SECTION II

Expression of the *ftsY* Gene is Controlled by Different Promoters in Vegetative and Sporulating Cells of *B. subtilis*, and functional analysis of FtsY protein.

SUMMARY

B. subtilis FtsY is essential for protein secretion and vegetative cell growth. The *ftsY* gene is expressed during both the logarithmic phase and sporulation. In vegetative cells, *ftsY* is transcribed along with two upstream genes, *rncS* and *smc*, that are under the control of the major transcription factor σ^A. During sporulation, *ftsY* is solely expressed from a σ^K and GerE controlled promoter that is located immediately upstream of *ftsY* inside the *smc* gene during sporulation. To examine the role of FtsY during sporulation, I established the *B. subtilis* ISR39 strain, another *ftsY* conditional mutant in which *ftsY* expression can be shut off during spore formation but not during the vegetative state. Spores of the ISR39 strain have the same resistance to heat and chroloform as the wild type, while the resistance to lysozyme was obviously reduced. Electron microscopy showed that the outer coat of ISR39 spores was not completely assembled. The coat protein profile of the *ftsY* mutant spores was different from that of wild type spores. The amounts of CotA, and CotE were reduced in spore coat proteins of FtsY mutant spores and the molecular mass of CotB was reduced. In addition, I showed that CotA, CotB, and CotE existed normally at T_8 of sporulation in *ftsY* mutant cells. In addition, immunoelectron microscopy localized FtsY on the inner and outer coats of wild type spores. These results suggest that FtsY has pivotal roles in assembling coat proteins onto the coat layer during spore morphgenesis.

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 (ER) in mammalian cells. Recent genetic and biochemical evidence indicates that targeting may also be SRP-mediated in bacteria (Lütcke, 1995, Schatz and Dobberstein, 1996, Fekkes and 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 (scRNA) 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). I cloned a gene for a homologue of the α-subunit of the SRP-receptor (SRα) and designated it *srb* (Section I and Oguro *et al.*, 1995). The *srb* gene was renamed *ftsY* in the *B. subtilis* genome project since the amino acid sequence of FtsY has 49.7% identity to that of *E. coli* FtsY (Kunst *et al.*, 1997). During the vegetative stage, *ftsY* is transcribed with the two 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* causes normal cell growth and leads to a substantial loss of β-lactamase translocation (Section I, Oguro *et al.*, 1995, Oguro *et al.*, 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 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 and Stragier, 1992; Stragier and Losick, 1996). During the assembly of the cortex and coat proteins in the forespore, a number of polypeptides and proteins would be synthesized within the mother cell and deposited on the forespore (Stragier and 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 from the σ^A promoter, *ftsY* is expressed solely at T_8 during sporulation, from a promoter that is controlled by σ^K and GerE and located immediately upstream of *ftsY* and inside the *smc* gene. To examine the role of FtsY during sporulation, I established the *B. subtilis* strain ISR39. The expression of *ftsY* in the ISR39 strain can be turned off during sporulation but not in vegetative cells. Electron microscopy showed that the outer coat of the *ftsY* mutant spores is composed of thin layers and immunoelectron microscopy localized FtsY in the coat. Moreover, the amount of several coat proteins, such as CotA and CotE, is reduced and molecular weight of CotB is significantly reduced in the outer coat of the FtsY mutant spores. These results suggest that FtsY is essential for the systematic assembly of spore coat proteins into the outer coat.

MATERIALS AND METHODS

Bacterial strains and media.

The *B. subtilis* and *E. coli* 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 ftsY*::pMT3ftsY) was constructed from *B. subtilis* 168*trpC2* 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 the plasmid origin of replication that functions only in *E. 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 amino acids) was synthesized by the polymerase chain reaction (PCR) using the synthetic oligonucleotides PS-1 (5'- ctatacagccaagcttgaattcgttcagtaacgagg -3', generating a *Hin*dIII restriction site) and PS-2 (5'- cgcattatggggatccgttttcccgacgccgtttac -3', generating a *Bam*HI restriction site) at positions 4915 to 4933 and 5330 to 5349, respectively, in the DNA sequence reported by Oguro *et al.* (1996). The amplified fragment was digested by *Hin*dIII and *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.

The oligonucleotide primers COTBM246 (5'- tggatcctcatggacccgtataaaaa-3') and COTB1139R (5'- cctcgagaatttacgtttccagtgatagtc -3') were used to amplify a 1385-bp segment including the *cotB* gene from the *B. subtilis* 168 chromosome. The PCR product was restricted at

the *Bam*HI and *Xho*I sites introduced by the primers and inserted into *Bam*HI and *Xho*I restricted pTUE1122 (Nakane *et al.* 1995) to create the plasmid pCOTB1. The oligonucleotide primers COTEM56 (5'- tgcactctagacaaatgccca -3') and COTE540R (5'- ttagatctttcaggatctcccactaa -3') were used to amplify a 596-bp segment including the *cotE* gene from the *B. subtilis* 168 chromosome. The PCR product was restricted at the *Xba*I and *Bgl*II sites introduced by the primers and inserted into *Xba*I and *Bgl*II restricted pTUE1122 to create the plasmid pCOTE1. The cloned genes in pTUE1122 encode products with an additional 6 histidine residues at their C-terminals.

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 and Losick (1986). Northern hybridization proceeded according to a modification of the method described by Sambrook et al. (1989). Total RNA (10 μg) was resolved by electrophoresis on 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 plus 0.09 M sodium citrate, 2 × Denhardt's reagent, 0.1 % sodium dodecyl sulfate, 100 μg of salmon sperm DNA ml-1). To isolate DNA probes for *ftsY*, a 1.0 kb DNA region of *ftsY* was amplified by PCR using the synthetic oligonucleotide pairs, PS-3 (5'- aaagaggttaaaagatgagctt -3') and PS-4 (5'- gcctatcaagtaagaagata -3') at positions 4935 to 4956 and 5995 to 5976, respectively, of the DNA sequence reported by SectionI. A 1.1 kb DNA of *cotYZ* was amplified using PC-1 (5'- atgatgtgtacgattgatta -3') and PC-2 (5'- atatatagacgttcacccac -3') at positions 2720 to 2701 and 1571 to 1590 of the sequence described by Zhang *et al.* (1993), and a 0.6 kb DNA of *cotZ* was amplified using PC-1 and PC-3 (5'- aaacacttgtaaagaggaat -3') at position 2151 to 2170 of the latter sequence (Zhang *et al.*, 1993). The PCR template was chromosomal DNA of *B. subtilis* 168*trpC2*. After purification by agarose gel electrophoresis, the amplified DNA fragments were labeled with ^{32}P using a random primer DNA labeling kit (Takara-Shuzo Co. Ltd., Kyoto, Japan)

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 to 4339 of the nucleotide sequence reported by Oguro *et al.* (1996). Total RNAs to be tested (40 μg) and 5 $\times 10^4$ c.p.m of ^{32}P-labeled 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 to 3295 and 4635 to 4615, respectively, in the DNA sequence reported by Section I. The amplified fragment was digested by *Dra*I and *Bam*HI and ligated into M13 digested with *Hinc*II and *Bam*HI.

Spore resistance.

B. subtilis cells were cultured in Schaeffer medium at 37 °C for 24h after the end of exponential growth (T_{24}) and spore resistance was assayed as follows. The cultures were heated at 80 °C for 30 min, digested with lysozyme (final concentration, 0.25 mg/ ml) at 37 °C for 10 min or extracted with 10% (vol/vol) chloroform at room temperature for 10 min as described by Nicholson and Setlow (1990). The processed cultures were then diluted in distilled water, plated on LB agar and incubated over night at 37 °C. Survival was determined by counting colonies.

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. Cells were lysed with lysozyme at a final concentration of 2mg ml-1 of frozen cells suspended in 100 μl of GTE (25mM Tris-HCl, pH 7.5,

50mM glucose, 10 mM EDTA) at room temperature for 5 min, then boiled in 0.4 M Tris-HCl, pH 6.8, 2 % SDS, 0.5 % mercaptoethanol and 10 % glycerol for 5 min, and separated by centrifugation. The supernatants were used as cell lysate preparations.

Electron microscopy.

Wild-type cells (168*trpC2*) and ISR39 harvested at T_{24} in Schaeffer medium 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.

Solublization of proteins from purified mature spores.

B. subtilis Wild-type cells (168*trpC2*) and ISR39 cultures in Schaeffer medium (5ml) were harvested at T_{24} of sporulation and washed with 10 mM sodium phosphate buffer (pH 7.2) containing 0.5 M sodium chloride. The pellets were suspended in 0.1 ml of lysozyme solution (10mM sodium acetate buffer at pH 7.2 with 1% (wt/vol) lysozyme) and incubated for 15 min at 37 °C. After adding 1.0 ml of 10 mM sodium phosphate buffer (pH 7.2) containing 0.5 M sodium chloride, the suspensions were separated by centrifugation. The pellets were suspended in 100 µl of buffer consisting 2% (wt/vol) sodium dodecyl sulfate (SDS), 5% (vol/vol) 2-mercaptoethanol, 10% (vol/vol) glycerol, 62.5 mM Tris-HCl (pH 6.8), and 0.05% (wt/vol) bromophenol blue and boiled for 5 min. Most of the spore coat proteins, including some core proteins, were solubilized by this procedure.

