Dynamic Regulation of Ribosomal Gene Expression During Adaptation to Fluctuating Nutritional Conditions

By

Hector L. Burgos

A dissertation submitted in partial fulfillment of

the requirements for the degree of

Doctor of Philosophy

(Microbiology)

at the

UNIVERSITY OF WISCONSIN-MADISON

2017

Date of final oral examination: Wednesday, August 16th, 2017

The dissertation is approved by the following members of the Final Oral Committee:

Richard L. Gourse, Professor, Bacteriology
Briana Burton, Professor, Bacteriology
James L. Keck, Professor, Biomolecular Chemistry
Robert L. Landick, Professor, Biochemistry
Karen M. Wassarman, Professor, Bacteriology

Dynamic Regulation of Ribosomal Gene Expression During Adaptation to Fluctuating Nutritional Conditions

Hector L. Burgos

Under the supervision of Professor Richard L. Gourse

University of Wisconsin-Madison

In *E. coli*, ribosome synthesis is tightly regulated with respect to nutritional conditions to ensure appropriate management of cellular resources during growth. To accomplish this the cell uses ppGpp and DksA to directly regulate transcription of rRNA and most r-protein promoters. In addition, r-proteins are regulated by translational feedback: when r-protein levels exceed the levels of free rRNA, repressor r-proteins bind to their own mRNAs and inhibit synthesis of their operon at the level of translation. Since ppGpp can directly regulate r-protein promoters and indirectly regulate r-protein translation through effects on rRNA synthesis, the focus of my thesis work was to determine the relative contributions of transcriptional control and translational feedback to regulation of r-protein synthesis with respect to nutritional conditions.

I designed a chromosomally-encoded reporter system to test regulation of r-protein promoters separately from translational feedback. I found that regulation of r-protein synthesis in response to changes in ppGpp levels during exponential growth and during entry into stationary phase happens primarily through translational feedback. On the other hand, r-protein promoters play a role in regulating r-protein expression during late stationary phase and during outgrowth in response to variations in NTP levels.

ppGpp and DksA can both inhibit and activate transcription depending on the promoter. Whereas the role of promoter sequences in inhibition of transcription by DksA and ppGpp have been studied previously, analogous studies of activation have not been performed. I studied the role

promoter sequences have in activation of P*iraP* transcription by DksA and ppGpp. I found that mutations in the -35, -10, and discriminator region of the *iraP* promoter affect activation of this promoter by DksA and ppGpp, presumably because these mutations affected the intrinsic kinetics of this promoter during transcription initiation.

Since previous literature show that ppGpp can regulate lipid metabolism, at both the enzymatic and transcriptional levels, I tested direct regulation by ppGpp and DksA of multiple promoters of genes involved in lipid metabolism. Preliminary results show that some promoters of genes involved in lipid metabolism are indeed regulated directly by ppGpp and DksA. These results show that ppGpp plays a central role in maintaining cellular homeostasis and regulating resource utilization in response to different environmental conditions.

Dedication

I dedicate this work to:

My Mother, Janette Robles, for her unwavering support and love, without which I would not be what I am now.

My Father, Hector I. Burgos, for teaching me to always try to make the best decisions I can throughout life.

My older brother, Enrique Vargas, for being a phenomenal role model and inspiring me to work hard to achieve my goals.

My younger brother, Emanuel F. Burgos, for infusing my life with just the right amount of joy.

Angel R. Del Valle-Echevarria, for his endless emotional support and comic relief that helped me persevere throughout my graduate studies.

Bradon McDonald, for all the intellectually-stimulating conversations we have had and all the ones that are yet to come.

Shelby Ann Sandstrom, for helping me develop into the best version of myself.

Acknowledgements

Endless gratitude to my advisor, Richard L. Gourse, for providing a supportive and rigorous learning environment in which I could develop as a scientist, teaching me to think objectively, and always having my best interests at heart.

Many thanks to Wilma Ross, for always being willing to discuss research and provide her ample technical and intellectual expertise.

I sincerely appreciate all the encouragement and constructive criticisms that members of my committee (Heidi Goodrich-Blair, James L. Keck, Robert L. Landick, and Karen M. Wassarman) have given me throughout Graduate School that contributed greatly to my development as a scientist.

Thanks to Janet Newlands, for her invaluable expertise and assistance on all things that should have a firewall.

Thanks to Tamas Gaal, for contributing a unique and expansive point of view that broadened my own perspective of science.

My gratitude to Abbey Thompson and Christina Hull, for their ever-present support and interest in all my endeavors.

Many thanks to Cathy Davis Gray and Kari Straus for their assistance in wading through the bureaucracy of academic research.

Finally, my appreciation goes out to all members of the Gourse lab, past and present, for being fantastic coworkers and friends. It has been my utmost pleasure to work with all of you.

Special thanks to Briana Burton, for her willingness to step in at the last minute and fill a vacancy in my Final Oral Committee.

Table of Contents

List of Figures

List of Tables

Chapter 1

Introduction

Bacteria regulate gene expression to adapt and survive

Microorganisms are ubiquitous across the biosphere. As such they must adjust to a plethora of different environmental conditions to thrive, including changing oxygen levels, metal and essential nutrients availability, temperature, pH, carbon and energy sources, water content, hydrostatic pressure, and intracellular microenvironments during disease and symbiosis. Bacteria have evolved multiple mechanisms that allow them to adapt to and survive in these varied ecosystems. These include biofilm formation, sporulation, persistence, quorum sensing, programmed cell death, and morphological changes such as the swarmer-to-stalk transition of *Caulobacter crescentus* and formation of fruiting bodies by *Myxococcus xanthus* (Dworkin, 1996; Engelberg-Kulka et al., 2006; Kussell et al., 2005; Miller and Bassler, 2001; O'Toole et al., 2000; Piggot and Coote, 1976; Shapiro, 1976).

While these processes result in different physiological outcomes, all of them rely on bacteria sensing environmental signals accurately and modulating gene expression accordingly. This places regulation of gene expression as one of the main strategies microbes employ to survive and, accordingly, years of study on this topic have shown that all stages of gene expression, from DNA supercoiling (Dorman, 1991) to post-translational modification of proteins (Cain et al., 2014), are subject to regulation.

Regulation of ribosome levels

One of the earliest studied systems for regulation of gene expression with respect to nutritional conditions was the ribosome. In bacteria, ribosomes are composed of three rRNA molecules, 5S, 16S, and 23S, and more than 50 different ribosomal proteins (r-proteins), all of which must be synthesized in the correct amounts to prevent ribosome assembly defects and in enough quantity to support the translational requirement of the cell (Nomura, 1999; Nomura et al., 1984). In

E. coli, the numbers of ribosomes per cell can vary from 7,000 up to 70,000 depending on growth rate (Bremer and Dennis, 1996). Due to the high amount of resources needed for generating and maintaining these levels of ribosomes and the energy consumed during translation, ribosomes are the biggest consumers of energy and nutrients in bacteria during fast growth and as such ribosome synthesis is tightly regulated with respect to nutritional conditions (Nomura et al., 1984). The protein elongation rate per ribosome remains constant at different growth rates (Nomura, 1999), therefore regulation of ribosome activity is primarily achieved by regulating the synthesis of its components, rRNA and r-proteins. During periods of very slow growth and in stationary phase the Ribosome Modulation Factor (RMF) causes dimerization of ribosomes, which form 100S particles that are catalytically inactive (Wada et al., 1995).

Regulation of ribosomal protein synthesis

Most of the more than 50 different r-protein genes in *E. coli* are organized within operons that are located in the half of the chromosome containing the origin of replication (Nomura et al., 1984). While their organization in operons may seem to partly explain the stoichiometric synthesis of r-proteins, early studies showed that synthesis of most r-proteins is regulated at the level of translation through a mechanism known as translational feedback (Dean and Nomura, 1980; Dennis and Fill, 1979; Fallon et al., 1979; Yates et al., 1980). During growth, newly synthesized r-proteins quickly bind to free rRNA and are assembled into the ribosome. However, when there is a decrease in the levels of free rRNA, excess r-proteins accumulate and several dual-function r-proteins act as repressors, binding to their own mono or polycistronic mRNA and inhibiting expression of the operon at the level of translation (Zengel and Lindahl, 1994). Each operon has one r-protein that acts as the repressor and is specific to its own operon (Fig. 1.1). Additional mechanisms have been described that account for the observation that when repressor r-proteins bind to a single site on the

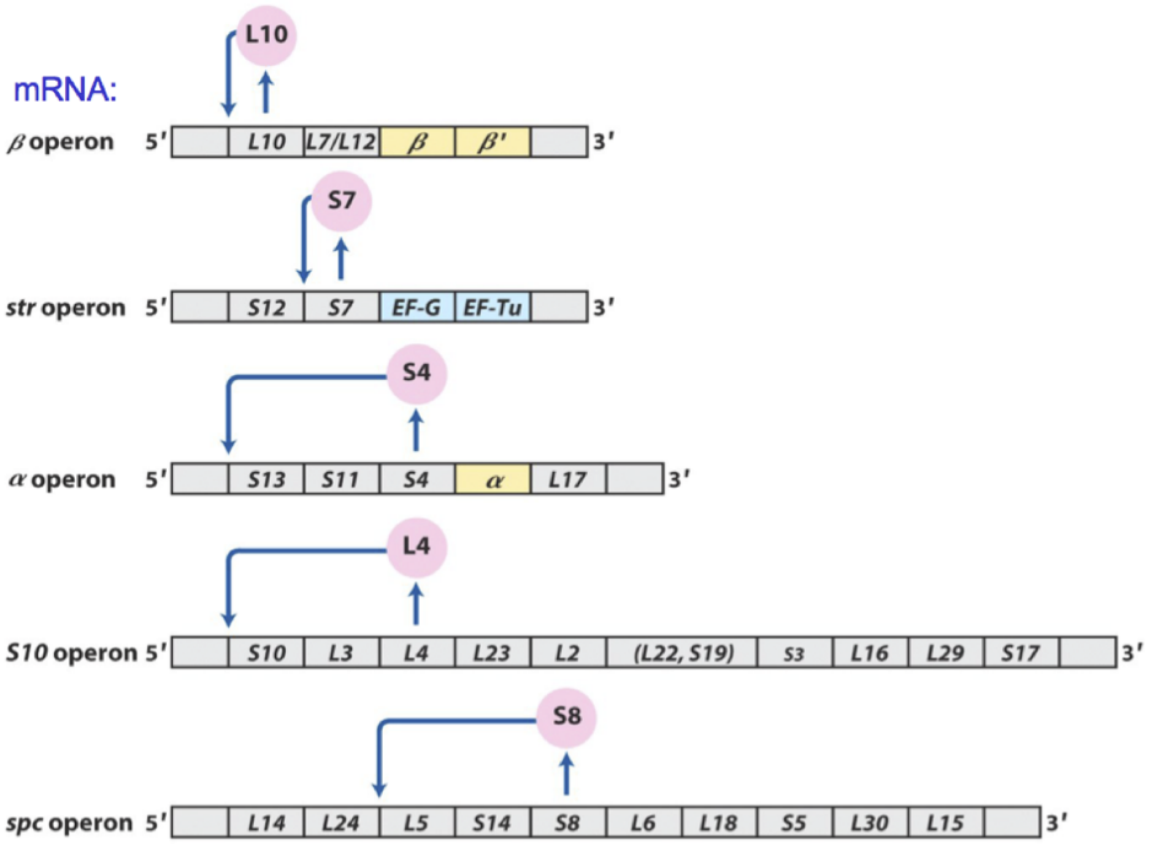

Figure 1.1 Ribosomal protein operons encode their own repressor. Schematic diagram of various ribosomal protein operons showing the genetic organization and highlighting the repressor and target site for each operon. Adapted from Nelson et al. (2008).

polycistronic mRNA, usually in the leader region of one of the cistrons, this results in inhibition of expression of every member of the operon (Nomura et al., 1984; Zengel and Lindahl, 1994).

Translational coupling, the mechanism where translation of downstream cistrons must be preceded by translation of upstream cistrons, has been shown for the L11-L1 and L35-L20 operons (Baughman and Nomura, 1983; Lesage et al., 1992). The current model for this mechanism is that there are secondary structures in the mRNA of these operons that occlude the ribosome binding site (RBS) of downstream ORFs until an incoming translating ribosome causes melting of those structures, increasing accessibility to the RBS and allowing expression of those downstream genes. Another mechanism, retroregulation, has been described for the *spc* operon, where binding of the repressor r-protein S8 to the *spc* mRNA near the RBS for the third cistron stimulates endonucleolytic cleavage of the message, leading to further degradation of the mRNA by exonucleases, which ultimately results in decreased expression of the first two cistrons, coding for L14 and L24 (Mattheakis et al., 1989). The L11-L1 mRNA is destabilized when the repressor L1 binds to its target site in the L11 leader due to translation inhibition and the subsequent loss of protection from nucleases provided by translating ribosomes (Cole and Nomura, 1986). Nonetheless, L1 binding to its target site is not thought to directly stimulate endonucleolytic cleavage of the L11-L1 mRNA as is thought to be the case during S8 binding of the *spc* mRNA. Finally, for the S10 operon, binding of the repressor L4 to the S10 leader causes inhibition of translation and early termination of transcription, resulting in tight regulation of the 11 member operon (Zengel and Lindahl, 1994).

Regulation by translational feedback has been demonstrated recently for several additional r-protein operons that had not been studied previously. The mRNA leader region of the single-member *rpsA* (S1) operon folds into a structure of three hairpins that form a non-contiguous ribosome binding site. S1 binding to the *rpsA* leader disrupts the three-hairpin structure eliminating

the RBS and inhibiting translation initiation (Boni et al., 2001). Both members of the *rpsB-tsf* operon were shown to be under translational feedback regulation by S2 binding to the *rpsB* leader region (Aseev et al., 2008). Interestingly, mutants that produce lower levels of S1 have impaired S2-dependent translational feedback regulation of the *rpsB-tsf* operon, suggesting S1 might work together with S2 for repression of *rpsB-tsf* (Aseev et al., 2008). The *rplY* (L25) and *rplM-rpsI* (L13-S9) operons are under translational feedback regulation by L25 and L13, respectively (Aseev et al., 2015; 2016). Finally, the *rpsF-priB-rpsR-rplL* operon is regulated by an S6:S18 complex that binds to the leader region of *rpsF* (Babina et al., 2015).

Mechanism of translational feedback inhibition

The competition of the repressor r-protein binding to rRNA versus the mRNA target led to the hypothesis that the mRNA binding site mimics the rRNA binding site (Nomura et al., 1984). Initial studies identified homologous regions in the primary and secondary sequences of the repressor mRNA target sites compared to their rRNA binding sites. However, those homologies were not always accurate in predicting the repressor binding site (Zengel and Lindahl, 1994) and instead it was found that it is the tertiary structure that is similar between the binding sites. Chemical footprinting of L4 bound to its target on the S10 mRNA leader showed that although there is no significant similarity in secondary structure, there are significant similarities in tertiary structure between the L4 binding sites on the S10 mRNA leader and on 23S rRNA (Stelzl et al., 2003). Furthermore, crystallographic studies showed that the structure of S8 bound to its target on the *spc* mRNA is similar to the structure of S8 bound to its 16S binding site, helix 21 (Fig. 1.2; Merianos et al., 2004). These findings support the model that structural mimicry between the mRNA and rRNA binding site of repressor r-proteins plays an important role in translational feedback regulation.

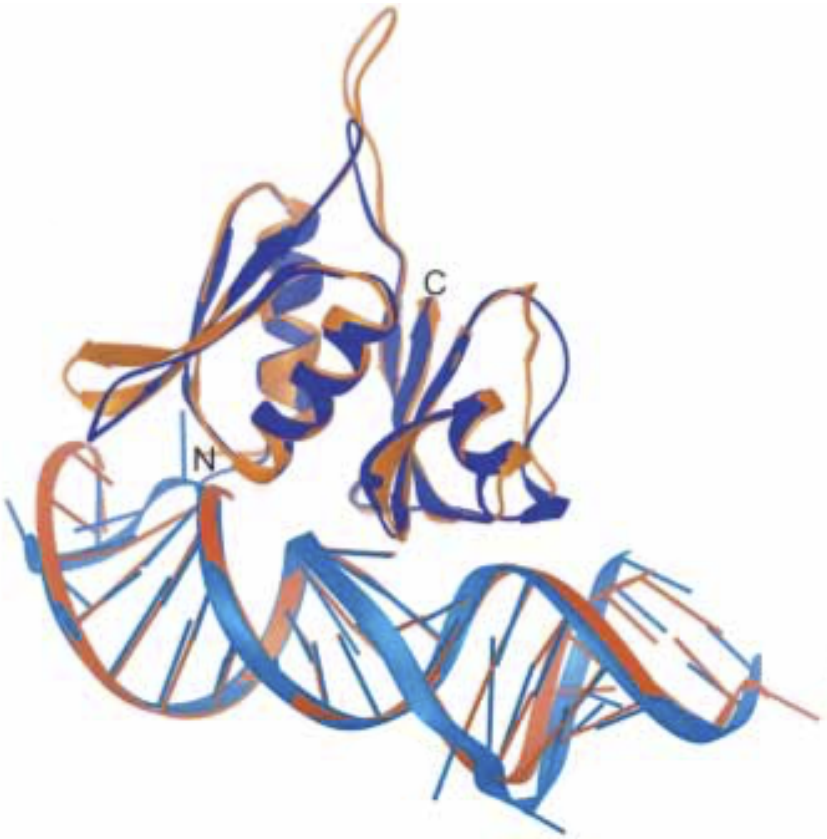

Figure 1.2 Structural mimicry between the S8 RNA binding sites. Crystallographic structure of S8 bound to helix 21 of *T. thermophilus* 16S rRNA (orange) superimposed with the structure of S8 bound to the *E. coli spc* operon mRNA target site (blue). While there are a few differences, it is apparent that the *spc* mRNA target mimics the tertiary structure of the 16S binding site for S8. Adapted from Merianos et al. (2004).

Even though early work showed that regulation through translational feedback occurs at the level of translation initiation, detailed studies on the specific mechanisms by which this happens have been performed for only a few r-protein operons. A recent study showed that regulation of the L35- L20 operon occurs through direct competition between the repressor r-protein L20 and 30S ribosomal subunit binding to the leader of the L35 ORF (Haentjens-Sitri et al., 2008). On the other hand, regulation of the *rpsO-pnp* operon by S15 occurs through an entrapment mechanism where repressor binding to its target site does not prevent 30S binding to the *rpsO-pnp* mRNA RBS, but instead traps the 30S-mRNA-$tRNA_f^{Met}$ ternary complex in an inactive conformation that prevents translation (Bénard et al., 1996). Regulation of the α operon by S4 occurs through this entrapment mechanism as well (reviewed in Schlax and Worhunsky, 2003).

We note that for the translational feedback mechanism to work, the repressor r-proteins must bind preferentially to rRNA vs. their mRNA target. Nonetheless, binding assays have shown that in some cases r-proteins bind to rRNA and their mRNA target with comparable affinities, suggesting that the strong preference of repressor r-proteins to bind rRNA is due to the sequestration of r-proteins into the ribosome assembly pathway, and not solely due to differences in binding affinity (Zengel and Lindahl, 1994). Therefore, the translational feedback mechanism essentially serves as an accurate sensor for the levels of rRNA, adjusting the output of r-protein correspondingly.

Regulation of r-protein transcription

In addition to translational feedback, there is substantial evidence that r-protein synthesis is also regulated at the level of transcription. Previously it was shown that ppGpp inhibits transcription *in vitro* from a DNA template carrying ~27 r-proteins (Lindahl et al., 1976). A separate study showed that ppGpp also inhibits *in vitro* transcription of S20 (Wirth et al., 1981). Furthermore, stringent

control of the S10 operon was shown to require only the S10 promoter (Freedman et al., 1985). Global transcriptome analysis using microarrays showed that expression of many r-protein genes are inhibited during amino acid starvation in a *relA*-dependent manner (Traxler et al., 2008). Experiments performed in our lab showed that indeed ppGpp and DksA can directly regulate promoter activity of many r-protein promoters (Lemke et al., 2011).

Recent work investigating r-protein operons that have not been studied before shows that transcription of the *rplM-rpsI*, *rpmB-rpmG*, *rplU-rpmA*, and *rplY* r-protein operons is regulated by DksA and ppGpp (Aseev et al., 2015; 2016; Maouche et al., 2016); however, of these r-protein operons only the *rplY* promoter has been shown to be directly regulated by ppGpp and DksA (Aseev et al., 2015). Finally, unpublished RNAseq data from our lab shows that *in vivo* transcription of all r-protein operons is strongly inhibited in response to induction of ppGpp synthesis (L3 is the most strongly inhibited at 10 min after induction of ppGpp synthesis [~16-fold] but all r-protein genes are inhibited more than 2-fold; Sanchez-Vazquez, P., and Gourse, R.L., *manuscript in preparation*). While this global dataset cannot differentiate between direct and indirect effects of ppGpp on r-protein transcription, the results are consistent with direct regulation of r-protein promoter activity by ppGpp and DksA as shown in Lemke et al. (2011) and with the ppGpp-dependent inhibition of r-protein transcription seen in Traxler et al. (2008).

Expression of rRNA genes

In *E. coli*, rRNA is expressed from 7 rRNA operons that are located in the half of the chromosome closest to the origin of replication (Blattner et al., 1997). During very fast growth, up to 85% of total RNA synthesis in *E. coli* is stable RNA synthesis, which is mainly tRNA and rRNA (Bremer and Dennis, 1996). Such high levels of rRNA synthesis is due primarily to the strength of the rRNA promoters, which are able to engage up to 80% of active RNAP in transcription of stable

RNA (Bremer and Dennis, 1996). Each rRNA operon is transcribed from two promoters, P1 and P2, that have near-consensus core promoter sequences, which together with sequences upstream of these core promoters explain the considerable strength of these promoters *in vivo* (Paul et al., 2004b).

The UP element is an A + T-rich region adjacent to the -35 hexamer that binds the αCTD subunit of RNAP and stimulates transcription initiation at rRNA promoters (Ross et al., 1993). In addition, multiple Fis-binding sites are present further upstream from the core rRNA promoters, with the specific number of sites varying from 3-5 depending on the operon and with distance of the binding sites from the P1 promoter ranging from -71 to -181 (Hirvonen et al., 2001). Fis binding to it target sites upstream of rRNA promoters has been shown to result in recruitment of RNAP and to facilitate later steps during transcription initiation, increasing transcription of rRNA especially during early log phase when the levels of Fis increase transiently but substantially (Paul et al., 2004b). Together, UP elements and Fis-binding sites increase transcription from rRNA promoters up to ~300-fold (Paul et al., 2004b).

Regulation of rRNA synthesis

Because r-protein synthesis is regulated with respect to rRNA levels through translational feedback, synthesis of rRNA is the rate-limiting step in ribosome biogenesis and as such is regulated tightly with respect to nutritional conditions (Nomura et al., 1984). The rate of rRNA synthesis is carefully regulated with respect to growth rate to ensure that ribosomes are generated quickly enough to support growth, but not to exceed the metabolic capacity of the cell and waste resources on unneeded ribosomes (Gourse et al., 1996). During the stringent response, a classical example of cellular adaptation to nutrient deprivation, ppGpp levels quickly increase in response to amino acid starvation (Gallant, 1979), resulting in inhibition of rRNA synthesis and increased expression of amino acid biosynthetic operons (Haugen et al., 2008). Ribosomal RNA synthesis is also subject to

feedback control, where any nutritional change that disrupts translation results in corresponding changes in rRNA expression, ensuring that ribosome biogenesis is properly balanced with the translational capacity of the cell (Paul et al., 2004b). Finally, the nucleoid-associated protein H-NS has been shown to play a role in decreasing rRNA expression as cells enter stationary phase (Afflerbach et al., 1998).

Early studies investigating the sequences involved in regulation of rRNA synthesis showed that ppGpp can enhance pausing within the rRNA leader *in vitro* (Kingston and Chamberlin, 1981), suggesting the *rrn* leader could play a role in stringent control. However, later experiments testing the role of these regions *in vivo* showed that the *rrn* leader is not required for stringent control (Gourse et al., 1983), whereas the core *rrn* P1 promoter is sufficient for stringent control and feedback regulation of rRNA transcription (Gourse et al., 1986). Nonetheless, these results do not preclude other regulatory mechanisms that might act through the *rrn* leader. The core *rrn* P2 promoters are also regulated by stringent control, feedback control, and growth rate-dependent control, but are generally weaker than the P1 promoters and their regulation is not as strong (Murray et al., 2003a). These results showed that regulation of rRNA synthesis happens at the level of transcription initiation.

Mechanism for regulation of rRNA transcription initiation

Transcription in bacteria is initiated by RNAP binding to the core promoter sequences on DNA, known as the -35 and -10 hexamers. After forming the RNAP-promoter complex (RP), a series of isomerization steps occur that result in DNA melting, positioning of the template strand +1 base (the transcription start site) in the active site, and forming the transcription-competent open complex (RP_O). Incoming NTPs can then access the active site within RP_O, anneal to the base positioned in the active site and initiate RNA synthesis that leads to transcription elongation.

The levels of ppGpp and NTPs regulate the activity of ribosomal RNA promoters due to their intrinsic kinetic properties and result in an unusually short-lived RP_O (reviewed in Haugen et al., 2008). ppGpp decreases the stability of the RP_O of all promoters tested, but this only results in inhibition of promoter activity for promoters that have intrinsically short-lived open complexes like *rrnB* P1 (Barker et al., 2001). On the other hand, also due to the transient nature of the RP_O, rRNA promoters require unusually high levels of their initiating NTPs (iNTPs) for maximal activity (Gaal et al., 1997). Even though regulation of rRNA promoter activity by ppGpp and iNTPs can be reconstituted in a purified system, the transcription factor DksA is required for regulation of rRNA synthesis *in vivo* (Paul et al., 2004a). DksA also destabilizes the RP_O, ensuring that the lifetime of the complex is short enough that it is sensitive to regulation by ppGpp and NTP levels (Haugen et al., 2008). DksA and ppGpp are also able to directly activate amino acid promoters, however the kinetic requirements of the promoters for activation are different than for inhibition; activated promoters have a stable RP_O but a high activation energy to get to that state (Paul et al., 2005).

ppGpp and DksA bind directly to RNAP to regulate transcription (Barker et al., 2001; Paul et al., 2004a). DksA binds in the secondary channel of RNAP and makes contacts near the RNAP active site that have been suggested to play a role in the mechanism of DksA-dependent regulation of transcription (Lennon et al., 2012). ppGpp binds to two sites on RNAP, one is at the interface of the ω and β' subunits (site 1; Ross et al., 2013), and the other is at the interface of DksA and the RNAP rim helices (site 2; Ross et al., 2016) (Fig. 1.3). The latter site is largely responsible for ppGpp-dependent regulation both *in vivo* and *in vitro*, and accounts for the synergistic relationship between DksA and ppGpp (Ross et al., 2016). Furthermore, while ppGpp alone can partially inhibit transcription of rRNA promoters through site 1, activation of transcription requires site 2 (Ross et al., 2016), consistent with the requirement for DksA in activating transcription *in vivo* and *in vitro* (Paul et al., 2005).

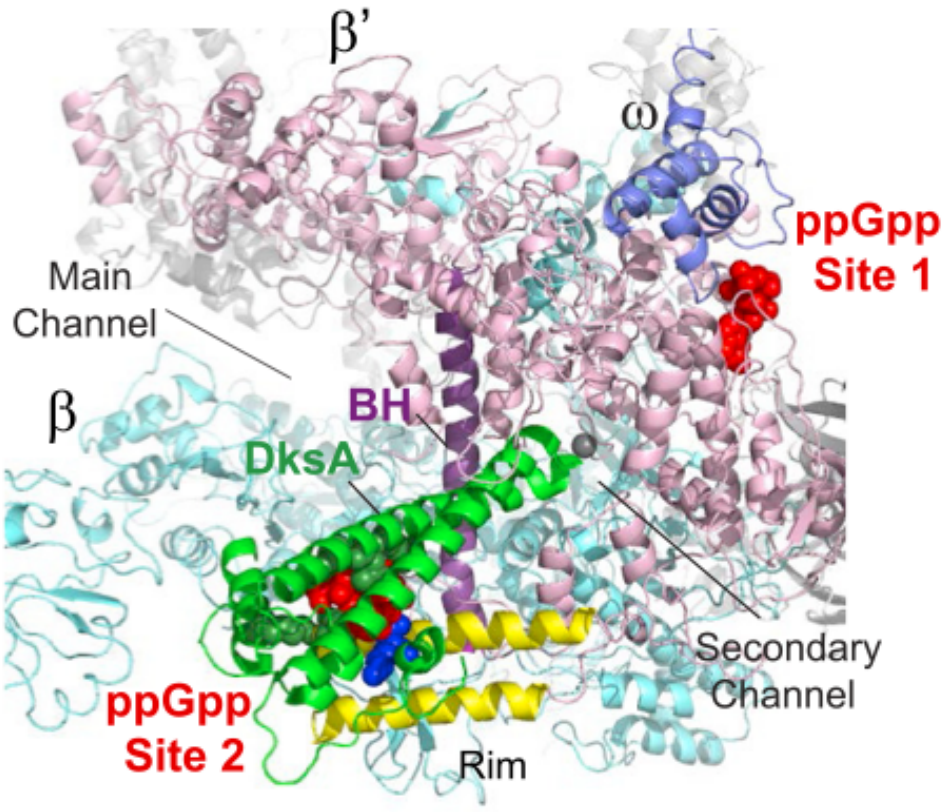

Figure 1.3 ppGpp binds to two sites on RNAP. Model showing the two binding sites for ppGpp on RNAP. DksA and ppGpp bound to site 2 were modeled into a structure of RNAP with ppGpp bound in site 1. Adapted from Ross et al. (2016).

