Journal of Experimental Microbiology and Immunology (JEMI) Vol. 17: 24 – 28
Copyright © April 2013, M&I UBC

Capsule Formation is Necessary for Kanamycin Tolerance in *Escherichia coli* K-12

Fatimah Al Zahrani, Morris Huang, Brandent Lam, and Rod Vafaei
Department Microbiology & Immunology, University of British Columbia

Capsules play an important role in protecting bacteria from harmful substances such as antibiotics, by hindering entry into the cell and ability to reach the bacterial cytosol. The study investigated the effects of sucrose non-ionic osmotic stress on kanamycin tolerance in *E. coli* K-12 through the induction of capsule formation. Through the use of *rcsB* deletion mutant strain of *E. coli* deficient in capsule formation pathway, we explored the protective effects of capsular presence against kanamycin under sucrose osmotic stress conditions. Presence of functional capsule allowed for higher tolerance to bacteriocidal effects of kanamycin in *E. coli,* relative to the strains unable to form capsules. No notable induction of kanamycin tolerance was observed within wild type *E. coli* between the different sucrose osmotic treatments used. Ultimately, the results suggested that though the presence of a capsule is necessary for some tolerance to kanamycin, at the treatment concentrations used, sucrose osmotic stress does not result in additional tolerance to kanamycin.

Escherichia coli is a Gram-negative, rod-shaped bacterium capable of forming a capsule. Capsule formation is functionally advantageous due to its importance in virulence, protection from desiccation, and as a barrier to external harmful entities such as toxins (1). Based on previous observations and studies, we anticipated that environmental stress such as osmotic stress treatments may induce increased capsule formation resulting in a tolerance to antimicrobial compounds such as kanamycin (2). Kanamycin, which was the antibiotic used in the study by Kuzhiyil *et al.*(2), is only expected to be stable for 24 hours at room temperature in a 0.9% solution of NaCl (3). This is approximately a 0.15 M solution. It was then reasoned that high NaCl concentrations may affect kanamycin function; therefore, NaCl may not be the best candidate to induce osmotic stress in *E. coli* cells prior to kanamycin treatment. This is because it might affect the expected MIC of 4 µg/ml for kanamycin treatment of *E. coli* K-12 strains in 0.2% glycerol M9 (4).

In this study, osmotic stress was expected to induce capsular thickening and increase tolerance through means of a physical barrier. This response is controlled by the Response Capsular Synthesis (Rcs) phosphorelay system (5). In this system, RscC is the sensor protein, YojN is the Hpt protein that relays the phosphoryl group, and RcsB is the receiver/effector that leads to the activation of the *cps* genes responsible for capsule formation (5, 6). RcsB activation of the *cps* genes is enhanced by RcsA protein (5). However, capsules can be synthesized through multiple pathways and by many different sets of enzymes (7) meaning, for the purposes of our study, it is not possible to eliminate capsule formation by deleting synthetic enzyme genes. A better approach is to target the regulator system responsible for the group-I capsular polysaccharides (8). Studies have shown that mutations in this system such as defective RcsB or YojN in *E. coli* K-12 result in hypersensitivity to osmotic stress (6) as expected with the inhibition of capsule formation. By this rationale, an *E. coli* K-12 *rcs*B deletion mutant was used in this study to hinder capsule formation and assess the necessity of capsules in kanamycin tolerance.

Effects of osmotic stress response capsule formation has been explored with ionic salt as an induction agent, however non-ionic stress inducers are poorly documented for *E. coli* K-12 strains. Sucrose was used as a non-ionic osmotic stress inducer due to the lack of metabolic capability towards sucrose in *E. coli* (9) focusing the study on tolerance against the aminoglycoside kanamycin.

