Journal of Experimental Microbiology and Immunology (JEMI) Vol. 16: 101 – 107

The down regulation of *E. coli* OmpF in response to sub-inhibitory concentrations of kanamycin is not mediated by MarA.

Yang Fei, Vivian Ma, Nina Maerkl, and Paul You
Department of Microbiology & Immunology, University of British Columbia

Previous studies have shown that transient resistance to aminoglycosides involved the downregulation of OmpF porins, a type of porin expressed during osmoregulation in *E. coli*. The MarA protein from the *marRAB* operon, implicated in maintaining a multi-antibiotic resistance phenotype, during an antibiotic challenge, was also found to be involved in OmpF downregulation. In order to determine the regulation of OmpF levels by MarA during an aminoglycoside challenge, OmpF protein levels within the BW25113 (wild type), JW5249 (Δ*marA*) and JW0912 (Δ*ompF*) after exposure to different concentrations of antibiotic were compared. The kanamycin cassette was removed in Δ*ompF* and Δ*marA* strains via pCP20 plasmid transformation, MICs were then determined using the microtitre broth dilution method and each strain was grown under 0x, 1/2x and 3x MIC kanamycin. Membrane protein isolation was then performed followed by SDS-PAGE and Western Blot band quantification of OmpF. After the successful removal of the kanamycin cassette, Δ*ompF* showed the lowest MIC at 4.5ug/ml kanamycin while WT and Δ*marA* both had MIC at 9.1µg/ml kanamycin. However, Δ*ompF* showed the most rapid growth under 1/2x MIC kanamycin, followed by WT and Δ*marA* strains. Western Blotting showed that Δ*ompF* had the highest OmpF protein levels. Meanwhile, Δ*marA*, Δ*ompF* and WT strains showed 88%, 73% and 68% decreases in OmpF protein levels, respectively, during the 1/2xMIC kanamycin challenge. Hence, these results indicated that MarA does not mediate OmpF downregulation during subinhibitory Kanamycin challenge.

The multiple antibiotic resistance locus (*mar*) of *Escherichia coli* has been given a considerable amount of attention in recent studies. The mar locus consists of two divergently transcribed operons surrounding the mar operator (13). One transcriptional unit, *marRAB*, encodes for the MarR repressor protein, which regulates the expression of the *mar* operon, and an activator protein (MarA). The function of the products of *marB* on the same transcriptional unit and *marC*, on the other transcriptional unit are unknown.

The MarA activator protein has been shown to alter the expression of more than sixty *E. coli* genes (13). These changes in gene expression have been shown to result in a partial antibiotic resistance. Although the observed level of antibiotic resistance that arises is not in itself very significant, it may facilitate the development of further antibiotic resistance (13). The majority of the antibiotic resistance stems from *marA* induced expression of the AcrAB-TolC efflux pump (11) while partial resistance may be linked to the downregulation of the expression of *E. coli* outer membrane porin OmpF (1, 10). OmpF mediates the movement of hydrophilic solutes, including kanamycin, through the outer membrane of *E. coli* and is expressed in response to environmental changes. The two porins share similar structure but differ in substrate specificity and diffusion rates. OmpF is the larger of the two and permits faster diffusion (13).

The downregulation of OmpF can occur though *marA* induced expression of *micF,* an antisense RNA which binds the 5' untranslated region of *ompF* mRNA preventing ribosome binding, and thus translation, in addition to promoting degradation of the mRNA message usually in response to environmental stresses (8).

It has been observed that transient antibiotic resistance is induced when *E. coli* cells are exposed to sub-inhibitory levels of kanamycin (4, 1, 10). Kanamycin is an aminoglycoside antibiotic that functions to stop protein synthesis through binding of the 16S ribosomal RNA (15). Many bacterial mechanisms of resistance to aminoglycosides have been observed including modification of the antibiotic or target, and change in bacterial membrane permeability most notably decreased inner membrane transport and active efflux. In addition to these, it has been hypothesized, but not conclusively proven, that a decrease in the expression of OmpF is partially responsible for the observed antibiotic resistance, since

it is thought to restrict antibiotic movement into the cell (4, 1, 10).

