

Heavy Involvement of Stringent Transcription Control Depending on the Adenine or Guanine Species of the Transcription Initiation Site in Glucose and Pyruvate Metabolism in *Bacillus subtilis*^{∇†}

Shigeo Tojo, Kanako Kumamoto, Kazutake Hirooka, and Yasutaro Fujita*

Department of Biotechnology, Faculty of Life Science and Biotechnology, Fukuyama University, Fukuyama, Japan

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In *Bacillus subtilis* cells, the GTP level decreases and the ATP level increases upon a stringent response. This reciprocal change in the concentrations of the substrates of RNA polymerase affects the rate of transcription initiation of certain stringent genes depending on the purine species at their transcription initiation sites. DNA microarray analysis suggested that not only the *rrn* and *ilv-leu* genes encoding rRNAs and the enzymes for synthesis of branched-chain amino acids, respectively, but also many genes, including genes involved in glucose and pyruvate metabolism, might be subject to this kind of stringent transcription control. Actually, the *ptsGHI* and *pdhABCD* operons encoding the glucose-specific phosphoenolpyruvate:sugar phosphotransferase system and the pyruvate dehydrogenase complex were found to be negatively regulated, like *rrn*, whereas the *pycA* gene encoding pyruvate carboxylase and the *alsSD* operon for synthesis of acetoin from pyruvate were positively regulated, like *ilv-leu*. Replacement of the guanine at position 1 and/or position 2 of *ptsGHI* and at position 1 of *pdhABCD* (transcription initiation base at position 1) by adenine changed the negative stringent control of these operons in the positive direction. The initiation bases for transcription of *pdhABCD* and *pycA* were newly determined. Then the promoter sequences of these stringent operons were aligned, and the results suggested that the presence of a guanine(s) and the presence of an adenine(s) at position 1 and/or position 2 might be indispensable for negative and positive stringent control, respectively. Such stringent transcription control that affects the transcription initiation rate through reciprocal changes in the GTP and ATP levels likely occurs for numerous genes of *B. subtilis*.

Stringent control is one of the most important adaptations which help bacteria survive under harsh conditions. Of the various occasions when stringent control results from the synthesis of GDP 3'-diphosphate (ppGpp) from GTP, which is catalyzed by the RelA protein associated with ribosomes, the most prominent is the repression of stable RNA synthesis (4). This control also affects the expression of certain genes, including genes involved in amino acid biosynthesis. However, *Bacillus subtilis* and *Escherichia coli* use different strategies for stringent transcription control (19). ppGpp may not inhibit *B. subtilis* RNA polymerase directly, whereas ppGpp decreases rRNA promoter activity by directly inhibiting *E. coli* RNA polymerase.

GTP and ATP are well-known gauges of the general energetic capacity and energy charge of cells, respectively. In *B. subtilis*, the GTP and ATP levels decrease and increase during a stringent response (e.g., amino acid starvation), respectively (20, 24, 31, 48), as shown in Fig. S1 in the supplemental material. The changes are mediated by ppGpp synthesized by RelA (52) during a stringent response, probably through its inhibition of IMP dehydrogenase, the first enzyme in the pathway leading to the biosynthesis of GTP (24). The inhibition of GTP synthesis results in a decrease in the GTP level and

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 also occur when *B. subtilis* cells are treated with decoyinine (19, 25, 48), a GMP synthase inhibitor (46), even in a *relA*-deficient strain because the inhibition is not mediated by ppGpp (48).

The decrease in the GTP level during a stringent response can be sensed by the CodY protein, a GTP-binding repressor of many genes, such as *dpp* encoding dipeptide permease (42) and *ilv-leu* encoding the enzymes for synthesis of branched-chain amino acids (41), that are normally quiescent when cells are grown in a nutrient-rich medium (14, 28, 34). CodY also functions as a transcriptional activator of certain genes, such as *ackA* involved in acetate formation (39). Thus, lowering the GTP concentration inactivates the CodY protein, leading to derepression or deactivation of these genes. Another mechanism underlying stringent control has recently emerged. The reciprocal changes in the concentrations of GTP and ATP, which are substrates of RNA polymerase, can be sensed through modulation of the rate of initiation of transcription of several stringent genes, such as *rrn* encoding rRNAs (19) and *ilv-leu* (20, 48). This CodY-independent mechanism involves the base, guanine or adenine, at the transcription initiation site (position 1 or 2; the base at position 1 is the transcription initiation base), which is related to negative and positive stringent control of *rrn* and *ilv-leu*, respectively. The downregulation and upregulation of the *rrn* and *ilv-leu* promoter activities are always correlated with the decrease and increase in the intracellular GTP and ATP concentrations, respectively. The two mechanisms underlying *B. subtilis* stringent transcription

* Corresponding author. Mailing address: Department of Biotechnology, Faculty of Life Science and Biotechnology, Fukuyama University, 985 Sanzo, Higashimura-cho, Fukuyama, Hiroshima 729-0292, Japan. Phone: 81 84 936 2111. Fax: 81 84 936 2023. E-mail: yfujita@bt.fubt.fukuyama-u.ac.jp.

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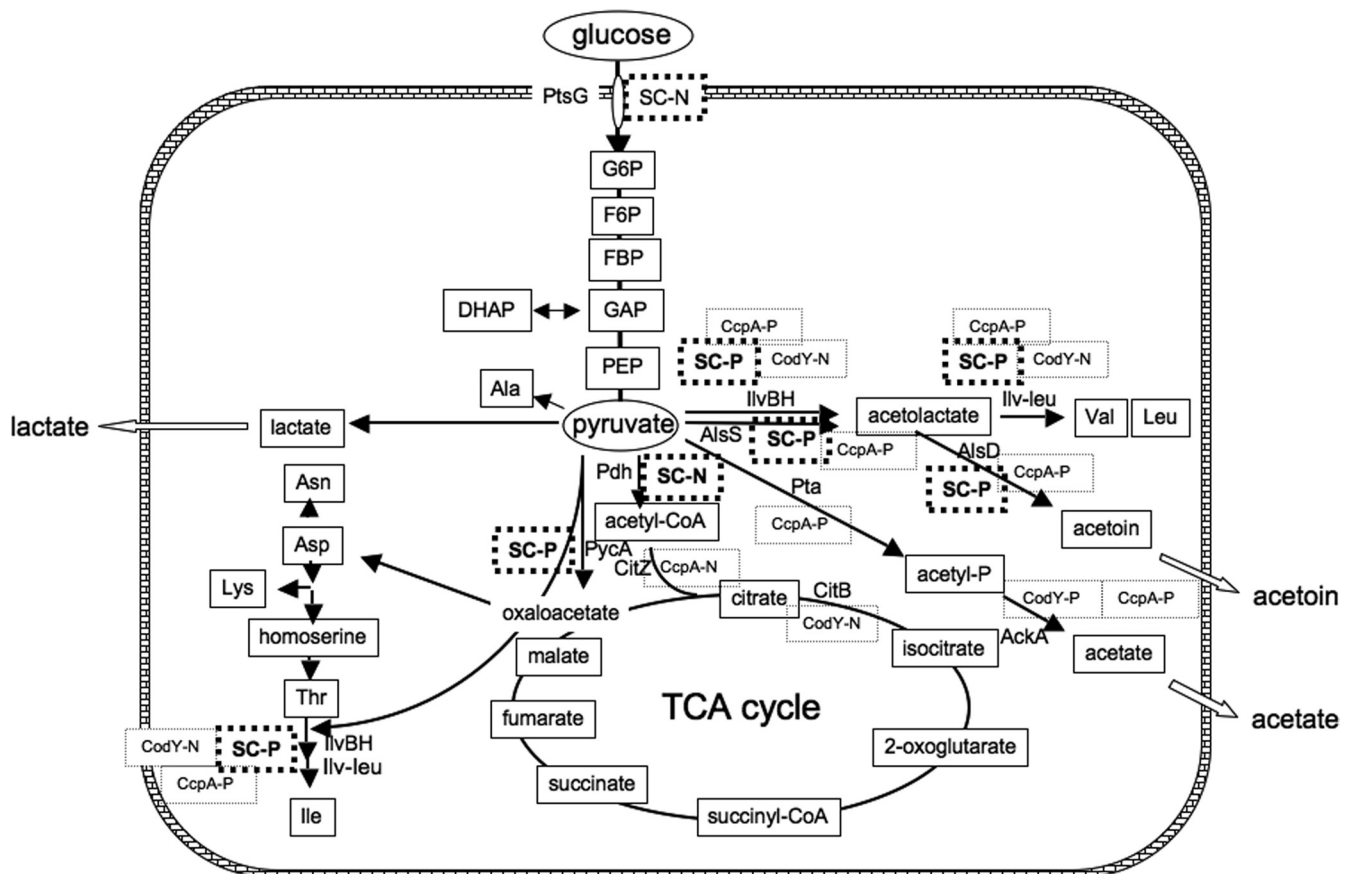


FIG. 1. Intermediates in carbon metabolism and CcpA and CodY regulation and CodY-independent stringent control of the genes involved in this metabolism. The glucose and pyruvate metabolism described in the text includes glycolysis; the formation of acetyl-CoA, oxaloacetate, and alanine; the secretory pathways for acetoin, acetate, and lactate; and the synthesis of branched-chain amino acids. CcpA-N and CcpA-P indicate negative and positive regulation by CcpA, respectively, and CodY-N and CodY-P indicate negative and positive regulation by CodY, respectively. The *ilv-leu* (40) and *citB* (16) genes are known to be repressed by CodY, whereas the *ackA* gene is activated (39). Also, the *citZ* gene is repressed by CcpA (17), whereas the *ilv-leu* (41, 49), *alsSD* (51), *pta* (33), and *ackA* (50) genes are activated by CcpA. SC-N and SC-P indicate negative and positive CodY-independent stringent control, respectively. Abbreviations: TCA, tricarboxylic acid; G6P, glucose-6-phosphate; F6P, fructose-6-phosphate; FBP, fructose-1,6-bisphosphate; DHAP, dihydroxyacetone phosphate; GAP, glyceraldehyde 3-phosphate; PEP, phosphoenolpyruvate.

control have been determined for the *ilv-leu* operon (see Fig. S1 in the supplemental material).

