*J. Biochem.* **136**, 387–397 (2004)
DOI: 10.1093/jb/mvh127

# Mutational Analysis of the Helix-Turn-Helix Region of *Bacillus subtilis* Response Regulator DegU, and Identification of *cis*-Acting Sequences for DegU in the *aprE* and *comK* Promoters

**Kana Shimane and Mitsuo Ogura***
*Department of Marine Science and Technology, Tokai University, 3-20-1 Shimizu-Orido, Shizuoka 424-8610*

Received June 7, 2004; accepted June 29, 2004

**The DegS-DegU two-component system in *Bacillus subtilis* regulates exoprotease production and competence development. Phosphorylated and unphosphorylated forms of DegU are required for activation of *aprE* and *comK*, respectively. Alanine-scanning mutagenesis of the helix-turn-helix region of DegU and *in vivo* examination of 27 DegU variants revealed five common mutants that showed severe reduction of gene expression of both *aprE* and *comK* because of reduced DNA-binding activity. This observation suggested that the DegU-recognized *cis*-sequences might not be considerably changed for either promoter. We identified a DegU-recognized inverted repeat in the *comK* promoter using various mutant *comK-lacZ* fusions. Inspection of the *aprE* promoter sequence revealed a tandem repeat consisting of short AT-rich sequences containing a consensus one, 5′-TAAAT-3′, which was found in the downstream half of the inverted repeat involved in *comK* activation. Oligonucleotide-directed replacement of the short AT-rich sequences located in the center of each motif decreased DegU-dependent *aprE* expression, implying that the repeat is required for the activation of *aprE*. Based on these results, it was concluded that DegU would function through the inverted repeat in the *comK* promoter and the tandem repeat in the *aprE* promoter.**

**Key words: alanine-scanning mutagenesis, *B. subtilis*, DegU, two-component system.**

Abbreviations: Cm, chloramphenicol; Km, kanamycin; Sp, spectinomycin; Amp, ampicillin; Trm, trimethoprim; Em, erythromycin.

To respond to environmental fluctuations, bacteria employ an elaborate tool for signal transduction, a large family of two-component system proteins. A classical two-component system is composed of a sensor kinase and its cognate response regulator (*1*, *2*). The catalytic part of the kinase phosphorylates its own histidine residue by responding to the signal input. The phosphoryl group is then transferred to a conserved aspartate residue on the cognate response regulator, which acts as a transcription factor in most cases. Given the many studies on how the response regulator regulates the output response through phosphorylation, it is not surprising that various strategies have been found (*1*). Upon phosphorylation, some regulators dimerize to be activated, while others are relieved from inhibition exerted by the N-terminal domain.

The response regulator DegU and its cognate histidine kinase DegS of *Bacillus subtilis*, a gram-positive soil bacterium, regulate many cellular processes, including exoprotease production and competence development (*3*–*7*). DegU belongs to the LuxR-FixJ family, whose members have a helix-turn-helix (HTH) structure at the C-terminus (*8*). It has been shown that the DegS-DegU system senses salt stress and mediates appropriate responses (*9*, *10*). Unphosphorylated DegU is required for competence development and binds to the promoter region of *comK* encoding a master regulator of competence development (*11*–*15*). ComK is a transcription factor for late competence operons, such as *comG* (*12*). DegU has been reported to facilitate the binding of ComK to its own promoter (*16*). No other targets of unphosphorylated DegU are known, whereas phosphorylated DegU is known to trigger the expression of many genes, including *aprE*, *nprE*, *sacB*, *sacX* and *degQ,* and repress that of *wapA* (*4*, *6*, *17*, *18*).

Since phosphorylated DegU triggers transcription of *aprE* and unphosphorylated DegU is required for transcription of *comK*, DegU is regarded as a molecular switch controlling cell fate (*3*). To date, the recognition sequences of DegU, multimerization state and affinity of DNA binding, resulting in specific activation of *aprE* but not *comK,* remain unknown. For instance, the DegU-binding sequences in both the *aprE* and *comK* promoters have not been fully determined, although the region protected by DegU from cleavage by DNase I in the *comK* promoter has been reported (*16*).

Expression of *aprE*, which encodes the major alkaline extracellular protease, is controlled through a complex regulatory network, including DegU and Spo0A, the latter of which is a master regulator of sporulation initiation (*19*, *20*). Phosphorylated DegU positively controls *aprE* expression and DegU has been shown to bind to the upstream region of *aprE,* although the sequence recognized by DegU is unknown (*21*). Three repressors have

*To whom correspondence should be addressed. Tel: +81-543-34-0411 Ext 2934, Fax: +81-543-34-9834, E-mail: oguram@scc.u-tokai.ac.jp

 

been shown to negatively regulate *aprE* expression. AbrB binds to the *aprE* promoter to repress *aprE* transcription in the growth phase. At the transition to the stationary phase, phosphorylated Spo0A represses *abrB* transcription, resulting in *aprE* transcription initiation (*20*). ScoC has been identified as a repressor for the expression of *aprE* and *nprE*, the gene encoding the major extracellular neutral protease (*22*). Finally, SinR is known to repress *aprE* expression (*23*).

In this study, we determined the positions of amino acids important for DegU binding to both the *comK* and *aprE* promoters through alanine-scanning analysis of the HTH region of DegU. Five amino acid positions are critical for binding to both promoters, suggesting that DegU might recognize similar *cis*-elements in both promoters. Further, mutational analysis of the *comK* promoter revealed an inverted repeat as the DegU-recognition sequence, while mutational analysis of the *aprE* promoter demonstrated that four direct repeats of the half-site detected in the inverted repeat sequence in the *comK* promoter are required for DegU-dependent expression of *aprE*.

## EXPERIMENTAL PROCEDURES

*Bacterial Strains and Culture Media*—All of the mutant strains used in this study are listed in Table 1 except for those shown in Figs. 1 and 5. Either one-step competence medium (MC, *24*) or Schaeffer's sporulation medium was used for determination of β-galactosidase activity (*25*). *E. coli* cells for DNA manipulation were grown in Luria-Bertani (LB) medium (*26*). The concentrations of antibiotics used were given previously (*27*).

*Materials*—Synthetic oligonucleotides were commercially prepared by Espec Oligo Service (Ibaraki, Japan) and are shown in Table 2. Sequencing was carried out with a 377 DNA Sequencer and a Dye Terminator Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA).

*Construction of Plasmids*—Table 3 lists all plasmids constructed in the present study. To construct pDG-His-degU, pHis-degU was treated with *Xho*I, subsequently with T4 DNA polymerase, and finally with *Hind*III (*21*). The resultant large fragment was cloned into pDG1730 treated with *Eco*RI, subsequently with T4DNA polymerase, and finally with *Hin*dIII (*28*). To construct ptrpBGI-K, the *trpE* fragment of ptrpBGI was replaced with a DNA carrying *Eco*RI-*Bam*HI-*Kpn*I-*Hin*dIII sites constructed through annealing of oligonucleotides *Bam*HI-1 and *Bam*HI-2 (*29*). To construct pComK-BGI, a PCR product obtained using ComK-3 and ComK-4 was treated with *Bam*HI and *Hin*dIII, and then cloned into ptrpBGI-K treated with the same restriction enzymes. To construct pAprE-BGI, a PCR product obtained using AprE-E and AprE-H was treated with *Eco*RI and *Hin*dIII, and then cloned into a large fragment of ptrpBGI treated with the same restriction enzymes, separated by a gel electrophoresis and purified from the gel.

*Construction of Strains and Site-Directed Mutagenesis*—To introduce alanine substitutions into DegU, site-directed mutagenesis of the cloned *degU* cassette in pDG-His-degU was carried out using a QuickChange site-directed mutagenesis kit (Stratagene, La Jolla, CA, USA) according to the instructions provided by the supplier. Introduction of the mutations was confirmed by sequencing of the plasmids. All the mutant *degU* cassettes were entirely sequenced. A plasmid bearing the

