Expression of the *ftsY* gene, encoding a homologue of the α subunit of mammalian signal recognition particle receptor, is controlled by different promoters in vegetative and sporulating cells of *Bacillus subtilis*

Hiroshi Kakeshita, Akihiro Oguro, Reiko Amikura, Kouji Nakamura and Kunio Yamane

Author for correspondence: Kunio Yamane. Tel: +81 298 53 6680. Fax: +81 298 53 6680.
e-mail: kyamane@sakura.cc.tsukuba.ac.jp

Institute of Biological Sciences, University of Tsukuba, Tsukuba-shi, Ibaraki 305, Japan

***Bacillus subtilis* FtsY (Srb) is a homologue of the α subunit of the receptor for mammalian signal-recognition particle (SRP) and is essential for protein secretion and vegetative cell growth. The *ftsY* gene is expressed during both the exponential phase and sporulation. In vegetative cells, *ftsY* is transcribed with two upstream genes, *rncS* and *smc*, that are under the control of the major transcription factor σ^A . During sporulation, Northern hybridization detected *ftsY* mRNA in wild-type cells, but not in sporulating cells of σ^K and *gerE* mutants. Therefore, *ftsY* is solely expressed during sporulation from a σ^K - and *GerE*-controlled promoter that is located immediately upstream of *ftsY* inside the *smc* gene. To examine the role of FtsY during sporulation, the *B. subtilis* strain ISR39 was constructed, a *ftsY* conditional mutant in which *ftsY* expression can be shut off during spore formation but not during the vegetative state. Electron microscopy showed that the outer coat of ISR39 spores was not completely assembled and immunoelectron microscopy localized FtsY to the inner and outer coats of wild-type spores.**

Keywords: *Bacillus subtilis*, FtsY (Srb), gene expression, σ^K and *GerE*, immunoelectron microscopy

INTRODUCTION

The signal-recognition particle (SRP) and the SRP receptor play a central role in targeting presecretory proteins to the membrane of the endoplasmic reticulum in mammalian cells. Recent genetic and biochemical evidence indicates that targeting may also be mediated by SRP in bacteria (Lütcke, 1995; Schatz & Dobberstein, 1996; Fekkes & Driessen, 1999). *Bacillus subtilis* is a Gram-positive bacterium that secretes high levels of extracellular enzymes into the culture medium. In *B. subtilis*, small cytoplasmic RNA and Ffh are homologues of SRP 7S RNA and SRP54 protein (a 54 kDa subunit of SRP), respectively (Honda *et al.*, 1993; Struck *et al.*, 1989). These are essential for protein translocation and the normal growth of *B. subtilis* (Honda *et al.*, 1993; Nakamura *et al.*, 1992). We cloned a gene for a homologue of the α subunit of the SRP receptor

(SR α) and designated it *srb* (Oguro *et al.*, 1995). The *srb* gene was renamed *ftsY* in the *B. subtilis* genome project since the amino acid sequence of Srb has 49.7% identity to that of *E. coli* FtsY (Kunst *et al.*, 1997). During the vegetative stage, *ftsY* is transcribed with the upstream genes, *rncS* (ribonuclease III) and *smc* (a homologue of the SMC family protein), under the control of the major transcription factor σ^A . Depleting *ftsY* in *Bacillus subtilis* inhibits normal cell growth and leads to a substantial loss of β -lactamase translocation (Oguro *et al.*, 1995, 1996), indicating that FtsY is essential for protein translocation.

B. subtilis generates a heat-resistant endospore under poor nutrient conditions. During sporulation, the forespore and mother cell each contain a chromosome and engage in a specific and genetic program via four compartment-specific σ subunits of RNA polymerase. Forespore-specific gene expression is controlled by σ^F and σ^G . Activation of σ^E in the mother cell is followed by the synthesis and activation of σ^K . In addition, two small

Abbreviation: SRP, signal recognition particle.

DNA-binding proteins, SpoIIID and GerE, activate or repress the transcription of many mother cell-specific genes. Mother-cell transcription factors form a hierarchical regulatory cascade in which the synthesis of each factor depends upon the activity of the prior factor, in the order σ^E , SpoIIID, σ^K and GerE (Losick & Stragier, 1992; Stragier & Losick, 1996). During the assembly of the cortex and coat proteins in the forespore, a number of polypeptides and proteins are synthesized within the mother cell and deposited on the forespore (Stragier & Losick, 1996). However, little is known about the role of the protein-secretion machinery in spore formation.

