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Mutation in *yaaT* Leads to Significant Inhibition of Phosphorelay during Sporulation in *Bacillus subtilis*

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In the course of a *Bacillus subtilis* functional genomics project which involved screening for sporulation genes, we identified an open reading frame, *yaaT*, whose disruptant exhibits a sporulation defect. Twenty-four hours after the initiation of sporulation, most cells of the *yaaT* mutant exhibited stage 0 of sporulation, indicating that the *yaaT* mutation blocks sporulation at an early stage. Furthermore, the mutation in *yaaT* led to a significant decrease in transcription from a promoter controlled by Spo0A, a key response regulator required for the initiation of sporulation. However, neither the level of transcription of *spo0A*, the activity of σ^H, which transcribes *spo0A*, nor the amount of Spo0A protein was severely affected by the mutation in *yaaT*. Bypassing the phosphorelay by introducing an *spo0A* mutation (*sof-1*) into the *yaaT* mutant suppressed the sporulation defect, suggesting that the *yaaT* mutation interferes with the phosphorelay process comprising Spo0F, Spo0B, and histidine kinases. We also observed that mutation of *spo0E*, which encodes the phosphatase that dephosphorylates Spo0A-P, suppressed the sporulation defect in the *yaaT* mutant. These results strongly suggest that *yaaT* plays a significant role in the transduction of signals to the phosphorelay for initiation of sporulation. Micrographs indicated that YaaT-green fluorescent protein localizes to the peripheral membrane, as well as to the septum, during sporulation.

Initiation of sporulation in *Bacillus subtilis* is regulated by a signal transduction pathway, the phosphorelay, which is a multicomponent phosphotransfer system that is switched on in response to environmental, cell cycle, and metabolic signals (3, 36). The processing and integration of these signals by the phosphorelay control the level of phosphorylation of the transcription factor, Spo0A. Environmental and cellular signals that favor sporulation activate autophosphorylation of the sensor kinases KinA, KinB, KinC, and KinD, leading to input of a phosphate group into the phosphorelay (1, 2, 12, 13, 18, 20, 33, 37, 46). In this relay, the phosphate group is subsequently transferred to a response regulator, Spo0F. The resulting molecule, Spo0F-P, serves as a substrate for the Spo0B protein, a phosphotransferase which finally activates Spo0A by transferring the phosphate to the Spo0A protein (2). Spo0A-P, the activated form of Spo0A, indirectly controls the transcription of a number of genes by regulating the level of other transcription regulators. Spo0A-P directly activates transcription of the genes for many regulatory proteins and sigma factors required for cell-type-specific gene expression. Spo0A-P is also known to stimulate axial filament formation and asymmetric polar septation, which give rise to two unequal cells, a larger mother cell and a forespore cell (21, 38).

Just after septation, gene expression is controlled by the RNA polymerase sigma factors, σ^F in the forespore and σ^E in the mother cell. Later in sporulation, when the forespore has become engulfed by the mother cell, σ^F and σ^E are replaced by σ^G and σ^K, respectively (reviewed in references 22, 38, and 45). The coordinated functions of this cascade of sigma factors eventually transform the cell into an environmentally resistant spore.

The phosphorelay, which is obviously a process that is indispensable for efficient sporulation in *B. subtilis*, is subject to a variety of complex controls involving the transfer of phosphate through its component proteins. Interestingly, although the pathway and regulating proteins have been identified and investigated, the signals and the effectors of the regulators remain unknown.

In this study, we identified a sporulation-deficient *yaaT* mutant obtained by screening disruptants with disruptions in all genes having unknown functions within the framework of the *B. subtilis* functional genomics project conducted by a Japanese consortium. Here we present evidence that *yaaT* plays a significant role in phosphorelay during initiation of sporulation in *B. subtilis*.

MATERIALS AND METHODS

Bacterial strains, plasmids, phages, and general methods. The bacterial strains, plasmids, and phages used in this study are listed in Table 1. The oligonucleotide primers used are shown in Table 2. Transformation of *B. subtilis* was performed by using the method described by Dubnau and Davidoff-Abelson (7). The efficiency of sporulation was measured by growing *B. subtilis* cells in Difco sporulation medium (DSM) (42) at 37°C for 24 h. The number of spores (CFU) per milliliter of culture was determined by determining the number of heat-resistant (80°C, 10 min) colonies on tryptose blood agar base. Plasmid construction was performed by using *Escherichia coli* JM105.

Plasmid, phage, and strain construction. Plasmid pJMyaaT was constructed with the internal fragment of *yaaT* amplified with primers yaaTF and yaaTR. The PCR product and plasmid pJM114 (35) used for construction were completely digested with *Pst*I and *Bam*HI and then ligated. The resulting construct was used

