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Escherichia coli K12 Group 2 capsular biosynthesis does not appear to be thermoregulated by an RpoS-dependent mechanism

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Capsule production in *Escherichia coli* strains K12 and K30 requires the transcription factor RfaH which is regulated by unknown mechanisms in *E. coli*. However, in *Salmonella enterica* serovar typhi, RfaH is regulated by the stress-response sigma factor RpoS. In *E. coli* low temperatures have been shown to increase both RpoS expression and capsular polysaccharide production. However, more recent studies of have reported conflicting results concerning the temperature dependence of capsule regulation with reports of both 2-fold and 10-fold increases in capsule synthesis at 21˚C compared to 37˚C. We hypothesized that RpoS would be up regulated at 21˚C compared to 37˚C, resulting in increased capsule production. To investigate this, we grew *E. coli* strains K12, K30, and Δ*rpoS* of K12 at 21˚C and 37˚C and compared capsule production quantitatively using an adapted phenol-sulphuric acid assay and qualitatively using Maneval's staining protocol and light microscopy. We observed low carbohydrate concentrations and variable results with the capsule quantification assay, but were able to confirm previous results showing that Δ(*wza*)$_{K30}$ mutant strain of *E. coli* K30 is capsule deficient. Microscopy showed thicker capsule associated with wild type K30 compared to Δ(*wza*)$_{K30}$, but we did not observe any differences in capsule between 21°C and 37°C. To investigate whether RpoS activity is thermoregulated, we transformed K12 and mutant Δ*rpoS E. coli* with a *dsrB-lacZ* β-galactosidase reporter plasmid. *dsrB* is a downstream gene regulated by RpoS, and was thus used as a readout for RpoS activity. Our results failed to demonstrate the expected thermoregulation of RpoS, and an RpoS mutant exhibited significantly greater β-galactosidase activity than a K12^{dsrB} at 37°C. β-galactosidase activity units were higher than expected, suggesting that using a multi-copy plasmid to introduce the *dsrB* promoter::LacZ translational fusion reporter is not a sensitive method for measuring rpoS transcription levels. Our results warrant additional experimentation to determine the relationship between RpoS, temperature, and capsule production in *E. coli* K12 and K30 strains. Furthermore, alternative methods of measuring capsule production and RpoS expression should be explored.

Bacterial capsules are protective structures on the outer surface of many bacteria that serve as virulence factors and play an important role in adherence (1). *Escherichia coli* strains K12 and K30 group 2 and group 1 capsule biosynthesis pathways translocate capsular polysaccharides (*cps*) to the cell surface (1). Gene expression from *E. coli* group 1 and 2 *cps* loci (*wza*, *wzb*, *wzc*) is driven from a constitutive promoter and requires the transcription factor RfaH for RfaH-dependent anti-termination (1). Without RfaH, capsule production is significantly reduced (1). In *Salmonella enterica* serovar typhi, Bittner *et al.* showed that RpoS, a sigma factor that activates genes responsible for the transition to a stress-resistant phenotype, positively regulates the *rfaH* promoter (2). RpoS is responsible for the global activation of many pathways involved in resistance to osmotic, temperature, and starvation-related stresses (3). Both RpoS and RfaH are expressed highly during stationary phase, when stress-resistant phenotypes are likely to be engaged (2, 4).

Low temperature has also been shown to cause increased expression of *rpoS* through the action of the small regulatory RNA DsrA (5). DsrA increases translation efficiency of *rpoS* mRNA by altering its secondary structure (6). In *E. coli* K12, White-Ziegler *et al.* identified 122 genes under RpoS control up-regulated at 23˚C compared to 37˚C, many of which are associated with biofilm formation, suggesting that temperature may be a primary environmental cue in biofilm development. We therefore hypothesized that up-regulation of RpoS at 21˚C is required for increased capsule biosynthesis. Previous reports have revealed conflicting results regarding the role of temperature in the regulation of capsule biosynthesis. Botros *et al.* and Parmar *et al.* demonstrated a 10-fold increase in *E. coli* K30 group 1 capsule production at 21˚C compared to 37˚C (7, 8). However, Dhanoa *et al*. did not observe increased capsule biosynthesis at 21˚C (9). In this study, no difference in carbohydrate levels between *E. coli* K12 and a capsule deficient K30 strain Δ(*wza*)$_{K30}$ was observed at 21˚C (9).

