Tetracycline Modulates the Valine Induced Stringent Response and Decreases Expressed RpoS in Escherichia coli

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The stringent response in Escherichia coli is a physiological response to stress characterized by accumulation of the alarmone (p)ppGpp and the down-regulation of various processes involved in cellular growth and protein synthesis. It has been previously shown that activation of the stringent response by amino acid starvation is able to confer protection against various classes of antibiotics, including tetracycline and ampicillin. However, no observations have been made on partial activation of stringency. This investigation attempted to repress the level of bacterial stringent response in E. coli CP78 through the use of tetracycline. Tetracycline is an antibiotic which has been shown to restrict the cellular accumulation of (p)ppGpp in CP78 Escherichia coli. The effectiveness of the starvation by valine was monitored by measuring turbidity. The modulation of stringent response was monitored by the level of RpoS, since RpoS production is known to be enhanced by activated stringent response. As measured by turbidity, we successfully established an amino acid starvation condition with valine treatment as seen in the inhibited growth of CP78 cells as compared to untreated cells. The valine inhibition of the growth of the bacteria appeared to be reduced by the addition of tetracycline. However, cultures treated with high concentrations of tetracycline seemed to become more turbid than the untreated control even though they had similar levels of protein. This suggested that the tetracycline might have been deteriorating and increasing the absorbance of the cultures. The increasing absorbance might have contributed to the observed turbidity readings. The expression of RpoS as determined by Western blots was barely detectable in the lysates; therefore, the effect of tetracycline on RpoS levels was difficult to interpret. However, slight upregulation of RpoS expression was observed at high tetracycline concentration when cells were treated for a prolonged period of time (2 hours), suggesting that tetracycline may be involved in modulating the expression of RpoS. Taken together, the results indicate that tetracycline may decrease the bacterial stringent response and that this model may prove to be effective to study antibiotic cross-resistance in the future.

When Escherichia coli are exposed to an environmental stress such as amino-acid starvation they initiate the stringent response, a regulatory reaction which allows the cells to divert resources away from growth and division and toward amino acid synthesis in order to survive until nutrient conditions improve. This response involves the ribosome associated enzyme, RelA. When amino-acyl tRNA species are low in concentration, the ribosome can be stalled. Consequently, deacylated tRNAs have an increased likelihood of binding to the A-site of the 30S ribosomal subunit. The subsequent binding of Rel A to the deacetylated tRNA-ribosome complex results in RelA activation and production of ppGpp (2). (p)ppGpp is an intracellular signaling alarmone which triggers the stringent response and enables bacteria to survive these periods of nutrient limitation (11). The (p)ppGpp binds directly to the bacterial RNA polymerase and changes the transcriptional activity of genes, including the up-regulation of genes encoding metabolic enzymes like the Sigma S factor (RpoS). RpoS binds to the core of the bacterial RNA polymerase and induces stress-response-specific gene expression (7). By comparing a relA knockout to wild-type E.coli, Cheng et al. showed that induction of stringent response by exposure to sub-lethal concentrations of antibiotics could confer resistance to antibiotics from other classes (3). It was hypothesized that the mechanism for antibiotic resistance was induction of stringent response and inhibit growth processes of the stressed cell (3), because bacteria which are down-regulating growth processes should be less affected by antibiotics. To synthesize this intermediate induction of stringent response, we devised a model to partially inactivate the stringent response.
It has been shown that excessive valine concentrations can limit isoleucine concentrations, resulting in stringent response. High valine concentrations have been observed to result in decreased protein and ribonucleic acid accumulation in *E. coli* (14). Observations made by Gladstone in *Bacillus anthracis* (10) suggested two distinct explanations for the growth-inhibitory effects of excess valine concentrations in culture. First, that excess of one amino acid (valine) may block incorporation of another due to similarity in structure (isoleucine). Second, that excess amino acid may prevent synthesis of isoleucine through acetylacetate synthase.

The antibiotic tetracycline has been observed to inhibit ppGpp synthesis *in vitro* (20) and ppGpp and protein degradation during amino acid starvation *in vivo* (20). Voelmy and Goldberg (20) have shown that subjecting *E. coli* to low concentrations of tetracycline during amino acid starvation produces a steady-state level of ppGpp. These results can be explained by tetracycline’s mechanism of action. Tetracycline inhibits protein synthesis by blocking the binding of aminocyl-tRNAs to the 30S ribosomal subunit A site (20). Since the binding of a decacylated tRNA to the A site is required for RelA activation, tetracycline should inhibit the induction of stringent response by downregulating ppGpp synthesis and protein degradation. Therefore, intermediate concentrations tetracycline will be used to partially inactivate stringent response.