The aim of this experiment was to investigate a potential link between exposure to sub-inhibitory levels of kanamycin and regulation of OmpF expression through the *mar* locus. This was done by comparing the expression of the OmpF porin in a wildtype *E. coli* strain to that of a *marA* deletion mutant and an *ompF* deletion mutant after exposure to kanamycin at a determined sub-inhibitory concentration. It was determined that the mar locus encoded marA activator did not play a significant role in the downregulation of OmpF expression under these conditions.

MATERIALS AND METHODS

Bacterial strains and media. All strains used for the study originated from the Keio collection (2). *E. coli* BW25113 (Parental strain) and the JW5249 strain (Δ*mar*A) were the primary strains used for this study. The JW0912 strain (Δ*omp*F) was used as a negative control for outer membrane protein expression. All strains were cultured in Luria Bertani (LB) broth containing 1% (w/v) tryptone (BD #211701), 0.5% (w/v) yeast extract (Difco #210929), 1% (w/v) NaCl (Fisher #7647-14-5), with pH adjusted to 7.0 with 5N NaOH, using a Fisher-Scientific Accumet 900 pH meter.

Preparation of antibiotic stock solutions. All antibiotics were prepared as 50 mg/ml working stocks. 100% ethanol was used as the solvent for Chloramphenicol (Sigma #C0378) and dH_2O was used for Ampicillin (Sigma #A9518) and Kanamycin monosulfate (Sigma #K4000) solutions. All antibiotic stock solutions were subsequently filter-sterilized using a 0.22 µm Millipore filter (#HAWP01300) before storage at 4°C.

Kanamycin resistance cassette removal using pCP20. For each of the JW5249 (Δ*mar*A) and JW0912 (Δ*omp*F) strains, deletion of the target gene was performed via an in-frame replacement of the gene with a kanamycin resistance cassette, hence conferring kanamycin resistance (2). The removal of the kanamycin cassette involved the transformation of pCP20 plasmids into each mutant strain since the kanamycin cassette is flanked by 2 FRT recognition sites (2). The pCP20 plasmids, previously isolated from *Bt340* cells, contain a flippase gene and an ampicillin cassette (5). Transformation of the plasmids was performed as described in "$CaCl_2$ transformation of *E. coli*" by Hancock (9) . After transformation, cultures were transferred to sterile test tubes containing 1 ml of LB, incubated for 1.5 h in a shaking water bath at 30°C after which cells were plated onto LB plates with 25 µg/ml ampicillin and incubated at 30°C overnight. The next day, transformed colonies were plated onto LB plates and incubated at 42°C overnight to induce flippase production and the loss of the pCP20 plasmid and kanamycin cassette. This procedure was repeated again to ensure complete loss of the plasmid. Two days after transformation, cells from the last LB plate were spot-plated on both gridded LB plates containing 50 µg/ml kanamycin, and on LB plates containing 50 µg/ml ampicillin and 25 µg/ml chloramphenicol to confirm plasmid loss along with the cassette.

Minimal inhibitory concentration (MIC) microdilution assay. MIC microdilution assay was performed in a 96 well polyethylene flat bottom plate as previously described (9). Serial dilution of kanamycin was performed in 100 µl of LB to achieve the final concentrations of 125, 62.5, 36.3, 18.2, 9.1 4.5, 2.3, 1.1, 0.6, 0.3 and 0 µg/ml. Overnight cultures of WT, Δ*marA* and Δ*ompF* strains were diluted to 10^4 cells/ml. 5 µl of these dilutions were added to each well in their respective rows and incubated at 37°C for 18-36 h. The plates were measured using a BIO-RAD 3550 model microplate reader and the MICs were determined by observing a direct decrease in OD_{595} to background levels.

