Journal of Experimental Microbiology and Immunology (JEMI) Vol. 20:7 – 11
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Crl and RpoS May Not Be Involved in Cross Protection against Tetracycline in *Escherichia coli* in Response to Heat Stress

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RpoS is the general regulator of stress response in *Escherichia coli* and is up-regulated under environmental stress conditions such as heat stress, low pH and starvation. Upon primary stress, *E. coli* develops increased resistance against a secondary unrelated stressor such as antibiotics. This phenomenon is termed cross protection. Crl, a transcription regulator, is involved during primary stress by working in concert with RpoS; however, it has yet to be investigated whether Crl is necessary during cross protection. In this study, the role of RpoS and Crl were investigated by subjecting wild-type (WT) *E. coli* strain BW25113, Δ*rpoS* strain JW5437-1, and Δ*crl* strain JW0230-1 to different durations of heat stress at 45°C. Following heat stress, resistance against tetracycline was determined using the minimum inhibitory concentration (MIC) assay. We could not detect an increase in tetracycline resistance following heat shock in the WT strain. In addition, the results of the MIC showed no difference in tetracycline resistance between the WT and knockout strains after increasing heat stress. This suggests that Crl and RpoS may not be involved in cross protection against tetracycline in response to prolonged primary heat stress.

Cross protection is a phenomenon observed in *Escherichia coli* where a primary stressor induces enhanced resistance to a secondary unrelated stressor. Chung *et al*. summarized a number of observations in which cells that have undergone primary environmental stress, such as heat, acid and starvation, become more resistant to a secondary stressor (1). Of particular interest is the induction of enhanced antibiotic resistance post-primary stress and its implications in *E. coli* infections with increased ability to resist antibiotic treatment.

RpoS, a stationary-phase alternative sigma factor, is a general regulator of primary stress response in *E. coli* and mediates the cross protection pathway. In the presence of an environmental stressor, RpoS is upregulated and controls the expression of stress-resistance genes, such as membrane proteins, efflux pumps, and nutrient scavenging proteins (2). RpoS expression increases after 30 minutes of heat stress and protein levels are maintained via a decrease in proteolysis of RpoS (3, 4). Accumulation of RpoS therefore prepares the cell for secondary stresses.

The cross protection relationship between tetracycline resistance and primary heat stress has been studied with conflicting results on whether this phenomenon actually occurs, and whether RpoS plays a central role (3, 5). Discrepancies in results may be due to other proteins that play a role in the cross protection pathway and were not controlled for. A potential candidate is Crl, a transcription regulator also known to upregulate genes related to stress response. Furthermore, in the presence of a primary environmental stressor, Crl binds and sequesters RpoS in the assembly of the RNA polymerase complex, favoring the use of RpoS over other sigma factors in driving transcription (6, 7). However, little is known about the role of Crl in the secondary stress response pathway and in conferring cross protection.

To elucidate the roles of Crl and RpoS in cross protection against tetracycline in response to prolonged primary heat stress, this study investigated the degree of tetracycline resistance in response to various durations (0-90 minutes) of heat stress in wild-type (WT), Δ*rpoS* and Δ*crl* strains of *E. coli*. We hypothesized that Crl is required in RpoS-mediated cross protection against tetracycline in response to prolonged (0-90 minutes) heat stress, and therefore without Crl, tetracycline resistance will decrease compared to the WT. We showed that in the presence of primary heat stress, the WT strain did not exhibit increased tetracycline resistance. In addition, both Δ*crl* and Δ*rpoS* strains were equally resistant to tetracycline compared to the WT, suggesting that Crl and RpoS may not play a central role in cross protection against tetracycline in response to prolonged heat stress.

MATERIALS AND METHODS

Bacterial strains and growth conditions. *Escherichia coli* K-12 strain BW25113 (wild-type control) was obtained from the MICB 421 bacterial culture collection in the Department of Microbiology and Immunology, University of British Columbia. The *E. coli* JW5437-1 (*ΔrpoS746::kan*) and JW0230-1 (*Δcrl-758::kan*) strains were obtained from the Coli Genetic Stock Centre (CGSC), Yale University. The genotype for all the strains are *Δ(araD-araB)567*, *ΔlacZ4787*(::rrnB-3), λ^-, *rph-1*, *Δ(rhaD-rhaB)568*, *hsdR514*. JW5437-1 strain is an *rpoS* deletion mutant and JW0230-1 is a *crl* deletion mutant. All strains were cultured in Lysogeny Broth (LB) (1% (w/v) tryptone (Fisher, Cat. #B211705), 0.5% (w/v) yeast extract (Fisher, Cat. #BP1422-500), and 1% (w/v) NaCl (Fisher, Cat. #BP358-1), pH 7.0. Overnight cultures of each strain were grown at 37°C with shaking at 150 rpm to reach stationary phase with an OD_{600} greater than 1.5.