MATERIALS AND METHODS

Cell strain, growth condition and media. The strains wild type *E. coli* K-12 BW28357 (6) and *rcsB* deletion mutant *E. coli* BW30009 (6) were used for the study. The cells were cultured on 0.2% glycerol M9 minimal liquid media and 0.2% glycerol M9 minimal media plates (10). Working final sucrose concentrations made to 2.36 mg/ml and 7.08 mg/ml. Cultures were incubated at 37°C in a shaking water bath. Plated cultures were incubated in the 37°C room for 48 hours. All media used in the subsequent experiments and assays used 0.2% glycerol M9 media.

Sensitivity of bacterial strains to kanamycin. This assay was performed to ensure that both wild type *E. coli* BW28357 and mutant *E. coli* BW30009strains obtained from the CGSC Keio library (11) were sensitive to kanamycin**.** Two M9 media plates were divided into four quadrants and each quadrant was streaked with positive control kanamycin sensitive *E. coli* B23, negative control kanamycin resistant *E. coli* JW2755, *RcsB* mutant *E. coli* K-12 BW30009 or wild type *E. coli* K-12 BW28357. At the center of the quadrants, a filter paper disc soaked in 50 µg/ml and 100 µg/ml of kanamycin was placed onto two different M9 media plate. The plates were incubated overnight and zones of inhibition were measured.

Kanamycin Minimal Inhibitory Concentration (MIC) assay. This assay was performed by Xu *et al.* (12) to test the MIC of kanamycin in wild type *E. coli* and mutant *E. coli* strains. The minimal inhibitor concentration (MIC) was determined for use in subsequent growth curve experiments. Overnight cultures for both the wild type *E. coli* and mutant *E. coli* strains were subjected to kanamycin of varying concentrations. A sterile 96-well plate was filled with serial dilutions to achieve a range of final kanamycin concentrations of 0, 1, 2, 4, 8, 16, 32, 64 and 128 µg/ml. The turbidity of the two bacterial cultures were measured and standardized turbidity to 0.1 OD_{500}. Five µl of culture was

Copyright © April 2013, M&I UBC

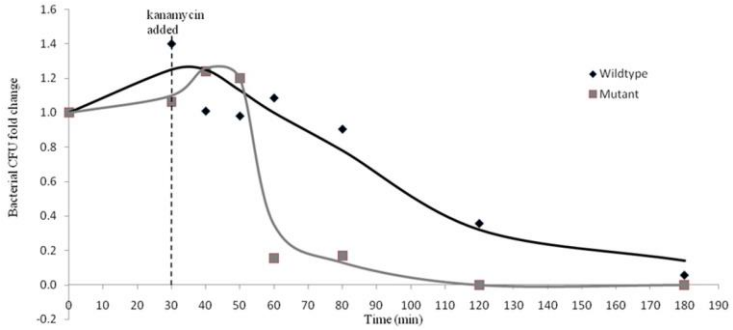


FIG 1 The *E. coli* wild type and *rcsB* mutant strains have similar growth curves in the absence of kanamycin with low osmotic stress induced by sucrose (2.36 mg/ml).

added to each well in order to yield a cell density of $5x10^5$ cells per well. The culture turbidity and presence of growth was visually observed after 24 hour incubation. The culture turbidity was visually re-evaluated after 72 hours of incubation to ensure the growth seen at 24 hours was accurate.

Effect of sucrose on kanamycin activity. This assay was designed to test the effects of our experimental osmotic sucrose treatments on kanamycin activity. The mutant strain was used and was previously determined to be positive for kanamycin sensitivity in all our treatment conditions. Four µg/ml of kanamycin was added to sucrose incorporated growth media at 2.36 mg/ml and 7.08 mg/ml (the experimental sucrose concentrations). The kanamycin and sterile sucrose combined solutions were first incubated for 3 hours at 37ºC in a shaking water bath mimicking growth conditions before the addition of mutant *E. coli* culture. After the initial turbidity OD_{500} was noted, the sucrose, kanamycin, and culture solution were incubated at 37ºC. The turbidity OD_{500} readings were then taken after 24 hours and noted to see if there was any growth as compared to a no kanamycin control and a M9/no sucrose control.

