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Multiple Copies of the *proB* Gene Enhance *degS*-Dependent Extracellular Protease Production in *Bacillus subtilis*

MITSUO OGURA,[1] MUTSUMI KAWATA-MUKAI,[2] MITSUHIRO ITAYA,[2] KOJI TAKIO,[3] AND TERUO TANAKA[1]*

School of Marine Science and Technology, Tokai University, Shimizu, Shizuoka 424,[1] Mitsubishi Kasei Institute of Life Sciences, Machida, Tokyo 194,[2] and RIKEN (Institute of Physical and Chemical Research), Wako, Saitama 350-01,[3] Japan

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***Bacillus subtilis* secretes extracellular proteases whose production is positively regulated by a two-component regulatory system, DegS-DegU, and other regulatory factors including DegR. To identify an additional regulatory gene(s) for exoprotease production, we performed a shotgun cloning in the cell carrying multiple copies of *degR* and found a transformant producing large amounts of the exoproteases. The plasmid in this transformant, pLC1, showed a synergistic effect with multiple copies of *degR* on the production of the extracellular proteases, and it required *degS* for its enhancing effect. The DNA region responsible for the enhancement contained the *proB* gene, as shown by restriction analyses and sequence determination. The *proB* gene encoding γ-glutamyl kinase was followed by the *proA* gene encoding glutamyl-γ-semialdehyde dehydrogenase at an interval of 39 nucleotides, suggesting that the genes constitute an operon. pLC1 contained the complete *proB* gene and a part of *proA* lacking the *proA* C-terminal region. It was also found that *proB* on the chromosome showed a synergistic effect with multiple copies of *degR*. We consider on the basis of these results that the metabolic intermediate, γ-glutamyl phosphate, would transmit a signal to DegS, resulting in a higher level of phosphorylated DegU. Possible involvement of DegR in this process is discussed.**

In the bacterial two-component regulatory system, the phosphorylated response regulator transmits the signal to the target gene(s), resulting in an appropriate output response. It has been postulated that the sensor kinase also functions as the phosphatase of the phosphorylated response regulator to control the half-life of the activated response regulator. The signaling mechanisms well studied so far include the regulation of the synthesis of porin proteins (1, 11, 17), chemotaxis proteins (14, 15, 48), and glutamine synthetase (22, 31, 47). Recently it was suggested that low-molecular-weight substances are involved in the regulation of the phosphate regulon of *Escherichia coli*, and indeed some response regulators were shown to phosphorylate themselves by using low-molecular-weight phospho-donors such as phosphoramidate, acetyl phosphate, and carbamoyl phosphate (9, 23, 25, 44). The newly detected signaling pathway may participate in subjecting the bacterial cells to fine-tuning of their physiological state, since the changes of the amounts of such metabolic intermediates may reflect the environmental or internal states.

The production of the extracellular proteases of *Bacillus subtilis* is subject to a complex network of positive and negative regulators. Multiple copies of the *degR* (30, 42, 50), *degQ* (2, 49), *senS* (46), and *tenA* (33) genes result in the overproduction of the extracellular proteases. On the other hand, the *abrB* (10), *hpr* (35), *sin* (12), and *pai* (16) loci are involved in negative regulation, and overproduction of the products of these genes inhibits the production of the exoproteases. In this complex regulatory network of exoprotease production, the two-component regulatory system, *degS-degU*, plays a central role (6, 28). In this system, it is thought that the sensor kinase, DegS, accepts environmental stimuli, autophosphorylates on its own histidine residue, and transfers the phosphate to the aspartate residue of the cognate response regulator DegU (34). DegS is also involved in the dephosphorylation of the phosphorylated DegU (6, 28). It has been demonstrated that the phosphorylated form of DegU is stabilized by multiple copies of *degR* (29) or mutations that cause overproduction of the exoproteases, such as *degS100*(Hy), *degS200*(Hy), *degU32*(Hy), and *degU9*(Hy) (6, 7, 41, 43).

We report in this paper that the *proB* gene enhances extracellular protease production with multiple copies of the *degR* gene in a synergistic mode and that the *proB* effect is dependent on the *degS* gene. We discuss the possibility that γ-glutamyl phosphate, the intermediate compound produced by the *proB* gene product in proline biosynthesis, could serve as a low-molecular-weight phospho-donor for the DegS-DegU two-component system.

MATERIALS AND METHODS

Materials. Restriction enzymes and T4 DNA ligase were purchased from Toyobo, Co. (Tokyo, Japan), a DNA blunting kit was bought from Takara Shuzo Co. (Kyoto, Japan), and IPTG (isopropyl-β-D-thiogalactoside) and ATP were from Sigma Chemical Co.

Media and antibiotics. The media used in this study were Luria-Bertani broth, Luria-Bertani agar medium (26), Luria-Bertani agar medium containing casein and gelatin (LBCG) (40), antibiotic medium 3 (Difco Laboratories), and Schaeffer's sporulation medium (37). Concentrations of the antibiotics added to the media were 10 μg/ml for trimethoprim and kanamycin, 15 μg/ml for tetracycline, and 5 μg/ml for chloramphenicol.

Bacterial strains and plasmids. The bacterial strains and plasmids used in this study are listed in Table 1. MTPB33 was constructed by insertion of the *cat* gene into the *Not*I site of pNEXT62 (20) and subsequent introduction of the constructed

* Corresponding author. Mailing address: Department of Marine Science, School of Marine Science and Technology, Tokai University, Orido 3-20-1, Shimizu, Shizuoka 424, Japan. Phone: 543-34-0411, ext. 2342. Fax: 543-34-5095.

TABLE 1

Strain or plasmid	Genotype and/or description	Source (reference)[a]
Strains		
B. subtilis		
MI112	*arg-15 leuB8 thr5 recE4 hsmM hsrM*	This laboratory
DB104	*his nprR2 nprE18 ΔaprE3*	R. H. Doi (21)
MT33	*his nprR2 nprE18 aprE'-'lacZ*	pSKD1→DB104
MTPB33	*his nprR2 nprE18 proB::cat aprE'-'lacZ*	This study
CU741	*trpC2 leuC7*	S. A. Zahler (45)
TT714	*trpC2 leuC7 degU'-'lacZ*	This laboratory (28)
TT715	*trpC2 leuC7 aprE'-'lacZ*	This laboratory (29)
TT7291	*trpC2 leuC7 ΔdegS aprE'-'lacZ*	This laboratory (29)
OM110T	*trpC2 leuC7 proA::tet*	pEPV1T→CU741
OM115	*trypC2 leuC7 proB'-'lacZ*	pSPB106→CU741
E. coli		
JM109	*recA1 Δ(lac-proAB) endA1 gyrA96 thi-1 hsdR17 relA1 supE44* [F *traD36 proAB lacI*^{q}*Z ΔM15*]	J. Messing (51)
Plasmids		
pNC6	Trimethoprim resistance	This laboratory (30)
pNC61	pNC6 carrying *degR*	This laboratory (30)
pNC61ΔH8	Derivative of pNC61	This laboratory (30)
pNC61ΔV10	Derivative of pNC61	This laboratory (30)
pNC61ΔR5	Derivative of pNC61	This laboratory (30)
pUBH1	Kanamycin resistance	R. H. Doi (21)
pLC1	pUBH1 carrying *proB* and N-terminal region of *proA*	This study
pLC221	Deletion plasmid of pLC1 lacking *Cla*I-*Bam*HI region	This study
pSKD1	Carries *aprE'-'lacZ* fusion	This laboratory (28)
pUC19	Ampicillin resistance	J. Messing (51)
pBEST304	Carries tetracycline resistance cassette	M. Itaya (19)
pEPV1T	Carries tetracycline resistance cassette and a part of *proA*	This study
pSPB106	Carries tetracycline resistance cassette and *proB'-'lacZ* fusion	This study

