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Complex Regulation of the *Bacillus subtilis* Aconitase Gene

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The roles of the CcpC, CodY, and AbrB proteins in regulation of the *Bacillus subtilis* aconitase (*citB*) gene were found to be distinct and to vary with the conditions and phase of growth. CcpC, a citrate-inhibited repressor that is the primary factor regulating *citB* expression in minimal-glucose-glutamine medium, also contributed to repression of *citB* during exponential-phase growth in broth medium. A null mutation in *codY* had no effect on *citB* expression during growth in minimal medium even when combined with *ccpC* and *abrB* mutations. However, a *codY* mutation slightly relieved repression during exponential growth in broth medium and completely derepressed *citB* expression when combined with a *ccpC* mutation. An *abrB* mutation led to decreased expression of *citB* during stationary phase in both broth and minimal medium. All three proteins bound in vitro to specific and partially overlapping sites within the *citB* regulatory region. Interaction of CcpC and CodY with the *citB* promoter region was partially competitive.

In *Bacillus subtilis*, genes that are turned on as cells leave the rapid exponential growth phase are often subject to multiple forms of regulation, and several kinds of signals are known to influence the expression of such genes. Growing cells excrete signaling compounds whose accumulation in the environment can be detected by other cells when the compounds reach a threshold concentration, an event that usually occurs late in exponential growth phase. In *B. subtilis*, certain oligopeptides have such activities (25). These so-called quorum-sensing peptides are typically recognized by membrane-bound sensors or by the oligopeptide transport system; their internalization may or may not be required for their activity (25). For instance, the PhrA pentapeptide enters the cell, binds to and inhibits the RapA phosphatase, and thereby unleashes a signal transduction pathway that leads to activation by phosphorylation of the transcription factor Spo0A (33, 34). The Spo0A activation pathway, also known as the phosphorelay, initiates when one of several histidine kinases phosphorylates itself and then transfers the phosphate to an aspartate residue on Spo0F (4). Spo0B then transfers the phosphate from Spo0F to an aspartate on Spo0A (4).

RapA is a Spo0F~P phosphatase; its activity therefore interrupts the phosphorelay, and PhrA relieves the blockage (33, 34). Spo0A~P acts as an essential positive regulator of sporulation genes (20). The genes that are turned on by Spo0A~P allow the cell to form the polar division septum characteristic of sporulating cells (26) and to activate a cascade of RNA polymerase sigma factors for transcription of all subsequently expressed sporulation genes (44).

A second kind of signaling depends on nutrient availability (43). It is unclear which specific nutrients in a complex mixture, such as nutrient broth, are sensed by cells, but exhaustion of these nutrients presumably leads to changes in intracellular pools of certain critical metabolites. Conditions that cause a drop in the GTP pool, for instance, are associated with the induction of stationary-phase genes and sporulation (12, 27, 28, 30). A major contributor to such regulation is CodY, a GTP-dependent pleiotropic repressor of stationary-phase genes (35, 39, 40, 42). When the GTP pool drops, CodY becomes inactivated, and its target genes can be expressed (35). Nutrient exhaustion, like peptide signaling, activates sporulation through the Spo0A phosphorelay (21).

One of the activities of Spo0A~P is to repress transcription of *abrB* (48), a gene whose product is a pleiotropic repressor of early-stationary-phase genes (46, 47). Since AbrB is rapidly turned over (15), inhibition of its synthesis leads to a large decrease in its intracellular concentration and induction of some stationary-phase genes.

The *B. subtilis* genes encoding the enzymes of the tricarboxylic acid branch of the Krebs citric acid cycle (citrate synthase, aconitase, and isocitrate dehydrogenase) are subject to at least two forms of regulation. During growth in minimal medium, these genes are repressed synergistically when the medium contains a rapidly utilizable carbon source, such as glucose, and a ready source of 2-ketoglutarate, such as glutamine or glutamate (37). When cells are grown in a poorer medium, e.g., with succinate or citrate as the sole carbon source, or when ammonium serves as the sole nitrogen source, the tricarboxylic acid branch genes are derepressed (37). This response fits well with the metabolic roles of the enzymes in ATP generation, production of reducing equivalents, and synthesis of critical biosynthetic intermediates derived from 2-ketoglutarate.