Sub-inhibitory kanamycin treatment of cell cultures. Overnight cultures of all three strains were diluted to 0.1 OD_{600} in 200 ml of LB and incubated with mild aeration at 37°C to 0.15 OD_{600}. 3x, 0.5x and 0x MIC of kanamycin was added into the cultures. The OD_{600} of each culture was monitored over a 4 h period and then immediately placed on ice to stop growth. 10% glycerol (BDH #B28454-76) was added to the samples which were stored at -80°C until use.

Isolation of outer membrane proteins. Treated cell cultures were harvested and protein isolation was performed as previously described by Carson *et al* (3). Cultures were spun down by centrifugation at 10,000 x *g* for 5 min in a Beckman J2-21 centrifuge with a JA-14 rotor. Pellets were resuspended in 4 ml resuspension buffer (10 mM Tris-HCl (Sigma #096K5405), 1 mM EDTA (FisherBiotech #BP120-1), 20% sucrose (Sigma #S-0389), 1 mg/ml lysozyme (Sigma #L-7651) pH 8.0) and lysed at 1500 psi as previously described (10). Lysates were centrifuged at 2500 x *g* for 25 min at 10 °C to remove any unbroken cells and debris. The supernatants were collected and subsequently centrifuged at 35,000 x *g* for 20 min with a JA-20 rotor. Pellets were washed twice with 10 mM Tris-HCl pH 8.0 and resuspended in 2 ml outer membrane porin resuspension buffer (2% Triton X-100 (BIO RAD

TABLE 1. Raw data of MIC determination based on bacterial concentrations at different kanamycin concentrations.

Kanamycin concentration (µg/ml)	Bacterial Concentrations (OD_{600} units)		
	WT	Δ*Mar*A	Δ*Omp*F
0	0.162*	0.174*	0.161*
125	0.152	0.169	0.160
62.5	0.166	0.166	0.146
36.3	0.154	0.162	0.145
18.2	0.142	0.158	0.147
9.1	0.139^	0.157^	0.149
4.5	0.425	0.373	0.146^
2.3	0.335	0.556	0.428
1.1	0.500	0.674	0.601
0.6	0.690	0.719	0.723
0.3	0.817	0.731	0.837
0	0.903	0.755	0.807

*represent background readings for which no cells were present in the wells.

^ ODs at which kanamycin concentrations were taken as MICs.

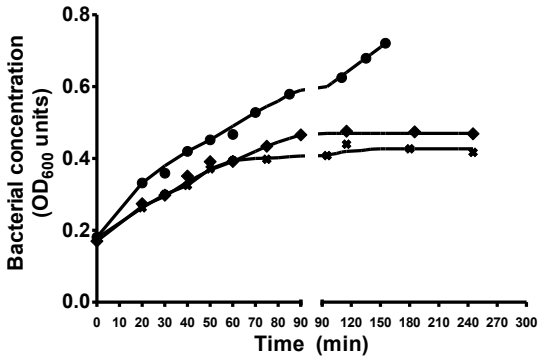

FIG 1. Effect of 1/2x MIC kanamycin on growth of different *E. coli* strains. ● indicate OD_{600} values for Δ*Omp*F culture (JW 0912), ✖ indicate OD_{600} values for the Δ*Mar*A (JW 5249) culture, and ◆ indicate OD_{600} values for the wildtype culture (BW 25113). Δ*Omp*F culture was taken out and placed on ice from t=155min onwards in order to prevent the OD_{600} value from exceeding 0.8, after which measurements may not be accurate.

#161-0407), 10 mM Tris HCl (Sigma #096K5405) pH 8.0).

Buffer replacement of isolated outer membrane protein samples. As previously described by Carson *et al*, SDS buffer (8 mM Tris HCl (Sigma #096K5405), 2% SDS (EM Science #DX2490-2) pH 8.0) was used to replace the OMP resuspension buffer (3). 1 ml of protein sample was added into a Centricon centrifugal filter device (Centricon YM-10; Catalog No. YM-10: 4205**)** and was spun down at 5000 x *g* at 25°C for 1.25 h in a Sorval RC-5B centrifuge with a SS-34 rotor. 350 µl of SDS buffer was added to the device and spun again at 5000 x *g* for 45 min twice in order to further dilute the original OMP buffer solution. The device was then inverted and spun at 1000 x *g* for 5 min into a collection tube and stored at -20°C until use.

