Possible function and some properties of the CcpA protein of *Bacillus subtilis*

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The *ccpA* mutations *alsA1* (*alsA1* is allelic to *ccpA*) and *ccpA::Tn917* completely abolished catabolite repression of gluconate kinase and sorbitol dehydrogenase synthesis in *Bacillus subtilis*, whereas they only partially affected the catabolite repression of inositol dehydrogenase, histidase and xylose isomerase synthesis. The *alsA1* mutation also partially affected catabolite repression of sporulation. Analysis of revertants from the *alsA1* mutant by direct sequencing indicated that this mutation comprises a base substitution of guanine at nucleotide –14 to adenine within the Shine–Dalgarno sequence of the *ccpA* gene (*ccpA* translation starts at nucleotide +1). A 1.37 kb *EcoRI* fragment carrying the *ccpA* gene was cloned into *Escherichia coli* plasmid pUC19 and *B. subtilis* plasmid pUB110, resulting in plasmids pCCPA19 and pCCPA110, respectively. The *ccpA* gene carried in pCCPA110 complemented the *alsA1* mutation. Western blotting revealed that the level of the CcpA protein in *B. subtilis* cells, which seemed to be constitutively synthesized, was approximately 10 times lower for the *alsA1* mutant than for the wild-type. The CcpA protein synthesized by either *E. coli* cells bearing pCCPA19 or *B. subtilis* cells bearing pCCPA110 was purified to over 90% homogeneity; the latter cells were grown in the presence of glucose. The molecular mass of the protein purified from *E. coli* was 74 kDa, suggesting that this protein exists as a dimer because its subunit molecular mass was 38 kDa as determined by SDS-PAGE. Gel retardation analysis indicated that the purified CcpA protein in both cases did not bind to the *cis* sequence for catabolite repression of the *gnt* operon, but it bound non-specifically to DNA.

Keywords: catabolite repression, gluconate metabolism, *Bacillus subtilis*, CcpA protein

INTRODUCTION

Several research groups have recently presented interesting findings which imply that *Bacillus subtilis* might possess a common but unknown negative regulatory mechanism underlying catabolite repression. The five *cis* sequences responsible for the catabolite repression of the *amyE* (Nicholson *et al.*, 1987), *gnt* (Miwa & Fujita, 1990, 1993), *xyl* (Jacob *et al.*, 1991), *hut* (Oda *et al.*, 1992) and *bglS* (Krüger *et al.*, 1993) operons have been determined; these sequences exhibit similarity with the *B. subtilis* catabolite repression consensus sequence, which was originally reported by Weickert & Chambliss (1990). Moreover, a *trans*-acting negative gene product (CcpA) which is involved in catabolite repression of the *amyE* gene was identified (Henkin *et al.*, 1991). The *crsA47*

mutation, which is allelic to the *sigA* gene encoding the σ^A factor of the *B. subtilis* RNA polymerase, affected catabolite repression of the *gnt* operon, which is mediated by the CcpA protein (Fujita & Miwa, 1994). The molecular mechanism underlying this negative common catabolite repression is obviously not one involving a cyclic AMP (cAMP) receptor protein–cAMP complex, such as that which has been well demonstrated in enteric bacteria (Botsford & Harman, 1992). Although the CcpA protein is likely to play a central role in this mechanism (Chambliss, 1993), this new regulation mechanism remains to be elucidated.

In this study, we analysed some properties of an *alsA1* mutant (*alsA1* is allelic to *ccpA*) and the purified CcpA protein from *Escherichia coli* and *B. subtilis* bearing the respective *ccpA*-encoding plasmids.

Abbreviations: CAT, chloramphenicol acetyltransferase.

METHODS

Bacterial strains and plasmids. *Escherichia coli* strains XL1-blue {*supE44 hsdR17 recA1 endA1 gyrA46 thi relA1 lac F'* [*proAB⁺ lacI^q lacZΔM15 Tn10(Tet^r)*]} and JM109 {*recA supE44 endA1 hsdR17 gryA96 relA1 thi Δ(lac-proAB) F'* [*traD36 proAB⁺ lacI^q lacZΔM15*]} were used. *Bacillus subtilis* strains 1A1 (*trpC2*), 1A250 (*trpC2 alsR1 ilvBΔ1*) and 1A147 (*alsA1 trpC2 alsR1 ilvBΔ1*) were obtained from the *Bacillus* Genetic Stock Center (Ohio State University). *B. subtilis* strain WLN-29 (*aroG932 trpC2 ccpA::Tn917*) was obtained from G. H. Chambliss (University of Wisconsin).

