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Organization and Expression of the *Bacillus subtilis sigY* Operon

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We investigated the organization and expression of the *Bacillus subtilis sigY* operon, the first gene of which codes for σ^Y, a member of the extracytoplasmic function (ECF) family of sigma factors. The *sigY* operon, comprising six genes (*sigY*, *yxlC*, *D*, *E*, *F*, and *G*), was induced upon nitrogen starvation; it was continuously transcribed from the 31st base upstream of *sigY* to a neighboring convergent gene, *yxlH*, resulting in a 4.2-kb mRNA. The expression of the *sigY* operon was also positively autoregulated through σ^Y, suggesting that its transcription is likely to be directed by σ^Y. Deletion analysis of the *sigY* promoter, which was localized by primer extension, revealed the promoter region of *sigY* with the "–10" and "–35" sequences of CGTC and TGAACG, respectively. The latter sequence was distinct from those recognized by σ^W, σ^X, and σ^M. The σ^Y-directed transcription of *sigY* was under negative regulation involving YxlD. *sigY* disruption affected sporulation induced by nitrogen starvation, but *sigY* induction upon nitrogen starvation was not associated with the sporulation process. The organization and function of the *sigY* operon are significantly conserved in several microorganisms living in adverse living environments.

Key words: *Bacillus subtilis*, extracytoplasmic function, negative regulation, nitrogen starvation, positive autoregulation, sigma factor, *sigY* operon, sporulation, transcription.

Abbreviations: ABC, ATP binding cassette; β-Gal, β-galactosidase; DIG, digoxigenin; ECF, extracytoplasmic function; IPTG, isopropyl-β-D-thiogalactopyranoside; LB, Luria-Bertani; OD, optical density; P*sigY*, *sigY* promoter; P*spac*, *spac* promoter; TBAB, tryptose blood agar base.

The soil bacterium *Bacillus subtilis* must adapt to and survive in various environmental conditions by adopting the pattern of genome expression characteristic of the respective conditions. Alternate sigma factors could be one means of alteration of gene expression in response to a change in the environment. Well-characterized alternate sigma factors, such as σ^H, σ^E, σ^F, σ^G, and σ^K, are involved in the sporulation process, σ^B in general stress, and σ^D in chemotaxis, motility and autolysis (*1*), the physiological functions of which have been extensively studied and elucidated.

In 1994, Lonetto *et al*. initially proposed the extracytoplasmic function (ECF) subfamily of sigma factors, a distinct subfamily within the sigma-70 family of regulators (*2*). Sequencing of the entire *B. subtilis* genome has revealed seven unidentified sigma factors (σ^X, σ^W, σ^M, σ^Y, σ^V, σ^Z, and σ^{YlaC}) that belong to the ECF subfamily (*3*). The complete genome sequences of dozens of bacteria have been determined, so the number of members of this family of regulators has logarithmically increased. ECF sigma factors typically control cell envelope-related functions such as the secretion or synthesis of exopolysaccharides, or ion import or efflux (*4*, *5*). Most studies performed to determine the roles of the ECF sigma factors of *B. subtilis* have concentrated on three of them, σ^X (*6*, *7*), σ^W (*6–10*), and σ^M (*10–12*), leading to a model in which these three ECF sigma factors function to help maintain cell envelope integrity by triggering the expression of genes that inactivate or detoxify antibiotics, or alter cell surface properties (*5*, *10*). On the other hand, the functions of the other four ECF sigma factors of *B. subtilis* including σ^Y have been scarcely investigated so far, although candidate target genes of all the ECF sigma factors of this organism have been searched for by means of DNA microarray analysis with a forced expression system for their genes very recently (*13*).

ECF sigma factors share some common features besides their cell envelope-related functions, as described above. In many cases, an ECF sigma factor is cotranscribed with a transmembrane anti-sigma factor with an extracytoplasmic sensory domain and an internal inhibitory domain (*5*, *14*). The promoter of the operon encoding the ECF sigma factor is often recognized by its own sigma factor. Our recent DNA microarray analysis also indicated that the promoters of the *B. subtilis* ECF sigma operons except for *sigZ* are likely recognized by the own sigma factors (*13*). This study focused on the organization and expression of the *sigY* operon encoding one of the ECF sigma factors not characterized well so far (Fig. 1).

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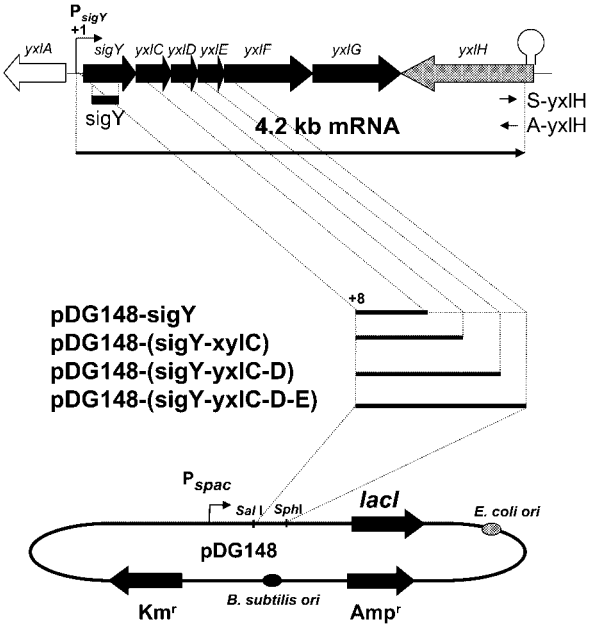

Fig. 1. **Organization and transcription of the *sigY* operon, and structures of derivatives of plasmid pDG148 carrying *sigY* and its downstream genes.** The gene organization of the *sigY* region was cited from the literature (*3*). The *sigY* operon, consisting of six genes (*sigY*, *yxlC*, *D*, *E*, *F*, and *G*), was transcribed from the 31st base upstream of the translation start base of *sigY* as a 4.2-kb mRNA extending into a convergent gene, *yxlH*, as described below. Derivatives of plasmid pDG148 carried a series of *sigY* regions from nucleotide +8 (+1 is the *sigY* transcription initiation base) to the stop codon of each gene of the *sigY* operon. These regions were placed downstream of P*spac* in plasmid pDG148 using the *Sal*I and *Sph*I sites. The *spac* promoter is negatively regulated by LacI so that IPTG addition induces the genes cloned. The locations of the probes (sigY, S-yxlH, and A-yxlH) used for Northern analysis are also shown.

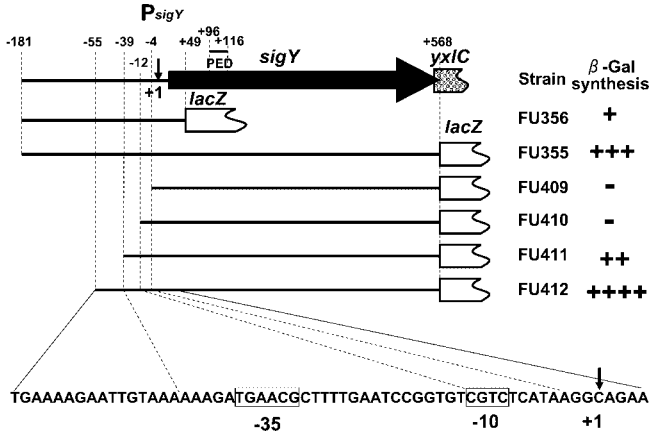

Fig. 2. **The promoter region of the *sigY* operon, and its deletion analysis.** The *B. subtilis* strains carried a series of *lacZ* fusions in the *amyE* locus, in which the *sigY* promoter regions indicated by bold lines were transcriptionally fused with *lacZ*. The relative level of β-Gal synthesis induced upon nitrogen starvation in the cells of each strain is shown. The location of the primer (PED) used for primer extension analysis to identify the 5′-end of the *sigY* transcript is also indicated. In the lower part of the figure, the nucleotide sequence of the *sigY* promoter is shown, where the *sigY* transcription initiation base (+1), and the "–10" and "–35" regions of the promoter are indicated.

Here, we report that the *sigY* operon consisting of six genes (*sigY*, *yxlC*, *D*, *E*, *F*, and *G*) was induced upon nitrogen starvation. Transcription of the *sigY* operon was triggered by σ^Y itself, which was under negative regulation involving YxlD. Primer extension and deletion analyses of the promoter region of this operon allowed us to identify a promoter sequence likely recognized by σ^Y.