Small molecules as signals of cellular metabolism

The levels of ppGpp and NTPs vary during growth to regulate rRNA transcription with respect to nutritional conditions (Fig. 1.4; Murray et al., 2003b). Changes in nutritional conditions during exponential growth result in a corresponding change in the intracellular levels of ppGpp that regulate ribosomal and amino acid gene expression and allow the cells to adapt to their new environmental conditions. As cells transition into stationary phase there is a transient increase in ppGpp levels and a decrease in NTP levels, both acting to decrease ribosome synthesis. During late stationary phase ppGpp levels steadily decrease down to background levels, however, transcription from rRNA promoters remains low because NTP levels are already low (Murray et al., 2003b). When nutritional conditions improve, a sharp increase in NTP levels results in a concurrent increase in rRNA transcription; ppGpp levels are already low in stationary phase, so a further decrease in ppGpp levels cannot account for the increase in rRNA synthesis (Murray et al., 2003b).

In *E. coli*, ppGpp is synthesized by the proteins RelA and SpoT (Potrykus and Cashel, 2008). RelA synthesizes ppGpp when uncharged tRNAs are present in the A-site of ribosomes during translation (Wendrich et al., 2002), in this way ppGpp serves as a signal of amino acid availability. SpoT can both synthesize and hydrolyze ppGpp (Potrykus and Cashel, 2008; Srivatsan and Wang, 2008). The activity of SpoT has been shown to be involved in responses to starvation of fatty acids, phosphate, carbon, and iron, in addition to osmotic shock and detergent stress (reviewed in Potrykus and Cashel, 2008; Srivatsan and Wang, 2008); nonetheless, the exact mechanism through which different signals regulate SpoT activity requires further study. ATP and GTP are consumed during protein synthesis and as such their levels serve as signals of translational activity and regulate ribosome biogenesis accordingly (Gaal et al., 1997).

Taken together, the adjustment and flux of the levels of ppGpp and NTPs work together with DksA to adjust ribosome biogenesis with respect to variations in nutritional conditions so that

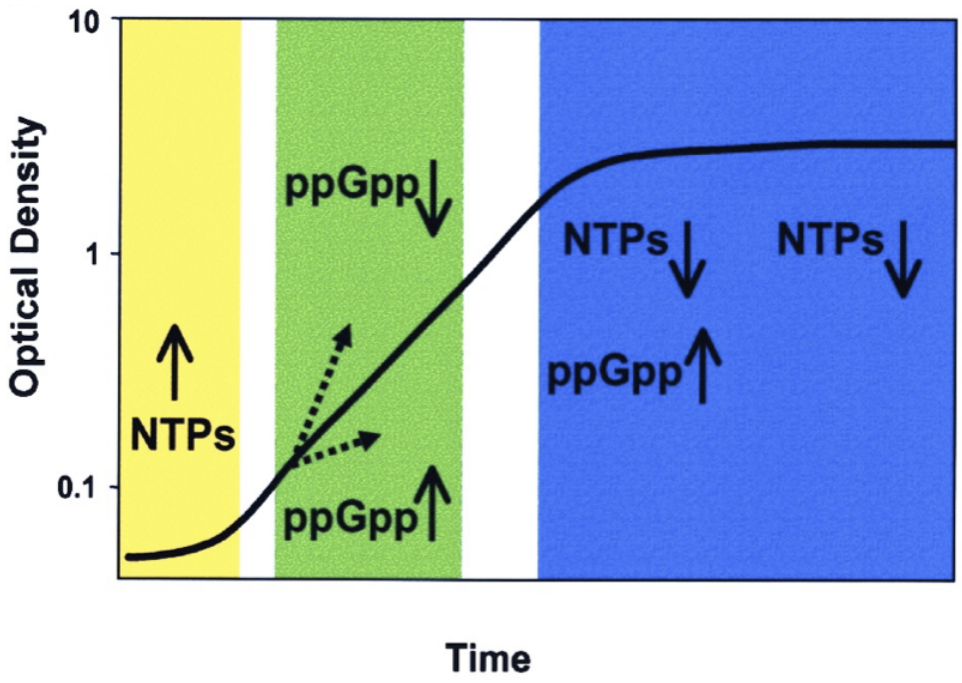

Figure 1.4 ppGpp and NTP levels vary during growth. Hypothetical growth curve showing respective changes in ppGpp and NTP levels during growth. The dotted lines on the exponential growth part of the curve represent either an increase or decrease in rRNA synthesis due to changes in ppGpp levels caused by sudden changes in nutritional conditions. During entry into stationary phase ppGpp levels increase and NTP levels decrease, however, ppGpp levels eventually decrease again while NTP levels remain low. Adapted from Murray et al. (2003b).

homeostasis can be maintained (Fig. 1.5; Paul et al., 2004b). In addition to its role in regulating global transcription, ppGpp has been shown to have a role in regulating DNA replication, translation elongation, and pathogenesis, consistent with its proposed role as a central regulator of bacterial metabolism and physiology (reviewed in Potrykus and Cashel, 2008; Srivatsan and Wang, 2008).

Regulation of ribosome synthesis in other organisms

Although most studies on regulation of ribosome synthesis have focused on *E. coli*, there is evidence for similar mechanisms acting in many other bacterial species. For example, the S10 operon from several bacteria related to *E. coli*, including five enterobacteria and two non-enteric members of the gamma subdivision, have mRNA secondary structure features in common and is feedback regulated by the repressor r-protein (L4) from *E. coli* (Allen et al., 1999). The S10 and *spc* operons in *Vibrio cholera*, a member of γ-proteobacteria, were shown to be under translational feedback regulation by L4 and S8, respectively (Allen et al., 2004). The regulatory region within the leader of the *rpsB-tsf* operon from *E. coli* is conserved in several γ-proteobacteria, and fusions of the *rpsB* leader from those bacteria to *lacZ* were regulated at the translational level by S2 (*rpsB*) (Aseev et al., 2009). Furthermore, a phylogenetic analysis of 10 RNA regulatory regions of r-protein operons from *E. coli* showed that 7 of them were conserved only within γ-proteobacteria, while the remaining three were found to varying extents in most other bacterial phyla (only acidobacteria had none of the 10 regulatory regions studied; Fu et al., 2013).

Even though regulation of r-protein synthesis has not been extensively studied in *Bacillus subtilis*, ribosome synthesis is also inhibited in response to amino acid starvation, and rRNA synthesis is the rate-limiting step for ribosome synthesis (Henkin, 2002). Synthesis of r-protein S4 has been shown to be under translational feedback control in *B. subtilis* (Grundy and Henkin, 1991),

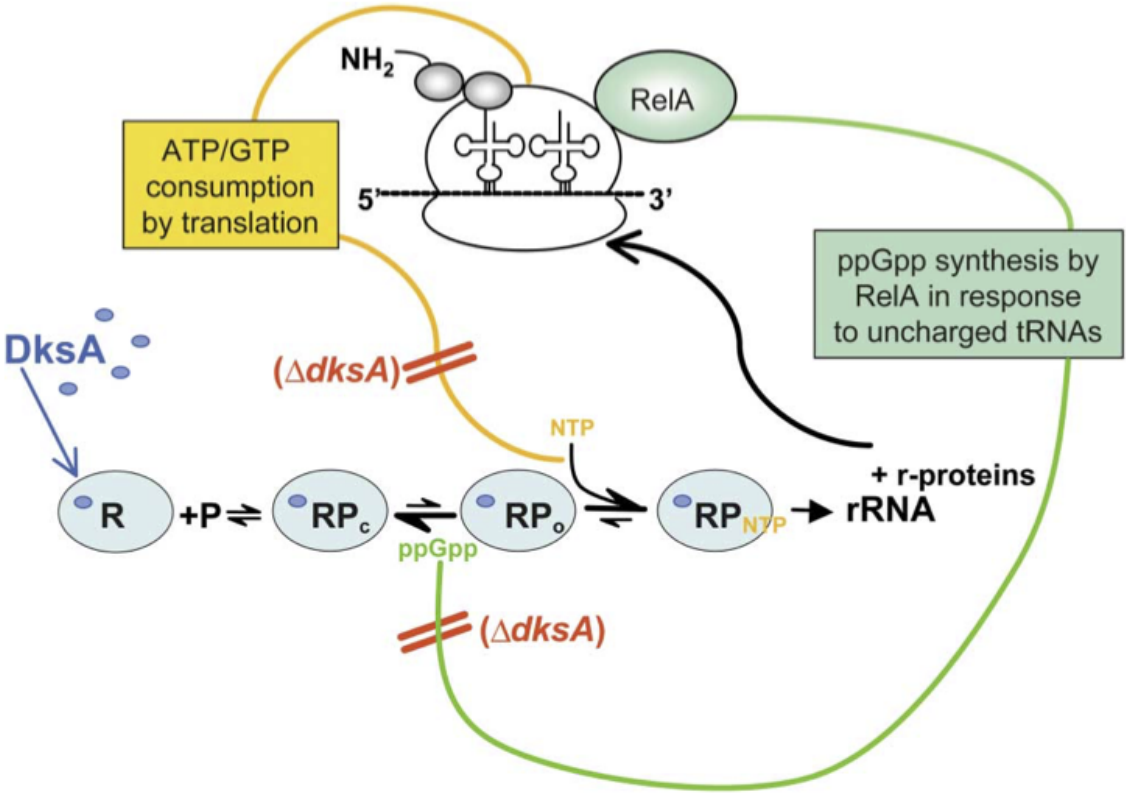

Figure 1.5 Feedback control of ribosome synthesis. ppGpp and NTPs, together with DksA, regulate synthesis of rRNA. ppGpp is synthesized by RelA in response to uncharged tRNAs, while ATP and GTP are consumed during the process of translation. Both molecules serve as signals of translational activity in the cell. DksA binds directly to RNAP and sensitizes rRNA promoters to regulation by ppGpp and iNTPs. Adapted from Paul et al. (2004a).

suggesting that mechanisms for regulation of r-protein synthesis in *E. coli* are conserved outside proteobacteria. However, inhibition of rRNA synthesis in *B. subtilis* is not due to direct binding of ppGpp to RNAP, rather ppGpp accumulation causes a decrease in the concentration of GTP, which is the initiating NTP for all rRNA operons in this organism, that results in a concurrent decrease of rRNA synthesis (Krásný and Gourse, 2004). Further studies showed that ppGpp decreases GTP levels by binding to and inhibiting the activity of various GTP biosynthetic enzymes in *B. subtilis* (Kriel et al., 2012).

Synthesis of r-proteins from the L1 operon in various archaeal species is regulated by a translational feedback mechanism involving a bi-functional repressor r-protein that responds to variations in rRNA levels (Kraft et al., 1999). Finally, mechanisms for translational control of ribosomal protein synthesis in response to different signals seem to be present in several eukaryotic organisms studied (Meyuhas et al., 1996). Taken together, these results indicate that regulation of ribosome synthesis with respect to nutritional conditions and regulation of r-proteins at the translational level are conserved features amongst most living organisms.

References

Afflerbach, H., Schröder, O., and Wagner, R. (1998). Effects of the *Escherichia coli* DNA-binding protein H-NS on rRNA synthesis *in vivo*. Mol. Microbiol. *28*, 641–653.

Allen, T., Shen, P., Samsel, L., Liu, R., Lindahl, L., and Zengel, J.M. (1999). Phylogenetic analysis of L4-mediated autogenous control of the S10 ribosomal protein operon. J. Bacteriol. *181*, 6124–6132.

Allen, T.D., Watkins, T., Lindahl, L., and Zengel, J.M. (2004). Regulation of ribosomal protein synthesis in *Vibrio cholerae*. J. Bacteriol. *186*, 5933–5937.

Aseev, L.V., Levandovskaya, A.A., Skaptsova, N.V., and Boni, I.V. (2009). Conservation of regulatory elements controlling the expression of the *rpsB-tsf* operon in γ-proteobacteria. Mol. Biol. *43*, 101-107.

Aseev, L.V., Bylinkina, N.S., and Boni, I.V. (2015). Regulation of the *rplY* gene encoding 5S rRNA binding protein L25 in *Escherichia coli* and related bacteria. RNA *21*, 851–861.

Aseev, L.V., Koledinskaya, L.S., and Boni, I.V. (2016). Regulation of Ribosomal Protein Operons *rplM-rpsI*, *rpmB-rpmG*, and *rplU-rpmA* at the Transcriptional and Translational Levels. J. Bacteriol. *198*, 2494–2502.

Aseev, L.V., Levandovskaya, A.A., Tchufistova, L.S., Scaptsova, N.V., and Boni, I.V. (2008). A new regulatory circuit in ribosomal protein operons: S2-mediated control of the *rpsB-tsf* expression *in vivo*. RNA *14*, 1882–1894.

Babina, A.M., Soo, M.W., Fu, Y., and Meyer, M.M. (2015). An S6:S18 complex inhibits translation of *E. coli rpsF*. RNA *21*, 1-8.

Barker, M.M., Gaal, T., Josaitis, C.A., and Gourse, R.L. (2001). Mechanism of regulation of transcription initiation by ppGpp. I. Effects of ppGpp on transcription initiation *in vivo* and *in vitro*. J. Mol. Biol. *305*, 673–688.

Baughman, G., and Nomura, M. (1983). Localization of the target site for translational regulation of the L11 operon and direct evidence for translational coupling in *Escherichia coli*. Cell *34*, 979–988.

Bénard, L., Philippe, C., Ehresmann, B., Ehresmann, C., and Portier, C. (1996). Pseudoknot and translational control in the expression of the S15 ribosomal protein. Biochimie *78*, 568–576.

Blattner, F.R., Plunkett, G., Bloch, C.A., Perna, N.T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J.D., Rode, C.K., Mayhew, G.F., et al. (1997). The complete genome sequence of *Escherichia coli* K-12. Science *277*, 1453–1462.

Boni, I.V., Artamonova, V.S., Tzareva, N.V., and Dreyfus, M. (2001). Non-canonical mechanism for translational control in bacteria: synthesis of ribosomal protein S1. Embo J. *20*, 4222–4232.

Bremer, H., and Dennis, P.P. (1996). Modulation of chemical composition and other parameters of the cell by growth rate. *Escherichia Coli* and *Salmonella*: Cellular and Molecular Biology *2*, 1553–1569.

Cain, J.A., Solis, N., and Cordwell, S.J. (2014). Beyond gene expression: the impact of protein post-translational modifications in bacteria. J. Proteomics *97*, 265–286.

Cole, J.R., and Nomura, M. (1986). Changes in the half-life of ribosomal protein messenger RNA caused by translational repression. J. Mol. Biol. *188*, 383–392.

Dean, D., and Nomura, M. (1980). Feedback regulation of ribosomal protein gene expression in *Escherichia coli*. Proc. Natl. Acad. Sci. U S A *77*, 3590-3594.

Dennis, P.P., and Fill, N.P. (1979). Transcriptional and post-transcriptional control of RNA polymerase and ribosomal protein genes cloned on composite ColE1 plasmids in the bacterium *Escherichia coli*. J. Biol. Chem. *254*, 7540–7547.

Dorman, C.J. (1991). DNA supercoiling and environmental regulation of gene expression in pathogenic bacteria. Infection and Immunity *59*, 745–749.

Dworkin, M. (1996). Recent advances in the social and developmental biology of the myxobacteria. Microbiol. Rev. *60*, 70–102.

Engelberg-Kulka, H., Amitai, S., Kolodkin-Gal, I., and Hazan, R. (2006). Bacterial programmed cell death and multicellular behavior in bacteria. PLoS Genet. *2*, 1518-1526.

Fallon, A.M., Jinks, C.S., Yamamoto, M., and Nomura, M. (1979). Expression of ribosomal protein genes cloned in a hybrid plasmid in *Escherichia coli*: gene dosage effects on synthesis of ribosomal proteins and ribosomal protein messenger ribonucleic acid. J. Bacteriol. *138*, 383–396.

Freedman, L.P., Zengel, J.M., and Lindahl, L. (1985). Genetic dissection of stringent control and nutritional shift-up response of the *Escherichia coli* S10 ribosomal protein operon. J. Mol. Biol. *185*,701-712.

Fu, Y., Deiorio-Haggar, K., Anthony, J., and Meyer, M.M. (2013). Most RNAs regulating ribosomal protein biosynthesis in *Escherichia coli* are narrowly distributed to Gammaproteobacteria. Nucleic Acids Res. *41*, 3491–3503.

Gaal, T., Bartlett, M.S., Ross, W., Turnbough, C.L., and Gourse, R.L. (1997). Transcription regulation by initiating NTP concentration: rRNA synthesis in bacteria. Science *278*, 2092–2097.

Gallant, J.A. (1979). Stringent control in *E. coli*. Annu. Rev. Genet. *13*, 393–415.

Gourse, R.L., de Boer, H.A., and Nomura, M. (1986). DNA determinants of rRNA synthesis in *E. coli*: growth rate dependent regulation, feedback inhibition, upstream activation, antitermination. Cell *44*, 197–205.

Gourse, R.L., Gaal, T., Bartlett, M.S., Appleman, J.A., and Ross, W. (1996). rRNA transcription and growth rate-dependent regulation of ribosome synthesis in *Escherichia coli*. Annu. Rev. Microbiol. *50*, 645–677.

Gourse, R.L., Stark, M.J., and Dahlberg, A.E. (1983). Regions of DNA involved in the stringent control of plasmid-encoded rRNA *in vivo*. Cell *32*, 1347–1354.

Grundy, F.J., and Henkin, T.M. (1991). The *rpsD* gene, encoding ribosomal protein S4, is autogenously regulated in *Bacillus subtilis*. J. Bacteriol. *173*, 4595–4602.

Haentjens-Sitri, J., Allemand, F., Springer, M., and Chiaruttini, C. (2008). A competition mechanism regulates the translation of the *Escherichia* coli *operon* encoding ribosomal proteins L35 and L20. J. Mol. Biol. *375*, 612–625.

Haugen, S.P., Ross, W., and Gourse, R.L. (2008). Advances in bacterial promoter recognition and its control by factors that do not bind DNA. Nat. Rev. Microbiol. *6*, 507–519.

Henkin, T.M. (2002). Ribosomes, protein synthesis factors, and tRNA synthetases. In *Bacillus Subtilis* and Its Closest Relatives: from Genes to Cells, A.L. Sonenshein, ed. (Washington, D.C.: ASM Press), pp.313-322.

Hirvonen, C.A., Ross, W., Wozniak, C.E., Marasco, E., Anthony, J.R., Aiyar, S.E., Newburn, V.H., and Gourse, R.L. (2001). Contributions of UP Elements and the Transcription Factor FIS to Expression from the Seven *rrn* P1 Promoters in *Escherichia coli*. J. Bacteriol. *183*, 6305–6314.

Kingston, R.E., and Chamberlin, M.J. (1981). Pausing and attenuation of *in vitro* transcription in the *rrnB* operon of *E. coli*. Cell *27*, 523–531.

Kraft, A., Lutz, C., Lingenhel, A., Gröbner, P., and Piendl, W. (1999). Control of ribosomal protein L1 synthesis in mesophilic and thermophilic archaea. Genetics *152*, 1363–1372.

Krásný, L., and Gourse, R.L. (2004). An alternative strategy for bacterial ribosome synthesis: *Bacillus subtilis* rRNA transcription regulation. Embo J. *23*, 4473–4483.

Kriel, A., Bittner, A.N., Kim, S.H., Liu, K., Tehranchi, A.K., Zou, W.Y., Rendon, S., Chen, R., Tu, B.P., and Wang, J.D. (2012). Direct regulation of GTP homeostasis by (p)ppGpp: a critical component of viability and stress resistance. Mol. Cell *48*, 231–241.

Kussell, E., Kishony, R., Balaban, N.Q., and Leibler, S. (2005). Bacterial persistence: a model of survival in changing environments. Genetics *169*, 1807–1814.

Lemke, J.J., Sanchez-Vazquez, P., Burgos, H.L., Hedberg, G., Ross, W., and Gourse, R.L. (2011). Direct regulation of Escherichia coli ribosomal protein promoters by the transcription factors ppGpp and DksA. Proc. Natl. Acad. Sci. U S A *108*, 5712–5717.

Lennon, C.W., Ross, W., Martin-Tumasz, S., Toulokhonov, I., Vrentas, C.E., Rutherford, S.T., Lee, J.-H., Butcher, S.E., and Gourse, R.L. (2012). Direct interactions between the coiled-coil tip of DksA and the trigger loop of RNA polymerase mediate transcriptional regulation. Genes Dev. *26*, 2634–2646.

Lesage, P., Chiaruttini, C., Graffe, M., Dondon, J., Milet, M., and Springer, M. (1992). Messenger RNA secondary structure and translational coupling in the *Escherichia coli* operon encoding translation initiation factor IF3 and the ribosomal proteins, L35 and L20. J. Mol. Biol. *228*, 366–386.

Lindahl, L., Post, L., and Nomura, M. (1976). DNA-dependent in vitro synthesis of ribosomal proteins, protein elongation factors, and RNA polymerase subunit α: inhibition by ppGpp. Cell *9*,

439–448.

Maouche, R., Burgos, H.L., My, L., Viala, J.P., Gourse, R.L., and Bouveret, E. (2016). Coexpression of *Escherichia coli* obgE, Encoding the Evolutionarily Conserved Obg GTPase, with Ribosomal Proteins L21 and L27. J. Bacteriol. *198*, 1857–1867.

Mattheakis, L., Vu, L., Sor, F., and Nomura, M. (1989). Retroregulation of the synthesis of ribosomal proteins L14 and L24 by feedback repressor S8 in *Escherichia coli*. Proc. Natl. Acad. Sci. U S A *86*, 448–452.

Merianos, H.J., Wang, J., and Moore, P.B. (2004). The structure of a ribosomal protein S8/*spc* operon mRNA complex. RNA *10*, 954–964.

Meyuhas, O., Avni, D., and Shama, S. (1996). Translational control of ribosomal protein mRNAs in eukaryotes. In Translational Control, J.W.B. Hershey, ed. (Cold Spring Harbor, NY: CSHL Press), pp. 363-409.

Miller, M.B., and Bassler, B.L. (2001). Quorum Sensing in Bacteria. Annu. Rev. Microbiol. *55*, 165–199.

Murray, H.D., Appleman, J.A., and Gourse, R.L. (2003a). Regulation of the *Escherichia coli rrnB* P2 promoter. J. Bacteriol. *185*, 28–34.

Murray, H.D., Schneider, D.A., and Gourse, R.L. (2003b). Control of rRNA expression by small molecules is dynamic and nonredundant. Mol. Cell *12*, 125–134.

Nelson, D.L., Lehninger, A.L., and Cox, M.M. (2008). Lehninger Principles of Biochemistry (Macmillan).

Nomura, M. (1999). Regulation of ribosome biosynthesis in *Escherichia coli* and *Saccharomyces* cerevisiae: diversity and common principles. J. Bacteriol. *181*, 6857–6864.

Nomura, M., Gourse, R., and Baughman, G. (1984). Regulation of the synthesis of ribosomes and ribosomal components. Annu. Rev. Biochem. *53*, 75–117.

O'Toole, G., Kaplan, H.B., and Kolter, R. (2000). Biofilm formation as microbial development. Annu. Rev. Microbiol. *54*, 49–79.

Paul, B.J., Barker, M.M., Ross, W., Schneider, D.A., Webb, C., Foster, J.W., and Gourse, R.L. (2004a). DksA: a critical component of the transcription initiation machinery that potentiates the regulation of rRNA promoters by ppGpp and the initiating NTP. Cell *118*, 311–322.

Paul, B.J., Berkmen, M.B., and Gourse, R.L. (2005). DksA potentiates direct activation of amino acid promoters by ppGpp. Proc. Natl. Acad. Sci. U S A *102*, 7823–7828.

Paul, B.J., Ross, W., Gaal, T., and Gourse, R.L. (2004b). rRNA transcription in *Escherichia coli*. Annu. Rev. Genet. *38*, 749–770.

Piggot, P.J., and Coote, J.G. (1976). Genetic aspects of bacterial endospore formation. Bacteriol.

Rev. *40*, 908–962.

Potrykus, K., and Cashel, M. (2008). (p)ppGpp: still magical? Annu. Rev. Microbiol. *62*, 35–51.

Ross, W., Gosink, K.K., Salomon, J., Igarashi, K., Zou, C., Ishihama, A., Severinov, K., and Gourse, R.L. (1993). A third recognition element in bacterial promoters: DNA binding by the alpha subunit of RNA polymerase. Science *262*, 1407–1413.

Ross, W., Sanchez-Vazquez, P., Chen, A.Y., Lee, J.-H., Burgos, H.L., and Gourse, R.L. (2016). ppGpp Binding to a Site at the RNAP-DksA Interface Accounts for Its Dramatic Effects on Transcription Initiation during the Stringent Response. Mol. Cell *62*, 811–823.

Ross, W., Vrentas, C.E., Sanchez-Vazquez, P., Gaal, T., and Gourse, R.L. (2013). The magic spot: a ppGpp binding site on *E. coli* RNA polymerase responsible for regulation of transcription initiation. Mol. Cell *50*, 420–429.

Schlax, P.J., and Worhunsky, D.J. (2003). Translational repression mechanisms in prokaryotes. Mol. Microbiol. *48*, 1157-1169.

Shapiro, L. (1976). Differentiation in the *Caulobacter* cell cycle. Annu. Rev. Microbiol. *30*, 377-407.

Srivatsan, A., and Wang, J.D. (2008). Control of bacterial transcription, translation and replication by (p)ppGpp. Curr. Opin. Microbiol. *11*, 100–105.

Stelzl, U., Zengel, J.M., Tovbina, M., Walker, M., Nierhaus, K.H., Lindahl, L., and Patel, D.J. (2003). RNA-structural mimicry in *Escherichia coli* ribosomal protein L4-dependent regulation of the S10 operon. J. Biol. Chem. *278*, 28237–28245.

Traxler, M.F., Summers, S.M., Nguyen, H.-T., Zacharia, V.M., Hightower, G.A., Smith, J.T., and Conway, T. (2008). The global, ppGpp-mediated stringent response to amino acid starvation in *Escherichia coli*. Mol. Microbiol. *68*, 1128–1148.

Wada, A., Igarashi, K., Yoshimura, S., Aimoto, S., and Ishihama, A. (1995). Ribosome modulation factor: stationary growth phase-specific inhibitor of ribosome functions from *Escherichia coli*. Biochem. Biophys. Res. Commun. *214*, 410–417.

Wendrich, T.M., Blaha, G., Wilson, D.N., Marahiel, M.A., and Nierhaus, K.H. (2002). Dissection of the mechanism for the stringent factor RelA. Mol. Cell *10*, 779–788.

Wirth, R., Kohles, V., and Böck, A. (1981). Factors modulating transcription and translation *in vitro* of ribosomal protein S20 and isoleucyl-tRNA synthetase from *Escherichia coli*. Eur. J. Biochem. *114*, 429–437.

Yates, J.L., Arfsten, A.E., and Nomura, M. (1980). *In vitro* expression of *Escherichia coli* ribosomal protein genes: autogenous inhibition of translation. Proc. Natl. Acad. Sci. U S A *77*, 1837–1841.

Zengel, J.M., and Lindahl, L. (1994). Diverse mechanisms for regulating ribosomal protein synthesis in *Escherichia coli*. Prog. Nucleic Acid Res. Mol. Biol. *47*, 331–370.

Chapter 2

Roles of Transcriptional and Translational Control Mechanisms in Regulation of Ribosomal Protein Synthesis in *Escherichia coli*

This chapter is in press (Burgos, H.L, O'Connor, K., Sanchez-Vazquez, P., and Gourse, R.L. [2017]. Roles of transcriptional and translational control mechanisms in regulation of ribosomal protein synthesis in Escherichia coli. J. Bacteriol.). Most of the experiments were performed by me. Kevin O'Connor performed the experiments shown in Figure 2.6, and Patricia Sanchez-Vazquez performed the experiment shown in Fig. 2.5B. Richard L. Gourse, Wilma Ross, and I wrote the manuscript.

Abstract

Bacterial ribosome biogenesis is tightly regulated to match nutritional conditions and to prevent formation of defective ribosomal particles. In *E. coli*, most ribosomal protein (r-protein) synthesis is coordinated with ribosomal RNA synthesis by a translational feedback mechanism: when r-proteins exceed rRNAs, specific r-proteins bind to their own mRNAs and inhibit expression of the operon. It was recently discovered that the second messenger nucleotide ppGpp, which directly regulates rRNA promoters, is also capable of regulating many r-protein promoters. To examine the relative contributions of the translational and transcriptional control mechanisms to the regulation of r-protein synthesis, we devised a reporter system that enabled us to genetically separate the cis-acting sequences responsible for the two mechanisms and to quantify their relative contributions to regulation under the same conditions. We show that the synthesis of r-proteins from the S20 and S10 operons is regulated by ppGpp following shifts in nutritional conditions, but most of the effect of ppGpp required the 5' region of the r-protein mRNA containing the target site for translational feedback regulation, not the promoter. These results suggest that most regulation of the S20 and S10 operons by ppGpp following nutritional shifts is indirect and occurs in response to changes in rRNA synthesis. In contrast, we found that the promoters for the S20 operon were regulated during outgrowth, likely in response to increasing NTP levels. Thus, r-protein synthesis is dynamic, with different mechanisms acting at different times.