The present study shows that, in addition to the expression of *ftsY* in vegetative cells by the σ^A promoter, *ftsY* is expressed solely at t_8 during sporulation (times are given as hours after the onset of sporulation; i.e. t_8 is 8 h after the onset of sporulation), from a promoter that is controlled by σ^K and GerE, and located immediately upstream of *ftsY* and inside the *smc* gene. Electron microscopy showed that the outer coat of the *ftsY* mutant spores is composed of thin layers and immunoelectron microscopy localized FtsY to the coat.

METHODS

Bacterial strains and media. The *B. subtilis* strains listed in Table 1 were maintained and cultured in Luria–Bertani (LB) medium. Bacterial cells were cultivated in Schaeffer medium (Schaeffer *et al.*, 1965) with vigorous shaking to induce sporulation. *B. subtilis* ISR39 (*trpC2* *srb*::pMT3ftsY) was constructed from *B. subtilis* 168 by homologous recombination

between the chromosome and the plasmid pMT3ftsY, carrying another truncated *ftsY* gene, as described below.

Plasmid construction. To construct *B. subtilis* ISR39, an *ftsY* conditional null mutant, pMT3ftsY was derived from pMutinT3 (Moriya *et al.*, 1998), which contains a plasmid origin of replication that functions only in *Escherichia coli*, the *spac-1* promoter, the *lacI* gene expressed by the *penP* promoter, the *ermC* gene and three ρ -independent transcriptional terminators in front of *spac-1*. A 434 bp DNA fragment containing the flanking and N-terminal portions of *ftsY* (134 aa) was synthesized by the PCR using the synthetic oligonucleotides PS-1 (5'-CTATACAGCCAAGCTTGAATTCGTTTCAGTAACGAGG-3', generating a *Hind*III restriction site) and PS-2 (5'-CGCATTATGGGGATCCGTTTTC-CCGACGCCGTTTAC-3', generating a *Bam*HI restriction site) at positions 4915–4933 and 5330–5349, respectively, in the DNA sequence reported by Oguro *et al.* (1996). The amplified fragment was digested with *Hind*III/*Bam*HI, then ligated into pMutinT3 that had been digested with the same enzymes. The construct in which the ribosome-binding sequence and the truncated *ftsY* gene were positioned downstream of three ρ -independent transcriptional terminators and the *spac-1* promoter was designated pMT3ftsY.

RNA preparation and Northern hybridization. Total RNAs of *B. subtilis* cells cultured in Schaeffer medium were extracted at various vegetative and sporulating stages as described by Igo & Losick (1986). Northern hybridization proceeded according to a modification of the method described by Sambrook *et al.* (1989). Total RNA (10 μ g) was resolved by electrophoresis through a 1.5% agarose gel containing 2.2 M formaldehyde, then transferred to Gene Screen Plus nylon membranes (NEN Research Products). Prehybridization and hybridization proceeded at 65 °C in hybridization buffer (0.9 M NaCl, 0.09 M sodium citrate, 2 $\times$ Denhardt's reagent, 0.1% SDS, 100 μ g salmon sperm DNA ml⁻¹). To isolate DNA probes for *ftsY*, a

Table 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Genotype or description	Source or reference
<i>B. subtilis</i> strains		
168	<i>trpC2</i>	Laboratory stock
ISR39	<i>ftsY</i> ::pMT3ftsY <i>trpC2</i>	This work
1G12	<i>gerE36 leu-2 trpC2</i>	BGSC*
MO718	<i>spoIIIG::kan trpC2</i>	P. Stragier†
MO719	<i>spoIIAC::kan trpC2</i>	P. Stragier†
MO1027	<i>spoIVCB::erm trpC2</i>	P. Stragier†
MO1781	<i>spoIIIGAB::kan trpC2</i>	P. Stragier†
<i>E. coli</i> strains		
JM109	<i>recA1</i> Δ (<i>lac-proAB</i>) <i>endA1</i> <i>gyrA9</i> <i>thi-1</i> <i>hsdR17</i> <i>relA1</i> <i>supE44</i> [F' <i>traD36 proA</i> ⁺ B ⁺ <i>lacI</i> ^q <i>lacZ</i> Δ M15]	Sambrook <i>et al.</i> (1989)
Plasmids		
pMutinT3	pBR322 derivative carrying <i>spac-1</i> promoter, <i>lacI</i> , <i>ermC</i> gene and three ρ -independent transcriptional terminators	Moriya <i>et al.</i> (1998)
pMT3ftsY	pMutinT3 carrying a 434 bp <i>Hind</i> III– <i>Bam</i> HI fragment of <i>B. subtilis</i> <i>ftsY</i>	This work

* BGSC, *Bacillus* Genetic Stock Center.

