SECTION I

Cloning and Characterization of the *Bacillus subtilis ftsY* (*srb*) Which Product is a Homologue of the α-Subunit of Mammalian Signal Recognition Particle Receptor

SUMMARY

I cloned a *Bacillus subtilis* gene, *ftsY* (*srb*) encoding a homologue of mammalian signal recognition particle (SRP) receptor α-subunit (SRα). The *ftsY* gene is 987 bp in length and encodes a 329-amino acid protein. The deduced amino acid sequence of the protein shared 26.6, 36.2 and 49.7% identity with those of mammalian SRα, archaebacterial DPα and *Escherichia coli* FtsY, respectively. There is an ρ-independent transcriptional terminator downstream from the stop codon of *ftsY*, but no promoter sequence upstream from the gene. To analyze the gene organization in the potential operon, I sequenced a 6098-bp DNA containing *ftsY*. Primer extension analysis and Northern blot analysis revealed that *ftsY* constitutes an operon with two additional upstream ORFs (*orf3* and *orf4*). The product of ORF3 (266 amino acids) shared 36.0% identity with *E. coli* ribonuclease III (RNase III) which is an endonuclease that specifically hydrolyzes double-stranded RNA. The product of ORF4 (1188 amino acids) shared 26.6% identity with yeast Smc1 which involved in chromosome compaction.

To analyze the biological function of FtsY, I established the *Bacillus subtilis* ISR38 strain in which *ftsY* expression was induced by IPTG. The depletion of FtsY caused a defect in the cell growth and the cells became filamentous, broken and twisted shape. I detected the accumulation of *B. subtilis*' β-lactamase derivatives of which the signal peptide region was replaced with that of *B. subtilis* AprE or an alkarophilic *Bacillus sp.* #1011 CGTase in the FtsY depleted cells. Furthermore, a fusion protein which has *B. subtilis* PBP5* signal peptide region and β-lactamase mature region was accumulated in the FtsY depleted cell. PBP5* involved in the synthesis of cortex is translated in mother cell and then transported to forespore. These results suggest that the FtsY protein is involved not only in the secretion of extracellular proteins but also in the localization of spore-forming proteins.

INTRODUCTION

The signal recognition particle (SRP) and its receptor play a central role in the protein translocation in eukaryotic cells. The SRP receptor mediates binding of SRP to the endoplasmic reticulum (ER) in mammalian cells (Gilmore *et al.*, 1982; Meyer *et al.*, 1982; Wiedmann *et al.*, 1987). Mammalian SRP consists of one RNA molecule (7S RNA) and six polypeptides of 9, 14, 19, 54, 68 and 72 kDa. The SRP receptor is a heterodimeric protein comprised of a 69 kDa subunit (SRα) and a 30 kDa subunit (SRβ) which are associated with the ER membrane.

In *Bacillus subtilis*, small cytoplasmic RNA (scRNA) and Ffh, which are homologues of 7S RNA and SRP54 protein of mammalian SRP, respectively, have been identified (Struck *et al.*, 1988; Nakamura *et al.*, 1992; Honda *et al.*, 1993). The scRNA and Ffh form a complex in vivo and the defect in them inhibits secretory protein translocation in *B. subtilis* (Nakamura *et al.*, 1992; Honda *et al.*, 1993). Therefore, it is suggested that the SRP-like particle, of which the structure and function are similar to those of mammalian SRP, is also present and functional in *B. subtilis*. In *Escherichia coli*, 4.5S RNA and Ffh associate to form a stable ribonucleoprotein complex, which interacts with FtsY, a homologue of mammalian SRα. *E. coli* FtsY which is a homologue of mammalian SRα was in part located at the cytoplasmic membrane and that depletion and overexpression of FtsY affects both cell morphology and protein export (Luirink *et al.*, 1994).

SRP54 and SRα contain related GTPase domains which are required for each function (Connolly and Gilmore, 1986, 1989; Bernstein *et al.*, 1989; Römisch *et al.*, 1989). The multiple sequence alignment revealed that the GTPase domain is highly conserved in their homologues (Althoff *et al.*, 1994). The GTPase domain of SRP54, SRα and each homologues are more closely related to each other than to other members of GTPase subfamily (Bourne *et al.*, 1990).

To confirm the presence of an SRP-SRP receptor mediated targeting system for secretory protein in *B. subtilis* in a manner similar to mammalian cells, I cloned the *B. subtilis*

ftsY gene encoding a homologue of mammalian SRα by using synthetic oligonucleotides for the GTP-binding consensus elements. Then I characterized the predicted motifs of its amino acid sequence. Furthermore, to analyze the gene organization of a putative operon and the regulation of transcription, I determined the nucleotide sequence upstream from *ftsY*, then analyzed the promoter and transcription start point by primer extension analysis and Northern blot analysis. I detected that the *ftsY* operon consists of three genes encoding RNase III, SMC protein and FtsY. The deduced amino acid sequence of FtsY shared 26.6, 36.2 and 49.7% identity with those of mammalian SRα, archaebacterial DPα and *E. coli* FtsY, respectively.

To investigate the function of FtsY *in vivo*, I constructed the mutant strain in which the expression of the *ftsY* gene was under the control of the IPTG-inducible promoter. Using this strain, I examined the effects of the depletion of FtsY on cell growth, morphology and translocation of the extracellular proteins.

Under starvation conditions, *B. subtilis* undergoes a differentiation process that culminates with the release of a dormant endspore. In the sporulation process, several proteins such as coat proteins, proteins invloved in the synthesis of cortex and so on are expressed in mother cell and then translocated to the forespore. In this study, I indicated that FtsY was concerning with the localization of spore-forming protein.

MATERIALS AND METHODS

Bacterial strains and plasmids.

E. coli strains used were JM109 [Δ(*lac-proAB*) *thi recA1 gyrA96 hsdR17 supE44 relA1* F' (*traD36 proAB+ lacIq lacZ*Δ*M15*)] for recombinant DNA manipulations and MN514 [*hsdR514*(r_k-m_k-) *argH galE galX strA lycB7* (*Hfl*+)] for the host of l phage. *B. subtilis* strain used was 168 *trpC2* which is the laboratory lebeled wild type strain. *E. coli* strains were cultured at 37°C in L-broth. *B. subtilis* strain was cultured at 37°C in L-broth. The antibiotic concentrations, when used, were 50 mg/ml for ampicillin, 5 mg/ml for chloramphenicol and 10 mg/ml for kanamycin.

Southern hybridization.

DNA digested with restriction enzymes were separated by agarose gel electrophoresis and transferred onto GeneScreen Plus nylon membranes (Dupont / NEN Research Products, Boston, MA). PCR amplified DNA fragment between GTP-binding element I and element II was labeled with [α-^{32}P] dCTP by a random primer DNA labeling kit (Takara Shuzo Co. Ltd., Kyoto, Japan). For hybridization at high stringency, the membranes were prehybridized for 1 h at 65°C in a solution of 1 M NaCl, 10% dextran sulfate, 0.5% sodium dodecyl sulfate (SDS) and denatured herring sperm DNA (0.5 mg/ml). Labeled probe was then added to the mixture and hybridized for 12 to 18 h at 65°C. The membranes were washed for 1 h at 65°C with 2 × SSC (1× SSC is 0.15 M NaCl plus 0.015 M sodium citrate) containing 1% SDS. Plaque hybridization was performed with the same method under high stringency after plaques were fixed to nylon membrane.

DNA sequencing.

DNA sequencing was performed by the dideoxy chain-termination method of Sanger *et*

al. (1977) with the Taq Dye primer Cycle Sequencing Kit and a model 373A DNA sequencer (Applied Biosystems Inc.).

General DNA manipulations.

DNA manipulation and *E. coli* transformation were accomplished by the methods outlined by Sambrook *et al.* (1989).

Construction of plasmid pTUE960 for the preparation of *B. subtilis* ISR38 strain.

To insert the *ftsY* gene into the chromosome under the control of the *spac-1* promoter, pTUE960 was constructed from pDH88 (Henner, 1990), which contains the pBR322 replication origin, the *spac-1* promoter, the *lacI* gene expressed by the *penP* promoter and the pC194 cat gene (Fig. 1). A 448-bp *Eco*RI-*Hin*dIII fragment carrying 11 bp of the 3' portion of *orf4* and 415 bp of the 5' portion of f*tsY* was inserted into the *Eco*RI-*Hin*dIII site of pBluescript II SK- to generate pTUE950. A 158-bp *Sma*I-*Sau*3AI fragment of pTUE950 was then inserted into the *Sma*I-*Bgl*II site of pDH88. The constructed plasmid, in which the ribosome-binding sequence and the truncated *ftsY* gene were positioned downstream of the *spac-1* promoter, was designated pTUE960. The plasmid was used in the preparation of the *B. subtilis* ISR38 strain.

