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Isolation and Characterization of a Sporulation Initiation Mutation in the *Bacillus subtilis secA* Gene

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A *Bacillus subtilis secA* mutant, *secA12*, which is blocked at an early stage of sporulation, is able to grow as well as the wild-type strain at all temperatures tested. Experiments with *lacZ* fusion genes showed that the induction of *kinA* expression, as well as the sporulation-specific transcription of the *spo0A* gene, was not observed in the *secA12* mutant. However, transcription of the *spo0H* gene (coding for σ^H, which is required for the transcription of *kinA* and *spo0A*) and accumulation of the σ^H protein were not affected in *secA12*. These results suggested that mutations in *secA* affect a factor required for efficient transcription of *kinA* as well as for the activation of the phosphorelay pathway.

A gene, *div*$^+$/*secA*$^+$, of *Bacillus subtilis* was first described as one of the genes controlling the initiation of cell division (15, 20, 21), and its nucleotide sequence was determined by Sadaie et al. (22). Because of its homology with the *Escherichia coli secA* gene, Takamatsu et al. (27) proposed to call this gene *secA*. The mutant form of this gene, *div341*/*secA341*, encodes proteins that exhibit pleiotropic phenotypes that affect cell division, secretion of extracellular enzymes, autolysis, development of competence, spore outgrowth, and sporulation (15, 20–22, 27).

We have recently shown using the *secA341* mutant that SecA is required during at least three different periods of spore development, $T_{-0.5}$ to $T_{0.5}$, T_1 to T_2, and T_3 to T_5, as shown by temperature shift-up and -down experiments (5). We have also shown that the transcription of *spo0A* from the sporulation-specific promoter, Ps (see below), is not induced in the *secA341* cells when they are exposed to nonpermissive temperatures from $T_{-0.5}$ to $T_{0.5}$ (5).

The product of the *spo0A* gene plays a key role in the initiation of sporulation (17, 24–26, 33). It is converted to an active and phosphorylated form, Spo0A~P, via the so-called phosphorelay pathway, consisting of sensor kinase KinA and the phosphotransfer proteins Spo0B and Spo0F (7, 12, 28). The *spo0A* gene has two promoters: a vegetative promoter (Pv; σ^A type) and a sporulation-specific promoter (Ps; σ^H type) (8). Ps-directed transcription depends absolutely on the *spo0A*, *spo0B*, and *spo0F* gene products as well as the *spo0H* product (a minor sigma factor, σ^H), required for the initiation of sporulation (8–10, 19, 31, 32) and partially on the *kinA* gene product (unpublished results).

The expression of *spo0H* coding for σ^H is regulated at post-translational as well as transcriptional levels (11, 19, 30). Healy et al. have proposed the existence of a posttranscriptional mechanism stabilizing the σ^H protein or increasing the amount of σ^H protein in the transition state between vegetative growth and sporulation (11). More recently we have shown that expression of the *kinA* gene, whose transcription is directed by RNA polymerase containing σ^H (19), required *spo0H*, but not the *spo0A*, *spo0B*, and *spo0F* genes and that the accumulation of σ^H protein occurred in *spo0A*, *spo0B*, and *spo0F* mutants (6). Thus, the transcriptional activation of the *kinA* gene is an immediate early sporulation initiation event, since its induction is not dependent on the phosphorelay pathway and is observed approximately 1 h earlier than that of *spo0A*-Ps transcription (6).

We have investigated whether the *secA* gene could play an important role in an early stage of sporulation and have isolated a sporulation-defective *secA* mutant in order to clarify the role of SecA during the initiation of sporulation.

Isolation of a new sporulation-defective *secA* mutant. The SecA proteins in both *E. coli* (13, 16) and *B. subtilis* (21, 27) play a role in the translocation of secreted proteins which are essential for vegetative growth. Whether the inability of *B. subtilis secA341* grown at 37°C to sporulate is due to a protein secretion defect or to another undetermined defect remains unclear, because the vegetative growth of this mutant is retarded appreciably at temperatures higher than 37°C, where sporulation is also blocked. It appears that the sporulation phenotype of the *secA341* strain is leaky at 37°C, as seen also from the incomplete inhibition of *kinA* and *spo0A*-Ps induction (data not shown). It is possible that *B. subtilis* SecA has two independent functions, one of which is required for sporulation but not for vegetative growth, while the other function is essential for both. We, therefore, tried to isolate a *B. subtilis secA* mutation which allowed vegetative growth at a higher temperature than the nonpermissive temperature for sporulation.