† Institut de Biologie Physico-Chimique, Paris, France.

1.0 kb DNA region of *ftsY* was amplified by PCR using the synthetic oligonucleotides PS-3 (5'-AAAGAGGTTAAAAG-ATGAGCTT-3') and PS-4 (5'-GCCTATCAAGTAAGAAG-ATA-3') at positions 4935–4956 and 5995–5976, respectively, of the DNA sequence reported by Oguro *et al.* (1996). A 1.1 kb fragment of *cotYZ* was amplified using PC-1 (5'-ATGATG-TGTACGATTGATTA-3') and PC-2 (5'-ATATATAGACG-TTCACCCAC-3') at positions 2720–2701 and 1571–1590 of the sequence described by Zhang *et al.* (1993), and 0.6 kb of *cotZ* was amplified using PC-1 and PC-3 (5'-AAACACTT-GTAAAGAGGAAT-3') at position 2151–2170 of the latter sequence (Zhang *et al.*, 1993). The PCR template was chromosomal DNA of *B. subtilis* 168. After purification by agarose gel electrophoresis, the amplified DNA fragments were labelled with 32 P using a random primer DNA labelling kit (Takara Shuzo) and used as hybridization probes.

Mapping the 5' terminus of *ftsY* mRNA during sporulation.

Primer extension proceeded using the synthetic oligonucleotide Pr (5'-ACCCTCTCAAACTCATCTAT-3') at position 4358–4339 of the nucleotide sequence reported by Oguro *et al.* (1996). The RNAs to be tested (40 µg) and 5×10^4 c.p.m. 32 P-labelled oligonucleotide primer were hybridized at 40 °C overnight. Rous-associated virus-2 reverse transcriptase was added and the mixture was incubated at 42 °C for 1 h. The reaction products were resolved on DNA sequencing gels. The 5' ends of *ftsY*-specific mRNAs were determined by comparison with sequencing ladders generated from an M13 clone that included a 1.6 kb DNA fragment of the upstream gene (*smc*) of *ftsY* using the Pr oligonucleotide primer. A 1.6 kb DNA fragment was synthesized by PCR using synthetic oligonucleotides PS-5 (5'-CCTCTGTATCAGGCACC-3') and PS-6 (5'-CAGGAGGATCCAGTTTTGCAG-3', generating a *Bam*HI restriction site) at positions 3279–3295 and 4635–4615, respectively, in the DNA sequence reported by Oguro *et al.* (1996). The amplified fragment was digested by *Dra*I/*Bam*HI and ligated into M13 digested with *Hinc*II/*Bam*HI.

Preparation of cell lysates from sporulating cells. Sporangia of *B. subtilis* growing in Schaeffer medium were harvested, washed once in TBS (25 mM Tris/HCl, pH 7.5, 135 mM NaCl, 2.7 mM KCl) and frozen at –70 °C until use. Frozen cells suspended in 100 µl GTE (25 mM Tris/HCl, pH 7.5, 50 mM glucose, 10 mM EDTA) were lysed with lysozyme at a final concentration of 2 mg ml⁻¹ at room temperature for 5 min, then boiled in 0.4 M Tris/HCl, pH 6.8, 2% SDS, 0.5% β-mercaptoethanol and 10% (v/v) glycerol for 5 min, and separated by centrifugation. The supernatants were used as cell-lysate preparations.

SDS-PAGE and immunoblotting. Protein samples were resolved by SDS-PAGE (10% acrylamide), and electrotransferred to a PVDF membrane (Immobilon; Millipore). The membrane was incubated overnight at room temperature in phosphate buffered saline/Tween 20 (8 mM sodium phosphate, pH 7.5, 150 mM NaCl, 0.1% Tween 20), containing 5% low-fat milk. The membrane was then incubated for 1 h at room temperature with an anti-FtsY antiserum (at a dilution of 1:5000), in phosphate-buffered saline/Tween 20, followed by an incubation with a secondary antibody conjugated to horseradish peroxidase (Amersham Biotech) at a 1:5000 dilution for 1 h. Immunoblots were washed and visualized using enhanced chemiluminescence reagents, as described by the manufacturer (Amersham Biotech).

Electron microscopy. Wild-type cells (168) and ISR39 grown in Schaeffer medium and harvested at t_{24} were fixed and embedded as described by Nishiguchi *et al.* (1994), then stained with 1% uranyl acetate for 30 min and Reynold's lead

(Hayat, 1972) for 30 min. Stained cells were examined using a JEOL 2000EXII electron microscope.

