DEVELOPMENT OF *IN VIVO* PPGPP REPORTERS IN *ESCHERICHIA COLI*

BY

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THESIS

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ABSTRACT

ppGpp is a small molecule that works as a global master regulator of the *E. coli* physiology, most notably during the stringent response. Systems biology studies have emphasized its role in understanding the host response to the burden of introduced synthetic genetic circuits. Traditionally, researchers rely on *in vitro* methods to measure the intracellular ppGpp levels. *In vivo* ppGpp reporters, however, would allow the possibility of closely monitoring the ppGpp levels during different processes, especially the host response given the functioning of synthetic circuits with relatively fast dynamics. In this study, a series of *in vivo* ppGpp reporters were developed in *E. coli* by placing a fluorescent protein under the control of promoters from genes known to be controlled by ppGpp levels. Reporter performance was tested by monitoring the fluorescence level with microscope during different stages of growth, induced stringent response, and different growth conditions. The four positive ppGpp reporters showed strong positive correlation with ppGpp levels, including two with particularly high sensitivity and signal intensity. The testing process also shed new light on the dynamics of ppGpp production during batch culture growth and under nutrient limitation. With further development, these *in vivo* ppGpp reporters promise to be very useful when studying the dynamics of host resource partitioning.

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Dedication

This paper is dedicated to my cat Maumee, for trying to be sillier than me. Keep trying.

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CHAPTER 1: INTRODUCTION

Bacteria have evolved sophisticated strategies to cope with different conditions. When nutrients are sufficient, they would grow rapidly, while when nutrients are limited, they would exhibit very low growth rates but rather focus the limited resources on maintenance and survival. This doesn't simply happen passively based on nutrient limitation itself but involves active global regulation of the gene expression landscape. In *Escherichia coli*, arguably the most important process in this respect involves the master regulator (p)ppGpp (guanosine pentaphosphate or tetraphosphate, hereafter collectively referred to as ppGpp) (Jin, Cagliero and Zhou, 2012; Hauryliuk *et al.*, 2015).

Intracellular ppGpp production is traditionally associated with stringent response, the process initiated when resources become limiting for bacterial growth and cells enter a state of low protein synthesis and low growth rate. More recent studies have revealed ppGpp plays a role in more than just the extreme cases (Balsalobre, 2011; Potrykus *et al.*, 2011; Traxler *et al.*, 2011; Gaca *et al.*, 2013). In general, the ppGpp level is inversely related to nutrient conditions and growth rate. Two enzymes, RelA and SpoT, are responsible for the intracellular ppGpp biosynthesis and degradation in *E. coli*, where RelA carries the majority of ppGpp synthesis, while SpoT is responsible for ppGpp degradation as well as part of its synthesis. These enzymatic activities are subject to regulation of a variety of stress signals. SpoT activity has been found to respond to many different signals including limitation in phosphate, iron, fatty acids, carbon source, and osmotic stress, while RelA mainly respond to amino acid starvation (Fig. 1A) (Hauryliuk *et al.*, 2015). Among them, the effects of amino acid starvation in *E. coli* represent the best-studied mechanism. When there is an amino acid shortage in the cell, namely the protein synthesis capacity is low, some tRNAs will not be charged with an amino acid (deacetylated tRNA); when the uncharged tRNA enters the

translation apparatus, translational stalling would occur. The complex of ribosome and deacetylated tRNA is recognized by RelA, which binds to the complex and becomes activated, catalyzing the synthesis of ppGpp from ATP and GTP/GDP (Hauryliuk *et al.*, 2015).

ppGpp can profoundly change the gene expression landscape. Quite a few mechanisms have been proposed for its regulation to take place. Among them, the direct alteration of RNA Polymerase (RNAP) conformation is seen as the major mode of action in *E. coli* (Fig. 1A). The *E. coli* RNAP has two binding sites for ppGpp, one to be bound by ppGpp alone, and the other by ppGpp together with the small regulatory protein DksA (Ross *et al.*, 2016). Binding by ppGpp either decreases or increases transcription initiation by RNAP depending on the promoters. During exponential growth, ribosome synthesis takes up the largest part of cellular resources, and rRNA promoters can be responsible for as much as 90% of the number of transcripts made in the cell. When ppGpp is at high level, however, the ratio can drop to as low as 25% (Gourse *et al.*, 1996; Dennis, Ehrenberg and Bremer, 2004). Among others this is the most important case where ppGpp-binding of RNAP decreases the initiation of transcription, leading to a decrease of ribosome number in the cell, and consequently slower protein synthesis and cell growth. In the meantime, transcription of many other genes is upregulated due to the RNAP conformation change from ppGpp binding. The most representative group of such genes is the amino acid biosynthesis genes, corresponding to the important cause of ppGpp increase, amino acid starvation (Potrykus and Cashel, 2008; Gourse *et al.*, 2018).

The importance of the dramatic host resource partitioning by ppGpp extends beyond the study of bacteria physiology in itself. As the field of synthetic biology develops further, more and more

recognition has been taken on the effects of host context on circuit performance. Taking a simple view, whenever a heterologous gene is introduced into the host cell, its expression would be subject to the availability and activity of RNAP, ribosome, nucleotides, amino acids, ATP, and cell division rate. In the meantime, the expression of heterologous genes would take up such resources, posing a burden to the host cell, and in turn altering the host physiological state (Scott *et al.*, 2010; Liao, Blanchard and Lu, 2017). In one example, when the non-toxic protein LacZ was inducibly expressed in *E. coli*, a clear negative correlation was observed between cellular growth rate and LacZ amount; in the extreme case where LacZ was induced to account for 30% of all proteins, ribosome destruction was observed, and cells stopped growing and lost viability after several hours (Dong, Nilsson and Kurland, 1995).

One study pointed to a close link between host-circuit interplay and the role of ppGpp. Shachrai *et al*. found that the cost of heterologous protein production was reduced in the later part of exponential growth, but when ppGpp level was kept higher by knocking out the *spoT* gene, this drop of cost would disappear. The authors argued that heterologous protein production causes significant growth slowdown mainly due to the limitation of ribosome number during initial cell cycles in exponential phase, as cells were transferred from stationary phase, but this limitation would be eased as ribosome became more abundant. When ppGpp level was kept higher in the spoT knockout starin, however, ribosome synthesis rate would be lower, and thus the constraint of ribosome abundance would carry on (Shachrai *et al.*, 2010a). Another study showed how a better understanding of the circuit effect on host physiology could benefit design of synthetic gene expression. Ceroni *et al.* looked beyond cell growth rate and considered cellular capacity, defined as the ability of cells to express a generic gene. Interestingly, they showed that cellular capacity

could be dramatically affected by heterologous gene expression even when the observed effect on growth rate were low. By quantifying the burden of heterologous gene expression on host gene expression capacity, they also demonstrated that ribosome availability could be the bottleneck, as seen by the increased burden when strong RBSs were used. With this knowledge, they showed that a combination of strong promoter with weaker RBS could achieve high heterologous gene expression level while keeping the burden on host gene expression capacity low (Ceroni *et al.*, 2015). While these studies focused on simple factors such as gene expression level and cell growth, it is reasonable to imagine that different effects on host physiology as a result of differences in circuit burden can lead to different circuit dynamics if more complex synthetic circuits are used. Indeed, when researchers changed the circuit burden of a toggle switch by reducing its copy number, extensive efforts of tuning were required to restore its toggle switch behavior (Lee *et al.*, 2016). This intricate relationship between host and circuit deserves sufficient understanding in the systems level as synthetic biology strives for higher predictability.

Despite the importance of ppGpp in the systems-level understanding of bacteria resource allocation, its measurement has largely relied on *in vitro* measurement using either isotope labeling or thin-layer chromatography. This is not only laborious to perform, but also limits the questions that can be answered related to the dynamics aspect. An *in vivo* ppGpp reporter, however, could ideally reflect the dynamics of intracellular ppGpp level in real time, without having the cells lysed. In this study, several ppGpp reporters were constructed (Fig. 1B), and their performances were tested during batch culture growth and under stringent response. Different sensitivity and signal intensity were shown by these reporters, and efforts were made to improve their properties.

CHAPTER 2: RESULTS

Design of ppGpp reporters

One set of ppGpp reporters were available at the start of this study: a negative ppGpp reporter and a positive ppGpp reporter. The negative reporter was constructed by putting *gfp-asv* (encoding a fast-degrading version of GFP) under control of the promoter *rrnBP1* (Shah *et al.*, 2006), whose strength is decreased by ppGpp at the transcription initiation step (Barker *et al.*, 2001). The positive reporter was constructed by translational fusion of *mCherry* with *rpoS*, forming *rpoS-mCherry* (Maisonneuve, Castro-Camargo and Gerdes, 2013). RpoS is the major stringent response sigma factor and was reported to be positively regulated by ppGpp, largely at the translational level (Girard *et al.*, 2017). Both of the ppGpp reporter constructs were integrated on the genome. After obtaining the strains harboring the two reporters, we tested them in different growth phases, and with induced stringent response. In some cases, we were able to see that for the strain with both reporters present, cells either displayed green fluorescence or red fluorescence, corresponding with either low or high ppGpp levels, respectively (data not shown). However, we found that the fluorescence level from positive *rpoS-mCherry* reporter was very low and barely above the background in most cases, including SHX treatment, except when in extremely harsh conditions (e.g., on agar plates stored in fridge for days) (data not shown), while the negative *rrnBP1-GFP-asv* reporter maintained high fluorescence well into stationary phase where ppGpp level is expected to be high. These results indicate that the two reporters have very low sensitivity to different ppGpp levels and may only work for very high ppGpp levels. Considering that different ppGpp levels can result in very different physiological responses in bacteria (Balsalobre, 2011;

Gaca *et al.*, 2013), these reporters may be very limited in helping understand the complexity of ppGpp-mediated regulation, especially in a quantitative or semi-quantitative way.