[a] Arrows indicate transformation of the plasmid DNAs into the respective strains.

plasmid into the *proB* gene by a double-crossover event. The pLC1 derivatives, pBCL1, pKPL3, and pNOL1, were constructed by self-ligation of pLC1 digested with *Bcl*I, *Kpn*I, and *Not*I, respectively, and treated with T4 DNA polymerase. pHDL1 was similarly made by filling in the *Hin*dIII site of pLC221, a deletion plasmid of pLC1 lacking the 350-bp *Cla*I-*Bam*HI fragment in the pUBH1 part (21). pHDL2, pHDL7, pSAL4, and pSAL5 were constructed by ligation of pLC1 digested with *Hin*dIII or *Sac*I. In pHDL2, the DNA region between the two *Hin*dIII sites, one in the insert and the other in the vector, is inverted, whereas it is deleted in pHDL7. pEPV1T is a pUC19 derivative carrying the tetracycline resistance gene cassette from pBEST304 (19) at the *Xba*I site and the blunt-ended *Pvu*II-*Eco*RI DNA fragment from the 3′ portion of the *proBA* region of pLC1 (see Fig. 4) at the *Sma*I site. pSPB106 was constructed by the following two steps. First, a tetracycline resistance cassette (19) was inserted into the *Sca*I site of pSK10Δ6 (52), and a *Hin*dIII linker was introduced into the *Sma*I site of the resulting plasmid. Second, the *Hin*dIII fragment carrying the N-terminal part of the *proB* gene from pLC1 was introduced into this plasmid.

Assay of extracellular proteases. For examination of the effects of plasmids, MI112 cells carrying the plasmids were plated with toothpicks onto the LBCG plates containing trimethoprim and kanamycin. The synergistic effect was determined on the basis of the size of the halo generated after 24 h.

Transformation. The polyethylene glycol-induced protoplast transformation procedure was performed by the method of Chang and Cohen (5). The transformed cells were selected with kanamycin and trimethoprim as described previously (40).

Nucleotide sequence analysis. DNA was sequenced by the dideoxynucleotide chain termination method. Sequence reactions were performed with a chemiluminescent DNA sequencing kit (Toyobo Co. Ltd.) or a dye terminator cycle sequencing kit (Applied Biosystems.).

β-Galactosidase assay. Cells containing plasmids were grown in the Schaeffer's sporulation medium and processed by the method of Ferrari et al. (10), except that the cells were grown at 37°C. Results shown in the figures are for typical experiments among those conducted two to three times.

Nucleotide sequence accession number. The nucleotide sequence data reported in this paper will appear in the DDBJ, NCBI, EMBL, and GSDB nucleotide sequence databases under accession number D26044.

RESULTS

Cloning of a gene(s) enhancing exoprotease production. We have previously shown that multiple copies of *degR* stabilize phosphorylated DegU, causing overproduction of exoproteases (30). In order to further isolate a gene(s) regulating the production of the extracellular proteases, we performed a shotgun cloning of *B. subtilis* DNA in the cells carrying pNC61, which contains the gene for *degR* on the trimethoprim resistance vector pNC6 (30). This cloning strategy has two advantages, i.e., (i) a gene(s) encoding a protein interacting with DegR may be cloned, and (ii) a gene whose product has a weak inhibitory effect and thus needs to be detected in cells already producing a higher level of the exoproteases may be cloned. One such example is the isolation of the N-terminal region of the *degU* gene, which shows an inhibitory effect on exoprotease production (40).

B. subtilis CU741 total DNA and the kanamycin resistance vector pUBH1 were digested with *Eco*RI, ligated, and transformed into the MI112 cells carrying pNC61 by protoplast transformation. Kanamycin- and trimethoprim-resistant transformants were screened for overproducers of the exoproteases by the halo assay (see Materials and Methods). Among the 2,500 transformants, one colony showed an extremely large halo compared with those of the cells carrying pNC61 and pUBH1. The plasmid from this transformant was designated pLC1.

Enhancing effect of pLC1 on expression of *aprE*. To quantitate the extent of the apparent synergistic effect of pLC1 on pNC61, we introduced pLC1 together with pNC61 or pNC6 into MT33 cells that carry a chromosomal *aprE'-'lacZ* fusion. Cells were grown in Schaeffer's sporulation medium, and the β-galactosidase activities in the cells were determined. As shown in Fig. 1A, the cells carrying both pNC61 and pLC1 showed about 50-fold higher levels of activity than the control cells carrying pNC6 and pUBH1 did. The activities in the cells

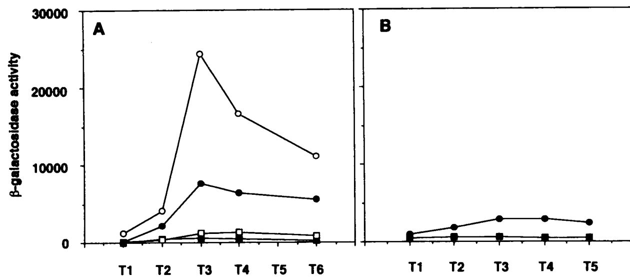

FIG. 1. Effect of pLC1 and pNC61 on *aprE'-'lacZ* expression. The expression of *aprE* was measured by monitoring the β-galactosidase activity derived from the *aprE'-'lacZ* translational fusions on the MT33 $proB^+$ chromosome (A) and the MTPB33 *proB* chromosome (B). Experimental conditions are described in Materials and Methods. Numbers on the *x* axis represent the growth times in hours relative to the end of vegetative growth (T_0). The β-galactosidase activity is expressed in Miller units. ○, cells carrying pLC1 and pNC61; ●, cells carrying pUBH1 and pNC61; □, cells carrying pLC1 and pNC6; ■, cells carrying pUBH1 and pNC6.

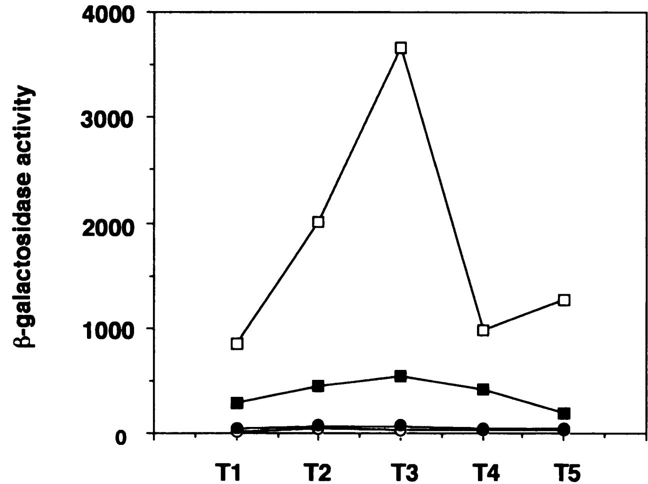

FIG. 3. Requirement of *degS* for the enhancement of *aprE* expression by pLC1. The expression of *aprE* was measured by monitoring the β-galactosidase activity produced from the chromosomal *aprE'-'lacZ* translational fusion. Experimental conditions are described in Materials and Methods. Numbers on the *x* axis represent the growth times in hours relative to the end of vegetative growth (T_0). □, TT715 ($degS^+$ *aprE'-'lacZ*) carrying pLC1; ■, TT715 carrying pUBH1; ○, TT7291 (Δ*degS aprE'-'lacZ*) carrying pLC1; ●, TT7291 carrying pUBH1.

carrying pNC61 plus pUBH1 and those carrying pNC6 plus pLC1 were estimated to be 12- and 2.5-fold higher at T_3 (i.e., 3 h after the end of vegetative growth) than that in the control cells, respectively. This shows that although pLC1 had a small positive effect on *aprE* expression, its effect became more prominent when it was present together with pNC61. From the magnitude of amplification, it can be seen that the effect of coexistence is synergistic but not additive.

