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37/661 (2), Fort P.O.
Trivandrum-695 023
Kerala, India

Escherichia coli and *Bacillus subtilis*: The Frontiers of Molecular Microbiology Revisited, 2012: 179-196
ISBN: 978-81-308-0492-7 Editors: Yoshito Sadaie and Kouji Matsumoto

6. Regulation of gene expression

6-1. Stringent transcription control of *Escherichia coli* and *Bacillus subtilis*

Yasutaro Fujita, Shigeo Tojo and Kazutake Hirooka

Faculty of Life Science and Biotechnology, Fukuyama University, 985 Sanzo, Higashimura-cho
Fukuyama, Hiroshima 729-0292, Japan

Abstract. ppGpp and pppGpp, generally referred to as (p)ppGpp, are produced in cells of many bacteria when they encounter adverse environmental conditions such as amino acid limitation. The representative Gram-negative and -positive bacteria *Escherichia coli* and *Bacillus subtilis* adopt distinct strategies for stringent transcription control involving (p)ppGpp. *E. coli* RNA polymerase is a target for the (p)ppGpp interaction with it, (p)ppGpp thereby regulating stringent genes positively and negatively, depending on their specific promoter sequences. DksA enhances (p)ppGpp's effect in this bacterium. *rrn* transcription is likely enhanced through an increase of the *in vivo* ATP concentration, as the transcription-initiation nucleotide of most P1 promoters of *rrn* is ATP. In contrast, *B. subtilis* RNA polymerase is not a (p)ppGpp target. Instead, (p)ppGpp indirectly affects the transcription of stringent genes, most likely through inhibition of IMP dehydrogenase. This inhibition causes reciprocal changes of a GTP decrease and an ATP increase *in vivo*, resulting in the repression

Correspondence/Reprint request: Dr. Yasutaro Fujita, Faculty of Life Science and Biotechnology, Fukuyama University, 985 Sanzo, Higashimura-cho, Fukuyama, Hiroshima 729-0292, Japan
E-mail: yfujita@bt.fubt.fukuyama-u.ac.jp

and activation of the negative and positive stringent promoters whose transcription-initiation nucleotides are GTP and ATP, respectively. The addition of decoyinine, a GMP synthase inhibitor, also causes these reciprocal changes of the ATP and GTP concentrations. There are many positive stringent genes, such as *ilv-leu*, *pycA*, *kinA*, and *kinB*, whereas the many negative ones include *rrn*, *pts*, and *pdh*. Besides, the GTP decrease also causes derepression of members of the CodY regulon. Hence, the onset of the (p)ppGpp-dependent and CodY-dependent stringent controls is able to trigger massive sporulation under various sporulation-favorable medium conditions.

Introduction

To respond to changing environmental conditions, bacteria have evolved a large number of cellular regulatory mechanisms. One of the most-studied global regulatory systems in this context is the stringent response, originally characterized as the rapid down regulation of stable RNA synthesis during amino acid limitation in *Escherichia coli*. The hallmark of the stringent response is the accumulation of the effector molecule 5'-diphosphate 3'-diphosphate guanosine (ppGpp), which is derived from 5'-triphosphate 3'-diphosphate guanosine (pppGpp) through hydrolysis. Collectively, pppGpp and ppGpp are termed (p)ppGpp. Currently, we know that (p)ppGpp signals nutritional stress, leading to adjustment of gene expression in most bacteria and plants. However, the (p)ppGpp pool not only increases during amino acid limitation, but also in response to other types of nutrient limitation and factors causing growth arrest. Substantial details of the effects of (p)ppGpp on transcription regulation as well as bacterial physiology have been revealed [13, 45, 50, 68, 74], nevertheless, many aspects remain unknown even in the best-studied bacteria such as *E. coli* and *Bacillus subtilis*. Yet, these two bacteria appear to use different strategies to control stringent genes such as those involved in the synthesis of rRNA and amino acids at the transcription level [46, 95].

