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The Effect of Osmotic Shock on RpoS Expression and Antibiotic Resistance in *Escherichia coli*

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The general stress response regulator σ^s, commonly known as RpoS, is a sigma subunit of RNA polymerase that is up-regulated when bacteria are exposed to environmental stresses such as osmotic shock, high temperature, UV radiation, and feast-famine conditions. Current knowledge is that a single stress condition can provide stress tolerance and can also lead to cross-protection against unrelated challenges. Exposure to sublethal levels of osmotic stress has been found to increase antibiotic resistance in *Escherichia coli*. In this study, we sought to elucidate whether this cross-protection is conferred through an RpoS-dependent manner. Wild type (WT) and Δ*rpoS E. coli* strains were subjected to continuous 0.3 M NaCl osmotic shock conditions followed by subsequent exposure to either ampicillin, chloramphenicol, or rifampicin to determine the minimum inhibitory concentration (MIC) of these antibiotics. RpoS levels were determined by Western immunoblotting with a monoclonal RpoS-specific antibody, however no difference was observed between levels of RpoS induction under normal and osmotic shock conditions. RpoS was found to have some effect on antibiotic resistance as the WT strain had greater antibiotic resistance under normal M9 conditions than the Δ*rpoS* strain. Mechanistic differences between modes of resistance for different antibiotics may also exist. No cross-protection against antibiotics with continuous osmotic shock via RpoS-dependent mechanisms was found as osmotic shock was seen to increase antibiotic susceptibility in both the Δ*rpoS* and WT strains.

Escherichia coli is a gram-negative enteric bacterium that like many microorganisms, is frequently exposed to environmental stresses such as temperature, UV radiation, osmotic shock, and feast-famine conditions (7, 13). These stressors have an adverse effect on the physiological welfare of bacterial cells and thus these cells must be able to adapt in order to survive.

To counteract the effects of these stressors, *E. coli* produces the general stress response regulator, σ^s (RpoS), which allows for the up-regulation of genes that aid in survival under adverse conditions (8). RpoS is a sigma subunit of RNA polymerase that is up-regulated in *E. coli* during stationary phase and upon exposure to other stresses including but not limited to those aforementioned (8). Current understanding is that RpoS directs RNA polymerase to alternative promoters, which result in the expression of new genes that are adaptive under conditions of stress (8). It has been shown that the exposure to a single stress condition can provide stress tolerance and can also lead to cross-protection against unrelated challenges since the subset of activated genes may affect multiple responses (10, 8).

Bacterial cells are believed to sense the actions of antibiotics as just another form of environmental stress and relationships have been suggested linking environmental stresses, such as those occurring in foods and the domestic environment and the development of antibiotic resistance (10). In an experiment investigating the effect of various environmental stresses (high/low temperature, osmotic, and pH) on antibiotic resistance in various food-related pathogens, McMahon et al. found sublethal levels of osmotic stress induced an increase in antibiotic resistance in *E. coli* (10). A separate finding by Rami et al. found that constitutive expression of the stationary phase alternative sigma factor RpoS in *E. coli* during the exponential phase partially suppressed drug sensitivity associated with an *acrAB* mutation that inactivates the major multidrug resistance pump (12). This suggests that RpoS might significantly contribute to multidrug resistance, underscoring yet another role for this important stress-related transcription factor. Thus, we postulate that there may be a direct link between environmental stressors, RpoS expression, and antibiotic resistance. In this study, we investigated whether the increased antibiotic resistance seen in *E. coli* after osmotic shock is an effect of cross-protection by the general stress response regulator RpoS. We used a Δ*rpoS* strain (JW5437-1) and its parent strain (BW25113) to investigate whether RpoS upregulation as triggered by exposure to osmotic stress increases antibiotic resistance in *E. coli*. Specifically, resistance

to three antibiotics was investigated. Chloramphenicol, ampicillin, and rifampicin are broad spectrum antibiotics that inhibit bacterial protein synthesis, cell wall synthesis, and bacterial transcription, respectively (1). These antibiotics were chosen for their clinical importance and to allow for more insight on the mechanistic details of resistance if a cross-protective correlation were to be found.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *E. coli* substrain BW25113 (WT positive control) was obtained from the University of British Columbia, Department of Microbiology and Immunology MICB 421 culture collection. Substrain JW5437-1 (*ΔrpoS746::kan*) was obtained from the Coli Genetic Stock Center, Yale University. JW5437-1 contains a deletion mutation in the *rpoS* gene and both strains are *F-, Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), LAM-, rph-1, Δ(rhaD-rhaB)568, hsdR514*.

