

# The *Bacillus subtilis* Response Regulator Gene *degU* Is Positively Regulated by CcpA and by Catabolite-Repressed Synthesis of ClpC

Hiroshi Ishii, Teruo Tanaka, Mitsuo Ogura

Institute of Oceanic Research and Development, Tokai University, Orido-Shimizu, Shizuoka, Japan

In *Bacillus subtilis*, the response regulator DegU and its cognate kinase, DegS, constitute a two-component system that regulates many cellular processes, including exoprotease production and genetic competence. Phosphorylated DegU (DegU-P) activates its own promoter and is degraded by the ClpCP protease. We observed induction of *degU* by glucose in sporulation medium. This was abolished in two mutants: the *ccpA* (catabolite control protein A) and *clpC* disruptants. Transcription of the promoter of the operon containing *clpC* (P<sub>clpC</sub>) decreased in the presence of glucose, and the disruption of *ccpA* resulted in derepression of P<sub>clpC</sub>. However, this was not directly mediated by CcpA, because we failed to detect binding of CcpA to P<sub>clpC</sub>. Glucose decreased the expression of *clpC*, leading to low cellular concentrations of the ClpCP protease. Thus, *degU* is induced through activation of autoregulation by a decrease in ClpCP-dependent proteolysis of DegU-P. An electrophoretic mobility shift assay showed that CcpA bound directly to the *degU* upstream region, indicating that CcpA activates *degU* through binding. The bound region was narrowed down to 27 bases, which contained a *cre* (catabolite-responsive element) sequence with a low match to the *cre* consensus sequence. In a footprint analysis, CcpA specifically protected a region containing the *cre* sequence from DNase I digestion. The induction of *degU* by glucose showed complex regulation of the *degU* gene.

Glucose is a very easily metabolized carbon source and thus is one of the preferred carbon sources for bacteria. Addition of glucose to growth medium has profound effects on bacterial cell physiology (1). The transcription factor CcpA plays a central role in catabolite regulation in low-GC Gram-positive bacteria (2, 3). CcpA is activated by association with P-Ser-Hpr, which is Ser-phosphorylated Hpr produced by HprK. Hpr is a histidine-containing phosphotransfer protein involved in sugar transport. Activation of HprK is triggered by accumulation of fructose-1,6-biphosphate, whose cellular concentrations are increased by the incorporation of a preferred sugar such as glucose. CcpA binds to a degenerate pseudopalindromic sequence known as the *cre* (catabolite-responsive element) sequence (4). CcpA functions negatively and positively for gene transcription, and its function is determined by the location of the *cre* sequence in many cases (2). Upstream binding of CcpA from the core promoter results in transcriptional activation, but it represses transcription when it binds to regions in the core promoter or at downstream locations, including within the gene open reading frame (ORF) (2). Many of the genes regulated by CcpA are related to steps in carbon metabolism, such as glycolysis, gluconeogenesis, and the tricarboxylic acid (TCA) cycle, and to nitrogen metabolism. Several global analyses, such as DNA microarray analyses, chromatin immunoprecipitation (ChIP) analyses, and *in silico* searching for *cre* sequences in the genome revealed that more than 300 genes are directly or indirectly regulated by CcpA (5–11). In DNA microarray analyses and chromatin immunoprecipitation analyses, many previously unidentified CcpA-dependent genes were detected, leading to expansion of the functional roles of CcpA. We note that *degU* was not identified as a CcpA-dependent gene in these analyses.

DegU is a response regulator that belongs to the NarL subfamily (12). It is activated by phosphorylation of a single Asp site on its receiver domain by the cognate kinase DegS (13). The *degU* gene resides within an operon with the upstream *degS* gene (14, 15). DegU, a DNA-binding protein that recognizes AT-rich octamers in a variety of arrangements (12, 16), controls many genes and

biological processes (17, 18). In its low-level phosphorylation state, including its nonphosphorylated state, DegU activates *comK* (a master regulator of genetic competence) and the *fla/che* operon (12, 14, 19, 20). In its high-level phosphorylation state, DegU activates *degU* itself and induces many target genes encoding extracellular degrading enzymes (20–25). Thus, *degU* is activated by an autoregulatory loop through the P3 promoter (26, 27). In addition, high levels of DegU-P result in repression of motility (25, 28). The P2 promoter, which is located upstream of the *degU* P3 promoter, has been known to be activated by the transcription factor TnrA either under nitrogen starvation conditions or in a disruptant of *glnA*, encoding glutamine synthetase (27, 29). These observations indicate that there is some nutrient-dependent regulation of *degU*. Furthermore, the AAA<sup>+</sup> protease ClpCP specifically degrades the phosphorylated form of DegU (DegU-P), leading to modulation of the DegU autoactivation loop (26, 30). DegU is also regulated by protein-protein interactions, including the RapG-PhrG system. RapG inhibits the binding of DegU to DNA, and the extracellular pentapeptide PhrG inhibits RapG activity (31). The other protein-protein interaction includes the small protein SwrA, which is a swarming motility regulator (32). SwrA interacts with the N-terminal region of DegU, leading to stabilization of the DegU dimer (16).

In this paper, we report that the addition of glucose induced expression of the *degU* gene, leading to increased expression of

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Address correspondence to Mitsuo Ogura, oguram@scc.u-tokai.ac.jp.

H.I. and M.O. contributed equally to this article.

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TABLE 1 Strains and plasmids used for this study