Characterization of the growth and morphology of *B. subtilis* ISR38.

B. subtilis ISR38 was cultured overnight at 37°C in L-broth containing chroramphenicol and 3 mM IPTG. Cells were harvested and washed twice with L-broth. They were then inoculated into L-broth supplemented with or without 3 mM IPTG in the presence of chloramphenicol and cultured at 37°C. To analyze the effect of the depletion of FtsY on the morphology of *B. subtilis*, cells were observed by electron microscopy.

Construction of plasmid pTUBE1234, pTUBE1235 and pTUBE1236.

Using pUC18 as the template for two oligonucleotides (5'-tgaagatcactcgagtgcacga-3'

and 5'-ggtctgacagagatctatgcttaat-3'), a DNA fragment (772 bp) encoding the mature region of β-lactamase (amino acid position from 40 to 283) was amplified by PCR, digested by *Xho*I and *Bgl*II and inserted into the *Xho*I-*Bgl*II site of pUC18H6 containing a DNA region for a hexa-histidine tag to construct plasmid pTUE1200 (Nakane *et al.*, 1995). Three DNA fragments encoding the signal peptide regions of *B. subtilis* alkarine protease (AprE), *B. subtilis* penicilin binding protein 5* (PBP5*) and *Bacillus sp.* #1011 cyclodextrin glucanotransferase (CGTase) were prepared using three sets of oligonucleotides (5'-tctagaaaaaggagagggtaaagaatgag-3' and 5'-ctcgaggctgtagaaccagtaggtcc-3', AprE), (5'-tctagaacaaggacgtgagcaaacatg-3' and 5'-ctcgatatgccgtcctttgaagccgt-3', PBP5*) and (5'-aaagcgacggacaggcctg-3' and 5'-gaaccggtcggtgaagatctga-3', CGTase). The three fragments generated (751 bp for AprE, 751 bp for PBP5* and 214 bp for CGTase), were digested by the appropriate restriction enzymes and inserted into pTUE1220 to obtain plasmids pTUE1226, pTUE1227 and pTUE1228 encoding the respective signal peptide region. Plasmid pHY300PLK (Takara shuzo Co., LTD., Kyoto, Japan) was digested by *Afl*II, T_4 DNA polymerase, and *Eco*RI to isolate the DNA fragment containing the origin of pAMα1 and tetracycline resistance gene. Plasmid pUC19 was digested by *Eco*RI and *Dra*I, and the DNA fragment containing *colE1* origin was isolated. These two DNA fragments were joined to construct plasmid pTUBE1200. Each of the *Sac*I-*Bgl*II digests of the pTUE1226, pTUE1227 and pTUE1228 was inserted into the *Sac*I-*Bgl*II site of the pTUBE1200. Plasmids pTUBE1234, pTUBE1235 and pTUBE1236 were generated. The structures of the constructed plasmids were confirmed by nucleotide sequencing.

Immunoblotting of β-lactamase derivatives.

I prepared the culture (O.D.A660 =400) of ISR38 harboring pTUBE1254, pTUBE1255 and pTUBE1256. The culture was precipitated and each pellets were boiled for 5 min in 0.4 M Tris-HCl (pH 6.8) containing 2% SDS, 5% merchaptoethanol and 10% glycerol, and resolved by SDS-PAGE (9% gel). Then the proteins were blotted onto polyvinilidene difluoride (PVDF) membranes (Millipore Corp., Bedford, MA). β-lactamase derivatives were detected by

immunoblotting using the rabbit anti- *E. coli* β-lactamase antiserum, respectively, followed by Enhanced Chemiluminescence (ECL) (Amersham Plc., U.K.).

Electron microscopy.

After centrifugation of cultures in L-broth at 37°C for 7 h, the pellets 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.

RESULTS

Cloning and sequencing of the *ftsY* gene and its upstream region from the *B. subtilis* chromosome.

To verify the existence of a *B. subtilis* gene encoding a homologue of mammalian SRα, Southern hybridization was performed on the *B. subtilis* chromosomal DNA using the *E. coli ftsY* gene as a probe after the DNA was digested with several restriction enzymes and electrophoresed on an agarose gel. No positive band, however, was identified. Therefore, I looked at to the GTP-binding consensus elements that are highly conserved in the SRP receptors (Fig. I-1). The amino acid sequences of element I of mammalian SRα, archaebacterial DPα and *E. coli FtsY* were completely identical, whereas they differed from those of SRP54 and Ffh proteins of *E. coli* and *B. subtilis*. Furthermore, their sequences in element II were also identical. Based on these amino acid sequences of element I and II, oligonucleotides designed in Fig. I-2A were synthesized. The polymerase chain reaction (PCR) using these synthetic DNA as primers was performed and a 262 bp DNA fragment was obtained as the major PCR product (Fig. I-2B). The nucleotide sequence of this fragment was confirmed and the putative amino acid sequence of this fragment had 47.2% identity with the region between element I and element II of *E. coli* FtsY. Therefore, this ^{32}P-labeled fragment was used as a probe to hybridize with the *B. subtilis* chromosomal DNA after digestion by several restriction enzymes. A single band of approximately 1.9 kb in the *Aor*51HI digest and two bands of approximately 0.4 kb and 1.9 kb in the *Pvu*II digest were revealed (Fig. I-3A). Preparative scales of the *Aor*51HI-digested and *Pvu*II-digested chromosomal DNA from *B. subtilis* 168 *trpC2* were resolved by agarose gel electrophoresis. Thereafter, 1.5 to 2 kb fragments of the two preparations were extracted from the gel, ligated with lgt10 arms, packaged *in vitro*, and then were used to infect *E. coli* NM514. Among 6,500 plaques, 64 positive phage clones carrying the 1.9-kb *Aor*51HI fragment and 36 positive phage clones carrying the 1.9-kb *Pvu*II fragment were isolated from 8,000 plaques.

The nucleotide sequences of the entire region of both 1876-bp *Aor*51HI and 1874-bp *Pvu*II fragment were determined. These two fragments overlapped in 779 bp and the total size was found to be 2971 bp (Fig. I-3B). Sequencing revealed the presence of two possible open reading frames (ORFs) in this region though the N-terminal portion of the first ORF was discarded because a suitable initiation site was not observed. In the second ORF, an identical sequence for the probe used for the hybridization was located. This deduced protein appears to have amino acid identity to mammalian SRα. This gene was therefore designated as *srb* (SRP receptor candidate of *B. subtilis*), renamed *ftsY*. Downstream of the stop codon of the *ftsY* gene, an ρ-independent terminator was observed. However, upstream of the initiation codon of the *ftsY* gene, there was a clear Shine-Dalgarno sequence but no promoter region, suggesting that the first ORF and *ftsY* constitute an operon. This suggests that *ftsY* consists of an operon with another upstream *orf*(s). Furthermore, I determined the nucleotide sequence upstream from *ftsY*. To obtain a DNA fragment upstream region of *ftsY*, I used inverse PCR against *B. subtilis* chromosome (Triglia *et al.*, 1988). The nucleotide sequence of the amplified fragment was determined and I got a 6098-bp sequence containing *ftsY*. In the 6098-bp sequence, only one putative σ^A promoter was identified at the nt 291 to 319 (TTGATA-17 bp-GATGCT; underline indicates the consensus) (Fig. I-4). No other σ promoter sequences were found. Two putative transcriptional terminators were located downstream of *orf2* and *ftsY* at nt 380 to 412 and 5939 to 5970, respectively (Fig. I-4 and I-5). These results suggested that *ftsY* constitutes an operon with *orf3* and *orf4*.

The deduced amino acid sequence of ORF3 (266 amino acid residues) shared 36.0, 26.6 and 30.8% identity with *E. coli* RNase III, *Saccharomyces cervisiae* Rnt1 and *Schizosaccharomyces pombe* Pac1, respectively.

The deduced amino acid sequence of ORF4 (1188 aa) were found to contain a structural motif of the SMC family, involved in chromosome compaction (Strunnikov *et al.*, 1993; Hirano *et al.*, 1995). ORF4 shared 26.6% identity with yeast Smc1.

Mapping of the transcription initiation site for the *ftsY* gene and gene organization.

