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*

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Two classes of mutations were identified in the degS and degU regulatory genes of Bacillus subtilis, leading either to deficiency of degradative enzyme synthesis (degS or degU mutations) or to a pleiotropic phenotype which includes overproduction of degradative enzymes and the loss of genetic competence (degS(Hy) or degU(Hy) mutations).

We have shown previously that the DegS protein kinase and the DegU response regulator form a signal transduction system in Bacillus subtilis. We now demonstrate that the DegS protein kinase also acts as a DegU phosphatase. We present evidence that the DegU response regulator has two active conformations: a phosphorylated form which is necessary for degradative enzyme synthesis and a nonphosphorylated form required for expression of genetic competence. The degU146-encoded response regulator, allowing expression of genetic competence, has been purified and seems to be modified within the putative phosphorylation site (D56→N) since it is no longer phosphorylated by DegS. Both the degU146 mutation as well as the degS220 mutation, which essentially abolishes DegS protein kinase activity, lead to deficiency of degradative enzyme synthesis, indicating the requirement of phosphorylated DegU for the expression of this phenotype. We also purified the degU32(Hy)-encoded protein and showed that this response regulator is phosphorylated by the DegS protein kinase in vitro. In addition, the phosphorylated form of the degU32(Hy)-encoded protein presented a strongly increased stability as compared with the wild type DegU protein, thus leading to hyperproduction of degradative enzymes in vivo.

The degS and degU genes of Bacillus subtilis encode proteins involved in the control of expression of different cellular functions, including degradative enzyme synthesis, competence for DNA uptake, and the presence of flagella. Two classes of mutations were identified in both genes. One class of mutations leads to deficiency of degradative enzyme synthesis (degS or degU mutations), and the second one leads to overproduction of degradative enzymes (degS(Hy) or degU(Hy) mutations). This second class of mutations is associated with a pleiotropic phenotype which includes the ability to sporulate in the presence of glucose, loss of flagella, and decreased genetic competence (1–4). This latter result may be interpreted as a negative effect of the degS(Hy) or degU(Hy) mutations on the expression of an essential component of the competence pathway. It has indeed been shown that the degU32(Hy) mutation down-regulates the expression of srfA, an essential competence regulatory gene (5).

The degS and degU gene products are histidine kinase and response regulator proteins, respectively (3, 6–9). A functional DegU protein is required for both expression of late competence genes and degradative enzyme synthesis in Bacillus subtilis (4, 6, 7, 10). A functional DegS protein is also required for degradative enzyme synthesis but is not required for competence (11). It has been shown that the DegS protein kinase activates the DegU response regulator through phosphoryl group transfer and that this activated form is needed for expression of degradative enzymes (8, 9). DegS also has phosphatase activity (this paper) and is consequently capable of inactivating DegU by dephosphorylation. The nonactivated (unphosphorylated) form of DegU appears to be required for competence (4, 9). In support of this hypothesis are the data obtained with the degU146 mutation which modifies the putative phosphorylation site in the DegU amino acid sequence by replacing an aspartate residue at position 56 by asparagine (4). In this paper, we show that the purified DegU146 protein is no longer phosphorylated by the DegS protein kinase. As expected, this mutation decreases degradative enzyme production but allows full expression of late competence genes. A similar phenotype is observed for a mutant carrying the degS220 mutation, which is deficient for DegS protein kinase activity, as shown in this paper. However, in the case of this mutant one cannot exclude a low level of phosphorylation of DegU by another histidine protein kinase due to a phenomenon described as "cross-talk" (12).