The 6.1-kb *Pvu*II fragment of ϕ105-*secA*$^+$ DNA (including the 4.1-kb *Cfr*13I fragment of the *B. subtilis* genome) (22) was cloned with *Pvu*II-digested pBR322, and the resulting plasmid was designated pBSA. *E. coli* MC4100 (F$^-$ [*argF-lacZ*]*U169 araD139 rpsL150 ptsF25 flbB5301 rbsR deoC relA1*) cells harboring pBSA were cultured in competence medium I, which was made by combining 10 ml of 1× minimal medium solution (3), 0.1 ml of 50% glucose, 0.05 ml of 1 M $MgSO_4$, 0.06 ml of 5% Casamino Acids (Difco), and required auxotrophic supplements. The cells were harvested when the optical density at 660 nm (OD_{660}) of the culture reached 0.25 and were treated in 1×

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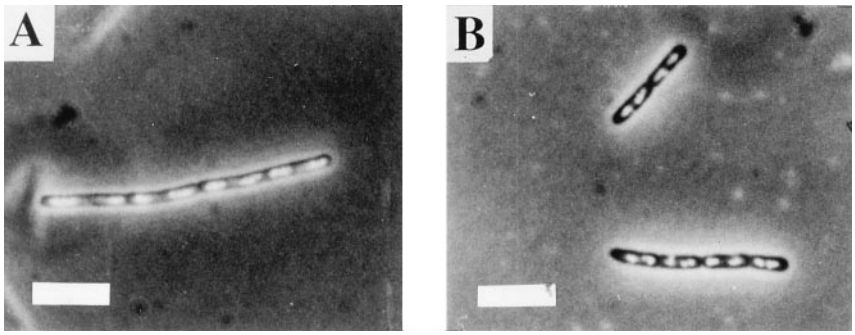

FIG. 1. Micrographs of vegetatively growing cells stained with 4′,6-diamidino-2-phenylindole. (A) UOT-1285 (*trpC2 lys1 aprEΔ3 nprR2 nprE18*); (B) UOT-1840 (*trpC2 lys1 aprEΔ3 nprR2 nprE18 secA12*). Bar, 10 μm.

minimal medium solution with 200 μg of *N*-methyl-*N*′-nitro-*N*-nitrosoguanidine per ml at 37°C for 30 min. The mutagenized cells were then grown in 5 ml of Luria-Bertani medium (23) overnight at 32°C. The plasmid whose DNA contained the *secA* gene, pBSA, was extracted from the mutagenized cells, was linearized with *Bam*HI, and was used to transform the *B. subtilis secA341* mutant. Then the cells were plated on sporulation medium supplemented with 0.1% (wt/vol) glucose (2× SG) (5) and were incubated at 42°C. Among approximately 10^4 temperature-resistant transformants, we obtained one sporulation-defective *secA* mutant, designated *secA12*. In order to show that the mutation in *secA* was actually causing the phenotype, we did a complementation test using ϕ105-*secA*$^+$. The sporulation deficiency observed in the *secA12* mutant was restored when ϕ105-*secA*$^+$ was lysogenized in this mutant.

Characterization of *secA12* mutant. The *secA12* mutant cells were able to grow in 2× SG sporulation medium at the same rate as the wild-type cells at all temperatures tested up to 42°C (data not shown), although the *secA12* cells formed slightly smaller colonies on plates at 42°C than those formed by the wild-type strain. Vegetative cells of the *spo*$^+$ and *secA12* mutant strains grown in 2× SG medium at 37°C were spread on a glass slide, fixed by flaming, and stained with 1 μg of 4′,6-diamidino-2-phenylindole per ml in saline solution, and photographs were taken with a combination of phase-contrast and fluorescence systems. The cell morphology, chromosome segregation, and cell division of the *secA12* mutant strain were similar to those of the wild-type strain (Fig. 1A and B). Nevertheless, at any temperature studied (32, 37, or 42°C), *secA12* could not form heat-resistant spores that retained the *secA12* phenotype after germination.