To overcome these challenges, we set out to develop new ppGpp reporters. While different mechanisms of ppGpp-mediated regulation have been reported, it is believed that the major mode of action is transcriptional, where ppGpp binds to the RNAP, singly or together with DksA, and in turn changes RNAP preferences to different promoters, either positively or negatively (Potrykus and Cashel, 2008). It thus makes sense to exploit promoters whose strengths are most dramatically affected by changes in ppGpp levels and use them to control fluorescent protein expression for *in vivo* ppGpp reporting. Up to the point when the study was being done, two sets of transcriptomic profiling were available as to the ppGpp-mediated response (Durfee *et al.*, 2008; Traxler *et al.*, 2008). We relied mainly on the one done by Durfee *et al*., as it contains the transcriptome response as soon as 5 min after ppGpp induction, where the majority of the effects were supposed to be direct. Three major criteria were used in selecting candidates: 1) there should be dramatic difference in the transcript levels after ppGpp induction in WT, but not in relA knockout strain; 2) the function of the gene should be relatively known; 3) the promoter should not be known to be under other modes of regulation. These criteria maximize the potential that the chosen promoters would show dramatic response to changes in ppGpp levels and ppGpp levels only, without complication of other factors. Based on the above-mentioned microarray results and these criteria, together with literature mining, four promoters considered positively regulated by ppGpp were chosen: *P_thr* (for operon *thrABC*) (Paul, Berkmen and Gourse, 2005), *P_ilv* (first promoter of operon *ilvLXGMEDA*), *P_liv* (*livKp2* was chosen instead of *livKp1* which is known to be regulated by Lrp) (Haney *et al.*, 1992), and *P_phoB* (see sequence in Fig. 2). The former three are all

promoters for amino acid biosynthesis/transport genes and are supposed to be regulated by ppGpp via its effect on RNAP preference. In the case of *P_phoB*, ppGpp is known to inhibit the activity of PPX, an enzyme that degrades poly-phosphate (polyP); this leads to accumulation of polyP, which in turn activates *P_phoB* (Ault-Riché *et al.*, 1998; Rao, Liu and Kornberg, 1998; Hauryliuk *et al.*, 2015). Considering that no transcription/translation step is involved in this regulation process to affect the response speed, *P_phoB* was also chosen as a candidate. As a well-studied control, *rrnBP1* (Barker *et al.*, 2001) was chosen which is negatively regulated by ppGpp.

To construct the ppGpp reporters, the candidate promoters were used to control mCherry expression on a high copy number (300-500) plasmid backbone (Fig. 3) (see details in Materials and Methods). The *mCherry* gene was a codon-optimized version for *E. coli*. All the four positive reporters and the *rrnBP1*-based negative reporter were successfully constructed. However, the *rrnBP1*-based reporter suffered from significant mutation rate due to the high strength of the promoter together with mCherry toxicity/burden, severely diminishing measurement reliability, and thus results for this reporter with high-copy plasmid backbone are not presented here. The *P_thr-mCherry* reporter bore a point mutation found closely upstream of the transcription start site (Fig. 2A). As performance test revealed desirable performance, this point mutation was not corrected and was present in all *P_thr*-based reporters built in this study.

Evaluation of ppGpp reporter performance in batch culture growth

Many studies done in different media with or without amino acids have demonstrated that during *E. coli* growth in batch culture, the intracellular level ppGpp continues to rise as culture OD increases and growth rate decreases, and cells approach and enter stationary phase from

exponential phase (Sarubbi, Rudd and Cashel, 1988; Traxler *et al.*, 2008; Cavanagh, Chandrangsu and Wassarman, 2010; Marisch *et al.*, 2013; Varik *et al.*, 2017). This strong relationship between ppGpp level and culture time serves as a convenient strategy to estimate relative intracellular ppGpp levels, and in turn to evaluate ppGpp reporter performance. Performance of the ppGpp reporters were thus first evaluated by monitoring red fluorescence of cells during batch liquid culture growth in LB inoculated from overnight culture (Fig. 4&5). In all cases, within the first one or two hours, cells displayed red fluorescence, which was considered to be either residual mCherry from the overnight stationary phase culture, or that the cell physiology was still adapting to the new media and ppGpp levels haven't be fully adjusted. Afterwards, red fluorescence becomes invisible under microscope as the cells grow in exponential phase. Red fluorescence reappears later and becomes brighter and brighter toward the end of the measurement (22 hr). It took the reporters 7-9 hours for the onset of red fluorescence, with *P_thr*-based reporter appearing to be the fastest, showing red fluorescence for some cells at 7-hr point, and all cells at 9-hr point (Fig. 4B). This indicates that, unless other factors are at play, *P_thr*-based reporter is the most sensitive to the increase in ppGpp level. Based on the correlation between batch culture stationary phase growth and increased ppGpp level, these results support the potential of the constructed ppGpp reporters to be functional, with differences in response sensitivity and intensity.

Evaluation of ppGpp reporter performance with induced stringent response

SHX is a serine analog which induces apparent serine starvation and stringent response, increasing ppGpp levels. Compared with stationary-phase growth, SHX-induced effects are expected to be more purely based on ppGpp-mediation, except for secondary effects resulting from difficulty in protein synthesis where serine is required (Durfee *et al.*, 2008). We treated *E. coli* cells growing

in exponential phase with 0.5 mg/ml SHX in LB liquid culture and monitored red fluorescence with microscope (Fig. 6&7). Samples were taken at 30 min, 100 min, 300 min, 6.5 hr, and 18 hr after SHX was added. In all cases, SHX treatment led to increase in red fluorescence level, but again different reporters showed differences in response sensitivity and intensity. Increase in red fluorescence level could be seen in *P_thr* and *P_ilv*-based reporters as early and 30 min after treatment (data not shown) and became considerable at 100 min (Fig. 6), while *P_liv* and *P_phoB*-based reporters showed response barely detectable at 100 min (Fig. 7). These results agree with the growth-based observation that *P_thr* and *P_ilv*-based ppGpp reporters have either higher sensitivity or are subject to more direct regulation by ppGpp levels.

Probing critical LB concentration to induce stringent response with ppGpp reporters

Using the *P_thr*-based ppGpp reporter, we tried to answer the question: what degree of amino acid depletion during growth in LB medium would trigger stringent response? It is reported that LB provide *E. coli* with carbon source in the form of amino acids but not sugars (Sezonov, Joseleau-Petit and D'Ari, 2007). Understandably some amino acids in LB can also be used directly for *E. coli* protein synthesis. When growing in minimal medium with no amino acid supplemented, even though *E. coli* can synthesize amino acids with the carbon (glucose in the case of M9) and nitrogen sources, stringent response would be induced due to the lack of provided amino acids. This is supported by our experiments, where *E. coli* cells growing in exponential phase in LB were spun down and transferred into M9 medium with no LB or other form of amino acid supplementation. Harboring the *P_thr*-based ppGpp reporter, red fluorescence became visible between 30 min and 1 hr after the medium change (Fig. 8A; fluorescence at 30 min point was insignificant and not shown). When LB was provided, however, even at very low concentration, stringent response

would be absent. We started by transferring exponentially growing cells to M9-based medium mixed with a gradient of LB at concentrations of 31.6%, 10%, 3.16%, 0%, as well as 100% LB, and cells showed no red fluorescence at any concentration of LB above 0 within the first two hours, even though growth rate showed strong positive correlation with the concentration of LB below 31.6%.

We further decreased the gradient of LB concentrations to 1%, 0.316%, 0.1%, 0.0316%, and 0%, and checked red fluorescence at the 1 hr and 2 hr points. Interestingly, at LB concentrations of 0%, 0.0316%, and 0.1% cells showed red fluorescence at the 1 hr and 2 hr (Fig. 8ABC)., while at LB concentration of 0.316% cells only showed red fluorescence at the 2 hr point but not 1 hr (Fig. 8D). No red fluorescence was seen within the 2 hours for LB concentration of 1% (Fig. 8E). Furthermore, when we measured the OD at 2 hr, and compared each other with their most adjacent concentration (each different by a factor of 3), OD in 0.316% LB was 2.2 times that in 0.1% LB, while the difference between all other groups were below 1.5 times (Fig. 9). This indicates that some amino acid(s) in LB reaches critical concentration between 0.1% and 0.316% of LB concentration, below which starvation for such amino acid(s) and in turn stringent response would be triggered. Combined with the dissected concentration of each amino acid in LB from literature (Sezonov, Joseleau-Petit and D'Ari, 2007) or vendor information (*BD Bionutrients* ^{TM} *Technical Manual*, no date), supplementation of specific amino acids can be used to pinpoint the one(s) responsible for amino acid starvation at low LB concentration. Similar methods can be used to determine the limitation of which amino acid(s) contributes to the initiation of stringent response at stationary growth of *E. coli* in LB. More generally, the non-linearity observed for ppGpp response/growth rate at LB concentration indicates the existence of a threshold of nutrient

limitation for triggering ppGpp production in *E. coli*. These experiments also further showed the effectiveness of the *P_thr*-based ppGpp reporter.

Interestingly, when we cultured *E. coli* (seeded from exponential phase) harboring the reporters on an agarose pad with 10% LB in M9 in a sealed dish (Young *et al.*, 2011), cells showed red fluorescence from very early on (within 1-2 hours). This may indicate that oxygen limitation could induce ppGpp production, or that nutrient diffusion on solid medium was slow enough to result in amino acid starvation.

Copy number change and degradation tag addition to improve reporter properties

When placed under control of *rrnBP1*, the expression of mCherry resulted in high toxicity which led to dramatic cell growth retardation and high mutation rate. As a first step to decrease these effects, we changed the plasmid backbone for the *rrnBP1*-based reporter along with the other four reporters from the high-copy backbone (300-500 per cell) to pSC101-based backbone (1-5 per cell). The decrease of copy number for *rrnBP1-mCherry* construct did lower the burden/toxicity to the cell, leading to improved cell growth and low mutation rate (no loss of red fluorescence was observed during batch culture and colony growth). However, the reporter didn't behave as expected, with cells showing red fluorescence all through the growth curve, with even higher intensity at the stationary phase. A large proportion of the cells also displayed elongated morphology indicating stress. We attribute the retainment and increase of fluorescence to the lack/low rate of degradation of mCherry. Assuming no degradation, mCherry level could only decrease by growth-based dilution; as cell growth stalls during stationary phase, any mCherry produced would continue to accumulate in the cell, whereas high dilution rate during exponential

phase would decrease mCherry level. The result was decreased fluorescence during exponential phase when *rrnBP1* is expected to be more active, yet increased fluorescence during stationary phase when *rrnBP1* is expected to have lower activity. To solve this problem, we added degradation tag to the C-terminal of mCherry. Two versions of ssrA-based degradation tags, one with high degradation rate (AANDENYALAA, designated as "LAA") and one with medium degradation rate (AANDENYNYADAS, designated as "DAS2") were used (McGinness, Baker and Sauer, 2006). The pSC101-*rrnBP1-mCherry*-LAA construct resulted in desirable characteristics, with visible fluorescence seen for all cells at the exponential phase but no fluorescence at the stationary phase (22 hr). Thus, combining a decrease of copy number to decrease mCherry toxicity level, and addition of a strong degradation tag, we were able to build a functional negative ppGpp reporter.