The degU(Hy) mutations are interpreted as leading to alterations that result in enhanced activation of DegU by DegS. This enhanced activation may simply be caused by an enhanced rate of phosphorylation of the DegU protein as we have suggested previously using crude extracts of degU24(Hy) and degU31(Hy) mutants. Indeed, a stronger phosphorylation signal was obtained in the case of these mutants as compared with the wild type strain under these conditions (9). However, overproduction of degradative enzymes in the degU32(Hy) mutant is not because of an enhanced rate of phosphorylation of DegU but instead a decreased rate of dephosphorylation. Indeed, we show in this paper that the DegU32 H12L modified response regulator is phosphorylated in vitro and that the

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1 D. van Sinderen, personal communication.
downstream from the degU146 and degU32 mutations. Plasmid pMD418 was constructed from pMD417 by replacing the 552-bp SacI-HpaI fragment containing the degU146 mutation with a 478-bp SacI-HpaI fragment containing the degU32 mutation (see Table I). The latter fragment was generated by polymerase chain reaction using chromosomal DNA of strain QB136 (carrying mutation degU32) as a template, in which a SacI site was introduced 72 bp upstream from the degU start codon. The DNA sequence of the polymerase chain reaction fragments cloned in plasmid pMD409, pMD410, or pMD418 was verified, showing that degS220, degS200, or degU32 was the only mutation present.

**Ge1 Electrophoresis**—Sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) on 12% gels was performed as described (9).

### Purification of DegS200 and DegS220 Proteins—Overproduction of DegS200 and DegS220 was achieved by growing 400 ml of E. coli strain TGI carrying plasmids pMD410 or pMD409, respectively, at 37 °C overnight to the stationary phase (A600 = 3). The DegS200 and DegS220 proteins were then purified from inclusion bodies using a denaturation-renaturation procedure, as described for the wild type DegS protein (9). The method can be briefly described as follows. (i) The inclusion bodies were dissolved in 6 M urea. (ii) QAE-Sephadex A-50 beads were added, and the suspension was diluted with low salt buffer B (50 mM Tris-HCl, pH 8.0, 50 mM potassium chloride, 1 mM EDTA, 1 mM dithiothreitol) leading to adsorption of the DegS protein to the resin. (iii) Soluble DegS protein was eluted from the QAE-Sephadex using buffer B containing 200 mM potassium chloride. (iv) The purified DegS protein was dialyzed against the latter buffer.

**Purification of the DegU32 and DegU146 Proteins**—E. coli strain JM109(DE3) was freshly transformed with plasmid pMD416 or pMD418 to produce the DegU146 or DegU32 proteins, respectively, and grown on Luria broth plates containing 100 μg/ml ampicillin at 30 °C. Colonies were immediately used to inoculate a 400-ml culture in Luria broth medium containing 100 μg/ml ampicillin and grown at 37 °C until A600 reached 0.35. Production of the purified DegU proteins was induced for 2 h with 100 μg/ml isopropyl-1-thio-β-D-galactopyranoside (IPTG). The cells were harvested by centrifugation at 5,000 × g for 10 min, washed twice with 50 ml of buffer A (25 mM Tris-HCl, pH 8.0, 200 mM potassium chloride, 1 mM magnesium chloride, 1 mM calcium chloride, 7 mM mercaptoethanol, 1 mM phenylmethylsulfonyl fluoride) and resuspended in 10 ml of the same buffer. After sonication, the DegU146 protein was found to be present in a soluble form in the crude extract and was purified as described above. The protein was estimated as being more than 90% pure from a Coomassie Blue-stained SDS-polyacrylamide gel.

In contrast with the DegU146 protein, the DegU32 protein was found to be an inclusion body, however, being found as inclusion bodies in the cell pellet after centrifugation of the cell extract at 10,000 × g for 15 min. The inclusion bodies were dissolved in 1 ml of buffer B containing 5 M urea and incubated for 1 h at 4 °C. After centrifugation in an Eppendorf centrifuge for 5 min the clear supernatant contained the DegU32 protein which was further purified by loading the supernatant onto a QAE-Sephadex column. The column was washed with buffer A and eluted with a linear gradient of buffer B containing 5 M urea. The same buffer was used for elution of the column. Fractions of 1 ml were collected and analyzed by SDS-PAGE. Purified DegU32 (approximately 90% pure) was found in one fraction which was dialyzed against buffer B containing 500 mM KCl with 50 mM Tris-HCl, pH 7.5, 500 mM sodium chloride, 50 mM magnesium chloride, 10 mM calcium chloride, 0.5 mM dithiothreitol, and 5 mM thio-β-D-galactopyranoside.

