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# Expression of *kinA* and *kinB* of *Bacillus subtilis*, Necessary for Sporulation Initiation, Is under Positive Stringent Transcription Control

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***Bacillus subtilis*** **cells were exposed to decoyinine to trigger stringent transcription control through inhibition of GMP synthase; amino acid starvation results in the same control through inhibition of GMP kinase by 5′-diphosphate 3′-diphosphate guanosine. The positive and negative transcription control of the stringent genes involves adenine and guanine at the transcription initiation sites, whereby they sense an increase and a decrease in the *in vivo* ATP and GTP pools, respectively. Decoyinine also induces sporulation in minimum medium. DNA microarray analysis revealed that decoyinine induced two major sensor kinase genes, *kinA* and *kinB*, involved in the phosphorelay leading to spore formation. *lacZ* fusion experiments involving the core promoter regions of *kinA* and *kinB*, whose transcription initiation bases are adenines, indicated that decoyinine induced their expression. This induction was independent of CodY and AbrB. When the adenines were replaced with guanines or cytosines, the induction by decoyinine decreased. The *in situ* replacement of the adenines with guanines actually affected this decoyinine-induced sporulation as well as massive sporulation in nutrient medium. These results imply that operation of the positive stringent transcription control of *kinA* and *kinB*, which is mediated by an increase in the ATP pool, is likely a prerequisite for the phosphorelay to transfer the phosphoryl group to Spo0A to initiate sporulation.**

Entry into the sporulation pathway is governed by a member of the response regulator family of transcription factors known as Spo0A (1) (Fig. 1). Most response regulators are phosphorylated directly by the respective cognate sensor kinases that carry out autophosphorylation at a histidine residue and then transfer a phosphoryl group to an aspartyl residue in the response regulator. Spo0A, in contrast, is indirectly phosphorylated by a multicomponent phosphorelay system involving at least two kinases called KinA and KinB (9). 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 (Fig. 1) (10). An increased level of Spo0A∼P results in repression of transcription of the *abrB* gene for AbrB (11), leading to derepression of transcription of the *sigH* (*spo0H*) gene, encoding $\sigma^H$, an alternative $\sigma$ subunit of RNA polymerase (RNAP), as well as of *kinB* (12–14). Accordingly, elevation of the concentration of $\sigma^H$ RNAP leads to triggering of the transcription of *kinA*, *spo0F*, and *spo0A* (Fig. 1) (1, 15). Additionally, Spo0A∼P is required for the induced transcription of *spo0F* and *spo0A* (12, 14, 16), thereby setting up a self-reinforcing closed cycle.

However, the key unresolved issue regarding the feedback regulation of the phosphorelay is identification of the first component to be activated upon starvation triggering phosphorelay. Currently, GTP is known as a metabolite whose intracellular level is monitored by a GTP-sensing repressor, CodY (17, 18). When cells are grown under nutrient-rich conditions, the cellular GTP level is elevated, and the 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 by genome-wide transcript analysis (5). However, CodY itself cannot be the primary factor initiating sporulation because a mutant lacking it does not exhibit massive sporulation during growth under usual medium conditions (17).

More than a quarter of a century ago, decoyinine was found to induce sporulation of *Bacillus subtilis* cells exponentially growing in the presence of rapidly metabolizable carbon, nitrogen, and phosphate sources (19). Decoyinine causes the reciprocal concentration changes of a decrease in GTP and an increase in ATP (Fig. 2A), both being substrates of RNAP, by inhibiting GMP synthase (21, 22, 24, 26). (The changes of the *in vivo* NTP concentrations after decoyinine addition are shown in Fig. 2B [24]; the changes of the CTP and UTP concentrations were less than 2-fold, which are relatively tolerant to decoyinine addition [24].) These reciprocal concentration changes of GTP and ATP were supposed to be also evoked by inhibition of IMP dehydrogenase by 5′-diphosphate 3′-diphosphate guanosine (ppGpp), whose synthesis is triggered by a stringent response (27, 28). However, it was very recently shown that ppGpp does not inhibit IMP dehydrogenase efficiently, but it severely inhibits GMP kinase to convert GMP to GDP, resulting in the decrease of the *in vivo* GTP concentration (20). It is known that the stringent response also induces sporulation (28, 29). These reciprocal changes can be sensed through increases and decreases in the rate of transcription initiation from numerous positive and negative stringent promoters depending on their first and/or second initiation bases at nucleotide + 1 and

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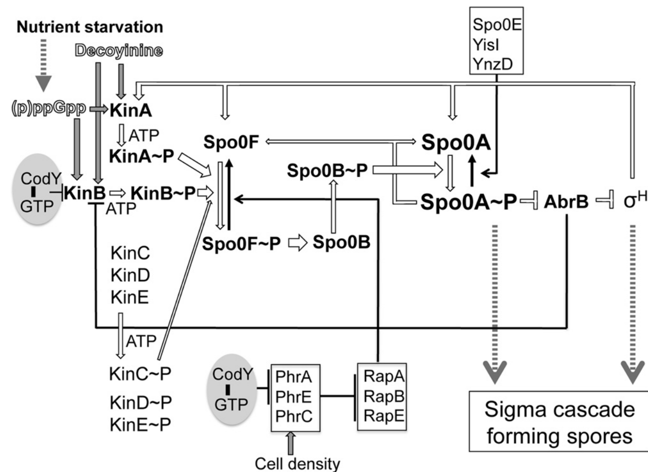


**FIG 1** Phosphorelay regulatory network leading to the formation of spores in *B. subtilis*. Upon nutrient starvation or decoyinine addition, two major sensor kinases (KinA and KinB) undergo autophosphorylation. In addition, three minor sensor kinases (KinC, KinD, and KinE) are considered to be involved in the phosphorylation of Spo0F (2). However, it was recently reported that not only KinC but also KinD and KinE are unlikely to be involved in this phosphorylation (3, 4). KinA∼P and KinB∼P provide phosphate input to the master transcriptional regulator, Spo0A, yielding Spo0A∼P via two additional regulators, i.e., the phosphorylated forms of Spo0F and Spo0B (Spo0F∼P and Spo0B∼P). Spo0A∼P becomes a positive or negative regulator for sporulation genes, including those for Spo0A itself, Spo0F, and the transition state transcription regulator AbrB. AbrB represses the transcription of the gene of $\sigma^H$, which is also essential for sporulation, as well as that of *kinB*. Thus, Spo0A∼P represses *abrB*, thereby stimulating $\sigma^H$ synthesis. *kinA* is transcribed with RNAP possessing $\sigma^H$. As a result, the transcription of the genes for KinA, Spo0F, and Spo0A is triggered in a closed-loop system. The accumulation of Spo0A∼P and $\sigma^H$ leads to the sigma cascade to form spores. The *kinB* gene is likely a target of CodY (5). The cell density is sensed by Phr peptides that are secreted, processed, and imported as pentapeptides back into the cell, where they inhibit the Rap proteins (RapA, RapB, and RapE) that cause dephosphorylation of Spo0F∼P. The *phrA* and *phrE* genes are CodY candidate targets (5). Spo0A∼P is susceptible to dephosphorylation through the action of Spo0E (6, 7) and two homologues, YisI and YnzD (8); expression of the last two proteins increases under nonsporulation conditions (8). Open and gray arrows and black arrows indicate forward and backward sporulation, respectively.

+2, i.e., adenine and guanine, respectively (Fig. 2). Therefore, this stringent transcription control involves no transcriptional regulators. Our DNA microarray analysis involving the Δ*codY* strain with and without decoyinine (22) revealed that the *kinA* and *kinB* promoters are likely included in numerous positive stringent promoters that are inducible upon decoyinine addition and possess adenines at their transcription initiation nucleotides (30, 31).