Similar procedures were done for the four positive ppGpp reporters. However, when moved from high-copy backbone to pSC101 backbone, the copy number drop resulted in large decrease of signal intensity, and consequently lowered reporter sensitivity. For example, in the case of P_ilv-based reporter, fluorescence stayed low stayed undetectable or very low during the first 18 hours of growth, though eventually becoming high at the 24 hr point. This contrasts the result with high-copy backbone where fluorescence became high as early as 11 hr. Addition of degradation tag to reporters on pSC101 backbone further decreased the signal intensity. When LAA tag was added to *P_thr*-based reporter on high-copy backbone, however, signal intensity did not drop beyond acceptable range, though more systematic analysis is yet to be done.

CHAPTER 3: DISCUSSION

By placing *mCherry* under control of ppGpp-responsive promoters, four potential ppGpp reporters were constructed, and their efficacy were tested based on the relationship between ppGpp levels and different growth phases, as well as SHX-induced stringent response. Results show increased mCherry expression level for all four reporters when the ppGpp level is expected to increase. Among them, the *P_thr* and *P_ilv*-based reporters showed especially high sensitivity to high-ppGpp situations, strongly indicating the effectiveness of these two reporters.

Recent reports have indicated that intracellular ppGpp can assume a large range of levels with very different physiological consequences (Traxler *et al.*, 2008; Shachrai *et al.*, 2010b; Balsalobre, 2011). Even during exponential phase where ppGpp is not traditionally thought of as a factor, it has recently been suggested that ppGpp may be playing an important role in regulating ribosome levels. This contrasts with traditional studies where the effects of ppGpp was only analyzed when elevated to a high level. Thus, having ppGpp reporters with different sensitivities, especially with high sensitivity to low ppGpp levels, can be very important in shedding new light on a comprehensive view of the role of ppGpp, and eventually a systems-level understanding of the resource portioning strategy of *E. coli* and other bacteria.

The main reference data for reporter candidate mining in this study came from microarray-generated transcriptome analysis. Microarray, however, has been known for having low sensitivity to small changes as well as low range of detectable fold-change, and in general suffers from relatively low reliability, when compared with its more recent counterpart RNA-seq (Consortium *et al.*, 2014). Future efforts in developing new ppGpp reporters can benefit a lot if RNA-seq-based

transcriptome data is available in order to identify new reporter candidates. Nevertheless, the chosen ppGpp reporter candidates chosen in this study showed good agreement with new RNA-seq results by Dr. Richard Gourse's group (University of Wisconsin-Madison; personal communication) (Sánchez-Vázquez, 2018).

The ppGpp reporters developed in this study can be improved in a few important ways. Firstly, calibration of reporter behavior with *in vitro* measurement of ppGpp levels can be done in a more rigorous and quantitative way. The current study used microscope-based single-cell level visualization for fluorescence level analysis but could benefit from using plate reader-based measurement to be more consistent and quantitative. Established *in vitro* ppGpp measurement can be used to map reporter fluorescence level to specific ppGpp levels (while taking into consideration potential delay of reporter behavior in response to changes in ppGpp level). More direct ways of manipulating intracellular ppGpp levels can also be considered when checking reporter performance, for example, by using inducible expression of the enzymatic domain of RelA (which catalyzes the ppGpp production and is constitutively active).

A codon-optimized version of mCherry was used as the reporter protein in this study. However, this protein resulted in considerable toxicity to the cells, most evidently shown when under control of the strong promoter *rrnBP1*, which resulted in dramatic growth retardation and high mutation rate. The fluorescent protein also suffered from low signal intensity when copy number was decreased in the case of the positive ppGpp reporters. The current study was limited in the choices of alternative fluorescent proteins due to instrument constraint and compatibility with relevant projects, but future studies should consider using less toxic *in vivo* reporter forms with high signal

intensity. Addition of degradation tag to reporter proteins decreases the effects of growth rate variation on reporter protein accumulation, and thus promises higher reporter fidelity in reflecting ppGpp levels.

The reporters developed in this study relies on the change of RNAP preference to different promoters, and thus isn't expected to work in another species or when there is changes to the RNAP. In a most recent study, a ppGpp-binding aptamer was discovered (Sherlock, Sudarsan and Breaker, 2018). Techniques have been developed to build riboswitches from aptamers to control gene expression (Qi *et al.*, 2012; Espah Borujeni *et al.*, 2016; Etzel and Mörl, 2017). Ideally, this process could decouple the riboswitch sensitivity from the choice of promoters, making it easy to suit the reporter construction to the strain context or desirable promoter strengths.

CHAPTER 4: MATERIALS AND METHODS

Strains, medium, growth conditions

Reporter construction was done using *E. coli* DH10B, while reporter characterization was done in MG1655. Both strains were from lab strain stock. Strains MG1655 rpoS-mcherry and MG1655-ASV, rpoS-mcherry (shared by Kenn Gerdes, University of Copenhagen) were used to evaluate the *rpoS-mCherry* and *rrnBP1-GFP* reporter performance (Maisonneuve, Castro-Camargo and Gerdes, 2013). LB, M9, or their mixture with the specific ratio was used as indicated in the context. LB was prepared according to established protocol. M9 minimal medium contains M9 salts, 2 mM $MgSO_4$, 0.1 mM $CaCl_2$, and 0.4% (w/v) glucose. Antibiotics were supplemented where appropriate, with final concentrations of Kanamycin at 50 mg/ml, and Chloramphenicol at 25 mg/ml. All liquid cultures were grown in 3-4 ml of media in 15 ml culture tubes at 37 °C with 250 rpm shaking.

Plasmid construction

Plasmid backbone for reporters were derived from either pLPT41 (high copy ColE1-derivative origin, 300-500/cell, Kanamycin resistance) (Potvin-Trottier *et al.*, 2016) or pChemoK (pSC101 origin, 1-5/cell, Chloramphenicol resistane) (Moon *et al.*, 2011a). The pLPT41 was initially reported to harbor ColE1 ori (15-20/cell) (Potvin-Trottier *et al.*, 2016), but our sequence analysis showed that its sequence doesn't contain the copy-number controlling *rop* gene, and is expected to be have similar copy number of 300-500 copies/cell.

Overlap PCR and Gibson assembly were used for plasmid construction. Briefly, primers bearing overlap with plasmid backbone and *mCherry* gene were used to amplify the candidate promoters

from MG1655 genome; a codon-optimized (for *E. coli*) version of *mCherry* was amplified from pCRISPReporter-mCherry (obtained from Addgene) (Cress *et al.*, 2015a), and the plasmid backbone was PCR amplified as well, both bearing suitable overlap sequence with adjacent fragments. Overlap PCR was performed first to obtain promoter-RBS-*mCherry* fusion fragment, which was then used for Gibson assembly with the plasmid backbone. The assembly products were transformed into *E. coli* DH10β competent cells, and colonies were picked, with which plasmids were extracted and sequence verified. Plasmids with the correct sequences were extracted and transformed into MG1655 for reporter evaluation.

Degradation tags were added by blunt-end ligation. The original untagged plasmids were first PCR amplified and linearized from the end of mCherry-coding sequencing while adding half of the degradation tag sequence on each primer. The PCR products were digested with DpnI to eliminate the original plasmids, then gel-extracted, phosphorylated by T4 PNK, and ligated by T4 DNA ligase.

ppGpp reporter evaluation in batch culture

For reporter characterization during batch culture growth, cells were inoculated into liquid media from single colonies on agar plates, grown overnight, and diluted 1:100 fold into fresh media before characterization.

ppGpp reporter evaluation with induced stringent response

For reporter response to SHX treatment, overnight cultures were diluted 1:100 into fresh media, grown to OD_{600nm} of 0.4-0.5, further diluted 1:2 fold into fresh medium, before adding SHX stock

solution (50 mg/ml) into medium to reach final concentration of 0.5 mg/ml. For control cultures, deionized water was added in place of SHX stock solution.

ppGpp reporter response with LB gradient

The LB gradient media were prepared by diluting LB liquid medium with M9 medium into specified concentrations. Overnight LB culture harboring the *P_thr*-based reporter was diluted 1:100 into fresh LB medium, grown to OD_{600nm} of 0.3-0.4, and aliquots of 400 ul culture were spun down; the cell pellets were resuspended into LB gradient media.

Image acquisition and analysis

1-2 ul of liquid cultures were taken at appropriate intervals and placed between a glass slide and a cover glass and imaged with an AMG EVOS FL microscope. Images were taken with transmitted and red fluorescence channels with 40X objective.

ImageJ was used to process and analyze the images. For better visualization of the pictures shown, the red fluorescence images were uniformly adjusted by thresholding minimum and maximum to be 20 and 100, and bright-field images 70 and 160.