SDS-PAGE and Immunoblotting.

Protein samples were resolved by SDS-PAGE (10% acrylamide), and electrotransferred to a polyvinyl difluoridine 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 1h at room temperature with an anti-FtsY antiserum (at a dilution of 1: 5,000), CotA (at a dilution of 1: 5,000) (Takamatsu, *et. al.*, 2000), CotB (at a dilution of 1: 5,000), or CotE (at a dilution of 1: 5,000) in phosphate-buffered saline-Tween 20, followed by an incubation with a secondary antibody conjugated to horseradish peroxidase (Amersham Biotech) at a 1: 5,000 dilution for 1h. Immunoblots were washed and visualized using enhanced chemiluminescence reagents, as described by the manufacturer (Amersham Biotech).

Preparation of recombinant CotB and CotE proteins.

E. coli transformants carrying pCOTB1 and pCOTE1 were cultured in 200 ml of LB medium supplemented with 50 μg of ampicillin per ml at 37 ℃ for 3 h. At the logarithmic growth phase, IPTG (final concentration 1 mM) was added to the culture and the cells were incubated for a further 3 h at 37 ℃. The His-tagged recombinant proteins, CotBH6 and CotEH6 were purified by affinity chromatography as described (Takamatsu, *et. al.*, 1999) and further purified by electroelution from an SDS-gel after SDS-PAGE as described (Abe, *et. al.*, 1995).

Preparation of antisera against His tag fusion proteins.

One milliliter of purified His-tagged protein (0.2 mg ml-1) and 16 mg of killed Mycobacterium tuberculosis cells (Difco) were mixed with 2 ml of complete Freund's adjuvant (Difco), then 3 ml of the emulsion was injected into a healthy rabbit. After 2 weeks, a mix of the same amount of protein with 2ml of incomplete Freund's adjuvant (Difco) was injected. Two weeks later, antisera against CotB and CotE were obtained.

N-terminal sequence determination.

N-terminal amino acid sequences were determined as described by Matsudaira (1987). The protein band in the SDS gel electrophoresis were electroblotted on to PVDF membranes, briefly strained with Coomassie brilliant blue and their N-terminal sequences were determined using an automatic amino acid sequencer (Type 492, Applied Biosystems Division, Perkin-

Elmer).

Immunoelectron microscopy.

Wild-type cells at the vegetative stage and during sporulation (T_{t8}) were harvested by centrifugation and suspended in 1.0 ml of phosphate-buffered Karnovsky's fixative (Karnovsky, 1965) at room temperature for 1.5 h. Fixed cells were washed once in 0.5 M NH_4Cl, 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 plased 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

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 (Section I, Oguro *et al.*, 1996). The *ftsY* gene forms an operon with *rncS* and *smc*. I 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*trpC2* (Fig. II-1A). Cultured 168*trpC2* 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. II-1A, lane 1). These results indicate that during a logarithmic phase, the *ftsY* gene is transcribed along 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. II-1A, lane 2) and it was undetectable in cultures 2 to 6 h after the end of the logarithmic phase of growth (T_2 to T_6) (Fig. II-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. II-1A, lane 6). I analyzed 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 *cotY* and *cotZ* are co-transcribed by σ^K-containing RNA polymerase from the PYZ promoter with a smaller *cotY* mRNA resulting from premature termination or RNA processing. I detected two bands (1.4 kb 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. II-1B, lanes 1 to 6). 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*.

I then analyzed the amounts of FtsY in lysates of *B. subtilis* 168*trpC2* by immunoblotting. Bands for FtsY were intense at T_{-2} and T_0. However, the density decreased after T_0 (Fig. II-1C lanes 3 to 5). At T_8, which is the period of *ftsY* expression (Fig. II-1A), the FtsY band was again detected, but at a density that was 2.5-fold higher than that at T_6 (Fig. II-1C lane 6). This result is consistent with the findings of the Northern hybridization (Fig. II-1A). After T_8, the amount of FtsY again decreased and the band was very faint at T_{10} (Fig. II-1C lane 7). On the other hand, at T_2 (Fig. II-1A), the amounts of FtsY protein were substantial, whereas *ftsY* mRNA is virtually absent (Fig. II-1A lane 3 and Fig. II-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. II-1A), I performed primer extension analysis using the synthetic oligonucleotide Pr (see Materials and Methods). The primer extension product is indicated by an arrow and two smaller minor products are noted. These minor products could have resulted from premature termination by the reverse transcriptase. The largest extension product indicated by an arrowhead (Fig. II-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* open reading frame, inside the *smc* gene (Fig. II-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. II-4A) (Roels and Losick, 1995; Zhang *et al.*, 1994; Zheng *et al.*, 1992). I identified putative GerE binding sequences (4110 to 4121 bp and 4160 to 4171 bp)

upstream from the PK promoter (Fig. II-3 and Fig. II-4B). These data suggested that *ftsY* is transcribed from PK promoters during sporulation as shown in the upper part of Fig. II-3.

Regulation of the *ftsY* gene during sporulation.

To examine which σ-factor is responsible for the *ftsY* transcription at T_8, RNAs from the sigma factor-deficient strains, MO1781 (*sigE*-), MO718 (*sigF*-), MO718 (*sigG*-), MO1027 (*sigK*-) and the GerE- deficient strain, 1G 12 (*gerE*-), were extracted at T_0 and T_8, and Northern hybridized using the 1061 bp DNA fragment encoding *ftsY* as the probe (Fig. II-5A). The 5.5 kb band found in *B. subtilis* 168*trpC2* (Fig. II-5A, lane 1), including the two upstream genes, was detected in all total RNA samples isolated at T_0 (Fig. II-5A, lanes 3, 5, 7, 9 and 11). The lower molecular weight bands may be degradation products of the 5.5 kb transcript. In contrast, the 1.7 kb band found in the RNA preparation of wild type cells at T_8 (Fig. II-5A, lane 2), was not detected in preparations of the σ^E, σ^F, σ^G, σ^K or *gerE* mutant cells sampled at T_8 (Fig. II-5A, lanes 4, 6, 8, 10 and 12). Under these conditions, I detected reduced levels of *cotY* and *cotZ* transcripts in RNA preparation derived from *gerE* mutant cells at T_8 (Fig. II-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. I 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.

Effect of depleting FtsY during sporulation on spore resistance.

To analyze the effect of FtsY-depletion upon sporulation, I 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. II-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 σ^K (PK) promoters. Expression of the intact *ftsY* gene in this strain should be regulated only by the IPTG-inducible promoter, *spac-1*, of which the nucleotide sequences of -35 and -10 regions are typical of an 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. I 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. II-6C, lanes 1 and 2). The growth of ISR39 cells was impaired in the absence of IPTG (data not shown), in agreement with published results showing that FtsY is essential for growth (Section I, Oguro *et al.*, 1996). I then investigated the expression of *ftsY* during logarithmic growth and during sporulation by Northern hybridization to define the size of the RNA product and determined when it appeared in *B. subtilis* ISR39 cells in the presence of 0.1 mM IPTG. I detected a 1.0 kb band at T_{-2} and T_0 (Fig. II-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 to T_8 (Fig. II-6B, lane 4 to 6). I monitored the level of FtsY protein by Immunoblotting during sporulation under the same condition (Fig. II-6C upper panel). In ISR39 cells, the FtsY protein was present at T_{-2} to T_2 (Fig. II-6C, lanes 1 to 3) and the level of FtsY decreased at T_4 to T_6 (Fig. II-6C, lanes 4 and 5). This timing is similar to that seen in the parent strain 168*trpC2* (Fig. II-1C). However, at T_8, FtsY was barely detectable in ISR39 cells. These results indicate that in the presence of IPTG, ISR39 cells do not express the *ftsY* gene that is under control of the σ^K promoter (Fig. II-6C, lane 7).

To examine the effect of FtsY depletion during sporulation, I analyzed the sporulation frequency of ISR39 cultured in the presence of 0.1 mM IPTG and harvested at T_{24}. The sporulation frequency of ISR39 cells appeared to be essentially identical to that of wild type

(Table. 1). To analyze the features of ISR39 spores that were formed under the conditions of FtsY depletion, resistance to heat, solvent, and lysozyme was examined as described in Material and Method. ISR39 and wild type spores were equally resistant to heat and solvent, whereas ISR39 resistance to lysozyme was reduced to 1/5 of that of the wild type. The absence of FtsY in the cells at T_8 was ascertained by immunoblotting (Table. 1). These results suggest that the coat of ISR39 spores is modified from the coat of wild type spores by FtsY deletion.

Effects of depletion of FtsY on spore morphology.

I examined the effect of FtsY depletion on the structure of coat layers using electron microscopy. In wild type 168*trpC2* spores, the coat appeared to consist of a thick, dense outer multilayer and a lamella inner coat (Fig. II-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. II-7B and D). At T_{48}, the *ftsY* mutant spores assumed the same form as they did T_{24} (Fig. II-8B and C). 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 similar to that typical of the inner coat layers. These results show that the outer coat from FtsY-depleted mutant spores was incomplete and appeared diffuse.