Gentry et al. observed that ppGpp, induced during starvation conditions, is a positive regulator of RpoS expression detected by immunoblotting (9); therefore, RpoS levels in the cell cultures were used to quantify the degree of stringent response. We hypothesized that cultures exposed to valine would activate stringent response, with full expression of RpoS; while untreated cultures would not express RpoS; and further, cultures co-treated with valine and tetracycline would partially activate stringent response with intermediate levels of RpoS.

We observed that tetracycline at low concentrations (5 μg/ml and 50 μg/ml) may inhibit stringent response activation. However, we found that tetracycline is prone to light sensitive and temperature sensitive instability, thus making this model a difficult one to work with.

**MATERIALS AND METHODS**

**Bacterial Strains.**  *E. coli* K12 CPT78 (F-, thr-1, leuB6(Am), fhuA2, gluV44(AS)?, gal-3, his-65, malT1(ΔR), xyl-7, mitA2, argH40, thi-1) was obtained from the MICB 421 culture collection from the Microbiology and Immunology Department at the University of British Columbia.

**Luria Broth growth curve culture conditions.** Cells were grown overnight in a 37°C Excella E24 air shaker (New Brunswick Scientific) in Luria Broth (LB). Overnight cultures were seeded into fresh LB to OD₆₀₀ = 0.246 and incubated in a shaking Gyrotory Water Bath Shaker (New Brunswick) at 200 rpm and 37°C for 5.5 h with turbidity measurements taken at 30 min intervals using the DU Series 500 Spectrophotometer (Beckman). Lysates were harvested at 1, 2, and 4 h.

**Preparation of M9 supplements.** M9 minimal salts media was supplemented with a final concentration of 1 μg/ml filter-sterilized thiamine (Sigma T-4625) and 80 μg/ml autoclaved leucine (Sigma L-8000), arginine (Sigma A-5006), histidine (Sigma H6034-25G), and threonine (Sigma T-8025). NH₄Cl solution was autoclaved separately, then combined aseptically and poured into petri dishes. Plates were then dried overnight at 37°C to allow the development of colored product. Each sample was read to a final volume of 100 μl of culture from each flask was spread plate onto supplemented M9 minimal media agar plates and incubated overnight at 37°C.

**Preparation of sterile tetracycline and valine.** 0.2 g tetracycline (Sigma T-3383) was dissolved in 20 mL of distilled water. 0.15 g valine (Sigma V-0500) was dissolved in 5 mL distilled water. Solutions were then filter sterilized using sterile Millipore 0.45 μm filter cups into 25 mL sterile polycarbonate test tubes. The tube containing tetracycline was wrapped in aluminum foil to prevent degradation of light-sensitive tetracycline and stored at -20°C. The tube containing valine was stored at 4°C.

**Preparation of supplemented M9 minimal media agar plates.** M9 minimal media was prepared in 2 times the concentration as above. An equal volume of 2X agar solution was made using 3% agar (Invitrogen). Each solution was autoclaved separately, then combined aseptically and poured into petri dishes. Plates were then dried overnight at 37°C in an incubator.

**Preparation of sterile tetracycline and valine.** Cells were grown overnight in 37°C air shaker at 200 rpm in M9 minimal media supplemented with amino acids and thiamine. Overnight cultures were seeded into fresh supplemented M9 minimal media and incubated at 37°C in a shaking water bath at 200 rpm for 2 h. Cultures were then split into 8 flasks and treated with 500 μg/ml valine to induce amino acid starvation along with varying concentrations of tetracycline and incubated for a further 15 to 120 min prior to cell lysate harvest. Throughout the incubation, turbidity readings were taking at predetermined intervals at OD₆₀₀ = 100 μl of culture from each flask was spread plate onto supplemented M9 minimal media agar plates and incubated overnight at 37°C.

**Cell breakage.** 25 ml of each treated culture was centrifuged at 7500 g at 4°C for 5 min using the J2-21 Centrifuge (Beckman). The cell pellet was washed in 10 mL cold Tris buffer (25 mM) and then spun at 7500 g at 4°C for 5 min. The cell pellet was resuspended in 1 mL Tris buffer (25 mM) and sonicated using the Microson Ultrasonic Cell Disruptor (Misonix) for 10 sec on setting 8 followed by 30 sec of cooling. Sonication was repeated 3 times in total. Samples were spun down at 14000 x g for 1 min at room temperature to collect cell debris using the 5415 D Centrifuge (Eppendorf). Supernatant was transferred to clean Eppendorf tubes and stored at -20°C.

