

# Slower Growth and Increased Biofilm Formation of *Escherichia coli* K-12 Stringent Response Mutant, *ΔrelA/spoT*, under Isoleucine Starvation

He Huang, Vivian Jiang, Yu Qing Zhou

Department of Microbiology and Immunology, University of British Columbia, Vancouver, British Columbia, Canada

**SUMMARY** Biofilm formation contributes to bacterial virulence and its formation is known to be affected by environmental condition and nutrient availability. Cells enter stringent response under nutrient-limiting conditions where guanosine 5'-triphosphate-3'-diphosphate and guanosine 5'-triphosphate-3'-diphosphate, collectively referred to as (p)ppGpp, are continually synthesized by the enzymes RelA and SpoT, and hydrolysis of (p)ppGpp is downregulated. (p)ppGpp regulates growth and other cellular processes including fimbriae expression. To examine the effect of stringent response on biofilm formation of *Escherichia coli* K-12, we constructed a growth curve and measured biofilm formation for wild type (WT) K-12 and *ΔrelA/spoT* mutant JKLL12W-2 under nutrient-rich and isoleucine-limiting conditions. While the growth of the two strains were similar in nutrient-rich lysogeny broth, *ΔrelA/spoT* mutant displayed slower growth under isoleucine-limiting conditions. The *ΔrelA/spoT* mutant had significantly greater biofilm formation in nutrient-rich media and formed higher level of biofilm under lower level of isoleucine. Since the *ΔrelA/spoT* mutant showed higher biofilm formation under lower isoleucine levels, stringent response in WT *E. coli* K-12 downregulates biofilm formation in a nutrient availability dependent manner. Our study of ppGpp<sup>0</sup> *E. coli* mutants behave similarly to *Pseudomonas putida* KT2440, another member of the gammaproteobacteria family, in biofilm formation and may suggest that the stringent response of these two bacteria follow similar mechanisms.

## INTRODUCTION

Bacteria often form community structures called biofilms (1). Biofilms grow attached to surfaces or interfaces (2) and have important medical implications as they can increase bacterial resistance to antibiotics and disinfectants (1). Biofilm formation is also a virulence factor in host colonization and disease propagation (3, 4). The extent of biofilm formation for a given organism depends on conditions such as temperature and nutrient availability in its environment (2). *In vitro* growth of *Escherichia coli* biofilm varies with growth conditions and media (2).

The stringent response is an adaptive response to nutritional and energy starvation and is highly conserved across bacteria (5). It involves a variety of transcriptional and translational changes which alter growth rate and metabolism, and have been linked to signaling pathways involved in biofilm formation (1, 6). During stringent response in *E. coli*, the enzymes RelA and SpoT synthesize guanosine 5'-diphosphate-3'-diphosphate (ppGpp) and guanosine 5'-triphosphate-3'-diphosphate (pppGpp)(6), collectively referred to as (p)ppGpp, a key secondary messenger(6). RelA synthesizes (p)ppGpp in response to amino acid starvation while SpoT does so in response to amino acid, carbon, iron, phosphate and lipid starvation (6). SpoT also hydrolyzes (p)ppGpp when cell stress is low, thus regulating (p)ppGpp levels in the cell (6).

Cau *et al.* (8) showed that under isoleucine limiting conditions, *E. coli* ppGpp<sup>0</sup> mutants showed limited growth. Balzer *et al.* studied biofilm formation of *E. coli* K-12 stringent response mutants and found that the mutant formed highest level of biofilm in LB when compared to the WT and ppGpp<sup>0</sup> mutants grown in minimal media (1). To add on these two

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Address correspondence to:  
<https://jemi.microbiology.ubc.ca/>

studies, we aim to investigate the effect of varying isoleucine concentration on growth and biofilm formation.

We hypothesize that media promoting greater cell growth should induce greater biofilm formation. For this study, the culturing media used were M63B1 minimal media supplemented with varying concentrations of isoleucine used in both growth curve generation and biofilm assay. We chose to vary concentrations of isoleucine to model a growth curve study done by Cau *et al.* (8). In addition, isoleucine is a potential target to induce nutrient starvation in *E. coli* as excess valine prevents the biosynthesis of isoleucine, results in the onset of growth arrest (9). Instead of adding excess valine, we decided to supplement with fewer amount of isoleucine to represent valine-induced isoleucine starvation. We did this because of the multiple amino acid auxotrophy associated with lack of (p)ppGpp in *E. coli* K-12 *AreIA/spoT* mutant (9). The growth curve of the wild type (WT) and the *AreIA/spoT* *E. coli* K-12 strains was generated over a 16-hour period at 37°C. Compared to the WT, we expect to see a decrease in the growth rate and a later onset of growth arrest in the *AreIA/spoT* mutant as the WT exhaust the isoleucine at a faster rate (9). A 24-hour biofilm assay was conducted using the WT, *AreIA/spoT*, and *AfimC* K-12 strains in order to quantitatively measure and compare the extent of biofilm formation. *AfimC* mutant was used as a negative control due its inability to form biofilm (10). *E. coli* K-12 belongs to the class of Gamma proteobacteria, stringent response mutants of various members of this class behaves differently. Some of these stringent response mutants have a trend of high biofilm formation compared to WT in rich media (11) whereas some are lower (1, 12, 13). Because of this trend, we expect highest biofilm formation in *AreIA/spoT* mutant grown in LB due to high nutrient levels (11). We also expected a positive correlation between the biofilm formation of *AreIA/spoT* and isoleucine level due to an increase in nutrient level (9). We believe that greater growth would be achieved with higher concentrations of isoleucine (9) and the biofilm would accumulate because the mutant can not disperse biofilm (12).