E. coli plasmid pUC19 and *B. subtilis* plasmid pUB110 were used as vectors for cloning of the *ccpA* gene. *E. coli* plasmid pGNT177 was constructed as described previously (Yoshida *et al.*, 1993). The 2 kb *EcoRI* fragment derived from plasmid pGNT41 (Miwa & Fujita, 1987) was cloned into the *HindIII* arm of plasmid pGNT177 after both fragments had been blunt-ended to yield plasmid pGNT61 (Fig. 1b).

Cloning of the *ccpA* gene. According to the nucleotide sequence of the *ccpA* gene reported by Henkin *et al.* (1991), we prepared a DNA probe [nucleotides +16 to +817; translation initiation nucleotide, +1 (Fig. 1a)] for cloning of the *ccpA* gene after amplification by means of PCR. *EcoRI* fragments of DNA of *B. subtilis* strain 1A1 were randomly cloned into pUC19 using *E. coli* strain XL1-blue, and then clones positive on colony hybridization with the above DNA probe were screened. A positive plasmid clone in which the *ccpA* gene was cloned in the same orientation as that of *lacZ* carried in pUC19 was designated as pCCPA19 (Fig. 1b). A 1.37 kb fragment isolated from an *EcoRI* digest of plasmid pCCPA19 was ligated with an *EcoRI* digest of pUB110, and then used for transformation of *B. subtilis* strain 1A147, with respect to kanamycin resistance, to yield plasmid pCCPA110. The sequence of the 1.37 kb fragment was confirmed by the dideoxy chain-termination method of Sanger *et al.* (1977).

Isolation of revertants of the *alsA1* mutation. Approximately 10^5 cells of strain 1A147 (*alsA1 trpC2 alsR1 ilvBΔ1*), obtained from one of single colonies on plates containing tryptose blood agar base (33 g l⁻¹, Difco) and 10 mM glucose, were inoculated into S6 medium (Fujita & Freese, 1981) containing 25 mM glucose and 50 µg ml⁻¹ each of tryptophan, isoleucine and valine, and allowed to grow overnight at 37 °C. The resulting culture was diluted 1000-fold with the same medium, and allowed to grow for 24 h. This dilution and cultivation were repeated at least six times until cells grew at the same rate as an isogenic wild-type strain, 1A250. Single colonies were isolated from each of 10 independent continuous cultivations. Three revertants out of 10 were tested and confirmed as to retention of the amino acids requirement and restoration of normal catabolite repression of gluconate kinase synthesis; the DNAs of these three revertants were directly sequenced using PCR as described below.

Identification of the base substitution introduced by the *alsA1* mutation. The chromosomal regions (nucleotides -279 to +1005; the *ccpA* structural gene comprises nucleotides +1 to +1002; Fig. 1) of strains 1A250 and 1A147, and revertants of strain 1A147, were sequenced directly by means of PCR and cycle sequencing using DNA Sequencer (373A) and Taq Dye Primer Cycle Sequencing Kit (Applied Biosystems), as follows. To prepare the templates for cycle sequencing, this region of their chromosomes was amplified by PCR using four sets of left and right primers containing the 21M13 and M13 reverse sequences, respectively. The cycle sequencing of the four templates (approximately 400 bp long) with the 21M13 and M13 reverse dye primers resulted in four sequences slightly over-

Fig. 1. (a) Location of the *alsA1* mutation. The 1.37 kb *EcoRI* fragment carrying the *ccpA* gene is shown. The initiation codon (ATG) of the *ccpA* gene was confirmed by determining the N-terminal amino acid of the CcpA protein synthesized by *E. coli*, and its adenine was assigned as nucleotide +1. As indicated, the *alsA1* mutation comprises a guanine substitution with adenine, which is located at nucleotide -14 within the Shine-Dalgarno sequence (SD) for the *ccpA* gene. The location of the DNA probe (nucleotides +16 to +817) used for cloning of the *ccpA* gene is also shown. (b) Structures of the two compatible plasmids, pCCPA19 and pGNT61, used for *in vivo* analysis of the function of the CcpA protein. pCCPA19 and pGNT61 carry, respectively, the replication origins *ori-coliE1* and *ori-177*, derived from pACYC177 (Chang & Cohen, 1978). pCCPA19 consists of the *EcoRI* arm of pUC19 and the 1.37 kb *EcoRI* fragment carrying the *ccpA* gene; the orientation of transcription from the *lac* promoter is the same as that for the *ccpA* gene. pGNT61 consists of the *HindIII* arm of plasmid pGNT177, and a 2.0 kb *EcoRI* fragment carrying the *gnt* promoter and the *gntR* gene; the *cat* gene is under the control of the *gnt* promoter. Ap and Tc represent ampicillin and tetracycline resistance genes, respectively.

lapping each other, which were connected to produce the nucleotide sequences corresponding to this region of their chromosomes.