Design of primer sets for PCR. Primers specific for the amplification of *rpoS* and *crl* were designed to flank the *rpoS* and *crl* genes, and bind beyond the sequences targeted by the primers used to generate the knockouts, based on the strain comments listed in the Yale CGSC strain report. The sequences for the *rpoS* primers are: 5'-TCGCTTGAGACTGGCCTTTCTG-3' (forward) and 5'-CGGAACCAGGCTTTTGCTTGAATG-3' (reverse). The sequences for the *crl* primers are: 5'-GACTGGATCGAAAAACGCTTGTG-3' (forward) and 5'-

ATGAATATTGCCGGATGTGATG-3' (reverse). These primers were ordered from Integrated DNA Technologies (IDT).\

Colony PCR to confirm knockout of *rpoS* and *crl* in *ΔrpoS* and *Δcrl* strains. Each reaction mixture consisted of 1X PCR buffer (Invitrogen, Cat. #10966-034), 1.5 mM $MgCl_2$ (Invitrogen, Cat. #10966-034), 0.2 mM dNTP mix (Invitrogen, Cat. #18427-013), 200 nM each of forward and reverse primer, 0.2 µl of Platinum *Taq* DNA Polymerase (Invitrogen, Cat. #10966-034), and made up to a final volume of 50 µl using sterile distilled water. Template DNA was added by transferring cells from an isolated colony on the plate culture directly into the 50 µl reaction mixture using a sterile pipette tip. Reactions were incubated in the Gene Cycler thermal cycler (Bio-Rad, Cat. #170-6700) at 94°C for 2 min, followed by 30 cycles of amplification: 94°C for 30 seconds, 60°C for 45 seconds, and 68°C for 1 min. The final extension was incubated at 68°C for 7 min. The PCR products were analyzed through agarose gel electrophoresis.

Gradient colony PCR to confirm knockout of *crl* in *Δcrl* strain. Reaction composition was prepared as described for colony PCR but in quintuplicate per strain. TGradient 96 thermal cycler (Whatman Biometra, Cat. #050-801) program was as described above for colony PCR but with the change of the 60°C annealing temperature to a gradient of annealing temperatures: 48.6°C, 52.2°C, 55.8°C, 59.3°C and 62.9°C.

Gel electrophoresis of PCR products. A 1.2% (w/v) gel was made by dissolving UltraPure agarose (Invitrogen, Cat. #10821-015) in SYBR Safe DNA Gel Stain in 0.5X TBE (Invitrogen, Cat. #S33100). 6X DNA loading dye (Thermo Scientific, Cat. #R0621) was added to PCR products prior to loading. The 100 bp DNA ladder (Invitrogen, Cat. #1526-050) was loaded along with the samples. The gel was run at 100 V for 90 min in 1X TBE buffer (89 mM Tris base (Fisher BioReagents, Cat. #BP1521), 89 mM boric acid (Fisher Chemicals, Cat. #A73-1), 2 mM EDTA (Fisher BioReagents, Cat. #BP2482-1), pH 8.0) and subsequently imaged using an Alpha Innotech AlphaImager.

Heat induction. After growing to stationary phase, the cultures were divided into 5 ml portions and induced for 0, 30, 60 and 90 min at 45°C in a water bath with shaking at approximately 150 rpm. Cultures with longer induction times were placed into the water bath before cultures with shorter induction times such that all samples were processed simultaneously.

Minimum inhibitory concentration (MIC) assay. The minimum inhibitory concentration (MIC) of tetracycline (Sigma, Cat. #T-3383) was determined by adding 100 µl of each heat induced culture to 100 µl of tetracycline diluted in LB in 96-well flat-bottom polypropylene plates (Costar, Cat. #3364). Final concentrations of tetracycline were 16, 8, 4, 2, 1, 0.5, 0.25 and 0.125 µg/ml. Cultures from each heat stress time point were diluted 1 in 10,000 in LB and plated in 2 sets of duplicates. Both sets of plates were incubated overnight – one at 37°C and the other at 42°C.

