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RpoS-Dependent Mechanisms are needed for Tetracycline Resistance but are Not Involved in the Cross-Protection against other Antibiotics in *Escherichia coli*

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RpoS, an alternate sigma subunit of RNA polymerase, is upregulated under stress conditions such as high heat, depletion of nutrient, high osmolarity, and UV radiation. Current understanding is that RpoS acts as a general stress response regulator to counter the effect of different types of stress, including antibiotics, when it is induced by a single stress. In this study, we sought to test the cross-protection ability of RpoS production in *E. coli* to antibiotics streptomycin, chloramphenicol, and tetracycline. A pBAD-*rpoS E. coli* strain was created by using PCR to amplify the *rpoS* gene before cloning it into the pBAD TOPO vector. The ligated vector was chemically transformed into Wild type (WT) bacteria to create the new strain. To ensure that arabinose successfully induced *rpoS*, western blotting and immunodetection were used. WT *E. coli* BW25113, JW5437-1Δ*rpoS*, and pBAD-*rpoS E. coli* strains were then tested for antibiotic resistance. It was observed that the pBAD-*rpoS* strain showed decreased susceptibility to tetracycline only. An *rpoS*-dependent mechanism is involved in the increased resistance to tetracycline; however, there was no cross-protection observed against other antibiotics.

σ^S, or RpoS, is a general stress response regulator produced by *Escherichia coli* and related bacteria to counter the effects of various stress conditions such as osmotic shock, high temperature, UV radiation, or depletion of nutrients (11). The mechanism by which the bacteria counter these negative stressors is by upregulating the *rpoS* and *rpoS*-related genes. RpoS is a sigma subunit of RNA polymerase that can replace the normal, or vegetative, sigma factor σ^{70} (RpoD) (9). When RpoS replaces RpoD, it provides the bacteria with the ability to survive various types of stresses. Moreover, it has been suggested that the RpoS does not limit protection to the specific type of stress that led to the upregulation of the gene; rather it provides protection for multiple types of stresses, including protection against antibiotics (6). An example of *rpoS* affecting antibiotic susceptibility is its regulation of penicillin binding protein production on the cell membrane (6, 15). This phenomenon is termed cross-protection.

It is believed that the reduced susceptibility to antibiotics after exposure to environmental stressors is an example of cross-protection. This is best shown by the linkage between susceptibility of food-related pathogens to antibiotics and preservative methods applied to them (15). McMahon *et al.* found that temperature, pH, and osmoticity had an effect on susceptibility of bacteria (15). However, it was unclear whether the difference in susceptibility was caused by the physical change in cells such as membrane fluidity or by RpoS (15). In an additional study by Huang et al., *E. coli* were grown under osmotic shock conditions to induce RpoS prior to MIC testing to see whether it caused cross-protection against antibiotics (10). Still, there was insufficient evidence that the osmotic shock upregulated RpoS. Also the effect of osmotic shock on cellular fitness was not investigated and this might have played a role in the resistance to antibiotics.

In order to separate the effect of environmental stressors on other aspects of cells during the upregulation of RpoS, we created a pBAD-*rpoS E. coli* strain (KAYA11W-1).We tested this strain with antibiotics and found that the clones did not provide cross-protection.

MATERIALS AND METHODS

Growth media, bacterial strains, and plasmids. Bacteria were grown in Luria Broth (LB) (1% (w/v) tryptone (Bacto™, Cat. #211705), 0.5% (w/v) yeast extract (Bacto™, Cat. #212750), and 1% (w/v) NaCl (Fisher Chemicals, Cat. #S271-3), pH 7.0) (12). Ampicillin (Amp) was used at 100 µg/ml. L-arabinose (Ara) (Sigma-Aldrich, Cat. #A3256) supplementation was at 0.2%. All liquid cultures were grown overnight on a shaking platform at 37°C at 200 rpm. *E. coli* K-12 strain BW25113 (wild-type control) and JW5437-1 (*ΔrpoS746::kan*) were obtained from the University of British