Using *secA12* cells lysogenized with ϕCAZ6 either carrying the *spo0A*-Ps-*lacZ* fusion (32) or with the *kinA-lacZ* fusion integrated (6), we tested whether or not the *secA12* mutation inhibited transcription induction by the *spo0A*-Ps and *kinA* promoters during early sporulation time periods. It is evident that little, if any, induction of both *spo0A*-Ps and *kinA* transcription was observed in *secA12* cells (Fig. 2A and B).

The *secA12* mutation affects neither *spo0H* induction nor σ^H accumulation. As stated above, the *secA12* mutation reduced the expression of *kinA*, which codes for one of the major kinases of the phosphorelay system (4, 18, 29). This result does not necessarily establish the direct involvement of the SecA protein in the induction of *kinA* transcription. Since *kinA* transcription is dependent on σ^H, whose expression is regulated at the posttranslational as well as transcriptional levels (11, 19, 30), there is a possibility that *secA12* somehow reduces σ^H activity, resulting in reduced *kinA* transcription. We therefore examined the effects of the *secA12* mutation on *spo0H* expression (Fig. 2C) and on the level of σ^H protein (Fig. 3) using the integrated *spo0H-lacZ* fusion and anti-σ^H antibody, respectively (6). The results showed that while *secA12* inhibited *kinA* induction, it affected neither the induction of *spo0H* expression nor the accumulation of σ^H at T_0 and T_1.

Observations similar to those noted above have been reported recently (6): in medium supplemented with excess glucose and glutamine, *kinA* induction was almost completely eliminated, though the accumulation of σ^H was observed. It is thus likely that there exists an additional factor that is controlled by SecA that regulates the expression of the *kinA* gene in response to nutritional conditions. It should be noted that, other than the *spo0H* mutant, only the *secA12* mutant has been shown to diminish *kinA* transcription at the onset of sporulation.

Mutation site of *secA12*. In *E. coli*, SecA is an essential cytoplasmic protein required for protein translocation, with the ability to recognize and interact with various proexoproteins (1, 2, 13, 16). Most of the *E. coli secA* mutants isolated to date showed a temperature-sensitive phenotype, and their mutations were localized in the ATP binding domain of the N-terminal region of the SecA protein (Fig. 4) (14). We sequenced the entire *B. subtilis secA* gene in the *secA12* mutant and found that the 515th codon of serine (TCA) was changed to that of leucine (TTA). The mutation site of *secA12* is located in the longest contiguous region of 23 amino acids conserved between *E. coli* and *B. subtilis* SecA proteins and near the

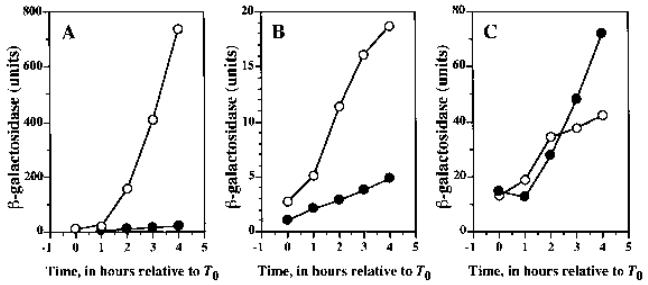

FIG. 2. Expression of β-galactosidase in *secA12* mutant strains carrying a *spo0A*-Ps-*lacZ* fusion (A), *kinA-lacZ* fusion (B), and *spo0H-lacZ* fusion (C). The cells were cultured in 2× SG medium at 37°C. The abscissa indicates the time preceding or following the end of exponential growth, which is designated as time zero. Samples of the culture were withdrawn for β-galactosidase activity determination as described previously (5, 6). One unit is $1{,}000 \times A_{420}/OD_{660}$/ml/min where A_{420} is the absorbance at 420 nm. Symbols: ○, UOT-1285 (*secA*$^+$); ●, UOT-1840 (*secA12*).