The *B. subtilis* tricarboxylic acid branch genes are also re-

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TABLE 1. *B. subtilis* strains used

Strain	Genotype or relevant characteristics	Source or reference
AF21	Δ*amyE*::Φ(*citBp21-lacZ cat*)	10
AF23	Δ*amyE*::Φ(*citBp23-lacZ cat*)	10
AF24	Δ*amyE*::Φ(*citBp24-lacZ cat*)	10
CJB9	*ccpC*::*spc* Δ*amyE*::Φ(*citBp21-lacZ cat*)	23
DZR143	*trpC2 pheA1* Δ*spo0A*::*erm*	D. Rudner
MAB160	*trpC2 pheA1 citB*::*spc*	5
PS56	*trpC2* Δ*amyE*::Φ(*dpp-lacZ neo*) *abrB*::(*cat*::*tet*)	P. Serror
PS258	*trpC2 codY*::*erm*	P. Serror
SJB67	*pheA1* Δ*citA*::*neo* Δ*citZ471*	22
HKB76	Δ*amyE*::Φ(*citBp21-lacZ cat*) Δ*citA*::*neo* Δ*citZ471*	AF21 × SJB67 DNA
HKB94	Δ*amyE*::Φ(*citBp21-lacZ cat*) *abrB*::(*cat*::*tet*)	AF21 × PS56 DNA
HKB95	Δ*amyE*::Φ(*citBp21-lacZ cat*) *codY*::*erm*	AF21 × PS258 DNA
HKB96	Δ*amyE*::Φ(*citBp21-lacZ cat*) *abrB*::*cat*::*tet codY*::*erm*	HKB94 × PS258 DNA
HKB97	Δ*amyE*::Φ(*citBp21-lacZ cat*) *ccpC*::*spc abrB*::(*cat*::*tet*)	CJB9 × PS56 DNA
HKB98	Δ*amyE*::Φ(*citBp21-lacZ cat*) *ccpC*::*spc codY*::*erm*	CJB9 × PS258 DNA
HKB99	Δ*amyE*::Φ(*citBp21-lacZ cat*) *ccpC*::*spc abrB*::(*cat*::*tet*) *codY*::*erm*	HKB97 × PS258 DNA
HKB125	Δ*amyE*::Φ(*citBp23-lacZ cat*) *codY*::*erm*	AF23 × PS258 DNA
HKB126	Δ*amyE*::Φ(*citBp24-lacZ cat*) *codY*::*erm*	AF24 × PS258 DNA
HKB165	Δ*amyE*::Φ(*citBp21-lacZ cat*) *citB*::*spc*	AF21 × MAB160 DNA
HKB166	Δ*amyE*::Φ(*citBp21-lacZ cat*) Δ*citA*::*neo* Δ*citZ471 citB*::*spc*	HKB76 × MAB160 DNA
HKB168	Δ*amyE*::Φ(*citBp21-lacZ cat*) *spo0A*::*erm*	AF21 × DZR143 DNA
HKB169	Δ*amyE*::Φ(*citBp21-lacZ cat*) *spo0A*::*erm citB*::*spc*	HKB165 × DZR143 DNA
HKB170	Δ*amyE*::Φ(*citBp21-lacZ cat*) *spo0A*::*erm citB*::*spc* Δ*citA*::*neo* Δ*citZ471*	HKB166 × DZR143 DNA

pressed during rapid exponential growth in broth medium but become induced as cells exhaust certain nutrients (7, 10, 37). All three enzymes of the tricarboxylic acid branch are required for efficient sporulation (5, 9, 13, 14, 22, 38, 53). The metabolic activities of the tricarboxylic acid branch enzymes appear to be as critical for sporulation as for growth in minimal medium, but aconitase may have an additional role in sporulation that is independent of its enzymatic activity (1).