We show in this communication that the replacement of these adenines of *kinA* and *kinB* with guanines or cytosines decreased their induction by decoyinine. Furthermore, when the adenine of either *kinA* or *kinB* was replaced with a guanine *in situ*, decoyinine-induced sporulation as well as massive sporulation in nutrient medium was well affected. These findings imply that the operation of the positive stringent control of transcription of *kinA* and *kinB*, which is enhanced by an increase in an RNAP substrate of ATP, is likely a prerequsite for the phosphorelay to transfer the phosphate group to Spo0A that eventually leads to the formation of spores.

## MATERIALS AND METHODS

**Bacterial strains and their construction.** The *B. subtilis* strains used in this work are listed in Table 1. The Δ*abrB*::*erm* strain FU1106 was constructed by means of recombinant PCR as follows. The regions upstream and downstream of the *abrB* gene were first amplified by PCR using DNA of the wild-type strain 168 as the template and primer pairs ABu-F/ABu-R and ABd-F/ABd-R (see Table S1 in the supplemental material), respectively. The *erm* cassette was amplified by PCR using DNA of plasmid pMUTIN2 (37) as the template and primer pair EM-F/EM-R. Second, recombinant PCR using primer pair ABu-F/ABd-R and three PCR fragments resulted in a PCR product covering the region upstream of *abrB*, the *erm* gene, and the region downstream of *abrB*. The resultant recombinant PCR product was used to transform strain 168 to erythromycin resistance (0.3 μg/ml) on tryptose blood agar base (Difco) plus 10 mM glucose (TBABG) plates to produce the Δ*abrB*::*erm* strain FU1106. The Δ*kinA*::*erm* and Δ*kinB*::*kan* strains FU1095 and FU1096 were provided by T. Sato (Hosei University) and K. Kobayashi (Nara Institute of Science and Technology), respectively. The Δ*kinA*::*erm* strain FU1095 was transformed to kanamycin resistance (5 μg/ml) with DNA of the Δ*kinB*::*kan* strain FU1096 to produce the Δ*kinA*::*erm* Δ*kinB*::*kan* strain FU1098. The *gid*::*spc* Δ*codY* strain PS37t was obtained by transformation of wild-type strain 168 with DNA of the *gid*::*spc* Δ*codY* strain PS37 to spectinomycin resistance (60 μg/ml). The *gid*::*spc* Δ*codY* Δ*kinA* and *gid*::*spc* Δ*codY* Δ*kinB* strains FU1063 and FU1064 were obtained by transformation of the *gid*::*spc* Δ*codY* strain PS37t with DNAs of the Δ*kinA* and Δ*kinB* strains FU1095 and FU1096 to erythromycin and kanamycin resistance, respectively. The presence of Δ*codY* in strains PS37t, FU1063, and FU1064 was confirmed by the appearance of the PCR product that was 250 bp shorter than that obtained for the $CodY^+$ strain, as described previously (38). Disruption of the *gid* gene, present in the Δ*codY* strain, did not affect the expression of the target genes in this work or sporulation efficiency.

To construct transcriptional promoter-*lacZ* fusion strains with the *kinA* and *kinB* promoters, their core regions comprising nucleotides −55 to +10 of *kinA* (30) and *kinB* (31) (nucleotide +1 is the transcription initiation nucleotide) were amplified using primer pairs KA-F1/KA-R1 and KB-F1/KB-R1 (Table S1) and DNA of wild-type strain 168 as the template, respectively. The PCR products were trimmed with XbaI and BamHI and then ligated with the XbaI-BamHI arm of plasmid pCRE-test2 (39). The ligated DNAs were used for transformation of *Escherichia coli* strain DH5α to ampicillin resistance (50 μg/ml) on Luria-Bertani medium plates (40). Correct construction of the fusions in the resulting plasmids was confirmed by DNA sequencing. The plasmids carrying the promoter regions of *kinA* and *kinB* were linearized with PstI and then used for double-crossover transformation of strain 168 to chloramphenicol resistance (5 μg/ml) on TBABG plates, which produced the $P_{kinA}$-*lacZ* and $P_{kinB}$-*lacZ* strains FU1087 and FU1115, respectively.

To construct strains carrying the *kinA* and *kinB* promoter-*lacZ* fusions with guanine or cytosine substitutions at nucleotide +1, the core promoter regions (nucleotides −55 to +10) were amplified using primer pairs KA-F1/KA-R1g and KB-F1/KB-R1g or KA-F1/KA-R1c and KB-F1/KB-R1c (Table S1), respectively, and chromosomal DNA of wild-type strain 168 as the template. The PCR products trimmed with XbaI and BamHI were cloned into plasmid pCRE-test2 in *E. coli* strain DH5α, as described above. Correct construction of the fusions in the resulting plasmids was confirmed by DNA sequencing. The plasmids that had a guanine or cytosine substitution at nucleotide +1 were each linearized with PstI and then used for transformation of strain 168, resulting in the $P_{kinA}$(A + 1G)-*lacZ* and $P_{kinB}$(A + 1G)-*lacZ* strains FU1088 and FU1116 or the $P_{kinA}$(A + 1C)-*lacZ* and $P_{kinB}$ (A + 1C)-*lacZ* strains FU1153 and FU1154, respectively.

To introduce Δ*spo0H*, Δ*abrB*, Δ*codY*, and Δ*spo0A* into the $P_{kinA}$-*lacZ*, $P_{kinB}$-*lacZ*, $P_{kinA}$ (A + 1G)-*lacZ*, and $P_{kinB}$(A + 1G)-*lacZ* strains FU1087, FU1115, FU1088, and FU1116, they were transformed with DNAs of the Δ*spo0H*::*erm*, Δ*abrB*::*erm*, *gid*::*spc* Δ*codY*, and Δ*spo0A*::*spc* strains ASK201, FU1106, PS37, and FU1121 to erythromycin and spectinomycin resistance, yielding the Δ*spo0H* strains FU1107, FU1117, FU1108, and FU1118; the Δ*abrB* strains FU1111, FU1122, FU1112, and FU1123; the Δ*codY* strains FU1109, FU1119, FU1110, and FU1120; and the Δ*spo0A*

