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Deficiency of the Initiation Events of Sporulation in *Bacillus subtilis clpP* Mutant Can Be Suppressed by a Lack of the Spo0E Protein Phosphatase

Hideaki Nanamiya,* Kouki Takahashi,* Masaya Fujita,† and Fujio Kawamura*,[1]

*Laboratory of Molecular Genetics, College of Science, Rikkyo (St. Paul's) University, Toshima-ku, Tokyo 171-8501, Japan; and †Department of Molecular and Cellular Biology, The Biological Laboratories, Harvard University, 16 Divinity Avenue, Cambridge, Massachusetts 02138

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Previous results have shown that the *Bacillus subtilis clpP* gene is required for developmental processes such as sporulation and competence development. Little is known about its function during the initiation of sporulation. We studied the effect of *clpP* mutation on the early events of sporulation. The expression of the *spo0A* and *spoIIG* genes, whose active transcription requires the phosphorylated Spo0A protein (Spo0A~P) as the transcription activator, was significantly decreased in the *clpP* mutant at the onset of sporulation. The expression of *spo0H* gene encoding σ^H protein was also greatly reduced. As expected from these results, the σ^H and Spo0A protein levels in the *clpP* mutant were also decreased during the initiation of sporulation, indicating that the accumulation of Spo0A~P was inhibited in the *clpP* mutant. We, therefore, introduced the mutation of the *spo0E* gene, which codes for the Spo0A~P-specific phosphatase, into the *clpP* mutant and found that this double mutant restored the expression of the *spo0A* as well as *spoIIG* genes. These results suggest that ClpP had an indirect influence on the intracellular concentration of Spo0A~P by regulating the activity of the Spo0E phosphatase during the initiation of sporulation.

***Key Words: Bacillus subtilis;* sporulation; cell differentiation; Clp protease; *clpP;* Spo0A; Spo0E phosphatase; σ^H.**

Sporulation in *Bacillus subtilis* is one of the pathways to cope with various growth-limiting conditions at the end of exponential growth (1, 2). At the initiation of sporulation, Spo0A protein is converted to the phosphorylated Spo0A protein (Spo0A~P) through the phosphorelay signal transduction system (3, 4) and acts as a positive, as well as a negative, transcriptional regulator on several early sporulation and sporulation-associated genes including *kinA*, *abrB*, *spo0F*, *spoIIA*, *spoIIG*, and *spo0A* itself (4–6). Among them, *kinA*, *spo0F* and *spo0A* are transcribed by σ^H containing RNA polymerase holo enzyme (7). σ^H is the sporulation initiation sigma factor encoded by the *spo0H* gene (8). It has been reported that the σ^H level began to increase at the initiation of sporulation and that the half-life of σ^H turnover increased several folds during sporulation compared with that during exponential growth (9). It has been recently shown that the accumulation of σ^H protein occurred to an appreciable level in a *spo0A* mutant despite the decrease in *spo0H* transcription at the onset of sporulation (10). These findings indicate that the increased level of σ^H activity at the onset of sporulation is mainly due to the regulation of a post-translational event. However, the detailed mechanism of σ^H regulation during spore development has remained unclear.

The Clp/Hsp100 proteins are highly conserved among eukaryotes and prokaryotes (11, 12). It has been shown that Clp proteins can either function as proteolysis regulators for the Clp protease, which comprises the ClpP proteolytic component (13) and regulatory ATPase component, or as molecular chaperones (14, 15). In the *B. subtilis* genome, three genes, *clpC*, *clpE*, and *clpX*, coding for ClpC, ClpE, and ClpX proteins, and *clpP* coding for ClpP protein have been identified (16). Our previous results have shown that ClpC contributes to σ^H instability at high temperature and in the late stages of sporulation (17). Recently, it has been shown that ClpX is essential for the induction of σ^H-dependent gene transcription *in vivo* (18). It has been also shown that *clpP* mutation severely inhibited spore development (19, 20). Taking these findings into consideration, we studied the effect of the *clpP* mutation on the regulation of σ^H protein during the initiation of sporulation. In this report, we describe the novel

[1] To whom correspondence and reprint requests should be addressed. Fax: 81-3-3985-2401. E-mail: kawamura@rikkyo.ne.jp.

