Restricted Transcription from Sigma H or Phosphorylated Spo0A Dependent Promoters in the Temperature-sensitive secA341 Mutant of Bacillus subtilis

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The temperature-sensitive secA341 mutation of Bacillus subtilis affects sporulation and sporulation-associated events as well as protein secretion and cell septation. With lacZ or bgab fusion genes, we examined the expression of the early sporulation genes in the mutant strain. Transcriptional expression of σH-dependent kinA, spo0A (Ps), phrC, spoVG, and citG (p2) genes was blocked by the secA341 mutation at 37°C. On the other hand, neither repression of the abrB gene nor induction of the spo0H (σH) gene was affected. Active RNA polymerase containing σH was, however, found to be produced in the mutant cells. Expression of the phosphorylated Spo0A-dependent spo11G operon was also blocked. Thus, the secA341 mutation blocks some step(s) or factor(s) required for σH-dependent transcription in vivo.

Key words: Bacillus subtilis; secA; σH promoter; sporulation

Sporulation is believed to start with phosphorylation of the Spo0A protein.1-3 Phosphorylated Spo0A protein represses transcription of the abrB gene,3 the product of which is postulated to be a negative regulator of spo0H gene expression,4 leading to derepression of spo0H expression. The product of the spo0H gene, σH, in turn directs transcription with the core enzyme of RNA polymerase from several promoters including that of kinA, a component gene of the phosphorelay leading to phosphorylation of Spo0A protein, and spo0A genes,7 thus resulting in production of sufficient amounts of phosphorylated Spo0A.

The kinA promoter is σH-dependent while spo0A has two promoters, one dependent on σN(Pv), and one on σH(Ps).9 spoVG and phrC genes, expressed during early sporulation stages, also have σH-dependent promoters. AcrB represses the spoVG gene, and the phrC gene codes for an extracellular competence-stimulating factor.9-14 A TCA cycle gene, citG, has a σH-dependent promoter (citGp2) used even during growth.15 Transcription from the spo11G (σH) promoter is done by a σH-containing RNA polymerase and is positively regulated by phosphorylated Spo0A.16,17 Transcription from the spo111G (σH) promoter depends on σH18 the expression of which requires σH and phosphorylated Spo0A.19,20

The temperature (42°C)-sensitive secA341 (originally ts341 or div-341) mutation in the secA gene21-26 of Bacillus subtilis makes a cell defective in competence, autolysis, spore outgrowth, protein secretion, and sporulation at a higher, slightly non-permissive temperature (37°C),27,28 where cell growth is almost intact. SecA is a protein translocation ATPase and plays the main role in exporting proteins into the membrane, periplasm, or medium29,30 and the above pleiotropic phenotypes may be caused by a defective translocation property of the mutated SecA341 protein, or the SecA protein may have another unknown regulatory function in gene expression.

This communication is aimed to examine the effects of the secA341 mutation on the lacZ or bgab31 expression of abrB-lacZ, spo0H-lacZ, kinA-lacZ, spo0A-lacZ, spo11G-lacZ, spo111G-lacZ, citG-bgalB, spoVG-bgalB, and phrC-bgalB fusion genes to discover the role of SecA protein in the expression of these genes involved in the early phase of sporulation.

Materials and Methods

Bacterial strains, plages, and plasmids. The Bacillus subtilis and Escherichia coli strains used are listed in Table 1. Phages φ105 and φ105CM32 were used for cloning the abrB-lacZ fusion together with plasmid pMC1871.33 Plasmid pDL,31 carrying a thermostable β-galactosidase gene (bgab) of B. steaothermophilus, was used to construct spoVG-bgalB, phrC-bgalB, and citG-bgalB fusions, integrated at the amyE locus of the
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E. coli strain

JMI109

recA1 endA1 gyrA96 thi hsdR17 supE44 relA1

λ−(lac-proAB)F' traD36 proAB lacIqZDM15

NIG1171 and NIG1172 were his' secA341 transformants of UOT1851 and UOT1852 with ts341 DNA respectively. cm, tc, neo, and ap are chloramphenicol, tetracycline, neomycin, and ampicillin respectively. NIG2003 was a Trp' SecA' transformant of NIG2001 with ts341 DNA.