**Bradford assay.** A standard curve was generated with bovine serum albumin (Sigma A-2153). Lysates were diluted appropriately with Tris buffer (Bio-Rad 500-0006) was diluted to 0% with distilled water and filtered using a Whatman #1 filter. 1 ml of filtered Bradford solution was added to each sample, vortexed, and incubated at room temperature for 5 min to allow the development of colored product. Each sample was read at A₁₀₀₅ nm.

**SDS-PAGE.** Equal amounts of protein were combined with 3X SDS-PAGE Sample Buffer (2.4% 1 M Tris-CI pH 6.8, 6% SDS, 30% glycerol, 16% β-mercaptoethanol, 0.06% w/v bromphenol blue). Samples were loaded onto an SDS-PAGE gel (12% running gel, 6% stacking gel) in a Bio-Rad SDS-PAGE Gel apparatus. The gel was run in running buffer (25 mM Tris base pH 6.8, 192 mM glycine, 1% SDS) at 100 V until the dye-front crossed the stacking buffer then increased to 120 V. Total time of electrophoresis was 2 h.
Western transfer and Western blot for immunodetection of RpoS. Proteins were transferred to Immobilon-P PVDF membrane (Millipore) in transfer buffer (25 mM Tris base pH 6.8, 192 mM glycine, 1% SDS, 20% methanol) and run at 100 V using a Bio-Rad western transfer apparatus. Between each step of the immunoblotting method, the membrane was washed with TBST (50 mM Tris, 150 mM NaCl, 0.2% KCl, 0.5% Tween 20, pH 7.4) 3 times for 10 minutes each at room temperature on a shaking platform. Following the transfer step, the membrane was incubated with Blocking Reagent (Roche 11 921 673 001) for 1 h shaking at room temperature. Primary polyclonal mouse anti-RpoS antibody (Neocline Biotechnology W0009) diluted 1:1000 in Western Blocking Reagent was hybridized to the membrane for 1 hr at room temperature. Secondary goat anti-mouse IgG (GibcoBRL 13864-012) diluted 1:5000 in Western Blocking Reagent for 1 h. Membrane was then washed with TBST as before and washed twice with AP substrate buffer (100 mM Tris pH 9.5, 100 mM NaCl, 5 mM MgCl₂) for 3 min each time. Following washes, membrane was treated with 10 ml BCIP/NCT (Sigma-Aldrich #B3804) and incubated until colourogenic detection of bands was evident.

Coomassie blue total protein staining. Following SDS-PAGE, gels were immersed in coomassie blue stain (45% methanol, 45% distilled water, 10% acetic acid, 0.25 g coomassie brilliant blue R-250 (GibcoBRL 15528-011) and placed on a shaking platform at room temperature overnight. Gels were then immersed in destaining solution (45% methanol, 45% distilled water, 10% acetic acid) and placed on a shaking platform at room temperature. Destaining solution was changed as needed until bands were clearly evident.

RESULTS

Growth rate of E. coli CP78 was slower in M9 than in Luria Broth. To evaluate whether the supplemented M9 minimal media could support growth of E.coli CP78 cells, the growth of E. coli CP78 was compared between M9 media and Luria Broth (LB). On average, growth in LB showed a doubling time of 75 min, while in supplemented M9 minimal media the E. coli CP78 had a doubling time of 120 min (Fig.1). This was expected as LB is a rich media which provides preformed cellular building blocks such that cells do not need to expend energy or time assimilating energy and electron sources for anabolism. M9 supported the growth of E. coli CP78 and therefore we were able to use it for our nutrient limitation studies.

Valine inhibited growth of CP78 cells. To verify the use of valine treatment as a viable method to induce starvation conditions for E. coli CP78, we compared the growth rates of valine-treated and untreated E. coli in M9 media. All valine-treated cultures had longer doubling times and decreased growth rates (k) compared to the untreated bacteria (Fig. 2A). Turbidity increased through the growth period for all cultures.

Intermediate concentrations of tetracycline increased turbidity of CP78 cultures. Since it was hypothesized that tetracycline inhibits the stringent response, an increase in turbidity was expected for valine-treated cultures because energy was not diverted from growth and division. This was observed in valine-treated cultures treated with 50 µg/ml of tetracycline, showing average higher OD readings throughout the growth curves compared to valine-treated cultures not treated with tetracycline (Fig. 2A).