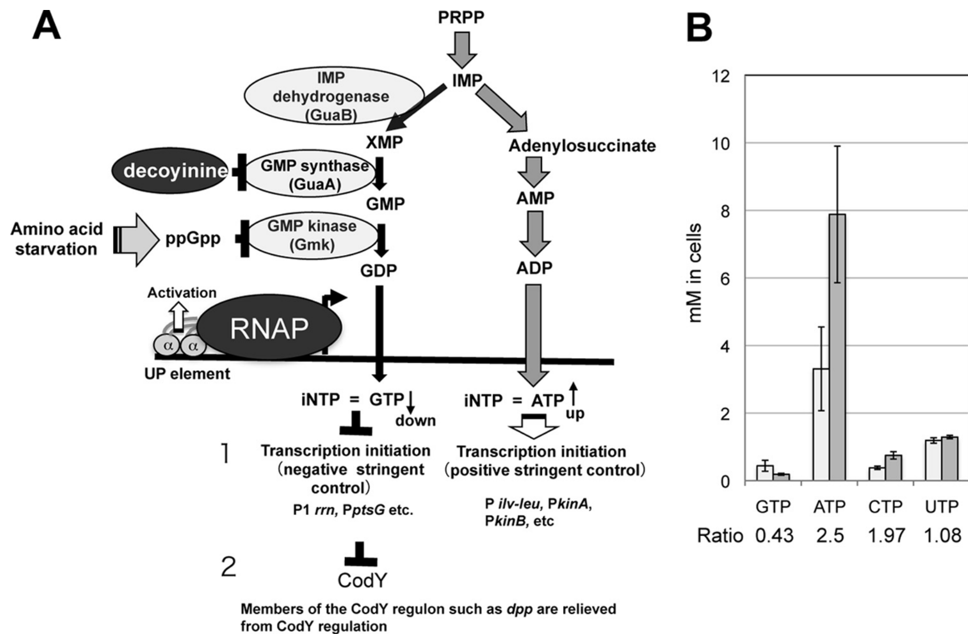


**FIG 2** Molecular mechanism underlying stringent transcription control in *B. subtilis*. (A) Illustration of its molecular mechanism. ppGpp is synthesized upon amino acid starvation, which is involved in inhibition of GMP kinase rather than IMP dehydrogenase (20). This inhibition results in the reciprocal 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$_{rrn}$ (21) and P$_{ptsGHI}$ (22) have guanines at their transcription initiation sites (nucleotides +1 and/or +2), whereas positive stringent promoters such as P$_{ilv-leu}$ (23, 24), P$_{kinA}$, and P$_{kinB}$ have adenines at these sites. So, the iNTPs are GTP and ATP for the negative and positive stringent promoters. Thus, the GTP decrease and ATP increase upon the stringent response result in downregulation of negative stringent promoters and upregulation of positive ones, respectively. The addition of decoyinine, a GMP synthase inhibitor, also evokes these reciprocal concentration changes of GTP and ATP. In addition, members of the CodY regulon such as *dpp* (25) are relieved from CodY regulation due to the decrease in GTP, a corepressor of CodY. Phosphoribosyl pyrophosphate (PRPP), IMP, and XMP are intermediates of purine biosynthesis. (B) *In vivo* concentration changes of NTP upon the stringent response in *B. subtilis*. *In vivo* NTP concentrations changed from the initial values (light gray bars) to the ones shown 30 min after decoyinine addition (darker gray bars); these values determined, by use of capillary electrophoresis mass spectrometry, as well as their standard deviations were taken from Table 3 in our previous publication (24). Ratios of values obtained with and without decoyinine are shown.

strains FU1124, FU1126, FU1125, and FU1127, respectively. The presence of Δ*codY* in strains FU1109, FU1119, FU1110, and FU1120 was confirmed as described above.

*In situ* replacement of adenines at nucleotide +1 of the *kinA* and *kinB* promoters with guanines or cytosines was carried out by means of positive selection using the *mazF* gene (36) (see Fig. S1 in the supplemental material). First, three PCR fragments carrying the spectinomycin resistance gene (*spc*), *lacI*, and *mazF* under the control of P$_{spac}$, and the respective *kinA* regions upstream and downstream of its promoter containing guanine instead of adenine at the *kinA* transcription initiation nucleotide, were prepared by means of PCRs using DNA of the *aprE*::(*spc lacI* P$_{spac}$-*mazF*) strain TM0310 as the template and primer pairs MF-F/MF-R, KA-F2/KA-R2g or KA-F2/KA-R2c, and KA-F3g/KA-R3 or KA-F3c/KA-R3 (Table S1), respectively. Second, recombinant PCR to combine the three fragments with themselves as templates and primer pair KA-F2/KA-R3 was carried out. Then, wild-type strain 168 was transformed to spectinomycin resistance (60 μg/ml) with the recombinant PCR fragment comprising the three fragments. The resulting spectinomycin-resistant cells were treated with isopropyl-β-D-thiogalactopyranoside (IPTG), and the IPTG-resistant and spectinomycin-sensitive transformants, which had resulted from the self-recombination between the short repeated regions including guanine and cytosine at the *kinA* transcription initiation site, were the *kinA* (A + 1G) strain FU1102 and the *kinA* (A + 1C) strain FU1156. Correct construction of the *kinA* (A + 1G) strain FU1102 and the *kinA* (A + 1C) strain FU1156 was confirmed by DNA sequencing. Similarly, the *kinB* (A + 1G) strain FU1113 was obtained by means of the same positive selection as described above, the first PCR resulting in the three fragments carrying the *spc*, *lacI*, and P$_{spac}$-*mazF* and the *kinB* regions upstream and downstream of its promoter containing guanine instead of adenine at the *kinB* transcription initiation site, using primer pairs MF-F/MF-R, KB-F2/KB-R2g, and KB-F3g/KB-R3 (Table S1), respectively, and the second recombinant PCR involving them as the templates and primer pair KB-F2/KB-R3. Although we attempted to similarly isolate the *kinB* (A + 1C) strain using primer pairs KB-F2/KB-R2c and KB-F3c/KB-R3 (Table S1) for the first PCR, we could not obtain the right *kinB* (A + 1C) strain due to the occurrence of various unexpected base substitutions and deletions in the *kinB* promoter region during the self-recombination to result in the IPTG-resistant and spectinomycin-sensitive transformants. Moreover, the *kinA* (A + 1G) strain FU1102, *kinA* (A + 1C) strain FU1156, and *kinB* (A + 1G) strain FU1113 were transformed with DNAs of the Δ*kinB*::*kan* and Δ*kinA*::*erm* strains FU1096 and FU1095 to kanamycin resistance (5 μg/ml) and erythromycin resistance (0.3 μg/ml) to yield the *kinA* (A + 1G) Δ*kinB* strain FU1103, *kinA* (A + 1C) Δ*kinB* strain FU1170, and *kinB* (A + 1G) Δ*kinA* strain FU1114, respectively.

The *kinA* (A + 1G) *kinB* (A + 1G) strain FU1155 was constructed as follows. The *kinA* (A + 1G) strain was transformed with the PCR product amplified by use of primer pair KB-F2/KB-R3 and a template DNA of an intermediate strain during the construction of the *kinB* (A + 1G) strain FU1102, which carries the *mazF* cassette (*spc lacI* P$_{spac}$-*mazF*) between the P$_{kinB}$ (A + 1G) repeated regions to result in spectinomycin resistance (60 μg/ml). The spectinomycin-resistant cells were treated with IPTG, and one of the IPTG-resistant and spectinomycin-sensitive transformants was the *kinA* (A + 1G) *kinB* (A + 1G) strain FU1155. Correct construction of strain FU1155 was confirmed by DNA sequencing.

**Cell cultivation, β-Gal assay, and spore titer.** The *lacZ* fusion strains were grown at 30°C overnight on TBABG plates containing the appropri-