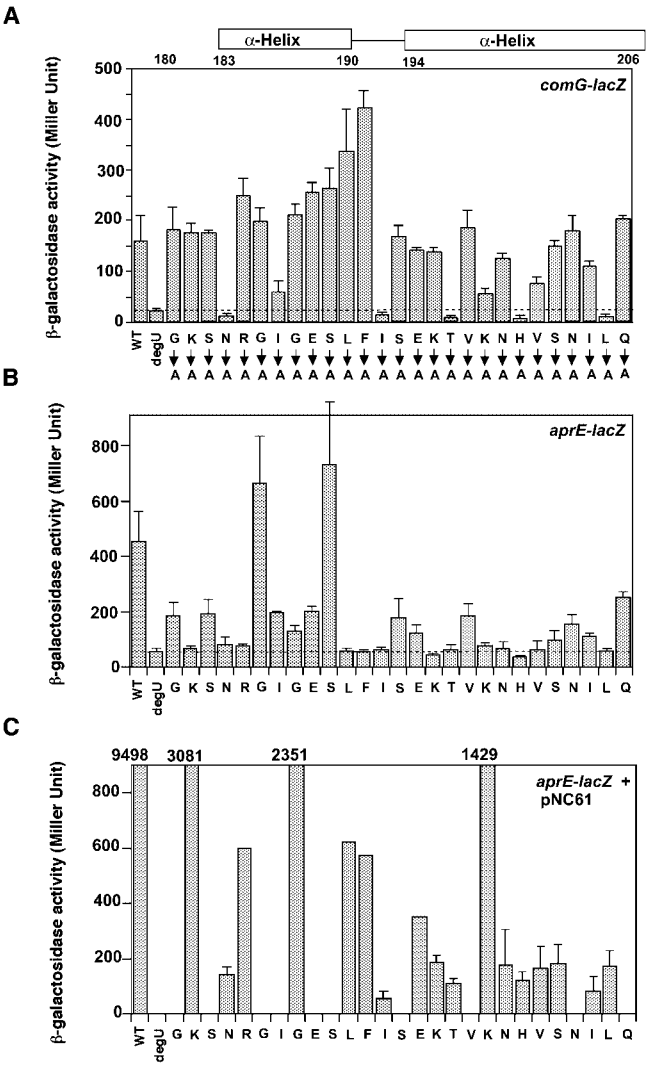


Fig. 1. **Alanine-scanning mutagenesis of the HTH region of DegU.** Cells were grown in modified competence medium (A) and Schaeffer·s sporulation medium (B and C), and β-galactosidase activity was determined as described under Experimental procedures. Data are the means ± SD of the peak values for strains carrying a single alanine substitution in Miller units. For the data with error bars, at least three independent experiments were performed in which samples were withdrawn at hourly intervals in the stationary phase. The letters under the panels are abbreviations for the amino acid residues in the wild-type sequence. The boxes over panel A depict the α-helix 8 and α-helix 9 comprising the HTH region of NarL (*50*). The numbers over panel A indicate the residue numbers of DegU. The dotted lines within panels A and B show the levels of the activities of OAM196 and OAM198, respectively. (A) The activities of WT and *degU* were determined using OAM195 and OAM196, respectively. All mutants are derivatives of OAM195 that bear the mutated *degU* cassette. (B) The activities of WT and degU were obtained with OAM197 and OAM198, respectively. All mutants are derivatives of OAM197 that bear the mutated *degU* cassette. (C) The strains used for activity measurements were the same as those shown in B except for the presence of pNC61 (multicopy *degR*, *33*). The activities outside the scale of the graph are shown as numbers over the graph.

Table 1. ***Bacillus subtilis*** **strains used in this study.**

| Strain | Relevant phenotype and description | Reference or source |
|---|---|---|
| CU741 | *trpC2 leuC7* | Laboratory stock |
| OGM100 | *trpC2 leuC7 comG'-'lacZ* ($Cm^r$) | *27* |
| TT715 | *trpC2 leuC7 aprE'-'lacZ* ($Cm^r$) | *53* |
| OAM195 | *trpC2 leuC7 amyE*::T5promoter-His6-*degU*($Sp^r$) *degU*::$Km^r$ *comG'-'lacZ*($Cm^r$) | This study |
| OAM196 | *trpC2 leuC7 amyE*::($Sp^r$) *degU*::$Km^r$ *comG'-'lacZ*($Cm^r$) | This study |
| OAM197 | *trpC2 leuC7 amyE*::T5promoter-His6-*degU*($Sp^r$) *degU*::$Km^r$ *aprE'-'lacZ*($Cm^r$) | This study |
| OAM198 | *trpC2 leuC7 amyE*::($Sp^r$) *degU*::$Km^r$ *aprE'-'lacZ*($Cm^r$) | This study |
| OAM199 | *trpC2 leuC7 amyE*::*comK'-'lacZ*(–255 to +54, $Cm^r$) | This study |
| OAM225 | *trpC2 leuC7 amyE*::*comK'-'lacZ*(–255 to +54, $Cm^r$) *degU*::$Km^r$ | This study |
| OAM145 | *trpC2 leuC7 amyE*::*aprE'-'lacZ*(–412 to +81, $Cm^r$) | This study |
| OAM207 | *trpC2 leuC7 amyE*::*aprE'-'lacZ*(–412 to +81, $Cm^r$) *degU*::$Km^r$ | This study |

The other strains used in this study are given in the figure legends.

substitution, K207A, could not be constructed for an unknown reason. The resultant mutant pDG-His-degU was introduced into the *B. subtilis* chromosome by transformation. To obtain mutants generated through a double crossover event, Em-sensitive and Sp-resistant colonies were chosen. To construct pComK-BGI carrying mutated *comK-lacZ* fusions, PCR-mediated mutagenesis was performed. PCR using the ComK-3 primer and a primer carrying nucleotide changes, and PCR using the ComK-4 primer and a primer with a sequence complementary to the mutated primer were performed, and the products were purified. Both PCR products were denatured, annealed, treated with T4 DNA polymerase (Takara Co., Shiga, Japan), and then each used as a template for PCR using primers ComK-3 and ComK-4. The PCR product was digested with *Bam*HI and *Hin*dIII, and then cloned into ptrpBGI-K treated with the same restriction enzymes. To construct pAprE-BGI carrying mutant *aprE-lacZ* fusions, similar PCRs to those for the construction of the mutant *comK-lacZ* fusions were performed with the exception of the use of AprE-E and AprE-H instead of ComK-3 and ComK-4. The final PCR product was digested with *Eco*RI and *Hin*dIII, and then cloned into ptrpBGI treated with the same restriction enzymes. All the derivatives of pComK-BGI and pAprE-BGI were introduced into the *amyE* locus in the *B. subtilis* chromosome by transformation after linearization by *Pst*I digestion. The oligonucleotides used for mutagenesis are shown in Table 2.

*Purification of His-Tagged DegU—E. coli* M15 cells carrying pRep4 and pDG-His-degU or its derivative were cultured in 100 ml of LB medium, and IPTG (final concentration, 0.5 mM) was added at the mid-log phase (Klett value, 50). After 5 h, the cells were harvested, collected by centrifugation and resuspended in 1 ml of TE buffer containing 1 mM phenymethylsulfonylfluoride (PMSF). The cells were disrupted with a French pressure cell (Aminco) and then centrifuged. Sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) analysis revealed that most His-tagged DegU was present in the pellet. Purification of His-tagged DegU from the pellet was performed as described previously (*21*).

*β-Galactosidase, Western Blot and Gel Retardation Assays*—Samples were withdrawn at hourly intervals for measurement of β-galactosidase activity as described previously (*30*). For a gel retardation assay a probe DNA was prepared by PCR using AprE-1 and AprE-2 or ComK-1 and ComK-2 as primers (*21*). The method for the gel retardation assay was described previously (*21*). The method used for Western blotting with Anti-DegU antibodies was described previously (*31*).

## RESULTS

*Identification of Amino Acid Positions of the DegU HTH Region Critical for comK Expression*—We first identified amino acid residues of the HTH region of DegU required for *comK* expression. For this purpose, a *degU* cassette with a six-histidine tag at its N-terminus was constructed in plasmid pDG-His-degU, which can be inserted into the chromosomal *amyE* locus via a double crossover event. Transcription of *comK* is regulated at two levels; at transcription itself by the transcription factors, and at the protein-protein interaction among MecA, ClpCP, ComS and ComK itself, and thus overall *comK* transcription is under the control of autoregulatory loop (*12*). Since expression of *comG* is a direct indicator of the ComK protein level that reflects *comK* transcription, *comG-lacZ* was selected for evaluation of possible effects of the mutant *degU* alleles. For the control experiment, the gene encoding wild-type His-tagged DegU was introduced into *amyE* of a strain carrying *comG-lacZ* with a *degU* disruption mutation, generating OAM195 (Table 1). It should be noted that *degU* gene expression is under the control of IPTG (isopropyl β-D-thiogalactopyranoside)–inducible promoter without *lacI*, so that *degU* expression was observed throughout growth without the addition of IPTG (data not shown). In this strain, expression of *comG-lacZ* is comparable to that observed in OGM100, in which the *degU* locus is in the natural context (Fig. 1A and see *27*). Thus, this experimental design was suitable for the screening of mutant alleles of *degU*. We constructed 27 strains carrying alanine substitutions in the HTH region of DegU and found for a strain deficient in *comG-lacZ* expression by means of multiple β-galactosidase assaying of cells growing in competence medium. As shown in Fig. 1A, five strains carrying a mutant *degU* allele with an amino acid substitution, N183A, I192A, T196A, H200A or L205A, did not sustain expression of *comG-lacZ*. We noted that in these mutants expression of *comG-lacZ* was lower than that in the strain carrying wild-type *degU*. Expression of *comK-lacZ* was also examined in strains carrying these five mutations, and β-galactosidase activity due to *comK-lacZ* was