CHAPTER 5: FIGURES AND TABLES

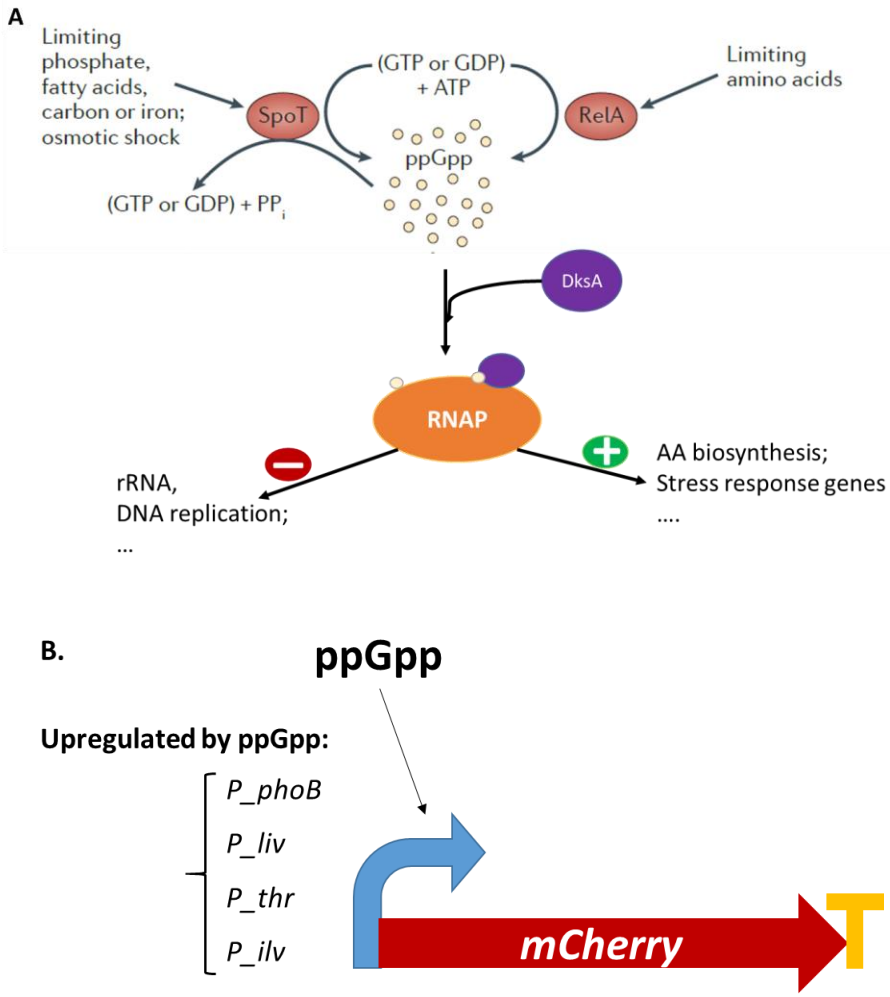

Figure 1. ppGpp-mediated global regulation and ppGpp reporter design. A) ppGpp mediated global regulation. RelA is responsible to the majority of ppGpp synthesis, while SpoT catalyzes ppGpp degradation and part of ppGpp synthesis. The two enzymes respond to different stress signals. ppGpp together with DksA binds to RNA polymerase to either increase or decrease the transcription of different genes. Figure adapted from Dalebroux and Swanson, 2012 with permission (Dalebroux and Swanson, 2012). B) Schematic of ppGpp reporter design. The gene for fluorescent protein mCherry was placed under control of promoters either positively or negatively regulated by ppGpp. The construct was introduced into *E. coli* on high-copy number plasmid backbone for most of the testing done in the study.

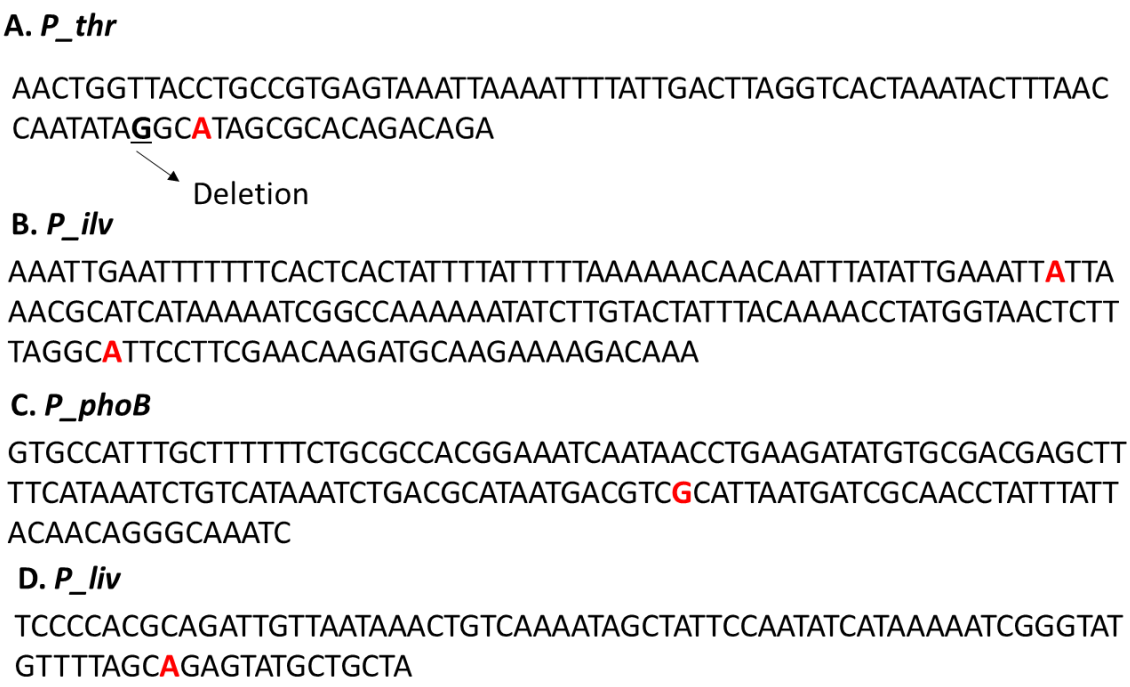

Figure 2. Sequence of promoters used for the ppGpp reporters. A) *P_thr*; B) *P_ilv*; C) *P_phoB*; D) *P_ilv*. Transcription start sites are bolded and marked in red. Site of mutations found are bolded and underlined, accompanied by the type of mutation. B) A point mutation was found closely upstream of the transcription start site of *P_thr-mCherry* reporter. As the resulting reporter still showed desirable performance, this point mutation was not corrected and was present in all *P_thr*-based reporters. C) *P_ilv* has two transcription start sites.

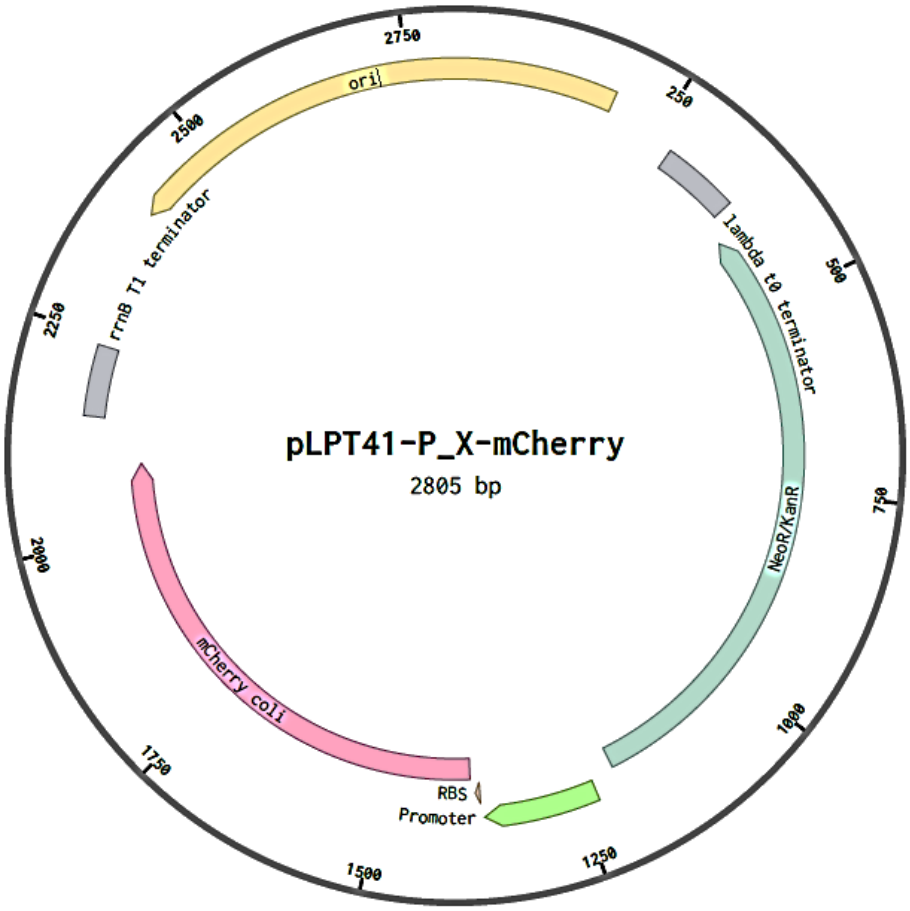

Figure 3. Representative plasmid map for ppGpp reporters in pLPT41 series. The origin of replication is a ColE1 derivative (300-500/cell). Candidate promoters were chosen to control mCherry expression. See Materials and Methods for details.

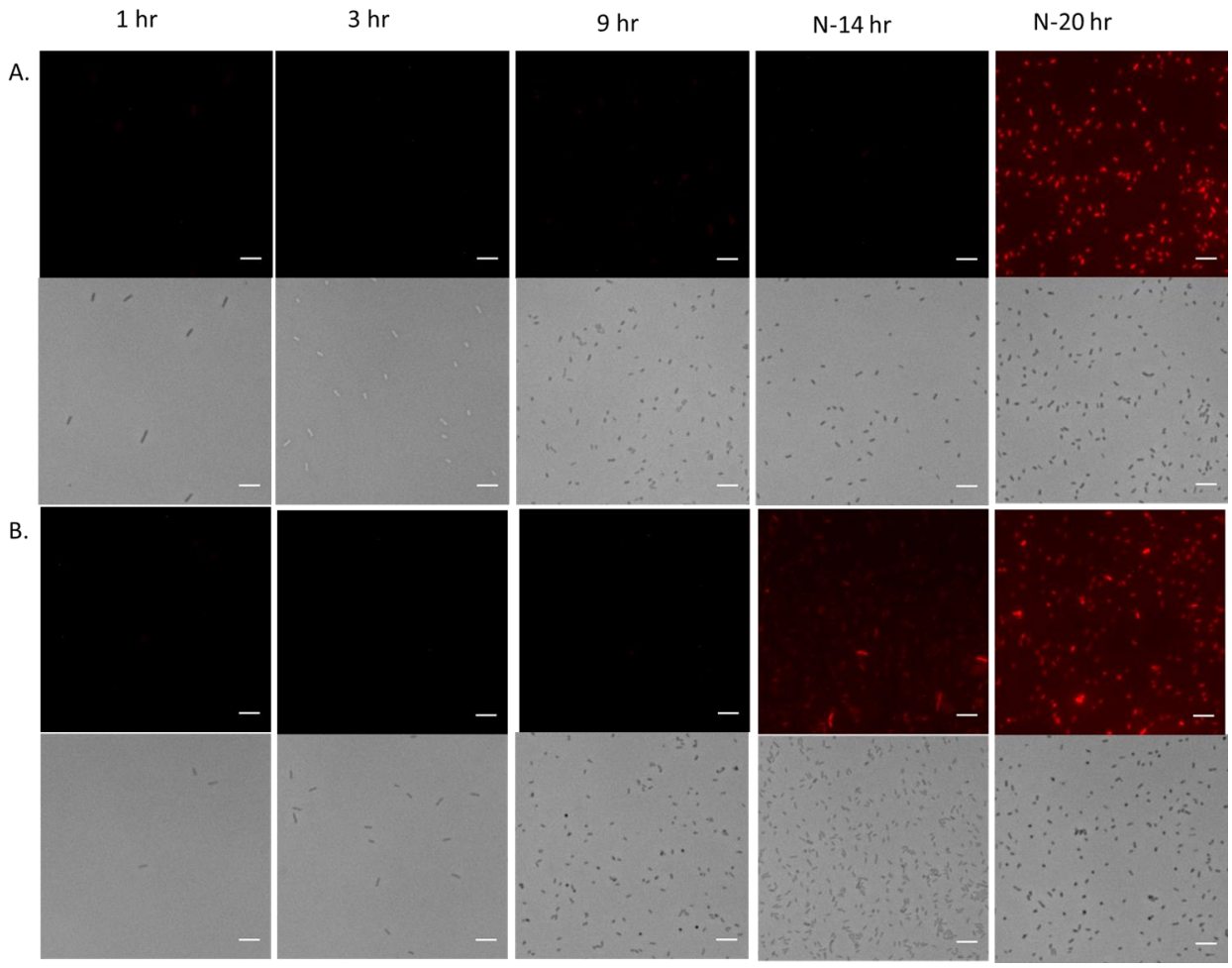

Figure 4. *P_thr* and *P_ilv*-based ppGpp reporter performance along growth curve. ppGpp reporters based on A) *P_thr*, B) *P_ilv*. Top panel: red fluorescence; bottom panel: brightfield. (Scale bar: 10 μm.) Images were taken and analyzed at twelve time points, but five are shown. 1-9 hr images were taken with cultures inoculated from overnight cultures with 1:100 dilution, and N-14 hr and N-20 hr images were taken with cultures inoculated from 1:100 dilution of the 9-hr cultures and grown for 14 hr and 20 hr, respectively. All images from the same channel were processed in same manner in ImageJ. See Materials and Methods for details.