Enzyme assay. For the assay of gluconate kinase, inositol dehydrogenase, histidase, xylose isomerase and sorbitol dehydrogenase, cells were grown at 37 °C to OD₆₀₀ 0.75 in S6 medium (Fujita & Freese, 1981) containing 0.5% Casamino acids (Difco) and 50 µg each of the required amino acids, with or without sodium gluconate (10 mM), *myo*-inositol (10 mM), histidine (5 mM), xylose (10 mM), and sorbitol (10 mM), and with each of the inducers and 10 mM glucose; for the histidase assay, 43 mM sodium glutamate was added instead of Casamino acids. Cell extracts (9 OD₆₀₀ units ml⁻¹ in 0.1 M Tris/HCl,

pH 8) were prepared by lysozyme treatment and brief sonication, as described previously (Nihashi & Fujita, 1984); for the xylose isomerase assay, 0.1 M borate buffer, pH 8, was used. These enzymes were assayed spectrophotometrically by published methods: gluconate kinase and inositol dehydrogenase (Nihashi & Fujita, 1984), histidase (Chasin & Magasanik, 1968), xylose isomerase (Horecker, 1974) and sorbitol dehydrogenase (Horwitz & Kaplan, 1964).

For the CAT assay, cells bearing two compatible plasmids were grown to OD₆₀₀ 0.6 in Luria-Bertani medium containing ampicillin (50 µg ml⁻¹), tetracycline (10 µg ml⁻¹) and chloramphenicol (10 µg ml⁻¹), with or without 10 mM gluconate, and with 10 mM each of gluconate and glucose. The cells were harvested and lysed by lysozyme treatment and brief sonication (8 OD₆₀₀ units of cells ml⁻¹) as described previously (Yoshida *et al.*, 1993). CAT was assayed spectrophotometrically as described by Shaw (1975).

Purification of the CcpA protein. The CcpA protein synthesized by *E. coli* strain XL1-blue bearing plasmid pCCPA19 was obtained as a 38 kDa polypeptide band on SDS-PAGE (Laemmli, 1970) during purification. Cells grown to the early stationary phase (OD₆₀₀ 3) in 300 ml Luria-Bertani medium containing ampicillin (50 µg ml⁻¹) were disrupted by sonication, and then the CcpA protein was partially purified by essentially the same procedures as those used for the *B. subtilis* GntR protein (Miwa & Fujita, 1988) (60% ammonium sulfate precipitation, and subsequent column chromatographies on Bio-Gel A-1.5 m and DEAE-cellulose, but without the phosphocellulose column chromatography). After the DEAE-cellulose chromatography, an aliquot of the CcpA preparation (40 mg total protein) in 20 mM Tris/HCl buffer, pH 7.9, containing 0.05 M KCl, 0.1 mM NaEDTA, pH 8, 0.1 mM dithiothreitol, 1 mM phenylmethylsulfonyl fluoride and 20% (v/v) glycerol, was applied to a calf thymus DNA-cellulose column (1.6 × 4.0 cm) prepared in the same buffer. The column was washed with 100 ml of the buffer and then eluted with a 100 ml linear KCl gradient (0.05–0.3 M). Fractions (0.9 ml) containing the CcpA protein (eluted at around 0.125 M KCl) were pooled, concentrated and dialysed against the buffer without KCl in a Collodion bag (Sartorius) to give 1 mg CcpA protein.

The CcpA protein synthesized by *B. subtilis* strain 1A250 bearing pCCPA110 was purified by the same procedures as described above. Cells were grown to OD₆₀₀ 0.75 in 3 litres of S6 medium containing 0.5% Casamino acids, 50 µg ml⁻¹ each of the required amino acids, 5 µg kanamycin ml⁻¹, and 10 mM each of gluconate and glucose. During CcpA purification, yielding 28 µg protein, this protein was pursued by Western blotting. After SDS-PAGE (10%, w/v, gel) and blotting, immunological detection was performed using rabbit antiserum against the CcpA protein purified from *E. coli* cells and then anti-rabbit IgG horseradish peroxidase conjugate (Bio-Rad). This rabbit antiserum was generated as described previously (Fujita & Freese, 1981).

Characterization of the CcpA protein. The molecular mass of the CcpA protein was determined by gel filtration on a Sephacryl S-300 column (Pharmacia, 0.5 × 115 cm) with molecular mass markers of thyroglobulin (669 kDa), catalase (232 kDa), gamma globulin (158 kDa), bovine serum albumin (67 kDa), ovalbumin (44 kDa), myoglobin (17 kDa), ribonuclease A (13.7 kDa) and cyanocobalamin (1.35 kDa).

