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The Rcs Phosphorelay May Regulate the *E. coli* Capsule Response to Sublethal Streptomycin Treatment

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The *Escherichia coli* colanic acid capsule produced by the *cps* gene confers resistance in the presence of sublethal antibiotic concentrations. However, the exact mechanism of capsule gene regulation in reaction to the presence of antibiotics is unknown. The two main proposed *cps* regulation pathways both involve the Rcs phosphorelay system, but differ regarding dependence on RpoS for *cps* transcription. In this study, we explored the changes in capsule formation and antibiotic resistance in response to treatment with sublethal concentrations of streptomycin using deletion mutants of the two proposed regulation pathways. Wild type, Δ *rcsB,* Δ *rpoS,* and Δ *cps. E. coli* strains were incubated in sublethal concentrations of streptomycin. When examined with MIC assays, the deletion mutants showed higher levels of antibiotic resistance compared to wild type strains of *E. coli.* We also demonstrated that capsule production was induced under sublethal streptomycin conditions with Maneval staining. Furthermore, qPCR detection of *cps* transcription indicated downregulation in Δ *rcsB* strains and upregulation in Δ *rpoS* after streptomycin treatment. Together, these results suggest that *E. coli* streptomycin resistance is not dependent on capsule production, and that *rcsB and rpoS* may both play a role in *cps* regulation when treated with sublethal doses of streptomycin.

Escherichia coli are rod shaped bacteria capable of producing a polysaccharide capsule. The capsule is important for protection from desiccation, survival during oxidative stress, bacterial virulence, and antibiotic resistance (1). In most non-pathogenic strains of *E. coli* , the *cps* operon is responsible for synthesis and maintenance of the capsule, which is composed of colanic acid, an extracellular polysaccharide common to *Enterobacteriaceae* . One proposed pathway of *cps* regulation is the Rcs (Regulator of Capsule Synthesis) system (Fig. 1) (2). The Rcs phosphorelay system in *E. coli* regulates the expression of stress response genes under conditions of desiccation (osmotic stress), low temperature, high Zn^{2+}, or presence of cationic peptides (3). Upon sensing stress, RcsC phosphorylates the central regulator of the system, RcsB (4). Phosphorylated RcsB is proposed to bind to the promoter regions of *cps* operon and promote gene transcription. Phosphorylated RcsB also initiates a signaling cascade that results in the upregulation of RpoS independent of the *cps* induction pathway (5). Castro *et al.* argued that RpoS does not play a central role in antibiotic tolerance based on experiments performed with RpoS deletion mutants (6).

Several studies have proposed models where *cps* activation is dependent on RpoS (Fig. 1). Relying on the Rcs system for sensing environmental signals and stress, phosphorylated RcsB upregulates *rprA* mRNA. The *rprA* mRNA stabilizes *rpoS* mRNA, allowing for increased RpoS production (7). RpoS is a sigma subunit of RNA polymerase that is induced under various stress conditions. Sigma factors competitively bind to RNA polymerase core proteins and increase their affinity for promoter sequences of specific genes (8). RpoS is proposed to upregulate the transcription of *cps* through increasing RNA polymerase binding affinity (9). Therefore, RpoS may be central to

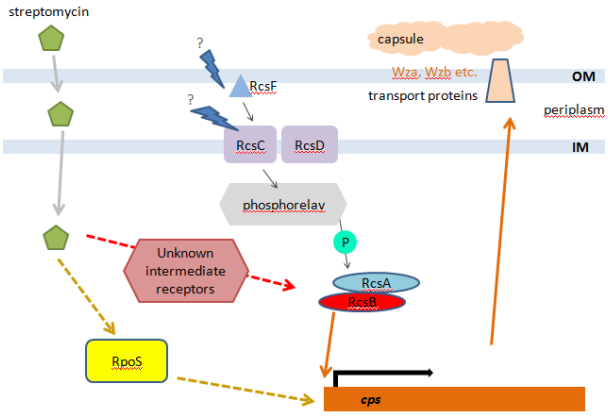

FIG. 1. Proposed pathways of *cps* regulation through RpoS and RcsB. Previous studies suggested that cps is activated via a phosphorelay system but the role of RpoS has not been confirmed. Because streptomycin translocates through the membrane to reach its active site, it is likely to act on RpoS, RcsB, or other intracellular receptors rather than transmembrane signalling components.

increasing aminoglycoside tolerance by upregulating *cps* and capsule formation in response to sublethal aminoglycoside exposure (6).