Table 2. **Oligonucleotides used in this study.**

| Name | Sequence | Product (strain or plasmid) |
|---|---|---|
| degU-180-U | 5′-GCTTGCAGACGCAAAAAGCAAC-3′ | pDG-HIs-degU(G180A) |
| degU-180-D | 5′-GTTGCTTTTTGCGTCTGCAAGC-3′ | pDG-HIs-degU(G180A) |
| degU-181-U | 5′-TGCAGACGGAGCAAGCAACCGC-3′ | pDG-HIs-degU(K181A) |
| degU-181-D | 5′-GCGGTTGCTTGCTCCGTCTGCA-3′ | pDG-HIs-degU(K181A) |
| degU-182-U | 5′-AGACGGAAAAGCCAACCGCGGT-3′ | pDG-HIs-degU(S182A) |
| degU-182-D | 5′-ACCGCGGTTGGCTTTTCCGTCT-3′ | pDG-HIs-degU(S182A) |
| degU-183-U | 5′-CGGAAAAAGCGCCCGCGGTATT-3′ | pDG-HIs-degU(N183A) |
| degU-183-D | 5′-AATACCGCGGGCGCTTTTTCCG-3′ | pDG-HIs-degU(N183A) |
| degU-184-U | 5′-AAAAAGCAACGCCGGTATTGGT-3′ | pDG-HIs-degU(R184A) |
| degU-184-D | 5′-ACCAATACCGGCGTTGCTTTTT-3′ | pDG-HIs-degU(R184A) |
| degU-185-U | 5′-AAGCAACCGCGCTATTGGTGAA-3′ | pDG-HIs-degU(G185A) |
| degU-185-D | 5′-TTCACCAATAGCGCGGTTGCTT-3′ | pDG-HIs-degU(G185A) |
| degU-186-U | 5′-CAACCGCGGTGCTGGTGAATCA-3′ | pDG-HIs-degU(I186A) |
| degU-186-D | 5′-TGATTCACCAGCACCGCGGTTG-3′ | pDG-HIs-degU(I186A) |
| degU-187-U | 5′-CCGCGGTATTGCTGAATCATTG-3′ | pDG-HIs-degU(G187A) |
| degU-187-D | 5′-CAATGATTCAGCAATACCGCGG-3′ | pDG-HIs-degU(G187A) |
| degU-188-U | 5′-CGGTATTGGTGCATCATTGTTT-3′ | pDG-HIs-degU(E188A) |
| degU-188-D | 5′-AAACAATGATGCACCAATACCG-3′ | pDG-HIs-degU(E188A) |
| degU-189-U | 5′-TATTGGTGAAGCATTGTTTATC-3′ | pDG-HIs-degU(S189A) |
| degU-189-D | 5′-GATAAACAATGCTTCACCAATA-3′ | pDG-HIs-degU(S189A) |
| degU-190-U | 5′-TGGTGAATCAGCGTTTATCAGT-3′ | pDG-HIs-degU(L190A) |
| degU-190-D | 5′-ACTGATAAACGCTGATTCACCA-3′ | pDG-HIs-degU(L190A) |
| degU-191-U | 5′-TGAATCATTGGCTATCAGTGAG-3′ | pDG-HIs-degU(F191A) |
| degU-191-D | 5′-CTCACTGATAGCCAATGATTCA-3′ | pDG-HIs-degU(F191A) |
| degU-192-U | 5′-ATCATTGTTTGCCAGTGAGAAA-3′ | pDG-HIs-degU(I192A) |
| degU-192-D | 5′-TTTCTCACTGGCAAACAATGAT-3′ | pDG-HIs-degU(I192A) |
| degU-193-U | 5′-ATTGTTTATCGCTGAGAAAACC-3′ | pDG-HIs-degU(S193A) |
| degU-193-D | 5′-GGTTTTCTCAGCGATAAACAAT-3′ | pDG-HIs-degU(S193A) |
| degU-194-U | 5′-GTTTATCAGTGCGAAAACCGTT-3′ | pDG-HIs-degU(E194A) |
| degU-194-D | 5′-AACGGTTTTCGCACTGATAAAC-3′ | pDG-HIs-degU(E194A) |
| degU-195-U | 5′-TATCAGTGAGGCAACCGTTAAA-3′ | pDG-HIs-degU(K195A) |
| degU-195-D | 5′-TTTAACGGTTGCCTCACTGATA-3′ | pDG-HIs-degU(K195A) |
| degU-196-U | 5′-CAGTGAGAAAGCCGTTAAAAAC-3′ | pDG-HIs-degU(T196A) |
| degU-196-D | 5′-GTTTTTAACGGCTTTCTCACTG-3′ | pDG-HIs-degU(T196A) |
| degU-197-U | 5′-TGAGAAAACCGCTAAAAACCAT-3′ | pDG-HIs-degU(V197A) |
| degU-197-D | 5′-ATGGTTTTTAGCGGTTTTCTCA-3′ | pDG-HIs-degU(V197A) |
| degU-198-U | 5′-GAAAACCGTTGCAAACCATGTC-3′ | pDG-HIs-degU(K198A) |
| degU-198-D | 5′-GACATGGTTTGCAACGGTTTTC-3′ | pDG-HIs-degU(K198A) |
| degU-199-U | 5′-AACCGTTAAAGCCCATGTCAGC-3′ | pDG-HIs-degU(N199A) |
| degU-199-D | 5′-GCTGACATGGGCTTTAACGGTT-3′ | pDG-HIs-degU(N199A) |
| degU-200-U | 5′-CGTTAAAAACGCTGTCAGCAAT-3′ | pDG-HIs-degU(H200A) |
| degU-200-D | 5′-ATTGCTGACAGCGTTTTTAACG-3′ | pDG-HIs-degU(H200A) |
| degU-201-U | 5′-TAAAAACCATGCCAGCAATATT-3′ | pDG-HIs-degU(V201A) |
| degU-201-D | 5′-AATATTGCTGGCATGGTTTTTA-3′ | pDG-HIs-degU(V201A) |
| degU-202-U | 5′-AAACCATGTCGCCAATATTTTA-3′ | pDG-HIs-degU(S202A) |
| degU-202-D | 5′-TAAAATATTGGCGACATGGTTT-3′ | pDG-HIs-degU(S202A) |
| degU-203-U | 5′-CCATGTCAGCGCTATTTTACAA-3′ | pDG-HIs-degU(N203A) |
| degU-203-D | 5′-TTGTAAAATAGCGCTGACATGG-3′ | pDG-HIs-degU(N203A) |
| degU-204-U | 5′-TGTCAGCAATGCTTTACAAAAA-3′ | pDG-HIs-degU(I204A) |
| degU-204-D | 5′-TTTTTGTAAAGCATTGCTGACA-3′ | pDG-HIs-degU(I204A) |
| degU-205-U | 5′-CAGCAATATTGCACAAAAAATG-3′ | pDG-HIs-degU(L205A) |
| degU-205-D | 5′-CATTTTTTGTGCAATATTGCTG-3′ | pDG-HIs-degU(L205A) |
| degU-206-U | 5′-CAATATTTTAGCAAAAATGAAT-3′ | pDG-HIs-degU(Q206A) |
| degU-206-D | 5′-ATTCATTTTTTGCTAAAATATTG-3′ | pDG-HIs-degU(Q206A) |
| ComK-1 | 5′-CCGGAATTCAGAATCCCCCCAATGCC-3′ | |
| ComK-2 | 5′-Biotin-CGGGATCCCAGTCTGTTTTCTGACTCATATT-3′ | |
| ComK-3 | 5′-ATTGGATCCGGAACAATTGTGAACGGATAA-3′ | pComK-BGI (OAM199) |
| ComK-4 | 5′-ATCAAGCTTCAGTCTGTTTTCTGACTCATATT-3′ | pComK-BGI (OAM199) |
| *Bam*HI-1 | 5′-AATTCTAGGATCCATGGTACCA-3′ | ptrpBGI-K |
| *Bam*HI-2 | 5′-AGCTTGGATCCATGGATCCTAG-3′ | ptrpBGI-K |