To analyze the transcription initiation site for the *ftsY* operon, I performed primer extension experiments using four primers (PE-1 to 4, nt 348 to 368, 576 to 595, 1410 to 1428 and 4985 to 5002, respectively, Fig. I-4 and I-5). Extension products were detected with the PE-1 primer, but no bands were detected with PE-2, PE-3 and PE-4. I analyzed extension products generated by PE-1 and found that the transcription initiation site was A 326 (Fig. I-6). Furthermore, using the PB-1 fragment (nt 317 to 374, Fig. I-4 and I-5) as a probe, I performed Northern blot analysis. A broad band that was about 6 kb and other shorter bands were detected (Fig. I-7). The same 6-kb bands were also detected by the other three probes, PB-2, PB-3 and PB-4 (nt 573 to 647, 1396 to 1455 and 4933 to 5002, respectively, Fig. I-4 and I-5) (Fig. I-7). The additional lower molecular weight band detected by PB-1 might be encoded by *orf1* and *orf2*, because the PB-1 fragment overlaps the 3' end of a transcript of the upstream operon. The products of *orf1* and *orf2* shared 66.7% identity with the C-terminus of *E. coli* 3-oxoacyl-ACP reductase and 63.9% identity with *E. coli* acyl carrier protein (ACP), respectively. These results indicates that transcription of the *srb* operon starts at the A 326, 7 bp downstream from -10 region of the putative promoter (nt 314 to 319) and proceeds to the transcriptional terminator downstream of *ftsY*.

Structural analysis of FtsY.

The amino acid sequence of the C-terminal two-thirds of FtsY containing element I, II and III (G-domain) shows a high degree of similarity with those from the other three sources (*E. coli*, archaebacterium and mammal), whereas the N-terminal portion of the FtsY protein is shorter (Fig. I-8). The similar region identity among the four proteins includes residues 1-329 of FtsY, residues 180-497 of *E. coli* FtsY, residues 48-369 of *Sulfolobus solfataricus* DPα and residues 291-638 of mammalian SRα. There is 49.7%, 36.2% and 26.6% sequence identity between FtsY and *E. coli* FtsY, *S. solfataricus* DPα and mammalian SRα, respectively. The amino acid sequence of element III of FtsY is TKLD that corresponds to the conserved sequence

(TKXD) in SRP54 family. Moreover, the sequence of element I is completely identical to the conserved sequence (GVNGVGKT/S) in SRP receptors.

Effect of the depletion of FtsY in *B. subtilis* on cell growth and morphology.

To analyze the functions of the FtsY protein in *B. subtilis*, *B. subtilis* ISR38 strain was constructed in which the expression of the *ftsY* gene is dependent on IPTG (Fig. I-9A). Plasmid pTUE960, in which the replication origin in *B. subtilis* is absent, was transferred into *B. subtilis* 168 *trpC2* by means of the competent-cell transformation (Wilson and Bott, 1968). No chloramphenicol-resistant transformants were obtained when IPTG was not added. Among the chloramphenicol-resistant transformants grown in the presence of IPTG, the *B. subtilis* ISR38 strain was selected for further study. Colony formation of *B. subtilis* ISR38 cells was exhibited in the presence and absence of IPTG (Fig. I-10A). The parent strain, 168 *trpC2*, grew well in either the presence or absence of IPTG. In contrast, the ISR38 strain could hardly grow when IPTG was absent. The growth of the ISR38 strain was also tested in L-broth (Fig. I-10B). In absence of IPTG, the cell growth was also impaired in comparison with that in the presence of IPTG. I confirmed by Western blotting that FtsY was not expressed in ISR38 grown in the absence of IPTG (Fig. I-9B).

To inspect cell morphology, ISR38 cells growing in the presence or absence of IPTG for 7 h after inoculation were observed by electron microscope (Fig. I-11A and B). Cells growing in the absence of IPTG became filamentous, broken and twisted shape, while those growing in the presence of IPTG exhibited the normal rod shape and normal length of *B. subtilis*. These results indicated that *ftsY* is an essential gene for normal cell growth.

Effect of the depletion of FtsY on the translocation of extracellular proteins.

To ascertain whether *B. subtilis* FtsY protein participates in the protein translocation of the extracellular protein, *B. subtilis* ISR38 transformant containing pTUB1234 and 1236 was cultured in the presence and absence of IPTG. Plasmid pTUBE1234 encoding a fusion protein

(AprE-Bla) constituted from a signal peptide region of *B. subtilis* alkaline protease at its N-terminal, a mature region of β-lactamase, together with plasmid pTUBE1236 encoding another fusion protein (CGT-Bla) constituted from a signal peptide of *Bacillus sp.* #1011 cyclodextrin glucanotransferase (CGTase), the mature region of β-lactamase. I determined the translocation efficiency of AprE-β-lactamase fusion protein (AprE-Bla) and CGTase-β-lactamase fusion protein (CGT-Bla) in FtsY depleted cells by Western blotting analysis (Fig. I-12 lanes 1-2, 3-4). FtsY was undetectable in the transformant in the absence of IPTG (Fig. I-12 lanes 7-8, 9-10). The lysate of ISR38 cells overexpressing AprE-Bla or CGT-Bla in the presence or absence of IPTG were resolved by SDS-PAGE gel and immunoblotted by anti-β-lactamase antiserum. In the absence of IPTG, the accumulation of the precursor form was observed in all proteins.

To test whether or not penicillin-binding protein 5* (PBP5*) -BlaH6 fusion protein translocation depends on FtsY protein like the secretion of extracellular enzymes such as a-amylase and alkaline protease, we examined the accumulation of PBP5*-Bla precursors in *B. subtilis*. PBP5* is spore specific protein which have signal sequence. In the presence of IPTG, PBP5*-Bla in *B. subtilis* ISR38-1235 was completely processed into the mature form (Fig. I-12, lane 5) whereas approximately 45% of the protein remained in the precursor form in the absence of IPTG (Fig. I-12, lane 6). FtsY was undetectable in the transformant in the absence of IPTG (Fig. I-12 lane 12). These results suggested that the translocation of PBP5* was dependent on the presence of FtsY as was the secretion of alkaline protease from vegetative cells.

DISCUSSION

By using synthetic oligonucleotides for the GTP-binding consensus elements, I cloned and sequenced the *B. subtilis ftsY* gene for a homologue of mammalian SRα and sequenced its upstream region. Then, I determined the structure of the *ftsY* operon that consists of *orf3*, *orf4* and *ftsY*. The ORF3 had homology to *E. coli* RNase III. *E. coli* RNase III is an endonuclease that specifically hydrolyzes ds-RNA (Robertson *et al.*, 1968). The domain structure of ORF4 is identity to that of SMC protein.

The amino acid sequence of the FtsY protein shared 26.6%, 36.2% and 49.7% identity with those of mammalian SRα, archaebacterial DPα and E. coli FtsY, respectively. Furthermore, the sequence in the G-domain of FtsY exhibited higher identity with the other three SRP receptors. Although the N-terminal portion of FtsY is shorter than that of other SRP receptors, FtsY has GTP-binding elements whose sequences are conserved in SRP receptors. The amino acid sequences of the three GTP-binding sites of FtsY are similar to the consensus GTP-binding elements, GXXXXGKT/S (element I), DXXG (element II) and NKXD (element III) (Dever et al., 1987), though one deviation from the consensus (N to T) was found in element III. This deviation is also found in the corresponding regions of SRP54, SRα and each homologues. Therefore, they may be grouped into the same subfamily of GTP-binding proteins.