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TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype, phenotype, and/or relevant characteristics	Source, reference, or construction[a]
E. coli JM105	*supE endA sbcB15 hsdR4 rpsL thi* Δ(*lac-proAB*)F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	50
B. subtilis strains		
168	*trpC2*	Laboratory stock
YAATd	*trpC2 yaaT*::pMUTyaaT	30
YABAd	*trpC2 yabA*::pMUTyabA	30
YAATK	*trpC2 yaaT*::pJMyaaT	pJMyaaT → 168
YAAT44	*trpC2 yaaT44*	pCAyaaT → 168
YAATGFP	*trpC2 yaaT*::pJMyaaTgfp	pJMyaaTgfp → 168
TtcGFP	*trpC2 yaaT*::pJMTtcgfp	pJMTtcgfp → 168
RL1740	*spoIIE*::*spoIIE-lacZ cat*	R. Losick
IIEZ	*trpC2 spoIIE*::*spoIIE-lacZ cat*	RL1740 → 168
RL388	*trpC2 pheA1* SPβ::*spoVG-lacZ cat*	R. Losick
VGZ	*trpC2* SPβ::*spoVG-lacZ cat*	RL388 → 168
UOT0531(ϕCAZ-1)	*trpC2* (ϕCAZ-1) *metB51 leuA8 nonB1*	49
0AZ	*trpC2* (ϕCAZ-1)	ϕCAZ-1—168
1L34	*trpC2 metB10 xin-1* (ϕ105dI:1t) SPβ(S)	BGSC[b]
1L34(ϕEDTA)	*trpC2 metB10 xin-1* (ϕEDTA) SPβ(S)	pEDTA → 1L34
YAATGFP(ϕEDTA)	*trpC2 yaaT*::pJMyaaTgfp (ϕEDTA)	ϕEDTA—YAATGFP
CD301	*trpC2* (ϕCD301)	ϕCD301—168
spoIIEΩpPE1	*spoIIEΩpPE1* (*spoIIE′-gfp* superglow)	17
JGFP	*trpC2 spoIIIJ*::pIIIJ-GFP	25
SOF[c]	*trpC2 leuA8 metB51 nonB1 Δspo0F sof-1*	16
SOJ	*trpC2 Δsoj-spo0J erm*	Laboratory stock
SDA	*trpC2 Δsda*::*spc*	This study
0ES	*trpC2 spo0E*::pUCS0E	This study
0ESIIEZ	*trpC2 spo0E*::pUCS0E *spoIIE*::*spoIIE-lacZ cat*	RL1740 → 0ES
T44SOJ	*trpC2 yaaT44 Δsoj-spo0J erm*	SOJ → YAAT44
T44SDA	*trpC2 yaaT44 Δsda*::*spc*	SDA → YAAT44
T440ES	*trpC2 yaaT44 spo0E*::pUCS0E	0ES → YAAT44
T440ESIIEZ	*trpC2 yaaT44 spo0E*::pUCS0E *spoIIE*::*spoIIE-lacZ cat*	0ESIIEZ → YAAT44
T44IIEZ	*trpC2 yaaT44 spoIIE*::*spoIIE-lacZ cat*	RL1740 → YAAT44
T44VGZ	*trpC2 yaaT44* SPβ::*spoVG-lacZ cat*	RL388 → YAAT44
T440AZ	*trpC2* (ϕCAZ-1) *yaaT44*	ϕCAZ-1—YAAT44
TKSOF	*trpC2 leuA8 metB51 nonB1 yaaT*::pJMyaaT *Δspo0F sof-1*	YAATK → SOF
Plasmids		
pUC19	*bla*	50
pCA191	*bla cat*	25
pJM114	*bla kan*	35
pBEST517A	*bla spc*	M. Itaya
pUCS192	*bla spc*	This study
pCAyaaT	*bla cat*	This study
pJMyaaT	*bla kan*	This study
pJMyaaTgfp	*bla kan*	This study
pJMTtcgfp	*bla kan*	This study
pUCS0E	*bla spc*	This study
pED405	*bla erm*	Laboratory stock
pEDTA	*bla erm*	This study

[a] The arrows indicate transformation from donor DNA to recipient strain. A dash indicates lysogenization of the host strain with phage.
[b] BGSC, *Bacillus* Genetic Stock Center, Ohio State University.
[c] The original designation was UOT0550.

to transform *E. coli* JM105 and was selected on ampicillin-supplemented Luria-Bertani solid media.

pUCS192 was constructed by cloning a *Bam*HI (blunted)-*Xba*I (blunted) fragment of pBEST517A containing the *spc* gene into the *Nde*I (blunted) site of pUC19 (50). To construct pUCS0E, a *Hin*dIII-*Bam*HI fragment bearing an internal fragment of *spo0E* was generated by PCR amplification with primers 0EF and 0ER and subcloned into the *Hin*dIII-*Bam*HI site of pUCS192.

Plasmid pJMyaaTgfp carrying the *yaaT-gfp* fusion gene was constructed with the oligonucleotide primers yaaTgfpF and yaaTgfpR to obtain a *Bam*HI- and *Pst*I-digestible PCR fragment. Next, *Pst*I- and *Xba*I-digestible fragments were generated by using the chromosomal DNA of *B. subtilis* spoIIEΩpPE1 (17) as the template with primers gfpsgF and gfpsgR. The insert was then ligated into the *Bam*HI-*Xba*I site of pJM114.

pEDTA was constructed with primers yaaTUF and yabAR by amplifying the *yaaT-yabA* coding region containing the putative promoter (as determined by Northern blot analysis as described at the BSORF website [http://bacillus.genome.ad.jp/]) by using chromosomal DNA of *B. subtilis* 168 as the template. The PCR products and pED405 (which contained the *erm* gene cassette in the *Pst*I-*Sma*I site of a 3.0-kb *Hin*dIII-*Eco*RI fragment of ϕ105 DNA) that were used for construction were completely digested with *Bam*HI and then ligated. pEDTA was cloned by the prophage transformation method in the temperate phage ϕ105dI:1t (9, 15). The recombinant phage was designated ϕEDTA.

Plasmid pJMTtcgfp carrying the P*yaaT-gfp* transcriptional fusion gene was constructed with oligonucleotide primers yaaTUF and yaaTUR to obtain a *Bam*HI- and *Pst*I-digestible PCR fragment. Next, *Pst*I- and *Xba*I-digestible fragments were generated by using chromosomal DNA of *B. subtilis* spoIIEΩpPE1 (17) as the template with primers gfpsgtc and gfpsgR. The insert was then ligated into the *Bam*HI-*Xba*I site of pJM114.