Table 2. **Continued.**

| Name | Sequence | Product (strain or plasmid) |
|---|---|---|
| ComK-36 | 5′-TTATTACTCTGCATTTAGT-3′ | OAM201 |
| ComK-37 | 5′-ACTAAATGCAGAGTAATAA-3′ | OAM201 |
| ComK-38 | 5′-ACTAGTCAGGGAGTACCAT-3′ | OAM202 |
| ComK-39 | 5′-ATGGTACTCCCTGACTAGT-3′ | OAM202 |
| ComK-40 | 5′-GTCATTTATGCCCATTAAA-3′ | OAM203 |
| ComK-41 | 5′-TTTAATGGGCATAAATGAC-3′ | OAM203 |
| ComK-42 | 5′-TTAGTACCCGGAAATATCA-3′ | OAM204 |
| ComK-43 | 5′-TGATATTTCCGGGTACTAA-3′ | OAM204 |
| ComK-30 | 5′-TTTAGTACCATTCCCTATCATTAAAAG-3′ | OAM205 |
| ComK-31 | 5′-CTTTTAATGATAGGGAATGGTACTAAA-3′ | OAM205 |
| ComK-32 | 5′-TACCATTAAACGCCATTAAAAGAT-3′ | OAM206 |
| ComK-33 | 5′-ATCTTTTAATGGCGTTTAATGGTA-3′ | OAM206 |
| ComK-44 | 5′-ATGACCATTTAGTATCATTAA-3′ | OAM217 |
| ComK-45 | 5′-TTAATGATACTAAATGGTACT–3′ | OAM217 |
| AprE-1 | 5′-biotin -CATGTCTATTTTCGTTC-3′ | |
| AprE-2 | 5′-GATCCACAATTTTTTGCTTC-3′ | |
| AprE-E | 5′-ATCGAATTCGGCCGCATCTGATGTCTTTG-3′ | pAprE-BGI (OAM145) |
| AprE-H | 5′-ATCAAGCTTGATCCACAATTTTTTGCTTCTCA-3′ | pAprE-BGI (OAM145) |
| AprE-3 | 5′-TTTTCTGTATGACGGTAGTTATTT-3′ | OAM208 |
| AprE-4 | 5′-AAATAACTACCGTCATACAGAAAA-3′ | OAM208 |
| AprE-13 | 5′-CTCTACGGGGGCAGCGAGAG-3′ | OAM209 |
| AprE-14 | 5′-CTCTCGCTGCCCCCGTAGAG-3′ | OAM209 |
| AprE-30 | 5′-TAAATAGAGATCCCCTCATCTCAAAA-3′ | OAM210 |
| AprE-31 | 5′-TTTTGAGATGAGGGGATCTCTATTTA-3′ | OAM210 |
| AprE-36 | 5′-ATGATATACCTCCCGAGAGATAAAATCA-3′ | OAM212 |
| AprE-37 | 5′-TGATTTTATCTCTGGGCAGGTATATCAT-3′ | OAM212 |
| AprE-38 | 5′-ATCATCTCAAATTTTTGGGTCTACTA-3′ | OAM214 |
| AprE-39 | 5′-TAGTAGACCCAAAAATTTGAGATGAT-3′ | OAM214 |
| AprE-40 | 5′-ATGGGTCTACTTTTTTATTATTCCAT-3′ | OAM216 |
| AprE-41 | 5′-ATGGAATAATAAAAAAGTAGACCCAT-3′ | OAM216 |

abolished in all of the strains (data not shown). These results were completely consistent with the results shown in Fig. 1A. We next examined the *in vivo* stability of the DegU variants by Western analysis, because the alanine substitution might cause loss of protein stability, leading to abolition of *comG-lacZ* expression. Figure 2A shows that similar levels of DegU in the wild-type strain were observed in all five mutants. Thus, we concluded that these five DegU mutants were deficient in some function required for *comK* expression but not protein stability.

*DegU Mutants Lost DNA-Binding Activity toward the comK Promoter*—To examine a possible change in DNA-binding activity of these mutants, His-tagged DegU proteins were produced in *E. coli* and all the His-tagged

Table 3. **Plasmids used in this study.**

| Plasmid | Description | Reference or source |
|---|---|---|
| pDG1730 | Em$^r$ Sp$^r$ Amp$^r$ | *28* |
| pHis-degU | Amp$^r$ T5 promoter-His6-*degU* | *21* |
| pDG-His-degU | Em$^r$ Sp$^r$ Amp$^r$ T5 promoter-His6-*degU* | This study |
| pNC61 | Trm$^r$ *degR* | *33* |
| ptrpBG1 | Cm$^r$ Amp$^r$ | *29* |
| ptrpBG1-K | Cm$^r$ Amp$^r$ | This study |
| pComK-BG1 | Cm$^r$ Amp$^r$ *comK′-′lacZ* (–255 to +54) | This study |
| pAprE-BG1 | Cm$^r$ Amp$^r$ *aprE′-′lacZ* (–412 to +81) | This study |

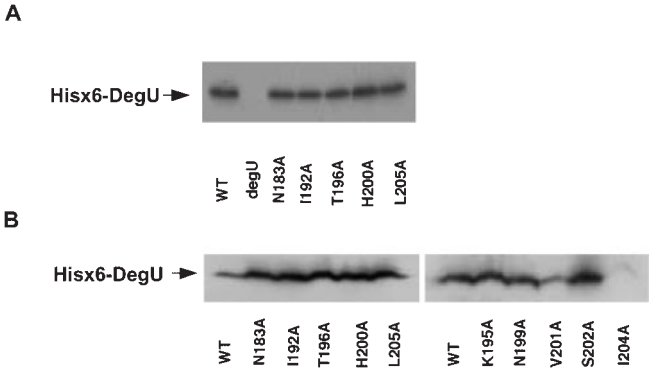


Fig. 2. **Western analysis of DegU mutants.** The mutation of each DegU is indicated under the panels. Cell-free extracts were made of derivatives of OAM195 carrying the mutated *degU* cassette. (A) Cells grown in 50 ml of modified competence medium (MC) were withdrawn 2 h after entry into the stationary phase, washed and resuspended in 1 ml of TE buffer (Tris-HCl 10 mM [pH 8.0], EDTA 1 mM), and then subjected to lysis with a French press. Equal amounts of protein (about 50 μg) were applied on a 14% SDS-polyacrylamide gel, and the DegU protein was detected as described under Experimental procedures. For the wild-type and null mutations of *degU*, OAM195 and OAM196 were used to prepare cell-free extracts, respectively. (B) Cells grown in 50 ml of Schaeffer·s sporulation medium were withdrawn at the same time as in A. Preparation of cell free extracts was carried out by the same method as described in the legend to A. For the wild-type, OAM197 was used to prepare cell-free extracts.

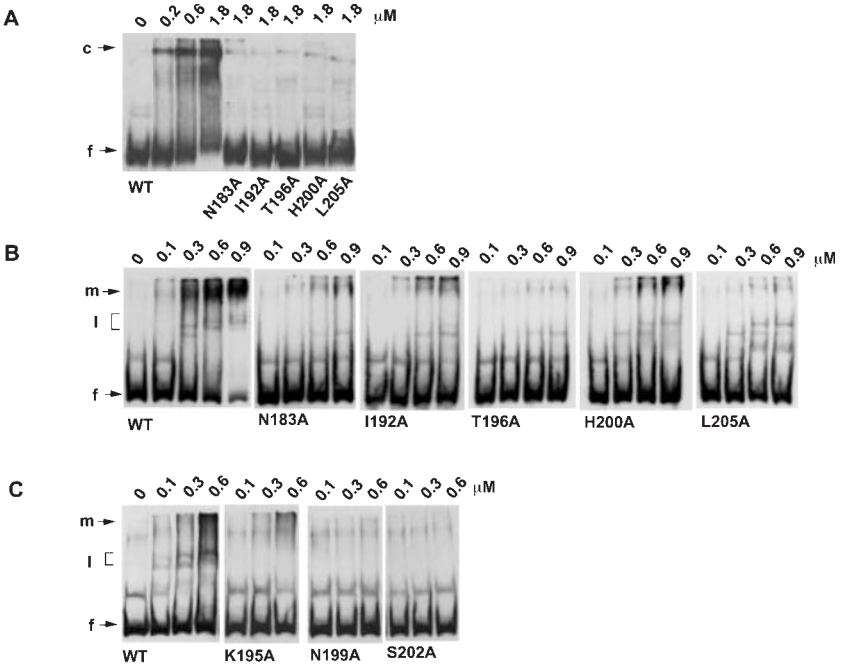


Fig. 3. **Gel retardation assaying revealed loss of binding of the DegU mutants to the *comK* and *aprE* promoters.** The gel retardation assay was performed as described under Experimental procedures. A 6% native polyacrylamide gel was used. The final concentrations of each protein are indicated at the top of the panel. The mutations of each DegU protein are indicated under the panels. (A) The *comK* probe DNA spanned positions –255 to +54 relative to the transcription start site, and was prepared by PCR using ComK-1 and ComK-2. The probe was the same as used in ref. *18*. c and f indicate the protein-DNA complex and free probe, respectively. All reaction mixtures contained poly dI-dC (1 μg/25 μl). (B) and (C) The *aprE* probe DNA spanned positions –146 to +81, and was prepared by PCR using AprE-1 and AprE-2. m, l and f indicate a multimer complex, ladder-like complexes and the free probe, respectively. All reaction mixtures contained poly dI-dC (1 μg/25 μl). The probes were incubated with DegU at increasing concentrations.

DegU proteins were purified from the insoluble fraction as a single band on SDS-PAGE analysis using Ni-affinity chromatography (*21* [wild-type DegU], data not shown for the mutants). Soluble forms of the denatured proteins were regenerated by removing urea from the protein fraction gradually. After the regeneration, we examined the secondary structures of the proteins by CD spectral analysis and observed that all the DegU variants have the same secondary structure (data not shown). The wild-type His-tagged DegU protein exhibited specific binding to the *comK* promoter (–255 to +55 relative to the transcription start site), as shown in Fig. 3A, since the reaction mixture contained a thousand-fold amount of poly dI-dC to the probe. It should be noted that the protein-DNA stoichiometry in the reaction is similar to that reported previously (*16*). A gel retardation assay revealed that all five mutants failed to generate comparable levels of a protein-DNA complex to that observed for the wild-type, indicating that these mutants had lost DNA-binding activity toward the *comK* promoter. These results accounted for the *in vivo* defect of *comG-lacZ* expression in the strains carrying the mutant *degU* cassette.