Maneval capsule staining. This assay was performed similar to the protocol outlined by Hughes and Smith (13) with minor modifications specified in Kuzhiyil *et al*. (2). The staining was used to confirm the presence of a capsule in the wild type *E. coli* strain and absence of a capsule in the mutant strain through morphological visualization. Overnight cultures for both the wild type and mutant strains were done. A few drops of Congo red (1% aqueous solution, Sigma Chemical Company C-6767) was mixed with culture and air dried onto a slide. Dried smears were counterstained for five minutes with two to three drops of Maneval's solution (0.0064% aqueous acid fuchsin, JT Baker Chemicals, A355-3; 0.51% aqueous ferric chloride, Fisher Scientific I-89; 0.77% aqueous glacial acetic acid, Acros, 42322-0025; 0.64% aqueous phenol solution, Invitrogen IS509-037). Excess stain was poured off. Slides were gently washed with dH_2O and air-dried. Slides were observed under 1000x magnification on a light microscope.

Osmotic stress induction and tolerance. This experiment was originally performed by Kuzhiyil *et al.* (6) with modifications to protocol in order to determine the effects of sucrose induced osmotic stress on kanamycin resistance in both wild type and mutant *E. coli* strains. Overnight cultures for both the wild type and mutant strains were made. Each culture was then diluted to obtain turbidity OD_{500} reading of 0.15.After induction of osmotic stress using a range of sucrose concentrations (0, 2.36, and 7.08 mg/ml), the cultures were incubated for 5 hours to allow for growth in osmotic stress conditions and respond by producing more capsule components. Five hours after osmotic stress induction (i.e. t = 0), the sucrose treated bacterial cultures were first diluted to obtain an OD_{500} reading of 0.15, then a sample was plated on M9 solid media at final plated dilutions of 5×10^{-7} and 5×10^{-8}. The cultures were placed in a shaking water bath at 37℃. When the logarithmic growth is achieved after 30 minutes, kanamycin at MIC concentrations (4 µg/ml) was added to the culture and sampled further. At subsequent time points samples were taken from the cultures and plated on M9 solid minimal media at the final plated dilutions of 5×10^{-7} and 5×10^{-8}. Colony counts were checked 48 hours after incubation at 37 ℃. The results were from two separate experiments and hybridized by normalizing all the data to the population size at t = 0 min.

RESULTS

Maneval's staining of *E. coli* K-12 strain. Staining of the two strains with Maneval reagent showed a distinct difference between the wild type *E. coli* strain and the *rcsB* mutant strain. The wild type displayed a smooth white halo as the outline of the bacterium. The white capsular halo estimated roughly 1/10 the width of the rod shaped bacterium. The *rcsB* mutant showed no continuous white halo and had an altered morphology. An estimation of the capsular thickness is impossible to determine as discontinuous outlines were rough and non-uniform the uneven, rough outline of the mutant strain was clear and distinguishable compared to the wild type strain. Data not shown.

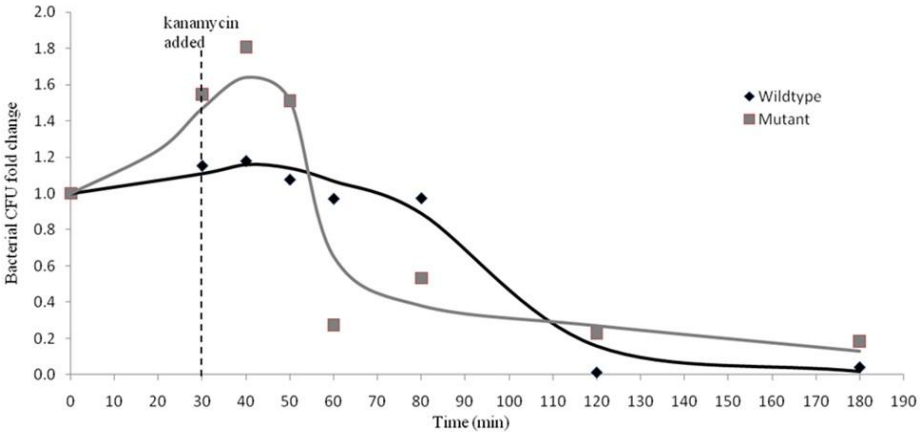
Kanamycin MIC determination assay. To elicit a bactericidal effect of kanamycin, the range of minimal inhibitory concentration yielded a 4 µg/ml for both wild type and mutant strain. The experimental wild type and mutant strains were also more sensitive to kanamycin compared to the tested *E. coli* B23 strain control that is sensitive to kanamycin. This suggested that the strains for the study is kanamycin sensitive.