Strain or plasmid	Genotype or description	Reference or source
<b>Strains</b>		
168	<i>trpC2</i>	Laboratory stock
TF10	<i>trpC2 ccpA</i> (Cm <sup>r</sup> )	33
OAM632	<i>trpC2 hprK</i> (Pm <sup>r</sup> )	This study
OAM588	<i>trpC2 clpC</i> (Tc <sup>r</sup> )	26
CU741T	<i>trpC2 leuC7 tnrA</i> (Cm <sup>r</sup> )	27
168 <i>glnA14</i>	<i>trpC2 glnA</i> (Sp <sup>r</sup> )	29
OAM415	<i>trpC2 amyE::degU-lacZ</i> U-WT (Cm <sup>r</sup> )	26
OAM633	<i>trpC2 amyE::degU-lacZ</i> U-del11 (Cm <sup>r</sup> )	This study
OAM634	<i>trpC2 amyE::degU-lacZ</i> U-M15 (Cm <sup>r</sup> )	This study
OAM516	<i>trpC2 amyE::degU-lacZ</i> U-del3 (Cm <sup>r</sup> )	26
OAM636	<i>trpC2 amyE::degU-lacZ</i> U-P2 (Cm <sup>r</sup> )	This study
OAM637	<i>trpC2 amyE::degU-lacZ</i> U-P2 (Cm <sup>r</sup> ) <i>glnA</i> (Sp <sup>r</sup> )	This study
OAM638	<i>trpC2 amyE::degU-lacZ</i> U-P2 (Cm <sup>r</sup> ) <i>glnA</i> (Sp <sup>r</sup> ) <i>tnrA</i> (Cm <sup>r</sup> ::Km <sup>r</sup> )	This study
OAM639	<i>trpC2 amyE::degU-lacZ</i> U-P2 (Cm <sup>r</sup> ::Tc <sup>r</sup> ) <i>glnA</i> (Sp <sup>r</sup> ) <i>ccpA</i> (Cm <sup>r</sup> )	This study
OAM420	<i>trpC2 amyE::degU-lacZ</i> U-WT (Cm <sup>r</sup> ) <i>clpC</i> (Tc <sup>r</sup> )	26
OAM416	<i>trpC2 amyE::degU-lacZ</i> U-WT (Cm <sup>r</sup> ) <i>degU</i> (Km <sup>r</sup> )	26
OAM640	<i>trpC2 thrC::degU-lacZ</i> U-WT (Sp <sup>r</sup> )	This study
OAM641	<i>trpC2 thrC::degU-lacZ</i> U-WT (Sp <sup>r</sup> ) <i>ccpA</i> (Cm <sup>r</sup> )	This study
OAM642	<i>trpC2 thrC::degU-lacZ</i> U-WT (Sp <sup>r</sup> ) <i>hprK</i> (Pm <sup>r</sup> )	This study
OAM643	<i>trpC2 thrC::degU-lacZ</i> U-WT (Sp <sup>r</sup> ) <i>tnrA</i> (Cm <sup>r</sup> )	This study
OAM371	<i>trpC2 amyE::sacB-lacZ</i> S-WT (Cm <sup>r</sup> )	20
OAM372	<i>trpC2 amyE::sacB-lacZ</i> S-WT (Cm <sup>r</sup> ) <i>degU</i> (Km <sup>r</sup> )	20
OAM367	<i>trpC2 amyE::bpr-lacZ</i> B-WT (Cm <sup>r</sup> )	23
OAM368	<i>trpC2 amyE::bpr-lacZ</i> B-WT (Cm <sup>r</sup> ) <i>degU</i> (Km <sup>r</sup> )	23
OAM644	<i>trpC2 amyE::PclpC-bgaB</i> (Cm <sup>r</sup> ::Tc <sup>r</sup> )	This study
OAM645	<i>trpC2 amyE::PclpC-bgaB</i> (Cm <sup>r</sup> ::Tc <sup>r</sup> ) <i>ccpA</i> (Cm <sup>r</sup> )	This study
OAM646	<i>trpC2 amyE::PclpC-bgaB</i> (Cm <sup>r</sup> ::Tc <sup>r</sup> ) <i>hprK</i> (Pm <sup>r</sup> )	This study
<b>Plasmids</b>		
pDG1729	Insertion vector for <i>thrC</i> , spectinomycin and erythromycin resistance genes, and <i>lacZ</i>	34
pDG1729-degU	pDG1729 carrying a promoter region of <i>degU</i> (positions -184 to +56)	26
pIS284	Insertion vector for <i>amyE</i> , chloramphenicol resistance gene, and <i>lacZ</i>	20
pIS284-degU-del11	pIS284 carrying a promoter region of <i>degU</i> (positions -151 to +56)	This study
pIS284-degU-M15	pIS284 carrying a promoter region of <i>degU</i> (positions -151 to +56), with CG-to-TT mutation (positions -135 and -136)	This study
pIS284-degU-P2	pIS284 carrying a promoter region of <i>degU</i> (positions -184 to -34)	This study
pPhl2	Phleomycin resistance	35
pPhl2-hprK	pPhl2 carrying part of <i>hprK</i>	This study
ECE75	Ampicillin resistance; Cm <sup>r</sup> ::Tc <sup>r</sup>	BGSC
ECE73	Ampicillin resistance; Cm <sup>r</sup> ::Km <sup>r</sup>	M. Steinmetz
His-CcpA	pQE30-based vector for production of CcpA-His	36
His-MecA	pQE8-based vector for production of MecA-His	This study

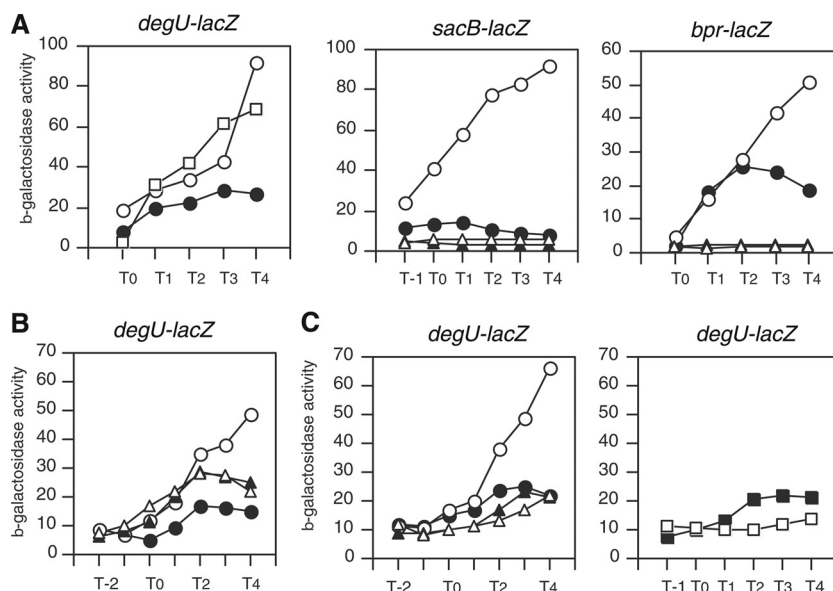
two DegU-P-driven genes. The utilization of the promoter of the operon containing *clpC* (*PclpC*) was subject to CcpA-dependent glucose repression. Glucose decreased the expression of *clpC*, leading to low cellular concentrations of the ClpCP protease. Thus, *degU* was induced through activation of autoregulation. On the other hand, CcpA bound directly to the *degU* upstream region in electrophoretic mobility shift assay (EMSA) and footprint analyses, indicating that CcpA activated *degU* by binding to it. These analyses revealed a *cre* sequence with a low match to the consensus sequence in *degU*. The *cre* sequence itself in *degU* showed a low affinity for CcpA, while in the more natural context (using a longer DNA fragment containing *cre* as a probe), the *cre* sequence in *degU* showed a stronger affinity for CcpA.

