

Transcription of *Bacillus subtilis* *degR* Is σ^D Dependent and Suppressed by Multicopy *proB* through σ^D

MITSUO OGURA AND TERUO TANAKA*

School of Marine Science and Technology, Tokai University, Shimizu, Japan

Received 14 September 1995/Accepted 31 October 1995

Production of *Bacillus subtilis* exoproteases is positively regulated by the DegS-DegU two-component regulatory system and other regulatory factors including DegR and ProB. It was shown that the expression of *degR* was virtually abolished in a *sigD* mutant and that the transcriptional initiation site in vivo is preceded by a sequence very similar to the consensus sequence of σ^D -recognized promoters. Alteration of the -10 sequence of the putative promoter greatly reduced the expression of *degR*. These results show that *degR* expression is driven by the alternative sigma factor, σ^D . It was found that *degR* expression was suppressed by multiple copies of *proB* on plasmid pLC1 and that this suppression was exerted at the transcriptional level through a target in the vicinity of the *degR* promoter. Furthermore, it was shown that the expression of another σ^D -directed gene, *hag*, was suppressed by pLC1. Suppression by pLC1 diminished when the sequence of the -10 element of the *degR* promoter was changed to a σ^A -like promoter sequence. pLC1, however, did not suppress *sigD* expression. On the basis of these results, we conclude that multicopy *proB* on pLC1 inhibits transcription from σ^D -driven promoters by affecting some posttranscriptional process of σ^D .

Bacteria grow exponentially in optimal conditions and finally reach the steady-state growth phase because of starvation of nutrients or high cell density. At the transition state from the exponential phase to the steady-state phase, bacterial cells are forced to choose one of several ways for their further survival.

Bacillus subtilis has several choices to make, such as competence development, acquisition of motility, sporulation, and production of extracellular degradative enzymes, toward the end of the exponential growth phase (7, 10, 17, 23). Such differentiation has been shown to be mediated by several specific regulatory proteins.

One of these phenomena, the production of extracellular proteases, is positively regulated by a two-component regulatory system, DegS-DegU (2, 3, 10, 17, 18). In the essential *degS-degU* system, the sensor kinase, DegS, is thought to accept certain environmental stimuli, autophosphorylate on its own histidine residue, and transfer the phosphate to the aspartate residue of the cognate response regulator DegU (2, 17, 18, 24). DegS is also involved in the dephosphorylation of the phosphorylated DegU (3, 27). In the regulation of the exoprotease production, other regulatory factors including DegR (19, 20, 28, 31) and ProB (21) act in concert with the DegS-DegU system. We have reported that multiple copies of the *B. subtilis* *proB* gene on pLC1 encoding γ -glutamyl kinase show a synergistic effect on the production of the exoproteases when *degR* is carried on the multicopy plasmid pNC61 (21). This effect of multicopy *proB* on plasmid pLC1 is dependent on *degS*, and we postulated that the metabolic intermediate, γ -glutamyl phosphate synthesized by ProB, might transmit a signal to DegS, resulting in a higher level of phosphorylated DegU (21).

Acquisition of motility requires coordinated expression of many genes involved in generation of the complex and multi-component organelle flagellum (23). The coordinated expression of the motility genes is dependent on an alternative σ factor, σ^D , which is specific for transcription of the *hag* gene,

the *flgMK*-containing operon, and the *motAB* operon (23). σ^D recognizes highly conserved -35 and -10 elements consisting of 5'-CTAAA-3' and 5'-GCCGATAT-3', respectively (4). It has been reported that the *degR* promoter is recognized by σ^D -containing RNA polymerase in vitro (6). The synthesis of σ^D factor is temporally regulated, and the factor itself is regulated by an anti- σ^D factor encoded by the *flgM* gene through protein-protein interaction (16).

The results described in this article show that the transcription of *degR* is driven by a σ^D factor and that multicopy *proB* inhibits the transcription of *degR* as well as another σ^D -dependent gene, *hag*. These results suggest that the regulation of the two adaptive responses, the production of exoproteases and acquisition of motility, partially overlaps at a process involving the σ^D factor.

MATERIALS AND METHODS

Materials. Restriction enzymes were purchased from Toyobo Co. (Tokyo, Japan). The PCR amplification kit, DNA blunting kit, DNA ligation kit, IPTG (isopropyl- β -D-thiogalactopyranoside), and X-Gal (5-bromo-4-chloro-3-indolyl- β -D-galactopyranoside) were bought from Takara Shuzo Co. (Shiga, Japan). The synthetic oligonucleotides were commercially prepared by Sawaday Technology Co. (Tokyo, Japan). The PCR apparatus was PJ2000 (Perkin-Elmer Cetus).