(p)ppGpp synthesis and breakdown in bacteria

In *E. coli*, the RelA and SpoT proteins are involved in stress-induced (p)ppGpp accumulation. RelA is a ribosome-associated (p)ppGpp synthase responding mainly to uncharged tRNAs that accumulate as a result of amino acid limitation [16,99] (Fig. 1A). SpoT is a bifunctional (p)ppGpp synthase and hydrolase, which presumably regulates the (p)ppGpp level in response to most conditions other than amino acid limitation, although the mechanism underlying SpoT activation is unknown [16,33,50]. In contrast, many other bacterial species including *B. subtilis* contain only one RelA-SpoT homologue, designated as Rel, which possesses both (p)ppGpp synthase and

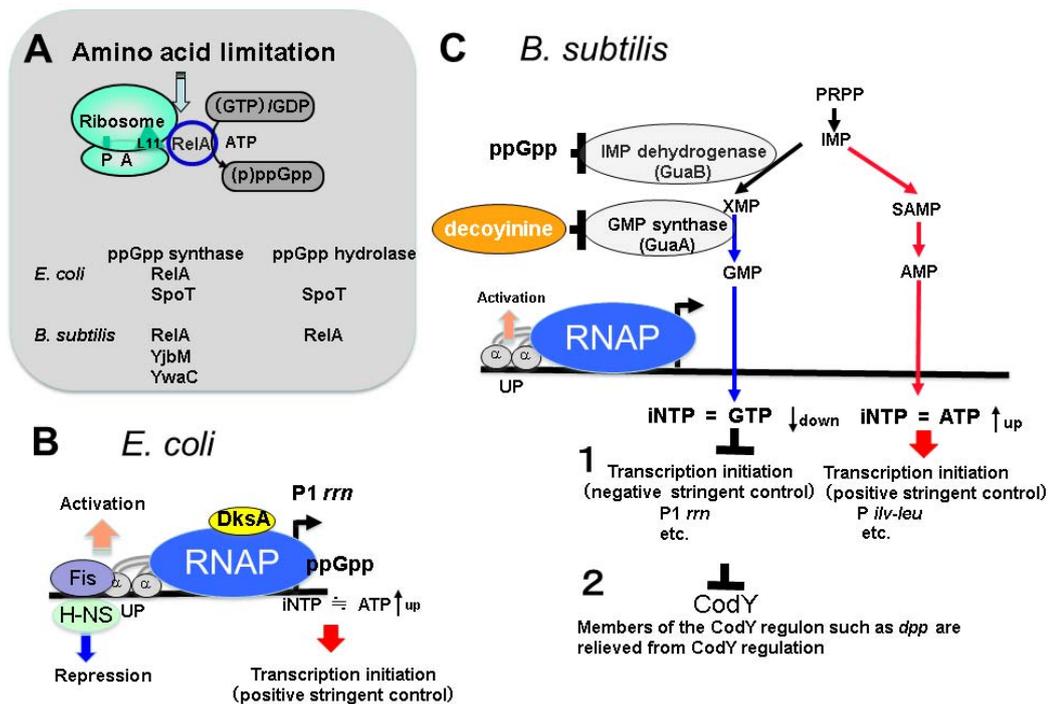


Figure 1. Distinct strategies for controlling stringent genes in *E. coli* and *B. subtilis*. **A.** Upon amino acid limitation, activation of RelA requires a cognate uncharged tRNA, a translating ribosome with an empty A site paused due to lack of a cognate charged tRNA, and ribosome-protein L11. Synthesis of (p)ppGpp from GDP or (GTP) involves pyrophosphoryl transfer from ATP. *E. coli* possesses RelA, which is only involved in (p)ppGpp synthesis, SpoT being engaged in both (p)ppGpp synthesis and hydrolysis, whereas *B. subtilis* possesses RelA, involved in both (p)ppGpp synthesis and hydrolysis, and two other (p)ppGpp synthases (YjbM and YwaC). **B.** In *E. coli*, (p)ppGpp interacts with RNAP to regulate its activity positively and negatively, depending on the target promoter sequence. DksK assists this activity regulation through (p)ppGpp. α CTD activates RNAP through its interaction with UP elements. Fis and H-NS activate and repress the RNAP activity, respectively. Besides, the activity of P1 promoters of *rrn*, whose transcription initiation bases are adenines, except for that of *rrnD*, increases when the concentration of an iNTP of ATP is elevated. **C.** (p)ppGpp does not interact with *B. subtilis* RNAP. Neither DksA, nor Fis and H-NS is involved in regulation of RNAP activity. Instead, (p)ppGpp is most likely involved in inhibition of IMP dehydrogenase. This inhibition results in the reciprocal changes of a GTP decrease and an ATP increase, which result in negative and positive regulation of numerous stringent promoters. Negative stringent promoters such as P1 *rrn* carry guanines at their transcription initiation sites [positions +1 and (or) +2], whereas the positive stringent promoters such as *Pilv-leu* carry adenines at such sites. Thus, the GTP decrease and ATP increase upon stringent response result in down regulation of negative stringent promoters and upregulation of positive stringent promoters, respectively. Decoyinine addition also evokes those reciprocal concentration changes of GTP and ATP. In addition, members of the CodY regulon such as *dpp* are relieved from CodY regulation due to the decrease in GTP, a corepressor of CodY.

hydrolase activity [57]. *B. subtilis* Rel is designated as RelA. Modulation of the (p)ppGpp level by such bifunctional enzymes occurs through two distinct active sites controlled by a reciprocal regulatory mechanism [4, 5, 42, 52, 53]. RelA-SpoT homologues have also been recently detected in plants [12, 34].