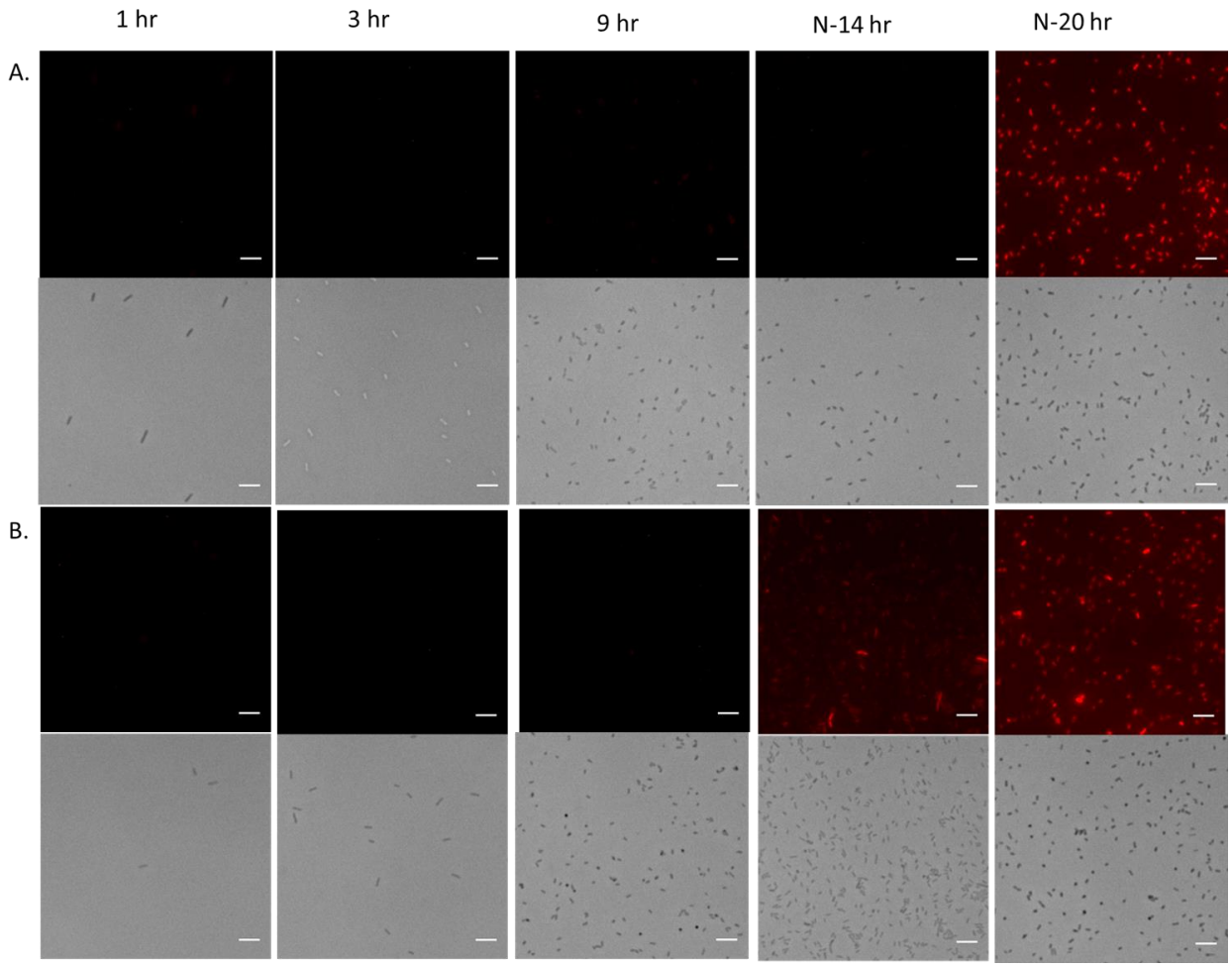

Figure 5. *P_phoB* and *P_liv*-based ppGpp reporter performance along growth curve. ppGpp reporters based on A) *P_phoB*, B) *P_liv*. Top panel: red fluorescence; bottom panel: brightfield. (Scale bar: 10 μm.) Images were taken and analyzed at twelve time points, but five are shown. 1-9 hr images were taken with cultures inoculated from overnight cultures with 1:100 dilution, and N-14 hr and N-20 hr images were taken with cultures inoculated from 1:100 dilution of the 9-hr cultures and grown for 14 hr and 20 hr, respectively. All images from the same channel were processed in same manner in ImageJ. See Materials and Methods for details.

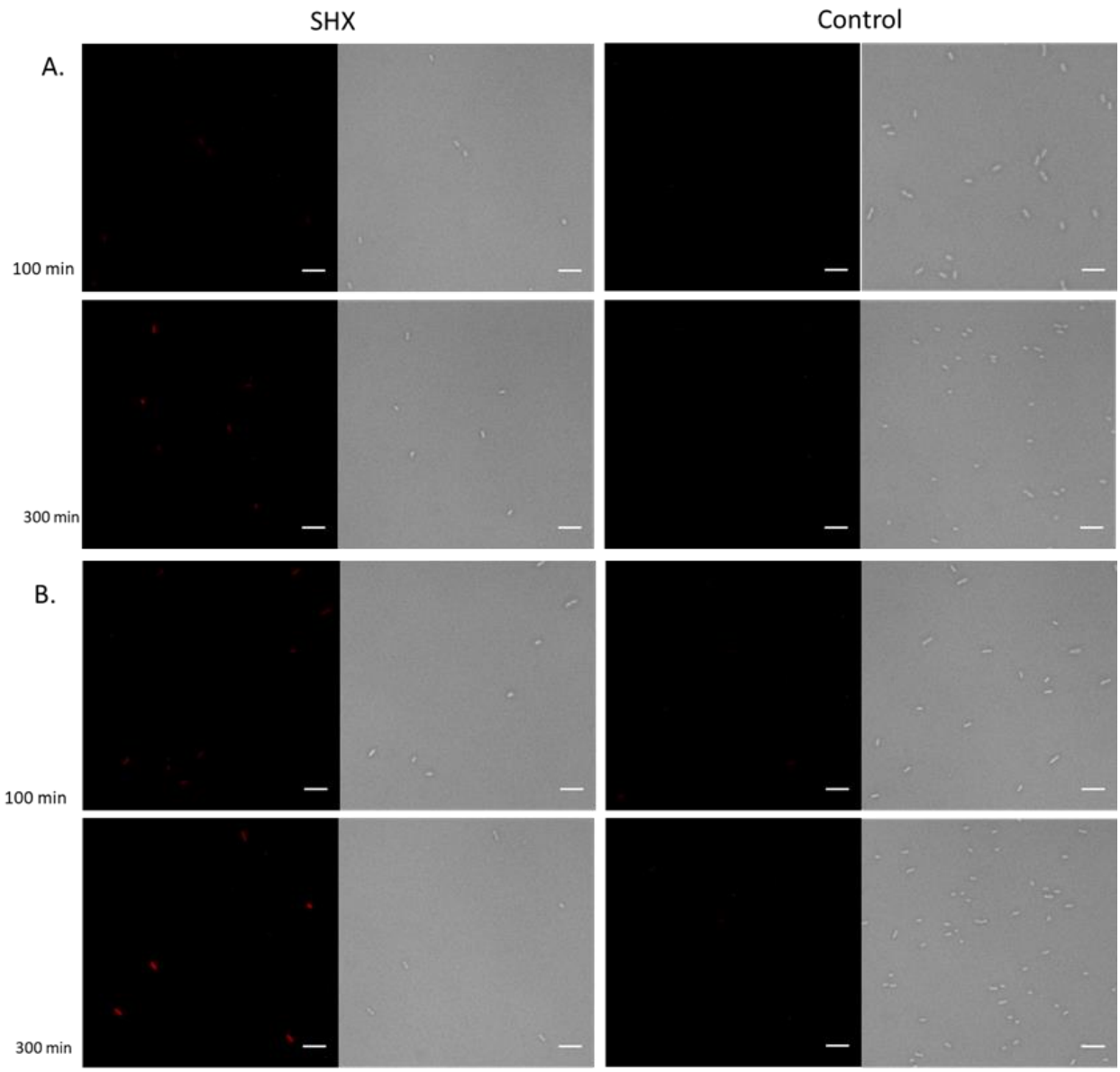

Figure 6. *P_thr* and *P_ilv*-based ppGpp reporter performance under stringent response induction with SHX. ppGpp reporters based on A) *P_thr*, B) *P_ilv*. Left panel: red fluorescence; right panel: brightfield. (Scale bar: 10 μm.) Images were taken at six time points after exponentially growing cells were treated with SHX, among which three are shown. All images from the same channel were processed in same manner in ImageJ. See Materials and Methods for details.