Importance

Bacterial cells have evolved complex and seemingly redundant strategies to regulate many high energy-consuming processes. In *E. coli*, synthesis of ribosomal components is tightly regulated with respect to nutritional conditions by mechanisms that act at both the transcription and translation steps. In this work, we conclude that both NTP and ppGpp concentrations can regulate

synthesis of ribosomal proteins, but most of the effect of ppGpp is indirect as a consequence of translational feedback in response to changes in rRNA levels. Our results illustrate how effects of seemingly redundant regulatory mechanisms can be separated in time and that even when multiple mechanisms act concurrently their contributions are not necessarily equivalent.

Introduction

The ribosome is one of the largest consumers of cellular resources during fast growth, and thus its biosynthesis must be regulated appropriately with respect to nutritional conditions. This represents a unique challenge as the cell has to synthesize three ribosomal RNA (rRNA) molecules and more than 50 different ribosomal proteins stoichiometrically and in ample quantities to support cell growth while simultaneously preventing wastage of resources and formation of defective ribosome particles (Dodd et al., 1991; Nomura et al., 1984). In *E. coli*, decades of research have shown that the synthesis of most r-proteins is regulated by feedback mechanisms in which specific r-proteins act as translational repressors when they accumulate in excess of rRNA, binding to their own mRNAs to inhibit synthesis of r-proteins from their respective operons (reviewed in Nomura et al., 1984; Zengel and Lindahl, 1994). Since binding of the repressor is to a single site on each mRNA, additional mechanisms such as translational coupling, retroregulation, and transcription attenuation are thought to account for regulation of other genes within polycistronic operons (Nomura et al., 1984; Zengel and Lindahl, 1994). The r-protein repressors have a higher affinity for rRNA than for their mRNA targets, ensuring that when free rRNA is available, ribosome assembly is favored over inhibition of r-protein synthesis, making rRNA the rate-limiting substrate for ribosome synthesis (Nomura et al., 1984).

Ribosomal RNA transcription is tightly regulated to balance ribosome synthesis with the cellular requirement for protein synthesis (Murray et al., 2003; Paul et al., 2004b). The cell adjusts

rRNA synthesis in large part by using NTP concentrations and ppGpp (for brevity, both the tetra- and penta-phosphate versions are referred to here as ppGpp) as signals of the nutritional state of the cell (Murray et al., 2003). For example, intracellular ppGpp levels increase dramatically in response to amino acid starvation, which results in a sharp inhibition of ribosome synthesis and an increase in amino acid synthesis, referred to as the stringent response (Potrykus and Cashel, 2008). ppGpp and NTP concentrations regulate rRNA synthesis primarily at the level of transcription initiation (reviewed in Paul et al., 2004b). rRNA promoters have evolved with intrinsic kinetic properties that result in a requirement for higher initiating NTP concentrations than most other promoters, as well as a high sensitivity to inhibition by ppGpp (Barker et al., 2001; Gaal et al., 1997; Murray et al., 2003). ppGpp regulates transcription initiation by binding directly to two separate sites on RNAP (Ross et al., 2016; 2013). The RNAP binding factor DksA, which contributes to formation of one of the ppGpp binding sites on RNAP (Ross et al., 2016), also increases the dependence of rRNA promoters on the initiating NTP concentration and acts synergistically with ppGpp to inhibit transcription initiation from these promoters (Paul et al., 2004a; Rutherford et al., 2009). Therefore, in contrast to most other promoters, rRNA promoters are strongly inhibited when NTP concentrations are low and/or when ppGpp concentrations are high, such as when cells are starved for nutrients or during the transition to stationary phase (Gaal et al., 1997; Murray et al., 2003; Paul et al., 2004a).

Direct transcriptional regulation of rRNA synthesis with respect to nutritional conditions, together with indirect regulation of r-protein synthesis through translational feedback, is theoretically sufficient to explain both the balanced synthesis of every ribosomal component and the coordination of ribosome synthesis with nutritional conditions (Nomura et al., 1984; Paul et al., 2004b). However, early work indicated that transcription of various r-proteins was inhibited by ppGpp *in vitro* (Lindahl et al., 1976), that the *rpsJ* (S10) r-protein promoter was stringently controlled

(Freedman et al., 1985), and that ppGpp inhibited transcription of the *rpsT* (S20) gene in a coupled transcription-translation system *in vitro* (Wirth et al., 1981), suggesting that at least some r-protein promoters are regulated by ppGpp. Nonetheless, in many cases it was shown that translational feedback accounted for stringent control of r-protein operons, e.g. in the L11, *spc*, and α r-protein operons (Cole and Nomura, 1986; Miura et al., 1981). Thus, when early global analyses showed that transcript levels of many r-protein operons were reduced during amino acid starvation (Traxler et al., 2008), the results were attributed to indirect effects on r-protein mRNA levels caused by translational feedback inhibition in response to ppGpp directly regulating rRNA synthesis. More recently, we reported that many r-protein promoters were regulated directly by ppGpp and DksA (Lemke et al., 2011), suggesting there might be redundancy in ppGpp-dependent regulation of r-protein synthesis. It was also reported recently that the *rplM-rpsI*, *rpmB-rpmG*, and *rplU-rpmA* operons were regulated at the transcription level by ppGpp and DksA, but only *rplM-rpsI* was regulated at the translation level as well (Aseev et al., 2016).

To evaluate the relative impact of the transcriptional and translational control mechanisms on r-protein regulation by ppGpp, we re-examined the regulation of two r-protein operons whose promoters were strongly regulated by ppGpp in the previous study (Lemke et al., 2011), the *rpsT* (S20) operon and the *rpsJ* (S10) operon, both of which were also documented as being regulated by translational feedback (Zengel and Lindahl, 1994). We show here that most regulation of the S20 operon following induction of ppGpp is indirect and likely through the translational feedback mechanism, whereas the promoters of the S20 operon are regulated during outgrowth, likely in response to increased NTP levels. Likewise, most regulation of the *rpsJ* operon by ppGpp also is promoter-independent. We propose that post-transcription initiation events are the main targets for regulators of r-protein synthesis in response to changes in nutritional conditions during exponential growth, whereas regulation of r-protein promoter activity by NTPs is important for preventing

expression during stationary phase and for quickly restarting ribosome synthesis during outgrowth from stationary phase. Thus, regulation of r-protein synthesis is dynamic, with different mechanisms playing roles at different times.

Results

Design of a reporter system for measuring S20 synthesis. Although the sequences required for translational feedback control of the *rpsT* mRNA were not defined exhaustively, and direct binding of S20 to the *rpsT* mRNA was never demonstrated (Donly and Mackie, 1988), there is a substantial literature regarding autoregulation of S20 synthesis at the level of translation (reviewed in Zengel and Lindahl, 1994). Gene dosage experiments showed that S20 expression is regulated at a post-transcriptional step (Parsons and Mackie, 1983), coupled transcription-translation experiments showed that S20 inhibits its own synthesis directly from a DNA template carrying the S20 structural gene (Wirth and Böck, 1980; Wirth et al., 1982), and *in vitro* translation experiments showed that excess 16S rRNA results in increased S20 synthesis (Wirth et al., 1981), presumably from derepression of translational feedback. Furthermore, overexpression of S20 resulted in inhibition of expression from an *rpsT* leader/RBS-*lacZ* fusion (Parsons et al., 1988), chemical and enzymatic probing showed that the *rpsT* mRNA segment encompassing the first 18 codons of S20 folds into a structure containing two hairpins (Mackie, 1992), and mutation of the UUG start codon to AUG eliminated autoregulation (Parsons et al., 1988). It was therefore proposed that the hairpins are part of the regulatory target for S20 and, along with the ribosome binding site, constitute the sequences needed for translational feedback inhibition (Mackie, 1992).

Based on this information, we designed a reporter system to detect effects of ppGpp on the *rpsT* promoters separately from effects on translation of S20. Schematics of the reporters are shown in Fig. 2.1A (details in legend). To create open reading frames (ORFs) of the same size for each

construct, the reporters contained the N-terminal 18 codons of the S20 gene (to include the two hairpins in the mRNA described above) fused in frame with the coding sequence for the Streptavidin-Binding Peptide (SBP) (Keefe et al., 2001) and the gene for β-galactosidase (*lacZ*). The SBP-tag was included to increase the size of the reporter protein slightly, resulting in its migration to a position devoid of other proteins when visualized in a discontinuous SDS-PAGE system (Neville, 1971) (Fig. 2.1B).

The reporters contained either the *rpsT* promoter(s) or the *lacUV5* promoter, as well as the leader region from either *rpsT* or *lacZ*. We use "*rpsT* leader" to refer to the *rpsT* mRNA region that starts immediately downstream of *rpsT* P2 and contains the first 18 codons of S20 fused to SBP-*lacZ* (Fig. 2.1A; reporters 1, 3, 5). The "*lacZ* leader" constructs (reporters 2, 4, 6, 7, 8) have the *lacZ* mRNA leader region and the first 18 codons of S20 fused to SBP-*lacZ*, but with an AUG start codon instead of a UUG (Parsons et al., 1988) as well as multiple silent mutations in the S20 (1-18) coding sequence to further ensure loss of regulation via translational feedback (MUT S20 [1-18]; details in Supplementary Materials and Methods). The remaining reporters (9, 10) are control constructs described below.

Since the reporter protein is too stable to measure rapid changes in synthesis rates by β-galactosidase activity assays, we instead measured synthesis by pulse-labeling with L-[^{35}S]methionine. To verify that the reporter protein band originated from the corresponding SBP-*lacZ* fusion, we compared its migration to that from a construct that encoded a smaller, SBP-less version of the reporter protein (Fig. 2.1A and 2.1B, reporters and lanes 9 and 10, respectively). A promoter-less reporter fusion and a strain without a reporter fusion did not show significant synthesis of the reporter protein (Fig. 2.1B, lanes 11 and 12, respectively), indicating that background levels of reporter synthesis were negligible. Our system made it possible to assay reporter synthesis in one step by phosphorimaging.

Figure 2.1

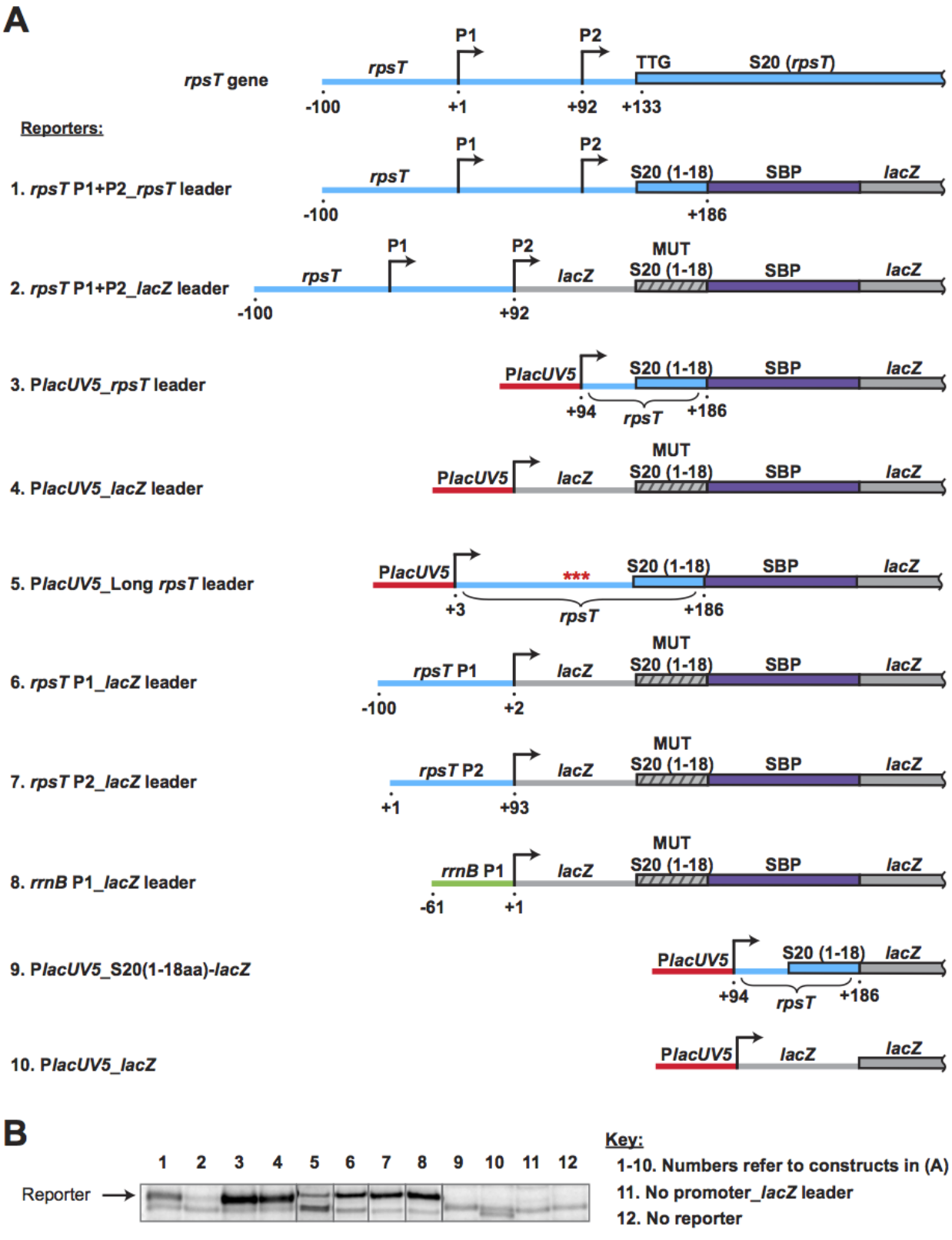
A
rpsT gene
rpsT
P1
P2
TTG
S20 (rpsT)
-100
+1
+92
+133
Reporters:
1. rpsT P1+P2_rpsT leader
S20 (1-18)
SBP
lacZ
+186
2. rpsT P1+P2_lacZ leader
MUT
3. PlacUV5_rpsT leader
PlacUV5
+94
4. PlacUV5_lacZ leader
5. PlacUV5_Long rpsT leader
***
+3
6. rpsT P1_lacZ leader
rpsT P1
7. rpsT P2_lacZ leader
rpsT P2
8. rrnB P1_lacZ leader
rrnB P1
-61
9. PlacUV5_S20(1-18aa)-lacZ
10. PlacUV5_lacZ
B
1 2 3 4 5 6 7 8 9 10 11 12
Reporter
Key:
1-10. Numbers refer to constructs in (A)
11. No promoter_lacZ leader
12. No reporter

Figure 2.1 Reporters for measuring regulation of S20 r-protein synthesis. (A) A diagram of the regulatory region in the S20 (*rpsT*) r-protein operon is shown above schematics of the reporter constructs. Reporters 1-8 contain a fusion of the first 54 nt of the *rpsT* ORF, coding for the first 18 aa of S20 (S20 [1-18]), in frame with an ORF encoding an SBP-*lacZ* fusion (S20 [1-18]-SBP-*lacZ*). We chose to fuse 54 nt (18 codons) of the *rpsT* ORF, instead of 18 nt (6 codons) like previous *rpsT* leader-*lacZ* fusions (Parsons et al., 1988), to allow formation of the two hairpins that had been previously characterized in this region because they might have a role in translational feedback (Mackie, 1992). The SBP (streptavidin-binding peptide)-*lacZ* fusion is described in Supplementary Materials and Methods. Constructs 1, 3, and 9 contain the wild-type *rpsT* leader sequence starting at the *rpsT* P2 transcription start site. Constructs 2, 4, 6-8 contain a *trp-lacZ* leader instead of the *rpsT* leader, as well as an S20 (1-18)-SBP-*lacZ* fusion in which mutations were introduced into the S20 translation initiation region to eliminate regulation through translational feedback (MUT S20 [1-18]; details in Supplementary Materials and Methods). Constructs 6, 7, and 8 contain the *rpsT* P1, *rpsT* P2, and *rrnB* P1 promoters, respectively, fused to the a *trp-lacZ* leader. Constructs 3-5, 9, and 10 contained the *lacUV5* promoter (Malan and McClure, 1984), a control promoter that is not regulated by ppGpp (Barker et al., 2001). Construct 5 contains the *lacUV5* promoter fused to the *rpsT* mRNA leader that corresponds to the leader transcribed from *rpsT* P1. Red asterisks represent 7 point mutations in the -35 and -10 elements of the *rpsT* P2 promoter designed to eliminate *rpsT* P2 activity (details in Supplementary Materials and Methods). The P*lacUV5* S20 (1-18)-*lacZ* and P*lacUV5*_*lacZ* reporters (constructs 9 and 10, respectively) contain the WT *lacZ* ORF and were used for size comparison to the S20 (1-18)-SBP-*lacZ* reporter protein. Numbers below the lines refer to positions relative to the *rpsT* P1 transcription start site. (B) Representative protein gels showing only the reporter protein products expressed from the constructs in (A). A construct encoding SBP-*lacZ* but

without a promoter was used to measure background levels of reporter protein expression (lane 11). The background strain used for reporter constructs, VH1000, is the "No reporter" control (lane 12).

Changes in S20 r-protein synthesis following ppGpp induction. Previous work showed that synthesis of r-proteins, including S20, is regulated in a *relA*-dependent manner (Dennis and Nomura, 1974). To test the role of ppGpp in r-protein synthesis, we used an inducible plasmid that encodes a constitutively active version of the ppGpp synthetase RelA (RelA') (Svitil et al., 1993) whose expression resulted in rapid accumulation of ppGpp (Fig. 2.2A). Quantification of the reporter protein band during ppGpp induction indicated that there was an ~8-fold reduction in synthesis from the construct containing the *rpsT* promoters and the *rpsT* leader sequence (construct 1) at 10 min after induction, whereas ppGpp induction had no effect on the control construct containing the *lacUV5* promoter and *lacZ* leader (construct 4; Fig. 2.2B-C). Expression of a catalytically inactive RelA had no effect on protein synthesis from either reporter strain (Fig. 2.2B-C).

Not surprisingly, ppGpp induction resulted in increases or decreases in synthesis of many proteins in the cell lysate (Fig. 2.2C), reflecting the global reprogramming of gene expression by ppGpp (reviewed in Magnusson et al., 2005). These changes in the cellular protein profile served as an internal control for ppGpp induction. Taken together, our results show that our system allowed measurement of rapid sequence-dependent changes in reporter protein expression in response to changes in nutritional conditions.

ppGpp-dependent regulation of S20 synthesis primarily requires the *rpsT* leader. S20 reporter synthesis from reporters containing the *rpsT* leader (Fig. 2.3A, constructs 1 and 3) declined quickly in response to ppGpp induction (8-fold and 12-fold at 10 min, and 12-fold and 8-fold at 20 min, respectively; blue and red curves). In contrast, ppGpp inhibited expression only slightly when the *rpsT* promoters were fused to the *lacZ* leader (construct 2, green curve), and not at all from the P*lacUV5_lacZ* leader construct (construct 4, purple curve). We also measured expression of the S20 reporter during entry into stationary phase (Fig. 2.3B), a transition when there is a temporary

Figure 2.2

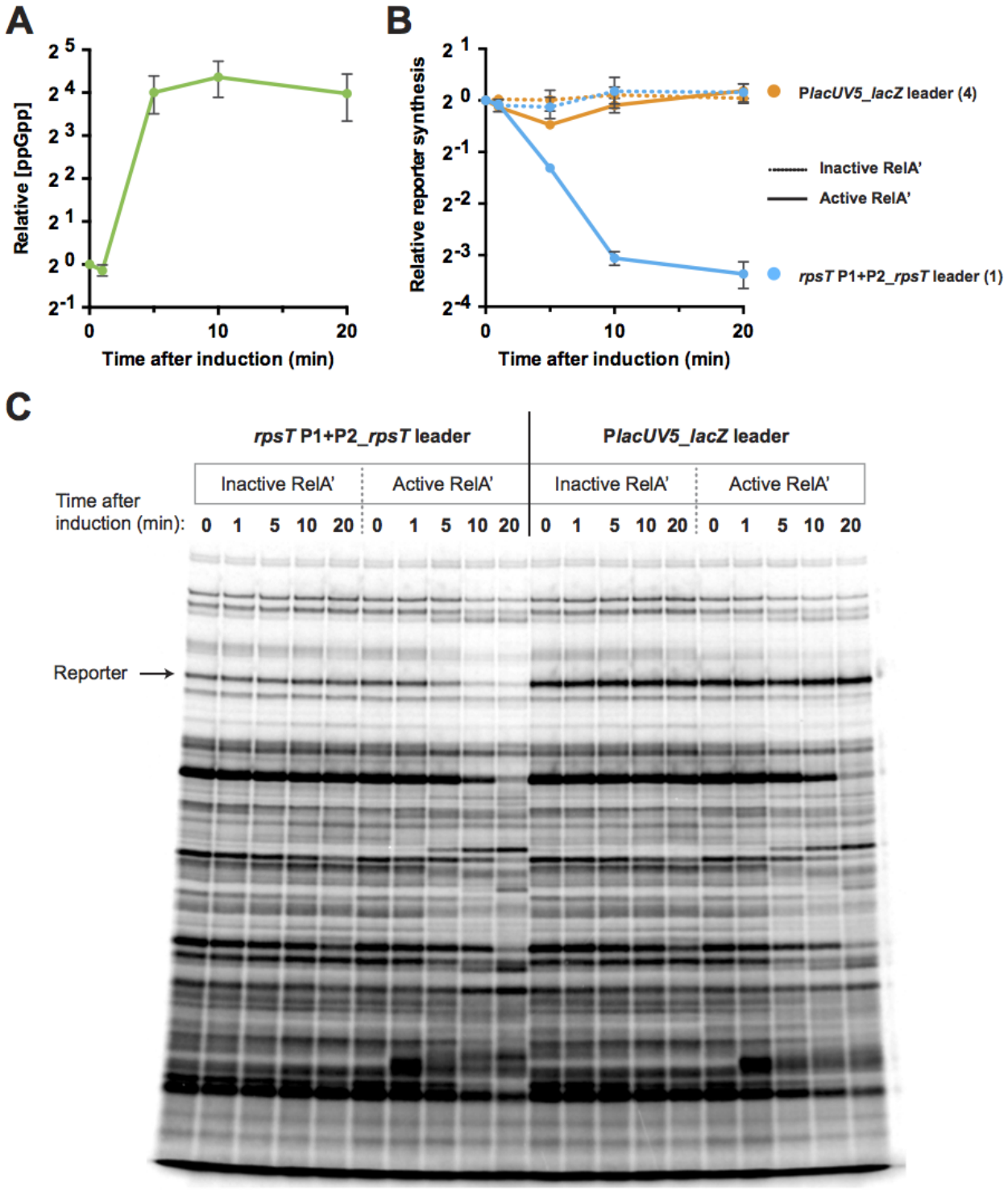
A
Relative [ppGpp]
Time after induction (min)
B
Relative reporter synthesis
Time after induction (min)
PlacUV5_lacZ leader (4)
Inactive RelA'
Active RelA'
rpsT P1+P2_rpsT leader (1)
C
rpsT P1+P2_rpsT leader
PlacUV5_lacZ leader
Inactive RelA'
Active RelA'
Time after induction (min):
0 1 5 10 20 0 1 5 10 20 0 1 5 10 20 0 1 5 10 20
Reporter

Figure 2.2 S20 synthesis during induction of ppGpp synthesis. (A) ppGpp levels were measured as described in the Materials and Methods. Error bars represent the range (n = 2). Data are from chromatogram shown in Fig. 2.5B, lanes 1-5. Relative increases in ppGpp concentration (Y-axis) are likely an underestimate, because the signal at time zero is close to background. (B) The reporter protein band from the strains containing the *rpsT* P1+P2_*rpsT* leader and P*lacUV5*_*lacZ* leader constructs (constructs 1 and 4 in Fig. 2.1A), and either the active or inactive RelA' plasmid, was quantified by phosphorimaging, corrected for background, and normalized to the reporter band at time zero for each strain. Error bars represent the range (n = 2). Numbers in parenthesis in the labels refer to the numbers of the reporter constructs shown in Fig. 2.1A. (C) Representative gel illustrating ^{35}S-pulse-labeled protein profiles following ppGpp induction as described in the text and Materials and Methods. Arrow shows the position of the reporter band.

Figure 2.3

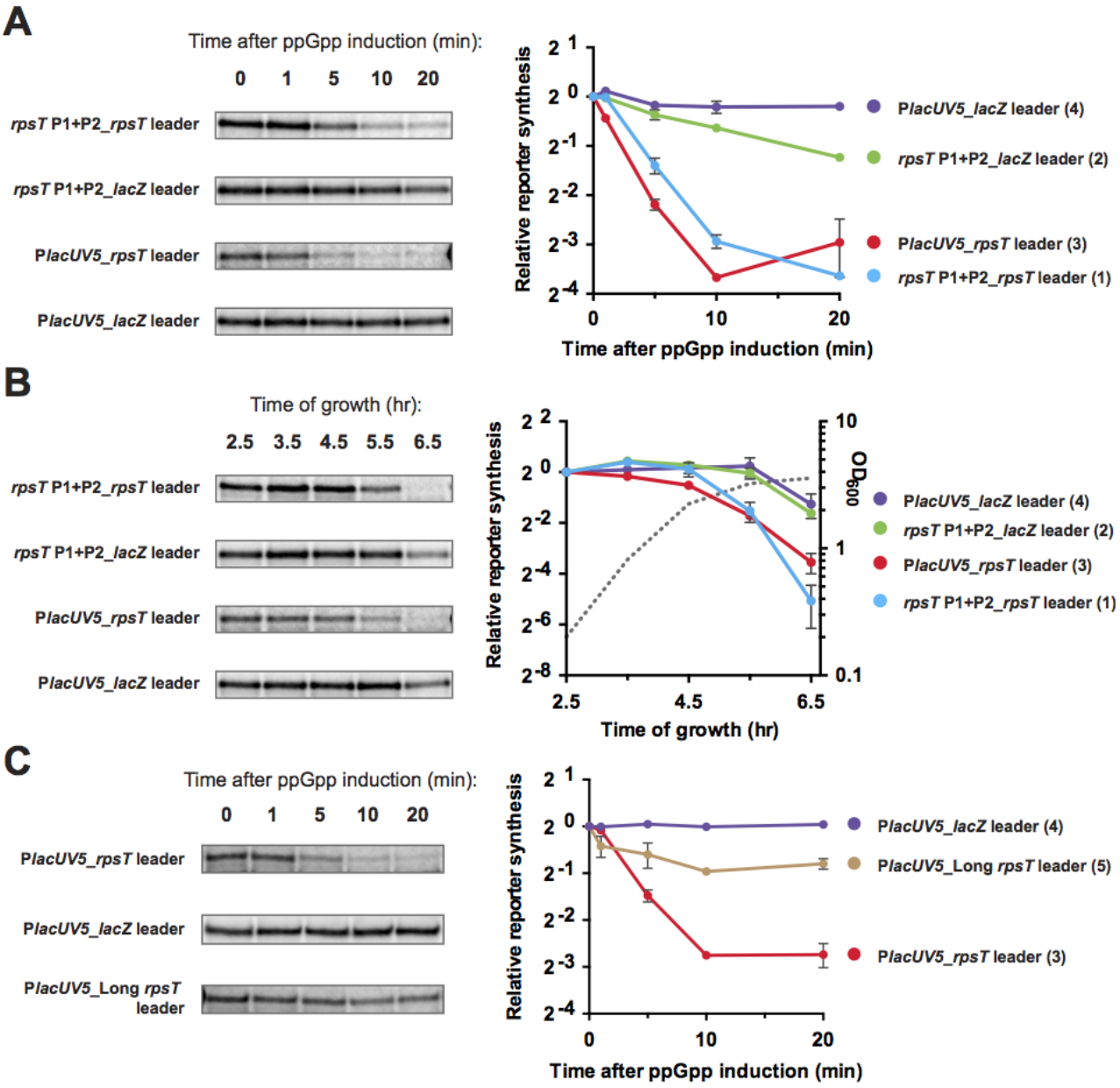
A
Time after ppGpp induction (min):
0 1 5 10 20
rpsT P1+P2_rpsT leader
rpsT P1+P2_lacZ leader
PlacUV5_rpsT leader
PlacUV5_lacZ leader
Relative reporter synthesis
Time after ppGpp induction (min)
PlacUV5_lacZ leader (4)
rpsT P1+P2_lacZ leader (2)
PlacUV5_rpsT leader (3)
rpsT P1+P2_rpsT leader (1)
B
Time of growth (hr):
2.5 3.5 4.5 5.5 6.5
rpsT P1+P2_rpsT leader
rpsT P1+P2_lacZ leader
PlacUV5_rpsT leader
PlacUV5_lacZ leader
Relative reporter synthesis
OD600
Time of growth (hr)
PlacUV5_lacZ leader (4)
rpsT P1+P2_lacZ leader (2)
PlacUV5_rpsT leader (3)
rpsT P1+P2_rpsT leader (1)
C
Time after ppGpp induction (min):
0 1 5 10 20
PlacUV5_rpsT leader
PlacUV5_lacZ leader
PlacUV5_Long rpsT leader
Relative reporter synthesis
Time after ppGpp induction (min)
PlacUV5_lacZ leader (4)
PlacUV5_Long rpsT leader (5)
PlacUV5_rpsT leader (3)

Figure 2.3 Regulation of S20 synthesis by ppGpp requires the *rpsT* leader. (A) ^{35}S reporter protein bands from a representative gel are shown from *rpsT* reporters following induction of ppGpp. Relative synthesis plots at the right are from multiple experiments. (B) Reporter protein bands and quantification from multiple experiments as in (A) during transition into stationary phase. Cells were grown in MOPS medium (Neidhardt et al., 1974) supplemented with 19 aa (no methionine), and glucose as the carbon source. Pulse-labeled samples were taken every hour starting at OD_{600} = 0.2 (see Materials and Methods). (C) Same as (A) but with the Long *rpsT* leader reporter (Fig. 2.1A, construct 5). Error bars in each graph indicate the range (n = 2). Numbers in parenthesis in the labels refer to the numbers of the reporter constructs shown in Fig. 2.1A.

increase in ppGpp concentration and a steady decrease in the levels of NTPs (Murray et al., 2003). Expression from constructs with the *rpsT* leader declined more rapidly than from those with the *lacZ* leader during entry into stationary phase (Fig. 2.3B). Thus, ppGpp-dependent regulation of S20 synthesis occurs primarily at the translation level after shifts during exponential phase and during entry into stationary phase.