Protein concentration assay. Recovered protein concentration was determined by performing a bicinchoninic acid (BCA) assay. Protein concentration standards were created using a 25 mg/ml bovine serum albumin stock solution diluted to 2000, 1500, 100, 750, 500, 250, 125, 25 and 0 µg/ml. 4 replicates of 25 µl diluted standards were added into their respective rows. 4 replicates of 25 µl 1/20 dilutions of the recovered protein lysates were added into their respective rows. 200 µl of BCA working reagent was added into all wells and allowed to incubate at room temperature for 30 min. Plates were read at A_{595} with a BIO-RAD 3550 model microplate reader.

SDS-PAGE and western blotting of the outer membrane protein samples. Sodium dodecyl sulfate polyacrylamide agarose gel electrophoresis (SDS-PAGE) was performed on all recovered protein samples as described by Hu *et al* with a 4% stacking gel and a 10% running gel. Multiple gels were run at 120 V for 1.5 h in parallel for different purposes. Gels for Coomassie staining contained 0.875 µg of protein while gels for blotting contained 50 µg of protein. All samples were mixed with equal parts of 2X sample buffer. Staining was done in Coomassie staining solution for 30 min and destained overnight. Two SDS-PAGE gels were used in the transfer to nitrocellulose membranes (Bio-RAD #162-0146) at 100 V for 1 h. Protein detection was then performed by incubating one membrane in blocking buffer (5% w/v skim milk powder, 1X TBST pH 7.5) for 1 h at room temperature followed by the primary antibody overnight at 4°C. The second membrane was washed in 1x TBST (0.1% v/v Tween 20 in 1X TBS) and left overnight as well. Both were washed 3 times for 1 min in TBST and then incubated in secondary antibody (Biorbyt orb #13626) for 45 min. The membranes were then washed 3 times for 10 min in TBST, followed by the addition of BCIP/NBT substrate (170-6432 Alkaline Phosphatase Conjugate Substrate) for approximately 15 min to allow for band development. The membranes were then placed in distilled water to wash off the substrate and then air dried. Band intensities were determined by using the ImageJ version 1.45 software as previously described (10).

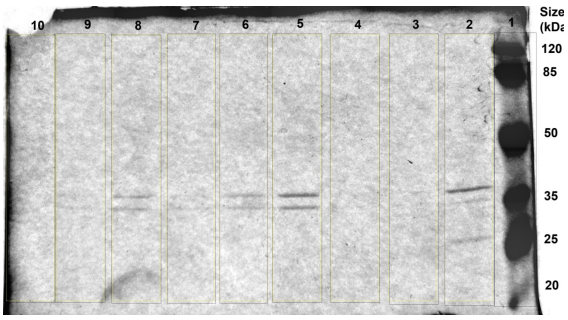

FIG. 2. Effect of kanamycin on the outer membrane protein profile of different *E. coli* strains. Outer membrane proteins were isolated from *E. coli* strains treated with different concentrations of kanamycin (0xMIC, ½x MIC and 3xMIC), and electrophoresed on a 10% SDS-PAGE as follows: lane 1, pre-stained molecular weight ladder; lanes 2,3&4, wild type(BW 25113) + kanamycin (0xMIC, ½x MIC and 3xMIC respectively); Lanes 5,6&7, Δ*Omp*F (JW0912) + kanamycin (0xMIC, ½x MIC and 3xMIC respectively); Lanes 8,9&10, Δ*Mar*A (JW5249) + kanamycin (0xMIC, ½x MIC and 3xMIC respectively)