Columbia, Department of Microbiology and Immunology MICB 421 culture collection. Both strains are F-, Δ*(araD-araB)567*, Δ*lacZ4787*(::rrnB-3), λ⁻, *rph-1*, Δ*(rhaD-rhaB)568*, *hsdR514*. *E. coli* K-12 strain JW5437-1 is an *rpoS* deletion mutant. OneShot® TOP10 Chemically Competent *E. coli* were supplied in the pBAD TOPO TA® Expression Kit (Invitrogen, Cat. #K4300-01) and were thawed on ice before transformation. The pBAD TOPO plasmid was supplied in the pBAD TOPO TA® Expression Kit which was used for transforming the supplied OneShot® TOP10 chemically competent *E. coli* with the *rpoS* gene. Plasmid pBAD TOPO is 4126 nucleotides long, containing an ampicillin resistance gene and an arabinose promoter with its regulatory elements (for arabinose-induced expression of the target gene).

PCR primer design for *rpoS*. Primers were designed to amplify *rpoS* from *E. coli* BW25113. The PCR product was used to clone the rpoS gene into a pBAD TOPO plasmid. The forward primer (TOPO *rpoS*-f-3gamma 11w) (5'-TGAGAGGATCACTACATGAGTCAGAATACG-3') contained an in-frame stop codon and translation reinitiation sequence consisting of a ribosome binding site to remove the N-terminal leader to allow for expression of native protein (IDT, Cat. #83466126). Reverse primer (TOPO *rpoS*-r-3gamma 11w) (5'– TTA AGC CTG CGG CTG AGT TAC AAC GTC TTT -3') included the native stop codon of *rpoS* to exclude the V5 epitope and polyhistidine region of the plasmid (IDT, Cat. #83466127). Both primers were resuspended in sterile distilled water to a final concentration of 100 µM.

Whole cell PCR amplification of *rpoS*. *E. coli* BW25113 was directly transferred from a plate culture using the end of a sterile pipette tip and was resuspended in 20 µl of sterile distilled water. The cells were heated for 10 minutes at 94°C, placed in a -80°C freezer for 10 minutes, thawed, and centrifuged. Six µl of the supernatant was used in the PCR reaction. The PCR reaction also included 1X PCR buffer (2.5 µl of 10X stock) (Invitrogen kit, Cat.#18038), 0.1 µl of the 100 µM forward and reverse *rpoS* primers, 2 µl of 25 mM $MgCl_2$, 0.5 µl of 10 mM dNTP mix and 0.125 µl of 5 U/µl Taq polymerase. The reaction volume was made up to a final volume of 25 µl using sterile distilled water. PCR reactions were incubated in Biometra® T Gradient Thermocycler at 94°C for 5 min, followed by 35 cycles of amplification: 30 seconds at 94°C, 45 seconds at 55°C, and 90 seconds at 72°C.The final extension was incubated for 10 min at 72°C, followed by storage at -20°C. The PCR products were analyzed in 1.0% (w/v) agarose electrophoresis gel to verify a 1kb band.

DNA gel electrophoresis. 1.0% (w/v) agarose or 1.3% (w/v) agarose (Bio-Rad, Cat. # 161-3101) was dissolved in 1X TAE buffer (40 mM Tris Base (Fisher Bioreagents, Cat. #BP152-1), 20 mM glacial acetic acid (Fischer Scientific, Cat. # 42322-0025), 1 mM EDTA (Fisher Chemical, Cat. #BP120-1), pH 8). One µl of GeneRuler™ 1 kb DNA Ladder (Fermentas, Cat. #SM0312) with 1X DNA loading dye (Fermentas, Cat. #R0611) was used to prepare the molecular weight standard. Samples were prepared by adding DNA loading dye before being loaded into wells. Gels were run at 120 V for 50 minutes in 1X TAE buffer. The gels were placed in a 0.5 µg/ml ethidium bromide solution for 20 minutes to stain the DNA for visualization under UV light.

