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Cross-Protection Against Tetracycline Conferred by Thermal Stress in *Escherichia coli* May be Independent of *rpo*S and *ots*A

Shivani Gounder, Yulia Merkulova, Kelsie Nixon, Tina Wang
Department Microbiology & Immunology, University of British Columbia

RpoS, a general stress response regulator, and trehalose had been implicated in helping the cells survive times of stress. Previous study had shown that when the cells were subjected to heat shock, they developed a more tetracycline-resistant state in a phenomenon known as cross-protection. While a role of RpoS has been suggested in the heat-induced cross-protection against tetracycline, the identity of the downstream effector(s) directly responsible for the phenomenon were not elucidated. We investigated if trehalose played a role in conferring tetracycline resistance and if *ots*A, one of the genes responsible for endogenous trehalose synthesis, was regulated by RpoS. Trehalose accumulation was assessed indirectly via *ots*A gene expression. The wild type, Δ*rpo*S, and Δ*ots*A strains were thermally stressed for various lengths of time. The cells were exposed to tetracycline of various concentrations to determine the resistance state of the cells. Expression of *rpo*S and *ots*A were quantified via qPCR. All three strains showed an increase in the level of resistance when exposed to heat shock for 20-30 minutes, while no distinct pattern of gene expression was observed. Our data indicates that *rpo*S and *ots*A expression may be independent and may not be involved in establishing heat-induced cross-protection against tetracycline.

Escherichia coli constantly modifies gene expression to adapt to the ever-changing environments and to survive the stress caused by stimuli such as temperature, pH, and osmolarity. They possess a DNA-depend RNA polymerase, a multi-subunit holoenzyme containing a core subunit that can couple to one of the seven sigma factors to regulate gene expression globally at the levels of transcription initiation. When the cells are stressed with an environmental stimulus such as heat, one of the stress response sigma factors, RpoS, generates a global stress response and allows for expression of various stress response genes to return the cells back to a state of homeostasis.

A reported RpoS-mediated phenomenon called cross-protection enables the bacteria to become resistant to other stressful treatments in addition to the stress they first encounter (1). Hui *et al* showed that in *E. coli*, when exposed to heat, elicited a response that also increased its resistance to the antibiotic tetracycline (2). This resistance to tetracycline was reported to be dependent on the presence of RpoS (2, 3). However, Hui *et al* also showed that the increase in tetracycline resistance was only observed in cells experiencing heat shock for less than an hour (2). An increase in RpoS expression as a result of prolonged heat shock did not augment but reversed and depressed the resistance state (2). This suggested that either another heat-induced sigma factor (RpoH or RpoE), or a downstream effector whose expression would more closely correlate to the state of resistance, was responsible for the heat-induced cross-protection. However, such effector remains to be identified.

Trehalose-6-phosphate synthase and trehalose-6-phosphate phosphatase, coded by *ots*A and *ots*B respectively, are required for the synthesis of trehalose, a disaccharide that protects proteins and cell membranes from disruption by stressors such as heat (4, 5, 6). Though trehalose can be sourced externally, only endogenously synthesized trehalose confers stress protection (7). Expression of *ots*AB operon is induced by RpoS after *E. coli* cells are exposed to heat shock (1) and this expression is necessary for RpoS-dependent thermo-tolerance during stationary phase (8, 9). Several methods through which trehalose may assist in preventing the accumulation of tetracycline in the cell have been suggested, including maintenance of the metal-tetracycline/H^+ antiporter and stabilization of the cell membrane (6, 10).

In this study, we investigated potential heat mediated increases in internal trehalose accumulation, as measured indirectly by *ots*A expression, occurred in an *rpo*S-dependent manner to confer cross-protection against tetracycline. Specifically, by using *rpo*S and *ots*A deletion mutants, we assessed the role of *rpo*S in conferring heat-induced cross-protection to tetracycline in *E. coli*, the necessity of *ots*A for the observed tetracycline resistance, and the dependence of *ots*A expression on *rpo*S.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *E. coli* BW25113 (wild type) and *E. coli* JW5437-1 (Δ*rpo*S) strains were obtained from the culture collection of the Microbiology and Immunology Department at the University of British Columbia. *E. coli* JW5312-3 (Δ*ots*A) obtained from the Coli Genetic Stock Center. Overnight cultures of each strain were grown in Luria-Bertani (LB), pH 7.0 (1.0% w/v tryptone, 0.5% w/v yeast extract, 0.5% w/v NaCl) at 37^o C at 150 rpm for 18-20 hours. A minimum OD_{460} of 1.5 was reached to ensure cells had reached the stationary phase so cells would be expressing *rpoS*.