TABLE 2. Oligonucleotide primers used in this study

Primer	Sequence (5′ to 3′)[a]	Description, location, and restriction site[b]
yaaTF	*AAAACTGCAG*ATGTAATTGGTGTCCGC	*yaaT* sense sequence, +24, *Pst*I
yaaTR	*CGCGGATCCC*GACCTGGCCGTACTCA	*yaaT* antisense sequence, +117, *Bam*HI
yaaTUF	*CGCGGATCC*AACAAAGACACAATGGCCG	*yaaT* sense sequence, −563, *Bam*HI
yaaTUR	*AAAACTGCAG*ACCTGGCCGTACTCAACGC	*yaaT* antisense sequence, +113, *Pst*I
yaaTDF	*AAAACTGCAG*GTAATTGCAAATAAACAGGTGG	*yaaT* sense sequence, +154, *Pst*I
yaaTDR	*CCCAAGCTT*TTCCAGAATATTTAGTCCG	*yaaT* antisense sequence, +708, *Hin*dIII
yabAR	*CGCGGATCC*TCATGTCCTCTGCCAGC	*yabA* antisense sequence, +457, *Bam*HI
0EF	*CCCAAGCTT*TGGGCGGTTCTTCTGAA	*spo0E* sense sequence, +18, *Hin*dIII
0ER	*CGCGGATCC*ACAATCCAGCTCCTGAC	*spo0E* antisense sequence, +116, *Bam*HI
yaaTgfpF	*CGCGGATCCC*GAGTCGACTTTAGAGAGCTTG	*yaaT* sense sequence, +388, *Bam*HI
yaaTgfpR	*AAAACTGCAG*AATCTGTGGTTTGTGCG	*yaaT* antisense sequence, +810, *Pst*I
gfpsgF	*GGATCCCCCGGGCTGCAG*GAATTCGATTAG	*gfp* sense sequence, −6, *Pst*I
gfpsgR	*GCTCTAGA*TTTGTATAGTTCATCCATGCC	*gfp* antisense sequence, +694, *Xba*I
gfpsgtc	*AAAACTGCAGAAGGTGGTGAACTACT*ATGAGTAAAGGAGAAGAACTTT	*gfp* sense sequence, +22, *Pst*I
sdaUF	TGCCAACTAATAAGATAGGGTTTC	*sda* sense sequence, −1149
sdaUR	**ACATGTATTCACGAACGAAAATCG**ATGCATATAAGAACAATCGTTCTG	*sda* antisense sequence, −127
sdaDF	**ATTTTAGAAAACAATAAACCCTTG**AGGGCCGGTTATTATGCTAACCAG	*sda* sense sequence, +310
sdaDR	ATGAAAGCAGAATGATATTCACTG	*sda* antisense sequence, +1304

[a] Additional sequences and restriction sites that do not correspond to the sequences of genes are indicated by italics and underlining, respectively. Sequences corresponding to the spectinomycin resistance gene are indicated by boldface type.

[b] The locations are the 3′ end positions of the primers corresponding to the numbers of nucleotides from the initiation codons of the genes.

pCAyaaT was constructed by cloning the *Bam*HI-*Pst*I-digested PCR fragment amplified with oligonucleotide primers yaaTUF and yaaTUR and the *Pst*I-*Hin*dIII-digested fragment amplified with primers yaaTDF and yaaTDR into *Bam*HI-*Hin*dIII-digested pCA191 (25). The resulting plasmid, pCAyaaT, contained a frameshift mutation in the *yaaT* gene at the *Pst*I site inserted at amino acid 44 (total number of amino acids, 275). The *yaaT44* frameshift mutation was constructed by the gene replacement method of Stahl and Ferrari (43) by using pCAyaaT.

An *sda* deletion mutant was created by using the long-flanking homology PCR strategy (47). The specific primers used for construction were primers sdaUF and sdaUR and primers sdaDF and sdaDR (Table 2). The resulting mutant contained a spectinomycin cassette between positions −127 and +310 of the *sda* gene.

β-Galactosidase assay. *B. subtilis* cells grown in hydrolyzed casein growth medium at 37°C were induced to sporulate by the resuspension method of Sterlini and Mandelstam (44), as specified by Nicholson and Setlow (28) and Partridge and Errington (32). The β-galactosidase activity was determined as previously described by the method of Miller (24) by using *o*-nitrophenyl-β-D-galactopyranoside as the substrate. The enzyme specific activity was expressed in nanomoles of substrate (*o*-nitrophenyl-β-D-galactopyranoside) hydrolyzed per milligram per minute.

Fluorescence microscopy. Cells were grown and sporulated at 37°C in DSM containing FM4-64 (final concentration, 0.5 μg/ml; Molecular Probes) for staining of the cell membrane (39). Five hundred microliters of the culture was centrifuged, and 400 μl of the supernatant was aspirated off. The cells were then resuspended in the remaining 100 μl. Portions (2 μl) of each sample were mounted on glass slides treated with 0.1% (wt/vol) poly-L-lysine (Sigma). Microscopy was performed with an Olympus BX50 phase-contrast and fluorescence microscope with a 100× UplanApo objective. Images were captured by using a SenSys charge-coupled device camera (Photometrics) and Metamorph 4.5 software (Universal Image). FM4-64 and green fluorescent protein (GFP) were visualized by using a WIG filter set (Olympus) and an FITC filter set (Olympus), respectively. Image processing was done with Adobe Photoshop 4.0.1J.