Overall, findings have been inconclusive in whether RpoS regulates *cps* directly or indirectly. Previously, Fowleri *et al.* showed that sublethal levels of streptomycin, an aminoglycoside antibiotic, can induce capsule formation which augmented bacterial resistance and survival against antibiotics (10). However, the mechanistic pathway for capsule regulation in response to aminoglycoside treatment remains unknown. We have observed a gap in knowledge on the mechanisms by which sublethal levels of antibiotics induce capsule formation. We hypothesize that RcsB

upregulates *cps* independently of RpoS under sublethal streptomycin treatment. In this study, we cultured wild- type (WT), Δ *rcsB,* Δ *rpoS,* and Δ *cps E. coli* strains in sublethal streptomycin concentrations and investigated capsule production and antibiotic resistance using, microscopy and quantitative polymerase chain reaction (qPCR) as well as minimum inhibitory concentration (MIC) assays, respectively. We show that Δ *rcsB,* Δ *rpoS,* and Δ *cps* deletion mutants have higher streptomycin resistance than WT *E. coli*, even though capsule staining indicated decreased capsule production in Δ *cps*. *cps* transcription is also downregulated in Δ *rcsB* and upregulated in Δ *rpoS* following streptomycin treatment. We observed that streptomycin resistance does not directly correlate with capsule formation and that *rcsB* and *rpoS* may both be involved in *cps* regulation.

MATERIALS AND METHODS

Cell strains. *Escherichia coli* DH5α was obtained from the MICB 421 culture collection (Department of Microbiology and Immunology, University of British Columbia), Δ *cpsB747 E. coli* JW2034 (Yale University Coli Genetic Stock Centre), Δ *rcsB E. coli* JW54371
(Yale University Coli Genetic Stock Centre), and Δ *rpoS746 E. coli* JW30009 (Yale University
Coli Genetic Stock Centre) were used in this study (Table S1). All experimental strains were derived from *E. coli* K12. Each strain was cultured in lysogeny broth (LB) (1.0% w/v tryptone and 0.5% w/v yeast extract) in Erlenmeyer flasks and grown in a 37 °C shaker to log phase (OD_{600} of 0.6 to 0.8) prior to inoculation in subsequent experiments.

Streptomycin minimum inhibitory concentration (MIC) assay. To assess MICs at 37°C, a colony of each of each *E. coli* strain was inoculated into 5 mL of LB broth containing 100, 50, 25, 12.5, 6.25, or 0 mg/L streptomycin and cultured overnight at 37°C in a shaking incubator. The MIC and sublethal concentrations of streptomycin were recorded the next day. To assess MIC at 21°C, a colony of each of the 4 strains were inoculated into 5 mL of LB broth containing 100, 50, 30, 20, 10, 5, 2, 1, or 0 mg/L streptomycin and incubated at 21°C without shaking. The MIC and sublethal concentration of streptomycin were assessed after 72 hours. MICs were defined as the lowest concentration at which cultures did not increase turbidity after overnight incubation, and the sublethal concentration was defined as that immediately below the MIC. Growth was assessed by measuring optical density using the an Ultrospec 3000 UV/Visible Spectrophotometer and also verified through visual inspection.