Turbidities varied depending on tetracycline concentrations. To evaluate the killing effect that the antibiotic tetracycline had on E.coli CP78, we measured the turbidity for increasing tetracycline concentrations. As expected, the cultures treated with 5 µg/ml and 50 µg/ml tetracycline grew more slowly than the untreated control (Fig. 2B). However, the growth rate for the negative control and 500 µg/ml tetracycline treatment were very similar (Fig. 2B), which did not support the expectation that using a higher concentration of tetracycline should reduce the growth rates proportionally.

500 µg/ml tetracycline increased turbidity of CP78 cultures. To determine the effect of tetracycline on starved E. coli CP78, the effect was compared in valine-treated and valine-untreated cultures. Compared to the negative (unstarved and untreated) control, cultures treated with 50 µg/ml tetracycline had lower OD values throughout the growth period. Also, cultures treated with 50 µg/ml tetracycline had higher OD readings when co-treated with valine (Fig. 2C). Unexpectedly, the 500 µg/ml tetracycline treatment yielded OD values that were on average 1.8X greater than the control and this same 1.8X increase in OD readings was also observed in the 500 µg/ml treatment with valine (Fig. 2C). However, these increased turbidity readings were not accompanied by increases in growth rate.

In order to explore the hypothesis that tetracycline may darken overtime at 37°C, thereby affecting turbidity measurements, tetracycline was incubated overnight with supplemented M9 minimal media unprotected from natural light. As expected, the sample appeared to have darkened in color (Data not shown). Also, the possibility of cell lysis causing increase light transmittance in the spectrophotometer was ruled out by microscopic analysis of the cultures showing intact cells with no morphological changes (Data not shown).
Cells were viable after treatment with valine and tetracycline. In order to assess whether cells were still viable after treatment with valine and/or tetracycline, each culture condition was spread plate on supplemented M9 minimal media agar plates. Confluent lawns were observed on all plates following incubation overnight at 37°C. However, less dense lawns were observed on the plates spread with cultures treated with 500 μg/ml tetracycline.

RpoS protein expression is growth-phase dependent. We confirmed that the western blot method has enough sensitivity to detect differential levels RpoS protein expression in CP78 cell lysates at different phases of cell growth. In Fig. 3, RpoS expression (42 kDa) steadily increased from early-exponential phase (Lane 2), through mid-exponential phase (Lane 3, OD460nm = 0.9), to stationary phase growth (Lane 4) when grown in nutrient-rich conditions of LB broth. This similar trend in RpoS protein accumulation is observed in cells growing under nutrient limiting conditions of M9 media, as seen in the slight increase in detected RpoS protein band intensities of CP78 cell lysates harvested 30 minutes apart at late-exponential phase (Lane 5 and Lane 7). A secondary band that is ubiquitously observed at 38 kDa through all samples has been reported to be a possible degradation product of RpoS (18) and is therefore not discussed in relation to RpoS expression.

RpoS protein expression is down-regulated in valine-treated cells. In an attempt to determine the basal level of RpoS protein expression in CP78 E. coli with induced stringent response by valine treatment, we detected a significant decrease in the RpoS protein band (42 kDa) intensity in valine-treated CP78 E. coli for two hours during exponential growth (Fig. 4, Lane 4; Fig. 5, Lane 4) as compared to the untreated control (Fig. 4, Lane 2; Fig. 2, Lane 2). However, this difference in RpoS protein expression between valine-treated and control cells was not evident in cells treated for 15 min during exponential growth (Fig. 4, Lanes 3 & 4). Additional banding pattern on the Western blot is likely due to the detection of non-specific interactions with the polyclonal RpoS antibody and is therefore not taken into consideration for analysis. Effects of amino acid starvation following valine treatment was confirmed by observed growth-rate decrease in treated cells compared to the untreated control through timed monitoring of culture turbidity (Fig. 1.).

Tetracycline can regulate the expression of RpoS. To explore the effects of tetracycline treatment (250 μg/mL) on CP78 E. coli activation of stringent response, we observed the up-regulated RpoS protein expression (42 kDa) regardless of valine-induced amino acid starvation status only following prolonged
treatment incubation time (Fig. 4, Lanes 7 & 10) (not visible due to blot scan quality). The induced expression remained at low levels in comparison to the level of expression seen in the untreated and unstarved control at late exponential growth (Fig. 4, Lane 3). At low concentrations of tetracycline (5 μg/ml & 50 μg/ml), no RpoS protein expression was detected following prolonged or short treatment incubation (Fig. 4 and Fig. 5). It is important to note that the resolution of Fig. 5 membrane is sub-optimal as compared to Fig. 4.

