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Stabilization of Phosphorylated *Bacillus subtilis* DegU by DegR

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The production of *Bacillus subtilis* extracellular proteases is under positive and negative regulation. The functional role of *degR*, one of the positive regulators, was studied in relation to the *degS* and *degU* gene products, which belong to the bacterial two-component regulatory system. Studies with a translational fusion between the *Escherichia coli lacZ* and the *Bacillus subtilis* subtilisin (*aprE*) genes indicated that the stimulatory site of DegR lay upstream of position −140, with the region upstream of position −200 being the major target. It was also found that *degS* and *degU* were epistatic to *degR*. These results suggested some relationship among the *degR*, *degS*, and *degU* gene products. The DegR protein was purified to homogeneity, and its in vitro effect on the phosphorylation reaction involving DegS and DegU was studied. For this purpose, a soluble-extract system in which the formation and dephosphorylation of DegU-phosphate could be examined was devised. The addition of DegR to the soluble-extract system enhanced the formation of DegU-phosphate. The enhancing effect was found to be due to the protection of DegU-phosphate from dephosphorylation. From these results, it was concluded that the positive effect of DegR on the production of the extracellular proteases is brought about by the stabilization of DegU-phosphate, which in turn may result in the stimulation of transcription of the exoprotease genes.

Bacillus subtilis excretes two major extracellular proteases, a neutral protease and an alkaline protease. Studies from several laboratories have demonstrated that the production of these enzymes is under positive and negative control. The presence of multiple copies of the *degR* (*prtR*), *degQ* (*sacQ*), *sen*, and *tenA* genes results in the overproduction of both enzymes, whereas the overproduction of the *hpr*, *sin*, and *pai* gene products leads to the inhibition of enzyme synthesis (4, 8, 19, 20, 21, 28, 30, 31, 32). Mutations in the *sacU* locus yield two phenotypes, SacU(Hy) and SacU$^-$, which show overproduction and reduced synthesis of both the extracellular proteases and levansucrase, respectively (13).

Among the genes so far described, *sacU* appears to play a central role in the regulation of exoprotease production. Studies by Henner et al. (6) and Kunst et al. (12) revealed that the *sacU* locus consists of two genes, *degS* and *degU*, the upstream and downstream genes, respectively, and that the gene set belongs to the bacterial family of two-component regulatory systems, in which one component accepts an environmental signal and transduces the information to the other component, resulting in activation of the target gene or cell machinery (23). Several such systems in both gram-negative (25) and gram-positive (1) bacteria have been shown to transduce the information through phosphorylation. Similarly, it has been demonstrated by us and others that DegS is autophosphorylated and then transfers the phosphate to DegU (2, 18). More recently, it was also shown that DegS is involved in the dephosphorylation of DegU-phosphate and that the mutant DegS proteins encoded by *degS200*(Hy) and *degS100*(Hy) were less active in the dephosphorylation reaction (25). These results suggest that the stabilization of DegU-phosphate leads to the enhanced production of the extracellular proteases.

It has been shown that mutations such as *hpr-97* and *degU32*(Hy) enhance the production of the extracellular proteases by increasing the mRNA levels of the exoprotease genes (5). The target site of *hpr-97* lies in a region upstream of position −200 with respect to the transcription initiation site of the *aprE* gene (the *B. subtilis* subtilisin gene), whereas the region between positions −164 and −141 is necessary for full stimulation by the *degU32*(Hy) and *degQ36*(Hy) mutations (5). The *hpr* gene encodes a negative regulator of exoprotease production (21), and the *hpr-97* mutation may cause inactivation of the negative regulator (7). The *degQ36*(Hy) mutation has been shown to be a promoter-up mutation of the *degQ* gene (31), whereas *degU32*(Hy) has been found to be due to a single amino acid change in the coding region of the *degU* gene (6).

We have shown that *degR* encodes a 60-amino-acid protein and that the presence of multiple copies of *degR* enhances mRNA synthesis for both *aprE* and *nprE* (the extracellular neutral protease gene) (19, 26). The present study was undertaken to reveal the target on which the *degR* gene product exerts its effect. It was concluded that DegR stabilizes DegU-phosphate, a form of DegU postulated to enhance the production of the extracellular proteases (17).

MATERIALS AND METHODS

Bacterial strains. The *B. subtilis* strains carrying various deletions upstream of the *aprE* promoter were donated by D. J. Henner (5). The deletion endpoints upstream of the transcription initiation site of *aprE* are as follows: −412 (SG35.18), strain BG4224; −340 (SG35.21), BG4226; −244 (SG35.20), BG4225; −200 (SG35.8), BG4197; −164 (SG35.8 25), BG4201; −141 (SG35.8 21), BG4199; −113 (SG35.8 23), BG4200; −104 (SG35.8 34), BG4202; −64 (SG35.8 6), BG4198; and −51 (SG35.5), BG4160. Other *B. subtilis* and *Escherichia coli* strains are listed in Table 1.

Materials. The DegS and DegU proteins were prepared as described previously (18). Isopropyl-β-D-thiogalactopy-

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype and/or description[a]	Source or reference[b]
Strains		
B. subtilis		
MI112	*arg-15 leuB8 thr-5 recE4 hsmM hsrM*	This laboratory
CU741	*trpC2 leuC7*	S. A. Zahler (29)
TT719	*trpC2 leuC7 ΔdegS*	This laboratory (18)
TT715	*trpC2 leuC7 aprE*::pSKD1	pSKD1→CU741
TT7291	*trpC2 leuC7 ΔdegS aprE*::pSKD1	pSKD1→TT719
TT7151	*trpC2 leuC7 degU*::Neo^r *aprE*::pSKD1	pSKD1 and pHIB2→CU741
E. coli JM103	*lacI*^{q} strain	16
Plasmids		
pKK223-3	Carries *E. coli tac* promoter	Pharmacia
pKK223-3-*degR*	pKK223-3 carrying *degR*	This study
pUBH1	Kanamycin resistance	R. H. Doi (10)
pSA1	pUBH1 carrying intact *degS* and N-terminal region of *degU*	This laboratory (24)
pSKD1	Carries *aprE'-'lacZ*	This laboratory (18)
pHIB2	Carries Neo^r at the *Bcl*I site in the *degU* gene	This study
pNC6	Trimethoprim resistance	This laboratory (19)
pNC61	pNC6 carrying *degR*	This laboratory (19)

