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Mutations in Catabolite Control Protein CcpA Showing Glucose-Independent Regulation in *Bacillus megaterium*

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We identified five single amino acid exchanges in CcpA that lead to permanent repression of the xylose utilization genes in the absence of glucose. Other proteins from the CcpA regulon also show glucose-independent regulation in the mutants. The mutant CcpA proteins bind to the DNA target catabolite responsive elements without the corepressor HPr-Ser-P.

Catabolite control protein CcpA is the central regulator of carbon catabolite repression (CCR) in *Bacillus megaterium*, *Bacillus subtilis*, and other gram-positive bacteria of low G+C content (5, 11, 12, 21, 22, 25). Genes and operons coding for the utilization of less favorable carbon sources, such as xylose, are regulated by CcpA on the level of transcription in the presence of rapidly metabolizable sugars like glucose or fructose (12). The mechanism of CCR is distinct from the one described for *Escherichia coli* (reviewed in reference 27). CcpA can either repress or activate transcription. Activation was shown in two cases, *ackA* in *B. subtilis* (31) and the *las* operon of *Lactococcus lactis* (22). Repression by CcpA was demonstrated for multiple genes and operons in *B. subtilis*, *B. megaterium*, and other gram-positive bacteria (10).

CcpA binds to DNA target sites termed catabolite responsive elements (CRE). Repression depends on the presence of HPr-Ser-P or Crh-Ser-P; the former is a component of the phosphoenolpyruvate:sugar phosphotransferase system whose phosphorylation state reflects glycolytic activity (30). The requirement for a corepressor for CcpA was confirmed by DNA footprinting studies involving CRE sequences from the *xyl* and *gnt* operons. In addition to HPr-Ser-P, glucose-6-phosphate also triggered CRE binding by CcpA in both systems in in vitro assays (7, 9, 24). Similar experiments with the *xynB* CRE showed that Crh-Ser-P can substitute for HPr-Ser-P as a corepressor, and both Crh-Ser-P and HPr-Ser-P can trigger CcpA-regulated CCR of the *lev* operon in vivo (8, 23). In contrast to these results, in vivo CCR of *amyE* is independent of phosphorylated HPr (14, 33), and even though CcpA-CRE interaction was strengthened by a combination of HPr-Ser-P and fructose-1,6-bisphosphate or NADP, it did not improve repression in in vitro transcription (15). Accordingly, CcpA is thought to receive signals from HPr-Ser-P or Crh-Ser-P and possibly from other effectors.

A direct interaction of CcpA with HPr-Ser-P has been demonstrated (3, 13), and a putative HPr-Ser-P binding site on CcpA was recently identified (17). CcpA is a member of the LacI-GalR family of bacterial repressors, and sequence similarities, limited proteolysis, and mutational data suggest a common three-dimensional fold for CcpA, LacI, and PurR (13, 16, 17, 34). On the other hand, HPr-Ser-P does not bind in the effector binding cleft where isopropyl-β-D-thiogalactopyranoside (IPTG) binds to LacI and hypoxanthine binds to PurR (see Fig. 4). It is therefore interesting to collect further evidence about the activation of CcpA for CRE binding.

CcpA mutagenesis and screen for glucose-independent repression. We have conducted a screen for CcpA variants which repress the xylose utilization genes of *B. megaterium* in the absence of a repressing carbon source. A plasmid library of mutated *ccpA* alleles was generated by in vitro mutagenesis with nitrite treatment, as described previously (18). It was transformed into *B. megaterium* WH353 [*lac* Δ*ccpA gdh2*Φ (*xylA-spoVG-lacZ*) Δ*xylR*], which carries an in-frame *ccpA* deletion and a *xylA-lacZ* fusion as a probe for catabolite repression activity. An additional chromosomal deletion in *xylR* ensured that repression of *xylA-lacZ* transcription is only caused by the plasmid-encoded CcpA. We screened the transformants on M9 minimal medium containing succinate, which is neutral in CCR, as a carbon source and 5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside (X-Gal) to identify CcpA mutants which permanently repress *xyl* expression, thus displaying the repression phenotype *ccpA*^{k}.

Screening about 12,000 colonies yielded 58 white or light blue colonies which were colony purified. The plasmids were isolated, passaged through *E. coli*, and retransformed into *B. megaterium* WH353. If the original phenotype was retained we quantified repression by determining β-galactosidase activities. A total of 28 of the original clones were regarded as glucose independent in CCR (*ccpA*^{k} phenotype), since they repressed *xylA-lacZ* expression in succinate to 50% or less of wild-type expression in the absence of glucose. Total cell extracts from the mutants were analyzed by immunoblotting with anti-CcpA antiserum as described previously (16, 19). All of the mutant proteins were present at levels similar to that of wild-type CcpA expressed from the same vector (data not shown).