Protoplasting, protein fraction, and Western immunoblot analysis. In order to detect the Spo0A protein by Western immunoblotting, *B. subtilis* cells were grown in hydrolyzed casein growth medium at 37°C and induced to sporulate by the resuspension method. Samples were taken at different times and centrifuged to collect cells. Cell pellets were protoplasted as described by Wu and Errington (48). Protein concentrations of samples were determined by the Bio-Rad protein assay (Bio-Rad), and 30-μg samples of proteins were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by Western immunoblotting with anti-Spo0A antibody (26). For Western immunoblot analysis of YaaT-GFP protein, *B. subtilis* cells were grown and sporulated in DSM at 37°C. Samples were taken at different times and centrifuged to collect cells. The cell pellets were protoplasted and fractionated as described by Wu and Errington (48). Then samples were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis and analyzed by Western immunoblotting with anti-GFP antibody (Molecular Probes). Protein concentrations of samples were determined by the Bio-Rad protein assay before fractionation.

RESULTS

Identification of the new sporulation gene *yaaT*. A *yaaT* plasmid insertion mutant, YAATd, was screened as a sporulation-defective phenotype from disruptant collections of genes having unknown functions in *B. subtilis* (30). Upstream and downstream of *yaaT* are the *holB* gene, which encodes a δ-subunit of DNA polymerase III, and the *yabA* gene, which encodes a protein that acts during initiation control on DNA replication. Both of these genes occur with the same direction of transcription on the chromosome DNA (19) (Fig. 1). YAATd exhibited significantly decreased production of heat-resistant spores (Table 3) in addition to a slow-growth phenotype (data not shown). We speculated that the slow growth of YAATd might be due to a polar effect on the downstream gene *yabA*. In order to test this possibility, we examined the phenotype of a *yabA* mutant, YABAdd. As expected, cells of the *yabA* mutant showed slow growth, but the *yabA* mutation had no significant effect on sporulation efficiency. Furthermore, in strain YAATd, in which *yaaT* was disrupted with the pMUTin1 vector, expression of the *yaaT* downstream gene *yabA* was ensured by an isopropyl-β-D-thiogalactopyranoside (IPTG)-inducible promoter, and the slow-growth phenotype was com-

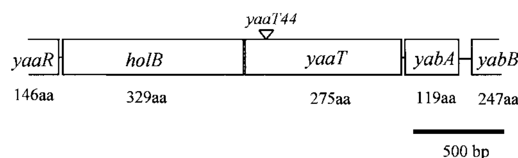


FIG. 1. Genetic organization of the *yaaT* region. The location of the *yaaT44* mutation is indicated. aa, amino acids.

TABLE 3. Sporulation frequencies of the *yaaT* mutants

Strain	CFU/ml		Frequency[b]
	Viable cells[a]	Spores	
168	4.0×10^8	3.8×10^8	0.95
YAATd	2.8×10^8	6.0×10^3	2.1×10^{-5}
YABAd	6.7×10^8	4.6×10^8	0.69
YAAT44	3.1×10^8	1.3×10^4	4.2×10^{-5}

[a] Cells were grown in DSM.
[b] The frequency is the ratio of the number of spores to the number of viable cells for each strain.

pletely restored by addition of IPTG. Moreover, Noirot-Gros et al. (29) have recently found a similar deficiency in a *yabA* mutant. We also constructed a frameshift *yaaT* mutant, YAAT44, whose mutation is not expected to affect the expression of *yabA*. This mutation caused a significant decrease in the production of heat-resistant spores (Table 3) but had no influence on cell growth. These results indicated that *yaaT* is involved in sporulation but is not required for cell growth.

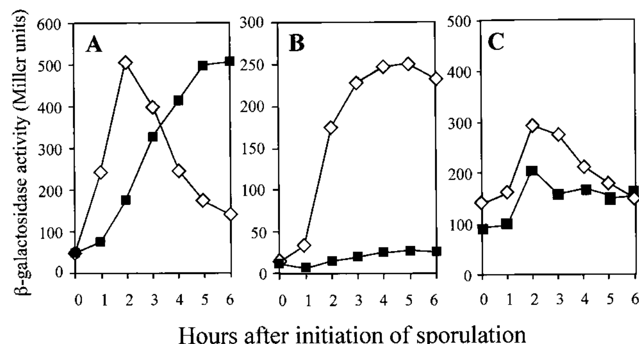


FIG. 3. Expression of the various early sporulation genes in the *yaaT* mutant. Various strains carrying the *lacZ* fusions were induced to sporulate, and the β-galactosidase activities were assayed. (A) *spoVG-lacZ* expression. Symbols: ◇, VGZ (wild type); ■, T44VGZ (*yaaT*). (B) *spoIIE-lacZ* expression. Symbols: ◇, IIEZ (wild type); ■, T44IIEZ (*yaaT*). (C) *spo0A-lacZ* expression. Symbols: ◇, 0AZ (wild type); ■, T440AZ (*yaaT*).