Capsule staining. A colony of each *E. coli* strain was inoculated in LB media with sublethal streptomycin concentrations of 5 mg/L for wild type, 20 mg/L for *Δcps* , 10 mg/L for *ΔrpoS* , and 10 mg/L for *ΔrcsB* based on the results from the MIC assay . The cultures were grown overnight at 37°C. Congo red stain (1% aqueous solution, Sigma Chemical Company C6767) was mixed with culture and airdried on a glass microscopy slide. Maneval's solution (0.0064% aqueous acid fuchsin, JT Baker Chemicals, A3553; 0.51% aqueous ferric chloride, Fisher Scientific I89; 0.77% aqueous glacial acetic acid, Acros, 423220025; 0.64% aqueous phenol solution, Invitrogen IS509037) was used as counterstain. Three drops of Maneval's solution was added to the slide and excess stain was drained. Slides were then gently washed with distilled water, air dried, and then observed under a light microscope at 100x magnification (11).

RNA extraction. LB broth was inoculated with 100 μL broth culture from all 4 strains and cultures were grown at 37°C until log phase of growth. Cultures were each then divided equally into 2 tubes, with streptomycin added to one of the two tubes at sublethal concentrations. Streptomycin concentrations were as follows: 5 mg/L for WT, 20 mg/L for *Δcps*, 10 mg/L for *ΔrpoS* , and 10 mg/L for *ΔrcsB* . Cultures with or without streptomycin were then grown overnight at 37°C. Total RNA was extracted from each of the 8 overnight cultures using the TRIzol reagent (Life Technologies #15596026).

cDNA conversion of RNA. 2 μg of each total RNA was treated with DNase I (Fermentas #EN0521) in a total reaction volume of 20 μL. Reactions were incubated at 37°C for 30 minutes, followed by the addition of 2 μL 50mM EDTA and incubation at 65°C for 10 minutes. DNase treated RNA samples were then used as template in cDNA conversion via SuperScript III FirstStrand Synthesis System for RT PCR (Invitrogen #18080051) according to the manufacturer's instructions. Random hexamers included in the kit were used as primers for cDNA conversion. After the cDNA conversion the reactions were heat- terminated at 85°C for 5 minutes and then treated with RNase H.

Measuring expression of the *cps* operon via quantitative PCR. Quantitative PCR primers were designed using Primer3 software (12). Primers were picked such that the oligo melting temperature was 60°C (between 58ºC-62ºC) and the product size range was 145- 160 bp. Two genes within the *cps* operon, *wzb* and *wzc* , were evaluated as indicators of *cps* expression. (Table S2) To compare *cps* expression levels between samples, qPCR was conducted on the CFX Connect RealTime System (BioRad) using the SsoAdvanced Universal SYBR Green Supermix (BioRad #1725274). The 10 μL reaction contained 100 ng of template cDNA with each primer at a concentration of 300 nM. qPCR conditions were as follows: 95°C for 2 minutes, 40 cycles of 94°C for 15 seconds and 55°C for 30 seconds, followed by a melt curve analysis.

RESULTS

Rcs pathway deletion mutants have higher streptomycin resistance in minimum inhibitory concentration assays (MICs). To determine the appropriate concentrations of streptomycin for inducing capsule formation in each of the four *E. coli* strains, we conducted minimum inhibitory concentration assays in both liquid media and agar based cultures. MIC assays were also carried out on WT, Δ *rcsB,* Δ *rpoS,* and Δ *cps* strains to test whether capsule pathway mutants would have decreased MICs compared to the WT due to capsule associated antibiotic resistance. A single colony of each strain was used to inoculate media with various streptomycin concentrations and grown at 37°C. The MIC was measured as the lowest concentration of streptomycin tested in which bacteria could not grow, and the sublethal concentration was the highest concentration tested in which bacteria grew (Fig. 2). In the overnight cultures, the WT strain (DH5α) had the lowest streptomycin resistance at both 21°C and 37°C. This was unexpected since there were no deletions in its capsule pathway, which was thought to confer antibiotic resistance. Surprisingly, the strain with the highest MIC was the *cps* deletion strain at 20 mg/L streptomycin. The MIC for Δ *cps* grown at 37 ° C was five times greater than WT, and 2.5 times greater than Δ *rcsB* and Δ *rpoS* . The sublethal concentration for *cps* deletion mutant was also significantly higher than all the

