The stationary phase sigma factor, RpoS, present in *Escherichia coli* is responsible for the phenomenon known as cross-protection where the cell acquires secondary resistance to a stress factor once exposed to an unrelated primary stress. Previous studies have shown contradictory observations based on the effects of RpoS overproduction where one study observed a positive outcome and another study observed an inhibitory outcome. Our study tested the possibility that low levels of RpoS contributed to positive cellular protection and higher levels contributed to inhibition of cell protection. RpoS was overexpressed in both a wild type strain, BW28357, and an RpoS, BW28465, *E. coli* strain though heat shock at 45°C for various lengths of time. Minimum inhibitory concentration (MIC) assays with tetracycline were performed in order to determine conferred levels of protection. RpoS was then isolated and quantified using Western blot. Western blot results revealed an increase in RpoS concentration with prolonged heat shock. Western blot analysis coupled with MIC results revealed that protection against tetracycline was diminished as RpoS expression was upregulated beyond a certain level.

**Materials and Methods**

**Bacterial Strains and Growth Conditions.** *E. coli* BW28465 and BW28357 were obtained from the MICB 421 bacterial culture collection in the Department of Microbiology and Immunology, University of British Columbia. Strain BW28357 contains a deletion in *rpoS* (derivatives of BW28357). Both strains were cultured in Tryptone Soy Broth (TSB) pH 7.0 (1.0% w/v Bactotryptone, 0.5% w/v Bacto yeast, 0.5% w/v NaCl). Overnight cultures of each strain were grown at 37°C and shaken at 150 rpm to reach stationary phase with an OD greater than 1.5.

**Heat Induction.** After reaching stationary phase, the master cultures were divided into 50 ml portions and induced for an allotted amount of time at 45°C while shaking at approximately 150 rpm. Cultures with the longest induction time were placed into the water bath first followed by specific time intervals between additions of other cultures in order to process all samples simultaneously at the end of 115 minutes to minimize RpoS degradation. All samples were kept on ice throughout the cell lysis process to retain the integrity of RpoS.

**Minimum Inhibitory Concentration (MIC) Assay.** The minimal inhibitory concentration (MIC) of heat induced cultures was determined by plating 100 µl of each culture with 100 µl of tetracycline (Sigma, #T-3383) at various concentrations. Prior to plating, cultures were diluted 1:100 in TSB and final concentrations of tetracycline at 16, 8, 4, 2, 1, 0.5, 0.25 and 0.125 µg/ml were made. Cultures from each heat shock time point were plated in triplicates. MIC plates were then incubated at 37°C.
overnight and analyzed by eye to determine the presence or absence of growth in each well.

**Cell Lysis.** After heat induction, samples were spun down at 10,000 rpm for 5 minutes. Pellets were harvested by resuspending in 500 µl of lysis buffer (PBS, 0.1% w/v, Lysozyme, 0.1% w/v, 0.06% DNase, 1% w/v MgCl₂, Protease Inhibitor Cocktail, Sigma-Aldrich, PF1465) and transferring to microcentrifuge tubes, which were then left at 4°C under constant agitation for 50 minutes. Half a gram of 0.1 mm diameter glass beads were then added into each solution. Subsequently, samples were placed in a bead beater (MPBio, FastPrep-24) for 6 rounds of cell lysis at 6.5 m/s for 45 seconds. Five hundred microliters of lysis buffer was then added to the sample before centrifugation at 5600 rpm for 10 minutes. Centrifugation was repeated after sample supernatants were transferred to new microcentrifuge tubes and protein samples were collected as part of the resulting supernatant.

**Bradford Assay.** Standard curve for the Bradford assay was prepared using bovine serum albumin. Samples were diluted at 1/200 and 1/500 added to a 96-well plate containing 100 ul Bradford reagent, and incubated at room temperature for 5 minutes. Protein concentrations of the samples were then determined by plate reading at 595nm and calculations were made using the standard curve.

**SDS-PAGE.** Lysed samples were boiled at 95°C for 10 min in 6x sample buffer (50 mM Tris pH 6.8, 100 mM dithiothreitol, 2% SDS, 0.1% Bromphenol blue, 10% glycerol). Approximately 10 mg of protein per sample, calculated from the Bradford assay results, was loaded into 12% SDS-polyacrylamide gels. Gels ran at 120 V for 1 hour in 1x running buffer (25mM Tris base pH 6.8, 192mM glycine, 1% SDS).

