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Involvement of Nitrogen Regulation in *Bacillus subtilis degU* Expression▽

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Bacillus subtilis **DegS-DegU belongs to a bacterial two-component system that controls many processes, including the production of exocellular proteases and competence development. It was found that when the glutamine synthetase gene *glnA*, which is involved in nitrogen regulation, was disrupted, the expression of the response regulator *degU* gene was increased. Deletion analysis and 5′-end mapping of the *degU* transcripts showed that the increase was caused by induction of a promoter (P2) located before the *degU* gene. Disruption of *tnrA*, a global regulator of nitrogen regulation, eliminated the P2 promoter induction by the *glnA* mutation. The fact that the P2 promoter is under nitrogen regulation was demonstrated by an increase in P2 expression with nitrogen-limited growth. It was also found by primer extension analysis that *degU* was transcribed by another promoter, P3, that is located downstream of P2. Efficient expression of P3 was dependent on phosphorylated DegU, as inactivation of the sensor kinase gene, *degS*, resulted in the loss of *degU* expression, although less efficient stimulation of *degU* expression was also observed with an enhanced level of DegU in a *degS*-deficient mutant. The promoter located upstream of the *degSU* operon, designated the P1 promoter here, was insensitive to *glnA* and *degU* mutations. These results suggest that *degU* expression is controlled by the three promoters under different growth conditions.**

The bacterial two-component regulatory system is a device to cope with changing environments around the cell. One such example is the *Bacillus subtilis* DegS-DegU pair, which controls various cellular processes. These include competence development (6, 10, 11, 25), swarming, flagellum formation (1, 16), biofilm formation (15, 35), osmotic response (29), poly-γ-glutamic acid synthesis (35), salt resistance (19), antibiotic synthesis (17), and the synthesis of extracellular degradative enzymes (21). It has been proposed that some of the events regulated by the DegS-DegU system are manifested by different extents of phosphorylation of the response regulator DegU by the DegS kinase (16, 40). Thus, the wide variety of processes regulated by the DegS-DegU system suggest that the signals activating DegU are transduced to DegS via different mechanisms.

Changes in nitrogen availability lead to the alteration of gene expression in *B. subtilis* (8). This nitrogen regulation is mediated by two transcriptional regulators, GlnR and TnrA. GlnR is a repressor of the *ureABC*, *glnR-glnA*, and *tnrA* genes, whereas TnrA is a global regulator that exerts positive and negative regulation of many genes (3, 7, 8, 42, 47). In nitrogen-rich environments, glutamine synthetase (GS), the gene product of *glnA*, is feedback inhibited, resulting in repression of *glnA* expression via GlnR. On the other hand, feedback-inhibited GS inhibits the global regulator TnrA by protein-protein interaction (44). Since GS is the only means for ammonium assimilation in *B. subtilis* (2) and inactivating mutations in this enzyme result in high-level expression of *glnRA* in medium containing excess nitrogen, GS has been proposed to be a monitor for the nitrogen status of the cell (31, 33). Thus, the genes under nitrogen regulation are regulated by either GlnR or TnrA through GS, which senses the nitrogen status in the cell (8, 42).

Production of the extracellular neutral and alkaline proteases, encoded by *nprE* and *aprE*, respectively, is subject to regulation by the DegS-DegU two-component system. Since the degradation products of these proteases could supply the cells with a nitrogen source, it may be possible that the expression of the *degS* and/or *degU* gene is subject to regulation by nitrogen metabolism. To investigate whether the *degS-degU* two-component system is influenced by nitrogen availability, we examined the effect of deletion of the *glnA* gene, whose gene product (glutamine) supplies nitrogen for the synthesis of about 25% of nitrogen-containing compounds in the cell (28).

In this report, we show that there are two promoters before the *degU* gene, namely, the upstream P2 and the downstream P3 promoters, and that they are under the regulation of GlnA-TnrA and DegS-DegU, respectively.

MATERIALS AND METHODS

Plasmids and plasmid construction. Plasmid pDLK2, a derivative of pDL2 (9) carrying the kanamycin resistance (Km^r) gene, was provided by K. Kobayashi. pDG148-degU was described previously (27).

Plasmid construction was performed with *Escherichia coli* JM103 (45). Plasmids carrying various upstream regions of *degU* were constructed by PCR amplification of the regions studied, followed by cleavage of the PCR products with EcoRI and BglII and subsequent cloning into pDLK2 that had been treated with EcoRI and BamHI. The plasmids thus constructed and the PCR primers used were as follows: for pAY4450, DGSU574F and DGSU880R; for pAY4434, DGSU574F and DGSU764R; for pAY4407 and pAY4407M, DGSU574F and DGSU737R; for pAY9707, DGSU627F and DGSU737R; for pAY9734 and pAY9734M, DGSU627F and DGSU764R; and for pAY3934, DGSU669F and DGSU764R. For construction of the plasmids below, the following synthetic DNAs were annealed and cloned into EcoRI- and BamHI-digested pDLK2: 669692F, 693750F, 750723R, and 722669R for pAY3920; 681710F, 711764F, 764737R, and 736681R for pAY5134; 692717F, 718764F, 764744R, and 743692R for pAY6234; and 711764F and 764711R for pAY8134. The cloned DNA regions were confirmed by sequence determination. Plasmid pBEST402, containing the blasticidin S resistance (Bs^r) gene,

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TABLE 1. Bacterial strains used in this study