## METHODS AND MATERIALS

**Bacterial strains, media, and growth conditions.** *E. coli* K-12 WT strain BW25113 (14), *relA* mutant strain JWLL12W1-1 (15), *AreIA/spoT* mutant strain JWLL12W1-2 (15), and *AfimC* mutant strain JW4279-1 (10) were used in the study. Strains BW25113 (WT), JWLL12W1-1 (*relA*) and JWLL12W1-2 (*AreIA/spoT*) were obtained from the UBC MICB 421/447 Strain bank. Strain JW4279-1 (*AfimC*) was purchased from Coli Genetic Stock Center. All culturing was done at 37 °C with shaking at 250 rpm in lysogeny broth (LB) for 16-18 hours (Excella E24; New Brunswick Scientific). The M63B1 minimal media (100 µM KH<sub>2</sub>PO<sub>4</sub>, 0.8 mM MgSO<sub>4</sub>, 3 µM Thiamine B1, pH 7.2) was prepared following Chalabaev *et al.* (13). The M63B1 minimal media was supplemented with 0.2% dextrose and 400 µM of the 19 L-proteinogenic amino acids excluding isoleucine (7) and was prepared with 0, 30, 60 and 400 µM of isoleucine to model starvation (0 µM) and nutrient limitation (30, 60 and 400 µM).

**Strain genotyping.** Bacterial genomic DNA was isolated with the PureLink genomic DNA extraction kit (Invitrogen K1820-01) as per manufacturer protocol. PCR reactions were

**Table 1 PCR Primers used in the study**

Gene target	Forward sequence (5'-3')	Reverse sequence (5'-3')
<i>relA</i>	CTCCAGTGACATTGTCGACG TC	CATGCCGATGGCGACGCTC
<i>spoT</i>	CAACCGTTGTATGACACCCAGG	GAACCGTCGCTGATCTGGCAG
<i>fimC</i>	CTTACCGGTAAAGAAGTTCAGGCC	CCTGTGCAGTACCACGAGCAATAC

performed in 25  $\mu$ L volumes with 500 ng of template DNA using Platinum Taq Polymerase (ThermoFisher 10966026) as per manufacturer protocol. Primers used for PCR amplification of *relA*, *spoT*, and *fimC* regions are listed in Table 1, flanking 4kb long regions in WT *E. coli* K-12 genome, bracketing the genes of interest. T1000 Thermocycler from BioRad was used, with an initial denaturation step of 94°C for 2 minutes, followed by 30 cycles of 94°C for 30 seconds, 55°C for 30 seconds, and 72°C for 5 minutes. Agarose gel electrophoresis checked for successful amplification of PCR products and was performed using a 1% agarose gel with 0.5x TBE and visualized using SYBR Safe (ThermoFisher S33101) on a BioRad ChemiDoc Imaging System. PCR products were purified with PureLink PCR Purification Kit (Invitrogen K310001) and sent to Genewiz for Sanger sequencing in both the forward and reverse direction for each PCR product. Sequencing primers are listed in Table 2, the sequences should begin 100 bp upstream of the start codon or 100 bp downstream of the stop codon of *relA*, *spoT*, and *fimC*, respectively for the forward and reverse primer. If the gene of interest was deleted, the PCR products should be smaller than 4kb and the forward sequencing primers should produce sequences downstream from the gene of interest, such as downstream genes or antibiotic resistance cassettes. Subsequently, the reverse sequencing primers that originate from downstream of the gene of interest should produce sequences from the upstream region.

**Growth Curve.** The *E. coli* cultures were grown overnight in LB at 37°C. The *E. coli* cultures were then harvested by centrifugation at 5000 X gravity for three minutes and washed with M63B1 minimal media supplemented with 0  $\mu$ M of L-isoleucine to remove excess amino acid from overnight culture medium. Resulting cells were diluted in LB or in M63B1 with 0, 30, 60 or 400  $\mu$ M of L-isoleucine to a final optical density at 600 nm ( $OD_{600}$ ) of 0.01. From the diluted samples, 200  $\mu$ L was transferred to a transparent flat bottom 96-well polystyrene microtiter plate (Sarstedt). Resulting cultures were incubated at 37°C in a microplate reader (Synergy H1; BioTek).  $OD_{600}$  was measured every 10 minutes over a 16-hour period, shaking for 3 seconds before measuring.

**Biofilm Assay.** The 96-well biofilm assay was done following protocol written by O'Toole (16). Overnight *E. coli* cultures were diluted with LB or with M63B1 containing 0, 30, or 60  $\mu$ M of L-isoleucine to a final  $OD_{600}$  of 0.05. 200  $\mu$ L of diluted culture was transferred to a flat bottom 96-well polystyrene microtiter plate (Sarstedt) and incubated at 37°C for 24 hours prior to staining. Prior to incubating, the peripheral wells of the plate were loaded with 200  $\mu$ L of distilled water to prevent edge effect, which is a phenomenon where the fluids in peripheral wells evaporate at a greater rate than others during incubation (17). Staining was done with 130  $\mu$ L of 0.1% crystal violet (CV) dissolved in 10% ethanol and distilled water for 20 minutes after discarding the overnight culture. CV was then discarded and the wells were washed four times by adding 200  $\mu$ L of distilled water using a P200 multichannel pipette. The plate was dried upside-down at room temperature overnight. After drying, a 20-minute incubation with 130  $\mu$ L of 30% acetic acid solubilized the CV and destained the wells. From each well, 100 $\mu$ L of the resulting acetic acid was transferred to another flat bottom 96-well polystyrene microtiter plate (Falcon) and absorbance at 590 nm was measured using a microplate reader (Epoch; BioTek).

