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Stringent Response Changes Cell Membrane Permeability in *Escherichia coli* but does not Develop Cross Tolerance to Kanamycin, Tetracycline and Ampicillin

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Stringent response induces many changes in the cell to provide a survival advantage in conditions of nutrient starvation. One of these changes is decreased susceptibility to kanamycin, tetracycline and ampicillin. In this study, we investigated changes in *Escherichia coli* membrane permeability induced by stringency to determine whether or not it would cause antibiotic resistance. Stringency was induced by addition of excessive valine. Phenol sulfuric carbohydrate quantification assay was used to measure changes in carbohydrate concentration after incubation with *E. coli* wild type and *rel*$^{-/-}$ after induction of stringency. A killing assay using 4x MIC of kanamycin, ampicillin and tetracycline after stringency induction was to determine if resistance was developed. Uptake of 2-deoxy-D-glucose was reduced while uptake of sucrose and dextran increased in wild type when compared with rel$^{-/-}$ mutant. Thus it suggests a decrease in permeability in plasma membrane while increasing permeability of the outer membrane. Antibiotic tolerance did not develop after 24 incubation under stringent response with either ampicillin, kanamycin or tetracycline as determined by a killing assay. Thus stringency is not sufficient to develop antibiotic cross resistance.

Bacteria exposed to stressful conditions are known to induce a stringent response characterized as a state of persistence (4). This is controlled by the accumulation of the alarmone guanosine tetraphosphate (ppGpp), a global regulator synthesized by ppGpp Synthase I (RelA) in response to amino acid starvation (9), which alters gene expression in order to shut down unnecessary protein synthesis and to decrease cellular growth rate (1, 11, 13). Stable RNA accumulation and phospholipid biosynthesis are coupled to the rate of protein synthesis and reduced under stringent conditions; (1, 9) this is speculated to be mediated by ppGpp as levels have be shown to increase while RNA accumulation had decreased (1,13). By measuring the accumulated RNA levels in amino acid starved cells it can be determined whether conditions of stringency have been reached.

In a study by Nunn and Cronan (18), the rate of phospholipid biosynthesis was shown to decrease 2 to 4 fold in relA^{WT} cells controlled by ppGpp accumulation, while no change was observed for mutant relA^{-} cells unable to accumulate ppGpp. Other studies have also shown that following amino acid starvation, *Escherichia. coli* CP78 (relA^{WT}) increase membrane proportions of saturated fatty acids and cyclopropoane fatty acids and decreased unsaturated fatty acid content following amino acid starvation, whereas relaxed (non-stringent) *E. coli* CP79 (relA^{-}) did not respond in this manner (10). This suggests a possible decrease in membrane fluidity resulting in lower permeability. A study by Greenway and England (11) had shown that accumulated ppGpp in stringent induced cells have a decrease in susceptibility to antibiotics following exposure, however the mechanism was unknown. Taken together, these data suggest that the stringent response causes a decrease in membrane fluidity resulting in lower permeability.

Findings suggest that ppGpp accumulation due to the stringent response may lower cell membrane permeability which can possibly aid in antibiotic cross-tolerance via antibiotic exclusion. Low membrane fluidity has been described as an intrinsic resistance mechanism used by bacteria to prevent intracellular diffusion of small molecules such as antibiotics into the cell (12, 17). The outer membrane (OM) of Gram negative bacteria, such as *E. coli*, acts as a selective permeability barrier ("molecular sieve") containing tightly packed, anionic lipopolysaccharide and hydrophilic diffusion channels called pores (12, 17). Pores allow the passage of small hydrophilic molecules of up to approximately 600 Daltons (16) while excluding any larger or hydrophobic molecules. Hence this is a common entry method into the periplasm for most antibiotics.

By testing the permeability levels of stringent induced *E. coli* CP78 and mutant *E. coli* CP79 under amino acid starved conditions, a link between ppGpp accumulation and membrane permeability can be

established. This can be executed by measuring the permeation of the sugars dextran, sucrose & 2-deoxy-D-glucose. Dextran is a large polymer of glucose (2 kD) unable to penetrate the cell wall (6), unlike the other two sugars. Therefore this sugar complex can be used to measure the interstitial space of the cells (7). In order to induce conditions of stringency and simulate amino acid starvation, inhibitory levels of the amino acid valine will be used to limit the synthesis of the amino acid isoleucine (22).

