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# RpoS is not Necessary to Increase Heat Tolerance in *Escherichia coli* BW25113 Following Sub-Inhibitory Kanamycin Pre-Treatment

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**RpoS, an alternative sigma subunit of RNA polymerase, is known to be involved in eliciting a general stress response in *Escherichia coli.* The aim of this study was to determine whether RpoS was necessary for the improved heat tolerance at 50°C previously observed in *Escherichia coli* pre-treated with sub-inhibitory kanamycin. Spread plating was done to assess cell viability before and after heat treatment in wildtype *Escherichia coli* and an isogenic Δ*rpo*S mutant. Western blotting was used to evaluate RpoS expression levels in both strains and to confirm the *rpo*S knockout. Our viability assay results showed reduced cell viability in both strains after kanamycin pre-treatment, and the Δ*rpo*S strain showed similar and higher percent viability compared to the wildtype in control and kanamycin treatment groups, respectively. Induction of RpoS was seen in the wildtype with pre-treatment of sub-inhibitory kanamycin and after heat treatment. Our findings indicate that RpoS was not necessary for inducing heat tolerance.**

RpoS (sigma factor 38 or $\sigma^{38}$) is an alternative sigma subunit of RNA polymerase expressed in bacteria subject to environmental stresses (1–3). When a bacterium is exposed to stress, a general stress response is initiated, in which RpoS competes with other sigma factors for RNA polymerase to upregulate genes that provide cellular protection (2, 3).

RpoS expression is highly regulated in *Escherichia coli*. During exponential phase, the level of cellular RpoS is reduced through immediate degradation by ClpXP, an ATP-dependent protease (4). This prevents the competitive binding of RpoS with sigma factor 70, which is responsible for the expression of housekeeping genes, to RNA polymerase. The *rpo*S transcript contains a 5' untranslated region which folds into a stem-loop structure, inhibiting ribosomal binding. RpoS expression is upregulated during stationary phase and in response to a variety of stress factors to initiate the general stress response (4, 5).

The expression of the RpoS regulon not only helps bacteria respond to the initial stress, but also offers cross-protection against other potential threats (6). Kanamycin is an aminoglycoside antibiotic that has been used for testing cross-protection in *E. coli* (6). Amenyogbe *et al* found that *E. coli* pre-treated with sub-inhibitory kanamycin resulted in increased heat tolerance, whereas the levels of cellular RpoS did not increase (6). In another study, a correlation between sub-inhibitory kanamycin pre-treatment and an increase in expression of the heat shock sigma factor RpoH was found (7). In response to heat stress, RpoH upregulates the expression of heat shock genes, which include chaperones and proteases that prevent protein aggregation and promote protein refolding (8). Hence, an increased tolerance to heat stress following sub-inhibitory kanamycin pre-treatment may be independent of RpoS.

Our study assessed whether RpoS was necessary for inducing heat tolerance in *Escherichia coli* pretreated with sub-inhibitory levels of kanamycin. We adapted the procedure devised by Amenyogbe *et al* using a wildtype strain and an isogenic *rpo*S knockout mutant of *E. coli*. We found that RpoS was induced in the wildtype strain with pre-treatment of sub-inhibitory kanamycin. After heat treatment, the knockout strain showed similar and higher cell viability in the control and kanamycin treatment groups, respectively, compared to the wildtype. Our results suggest that RpoS may not be integral to the heat stress response after kanamycin pre-treatment.

## MATERIALS AND METHODS

**Bacterial strains and culture growth.** *E. coli* strains from the Keio collection held at the Coli Genetic Stock Centre (CGSC) (9) were obtained through the MICB 421 culture collection from the Microbiology and Immunology Department at the University of British Columbia. *E. coli* BW25113 (wildtype, CGSC #7636) and BW28465 (Δ*rpo*S1271, CGSC #8000) were grown overnight at 37°C with mild aeration in M-9 minimal salts medium (0.05% w/v NaCl, 0.7% w/v $Na_2HPO_4$, 0.3% w/v $KH_2PO_4$, 0.1% w/v $NH_4Cl$, 0.02% w/v $MgSO_4 \cdot 7H_2O$, and 0.2% w/v glycerol adjusted to pH 7.4).