**Table 2 Sequencing Primers used in the study**

Gene target	Forward sequence (5'-3')	Reverse sequence (5'-3')
<i>relA</i>	GCAGGTCTGGTCCCTAAAG	CCTCAAACCGCTATCATATGTAG
<i>spoT</i>	GTTACCGCTATTGCTGAAGGTC	GCAGATGCGTGCATAACGTGTTG
<i>fimC</i>	CCTATCAGTAATTGTTTCAGCAGAT	CCCGGCAGTCAATTCTTTTATTC

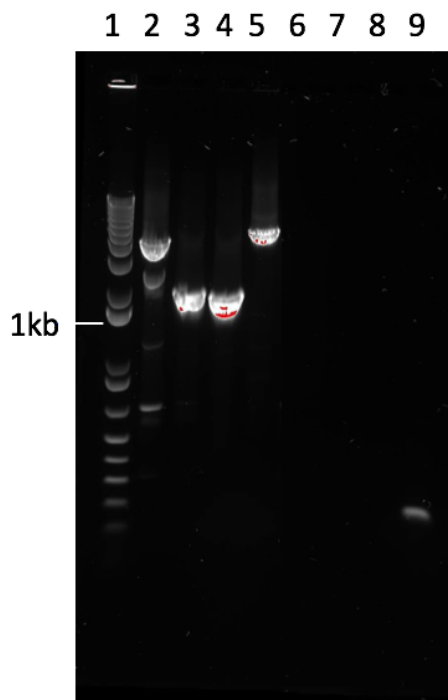
## RESULTS

**Confirmation of gene deletion in strains used via PCR and Sanger sequencing.** PCR amplification of gene deletion regions is successful as seen in Figure 1. Some contamination is visible in lane 2, possibly from non-specific binding of primers to the genomic DNA template. Since no contamination is seen in lane 6, we can rule out primer dimer formation and possible contamination in PCR master mix (Fig. 1). Regardless of the contamination, the size of the brightest band in lane 2 also raises questions (Fig. 1). Since JKLL12W-1 and JKLL12W-2 both has *relA* gene and kanamycin (KAN) cassette removed, after PCR amplification with identical primer set, they should both yield PCR products 2kb in size. In this case, lane 2 and lane 3 respectively showed bands 3.5kb and 1.5kb in size (Figure 1). We were unsure why this is, whether it is because the KAN cassette was not removed, or the *relA* gene is still present, or whether there is a mix-up at some point before PCR amplification. We followed through and sequenced purified PCR products from lanes 2,3,4,and 5 (Fig. 1). All 4 PCR products are sequenced in both the forward and reverse direction of gene of interest. This acts as insurance in case sequencing does not perform optimally in either direction.

Sequencing in reverse direction of *relA* region in JKLL12W-2 gave sequences of the upstream gene *mazE* (Fig. S1C). Sequencing in forward direction of *spoT* region in JKLL12W-2 gave sequences of the downstream gene *trmH* (Fig. S1C). Sequencing in forward direction of *fimC* region in JW4279-1 gave sequences of the KAN cassette (Fig. S1D). Based on the sequencing results (Fig. S1C-E), the deletion of *relA* and *spoT* in JKLL12W-2 and the deletion of *fimC* in JW4279-1 are confirmed. However the sequencing results for JKLL12W-1 came back negative as it appears to still contain the *relA* gene. Sequencing in the forward direction of *relA* region in JKLL12W-1 gave sequences starting at the 6th codon in the *relA* gene, followed by the rest of the *relA* gene (Fig. S1A). We checked by sequencing in the reverse direction, which showed the sequences of the *relA* gene. No KAN cassette was found next to the *relA* gene. Due to time constraints, we dropped JKLL12W-1 strain from our study and continued with the other three confirmed strains.