For determination of the N-terminal amino acid sequence of the CcpA protein, the purified protein was electrophoresed on a 10% SDS-polyacrylamide gel, and then this protein on the gel was blotted onto a PVDF protein sequencing membrane (Bio-Rad) using Bio-Rad Trans-blot SD according to the instruction manual. After deformylation of the CcpA protein (approx-

mately 3 µg) on the membrane in 0.6 M HCl at 25 °C for 24 h, it was washed with distilled water, dried and then applied to a protein sequencer (PPSQ-10, Shimadzu, Kyoto).

Gel retardation analysis of DNA binding of the CcpA protein was performed as described previously (Yoshida *et al.*, 1993). Three DNA fragments were isolated from an *Hpa*II digest of a 742 bp *Hind*III fragment containing the *gnt* promoter (nucleotides -202 to +541; *gnt* transcription initiation base, +1) (Fujita & Fujita, 1986), labelled with [α -³²P]dCTP (Amersham) by means of the filling-in reaction, and then added to the binding reaction mixture; these three fragments, of 291, 113 and 338 bp, carried *gnt* sequences of -202 to +90, +91 to +203, and +204 to +541, respectively.

RESULTS

Effect of the *ccpA* mutations on catabolite repression of adaptive enzyme syntheses and sporulation

Table 1 shows that the *alsA1* mutation, which is allelic to *ccpA*, and the *ccpA*::Tn917 mutation (Zahler *et al.*, 1990; Henkin *et al.*, 1991) affected not only catabolite repression of the synthesis of gluconate kinase, but also that of the synthesis of inositol dehydrogenase, histidase, xylose isomerase and sorbitol dehydrogenase. Although the catabolite repression of synthesis of gluconate kinase and sorbitol dehydrogenase was completely abolished by these two *ccpA* mutations, that of the synthesis of inositol dehydrogenase, histidase and xylose isomerase was only partially affected by them, suggesting that catabolite repression(s) not involving the CcpA protein as well as that mediated by this protein might operate in the latter three enzyme syntheses.

As shown in Table 2, the *alsA1* mutation also affected sporulation, but the effect was observed only when catabolite repression of sporulation of strain 1A147 was compared with that of strain 1A250; strain 1A147 could sporulate efficiently even in the presence of 0.8% glucose, whereas strain 1A250 could not sporulate normally in the presence of 0.5% glucose. We did not have an isogenic strain of strain WLN-29, so it was difficult to judge whether the *ccpA*::Tn917 mutation affected sporulation. Thus, the CcpA protein was considered to be only partially involved in catabolite repression of sporulation. The finding that the CcpA protein was involved in catabolite repression of not only adaptive enzyme syntheses but also sporulation implies that an unknown mechanism involving this protein may play a central role in *B. subtilis* catabolite repression.

Identification of the base substitution present in the *alsA1* mutation

Since the *alsA1* mutation is a very stable *ccpA* mutation, and strain 1A147 carrying this mutation has a good isogenic strain (1A250), we attempted to identify the base substitution introduced in the *alsA1* mutation. According to the nucleotide sequence of the 1.37 kb *Eco*RI fragment containing the *ccpA* gene (Henkin *et al.*, 1991), we designed and synthesized several primers for PCR amplification using DNAs of strains 1A250 and 1A147; this amplification could cover the region from nucleotide -279 to +1005 (translation of *ccpA* starts at +1: Fig. 1a)

Table 1. Effect of the *ccpA*::Tn917 and *alsA1* mutations on catabolite repression of synthesis of gluconate kinase, inositol dehydrogenase, histidase, xylose isomerase and sorbitol dehydrogenase

Cell growth, preparation of the crude extracts and assaying of the enzymes are described in the text. The experiments were each performed three times. Representative results are shown.

Strain	Repression ratio of enzyme activity without glucose to that with glucose				
	Gluconate kinase	Inositol dehydrogenase	Histidase	Xylose isomerase	Sorbitol dehydrogenase
1A250 (<i>ccpA</i> ⁺)	> 27	> 317	> 34	> 26	8.4
1A147 (<i>alsA1</i>)	0.8	4.9	2.6	10.5	1.0
WLN-29 (<i>ccpA</i> ::Tn917)	1.0	1.8	1.5	13.9	1.0

Table 2. Effect of the *alsA1* mutation on catabolite repression of sporulation

Cells were allowed to sporulate in nutrient sporulation medium (Fujita & Freese, 1981) at 37 °C for 24 h. For titration of spores, the cultures were treated at 75 °C for 15 min. The experiments were repeated at least twice; representative results are shown. Sporulation percentage is defined as [(no. of spores)/(no. of viable cells)] × 100.