**MIC assay.** Kanamycin stock solution was prepared by dissolving kanamycin sulfate (Sigma-Aldrich, Cat No. K1377) in $dH_2O$ to a final concentration of 2 mg/ml and filter sterilizing the solution through a 0.22 µm filter. The stock was diluted to final concentrations of 0, 0.4, 0.6, 0.8, 1.0, 1.2, 1.6, and 2.0 µg/ml in M-9 medium, and 100 µl of each concentration was plated in triplicate on a polycarbonate 96-well plate. Overnight cultures of each strain were diluted 1/100 and 100 µl of diluted culture was added to kanamycin treatment wells. The plate was incubated overnight at 37°C. Contents of each well were transferred to a polystyrene 96-well plate and the MIC of kanamycin for each strain was assessed by eye.

**Growth curve construction.** The growth curve construction method was adapted from Amenyogbe *et al* (6). Overnight cultures of each strain were split into control and sub-inhibitory kanamycin treatment flasks and were seeded at an $OD_{500}$ of 0.1. The appropriate amount of kanamycin stock solution was added to the treatment flasks to attain a sub-inhibitory kanamycin concentration, defined as half the MIC. Each culture was incubated in a 37°C shaking water bath and the $OD_{500}$ was

monitored regularly until an $OD_{500}$ of 0.5 was obtained.

**Heat stress treatment and viability assay.** Immediately after growth curve construction, each culture was incubated in a 50°C shaking water bath for 45 minutes, mimicking conditions used by Amenyogbe *et al* (6). Prior to and after 45 minutes of heat treatment, samples were taken from each flask for spread-plating (10 µl) and for SDS-PAGE and western blot analysis (30 ml). Samples (100 µl) were plated in duplicate on Luria-Bertani (LB) agar plates (1% w/v tryptone, 0.5% w/v yeast extract, 1% w/v NaCl, 1.5% w/v agar). After the plates were incubated at 37°C overnight, colonies were counted.

**Cell lysis and TCA precipitation.** The SDS-PAGE and western blot sample aliquots were centrifuged at 7500 x g for 10 minutes and resuspended in 5 ml sonication buffer (50 mM Tris-HCl, 100 mM NaCl, 5 mM DTT, protease inhibitor cocktail [Sigma-Aldrich, Cat No. P8465], at pH 7.5). Glass beads were added and lysis was carried out using the FastPrep®-24 instrument (MP Biomedicals) at a velocity of 6.0 m/s for 40 seconds. The samples were centrifuged at 8,000 x g for 5 minutes, and the supernatants (1 ml) were added to 1 ml of 10% TCA. The solutions were incubated on ice for 10 minutes and centrifuged at 9,000 x g for 10 minutes. The supernatant was discarded and the protein pellets were resuspended in 56 µl of 0.33 M NaOH and stored at 4°C until further use.

**Bradford assay.** A bovine serum albumin standard curve was prepared with a range from 0 to 1 mg/ml. All samples (diluted 1/25) in a final volume of 100 µl and standards were plated in triplicate on a 96-well plate containing 100 µl of Bradford reagent. The plate was incubated at room temperature for 10 minutes and read at 595 nm on a BioTek Epoch microplate spectrophotometer. Protein concentrations in the samples were calculated using the standard curve.

**Reducing SDS-PAGE.** Lysed samples were boiled at 95°C for 10 min in 4x sample buffer (0.125 M Tris pH 6.8, 5% v/v β-mercaptoethanol, 2% w/v SDS, 0.02% w/v bromophenol blue, 10% w/v glycerol). The lysates (20 µg protein per lane) were loaded onto 12.5% SDS gels along with molecular weight standards. The gels were run at 200 V for 35 minutes in running buffer (25 mM Tris base, 192 mM glycine, 0.1% w/v SDS at pH 8.3).