**Western Blot.** SDS-PAGE proteins were transferred onto nitrocellulose membranes using 1x transfer buffer (25 mM Tris base pH 6.8, 192 mM glycine, 20% methanol) at 90 V for 1 hour. Membranes were then blocked using 0.3% milk TBS-T (50mMTris, 150mMNaCl, 0.2% KCl, 0.5% Tween, pH 7.5) for approximately 30 minutes. Incubation of membrane at 4°C occurred for one hour in a 1:1000 dilution of mouse anti-sigmaS antibodies (Neoclon, W0009) in 0.3% milk TBS-T. Three washes with milk TBS-T for 5 minutes each were made, then the membrane was incubated at room temperature in a 1:5000 dilution of alkaline phosphatase labeled goat anti-mouse IgG (Life Technologies, 13864-012) in milk TBS-T. This was followed by three washes for 5 minutes per wash in clear 1x TBS. Detection included the addition of 1ml of NBT/BCIP solution (Sigma Aldrich, #B1911) directly over the membrane in the dark for 30 minutes.

**RESULTS**

**RpoS production increased with prolonged heat induction in wild-type strain.** To determine whether overproduction of RpoS occurred with increased heat exposure, protein extracted from heat induced E. coli BW28357 cultures were normalized and analyzed through Western blot. Relative band intensities indicated increased levels of RpoS expression over time of heat exposure (Fig 1 WT), while absence of the heat induction revealed low to undetectable amounts of RpoS (Fig 1 WT 0 min). A steady increase in RpoS expression could be seen initially as shown by the increasing intensity of bands (Fig 1 WT 30-115 min). However, band intensities leveled off after about 100 minutes of heat induction (Fig 1 WT) indicating that the amount of RpoS overproduced in cells had become constant.

**Loss of protection against tetracycline in WT E. coli BW28357 after prolonged heat induction.** To investigate the efficacy of protection after various times of heat induction, an MIC assay was used to determine the resistance of E. coli BW28357 cultures against serial diluted concentrations of tetracycline. Triplicates were used to ensure reproducibility of MIC assay results since there is an uncertainty of ±1 well. An untreated value at zero time was also used to determine its baseline resistance against tetracycline (Fig 2). While the baseline resistance was found to be 2 µg/ml (Fig 2, BW28357 0 min), results indicated a 2-fold increase in resistance from 2 µg/ml to 4 µg/ml as RpoS was overproduced at 30 min (Fig 1 WT). However, this improved resistance was lost as time of heat induction increased, until the MIC decreased to 1µg/ml, which is two times less than the pre-treated MIC (Fig 2, BW28357, 70-115 min).

**Verification that RpoS was responsible for cross-protection.** To verify that RpoS was responsible for the increased levels of cross-protection against tetracycline seen in the wild-type strain, the ΔrpoS E. coli strain, BW28465, was heat induced and treated under identical conditions. Proteins extracted were analyzed through Western blot. Results revealed the expected lack of RpoS expression indicated by the absence of bands (Fig 1, WT). Heat induced cultures of the mutant strain were also used in MIC assays where cross-protection against tetracycline was found to be absent as the MIC of all samples remained at 1µg/ml regardless of heat induction (Fig 2, BW28465).

**DISCUSSION**

In order to explain our findings, we refer to two possible mechanisms. The first mechanism is based on the observation that different sigma subunits compete with one
in conferring resistance, this ample for the wild type compared ing enough to result in MICs rpoS, Mut r folding of its induced moters. Levels of RpoS are rom previous studies by support ing the role of Rpo results indicated the MIC to be constant at 1µg/ml. Besides absence of RpoS expression (Fig 1 same conditions. Western blot results confirmed the BW28465, of the wild mutant, ΔRpoS strain. Thus, ΔRpoS expression was also important in determining if the protein overproduced (Fig 2, 0-70min). This lack of data reduced the detail of the observed trend, however, does not controvert its existence.