Growth conditions and induction of stress. Cells were grown in M9 minimal media (0.4% glycerol, 0.5% Bacto Tryptone, and 0.25% Bacto Yeast Extract) at 37°C shaken at 200 rpm. Cells were seeded from overnight culture at a dilution of 1/16, and after 4 hours, re-seeded again at a dilution of 3/25. After 45 min (OD_{460} ~0.3), WT and Δ*rpoS* cultures were divided in two aliquots, to one of which an osmotic upshift to a concentration of 0.3 M NaCl was done.

Determination of minimum inhibitory concentration. After 120 min of growth in either normal conditions or osmotically upshifted conditions, 75 µl of each bacterial sample was added in duplicate to serially diluted ampicillin, chloramphenicol, and rifampicin in 96 well plates to a total volume of 150 µl per well and a final antibiotic concentration of (0, 0.25, 0.5, 1, 2, 4, 8, 16, 32, 64, and 128 µg/ml). Plates were incubated at 37°C shaking mildly overnight. Minimum inhibitory concentration of each antibiotic was determined by eye the next day as the minimum concentration of antibiotic in which no visible growth is observed.

Cell lysis. At 0, 15, 30, 120 min and overnight growth, 10 ml of each sample was centrifuged at 7,500 x g in a J2-21 centrifuge (Beckman) at room temperature for 10 min. Cells were washed with 10 ml Tris buffer (10 mM, pH 8) and re-centrifuged before re-suspending cell pellets in 1 ml Tris buffer for sonication. Lysis was then performed by pulse sonification with a Microson Ultrasonic Cell Disruptor (Misonix, Farmingdale, NY) for 2 min. Lysates were microfuged at 14,000 x g in a 5415D centrifuge (Eppendorf, Germany) at 4°C and stored at -20°C for further use.

Total protein assay. Lysates were analyzed for protein concentration by the Bradford assay as previously described (3). Chicken egg albumin was used as a protein standard.

SDS-PAGE. Lysates were normalized for protein content according to results from the Bradford assay and equal amounts of proteins were combined with NuPage 4X sample buffer (Invitrogen). Samples were then heated to 90°C for 3 min and microfuged at 14, 000 x g at room temperature for 10 s. Samples were loaded onto a 12% SDS-polyacrylamide gel (Bio-Rad) and run vertically in running buffer (25 mM Tris base pH 6.8, 192 mM glycine, 1% SDS) at 120 V in a Bio-Rad PAGE apparatus for 60 min on ice.

Western blotting and immunodetection of RpoS. Following SDS-PAGE, proteins were blotted onto a Hybond™-P membrane (Amersham Biosciences) in transfer buffer (25 mM Tris base, 192 mM glycine, 20% methanol) and run at 100 V in a chilled Bio-Rad electroblotting apparatus. Membranes were washed 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 (Roche, cat. # 11 921 673 001) for 1 hr. Following blocking, membrane was hybridized with a primary mouse anti-RpoS antibody (Neoclone Biotechnology, cat. # W0009) diluted 1/1000 in Western blocking reagent for 1 hr at room temperature on a shaking apparatus. Following three additional washes with TBS-Tween 20, hybridization with a secondary alkaline peroxidise-linked goat anti-mouse Ig (Chemicon International, cat. # AP124A) diluted 1/3000 in Western blocking reagent was performed for 1 hr. The membrane was washed three more times with TBS-Tween 20 and twice with substrate buffer (100 mM Tris pH 9.5, 100 mM NaCl, 50 mM $MgCl_2$) before the addition of BCIP/NBT substrate (Sigma-Aldrich cat. # B3804) for colorogenic detection of RpoS.

RESULTS

Effects of RpoS and osmotic shock on antibiotic resistance. *E. coli* strains BW25113 WT and JW5437-1 Δ*rpoS* were grown in serially diluted concentrations of ampicillin, chloramphenicol, and rifampicin in minimal growth (M9 media) and osmotic shock conditions (M9 + 0.3 M NaCl). As shown in Table 1, the effects of RpoS on antibiotic resistance vary depending upon the antibiotic used.

Under normal growth conditions, the Δ*rpoS* strain was much more sensitive to ampicillin, exhibiting a two-fold decrease in the MIC compared to the WT strain. The application of an osmotic shock condition also increased ampicillin sensitivity of the WT strain and Δ*rpoS* strain, resulting in a four-fold and two-fold decrease in MIC respectively. It was also observed that both Δ*rpoS* and WT strains displayed an equal degree of antibiotic sensitivity when exposed to osmotic shock (MIC=4.0 µg/ml).

