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Regulation of *Bacillus subtilis aprE* Expression by *glnA* through Inhibition of *scoC* and σ^D-Dependent *degR* Expression▽

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Expression of the gene for the extracellular alkaline protease (*aprE*) of *Bacillus subtilis* is subject to regulation by many positive and negative regulators. We have found that *aprE* expression was increased by disruption of the glutamine synthetase gene *glnA*. The increase in *aprE* expression was attributed to a decreased in expression of *scoC*, which encodes a negative regulator of *aprE* expression. The *glnA* effect on *scoC* expression was abolished by further disruption of *tnrA*, indicating that *aprE* expression is under global regulation through TnrA. In the *scoC* background, however, *aprE* expression was decreased by *glnA* deletion, and it was shown that the decrease was due to a defect in positive regulation by DegU. Among the genes that affect *aprE* expression through DegU, the expression of *degR*, encoding a protein that stabilizes phosphorylated DegU, was inhibited by *glnA* deletion. It was further shown that the decrease in *degR* expression by *glnA* deletion was caused by inhibition of the expression of *sigD*, encoding the σ^D factor, which is required for *degR* expression. In accordance with these findings, the expression levels of *aprE-lacZ* in *glnA scoC degR* and *scoC degR* strains were identical. These results led us to conclude that *glnA* deletion brings about two effects on *aprE* expression, i.e., a positive effect through inhibition of *scoC* expression and a negative effect through inhibition of *degR* expression, with the former predominating over the latter.

Bacillus subtilis produces a wide variety of extracellular degradative enzymes such as proteases, α-amylase, levansucrase, and others (1, 19, 27). The extracellular proteases are produced after the end of the exponential growth phase, and among those enzymes, the neutral and alkaline proteases encoded by *nprE* and *aprE*, respectively, are the major ones (27). The mechanism of *aprE* expression has attracted interest in terms of gene expression, since it is temporally controlled and subject to regulation by a large number of positive and negative regulators, apparently for timely and effective use of the enzyme in the habitat (18, 19).

The primary regulators that directly affect *aprE* expression include the four DNA-binding proteins ScoC, SinR, AbrB, and DegU. ScoC, SinR, and AbrB are negative transcriptional regulators, while DegU constitutes a two-component regulatory system with DegS and exerts a positive effect on *aprE* transcription (Fig. 1). These regulators play their roles by binding to either upstream regions (ScoC, SinR, and DegU) of the transcriptional initiation point or the transcriptional initiation region (AbrB) of *aprE* (8, 13, 30, 33). The *scoC*, *sinR*, and *abrB* genes are under the control of the *spo0A* gene product, and it has been shown that only the cells containing threshold levels of the phosphorylated form of both DegU and Spo0A exhibit *aprE* expression (35). In addition to these four factors, there are many positive and negative regulators that affect *aprE* expression indirectly (Fig. 1). The regulators DegQ, DegR, TenA, ProB, RapG, and RelA affect *aprE* expression through the DegS-DegU route; SenS and SalA do so by affecting transcription of *scoC*; and SinI does so by inhibiting the SinR function (2, 9, 14, 15, 20, 21, 22, 23, 26). In addition, another negative factor, PaiA, is known, but its mode of action on *aprE* expression has not been studied since its discovery (12).

The large amounts of the secreted proteases (the gene products of *aprE* and *nprE*) and the large number of regulatory factors controlling *aprE* expression suggest the importance of these exocellular proteases for the host *B. subtilis* cells to survive the harsh natural environments. One possible explanation for such high production of the proteases is that they are used to degrade insoluble proteins that happen to be present around the cells in the natural habitats. This may result in the supply of oligopeptides and/or amino acids, from which nitrogen-containing compounds may be derived. However, since the production of the enzymes in large amounts may be a burden to the cell, strict control in response to the nutritional status of the cell must be necessary. One possible candidate for such a regulator is TnrA, which receives information for nitrogen availability in the cell through interaction with feedback-inhibited glutamine synthetase, the *glnA* gene product (38). On the assumption that the role of the alkaline protease is to degrade high-molecular-weight proteins to supply nitrogen sources, it may be possible that *aprE* is also under nitrogen regulation through the GlnA-TnrA pathway. In this sense, a nitrogen-replete status in the cell may be a situation where TnrA is inhibited by complex formation with feedback-inhibited GlnA. Conversely, disruption of *glnA* leading to the release of TnrA from the feedback-inhibited GlnA may mimic a situation where the nitrogen source is scarce.

We have previously shown that *glnA* deletion results in overexpression of *degU* and that this was caused by induction of the P2 promoter present in a 3′ region of the *degS* gene, with which the *degU* gene constitutes an operon (42). In an attempt to examine whether the signal transduction through GlnA and

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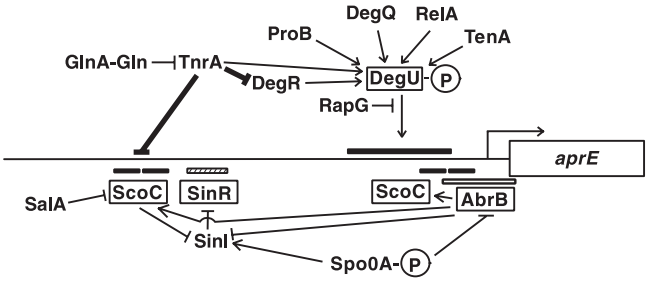

FIG. 1. Regulatory network in *aprE* expression. The four regulators, which bind upstream regions of *aprE*, are enclosed by rectangles. The binding sites of ScoC (positions −324 to −295, −292 to −267, −79 to −59, and −35 to −14 relative to the transcription initiation site of *aprE*), SinR (positions −268 to −220), and AbrB (positions −59 to +25) are shown by the solid, hatched, and open bars, respectively (8, 13, 33). DegU exerts positive effects on the region between positions −164 and −113 (11), and a binding site of DegU was demonstrated within a region spanning positions −146 to +86 (30). GlnA with an asterisk indicates the feedback-inhibited form of GlnA (31, 39). For simplicity, only the phosphorylated form of DegU is shown. The arrows and T-bars show stimulation and inhibition by the regulators, respectively, and the thick lines show the results obtained in this study. The bent arrow depicts the transcription from the *aprE* promoter. The map is not drawn to scale.