pBAD TOPO TA cloning of *rpoS* into pBAD TOPO vector. The cloning and transformation of the *rpoS* PCR product into the pBAD vector was performed as described in the pBAD TOPO® TA Expression Kit manual (12) (Invitrogen, Cat. #K4300-40). Fifty µl of the transformed cells were spread on LB agar plates supplemented with ampicillin (100 µg/ml) (LB+Amp) to select for transformants.

pBAD vector isolation. Plasmid isolation was done on overnight cultures of 5 ml of TOP10 *E. coli* using the instruction the GeneJET™ Plasmid Miniprep Kit (Fermentas, K0502). The samples were stored at -20°C**.** The plasmid quantity and purity was assessed using the A_{280} and A_{260} values measured with the Nanodrop 2000 (Thermos Scientific)

Plasmid insert orientation analysis. Using the isolated plasmid, restriction enzyme digests were carried out using 2 U of StyI (NEB, #R0500L), 1X NEBuffer 3 (NEB, # B7003S), 2 µg of plasmid DNA, 1X BSA solution, and sterile distilled water to make up to final volume of 20 µl. A digest was also carried out using 2 U of XmnI (NEB, # #R0194L), 1X NEBuffer 4 (NEB, # B7004S), 2 µg of plasmid DNA, 1X BSA solution, and sterile distilled water to make up to final volume of 20 µl. The digestions were incubated at 37°C for 1 hour. Samples were analyzed by running the products on a 1.3% agarose gel.

Preparation of chemically competent *E. coli* BW25113. *E. coli* BW25113 was made chemically competent using the methods described in Current Protocols in Molecular Biology (17). The competent *E. coli* BW25113 cells were stored at -80°C then centrifuged at 4°C at 4000xg.

Transformation of chemically competent *E. coli* BW25113. 2 µl of the correctly orientated isolated plasmid was mixed gently with the chemically competent *E. coli* BW25113 and incubated on ice for 10 minutes. The reaction vial was then heat shocked at 42°C for 30 seconds. 250 µl of LB medium was added to the reaction vial and incubated at 37°C in a shaking incubator at 200 rpm for 1 hour. Following this incubation, 50 µl of the cells were spread on LB agar plates supplemented with ampicillin (100 µg/ml) to select for transformants. Untransformed cells were also plated as a negative control. Prior to transformation with the *rpoS* clone, 0.5 µl of pBR322 was transformed and plated to see if the *E. coli* cells were competent.

Arabinose optimal concentration test. The test for the optimum concentration was performed as outlined in the pBAD TOPO TA® Expression Kit Manual (12). Transformed cells were grown in LB medium with 10-fold dilutions of arabinose (0.002%, 0.02%, 0.2%, and 2%) to test which concentration of arabinose was optimal for growth based on the OD_{600} values after 4 hours of growth at 37°C. The optimal concentration was determined by the culture conditions that had the greatest OD_{600} value after 4 hours.

Total protein assay. Lysates grown in LB media supplemented with arabinose were analyzed for protein concentration using the Bradford assay as previously described (2). Chicken egg albumin was used in place of bovine serum albumin as a protein standard. The total protein content was standardized before loading onto a polyacrylamide gel.

SDS-PAGE. The lysates of wild-type *E. coli* BW25113, the *rpoS*-knockout JW5437-1, and the pBAD-*rpoS* grown in LB medium supplemented with 0.2% arabinose were combined with NuPage 4X sample buffer (Invitrogen, # NP0007). Samples were then heated to 99°C for 5 minutes and loaded onto a 15% SDS-polyacrylamide gel (Bio-Rad, #161-0156) and run in Tris-glycine running buffer (25 mM Tris base pH 6.8, 192 mM glycine, 1% SDS) at 110 V in a Bio-Rad PAGE apparatus for 75 minutes. Prestained Protein Molecular Weight Marker (Fermentas, # SM0441) was used as the molecular weight standard.