For the *B. subtilis* aconitase (*citB*) gene, only one mechanism of regulation has been uncovered to date. Repression of *citB* during growth in minimal medium containing glucose and glutamine is primarily due to the activity of CcpC, a LysR family transcription factor (23). CcpC binds to the *citB* promoter region in two locations. The primary binding site is a dyad symmetry element centered at position −66 with respect to the transcription start site (10, 11, 23). A secondary binding site, whose utilization is dependent on the −66 dyad, is located near position −30 (23; S.-I. Kim, C. Jourlin-Castelli, S. R. Wellington, and A. L. Sonenshein, submitted for publication). When CcpC is bound to both sites, the DNA between them bends, occluding access by RNA polymerase (23; Kim et al., submitted). Mutations in the −66 and −30 regions reduce binding of CcpC and derepress *citB* transcription (10, 11; Kim et al., submitted). In the presence of the inducer, citrate, binding to the −30 region is inhibited, bending is partially relieved, and RNA polymerase enters the promoter and initiates transcription (23; Kim et al., submitted). Glucose affects *citB* expression by indirectly activating CcpA, a repressor of the citrate synthase (*citZ*) gene (24), and by inhibiting citrate uptake (49, 50, 52). Glutamine serves as a source of 2-ketoglutarate, which in turn inhibits the activity of the small amount of citrate synthase enzyme that is made in the presence of glucose (H.-J. Kim, unpublished results). Thus, when cells are growing in glucose-glutamine medium, there is insufficient accumulation of citrate to inactivate CcpC, and *citB* is repressed.

In cells growing in broth medium, however, neither interruption of the *ccpC* gene nor mutation of the −66 dyad element derepresses *citB* expression (10). Thus, there must be at least one additional *citB* regulatory protein that is active in broth-grown cells. Since *citB* expression is induced in late-exponential-phase cells in broth culture (7, 10, 37) and by treatment of rapidly growing cells with decoyinine, an inhibitor of guanine nucleotide synthesis (7, 30), we suspected that either AbrB or CodY might be the second regulator. We show here that CodY and AbrB as well as CcpC bind to the *citB* promoter region and that mutations that inactivate CcpC and CodY have small derepressing effects individually on *citB* expression in broth-grown cells but give nearly total derepression when combined. While AbrB was also capable of binding to the *citB* promoter region in vitro, an *abrB* mutation did not contribute to derepression either alone or in combination with *codY* and *ccpC* mutations. In fact, disruption of *abrB* led to a small decrease in *citB* expression.

MATERIALS AND METHODS

Growth of bacteria and assay of β-galactosidase. Bacterial strains used are listed in Table 1. *B. subtilis* strains were grown in TSS minimal medium (10) or in a nutrient broth-based medium (DS), in which cells grow and then sporulate when they reach stationary phase (10). For measurements of β-galactosidase activity in *lacZ* fusion strains, 1-ml culture samples were harvested and assayed as previously described (24). The data shown in Fig. 1 to 4 are representative of two to three trials with independently prepared samples.

Protein overexpression and purification. CcpC (23, 24) and AbrB (47) were purified as described previously.

A C-terminal, 6× histidine-tagged version of CodY (CodY-His_6) was expressed in *Escherichia coli* from plasmid pKT1 under the control of the *araBAD* promoter. To construct pKT1, the *codY* gene was amplified from the *B. subtilis* chromosome by PCR with a forward primer that incorporated a *Sac*I site, the *codY* ribosome-binding site, and the *codY* start codon and a reverse primer that included the last six codons of *codY* (including a C-terminal histidine), an additional five histidine codons, a stop codon, and an *Sph*I site. After digestion with *Sac*I and *Sph*I, the PCR product was ligated to *Sac*I- and *Sph*I-treated pBAD30,

a vector useful for placing genes under the control of the *araBAD* promoter (19). An ampicillin-resistant transformant of *E. coli* strain KS272 (45) had the appropriate insert, as verified by DNA sequencing of the *codY* open reading frame.