This study investigates changes in permeability as a consequence of the stringent response by testing uptake of dextran, sucrose and 2-deoxy-D-glucose. To determine whether there were differences in cross-tolerance to these antibiotics due to the stringent response in *E. coli* CP78 and the relaxed response in *E. coli* CP79 samples were subjected to time-dependant killing assays following permeability assays in attempt to correlate the two processes. A short term decrease in susceptibility to antibiotics was expected to occur in the stringent wild type CP78 strain while also exhibiting a decrease in permeability.

MATERIALS & METHODS

Bacterial strains and growth conditions. Isogenic strains of *E. coli* K-12 CP78 (F-, thr-1, leuB6(Am), fhuA2, glnV44(AS), gal-3, his-65, malT1(λR), xyl-7, mtlA2, argH46, thi-1) and CP79 (F-, thr-1, leuB6(Am), fhuA2, glnV44(AS), gal-3, his-65, relA2, malT1(λR), xyl-7, mtlA2, argH46, thi-1) were obtained from MICB 421 culture collection from the Microbiology and Immunology Department at University of British Columbia. Cultures were grown on Luria-Bertani (LB) agar plates at 37 ºC and stored at 4 ºC for subculture. For all experimental assays, broth cultures were grown at 37 ºC with aeration at 100rpm in M-9 minimal salt media supplemented with 0.6% (w/v) glycerol, 100 µg/ml each of L-leucine (Sigma, T-8625), L-histamine (Sigma, A-5006), L-arginine (Sigma, H6034-256), L-threonine (Sigma, L-8000), and 1 µg/ml L-thiamine (Sigma, T-4625). To induce stringency, subcultures were treated with L-valine (Sigma, V4638) when applicable.

Growth curve determination and radioactive uracil incorporation assay. Overnight cultures were used to inoculate fresh cultures on the day of the experiment, and grown until reaching an OD_{460} (Spectronic 20D+) reading between 0.15-0.3, and then adjusted to the same cell density using M9 minimal salt media. Each culture flask was incubated at 37 ºC in a waterbath for 29 min to reach log phase, after which duplicate aliquots from each culture sample were distributed into four separate 125 ml Erlenmeyer flasks containing uracil at a final concentration of 2 µg/ml. To one of each CP78 and CP79 culture flask was added valine (Sigma, V4638) at a final concentration of 100 µg/ml. At 30 min, duplicate samples of 50µl from each culture flask were transferred onto 8 individual filter paper disks, air dried for 15-30 seconds and then treated with chilled 5% trichloroacetate (TCA) for the negative controls. At T=0, the OD_{460} was read for each culture flask, and a 1 ml sample of each culture was transferred into four separate test tubes each containing 0.25uCi [14C]-uracil, swirled to mix, 50 µl duplicates of sample were then transferred again onto filter paper disks, air dried for 15-30 seconds, and treated with chilled 5% TCA. This process was repeated in duplicate for T = 5, 15, 25, 35, 45 and 60 min. Turbidity measurements for each culture flask were also taken at each time interval. At T=70 min the TCA solution was decanted and the filters were washed with 50 ml chilled 5% TCA solution. At T=80 min, TCA solution was decanted and the filters were washed with 20 ml chilled 95% ethanol to remove residual TCA. At T=90 min the ethanol was decanted and the filters were transferred to foiled try with paper toil lining and backed overnight at 100 ºC. After 24 hrs each filter (56 total) were transferred to scintillation vials with 4 ml of scintillation fluid in order to measure radioactivity incorporation using the scintillation counter (Beckman, LS6500).