RESULTS

Confirmation of *rpoS* knockout in JW5347-1 (Δ*rpoS*). To determine if *rpoS* was knocked out in JW5347-1, colony PCR was performed to confirm the absence of *rpoS* in the Δ*rpoS* strain, and presence of *rpoS* in the WT and Δ*crl* strains. A primer set designed to bind to the regions flanking *rpoS* was used for amplification. Colony PCR products from the WT strain served as a positive control for strain phenotype, demonstrating the presence of a 1084 bp band corresponding to the expected size of *rpoS* (Fig. 1, lane 1). A 1084 bp band corresponding to *rpoS* was observed in the Δ*crl* strain (Fig. 1, lane 1). A ~1.4 kb band corresponding to

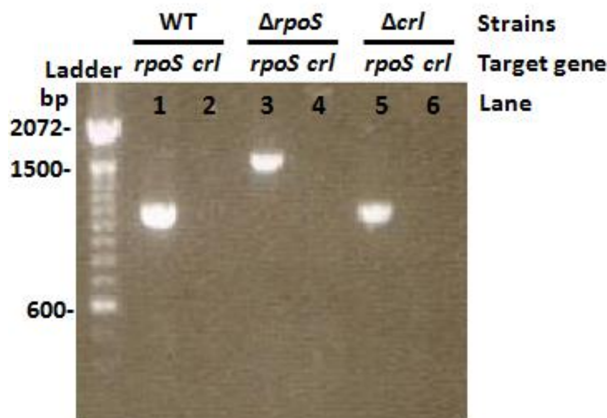

FIG 1 Agarose gel electrophoresis of colony PCR products confirming knockout of *rpoS* in Δ*rpoS* strain.

the *kan* cassette was observed in the Δ*rpoS* strain (Fig. 1, lane 3), confirming knockout of *rpoS* in the Δ*rpoS* strain.

Confirmation of *crl* knockout in JW0230-1 (Δ*crl*). To determine if *crl* was knocked out in JW0230-1, gradient colony PCR was performed to confirm the absence of *crl* in the Δ*crl* strain, and presence of *crl* in the WT and Δ*rpoS* strains. A primer set designed to bind to the regions flanking *crl* was used for amplification. The following annealing temperatures were tested for each strain: 48.6°C, 52.2°C, 55.8°C, 59.3°C and 62.9°C. Colony PCR products obtained from a single WT strain colony using primer sets designed for *rpoS* amplification served as a positive control (Fig. 2, lane 16). A colony PCR reaction identical to the positive control, less the WT colony to serve as template DNA, served as a negative control (Fig. 2, lane 17). The optimal annealing temperature of the *crl* primer set was found to be 62.9°C. A 525 bp band corresponding to *crl* was observed in the WT and Δ*rpoS* strains (Fig. 2, lane 5 and 10). A ~1.4 kb band corresponding to the *kan* cassette was observed in the Δ*crl* strain (Fig. 2, lane 15), confirming knockout of *crl* in the Δ*crl* strain.

No significant difference in MIC between WT, Δ*rpoS* and Δ*crl* strains in response to different durations of heat stress. To determine whether Crl is involved in RpoS-mediated upregulation of tetracycline resistance genes in response to prolonged heat stress, WT, Δ*rpoS*, and Δ*crl* strains were subjected to primary heat stress and their sensitivity to tetracycline was measured through the MIC assay. Cultures were exposed to heat stress (45°C) for 90, 60 or 30 min in an attempt to upregulate tetracycline resistance genes. Cultures were then plated over a range of tetracycline concentrations and incubated overnight at 37°C and 42°C. The MIC was taken as the lowest concentration of tetracycline that resulted in inhibition of growth, indicated by the absence of visible turbidity.

The following within-plate assay controls were implemented: cultures were plated in a final concentration of 16 µg/ml tetracycline to serve as a positive control for inhibition of growth; cultures were plated in LB to serve as a negative control for inhibition of growth; wells containing