**Western blot.** A western blot procedure to detect RpoS was adapted from Hui *et al* (2). Proteins were transferred from the SDS gel to Trans-Blot nitrocellulose membranes (Bio-Rad, Cat No. 9846) at 90 V for 1 hour in transfer buffer (25 mM Tris, 192 mM glycine, 10% v/v methanol). The membrane was blocked in milk TBS-T (0.3% skim milk powder, 50 mM Tris, 150 mM NaCl, 0.2% w/v KCl, 0.5% v/v Tween-20 at pH 7.5) for 1 hour for the first replicate and 2 hours for the second replicate. The blots were incubated in mouse anti-RpoS primary antibody (Neoclone, Cat No. W0009) at a 1:1000 dilution in blocking buffer for 1 hour. After the primary antibody was discarded, the membrane was washed in TBS 3 times for 5 minutes per wash, and then incubated in an alkaline phosphatase labeled goat anti-mouse IgG secondary antibody (Novex, Cat No. G-21060) for 1 hour. After discarding the secondary antibody, the membrane was washed in TBS 3 times for 5 minutes per wash. Detection was carried out by adding BCIP/NBT (Sigma-Aldrich, Cat No. B1911) to the membrane and incubating for 1 hour at room temperature.

## RESULTS

**The MIC of kanamycin was identical for wildtype and Δ*rpo*S *E. coli*.** The calculated sub-MIC of kanamycin for sub-inhibitory kanamycin pre-treatment was 0.5 µg/ml for both wildtype and Δ*rpo*S strains, and was twice the concentration used by Amenyogbe *et al* (6).

**The growth patterns for wildtype and Δ*rpo*S cultures were inconsistent between the two replicates.** In the first replicate, the growth patterns were consistent among all cultures (Fig 1a). The duration of exponential growth was 3 hours, where a curve fit showed a similar slope in all treatment cultures. After 3 hours, the growth slowed as indicated by a decline in the slope of the curves. In the second replicate, growth appeared to halt in the kanamycin-treated wildtype culture considerably earlier than the other cultures (Fig 1b). After heat treatment, the

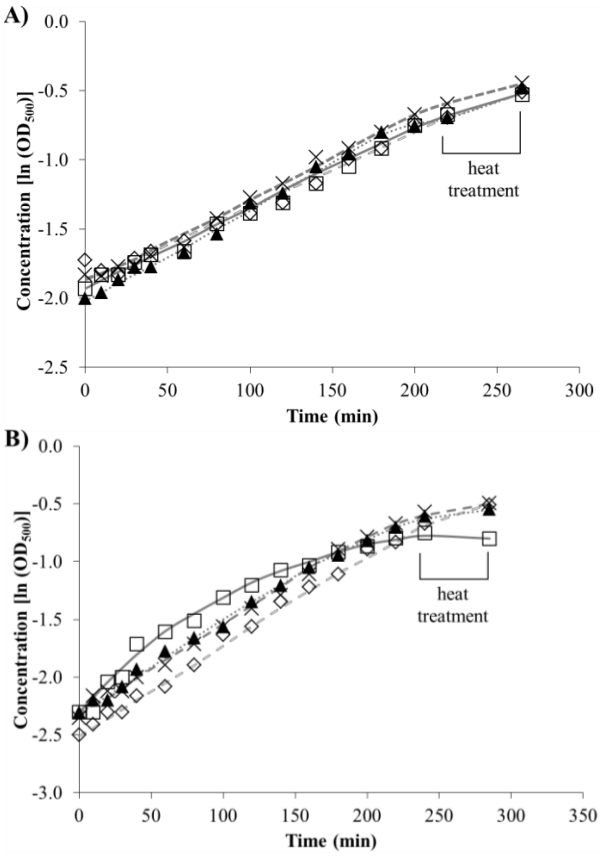


**FIG 1** Growth curves for wildtype and Δ*rpo*S *E. coli* control and sub-inhibitory kanamycin treated cultures. Graphs shown represent data for A) replicate 1 and B) replicate 2. Treatment groups include wildtype treated with kanamycin (□), wildtype control (◇), Δ*rpo*S treated with kanamycin (▲) and Δ*rpo*S control (×).