[a] Neo^r, neomycin resistance determinant.
[b] Arrows designate transformation of the plasmid DNA into the respective strain. For construction of TT7151, pHIB2 was linearized to avoid chromosomal integration by Campbell-type recombination.

ranoside (IPTG) was purchased from Sigma Chemical Co. Plasmid pKK223-3, Sephacryl S-200 (superfine), and DEAE-Sephacel were obtained from Pharmacia LKB Biotechnology Inc. Two sets of molecular weight markers were used for sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) systems; one, obtained from Amersham, consists of myosin, phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase, trypsin inhibitor, and lysozyme, and the other, obtained from Sigma, consists of myoglobin and its fragments.

Plasmids and plasmid construction. The plasmids used in this study are listed in Table 1. Plasmid pKK223-3-*degR* was constructed by insertion of the *Eco*RI fragment of pNC61 (19) into the *Eco*RI site of pKK223-3 so that the expression of the *degR* gene was under the control of the *tac* promoter (Fig. 1).

Preparation of *B. subtilis* soluble cell extracts. *B. subtilis* MI112 carrying either pUBH1 or pSA1 was grown in Luria-Bertani broth (1 liter) containing kanamycin (10 μg/ml) at 37°C. Two hours after the end of the logarithmic growth phase, cells were chilled in ice water, collected by centrifugation, and washed once with cold buffer consisting of 20 mM Tris-HCl (pH 8.0), 0.5 mM Na-EDTA, and 50 mM KCl (buffer A). The following steps were carried out on ice or at 4°C. One gram of wet cells was suspended in 2.5 ml of buffer A. After the addition of 0.1 g of glass beads (controlled-pore glass; CPG-10-1000; Electro-nucleonics, Inc.) and phenylmethylsulfonyl fluoride to a concentration of 1 mM, the suspension was sonicated to disrupt the cells. The glass beads and the cell debris were removed by centrifugation at 8,500 rpm for 15 min with a Hitachi RPR20-2 rotor, and the supernatant was further centrifuged at 40,000 rpm (100,000 × *g*) for 30 min. The supernatant was divided into 50-μl quantities and stored at −80°C (soluble cell extract).

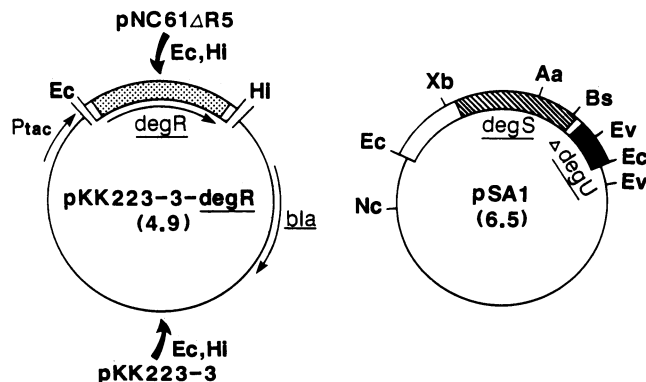

FIG. 1. Plasmids used in this study. Numbers in parentheses represent the sizes of the plasmids in kilobases. Abbreviations: Aa, *Aat*I; Bs, *Bst*BI; Ec, *Eco*RI; Ev, *Eco*RV; Hi, *Hin*dIII; Nc, *Nco*I; Xb, *Xba*I.

Purification of the DegR protein. *E. coli* JM103 carrying pKK223-3-*degR* was grown at 37°C in 0.5 liter of Luria-Bertani broth containing ampicillin (50 μg/ml). At the mid-log phase of growth, IPTG was added to a concentration of 0.4 mM, and the cells were grown for a further 3 h. All subsequent steps were performed on ice or at 4°C. The cells were pelleted by centrifugation, washed once with 20 mM Tris-HCl buffer (pH 7.5) containing 0.1 M NaCl (buffer B), and resuspended in 7.5 ml of the same buffer. The suspension was sonicated to disrupt the cells and centrifuged at 40,000 rpm (100,000 × *g*) for 45 min. Solid ammonium sulfate was added to the supernatant to yield 30% saturation, and the precipitates were discarded. After the supernatant was brought to 60% ammonium sulfate saturation and kept on ice for 30 min, the precipitates formed were collected by centrifugation and dissolved in 0.5 ml of buffer B. The sample was applied to a Sephacryl S-200 column (1.6 by 88 cm) that had been equilibrated with buffer B, and DegR was eluted with the same buffer. The elution profile was monitored by SDS-glycerol-PAGE (22), and the fractions containing DegR were pooled. After twofold dilution with water, the sample was applied to a DEAE-Sephacel column (1.0 by 12 cm) that

had been equilibrated with 10 mM Tris-HCl buffer (pH 7.5) containing 50 mM NaCl. Proteins were eluted with a linear NaCl gradient (50 to 200 mM) in 10 mM Tris-HCl buffer (pH 7.5) in a total volume of 120 ml. The fractions containing the purified DegR protein were stored on ice.

Estimation of the relative molecular mass of the purified DegR protein was performed by use of a high-pressure liquid chromatography (HPLC) system with a gel filtration column (TSK G3000 SWXL; 7.6 by 300 mm; Toso, Tokyo, Japan). The buffer used for column equilibration and elution of the protein was 0.1 M Na-phosphate buffer (pH 7.0) containing 0.1 M Na_2SO_4. The retention time for DegR was compared with those for aldolase (160 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), chymotrypsinogen (25 kDa), and cytochrome *c* (12 kDa). The relative molecular mass of the SDS- and mercaptoethanol-treated DegR protein was determined by SDS-glycerol-PAGE and SDS-urea-PAGE (9) with the two sets of molecular weight markers described above.