Sequence analysis revealed that 13 of the 28 *ccpA*^{k} alleles carried distinct mutations and that most of them caused multiple amino acid substitutions. They were separated by subcloning utilizing unique restriction sites in *ccpA*. The subclones were rescreened as described above. From the original screen and the subcloning we obtained the following five CcpA mutants, each with a single amino acid substitution, which exert permanent, glucose-independent repression: CcpA^{k}E77L, CcpA^{k}I227V, CcpA^{k}D275G, CcpA^{k}M282, and CcpA^{k}T306I.

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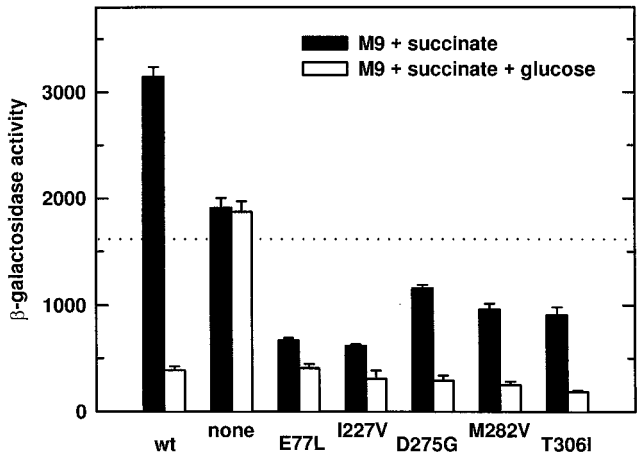

FIG. 1. β-Galactosidase activities expressed from *xylA-lacZ* in *B. megaterium* WH353 regulated by different CcpA mutants. Cells were grown in M9 medium with 0.5% succinate or with 0.5% succinate plus 0.2% glucose. The dotted line marks 50% of the wild-type (wt) expression with succinate. The amino acid substitutions in CcpA are shown under the respective columns.

None of the mutants represses *xyl* expression to the level obtained by the wild type with glucose, and they all show increased repression upon addition of glucose (Fig. 1). Thus, the mutants are only partially independent of glucose. We have observed severe growth deficiencies in all of the *ccpA*^{k} strains (data not shown). A more complete, permanent regulation phenotype might not show up in our screen if the phenotype is linked to poor growth.

2D gel electrophoresis of protein extracts from wild-type and *ccpA*^{k} mutant cells. To analyze the effects of the *ccpA*^{k} mutations on the entire CcpA regulon we used two-dimensional (2D) gel electrophoresis. Total soluble protein extracts were prepared from the *B. megaterium ccpA* deletion mutant WH353 carrying the empty vector pWH1509K (26) or derivatives of pWH2051 carrying the genes for wild-type CcpA or one of the five single amino acid CcpA^k mutants. Protein extract (100 mg) was then subjected to 2D protein electrophoresis as described by Völker et al. (32), and the protein profiles of the different strains were compared after silver staining. A typical gel is shown in Fig. 2 (left panel).

If a strain expressing wild-type CcpA is grown in glucose, spots for proteins whose expression is CCR dependent should be absent or reduced in intensity compared to those of the same strain grown in succinate, i.e., without the repressing carbon source. A comparison with a strain carrying only the vector revealed high expression of those proteins in the presence of glucose (data not shown), demonstrating that the regulation was mediated by CcpA. Using this approach, we recognized 39 spots showing the pattern for CcpA-dependent repression. Examples are shown in Fig. 2 (small panels, top row). Furthermore, we identified four proteins showing CcpA-dependent activation of expression (Fig. 2, small panels, bottom row). These spots were almost undetectable in the absence of glucose or if the cells were lacking CcpA. These numbers of proteins do not reflect the total size of the CcpA regulon because the expression of many proteins needs to be specifically induced, and we only looked at soluble proteins in the absence of inducers. The limited detectability of intensity differences in silver-stained gels further reduces the number of regulated proteins that are recognized.