### *E. coli* K-12 $\Delta relA/spoT$ mutant experiences slower growth in nutrient-limiting media.

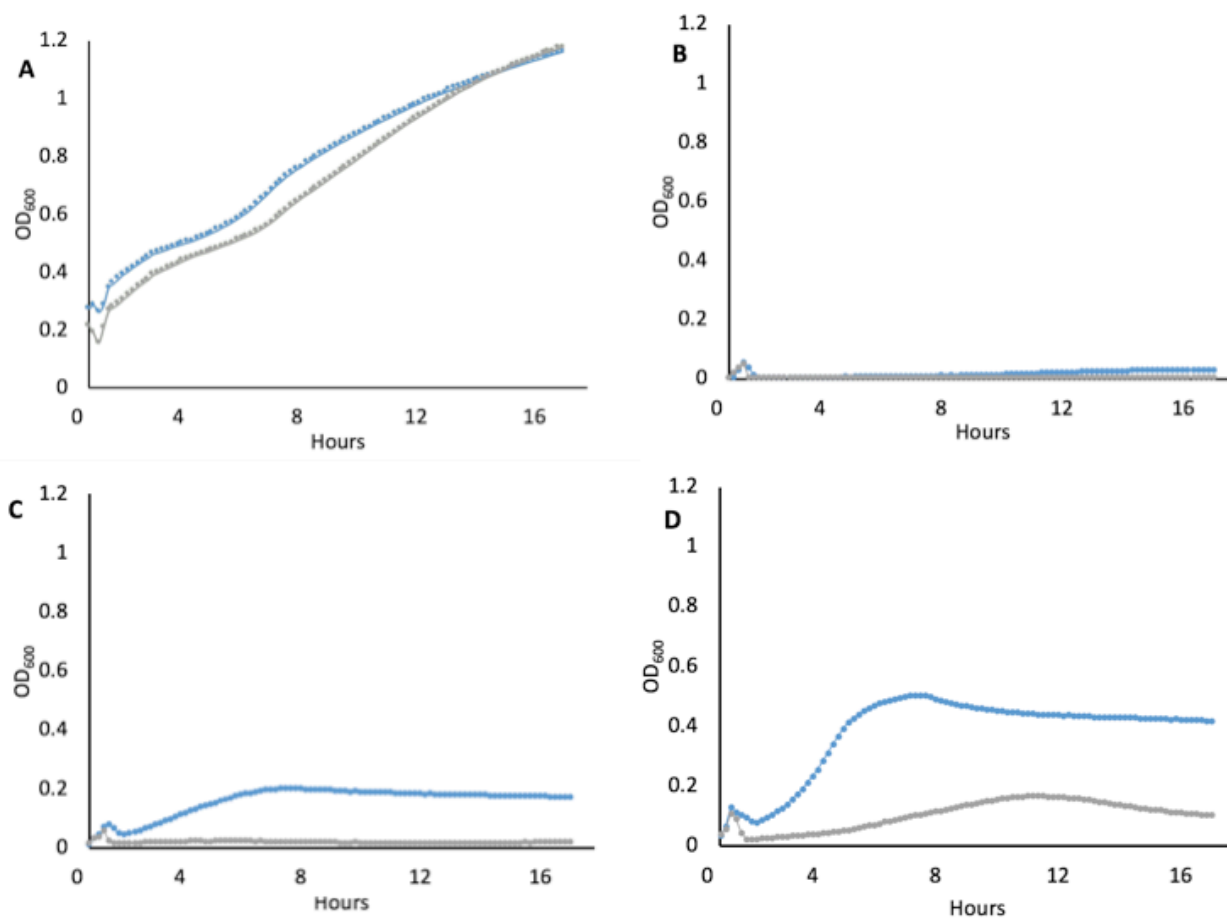
We generated a growth curve for both the WT strain BW25113 and the  $\Delta relA/spoT$  strain JKLL12W-2 over a period of 16 hours to determine effect of isoleucine starvation on growth. From this, we were able to directly compare the two strains, and investigate the



**FIG. 1** Confirmation of PCR amplification of regions flanking  $\Delta relA$ ,  $\Delta spoT$  in JKLLW1-2 and  $\Delta FimC$  in JW4279-1. Lane 1 is the 1kb Ladder. Lanes 2-5 are PCR products that amplified regions of interest, respectively from JKLL12W-1 ( $\Delta relA$ ), JKLL12W-2 ( $\Delta relA$ ), JKLL12W-2 ( $\Delta spoT$ ) and JW4279-1 ( $\Delta fimC$ ). Lanes 5-7 are primer only negative controls for:  $\Delta relA$ ,  $\Delta spoT$  and  $\Delta fimC$ . Lane 8 is the pUC19 positive control.

phenotypical growth changes caused by the absence of *relA* and *spoT*. When grown in nutrient-rich media such as LB, both strains displayed similar growth pattern (Fig. 2A). After 16 hours of incubation in LB, both the WT and the  $\Delta relA/spoT$  reached the same absorbance,  $OD_{600} \sim 1.2$  (Fig. 2A). Growing the strains in LB did not seem to affect the growth rate of the two strains. When grown in minimal media supplemented with varying concentrations of isoleucine, the  $\Delta relA/spoT$  mutant displayed slower growth compared to the WT (Fig. 2B-D). When grown in media supplemented with 60  $\mu\text{M}$  of isoleucine for 16 hours, WT achieved higher growth compared to the  $\Delta relA/spoT$  mutant,  $OD_{600} \sim 0.50$  and 0.17 respectively (Fig. 2D). For strains that grew in media supplemented with 30  $\mu\text{M}$  of isoleucine, similar growth pattern was observed (Fig. 2C). Compared to the WT, the  $\Delta relA/spoT$  mutant experienced later growth arrest. When grown in minimal media supplemented with 60  $\mu\text{M}$ , the WT entered stationary phase after 6 hours of incubation whereas the  $\Delta relA/spoT$  mutant entered stationary phase after 10 hours of incubation (Fig. 2D). The same trend was shown for strains that grew in minimal media supplemented with 400  $\mu\text{M}$  of isoleucine where the WT and the  $\Delta relA/spoT$  mutant experienced growth arrest after 4.9- and 5.8- hours of incubation respectively (Fig. S2). Based on the results collected, we know that  $\Delta relA/spoT$  mutant experiences slower growth compared to the WT and they also enter stationary phase at a later time.