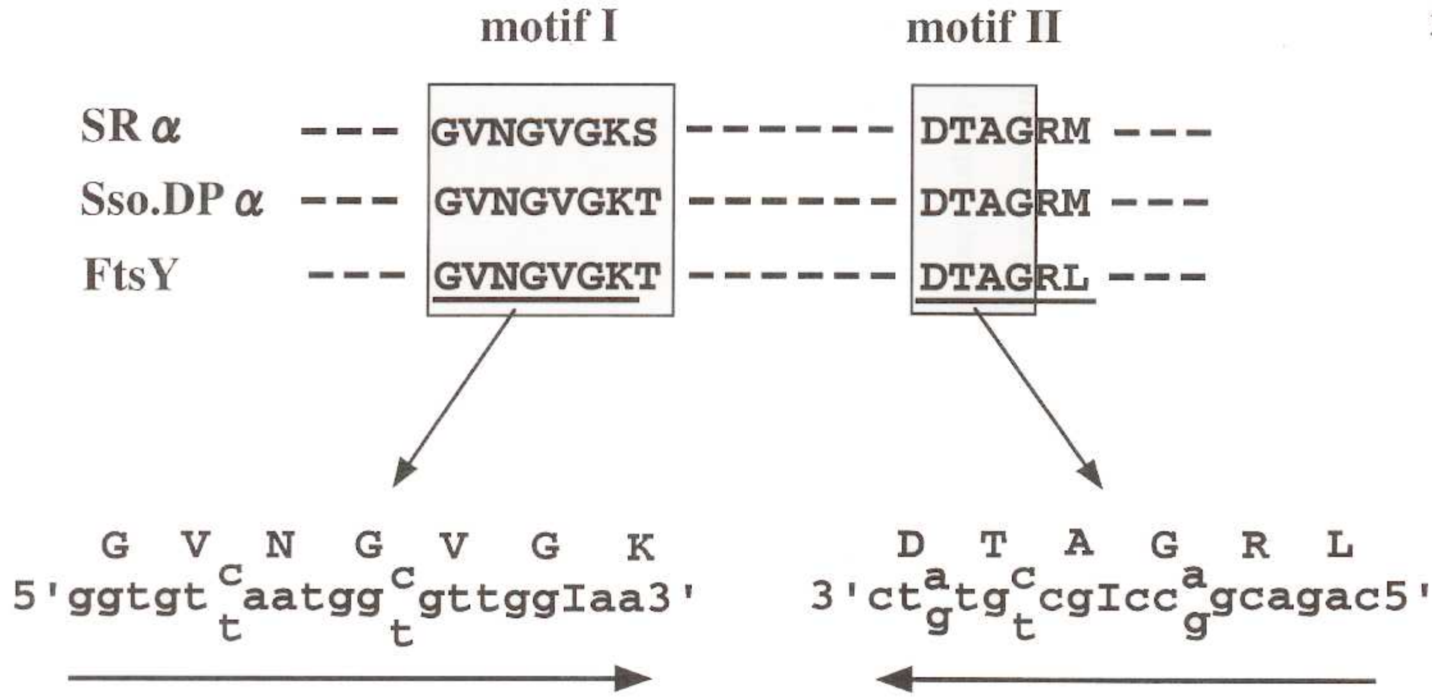
I showed that the depletion of the FtsY protein in *B. subtilis* caused a defect in normal cell growth and an aberrant morphology. Furthermore, depletion of FtsY in *B. subtilis* led to a defect in the translocation of extracellular proteins β-lactamase derivatives. The morphological changes were remarkably similar to those observed in scRNA depleted cells and *B. subtilis* Ffh depleted cells. In addition, it has been previously reported that the scRNA or *B. subtilis* Ffh depleted cells also showe a defect in the translocation of extracellular proteins (Nakamura *et al.*, 1992; Honda *et al.*, 1993). These suggests that *ftsY* is an essential gene for normal cell growth and morphogenesis and an SRP-SRP receptor mediated targeting system has a pivotal role in protein secretion in *B. subtilis*.

Fig. I-1. Alignment of the GTP-binding consensus elements of SRP54 family; mammalian SRa (SRα), *S. solfataricus* DPα (DPα) *E. coli* FtsY (FtsY), mammalian SRP54 (SRP54), *E. coli* Ffh (Ffh) and *B. subtilis* Ffh (Ffh). Numbers in the sequence refer to amino acid position in the respective protein. Identical amino acids are in black.

GTP-binding consensus	element I		element II		element III	
consensus	-------GXXXXGKT/S	---------	DXXG	-------	NKXD	-----
SRα (mammal)	--425GVNGVGKS432	-------	520DTAG523	---	588TKFD591	---
DPα (*S.solfataricus*)	--179GVNGVGKT186	-------	261DTAG264	---	319TKVD322	---
FtsY (*E.coli*)	--300GVNGVGKT307	-------	382DTAG385	---	446TKLD449	---
SRP54 (mammal)	--108GLQGAGKT115	-------	190DTSG193	---	248TKLD251	---
Ffh (*E.coli*)	--107GLQGAGKT114	-------	190DTAG193	---	248TKVD251	---
Ffh (*B.subtilis*)	--108GLQGAGKT115	-------	191DTAG194	---	249TKLD252	---

Fig. I-2. Sequence of synthesized oligonucleotides for primers to use for PCR (A) and PCR product (B). (A) Based on the conserved amino acid sequences in the SRP receptors, oligonucleotides used for PCR were designed and synthesized. Arrows under the DNA sequence indicate the direction of 5' to 3'. (B) The PCR product using the synthesized DNA as primers was electrophoresed on a 2.0% agarose gel and stained by ethidium bromide. The size markers (in bp) are *Hin*dIII- and *Eco*RI-digested lambda phage DNA fragments.

(A) Sequence of synthesized DNA



(B) PCR product

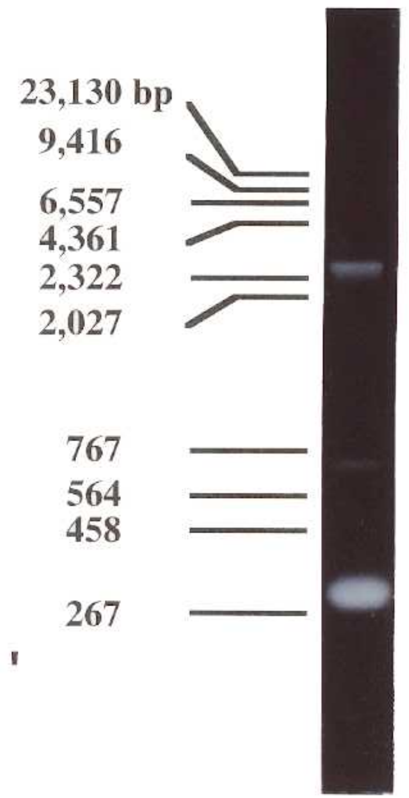
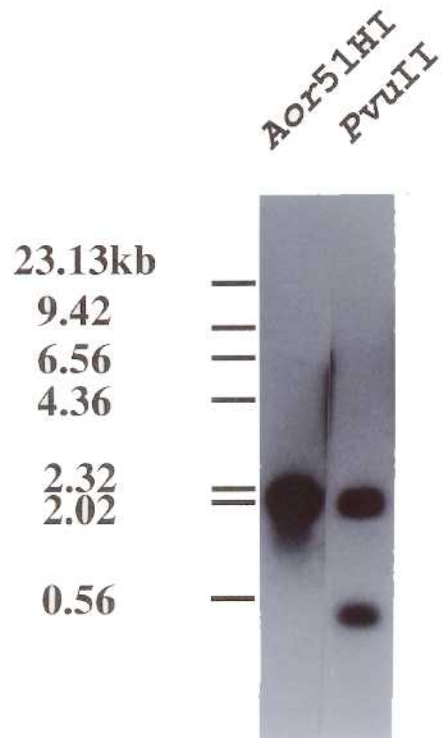


Fig. I-3. Southern hybridization of *B. subtilis* chromosomal DNA (A) and physical map and location of ORFs in the sequenced 2971 bp fragment (B). (A) The DNA sample (10 mg) was digested with the restriction enzymes *Aor*51HI or *Pvu*II, electrophoresed on a 0.8% agarose gel, transferred onto a GeneScreen Plus membrane, hybridized with an α-^{32}P-labeled 262-bp PCR product under stringent condition, and then autoradiographed. The size markers (in kb) were *Hin*dIII-digested lambda phage DNA fragments. (B) The ORFs are represented by the gene products, which were predicted from the nucleotide sequence in Fig. I-4. A thick bar indicates the position of PCR product used as the probe. Arrows indicate the direction of transcription and translation in each ORF. A, *Aor*51HI site; E, *Eco*RI site; P, *Pvu*II site.

(A) Southern hybridization



(B) Physical map and localization of ORFs

orf4
ftsY
probe

P A A E P E P A
2979bp
P P
1882bp
779bp
A A
1876bp
200bp

A, *Aor*51HI
E, *Eco*RI
P, *Pvu*II

Fig. I-4. The nucleotide and deduced amino acid sequence of *B. subtilis ftsY* operon and the preceding *orfs*. A putative promoter region is indicated as -35 and -10. SD preceding each ORF are indicated. Inverted repeat structures are indicated by converging arrows. Two terminators are indicated at nt 380 to 412 and 5939 to 5970. , a possible transcription start site of *ftsY* operon determined by primer extension (shown in Fig. I-6). The nt sequences complementary to the synthetic oligos (PE-1, PE-2, PE-3 and PE-4) used in the primer extension experiments are indicated by arrows over the sequence. The nt fragments (PB-1, PB-2, PB-3 and PB-4) used as probes for Northern blot analysis are double underlined. The three GTP-binding elements (boxes I to III) are indicated.

orf1—— orf2——

GAGCTCGTTATATGACAGGCCAAACGCTTCATATTGACGGCGGAATGGTGATGTAAGTTTTTTCTCGAAAATTTCATCGTAGTTTCTCTAGTTTTTTAAAAACGAATCCACTATGATACTTGAGGGGAGGTGAATTGCTATGGCAGACAC 150
A R Y H T G Q T L H I D G G H V H * SD 17
M A D T 4