Recently, two genes of *B. subtilis*, *yjbM* and *ywaC*, were identified [60]. They code for a novel type of (p)ppGpp synthase that corresponds to the synthase domain of RelA-SpoT family members. These proteins appear to function in the biosynthesis of (p)ppGpp with a mode of action distinct from that of RelA-SpoT homologues.

Distinct strategies for controlling stringent genes in *E. coli* and *B. subtilis*

The numerous effects of (p)ppGpp on metabolism and physiology are complex and seem to differ a great deal in various organisms. Here, we focus on stringent transcription control, which is mediated by (p)ppGpp, in *E. coli* and *B. subtilis*.

1. (p)ppGpp action at the transcription level in *E. coli*

Inhibition of rRNA synthesis is the classical feature of the stringent response; many hypotheses have been put forward to explain this event. The understanding of (p)ppGpp regulation of transcription currently seems based on five key features (Fig. 1B): (a) shared characteristics of promoters affected by (p)ppGpp, (b) genetic and structural evidence that RNA polymerase (RNAP) is the target of (p)ppGpp, (c) the DksA protein enhances (p)ppGpp regulation, (d) regulation of *rrn* P1 promoters through interaction of the C-terminal domains of α subunits (α CTDs) with A + T-rich sequences adjacent to -35 hexamers (UP elements), and by Fis and H-NS, and (e) the stringent control also involves transcription initiation nucleoside triphosphates (iNTP). Moreover, most of these hypotheses are also applicable to the features of the positive transcription regulation of stringent genes such as those involved in amino acid synthesis.

(a) Characteristics of promoters affected by (p)ppGpp

One of the key elements of promoters that are inhibited by (p)ppGpp is the presence of a GC-rich discriminator, which is defined as the region between the -10 hexamer and position +1 (+1 is the transcription initiation base) [98]. In addition, the discriminator's activity is dependent on the -35 and -10 sequences, as well as the -10/-35 spacer length [65].

Promoters negatively controlled by (p)ppGpp have a 16-bp linker, in contrast with the general 17-bp consensus. Promoters that are activated by (p)ppGpp appear to have an AT-rich discriminator and longer linkers (for example, the *his* promoter linker is 18 bp long). There is also evidence that the sensitivity to supercoiling influences (p)ppGpp responses [24].

(b) RNAP is a target of (p)ppGpp

Although it is plausible that transcription of rRNA has to be controlled by (p)ppGpp during the stringent response, genetic evidence suggesting that RNAP is a target of (p)ppGpp came from the discovery that M⁺ mutants (also called stringent RNAP mutants) mimic in vitro and in vivo physiology and transcription control given by (p)ppGpp, even in its absence. The cross-linking of ppGpp to RNAP strengthened this notion, although different contacts were deduced [17, 93]. The structural details of the association between ppGpp and RNAP came from the investigation of cocrystals that positioned ppGpp in the secondary channel of RNAP near the catalytic center [3]. This channel provides access to the catalytic center for NTP substrates during polymerization and an entry point for derailed backtracked, nascent RNA in RNAP arrested during elongation. The (p)ppGpp target could be defined by direct contacts with appropriate RpoB and RpoC residues. Nevertheless, sequence changes in M⁺ mutants, chemical cross-linking, and cocrystallization indicate different target locations.

(c) DksA enhances regulation

DksA has various regulatory functions in addition to its ability to restore thermo-tolerance to a *dnaK* mutant when overexpressed [44]. Among these functions is a requirement for DksA and (p)ppGpp to stimulate the accumulation of RpoS during the early stationary phase of growth [14]. The regulatory interrelation between (p)ppGpp and DksA became clear when DksA was found to be necessary for the stringent response. This finding was followed by the discovery that DksA enhanced (p)ppGpp regulation generally in vitro and in vivo in studies of inhibition of a rRNA promoter or activation of selected amino acid biosynthetic promoters [66, 67]. Because DksA enhances (p)ppGpp's effect, whether inhibition or activation, it is called a (p)ppGpp cofactor. DksA binds in the secondary channel of *E. coli* RNAP [69], stabilizes the interaction of RNAP with ppGpp, and decreases the open complex lifetime, putting rRNA promoters into a kinetic range in vivo where they are susceptible to changes in the (p)ppGpp and iNTP concentrations [66].