Strains and plasmids. Bacterial strains and plasmids are listed in Table 1. The *degR'*-*lacZ* derivatives described in this article were constructed by linking the 26th codon of *degR* with the *lacZ* gene lacking the first 8 codons in plasmid ptpBG1 (26). Plasmid pdeg11 was constructed by PCR amplification of the *B. subtilis* CU741 chromosomal DNA using primers *dgr-1* and *dgr-2*, followed by digestion of the resultant DNA fragment with *Hind*III and *Eco*RI and insertion into ptpBG1 which had been digested with *Hind*III and *Eco*RI. Plasmids pdeg12 and pdeg13 were constructed by the same procedure except that primers *dgr-3* and *dgr-2* and primers *dgr-4* and *dgr-2* were used, respectively. Plasmid pdeg101 was constructed as follows. A DNA region flanking the *degR* promoter was PCR amplified by using the CU741 chromosomal DNA and either primers *dgr-1* and *dgr-2* or primers *dgr-2* and *dgr-m1* (Table 2). The synthesized DNAs were heat denatured, annealed, and treated with T4 DNA polymerase, and the resulting DNA was used as the template for PCR amplification with the *dgr-1* and *dgr-2* primers. The mutated DNA thus obtained was inserted into the *Eco*RI- and *Hind*III-treated ptpBG1 after digestion with the same enzymes. Plasmids pdeg21, pdeg22 and pdeg14 were similarly constructed by using primers *dgr-m3* and *dgr-m4*, primers *dgr-m5* and *dgr-m6*, and primers *dgr-d1* and *dgr-d2*, respectively. The changes introduced were confirmed by DNA sequencing. Plasmid pUKM504 was constructed by insertion at the *Sca*I site of pUC18 (32) of the kanamycin resistance (*Km^r*) gene obtained by digestion of pBEST509 (8) with *Pst*I. The pUKS04 plasmid carrying an internal part of the *sigD*-coding region

* Corresponding author. Mailing address: Department of Marine Science, School of Marine Science and Technology, Tokai University, Orido 3-20-1, Shimizu, Shizuoka 424, Japan. Phone: 543-34-0411, ext. 2933. Fax: 543-34-9834.

TABLE 1. Bacterial strains and plasmids used in this study

Strain or plasmid	Relevant phenotype and description	Reference or source ^a
<i>B. subtilis</i>		
CU741	<i>trpC2 leuC7</i>	30
ODM50	<i>trpC2 leuC7 amyE::(degR1'-lacZ Cm^r)</i>	pdeg11→CU741
ODS100	<i>trpC2 leuC7 sigD::pUKS504</i>	pUKS504→CU741
ODS101	<i>trpC2 leuC7 sigD::pUKS504 amyE::(degR1'-lacZ Cm^r)</i>	pdeg11→ODS100
1A201	<i>trpC2 hisA1 sacA321 degS42</i>	BGSC ^b
ODM601	<i>trpC2 degS42 amyE::(degR1'-lacZ Cm^r)</i>	1A201→ODM50
ODM40	<i>trpC2 leuC7 amyE::(degR3'-lacZ Cm^r)</i>	pdeg12→CU741
ODM20	<i>trpC2 leuC7 amyE::(degR4'-lacZ Cm^r)</i>	pdeg13→CU741
ODM50D	<i>trpC2 leuC7 amyE::(degRd1'-lacZ Cm^r)</i>	pdeg14→CU741
ODM501	<i>trpC2 leuC7 amyE::(degRm1'-lacZ Cm^r)</i>	pdeg101→CU741
CB123	<i>trpC2 pheA5 hag'-lacZ</i>	1
ODF200	<i>trpC2 leuC7 hag'-lacZ</i>	CB123→CU741
ODM511	<i>trpC2 leuC7 amyE::(degRm3'-lacZ Cm^r)</i>	pdeg21→CU741
ODM512	<i>trpC2 leuC7 amyE::(degRm5'-lacZ Cm^r)</i>	pdeg22→CU741
ODM612	<i>trpC2 leuC7 sigD amyE::(degRm5'-lacZ Cm^r)</i>	pUKS504→ODM512
AC327SL	<i>his1 smo1 purB sigD'-lacZ</i>	11
ODS200	<i>trpC2 leuC7 sigD'-lacZ</i>	AC327SL→CU741
CB25	<i>trpC2 phe1</i>	16
CB149	<i>trpC2 phe1 flgMΔ80</i>	16
OBN25	<i>trpC2 phe1 (degR1'-lacZ Cm^r)</i>	pdeg11→CB25
OBN149	<i>trpC2 phe1 flgMΔ80 (degR1'-lacZ Cm^r)</i>	pdeg11→CB149
<i>E. coli</i> JM109		
	<i>recA1 Δ(lac-proAB) endA1 gyrA96 thi-1 hsdR17 relA1 supE44 [EtraD36 proAB lacI^qZ ΔM15]</i>	32
Plasmids		
pTrpBG1	Cm ^r Ap ^r	26
pdeg11	Cm ^r Ap ^r <i>degR1'-lacZ</i>	This work
pdeg12	Cm ^r Ap ^r <i>degR3'-lacZ</i>	This work
pdeg13	Cm ^r Ap ^r <i>degR4'-lacZ</i>	This work
pdeg14	Cm ^r Ap ^r <i>degRd1'-lacZ</i>	This work
pdeg101	Cm ^r Ap ^r <i>degRm1'-lacZ</i>	This work
pUKM504	pUC18 carrying Km ^r	This work
pUKS504	pUKM504 carrying a part of <i>sigD</i>	This work
pdeg21	Cm ^r Ap ^r <i>degRm3'-lacZ</i>	This work
pdeg22	Cm ^r Ap ^r <i>degRm5'-lacZ</i>	This work
pUBH1	Km ^r Pm ^r	9
pLC1	Km ^r Pm ^r <i>proB</i>	21
pNC6	Tm ^r	20
pNC61	Tm ^r <i>degR</i>	20

^a Arrows indicate transformation of the chromosomal or plasmid DNAs into the respective strains.