TnrA is involved in *aprE* expression, we found that disruption of the *glnA* gene resulted in an increase in *aprE* expression, suggesting a link between *aprE* expression and the GlnA-TnrA system. We show here that a decrease in *scoC* expression by *glnA* deletion is the basis for the increase in *aprE* expression. We also show that an increase in *degU* expression by the *glnA* mutation does not contribute to stimulation of *aprE* expression, because *glnA* deletion inhibits the expression of *sigD*, encoding the σ^D factor, which in turn inhibits σ^D-dependent expression of *degR*, whose gene product stabilizes the phosphorylated form of DegU (20).

MATERIALS AND METHODS

Bacterial strains. The bacterial strains used in this study are listed in Table 1. *Bacillus subtilis* strain AY741TN was constructed by replacing chloramphenicol resistance (Cm^r) in strain AY741T with neomycin resistance (Nm^r) by transformation of a switching plasmid, pCm::Nm (32), provided by the Bacillus Genetic Stock Center.

Plasmids and plasmid construction. The plasmids used in this study are listed in Table 1. Plasmids carrying various upstream regions of *scoC* were constructed by PCR amplification of the regions studied, followed by cleavage of the PCR products with both EcoRI and BamHI and subsequent cloning into pIS284 that had been treated with the same restriction enzymes. The plasmids thus constructed and the PCR primers used (Table 2) were as follows: for pISCO256, ScoC256 and ScoCR; for pISCO279, ScoC 279 and ScoCR; for pISCO301, ScoC301 and ScoCR; and for pISCO350, ScoC350 and ScoCR. Plasmid pSCO256M was created by a two-step procedure. First, two PCR fragments were prepared with YhaHF plus ScoCMR and ScoCMF plus ScoCR. Second, the resultant PCR fragments were fused by a second PCR in the presence of primers YhaH and ScoCR, followed by cleavage with EcoRI and BamHI and cloning into pIS284 treated with the same restriction enzymes.

Media. LB (Lennox) was purchased from Difco Co. Ltd. Schaeffer's sporulation medium (SSM) was prepared as described previously (28). LBG and SSMG were LB and SSM supplemented with 0.2% glutamine, respectively. The concentrations of antibiotics added to media were 5 μg/ml, 15 μg/ml, and 500 μg/ml for Cm, Nm, and blasticidin S (Bs), respectively. X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside) was added at a concentration of 100 μg/ml.

Determination of β-galactosidase activities. Cells from stock culture were spread on LBG plates containing appropriate antibiotics and X-Gal and grown overnight. The colonies formed were transferred to LBG medium and incubated overnight, and the cultures were then inoculated into SSMG at a concentration of 1%. The levels of β-galactosidase activity (in Miller units) were determined for the samples taken from T−1 (1 hour before the end of exponential growth phase) to T5 as described previously (22). In the experiments for Fig. 6A, determination started from T−1.5. Along with this quantitative assay, expression of *lacZ* fusions was also confirmed qualitatively by the blue color developed by colonies on X-Gal-containing plates.

Primer extension analysis. RNA was isolated from strains CU741 and AY741G grown in SMMG as described previously (43). Determination of the transcriptional start site was performed according to the procedure described previously (42), except that the biotinylated primer was SCBio47 and the sequencing ladders were prepared by using a PCR product made with primers YhaHF and ScoCR as a template.

RESULTS

Stimulation of *aprE* expression by *glnA* disruption. In order to investigate whether expression of the extracellular protease gene *aprE* is influenced by nitrogen regulation, we estimated *aprE* expression in strains with and without the intact *glnA* gene. Figure 2A shows that *aprE* expression increased about fourfold by deletion of the *glnA* gene (AY145G) compared to that in the wild-type strain (AY145). Since GlnA regulates the activity of TnrA (39), we also examined the effect of *tnrA* deletion on *aprE* expression in those strains. Stimulation of *aprE* expression was no longer seen in a *glnA tnrA* double mutant, AY145GT, whereas there was no effect of *tnrA* disruption alone on *aprE* expression (AY145T). The *glnA* gene constitutes an operon with its upstream gene *glnR*, which also encodes a regulator that controls the expression of the genes for nitrogen regulation (6). It was shown that an in-phase deletion of the *glnR* gene (*glnR57* mutation [29]) did not affect the expression of *aprE-lacZ* (data not shown). These results show that deletion of the *glnA* gene resulted in a positive effect on *aprE* expression through TnrA.

Effect of *glnA* deletion on expression of the four genes encoding primary regulators of *aprE* expression. Expression of *aprE* is known to be controlled directly by the four regulators ScoC, SinR, DegU, and AbrB, which bind to various sequences in the control region of the *aprE* gene (Fig. 1). To identify the regulator(s) through which the *glnA* disruption mutation exerts its effect, we examined the influence of the *glnA* mutation on *aprE-lacZ* expression in strains carrying deletions upstream of the transcription initiation site of *aprE*. The strains we used for the experiments for Fig. 2A carried the control region up to position −412 relative to the transcription initiation site of *aprE*. When this region was deleted to position −299, the *glnA* mutation caused a 60% reduction of *aprE* expression (Fig. 2B), in contrast to the stimulation seen in AY157G (Fig. 2A). It was demonstrated previously that the negative effect of ScoC on *aprE* expression is lost when the sequence upstream of position −299 is removed (22), suggesting that ScoC is involved in the stimulation of *aprE* expression in the *gln*A mutant, although the 60% reduction cannot be explained on this basis. On the other hand, there was no effect of *glnA* deletion in strain AY241G, in which the upstream region of *aprE* is deleted to position −113 (Fig. 2C). It was shown previously that the target of AbrB resides at positions −59 to +25 upstream of *aprE* (33), suggesting that *abrB* is not the target of *glnA* disruption. This notion was further substantiated by the observation that the enhancing effect of *glnA* deletion on *aprE* expres-