Given the lack of clear evidence demonstrating the role of temperature on RpoS and capsule polysaccharide biosynthesis, we attempted to examine thermoregulation of RpoS and its effect on capsule production. We examined capsule biosynthesis in the *E. coli* K12 mutant strain JW5437-1 (*ΔrpoS746::kan*) as well as its parental wild-type (WT) strain BW25113 (10). We quantified capsule carbohydrate in both strains and indirectly measured RpoS activity levels using an RpoS regulated *dsrB* promoter*::LacZ* translational fusion reporter plasmid (5). In our hands, the phenol-sulphuric acid carbohydrate quantification assay detected low carbohydrate levels and inconsistent results. An attempt was made to measure *rpoS* transcription levels at different temperatures by introducing the multi-copy *dsrB* promoter*::LacZ* fusion reporter

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plasmid in an Δ*rpoS E.coli* K12. We did not observed the previously reported suppression of *dsrB* promoter activity obtained when this construct was transformed into a similar Δ*rpoS* mutant *E.coli* K12 strain as a single-copy reporter (5). Additionally, we observed no difference between the thickness of capsule when comparing K12 and Δ*rpoS* strains grown at either 21°C or 37°C by microscopy. Overall, our results suggest that RpoS may not play a key role in the thermoregulation of *E. coli* group 2 K12 capsule polysaccharide biosynthesis.

MATERIALS AND METHODS

Bacteria Strains, Preparation of Growth Media, and Conditions. *E. coli* K12 strains BW25113 and JW5437-1 were obtained from the MICB 447 bacterial culture collection in the Department of Microbiology and Immunology, University of British Columbia. JW5437-1 contains a deletion in *rpoS* (*ΔrpoS746::kan*). All other genetic mutations are shared in the two strains, including Δ(araD-araB)567, ΔlacZ4787(::rrnB-3), λ-, Δ(rhaDrhaB)568 and hsdR514 (Kieo). *E. coli* K30 control strains E69 (serotype: O9a:K30:H12), CWG655 [$wza_{22\ min}$*::aadA* Δ(*wza-wzb-wzc*)$_{K30}$*::aphA3* Km^r Sp^r], and CWG281 (E69 wza_{K30}*::aaC1*, $wza_{22\ min}$*::aadA*; Gm^r, Sp^r) were obtained from Dr. Chris Whitfield's laboratory (Department of Molecular and Cellular Biology, Univeristy of Guelph). CWG655Δ [*wza-wzb-wzc*$_{K30}$] and CWG281 have a polar aadA insertion in the *wza* locus corresponding to 22 minutes on the *E. coli* K12 lineage map that eliminates expression of this copy of the *wza-wzb-wzc* locus (11). The second loci of *wza-wzb-wzc* (CWG655) or *wza* alone (CWG281) have been inactivated using PCR amplification and cloning into the suicide vector pWQ173, which was used to excise parts of *wza* and *wzc* as well as all of *wzb* (11). DH5α was utilized as a positive control for the Miller β-galactosidase assays. In this paper, strains BW25113 and JW5437-1 are referred to as WT-K12 and RpoS-/- respectively, while E69, CWG281 are referred to as WT-K30, and $wza^{-/-}$ respectively. Liquid cultures were incubated on a shaker contained in either a 37˚C incubator or at room temperature (approximately 21˚C). Plates were incubated in either a 37˚C incubator or at room temperature (approximately 21˚C). Bacterial cultures were grown in Luria Bertani (LB) broth (1.0% w/v tryptone, 0.5% w/v yeast extract, 0.5% w/v NaCl, pH 7) or LB (1.5% agar) plates for capsule isolation and staining. For the miller β-galactosidase assay, bacterial cells were grown on LB plates.