Immunoelectron microscopy. Wild-type cells at the vegetative stage and during sporulation (t_{18}) were harvested by centrifugation and suspended in 1.0 ml phosphate-buffered Karnovsky's fixative (Karnovsky, 1965) at room temperature for 1.5 h. Fixed cells were washed once in 0.5 M NH₄Cl, suspended in hot solubilized 1% Bacto agar in water, gelatinized, then sequentially dehydrated at 4 °C for 15 min each in 50, 70, 80, 90 and 95% ethanol, followed by twice in 100% ethanol at –20 °C for 30 min. Thereafter, the cells were washed twice with 100% acetone at –20 °C for 30 min, then placed in Lowicryl HM20/acetone (1:3, 1:1, 3:1) at –50 °C for 1 h each, followed by 100% Lowicryl HM20 at –50 °C overnight. After adding fresh resin, blocks were polymerized by UV irradiation at –50 °C in a gelatinous capsule overnight. The blocks were thin-sectioned (gold-silver sections) using a diamond knife and placed on nickel grids that were subsequently placed on droplets of 1% glycine, 1% gelatin for 30 min, then onto a 1:200 dilution of rabbit anti-FtsY antibody overnight in a hydrated chamber. The grids were then washed five times by floating on droplets of 10 mM Tris/HCl (pH 8.0), 0.1 mM EDTA for 10 min and incubated with a 1:100 dilution of goat anti-rabbit antibodies conjugated to 15 nm gold particles (Bio-Rad) for 1 h. After a second wash, cells were stained with 1% uranyl acetate followed by Reynold's lead (Hayat, 1972) for 30 min each, then examined using a JEOL 2000EXII electron microscope.

RESULTS AND DISCUSSION

Sporulation-specific transcript of the *ftsY* gene

FtsY is one component of the protein-secretion machinery of *B. subtilis*. Depletion of FtsY leads to defective cell growth and the accumulation of secretory-protein precursors (Oguro *et al.*, 1996). The *ftsY* gene forms an operon with *rncS* and *smc*. We investigated *ftsY* expression during sporulation by Northern hybridization and determined the size of the RNA product as well as the time it appeared in *B. subtilis* 168 (Fig. 1a). Cultured 168 cells were harvested at various developmental stages and total RNA was extracted for Northern hybridization. The total RNA isolated from cells during vegetative growth, at t_{-2} , contained a band of approximately 5.5 kb that corresponded to a transcript which included three genes (Fig. 1a, lane 1). These results indicated that the three genes are simultaneously transcribed during exponential phase with two upstream genes (*rncS* and *smc*) by a putative σ^A -containing RNA polymerase.

At t_0 , the 5.5 kb band had disintegrated (Fig. 1a, lane 2) and it was undetectable in cultures 2–6 h after the end of the exponential phase of growth (t_2 – t_6) (Fig. 1a, lanes 3 to 5). This rapid disappearance of the 5.5 kb band may be caused by specific degradation after t_0 , in addition to reduced RNA production, since no obvious breakdown of 16S and 23S rRNAs was evident in the same samples (data not shown). At t_8 on the other hand, a 1.7 kb band containing *ftsY* mRNA appeared (Fig. 1a, lane 6). We analysed transcripts of spore-coat proteins expressed during sporulation by Northern hybridization to compare the timing of *ftsY* expression with that of *cotY* and *cotZ* as markers, since Zhang *et al.* (1994) reported that

Fig. 1. Expression of the *ftsY* gene. (a) Northern hybridization of *ftsY* mRNA. Total RNA was extracted from wild-type (*B. subtilis* 168) cells in Schaeffer medium cultured for t_{-2} (lane 1), t_0 (lane 2), t_2 (lane 3), t_4 (lane 4), t_6 (lane 5) and t_8 (lane 6). Total RNA (10 μ g) was analysed using a radiolabelled, nick translated 1061 bp DNA fragment of *ftsY* as the probe. The size of *ftsY* mRNA is indicated at the left of the figure. (b) Expression of *cotYZ* and *cotZ* during sporulation. Since *cotYZ* and *cotZ* are specifically expressed during sporulation (Zhang *et al.*, 1994), total RNAs of wild-type cells cultured until t_7 (lanes 1 and 4), t_8 (lanes 2 and 5) and t_9 (lanes 3 and 6) were extracted, blotted and probed with a 1150 bp DNA fragment of *cotYZ* and a 569 bp DNA of *cotZ*, respectively. (c) Immunoblot of FtsY expressed in *B. subtilis* 168. *B. subtilis* 168 was cultured in Schaeffer medium harvested at t_{-2} (lane 1), t_0 (lane 2), t_2 (lane 3), t_4 (lane 4), t_6 (lane 5), t_8 (lane 6) and t_{10} (lane 7) and lysed. Total proteins (20 μ g) from each preparation were resolved by SDS-PAGE and immunoblotted against anti FtsY antiserum. The arrow indicates the position of FtsY. The lower part of (c) indicates the relative amount of each FtsY band when the t_0 band density corresponds to 100.