## MATERIALS AND METHODS

**Bacterial strains, culture media, and general methods.** All of the *Bacillus subtilis* strains used for this study are listed in Table 1. Schaeffer's nutrient

broth-based sporulation medium and Luria-Bertani medium (LB medium; Lennox, Difco, MI) were used for the determination of  $\beta$ -galactosidase activity (1). For *B. subtilis* transformation, modified competence medium was used (13). *Escherichia coli* JM103 cells for DNA manipulation were grown in LB medium. The concentrations of antibiotics used were described previously (35). Synthetic oligonucleotides were prepared commercially by Tsukuba Oligo Service (Ibaraki, Japan) and are listed in Table S1. KOD+-neo DNA polymerase I (Toyobo, Osaka, Japan) with high fidelity was used for PCR.

**Plasmid construction.** The plasmids used in this study are listed in Table 1. To construct pPhl2-hprK, a PCR product was prepared using the relevant primer pair (see Table S1 in the supplemental material), and the DNA fragment was digested with BamHI and BglII and ligated into the BamHI-digested pPhl-2 plasmid (35). To construct pIS284-degUdel11, -degUM15, and -degU-P2, PCR products were prepared using the relevant primer pairs (see Table S1). The DNA fragments were digested with BamHI and HindIII and then ligated into the similarly digested pIS284 plasmid (20). To construct His-MecA, a PCR product was prepared using



**FIG 1** Glucose induction of expression of DegU-dependent genes and abolishment of induction in *ccpA* and *clpC* mutants. Cells were grown in sporulation medium without (closed symbols) or with (open symbols) 2% (wt/vol) glucose. Cells were sampled hourly.  $\beta$ -Galactosidase activities are shown in Miller units. The x axis represents the growth time (h) relative to the end of vegetative growth (T0). (A) Glucose induction of DegU-dependent gene expression. Each gene fusion is indicated above the relevant panel. (Left) Squares, 1% glucose; open circles, 2% glucose. (Middle and right panels) Circles, wild-type cells; triangles, *degU* cells. (B) Expression of wild-type *degU-lacZ* fusion in the *clpC* strain (triangles). Data for wild-type cells are shown in circles. (C) Expression of wild-type *degU-lacZ* fusion in *ccpA* (left) and *hprK* (right) strains. Circles, wild type; triangles, *ccpA* strain; squares, *hprK* strain.

the relevant primer pair (see Table S1), and the DNA fragment was digested with BamHI and then ligated into the BamHI-digested pQE8 plasmid (Qiagen, Hilden, Germany). The sequences cloned into all plasmids were confirmed.

**$\beta$ -Galactosidase assays.** Samples were collected at hourly intervals, and the level of  $\beta$ -galactosidase activity was determined as described previously (35).

**Protein purification.** The procedures used to induce protein production and purify proteins from *E. coli* were described previously (31). His-tagged CcpA was expressed as a soluble protein in *E. coli* M15 cells carrying pRep4 and was purified by stepwise elution with imidazole from a Ni affinity column (36). After SDS-PAGE analysis of the fractions, the purified protein was dialyzed against TEDG buffer (37).

**Western blot assay.** Cells were harvested and lysed using a French press in Tris-EDTA (TE) buffer containing 1 mM phenylmethylsulfonyl fluoride. The whole-cell lysate was separated into supernatant and pellet by centrifugation. Soluble proteins were separated by 12.5% SDS-PAGE. An anti-MecA polyclonal antibody was raised against purified His-MecA in rabbits and prepared by JBioS Inc. (Saitama, Japan). The Western blot assay was conducted using a BM chemiluminescence Western blotting kit (Roche, Basel, Switzerland). We used polyclonal anti-DegU (38), anti-ClpP (39), and anti-ClpC (40) antibodies for analyses. Quantification of band intensities was described previously (26).

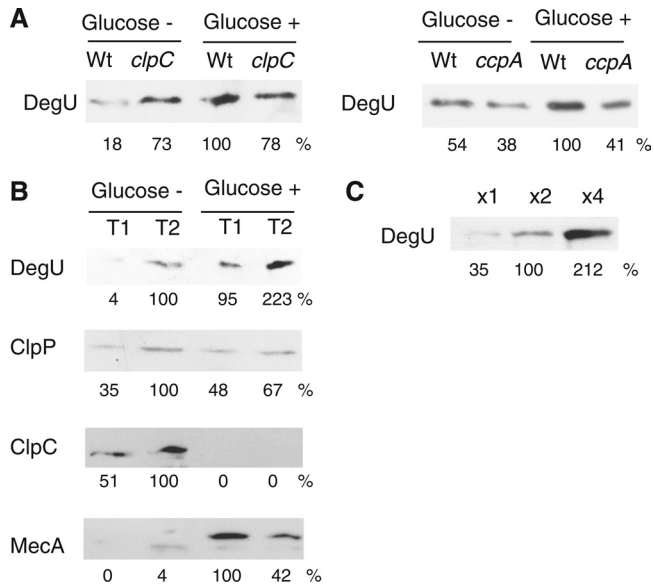
**EMSA and DNase I footprint assay.** Probes were prepared by PCR amplification using an appropriate primer and the biotinylated primer shown in Table S1 in the supplemental material. For EMSA, the indicated amounts of His-CcpA were incubated with 5 nM DNA probe in 12  $\mu$ l of 0.5 $\times$  TEDG buffer containing 20 mM KCl, 1 mM MgCl<sub>2</sub>, and an appropriate amount of poly(dI-dC) as a nonspecific competitor (GE Healthcare, United Kingdom) for 15 min at 37°C. After addition of 2  $\mu$ l of loading buffer (26), the samples were separated in polyacrylamide gels containing the indicated concentrations of acrylamide. Electrophoresis was performed in 0.25 $\times$  Tris-borate-EDTA (TBE) buffer at 4°C. For the footprint assay, the reaction mixture was similar to that used for the EMSA, except that 30 nM probe DNA was included in the mixture. After incubation for 15 min at 37°C, 0.05 unit of DNase I (TaKaRa, Kyoto,

Japan) was added, and the mixture was incubated for 3 min at 28°C. After addition of 25  $\mu$ l TE buffer, phenol-chloroform-isoamyl alcohol (25:25:1) extraction was performed, followed by ethanol precipitation with carrier tRNA. The samples were separated in a 6% polyacrylamide gel containing 7 M urea. Electrophoresis was performed in a TBE buffer. Biotin-labeled DNA was detected as described previously (35).