### Phosphorylation of DegS and DegU Proteins

**In vitro phosphorylation assays** were carried out as described previously (9). The phosphorylation reaction mixtures (50 μl) contained 20 μg of purified DegS, 30 μg of DegS200, or 40 μg of DegS220 protein, respectively, in buffer C (100 mM Tris-HCl, pH 8.0, 200 mM KC1, 4 mM MgCl2, 4 mM CaCl2, 0.5 mM dithiothreitol, 0.1 mM EDTA, 5% glycerol (v/v)). The reaction was initiated by addition of 3 μCi of [γ-32P]ATP (Amersham International, Buckinghamshire, England) diluted with nonlabeled ATP to give a final concentration of 2.5 μM. The reaction mixture was then incubated at 30 °C for 2 min at room temperature, and the samples were subjected to trypsin phosphorylation reactions as described below. Alternatively, the autophosphorylation reaction was stopped by adding 0.1 volume of a solution containing 10% SDS and 0.02% bromphenol blue and analyzed by 12% SDS-PAGE followed by autoradiography.

The apparent Kₐ values of purified DegS200 or DegS220 proteins were determined as described (9). The catalytic constant (kₐ) was calculated from the Vₐₐ₉ value (pmol/min) and the amount of DegS protein (pmol) used in the reaction.

<table>
<thead>
<tr>
<th>Strain</th>
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<td>pMD416</td>
<td>degU146</td>
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* For detailed description of construction, see "Experimental Procedures."  
* For detailed description of the mutations see (3, 4).
Isolation of Phosphorylated DegS—The DegS protein (80 µg) was autophosphorylated as described above, in a 200-µl reaction mixture containing 90 µCi of [γ-32P]ATP. Phosphorylated DegS was precipitated in the presence of 200 µg of carrier bovine serum albumin (Miles; 3 x crystallized) by adding solid ammonium sulfate to 70% saturation. After a 15-min incubation at 4°C, the suspension was centrifuged for 10 min at 12,000 x g at 4°C. The precipitate was dissolved in buffer B containing 200 mM potassium chloride, precipitated again, and washed with 70% saturated ammonium sulfate. The pellet containing phosphorylated DegS was then dissolved in 50 µl of buffer B containing 200 mM potassium chloride, and remaining ammonium sulfate was eliminated by dialysis for 2 h against 100 ml of same buffer (with one change), on a millipore VMWP filter (0.05 µm pore size). Phosphorylated DegS was essentially free of [γ-32P]ATP as determined by SDS-PAGE and autoradiography (data not shown).

Phosphorylation of DegU Proteins—Wild type DegS or the modified DegS200 and DegS220 proteins were incubated for 2 min in buffer E containing [γ-32P]ATP to produce autophosphorylated 32P-labeled protein kinases as described above. 4 µg of phosphorylated wild type DegS, 12 µg of phosphorylated DegS200, or 16 µg of phosphorylated DegS220 proteins, respectively, were added to a reaction mixture containing 2 µg of purified DegU, unlabeled ATP (0.6 mM final concentration), and buffer E in a final volume of 100 µl. Similar reactions using modified response regulators contained 20 µg of purified DegU32 or 10 µg of purified DegU146. After incubation at 25°C for the indicated times, 20-µl aliquots were removed, in which the phosphorylation reaction was stopped by adding 5 µl of 10% SDS, 0.02% bromphenol blue, and analyzed by SDS-PAGE.

Isolation of Phosphorylated DegU—6 µg of [32P]DegU, was separated from ATP as described above, was incubated with 5 µg of purified DegU in buffer E in a final volume of 100 µl. After 5 min of incubation at 25°C, the reaction mixture was immediately loaded on an Ultrogel AcA 44 column (5.5 x 0.5 cm) allowing separation of DegU (26 kDa) from DegS (45 kDa). The column was washed with buffer B containing 50 mM KCl, and the collected fractions (100 µl) were frozen at 70°C. Aliquots were analyzed by SDS-PAGE and autoradiography. Fractions containing only [32P]DegU were used for further study.