Strain	Genotype or description	Reference or source
B. subtilis strains		
CU741	*trpC2 leuC7*	41
AY741G	*trpC2 leuC7 glnA*::Bs^r	This study
CU741T	*trpC2 leuC7 tnrA*::Cm^r	This study
TT711	*trpC2 leuC7 degU*::Cm^r	38
HJS31	Δ*glnR57*	32
CU741SN	*trpC2 leuC7 degS*::Nm^r	pDBNM1 × CU741
CU741S	*trpC2 leuC7 degS619*; carries an in-phase deletion of *degS* (codons 11 to 170)	This study
AY101	*trpC2 leuC7 amyE*::*lacZ* (no promoter)	pDLK2 × CU741
AY4450	*trpC2 leuC7 amyE*::*degU-lacZ*(1044-1350) (Nm^r)	pAY4450 × CU741
AY4450G	*trpC2 leuC7 amyE*::*degU-lacZ*(1044-1350) (Nm^r) *glnA*::Bs^r	AY741G × AY4450
AY4434	*trpC2 leuC7 amyE*::*degU-lacZ*(1044-1234) (Nm^r)	pAY4434 × CU741
AY4434G	*trpC2 leuC7 amyE*::*degU-lacZ*(1044-1234) (Nm^r) *glnA*::Bs^r	AY741G × AY4434
AY4407	*trpC2 leuC7 amyE*::*degU-lacZ*(1044-1207) (Nm^r)	pA4407 × CU741
AY4407G	*trpC2 leuC7 amyE*::*degU-lacZ*(1044-1207) (Nm^r) *glnA*::Bs^r	AY741G × AY4407
AY4407T	*trpC2 leuC7 amyE*::*degU-lacZ*(1044-1207) (Nm^r) *tnrA*::Cm^r	CU741T × AY4407
AY4407GT	*trpC2 leuC7 amyE*::*degU-lacZ*(1044-1207) (Nm^r) *glnA*::Bs^r *tnrA*::Cm^r	CU741T × AY4407G
AY4407U	*trpC2 leuC7 amyE*::*degU-lacZ*(1044-1207) (Nm^r) *degU*::Cm^r	TT711 × AY4407
AY4407GU	*trpC2 leuC7 amyE*::*degU-lacZ*(1044-1207) (Nm^r) *glnA*::Bs^r *degU*::Cm^r	TT711 × 4407G
AY9707	*trpC2 leuC7 amyE*::*degU-lacZ*(1097-1207) (Nm^r)	pAY9707 × CU741
AY9707G	*trpC2 leuC7 amyE*::*degU-lacZ*(1097-1207) (Nm^r) *glnA*::Bs^r	AY741G × AY9707
AY4407M	Same as AY4407 except for carrying sequence alterations	This study
AY4407MG	Same as AY4407G except for carrying sequence alterations	AY741G × AY4407M
AY9734	*trpC2 leuC7 amyE*::*degU-lacZ*(1097-1234) (Nm^r)	pAY9734 × CU741
AY9734G	*trpC2 leuC7 amyE*::*degU-lacZ*(1097-1234) (Nm^r) *glnA*::Bs^r	AY741G × AY9734
AY9734M	Same as AY9734 except for a mutation at the P3 promoter	This study
AY9734MG	Same as AY9734G except for a mutation at the P3 promoter	AY741G × AY9734M
AY3934	*trpC2 leuC7 amyE*::*degU-lacZ*(1139-1234) (Nm^r)	pAY3949 × CU741
AY3934G	*trpC2 leuC7 amyE*::*degU-lacZ*(1139-1234) (Nm^r) *glnA*::Bs^r	AY741G × AY3934
AY3920	*trpC2 leuC7 amyE*::*degU-lacZ*(1139-1220) (Nm^r)	pAY3920 × CU741
AY3920G	*trpC2 leuC7 amyE*::*degU-lacZ*(1139-1220) (Nm^r) *glnA*::Bs^r	AY741G × AY3920
AY5134	*trpC2 leuC7 amyE*::*degU-lacZ*(1151-1234) (Nm^r)	pAY5134 × CU741
AY5134G	*trpC2 leuC7 amyE*::*degU-lacZ*(1151-1234) (Nm^r) *glnA*::Bs^r	AY741G × AY5134
AY5134T	*trpC2 leuC7 amyE*::*degU-lacZ*(1151-1234) (Nm^r) *tnrA*::Cm^r	CU741T × AY5134
AY5134GT	*trpC2 leuC7 amyE*::*degU-lacZ*(1151-1234) (Nm^r) *glnA*::Bs^r *tnrA*::Cm^r	CU741T × AY5134G
AY5134U	*trpC2 leuC7 amyE*::*degU-lacZ*(1151-1234) (Nm^r) *degU*::Cm^r	TT711 × AY5134
AY5134GU	*trpC2 leuC7 amyE*::*degU-lacZ*(1151-1234) (Nm^r) *glnA*::Bs^r *degU*::Cm^r	TT711 × AY5134G
AY5134S	*trpC2 leuC7 amyE*::*degU-lacZ*(1151-1234) (Nm^r) *degS619*	AY5134 × CU741S
AY5134GS	*trpC2 leuC7 amyE*::*degU-lacZ*(1151-1234) (Nm^r) *glnA*::Bs^r *degS619*	AY741G × AY5134S
AY5134HY	*trpC2 leuC7 amyE*::*degU-lacZ*(1151-1234) (Nm^r) *degS200*(Hy)	This study
AY6234	*trpC2 leuC7 amyE*::*degU-lacZ*(1162-1234) (Nm^r)	pAY6234 × CU741
AY6234G	*trpC2 leuC7 amyE*::*degU-lacZ*(1162-1234) (Nm^r) *glnA*::Bs^r	AY741G × AY6234
AY6234U	*trpC2 leuC7 amyE*::*degU-lacZ*(1162-1234) (Nm^r) *degU*::Cm^r	TT711 × AY6234
AY8134	*trpC2 leuC7 amyE*::*degU-lacZ*(1181-1234) (Nm^r)	pAY8134 × CU741
AY8134G	*trpC2 leuC7 amyE*::*degU-lacZ*(1181-1234) (Nm^r) *glnA*::Bs^r	AY741G × AY8134
AYDS11	*trpC2 leuC7 amyE*::*degS-lacZ*	pDegS12 × CU741
AYDS11G	*trpC2 leuC7 amyE*::*degS-lacZ glnA*::Bs^r	AY741G × AYDS11
AYDS11U	*trpC2 leuC7 amyE*::*degS-lacZ degU*::Cm^r	TT711 × AYDS11
E. coli strain		
JM103	Δ*lac-pro thi rpsL supE sbcB hsdR4* F′ [*traD36 proAB*$^+$ *lacI*^{q} *lacZ*Δ*M15*]	45

was described previously (13). Plasmid pDegS12 carrying a *degS-lacZ* fusion was created as follows: a 798-bp EcoRI-BamHI fragment containing the promoter and the N-terminal 22 codons of *degS* was excised from pHAW3 (22) and ligated to EcoRI- and BamHI-digested pDLK2. pDSNM1 was constructed by insertion of a 1.4-kb SmaI fragment containing the neomycin resistance (Nm^r) gene from pBEST509 (M. Itaya, unpublished data) into the AflII site of the *degS* gene in pOU1 (38) that had been blunted with T4 DNA polymerase. pODELS2 was created by insertion of annealed, synthetic DNAs (DSBSAF and DSBSAR) between the AflII and BstXI sites of pOU1. pHDCS2, pHV33, and pDES200 carrying the *degS* gene derived from the *degS200*(Hy) mutant have been described previously (4, 37, 39).

Bacterial strains and media. The strains used in this study are listed in Table 1. To construct a *glnA* disruption mutant, the AY741G mutant, by insertion of the Bs^r gene, two PCR fragments derived from the N- and C-terminal regions of *glnA* and a fragment containing the Bs^r gene were prepared using the PCR primer pairs GLNA3 plus GLNAR617, GLNAF679 plus GLNA1329, and BSF plus BSR, respectively (Table 2). The templates used were the chromosomal DNA of strain CU741 for the *glnA* fragments and pBEST402 for the Bs^r gene-containing fragment. The three fragments were fused by PCR using the primers GLNA3 and GLNA1329, followed by transformation into CU741. By this procedure, the DNA region between codons 214 and 226 in *glnA* was replaced by the Bs^r gene. Strain CU741T was made by insertion of the *tnrA*::Cm^r (chloramphenicol resistance) DNA provided by K. Kobayashi into CU741. Construction of CU741S was performed in a two-step procedure using the gene conversion method (36). First, strain CU741SN (Table 1) was transformed with pODELS2 and pHDCS2, selecting for Nm-sensitive and Cm-resistant cells. After single-colony isolation, segregationally unstable pHDCS2 was removed from the cell by growth in antibiotic-free LB Lenox medium. Strain AY5134HY was constructed by the same gene conversion method, except that plasmids pDES200 and pHDCS2 were used. The *lacZ* fusions at the *amyE* locus were constructed by transformation of linearized plasmids into strain CU741.

