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Effect of *degS-degU* Mutations on the Expression of *sigD*, Encoding an Alternative Sigma Factor, and Autolysin Operon of *Bacillus subtilis*

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Primer extension analysis of transcripts of the *Bacillus subtilis* autolysin (*cwlB*) operon indicated that SigD-dependent transcripts from the Pd promoter are missing in the *degU32*(Hy) and *degS200*(Hy) mutants. The *degU32*(Hy) mutation caused a 99% reduction in the expression of a *sigD-lacZ* translational fusion gene constructed in the *B. subtilis* chromosome. The phosphorylated form of the DegU protein seems to be a regulator for expression of the *sigD* gene.

Bacillus subtilis produces two major autolysins (*N*-acetylmuramoyl-L-alanine amidase and endo-β-*N*-acetylglucosaminidase) during the vegetative growth phase (7, 8, 12, 28). A gene encoding the former protein (CwlB) has been cloned by us and others (15, 18), and it is the third gene in an operon consisting of three genes which encode a putative lipoprotein (LppX), a modifier protein (CwbA), and CwlB, in that order (14, 15, 18). Transcription of the *cwlB* operon mainly depends on expression of the SigD protein, which is responsible for cell motility and chemotaxis (16, 20). Recently, we cloned a *cwlG* gene encoding an endo-β-*N*-acetylglucosaminidase and constructed a double mutant deficient in the two above-mentioned autolysins. The double mutant exhibited greatly impaired motility on a swarm plate, whereas single mutants were motile (27). Margot et al. have suggested that *cwlG* (*lytD*) is also transcribed by the SigD form of RNA polymerase (19).

One of the pleiotropic genes, *sin(flaD)*, is involved in the control of many late-growth developmental processes (32). The *sin(flaD)* mutation results in an increase in alkaline protease, a filamentous cell morphology, poor development of competence, loss of motility, and a decreased level of autolysin (30, 32). We previously focused on the positive function of *sin(flaD)* in autolysin production (16, 30). A mutation in the C-terminal region of Sin(SinR, FlaD) (3) significantly decreased the expression level of the *sigD* gene (16). Primer extension analysis indicated that transcription from the sigma D promoter (Pd) of the *cwlB* operon does not occur in the case of the *sin(flaD1)* mutant. Moreover, transcription from the sigma A promoter (Pa) also did not occur (16).

The *degS* and *degU* genes form an operon encoding a two-component system (11, 13, 23, 24, 33). Two classes of mutation that have been found in both the *degS* and *degU* genes lead either to a deficiency of degradative enzyme production or a pleiotropic Hy phenotype, which includes hyperproduction of degradative enzymes, the ability to sporulate in the presence of glucose, poor development of competence, a filamentous cell morphology, and loss of flagella and motility (2, 11, 23, 31, 33). The DegU response regulator appears to have two active conformations regulated by DegS protein kinase. A phosphorylated form of DegU is necessary for degradative enzyme production and appears to act as a repressor of essential components of the competence development pathway encoded by the *srf* operon (5, 9, 24). A nonphosphorylated form of DegU is also required for the development of competence, i.e., late competence genes such as *comG* (6, 29). It has been suggested that the *degU*(Hy) allele may regulate some step(s) upstream from *hag* (structural gene for flagellin) (22) and *degR* (accessory regulatory peptide for DegS-DegU) (26, 34, 35). We report here that the *degU*(Hy) mutation depresses the expression of the *sigD-lacZ* fusion gene and prevents the SigD-dependent transcription of the *cwlB* operon and the phosphorylated form of the DegU protein may regulate the level of the SigD protein.

To determine whether the *cwlB* operon is transcribed in *B. subtilis* 327UH, 327SH, 327UN, and 327SN carrying the *degU32*(Hy), *degS200*(Hy), *degU146*, and *degS42* mutations, respectively (Table 1), we performed primer extension analysis of the *cwlB* operon transcripts as described previously (16), except for the use of ISOGEN (Nippon Gene) for the isolation of RNA (Fig. 1). Pd and relatively weak Pa transcripts were detected during the late stage of and at the end of the exponential growth phase (T_{-1} and T_0, respectively) (Fig. 1) but not during the stationary phase for the wild-type AC327 strain (16). At T_{-1} and T_0, transcript activity from the Pd promoter was undetectable in the *degU32*(Hy) and *degS200*(Hy) mutants and slightly decreased in the *degU146* and *degS42* mutants, whereas the Pa transcript activity (probably transcribed by E-σ^A) was approximately the same as that in the wild type, AC327. Therefore, the DegS-DegU proteins, as well as the SigD protein (16), mainly control the Pd transcription. On the other hand, both the Pd and Pa transcripts were missing in the *sin(flaD1)* mutant (16). Therefore, the DegS-DegU regulatory protein seems to be involved in a different pathway controlling the *cwlB* transcripts from Sin(FlaD). Previously, we demonstrated that the *sin(flaD1)* mutation interferes with transcription from the Pd promoter by depressing the expression of *sigD* (16). To determine whether this is also the case with the *degU* mutants, we measured the expression of the *sigD* gene in the *degU* mutants by introducing the *degU* mutations into AC327SL having a *sigD-lacZ* fusion gene in its chromosome. The β-galactosidase activities of *B. subtilis* AC327SL, 327UNSL, and 327UHSL (Table 1) are shown in Fig. 2. The *degU32*(Hy) mutation caused a 99%