Minimal inhibition concentrations (MIC) assays. 100 µl of complete M-9 minimal salt media was transferred to the top 6 rows (A-F) of two sterile 96-well culture plates. 3 ml sterile solutions of tetracycline (Sigma, T-3383), ampicillin (Sigma, A-9518) and kanamycin (Sigma, K-4000) were prepared at a concentration of 1.024 mg/ml in complete M9 minimal salt media. Sterile solution is poured into a multi-channel pipette reservoir, using a multi-channel pipette, 1/2 serial dilution of the kanamycin antibiotic solution was performed across the rows A and B of each 96-well plate up to wells A11 and B11 to a final concentration of 0.5 µg/ml. The same serial dilutions were performed for ampicillin solution in rows C and D, and for tetracycline solutions in rows E and F. Row G contained M-9 minimal salt media alone as a negative control to ensure no contamination and row H contained bacterial cells and M-9 minimal salt media only as a positive control. Overnight cultures of *E. coli* CP78 and CP79 strains were diluted with complete M9 minimal salt media to achieve an OD460nm reading of 0.01. 100µl of the diluted cultures were transferred to the top 6 rows of each 96-wells culture plate using a multi-channel pipette. The plates were incubated in a 37 ºC incubator and assessed for visible bacterial growth after 24 hours. MIC's of each antibiotic for each strain were determined by the lowest antibiotic concentration which inhibited bacterial growth.

Carbohydrate permeability assays. Standard curves for each carbohydrate, 2-deoxy-D-glucose (NBC, 4024), D-sucrose (Sigma, S-0389) and dextran T2000 (BDH, 38015) were plotted to correlate absorbance with sugar concentrations using a phenol-sulfuric acid assay (7). Briefly, 2-deoxy-D-glucose was serially diluted in 1 ml of complete M9 minimal salt media from 1mM to 0mM at 0.1mM intervals. Similarly, D-sucrose was diluted from 0.5mM to 0.05mM at 0.05mM intervals, and dextran T2000 was diluted from 1000µg/ml to 100µg/ml at 100µg/ml intervals. 25µl of 80% liquefied phenol (Fischer, A931-1) was added to each sample, and then concentrated sulfuric acid (BDH, ACS 897-43) was added at a final concentration of 13 M. Sulfuric acid was added quickly and directed perpendicular to the liquid surface in the test tube to promote mixing. The samples were allowed to stand for 10 minutes, then shaken and incubated at 30 ºC for 20 minutes. Absorbance readings (A490) were taken with the Spectronic20 spectrophotometer. A starter culture of CP78 and CP79 prior to the ON culture was prepared by inoculating from LB plates into flasks containing 10 ml of complete M-9 minimal salt media for each strain. Overnight cultures were grown, and then split into two equal volumes into four 250 ml fixed-angle centrifuge tubes and cells were pelleted at 9000 x g for 20 min (Sorvall RC-5B Plus, SLA-1500 rotor). The supernatant was discarded and pellets were resuspended in 250 ml of fresh M-9 minimal salt media and then transferred to 2 sterile flasks for each CP78 and CP79. Cultures were incubated for 30 min, until reaching log phase in order to further concentrate the cells. Cultures were then centrifuged as described above, and pellets were again resuspended in 250 ml fresh M-9 minimal salt media and the initial turbidity was measured at 460nm. Valine was added to one of the two flasks of both CP78 and CP79 at a final concentration of 100 µg/ml, and cultures were incubated under the same conditions for 1 hr to induce stringency. The turbidity was measured at 460nm and cultures were centrifuged under the same conditions again. Pellets (~250 mg cells per pellet) were resuspended in 3 ml fresh M-9 minimal salt media and each of the 4 sample cultures were equally split (1 ml each) into three microfuge tubes and cells were obtained by centrifugation at 12,000 rpm for 2 min. Supernatant was discarded and sample pellets were weighted in their respective tubes. One pellet of each the 3 replicates of the 4 sample cultures was resuspended in 250 µl of each carbohydrate, 4mM 2-deoxy-D-glucose, 2 mM D-sucrose and 150 µg/ml dextran. Samples were incubated at room temperature for 15 min to allow diffusion and transport of sugars to ensure that equilibrium has been reached, and

then were centrifuged again at 12,000 rpm for 2min. 200µl of supernatant from each tube was transferred into a new microfuge tube. Each sample was diluted 1/50 with M-9 minimal salt media and transferred to 12 Spectronic20 cuvettes, and a control of each carbohydrate solution in M-9 minimal salt media alone were made. The phenol-sulfuric acid assay was performed in the exact same manner as the generation of the standard curves with 25 µl 80% phenol and 2.5 ml concentrated sulfuric acid, shaken then incubated at 30 ºC for 20 min. Turbidity readings were obtained at 490 nm and were normalized to cell pellet weight. This assay was repeated three independent times to ensure confidence in the results.

