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

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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 tricarboxylic acid cycle. Furthermore, the AAA protease ClpCP specifically degrades the phosphorylated form of DegU (DegU-P), leading to derepression of the DegU operon containing clpC (PclpC) decreased in the presence of glucose, and the disruption of ccpA resulted in derepression of PclpC. However, this was not directly mediated by CcpA, because we failed to detect binding of CcpA to PclpC. Glucose decreased the expression of clpC, leading to low cellular concentrations of the ClpC protease. Thus, degU is induced through activation of autoregulation by a decrease in ClpC-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.

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+ protease ClpCP specifically degrades the phosphorylated form of DegU (DegU-P), leading to modulation of the DegU autoreactivation 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...
showed a stronger affinity for CcpA. On the other hand, CcpA bound directly to the degU sequence in analyses revealed a affinity for CcpA, while in the more natural context (using a lon-generating DNA fragment containing affinity for CcpA, while in the more natural context (using a lon-generating DNA fragment containing

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Plasmids

- **pDG1729**: Insertion vector for thrC, spectinomycin and erythromycin resistance genes, and lacZ
- **pDG1729-degU**: pDG1729 carrying a promoter region of degU (positions -184 to +56)
- **pIS284**: Insertion vector for amyE, chloramphenicol resistance gene, and lacZ
- **pIS284-degU-del11**: pIS284 carrying a promoter region of degU (positions -151 to +56)
- **pIS284-degU-M15**: pIS284 carrying a promoter region of degU (positions -151 to +56), with CG-to-CT mutation (positions -135 and -136)
- **pIS284-degU-P2**: pIS284 carrying a promoter region of degU (positions -184 to -34)
- **pPh12**: Phleomycin resistance
- **pPh12-hprK**: pPh12 carrying part of hprK
- **EC73**: Ampicillin resistance; Cm::Tc
- **EC73**: Ampicillin resistance; Cm::Km
- **His-CcpA**: pQE30-based vector for production of CcpA::His
- **His-MecA**: pQE8-based vector for production of MecA::His

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 ClpC 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 β-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 1 (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 pPh12-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 BgII and ligated into the BamHI-digested pPh12 plasmid (35). To construct pIS284-degU-del11, -degU-M15, 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

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CcpA-Dependent Regulation of degU

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.

β-Galactosidase assays. Samples were collected at hourly intervals, and the level of β-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. Western blot assay was conducted using a BM chemiluminescence Western blotting kit (Roche, Basel, Switzerland). We used polyclonal anti-DegU (38), anti-MecA polyclonal antibody was raised against purified His-MecA in rabbits and prepared by JBioS Inc. (Saitama, Japan). The Western blot analysis of the fractions, the purified protein was dialyzed against TEDG buffer (37).

EMSAs 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 μl of 0.5× TEDG buffer containing 20 mM KCl, 1 mM MgCl2, 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 μl of loading buffer (26), the samples were separated in polyacrylamide gels containing the indicated concentrations of acrylamide. Electrophoresis was performed in 0.25× 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 μl TE buffer, phenol-chloroform-isooamy 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. S1 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 cpa 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 1 Glucose induction of expression of DegU-dependent genes and abolishment of induction in cpa and clpC mutants. Cells were grown in sporulation medium without (closed symbols) or with (open symbols) 2% (wt/vol) glucose. Cells were sampled hourly. β-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 cpa (left) and hprK (right) strains. Circles, wild type; triangles, cpa strain; squares, hprK strain.
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 production of P-Ser-Hpr (2). Thus, we examined the expression of degU-lacZ in the absence of glucose (Fig. S1A in the supplemental material). This is because in the presence of glucose, the levels of degU expression 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 ClpP 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).

**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).

**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
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 promoter. 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 F3 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. 4C). 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 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-
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 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 β-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 β-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 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 (1,750 nM; −++, 150 nM) in buffer containing poly(dI-dC) (0.5 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.

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. 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.

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.
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 (TGGAAGGACGAGTGACA; 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 β-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 β-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 (acytly-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-
sequence 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 creates specific binding includes its sliding on DNA adjacent to a specific binding site through nonspecific binding (36). 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, cfrT, phoP, and resD, in two-component regulatory systems (49, 55, 58). These 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, CrvY 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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REFERENCES


DegU via its interaction with N-terminal domain of DegU. J. Biochem. 151:643–655.