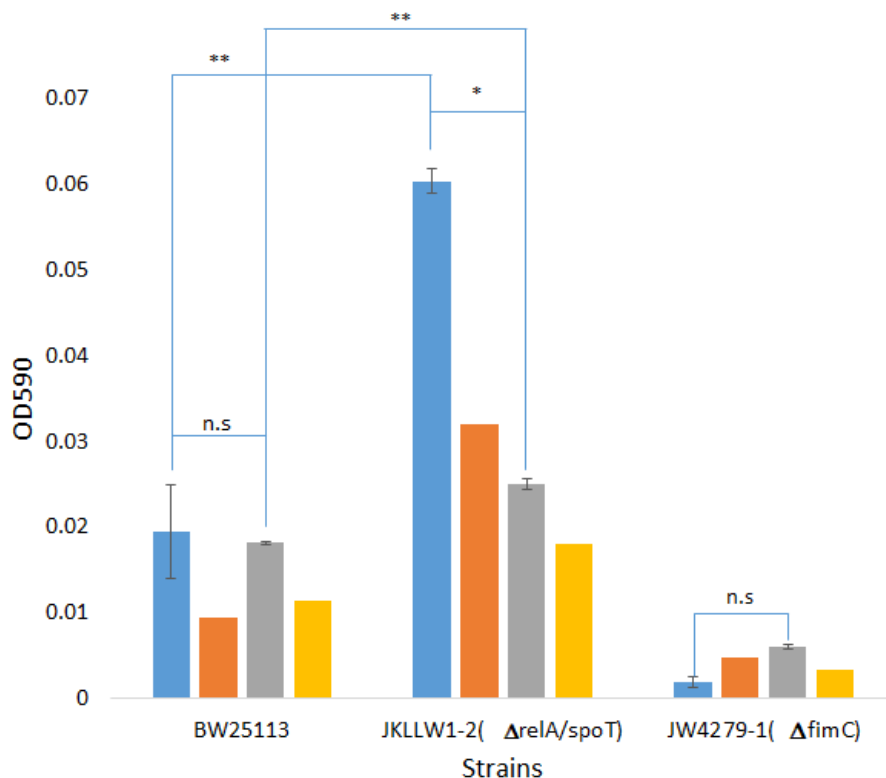
***E. coli* K-12 WT and  $\Delta relA/spoT$  mutant show an isoleucine concentration dependent growth rate in minimal media.** For both the WT and the  $\Delta relA/spoT$  mutant, there was an isoleucine dependent increase in growth in nutrient limiting conditions. When grown in minimal media supplemented with 30  $\mu\text{M}$  of isoleucine, the highest absorbance of the WT and the  $\Delta relA/spoT$  mutant were  $OD_{600} \sim 0.2$  and 0.02 respectively (Fig. 2C). After doubling



**FIG. 2** Growth profile of BW25113 (blue) and JKLLW1-2 (grey) in LB (A), M63B1 with 0  $\mu\text{M}$  L-Ile (B), 30  $\mu\text{M}$  L-Ile (C), 60  $\mu\text{M}$  L-Ile (D). Concentration dependent growth inhibition and early onset growth arrest in minimal media with decreasing amounts of L-Ile.

the isoleucine concentrations, the highest absorbance of the WT and the *ΔrelA/spoT* mutant were OD<sub>600</sub> ~0.40 and 0.17 respectively (Fig. 2D). When compared to minimal media supplemented with 0 μM of isoleucine, the WT and the *ΔrelA/spoT* mutant had the lowest amount of growth, OD<sub>600</sub> ~0.03 and 0.004. Altogether, with an increase in the amount of isoleucine supplemented in the minimal media, there was an increase in growth for both *E. coli* K-12 WT strain and *ΔrelA/spoT* mutant.

***ΔrelA/spoT* mutant has higher level of biofilm formation compared to the WT.** We conducted a 96-well microtiter biofilm assay to compare the level of biofilm formed for the WT and the *ΔrelA/spoT* mutant strains in different media. This allows us to gain insight as to how the absence of the *relA* and the *spoT* genes affect biofilm formation. Our negative control, *ΔfimC* mutant JW4279-1, displayed the lowest level of biofilm formation, OD<sub>590</sub> values around 0.002-0.006 (Fig. 3). Similar levels of biofilm was formed by the *ΔfimC* mutant in LB and minimal media supplemented with 30 μM of isoleucine (p>0.05). This shows that the *ΔfimC* mutant successfully functions as a negative control and the data collected for the other tested strains are reliable. Regardless of the media used, the *ΔrelA/spoT* mutant formed more biofilm in comparison to the WT strain. When grown in LB, a nutrient rich media, for 24 hours, the amount of biofilm formed by the *ΔrelA/spoT* mutant was three times greater than the amount of biofilm formed by the WT (p<0.05), OD<sub>590</sub> ~0.6 and 0.2 respectively (Fig. 3). When grown in minimal media supplemented with 30 μM of isoleucine, the *ΔrelA/spoT* mutant still displayed higher level of biofilm, however not as high as its levels in LB media (p<0.05). We were not able to make a confident comparison between the WT and the *ΔrelA/spoT* mutant for their level of biofilm formation in minimal media supplemented with 0 and 60 μM of isoleucine due to the lack of biological replicates (n=1). However, compared to the WT, the *ΔrelA/spoT* mutant formed three times more biofilm in media supplemented with 0 μM of isoleucine and two times in media with 60 μM of isoleucine (Fig. 3). Altogether, our observation shows that the *ΔrelA/spoT* mutant forms higher level of biofilm than the WT in both nutrient-rich and limiting media.