CcpA-mediated regulation should be independent of glucose in the CcpA^k mutants. Spot intensities of cells of CcpA^k mutants grown in succinate resemble those found with cells of the wild type grown in glucose. Examples obtained with CcpA^{k}E77L are shown in Fig. 2 (rightmost small panels). The CcpA^{k}E77L strain showed the expected pattern for 37 of 39 repressed proteins and for 3 of 4 activated proteins identified in the wild type. Similar results were obtained with the strains with other single amino acid mutations, as follows: for CcpA^{k}I227V, 35 of 39 and 4 of 4; for CcpA^{k}D275G, 31 of 39 and 4 of 4; for CcpA^{k}M282V, 30 of 39 and 4 of 4; and for CcpA^{k}T306I, 35 of 39 and 3 of 4 (results are the numbers of repressed and activated proteins, respectively). In summary, more than 75% of the 43 proteins regulated in a glucose-dependent fashion in the wild type were regulated in the absence of glucose in each of the CcpA^k strains. Therefore,

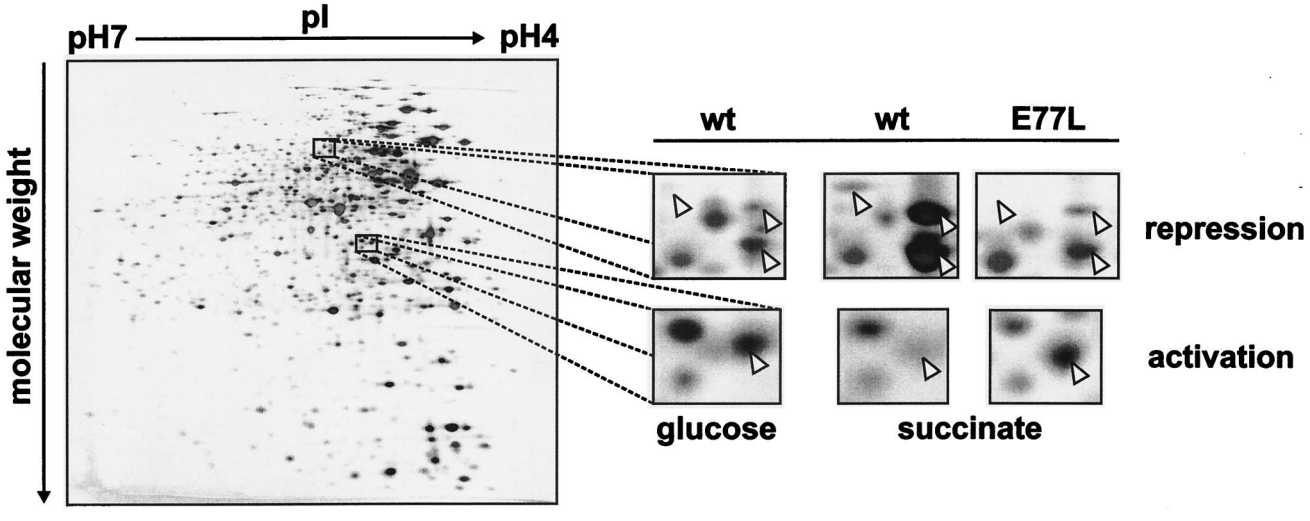

FIG. 2. CcpA-mediated changes in the protein synthesis profile of *B. megaterium*. The panel on the left shows a silver nitrate-stained 2D gel loaded with 100 μg of crude protein extract from *B. megaterium* WH353(pWH2051) expressing wild-type (wt) CcpA which had been grown on glucose. The small panels on the right display enlarged regions from this gel and the corresponding regions from gels prepared with extracts of the same strain grown on succinate and of CcpA^{k}E77L grown on succinate. Arrowheads indicate proteins showing the expected CCR or carbon catabolite activation pattern, and glucose-independent regulation in CcpA^{k}E77L.

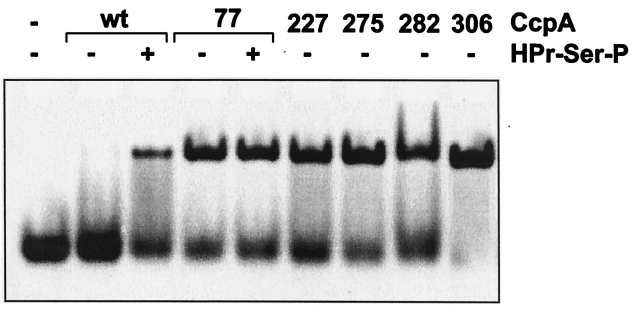

FIG. 3. Retardation of CRE by wild-type (wt) and mutant CcpA. Purified CcpA and HPr-Ser-P were combined with a 26mer oligonucleotide containing CRE as indicated above the lanes. Numbers indicate the position of the amino acid substitution in each CcpA mutant. Samples were incubated at 37°C for 10 min prior to loading and run on a non-denaturing 5% polyacrylamide gel. DNA was visualized with ethidium bromide.

permanent, glucose-independent repression by the five CcpA variants is not limited to *xylA*.