Synergistic effect of pLC1 on *degR*. We next examined whether the observed synergistic effect of pLC1 is dependent on the *degR* gene on pNC61. The cells carrying various pNC61 derivatives were examined for extracellular protease production by the halo assay on the LBCG agar plates, and the results are shown in Fig. 2. The intact *degR* gene is carried on pNC61ΔH8 and pNC61ΔR5, whereas it is deleted in pNC61ΔV10 (30). It was found that the cells carrying pLC1

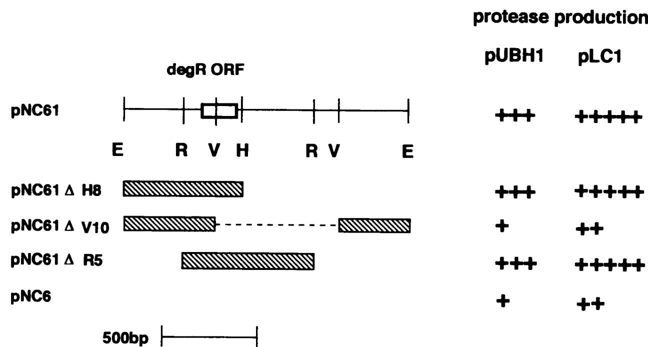

FIG. 2. Determination of the DNA region on pNC61 necessary for the synergistic effect of pLC1. The restriction map of the 1.6-kb DNA insert in pNC61 is shown. The open box represents the *degR* gene. Abbreviations: E, *Eco*RI; H, *Hin*dIII; R, *Rsa*I; V, *Eco*RV. The hatched boxes in the deletion plasmids represent the DNA regions remaining in the plasmids. The sizes of the halos produced by the MI112 cells harboring either pLC1 or pUBH1 together with pNC61 or its derivatives were measured as described in Materials and Methods. The numbers of plus signs represent the sizes of the halos relative to that produced by the control cells carrying pUBH1 and pNC6. The approximate diameters of the halos in a typical experiment were as follows: +, 9.0 mm; ++, 9.0 to 10.5 mm; +++, 10.5 to 12.0 mm; +++++, 15.0 mm.

plus pNC61ΔH8 and those carrying pLC1 plus pNC61ΔR5 produced halos as large as that observed for the cells carrying pLC1 and pNC61. Conversely, the cells carrying pLC1 and pNC61ΔV10 showed a halo similar in size to the one observed for those carrying pLC1 and pNC6. On the basis of these data we concluded that the synergistic action of pLC1 requires the *degR* gene.

The synergism between the gene on pLC1 and *degR* seems to be observed only when *degR* was present on a multicopy plasmid, since the extent of the enhanced production of the exoproteases by pLC1 was not changed irrespective of whether the chromosomal *degR* was intact or disrupted (data not shown). It should be noted that a disruption of the chromosomal *degR* had no effect on exoprotease production (50), showing that the synergism depends on the amount of DegR in the cell.

To examine whether pLC1 also exerts its synergistic effect on another positive regulator, *degQ*, we constructed plasmid pNQ602, in which the *degQ* gene carried on the 2.9-kb fragment insert of pBQ1 (2) had been transferred to a trimethoprim resistance vector, and introduced it into MT33 cells. We found no synergistic effect of pLC1 on pNQ602 (32), suggesting that the synergistic effect of pLC1 is specific to DegR.

Taking account of these facts, we concluded that pLC1 cooperatively works with pNC61 in the production of the exoproteases.

Effect of *degS* deletion on enhancing activity of pLC1. The positive regulation by *degR* requires intact *degS* and *degU* (29). To examine whether the effect of pLC1 depends on the *degS* gene product, the plasmid was introduced into strains TT715 ($degS^+$) and TT7291, a strain carrying a deletion in the *degS* gene, and the expression of *aprE* was determined by the β-galactosidase activity produced from the *aprE'-'lacZ* fusion on the chromosome. It was found that the level of β-galactosidase activity in TT715 carrying pLC1 was sixfold higher at T_3 than that in TT715 carrying pUBH1 (Fig. 3). The slightly higher level of enhancing activity observed at T_3 in this

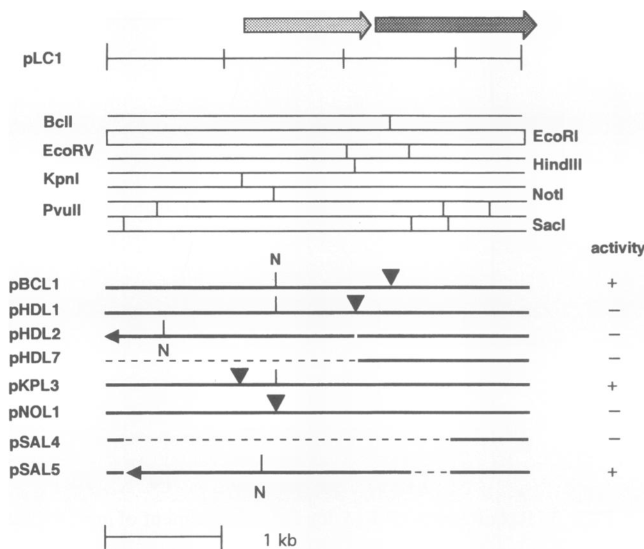

FIG. 4. Restriction map of the 3.5-kb insert in pLC1 (upper half) and structures of pLC1 derivatives (lower half). The light and dark shaded arrows represent ORFs and the direction of translation. Dashed lines in pHDL7, pSAL4, and pSAL5 indicate the regions deleted from pLC1. N, *Not*I site. The arrows in the lower half indicate the inversion of the respective DNA fragments. In pHDL2, an extra sequence of 29 nucleotides carried over from the vector during construction is added at the rightmost part of the arrow (see Materials and Methods). Construction of the derivatives is described in Materials and Methods. Closed triangles indicate the frameshift mutations introduced, and plus and minus signs show the presence and absence of the enhancing effect on exoprotease production, respectively, as determined by the halo assay with the cells carrying pNC61. Plus and minus signs correspond to +++++ and +++ in Fig. 2, respectively.

experiment compared with the level of enhancement observed when MT33 was used as the host strain (see above) may be due to the strain difference. The deletion of *degS* in strain TT7291 carrying pUBH1 caused a 10-fold reduction of *aprE* expression compared with that of TT715 carrying pUBH1, and introduction of pLC1 into TT7291 did not enhance the expression of the *aprE* gene appreciably (Fig. 3). From these results, we concluded that the gene on pLC1 requires the DegS protein for its enhancing activity.

Restriction analyses and determination of the sequence of the gene responsible for the synergistic effect on exoprotease production. A physical map of the 3.5-kb DNA insert in pLC1 was made, and various derivatives were constructed as shown in Fig. 4. The production of the exoproteases by MI112 cells carrying pNC61 and the respective constructed plasmids was investigated by the halo assay on LBCG agar plates. The frameshift mutations introduced into the unique *Bcl*I (pBCL1) and *Kpn*I (pKPL3) sites did not affect the enhancing activity of pLC1, whereas the frameshift mutations at the unique *Not*I (pNOL1) and *Hin*dIII (pHDL1) sites eliminated this activity. Plasmid pSAL5 containing the inverted 2.5-kb *Sac*I fragment retained the enhancing activity. Inversion or deletion of the *Hin*dIII fragment (pHDL2 and pHDL7) and deletion of the two *Sac*I fragments (pSAL4) resulted in the loss of activity. These results show that the 1.4-kb *Kpn*I-*Bcl*I region is responsible for the enhancing activity.