TABLE 1. Bacterial strains and plasmids used in this study

Species and strain or plasmid	Description	Source or reference
B. subtilis		
CU741	*trpC2 leuC7*	Laboratory stock
AY741G	*trpC2 leuC7 glnA*::Bs^r	42
AY741T	*trpC2 leuC7 tnrA*::Cm^r	42
AY741TN	*trpC2 leuC7 tnrA*::Nm^r	pCm::Nm × AY741T
TT7151	*trpC2 leuC7 degU*::Nm^r *aprE*::pSKD1	20
AY741U	*trpC2 leuC7 degU*::Nm^r	TT7151 × CU741
AY741L	*trpC2 leuC7 amyE*::*lacZ* (Cm^r) (no promoter)	pIS284 × CU741
OAM145	*trpC2 leuC7 amyE*::*aprE-lacZ*(−412, Cm^r)	22
AY145G	*trpC2 leuC7 amyE*::*aprE-lacZ*(−412, Cm^r) *glnA*::Bs^r	AY741G × OAM145
AY145T	*trpC2 leuC7 amyE*::*aprE-lacZ*(−412, Cm^r) *tnrA*::Nm^r	AY741TN × OAM145
AY145U	*trpC2 leuC7 amyE*::*aprE-lacZ*(−412, Cm^r) *degU*::Nm^r	AY741U × OAM145
AY145GT	*trpC2 leuC7 amyE*::*aprE-lacZ*(−412, Cm^r) *glnA*::Bs^r *tnrA*::Nm^r	AY741TN × AY145G
OAM147	*trpC2 leuC7 amyE*::*aprE-lacZ*(−299, Cm^r)	22
AY147G	*trpC2 leuC7 amyE*::*aprE-lacZ*(−299, Cm^r) *glnA*::Bs^r	AY741G × OAM147
AY241	*trpC2 leuC7 amyE*::*aprE-lacZ*(−113, Cm^r)	BG4200 × CU741
AY241G	*trpC2 leuC7 amyE*::*aprE-lacZ*(−113, Cm^r) *glnA*::Bs^r	AY741G × AY241
TSU2	*trpC2 leuC7 amyE*::*scoC-lacZ*(−422, Cm^r)	14
TSU2G	*trpC2 leuC7 amyE*::*scoC-lacZ*(−422, Cm^r) *glnA*::Bs^r	AY741G × TSU2
TSU2T	*trpC2 leuC7 amyE*::*scoC-lacZ*(−422, Cm^r) *tnrA*::Nm^r	AY741TN × TSU2
TSU2GT	*trpC2 leuC7 amyE*::*scoC-lacZ*(−422, Cm^r) *glnA*::Bs^r *tnrA*::Nm^r	AY741TN × TSU2G
TSU31	*trpC2 leuC7 amyE*::*scoC-lacZ*(−167, Cm^r)	pSCO256 × CU741
TSU31G	*trpC2 leuC7 amyE*::*scoC-lacZ*(−167, Cm^r) *glnA*::Bs^r	AY741G × TSU31
TSU32	*trpC2 leuC7 amyE*::*scoC-lacZ*(−144, Cm^r)	pSCO279 × CU741
TSU32G	*trpC2 leuC7 amyE*::*scoC-lacZ*(−144, Cm^r) *glnA*::Bs^r	AY741G × TSU32
TSU33	*trpC2 leuC7 amyE*::*scoC-lacZ*(−122, Cm^r)	pSCO301 × CU741
TSU33G	*trpC2 leuC7 amyE*::*scoC-lacZ*(−122, Cm^r) *glnA*::Bs^r	AY741G × TSU33
TSU34	*trpC2 leuC7 amyE*::*scoC-lacZ*(−73, Cm^r)	pSCO350 × CU741
TSU34G	*trpC2 leuC7 amyE*::*scoC-lacZ*(−73, Cm^r) *glnA*::Bs^r	AY741G × TSU34
TSU2M	TSU2 carrying sequence alterations at the putative TnrA target site	pSCO256 M × CU741
TSU2MG	Same as TSU2 M except for carrying *glnA*::Bs^r	AY741G × TSU2 M
TSU2MT	Same as TSU2 M except for carrying *tnrA*::Nm^r	AY741TN × TSU2 M
TSU2MGT	Same as TSU2 M except for carrying *glnA*::Bs^r and *tnrA*::Nm^r	AY741TN × TSU2MG
OAM157	*trpC2 leuC7 amyE*::*aprE-lacZ*(−412, Cm^r) *scoC*::Em^r Tc^r	22
AY157G	*trpC2 leuC7 amyE*::*aprE-lacZ*(−412, Cm^r) *scoC*::Em^r Tc^r *glnA*::Bs^r	AY741G × OAM157
AY157U	*trpC2 leuC7 amyE*::*aprE-lacZ*(−412, Cm^r) *scoC*::Em^r Tc^r *degU*::Km^r	AY741U × OAM157
AY157GU	*trpC2 leuC7 amyE*::*aprE-lacZ*(−412, Cm^r) *scoC*::Em^r Tc^r *glnA*::Bs^r *degU*::Km^r	AY741U × AY157G
ODM50	*trpC2 leuC7 amyE*::*degR1-lacZ*(−352, Cm^r)	24
AY50G	*trpC2 leuC7 amyE*::*degR1-lacZ*(−352, Cm^r) *glnA*::Bs^r	AY741G × ODM50
AY50T	*trpC2 leuC7 amyE*::*degR1-lacZ*(−352, Cm^r) *tnrA*::Nm^r	AY741TN × ODM50
AY50GT	*trpC2 leuC7 amyE*::*degR1-lacZ*(−352, Cm^r) *glnA*::Bs^r *tnrA*::Nm^r	AY741TN × AY50G
ODM612	*trpC2 leuC7 sigD amyE*::*degR*m5-*lacZ*(Cm^r)	24
AYM612G	*trpC2 leuC7 sigD amyE*::*degR*m5-*lacZ*(Cm^r) *glnA*::Bs^r	AY741G × ODM612
ODS200	*trpC2 leuC7 sigD-lacZ*(Cm^r)	24
AYS200G	*trpC2 leuC7 sigD-lacZ*(Cm^r) *glnA*::Bs^r	AY741G × ODS200
AYS200T	*trpC2 leuC7 sigD-lacZ*(Cm^r) *tnrA*::Nm^r	AY741TN × ODS200
AYS200GT	*trpC2 leuC7 sigD-lacZ*(Cm^r) *glnA*::Bs^r *tnrA*::Nm^r	AY741TN × AYS200G
ODF200	*trpC2 leuC7 hag-lacZ*(Cm^r)	24
AYF200G	*trpC2 leuC7 hag-lacZ*(Cm^r) *glnA*::Bs^r	AY741G × ODF200
BG4136	*trpC2 hisA1 thr-5 degR*::Em^r	40
AY145R	*trpC2 leuC7 amyE*::*aprE-lacZ*(−412, Cm^r) *degR*::Em^r	BG4136 × OAM145
AY157R	*trpC2 leuC7 amyE*::*aprE-lacZ*(−412, Cm^r) *scoC*::Em^r Tc^r *degR*::Em^r	OAM157 × AY145R
AY157GR	*trpC2 leuC7 amyE*::*aprE-lacZ*(−412, Cm^r) *scoC*::Em^r Tc^r *degR*::Em^r *glnA*::Bs^r	AY741G × AY157R
E. coli JM103	Δ(*lac-pro*) *thi rpsL supE sbcB hsdR4* F′ [traD36 *proAB*$^+$ *lacI*^{q} *lacZ*ΔM15]	41
Plasmids		
pIS284	Cm^r, *E. coli* plasmid for insertion of *lac*Z fusions into *B. subtilis amyE* locus	I. Smith
pCm::Nm	*E. coli* plasmid to change Cm^r to Nm^r	M. Steinmetz
pSCO256	pIS284 carrying positions −167 to +139 of the *scoC* promoter	This study
pSCO279	pIS284 carrying positions −144 to +139 of the *scoC* promoter	This study
pSCO301	pIS284 carrying positions −122 to +139 of the *scoC* promoter	This study
pSCO350	pIS284 carrying positions −73 to +139 of the *scoC* promoter	This study
pSCO256 M	pSCO256 carrying altered nucleotides upstream of *scoC*	This study