**TABLE 1** *B. subtilis* strains used in this work

| Strain | Genotype | Reference or source |
|---|---|---|
| 168 | *trpC2* | 32 |
| PS37 | *trpC2 gid*::*spc* Δ*codY* | 33 |
| PS37t | *trpC2 gid*::*spc* Δ*codY* | This work |
| FU1095 | *trpC2* Δ*kinA*::*erm* | T. Sato, this work |
| FU1096 | *trpC2* Δ*kinB*::*kan* | K. Kobayashi, this work |
| FU1063 | *trpC2 gid*::*spc* Δ*codY* Δ*kinA*::*erm* | This work |
| FU1064 | *trpC2 gid*::*spc* Δ*codY* Δ*kinB*::*kan* | This work |
| FU1098 | *trpC2* Δ*kinA*::*erm* Δ*kinB*::*kan* | This work |
| ASK201 | *trpC2* Δ*spo0H*::*erm* | 34 |
| FU1121 | *trpC2* Δ*spo0A*::*spc* | 35 |
| TM0310 | *trpC2 aprE*::[*spc lacI* $P_{spac}$-*mazF*] | 36 |
| FU1106 | *trpC2* Δ*abrB*::*erm* | This work |
| FU1087 | *trpC2 amyE*::[*cat* $P_{kinA}$ (−55/+10)-*lacZ*] | This work |
| FU1088 | *trpC2 amyE*::[*cat* $P_{kinA}$ (−55/+10)(A + 1G)-*lacZ*] | This work |
| FU1115 | *trpC2 amyE*::[*cat* $P_{kinB}$ (−55/+10)-*lacZ*] | This work |
| FU1116 | *trpC2 amyE*::[*cat* $P_{kinB}$ (−55/+10)(A + 1G)-*lacZ*] | This work |
| FU1107 | *trpC2* Δ*spo0H*::*erm amyE*::[*cat* $P_{kinA}$ (−55/+10)-*lacZ*] | This work |
| FU1108 | *trpC2* Δ*spo0H*::*erm amyE*::[*cat* $P_{kinA}$ (−55/+10) (A + 1G)-*lacZ*] | This work |
| FU1117 | *trpC2* Δ*spo0H*::*erm amyE*::[*cat* $P_{kinB}$ (−55/+10)-*lacZ*] | This work |
| FU1118 | *trpC2* Δ*spo0H*::*erm amyE*::[*cat* $P_{kinB}$ (−55/+10) (A + 1G)-*lacZ*] | This work |
| FU1109 | *trpC2 gid*::*spc* Δ*codY amyE*::[*cat* $P_{kinA}$ (−55/+10)-*lacZ*] | This work |
| FU1110 | *trpC2 gid*::*spc* Δ*codY amyE*::[*cat* $P_{kinA}$ (−55/+10)(A + 1G)-*lacZ*] | This work |
| FU1119 | *trpC2 gid*::*spc* Δ*codY amyE*::[*cat* $P_{kinB}$ (−55/+10) -*lacZ*] | This work |
| FU1120 | *trpC2 gid*::*spc* Δ*codY amyE*::[*cat* $P_{kinB}$ (−55/+10)(A + 1G)-*lacZ*] | This work |
| FU1111 | *trpC2* Δ*abrB*::*erm amyE*::[*cat* $P_{kinA}$ (−55/+10)-*lacZ*] | This work |
| FU1112 | *trpC2* Δ*abrB*::*erm amyE*::[*cat* $P_{kinA}$ (−55/+10) (A + 1G)-*lacZ*] | This work |
| FU1122 | *trpC2* Δ*abrB*::*erm amyE*::[*cat* $P_{kinB}$ (−55/+10)-*lacZ*] | This work |
| FU1123 | *trpC2* Δ*abrB*::*erm amyE*::[*cat* $P_{kinB}$ (−55/+10) (A + 1G)-*lacZ*] | This work |
| FU1124 | *trpC2* Δ*spo0A*::*spc amyE*::[*cat* $P_{kinA}$ (−55/+10)-*lacZ*] | This work |
| FU1125 | *trpC2* Δ*spo0A*::*spc amyE*::[*cat* $P_{kinA}$ (−55/+10) (A + 1G)-*lacZ*] | This work |
| FU1126 | *trpC2* Δ*spo0A*::*spc amyE*::[*cat* $P_{kinB}$ (−55/+10)-*lacZ*] | This work |
| FU1127 | *trpC2* Δ*spo0A*::*spc amyE*::[*cat* $P_{kinB}$ (−55/+10) (A + 1G)-*lacZ*] | This work |
| FU1102 | *trpC2 kinA* (A + 1G) | This work |
| FU1103 | *trpC2 kinA* (A + 1G) Δ*kinB*::*kan* | This work |
| FU1113 | *trpC2 kinB* (A + 1G) | This work |
| FU1114 | *trpC2 kinB* (A + 1G) Δ*kinA*::*erm* | This work |
| FU1153 | *trpC2 amyE*::[*cat* $P_{kinA}$ (−55/+10)(A + 1C)-*lacZ*] | This work |
| FU1154 | *trpC2 amyE*::[*cat* $P_{kinB}$ (−55/+10)(A + 1C)-*lacZ*] | This work |
| FU1155 | *trpC2 kinA* (A + 1G) *kinB* (A + 1G) | This work |
| FU1156 | *trpC2 kinA* (A + 1C) | This work |
| FU1170 | *trpC2 kinA* (A + 1C) Δ*kinB*::*kan* | This work |

**TABLE 2** DNA microarray analysis of induction of the *spo* genes involved in sporulation phosphorelay upon addition of decoyinine[a]

| *spo* gene | Fold expression (with decoyinine/ without decoyinine) |
|---|---|
| *kinA* | 3.33 |
| *kinB* | 1.96 |
| *kinC* | 0.657 |
| *kinD* (*ykvD*) | 1.53 |
| *kinE* (*ykrQ*) | 4.25 |
| *spo0F* | 8.09 |
| *spo0B* | 0.854 |
| *spo0A* | 4.31 |
| *abrB* | 0.546 |
| *sigH* | 1.92 |
| *rapA* | 14.4 |
| *rapB* | 4.84 |
| *rapE* | 2.06 |
| *spo0E* | 2.28 |
| *ynzD* | 0.560 |
| *yisI* | 0.752 |

[a] DNA microarray analysis was performed previously (22). Also, the data were deposited in the KEGG Expression Database (http://www-genome.jp/kegg/expression).

ate antibiotic(s), chloramphenicol (5 μg/ml), spectinomycin (60 μg/ml), and/or erythromycin (0.3 μg/ml). The cells were inoculated and grown in 50 ml of S6 medium (41) containing 25 mM glucose and 50 μg of tryptophan per ml. When the cells reached to an optical density at 600 nm ($OD_{600}$) of 0.5, 10-ml aliquots were distributed into two flasks, and decoyinine was added to one culture to obtain a final concentration of 500 μg/ml (18 mM). The cultures with and without decoyinine were further incubated. During incubation before and after decoyinine addition, 1-ml aliquots of the culture were withdrawn at 30-min intervals, and the β-galactosidase (β-Gal) activity in crude cell extracts was measured spectrophotometrically as described previously (37). At 10 h after decoyinine addition (T10), the titers of viable cells (*V*) and spores (*S*) that were heat resistant (75°C for 20 min) were measured to obtain the sporulation percentage ($S/V \times 100$). The sporulation percentage at T20 (20 h after the entry into the stationary cell phase) was also determined using nutrient sporulation medium (NSMP) (41).

## RESULTS

**DNA microarray analysis of stringent transcription control of the genes involved in phosphorelay that initiates sporulation.** Decoyinine has been known for a long time to induce the sporulation of cells exponentially growing in the presence of rapidly metabolizable carbon, nitrogen, and phosphate sources (19). However, CodY cannot be the primary factor initiating the massive sporulation, as discussed above. So, we examined the levels of induction by decoyinine of the genes involved in the sporulation phosphorelay (Fig. 1) by using the data from the previous DNA microarray analysis involving the Δ*codY* strain with and without decoyinine to eliminate expression disturbance by decoyinine induction of members of the CodY regulon (22) (see the DNA microarray data deposited in the KEGG Expression Database [http://www.genome.jp/kegg/expression]). This examination revealed that out of the sporulation sensor kinase genes (*kinA* to *kinE*) (Fig. 1), *kinA*, *kinB*, *kinD*, and *kinE* are likely included in numerous positive stringent promoters that are inducible upon decoyinine addition (Table 2). Among the five sensor kinases, KinA and KinB are likely major sensor kinases involved in the phosphorelay that initiates sporulation (31, 42). Recently, KinC and KinD were found to be involved in biofilm formation rather than sporulation

(4, 43). Moreover, KinD and KinE are unlikely to be involved in the phosphorelay leading to the formation of spores (3). Therefore, *kinA* and *kinB* were targets of the current study on metabolic regulation of sporulation initiation, because KinA and KinB are major sensor kinases to have the phosphoryl group in sporulation phosphorelay and their genes are likely under positive stringent transcription control involving the adenines at their transcription initiation nucleotides (30, 31).

Expectedly, the DNA microarray analysis had also revealed that *spo0F*, *spo0A*, and *sigH*, besides *kinA* and *kinB*, were induced upon decoyinine addition (Table 2). The increased level of Spo0A~P upon the onset of the phosphorelay results in repression of transcription of the *abrB* gene for AbrB (11), leading to the derepression of transcription of the *sigH* (*spo0H*) gene, encoding $\sigma^H$, as well as of *kinB* (12–14), and RNAP containing $\sigma^H$ transcribes *kinA*, *spo0A*, and *spo0F* (Fig. 1) (1, 15, 30). Also, Spo0A~P is required for the induced transcription of *spo0F* and *spo0A* (12, 14, 16).