Confirmation of Deletion Mutants. Genotype of deletion mutants was confirmed through colony PCR and subsequent restriction endonuclease digestion of PCR products. The following primers were used for *ots*A (forward: 5' -CTTTTGCGCTGCATTATCGT-3', reverse: 5'-CTATCGCCAGGCCAGTAAAG-3') and *rpo*S (forward: 5'-CCCATAACGACACAATGCTG-3', reverse:

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5'-TATCTGGGGTTGTCGGTAGC-3'). To minimize contamination of PCR with medium, colonies were lightly poked with a stab and diluted in 20 µl distilled water, incubated at 95°C for 5 minutes, chilled on ice and centrifuged at 4°C to pellet agar and debris. Supernatant was used as DNA template. Invitrogen AccuPrime DNA polymerase was used for amplification and reactions were set up according to the manufacturer's protocols. 1.25 µl of DMSO was included in each 25 µl PCR reaction to destabilize secondary structures. PCR cycling conditions were as follows: 94°C denaturation for 5 min; 34 cycles of 94°C denaturation for 30 s, 49°C annealing for 30 s, 68°C extension for 2 min; and a final extension at 68°C for 5 min. Due to similar PCR product sizes, *ots*A primer PCR products from the wild type and the Δ*ots*A strains were digested for 1 hour at 37°C with NcoI in NEBuffer 4 (New England BioLabs®), followed by 2% agarose gel electrophoresis to determine fragment sizes and identify the potential products. The amplicon from the Δ*ots*A strain contained a NcoI cleavage site while the wild type did not.

Heat Shock. Overnight cultures were divided into 5 ml aliquots and heat shocked at 45°C for the specified amount of time in a water bath shaking at 150 rpm. In trials 1 and 2, samples concluded their heat shock treatments simultaneously and were subsequently processed for plating on microtiter plates for the MIC assay. In Trials 3 and 4, all samples began heat shock treatments simultaneously, and individual samples were processed immediately following the allotted heat shock durations. This modification to the protocol was made to keep the time lag due to pipetting the samples constant, and to minimize potential physiological changes in the cells.

Minimum Inhibitory Concentration (MIC) Assay. Each heat shock culture sample was diluted 1:100 in LB medium. One hundred µl of each dilution was plated to a 96 well polystyrene microtiter plate with 100 µl of tetracycline with final concentrations of 8, 4, 2, 1, and 0.5 µg/ml. Each sample was plated in duplicate. MIC plates were incubated for 48 hours at 37° C at 150 rpm and growth was assessed visually. Visual assessment was conducted independently by each of the four team members and was done blind without prior knowledge of the identity of the samples. MIC data was analyzed by comparing the results of trials 1 and 2 together, and 3 and 4 together.

RNA Isolation and cDNA Synthesis. Following the heat shock, $6x10^8$ cells were suspended in TRIzol® Reagent (Invitrogen™), and total RNA was isolated following the manufacturer's protocol. RNA concentration was measured using NanoDrop spectrophotometer. A total of 1 µg of RNA template was used in each sample for cDNA synthesis. SuperScript® VILO™ cDNA Synthesis Kit (Invitrogen™) was used following the manufacturer's protocol. Negative control samples containing RNA but no reverse transcriptase (RT) were set up in parallel.

Analysis of Gene Expression. Gene expression of *rpo*S and *ots*A were analyzed through qPCR using the Sso Advanced™ SYBR® Green Supermix (Bio-Rad), following the manufacturer's protocol. The following primers were used for *rpo*S (forward: 5'-CCACGCAAGATGACGATATG-3', reverse: 5'-GAGGCCAATTTCACGACCTA-3'), *ots*A (forward: 5'-GGTGAAACAGGGAATGAGGA-3', reverse: 5'-AGAACGGCATTGGAGAATTG-3'), and the reference gene *gap*A (forward:5'-AGGTCTGATGACCACCGTTC-3', reverse: 5'-GGAACGCCATACCAGTCAGT-3'). Each reaction underwent a 3-minute denaturation and enzyme activation period at 95° C before 40 cycles of denaturing at 95°C for 10 seconds, annealing at 55°C for 30 seconds, and extension at 72°C for 30 seconds. Melt curve data was collected between 55°C and 95°C at 0.5°C increments. RNA controls without RT were run in parallel. Only trial 4 samples were used for gene expression analysis due to significant DNA contamination in RNA samples isolated from trials 1, 2, and 3. Data was analyzed by ΔΔCq method and expression of *rpo*S and *ots*A were normalized with respect to *gap*A. Amplification observed in the negative no-RT controls was subtracted from gene expression in samples. Standard deviation of technical replicates was propagated throughout the calculations to obtain 95% confidence intervals in gene expression values.