In order to answer an interesting question, whether such CodY-independent stringent control occurs in the regulation of various operons, including catabolic and anabolic operons, we performed a DNA microarray analysis using wild-type and $\Delta codY$ strains grown with and without decoyinine. This analysis suggested that in addition to the genes for protein synthesis, such as *rm*, many metabolic operons, including operons involved in critical stages of metabolic regulation, might also be under stringent control, and tens of these operons might be subject to CodY-independent stringent control. Among these operons, we focused on the genes involved in glucose and pyruvate metabolism (Fig. 1) in whose regulation a catabolite control protein (CcpA) and CodY are known to be the main proteins involved. Among the operons involved in this metabolism, only the *ptsGHI* (10, 45) and *pdhABCD* (9, 13) operons encoding the glucose-specific phosphoenolpyruvate:sugar phosphotransferase system (PTS) and the pyruvate dehydrogenase complex, respectively, were severely downregulated. In contrast, not only *ilv-leu* (11, 49) but also *pycA* encoding pyru-

vate carboxylase (5) and *alsSD* (35) involved in synthesis of acetoin from pyruvate were substantially upregulated. We demonstrated that these operons were actually under CodY-independent stringent control. The guanines located at positions 1 and 2 of the transcription initiation sites of *ptsGHI* and *pdhABCD* are essential for the CodY-independent negative stringent control, whereas adenines are located at position 1 or 2 of the transcription initiation sites of the *pycA* and *alsSD* operons, as they are in *ilv-leu*.

MATERIALS AND METHODS

Bacterial strains and construction of strains. The *B. subtilis* strains used in this work are listed in Table 1. Strain FU808 was constructed by transformation of strain 1A765 with chromosomal DNA of strain PS37 to spectinomycin resistance (60 μ g/ml) on plates containing tryptone blood agar base (Difco) with 10 mM glucose (TBABG). The presence of $\Delta codY$ in the resulting transformant was confirmed by the appearance in the $\Delta codY$ strain of a PCR product that was 250 bp shorter than that obtained for the *codY*⁺ strain, as described previously (28). Disruption of the *gid* gene, present in the $\Delta codY$ strain, did not affect expression of the target genes in this work. To construct transcriptional promoter-*lacZ* fusion strains with *ptsGHI*, *pycA*, *alsSD*, and *pdhABCD*, the promoter regions comprising nucleotides -55 to 26 and -47 to 167 were amplified using primer

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

| Strain | Genotype | Reference or source |
|-----------------------------|--|---------------------|
| 168 | <i>trpC2</i> | 1 |
| 1A765 (= BR16) ^a | <i>trpC2 lys</i> | 47 |
| 1A766 (= BR17) ^a | <i>trpC2 lys relA1</i> | 47 |
| PS29 | <i>trpC2 gid::spec</i> | 38 |
| PS37 | <i>trpC2 gid::spec ΔcodY</i> | 38 |
| FU807 | <i>trpC2 lys relA1 gid::spec ΔcodY</i> | This study |
| FU808 | <i>trpC2 lys gid::spec ΔcodY</i> | This study |
| FU906 | <i>trpC2 lys amyE::[cat PptsG(-55/26)-lacZ]</i> | This study |
| FU1025 | <i>trpC2 lys relA1 amyE::[cat PptsG(-55/26)-lacZ]</i> | This study |
| FU1029 | <i>trpC2 lys gid::spec ΔcodY amyE::[cat PptsG(-55/26)-lacZ]</i> | This study |
| FU1019 | <i>trpC2 lys amyE::[cat PpdhA(-47/167)-lacZ]</i> | This study |
| FU1021 | <i>trpC2 lys relA1 amyE::[cat PpdhA(-47/167)-lacZ]</i> | This study |
| FU1031 | <i>trpC2 lys gid::spec ΔcodY amyE::[cat PpdhA(-47/167)-lacZ]</i> | This study |
| FU977 | <i>trpC2 lys amyE::[cat PpycA(-55/26)-lacZ]</i> | This study |
| FU1023 | <i>trpC2 lys relA1 amyE::[cat PpycA(-55/26)-lacZ]</i> | This study |
| FU1027 | <i>trpC2 lys gid::spec ΔcodY amyE::[cat PpycA(-55/26)-lacZ]</i> | This study |
| FU937 | <i>trpC2 lys amyE::[cat PalsS(-55/26)-lacZ]</i> | This study |
| FU1026 | <i>trpC2 lys relA1 amyE::[cat PalsS(-55/26)-lacZ]</i> | This study |
| FU1041 | <i>trpC2 lys gid::spec ΔcodY amyE::[cat PalsS(-55/26)-lacZ]</i> | This study |
| FU934 | <i>trpC2 lys amyE::[cat PptsG(-55/26)(G2A)-lacZ]</i> | This study |
| FU1042 | <i>trpC2 lys amyE::[cat PptsG(-55/26)(G1A)-lacZ]</i> | This study |
| FU1045 | <i>trpC2 lys amyE::[cat PptsG(-55/26)(G1A, G2A)-lacZ]</i> | This study |
| FU1044 | <i>trpC2 lys amyE::[cat PpdhA(-47/167)(G1A)-lacZ]</i> | This study |
| FU1060 | <i>trpC2 lys amyE::[cat PpycA(-55/26)(A1G)-lacZ]</i> | This study |
| FU1061 | <i>trpC2 lys amyE::[cat PalsS(-55/26)(A1G)-lacZ]</i> | This study |

^a Strain obtained from the *Bacillus* Genetic Stock Center (Columbus, OH).

pairs PTS-F/PTS-R, PYC-F/PYC-R, ALS-F/ALS-R, and PDH-F/PDH-R (see Table S1 in the supplemental material), respectively, and DNA of strain 168 as the template. The PCR products were trimmed with XbaI and BamHI and then ligated with the XbaI-BamHI arm of plasmid pCRE-test2 (27). The ligated DNAs were used for transformation of *E. coli* strain DH5α to ampicillin resistance (50 μg/ml) on Luria-Bertani medium plates (36). Correct construction of the fusions in the resulting plasmids was confirmed by DNA sequencing. The plasmids carrying the promoter regions of *ptsGHI*, *pycA*, *alsSD*, and *pdhABCD* were linearized with PstI and then used for double-crossover transformation of strains 1A765, 1A766 (*relA1*), and FU808 (*ΔcodY*) to chloramphenicol resistance (5 μg/ml) on TBABG plates, which produced strains FU906, FU1025, and FU1029, strains FU977, FU1023, and FU1027, strains FU937, FU1026, and FU1041, and strains FU1019, FU1021, and FU1031, respectively.

To construct strains FU934, FU1042, and FU1045 carrying the *ptsGHI* promoter-*lacZ* fusion with an adenine substitution(s) at position 1 and/or 2 in the *ptsGHI* promoter (guanine at position 2 changed to adenine [G2A, GA], guanine at position 1 changed to adenine [G1A, AG], and guanine at positions 1 and 2 changed to adenine [G2A G1A, AA]), the promoter regions (nucleotides -55 to 26) were amplified using primer pairs PptsG-F2/PptsG-GA-R, PptsG-F2/PptsG-AG-R, and PptsG-F2/PptsG-AA-R (see Table S1 in the supplemental material), respectively, and chromosomal DNA of strain 168 as the template. The PCR products trimmed with XbaI and BamHI were cloned into plasmid pCRE-test2 (27) 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 base substitution at position 1 and/or 2 (GA, AG, and AA) were each linearized with PstI and then used for transformation of strain 1A765, resulting in strains FU934, FU1042, and FU1045, respectively.

To construct strain FU1044 carrying a *pdhABCD* promoter-*lacZ* fusion with adenine substituted for guanine at position 1 in the *pdhABCD* promoter (G1A), the upstream and downstream parts of the promoter region (nucleotides -47 to 167) were separately amplified with two primer pairs (PpdhA-A1-F/PpdhA-A1-R and PpdhA-A2-F/PpdhA-A2-R) (see Table S1 in the supplemental material) using chromosomal DNA of strain 168 as the template. Next, the two PCR products were mixed, and extension reactions were carried out without any primer. PCR with the resultant fragment as the template and primers PpdhA-A1-F and PpdhA-A2-R was performed to amplify the combined DNA fragment, which was then trimmed with XbaI and BamHI and cloned into plasmid pCRE-test2 (27) in *E. coli* strain DH5α, as described above. Correct base substitution was confirmed by DNA sequencing. The constructed plasmid was linearized with

PstI and then used for transformation of strain 1A765, resulting in strain FU1044.

To construct strains FU1060 and FU1061 carrying *pycA* and *alsSD* promoter-*lacZ* fusions with guanine substituted for adenine at position 1 in the *pycA* and *alsSD* promoters, the promoter regions (nucleotides -55 to 26) were amplified using primer pairs PpycA-F2/PpycA-G-R and PalsS-F2/PalsS-G-R (see Table S1 in the supplemental material), respectively, and chromosomal DNA of strain 168 as the template. The PCR products were trimmed with XbaI and BamHI and were cloned into plasmid pCRE-test2 (27) in *E. coli* strain DH5α, as described above. Correct construction of the fusions in the resulting plasmids was confirmed by DNA sequencing. The plasmids with the base substitutions at position 1 in the *pycA* and *alsSD* promoters were linearized with PstI and then used for transformation of strain 1A765, resulting in strains FU1060 and FU1061, respectively.

