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 (Miw a & 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 σA 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 gyrA96 thiA relA1 lac F' [proAB' lacIq XylSlacZM15 Tn10(Tet')]) and JM109 (recA supE44 hsdR17 gyrA96 relA1 thiA [lac-proAB') F' [traD36 proAB' lacIq XylS lacZM15]) were used. Bacillus subtilis strains 1A1 (trpC2, 1A250 (trpC2 alsR1 ilvBA1) and 1A147 (alsA1 trpC2 alsR1 ilvBA1) were obtained from the Bacillus Genetic Stock Center (Ohio State University). B. subtilis strain WLN-29 (aroG932 trpC2 ccrA+) was isolated by Dr. Miwa (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 was 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 plasmid 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 10³ cells of strain 1A147 (alsA1 trpC2 alsR1 ilvBA1), obtained from one of 100 single colonies on plates containing tryptophan blood agar base (33 g l⁻¹, Difco) and 10 mM glucose, were inoculated into S6 medium (Fujita & Freese, 1981) containing 0.5 mM glutamate and 50 μM α-ketoglutarate, 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-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 ml⁻¹ of 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.

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Purification of the CcpA protein. The CcpA protein synthesized by E. coli strain XL1-blue bearing plasmid pCPPA19 was obtained as a 38 kDa polypeptide band on SDS-PAGE (Laemmli, 1970) during purification. Cells grown to the early stationary phase (OD$_{600}$ 3) in 300 ml 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 glucose, and with 10 mM each of gluconate and glucose. The cells were harvested and lysed by lysozyme treatment and brief sonication (8 OD$_{600}$ units of cells ml$^{-1}$) as described previously (Yoshida et al., 1993), CAT was assayed spectrophotometrically as described by Shaw (1975).

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 EcoRI 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 $-270$ to $+1005$ (translation of ccpA starts at +1: Fig. 1a)

B. subtilis CcpA protein

pH 8) were prepared by lysozyme treatment and brief sonication, as described previously (Nishashi & Fujita, 1984); for the xylose isomerase assay, 0·1 M borate buffer, pH 8, was used. These enzymes were assayed spectrophotometrically by published methods: glucokinase and inositol dehydrogenase (Nishashi & Fujita, 1984), histadase (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 OD$_{600}$ 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 OD$_{600}$ units of cells ml$^{-1}$) as described previously (Yoshida et al., 1993). CAT was assayed spectrophotometrically as described by Shaw (1975).

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 × 15 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 cytochrome b5 (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 deamidylation of the CcpA protein (approximately 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 (PPSEQ-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 HpaII digest of a 742 bp HindIII 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 336 bp, carried gnt sequences of $-202$ to $+90$, $+91$ to $+203$, and $+204$ to $+541$, respectively.

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 EcoRI 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 $-270$ to $+1005$ (translation of ccpA starts at +1: Fig. 1a)
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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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Repression ratio of enzyme activity without glucose to that with glucose</th>
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<tr>
<td></td>
<td>Gluconate kinase</td>
</tr>
<tr>
<td>1A250 (ccpA)</td>
<td>&gt; 27</td>
</tr>
<tr>
<td>1A147 (alsA1)</td>
<td>0.8</td>
</tr>
<tr>
<td>WLN-29 (ccpA::Tn917)</td>
<td>1.0</td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>Strain</th>
<th>Sporulation percentage</th>
</tr>
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<tr>
<td></td>
<td>Glucose concn (%)</td>
</tr>
<tr>
<td></td>
<td>0.2</td>
</tr>
<tr>
<td>1A250 (ccpA)</td>
<td>90.0</td>
</tr>
<tr>
<td>1A147 (alsA1)</td>
<td>77.0</td>
</tr>
</tbody>
</table>

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 SFβ 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 deformylated. This deformylation 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 pCCPAl10 (ccpA+)

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Gluconate kinase (nmol min⁻¹ mg⁻¹)</th>
<th>Repression ratio (Gnt/Gnt + Glc)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>No Gnt</td>
<td>Gnt</td>
</tr>
<tr>
<td>1A250 (ccpA⁺)</td>
<td>pUB110</td>
<td>&lt; 1.0</td>
<td>44.8</td>
</tr>
<tr>
<td></td>
<td>pCCPA110</td>
<td>&lt; 1.0</td>
<td>32.6</td>
</tr>
<tr>
<td>1A147 (alsA1)</td>
<td>pUB110</td>
<td>&lt; 1.0</td>
<td>52.9</td>
</tr>
<tr>
<td></td>
<td>pCCPA110</td>
<td>&lt; 1.0</td>
<td>34.7</td>
</tr>
</tbody>
</table>

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 pCCPAl10 (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.

Fig. 3. Western blotting for determination of the CcpA protein levels in B. subtilis cells. Cells were grown in 56 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 pCCPAl10 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

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).
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-Dalgarano 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 but, 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 HindIII fragment (Fujita & Fujita, 1986) were added; the 113 and 291 bp fragments carry the cis sequence for 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.

<table>
<thead>
<tr>
<th>Plasmid set in cells</th>
<th>CAT activity (nmol min⁻¹ per 10⁸ copies of pGNT61)</th>
<th>Repression ratio (Gnt/Gnt + Glc)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No Gnt</td>
<td>Gnt</td>
</tr>
<tr>
<td>pGNT61, pUC19</td>
<td>25.1</td>
<td>102.3</td>
</tr>
<tr>
<td>pGNT61, pCCPA19</td>
<td>20.1</td>
<td>143.9</td>
</tr>
</tbody>
</table>

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 sublustin 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 extracellular 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 *bgI5* (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), *bgI5* (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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