Strain	Sporulation percentage				
	Glucose concn (%):				
	0.2	0.5	0.8	1.2	2.0
1A250 (<i>ccpA</i> ⁺)	90.0	3.1	0.9	0.6	0.7
1A147 (<i>alsA1</i>)	77.0	71.0	20.0	2.0	1.3

containing the putative *ccpA* promoter and its structural gene, encoding 334 amino acids. Cycle sequencing of the DNA fragments amplified with DNA of strain 1A250 revealed that the sequence determined with DNA of strain 1A250 completely coincided with the reported sequence of this region. However, we detected one mismatch with DNA of strain 1A147, namely a base substitution of guanine at nucleotide -14 to adenine within the Shine-Dalgarno sequence for the *ccpA* gene, indicating that the *alsA1* mutation might be due to this base substitution (Fig. 1a).

Strain 1A147 grew more slowly in S6 medium containing glucose as the sole carbon source than did strain 1A250; their doubling times were 2.2 and 1.6 h, respectively. We isolated 10 independent revertants of strain 1A147 which grew at the same rate as 1A250 after successive cultivations under these growth conditions. Three of these revertants were examined as to catabolite repression of gluconate kinase synthesis, and all of them exhibited normal catabolite repression (data not shown). The base substitutions which had occurred in the DNA of these strains were identified by means of DNA amplification and cycle sequencing; all were found to be reversion of adenine to guanine at nucleotide -14, so we concluded

that the *alsA1* mutation does indeed comprise adenine substitution of guanine at nucleotide -14 within the Shine-Dalgarno sequence for the *ccpA* gene.

Cloning and expression of the *ccpA* gene in *E. coli* and *B. subtilis* plasmids, and purification of the CcpA protein

The 1.37 kb *EcoRI* fragment containing the *ccpA* gene was cloned into *E. coli* plasmid pUC19 to produce *E. coli* strain XL1-blue bearing pCCPA19 by means of colony hybridization using a PCR-amplified fragment (nucleotides +16 to +817) as a probe (Fig. 1). The 1.37 kb fragment derived from pCCPA19 was also cloned into *B. subtilis* plasmid pUB110 to produce strains 1A147 and 1A250 bearing plasmid pCCPA110. Table 3 shows that normal catabolite repression of gluconate kinase synthesis was observed in strain 1A147 bearing pCCPA110, but not in strain 1A147 bearing pUB110, indicating that the *ccpA* gene in the 1.37 kb fragment cloned into pUB110 complemented the *alsA1* mutation; the *ccpA* gene cloned in SPβ prophage was also reported to complement this mutation (Henkin *et al.*, 1991). However, the over-produced CcpA protein seemed to be somewhat harmful to *B. subtilis* cells, because the induction of gluconate kinase (Table 3) and the growth rate (data not shown) were affected by the presence of pCCPA110 in either strain 1A250 or 1A147; 1A250 and 1A147 cells bearing pCCPA110 grew at approximately two-thirds the rates of the respective strains bearing pUB110.

E. coli strain XL1-blue bearing pCCPA19 could constitutively synthesize a protein, which accounted for approximately 5% of the total protein, that had the electrophoretic mobility of a polypeptide of approximately 38 kDa on SDS-PAGE; strain XL1-blue bearing pUC19 did not synthesize the protein. This putative CcpA protein was purified to approximately 95% homogeneity, as shown in Fig. 2. The N-terminal sequence of the purified protein was determined with an automatic sequencer after the protein had been deacylated. This deacylation enhanced the yield of each hydrolysed residue by approximately 1.4-fold, suggesting that the first methionine might be partially formylated. Although the N-terminal meth-

Table 3. Complementation of the chromosomal *alsA1* mutation with pCCPA110 (*ccpA*⁺)

Cell growth, preparation of the crude extracts and assaying of gluconate kinase are described in the text. Representative results from three independent experiments are shown. Gnt, gluconate; Glc, glucose.

Strain	Plasmid	Gluconate kinase (nmol min ⁻¹ mg ⁻¹)			Repression ratio (Gnt/Gnt + Glc)
		No Gnt	Gnt	Gnt + Glc	
1A250 (<i>ccpA</i> ⁺)	pUB110	< 1.0	44.8	< 1.0	> 44
	pCCPA110	< 1.0	32.6	< 1.0	> 32
1A147 (<i>alsA1</i>)	pUB110	< 1.0	52.9	49.1	1.1
	pCCPA110	< 1.0	34.7	< 1.0	> 34