**DISCUSSION**

Cohen (5) showed that the addition of valine to an exponentially growing culture of *E. coli* did not result in an abrupt cessation of growth; instead the optical density of the culture continued to increase at a linear rate (15). The continued slow increase in growth was attributed to enzymes formed during this period being less active because they are formed by incorporating valine in place of isoleucine.

Addition of tetracycline to a starved culture would be expected to cause an increase in turbidity values, since tetracycline inhibits the cells ability to divert energy away from growth toward amino acid synthesis when exposed to isoleucine starvation. However, addition of tetracycline to an unstarved culture would result in a decrease in turbidity values as tetracycline inhibits protein synthesis (4). Combining these observations, it was predicted that treatment of starved CP78 cells with 50 μg/ml of tetracycline would yield lower turbidity (cell density) than unstarved cells treated with the same tetracycline concentration. In Fig. 2C, it is clear that the growth rates and doubling times of the above-mentioned treatments are similar and the average OD readings for the 50 μg/ml tetracycline treatment of starved cells were consistently lower than 50 μg/ml tetracycline treatment of unstarved cells. This is consistent with the expected result.

Since tetracycline inhibits protein synthesis, a decrease in cell growth rate and turbidity would be expected (5). This was observed in the cultures treated with 5 μg/ml and 50 μg/ml of tetracycline, but not in those treated with 500 μg/ml of tetracycline (Fig. 2B). Contrarily, the 500 μg/ml tetracycline treatments yielded OD values that were greater than the control (Fig. 2C). However, these increased turbidity readings were not accompanied by increases in growth rate, suggesting turbidity may not be accurately reflecting cell density. The data for 500 μg/ml treatments was not consistent with expected results and it must be concluded that tetracycline structure was interfering with turbidity measurements by increasing light transmittance.

The elevated OD readings in the 500 μg/ml tetracycline culture as observed in Fig. 2C could be due to the increased concentration of the tetracycline molecules in the solution interfering with the light transmittance in the Beckman Spectrophotometer. Tetracycline has a hydronaphthalene nucleus with 4 fused rings that can exist in either a non-ionized lipophilic form or a zwitterionic hydrophilic form (17). The conditions of the media and the heat of the water bath could have caused conformational changes in the tetracycline molecules resulting in higher light transmittance. Also, tetracycline can darken when exposed to sunlight (17) which could affect absorbance readings as tetracycline concentration increases and such an effect is amplified. This darkening phenomenon was observed in this experiment when tetracycline was incubated overnight with media, unprotected from natural light. In addition, when tetracycline is taken up by the cells it can form a magnesium-tetracycline chelate to which the cell membrane is impermeable and the tetracycline can be trapped and accumulate in the cells resulting in abnormally high OD readings (17).
The expected increase in turbidity as a result of tetracycline stringent response inhibition was not observed in valine-treated cultures treated with 5 μg/mL of tetracycline. This sample showed an overall lower OD than the valine-treated culture (Fig.2A). This suggests that there is a threshold concentration between 5 μg/ml and 50 μg/ml which inhibits stringent response.

RpoS expression was used in this experiment as an indicator of the degree of stress cells were undergoing during stringent response induction. RpoS expression increases from basal levels in exponential phase to stationary phase growth in both LB and M9 minimal media. This is consistent with results described in Jishage et al. (12), who demonstrated that sigma S, or sigma 38, increases during the transition from exponential phase to stationary phase and is maintained at these higher levels (12). This indicates that RpoS is required in stationary phase to regulate growth in a limited nutrient environment and has also been shown to be expressed during stringent response.

Supplemented M9 minimal media treated with valine induced less RpoS than the untreated culture as seen in Fig. 3 and Fig. 4. At first glance, this observation appears to be contrary to our expectations as valine induces stringent response by amino acid starvation. However, it is important to note that the high levels of RpoS protein expression is only observed in un-starved cells harvested after prolonged growth incubation time (4 hours total). As indicated in Fig. 1, it is clear that at 120 min, E. coli CP78 has entered late-exponential growth phase and begin to accumulate RpoS protein in preparation for stationary phase survival. We hypothesize that the low level of RpoS expression in valine-treated cells can be attributed to transcriptional inhibitory effects of amino acid starvation, slowing down the process of protein synthesis in order to conserve cellular resources during nutrient-limited growth. Due to the sensitive nature of RpoS expression levels as determined by phase of cell growth it is unwise to make conclusions based on direct comparisons between the starved and unstarved samples in Fig. 3 and Fig. 4 since they are in different phases of growth. Our hypothesis is further confirmed in Fig. 5 where the difference in RpoS expression between starved and unstarved samples cannot be distinguished when both samples were still within early-exponential phase growth.