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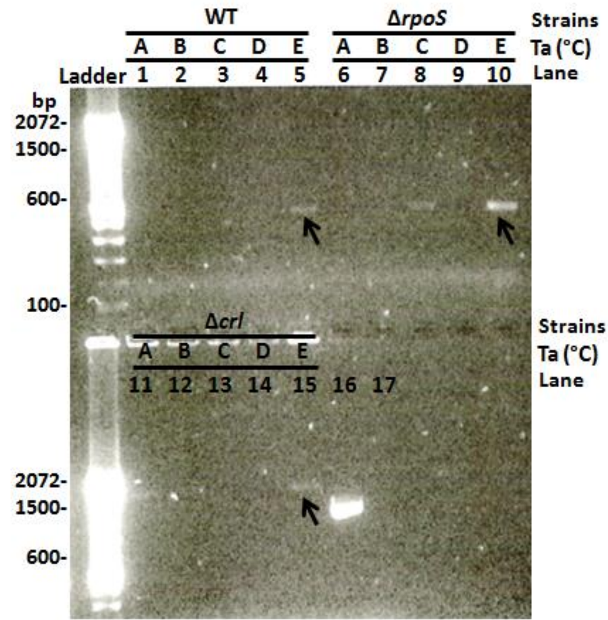

FIG 2. Agarose gel electrophoresis of gradient colony PCR products confirming knockout of *crl* in Δ*crl* strain. The following primer annealing temperatures (Ta) (°C) were tested: A = 48.6, B = 52.2, C = 55.8, D = 59.3 and E = 62.9. Primer set designed for *crl* amplification was used in lanes 1-15. Lane 16 contains colony PCR products obtained from a single WT strain colony using primer sets designed for *rpoS* amplification as a positive control. Lane 17 contains identical components as lane 16, less the WT colony to serve as template DNA, serving as a negative control.

LB alone served as additional negative controls and sterility controls. The WT strain served as a phenotype control for comparison when analyzing MIC data obtained from the Δ*rpoS* and Δ*crl* strains. Cultures that were not exposed to heat stress (time = 0 min) served as a control for MIC comparisons with increasing heat stress duration. The set of plates incubated at 37°C served as a control to the set of plates incubated at 42°C to determine whether prolonged heat stress at 42°C resulted in a difference in MIC.

Baseline tetracycline resistance (Table 1, time = 0 min) of all strains was 4 μg/ml, with the exception of the Δ*crl* strain, which had a baseline resistance of 2 μg/ml. In all strains, tetracycline resistance after heat stress (time = 30, 60, 90 min, per strain) was unchanged compared to baseline resistance. Moreover, there was no significant difference in the MIC of tetracycline between plates incubated at 37°C and 42°C overnight for 18 hours (Table 1). Taken together, these results suggest that different durations (0-90 min) of heat stress do not affect MIC of tetracycline within any particular strain. As well, the presence of RpoS and Crl in the WT strain does not contribute to increased tetracycline resistance. Finally, the presence of prolonged heat stress during the overnight incubation does not affect the ability of the strain to confer tetracycline resistance.

Table 1: Tetracycline minimum inhibitory concentrations of the WT, Δ*rpoS*, and Δ*crl* strains after exposure to varying heat stress durations and incubation conditions.

Heat shock duration (min)	MIC concentration (μg/ml)					
	WT		Δ*rpoS*		Δ*crl*	
	37°C	42°C	37°C	42°C	37°C	42°C
0	4	4	4	4	4	2
30	4	4	4	4	4	2
60	4	4	4	4	4	2
90	4	4	4	4	4	2

DISCUSSION

Based on the observation that RpoS mediates heat stress-induced cross protection, we hypothesized that the presence of RpoS and Crl contributes to cross protection of *E. coli* against tetracycline in the presence of prolonged primary heat stress, whereas the absence of the two proteins would inhibit the ability to do so (1). The data we collected, however, suggest that the WT strain does not demonstrate increased resistance to tetracycline after primary heat stress, and RpoS and Crl may not play a central role in increasing resistance against tetracycline after primary heat stress.

Tetracycline MIC assays were performed on three different strains of *E. coli* to test for the presence of cross protection. Because MIC is a qualitative interpretation, there is a degree of error associated with interpretation of the results. The determined MIC is given an uncertainty of +/- 1 well, where it is possible the true MIC lies in the neighbouring well to each side of the well taken as the determined MIC. The determined MICs for all strains, across all conditions tested fell in the range of 2 to 4 μg/ml tetracycline. Because wells containing 2 and 4 μg/ml tetracycline are neighbouring wells in the assay plate, we do not consider the MICs of 2 and 4 μg/ml to be notably different.

Based on this interpretation, we observed no differences in the degree of tetracycline resistance across the WT, Δ*rpoS* and Δ*crl* strains in the stationary phase, after different durations of heat stress (Table 1). The first conclusion we can draw from these results is that duration of primary heat stress does not affect tetracycline resistance in the strains tested. Because heat stress is known to induce the production and accumulation of RpoS, the WT strain was expected to have increased levels of RpoS, which would increase expression of tetracycline resistance genes over longer durations of heat stress (4). This should result in decreased susceptibility to tetracycline over time, yielding a higher MIC. However, our results show that the strains were equally resistant to tetracycline whether or not they were heat stressed. This suggests that cross

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protection against tetracycline is not a response to primary heat stress in the strains tested.

