Journal of Experimental Microbiology and Immunology (JEMI) Vol. 12:45-49

The Role of CpsG in Capsular Carbohydrate Accumulation in Response to Aminoglycoside Stimulation

Yiannis Himaras, Frank Lin, Shin Kim and Peter Cao
Department of Microbiology and Immunology

***Escherichia coli* had been shown to increase its capsular polysaccharide (CPS) production upon stimulation with kanamycin and streptomycin. In this study, an attempt was made to investigate whether or not CpsG, a phosphomannomutase responsible for part of the colanic acid synthesis pathway, is involved in such response. Our study showed that upon one hour of kanamycin or streptomycin stimulation, Δ*cpsG E. coli* was still able to increase its CPS concentration, implying that CpsG might not play a role in such response. Silver staining following PAGE electrophoresis indicated that CpsG was not sufficiently abundant in *E. coli* to be visible as a difference between the Wild Type and Δ*cpsG* strains. Absence of CpsG did not affect CPS accumulation as if such accumulation is independent of the CpsG pathway.**

Capsular polysaccharides (CPS) that are produced by certain species of bacteria such as *Escherichia coli* appear to slow down the diffusion of potentially harmful substances such as antibiotics into the cell (10). Therefore, it is logical to expect that CPS producing bacteria such as *E. coli* will increase CPS production upon antibiotic stimulation, and this increase has been observed in prior research (7). For instance, exposure to sub-lethal doses of kanamycin and streptomycin caused an increase of CPS in *E. coli* (7). This finding implied that aminoglycosides may have secondary effects that influence the *cps* operon, which is responsible for the synthesis of colanic acid (CA), the extracellular polysaccharide produced by most strains of *E. coli* (8, 16). Among the genes in the *cps* operon, *cpsG*, also known as *manB*, encodes for a phosphomannomutase, which is responsible for an upstream step in the CA biosynthesis pathway (16).

The *cps* operon is responsible for the production and transportation of colanic acid which is principally used in capsular polysaccharide (CPS) formation (17). The *cps* operon consists of enzymes along with transporters which modify and assemble existing cytoplasmic sugars into colanic acid subcomponents. One of the essential genes required for the successful production of colanic acid is *cpsG*. *cpsG* is synonymous to *manB*, and functions as a phosphomannomutase responsible for converting a-D-mannose 1-phosphate (M1P) to D-mannose-6-phosphate (M6P) which is required in the colanic acid biosynthesis and GDP mannose metabolism pathways (1). M6P is the first sugar required in the synthesis of colanic acid. *CpsG* was selected as a candidate for knocking out the overall effect of the *cps* operon because of its essential role in initial sugar conversion. Without *cpsG*, it is assumed that M6P cannot be synthesized, and consequently all subsequent pathway functions will be inhibited. In this study, we investigated if CpsG plays a role in the increase of CPS in *E. coli* following exposure to kanamycin and streptomycin. This hypothesis was tested by comparison of CPS production between a wild type and a *cpsG* mutant strain of *E. coli*.