^b BGSC, *Bacillus* Genetic Stock Center.

was constructed as follows. The 817-bp DNA fragment containing the entire *sigD*-coding region was prepared by PCR amplification using the CU741 chromosomal DNA and primers sgd-1 and sgd-2. The 189-bp *Sau3AI-HindIII* DNA fragment that was originated from an internal part of the *sigD*-coding region and was used for *sigD* disruption by Helmann et al. (5, 13) was cloned into *Bam*HI- and *Hind*III-digested pUKM504.

Media and antibiotics. The media used were Luria-Bertani broth, Luria-Bertani agar medium, antibiotic medium 3 (Difco Laboratories), modified competence medium, and Schaeffer's sporulation medium (10, 12, 25). The concentrations of the antibiotics added to the media were 15 μg/ml for tetracycline, 10 μg/ml for kanamycin and trimethoprim, and 5 μg/ml for chloramphenicol and phleomycin.

Isolation of RNA. Cells from the late logarithmic phase of growth (20 ml) in Schaeffer's sporulation medium were collected by centrifugation at 0°C, resuspended in 1 ml of solution A (15 mM Tris-HCl [pH 8.0], 6 mM EDTA, 0.45 M sucrose, 2 mg of lysozyme per ml), and incubated at 0°C for 30 min. RNA was isolated with the Isogen RNA isolation kit (Nippon Gene Co., Tokyo, Japan) according to the manufacturer's recommendation and dissolved in 100 μl of distilled water.

Primer extension analysis. Total RNA (40 μg) and 4 pmol of the 5'-biotinated 30-mer oligonucleotide primer representing nucleotides 440 to 411 (5'-CAACT TCAAGTCTTTATCATCCATAGCTGC-3' [20, 31]) were mixed in a hybridization buffer (40 mM Tris-HCl [pH 7.5], 50 mM NaCl) in a total volume of 30 μl, heated for 90°C for 3 min, and incubated at 55°C for 60 min. The nucleic acids were precipitated with cold ethanol and treated with avian myeloblastosis virus reverse transcriptase by using the First-Strand cDNA synthesis kit (Life Sciences,

Inc.) according to the manufacturer's instruction. Extension products were phenol extracted, precipitated with ethanol, and resuspended in 10 μl of 0.1 N NaOH. After heat treatment at 95°C for 3 min, 5 μl of 95% formamide containing 0.05% each bromophenol blue and xylene cyanol was added. The samples were subjected to gel electrophoresis (6% polyacrylamide sequencing gel) alongside sequencing ladders obtained with the same primer. The extended DNA and sequence ladders were visualized with the nonradioisotope DNA detection kit (Chemiluminescence; Toyobo Co.).

β-Galactosidase assay. Cells were grown in Schaeffer's sporulation medium as described in the figure legends and processed by the method described previously (21). The results shown in the figures are those of experiments representative of one or two other experiments.

RESULTS AND DISCUSSION

Transcription of *degR* is dependent on σ^D . It has been reported that the *degR* gene serves as a good template for in vitro transcription by E- σ^D (6). To test whether in vivo transcription of *degR* is also driven by E- σ^D , we examined *degR'-lacZ* expression in both a wild-type strain and a *sigD* knockout strain. The result showed that the expression of *degR* was transient with a maximum shortly before time zero (end of vegetative growth) in the parental strain (ODM50), an observation sim-

TABLE 2. Oligonucleotides used for PCR-mediated mutagenesis

Oligonucleotide	Sequence ^a	Location ^b
dgr-1	5'-CGAATTCATCCAGCTGATGCT-3'	-352 to -331
dgr-2	5'-GAAGCTTATCGGCCAGTTCTTCTAAAT-3'	+142 to +123
dgr-3	5'-CGAATTCCTTAGCATCCTCAAT-3'	-253 to -236
dgr-4	5'-CGAATTC AATTTATGTACCAAAAATA-3'	-52 to -34
dgr-m1	5'-AAAAATAAGAGCTTATAACT-3'	-23 to -4
dgr-m2	5'-AGTTATAAGCTCTTATTTTT-3'	-4 to -23
dgr-d1	5'-CCCCTTCTCTATCAATTC AATAGTTATATATCGG-3'	+55 to +41 and +3 to -14
dgr-d2	5'-TTGATAGAGAAGGGGAA-3'	+41 to +55
dgr-m3	5'-TAAAAATAAGCTATAATAACTATTG-3'	-24 to +1
dgr-m4	5'-CAATAGTTATTTATAGCTTATTTTTA-3'	+1 to -24
dgr-m5	5'-TAAAAATAATGTATAATAACTATTG-3'	-24 to +1
dgr-m6	5'-CAATAGTTATTTATACATTATTTTTA-3'	+1 to -24
sgd-1	5'-TCCCGGGTATTAGGGGGATAACAATGC-3'	1,160 to 1,179 ^c
sgd-2	5'-ATCTAGATCACTCGCTAACCATGAAATT-3'	1,962 to 1,942

^a Sequences added to introduce restriction sites are underlined. Nucleotides added for sequence alteration are boldfaced.