Kanamycin stability in Sucrose. Kanamycin dissolved in sucrose M9 media displayed no significant decrease in kanamycin activity. No growth was observed in any of the samples treated with kanamycin at MIC (4 µg/ml), even after incubation of the kanamycin in the sucrose M9 solutions. The untreated control registered an OD_{500} of 0.630.

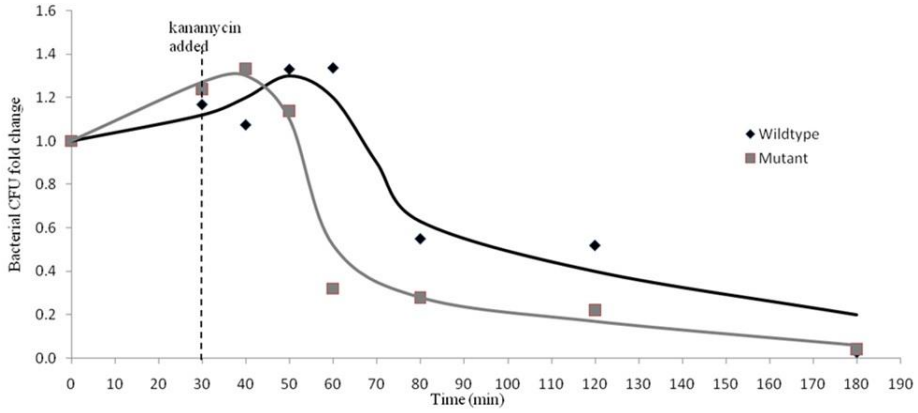
Growth and tolerance of *E. coli* strain in sucrose stress. There were no negative impacts to growth of both wild type and *rcsB* mutant *E. coli* strains when osmotic stress was introduced through the use of transient sucrose in growth medium (Fig. 1). Bacterial growth showed

Copyright © April 2013, M&I UBC

A.



B.



C.

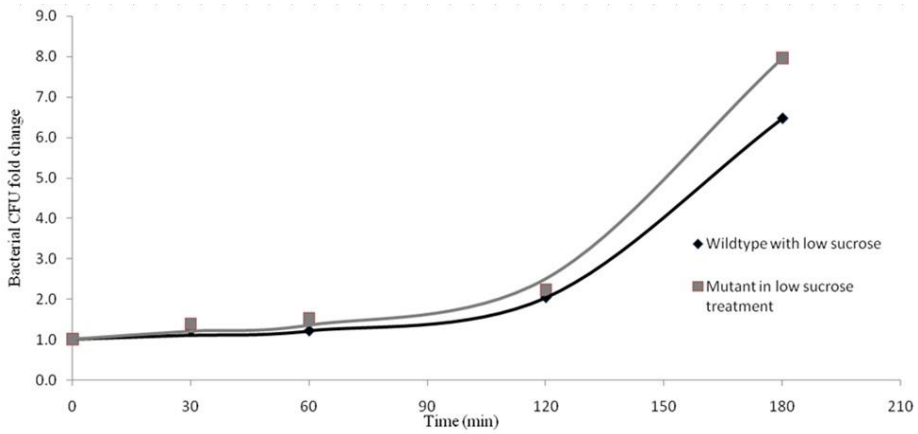


FIG 2 Wild type *E. coli* shows temporary tolerance to kanamycin as compared to *rcs*B mutant *E. coli* in M9 minimal media. (A) No sucrose. (B) Low sucrose osmotic stress (2.36 mg/ml). (C) High sucrose osmotic stress (7.08 mg/ml).

similar curves at all sucrose osmotic treatment concentrations (0 mg/ml, 2.36 mg/ml and 7.08 mg/ml) (Data not shown).