Determination of the sequence of the 2.3-kb *Kpn*I-*Eco*RI DNA fragment revealed a complete open reading frame (ORF) followed by another ORF with its C-terminal region truncated (Fig. 4 and 5). A homology search of the PIR database with the FASTA program (24) showed that the amino acid sequences of the complete and truncated ORFs are homologous to those of ProB and ProA proteins of *E. coli* (8), respectively. The theory that the cloned genes were indeed the *B. subtilis* counterparts of the *pro* genes was verified by the following two experiments. First, insertion of the *cat* gene at the *Not*I site in the putative *proB* gene (Fig. 4 and 5) followed by transformation of the resulting construct into the *B. subtilis* CU741 chromosome produced chloramphenicol-resistant cells with the Pro^- phenotype. Second, when the *Pvu*II-*Eco*RI fragment lying at the extreme 3′ end of the 3.5-kb insert (Fig. 4 and 5) was cloned into a pUC19 derivative carrying the tetracycline resistance gene, and the resulting plasmid, pEPV1T, was transformed into the CU741 chromosome by Campbell-type recombination, the tetracycline-resistant transformants obtained showed the Pro^- phenotype. One of the transformants was designated OM110T. To complete the *proA* sequence, the missing C-terminal region of *proA* was cloned by chromosomal walking and sequenced. The sequence thus obtained is also included in Fig. 5.

The translational initiation codon of the *proB* gene was determined by cloning the *Kpn*I-*Sac*I 1.5-kb DNA fragment downstream from the T7 promoter in pGEM2 (Promega Co.) and then purifying the Pro protein and determining the sequence of the N-terminal end (data not shown). The initiation codon was found to be the ATG starting at nucleotide 84, which is preceded by a putative Shine-Dalgarno (SD) sequence (27). On the other hand, the initiation codon of *proA* was deduced from the nucleotide sequence to be the ATG starting at nucleotide 1188, since this codon is preceded by a row of 6 nucleotides that can be a good candidate for the SD sequence (27) (Fig. 5).

No candidates for the ORF could be identified in the complementary strand of the *Kpn*I-*Eco*RI region, since there were no possible initiation codons followed by appropriate SD sequences.

Taking account of these results, we concluded that multiple copies of the *proB* gene are responsible for the enhancing effect of pLC1 on the production of the extracellular proteases.

Synergism between *degR* on pNC61 and single-copy *proB* gene. To test whether the *proB* gene on the chromosome also shows the synergistic effect with the multicopy *degR* gene, we examined the effect of disruption of *proB* on the enhancing effect of pNC61. As shown in Fig. 1B, the stimulatory effect of pNC61 was found to be 5-fold at T_3 and T_4 in the strain carrying a *proB* disruption, compared with the 12-fold stimulation observed when the *proB* gene was intact (Fig. 1A). This is consistent with the observation that the enhancing effect of pNC61 on exoprotease production was reduced when the *proB* gene was disrupted (32). There was no detectable difference in the expression of *aprE′-′lacZ* in the wild-type and the *proB*-disrupted strains carrying the vectors alone (compare Fig. 1A and B). These results show that a synergism between multicopy *degR* and the single-copy *proB* on the chromosome was also observed.

The *proA* gene on pLC1 lacking its C-terminal region encodes inactive glutamyl-γ-semialdehyde dehydrogenase. pLC1 contains the complete *proB* gene and a part of the *proA* gene lacking the *proA* C-terminal 31 codons as described above. Therefore, if the glutamyl-γ-semialdehyde dehydrogenase encoded by the truncated *proA* is inactive, the γ-glutamyl phosphate produced by γ-glutamyl kinase (the *proB* gene product) would accumulate. When pLC1 was introduced into strain OM110T, the transformants showed the Pro^- phenotype, indicating that the multiple copies of the truncated *proA*

KpnI
GGTACCACGGAAACCCATTTGCTCCTTATGAAATCAGGATGAAATGGGTTTTTTATTGTAGAAGAGCCGCGGAGGAGAA 80

TAA ATG AAA AAA CAA AGA ATA GTA GTG AAA ATA GGA AGC AGT TCG CTC ACG AAT AGC AAA 140
Met Lys Lys Gln Arg Ile Val Val Lys Ile Gly Ser Ser Ser Leu Thr Asn Ser Lys

GGA AGC ATT GAT GAG CAA AAT CAG AGA GCA TGC TCA GCG ATT TCC GTG TTA AAA AAA GCG 200
Gly Ser Ile Asp Glu Gln Asn Gln Arg Ala Cys Ser Ala Ile Ser Val Leu Lys Lys Ala

GGG CAT GAA ATG ATT CTG ATT ACC TCG GGT GCC GTA GCG GCG GGG TTT TCC AGC CTC GGT 260
Gly His Glu Met Ile Leu Ile Thr Ser Gly Ala Val Ala Ala Gly Phe Ser Ser Leu Gly

NotI
TAT CCA TCC CGT CCC GTT ACC ATC AAA GGA AAA CAG GCG GCG GCC GCG GTC GGA CAA ACA 320
Tyr Pro Ser Arg Pro Val Thr Ile Lys Gly Lys Gln Ala Ala Ala Ala Val Gly Gln Thr

CTG TTA ATG CAG CAA TAT ATG AAT CAA TTT AAA CAA TAC TCA CTG ACT CCG GGA CAA ATC 380
Leu Leu Met Gln Gln Tyr Met Asn Gln Phe Lys Gln Tyr Ser Leu Thr Pro Gly Gln Ile

CTT TTA ACG AGA AAT GAT TTT TCG AAA AGA GAA CGG TAT CGA AAC GCG TAT GCT ACG ATA 440
Leu Leu Thr Arg Asn Asp Phe Ser Lys Arg Glu Arg Tyr Arg Asn Ala Tyr Ala Thr Ile

ATG GAA TTA TTA GAG CGG GGC GTC ATT CCG ATT ATC AAC GAG AAC GAT TCT ACA TCT GTT 500
Met Glu Leu Leu Glu Arg Gly Val Ile Pro Ile Ile Asn Glu Asn Asp Ser Thr Ser Val

GAA GAA TTG ACA TTC GTA GAT AAT GAT ATG CTT TCT GCG TTA GTC AGC GGA TTG ATT CAC 560
Glu Glu Leu Thr Phe Gly Asp Asn Asp Met Leu Ser Ala Leu Val Ser Gly Leu Ile His

GCG GAC CAG CTC ATG ATT CTC ACT GAC ATT AAC GGA CTG TAT GAT GCC AAT CCA AAT GAA 620
Ala Asp Gln Leu Met Ile Leu Thr Asp Ile Asn Gly Leu Tyr Asp Ala Asn Pro Asn Glu

ATC CTG AGC GAA CGA TTT GAT TAT TTG CCA GAG ATC ACG CCT GAA TTG CTT GGA TAT GCA 680
Ile Leu Ser Glu Arg Phe Asp Tyr Leu Pro Glu Ile Thr Pro Glu Leu Leu Gly Tyr Ala