Killing Assay. Sterile 5 ml antibiotic solutions of kanamycin (4 µg/ml), tetracyline (4 µg/ml) and ampicillin (16 µg/ml) were prepared at concentrations 4-fold greater than the determined MIC. Overnight cultures of CP78 and CP79 were prepared then split into two each and brought into log phase the following day using fresh M-9 minimal salt media and incubating at 37 ºC in a mildly aerated waterbath. 100 µg/ml of valine was added to one of each CP78 and CP79 flasks for amino acid starvation and incubated for 1 hour under the same conditions. Each culture sample was then diluted with M-9 minimal salt media to a final volume of 10 ml to achieve an OD460nm reading of 0.01. On a 96 well plate, 100 µl of ampicillin (16 µg/ml) solution was added to wells A1 – A7, and D1 to D7, 100 µl of kanamycin (4 µg/ml) to wells B1 to B7 and E1 to E7, and 100 µl of tetracycline (4 µg/ml) to wells C1 to C7 and F1 to F7. 100 µl of diluted control *E. coli* CP78 was then added to wells A2-A4, B2-B4, and C2-C4, and 100 µl of *E. coli* CP79 to wells D2-D4, E2-E4, and F2-F4. 100 µl of valine treated *E. coli* CP78 was added to wells A5-A7, B5-B7 and C5-C7 and 100 µl of valine treated *E. coli* CP79 to wells D5-D7, E5-E7 and F5-F7. The 96 well plate was incubated at 37 ºC for 24 hours. After incubation, the plate was assessed for growth.

RESULTS

In order to study the rule of stringency in membrane permeability, the stringent response was induced with the addition of valine to create amino acid starvation conditions. The results of this assay, illustrated in Figure 1A show the induction of stringent response upon addition of valine in excess. The rate of RNA synthesis, which correlates with the incorporation of radioactive uracil, was normalized to growth of corresponding culture as determined by turbidity. In CP78 strain, incorporation of radioactive uracil was observed in conjunction to increasing cell growth. With the addition of valine, radioactive uracil incorporation becomes limited even in the presence of continued cell growth. As can be seen in figure 1B, CP79 continued to incorporate radioactive uracil in the presence of valine, indicating failure to induce stringent response, as expected. Both untreated and valine treated samples incorporated radioactive uracil at a similar rate in conjunction to cell growth.

Table 1 shows the minimal inhibitory concentrations of kanamycin, ampicillin and tetracycline determined for *E. coli* strains CP78 and CP79. The two strains display the same relatively high sensitivity to tetracycline and relatively low sensitivity to ampicillin. CP79 also has relatively high sensitivity to kanamycin while CP78 was only moderately sensitive to kanamycin.

TABLE 1. Minimum inhibitory concentration of kanamycin, ampicillin and tetracycline on *E. coli* CP78 and CP79.

E. coli Strain	MIC (µg/ml)		
	Kanamycin	Ampicillin	Tetracycline
CP78	2	4	1
CP79	1	4	1

After induction of stringency, permeability changes were apparent in wild type and *rel*$^{-/-}$ mutant. Changes in carbohydrates uptake upon valine treatment are presented as a ratio of the decrease in carbohydrate concentration in valine treated sample to untreated sample in Figure 2. Both CP78 and CP79 experienced decreased uptake of 2-deoxy-D-glucose and increased uptake of D-sucrose and Dextran T-2000 following valine treatment. Despite the same trends in uptake and exclusion of the three carbohydrates upon valine addition, the changes were much more prominent in CP78 than in CP79, which showed very minor changes for all three carbohydrates. Thus RelA induced stringency is sufficient to induce permeability changes regarding carbohydrate uptake. The permeability changes may also affect uptake and exclusion of other compounds such as antibiotics.