Purification of mutant proteins and PAGE DNA retardation assays. We tested DNA binding of the mutant proteins with and without the corepressor HPr in vitro. For this, the five *ccpA*^{k} alleles leading to single amino acid substitutions were cloned into overexpression vectors, and the proteins were overproduced in *B. megaterium* and purified by column chromatography (data not shown) as has been described for the wild-type protein (9). The apparently homogeneous preparations were then used for DNA retardation experiments. To obtain the corepressor HPr-Ser-P, the gene for HPr from *B. megaterium* was cloned into the same overexpression system, and overproduced in *B. megaterium* (33a). Purification was carried out essentially as described previously for HPr from *Staphylococcus aureus* (1). The protein was subsequently phosphorylated at Ser46 with partially purified HPr kinase from *B. megaterium*; the protocol was taken from Deutscher and Saier, Jr. (4), with minor adaptations. The preparation contained more than 90% HPr-Ser-P as estimated by nondenaturating polyacrylamide gel electrophoresis (PAGE) (data not shown).

For the PAGE DNA retardation assays, a synthetic double-stranded oligonucleotide containing CRE (26mer, as described in reference 13) was mixed with purified CcpA and HPr-Ser-P at concentrations of 5 μM (DNA), 10 μM (CcpA), and 10 μM (HPr-Ser-P) in a buffer containing 100 mM Tris-HCl (pH 7.5), 1 mM EDTA, and 10% glycerol. The mixture was incubated at 37°C for 10 min prior to the loading of 5 μl of it on a nondenaturing 5% polyacrylamide minigel (9 by 6 cm). The gel was run in 100 mM Tris-HCl (pH 7.5)–1 mM EDTA at 110 V for 45 min and stained with ethidium bromide.

Under the conditions employed, the wild-type CcpA protein could only form a complex with the DNA fragment if the corepressor HPr-Ser-P was present, as shown in Fig. 3. This effect is specific as it cannot be induced by the addition of unphosphorylated HPr (data not shown). Figure 3 shows that the same fragment is complexed by all five CcpA^k proteins in the absence of the corepressor. The addition of HPr-Ser-P does not increase the amount of complex formed (only shown for CcpA^{k}E77L). Thus, all CcpA^k proteins exhibit HPr-Ser-P-independent DNA binding in vitro.

Position of the mutations in the three-dimensional structure. It was surprising that the five amino acid substitutions causing the *ccpA*^{k} phenotype are located at distant positions on the protein chain. To further assess their location we took advantage of the putative common three-dimensional fold of CcpA and the LacI-GalR family of bacterial regulators (13, 16). Sequence comparisons of CcpA proteins to other family members showed that they constitute a distinct subgroup among LacI-GalR regulators (17), but overall structural similarities should be sufficient to evaluate amino acid location. The model of CcpA shown in Fig. 4 is based on the three-dimensional structure of PurR (29) (Protein Data Bank entry 1PNR). The positions mutated in the CcpA^k proteins are indicated. The most striking common feature is their location in the protein core and not in the DNA binding heads. They highlight different regions of the protein with possible functional importance: the dimerization surface, the effector binding cleft, and the corepressor binding site.

Two of the mutations, those in CcpA^{k}I227V and CcpA^{k}M282V, affect residues whose analogs in LacI (Phe226 and Tyr282) and PurR (Tyr227 and Tyr282) play a role in the dimerization of the C-terminal subdomains of the protein core (6, 29). The structure and relative positions of the C-terminal subdomains do not change significantly upon switching between the DNA binding and nonbinding conformations. They form the support for a hinge movement of the two N-terminal subdomains which opens and closes the effector binding clefts (20, 28). It is therefore not obvious how a mutation in this region could lead to permanent repression by CcpA. The mutations introduce no drastic changes in the chemical properties of the affected residues in terms of size or hydrophobicity. These small changes might be the reason that dimerization is still possible, and their effector-independent DNA-binding implies a function of the C-terminal CcpA subdomain more pronounced than those recognized for LacI and PurR.

