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Bacillus subtilis ccpA Gene Mutants Specifically Defective in Activation of Acetoin Biosynthesis

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A large number of carbon source utilization pathways are repressed in *Bacillus subtilis* by the global regulator CcpA, which also acts as an activator of carbon excretion pathways during growth in media containing glucose. In this study, CcpA mutants defective in transcriptional activation of the *alsSD* operon, which is involved in acetoin biosynthesis, were identified. These mutants retained normal glucose repression of *amyE*, encoding α-amylase, and *acsA*, encoding acetyl-coenzyme A synthetase, and normal activation of *ackA*, which is involved in acetate excretion; in these *ccpA* mutants the CcpA functions of activation of the acetate and acetoin excretion pathways appear to be separated.

The CcpA protein is a key central regulator of carbon metabolism in *Bacillus subtilis* and other gram-positive organisms (7). CcpA is a member of the LacI/GalR family of transcriptional regulatory proteins and binds to conserved *cre* sites in the promoter regions of its target genes (8, 9, 11, 24). The response to glucose is mediated at least in part by the HPr/Crh signaling pathway (1, 3, 10, 16). While the role of CcpA as a repressor of genes involved in utilization of secondary carbon sources is well established, CcpA is also required for activation of carbon excretion pathways, including those for production of acetate, acetoin, and glycogen, during growth in glucose (5, 15, 18; C. Moran, personal communication).

The *ackA*, *pta*, and *glg* genes all contain *cre* sites upstream of the promoter which are required for transcriptional activation. However, no *cre* site was found in the *alsSD* operon, which encodes acetolactate synthase and acetolactate decarboxylase, enzymes involved in the biosynthesis of acetoin (17, 18). The *alsR* gene, which encodes a LysR family transcriptional regulator, is transcribed divergently from *alsSD* and has been proposed to act as an activator of *alsSD* transcription. Acetate has been implicated as the effector controlling AlsR-dependent activation, since addition of exogenous acetate increased *alsSD* transcription during vegetative growth (17, 18). This suggested the possibility that the effect of CcpA on *alsSD* was indirect, perhaps mediated via acetate accumulation due to dependence of the *ackA*/*pta* pathway on CcpA.

Isolation of *ccpA* mutants defective in activation of *alsS* transcription. An insertion of Tn*917lac* into the *alsS* gene was isolated in a search for genes induced during growth in glucose (21). *ccpA* mutants defective in activation of *alsS* transcription were then identified. Multiple independent pools of ethyl methanesulfonate-mutagenized SPβ phage carrying the intact *ccpA* gene as well as a selectable *cat* gene were introduced into strain ZB449ccpTn*2* containing the *ccpA*::*spc* null allele and the *alsS*::Tn*917lac* insertion. Transductants (4,000) were patched onto tryptose blood agar base (TBAB) plates (Difco) containing X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside; 40 μg/ml) and 1% glucose, and 17 white colonies were identified. To eliminate mutations in *ccpA* that resulted in a loss of function, repression of α-amylase production during growth on glucose was tested by monitoring starch hydrolysis (8). Three independently derived isolates retained normal glucose repression of *amyE*. Two of the *ccpA* mutants, designated CM48RC and CM286PL, exhibited very low *alsS-lacZ* activity and wild-type colony morphology, while the third, designated CM18AV, exhibited no detectable *alsS-lacZ* activity on plates and increased colony size during growth on TBAB medium containing glucose.

A DNA fragment containing the entire *ccpA* gene from the SPβ prophage was amplified by PCR and sequenced. Mutant CM18AV contained a substitution of a valine for an alanine at amino acid 18 of CcpA. CM48RC contained a substitution of a cysteine for an arginine at amino acid 48, and CM286PL contained a substitution of a leucine for a proline at amino acid 286. The mutations at amino acid positions 18 and 48 are within regions which interact with DNA in the CcpA homolog PurR (20), while the mutation at position 286 is within a less conserved region of proteins in this family. Since the mutants all retained the ability to repress *amyE* expression during growth in glucose, DNA binding activity, at least at the *amyE cre* site, must have been retained.