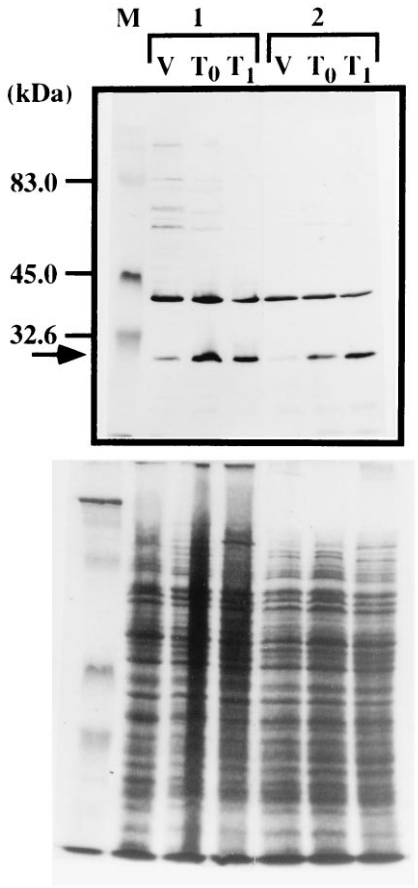

FIG. 3. Western blot analysis of σ^H protein levels in extracts (50 μg of total proteins) from cells of *secA12* mutant strains. Cells grown in 2× SG medium were collected at the end of (T_0), and 1 h after the end of (T_1), the exponential growth phase during the vegetative growth phase (V; OD_{660} = 0.25), about 1 h before the end of exponential growth. Samples were prepared, and Western blot analysis was performed as described previously (6). The lower panel represents Coomassie brilliant blue-stained total protein; the lanes correspond to those in the upper panel. The arrow indicates the position of σ^H. Lanes: M, molecular mass markers (kaleidoscope prestained standards [Bio-Rad]); 1, UOT-1285 (*secA*$^+$); 2, UOT-1840 (*secA12*).

secA341 mutation site in the middle of the SecA protein (Fig. 4) (22, 27).

It is of particular interest that the *secA* mutation sites of *E. coli* and those of *B. subtilis* are localized in separate portions of the *secA* gene. The *secA* gene might comprise two domains, one of which is in the middle of the gene and is indispensable specifically for sporulation. The *secA12* mutation of *B. subtilis* causes a defect in the sporulation-specific SecA function. One, at least, of the results of this mutation is the reduction in transcription of the *kinA* gene coding for the major kinase of the sporulation-specific phosphorelay. On the other hand, the conservation of the amino acid sequence surrounding the *secA12* mutation suggests that this region may code for an important functional domain in *E. coli* and *B. subtilis*. In addition, we were unable to isolate intergenic suppressor mutations that restored the ability of the *secA341* mutant to sporulate, probably because the *secA341* mutation affects three different stages of sporulation (5). Although some intergenic suppressor mutations of *secA12* can be isolated (unpublished data), the results suggest that the *secA12* mutation blocks only the initiation stage of sporulation. The *secA12* mutant, therefore, offers a useful tool not only for studies of the immediate early functions during sporulation of *B. subtilis* but also of as yet unknown SecA functions in bacteria. We note that an alternate explanation for the *secA12* effects could be that this mutation results in a "toxic" form of SecA that interferes with early sporulation events in a manner that is unrelated to the normal function, if any, of SecA during development.

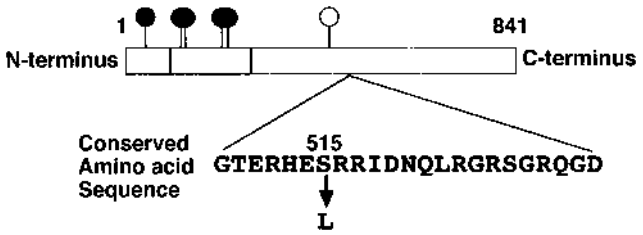

FIG. 4. Comparison between sites of known mutations in the *secA* genes of *E. coli* and *B. subtilis*. Numbers indicate the order of amino acids in the *B. subtilis* SecA protein (22). Solid and open circles indicate mutation locations of *E. coli* *secA* temperature-sensitive (14) and *B. subtilis secA341* mutants, respectively. An arrow indicates the location of *secA12*. The shaded box shows the location of the essential ATP binding domain (14).

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