To determine whether or not resistance was developed as a result of stringency, wild type and *rel*$^{-/-}$ mutants were subjected to a killing assay. The killing assay (Fig. 3) measured death of cultures after 24 hours incubation in the presence 4x the MIC of ampicillin, kanamycin or tetracycline, with or without valine treatment. Cell death was measured by the absolute decrease in optical density, after correcting for the background absorbance of antibiotics. Untreated samples for both CP78 and CP79 strains experienced a minor decrease in optical density after 24 hour incubation with antibiotics. However, both CP78 and CP79 experienced a greater decrease in optical density following 24 hours incubation in the presence of antibiotics and valine. The degree of cell death induced by kanamycin, ampicillin and tetracycline were comparable. Both *E. coli* strains experienced similar levels of antibiotics induced cell death, in the presence or absence of valine. Thus the results indicate that under our experimental conditions, resistance was not developed under the stringent response.

DISCUSSION

Stringency has been show to affect multiple processes within the cell. The aim of this study was to investigate stringency dependent changes in membrane

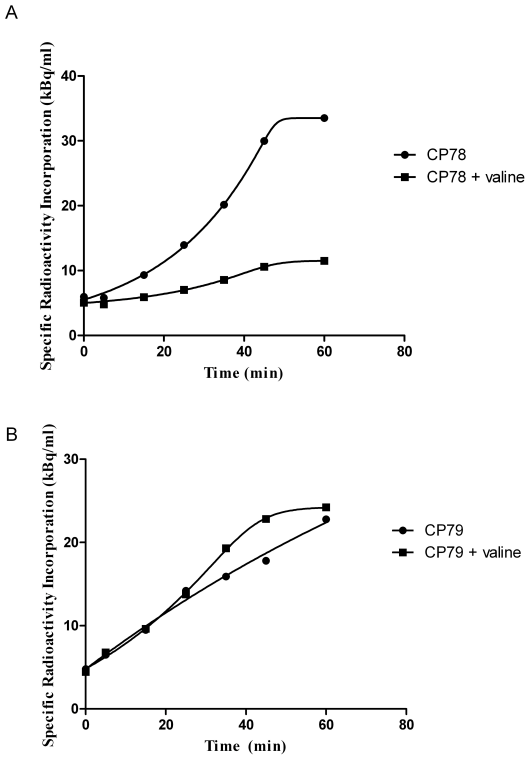

FIG. 1. Effect of valine on the rate of RNA synthesis in *E. coli* CP78 (A) and CP79 (B). Excess valine was added at Time = -30 min.

permeability and antibiotic resistance. RelA is required for inducing stringent response under nutrient limiting conditions. Excess valine inhibits isoleucine synthesis thus lead to amino acid starvation and activation of the stringent response (22). The data in figure 1 suggests that a proper stringent response, indicated by limited accumulation of RNA synthesis, was induced in CP78 strain by addition of excess valine. CP79, a relA knock out mutant, was unable to enter stringency and continued to grow and synthesize RNA unaffected by addition of excess valine. Taken together, these data confirm the phenotypes of these strains.

The antibiotics in this experiment, ampicillin (β-lactam), kanamycin (aminoglycoside) and tetracycline, are all small hydrophilic molecules that follow similar uptake into the cell (or periplasm for the ampicillin). β-lactams target the periplasm by simple diffusion (23) and interfere with peptidoglycan cell wall synthesis resulting in lysis. Greenway and England (11) report that increased levels of ppGpp and the induction of the stringent response has exhibited tolerance to β-lactam antibitotics (11). This may be explained by the lack of growth and/or replication of the cells under stringency which would not be undergoing copious peptidoglycan synthesis thereby blocking β-lactam activity, unlike relaxed cells (relA$^-$) which would still be actively

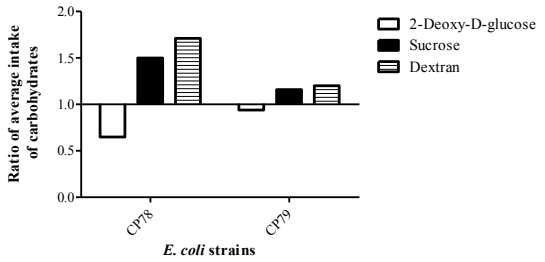

FIG. 2. Membrane permeability of *E. coli* to carbohydrates after the induction of stringency. Carbohydrate intake expressed as a ratio between valine treated and untreated *E. coli* samples for induced conditions of stringency.

increasing in size and numbers. Diffusion of ampicillin into the periplasm will be detected for change under stringent conditions.