Phosphorylation experiments. The standard reaction mixture for testing the phosphorylation of DegS and DegU was described previously (18). The reaction was started by the addition of [γ-^{32}P]ATP (2 Ci/mmol), and incubation was carried out at 37°C. At various times, samples of 7.5 μl were removed and the radioactivity incorporated into DegS and DegU was determined by autoradiography after SDS-PAGE (14); the radioactive bands were then quantitated as described previously, and the radioactivity was expressed as PSL (photostimulated luminescence), an arbitrary unit (18).

RESULTS

Location of the target site of *degR* upstream of *aprE*. Henner et al. constructed a series of *B. subtilis* strains in which the *E. coli lacZ* gene had been translationally fused to the coding region of *aprE* and various lengths of deletion had been introduced upstream of the *aprE* gene (5). Expression of the *lacZ* gene in those strains started at about 2 h after the cessation of the logarithmic growth phase (T_2), and the β-galactosidase activity reached the maximum level at about T_3 or T_4 for all the strains used, except for the strain with deletion SG35.5, in which the activity continued to increase until at least T_5 (data not shown). We chose to measure β-galactosidase activities at T_4 for the present study. To locate the target site of *degR*, we plotted β-galactosidase activities in the deletion strains carrying either pNC6 (vector) or pNC61 (pNC6 plus *degR*) against the deletion endpoints with respect to the transcription initiation site of *aprE* (Fig. 2). The profile of β-galactosidase activities observed for the pNC6-carrying strains (Fig. 2A) was similar to the profile reported by Henner et al., who measured the initial rate of β-galactosidase synthesis (5). The β-galactosidase activities in the cells carrying pNC61 were consistently higher than those in the cells carrying pNC6 (Fig. 2A). When the magnitude of enhancement by pNC61 was plotted against the deletion endpoints, a profile of stimulation (Fig. 2B) was obtained; i.e., the level of enhancement by pNC61 was about 25-fold for the region upstream of −340, 10-fold for the region between −200 and −164, and 2- to 3-fold for the region between −141 and −51. The level of stimulation at −244 in the pNC61-carrying cells seemed to be underestimated, since the activity in strain BG4225 (−244) carrying pNC6 was already high (Fig. 2A) and, therefore, the enhancing effect of pNC61 could not have been fully manifested. A similar observation was made previously for this strain (5). These results suggest that there are at least three sites (−244 to −200, −164 to −141, and a region downstream from −51)

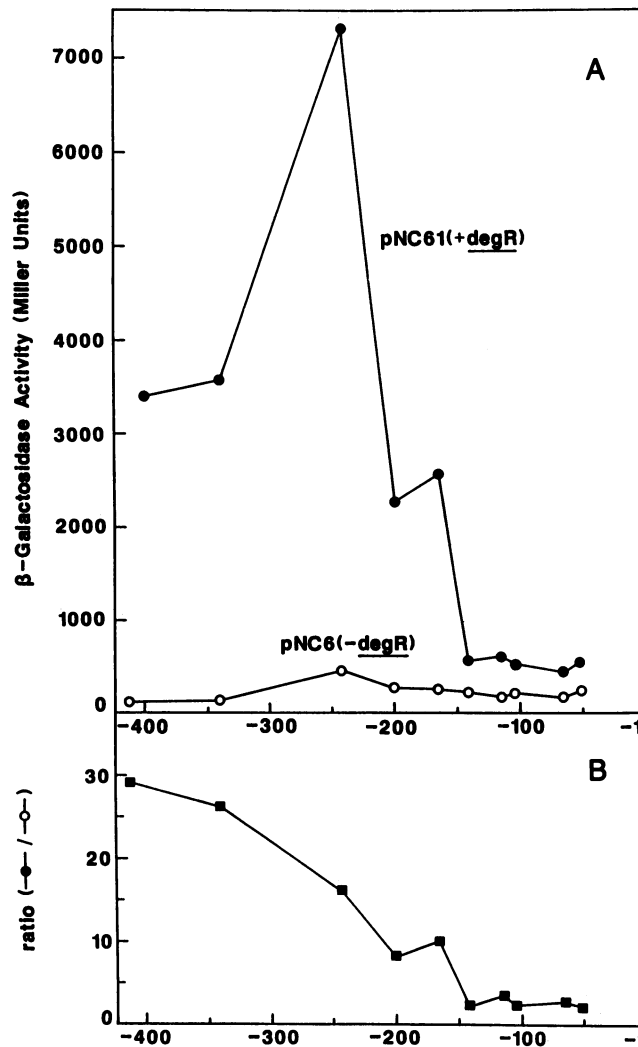

FIG. 2. Effect of deletion of the upstream region of *aprE* on the stimulation of *aprE* expression by multiple copies of *degR*, as monitored by β-galactosidase activity. Cells containing pNC6 or pNC61 were processed by the method of Ferrari et al. (3), except that the cells were grown at 37°C. Numbers on the abscissa indicate the distances in base pairs from the transcription initiation site (+1) of *aprE*. (A) β-Galactosidase activity in cells harboring pNC6 or pNC61. (B) Stimulation of *aprE* expression by multiple copies of *degR*, as shown by the ratio of β-galactosidase activity in cells carrying pNC61 to that in cells carrying pNC6.

at which the *degR* gene product exerts its effect in the upstream region of *aprE*, although we cannot rule out the possibility that the weak stimulation in the most downstream region is due to a secondary effect.