*Identification of Amino Acid Residues of the DegU HTH Region Required for aprE Expression*—To examine possible effects of alanine substitution of DegU on *aprE-lacZ* expression, we adopted the same experimental system as that used for the screening of mutants in which *comG-lacZ* expression was decreased. As is the case for *comG-lacZ*, the *degU* allele encoding wild-type His-tagged DegU was able to drive *aprE-lacZ* expression, as shown in Fig. 1B. Thus, we constructed derivatives of OAM197 (*aprE-lacZ degU amyE*::T5 promoter-His6-*degU*) and examined the β-galactosidase activity of the strains growing in sporulation medium. Of the 27 mutants thus constructed, 17 showed more than a 70% decrease in *aprE-lacZ* expression (Fig. 1B). Among these mutants there might be a strain carrying a *degU* mutant whose product is less active as to DegS-dependent phosphorylation. In such a strain *aprE-lacZ* expression would be enhanced under the genetic conditions causing stabilization of phosphorylated DegU. To exclude such a mutant from further analysis, multicopy *degR*, which is known to enhance *aprE-lacZ* expression through stabilization of phosphorylated DegU, was introduced into these 17 strains, and *aprE-lacZ* expression was observed in the resultant strains (*32*–*33*). Ten strains with substitutions, N183A, I192A, K195A, T196A, N199A, H200A, V201A, S202A, I204A and L205A, still showed low levels of *aprE-lacZ* expression (Fig. 1C). Next, we examined the protein stability of these DegU mutants by Western analysis of cells growing in sporulation medium (Fig. 2B). A reduced and no protein band were observed for V201A and I204A, respectively, indicating that instability of the mutants caused a decrease in *aprE-lacZ* expression. The remaining eight mutants showed a steady-state level of DegU, demonstrating that these eight proteins were stable *in vivo*.

*Eight DegU Mutants Showed Reduced DNA Binding to the aprE Promoter*—Three mutants were purified as His-tagged proteins using a Ni-affinity column in addition to the five for the *comK* promoter. We determined the DNA-binding activities of these mutants by a gel retardation assay using the *aprE* promoter (–146 to +81 relative to the transcription start site). The *aprE* promoter is a target of phosphorylated DegU according to a former study, however, we used unphosphorylated DegU in this experiment, because of the difficulty of purification of the active form of DegS kinase. Moreover, the addition of acetyl-phosphate, which could phosphorylate some response regulators, such as ComA, without the cognate kinase (*34*), did not affect DNA binding of DegU (data not shown). The addition of wild-type DegU to the reaction mixture generated ladder-like signals and a highly shifted multimer complex, as reported previously (Fig. 3B, *21*). We analyzed the mutant proteins by a gel retardation assay. All eight DegU mutants showed reduced binding activity, although the extent of the decrease var-

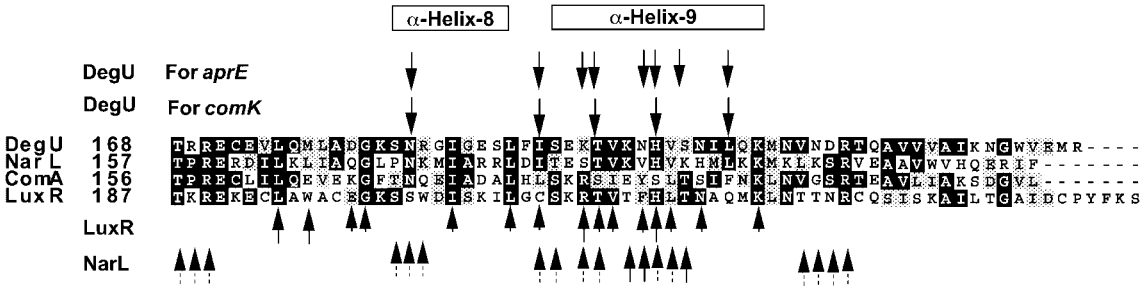


Fig. 4. **Results of mutational analysis and alignment of the C-terminus containing the HTH region of the LuxR-FixJ family.** The numbers indicate the positions of amino acids starting from the first amino acid of each protein. Black and gray backgrounds indicate identical and similar amino acids, respectively. Multiple alignment was carried out with CRUSTAL W (*51*), and the figure was made with BOXSHADE. The boxes over the alignment depict α-helix 8 and α-helix 9 in the NarL HTH region (*50*). The results shown in Figs. 1A, C and 3 are summarized, that is, amino acids critical for DegU binding to the *comK* and *aprE* promoters are shown with arrows. Amino acids whose substitution had strong and weak effects on DNA binding of LuxR are indicated by long and short arrows, respectively (*47*). According to structural analysis of a NarL-DNA complex, amino acids with side-chains in contact with the major groove floor are indicated by solid arrows (*39*). Those in contact with DNA via peptide chains or in contact with the sugar-phosphate backbone are shown by dotted arrows.

ied with the mutant (Fig. 3B and C). These results were consistent with the *in vivo* effects of the mutations on *aprE-lacZ* expression, and are summarized in Fig. 4. We noted that five that did not sustain expression of *comG-lacZ* and binding to the *comK* promoter also showed reduced binding to the *aprE* promoter. These results suggested that the cis-sequences for DegU of *comK* and *aprE* might not be significantly different although the amino acid(s) that contributes to sequence-specific recognition of DegU via binding to bases through hydrogen bonding remains unknown.

*Precise Mapping of the DegU-Recognized Sequence in the comK Promoter*—A 30-base pair region of the *comK* promoter protected by DegU from DNase I cleavage has been reported, and an inverted repeat located in this region, 5′-TACTA-N6-TAGTA-3′ (shown as a pair of dotted arrows in Fig. 5A), was recognized previously as a putative DegU recognition sequence (*16*). To accurately identify the DegU-recognized sequence, we constructed seven strains with *comK-lacZ* fusions with substitution of blocks of two to three bases and then measured the β-galactosidase activities of the mutant fusions. As shown in Fig. 5A, the β-galactosidase activities of OAM201, OAM202, OAM204, OAM205 and OAM217 were decreased, but those of the other fusions were not, compared to that observed for the wild-type, OAM199. Consideration of these results indicated the probability that DegU recognizes an inverted repeat, 5′-G-N2-ATTTA-N7-TAAAT-N2-C-3′, but not 5′-TACTA-N6-TAGTA-3′. We note that in OAM217 a fusion carrying a mutation that creates the complete direct repeat of 5′-CATTTAG-3′ but disrupts the identified inverted repeat showed decreased expression. In addition, a direct repeat of the sequence 5′-TCATT-3′ was found (tandem dotted arrows in Fig. 5A). The promoter-proximal half-site of this direct repeat, however, is out of the region protected from DNase I cleavage, and therefore it is unlikely that this direct repeat is important for DegU binding.

*In Vivo Examination of the Direct Repeat in the aprE Promoter*—To identify DegU-acting sequences in the *aprE* promoter, we inspected the *aprE* promoter sequence in the DNA fragment (–146 to +81), which is sufficient for DegU binding in a gel shift experiment, and identified six repeats of the 5′-TAAAT-3′–like motif. To determine the role of each of the motifs, which are sequentially designated as site I through VI (Fig. 5B), we constructed strains that carry an *aprE-lacZ* fusion bearing a substitution of the motif, and examined the effects of the mutations on expression of the fusions. Mutations of the promoter distal sites, sites I and II, did not cause significant decreases in the promoter activity, indicating that these sites do not play important roles in activation of the *aprE* promoter by DegU. The –35 region is located between sites V and VI, which should be AT-rich for the basal activity of the promoter (*35*). Indeed, independent substitution of these two sites by GC stretches resulted in abolition of the promoter activity (data not shown), and thus these two sites were substituted by AT stretches. Mutation of sites III, IV, V and VI resulted in reduction to 30%, 37%, 35% and 30% of that for the wild-type fusion, respectively, suggesting that these sites play important roles in activation of the *aprE* promoter. Next, to examine the *degU*-independent expression of the fusions, the *degU*::Km cassette was introduced into each strain and then we determined the β-galactosidase activity of each strain. Expression of the wild-type *aprE-lacZ* fusion in the *degU* background was decreased to 15% of that observed in the wild-type background, demonstrating the high dependency of the wild-type fusion on *degU*. On the other hand, the *degU* disruption allowed the mutated fusions as to sites III, IV, V and VI to express 90%, 71%, 42% and 31% of the β-galactosidase activities observed for the corresponding reference strains, respectively, indicating that the mutations released *aprE-lacZ* expression from requirement of the *degU* gene to different extents (Fig. 4B). In particular, mutation of sites III and IV caused severe reduction of the *degU*-dependency of *aprE-lacZ* expression, indicating that the tandem repeat of two motifs plays a major role in DegU-dependent *aprE* expression. We noted that the *degU*-independent expression of these two fusions was two-fold higher than that observed for the wild-type fusion. This enhancement might be caused by possible alleviated binding of ScoC or AbrB to the mutated *aprE* promoters because the substitutions were located in regions near the binding sites for these repressors (see Fig. 6). Furthermore, to examine