MATERIALS AND METHODS

Bacterial Strains and Plasmids, and their Construction—The bacterial strains and plasmids used in this work are listed in Table 1. To construct strains FU355, FU356, FU409, FU410, FU411, and FU412 carrying fusions of various *sigY* regions (–181/+568, –181/+49, –4/+568, –12/+568, –39/+568, and –55/+568; +1 is the transcription initiation base of the *sigY* operon, as mentioned below) with *lacZ* in the *amyE* locus (Fig. 2), the *sigY* regions were amplified with a primer pair (Table 2; SU/SD2, SU/SD1, F09U/SD2, F10U/SD2, F11U/SD2, and F12U/SD2), respectively and DNA of strain 168 as a template, respectively. The PCR products possessing 5′ and 3′ flanking *Xba*I and *Bam*HI sites were trimmed with the two enzymes, and then ligated with the *Xba*I–*Bam*HI arm of plasmid pCRE-test2 (*19*) after elimination of the *spac* promoter (P*spac*) from the plasmid by double digestion. The ligated DNAs were used for the transformation of *Escherichia coli* strain DH5α to ampicillin-resistance (50 μg/ml) on Luria-Bertani (LB) plates (*15*). The correct construction of the fusions in the resulting plasmids was confirmed by sequencing. The plasmids were linearized with *Pst*I, and then used for double-crossover transformation of strain 168 to chloramphenicol-resistance (5 μg/ml) on tryptose blood agar base (Difco) plates containing 10 mM glucose (TBABG).

Strain ASK450 carrying one of the above fusions [P*sigY* (*sigY* promoter)-*sigY′* (–181/+49)-*lacZ*] at the *amyE* locus and a deletion of the *sigY* operon [Δ(P*sigY-sigY-yxlC-D-E-F-G*)::erm] was constructed as follows. To delete the *sigY* operon, the PCR fragment in which the regions flanking the *sigY* operon sandwich the *erm* gene was prepared by means of long-flanking homology PCR (*21*). The two regions were amplified by PCR using primer pairs (YuU/YuD and GdU/GdD for the regions upstream of *sigY* and downstream of *yxlG*, respectively; Table 2) and DNA of strain 168 as a template. In addition, the erythromycin-resistance gene (*erm*) encoded by plasmid pMUTIN (*18*) was amplified by PCR using a primer pair (EmU/EmD; Table 2) and DNA of strain YF316 as a template. A joint PCR procedure (*21*) involving the three above PCR products did not work well, so the PCR joining of the upstream fragment and the *erm* cassette was followed by further joining of the downstream fragment. The resulting PCR fragment was used for the transformation of strain FU356 to erythromycin-resistance (0.3 μg/ml) on TBABG plates. Correct deletion of the *sigY* operon from strain FU356 was confirmed by means of PCR and sequencing.

To construct a series of plasmids, pDG148-sigY, pDG148-(sigY-yxlC), pDG148-(sigY-yxlC-D), and pDG148-(sigY-yxlC-D-E), each carrying part of the *sigY* operon under

Table 1. **Bacterial strains and plasmids used in this work.**

Strain or plasmid	Genotype or description	Source
Strain		
E. coli		
DH5α	*susE44* Δ*lacU169* (ϕ80*lacZ*ΔM15) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	*15*
JM109	*recA1 supE44 endA1 hsdR17 gryA96 relA1 thi* Δ(*lac-proAB*) F′ [*traD36 proAB*$^+$ *lac*^{q} *lacZ* ΔM15]	*15*
C600	*supE44 hsdR thi-1 thr-1 leuB6 lacY1 tonA21*	*15*
B. subtilis		
168	*trpC2*	*16*
ASK431	*trpC2 yxlC*::pMUTINΔ*lacZ* (P*spac-yxlC-D-E-F-G*)	This work
ASK432	*trpC2 yxlD*::pMUTINΔ*lacZ* (P*spac-yxlD-E-F-G*)	This work
ASK433	*trpC2 yxlE*::pMUTINΔ*lacZ* (P*spac-yxlE-F-G*)	This work
YF315(=SIGYp)	*trpC2 sigY*::pMUTIN (P*spac-sigY-yxlC-D-E-F-G*)	This work
YF316 (=SIGYd)	*trpC2 sigY*::pMUTIN	*16*
YXLHd	*trpC2 yxlH*::pMUTIN	*16*
FU355	*trpC2 amyE*::[*cat* P*sigY-sigY* (–181/+568)-*lacZ*]	This work
FU356	*trpC2 amyE*::[*cat* P*sigY-sigY*′(–181/+49)-*lacZ*]	This work
ASK201	*trpC2 spo0H*::erm	*17*
ASK202	*trpC2 spoIIAC*::kan	*17*
ASK203	*trpC2 spoIIGAB*::kan	*17*
ASK204	*trpC2 spoIIIG*::kan	*17*
ASK205	*trpC2 spoIVCB*::erm	*17*
FU357	*trpC2 amyE*::[*cat* P*sigY-sigY* (–181/+568)-*lacZ*] *spo0H*::erm	This work
FU358	*trpC2 amyE*::[*cat* P*sigY-sigY* (–181/+568)-*lacZ*] *spoIIAC*::kan	This work
FU359	*trpC2 amyE*::[*cat* P*sigY-sigY* (–181/+568)-*lacZ*] *spoIIGAB*::kan	This work
FU360	*trpC2 amyE*::[*cat* P*sigY-sigY* (–181/+568)-*lacZ*] *spoIIIG*::kan	This work
FU361	*trpC2 amyE*::[*cat* P*sigY-sigY* (–181/+568)-*lacZ*] *spoIVCB*::erm	This work
FU409	*trpC2 amyE*::[*cat* ΔP*sigY-sigY* (–4/+568)-*lacZ*]	This work
FU410	*trpC2 amyE*::[*cat* ′P*sigY-sigY* (–12/+568)-*lacZ*]	This work
FU411	*trpC2 amyE*::[*cat* P*sigY-sigY* (–39/+568)-*lacZ*]	This work
FU412	*trpC2 amyE*::[*cat* P*sigY-sigY* (–55/+568)-*lacZ*]	This work
ASK450	*trpC2* ΔP*sigY-yxlC-D-E-F-G*::erm *amyE*::[*cat* P*sigY-sigY*′ (–181/+49)-*lacZ*]	This work
Plasmid		
pMUTIN	Em^r *lacI* P*spac lacZ*	*18*
pMUTINΔlacZ	Em^r *lacI* P*spac*	This work
pCRE-test2	Cm^r *lacZ 'amyE amyE*′	*19*
pDG148	Km^r *lacI* P*spac*	*20*
pDG148-*sigY*	pDG148 carrying P*spac sigY* (+8/+568)	This work
PDG148-(sigY-yxlC)	pDG148 carrying P*spac sigY-yxlC* (+8/+890)	This work
pDG148-(sigY-yxlC-D)	pDG148 carrying P*spac sigY-yxlC-D* (+8/+1096)	This work
pDG148-(sigY-yxlC-D-E)	pDG148 carrying P*spac sigY-yxlC-D-E* (+8/+1271)	This work
pMUTIN-yxlH	pMUTIN carrying part of *yxlH* used for construction of strain YXLHd	*16*

the control of P*spac* (Fig. 1), the corresponding regions were amplified by PCR using a primer pair (Table 2; UY/DY, UY1/DC, UY1/DD, and UY1/DE) and DNA of strain 168 as a template, respectively. The PCR products were digested with *Sal*I and *Sph*I, and then ligated with the *Sal*I–*Sph*I arm of plasmid pDG148 (*20*). The ligated DNAs were used for the transformation of *E. coli* strain C600 to ampicillin-resistance. The correct cloning of each of the *sigY* regions was confirmed by sequencing. The resultant plasmids were transferred to *B. subtilis* strain ASK450 by selecting the transformants exhibiting kanamycin-resistance (10 μg/ml).