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TABLE 1

Bacillus subtilis Strains Used in This Study

Strain	Relevant genotype	Source or reference
UOT1285	*trpC2 lys1 aprEΔ3 nprR2 nprE18*	Laboratory stock
RIK10	*trpC2 lys1 aprEΔ3 nprR2 nprE18 amyE::spo0A-bgaB cat*	(17)
RIK50	*trpC2 lys1 aprEΔ3 nprR2 nprE18 amyE::spo0H-bgaB cat*	(17)
RIK75	*trpC2 lys1 aprEΔ3 nprR2 nprE18 amyE::spoIIG-bgaB cat*	This study
RIK731	*trpC2 lys1 aprEΔ3 nprR2 nprE18 clpP::spc*	This study
RIK732	RIK 731 *amyE::spo0A-bgaB cat*	This study
RIK733	RIK 731 *amyE::spo0H-bgaB cat*	This study
RIK735	RIK 731 *amyE::spoIIG-bgaB cat*	This study
RIK737	*trpC2 lys1 aprEΔ3 nprR2 nprE18 spo0E::erm*	This study
RIK738	RIK 737 *amyE::spo0A-bgaB cat*	This study
RIK739	RIK 737 *amyE::spo0H-bgaB cat*	This study
RIK740	RIK 737 *amyE::spoIIG-bgaB cat*	This study
RIK741	*trpC2 lys1 aprEΔ3 nprR2 nprE18 spo0E::erm clpP::spc*	This study
RIK742	RIK 741 *amyE::spo0A-bgaB cat*	This study
RIK743	RIK 741 *amyE::spo0H-bgaB cat*	This study
RIK744	RIK 741 *amyE::spoIIG-bgaB cat*	This study

findings of the ClpP function that controls the negative regulator of Spo0A~P, Spo0E phosphatase, at the onset of sporulation.

MATERIALS AND METHODS

Bacterial strain. The *Bacillus subtilis* strains used in this study are listed in Table 1. All strains are isogenic with UOT1285 (*trpC2 lys1 aprEΔ3 nprR2 nprE18).* RIK731 carrying *clpP* disrupted mutation was constructed as follows. Oligonucleotide primers are used to amplify the *clpP* gene by the PCR procedure. The upstream and downstream primers were CPF (5′-TTCACCGGATCCGGTGAAGGAGGAGCATTATG-3′) and CPR (5′-GATAACGGATCCGTTATCACGGTCTGTGTCGC-3′), respectively. The underlined sequences denote *Bam*HI restriction sites. The fragment obtained by PCR was digested with *Bam*HI and inserted into the plasmid pCP112 (21). The recombinant plasmid, named pRIK710, contains a *Sma*I restriction site in the cloned *clpP* gene. A spectinomycin resistance cassette, a derivative of pBEST517A (22), was inserted into the *Sma*I site of the pRIK710. The resulting plasmid, pRIK711, was linearized by *Sca*I and integrated into the *clpP* site of the chromosome of *B. subtilis* UOT1285 by a double-crossover recombination and selection for spectinomycin resistance (Spc^r). Proper integration was confirmed by PCR amplification and by checking the phenotype that cannot grow at over 50°C (19, 20). RIK737 carrying the *spo0E* disrupted mutation was constructed as follows. The upstream and downstream regions of the *spo0E* gene were amplified by PCR procedures with the primers OEUF (5′-CAGCGAATTCGCTGCATCGTCAATGAATCC-3′) and OEUR (5′-CACGCCCGGGCGTGTCATGCCCTGTAAATCC-3′) for the upstream region or OEDF (5′-TGCGCCCGGGCGCAGAGATGTTATGCCAGC-3′) and OEDR (5′-TGCCAAGCTTGGCATTGCCATTCGATTTGC-3′) for the downstream region, respectively. The underlined sequences denote *Eco*RI and *Sma*I restriction sites for the upstream fragment, or *Sma*I and *Hin*dIII restriction sites for the downstream fragment, respectively. These fragments obtained by PCR were cut with *Eco*RI and *Sma*I for the upstream fragment, or *Sma*I and *Hin*dIII for the downstream fragment respectively, and ligated simultaneously with pUC18 *Eco*RI/*Hin*dIII digest. The resulting plasmid, pRIK0Ed, contained the 999-bp DNA fragments derived from the *spo0E* region, but 114 bp within the *spo0E* gene (between 106 and 210 bp from the start codon) were deleted. This plasmid was cleaved with *Sma*I and ligated with the erythromycin resistance cassette derived from pAE41 (23) *Pvu*II digestion. The recombinant plasmid, pRIK0Ee, was linearized by *Sca*I and integrated into the *spo0E* site of the chromosome of *B. subtilis* UOT1285 by a double-crossover recombination and selection for erythromycin resistance (Em^r). Proper integration was confirmed by PCR amplification. Transcriptional *bgaB* fusion to *spoIIG* was constructed by cloning *Eco*RI-*Bam*HI-digested PCR fragment amplified by means of primers IIGPF (5′-CCGGAATTCCTTCCTCGACAAATTAAGCAG-3′) and IIGPR (5′-CGCGGATCCCTTTCTTTCTTGCCTCACGC-3′), into pDLd (17). The recombinant plasmid was linearized by *Pst*I and transformed into *B. subtilis* 168 for integration at the *amyE* site of the chromosome by a double-crossover recombination. Proper integration was confirmed by monitoring Amy^- phenotype and by PCR amplification. Chromosomal DNA was extracted from the strain and was used to transform UOT1285 to construct RIK75. Chromosomal DNA extracted from RIK731 was used to transform RIK10 (*amyE::spo0A-bgaB cat*), RIK50 (*amyE::spo0H-bgaB cat*), and RIK75 (*amyE::spoIIG-bgaB cat*) to obtain RIK732 (*clpP::spc amyE::spo0A*Ps-*bgaB cat*), RIK733 (*clpP::spc amyE::spo0H-bgaB cat*), and RIK735 (*clpP::spc amyE::spoIIG-bgaB cat*) by Cm^r Spc^r phenotype. Strains RIK738 (*spo0E::erm amyE::spo0A*Ps-*bgaB cat*), RIK739 (*spo0E::erm amyE::spo0H-bgaB cat*), and RIK740 (*spo0E::erm amyE::spoIIG-bgaB cat*) were constructed in a similar way by using the chromosomal DNA extracted from RIK737 and selection for Cm^r Em^r phenotype. Chromosomal DNA extracted from RIK731 was used to transform RIK738, RIK739 and RIK740 to obtain RIK742 (*clpP::spc spo0E::erm amyE::spo0A*Ps-*bgaB cat*), RIK743 (*clpP::spc spo0E::erm amyE::spo0H-bgaB cat*) and RIK744 (*clpP::spc spo0E::erm amyE::spoIIG-bgaB cat*) by Cm^r Spc^r Em^r phenotype.