## RESULTS

**Glucose-induced *degU* expression.** During the course of studying the expression of *degU* driven by its own promoter, we observed that *degU-lacZ* was induced by addition of glucose to the sporulation medium (Fig. 1A, left panel). Thereafter, we always incorporated 2% (wt/vol) glucose into the medium. This effect was dependent on the composition of the medium, as the expression of *degU-lacZ* in LB medium was slightly reduced by glucose (see Fig. S1A in the supplemental material). Next, we examined the expression of DegU-driven genes in the presence of glucose to confirm whether the positive effect was observed ubiquitously among genes regulated by DegU. As reported previously, there are more than 120 DegU regulon genes, and many of them are under complex regulation by multiple transcription factors, such as that encoded by *aprE* (18, 41). Thus, we chose the following two genes for analyses: the *bpr* gene, which is regulated only by DegU (23), and the *sacB* gene, whose *cis* element (which is responsible for sucrose induction) was deleted, resulting in activation of the *sacB-lacZ* fusion solely by DegU (20). As shown in Fig. 1A, glucose induced expression of both fusions. In addition, the induction of both genes was abolished by disruption of *degU* (Fig. 1A). These observations confirmed that the *sacB* and *bpr* genes were induced in a DegU-dependent manner by glucose.

**Disruption of *clpC* and *ccpA* abolishes glucose induction of *degU*.** Previously, we reported that ClpCP degrades DegU-P. Thus, the expression of *degU* is enhanced in the *clpC* strain be-



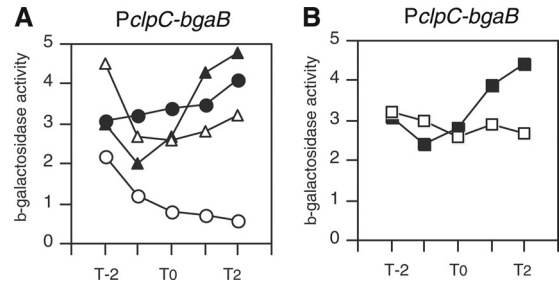
**FIG 2** Western blot analysis of DegU and ClpCP, with and without glucose. Cells were grown in sporulation medium. Times of sample collection and addition of 2% (wt/vol) glucose are indicated above the panels. In each panel, relative band intensities are indicated under the panel. In the sets of experiments, equal amounts of proteins were applied to the lanes. (A) DegU in *clpC* and *ccpA* strains. (B) Effects of glucose on DegU, ClpP, ClpC, and MecA. (C) Relationship between protein amounts and band intensities.

cause DegU-P is not degraded in that strain (26). Based on these regulatory mechanisms, it is possible that addition of glucose somehow affects ClpCP-dependent regulation of *degU*. To test this possibility, we examined the expression of *degU* in the *clpC* strain in medium containing glucose. Addition of glucose did not further enhance the increased expression of *degU* in the *clpC* strain (Fig. 1B). The level of *degU* expression in the *clpC* strain did not reach that in the wild-type strain in the presence of glucose. This suggested that another mechanism(s) besides the ClpCP system affected expression.

The transcription factor CcpA is one of the primary regulators of glucose induction, and HprK produces active CcpA through production of P-Ser-Hpr (2). Thus, we examined the expression of *degU* in the presence of glucose in the *ccpA* strain. As expected, the induction of *degU* by glucose was abolished by disruption of *ccpA* (Fig. 1C, left panel), indicating that CcpA was involved in this phenomenon. Without glucose, disruption of *ccpA* resulted in a slight decrease in *degU* expression. We obtained a similar result with the *hprK* strain (Fig. 1C, right panel), confirming the involvement of HprK-activated CcpA in the glucose induction of *degU*.

To further confirm the glucose effect on *degU* expression, we used the synthetic competence medium MC with the addition of either glucose or glycerol. Compared to the expression of *degU-lacZ* in glucose-based competence medium, the levels of *degU* expression were lower in glycerol-based competence medium (see Fig. S1A in the supplemental material). This is because in the glycerol-based medium, CcpA is inactivated to derepress glycerol incorporation (42). This result again confirmed the CcpA-dependent control of *degU* expression.

Since glucose had no effect on *degU* expression in the *clpC* mutant, the simplest explanation is that the effects of the CcpA and ClpC proteins are interdependent. To test this idea, we con-



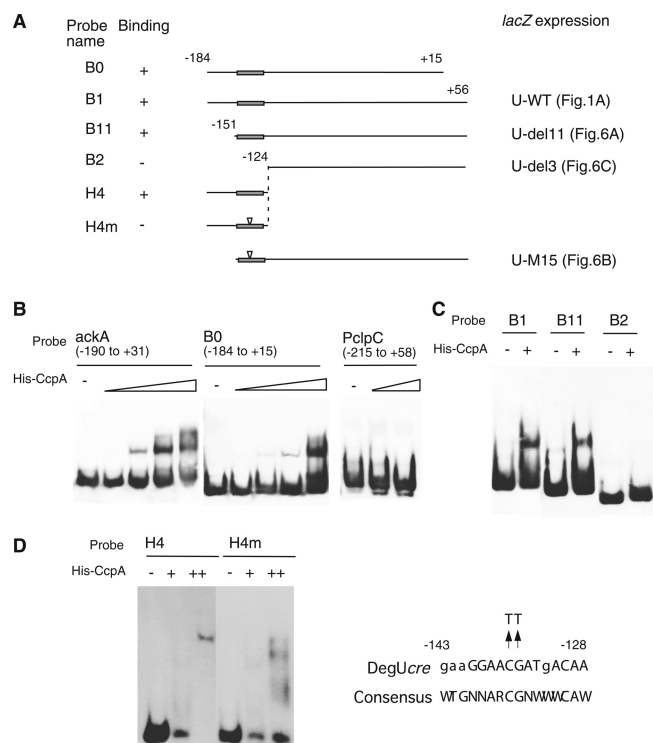
**FIG 3** CcpA-dependent glucose repression of PclpC. Cells were grown in sporulation medium without (closed symbols) or with (open symbols) 2% (wt/vol) glucose. Cells were sampled hourly.  $\beta$ -Galactosidase activities are shown in Miller units. The x axis represents the growth time (h) relative to the end of vegetative growth (T0). (A) Circles, wild type; triangles, *ccpA* strain. (B) Squares, *hprK* strain.

structed a *ccpA* and *clpC* double mutant and examined its  $\beta$ -galactosidase activity. As shown in Fig. S1B in the supplemental material, the *clpC* mutation did not enhance the expression of wild-type *degU-lacZ* compared to that observed in the *ccpA* strain. This suggested that CcpA and ClpC are somehow interdependent through an unknown mechanism.