B. subtilis chromosome.

Medium for sporulation. B. subtilis cells were grown in Schaeffer's spore broth (DS medium) or 2 X SG medium consisting of 2 X Schaeffer's spore broth and 0.1% glucose. Minimal glucose medium containing 20 μM MnCl₂ was used when citGp2-bgaB fusion was examined. Fresh cells grown on nutrient broth agar at 30°C or overnight culture in LB broth at 30°C were transferred to fresh medium. E. coli cells were grown in LB broth.

Construction of the lacZ or bgaB fusion genes and measurement of β-galactosidase activity. The abrB-lacZ translational fusion gene was constructed by ligating Smal digested pMC1871 and the abrB promoter DNA; the latter was obtained with PCR amplification of the abrB promoter region using the PCR kit of Takara (Takaya, Kyoto, Japan) and JH642 chromosomal DNA. Primers used were 5′atccgggagatctgttattctcggt3' and 5′atccgggagatctgttattctcggt3'. The PCR product contained the first 202 bases of DNA flanked by atccgg and gecct at the 5′ and 3′ ends, respectively. The fusion gene contained the promoter region and the first 7 codons of abrB with a substitution of CCC for GGT in the 7th codon. Ligated DNA was used to transform E. coli JMI109 to obtain blue colonies on LB agar containing 5 μg/ml tetracycline (Sigma) and 0.02% X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, Takara). Plasmid DNA containing the abrB promoter region was purified and digested with PstI and made blunt with a blunting kit containing T4 polymerase (Takara). It was ligated with CM DNA, treated with BamHI and the blunt end kit, and used to transform into NIG1121 lysogenic for φ105 to obtain a temperate phage, φCMabrBlacZ, as described elsewhere. The recombinant phage was induced with mitomycin C (Kyowa Hakko, Tokyo) and propagated in the secA341 mutant strain.

The spoVG-bgaB and phrC-bgaB transcriptional fusions were constructed by cloning in the BamHI site of the pdL plasmid PCR-synthesized promoter DNAs prepared with primer pairs containing BamHI sites, followed by digestion with BamHI. spoVG primers were designed from the spoVG promoter sequence: ggcggatcacaagctttatcatcaatgagcgttagctgaagacaccggatcacaagctttatcatcaatgagcgttagctga. phrC primers were designed from the phrC promoter: ggcggatcacaagctttatcatcaatgagcgttagctgaagacaccggatcacaagctttatcatcaatgagcgttagctga.
For the construction of the citG-bgaB transcriptional fusion, BamHI digested pDL was treated with T4 polymerase to produce blunt ends. It was ligated with PCR product containing SmaI sites. citG primers were designed from the citGp2 promoter: atccgggagaatc, cgggaggaggcggccttatcagagaggagc. The constructed plasmids were used to transform E. coli JM109 cells to Amp (50 μg/ml) and transformed colonies were examined for insertions by PCR. Candidate plasmids were used to transform B. subtilis cells to Cm (5 μg/ml). Transformant DNA was examined by PCR to find whether the bgaB fusion gene was integrated at the amyE locus or not.

The other lacZ fusions were all translational fusions. The spo0H-lacZ fusion contained the first 65 codons of the spo0H gene with a substitution of Pro for Gln at the 65th codon and integrated into the spo0H region of the chromosome. The kinA-lacZ fusion contained the first 599 codons of the kinA gene and was integrated into the kinA region of the chromosome. The spo0A-lacZ fusion contained the first 32 codons of the spo0A gene. The spo0H-lacZ fusion and the spo0H-lacZ fusion contained the first 20 and 32 codons of the spo0H and spo0H genes, respectively (Masuda and Kobayashi, unpublished result). Yamada and Kobayashi, unpublished result). spo0A-, spo0H-, and spo0H-lacZ fusion genes were cloned on the φCM temperate phage.

The activity of β-galactosidase was measured as described elsewhere. For measurement of the bgaB activity, the sample was incubated at 60°C for 5 min.

**In vitro transcription of the abrB and spoVG promoters.** Purification of RNA polymerase holoenzymes containing a histidine-tagged β′ subunit with a nickel (Ni2+-nitrilo-tri-acetate) column, and transcription in vitro will be described elsewhere (Fujita and Sadaie, Gene, in press).