METHODS AND MATERIALS

Cell culture preparation and growth conditions. Unless otherwise specified, the cell cultures were grown in Luria broth (0.5% w/v Bacto Yeast Extract, 1% w/v Bacto Tryptone, 0.5% w/v NaCl, pH 7) media or on LB plates (LB broth with 1.5% agar) at 37ºC. The *Escherichia coli* strains used in this experiment were kindly provided by Coli Genetic Stock Center of Yale University. The strains included *E. coli* BW25113 (genotype Δ(araD-araB)567, lacZ4787(del)(::rrnB-3), LAM-, rph-1, Δ(rhaD-rhaB)568, hsdR514) , *E. coli* JW2033-1 (genotype identical to BW25113 with addition of cpsG746(del)::kan) and *E. coli* BT340 (contains pCP20 plasmid). *E. coli* BW25113 and *E coli* JW2033-1 will be referred to as WT and Δ*cpsG* cells, respectively. WT cells and Δ*cpsG* cells are genetically identical except that Δ*cpsG* strain does not contain the *cpsG* gene. The Δ*cpsG E. coli* contained a kanamycin-resistance transposon. The transposon was removed using the pCP20 plasmids (ts-rep, [cl857](lambda)(ts), bla(ApR), cat, FLP(II), ampicillin-resistant, chloroamphenicol-resistant) derived from *E. coli* BT340 cells. The plasmids were isolated using Invitrogen PureLink Quick Plasmid Miniprep, Cat. No. K2100-10. Isolated pCP20 plasmids were transformed to Δ*cpsG* cells by electroporation as described by Hinze (19). Following the transformation, the cells were spread on LB plates and incubated at 30ºC overnight. 20 colonies from the overnight plates were transferred onto fresh LB grid plates and incubated at 42ºC overnight to activate the flippase in the pCP20, excise the kanamycin-resistant transposon, and eliminating the

temperature sensitive pCP20 plasmids. Colonies from the 42ºC overnight LB plates were replica plated to one fresh LB plate, one LB + ampicillin (100 µg/ml) plate and one LB + kanamycin (30 µg/ml) plate and incubated at 42ºC overnight. A kanamycin/ampicillin sensitive colony was then streaked on a fresh LB plate and incubated at 37ºC. This was the Δ*cpsG* cells used for the experiment.

Antibiotic stimulation. 20 ml of overnight WT and Δ*cpsG* cells were grown in 500 ml of fresh LB broth in 37ºC shaking waterbath for 4 hours. Each fresh culture was then split into three 150 ml cultures. One of the three 150 ml cultures was treated with kanamycin (25 µg/ml), another one was treated with streptomycin (25 µg/ml), while the last one was left untreated as a control. The cultures were then incubated in 37ºC shaking water bath for one hour. Turbidity values were then taken for each cell culture at 460 nm.

Capsular polysaccharide isolation. This protocol was adopted from the capsule isolation method described by Ganal et al. (4). In brief, the antibiotic stimulated cells were centrifuged at 17,000 x g for 20 min, and the pellets were resuspended in 50 ml of PBS. Five ml of each cell suspension was aliquoted for silver staining. The remaining cells were blended with a bead basher without beads (BioSpec BeadBeater™) for 5 min to release the capsular carbohydrate. The blended mixtures were then centrifuged at 17,000 x g for 10 min. The supernatants were transferred to fresh centrifuge bottles containing 60 ml of ice-cold acetone. The solutions were centrifuged again at 6,000 x g for 10 min. The pellets were then resuspended in 5 ml of distilled deionized water (dH_2O) and then enclosed in Spectra/Por^R Molecularporous membrane (MW cut-off of 6,000 – 8,000 kDa) with 100 µl of chloroform. These samples were dialysed against 1L of dH_2O for 48 hours. The dialysis solution was changed twice a day. The final dialysed samples were dried overnight with a lyophilizer. The dried samples were resuspended in 20 ml of $MgCl_2$ (10 mM). DNaseI (5 µg/ml; Invitrogen) and RNaseA (100 µg/ml; Invitrogen) were added. The samples were incubated for 5 hours at 37ºC, followed by incubation with pronase (100 µg/ml) for 20 hours. The samples were then heated at 80ºC for 30 minutes and centrifuged at 17,000 x g for 20 min. The supernatants were dialysed using the same membrane as above against 2L of dH_2O and lyophilized for 48 hours. The dried samples were assayed for carbohydrate concentration with the anthrone assay.