TABLE 2. DNA primers used in this study

Primer	Nucleotide sequence (5′→3′)[a]
YhaHF	AGTTGAATTCGCACCTTCCTCAGGAAAGC
ScoC256	AGTTGAATTCAGCTGGAGAAAACCTTAC
ScoC279	AGTTGAATTCTAATATCCTATTCAAAAGAAA
ScoC301	AGTTGAATTCAAAATGCGGGCCAAAATTGG
ScoC350	AGTTGAATTCTTCGTCGCAATGGTTTGTGA
ScoCMF	GAGAAAATCAGACAGCTGGCGTCGCCTGTT ACAAACTAATATCCTATTCAAAAGAAAAA
ScoCMR	TTTTTCTTTTGAATAGGATATTAGTTTGTAA CAGGCGACGCCAGCTGTCTGATTTTCTC
ScoCR	AGTTGGATCCTTCTCGATCGATTTCC
SCBio47	XGCTGAGCCATTTTCTGGGTG

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

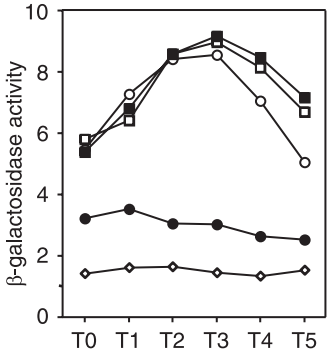

FIG. 3. Inhibition of *scoC-lacZ* expression by *glnA* deletion and recovery by a *tnrA* null mutation. Open circles, strain TSU2 (wild type); solid circles, TSU2G (*glnA*); open squares, TSU2T (*tnrA*); solid squares, TSU2GT (*glnA tnrA*); diamonds, AY741L (no *lacZ* promoter). The data set of β-galactosidase activities was from one of two experiments, in which the variations of the enzyme levels were within 15%.

sion was not affected by mutations in *abrB* or *spo0A*, which encodes the repressor of *abrB* (data not shown).

It is possible from these results that *scoC* is a candidate for the target of *glnA* deletion. To test this notion, we examined *scoC-lacZ* expression in the *glnA* background. The results showed that *glnA* deletion caused a decrease in *scoC-lacZ* expression in the *glnA* mutant TSU2G compared to the wild-type strain TSU2 (Fig. 3). The reduced expression of *scoC-lacZ* in strain TSU2G was restored to the wild-type level in strain TSU2GT carrying both *glnA* and *tnrA* mutations, whereas the *tnrA* mutation alone did not affect *scoC-lacZ* expression (TSU2T). Since ScoC is a negative regulator of *aprE* expression, these findings with the *glnA* and *tnrA* mutants are in parallel with those for *aprE-lacZ* expression shown in Fig. 2A and confirm that *scoC* is a target of *glnA* deletion.

It was also shown that there was no effect of *glnA* deletion on *sinR-lacZ* or *sinI-lacZ* expression (data not shown), indicating that the *glnA* deletion effect is not via the SinI-SinR route.

We have already shown that *degU* expression is under positive regulation of TnrA in the *glnA* background (42). All of these results show that *scoC* and *degU* are the targets of *glnA* deletion among the four primary regulators.

Analysis of the upstream region of *scoC*. We next investigated the target of *glnA* deletion in the upstream region of *scoC*. First, we determined the transcriptional initiation site of the *scoC* gene. By referring to the profiles of *scoC* expression in Fig. 3, we isolated RNA samples at T2 from both the wild-type and *glnA* strains and used them for primer extension analysis. As shown in Fig. 4A, the reverse transcriptase product of the RNA prepared from the wild-type strain CU741 was more intense than that from the *glnA* mutant CU741G and migrated with a sequence ending in T that corresponds to A in the sense strand located 37 bases upstream of the *scoC* start codon (Fig. 4B). The transcriptional start site is preceded by putative −35 and −10 regions recognized by σ^A-type RNA polymerase (10) (Fig. 4B).