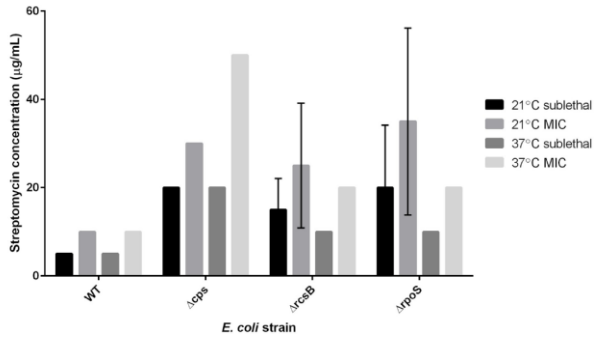

FIG. 2. Sublethal and minimum inhibitory concentrations of streptomycin by culture conditions. MIC assays were conducted on broth cultures incubated separately at both 21 ℃ and 37 ℃. Relative streptomycin resistance of each strain was consistent across both culture temperatures. Error bars indicate standard deviation. WT=strain DH5α, *Δcps* =strain JW2034, *ΔrcsB* =strain JW54371, *ΔrpoS*= strain BW30009.

other strains (Fig. 2). The MICs and sublethal concentrations for Δ *rcsB* and Δ *rpoS* are both 2 times greater than WT *E. coli*. Agar plate-based assays were consistent with the liquid media results but were slightly higher, perhaps due to degradation of streptomycin on the exposed agar surface (data not shown).

Previous studies suggested that capsule formation and antibiotic resistance were enhanced at 21°C for all strains (Fig. 2) (13). In order to confirm if incubation temperature had an effect on capsule formation, we also conducted the same MIC assays at 21°C. For all strains other than the *Δcps* strain, there was no significant difference between MICs at 21°C and 37°C. The *Δcps* strain had significantly lower MIC at 21°C than at 37°C. However, since the sublethal concentrations are identical, this difference may be due to the range and increments of streptomycin concentrations used for the assay. Overall, the MICs and streptomycin resistance for the deletion mutants are higher than those for WT *E. coli* independent of growth temperature. No difference in MIC was observed in the WT strain between the two temperatures. The *rpo*S mutant showed slightly higher MICs than the *cps* mutant at both temperatures, which suggests that enhanced antibiotic resistance may be obtained through *cps* and possibly *rpoS*- independent pathways.

Growth assays showed that WT strain DH5α, had the lowest resistance whereas the capsule mutant strains had higher resistance. This was surprising as we expected that the inability to produce capsule may lead to decreased antibiotic resistance. For the assay at 21°C, spectrophotometric readings of cultures grown at sublethal concentrations were slightly lower than in the 37°C assay but remained distinguishable from MIC cultures.

Sublethal streptomycin induced capsule formation in deletion mutants. To confirm capsule formation for the various strains under the induction of sublethal streptomycin concentrations, Maneval capsule staining was used to distinguish capsule. The cells were grown in various streptomycin concentrations or in LB alone. The Maneval solution stains the background and the bacterial cell, but not the capsule, allowing for visualization of the capsule (Fig. 3). Positive controls consisted of mixed cultures of non-capsule- forming cells and capsule- forming cells. In broth alone, no capsule or very thin capsules were observed in *cps* mutants, indicating that the *cps* deletion resulted in inability to produce capsule. However, capsules were observed in all strains after 48 hours incubation at 37°C in the presence of sublethal streptomycin. Thus the presence of streptomycin induced capsule formation. Based on visual observation, the thinnest capsule was observed for *Δcps* (Fig. 3B), which is consistent with the decreased ability to produce capsule due to deletion of *cps*. Surprisingly, *Δcps* strain appeared to be capable of producing capsule or other extracellular matrix material. No capsules or cells were observed in stained cultures grown at the minimum inhibitory concentration (data not shown).