To construct plasmid pMUTINΔ*lacZ*, pMUTIN2 (*18*) was digested with *Cla*I and *Sac*I, blunt-ended with T4 DNA polymerase, and then ligated. Strains ASK431, ASK432 and ASK433 were constructed essentially as described previously (*16*), with pMUTINΔ*lacZ* integration into *yxlC*, *D* and *E*, placing *yxlC-D-E-F-G*, *yxlD-E-F-G*, and *yxlE-F-G* under the control of P*spac*, respectively. The PCR products were amplified using a primer pair (Table 2; A1U/A1D, A2U/A2D, and A3U/A3D) for construction of strains ASK431, ASK432 and ASK433, respectively, trimmed with *Eco*RI (or *Hin*DIII) and *Bam*HI, and cloned into pMUTINΔ*lacZ*. The resulting plasmids were integrated into the respective target genes through a single crossover event, erythromycin-resistant integrants being selected in the presence of 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG).

To construct strains FU357, FU358, FU359, FU360, and FU361 carrying the P*sigY-sigY* (–181/+568)-*lacZ* fusion and a disrupted sporulation-specific sigma factor gene, strains ASK201, ASK202, ASK203, ASK204, and ASK205 were transformed with DNA of strain FU355 carrying the fusion with chloramphenicol-resistance (5 μg/ml) on TBABG plates containing erythromycin (0.3 μg/ml) or kanamycin (10 μg/ml).

Table 2. **Oligonucleotides used in this work.**

Name	Sequence[a]
SU	5′-AGTCGCTAGTCTAGATGCATCGTCCTCCATATT-3′
F09U	5′- AGTCGCTAGTCTAGAAAGGCAGAAAAACAAAAA-3′
F10U	5′-AGTCGCTAGTCTAGACGTCTCATAAGGCAGAAA-3′
F11U	5′-AGTCGCTAGTCTAGAAAAGATGAACGCTTTTGA-3′
F12U	5′-AGTCGCTAGTCTAGATGAAAAGAATTGTAAAAA-3′
SD1	5′-CATGGTCGCGGATCCTTATTCTTGTGTATCCAA-3′
SD2	5′-TTAGATCGCGGATCCTTATTCATCATCCCACTC-3′
EmU	5′-CTTAAGAGTGTGTTGATAGTGC
EmD	5′-CTAGGGACCTCTTTAGCTCC
YuU	5′-GCCACTAGGAAAATTGGCAG
YuD	5′-TTTTAAGATACTGCACTATCAACACACTCTTAAGGCCATTGACAGATCAGGC
GdU	5′-CAGCTTCCAAGGAGCTAAAGAGGTCCCTAGGCTATTAATTGATCATGTTACG
GdD	5′-CATTTAGGGAGGCAGGTTCC
PEU	5′-ATGCATATTAGCCCCGAACC-3′
PED	5′-AGTGATAGTGGAAAAGGGCTG-3′
NU	5′-AAACGACGAGGCGTTTACAG-3′
ND	5′-CCCACTCCTTTCTGATTTGC-3′
SP6-F	5′-ATTTAGGTGACACTATAGAATACGTGTGGAATTGTGAGCGG-3′
T7-R	5′-TAATACGACTCACTATAGGGCGAAGTGTATCAACAAGCTGG-3′
UY	5′-ATTCTACATCGCGTCGACACAAAAAAGGGGGGATCAC-3′
DY	5′- GTTTGACCCGTAGCATGCTTATTCATCATCCCACTC-3′
UY1	5′- GTCGTCGACACAAAAAAGGGGGGATCAC-3′
DC	5′- GCAGCATGCTGTTTGTGTCATCCGCG-3′
DD	5′-GCAGCATGCCCAAGAAATATTCATTGC-3′
DE	5′- GCAGCATGCTTACCTTTGCTTTCTGC-3′
A1U	5′-GAAGAATTCCAGCAAATCAGAAAGGAG-3′
A1D	5′-GGAGGATCCTTGAAGAAGTTTTTTTCTGTATTC-3′
A2U	5′-AAGAAGCTTGCGAATGCGAGGTGAAAC-3′
A2D	5′-GGAGGATCCGACAATCCCCCAAAC-3′
A3U	5′-AAGAAGCTTAGGCCGGATCGGAAGA-3′
A3D	5′-GGAGGATCCACTTTGGCCCGTTC-3′

[a]The underlined sequences are the sites for restriction enzymes used for cloning of the resulting PCR products.

Cell Growth and β-Galactosidase (β-Gal) Assay—*B. subtilis* cells were grown at 30°C on TBABG containing chloramphenicol (5 μg/ml) (and an additional antibiotic if necessary) overnight. The cells were inoculated into 50 ml NSMP (*22*) or MM medium (*16*) containing tryptophan (50 μg/ml), and then incubated at 37°C. When the cells were subjected to nitrogen starvation, the concentration of glutamine in the MM medium was reduced to 0.02%. During growth and nitrogen starvation, 1 ml aliquots of the culture were withdrawn, and the β-Gal activity in the cells was spectrophotometrically assayed, as described previously (*16*).

The cells with a derivative of plasmid pDG148 were inoculated into LB medium, and then grown to the early logarithmic phase [optical density at 600 nm (OD_{600}) = 0.2]. One half of the culture was exposed to 1 mM IPTG for 1 h. 1 ml aliquots of the culture with and without IPTG were withdrawn, and the β-Gal activity in the cells was determined, as described above.

DNA Microarray Analysis—DNA microarrays were prepared as described previously (*23*). Total RNA was extracted and purified from *B. subtilis* cells as described previously (*23*). The fluorescently labeled cDNA probes used for hybridization to DNA microarrays were prepared by a two-step procedure, as described previously (*24*). The hybridization and microarray analyses were performed as described previously (*23*, *25*).

Northern and Primer Extension Analyses—Total RNA was extracted and purified as described above. For Northern analysis, RNA was electrophoresed in a glyoxal gel and then transferred to a Hybond-N membrane (Amersham) (*15*). To prepare a probe (sigY) for detection of transcripts carrying *sigY*, the product amplified by PCR using a primer pair (Table 2; NU/ND) and chromosomal DNA of strain 168 as a template was labeled with a *Bca*BEST labeling kit (Megalabel; Takara Shuzo, Kyoto) and [α-^{32}P]dCTP (Amersham). Hybridization was carried out as described (*15*). To prepare sense and antisense RNA probes (S-yxlH and A-yxlH) for Northern blotting to verify that *sigY* transcription extended into the coding region for *yxlH* oppositely oriented to *sigY*, a DNA fragment containing part of *yxlH*, with flanking SP6 and T7 RNA polymerase promoter tags at the upstream and downstream ends, respectively, was amplified with a primer pair (Table 2; SP6-F/T7-R) and DNA of plasmid pMUTIN-yxlH, previously used for the construction of strain YXLHd (*16*), as a template. The PCR product was subjected to *in vitro* transcription driven by SP6 and T7 RNA polymerases to obtain digoxigenin (DIG)-labeled RNA probes (S-yxlH and A-yxlH), respectively (*17*). After hybridization with a membrane, the chemiluminescent signal on the membrane was detected with a DIG luminescence detection kit (Roche Diagnostics).

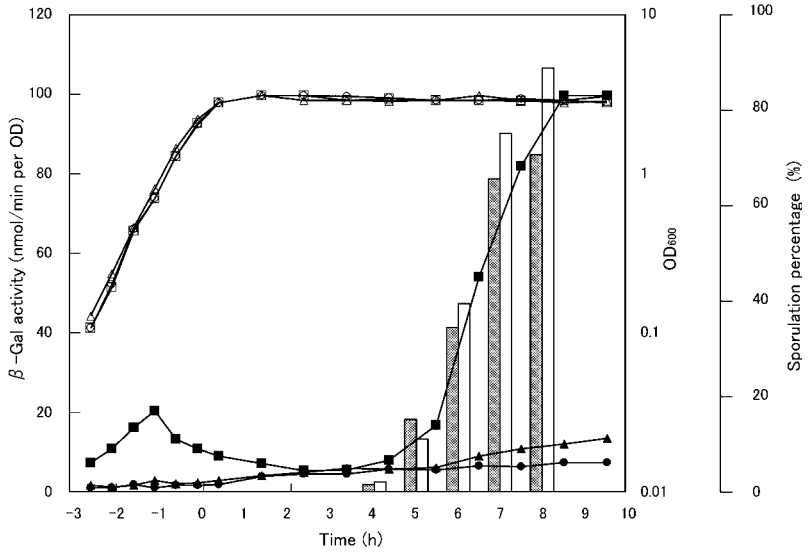

Fig. 3. **Expression of *sigY* during growth and sporulation.** Cells of strains 168 (wild-type) (circles), FU355 (P*sigY-sigY-lacZ*) (squares), and FU356 (P*sigY-lacZ*) (triangles) were grown and sporulated in NSMP medium (*22*), and OD_{600} (open symbols) and β-Gal synthesis (closed symbols) were monitored. The β-Gal assay was performed as described in the text. The heat-resistant spore formation by strains FU355 (open bars) and FU356 (grey bars) was also examined.