(d) Regulation of *rrn* P1 promoters through interaction of α CTDs with UP elements, and by Fis and H-NS

Sequences upstream of the *rrn* P1 core promoters explain their exceptional strength, transcription being increased as much as 300-fold [36, 40]. UP elements bind to α CTDs of RNAP and account for most of this increase, the two stimulating the promoter activity 20- to 50-fold [19,40,78]. UP elements in *E. coli* promoters include binding sites (promoter proximal and distal subsites) for either one or both α CTDs [20]. The *rrn* P1 promoters possess good binding sites for both α CTDs, whereas the P2 promoters seem to have good binding sites for only α CTD at the proximal position [59,77]. Not only have the *rrn* P1 promoters evolved to possess α CTD binding sites, the sequences of which are relatively good matches to the UP element consensus sequence [19, 20], but also these UP element sequences are optimally positioned relative to the sigma binding site [55].

Fis acts as a classical activator of *rrn* P1 promoters, binding to specific sites upstream of the promoters and enhancing transcription. The level of Fis parallels the conditions when it is necessary for enhancement of rRNA transcription. The Fis concentration increases when cells are introduced into rich, fresh medium, remains high during exponential growth, and then decreases as cells enter the stationary phase [2,6,62]. The Fis level also increases proportionally with the growth rate [2], and transcription of the *fis* gene is inhibited by amino acid limitation [51,64].

The nucleoid-associated protein H-NS possesses some of the characteristics of a classic negative regulator. Its level is lowest when rRNA transcription is most active, and the H-NS concentration increases during entry into the stationary phase. Accordingly, it inhibits *rrn* P1 activity most during the stationary phase [1].

(e) (p)ppGpp-independent stringent control of *rrn* transcription involving iNTPs

When Cashel and others constructed a strain deficient in the biosynthetic genes for (p)ppGpp (*relA* and *spoT*), and it was found that this $\Delta relA \Delta spoT$ strain retained many of the properties of the wild-type strain as to regulation of rRNA and some tRNA promoters [8, 11, 31, 73], it became evident that (p)ppGpp was insufficient to explain the regulation of stable RNA transcription. This led to a search for additional regulators of *rrn* P1 promoter activity.

Promoters require higher concentrations of NTPs for initiation than for elongation, but *rrn* P1 promoters indeed require much higher concentrations

of their iNTPs than other promoters in order to attain maximal activity *in vitro* [30]. Six of the seven *rrn* P1 promoters initiate transcription with ATP nine bases downstream of the -10 hexamer, whereas the seventh (*rrnD* P1) initiates it with GTP.

Support for the hypothesis that the NTP concentration might not be saturating for rRNA promoters *in vivo* and thus might play a role in rRNA regulation originated from the isolation of RNAP mutants requiring higher concentrations of the iNTPs for transcription *in vitro* and of promoter mutants that no longer required high concentrations of NTPs for initiation *in vitro*. These RNAP and rRNA promoter mutants exhibited altered rRNA regulatory properties *in vivo* [7, 10, 30]. Furthermore, when ATP and GTP syntheses were uncoupled so that their concentrations no longer changed in parallel *in vivo*, the activities of *rrn* P1 promoters coincided with the identity of their iNTPs [79]. These experiments revealed that *rrn* P1 promoters had the potential to be regulated through changes in the iNTP concentration.

As noted for the models of regulation by (p)ppGpp, the short-lived nature of the open complex was proposed to be responsible for making rRNA promoters sensitive to the concentrations of their iNTPs [9, 30]. In this model, the iNTPs transiently increases the lifetime of the open complex, very greatly increasing transcription [8]. Alternatively, it has been proposed that the iNTPs cause a conformational change in RNAP, converting a “short-lived intermediate” that forms at rRNA promoters into a complex capable of transcription [47]. Anyway, the concentration of the first iNTP plays a major role in regulation. The concentration of a second iNTP can also influence transcription, in spite of a lesser extent [47].