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TABLE 1. *B. subtilis* strains

Strain	Genotype	Source or reference
AC327	*purB his-1 smo-1*	1, 16
AC327SL	*purB his-1 smo-1 sigD*::pSD3 (*sigD-lacZ cat*)	16
QB4414	*degU146 trpC2*	F. Kunst, 4
1A165	*degU32*(Hy) *trpC2*	BGSC[a]
1A199	*degS200*(Hy) *leuA8 trpC2*	BGSC (23)
1A201	*degS42 hisA1 trpC2 sacA321*	BGSC (23)
327UN	*degU146 purB smo-1*	QB4414→AC327[b]
327UH	*degU32*(Hy) *his-1 smo-1*	1A165→AC327
327SN	*degS42 purB smo-1*	1A201→AC327
327SH	*degS200*(Hy) *his-1 smo-1*	1A199→AC327
327UNSL	*degU146 purB smo-1 sigD*::pSD3 (*sigD-lacZ cat*)	QB4414→AC327SL
327UHSL	*degU32*(Hy) *purB smo-1 sigD*::pSD3 (*sigD-lacZ cat*)	1A165→AC327SL
327UDSL	Δ*degU*::*tet his-1 purB smo-1 sigD*::pSD3 (*sigD-lacZ cat*)	This study

[a] BGSC, Bacillus Genetic Stock Center, The Ohio State University.

[b] Arrows indicate construction by transformation. *degU146*, *degS42*, and Δ*degU*::*tet* were confirmed by the decreased level of extracellular protease and levansucrase, as judged from the lack of opaque halos around colonies growing on Luria-Bertani (LB) plates containing 1% casein (37°C, 16 h) and from the lack of levan production on growth on LB plates containing 4% sucrose (37°C, 24 h), respectively (17). *degU32*(Hy) and *degS200*(Hy) were confirmed by the overproduction of extracellular proteases (17).

reduction in the expression of a *sigD-lacZ* translational fusion gene. This suggests that the *degU32*(Hy) mutation may depress the Pd transcription of the *cwlB* operon by causing a decrease in the SigD protein. However, no biochemical evidence for specific binding of DegU to target sites yet exists; thus, it remains possible that the DegU protein directly regulates the Pd transcription of the *cwlB* operon.

To determine whether the phosphorylated form of DegU reduces the expression of the SigD protein or whether the unphosphorylated form of DegU is necessary for the expression of the SigD protein, we constructed an in vitro-derived deletion mutant of *degU* as follows. A 2.4-kb *Eco*RI fragment containing *degS* and truncated *degU* genes was isolated from pDH50 (Bacillus Genetic Stock Center) and then inserted into the *Eco*RI site of pUC119, the resultant plasmid being designated pUC50. A 1.5-kb blunt-ended *Eco*RI-*Hin*dIII fragment containing a *tet* (tetracycline resistant) gene from pUCTC (27) was inserted into the *Eco*RV site of pUC50, which is located 169 bp downstream of the 5′ terminus of the *degU* gene (11). The resultant plasmid, pEV3, was linearized with *Hin*dIII and then used to transform *B. subtilis* 327SL, with Tc^r transformants being selected. A transformant, designated 327UDSL, exhibited a deficiency in degradative enzyme production (17). This phenotype is as expected for a *degU* deletion mutant (23, 33). Although deletion of the *degU* gene has been believed to have no effect on the *sigD*-dependent function (24), we show here that it decreases *sigD* expression by two-thirds. The *degU146* mutation (deficiency in DegU phosphorylation) (4, 23) also decreases *sigD* expression by two-thirds. From this point, the phosphorylated form of DegU seems to be a weak positive regulator for *sigD* expression. Moreover, the result is supported by the reduced amounts of Pd transcripts in the *degU146* and *degS42* mutants (Fig. 1).