growth curve plateaued for the kanamycin pre-treated wildtype culture, indicating that the culture stopped growing. All other treatments remained in slower exponential growth after heat shock. Deviations from the real trend associated with the turbidity measurements may explain the variations seen in the growth for the two replicate experiments. The interpreted trends rely on one datum point per time point, so the curve fits were plotted under the assumption that the single values were representative of the actual growth trends for each culture.

**Sub-inhibitory kanamycin pre-treatment reduced the viability in both wildtype and Δ*rpo*S strains, where the**

**Δ*rpo*S strain showed similar or increased viability compared to the wildtype.** For the control groups, the Δ*rpo*S strain exhibited a 1.5-fold increase in viability compared to the wildtype (Fig 2). There was no significant difference in viability between kanamycin pre-treated Δ*rpo*S and wildtype strains after heat treatment. These observations support our hypothesis that RpoS is not necessary in increasing heat tolerance in *E. coli*. The large standard deviations may be attributed to the differences seen in the growth patterns for each replicate (Fig 1).
The reduction in percent viability of kanamycin pre-treated cells compared to the untreated cells was significant for both strains. Within the wildtype and Δ*rpo*S strains, a 2.7 and 4.5-fold reduction, respectively, was observed (Fig 2). Kanamycin pre-treatment did not increase heat tolerance in the wildtype strain, contrary to our expectations and to the observations made by Amenyogbe *et al* (Fig 2).

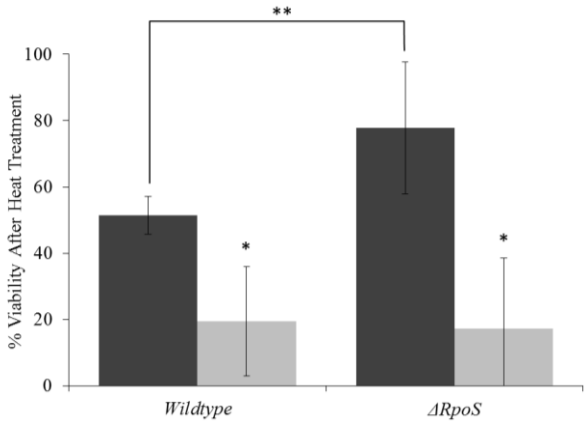


**FIG 2** Differences in percent survival in control and sub-inhibitory kanamycin treatments for wildtype and Δ*rpo*S *E. coli* after 45 minutes of heat treatment. Data is presented as mean ± SD (n=2). Dark grey bars represent control groups and light grey bars represent kanamycin-treated groups. * represents a significant difference compared to the control for the respective strains. ** represents a significant difference between wildtype and Δ*rpo*S strains for the control groups. Percent viability was calculated by normalizing colony counts made after heat shock to counts made prior to heat shock.

**The *rpo*S is expressed in the wildtype strain when pre-treated with sub-inhibitory kanamycin and when subjected to heat shock.** Western blot results from the first replicate are shown (Fig 3). An absence of bands in the Δ*rpo*S samples indicated a lack of RpoS expression and provided evidence that the strain used contained an *rpo*S deletion. For the wildtype strain, bands corresponding to 38 kDa RpoS were seen in kanamycin pre-treatment samples prior to and after heat shock, and in the control after heat treatment.

The increased intensity of the RpoS band in the kanamycin-treated wildtype culture after heat treatment implied an increase in *rpo*S expression after exposure to heat stress. However, the intense band at the top of the lane suggested a possible overloading of protein, which reduces the reliability of a comparison of band intensity between lanes. Three additional bands corresponding to sizes smaller than RpoS (37, 32, 25 kDa) were also present in the kanamycin-treated wildtype culture after heat treatment and were attributed to RpoS breakdown products.