Cell growth and β-Gal assay. The *lacZ*-fusion strains were grown at 30°C overnight on TBABG plates containing the appropriate antibiotic(s), chloramphenicol (5 μg/ml) and/or spectinomycin (60 μg/ml). The cells were inoculated into 100 ml of minimal medium comprising 0.4% glucose, 0.2% glutamine, and 50 μg/ml tryptophan (MM medium) (57) supplemented with a mixture of 16 amino acids (glutamine, histidine, tyrosine, and asparagine were omitted) (2) at an optical density at 600 nm (OD₆₀₀) of 0.08 and then incubated at 37°C. When the OD₆₀₀ reached approximately 0.5, 45-ml aliquots of the culture were harvested, and the cells were centrifuged at 25°C (2,000 × g for 10 min). The cells were suspended in 45 ml of MM medium supplemented with the amino acid mixture described above with and without lysine, and then the cultures were incubated further. During incubation before and after cell resuspension, 1-ml aliquots of the culture were withdrawn at 15-min intervals at most, and the β-galactosidase (β-Gal) activity in crude cell extracts was measured spectrophotometrically as described previously (56).

When the effect of decoyinine on *lacZ* expression in the fusion strains was examined, a culture was divided into two 45-ml portions when the OD₆₀₀ of the culture reached approximately 0.5, and then decoyinine (500 μg/ml) was added to one of the portions. The β-Gal activity was monitored as described above.

DNA microarray analysis. DNA microarray analysis using microarrays consisting of 3,941 protein genes was performed as described previously (57). Cells of strains 1A765 and FU808 were grown in two 200-ml cultures using MM medium supplemented with the 16 amino acids described above at 37°C until the OD₆₀₀ was 0.7, and then decoyinine was added to one of the cultures at a final concentration of 500 μg/ml. After cultivation for an additional 15 min, the cells

were harvested by centrifugation, and then total RNA was extracted and purified for synthesis of cDNA labeled with a fluorescent dye (Cy5 for the sample to which decoyinine was added and Cy3 for the control).

Metabolome analysis. The *in vivo* concentrations of metabolites, including nucleotides, were determined by capillary electrophoresis-mass spectrometry (CE-MS) after extraction with methanol, essentially as described previously (43). Cells of strain 1A765 were subjected to decoyinine treatment as described above. The cells were collected on a Millipore Isopore membrane filter (HTTP; pore size, 0.4 μ m; diameter, 47 mm). Each of the cell-bearing membranes was put into a heat-sealable plastic bag containing 2 ml of ice-chilled methanol, and then the cells were detached from the membrane and the bag was sealed. The sealed bags were incubated at 70°C for 1 h. After the cell suspensions were centrifuged to remove cell debris, the supernatants were treated with chloroform to remove lipids. After the upper layer was subjected to ultrafiltration (Millipore Ultrafree-MC; 5,000 nominal molecular weight limit [NMWL]), the filtrates were freeze-dried. After the pellets were dissolved in distilled water, samples were subjected to CE-MS to identify the metabolites in them and to measure the concentrations of the metabolites, as described previously (43). (This metabolome analysis was carried out by Human Metabolome Technologies, Inc., Japan.) The *in vivo* molar concentrations of metabolites were calculated by assuming that the aqueous volume of 1 OD₆₀₀ unit (OD₆₀₀ \times ml) corresponded to 0.83 μ l (8).

Primer extension analysis. Primer extension analysis was performed as described previously (55). RNA samples were prepared as described previously (57) using cells of strain 1A765 that had been grown in MM medium containing the 16-amino-acid mixture described above. Reverse transcription using an RNA sample was initiated with primers PptsG-R, PpdhA-R, and PpycA-R for the *ptsGHI*, *pdhABCD*, and *pycA* transcripts, respectively (see Table S1 in the supplemental material), which had been labeled at the 5' end by using a Megalabel kit (Takara-Bio, Kyoto, Japan) and [γ -³²P]ATP (MP Biomedicals). A template for the dideoxy sequencing reaction for ladder preparation starting from each of the same end-labeled primers was prepared by PCR using primer pairs PptsG-F1/PptsG-R, PpdhA-F/PpdhA-R, or PpycA-F/PpycA-R (see Table S1 in the supplemental material) and DNA from strain 168 as the template.

Northern analysis. Total RNA was extracted from cells of strain 1A765 and purified as described previously (57). The RNA was electrophoresed in a glyoxal gel and then transferred to a Hybond-N membrane (GE Healthcare) (36). The RNA transferred to the membrane was stained with 0.003% methylene blue to check its quality before hybridization. To prepare probes for detection of the *ptsGHI* and *pycA* transcripts, the corresponding products amplified by PCR using primer pairs LptsG-F/LptsG-R, ptsG-F/ptsG-R, ptsH-F/ptsH-R, and pycA-F/pycA-R (see Table S1 in the supplemental material) and chromosomal DNA of strain 168 as the template were labeled with a BcaBEST labeling kit (Takara-Bio, Kyoto, Japan) and [α -³²P]dCTP (MP Biochemicals). Hybridization and transcript detection were carried out as described previously (36).

RESULTS

Stringent control of the genes involved in glucose and pyruvate metabolism. Amino acid starvation and exposure of cells to decoyinine result in a decrease in the GTP level and an increase in the ATP level, which depend on and are independent of RelA, respectively (15, 20, 24, 48). Each of these treatments triggers CodY-dependent and CodY-independent stringent control of many genes, including the *ilv-leu* genes (48). In order to find genes that are under stringent transcription control, we performed a DNA microarray analysis with strains 1A765 (*lys*) and FU808 (*lys* Δ *codY*) which had been grown on MM medium with the amino acid mixture described above to the mid-logarithmic phase and incubated for 15 min with and without decoyinine (decoyinine treatment) in the wild-type and Δ *codY* genetic backgrounds. We detected hundreds of genes that are under negative and positive stringent control triggered by decoyinine addition (>3-fold change in the wild-type background level) (see the DNA microarray data deposited in the KEGG Expression Database [http://www.genome.jp/kegg/expression]). As expected, the *rpl* and *rps* genes coding for ribosomal proteins derived from the large and small subunits, respectively (22), were severely downregulated.

TABLE 2. DNA microarray analysis of CodY-dependent and -independent stringent control induced by decoyinine addition

| Gene ^a | Ratio of expression with decoyinine to expression without decoyinine in the CodY ⁺ background ^b | Ratio of expression with decoyinine to expression without decoyinine in the CodY ⁻ background ^b | CodY ⁻ /CodY ⁺ expression ratio ^{b,c} |
|-------------------|---|---|--|
| <i>alsS</i> | 21 | 7.5 | 1.0 |
| <i>alsD</i> | 13 | 7.8 | 0.54 |
| <i>pdhA</i> | 0.13 | 0.09 | 0.70 |
| <i>pdhB</i> | 0.10 | 0.06 | 0.54 |
| <i>pdhC</i> | 0.13 | 0.16 | 0.73 |
| <i>pdhD</i> | 0.16 | 0.31 | 0.75 |
| <i>ilvB</i> | 4.8 | 1.7 | 38 |
| <i>ilvN</i> | 5.9 | 1.9 | 44 |
| <i>ilvC</i> | 8.0 | 1.9 | 49 |
| <i>leuA</i> | 8.7 | 1.8 | 47 |
| <i>leuB</i> | 15 | 1.6 | 46 |
| <i>leuC</i> | 24 | 1.50 | 46 |
| <i>leuD</i> | ND ^d | ND | ND |
| <i>ptsG</i> | 0.01 | 0.03 | 0.88 |
| <i>ptsH</i> | 0.60 | 0.24 | 0.81 |
| <i>ptsI</i> | 0.45 | 0.33 | 0.70 |
| <i>pycA</i> | 3.1 | 2.2 | 0.96 |

^a Of the genes that are involved in glucose and pyruvate metabolism (Fig. 1), those whose expression was altered more than 3-fold upon addition of decoyinine in the wild-type background are included. The *ptsHI* genes are also included.

^b The standard deviations of the expression ratios were less than 15%.

^c The expression ratios were obtained by a DNA microarray analysis performed previously (28).

^d ND, the expression signal of *leuD* was not detectable due to unknown technical problems with our DNA microarray analysis.

Of the operons involved in glucose and pyruvate metabolism shown in Fig. 1, only the *ptsGHI* (10, 45) and *pdhABCD* (9, 13) operons were severely downregulated upon decoyinine treatment. In contrast, *ilv-leu* (11, 48), *pycA* (5), and *alsSD* (35) were upregulated substantially. Table 2 shows the expression ratios (expression with decoyinine/expression without decoyinine) for these genes that were upregulated and downregulated upon decoyinine treatment. Table 2 also shows that the expression ratios (expression in Δ *codY* strain PS37/expression in wild-type strain PS29) obtained in a previous DNA microarray analysis (28). In addition to expression of the genes in the *ilv-leu* operon, a direct target of CodY, *ptsG* expression also changed more than 2-fold upon *codY* disruption. However, we do not know whether the *ptsG* gene is a direct or indirect target of CodY. For stringent control, the expression ratios of the upregulated and downregulated genes in the wild-type background in general were higher and lower than the expression ratios in the Δ *codY* background, respectively. This could be explained by relief from CodY repression, as observed for *ilv-leu*. Also, some enhancement of the CodY-independent stringent control of these genes might occur in the wild-type background, as previously observed for *ilv-leu* (48).