Sucrose osmotic stress induced kanamycin tolerance. Wild type and capsule deficient *rcsB* mutant strains of *E. coli* displayed clear differences in tolerating kanamycin under different sucrose osmotic stress levels. The cultures were stressed under sucrose containing media and re-established into logarithmic growth for 30 minutes.

In the absence of osmotic stress, a sudden increase in death rate was evident in the mutant strain between 50 and 60 minutes (Fig. 2A). However, the wild type strain displayed a gradual death rate beginning at the same time as the mutant strain (50 minutes), with an increase of death rate at 80 minutes.

Copyright © April 2013, M&I UBC

A similar trend is seen when a low level of sucrose is used to induce osmotic stress (2.36 mg/ml) in the culture. The same treatment following displayed a sharp death rate in the mutant strain between 50 and 60 minutes (Fig. 2B), while the wild type strain displayed a more gradual death rate beginning at the same time as the mutant strain (50 minutes) that increased after 80 minutes.

When the strains were treated under high sucrose osmotic stress level (7.08 mg/ml) a different relationship was observed. Under the same treatment as other sucrose osmotic stress levels, the mutant strain displayed the same trend as previously seen, with a sharp death rate at 50 to 60 minutes. However, the wild type strain did not show a decrease in population at 50 minutes but rather its death rate began at 60 minutes (Fig. 2C). The extension of the death phase compared to lower sucrose osmotic stress treatment was 10 minutes. A sharp death rate was also observed for the wild type strain under high sucrose osmotic stress rather than a gradual death rate as seen in lower level of osmotic stress.

However, in the region of 50 to 80 minutes, there was variability in the data collected. The difference in the wild type strain under different osmotic stress can be seen to have a minor effect. The high osmotic stress condition when compared to the lower stress conditions displayed a alternative curvature that may be dependent on various variability in the data. Nonetheless the delayed death of *E. coli* wild type with a functional capsule indicated better tolerance to kanamycin for a prolonged period of time when stressed osmotically compared to the mutant strain deficient in regular capsule covering; showing no enhanced effects in kanamycin tolerance when osmotically stressed with sucrose.

DISCUSSION

The resulting kanamycin tolerance by *E. coli* K-12 has a strong correlation to the presence of capsule. The *rcsB* experiment where capsule formation was induced using sucrose, it was found that concentrations of 15% w/v solutions of sucrose (0.15 g/ml) successfully induced capsule formation with similar kinetics as NaCl solutions of 0.1 M - 0.5 M (17). The use of a sucrose treatment with a concentration that is approximately 20 fold less than 0.15 g/ml could have then failed to induce capsule formation. However, treatment of bacteria under such extensively concentration of solutes may not be feasible for this study either. High concentrations of sucrose may alter the action of kanamycin (7) and the resulting death of bacteria may not be due to a single factor anymore (6); impacting any results that would be obtained. By changing the concentration of sucrose treatment to that of 15% w/v we may be able to clearly identify the trend and possible induced tolerance to kanamycin with respect to capsule formation. Another possibility for why we do not see any notable induced tolerance at low sucrose concentrations could be related to the temperature at which this experiment was conducted. It has been shown that at 37^{o}C, RcsA activity can be hindered which reduces the Rcs pathway induction of *cps* genes by 3 fold or more (17). All mutant showed a relatively immediate and steep death curve after the addition of kanamycin to the growth medium. On the other hand, the wild type strain that has a proper capsule forming pathway showed a gradual decrease and delayed death (Fig. 2A and Fig. 2B), especially between the time of 60 min to 110 min time frame. The capsule likely acts as a physical barrier to the antibiotic which is only effective in killing bacteria when inside the bacterial cytosol (14, 15). Our findings indicated that having the ability to form a capsule through pathways downstream of RcsB is advantageous in terms of providing some temporary tolerance to kanamycin at 4 μg/ml; our findings are consistent with previous findings in which capsular material was shown to provide protection from aminoglycosides such as kanamycin (2, 16).