GGT TCA GCT GGA TCA AAG GTC GGC ACC GGC GGA ATG AAA TCA AAG CTG TTA GCC ACA CAA 740
Gly Ser Ala Gly Ser Lys Val Gly Thr Gly Gly Met Lys Ser Lys Leu Leu Ala Thr Gln

ACC GCG CTG TCT CTG GGA GTG AAA GTA TTC ATT GGA ACT GGC AGC GGA GAG CAA AAA CTT 800
Thr Ala Leu Ser Leu Gly Val Lys Val Phe Ile Gly Thr Gly Ser Gly Glu Gln Lys Leu

GCG GAC ATT TTG GAC GGC AGG GGA GAC GGC ACT TAT ATC GGA GAC AAA GAA CTA TCT TCG 860
Ala Asp Ile Leu Asp Gly Arg Gly Asp Gly Thr Tyr Ile Gly Asp Lys Glu Leu Ser Ser

GTT AAC AAC ACA AGG CAG TGG ATT CAG TTC CAC TCG CCG ATA TCG GGA GAA ATT ATC ATT 920
Val Asn Asn Thr Arg Gln Trp Ile Gln Phe His Ser Pro Ile Ser Gly Glu Ile Ile Ile

GAT GCG GGT GCC GAA GAG GCG ATG ATC CAT AAT GGA AGC AGC CTT TTG CCC GCT GGA GTT 980
Asp Ala Gly Ala Glu Glu Ala Met Ile His Asn Gly Ser Ser Leu Leu Pro Ala Gly Val

HindIII
GTG GGC GTG AAC GGA AGC TTT CCG AAA GGG GCG GTT GTG GAA GTC AGG GGA CCG GGC GGC 1040
Val Gly Val Asn Gly Ser Phe Pro Lys Gly Ala Val Val Glu Val Arg Gly Pro Gly Gly

GTG ATC GGC AAA GGC CAA ACT CAT TAC TCC TCC GAG GAG ATT ATG GAG GCT AAA GGC AAA 1100
Val Ile Gly Lys Gly Gln Thr His Tyr Ser Ser Glu Glu Ile Met Glu Ala Lys Gly Lys

CGC AGT GAC GAA CTT GAT TTT GAG AAA ACG TTT GAG GTT ATT CAT TAG GAATGACTGGGTCAAT 1164
Arg Ser Asp Glu Leu Asp Phe Glu Lys Thr Phe Glu Val Ile His ***

GTAAAAGACTAGGAGGCGGAAAA ATG AGT GAA GTT TCT GTA AAA GCG AAG CTG GCG AAA GAA GCA 1229
Met Ser Glu Val Ser Val Lys Ala Lys Leu Ala Lys Glu Ala

BclI
GCA GCC GAA ATG ATC ATG AAA ACA ACA GCC GAG AAG GAT TAG GCG CTC AGC CTC ATT GCA 1289
AlA Ala Glu Met Ile Met Lys Thr Thr Ala Glu Lys Asp Glu Ala Leu Ser Leu Ile Ala

AAC GGA CTC CGC AAA GAA CTG GAT TTT CTC TTG GCG GAG AAT GCA AAA GAC ATT GTG AAC 1349
Asn Gly Leu Arg Lys Glu Leu Asp Phe Leu Leu Ala Glu Asn Ala Lys Asp Ile Val Asn

GGA AAA GAG AAT GGT TTA ACA CCG GAC ATC ATT GAC CGT CTC TCA TTG GAT GAG AAA CGG 1409
Gly Lys Glu Asn Gly Leu Thr Pro Asp Ile Ile Asp Arg Leu Ser Leu Asp Glu Lys Arg

SacI
ATA CGC GAT ATC GCG GAC GCG GTG GAG CTC TTA ATC GAC TTA GCG GAC CCA ATC GGC GAC 1469
Ile Arg Asp Ile Ala Asp Ala Val Glu Leu Leu Ile Asp Leu Ala Asp Pro Ile Gly Asp

TCT CTT GAA ACG ATT GAA AAA GAA AAC GGC CTG TTT ATT CAA AAA ATC CGT GTG CCG CTC 1529
Ser Leu Glu Thr Ile Glu Lys Glu Asn Gly Leu Phe Ile Gln Lys Ile Arg Val Pro Leu

GGT GTC GTA GGA ATG ATT TAT GAG GCG AGG CCA AAC GTC ACA GTT GAT GCG GCT ACC CTT 1589
Gly Val Val Gly Met Ile Tyr Glu Ala Arg Pro Asn Val Thr Val Asp Ala Ala Thr Leu

TGC CTG AAG ACA GGG AAC GCG GTT GTG CTG CGG GGA AGC TCC TCA GCC ATC CAC AGC AAC 1649
Cys Leu Lys Thr Gly asn Ala Val Val Leu Arg Gly Ser Ser Ser Ala Ile His Ser Asn

AAA GCG CTC GTC AGT GTC ATT TAC AGA GCA CTT GAG CAA TCA GCG CTT CCG ATT CAC ACT 1709
Lys Ala Leu Val Ser Val Ile Tyr Arg Ala Leu Glu Gln Ser Ala Leu Pro Ile His Thr

PvuII SacI
GTG CAG CTG ATT GAG GAT ACG AGC AGA GAG ACA GCA AAA GAG CTC TTT ACG TTA AAT GAT 1769
Val Gln Leu Ile Glu Asp Thr Ser Arg Glu Thr Ala Lys Glu Leu Phe Thr Leu Asn Asp

GGC TTA GAC GTA TTG ATT CCG CGC GGA GGC AAG AAA CTG ATT GAT CTC GTT GTG AGA GAA 1829
Gly Leu Asp Val Leu Ile Pro Arg Gly Gly Lys Lys Leu Ile Asp Leu Val Val Arg Glu

TCA ACA GTT CCT GTA TTA GAA ACG GGA GCA GGA AAC TGC CAC ATA TTC ATT GAC GAA ACA 1889
Ser Thr Val Pro Val Leu Glu Thr Gly Ala Gly Asn Cys His Ile Phe Ile Asp Glu Thr

GCC AAA CCG CAA ATG GCA GAA AAG GTT GTT GTA AAT GCC AAA ACG CAG CGT CCT TCT GTA 1949
Ala Lys Pro Gln Met Ala Glu Lys Val Val Val Asn Ala Lys Thr Gln Arg Pro Ser Val

TGC AAC GCG ATT GAA TCA TTG CTG ATT CAC AAG GCT TGG GCA AGA CAG AAC GGA AAA GAA 2009
Cys Asn Ala Ile Glu Ser Leu Leu Ile His Lys Ala Trp Ala Arg Gln Asn Gly Lys Glu

PvuII
TTG CTG GAC CAG CTG GAA AAC GCG GGA GTT GAA ATT CGC GGT GAT GAA TTG GTA TGT GAA 2069
Leu Leu Asp Gln Leu Glu Asn Ala Gly Val Glu Ile Arg Gly Asp Glu Leu Val Cys Glu

CTT CAT CCT TCA AGC AAA CAA GCA TCA AAA GAA GAT TGG GAA ACC GAA TTT TTA GCG CCT 2129
Leu His Pro Ser Ser Lys Gln Ala Ser Lys Glu Asp Trp Glu Thr Glu Phe Leu Ala Pro

GTC CTC AGC GTA AAG ACG GTT GAA AAC GTC CAA GAA GCT GTA AAG CAT ATC CAA CAA TAC 2189
Val Leu Ser Val Lys Thr Val Glu Asn Val Gln Glu Ala Val Lys His Ile Gln Gln Tyr