TABLE 2. Primers used in this study

Primer	Nucleotide sequence (5′–3′)[a]
GLNA3	GGCAAAGTACACTAGAGAAG
GLNAR617	CACTTCATAACCGATATTAGGGTTTGGATGTCATCACAAGAGC
GLNAF679	TACCCGAAATTAAAAGTTTTACCACTGCATGCGACATTTATGCC
GLNA1329	CTGAGACATATACRGTTCGC
BSF	CCTAATATCGGTTATGAAGTG
BSR	GGTAAAACTTTTAATTTCGGGTA
DegS618	CGAGCGGATTTTCCGTG
DegUF	GTAAAGCTTGACCGAATGCTAGAGTATATAG
DegUR	GTAGTCGACTAGTAAAAGGCAAGTCTCC
DGSU574F	AGTTGAATTCGGCTTGCTGGGCATG
DGSU880R	AGTTAGATCTTCATCACCTTCGGCTACCAC
DGSU764R	AGTTAGATCTCGCCTCCTTGTATTATTGTTC
DGSU737R	AGTTAGATCTCTAGCATTCGGTCAATATG
DGSU627F	AGTTGAATTCAATAGATTCGAAAATAGGTCTTGG
DGSU669F	AGTTGAATTCAGGTTCCGTTATCTCTTTGACT
MU14F	GAGTAGATTTATTGGACGGAACGACGGTTATAGATTCGAAAATAGGTCTTG
MU14R	CAAGACCTATTTTCGAATCTATAACCGTCGTTCCGTCCAATAAATCTACTC
669692F	AATTCAGGTTCCGTTATCTCTTTGACTAT
693750F	GATTTGTAAAATAGAGCCAAAAGGCATATTGACCGAATGCTAGAGTATATAGAACAAT
750723R	GATCATTGTTCTATATACTCTAGCATTCGGTC
722669R	AATATGCCTTTTGGCTCTATTTTACAAATCATAGTCAAAGAGATAACGGAACCTG
681710F	AATTCTCTTTGACTATGATTTGTAAAATAGAGCC
711764F	AAAAGGCATATTGACCGAATGCTAGAGTATATAGAACAATAATACAAGGAGGCGA
764737R	GATCTCGCCTCCTTGTATTATTGTTCTATATAC
736681R	TCTAGCATTCGGTCAATATGCCTTTTGGCTCTATTTTACAAATCATAGTCAAAGAG
692717F	AATTGATTTGTAAAATAGAGCCAAAAGGC
718764F	ATATTGACCGAATGCTAGAGTATATAGAACAATAATACAAGGAGGCGA
764744R	GATCTCGCCTCCTTGTATTATTGTTC
743692R	TATATACTCTAGCATTCGGTCAATATGCCTTTTGGCTCTATTTTACAAATC
711764F	AATTAAAAGGCATATTGACCGAATGCTAGAGTATATAGAACAATAATACAAGGAGGCGA
764711R	GATCTCGCCTCCTTGTATTATTGTTCTATATACTCTAGCATTCGGTCAATATGCCTTTT
DSBSAF	TTAACTTCCCGGGAAGGTACCTTAGATCCACT
DSBSAR	GATCTAAGGTACCTTCCCGGGAAG
DUBio866	XTACCACTTCAAAGGTAGGTTC
DUBio1	XCTGATGGTCGTCGATAATAAC

[a] X, biotin attached to the nucleotide at the 5′ end.

LB Lenox medium and antibiotic medium III were obtained from Difco Co. Modified competence medium and Shaffer's sporulation medium were prepared as described previously (18, 30). These media contained glutamine at a final concentration of 0.2%. BSS medium was made by the procedure of Chasin and Magasanik (5).

Northern analysis. Cells grown in Schaeffer's medium were collected from 40-ml cultures at the indicated times, and total RNAs were isolated as previously described (46). The RNA samples (20 μg) were denatured with formamide and electrophoresed in a 1.2% agarose gel, and *degU* mRNA was detected as described previously, using a DIG luminescent detection kit (Boehringer Mannheim) (24). A digoxigenin-labeled DNA probe for the entire *degU* gene was prepared by PCR with a DIG probe synthesis kit (Boehringer Mannheim) and the primer pair DegUF plus DegUR. The RNA size marker set used was RNA molecular weight marker III, obtained from Roche Diagnostics.

Primer extension analysis. Primer extension was performed with an avian myeloblastosis virus reverse transcriptase cDNA synthesis kit obtained from Life Sciences, Inc. The reaction mixture contained 10 μg of RNA and the biotinylated primer DUBio866 or DUBio1. The reaction products were run in sequencing gels together with sequencing ladders prepared by using the same primers and a PCR fragment prepared with primers DegS618 and DegUR as a template.

Site-directed mutagenesis. Introduction of nucleotide changes into the putative TnrA recognition site was performed as follows. Two PCR fragments, amplified using primer pairs DGSU574F plus MU14R and MU14F plus DGSU737R, were prepared with CU741 DNA as a template, and after purification, they were mixed and subjected to a second PCR with primers DGSU574F and DGSU737R. The resultant PCR fragment was purified, digested with EcoRI and BamHI, and cloned between the EcoRI and BanHI sites of pDLK2.

β-Galactosidase assays. Cells from frozen stock cultures were spread on glutamine-supplemented LB plates containing appropriate antibiotics 1 day before experiments, and the colonies formed were transferred to glutamine-containing LB medium and incubated overnight. The cultures were then inoculated into glutamine-containing Schaeffer's medium at a concentration of 1%. For experiments with synthetic medium, cells were grown overnight in BSS medium without NH_4Cl and then inoculated at 1% into fresh BSS medium with or without NH_4Cl. Samples were withdrawn at hourly intervals, and β-galactosidase activities (in Miller units) were determined as described previously (24). The enzyme levels in all experiments were determined for the samples taken from T−1 (1 hour before the end of exponential growth phase) to T5 (5 hours after the end of exponential growth phase). The experiments were repeated at least twice, and the variations of the enzyme levels determined were within 20%. The data shown in this work are those from a typical experiment.

Transformation. Cells were made competent in MC medium by a previously described method (26).