Fig. 2. Determination of the transcription-initiation site by primer-extension analysis. Total RNAs (40 μ g) from *B. subtilis* 168 cultured in Schaeffer medium at t_8 (lane 1) and t_9 (lane 2) were hybridized with a labelled Pr primer that was complementary to nucleotides 4339–4358 in the sequence shown in Fig. 3. Primer-extended products obtained with reverse transcriptase were resolved by electrophoresis through 8% polyacrylamide sequencing gels, then visualized by autoradiography. DNA sequencing reaction mixtures containing the Pr primer and a single stranded DNA from the M13 derivative as the template, were resolved by electrophoresis in parallel (lanes A, C, G and T). Positions of the major product is indicated by an arrowhead. The asterisk in the sequence shows the estimated position of the transcription-initiation site.

cotY and *cotZ* are co-transcribed by σ^K -containing RNA polymerase from the P_{YZ} promoter with a smaller *cotY* mRNA resulting from premature termination or RNA processing. We detected two bands (1.4 and 0.6 kb) in total RNAs at t_7 , t_8 and t_9 using the 1150 bp DNA probe for *cotYZ*, but only one 1.4 kb band using the 569 bp DNA probe for *cotZ* (Fig. 1b). The density of the 1.4 and 0.6 kb bands indicated that *cotYZ* expression was maximal at t_8 under our culture conditions. This period of *cotYZ* expression coincided with that of *ftsY*.

We then analysed the amounts of FtsY in lysates of *B. subtilis* 168 by immunoblotting. Bands for FtsY were intense at t_{-2} and t_0 . However, the density decreased after t_0 (Fig. 1c, lanes 3–5). At t_8 , which is the period of *ftsY* expression (Fig. 1a), the FtsY band was again detected, but at a density that was 2.5-fold higher than that at t_6 (Fig. 1c, lane 6). This result is consistent with the findings of the Northern hybridization (Fig. 1a). After t_8 , the amount of FtsY again decreased and the band was very faint at t_{10} (Fig. 1c, lane 7). On the other hand, at t_2 (Fig. 1a), the amounts of FtsY protein were substantial, whereas *ftsY* mRNA is virtually absent (Fig. 1a, lane 3 and Fig. 1c, lane 3). These data suggest that the half-life of FtsY protein is relatively long.

Mapping the 5' terminus of *ftsY* mRNA expressed during sporulation

To define the 5' terminus of the 1.7 kb transcript of *ftsY* found at t_8 (Fig. 1a), we performed primer-extension analysis using the synthetic oligonucleotide Pr (see Methods). The primer-extension product is indicated by

Fig. 3. Schematic representation of the *ftsY* gene expression. The third gene of the *ftsY* operon is *ftsY* and its expression is controlled by two promoters (PA and PK) as indicated by Northern hybridization (Fig. 1a) and by the primer-extension analysis of the 1.7 kb *ftsY* mRNA (Fig. 2). The positions and lengths of each mRNA are controlled by the two promoters shown above. Nucleotide sequences of the putative PK promoter and the transcription-initiation site are shown below. Nucleotide numbers are as reported by Oguro *et al.* (1996). Pr is a synthetic oligonucleotide used as the primer to map the 5' terminus of *ftsY* mRNA. Putative GerE-binding regions are double underlined.