Changes in the *in vivo* concentrations of the intermediates of glucose and pyruvate metabolism upon decoyinine addition. In addition to the results of the DNA microarray analysis described above (Table 2), the results of a metabolome analysis of the changes in the *in vivo* concentrations of the intermediates of glucose and pyruvate metabolism (Fig. 1) upon decoyinine treatment in the CodY⁺ background of strain 1A765 (*lys*)

TABLE 3. Metabolome analysis to determine the change in *in vivo* metabolite concentrations after decoyinine addition

| Compound | <i>In vivo</i> concn (mM) without decoyinine addition ^a | <i>In vivo</i> concn (mM) with decoyinine addition ^a |
|----------------------------|--|---|
| Glucose-6-phosphate | 6.3 | 7.7 |
| Fructose-6-phosphate | 2.8 | 3.6 |
| Fructose-1,6-bisphosphate | 4.5 | 0.8 |
| Dihydroxyacetone phosphate | 0.5 | 0.2 |
| Glyceraldehyde 3-phosphate | 46 | 36 |
| Phosphoenolpyruvate | 22 | 20 |
| Lactate | 270 | 410 |
| Acetyl-CoA | 7.7 | 7.3 |
| Citrate | 1.7 | 7.1 |
| Isocitrate | 0.3 | 0.4 |
| 2-Oxoglutarate | 0.5 | 1.4 |
| Succinate | 54 | 38 |
| Fumarate | 4.3 | 4.2 |
| Malate | 1.7 | 2.6 |
| Ala | 150 | 190 |
| Val | 9.9 | 40 |
| Homoserine | 3.9 | 3.7 |
| Thr | 21 | 44 |
| Ile | 17 | 35 |
| Leu | 11 | 31 |
| Asn | 0.8 | 1.4 |
| Asp | 25 | 200 |
| Lys | 12 | 21 |
| GTP | 0.4 | 0.19 |
| ATP | 3.1 | 6.6 |
| ppGpp | ND ^b | ND |

^a The standard deviations of the values were less than 20%.

^b ND, not determined.

that was part of a previous metabolome analysis (48) are shown in Table 3. In this analysis we were able to determine the *in vivo* concentrations of only some of the intermediates of carbon metabolism shown in Fig. 1, those listed in Table 3. Upon decoyinine addition, the concentrations of GTP and ATP decreased and increased, respectively. But ppGpp was undetectable when decoyinine was added, in contrast to its detection upon lysine starvation (48). The only intermediates whose concentrations decreased more than 2-fold were fructose-1,6-bisphosphate (FBP) and dihydroxyacetone phosphate (DHAP) in the glycolytic pathway, which likely resulted from the shutdown of the PTS for glucose transport triggered by decoyinine treatment. On the other hand, the compounds whose concentrations increased more than 2-fold were amino acids derived from oxaloacetate (Val, Thr, Ile, Leu, Asp, and Lys), and the increase in the Asp concentration was the most prominent increase. This could be explained by the induction of *pycA* and *ilv-leu* under stringent control triggered by addition of decoyinine. Moreover, the concentrations of citrate and 2-oxoglutarate in the tricarboxylic acid (TCA) cycle also increased more than 2-fold, and the concentration of isocitrate also increased. This might be explained by the finding that CcpA could not repress the *citZ* gene encoding citrate synthase that condenses acetyl coenzyme A (acetyl-CoA) with oxaloacetate (17). (The complex consisting of CcpA and P-Ser-HPr was not formed well with a low *in vivo* concentration of FBP [7], which resulted from strong negative stringent control of *ptsGHI*.) Actually, *citZ* expression increased 2.4-fold upon decoyinine addition in the wild-type background as determined by the DNA microarray analysis described above.

Negative stringent control of the *ptsGHI* operon. DNA microarray analysis indicated that the *ptsGHI* operon was severely downregulated upon decoyinine addition, suggesting that it might be under strong negative stringent control. Transcription and regulation of the *ptsGHI* operon were studied previously by Stülke et al. (45). As shown in Fig. 2A, there are the two transcription promoters for the *ptsGHI* operon; one of these promoters is P_{*ptsGHI*} for *ptsGHI* transcription, and the other is P_{*ptsHI*} for *ptsHI* transcription. GlcT, an antiterminator belonging to the BglG/SacY family, binds to a ribonucleic antiterminator upstream of *ptsG* in the presence of glucose. This prevents formation of an overlapping transcriptional terminator. Northern analysis of the *pts* transcripts with the corresponding probes for the *ptsG* leader (ORF), and the *ptsH* ORF (Fig. 2B) indicated that the transcription from P_{*ptsGHI*} was severely downregulated upon lysine starvation (Fig. 2B, lanes 2, 5, and 8), as well as upon decoyinine addition (lanes 3, 6, and 9), whereas the transcription from P_{*ptsHI*} was downregulated only moderately. The former downregulation was likely due to CodY-independent stringent control, as verified below. The mechanism underlying the latter moderate downregulation cannot be explained properly at present. We also performed a primer extension analysis to identify the same transcription initiation guanine residue of *ptsGHI* (336 nucleotides upstream of the translation initiation site of *ptsG*) that was determined previously by Stülke et al. (45) (Fig. 2C). This analysis indicated that the transcription from this guanine was downregulated upon lysine starvation (Fig. 2C, lanes + and -).

In order to characterize the stringent control of the *ptsGHI* promoter, the core promoter region (nucleotides -55 to 26) was placed upstream of *lacZ* in the wild-type, *relA1*, and Δ *codY* backgrounds, and the β -Gal synthesis under control of the core *ptsGHI* promoter was monitored upon lysine starvation and decoyinine treatment (Fig. 3A). When lysine was depleted from the medium, the cells stopped growing, and β -Gal synthesis decreased in the wild-type background. (Cell growth and β -Gal synthesis were maintained for more than 1 h if lysine was not depleted.) A decrease in β -Gal synthesis was not observed in the *relA1* background but was observed in the Δ *codY* background. When decoyinine was added to the medium, β -Gal synthesis decreased to almost the same extent in the wild-type, *relA1*, and Δ *codY* backgrounds. The results indicate that the negative regulation was independent of CodY and suggest that it was likely mediated by a decrease in the GTP level caused by IMP dehydrogenase inhibition by ppGpp synthesized by RelA upon lysine starvation or by GMP synthase inhibition by decoyinine.

Replacement of guanines at the transcription initiation sites (positions 1 and 2) of the *ptsGHI* operon by adenine. According to the results of the primer extension analyses (45) (Fig. 2C), the base at the transcription initiation site (position 1) of the *ptsGHI* operon was a guanine. It was reported previously that the initiating base for transcription from *B. subtilis* rRNA promoters is a guanine and that changes in the promoter activity always correlate with changes in the intracellular GTP concentration (19). Also, adenine at the transcription initiation site of the *ilv-leu* operon is critical for positive stringent control of this operon, which likely correlates with changes in the intracellular ATP concentration (20, 48). To determine if adenine substitution for guanine at positions 1 and 2 affects the

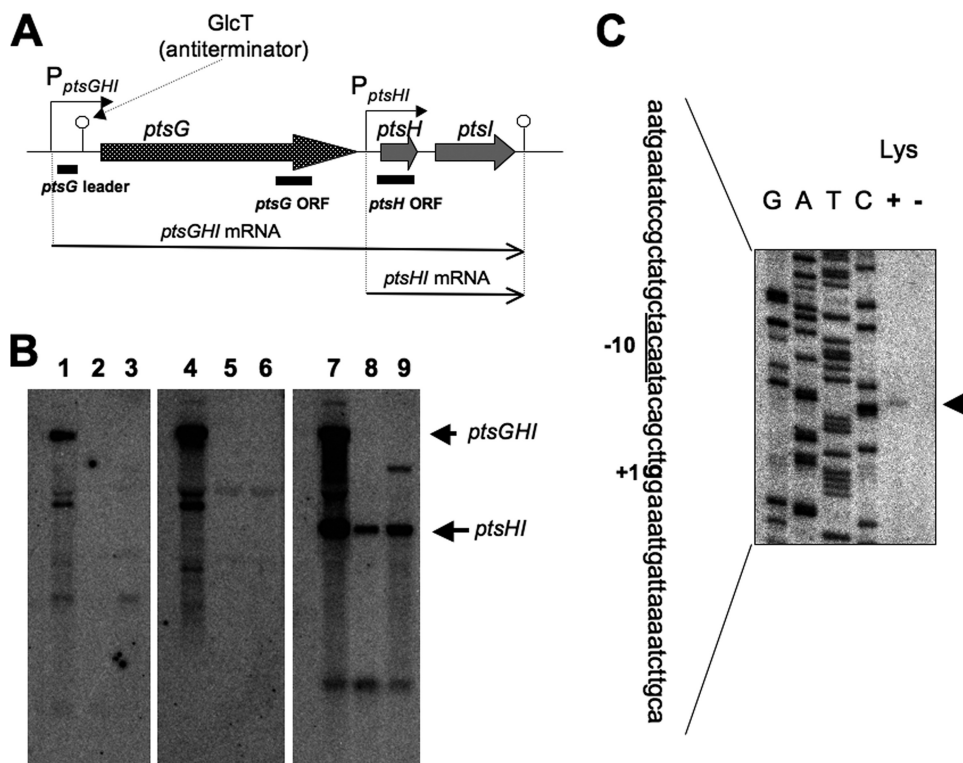


FIG. 2. Downregulation of *ptsGHI* transcription upon lysine starvation and decoyinine treatment. (A) The *ptsGHI* operon has two promoters, P_{ptsGHI} for *ptsGHI* transcription and P_{ptsHI} for *ptsHI* transcription (45). GlcT, an antiterminator belonging to the BglG/SacY family, binds to a ribonucleic antiterminator upstream of *ptsG* in the presence of glucose. This prevents formation of an overlapping transcriptional terminator. The *ptsGHI* and *ptsHI* transcription terminators are also located downstream of *ptsI*. (B) Northern analysis of the stringent control of *ptsGHI* transcription. RNA samples were prepared from cells of strain 1A765 (*lys*) that had been subjected to lysine starvation for 30 min (lanes 2, 5, and 8) or to decoyinine treatment (500 μ g/ml) for 30 min (lanes 3, 6, and 9) and from cells that were not subjected to these stresses (lanes 1, 4, and 7). The transcripts in the left, middle, and right panels were detected with the probes for the *ptsG* leader, *ptsG* ORF, and *ptsH* ORF (indicated in panel A), respectively, whose preparation is described in Materials and Methods. The positions of the *ptsGHI* and *ptsHI* transcripts are indicated by the arrows on the right. (C) Primer extension analysis for mapping of the 5' end of the *ptsGHI* transcript. Total RNA from cells of strain 1A765 (*lys*) which had been subjected to lysine starvation for 30 min (lane -) and total RNA from cells of strain 1A765 which had not been subjected to this nutrient stress (lane +) were annealed with the PptsG-R primer (see Table S1 in the supplemental material), and then primer extension was performed as described in Materials and Methods. Lanes G, A, T, and C contained the products of the corresponding dideoxy sequencing reactions with the PCR product as the template, as described in Materials and Methods. The part of the nucleotide sequence of the coding strand corresponding to the ladder is shown on the left; the transcription initiation base (+1) is indicated by bold type, and the corresponding -10 region for the *ptsGHI* promoter is underlined. The position of the runoff cDNA is indicated by the arrow on the right.