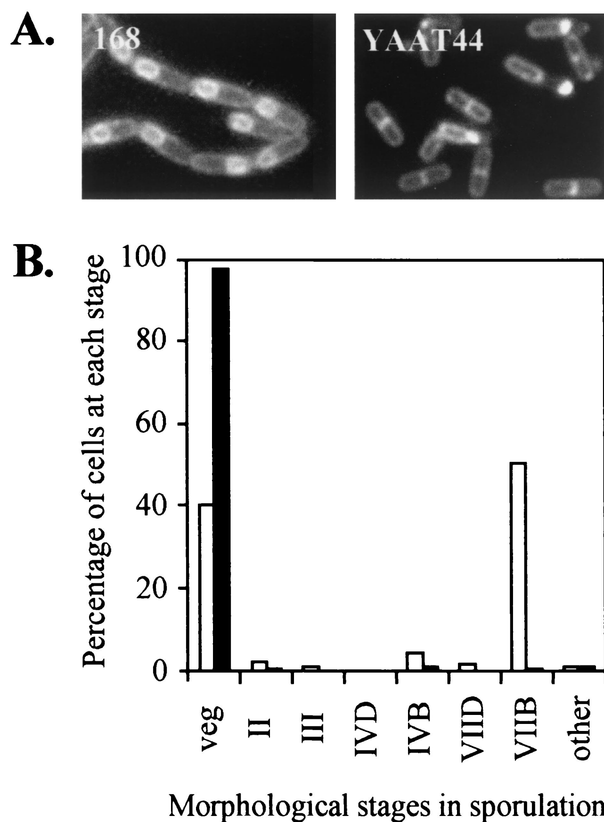


FIG. 2. Effect of *yaaT* mutation on sporulation. (A) Fluorescence microscopy of wild-type cells and YAAT44 cells stained with FM4-64 6 h after inoculation into DSM. (B) Quantification of morphological stages in the cell population of the *yaaT* mutant in DSM at T_{24}. Open bars, wild type; solid bars, YAAT44. veg, vegetative cells and stage 0 cells (no asymmetric septa); II, stage II cells with asymmetric septa; III, stage III cells with spore protoplasts (forespores) within the rod-shaped mother cells; IVD and IVB, stage IV cells with forespores becoming phase dark and progressively phase bright, respectively; VIID and VIIB, spore bodies becoming phase dark and phase bright, respectively, with no surrounding rod-shaped mother cells. Altogether, 455 wild-type cells and 406 YAAT44 mutant cells were counted.

YaaT is required for the early stage of sporulation. To determine the morphological stage at which the *yaaT* disruptant is blocked in the sporulation process, we observed the cells by phase-contrast and fluorescence microscopy by using the membrane-staining method and FM4-64 (39). Because FM4-64 is a membrane-impermeable fluorescent membrane stain, the culture was grown in DSM containing FM4-64 and sampled 6 h after the onset of sporulation (T_6), at which point internal membrane structures derived from the cytoplasmic membrane were also stained. Figure 2A shows that the frameshift *yaaT* mutant (YAAT44) could not proceed to the late stage of sporulation. Furthermore, analysis of FM4-64-stained T_{24} cells by fluorescence microscopy showed that in approximately 98% of the *yaaT* mutant cells sporulation was arrested early, before asymmetric septum formation (Fig. 2B). Based on this observation, we classified *yaaT* as a stage 0 gene and concluded that the *yaaT* gene product is required for the early stage of sporulation.

Effect of *yaaT* mutation on Spo0A activation. The initiation of sporulation is controlled by phosphorylation of a single transcription regulator, Spo0A, and stabilization of the early-stage-specific sigma factor σ^H (23). Since the morphological stage of blockage of the *yaaT* mutant is stage 0, the *yaaT* mutation was expected to interfere with the expression of σ^H- or Spo0A- and σ^A-dependent genes. To study the effect of the *yaaT* mutation on σ^H and Spo0A activities, a *spoVG-lacZ* transcriptional fusion, which originated from a σ^H-dependent promoter, and a *spoIIE-lacZ* transcriptional fusion, which originated from a σ^A-dependent promoter requiring Spo0A proteins, were used. In the wild-type cells, expression of *spoVG-lacZ* was dramatically elevated and reached the maximum level at T_2 and then decreased. In the *yaaT* mutant, expression slowly increased and a reached maximum level at T_5 with a 1-h delay compared to the wild type, but the maximum level of expression was almost the same as that of the wild type (Fig. 3A). On the other hand, expression of the *spoIIE-lacZ* fusion was induced shortly after the initiation of sporulation

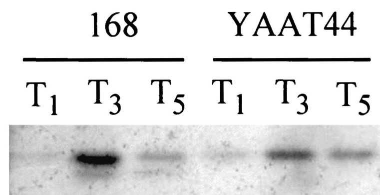


FIG. 4. Western blot analysis of Spo0A protein in the *yaaT* mutant. The cells were induced to sporulate and collected at different times. Western blots of whole-cell extracts were detected with an antibody that recognizes Spo0A.

and reached the maximum level at T_4 to T_5 in wild-type cells. Conversely, the expression in the *yaaT* mutant was strongly inhibited (Fig. 3B). We also examined the levels of expression of *spo0A* in the *yaaT* mutant. *spo0A* is transcribed during vegetative growth from two promoters, a σ^A-dependent promoter (P_V) that is turned off around T_0 and a σ^H-dependent promoter that is substantially activated at the onset of the stationary phase (49). In the *yaaT* mutant, the level of expression of *spo0A* during sporulation was 70 to 80% of the wild-type level (Fig. 3C). Furthermore, we examined the amount of Spo0A protein during sporulation by performing a Western immunoblot analysis with anti-Spo0A antibody. In the wild type, a 29.5-kDa band of Spo0A protein was detected at T_1 to T_5, specifically at T_3 (Fig. 4). On the other hand, in the *yaaT* mutant the intensity of the Spo0A protein signal was 50 to 60% of the wild-type intensity. This pattern of accumulation of the Spo0A protein closely correlated with expression of *spo0A* in the *yaaT* mutant (Fig. 3C). The point to be emphasized here is that the level of production of Spo0A was not substantially lower than the level of expression of Spo0A-P-dependent genes after introduction of the *yaaT* mutation, suggesting that the *yaaT* mutation interferes with the phosphorylation of Spo0A through a phosphorelay but does not interfere with the production of Spo0A.