Western blotting and immunodetection of RpoS. Following SDS-PAGE, a Hybond™-P membrane (Amersham Biosciences, #RPN2020F) was soaked in 100% methanol and the proteins were transferred onto the Hybond™-P membrane in Western transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol) and run at 30 V overnight in a Bio-Rad electroblotting apparatus at 4°C. Membranes were washed 3 times with TBS-Tween 20 (50 mM Tris, 150 mM NaCl, 0.2% KCl, 0.5% Tween 20, pH 7.5) and treated with Western blocking reagent (Sigma Aldrich, #B6429-500ML) for 1 hr. Following blocking, the membrane was hybridized for 1 hour at 20°C on a shaking apparatus with a primary mouse anti-RpoS antibody (Neoclone Biotechnology, cat. # W0009) diluted 1/1000 in Western blocking reagent. Three additional washes with TBS-Tween 20 were done before hybridization for 1 hour on a shaking platform with a secondary alkaline phosphatase-linked goat anti-mouse Ig (Chemicon International, cat. #AP124A) diluted 1/3000 in Western blocking

reagent. The membrane was washed three times with TBS-Tween 20 and three times with substrate buffer (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) before the addition of 10 ml of BCIP/NBT substrate (Sigma-Aldrich cat. #B3804) for the detection of RpoS. A picture was taken after colour formation. The membrane was washed with distilled water and stored at -20°C

Minimum inhibitory concentration assay. The minimal inhibitory concentration (MIC) was determined by the microtitre broth dilution method as described in the Hancock Laboratory Methods (8). *E. coli* strains were grown in the LB medium supplemented with arabinose diluted to a concentration of 10^4 to 10^5 CFU/ml. Streptomycin, chloramphenicol, and tetracycline were used to test for the MIC of each antibiotic in triplicates. A two-fold dilution series was made to make the desired final antibiotic concentration of 0, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, and 128 µg/ml in 100 µl of LB medium supplemented with arabinose. Five µl of the diluted culture was added to each well. When satisfactory growth was obtained after 24-36 hours, the plates were analyzed by eye by determining the boundary between growth and no growth.

RESULTS

PCR amplification of *rpoS*. The PCR amplification of the *rpoS* gene isolated from *E. coli* BW25113 was performed. There was an approximate 1000 bp fragment on an agarose gel in Figure 1. A 1008 bp fragment was expected which corresponded to an in-frame stop codon, a translation reinitiation sequence and the *rpoS* gene.

Transformation of chemically competent TOP10 *E. coli* with pBAD-TOPO vector. The success of the ligation reaction was assessed by the formation of colonies recovered on LB medium supplemented with ampicillin. The colonies formed on the plates were numerous, indicating that the transformation reaction was efficient. A control plate was inoculated with untransformed *E. coli* BW25113 on a LB+Amp agar. There was no growth which indicated that the ampicillin supplementation was sufficient for selecting transformants. The six transformants that were tested were designated pKAYA11W1-pKAYA11W6

Analysis of the orientation of the insert. To determine whether the transformant had the correct orientation of the PCR gene product, a restriction digest analysis was done using restriction enzymes, StyI and XmnI. The overall size of the pBAD vector was 4126 nucleotides, whereas the pBAD-*rpoS* recombinant plasmid was 5134 nucleotides. The molecular weights of the bands yielded by pKAYA11W1, pKAYA11W2, pKAYA11W3, pKAYA11W4, and pKAYA11W5 had band sizes when cut with StyI were around 4.8 kbp and 250 bp; there were band sizes of 4.2 kbp 700 bp and 200 bp when cut with XmnI as shown in Figure 2. pKAYA11W4 had an extra band when digested with XmnI around 3.5 kbp as shown in Lane 11. pKAYA11W6 had an entirely different banding pattern