Silver staining of PAGE electrophoresis gel. The samples were kept chilled at all time to prevent proteolysis. The 5 ml cell aliquots were centrifuged at 17,000 x g for 20 min at 4ºC, and the pellets were resuspended in 1 ml of Tris-HCl (20 mM, pH 7.5). The cell suspensions were lysed mechanically using Sigma glass beads and the FastPrep machine (Q-bio gene) at a setting of 6 for 40 sec. Immediately after bead bashing, the cells were chilled for 2 minutes and then bead bashed again using the same settings. The cell lysates were centrifuged at maximum speed in a microfuge for 5 min, and the supernatants were collected and centrifuged again using the same settings. The supernatants were transferred to new microfuge tubes, and final nuclease concentration of 2.4 µg/ml was added to each supernatant. The sample mixtures were incubated on ice for 5 min, and SDS sample buffer (SB) (19) was added to the samples to reach a final concentration of 1X SB. Twenty-five µl of each sample was loaded and electrophoresed on a 10 % polyacrylamide gel in 1X running buffer (19) at 150 V for 1.5 hours. The standard molecular weight marker used was Invitrogen HiMarkTM Unstained Protein Standard (Cat. No. LC5688). Immediately following gel electrophoresis, silver staining was performed according to the protocol described in BioRad Silver stain plus kit (Cat. No. 161-0449).

Anthrone assay. The anthrone reagent for carbohydrate assay was prepared by first dissolving 0.2 g of anthrone in 5 ml of absolute ethanol, followed by addition of 95 ml of concentrated sulphuric acid. The lyophilized capsular polysaccharide samples were resuspended in 0.5 ml of dH_2O. Half ml of standards contained 0, 20, 40, 60, 80 and 100 µg/ml of glucose. All the samples and standards were mixed with 4 ml of anthrone reagent and chilled in an ice bath before mixing. The chilled samples were then placed in a boiling water bath for 15 min then chilled to room temperature before measuring the concentration at A_{625}.

RESULTS

Capsule polysaccharide induction. Since the cells were going to be treated with kanamycin, it was necessary to remove the kanamycin-resistant transposon in the Δ*cpsG* cells. As shown in Figure 1, the WT *E.coli* cells showed increased CPS when treated with kanamycin and streptomycin in comparison to the control, although the difference appears to be statistically insignificant, which does not agree with previous findings (4). Among the Δ*cpsG* cells, the streptomycin treated sample showed the highest amount of CPS, followed by the control sample and kanamycin treated sample, but the difference in the CPS content were statistically insignificant. For both WT and Δ*cpsG* cells, kanamycin induced less CPS than than streptomycin. Suprisingly, Δ*cpsG* cells produced more overall CPS than wildtype cells.

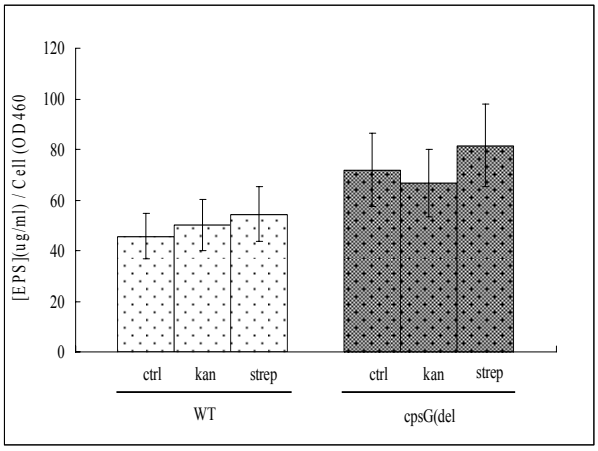

FIG. 1. Effect of aminoglycoside treatment on the concentration of CPS accumulated by WT and Δ*cpsG* cells. Each cell type was stimulated with or without the indicated aminoglycoside at 25 ug/ml for one hour. The CPS concentration was measured by Anthrone assay. The error estimated from the standard curve was used to determine the error in sample estimation (data not shown).