**Enzymes and chemicals.** Restriction endonucleases and enzymes were purchased from Takara Shuzo Co. Ltd., Kyoto and Sigma. All other chemicals were purchased from Wako Chemicals Co. Ltd. Tokyo. Nutrient broth, tryptone, and yeast extracts were from Difco, and agar was purchased from Koseka Kako (Tokyo).

**Results**

The effects of the secA341 mutation on the expression of genes the transcription of which depends upon σH and/or phosphorylated Spo0A

To see at which stage sporulation is blocked in the secA341 mutant cells at 37°C, the expression of lacZ or bgaB (coding for the thermostable β-galactosidase of B. stearothermophilus) fused to the abrB, spo0H, kinA, spo0A, spoVG, phrC, citG, spoIIIG, and spoIIIG genes was examined. As shown in Fig. 1, transcription from abrB in the secA341 mutant strain at 37°C decreased toward the end of growth as in the wild-type strain (Fig. 1A) and induction of the spo0H gene was observed upon sporulation both in the secA341 mutant and the wild-type strain at 37°C (Fig. 1B). However, transcription from σH-dependent kinA (Fig. 1C) and the spo0A spore promoter (Ps) (Fig. 2B) was greatly reduced in the secA341 mutant cells at 37°C during the early sporulation stage, while transcription from the σA-dependent spo0A promoter (Ps) was not affected by the secA341 mutation at 37°C (Fig. 2A). Ps + Ps promoter was used in Fig. 2A and the promoter containing only Ps was used in Fig. 2B. Similar reduced expression from a σH-dependent spo0A (Ps) promoter has been obtained, by heat treatment (39°C for 60 min) or temperature shift from 32°C to 39°C of the mutant cells.

Transcription of the σH-dependent promoters of spoVG (Fig. 3A) and phrC (Fig. 3B) was also suppressed in the secA341 mutant strain at 37°C during the early sporulation stage. Repression by AbrB of the spoVG gene was presumably released as in the case of spo0H due to the repressed expression of abrB described above. phrC has another distal promoter used at the

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**Fig. 1.** Effects of the secA341 Mutation at 37°C on the Expression of abrB-lacZ, spo0H-lacZ, and kinA-lacZ Fusion Genes of B. subtilis during Sporulation in DS Medium.

A, abrB-lacZ. Open circle, wild-type strain NIG1121 (φCMbrBlacZ). Closed circle, secA341 strain NIG1152 (φCMbrBlacZ). B, spo0H-lacZ. Open circle, wild-type strain UOT1851. Closed circle, secA341 strain NIG1172. C, kinA-lacZ. Open circle, wild-type strain UOT1851. Closed circle, secA341 strain NIG1171. T4 indicates the hour of incubation. n = 0 at the end of logarithmic phase of growth.
Fig. 2. Effects of the secA341 Mutation at 37°C on the Expression of spo0A Pr, Ps-lacZ, and spo0A Ps-lacZ Fusion Genes of B. subtilis during Sporulation in DS Medium.
A, spo0A Pr, Ps-lacZ. Open circle, wild-type strain NIG1121 (gCMZ1, Pr and Ps). Closed circle, secA341 strain NIG1152 (gCMZ1, Pr and Ps). B, spo0A Ps-lacZ. Open circle, wild-type strain NIG1121 (gCMZ6, Ps). Closed circle, secA341 strain NIG1152 (gCMZ6, Ps). Tn indicates the hour of incubation. n = 0 at the end of logarithmic phase of growth.

Fig. 3. Effects of the secA341 Mutation at 37°C on the Expression of spoVG-bgaB and phrC-bgaB Fusion Genes of B. subtilis during Sporulation in 2 × SG medium.
A, spoVG-bgaB. Open circle, wild-type strain NIG1407. Closed circle, secA341 strain NIG1408. B, phrC-bgaB. Open circle, wild-type strain NIG1405. Closed circle, secA341 strain NIG1406. Tn indicates the hour of incubation. n = 0 at the end of logarithmic phase of growth.

Fig. 4. Effects of the secA341 Mutation at 37°C on the Expression of citGp2-bgaB Fusion Gene of B. subtilis during Sporulation in 2 × SG medium or during Growth in Minimal Glucose Medium.
A, 2 × SG medium. B, Minimal glucose medium. Glucose (0.5%), MnCl2 (20 μM), casamino acids (0.02%), methionine (50 μg/ml), and histidine (50 μg/ml) were added to the minimal salts (36). Open circle, wild-type strain NIG1451. Closed circle, secA341 strain NIG1452. Tn indicates the hour of incubation. n = 0 at the end of logarithmic phase of growth.