E. coli KS272(pKT1) was grown at 37°C in 2 liters of L broth supplemented with 50 μg of ampicillin per ml. When the culture reached an optical density at 600 nm (OD_{600}) of approximately 0.6, expression of CodY-His_6 was induced by the addition of arabinose to a final concentration of 0.2%. After incubation for 6 to 8 h, cells were harvested by centrifugation at 3,800 × *g* at 4°C for 10 min. The cell pellets were washed once with 15 ml of solution A (50 mM Tris-HCl [pH 8.0], 2 mM disodium EDTA, 1 mM dithiothreitol) supplemented with 1 mM phenylmethylsulfonyl fluoride and resuspended in 15 ml of sonication buffer (200 mM KCl, 50 mM Tris-Cl [pH 7.5], 10% glycerol, 0.1% Igepal [Nonidet P-40], 1 mM phenylmethylsulfonyl fluoride, 2 μM pepstatin A, 0.5 mM dithiothreitol, 0.2 mM disodium EDTA). The cells were lysed by freezing and thawing and then subjected to sonication with a Branson Sonifier cell disrupter, model 200, for five cycles of 30 s each with 15-s rests. Cell debris was removed by centrifugation at 20,000 × *g* for 20 min at 4°C. The supernatant fluids were collected, and DNA and ribosomes were precipitated by the addition of streptomycin sulfate (0.01 g/ml) with stirring for 2 h at 4°C. The suspension was centrifuged at 20,000 × *g* at 4°C for 15 min, and the supernatant fluids were dialyzed against 1 liter of sonication buffer without dithiothreitol or disodium EDTA for 2 h at 4°C, with buffer replacement after 1 h.

The dialyzed lysates were mixed with 2 ml of Talon (Clontech) metal (Co^+) affinity resin that had been equilibrated with buffer I (20 mM Tris-HCl [pH 8.0], 5 mM β-mercaptoethanol, 10% glycerol, 1 mM phenylmethylsulfonyl fluoride, 2 μM pepstatin A, and 0.1% Igepal) supplemented with 150 mM KCl and incubated for 20 min at 4°C with tumbling. The resin and bound His-tagged protein were collected by centrifugation at 1,000 × *g* for 10 min at 4°C and washed sequentially at 4°C with 15 ml each of buffer I containing 500 mM KCl and 5 mM imidazole and buffer I containing 125 mM KCl and 5 mM imidazole. The resin slurry was then added to a 5-ml disposable column (Clontech) and allowed to settle to form a 2-ml column. CodY-His_6 was eluted with 5 ml of buffer I containing 125 mM KCl and 50 mM imidazole but lacking phenylmethylsulfonyl fluoride and pepstatin A. Fractions (0.5 ml) were collected and mixed with 5 μl of 100 mM dithiothreitol–100 mM EDTA. All fractions were subjected to sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). The fractions containing CodY-His_6 (estimated purity, >95%) were concentrated by ultrafiltration with an Ultrafree- 10,000 NMWL membrane (Millipore) and stored at −20°C (CodY-His_6 is stable for more than 2 months at −20°C, as judged by SDS-PAGE and the ability to bind to the *dpp* promoter).

DNase I footprinting assays. Three different radioactive DNAs were used for footprinting assays. For the experiment shown in Fig. 5, a PCR product corresponding to positions −320 to +202 relative to the *citB* transcription start site was synthesized with a ^{32}P-labeled oligonucleotide that primed synthesis of the template (noncoding) strand, an unlabeled oligonucleotide for the complementary strand, and chromosomal DNA as the template. Primer labeling was achieved with T4 polynucleotide kinase (Gibco BRL, Life Technologies) and 150 μCi of [γ-^{32}P]ATP (6,000 Ci/mmol; New England Nuclear). For Fig. 6, plasmid pKSI64 (Kim et al., submitted), which contains the *citB* regulatory region from positions −166 to +123 cloned in pSK− (Stratagene), was the template. The PCR also contained the ^{32}P-labeled T3 primer and the unlabeled T7 primer. As a result, the nontemplate (coding) strand was labeled. The PCR product was purified from a 1% agarose gel with the QIAquick gel extraction kit (Qiagen). For Fig. 7, the target DNA was an *Eco*RI-*Hin*dIII fragment from pAF13 (10) containing the *citB* promoter region from positions −84 to +36. The DNA fragment was labeled at the 3′ end at either the *Eco*RI or *Hin*dIII site with [α-^{32}P]dATP and the Klenow enzyme.