^b Relative to the transcriptional start point of *degR* except in the case of *sgd-1* and *sgd-2*.

^c From reference 13.

ilar to that reported for the expression of *sigD* (11), whereas this expression was virtually abolished when a *sigD* mutant strain, ODS101, was used (Fig. 1).

We next determined the transcription start site of *degR* by primer extension. We isolated RNA both at 30 min before time zero ($T - 0.5$) and at time zero to avoid missing the optimal time of RNA isolation, since the expression of *degR* is transient, as shown in Fig. 1. The results showed that a single band was detected with the RNA obtained at both time points from the cells carrying pUBH1 (Fig. 2, lanes 5 and 9). This start site corresponds to G at nucleotide 352 in the published sequence of *B. subtilis degR* and is 64 bp upstream of the AUG start codon of *degR* (20, 31). By comparison with known *B. subtilis* promoters, the two upstream sequences 5'-GAAA-3' (nucleotides 319 to 322) and 5'-GCCGATAT-3' (nucleotides 338 to 345 [20, 31]) were found to be closely related to the consensus -35 and -10 sequences of the E- σ^D recognizable promoter (23), respectively. When the primer extension experiments were performed with RNA from ODS100 (*sigD*) carrying either pUBH1 or pLC1, no extension product was detected (Fig. 2, lanes 7, 8, 11, and 12). These results are in line with the in vitro transcription of *degR* (6).

Strain ODM501 carries a -10 element of the putative σ^D -recognized promoter altered by four base changes. The expres-

sion of *degR* in ODM501 was found to be greatly reduced, as shown in Fig. 3A.

From these results we conclude that the in vivo expression of *degR* is dependent on σ^D at least in the cells growing in Schaeffer's sporulation medium (25), although several transcripts were detected in the Luria-Bertani medium-cultured cells (20).

Multiple copies of *proB* suppress *degR* expression. The expression of the chromosomal *degR* gene was greatly reduced in strain ODM50 harboring pLC1 (Fig. 3B). This observation is consistent with the results that a transcript of *degR* was not detectable in the primer extension analysis using total RNA obtained from the CU741 cells carrying pLC1 (Fig. 2, lanes 6

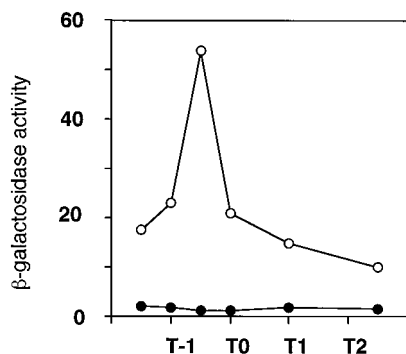


FIG. 1. Disruption of *sigD* abolishes *degR* expression. Cells were grown in Schaeffer's sporulation medium containing chloramphenicol, and β -galactosidase activities were determined as described previously (21) and are shown in Miller units. Numbers on the x axis represent the growth time in hours relative to the end of vegetative growth (T0). Open and closed circles, ODM50 (*degR*'-*lacZ*) and ODS101 (*degR*'-*lacZ sigD*), respectively.

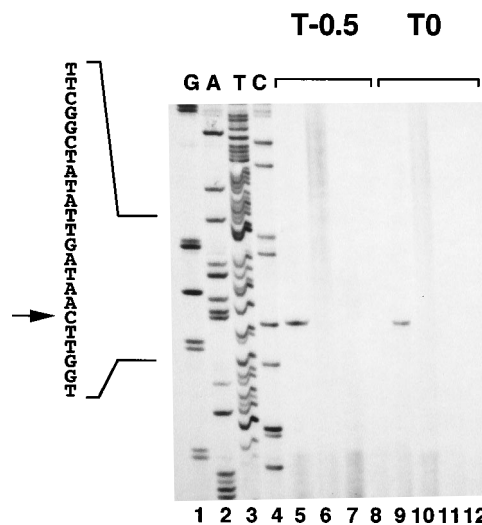


FIG. 2. Determination of the 5' end of the *degR* transcript by the primer extension method and analysis of in vivo transcripts of *degR* in *sigD* cells carrying pUBH1 or pLC1. The method used for primer extension is described in Materials and Methods. The primer consisted of the oligonucleotide representing nucleotides 411 to 440 (20, 31). The primer extension reaction was done with RNA isolated from strains CU741(pUBH1) (lanes 5 and 9), CU741(pLC1) (lanes 6 and 10), ODS100(pUBH1) (lanes 7 and 11), and ODS100(pLC1) (lanes 8 and 12). Cells were grown in Schaeffer's sporulation medium containing kanamycin for CU741(pUBH1 or pLC1) and both kanamycin and phleomycin for ODS100(pUBH1 or pLC1). Lanes 1 to 4, sequence ladders of the coding strand obtained with the same primer as that used for primer extension. Arrow, transcriptional start of *degR*.