For high sucrose treatment of wild type *E. coli*, there is a small delay in the observed reduction in cell count after kanamycin addition, as compared to the other sucrose treatment conditions. Considering this deviation of the curve is a result of a single data point from one replicate, it is hard to validate this observation. It is appreciated that osmotic stress causes a sensor-response mechanism where RcsC (a membrane bound protein) senses the environment and phosphorylates RcsB. In turn, activation of RcsB leads to the transcriptional activation of *cps* gene cluster resulting in the increase in capsule production (17). The resulting increase in capsule material production and incorporation delays the entry of kanamycin (16). Overall, the sucrose treatments in this study did not result in a strong enhancement of kanamycin tolerance for wild type *E. coli* K-12. This is also evident when compared to the observed increase in MIC reported by other groups working on induced kanamycin tolerance through capsule formation and osmotic shock (2).

The weak response to sucrose osmotic shock could have been due to several factors. Firstly, in a previous our assays were performed at 37^{o}C, so it is plausible that temperature hindered the capsule forming response. A third variable is the amount of antibiotic used in the experiment. The 4 ug/ml concentration of kanamycin used was determined by a MIC range test that showed bactericidal effects between 2 - 4 ug/ml. The use of a lower subinhibitory concentration display a better kanamycin tolerance trend correlative to different osmotic treatments.

When comparing *rcsB* mutants to the wild type *E. coli*, a temporary advantage in tolerance to kanamycin in the wild type *E. coli* over the mutant was evident (Fig. 2A - Fig. 2C), indicating the capsule formation pathway is necessary for temporary tolerance to kanamycin. Sucrose as an osmotic treatment may induce further tolerance, or even resistance to kanamycin, but this was not strongly evident in the data. Delayed bacteriocidal effects observed under high sucrose treatment cannot confidently indicate a correlation between osmotic sucrose stress and tolerance to kanamycin. Further experimentation is needed to confirm this correlation.

Copyright © April 2013, M&I UBC

FUTURE DIRECTIONS

While our experiments showed that capsule formation pathway component, *rcs*B, plays an important role in induced kanamycin tolerance, there were some issues with regards to how clearly the data showed osmotic induction of kanamycin within strains. The question of why the sucrose osmotic treatments in our experiments did not induce a higher level of kanamycin tolerance, while previous experiments with NaCl osmotic treatments did succeed in showing what looked like kanamycin tolerance, needs to be answered. The best approach to doing this might be to address the most likely problems that may have hindered our results first, such as increasing the concentration of sucrose in the treatments to see if that is a key factor.

For future studies on this topic, it may be important to track capsule formation. One method could be to stain capsular components using affinity staining (for example, lectin stains) and run the samples through a flow cytometer to track any increase in capsular expression on the bacterial surfaces. Doing this provides insight on the extent of capsular formation for the osmotic treatments used and how well the relative quantity of capsular components correlates with any resistance or tolerance that was observed.

ACKNOWLEDGEMENTS

This project was conducted at the Department of Microbiology and Immunology at the University of British Columbia. We would like to thank Dr. William Ramey for all his support and assistance throughout these experiments. We would like to extend our sincere gratitude to Lando Robillo, Nick Cheung and others at the media room for providing us with all the necessary laboratory supplies and laboratory technique tips. Lastly, we would also like to thank Jennifer Geddes for her constant guidance throughout the term.

