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Heat-shock Increases RpoD Dependent β-galactosidase Activity in the *Escherichia coli* Strains BD792 and B23

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RpoD is a primary sigma factor in *Escherichia coli* responsible for expression of *lacZ*. RpoS is an alternative sigma factor that is upregulated under environmental stress. RpoS competitively binds to RNA polymerase core proteins, displacing RpoD so that stress-related genes can be transcribed instead. This causes a decreased expression of RpoD-dependent genes. There have been contradictory findings on the relationship between heat-induced stress and RpoS concentrations in *Escherichia coli*. One study found that RpoS concentrations decrease upon heat-induced stress, while another found the opposite. To address this issue, we have tested whether heat-shock causes a decrease in RpoS levels and a concomitant increase in RpoD-dependent gene expression, indirectly using a *lacZ* reporter system. An increase in RpoD-dependent lacZ gene expression would indicate a decrease in RpoS activity. *Escherichia coli* wild-type B23 and *rpoS$^-$* BD792 strains were subjected to heat-shock treatment and β-galactosidase activities were measured using Miller assays as indication of *lacZ* expression. In the wild-type strain, β-galactosidase activity significantly increased upon heat-shock. Similarly, β-galactosidase activity significantly increased in the *rpoS$^-$* strain, but the amount of β-galactosidase activity was significantly higher than that of the wild-type strain upon 15 and 60 minutes of heat-shock. Our data show that heat-shock increases the levels of RpoD-dependent β-galactosidase activity, suggesting a decrease in RpoS activity. Since an increase in β-galactosidase activity was also observed in the *rpoS$^-$* strain upon heat shock, our data imply that there may be another protein involved in regulating RpoD-dependent β-galactosidase activity.

Bacteria such as *Escherichia coli* (*E. coli)* are continuously subjected to environmental stressors such as changes in temperature and exposure to UV light (1, 2). In response to such changes in the environment, bacterial cells regulate specific genes essential to survival under different conditions through differential expression of various sigma factors (3). Sigma factors are proteins responsible for regulating transcription of genes involved in bacterial growth and survival (4). These factors competitively bind to core proteins that make up RNA polymerase (RNAP) and increase their affinity for promoter sequences of the specific genes to be transcribed (5). Sigma factor binding to the same RNAP core defines the genes that are transcribed in response to the survival and growth requirements of bacteria (4). The primary sigma factor, RpoD, for example, predominates in *E. coli* under optimal growth conditions with no environmental stressors (4). RpoD is responsible for transcribing *lacZ*, which is needed when lactose is present as the carbon source required for bacterial growth and survival (6). The *lacZ* gene encodes for the ß-galactosidase protein, which cleaves lactose into galactose and glucose when environmental lactose is present and glucose is absent (6). In addition, RpoD has also been found to be responsible for genes that confer tetracycline resistance (6). However, RpoD can be outcompeted by other sigma factors when growth conditions change when growth conditions change, other sigma factors can be upregulated which outcompete RpoD for the RNAP core complex (6).

In response to environmental stressors, the expression of the alternative sigma factor RpoS is upregulated due to temperature-sensitive regulators at the translational level (7). At the translational level, small noncoding RNAs (sRNAs) are induced by environmental changes and increase *rpoS* mRNA translation to allow cells to adjust to external stress (7). One such sRNA, DsrA, contains a temperature-sensitive transcription initiation thermocontrol which is repressed at high (42˚C) temperatures, but is active at lower temperatures (25˚C) (8). As a result, high temperatures interfere with the initiation of the DsrA promoter, which in turn causes a decrease in translation of RpoS due to the down regulation of DsrA (7). Through this translational control mechanism, this alternative sigma factor is able to outcompete the primary sigma factor RpoD in binding to RNAP during low temperature conditions (6–8). This competitive displacement of RpoD by RpoS allows bacterial cells to survive changes in environmental temperatures (7, 8).