Media. For sporulation medium, two strengths Schaeffer's sporulation medium supplemented with 0.1% glucose (24), 2xSG, was used. Chloramphenicol (5 μg/ml), spectinomycin (100 μg/ml), and erythromycin (0.5 μg/ml) was added as required. Solid medium consisted of Luria broth (LB) (25) agar supplemented with antibiotics as required.

Assay of β-galactosidase activity. The expression of the *bgaB-spo0A*Ps, *-spo0H*, and *-spoIIG* fusions was monitored by following the thermostable β-galactosidase (BgaB) activity (26). Cells were grown in 2xSG medium and aliquots of 0.5 to 1.5 ml were collected at the indicated times for the assay of thermostable β-galactosidase activity as described previously (17). One unit of BgaB activity was expressed as $1000 \times ABS_{420\ nm}/OD_{660\ nm}$/ml/min as described previously (17).

Western blot analysis. Cells were grown in the 2xSG medium and samples of 1.5 to 4 ml were taken at the indicated times and centrifuged

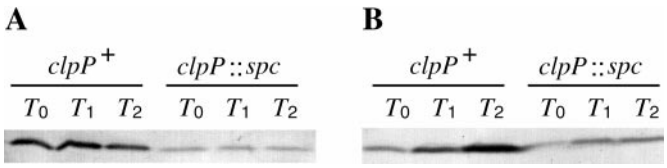

FIG. 1. Western blot analysis. (A) σ^H protein and (B) Spo0A protein in wild-type and *clpP* mutant strains during sporulation at 37°C. The cells were grown in 2xSG at 37°C and collected at the time (expressed in hours) before or after the end of exponential growth, which is designated as *T*0. Western blotting was performed as described under Materials and Methods.

to collect cells. Cell pellets were frozen and stored at −70°C. Pellets were resuspended with ice cold buffer (10 mM Tris–HCl pH 8.0, 1 mM EDTA, 10% glycerol, 0.3 mM dithiothreitol [DTT], 2 mM phenylmethylsulfonyl fluoride [PMSF], and 0.1 mg/ml of Lysozyme) and disrupted by sonication on ice. 15 μg of the cell extracts, whose concentrations were determined by the Bio-Rad protein assay (Bio-Rad), were electrophoresed through sodium dodecyl sulfate (SDS)–12.5% (w/v) polyacrylamide minigels. The proteins were transferred to polyvinylidene difluoride (PVDF) membrane (Millipore), and immunodetection procedures were carried out as previously described (10) whichever the cases using anti-σ^H antibody (10), anti-Spo0A antibody (17).