Effect of *sof-1* mutation on *yaaT* mutant. In the wild-type cells, the phosphorylation pathway consists of at least four histidine kinases (KinA, KinB, KinC, and KinD), Spo0F, Spo0B, and Spo0A (reviewed in reference 3). A mutation in *spo0A*, *sof-1*, suppresses the sporulation defect caused by *spo0F* or *spo0B* mutations (11, 16). This suppression depends on *kinC*, indicating that $Spo0A^{sof-1}$ can receive phosphate directly from KinC (18, 20). If a mutation in *yaaT* affects Spo0A-P production by inhibiting the phosphorelay, it is expected that the sporulation frequency in the *yaaT* mutant would be suppressed by introduction of the *sof-1* mutation. We therefore introduced the *sof-1* mutation into the *yaaT* mutant and checked whether the *sof-1* mutation suppressed the sporulation defect in the *yaaT* mutant. As shown in Table 4, the sporulation defect in the *yaaT* mutant was completely suppressed by the *sof-1* mutation, confirming that *yaaT* is involved in some step of the phosphorelay during activation Spo0A.

TABLE 4. *sof-1* mutation completely suppresses sporulation inefficiency in the *yaaT* mutant

Strain	CFU/ml		Frequency[b]
	Viable cells[a]	Spores	
168	4.0×10^8	3.8×10^8	0.95
YAATK	2.9×10^8	5.0×10^3	1.7×10^{-5}
SOF	1.5×10^8	1.5×10^8	1.00
TKSOF	1.2×10^8	1.2×10^8	1.00

[a] Cells were grown in DSM.
[b] The frequency is the ratio of the number of spores to the number of viable cells for each strain.

TABLE 5. Effects of mutations in genes that negatively affect sporulation on *yaaT* mutants

Strain	CFU/ml		Frequency[b]
	Viable cells[a]	Spores	
168	4.0×10^8	3.8×10^8	0.95
YAAT44	3.1×10^8	1.3×10^4	4.2×10^{-5}
SOJ	1.9×10^8	1.9×10^8	1.00
SDA	4.1×10^8	3.2×10^8	0.78
0ES	4.5×10^8	4.1×10^8	0.85
T44SOJ	2.9×10^8	5.5×10^3	1.9×10^{-5}
T44SDA	2.2×10^8	4.8×10^3	2.2×10^{-5}
T440ES	2.8×10^8	1.4×10^8	0.50

[a] Cells were grown in DSM.
[b] The frequency is the ratio of the number of spores to the number of viable cells for each strain.

***yaaT* is probably involved in activation of Spo0A through *spo0E*.** It is known that several proteins that repress the phosphorelay and their specific repressors control the initiation of sporulation. This mechanism ensures that sporulation is not initiated unless conditions seem proper (reviewed in reference 3). It is known that the Soj, Sda, and Spo0E proteins are the negative factors of the phosphorelay (4, 5, 31, 34, 40). If the *yaaT* gene product affects the phosphorelay by inhibiting these negative factors, then the *yaaT* mutation is expected to suppress sporulation due to introduction of a mutation of these factors. To determine whether *yaaT* is related to the phosphorelay through *soj*, we introduced the *soj* mutation into the *yaaT* mutant and examined the sporulation frequency in the *yaaT soj* double mutant. As shown in Table 5, the sporulation deficiency in the *yaaT* mutant was not suppressed by the *soj*

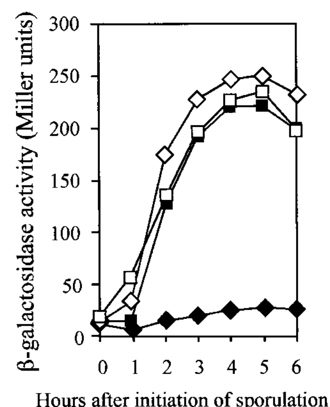
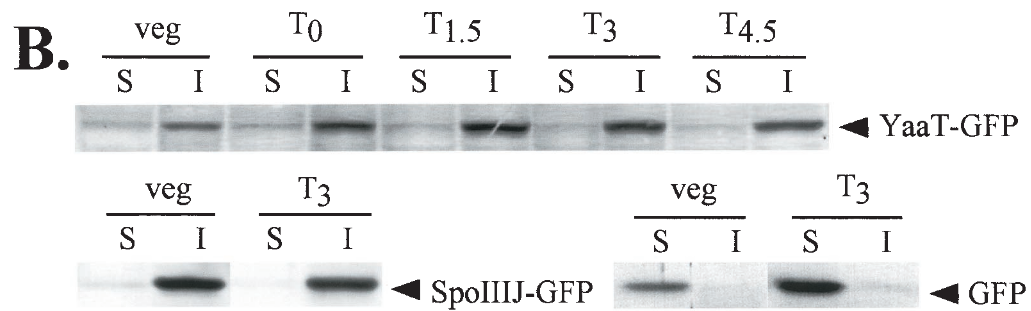


FIG. 5. Expression of *spoIIE-lacZ* fusion in the *yaaT spo0E* double mutant. Various strains carrying the *lacZ* fusions were induced to sporulate, and the β-galactosidase activities were assayed. Symbols: ◇, IIEZ (wild type); □, 0ESIIEZ (*spo0E*); ◆, T44IIEZ (*yaaT*); ■, T440ESIIEZ (*yaaT spo0E*).

A.



mutation, indicating that *yaaT* is not involved in the pathway through *soj* and *spo0J*. Likewise, to determine whether *yaaT* is involved in the DNA replication pathway through the *sda* gene, in which a mutation results in restoration of the sporulation deficiency of *dna* mutants (14), we introduced an *sda* mutation into the *yaaT* mutant and then examined the sporulation frequency in the *yaaT sda* double mutant. The *sda* mutation also did not suppress the sporulation deficiency in the *yaaT* mutant, implying that *yaaT* does not participate in a DNA replication pathway through *sda* (Table 5).