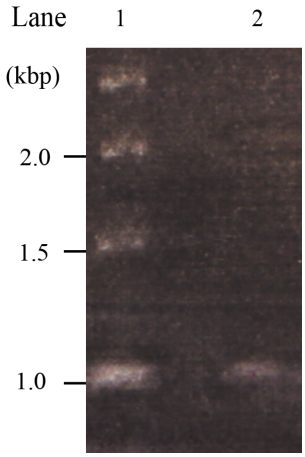

FIG. 1. PCR amplification of the *rpoS* gene. Lane 1: GeneRuler™ 1 kb DNA Ladder, Lane 2: PCR product amplified by primers TOPO *rpoS*-f-3gamma 11w and TOPO *rpoS*-r-3gamma 11w.

with band sizes of 4.2 kbp and 900 bp when cut with StyI, and 3.4 kbp, 1050 bp and 700 bp when cut with XmnI. pKAYA11W2, pKAYA11W3, and pKAYA11W5 had the expected banding pattern of a correctly orientated insert.

The growth of pBAD-*rpoS* clones was greatest when supplemented with 0.2% arabinose. To determine the optimum concentration of arabinose supplementation, a growth assay was performed comparing the OD_{600} of serially diluted LB supplemented with arabinose after 4 hours. The cells grown in 0.2% arabinose showed 5.1% greater growth than the second most viable growth condition. This concentration of arabinose was used for the growth of the WT *E. coli* BW25113, *rpoS*-knockout JW5437-1, and the BW25113 + pBAD-*rpoS* (KAYA11W1), the banding pattern of the lysates are shown in Figure 3.

Verification of the RpoS production in the pBAD-*rpoS* clone. To verify that the KAYA11W1 clone induced RpoS production in the presence of arabinose a western blot was performed (Fig. 3). The results of this procedure indicated that the *E. coli* KAYA11W1 was a pBAD-*rpoS* clone as the *rpoS* gene product, about 38 kDa in size, was detectable after the addition of the BCIP/NBT substrate. The difference in RpoS production between the three strains has also been demonstrated in Figure 3. As expected, the *rpoS* clone had the greatest expression of RpoS and wild-type BW25113 had a detectable amount of RpoS, albeit not as much as KAYA11W1. The JW5437-1Δ*rpoS* knockout served as the negative control; there was no

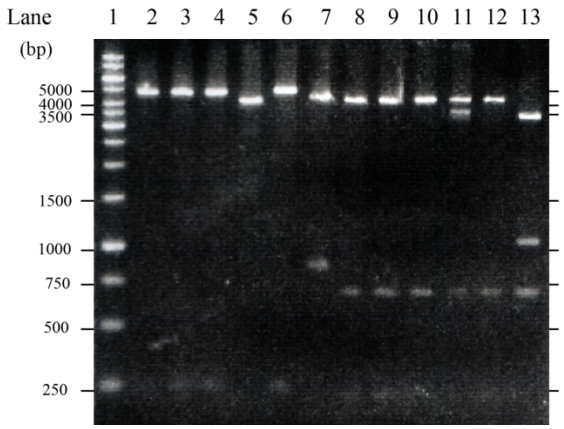

FIG. 2. Restriction digest analysis of TOPO TA ligation plasmids to verify orientation of *rpoS* gene in pBAD-TOPO vector. Lane 1: GeneRuler™ 1 kb DNA Ladder, Lane 2 to 7: StyI digested pKAYA11W1 to pKAYA11W6, Lane 8 to 13: XmnI digested pKAYA11W1 to pKAYA11W6.

band in lane 3 which suggests that the western was successful. Also, the lack of other bands indicates that there were no unexpected problems associated with the nonspecific binding of the secondary antibody.

MIC assay. The *E. coli* strains, wild-type *E. coli* BW25113, *rpoS*-knockout JW5437-1, and KAYA11W1, were subjected to serial diluted concentrations of streptomycin, chloramphenicol, and tetracycline grown in LB medium supplemented with 0.2% arabinose. The MIC of the strains to these antibiotics are shown in Table 1. All of the antibiotics tested had low MIC for each of the three strains suggesting that the antibiotics were effective in inhibiting the growth of cells. The three strains showed the highest sensitivity to chloramphenicol, which inhibited growth of bacteria at a concentration of 1 µg/ml, and the lowest sensitivity to tetracycline. The MIC values of the *E. coli* BW25113 and *E. coli* JW5437-1Δ*rpoS* were the same throughout the assay. The KAYA11W1 strain had similar MIC values to *E. coli* BW25113 and *JW5437*-1Δ*rpoS* in the presence of streptomycin and chloramphenicol, but showed a 2-fold decrease in the susceptibility to tetracycline (32 µg/ml compared to 16 µg/ml) likely due to the induced RpoS production. Since the uncertainty of the MIC assay is ± one well, several replicates were done such that the MIC for tetracycline was reproducible.