RESULTS AND DISCUSSION

Effects of the clpP Mutation on the Accumulation of σ^H Protein

To investigate the ClpP function during sporulation, we constructed *clpP* disruptant (RIK731: *trpC2 lys1 aprEΔ3 nprR2 nprE18 clpP::spc)* as described under Materials and Methods and studied its effect on the level of the σ^H protein during the initiation of sporulation by Western blot analysis. As shown in Fig. 1A, σ^H protein was rapidly increased at *T*1, 1 h after the end of the exponential growth, in the wild type strain UOT1285 (*trpC2 lys1 aprEΔ3 nprR2 nprE18*), which has been used as the wild-type strain in our laboratory. In contrast, the σ^H level was extremely low in the *clpP* disruptant during the early stages of sporulation (Fig. 1A). It has been shown that the σ^H level in the *clpC* disruptant was comparable to that in the wild type (17). It is thus most likely that ClpP would be involved in regulating the intracellular level of σ^H protein in concert with the other Clp protein(s) except for ClpC. We next examined the effect of *clpP* disrupted mutation on the expression of the *spo0H* gene by monitoring the *spo0H-bgaB* directed thermostable β-galactosidase activity. As shown in Fig. 2A, the expression of the *spo0H* gene was greatly reduced in the *clpP* disruptant compared with that in the wild type. These results suggest that extremely low expression of the *spo0H* gene would cause the low level of the σ^H protein in the *clpP* mutant at the onset of sporulation.

Effects of the clpP Mutation on the Accumulation of Spo0A Protein

Transcription of the *spo0H* gene, directed by σ^A-containing RNA polymerase holo enzyme, is repressed by a DNA-binding protein, AbrB, during vegetative growth and increases during the early stages of sporulation, at which time the *abrB* gene is repressed by the

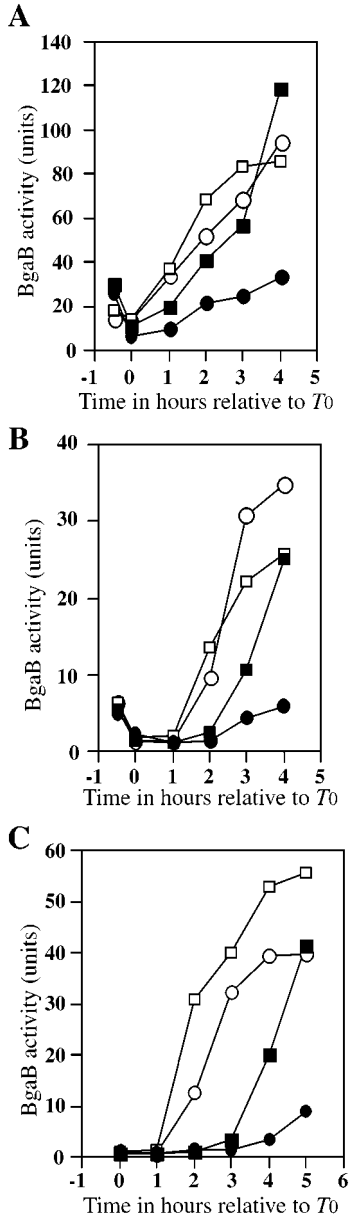

FIG. 2. Expression of the various early sporulation genes in the *clpP, spo0E,* and *clpP spo0E* double mutant. Wild-type and these mutants carrying the indicated bgaB fusions were grown in 2xSG medium with shaking at 37°C. Cells were collected at the indicated time (expressed in hours) before or after the end of exponential growth, which is designated as *T*0. (A) Expression of *spo0H-bgaB* directed thermostable β-galactosidase. Symbols: ○, RIK50 (wild type); □, RIK739 (*spo0E::erm*); ●, RIK733 (*clpP::spc*); ■, RIK743 (*clpP::spc spo0E::erm*). (B) Expression of *spo0A*Ps-*bgaB* directed thermostable β-galactosidase. Symbols: ○, RIK10 (wild type); □, RIK738 (*spo0E::erm*); ●, RIK732 (*clpP::spc*); ■, RIK742 (*clpP::spc spo0E::erm*). (C) Expression of *spoIIG-bgaB* directed thermostable β-galactosidase. Symbols: ○, RIK75 (wild type); □, RIK740 (*spo0E::erm*); ●, RIK735 (*clpP::spc*); ■, RIK744 (*clpP::spc spo0E::erm*).