Growth Curves. *E. coli* WT-K12 and Δ*rpoS* strains were grown in LB broth overnight at either 21˚C or 37˚C. 10 ml LB broth cultures were inoculated to OD_{660} of 0.1-0.15 to initiate lag phase growth. OD_{660} was recorded every hour until stationary growth phase was achieved.

Qualitative Validation of Δ*rpoS* strain. *E. coli* WT-K12 and Δ*rpoS* experimental strains were grown in two replicates on LB agar at either 21˚C or 37˚C as an ~3 cm diameter smear. These colonies were tested with two different qualitative readouts of RpoS activity. The catalase test was performed by dropping 200 µl of 32% hydrogen peroxide (H_2O_2) onto the centre of the smear, and monitoring any subsequent bubble formation (12). Iodine assays were performed by dropping 5 drops of iodine onto smears and observing the presence or absence of a brown precipitate (13).

Transformation of Chemically Competent *E. coli* BW25113 and JW5437-1. *E. coli* WT-K12 and Δ*rpoS* strains were made chemically competent using the $CaCl_2$ transformation method described in the Hancock Lab Protocols (14). Additional aliquots of competent *E. coli* BW25113 and JW5437-1 cells were stored at -80˚C. 2 µl of a *dsrB* promoter*::LacZ* translational fusion RpoS activity reporter obtained from Sledjeski *et al*. was mixed gently with chemically competent *E. coli* BW25113 and JW5437-1 cells and incubated on ice for 30 minutes. Cells were then heat shocked at 42°C for 45 seconds, and returned to ice for 1 minute. 1.2 ml of LB medium was added to the reaction vial and incubated at 37˚C in a shaking incubator at 200 rpm for a 1-hour recovery period. 100 µl of the transformed cells were then spread on LB agar plates supplemented with ampicillin (100 µg/mL) to select for transformants. We denote the multi-copy transformed WT-K12 and Δ*rpoS* strains as WT-K12^{dsrB} and Δ*rpoS*^{dsrB} respectively.

Capsule Extraction and Quantification by Phenol-Sulphuric Acid Assay. Surface polysaccharides were extracted and quantified using a modified version of the phenol-sulphuric acid assay protocol developed by Brimacombe *et al.* (15). *E. coli* strains were grown at 37˚C overnight and at 21˚C for two nights to reach stationary phase. Overnight cultures were diluted 1/10, and the OD_{650} was recorded. 1 ml of each culture was centrifuged at 14,600 x g for 5 minutes using an Eppendorf 5414D Microcentrifuge. The cells were then resuspended in 700 µl of 50 mM NaCl and centrifuged at 14,600 x g for 3 minutes. The washing step was repeated 3 times. The final cell pellet was resuspended in 1 ml of 50 mM EDTA and incubated at 37 ˚C for 60 minutes to dissociate capsule and LPS from the cells. Cells were then spun at 14,600 x g for 3 minutes, and the supernatant (containing any tightly-bound capsule and LPS) was collected in clean microcentrifuge tubes. Carbohydrate was then subjected to a phenol-sulphuric acid carbohydrate quantification assay as follows: 200 µl of each sample was added to glass tubes, and 200 µl of 50 mM EDTA was added to an additional tube as a control. In the fume hood, 200 µl of 5% phenol and 1 ml of 93% sulphuric acid were added simultaneously. Colour was allowed to develop for 10 minutes at room temperature after which optical density was measured at 490 nm using a Spectronic 20+ spectrophotometer. A standard curve was generated using a 1 mg/ml carbohydrate stock solution (1:1 sucrose:fructose).The absorbance readings of the samples were normalized to the carbohydrate standards to calculate carbohydrate concentration. The concentration was divided by the original OD_{650} of the overnight culture to generate a normalized carbohydrate concentration for each sample.

