Temperature-Sensitive Sporulation Caused by a Mutation in the Bacillus subtilis secY Gene

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A thermosensitive sporulation mutant of Bacillus subtilis containing a mutation in the secY gene was isolated and characterized. No asymmetric septum specific to the sporulation was detected by electron microscopy at the nonpermissive temperature, indicating that the block occurred at a very early stage of sporulation. Furthermore, competence development in the mutant cell was affected even at the sporulation-proficient temperature. It is assumed that the SecY protein of B. subtilis has multiple roles both in the regulation of sporulation and in stationary-phase-associated phenomena.

Under conditions of nutrient limitation, Bacillus subtilis cells initiate a differentiation process leading to the production of dormant spores (15, 25). Cells entering stationary phase also express a variety of responses including the induction of chemotaxis and motility, development of genetic competence, and production of extracellular enzymes and antibiotics. The transition from the vegetative to the sporulation phase requires at least seven genes known as spol. spo0 mutants cannot form an asymmetric septum, which is the first morphological change in the sporulation process. Some of the most crucial questions concerning the initiation of sporulation are what kinds of environmental signals trigger the process, how they are sensed by the vegetative growing cells, and how the cells respond to those signals. Among spo0 genes, spo0A is thought to play a central role in the initial stage of sporulation since spo0A mutants exert the most pleiotropic effects on the sporulation-associated phenotypes described above (24).

We have previously reported (6, 32) that the expression of the spo0A gene is regulated at the transcription level by switching two discrete promoters, Pv and Ps. Transcription from the upstream Pv promoter occurs at a low level in the vegetatively growing cells and ceases at the initial stage of the sporulation process (0.5 h after the end of log-phase growth [T0.5]). On the other hand, transcription from the downstream promoter is essentially null in vegetatively growing cells but is induced at T0.5 and continues to increase until T2.5. In the presence of high concentrations of glucose (e.g., 2% glucose), the promoter switching from the Pv-directed transcription to the Ps-directed one does not occur, which results in markedly reduced sporule formation. It is known that a catabolite-resistant sporulation mutant carrying the crsA47 (sigA47) mutation (28) within the gene encoding a1 (13) suppresses the glucose inhibition of the promoter switching from Pv to Ps.

Spo0A protein and Spo0F protein share sequence homology with receiver proteins (29, 35) of prokaryotic two-component signal transduction systems (24, 26). It has been shown that Spo0A protein is a phosphoreceptor which accepts a phosphate moiety from the phosphorylated Spo0F protein by Spo0B phosphomessenger that is phosphorylated by kinases which function depending on the particular environmental signals (4). The series of phosphate transfer reactions in the initial stage of sporulation process is called a phosphorelay (4).

We have previously isolated a B. subtilis gene homologous to Escherichia coli secY (18, 34). The secY (prlA) gene in E. coli is essential and encodes a 49-kDa integral membrane protein (2, 3, 5). Its temperature-sensitive mutation secY24 results in pleiotropic accumulation of the precursor forms of secretory proteins (23, 31), and the alleles of prlA suppress the translocation defects of signal sequence mutations (7). The SecY protein has been postulated to form a channel for polypeptide translocation (1, 2). B. subtilis secY encodes a polypeptide of 431 amino acid residues that has 41% identity with the E. coli SecY. On the basis of the similarity of the distribution of hydrophobic amino acids, B. subtilis SecY protein is predicted to be an integral membrane protein with 10 membrane-spanning segments and also to be a component of the protein export apparatus (18, 27).

In this paper, we describe the isolation and characterization of a sporulation-deficient mutant having a mutation site in the secY gene. Sadaie and Kada (20) reported previously that strains carrying septum-initiation mutations were defective in sporulation and competence development. One of these genes was recently found to be B. subtilis secA (22). E. coli SecA is a peripheral membrane protein involved in the translocation of secretory proteins (19) and has been suggested to interact with SecY (8). Therefore, it might be generally considered that the protein transport apparatus is involved in the regulation of sporulation. The mechanism of protein transport in B. subtilis is poorly understood, and no detailed study on the function of each component in the transport apparatus has been reported. Sporulation of B. subtilis is a very complex process in which a number of genes are involved. Since E. coli does not possess sporulation ability, it is interesting to analyze the function of secY in the regulation of sporulation in order to comprehend the nature of the SecY protein. It may also provide a new insight into the initiation mechanism of B. subtilis sporulation.

Selection of sporulation-deficient mutants. We have previously isolated a B. subtilis gene homologous to E. coli secY (34). To isolate a DNA fragment containing the secY gene, we utilized the rpsE2 marker, which is a mutation in the ribosomal protein S5 gene located closely upstream of the
secY gene. Therefore, the _Agt11_ recombinant phage carrying the secY gene also contains _rpsE2_ gene, which confers spectinomycin resistance (Spc^c) to the cell. To obtain sporulation-deficient mutations occurring in the secY gene, _E. coli_ LE392 (16) lysogenized with this recombinant λ phage was mutagenized with _N_-methyl- _N''_-nitro- _N''_-nitrosoguanidine. DNA was isolated from the mutagenized cells and used to transform _B. subtilis_ UOT-1248 (trpC2 _lys^-1_) to Spc^e. The Spc^e transformants were found to be oligosporogenic on plates containing spectinomycin but were sporulation proficient in the absence of the drug. Therefore, colonies that appeared were replica plated on a 2× SG plate (14) without spectinomycin and incubated at various temperatures. Of about 10,000 transformants examined, 4 exhibited the temperature-sensitive sporulation phenotype. These isolates sporulated normally at 37°C but were oligosporogenic at 45°C.