Effect of multiple copies of *degR* on the expression of *aprE* in *degS* and *degU* mutants. To examine the functional relationship among the *degS*, *degU*, and *degR* genes, we studied the effect of disruption of the former two genes on the stimulation of *aprE* expression by *degR*. Strain TT7291 carries a deletion removing 90% of the *degS* coding region, whereas in strain TT7151, the *degU* gene is disrupted by insertion of the Neo^r (neomycin resistance) gene at the *BclI* site in the *degU* gene (18, 24). These strains carry an *aprE'*-*'lacZ* fusion (Table 1). In strain TT719 (Δ*degS degU*$^+$), the parental

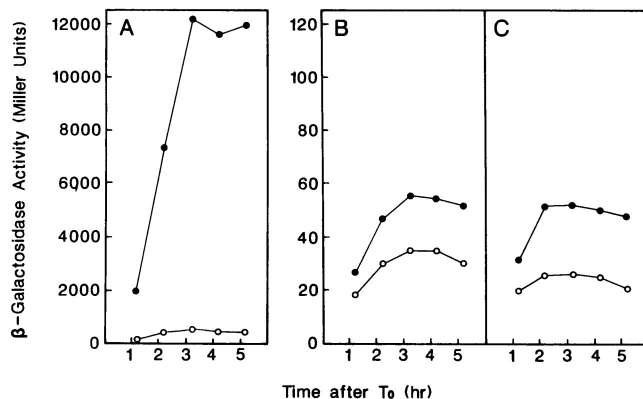

FIG. 3. Effect of multiple copies of *degR* on the expression of *aprE'-'lacZ* in strains carrying a disruption in either *degS* or *degU*. Panels A, B, and C show β-galactosidase activities in strains TT715 (*degS*$^+$ *degU*$^+$), TT7291 (Δ*degS degU*$^+$), and TT7151 (*degS*$^+$ *degU*::Neo^r), respectively, harboring pNC61 (●) or pNC6 (○). β-Galactosidase activity was determined as described in the legend to Fig. 2.

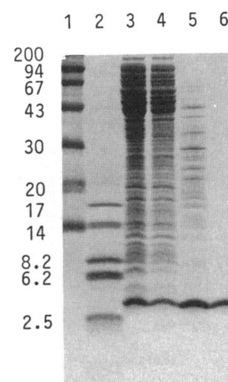

FIG. 4. Purification of the DegR protein. The purification procedures are described in Materials and Methods. Samples from each step were subjected to SDS-glycerol-PAGE. Lanes: 1 and 2, molecular weight markers; 3, total cell lysate; 4, ammonium sulfate fraction; 5, Sephacryl S-200 fraction; 6, DEAE-Sephacel fraction. The numbers on the left show the molecular masses of the marker proteins (expressed in kilodaltons). These were myosin (200 kDa), phosphorylase *b* (94 kDa), bovine serum albumin (67 kDa), ovalbumin (43 kDa), carbonic anhydrase (30 kDa), trypsin inhibitor (20 kDa), myoglobin (17 kDa), lysozyme (14 kDa), myoglobin fragments I and II (14 kDa), myoglobin fragment I (8.2 kDa), myoglobin fragment II (6.2 kDa), and myoglobin fragment III (2.5 kDa).

strain of TT7291, the expression of the downstream *degU* gene is about 60% compared with that of the *degU* gene downstream of intact *degS* in the wild-type strain (18). Plasmids pNC6 and pNC61 were introduced into strains TT7151, TT7291, and CU741 by protoplast transformation, and the expression of *aprE* was determined by measuring β-galactosidase activity. The presence of multiple copies of *degR* enhanced β-galactosidase synthesis in CU741 25-fold, whereas it enhanced this synthesis only 2-fold or less in strains TT7291 and TT7151 (Fig. 3). This determination was one of three and was reproducible. These results would be expected if disruption of the *degS* and *degU* genes on the chromosome reduced the expression of the *degR* gene on pNC61. To examine this possibility, we introduced pNZ2, a derivative of pNC61 carrying *degR'-'lacZ* (19), into TT719 (Δ*degS degU*$^+$) and TT711 (*degS*$^+$ *degU*::*cat*) and studied the expression of *degR*. Disruption of the *degS* and *degU* genes had no effect on the expression of the *degR* gene on the multicopy plasmid (data not shown). These results show that *degS* and *degU* are epistatic to *degR*.

The results obtained suggested the following three possibilities: first, DegR exerts its effect through a direct interaction with the *degS* and/or *degU* gene product; second, it affects an unknown step prior to the site of action of this gene product(s); and third, DegR acts in parallel to DegS and DegU, as an accessory transcriptional enhancer, on a separate but nearby target of the *degS* and *degU* genes. We examined the first possibility, i.e., the effect of DegR on the phosphorylation reaction carried out by the *degS* and *degU* gene products.

Purification of the DegR protein. To examine the function of the *degR* gene product in vitro, we purified the DegR protein after amplification in *E. coli* as follows. First, the *degR* gene was placed under the control of the *E. coli tac* promoter present in plasmid pKK223-3 and inducible by the addition of IPTG (Fig. 1). The purification procedures are described in Materials and Methods, and SDS-glycerol-PAGE patterns at each purification step are shown in Fig. 4. A homogeneous preparation of the putative DegR protein was obtained at the final purification step. In the SDS-glycerol-PAGE system used for the resolution of small proteins, the purified DegR protein was found to have a molecular weight of 3,500 (Fig. 4), although its calculated molecular weight is 7,109, determined on the basis of the nucleotide sequence of *degR* (19). The same molecular weight was estimated when the purified protein was subjected to SDS-urea-PAGE (data not shown). The N-terminal amino acid sequence of up to 20 residues was found to be identical with that deduced from the nucleotide sequence of the *degR* gene, and the amino acid composition was found to be in good agreement with that of DegR (data not shown). From these results, we conclude that the purified protein is DegR. The reason for the lower molecular weight observed in the SDS gel systems is not known.