**Western analysis of DegU.** Next, we confirmed the glucose induction of *degU* at the protein level. The relationship between band intensities and protein amounts (Fig. 2C) showed a rough linearity, which justified quantification of the observed bands. As shown in Fig. 2A and B, addition of glucose increased the amount of the DegU protein to various extents, consistent with the results of *degU-lacZ* expression. In both the *clpC* and *ccpA* strains, addition of glucose did not increase the amount of DegU (Fig. 2A). Using the same soluble extracts of whole-cell lysates, we investigated the protein levels of the ClpCP degradation machinery associated with MecA. Surprisingly, the ClpC protein was not detected in extracts from the culture medium containing glucose, while similar levels of the ClpP protein were detected in extracts both with and without glucose (Fig. 2B). Moreover, the amount of MecA, a substrate for ClpCP (43), was clearly enhanced. In addition, there was only a slight increase in *mecA* transcription (see Fig. S2 in the supplemental material). Thus, the increased amount of MecA was consistent with the disappearance of ClpC. Consequently, when glucose was added to the sporulation medium, ClpCP was not functional because of the lack of ClpC, leading to enhanced *degU* expression. Like MecA, ClpE is a substrate for ClpCP (44). Thus, there was abundant ClpE in the ClpC-depleted cells. We note that the anti-ClpC antibody cross-reacted with ClpE under strong detection conditions, including when high concentrations of secondary antibody were used (data not shown).

**CcpA-dependent catabolite repression of *clpC*.** According to the Western blot analysis of ClpC, we speculated that CcpA may downregulate the expression of *clpC* driven by the promoter of the four-gene *ctsR-mcsA-mcsB-clpC* operon (45, 46). We analyzed a PclpC-*bgaB* fusion to explore this possibility. As shown in Fig. 3A, PclpC-*bgaB* was repressed by addition of glucose, and disruption of *ccpA* at least partially derepressed the expression of the fusion in the presence of glucose. The disruption of *hprK* also derepressed PclpC-*bgaB* from glucose repression (Fig. 3B). These results clearly demonstrated that the *clpC* operon is repressed by glucose in a CcpA-dependent manner. It was reported that CtsR represses





**FIG 4** Binding of His-CcpA to *degU*. Various probes were prepared by PCR amplification using the primers shown in Table S1 in the supplemental material. (A) Structures of probes. Solid rectangles on lines show *cre* sites. Numbers indicate nucleotide positions relative to the transcription start site. Relationships between probes and fusions with corresponding regions fused to *lacZ* are indicated. (B to D) Each reaction mixture contained poly(dI-dC) (1  $\mu$ g [B], 0.7  $\mu$ g [C], or 0  $\mu$ g [D]) as a nonspecific competitor and probe. Samples were analyzed by nondenaturing polyacrylamide gel electrophoresis on 4.5% gels (B and C) or 11% gels (D). In each panel, all lanes shared the same gel electrophoresis conditions. For panel B, probes were incubated with increasing amounts of His-tagged CcpA (0, 12.5, 25, 50, and 100 nM for *ackA* and B0 and 0, 50, and 100 nM for PclpC). For panels C and D, probes were incubated with His-tagged CcpA (+, 75 nM; ++, 150 nM).

expression of its own operon (45). Therefore, we examined the effects of glucose and disruption of *ccpA* in a *ctsR* disruptant. We observed essentially the same results. In the *ctsR* background, the expression of *clpC-bgaB* was enhanced to about 25 Miller units without glucose and decreased to about 5 Miller units with glucose (data not shown). The repression was not observed in the *ccpA* strain (data not shown). These results again demonstrated the CcpA-dependent repression of the operon and showed that this regulation was independent of CtsR.

These observations raised the possibility that CcpA might bind to the regulatory region of PclpC. Thus, we used the same region of PclpC cloned into the PclpC-*bgaB* fusion (positions -215 to +58 relative to the transcription start site) as a probe for EMSA. Unfortunately, we did not observe any binding under the conditions used (Fig. 4B), suggesting an indirect regulation of PclpC by CcpA.

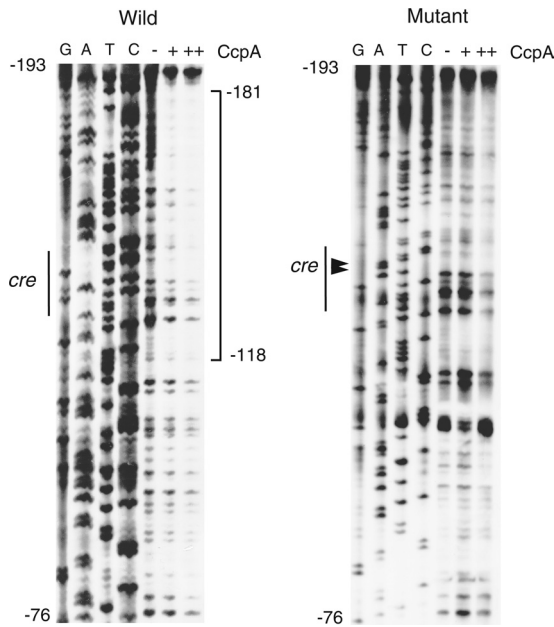
**Direct binding of CcpA to the *degU* promoter region.** The results of the epistatic analyses using the *degU-lacZ* fusion (Fig. 1C) suggested that CcpA might be involved in glucose induction of *degU* through its binding to *degU*. To investigate this possibility, we examined the direct binding of CcpA to the *degU* pro-