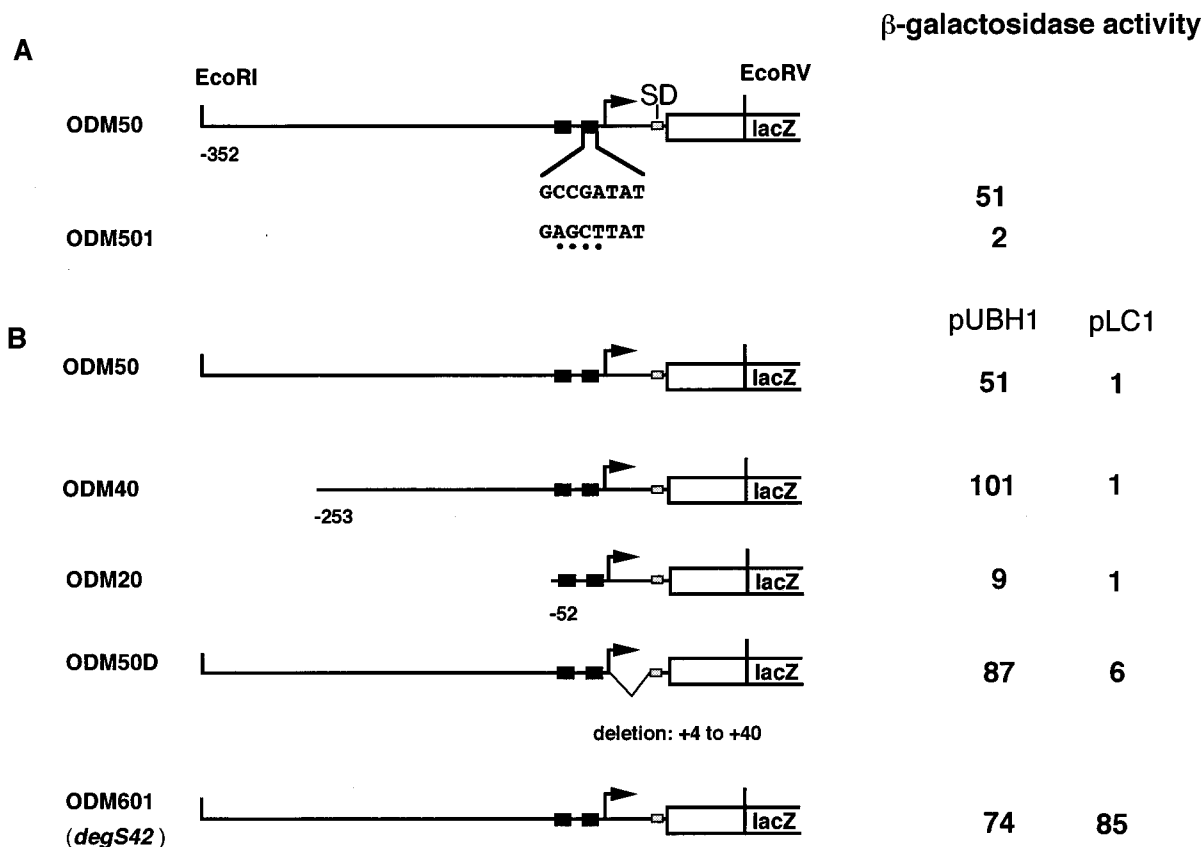


FIG. 3. *degR* expression is dependent on the σ^D promoter and subject to suppression by pLC1. Growth conditions and measurement of β -galactosidase activities were identical to those described in the legend to Fig. 1, except that the culture media contained chloramphenicol (A) or both kanamycin and chloramphenicol (B). The β -galactosidase activities shown were taken at $T - 0.5$. Solid boxes, -35 and -10 elements of the σ^D recognizable promoter; shaded box, Shine-Dalgarno sequence (SD). Nucleotides introduced for mutation are indicated (dots). Open box, N-terminal region of *degR* (26 amino acids) fused to that of *lacZ*. Arrows, transcription start point of *degR*. Numbers below the lines indicate the deletion endpoints or the deleted region relative to the transcription start site of *degR*.

and 10). These results show that pLC1 inhibits *degR* expression at the transcription level.

There are intact *proB* and the N-terminal part of *proA* in the upstream and downstream regions in pLC1, respectively (21). Disruption of *proB* at the *NotI* site but not *proA* at the *BclI* site (21) abolished the inhibitory effect of pLC1, indicating that *proB* is responsible for the activity (22).

To examine which DNA region on *degR* is subject to suppression by pLC1, *degR* expression was examined in strains ODM40, ODM20, and ODM50D carrying various deletions upstream of the *degR*-coding region. The results shown in Fig. 3B indicate that, although the expression level was reduced, the DNA region from -52 to $+3$ with respect to the transcription start site was sufficient for the expression of *degR* and that the target of pLC1 was either the region between these nucleotides or the region downstream from $+41$.

We have previously reported that the extent of enhancement of *aprE* expression by plasmid pNC61 was reduced to about 40% in a *proB* background (21). In contrast the severalfold enhancement of *aprE* expression by pLC1 did not change in a *degR* strain (21). One interpretation of these results would be that there may be a regulatory cascade, *degR-proB-degSU*.