***rcs*B and *rpo*S may both be involved in *cps* regulation as measured by quantitative PCR.** Since *cps* transcription corresponds with capsule synthesis, we measured *cps* transcription under streptomycin induction for the various deletion mutants by performing qPCR on the *wzc* and *wzb* mRNA. *wzc* and *wzb* are genes in the *cps* operon and are transcribed from the same promoter. Primers for two genes were validated. GAPDH, a highly- conserved metabolic gene, was successfully validated as an internal control (data not shown). Melt curve assays of each gene showed 1 single peak/dissociation curve and it is unlikely that the primer bound to itself or to nonspecific regions during amplification.

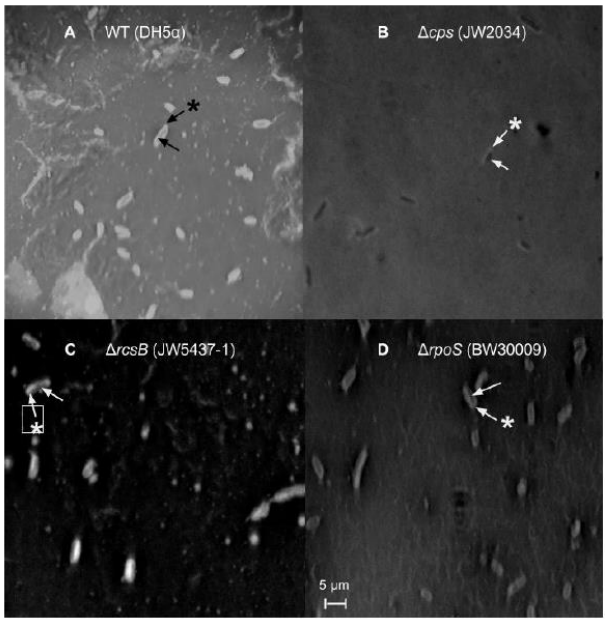

FIG. 3. Bacterial capsules were successfully visualized by Maneval staining. Sample background and the cell interior were dyed, leaving the capsule unstained and distinguishable as a light-coloured halo around cells. Capsules were observed in all strains after 48h incubation at 37°C in the presence of sublethal streptomycin. Arrows indicate cells, while asterisks (*) indicate capsule.

The data was normalized against capsule production of bacteria cultured in Luria Bertani broth (Fig. 4). In this study relative expression greater than 1 was deemed indicative of upregulated transcription and less than 1 is indicative of downregulation. Results are consistent with the expectation that streptomycin is sufficient for capsule production, as WT capsule genes showed significantly higher relative expression levels, indicative of upregulation. The *cps* mutant showed no difference in either *wzb* or *wzc* expression between growth in LB only or LB with sublethal streptomycin induction (Fig. 4). This result is expected as there is no *cps* to transcribe regardless of growth conditions. In the *rcsB* mutant, both genes had significantly lowered expression presence of streptomycin. Thus *rcsB* may be involved in *cps* regulation. The *rpoS* mutant had *wzc* upregulated in the presence of streptomycin, consistent with the observed capsule production via Maneval staining, but *wzb* was slightly downregulated (Fig. 4). The *rpoS* deletion resulted in a higher *wzc* transcription rate than WT strain. Therefore *rpoS* may be involved in both upregulation and downregulation of *cps* operon genes. Expression levels of *rcsB* and *rcsC* generally showed the same relationship across the 4 strains, but there was no consistent pattern of relative *wzb* and *wzc* levels within each strain.