Analysis of proteins extracted from *ftsY* mutant spores.

I determined whether or not the spore coat profile is modified in ISR39 mutant spores by analyzing the composition of spore coat protein by SDS-PAGE. Proteins were solubilized from mutant and wild type spores that were purified from 5ml of culture medium each at T_{24}. The protein profile of the *ftsY* mutant and wild type spores on SDS-PAGE significantly differed (Fig. II-9). Proteins with molecular masses of 65, 61, 27, and 21 kDa were absent or reduced in *ftsY* mutant spores. In contrast, a 57 kDa protein was absent from wild type spores, but present in the mutant. Analysis of the NH_2-terminal sequence of the wild type 65 kDa protein revealed the

sequence TLEKFVDALPI, which corresponded to the region from Thr-2 to Ile-12 of CotA (Donovan, *et al.* 1987). The NH_2-terminal sequence of 61 kDa protein consisted of SKRRMKYHSNN, which corresponded to the region from Ser-2 to Asn-12 of CotB (Donovan, *et al.* 1987). The NH_2-terminal sequence of 21 kDa protein yielded SEYREIITKAV, which corresponded to the region from Ser-2 to Val-12 of CotE (Zheng, *et al.* 1987). In addition, the NH_2-terminal sequence of 57 kDa protein of the mutant gave SKRRMKYHSNN, which corresponded to the region from Ser-2 to Asn-12 of CotB. I could not determine the N-terminal sequence of the 27 kDa protein (table. 2).

I ascertained which bands differed between the mutant and wild type by immunoblotting (Fig. II-10A lanes 1-2, 5-6, 9-10). Densitometric analysis demonstrated that the amounts of CotA and CotE in the mutant spores were reduced to 40 and 10 % of that of the wild type. Since the wild type 61 kDa spore protein and the mutant 57 kDa spore protein cross-reacted with the anti CotB antiserum and had the same amino acid sequence, CotB protein in the mutant spore was possibly modified in the C-terminal region by an unknown mechanism(s).

To examine whether or not the reduced amount of CotA and CotE in the mutant spores is caused by the limited expression of each gene. I compared the total levels of CotA, CotB and CotE in extracts of sporangia from the wild type and the FtsY mutant that was harvested at the T_8 (Fig. II-10A lanes 3-4, 7-8, 9-10). Densitometric analysis showed that the amounts of these coat proteins were almost identical. Furthermore, the molecular size of CotB in ISR39 was the same as that in wild type cells (lane 4). These data suggest that FtsY is involved in spore coat assembly. Northern blotting confirmed that the amounts and molecular size of the transcripts of *cotA*, *cotB* and *cotE* during sporulation from ISR39 mutants cells were the same as those from the wild type (Fig. II-10B).

Immunocytochemical localization of FtsY.

To analyze the subcellular localization of FtsY proteins in vegetative cells and spores, *B. subtilis* 168*trpC2* 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 both in the cytoplasm and on cytoplasmic membranes of vegetative wild-type cells (Fig. II-11A). Gold-granules were not found in vegetative cells of ISR39 in the absence of IPTG (data not shown). I counted gold granules in the cytoplasm and on the membrane of 25 cells. FtsY was localized in the cytoplasm and on the 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 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. II-11B and C) where they would have been brought to the forespores from mother cells. In ISR39 spores cultured in the presence of 0.1mM IPTG, gold granules were not localized on the coat regions (Fig. II-11D). In contrast, gold granules located in the core region would be expressed before polar septum is formed and would have remained in the core region during sporulation.

DISCUSSION

FtsY is one component of the protein secretion machinery of *B. subtilis*. Depletion of FtsY leads to defective cell growth and the accumulate of precursors of secretory proteins (as described in Section I). The present study found that the expression of *ftsY* is differently regulated in vegetative and sporulating cells. In vegetative cells, *ftsY* is transcribed with the two upstream genes, *mcsS* and *smc*, under the control of the major transcription factor σ^A : an mRNA of approximately 5.5 kb was detected (Fig. II-1A lane 1) as reported by Oguro *et al.* (Oguro *et al.*, 1996). In contrast, a 1.7 kb transcript for *ftsY* was detected at the T_8 stage during sporulation, but the 5.5 kb band was not. This stage coincides with the maximum expression of *cotYZ* and *cotZ*. The 1.7kb band was not detected in the total RNA preparation obtained from the σ^F, σ^G, σ^E, σ^K and GerE mutants by Northern hybridization (Fig. II-5A), suggesting that *ftsY* expression depends upon both σ^K and GerE. I determined the transcription start site of *ftsY* during sporulation by primer extension (Fig. II-2 and II-3) using total RNA preparations from sporulating cells at T_8 and T_9. The sequence upstream of the start site was similar to the consensus sequences of promoters (-35 and -10 regions) that are recognized by σ^K. Furthermore two putative GerE binding regions were found in the upstream region of the PK promoter (Fig. II-3). Figure II-4 (A) shows that the nucleotide sequences of GerE-independent promoters closely match the consensus sequence whereas the sequences of GerE-dependent promoters generally have little resemblance (Roels and 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.

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 to 4 h of sporulation (T_3 to T_4) and are individually deposited on the surface of the prespore. The finding that the 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. Because of the physiological function of FtsY in sporulation, I constructed a conditional mutant in which transcription from the RNA polymerase containing σ^A is blocked by three tandem terminators but that directed by σ^K can proceed (Fig. II-7). *ftsY* mutant spore have the same resistance to heat and chroloform as the wild type, while its resistance to lysozyme was obviously. The assembly of spore coat proteins was aberrant in mutant spores, resulting in a thin outer coat layer (Fig. II-8B and D), such as the *cotXYZ* triple mutant and *cotM* mutant spores (Henriques *et al.*, 1997; Zhang *et al.*, 1993). Subsequently, at T_{48}, the mutant spores assume the same form as they do at T_{24} (Fig. II-11). This is not due to delayed sporulation in mutant cells. The coat protein profile of the *ftsY* mutant spores was different from that of wild type spores. The amounts of CotA, and CotE were reduced in spore coat proteins of FtsY mutant spores and the molecular mass of CotB was reduced. In addition, I showed that CotA, CotB, and CotE existed normally at T8 of sporulation in ftsY mutant cells. These results suggested that FtsY has pivotal roles in assembling coat proteins onto the coat layer during spore morphgenesis. CotA and CotB are outer coat proteins (Dricks, 1999; Zheng, *et al.*, 1988; Donovan *et al.*, 1987). CotE is a prominent structural component of the coat (Dricks, 1999; Stevens *et al.*, 1992, Zheng, *et al.*, 1988; Bauer, *et al.*, 1999). A CotE band was absent from soluble coat fraction of FtsY mutant spores. This result is consistent with the finding of lysozyme resistance (Zheng, *et al.*, 1988). Moreover, immunoelectron microscopy localized FtsY in the inner and outer spore coat layers as well as in the cytoplasm of mother cells. These results suggest that FtsY participates in spore coat assembly or, that FtsY function is needed for the assembly of other protein.

Table 1

Resistance of the *ftsY* conditional null mutant spores

Strain	Addition of IPTG	FtsY[a)]		Viability (CFU/ml) after the following treatment			
		Veg	Spo	None	Heat	Lysozyme	Chloroform
168	0	+	+	$1.3\,(\pm0.2)\times10^{8}$	$1.0\,(\pm0.2)\times10^{8}$	$9.7\,(\pm1.9)\times10^{7}$	$8.5\,(\pm1.6)\times10^{7}$
168	0.1 mM	+	+	$1.3\,(\pm0.2)\times10^{8}$	$1.1\,(\pm0.2)\times10^{8}$	$9.7\,(\pm2.0)\times10^{7}$	$8.6\,(\pm1.5)\times10^{7}$
ISR39	0.1 mM	+	-	$1.1\,(\pm0.2)\times10^{8}$	$7.4\,(\pm2.1)\times10^{7}$	$1.8\,(\pm1.2)\times10^{7}$	$8.8\,(\pm1.2)\times10^{7}$

[a)]The presence (+) or absence (-) of FtsY in the cells was determined by the Western blotting method after each cell-lysate was electrophoresed in 0.1 % SDS. Veg, cells in vegetative stage at T_{-2}; Spo, cells in sporulation stage at T_8. Standard deviation is calculated from ten independent experiments.