Effect of *ccpA* mutations on *alsS-lacZ* expression. Expression of *alsS-lacZ* was quantitated during growth in TSS medium (2) with 1% Casamino Acids in the presence or absence of glucose (1%) (Table 1). A derivative of ZB449ccpTn*2* containing the wild-type *ccpA* locus on an SPβ prophage was constructed as a control. Expression was very low in all strains in the absence of glucose and was induced 45-fold during growth in glucose in the strain containing wild-type *ccpA*. No induction was observed in strain ZB449ccpTn*2* containing the null allele of *ccpA*, while a small increase in activity was detected in strains carrying *ccpA* genes with point mutations (*ccpA* point mutants). This is consistent with the phenotypes observed during the mutant screening, although under those conditions it appeared that CM18AV exhibited the most severe phenotype. The phenotype of the *ccpA* point mutants therefore resembles that of the *ccpA* null mutant in having nearly complete loss of *alsS* expression. Similar effects on *alsS-lacZ* expression were observed in other growth media, including NSM (19) and Luria-Bertani (LB) medium (13; data not shown).

Effect of *ccpA* mutations on *ackA-lacZ* expression. The SPβ::*ccpA* phage from the wild-type and mutant strains was transferred into strain BR151MAccp::spc, and an *ackA-lacZ*

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TABLE 1. Expression of *alsS-lacZ* fusion in *ccpA* mutants[a]

Strain	*ccpA* allele	β-Galactosidase activity		Ratio
		− Glucose	+ Glucose	
ZB449Tn*2*CWT	Wild type	0.77	35	45
CM18AV	*ccpA*18AV	0.63	1.0	1.6
CM48RC	*ccpA*48RC	0.63	1.6	2.5
CM286PL	*ccpA*286PL	0.65	0.94	1.5
ZB449ccpTn*2*	*ccpA*::*spc*	0.66	0.73	1.1

[a] All strains contained the Tn*2* *alsS*::Tn*917lac* insertion (21). Cells were grown in TSS medium (4) containing 1% Casamino Acids (− glucose) or TSS medium containing 1% Casamino Acids and 1% glucose (+ glucose). β-Galactosidase activities are expressed in Miller units (13) and indicate activity 2 h after the time of entry of the culture into stationary phase. The ratio indicates the level of induction during growth in the presence of glucose. All growth experiments were carried out at least twice, and samples were assayed in duplicate; variation was less than 10%.

TABLE 3. Expression of *acsA-lacZ* fusion in *ccpA* mutants[a]

Strain	*ccpA* allele	β-Galactosidase activity		Ratio
		− Glucose	+ Glucose	
BR151MACWT	Wild type	54	2.5	22
CPC18AV	*ccpA*18AV	46	10	4.6
CPC48RC	*ccpA*48RC	51	11	4.6
CPC286PL	*ccpA*286PL	45	9.0	5.0
BR151MAccp::spc	*ccpA*::*spc*	54	46	1.2

[a] All strains contained an *acsZ-lacZ* transcriptional fusion (4). Cells were grown in NSM medium (19) in the absence (− glucose) or presence (+ glucose) of glucose (1%). β-Galactosidase activities are expressed in Miller units (13) and indicate activity 1 h after the time of entry of the culture into stationary phase. The ratio indicates the level of repression during growth in the presence of glucose. All growth experiments were carried out at least twice, and samples were assayed in duplicate; variation was less than 10%.

transcriptional fusion (5) was introduced into the resulting strains. Cells were grown in TSS medium with 1% Casamino Acids with or without glucose (1%), and β-galactosidase activity was measured (Table 2). While the expression of *ackA* during growth in the absence of glucose was reduced somewhat in the strains containing *ccpA* with point mutations compared to that of the strain containing the wild-type allele of *ccpA*, expression during growth in glucose was unaffected. The *ccpA* null allele resulted in both reduction of basal expression and loss of glucose induction, as previously reported (5). It therefore appears that the *ccpA* point mutations separate the functions of CcpA in transcriptional activation of *ackA* and *alsS*. This result also makes it unlikely that CcpA mediates its effect on *alsS* expression only through its effect on *ackA*, since in these mutants *ackA* expression was unaffected during growth in glucose.