GGC ACC AAT CAT TCT GAA GCG ATT TTA ACT GAA AAT GAC AAA AAT GCG GTA TAT TTT CAA 2249
Gly Thr Asn His Ser Glu Ala Ile Leu Thr Glu Asn Asp Lys Asn Ala Val Tyr Phe Gln

ACG GCT GTC GAT GCT GCC GCT GTC TAT CAT AAC GCG TCA ACC CGC TTT ACC GAC GGC TTT 2309
Thr Ala Val Asp Ala Ala Ala Val Tyr His Asn Ala Ser Thr Arg Phe Thr Asp Gly Phe

EcoRI HindIII
GGA ATT CGG CTA CGG AGC CGA AAT CGG CAT CAG CAC GCA AAA GCT TCA TGC AAG AGG ACC 2369
Gly Ile Arg Leu Arg Ser Arg Asn Arg His Gln His Ala Lys Ala Ser Cys Lys Arg Thr

GAT GGG GCT TCC TGC ACT GAC TTC TAC AAA ATA CAT CAT TAA AGGAACTGGGCAAATCCGTGAATA 2435
Asp Gly Ala Ser Cys Thr Asp Phe Tyr Lys Ile His His ***

HindIII HindIII
GCGGGGTAATGTTCAATGAAAAAGCTTATCTCATTACGAGATAAGCTTTTTTATACGCGAAAAATTGACATTTCAAATTT 2515

AATTGTGTACAATTTAATTGTATAAAATATATTGGGGAATGAAAAATGAGTCAGCCATTATTTACCGCAACTGTTTCAG 2595

CGGTAGGAGGAAGAGAAGGAAAGGTCATTTCATCAGACCGCGTTCTTGAGCTTGATGTCGCAATGCCGGGGACACCGAGA 2675

GCCAAGAAAATTAGAAAAAGCGACAAAATCCAGAGCAGCTTGTTTG 2721

FIG. 5. Nucleotide sequences of the *proB* and *proA* genes and their flanking region. The sequences with thick and thin underlines represent the putative SD sequences and ρ-independent transcription terminators, respectively. The shaded nucleotides show the restriction endonuclease sites relevant to the present study. The amino acid sequence determined by the N-terminal analysis is boxed.

gene on pLC1 do not code for active glutamyl-γ-semialdehyde dehydrogenase.

Effects of *proB* and *degR* on expression of *degU* and effect of *degR* on expression of *proB*. The *degS* and *degU* genes are the key elements positively controlling the production of the exoproteases (6, 28). It was found that multicopy *proB* had little effect on the expression of *degU*, whereas multicopy *degR* produced a slight stimulation (Fig. 6A and B). The extent of the observed *degR* effect, however, cannot explain the level of enhancement of *aprE* expression (Fig. 1) (29) and thus may be the secondary effect.

Multicopy *degR* did not affect the expression of *proB* appreciably, as shown in Fig. 6C.

These results show that the enhanced expression of *aprE* caused by *proB* and *degR* is not due to either the overexpression of *degU* or the enhancement of *proB* expression by *degR*.

Physical mapping of the cloned DNA fragment. pLC1 contains a 3.5-kb *Eco*RI insert carrying a *Not*I site (Fig. 5). We found that the *Eco*RI insert was identical to the one carried in pNEXT62, a *Not*I linking clone (20). This finding automatically places the locus of the *proB* and *proA* region at 1,375 kb on the *B. subtilis* physical map (20). The observation that disruption of the *Not*I site in the 3.5-kb insert resulted in the Pro^- phenotype (see above) is in line with the previous report that the *pro(BA)* locus determined by genetic mapping is located at 115° on the *B. subtilis* genetic map (4, 36).

DISCUSSION

We demonstrated that multiple copies of *proB* enhanced the expression of *aprE* severalfold and that the level of enhancement by *proB* was elevated when multiple copies of *degR*, but not *degQ*, coexisted in the same cell. The enhancing effect of *proB* and *degR* was found to be synergistic, suggesting that both gene products work in the same regulatory pathway. We have also obtained the following results by using translational fusions of the respective genes with *lacZ*. Neither the multiple copies of *proB* nor those of *degR* stimulated the expression of the *degS-degU* operon significantly, and the multiple copies of *degR* did not stimulate the expression of *proB*. Furthermore, the

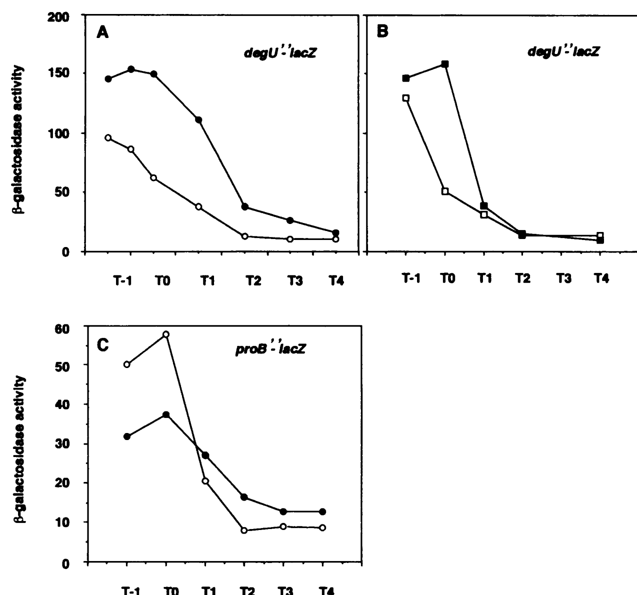

FIG. 6. Effects of multicopy *degR* and *proB* on gene expression. The expression of each gene was determined by measuring the β-galactosidase activities directed by the gene fusions shown. Experimental conditions are described in Materials and Methods. Numbers on the *x* axis represent the growth times in hours relative to the end of vegetative growth (T_0). (A) ●, TT714 carrying pNC61; ○, TT714 carrying pNC6; (B) ■, TT714 carrying pLC1; □, TT714 carrying pUBH1. (C) ●, OM115 carrying pNC61; ○, OM115 carrying pNC6.

multiple copies of the *proB* gene reduced the expression of *degR* (32). From these results, we presume that the synergism is not effected at the transcriptional or translational step of the three transcriptional units but is caused by direct or indirect interaction of the ProB and DegR proteins with DegS.

proA was found downstream from *proB* as revealed by the sequence determination and the disruption of the corresponding gene on the chromosome. The *proB* gene encodes γ-glutamate kinase that converts glutamate to γ-glutamyl phosphate, and the *proA* gene product, glutamate-γ-semialdehyde dehydrogenase, converts the latter compound to glutamate-γ-semialdehyde (3, 8, 13). It is possible that γ-glutamyl phosphate is accumulated in cells carrying pLC1, since the *proA* gene on this plasmid is truncated and inactive. In fact, a *proA* mutation in *E. coli* results in the accumulation of γ-glutamyl phosphate (13, 18). γ-Glutamyl phosphate is unstable and remains bound to γ-glutamate kinase (3, 38). It has been shown that γ-glutamate kinase and glutamate-γ-semialdehyde dehydrogenase form a complex in *E. coli* and that the kinase activity is not detectable in the absence of the dehydrogenase (38). According to our data, the derivative plasmids, pSAL5 and pBCL1, containing the complete *proB* gene and part of the *proA* gene, showed activity that enhanced exoprotease production, indicating that the enhancing effect does not require the truncated *proA* gene on pLC1.