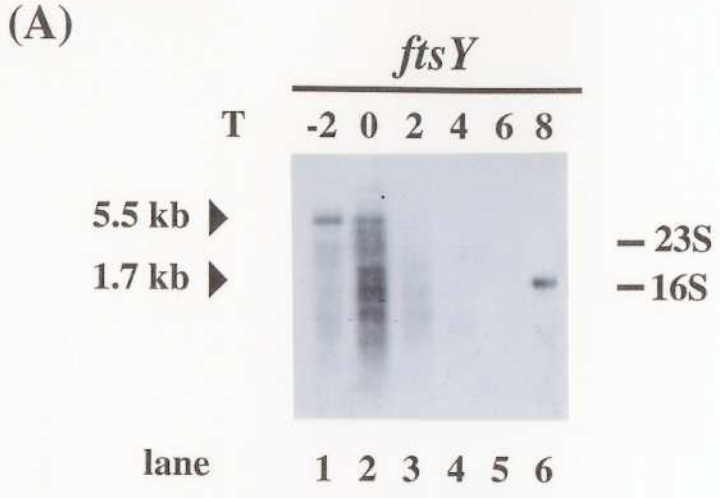
Table 2

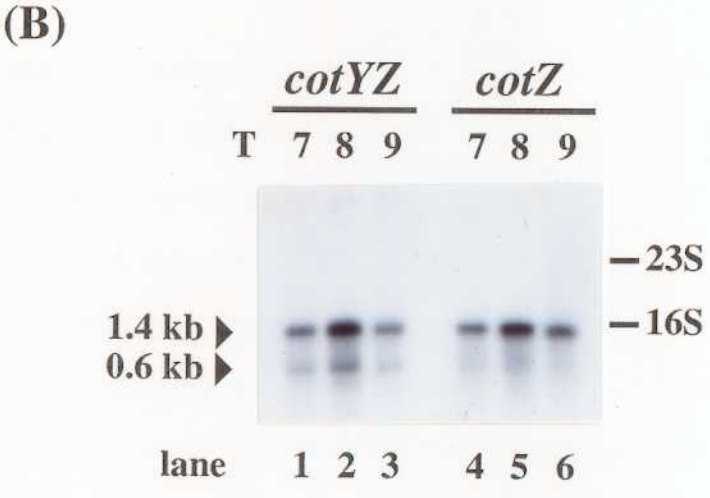
Amino acid Sequences of spore proteins

Molecular mass (kDa)	N-terminal sequence	Candidate (predicted sequence)
65	TLEKFVDALPI	CotA (^{2}TLEKFVDALPI^{12})
61	SKRRMKYHSNN	CotB (^{2}SKRRMKYHSNN^{12})
57	SKRRMKYHSNN	CotB (^{2}SKRRMKYHSNN^{12})
22	SEYREIITKAV	CotE (^{2}SEYREIITKAV^{12})

N-terminal amino acid sequences of the spore proteins blotted onto a PVDF membrane were determined as described in Material methods. Proteins including the corresponding sequence are shown. Numbers in the sequence indicate aminoacid proteins. Underlining indicates an incompatible residue.

Fig. II-1. Expression of the *ftsY* gene. (A) Northern hybridization of ftsY mRNA. Total RNA was extracted from wild type (*B. subtilis* 168*trpC2*) 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 μg) was analyzed using a radiolabeled, 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, Northern hybridized 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*trpC2*. *B. subtilis* 168*trpC2* (lanes 1 to 7) 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 μg) from each preparation were resolved by SDS-PAGE and immunoblotted against anti FtsY antiserum. Arrow indicates the position of FtsY. T, culture periods. Lower part of (C) indicates relative amount of each FtsY band when T_0 band density corresponds to 100.


(A)
ftsY
T -2 0 2 4 6 8
5.5 kb
1.7 kb
23S
16S
lane 1 2 3 4 5 6


(B)
cotYZ
cotZ
T 7 8 9 7 8 9
23S
16S
1.4 kb
0.6 kb
lane 1 2 3 4 5 6

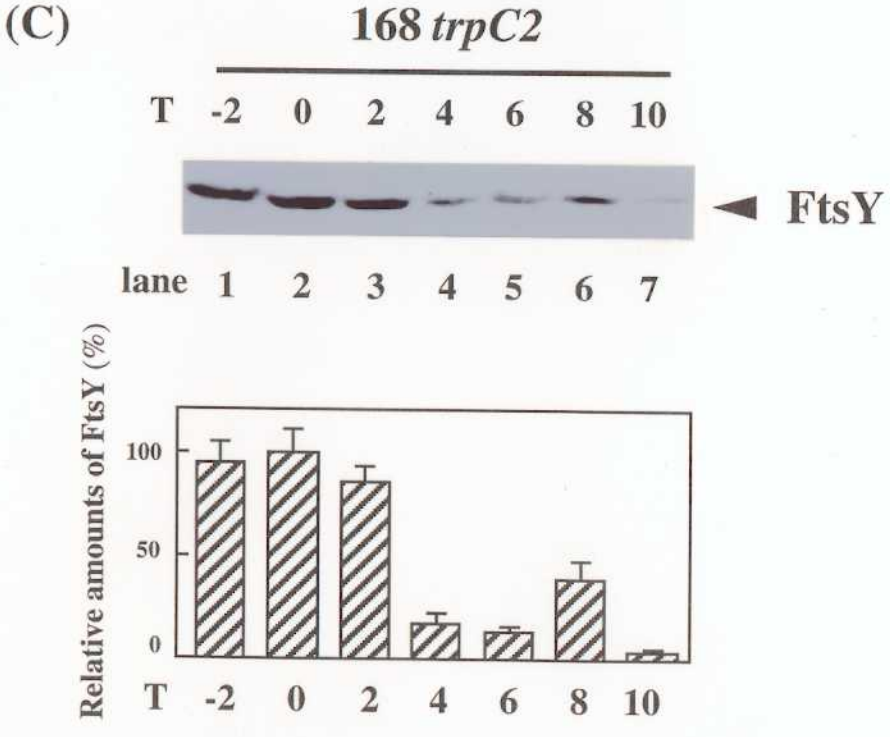

(C)
168 trpC2
T -2 0 2 4 6 8 10
FtsY
lane 1 2 3 4 5 6 7
Relative amounts of FtsY (%)
100
50
0
T -2 0 2 4 6 8 10

Fig. II-2. Determination of the transcriptional initiation site by primer extension analysis. Total RNAs (40 μg) from *B. subtilis* 168*trpC2* cultured in Schaeffer medium at T_8 (lane 1) and T_9 (lane 2) were hybridized with a labeled Pr primer, that was complementary to nucleotides (4339 to 4358) in the sequence shown in Fig. II-3. Primer-extended products obtained with reverse transcriptase were resolved by electrophoresis in 8 % polyacrylamide sequencing gels, then visualized by autoradiography. Dideoxy 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 products are indicated by arrowhead. Asterisk in the sequence shows estimated position of the transcriptional initiation site.

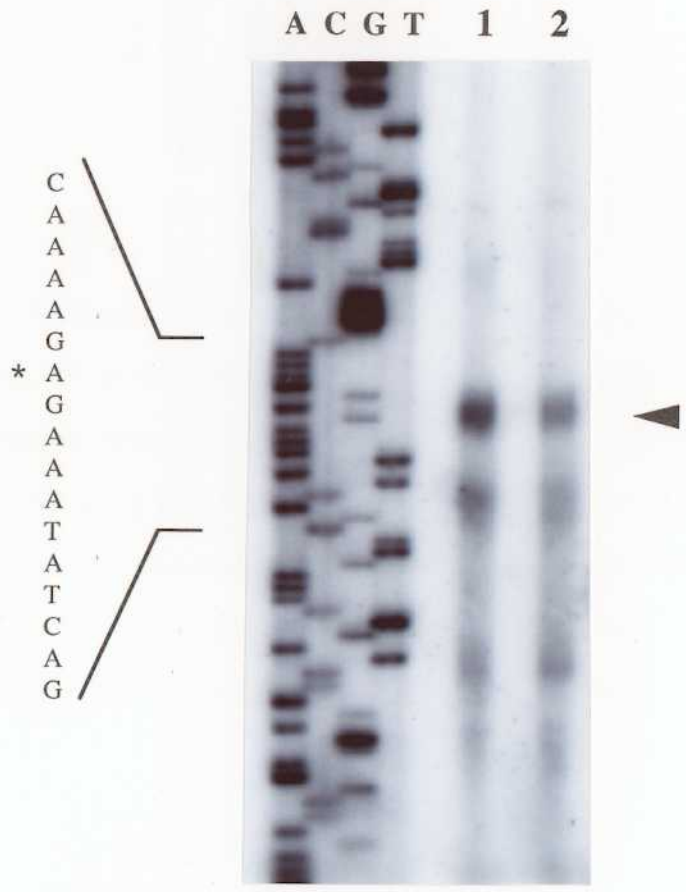
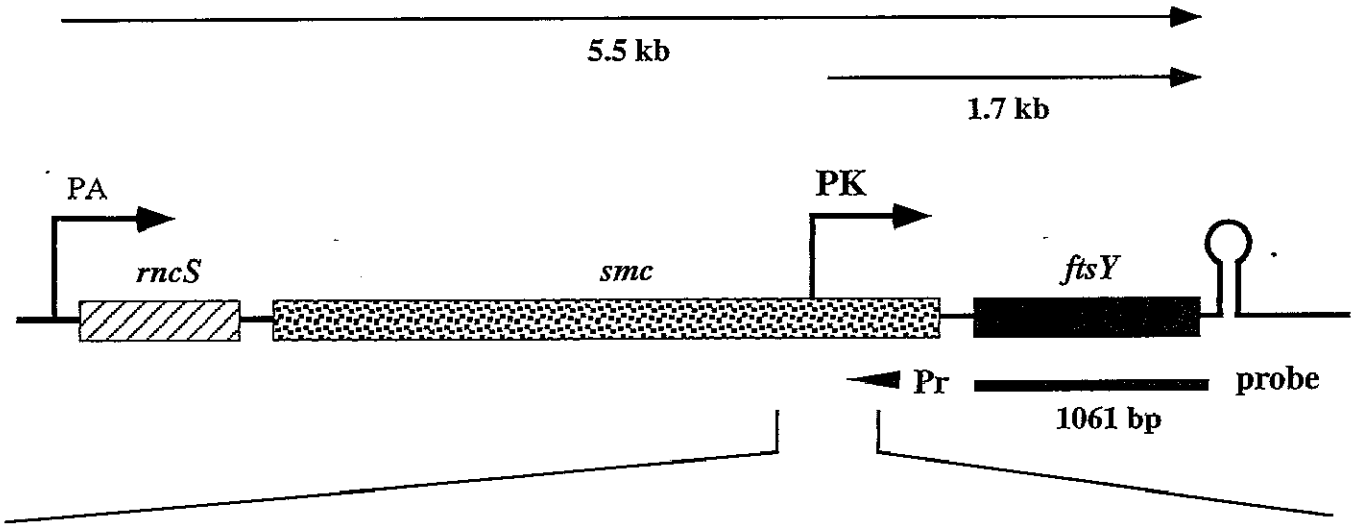