Free PsrA (a TetR family member) has been shown to increase the RpoS expression in Pseudomonas aeruginosa (13). TetR is released in the presence of tetracycline, suggesting that tetracycline can in fact increase the RpoS expression. We hypothesize that a similar mechanism is responsible for the increase in RpoS expression in E. coli CP78.

While it is possible that high doses of tetracycline were able to efficiently block ppGpp synthesis as reported by Voellmy et al. (20), the downstream effect on RpoS was negligible in comparison to any possible effect of TetR regulation of RpoS expression. Therefore RpoS is not a good indicator of inactivation of stringent response by tetracycline at high concentrations. Instead, ppGpp synthesis should be directly monitored using radio-labelled 32P to determine the effect of high doses of tetracycline independently of the induction of RpoS.

The reduced growth rate of valine-treated cultures compared to the unstarved control indicated that amino acid starvation by inhibition of isoleucine synthesis was successful (Fig. 2A). However, this is contradicted by the observation that valine treatment inhibits expression of RpoS (Fig. 4). Because the turbidity measurements were confounded by tetracycline, it was inconclusive as to whether tetracycline truly enhanced growth of a starved culture, or whether it was solely the action of tetracycline which was increasing the turbidity readings. However, taken together with the fact that tetracycline diminishes the expression of RpoS (Fig. 5), we can propose with greater confidence that the tetracycline may partially inactivate stringent response.

In conclusion, according to turbidity measurements, valine effectively starved and induced stringent response by E. coli CP78 grown in supplemented M9 media, while 50 μg/ml tetracycline may have partially inactivated stringent response. However, because of the instability of tetracycline at 37°C, it is difficult to ascertain whether the increase in turbidity was an artifact of change in conformation of tetracycline, or a genuine inactivation of stringent response. Tetracycline at concentrations 5 μg/ml, and 50 μg/ml were observed to inhibit RpoS expression in valine-starved cells. Taking both turbidity measurements into account, we conclude that intermediate levels of tetracycline may partially inactivate stringent response and since concentrations of tetracycline used in this experiment yielded viable cells after treatment, however it must be noted that this model is problematic due to the limitation of tetracycline degradation as mentioned previously.

**FUTURE DIRECTIONS**

In this study, we have made some interesting observations in regards to the regulation of stringent response activation by tetracycline treatment. To further this research, we believe that it is of foremost importance to optimize the growth conditions of E. coli CP78. Throughout this investigation, we have observed suboptimal growth rates in M9 minimal media. The growth rate observed in this investigation is two times longer than the growth rate reported by Cheng et al. (3) using the same bacterial strain and similar growth conditions. It has been reported that suboptimal growth
rates of \textit{E. coli} in culture is associated with unfavourable carbon substrates (8). Lower growth rate could result in decreased rRNA expression therefore affecting \textit{E. coli} protein expression and stringent response activation thus confounding our experimental results. To mitigate this problem, we suggest for future groups to select an alternative heterotrophic \textit{E. coli} strain to work with. Also, optimization of the western blot protocol for RpoS detection should be performed. The detection of RpoS on the western blots is limited by the protein concentration present in the bacterial lysates and also by the sensitivity of the immunoblotting signal detection system/method. An additional experimental step to concentrate the protein in the lysates could be performed to increase the amount of total protein that will be loaded into the gels. It is also worthwhile to use a chemi-fluorescence western blot detection system such as ECL instead of the NBT/BCIP AP substrate system since the ECL system is reported to detect product amounts in the picogram and femtogram ranges. Furthermore, alternative methods to monitor the activation of stringent response must be explored since RpoS protein expression is regulated at many levels, from mRNA stability via translation to protein stability and proteolysis, it is therefore sufficient to postulate that small regulatory changes resulting from tetracycline treatment may be masked by other processes that regulate RpoS expression (16). A direct way to measure stringent response activation can be achieved directly through the monitoring of (p)ppGpp accumulation by incorporation of radio-labeled $^{32}$P and visualization with thin-plate chromatography. (p)ppGpp is a stringent response alarmone that is directly upregulated when

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**REFERENCES**