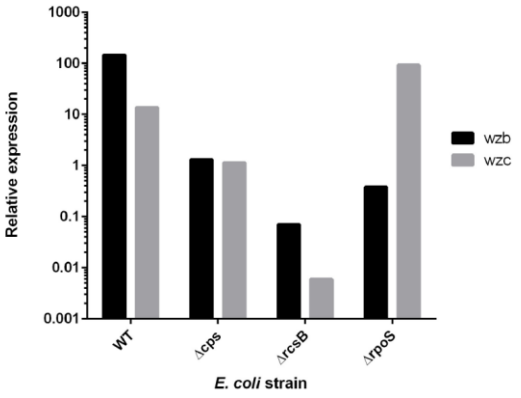

FIG. 4. Capsule gene expression at sublethal streptomycin concentrations. Expression of capsule genes *wzb* and *wzc* for all 4 experimental strains was evaluated relative to GAPDH. All strains were cultured at sublethal streptomycin levels determined in the MIC assays. Due to technical issues, no replicate data was obtained (n=1).

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DISCUSSION

MIC results were generally well supported and the effect of temperature on capsule production was consistent with previous studies, but the increased resistance of Rsc pathway deletion mutants compared to the WT strain was unexpected. More consistent incubation time as well as consistent intervals between the tested concentrations would enable verification of how much resistance differs between strains, as the structure of serial dilutions may make differences in MICs artificially large. The temperature independent antibiotic resistance is inconsistent with existing studies where MICs are generally higher at 21°C, due to decreased growth rate and membrane permeability at the lower temperature, leading to an overall decreased streptomycin uptake (13, 14).

Maneval staining made it possible to visually distinguish capsule presence and size variations across the 4 strains. In the future, more controls could be used since some *E. coli* strains may form capsule even when cultured in the absence of streptomycin or other antibiotics. There is also no established method to consistently induce capsule formation and little to no literature on the consistent preparation of capsule stain. Additional replicates should be conducted alongside strains known to consistently produce capsule or known not to produce any capsule cells. Addition of Anthony's solution may also enhance microscopy as the capsule itself is stained (15).

Quantitative PCR results are preliminary due to limited replicates, but the differences observed in *wzb* and *wzc* expression and are consistent with differences in microscopically observed capsule production levels. Analysis of genes that encode proteins in capsule subunits may be more representative of capsule production levels (16). It is unclear whether *wzb* or *wzc* is more effective as an indication of cps expression. Since *wzb*/*wzc* encode transporter proteins, it is likely that these are expressed early during capsule production and may be less indicative of capsule production in cultures incubated for a longer period of time (17, 18). It is also unknown whether multiple *cps* genes or the entire operon was deleted in the mutant strains, so the relevant regions in each experimental strain should also be sequenced to determine if reversion of capsule pathway functionality was present.

The observations strongly suggest that streptomycin exposure induces capsule formation in cells with functioning capsule expression pathways. However, capsule does not appear necessary for streptomycin resistance. The *cps* operon may be required for capsule formation but capsule formation may occur through other pathways. We found that the *cps* mutant may form some capsule but this ability is significantly decreased compared to the wild type strain.

The *cps* mutants may retain streptomycin resistance through some other mechanism, and *rpoS* and *rcsB* may or may not be necessary for streptomycin resistance. It is likely that *rpoS* and *rcsB* are not required for capsule formation. Expression levels of *wzb* and *wzc* determined via qPCR are positively correlated to the amount of colanic acid capsule production. Overall, these data support our proposed model, specifically in that RpoS is not necessary for capsule formation (Fig. 1).

FUTURE DIRECTIONS

While our experiments demonstrated that capsule production was correlated to *cps* expression, there were

some issues with regards to accurate identification of the capsule induction and formation under sublethal streptomycin conditions for the various strains. Future studies on this topic should prioritize accurate evaluation of capsule production. Carbohydrate assays may be used to better quantify capsule induction. We attempted to evaluate capsule formation using the phenolsulfuric acid assay but were unable to gather useful results due to technical issues. There are alternate approaches to measuring *cps* output, including use of reporter genes, such as a *lac* reporter fusion to the *cps* gene, to indirectly measure *cps* transcription. Additional verification of qPCR primers for *rcsB* and *rpoS* genes should be performed (Table S3). The effects of other aminoglycoside antibiotics should also be considered.