Second, we searched for the DNA region upstream of *scoC* where the *glnA* mutation exerts its effect. We constructed at the *amyE* locus *lacZ* fusions carrying various deletions upstream of the transcriptional initiation site of *scoC* and examined β-galactosidase levels in these strains. In strains with the 5′ end points of −422 and −167 (Fig. 4B), the expression of *scoC-lacZ* was reduced in the *glnA* mutants (Table 3, compare strains TSU2 and TSU31with TSU2G and TSU31G, respectively), whereas *glnA* deletion did not affect *scoC-lacZ* expression in strains TU32G, TU33G, and TU34G, carrying the 5′ end points of −144, −122, and −73, respectively, indicating that *glnA* deletion affects the DNA region between positions

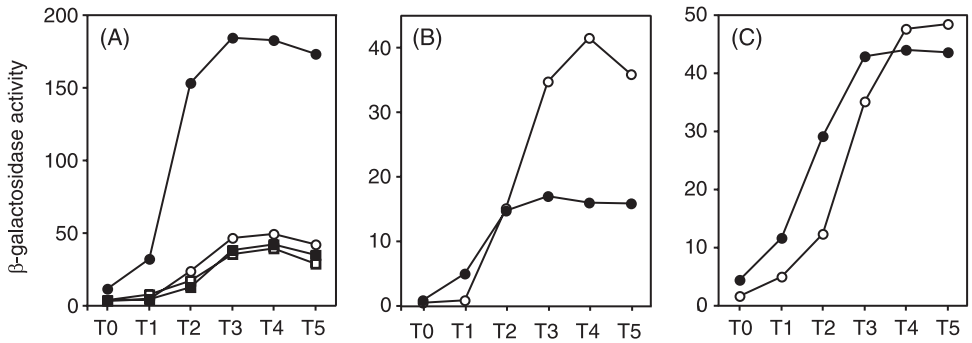

FIG. 2. Expression of *aprE-lacZ* fusions containing upstream regions of *aprE* up to positions −412 (A), −299 (B), and −113 (C) relative to the transcription start site of *aprE*. (A) Open circles, strain OAM145 (wild type); solid circles, AY145G (*glnA*); open squares, AY145T (*tnrA*); solid squares, AY145GT (*glnA tnrA*). (B) Open circles, OAM147 (wild type); solid squares, AY147G (*glnA*). (C) Open circles, AY241 (wild type); solid circles, AY241G (*glnA*). Cell growth and β-galactosidase activities were determined as described in Materials and Methods. Each data set is from one of two experiments, in which the variations of the enzyme levels were within 15%.

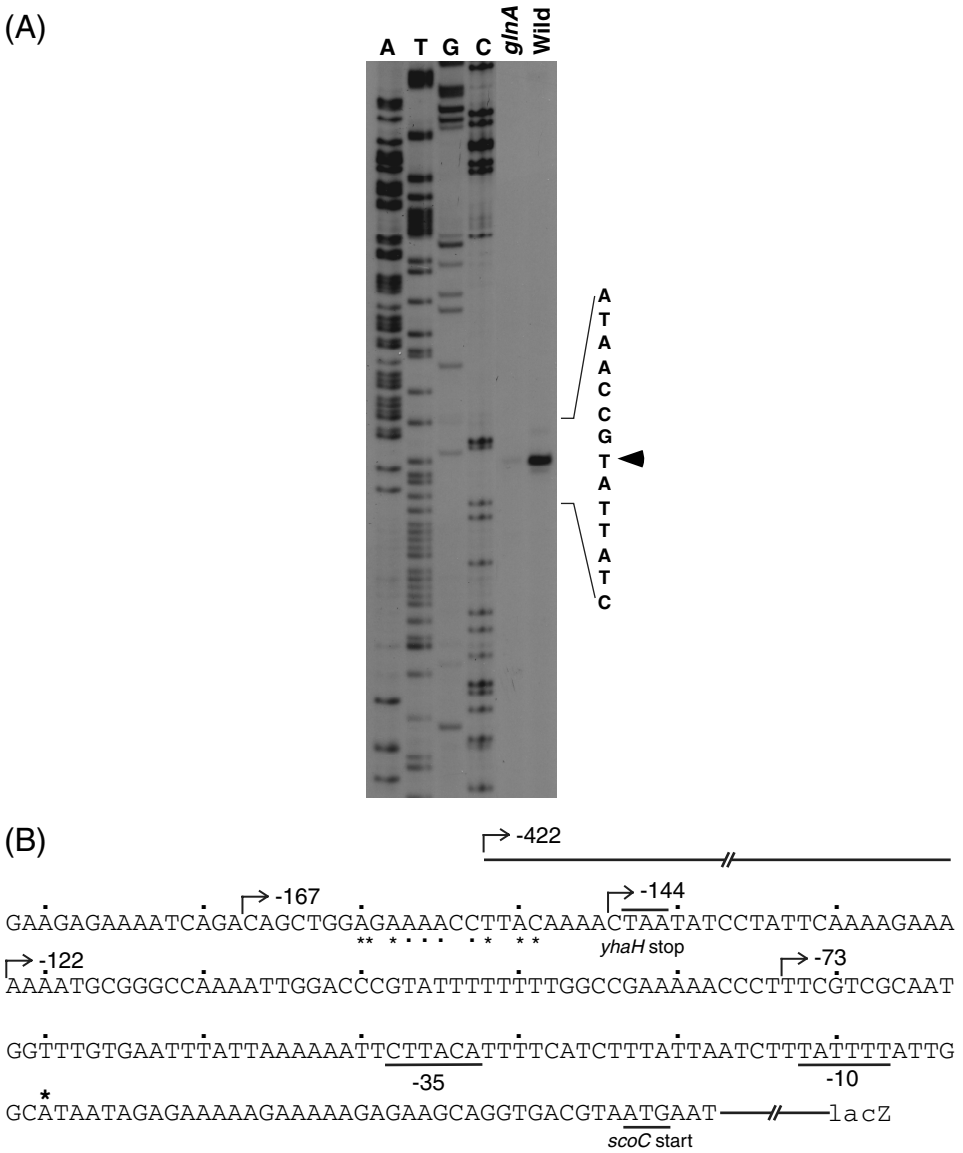

FIG. 4. Determination of the 5′ end of *scoC* mRNA by primer extension analysis (A) and the sequence upstream of the *scoC* gene (B). (A) RNAs were isolated from strains CU741 (wild type) and CU741G (*glnA*) at T2 and used for primer extension analysis as described in Materials and Methods. The arrowhead indicates the transcriptional start site. (B) The asterisk and bent arrows above the sequence indicate the transcriptional start site and the 5′ ends of deletion mutations, respectively. The asterisks and dots under the sequence show the nucleotides that show similarity to the consensus sequence of the TnrA recognition site. The dots above the sequence show the positions introduced at every 10 nucleotides from position −1 relative to the transcriptional initiation site.