negative stringent control of *ptsGHI*, we constructed strains FU934, FU1042, and FU1045, in which the original GG (positions 1 and 2) was replaced by GA, AG, and AA, respectively, in the core *ptsGHI* promoter region (nucleotides -55 to 26), which had been placed upstream of *lacZ* in the wild-type genetic background (Fig. 4A).

Although negative stringent regulation of the core *ptsGHI* promoter was observed upon lysine starvation in strain FU906 carrying the original GG residues (Fig. 4B, left panel), this regulation was somehow quenched in strain FU1042 (AG) and was almost completely absent in strain FU934 (GA). When the two guanines at positions 1 and 2 were replaced by adenines, resulting in strain FU1045 (AA), the *lacZ* expression under control of this variant of the core *ptsGHI* promoter was quite low, but the original negative stringent regulation was clearly converted to positive regulation. When cells of these strains were exposed to decoyinine (Fig. 4B, right panel), the original negative regulation in strain FU906 (GG) was converted to rather positive regulation in strains FU1042 (AG) and FU934

(GA). Also, the core *ptsG* promoter of strain FU1045 (AA) responded most positively to decoyinine treatment, although its activity was quite low. These results suggest that guanines at positions 1 and 2 in the *ptsGHI* transcription initiation site are indispensable for negative stringent control and that the original negative regulation was easily converted to positive stringent regulation in the order AG-GA-AA when the guanines were replaced by adenines.

Negative stringent control of the *pdhABCD* operon. DNA microarray analysis suggested that the *pdhABCD* operon encoding the four subunits of the pyruvate dehydrogenase multienzyme complex (9, 13) might also be under strong negative stringent control (Table 2). This operon is transcribed as a 5.2-kb transcript from the promoter upstream of *pdhA* (Fig. 5) (13). The promoter for *pdhABCD* was designated P1, although its transcription initiation base was not determined (13). This putative promoter was found to be unable to function in *lacZ* fusion experiments (data not shown), so we performed a primer extension analysis to determine the transcription initi-

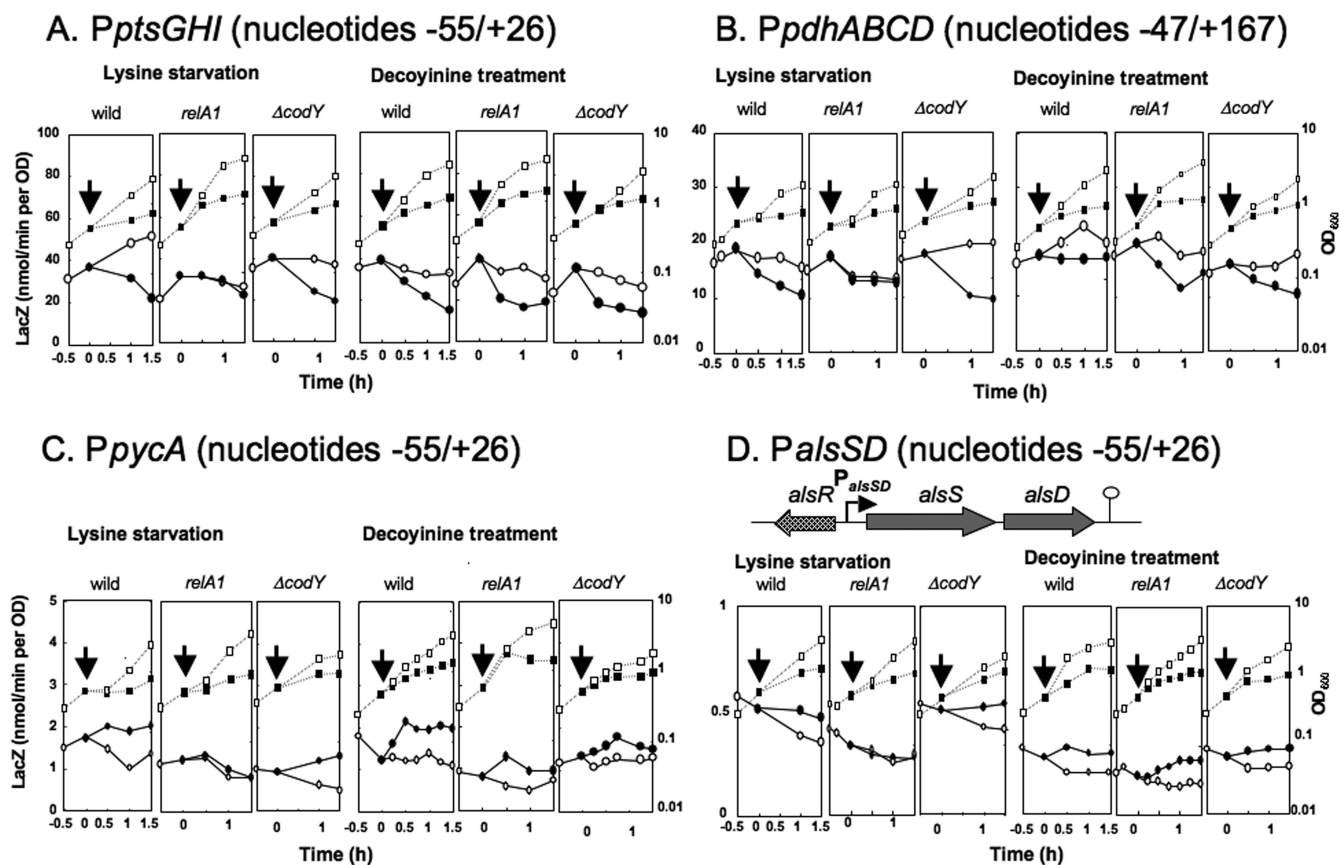


FIG. 3. *lacZ* expression under control of the promoters of the stringent operons. The core *ptsGHI*, *pycA*, and *alsSD* promoters (nucleotides -55 to 26), as well as the *pdhABCD* promoter (nucleotides -47 to 164), were placed upstream of *lacZ*, and each of the resultant *lacZ* fusions was integrated into the *amyE* locus of the wild-type, *relA1*, and $\Delta codY$ strains. *lacZ* expression under control of the *ptsGHI* (A), *pdhABCD* (B), *pycA* (C), and *alsSD* (D) promoters (P_{ptsGHI} , $P_{pdhABCD}$, P_{pycA} , and P_{alsSD}) was monitored using strains FU906, FU1025, and FU1029, strains FU1019, FU1021, and FU1031, strains FU977, and FU1023, and FU1027, and FU1027, and strains FU937, FU1026, and FU1041, respectively, upon lysine starvation and decoyinine treatment, as described in Materials and Methods. OD₆₀₀ values and LacZ activities are indicated by squares and circles; filled and open symbols indicate treatments with and without lysine starvation and decoyinine, respectively. Arrows indicate the onset of these stresses. In the upper part of panel D, *alsSD* transcription, whose activation is mediated by AlsR, is shown.

ation base of *pdhABCD*. We found that the transcription initiation base is a guanine located 211 bases upstream of the translation initiation base of the *pdhA* gene (Fig. 5), which enabled us to determine its -35 and -10 regions likely recognized by σ^A RNA polymerase. The transcription from this guanine was severely downregulated upon lysine starvation (Fig. 5, lanes + and $-$). To examine this negative regulation of the *pdhABCD* promoter, the promoter region (nucleotides -47 to 167) was placed upstream of *lacZ* in the wild-type, *relA1*, and $\Delta codY$ backgrounds. The β -Gal synthesis under control of the *pdhABCD* promoter was monitored upon lysine starvation and decoyinine treatment (Fig. 3B). A decrease in β -Gal synthesis upon lysine starvation was observed in the wild-type and $\Delta codY$ backgrounds, but not in the *relA1* background. When decoyinine was added to the medium, β -Gal synthesis decreased to almost the same extent in the wild-type, *relA1*, and $\Delta codY$ backgrounds. The results indicate that this negative regulation was also independent of CodY.

The transcription initiation base was found to be a guanine (Fig. 5). To determine if replacement of this guanine by adenine affects the negative stringent control of *pdhABCD*, we constructed

strain FU1044 (with A) with this replacement in the *pdhABCD* promoter region (nucleotides -47 to 167) upstream of *lacZ* in the wild-type genetic background (Fig. 6A). Although negative stringent control of the *pdhABCD* promoter was observed upon lysine starvation in strain FU1019 with the original guanine residue (Fig. 6B, left panel), it was almost completely absent in strain FU1044 with A (Fig. 6B, right panel). When cells of these strains were exposed to decoyinine, the original negative regulation observed in strain FU1019 (with G) was completely quenched in strain FU1044 (with A) (Fig. 6B, right panel). These results suggest that the guanine at position 1 in the *pdhABCD* transcript is indispensable for this negative stringent control.