CpsG protein Visualization. From Figure 2, we were not able to localize the band corresponding to CpsG protein (50 kDa) due to high backgrounds since we loaded the total cell lysates. The expected band corresponding to CpsG protein should be present in the WT cells but absent in the Δ*cpsG* cells between the 45 kDa and 66 kDa molecular weight markers. Therefore, silver staining, at least in this case, was not helpful in visualizing CpsG. Lanes 5 and 6 appeared less intense compared to other lanes, but this does not represent lower amounts of proteins due to reasons that will be addressed in the discussion. In lane 5 at the positions marked by arrows, there appeared to be less proteins compared to other lanes.

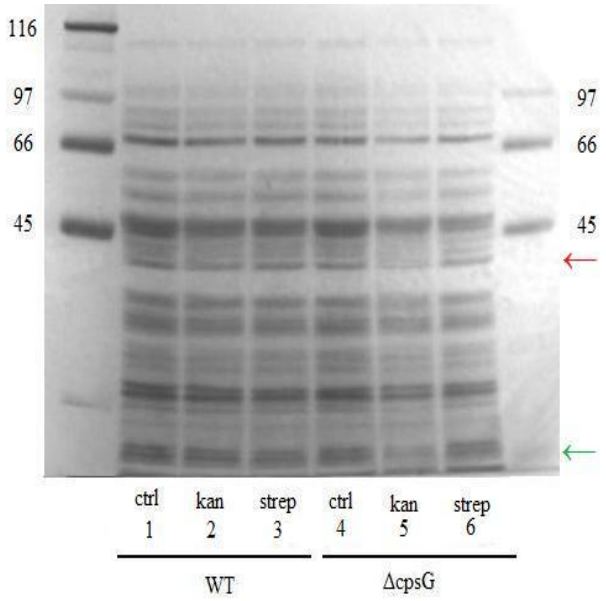

FIG. 2. The effect of aminoglycoside treatment on the accumulation of proteins in WT and Δ*cpsG* cells. The WT and Δ*cpsG* cells were stimulated with or without aminoglycosides (25 μg/ml) as indicated for 1 hour. The total cell lysates were run on a 10% PAGE gel at 150 V for 1.5 hours and the gel was silver stained. The target band (50 kDa CpsG) was masked by massive amount of proteins of similar molecular weight. The far right and left lanes are standard molecular weight markers. The arrows indicate the bands that show decreased amount of proteins in the kanamycin-treated Δ*cpsG* samples.

DISCUSSION

Ganal et al. (4) observed that exposure of *E. coli* with streptomycin and kanamycin at the minimal inhibitory concentration caused a notable increase synthesis of colanic acid. In this experiment, the *cpsG* knock out strain should have decreased CPS content but this was not observed (see Figure 1). This might suggest that the effects of kanamycin and streptomycin are not due to interact with the *cps* pathway which contains *cpsG*. Other mechanisms could explain this increase in CPS. One possibility is that the regulators of the *cps* operon, RcsA, are able to activate CPS production elsewhere in the cell independent of colanic acid synthesis (5). It is known that RcsA protein activates the production of exopolysaccharide O antigen for LPS, M antigen (colanic acid), and K antigen (2). Kanamycin and streptomycin could have potentially activated other exopolysaccharide synthesis pathways bypassing the *cps* operon in colanic acid production. To distinguish this possibility would require more specific analysis of the type of sugar accumulated.