A recent study contradicted these results and instead correlated high temperatures solely with increased RpoS (9). This study found that tetracycline resistance gene expression, which depends on RpoD-mediated gene transcription, decreased when environmental temperature was increased by heat-shock (9). Therefore, after quantifying the amount of RpoS, they concluded that heat-shock caused an increase in RpoS, which competitively bound to RNAP and decreased RpoD-mediated gene transcription (9). The observed phenotype after heat-shock was a decrease in RpoD-dependent tetracycline resistance (9).

The purpose of this paper is to resolve this contradiction between low and high RpoS expression at high temperature. Since *lacZ* expression is also dependent on RpoD, we hypothesize that RpoS would decrease following heat-shock of *E. coli* cells and *lacZ* expression would increase. This would support a model in which an increase in RpoD-mediated transcription of *lac*Z is due to less RpoS competitively binding to RNAP. We found that our results

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support the model that states: RpoS expression is inhibited at high temperatures and RpoD expression is upregulated.

MATERIALS AND METHODS

Bacterial Strains and Growth Conditions. *E. coli* strains B23 (*lacZ*^{WT} *rpoS*$^{+}$) and BD792 (*lacZ*^{WT} *rpoS*$^{-}$) were obtained from MICB 421 bacterial stock collection in the Department of Microbiology and Immunology, University of British Columbia. BD792 is a derivative of B23 wild-type strain and contains an inactivating base pair mutation *rpoS396(Am)* (6). *E. coli* strains 285c (*lacZ*^{WT} *rpoD*$^{-}$) and P90A5c (*lacZ*^{WT} *rpoD*$^{+}$) were a gift from Dr. Richard Calendar from University of California, Berkley. Strain 285c, derived from P90A5c, contains a temperature-sensitive mutation in the *rpoD* gene, which inhibits growth at 37ºC (10). The parent strain P90A5c is a temperature-sensitive strain derived from unknown mutations that arose after crossing P90 and AT12-55 strains (10).

Strains B23, BD792, and P90A5c were grown in Luria-Bertani (LB) medium, while 285c was grown in LB medium supplemented with 25 µg/µl streptomycin to prevent reversion mutation (11). Cultures were grown at 37℃ for B23 and BD792, and at 30℃ for P90A5c and 285c overnight in shaking incubators at 150 rounds per minute (rpm). These are specific optimal growth conditions for each strain.

After incubating overnight, cultures were diluted 1 in 50 by adding 100 µl of cultures to 4.9 ml LB medium. 12.5 µl of 0.8 M isopropyl β-D-1-thiogalactopyranoside (IPTG) was added to the diluted cultures to induce *lacZ* expression before returning the cultures back to shaking incubators (150 rpm, 37℃ for B23 and BD792, 30℃ for P90A5c and 285c). Cultures were removed from shaking incubators when optical density (OD) readings (Biochrom Ultrospec 3000) were in the range of 0.6-1.0 at 600 nm, which is indicative of stationary phase (12, 13). Stationary phase bacterial cultures for each strain were diluted to 0.6 OD_{600} using LB medium as diluent to normalize cell concentrations for all samples. This 0.6 OD_{600} value would be used in the Miller assay equation as absorbance (Abs_{600}) (14).

Heat-shock. 0.6 OD600 diluted stationary phase cultures of B23 and BD792 strains were incubated for one of 0, 15, 30, or 60 minutes in the 42ºC incubator. After the specified time, the cultures were removed from the incubator and used in Miller β-galactosidase assays.

Miller β-galactosidase Assays. OD_{600} readings of Permeabilization Solution (100 mM Na_2HPO_4, 20 mM KCl, 2 mM $MgSO_4$, 0.8 mg/ml hexadecyltrimethylammonium bromide, 0.4 mg/ml sodium deoxycholate, 5.4 µl/ml β-mercaptoethanol) were taken in order to determine the baseline absorbance values for the assays. 20 µl each of heat-shocked B23 and BD792 cultures and non-heat-shocked P90A5c and 285c stationary phase cultures were mixed with 80 µl aliquots of Permeabilization Solution in 2.0 ml microfuge tubes. This 20 µl volume of cell cultures mixed with Permeabilization Solution would be used in the Miller assay equation after conversion to millilitres (14).