Fig. 2. The CcpA proteins purified from *B. subtilis* and *E. coli* cells. The purified CcpA proteins (1.5 µg each) synthesized by *B. subtilis* strain 1A250 bearing pCCPA110 (lane 1) and *E. coli* strain XL1-blue bearing pCCPA19 (lane 2) were applied to a 10% SDS-polyacrylamide gel. Lane M contains standard proteins used as molecular mass markers: phosphorylase *b* (92.5 kDa), bovine serum albumin (66.2 kDa), ovalbumin (45.0 kDa), carbonic anhydrase (31.0 kDa) and soybean trypsin inhibitor (21.5 kDa). The CcpA protein (38 kDa) is indicated by an arrow.

ionine, which should appear at the first cycle, was scarcely detected, the second to eleventh amino acid terminal residues were the same as reported by Henkin *et al.* (1991), indicating that the purified protein was the CcpA protein which had been translated from an initiation codon, ATG, starting from nucleotide +1 (Fig. 1a). Since we had identified the initiation codon of the *ccpA* gene, we could conclude that the *alsA1* mutation comprises a substitution of guanine to adenine at nucleotide -14 within the Shine-Dalgarno sequence for the *ccpA* gene (Fig. 1a).

Fig. 3. Western blotting for determination of the CcpA protein levels in *B. subtilis* cells. Cells were grown in S6 medium containing 50 µg each of the required amino acids, with or without 10 mM gluconate or with gluconate and glucose. Cell extracts from 190 OD₆₀₀ units of cells were prepared by the same method as the first step for purification of the CcpA protein using sonication; the extracts contained approximately 60 mg total protein ml⁻¹. Western blotting was performed as described in the text. Lanes 1, 2, 3 and 4 contained, respectively, 45, 90, 180 and 360 ng CcpA protein purified from *E. coli*. Lanes 5 to 10 and lanes 11 to 13 contained cell extracts from strains 1A250 (wild-type) and 1A147 (*alsA1*), respectively. Lanes 5, 7, 9, 11, 12 and 13; and lanes 6, 8 and 10 contained 270 and 90 µg of total protein, respectively. Lanes 5, 6 and 11; lanes 7, 8 and 12; and lanes 9, 10 and 13 contained, respectively, extracts from cells grown without gluconate; with gluconate; and with gluconate and glucose.

The molecular mass of the CcpA protein purified from *E. coli* cells was determined by gel filtration to be 74 kDa. The molecular mass of the subunit of the CcpA protein is 38 kDa, so the CcpA protein probably exists as a dimer.

The CcpA protein synthesized by *B. subtilis* strain 1A250 bearing pCCPA110 was also purified to over 90% homogeneity by the same purification procedures as those used for the *E. coli* cell extract. We purified the CcpA

Fig. 4. DNA binding ability of the CcpA protein. Gel retardation analysis for DNA binding of the purified CcpA protein was performed as described previously (Yoshida *et al.*, 1993). Three DNA fragments (113, 291 and 338 bp) (100 pM each) were added to the reaction mixtures; the 291 and 113 bp fragments contain the *gnt* promoter and the *cis* sequence for catabolite repression of *gnt*, respectively, while the 338 bp fragment was an internal control for this analysis. The preparation of these fragments was described in the text. (a) Lane 1 contained no protein; lanes 2 to 4, CcpA protein synthesized by *B. subtilis*; lanes 5 to 7, CcpA protein synthesized by *E. coli*; and lanes 8 to 10, ovalbumin. The concentrations of the added protein in the binding reaction mixture were as follows: lanes 2, 5 and 8, 80 nM; lanes 3, 6 and 9, 160 nM; lanes 4, 7 and 10, 320 nM. (b) The CcpA proteins (320 nM) purified from *B. subtilis* (lanes 1 to 3) and *E. coli* (lanes 4 to 6) were added to the binding mixture. Lanes 1 and 4 contained no competitive DNA; lanes 2 and 5 contained 2.5 µg sonicated calf thymus DNA of 500 bp in average length; lanes 3 and 6 contained 100 nM of the 338 bp fragment.

protein from an extract of cells grown in the presence of glucose, that is, under conditions exerting catabolite repression. However, we could not distinguish it from that purified from *E. coli* cell extracts on SDS-PAGE (Fig. 2), indicating that no protein modification could be detected by this technique.

CcpA protein levels in cells of strains 1A250 and 1A147

Strains 1A250 and 1A147 were cultivated under three growth conditions: where gluconate kinase was not induced, where it was induced by gluconate, and where this induction was repressed by glucose. The levels of the CcpA proteins in crude extracts of these cells were measured by Western-blotting (Fig. 3). The results indicated that this protein was probably synthesized constitutively under all three growth conditions. Cell extracts of strain 1A250 contained approximately 2 µg CcpA protein per mg total protein (Fig. 3), which corresponds to roughly 3000 molecules per cell on the assumption that proteins were completely extracted from the cells. The CcpA level in strain 1A147 (*alsA1*) was approximately 10-fold lower than that in strain 1A250 (*alsA*⁺) (Fig. 3), probably due to a decrease of CcpA synthesis in this mutant caused by the poor binding of ribosomes to the mutated binding site within the Shine-Dalgarno sequence for the *ccpA* gene.