Sailer et al. (13) exposed a *cps*/LacZ mutation *E. coli* to several different types of antibiotics and observed no activation within the *cps* system. In a similar experiment, Rachid et al. (10) exposed *Staphylococcus epidermidis* to varying MIC levels of antibiotics. The results obtained from gentamycin, an aminogylcoside related to streptomycin and kanamycin, produced no increase in polysaccharide adhesion molecules. From Figure 1, it can be seen that contrary to the findings of Ganal et al. (4), there was no significant increase in total exopolysaccharide concentration upon treatment with aminogylcosides compared to the control. As can be clearly seen in figure 1, the samples from the WT strain appeared to show substantially less total carbohydrate in all isolates when compared to the samples from the *cpsG* knockout strain. The most logical explanation for this occurrence is due to the fact that significant quantities of material from all three WT samples was lost during macromolecule isolation and were unrecoverable. Furthermore, the actual volume containing the cellular material, including the carbohydrates, was unknown because no value was recorded for the true amount of material used during the carbohydrate isolation. As such, normalization of carbohydrate levels in WT samples was done using overestimated values for turbidity. Due to this overestimation, the reported normalized levels of WT carbohydrate present in this report are actually lower than what the levels were in reality. Thus, the results seen in Figure 2 are primarily the result of experimental error and not the actual relationship between the carbohydrate levels of WT and Δ*cpsG* cells. This could also possibly explain the contrast between Ganal et al. (4) study that showed a increased levels of total CPS when cells were exposed to aminoglycoside antibiotics. Since figure 2 does not represent the true situation we cannot conclude that this experiment actually did produce results different from that of Ganal et al (4).

Despite the inherent inaccuracy caused by experimental error, it is possible that the WT and Δ*cpsG* cells actually did produce carbohydrate levels that were similar. It has been shown that the *cps* system will only become activated in the presence of specific β-lactams (13). It was shown that *cps* strain was not stimulated to produce colanic acid when the cells are exposed to either streptomycin or kanamycin (13). Thus, theoretically, there should be no real difference in carbohydrate levels in either the WT or Δ*cpsG* cells as neither of the antibiotics used can stimulate *cps* activity since *cpsG* does not play any other role beyond synthesis of substrate for colanic acid and an increase should not be occurring with the antibiotics used in this experiment. In addition to this, *E. coli* possesses alternative metabolic pathways that can synthesize the M6P independently of the cpsG pathway (16). As such, even if *cpsG* was active during antibiotics treatment in the WT strain, it is possible that the Δ*cpsG* cells would still be able to carry out at least a limited synthesis of

M6P and thus colanic acid (3). The alternate path to M6P utilizes an ADP-sugar diphosphatase instead of *cpsG*, thus providing for the potential to overcome the *cpsG* defect even if *cpsG* was active in the antibiotic stress, despite other literature stating that this is not occurring (3).

Another finding (Figure 1) is that in the WT cells, streptomycin and kanamycin treated samples superficially appeared to have induced more CPS in comparison to the control which is in accordance with prior research findings (1,7). However, in the Δ*cpsG* samples, the control produced more CPS than kanamycin treated sample. This may be attributed to experimental errors in performing the anthrone assay. The error analysis on Figure 1 suggests that all these increases in CPS might be due to biological noise and are not significant. Alternatively, it is important to realize that the intracellular environment of Δ*cpsG* samples is not natural in comparison to the WT samples therefore unexpected variations are somewhat expected.

Also from Figure 1, it appears as though streptomycin induced more CPS production than kanamycin for both wildtype and Δ*cpsG* strains of *E.coli* K12. One possible explanation for this observation is that kanamycin is a slightly stronger antibiotic than streptomycin in handling Enterobacteriaceae such as *E. coli* or *Salmonella*. For instance, prior findings seem to indicate that kanamycin can be up to 90% effective against 351 clinical isolates of Enterobacteriaceae whereas streptomycin was only 80% effective at a minimal inhibitory concentration of 16 mg/L (9). In addition, it has been observed that the total protein concentration in kanamycin treated sample of *E. coli* B23 was less than streptomycin treated sample (1). Therefore, it is possible that the stronger antimicrobial activity of kanamycin may have reduced protein synthesis to a greater extent than streptomycin. This, in turn, may have prevented kanamycin treated sample from producing biosynthetic enzymes involved in colanic acid (CA) synthesis, resulting in lower concentration of CPS concentration. In contrast, streptomycin treated cells in both WT and Δ*cpsG* conditions may have been subjected to lower inhibition of protein synthesis than the cells under the influence of kanamycin, due to the weaker antibiotic activity of streptomycin than kanamycin (14,15). As a result, streptomycin treated cells may have been better able to produce biosynthetic enzymes involved in CA synthesis, hence producing more CPS.