Effect of *ccpA* mutations on repression of *acsA-lacZ* expression. The ability of the CcpA variants to repress *acsA*, another known target of CcpA (4, 6), was examined, since perturbations in levels of acetyl-coenzyme A (CoA) synthetase, which converts acetate to acetyl-CoA, might affect *alsS* expression. As shown in Table 3, each of the mutant alleles resulted in significant repression of *acsA-lacZ* expression during growth in glucose, while the *ccpA* null mutant exhibited total loss of repression, as previously reported (4). The efficiency of repression of *acsA-lacZ* was somewhat reduced in the *ccpA* point mutants relative to that in strains carrying the wild-type allele of *ccpA*, suggesting that although the mutant variants of CcpA are able to bind DNA, their affinity for the *acsA cre* site may be lower than that of wild-type CcpA. It is also possible that the reduced repression is due to an effect on other factors involved in *acsA* regulation.

Acetate production in *ccpA* mutant strains. Acetate has been implicated as the effector for induction of *alsS* transcription by its activator AlsR (17, 18). Although *ackA* expression was unimpaired in the *ccpA* point mutants during growth in glucose, it remained possible that acetate production was reduced because of other effects on carbon metabolism. In addition, the partial derepression of *acsA* could result in a reduction of acetate accumulation. Acetate concentrations in the culture supernatants were therefore directly measured (Table 4). The *ccpA*::*spc* null allele resulted in a twofold drop in acetate levels during growth in glucose, compared to the acetate level for the wild-type strain; the residual acetate production is presumably due to the basal level of *ackA* transcription in the absence of CcpA-dependent activation (22). The *ccpA* point mutants CPC18AV and CPC286PL exhibited a small decrease in acetate accumulation, while CPC48RC produced wild-type levels of acetate. It therefore appears that a reduction in acetate accumulation is unlikely to be responsible for the effect of these mutants on *alsS* expression.

Effect of addition of acetate on *alsS-lacZ* expression. Addition of acetate has been shown to activate *alsS* transcription during exponential growth in LB medium (17). Since the accumulation of acetate was partially reduced in the *ccpA* mutants, the effect of addition of exogenous acetate was tested. Cultures were grown in LB medium containing 1% glucose in the presence or absence of potassium acetate (50 mM). The addition of acetate at either pH 7.0 (Fig. 1) or pH 6.0 (data not shown) activated transcription of *alsS-lacZ* during the exponential-growth phase in the control strain containing the wild-type *ccpA*. Acetate addition conferred a small increase in *alsS-lacZ* expression in the *ccpA* point mutants, but had no effect on

TABLE 2. Expression of *ackA-lacZ* fusion in *ccpA* mutants[a]

Strain	*ccpA* allele	β-Galactosidase activity		Ratio
		− Glucose	+ Glucose	
BR151MACWT	Wild type	44	160	3.6
CPC18AV	*ccpA*18AV	28	150	5.4
CPC48RC	*ccpA*48RC	32	170	5.3
CPC286PL	*ccpA*286PL	27	140	5.2
BR151MAccp::spc	*ccpA*::*spc*	24	27	1.1

[a] All strains contained an *ackA-lacZ* transcriptional fusion (5). Cells were grown in TSS medium containing 1% Casamino Acids (− glucose) or TSS medium containing 1% Casamino Acids and 1% glucose (+ glucose). β-Galactosidase activities are expressed in Miller units (13) and indicate activity 30 min prior to the time of entry of the culture into stationary phase. The ratio indicates the level of induction during growth in the presence of glucose. All growth experiments were carried out at least twice, and samples were assayed in duplicate; variation was less than 10%.

TABLE 4. Acetate concentrations in cultures of *ccpA* mutants

Strain	*ccpA* allele	Acetate concn (mM)[a]	
		− Glucose	+ Glucose
BR151MACWT	Wild type	0.50	13
CPC18AV	*ccpA*18AV	0.65	9.9
CPC48RC	*ccpA*48RC	0.57	12
CPC286PL	*ccpA*286PL	0.65	11
BR151MAccp::spc	*ccpA*::*spc*	0.75	7.3

[a] Cells were grown in TSS medium containing 1% Casamino Acids (− glucose) or containing 1% Casamino Acids and 1% glucose (+ glucose). Samples were taken 30 min prior to the time of entry of the culture into stationary phase.