Positive stringent control of the *pycA* gene. The *pycA* gene encodes a pyruvate carboxylase that converts pyruvate to oxaloacetic acid through CO₂ fixation. This enzyme is rather constitutively synthesized and strongly activated by acetyl-CoA (5). *pycA* mRNA is 3.4 kb long (Fig. 7A), indicating that this gene is monocistronically transcribed. Northern analysis indicated that *pycA* expression was induced approximately 3-fold upon lysine starvation (Fig. 7A, lanes + and $-$). Primer extension analysis was performed to determine the transcription

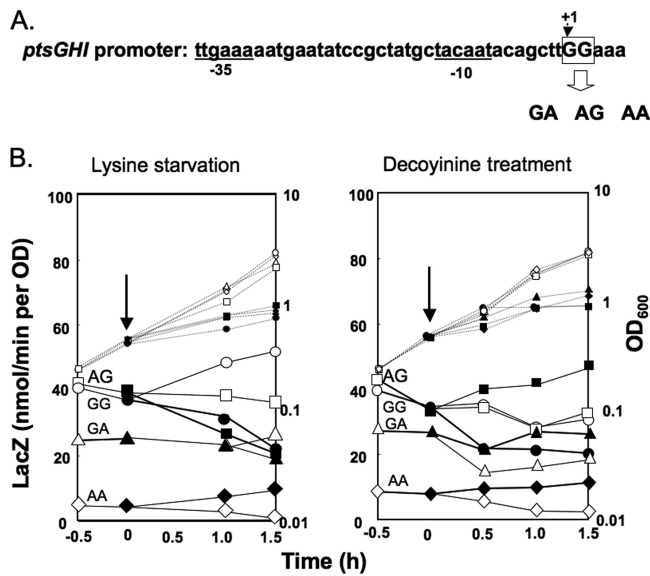


FIG. 4. Effect of replacement of guanines at the transcription initiation bases (positions 1 and 2) of the *ptsGHI* operon by adenine on negative stringent control of this operon. (A) The two guanines (GG) at the transcription initiation site (positions 1 and 2) of the *ptsGHI* promoter (nucleotides -55 to 26) were replaced by GA, AG, and AA, as described in Materials and Methods. The -35 and -10 regions of the *ptsGHI* promoter are underlined. (B) The mutant core *ptsGHI* promoters were placed upstream of *lacZ* in the *relA*⁺ and *codY*⁺ backgrounds. *lacZ* expression in the resulting strains (FU934 with GA, FU1042 with AG, and FU1045 with AA), as well as in strain FU906 with GG, was monitored upon lysine starvation and decoyinine treatment, as described in Materials and Methods. OD₆₀₀ values (small symbols) and *LacZ* activities (large symbols) are indicated by circles for FU906 with GG, by squares for FU1042 with AG, by triangles for FU934 with GA, and by diamonds for FU1045 with AA; filled and open symbols indicate results obtained with and without lysine starvation and decoyinine treatment, respectively. The arrows indicate the onset of the stresses.

initiation base of *pycA* (Fig. 7B). There were two bands corresponding to adenines (positions 1 and 3); the transcription initiation adenine for the longer transcript was assigned to position 1 (30 nucleotides upstream of the translation initiation base of *pycA*), which enabled us to assign its -35 and -10 regions likely recognized by σ^A RNA polymerase. These transcripts were induced approximately 5-fold upon lysine starvation (Fig. 7B, lanes - and +).

To examine the positive regulation of the *pycA* promoter, the core promoter region (nucleotides -55 to 26) was placed upstream of *lacZ* in the wild-type, *relA1*, and Δ *codY* backgrounds. The β -Gal synthesis under control of the *pycA* promoter was monitored upon lysine starvation and decoyinine treatment (Fig. 3C). An increase in β -Gal synthesis upon lysine starvation was observed in the wild-type and Δ *codY* backgrounds, but not in the *relA1* background. When decoyinine was added to the medium, β -Gal synthesis increased to almost the same extent in the wild-type, *relA1*, and Δ *codY* backgrounds. The results indicate that this positive regulation was also independent of CodY. Thus, the positive regulation of the presumably constitutive core *pycA* promoter was most likely due to the CodY-independent stringent control enhancing the transcription initiation rate, as observed for *ilv-leu* (20, 48).

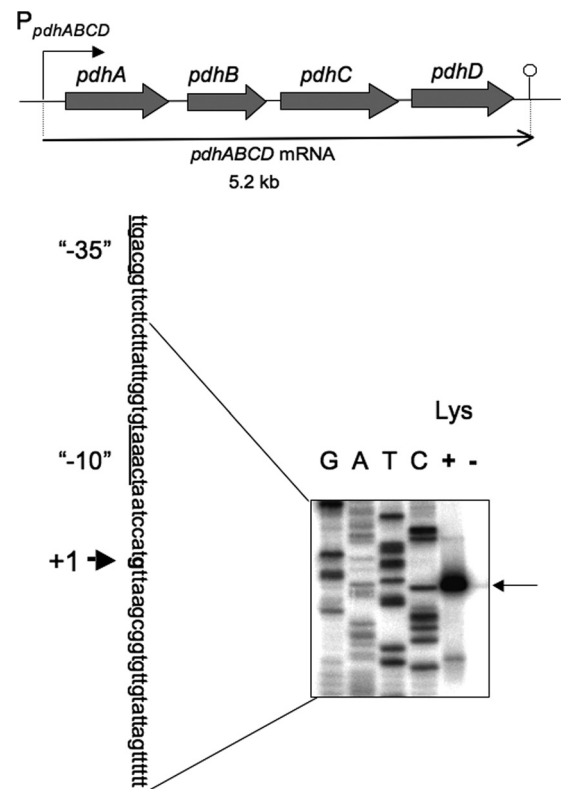


FIG. 5. Transcription of the *pdhABC* operon. The genes in the *pdhABC* operon encode the pyruvate decarboxylase E1 α and E1 β , dihydrolipoamide acetyltransferase E2, and dihydrolipoamide dehydrogenase E3 subunits of the pyruvate dehydrogenase multienzyme complex (PDH), respectively. Transcription of this operon results in a 5.2-kb *pdhABC* mRNA (13). The 5' end of the *pdhABC* transcript was determined by primer extension analysis (lower panel). Total RNAs from cells of strain 1A765 (*lys*) that had been subjected to lysine starvation for 30 min (lane -) and from cells not subjected to this nutrient stress (lane +) were annealed with the PpdhA-R primer (see Table S1 in the supplemental material), and then primer extension was performed, as described in Materials and Methods. Lanes G, A, T, and C contained the products of the corresponding dideoxy sequencing reactions performed with the PCR product as the template, as described in Materials and Methods. The nucleotide sequence of the coding strand corresponding to the ladder is shown; the transcription initiation base (+1) is indicated by bold type, and the -35 and -10 regions of the *pdhABC* promoter are underlined. The position of the runoff cDNA is indicated by the arrow on the right.

The transcription initiation bases were found to be adenines (positions 1 and 3) (Fig. 7B). To determine if replacement of the adenine at position 1 by guanine affects the positive stringent control of *pycA*, we constructed strain FU1060 with this substitution in the *pycA* promoter region (nucleotides -55 to 26) upstream of *lacZ* in the wild-type genetic background. The positive stringent control of the *pycA* promoter that was observed upon lysine starvation in strain FU977 with the original adenine residue was slightly but significantly quenched in strain FU1060 with the guanine replacement (data not shown). When cells of these strains were exposed to decoyinine, the original positive regulation observed in strain FU977 (with A) was largely quenched in strain FU1060 (with G) (Fig. 8, left panel). These results suggest that the adenine at position 1 in the *pycA* transcript is involved in this positive stringent control.

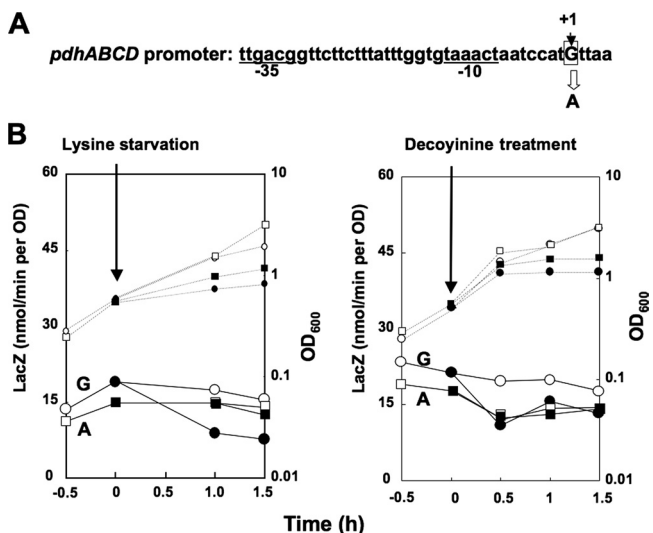


FIG. 6. Effect of replacement of guanine at the transcription initiation base (+1) of the *pdhABCD* operon with adenine on the negative stringent control of this operon. (A) The guanine at the transcription initiation site (+1) of the *pdhABCD* promoter (nucleotides -47 to 167) was replaced by adenine. The -35 and -10 regions of the *pdhABCD* promoter are underlined. (B) The mutant *pdhABCD* promoter was placed upstream of *lacZ* in the *relA*⁺ and *codY*⁺ backgrounds. *lacZ* expression in the resulting strains (FU1019 [wild type] and FU1044 [mutant]) was monitored upon lysine starvation and decoyinine treatment, as described in Materials and Methods. OD₆₀₀ values (small symbols) and LacZ activities (large symbols) are indicated by circles for FU1019 and by squares for FU1044; filled and open symbols indicate results obtained with and without lysine starvation and decoyinine treatment, respectively. The arrows indicate the onset of these stresses.