Inability of the CcpA protein to bind to the *cis* sequence for catabolite repression of the *gnt* operon

It has been proven in the systems of *amyE*, *bglS* and *gnt*, which are now known to include *xyl* and *hut*, that catabolite repression requiring the consensus *cis* sequence

is mediated by the CcpA protein. Furthermore, the CcpA protein could bind non-specifically to DNA-cellulose, suggesting that it might be a DNA-binding protein. Thus, we examined by means of gel retardation analysis whether the purified CcpA protein could bind to the *cis* sequence for catabolite repression of the *gnt* operon, which is located downstream of the *gnt* promoter (Miwa & Fujita, 1993). In our gel retardation analysis (Fig. 4), 100 pM each of three labelled DNA fragments derived from a 742 bp *Hind*III fragment (Fujita & Fujita, 1986) were added; the 113 and 291 bp fragments carry the *cis* sequence for *gnt* catabolite repression and the *gnt* promoter, respectively, while the 338 bp fragment, which was added as an internal control, contains no known specific sequence. Even when the CcpA protein purified from either *B. subtilis* or *E. coli* cells was added in an amount corresponding to 400 times higher molarity than that of the DNA fragments, the mobility of the latter did not change at all (data not shown). When more of this protein was added, the DNA fragments started to shift, as shown in Fig. 4(a): the 291 bp fragment began to shift on the addition of CcpA protein at 800 times higher molarity (lanes 2 and 5), and the 338 bp fragment shifted on the addition of 1600 times higher molarity (lanes 3 and 6); however, the 113 bp fragment did not shift well even on the addition of 3200 times higher molarity (lanes 4 and 7). We do not know the reason why these three fragments exhibited different shifts, though it might be partially due to their sizes. Anyway, these shifts were not observed on the addition of ovalbumin as a control (lanes 8 to 10, Fig. 4a). When either 100 nM of the unlabelled 338 bp fragment (1000 times molarity compared to that of the three labelled fragments) or 2.5 µg of sonicated calf thymus DNA was added to the binding reaction mixture,

Table 4. CAT synthesis under the control of the *gnt* promoter in the presence of the *ccpA* gene in *E. coli*

Cell growth, preparation of the crude extracts and assaying of CAT are described in the text. The amounts of pGNT61 in the crude plasmid preparations were determined densitometrically by agarose gel electrophoresis; then the copy numbers per mg protein in crude extracts were calculated on the assumption that the plasmid yield from the same amount of cells from which the crude extract had been prepared was almost 100%. The experiments were repeated at least twice; representative data are shown. Gnt, gluconate; Glc, glucose.

Plasmid set in cells	CAT activity (nmol min ⁻¹ per 10 ¹⁰ copies of pGNT61)			Repression ratio (Gnt/Gnt + Glc)
	No Gnt	Gnt	Gnt + Glc	
pGNT61, pUC19	25.1	102.3	103.2	0.99
pGNT61, pCCPA19	20.1	143.9	174.8	0.82

none of the labelled DNA fragments shifted (Fig. 4b). These results clearly indicated that neither the CcpA protein from *E. coli* cells nor that from *B. subtilis* cells grown with glucose binds to the *cis* sequence for catabolite repression of the *gnt* operon, but both proteins bind non-specifically to DNA.

Our observation that the CcpA protein only bound non-specifically to DNA might be due to artifacts such as inactivation of the CcpA protein during its purification and/or the conditions for the gel retardation analysis. Thus, we attempted to reconstruct catabolite repression of *gnt* in *E. coli* cells by using the *cat* gene under *gnt* regulation and the CcpA protein synthesized *in vivo*. *E. coli* strain JM109 was transformed with two compatible plasmids, pGNT61 and pUC19 (or pCCPA19), which carry *ori-177* and *ori-colE1* for their replication, respectively (Fig. 1b); pGNT61 carries the *cat* gene under the control of the *gnt* induction system. We have already reported that in *B. subtilis*, the syntheses of CAT and subtilisin under the direction of this *gnt* promoter construct using the respective promoter-probe plasmids are subject to glucose repression (Fujita & Fujita, 1987; Miwa & Fujita, 1990). As shown in Table 4, CAT synthesis was induced in cells bearing plasmids pGNT61 and pUC19 upon the addition of gluconate to the medium. When pCCPA19 was introduced instead of pUC19, CAT synthesis was not affected by the synthesized CcpA protein even in cells grown with glucose (Table 4). (The CAT activities shown in this table were normalized per 10¹⁰ copies of pGNT61 because the copy number of pGNT61 in cells bearing both pGNT61 and pCCPA19 was decreased in comparison with that in cells bearing pGNT61 and pUC19, which was caused by the expression of the *ccpA* gene. We do not know why the CcpA protein decreased the copy number of plasmid pGNT61.) The results suggest that the signalling system from extra-cellular glucose to catabolite repression of the *gnt* operon mediated by the CcpA protein might not work in *E. coli*. However, this observation implies that the CcpA protein by itself might not bind to the *cis* sequence for catabolite repression of the *gnt* operon, because CAT synthesis