The mixtures were incubated in 30ºC incubator for 30 minutes. 600 µl of warm (30ºC) Substrate Solution (60 mM Na_2HPO_4, 40 mM NaH_2PO_4, 1mg/ml o-nitrophenyl-β-D-Galactoside (ONPG), 2.7µl/ml β-mercaptoethanol) was added to each mixture at the end of the 30-minute incubation and the time of addition was noted. When yellow colour became clearly visible in the mixtures, 700 µl of Stop solution was added. The exact stop time for each mixture was recorded. This total amount of time for the mixtures to turn yellow after addition of Substrate Solution would be used in the Miller assay equation (14).

After stopping the reaction, the tubes containing the mixtures were spun at room temperature at 12,000×g using a microcentrifuge (Eppendorf Centrifuge 5424) for 10 minutes. The supernatants were then transferred to cuvettes and the absorbance values measured at 420 nm using a UV/visible spectrophotometer (Biochrom Ultrospec 3000). The Abs_{420} values were then converted to Miller Units. The Miller Unit is an arbitrary unit used to represent and compare β-galactosidase expression (14). Miller Unit was calculated using the equation below according to the method of Miller (14, 15):

$$\text{Miller Unit} = \frac{(Abs_{420})}{(Abs_{600}) \text{ x (volume) x (reaction time)}}$$

RESULTS

β-galactosidase (β-gal) activity was lower in the *E. coli* *rpoD*$^{-}$ 285c strain compared to *rpoD*$^{+}$ P90A5c strain under optimal growth conditions. To determine if *lacZ* expression was dependent on RpoD, we compared the β-gal activity of the *rpoD-* strain 285c to the *rpoD*$^{+}$ strain P90A5c. The latter was a positive control (Fig 1a). In addition, to determine if the presence of RpoS affects *lacZ* expression, we compared the β-gal activity of the *rpoS*$^{-}$ strain BD792 (used as a negative control) to the *rpoS*$^{+}$ wild-type strain, B23 (Fig 1b). We chose the Miller assay to study β-gal activity because it is quick, easy, and gives robust results for the majority of *E. coli* strains (14).

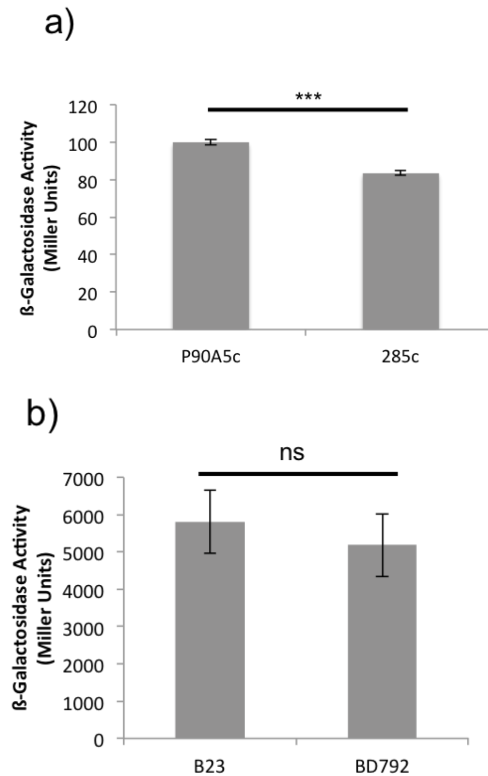

FIG 1 (a) β-galactosidase activity of *rpoD*$^{+}$ P90A5c and *rpoD*$^{-}$ 285c strains under optimal growth conditions (30℃ overnight in LB for P90A5c, LB with 25 µg/ul streptomycin for 285c, 150 rpm) (Left). (b) β-galactosidase activity of *rpoS*$^{+}$ B23 and *rpoS*$^{-}$ BD792 strains under optimal growth conditions (37℃ overnight in LB, 150 rpm) (Right). n ≥ 3 repetitions per strain tested. Mean ±SEM (standard error of mean) is shown. ***P<0.001 determined using Student's paired two-tailed t-test. ns = not significant.