We based the upstream limit of the *rpsT* mRNA sequence in the P*lacUV5*_*rpsT* leader reporter (construct 3) on the *rpsT* P2 transcription start site, because this promoter was the more active of the two *rpsT* promoters *in vivo* (Mackie and Parsons, 1983) and because the resulting mRNA contained the sequences necessary for regulation by translational feedback (Parsons et al., 1988). However, the mRNA derived from the *rpsT* P1 promoter could in theory be regulated differently from that starting at P2, e.g., the sequences between *rpsT* P1 and P2 could potentially alter the secondary structure of the region responsible for translational feedback. To test regulation of the *rpsT* leader starting from *rpsT* P1, we constructed a reporter with the *lacUV5* promoter fused to the *rpsT* leader starting at +3 relative to *rpsT* P1 and with mutations that eliminated activity of the *rpsT* P2 promoter (P*lacUV5*_Long *rpsT* leader; Fig. 2.1A, construct 5; details in Supplementary Materials and Methods).

ppGpp inhibited S20 synthesis about 2-fold from the reporter derived from the mRNA starting at *rpsT* P1, in contrast to the 8-fold inhibition observed with the reporter derived from the mRNA starting at *rpsT* P2 (Fig. 2.3C, constructs 5 and 3, respectively). We conclude that both mRNAs are regulated by translational feedback in response to ppGpp induction, but the *rpsT* P2-derived mRNA appears to be more sensitive to regulation than the *rpsT* P1-derived mRNA. In theory, the reduced translational feedback of the P1 transcript could derive from a difference in mRNA folding that interferes with S20 binding, from increased ribosome loading on the P2-derived transcript, or from a shorter mRNA lifetime. Furthermore, we cannot exclude the possibility that the

mutations introduced to inactivate *rpsT* P2 alter the structure of the mRNA and thus its regulation. In any case, the increased sensitivity of both transcripts to ppGpp was not a property of the promoter.

The *rpsT* promoters are regulated *in vivo*, but to a lesser extent than rRNA promoters. The results shown in Figure 2.3A-B indicated that ppGpp regulates S20 expression primarily at the translational level rather than by regulating *rpsT* promoter activity. However, using different methods for measuring promoter activity and for inducing ppGpp accumulation than used here (Lemke et al., 2011), we showed previously that the individual *rpsT* P1 and P2 promoters were regulated directly by ppGpp (see Discussion). Using the same reporter system and ppGpp induction conditions as in Fig. 2.3A, we found that there was no effect of ppGpp on the *rpsT* P1 promoter (Fig. 2.4A, construct 6) and a two-fold effect on *rpsT* P2 (Fig. 2.4A, construct 7). This contrasts with the 8 to 12-fold effect of ppGpp on expression when the *rpsT* leader was present (Fig. 2.3A, constructs 1 and 3) and the 16-fold effect of ppGpp on the *rrnB* P1 promoter (Fig. 2.4A, construct 8). Thus, under conditions in which effects of ppGpp on the *rpsT* promoters and the translational feedback mechanism could be compared directly, effects at the translation level dominated the *rpsT* response to ppGpp. The magnitude of ppGpp-dependent inhibition from constructs that contain the *rpsT* leader is similar to that observed with the *rrnB* P1 promoter (compare Fig. 2.3A, constructs 1 and 3, to Fig. 2.4A, construct 8), supporting a model in which direct regulation of rRNA transcription results in indirect regulation of r-protein synthesis through translational feedback (see Discussion).

We next tested if regulation of the *rpsT* promoters might play a larger role in regulation of S20 synthesis during outgrowth from stationary phase, a condition when rRNA promoter activities are dependent almost exclusively on a surge in intracellular NTP pools rather than on decreases in the already very low concentration of ppGpp (Murray et al., 2003). Using RT-qPCR to measure

Figure 2.4

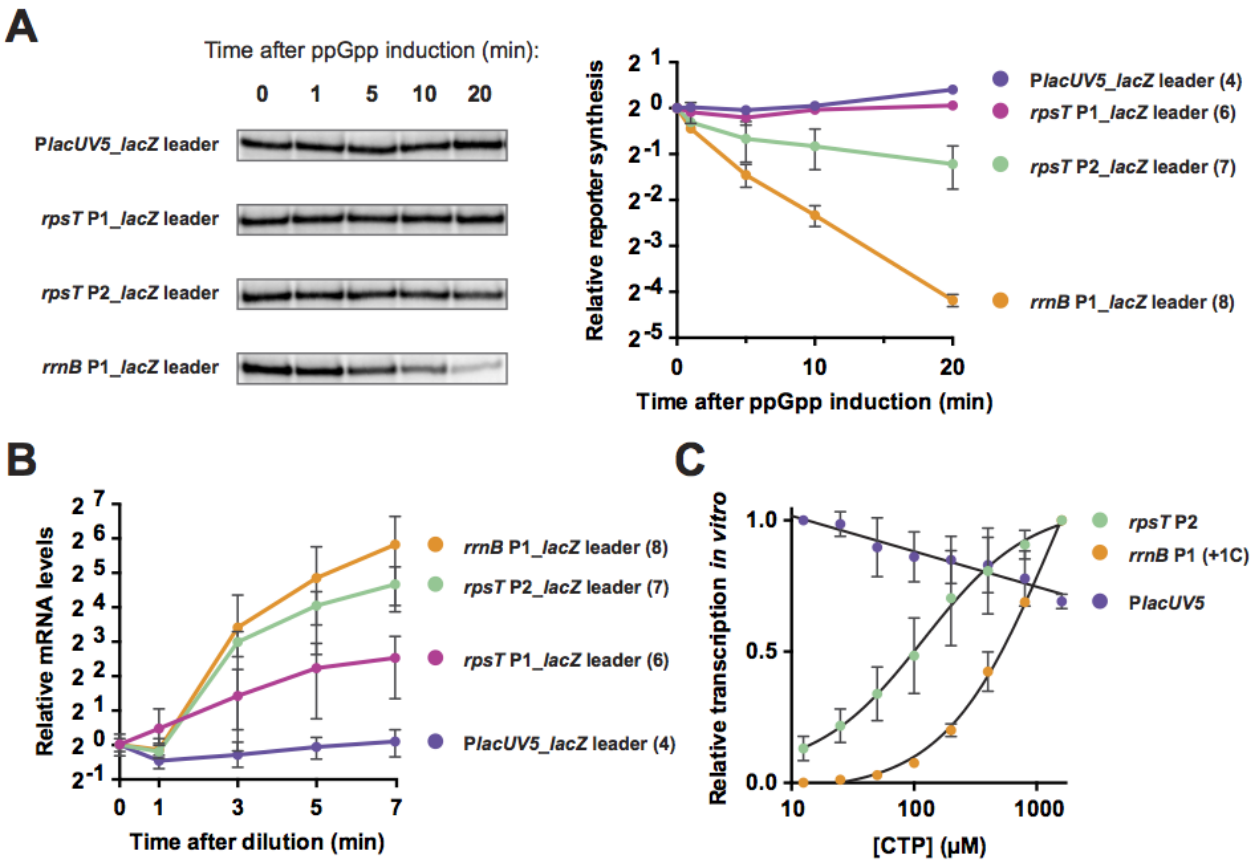
A
Time after ppGpp induction (min):
0 1 5 10 20
PlacUV5_lacZ leader
rpsT P1_lacZ leader
rpsT P2_lacZ leader
rrnB P1_lacZ leader
Relative reporter synthesis
Time after ppGpp induction (min)
PlacUV5_lacZ leader (4)
rpsT P1_lacZ leader (6)
rpsT P2_lacZ leader (7)
rrnB P1_lacZ leader (8)
B
Relative mRNA levels
Time after dilution (min)
rrnB P1_lacZ leader (8)
rpsT P2_lacZ leader (7)
rpsT P1_lacZ leader (6)
PlacUV5_lacZ leader (4)
C
Relative transcription in vitro
[CTP] (μM)
rpsT P2
rrnB P1 (+1C)
PlacUV5

Figure 2.4 Regulation of *rpsT* promoters. (A) ^{35}S reporter protein bands from representative gel and quantification from multiple experiments showing changes in reporter synthesis from transcriptional fusions (Fig. 2.1A, constructs 4, 6-8) following induction of ppGpp *in vivo*. Promoter endpoints of the transcriptional fusions are P*lacUV5* (-59/+1), *rpsT* P1 (-100/+2), *rpsT* P2 (-90/+2), and *rrnB* P1 (-61/+1), where +1 is the transcription start site. Error bars represent the range (n = 2). (B) Promoter activity during outgrowth was measured by RT-qPCR and was initiated by a 1:10 dilution of a stationary phase culture into fresh medium. Error bars represent the range (n = 2). (C) Promoter activity measured by *in vitro* transcription at different CTP concentrations. Promoter fragments with the indicated endpoints relative to the transcription start site (*rpsT* P2, -89/+50; *rrnB* P1+1C, -66/+9; and P*lacUV5,* -59/+38) were cloned into pRLG770, resulting in plasmids pRLG9237, pRLG3735, and pRLG2222, respectively (for details see Table 2.S1). Error bars represent the SD (n = 3). Numbers in parenthesis in the labels refer to the numbers of the reporter constructs shown in Fig. 2.1A.

mRNA levels from the reporter constructs used in Fig. 2.4A, we found that the activities of the *rpsT* P1 and P2 promoters, like that of the *rrnB* P1 promoter, increased rapidly after dilution of overnight cultures into fresh medium (Fig. 2.4B). *rrnB* P1 increased the most, *rpsT* P2 was next, then *rpsT* P1, and P*lacUV5* increased little if at all, following the same rank order of the responses of these constructs to ppGpp induction (Fig. 2.4A).

Since sensitivity to the concentration of its initiating nucleotide is responsible for the increase in *rrnB* P1 activity during outgrowth (Murray et al., 2003), we tested the dependence of *rpsT* P2 activity on initiating NTP (iNTP) concentration *in vitro*. *rpsT* P2 starts predominantly with CTP (Mackie and Parsons, 1983); therefore we used an *rrnB* P1 mutant that starts with CTP (instead of ATP) for comparison. *rpsT* P2 activity depended on CTP concentration, unlike the control promoter *lacUV5*, but its dependence was not as great as for *rrnB* P1 (+1C) (Fig. 2.4C). Since the concentration of CTP increases during outgrowth like the other NTPs (Murray and Gourse, 2004; Murray et al., 2003), we suggest that the dramatic increase in expression of *rpsT* P2 during outgrowth results, at least in part, from the increase in iNTP concentration.

Control of S20 synthesis requires regulation of rRNA transcription. The most straightforward explanation for the observed *rpsT* leader-dependent regulation of S20 synthesis by ppGpp is that S20 regulates its own synthesis at the level of translation in response to changes in free rRNA levels that titrate away S20 during ribosome assembly (Parsons and Mackie, 1983). In this model, the effect of ppGpp on S20 synthesis is indirect, a consequence of the direct regulation of rRNA synthesis by ppGpp. Nevertheless, it remained a formal possibility that ppGpp could regulate S20 synthesis by some other mechanism that did not require regulation of rRNA transcription.

Therefore, we tested the effect of ppGpp on S20 synthesis in a strain in which rRNA was derepressed because it lacked DksA (Δ*dksA*) (Paul et al., 2004a; Ross et al., 2016). In agreement with the results shown in Figs. 2.3 and 2.4, synthesis of the reporter from the construct containing the

rpsT leader was strongly decreased by 20 min after induction of ppGpp synthesis in a wild-type strain (Fig. 2.5A; solid red curve), but there was only a small decrease in the strain lacking *dksA* (Fig. 2.5A; red dotted curve). As expected, ppGpp induction had no effect on the P*lacUV5_lacZ* leader construct in the wild-type or Δ*dksA* strain. Not surprisingly, DksA affected the synthesis of many other cellular proteins (Fig. 2.S1), consistent with the role of ppGpp/DksA in global reprogramming of gene expression (Paul et al., 2004).

There was a formal possibility that the lack of strong effects of ppGpp induction in the Δ*dksA* strain resulted from a failure to induce ppGpp synthesis, not from a defect in regulation of rRNA synthesis. Therefore, we measured ppGpp induction directly by ^{32}P-orthophosphate incorporation and thin layer chromatography. ppGpp levels were similar or identical to those in the wild-type strain (Fig. 2.5B). Taken together, the data suggest that effects of ppGpp on reporters containing the *rpsT* leader occurred indirectly, likely through translational feedback inhibition of S20 synthesis, in response to inhibition of rRNA synthesis by ppGpp/DksA.

The *rpsJ* promoter is dispensable for ppGpp-dependent regulation of S10 synthesis. Previously we reported that the *rpsJ* (S10) promoter was one of the r-protein promoters most strongly affected by DksA and ppGpp *in vitro* and by the absence of DksA in stationary phase (Lemke et al., 2011). The S10 operon is regulated by the r-protein L4, which binds to the leader region of its mRNA (Stelzl et al., 2003), causing inhibition of translation initiation and premature transcription termination (reviewed in (Zengel and Lindahl, 1994). We used the same strategy as with *rpsT* but using reporters based on *rpsJ* (Fig. 2.6A) to examine the relative contribution of the *rpsJ* promoter versus the leader region to regulation by ppGpp. Reporter synthesis was even more strongly and rapidly inhibited in response to ppGpp induction when the reporter contained the *rpsJ* leader than when the reporter contained the *rpsT* leader (Fig. 2.6B; compare with Fig. 2.3A), whether or not the reporters contained the *rpsJ* promoter (Fig. 2.6B). We conclude that the S10 promoter

Figure 2.5

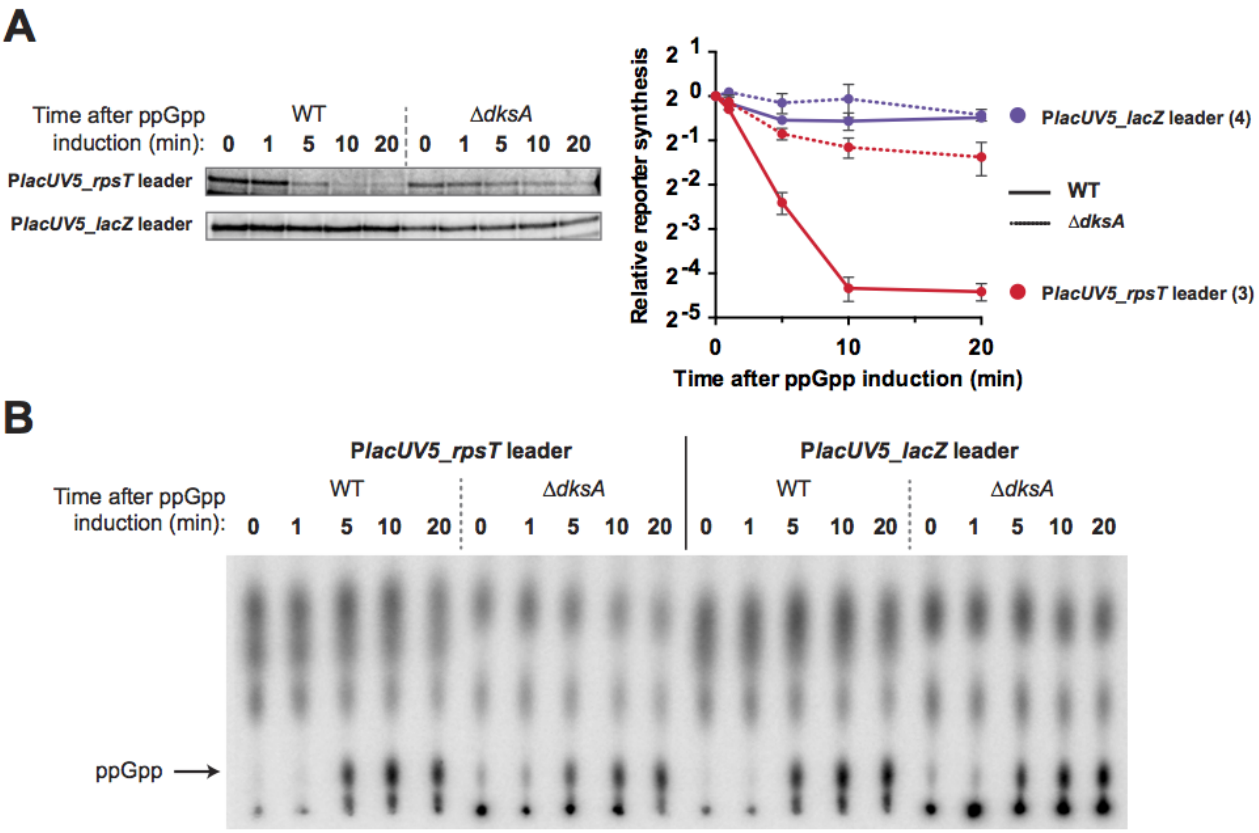
A
Time after ppGpp induction (min):
WT
ΔdksA
0 1 5 10 20 0 1 5 10 20
PlacUV5_rpsT leader
PlacUV5_lacZ leader
Relative reporter synthesis
2 1
2 0
2 -1
2 -2
2 -3
2 -4
2 -5
0
10
20
Time after ppGpp induction (min)
PlacUV5_lacZ leader (4)
WT
ΔdksA
PlacUV5_rpsT leader (3)
B
PlacUV5_rpsT leader
PlacUV5_lacZ leader
Time after ppGpp induction (min):
WT
ΔdksA
0 1 5 10 20 0 1 5 10 20 0 1 5 10 20 0 1 5 10 20
ppGpp

Figure 2.5 DksA is required for ppGpp-dependent regulation of S20 synthesis. (A) Left panel is a representative gel showing ^{35}S reporter bands from the P*lacUV5_rpsT* leader and P*lacUV5_lacZ* leader constructs at times after ppGpp induction in either a WT or Δ*dksA* background. Right panel shows the quantification of reporter protein synthesis from multiple experiments (n = 2). Numbers in parenthesis in the labels refer to the numbers of the reporter constructs shown in Fig. 2.1A. Error bars represent the range (n = 2). (B) Thin layer chromatogram shows measurement of ppGpp levels following induction of *relA*' expression from pALS13 in the strains used in panel (A) (n = 2). ppGpp indicates the region of the plate where ppGpp and pppGpp run together.

Figure 2.6

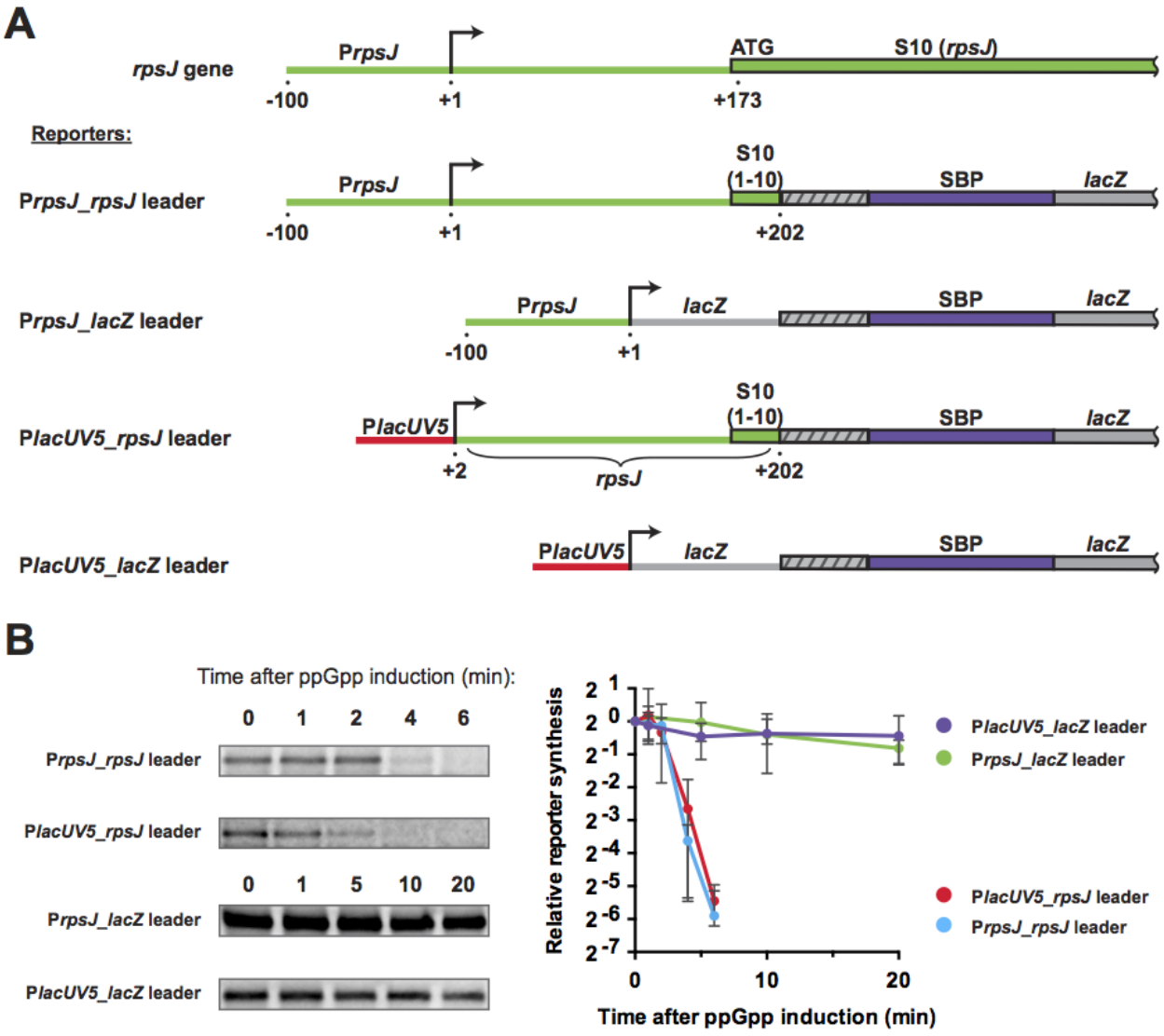
A
rpsJ gene
PrpsJ
ATG
S10 (rpsJ)
-100
+1
+173
Reporters:
PrpsJ_rpsJ leader
S10 (1-10)
SBP
lacZ
+202
PrpsJ_lacZ leader
PlacUV5_rpsJ leader
PlacUV5
+2
rpsJ
PlacUV5_lacZ leader
B
Time after ppGpp induction (min):
0 1 2 4 6
0 1 5 10 20
Relative reporter synthesis
Time after ppGpp induction (min)

Figure 2.6 Regulation of the S10 operon is independent of the *rpsJ* promoter. (A) Schematic diagrams of the 5' region of the S10 operon and the *rpsJ* reporters. Numbering below diagrams is relative to the P*rpsJ* transcription start site (Olins and Nomura, 1981). Reporters containing the *rpsJ* leader are N-terminal fusions of the *rpsJ* leader and the first 30 nt of the *rpsJ* ORF, which code for the first 10 aa of S10 (S10 [1-10]) to the MUT S20 (1-18)-SBP-*lacZ* reporter (described in Supplementary Materials and Methods). The *lacUV5_lacZ* leader construct is the same one shown in Fig. 2.1A. (B) ^{35}S-reporter protein bands from representative gel and quantification from multiple experiments following induction of ppGpp. Error bars represent the standard deviation (n = 3).

complex can be directly inhibited by ppGpp (Lemke et al., 2011), but the initial and predominant effect of ppGpp during nutritional shifts is indirect, stemming from effects of ppGpp on rRNA synthesis and L4-mediated inhibition through attenuation and translational feedback.

Discussion

Regulation of ribosomal protein synthesis in *E. coli* occurs through a translational feedback mechanism that matches r-protein output to rRNA synthesis rates (reviewed in Lindahl and Zengel, 1986; Nomura et al., 1984; Zengel and Lindahl, 1994). Nevertheless, there is considerable evidence that transcription of many r-protein operons is regulated directly by DksA and ppGpp (Lemke et al., 2011). Here we attempted to determine whether these systems are redundant, with each one capable of providing full regulatory function when the other is inactivated, or whether the two systems serve different functions, with both mechanisms retained in evolution because they regulate r-protein synthesis at different times in growth or under different nutritional conditions. We studied regulation of the S20 and S10 operons as test cases because it was shown previously that they were regulated by both transcriptional and translational mechanisms, and because the sequences required for each type of regulatory mechanism were known (Lemke et al., 2011; Zengel and Lindahl, 1994).

Even though the *rpsT* and *rpsJ* promoters were among the r-protein promoters most strongly inhibited by ppGpp and DksA in our previous study (Lemke et al., 2011), we found that the sequences responsible for translational feedback were actually the ones most required for regulation of r-protein synthesis; these sequences were both necessary and sufficient for rapid changes in r-protein synthesis in response to ppGpp. Nevertheless, the r-protein promoters were regulated but to a smaller degree than rRNA promoters. Following production of ppGpp by induction of RelA' synthesis from a plasmid, *rrnB* P1 was 7.2-fold more inhibited by ppGpp than *rpsT* P2 *in vivo* (Fig. 2.4A), consistent with the different sensitivities of r-protein and rRNA promoters to regulation by

DksA *in vitro* that we reported previously (50% inhibitory concentration [IC_{50}] for DksA in inhibiting transcription from *rrnB* P1 was ~5-fold lower than the IC_{50} for *rpsT* P2) (Lemke et al., 2011). Thus, the emerging model for responses to rapid shifts in nutrient conditions in exponentially growing cells is that r-protein promoters respond to ppGpp *in vivo*, but this response is fairly small compared to the responses of rRNA promoters to ppGpp, which in turn lead to regulation of r-protein synthesis through translational feedback (Lemke et al., 2011).

When considered in the context of the much greater extent of inhibition when the *rpsT* leader is present (and therefore subject to translational control), the effects of ppGpp/DksA on the *rpsT* promoters are small. However, they are not insignificant. The extent of regulation of the promoters was quite similar in Lemke et al. (2011) to that reported here. There was ~3-fold inhibition of *rpsT* P2 upon ppGpp induction in Figure 2.2E in Lemke et al. (2011) versus ~2-fold here in Figure 2.4A. For *rpsT* P1, there was little or no effect of ppGpp induction on *rpsT* P1 here (Fig. 2.4A) and a ~3-fold effect in Figure 2.2D of Lemke et al. (2011). These differences are attributable, at least in part, to differences in the levels of ppGpp in the two studies: ppGpp levels are about 1.5-fold greater after amino acid starvation as performed in ref. 19 (~900 pmol/A_{600} [Fiil et al., 1972], compared to ~600 pmol/A_{600} from utilizing an inducible *relA'* plasmid like that used here [Schreiber et al., 1991]).

Likewise, regulation of the *rpsJ* operon during log phase was dependent on transcript leader sequences rather than on the promoter (Fig. 2.6B), consistent with the absence of an effect of DksA on regulation of the *rpsJ* promoter during log phase (Fig. 2.1B in [Lemke et al., 2011]). As noted previously (Lemke et al., 2011), the *rpsJ* promoter was regulated by ppGpp/DksA in stationary phase and *in vitro*, phenomena that will require further study.

There are times during growth when r-protein promoters, including those for the *rpsT* and *rpsJ* operons, play an important role in regulation of r-protein synthesis; namely during stationary

phase and outgrowth from stationary phase (Lemke et al., 2011). We found hat *rpsT* P2 promoter activity rapidly increased during outgrowth and was dependent on high levels of the initiating NTP *in vitro* (Fig. 2.4B-C), as reported previously for rRNA promoters (Murray et al., 2003). We suggest that the rapid increase in NTP levels that occurs during outgrowth is responsible for turning on r-protein synthesis, which together with increased rRNA synthesis results in rapid synthesis of ribosomes when nutritional conditions improve.

Two different mechanisms regulate *E. coli* rRNA promoter activity during growth. rRNA promoters are regulated by ppGpp during nutritional shifts in log phase and by NTP concentrations during stationary phase and outgrowth from stationary phase (Murray et al., 2003). The rationale for having two different mechanisms is that NTP concentrations are saturating in log phase; they are too high and buffered against fluctuations and thus cannot account for the rapid responses in rRNA promoter activity that occur during nutritional shifts in log phase cells (Schneider and Gourse, 2004). Conversely, ppGpp is a negative regulator of rRNA promoters, but the concentration of ppGpp is too low in stationary phase to effectively regulate transcription of rRNA. Thus, a further decrease in ppGpp concentration cannot account for the rapid increase in promoter activity during outgrowth (Murray et al., 2003). We propose that having two mechanisms for regulation of r-protein synthesis has a similar basis as for rRNA promoters: ppGpp is responsible for rapid changes in r-protein synthesis following nutritional shifts in log phase and NTP concentrations regulate r-protein expression during stationary phase and outgrowth.