increased level of the Spo0A~P in the cell (8, 27–29). Moreover, transcription of the *spo0A* gene from the Ps promoter during early stages of sporulation is dependent on Spo0A~P itself as well as the σ^H-containing RNA polymerase holo enzyme (7, 30). We therefore next examined the levels of Spo0A and the expression of *spo0A*Ps-*bgaB* in the *clpP* mutant during the initiation of sporulation. The expression of the *spo0A* gene from the Ps promoter and the *spoIIG* gene, both require the Spo0A~P as the transcription activator, did not increase in the *clpP* mutant at the onset of sporulation, nor did the Spo0A level (Figs. 1B, 2B, and 2C). These results indicated that the intracellular concentration of Spo0A~P was extremely low in the *clpP* mutant. There are, at least, two possibilities that the phosphorelay system, which generates Spo0A~P, does not sufficiently operate in the *clpP* mutant or that Spo0A~P is destabilized, degraded and/or dephosphorylated, in the *clpP* mutant. To examine the first possibility, we introduced the *sof-1* mutation, which is the allelic gene of *spo0A* (31, 32), into the *clpP* mutant and found that the expression of *spo0A* from the Ps promoter was not significantly induced in the *clpP sof-1* double mutant (data not shown). Since altered Spo0A protein in the *sof-1* mutant can be phosphorylated without the phosphorelay function (33, 34), this result strongly suggested that ClpP was involved in the stabilization of Spo0A~P by presumably protecting it from degradation and/or dephosphorylation during the early stages of sporulation.

spo0E Mutation Could Bypass the Spo0A~P-Dependent Gene Expression in the clpP Mutant

It has been reported that phosphorylated Spo0A protein is negatively regulated by the Spo0E protein (35, 36). Spo0E protein is identified as a phosphatase that can dephosphorylate Spo0A~P specifically (35). It is thus possible that ClpP might increase the Spo0A~P level by controlling the Spo0E activity during the initiation of sporulation. To test the possibility, we monitored the expression of the *spo0A*Ps-, *spoIIG*-directed BgaB activity in the *clpP spo0E* double mutant as described under Materials and Methods. As shown in Figs. 2B and 2C, the expression of both genes was clearly restored in the *clpP spo0E* double mutant whereas their induction times were delayed compared with those in the wild type. Moreover, the expression of the *spo0H* gene was also induced in the *clpP spo0E* double mutant (Fig. 2A). These results support the idea that ClpP would play an important role for the Spo0A~P activity by regulating the Spo0E function. To examine whether the *clpP* mutation affects the Spo0E function at the transcription level or at the posttranscription level, we examined the expression of the *spo0E* gene by monitoring the *spo0E-bgaB* directed thermostable β-galactosidase. The expression level and pattern of the *spo0E* gene in the *clpP* mutant was almost the same as that in the wild type (data not shown). It is, thus, most likely that ClpP is involved in the regulation of Spo0E protein at the posttranscription level.

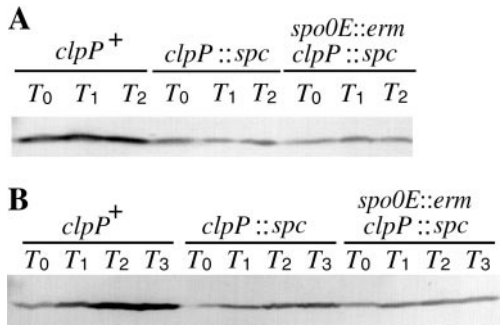

FIG. 3. Western blot analysis. (A) σ^H protein and (B) Spo0A protein in wild-type and *clpP* mutant strains during sporulation at 37°C. Experimental procedures were performed as described in the legend to Fig. 1.

As shown in Figs. 3A and 3B, the intracellular concentration of both Spo0A protein and σ^H protein remarkably could not be restored in the *clpP spo0E* double mutant. Recently, Zuber *et al.* made several important reports on the post-translational regulation of σ^H during the early stages of sporulation (18, 37). They found that the concentration and activity of σ^H were negatively affected by low environmental pH conditions (37). They also found that the ATP dependent proteases LonA and LonB and the regulatory ATPase ClpX functioned in the posttranslational regulation of σ^H (18). It is thus suggested that the overexpression of these genes might be caused in the *clpP* background and result in the low levels of σ^H and Spo0A. Moreover, the *clpP spo0E* double mutant could not sporulate at all (data not shown), like the *clpP* mutant as previously reported by Msadek *et al.* (19). The disruption of the *spo0E* gene can restore only the early events of sporulation in the *clpP* mutant but cannot restore its ability to sporulate, indicating that ClpP function is essential for not only early but also late stages of sporulation.

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