Capsule Staining. Control strains were grown overnight at either 21˚C or 37˚C on either LB plates or in LB broth. Colonies were taken from the plates using a sterilized loop and suspended in 250 ul of sterile saline. Capsule staining was performed using a modified version of the Maneval's capsule staining method described by Hughes and Smith (16). Cell suspensions were mixed with 250 µl of Congo Red (1% aqueous solution, Sigma C-6767), spread onto a glass microscope slide using a sterilized loop, and air-dried for 5-10 minutes. Maneval's solution (0.047% w/v acid fuchsin, JT Baker Chemicals, A355-3; 2.8% w/v ferric chloride, Fisher Scientific I-89; 4.8% v/v aqueous glacial acetic acid, Acros, 42322-0025; 3.6% v/v aqueous phenol solution, Invitrogen IS509-037) was then pipetted onto the dried smears and allowed to sit for approximately 2 minutes. The counterstain was washed off with dH_2O and slides were air dried prior to viewing using an Axiostar plus light microscope at 1000x magnification with oil immersion. The images were captured using Axiovision LE64 software.

Miller β-galactosidase Assays. β-galactosidase (β-gal) assays were performed using methods based on the original Miller publication describing the development of this assay (17). Overnight solid LB media cultures of WT-K12^{dsrB} and Δ*rpoS*^{dsrB} strains were grown at 21˚C and 37˚C. Additionally, *E. coli* control strains DH5α and WT-K12 were grown on LB plates overnight at 37°C as positive and negative controls for β-gal activity, respectively. Using sterile swabs, turbid cell solutions were prepared in 2 ml of freshly prepared, chilled Z-buffer (0.6 M Na_2HPO_4, 0.4 M NaH_2PO_4, 0.01M KCl, 1 mM $MgSO_4$, 0.27 % (v/v) β-mercaptoethanol, pH 7). OD_{600} of the resuspended cell

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solution was then measured by blanking against Z-buffer. 10 µl Z-buffer cell suspension was added to 990 µl of Z-buffer. The cells were then permeabilized by adding 100 µl chloroform and 50 µl 0.01% (w/v) SDS. Samples were vortexed for 5 seconds and equilibrated for 5 minutes at 30˚C. 200 µl of freshly prepared substrate solution (4 mg/ml o-nitrophenyl β-D-galactopyranoside in 0.6 M Na_2HPO_4, 0.4 M NaH_2PO_4, pH 7 phosphate buffer) was added to start the reaction at 30°C. Stop solution (0.5 ml of 1M Na_2CO_3) was added after sufficient yellow color (similar to undiluted LB) had developed. 1 ml of the stopped reaction was transferred to a 1.5 ml eppendorf tube and spun for 5 minutes to remove debris and chloroform. $Absorbance_{420}$ and $Absorbance_{550}$ were recorded and Miller Activity Units were calculated using the equation below.

$$Miller\ Units = 1000 \times (OD_{420} - 1.75 \times OD_{550})/(T \times V \times OD_{600})$$

Statistical Analysis. Statistical analysis was performed for the phenol-sulphuric acid capsule carbohydrate quantification assay and the RpoS activity β-gal reporter assay. Statistical significance was determined using an unpaired, two-tailed t-test ($p<0.05$). For the phenol-sulphuric acid capsule carbohydrate quantification assay, comparisons were made between WT-K30, Δ(*wza*)$_{K30}$, WT-K12, and Δ*rpoS* strains at both 21˚C and 37˚C. Comparisons were also made between 21˚C and 37˚C for the WT-K30, Δ(*wza*)$_{K30}$, WT-K12, and Δ*rpoS* strains. For the β-gal reporter assay, comparisons were made between WT-K12^{dsrB} and Δ*rpoS*^{dsrB} at both 21˚C and 37˚C and between the 21˚C and 37˚C groups for each respective experimental strain.