Although Figure 1 showed that Δ*cpsG* cells were still able to respond to streptomycin treatment, this does not exclude the possibility that CpsG concentration has changed upon streptomycin treatment. In order to visualize any change in CpsG concentration upon aminoglycoside stimulation, silver stain was performed on the cell lysates of both WT and Δ*cpsG* cells. However, silver stain did not serve the purpose of visualizing CpsG proteins because the 50 kDa bands were too intense (Figure 2). This result was not surprising, as a search for proteins ranging from 45 to 50 kDa in *E. coli* on EcoProDB returned about 67 proteins, many of which are highly abundant in *E. coli* (3). Due to great amounts of background proteins, bands would appear at 50 kDa position in Δ*cpsG* cell lysate lanes regardless of the presence or absence of CpsG. The fact that the 50 kDa band intensity in Δ*cpsG* cell lysates did not decrease by an observable level indicates that CpsG was not of high abundance in the cells.

While the amount of Δ*cpsG*-kan and –strep samples loaded on to the gel appears to be less compared to the other samples (Figure 2), it could be due to technical issues on the lighting within the gel photographing stage. As shown in Figure 2, the top right corner appears to have a light source which distributed light unevenly across the gel, leading to the appearance that the three lanes on the right hand side were brighter. Nonetheless, the thickness of the bands on the gel should be comparable.

Two bands that are clearly down regulated reside in lane 5 adjacent to the arrows. These two bands seem to contain proteins which are down regulated in the presence of kanamycin when CpsG is absent. In other words, CpsG might be shielding these proteins against inhibition by kanamycin. However, given that the current research (6,16) indicate that CpsG is a phosphomannomutase, which has a main role of converting mannose-6-phosphate to mannose-1-phosphate, it is difficult to imagine CpsG playing a protective role for certain proteins.

The data generated from this experiment does not exclude the possible effects that kanamycin and streptomycin might have on the *cps* operon. Capsular biosynthesis is regulated by a complex regulatory network, and with current knowledge available, it is difficult to study the effects of *cps* operon while excluding all the other capsule-related genes. Therefore, while the data drawn from this experiment may reject our original hypothesized relationship that stimulation with aminoglycosides activates the *cps* operon and, this conclusion must be further investigated.

FUTURE EXPERIMENTS

Due to the presence of at least one alternative pathway that appears to abrogate the disruption of CpsG function, further experimentation into the effects CpsG has on capsule carbohydrate levels is unwarranted. Instead, the role of the whole Cps operon in capsule carbohydrate regulation and synthesis should be investigated. One possible method that could be used to explore the role of the Cps operon would be to

use mutant cells that contained Cps promoters that were unable to interact with RcsAB, a transcription factors that positively regulates the expression of the Csp operon (16). Using such a mutant, the role of the Cps operon in the secondary response an aminoglycoside induces in a cell could be analyzed.

ACKNOWLEDGEMENT

We would like to thank Dr. William Ramey for all his consultation and advice as well the members of the Wesbrook media room for all their support, especially Lando Robillo for the sample lyophilization he carried out for us. We would also like to show our appreciation to the members of the Gold lab for allowing us access to their equipment and technical advice. Lastly, we wish to extend a warm thanks to Karen Smith and Jennifer Sibley for their kind support through the numerous problems we encountered.