**Mapping of the sporulation-deficient mutation within the secY gene.** DNAs from the sporulation-deficient mutants described above were used to transform UOT-1248 to Spc^e. A total of 70 to 80% of the Spc^e transformants were oligosporogenic at 45°C. The very high cotransformation frequency between the Spc^e and sporulation-deficient (oligosporogenic) phenotypes indicated that the sporulation-deficient mutations were closely linked to the Spc^e site. To determine whether the sporulation-deficient mutations were located within the secY gene, a plasmid containing the wild-type secY region of _B. subtilis_ was constructed. This plasmid carried a polymerase chain reaction (PCR)-derived fragment which was amplified from UOT-1248 DNA with primers corresponding to regions just upstream and downstream of the secY gene. The sporulation-deficient mutants were transformed with this plasmid to the sporulation-proficient phenotype. To obtain sporulation-proficient transformants at 45°C, the recipient cells after contact with the donor DNA, were incubated at 45°C for 20 h in 2× SG medium. Heat-resistant spores produced in the primary culture were plated on a Schaeffer's sporulation plate to identify sporulation-proficient transformants. Thus, the two of four sporulation-deficient mutants described above were transformable to sporulation proficiency with the wild-type secY DNA; that is, these sporulation-deficient mutations are located within the secY gene.

**Determination of the nucleotide sequences of sporulation-deficient secY mutations.** The nucleotide sequence of the entire secY gene of one of the two sporulation-deficient mutants was determined. Only one base in this region was different from that of wild-type strains: the cytosine (C) residue at position 1441 of the wild-type DNA (the position number is the nucleotide position in reference 34) was changed to thymidine (T), which changed the proline (P) residue at the 76th amino acid of the SecY protein to leucine (L). Accordingly, we refer to this mutation as secY76. A partial nucleotide sequence determination for the other mutant revealed the same base change at the same position. The secY76 mutation was transferred to UOT-1248 by congermination transformation with _lys^+ _DNA. The resultant strain was named HR71 (trpC2 secY76) and used for further analysis.

**Sporulation frequency by HR71.** Growth capabilities and sporulation frequencies of strains HR71 and UOT-1248 were tested at various temperatures. HR71 could grow in liquid culture at temperatures between 30 and 45°C. Although the colonies on the 2× SG plate incubated at 49°C were relatively smaller than those of UOT-1248, we concluded that the secY76 mutation did not affect the vegetative growth. HR71 sporulates normally at both 30 and 37°C but sporulates at a reduced frequency at higher temperatures. At 45°C, at which UOT-1248 sporulates at the normal frequency, the HR71 strain showed markedly reduced sporulation (Table 1).

**Temperature shift-up and shift-down experiments.** To determine the period during which SecY protein is required for the sporulation process, temperature shift-up and shift-down experiments were carried out (Fig. 1). When the cells were shifted from 37 to 45°C after _T_3, the sporulation frequencies

### Table 1. Sporulation frequencies of _B. subtilis_ strains at various temperatures

<table>
<thead>
<tr>
<th>Strain</th>
<th>Frequencya at:</th>
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<tbody>
<tr>
<td></td>
<td>30°C</td>
</tr>
<tr>
<td>UOT-1248</td>
<td>8.6 x 10^-1</td>
</tr>
<tr>
<td>HR71</td>
<td>8.2 x 10^-1</td>
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- Expressed as the ratio of spores to viable cells. Numbers of viable cells did not vary significantly at the temperatures examined.

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**FIG. 1.** Sporulation frequencies of HR71 after temperature shift. Cells were grown in 2× SG medium containing 0.1% glucose (14) at either 37 or 45°C. At the indicated time, a portion of each culture was taken and incubated at the other temperature. After 20 h, heat-resistant spores were scored by heating the cells at 80°C for 10 min and then plating them. Time is expressed in hours, with _T_6 representing the end of exponential growth.
FIG. 2. Electron microscopy of HR71. The cells were grown in 2× SG at 37°C until $T_2$ (A) or at 45°C until $T_2$ (B) or $T_4$ (C) and analyzed as described previously (30) with a JEOL200 CX electron microscope at 100 kV.
were close to the wild-type level, and similar frequencies were obtained when the cells were shifted from 45 to 37°C before T_{-0.5}. The frequencies of wild-type UOT-1248 were not affected by these temperature shifts (data not shown). These results indicated that the mutant cells must be at the permissive temperature from T_{-0.5} through T_5 to sporulate normally.