Of the three antibiotics tested, *E. coli* displayed the least amount of sensitivity to chloramphenicol. Under normal growth conditions, the WT strain did not exhibit measurable growth inhibition at the antibiotic concentrations tested. Upon exposure to osmotic shock, bacteria exhibited an increase in sensitivity, with an observed 128 µg/ml MIC. The Δ*rpoS* strain displayed a very different pattern of inhibition; exposure to osmotic shock appears to have increased the resistance of Δ*rpoS* strain to chloramphenicol. The Δ*rpoS* strain grown in M9 minimal media showed a decrease in antibiotic resistance compared to the WT.

TABLE 1. The effect of osmotic shock by 0.3 M NaCl in minimal media on antibiotic resistance in WT and Δ*rpoS E. coli* strains.

Strain/Culture Condition	Ampicillin MIC (µg /ml)	Chloramphenicol MIC (µg/ml)	Rifampicin MIC (µg /ml)
BW25113 WT Minimal M9	16.0	>128.0	64.0
BW25113 WT Osmotic shock	4.0	128.0	8.0
JW5437-1Δ*rpoS* Minimal M9	8.0	96.0	32.0
JW5437-1Δ*rpoS* Osmotic shock	4.0	128.0	16.0

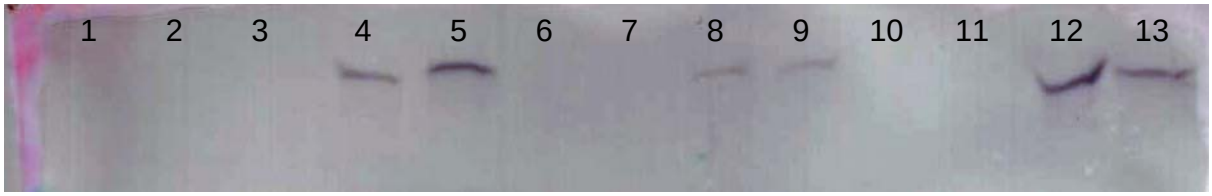

FIG. 1. Detection of RpoS protein from *E. coli* JW5437-1 Δ*rpoS* and BW25113 WT lysates by Western immunoblotting (12% SDS-PAGE). 1: Ladder (Invitrogen MultiMark Multicoloured standard), 2: 0 min JW5437-1 Δ*rpoS* M9, 3: 0 min JW5437-1 Δ*rpoS* 0.3 M NaCl, 4: 0 min BW25113 WT M9, 5: 0 min BW25113 WT 0.3 M NaCl, 6: 15 min JW5437-1 Δ*rpoS* M9, 7: 15 min JW5437-1 Δ*rpoS* 0.3 M NaCl, 8: 15 min BW25113 WT M9, 9: 15 min BW25113 WT 0.3 M NaCl, 10: 30 min JW5437-1 Δ*rpoS* M9, 11: 30 min JW5437-1 Δ*rpoS* 0.3 M NaCl, 12: 30 min BW25113 WT M9, 13: 30 min BW25113 WT 0.3 M NaCl.

However, under osmotic shock conditions, both strains showed the same level of resistance.

Experiments done using rifampicin yielded results similar to those found in ampicillin experiments. When exposed to rifampicin, Δ*rpoS* strains had a two-fold decrease in MIC compared to the WT strain under normal growth conditions. Osmotic shock increased the sensitivity of both WT and Δ*rpoS* strains to rifampicin by eight-fold and two-fold respectively. Interestingly, when exposed to osmotic shock, Δ*rpoS* strains appear to have greater resistance to rifampicin than bacteria from the WT strain.

Verification of Δ*rpoS* mutant *E. coli*. In order to verify that the Δ*rpoS* strain of *E. coli* was actually negative for RpoS, western blot experiments were performed (FIG. 1). Results from the western blot confirm that *E. coli* strain JW5437-1 Δ*rpoS* was indeed a Δ*rpoS* strain (lanes 2, 3, 6, 7, 10, 11), and that BW25113 WT express RpoS protein in detectable quantities (lanes 4, 5, 8, 9, 12, 13). Initially, the purpose of the western immunoblots was to characterize the shifts in RpoS protein levels upon osmotic shock. Ponceau staining of the nitrocellulose membrane was performed in an attempt to visualize RNA polymerase as a loading control. However, low protein concentration and poor resolution of staining prevented individual bands from being identified; as even protein loading could not be verified, it is not possible to determine whether or not RpoS protein levels changed between treatments.