Primer extension analysis was performed as described previously (*26*). Reverse transcription was initiated from the PED primer corresponding to nucleotides +96 to +116 (Fig. 2 and Table 2), which had been labeled at its 5′ end by use of a Megalabel kit (Takara Shuzo) and [γ-^{32}P]ATP (Amersham). A template for the dideoxy sequencing reactions for ladder preparation starting from the same end-labeled primer was prepared by PCR using a primer pair (Table 2; PEU/PED) and DNA from strain 168 as a template.

RESULTS

Phenotype of a sigY Disruptant and Regulation of sigY Expression—Within the framework of the international project in Japan and Europe, 'The functional analysis of the *B. subtilis* genome', we have disrupted 125 genes including *sigY* in the *gntZ-ywaA* region through integration of plasmid pMUTIN (*16*, *18*), and the resulting disruptants including strain YF316 (=SIGYd) carrying *sigY*::pMUTIN have been subjected to systematic screening for categorized phenotypes as to motility, sporulation, carbon source utilization and so on. Although nearly half of the disruptants exhibited somewhat altered phenotypes compared to strain 168, strain YF316 did not exhibit a clearly altered phenotype, as can be seen on the web site of the Japan Functional Analysis Network for *B. subtilis* [JAFAN (http://bacillus.genome.ad.jp/)].

The *sigY* operon is likely to be positively regulated by its own product, σ^Y, as described very recently (*13*). To further examine the regulation of *sigY* expression, we constructed strains FU355 and FU356, which carried the P*sigY-sigY-lacZ* and P*sigY-lacZ* fusions in the *amyE* locus, respectively (Fig. 2). Strains FU355 and FU356 were grown and sporulated in NSMP medium (*22*), β-Gal expression in the cells being followed (Fig. 3). β-Gal synthesis in the cells of strain FU355 increased slightly on the transition from glycolytic to gluconeogenic growth in the middle of the exponential growth phase, and it increased dramatically from T5 [T0 is the time (h) from the transition of the logarithmic phase to the stationary one], when heat-resistant spores began to appear. However, β-Gal synthesis in FU356 increased only slightly but significantly from T5. The results implied that *sigY* expression might be not only under positive autoregulation through σ^Y itself but also under cell phase-dependent regulation. Furthermore, the *sigY* gene *in situ* on the chromosome of strain FU356 was also considered not to be expressed well, even though the cells entered the phase after T5, because β-Gal synthesis was not induced well in this phase. This low expression of *sigY* implied the occurrence of some negative regulation of *sigY* expression.

sigY expression in strain FU355 (P*sigY-sigY-lacZ*) increased dramatically from T5, as described above. When we reexamined the sporulation efficiency of strain YF316 (*sigY*::pMUTIN), using NSMP medium, in comparison with that of strain 168 (wild type), the *sigY* disruption was found to affect sporulation only slightly; the sporulation percentages for strains 168 and YF316 were 94 and 34% with standard deviations of 15 and 18%, respectively, when the cells reached T8.

Positive Autoregulation of sigY Expression through σ^Y and Its Negative Regulation Involving YxlD—As described above, expression of the *sigY* gene was considered to be negatively regulated without the supply of an additional *sigY* gene under its promoter, even though the cells entered the late sporulation phase. To examine the possibility that σ^Y-activated transcription might be repressed by a product(s) of gene(s) downstream of *sigY*, we attempted to construct strains carrying a series of *lacZ* fusions with P*sigY-sigY* and downstream gene(s) at the *amyE* locus, but we did not succeed in such construction for unknown reasons, despite several attempts. Moreover, we could not disrupt *yxlC* or *yxlD* by integration of plasmid pMUTIN derivatives through a single-crossover event. [Strains YXLCdd and YXLDdd, which had been constructed by integration of pMUTIN derivatives into *yxlC* and *yxlD* through a double-crossover event previously (*16*), suffered from severe DNA rearrangement at the integration site during the storage process.]

Thus, we alternatively constructed derivatives of plasmid pDG148 carrying *sigY*, *sigY-yxlC*, *sigY-yxlC-D,* and *sigY-yxlC-D-E* under the control of P*spac* (Fig. 1) to determine if the gene(s) downstream of *sigY* encode negative

Table 3. ***lacZ* expression driven from the *sigY* promoter upon induction of *sigY* and its downstream genes.** Cells of strain ASK450 bearing each plasmid were incubated for 1 h with and without IPTG, and then β-Gal assaying was performed, as described in the text.

Plasmid[a]	IPTG addition	β-Gal activity (nmol/min per OD)
pDG148-sigY	+	147.1
	–	21.3
pDG148-(sigY-yxlC)	+	116.8
	–	19.8
pDG148-(sigY-yxlC-D)	+	0.15
	–	0.10
pDG148-(sigY-yxlC-D-E)	+	0.10
	–	0.13
pDG148	+	0.09
	–	0.12

[a]The copy number of each plasmid in the cells was roughly determined by means of agarose gel electrophoresis, which indicated that it did not significantly vary under the experimental conditions adopted.

regulators for *sigY* expression. The constructed plasmids were transferred into strain ASK450 carrying the fusion born by strain FU356 (P*sigY- lacZ*) at the *amyE* locus and a large deletion of the *sigY* to *yxlG* genes, which had been constructed by the transformation of strain FU356 with the product of long-flanking homology PCR (*21*). Cells of strain ASK450 bearing the respective plasmids, which had been grown to the early logarithmic phase, were further incubated for 1 h with and without IPTG, and then the β-Gal activity in the cells was determined (Table 3). The β-Gal synthesis increased dramatically in the cells bearing plasmid pDG148-sigY exposed to IPTG, whereas it increased only slightly in the cells incubated without IPTG, which clearly confirmed that transcription from P*sigY* was directed by σ^Y. The β-Gal synthesis in the cells bearing plasmid pDG148 -(sigY-yxlC) exposed to IPTG was 79.4% that in the cells bearing plasmid pDG148-sigY, implying that YxlC might not function as a negative regulator for this σ^Y-directed transcription. However, when the cells bearing either plasmid pDG148-(sigY-yxlC-D) or pDG148-(sigY-yxlC-D-E) were exposed to IPTG, almost no β-Gal synthesis was observed, clearly indicating that YxlD was involved in negative regulation of σ^Y-directed transcription of *sigY*, but that YxlE was not. We could not exclude the possibility that YxlD functioned as a negative regulator only in the presence of YxlC. The results coincided well with the observation that β-Gal synthesis was not induced well in the cells of strain FU356 (P*sigY- lacZ*) sporulated in NSMP medium, in contrast to the good expression of it in strain FU355 (P*sigY-sigY-lacZ*) (Fig. 3); the negative regulation was strong enough to repress *sigY* expression in strain FU356 containing only one copy of *sigY* on the chromosome.