RESULTS

Transcriptional activation of *degU* by *glnA* deletion. In an attempt to examine whether *degU* expression is under nitrogen regulation, we introduced a *glnA* disruption mutation into a strain carrying a transcriptional *degU-lacZ* fusion at the *amyE* locus (strain AY4450) (Fig. 1) and determined the β-galactosidase activity. The DNA region that we examined spans nucleotides (nt) 1,044 to 1,350; we define nt 1 as the first nucleotide of the *degS* coding sequence, and by this definition, the *degU* gene starts at nt 1,241 (Fig. 1). It was shown that β-galactosidase activities in the *glnA* mutant (AY4450G) were increased 12- to 7-fold compared with those in the wild-type strain before and around the end of exponential growth (T−1

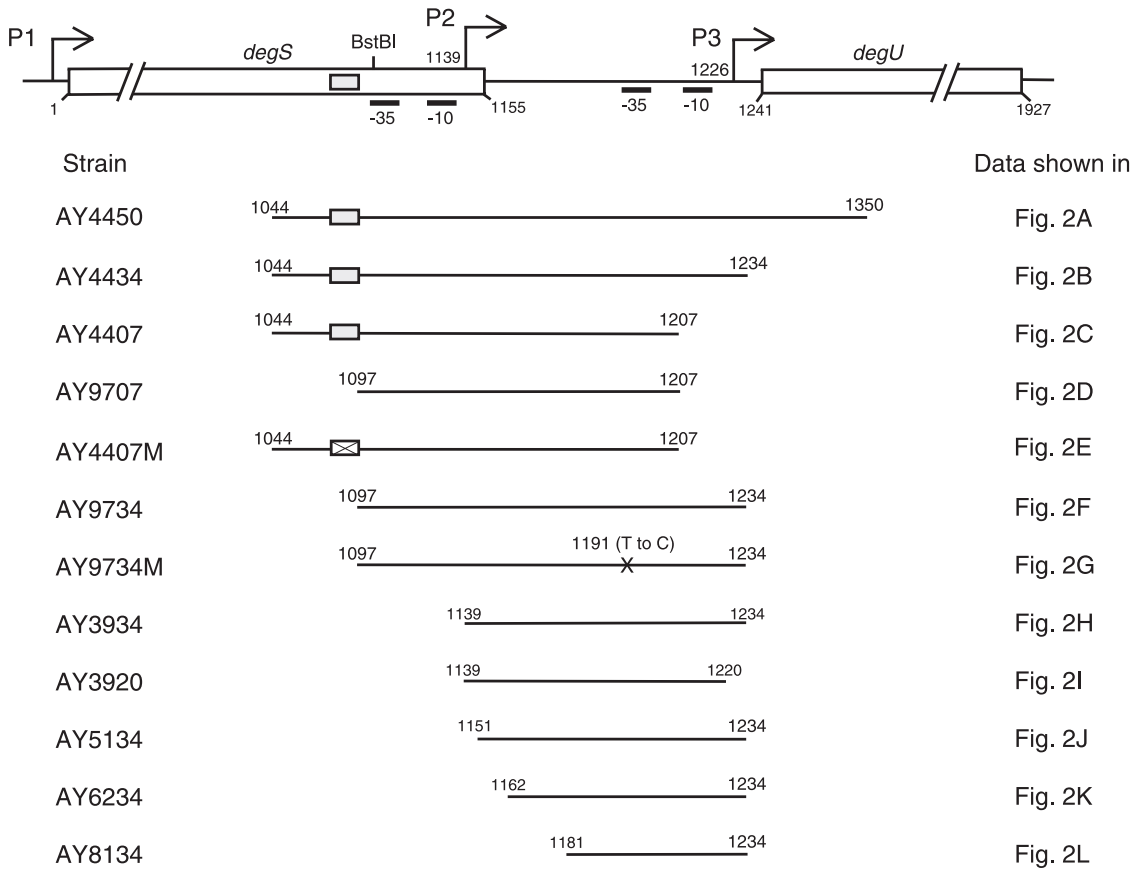

FIG. 1. Search for *degU* promoters by deletion analysis. The numbers indicate the nucleotide positions in the *degS-degU* region, with nt 1 being the first nucleotide of the *degS* coding sequence. Bent arrows depict the transcriptional initiation sites, which are shown by nucleotide numbers. The gray and crossed boxes depict the wild-type and mutant TnrA boxes, respectively. The map is not drawn to scale.

to T1), and the expression level remained 2- to 3-fold higher thereafter (Fig. 2A).

To confirm that the enhanced expression of the *lacZ* fusion was caused at the transcriptional level, we performed Northern analysis, using RNAs obtained at different culture times and a digoxigenin-labeled *degU* probe. An RNA band with a size of 0.7 to 0.8 kb was detected in the samples from the wild-type cells (Fig. 3, bottom arrow). In contrast, the RNA band from samples of the *glnA* mutant was much more intense and accompanied by possible degradation products. Since the RNA band covers the entire *degU* coding sequence (690 bp), we concluded that the transcription of *degU* was enhanced by the deletion of *glnA*. It should be noted that Kobayashi, using an undomesticated strain (16), found RNA bands with sizes larger than the 0.7-kb *degU* band at the end of exponential growth, which were probably the degradation products of the *degSU* operon mRNA originating from the promoter upstream of *degS*, suggesting that transcription from the *degS* promoter extends into the *degU* region. No such bands, however, were detectable at the positions expected for the sizes of the *degSU* mRNA (Fig. 3, top arrow) and its degradation products in our hands, probably reflecting the low-level synthesis and degradation of the *degSU* mRNA.

Search for promoters of *degU* expression. In order to locate the site of stimulation by the *glnA* mutation, we introduced deletions into the upstream region of *degU* and estimated β-galactosidase activities derived from the *degU-lacZ* fusions in the presence and absence of the *glnA* disruption. The DNA regions tested are depicted in Fig. 1. There was essentially no change of the overall profile of β-galactosidase activities when the deletion extended from the 3′ end (nt 1,350) to nt 1,234 (strains AY4434 and AY4434G) (Fig. 1 and 2B). However, for strain AY4407, carrying a further deletion to nt 1,207 (Fig. 1), the expression of the *lacZ* fusion was observed only in the *glnA* disruptant (AY4407G), and the expression level was higher around the end of exponential growth phase (Fig. 2C). On the other hand, for strain AY9707, in which a region spanning nt 1,097 to 1,207 was fused to *lacZ*, there was no detectable β-galactosidase activity irrespective of the *glnA* disruption (Fig. 2D). These results suggested that there are at least two promoters between nt 1,044 and nt 1,234, with the upstream and downstream promoters being completely dependent and partially dependent on *glnA* deletion for expression, respectively.

We then successively deleted nucleotides from the 5′ end (nt 1,097) to nt 1,181 (Fig. 1). It was found that similar profiles and levels of β-galactosidase activities were exhibited in strains AY9734, AY3934, AY5134, and AY6234 and their *glnA* derivatives (Fig. 2F, H, J, and K, respectively), but a further deletion to nt 1,180 (AY8134) resulted in a total loss of *lacZ* fusion