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REFERENCES

1. **Zahrani FA, Huang M, Lam B, Vafaei B.** 2013. Capsule formation is necessary for kanamycin tolerance in *Escherichia coli* K12. J Exp Microbiol Immunol **17** :2428.
2. **Gottesman S, Stout V.** 1991. Regulation of capsular polysaccharide synthesis in Escherichia coli K12. Mol Microbiol **5(7):** 1599606.
3. **Laubacher ME, Ades SE.** 2008. The Rcs phosphorelay is a cell envelope stress response activated by peptidoglycan stress and contributes to intrinsic antibiotic resistance. J Bacteriol 190(6):20652074.
4. **Latasa C, García B, Echeverz M, Toledo Arana A, Valle J, Campoy S, Garcíadel Portillo F, Solano C, Lasa I.** 2012. Salmonella biofilm development depends on the phosphorylation status of RcsB. J Bacteriol **194(14):** 370822.
5. **Majdalani N, Gottesman S.** 2005. The Rcs phosphorelay: a complex signal transduction system. Annu Rev Microbiol **59**:379405.
6. **Amirault M, Kuo C, Sage B.** 2014. Expression of rprA, a regulator of RpoS translation, increases tolerance to kanamycin in RcsB- deficient *Escherichia coli* K12. J Exp Microbiol Immunol **18**:1217.
7. **Castro R, Dougan M, Rioux L, Zhang B.** 2014. RpoS may not play a central role in coordinating heat shock stress response and antibiotic tolerance. J Exp Microbiol Immunol **18**:2429.
8. **Hengge- Aronis R.** 2002. Signal Transduction and Regulatory Mechanisms Involved in Control of the σS (RpoS) Subunit of RNA
9. Polymerase. Microbiol Mol Biol Rev **66(3):** 373–395.
10. **Griffith KL, Wolf RE Jr.** 2002. Measuring b- galactosidase activity in Bacteria: Cell Growth, Permeabilization, and Enzyme Assays in 96 Well Arrays. Biochem Biophys Research Commun **290(1)**: 397402.
11. **Fowler D, Hu J, Hou P, Wong C.** 2009. The Effect of Sub-Inhibitory Streptomycin on Capsular Polysaccharide Production and Streptomycin Resistance in *Escherichia coli*. J Exp Microbiol Immunol **13** :4752.
12. **Maneval WE**. 1931. The Staining of Flagella of Bacteria, with Special Reference to Mordants. J Bacteriol **21(5):** 313–321.
13. **Rozen S, Helen J, Skaletsky HJ.** 1998. Primer3. Code available at
14. http://www.genome.wi.mit.edu/genome_software/other/primer3.html.
15. **Parmar S, Rajwani A, Sekhon S, Suri K.** 2014. The *Escherichia coli* K12 capsule does not confer resistance to either tetracycline or streptomycin. J Exp Microbiol Immunol **18:** 7681.
16. **Kamensek S, Zgur- Bertok D.** 2013. Global transcriptional responses to the bacteriocin colicin M in *Escherichia coli*. BMC Microbiol **13** :42.
17. **Rahn A, Drummelsmith J, Whitfield C.** 1999. Conserved organization in the cps gene clusters for expression of *Escherichia coli* group 1 antigens: relationship to the colanic acid biosynthesis locus and the cps genes from *Klebsiella pneumoniae*. J Bacteriol **181(7):** 23072313.
18. **Hughes RB, Smith AC.** 2007. Capsule Stain Protocols. ASM Microbe Library. Accessed 15 Mar 2015.
19. **Beggs WH, Williams NE.** 1971. Streptomycin uptake by *Mycobacterium tuberculosis* . Appl Microbiol **21(4):** 751753.
20. **Rahn A, Whitfield C.** 2003. Transcriptional organization and regulation of the *Escherichia coli* K30 group 1 capsule biosynthesis (cps) gene cluster. Mol Microbiol **47(4):** 10451060.