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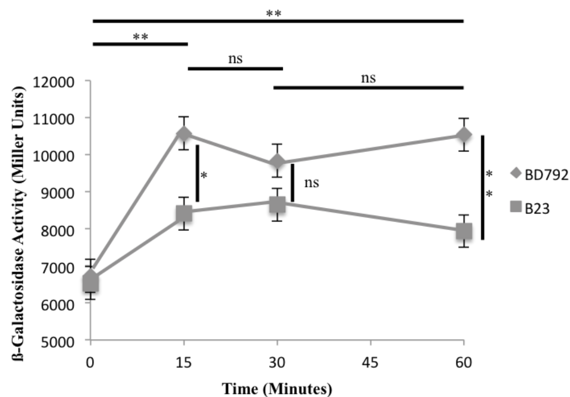

FIG 2 β-galactosidase activity of *rpoS*⁺ B23 and *rpoS*⁻ BD792 strains under various times of heat-shock. n ≥ 3 repetitions per strain tested. Mean ±SEM (standard error of mean) is shown. **P<0.05 and **P<0.01 determined using Student's paired two-tailed t-test. ns = not significant.

The results of the Miller assay show that *rpoD*⁻ 285c strain, when fully induced with 10 mM IPTG to induce the expression of *lacZ*, displays significantly reduced β-gal activity compared to the *rpoD*⁺ P90A5c strain (Fig 1a). Concurrent with previously published data that determined *lacZ* expression was solely dependent on RpoD, the *rpoS*⁺ B23 and the *rpoS*⁻ BD792 strains did not show significant differences in β-gal activity when induced with IPTG (Fig 1b) (3, 6). We wished to determine if ß-gal activity was solely dependent on RpoD and we expected that the *rpoD*⁺ P90A5c strain would have the same β-gal activity as *rpoS*+ B23 strain because both strains possess wild-type *rpoD*. However, we saw that β-gal activities for the *rpoS*⁺ B23 and *rpoS*⁻ BD792 strains were higher than the activities for *rpoD*⁺ P90A5c and *rpoD*⁻ 285c strains by factors of 50 (Fig 1a and Fig 1b). This variance may be attributed to strain differences between wild-type B23 and *rpoD*⁺ P90A5c, since these are not isogenic (16). These results indicated that under IPTG induction, RpoS was not involved in regulating β-gal activity and RpoD may not be the sole regulator of *lacZ*.

***E. coli rpoS*⁻ BD792 strain exhibited significantly higher β-galactosidase activity than *rpoS*⁺ B23 strain at 15 and 60 minutes of heat-shock at 42ºC.** To test our hypothesis that decreasing RpoS through heat-shock would increase β-gal activity, *rpoS*⁺ B23 strain and *rpoS*⁻ BD792 negative control strain were induced with IPTG during logarithmic growth phase, exposed to heat, and then measured with Miller assays for β-gal activity. The *rpoS*⁻ BD792 strain showed higher β-gal activity compared to *rpoS*⁺ B23 strain after heat-shock at 15 minutes and 60 minutes of heat-shock but not at 30 minutes of heat-shock (Fig 2).

Based on our hypothesis, we expected the *rpoS*⁻ BD792 β-gal activity to be significantly higher than *rpoS*⁺ B23 initially, but for it to converge with that of B23 as heat-shock duration increased and RpoS levels in B23 decreased, resulting in higher β-gal activity in B23. However, while the β-gal activity increased after the initial heat-shock at 0 min for both B23 and BD792, the β-gal activities from 15 min to 60 min were statistically constant with no significant differences between the different time-points (Fig 2). In addition, the increase in β-gal activity upon heat-shock for both *rpoS*⁺ and *rpoS*⁻ strains indicated that regardless of presence of *rpoS*, heat-shock caused β-gal activity to increase (Fig 2). These results revealed that there must be other underlying mechanism responsible for *lacZ* expression apart from RpoD (Fig 3).

