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Pre-treatment with Sub-Inhibitory Kanamycin has no Effect on RpoS Expression but Increases Heat Tolerance of *Escherichia coli* BW25113

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The RpoS sigma factor mediates the induction of the general stress response in *Escherichia coli,* which offers protection against a wide array of external stresses. Other studies have shown that pre-treatment of *E. coli* with sub-inhibitory antibiotics conferred persisting antibiotic resistance against the same and other antibiotics, and pre-treatment with high temperature also enabled resistance to low pH media. Consequently, the aim of this study was to determine whether treatment of *E. coli* BW25113 with sub-inhibitory kanamycin would elicit elevated RpoS expression in exponential phase cells, and subsequently result in improved survival in conditions of high temperature (50°C) and/or high acidity (pH 2). Plating was done to assess cell viability at 5, 20, and 45 minutes post-incubation in stress conditions. Western blotting was used to compare RpoS expression levels of different treatments. No difference in RpoS expression was obtained between control and kanamycin treatments. Neither culture survived the pH 2 treatment in M9 minimal medium, however, after 45 minutes of treatment at high temperature, over 10-fold higher cell survival was observed in cells pre-treated with kanamycin. This indicates that sub-inhibitory kanamycin was sufficient to elicit some additional thermal resistance in *E. coli*, but did not induce the acidic resistance systems under the conditions tested. The involvement of RpoS in stimulating the protective response could not be concluded in this experiment.

Escherichia coli can communicate with its environment through versatile sensory systems and signaling cascades. One such system responds to environmental stresses such as low pH, high heat, high osmolarity, and nutrient deprivation. *E. coli* can respond to these environmental stresses by synthesizing proteins that render it more resistant to the same stress later on, a degree of stress that would otherwise be lethal (1).This general stress response is characterized by the activation of alternative sigma factor RpoS (σ^{38}). Negative regulation of RpoS is removed as cells enter stationary phase or when exponentially-growing cells encounter an environmental stress. RpoS then guides RNA polymerase to upregulate expression of some 100 genes that help the cell respond to not only the initial stress encountered, but also offers cross-protection against other potential threats (13). As an example, cells that are grown in carbon starvation conditions are subsequently better able to survive in low pH or high temperature conditions (1) while heat-shocked cells are better able to survive hyperosmolarity (10).

Aside from sensing environmental stresses, various bacteria have been observed to phenotypically modify themselves in the presence of antibiotics in a dose-dependent manner. When grown at sub-inhibitory concentrations of kanamycin, *E. coli* are subsequently resistant to otherwise lethal doses of streptomycin, tetracycline, and ampicillin (4, 5, 12). Antibiotics have been shown to exhibit hormesis. Microbe-derived animoglycosideantibiotics such as kanamycin may serve as quorum-sensing molecules when found in diffuse quantities in the natural environment (18), for example, inducing biofilm formation in *E. coli* (10). Therefore, cells may register these antibioticsas potential threats from the environment just as they do with osmotic and temperature-related stresses. Here, exposure to sub-minimal inhibitory concentration (MIC) antibiotics may prime cells to prepare for subsequent environmental stresseswhich can include, but is not limited to, cross-protection against other antibiotics. If this cellular response is analogous to the general stress response to environmental threats, it can be predicted that RpoS would be upregulated during exponential growth in cells exposed to sub-MIC antibiotics.

In this study, we assessed whether treating *E. coli* BW25113 with sub-inhibitory concentrations of kanamycin rendered the cells better able to survive other generalized environmental stressors, specifically, low pH or high temperature. We also assessed whether RpoS was upregulated during exponential phase growth in the kanamycin pretreated culture when compared to the untreated culture. While we found that RpoS was not differentially expressed between untreated and kanamycin pretreated cultures, the pretreated cells were better able to survive heat-shock one hour into heat treatment, however, no benefit was observed in low pH. As such, no inferences can be made on the effect of kanamycin pre-treatment on acid tolerance, and the involvement of RpoS in eliciting heat protection remains unclear.

MATERIALS AND METHODS

Bacterial strains and culture growth.*E. coli* K-12 BW25113 Δ*(araD-araB)567*, Δ*lacZ4787*(::rrnB-3), lambda$^-$, *rph-1*, Δ*(rhaD-rhaB)568*, *hsdR514)* was obtained from the MICB 421 culture collection (Department of Microbiology and Immunology, University of British Columbia). The cultures were grown in M9 minimal salts medium (0.05% w/v NaCl, 0.7% w/v Na_2HPO_4, 0.3% w/v KH_2PO_4, 0.1% w/v NH_4Cl, 0.02% w/v $MgSO_4 \cdot 7H_2O$, 0.2% w/v glycerol adjusted to pH 7.4) overnight at 37°C with gentle aeration.