−167 and −144. A computer search revealed a sequence similar to the TnrA recognition sequence, TGTNANAWWW TMTNACA (44), located in this DNA region (CTG GAGAAAACCTTACA [underlined nucleotides match the requirement], see also Fig. 4B). To examine whether this sequence is involved in the regulation of *scoC* expression, we constructed strain TSU2M, in which the putative TnrA target sequence in strain TSU2 was changed to CTGGCGTCGCCT GTTAC (underlined nucleotides were changed) and compared the *scoC-lacZ* expression levels in strain TSU2M and its *glnA* mutant, TSU2MG. As shown in Table 3, the expression of *scoC-lacZ* in strain TSU2MG was not inhibited by the *glnA* mutation but was rather high compared to the level found in strain TSU2M. Disruption of *tnrA* did not affect the expression level (TSU2MT) but caused a decrease in the expression level in strain TSU2MG (*glnA*), suggesting participation of TnrA in the slight stimulation. Although the reason for this slight increase is not known at present, the results strongly suggest that the nucleotides that we changed are the constituents of the TnrA target involved in *scoC* regulation. It remains to be studied whether purified TnrA protein binds to this sequence.

Inability of overexpressed *degU* to stimulate *aprE* expression in the *glnA* background. We have previously shown that *degU* expression is stimulated by *glnA* deletion and that this is due to positive regulation of *degU* expression by TnrA (42). It is known that *aprE* expression is stimulated by the phosphory-

TABLE 3. Effect of deletion or sequence alteration of the *scoC* upstream region on *scoC-lacZ* expression

Strain	5′ End point	β-Galactosidase activity (Miller units)[a]
TSU2	−422	8.7
TSU2G (*glnA*)	−422	3.6
TSU31	−167	15
TSU31G (*glnA*)	−167	5.8
TSU32	−144	41
TSU32G (*glnA*)	−144	43
TSU33	−122	49
TSU33G (*glnA*)	−122	53
TSU34	−73	41
TSU34G (*glnA*)	−73	38
TSU2 M	−422	26
TSU2MG (*glnA*)	−422	39
TSU2MT (*tnrA*)	−422	26
TSU2MGT (*glnA tnrA*)	−422	27
AY741L (no fusion)		1.4

[a] Cells were grown in SSMG as described in Materials and Methods. β-Galactosidase activities from T1 to T5 were determined, and the highest values attained at T3 (except for strain TSU2G) (see Fig. 3) are shown.

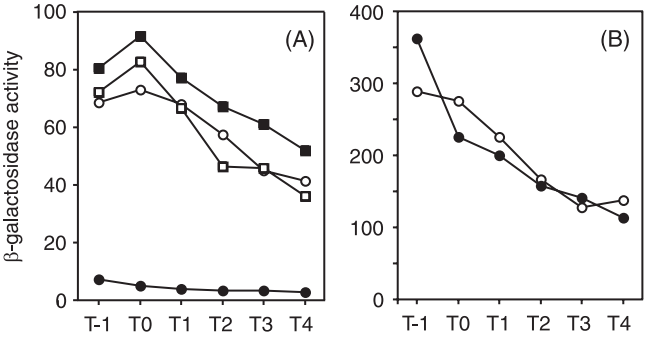

FIG. 5. Effect of *glnA* disruption on *degR-lacZ* driven by σ^D (A)- and σ^A (B)-dependent RNA polymerase. (A) Open circles, ODM50 (wild type); solid circles, AY50G (*glnA*); open squares, AY50T (*tnrA*); solid squares, AY50GT (*glnA tnrA*). (B) Open circles, ODM612 (wild type); solid circles, AYM612 (*glnA*). β-Galactosidase activities were determined as described in Materials and Methods. The data set shown is from one of two experiments, and the variations of the enzyme levels between the experiments were within 15%.

lated form of DegU (19). Thus, if the DegU protein produced in the *glnA* mutant is phosphorylated, *aprE* expression is expected to increase accordingly. To examine this possibility, we used *scoC* knockout mutants to eliminate the effect of *glnA* on *scoC* expression. The results in Table 4 show that under the conditions where *glnA* deletion caused a threefold increase in *aprE* expression in the *scoC*$^+$ strains (compare strains OAM145 and AY145G), the level of *aprE-lacZ* expression in strain AY157G (*scoC glnA*) was much lower than that in strain OAM157 (*scoC*), indicating that the increased expression of *degU* in the *glnA* mutant does not contribute to positive regulation of *aprE*.

Since *glnA* disruption influences the expression of only the *scoC* and *degU* genes among the four DNA-binding factors that regulate *aprE* expression (see above), we expected that the effect of *glnA* deletion on *aprE* expression would not be seen in a *scoC degU* double mutant, and this was indeed the case (Table 4, compare strains AY157U and AY157GU).

These results suggested that although the expression level of *degU* is high in the *glnA* background, the level of functional DegU for *aprE* expression, i.e., the phosphorylated form of DegU, might be low. This notion prompted us to examine the factors that affect the expression of *aprE* through the DegS-DegU route.

TABLE 4. Effect of *glnA*, *scoC*, and *degU* mutations on *aprE* expression

Strain	Relevant genotype	β-Galactosidase activity (Miller units)[a]
OAM145	*aprE-lacZ*	49
AY145G	*aprE-lacZ glnA*	156
AY145U	*aprE-lacZ degU*::Nm^r	15
OAM157	*aprE-lacZ scoC*	484
AY157G	*aprE-lacZ scoC glnA*	256
AY157U	*aprE-lacZ scoC degU*	40
AY157GU	*aprE-lacZ scoC degU glnA*	36

[a] Cell growth and determination of β-galactosidase activities are described in the footnote *a* of Table 3. The highest values attained at either T3 or T4 are shown.

Inhibition of *degR* expression by *glnA* deletion. There are six such factors currently known (Fig. 1). Among these factors, *degR* and *degQ* exhibit larger effects than others in a single-copy state: disruption of *degR* and *degQ* resulted in 66 and 79% reduction in *aprE-lacZ* expression, respectively, in SSM, which we used in this study (25). To examine whether *glnA* deletion affects *aprE* expression through any of these genes, we determined the expression levels of their *lacZ* fusions in the *glnA* background. The results showed that there was no *glnA* effect on the expression of *degQ*, *relA*, *tenA*, and *rapG*, whereas less than 30% inhibition was observed for *proB* expression (data not shown). In contrast, the expression of *degR-lacZ* was greatly reduced in a *glnA* mutant, AY50G, compared to that in the wild-type strain ODM50 (Fig. 5). The expression level of *degR-lacZ* in the *glnA* background was restored to the wild-type level by an additional mutation in *tnrA* (AY50GT), indicating that the effect of *glnA* deletion is through TnrA. The *tnrA* mutation alone did not affect *degR* expression (AY50T).