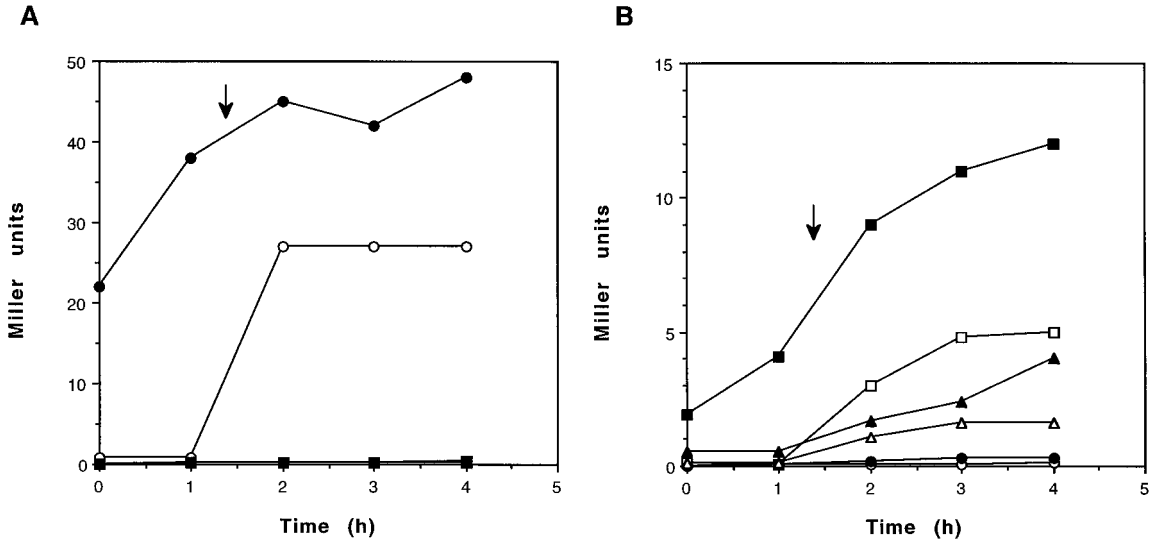

FIG. 1. Effect of addition of acetate on *alsS-lacZ* expression in the *ccpA* mutants. All strains contained the Tn*2 alsS*::Tn*917lac* insertion (21). Cultures were grown in LB medium (13) containing 1% glucose in the presence (filled symbols) or absence (open symbols) of potassium acetate (50 mM) (pH 7.0). Arrow, time of entry of cultures into stationary phase. β-Galactosidase activities are expressed in Miller units (13). (A) ZB449Tn2CWT (*ccpA* wild-type; circles) and ZB449ccpTn*2* (*ccpA*::*spc*; squares). (B) CM18AV (circles), CM48RC (squares), and CM286PL (triangles). Cultures were grown concurrently and are presented in two panels for clarity.

the *ccpA*::*spc* null mutant. These results indicate that addition of acetate is not sufficient to restore normal *alsS* expression in the *ccpA* mutants, although it clearly influences *alsS* transcription. These results support the hypothesis that CcpA plays a role in *alsS* regulation in addition to its role in activating *ackA* expression.

Effects on the pH of the culture supernatant. Growth of *B. subtilis* in the presence of glucose results in a reduction in the pH of the culture medium. The pH generally reaches its lowest point at the end of the exponential phase, which correlates with the time of activation of *alsSD* transcription. Careful buffering of the medium to pH 7.0 eliminated acetoin production (data not shown), suggesting that a reduction in pH may be required for *alsS* transcriptional activation, possibly because of effects on acetate transport. The pH of the culture medium was therefore measured during growth in NSM with or without glucose (1%) (Fig. 2). All *ccpA* mutants exhibited a drop in pH levels during growth in glucose. The *ccpA* null mutant displayed a defect in both the timing and magnitude of the pH decrease, while the CM18AV mutant exhibited a phenotype intermediate between that of the null mutant and the wild-type strain; the other point mutants exhibited patterns similar to that of the wild-type strain, although the pH increased more rapidly in stationary phase. The modest effects of the *ccpA* point mutants on the pH profiles make it unlikely that this plays a major role in the major defect in *alsS* transcription.