-35
ATTAGAGCGTGTAACGAAAATCATCGTAGATCGCCTTGGCGTTGATGAAGCAGACGTCAAACTTGAAGCTTCTTTCAAGGAAGACTTAGGTGCTGATTCCCTAGATGTAGTTGAGCTTGTTATGGAACTTGAAGACGAGTTTGATATGGA 300
L E R V T K I I V D R L G V D E A D V K L E A S F K E D L G A D S L D V V E L V H E L E D E F D M E 54

-10 PB-1
GATTTCTGACGAAGATGCTGAAAAGATTGCAACAGTCGGCGACGCTGTGAACTACATACAAAACCAGCAATAAGCTGATGCTAAAAGTCCCGCCGAAACGCGGGGCTTTAGCCCCTTTATTCGTGCAGTTATTACAGCATGCCGCTTTTAC 450
I S D E D A E K I A T V G D A V N Y I Q N Q Q * 77

PB-1 orf3—— PB-2
GCGCGCTGTATCCGGTTTAGTCAGAGAAGCGCGGTGAACCTGATGTGCCTATGGAGGTTACTATGTCAAAACACTCACATTATAAAGATAAAAAAAAAGTTCTATAAAAAAGTAGAACAATTTAAAGAGTTTCAAGAACGGATTTCGGTTC 600
SD M S K H S H Y K D K K K F Y K K V E Q F K E F Q E R I S V H 30

ACTTTCAAAATGAAAAGCTTTTGTATCAAGCATTTACACATTCATCTTATGTGAATGAGCATCGGAAAAAGCCGTATGAAGATAATGAAAGGCTTGAATTTTTAGGTGACGCTGTTTTGGAACTGACGATCTCCAGATTCTTATTTCCCA 750
F Q N E K L L Y Q A F T H S S Y V N E H R K K P Y E D N E R L E F L G D A V L E L T I S R F L F P K 80
PB-2

AATACCCGGCTATGAGTGAAGGAGATTTGACGAAATTGAGAGCCGCAATTGTTTGCGAACCGTCTCTCGTTTCATTGGCTCACGAGCTGTCATTCGGCGATCTTGTCCTGTTGGGTAAAGGCGAGGAAATGACAGGCGGAAGAAAGCGTC 900
Y P A M S E G D L T K L R A A I V C E P S L V S L A H E L S F G D L V L L G K G E E M T G G R K R P 130

CTGCTCTATTGGCGGATGTTTTTAAGGCATTTATCGGAGCCTTGTACCTTGACCAAGGATTAGAGCCGGTCGAAAGTTTCTTAAAAGTTTATGTGTTCCCTAAAATTAACGATGGTGCTTTTCCTCATGTGATGGATTTCAAAAGCCAGC 1050
A L L A D V F K A F I G A L Y L D Q G L E P V E S F L K V Y V F P K I N D G A F P H V M D F K S Q L 180

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Q E Y V Q R D G K G S L E Y K I S N E K G P A H N R E F E A I V S L K G E P L G V G N G R S K K E A 230

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E Q H A A Q E A L A K L E K H H T K Q L N P P Y D S G G P Q Y V C R L I * 266

orf4—— PB-3
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SD M F L K R L D V I G F K S F A E R I S V D F V K G V T A V V G P N G S G K S N I T D A I R W 46
PB-3

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V L G G Q S A R S L R G G K M E D I I F A G S D S R K R L N L A E V T L T L D N D D H F L P I D F H 96

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E V S V T R R V Y R S G E S E F L I N N Q P C R L K D I I D L F M D S G L G K E A F S I I S Q G K V 146

GAAGAGATCCTGAGCAGCAAAGCGGAGGATCGCCGCAGTATCTTTGGAGAAGCGGCCGGGGTGCTTAAATATAAAACCAGAAAGGAAAAAAGCAGGAAATAAACTGTTTGAGACACAGGACAATCTAAATCGGGTAGGAGATATATTACAT 1950
E E I L S S K A E D R R S I F G E A A G V L K Y K T R K E K A G N K L F E T Q D N L N R V G D I L H 196

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E L E G Q V E P L K I Q A S I A K D Y L E K K K E L E H V E I P L T A Y D I E E L H G K W S T L K E 246

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K V Q M A K E E E L A E S S A I S P K E A K I E G T R D K I Q A L D E S V D E L Q Q V L L V T S E E 296

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L E K L E G R K E V L K D R K K N A V Q N Q E Q L E E A I V Q F Q Q K E T V L T R R A F E A E A V F 346

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E T L Q A E V K Q L R A Q V K E K Q H A L S L H N E N V E E K I E Q L K S D Y F E L L N S Q A S I R 396

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N E L Q L L D D Q M S Q S A V T L Q R L A D N N E K H L Q E R H D I S A R K A A C K T E F A R F E Q 446

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E I H S Q V G A Y R D M Q T K Y E Q K K R Q Y E K N E S P L Y Q A Y Q Y V Q Q A R S K K D M L D T M 496

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Q G D F S G F Y Q G V K E V L K A K D R L G G I R G A V L E L I S T E Q K Y E T A I E I A V G A S P 546

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Q H V V T D D E Q S A R K A I Q Y L K Q N S F G R A T F L P L S V I R D R Q L S K P L R G N S G P A 596

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F I I S F G V A S E L V T F D P A Y R S V I Q N L L G N R S D Y R G L K G G A N E L A K L L G H R Y 646

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R I V T L E G D V V N P G G S M T G G S V K K K N N S L L G R T R E L G D V T K R L A E M E E K T S 696

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L L E Q E V Q T L K H S I Q D M E K K L A D L R E T G E G L R V K Q Q D V K G Q L Y E P Q F A E K N 746

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I N T H L E L Y D Q E K S A L S E S D E E R K V R K R K L E E E L S A V S E K M K Q L E E D I D R L 796

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T K Q K Q T Q S S T K E S L S N E L T E L K I A A A K K E Q A C K G E E D N L A R L K K E L T E T E 846

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L A L K E A K E D L S F L T S E M S S S T S G E E K L E E A A K H K L N D K T K T I E L I A L R R D 896

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Q R I K L Q H G L D T Y E R E L K E M K R L Y K Q K T T L L K D E E V K L G R M E V E L D N L L Q Y 946

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L R E E Y S L S F E G A K E K Y Q L E T D P E E A R K R V K L I K L A I E E L G T V N L G S I D E F 996

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E R V N E R Y K F L S E Q K E D L T E A K N T L F Q V I E E M D E E M T K R F N D T F V Q I R S H F 1046

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D Q V F R S L F G G G R A E L R L T D P N D L L H S G V E I I A Q P P G K K L Q N L N L L S G G E R 1096

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A L T A I A L L F S I L K V R P V P F C V L D E V E A A L D E A N V F R F A Q Y L K K Y S S D T Q F 1146

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I V I T H R K G T M E E A D V L Y G V T M Q E S G V S K V I S V K L E E T K E F V Q * SD 1188
M 1

srb—— PE-4
GAGCTTTTTTAAAAAATTAAAAGAGAAAATCACAAAACAGACAGATTCCGTATCTGAAAAGTTTAAGGATGGCCTTGAAAAAACAAGAAACTCCTTTCAAAACAAAGTGAATGATCTTGTATCCCGTTACCGTAAAGTGGATGAGGATTT 5100
S F F K K L K E K I T K Q T D S V S E K P K D G L E K T R N S P Q N K V N D L V S R Y R K V D E D F 51
PB-4

CTTCGAAGAGCTTGAAGAGGTTCTTATCAGCGCGGATGTCGGTTTTACAACCGTTATGGAATTAATAGATGAGCTGAAAAAAGAAGTCAAACGCAGAAATATTCAAGATCCAAAGGAAGTCCAGTCAGTGATTTCTGAGAAACTGGTCGA 5250
F E E L E E V L I S A D V G F T T V H E L I D E L K K E V K R R N I Q D P K E V Q S V I S E K L V E 101

GATTTATAACAGCGGAGATGAGCAAATTTCAGAACTGAACATCCAGGATGGGCGTTTAAACGTAATCCTTCTGGTAGGTGTAAACGGCGTCGGGAAAACAACAACGATCGGAAAGCTTGCTCATAAAATGAAACAAGAAGGAAAATCTGT 5400
I Y N S G D E Q I S E L N I Q D G R L N V I L L V G V N G V G K T T T I G K L A H K M K Q E G K S V 151

TGTACTTGCCGCCGGAGACACTTTTAGAGCGGGAGCCATTGAACAGCTGGAAGTATGGGGAGAGCGTACAGGAGTGCCTGTCATTAAGCAGACGGCAGGAAGCGATCCGGCGGCTGTCATCTACGATGCTGTTCATGCTGCGAAAGCAAG 5550
V L A A G D T F R A G A I E Q L E V W G E R T G V P V I K Q T A G S D P A A V I Y D A V H A A K A R 201