A C G T 1 2
C
A
A
A
G
A
G
A
A
A
T
A
T
C
A
G
*

Fig. II-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. II-1A) and by the primer extension analysis of the 1.7 kb *ftsY* mRNA (Fig. II-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 Section I. Pr is a synthetic oligonucleotide used as the primer to map the 5' terminus of *ftsY* mRNA. Putative GerE binding regions are double underlined.



AGAAATGAAACGCCTGTATAAACAAAAAACAACGCTCTTAAAAGACGAAGAAGTCAAACT 4160

- 35

TGGACGAATGGAAGTCGAGCTTGATAATTTACTCCAATACTTACGGGAGGAATACAGCTT 4220

- 10

GTCCTTTGAGGGGGCAAAAGAGAAATATCAGCTTGAAACTGATCCAGAGGAAGCCAGAAA 4280

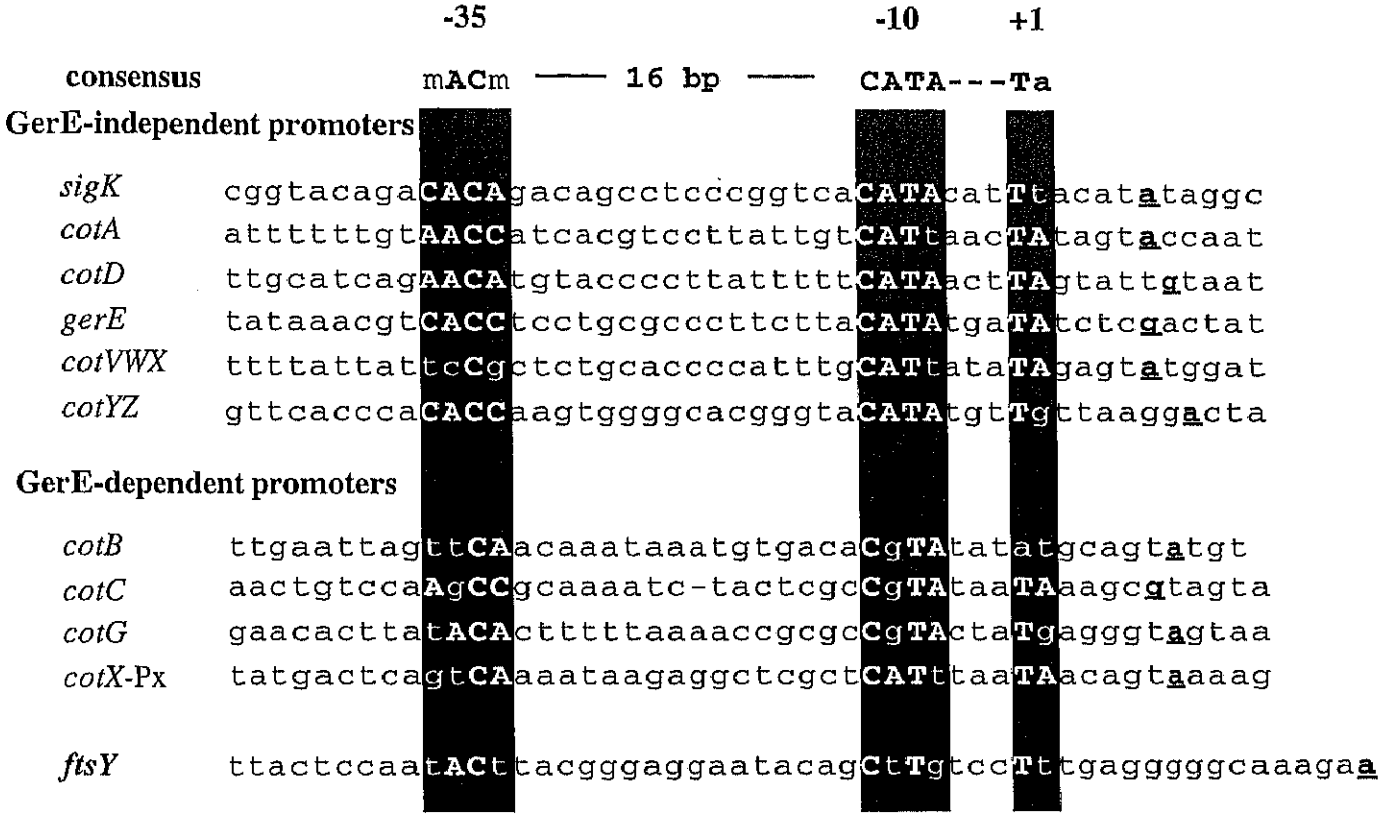
GCGCGTGAAGCTGATTAAACTCGCAATTGAAGAGCTGGGTACCGTGAACCTCGGAAGCAT 4340

Pr

AGATGAGTTTGAGAGGGTCAACGAACGGTACAAGTTTCTGTCGGAACAAAAAGAAGATTT 4400

Fig. II-4. Sequence comparison of 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 transcription start point. Sequences for PK putative promoter region for *ftsY* (Fig. II-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 PK promoter region of *ftsY* are shown (4108 to 4119 and 4158 to 4169) (below). R, A or G; W, A or T; Y, T or C.

(A)

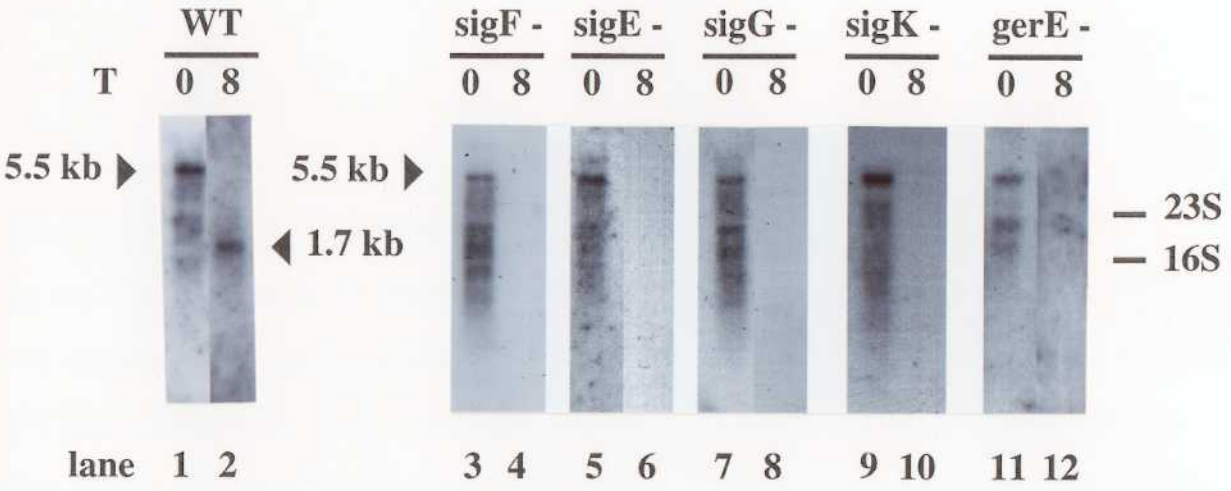


(B)

Consensus			RWWTRGGY--YY (AAA A C CC /// T/GG/ -- // GTT G T TT)	
cotB		-79	**AAATGGGTatTC**	-68
cotB		-53	**AATTAGGCtaTT**	-42
cotC	site 1	-140	**GTTTGGGCcgaT**	-129
cotC	site 2	-74	**ATTTGGaCagCC**	-63
cotVWX		-53	**AAATtGGTtaTT**	-42
cotX		-66	**AAATAGGgttCT**	-55
cotX		-32	**GAcTGaGTcaTa**	-43
cotYZ		-52	**ATATAGaCgtTC**	-41
cotYZ		-33	**GTgTGGGTgaaC**	-44
ftsY	(4158)	-83	**AcTTGGaCgaaT**	-72 (4169)
ftsY	(4119)	-121	**ATAcAGGCgtTT**	-133 (4108)

Fig. II-5. Transcription of *ftsY* in *B. subtilis* wild-type strain 168*trpC2* 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*trpC2*) (lanes 1 and 2), MO719 (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). T, culture periods. (B) Northern hybridization of *cotYZ* and *cotZ* mRNA in *gerE* mutants. Total RNA extracted from wild type (*B. subtilis* 168*trpC2*) (lanes 1 and 2) and 1G12 (GerE-) (lanes 3 and 4) cells was Northern hybridized and probed with a 1.1 kb DNA fragment of *cotYZ*.