MIC assay. Chen *et al.*'s MIC procedure was used (4). An antibiotic stock solution was prepared by dissolving kanamycin monosulfate (Sigma, cat #K4000) in distilled water to a final concentration of 20 mg/ml, filter sterilizing the solution, and storing it at 4°C until needed. Antibiotic working solutions were made by diluting the kanamycin stock solution in M9 medium to a final concentration of 0, 0.5, 1.0, 2.0, 4.0, 8.0, 16.0, and 32.0μg/ml in a volume of 1 ml each. The plate was inspected both visually and with the Bio-RAD Model 3550 Microplate reader the next day.

Treatment with sub-inhibitory kanamycin and growth curve construction. The overnight culture was split into control and kanamycin-treated flasks and used to seed a starting culture with an OD_{500} of 0.1, diluted in M9 medium. Kanamycin equal to 0.5x MIC was used as the sub-inhibitory treatment. The cultures were then incubated in a 37°C shaking water bath and OD_{500} readings were taken periodically to monitor culture growth until an OD_{500} of 0.5 was reached.

Secondary stress treatment and viability assay. 750 μl of the 0.5 OD_{500} cultures was added to 75 ml of regular M9 medium and pH 2 M9 medium (pH adjusted using concentrated HCl). The heat treatment flasks were subsequently incubated in the 50°C water bath and the pH treatment flasks placed in the 37°C water bath. Samples were taken for plating at 5, 20, and 45 minutes after the start of incubation. 100 μl samples of 10^{-1}, 10^{-2}, and 10^{-3} dilutions were plated on Luria-Bertani agar plates (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl, and 1.5% w/v agar). The plates were incubated overnight at 37°C, and the colonies counted the following day.

Cell lysis. Once the seeded cultures have reached 0.5 OD_{500}, 30 ml of each culture (+/- kanamycin) along with 30 ml of the overnight culture (i.e. stationary phase) were centrifuged at 7500 x g for 10 min. The pellets were washed with 10 ml of 10 mMTris, pH 8 and re-centrifuged. The cell pellets were re-suspended in 1 ml of 10 mMTris buffer, pH 8. The cells were lysed by pulse sonicationwith the Ultrasonic Cell Disruptor at the setting of 8. Sonication was done in 3 cycles of 10 sec sonication followed by 30 sec pause. The lysates were centrifuged at 14,000 x g at 4°C and the supernatants were transferred to fresh microcentrifuge tubes and stored at -20°C until further use.

Bradford Assay. Total protein concentration was determined using the Bradford Assay (2). Bovine serum albumin (FermentasBioLabsNew England, cat #B90015) was used as the protein standard. The assay was conducted using the Bio-Rad Bradford Reagent (Bio-Rad, #500-0006) by mixing 20.6 mL of Bio-Rad Bradford Reagent with 50.4 mL of distilled water.

SDS-PAGE. The cell lysates were normalized for protein content according to results from the Bradford assay. 10μl of the lysates were combined with 10μl 2x SDS sample buffer[50 mMTris pH 6.8, 100mM DTT, 2% w/v SDS, 0.1% w/v bromophenol blue (J.T.Baker Chemical Co.,cat #D293-03), 10% w/v glycerol]. The samples were heated for 2 minutes at 95 °C (briefly spun after), loaded onto 12% SDS-polyacrylamide gel and ran vertically in 1x Running buffer (25 mMTris base pH 6.8, 192 mM glycine, 1% w/v SDS) at 120 V for 60 min. 10 μl of BLUeyePrestained Protein Ladder (Gendirex, Cat. # PM007-0500) was loaded as well.