RESULTS

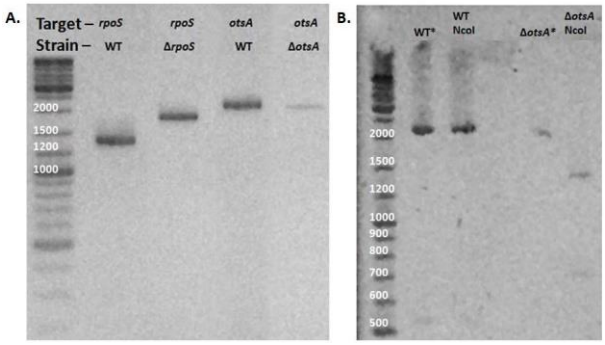

FIG 1A PCR amplicons of the *rpoS* and *otsA* loci, confirming the identify of both mutants. **B** Restriction enzyme digest of PCR amplicon spanning the *otsA* locus of the WT or Δ*otsA* mutant using NcoI. Undigested samples are identified with *. WT – wild type, *ΔrpoS*, *ΔotsA*.

PCR confirmation of deletion mutants. Deletions of the *rpo*S and of *ots*A were confirmed to ensure that any of the observations made in these strains were not due to incomplete deletion of the gene. For the Δ*rpo*S strain, a 1934 bp PCR product was expected, while a 1568 bp product was expected for the wild type. We observed a band at approximately 1500 bp for the wild type and 2000 bp for the Δ*rpo*S strain (Fig. 1A). For the Δ*ots*A strain, a 2109 bp product spanning the *ots*A gene was expected, and a 2288 bp product for the wild type strain. As expected, a slightly larger product was observed with the wild type strain compared to the Δ*ots*A strain (Fig. 1A). However, since PCR products around 2000 bp were expected for both strains (Fig. 1A), restriction endonuclease digest of the amplification product with NcoI was performed to confirm the deletion of *ots*A in the Δ*ots*A strain. While NcoI did not cleave the wild type PCR product, the

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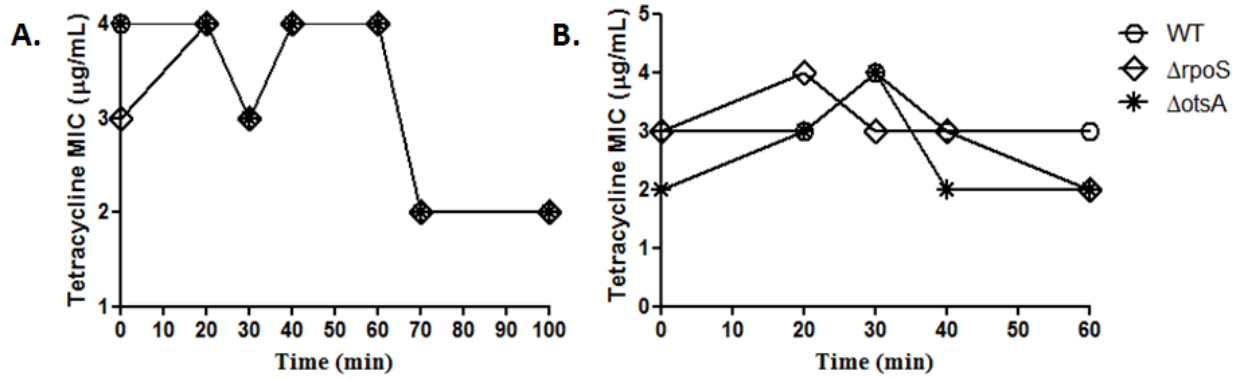

FIG 2 Reproducibility of the MIC assay for tetracycline at different durations of heat shock in four independent trials. **A** Trials 1 and 2. **B** Trials 3 and 4.

Δ*ots*APCR product was cleaved to give the expected 1300 bp and 700 bp (faint band) products, further verifying the deletion mutant (Fig. 1B).