Finally, we examined the sporulation frequency and monitored expression of the *spoIIE-lacZ* activity in the *yaaT spo0E* double mutant. As shown in Table 5, the sporulation deficiency caused by the *yaaT* mutation was almost completely suppressed by the *spo0E* mutation. In addition, expression of *spoIIE* was completely restored in the double mutant (Fig. 5). These results suggest that YaaT plays an important role in the function of Spo0A-P by regulating the activity of Spo0E. It is thus possible that YaaT might increase the Spo0A-P level by controlling the Spo0E activity during the initiation of sporulation.

Localization of YaaT. The *yaaT* product was predicted to be a cytoplasmic protein based on its amino acid sequence, as analyzed by the SOSUI prediction system. In order to determine the localization of YaaT, we constructed a translational fusion of *yaaT* to the *gfp* (GFP) gene. To avoid inhibition of cell growth due to prevention of expression of *yabA* caused by integration of pJMyaaTgfp, we introduced φEDTA, which contains an intact copy of the *yabA* gene, into YAATGFP. The activity of YaaT was not inhibited by fusion to GFP, since this strain was able to sporulate as efficiently as the wild type (data not shown). A culture of YAATGFP(φEDTA) in DSM was grown in the presence of the vital membrane stain FM4-64, and samples were taken at the vegetative and sporulation phases. We then observed the membrane morphology and location of the YaaT-GFP fusion protein in *B. subtilis* by fluorescence microscopy (Fig. 6A). In the vegetative phase, YaaT-GFP localized throughout the periphery of the cell and the division septum. In the sporulation stages, fluorescence of the YaaT-GFP was observed throughout the periphery of the cell; however, in 80% of the cells two fluorescent dots were observed at the sides of an asymmetric septum and at the edges of the forespore ($T_{1.5}$ to T_3). The fluorescence diminished at the late stages of sporulation ($T_{4.5}$).

We analyzed cellular distribution of the YaaT-GFP fusion protein with SpoIIIJ-GFP (a membrane protein [25]) and the native GFP (a typical soluble protein), which were used as controls in Western immunoblotting experiments performed with antibody against GFP (Fig. 6B). The localization of these proteins was analyzed by the cell fractionation method (48). As expected, YaaT-GFP and SpoIIIJ-GFP were detected mainly in the insoluble fractions, whereas GFP was detected in the soluble fraction, suggesting that YaaT is membrane associated, as predicted.

DISCUSSION

Initiation of sporulation in *B. subtilis* is governed by a complex phosphorylation mechanism. Identification of a new gene involved in phosphorylation is essential for deciphering the phosphorelay process. We describe here identification of the new sporulation gene *yaaT*, which is essential for the phosphorelay during initiation of sporulation. The sequence of the *yaaT* gene is widely conserved in prokaryotes (some gram-positive bacteria and archaea), but the functions of the gene are unknown (Fig. 7). Interestingly, no *yaaT* homologue exists in gram-negative bacteria typically, implying that the function of YaaT in sporulation has developed through an evolutionary process within the gram-positive bacteria. The *yaaT* gene is located downstream of *holB*, which encodes the δ-subunit of DNA polymerase III, and upstream of *yabA*, which encodes a negative regulator of initiation of DNA replication (29). Considering the information on the adjacent open reading frames, it is possible that the *yaaT* gene product is also related to DNA replication. Burkholder et al. (4) proposed that the *sda* gene is involved in the phosphorylation pathway, which receives a signal from the DNA replication cycle. However, we have shown that the *sda* mutation does not suppress the *yaaT* mutation, suggesting that there is no relationship between *sda* and *yaaT*. Therefore, *yaaT* is not likely to be involved in DNA replication, at least not through the pathway involving *sda*.

Spo0E is a protein phosphatase that dephosphorylates active Spo0A-P to inactive Spo0A. The *spo0E* gene is transcribed by the σ^A form of RNA polymerase and is repressed by AbrB. The time of expression is from T_0 to T_3 (34). It has been predicted that Spo0E is involved in sensing some inhibitory signals unfavorable for sporulation. One of the protein Spo0E mutants, Spo0E11, which has a deletion at its C-terminal end, is a hyperactive phosphatase, and the *spo0E11* mutant exhibits sporulation deficiency (31). Perhaps this mutant protein has lost a controlling site and is unable to respond to a signal that modulates Spo0E phosphatase activity (31). This region of the molecule might be responsible for signal interpretation that may take the form of a discrete signal or may delineate a region that interacts with another compartment of the phosphorelay (31). In this work, we showed that the *spo0E* mutation suppressed the sporulation deficiency and restored the transcriptional level of *spoIIE-lacZ* in the *yaaT* mutant. Although it is not clear if YaaT and Spo0E interact directly, it is possible that YaaT controls the Spo0A-P level through Spo0E activity during sporulation. In this light, *yaaT* may inhibit the activity of Spo0E, which dephosphorylates the Spo0A-P protein and then

FIG. 6. Localization of YaaT-GFP fusion protein. (A) Typical phase-contrast (panels a, d, g, j, and m), membrane-stained (FM4-64) (panels b, e, h, k, and n), and GFP fluorescence (panels c, f, i, l, and o) micrographs. Strains carrying YaaT-GFP were observed in the vegetative stage (panels a, b, and c) and at T_0 (panels d, e, and f), $T_{1.5}$ (panels g, h, and i), T_3 (panels j, k, and l), and $T_{4.5}$ (panels m, n, and o). (B) Western blot analysis of fractionated YaaT-GFP, SpoIIIJ-GFP, and GFP. YAATGFP(φEDTA), JGFP, and TtcGFP cells were grown in DSM at 37°C and collected at different times. Each cell extract was fractionated into soluble and insoluble fractions and examined with an antibody that recognizes GFP. veg, vegetative cells; S, soluble fraction, I, insoluble fraction.