RESULTS

Determination of kanamycin MIC. Knockout strains acquired from the Keio collection were created by inserting a kanamycin resistance cassette into the genes of interest (2). In order to clearly demonstrate kanamycin transient resistance conferred by the Mar locus through OmpF porin regulation, kanamycin cassettes were removed by inserting a plasmid, pCP20, containing a temperature sensitive flippase. Successful removal of the plasmid was verified by the lack of growth on ampicillin and chloramphenicol LB plates for which the pCP20 contains resistance genes. After the successful removal of the kanamycin resistance cassette, the MIC of each strain was determined. Based on turbidity readings taken from our plate, the *Δomp*F strain unexpectedly showed the highest sensitivity to kanamycin with a MIC of 4.5 µg/ml. *Δmar*A and WT both showed a MIC of 9.1 µg/ml. MICs were determined to be the kanamycin concentration at which OD_{595} levels decreased to background (Table 1). The discrepancies found within our MICs may be due to the mislabeling of 96-well plate. Due to time constraints, these kanamycin concentrations were used for the remainder of the experiment and the MIC determination was not replicated.

Effect of sub-inhibitory levels of kanamycin on the growth of the different strains. In order to determine whether the different strains showed dissimilar growth curves upon treatment with sub-inhibitory levels of kanamycin, OD_{600} of each culture was monitored over a 4 h period. As expected, cells treated with no antibiotic showed similar exponential growth across all three strains, whereas cultures treated at 3x MIC all showed inhibited growth after 65 min. On the other hand, 1/2x MIC cultures, showed clear differences in growth patterns. Δ*omp*F culture showed a significantly higher ability to grow as compared to the WT culture while Δ*mar*A cultures began to diverge from the WT curve after 70 min (Fig 1). By 120 min, Δ*mar*A cultures asymptoted at an OD_{600} of 0.33 and WT cultures asymptoted at an OD_{600} of 0.47 (Fig. 1). However, Δ*omp*F cultures did not asymptote even at 160 min and had to be placed on ice after reaching OD_{600} of 0.7 (Fig. 1). Taken together, these data indicated that a lack of OmpF in the outer membrane conferred resistance to the treatment of kanamycin while the deletion in the *marA* conferred a slightly higher sensitivity to it.

Detection, quantification and the effects of MarA on OmpF expression. The SDS-PAGE of the isolated membrane fraction of each strain following the kanamycin treatment showed that the band intensities at about the 40 kDa range were clearly lower in response to increased antibiotic concentrations for each strain (Fig. 2). This replicated the results seen in a previous study by Hu *et al* in that it may be indicative of lower OmpF expression in response to sub-inhibitory levels of kanamycin (10). However, the results contradict the *ompF* gene removal in Δ*omp*F mutants where no band intensity changes were expected due to complete

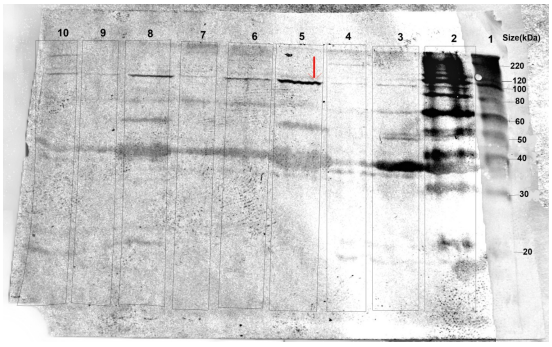

FIG. 3. Effect of treatment with kanamycin on the outer membrane protein levels of OmpF protein in *E. coli* strains. Outer membrane proteins were isolated from *E. coli* strains treated with different concentrations of kanamycin (0xMIC, ½x MIC and 3xMIC), and electrophoresed using a 10% SDS- PAGE followed by a Western blot: lane 1, pre-stained molecular weight ladder; lanes 2,3&4, wild type(BW 25113) + kanamycin (0xMIC, ½x MIC and 3xMIC respectively); Lanes 5,6&7, Δ*Omp*F (JW0912) + kanamycin (0xMIC, ½x MIC and 3xMIC respectively); Lanes 8,9&10, Δ*Mar*A (JW5249) + kanamycin (0xMIC, ½x MIC and 3xMIC respectively)

deletion of the *ompF* gene (2). In order to confirm the protein identity of the band, OmpF levels were directly assessed with a western blot assay. Unexpectedly, there was an absence of clear bands around the 40 kDa region, but distinct bands were observed slightly below the 100 kDa marker in all lanes (Fig. 3). These bands could be identifying OmpF proteins with OmpF subunits at 40kDa and trimers at 120kDa. However, it was difficult to quantify or identify the proteins due to a lack of clarity, especially at the 40kDa level. A Western Blot control, ran without proteins was performed and indicated little background.