Effect of *ccpA* mutations on growth. A null mutation in *ccpA* results in a defect in growth in minimal media with glucose as the sole carbon source (7, 25); this is due at least in part to derepression of nitrogen metabolism genes, in particular *rocG*, encoding glutamate dehydrogenase (B. R. Belitsky and A. L. Sonenshein, personal communication). The effect of the *ccpA* point mutations on growth was therefore examined. While the null mutant exhibited no growth on agar plates under the conditions tested, the point mutants all exhibited some growth on glucose minimal medium; however, the growth was clearly defective in comparison to that of the wild-type strain (data not shown). The point mutations therefore are likely to confer a partial defect in regulation of nitrogen metabolism. Growth rates in TSS medium with 1% Casamino Acids with or without glucose were also determined. The *ccpA*::*spc* null allele resulted in a significant decrease in growth rate in the presence of glucose, relative to the growth rate of the wild-type strain, presumably reflecting the major role of CcpA in the control of carbon metabolism; in contrast, the point mutants exhibited growth rates indistinguishable from that of the wild-type parent strain (data not shown).

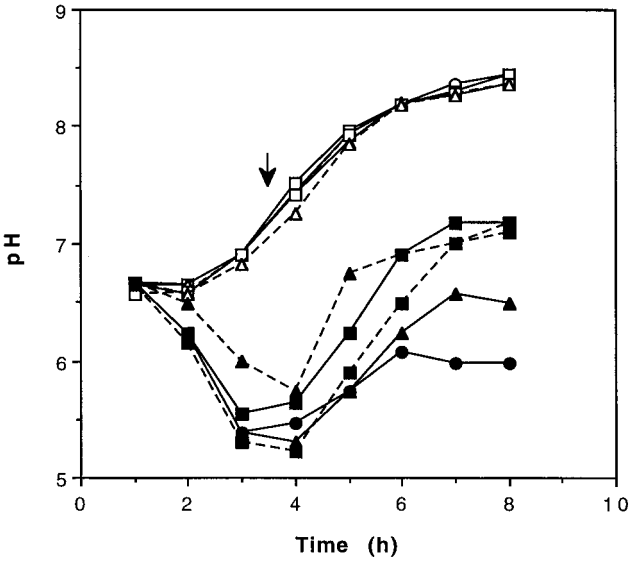

FIG. 2. pH profiles of cultures of *ccpA* mutants. Cultures were grown in NSM broth (19) in the presence (filled symbols) or absence (open symbols) of glucose (1%). Arrow, time of entry of the cultures into stationary phase. BR151MACWT (*ccpA* wild type), circles; CPC18AV, squares with solid lines; CPC48RC, triangles with solid lines; CPC286PL, squares with dashed lines; BR151MAccp::spc (*ccpA* null), triangles with dashed lines.

Summary. These studies indicate that certain *ccpA* point mutations can separate CcpA's function of activating the acetoin biosynthesis pathway from its other functions in the cell. Since neither *ackA* transcription nor acetate accumulation was affected during growth in glucose and since the defect in *alsSD* transcription could not be bypassed by addition of exogenous acetate, the effect of CcpA on *alsSD* transcription must involve factors other than acetate as an effector. The mechanism of *alsSD* transcriptional activation by CcpA remains to be determined.

Two of the mutations in *ccpA* identified in this study mapped to regions highly conserved in the LacI/GalR family of transcriptional regulators (14, 23). The *ccpA*18AV allele confers an amino acid substitution within the recognition helix of the helix-turn-helix motif, which interacts with the operator site in the major groove of DNA (20). The *ccpA*48RC allele resulted in the substitution of a cysteine for an arginine approximately 30 amino acids downstream of the recognition helix. The altered amino acid is not highly conserved but is flanked by two highly conserved residues. This substitution maps to a site which in LacI and PurR forms a tight bend and is believed to interact with the operator site in the minor groove (20). Mutation of arginine 48 to serine in *Bacillus megaterium* CcpA resulted in loss of catabolite repression of the *xyl* operon and loss of DNA binding at the *xyl cre* site (12). The 18AV and 48RC substitutions in *B. subtilis* CcpA appeared to have little effect on operator site recognition, demonstrated by repression of *amyE* and *acsA*, and activation of *ackA*. It is possible that these amino acid substitutions alter operator site recognition but that interactions with all *cre* sites are not equivalent. These alterations may affect the interaction with an unidentified sequence at the *alsSD* or *alsR* promoter regions or at an unknown target gene, the product of which in turn affects *alsSD* transcription, without altering recognition of the *cre* sites at *ackA*, *amyE*, and *acsA*. Further analysis of *alsSD* regulation will be required to clarify this issue.

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