RESULTS

The phenol-sulphuric acid capsule quantification method yielded low capsule concentrations and inconsistent results for WT-K12, ΔRpoS, and WT-K30 *E. coli* strains. To quantify the amount of capsule produced by each bacterial strain at 21˚C and 37˚C, WT-K12, *ΔrpoS*, and Δ(*wza*)$_{K30}$, and WT-K30 cells were grown overnight on LB plates, and capsule was isolated using an adapted phenol-sulphuric acid assay (15). As expected, the Δ(*wza*)$_{K30}$ strain, which has been described as capsule deficient (18), showed a negative phenotype in our assay (Fig. 1). The WT-K12 strain exhibited higher capsule production at 21˚C compared to 37˚C (Fig. 1), similar to results described previously (7, 8). However, while Botros *et al.* and Parmar *et al.* observed a 10-fold increase in capsule production at 21˚C, we observed only a 2 to 4-fold increase at 21^{0}C compared to 37^{0}C, which was not a statistically significant difference. The Δ*rpoS* strain showed increased capsule production at 37˚C compared to 21˚C (Fig. 1). The WT-K30 strain also showed higher capsule production at 37˚C compared to 21˚C (Fig. 1), but these results were also not statistically significant. Overall, the assay yielded inconsistent results and did not show the decrease in capsule production in the Δ*rpoS* mutant that we expected. It is possible that the variability could be a result of the low carbohydrate concentrations obtained from these strains of *E. coli*, as the measured values were near the detection limit for the spectrophotometer.

***E. coli* Δ*rpoS* strain exhibits reduced catalase activity and glycogen levels.** We confirmed the phenotype of our Δ*rpoS* strain using catalase and iodine assays. RpoS-deficient strains are known to exhibit suppressed catalase expression, and thus do not form bubbles (12), as we observed (Fig. S1). Additionally, RpoS-deficient strains do not produce glycogen, and thus do not turn yellow during the colourimetric iodine-glycogen assay (Fig. S2) (13). We generated growth curves for all experimental and control strains (Fig. S2) to obtain an accurate estimate of stationary phase for future experiments. All strains exhibited characteristic bacterial growth, and cultures grown at 21˚C demonstrate slowed growth compared to those grown at 37˚C.

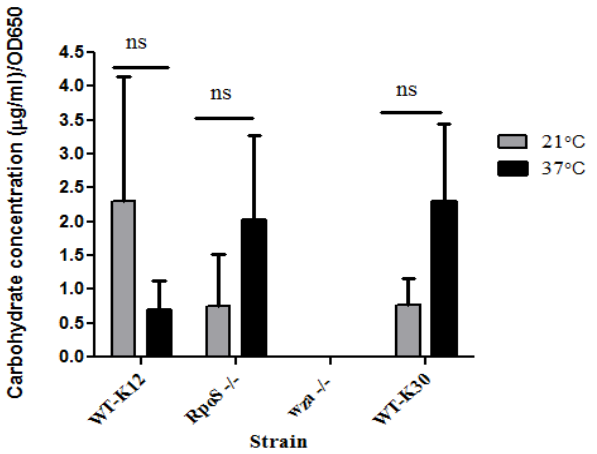

FIG 1. Concentrations of carbohydrate isolated from each strain grown on LB-agar plates at 21 and 37 ^{0}C. Optical density was read at $O.D._{490}$, and normalized to the $O.D._{650}$ of the culture suspension. n=2 for all strains and conditions. Mean ±SEM (Standard Error of mean) is shown. ns indicates that the differences shown are not significant.

WT-K12 and Δ*rpoS* strains do not exhibit increased capsule production by microscopy visualization at 21˚C. To confirm previously observed increases in capsule thickness in WT-K30 compared to Δ(*wza*)$_{K30}$ at 21˚C, we performed capsule staining using a modified version of Maneval's capsule staining protocol (16), and assessed capsule size by light microscopy. As expected, the WT-K30 strain demonstrated thicker capsule compared to Δ(*wza*)$_{K30}$ (Fig. 2 A, B). However, we did not observe any differences in capsule staining between WT-K12 and Δ*rpoS* grown at 21°C and 37°C, consistent with our findings from the phenol-sulphuric acid biochemical capsule quantification assay (Fig. 2 C, F).