## DISCUSSION

*E. coli* pre-treated with sub-inhibitory kanamycin results in heat tolerance, whereas the levels of cellular RpoS do not increase (6). Our results suggested that RpoS was not necessary for increasing heat tolerance in *E. coli*, because the Δ*rpo*S mutant had similar or better viability than the wildtype after heat treatment (Fig 2). RpoS competes with other sigma factors for RNA polymerase to upregulate genes involved in cellular protection (2, 3, 10, 11). One such sigma factor is RpoH, which is specifically upregulated in response to heat stress and initiates transcription of heat shock proteins (12). Because RpoH is specific for the heat stress response while RpoS is involved in responding to multiple general stresses, RpoH is perhaps more integral in regulating the heat stress response than RpoS. As a consequence, the lack of RpoS expression in the Δ*rpo*S mutant might eliminate the competitive binding between RpoS and RpoH to RNA polymerase. This may result in an increased binding of RpoH to the RNA polymerase, therefore promoting an increase in transcription of heat shock proteins and the survival of the Δ*rpo*S mutant when subjected to heat stress.

Rahman *et al* determined through microarray analysis that the expression of many metabolic genes are altered in an *rpo*S knockout mutant relative to its wildtype counterpart (13). The expression of genes under particular functional categories, such as those involved in an adaptation to stress, did not appear biased towards up or down regulation (13). Investigating the effects of an *rpoS* deletion on genes coding for proteins involved in the heat shock cascade would help explain the increased survivability of the Δ*rpo*S mutant relative to the wildtype strain.

Our Western blot indicated that RpoS expression was induced in the wildtype strain due to external stresses from kanamycin and heat treatment (Fig 3). Thus, kanamycin and heat stresses applied together could result in a cumulative increase in RpoS expression, as suggested by the relatively more intense band in the wildtype strain after both kanamycin and heat treatment (Fig 3), but this may be due to possible protein overloading. As *E. coli* is often exposed to multiple environmental stresses, it may be worth elucidating the potential of multiple stresses inducing variability in the level of RpoS expression. In addition, RpoS breakdown products were seen on the western blot only after heat treatment in the wildtype strain. This observation suggests that RpoS may have been degraded due to the heat treatment.

We found that RpoS was not induced in the control while in slowed exponential phase. We speculated that