moter. In most cases, CcpA does not bind specifically to its target if it is not associated with P-Ser-Hpr (2, 47). In several experiments, however, His-tagged CcpA bound specifically to its target without P-Ser-Hpr, probably because of the positive charge of histidines and/or structural changes in the protein (36, 48–50). Thus, we used His-CcpA for the analysis. Using the *ackA* promoter and PclpC as positive and negative controls, respectively, we conducted an EMSA using His-CcpA and the *degU* promoter region (positions -184 to +15 relative to the transcription start site of the P3 promoter). As shown in Fig. 4B, CcpA bound to the *degU* probe in the presence of the nonspecific competitor poly(dI-dC). To narrow down the binding region, we used several 5'- and 3'-deleted probes (Fig. 4A). CcpA bound to the B1 and B11 probes but not to the B2 probe, indicating that the region from positions -151 to -124 was required for binding (Fig. 4C). This was further confirmed by the binding of CcpA to the H4 probe (positions -184 to -124). The region spanning positions -151 to -124 contained a *cre*-like sequence, although this sequence showed a low match to the consensus *cre* sequence (Fig. 4A). We introduced mutations into the core CG nucleotides in this *cre* sequence in the H4 probe, creating the H4m probe. In EMSAs, the H4m probe did not show a discrete band but instead showed a smeared pattern, suggesting dissociation of CcpA from the DNA-protein complex during electrophoresis. This observation demonstrated the low affinity of the H4m probe for CcpA compared with that of the H4 probe (Fig. 4D). This further confirmed the specific binding of CcpA to the *cre* sequence in *degU*. Next, we performed an EMSA using this sequence itself as a probe. CcpA bound efficiently to the *cre* sequence of *ackA*, while it bound rather poorly to the *degU*-derived probe (see Fig. S3 in the supplemental material). However, the binding to the *cre* sequence in *degU* was considered specific, because the probe spanning positions -172 to -152 in *degU* did not bind (see Fig. S3). In summary, CcpA bound efficiently to the *cre* sequence in *degU* when nonspecific DNA was present beside the *cre* sequence, and binding occurred irrespective of the sequence on the 5' or 3' side. This is based on the appearance of discrete bands in EMSAs with the H4 and B11 probes.

#### Footprint analysis of CcpA binding to *cre* sequence in *degU*.

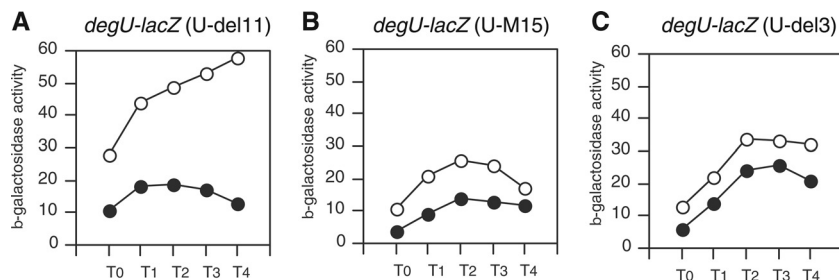
To further analyze CcpA binding to *degU*, we performed a footprint experiment. As shown in Fig. 5, CcpA clearly protected the region corresponding to the H4 probe from DNase I attack. This result was consistent with the results of EMSA using the H4 probe. In previous binding studies (48–51), CcpA protected the relatively narrow regions containing the *cre* sequence, except for the *cre* sequence in the *lev* gene, for which a long footprint pattern was observed (about 65 bases). Thus, our results showed some differences from those of previous studies. We do not know the stoichiometry that underlies these results, that is, whether one dimer binds nonspecifically upstream of the *cre* sequence and the other binds specifically to the *cre* sequence or one dimer binds to the *cre* sequence while concomitantly interacting with the upstream region, protecting it from DNase I attack. We introduced the same mutations for H4m (replacement of the core CG nucleotides) into the probe and conducted another footprint experiment. We observed much more reduced binding, again demonstrating the importance of the *cre* sequence in *degU* in CcpA binding (Fig. 5).

**Mutational analysis of *cre* sequence in *degU*, using *degU-lacZ*.** To confirm the *in vitro* results *in vivo*, we used *degU-lacZ* fusions with deletions or mutations. As expected, the expression of B11, whose 5' end was position -151, was induced in the pres-

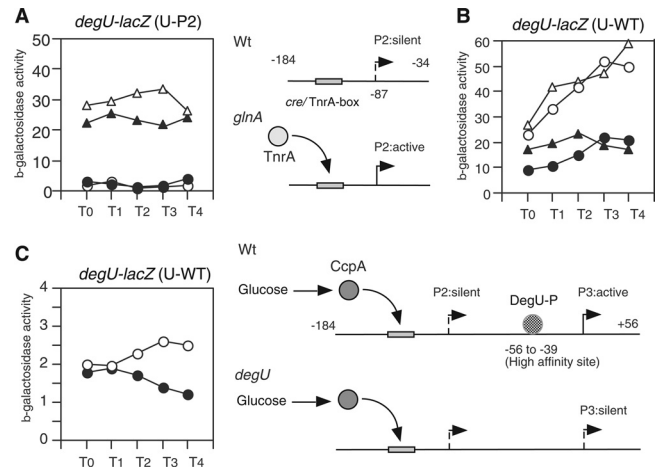


**FIG 5** Footprint analysis of His-CcpA binding to *degU*. To analyze the wild-type template strand, oligonucleotide pairs DegU-Pro-Biotin3/DegU-pIS-B1 and DegU-pIS-H3/DegU-pIS-B1 were used for PCR amplification to generate the probe and sequencing ladder template, respectively. To analyze the mutant template strand, the oligonucleotide pair DegU-Pro-Biotin3/DegU-pIS-B1 in addition to mCre-degU-F/mCre-degU-R was used to create a mutant probe for PCR-mediated mutagenesis (35). DegU-pIS-H3 and DegU-pIS-B1 were used for PCR amplification of the resultant mutant probe to generate the sequencing ladder template. The probe spanning positions  $-184$  to  $+15$  relative to the transcription start site, which carries the additional nine bases derived from the PCR primer, was incubated with His-CcpA (+, 75 nM; ++, 150 nM) in buffer containing poly(dI-dC) ( $0.5 \mu\text{g}$ ) as a nonspecific competitor (the buffer had the same composition as the buffer used for EMSA) and subjected to DNase I cleavage. A sequencing ladder is shown, with G, A, T, and C lanes. The square bracket shows the protected region. The two mutated nucleotides are indicated by arrowheads.