Western Blot. Following SDS-PAGE, proteins were blotted onto a BioTrace NT transfer membrane (Pall Co., cat #P/N 66485) in a chilled transfer buffer (25 mMTris base, 192 mM glycine, 20% v/v methanol) and ran at 100 V in an electro-blotting apparatus for 60 min. The membrane was washed for 5 minutes with 15 ml TBS-Tween 20 (50 mMTris pH 7.5, 150 mM NaCl, 0.1% v/v Tween 20) and incubated in 15 ml of blocking buffer [5% w/v skim milk powder and 0.1% v/v TBS-Tween 20 (50 mMTris pH 7.5, 150 mM NaCl)] for 1 hour. Following the blocking, the membrane was washed with 15 ml TBST for 5 minutes (3 times) and hybridized with 10 ml of the primary antibody [mouse anti-RpoS(Neoclone Biotechnology, cat. #W0009) diluted 1:1000 in blocking buffer] overnight at 4 °C on a shaking apparatus. After discarding the primary antibody, the membrane was washed with 15 ml of TBST for 5min(3 times). After the washing, the membrane was hybridized with a secondary antibody (peroxidase-linked anti-mouse sheep Ig(Chemicon International, cat. # AP124A) diluted 1:5,000 in blocking buffer) for 1hat room temperature on a shaking apparatus. After discarding the secondary antibody, the membrane was washed for 5minwith 15 ml of TBST (3 times). The membrane was taken to the Life Sciences Center (UBC) to perform the RpoS detection.50μl of aluminol-based chemiluminescent substrate (house recipe of the Beatty Lab) was added to the membrane and incubated for 2 minutes in a dark room. The emitted light was captured on an X-ray film (1 minexposure) and the protein band corresponding to ~38 kDa was detected as RpoS.

RESULTS

RpoS expression wasunchanged in exponentially growing *E. coli* BW25113 in the presence or absence of kanamycin. The MIC assay revealed that *E. coli* BW25113 didnot grow in concentrations of kanamycin above 0.5 μg/ml. This was used as the MIC for this strain, and the sub-MIC concentration used to pre-treat newly-inoculated cultures was 0.25 μg/ml. RpoS expression was determined for stationary phase cells, and exponentially-growing cells in the presence or absence of kanamycin. Western-blot detection of RpoS from cell lysate revealed that the protein was expressed in all three samples, as indicated by the high-intensity bands at 38 kDa in Figure 1. However, the Western blot contained several nonspecific bands (data not

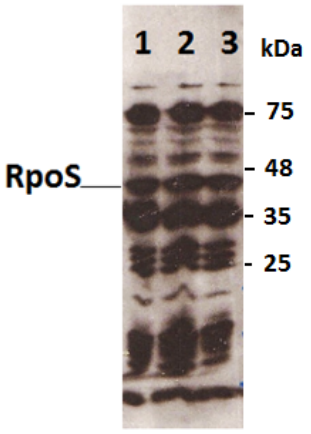

FIG. 1.Western blot visualization of RpoS protein band (~38 kDa) of *E. coli* BW25113 cell lysates. *E. coli* cells were treated and loaded on lanes as follows: Lane 1) control, kanamycin-untreated, exponential phase cells; Lane 2) kanamycin-treated, exponential phase cells; Lane 3) stationary phase, non-treated.

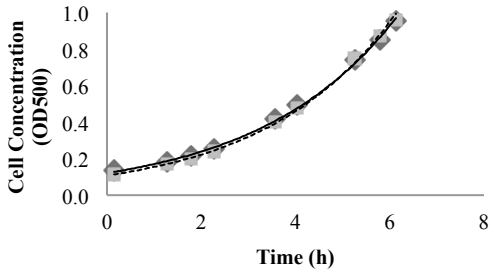

FIG. 2.Growth curve of control and kanamycin-treated cultures.♦ = control (solid line); ■ = kanamycin (dashed line).

shown). The nonspecific banding patterns were identical for each sample, and the band of highest intensity was at 38 kDa, the expected size for RpoS protein.

***E. coli* BW25113 grown in the presence of kanamycin wasmore resistant to death by heat-shock.** The control and kanamycin-treated cultures had similar growth rates at exponential phase, with a generation time of approximately 2 h(Fig. 2). After exponentially-growing *E. coli* BW25113 was transferred to fresh media at 50 °C , the decline in viable cell concentration in the heated culture was similar for the pretreated and untreated cells at 5 and 30 minutes into the treatment. However, after 45 min, there was a significant decrease in viable cell concentration in the untreated where only 0.6 % of the population remained while 9.6% of the population remained in the pretreated culture (Fig. 3).

***E. coli* BW25113 wasunable to tolerate incubation in media at a pH of 2.** An identical experiment was performed to the heat treatment, where exponentially-growing cells in the presence or absence of sub-MIC kanamycin were transferred to M9 media at a pH of 2 and 37^{o}C. No viable cells were recovered from any of the low pH cultures at 5 min post inoculation, or any later time points (data not shown).