Stability of Phosphorylated DegU Proteins—5 µg of purified wild type DegU or 20 µg of purified DegU32 was phosphorylated as described above, by incubating these proteins for 20 min at room temperature in a 100-µl reaction mixture containing DegU phosphorylated in the presence of [γ-32P]ATP. Following the addition of unlabeled ATP to a final concentration of 2 mM, 20-µl aliquots were removed at the indicated times, and the transphosphorylation reactions were stopped and analyzed by SDS-PAGE. These experiments were repeated in the absence of ATP, using [32P]DegU, purified as described above.

In another set of reactions [32P]DegU was separated from DegS as described above, and the stability of the phosphorylated protein in the presence or absence of 4 µg of purified DegS was examined by SDS-PAGE.

Quantitative analysis of radioactive protein bands was carried out using a Betagen Betascope 603 Blot Analyzer.

RESULTS

Cloning of Mutant degS Genes and Purification of the Corresponding Gene Products—We described previously the construction of plasmid pMD404 containing the strong B. subtilis degS promoter (9). Recombinant plasmids, which express in E. coli the degS200(Hy) or degS220 genes under control of the degS36 promoter, were constructed from plasmid pMD404 (see "Experimental Procedures").

The conditions for overproduction of the DegS200 and DegS220 proteins were the same as earlier described for the wild type DegS protein (9). After sonication and centrifugation of the cells, overproduced DegS200 and DegS220 proteins were found as inclusion bodies in the pellet. The DegS200 and DegS220 proteins were then purified as described and estimated as being more than 90% pure as judged from Coomassie Blue-stained gels (see "Experimental Procedures").

Cloning of the Mutant degU Genes and Purification of the Corresponding Gene Products—The degU gene product presents toxicity to E. coli. For this reason, we adopted a strategy allowing cloning of toxic genes in E. coli. The mutant degU genes were placed downstream from a T7 promoter in plasmid pTT5–5. Recombinant plasmids pMD416 and pMD418, derived from pTT5–5 and containing the degU146 or degU32 genes, respectively, were then introduced in strain T6, which does not express these genes since it does not contain the T7 RNA polymerase gene. The plasmids were then transferred to strain JM109(DE3) which contains a chromosomal copy of an isopropyl 1-thio-β,β-d-galactopyranoside-inducible T7 RNA polymerase gene. Freshly transformed JM109(DE3) clones were used to inoculate a preculture which was grown overnight at 30°C. After isopropyl 1-thio-β,β-d-galactopyranoside induction the mutant proteins were overproduced and purified as described in "Experimental Procedures." The DegU146 protein was present in a soluble form in the crude extract and was purified as described earlier for the wild type DegU protein up to the AcA 44 gel filtration step (9). At this stage, this protein has an estimated purity superior to 90% and was used as such in phosphorylation reactions (see below).

In contrast to the DegU146 protein, the DegU32 protein was found as inclusion bodies in the cell pellet obtained after centrifugation of the sonicated extract. This pellet was dissolved in 5 M urea and centrifuged again. The clear supernatant was loaded on an Ultragel AcA 44 column equilibrated with buffer B containing 5 M urea, and the same buffer was used for elution of the column. Fractions containing DegU32, which was more than 90% pure, were dialyzed against buffer B containing 500 mM potassium chloride, since lower salt concentrations (<200 mM potassium chloride) led to precipitation of the DegU32 protein.