Tn. Transcription from this promoter was also inhibited in the secA341 mutant cells at 37°C.

Transcription from the σH-dependent citG promoter (citGp2) (Fig. 4A) was not affected drastically during the early sporulation stage. However, transcription from the citGp2 promoter during growth and the early sporulation stage was more restricted by the secA341 mutation at 37°C when cells were grown in a minimal glucose medium (Fig. 4B).

Expression of spoIIIG (Fig. 5A) and spoIIG (Fig. 5B) was also blocked at 37°C in the secA341 (ts) mutant cells. Transcription from the promoter of the former gene depends on σε and requires phosphorylated Spo0A as an activator while that of the latter gene depends on σH, the efficient expression of which depends on σH and phosphorylated Spo0A.

As transcription from the promoters described above at 30°C in the mutant cells was comparable to that of the wild-type strain, slight inactivation of the mutant SecA341 protein at 37°C would result in defective transcription from σH and/or phosphorylated Spo0A-dependent promoters during the early sporulation stage.

Active sigma H protein was produced in the secA341 mutant cell at 37°C

Since σH-dependent promoters were not transcribed in the secA341 mutant cells at 37°C as described above, we partially purified RNA polymerase holoenzyme containing a histidine-tagged β subunit by a nickel column from wild-type cells and the mutant cells at 37°C and did in vitro transcription of the σε-dependent abdB and σH-dependent spoVG promoters. This RNA polymerase preparation contained σε- and σH-holoenzyme and transcribed both promoters, and the enzyme prepared from the spo0H mutant cells did not transcribe the σH-dependent spoVG promoter (Fujita and Sadaie, unpublished...
Fig. 5. Effects of the secA341 Mutation at 37°C on the Expression of spoIIG-lacZ and spoIIIG-lacZ Fusion Genes of B. subtilis during Sporulation in DS Medium.
A, spoIIG-lacZ. Open circle, wild-type strain NIG1121 (αCMspoIIGlacZ). Closed circle, secA341 strain NIG1152 (αCMspoIIGlacZ). B, spoIIIG-lacZ. Open circle, wild-type strain NIG1121 (αCMspoIIGlacZ). Closed circle, secA341 strain NIG1152 (αCMspoIIGlacZ). T_i indicates the hour of incubation. n = 0 at the end of the logarithmic phase of growth.

Fig. 6. In Vitro Transcription of abrB and spoVG Promoters with Partially Purified RNA Polymerases of B. subtilis.
RNA polymerase was extracted from cells grown in 2 x SG medium at 37°C to the end of the logarithmic phase of growth. A, In vitro transcription of abrB and spoVG promoters with RNA polymerases partially purified through a nickel (Ni^{2+}-nitritol-tri-acetate) column. Reaction mixture contained ATP, GTP, CTP, UTP, [γ-32P]UTP, heparin, RNA polymerase (5 μg), and promoter DNA (0.1 pmol) synthesized by PCR. Transcripts were analyzed by electrophoresis on polyacrylamide gel. Gels were analyzed with a Bioimage-analyser BAS2000 (Fuji Film Co., Ltd, Tokyo). 136-base and 61-base long transcripts were expected for abrB and spoVG, respectively. Lane 1, MW. Lane 2, wild type NIG2001. Lane 3, secA341 strain NIG2003. B, Western blot analysis of σ^H proteins of partially purified RNA polymerases (5 μg) described above. The sample was heat denatured and put on SDS gel electrophoresis. Anti σ^H antibody was custom-made with purified σ^H protein (Fujita and Sadaie, unpublished result). Lane 1, wild-type NIG2001. Lane 2, secA341 strain NIG2003.

result). As shown in Fig. 6, the ratio of the amount of in vitro transcripts, spoVG/abrB, was similar in the wild type and mutant strains (Fig. 6A), and similar amounts of σ^H protein were produced in the wild type and mutant strains (Fig. 6B). Thus, the results obtained clearly indicated that assembly of the core and activity of the σ^H protein were not defective in the secA341 mutant cells at 37°C.