Fig. 4. Sequence comparison of the PK promoter of *ftsY* with promoter regions transcribed by σ^K -containing RNA polymerase. (a) Alignment of nucleotide sequences for promoter regions transcribed by σ^K -containing RNA polymerase. Promoters for six genes transcribed in the absence of GerE and four genes for which transcription required GerE in addition to σ^K are shown separately. Nucleotides in each promoter that match the consensus sequence (bold face and capital letters) are shown between the groups (m = C or A). Bold face and underlined nucleotides correspond to the transcription-start point. Sequences for the PK putative promoter region for *ftsY* (Fig. 3) are shown with matches to the consensus indicated by capital letters. (b) Alignment of nucleotide sequences of GerE-binding sites upstream of *cotB*, *cotC*, *cotVWX*, *cotX* and *cotYZ* promoter (Zhang *et al.*, 1994; Zheng *et al.*, 1992). Sequences thought to be GerE-binding sites for the PK promoter region of *ftsY* are shown (4108–4119 and 4158–4169). R = A or G; W = A or T; Y = T or C.

an arrow and two smaller minor products are visible in Fig. 2. These minor products could have resulted from premature termination by the reverse transcriptase. The largest extension product indicated by an arrowhead (Fig. 2) corresponded to the 5' terminus of the 1.7 kb transcript of *ftsY* mRNA during sporulation. This transcript was more abundant in RNA from cells harvested at t_8 than at t_9 . The 5' terminus of the *ftsY* mRNA was located 705 bp upstream of the translation-initiation site for the *ftsY* ORF, inside the *smc* gene (Fig. 3). These results indicated that *ftsY* is transcribed solely

via the putative promoter (PK) during sporulation, since the *ftsY* gene is 987 bp long and a ρ -independent transcriptional terminator is located downstream of the stop codon of *ftsY*. The nucleotide sequence around the PK promoter (4199–4226 region) was similar to the consensus sequence of the -35 (AC) and -10 (CATA---Ta) promoter region recognized by *B. subtilis* RNA polymerase containing σ^K (Fig. 4a) (Roels & Losick, 1995; Zhang *et al.*, 1994; Zheng *et al.*, 1992). Fig. 4(a) shows that the nucleotide sequences of GerE-independent promoters closely match the consensus

Fig. 5. Transcription of *ftsY* in *B. subtilis* wild-type strain 168 and sigma mutants. (a) Northern hybridization of *ftsY* mRNA in σ^E , σ^F , σ^G , σ^K and *gerE* mutants. Total RNA was extracted from wild-type (*B. subtilis* 168) (lanes 1 and 2), MO1719 (SigF^-) (lanes 3 and 4), MO1781 (SigE^-) (lanes 5 and 6), MO718 (SigG^-) (lanes 7 and 8), MO1027 (SigK^-) (lanes 9 and 10) and 1G12 (*GerE*⁻) (lanes 11 and 12) cells cultured in Schaeffer medium for t_0 (lanes 1, 3, 5, 7, 9 and 11) and t_8 (lanes 2, 4, 6, 8, 10 and 12). (b) Northern hybridization of *cotYZ* and *cotZ* mRNA in *gerE* mutants. Total RNA extracted from wild-type (*B. subtilis* 168) (lanes 1 and 2) and 1G12 (*GerE*⁻) (lanes 3 and 4) cells was blotted and probed with a 1.1 kb DNA fragment of *cotYZ*.

sequence, whereas the sequences of GerE-dependent promoters generally have little resemblance (Roels & Losick, 1995). The nucleotide sequence of the -10 region of the PK promoter has low identity with the consensus sequence of the σ^K promoter, which is consistent with the fact that *ftsY* transcription during sporulation is regulated in a GerE-dependent manner. We identified putative GerE-binding sequences (4110–4121 bp and 4160–4171 bp) upstream from the PK promoter (Figs 3 and 4b). These data suggest that *ftsY* is transcribed by PK promoters during sporulation as shown in the upper part of Fig. 3.

Regulation of the *ftsY* gene during sporulation

To examine which σ factor relates to *ftsY* transcription at t_8 , RNAs from the sigma factor-deficient strains MO1781 (SigE^-), MO719 (SigF^-), MO718 (SigG^-), MO1027 (SigK^-) and the GerE-deficient strain, 1G12, were extracted at t_0 and t_8 , and hybridized using the 1061 bp DNA fragment encoding *ftsY* as the probe (Fig. 5a). The 5.5 kb band found in *B. subtilis* 168 (Fig. 5a, lane 1), including the two upstream genes, was detected in all total RNA samples isolated at t_0 (Fig. 5a, lanes 3, 5, 7, 9 and 11). The lower-molecular-mass bands may be degradation products of the 5.5 kb transcript. In contrast, the 1.7 kb bands found in the RNA preparation of wild-type cells at t_8 (Fig. 5a, lane 2), were not detected in preparations of the σ^E , σ^F , σ^G , σ^K and *gerE* mutant cells sampled at t_8 (Fig. 5a, lanes 4, 6, 8, 10 and 12). Under these conditions, we detected reduced levels of *cotY* and *cotZ* transcripts in RNA preparations derived from *gerE* mutant cells at t_8 (Fig. 5b), compared with the wild-type. Zhang *et al.* (1994) reported that the expression of *cotY* and *cotZ* is under the control of σ^K -containing RNA polymerase and GerE. No obvious bands corresponded to *cotY* and *cotZ* in the σ^K mutant. However, lower levels of *cotY* and *cotZ* transcripts were detected in the