REFERENCES

1. **Chung, C., G. Hung, C. Lam, L, Madera**. 2006. Secondary effects of streptomycin and kanamycin on macromolecular composition of *Escherichia coli* B23 cell. J. Exp. Microbiol. Immunol. 2006. **9:**11-15.
2. **EcoCyc**. April 9th 2008 revision date. (online) http://biocyc.org/ECOLI/NEW-IMAGE?type=NIL&object=PC00084
3. **EcoProDB**. November 16 2006 revision date. (online) http://eecoli.kaist.ac.kr/search?term=45000+%7E+50000&category=mw
4. **Ganal, S., C., Gaudin, K., Roensch, and M., Tran**. 2007. Effects of streptomycin and kanamycin on the production of capsular polysaccharides in Escherichia coli B23 cells. J. Exp. Microbiol. Immunol. **11**: 54 – 59.
5. **Gottesman, S., P. Trisler, and A. Torres-Cabassa.** 1985. Regulation of capsular polysaccharide synthesis in *Escherichia coli* K-12: characterization of three regulatory genes. J. Bacteriol. **162:** 1111-1119.
6. **Hagiwara, D., M. Sugiura, T. Oshima, H., Mori, H., Aiba, T., Yoshimoto and T., Mizuno.** 2003. Genome-wide analyses revealing a signaling network of the RcsC-YojN-RcsB phosphorelay system in Escherichia coli. J. Bacteriol. **185**: 5735 – 5746.
7. **Kestell, D., S. Lai, G. Liang, S. Waters, and A. Wladichuk.** 2002. Effects of kanamycin and streptomycin on the macromolecular composition of streptomycin-sensitive and resistant *Escherichia coli* strains. J. Exp. Microbiol. Immunol. **2:**177-181.
8. **Lortholary, O., M. Tod, Y. Cohen, and O. Petitjean**. 1995. Aminoglycosides. Med Clin North Am. **79**: 761-87.
9. **Phillips, I., S. Eykyn, B. A. King, C. Jenkins. 1977**. The *in vitro* antibacterial activity of nine aminoglycosides and spectinomycin on clinical isolates of common Gram-negative bacteria. J. Antimicrob. Chemother. **3**:403-410.
10. **Rachid, S., K. Ohlsen, W. Witte, J. Hacker and W. Ziebuhr.** 2000. Effect of Subinhibitory Antibiotic Concentration on Polysaccharide Intercellular Adhesin Expression in Bio-film Forming *Staphylococcus epidermidis*. Antimicrob Agents Chemother. **44**: 3357-3363.
11. **Ramey, W. D. 2005.** Microbiology 421: Manual of Experimental Microbiology. University of British Columbia, Vancouver, B.C.
12. **Rudd, K.E.**, EcoGene: a genome sequence database for Escherichia coli K-12, Nucleic Acids Res., 28:60-64, 2000.
13. **Sailer, F. C., B. M. Meberg, and K. D. Young.** 2003. ß-Lactam induction of colanic acid in gene expression in *Escherichia coli*. FEMS Microbiol. Lett. **226**: 245-249
14. **Schroeder, R., C. Waldsich, and H. Wank.** 2000. Modulation of RNA function by aminoglycoside antibiotics. EMBO. **19:** 1-9.
15. **Slack, M. P. E., and W. W. Nichols**. 1982. Antibiotic penetration through bacterial capsules and exopolysaccarides. J. Antimicrob. Chemother. **10**: 368-372.
16. **Steveson, G., A. Kanella, Hobbs, M., and Reeves, P. R**. 1996. Organization of the Escherichia coli K-12 gene cluster responsible for production of the extracellular polysaccharide colonic acid. J. Bacteriol. **178**: 4885 – 4893.
17. **Whitfield, C.** 2006. Biosynthesis and Assembly of Capsular Polysaccharides in *Escherichia coli*. Annu. Rev. Biochem. **75**: 39-68.
18. **Wilson, J. W., M. J. Schurr, C. L. LeBlanc, R. Ramamurthy, K. L. Buchanan, and C. A. Nickerson.** 2002. Mechanism of bacterial pathogenicity. Postgrad Med. J. **78:** 216-224.
19. **Hinze, E.** 2006. Microbiology 323: Microbiological Techniques II Laboratory Manual. University of British Columbia. 36 – 126.