The inhibitory effect of *glnA* deletion on *degR* expression was also observed when the upstream region of *degR* was deleted from position −422 (ODM50) to −52 (ODM20) (24) with respect to the transcriptional initiation site and also when extensive sequence alterations were introduced between positions −46 and −30, where there is a sequence showing a low similarity to the TnrA recognition sequence (data not shown). These results show that the sequence upstream of position −30 is not subject to regulation by GlnA and raised another possibility that transcription of *degR* driven by σ^D-RNA polymerase (24) might be affected. We have previously reported a strain, ODM 612, in which the recognition sequence of σ^D-RNA polymerase in the *degR* promoter is changed to that of σ^A-RNA polymerase and the *sigD* gene is deleted (24). When this strain was used to examine the effect of *glnA* deletion, no inhibition of *degR-lacZ* expression was observed (Fig. 5B), suggesting strongly that the *glnA* effect on *degR* expression is exerted via σ^D-dependent transcription.

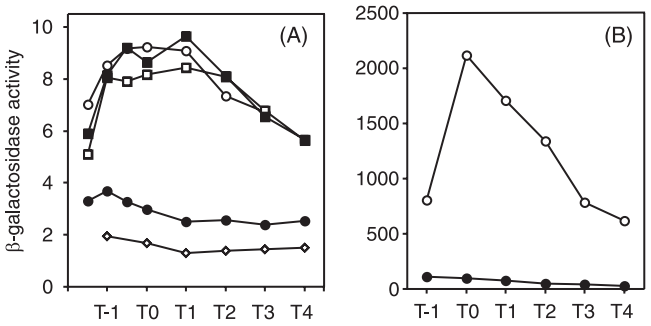

FIG. 6. Inhibition of *sigD-lacZ* (A) and *hag-lacZ* (B) expression by *glnA* deletion. (A) Open circles, ODS200 (wild type); solid circles, AYS200G (*glnA*); open squares, AYS200T (*tnrA*); solid squares, AYS200GT (*glnA tnrA*); diamonds, AY741L (no *lacZ* promoter). The data are from one of three experiments, in which the variations of the enzyme levels were within 10%. (B) Open circles, ODF200 (wild type); solid circles, AYF200G (*glnA*). Results from a typical experiment are shown. β-Galactosidase activities were determined as described in Materials and Methods.

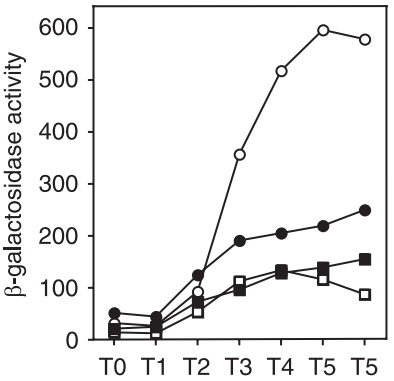

FIG. 7. Effect of *glnA* deletion on *aprE-lacZ* expression in the *scoC* and *scoC degR* background. Cells were grown under the same conditions as described in the legend to Fig. 2. Open circles, OAM157 (*scoC*); solid circles, AY157G (*scoC glnA*); open squares, AY157R (*scoC degR*); solid squares, AY157GR (*scoC degR glnA*). The data points are the means of values obtained from three determinations. The variations of the enzyme levels among the experiments were within 20%.

Inhibition of *sigD* and *hag* expression by *glnA* deletion. The above results prompted us to examine the effect of *glnA* deletion on the expression of the *sigD* gene, encoding the σ^D protein. As shown in Fig. 6A, *sigD-lacZ* expression was greatly reduced in the *glnA* mutant AY200G compared to the wild-type strain ODS200, and furthermore, the inhibition was lost by further addition of a *tnrA* deletion. It is apparent from these results that the inhibitory effect of *glnA* deletion on *degR* expression was caused by inhibition of *sigD* expression, which resulted in a reduction of the σ^D level.

It is known that the flagellin gene *hag* is transcribed by σ^D-RNA polymerase (3, 16). Therefore, if *glnA* deletion causes inhibition of *sigD* expression, it is expected that *hag* expression will also be inhibited by *glnA* deletion, and this was indeed the case, as shown in Fig. 6B. Furthermore, the inhibition of *hag-lacZ* expression was abolished by the introduction of the *tnrA* disruption mutation (data not shown), indicating that the *glnA* effect on *hag* expression is exerted through TnrA.

Regulation of *aprE* expression by *glnA* deletion through inhibition of *scoC* and *degR* expression. It was shown previously that enhanced expression of *aprE* by multicopy *degR* is caused by stabilization of phosphorylated DegU, possibly through inhibition of the dephosphorylation activity of DegS (20, 21). It thus appeared that deletion of *glnA* might reduce the functional activity of DegU through DegR, resulting in reduced *aprE* expression in the *scoC* background. As the inhibition of *aprE* expression by *glnA* deletion was not seen in the *scoC degU* background (Table 4), we presumed that the reduction of *aprE* expression in the *scoC glnA* strain compared the *scoC* mutant (Table 4) might be caused by a reduction in the level of phosphorylated DegU. To examine this possibility, we investigated the effect of deletion of *degR* on the expression of *aprE* in strains carrying *scoC* and *scoC glnA* mutations. If the negative *glnA* effect on *aprE* expression in the *scoC* background is exerted via *degR*, deletion of the *degR* gene would result in similar levels of *aprE-lacZ* expression in both the *scoC and scoC glnA* mutants. It was found that under the condition where *glnA* deletion caused 60% inhibition of *aprE-lacZ* expression in the *scoC* background (Fig. 7, compare OAM157 and AY157G), the inhibitory effect of *glnA* deletion was no longer seen in the *scoC degR* background (Fig. 7, compare AY157R and AY157GR). These results show that inhibition of *degR* by *glnA* deletion is responsible for the decrease in *aprE* expression in the *scoC* background.