The primary difference between regulation of rRNA and r-protein synthesis is that the effect of ppGpp on r-protein synthesis during nutritional shifts in log phase is indirect, occurring primarily through translational feedback in response to changes in rRNA synthesis. However, translational feedback would be impractical for maintaining inhibition of r-protein synthesis during metabolic dormancy, because there is a lack of robust translation during stationary phase (Navarro Llorens et

al., 2010) and most free r-proteins are unstable when not incorporated into ribosomes (Dennis, 1974), which together would result in the concentration of repressor r-proteins being too low to inhibit r-protein synthesis. Thus, we propose that low NTP levels are responsible for maintaining inhibition of r-protein expression during stationary phase, with NTP levels increasing rapidly to turn on ribosome synthesis when nutritional conditions improve, as with rRNA promoters (Murray et al., 2003).

We have attributed the requirement for the *rpsT* (S20) leader in regulation by ppGpp to its role as the target site for translational feedback inhibition by S20 (Parsons et al., 1988; Zengel and Lindahl, 1994), a model in which the requirement for DksA (Fig. 2.5A) is explained by its role in regulation of rRNA transcription initiation. It was reported previously that overexpression of S20 from a plasmid reduced expression from an *rpsT* leader/RBS-*lacZ* fusion and that S20 inhibited its own synthesis in a coupled transcription-translation system (Wirth and Böck, 1980; Wirth et al., 1982). Mutations in the *rpsT* leader and start codon affected translational feedback (Parsons et al., 1988), suggesting that this inhibition occurred at the translation level (Wirth et al., 1981) and consistent with the simple model that S20 regulates translation from its own mRNA. However, we note that there is currently no evidence for direct binding of S20 by itself to its mRNA (Donly and Mackie, 1988). Alternative models consistent with our data are that S20 could bind to the *rpsT* mRNA as a complex with other proteins, that S20 could target the translation initiation complex rather than compete with ribosome binding, or that feedback inhibition could result from S20-dependent transcription attenuation. Discriminating among these possible mechanisms will require further investigation.

Materials and Methods

Strain constructions. See Supplementary Materials and Methods for more details. Strains, plasmids, oligonucleotides, and synthetic gene fragments (gBlocks) used in this study are listed in Tables 2.S1 and 2.S2. Synthetic DNAs were obtained from Integrated DNA Technologies (IDT). Reporter fusions were constructed in strain DY330 by recombineering and then transferred into VH1000 by P1 transduction (Thomason et al., 2007; 2014). The *dksA* gene was deleted by infection of reporter strains with a P1 lysate grown on RLG6632 (*dksA*::*kan*) and selection for kanamycin-resistance. The strains were transformed with plasmids as described (Chung et al., 1989).

Reporter protein synthesis. Cells were grown at 37°C with aeration in MOPS medium supplemented with 0.4% glucose, 80 μg/ml 19 amino acids (no methionine), and 10 μg/ml thiamine (Neidhardt et al., 1974). Experiments with Δ*dksA* strains were performed with the same medium as above, but amino acids were added from 5X Supplement EZ Minus Methionine (Teknova; #M2109). For measuring regulation of reporter protein synthesis during ppGpp induction, cells carrying either the pALS13 or pALS14 plasmids (Svitil et al., 1993), maintained with 100 μg/ml ampicillin, were grown to mid-log phase (optical density at 600 nm [OD_{600}] of ~0.2), induced with 1 mM IPTG, and samples for pulse labeling were taken immediately before and at the indicated times after addition of IPTG. Cells were pulse-labeled by incubating 0.5 ml aliquots of the cultures with 10 μCi of L-[^{35}S]methionine (PerkinElmer; NEG709A) for 10 min at 37°C. Under these conditions, cells saturated for incorporation of radioactivity into protein within 30 s of L-[^{35}S]methionine addition (HLB and RLG, unpublished). Cells were pelleted by centrifugation, suspended in sample buffer containing SDS (Neville, 1971), and lysed by heating to 65°C for 10 min. The cell lysate was then vortexed vigorously to shear genomic DNA and cleared by centrifugation. The cleared lysate was run in an 8% acrylamide discontinuous buffer SDS-PAGE system (Neville, 1971) and visualized by phosphorimaging.

For measuring reporter protein synthesis during entry into stationary phase, cultures were inoculated at an OD_{600} ~0.01 and grown in the same medium as described above. A sample for pulse-labeling was taken when the cells reached OD_{600} ~0.2 and every hour after. The volume of culture harvested at each time point was adjusted to the same OD_{600} to ensure that labeling was performed with the same number of cells. Radiolabeled proteins were visualized and measured as described above.

Analysis of mRNA levels during outgrowth. Cells were grown at 37°C with aeration in MOPS defined medium supplemented with 0.4% glucose, 80 μg/ml of all 20 amino acids, and 10 μg/ml thiamine (Neidhardt et al., 1974). To measure regulation of promoter activity during outgrowth, we inoculated cultures at an OD_{600} of ~0.001, allowed them to grow for 24 hr, then initiated outgrowth by diluting the stationary phase culture 1:10 into fresh medium pre-warmed to 37°C. RNA extraction and RT-qPCR was performed as described previously (Ross et al., 2016). Briefly, samples for RNA extraction were removed from the stationary phase culture and at the indicated times after dilution into fresh medium by transferring aliquots into an ice-cold 95% ethanol/5% phenol stop solution that inactivates RNases (Khodursky et al., 2003). Cells were lysed in TE buffer (10 mM Tris-Cl, pH 8.0, 1 mM EDTA) containing lysozyme (Epicentre), 1% SDS, and total RNA from strain RLG11387 (contains plasmid P*lacUV5*_GFP) as a marker for RNA loss during extraction. RNA was extracted from the cells using the hot phenol method (Khodursky et al., 2003) and digested with DNase I (NEB). cDNA was synthesized from 0.5 μg of total RNA using the iScript cDNA synthesis kit (BioRad 170-8891). Quantitative PCR was performed with the cDNA using the iTaq Universal SYBR Green Supermix (BioRad 172-5122) on a CFX Connect Real-Time PCR Detection System (BioRad). The mRNA originating from the reporter fusion was detected using oligos 7664 and 7665, and the *gfp* mRNA was detected with oligos 7608 and 7609

(Table S2). The amount of reporter mRNA was normalized to the *gfp* mRNA and to the pre-shift samples using the $2^{-\Delta\Delta C}{}_T$ method (Livak and Schmittgen, 2001).

***In vitro* transcription.** Multiple-round *in vitro* transcription reactions were performed essentially as described previously (Ross and Gourse, 2009). Reactions were carried out at 30°C and contained 170 mM KCl, 10 nM Eσ^{70} RNAP, 1 nM of supercoiled plasmid template, 200 μM of ATP and GTP, 10 μM UTP, and ~2 μCi of [γ-^{32}P]UTP. CTP was added to the reactions in varying concentrations ranging from 12.5 μM to 1.6 mM. The promoters of interest were contained in the pRLG770 transcription vector (plasmids are listed in Table 2.S1). Transcripts were resolved by electrophoresis in a 5.5% polyacrylamide, 7 M urea gel and visualized by phosphorimaging.

Measurement of ppGpp levels. Cells were grown in the same medium as described above in "Reporter protein synthesis" for Δ*dksA* strains, supplemented with 100 μg/ml ampicillin to maintain the pALS13 plasmid and 10 μCi/ml of [^{32}P]orthophosphate. At OD_{600}, ~0.2 cells were induced with 1 mM IPTG, and samples were harvested before and at the indicated times after induction. Samples were processed by extraction with formic acid and analyzed by thin-layer chromatography in 0.85 M KH_2PO_4 (pH 3.4) buffer as described previously (Ross et al., 2016).

Acknowledgements

We thank Wilma Ross for comments on the manuscript. Work in our laboratory was supported by National Institutes of Health grant R37 GM37048 to RLG. HLB and PSV were supported in part by predoctoral fellowships from Molecular Biosciences Training Grant NIH T32 GM007215 and Advanced Opportunity Fellowships through SciMed Graduate Research Scholars and the Wisconsin Alumni Research Foundation at UW-Madison. We thank Sarah Ades for gifts of materials.

References

Aseev, L.V., Koledinskaya, L.S., and Boni, I.V. (2016). Regulation of ribosomal protein operons *rplM-rpsI*, *rpmB-rpmG*, and *rplU-rpmA* at the transcriptional and translational levels. J. Bacteriol. *198*, 2494–2502.

Barker, M.M., Gaal, T., Josaitis, C.A., and Gourse, R.L. (2001). Mechanism of regulation of transcription initiation by ppGpp. I. Effects of ppGpp on transcription initiation *in vivo* and *in vitro*. J. Mol. Biol. *305*, 673–688.

Chung, C.T., Niemela, S.L., and Miller, R.H. (1989). One-step preparation of competent *Escherichia coli*: transformation and storage of bacterial cells in the same solution. Proc. Natl. Acad. Sci. U S A *86*, 2172–2175.

Cole, J.R., and Nomura, M. (1986). Translational regulation is responsible for growth-rate-dependent and stringent control of the synthesis of ribosomal proteins L11 and L1 in *Escherichia coli*. Proc. Natl. Acad. Sci. U S A *83*, 4129–4133.

Dennis, P.P., and Nomura, M. (1974). Stringent control of ribosomal protein gene expression in *Escherichia coli*. Proc. Natl. Acad. Sci. U S A *71*, 3819–3823.

Dennis, P.P. (1974). Synthesis and stability of individual ribosomal proteins in the presence of rifampicin. Mol. Gen. Genet. *134*, 39–47.

Dodd, J., Kolb, J.M., and Nomura, M. (1991). Lack of complete cooperativity of ribosome assembly in vitro and its possible relevance to *in vivo* ribosome assembly and the regulation of ribosomal gene expression. Biochimie *73*, 757–767.

Donly, B.C., and Mackie, G.A. (1988). Affinities of ribosomal protein S20 and C-terminal deletion mutants for 16S rRNA and S20 mRNA. Nucleic Acids Res. *16*, 997–1010.

Freedman, L.P., Zengel, J.M., and Lindahl, L. (1985). Genetic dissection of stringent control and nutritional shift-up response of the *Escherichia coli* S10 ribosomal protein operon. J. Mol. Biol. *185*, 701–712.

Gaal, T., Bartlett, M.S., Ross, W., Turnbough, C.L., and Gourse, R.L. (1997). Transcription regulation by initiating NTP concentration: rRNA synthesis in bacteria. Science *278*, 2092–2097.

Keefe, A.D., Wilson, D.S., Seelig, B., and Szostak, J.W. (2001). One-step purification of recombinant proteins using a nanomolar-affinity streptavidin-binding peptide, the SBP-Tag. Protein Expr. Purif. *23*, 440–446.

Khodursky, A.B., Bernstein, J.A., Peter, B.J., Rhodius, V., Wendisch, V.F., and Zimmer, D.P. (2003). *Escherichia coli* spotted double-strand DNA microarrays: RNA extraction, labeling, hybridization, quality control, and data management. In Functional Genomics, (New Jersey: Humana Press), pp. 61–78.

Lemke, J.J., Sanchez-Vazquez, P., Burgos, H.L., Hedberg, G., Ross, W., and Gourse, R.L. (2011). Direct regulation of *Escherichia coli* ribosomal protein promoters by the transcription factors ppGpp

and DksA. Proc. Natl. Acad. Sci. U S A *108*, 5712–5717.

Lindahl, L., and Zengel, J.M. (1986). Ribosomal genes in *Escherichia coli*. Annu. Rev. Genet. *20*, 297–326.

Lindahl, L., Post, L., and Nomura, M. (1976). DNA-dependent in vitro synthesis of ribosomal proteins, protein elongation factors, and RNA polymerase subunit α: inhibition by ppGpp. Cell *9*, 439–448.

Livak, K.J., and Schmittgen, T.D. (2001). Analysis of relative gene expression data using real-time quantitative PCR and the $2^{-\Delta\Delta C}{}_{T}$ Method. Methods *25*, 402–408.

Mackie, G.A. (1992). Secondary structure of the mRNA for ribosomal protein S20. Implications for cleavage by ribonuclease E. J. Biol. Chem. *267*, 1054–1061.

Mackie, G.A., and Parsons, G.D. (1983). Tandem promoters in the gene for ribosomal protein S20. J. Biol. Chem. *258*, 7840–7846.

Magnusson, L.U., Farewell, A., and Nyström, T. (2005). ppGpp: a global regulator in *Escherichia coli*. Trends Microbiol. *13*, 236–242.

Malan, T.P., and McClure, W.R. (1984). Dual promoter control of the *Escherichia coli* lactose operon. Cell *39*, 173–180.

Miura, A., Krueger, J.H., Itoh, S., de Boer, H.A., and Nomura, M. (1981). Growth-rate-dependent regulation of ribosome synthesis in *E. coli*: expression of the *lacZ* and *galK* genes fused to ribosomal promoters. Cell *25*, 773–782.

Murray, H.D., and Gourse, R.L. (2004). Unique roles of the *rrn* P2 rRNA promoters in *Escherichia coli*. Mol. Microbiol. *52*, 1375–1387.

Murray, H.D., Schneider, D.A., and Gourse, R.L. (2003). Control of rRNA expression by small molecules is dynamic and nonredundant. Mol. Cell *12*, 125–134.

Navarro Llorens, J.M., Tormo, A., and Martínez-García, E. (2010). Stationary phase in gram-negative bacteria. FEMS Microbiol. Rev. *34*, 476–495.

Neidhardt, F.C., Bloch, P.L., and Smith, D.F. (1974). Culture Medium for Enterobacteria. J. Bacteriol. *119*, 736–747.

Neville, D.M. (1971). Molecular weight determination of protein-dodecyl sulfate complexes by gel electrophoresis in a discontinuous buffer system. J. Biol. Chem. *246*, 6328–6334.

Nomura, M., Gourse, R., and Baughman, G. (1984). Regulation of the synthesis of ribosomes and ribosomal components. Annu. Rev. Biochem. *53*, 75–117.

Olins, P.O., and Nomura, M. (1981). Regulation of the S10 ribosomal protein operon in *E. coli*: nucleotide sequence at the start of the operon. Cell *26*, 205–211.

Parsons, G.D., and Mackie, G.A. (1983). Expression of the gene for ribosomal protein S20: effects of gene dosage. J. Bacteriol. *154*, 152–160.

Parsons, G.D., Donly, B.C., and Mackie, G.A. (1988). Mutations in the leader sequence and initiation codon of the gene for ribosomal protein S20 (*rpsT*) affect both translational efficiency and autoregulation. J. Bacteriol. *170*, 2485–2492.

Paul, B.J., Barker, M.M., Ross, W., Schneider, D.A., Webb, C., Foster, J.W., and Gourse, R.L. (2004a). DksA: a critical component of the transcription initiation machinery that potentiates the regulation of rRNA promoters by ppGpp and the initiating NTP. Cell *118*, 311–322.

Paul, B.J., Ross, W., Gaal, T., and Gourse, R.L. (2004b). rRNA transcription in *Escherichia coli*. Annu. Rev. Genet. *38*, 749–770.

Potrykus, K., and Cashel, M. (2008). (p)ppGpp: still magical? Annu. Rev. Microbiol. *62*, 35–51.

Ross, W., and Gourse, R.L. (2009). Analysis of RNA polymerase-promoter complex formation. Methods *47*, 13–24.

Ross, W., Sanchez-Vazquez, P., Chen, A.Y., Lee, J.-H., Burgos, H.L., and Gourse, R.L. (2016). ppGpp binding to a site at the RNAP-DksA interface accounts for its dramatic effects on transcription initiation during the stringent response. Mol. Cell *62*, 811–823.

Ross, W., Vrentas, C.E., Sanchez-Vazquez, P., Gaal, T., and Gourse, R.L. (2013). The magic spot: a ppGpp binding site on *E. coli* RNA polymerase responsible for regulation of transcription initiation. Mol. Cell *50*, 420–429.

Rutherford, S.T., Villers, C.L., Lee, J.H., Ross, W., and Gourse, R.L. (2009). Allosteric control of *Escherichia coli* rRNA promoter complexes by DksA. Genes Dev *23*, 236–248.

Schneider, D.A., and Gourse, R.L. (2004). Relationship between Growth Rate and ATP Concentration in *Escherichia coli*: a bioassay for available cellular ATP. J. Biol. Chem. *279*, 8262–8268.

Stelzl, U., Zengel, J.M., Tovbina, M., Walker, M., Nierhaus, K.H., Lindahl, L., and Patel, D.J. (2003). RNA-structural mimicry in *Escherichia coli* ribosomal protein L4-dependent regulation of the S10 operon. J. Biol. Chem. *278*, 28237–28245.

Svitil, A.L., Cashel, M., and Zyskind, J.W. (1993). Guanosine tetraphosphate inhibits protein synthesis *in vivo*. A possible protective mechanism for starvation stress in *Escherichia coli*. J. Biol. Chem. *268*, 2307–2311.

Thomason, L.C., Costantino, N., and Court, D.L. (2007). *E. coli* genome manipulation by P1 transduction. Curr. Protoc. Mol. Biol. *Chapter 1*, Unit1.17.

Thomason, L.C., Sawitzke, J.A., Li, X., Costantino, N., and Court, D.L. (2014). Recombineering: genetic engineering in bacteria using homologous recombination. Curr. Protoc. Mol. Biol. *106*, 1.16.1–1.16.39.

Traxler, M.F., Summers, S.M., Nguyen, H.-T., Zacharia, V.M., Hightower, G.A., Smith, J.T., and

Conway, T. (2008). The global, ppGpp-mediated stringent response to amino acid starvation in *Escherichia coli*. Mol. Microbiol. *68*, 1128–1148.

Wirth, R., and Böck, A. (1980). Regulation of synthesis of ribosomal protein S20 *in vitro*. Mol. Gen. Genet. *178*, 479–481.

Wirth, R., Kohles, V., and Böck, A. (1981). Factors modulating transcription and translation *in vitro* of ribosomal protein S20 and isoleucyl-tRNA synthetase from *Escherichia coli*. Eur. J. Biochem. *114*, 429–437.

Wirth, R., Littlechild, J., and Böck, A. (1982). Ribosomal protein S20 purified under mild conditions almost completely inhibits its own translation. Mol. Gen. Genet. *188*, 164–166.

Zengel, J.M., and Lindahl, L. (1994). Diverse mechanisms for regulating ribosomal protein synthesis in *Escherichia coli*. Prog. Nucleic Acid Res. Mol. Biol. *47*, 331–370.

Supplemental Data

Figure 2.S1

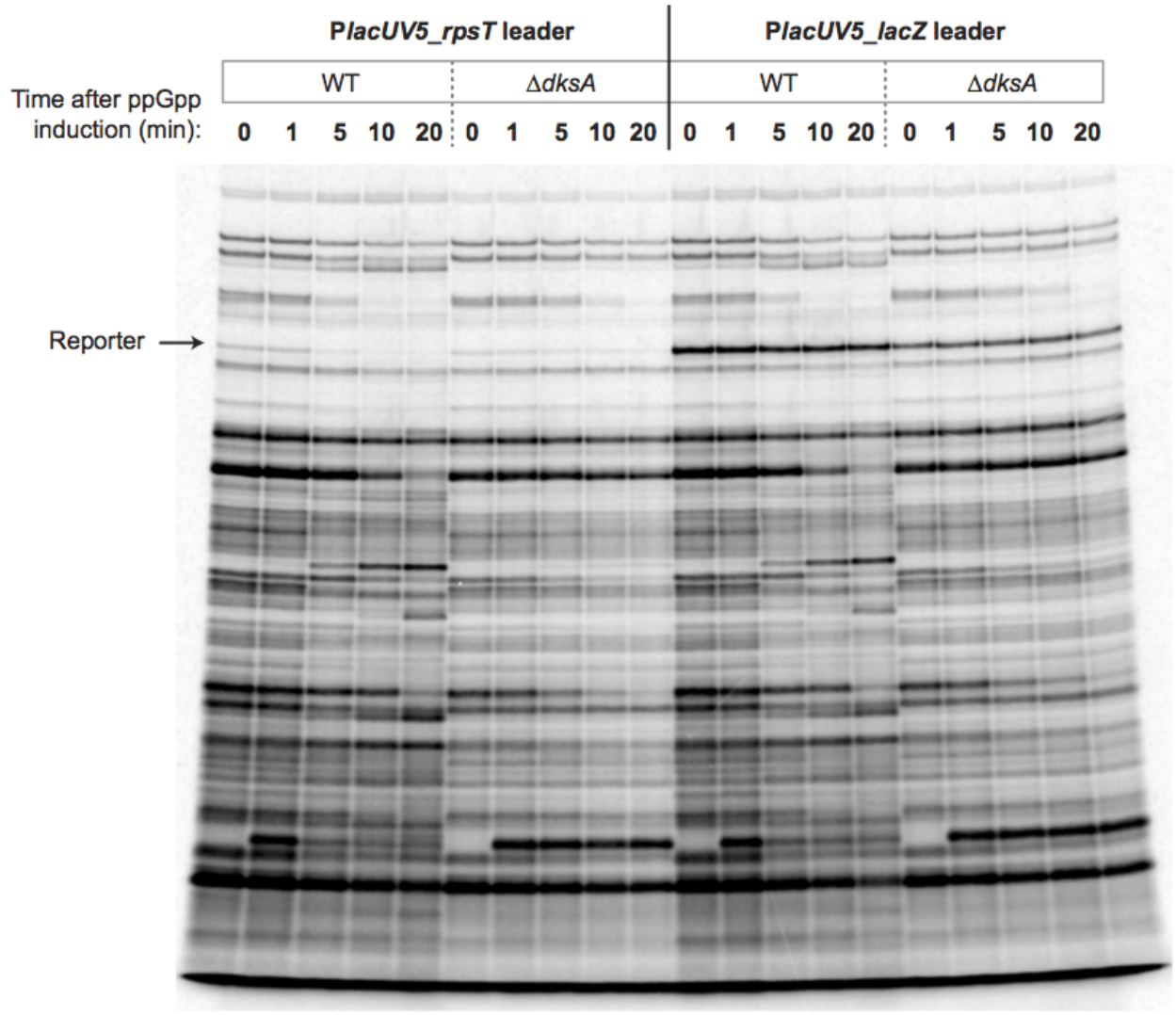

Figure 2.S1 DksA is required for ppGpp-dependent changes in global gene expression. Representative gel from two independent experiments showing changes in protein synthesis from the P*lacUV5_rpsT* leader and P*lacUV5_lacZ* leader reporter constructs (Fig. 2.1A; constructs 3 and 4, respectively) in either a WT or Δ*dksA* background following induction of ppGpp synthesis. Effects of ppGpp on the reporter fusion containing the *rpsT* leader were dependent on *dksA*, indicating that ppGpp was induced (as shown in Fig. 2.5B), but its effects were indirect (likely resulting from direct effects of DksA and ppGpp on rRNA synthesis).

Table 2.S1 Bacterial strains and plasmids

Strain No.	Strain name	Genotype	Source	PCR components			P1 lysate donor source
				FO oligo	RE oligo	Template DNA	
RLG3499	VH1000	MG1655 *lacZ*-, *lacI*-, *pyrE*+	(Gaal et al., 1997)	N/A	N/A	N/A	N/A
RLG4998	P*lacUV5*_*lacZ*	VH1000 P*lacUV5*(-59/+36)-*lacZ*	(Barker et al., 2001)	N/A	N/A	N/A	N/A
RLG6341	DY330	W3110 Δ*lacU169 gal490* λ*cI857* Δ(*cro-bioA*)	(Yu et al., 2000)	N/A	N/A	N/A	N/A
RLG9933	MG1655	K-12 F⁻ λ⁻ *ilvG⁻ rfb-50 rph-1*	(Blattner et al., 1997)	N/A	N/A	N/A	N/A
RLG11387	pP*lacUV5*_GFP	VH1000 with P*lacUV5*-GFP plasmid	This work	N/A	N/A	N/A	N/A
RLG11397	*frt-kan* in DY330	DY330 *ygfF-gcvP*<>*frt-kan*	This work	6668	6669	RLG10081	N/A
RLG11398	*frt-kan-cat-sacB* in DY330	DY330 *ygfF-gcvP*<>*frt-kan-cat-sacB*	This work	6673	6674	RLG10405	N/A
RLG11399	*tetAR*_*cat-sacB* in DY330	DY330 *ygfF-gcvP*<>*tetAR-cat-sacB*	This work	6773	6774	RLG10077	N/A
RLG13665	*tetAR*_P*lacUV5*_*lacZ*_inactive RBS in DY330	DY330 *ygfF-gcvP*<>*tetAR*-P*lacUV5-lacZ-rrnB* T1	This work	6779	6780	pRLG13662	N/A
RLG13667	*tetAR*_P*lacUV5*_*cat-sacB*_inactive RBS_*lacZ* in DY330	DY330 *ygfF-gcvP*<>*tetAR*-P*lacUV5-cat-sacB*-inactive RBS-*lacZ-rrnB* T1	This work	6781	6825	RLG10405	N/A
RLG13808	*tetAR*_P*lacUV5* with *trpA-lacZ* leader in DY330	DY330 *ygfF-gcvP*<>*tetAR*-P*lacUV5-trpA'-lacZ* leader-*lacZ-rrnB* T1	This work	6908	6909	RLG9459	N/A
RLG13812	*tetAR*_P*lacUV5*_*cat-sacB*_*trpA-lacZ* leader_LacZ in DY330	DY330 *ygfF-gcvP*<>*tetAR*-P*lacUV5-cat-sacB-trpA'-lacZ* leader-*lacZ-rrnB* T1	This work	6910	6825	RLG13667	N/A
RLG13903	P*lacUV5*_S20(1-18)/*lacZ* in DY330	DY330 *ygfF-gcvP*<>*tetAR*-P*lacUV5-rpsT* leader-S20(1-18aa)/LacZ-*rrnB* T1	This work	7026	7020	RLG9933	N/A
RLG13918	P*lacUV5*_S20(1-18)/*lacZ*	VH1000 *ygfF-gcvP*<>*tetAR*-P*lacUV5-rpsT* leader-S20(1-18aa)/LacZ-*rrnB* T1	This work	N/A	N/A	N/A	RLG13903
RLG14029	P*lacUV5*(C-14A)_*lacZ* leader in DY330	DY330 *ygfF-gcvP*<>*tetAR*-P*lacUV5*(C-14A)-*trpA'-lacZ* leader-S20(mut.1-18aa)/SBP/LacZ-*rrnB* T1	This work	7071	N/A	N/A	N/A
RLG14082	P*lacUV5_cat-sacB_lacZ* leader in DY330	DY330 *ygfF-gcvP*<>*tetAR*-P*lacUV5-cat-sacB-trpA'-lacZ* leader-S20(mut.1-18aa)/SBP/LacZ-*rrnB* T1	This work	6825	6910	RLG13667	N/A
RLG14110	P*rpsT_rpsT* leader in DY330	DY330 *ygfF-gcvP*<>*tetAR-rpsT* P1+P2-*rpsT* leader-S20(1-18aa)/SBP/LacZ-*rrnB* T1	This work	7017	7110	RLG9933	N/A
RLG14111	P*lacUV5_rpsT* leader in DY330	DY330 *ygfF-gcvP*<>*tetAR*-P*lacUV5-rpsT* leader-S20(1-18aa)/SBP/LacZ-*rrnB* T1	This work	7026	7110	RLG9933	N/A
RLG14112	P*rpsT_lacZ* leader in DY330	DY330 *ygfF-gcvP*<>*tetAR-rpsT* P1+P2-*trpA'-lacZ* leader-S20(mut.1-18aa)/SBP/LacZ-*rrnB* T1	This work	7017	7018	RLG9933	N/A