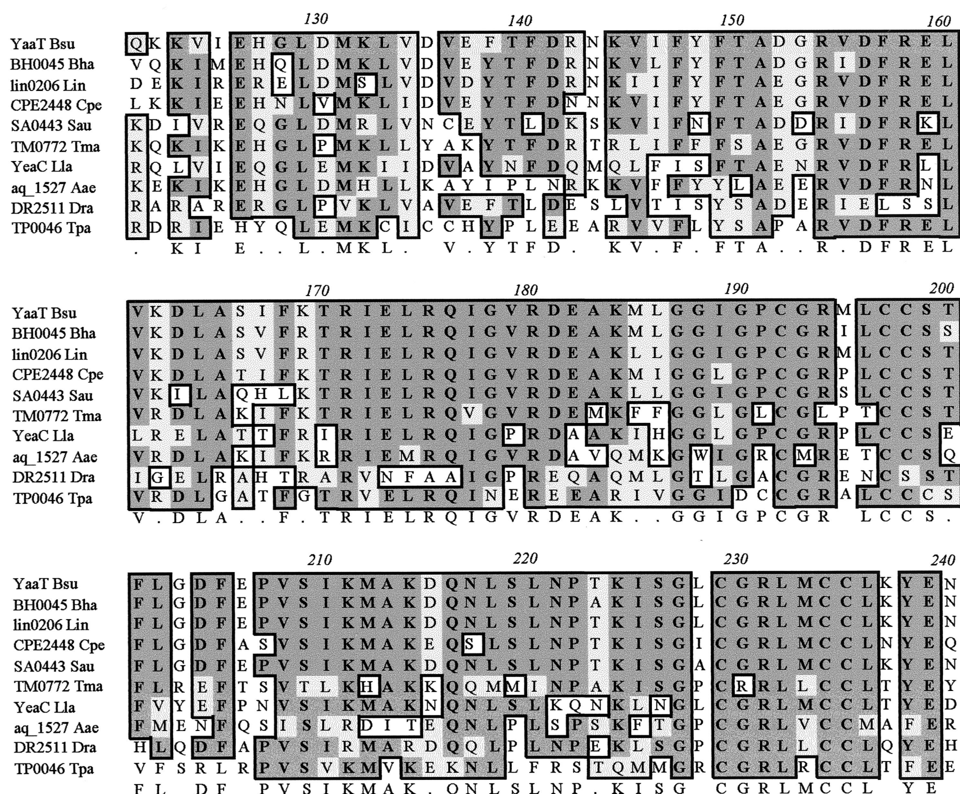


FIG. 7. Comparison of the amino acid sequences of the YaaT homologues from *B. subtilis* (YaaT Bsu), *Bacillus halodurans* (BH0045 Bha), *Listeria innocua* (lin0206 Lin), *Clostridium perfringens* (CPE2448 Cpe), *Staphylococcus aureus* (SA0443 Sau), *Thermotoga maritima* (TM0772 Tma), *Lactococcus lactis* (YeaC Lla), *Aquifex aeolicus* (aq_1527 Aae), *Deinococcus radiodurans* (DR2511 Dra), and *Treponema pallidum* (TP0046 Tpa).

supports accumulation of Spo0A-P during the early stage of sporulation. However, it has been shown that a null mutation of *spo0E* does not lead to a significant increase in the activity of Spo0A-P compared with the activity in the wild type, meaning that Spo0E can dephosphorylate Spo0A-P only at a low level (34, 41) (Fig. 4). Alternatively, some unknown inhibitory signals may inactivate YaaT or the interaction between YaaT and Spo0E at a later stage of sporulation, making it possible for Spo0E to perhaps inhibit excess accumulation of Spo0A-P. Interestingly, Nanamiya et al. (27) reported that ClpP also controlled Spo0A-P activity by negatively regulating the Spo0E function.

Two components of the phosphorelay, KinB and KinC, are both predicted to be integral membrane proteins, and they localize to the cell membrane and sense signals for initiation of sporulation. In our study we observed that YaaT-GFP localized throughout the periphery of the cell and the division septum. In the sporulation stages, the fluorescence of YaaT-GFP was observed as two dots at the side or edge of the engulfing asymmetric septum and the forespore. The *yaaT* product was predicted to be a cytoplasmic protein based on its amino acid sequence. We therefore speculate that YaaT is associated with the cell membrane along with another unknown protein that localizes to the cell membrane. We also speculate that YaaT directly or indirectly senses the signals for sporulation at the surface of the cell. However, it is still not known why YaaT-GFP localizes as two dots at the side or edge of the engulfing asymmetric septum and the forespore. Eichenberger et al. (8) observed localization of the SpoIIM-GFP fusion protein as a spherical focus at the forespore and as two dots at the distal polar division site of the cell, suggesting that the dots represent in cross section a ring that encircles the inside surface of the cell. The division proteins DivIB, DivIC, and PBP2B localize as a two-dot pattern before septum formation (6, 10). Harry and Wake (10) proposed that the two-dot pattern of DivIB represents an encircling ring of molecules attached to the cell membrane, that the two dots were located on the edge of the cell, and that the top and bottom regions of the ring could not be visualized by changing the focus. Therefore, it is possible that YaaT-GFP also forms a ring-like structure. However, most probably YaaT acts at stage 0 of sporulation, since inhibition of sporulation by the *yaaT* mutation was almost completely suppressed by the *sof-1* mutation and the *spo0E* mutation. The two-dot pattern of localization of YaaT-GFP after asymmetric septation might have just a minor role or no role in sporulation.

Identification of the new sporulation gene *yaaT*, mutation of which results in significant inhibition of phosphorylation and sporulation of cells, provides another major piece of information concerning the complex phosphorylation mechanism during sporulation. Further work on YaaT might lead to greater understanding of sporulation in *B. subtilis*.

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