanamycin pre-treatment. The OmpF- and OmpC- mutant strains showed a 20% increase in

resistance to ampicillin after pre-treatment, which is only slightly different than the resistance demonstrated by the wildtype. After taking into account potential measurement and sampling error, we cannot conclusively say that the OmpC porin does not have an effect on cross-resistance. The data suggests that OmpF and OmpC are not the only factors responsible for induced transient cross-resistance.

The effect of kanamycin pre-treatment had a minimal effect on kanamycin resistance and transient cross-resistance in our experiment. This is in contrast to a previous study which found a near threefold increase in growth rate in *E. coli* B23 when subjected to 4 $\mu\text{g/ml}$ ampicillin after kanamycin pre-treatment (1). It should be noted however, that we obtained MIC values two times greater than what Chen *et al.* considered lethal levels (1). Additionally, Chen *et al.* found a 2.5 fold increase in growth rate when cells were subjected to 16 $\mu\text{g/ml}$ of kanamycin after a 4 $\mu\text{g/ml}$ kanamycin pre-treatment (1), whereas we found no inducible resistance with regards to kanamycin.

Possible problems with our experiment that may have affected the outcome include the fact that we did not account for equalizing the amount of kanamycin added to the treated cultures with the untreated cultures. This should have been done in order to make conditions as similar as possible for our untreated controls. However, the effects are assumed to be minor due to the high concentration of ampicillin and kanamycin that the cultures were subjected to for the 160 min incubation. Another problem to consider is the issue of not knowing the lethal concentration of ampicillin for the strains. An inhibitory concentration of 100 $\mu\text{g/ml}$ was picked from a range of tested concentrations in our MIC assay because it showed the maximum difference between the treated and untreated lysis rates across all strains.

Due to time constraints, not enough replicates of the experiment were done to obtain conclusive and repeatable data. This should be a strongly considered when evaluating the results of the experiment. Also, the method used to calculate the lysis rates presents another problem. Only two data points were picked and the instantaneous lysis rates at these time points were averaged to represent the average lysis rate for the entire treatment. Although, the curves were estimated to be linear between these two time points, some of the complexity of the lysis curves was lost in order to make the data comparable across strains.

While our data would seem to suggest that OmpF does not play a role in the mechanism of kanamycin-induced transient resistance and cross-resistance, we cannot make this conclusion. The lysis rates observed in 100 $\mu\text{g/ml}$ ampicillin were low to begin with, so any variance observed between treatments seemed proportionally large. For example, a decrease in lysis

rate of only 0.013 OD₅₉₅/hr represented a 20% difference in lysis rate. This is further complicated by the fact that we observed a degree of variance in our readings between duplicate samples and different time points. The unexpectedly high MIC values we obtained and the lack of any noticeable change in ampicillin MICs between strains makes our results even less reliable and may indicate intrinsic resistance. It is also possible that the mechanism of resistance may be strain-dependent and is present in *E. coli* B23 but not *E. coli* K12, since we did not see any significant correlation between pre-treated and untreated cultures in the response to either kanamycin or ampicillin.

FUTURE EXPERIMENTS

Since previous experiments made use of *E. coli* B23 strains in studying induced transient cross resistance after exposure to kanamycin, it would be useful to look at B23 strains which have been made OmpF-, rather than using K12 strains in order to look at what role OmpF plays in transient cross-resistance in B23 cells. Additionally, this experiment could be repeated with phenotypically similar K12 cells with regards to OmpF, which possess lower, more typical MIC values. This would clarify what role OmpF plays in previously observed transient cross-resistance in B23 cells as well as determine if this mechanism of resistance is specific to B23 strains.

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