Autophosphorylation of the DegS200 and DegS220 Proteins—Synthesis of the DegS200 protein leads to overproduction of degradative enzymes in B. subtilis, whereas synthesis of the DegS220 protein, on the contrary, leads to deficiency of degradative enzyme production. These opposite phenotypes could be the consequence of modifications of DegS kinase activity. Therefore the apparent K_a and k_m values for the modified enzymes were determined and compared with the values of the wild type DegS enzyme. The apparent K_a was deduced from initial rates of phosphorylation as a function of the ATP concentration; the k_m was deduced from the maximum velocity (V_max) and the enzyme concentration (data not shown). The apparent K_m values determined for wild type DegS and DegS200 were in the same range (18 and 22 µM ATP, respectively), whereas the apparent K_m for DegS220 was found to be higher (45 µM ATP). However, the main difference observed was a strong reduction of the k_m values determined for DegS200 (5.7 x 10^-9 min^-1) and DegS220 (7.6 x 10^-9 min^-1) as compared with the wild type DegS protein (1.3 x 10^-3 min^-1).

Significantly, the DegS200 and DegS220 proteins had rather similar catalytic properties as far as the autophosphorylation reaction was concerned; yet these two proteins lead to the expression of clearly distinct phenotypes in B. subtilis. We therefore examined the capacity of both proteins to phosphorylate the DegU protein.

Phosphorylation of the DegU Response Regulator by Modified DegS Kinases—Although both the DegS200 and DegS220 proteins retain their autophosphorylation activity, they behave quite differently with regard to phosphorylation of the DegU response regulator, since the DegS220 protein has essentially lost its protein kinase activity.

To determine whether the DegS200 and DegS220 proteins catalyze phosphorylation of the DegU response regulator, two-step reactions were carried out. Purified DegS kinases were
autophosphorylated in the presence of [γ-32P]ATP as described under "Experimental Procedures." The reaction mixture containing [32P]DegS was then added to the purified DegU protein in the presence of an excess of unlabeled ATP (0.6 mM). Transfer of 32P from DegS to DegU was examined using SDS-PAGE followed by autoradiography. After 5 min of incubation of the DegU protein in the presence of [32P]DegS200, the DegU phosphorylation signal was diminished as compared with the control reaction carried out using wild type [32P]DegS (Fig. 1). Since the concentrations of [32P]DegS200 and [32P]DegS220 used were 3-4-fold higher than [32P]DegS concentration, it appears that DegS200 is less efficient in DegU phosphorylation as compared with wild type DegS. However, the DegS220 protein is essentially deficient for protein kinase activity since phosphorylation of DegU by DegS220 was nearly undetectable as compared with phosphorylation by DegS200 or wild type DegS (Fig. 1).

Phosphorylation of Modified DegU Response Regulators by Wild Type DegS Kinase—In contrast with strains containing a disrupted degU gene, the degU146 mutant of B. subtilis, which is thought to produce a nonphosphorylatable response regulator, is competent for DNA uptake (4). Indeed, the degU146 mutation modifies the putative phosphoryl acceptor site of DegU (D56+N), which led us to suggest that the unphosphorylated form is required for the expression of late competence genes (4).

Purified DegU146 protein was incubated with phosphorylated [32P]DegS, effectively demonstrating that this modified response regulator can no longer be phosphorylated (Fig. 2).

We previously interpreted the degU(Hy) mutations as alterations in the response regulators that result in enhanced activation by DegS via phosphoryl group transfer (see the Introduction and Ref. 9). To our surprise, however, no phosphorylation of the degU32(Hy) encoded response regulator could be detected with a rapid screening assay based on the use of crude cell extracts (9). Apparently, this previous assay was not sensitive enough, since we now demonstrate that the purified DegU32 protein is indeed phosphorylated by DegS (Fig. 2). However, the phosphorylation signal obtained with the DegU32 protein was weaker than that obtained with the wild type protein even though the DegU32 protein concentration in the transphosphorylation reactions was approximately 2-fold higher (Fig. 2).

Based on our model, we predict that the hyperproduction phenotype caused by the degU32(Hy) mutation is the consequence of either an increased rate of phosphorylation of the DegU32 response regulator or an increased stability of phosphorylated DegU32 as compared with phosphorylated wild type DegU. Since the result presented above excludes the first hypothesis, we examined the stability of the phosphorylated DegU32 response regulator.