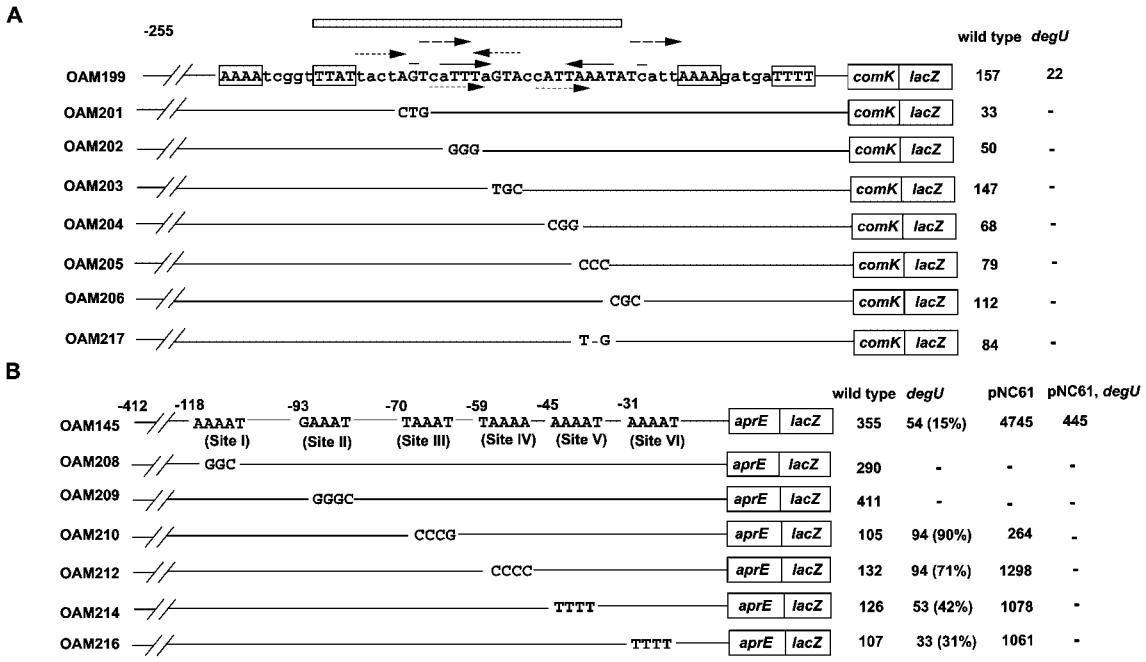


Fig. 5. ***In vivo* examination of the cis-acting sequence for DegU.** The lines and boxes indicate the regions containing the promoter of *comK* (A) or *aprE* (B), and the fusions between an N-terminal region of that and *lacZ*, respectively. The numbers over the control fusions (OAM199 in A and OAM145 in B) indicate the nucleotides relative to the transcriptional start sites of *comK* and *aprE*, respectively. Cells were grown in modified competence medium (A) and Schaeffer's sporulation medium (B), and β-galactosidase activity was determined as described under Experimental procedures. At least three independent experiments were performed in which samples were withdrawn at hourly intervals in the stationary phase. The averages of the peak values are shown. The standard deviations were within 15% (A) and 20% (B) in all experiments. (A) The partial nucleotide sequence of the wild-type *comK-lacZ* fusion in OAM199 is shown. The box and four pairs of solid or dotted arrows indicate the region protected by DegU from DNase I cleavage (*16*), and the inverted or direct repeats, respectively. The pair of solid arrows shows the DegU-recognition sequence identified in this study. The capital letters in the nucleotide sequence indicate the substituted nucleotides in the mutated fusions. The overlapping boxes in the sequence show the *comK* boxes. The numbers in the column show β-galactosidase activity expressed in Miller units. OAM201, OAM202, OAM203, OAM204, OAM205, OAM206 and OAM217 are derivatives of OAM199 (Table 1), carrying the indicated mutations on the *comK* promoter. (B) The structure and six half-sites of the DegU-recognized inverted repeat of the wild-type *aprE-lacZ* fusion in OAM145 are shown. The numbers in the columns show β-galactosidase activity expressed in Miller units. Those in parentheses in the second column show the ratios of the activities of the *degU* strains to those of the reference strains. OAM208, OAM209, OAM210, OAM212, OAM214 and OAM216 are derivatives of OAM145 (Table 1), carrying the indicated mutations on the *aprE* promoter. OAM145, OAM210, OAM212, OAM214 and OAM216 were transformed with the *degU*::Km$^r$ cassette, generating OAM207 (Table 1), OAM211, OAM213, OAM215 and OAM226, respectively.

the effects of an increase in the amount of phosphorylated DegU on these mutated promoter fusions, we introduced plasmid pNC61 (multicopy *degR*) into the strains and then measured β-galactosidase activities. As a result, the enhancing effect of pNC61 was almost completely abolished in OAM210 (the site III-mutated fusion), and the effects were reduced in the other three mutants. These results indicated that the direct repeat is important for phosphorylated-DegU-dependent *aprE* expression.

Taken together, these results indicated that the direct repeat composed of the four sites is involved in DegU-dependent expression of *aprE*.

## DISCUSSION

In this study, we determined the DegU-acting regions in the *comK* and *aprE* promoters. DegU would bind to the inverted repeat in the *comK* promoter, and function through the direct repeat of the half-site in the *aprE* promoter. Response regulators that belong to the FixJ-LuxR family, including DegU, GerE, NarL, LuxR and UhpA, recognize an inverted repeat sequence in general (*8*, *36*–*39*). On the other hand, a direct repeat sequence has been demonstrated to be recognized by response regulators that belong to the OmpR family, characterized by a winged HTH structure (*40*). Therefore, it may be peculiar to some extent that DegU can bind to both inverted and direct repeats. This case, however, is not unprecedented. NarL binds a target through two different combinations of the same motifs, *i.e.*, head-to-head and head-to-tail arrangements, and the difference in arrangement corresponds to its function, that is, repression or activation (*41*). The RepA protein of *Pseudomonas* plasmid pPS10 bound to an inverted repeat and its half-site as both dimer and monomer forms, respectively (*42*). In addition, the *Escherichia coli* mini-F plasmid initiator RepE functions as a monomer and a dimer for replication initiation and gene repression, respectively (*43*).

The identified nucleotide sequences required for DegU-dependent regulation for both the *comK* and *aprE* promoters are short AT-rich sequences, which are present in many promoter regions in the *B. subtilis* chromosome. Therefore, additional nucleotides might be required for