DISCUSSION

It has previously been observed that *E. coli* grown in the presence of sub-MIC kanamycin was subsequently more resistant to the same and other unrelated antibiotics at concentrations inhibitory to untreated cells (4). It was also revealed that the pre-incubation of *E. coli* at high temperature increased its tolerance to acidic environments (11). In the present study, we demonstrated that incubating *E. coli* in sub-MIC kanamycin may also render cells more tolerant to heat shock.

We hypothesized that the presence of sub-MIC kanamycin in the growth environment would act as a danger signal to *E. coli* in the same manner as physical stresses like low pH or high osmolarity. *E. coli* has been previously shown to upregulateRpoS during exponential phase growth when exposed to an initial nonlethal stress, leading to expression of several genes that render the cell more resistant to subsequent, otherwise lethal, unrelated stresses (11, 13). We did not, however, observe a difference in RpoS expression between the cultures grown in the presence or absence of kanamycin. Western blot immunodetection revealed identical levels of RpoS in the cell lysate of stationary phase cells and the two exponentially-growing cultures (Fig. 1). It should be noted that there may indeed be a difference in RpoS expression that may not be captured by Western blot. In a previous study, RpoS expression in *E. coli* was assessed during exponential phase growth in M63 media (7). While Western blot detection of RpoS showed no difference in cultures after an OD_{600} of 0.6, microarray analysis revealed that its expression was higher in stationary phase cells than exponentially-growing cells (7). We were not expecting to detect high levels of RpoS during exponential phase because its expression has been previously reported to be low during this phase of growth (1). Before concluding that RpoS expression is identical between each treatment, it must first be ruled out that the observed bands are not a relic of sub-optimized immunoblotting protocols. Extensive nonspecific banding was observed in each sample. These may be due to nonspecific binding of the

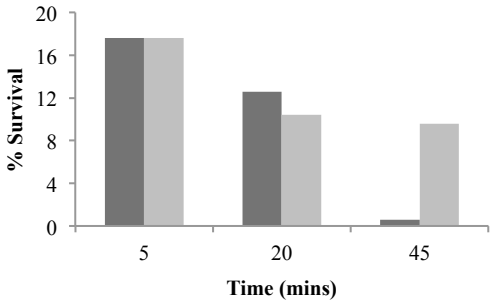

FIG. 3. Comparison of control and kanamycin treatment survival rates when incubated at 50°C. Cells harvested after 4 hours of incubating the seeded cultures. % survival calculated as a proportion of viable cells inoculated initially with a starting OD_{500} of 0.005, assumed to be ~$5x10^6$ cells/ml. Numbers are based on single plate counts. Dark grey indicates control; light grey indicates kanamycin treatment.

secondary antibody, inadequate membrane blotting before incubating with the primary antibody, or insufficient membrane washes. Here, the bands identified as RpoS may appear saturated due to surrounding nonspecific bands making relative difference of intensity between treatments impossible to detect.

Alternatively, growth conditions may have a drastic impact on the observed phenotype of the cells. Previous studies comparing RpoS expression in *E. coli* growing in rich or minimal media revealed that RpoS expression was much higher in early exponential phase in minimal media when compared to LB. This difference was exacerbated when the carbon source used was glycerol rather than glucose, the preferred carbon source (7).

Sub-MIC levels of kanamycin did not improve the acid tolerance of *E. coli* in pH 2 M9 medium, which is in contrast to other studies using the same strain of *E. coli* grown in LB media at pH 2-2.5 (11, 19). *E. coli* is generally known to be tolerant to highly acidic environments (15), naturally colonizing the gastrointestinal tracts of mammals, therefore this result was unexpected. There are at least four RpoS-dependent acid resistance (AR) systems known to exist in *E. coli*, but the regulators that they stimulate may be dependent on the growth medium, the growth phase, and the strain used (16). For instance, several strains of *E. coli* were shown to have been unable to survive an acid challenge of pH 2.5 in non-supplemented minimal media, but conferred resistance when challenged in minimal media supplemented with glutamate and arginine (12). It is now known that the majority of the AR systems are dependent on amino acids for proper function (9). As minimal media was used in this experiment, although RpoS was being expressed, the amino acid-deficient environment that the cells were grown and challenged in may not have provided the substrates necessary to activate the acid resistance systems.