Aminoglycosides also diffuse through the OM of bacteria in a process that occurs as rapid as some hydrophilic, uncharged hexoses and disaccharides, which is not expected due to the larger molecular weights of these antibiotics (15, 21). Crossing of the cytoplasmic membrane occurs in an uptake process called Energy-Dependent Phase I and II (EDPI and EDPII), although a transport carrier has yet to be identified (12, 21). These antibiotics target the cytoplasm, specifically the 30S ribosomal subunit at which they bind to and inhibit ribosomal translocation along the messenger RNA resulting in protein mis-translation and ribosome stalling (8). We hypothesize that upon kanamycin exposure stalling of the ribosome may mimic what occurs due to the lack of available aminoacyl-tRNA's during periods of amino acid starvation. In response, this may activate RelA and the stringent response possibly leading to cross-tolerance of the antibiotic. Tetracycline is the third antibiotic to be tested and is yet another diffusible one that targets the cytoplasm, specifically the 30S ribosomal subunit (3) with a similar mechanism to that of kanamycin.

Stringency did affect permeability of the three carbohydrates. Changes in permeability upon valine treatment can be observed in figure 2. The changes are suggestive that permeability for 2-deoxy-D-glucose decreases in CP78 dramatically when compared to CP79. Decrease in permeability to 2-deoxy-D-glucose was also observed in CP79 but the changes were negligible, and likely due to experimental variation. This suggests that the cellular membrane changes to be less permeable and exclude compounds from entering the cell. Increased permeability changes for sucrose and dextran were unexpected since sucrose can only cross the outer membrane and enter the periplasmic space while dextran cannot cross the outer

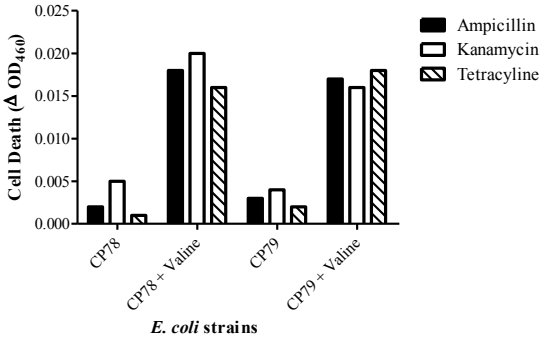

FIG. 3. Cell death induced by ampicillin, kanamycin or tetracycline, in the presence or absence of excess valine. Absolute changes in OD_{460} were calculated after correcting for background absorbance influenced by presence of antibiotics, which were added at 4x MIC.

membrane and should remain excluded from the cell. Our data suggests that contrary to our initial predictions, stringency might induce changes to permeability to the outer membrane resulting in increased intake of sucrose and dextran. Our hypothesis arose from the fact that upon induction of the stringent response, cells upregulate lipid biosynthesis and enter a protective state to increase likelihood of survival (18). However, other factors such as expression of porins could be at play to affect the rate of carbohydrates diffusion across outer membrane (16). An increase uptake of sucrose and dextran was also observed in CP79, but again the changes were much more subtle in CP79 relative to CP78. This observation is consistent with the fact that CP79 is incapable of inducing stringent response, thus CP79 was relatively insensitive to valine treatment. Our data were not without error; there were considerable variation between replicates of assay. Further optimization of the permeability assay may be required to more accurately conclude whether or not changes in membrane permeability were induced by stringency.

Stringency has been shown to affect tolerance to antibiotics. Low level antibiotic resistance is defined by an increased in MIC. The killing assay in figure 3 showed evidence contradicting this fact. CP78 and CP79 without valine treatment displayed minor changes in death when compared to valine treatment of respective strains. In both strains, an increase in antibiotic sensitivity was observed with addition of valine. Unexpectedly, CP79 behaved differently with and without valine treatment. Since the CP79 strain is incapable of inducing stringency but the same trend in antibiotics induced cell death was seen between CP78 and CP79 in the presence of valine, our data suggest the possibility that stringency may not be involved in the observed increase in antibiotic sensitivity. Another possibility is that the combination of valine and antibiotics may be responsible for inducing the observable effect. This effects of antibiotics might have overshadowed the effect of the stringent response thus prevented the observation of our expected result of CP79 treatment behaving similarly to CP79 untreated sample. A modified killing assay should be performed to look at the effects after stringency is induced and valine is removed before introduction of antibiotics. Replicates of the killing assay were performed simultaneously, thus the data presented here may not accurately reflect errors associated with the assay. Further optimization may be required to reproduce data in a more consistent manner.