under the control of the *gnt* regulation involving this *cis* sequence was not affected negatively by the CcpA protein in *E. coli* cells.

DISCUSSION

Catabolite repression mediated by the CcpA protein is likely to be a global regulatory system because the *ccpA* mutation has pleiotropic effects on catabolite repression of various adaptive enzyme syntheses. This protein was found to be involved not only in catabolite repression of α -amylase synthesis (Henkin *et al.*, 1991), but also in that of levanase (Martin *et al.*, 1989), β -glucanase (Krüger *et al.*, 1993) and gluconate kinase (Fujita & Miwa, 1994) syntheses. In addition the *ccpA* mutations affected the catabolite repression of inositol dehydrogenase, histidase, xylose isomerase and sorbitol dehydrogenase syntheses, as well as that of sporulation (Tables 1 and 2). Furthermore, the production of acetoin is abolished by the *alsA1* mutation (Zahler *et al.*, 1990), and the stimulation of *ackA* expression by glucose was blocked in a *ccpA* mutant (Grundy *et al.*, 1993). These observations suggest that the *ccpA* gene plays an important role in coordinating global gene expression in response to rapidly metabolizable carbohydrates in the medium.

It has been reported that catabolite repression of the *amyE* (Nicholson *et al.*, 1987), *gnt* (Miwa & Fujita, 1990, 1993), *xyl* (Jacob *et al.*, 1991), *but* (Oda *et al.*, 1992) and *bglS* (Krüger *et al.*, 1993) operons involves the respective five *cis* sequences, which exhibit similarity with the *B. subtilis* catabolite repression consensus sequence (Weickert & Chambliss, 1990). Among these systems, the CcpA protein has been reported to be involved in catabolite repression of *amyE* (Henkin *et al.*, 1991), *bglS* (Krüger *et al.*, 1993) and *gnt* (Fujita & Miwa, 1994). In the present study, we showed that this protein was involved in catabolite repression of the *but* and *xyl* operons (Table 1). These facts suggest that *B. subtilis* catabolite repression involving the consensus *cis* sequence seems to be generally mediated by a negative regulator of the CcpA protein. However, the CcpA protein seemed to be synthesized constitutively in *B. subtilis* cells (Fig. 3). Our gel retardation analysis

involving the CcpA proteins purified from both *B. subtilis* and *E. coli* indicated that the CcpA protein was a non-specific DNA-binding protein (Fig. 4). Not only the CcpA protein purified from *E. coli* cells but also that from *B. subtilis* cells grown under conditions exerting catabolite repression did not bind specifically to the *cis* sequence for catabolite repression of the *gnt* operon, as judged by gel retardation analysis (Fig. 4). We do not think that the specific binding activity of this protein to the *cis* sequence was specifically lost during purification because the purified CcpA proteins retained the non-specific binding ability. We could not detect any specific binding ability in protein preparations from any step of our purification procedures (data not shown). Further evidence that the CcpA protein by itself probably does not interact with the *cis* sequence for *B. subtilis* catabolite repression in *E. coli* cells comes from the observation that this protein did not affect the expression of the *cat* gene under the control of the *gnt* induction system (Table 4); it remains a possibility that *gnt* transcription by *E. coli* RNA polymerase might not be affected even if the CcpA protein bound to the *cis* sequence for *B. subtilis* catabolite repression.

Although we cannot exclude the possibility that the above results might be due to unexpected artifacts in our *in vivo* and *in vitro* experiments, they can be reasonably explained as follows: (i) the CcpA protein is indirectly involved in catabolite repression through regulation of another regulatory gene whose product can recognize the consensus *cis* sequence for catabolite repression; or (ii) this protein requires a conformational change in its three-dimensional configuration for specific binding to the consensus *cis* sequence for *B. subtilis* catabolite repression. This change can be induced by a protein modification such as proteolysis or protein phosphorylation, which could not be detected on SDS-PAGE (Fig. 2), or by association with some factor (protein or metabolite).

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