2. (p)ppGpp indirectly regulates the stringent genes in *B. subtilis*

(a) Strategy for controlling rRNA synthesis in *B. subtilis*

B. subtilis rRNA core promoters contain -10 and -35 hexamers exhibiting excellent matches to the proposed consensus sequences for recognition by the σ subunit of RNAP, and in contrast to *E. coli* rRNA core promoters, they also contain sequences characteristic of extended -10 elements, have A-T-rich sequences between the -10 hexamer and position +1, and some have consensus -10/-35 spacer lengths (17 bp). These properties likely account for the intrinsic strength of *B. subtilis* rRNA promoters. That is, the stimulation of *B. subtilis* *rrn* core promoter activity by upstream sequences in *B. subtilis* is moderate in comparison to that in *E. coli*. No gene paralogous to *fis* was apparent on examination of the *B. subtilis* genome sequence. Most or all of the modest effect of the *rrn* upstream sequences could be due to α CTD-UP element interactions. UP elements are common in *B. subtilis* promoters [25,

38, 54]. DksA was recently identified as a key transcription factor for regulation of rRNA promoters in *E. coli* [66], but there are no *B. subtilis* paralogues exhibiting strong similarity to DksA.

The P1 and P2 promoters of the *B. subtilis* *rrnB* and *rrnO* operons initiate transcription with GTP [45]. All of the P1 and P2 promoters of the other *rrn* operons also initiate transcription with GTP [61]. The iNTP for transcription from *B. subtilis* rRNA promoters is GTP, which is in clear contrast to the case of *E. coli*. Changes in promoter activity always correlate with changes in the intracellular GTP concentration, and they are usually dependent on RelA [45]. In contrast to the situation for *E. coli*, where (p)ppGpp decreases rRNA promoter activity by directly inhibiting RNAP [7], (p)ppGpp does not inhibit *B. subtilis* RNAP directly [45]. Rather, an increase in the (p)ppGpp concentration reduces the available GTP pool, thereby modulating rRNA promoter activity indirectly, as described below.

A comparison of *rrn* P1 promoters from various bacterial species revealed two main types of sequences: (i) *B. subtilis*-like *rrn* P1 promoters with an A/T-rich region between -10 and +1 and, in many species, with a T at -5 [84]. This sequence type appears to be typical of Firmicutes. (ii) *E. coli*-like *rrn* P1 promoters with a G/C-rich region between -10 and +1 [98]. This sequence type appears to be specific to Gram-negative bacteria. Thus, the sequences of *rrn* P1 promoters of Gram-positive and -negative bacteria demonstrate that the same type of regulation involving iNTPs can be achieved with strikingly different sequences in phylogenetically distant species.

(b) Mechanisms underlying stringent transcription control in *B. subtilis*.

GTP and ATP are well known as gauges of the general energetic capacity and energy charge of cells, respectively. In *B. subtilis*, the GTP and ATP levels decrease and increase with the stringent response (e.g., amino acid limitation), respectively [46, 48, 63, 96], as illustrated in Fig. 1C. This change is mediated by (p)ppGpp synthesized by RelA upon stringent response [100], probably through its inhibition of IMP dehydrogenase, the first enzyme in the pathway leading to the biosynthesis of GTP [48]. This inhibition of GTP synthesis causes a decrease in the GTP level and the accumulation of IMP, which is also a precursor of ATP, resulting in the increase in the ATP level. These reciprocal changes in the ATP and GTP levels are also observed on treatment of *B. subtilis* cells with decoyinine [45, 49, 96], a GMP synthase inhibitor, even in a *relA*-deficient strain, because this inhibition is not mediated by (p)ppGpp [96].

The two mechanisms underlying the *B. subtilis* stringent transcription control are shown in Fig. 1C. The decrease in the GTP level with the

stringent response can be sensed by the CodY protein, a GTP-binding repressor of many genes, such as *dpp* encoding dipeptide permease [83] and *ilv-leu* encoding the enzymes for the synthesis of branched-chain amino acids [81, 82, 97], which are normally quiescent when cells are grown in a nutrient-rich medium [43, 58, 75]. CodY also functions as a transcriptional activator of certain genes such as *ackA* involved in acetate formation [80]. Thus, lowering of the GTP concentration inactivates the CodY protein, leading to deregulation of these genes. Another mechanism underlying stringent control has recently emerged. The reciprocal concentration changes in GTP and ATP, substrates of RNAP, can be sensed through the modulation of the rate of transcription-initiation of several stringent genes such as *rrn* [45], *ilv-leu*, *tufA* (encoding elongation factor Tu), and *ywaA* [46, 96]. This CodY-independent mechanism involves the purine base species, guanine and adenine, of the transcription initiation site [positions +1 and (or) +2], which is related to the negative and positive stringent control of *rrn*, and *ilv-leu*, *tufA*, and *ywaA*, respectively. The downregulation and upregulation of such genes are always correlated with the decrease and increase in the concentrations of GTP and ATP, which are the first iNTP and (or) second iNTP, respectively.