Estimation of the molecular weight of DegR in the absence of denaturing reagents was performed with a gel filtration HPLC column, and it was found that DegR eluted between ovalbumin and chymotrypsinogen (see Materials and Methods). The retention time corresponded to a molecular weight of 28,000 (data not shown). Since the calculated molecular weight of DegR is 7,109, the results suggest that the DegR protein exists as a tetramer.

Phosphorylation of DegU in a soluble cell extract. We next examined the effect of DegR on the in vitro phosphorylation of DegS and DegU. The experimental system that we used consisted of purified DegU, [γ-^{32}P]ATP, and a soluble extract prepared from *B. subtilis* carrying *degS* on a multicopy plasmid. This system is based on the ideas that the soluble extract from such cells may contain enough DegS protein to carry out the phosphorylation of DegU and that under this experimental condition the DegR protein may exert its effect.

Soluble extracts were prepared from MI112 cells carrying pUBH1 (used as a vector) or pSA1 (pUBH1 plus the *degS* gene; 22) as described in Materials and Methods and incubated with [γ-^{32}P]ATP and with or without purified DegU. When extracts from cells carrying either pUBH1 or pSA1 were used, several radioactive bands were observed; the intensity of most bands increased with incubation time, but the radioactivity in some bands became no more visible

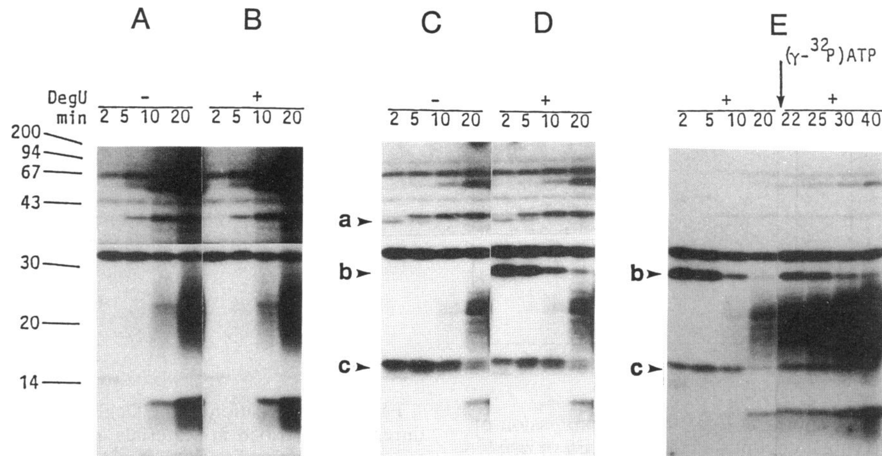

FIG. 5. Phosphorylation of DegS, DegU, and the N-terminal DegU in soluble extracts. Soluble extracts prepared from pUBH1-carrying cells (A and B) or from pSA1-carrying cells (C, D, and E) were incubated with (B, D, and E) or without (A and C) purified DegU as described in Materials and Methods. The concentrations of DegU and soluble extract were 0.6 μM and 1.2 mg of protein per ml, respectively. Samples were removed at the indicated times and processed as described in Materials and Methods. In panel E, [γ-^{32}P]ATP (0.1 mM) was added again after 20 min, and the reaction was continued. Arrowheads a, b, and c indicate the phosphorylated forms of DegS, DegU, and N-terminal DegU, respectively. The upper portions of panels A, B, C, and D, corresponding to proteins with molecular weights higher than 35,000, were overexposed to reveal the phosphorylated DegS band. + and − indicate the presence and absence of DegU, respectively. The numbers on the left show the mobilities of the molecular mass markers (expressed in kilodaltons).

within 5 min (Fig. 5A and C). The addition of DNase and RNase to the reaction mixture did not change the banding pattern, whereas the bands were not visible when pronase was added, indicating that the bands were due to radioactive proteins. Overall banding patterns were similar for extracts from cells carrying pUBH1 and pSA1, except that there were two extra bands in the sample prepared from pSA1-carrying cells (arrowheads a and c in Fig. 5C), one with a molecular weight of 41,000 and the other with a molecular weight of 16,500. The mobility of the 41-kDa protein band was identical to that of the DegS protein (see below), and this band was not observed when an extract from cells carrying pAA1 (24), a deletion plasmid of pSA1 that lacks two-thirds of the coding region of *degS*, was used (data not shown). The 16.5-kDa band was characterized as follows. When the soluble extracts from the pUBH1- and pSA1-carrying cells were subjected to SDS-PAGE and the gel was stained with Coomassie brilliant blue, a protein band corresponding in mobility to the 16.5-kDa band was detected only in the extract prepared from the pSA1-carrying cells. We isolated the 16.5-kDa protein from an SDS-polyacrylamide gel, determined the N-terminal amino acid sequence, and found that the sequence of the 15 N-terminal amino acids was identical to that of the DegU protein, which was deduced from the nucleotide sequence (data not shown). These results indicate that the 16.5-kDa protein consists of 139 amino acids derived from the N-terminal region of DegU and 8 amino acids derived from the vector (see pSA1 in Fig. 1). This protein is referred to as N-terminal DegU.

Upon addition of the purified DegU protein to the incubation mixture, a new radioactive band (band b) appeared only when the extract prepared from the pSA1-carrying cells was used (compare Fig. 5B and D). The new band in Fig. 5D was indistinguishable in mobility from DegU, as revealed by staining of the same gel with Coomassie brilliant blue (data not shown), indicating that the added DegU protein was phosphorylated by DegS encoded by the *degS* gene on pSA1. After prolonged exposure of the gel in Fig. 5C to an X-ray film, a faint band corresponding in mobility to DegU appeared, but no such radioactive band was observed when an extract prepared from TT711, a strain in which *degU* is disrupted by the insertion of *cat* (24), carrying pSA1 was used (data not shown), suggesting strongly that DegU encoded by *degU* on the chromosome was phosphorylated by DegS encoded by *degS* on pSA1. Phosphorylation of DegU resulted in a decrease in the phosphorylation of the 16.5-kDa protein (compare Fig. 5C and D). This result is most likely due to competition between the added DegU and the N-terminal DegU for the DegS-phosphate formed in the extract.