DISCUSSION

One of the major limitations to our study was that we were unable to determine whether RpoS was upregulated after being subjected to the 0.3 M NaCl osmotic shock conditions. Although the Bradford assay was used to normalize the amount of protein loaded, the poor resolution of the ponceau staining and the immunodetection along with the inability to load sample replicates (we wanted to have all samples on one gel for valid comparison) did not allow us to accurately assess the amount of RpoS produced. One additional limition is that the biological fitness of the two strains was not empirically assessed.

Additionally, we did not feel that the MIC results for chloramphenicol were valid. The MIC for the WT strain in M9 media exceeded our maximum range of 128 µg/ml and even the lowest MIC was questionably high at 96 µg/ml for the Δ*rpoS* strain in the osmotic shock condition. Literature value for the MIC of chloramphenicol for *E. coli* is 5.3 µg/ml, 24-fold less than the MICs seen in our assay (4). Both the WT and the Δ*rpoS* strains do not contain any chloramphenicol resistance genes and thus resistance should not be seen to as high as 128 µg/ml. Thus it is likely that the chloramphenicol used was at least partially inactive or that a dilution error may have occurred. For future discussions, the results from the chloramphenicol condition will not be considered. The literature MIC values for ampicillin (2-4 µg/ml) and rifampicin (16 µg/ml) are closer to our determined MIC values (Table 1), although our values were still a little higher (2). This discrepancy may arise from slight differences in strains, incubation conditions/media and/or suboptimal functioning of the antibiotic; the values, however, were close enough to literature values to accept validity. An additional explanation for this discrepancy may be due to the high initial inoculum used; high inoculums can result in significantly higher MICs.

When comparing the sensitivity of *E. coli* to antibiotics between normal M9 minimal media and continuous osmotic shock of 0.3 M NaCl conditions, both Δ*rpoS* and WT strains showed an increased susceptibility to the antibiotics (ampicillin and rifampicin) under shock conditions. It has previously been shown that sublethal osmolarity conditions (defined by the authors as the salt concentration at which 95% of the bacterial population is killed) increases antibiotic resistance in *Escherichia coli* (10). Brief shocks to environmental stressors such as temperature, pH, osmolarity and detergents have also been shown to increase resistance to subsequent challenge against both related and unrelated stressors

such as antibiotics due to the activation of the same set of protective genes by global regulators (11, 7, 8). The conflict of our result with literature may be due to the nature of the stress exposure. In contrast to the previous experiments, our initial osmotic shock was not at sublethal levels and the presence of the shock was sustained. The continuous exposure to the dual shocks may have overloaded the coping mechanisms of the cells. In hindsight, we recognize that the challenge of growing under both antibiotic and osmotic shock simultaneously is different from that of growth with antibiotics after prior exposure to an osmotic shock. Under osmotic shock, bacteria must make compatible solutes, osmoprotectants and various proteins in order to ensure proper turgor pressure of the cell (5). In the case where this stress is removed prior to the addition of a second stressor (antibiotic exposure), the cells do not have to use resources to produce the molecules and proteins for sustained osmotic regulation, therefore cells can direct resources to growth and survival in antibiotics. In the case of sublethal conditions, completely different mechanisms may be at work in which the resistance to antibiotics observed may have risen from the selection for hypermutability (10). Such populations are likely to have increased resistance when faced with other environmental stressors as well.