(A)


WT
sigF -
sigE -
sigG -
sigK -
gerE -
T
0 8
0 8
0 8
0 8
0 8
0 8
5.5 kb
5.5 kb
1.7 kb
23S
16S
lane 1 2 3 4 5 6 7 8 9 10 11 12

(B)

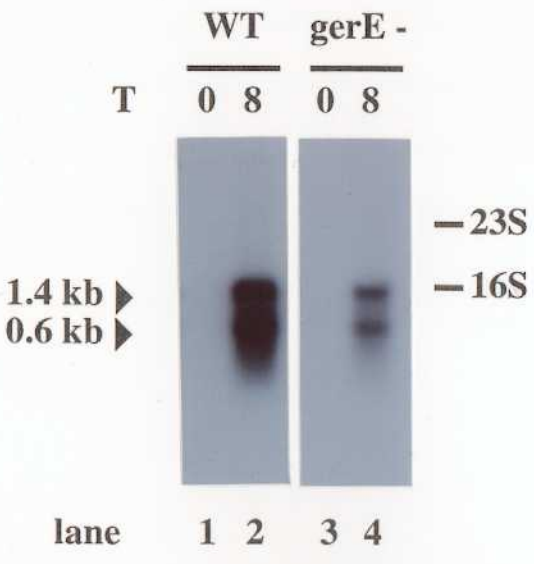
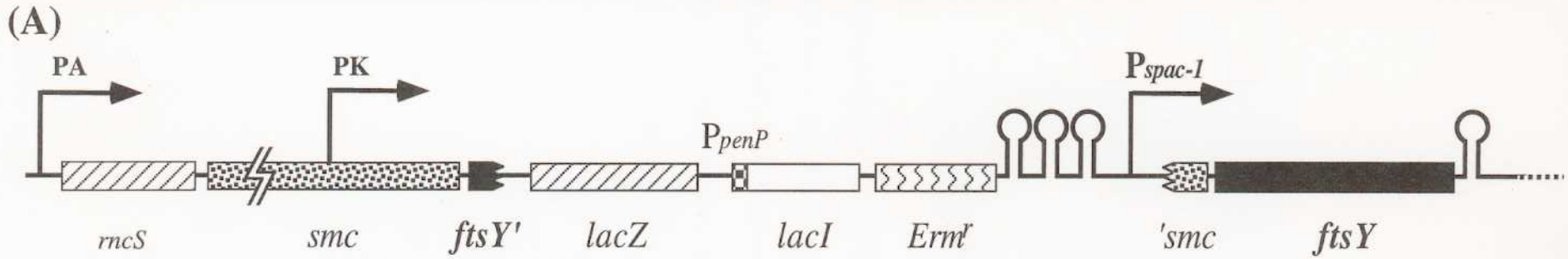
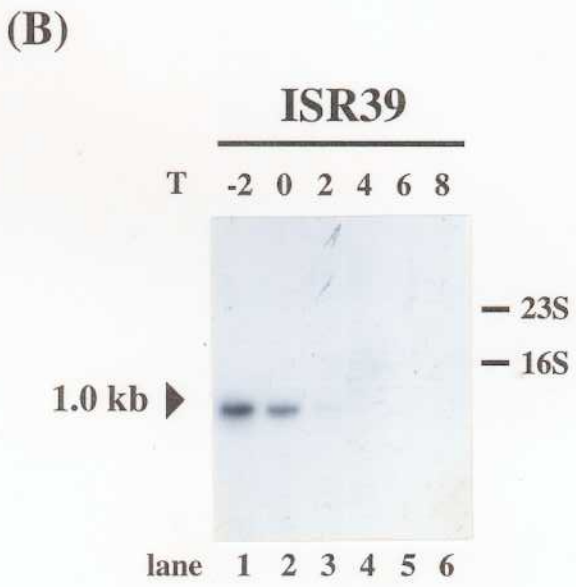

WT
gerE -
T
0 8
0 8
23S
16S
1.4 kb
0.6 kb
lane 1 2 3 4

Fig. II-6. Construction 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 located by DNA-DNA hybridization and by examining PCR products. Integrating the *E. coli* plasmid, pMT3FtsY, into the *ftsY* locus results in a truncated *ftsY* (*ftsY'*) under control of authentic PA and PK and an intact copy of *ftsY* (*ftsY*) under the control of the *spac-1* promoter (P*spac-1*). ρ-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 (lanes 1 to 7) 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). Arrow indicates the position of FtsY. T, culture periods.



***B. subtilis* ISR39 chromosome**



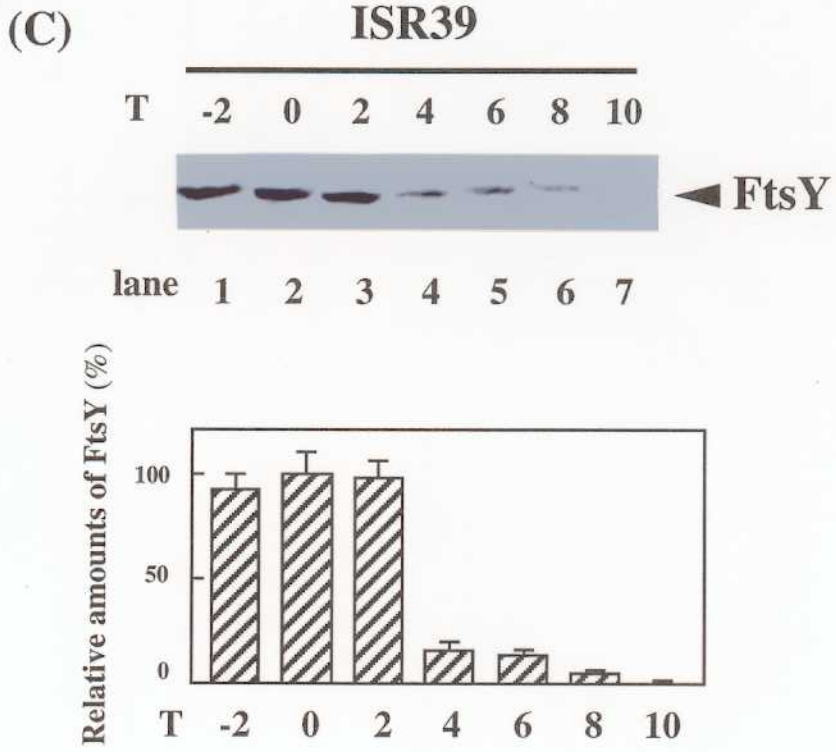
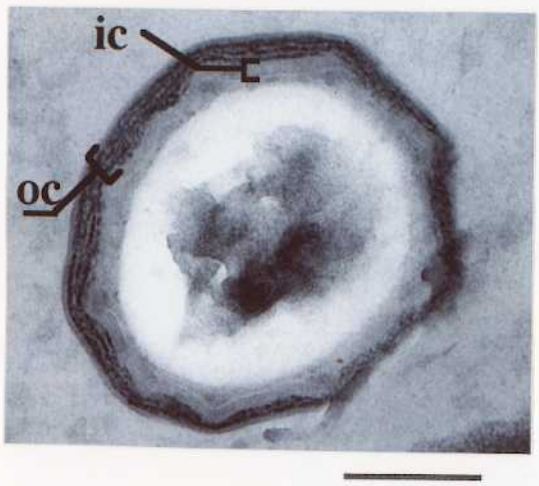
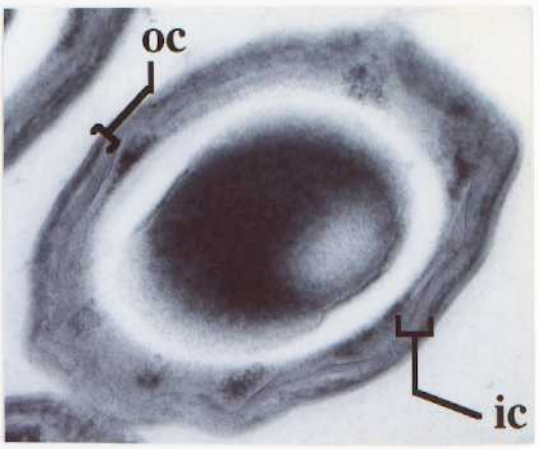


Fig. II-7. Electron microscopy of spores of 168*trpC2* and ISR39. Electron micrographs show sections of 168*trpC2* (A and C) and ISR39 (the *ftsY* conditional null mutant, B and D). ISR39 spores have an anomalous morphological arrangement. Densely stained outer coat (oc) is incomplete in ISR39. In contrast, the inner coat (ic) of wild type and mutant spores are similarly constructed. Cells cultured in sporulation medium were harvested at T_{24}. Bar, 200 nm.