The amount of DegU-phosphate produced in the soluble extract decreased rather rapidly during the incubation period (Fig. 5D) compared with the amount of DegU-phosphate produced by purified DegS (18). The DegU protein seems to be stable at least during the incubation period, since the intensity of the DegU bands remained the same, as revealed by Coomassie brilliant blue staining (data not shown). The addition of [γ-^{32}P]ATP to the reaction mixture after 20 min resulted in a reappearance of DegU-phosphate (Fig. 5E), indicating that DegS was still active at least in part in the reaction mixture after 20 min. From these results, we conclude that the rapid disappearance of DegU-phosphate formed in the soluble extract can be explained in two ways: one is that [γ-^{32}P]ATP is lost rapidly, resulting in a low concentration of the radioactive compound, and the other is that DegU-phosphate, once formed, is dephosphorylated in the extract.

It was demonstrated that the dephosphorylation of DegU-phosphate was reduced in a reaction mixture containing purified DegS proteins encoded by the *degS100* and *degS200* genes (25). It was also shown that DegS encoded by the *degS42* gene lacked the ability to autophosphorylate and to phosphorylate DegU. The soluble extracts prepared from the cells carrying these mutations on pSA1 exhibited behaviors (data not shown) similar to those of the purified mutant DegS proteins (27), thus validating the soluble extract method for examining the fate of DegU-phosphate.

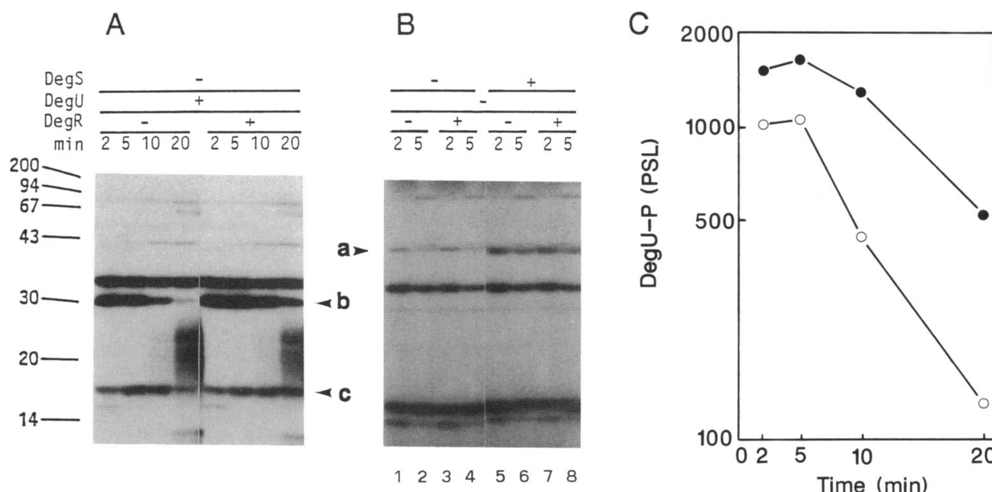

FIG. 6. Stimulation of DegU-phosphate formation by DegR. (A) The soluble extract (1.2 mg of protein of ml) from the pSA1-carrying cells and DegU (1 μM) were incubated with or without DegR (5 μM). (B) The same soluble extract was incubated with or without DegS (8 μM) and DegR (5 μM) in various combinations as shown. The phosphorylated proteins were detected by SDS-PAGE as described in Materials and Methods. + and − indicate the presence and absence of the factors shown on the left, respectively. The numbers on the left show the positions of the molecular mass markers (expressed in kilodaltons). The X-ray film in panel B was overexposed to reveal the DegS-phosphate bands. Arrowheads a, b, and c show the phosphorylated forms of DegS, DegU, and N-terminal DegU, respectively. (C) The DegU-phosphate (DegU-P) formed in panel A was quantitated as described in Materials and Methods. Symbols: ○, without DegR; ●, with DegR.

Stimulation of DegU phosphorylation by DegR in a soluble extract. The soluble extract from the pSA1-carrying cells and purified DegU were incubated with [γ-^{32}P]ATP in the presence or absence of purified DegR, and the extent of phosphorylation of DegU was examined after various times of incubation. The extent of DegU phosphorylation was higher in the presence of DegR than in its absence throughout the incubation (Fig. 6A). The level of radioactivity in the N-terminal DegU fragment was also elevated by DegR at 20 min (Fig. 6A). We note that, for the several radioactive bands, only the phosphorylation of DegU and the N-terminal fragment of DegU was stimulated by DegR, indicating that the effect of DegR was specific to the production of DegU-phosphate. The radioactive DegU bands in Fig. 6A were quantitated and plotted against time (Fig. 6C). Under the condition used, enhancement at the earlier times (2 and 5 min) was about 1.5-fold, but the difference in the amounts of DegU-phosphate became larger as the incubation time was increased (4-fold at 20 min) (Fig. 6C). Similar experiments were carried out several times, and the enhancing effect of DegR was always observed. These results show that the DegU-phosphate formed in the presence of DegR is more stable than that formed in the absence of DegR.

The enhancing effect of DegR on DegU phosphorylation could have been due to the enhancement of the autophosphorylation of DegS by DegR, which may have resulted in the enhanced phosphorylation of DegU. To examine this possibility, we incubated the soluble extract from the pSA1-carrying cells and [γ-^{32}P]ATP with or without DegR and examined the resultant radioactive bands by SDS-PAGE and then autoradiography. The intensity of band a did not change upon the addition of DegR (Fig. 6B, lanes 1 through 4). The addition of purified DegS increased the intensity of band a (Fig. 6B, lanes 5 and 6), and there was little effect of added DegR on the intensity of this band (Fig. 6B, lanes 7 and 8). The mobility of band a was indistinguishable from that of the DegS protein, as revealed by Coomassie brilliant blue staining (data not shown). On the other hand, there was no stimulation of DegS-phosphate formation when purified DegS, DegU, and DegR were incubated under the condition in which DegR stimulated the formation of DegU-phosphate by a factor of 1.6 (data not shown). These results show that band a corresponds to the phosphorylated form of DegS and that DegS phosphorylation is not stimulated by DegR.