RLG14113	P*lacUV5_lacZ* leader in DY330	DY330 *ygfF-gcvP*<>*tetA*R-P*lacUV5-trpA'-lacZ* leader-S20(mut.1-18aa)/SBP/LacZ-*rrnB* T1	This work	6909	7111	N/A	N/A
RLG14114	*rrnB* P1_*lacZ* leader in DY330	DY330 *ygfF-gcvP*<>*tetA*R-*rrnB* P1-*trpA'-lacZ* leader-S20(mut.1-18aa)/SBP/LacZ-*rrnB* T1	This work	7021	7022	RLG9933	N/A
RLG14115	No promoter_*lacZ* leader in DY330	DY330 *ygfF-gcvP*<>*tetA*R-*rrnB* dead-*trpA'-lacZ* leader-S20(mut.1-18aa)/SBP/LacZ-*rrnB* T1	This work	7024	7025	RLG13907	N/A
RLG14153	P*rpsT_rpsT* leader	VH1000 *ygfF-gcvP*<>*tetA*R-*rpsT* P1+P2-*rpsT* leader-S20(1-18aa)/SBP/LacZ-*rrnB* T1	This work	N/A	N/A	N/A	RLG14110
RLG14154	P*lacUV5_rpsT* leader	VH1000 *ygfF-gcvP*<>*tetA*R-P*lacUV5-rpsT* leader-S20(1-18aa)/SBP/LacZ-*rrnB* T1	This work	N/A	N/A	N/A	RLG14111
RLG14155	P*rpsT_lacZ* leader	VH1000 *ygfF-gcvP*<>*tetA*R-*rpsT* P1+P2-*trpA'-lacZ* leader-S20(mut.1-18aa)/SBP/LacZ-*rrnB* T1	This work	N/A	N/A	N/A	RLG14112
RLG14156	P*lacUV5_lacZ* leader	VH1000 *ygfF-gcvP*<>*tetA*R-P*lacUV5-trpA'-lacZ* leader-S20(mut.1-18aa)/SBP/LacZ-*rrnB* T1	This work	N/A	N/A	N/A	RLG14113
RLG14157	*rrnB* P1_*lacZ* leader	VH1000 *ygfF-gcvP*<>*tetA*R-*rrnB* P1-*trpA'-lacZ* leader-S20(mut.1-18aa)/SBP/LacZ-*rrnB* T1	This work	N/A	N/A	N/A	RLG14114
RLG14158	No promoter_*lacZ* leader	VH1000 *ygfF-gcvP*<>*tetA*R-*rrnB* dead-*trpA'-lacZ* leader-S20(mut.1-18aa)/SBP/LacZ-*rrnB* T1	This work	N/A	N/A	N/A	RLG14115
RLG14360	P*rpsT_rpsT* leader, pALS13	VH1000 *ygfF-gcvP*<>*tetA*R-*rpsT* P1+P2-*rpsT* leader-S20(1-18aa)/SBP/LacZ-*rrnB* T1	This work	N/A	N/A	N/A	N/A
RLG14361	P*lacUV5_rpsT* leader, pALS13	VH1000 *ygfF-gcvP*<>*tetA*R-P*lacUV5-rpsT* leader-S20(1-18aa)/SBP/LacZ-*rrnB* T1	This work	N/A	N/A	N/A	N/A
RLG14362	P*rpsT_lacZ* leader, pALS13	VH1000 *ygfF-gcvP*<>*tetA*R-*rpsT* P1+P2-*trpA'-lacZ* leader-S20(mut.1-18aa)/SBP/LacZ-*rrnB* T1	This work	N/A	N/A	N/A	N/A
RLG14363	P*lacUV5_lacZ* leader, pALS13	VH1000 *ygfF-gcvP*<>*tetA*R-P*lacUV5-trpA'-lacZ* leader-S20(mut.1-18aa)/SBP/LacZ-*rrnB* T1	This work	N/A	N/A	N/A	N/A
RLG14364	*rrnB* P1_*lacZ* leader, pALS13	VH1000 *ygfF-gcvP*<>*tetA*R-*rrnB* P1-*trpA'-lacZ* leader-S20(mut.1-18aa)/SBP/LacZ-*rrnB* T1	This work	N/A	N/A	N/A	N/A
RLG14365	P*rpsT_rpsT* leader, pALS14	VH1000 *ygfF-gcvP*<>*tetA*R-*rpsT* P1+P2-*rpsT* leader-S20(1-18aa)/SBP/LacZ-*rrnB* T1	This work	N/A	N/A	N/A	N/A
RLG14368	P*lacUV5_lacZ* leader, pALS14	VH1000 *ygfF-gcvP*<>*tetA*R-P*lacUV5-trpA'-lacZ* leader-S20(mut.1-18aa)/SBP/LacZ-*rrnB* T1	This work	N/A	N/A	N/A	N/A

RLG14531	P*rpsJ_rpsJ* leader in DY330	DY330 *ygfF-gcvP*<>*tetA*R-P*rpsJ-rpsJ* leader-S10(10aa)/S20(mut.1-18aa)/SBP/LacZ-*rrnB* T1	This work	7397	7398	RLG9933	N/A
RLG14532	P*rpsJ_lacZ* leader in DY330	DY330 *ygfF-gcvP*<>*tetA*R-P*rpsJ-trpA'-lacZ* leader-S20(mut.1-18aa)/SBP/LacZ-*rrnB* T1	This work	7397	7399	RLG9933	N/A
RLG14541	P*lacUV5*_Long *rpsT* leader in DY330	DY330 *ygfF-gcvP*<>*tetA*R-P*lacUV5*-Long *rpsT* leader (-130 from start codon)-S20(1-18aa)/SBP/LacZ-*rrnB* T1	This work	7395	N/A	N/A	N/A
RLG14559	P*lacUV5*_Long *rpsT* leader	VH1000 *ygfF-gcvP*<>*tetA*R-P*lacUV5*-Long *rpsT* leader (-130 from start codon)-S20(1-18aa)/SBP/LacZ-*rrnB* T1	This work	N/A	N/A	N/A	RLG14541
RLG14568	*rpsT* P1_*lacZ* leader in DY330	DY330 *ygfF-gcvP*<>*tetA*R-*rpsT* P1-*trpA'-lacZ* leader-S20(mut.1-18aa)/SBP/LacZ-*rrnB* T1	This work	7017	7197	RLG9933	N/A
RLG14569	*rpsT* P2_*lacZ* leader in DY330	DY330 *ygfF-gcvP*<>*tetA*R-*rpsT* P2-*trpA'-lacZ* leader-S20(1-18aa)/SBP/LacZ-*rrnB* T1	This work	6784	7018	RLG9933	N/A
RLG14572	P*lacUV5*_Long *rpsT* leader, pALS13	VH1000 *ygfF-gcvP*<>*tetA*R-P*lacUV5*-Long *rpsT* leader (-130 from start codon)-S20(1-18aa)/SBP/LacZ-*rrnB* T1	This work	N/A	N/A	N/A	N/A
RLG14582	*rpsT* P1_*lacZ* leader	VH1000 *ygfF-gcvP*<>*tetA*R-*rpsT* P1-*trpA'-lacZ* leader-S20(mut.1-18aa)/SBP/LacZ-*rrnB* T1	This work	N/A	N/A	N/A	RLG14568
RLG14583	*rpsT* P2_*lacZ* leader	VH1000 *ygfF-gcvP*<>*tetA*R-*rpsT* P2-*trpA'-lacZ* leader-S20(mut.1-18aa)/SBP/LacZ-*rrnB* T1	This work	N/A	N/A	N/A	RLG14569
RLG14584	P*lacUV5_rpsJ* leader in DY330	DY330 *ygfF-gcvP*<>*tetA*R-P*lacUV5-rpsJ* leader-S10(10aa)/S20(mut.1-18aa)/SBP/LacZ-*rrnB* T1	This work	7398	7442	RLG9933	N/A
RLG14594	P*lacUV5_rpsT* leader, Δ*dksA*	VH1000 *dksA*::*kan*, *ygfF-gcvP*<>*tetA*R-P*lacUV5- rpsT* leader-S20(1-18aa)/SBP/LacZ-*rrnB* T1	This work	N/A	N/A	N/A	RLG6632
RLG14596	P*lacUV5_lacZ* leader, Δ*dksA*	VH1000 *dksA*::*kan*, *ygfF-gcvP*<>*tetA*R-P*lacUV5-trpA'-lacZ* leader-S20(mut.1-18aa)/SBP/LacZ-*rrnB* T1	This work	N/A	N/A	N/A	RLG6632
RLG14601	P*rpsJ_rpsJ* leader	VH1000 *ygfF-gcvP*<>*tetA*R-P*rpsJ-rpsJ* leader-S10(10aa)/S20(mut.1-18aa)/SBP/LacZ-*rrnB* T1	This work	N/A	N/A	N/A	RLG14531
RLG14602	P*rpsJ_lacZ* leader	VH1000 *ygfF-gcvP*<>*tetA*R-P*rpsJ-lacZ* leader-S20(mut.1-18aa)/SBP/LacZ-*rrnB* T1	This work	N/A	N/A	N/A	RLG14532
RLG14604	P*lacUV5_rpsJ* leader	VH1000 *ygfF-gcvP*<>*tetA*R-P*lacUV5-rpsJ* leader-S10(10aa)/S20(mut.1-18aa)/SBP/LacZ-*rrnB* T1	This work	N/A	N/A	N/A	RLG14584
RLG14614	P*lacUV5_rpsT* leader, Δ*dksA,* pALS13	VH1000 *dksA*::*kan*, *ygfF-gcvP*<>*tetA*R-P*lacUV5- rpsT* leader-S20(1-18aa)/SBP/LacZ-*rrnB* T1	This work	N/A	N/A	N/A	N/A

RLG14616	P*lacUV5_lacZ* leader, Δ*dksA,* pALS13	VH1000 *dksA*::*kan*, *ygfF-gcvP*<>*tetA*R-P*lacUV5-trpA'-lacZ* leader-S20(mut.1-18aa)/SBP/LacZ-*rrnB* T1	This work	N/A	N/A	N/A	N/A
RLG14632	P*rpsJ_rpsJ* leader, pALS13	VH1000 *ygfF-gcvP*<>*tetA*R-P*rpsJ-rpsJ* leader-S10(1-10aa)/S20(mut.1-18aa)/SBP/LacZ-*rrnB* T1	This work	N/A	N/A	N/A	N/A
RLG14634	P*rpsJ_lacZ* leader, pALS13	VH1000 *ygfF-gcvP*<>*tetA*R-P*rpsJ-lacZ* leader-S20(mut.1-18aa)/SBP/LacZ-*rrnB* T1	This work	N/A	N/A	N/A	N/A
RLG14638	P*lacUV5_rpsJ* leader, pALS13	VH1000 *ygfF-gcvP*<>*tetA*R-P*lacUV5-rpsJ* leader-S10(1-10aa)/S20(mut.1-18aa)/SBP/LacZ-*rrnB* T1	This work	N/A	N/A	N/A	N/A
RLG14705	*rpsT* P1_*lacZ* leader, pALS13	VH1000 *ygfF-gcvP*<>*tetA*R-*rpsT* P1-*trpA'-lacZ* leader-S20(mut.1-18aa)/SBP/LacZ-*rrnB* T1	This work	N/A	N/A	N/A	N/A
RLG14707	*rpsT* P2_*lacZ* leader, pALS13	VH1000 *ygfF-gcvP*<>*tetA*R-*rpsT* P2-*trpA'-lacZ* leader-S20(mut.1-18aaWT)/SBP/LacZ-*rrnB* T1	This work	N/A	N/A	N/A	N/A

Plasmid	**Relevant properties**	**Source**
pALS13	P*tac*-truncated *relA*, active protein, Amp^r	(Svitil et al., 1993)
pALS14	P*tac*-truncated *relA*, inactive protein, Amp^r	(Svitil et al., 1993)
pRLG770	Transcription vector	(Ross et al., 1990)
pRLG2222	pRLG770 containing P*lacUV5* (-59/+38)	(Chandrangsu et al., 2011)
pRLG3735	pRLG770 containing *rrnB* P1 (+1C; -66/+9)	(Murray and Gourse, 2004)
pRLG9237	pRLG770 containing *rpsT* P2 (-89/+50)	(Lemke et al., 2011)

Table 2.S2 Oligos and gBlocks for recombineering and qPCR

Oligo #	Sequence	Purpose
6668	tgccggcctgttcccctcaccctaaccctctccccaaaggtg taggctggagctgcttcg	Forward oligo used to amplify the *frt-kan* cassette with flanking homology to the *ygfF-gcvP* intergenic region.
6669	gcttcggcgccttttttagtcagatgacaaagtacaaaagtatt ccggggatccgtcgacc	Reverse oligo used to amplify the *frt-kan* cassette with flanking homology to the *ygfF-gcvP* intergenic region.
6673	tcagggagagggccggggtgagggtaaatattcgcgccag atcaaagggaaaactgtcca	Forward oligo used to amplify the *cat-sacB* cassette with flanking homology to the *ygfF-gcvP* intergenic region.
6674	cctttggggagagggttagggtgaggggaacaggccggca aaaatgagacgttgatcggc	Reverse oligo used to amplify the *cat-sacB* cassette with flanking homology to the *ygfF-gcvP* intergenic region.
6773	tgccggcctgttcccctcaccctaaccctctccccaaaggtg ccctcttgggttatcaagtgccggcctgttcccctcaccctaa ccctctccccaaaggtgccctcttgggttatcaag	Forward oligo used to amplify the *tetAR* cassette with flanking homology to the *ygfF-gcvP* intergenic region.
6774	gcttcggcgccttttttagtcagatgacaaagtacaaaagtgta cgttggagccgcattatgcttcggcgccttttttagtcagatga caaagtacaaaagtgtacgttggagccgcattat	Reverse oligo used to amplify the tetAR cassette with flanking homology to the *ygfF-gcvP* intergenic region.
6779	agagggccggggtgagggtaaatattcgcgccagtcctact caggagagcgttcaccgacagagggccggggtgagggtaa atattcgcgccagtcctactcaggagagcgttcaccgac	Reverse oligo used to amplify P*lacUV5_lacZ_rrnB* T1 with flanking homology to the *ygfF-gcvP* intergenic region.
6780	ttggggagagggttagggtgaggggaacaggccggcagcg tatcacgaggccctttcgtcttggggagagggttagggtgag gggaacaggccggcagcgtatcacgaggccctttcgtc	Forward oligo used to amplify P*lacUV5_lacZ_rrnB* T1 with flanking homology to the *ygfF-gcvP* intergenic region.
6781	cctctttaatgaattacccggggaattcggtaccggatccatc aaagggaaaactgtccacctctttaatgaattacccggggaa ttcggtaccggatccatcaaagggaaaactgtcca	Reverse oligo used to amplify *cat-sacB* cassette with flanking homology to the *lacZ* leader.
6784	ggcagcgtatcacgaggccctttcgtcttcacctcgagccat cactacgtaacgagtgcc	Forward oligo used to amplify *rpsT* P2, starting at -89 relative to the TSS, with flanking homology to the *tetA*R cassette.
6825	ggctttacactttatgcttccggctcgtataatgtgtggaaaaa tgagacgttgatcggc	Forward oligo used to amplify *cat-sacB* cassette with flanking homology to the *lacUV5* promoter.
6908	aaacgacggccagtgaatccgtaatcatggtcatagctgtttc ctgtgtgataaagaaag	Reverse oligo used to amplify the *trpA*'-*lacZ* leader from a lamba lysogen reporter system (System II, Gourse lab collection) with flanking homology to *lacZ*.
6909	caggctttacactttatgcttccggctcgtataatgtgtggaac aaccgatgaaagcggc	Forward oligo used to amplify the *trpA*'-*lacZ* leader from a lamba lysogen reporter system (System II, Gourse lab collection) with flanking homology to the *lacUV5* promoter.
6910	gctgtgggattaactgcgcgtcgccgctttcatcggttgtatc aaagggaaaactgtcca	Reverse oligo used to amplify the *cat-sacB* cassette with flanking homology to the *trpA*'-*lacZ* leader.
7017	gcagcgtatcacgaggccctttcgtcttcacctcgagtcattg ccatggcgcaaatcacg	Forward oligo used to amplify *rpsT*, starting from -100 relative to the *rpsT* P1 TSS, with flanking homology to the *tetA*R cassette.
7018	tgggattaactgcgcgtcgccgctttcatcggttgtggccga ggaatatgccctttagc	Reverse oligo used to amplify the *rpsT* promoters, starting from +2 relative to the *rpsT* P2 TSS, with flanking homology to the *trpA*'-*lacZ* leader.
7020	tgtaaaacgacggccagtgaatccgtaatcatggtacgagcc ttttcagactgaatggcg	Reverse oligo used to amplify the *rpsT* leader, starting from the 18 codon of *rpsT*, with flanking homology to the *lacZ* ORF; this oligo creates an in-frame fusion between S20 (1-18aa) and LacZ.
7021	ccggcagcgtatcacgaggccctttcgtcttcacctcgagtc agaaaattattttaaatttcctcttgtcaggccgg	Forward oligo used to amplify *rrnB* P1, starting at -61 from the TSS, with flanking homology to the *tetA*R cassette.
7022	gggattaactgcgcgtcgccgctttcatcggttgttggtggcg cattatagggagttatt	Reverse oligo used to amplify *rrnB* P1, starting at +1 from the TSS, with flanking homology to the *trpA'-lacZ* leader.
7024	ccggcagcgtatcacgaggccctttcgtcttcacctcgagtc agaaaattattttaaatttcctcgactcaggccgg	Forward oligo used to amplify the *rrnB* dead promoter (*rrnB* P1 mutant that has no RNAP binding activity; pRLG11826 from Gourse lab strain collection), starting from -61 relative to *rrnB* P1 TSS, with flanking homology to the *tetA*R cassette.
7025	gcggctgtgggattaactgcgcgtcgccgctttcatcggttgt tggtggcgccttaccgg	Reverse oligo used to amplify the *rrnB* dead promoter (*rrnB* P1 mutant that has no RNAP binding activity; pRLG11826 from Gourse lab strain collection), starting from +1 relative to *rrnB* P1 TSS, with flanking homology to the *trpA'-lacZ* leader.
7026	ggctttacactttatgcttccggctcgtataatgtgtggatttg aattgtccatatagaacacatttgggagttgg	Forward oligo used to amplify the *rpsT* leader, starting from +3 relative to the *rpsT* P2 TSS, with flanking homology to the *lacUV5* promoter.

7110	gtgaccaccacgccaaccggtggtttttttcgtccatacgagc cttttcagactgaatggc	Reverse oligo used to amplify the *rpsT* leader, starting from the 18th codon of S20, with flanking homology to the SBP-tag sequence.
7111	gctgtgggattaactgcgcgtcgccgctttcatcggttgt	Reverse oligo containing the *trpA'-lacZ* leader sequence. The 3' base of this oligo is complementary with the first nucleotide of the *trpA'-lacZ* leader.
7197	tgtgggattaactgcgcgtcgccgctttcatcggttgtgcgca ggattctaccagcttgc	Reverse oligo used to amplify *rpsT* P1, starting from +2 relative to the TSS, with flanking homology to the *trpA'-lacZ* leader.
7397	ccggcagcgtatcacgaggccctttcgtcttcacctcgaggt gtcaaaaatgcactgaacgaacttgagag	Forward oligo used to amplify *rpsJ*, starting from -100 relative to the *rpsJ* promoter's TSS, with flanking homology to the *tetAR* cassette.
7398	actgtatagcacgttttttagcagatttgatgttagccatcagg cggatacggattctttggttc	Reverse oligo used to amplify the *rpsJ* leader, starting from the 10th codon of S10, with flanking homology to the S20 (mut1-18aa) and SBP-tag region.
7399	gctgtgggattaactgcgcgtcgccgctttcatcggttgtccg cgcattatacatacttaaccaccgaac	Reverse oligo used to amplify the *rpsJ* promoter, starting at +1 relative to the TSS, with flanking homology to the *trpA'-lacZ* leader.
7442	tttacactttatgcttccggctcgtataatgtgtggagcttgtc gtagttgacagcaggt	Forward oligo used to amplify the *rpsJ* leader, starting at +2 relative to the *rpsJ* promoter's TSS, with flanking homology to the *lacUV5* promoter.
7608	gggtgaaggtgatgctacaa	Forward oligo for detection of superfolder GFP by qPCR.
7609	gaacaccataggtcagagtagtg	Reverse oligo for detection of superfolder GFP by qPCR.
7664	aaacagctatggctaacatcaaatc	Forward oligo for detection of the *lacZ*m5'-SBP-tag region by qPCR.
7665	agaccttcaacaacgtgacc	Reverse oligo for detection of the *lacZ*m5'-SBP-tag region by qPCR.
gBlocks	**sequence**	**Purpose**
7071	aggcaccccaggctttacactttatgcttccggctcgtataat gtgtgga**acaaccgatgaaagcggcgacgcgcagtt aatcccacagccgccagttccgctggcggcatttta actttctttatcacacaggaaacagct**atggctaacatc aaatctgctaaaaaacgtgctatacagtctgaaaaagctcgta tggacgaaaaaaccaccggttggcgtggtggtcacgttgttg aaggtctggct	Double stranded DNA fragment containing the *lacUV5* promoter, *trpA'-lacZ* leader (sequence in bold), MUT S20 (1-18) (mutations described in Supplementary Materials and Methods), and the sequence coding for the Streptavidin-binding peptide (SBP), was fused in frame to the first few codons of *lacZ* and used to clone the first 18 aa of S20, with silent mutations that eliminate regulation through translational feedback, and SBP fused to the NTD of β-galactosidase by recombineering.
7395	ggctttacactttatgcttccggctcgtataatgtgtggacatc actacgtaacgagtgccggcacattaacggcgcttatttgca caaatccagccacaaaagaaggctaaaaggggctctacctc ggcctttgaattgtccatatagaacacatttgggagttggacc ttggctaatatcaaatcagctaagaagcgcgccattcagtctg aaaaggctcgtatggacgaaaaaaccaccggttggcg	Double stranded DNA fragment containing the *lacUV5* promoter, the *rpsT* leader starting at +3 relative to the *rpsT* P1 TSS, and mutations eliminating the activity *rpsT* P2 promoter (mutations are described in Supplementary Materials and Methods), as well as the coding sequence for the first 18aa of S20, and the SBP-tag. This fragment was used for recombineering to create the P*lacUV5*_Long *rpsT* leader construct.

Supplemental Materials and Methods

Strain construction. Reporter fusions were constructed in DY330 (Yu et al., 2000) by recombineering (Thomason et al., 2014). Briefly, cells were grown in LB at 30°C with aeration to OD_{600} = 0.4-0.6, λ Red recombination functions were induced by heat-shock at 42°C for 15 min, and cells were made electrocompetent by multiple rounds of washing with cold-H_2O (Thomason et al., 2014). Double-stranded DNA fragments containing the sequences of interest were electroporated into cells where they recombined with the chromosome by homology. Recombinants were selected by antibiotic resistance or counter-selected by plating the recombinants on LB without NaCl, supplemented with 6% sucrose, and growing at 30°C (Blomfield et al., 1991). Double-stranded DNAs were obtained by PCR with Phusion high-fidelity DNA polymerase (New England BioLabs M0530) following the manufacturer's protocol and using the oligos and template DNA listed in Table 2.S1.

To construct reporter fusions, an *frt-kan* cassette was cloned into the *ygfF-gcvP* intergenic region (genomic position 3044022…3044161 in the MG1655 DNA sequence; accession number NC_000913.3) of DY330 (Yu et al., 2000) (strain RLG6341, lab collection), downstream from the rho-independent terminator of *gcvP*, resulting in strain RLG11397. This genomic location was chosen because *ygfF* is not an essential gene, and its deletion does not have effects detrimental to cell growth (Baba et al., 2006), suggesting that any unintended effects on expression of *ygfF* should not affect the reporter fusion. In addition, the Rho-independent terminator in *gcvP* should decrease background from transcription originating upstream of the reporter region. A *cat-sacB* cassette was inserted downstream *frt-kan* of RLG11397, forming a *frt-kan-cat-sacB* fusion (RLG11398). The *frt-kan* cassette in RLG11398 was then replaced by a *tetAR* cassette (RLG11399) to further reduce potential leaky transcription from upstream of the reporter; in the absence of tetracycline expression of *tetA* is repressed by TetR (Altenbuchner et al., 1983).

Using counter-selection, we substituted the *cat-sacB* cassette from RLG11399 with a DNA fragment containing a P*lacUV5-lacZ-rrnB* T1 fusion downstream of *tetAR* (RLG13665). We reinserted the *cat-sacB* cassette between P*lacUV5* and *lacZ* of RLG13665, forming RLG13667. Initial tests with reporter fusions based on RLG13667 showed that the synthetic *lacZ* ribosome binding site (RBS) in that fusion was too weak for measuring reporter protein expression (data not shown). Therefore, we substituted the RBS in RLG13667 with the *trpA'-lacZ* leader (derived from the λ-based *lacZ* fusion described as System II in (Rao et al., 1994) to form RLG13808. The *cat-sacB* cassette was then inserted between P*lacUV5* and the *trpA'-lacZ* leader of strain RLG13808 to form RLG13812. To examine regulation by the *rpsT* 5'-mRNA region, we substituted the *trpA'-lacZ* leader with the *rpsT* leader, including the first 18 amino acids of S20, into RLG13812 downstream of P*lacUV5* and fused in-frame to *lacZ*, resulting in RLG13903. However, the reporter protein band in RLG13903 (the S20 (1-18aa)/*lacZ* fusion) migrated to the same position as another protein band (Fig. 2.1B, lane 9).

To create a reporter protein that migrated to a clearer area of the gel, we used a synthetic DNA fragment (IDT) and RLG13812 to create a fusion between the first 18 amino acids of S20 (with silent mutations that eliminated regulation by translational feedback; see Fig. 2.1 legend for details), the Streptavidin-binding peptide (SBP; MDEKTTGWRGGHVVEGLAGELEQLRARLEHHPQGQREP) (Keefe et al., 2001) to the N-terminus of *lacZ* (SBP-*lacZ*), resulting in RLG14029. During cloning of strain RLG14029, a mutation was erroneously introduced into the *lacUV5* promoter (C-14A). This mutation was corrected concurrent with insertion of the *cat-sacB* cassette between P*lacUV5* and the *trpA'-lacZ* leader of strain RLG14029 to create RLG14082. All reporter fusions were constructed in RLG14082 and then transferred into VH1000 by P1 transduction (Thomason et al., 2007).

Constructs 2, 4, 6-8 (Fig. 2.1A) containing the *trpA'-lacZ* leader fused to the S20 (1-18)-SBP-*lacZ* coding region also contained mutations in the *rpsT* sequence (MUT S20 [1-18]) designed to eliminate potential translational feedback regulation (T+133A, T+141C, A+150T, G+156A, G+159A, C+162T, C+165T, T+168A, and G+180A; numbering is relative to the transcription start site of *rpsT* P1) (Mackie, 1989). The T+133A mutation changes the start codon from UUG to AUG and eliminates translational feedback regulation of the *rpsT* leader (Parsons et al., 1988), while the remaining changes are silent mutations intended to disrupt the mRNA secondary structure of this region (Mackie, 1992).

The reporters with the *lacUV5* promoter (constructs 3-5, 9, and 10) contained DNA sequences corresponding to -59/+1 with respect to the transcription start site from the *lac* promoter region (the *lacUV5* mutation is a substitution of 2 bp in the -10 region eliminating the requirement for CRP-cAMP) (Malan and McClure, 1984).

To eliminate *rpsT* P2 promoter activity, construct 5 (Long *rpsT* leader) contained 7 point mutations in the putative -35 and -10 elements (T-35G, T-34C, G-33C, C-12G, A-11C, A-9C, and T-7A; numbering relative to the *rpsT* P2 transcription start site [Mackie and Parsons, 1983]; represented by asterisks in Fig. 2.1A).

Supplemental References

Altenbuchner, J., Schmid, K., and Schmitt, R. (1983). Tn1721-encoded tetracycline resistance: mapping of structural and regulatory genes mediating resistance. J. Bacteriol. *153*, 116–123.

Baba, T., Ara, T., Hasegawa, M., Takai, Y., Okumura, Y., Baba, M., Datsenko, K.A., Tomita, M., Wanner, B.L., and Mori, H. (2006). Construction of *Escherichia coli* K-12 in-frame, single-gene knockout mutants: the Keio collection. Mol. Syst. Biol. *2*, 2006.0008–.0011.

Barker, M.M., Gaal, T., Josaitis, C.A., and Gourse, R.L. (2001). Mechanism of regulation of transcription initiation by ppGpp. I. Effects of ppGpp on transcription initiation *in vivo* and *in vitro*. J. Mol. Biol. *305*, 673–688.

Blattner, F.R., Plunkett, G., Bloch, C.A., Perna, N.T., Burland, V., Riley, M., Collado-Vides, J., Glasner, J.D., Rode, C.K., Mayhew, G.F., et al. (1997). The complete genome sequence of *Escherichia coli* K-12. Science *277*, 1453–1462.

Blomfield, I.C., Vaughn, V., Rest, R.F., and Eisenstein, B.I. (1991). Allelic exchange in *Escherichia coli* using the *Bacillus subtilis sacB* gene and a temperature-sensitive pSC101 replicon. Mol. Microbiol. *5*, 1447–1457.

Chandrangsu, P., Lemke, J.J., and Gourse, R.L. (2011). The *dksA* promoter is negatively feedback regulated by DksA and ppGpp. Mol. Microbiol. *80*, 1337–1348.

Gaal, T., Bartlett, M.S., Ross, W., Turnbough, C.L., and Gourse, R.L. (1997). Transcription regulation by initiating NTP concentration: rRNA synthesis in bacteria. Science *278*, 2092–2097.

Keefe, A.D., Wilson, D.S., Seelig, B., and Szostak, J.W. (2001). One-step purification of recombinant proteins using a nanomolar-affinity streptavidin-binding peptide, the SBP-Tag. Prot. Expr. Purif. *23*, 440–446.

Lemke, J.J., Sanchez-Vazquez, P., Burgos, H.L., Hedberg, G., Ross, W., and Gourse, R.L. (2011). Direct regulation of *Escherichia coli* ribosomal protein promoters by the transcription factors ppGpp and DksA. Proc. Natl. Acad. Sci. U S A *108*, 5712–5717.

Mackie, G.A. (1989). Stabilization of the 3' one-third of *Escherichia coli* ribosomal protein S20 mRNA in mutants lacking polynucleotide phosphorylase. J. Bacteriol. *171*, 4112–4120.

Mackie, G.A. (1992). Secondary structure of the mRNA for ribosomal protein S20. Implications for cleavage by ribonuclease E. J. Biol. Chem. *267*, 1054–1061.

Malan, T.P., and McClure, W.R. (1984). Dual promoter control of the *Escherichia coli* lactose operon. Cell *39*, 173–180.

Murray, H.D., and Gourse, R.L. (2004). Unique roles of the *rrn* P2 rRNA promoters in *Escherichia coli*. Mol. Microbiol. *52*, 1375–1387.