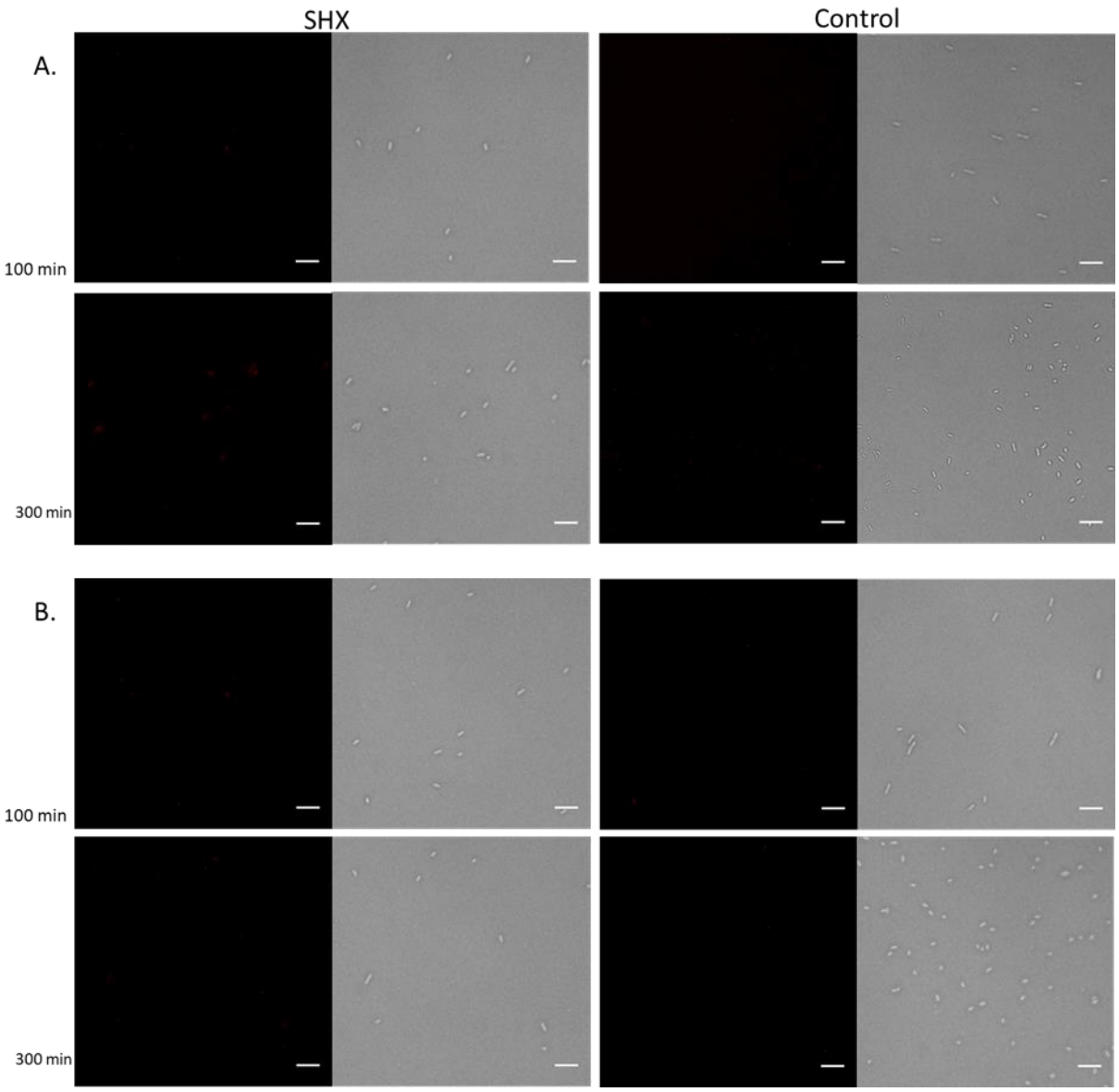

Figure 7. *P_phoB* and *P_liv*-based ppGpp reporter performance under stringent response induction with SHX. ppGpp reporters based on A) *P_phoB*, B) *P_liv*. Left panel: red fluorescence; right panel: brightfield. (Scale bar: 10 μm.) Images were taken at six time points after exponentially growing cells were treated with SHX, among which three are shown. All images from the same channel were processed in same manner in ImageJ. See Materials and Methods for details.

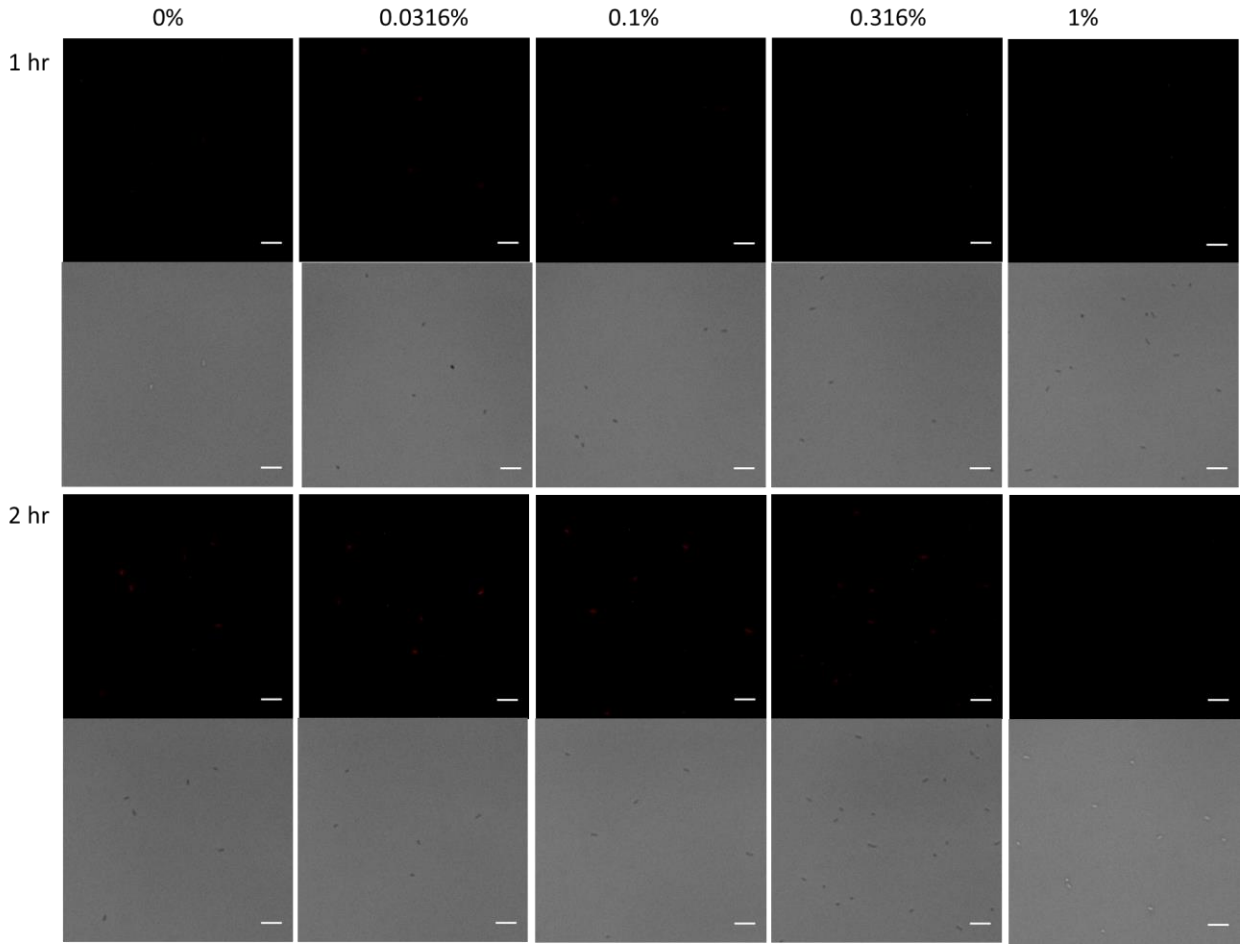

Figure 8. ppGpp reporter response given varying LB concentrations in M9 medium. Scale bar: 10 μm. Exponentially growing cells in LB were spun down and transferred to media containing specific concentrations of LB (as labeled) diluted in M9 minimal medium. All images from the same channel were processed in same manner in ImageJ. See Materials and Methods for details.

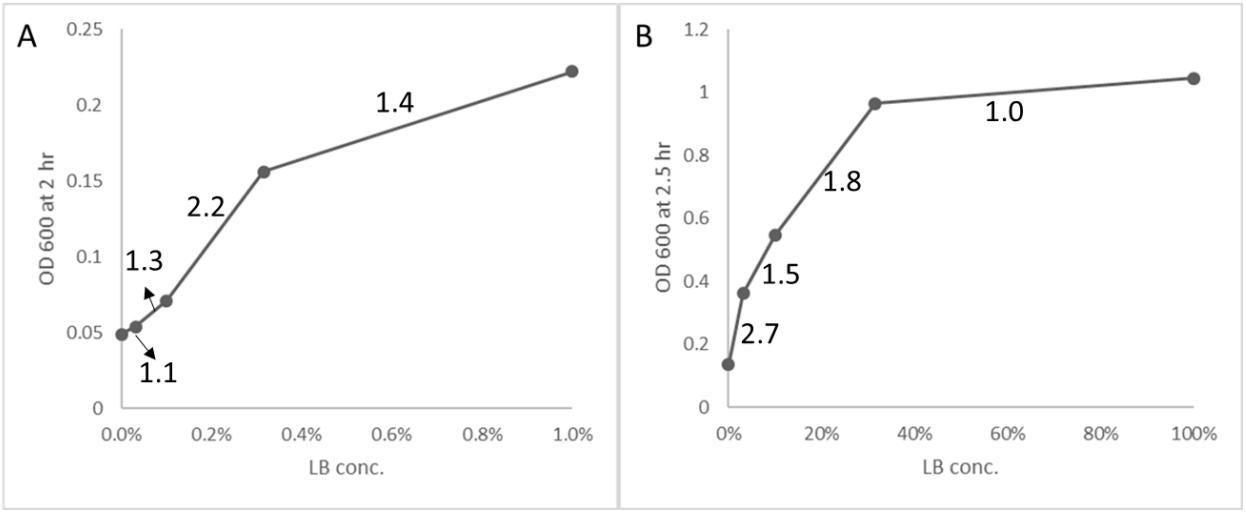

Figure 9. Culture optical density snapshot given varying LB concentrations in M9 medium. Exponentially growing cells in LB were spun down and transferred to media containing specific concentrations of LB (as labeled) diluted in M9 minimal medium. Optical densities at 600 nm were taken at A) 2 hr point after dilution for LB concentrations between 0 and 1%, and at B) 2.5 hr point after dilution for LB concentrations between 0 and 100%. Each data point has 3-fold difference in LB concentration with adjacent point. The fold-difference in OD between each adjacent pair was labeled. Note the sharper difference of OD between LB concentrations of 0.1% and 0.316%, which coincides with the transition range between presence and absence of *P_thr*-based ppGpp reporter response.