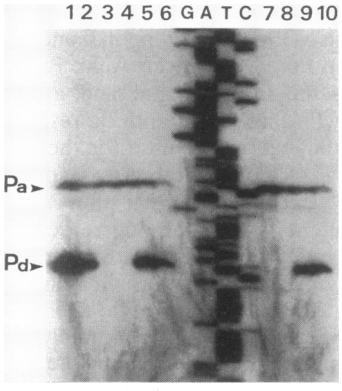

FIG. 1. Primer extension analysis of *cwlB* operon transcripts in *degS-degU* mutants. The 5′-end-labeled primer, which is complementary to the 5′ region of the *cwlB* operon, and the conditions for the extension with reverse transcriptase and electrophoresis have been described previously (16). Lanes: G, A, T, and C, four reactions in the M13 sequencing with the same primer (16); 1 and 2, 3 and 4, 5 and 6, 7 and 8, and 9 and 10, RNAs (20 μg each) from *B. subtilis* AC327, 327UH[*degU32*(Hy)], 327UN[*degU146*], 327SH[*degS200*(Hy)], and 327SN[*degS42*], respectively. Odd- and even-numbered lanes, RNAs from cells at T_{-1} and T_0, respectively. The transcriptional start sites of Pa and Pd promoters are indicated by arrowheads. Autoradiography was performed for 63 h.

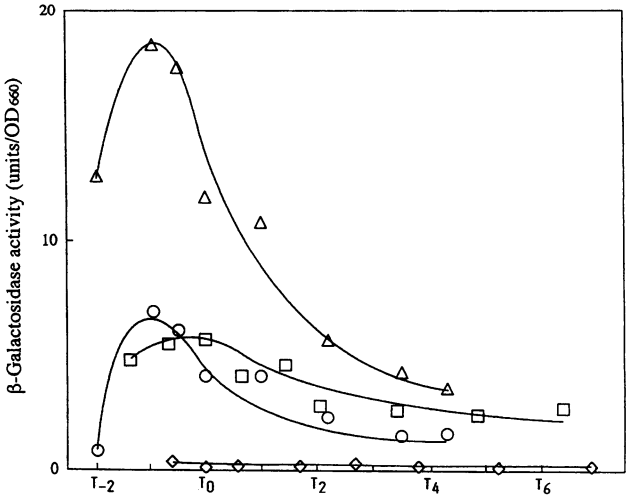

FIG. 2. Effects of *degU* mutations on production of *sigD-lacZ* translational fusion proteins. The *sigD*-directed β-galactosidase activity was determined at the indicated times relative to the end of the exponential growth phase (T_0) in *B. subtilis* AC327SL(*sigD-lacZ*) (△), 327UNSL(*degU146 sigD-lacZ*) (□), 327UHSL[*degU32*(Hy) *sigD-lacZ*] (◇), and 327UDSL(Δ*degU sigD-lacZ*) (○). OD_{660}, optical density at 660 nm.

Márquez et al. (20) and Márquez-Magana and Chamberlin (21) reported that *sigD* may be part of a large operon containing several genes upstream of the *sigD* gene, and they showed that an upstream integrant in the *sigD* locus produced 7% of the wild-type level of the SigD protein and exhibited the synthesis of 64% of the wild-type flagellin and a decrease in swarm size (55% ± 10% of the wild-type level). Thus, the disruption of the *degU* gene and deficiency in DegU phosphorylation may not significantly affect motility, as other investigators had noted (24). On the contrary, the *degU32*(Hy) mutation leads to accumulation of the phosphorylated form of DegU on account of the lower rate of dephosphorylation of DegU than

that of the wild-type DegU protein, the half-life being 120 min rather than 18 min (4), and it dramatically reduced *sigD* expression. In this case, the phosphorylated form of DegU seems to be a negative regulator for the *sigD* expression. Although it is unknown whether the phosphorylated form of DegU directly acts as a repressor of the *sigD* gene, the phosphorylated form of DegU is more likely to be a negative regulator for *sigD* expression. The *degS-degU* two-component system may be very complicated because of the presence of possible crosstalking modulators (24).

DegR is a 60-amino-acid protein which enhances the production of degradative enzymes when overproduced (26, 35). Nagami and Tanaka found that DegR-overproducing cells exhibited cellular filamentation which may be accompanied by a decreased autolysin level in *B. subtilis* (26). The effect of DegR on the production of extracellular proteases is due to stabilization of the phosphorylated form of DegU (25). A sequence very similar to those of promoters recognized by E-σ^D was observed upstream of the transcription start site of the *degR* gene (26), and in vitro transcription studies showed that *degR* was a good template for E-σ^D (10, 32). The *degU32*(Hy) mutation prevents *degR* expression (24). Assuming that E-σ^D transcribes *degR* in vivo, the accumulation of the phosphorylated form of DegU might affect the *degR* expression through the level of the SigD protein.

It is known that *sin(flaD)* is one of the transition state regulators (32). Once cells are committed to sporulate, SinI counters the action of SinR at the posttranslational level (3) thereby causes a reduced level of SinR, but, on the contrary, competence genes and SigD-dependent functions, including autolysin production, are repressed (16, 32). Similarly, the DegS-DegU regulatory pair could be involved in the choice between producing degradative enzymes and expressing competence and SigD-dependent genes.

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