The above results show that the *glnA* effect on *aprE* expression was due to repression of *scoC* and *degR* expression and that the reduced expression of *proB* by *glnA* deletion (see above) was not involved. We have shown previously that a null mutation of *proB* resulted in 40% inhibition of *aprE* expression (25). Since this *glnA* effect on *proB* expression is small and, in addition, the effect of *proB* on *aprE* expression is indirect, i.e., through DegS-DegU (21), it is most likely that the inhibitory effect of *proB* on *aprE* expression in the *glnA* background was negligible, resulting in no difference in the *aprE* expression levels between strains AY157GR (*scoC degR glnA*) and AY157R (*scoC degR*) (Fig. 7).

DISCUSSION

It has been well documented that GlnA and TnrA are involved in nitrogen regulation and that the feedback-inhibited GlnA controls TnrA by complex formation, resulting in regulation of various cellular processes (6, 7, 39, 44). To examine whether the expression of the *B. subtilis* exocellular protease genes is under nitrogen regulation, we studied *aprE* expression in cells carrying a deletion in the *glnA* gene and indeed found that it is subject to nitrogen regulation, as demonstrated by the experiments in which *glnA* deletion caused an increase in *aprE* expression (Fig. 2A). It was also shown that the positive effect of *glnA* deletion was through inhibition of the expression of the negative regulator gene *scoC* (Fig. 3), and this was confirmed by primer extension analysis, deletion analysis of the upstream region of the *scoC* gene (Fig. 4), and sequence alteration of the putative TnrA binding site (Table 3).

It was shown previously that the expression of *degU*, encoding a positive regulator of *aprE* expression, was enhanced by *glnA* deletion (42), but this increase did not contribute to

stimulation of *aprE* expression (Table 4). Among the regulators that affect *aprE* expression in a DegS-DegU-dependent manner, the expression of *degR* was severely inhibited by *glnA* deletion (Fig. 5). DegR stabilizes the phosphorylated form of DegU (20). Since *degR* expression was reduced in the *glnA* background, the inability of increased *degU* expression to enhance *aprE* expression was most likely due to the inactive (unphosphorylated) form of DegU. This notion was supported by the experiments in which there was no effect of *glnA* deletion on *aprE* expression in the *scoC degR* background (Fig. 7). These observations led us to conclude that *aprE* expression is under positive and negative nitrogen regulation by the GlnA-TnrA route and that the positive effect through *scoC* repression exceeds the negative effect through repression of *degR* expression.

In the cells carrying a deletion up to position −299 relative to the transcription start point of *aprE*, the expression of *aprE* was substantially reduced in the *glnA* background (AY147G) compared to its wild-type strain OAM147 (Fig. 2B), whereas there was no difference between strains AY241 and AY241G (*glnA*) carrying a deletion up to position −113 (Fig. 2C). These results can be explained on the basis of the presence or absence of the target sites of ScoC and DegU through which DegR exerts its effect. Previous deletion analyses have shown that the target sites of ScoC and DegU are located upstream of position −299 and between positions −164 and −113, respectively (11, 22), indicating that the DegU but not the ScoC target site is present in strains AY147 and AY147G. We conclude, therefore, that the decrease in *aprE* expression in AY147G is due to the negative effect of *glnA* deletion on *degR* expression that results in a decrease in the level of phosphorylated DegU.

We showed that the expression of *degR* and *hag*, both of which are transcribed by σ^D-RNA polymerase, was subject to regulation by the GlnA-TnrA route (Fig. 5A and 6B). In addition, the *sigD* gene, encoding the σ^D factor, was also found to be under the regulation of GlnA-TnrA (Fig. 6A). Since the DNA region upstream of position −30 relative to the transcription initiation site of *degR* is not involved in the GlnA-TnrA regulation (see above), it seems unlikely that the regulation is exerted by direct binding of TnrA to the regulatory region of *degR.* It seems more likely that the direct consequence of *glnA* deletion is repression of *sigD* expression, which then results in a decrease in the σ^D level, leading to inhibition of *degR* expression.

The *sigD* gene is transcribed by at least three promoters, P_{D-3}, *fla*/*che* P_A, and P_{sigD}, and among them transcription only from the *fla*/*che* P_A promoter supplies enough transcripts to support the expression of the *hag* gene (36). As *glnA* deletion causes a decrease in *hag* expression (Fig. 6B), it is possible that transcription from the *fla*/*che* P_A promoter is the target of the GlnA-TnrA route. A future experiment will include study of the binding of the TnrA protein to this region as well as the putative target site upstream of *scoC* found in this study.

The two major global regulators TnrA and CodY play their roles according to the nitrogen status and the GTP level reflecting the energy in the cell, respectively (7, 31). Among the numerous genes regulated by these regulators, some exhibit regulation by both of them. These include *ilv-leu* (34) for the catabolic pathways of branched-chain amino acids, *ureABC* (37) for the degradation of urea, and *gabP* (5) for the transport of gamma-aminobutyrate. It was demonstrated previously that *hag* and the *fla*/*che* operon containing *sigD* are under the control of CodY (4, 17). We showed here that the expression of the *hag* and *sigD* genes is regulated by TnrA, indicating that these genes are new members of the group under the control of both the TnrA and Cod Y regulators.

The experimental condition that we used in this study, i.e., the absence of the glutamine synthetase gene *glnA*, may mimic the situation where the signal transduction through the GlnA-TnrA route is shut off. In other words, it may represent a condition in which TnrA is fully active due to the absence of feedback inhibition by GlnA (39). In this situation, it was found that *aprE* expression was stimulated by repression of the negative regulator gene, *scoC*, while the σ^D-dependent transcription of the *hag* and *degR* genes was repressed (Fig. 5 and 6B). Inhibition of *hag* expression by *glnA* deletion (Fig. 6B) will result in a reduction of the flagellin protein content in the cell, most likely leading to the generation of immobile cells. In an environment where cells secrete a large amount of proteases, it is conceivable that they stay there in order to take up the degradation products and that there is no need for them to elaborate flagella to swim away from the nutrients (18).

The above interpretation is contradictory, however, when the inhibitory effect of *glnA* deletion on *degR* expression is taken into consideration, since DegR is a positive regulator of *aprE* expression. The increased expression of *degU* and the decrease in *degR* expression may result in an increased level of unphosphorylated DegU. DegU is a molecular switch controlling the synthesis of degradative enzymes and competence development in its phosphorylated and unphosphorylated form, respectively (19). We speculate, therefore, that one consequence of *glnA* deletion might be stimulation of competence development, which may be useful for the cell to incorporate DNA for nutrients. A preliminary result has shown that *glnA* deletion affects *comK* expression (data not shown).

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