Table 1. List of primers used in this study

Primer	Sequence
RBS-mCherry-F	ATAAAGGAGGTAAAatatggtttcaaaaggcgaagaagacaacatggcgattatcaagga
ColE1-mCherry-R	catgggatcccccgggctgcaggaattcgatatcaagcttttatttgtacagttcatccataccgccggtag
pLPT41-F	aagcttgatatcgaattcctgcagcccg
pLPT41-R	tgatcaagagacaggatgaggatcgtttcg
LAA-pLPT41-F	AAAATTACGCGCTTGCAGCAtaaaagcttgatatcgaattcctgcagcccg
PrrnB-pLPT41-R	ATTTTCTGACCGCGCATTTTTTATTCTTTAtgatcaagagacaggatgaggatcgtttcg
pLPT41-PrrnB-F	cgaaacgatcctcatcctgtctcttgatcaTAAAGAATAAAAAATGCGCGGTCAGAAAAT
mCherry-PrrnB-R	cttcgccttttgaaaccatatTTTACCTCCTTTATGTGTCAGTGGTGGCGCATTATAGGG
pLPT41-PflgI-F	tcatgcgaaacgatcctcatcctgtctcttgatcaTGACCATCAACGGCATAAATAGCGA
mCherry-PflgI-R	cttcgccttttgaaaccatatTTTACCTCCTTTATATCTCCTCCGCAGGTATCAAAATTC
pLPT41-PphoB-F	atcatgcgaaacgatcctcatcctgtctcttgatcaGTGCCATTTGCTTTTTTCTGCGCC
mCherry-PphoB-R	ccttttgaaaccatatTTTACCTCCTTTATGATTTGCCCTGTTGTAATAAATAGGTTGCG
pLPT41-pliv-F	catgcgaaacgatcctcatcctgtctcttgatcaTCCCCACGCAGATTGTTAATAAACTG
mCherry-Pliv-R	tcgcctttgaaaccatatTTTACCTCCTTTATTAGCAGCATACTCTGCTAAAACATACC
pLPT41-Pthr-F	tcatgcgaaacgatcctcatcctgtctcttgatcaAACTGGTTACCTGCCGTGAGTAAAT
mCherry-Pthr-R	ttcgcctttgaaaccatatTTTACCTCCTTTATTCTGTCTGTGCGCTATGCCTATATTG
ChemoK-reporter-F	acctgcgtgcaatccatcttgt
ChemoK-reporter-R	cgaaggctgataccgctcgccgc
reporter-ChemoK-R	acctagggcgttcggctgcggcgagcggtatcagccttcggcacgggcaaattgc
reporter-ChemoK-F	ggatcgtttcgcatgattgaacaagatggattgcacgcaggtggctcactcaaaggcggt
tag-mCherry-R	ATAGTTTTCATCGTTGGCTGCtttgtacagttcatccataccgccgg
DAS2-mCherry-F	AATTACGCGGATGCCAGTtaaaagcttgatatcgaattcctgcagcc
LAA-mCherry-F	gcactggctgcttaaaagcttgatatcgaattcctgcagcc

Table 2. List of plasmids used in this study

Plasmid	Description	Origin of replication	Selection marker	Source/reference
pLPT41	Backbone for pLPT41-series plasmids	ColE1 derivative	KanR	(Potvin-Trottier *et al.*, 2016)
pChemoK	Backbone for pSC101-series plasmids	pSC101	CmR	(Moon *et al.*, 2011b)
pCRISPReporter-mCherry	Template for E. coli codon-optimized mCherry	pBR322	AmpR	(Cress *et al.*, 2015b)
pLPT41-P_thr-mCherry	pLPT41-P_thr-mCherry	ColE1 derivative	KanR	This study
pLPT41-P_ilv-mCherry	pLPT41-P_ilv-mCherry	ColE1 derivative	KanR	This study
pLPT41-P_liv-mCherry	pLPT41-P_liv-mCherry	ColE1 derivative	KanR	This study
pLPT41-P_phoB-mCherry	pLPT41-P_phoB-mCherry	ColE1 derivative	KanR	This study
pSC101-Pthr-mCherry	pSC101-Pthr-mCherry	pSC101	CmR	This study
pSC101-P_ilv-mCherry	pSC101-P_ilv-mCherry	pSC101	CmR	This study
pSC101-rrnBP1-mCherry	pSC101-rrnBP1-mCherry	pSC101	CmR	This study
pSC101-P_phoB-mCherry	pSC101-P_phoB-mCherry	pSC101	CmR	This study
pSC101-P_liv-mCherry	pSC101-P_liv-mCherry	pSC101	CmR	This study
pLPT41-P_thr-mCherry-LAA	pLPT41-P_thr-mCherry-LAA	ColE1 derivative	KanR	This study
pLPT41-P_thr-mCherry-DAS2	pLPT41-P_thr-mCherry-DAS2	ColE1 derivative	KanR	This study
pSC101-Pthr-mCherry-LAA	pSC101-Pthr-mCherry-LAA	pSC101	CmR	This study
pSC101-P_ilv-mCherry-LAA	pSC101-P_ilv-mCherry-LAA	pSC101	CmR	This study
pSC101-rrnBP1-mCherry-LAA	pSC101-rrnBP1-mCherry-LAA	pSC101	CmR	This study
pSC101-Pthr-mCherry-DAS2	pSC101-Pthr-mCherry-DAS2	pSC101	CmR	This study
pSC101-P_ilv-mCherry-DAS2	pSC101-P_ilv-mCherry-DAS2	pSC101	CmR	This study
pSC101-rrnBP1-mCherry-DAS2	pSC101-rrnBP1-mCherry-DAS2	pSC101	CmR	This study

Table 3. List of strains used in this study

Strain	Description	Parental strain	Source/reference
MG1655-ASV, rpoS-mcherry	rpos-mCherry::frt, attB::rrnBP1::gfpASV (AmpR)	MG1655	(Maisonneuve, Castro-Camargo and Gerdes, 2013)
MG1655 rpoS-mcherry	rpoS-mCherry::frt	MG1655	(Maisonneuve, Castro-Camargo and Gerdes, 2013)
WL12	pLPT41-P_thr-mCherry	MG1655	This study
WL13	pLPT41-P_ilv-mCherry	MG1655	This study
WL14	pLPT41-P_liv-mCherry	MG1655	This study
WL15	pLPT41-P_phoB-mCherry	MG1655	This study
WL27	pSC101-Pthr-mCherry	MG1655	This study
WL28	pSC101-P_ilv-mCherry	MG1655	This study
WL29	pSC101-rrnBP1-mCherry	MG1655	This study
WL30	pSC101-P_phoB-mCherry	MG1655	This study
WL31	pSC101-P_liv-mCherry	MG1655	This study
WL32	pLPT41-P_thr-mCherry-LAA	MG1655	This study
WL33	pLPT41-P_thr-mCherry-DAS2	MG1655	This study
WL34	pSC101-Pthr-mCherry-LAA	MG1655	This study
WL35	pSC101-P_ilv-mCherry-LAA	MG1655	This study
WL36	pSC101-rrnBP1-mCherry-LAA	MG1655	This study
WL37	pSC101-Pthr-mCherry-DAS2	MG1655	This study
WL38	pSC101-P_ilv-mCherry-DAS2	MG1655	This study
WL39	pSC101-rrnBP1-mCherry-DAS2	MG1655	This study

REFERENCES

Ault-Riché, D. *et al.* (1998) 'Novel assay reveals multiple pathways regulating stress-induced accumulations of inorganic polyphosphate in Escherichia coli.', *Journal of bacteriology*, 180(7), pp. 1841–7. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9537383 (Accessed: 9 December 2018).

Balsalobre, C. (2011) 'Concentration matters!! ppGpp, from a whispering to a strident alarmone', *Molecular Microbiology*. John Wiley & Sons, Ltd, 79(4), pp. 827–829. doi: 10.1111/j.1365-2958.2010.07521.x.

Barker, M. M. *et al.* (2001) 'Mechanism of regulation of transcription initiation by ppGpp. I. Effects of ppGpp on transcription initiation in vivo and in vitro', *Journal of Molecular Biology*. Academic Press, 305(4), pp. 673–688. doi: 10.1006/JMBI.2000.4327.

BD Bionutrients ™ Technical Manual (no date). Available at: https://www.bdbiosciences.com/documents/bionutrients_tech_manual.pdf (Accessed: 2 December 2018).

Cavanagh, A. T., Chandrangsu, P. and Wassarman, K. M. (2010) '6S RNA regulation of relA alters ppGpp levels in early stationary phase.', *Microbiology (Reading, England)*. Microbiology Society, 156(Pt 12), pp. 3791–800. doi: 10.1099/mic.0.043992-0.

Ceroni, F. *et al.* (2015) 'Quantifying cellular capacity identifies gene expression designs with reduced burden', *Nature Methods*. Nature Publishing Group, 12(5), pp. 415–418. doi: 10.1038/nmeth.3339.

Consortium, S.-I. *et al.* (2014) 'A comprehensive assessment of RNA-seq accuracy, reproducibility and information content by the Sequencing Quality Control Consortium', *Nature*

Biotechnology. Nature Publishing Group, 32(9), pp. 903–914. doi: 10.1038/nbt.2957.

Cress, B. F. *et al.* (2015a) 'CRISPathBrick: Modular Combinatorial Assembly of Type II-A CRISPR Arrays for dCas9-Mediated Multiplex Transcriptional Repression in *E. coli*', *ACS Synthetic Biology*, 4(9), pp. 987–1000. doi: 10.1021/acssynbio.5b00012.

Cress, B. F. *et al.* (2015b) 'CRISPathBrick: Modular Combinatorial Assembly of Type II-A CRISPR Arrays for dCas9-Mediated Multiplex Transcriptional Repression in *E. coli*', *ACS Synthetic Biology*, 4(9), pp. 987–1000. doi: 10.1021/acssynbio.5b00012.

Dalebroux, Z. D. and Swanson, M. S. (2012) 'ppGpp: magic beyond RNA polymerase', *Nature Reviews Microbiology*, 10(3), pp. 203–212. doi: 10.1038/nrmicro2720.

Dennis, P. P., Ehrenberg, M. and Bremer, H. (2004) 'Control of rRNA Synthesis in Escherichia coli: a Systems Biology Approach †', *MICROBIOLOGY AND MOLECULAR BIOLOGY REVIEWS*, 68(4), pp. 639–668. doi: 10.1128/MMBR.68.4.639-668.2004.