The synergistic effect of pLC1 carrying *proB* and pNC61 carrying *degR* on the expression of *aprE'-lacZ* (21) is apparently contradictory in light of the transcription inhibition of *degR* by pLC1. We presume that the amount of DegR is critical for the synergism to be seen in the presence of a large amount

of ProB. Thus, in the cells carrying the vector pNC6 and pLC1, the amount of DegR directed from the chromosomal *degR* would be too small to show synergism because *degR* expression is suppressed by pLC1, whereas when *degR* is present on pNC61 the amount of DegR would be relatively high and sufficient to show synergism even under the condition that *degR* expression is suppressed by pLC1. On the other hand, in the cells containing pNC61 and the vector pUBH1, an excess amount of DegR would be able to show low-level synergism with ProB produced from the chromosomal *proB* gene, and this synergism would become negligible in *proB* cells. This interpretation is consistent with the observations described above.

The suppressive effect of multicopy *proB* on *degR* expression is dependent on DegS. We have previously shown that the enhancement of *aprE* expression by multicopy *proB* on pLC1 was dependent on intact *degS* (21). We therefore investigated whether the suppression of *degR* expression by pLC1 also requires DegS. Strain ODM601 is isogenic to strain ODM50 except that it carries the *degS42* mutation, a mutation that causes deficiency in autophosphorylation of DegS and subsequent phosphorylation of DegU (27). Results showed that pLC1 had no inhibitory effect on the transcription of *degR* in the *degS42* background (Fig. 3B), indicating that DegS is necessary for the suppressive effect of pLC1 on *degR* expression. Moreover, deletion of either *degS* or *degU* abolished the sup-

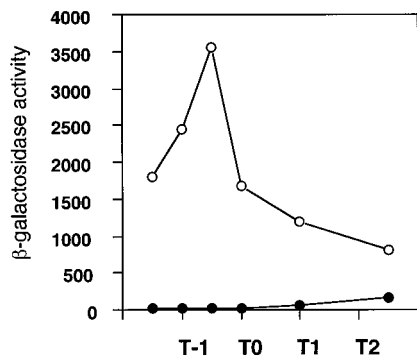


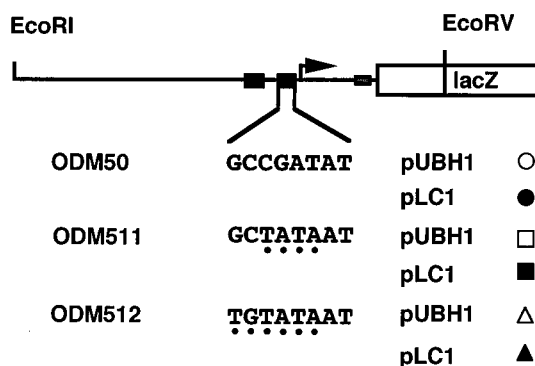
FIG. 4. Multicopy *proB* suppresses expression of *hag'*-*lacZ*. Numbers on the x axis represent the growth time in hours relative to the end of vegetative growth (T0). Growth condition and measurement of β -galactosidase activities were identical to those for Fig. 1 except that the culture media contained both chloramphenicol and kanamycin. ○, ODF200(pUBH1); ●, ODF200(pLC1).

pressive effect of pLC1 (22). These results show that the inhibitory effect of pLC1 requires intact DegS.

Effect of pLC1 on expression of σ^D -directed *hag'*-*lacZ*. To examine whether the suppressive effect of pLC1 on the *degR* promoter is also seen for other σ^D -dependent genes, we studied the expression of *hag'*-*lacZ* (1, 15). As shown in Fig. 4, the expression of *hag'*-*lacZ* was almost completely suppressed when pLC1 was present in the cell. An essentially similar result was obtained with the fusion *cwlB'*-*lacZ*, whose expression is dependent on both σ^D and σ^A RNA polymerases (11, 22). On the basis of these results, we suggest that the suppressive effect of multicopy *proB* is general for σ^D -directed promoters but not specific for *degR* transcription.

No significant homology between the sequences of *degR* (downstream from -52) and *hag* could be detected except for their promoters (22). This observation together with the results of locating the pLC1 target described above confined the inhibitory effect of ProB to the promoter region.