Similar band intensity patterns were observed for these bands as compared to our stained gel at the 40 kDa marker (Fig. 3). Changes in band intensity, on the western blot, quantified using the ImageJ software estimated the relative levels of OmpF porin production. For the Δ*mar*A, Δ*omp*F and WT strains, growth under 1/2x MIC kanamycin resulted in an 88%, 73% and 68% decrease in OmpF porin production compared to growth without kanamycin (Fig. 4). This quantitatively demonstrates that OmpF porin is downregulated in response to the addition of kanamycin.

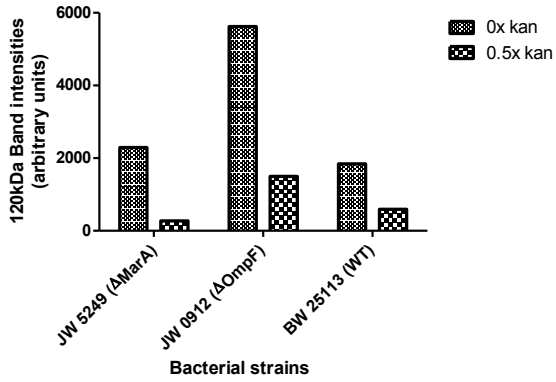

FIG 4: The effect of kanamycin on the differential OmpF outer membrane protein levels across E. coli strains. Densitometry was carried out on the 120kDa band from Fig 3. Band intensities are calculated using ImageJ. Coarse checkered patterns indicate that cultures have been incubated with kanamycin at 1/2x MIC while fine checkered patterns indicate that cultures have been incubated without kanamycin.

DISCUSSION

Previous studies (1, 10) have focused on determining the role of OmpF downregulation on observed transient resistance to aminoglycosides when pretreated with sub-inhibitory levels of kanamycin using strains obtained from the Keio collection. The knockout strains that were used in these previous studies, as well as our own, were created by replacing the gene of interest with a kanamycin resistance cassette (2). In order to fully explore the possible mechanisms, through MarA, behind the downregulation of OmpF, these cassettes must first be removed. In our study we were able to demonstrate that the removal of the resistance cassettes was successful. The MICs that we determined were significantly lower than what was previously stated by these studies in which the cassettes were not removed (1, 10).

Since the protein quantity used in the SDS-PAGE assay as previously described was low (0.875 µg), definitive quantification of OmpF could not be achieved (10). Therefore in this study, we attempted to quantify demonstrated the downregulation of OmpF, occurring in response to kanamycin, by using a western blot with specific OmpF antibodies. The observed bandings at higher molecular weights on the western blot are likely trimeric forms of OmpF protein. Experiments have shown that trimeric OmpF is fairly stable and that boiling at 70˚C for 10 min, along with the appropriate detergent concentrations, is necessary to begin denaturing it into its monomeric subunit so that can be observed on a SDS-PAGE gel (12). Though we did boil our samples at 95˚C, it was only done for 3-5 min. As a result, insufficient denaturing time may have resulted in partially denatured trimers and explain the absence of banding at 40 kDa and presence of bands slightly lower than 100 kDa. Further optimization and refinement of the western blot is required to fully determine if OmpF is actually present. If not, investigation in the methodology of OmpF isolation must be performed as this indicates low yields of this protein.