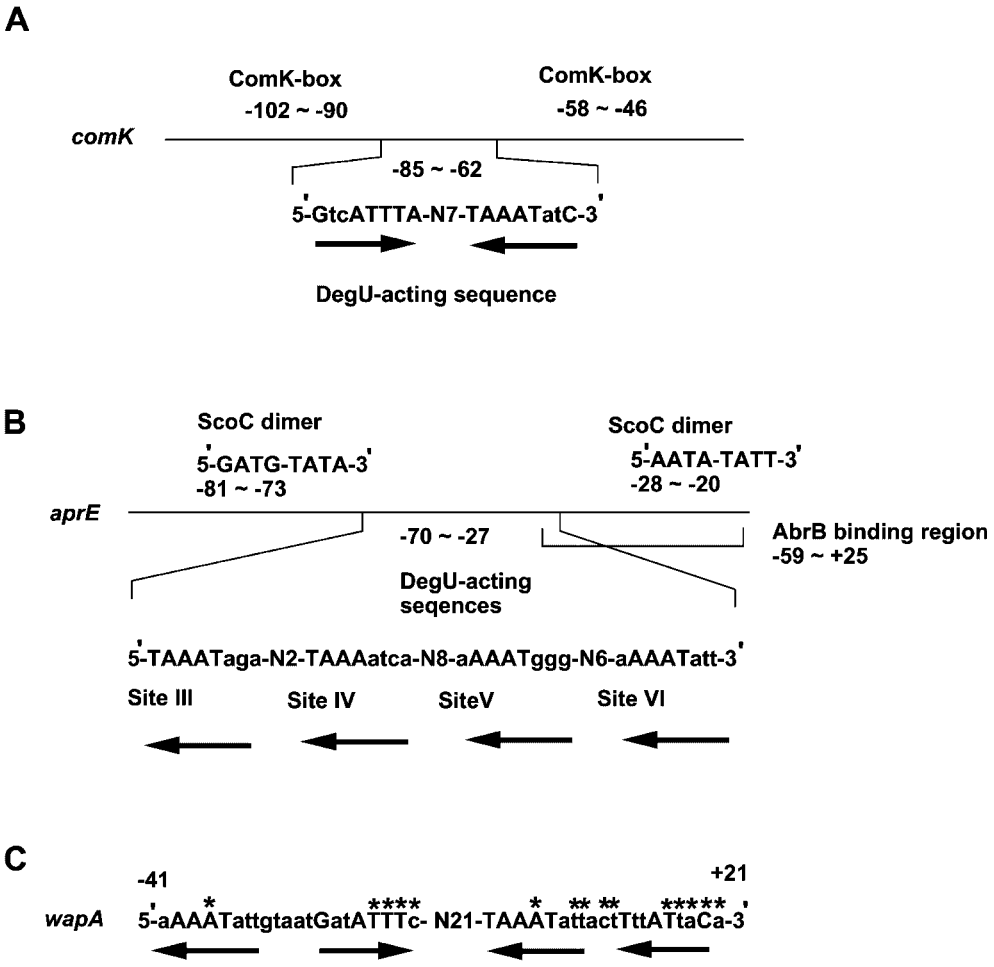


Fig. 6. **Schematic representation of the cis-acting sequences for DegU.** Lines show the indicated promoter region. Under the lines, the cis-acting sequences for DegU are shown, the numbers indicating the nucleotide positions relative to the transcription start site. Arrows denote the *cis*-acting sequences for DegU. Large characters in the sequence show nucleotides that are complementary to each other in the inverted repeat in the *comK* promoter. (A) The *comK* promoter is shown. The other DNA-binding proteins known to interact with *comK* are omitted and the ComK-box is shown. (B) Part of the control region of *aprE* is shown. ScoC binding sequences are also shown (*52*). The region protected by AbrB from DNase I cleavage is shown by the bracket because the precise AbrB binding sites are not known for the *aprE* promoter. (C) The *wapA* promoter sequence is depicted. Asterisks indicate the nucleotides whose substitution resulted in deregulation of phosphorylated-DegU–dependent repression when cells were grown under high salt conditions (*7*).

DegU-recognition. Alternatively, some secondary structures around the motifs might contribute to the specific recognition of DegU. Recently, recognition sequences for *B. subtilis* response regulator ResD were reported, the sequences being short AT-rich ones as in the case of DegU (*44*).

DegU has many targets to be controlled, however, except for *aprE*, the only gene whose regulation has been analyzed in detail is the *wapA* gene, which encodes a cell wall–associated protein. For instance, *sacB* is known to be regulated by phosphorylated DegU, however, the binding site in the *sacB* promoter remains to be determined (*45*). Expression of *wapA* is downregulated in the presence of high salt in a DegU-dependent manner (*4*). This regulation requires the *degS* gene, indicating that phosphorylated DegU participates in the salt regulation. Mutations conferring a deregulation phenotype as to DegU-dependent repression by high salt have been isolated and are shown in Fig. 6C (*4*). Most of these mutations could be assigned to the putative *cis*-acting motifs for DegU. The different arrangement of the motifs in *wapA* from that in *aprE* might correspond to repression by phosphorylated DegU instead of activation. Moreover, sequences resembling the identified *cis*-acting sequences for DegU were detected in the region required for DegU regulation in the *sacB*, *sacX* and *degQ* promoters (*17*, *18*, *45*), however, we could not determine whether these sequences indeed function in DegU-dependent regulation.

A previous study involving the *degU32*(Hy) mutation, which renders phosphorylated DegU resistant to dephosphorylation by DegS, causing enhanced expression of *aprE* (*24*, *46*), revealed the target region of this mutation to be nucleotide positions –164 to –113 relative to the transcription start site. This is different from our results. The mutation of site III almost completely abolished the *degU*-dependency of the *aprE-lacZ* fusion with the promoter region spanning from –412 to +54, indicating that site III but not the –164 to –113 region is critical for *aprE* expression. This discrepancy may be due to the use of the *degU32*(Hy) mutation instead of wild-type *degU*. For

instance, it is possible that the preferred binding site for the DegU32 mutant protein may be located in the –164 to –113 region.

We used unphosphorylated DegU for the binding assay because of the difficulty in obtaining a fully active DegS kinase, but we were able to observe DegU binding to the *aprE* promoter. In many cases, an unphosphorylated response regulator could bind to the same target DNA that the phosphorylated regulator recognizes, such as UhpA, ComA and Spo0A, although phosphorylation of the response regulator resulted in a more extended region being protected by DNase I cleavage or higher affinity of binding to DNA (*19*, *34*, *37*). Experiments involving unphosphorylated DegU, therefore, can provide important information on DegU binding. In addition, the results of the binding assay involving the *aprE* promoter were consistent with the observation that overexpression of DegU circumvented the absence of DegS, *i.e.*, requirement of phosphorylation (*7*).

We compared the results of mutational analysis of the HTH region of DegU to those with LuxR (*47*, *48*). A structural study of a co-crystal of the C-terminal region of NarL and the DNA containing the NarL-recognized inverted repeat revealed the amino acids required for binding to the base and for that to the phosphate-sugar backbone (*39*). Judging from this comparison, I192, T196 and H200 of DegU have a corresponding amino acid required for DNA binding in both LuxR and NarL. However, it should be noted that amino acids located at similar positions in homologous proteins did not necessarily act in a similar way, because each homologous DNA-binding protein might bind to each target DNA in a different manner (*49*).

We are indebted to N. Uchida for β-galactosidase assaying of the mutants. This work was supported by a Grant-in-Aid for Scientific Research (C) from the Ministry of Education, Science and Culture of Japan.

## REFERENCES

1. Robinson, V.L., Buckler, D.R., and Stock, A.M. (2000) A tale of two components: a novel kinase and a regulatory switch. *Nat. Struct. Biol.* **7**, 626–633
2. Stock, A.M., Robinson, V.L., and Goudreau, P.N. (2000) Two-component signal transduction. *Annu. Rev. Biochem.* **69**, 183–215
3. Dahl, M.K., Msadek, T., Kunst, F., and Rapoport, G. (1992) The phosphorylation state of the DegU response regulator acts as a molecular switch allowing either degradative enzyme synthesis or expression of genetic competence in *Bacillus subtilis*. *J. Biol. Chem.* **267**, 14509–14514
4. Dartois, V., Debarbouille, M., Kunst, F., and Rapoport, G. (1998) Characterization of a novel member of the DegS-DegU regulon affected by salt stress in *Bacillus subtilis*. *J. Bacteriol.* **180**, 1855–1861
5. Mader, U., Antelmann, H., Buder, T., Dahl, M.K., Hecker, M., Homuth, G. (2002) *Bacillus subtilis* functional genomics: genome-wide analysis of the DegS-DegU regulon by transcriptomics and proteomics. *Mol. Genet. Genomics* **268**, 455–467
6. Msadek, T., Kunst, F., and Rapoport, G. (1993) Two-component regulatory systems in *Bacillus subtilis* and other Gram-positive bacteria in *Biochemistry, Physiology, and Molecular Genetics* (Sonenshein, A.L., Hoch, J.A., and Losick, R., eds.) pp. 729–745, ASM Press, Washington, DC
7. Ogura, M., Yamaguchi, H., Yoshida, K., Fujita, Y., and Tanaka, T. (2001) DNA microarray analysis of *Bacillus subtilis* DegU, ComA and PhoP regulons: an approach to comprehensive analysis of *B. subtilis* two-component regulatory systems. *Nucleic Acids Res.* **29**, 3804–3813
8. Pao, G.M., Tam, R., Lipschiz, L.S., and Saier, J. (1994) Response regulators: structure, function and evolution. *Res. Microbiol.* **145**, 356–362
9. Kunst, F. and Rapoport, G. (1995) Salt stress is an environmental signal affecting degradative enzyme synthesis in *Bacillus subtilis*. *J. Bacteriol.* **177**, 2403–3407
10. Steil, L., Hoffmann, T., Budde, I., Volker, U., and Bremer, E. (2003) Genome-wide transcriptional profiling analysis of adaptation of *Bacillus subtilis* to high salinity. *J. Bacteriol.* **185**, 6358–6370
11. Berka, R.M., Hahn, J., Albano, M., Draskovic, I., Persuh, M., Cui, X., Sloma, A., Widner, W., and Dubnau, D. (2002) Microarray analysis of the *Bacillus subtilis* K-state: genome-wide expression changes dependent on ComK. *Mol. Microbiol.* **43**, 1331–1345
12. Dubnau, D. and Lovett, C.M., Jr. (2002) Transformation and recombination in *Bacillus subtilis and Its Closest Relatives* (Sonenshine, A.L., Hoch, J.A., and Losick, R. eds.) pp. 453–472, ASM Press, Washington, DC
13. Grossman, A.D. (1995) Genetic networks controlling the initiation of sporulation and the development of genetic competence in *Bacillus subtilis*. *Annu. Rev. Genet.* **29**, 477–508.
14. Hamoen, L.W., Smits, W.K., de Jong, A., Holsappel, S., and Kuipers, O.P. (2002) Improving the predictive value of the competence transcription factor (ComK) binding site in *Bacillus subtilis* using a genomic approach. *Nucleic Acids Res.* **30**, 5517–5528
15. Ogura, M., Yamaguchi, H., Fujita, Y., Kobayasi, K., Ogasawara, N., and Tanaka, T. (2002) Whole-genome analysis of genes regulated by the *Bacillus subtilis* competence transcription factor ComK. *J. Bacteriol.* **184**, 6244–6253
16. Hamoen, L.W., Van Werkhoven, A.F., Venema, G., and Dubnau, D. (2000) The pleiotropic response regulator DegU functions as a priming protein in competence development in *Bacillus subtilis*. *Proc. Natl. Acad. Sci. USA* **97**, 9246–9251
17. Crutz, A.-M. and Steinmetz, M. (1992) Transcription of the *Bacillus subtilis* sacX and sacY genes, encoding regulators of sucrose metabolism, is both inducible by sucrose and controlled by the DegS-DegU signalling system. *J. Bacteriol.* **174**, 6087–6095
18. Msadek, T., Kunst, F., Klier, A., and Rapoport, G. (1991) DegS-DegU and ComP-ComA modulator-effector pairs control expression of the *Bacillus subtilis* pleiotropic regulatory gene *degQ*. *J. Bacteriol.* **173**, 2366–2377
19. Hoch, J.A. (1993) *spo0* genes, the phosphorelay, and the initiation of sporulation in *Bacillus subtilis* and other Gram-positive bacteria in *Biochemistry, Physiology, and Molecular Genetics* (Sonenshine. A.L., Hoch, J.A. and Losick. R., eds.) pp. 747–756, ASM Press, Washington, DC
20. Strauch, M.A. and Hoch, J.A. (1993) Transition state regulators: sentinels of *Bacillus subtilis* post-exponential gene expression. *Mol. Microbiol.* **7**, 337–342
21. Ogura, M., Shimane, K., Asai, K., Ogasawara, N., and Tanaka, T. (2003) Binding of response regulator DegU to the *aprE* promoter is inhibited by RapG, which is counteracted by extracellular PhrG in *Bacillus subtilis*. *Mol. Microbiol.* **49**, 1685–1697
22. Perego, M. and Hoch, J.A. (1988) Sequence analysis and regulation of the *hpr* locus, a regulatory gene for protease production and sporulation in *Bacillus subtilis*. *J. Bacteriol.* **170**, 2560–2567
23. Gaur, N.K., Oppenheim, J., and Smith, I. (1991) The *Bacillus subtilis sin* gene, a regulator of alternate developmental processes, codes for a DNA-binding protein. *J. Bacteriol.* **173**, 678–686
24. Kunst, F., Msadek, T., and Rapoport, G. (1994) Signal transduction network controlling degradative enzyme synthesis and competence in *Bacillus subtilis* in *Regulation of Bacterial Dif-*