***lacZ* expression under the control of the promoters of *kinA* and *kinB* upon decoyinine addition.** To confirm the DNA microarray data showing that the *kinA* and *kinB* promoters are under positive stringent control in response to decoyinine addition (Table 2), the *kinA* and *kinB* core promoter regions (nucleotides −55 to +10) were placed upstream of *lacZ* in the *amyE* locus to yield the $P_{kinA}$-*lacZ* and $P_{kinB}$-*lacZ* strains FU1087 and FU1115, respectively. (The *B. subtilis* strains are listed in Table 1.) As shown in Fig. 3 (refer to WT), synthesis of β-Gal in the cells growing in a synthetic medium (S6 medium) (41), which is encoded by *lacZ* under the control of the *kinA* and *kinB* promoters, significantly increased after addition of decoyinine in the wild-type background. To demonstrate that each adenine at the initiation nucleotide for transcription of *kinA* and *kinB* is involved in this positive stringent control, they were replaced with guanines to yield the $P_{kinA}$ (A + 1G)-*lacZ* and $P_{kinB}$ (A + 1G)-*lacZ* strains FU1088 and FU1116. (Adenine replacement with guanine at nucleotide +1 of the positive stringent promoter always diminishes its positive regulation, but the extent of the diminution [sometimes entering negative regulation] depends on the delicate nucleotide sequence close to nucleotide +1 of the positive stringent promoter [22].) As shown in Fig. 3 (refer to WT), this replacement did not significantly reduce the *kinA* and *kinB* promoter activities, but the positive stringent control of these promoters was diminished [$P_{kinA}$ (A + 1G)] or abolished [$P_{kinB}$ (A + 1G)]. To confirm the results of the adenine replacement with guanine, the adenines were also replaced with cytosines to yield the $P_{kinA}$ (A + 1C)-*lacZ* and $P_{kinB}$ (A + 1C)-*lacZ* strains FU1153 and FU1154, as the change of the CTP concentration is relatively tolerant to decoyinine addition (Fig. 2B) (24). As shown in Fig. 3 (WT), this replacement did not reduce the *kinA* promoter strength, but it reduced the *kinB* promoter strength largely. The *lacZ* expression from the recombinant promoter [$P_{kinA}$(A + 1C)] was slightly induced, although it was less induced than that from the recombinant promoter [$P_{kinA}$ (A + 1G)] (Fig. 3A; refer to WT). The positive transcription control of $P_{kinB}$ (A + 1C) promoter was not significantly detected, that is, within the standard deviations (Fig. 3C). As cytosine at nucleotide + 1 is a rare phenomenon in *B. subtilis* (44), substituting +1 A/G for C leads to a decrease in the promoter activity as seen with the *kinB* promoter. In the *kinA* promoter, nucleotide +2 is a G, and it is possible that by replacing the +1 A with C moves the transcription start site 1 bp downstream so that it now starts with G. This would explain the still relatively high activity of this promoter version as well as its inability to increase transcription after decoyinine treatment. These overall results of the adenine replacements with guanine and cytosine indicated that the adenines are actually involved in the positive stringent transcription control of the *kinA* and *kinB* promoters.

*kinA* and *kinB* are known to be transcribed by RNAP possessing $\sigma^H$ and $\sigma^A$ (30, 31), so the $P_{kinA}$-*lacZ*and $P_{kinB}$-*lacZ* fusions were placed in the Δ*sigH* genetic background to yield the $P_{kinA}$-*lacZ* Δ*sigH* and $P_{kinB}$-*lacZ* Δ*sigH* strains FU1107 and FU1117, respectively. As expected, the induction of *lacZ* under the control of $P_{kinA}$ after addition of decoyinine was not observed in the Δ*sigH* genetic background (Fig. 3A), but *lacZ* under the control of $P_{kinB}$ was expressed in the Δ*sigH* genetic background as in the wild-type background (Fig. 3B). The results indicate that *lacZ* transcription from $P_{kinA}$, which increased upon decoyinine addition, is actually conducted by RNAP containing $\sigma^H$, whereas that from $P_{kinB}$ is most likely by RNAP containing $\sigma^A$. The *kinB* gene is a candidate of the CodY targets, as revealed by genome-wide transcript analysis (5). So, the observed positive stringent regulation of the *kinA* and *kinB* promoters might be explained by derepression due to CodY inactivation through a decrease in the *in vivo* GTP pool upon decoyinine addition. To determine if the *lacZ* expression from the *kinA* or *kinB* promoter is affected by *codY* deletion, the $P_{kinA}$-*lacZ* and $P_{kinB}$-*lacZ* fusions were placed in the Δ*codY* background to yield the $P_{kinA}$-*lacZ* Δ*codY* and $P_{kinB}$-*lacZ* Δ*codY* strains FU1109 and FU1119, respectively. As shown in Fig. 3, *lacZ* expression from neither $P_{kinA}$ nor $P_{kinB}$ was affected by Δ*codY*. Hence, it is considered that CodY is not involved in the positive stringent control of *kinA* and *kinB*. Even if *kinB* is a direct target of CodY, which is inactivated upon decoyinine addition, the binding site of CodY in the *kinB* promoter region is supposed to be located outside the core *kinB* promoter region (nucleotides −55 to +10). It is known that *kinB* is repressed by AbrB and that its binding site is located between nucleotides −34 and −6 (Fig. 3B) (13). To determine if the *lacZ* expression from the *kinA* or *kinB* promoter is affected by *abrB* deletion, the $P_{kinA}$-*lacZ* and $P_{kinB}$-*lacZ* fusions were placed in the Δ*abrB* background to yield the $P_{kinA}$-*lacZ* Δ*abrB* and $P_{kinB}$-*lacZ* Δ*abrB* strains FU1111 and FU1122, respectively. As shown in Fig. 3, *lacZ* expression from neither $P_{kinA}$ nor $P_{kinB}$ was affected by Δ*abrB*. Hence, it is considered that AbrB is not involved in the positive stringent control of *kinA* and *kinB*. Although the fact that the transcription from the *kinB* promoter is not affected by Δ*abrB* cannot be properly explained, it is possible to consider that AbrB is too rapidly repressed after decoyinine addition by Spo0A~P, which increased upon the onset of the phosphorelay, to be detected even in the *abrB*$^+$ genetic background.

Furthermore, *kinA* is reported to be negatively regulated by Spo0A~P (12), but its Spo0A box is located outside the core *kinA* promoter (nucleotides −55 to +10). However, *kinA* is transcribed by RNAP possessing $\sigma^H$, and the expression of *sigH* encoding $\sigma^H$ is activated by Spo0A~P through repression of AbrB (Fig. 1), so it is expected that *kinA* is not transcribed in the Δ*spo0A* genetic background. To determine if the *lacZ* expression from the *kinA* or *kinB* promoter is affected by the *spo0A* deletion, the $P_{kinA}$-*lacZ* and $P_{kinB}$-*lacZ* fusions were placed in the Δ*spo0A* background to yield the $P_{kinA}$-*lacZ* Δ*spo0A* and $P_{kinB}$-*lacZ* Δ*spo0A* strains FU1124 and FU1126, respectively. As expected, *lacZ* expression from $P_{kinA}$ was not induced by decoyinine addition in the *spo0A*