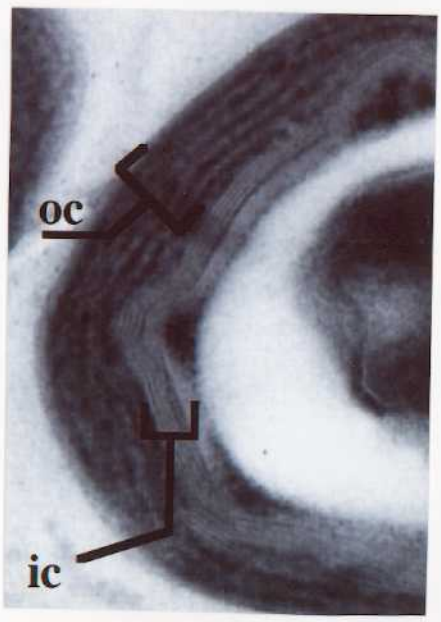
(A)



(B)



(C)



(D)

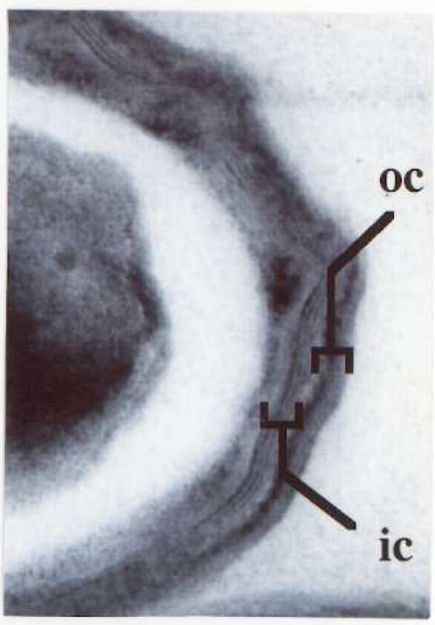
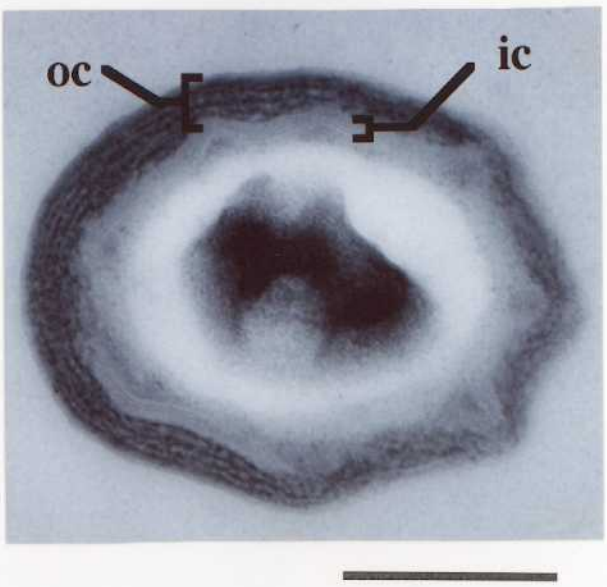
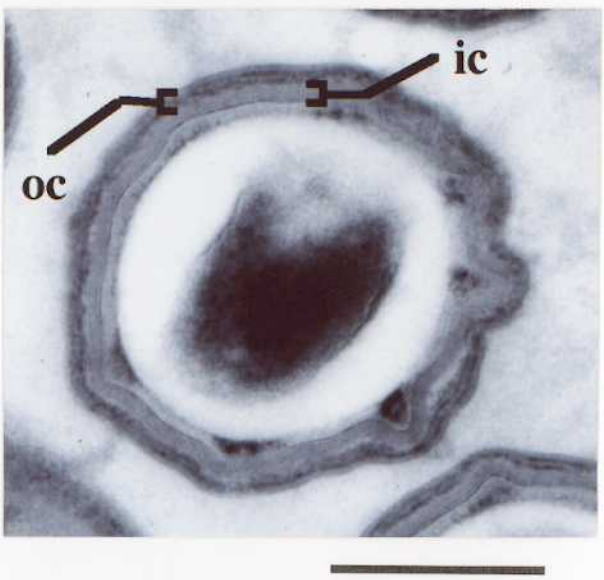


Fig. II-8. Electron microscopy from spores of 168 and ISR39. Electron micrographs show sections of spores of 168 at T24 (A) and ISR39 at T24 (B) and T48 (C). Morphological arrangement ISR39 spores is anomalous. Densely stained outer coat (oc) is incomplete in ISR39. In contrast, the inner coat (ic) of wild type and mutant spores are similarly constructed. Cells cultured in sporulation medium were harvested at T24 or T48. Bar, 200 nm.

(A)



(B)



(C)

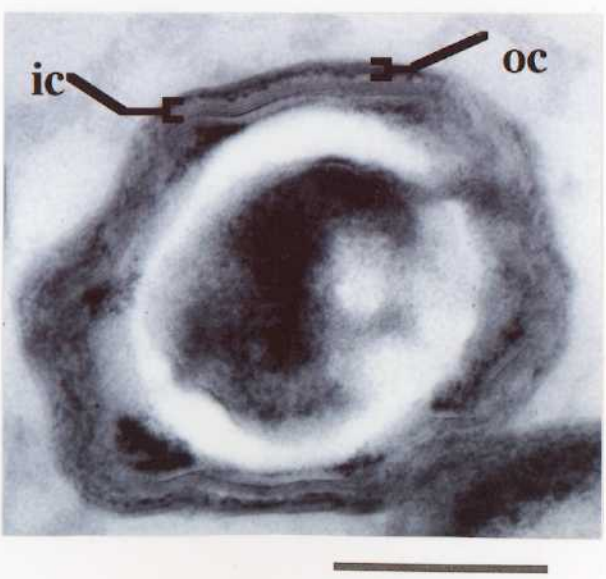


Fig. II-9. SDS-PAGE analysis of proteins solubilized from spores. Spores were purified from T24 sporulating cells. Proteins were solubilized boiling in SDS containing 2-mercaptoethanol and resolved by SDS-PAGE (15% polyacrylamide gel) prepared from Wild-type (lane 1) and *ftsY* mutant spores (lane 2). Molecular weights indicated in the right hand of the figure show that amount of bands significantly increased or decreased in the protein extract of *ftsY* conditional mutant spores.

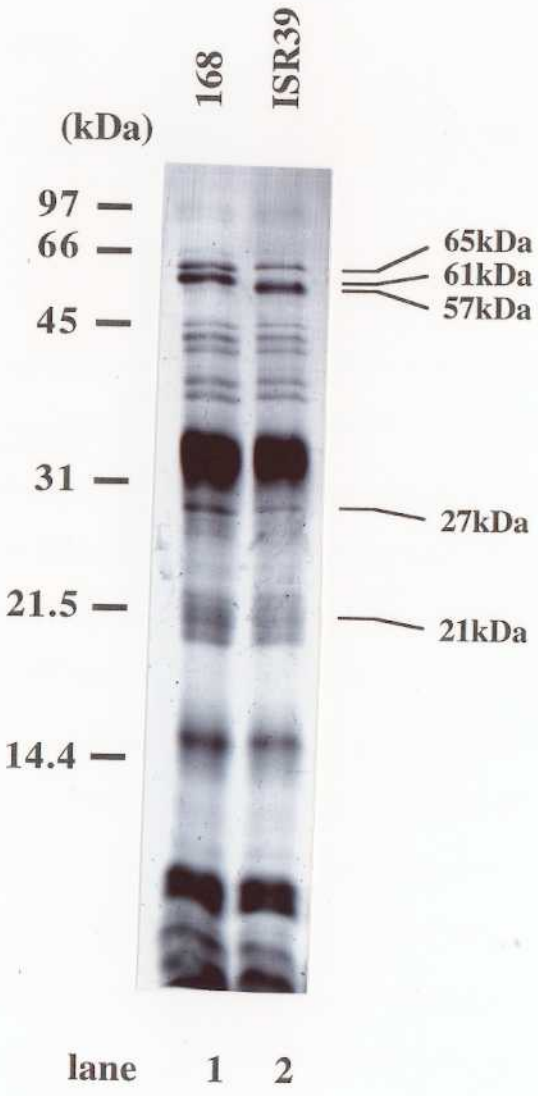

168
ISR39
(kDa)
97
66
45
31
21.5
14.4
65kDa
61kDa
57kDa
27kDa
21kDa
lane 1 2

Fig. II-10. Detection of CotA, CotB, and CotE proteins in spores at T_{24} and sporangia at T_8 by immunoblotting and expression of the *cotA*, *cotB* and *cotE* genes. (A) Immunoblotting of CotA, CotB, and CotE proteins in spore coat proteins (T_{24}) and sporangia (T_8) of 168 and ISR39 cells harvested from Schaeffer medium at T_{24} and T_8. Coat proteins were solubilized from spores (10^7 cells), resolved by SDS-PAGE (15% gel) and imunoblotted using antiserum against CotA (lanes 1-2), CotB (lanes 5-6), or CotE (lanes 9-10). Total proteins (20 μg each) of 168 and ISR39 sporangia at T_8 were immunoblotted using antiserum against CotA (lanes 3-4), CotB (lanes 7-8), or CotE (11-12). *B. subtilis* 168 (lanes 1, 3, 5, 7, 9 and 11) and ISR39 (lanes 2, 4, 6, 8, 10 and 12). (B) Northern hybridization of *cotA*, *cotB* and *cotE* transcripts. Total RNA (10 μg each) extracted from wild type (lane 1, 3, 5) and mutant cells (lane 2, 4, 6) in Schaeffer medium at T_8 was analyzed using radiolabeled DNA fragments of *cotA*, *cotB* or *cotE* as probes. The size of the *cotA*, *cotB* and *cotE* transcripts are indicated at the left of the figure.