WT-K12^{dsrB} and Δ*rpoS*^{dsr} multi-copy reporter strains demonstrate variable β-galactosidase activity. To determine if RpoS activity is thermoregulated, we compared the β-gal activity of a WT-K12^{dsrB} reporter strain grown to stationary phase at either 21˚C or 37˚C. In addition, we compared the β-gal activity levels of Δ*rpoS*^{dsrB} and WT-K12^{dsrB} strains grown at both temperatures to replicate finding by Sledjeski *et al*. showing that *rpoS* mutants are an optimal negative control for the single copy version of this reporter assay. We chose to utilize the Miller assay to study β-gal activity levels due to its well characterized reproducibility and ease of implementation in *E. coli* strains

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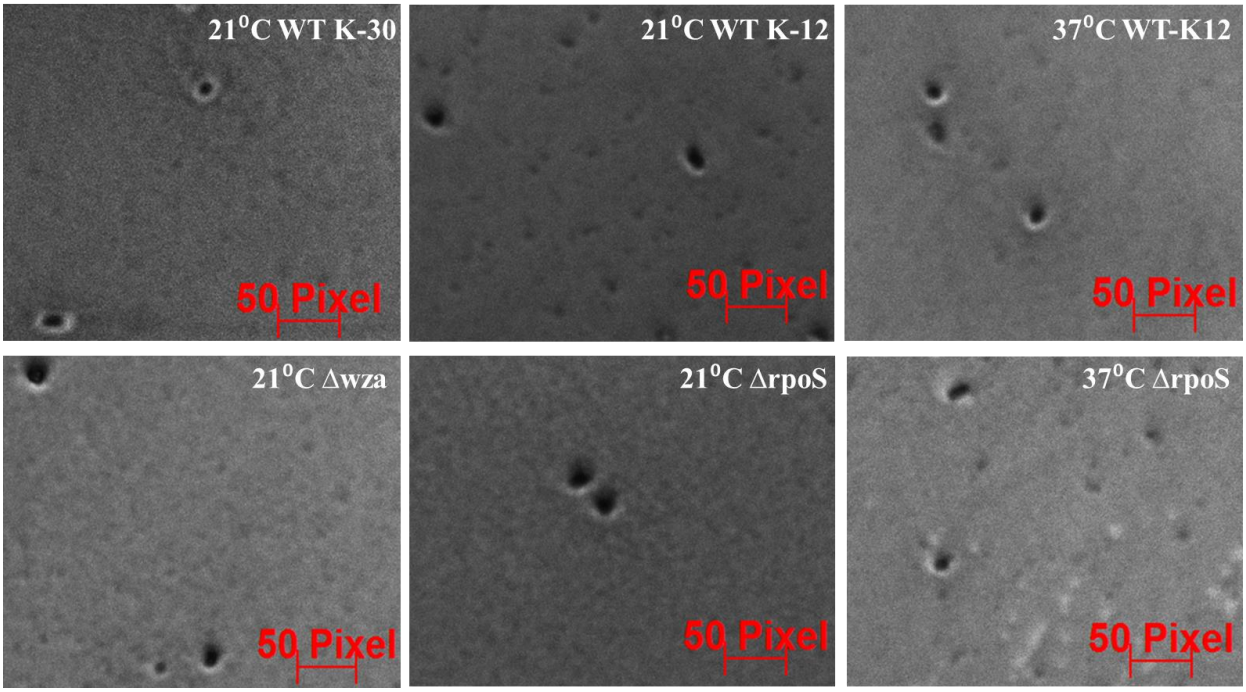

FIG 2. Similar levels of capsule thickness in WT-K12 and ΔrpoS strains grown in LB broth at 21^0 and 37^0. (A) E69 WT-K30 cells grown in LB broth at 21^0. (B) Δ*wza* cells grown in LB broth at 21^0. (C) WT-K12 cells grown in LB broth at 21^0. (D) WT-K12 cells grown in LB broth at 37^0. (E) Δ*rpoS* cells grown in LB broth at 21^0. (F) Δ*rpoS* cells grown in LB broth at 37^0.

(17). The results of the Miller β-gal assay failed to demonstrate the expected observed suppression of *dsrB* promoter activity in RpoS mutants, with the Δ*rpoS*^{dsrB} strain exhibiting significantly greater β-gal activity than the WT-K12^{dsrB} at 37˚C (Fig. 3). At 21˚C however, there was no significant difference obtained between the WT-K12^{dsrB} and Δ*rpoS*^{dsrB} strains β-gal activity (Fig. 3). The high range of the observed β-gal activities (10,000-20,000) in this assay is characteristic of a multi-copy β-gal reporter assay because fully induced single copy LacZ expression is known to be approximately 1500 activity units (17). These results suggest that multi-copy implementation of this *dsrB* promoter::*LacZ* translational fusion reporter is not a reliable method for measuring RpoS activity.