(c) Characterization and alignment of *B. subtilis* stringent promoters

DNA microarray analysis involving the wild-type and Δ *codY* strains grown with and without decoyinine revealed that the CodY-independent stringent control participates in the regulation of various operons including catabolic and anabolic ones [95]. In addition to *rrn*, several metabolic operons involved in critical stages of metabolic network, such as the *ptsGHI* encoding the glucose-specific phosphoenolpyruvate:sugar phosphotransferase system (PTS) [35, 92] and *pdhABCD* encoding the pyruvate dehydrogenase complex [32, 39], were severely downregulated, suggesting that they might be subject to negative CodY-independent stringent control [95]. In contrast, not only *ilv-leu* [37, 97], but also *pycA*, encoding pyruvate carboxylase [18, 95], and *alsSD* [76], responsible for the synthesis of acetoin from pyruvate, were well upregulated [95].

The nucleotide sequences of the promoter regions of the above *ptsGHI*, *pdhABCD*, *ilv-leu*, *pycA*, and *alsSD* operons together with those of the *rrnB*, *tufA*, *kinA*, and *kinB* promoters are aligned in Fig. 2. The guanines at positions +1 and (or) +2 of the negative stringent promoters (*P1rrnB*, *PtufA*, *PptsGHI*, and *PpdhABCD*) were replaced with adenines to convert negative stringent control to neutral or positive [45, 95], whereas the adenines at positions +1 and (or) +2 of the positive promoters (*Pilv-leu*, *PpycA*, and *PalsSD*) were replaced with guanines to convert positive stringent control to

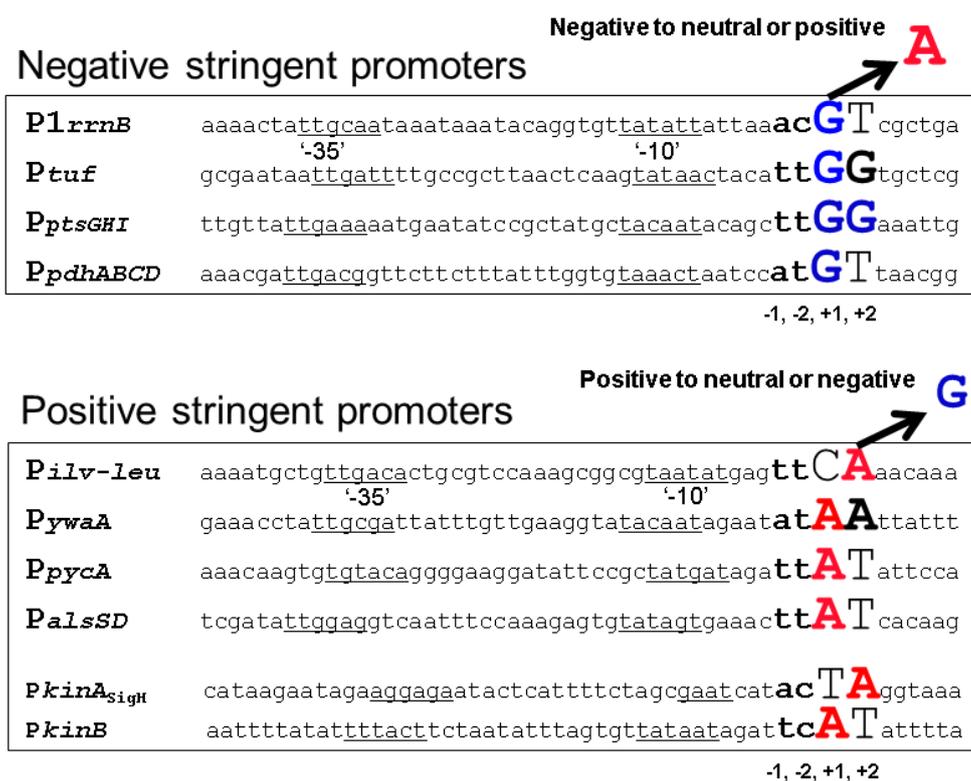


Figure 2. Regulation of *B. subtilis* stringent promoters depending on the base species at transcription initiation sites. The negative and positive stringent promoters listed are those in which purine bases at the respective transcription initiation sites [positions +1 and (or) +2] were actually replaced from guanine to adenine or vice versa, respectively; the negative ones are P1 *rrnB* [45], *PtuF* [46], *PptsGHI* [95], and *PpdhABCD* [95], and the positive ones are *Pilv-leu* [46, 96], *PywaA* [46], *PpycA* [95], *PalsSD* [95], and *PkinA* and *PkinB* (Tojo, S. and Fujita, Y., unpublished). The large capital Gs (guanines, blue) were replaced with As (adenines), which resulted in a change of transcription regulation from negative to neutral or positive, whereas the large bold-faced capital As (red) were replaced with Gs, which resulted in a change of transcription regulation from positive to neutral or negative. The bases at positions -1 and -2 are in large lower-case bold-faced letters.