Absence of *rpo*S and *ots*A did not compromise heat-induced cross-protection against tetracycline. MIC assays were performed to assess the degree of protection conferred against tetracycline in each of the three strains following heat shock treatment. Results of the MIC in trials 1 and 2 showed no correlation between the presence of *rpo*S or *ots*A and tetracycline resistance as all three strains followed a similar MIC pattern (Fig. 2A). All three strains showed similar level of resistance after 20 minutes of heat shock exposure. Consistent with previous studies (2), the MIC dropped, from 4 µg/ml to 2 µg/ml, after being thermally stressed for 60 minutes or longer. However, contrary to prior observations of an increase in resistance at around 30 minutes of heat shock treatment (2), all three strains experienced a decrease in resistance at 30 minutes. In addition, the Δ*rpoS* mutant was found to have a lower baseline resistance (3 µg/ml) than the wild type and the Δ*ots*A mutant strain, and to have experienced an increase in resistance from time 0 to 20.

In trials 3 and 4, samples were exposed to the heat shock treatment at the same time as trials 1 and 2 to minimize time lag between preparing the different samples for plating. A different pattern of MIC was observed as an increase in the level of resistance was observed in all three strains (Fig. 2B). As expected, in the wild type strain, the level of resistance peaked after 30 minutes of heat shock treatment, and showed an decrease when the cells were stressed for a longer period of time. Unexpectedly, in the Δ*ots*A mutant, the level of resistance also peaked at 30 minutes of exposure to heat shock. Similar to what we observed during trials 1 and 2, the Δ*rpo*S mutant started with a baseline resistance of 3 µg/ml, and the level of resistance peaked when the cells were thermally stressed for 20 minutes. In both cases with the deletion strains, the maximum level of protection conferred (4 µg/ml) matched that as observed with the wild type strain.

In all trials, the pattern of MIC depicting an increase followed by a decrease in the level of resistance was observed amongst all three strains. This suggested that absence of *rpo*S and *ots*A did not compromise the cells from being able to withstand the tetracycline challenge.

Inductions of *rpo*S and of *ots*A were not needed for heat-induced cross-protection against tetracycline. Because no difference in the level of conferred protection was found between the three strains, the expression state of *rpo*S and *ots*A following the heat shock treatment was examined by qRT-PCR. While heat shock treatment resulted in an induction of *rpo*S in the wild type strain, *rpo*S was down-regulated between 0 and 40 minutes in the Δ*ots*A strain (Fig. 3A). As expected, no *rpo*S transcript was detected in the Δ*rpo*S mutant (Fig. 3A). Conversely, *ots*A was down-regulated by two-fold in the wild type strain between 0 and 30 minutes (Fig. 3B). In the Δ*rpo*S mutant, no induction of *ots*A was observed (Fig. 3B). No *ots*A expression was observed in the Δ*ots*A mutant, as expected (Fig.3B). Since induction of *rpo*S was observed only in the wild type strain, the induction was unlikely to have played a significant role in conferring tetracycline resistance. As with what Hui *et al*. had observed (2), a steady increase in the expression of *rpo*S did not correlate with the MIC pattern. Since *ots*A was not induced in all three strains, it was unlikely that its expression played a role in conferring tetracycline resistance.

Dependency of *ots*A on *rpo*S remained to be elucidated. To assess if *ots*A expression is influenced by activation of *rpo*S, the gene expression of *ots*A in the Δ*rpo*S mutant was examined. No induction of *ots*A was observed (Fig. 3B), thus it remained inconclusive whether *ots*A expression depended on *rpo*S.

DISCUSSION

Contrary to our expectations, the lack of *ots*A induction in the wild type strain and the increase in the level of resistance in the Δ*ots*A mutant suggested that *ots*A does not play a role in conferring tetracycline resistance after exposure to heat shock treatment. In the wild type strain, the maximum level of tetracycline resistance was observed after being thermally stressed for 30 minutes; this corresponded with the lowest expression level of *ots*A. A decrease in the level of