Several low-molecular-weight phospho-compounds have been shown to serve as phospho-donors for response regulators such as CheY, CheB, and NtrC. Thus, both CheY and CheB can be phosphorylated by the N-phosphoryl group of phosphoramidate, whereas CheY is also phosphorylated by acetyl phosphate and carbamoyl phosphate (25). NtrC is phosphorylated by all three of these phospho-compounds (9). These in vitro results show that the individual response regulator has its own spectrum of low-molecular-weight phospho-compounds being used as phospho-donors. Moreover, multiple copies of the acetate kinase gene or accumulation of acetyl phosphate by mutation can restore the expression of the *pho* regulon in a strain lacking both the PhoM and PhoR sensor kinases, suggesting that the phospho-compounds phosphorylate the response regulators without their cognate sensor kinases in vivo (23, 44).

DegR

```
  MDDKDLKLILHKTFIEIYSDLEELADIAKKGKPSMEKYVEEIEQRCKQNILAIEIQMKIK
MNKTKMDSKVLDSILMKMLKTVDGSKDEVFQIGEQSRQQYEQLVEELKQIKQQVYEVIELGDKLE
```

N-terminal region of DegS

FIG. 7. Homology of amino acid sequences between DegR and the N-terminal region of DegS. Double and single dots indicate identical amino acids and conservative changes of amino acids, respectively. A homology search was performed with the FASTA program (24).

Acylphosphates are known to be high-energy compounds. Therefore, it is tempting to speculate that γ-glutamyl phosphate could also serve as a low-molecular-weight phospho-donor and that it is involved in the phosphorylation of the response regulator DegU. Since the enhancing activity of ProB requires *degS*, phosphorylation of DegU might occur via phosphorylation of DegS by γ-glutamyl phosphate. Our current hypothesis of the synergistic effect of *proB* and the multiple copies of *degR* is as follows. γ-Glutamyl phosphate would phosphorylate DegS directly or indirectly, increasing the level of phosphorylated DegS in the cell. This would result in a higher concentration of phosphorylated DegU, which would then be stabilized by DegR. There is a strong homology between the amino acid sequences of DegR and the N-terminal region of DegS (30% identical and 40% similar amino acids; Fig. 7). One possibility of how such stabilization is effected would be that the N-terminal region of DegS is involved in the dephosphorylation of DegU phosphate and that in some way DegR inhibits this reaction by competition. Higher levels of DegU phosphate could possibly also be achieved by the regulation of DegS phosphatase activity by γ-glutamyl phosphate. However, in addition to these possibilities, direct involvement of the ProB protein itself in this signal transduction pathway could be possible, since the synergistic effect was still observed with the *proB* gene in the single-copy state (Fig. 1A and B), a situation in which the γ-glutamyl phosphate would not be at a level high enough to allow it to be transferred to the signal transduction pathway.

Exoprotease production is also positively regulated by *spoOA* (39). If γ-glutamyl phosphate is also involved in the signal transduction pathway involving SpoOA, the enhancing effect of multicopy *proB* on *aprE* expression should be detectable in the *spoOA*$^+$ background. However, this was not the case (Fig. 3). Although these data appear to show that *proB* does not affect the SpoOA pathway, more rigorous experiments are necessary to draw a conclusion. It is, therefore, an open question whether the positive effect of *proB* on *aprE* expression is specific to the DegS-DegU system.

The DegS-DegU two-component system also regulates competence development, i.e., the nonphosphorylated form of DegU is necessary for the activation of competence genes (7). Therefore, it is expected that the higher level of extracellular protease production caused by a higher concentration of phosphorylated DegU should result in a lower level of competency. We observed that the cells carrying pLC1 showed competency reduced to a level that was 1/5 to 1/10 of that of

the wild-type strain (32), a result in line with the interpretation that multiple copies of the *proB* gene enhance phosphorylation of DegU.

It is of interest that a housekeeping gene, *proB*, enhances exoprotease production in a *degS*-dependent manner and in a synergistic way with *degR*. Although the presence of multiple copies of *proB* in the cell is not a physiological condition, it may not be unreasonable to assume that the level of γ-glutamyl phosphate could increase under certain circumstances, such as metabolic unbalance, in the wild, since γ-glutamyl phosphate is also produced as an intermediate in at least one other biosynthetic pathway, i.e., the glutamine pathway.