It has been reported that negative regulators for σ^M -directed transcription (YhdL and YhdM), presumably constituting anti-σ^M, are essential for growth in a nutrient broth (*11*). Although only YxlD was found to function as a negative regulator for σ^Y-directed transcription, we could not disrupt *yxlC* or *yxlD* through integration of plasmid pMUTIN derivatives; we had no problem disrupting *sigY* and *yxlE*. To determine if the *yxlC* and *D* genes are essential for growth, we constructed pMUTIN-Δ*lacZ*-integrated strains, ASK431, ASK432, and ASK433, placing *yxlC-D-E-F-G*, *yxlD-E-F-G*, and *yxlE-F-G* under the control of P*spac*, respectively, by selecting erythromycin-resistant transformants in the presence of IPTG. The constructed strains as well as strain YF316 (*sigY*::pMUTIN) were examined as to their growth in LB medium with and without 1 mM IPTG. All the strains normally grew in liquid LB medium just like strain 168, even in the absence of IPTG (data not shown). However, strains ASK431 and ASK432 scarcely grew on LB plates without IPTG, in contrast to the good growth of strains YF316 and ASK433, in spite of that they grew well on LB plates with 1 mM IPTG (data not shown). The cells of strains ASK431 and ASK432 that had been grown in liquid LB medium exhibited the same growth defect on LB plates. These results indicate that the *yxlC* and *yxlD* genes are only essential for growth on LB plates when *sigY* is intact, and explain why we could not disrupt the *yxlC* and *yxlD* genes by integration of plasmid pMUTIN derivatives through a single-crossover event.

sigY Induction by Nitrogen Starvation—In order to examine the phase-dependent regulation associated with sporulation, we searched for growth conditions under which *sigY* was well expressed by monitoring the β-Gal synthesis in strain FU355 (P*sigY-sigY-lacZ*), using various nutrient-limited forms of solid MM medium. When various limitation conditions were tested, we obtained evidence that nitrogen limitation might induce *sigY* expression (data not shown). Thus, we reduced the concentration of glutamine (0.2%) in liquid MM medium to 0.02% for the cells to be subjected to nitrogen starvation. As shown in Fig. 4A and B, the cells of strain FU355 grew to OD_{600} = 5 in MM medium, but they ceased growing in the medium containing 0.02% glutamine after the OD_{600} reached approximately 1.0 and were subjected to nitrogen starvation. Although there was no significant induction of β-Gal synthesis even if the cells were incubated up to T12 in MM medium (Fig. 4A), β-Gal synthesis started to increase dramatically after T3 on cultivation in MM medium containing only 0.02% glutamine, implying that *sigY* might be induced upon nitrogen starvation (Fig. 4B). This induction suddenly stopped at about the same time as the cells recovered the ability to grow upon the addition of extra nitrogen source (glutamine or ammonium) at T2 (Fig. 5A), indicating that *sigY* was actually induced by nitrogen starvation. When β-Gal synthesis in the cells of strain FU356 (P*sigY-lacZ*) was followed under the same nitrogen excess and starvation conditions, it was also found to begin to increase at T3 only under nitrogen starvation conditions, although approximately 100 times less synthesis than that in strain FU355 was observed (Fig. 4C and D). This difference in the amount of β-Gal synthesized was almost the same as that observed in strains FU355 and FU356 during sporulation in NSMP (Fig. 3). Thus, it is possible to infer that the *sigY* induction observed after T5 during cultivation in NSMP might result from depletion of the nitrogen source in the medium.

sigY Induction was not Associated with the Sporulation Process—As described above, *sigY* disruption affected sporulation in NSMP only slightly. When cells of strain 168 were subjected to nitrogen starvation, a portion of

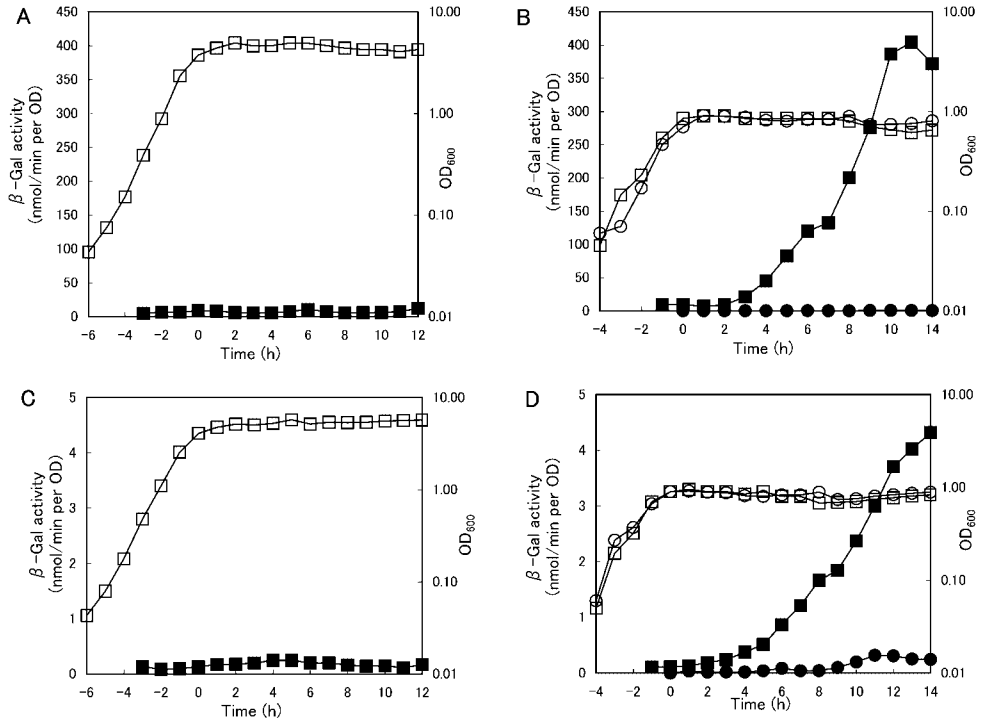

Fig. 4. **Induction of *sigY* upon nitrogen starvation.** Cells of strains FU355 (P*sigY-sigY-lacZ*) (A, B) and FU356 (P*sigY-lacZ*) (C, D) (squares) were grown in MM medium (*16*), the nitrogen source in which was 0.2% (A, C) or 0.02% (5 mM) (B, D) glutamine. The cells suffered from nitrogen starvation after the growth on 0.02% glutamine had reached approximately OD_{600} = 1. Cells of strain 168 (circles) were also grown in MM medium containing 0.02% glutamine (B, D). β-Gal was assayed as described in the text. Open and closed symbols denote OD_{600} and β-Gal activity, respectively.

the cells could enter the sporulation stage, resulting in roughly 10 times less heat-resistant spores than in NSMP at T8. This low sporulation efficiency was clearly reduced by the *sigY* disruption in strain YF316 by approximately 30-fold. However, this disruption did not affect the viability of cells even when the cells were subjected to nitrogen starvation for 20 h (data not shown). To determine if the *sigY* induction upon nitrogen starvation is associated with the sporulation process, we constructed a series of strains (FU357, FU358, FU359, FU360, and FU361) deficient in each of the sporulation-specific sigma factors (σ^H, σ^F, σ^E, σ^G, and σ^K), carrying the P*sigY-sigY-lacZ* fusion at *amyE*. As shown in Fig. 5B, β-Gal synthesis in strain FU359 [*spoIIGAB* (*sigE*)::kan P*sigY-sigY-lacZ*] began at T3 when it was submitted to nitrogen starvation, just as in strain FU355 (P*sigY-sigY-lacZ*). β-Gal induction was also observed in the cells of strain FU357 (*spo0H*::erm P*sigY-sigY-lacZ*) after T3 upon nitrogen starvation, but the level of β-Gal synthesis in strain FU357 was higher throughout growth and nitrogen starvation than in strains FU355 and FU359 (Fig. 5B). Moreover, β-Gal synthesis in the other strains, FU358, FU360, and FU361, was induced upon nitrogen starvation, just as in strain FU355 (data not shown). These results indicated that the induction of *sigY* upon nitrogen starvation was not associated with the sporulation process itself.

Induction of the sigY Operon upon Nitrogen Starvation—As shown in Fig. 1, the *sigY* operon is likely to consist of six genes (*sigY*, *yxlC*, *D*, *E*, *F* and *G*) (*3*). To determine if the six genes are simultaneously induced upon nitrogen starvation, we performed DNA microarray analysis using cells of strain 168 before and after exposure to nitrogen starvation. The cells were harvested in the middle of the logarithmic growth phase, and at T0, T6, and T12 after the cells had entered the nitrogen starvation phase. Then, their RNAs were subjected to DNA microarray analysis, and the gene expression intensities obtained with RNAs from cells at T0, T6, and T12 were divided by that obtained with RNA from cells in the middle of the logarithmic growth phase, respectively, which yielded three expression ratios (T0/mid-log, T6/mid-log, and T12/mid-log). The DNA microarray data are available on the KEGG Expression Database website (http://www.genome.ad.jp/kegg/expression). Figure 6 shows the expression ratios (T0, T6, and T12/mid-log) of *sigY*, *yxlC*, *D*, *E*, *F*, and *G*, the supposed constituents of the *sigY* operon, as well as those of *yxlH*, which is downstream of *yxlG* but oppositely oriented. As expected, the *sigY*, *yxlC*, *D*, *E*, *F*, and *G* genes were actually induced at T6 and more at T12 upon nitrogen starvation, suggesting that the *sigY* operon (*sigY*, *yxlC*, *D*, *E*, *F*, and *G*) was induced by nitrogen starvation. The results well coincided with those of the *lacZ* fusion experiments (Fig. 4, B and D), especially with those of that involving strain FU356 carrying only P*sigY-lacZ* (Fig. 4D). The reasons why unexpected induction of *yxlH* upon nitrogen starvation was observed are given below.