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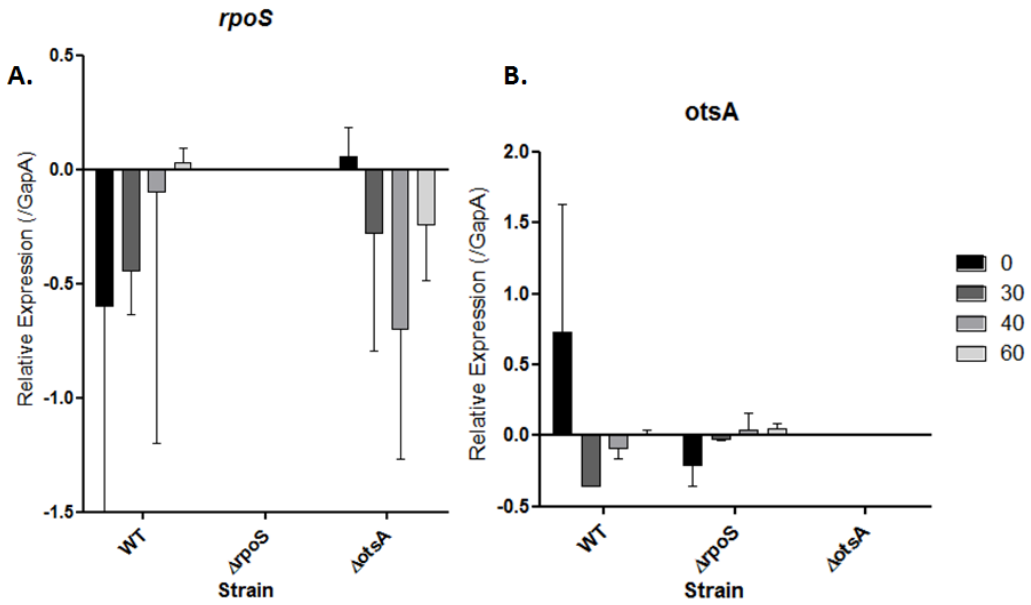

FIG 3 Effect of time on the relative expression of *rpo*S **(A)** and *ots*A **(B)** following heat shock. WT 0 min sample was set as reference and each sample was normalized by using *Gap*A, used as a housekeeping gene to give relative expression. Observed expression values were corrected to zero by subtracting the PCR expression in control samples without reverse transcriptase to account for genomic DNA contamination of RNA preparations. Error bars represent 95% confidence intervals. WT – wild type, *ΔrpoS* - JW5437-1, *ΔotsA* - JW5312-3.

resistance when thermally stressed for longer than 30 minutes corresponded with a slight increase in *ots*A expression relative to the expression at 30 minutes. In addition, the Δ*ots*A mutant exhibited an increase in the level of resistance when exposed to heat shock for 30 minutes, as with the wild type, and showed a similar level of resistance as the wild type, being able to tolerate 4 µg/ml of tetracycline, despite a lack of *ots*A expression. A similar increase in resistance paired with lack of *ots*A induction was also observed in the Δ*rpo*S mutant. This suggested that *ots*A expression was not necessary in conferring resistance to tetracycline.

However, the lack of *ots*A expression did not completely eliminate the possibility that trehalose conferred some level of protection against tetracycline. As trehalose synthesis can occur by two other pathways other than the *ots*AB pathway (5), it was still possible that trehalose accumulated. A direct assay for the presence of trehalose would be needed to verify its necessity. If trehalose did indeed accumulate with respect to the level of resistance, the biosynthesis pathway would be independent of the proposed *rpo*S-*ots*AB pathway. This would also suggest that heat-induced cross-protection was not dependent on the proposed *rpo*S-*ots*AB pathway. Although two other biosynthesis pathways have been documented such as those observed in *Mycobacterium smegmatis* and in the *Sulfolobus* genus, they are not well documented in *E. coli* (5).

With respect to *rpo*S, as with what Hui *et al.* had demonstrated, we observed a slight increase in the expression of *rpo*S when the wild type cells were subjected to heat shock for up to 60 minutes (2). This corresponded with a peak in the level of resistance at 30 minutes and a decrease thereafter, which also agreed with the data from Hui *et al* (2). However, the induction of *rpo*S, approximately half a fold increase, was slight in the wild type. It was unlikely that an overexpression of *rpo*S had an inhibitory effect on the downstream effector genes responsible for conferring tetracycline resistance, as previously suggested (2), since in the Δ*ots*A mutant strain where a similar MIC pattern as the wild type was observed, instead of an increase in *rpo*S expression, a decrease of *rpo*S expression was observed. Specifically, the lowest level of expression was observed with 40 minutes of heat shock treatment, where the observed level of resistance had already decreased from 4 to 2 µg/ml of tetracycline. In addition, Behmardi *et al.* demonstrated that overexpression of RpoS through transformation with a pBAD-*rpo*S was correlated with a two-fold increase in the level of tetracycline resistance (3).