neutral or negative [46, 95]. Furthermore, we also aligned the promoter sequences of the operons, the expression of whose constituent genes was found to be altered more than 10-fold upon decoyinine addition on DNA microarray analysis, and whose transcription initiation bases are known [95]. These base replacements and sequence alignments suggest that guanines and not adenines are located at positions +1 and (or) +2 for negative stringent control, and that adenines and not guanines are located at positions +1 and (or) +2 for positive stringent control. Moreover, positions of -1 and -2 are AT-rich (37 out of 48), especially T-rich (23 out of 48). These alignments imply that CodY-independent stringent control involving modulation of the

transcription initiation rate might occur for hundreds of stringent genes, which were detected on the above DNA microarray analysis, other than those investigated so far by us [95, 96] and by Krásný et al.[45, 46].

The presence of either guanine or adenine at the transcription initiation site [positions +1 and (or) +2] is likely indispensable for the negative and positive stringent control affecting the transcription initiation rate. Currently, we have no data showing that any other sequence conservation or requirement is necessary for this stringent control. Interestingly, the fact that the stringent transcription controls involving iNTP of *E. coli* and *B. subtilis* use the first iNTP and the first and second iNTPs, respectively, might be related to the difference in the stabilities of the open complexes of the two microorganisms previously noticed by Whipple and Sonenshein [101]. The open complex formed with *E. coli* RNAP before formation of the elongation complex for transcription is stable before formation of any phosphodiester bond, whereas the open complex formed with *B. subtilis* RNAP in the absence of nucleotide triphosphate is unstable. This implies that in *B. subtilis* a rate-limiting transcription initiation step might involve the formation of the first phosphodiester bond between the nucleotides at positions +1 and +2, which is assumed to be most affected by the concentrations of GTP and ATP, which change greatly when there is a stringent response. The details of the molecular mechanism underlying the stringent control affecting the transcription initiation rate, including the hypothesis described above, remain to be determined.

(d) Sporulation triggered by induction of stringent genes of *kinA* and *kinB* upon decoyinine addition

Entry into the sporulation pathway is governed by a member of the response regulator family of transcription factors known as Spo0A [41] (Fig. 3). Like other response regulators, Spo0A is activated by phosphorylation at an aspartyl residue located in the N-terminal portion of the protein, which enhances the capacity of Spo0A to bind its recognition sequence (0A-box) in DNA, via its DNA-binding domain in the C-terminal portion of the protein [87]. Most response regulators are phosphorylated directly by cognate sensor kinase that carries out autophosphorylation at a histidine residue and then transfers the phosphoryl group to the aspartyl residue in the response regulator. Spo0A, in contrast, is indirectly phosphorylated by a multicomponent phosphorelay system involving at least three kinases called KinA, KinB, and KinC [87] (Fig. 3); KinD and KinE are unlikely to be involved in this phosphorelay leading to form spores [27]. The kinases phosphorylate Spo0F, and the resulting Spo0F~P, in turn, transfers the

phosphoryl group to Spo0B. Finally, Spo0B~P transfers the phosphoryl group to, and thereby activates, Spo0A [15] (Fig. 3). An increased level of Spo0A~P results in repression of transcription of the *abrB* gene for AbrB [29], leading to the derepression of transcription of the *sigH* (*spo0H*) gene, encoding σ^H , an alternate σ subunit of RNAP as well as of *kinB* [28, 88, 90]. Accordingly, elevation of the concentration of σ^H RNAP leads to stimulation of the transcription of *kinA*, *spo0F*, and *spo0A* [41, 89] (Fig. 3). Additionally, Spo0A~P is required for the induced transcription of *spo0F* and *spo0A* [28, 90, 91], thereby setting up a self-reinforcing closed cycle.