ence of glucose (Fig. 6A). This was expected because the fusion contained the *cre* sequence. However, the expression of the other two fusions, B11m and B2, was not fully induced in the presence of glucose (Fig. 6B and C), because B11m carried a mutated *cre* sequence and B2 had no *cre* sequence. These results were consistent with those of the *in vitro* binding study and demonstrated the *in vivo* functionality of the *cre* sequence in glucose induction of *degU*. The partial induction of the B11m and B2 fusions in the presence of glucose can be attributed to the lack of ClpCP degradation



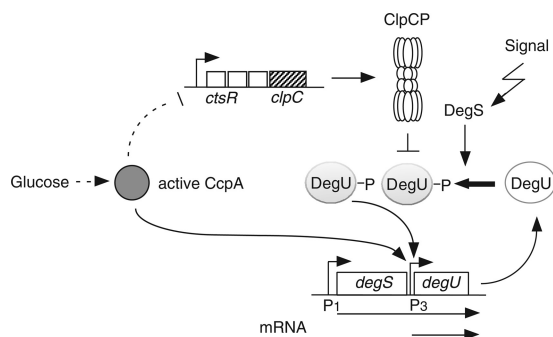
**FIG 6** Effects of deletion/mutation of *cre* on expression of *degU-lacZ*. Cells were grown in sporulation medium without (closed symbols) or with (open symbols) 2% (wt/vol) glucose. Cells were sampled hourly.  $\beta$ -Galactosidase activities are shown in Miller units. The x axis represents the growth time (h) relative to the end of vegetative growth (T0).



**FIG 7** Effects of glucose addition on P2 promoter. Cells were grown in sporulation medium without (closed symbols) or with (open symbols) 2% (wt/vol) glucose. Each fusion is indicated above the relevant panel. (A) Circles, wild-type cells; triangles, *glnA* cells. Schematic representations of the structure of U-P2 and the regulation of P2 are shown alongside the panel. Numbers indicate nucleotide positions relative to the transcription start site for P3. (B) Circles, wild-type cells; triangles, *tnrA* cells. (C) Circles, *degU* cells. Schematic representations of the regulation of the U-WT fusion bearing the P2 and P3 promoters are shown alongside the panel. Numbers indicate nucleotide positions relative to the transcription start site for P3.

machinery resulting from glucose addition (Fig. 2B). When the *ccpA* gene was disrupted, ClpCP no longer affected the expression of *degU-lacZ* (see Fig. S1B in the supplemental material). Thus, these two observations seem to contradict each other. Perhaps the *ccpA* disruption and deletion/mutation of *cre* in *degU* result in different regulatory effects.

**The P2 promoter is silent and is not induced by glucose.** As reported previously (27), the *degU* gene is preceded by the transcription factor TnrA-dependent promoter P2, which is activated by the disruption of *glnA* (27). To examine the involvement of the P2 promoter in glucose induction of *degU*, we constructed a fusion bearing the P2 promoter alone (U-P2) (Fig. 7A). U-P2 showed no  $\beta$ -galactosidase activity in the presence of 2% glucose, indicating that glucose did not activate the P2 promoter (Fig. 7A). As a control, we introduced the *glnA* disruption into the strain bearing this fusion, and as expected, the resultant strain showed  $\beta$ -galactosidase activity. This expression was dependent on TnrA, as the further introduction of a *tnrA* disruption into the strain abolished the P2 expression (data not shown). This is consistent



**FIG 8** Schematic representation of glucose induction mechanism of *degU*. Arrows and the T bar indicate activation and inhibition, respectively. Bent arrows indicate promoters. The dotted line indicates an indirect effect.

with former observations (27). The addition of glucose slightly enhanced the expression of TnrA-dependent expression of P2 (Fig. 7A). This enhancement is thought to also be CcpA dependent, since this slight enhancement of the expression of P2 was not observed in the *glnA* and *ccpA* double mutant (data not shown). It should be noted that the putative TnrA box (TGGAAGGAACGA TGACA; positions  $-145$  to  $-129$  relative to the transcription start site of P3) and the *cre* site overlap at the same region. It is not known at present, however, whether CcpA and TnrA might bind to the same sequence concomitantly. Moreover, we examined the expression of the *degU* fusion bearing the P2 and P3 promoters in a *tnrA* disruptant. As shown in Fig. 7B, the disruption of *tnrA* did not affect the glucose induction ratio or the expression levels of the fusion. This result further demonstrated that P2 is not related to the glucose induction of *degU*. The P3 promoter is activated by DegU-P, and thus the fusion bearing only P3 and the DegU-binding site (U-del5) showed no  $\beta$ -galactosidase activity in the *degU* disruptant (26). This indicated that P3 was solely dependent on DegU-P. In addition, we note that P2 is not influenced by *degU* disruption (27). To confirm that P2 is silent under wild-type conditions, we examined the  $\beta$ -galactosidase activity of the U-WT fusion in the *degU* background. Only marginal activities were observed without glucose, and a minor enhancement of activities was seen in the presence of glucose (Fig. 7C). This finally demonstrated that P2 is silent and that P3 but not P2 contributes to glucose induction. The slight enhancement of expression of the fusion is due to CcpA's action on the residual P3 promoter activity.

## DISCUSSION

In this report, we present the direct and indirect regulatory mechanisms of glucose induction of *degU* (Fig. 8). Evidence is presented that CcpA activates *degU* transcription by binding to its upstream region. Moreover, catabolite repression of *clpC* reduces the amount of ClpC, leading to activation of the autoregulatory loop of *degU*. The effects of *ccpA* mutation and/or addition of glucose to various media on bacterial growth have been analyzed in several genomewide global analyses (5–8, 10, 11). None of the previous studies reported induction of the expression of DegU-driven genes, including the *degU* gene itself, by glucose and CcpA binding to *degU*. There are several possible reasons for this. The previous studies did not use the same conditions as those in our study, that is, growth in sporulation medium supplemented with glucose and serial sampling from log phase to early stationary phase. Thus,

induction of the expression of DegU-driven genes is dependent on the medium conditions and sampling points. For example, in LB medium, the expression of *degU-lacZ* was slightly repressed by the addition of glucose, while in competence medium the induction by glucose was clearly observed in early stationary phase (see Fig. S1A in the supplemental material). In the ChIP analysis, CcpA did not bind to the control region of *degU* or to those of the genes for which CcpA-dependent direct regulation has been verified (2, 6). This might be due to the fact that the ChIP analysis provided a snapshot of the CcpA-binding pattern of cells in the exponential growth phase in M9 medium containing glucose and malate (6).