Previous studies on *E.coli'*s response to environment stimuli, including temperature stress, revealed certain changes in its heat resistance ability depending on which growth phase *E.coli* passes through. In general, as the growth rate diminishes, there is a corresponding rise in heat resistance until its peak in the stationary phase (3, 8). For example, *E.coli* had shown a very low survival rate (1-2%) when the cells were heat-treated during the exponential growth phase compared to various points during the stationary phase (20-80%) (8).Inour experiment, the cells were in the exponential growth phase when heat-shocked. Thus, as expected, the control culture (non-kanamycin treated) experienced a steady decline in the survival rate throughout the heat shock treatment, reaching less than 1% within 40 minutes (Fig. 2).The kanamycin pre-treated culture also experienced a steady decline in the viable cells at the beginning of the heat treatment. However, after 20 minutes into the treatment, the survival rate stopped decreasing and stayed approximately constant (~10 %) within the next 20 minutes (Fig. 2). The data demonstrated that *E.coli* pre-treated with kanamycin could better adapt and survive heat-shock at 50 °C, and that this effect was not seen until 20 minutes into heat-shock treatment. Our RpoS expression results, however, showed no difference in the amount of RpoS present in all the samples tested, thus making it impossible to attribute the increased heat tolerance in the kanamycin pretreated culture toRpoS-induced cross-protection. Our RpoS expression results also contradicted with the previously observed upregulation of RpoS in *E.coli*during the stationary growth phase compared to exponential phase (1, 7). Thus, before drawing any conclusion regarding correlation between RpoS expression and the induced cross-protection against heat, the possible issues with our Western blot detection of RpoS and the possible influence of the growth conditions on RpoS expression discussed above must be addressed.

In *E. coli*, the response to temperature upshift consists of the rapid induction of the synthesis of more than 20 heat shock proteins,the regulation of whichis dependent on the sigma factor σ32 or RpoH (17). The heat shock proteins (that are mainly molecular chaperones and ATP-dependant proteases) are essential

for cell survival since they degrade or refold misfolded proteins under both stressed and unstressed conditions. Increase in RpoH has been shown to be triggered not only by heat, but also by ethanol, viral infection, methylating and alkylating agents, hydrogen peroxide, starvation, and certain antibiotics (17).Thus, sub-MIC kanamycin pre-treatment might have influenced the increasedresistanceto heat-shock independent of RpoS, but rather, via other sigma factors such as RpoH.

Sub-MIC antibiotics may act on cell phenotypes in ways we have yet to appreciate. Aminoglycosides have already demonstrated the ability to alter gene expression in *E. coli* in ways distinct from non-aminoglycoside inhibitors of the 30S ribosomal subunit or inhibitors of the 50S subunit (6). Small molecules that bind the ribosome have previously been associated with sigma factors. Through x-ray crystallography, aminoglycosides were observed to bind RNA sequences and ribosomal proteins. Other well-studied small molecules such as guanosine triphosphate are known to promote interaction between RpoS and RNAP through this binding (1). Sub-MIC kanamycin may therefore affect the binding of specific sigma factors to RNAP. Which sigma factor would depend on antibiotic concentration and cell-type.If RpoS did not play a role in the increased resistance to heat shock, then perhaps the activity of a separate sigma factor like RpoH is behind this effect.

In conclusion, it remains unclear whether *E. coli* responds to sub-MIC kanamycin treatment in the same way it does to environmental stresses like low osmolarity or high temperature by upregulating global stress response factor RpoS. However, *E. coli* exposed to sub-MIC kanamycin were better equipped to survive heat shock. This suggests that antibiotics found in minimal concentrations may offer a cross-protection to some environmental stresses like heat, but perhaps not others like low pH environments. The cellular mechanism through which kanamycin exerts this effect remains unclear.

FUTURE DIRECTIONS

Repeating the experiment using LB media in place of minimal media may allow for the effect kanamycin has on RpoS expression to be better visualized as well as help avoid the possible stress condition that minimal media induces. Before the role of other sigma factors is considered in the observed protection of heat-mediated killing, western-blot detection of RpoS in these procedures would have to be more conclusive. Using a lower concentration of protein in SDS-PAGE, and increasing Western blotting stringency may produce less ambiguous results with lower background. Experiments repeated using LB media and an optimized Western blotting procedure should not reveal much RpoS expression in kanamycin untreated cultures, when sampled in early exponential phase growth. Thus, if the pretreated culture demonstrates similar RpoS expression to control, it would be safer to conclude that RpoS is not involved in the cross protection against heat shock.