Transcript Analysis of the sigY Operon—We performed Northern analysis of *sigY* transcripts induced upon nitrogen starvation (Fig. 7). RNA samples prepared from cells of strain 168 harvested at T1 and T6 upon nitrogen starvation were subjected to Northern blotting using a *sigY*-specific probe. As shown in Fig. 7A, we detected two transcripts of 4.2 kb and 4.8 kb only in the cells harvested at T6 (lane 2). Primer extension analysis using the same RNA samples mapped the 5′-end of the *sigY* transcripts (Fig. 8), suggesting that *sigY* transcription likely started from the 31st base upstream of the translation start base of *sigY*. Since the distance from this *sigY* transcription initiation base to the translation start base of the convergent *yxlH* gene was estimated to be 4,133 bp, the domi-

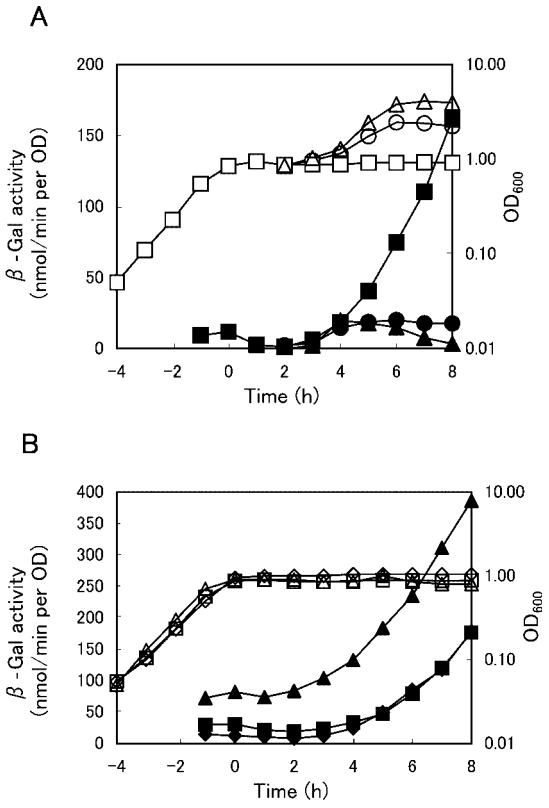

Fig. 5. **Characterization of *sigY* induction upon nitrogen starvation.** (A) Suppression of *sigY* induction on the addition of a nitrogen source. Cells of strain FU355 (P*sigY-sigY-lacZ*) were grown and subjected to nitrogen starvation in MM medium (0.02% glutamine). The culture was divided into three portions at T2, to which nothing (squares), and 5 mM glutamine (triangles) or ammonium chloride (circles) was added, respectively. (B). No effect of *spo0H*::erm and *spoIIGAB*::kan on *sigY* induction upon nitrogen starvation. Cells of strains FU355 (*spo*$^+$) (squares), FU357 (*spo0H*::erm) (triangles), and FU359 (*spoIIGAB*::kan) (diamonds) were grown and subjected to nitrogen starvation in MM medium (0.02% glutamine). β-Gal was assayed as described in the text. Open and closed symbols denote OD_{600} and β-Gal activity, respectively.

nant 4.2-kb transcript detected on Northern analysis (Fig. 7A) was long enough to cover *sigY*, *yxlC*, *D*, *E*, *F*, and *G,* and to extend into the coding region of *yxlH* (Fig. 1). The 4.8-kb transcript appeared to be one that escaped termination at the beginning of the *yxlH* gene.

To determine if the *sigY* transcripts carried the anti-sense strand of *yxlH*, we performed Northern blotting with two kinds of *yxlH*-specific RNA probes, S-yxlH and A-yxlH, which are sense and anti-sense RNAs corresponding to the 5′-part of *yxlH*, respectively. We could not detect transcripts in the RNA sample (T6) used for the above Northern analysis (Fig. 7A), probably due to their low amounts. So, we prepared RNA samples from the cells of strain 168 bearing plasmid pDG148-sigY grown with and without IPTG, because IPTG addition caused forced production of σ^Y encoded by the plasmid, leading to induction of the constituents of the *sigY* operon and *yxlH*, as described previously (*13*). Fig. 7B shows that the 4.2-kb transcript induced with IPTG only hybridized with S-yxlH (lane 1), indicating that *sigY* transcription extended into the convergent *yxlH*. We found a typical ρ-independent terminator located between 4,066 and 4,099 bases from the initiation base of *sigY* transcription, which might be its terminator (Fig. 1). This finding clearly explained why *yxlH* was detected on DNA microarray analysis with the DNA microarray onto which double-stranded PCR products for *B. subtilis* genes had been spotted (Fig. 6; 23).

It is quite unusual that *sigY* transcription extends into the convergent *yxlH* without any termination between *yxlG* and *yxlH*. A similarity search as to YxlH in protein databases with the BLASTP program (*27*) revealed that this protein exhibits significant similarity to multidrug-efflux proteins of various microorganisms. Also, we could not assign any meaningful open reading frame in the same direction as the transcription of the *sigY* operon in the *yxlH* region (data not shown). Thus, it was considered that the *sigY* transcript extending into *yxlH* might inhibit *yxlH* translation through hybridization between *sigY* and *yxlH* mRNAs. We examined whether or not *yxlH* was expressed during growth and nitrogen starvation by monitoring the β-Gal synthesis in cells of strain YXLHd, a pMUTIN-integrant as to *yxlH*. However, we observed no synthesis of β-Gal in cells of this strain during growth and nitrogen starvation under the conditions we adopted (data not shown).

Deletion Analysis of the sigY Promoter Region—We determined the 5′-end of *sigY* transcripts by means of a primer extension experiment (Fig. 8). To confirm that this site was the transcription initiation one of the *sigY* operon, we performed deletion analysis of the *sigY* pro-

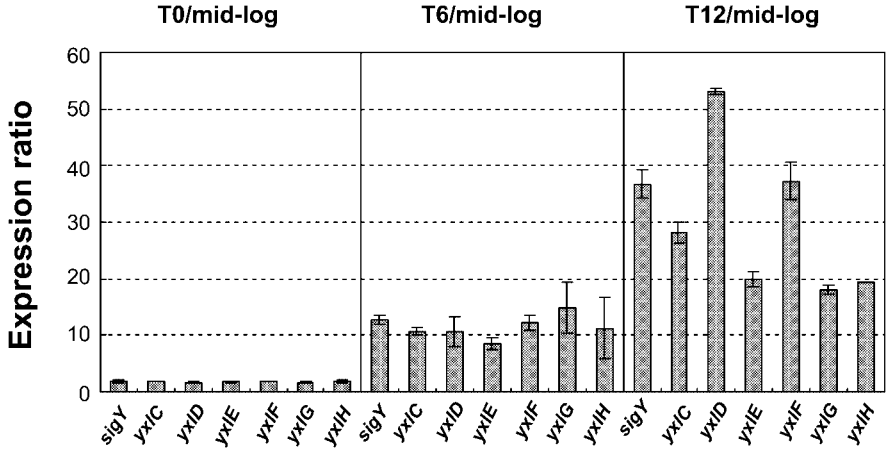

Fig. 6. **DNA microarray analysis of the induction of the *sigY* operon.** Total RNAs were prepared from strain 168 cells harvested 2 h before T0 [mid-logarithmic phase (mid-log)], and T0, T6, and T12. DNA microarray analysis was performed as described in the text. The gene expression intensities (Cy5) obtained with RNAs from the cells at T0, T6, and T12 were divided by those at the mid-logarithmic phase, which yielded the expression ratios, T0/mid-log, T6/mid-log, and T12/mid-log, respectively. The expression ratio for each gene was taken as the average of two values obtained for duplicate expression profiles, the standard deviation of which is also shown.