A



B

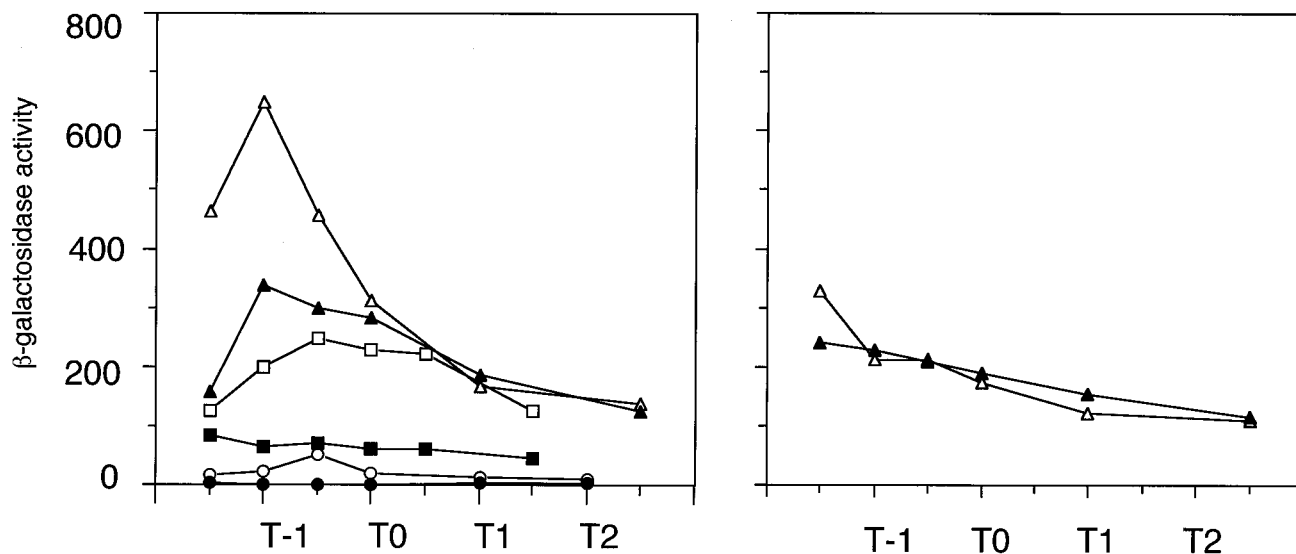


FIG. 5. Effect of nucleotide alteration from a σ^D - to a σ^A -type -10 sequence. (A) Reduction of the suppressive effect of pLC1 on *degR* expression; (B) inability of pLC1 to suppress *degR* expression in a *sigD* mutant. The scales on the y axes in the two panels are the same. The details of the drawings are described in the legend to Fig. 3. The numbers on the x axis represent the growth time in hours relative to the end of vegetative growth (T0). Growth conditions and measurement of β -galactosidase activities were identical to those in the legend to Fig. 1 except that the culture media contained both kanamycin and chloramphenicol (A) or kanamycin, chloramphenicol, and phleomycin (B).

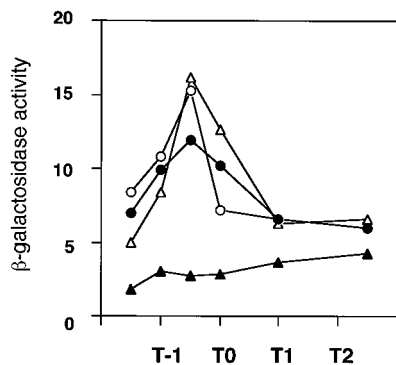


FIG. 6. Effects of pLC1 and pNC61 on *sigD*'-*lacZ* expression. Numbers on the x axis represent the growth time in hours relative to the end of vegetative growth (T0). Growth conditions and measurement of β -galactosidase activities were identical to those in the legend to Fig. 1 except that the culture media contained chloramphenicol plus either kanamycin or trimethoprim. ○, ODS200 (pUBH1); ●, ODS200(pLC1); △, ODS200(pNC6); ▲, ODS200(pNC61).

Substitution of the σ^D -recognized sequence by a σ^A -type consensus sequence on the *degR* promoter. If the transcription of *degR* depends on $E\text{-}\sigma^D$, the inhibition by pLC1 is expected to be relieved by changing the promoter sequence of *degR*. To test this notion, we constructed strains carrying the *degR*'-*lacZ* fusion whose consensus -10 sequence had been changed to σ^A -type consensus sequences. In strain ODM511 four base changes were introduced in the -10 sequence, whereas in strain ODM512 further two base changes were added to those present in strain ODM511. It was found that the expression of *degR* in strain ODM511(pUBH1) was increased fivefold at around time zero compared with that in the wild-type strain ODM50(pUBH1) (Fig. 5A). The presence of pLC1 in ODM511 inhibited *degR* expression 3-fold at $T - 0.5$, in sharp contrast to the 50-fold inhibition by pLC1 in ODM50 (Fig. 5A). Moreover, strain ODM512(pUBH1) showed a further 3.5-fold increase in *degR* expression versus strain ODM511 (pUBH1), and the level of inhibition by pLC1 was reduced as much as twofold at $T - 0.5$ and became negligible toward the end of the culture (Fig. 5A). These results show that as the promoter sequence changes from one containing the consensus -10 sequence of σ^D to one containing the -10 sequence of σ^A , the inhibition level of *degR* expression is decreased. The partial inhibition by pLC1 in ODM511 and ODM512 (Fig. 5A) could be attributed to the inhibitory effect on σ^D -driven transcription from the promoter in which the -35 element of the σ^D -type promoter still remained intact. It is, therefore, expected that if ProB from pLC1 suppresses *degR* expression through σ^D , the pLC1 effect should be no longer seen in a *sigD* background. To test this possibility, *degR* expression was examined in ODM612, a *sigD* derivative of ODM512. As shown in Fig. 5B, no significant difference in *degR*'-*lacZ* expression was observed for ODM612(pUBH1) and ODM612(pLC1), in agreement with the above notion.