Based on the fact that bands were seen in all strains, OmpF seemed to be clearly present in the Δ*ompF* strain. This is puzzling as the Keio collection clearly states that the genes of interest are replaced by the kanamycin resistant cassette (2). Therefore, after the removal of this cassette no detectable levels of OmpF indicative bands should be present (2). It is possible that the knockout strain were created by a partial deletion of *ompF* or simply inserting the cassette in the middle of the gene causing an insertional deletion and therefore the removal of this cassette would revert the strain back to the wild type phenotype. However, the construction records show that the insertion targeted the flanking regions on the ompF gene (2) so an incomplete insertion is unlikely. An alternative trivial possibility was that the strains were mixed up or that parental strains survived the selection. With more time these

alternatives could have been assessed by repeating the assays with a new isolate after confirming the genetic deletion and the absence of an *omp*F sequence in the genome.

In order to determine whether MarA mediates the downregulation of OmpF during aminoglycoside stress, differential band intensities were examined on our western blot. The significant change in band intensity for the *ΔmarA* strains led us to conclude that MarA does not play a large role in down regulating OmpF, via up regulation of *micF*, in response to the sub-inhibitory levels of kanamycin. Another unknown process may be responsible for regulating OmpF expression in the presence of antibiotic stress. Multiple studies have shown that MarA is responsible for the downregulation of OmpF during a variety of stress-induced responses (6, 7). However, none have looked into whether aminoglycosides have the ability to induce MarA expression. MarA has been well documented to regulate the AcrAB-TolC efflux pump system involved in multiple drug resistance in *E. coli* (11). Though AcrB does have a wide specificity towards different stressors, it does not target aminoglycosides (11). Instead, AcrD, an AcrB homolog, has been shown to be the main transporter for aminoglycosides including kanamycin (14). AcrD is not regulated by MarA, but by BaeR which is another DNA binding regulator shown to induce multiple drug resistance (14). BaeR is a part of a two-component system, along with the sensor kinase BaeS, which has been shown to be able to detect both external and internal stressors (14). Since MarA does not seem to be able to mount an OmpF-dependent response against aminoglycosides, it is possible that it is not fully expressed during exposure to sub-inhibitory levels of kanamycin and that other pathways are responsible for this phenotype. In the absence of MarA, a slightly higher sensitivity towards kanamycin was observed based on our growth curve (FIG. 1). This may show that MarA still plays an essential role in producing immediate resistance to kanamycin through other mechanisms yet to be determined.

In this study, even though we were not able to conclusively quantify the amount of expressed OmpF in response to sub-inhibitory levels of kanamycin within our strains, we quantified the relative decrease in OmpF production during exposure to 1/2x MIC of kanamycin. We were also able to determine that MarA does not play a large role in the downregulation of OmpF based on the data produced from our western blot. Other pathways are likely responsible during the response to exposure to kanamycin. Hence, the exact mechanisms of OmpF protein regulation in aminoglycoside resistance warrant further research.

FUTURE EXPERIMENTS

It should be made a priority to definitively determine whether the removal of the kanamycin resistance cassette results in a full or partial deletion of *ompF* or reverts the Δ*ompF* back to WT. This can be done by using PCR and primers, found within the MICB 424 primer collection, flanking the *ompF* gene. A 1 kb PCR product is expected if the complete deletion of the *ompF* gene and cassette was successful. PCR products of the genomes still containing the cassette is expected to be 2 kb in size. If these are the correct strains, future groups should seek to conclusively quantify the level of OmpF expression utilizing the *ompF* antibody used. The membrane sample should be treated at 95˚C for 10 min to be certain that any OmpF trimers are fully denatured prior to the SDS-PAGE and western blot. Optimization in the western blot protocol is required. It may also be wise to investigate different outer membrane isolation protocols as it is possible that our stated method yielded low OmpF concentrations.

Subsequently, to conclude whether or not MarA is expressed during exposure to kanamycin or other aminoglycosides, groups may seek to measure MarRAB promoter activity through the use of a reporter gene fused to the promoter. If the MarRAB promoter is cloned and ligated into a conjugative plasmid upstream of a promoterless *lacZ* (B-galactosidase gene) or *gfp* gene (green fluorescence protein gene), the activity of the MarRAB promoter in transformed *E. coli* can be measured through B-galactosidase activity or fluorescence upon exposure to kanamycin. No activity should be observed if *marRAB* activity is not induced during aminoglycosides induced stress.