To assess the role of RpoS and Crl in the cross protection pathway linking secondary tetracycline resistance to primary heat stress, we compared tetracycline sensitivity of RpoS and Crl knockout strains to the WT strains using the MIC assay. If RpoS and Crl were involved in the upregulation of tetracycline resistance genes in response to primary heat stress, Δ*crl* and Δ*rpoS* strains would be expected to show increased susceptibility to tetracycline (lower MIC) compared to the WT strain. However, our results show that Δ*crl* and Δ*rpoS* strains showed similar resistance towards tetracycline compared to the WT strain, regardless of whether the strains had been subjected to heat shock or not. This suggests that RpoS and Crl may not play central roles in the cross protection against tetracycline in response to prolonged primary heat stress.

To control for the possibility that cells no longer in the presence of heat stress may revert to their pre-stress phenotype, we incubated identical sets of plates overnight at 37°C and at 42°C, so that the latter set was under prolonged heat stress. No notable differences in MIC were observed between all strains incubated at 37°C compared to 42°C, suggesting that cell phenotype reversion is not a major concern. Although there is a degree of uncertainty associated with determining the MIC, it is interesting to note that the Δ*crl* strain seemed to be more tetracycline sensitive at all heat stress durations when incubated at 42°C, suggesting that Crl may affect the overall viability of cells when grown under prolonged heat stress conditions.

Taken together, our results do not support the involvement of Crl and RpoS in cross protection against tetracycline in *E. coli* in response to prolonged primary heat stress. Our findings do not agree with the observation of reduced tetracycline resistance upon heat stress-induced overproduction of RpoS in *E. coli* reported by Hui *et al*. (3). Our findings support those of Castro *et al*., who observed that RpoS may not play a role in coordinating heat shock stress and tolerance to tetracycline (5). The discrepancy between the findings of Hui *et al.* and Castro *et al.* may be because the mechanism of coordination between heat stress-induced RpoS levels and the upregulation of tetracycline resistance genes is not well described. Tetracycline resistance in *E. coli* is mediated primarily through an efflux pump mechanism tightly regulated by the tetracycline repressor and operator, *tetR* and *tetO*, which repress the production of proteins involved in the efflux pump in the absence of tetracycline (8). Although Hui *et al.* have observed that RpoS increases and accumulates upon heat stress, perhaps RpoS and Crl are not involved in the regulation of *tetR* and *tetO*, and other downstream tetracycline resistance genes (3).

FUTURE DIRECTIONS

This study suggests that RpoS and Crl may not be involved in cross protection against tetracycline in response to primary heat stress. This conclusion is based on the assumption that RpoS and Crl were upregulated in response to primary heat stress; however, RpoS and Crl levels were not determined in our study. To investigate whether the heat-stress used was sufficient to upregulate Crl and RpoS, protein and mRNA levels of these genes should be compared at each heat stress time point. The upregulation of these genes can be assessed via qPCR to quantify mRNA levels and via Western blotting to quantify protein levels.

Furthermore, the heat shock conditions for upregulation of RpoS were previously determined and RpoS levels were expected to be upregulated, as our experiment was conducted with identical conditions (3). However, this experiment did not investigate the optimal conditions for upregulation of Crl. Given that Crl is a low temperature induced protein, cultures could be incubated at 30°C overnight instead of 37°C until stationary phase prior to heat stress to allow upregulation of Crl and accumulation of RpoS (9).

Our results suggest that heat stress does not upregulate Tet resistance genes and induce cross protection against tetracycline. Since cross protection has been observed with different stressors, future groups could investigate the relationship between stressors such as acid, starvation, or osmolarity, and exposure to tetracycline or other antibiotics (1). It would be more efficient to first confirm upregulation of RpoS levels in response to the primary stressor and whether cross protection is observable in the WT strain, before investigating whether Crl is involved.

ACKNOWLEDGEMENTS

We would like to thank Dr. David Oliver, Dr. Danielle Krebs, Celine Michiels and Andrew Santos for their guidance and technical support throughout the course of this study, as well as the media room personnel for supplying the lab glassware used in the experiments. This study was supported by the Department of Microbiology and Immunology at the University of British Columbia.

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