Dong, H., Nilsson, L. and Kurland, C. G. (1995) 'Gratuitous overexpression of genes in Escherichia coli leads to growth inhibition and ribosome destruction.', *Journal of bacteriology*. American Society for Microbiology Journals, 177(6), pp. 1497–504. doi: 10.1128/JB.177.6.1497-1504.1995.

Durfee, T. *et al.* (2008) 'Transcription profiling of the stringent response in Escherichia coli.', *Journal of bacteriology*. American Society for Microbiology Journals, 190(3), pp. 1084–96. doi: 10.1128/JB.01092-07.

Espah Borujeni, A. *et al.* (2016) 'Automated physics-based design of synthetic riboswitches from diverse RNA aptamers', *Nucleic Acids Research*. Oxford University Press, 44(1), pp. 1–13. doi: 10.1093/nar/gkv1289.

Etzel, M. and Mörl, M. (2017) 'Synthetic Riboswitches: From Plug and Pray toward Plug and

Play', *Biochemistry*. American Chemical Society, 56(9), pp. 1181–1198. doi: 10.1021/acs.biochem.6b01218.

Gaca, A. O. *et al.* (2013) 'Basal levels of (p)ppGpp in Enterococcus faecalis: the magic beyond the stringent response.', *mBio*. American Society for Microbiology, 4(5), pp. e00646-13. doi: 10.1128/mBio.00646-13.

Girard, M. E. *et al.* (2017) 'DksA and ppGpp Regulate the σS Stress Response by Activating Promoters for the Small RNA DsrA and the Anti-Adapter Protein IraP.', *Journal of bacteriology*. American Society for Microbiology Journals, 200(2), pp. e00463-17. doi: 10.1128/JB.00463-17.

Gourse, R. L. *et al.* (1996) 'rRNA TRANSCRIPTION AND GROWTH RATE–DEPENDENT REGULATION OF RIBOSOME SYNTHESIS IN *ESCHERICHIA COLI*', *Annual Review of Microbiology*. Annual Reviews 4139 El Camino Way, P.O. Box 10139, Palo Alto, CA 94303-0139, USA , 50(1), pp. 645–677. doi: 10.1146/annurev.micro.50.1.645.

Gourse, R. L. *et al.* (2018) 'Transcriptional Responses to ppGpp and DksA', *Annual Review of Microbiology*. Annual Reviews , 72(1), pp. 163–184. doi: 10.1146/annurev-micro-090817-062444.

Haney, S. A. *et al.* (1992) 'Lrp, a leucine-responsive protein, regulates branched-chain amino acid transport genes in Escherichia coli.', *Journal of bacteriology*, 174(1), pp. 108–15. Available at: http://www.ncbi.nlm.nih.gov/pubmed/1729203 (Accessed: 9 December 2018).

Hauryliuk, V. *et al.* (2015) 'Recent functional insights into the role of (p)ppGpp in bacterial physiology', *Nature Reviews Microbiology*. Nature Publishing Group, 13(5), pp. 298–309. doi: 10.1038/nrmicro3448.

Jin, D. J., Cagliero, C. and Zhou, Y. N. (2012) 'Growth rate regulation in *Escherichia coli*', *FEMS Microbiology Reviews*. Oxford University Press, 36(2), pp. 269–287. doi: 10.1111/j.1574-

6976.2011.00279.x.

Lee, J. W. *et al.* (2016) 'Creating Single-Copy Genetic Circuits', *Molecular Cell*, 63(2), pp. 329–336. doi: 10.1016/j.molcel.2016.06.006.

Liao, C., Blanchard, A. E. and Lu, T. (2017) 'An integrative circuit–host modelling framework for predicting synthetic gene network behaviours', *Nature Microbiology*. Nature Publishing Group, 2(12), pp. 1658–1666. doi: 10.1038/s41564-017-0022-5.

Maisonneuve, E., Castro-Camargo, M. and Gerdes, K. (2013) 'RETRACTED: (p)ppGpp Controls Bacterial Persistence by Stochastic Induction of Toxin-Antitoxin Activity', *Cell*. Cell Press, 154(5), pp. 1140–1150. doi: 10.1016/J.CELL.2013.07.048.

Marisch, K. *et al.* (2013) 'Evaluation of three industrial Escherichia coli strains in fed-batch cultivations during high-level SOD protein production', *Microbial Cell Factories*. BioMed Central, 12(1), p. 58. doi: 10.1186/1475-2859-12-58.

McGinness, K. E., Baker, T. A. and Sauer, R. T. (2006) 'Engineering Controllable Protein Degradation', *Molecular Cell*. Cell Press, 22(5), pp. 701–707. doi: 10.1016/J.MOLCEL.2006.04.027.

Moon, T. S. *et al.* (2011a) 'Construction of a genetic multiplexer to toggle between chemosensory pathways in Escherichia coli.', *Journal of molecular biology*. NIH Public Access, 406(2), pp. 215–27. doi: 10.1016/j.jmb.2010.12.019.

Moon, T. S. *et al.* (2011b) 'Construction of a genetic multiplexer to toggle between chemosensory pathways in Escherichia coli.', *Journal of molecular biology*. NIH Public Access, 406(2), pp. 215–27. doi: 10.1016/j.jmb.2010.12.019.

Paul, B. J., Berkmen, M. B. and Gourse, R. L. (2005) 'DksA potentiates direct activation of amino acid promoters by ppGpp', *Proceedings of the National Academy of Sciences*, 102(22),

pp. 7823–7828. doi: 10.1073/pnas.0501170102.

Potrykus, K. *et al.* (2011) ‘ppGpp is the major source of growth rate control in E. coli’, *Environmental Microbiology*. Wiley/Blackwell (10.1111), 13(3), pp. 563–575. doi: 10.1111/j.1462-2920.2010.02357.x.

Potrykus, K. and Cashel, M. (2008) ‘(p)ppGpp: Still Magical? *’. doi: 10.1146/annurev.micro.62.081307.162903.

Potvin-Trottier, L. *et al.* (2016) ‘Synchronous long-term oscillations in a synthetic gene circuit’, *Nature*. Nature Publishing Group, 538(7626), pp. 514–517. doi: 10.1038/nature19841.

Qi, L. *et al.* (2012) ‘Engineering naturally occurring trans -acting non-coding RNAs to sense molecular signals’, *Nucleic Acids Research*. Oxford University Press, 40(12), pp. 5775–5786. doi: 10.1093/nar/gks168.

Rao, N. N., Liu, S. and Kornberg, A. (1998) ‘Inorganic polyphosphate in Escherichia coli: the phosphate regulon and the stringent response.’, *Journal of bacteriology*, 180(8), pp. 2186–93. Available at: http://www.ncbi.nlm.nih.gov/pubmed/9555903 (Accessed: 9 December 2018).

Ross, W. *et al.* (2016) ‘ppGpp Binding to a Site at the RNAP-DksA Interface Accounts for Its Dramatic Effects on Transcription Initiation during the Stringent Response’, *Molecular Cell*. Cell Press, 62(6), pp. 811–823. doi: 10.1016/J.MOLCEL.2016.04.029.

Sánchez-Vázquez, P. (2018) *Genome-wide effects of ppGpp binding to RNA Polymerase on E. coli gene expression*. Available at: https://search.proquest.com/pqdtglobal/docview/2070598050/fulltextPDF/2150D77B76E94B0DPQ/4?accountid=14553 (Accessed: 9 December 2018).

Sarubbi, E., Rudd, K. E. and Cashel, M. (1988) *Basal ppGpp level adjustment shown by new spoT mutants affect steady state growth rates and rrnA ribosomal promoter regulation in*

Escherichia coil. Available at: https://link.springer.com/content/pdf/10.1007%2FBF00339584.pdf (Accessed: 11 December 2018).

Scott, M. *et al.* (2010) 'Interdependence of cell growth and gene expression: origins and consequences.', *Science (New York, N.Y.)*. American Association for the Advancement of Science, 330(6007), pp. 1099–102. doi: 10.1126/science.1192588.

Sezonov, G., Joseleau-Petit, D. and D'Ari, R. (2007) 'Escherichia coli physiology in Luria-Bertani broth.', *Journal of bacteriology*. American Society for Microbiology Journals, 189(23), pp. 8746–9. doi: 10.1128/JB.01368-07.

Shachrai, I. *et al.* (2010a) 'Cost of Unneeded Proteins in E. coli Is Reduced after Several Generations in Exponential Growth', *Molecular Cell*. Cell Press, 38(5), pp. 758–767. doi: 10.1016/J.MOLCEL.2010.04.015.

Shachrai, I. *et al.* (2010b) 'Cost of Unneeded Proteins in E. coli Is Reduced after Several Generations in Exponential Growth', *Molecular Cell*. Cell Press, 38(5), pp. 758–767. doi: 10.1016/J.MOLCEL.2010.04.015.

Shah, D. *et al.* (2006) 'Persisters: a distinct physiological state of E. coli', *BMC Microbiology*. BioMed Central, 6(1), p. 53. doi: 10.1186/1471-2180-6-53.

Sherlock, M. E., Sudarsan, N. and Breaker, R. R. (2018) 'Riboswitches for the alarmone ppGpp expand the collection of RNA-based signaling systems.', *Proceedings of the National Academy of Sciences of the United States of America*. National Academy of Sciences, 115(23), pp. 6052–6057. doi: 10.1073/pnas.1720406115.

Traxler, M. F. *et al.* (2008) 'The global, ppGpp-mediated stringent response to amino acid starvation in Escherichia coli', *Molecular Microbiology*. Wiley/Blackwell (10.1111), 68(5), pp.

1128–1148. doi: 10.1111/j.1365-2958.2008.06229.x.

Traxler, M. F. *et al.* (2011) 'Discretely calibrated regulatory loops controlled by ppGpp partition gene induction across the "feast to famine" gradient in Escherichia coli', *Molecular Microbiology*. Wiley/Blackwell (10.1111), 79(4), pp. 830–845. doi: 10.1111/j.1365-2958.2010.07498.x.

Varik, V. *et al.* (2017) 'HPLC-based quantification of bacterial housekeeping nucleotides and alarmone messengers ppGpp and pppGpp', *Scientific Reports*. Nature Publishing Group, 7(1), p. 11022. doi: 10.1038/s41598-017-10988-6.

Young, J. W. *et al.* (2011) 'Measuring single-cell gene expression dynamics in bacteria using fluorescence time-lapse microscopy.', *Nature protocols*. NIH Public Access, 7(1), pp. 80–8. doi: 10.1038/nprot.2011.432.