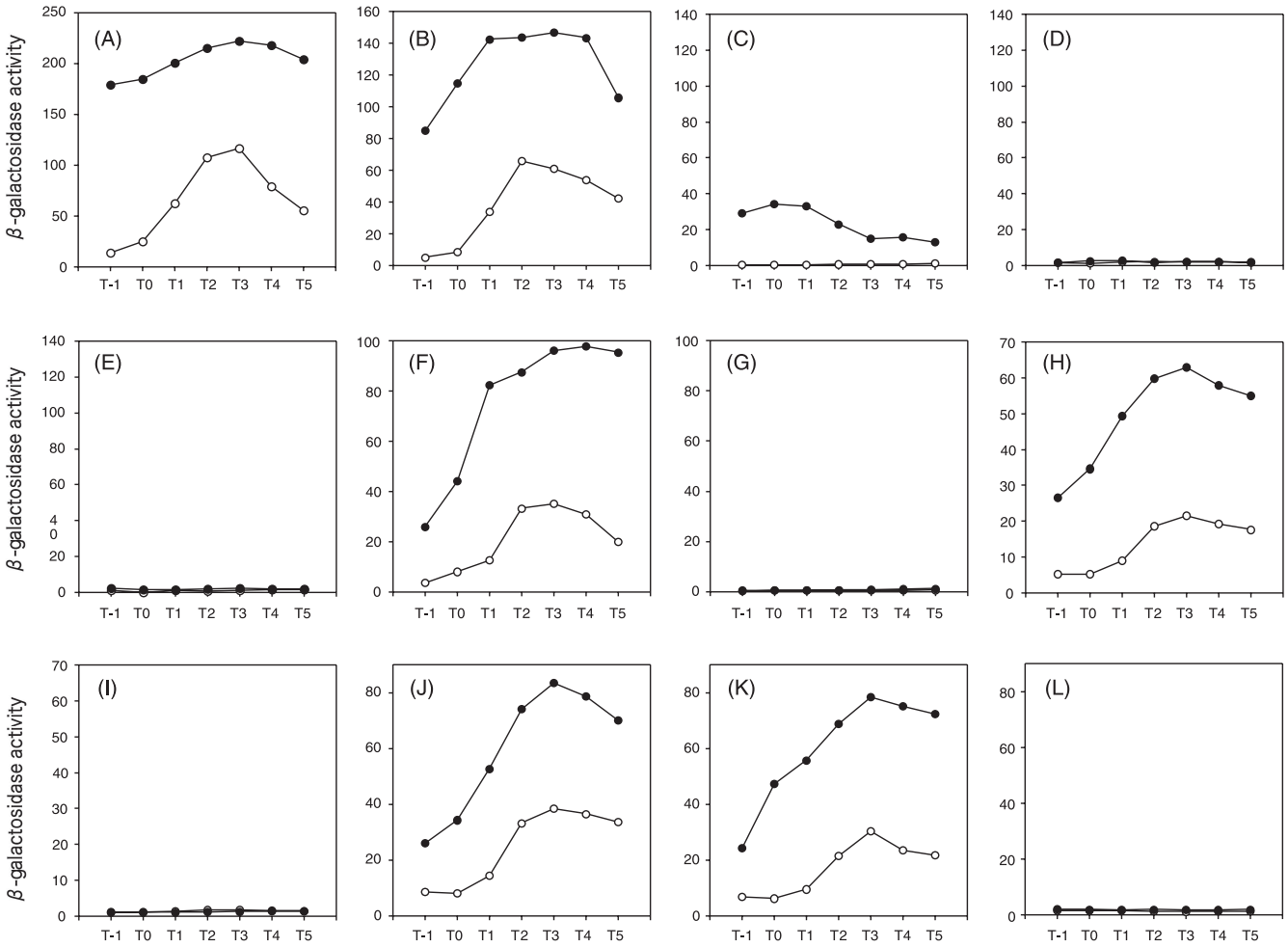

FIG. 2. Effects of *glnA* disruption on expression of *lacZ* fused to various regions upstream of *degU.* Cells grown overnight in glutamine-containing LB medium were inoculated into glutamine-containing Schaeffer's sporulation medium, and β-galactosidase activities were determined as described in Materials and Methods. Open circles, wild type; solid circles, *glnA* mutant. (A) Strains AY4450 and AY4450G; (B) AY4434 and AY4434G; (C) AY4407 and AY4407G; (D) AY9707 and AY9707G; (E) AY4407M and AY4407MG; (F) AY9734 and AY9734G; (G) AY9734M and AY9734MG; (H) AY3934 and AY3934G; (I) AY3920 and AY3920G; (J) AY5134 and AY5134G; (K) AY6234 and AY6234G; (L) AY8134 and AY8134G.

expression (Fig. 2L), irrespective of *glnA* deletion. It was also shown that strain AY3920, in which the 14 nucleotides at the 3′ end of the sequence in AY3934 were deleted (Fig. 1), showed no detectable β-galactosidase activity (Fig. 2I).

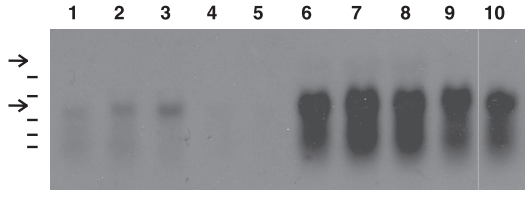

FIG. 3. Stimulation of *degU* mRNA synthesis by *glnA* disruption. RNAs were isolated from CU741 (lanes 1 through 5) and CU741G (*glnA* mutant) (lanes 6 through 10) cells at different time points and used for Northern analysis as described in Materials and Methods. The upper and lower arrows indicate the positions of the expected transcript sizes of the *degSU* operon and the *degU* gene, respectively. Lanes 1 and 6, T0; lanes 2 and 7, T1; lanes 3 and 8, T2; lanes 4 and 9, T3; lanes 5 and 10, T4. The lines on the left are RNA size markers, with the following sizes (bases), from the top: 1,517, 1,049, 575, 438, and 310.

Transcriptional initiation sites of *degU*. To correlate *degU-lacZ* expression with the DNA sequence, we searched for promoters upstream of *degU*. The RNA samples from T2 used for Northern analysis were subjected to primer extension analysis to determine the transcriptional initiation sites of *degU*. With the RNA obtained from the wild-type cells and a biotin-labeled primer, DUBio866 (Fig. 4B), we detected primer extension products that were separated by 1 nt (Fig. 4A, left panel). Since the faster-moving band was most likely produced by nibbling, we assigned the initiation site of *degU* in the wild-type cell to the nucleotide 15 bases upstream of the *degU* start codon (Fig. 4B). We designated the promoter for this transcription initiation site P3. With the RNA from the *glnA* cells, another discrete band was detected upstream of the P3 start site (Fig. 4A, left panel). To map this site more precisely, we used another biotin-labeled primer, DUBio1, and found that the transcriptional start site was 102 nucleotides upstream of the *degU* start codon (Fig. 4A, right panel, and B). We designated this promoter P2. The faint band seen between the two bands with the RNA from the *glnA* cells (Fig. 4A, left panel) may not repre-