To lend support to the above, an *rpoS*knockout strain derived from *E. coli* BW25113 can be pre-treated and heat-shocked. Not only will immunodetection of RpoS in lysate derived from this strain serve as a necessary negative contol missing from this study, but if the same cross-protection is observed in the absence of *rpoS*then it can be safely concluded that this sigma factor is not the one responsible for kanamycin-induced heat tolerance. The role of alternative sigma factors such as RpoH could then be considered in future experiments searching for the regulation systems affected by sub-MIC kanamycin.

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REFERENCES

1. **Battesti A., N. Majdalani, and S. Gottesman.** 2011. The RpoS-mediated general stress response in *Escherichia coli*. Annu. Rev. Microbiol. **65:**189-213.
2. **Bradford, M. M**. 1976. A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Anal. Biochem. **72**:248-254.
3. **Berney, M., H. Weilenmann, J. Ihssen, C. Bassin, and T. Egli.** 2006.Specific growth rate determines the sensitivity of *Escherichia coli* to thermal, UVA, and solar disinfection. Appl. Env. Microbiol.**72:**2586-2593.
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. **Chiang, V., B. Wong, C. Wong, and J. Yu**. 2011. On the limited role of relA in kanamycin and amino acid starvation induced stringency and subsequent antibiotic cross-protection in *Escherichia coli*. J. Exp. Microbiol. Immunol. **15:**59-63.
6. **Davies J., G. B. Spiegelman, and G. Yim**. 2006. The world of subinhibitory antibiotic concentrations. Curr. Opin. Microbiol. **9**:445-453.
7. **Dong, T., and H. E. Schellhorn**. 2009. Control of RpoS in global gene expression of *Escherichia coli* in minimal media. Mol. Genet. Genomics.**281**:19-33.

8. **Elliker, P. R., and W. C. Frazier.** 1938. Influence of time and temperature of incubation on heat resistance of *Escherichia coli*. J. Bacteriol. **36:**83-98.
9. **Foster, J. W.** 2004. *Escherichia coli* acid resistance: tales of an amateur acidophile. Nat. Rev. Microbiol. **2:**898-907.
10. **Hoffmann L.R., D. A. D'Argenio, M. J. MacCoss, Z. Zhang, R. A. Jones, and S. I. Miller.**2005. Aminoglycoside antibiotics induce bacterial biofilm formation. Nature **436:** 1171-1175.
11. **Hsu, Y., L. Lam, J. Lee, and A. Sharma.** 2009. Effect of sequential treatment with high temperature and hyperosmolarity or low pH on RpoS expression and cell viability in *Escherichia coli*B23. J. Exp. Microbiol. Immunol.**13:**7-12.
12. **Lin, J., I. S. Lee, J. Frey, J. L. Slonczewski, and J. W. Foster.** 1995. Comparative analysis of extreme acid survival in *Salmonella typhimurium, Shigellaflexneri*, and *Escherichia coli*. J. Bacteriol.**177:**4097-4104.
13. **Liu, H., M. Zhu, and S. Zhu.** 2011. Persistence of antibiotic resistance and capsule in *E. coli* B23 after removal from sublethal kanamycin treatment. J. Exp. Microbiol. Immunol. **15:**43-46.
14. **Patten C.L., M. G. Kirchhof, M. R. Schertzbert, R. A. Morton, and H. E. Schellhorn.** 2004. Microarray analysis of RpoS-mediated gene expression in *Escherichia coli* K-12. Mol. Gen. Genomics.**272:**580-591.
15. **Smith. J. L.** 2003. The role of gastric acid in preventing foodborne disease and how bacteria overcome acid conditions. J. Food Prot.**66:**1292-1303.
16. **Stincone A., N. Daudi, A. S., Rahman, P. Antczak, I. Henderson, J. Cole, M. D. Johnson, P. Lund, and F. Falciani.** 2011. A systems biology approach sheds new light on *Escherichia coli* acid resistance. Nucleic Acids Res. **39:**7512-7528.
17. **Wick L. M. and T. Egli.** 2004. Molecular components of physiological stress responses in *Escherichia coli.* Adv. Biochem. Engin. Biotechnol. **89:**1-45.
18. **Yim G., H. H. Wang, and J. Davies.** 2006. The truth about antibiotics. Int. J. Med. Microbiol. **296:**163-170.
19. **Zhang, X., R. Garcia-Contreras, and T. K. Wood.**YcfR (BhsA) influences *Escherichia coli* biofilm formation through stress response and surface hydrophobicity. J. Bacteriol. **189:**3051-3062.