AAATGCCGATGTATTAATTTGTGATACGGCAGGGCGTCTCCAAAACAAAGTAAATCTCATGAAAGAGCTTGAAAAAGTAAAACGTGTTATCGAAAGAGAAGTTCCTGAAACTCCGCATGAGGTGCTGCTTGCCCTTGATGCCACGACCGG 5700
N A D V L I C D T A G R L Q N K V N L M K E L E K V K R V I E R E V P E T P H E V L L A L D A T T G 251

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Q N A M A Q A K E F S K A T N V T G I A L T K L D G T A K G G I V L A I R N E L H I P V K L V G L G 301

AGAAAAAGTTGATGACCTTCAGGAATTTGATCCAGAATCCTATGTGTACGGACTCTTTTCAGATTTAGTGGAAAAAGCCGACGATTAAGAAAAAAGGCCCCAACATCTTGGGGCCTTTTTCTTTTTTATCTTCTTACTTGATAGGCGAAAT 6000
E K V D D L Q E F D P E S Y V V Y G L F S D L V E K A D D * 329

GATAAAGGCTGTTATCAGTGGATACCAGTCTTGACTCACCAGAAAAAACTCTGAATGGGATGATGTCATAGTAATGAACGGAAACAGATGTGTAATAC 6098

Fig. I-5. Operon organization in a 6098-bp sequence. Black boxes indicate location of primers (PE-1 to 4) used in primer extension. Hatched boxes indicate location of probes (PB-1 to 4) used in Northern blot analysis. Converging arrows indicate a putative stem-loop structure. indicates terminators.

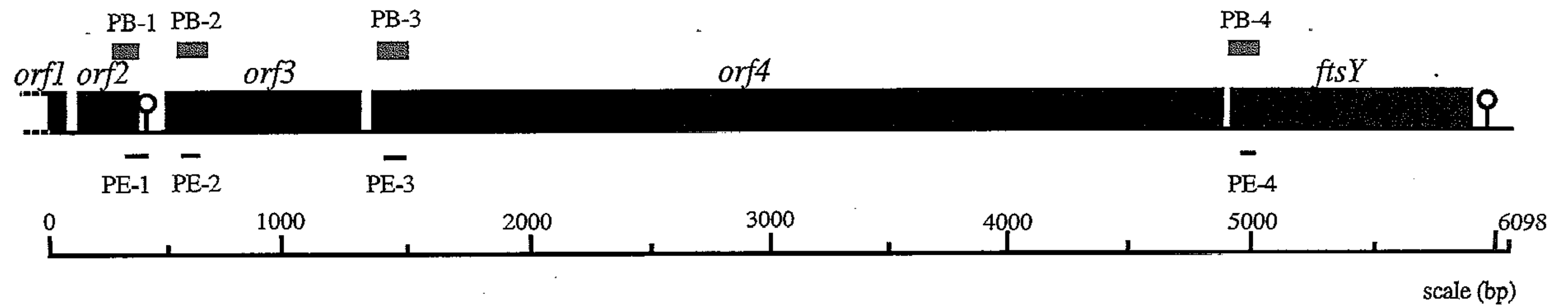

PB-1
PB-2
PB-3
PB-4
orf1
orf2
orf3
orf4
ftsY
PE-1
PE-2
PE-3
PE-4
0
1000
2000
3000
4000
5000
6098
scale (bp)

Fig. I-6. Mapping of the transcription start site of the *ftsY* operon by primer extension. The synthetic oligo PE-1 was 5' end labeled with polynucleotide kinase and [γ-^{32}P]ATP. The labeled primer (5 x 10^4 cpm) was annealed to 40 mg of total RNA isolated from cells harvested at late log phase and extended by Rous associated virus-2 reverse transcriptase for 1 h at 42°C. Using the same primer, a pUC18 clone of the 5' leader region of the *ftsY* operon was sequenced (the lanes marked CTAG). The samples were resolved by 8 M urea-6% polyacrylamide gel electrophoresis and visualized by autoradiography. Asterisk marks the transcription start site. Arrow indicates the extension product.

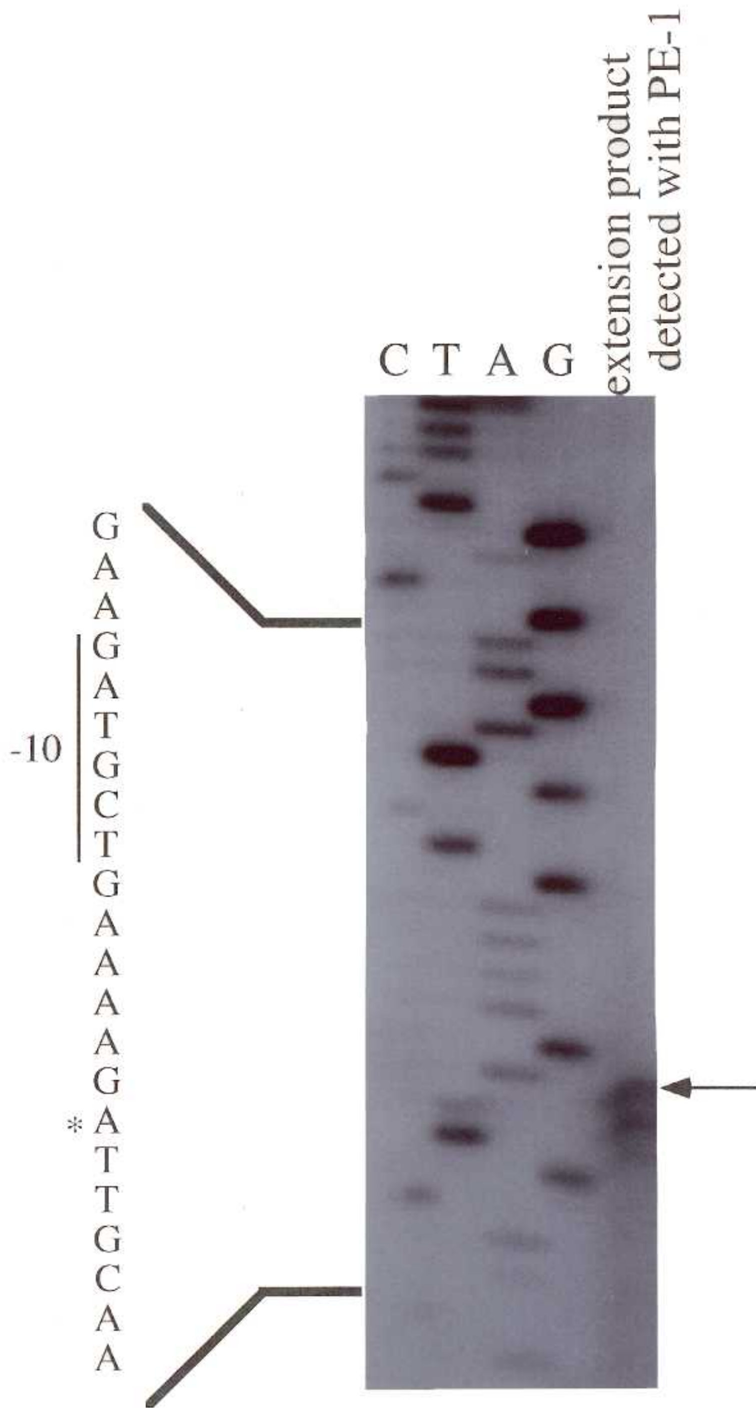

C T A G
extension product detected with PE-1
G
A
A
G
A
T
G
C
T
G
A
A
A
A
G
* A
T
T
G
C
A
A
-10

Fig. I-7. Northern blot analysis of *B. subtilis* RNAs by four probes (PB-1 to 4). RNA isolated from *B. subtilis* 168 at late log phase was electrophoresed on the 2 M formaldehyde-1% agarose gel and transferred to GeneScreen Plus. The membranes were then hybridized with the ^{32}P-labeled probes (PB-1 to 4). Arrow indicates a putative transcript of *ftsY* operon. Asterisk indicates a putative transcript of upstream.