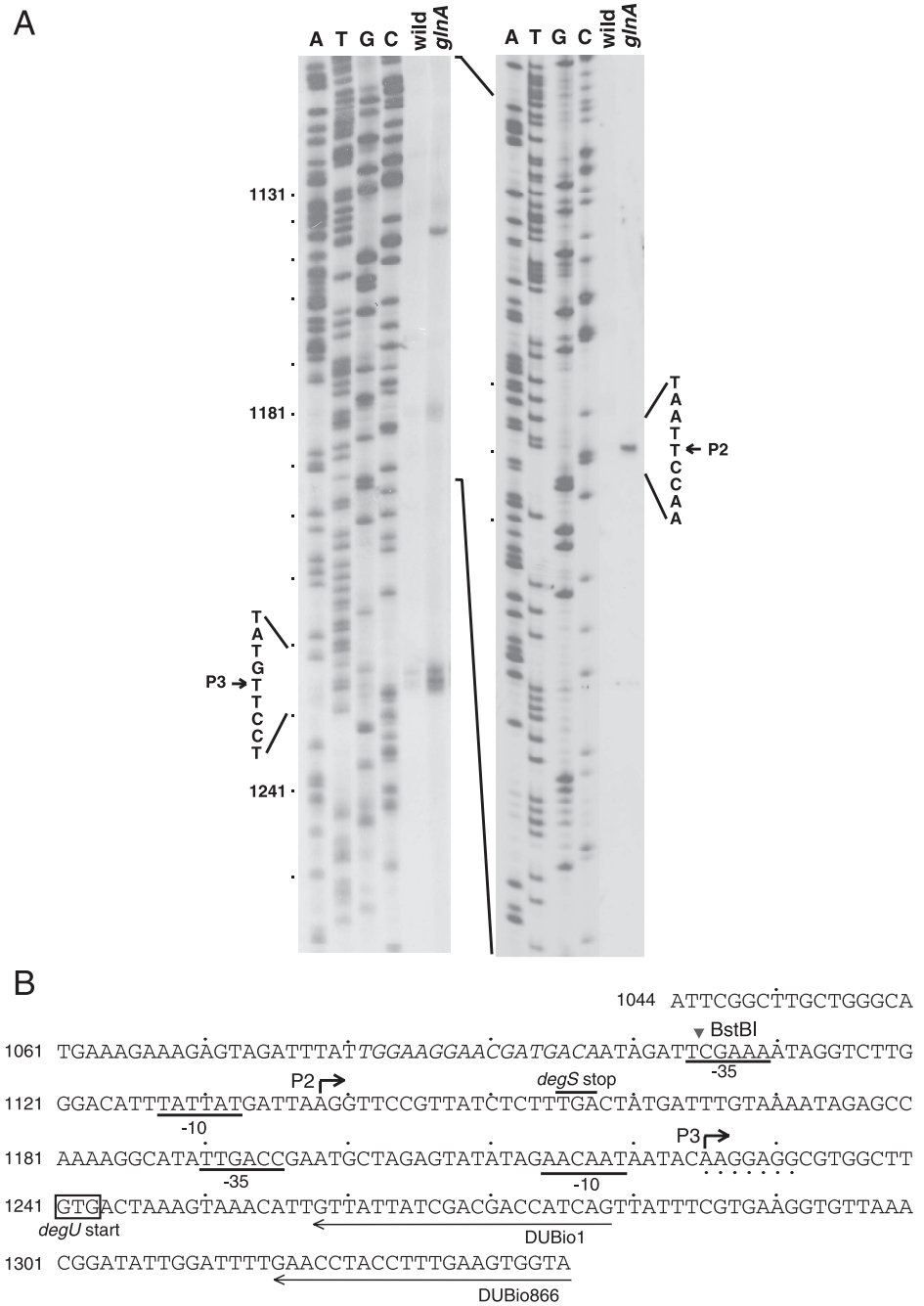

FIG. 4. Determination of transcriptional start sites of *degU.* (A) Primer extension analysis of *degU* promoters. The numbers on the left indicate the positions of the nucleotides relative to the first nucleotide of the *degS* coding sequence. RNAs obtained at T2 from CU741 (wild type) and CU741G (*glnA* mutant) were subjected to primer extension analysis with either primer DUBio866 or primer DegUBio1 (left or right panel, respectively). The arrowheads indicate the nucleotides at which transcription initiates. The experimental conditions are described in Materials and Methods. (B) Nucleotide sequence around the *degS-degU* intercistronic region. The nucleotides shown in italics and bent arrows denote the TnrA box and transcriptional start sites, respectively.

sent the promoter function, since it was not detected with primer DUBio1 (Fig. 4A, right panel). The two transcriptional initiation sites are preceded by putative −10 and −35 regions recognized by σ^A-type RNA polymerase (Fig. 4B).

The *degU-lacZ* fusion in strain AY9734M did not show promoter activity (Fig. 2G). Sequence analysis revealed a T-to-C mutation at nt 1,191, which is within the −35 region of the P3 promoter. In strain AY3920, the deletion had extended from the 3′ end to nt 1,221, which is adjacent to the −10 sequence of the P3 promoter, and this sequence organization may explain the loss of *degU-lacZ* expression shown in Fig. 2I. Expression of *degU-lacZ* was observed in strain AY6234 (Fig. 2K) but not in AY8134 (Fig. 2L). The 5′ end of the *degU* region contained in these constructs is at positions −64 and −45,

TABLE 3. Effects of *glnA* and *tnrA* deletion on *degU-lacZ* expression[a]

Strain	Relevant genotype	β-Galactosidase activity
AY5134	Wild type	35
AY5134G	*glnA*::Bs^r	81
AY5134T	*tnrA*::Cm^r	40
AY5134GT	*glnA*::Bs^r *tnrA*::Cm^r	39
AY4407	Wild type	1.1
AY4407G	*glnA*::Bs^r	33
AY4407T	*tnrA*::Cm^r	1.0
AY4407GT	*glnA*::Bs^r *tnrA*::Cm^r	1.0

[a] Cells were grown in glutamine-containing Schaeffer's medium as described in Materials and Methods. β-Galactosidase activities from T−1 to T5 were determined (Miller units), and the highest values attained are shown.

respectively, with respect to the P3 promoter. It is possible that the loss of the promoter activity in AY8134 was caused by the deletion of a sequence with which some positive regulator interacts, and we indeed found that the candidate for the regulator is most likely DegU or phosphorylated DegU (see below).

It has been shown previously by *degU-lacZ* fusion analysis that there are two promoters for the expression of the *degSU* operon, i.e., the major and minor promoters, located upstream of *degS* and in a 3′ region of the *degS* coding region, respectively, and the transcriptional initiation site of the major promoter was determined by primer extension analysis (20). We term this promoter P1 in this paper.

Involvement of TnrA in P2 and P3 promoter expression. From the results showing that *degU-lacZ* expression was observed in the *glnA* disruption mutant AY4407G but not in AY9707G (Fig. 2C and D) and that the DNA region at the *amyE* locus in AY4407G contains only the P2 promoter (Fig. 1), we concluded that the P2 promoter is under *glnA* regulation.

We next studied how the *glnA* mutation affects *degU* expression through the P3 promoter, as exemplified in AY5134 and AY5134G. It is well documented that the *glnA* gene product regulates the activities of GlnR and TnrA, through which many genes are regulated (8, 42). To investigate whether the *glnA* effect on *degU* expression from the P3 promoter occurs through any of these regulatory proteins, we first examined the effect of the *glnR57* mutant, which carries an in-phase deletion in *glnR* (32), and found that it had no effect on *degU-lacZ* expression in strain AY5134 (data not shown). On the other hand, the elevated expression of *degU-lacZ* in the *glnA*-deleted AY5134G strain was diminished to the level seen in the wild-type strain, AY5134, by additional disruption of *tnrA* (AY5134GT) (Table 3). The *tnrA* deletion alone in strain AY5134T did not affect *degU-lacZ* expression. We concluded from these results that the *degU-lacZ* fusion in AY5134G is expressed via two pathways, with one being the P3 promoter and the other a direct or indirect stimulatory effect on P3 through the GlnA-TnrA route.