**Electron microscopy observation.** To study the morphological phenotype of HR71, T_2 cells of HR71 grown at either 37 or 45°C were analyzed by electron microscopy as described previously (30). Both vegetative and spor septe were visible in the cells grown at 37°C, and a typical photograph of the spore septum is shown in Fig. 2A. On the other hand, we observed no spore septum among about 100 cells grown at 45°C (Fig. 2B). Even when the cells were cultivated for a prolonged period until T_4, no spore septum was observed (Fig. 2C). While the wild type UOT-1248 cells showed similar morphology at either temperature and spore septa were observed even in the T_2 cells grown at 45°C (data not shown).

**Competence of HR71.** The transformation frequency of HR71 was compared with that of UOT-1248 by using UOT-0278 (hisA1 metB51) chromosomal DNA as a trp^{+} donor and plasmid pUB110 as a Km^{+} DNA. The numbers of transformants of HR71 per milliliter per microgram of DNA were 9.5 \times 10^{6} with trp^{+} and 1.5 \times 10^{5} with Km^{+} DNA, values which are more than 100-fold lower than those for UOT-1248 (3.0 \times 10^{6} and 1.0 \times 10^{5} transformants per ml per \mu g of DNA, respectively). These results indicate that HR71 has a deficiency in some step of DNA uptake or recombination.

**S1 nuclease protection assay of spo0A transcript.** The results of electron microscopy analysis showed that the sporulation was blocked at a very early stage, since no asymmetric spore septum formation was visible at the high temperature. This morphology is the same as that of spo0A mutants; therefore, we analyzed the expression of spo0A in the mutant cells. HR71 cells were grown in 2× SG medium at either 37 or 45°C, and total cellular RNA was extracted at the vegetative (T_{-0.5}) and stationary (T_5) phases. An S1 nuclease protection assay using a 32P-labeled anti-spo0A RNA probe (A1 probe [6]) was carried out. As reported previously (6), the differential expression of the spo0A gene is regulated by switching the promoter from Pv to Ps during the initial stage of sporulation. When the HR71 cells were grown at 37°C, the promoter switching was clearly seen; on the other hand, the Ps promoter was hardly activated in the cells grown at 45°C (Fig. 3). Since the promoter switching of wild-type UOT-1248 cells grown at 45°C could be observed just as it was in the cells grown at 37°C (data not shown), these results indicated that at least a part of the role of the SecY protein is in the activation of spo0A Ps promoter and may explain the very early block in the sporulation process at 45°C.

**Conclusions.** The regulation of spo0A gene expression is complex (24). In particular, the expression from the Ps promoter, which is thought to be one of the most important steps in the initiation of sporulation, is regulated by many gene products (4, 6, 32, 33). These gene products may sense the cellular environment and determine whether the cells should proceed to sporulation or keep growing. There may be several checkpoints in turning on successive gene expression before the spo0A Ps promoter is stimulated. The results presented here strongly suggest that the secY gene product is also involved in the regulatory network of spo0A gene expression. The crsA47 or sigF-1 (sigA47), mutation, which is known to bypass the requirement of the Spo0F, Spo0B, or Spo0E protein in the phosphorelay system leading to the phosphorylation of Spo0A (10, 12), could not overcome the sporulation deficiency of the secY76 mutation (data not shown). Therefore, the SecY protein has a role in the regulation of the spo0A gene somewhere downstream of the phosphorelay system or through a completely different pathway. Although we do not know the precise point at which the SecY protein functions, it is unlikely that SecY directly regulates spo0A transcription. Since SecY is considered a component of the protein translocation apparatus, the secY76 mutation may affect the transport of an exoprotein that is involved in the initiation of sporulation and competence. The mutant cell has to be at a permissive temperature from T_{-0.5} through T_5 to proceed to a normal level of sporulation, and thus, the SecY protein should function throughout this period. The function of the spo0A gene has been shown not to be required for sporulation after T_{2.5} (13). These results indicate that the SecY protein functions in at least two steps, one corresponding to activation of spo0A gene expression and another after the spo0A gene is activated. The time T_5 corresponds to the completion of forespore development.

The mutation site of secY76 is located in the putative second membrane-spanning domain in the SecY protein. This site is also located in the highly homologous region reported among SecY proteins (9). Indeed, the proline residue that is mutated to leucine in the HR71 strain is conserved in 14 SecY proteins, ranging from eukaryotic to mycoplasma proteins (9, 11, 17).

Another feature of this mutant is the deficiency of genetic competence. Although the transformation experiments were carried out at 37°C, at which they sporulate normally, the frequency was 100-fold lower than that of wild-type cells (see above). As determined by analysis with a lacZ fusion, the expression of the spo0A gene in HR71 was reduced even at 37°C (data not shown). The development of competence is known to require the spo0A gene product (21), and this may explain the low competence of HR71, while the residual activity of Spo0A is sufficient for sporulation to proceed at this temperature.

The effect of the secY76 mutation on protein secretion is not yet well characterized. However, the production of α-amylase or extracellular protease is not retarded in HR71 at either 37 or 45°C (data not shown). Preliminary results showed similar rates of precursor processing for α-amylase, indicating that the secY76 mutation does not affect secretion of this enzyme.

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