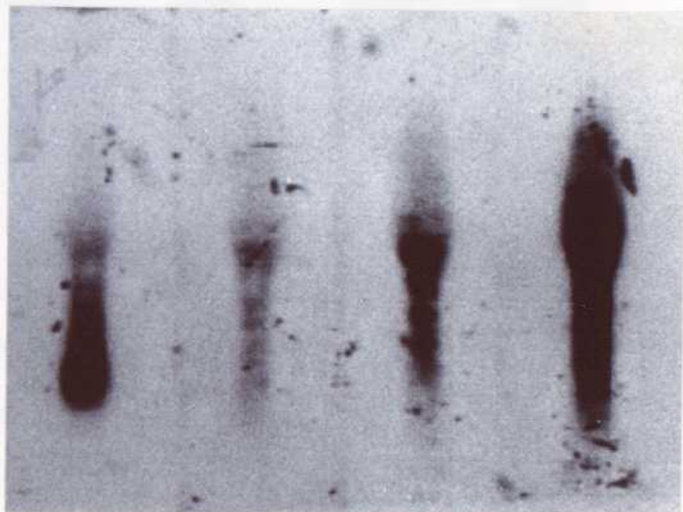

PB-1
PB-2
PB-3
PB-4
*

Fig. I-8. Homology of the putative *ftsY* gene product to other proteins of SRP receptors. The alignment of the primary sequences of *B. subtilis* FtsY (FtsY), *E. coli* FtsY (FtsY), *S. solfataricus* DPα (DPα) and mammalian SRα (SRα) is indicated. Putative GTP-binding elements (boxes I to III) are indicated. The position in the amino acid sequence of the first amino acid on every line is shown. The amino acid residues showing identity to FtsY are indicated by a black background. Gaps for the maximum matching are indicated by dashes. Asterisks indicate the ends of amino acid sequences.

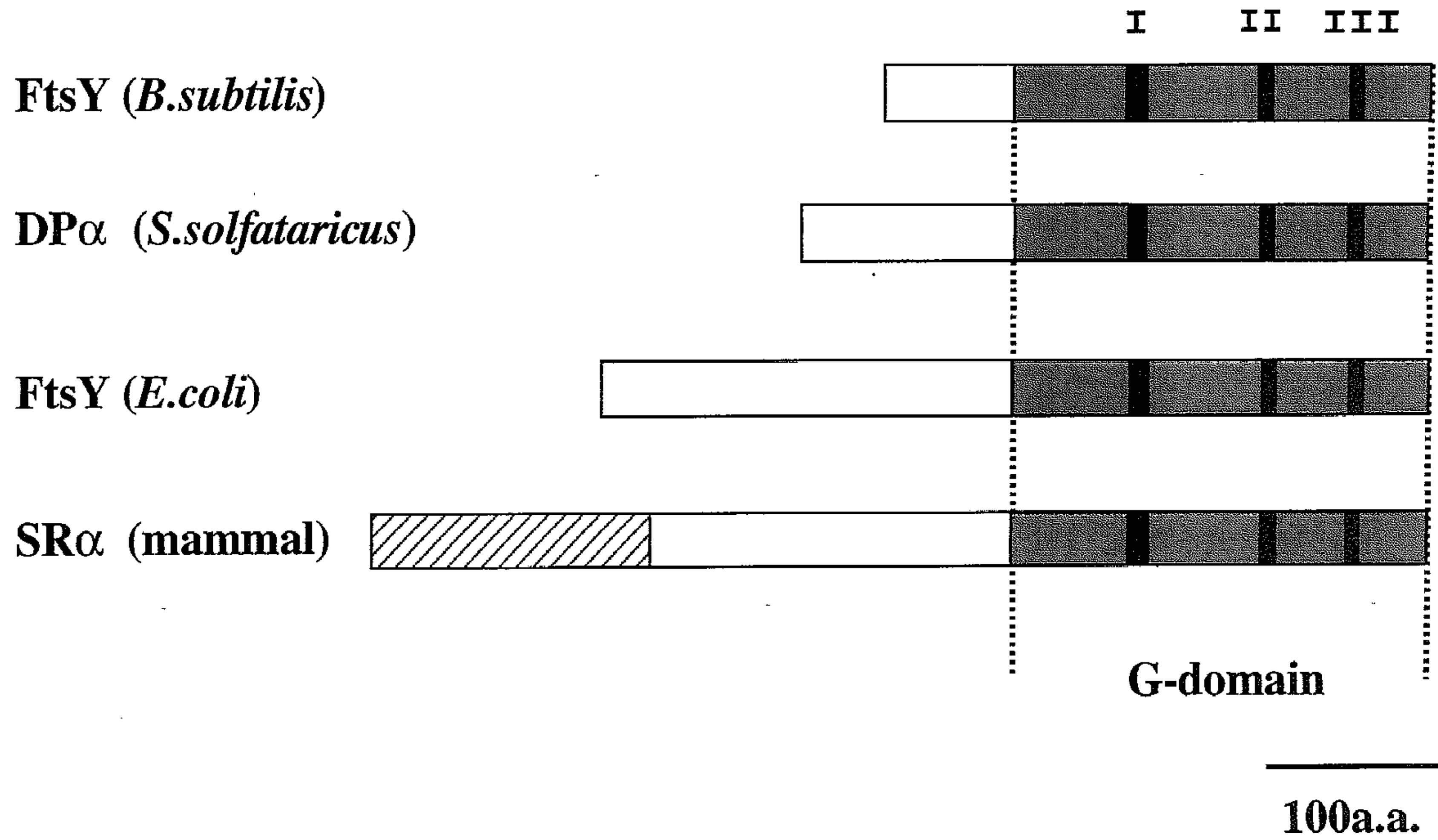

I
II
III
FtsY (B.subtilis)
DPα (S.solfataricus)
FtsY (E.coli)
SRα (mammal)
G-domain
100a.a.

Fig. I-9. Construction of *B. subtilis* ISR38 in which the expression of *ftsY* is dependent upon IPTG. (A) pTUE960 plasmid [a derivative of pDH88 (Henner, 1990) carrying 292-bp *Eco*RI-*Sau*3AI(nt 4915 to 5206) fragment] was integrated to transform *B. subtilis* chromosome by homologous recombination and then *B. subtilis* ISR38 was selected by Cm resistance. *B. subtilis* ISR38 chromosome results in a truncated copy of *ftsY* (*ftsY'*) under the control of the authentic promoter and an intact copy of *ftsY* under the control of the *spac-1* promoter (P*spac-1*). (B) Immunological detection of FtsY in *B. subtilis* wild type strain and ISR38 strain. Lyates from wild type (168 *trpC2*) strain cells (lane 1), ISR38 strain cells cultured in the presence (lane 2) or absence (lane 3) of IPTG were analyzed by immunoblotting with he anti-FtsY antibody.

(A)

pC194*cat*

pTUE960

Amp^r

lacI

P$_{spac\text{-}1}$ P$_{penP}$

'*smc* *ftsY'*

Homologous Recombination

Eco RI *Sau* 3AI

B. subtilis 168 chromosome

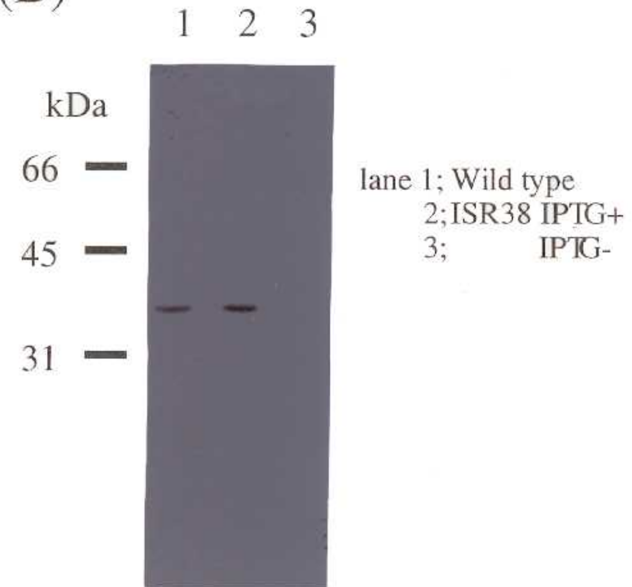
smc *ftsY*

P$_{penP}$ P$_{spac\text{-}1}$

smc *ftsY'* *lacI* pC194*cat* *Amp*^{r} '*smc* *ftsY*

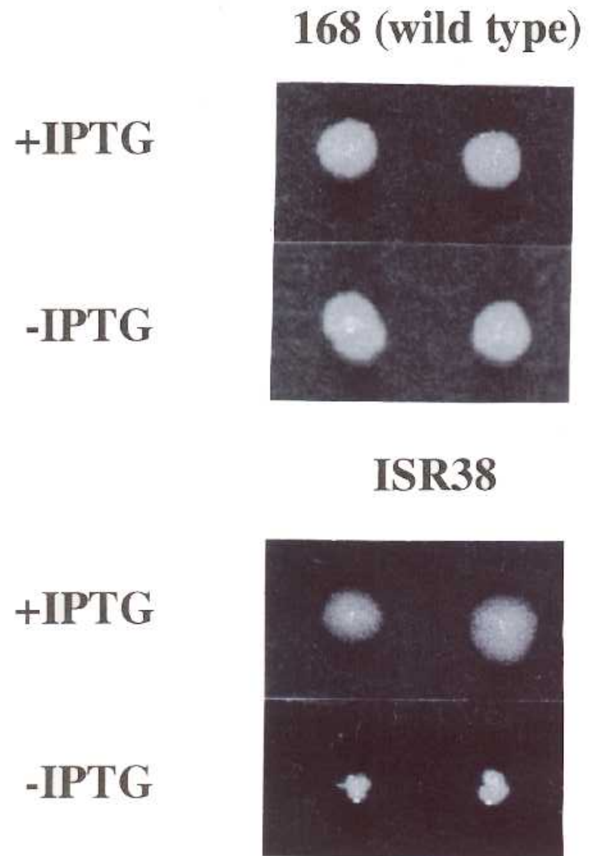
B. subtilis **ISR38 chromosome**

(B)



lane 1; Wild type
2; ISR38 IPTG+
3; IPTG-

Fig. I-10. Effects of depletion of FtsY on growths on L-broth agar plates (A) and in liquid cultures (B). (A) Cells were grown for 12 h at 37°C on L-broth agar plates with or without 3 mM IPTG. (B) ISR38 was cultured in L-broth in the presence (●) or absence (▲) of 3 mM IPTG.