However, the key unanswered question regarding the feedback regulation of the phosphorelay is what is the first component to be activated upon starvation triggering phosphorelay. Currently, GTP is known as the only metabolite whose intracellular level is monitored by a GTP-sensing repressor, CodY [75, 86]. When cells are growing under nutrient-rich conditions, the cellular GTP level is elevated, and genes under the control of CodY are repressed. Conversely, when cells have limited nutrients, the GTP level is low, resulting in the derepression of CodY-regulated genes. Actually, the *kinB* gene is a candidate target of CodY, as revealed on genome-wide transcript analysis [58]. However, CodY cannot be the primary factor initiating sporulation because a mutant lacking the GTP sensor does not exhibit massive sporulation during growth [75].

One of the difficulties in solving this problem is that there are many possible pathways that affect the activity of the sporulation initiation network in wild-type cells [85]. To overcome these limitations, a simplified approach, in which the network is decoupled into subsystems, was taken [23, 27]. This approach led to the finding that sporulation can be triggered with high efficiency in cells in the exponential phase of growth in rich medium and also in ones under starvation conditions by artificial induction of the synthesis of the histidine kinases with isopropyl- β -D-thiogalactopyranoside (IPTG) [22, 23, 27]. This suggests that the total histidine kinase activity of KinA, KinB, and KinC toward Spo0F was critical for triggering sporulation under any growth conditions.

Decoyinine was found to induce sporulation of cells exponentially growing in the presence of rapidly metabolizable carbon, nitrogen and phosphate sources in 1977 [56]. As described above, decoyinine causes the reciprocal concentration changes of a decrease in GTP and an increase in ATP, both being substrates of RNAP, by inhibiting GMP synthase. This can be sensed through the increases and decreases of the rate of transcription initiation from numerous stringent promoters depending on their first and second initiation bases, i.e., guanine and adenine, respectively (Fig. 1B). Our

DNA microarray analysis involving the *ΔcodY* strain with and without decoyinine [95] revealed that the *kinA* and *kinB* promoters are likely included in numerous positive stringent promoters that are inducible upon decoyinine addition, which have adenines in their transcription initiation sites (Fig. 2). The replacement of these adenines of *kinA* and *kinB* with guanines decreased their induction by decoyinine (Fig. 2) (Tojo, S., and Fujita, Y., unpublished observations). Furthermore, when the adenine either of *kinA* or *kinB* was replaced with guanine *in situ*, decoyinine could not induce sporulation any more. These findings strongly indicate that CodY-independent positive stringent control of *kinA* and *kinB* is involved in triggering of phosphorelay that eventually leads to the formation of spores without the involvement of any transcription regulators. In other words, sporulation is most likely triggered by the increase of ATP which is caused by nutrient limitation in sporulation-favorable medium conditions. The ATP increase is sensed by putting RNAP reaction involving ATP as an iNTP of *kinA* and *kinB* transcription into kinetic range, leading their activation to trigger phosphorelay to induce sporulation.

Conversely, phosphoryl groups are drained from the phosphorelay system mentioned above through the action of dedicated phosphatases; RapA, RapE, and RapB dephosphorylate Spo0F~P by sensing low cell density, and Spo0E, YisI, and YnzD dephosphorylate Spo0A~P [71, 72] (Fig. 3). The opposing actions of the kinases and the phosphatases are believed to integrate environmental and physiological signals for the decision to sporulate by governing flux through the relay system and hence the level of Spo0A~P, which must reach a threshold concentration to trigger sporulation [26]. Therefore, negative regulation of CodY-dependent and -independent stringent genes, which is evoked by stringent conditions such as decoyinine addition, contributes to the inhibition of dephosphorylation of Spo0F~P and Spo0A~P through decreases or inhibition of the phosphatases.

We assume that this positive and negative stringent control is evoked by the reciprocal changes of a GTP decrease and an ATP increase triggers sporulation, the changes being caused by the inhibition of IMP dehydrogenase by (p)ppGpp or GMP synthase by decoyinine, or through alteration of purine metabolism due to unknown nutrient limitation, and it most likely operates not only in amino acid limitation but also in various starvation conditions to induce sporulation. However, it is notable that the efficiency of the induction of sporulation, which is induced by the stringent response, likely depends on the degree of the down regulation of the phosphatases that interfere the phosphorelay system that leads to the formation of spores, which is affected by the medium constituents; for example, decoyinine addition did not cause massive sporulation in the

presence of good nitrogen sources such as ammonium and glutamate or glutamine in the medium in spite of sufficient induction of *kinA* and *kinB* to likely induce sporulation (Tojo, S. and Fujita, Y., unpublished observations).

Acknowledgments

Recent research in the laboratory of the corresponding author was supported by a Grant-in-Aid for Scientific Research (B) and a Strategic Support Project of Research Infrastructure Formation for Private Universities, from the Ministry of Education, Culture, Sports, Science, and Technology of Japan.

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