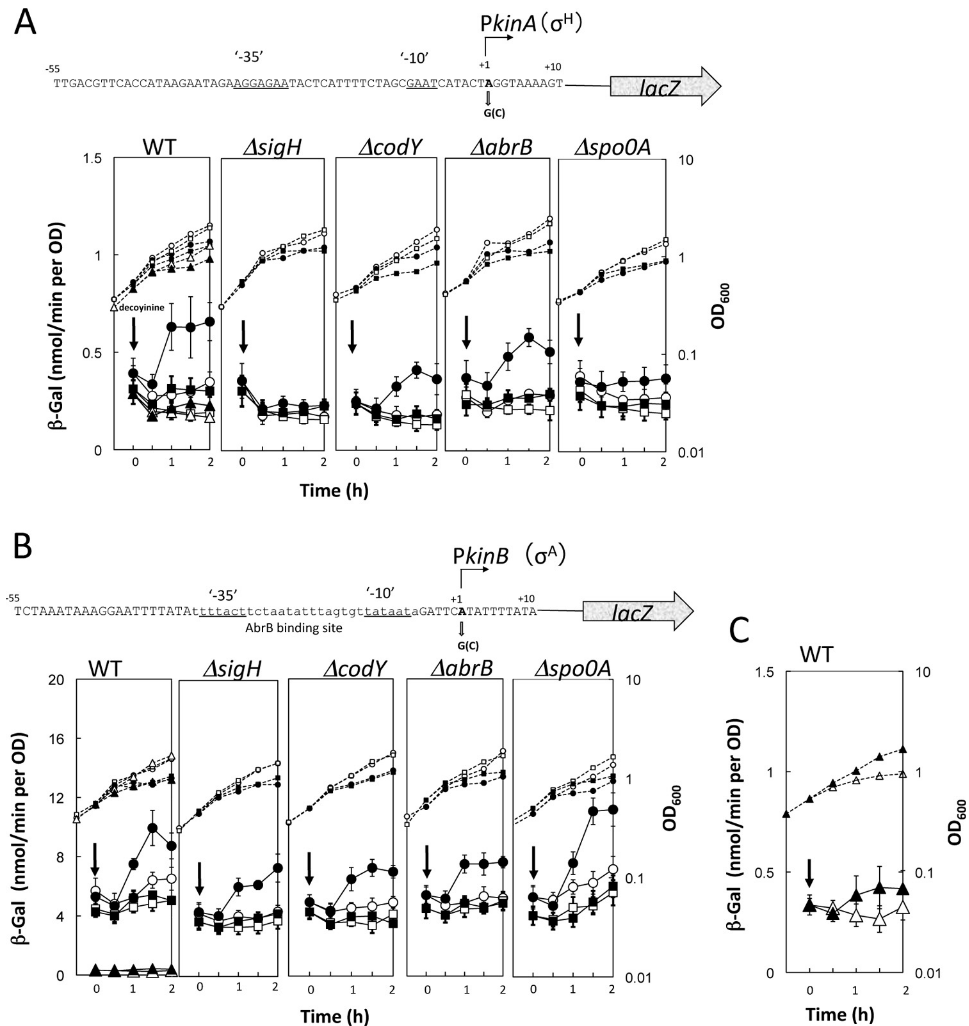


**FIG 3** Activation of the *kinA* and *kinB* core promoters upon addition of decoyinine. (A) The nucleotide sequence of the core *kinA* promoter region (nucleotides −55 to +10) is shown in the upper part, along with the transcription initiation adenine (boldfaced A at nucleotide +1) and the −10 and −35 regions that are recognized by RNAP possessing $\sigma^H$ (30). This promoter region was transcriptionally fused with *lacZ*. The adenine at nucleotide +1 was replaced with a guanine. The synthesis of β-Gal encoded by *lacZ* under the control of the wild-type and guanine substitution *kinA* promoters was monitored after the addition of decoyinine in the wild-type (WT), Δ*sigH*, Δ*codY*, Δ*abrB*, and Δ*spo0A* backgrounds. Closed and open symbols indicate the presence and absence of decoyinine addition; circles, squares, and triangles represent the *kinA* promoters possessing adenine, guanine, and cytosine at nucleotide +1, respectively. Large and small symbols denote β-Gal activity and $OD_{600}$, respectively. (B) The nucleotide sequence of the core *kinB* promoter region (nucleotides −55 to +10) is shown, along with the transcription initiation adenine (boldfaced A at nucleotide +1) and the −10 and −35 regions (underlined) that are most likely recognized by RNAP possessing $\sigma^A$ (31). This promoter region containing the AbrB binding site (lowercase letters) (13) was fused with *lacZ*, whose transcription initiation adenine was replaced with guanine. β-Gal synthesis under the control of the WT and guanine substitution *kinA* promoters was monitored after the addition of decoyinine in the WT, Δ*sigH*, Δ*codY*, Δ*abrB*, and Δ*spo0A* backgrounds. The symbol assignment is the same as in panel A. (C) β-Gal synthesis under the control of the WT and cytosine substitution *kinB* promoter was monitored after the addition of decoyinine in the WT, where a scale of the vertical axis (WT in panel B) for the β-Gal synthesis is enlarged to the maximal value of 1.5. In all panels, the standard deviations of the average β-Gal activity values from the duplicated or triplicate experiments are shown by error bars. Thick and thin error bars are used for the adenine and guanine at nucleotide +1 of *kinA* and *kinB* (A and B), respectively. The error bars for the cytosine at nucleotide +1 of *kinA* and *kinB* are not shown in panel A or B (WT) to avoid unnecessary complication, but they are shown in panel C.

background, but that from $P_{kinB}$ was not affected by the *spo0A* deletion (Fig. 3).

Even when $P_{kinA}$ (A + 1G)-*lacZ* and $P_{kinB}$ (A + 1G)-*lacZ* were introduced into the Δ*sigH*, Δ*codY*, Δ*abrB*, and Δ*spo0A* backgrounds, no significant induction of *lacZ* expression was observed, as in the wild-type background. The overall results indicate that the positive stringent control of $\sigma^H$- and $\sigma^A$-dependent transcription from the respective *kinA* and *kinB* promoters, which is evoked by decoyinine addition, involves the adenines at their transcription initiation nucleotides and that this positive stringent control is most likely independent of either CodY or AbrB.

**Sporulation efficiency of *kinA* and *kinB* mutants with replacement of adenine with guanine (or cytosine) at the transcription initiation nucleotide.** *lacZ* expression under the control of either the $P_{kinA}$ or $P_{kinB}$ promoter carrying adenine at the transcription initiation nucleotide was induced by the addition of decoyinine, but it was not significantly induced when the adenine was replaced with guanine (Fig. 3). To examine the effect of this

**TABLE 3** Effects of variable mutations, including the substitution mutations of the respective adenines at nucleotide +1 of $P_{kinA}$ and $P_{kinB}$ with guanines, on sporulation efficiency

| Strain | Sporulation percentage (%)[a] | |
|---|---|---|
| | Decoyinine induced, S6 medium, T10 | Nutrient medium (NSMP), T20 |
| Wild-type 168 | 19 | 71 |
| PS37t (Δ*codY*) | 36 | 29 |
| FU1106 (Δ*abrB*) | 0.40 | 34 |
| FU1121 (Δ*spo0A*) | $<5 \times 10^{-6}$ | $<5 \times 10^{-6}$ |
| ASK201 (Δ*spo0H*) | $<5 \times 10^{-6}$ | $<5 \times 10^{-6}$ |
| FU1095 (Δ*kinA*) | 0.26 | 7.9 |
| FU1063 (Δ*kinA* Δ*codY*) | 7.3 | 8.5 |
| FU1096 (Δ*kinB*) | 0.67 | 22 |
| FU1064 (Δ*kinB* Δ*codY*) | 1.5 | 12 |
| FU1098 (Δ*kinA* Δ*kinB*) | $<5 \times 10^{-6}$ | $<5 \times 10^{-6}$ |
| FU1102 [*kinA* (A + 1G)] | 3.5 | 36 |
| FU1103 [*kinA* (A + 1G) Δ*kinB*] | $6.2 \times 10^{-4}$ | 0.055 |
| FU1113 [*kinB* (A + 1G)] | 1.7 | 25 |
| FU1114 [Δ*kinA* *kinB* (A + 1G)] | $2.1 \times 10^{-5}$ | 0.0019 |
| FU1155 [*kinA*(A + 1G) *kinB* (A + 1G)] | 0.0011 | 2.3 |
| FU1156 [*kinA* (A + 1C)] | 4.2 | 27 |
| FU1170 [*kinA* (A + 1C) Δ*kinB*] | $7.6 \times 10^{-5}$ | 0.0052 |

[a] The sporulation experiments were repeated at least three times. Representative values are presented. The standard deviations were less than 15% of the values shown.

replacement on decoyinine-induced sporulation as well as on the massive sporulation using NSMP (41) as a control experiment, the transcription initiation bases, adenines, of the *kinA* and *kinB* promoters were replaced with guanines *in situ* by means of positive selection using the *mazF* gene (36), as described in Materials and Methods and illustrated in Fig. S1, to yield the *kinA* (A + 1G) and *kinB* (A + 1G) strains FU1102 and FU1113, respectively.