Stabilities of the Phosphorylated DegU and DegU32 Response Regulators—A parameter that plays an important role in DegS/DegU-mediated regulation is the maintenance of the DegU response regulator in its activated state, i.e. retention of the phosphoryl group by DegU. We show here that the DegS protein has DegU phosphatase activity, since the rate of DegU dephosphorylation is increased in the presence of DegS as compared with the spontaneous dephosphorylation of DegU in the absence of DegS (Fig. 3). An apparent half-life of 80 min was found for the phosphorylated form of DegU in the absence of DegS. Addition of DegS to the reaction...
mixture increased the dephosphorylation rate of DegU phosphate, giving an apparent half-life of 40 min (Fig. 3). Adding an excess of unlabeled ATP to the reaction mixture further increased the dephosphorylation rate of DegU (half-life of 20 min) (Fig. 3). These results are similar to those obtained for the EnvZ-OmpR regulatory pair (20).

The latter set of conditions, leading to an optimal dephosphorylation rate of DegU, was chosen for the comparison of the stabilities of the phosphorylated DegU32 and wild type DegU proteins. The purified DegU and DegU32 proteins were thus preincubated with [32P]DegS for 20 min, and an excess of unlabeled ATP was subsequently added to the reaction mixture. The DegS-catalyzed dephosphorylation of the modified DegU32 protein was found to be considerably slower than that of the wild type DegU response regulator (Fig. 4). An apparent half-life of 20 min was found for wild type DegU phosphate, whereas the half-life of DegU32 phosphate was strongly increased and found to be 140 min under the same conditions. The increased stability of the phosphorylated DegU32 response regulator is presumably responsible for the in vivo effect of the degU32(Hy) mutation which leads to hyperproduction of degradative enzymes.

**DISCUSSION**

The degS and degU genes of *B. subtilis* encode a protein kinase and response regulator, respectively. This signal transduction pathway controls the expression of two classes of genes in *B. subtilis*: late competence genes involved in DNA uptake and genes encoding degradative enzymes. A functional degU gene is required for the expression of both cellular functions (4, 7, 10), whereas a functional degS gene is only required for the expression of degradative enzymes (11). These genetic data suggested to us that DegU may be an ambivalent regulator, which is required for competence in its unphosphorylated form, whereas the phosphorylated form is necessary for production of degradative enzymes (4).

In this paper we present conclusive evidence in support of this hypothesis.

We reported earlier that degS and degU mutants could be divided in two classes on the basis of their phenotypes: (i) mutants which are deficient for degradative enzyme synthesis but present a near wild type level of genetic competence, e.g. degS220 or degU146; (ii) mutants which are hyperproducers of degradative enzymes, but are deficient for genetic competence, e.g. degS200(Hy) or degU32(Hy).

In the present paper we describe the purification and study of the properties of the corresponding mutationaly altered proteins, DegS220, DegU146, and DegU32.

We showed here that the purified DegU146 protein, containing a D56N amino acid substitution modifying the putative phosphorylation acceptor site, is no longer phosphorylatable by the DegS protein kinase. This result is comparable to the data obtained by Burbulys et al. (1991) (21), Lukat et al. (1991) (22) and Jin et al. (1990) (23) who showed that the CheY D57N, SpoOA D56N, and VirG D52N response regulators modified at equivalent positions are no longer phosphorylated by their cognate protein kinases. The DegU146 protein has lost the capacity to stimulate degradative enzyme synthesis but still allows expression of late competence genes such as comC. This is in sharp contrast with mutants in which the degU gene is disrupted, leading to absence of competence (4, 7, 10).

Mutations in degS leading to the inability to phosphorylate the DegU response regulator have a phenotype which is comparable to that of the degU146 mutant. An example of such a mutant is degS42, encoding a modified protein with an E300K amino acid substitution (4). Tanaka et al. (24) recently showed that the DegS42 protein is deficient for both auto-phosphorylation and phosphorylation of the DegU response regulator. The DegS220 protein described in this paper is also deficient in DegU phosphorylation activity. However, an interesting difference between the DegS42 and DegS220 proteins is that DegS220 retains autophosphorylation activity. The reduced DegU phosphorylation activity is presumably the reason that the degS220 mutation was initially identified as an extragenic suppressor of the hyperproduction phenotype of the degU32 mutant. Indeed, the DegU32 protein remained unphosphorylated when incubated with the DegS220 kinase in the presence of ATP (results not shown).