Effect of DegR on the dephosphorylation of DegU-phosphate. The results described so far have suggested that the increased phosphorylation of DegU by DegR is due to the stabilization of DegU-phosphate by DegR. To examine this possibility further, we chased the radioactive phosphate incorporated into DegU by adding unlabeled ATP to the phosphorylation reaction mixture. Thus, the soluble extract from pSA1-carrying cells and the purified DegU protein were incubated with [γ-^{32}P]ATP in the presence and absence of DegR, and after 2 min, a 30-fold molar excess of unlabeled ATP was added. As shown in Fig. 7A, the addition of DegR slowed the rate of dephosphorylation of DegU-phosphate, and a quantitative analysis of the autoradiogram revealed that the apparent half-life of DegU-phosphate was 3.6-fold longer in the presence of DegR than in its absence (Fig. 7B, left panel).

The results described above would have been obtained if DegR protected [γ-^{32}P]ATP in the reaction mixture from hydrolysis, since the concentration of [γ-^{32}P]ATP is a limiting factor for the phosphorylation of DegU, as shown in Fig. 5E, and a higher concentration of the radioactive compound may lead to the production of a higher level of DegU-phosphate. However, direct examination of the ATP level revealed that there was no detectable difference between the reaction mixtures with and without DegR. The similar ATP levels in the two reaction mixtures were reflected in the following observations. First, except for the DegU and the N-terminal DegU proteins, the proteins were phosphorylated to the same extent (Fig. 6A). Second, we have shown that the autophosphorylation of purified DegS is dependent on the concentration of ATP (18). As shown in Fig. 5B, the addition of DegR did not increase the DegS-phosphate level.

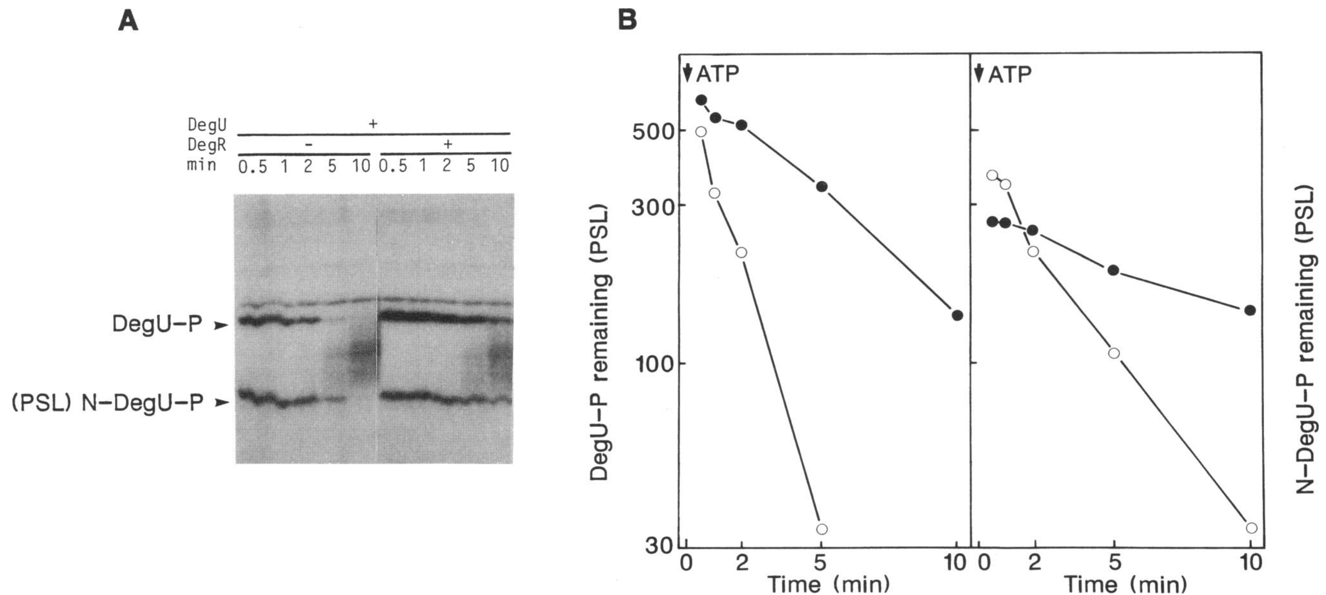

FIG. 7. Dephosphorylation of DegU-phosphate (DegU-P) and N-terminal DegU–phosphate (N-DegU-P) in the soluble extract in the presence and absence of DegR. After the reaction mixture with or without added DegR was incubated for 2 min (see the legend to Fig. 6A), a 30-fold molar excess of unlabeled ATP was added. At the indicated times, samples were removed and subjected to SDS-PAGE and then autoradiography. (A) Autoradiogram. + and − indicate the presence and absence of DegU or DegR, respectively. (B) Amount of radioactivity remaining in DegU and N-terminal DegU (left and right panels, respectively). Symbols: ●, with DegR; ○, without DegR.

From all of these data, we conclude that DegR stabilizes the phosphorylated DegU protein.

The rate of dephosphorylation of phosphorylated N-terminal DegU (N-terminal DegU–phosphate) was also slowed by the addition of DegR (Fig. 7A and B, right panel). The apparent half-life of N-terminal DegU–phosphate was longer than that of DegU-phosphate, suggesting that the C-terminal portion of DegU affects either the dephosphorylation of DegU-phosphate or the stability of the DegU protein.