Discussion
The results described in the text indicate that neither the production nor assembly of σ^H-containing RNA polymerase is blocked in the secA341 mutant cell at 37°C. However, the secA341 mutation affected some step(s) or factor(s) required for σ^H-dependent transcription in vivo. The possible defects caused by this mutation are discussed as follows.

Purified SecA protein did not stimulate in vitro transcription of the spoVG promoter with Eσ^H RNA polymerase (data not shown). Some signals or activators produced during the early sporulation stage and required for transcription from σ^H-dependent, early expressing kinA, spo0A(Ps), spoVG, and phrC promoters may be missing in the secA341 mutant cell at 37°C, or some negative regulatory factor(s) such as SinR^{H} may not be eliminated. These signals or factor(s) may be lost during preparation of RNA polymerase. Extracellular AprE expression was also repressed in the secA341 mutant cell during the early sporulation stage and its repression was at least partially due to the defective expression of some positive factors such as DegR the expression of which depends on σ^D, a sigma factor required also for the expression of flagellar protein gene. In this case, disrup-
tion of the anti-σ^D gene restored degR expression in the secA341 mutant, suggesting that anti-σ^D was not diluted in the mutant cell (Sadaie, Biosci. Biotechnol. Biochem. in press).

As the citGp2 promoter is transcribed by the σ^H-containing RNA polymerase even during growth, depending on the medium composition or when the TCA cycle enzyme activity is high, this promoter may be regulated differently from other σ^H-dependent promoters described above although a typical σ^H consensus −35 and −10 sequence occurs in the citGp2 promoter.\(^{(15)}\) This may be the reason why bgaB expression from the citGp2-bgaB fusion genes in the secA341 mutant cells at 37°C was not affected strongly during sporulation in a complex medium, but was affected during growth and early sporulation stage in a minimal medium. This may suggest that the activators or inhibitors postulated above are not produced or eliminated even during growth in the mutant cell at 37°C. Defective activity of the TCA cycle may cause defective sporulation\(^{(49)}\) in the secA341 mutant cell at 37°C.

The newly isolated secA12 mutation\(^{(20)}\) also arrested σ^H-dependent expression without any defects in σ^H protein accumulation. The secA341 and secA12 are P431L and S515L mutations, respectively, located in conserved regions between B. subtilis and E. coli SecA; the latter location corresponds to the low affinity ATP binding region of E. coli SecA.\(^{(20)}\) Mutated SecA341 or SecA12 proteins may both interfere with σ^H (or σ^D) protein in vivo, but this may not be the case, as both mutations were located in different positions, and the div-341 mutant protein was more unstable than wild type protein in vivo, probably because of increased susceptibility to protease.\(^{(26)}\)

Defective (50%) expression of the rapA/prhA operon\(^{(33)}\) measured as bgaB activity of a rapA-bgaB transcriptional fusion (data not shown) in the secA341 mutant cell at 37°C may be one of the reasons why sporulation signals are missing in the mutant cells. RapA is the phosphatase of Spo0F-P and PrhA is the extracellular inhibitor of RapA. Transcription of the rapA/prhA operon is presumably σ^D dependent, and requires phosphorylated ComA mediated by the competence-stimulating factor, which is a C-terminal pentapeptide of extracellular PhrC. The expression of prhC is σ^H-dependent and is missing in the secA341 mutant strain as described in the text. Defective prhC expression may be one of the reasons for poor sporulation and poor competence of the secA341 mutant strain\(^{(27,28)}\) at 37°C through defective activation of ComA protein.\(^{(11,12)}\)

The phosphorylated form of the Spo0A protein must be produced in a small but sufficient amount to repress the abrB gene in the secA341 (ts) mutant cells at 37°C, since the repression of abrB expression and the induction of spo0H expression were observed in the secA341 mutant cell at 37°C. On the other hand, the production of large amounts of Spo0A and hence its phosphorylated form depending on σ^H activity through increased expression of the component genes of the phosphorelay such as kinA and spo0A (Ps) must be blocked in the mutant cell at 37°C. As the induction of spoIIG expression at around T_1 occurred one hour later than the repression of abrB expression at around T_0, the phosphorylated form of Spo0A protein may be required in a great amount for activation of the spoIIG promoter. This may be the reason why phosphorylated Spo0A dependent spoIIG expression was inhibited in the mutant cell at 37°C.

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References

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