(A)
168 (wild type)
+IPTG
-IPTG
ISR38
+IPTG
-IPTG

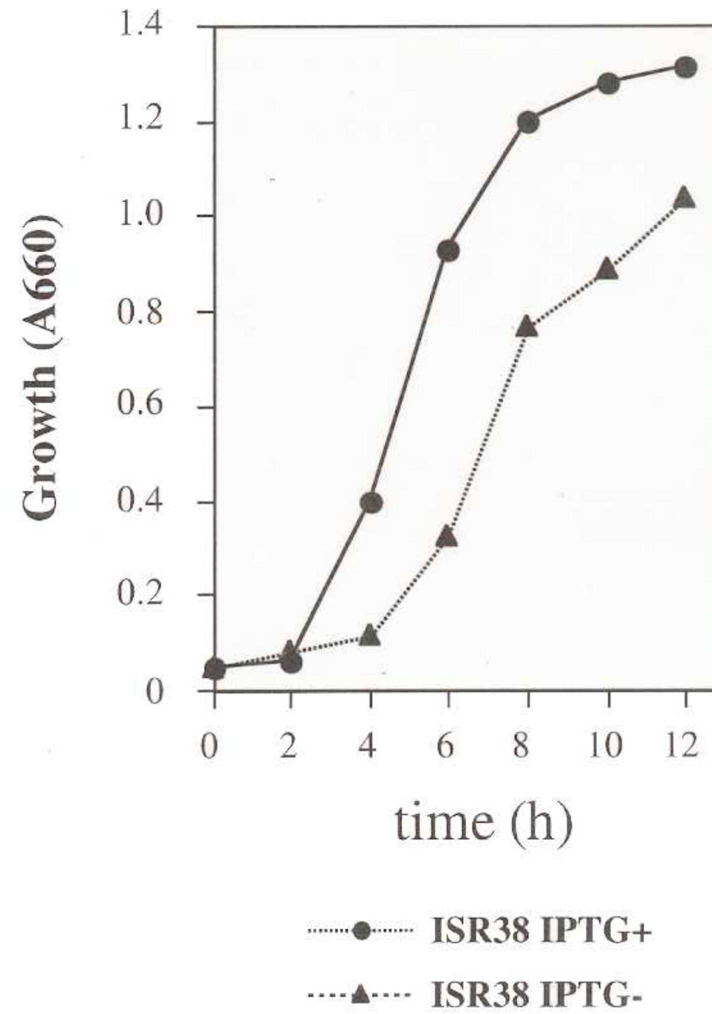
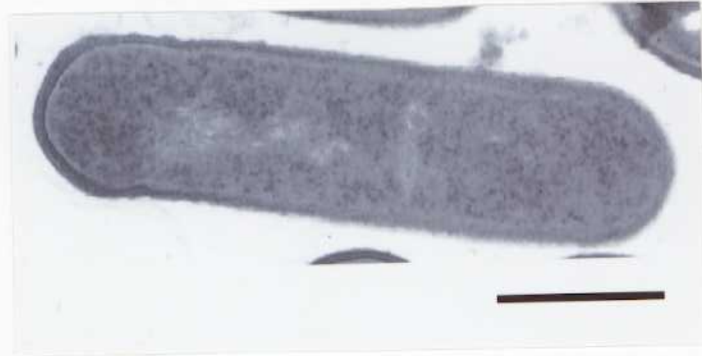

(B)
Growth (A660)
1.4
1.2
1.0
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
time (h)
ISR38 IPTG+
ISR38 IPTG-

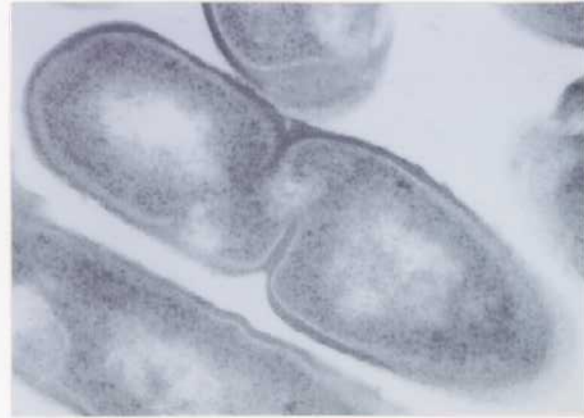
Fig. I-11. Transmission of electron microscopy of ISR38 which was cultured in the presence (A) or absence (B) of IPTG. (A) In the presence of IPTG, ISR38 shows a normal rod shape just as wild type cell. (B) In the absence of IPTG, ISR38 shows a twisted (upper) and/or broken (lower) cell shape.

(A) ISR38 IPTG +

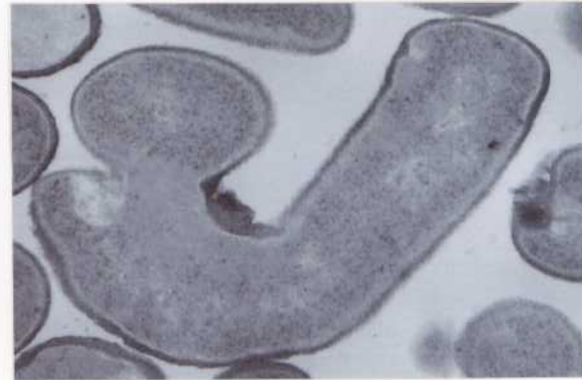


500nm

(B) ISR38 IPTG -



500nm

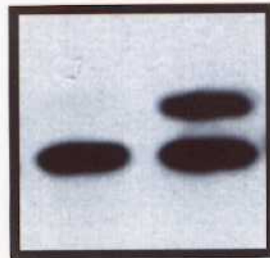


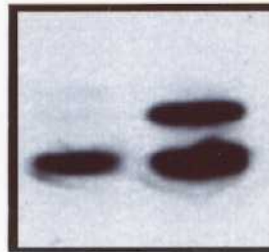
500nm

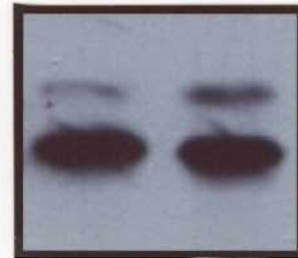
Fig. I-12. Effects of the depletion of FtsY on the translocation of *B. subtilis* β-lactamase derivatives. The signal peptide region of β-lactamase was replaced by that of *B. subtilis* alkaline protease (AprE), *B. subtilis* Penicillin binding protein 5* (PBP5*) or alkalophilic *Bacillus cyclodextrim* glucanotransferase (CGTase). Lysates from AprE-β-lactamase fusion protein (AprE-BlaH6), PBP5*-β-lactamase fusion protein (PBP5*-BlaH6) or CGTase-β-lactamase fusion protein (CGT-BlaH6) overexpressing ISR38 which were cultured in the presence (lane +) or absence (lane -) of IPTG were analyzed by immunoblotting with the anti-β-lactamase antiserumand (lanes 1-6) and anti FtsY antiserumand (lanes 7-12). Arrowheads indicate precursor and mature forms of the fusion proteins (lanes 1 to 6) and FtsY (lanes 7 to 12).

B.subtilis ISR38-1234 (AprE-BlaH6) *B.subtilis* ISR38-1235 (PBP5*-BlaH6) *B.subtilis* ISR38-1236 (CGTase-BlaH6)

IPTG + - + - + -







precursor

mature

lane 1 2 3 4 5 6

IPTG + - + - + -

FtsY

FtsY

FtsY

lane 7 8 9 10 11 12