Many biosynthetic enzymes are targets for Clp-dependent degradation in the glucose-starved stationary phase (9). Thus, it is possible that ClpCP protease activity is enhanced after glucose starvation. Although this hypothesis remains to be verified, our finding that the transcription of the *clpC* gene is repressed by glucose is consistent with the pattern of regulation under glucose starvation conditions. Moreover, a global transcriptome analysis reported that transcription of the *clpC* gene was enhanced in a CcpA-dependent manner in the absence of glucose (39), which is consistent with our findings. In the current study, our results showed that repression of *clpC* is indirectly regulated by CcpA, because CcpA did not bind to the PclpC probe. Since partial derepression of *clpC* in the *ccpA* strain was observed, we investigated the effects of other *trans*-acting factors, such as CggR, CcpB, CcpC, and CcpN, which might be responsible for glucose-dependent regulation of PclpC expression (2, 6). Disruption of these genes did not affect reduced expression from PclpC in the presence of glucose (M. Ogura and H. Ishii, unpublished results). These results indicated that there might be an as yet unknown direct *trans*-acting factor for regulation of *clpC*. Otherwise, PclpC might be regulated indirectly, for example, by a change in metabolic state.

There are a few genes, such as *ackA*, *pta*, and the *ilv-leu* operon, that are directly activated by CcpA (36, 47, 52, 53). Their gene products are involved in consumption of acetyl-coenzyme A (acetyl-CoA) produced by accumulated pyruvate (2). Genes encoding polyketide synthase for bacillaene synthesis have been detected among the DegU-activated genes (14, 18, 54). This might be one reason for glucose activation of *degU*, because polyketide synthesis consumes acetyl-CoA (54). The *cre* sequences in these genes are located upstream of the core promoter, which enables these *cre* sequences to function as positive *cis* sequences. Thus, it is reasonable that the *cre* sequence in *degU* is located at positions  $-143$  to  $-128$  relative to the transcription start site, assuming DNA looping mediated by some DNA-binding proteins, e.g., DegU-P and/or CcpA, which would enable an interaction of CcpA with RNA polymerase over a long distance. In our previous study using various 5'-deleted *degU-lacZ* fusions, we observed a 3-fold decrease in *degU-lacZ* expression after deletion of the region from positions  $-184$  to  $-124$  when cells were grown in a glucose-based competence medium (26). Thus, we speculated that an as yet unknown transcription factor is involved in *degU* regulation. This unknown factor could be CcpA.

Experimental analyses of the relationship between *cre* sequences and CcpA-dependent regulation of corresponding genes have shown the intrinsic degenerative nature of the *cre* sequence (2) (Table 2). The *cre* sequences in Table 2 are examples of those with this degenerative characteristic, and all have had their CcpA-binding properties validated experimentally. Although the *cre* se-



TABLE 2 Comparison of *cre* sequences with low-level matching

Gene name or consensus	<i>cre</i> sequence <sup>a</sup>	No. of matching nt	<i>In vivo</i> binding <sup>b</sup>	Reference
Consensus	<u>WTGNNARC</u> <u>GNW</u> <u>WCAW</u>			4
<i>cydA</i>	<u>T</u> TGAAAtgaATccttg	8	+	50
<i>resA</i>	gTaAAAACGCTTTCTA	13	–	55
<i>citZ</i>	ATGTAAGCaTTTTCtT	14	+	48
<i>degU</i>	gaaGGAACGATgACAA	12	–	This study

<sup>a</sup> Underlining shows the most conservative nucleotides. Lowercase letters show mismatched nucleotides.

<sup>b</sup> Data from ChIP-chip experiments in reference 6.

quence in *degU* showed a low-level match to the consensus *cre* sequence, our results show that it can function as a CcpA-binding site and a positive *cis* sequence if some “scaffold” sequence is available. The “scaffold” might strengthen the searching efficiency of the binding site by CcpA through nonspecific binding of CcpA. A recent idea on how a transcription factor achieves specific binding includes its sliding on DNA adjacent to a specific binding site through nonspecific binding (56). This observation might increase the number of possible CcpA-binding sites in the *B. subtilis* genome, which has been estimated to be approximately 150 sites (2). Thus, these findings are important for further research on the function and binding modes of CcpA. Moreno et al. pointed out that in their transcriptome analyses, recognizable *cre* sites were virtually absent from the operons that are activated by glucose in a CcpA-dependent manner, although a large majority of the genes subject to catabolite repression were found in operons bearing one or more *cre* sites (10). Our results may partly explain this observation.

We used His-CcpA instead of intact CcpA with P-Ser-Hpr. The two proteins have similar DNA affinities, with  $K_d$  (dissociation constant) values of approximately 5 to 10 nM for the *cre* sequence in the *ackA* gene (4, 36). Both types of CcpA protein at similar concentrations were also reported to achieve protection from DNase I digestion in the *ilvB* promoter (52, 53). In addition, there are several reports of specific DNA binding of His-CcpA to target sequences (36, 48–50), indicating the full functionality of *in vitro* DNA binding of His-CcpA. In fact, the His tag in His-CcpA increases DNA binding about 6-fold over that of CcpA in the absence of P-Ser-Hpr, while there is no stimulatory effect of P-Ser-Hpr addition (57). Thus, our result that His-CcpA bound to the *cre* sequence in *degU* is reasonable, because His-CcpA seems to have authentic sequence specificity.

CcpA indirectly regulates many genes through its effect on regulators. CcpA directly regulates three response regulator genes, *citT*, *phoP*, and *resD*, in two-component regulatory systems (49, 55, 58). These three regulators are subject to catabolite repression, while the *degU* gene is activated by glucose. The rational explanation for the difference is an issue to be elucidated in the future.

The CcpA-binding site is located upstream of the low-affinity DegU-P-binding site (positions –104 to –87), which raises the possibility that CcpA may interact with DegU-P. Recent reports discussed possible interactions between CcpA and other transcription factors, for example, CodY on the *ilv-leu* operon and *ackA* promoters (52, 53, 59). Further research is required to clarify the possible interaction between CcpA and DegU-P.

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