Furthermore, the replacement of the adenine with cytosine at the transcription initiation nucleotide rendered only the *kinB* promoter inefficient (Fig. 3B and C), but decoyinine did not induce β-Gal synthesis under the control of $P_{kinA}$ (A + 1C) and $P_{kinB}$ (A + 1C) significantly (Fig. 3). We also attempted to replace the adenine with cytosine *in situ* as described above. We successfully introduced the adenine replacement with cytosine at the transcription initiation nucleotide of the *kinA* promoter to obtain the *kinA* (A + 1C) strain FU1156. However, we were unable to introduce this replacement at nucleotide +1 of the *kinB* promoter in spite of our numerous trials due to the occurrence of various unexpected base substitutions and deletions in the *kinB* promoter region at the final stage of the nucleotide replacement protocol (see Fig. S1 in the supplemental material). This might possibly be related to the fact that the replacement of the adenine with cytosine severely affected this *kinB* promoter strength (Fig. 3B and C).

We examined the sporulation efficiencies of various wild-type and mutant strains under decoyinine-induced sporulation conditions using S6 medium as well as under the nutrient sporulation conditions using NSMP (Table 3). When the cells of wild-type strain 168 exponentially growing in the S6 medium were exposed to decoyinine and further incubated for 10 h, they sporulated at the sporulation percentage of 19%. As a control, the wild-type cells were not exposed to decoyinine and were further incubated for 10 h, their sporulation percentage was 0.37%, indicating that decoyinine induced sporulation 51-fold. This decoyinine-induced sporulation was completely blocked in the Δ*spo0A*, Δ*spo0H*, or Δ*kinA* Δ*kinB* genetic background (less than $5 \times 10^{-6}$%, the experimental limit to count heat-resistant spores), whereas it was only partially affected by Δ*kinA* (0.26%) or Δ*kinB* (0.67%) (Table 3). Interestingly, Δ*codY* enhanced this sporulation (36%). Such enhancement with Δ*codY* was also observed in the genetic background of Δ*kinA* (7.3%) or Δ*kinB* (1.5%) (Table 3). In contrast, Δ*abrB* well inhibited this sporulation (0.40%) (Table 3). Thus, Δ*codY* or Δ*abrB* well affected the metabolic network leading to the sporulation in S6 medium. It is reasonable that Δ*codY* allows the decoyinine-induced sporulation with S6 medium to proceed, because *kinB*, *phrA*, and *phrE* are probable targets of CodY (5), whose CodY derepression is favorable for sporulation initiation. However, it cannot be properly explained at present why Δ*abrB* affected sporulation in S6 medium in spite of possibly enhancing sporulation due to derepression of *sigH*.

Sporulation of wild-type strain 168 in NSMP (71%), which was perfomed as a control experiment for decoyinine-induced sporulation, was completely blocked in the Δ*spo0A*, Δ*spo0H*, or Δ*kinA* Δ*kinB* genetic background (less than $5 \times 10^{-6}$ %), whereas it was only partially affected by Δ*kinA* (7.9%) or Δ*kinB* (22%) (Table 3). These results indicate that the sporulation phosphorelay involving *kinA* and *kinB* is similarly operated in the massive sporulation in NSMP as in the decoynine-induced sporulation. However, Δ*codY* and Δ*abrB* only slightly reduced the wild-type sporulation percentage of 71% to 29 and 34%, respectively, in contrast to their considerable effect on the decoyinine-induced sporulation as described above.

The *kinA* (A + 1G) and *kinB* (A + 1G) strains FU1102 and FU1113 with adenine replacement with guanine at the transcription initiation nucleotide affected decoyinine-induced sporulation, reducing the wild-type sporulation of 19% to 3.5% and 1.7%, respectively (Table 3). Also, the *kinA* (A + 1C) strain FU1155 with adenine replacement with cytosine at nucleotide +1 affected decoyinine-induced sporulation, reducing the wild-type sporulation of 19% to 4.2%, which is essentially indistinguishable from the effect of the adenine replacement with guanine at nucleotide +1 in spite of slightly lower induction of β-Gal synthesis by decoyinine than in the *kinA* (A + 1G) strain (Fig. 3A). The *kinA* (A + 1G) Δ*kinB*, *kinA* (A + 1C) Δ*kinB*, and Δ*kinA* *kinB* (A + 1G) strains FU1103, FU1170, and FU1114 were able to sporulate only at very low frequencies ($6.2 \times 10^{-4}$, $7.6 \times 10^{-5}$, and $2.1 \times 10^{-5}$%), whereas the Δ*kinB* and Δ*kinA* strains FU1096 and FU1095 formed considerable numbers of spores (0.67 and 0.26%, respectively). Also, the *kinA* (A + 1G) *kinB* (A + 1G) strain FU1155 sporulated at a comparatively low frequency, 0.0011%.

Similarly, these replacements, *kinA* (A + 1G), *kinA* (A + 1C), and *kinB* (A + 1G), affected the massive sporulation in NSMP moderately, reducing the wild-type sporulation percentage of 71% to 36%, 27%, and 25%, respectively (Table 3). The *kinA* (A + 1G) Δ*kinB*, *kinA* (A + 1C) Δ*kinB*, and Δ*kinA* *kinB* (A + 1G) strains FU1103, FU1170, and FU1114 sporulated at low frequencies (0.055, 0.0052, and 0.0019%, respectively), whereas the Δ*kinB* and Δ*kinA* strains FU1096 and FU1095 formed considerable numbers of spores (22 and 7.9%, respectively). Also, the *kinA* (A + 1G) *kinB* (A + 1G) strain FU1155 formed a low but considerable number of spores (2.3%). These results clearly indicate that the respective adenine replacements with guanine at the transcription initiation nucleotides of *kinA* and *kinB* as well as the adenine replacement with cytosine at nucleotide +1 of *kinA* actually affect the decoyinine-induced sporulation in S6 medium as well as the massive sporulation in NSMP.

## DISCUSSION

Decoyinine, a GMP synthase inhibitor, induces the sporulation of exponentially growing wild-type cells in the presence of rapidly metabolizable carbon, nitrogen, and phosphate sources (Table 3) (19) and also causes the reciprocal concentration changes of an increase in ATP and a decrease in GTP, which can be sensed through increases and decreases in the rate of transcription initiation from positive and negative stringent promoters depending on their transcription initiation bases, i.e., adenine and guanine, respectively (22–24). DNA microarray analysis indicated that out of the genes involved in sporulation phosphorelay, decoyinine induced *kinA*, *kinB*, *kinD*, *kinE*, *spo0F*, *spo0A*, *sigH*, *rapA*, *rapB*, *rapE*, and *spo0E* more than 1.5-fold and repressed *kinC*, *abrB*, and *ynzD* the same amount (Table 2). The former genes, except *rap* and *spo0E*, were expected to be induced according to the sporulation phosphorelay (Fig. 1). KinC and KinD are involved in biofilm formation rather than sporulation (4, 43). The repression of *ynzD* and *yisI* (1.3-fold) coincided with the fact that YnzD and YisI increased under nonsporulation conditions (8).