DISCUSSION

To determine whether the transformants had the correct orientation of the PCR gene product, a restriction digest analysis was done using two restriction enzymes, StyI and XmnI. Since the pBAD TOPO TA vector system ligates a PCR product using the A/T overhang, the directionality of the gene within the vector may not be correct and subsequently would not express the protein of interest. StyI and XmnI were selected as restriction enzymes as it had restriction sites on the vector and in the insert which could be used to identify the directionality of the insert. The molecular weights of the bands yielded by pKAYA11W1, pKAYA11W2, pKAYA11W3, pKAYA11W5 were consistent with the expectations of a correctly orientated insert as shown in Figure 2. The banding pattern of pKAYA11W4 was not consistent to the expectations since the band sizes were smaller than expected. pKAYA11W4 had an extra band when digested with XmnI around 3500 bp as shown in Lane 11 which may suggest that the fidelity of the Taq polymerase was not high enough such that an additional restriction site was introduced.

The cells were treated at 0.2% arabinose to compare the *rpoS* expression among different as the cells had the greatest growth at that concentration. It is known that too much expression of *rpoS* can cause cell death by destruction of rRNA and ribosome due to loss of protein synthesis capacity (5). The intent of this study was to distinguish differences in antibiotic resistance that could be attributed solely to the change

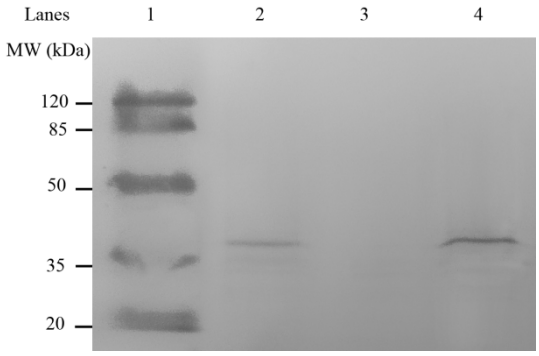

FIG. 3. Detection of RpoS protein from *E. coli* BW25113, JW5437-1Δ*rpoS*, and KAYA11W-1 by western immunoblotting. Lane 1: Fermentas Prestained Protein Molecular Weight Marker, Lane 2: wild-type BW25113, Lane 3: JW5437-1Δ*rpoS*, Lane 4: KAYA11W-1

in level of RpoS. Thus, it was expected that if there was indeed an *rpoS* dependent cross-protection mechanism, the transformants containing the *rpoS*-TOPO plasmid would have higher minimum inhibitory concentration to all three antibiotics than the wild-type and knockout strains. A limitation to our study was that, much like prior studies, we were unable to determine the effect of transformation on the general fitness of the cells. The transformation of KAYA11W1 cells may have had an increase in resistance to antibiotics due to the *rpoS*, but it may also have decreased the fitness by subjecting the cells to a transformation protocol.