While the presence of RpoS did not confer increased antibiotic resistance under continuous osmotic shock, it did under normal M9 conditions. This observation indicates that RpoS is involved with antibiotic resistance to some degree. When grown in normal M9 media, the WT strain exhibited two-fold and 1.3-fold increases in MIC values when compared to the Δ*rpoS* strain in ampicillin and rifampicin, respectively. This shows that even basal levels of RpoS contribute to antibiotic resistance. One reason for the resistance may be due to the expression of efflux pumps. The enhanced expression of the broad-specificity AcrAB efflux pump is known to increase resistance to environmental stressors such as some antibiotics and salts (14). Since RpoS is a sigma factor that can turn on 50-100 genes involved in multiple stress resistance, it may have an effect on *acrAB* expression (12). For ampicillin in particular, RpoS may also play a role in the susceptibility of penicillin binding proteins (PBPs) to the effects of beta-lactam antibiotics (6). In a study, it was found that wild type *E. coli* showed an increase of PBP 6 but a decrease in PBP 3 as the cells went into stationary phase, but this result was not present in an Δ*rpoS* strain, which resulted in cells that did not transition into shorter rods (6). The study also found that WT strains had an increased resistance to ampicillin compared to Δ*rpoS* strains which they proposed to be an effect of the change in PBP expression patterns (6). Therefore it is possible that the cells in our MIC assay showed the same difference in PBP expression which affected ampicillin sensitivity. This additional antibiotic specific mechanism may explain why the conferred resistance was greater in ampicillin than in rifampicin. For both antibiotic cases, the difference in MIC between normal and shock treatments was more pronounced in the WT strain than in the Δ*rpoS* strain, corresponding to a greater drop down to baseline level (in which no observable resistance was conferred under shock conditions). This observation is in line with the idea that RpoS has some effect on antibiotic resistance. One oddity to note is that the MIC for rifampicin was higher in the Δ*rpoS* strain than in the WT strain (Table 1). While the difference in concentration is two-fold, the values of the concentrations themselves are quite low, making the reliability of the difference questionable.

The observation that an osmotic shock of 0.3 M NaCl did not confer greater resistance of the WT compared to the Δ*rpoS* strain suggests that osmotic shock does not appear to offer cross-protection towards antibiotics through an RpoS-dependent mechanism. However, there is the possibility that the concentration of NaCl used was not sufficient to upregulate RpoS expression beyond basal levels to produce the hypothesized effect of cross-protection, especially under continuous exposure to both stressors. While one study found that an osmotic shock of 0.3 M NaCl was capable of inducing an RpoS up-regulation (11), another study found that a shock of 0.8 M NaCl was still insufficient to induce up-regulation (9). Since the loaded protein amounts for our western blot could not be verified, we were unable to confirm whether RpoS was upregulated. In the study where osmotic stress did confer antibiotic resistance, a sublethal osmotic shock of 4.5% (wt/vol) was determined and used (10). While a shock of 0.3 M NaCl was not enough to stimulate RpoS to confer cross-protection, perhaps a higher concentration would have been able to show cross-protection for the RpoS WT strain. However, the possibility that the increased antibiotic resistance seen under sublethal osmotic shock may be a result of stress-induced genetic plasticity rather than the expression of shock proteins cannot be ignored (10).

Our results suggest that RpoS has some effect on antibiotic resistance and that there may be mechanistic differences between modes of resistance for different antibiotics. Contrary to our original hypothesis, however, we did not observe cross-protection against antibiotics with continuous osmotic shock via RpoS-dependent mechanisms. In fact, osmotic shock was seen to increase antibiotic susceptibility in both the Δ*rpoS* and WT strains. This lack of stress tolerance upon prior exposure to another stress, regardless of RpoS dependency, is inconsistent with known facts and we have proposed some ideas to explain the discrepancy. Further experimentation is required to

elucidate the validity of these explanations.

FUTURE EXPERIMENTS

Since RpoS is a general stress response regulator, it would be important to investigate whether the difference observed in antibiotic resistance between the WT and Δ*rpoS* strains under normal conditions is in fact due to some RpoS-dependent mechanism, or is due to a result of a decrease in general fitness of the Δ*rpoS* strain. Thus in a future experiment, it may be useful to compare the growth rate of WT and Δ*rpoS* bacteria in normal media. Furthermore, a different experiment could use heat shock rather than an osmotic shock to upregulate RpoS expression. Since a temperature shock could easily be removed, it would help in analyzing whether the presence of two continuous shocks was truly a confounding factor.

In our experiment, we were unable to definitively induce an increase in RpoS expression by osmotic shock. Thus, in order to further confirm the role of RpoS in conferring antibiotic resistance, the upregulation of RpoS can be induced through a plasmid construct containing RpoS under a *pBAD* promoter. This would allow the determination of whether upregulation of RpoS confers antibiotic resistance when compared to basal expression of RpoS in *E. coli* grown in normal media.

Two proposed mechanisms of the observed increase in antibiotic resistance of the WT strain grown in normal media are the effects of RpoS on the enhanced expression of the AcrAB efflux pumps (12) and the altered expression of penicillin binding protein subtypes (6). In order to elucidate whether RpoS confers antibiotic resistance through these mechanisms, the level of AcrAB efflux pumps and the different subtypes of penicillin binding proteins could be measured by Western blot or qRT-PCR in Δ*rpoS* and WT strains and correlated to the level of antibiotic resistance.

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