A comparison of the β -galactosidase activities revealed that the peak observed around $T - 1$ for ODM512 (pUBH1) (Fig. 5A) was not seen for ODM612(pUBH1) (Fig. 5B), and similar levels of β -galactosidase activity were found after $T 1$ in both strains. These results show that the difference in the expression levels between the two strains is due to the transcription from the σ^D -type promoter and that the activities in ODM612(pUBH1) are most likely due to transcription from the newly constructed σ^A -type promoter.

We note that the difference in β -galactosidase activities in

ODM512(pUBH1) and ODM612(pUBH1) is severalfold higher than the β -galactosidase activities in ODM50(pUBH1) (Fig. 5). The transcription start site of the *degR*'-*lacZ* fusion in ODM612(pUBH1) was found to be the same as that of *degR* in CU741(pUBH1) (22), indicating that the sequence alteration of the -10 element induced both σ^D -type transcription and presumptive σ^A -type transcription.

Multiple copies of *proB* did not affect *sigD* expression. It was previously reported that the expression of *sigD* was completely repressed in cells carrying the *degU32*(Hy) mutation (29), a mutation known to stabilize the phosphorylated form of DegU (17). A similar result was expected for pLC1, since one possible explanation for the enhancing effect of pLC1 on *aprE* expression is the accumulation of phosphorylated DegU (21), which in turn may cause repression of *sigD*. It was found, however, that pLC1 did not affect *sigD*'-*lacZ* expression (Fig. 6). On the other hand, plasmid pNC61 carrying *degR* showed significant repression (Fig. 6). The pNC61 effect was not unexpected, since DegR was shown to stabilize the phosphorylated form of DegU (19) and the stabilization may result in inhibition of *sigD* expression as inferred from the result of the *degU32*(Hy) mutation. Therefore, taking into account these results and the observation that pLC1 inhibited σ^D -dependent expression of at least three genes as described above, we conclude that ProB inhibits some posttranscriptional process of σ^D .

We speculated previously that the level of the phosphorylated DegU in cells carrying pLC1 would be increased (21). Assuming that this is the case, one explanation for the failure of pLC1 to inhibit *sigD*'-*lacZ* would be that the concentration of phosphorylated DegU is less than that in the cells carrying pNC61, as deduced from the levels of the enhancement of *aprE* expression by the two plasmids (21). Such a low level of the phosphorylated DegU might act primarily as an inhibitor of the posttranscriptional regulation of σ^D but not as an inhibitor of the transcription of *sigD*.

Effect of pLC1 on *degR* expression in a Δ *flgM* strain. The *flgM* gene encodes an anti- σ^D protein (16). To examine the relationship between *flgM* and multicopy *proB*, the pLC1 effect was tested in a *flgM* background. The expression of *degR* was fivefold higher in OBN149 (*flgM*) than that in OBN25 (*flgM*⁺), in agreement with the nature of FlgM (Fig. 7). In contrast to the nearly complete inhibition of *degR* expression by pLC1 in OBN25, the inhibition was partial in OBN149 (Fig. 7). These results indicate that the pLC1 effect is not solely mediated by FlgM. Although several mechanisms with or without the in-

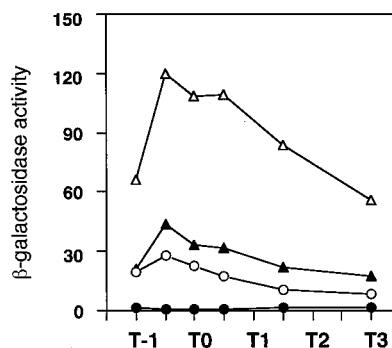


FIG. 7. Effects of pLC1 on *degR*'-*lacZ* expression in a *flgM* background. Growth conditions and measurement of β -galactosidase activities were identical to those for Fig. 1 except that the culture media contained both chloramphenicol and kanamycin. ○, OBN25(pUBH1); ●, OBN25(pLC1); △, OBN149(pUBH1); ▲, OBN149(pLC1).

involvement of FlgM can be envisaged, further investigation is required to draw a conclusion. It should be noted that Spo0K negatively regulates the expression of the σ^D -dependent *hag* gene without effect on the quantity of σ^D in the cell (14) and that we have found a gene that showed a negative effect on *degR* expression (22).

Two adaptive responses, the acquisition of motility and production of the exoproteases, were shown to intersect at the level of σ^D regulation through the suppressive effect of pLC1. It may be possible that the *proB* gene transmits a metabolic signal(s) to the machinery regulating σ^D under certain circumstances such as metabolic imbalance, since γ -glutamyl kinase encoded by *proB* is an enzyme involved in proline biosynthesis. In the cells carrying pLC1, the production of the exoproteases is stimulated severalfold (21), whereas flagellum formation is likely to be repressed through the inhibition of σ^D . Such a situation might be profitable for cells, since they would be able to obtain nutrition without chemotactic movement by flagella, whose formation requires a large amount of energy in the cell.

ACKNOWLEDGMENTS

We thank J. Sekiguchi, M. J. Chamberlin, and A. Galizzi for bacterial strains. We also thank K. Shimura, Y. Ohshiro, and J. Monden for technical assistance.

This work was supported by a grant-in-aid for scientific research from the Ministry of Education, Science and Culture of Japan.

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