Contrary to what Hui *et al.* had demonstrated (2), our data suggested that *rpo*S was unlikely to be involved in conferring tetracycline resistance. While Hui *et al.* observed a lack of increase in the level of tetracycline resistance in the Δ*rpo*S strain, we demonstrated that heat was capable of inducing a stress response in the Δ*rpo*S strain in a manner that was independent of *rpo*S to induce tetracycline resistance. This was shown with an increase in MIC from 3 to 4 µg/ml of tetracycline when exposed to heat for 20 minutes. Again, the maximal level of resistance, 4 µg/ml, was found to be comparable to the maximal level of resistance observed

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in the wild type strain. This suggested that other heat-induced regulators such as RpoE or RpoH may be involved in conveying heat-induced cross-protection (11, 12, 13).

It was possible that the timing played a role in the outcome of the MIC assay. Trials 1 and 2 were done following the protocol as outlined by Hui *et al.* but trials 3 and 4 were modified to stagger the plating of the samples to minimize time lag. Samples in Trials 1 and 2 were subjected to approximately an hour lag between the end of heat induction and plating on tetracycline. Overall, the peak in the level of tetracycline resistance when the cells were exposed to heat shock for 20-30 minutes was absent in trials 1 and 2. During the time lag, it was possible that the cells had cooled off slightly to have abrogated the protective effect of the stress response. Stress response sigma factors are under tight regulation to prevent one factor from dominating the RNA polymerase (1, 14). While RpoS protein levels had been observed to persist for 2 hours following heat shock during stationary phase (15), once the thermal stress had been removed, the expression level of the stress response sigma factors and the stress response were reversed back to the level of the unstressed cells (14).

Though Behmardi *et al*. concluded that RpoS was necessary in conferring tetracycline resistance, the cells in their study were not thermally stressed to elicit the possible stress response in the *E. coli* cells (3). RpoS may play a role in augmenting the level of tetracycline resistance, but not in the context of conveying heat-induced cross-protection against tetracycline. It should be pointed out that while Behmardi *et al*. observed an increase in the level of resistance when they overexpressed RpoS, the Δ*rpo*S mutant cells were not more sensitive to tetracycline than the wild type due to the absence of RpoS (3).

Though it had been suggested that *ots*AB expression was necessary for RpoS-dependent thermal tolerance (8, 9), it remained inconclusive whether *ots*AB was under the regulatory control of *rpo*S. Gene expression of *rpo*S and *ots*A in the wild type strain were examined. While an induction of *rpo*S was found between 0 and 30 minutes, a down-regulation of *ots*A was observed, though at time points 30 minutes and beyond, a slight induction of *ots*A was observed following the inductive trend of *rpo*S. In the Δ*rpo*S mutant, no induction of *ots*A was observed, suggesting that the absence of *rpo*S may still account for a lack of *ots*A expression as induction of *ots*A would have suggested that *ots*A expression was independent of *rpo*S.

In our study, we demonstrated that both the Δ*rpo*S and the Δ*ots*A strains were capable of increasing their level of tetracycline resistance following the heat shock treatment much like the wild type strain. The gene expression patterns of *rpo*S and *ots*A were found to poorly correlate to the resistance level observed. The relationship between *rpo*S and *ots*A remained inconclusive and may be independent of each other. In conclusion, *rpo*S and *ots*A were unlikely to be involved in conveying heat-induced cross-protection against tetracycline in *E. coli*.

FUTURE DIRECTIONS

Our MIC data shows no consistency or agreement with previous experiments and needs to be repeated. Furthermore, the lack of reproducibility limits the understanding of the effect on cross protection. The lag time between the end of heat shock and the time of plating seems to affect the MIC of all strains, and its effect needs to be investigated in future studies in order to limit the variation in patterns which subsequently limits interpretations of tests and correlations.

The results of qPCR lacked both significance and difference and need to be verified. In this study, RNA samples were not treated with DNAse to remove genomic DNA contamination which contributed to high background amplification in our qPCR and may have affected the observed trends. DNAse should be used in the future to avoid this technical error. Biological replicates should also be included to ensure reproducibility. In future experiments, a PCR control using isolated RNA should be run to see whether a PCR product is produced, indicating DNA contamination.

In the future, the role of other heat-induced sigma factors such as RpoH or RpoE in conferring cross-protection to tetracycline could be similarly investigated (11, 12, 13).

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