Alba et al. (25) described a class of mutations affecting the *E. coli* sensor kinase EnvZ or the OmpR response regulator leading to a defect in dephosphorylation of the response regulator. The degS200(Hy) and degU32(Hy) mutations are probably similar mutations affecting the DegS/DegU regulatory pair. These mutations were initially interpreted as alterations of the DegU and DegS proteins leading to increased phosphorylation or decreased dephosphorylation of the DegU protein. Tanaka et al. (24) showed that the degS200(D300) mutation leads to a decreased rate of dephosphorylation of the DegU protein. This result suggests that DegS has DegU phosphatase activity and that this activity seems to be deficient in the case of the DegS200 protein. We showed in this paper that DegS does indeed exhibit a DegU phosphatase activity and that the purified DegU32 protein has a significantly decreased rate of dephosphorylation as compared with the wild type DegU protein (see Figs. 3 and 4). We also showed that the DegS200- and DegS220-modified protein kinases have lost this DegU phosphatase activity (results not shown).

The data presented here concerning modifications of either the DegS protein kinase or the DegU response regulator
confirm our earlier hypothesis concerning the alternative active forms of the DegU response regulator, phosphorylated and unphosphorylated (4, 9). We suggested that the DegU response regulator acts as a genetic switch enabling \textit{B. subtilis} to choose between expressing competence genes or turning on degradative enzyme production, by dephosphorylating or phosphorylating DegU, respectively (Fig. 5). The phosphorylation of the response regulator is strongly diminished or nondetectable in the \textit{degS42}, \textit{degS220}, and \textit{degU146} mutants, allowing competence for DNA uptake but leading to deficiency for degradative enzyme production.

Two other mutations, \textit{degS200}(H\text{y}) and \textit{degU32}(H\text{y}), have several properties in common: (i) they lead to a moderate decrease in DegU phosphorylation activity; (ii) they both lead to slower dephosphorylation of DegU; (iii) they lead to hyperproduction of degradative enzymes and to deficiency of competence. These data strengthen the hypothesis that the effect of the \textit{degU32}(H\text{y}) mutation may be interpreted as previously proposed for the \textit{degS200}(H\text{y}) mutation (24), in one of the following ways. (i) The increased half-lives of DegU-phosphate in the \textit{degS200}(H\text{y}) and \textit{degU32}(H\text{y}) mutants lead to a higher steady-state level of DegU phosphate in vivo. (ii) The increased stability of DegU phosphate increases the time of interaction with the target genes encoding degradative enzymes. (iii) The role of phosphorylation in the activation of DegU may only be indirect (for a review, see 26), and the \textit{degS200} and \textit{degU32} mutations may lead to stabilization of the active conformation of the DegU response regulator.

We are presently trying to identify direct targets for the DegU response regulator, which is thought to bind to DNA, since it contains a putative helix-turn-helix motif (4). Preliminary evidence obtained by Mukai et al. (8) and by our laboratory suggests that DegU may not bind directly upstream from genes encoding degradative enzymes. This is also the case for the \textit{B. subtilis} ComP/ComA two-component system which also controls the expression of late competence genes. Indeed, several genes such as \textit{sfIA} (\textit{comL}), \textit{comK}, and \textit{mecA} seem to form regulatory intermediates between ComA and the late competence genes (5). One possible intermediate between DegU and genes encoding degradative enzymes appears to be the \textit{mecA} gene. Indeed, mutations in this gene bypass the need of both ComA and DegU for the expression of late competence genes, and we have shown recently that the \textit{mecA} mutation can also bypass the need of DegS/DegU for degradative enzyme production (5).

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REFERENCES