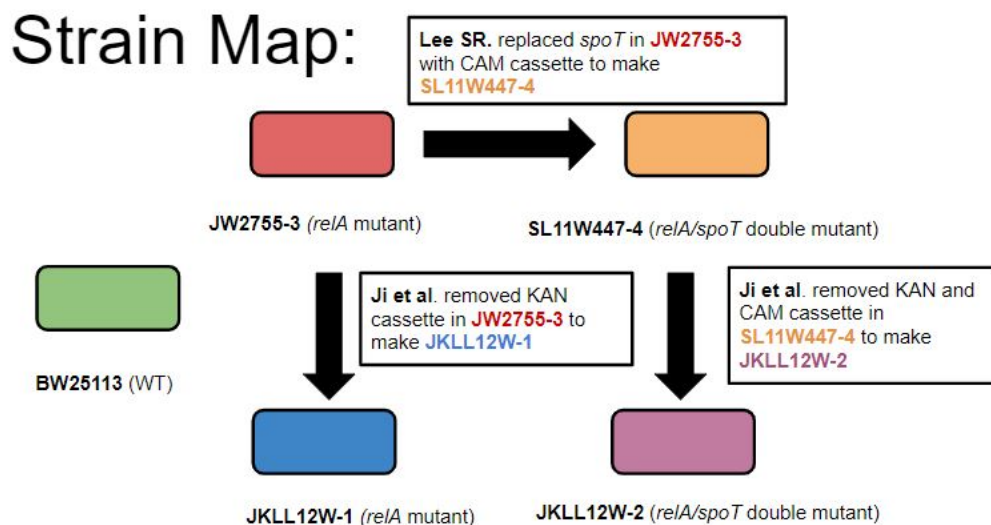
**FIG. 3** Biofilm formation after 24 hours at 37°C. LB (n = 3; blue) and M63B1 with 0 μM L-Ile (n = 1; orange), 30 μM L-Ile (n = 2; grey) and 60 μM L-Ile (n = 1; yellow). n.s. = not significant, \* = p < 0.05 and \*\* = p < 0.01.

***ArelA/spoT* mutant has higher biofilm in minimal media with limiting isoleucine and promoted slower growth.** With the biofilm assay we studied how the concentrations of isoleucine affected the biofilm formation of WT and *ArelA/spoT* mutant strain. Comparing the biofilm formed by the WT in LB (nutrient-rich media) and minimal media with 30  $\mu$ M of isoleucine, we found that nutrient level does not affect the formation of biofilm ( $p > 0.05$ ) (Fig. 3). Although we were not able to compare the data against the other two minimal media (0 and 60  $\mu$ M of isoleucine), the biofilm level was within the range of  $OD_{590} \sim 0.01-0.02$ . Based on the data we collected, the amount of biofilm formed by WT in 24 hours remains constant despite the nutrients available in the media ( $p > 0.05$ ). On the other hand, the *ArelA/spoT* mutant displayed changing biofilm level when grown in different media. Compared to the *ArelA/spoT* mutant grown in LB, the amount of biofilm formed by mutant grown in minimal media with 30  $\mu$ M of isoleucine was significant ( $p < 0.05$ ) (Fig. 3). This showed that a difference in media can result in difference in growth. We were not able to statistically analyze how isoleucine concentrations affected biofilm formation ( $n=1$  for minimal media with 0 and 60  $\mu$ M of isoleucine). However, we found a decreasing trend in biofilm with increasing isoleucine (Fig. 3). Amongst the three minimal media supplemented with varying concentrations of isoleucine, 0  $\mu$ M allowed the the highest level of biofilm formed ( $OD_{590} \sim 0.032$ ), whereas 60  $\mu$ M induced the lowest level of biofilm ( $OD_{590} \sim 0.018$ ) (Fig. 3). Minimal media supplemented with 30  $\mu$ M of isoleucine came in the middle ( $OD_{590} \sim 0.025$ ) (Fig. 3). Overall, our results showed that higher level of biofilm is correlated to minimal media with lower level of isoleucine. This pattern does not support our hypothesis as we believed that higher level of biofilm was correlated to higher level of isoleucine due to greater growth.

## DISCUSSION

In order to reproduce the growth curve data from Cau *et al.*, we initially wanted to study the same *E. coli* K-12 strains: WT strain BW25113, *ArelA* strain JKLL12W-1, and *ArelA/spoT* strain JKLL12W-2 (8). Our study initially aimed to observe any growth and biofilm formation differences between WT *E. coli* K-12, strain with *relA* single deletion and  $ppGpp^0$  *relA*, *spoT* double deletion. This will allow us to comment on the role *relA* plays in mediating stringent response and how its absence affects a cell in nutrient limiting conditions. In order to have a more robust biofilm assay, we included a *AfimC* strain, JW4279-1, from Coli Genetic Stock Center and used it as our negative biofilm control (18).