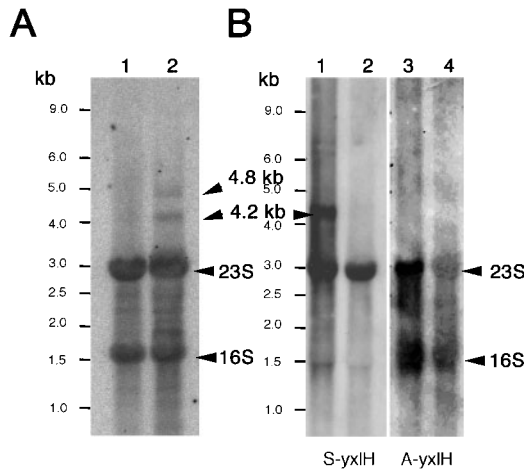

Fig. 7. **Northern analysis of the *sigY* transcript.** (A) RNA samples prepared from strain 168 cells harvested at T1 (lane 1) and T6 (lane 2) after nitrogen starvation were subjected to Northern analysis using a *sigY*-specific ^{32}P-labeled DNA probe, as described in the text. Each lane contained 5 μg RNA. The positions of size markers are indicated on the left. Two arrowheads indicate the 4.2- and 4.8-kb *sigY* transcripts, respectively. The two dense bands denoted as 23S and 16S resulted from unspecific hybridization of the probe to abundant 23S and 16S rRNAs. (B) RNA samples prepared from strain 168 cells bearing plasmid pDG148-sigY grown to the mid-logarithmic phase with 1 mM IPTG (lanes 1 and 3) and without IPTG (lanes 2 and 4) were subjected to Northern analysis using two kinds of *yxlH*-specific DIG-labeled RNA probes [S-yxlH (lanes 1 and 2) and A-yxlH (lanes 3 and 4) carrying sense and anti-sense RNA sequences, respectively], as described in the text. Each lane contained 15 μg RNA. The same size markers as for Northern analysis (A) were used, and the arrowhead indicates the 4.2-kb transcript covering from *sigY* to convergent *yxlH*.

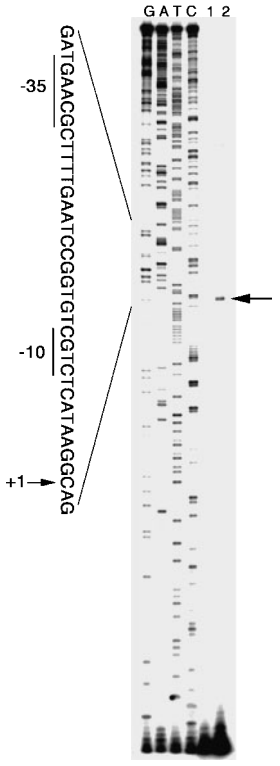

Fig. 8. **Primer extension analysis to identify the 5′-end of the *sigY* transcript.** RNA samples prepared from strain 168 harvested at T1 (lane 1) and T6 (lane 2) after nitrogen starvation were subjected to primer extension analysis using the primer (Table 2; PED) ^{32}P-labeled at its 5′ end. The arrow indicates the position of the reverse transcript. Lanes G, A, T, and C contained the products of the respective dideoxy sequencing reactions performed with the same labeled primer, using the PCR product amplified with a primer pair (PEU/PED) as a template. The part of the nucleotide sequence of the noncoding strand corresponding to the ladder is shown with the "–35" and "–10" regions of P*sigY*, which were deduced from the location of the 5′-end of the *sigY* transcript determined on this analysis (+1) and the results of deletion analysis of the *sigY* promoter region (Fig. 9).

moter region using a series of deletion derivatives of the P*sigY-sigY* (–181/+568)-*lacZ* fusion present in strain FU355. As shown in Figs. 2 and 9, β-Gal synthesis was observed in strains FU355, FU411, and FU412, whereas no synthesis was observed in strain FU409 or FU410. The β-Gal activities detected in strains FU412 and FU411 were approximately 2- and 1/5-fold that in FU355, implying that unknown repression and activation sequences for the *sigY* promoter might be located at nucleotides –181 to –56, and –55 to –40, respectively. However, it is unlikely that these sequences are involved in the *sigY* induction upon nitrogen starvation, because the induction profiles of strains FU412 and FU411 were similar to that of strain FU355 (Fig. 9). The fact that β-Gal synthesis was observed in strain FU411 suggests that the *sigY* promoter might be located at nucleotides –39 to +1 (Figs. 2 and 8).

Alignment of the *sigY* promoter region comprising nucleotides –39 to +1 with the corresponding promoter regions of known σ^X- and σ^W-targets (*5*, *28*)(data not shown) suggested that the "-10" and "-35" sequences of the *sigY* promoter might be CGTC and TGAACG, respectively (Figs. 2 and 8).

DISCUSSION

The *B. subtilis sigY* operon, comprising six genes (*sigY*, *yxlC*, *D*, *E*, *F*, and *G*), was induced upon nitrogen starvation (Figs. 4 to 8). This operon was continuously transcribed from the 31st base upstream of the translation initiation base of the *sigY* gene into *yxlH*, a neighboring convergent gene, resulting in a 4.2-kb mRNA (Figs. 1, and 6 to 8). Besides induction of the *sigY* operon upon nitrogen starvation, it was found to be positively autoregulated through σ^Y, as shown in Figs. 3 and 4, and Table 3, suggesting that its transcription is likely directed by its own sigma factor, σ^Y. This σ^Y-directed transcription was under negative regulation involving the YxlD protein (Figs. 3 and 4, and Table 3). There was a possibility that the *sigY* transcript extending into *yxlH* might inhibit *yxlH* translation through hybridization between *sigY* and *yxlH* mRNAs. However, we could not observe *yxlH* expression under the current growth and starvation conditions.

Primer extension to identify the 5′-end of the *sigY* transcript (Fig. 8), deletion analysis of the *sigY* promoter region (Figs. 2 and 9), and subsequent alignment of the *sigY* promoter with those of the target genes of σ^W, σ^X, and σ^M (*5*, *28*) suggested that the "–10" and "–35" sequences of the *sigY* promoter might be CGTC and

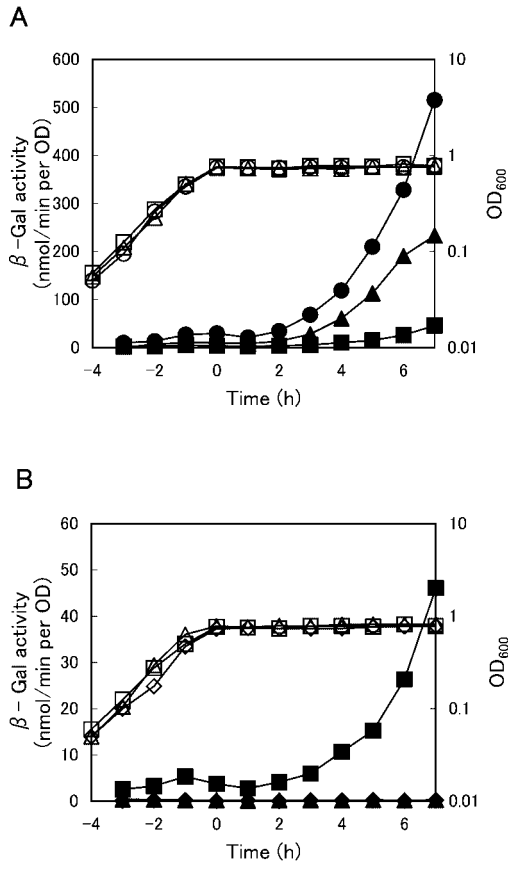

Fig. 9. **Deletion analysis of the *sigY* promoter region.** The structures of the strains used are given in Fig. 2. OD_{600} (open symbols) and β-Gal activity in the cells (closed symbols) were monitored during growth and nitrogen starvation. The β-Gal assay was performed as described in the text. (A) Cells of strains FU412 [P*sigY-sigY* (–55/+568), circles], FU355 [P*sigY-sigY* (–181/+568), triangles], and FU411 [P*sigY-sigY* (–39/+568), squares] were grown on 0.02% glutamine and subjected to nitrogen starvation. A large scale is adopted for the vertical axis. (B) Cells of strain FU411 (squares), FU409 [ΔP*sigY-sigY* (–4/+568), diamonds], and FU410 [P*sigY-sigY* (–12/+568), triangles] were grown and subjected to nitrogen starvation. A fine scale is adopted for vertical axis.