*ferentiation* (Piggot, P.J., Moran C.P., Jr., and Youngman, P. eds.) pp. 1–20, ASM Press, Washington, DC
25. Schaeffer, P.J., Millet, J., and Aubert, J. (1965) Catabolic repression of bacterial sporulation. *Proc. Natl. Acad. Sci. USA* **54**, 704–711
26. Sambrook, J., Fritsch, E.F., and Maniatis, T. (1989) *Molecular Cloning: A Laboratory Manual* 2nd ed., Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY
27. Ogura, M., Ohshiro, Y., Hirao, S., and Tanaka, T. (1997) A new *Bacillus subtilis* gene, *med*, encodes a positive regulator of *comK*. *J. Bacteriol.* **179**, 6244–6253
28. Guerout-Fleury, A.M., Frandsen, N., and Stragier, P. (1996) Plasmids for ectopic integration in *Bacillus subtilis*. *Gene* **180**, 57–61
29. Shimotsu, H. and Henner, D. (1986) Construction of a single copy integration vector and its use in analysis of regulation of the *trp* operon of *Bacillus subtilis*. *Gene* **33**, 103–119
30. Ogura, M., Kawata-Mukai, M., Itaya, M., Takio, K., and Tanaka, T. (1994) Multiple copies of the *proB* gene enhance *degS*-dependent extracellular protease production in *Bacillus subtilis*. *J. Bacteriol.* **176**, 5673–5680
31. Hata, M., Ogura, M., and Tanaka, T. (2001) Involvement of stringent factor RelA in expression of the alkaline protease gene *aprE* in *Bacillus subtilis*. *J. Bacteriol.* **183**, 4648–4651
32. Mukai, K., Kawata-Mukai, M., and Tanaka, T. (1992) Stabilization of phosphorylated *Bacillus subtilis* DegU by DegR. *J. Bacteriol.* **174**, 7954–7962
33. Nagami, Y. and Tanaka, T. (1986) Molecular cloning and nucleotide sequence of a DNA fragment from *Bacillus natto* that enhances production of extracellular proteases and levansucrase in *Bacillus subtilis*. *J. Bacteriol.* **166**, 20–28
34. Roggiani, M. and Dubnau, D. (1993) ComA, a phosphorylated response regulator protein of *Bacillus subtilis*, binds to the promoter region of *srfA*. *J. Bacteriol.* **175**, 3182–3187
35. Jarmer, H., Larsen, T.S., Krogh, A., Saxild, H.H., Brunak, S., and Knudsen, S. (2001) Sigma A recognition sites in the *Bacillus subtilis* genome. *Microbiology* **147**, 2417–2424
36. Crater, D.L. and Moran, C.P. Jr. (2001) Identification of a DNA binding region in GerE from *Bacillus subtilis*. *J. Bacteriol.* **183**, 4183–4189
37. Dahl, J.L., Wei, B.Y., and Kadner, R.J. (1997) Protein phosphorylation affects binding of the *Escherichia coli* transcription activator UhpA to the *uhpT* promoter. *J. Biol. Chem.* **272**, 1910–1919
38. Egland, K.A. and Greenberg, E.P. (2000) Conversion of the *Vibrio fischeri* transcriptional activator, LuxR, to a repressor. *J. Bacteriol.* **182**, 805–811
39. Maris, A.E., Sawaya, M.R., Kaczor-Grzeskowiak, M., Jarvis, M.R., Bearson, S.M., Kopka, M.L., Schroder, I., Gunsalus, R.P., and Dickerson, R.E. (2002) Dimerization allows DNA target site recognition by the NarL response regulator. *Nat. Struct. Biol.* **9**, 771–778
40. Kenney, L. (2002) Structure/function relationships in OmpR and other winged-helix transcription factors. *Curr. Opin. Microbiol.* **5**, 135–141
41. Darwin, A.J., Tyson, K.L., Busby, S.J., and Stewart, V. (1997) Differential regulation by the homologous response regulators NarL and NarP of *Escherichia coli* K-12 depends on DNA binding site arrangement. *Mol. Microbiol.* **25**, 583–595
42. Giraldo, R., Andreu, J.M., and Diaz-Orejas, R. (1998) Protein domains and conformational changes in the activation of RepA, a DNA replication initiator. *EMBO J.* **17**, 4511–4526
43. Ishiai, M., Wada, C., Kawasaki, Y., and Yura, T. (1994) Replication initiator protein RepE of mini-F plasmid: Functional differentiation between monomers (initiator) and dimers (autogenious repressor). *Proc. Natl. Acad. Sci. USA* **91**, 3839–3843
44. Geng, H., Nakano, S., and Nakano, M.M. (2004) Transcriptional activation by *Bacillus subtilis* ResD: Tandem binding to target elements and phosphorylation-dependent and -independent transcriptional activation. *J. Bacteriol.* **186**, 2028–2037
45. Klier, A., Fouet, M., Debarbouille, M., Kunst, F., and Rapoport. (1987) Distinct control sites located upstream from the levansucrase gene of *Bacillus subtilis*. *Mol. Microbiol.* **1**, 233–241
46. Henner, D.J., Ferrari, E., Perego, M., and Hoch, J.A. (1988) Location of the targets of the *hpr-97*, *sacU32(Hy)*, and *sacQ36(Hy)* mutations in upstream regions of the subtilisin promoter. *J. Bacteriol.* **170**, 296–300
47. Egland, K.A. and Greenberg, E.P. (2001) Quorum sensing in *Vibrio fischeri*: analysis of the LuxR DNA binding region by alanine-scanning mutagenesis. *J. Bacteriol.* **183**, 382–386
48. Trott, A.E. and Stevens, A.M. (2001) Amino acid residues in LuxR critical for its mechanism of transcriptional activation during quorum sensing in *Vibrio fischeri*. *J. Bacteriol.* **183**, 387–392
49. Garvie, C.W. and Wolberger, C. (2001) Recognition of specific DNA sequence. *Cell* **8**, 937–946
50. Baikalov, I., Schroder, I., Kaczor-Grzeskowiak, M., Grzeskowiak, K., Gunsalus, R.P., and Dickerson, R.E. (1996) Structure of the *Escherichia coli* response regulator NarL. *Biochemistry* **35**, 11053–11061
51. Hompson, J.D., Higgins, D.G., and Gibson, T.J. (1994) CLUSTAL W: improving the sensitivity of progressive multiple sequence alignment through sequence weighting, positions-specific gap penalties and weight matrix choice. CLUSTAL W. *Nucleic Acids Res.* **22**, 4673–4680
52. Kallio, P.T., Fagelson, J.E., Hoch, J.A., and Strauch, M.A. (1991) The transition state regulator Hpr of *Bacillus subtilis* is a DNA-binding protein. *J. Biol. Chem.* **266**, 13411–13417
53. Mukai, K., Kawata, M., and Tanaka, T. (1990) Isolation and phosphorylation of the *Bacillus subtilis degS* and *degU* gene products. *J. Biol. Chem.* **265**, 824–834