Both JKLL12W-1 and JKLL12W-2 are JEMI generated, deriving from JW2755-3 (*ArelA*), which was obtained from the KEIO library (8). JW2755-3 contains a KAN cassette in place of the *relA* gene (8). Lee *et al.* replaced the *spoT* gene in JW2755-3 with a chloramphenicol (CAM) cassette to create the *ArelA/spoT* double mutant strain SL11W447-



**FIG. 4** Source of the strains used in this study.

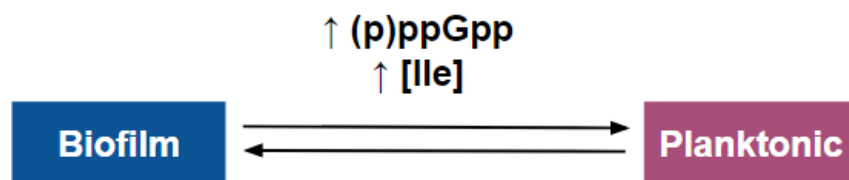
4 (Fig. 4) (19). Ji *et al.* removed the antibiotic cassettes from JW2755-3 and SL11W447-4 to create JKLL12W-1 and JKLL12W-2 respectively (Fig. 4) (15).

In our study, we first aimed to confirm the deletion of *relA*, *spoT*, and *fimC* genes in the various strains through PCR amplification followed by Sanger sequencing. We were able to confirm the deletion of *relA* and *spoT* in JKLL12W-2, and the deletion of *fimC* in JW4279-1 however JKLL12W-1 still contained the *relA* gene. Because JKLL12W-1 and JKLL12W-2 were both generated from the *ArelA* strain JW2755-3 from the Keio library, and *relA* was confirmed to be deleted from JKLL12W-2, the source of error must have occurred after JW2755-3. The error may have been caused by a mislabelling in MICB 421/447 Strain bank, during PCR sample preparation, or genomic isolation. Due to time constraints, we focused on JKLL12W-2 and studied the growth and biofilm formation of the ppGpp<sup>0</sup> mutant. Future studies should locate the source of error and confirm gene deletion before using JKLL12W-1.

For the growth curve experiment, we repeated it based on the same experimental conditions in Cau *et al.* (8), adding additional media with varying isoleucine concentrations as testing condition. Similar to their results (8), we saw slower growth rate for the *ArelA/spoT* mutant in isoleucine limiting conditions compared to the WT (Fig. 2B-D). For their study, Cau *et al.* used supplemented the minimal media with 60  $\mu$ M isoleucine (8). Expanding on their study, we used various concentrations of isoleucine, 0, 30, 60 and 400  $\mu$ M, and noticed a isoleucine dependent growth of the *ArelA/spoT* mutant (Fig. 2C-D, Fig. S2). The same pattern of lower growth in *ArelA/spoT* mutant compared to the WT is consistent among these nutrient-limiting media with varying isoleucine (Fig. 2 B-D). Based on our results, we propose that the *relA* and *spoT* genes allow the bacteria to grow faster and sustain a higher population in conditions with limiting isoleucine. We believe that the *relA* and *spoT* genes allow the bacteria to consume isoleucine at a faster rate, thus allowing faster growth and early growth arrest of the WT. The proteins RelA and SpoT are responsible of synthesizing (p)ppGpp, which is responsible for multiple metabolic pathways (9). For the *ArelA/spoT* mutant, it is possible that some of the pathways are altered due to the absence of (p)ppGpp.

Previous study done by Traxler *et al.* with the same media as our study (supplemented with 60  $\mu$ M of isoleucine) found that bacterial cells enter growth arrest when the isoleucine becomes exhausted (9). Similar to our results, they noticed that the WT utilized the isoleucine at a faster rate compared to the *ArelA/spoT* mutant, isoleucine exhausted after 425 and 500 minutes of incubation respectively (9). This showed that the *relA* and the *spoT* genes allowed the WT to have faster growth rate through a faster consumption of isoleucine. Due to the accelerated growth rate and exhaustion of isoleucine, the WT entered growth arrest at an earlier time which agreed with our findings of slower growth and later growth arrest of the *ArelA/spoT* mutant.

Compared to the WT *E. coli* K-12 *ArelA/spoT* mutant has been found to experience aberrant expression of genes for the metabolic pathways (9). Traxler *et al.* noticed that multiple anabolic pathways of the *ArelA/spoT* mutant, such as LPS/fatty acid biosynthesis, were upregulated compared to the WT (by more than 2-fold) (9). The upregulation of these anabolic genes in the *ArelA/spoT* mutant require extra energy compared to the WT to sustain the same level of growth. This may explain the slower growth shown by our



*ArelA/spoT* mutant and why the final optical density was lower despite grown in the same

**FIG. 5** Model of *E. coli* K-12 WT in minimal media with varying isoleucine concentrations. With increase in isoleucine concentration, *E. coli* K-12 shift to a planktonic lifestyle to growth. As isoleucine becomes depleted, *E. coli* K-12 changes phenotype to synthesize biofilm. With increase in (p)ppGpp, the biofilm disperses and *E. coli* K-12 goes back to being planktonic.FXT



minimal media over the same amount of time.