DISCUSSION

Stimulation of the transcription of the *aprE* gene by multiple copies of the *degR* gene depends on regions upstream of position −200, between positions −164 and −141, and downstream of position −51, with respect to the transcription initiation site, with the major target being upstream of position −200. The stimulatory effect of the *degU32*(Hy) mutation also depends on the latter two regions, although the stimulation in the most downstream region could be due to a secondary effect (5). Since the stimulatory effect of *degU32*(Hy) at sites upstream of position −200 has not been reported, a direct comparison of the targets of *degR* and *degU32*(Hy) is impossible. However, our results could be interpreted to mean that the targets of DegU and DegR overlap at least in part. On the other hand, when either the *degS* gene or the *degU* gene was inactivated, multiple copies of the *degR* gene failed to enhance the expression of the *aprE* gene (Fig. 3). This result shows that *degS* and *degU* are epistatic to *degR* and suggests that the phosphorylated form of DegU is necessary for DegR to exert its effect. One interpretation would be that DegR affects a process involving DegS and/or DegU directly or indirectly. This notion prompted us to examine the effect of DegR on the reaction involving DegS and DegU, i.e., autophosphorylation of DegS in the presence of ATP and subsequent phosphate transfer from DegS-phosphate to DegU (2, 18).

In this study, a soluble-extract system in which the phosphorylation of DegU by DegS, encoded by multiple copies of *degS*, could be monitored rather easily was developed. The addition of purified DegR to the soluble extract enhanced the extent of DegU phosphorylation (Fig. 6), and the enhancement was attributed to the stabilization of DegU-phosphate (Fig. 7). In these experiments, unlabeled ATP was added to dilute [γ-^{32}P]ATP in the reaction mixture. One might argue that this addition may have resulted in the exchange of phosphate between nonradioactive ATP and the radioactive phosphate present in DegU and that it was this reaction that DegR interfered with. However, the observation that the apparent half-life of DegU-phosphate was prolonged when DegR was present in the soluble extract without the addition of unlabeled ATP (Fig. 6) strongly suggests that this was not the case. In addition, the stabilization of DegU-phosphate by DegR was also observed when purified DegS-phosphate free from ATP was used; i.e., the addition of DegR increased the half-life by a factor of 1.6 (data not shown).

There may be two reasons for the difference between the soluble-extract and purified protein systems in the extent of stabilization of DegU-phosphate by DegR. One is that although DegS carries the dephosphorylating activity of DegU-phosphate, the DegS protein purified in the presence of 8 M urea was not sufficiently renatured to be affected by DegR. The other possibility is that the soluble extract contains a factor(s) that dephosphorylates DegU-phosphate and that DegR interferes with this activity. However, the latter possibility is unlikely since, when a soluble extract prepared from MI112 cells carrying pUBH1 (namely, without multiple copies of *degS*) was added to a phosphorylation reaction mixture containing purified DegS and DegU, there

was no further effect of DegR on the stabilization of DegU-phosphate than the effect observed when DegR was incubated only with purified DegS and DegU (data not shown). Therefore, we favor the first possibility, and the difference in the extent of stabilization by DegR may be due to the difference in the quality of the DegS protein itself. From these results, we conclude that DegR stabilizes DegU-phosphate. Clarification of how DegR stabilizes DegU-phosphate awaits further study.

Strains carrying the *degR* gene on a multicopy plasmid, such as pNC61, showed the same phenotypes as *degS*(Hy) and *degU*(Hy) mutant strains. Competence was reduced to about 1/20, sporulation occurred in Schaeffer sporulation medium containing glucose, and cells grew in long chains. These phenotypes and the extension of the half-life of DegU-phosphate by DegR are in keeping with the hypothesis that the phosphorylated form of DegU is responsible for the production of exoproteases and that the unphosphorylated form is responsible for competence development (17).

It is interesting that the truncated DegU protein, consisting of the N-terminal 139 amino acids (N-terminal DegU), was still able to be phosphorylated (Fig. 5) and that the rate of dephosphorylation was retarded by DegR (Fig. 7). The results show that this part of DegU has a conformation recognized by DegS and that its phosphorylated form is stabilized by DegR. We note that the dephosphorylation of DegU-phosphate was faster than that of N-terminal DegU-phosphate (Fig. 7) in both the presence and the absence of DegR. This result suggests either that the rate of dephosphorylation of DegU-phosphate depends on the conformation of DegU or that N-terminal DegU is more stable than DegU in the reaction mixture. We previously showed that multiple copies of the N-terminal region of *degU* inhibit the production of exoproteases in the presence and absence of *degR* on a multicopy plasmid (24). One interpretation of this result is that the inhibition was caused by competition between DegU and N-terminal DegU for phosphorylated DegS. As for other response regulators, phosphorylation of the N-terminal region has been reported for NtrC and CheB (11, 15).

The sensor proteins in the two-component regulatory systems are thought to recognize signals that are produced inside the cell or accepted on the surface of the membrane and to transduce the information to the effector protein through phosphorylation, resulting in the activation of transcription or of cell movement (23). DegR is a novel factor in that it stabilizes the activated form of the effector, DegU-phosphate, as described in this paper. How is this observation correlated with exoprotease production? The *degR* gene in the multicopy state has been shown to enhance the production of the extracellular proteases and levansucrase (19). Although the positive effect of the *degR* gene is seen only when it is on a multicopy plasmid and no effect on *aprE* or *sacB* expression is seen when the *degR* gene is deleted (32), the *degR* gene may have some role under certain physiological conditions. Overexpression of the single *degR* gene on the chromosome would result in the same effect as that observed with the *degR* gene on a multicopy plasmid. Therefore, it may not be unreasonable to assume that, in addition to signal transduction through DegS, there is another pathway in which the expression of the *degR* gene is enhanced, leading to overproduction of the extracellular proteases and levansucrase.

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