DISCUSSION

Previous studies indicate that the majority of genes in *E. coli* (approximately 40%) expressed at low temperatures (18-23°C) are regulated by RpoS (6, 19). Of the genes with increased transcription at low temperatures, the category of genes specifically noted were those associated with biofilm development. Previous reports by Botros *et al.* and Parmar *et al.* also demonstrated 10-fold increases in WT *E. coli* K30 group 1 capsule production at 21˚C compared to 37˚C (7, 8). These observations broadly support the model that low temperature is a primary environmental cue that triggers the general stress response. We therefore hypothesized that RpoS may regulate increased group 2 capsule biosynthesis at 21˚C compared to 37˚C and that deletion of *rpoS* may lead to decreased capsule production at 21˚C.

Biochemical quantification of capsule production using the phenol-sulphuric acid assay and microscopic analysis indicated no significant difference in capsule biosynthesis between Δ*rpoS* and WT strains at either temperature (Fig. 1 and Fig. 2). Δ(*wza*)$_{K30}$ was used as a negative control and showed minimal capsule production, as expected. This result is consistent with Dhanoa *et al.*'s findings, which failed to demonstrate increased capsule biosynthesis at 21˚C, showing no difference in carbohydrate levels between WT *E. coli* K12 and a capsule deficient K30 strain Δ(*wza*)$_{K30}$ (9). The lack of significant difference and the inconsistencies between our findings and those reported by previous studies may be explained by differing protocol optimization employed in the present study and influence of different K30 and K12 strain backgrounds.

Surface polysaccharides were extracted and quantified using a modified version of the phenol-sulphuric acid assay protocol developed by Brimacombe *et al.* (15). Among many colorimetric methods for carbohydrate determination, the phenol–sulfuric acid method is the simplest and most reliable method for measuring neutral sugars in oligosaccharides, proteoglycans, glycoproteins, and glycolipids (20). Important phenol-sulphuric acid assay protocol optimization steps unique to our study were the isolation of carbohydrate from cultures grown on LB plates, time-sensitive preparation of phenol and sulphuric acid reagents, and the sequence of addition of

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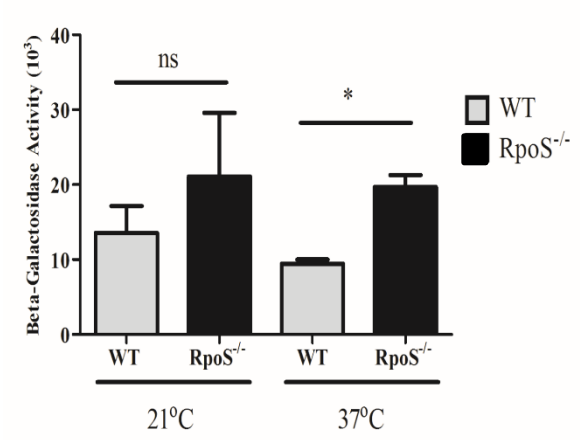

FIG 3 Multi-copy *dsrB*::LacZ fusion reporter exhibits increased activity in ΔrpoS strain at 37°C. β-galactosidase activity of WT-K12^{dsrB} and Δ*rpoS*^{dsr} grown on solid LB-amp media under low (21°) and high (37°) temperature conditions. n=2 replications per strain/condition tested. Mean ± SEM (standard error of mean) is shown. * indicates $p<0.05$, ns indicates not significant.

reagents prior to spectrophotometric reading. Previous studies by Dhanoa *et. al.,* Botros *et. al.,* and Parmar *et. al* isolated capsule from cultures grown in LB broth (7-9). To determine if the media impacted capsule biosynthesis we performed a simultaneous capsule quantification from solid and liquid cultures and showed no difference in capsule biosynthesis between the two (Fig. S3).