Compared to the WT, we found that the *ΔrelA/spoT* mutant had higher biofilm for each of the corresponding growth media. Biofilm formation was highest for *ΔrelA/spoT* mutant in LB. This result is in agreement with an earlier study done on *E. coli* stringent response mutant (1) and showed similar patterns to stringent response mutants of another Gamma proteobacteria *Pseudomonas putida* (12, 13). Based on the results, we noticed that the level of biofilm in WT was lower than the *ΔrelA/spoT* mutant ( $p < 0.05$  for both LB and minimal with 30  $\mu\text{M}$  of isoleucine). Since biofilm level of the WT was maintained between LB and minimal media with 30  $\mu\text{M}$  of isoleucine ( $p > 0.05$ ), we suspect that the (p)ppGpp synthesized by RelA and SpoT are responsible of maintaining level of biofilm. Since the same pattern was shown for a study with *P. putida* (12, 13), we suspect that that *E. coli* K-12 and *P. putida* KT2440 stringent response mutants behave similarly. In their study, Díaz-Salazar *et al.* found that the level of biofilm of the (p)ppGpp-synthesizing strains and the *ΔrelA/spoT P. putida* peaked during the stationary phase ( $\text{OD}_{620} = 1$  and 3 respectively) and either decreased to zero (WT) or remained constant (*ΔrelA/spoT*) (12). Liu *et al.* showed the same result where only the *P. putida* stringent response mutant sustained its biofilm level (13). The model created by Díaz-Salazar *et al.* showed that the (p)ppGpp synthesized by RelA and SpoT induce the proteolysis of a protein responsible for biofilm formation, thus leading to dispersal (12). This potentially explains why the biofilm formation of our WT remained at a low level and lower than the *ΔrelA/spoT* mutant despite the growth media used. The biofilm formed by the *ΔrelA/spoT* mutant never dispersed and accumulated over time.

After knowing that *ΔrelA/spoT* mutant does not disperse biofilm, we found that there is a higher level of biofilm for mutant grown in minimal media supplemented with fewer isoleucine (promote slower growth). Biofilm is a community of microbes where nutrients shared interchangeably and occurs predominantly when the cells are present in environments with limited nutrients (20). When the environment is high in nutrients, there is the tendency for the microbes to be planktonic, free flowing, and increase in growth (20). The biofilm may also be dispersed by (p)ppGpp when the surrounding nutrient is high and phenotypically change to planktonic (20). In the context of our study, minimal media with 0  $\mu\text{M}$  of isoleucine prevented the *E. coli* K-12 strains from growing. When incubated in this media, the *E. coli* K-12 strains are phenotypically favored for biofilm formation (20) and should not grow (9). Minimal media with increasing levels of isoleucine create an environment where majority of the seeded microbes are be planktonic, allowing them to grow (20). As the isoleucine depletes, more cells adhere to one another as part of the biofilm community. However, for media with higher concentrations of isoleucine, most of the nutrient was likely used for growth, thus decreasing its availability for biofilm formation. Whereas for the *E. coli* K-12 strains grown in media with lower isoleucine concentrations, the microbes will likely be in the biofilm phenotype and use more nutrient for biofilm formation. Since the *ΔrelA/spoT* mutant cannot synthesize (p)ppGpp, there is no dispersal biofilm (12). This indicates that the *ΔrelA/spoT* mutant within the biofilm will not become planktonic (20) and the biofilm will accumulate over time (12). Figure 5 shows our proposed model for *E. coli* K-12 WT grown in media with varying isoleucine concentrations.

**Limitations** We initially planned to investigate the growth and biofilm formation for all *E. coli* K-12 stringent response mutants, *ΔrelA* and *ΔrelA/spoT*. However, the results for our strain confirmation came back and JKLL12W-1 contained the *relA* gene (Supplementary X). Due to time constraints we were not able to troubleshoot this issue and determine the source of contamination. With regards to biofilm formation, we only know the biofilm formation in ppGpp<sup>0</sup> mutant, *ΔrelA/spoT* compared to the biofilm behavior of *relA* mutant. The growth curve and biofilm assay were both performed in 96-well plates which has limited aeration. For our growth curve experiment, the three technical replicates had no distinguishable outlier when measured at  $\text{OD}_{600}$ . However, only one biological replicate was performed for this experiment which decreases the reliability of ours results. For our biofilm experiment, we only have one biological replicate for strains grown in M63B1 minimal media supplemented with 0 and 60  $\mu\text{M}$  of isoleucine. If we had at least one more biological

replicate, it would increase our confidence and allow us to look at the significance in biofilm formation among the media.

**Conclusions** This study examined the growth and biofilm formation of *E. coli* K-12 *ArelA/spoT* stringent response mutant JKLL12W-2 under isoleucine starvation. We found that when grown in M63B1 minimal media, *ArelA/spoT* mutant experienced faster growth and a decrease in biofilm formation with increasing isoleucine concentration. Our findings on biofilm formation agree with previous studies on stress response mutants of another member of the gammaproteobacteria class, *P. putida* KT2440. The effect of increasing isoleucine concentration has not been studied before and we hope our findings can introduce a new aspect of looking at this topic.

**Future Directions** Future studies can study both stringent response mutants, *ArelA* and *ArelA/spoT*, and expand on our study by monitoring the amount of biofilm formation at shorter time intervals during the 24 hours of incubation. By doing this, we can see changes in biofilm levels over time and determine if the stringent response mutants of *E. coli* K-12 behave similarly to what is described in *P. putida* KT2440 (12) in terms of biofilm formation. In addition, the level of (p)ppGpp can also be measured in accordance with the biofilm and see the relationship between the two. For our study, the explanations and supporting evidence we found were mainly from previous biofilm studies done on *P. putida* KT2440 (12, 13). We were not able to find similar studies done for *E. coli* K-12. We encourage fellow researchers to look into this matter and broaden the field of knowledge for the biofilm formation in *E. coli*.

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