ACKNOWLEDGEMENTS

This study was supported by the Department of Microbiology and Immunology, University of British Columbia. We wish to thank Dr. William Ramey and Matt Mayer for their guidance in the design and execution of the experiment. In addition, we would also like to extend thanks to Manjeet Bains for providing use of equipment necessary to the study.

REFERENCES

1. **Agafitei, O., E. J. Kim, T. Maguire, and J. Sheridan.** 2010. The role of *Escherichia coli* porins OmpC and OmpF in antibiotic cross resistance induced by subinhibitory concentrations of kanamycin. J. Exp. Microbiol. Immunol. **14**:34-39.
2. **Baba, T. et al.** 2006. Construction of *Escherichia coli* K-12 in-frame single-gene knockout mutants: the Keio collection. Mol. Syst. Biol. **2**:2006.0008. doi:10.1038/msb4100050.

3. **Carson, J., and A. Lee.** 2010. Determination of the relative OmpA expression and membrane integration in an OmpA-deficient *E. coli* strain complemented with a plasmid containing an *ompA* gene. J. Exp. Microbiol. Immun. **14**:48-50
4. **Chen, X. L., S. He, C. Li, and J. Ryu.** 2009. Sublethal kanamycin induced cross resistance to functionally and structurally unrelated antibiotics. J. Exp. Microbiol. Immunol. **13**:53-57.
5. **Cherepanov, P. P., and W. Wackernagel.** 1995. Gene disruption in *Escherichia coli*: TcR and KmR cassettes with the option of Flp-catalyzed excision of the antibiotic-resistance determinant. Gene. **158**:9-14.
6. **Chubiz, L. M., and C. V. Rao.** 2011. Role of the *mar-sox-rob* regulon in regulating outer membrane porin expression. J. Bacteriol. **193**:2252-2260.
7. **Delcour, A. H.** 2009. Outer membrane permeability and antibiotic resistance. Biochim. Biophys. Acta. **1794**:808-816.
8. **Delihas, N., and S. Forst.** 2001. *MicF*: an antisense RNA gene involved in response of *Escherichia coli* to global stress factors. J. Mol. Biol. **313**:1-12.
9. **Hancock, R. E. W.** September 19 1999, posting date. [Online.] Hancock Laboratory Methods. Department of Microbiology and Immunology, University of British Columbia, British Columbia, Canada. http://www.cmdr.ubc.ca/bobh/methods.htm ("$CaCl_2$ Transformation of *E. coli* " and "MIC determination by microtitre broth dilution method protocols")
10. **Hu, W., R. MacDonald, J. L. Oosthuizen, and M. van Soeren.** 2011. Sub-inhibitory kanamycin changes outer membrane porin ratios in *Escherichia coli* B23 by increasing the level of OmpC. J. Exp. Microbiol. Immunol. **15**:96-102.
11. **Okusu, H., D. Ma, and H. Nikaido.** 1996. AcrAB efflux pump plays a major role in the antibiotic resistance phenotype of *Escherichia coli* multiple-antibiotic-resistance (Mar) mutants. J. Bacteriol. **78**:306-308.
12. **Phale, P.S, A. Philippse, T. Kiefhaber, R. Koebnik, V.P. Phale, T. Schirmer and J.P. Rosenbusch** 1998. Stability of trimeric OmpF porin: the contributions of the latching loop L2. Biochem. **37**:15663-15670
13. **Randall, L. P., and M. J. Woodward.** 2002. The multiple antibiotic resistance (*mar*) locus and its significance. Res. Vet. Sci. **72**:87-93.
14. **Rosenberg E. Y., D. Ma, and H. Nikaido.** 2000. AcrD of *Escherichia coli* is an aminoglycoside efflux pump. J. Bacteriol. **182**:1754–1756.
15. **Shakil, S., R. Khan, R. Zarrilli, and A. Khan.** 2008. Aminoglycosides versus bacteria – a description of the action, resistance mechanism, and nosocomial battleground. J. Biomed. Sci. **15**:5-14.