gerE mutant compared with the wild-type. These results are all in good agreement. We detected transcripts of *cotY* and *cotZ* in RNA preparations derived from *gerE* mutant cells at t_8 , indicating that the disappearance of 1.7 kb band corresponding to *ftsY* is not due to substantial degradation of RNA by RNases during preparation.

Effects of depletion of FtsY on spore morphology

To analyse the effect of FtsY depletion upon sporulation, we prepared a conditional null mutant of *ftsY* that expresses *ftsY* during the vegetative stage, but not during sporulation. Plasmid pMT3FtsY, which does not have a replication origin for *B. subtilis*, was integrated into the *B. subtilis* chromosome by single reciprocal recombination. The gene organization around *ftsY* of transformant strain ISR39 (Fig. 6a) was determined by Southern hybridization and PCR (data not shown). The strain ISR39 has three ρ -independent transcriptional terminators upstream of the *spac-1* promoter to avoid transcription of the *ftsY* gene from both the σ^A (PA) and PK promoters. Expression of intact *ftsY* gene in this strain should be regulated by only the IPTG-inducible promoter *spac-1*, of which the nucleotide sequences of -35 and -10 regions are typical of a σ^A promoter. Therefore, in the presence of a low concentration of IPTG, the *ftsY* gene can be expressed during the vegetative stage, but not during sporulation. We measured the amount of FtsY and the growth of ISR39 cells cultured in the presence of 0.1 mM IPTG. Immunoblotting detected normal levels of FtsY during logarithmic cell growth when cells were cultured in the presence of 0.1 mM IPTG. (Fig. 6c, lanes 1 and 2). The growth of ISR39 was impaired in the absence of IPTG (data not shown), in agreement with published results showing that FtsY is essential for growth (Oguro *et al.*, 1996). We then investigated the expression of *ftsY*

Fig. 6. Structure of *B. subtilis* ISR39 strain in which *ftsY* expression is dependent on IPTG, and expression of the *ftsY* gene in ISR39. (a) Schematic representation of the gene structure around *ftsY* in the *B. subtilis* ISR39 chromosome. Inserted genes were localized by DNA–DNA hybridization and by examining PCR products. Integrating the *E. coli* plasmid, pMT3FtsY, into the *ftsY* locus results in a truncated *ftsY* gene (*ftsY'*) under the control of authentic PA and PK and an intact copy of *ftsY* under the control of the *spac-1* promoter (*Pspac-1*). Ω , *p*-independent transcriptional terminator. (b) Northern hybridization of *ftsY* mRNA in *B. subtilis* ISR39. Total RNA was extracted from ISR39 cells cultured in Schaeffer medium in the presence of 0.1 mM IPTG. Conditions were as described in the legend to Fig. 1. The size of the predicted *ftsY* mRNA is indicated on the left. (c) Immunoblots of FtsY expression in ISR39. *B. subtilis* ISR39 cells were cultured in Schaeffer medium and harvested at t_{-2} (lane 1), t_0 (lane 2), t_2 (lane 3), t_4 (lane 4), t_6 (lane 5), t_8 (lane 6) and t_{10} (lane 7). The arrow indicates the position of FtsY.

during exponential growth and sporulation by Northern hybridization, to define the size of the RNA product and determine when it appeared in *B. subtilis* ISR39 in the presence of 0.1 mM IPTG. We detected a 1.0 kb band at t_{-2} and t_0 (Fig. 6b, lanes 1 and 2). This product is the expected size resulting from transcription from the *spac-1* promoter. However, the *ftsY* transcript was not detected at t_4 – t_8 (Fig. 6b, lanes 4–6). We monitored the level of FtsY protein by immunoblotting during sporulation under the same conditions (Fig. 6c, upper panel). In ISR39 cells, FtsY protein was present at t_{-2} – t_2 (Fig. 6c, lanes 1–3) and the level of FtsY decreased at t_4 – t_6 (Fig. 6c, lanes 4 and 5). This timing is similar to that seen in the parent strain 168 (Fig. 1c). However, at t_8 , FtsY was barely detectable in ISR39. These results indicated that in the presence of IPTG, ISR39 cells do not express the *ftsY* gene which is under control of the σ^K promoter (Fig. 6c, lane 7).