DISCUSSION

The transcription of bacterial DNA into mRNA is dependent on the biochemical characteristics of individual sigma factors (1, 2). Each sigma factor must bind to RNAP core proteins to increase their affinity for specific promoter regions of DNA (5). The up-regulation and down-regulation of sigma factors is dependent on the environment conditions the bacteria experiences (3). The exception is RpoD, the "housekeeping" sigma factor (6). While RpoD expression does not change in different environments, it can be out-competed by other sigma factors with greater affinity for RNAP (6). For instance, when environmental temperature is less than 42ºC, RpoS outcompetes RpoD for the RNA polymerase core (6–8). Our study intended to assess the connection between RpoS and RpoD-dependent β-gal activity at high temperatures. RpoD is responsible for translation of LacZ proteins; by using β-gal activity as a reporter, we were able to study RpoD activity.

To demonstrate that the sigma factor RpoD is necessary for *lacZ* expression and that RpoS is not involved, we acquired *rpoS*⁺ B23, *rpoS*⁻ BD792, *rpoD*⁻ 285c, and *rpoD*⁻ BD792 strains. Our experiments showed that knocking out *rpoS* without changing *rpoD*, as in the *rpoS*⁻ BD792 strain, had no significant impact on the β-gal activity (Fig 1b). However, when the *rpoD* gene was non-functional, as in the *rpoD*⁻ 285c strain, there was a significant decrease in β-gal activity (Fig 1a). These results indicated that under optimal growth conditions, RpoS was not involved in *lacZ* expression while RpoD was. This supports our hypothesis that RpoD is important for β-gal activity.

To study the activity of RpoD under the presence and absence of *rpoS*, the heat-shock method was applied to *rpoS*⁺ B23 and *rpoS*⁻ BD792 strains. In the absence of heat shock, the β-gal activity of both B23 and BD792 strains were equal (Fig 2). However, our results showed that upon 15 minutes of heat-shock, the β-gal activity for both B23 and BD792 strains increased (Fig 2). According to previous studies, when the cellular concentration of RpoS was lowered due to down-regulation at high temperatures, the competition on RpoD for the RNAP core was relieved and β-gal activity increased (6–8). While this explanation satisfied the result for the *rpoS*⁺ B23 strain, it does not explain why the *rpoS*⁻ BD792 strain also experienced this increase due

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to its lack of *rpoS*. Therefore, heat-shock must have had another effect on the bacteria, independent of RpoS, which caused β-gal activity to increase (Fig 3). In addition, after 15 minutes of heat-shock, the β-gal activities for both strains plateaued and the differences observed between 15 minutes and 60 minutes were not statistically significant (Fig 2). The higher β-gal plateau values for *rpoS*$^-$ BD792 compared to those of *rpoS*$^+$ B23 indicated that high levels of β-gal activity was dependent on *rpoS* absence. However, we expected that the plateaus would converge by 60 minutes because exposing B23 to 42ºC heat-shock would decrease its cellular RpoS to a level that is close to that of BD792 (6–8). Since this convergence did not happen, the observed results can be attributed to two possible reasons. The first possible explanation is that the heat-shock duration was not long enough to down-regulate RpoS completely. A second possible explanation is that the B23 strain expresses several proteins of which BD792 lacks due to the absence of RpoS-mediated transcription. There is a possibility that those downstream proteins could cause lower β-gal activity. The first reason is less supported because previous studies have shown that 60 minutes was sufficient to down-regulate RpoS (17, 18). Therefore, we speculate that the second possibility is more likely since RpoS is responsible for the transcription of many genes (18). These genes that are expressed as a result of RpoS activity can potentially cause β-gal activity to decrease by sequestering the RNAP core or reallocating the bacteria's cellular resources under the heat-shock condition, amongst many other possibilities. However, further studies are necessary to give evidence for this speculation.