This study was intended to observe general permeability changes in connection to antibiotic resistance induced by stringency. Under the effects of stringency, the cell would decrease permeability for increase in survival against antibiotics. Stringency was induced in CP78 strain but not CP79 strain and permeability appears to increase in the outer membrane but decreases for cytoplasmic membrane in CP78. The killing assay showed no increase in antibiotic sensitivity. The results show that stringency was sufficient to cause permeability changes but the permeability changes were not sufficient to account for antibiotic cross resistance.

FUTURE DIRECTIONS

From our results, the increase in permeability of the carbohydrates appears to be of interest. The increase of uptake of dextran and sucrose was evident but accumulation within the periplasm or cytoplasm is unclear. Fluorescent carbohydrates such as dextran-FITC and fluorescent sucrose could be used to visually conclude this possibility by monitoring increase in fluorescence in cells after induction of stringency and incubation with the mentioned fluorescent carbohydrates. This method will also determine the extent of permeability into the cell and confirm how each barrier is changing in terms of permeability. Optimization of the permeability assay would also need to be performed to obtain consistent data.

Further investigation should look at what aspect of permeability is altered. To do this, one should assess the level of expression of porins in the outer membrane under normal and stringent conditions with the strains used in this study. One approach is to treat both *E. coli* CP78 and CP79 with or without valine to induce no response or stringent response in both strains. Then the cells are centrifuged and the resulting cell pellet is collected and lysed. The levels of porins such as OmpA, OmpC and OmpF as well as other possible porin molecules can be assessed using western blotting

technique. Increased porin levels would indicate increase in permeability and decrease in porins would indicate reduction in permeability.

The difference between kanamycin induced stringency and excessive valine induced stringency will also need to be determined with respect to membrane permeability. It is possible that kanamycin can induce a stronger stringent response or even bind to secondary targets within the cell to induce the effects of antibiotic tolerance. First, to assess change in permeability from kanamycin induced stringency, one would perform the same experiment with excessive valine as above and repeat it by replacing excess valine with kanamycin. After induction of stringent response the permeability assay must be conducted again to determine if the level of stringency induced by the two methods are equivalent. If the permeability results remain the same between valine and kanamycin treated samples it can be suggested that the stringent response induced by both treatment methods affect permeability similarly. However if the results are different from each other it is clear that both kanamycin is operating stringency through a different method which may be responsible for development of antibiotic resistance. To determine what else is affected differently by kanamycin and valine a microarray analysis can be performed on the harvest pellets after extracting the RNA from the cells. The results will give an overall picture of how differently and what aspects of cellular functions are affected by kanamycin and valine. Looking through the data collected it may also be possible to determine which changes induced by stringency may result in antibiotic tolerance at the genetic level.