KinA and KinB, whose synthesis was found to be under positive stringent transcription control in this study, are likely major sensor kinases that initiate sporulation (31, 42). Recently, it was reported that using an IPTG-inducible promoter, the induction of the synthesis of KinA beyond a certain level leads to the entry of the irreversible process of sporulation irrespective of nutrient availability (45) and that the primary role of the N-terminal domain of KinA is to form a functional tetramer that is necessary for the kinase activity catalyzed by the C-terminal domain (46). These facts imply that KinA as well as KinB is synthesized in an active form, suggesting that the threshold autophosphorylation level of the KinA (plus KinB) protein governs entry into sporulation under usual sporulation conditions.

The experiments involving the *lacZ* fusions of the promoter regions of *kinA* and *kinB* confirmed their induction by decoyinine on DNA microarray analysis. As shown in Fig. 3, the positive stringent transcription control of $\sigma^H$- and $\sigma^A$-dependent transcription from the respective *kinA* and *kinB* promoters involves the adenine at their transcription initiation nucleotides, and this positive control is most likely independent of either CodY or AbrB. Either Δ*spo0A* or Δ*sigH* only affected *kinA* expression, because *kinA* is mainly transcribed by RNAP possessing $\sigma^H$, and *sigH* is indirectly activated by Spo0A through repression of AbrB (Fig. 1). Transcription of *kinA* and *kinB* is initiated at an adenine, so the initiation nucleotide triphosphate (iNTP) of *kinA* and *kinB* transcription is ATP. The increase in *in vivo* ATP concentration, which is evoked by decoyinine addition, enhances transcription initiation through RNAP, causing *kinA* and *kinB* activation. As far as we know, the positive stringent transcription control is only one device to activate the transcription of *kinA* and *kinB* by the modulation of the medium conditions, except that *kinA* transcription is driven by RNAP possessing $\sigma^H$ inducible upon the onset of the phosphorelay.

It is possible to speculate that the *kinA* induction by decoyinine might reflect the derepression of *sigH* through AbrB repression by Spo0A~P. However, this is unlikely, because the *kinA* gene was induced by decoyinine in an *abrB*$^+$ genetic background essentially in the same fashion as in a Δ*abrB* background (Fig. 3B). This observation suggests that AbrB could be rapidly repressed by Spo0A~P, which is formed through the phosphorelay triggered by KinA and KinB, leading to the immediate formation of $\sigma^H$.

Our recent study suggested that the presence of a guanine(s) and an adenine(s) at nucleotide +1 and nucleotide +2 might be indispensable for negative and positive stringent control, respectively (22). However, nucleotide +2 of *kinA* is guanine, which likely represses transcription initiation. It is possible to assume that the purine base at nucleotide +1 might be mainly involved in the formation of the transcription initiation complex in the case of the transcription conducted by RNAP possessing $\sigma^H$.

Spo0A, AbrB, CodY, and $\sigma^H$ are all transcriptional regulators, and $\sigma^H$ is the only sigma factor, involved in sporulation phosphorelay (Fig. 1). Expectedly, Δ*spo0A* and Δ*sigH* blocked sporulation completely under the two sporulation conditions (decoyinine-induced sporulation in S6 medium and massive sporulation in NSMP) (Table 3). Δ*codY* did not affect the sporulation in NSMP largely, but it enhanced the decoyinine-induced sporulation in S6 medium (Table 3). CodY negatively regulates *kinB* and *phrAE* (21); PhrA and PhrE inhibit RapA and RapE, respectively (Fig. 1), so Δ*codY* was likely to suppress this negative effect on the sporulation. However, it is conceivable that Δ*codY* affects the metabolic network leading to the sporulation in S6 medium more than in NSMP.

*kinA* (A + 1G), *kinA* (A + 1C), and *kinB* (A + 1G) mutants with adenine replacement with guanine or cytosine at the transcription initiation nucleotides of *kinA* and *kinB* were constructed (see Fig. S1 in the supplemental material); the construction of *kinB* (A + 1C) failed in spite of numerous trials. With respect to iNTP, the reciprocal changes of ATP increase and GTP decrease were provoked upon decoyinine addition, but CTP remained relatively unchanged (Fig. 2B) (24). The *kinA* (A + 1G) and *kinA* (A + 1C) mutants exhibited sporulation percentages between those of wild-type strain 168 and Δ*kinA* strains for the decoyinine-induced sporulation in S6 medium as well as for the massive sporulation in NSMP (Table 3). Also, the *kinB* (A + 1G) mutant exhibited sporulation percentages between those of the wild-type and Δ*kinB* strains under these two sporulation conditions (Table 3). Moreover, the *kinA* (A + 1G) *kinB* (A + 1G) strain sporulated at a lower frequency than that of either the *kinA* (A + 1G) or *kinB* (A + 1G) strain. Consequently, almost the same sporulation initiation mechanism, involving *kinA* and *kinB*, most likely operates for the decoyinine-induced sporulation in S6 medium and for the massive sporulation in NSMP. Although the Δ*kinA* and Δ*kinB* strains formed considerable numbers of spores under these two sporulation conditions, the Δ*kinA kinB* (A + 1G), Δ*kinA kinB* (A + 1C), and Δ*kinB kinA* (A + 1G) strains were scarcely able to form spores. These findings strongly indicated that the positive stringent transcription control of *kinA* and *kinB* without the involvement of any other transcription regulators is involved in enhancement of the phosphorelay that eventually leads to the formation of spores. In other words, operation of the positive stringent transcription control of *kinA* and *kinB* is a prerequisite to enter sporulation, whereby it can be gauged whether the energy charge of the cells, nearly parallel to *in vivo* ATP concentration, would be enough for sporulation to proceed. The ATP increase is sensed by putting the RNAP reaction with ATP as an iNTP of *kinA* and *kinB* transcription into the threshold kinetic range, leading to activation of the phosphorelay. In addition, an increase in the *in vivo* ATP pool likely facilitates the autophosphorylation of KinA and KinB for sporulation phosphorelay to proceed.

Conversely, the phosphoryl group is drained from the phosphorelay system through the actions of dedicated phosphatases; RapA, RapE, and RapB dephosphorylate Spo0F~P by sensing a low cell density, and Spo0E, YisI, and YnzD dephosphorylate Spo0A~P (Fig. 1) (2, 6). The opposing actions of the kinases and the phosphatases are believed to integrate environmental and physiological signals for the decision to sporulate by governing the flux through the relay system and hence the level of Spo0A~P, which must reach a threshold concentration to trigger sporulation (47). Therefore, the negative stringent transcription control and the CodY-mediated repression of the stringent genes, both of which are evoked by stringent conditions such as decoyinine addition, contribute to the inhibition of dephosphorylation of Spo0A~P and Spo0F~P through decreases in and inhibition of the phosphatases such as YisI and YnzD and RapA and RapE, respectively (Fig. 1 and Table 2). 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 downregulation of the phosphatases that interfere with the phosphorelay system leading to the formation of spores, which is affected by the medium constituents; for example, decoyinine addition did not cause the efficient 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* (S.Tojo and Y. Fujita, unpublished observations).

In conclusion, we suggest that operation of the positive stringent transcription control is a prerequisite for phosphorelay to transfer a phosphate group to Spo0A, leading to spore formation. However, this does not necessarily mean that the operation of the positive stringent transcription regulation triggers the sporulation to proceed. The delicate balance of the input of the phosphate group into the phosphorelay by KinA and KinB and its drain from it by the Rap phosphatase determines whether the cell can enter sporulation. Thus, it would be possible that a decrease of the Rap phosphatase and *kinB* derepression, which might be evoked by CodY inactivation probably through a GTP decrease, could trigger sporulation during the low but constant operation of the positive stringent control under certain sporulation conditions.

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