Parsons, G.D., Donly, B.C., and Mackie, G.A. (1988). Mutations in the leader sequence and initiation codon of the gene for ribosomal protein S20 (*rpsT*) affect both translational efficiency and

autoregulation. J. Bacteriol. *170*, 2485–2492.

Rao, L., Ross, W., Appleman, J.A., Gaal, T., Leirmo, S., Schlax, P.J., Record, M.T., and Gourse, R.L. (1994). Factor independent activation of *rrnB* P1. An "extended" promoter with an upstream element that dramatically increases promoter strength. J. Mol. Biol. *235*, 1421–1435.

Ross, W., Thompson, J.F., Newlands, J.T., and Gourse, R.L. (1990). *E. coli* Fis protein activates ribosomal RNA transcription *in vitro* and *in vivo*. Embo J. *9*, 3733–3742.

Svitil, A.L., Cashel, M., and Zyskind, J.W. (1993). Guanosine tetraphosphate inhibits protein synthesis *in vivo*. A possible protective mechanism for starvation stress in *Escherichia coli*. J. Biol. Chem. *268*, 2307–2311.

Thomason, L.C., Costantino, N., and Court, D.L. (2007). *E. coli* genome manipulation by P1 transduction. Curr. Protoc. Mol. Biol. *79*, 1.17.1-1.17.8.

Thomason, L.C., Sawitzke, J.A., Li, X., Costantino, N., and Court, D.L. (2014). Recombineering: genetic engineering in bacteria using homologous recombination. Curr. Protoc. Mol. Biol. *106*, 1.16.1–1.16.39.

Yu, D., Ellis, H.M., Lee, E.C., Jenkins, N.A., Copeland, N.G., and Court, D.L. (2000). An efficient recombination system for chromosome engineering in *Escherichia coli*. Proc. Natl. Acad. Sci. U S A *97*, 5978–5983.

Chapter 3

Conclusions and Future Directions

Conclusions and future directions

Decades of research have made apparent that bacterial cells have evolved incredibly complex metabolic systems that are finely-tuned to integrate environmental signals and efficiently adapt to changing conditions. ppGpp has emerged as a key secondary messenger that serves as a node to regulate various metabolic and physiological bacterial processes including ribosome biogenesis, DNA replication, GTP biosynthesis, phage replication and development, survival from starvation, motility, translation elongation and initiation, fatty acid synthesis, pathogenesis, symbiosis, and multicellular development (Braeken et al., 2006; Potrykus and Cashel, 2008; Srivatsan and Wang, 2008). Even though much research has focused on its role in regulating gene expression at the transcriptional level, it is thought that ppGpp can also directly regulate the activity of various proteins involved in several aspects of nucleotide, lipid, and general metabolism (Kanjee et al., 2012). Regardless of differences in mechanisms, ppGpp has been shown to be intimately involved in numerous processes within the bacterial kingdom and will likely remain the subject of future studies looking to expand our knowledge of the mechanisms bacteria employ to survive in ever-changing environments. The work presented here clarifies the role of small molecules, ppGpp and NTPs, in maintaining cellular homeostasis by regulating the synthesis of one of the most energy consuming molecules in bacteria, the ribosome.

What is the mechanism for feedback regulation of S20?

Studies on feedback regulation of S20 synthesis have suggested that binding of the repressor to the *rpsT* mRNA target site results in inhibition at the level of translation initiation (Parsons et al., 1988). Nonetheless, direct interaction between S20 and its mRNA target has not been detected experimentally (Donly and Mackie, 1988). Preliminary experiments done in our lab to either increase or decrease intracellular levels of S20 and measure concomitant changes in expression from the *rpsT*

leader have failed to confirm translational feedback regulation of this operon *in vivo* (Burgos and Gourse, unpublished data). We also measured changes in transcript levels from the *rpsT* leader reporter during induction of ppGpp synthesis and saw that the mRNA levels mimicked the changes in protein synthesis observed from the same reporter (Burgos and Gourse, unpublished data).

Since S20 protein synthesis and mRNA levels are decreasing in response to increasing ppGpp levels, we hypothesized that there are two possible mechanisms for feedback regulation of S20 synthesis that could explain these results: feedback regulation happens by S20 inhibiting translation initiation at the *rpsT* leader, which then results in decreased mRNA levels due to the loss of protection from nucleases that translating ribosomes provide (Deana and Belasco, 2005), or feedback regulation involves attenuation of transcription at the *rpsT* leader in response to S20 binding, resulting in reduction of mRNA levels and consequently protein synthesis. One difference between these models is that in the first case, S20 binding to the *rpsT* leader should result in a decrease in the *rpsT* mRNA half-life, while in the other case the reduction in *rpsT* mRNA levels is due to reduced transcription and would not necessarily involve destabilization of the message.

Preliminary experiments performed in our lab to measure changes in stability of the *rpsT* mRNA in response to inducing ppGpp synthesis did not detect a change in the half-life of the *rpsT* leader mRNA when repression was induced (Burgos and Gourse, unpublished data). This result suggests that maybe S20 synthesis is regulated by a transcription attenuation mechanism. However, we caution that because this experiment has only been performed a couple of times these results should be taken as preliminary. Nonetheless, it is apparent that further research is needed to elucidate the mechanism for feedback regulation of S20 synthesis.

Are other ribosomal protein promoters regulated by NTP levels?

Even though previous research had shown regulation of r-protein promoter activity in response to several conditions (Freedman et al., 1985; Lemke et al., 2011; Lindahl et al., 1976; Wirth et al., 1981), to our knowledge our work with *rpsT* P2 is the first time that regulation of r-protein promoter activity by initiating NTP levels has been demonstrated (chapter 2). Regulation of rRNA promoters by iNTP levels was demonstrated 20 years ago (Gaal et al., 1997) and it has been shown to play an important role in maintaining homeostasis in the cell by regulating rRNA synthesis during late stationary phase and outgrowth (Murray et al., 2003; Paul et al., 2004). Regulation of r-protein promoter activity by iNTP levels makes sense physiologically; it would be wasteful to allow transcription of r-protein promoters under conditions where NTP levels are too low to sustain productive synthesis of rRNA. Furthermore, because regulation by ppGpp and iNTPs has similar requirements in terms of the intrinsic kinetic properties of the regulated promoters (Barker et al., 2001; Gaal et al., 1997), it is likely that many r-protein promoters that were shown to be directly regulated by ppGpp and DksA (Lemke et al., 2011) will also be regulated by iNTP levels in a similar way to *rpsT* P2. Nonetheless, determining regulation of the plethora of r-protein promoters by iNTP levels requires further study.

Contributions to other projects

In addition to my work studying regulation of r-protein synthesis, I played a role in several other projects. These include investigating the role of promoter sequences in activation of transcription by DksA and ppGpp (Appendix A), testing direct regulation of fatty acid promoters by ppGpp and DksA (Appendix B), determining direct regulation of r-proteins by ppGpp and DksA (Lemke et al., 2011), and studying regulation of expression of the *rplU-rpmA-obgE-yhbE* operon (Maouche et al., 2016).

How do ppGpp and DksA together activate transcription?

In addition to inhibiting transcription, ppGpp and DksA are able to directly activate transcription from amino acid biosynthetic promoters (Paul et al., 2005). The prevailing hypothesis is that activated and inhibited promoters have a different rate-limiting step during transcription initiation that results in their varied responses to ppGpp and DksA (reviewed in Haugen et al., 2008); for inhibited promoters the equilibrium between RP_C and RP_O is shifted towards RP_C, while activated promoters have stable RP_Os but have a high activation energy to reach that state. If ppGpp and DksA stabilize an intermediate complex during transcription initiation, depending on whether the rate-limiting step during transcription initiation for a given promoter is before or after the ppGpp/DksA-stabilized complex, their effect will result in activation or inhibition, respectively. Consistent with this hypothesis, our lab showed in previous work that DksA stabilizes a complex of *rpsT* P2 with RNAP that forms after nucleation (this is the first step in DNA melting where the -11A base flips out of the duplex and into a binding pocket on RNAP) but before complete melting of promoter DNA (RP_O) (Winkelman, 2015). However, detailed studies on the mechanism of activation and how promoter sequences affect regulation are lacking.

To gain more information on the mechanism of activation of transcription by DksA and ppGpp, we studied the *iraP* promoter as an example of an activated promoter. We show that DksA and ppGpp directly activate P*iraP* activity (Appendix A; Ross et al., 2016). Furthermore, mutations in various *iraP* promoter regions and higher temperatures that make the transition to the open complex more amenable result in a bypass of the requirement for ppGpp and DksA to activate transcription (Appendix A). These results are consistent with the model that ppGpp and DksA are stabilizing an intermediate complex that forms during transcription initiation that is within the pathway of promoter melting. However, further studies are required to investigate the specific

molecular interactions that result in this effect. Albert Chen is continuing with this project to identify the mechanism for direct activation of promoter activity by DksA and ppGpp.

What is the role of ppGpp in regulation of fatty acid metabolism?

Previous work has shown that ppGpp plays a role in regulation of fatty acid metabolism (Appendix B). Preliminary results from our lab show that some promoters for genes related in fatty acid synthesis and degradation are regulated directly by ppGpp and DksA. In this work, we continued testing ppGpp/DksA-dependent regulation of promoters for genes related to lipid metabolism. Preliminary results show that two more lipid metabolism promoters are regulated by DksA and ppGpp (Appendix B). Taken together, these results show that ppGpp plays an important role in regulating fatty acid metabolism with respect to nutritional conditions. Because these experiments are preliminary, having been performed only once in many cases, a future direction for this project is to perform a systematic and reproducible analysis of all the lipid promoters that have been shown to have some regulatory connection with ppGpp. Additionally, to gain a better understanding of their regulation *in vivo*, regulation should be measured using lipid-promoter-*lacZ* fusions during induction of ppGpp synthesis.

References

Barker, M.M., Gaal, T., Josaitis, C.A., and Gourse, R.L. (2001). Mechanism of regulation of transcription initiation by ppGpp. I. Effects of ppGpp on transcription initiation *in vivo* and *in vitro*. J. Mol. Biol. *305*, 673–688.

Braeken, K., Moris, M., Daniels, R., Vanderleyden, J., and Michiels, J. (2006). New horizons for (p)ppGpp in bacterial and plant physiology. Trends Microbiol. *14*, 45–54.

Deana, A., and Belasco, J.G. (2005). Lost in translation: the influence of ribosomes on bacterial mRNA decay. Genes Dev. *19*, 2526–2533.

Donly, B.C., and Mackie, G.A. (1988). Affinities of ribosomal protein S20 and C-terminal deletion mutants for 16S rRNA and S20 mRNA. Nucleic Acids Res. *16*, 997–1010.

Freedman, L.P., Zengel, J.M., and Lindahl, L. (1985). Genetic dissection of stringent control and nutritional shift-up response of the *Escherichia coli* S10 ribosomal protein operon. J. Mol. Biol. *185*, 701–712.

Gaal, T., Bartlett, M.S., Ross, W., Turnbough, C.L., and Gourse, R.L. (1997). Transcription regulation by initiating NTP concentration: rRNA synthesis in bacteria. Science *278*, 2092–2097.

Haugen, S.P., Ross, W., and Gourse, R.L. (2008). Advances in bacterial promoter recognition and its control by factors that do not bind DNA. Nat. Rev. Microbiol. *6*, 507–519.

Kanjee, U., Ogata, K., and Houry, W.A. (2012). Direct binding targets of the stringent response alarmone (p)ppGpp. Mol. Microbiol. *85*, 1029–1043.

Lemke, J.J., Sanchez-Vazquez, P., Burgos, H.L., Hedberg, G., Ross, W., and Gourse, R.L. (2011). Direct regulation of *Escherichia coli* ribosomal protein promoters by the transcription factors ppGpp and DksA. Proc. Natl. Acad. Sci. U S A *108*, 5712–5717.

Lindahl, L., Post, L., and Nomura, M. (1976). DNA-dependent in vitro synthesis of ribosomal proteins, protein elongation factors, and RNA polymerase subunit α: inhibition by ppGpp. Cell *9*, 439–448.

Maouche, R., Burgos, H.L., My, L., Viala, J.P., Gourse, R.L., and Bouveret, E. (2016). Coexpression of *Escherichia coli obgE*, Encoding the Evolutionarily Conserved Obg GTPase, with Ribosomal Proteins L21 and L27. J. Bacteriol. *198*, 1857–1867.

Murray, H.D., Schneider, D.A., and Gourse, R.L. (2003). Control of rRNA expression by small molecules is dynamic and nonredundant. Mol. Cell *12*, 125–134.

Parsons, G.D., Donly, B.C., and Mackie, G.A. (1988). Mutations in the leader sequence and initiation codon of the gene for ribosomal protein S20 (*rpsT*) affect both translational efficiency and autoregulation. J. Bacteriol. *170*, 2485–2492.

Paul, B.J., Berkmen, M.B., and Gourse, R.L. (2005). DksA potentiates direct activation of amino acid promoters by ppGpp. Proc. Natl. Acad. Sci. U S A *102*, 7823–7828.

Paul, B.J., Ross, W., Gaal, T., and Gourse, R.L. (2004). rRNA transcription in *Escherichia coli*. Annu. Rev. Genet. *38*, 749–770.

Potrykus, K., and Cashel, M. (2008). (p)ppGpp: still magical? Annu. Rev. Microbiol. *62*, 35–51.

Ross, W., Sanchez-Vazquez, P., Chen, A.Y., Lee, J.-H., Burgos, H.L., and Gourse, R.L. (2016). ppGpp Binding to a Site at the RNAP-DksA Interface Accounts for Its Dramatic Effects on Transcription Initiation during the Stringent Response. Mol. Cell *62*, 811–823.

Srivatsan, A., and Wang, J.D. (2008). Control of bacterial transcription, translation and replication by (p)ppGpp. Curr. Opin. Microbiol. *11*, 100–105.

Winkelman, J.T. (2015). Mapping the path of DNA in transcription initiation intermediates (Doctoral dissertation). University of Wisconsin-Madison.

Wirth, R., Kohles, V., and Böck, A. (1981). Factors modulating transcription and translation in vitro of ribosomal protein S20 and isoleucyl-tRNA synthetase from *Escherichia coli*. Eur. J. Biochem. *114*, 429–437.

Appendix A

The Role of Promoter Sequences in Direct Regulation of P*iraP* Activity by DksA and ppGpp

I performed the experiments presented in this chapter. Some of the experiments were repeated by Wilma Ross and published (Ross, W., Sanchez-Vazquez, P., Chen, A.Y., Lee, J.H., Burgos, H.L., and Gourse, R.L. [2016]. ppGpp binding to a site at the RNAP-DksA interface accounts for its dramatic effects on transcription initiation during the stringent response. Mol. Cell *62*, 811-823).

Introduction

In addition to their role in inhibition of rRNA and r-protein transcription, ppGpp and DksA can directly activate transcription of amino acid promoters (Paul et al., 2005). ppGpp and DksA bind directly to RNAP and not DNA, therefore, the divergent but specific effects seen during transcription initiation are thought to be due to the intrinsic kinetic properties of the promoters and not to factors that bind DNA sequence-specifically (reviewed in Haugen et al., 2008). Promoters that are directly activated by ppGpp and DksA have stable open complexes (RP_Os) but have a high-energy barrier to isomerize to the RP_O during transcription initiation; DksA and ppGpp are proposed to decrease this energy barrier, allowing these promoters to more easily reach RP_O and begin RNA synthesis (Paul et al., 2005). Because transcription initiation is a multi-step process resulting from specific interactions between promoter DNA and RNAP, the kinetics of the complexes formed during this process depend on the sequences of the promoter DNA (Haugen et al., 2008). While many studies have looked at the role of promoter sequences in inhibition by DksA and ppGpp (Josaitis et al., 1995), the role of promoter sequences in activation of transcription has not been rigorously investigated.

In *E. coli*, the IraP protein is an anti-adaptor protein involved in stabilizing RpoS (σ^S) during phosphate starvation; it does this by binding to and titrating away RssB whose role is to bind σ^S and cause its degradation by the ClpXP protease (Bougdour et al., 2006). Bougdour and Gottesman (2007) showed that the *iraP* promoter (P*iraP*) is activated in response to phosphate starvation in a manner dependent on ppGpp. Furthermore, they showed that replacing the AT-rich discriminator region of P*iraP* with a GC-rich discriminator eliminated ppGpp-dependent activation of P*iraP* activity during phosphate starvation (Bougdour and Gottesman, 2007); suggesting that certain promoter sequences are involved in activation of transcription by ppGpp and DksA. These results implicate ppGpp as an important player in activation of the RpoS-dependent general stress response

in response to different signals. Nonetheless, it remained unclear whether activation of the *iraP* promoter by ppGpp was direct or indirect (Bougdour and Gottesman, 2007) and how other promoter sequences affected activation of transcription by DksA and ppGpp.

In this chapter, I show that P*iraP* is directly activated by ppGpp and DksA. Furthermore, I use P*iraP* as an example of an activated promoter to study the role that different promoter sequences have in activation of promoter activity by DksA and ppGpp. The results presented here are preliminary.

Results

To test ppGpp-dependent activation of P*iraP* and the role promoter sequences have during activation I cloned the *iraP* promoter, both WT and containing multiple mutations in several promoter regions (Fig. A.1), into the transcription vector p770 and tested transcription *in vitro* in the presence and absence of ppGpp and DksA. DksA and ppGpp increase transcription from P*iraP* WT by ~3-fold (Fig. A.2), showing that the regulation of this promoter by ppGpp is direct. Mutating the -35 and -10 sequences of P*iraP* so that they match the consensus promoter sequence (P*iraP* [c-35] and P*iraP* [c-10], respectively; Fig. A.1) results in much higher levels of basal transcription and precludes activation of P*iraP* transcription by DksA and ppGpp (Fig. A.2). These results show that increasing the strength of either the -35 or -10 elements of P*iraP* increases promoter activity substantially and bypasses the requirement of ppGpp and DksA for maximal promoter activity.

Mutating the P*iraP* discriminator region to make it GC-rich (P*iraP* [dis2]; Fig. A.1) eliminated ppGpp-dependent activation of P*iraP* *in vivo* during phosphate starvation (Bougdour and Gottesman, 2007). Furthermore, the GC-content in the discriminator region of promoters has been previously implicated in regulation by ppGpp and DksA (Barker et al., 2001; Travers, 1984). *In vitro*, increasing the G+C content of the discriminator (P*iraP* [dis2]) strongly reduces basal transcription

Figure A.1 Sequence of the *iraP* promoter and its mutants used in this study. The -35 and -10 core promoter hexamers are underlined and in bold. Bases in the promoter that match the consensus promoter sequence are capitalized; consensus promoter elements are TTGACA and TATAAT for the -35 and -10 hexamers, respectively. Red and blue colors are used to indicate where changes have been made to the WT *iraP* promoter sequence. The transcription start site is indicated as in Bougdour and Gottesman (2007).

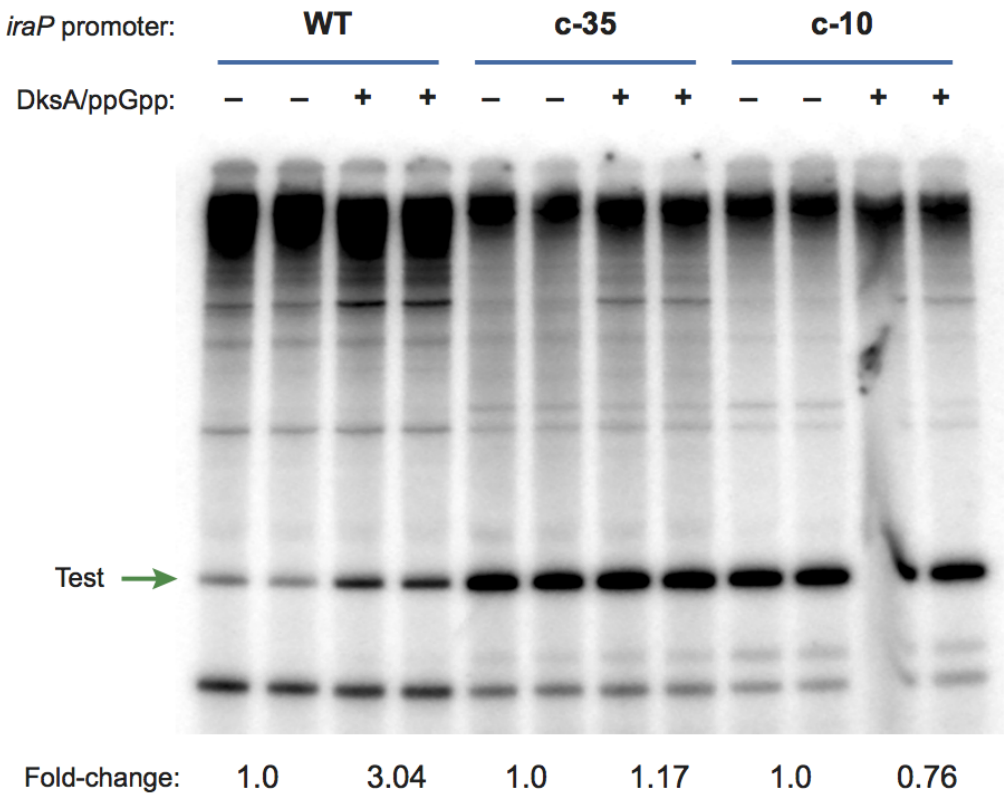

Figure A.2 Increasing the strength of the -35 and -10 core elements of P*iraP* bypasses ppGpp/DksA-dependent activation of transcription. Gel showing transcripts from the indicated promoters performed as described in Materials and Methods. Reactions were performed in duplicate.

compared to P*iraP* WT, and these mutations also eliminated the response to ppGpp and DksA (Fig. A.3). Combining a consensus -10 mutation with the dis2 mutation (PiraP [c-10/dis2]; Fig. A.1) resulted in much stronger basal transcription and again bypassed the need for ppGpp and DksA to activate transcription (Fig. A.3). These results suggest that GC-content in the discriminator region does play a role in regulation by ppGpp and DksA, but only in certain promoter sequence contexts, e.g., when the -10 and -35 sequences are relatively weak.

There is a specific interaction that can be formed between a G positioned one or two bases downstream of the -10 element in the non-template strand of a promoter and σ^{70} region 1.2 that results in highly increased stability of the RP_O (Haugen et al., 2008; Zhang et al., 2012). Transcription from an *iraP* promoter containing a -7GGG-5 mutation (P*iraP* [-7GGG-5]; Fig. A.1), which should establish an interaction with σ^{70} region 1.2, is activated by ppGpp and DksA to a similar extent as the WT promoter (Fig. A.4). Perhaps because the RP_O of P*iraP* is already stable because it has a very A+T-rich discriminator region, creating a contact with sigma region 1.2 does not further increase basal promoter activity and does not affect its ability to be activated by DksA/ppGpp. Basal transcription from P*iraP* (dis2/-7GGG-5) does not decrease as much as P*iraP* (dis2) and is no longer activated by ppGpp and DksA (Fig. A.4), suggesting that the effect of the dis2 mutation is dominant over the -7GGG-5 change with respect to regulation by ppGpp and DksA.

Discussion

The results presented here show that ppGpp and DksA directly activate transcription of the *iraP* promoter. Studies looking at the role of ppGpp during stress adaptation have focused mostly on the physiological response to amino acid starvation and the resulting increase in ppGpp levels and inhibition of ribosome synthesis. This work expands the number of known direct targets for ppGpp

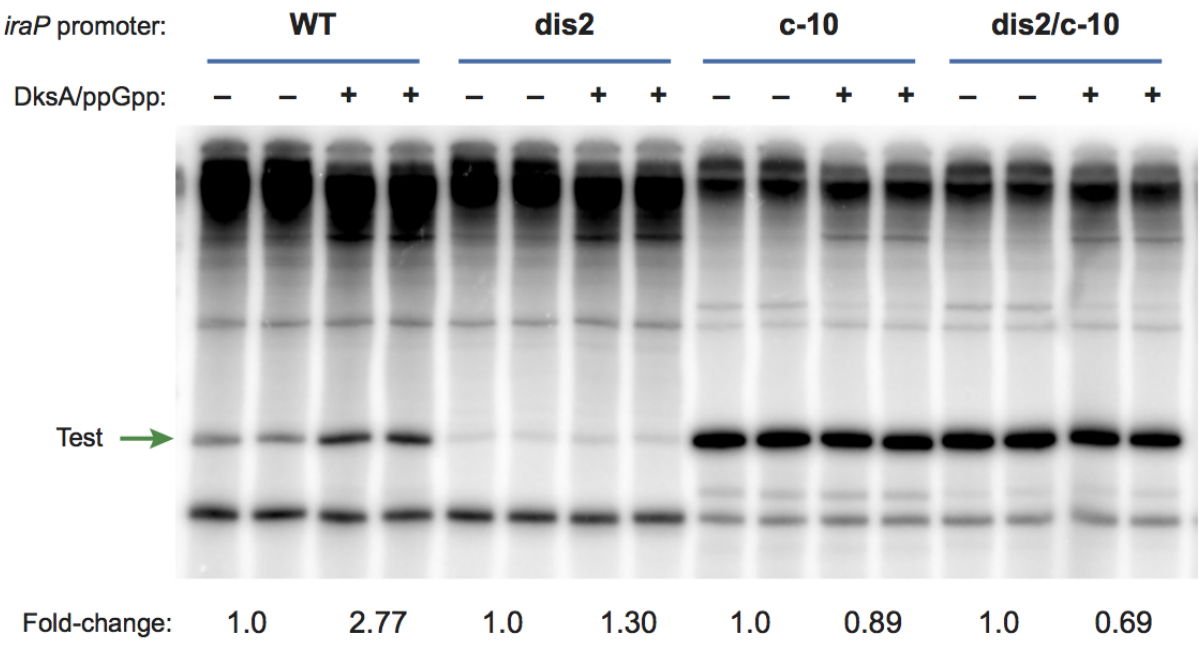

Figure A.3 GC-content of the discriminator region affects DksA/ppGpp-dependent activation of transcription. Gel showing transcripts from the indicated promoters performed as described in Materials and Methods. Reactions were performed in duplicate.

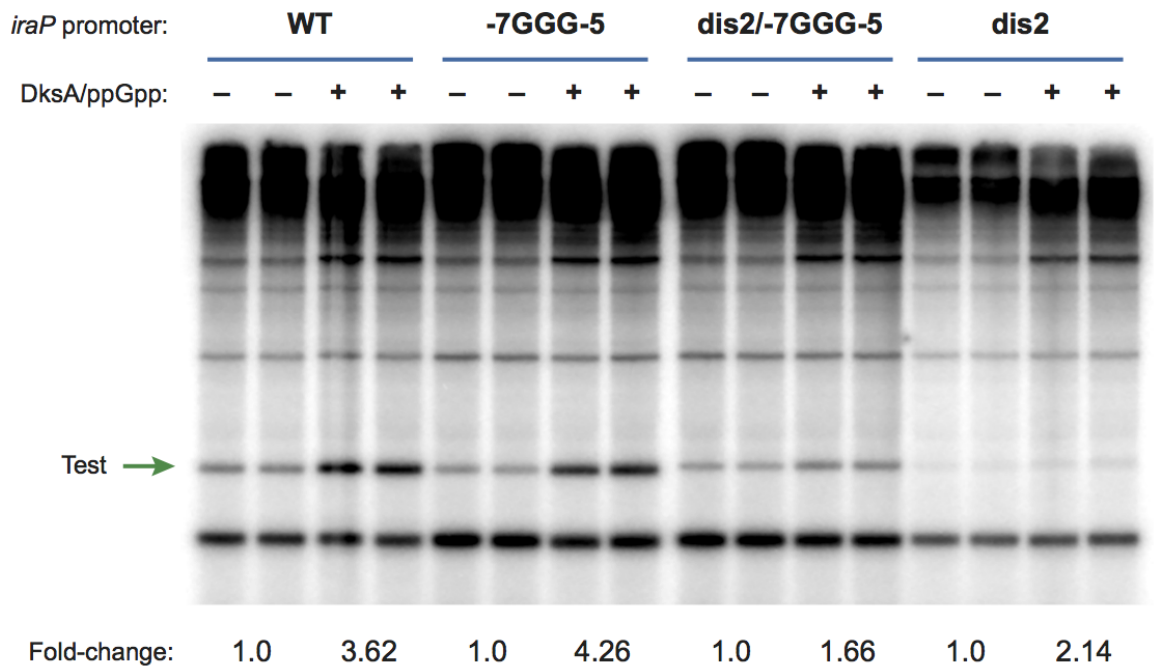

Figure A.4 An interaction between the P*iraP* discriminator region and σ^{70} region 1.2 does not interfere with activation of transcription by ppGpp and DksA. Gel showing transcripts from the indicated promoters performed as described in Materials and Methods. Reactions were performed in duplicate.

regulation, suggesting that ppGpp might have a role in activating the general stress response through RpoS in response so varying stress conditions by increasing IraP synthesis.

The result that mutating the -35 and -10 elements of the *iraP* promoter towards consensus results in bypassing the need for ppGpp and DksA to activate transcription is not surprising. It has long been recognized that having strong promoter sequences, especially the -35 and -10 hexamers, increases RNAP binding to promoter DNA, the isomerization rate of RP_O formation, and the stability of the RP_O (Haugen et al., 2008). Because DksA and ppGpp are proposed to activate transcription by increasing the isomerization rate towards RP_O formation (Paul et al., 2005), by changing the promoter sequences so that isomerization toward RP_O is not rate-limiting, the *iraP* promoter no longer requires DksA and ppGpp for maximal activity.