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REFERENCES

1. **Atherly, A. G.** 1974. Ribonucleic acid regulation in amino acid-limited cultures of *Escherichia coli* grown in a chemostat. J. Bacteriol. **120**:1322-1330.
2. B**everidge, T.J., J.A. Breznak, G.A. Marzluf, T.M. Schmidt, and L.R. Snyder.** 2007. Permeability and Transport, p. 518-530. *In* Methods for General and Molecular Microbiology, 3rd ed. Editor in Chief, C. A. Reddy. ASM Press, Washington, DC.
3. **Brodersen, D. E., W. M. Clemons, A. P. Carter, R. J. Morgan-Warren, B. T. Wimberly, and V. Ramakrishnan.** 2000. The structural basis for the action of the antibiotics tetracycline, pactamycin, and hygromycin B on the 30S ribosomal subunit. Cell **103**:1143-1154.
4. **Chen, L. X., 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. **Cheng, K., L. Ren, and K. Tsai.** 2010. Inhibitory concentrations of kanamycin in the presence of ppGpp synthase RelA confer protection against subsequent lethal antibiotic assaults in *E. coli* CP78. J. Exp. Microbiol. Immunol. **14**:51-56.
6. **Decad, G. M., H. Nikaido.** 1976. Outer membrane of gram-negative bacteria XII. Molecular-sieving function of cell wall. J. Bacteriol. **128**:325-336.
7. **Dubois, M., K. A. Gilles, J. K. Hamilton, P. A. Rebers, and F. Smith.** 1956. Colorimetric method for determination of sugars and related substances. Anal. Chem. **28**:350-356.
8. **Fourmy, D., M. I. Recht, S. C. Blanchard, and J. D. Puglisi.** 1996. Structure of the A site of *Escherichia coli* 16S ribosomal RNA complexed with an aminoglycoside antibiotic. Science **274**:1367-1371.
9. **Gallant, J. A.** 1979. Stringent control in *Escherichia coli*. Annu. Rev. Genet. **13**:393-415.
10. **Gitter, B., R. Diefenbach, H. Keweloh, and D. Riesenberg.** 1995. Influence of stringent and relaxed response on excretion of recombinant proteins and fatty-acid composition in *Escherichia coli*. Appl. Microbiol. Biotechnol. **43**:89-92.
11. **Greenway, D. L. A. and R. R. England.** 1999. The intrinsic resistance of *Escherichia coli* to various antimicrobial agents requires ppGpp and sigma(s). Lett. Appl. Microbiol. **29**:323-326.
12. **Hancock, R. E. W.** 1997. The bacterial outer membrane as a drug barrier. Trends Microbiol. **5**:37-42.
13. **Höltje, J. V.** 1979. Regulation of polyamine and streptomycin transport during stringent and relaxed control in *Escherichia coli*. J. Bacteriol. **137**:661-663.
14. **Lengeler, J. W., R. J. Mayer, and K. Schmid.** 1982. Phosphoenolpyruvate-dependent phosphotransferase system Enzyme-III and plasmid-encoded sucrose transport in *Escherichia coli* K-12. J. Bacteriol. **151**:468-471.
15. **Nakae, R. and T. Nakae.** 1982. Diffusion of aminoglycoside antibiotics across the outer-membrane of *Escherichia coli*. Antimicrob. Agents Chemother. **22**:554-559.
16. **Nakae, T., J. Ishii, and M. Tokunaga.** 1979. Subunit structure of functional porin oligomers that form permeability channels in the outer membrane of *Escherichia coli*. J. Biol. Chem. **254**:1457-1461.
17. **Nikaido, H.** 1994. Prevention of drug access to bacterial targets - permeability barriers and active efflux. Science **264**:382-388.
18. **Nunn, W. D. and J. E. Cronan.** 1976. Regulation of membrane phospholipid synthesis by *relA* gene - dependence on ppGpp levels. Biochemistry (N.Y.) **15**:2546-2550.
19. **Schmid, K., M. Schupfner, and R. Schmitt.** 1982. Plasmid-mediated uptake and metabolism of sucrose by *Escherichia coli* K-12. J. Bacteriol. **151**:68-76
20. S**chmid, K., R. Ebner, J. Altenbuchner, R. Schmitt, and J. W. Lengeler.** 1988. Plasmid-mediated sucrose metabolism in *Escherichia coli* K-12 - Mapping of the *scr* genes of pUR400. Mol. Microbiol. **2**:1-8.
21. **Taber, H. W., J. P. Mueller, P. F. Miller, and A. S. Arrow.** 1987. Bacterial uptake of aminoglycoside antibiotics. Microbiol. Rev. **51**:439-457.
22. **Umbarger, H. E. and B. Brown.** 1955. Isoleucine and valine metabolism in *Escherichia coli* V. antagonism between isoleucine and valine. J. Bacteriol. **70**:241-248
23. **Zimmermann, W. and A. Rosselet.** 1977. Function of outer membrane of *Escherichia coli* as a permeability barrier to beta-lactam antibiotics. Antimicrob. Agents Chemother. **12**:368-372.