The E77L and D275G substitutions are close to the effector binding cleft (Fig. 4) which is involved in ligand recognition and binding in PurR and LacI and undergoes structural changes in switching between the DNA binding and nonbinding states (20, 28). The side chain of Glu77 faces the effector binding cleft. It is flanked by other residues making direct contact with the effectors such as the analogous residues to Asp275 of CcpA which is mutated in CcpA^{k}D275G. Asp is well conserved at this position among the members of the LacI-GalR family and a general role in ligand binding is assumed (17, 34). The occurrence of *ccpA*^{k} mutations in the effector binding cleft indicates that low-molecular-weight effectors such as glucose-6-phosphate and NADP (9, 15, 24) could bind in that region, but a direct interaction of these compounds with CcpA and their physiological function in this regulation remain to be proven.

Thr306, which is changed to Ile in CcpA^{k}T306I, is in a solvent, exposed position, neighboring the proposed binding surface for HPr-Ser-P (17). Amino acid exchanges in that surface result in a loss of CCR in vivo and no or reduced interaction with HPr-Ser-P (17). In contrast, CcpA^{k}T306I leads to permanent repression and binding of DNA. The amino acid at position 306 is conserved among CcpA-like proteins but not among other members of the LacI-GalR family (17). Only one protein of the CcpA subfamily, RegA from *Clostridium acetobutylicum*, carries an Ile at this position, as does CcpA^{k}T306I. Interestingly, RegA complements a *B. subtilis ccpA* mutant to constitutive repression of *amyE* (2). There is no explanation why a change of the hydrophilic Thr to the hydrophobic Ile at this surface position renders CcpA regulation glucose independent, but CcpA^{k}T306I gives further evidence for the functional importance of this region.

In summary, the five amino acid substitutions conferring permanent, glucose-independent regulation by CcpA indicate that mutations in three different regions of the protein can alter DNA binding. Mutations in the corepressor binding cleft

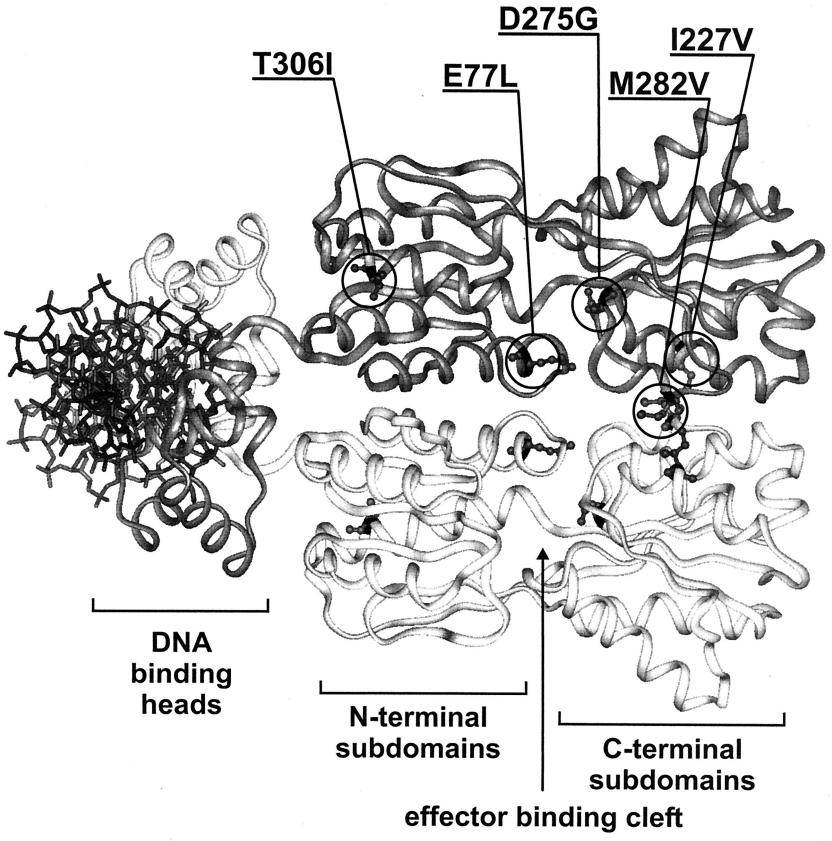

FIG. 4. Ribbon representation of a model of CcpA based on the PurR structure. One monomer is dark grey, and the other one is white; a stick model of the complexed DNA is shown in black, and the functional domains are named underneath. Circles in the darker monomer mark positions mutated in CcpA^k variants, and the respective amino acid substitutions are shown above the model. The side chains of the original amino acids at these positions are depicted as ball-and-stick models in both monomers.

are consistent with the assumption that CcpA may be triggered by low-molecular-weight effectors. The binding site for HPr-Ser-P has no equivalent in related repressors, but CcpA-specific conservation and the gain-of-function mutation characterized here emphasize its special role for CcpA-mediated regulation.

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