This study addressed the question of whether RpoD-dependent β-gal activity was enhanced when RpoS was down-regulated by heat-shock or was knocked-out as in *rpoS*$^-$ BD792. The results indicated that without heat-shock, RpoS did not have an effect on β-gal activity while RpoD was necessary (Fig 1a and Fig 1b). With heat-shock, while both B23 and BD792 strains showed increased β-gal activity, the presence of RpoS in B23 was correlated with a more moderate increase (Fig 2). This pattern indicated that heat-shock decreased the amount of RpoS that was in competition with RpoD in binding to the RNAP core, resulting in an increase in RpoD-dependent β-gal activity. Our results are in direct contradiction of previous research that stated heat-shock on *E. coli* increased RpoS and decreased RpoD-dependent gene expression (9). However, we are in agreement with other previously published research findings (7, 17). Our data also suggest there may be another protein involved in the regulation of RpoD-dependent β-gal activity that is indirectly linked to RpoS (Fig 3). This would explain the differences we observed between the *rpoS*$^+$ and *rpoS*$^-$ strains upon heat-shock.

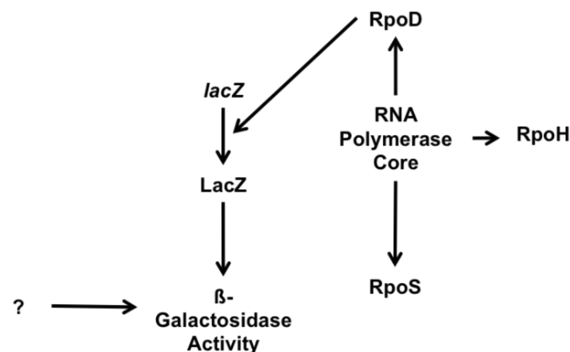

FIG 3 Proposed model of interactions that are involved in regulating β-galactosidase activity in *E. coli*. RNAP can be sequestered by any sigma factor (RpoD, RpoS, or RpoH). RpoD-RNAP transcribes the lacZ gene. Based on our study we theorize that there must be another component that affects ß-gal activity or RpoH-RNAP sequestering limits RpoD-RNAP activity.

FUTURE DIRECTIONS

While the Miller assay revealed significant β-gal activities for our strains under heat-shock and non-heat-shock conditions, we cannot clearly elucidate the cause of these differences. The results from our experiments indicate that there is another protein responsible for β-gal activity in addition to RpoS and RpoD. However, to fully determine how RpoS in being affected by heat-shock and if RpoS is responsible for the differences observed between B23 and BD792, we need to study *rpoS* mRNA levels. To assay what occurs at the mRNA level, we could conduct a qRT-PCR after heat-shock. Based on our results we would expect that *rpoS*$^+$ B23 would have more *rpoS* mRNA before the heat-shock.

In addition, we can transform a *rpoS*$^+$ plasmid into *rpoS*$^-$ BD792 strain to see if *rpoS*$^+$ phenotypes can be recovered. Following transformation, the BD792- *rpoS*$^+$ strain can be heat-shocked before performing the Miller assay. This experiment will determine if the *rpoS*+ B23 phenotype can be recovered by transforming in a *rpoS*$^+$ plasmid. If it can, then we can say with confidence that the absence of *rpoS* in BD792 accounted for the differences from B23 we observed in this study.

Once these tests confirm that the RpoS is the only difference between B23 and BD792, other sigma factors can be studied. One such sigma factor is RpoH, which is the heat-shock sigma factor that has higher levels at high temperatures (19). At high temperatures, RpoH sequesters the RNAP core (19). To conduct an experiment to examine the effects of this sigma factor on RpoD-dependent β-gal activity, a *rpoH*$^-$ *E. coli* strain can be compared with *rpoH*$^+$ BD792 and B23 strains using the same heat-shock and Miller assay procedures described in this paper. We expect that *rpoH*$^-$ *E. coli* strain would express greater RpoD-dependent β-gal activity than the *rpoH*$^-$ *E. coli* strain.

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