This partial effect of substitution of guanine for adenine at position 1 on the positive stringent response had been expected because the other transcription initiation from the adenine at position 3 appeared to occur in this mutant promoter.

Positive stringent control of the *alsSD* operon. The *alsSD* genes, which comprise an operon, encode acetolactate synthase and acetolactate decarboxylase, respectively, whereas a positive regulator of AlsR, encoded by a gene divergent from *alsSD*, is involved in the induction of *alsSD* in the postexponential growth phase (35) (Fig. 3D, upper panel). It is also known that CcpA is a positive transcriptional regulator of *alsSD*. However, CcpA activation appears to be indirect because a catabolite-responsive element of *cre* is unlikely to be present in the *alsSD* promoter region (7, 51). Transcription of this operon starts at an adenine that is 51 bases upstream of the translation initiation base of *alsS* (35). DNA microarray analysis suggested that *alsSD* might be under CodY-independent positive stringent control. To examine this positive regulation of the *alsSD* promoter, the core promoter region (nucleotides -55 to 26) was placed upstream of *lacZ* in the wild-type, *relA1*, and Δ *codY* backgrounds. The β -Gal synthesis under control of this promoter was monitored upon lysine starvation and decoyinine treatment (Fig. 3D). The β -Gal synthesis during the exponential growth phase without these stringent stresses, which was quite low, presumably due to a lack of positive regulation by AlsR, decreased continuously; we currently cannot explain this decrease. Thus, β -Gal synthesis upon lysine

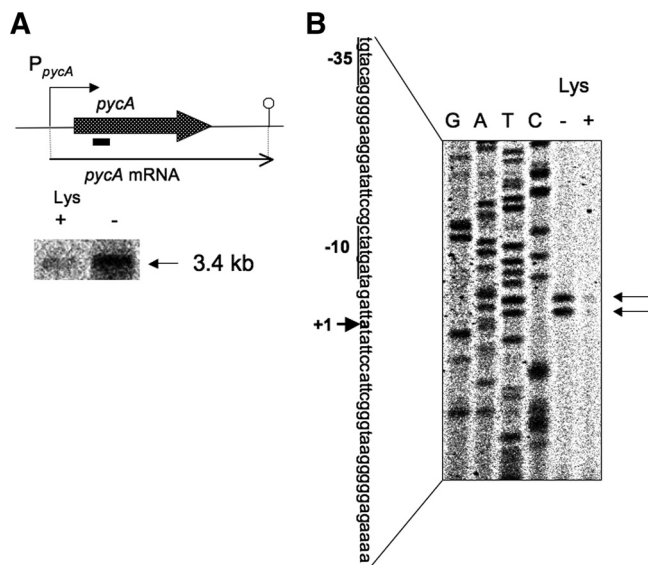


FIG. 7. Transcription of the *pycA* gene. (A) The *pycA* gene encodes pyruvate carboxylase. Its transcription results in a 3.4-kb monocistronic *pycA* mRNA. Northern analysis of the stringent control of *pycA* transcription was performed. RNA samples were prepared from cells of strain 1A765 (*lys*) that had been subjected to lysine starvation for 30 min and from cells that had not been starved. The 3.4-kb transcript was detected with the *pycA* probe (indicated by a bar). (B) The 5' end of each of the *pycA* transcripts was mapped. Total RNAs from cells of strain 1A765 (*lys*) that had been subjected to lysine starvation for 30 min (lane -) and cells that had not been subjected to this nutrient stress (lane +) were annealed with the PpycA-R primer (see Table S1 in the supplemental material), and then primer extension was performed, as described in Materials and Methods. Lanes G, A, T, and C contained the products of the corresponding dideoxy sequencing reactions performed with the PCR product as the template, as described in Materials and Methods. The nucleotide sequence of the coding strand corresponding to the ladder is shown; the transcription initiation site (+1) is indicated by bold type (the upper initiation base in lane -), and another initiation base was observed in the same lane (lower base). The corresponding -35 and -10 regions of the *pdhABCD* promoter are underlined. The two positions of the runoff cDNA are indicated by the arrows on the right.

starvation was maintained in the wild-type and Δ *codY* backgrounds, but not in the *relA1* background, in contrast to the decrease observed without lysine starvation. When decoyinine was added to the medium, β -Gal synthesis was maintained at almost the same level in the wild-type, *relA1*, and Δ *codY* backgrounds, in contrast to the decrease in the absence of decoyinine. The results indicate that the CodY-dependent positive regulation was also dependent on RelA upon lysine starvation and independent of RelA upon decoyinine treatment. The region upstream of nucleotide -55 is truncated in the core *alsSD* promoter, so it is very unlikely that AlsR or CcpA is responsible for this positive regulation. Thus, this positive regulation is most likely due to CodY-independent stringent control, as observed for *ilv-leu* (20, 48).

The transcription initiation base of the *alsSD* operon was reported to be an adenine (position 1). To determine if replacement of this adenine by guanine affects the positive stringent control of *alsSD*, we constructed strain FU1061 with this substitution in the *alsSD* promoter region (nucleotides -55 to 26) upstream of *lacZ* in the wild-type genetic background. The

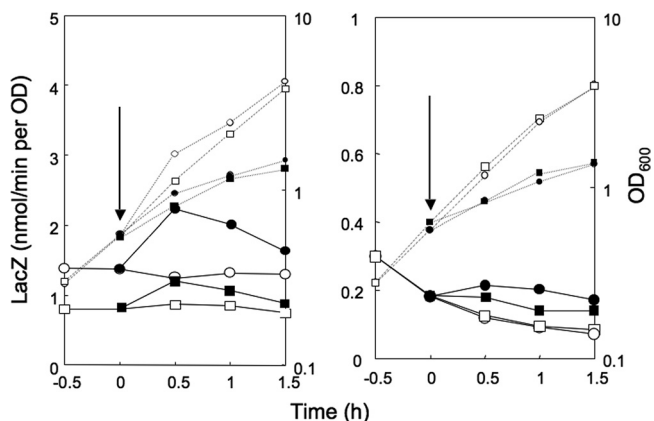


FIG. 8. Effect of replacement of adenine at each of the transcription initiation bases (position 1) of the *pycA* (left panel) and *alsSD* (right panel) operons by guanine on positive stringent control. The adenine at the transcription initiation site (+1) of the *pycA* and *alsSD* promoters (nucleotides -55 to 26) was replaced by guanine. The mutant *pycA* and *alsSD* promoters were placed upstream of *lacZ* in the *relA*⁺ and *codY*⁺ background. *lacZ* expression in wild-type strains FU977 (*pycA*) and FU937 (*alsSD*) and mutant strains FU1060 (*pycA* mutant) and FU1061 (*alsSD* mutant) was monitored upon decoyinine treatment, as described in Materials and Methods. OD₆₀₀ values (small symbols) and *LacZ* activities (large symbols) are indicated by circles for FU977 and FU937 and by squares for FU1060 and FU1061; filled and open symbols indicate results obtained with and without decoyinine treatment, respectively. The arrows indicate the onset of decoyinine treatment.

positive stringent control of the *alsSD* promoter, which was observed upon lysine starvation in strain FU937 with the original adenine, was slightly but significantly quenched in strain FU1061 with the guanine substitution (data not shown). When cells of these strains were exposed to decoyinine, the original positive regulation observed in strain FU937 (with A) was partially quenched in strain FU1061 (with G) (Fig. 8, right panel). These results suggest that the adenine at position 1 in the *alsSD* transcript is only partially involved in this positive stringent control. However, this does not necessarily mean that positive transcription regulation other than the CodY-independent positive stringent control is involved in this positive regulation, because the introduction of guanine at position 1 may cause a shift of the transcription initiation base from guanine at position 1 to other bases, such as cytosine at position 2 and adenine at position 3. Such a shift of the transcription initiation base upon introduction of the base substitution into the transcription initiation site was actually observed for *ilv-leu* (48).

DISCUSSION

The stringent transcription control of the *ilv-leu* operon was investigated in detail previously (20, 48), which revealed the molecular mechanisms underlying this control (see Fig. S1 in the supplemental material). Upon amino acid (lysine in our study [48]) starvation, the RelA protein synthesizes ppGpp, a probable inhibitor of IMP dehydrogenase. The resulting inhibition causes a decrease in the GTP level, whereas it increases the ATP level. Decoyinine treatment also induced these reciprocal changes in the GTP and ATP levels by inhibiting GMP

synthase. Thus, two mechanisms underlying the positive stringent control of the *ilv-leu* operon in response to changes in the GTP and ATP levels have been proposed (20, 48). Mechanism 1, a CodY-dependent mechanism, involves GTP, a corepressor of CodY, so a decrease in the GTP level results in detachment of CodY from its binding sites, resulting in positive regulation of *ilv-leu*. In mechanism 2, a CodY-independent mechanism, the *ilv-leu* promoter is activated by an increase in the ATP level. *In vitro* transcription analysis indicated that initiation of *ilv-leu* transcription tolerates a decrease in the level of GTP to a level in the range of the *in vivo* concentrations upon lysine starvation and decoyinine addition (20, 48). Also, an increase in the ATP level actually enhances the initiation of *ilv-leu* transcription *in vitro* (20). Conservation of adenine at the transcription initiation site is critical for this positive regulation. When the adenine was replaced by guanine, the positive regulation of the core *ilv-leu* promoter changed to negative regulation. However, whether this adenine is located at position 1 or position 2 is unclear, because there is a difference between the two reports (20, 48).