TGAACG, respectively. It is interesting to note that either σ^X, σ^W, or σ^M can recognize this CGTC sequence (*5*, *8–11*, *28–30*), but the consensus sequence of the –35 region recognizable to these three sigma factors is supposed to be tGaAAC (*5*, *8–10*, *29*, *30*), which is distinct from TGAACG recognized by σ^Y; there are only two As between TG and CG in the sequence of the "–35" region of P*sigY*, although the "AAC" motif is conserved.

To examine the conservation of the amino acid sequences of the products of the constituent genes of the *sigY* operon (*sigY*, *yxlC*, *D*, *E*, *F*, and *G*) and their organization in various organisms as well as their function, we subjected their amino acid sequences to a protein similarity search using the BLASTP 2.2.3 program (*27*). One of the ECF-type sigma factors, σ^Y, exhibited similarities to the sigma factors of various microorganisms, the highest score [100] being for RpoE5 of *Thermoanaerobacter tengcongensis*, a rod-shaped, Gram-negative, anaerobic eubacterium (*31*), whereas YxlF showed high similarity to numerous ATP-binding proteins of the ABC transporter family in various organisms (*32*), the first, second, third and fourth highest scores [241, 238, 237, and 198] being for BH0290 of *Bacillus halodurans*, an alkaliphilic bacterium (*33*), AP004596_43 of *Oceanobacillus iheyensis*, an alkaliphilic and extremely halotolerant bacterium (*34*), LMO2227 of *Listeria monocytogenes*, a food-borne pathogen (*35*), and CcmA12 of *T. tengcongensis*, respectively. YxlC and D did not show a higher score than 50 as to any protein, the score of YxlD as to a conserved hypothetic protein of *T. tengcongensis* (TTE2180) being the highest [43.5]. YxlE showed the first, second and third highest similarities to TEE2181 of T. *tengcongensis* (score 52), LMO2228 of *L. monocytogenes* (score 49), and AP004596_44 of *O. iheyensis* (score 45), respectively. Also, YxlG exhibited similarities to several conserved hypothetic proteins of bacteria, the first, second, third and fourth highest scores [145, 121, 117, and 65] being for LMO2226 of *L. monocytogenes*, AP004596_42 of *O. iheyensis*, BH0291 of *B. halodurans*, and TTE2183 of *T. tengcongensis*, respectively. These results indicated that the gene organization of the *sigY* operon was quite similar to that of the *RpoE5* operon likely composed of the *rpoE5*, TTE2179, TTE2180, TTE2181, *ccmA12*, and TE2183 genes (*31*), each of which is a homolog of the corresponding gene of the *sigY* operon except TTE2179. Furthermore, the gene order of *yxlE*, *F* and *G* is the same as that of the homolog genes of *L. monocytogenes* and *O. iheyensis*. Also, the two *B. halodurans* gene homologs as to *yxlF* and *yxlG* were also neighboring. The fact that the homologs of the constituent genes of the *sigY* operon are similarly organized in three bacteria living in extremely adverse environments implies that the function of the *sigY* operon might be related to some cellular function that enables them to tolerate such environments. In fact, the *B. subtilis sigY* operon was induced upon nitrogen starvation (Figs. 4 to 8), one of the undesirable conditions for this organism. Furthermore, the above conservation of the *sigY* operon in these four bacteria suggests that its constituent genes might be closely related evolutionarily, or that they might have been horizontally transmitted on an evolutionary time scale.

The above similarity search for YxlF revealed that this protein is highly likely an ATP-binding protein for one of the ATP binding cassette (ABC) transporter systems. Quentin *et al.* (*36*) performed an inventory and assembly of the ABC transporter systems in the complete genome of *B. subtilis*, and suggested that *B. subtilis* contains at least 78 ABC transporters. They also implied that YxlF and G, the genes of which are cotranscribed (Figs. 6 and 7), might comprise an ABC transporter, which was predicted to be an extruder related to antibiotic resistance systems. The similarity search also revealed that *L. monocytogenes* and *O. iheyensis* possess homologs of YxlE as well as YxlF and G, the gene orders of which are the same as in the *B. subtilis sigY* operon. This implies that the three proteins (YxlE, F, and G) might comprise an ABC transporter.

The *lacZ* fusion analysis indicated that *sigY* expression began to be induced 3 h after the onset of nitrogen starvation (Figs. 4 and 5). The expression was found to be nearly 100 times higher in the cells carrying the P*sigY-sigY-lacZ* fusion than in those carrying P*sigY-lacZ*, although the expression in the latter cells continued to

increase at least for a further 9 h (Fig. 4). The very high *sigY* expression in the former cells could be due to the doubling of the *sigY* gene. This fact also implies that the *sigY* operon *in situ* in the chromosome was not efficiently induced even when the cells were exposed to nitrogen starvation, although clear induction was observed on DNA microarray and Northern analyses (Figs. 6 and 7). This poor induction was possibly due to the presence of negative regulation for *sigY* expression involving YxlD (Table 3). The finding that prolonged exposure of cells to nitrogen starvation induced greater expression of the *sigY* operon (Figs. 4 and 6) implied that the longer the cells were exposed to nitrogen starvation, the more effectively the negative regulation involving YxlD might be repressed. Thus, it is conceivable that enormously adverse environmental conditions related to but severer than nitrogen starvation, which we do not know yet, might efficiently repress negative regulation, because the genes of the *sigY* operon and their organization, and the genes encoding a presumed ABC transporter and their organization are conserved in *T. tengcongensis*, and *B. halodurans* and *O. iheyensis*, respectively. The fact that *sigY* disruption affected the sporulation induced by nitrogen starvation might be related to the survival of *B. subtilis* under adverse environmental conditions. Furthermore, the fact that the *yxlD gene* as well as *yxlC* are only essential for growth on solid LB medium when *sigY* is intact, *i.e.* not in liquid LB, implies that σ^Y might play an unknown physiological role in growth on solid medium, which more resembles natural growth conditions such as in soil.

Recently, we identified 17 candidate members of the σ^Y regulon by means of DNA microarray analysis, using a conditional overproduction system for σ^Yin which cells of strain 168 carrying plasmid pDG148-sigY had been grown in LB medium with and without IPTG (*13*). We also performed DNA microarray analysis using this conditional σ^Y overproduction system in which the cells had been grown in Difco sporulation medium (*23*) with and without IPTG, and identified 10 candidate members of the σ^Y regulon. Furthermore, we performed DNA microarray analysis to compare the transcriptomes of the *sigY* disruptant and wild-type cells, and searched for 62 candidate members of the σ^Y regulon. The data obtained on these two additional microarray analyses are also available on the KEGG Expression Database website (http://www.genome.ad.jp/kegg/expression). However, there was no overlapping of the candidate genes found in the three DNA micraoarray analyses except for that of the *sigY*, *yxlC*, *D*, *E*, *F*, and *G* genes, and a false positive results for *yxlH*. Although we did not thoroughly search for additional targets besides the *sigY* operon, DNA microarray analyses with a *sigY* disruptant as well as a conditional overproducer of σ^Y revealed that the members of this regulon are likely very limited.

Very recently, Cao *et al.* (*37*) published a paper on expression of the *B. subtilis sigY* operon and identification of target promoters of σ^Y after completion of this work. They demonstrated that the *sigY* operon is expressed from an autoregulatory promoter site, which is under negative regulation involving YxlD and E. They found another target promoter of σ^Y for *ybgB*, although we missed this target because we failed to spot this gene on our DNA microarrays owing to some trouble in amplifying it. There is an obvious contradiction between the two works in that we demonstrated that YxlE did not participate in the negative regulation involving YxlD. Moreover, we cannot exclude the possibility that YxlD might function as a negative regulator only in the presence of YxlC. Further characterization of this negative regulation remains to be carried out.

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