A recent review (Driks, 1999) demonstrated four steps in spore-coat assembly that proceed in a defined temporal order and these are mainly regulated by the successive appearance of the regulatory proteins σ^E , spoIIID, σ^K

and GerE. Spore-coat polypeptides are synthesized only in the mother-cell compartment, starting after 3–4 h of sporulation (t_3 – t_4) and are individually deposited on the surface of the prespore. The finding that transcription of *ftsY* depends on both σ^K and GerE suggested that FtsY protein is required for inner and outer coat layer assembly that includes post-assembly modification of the coat protein. We examined the ultrastructure of the *ftsY* mutant spores by electron microscopy. In wild-type 168 spores, the coat appeared to consist of a thick, dense outer multilayer and a lamella inner coat (Fig. 7a and c). Compared with spores produced by the parental strain, 80% of 150 FtsY-depleted mutant spores had a thin and somewhat disorganized outer coat structure (Fig. 7b and d). These morphological changes have also been found in *cotXYZ* triple mutant and *cotM* mutant spores (Henriques *et al.*, 1997; Zhang *et al.*, 1993). At t_{48} , the *ftsY* mutant spores assumed the same form as they did at t_{24} (data not shown). This is not due to delayed sporulation in the *ftsY* mutant cells. Considerably less material appeared to be assembled in the surface layers of the outer coat of ISR39 spores. The appearance of this lamella-type structure of lower electron density was very

Fig. 7. Electron microscopy of 168 and ISR39 spores. Electron micrographs show sections of 168 (a and c) and ISR39 (the *ftsY* conditional null mutant, b and d). ISR39 spores have an anomalous morphological arrangement. Densely stained outer coat (oc) is incomplete in ISR39. In contrast, the inner coat (ic) of wild-type and mutant spores are similarly constructed. Cells cultured in sporulation medium were harvested at t_{24} . Bars, 200 nm.

Fig. 8. Subcellular localization of FtsY in vegetative cells and spores. Vegetative cells of wild-type at t_2 (a), sporulating cells of wild-type at t_{18} (b and c) and the *ftsY* conditional null mutant at t_{18} (d) were thin-sectioned and incubated with rabbit anti-FtsY antibody followed by a gold-conjugated secondary antibody, as described in Methods. Dark specks on electron micrographs are gold particles. Bars, 200 nm. Arrowheads indicate FtsY on the coat.

similar to that typical of the inner coat layers. In addition, we analysed the effect of *ftsY* upon sporulation by measuring the sporulation frequency of ISR39 cells that were cultured in the presence of 0.1 mM IPTG and harvested at t_{24} . Samples were plated following with or

without heating at 80 °C for 15 min. The sporulation frequency of the mutant appeared to be almost identical to that of the wild-type (data not shown).

Immunocytochemical localization of FtsY

To analyse the subcellular localization of FtsY proteins in vegetative cells and spores, *B. subtilis* 168 and ISR39 cells at the vegetative stage and at t_{18} were thin-sectioned. FtsY proteins were then observed by immunoelectron microscopy using rabbit anti-FtsY antiserum and goat antibodies conjugated to gold particles. Gold granules were found in both the cytoplasm and cytoplasmic membranes of vegetative wild-type cells (Fig. 8a). Gold granules were not found in vegetative cells of ISR39 in the absence of IPTG (data not shown). We counted gold granules in the cytoplasm and membrane of 25 cells. FtsY was localized in the cytoplasm and membrane at an approximate ratio of 2:3. This result was similar to that found by cell fractionation (data not shown). In *E. coli*, FtsY, a homologue of the α subunit of the mammalian SRP receptor, is functional at both the cytoplasm and membrane at an approximate ratio of 1:1 (Luirink *et al.*, 1994). FtsY in spores was predominantly located on the inner and outer coats (Fig. 8b and c) where they would have been brought to the forespores from mother cells. In ISR39 spores cultured in the presence of 0.1 mM IPTG, gold granules were not localized on the coat regions (Fig. 8d). In contrast, gold granules located in the core region would be expressed before the polar septum is formed and would have remained in the core region during sporulation.

These results suggest that FtsY participates in spore-coat assembly or that FtsY function is needed for the assembly of other proteins. Further study is necessary to determine the physiological roles of FtsY in spore-coat-protein assembly.

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