DNA microarray analysis involving the CodY⁺ and CodY⁻ strains that had been exposed and had not been exposed to decoyinine revealed that of the operons involved in glucose and pyruvate metabolism (Fig. 1 and Table 2), the *ptsGHI* (10, 45) and *pdhABCD* (9, 13) operons and the *pycA* (5) and *alsSD* (35) operons in addition to the *ilv-leu* operon are under negative and positive stringent control, respectively. Northern analysis showed that transcription from the *ptsGHI* promoter, but not transcription from the *ptsHI* promoter, was subject to the negative stringent control (Fig. 2). The *lacZ* fusion experiments revealed that the negative stringent control of the core *ptsGHI* promoter (nucleotides -55 to 26) was independent of CodY and mediated by ppGpp upon lysine starvation but not upon decoyinine addition (Fig. 3A). The guanine residues at the transcription initiation site (positions 1 and 2) of the *ptsGHI* operon were found to be indispensable for this negative regulation (Fig. 4). Similar experiments showed that the negative stringent control of the newly assigned *pdhABCD* promoter was independent of CodY and likely mediated by the decrease in the GTP level resulting from lysine starvation and decoyinine addition (Fig. 3B). The base substitution experiments revealed that the guanine at position 1 in the *pdhABCD* transcript was indispensable for the negative stringent control (Fig. 6). These results indicate that the negative stringent control of the *ptsGHI* and *pdhABCD* operons was dependent on the guanine residues at the transcription initiation site (positions 1 and 2) and was most likely triggered by the decrease in the GTP level caused by lysine starvation or decoyinine addition, as reported previously for the *B. subtilis* *rrnB* and *rrnO* operons (19). It is amazing that the entrance genes for glycolysis and the TCA cycle encoded by *ptsGHI* and *pdhABCD* are most likely subject to the same negative stringent control as *rrn*, *rps*, and *tufA* involved in protein synthesis (19, 20).

Not only the *ilv-leu* operon but also the *pycA* and *alsSD* operons involved in pyruvate metabolism are under CodY-independent positive stringent control caused by lysine starvation or decoyinine addition (Fig. 1 and Table 2). The *lacZ* fusion experiments indicated that the positive stringent control of *pycA* and *alsSD* is dependent on RelA but independent of

CodY (Fig. 3C and 3D), as observed for the *ilv-leu* operon (20, 48). Since the core *pycA* and *alsSD* promoter regions (nucleotides –55 to 26) were placed upstream of *lacZ* in these fusion experiments, it is very unlikely that this positive regulation of *pycA* and *alsSD* is caused by another, unknown transcription activator because of the probable elimination of the *cis*-acting site upstream of the –35 promoter regions if there is any such site. Moreover, it is also unlikely that an additional global regulator, such as CodY, is involved in this positive stringent control of the core *ilv-leu* (48), *pycA*, and *alsSD* promoters (nucleotides –55 to 26). Thus, the promoter activity of *pycA* and *alsSD* was likely triggered by the increase in the ATP level caused by lysine starvation or decoyinine addition, as observed for *ilv-leu* (20). As expected, the transcription initiation bases of *pycA* and *alsSD* are adenines; the former was identified in this study (Fig. 7), and the latter was described previously (35). When these adenines at position 1 of *pycA* and *alsSD* were replaced by guanine, the base substitutions only partially affected the positive stringent control of the two promoters, which revealed the involvement of the adenines. However, this partial involvement in the positive stringent control of *pycA* and *alsSD* does not necessarily mean that other transcriptional regulation by unknown regulatory factors is involved in the positive control. This is because transcription initiation of *pycA* occurs at two adenines (positions 1 and 3) (Fig. 7B) and because the base substitution may have shifted the transcription initiation base, as observed for *ilv-leu* (48). Thus, the elaborate stringent control of pyruvate metabolism involving not only positive regulation of *ilv-leu*, *pycA*, and *alsSD* but also negative regulation of *pdhABCD* reminds us that the fate of pyruvate is very important to the cell due to its location at the center of carbon metabolism (37).

In Fig. 9, the nucleotide sequences of the promoter regions of the *ptsGHI*, *pdhABCD*, *ilv-leu*, *pycA*, and *alsSD* operons are aligned to determine any sequence conservation in them; the first two operons and the last three operons are under negative and positive stringent control, respectively. The GG at positions 1 and 2 of the transcription initiation site of the *ptsGHI* operon was changed to GA, AG, and AA. All of the base substitutions shifted the negative stringent regulation in the positive direction in the order GA-AG-AA (Fig. 4). This fact indicates that both of the guanines at positions 1 and 2 are involved in the negative stringent control of *ptsGHI*. Also, adenine substitution for guanine at position 1 of *pdhABCD* eliminated its negative regulation (Fig. 6). Guanine substitution for adenine at position 1 of *pycA* and *alsSD* partially affected the positive stringent control of these operons (Fig. 8). Moreover, we previously reported that when the adenine at position 2 of the transcription initiation site of *ilv-leu* was changed to guanine, the positive stringent regulation became negative stringent regulation. Interestingly, position –1 is occupied by thymine at all five transcription initiation sites (Fig. 9). These results, as well as the sequence alignment (Fig. 9), suggest that a guanine and not an adenine is located at either position 1 or 2 or at both positions for negative stringent control and that an adenine and not a guanine is located at either position 1 or 2 or at both positions for positive stringent control. If guanine and adenine are present at positions 1 and 2 of the same site, which is the case in many operons (12; T. Tojo, unpublished data) and as actually site-directed mu-



FIG. 9. Alignment of the nucleotide sequences of the promoters under CodY-independent stringent control. The nucleotide sequences of the promoters of the *ptsGHI* and *pdhABCD* operons (P_{ptsGHI} and $P_{pdhABCD}$) under CodY-independent negative stringent control and the nucleotide sequences of the *ilv-leu*, *pycA*, and *alsSD* operons ($P_{ilv-leu}$, P_{pycA} , and P_{alsSD}) under CodY-independent positive stringent control are aligned. The transcription initiation base of the *ptsGHI* operon determined previously (45) was confirmed in this work (Fig. 2C). The initiation bases of the *ilv-leu* operon were reported previously to be cytosine at position 1 (48) and adenine at position 2 (20), a difference which has not been resolved. The initiation bases of the *pdhABCD* (Fig. 5) and *pycA* (Fig. 7B) operons were determined in this work; there are two initiation adenines for *pycA*, and the upstream position is designated position 1. The initiation base of the *alsSD* operon was determined previously (35). The sequences of the –35 and –10 regions of the promoters for these operons are underlined. The bases at positions 1 and 2 are indicated by large uppercase letters, and adenine and guanine are also indicated by bold type. The thymine at position –1 is indicated by a large lowercase letter. Conversion of guanine at position 1 and/or 2 of *ptsGHI* and *pdhABCD* to adenine alters the original negative stringent regulation to less negative or positive stringent regulation, while conversion of A at position 2 of *ilv-leu* to G changes the positive regulation to negative regulation (48). Moreover, conversion of adenine at position 1 of *pycA* and *alsSD* to G partially quenched the positive stringent control of these operons.

tagenized in the case of *ptsGHI*, the positive stringent control and negative stringent control of such operons are assumed to be neutralized or to cancel each other out.

DNA microarray analysis revealed that 371 and 317 protein genes are negatively and positively regulated more than 3-fold upon decoyinine addition even in the $\Delta codY$ genetic background. However, these genes are not always considered to be under the stringent control regulating the transcription initiation rate, as inferred from the concentrations of metabolites such as FBP, a signal compound in carbon catabolite regulation in *B. subtilis* (7), which are substantially changed upon decoyinine treatment, as observed in the metabolome analysis (Table 3). Thus, various transcriptional repressor and activators are thought to affect the expression of many genes when there are changes in the levels of the signal metabolites. Nevertheless, we 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 in a DNA microarray analysis and whose transcription initiation bases are known (see Table S2 in the supplemental material). The downregulated operons were the S10 (23), *str* (21), *pyrG* (26), and *pur* (6) operons, whereas the upregulated operons were

the *metIC* (3), *ureABC* (54), and *rapA* (29) operons. Also, we aligned the promoter sequences of the *rmB* (P1) (44), *rmO* (P1) (32), and *tufA* (21) operons that are downregulated and the *appDFABCA* (18) and *ywaA* (20) operons that are upregulated and are known to be subject to the stringent control mediated by modulation of the translation initiation rates (19, 20). As Table S2 in the supplemental material shows, there are adenines or guanines at positions 1 and 2 depending on whether the regulation is positive or negative. Moreover, all of the transcription initiation bases at position 1 for the P1 and P2 promoters of the 7 *rm* operons are guanines, and the bases at position 2 are all thymines, except for cytosine in the case of the *rmB* P2 promoter (30). However, the thymine at position -1 shown in the alignment in Fig. 9 is not well conserved in the promoter sequences shown in Table S2 in the supplemental material. This implies at least that the CodY-independent stringent control involving modulation of the transcription initiation rate might occur in numerous stringent operons other than those investigated so far by us (48; this study) and by Krásný et al. (19, 20).

The presence of either guanine or adenine in the transcription initiation site (positions 1 and 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 *B. subtilis* and *E. coli* use different strategies to control the operons under stringent control might be related to the difference in the stabilities of open complexes of the two microorganisms previously noticed by Whipple and Sonenshein (53). The open complex formed with *E. coli* RNA polymerase before the formation of the elongation complex for transcription is stable before the formation of any phosphodiester bond, whereas the open complex formed with *B. subtilis* RNA polymerase in the absence of nucleotide triphosphate is unstable. This implies that 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.

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