REFERENCES

1. **Locke JB**, **Colvin KM**, **Datta AK**, **Patel SK**, **Naidu NN**, **Neely MN**, **Nizet V**, **Buchanan JT**. 2007. *Streptococcus iniae* capsule impairs phagocytic clearance and contributes to virulence in fish. J. Bacteriol. **189**:1279–1287.
2. **Kuzhiyil A, Lee Y, Shim A, Xiong A.** 2012. Osmotic stress induces kanamycin resistance in *Escherichia coli* B23 through increased capsule formation. J ExpMicrobiol.Immunol.**16:**5-10.
3. **McEvoy GK.** 1997. American Hospital Formulary Service - Drug Information. Bethesda, MD: Amer. Soc. of H-Sys Pharm, Inc.. **1**:65-66.
4. **Garrett ER, Won CM**. 1973. Kinetics and mechanisms of drug action on microorganisms XVII: Bactericidal effects of penicillin, kanamycin, and rifampin with and without organism pretreatment with bacteriostatic chloramphenicol, tetracycline, and novobiocin. J. Pharm. Sci. **62**:1666-1673.
5. **Stout V, Gottesman S.** 1990. RcsB and RcsC: a two-component regulator of capsule synthesis in *Escherichia coli*. J Bacteriol.**172**:659-669.
6. **Kuzhiyil A, Lee Y, Shim A, Xiong A.** 2012. Osmotic stress induces kanamycin resistance in *Escherichia coli* B23 through increased capsule formation. J ExpMicrobiol.Immunol.**16:**5-10.
7. **Whitfield C.** 2006. Biosynthesis and assembly of capsular polysaccharides in *Escherichia coli*.Annu. Rev Biochem. **75**:39-68.
8. **Jayaratne P, Keenleyside WJ, MacLachlan PR, Dodgson C, Whitfield C.** 1997. Characterization of rcsB and rcsC from *Escherichia coli* O9:K30:H12 and examination of the role of the rcs regulatory system in expression of group I capsular polysaccharides.J Bacteriol.**175**:5384-5394.
9. **Drayson R, Leggat T, Wood M.** 2011. Increased antibiotic resistance post-exposure to sub-inhibitory concentrations is independent of capsular polysaccharide production in *Escherichia coli*. J. Exp. Microbiol. Immunol. **15**:36-42.
10. **Zimbro M, Power D.** 2003. Difco& BBL manual.Manual of microbiological culture media. Becton, Dickenson and Company, Sparks, Md.
11. **Baba T, Ara T, Haseqawa M, Takai Y, Okumura Y, Baba M, Datsenko KA, Tomita M, Wanner BL, Mori H.** 2006 Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio Collection. Mol. Syst. Biol. **2**:2006-2008
12. **Xu C, Lin X, Ren H, Zhang Y, Wang S, Peng X.** 2006. Analysis of outer membrane proteome of *Escherichia coli* related to resistance to ampicillin and tetracycline. Prot. **6**:462-473.
13. **Hughes RB, Smith A.** 2011. Capsule stain protocols. ML Microbe Library, ASM. Accessed on 13 March 2013 at http://www.microbelibrary.org/component/resource/laboratorytest/3041-capsule-stain-protocols.
14. **Hancock RE.** 1981. Aminoglycoside uptake and mode of action--with special reference to streptomycin and gentamicin. J Antimicrob.Chemother.**8**:249-276.
15. **Jana S, Deb JK**. 2006. Molecular understanding of aminoglycoside action and resistance. Appl. Microbiol. Biotechnol.**70**:140-150.
16. **Mingeot-Leclercq MP, Glupczynski Y, Tulkens PM.** 1999. Aminoglycosides: activity and resistance. Antimicrob.Agents Chemother.**43**:727–737.
17. **Sledjeski DD, Gottesman S.** 1996. Osmotic shock induction of capsule synthesis in *Escherichia coli* K-12. J. Bacteriol. **178**:1204-120.