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REFERENCES

1. **Aiba, H., T. Mizuno, and S. Mizushima.** 1989. Transfer of phosphoryl group between two regulatory proteins involved in osmoregulatory expression of the *ompF* and *ompC* genes in *Escherichia coli*. J. Biol. Chem. **264:**8563–8567.
2. **Amory, A., F. Kunst, E. Aubert, A. Klier, and G. Rapoport.** 1987. Characterization of the *sacQ* genes from *Bacillus licheniformis* and *Bacillus subtilis*. J. Bacteriol. **169:**324–333.
3. **Baich, A.** 1969. Proline synthesis in *Escherichia coli*: a proline-inhibitable glutamic acid kinase. Biochim. Biophys. Acta **192:**462–467.
4. **Buxton, R. S.** 1980. Selection of *Bacillus subtilis* 168 mutants with deletions of the PBSX prophage. J. Gen. Virol. **46:**427–437.
5. **Chang, S. C., and S. N. Cohen.** 1979. High frequency transformation of *Bacillus subtilis* protoplast by plasmid DNA. Mol. Gen. Genet. **168:**111–115.
6. **Dahl, M. K., T. Msadek, F. Kunst, and G. Rapoport.** 1991. Mutational analysis of the *Bacillus subtilis* DegU regulator and its phosphorylation by the DegS protein kinase. J. Bacteriol. **173:** 2539–2547.
7. **Dahl, M. K., T. Msadek, F. Kunst, and G. Rapoport.** 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.
8. **Deutch, A. H., K. E. Rushlow, and C. J. Smith.** 1984. Analysis of the *Escherichia coli proBA* locus by DNA and protein sequencing. Nucleic Acids Res. **12:**6337–6355.
9. **Feng, J., M. R. Atkinson, W. McCleary, J. B. Stock, B. L. Wanner, and A. J. Ninfa.** 1992. Role of phosphorylated metabolic intermediates in the regulation of glutamine synthetase synthesis in *Escherichia coli*. J. Bacteriol. **174:**6061–6070.
10. **Ferrari, E., D. J. Henner, M. Perego, and J. A. Hock.** 1988. Transcription of *Bacillus subtilis* subtilisin and expression of subtilisin in sporulation mutants. J. Bacteriol. **170:**289–295.
11. **Forst, S., J. Delgado, and M. Inouye.** 1989. Phosphorylation of OmpR by the osmosensor EnvZ modulates the expression of the *ompF* and *ompC* genes in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **86:**6052–6056.
12. **Gaur, N. K., E. Dubnau, and I. Smith.** 1986. Characterization of a cloned *Bacillus subtilis* gene that inhibits sporulation in multiple copies. J. Bacteriol. **168:**860–869.
13. **Hayzer, D. J., and T. Leisinger.** 1980. The gene-enzyme relationships of proline biosynthesis in *Escherichia coli*. J. Gen. Microbiol. **118:**287–293.
14. **Hess, J. F., K. Oosawa, N. Kaplan, and M. I. Simon.** 1988. Phosphorylation of three proteins in the signaling pathway of bacterial chemotaxis. Cell **53:**79–87.
15. **Hess, J. F., K. Oosawa, P. Matsumura, and M. I. Simon.** 1987. Protein phosphorylation is involved in bacterial chemotaxis. Proc. Natl. Acad. Sci. USA **84:**7609–7613.
16. **Honjo, M., A. Nakayama, K. Fukazawa, K. Kawamura, K. Ando, M. Hori, and Y. Furutani.** 1990. A novel *Bacillus subtilis* gene involved in negative control of sporulation and degradative-enzyme production. J. Bacteriol. **172:**1783–1790.
17. **Igo, M. M., A. J. Ninfa, and T. J. Silhavy.** 1989. A bacterial environmental sensor that functions as a protein kinase and stimulates transcriptional activation. Gene **3:**598–605.
18. **Inuzuka, M., H. Miyano, and M. Tomoeda.** 1976. Specific action of 4-nitropyridine 1-oxide on *Escherichia coli* K-12 Pro$^+$ strains leading to the isolation of proline-requiring mutants: isolation and characterization of Pro$^-$ mutants. Antimicrob. Agents Chemother. **10:**325–332.
19. **Itaya, M.** 1992. Construction of a novel tetracycline resistance gene cassette useful as a marker on the *Bacillus subtilis* chromosome. Biosci. Biotechnol. Biochem. **56:**685–686.
20. **Itaya, M., and T. Tanaka.** 1991. Complete physical map of the *Bacillus subtilis* 168 chromosome constructed by a gene-directed mutagenesis method. J. Mol. Biol. **220:**631–648.
21. **Kawamura, F., and R. H. Doi.** 1984. Construction of a *Bacillus subtilis* double mutant deficient in extracellular alkaline and neutral proteases. J. Bacteriol. **160:**442–444.
22. **Keener, J., and S. Kutsu.** 1988. Protein kinase and phosphoprotein phosphatase activities of nitrogen regulatory proteins NtrB and NtrC of enteric bacteria: roles of conserved amino-terminal domain of NtrC. Proc. Natl. Acad. Sci. USA **85:**4976–4980.
23. **Lee, T.-Y., K. Makino, H. Shinagawa, and A. Nakata.** 1990. Overproduction of acetate kinase activates the phosphate regulon in the absence of the *phoR* and *phoM* functions in *Escherichia coli*. J. Bacteriol. **172:**2245–2249.
24. **Lipman, D. J., and W. R. Pearson.** 1985. Rapid and sensitive protein similarity searches. Science **227:**1435–1441.
25. **Lukat, G. S., W. R. McCleary, A. M. Stock, and J. B. Stock.** 1992. Phosphorylation of bacterial response regulator proteins by low molecular weight phospho-donors. Proc. Natl. Acad. Sci. USA **89:**718–722.
26. **Maniatis, T., E. F. Fritsch, and J. Sambrook.** 1982. Molecular cloning: a laboratory manual. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
27. **McLaughlin, J. R., C. L. Murray, and J. C. Rabinovich.** 1981. Unique features in the ribosome-binding site sequence of the gram-positive *Staphylococcus aureus* β-lactamase gene. J. Biol. Chem. **256:**11283–11291.
28. **Mukai, K., M. Kawata, and T. Tanaka.** 1990. Isolation and phosphorylation of the *Bacillus subtilis degS* and *degU* gene products. J. Biol. Chem. **265:**824–834.
29. **Mukai, K., M. Kawata-Mukai, and T. Tanaka.** 1992. Stabilization of phosphorylated *Bacillus subtilis* DegU by DegR. J. Bacteriol. **174:**7954–7962.
30. **Nagami, Y., and T. Tanaka.** 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.
31. **Ninfa, A., and B. Magasanik.** 1986. Covalent modification of the *glnG* product, NRI, by the *glnL* product, NRII, regulates the transcription of the *glnALG* operon in *Escherichia coli*. Proc. Natl. Acad. Sci. USA **83:**5909–5913.
32. **Ogura, M., and T. Tanaka.** Unpublished data.
33. **Pang, A. S.-H., S. Nathoo, and S.-L. Wong.** 1991. Cloning and characterization of a pair of novel genes that regulate production of extracellular enzymes in *Bacillus subtilis*. J. Bacteriol. **173:**46–54.
34. **Parkinson, J. S.** 1993. Signal transduction schemes of bacteria. Cell **73:**857–871.
35. **Perego, M., and J. A. Hoch.** 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.
36. **Piggot, J. P., and J. A. Hoch.** 1985. Revised genetic linkage map of *Bacillus subtilis*. Microbiol. Rev. **49:**158–179.
37. **Schaeffer, P. J., J. Millet, and J. Aubert.** 1965. Catabolite repression of bacterial sporulation. Proc. Natl. Acad. Sci. USA **54:**704–711.
38. **Smith, C. J., A. H. Deutch, and K. E. Rushlow.** 1984. Purification and characteristics of a γ-glutamyl kinase involved in *Escherichia coli* proline biosynthesis. J. Bacteriol. **157:**545–551.
39. **Strauch, M. A.** 1993. AbrB, a transition state regulator, p. 757–764. *In* A. L. Sonenshein, J. A. Hoch, and R. Losick (ed.), *Bacillus subtilis* and other gram-positive bacteria: biochemistry, physiology,

and molecular genetics. American Society for Microbiology, Washington, D.C.

40. **Tanaka, T., and M. Kawata.** 1988. Cloning and characterization of *Bacillus subtilis iep*, which has positive and negative effects on production of extracellular proteases. J. Bacteriol. **170:**3593–3600.
41. **Tanaka, T., M. Kawata, and K. Mukai.** 1991. Altered phosphorylation of *Bacillus subtilis* DegU caused by single amino acid changes in DegS. J. Bacteriol. **173:**5507–5515.
42. **Tanaka, T., M. Kawata, Y. Nagami, and H. Uchiyama.** 1987. *prtR* enhances the mRNA level of the *Bacillus subtilis* extracellular proteases. J. Bacteriol. **169:**3044–3050.
43. **Tanaka, T., and M. Kawata-Mukai.** 1994. Stabilization of phosphorylated form of *Bacillus subtilis* DegU caused by *degU9* mutation. FEMS Microbiol. Lett. **115:**93–96.
44. **Wanner, B. L., and M. R. Wilmes-Riesenberg.** 1992. Involvement of phosphotransacetylase, acetate kinase, and acetyl phosphate synthesis in control of the phosphate regulon in *Escherichia coli*. J. Bacteriol. **174:**2124–2130.
45. **Ward, J. B., Jr., and S. A. Zahler.** 1973. Genetic studies of leucine biosynthesis in *Bacillus subtilis*. J. Bacteriol. **116:**719–726.
46. **Wong, S.-L., L.-F. Wang, and R. H. Doi.** 1988. Cloning and nucleotide sequence of *senN*, a novel *Bacillus natto* (*B. subtilis*) gene that regulates expression of extracellular protease genes. J. Gen. Microbiol. **139:**3264–3276.
47. **Weiss, Y., and B. Magasanik.** 1988. Phosphorylation of nitrogen regulator I (NRI) of *Escherichia coli*. Proc. Natl. Acad. Sci. USA **85:**8919–8923.
48. **Wylie, D., A. Stock, C.-Y. Wong, and J. Stock.** 1988. Sensory transduction in bacterial chemotaxis involves phosphotransfer between Che proteins. Biochem. Biophys. Res. Commun. **151:** 891–896.
49. **Yang, M., E. Ferrari, E. Chen, and D. J. Henner.** 1986. Identification of the pleiotropic *sacQ* gene of *Bacillus subtilis*. J. Bacteriol. **166:**113–119.
50. **Yang, M., H. Shimotsu, E. Ferrari, and D. J. Henner.** 1987. Characterization and mapping of the *Bacillus subtilis prtR* gene. J. Bacteriol. **169:**434–437.
51. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.
52. **Zuber, P., and R. Losick.** 1987. Use of a *lacZ* fusion to study the role of the *spoO* genes of *Bacillus subtilis* in development regulation. Cell **35:**275–283.