Mutating the normally AT-rich discriminator region of P*iraP* to a GC-rich discriminator (dis2) reduces basal transcription and eliminates activation of transcription by DksA and ppGpp (Fig. A.3 and A.4). A GC-rich discriminator region is thought to make it more difficult to melt the DNA strands during formation of the open complex and would destabilize the RP_O by increasing the likelihood of strand re-annealing (Haugen et al., 2006; Lamond and Travers, 1985). We suggest that DksA and ppGpp are still able to increase the rate of RP_O formation at P*iraP*, but the reduction in the stability of RP_O complexes due to the dis2 mutation makes the promoter complex susceptible to the negative effects of DksA and ppGpp. The result is that the promoter too inactive even when the factors are added.

Establishing a stronger contact between the discriminator region of P*iraP* and σ^{70} region 1.2 by making the -7GGG-5 mutation did not entirely eliminate activation of transcription by ppGpp and DksA (Fig. A.4). Because the open complex of P*iraP* WT is inherently long-lived, it is not surprising that stabilizing the RP_O further through the -7GGG-5 change has no effect on activation of transcription; the rate-limiting step is still preceding RP_O formation. On the other hand,

combining the -7GGG-5 and dis2 mutations together virtually eliminated activation of P*iraP* activity by DksA and ppGpp (Fig. A.4). While we have not yet performed kinetic measurements of transcription initiation complexes formed with these promoters, we propose that this result can be explained by the dominant effect that dis2 has on bubble collapse. Even though the σ^{70} region 1.2-discriminator interaction can form with P*iraP* (dis2/-7GGG-5), the very high GC-content of the discriminator will make it very difficult to not only melt promoter DNA to form the open complex but also to maintain strand-separation in the RP_O. This would result in a very-short-lived RP_O that combined with a slow isomerization rate of RPO formation ensures that transcription from the dis2/-7GGG-5 iraP promoter remains low even in the presence of ppGpp and DksA.

A dominant effect of DksA and ppGpp on bubble collapse is consistent with results from Jared Winkelman and Mike Maloney in our lab. They showed that the *rpsT* P2 promoter complex is open with RNAP alone, but addition of DksA and ppGpp forces the complex into a partially open state in which the upstream part of the -10 hexamer is single-stranded but the downstream part of the -10 hexamer is double-stranded (Winkelman, 2015).

Some of my conclusions are preliminary as the experiments have only been performed once. Further work is required to measure the kinetics of these *iraP* promoters and to determine the mechanism of activation of transcription by DksA and ppGpp. Albert Chen in our lab is currently working on determining the mechanism of activation of transcription by ppGpp and DksA.

Materials and Methods

Constructing *iraP* promoter mutants. The plasmids used in this chapter are listed in Table A.1. Promoter mutants were made as described previously (Gaal et al., 1989). Briefly, pairs of synthetic oligos containing the *iraP* promoter with the mutations of interest were obtained from Integrated DNA Technologies (IDT) so that the oligos annealed to each other within the -35 and -

10 spacer regions. The 3' ends were extended using Sequenase DNAP (Thermo Fischer Scientific) to form double stranded DNA fragments containing the promoters of interest flanked by EcoRI and HindIII sites. The promoters were then cloned into the p770 transcription vector through restriction enzyme cloning and verified by sequencing.

***In vitro* transcription.** Multiple-round *in vitro* transcription reactions were performed essentially as described previously (Ross and Gourse, 2009). Reactions were carried out at 30°C for 10 min and contained 150 mM NaCl, 10 nM Eσ70 RNAP, 1 nM of supercoiled plasmid template, 200 μM of ATP, GTP, and CTP, 10 μM UTP, and ~2 μCi of [γ-^{32}P]UTP. When added, DksA was at 2 μM and ppGpp at 100 μM. Transcripts were resolved by electrophoresis in a 5.5% polyacrylamide, 7 M urea gel and visualized by phosphorimaging.

Table A.1 Plasmids used in this study

Plasmid	Relevant properties	Source
p770	Transcription vector	(Ross et al., 1990)
pRLG11356	p770 containing P*iraP* WT (-64/+15)	This work
pRLG11364	p770 containing P*iraP* (c-35) (-64/+15)	This work
pRLG11358	p770 containing P*iraP* (c-10) (-64/+15)	This work
pRLG11357	p770 containing P*iraP* (dis2) (-64/+15)	This work
pRLG11359	p770 containing P*iraP* (c-10/dis2) (-64/+15)	This work
pRLG11362	p770 containing P*iraP* (-7GGG-5) (-64/+15)	This work
pRLG11363	p770 containing P*iraP* (dis2/-7GGG-5) (-64/+15)	This work

References

Barker, M.M., Gaal, T., Josaitis, C.A., and Gourse, R.L. (2001). Mechanism of regulation of transcription initiation by ppGpp. I. Effects of ppGpp on transcription initiation *in vivo* and *in vitro*. J. Mol. Biol. *305*, 673–688.

Bougdour, A., and Gottesman, S. (2007). ppGpp regulation of RpoS degradation via anti-adaptor protein IraP. Proc. Natl. Acad. Sci. U S A *104*, 12896–12901.

Bougdour, A., Wickner, S., and Gottesman, S. (2006). Modulating RssB activity: IraP, a novel regulator of sigma(S) stability in *Escherichia coli*. Genes Dev. *20*, 884–897.

Gaal, T., Barkei, J., Dickson, R.R., deBoer, H.A., deHaseth, P.L., Alavi, H., and Gourse, R.L. (1989). Saturation mutagenesis of an *Escherichia coli* rRNA promoter and initial characterization of promoter variants. J. Bacteriol. *171*, 4852–4861.

Haugen, S.P., Berkmen, M.B., Ross, W., Gaal, T., Ward, C., and Gourse, R.L. (2006). rRNA promoter regulation by nonoptimal binding of sigma region 1.2: an additional recognition element for RNA polymerase. Cell *125*, 1069–1082.

Haugen, S.P., Ross, W., and Gourse, R.L. (2008). Advances in bacterial promoter recognition and its control by factors that do not bind DNA. Nat. Rev. Microbiol. *6*, 507–519.

Josaitis, C.A., Gaal, T., and Gourse, R.L. (1995). Stringent control and growth-rate-dependent control have nonidentical promoter sequence requirements. Proc. Natl. Acad. Sci. U S A *92*, 1117–1121.

Lamond, A.I., and Travers, A.A. (1985). Stringent control of bacterial transcription. Cell *41*, 6–8.

Paul, B.J., Berkmen, M.B., and Gourse, R.L. (2005). DksA potentiates direct activation of amino acid promoters by ppGpp. Proc. Natl. Acad. Sci. U S A *102*, 7823–7828.

Ross, W., Thompson, J.F., Newlands, J.T., and Gourse, R.L. (1990). *E. coli* Fis protein activates ribosomal RNA transcription *in vitro* and *in vivo*. Embo J. *9*, 3733–3742.

Ross, W., and Gourse, R.L. (2009). Analysis of RNA polymerase-promoter complex formation. Methods *47*, 13–24.

Travers, A.A. (1984). Conserved features of coordinately regulated *E. coli* promoters. Nucleic Acids Res. *12*, 2605–2618.

Winkelman, J.T. (2015). Mapping the path of DNA in transcription initiation intermediates (Doctoral dissertation). University of Wisconsin-Madison.

Zhang, Y., Feng, Y., Chatterjee, S., Tuske, S., Ho, M.X., Arnold, E., and Ebright, R.H. (2012). Structural basis of transcription initiation. Science *338*, 1076–1080.

Appendix B

Testing Direct Regulation by ppGpp and DksA of Promoters Involved in Lipid Metabolism

I performed some of this work in the laboratory of Emmanuelle Bouveret at the National Center for Scientific Research in Marseille, France.

Introduction

Decades of research have shown that in *E. coli* fatty acid (FA) biosynthesis is regulated at multiple levels to ensure that the ratio of fatty acid content to cell mass remains constant regardless of growth rate and that lipid composition matches the physiological requirements of the cell (reviewed in Magnuson et al., 1993). Consistent with its central role in maintaining cellular homeostasis in response to environmental conditions, ppGpp has been shown to play a role in regulating FA biosynthesis. Amino acid starvation results in inhibition of lipid accumulation concurrent with an increase in ppGpp levels (Pizer and Merlie, 1973), and FA starvation causes SpoT-dependent accumulation of ppGpp (Seyfzadeh et al., 1993).

SpoT has been shown to interact directly with Acyl Carrier Protein (ACP), a protein central to lipid metabolism (Magnuson et al., 1993), and this interaction is necessary for ppGpp accumulation in response to fatty acid starvation (Battesti and Bouveret, 2006). ppGpp also directly inhibits activity of the PlsB and FabA enzymes, which are involved in phospholipid and fatty acid biosynthesis (Heath et al., 1994; Stein and Bloch, 1976). Furthermore, ppGpp plays a role in regulating the stability of LpxC, an LPS biosynthetic enzyme, even though the mechanism by which this happens is still unclear (Schakermann et al., 2013).

A previous study showed that the *fabH* promoter, which transcribes the *fabHDG* operon, is inhibited during the strignet response (Podkovyrov and Larson, 1996). Global transcriptome experiments showed that expression of several genes involved in lipid metabolism are also inhibited during the stringent response (Durfee et al., 2008; Traxler et al., 2008). These results prompted preliminary experiments in our lab, both *in vivo* and *in vitro*, that suggested that multiple promoters for genes involved in lipid metabolism are directly regulated by ppGpp and DksA (Lemke, 2010). More recently it was shown that ppGpp directly inhibits the activity of the *fabH* and *fadR* promoters (My et al., 2013). The *fadR* gene codes for the FadR transcriptional regulator that has been shown to

activate transcription of most genes involved in fatty acid synthesis (My et al., 2015), suggesting ppGpp can indirectly affect synthesis of many fatty acid biosynthesis enzymes.

Experiments performed in our lab using RNAseq to detect global transcriptional responses during induction of ppGpp synthesis showed major effects on expression, both increasing and decreasing expression of multiple genes involved in fatty acid degradation, fatty acid synthesis, and phospholipid synthesis (Fig. B.1; Sanchez-Vazquez, P, and Gourse, R.L., *manuscript in preparation*). It was possible that some or most of these effects were indirect, so we revisited regulation of lipid metabolism genes by ppGpp and DksA *in vitro*.

Results

To test direct regulation of promoters involved in lipid metabolism I picked several promoters expressing genes that showed a large effect in the RNAseq data (Fig. B.1) and, using data for global mapping of transcription start sites (Thomason et al., 2015), cloned the promoters into the p770 transcription vector (Table B.1). I also used some of the lipid promoters cloned by Lemke (2010) and included *rrnB* P1 and P*lacUV5* as controls. All promoters except *ybhP*p produced a transcript in an *in vitro* transcription reaction (Fig. B.2). DksA and ppGpp activated transcription from the *clsA*p 2.6-fold (Fig. B.3), and transcription of *plsB*p2 was inhibited ~2-fold by ppGpp and DksA. However, because our control promoter P*lacUV5* was also inhibited ~2-fold in this experiment, we could not be certain if this represented physiologically relevant regulation. As expected, *rrnB* P1 was inhibited by ppGpp and DksA ~3-fold (Fig. B.3). Unfortunately, unexpected technical difficulties precluded obtaining usable data from the remaining lipid promoters.

Figure B.1

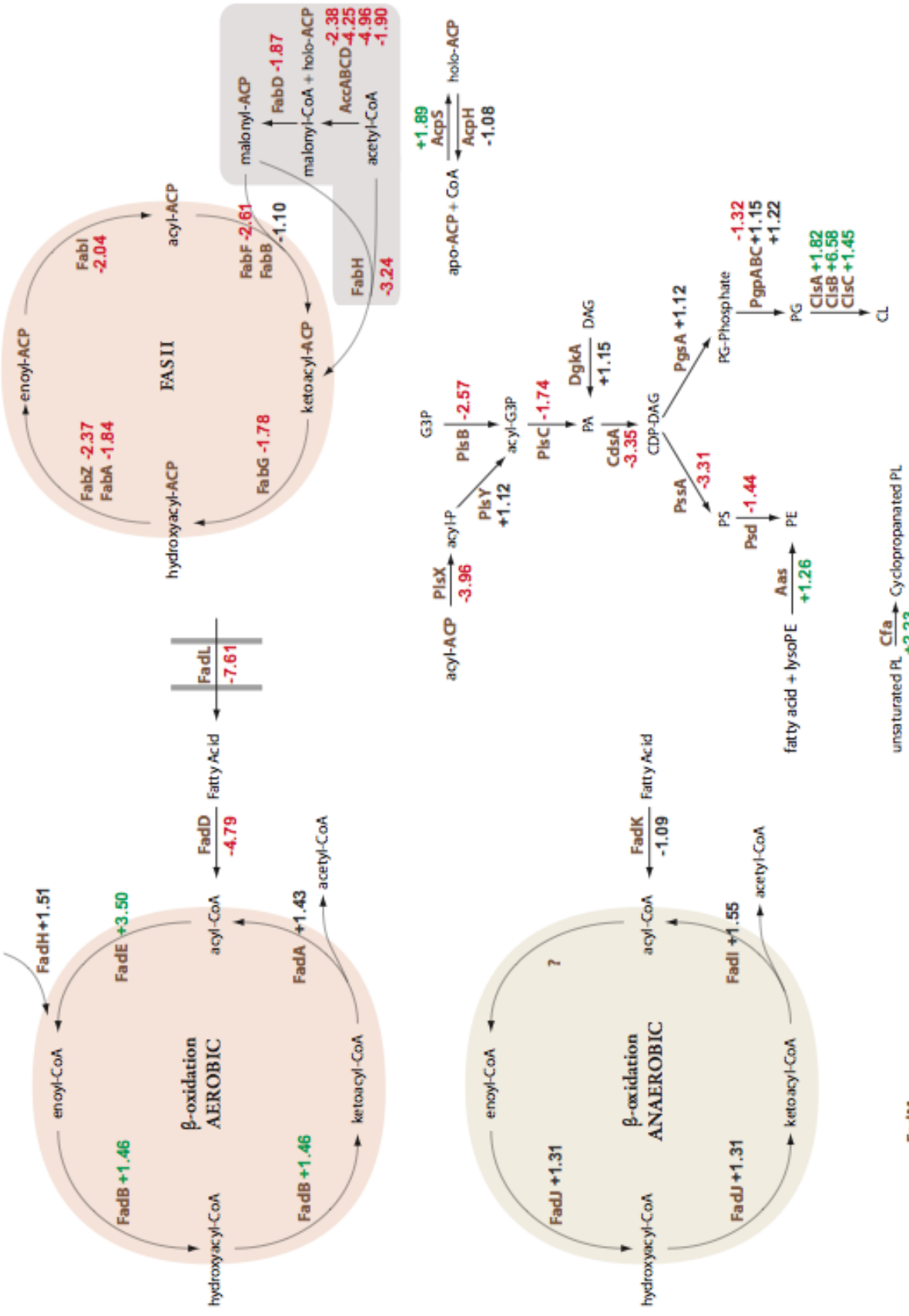
FASII
enoyl-ACP
hydroxyacyl-ACP
acyl-ACP
ketoacyl-ACP
FabI -2.04
FabZ -2.37
FabA -1.84
FabF -2.61
FabB -1.10
FabG -1.78
FabH -3.24
malonyl-ACP
malonyl-CoA + holo-ACP
acetyl-CoA
FabD -1.87
AccABCD -2.38 -4.25 -4.96 -1.90
+1.89 AcpS
AcpH -1.08
holo-ACP
apo-ACP + CoA
FadL -7.61
Fatty Acid
FadD -4.79
acyl-CoA
FadE +3.50
FadH +1.51
enoyl-CoA
β-oxidation AEROBIC
hydroxyacyl-CoA
FadB +1.46
FadA +1.43
FadB +1.46
ketoacyl-CoA
acetyl-CoA
G3P
PlsB -2.57
acyl-G3P
PlsY +1.12
acyl-ACP
PlsX -3.96
acyl-P
PlsC -1.74
PA
DgkA +1.15
DAG
CdsA -3.35
CDP-DAG
PgsA +1.12
PssA -3.31
PS
Psd -1.44
PE
fatty acid + lysoPE
Aas +1.26
PG-Phosphate
-1.32
PgpABC +1.15
+1.22
PG
ClsA +1.82
ClsB +6.58
ClsC +1.45
CL
unsaturated PL
Cfa +2.23
Cyclopropanated PL
FadK -1.09
Fatty Acid
acyl-CoA
?
enoyl-CoA
β-oxidation ANAEROBIC
hydroxyacyl-CoA
FadJ +1.31
FadJ +1.31
FadI +1.55
acetyl-CoA
ketoacyl-CoA
acyl-CoA
FadM +1.78
fatty acid + CoA

Figure B.1 ppGpp regulates expression of many genes involved in lipid metabolism. Starting at the top left and going clockwise, pathways for fatty acid degradation during aerobic growth, fatty acid synthesis through the FAS II pathway, synthesis of phospholipids and cardiolipin, and fatty acid degradation during anaerobic growth. The numbers represent the linear change in expression, either increasing or decreasing (green and red, respectively), during induction of ppGpp synthesis (Sanchez-Vazquez, P., and Gourse, R.L., *manuscript in preparation*). The colored numbers represent statistically significant changes, while the gray numbers are not statistically significant. Figure made by Emmanuelle Bouveret and modified by me.

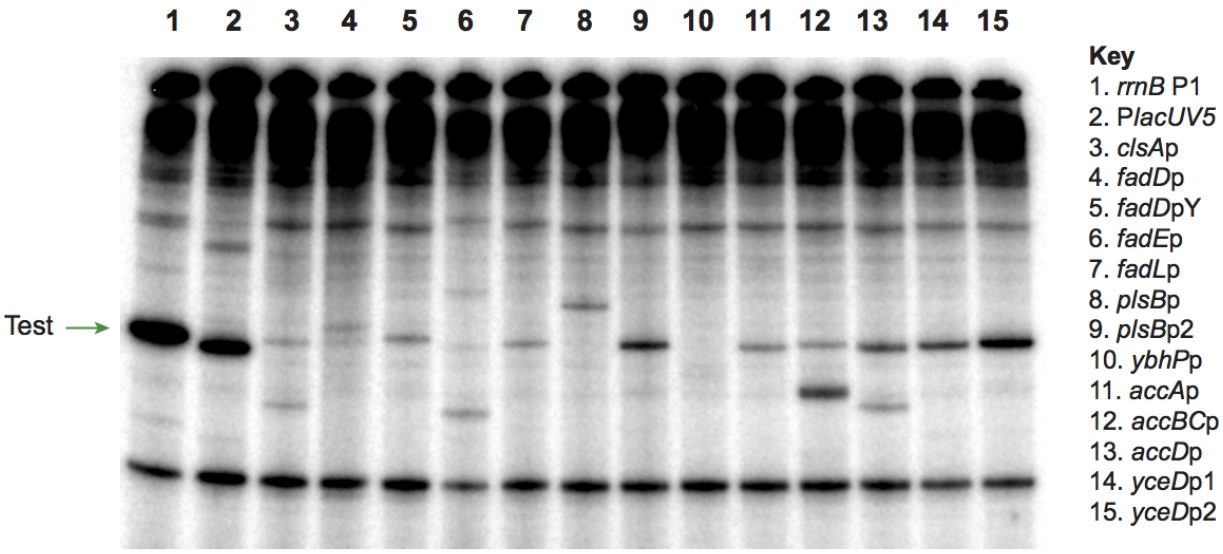

Figure B.2 Basal transcription *in vitro* from various lipid promoters. Gel showing transcripts from the indicated promoters performed as described in Materials and Methods.

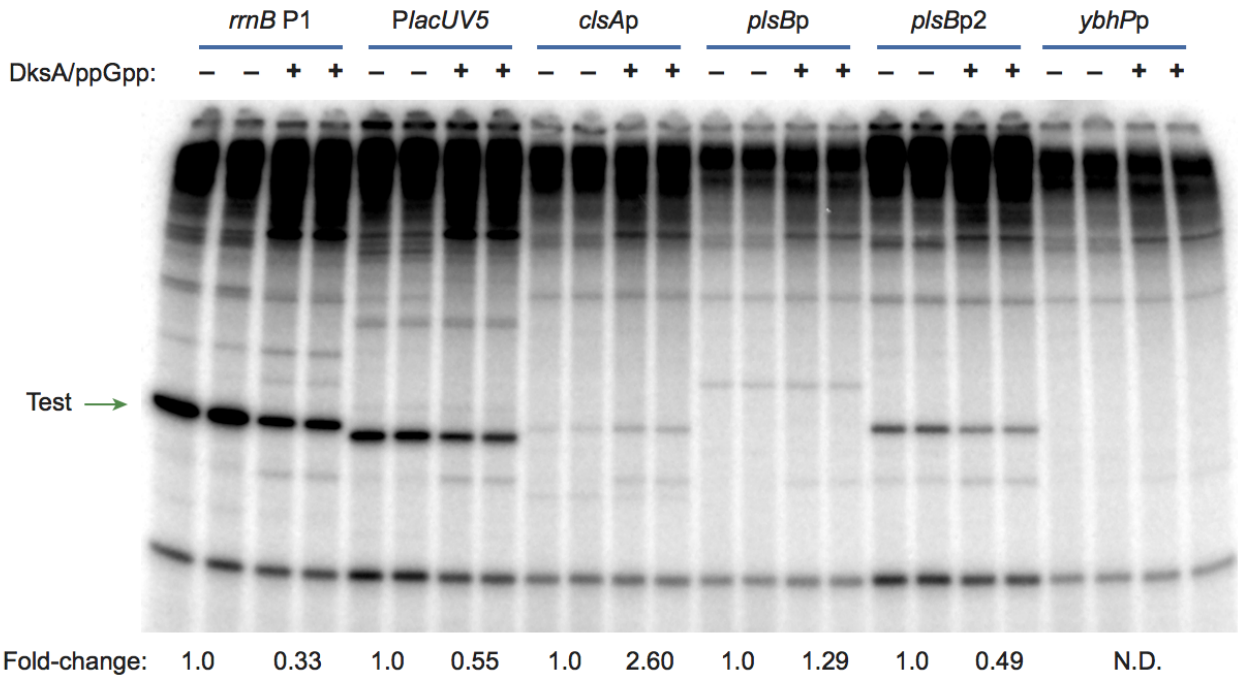

Figure B.3 DksA and ppGpp regulate activity of some promoters involved in lipid metabolism. Gel showing transcripts from the indicated promoters in the presence and absence of ppGpp and DksA, performed as described in Materials and Methods.

Discussion

A substantial literature has shown that lipid metabolism is under stringent control. To gain more information about the mechanism of this regulation I tested direct regulation of several promoters for lipid metabolism genes shown to be significantly regulated in response to ppGpp synthesis in an RNA-seq experiment (Fig. B.1). The results shown in this chapter suggest that at least some lipid promoters are regulated directly by DksA and ppGpp. Furthermore, regulation could be either positive or negative, as for *clsA*p and *plsB*p2, respectively (Fig. B.3). The *in vitro* results for these promoters were consistent with the direction of transcriptional regulation observed in the RNAseq data (compare Fig. B.3 with Fig. B.1). Taken together with the data from Lemke (2010), it appears that multiple promoters involved in lipid metabolism are regulated by ppGpp and DksA. Nonetheless, these results are preliminary and further studies are needed to better understand the role of ppGpp and DksA in regulating fatty acid content and composition during growth.

Materials and Methods

***In vitro* transcription.** The plasmids used in this chapter are listed in Table B.1. Multiple-round *in vitro* transcription reactions were performed essentially as described previously (Ross and Gourse, 2009). Reactions were carried out at 30°C for 10 min and contained 60 mM NaCl, 10 nM Eσ70 RNAP, 1 nM of supercoiled plasmid template, 200 μM of ATP, GTP, and CTP, 10 μM UTP, and ~2 μCi of [γ-^{32}P]UTP. When added, DksA was at 2 μM and ppGpp at 100 μM. Transcripts were resolved by electrophoresis in a 5.5% polyacrylamide, 7 M urea gel and visualized by phosphorimaging.

Table B.1 Plasmids used in this study

Plasmid	Relevant properties	Source
p770	Transcription vector	(Ross et al., 1990)
pRLG13065	p770 containing *rrnB* P1 (-88/+50)	(Ross et al., 1990)
pRLG11394	p770 containing P*lacUV5* (-59/+38)	(Chandrangsu et al., 2011)
pRLG15231	p770 containing *clsA*p (-100/+50)	This work
pRLG15232	p770 containing *fadD*p (-100/+50)	This work
pRLG15233	p770 containing *fadD*pY (-100/+50)	This work
pRLG15234	p770 containing *fadE*p (-100/+66)	This work
pRLG15235	p770 containing *fadL*p (-100/+50)	This work
pRLG15236	p770 containing *plsB*p (-100/+50)	This work
pRLG15237	p770 containing *plsB*p2 (-95/+50)	This work
pRLG15238	p770 containing *ybhP*p (-100/+50)	This work
pRLG9882	p770 containing *accA*p (-100/+50)	(Lemke, 2010)
pRLG9883	p770 containing *accBC*p (-100/+50)	(Lemke, 2010)
pRLG9884	p770 containing *accD*p (-100/+50)	(Lemke, 2010)
pRLG9887	p770 containing *yceD*p1 (-100/+50)	(Lemke, 2010)
pRLG9888	p770 containing *yceD*p2 (-100/+50)	(Lemke, 2010)

References

Battesti, A., and Bouveret, E. (2006). Acyl carrier protein/SpoT interaction, the switch linking SpoT-dependent stress response to fatty acid metabolism. Mol. Microbiol. *62*, 1048–1063.

Chandrangsu, P., Lemke, J.J., and Gourse, R.L. (2011). The dksA promoter is negatively feedback regulated by DksA and ppGpp. Mol. Microbiol. *80*, 1337–1348.

Durfee, T., Hansen, A.-M., Zhi, H., Blattner, F.R., and Jin, D.J. (2008). Transcription profiling of the stringent response in *Escherichia coli*. J. Bacteriol. *190*, 1084–1096.

Heath, R.J., Jackowski, S., and Rock, C.O. (1994). Guanosine tetraphosphate inhibition of fatty acid and phospholipid synthesis in *Escherichia coli* is relieved by overexpression of glycerol-3-phosphate acyltransferase (*plsB*). J. Biol. Chem. *269*, 26584–26590.

Lemke, J.J. (2010). Probing regulatory targets of DksA/ppGpp in *Escherichia coli* (Doctoral dissertation). University of Wisconsin-Madison.

Magnuson, K., Jackowski, S., Rock, C.O., and Cronan, J.E. (1993). Regulation of fatty acid biosynthesis in *Escherichia coli*. Microbiol. Rev. *57*, 522–542.

My, L., Ghandour Achkar, N., Viala, J.P., and Bouveret, E. (2015). Reassessment of the Genetic Regulation of Fatty Acid Synthesis in *Escherichia coli*: Global Positive Control by the Dual Functional Regulator FadR. J. Bacteriol. *197*, 1862–1872.

My, L., Rekoske, B., Lemke, J.J., Viala, J.P., Gourse, R.L., and Bouveret, E. (2013). Transcription of the *Escherichia coli* fatty acid synthesis operon *fabHDG* is directly activated by FadR and inhibited by ppGpp. J. Bacteriol. *195*, 3784–3795.

Pizer, L.I., and Merlie, J.P. (1973). Effect of serine hydroxamate on phospholipid synthesis in *Escherichia coli*. J. Bacteriol. *114*, 980–987.

Podkovyrov, S.M., and Larson, T.J. (1996). Identification of promoter and stringent regulation of transcription of the *fabH*, *fabD* and *fabG* genes encoding fatty acid biosynthetic enzymes of *Escherichia coli*. Nucleic Acids Res. *24*, 1747–1752.

Ross, W., Thompson, J.F., Newlands, J.T., and Gourse, R.L. (1990). *E. coli* Fis protein activates ribosomal RNA transcription *in vitro* and *in vivo*. Embo J. *9*, 3733–3742.

Ross, W., and Gourse, R.L. (2009). Analysis of RNA polymerase-promoter complex formation. Methods *47*, 13–24.

Schakermann, M., Langklotz, S., and Narberhaus, F. (2013). FtsH-Mediated Coordination of Lipopolysaccharide Biosynthesis in *Escherichia coli* Correlates with the Growth Rate and the Alarmone (p)ppGpp. J. Bacteriol. *195*, 1912–1919.

Seyfzadeh, M., Keener, J., and Nomura, M. (1993). *spoT*-dependent accumulation of guanosine tetraphosphate in response to fatty acid starvation in *Escherichia coli*. Proc. Natl. Acad. Sci. U S A *90*, 11004–11008.

Stein, J.P., and Bloch, K.E. (1976). Inhibition of *E. coli* beta-hydroxydecanoyl thioester dehydrase by ppGpp. Biochem. Biophys. Res. Commun. *73*, 881–884.

Thomason, M.K., Bischler, T., Eisenbart, S.K., Förstner, K.U., Zhang, A., Herbig, A., Nieselt, K., Sharma, C.M., and Storz, G. (2015). Global transcriptional start site mapping using differential RNA sequencing reveals novel antisense RNAs in *Escherichia coli*. J. Bacteriol. *197*, 18–28.

Traxler, M.F., Summers, S.M., Nguyen, H.-T., Zacharia, V.M., Hightower, G.A., Smith, J.T., and Conway, T. (2008). The global, ppGpp-mediated stringent response to amino acid starvation in *Escherichia coli*. Mol. Microbiol. *68*, 1128–1148.