To examine whether TnrA is also involved in P2 promoter expression, strain AY4407 and its derivatives carrying a *glnA* and/or *tnrA* deletion were examined. It was shown that P2 promoter expression was dramatically increased in the *glnA* strain AY4407G, in contrast to that in the wild-type strain, where no enzymatic activity was observed (Table 3). The enhanced expression in AY4407G was abolished by additional disruption of *tnrA*, as shown in strain 4407TG (Table 3), indicating that P2 is regulated by the GlnA-TnrA route.

Mutational analysis of the possible TnrA recognition sequence upstream of the P2 promoter. The involvement of TnrA in P2 promoter expression and the loss of its activity by deletion of the sequence between nt 1,044 and nt 1,097 (strains AY4407 and AY9707 in Fig. 1 and 2C and D) suggested a TnrA target in this region. A computer search for the recognition sequence of TnrA, TGTNAN$_7$TNACA (23, 42), or its updated version, TGTNANAWWWTMTNACA (47), revealed a sequence with partial homology (TGGAAGGAACGATGACA [underlined nucleotides match the requirement]) spanning nt 1,081 to 1,097 (positions −57 to −41 with respect to the transcriptional start site of P2) (Fig. 4B). When the nucleotide sequence was changed to TGGACGGAACGACGGTT (underlined nucleotides were changed), the promoter activity was lost in the mutant strain AY4407M and its *glnA* derivative (Fig. 1 and 2E), suggesting that this nucleotide sequence is the target of TnrA. It has been shown that transcriptional activation by TnrA depends on the target DNA sequence located closely to and upstream of the −35 sequence (43). The above result is in accordance with this precedent, although the nucleotide sequence in this case somewhat deviates from the updated version of the consensus sequence.

Expression of P2 promoter under nitrogen-limited conditions. Since the P2 promoter is activated by TnrA, it may also be stimulated in nitrogen-limiting medium. To test this notion, we grew AY4407 carrying the P2-*lacZ* fusion in glutamate-containing BSS medium, with or without the addition of NH_4Cl, and estimated the β-galactosidase activity. As shown in Fig. 5, the expression of the P2-*lacZ* fusion was elevated only under the nitrogen-limiting conditions, and this enhanced expression was abolished by *tnrA* deletion. These results show that the P2 promoter is nitrogen regulated.

Involvement of DegU in P3 but not in P2 promoter expression. It was proposed that *degU* expression is under positive autoregulation by its own gene product (16). We thus examined the effect of *degU* deficiency in strains AY4407 and AY5134, carrying the P2 and P3 promoters, respectively. The results showed that the elevated expression of the P2-*lacZ* fusion by *glnA*

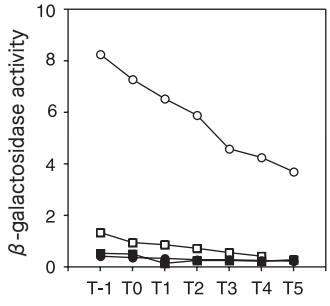

FIG. 5. Expression of P2 promoter during nitrogen-limited growth. Cells were grown overnight in BSS medium containing 0.2% glutamate and Nm at 10 μg/ml and transferred as a 1% inoculum to the same medium, with (solid symbols) and without (open symbols) the addition of NH_4Cl. Experimental procedures are described in Materials and Methods. Open circles, AY5134; solid circles, AY5134; open squares, AY5134T (*tnrA* mutant); solid squares, AY5134T (*tnrA* mutant).

TABLE 4. Effects of *degU* deletion on P2 and P3 promoter expression[a]

Strain	Relevant genotype	β-Galactosidase activity
P2-*lacZ* strains		
AY4407	Wild type	1.1
AY4407G	*glnA*::Bs^r	36
AY4407U	*degU*::Cm^r	1.3
AY4407GU	*glnA*::Bs^r *degU*::Cm^r	45
P3-*lacZ* strains		
AY5134	Wild type	36
AY5134G	*glnA*::Bs^r	83
AY5134U	*degU*::Cm^r	2.1
AY5134GU	*glnA*::Bs^r *degU*::Cm^r	2.3
AY6234	Wild type	30
AY6234U	*degU*::Cm^r	2.2

[a] Cells were grown in glutamine-containing Schaeffer's medium as described in Materials and Methods. The table contains two sets of data obtained from two separate experiments, one for strains carrying the P2-*lacZ* fusion and the other for those containing the P3-*lacZ* fusion. β-Galactosidase activities from T−1 to T5 were determined (Miller units), and the highest values attained are shown.

disruption (AY4407G) was not affected by *degU* disruption (AY4407GU), indicating that P2 promoter expression is not regulated by DegU. In contrast, P3-*lacZ* expression in both the wild-type and *glnA* strains (AY5134 and AY5134G, respectively) was reduced to the background level by *degU* disruption (AY5134U and AY5134GU) (Table 4). These results show that the expression of the P3 promoter is dependent on DegU and rule out the possibility that the stimulation of the P3 promoter by the *glnA* mutation in strain AY5134 (Table 3) is due to the effect of TnrA. Thus, it can be concluded that the expression of P3-*lacZ* at the *amyE* locus is under positive regulation of DegU irrespective of whether the transcription is stimulated or not by *glnA* deletion, whereas the P2-*lacZ* construct placed at the *amyE* locus is expressed from its own promoter, independent of DegU.

It should be noted that a deletion up to nt 1,161 carried in strain AY6234 is also subject to DegU regulation (Table 4), indicating that the DegU target is located downstream of this nucleotide.

Involvement of DegS in *degU* expression. We next investigated whether DegS is involved in *degU* expression. Kobayashi reported that phosphorylated DegS regulates *degU* expression, using a nonsense *degS* mutant and a *degU* mutant carrying a D-to-A mutation at codon 56, the site of phosphorylation (16). The *degS* mutant we used carried an in-phase *degS* deletion, *degS619*, in which codons 11 through 170 are deleted, eliminating a possible polar effect on the expression of the downstream *degU* gene. As shown in Table 5, there was almost no *degU-lacZ* expression in the *degS619* mutant (AY5134S) compared with the expression seen in the wild-type strain (AY5134), confirming the previous results of Kobayashi.

To investigate whether the increase in *degU* expression by *glnA* deletion has any effect on *degU-lacZ* expression in the *degS619* background, we introduced the *glnA*::Bs^r mutation into the strain carrying *degS619* to construct AY5134GS, and it was shown that there was a low but significant increase in *degU-lacZ* expression (Table 5). These results suggested that DegU might stimulate its own expression without phosphorylation by DegS. In order to examine this possibility further, we amplified DegU in the *degS619* strain AY5134S carrying pDG148-degU, in which *degU* expression is under the control of the IPTG (isopropyl-β-D-thiogalactopyranoside)-inducible P_{spac} promoter. As shown in Table 5, the addition of IPTG resulted in >100-fold increased expression of *degU*.

TABLE 5. Effects of *degS* deletion and overexpression of *degU* on *degU-lacZ* expression[a]

Strain	Relevant genotype	IPTG induction	β-Galactosidase activity
AY5134	Wild type		35
AY5134S	*degS619*		2.2
AY5134GS	*degS619* *glnA*::Bs^r		7.7
AY5134S(pDG148-degU)	*degS619*	−	2.8
AY5134S(pDG148-degU)	*degS619*	+	354
AY5134HY	*degS200*(Hy)		253
AY101	*amyE*::*lacZ*		1.2

[a] Cells were grown in glutamine-containing Schaeffer's medium as described in Materials and Methods. β-Galactosidase activities from T−1 to T5 were determined (Miller units), and the highest values attained are shown. IPTG (0.2 mM), when used, was added at the beginning of culture.