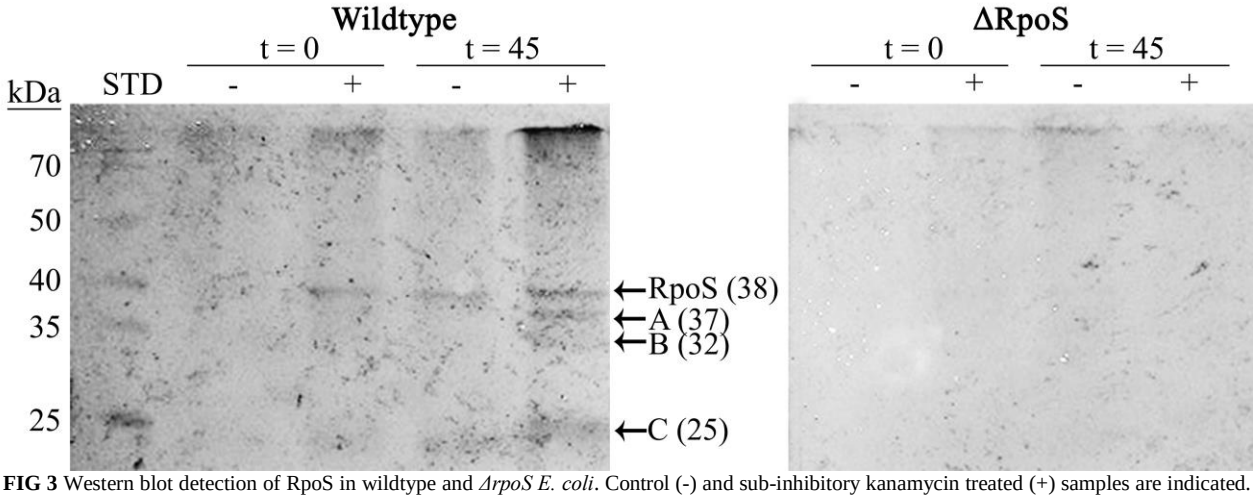


**FIG 3** Western blot detection of RpoS in wildtype and *ΔrpoS E. coli*. Control (-) and sub-inhibitory kanamycin treated (+) samples are indicated. A lack of bands in *ΔrpoS E. coli* (right) samples confirm the *rpoS* knockout. Wildtype *E. coli* sample (left) displays bands for RpoS (38 kDa) and possibly degraded RpoS or other proteins (A, B, and C) after heat treatment.

RpoS expression may only be induced after the culture has reached stationary phase, at which RpoS levels are relatively higher compared to the other phases. In contrast, Amenyogbe *et al* found that stationary and exponential phase wildtype cultures did not vary in relative RpoS levels (6), which was unexpected, as RpoS levels are downregulated during exponential phase (4). As their blot appeared to be overloaded with protein, a comparison of RpoS levels between the lanes would not be appropriate. As well, due to the high non-specific binding observed in their blot, we speculated that perhaps other proteins 38 kDa in size were probed in addition to RpoS, which could have contributed to the intensity of their RpoS bands.

Carrying out detection directly on the membrane using NBT/BCIP detection yielded high background due to possible contamination from the blocking and wash buffers and general handling of the membrane. The NBT/BCIP assay could be optimized to reduce background noise by increasing the blocking time, increasing the antibody dilutions, or decreasing the antibody incubation times.Differences in the sub-MIC level of kanamycin used, as well as the calculation of percent viability, could explain the observed discrepancy in the cell viability results between our study and Amenyogbe *et al*'s study. First, the concentration of kanamycin used in our study was two times greater than was used by Amenyogbe *et al* and may have been inhibitory to the cultures rather than inducing protection against heat stress. Our MIC may have been higher for our cultures due to biological variation. As well, the use of microplates with low well volumes in our MIC assay may have led to ambiguity in discerning the MIC, and this may be improved using test tubes to determine the MIC in a larger volume. Second, we calculated percent viability for each culture by normalizing the colony counts made after heat stress to counts made just prior to heat treatment. In contrast, Amenyogbe *et al* assumed a constant initial viability for all cultures, where the colony counts made post-treatment were normalized to an estimated number of total cells just after inoculation. As the growth patterns of microbial cultures may vary when grown in individual flasks for a lengthened period of time, our method of calculating cell viability should yield more representative results.

In our study, the control Δ*rpo*S strain had increased viability compared to the control wildtype strain after heat shock at 50°C, while pre-treatment with kanamycin unexpectedly reduced viability in both the wildtype and the Δ*rpo*S strains. Moreover, RpoS expression increased after both kanamycin pre-treatment and heat shock in the wildtype strain. Although our results deviated from previous studies, we deduced that RpoS is not necessary for heat tolerance.

## FUTURE DIRECTIONS

To clarify the discrepancy seen in the viability of the wildtype strain between our results and Amenyogbe *at al*'s results, this study should be repeated using the sub-MIC level kanamycin reported by Amenyogbe *at al*. If the results are consistent with our study, this would indicate that our viability assay is more robust. However, if the results are consistent with Amenyogbe *et al*'s study, the sub-MIC kanamycin level used in our current study may have been too high and may have skewed our results.

Our speculation that the elimination of *rpo*S reduces competitive binding to the RNA polymerase can be tested by repeating our experiment and looking at the expression of key heat-stress proteins in the RpoH regulon. If these proteins are expressed to a higher

degree in the Δ*rpo*S strain compared to the wildtype strain, this would support our notion that the removal of *rpo*S decreases the amount of competitive binding between RpoS and RpoH to the RNA polymerase.

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