An alternative proposed mechanism is based on one of the genes controlled by RpoS, DnaK (8). DnaK is induced during heat shock (8). DnaK assists in the folding of nascent peptides and binds to misfolded peptides in order to reduce their presence within the cell (8). DnaK undergoes a negative feedback loop with correlation to the levels of heat shock factors (HSF) that are produced in response to RpoS (9). As RpoS and HSF levels increase due to prolonged heat induction, DnaK levels diminish (9). RpoS folding as well as proper folding of its induced proteins are dependent on the functionality of DnaK (9). Therefore, a loss of DnaK results in an accumulation of misfolded RpoS-dependent proteins. The reduction in functioning RpoS-dependent proteins parallels the loss of RpoS function (9). Thus, although levels of RpoS protein continue to accumulate (Fig 1, WT), RpoS-induced activities such as cross-protection could be reduced.

To demonstrate that resistance against tetracycline was facilitated by the presence of RpoS, the ΔrpoS mutant, BW28465, of the wild-type strain was treated under the same conditions. Western blot results confirmed the absence of RpoS expression (Fig 1, Mut) while MIC assay results indicated the MIC to be constant at 1µg/ml. Besides supporting the role of RpoS in conferring resistance, this also shows that no other factor was responsible for the observed results. The basal resistance at 1µg/ml tetracycline (Fig 2) is a contribution of other sigma factors such as RpoD and RpoH, which are also heat induction proteins (9,10). Although other sigma factors result in heat protection, cross-protection is a rare occurrence (9). Significant RpoS levels are still required to induce cross-protection. The fact that a higher level of protection was observed in the control sample for the wild type compared to the mutant (Fig 2) may be a result of low levels of RpoS already present in the cell providing amplified resistance.

A potential contributor of error in our study pertains to the stability of RpoS, which is readily turned-over. In order to minimize protease activity, samples were processed and stored on ice. However, handling time of samples at room temperature may have resulted in significant degradation of RpoS. Therefore, the levels of RpoS may not be representative of the maximum induced levels initially within the cells. The most prominent limitation in our results includes the lack of time points when RpoS was overproduced (Fig 2, 0-70min). This lack of data reduced the detail of the observed trend, however, does not controvert its existence.

This study addressed the question of whether an inhibitory effect was seen at overproduced levels of RpoS. Results indicated that as RpoS levels increased due to prolonged heat induction, cellular protection was lost, thus corresponding with our hypothesis. Our findings also showed that aside from the presence of RpoS, the level of expression was also important in determining if the protein conferred cross-protection. This observation would explain the discrepancy in the results from previous studies by Hung (5) and Behmardi et al. (4) since different quantities of RpoS protein may have been induced under differentiated conditions.

FUTURE DIRECTIONS
In this experiment, the intensity of the protein bands in the Western blot was not distinctive enough to show an obvious increase in RpoS levels between some time points. This may be due to the actual proteins in each sample, or it may be due to the detection method. To avoid this in the future, a larger volume of each cell lysate sample can be loaded into SDS-PAGE gels to increase the amount of protein on the membrane and therefore a better detection of the protein.

Results from this study indicated a decrease in protection against tetracycline at high levels of RpoS expression. A proposed explanation for this was that an accumulation of RpoS competed with other sigma factors for the RNA polymerase core, thereby, downregulating the transcription of important survival proteins that are upregulated by other sigma factors (1). To investigate the effect of high levels of RpoS expression, the transcriptional level of other proteins that are dependent on other sigma factors, such as the dependence of lacZ on RpoD (11), could be accessed through Southern blot. In minimal medium containing isopropyl β-D-1-thiogalactopyranoside (IPTG), there should be a high level of lacZ transcription. If RpoS does have an inhibitory effect on RpoD binding to the RNA polymerase core, the amount of lacZ transcript would
decrease significantly in high RpoS concentration. An alternative approach to Southern blot is to measure galactosidase activity directly by using an enzyme assay or antibody detection.

Another proposed explanation is that RpoS misfolds when the folding protein DnaK diminishes from the negative feedback effect caused by an increase in HSF levels induced by RpoS (9). This mechanism could be investigated by tracing RpoS activity as well as HSF and DnaK levels within the cell when RpoS is overexpressed. The HSF and DnaK levels can be measure via an enzyme assay or Western blot.

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