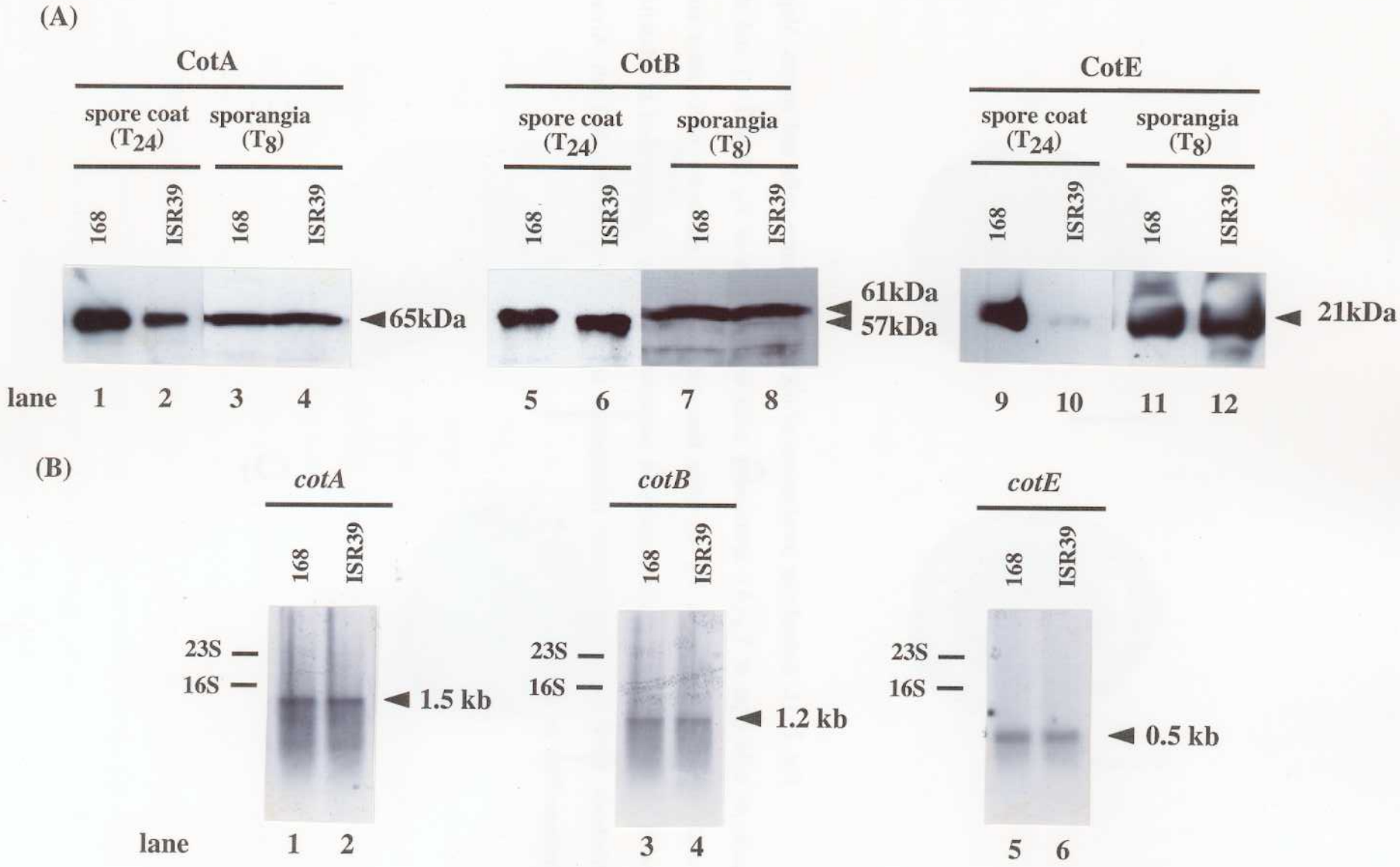
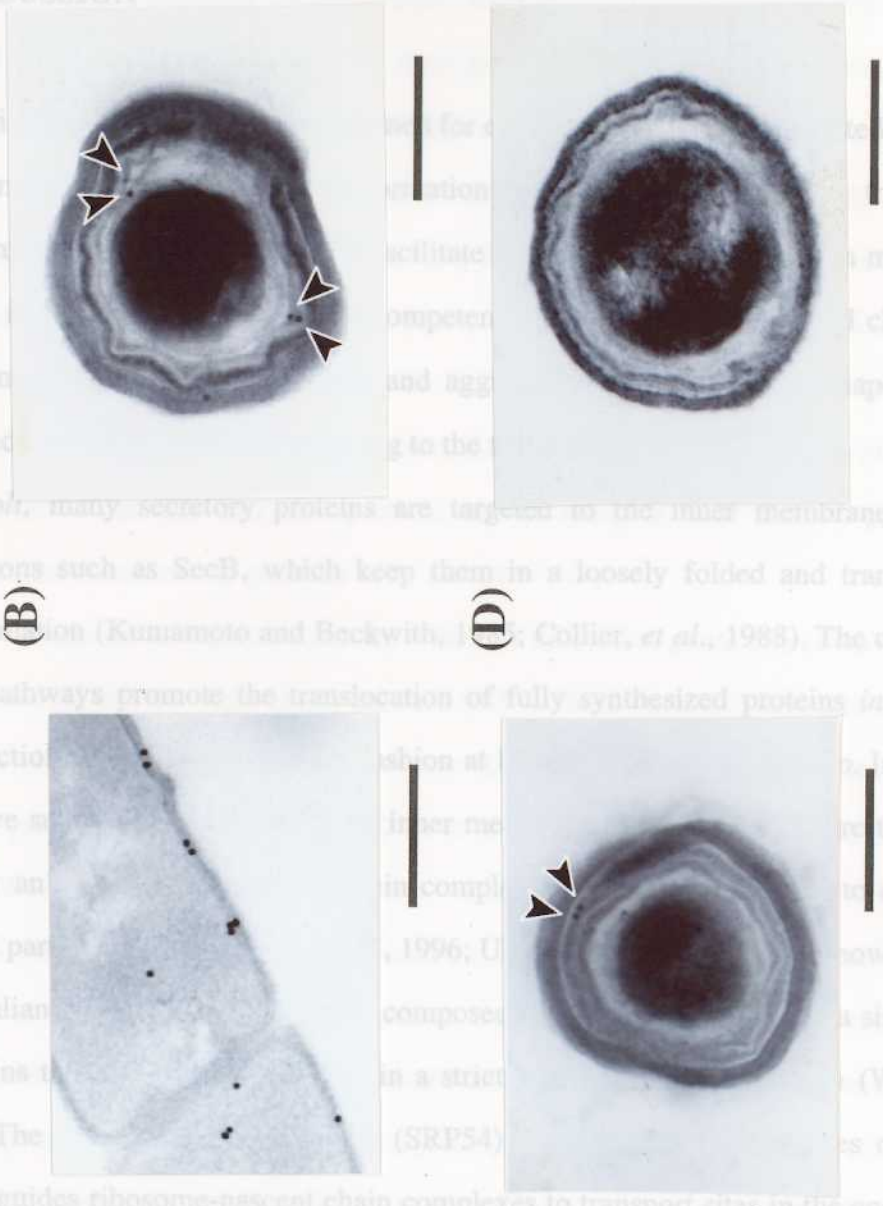

(A)
CotA
CotB
CotE
spore coat (T_{24})
sporangia (T_8)
168
ISR39
65kDa
61kDa
57kDa
21kDa
lane 1 2 3 4 5 6 7 8 9 10 11 12
(B)
cotA
cotB
cotE
23S
16S
1.5 kb
1.2 kb
0.5 kb
lane 1 2 3 4 5 6

Fig. II-11. 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 Materials and Methods. Dark specks on electron micrographs are gold particles. Bar, 200 nm. Arrowheads indicate FtsY on the coat.


(B)
(D)
(A)
(C)