It was also expected that the wild-type would have greater antibiotic resistance than the knockout as there was some RpoS production as shown in Figure 3. This however was not the case. At the level of RpoS produced by the wild-type cells there seemed to be no mechanism of *rpoS*-mediated antibiotic resistance because the wild-type and knockout had similar MICs. The MICs of all 3 strains to streptomycin and chloramphenicol were 4 µg/ml and 1 µg/ml, respectively, which were lower than literature values of 16 µg/ml (18) and 5.3 µg/ml (4). Since the relative MIC between the strains was being observed rather than the quantitative MIC, we felt that the results were valid. It was observed that the transformants had an increased resistance to tetracycline (Table 1). MIC to tetracycline for transformed cell was 32 µg/ml compared to the wild-type and *rpoS*-knockout which showed a MIC of 16 µg/ml. The resistance to tetracycline antibiotics in *E. coli* has been documented to depend on a decreased permeability of the antibiotic (13). This low level of resistance to tetracycline appears to result from the decreased uptake (13). One of the roles of *rpoS* is to change the cell membrane permeability by expressing *rpoS*-dependent genes that belong to the *Osm* family genes (3). The outer membrane lipoprotein is encoded from *osmB* gene (3). The OsmB lipoprotein cross-links the outer membrane of the cell with the peptidoglycan to increase survival under hyperosmotic condition (3). By increasing the cross-linking, it is speculated that tetracycline cannot penetrate the cell membrane to inhibit the protein synthesis inside the cell. Streptomycin and chloramphenicol did not show any change in resistance in transformed cells (Table 1). For these antibiotics, it is expected that they do not interact with *rpoS*-induced genes to increase the resistance of the cell. For streptomycin, the studies have found that it is an aminocyclitol aminoglycoside that directly interacts with the small ribosomal subunit and inhibit protein synthesis by disrupting the 30S and 50S ribosomal subunits interaction (14). One of the possible sources of resistance to streptomycin is the presence of linked *strA-strB* in the cell that inactivated streptomycin by two phosphotransferase enzyme (18). Perhaps each *E. coli* strain possessed this gene that produced similar MIC level in each of the strains, independent of an *rpoS* dependent mechanism. Chloramphenicol is a small lipid soluble molecule that can penetrate into the outer-membrane and effectively interfere with protein synthesis by binding amino acyl-tRNA to the A site on the 50S subunit of the ribosome (16). It is thought that the small size of the antibiotic allows it to escape the mechanisms involved in the increase in tetracycline resistance.

TABLE 1. Summary of the minimum inhibitory concentrations of wild-type, Δ*rpoS*, and pBAD-*rpoS E. coli* strains.

Strain	Streptomycin MIC (µg/ml)	Chloramphenicol MIC (µg/ml)	Tetracycline MIC (µg/ml)
Wild Type BW25113	4.0	1.0	16.0
JW5437-1Δ*rpoS*	4.0	1.0	16.0
KAYA11W-1 (pBAD-*rpoS*)	4.0	1.0	32.0

Contrary to our hypothesis, our results showed that there is no cross-protection against antibiotic challenges conferred by *rpoS* alone, as the overproduction of RpoS did not increase the resistance to streptomycin and chloramphenicol. Although *rpoS*-dependent mechanisms offer no cross-protection to antibiotic challenges, the elevated levels of RpoS in the KAYA11W1 strain increased the resistance to tetracycline. This suggests that the *rpoS*-dependent pathway might be involved in the low level resistance to only some antibiotics.

FUTURE DIRECTIONS

In our experiment a difference in tetracycline resistance was observed between the wild type and transformed cells. This could be due to a higher amount of RpoS production in transformed cells compared to the wild type strains or as a result of increase in general fitness of the transformed cell. In future experiments it may be useful to measure the MIC in the presence and in the absence of arabinose. In the presence of arabinose the pBAD promoter will be induced to express RpoS. In the absence of arabinose *rpoS* will not be induced, rather the KAYA11W1 strain should behave as the parental BW25113 strain. Performing this

experiment will give insight to the effect of *rpoS* solely on the antibiotic resistance. This will test whether KAYA11W1 behaves as its parental strain when the *rpoS* gene is not induced. If not, this indicates that the procedure involved in obtaining the strain may have conferred the increased resistance in a manner independent to the levels in RpoS.

Experiments testing why there were observed difference in tetracycline resistance may also be useful. Testing whether the *osm* family genes or other upregulated genes are involved in a mechanistic resistance to antibiotics would be beneficial to look into as it would give insight on a possible *rpoS*-mediated increase in antibiotic resistance

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