Our study also optimized the sequence of addition of reagents prior to spectrophotometric reading. Phenol and sulphuric acid were prepared fresh immediately prior to each experiment and were added simultaneously to the sample. We observed that over time, the 93% sulphuric acid begins to oxidize and turn yellow in colour. Since the spectrophotometric read-out of the assay is sensitive to optical density, the use of old, oxidized sulphuric acid could potentially increase these readings. In support of this, Parmar *et al.* and Botros *et al.* calculated carbohydrate concentrations of 140 (µg/ml)/OD_{660} and 350 (µg/ml)/OD_{660}, respectively (7, 8). These values are considerably higher than the carbohydrate concentration of 127.7(µg/ml)/OD_{650} for *Rhodobacter capsulatus* obtained by Brimbacombe *et. al*. since *R. capsulatus* produces significantly more capsular carbohydrates than *E. coli* (personal communication, Brimacombe). Therefore, the high readings obtained by previous groups may be an artefact of sulphuric acid oxidation and may explain why we obtained lower carbohydrate readings compared to previous groups.

To assess whether RpoS activity is thermoregulated, we used a *dsrB* promoter::*lacZ* translational fusion reporter obtained from Sledjeski *et al.* as an indirect measure of RpoS activity. The dsrB promoter is regulated by RpoS, and consequently, all expression of β-galactosidase from the dsrB::LacZ fusion is dependent on RpoS (5). Previous studies show that a *rpoS* mutation abolished expression of the single copy dsrB::LacZ fusion in stationary phase and decreased it significantly in exponential phase (5). In the present study, Δ*rpoS* transformed with the multi-copy dsrB:LacZ fusion reporter failed to demonstrate significant suppression of *dsrB* promoter activity in RpoS mutants, with no significant difference obtained between the WT-K12^{dsrB} and Δ*rpoS*^{dsrB} strains β-galactosidase activity at 21˚C (Fig. 3). Unexpectedly, the Δ*rpoS*^{dsrB} strain exhibited significantly greater β-galactosidase activity than the WT-K12^{dsrB} at 37˚C (Fig. 3). The reporter employed by Sledjeski *et al.* was a single copy reporter incorporated into the bacterial genome via phage-mediated lysogenesis (5), which may explain this discrepancy. The use of this reporter as a multi-copy plasmid is evident in the high range of observed β-gal activities (10,000-20,000) in this assay (Fig. 3). These values are characteristic of a multi-copy β-gal reporter, as fully induced single copy LacZ expression is known to be approximately 1500 activity units (17). This suggests that using a multi-copy plasmid to introduce the *dsrB* promoter::*lacZ* translational fusion reporter is not a reliable method for measuring RpoS activity.

In summary, our work suggests that RpoS may not play a key role in the thermoregulation of *E. coli* group 2 K-12 capsule polysaccharide biosynthesis. Biochemical and microscopic analysis demonstrate that capsule biosynthesis in the Δ*rpoS* strain is not significantly different compared to WT K-12 at low temperatures. In our hands, *dsrB* promoter::*LacZ* fusion reporter analysis of β-gal activity was inconclusive.

FUTURE DIRECTIONS

Although our evidence demonstrates that RpoS may not be involved in the thermoregulation of *E. coli* capsule biosynthesis, the variability of our results suggests that additional replicate experiments are necessary to obtain significant differences between our experimental conditions. This could be conducted using the now optimized phenol-sulphuric biochemical capsule quantification assay or an alternate measure of capsule production, such as the previously attempted biofilm growth assay (9). Additionally, the limited resolution of capsule size at 100x magnification makes it difficult to accurately demonstrate variation in capsule production. Therefore, we propose that future projects aim to implement transmission electron microscopy strategies to more accurately quantify capsule biosynthesis.

Future studies could also look directly at RpoS expression in response to temperature and other environmental stresses. We obtained an *rpoS* promoter::LacZ translational fusion reporter plasmid, however, we were unable to test it due to lack of time. Due to our inability to replicate the previously observed down regulation of *dsrB* promoter activity in a Δ*rpoS* strain in a multicopy format, we also recommend that future groups develop single copy reporter systems from these constructs to eliminate background LacZ expression

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and more accurately quantify the thermoregulation of RpoS activity/expression.

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