DegS has both phosphorylation and dephosphorylation activities toward DegU and DegU-phosphate, respectively, and the latter activity in the *degS200*(Hy) mutant is greatly reduced, leading to stabilization of DegU-phosphate in the cell (6, 39). We therefore expected that the mutation might cause an increase in the *degU* expression level, and the result shown in Table 5 indicates that this was indeed the case.

It can be concluded from these results that although phosphorylation of DegS causes positive autoregulation of *degU*, unphosphorylated DegU has the same capacity, although it is less efficient at low concentrations.

In strain AY6234 carrying pDG148-degU, the addition of IPTG also stimulated *degU-lacZ* expression to the same extent as that exhibited by AY5134 carrying the same plasmid, whereas no stimulation was observed in strain AY8134 (data not shown). These results and the data in Table 4 indicate that the target of DegU is present in a DNA region downstream of nt 1,162 but upstream of or including nt 1,181.

P1 promoter without *glnA* or *degU* regulation. To investigate whether the P1 promoter before the *degS* gene is subject to regulation by the *glnA* or *degU* gene product, we created a *degS-lacZ* fusion at the *amyE* locus and introduced this mutation into the constructed strain (see Materials and Methods). It was shown that disruption of neither *glnA* nor *degU* affected *degS-lacZ* expression (Table 6), indicating that unlike the P2 and P3 promoters, the P1 promoter is not subject to regulation by these gene products.

TABLE 6. Effects of *glnA* and *degU* mutations on expression of *degS-lacZ* placed at the *amyE* locus[a]

Strain	Relevant genotype	β-Galactosidase activity
AYDS11	Wild type	8.6
AYDS11G	*glnA*::Bs^r	8.0
AYDS11U	*degU*::Cm^r	8.6

[a] Experimental procedures are the same as those described in the footnote to Table 3.

DISCUSSION

We have shown in this study that *degU* expression is driven by two promoters, P2 and P3, located within the C-terminal region of the *degS* coding sequence and upstream of *degU*, respectively, and that the expression of P2- and P3-*lacZ* fusions placed at the *amyE* locus is stimulated by *glnA* deletion. It was also shown that *degU* expression was abolished by deletion of *degS* or *degU* and stimulated by an increased level of DegU or phosphorylated DegU.

Msadek et al. reported previously that there are two promoters for the expression of the *degSU* operon, with the major promoter located upstream of *degS* (here we call it P1) and the minor one located upstream of the BstBI site within the *degS* sequence (20) (Fig. 4B). The minor promoter activity is lost when the DNA region upstream of the BstBI site is removed (20). This upstream region is necessary for the P2 promoter in our study, suggesting that their minor promoter corresponds to the P2 promoter. On this basis, the P3 promoter found in this study is one that has not been detected previously. However, since the discrepancy could have arisen by a strain difference and/or the medium used, the relationship between the minor promoter and those found here, the P2 and P3 promoters, is not clear at present.

We have observed that the P2 and P3 promoters are activated by *glnA* mutation (Fig. 2; Tables 3 and 4). We interpret these results as follows. The deletion of *glnA* resulted in activation of TnrA, which then induced the P2 promoter activity, probably by binding to the target sequence upstream of the P2 promoter (Fig. 2E and 4B). An increase in the *degU* transcript from the P2 promoter contributed to amplification of *degU* expression, resulting in increased levels of DegU. In strains carrying the P3-*lacZ* fusion at the *amyE* locus, these events occurring at the native, chromosomal *degU* locus supplied increased amounts of DegU to the P3 promoter, resulting in enhanced expression of the P3 promoter, since it is positively regulated by DegU (Table 4). When the P2 promoter is also present before the *degU-lacZ* construct (strains AY4450 and AY4434), the transcription from the P2 promoter supplies additional *degU* transcripts, which results in a further increase in *degU* expression. Since the expression of the P2 promoter was higher at the end of the exponential growth phase (Fig. 2C), it seems reasonable that the *degU-lacZ* expression levels in strains AY4450 and AY4434 were higher during and at the end of exponential growth phase than those in the cells carrying only the P3-*lacZ* fusion at the *amyE* locus (compare Fig. 2A and B with Fig. 2F, H, J, and K).

It is tempting to speculate that the P2 promoter works in nitrogen-limited environments, since the limited ammonium supply in the medium caused an increase in expression of this promoter (Fig. 5). However, the low level of increase in *degU* expression may not be sufficient to increase the expression of *aprE*, since even the high-level expression of *degU* driven by IPTG induction of pDG148-degU used in this study failed to enhance *aprE* expression (27). It would rather suggest that *degU* expression through the P2 promoter might play some role in competence development, as shown by an increase in *comK* expression (data not shown) and the transformation efficiency during nitrogen-limited growth (14). Since the P3 promoter activity is increased by the presence of the intact *degS* and, even more dramatically, *degS200*(Hy) genes (Table 5) and since high levels of phosphorylated DegU are necessary for *aprE* expression (40), the role of the P3 promoter may be to supply phosphorylated DegU to the target genes through the DegS-DegU phosphorylation system. However, quantitative studies are required to know the extents to which the three promoters contribute to *aprE* expression and competence development, which require phosphorylated and unphosphorylated DegU, respectively.

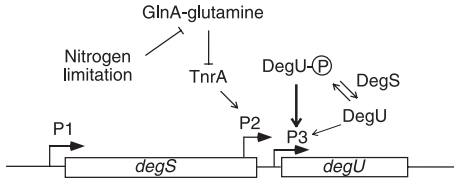

FIG. 6. Schematic representation of *degSU* operon regulation. For details, see Discussion. The bent arrows show the three promoters. The arrows and T bars indicate positive and negative regulation of the targets, respectively. The dark and light arrows pointing to P3 depict positive regulation with high and low efficiencies, respectively. The arrows are also used to show phosphorylation and dephosphorylation of DegU by DegS. The map is not drawn to scale.

A current model for *degU* expression is presented in Fig. 6, which is based on the present findings and the model presented by Kobayashi (16). Transcription from the P1 promoter without regulation by the GlnA-TnrA and DegS-DegU systems covers the entire *degSU* operon, probably without attenuation at the intercistronic region. When the cell encounters nitrogen-limiting conditions, the P2 promoter is stimulated, resulting in enhanced expression of *degU*. The P3 promoter is under positive autoregulation of DegU. Efficient autoregulation requires phosphorylated DegU, which binds to the promoter region of *degU* (16), although its unphosphorylated form also has some enhancing activity, as shown by an increase in expression of *degU-lacZ* in *glnA*- and *degS*-deficient cells and in *degS* cells containing high levels of unphosphorylated DegU (Table 4). In the *degS200*(Hy) mutant, in which the dephosphorylation activity of DegU-phosphate is low, the phosphorylated DegU molecules activate the P3 promoter to increase the *degU* mRNA level and enhance *aprE* expression by binding to the upstream region of the *aprE* gene (12, 34).

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