Labeled DNA probes (10,000 cpm per reaction), obtained as described above, were incubated with various amounts of CcpC or CodY proteins for 15 min at room temperature in 20-μl reactions in a buffer containing 20 mM Tris-Cl (pH 8.0), 50 mM KCl, 2 mM $MgCl_2$, 1 mM EDTA, 1 mM dithiothreitol, 0.05% (vol/vol) Igepal, calf thymus DNA (25 μg/ml), and 5% glycerol. In some reactions, GTP was added to 2 mM. After adjusting the $MgCl_2$ and $CaCl_2$ concentrations to 6 mM, the DNA-protein complexes were treated with 0.2 U of RQ1 DNase I (Promega) for 60 s at room temperature, the reaction was stopped by addition of EDTA to 20 mM, and the mix was diluted to 100 μl with distilled H_2O. The samples were extracted with an equal volume of phenol-chloroform-isoamyl alcohol and then with chloroform, precipitated with 3 volumes of ethanol, and resuspended in loading buffer [95% (vol/vol) formamide, 20 mM EDTA, 0.05% (wt/vol) bromophenol blue, 0.05% (wt/vol) xylene cyanol FF]. After heating for 5 min at 80°C, the samples were loaded onto an 8 M urea–5% polyacrylamide gel. Sanger sequencing reactions were carried out with unlabeled T3 primer, pKSI64, the Sequenase kit (United States Biochemical Corp.), and [α-^{35}S]dATP, and loaded on the same gel. After electrophoresis, the gel was dried and analyzed by autoradiography.

DNase I footprinting of AbrB binding to the *citB* promoter region was assayed as described previously (47) except that the binding and digestion reactions were carried out at room temperature. Maxam-Gilbert sequencing reactions were performed by standard procedures (29).

TABLE 2. β-Galactosidase activity of the *citBp21-lacZ* fusion in minimal medium[a]

Strain	Relevant genotype	β-Galactosidase activity (Miller units)
AF21	wild type	0.8
CJB9	*ccpC*	31
HKB94	*abrB*	0.5
HKB95	*codY*	1.4
HKB96	*abrB codY*	0.4
HKB97	*ccpC abrB*	19
HKB98	*ccpC codY*	29
HKB99	*ccpC abrB codY*	17

[a] Strains were grown in TSS minimal medium containing 0.5% glucose and 0.2% glutamine as the carbon and nitrogen sources. Each value is the average of at least two determinations that varied by less than 20%.

RESULTS

Effects of *ccpC, codY,* and *abrB* mutations on *citB* expression in defined medium. Confirming prior results (10, 24), expression of a *citB-lacZ* fusion was very low when wild-type cells were in steady-state exponential growth in a defined medium (TSS) containing glucose and glutamine as the sole carbon and nitrogen sources (Table 2). Introduction of a *ccpC* mutation greatly derepressed expression (Table 2). A *codY* mutation had no effect, either alone or in combination with *ccpC* (Table 2). Inactivation of *abrB* reduced *citB-lacZ* expression about twofold in an otherwise wild-type strain or in combination with a *codY* or *ccpC* mutation (Table 2). Thus, when cells are growing in a defined minimal medium, CcpC is the principal regulator of *citB*, AbrB plays a minor, positive role, and CodY makes no apparent contribution to *citB* expression.

Effects of *codY, ccpC,* and *abrB* mutations on *citB* expression in broth medium. When wild-type cells carrying a *citB-lacZ* fusion were grown in DS, a nutrient broth-based medium that supports growth and sporulation, β-galactosidase activity was low during the early exponential growth phase but increased substantially as cells approached the stationary phase (10) (Fig. 1A). This induction was transient, however; expression decreased rapidly after the cells entered stationary phase (Fig. 1A). Introduction of a *ccpC* mutation into these cells caused only limited derepression during the exponential growth phase (Fig. 1A). A *codY* mutation also caused a small increase in *citB-lacZ* expression during the exponential growth phase but decreased the induced level of expression in the stationary phase. When the *ccpC* and *codY* mutations were combined, they proved to have synergistic effects during growth. The double mutant strain showed highly derepressed expression of *citB-lacZ* during exponential phase (Fig. 1A). Moreover, the inhibitory effect of a *codY* single mutation on *citB-lacZ* expression in stationary-phase cells was suppressed in the *codY ccpC* double mutant. Thus, either CcpC or CodY alone is able to provide substantial repression of *citB* during the exponential growth phase. Both proteins have to be inactivated in order to

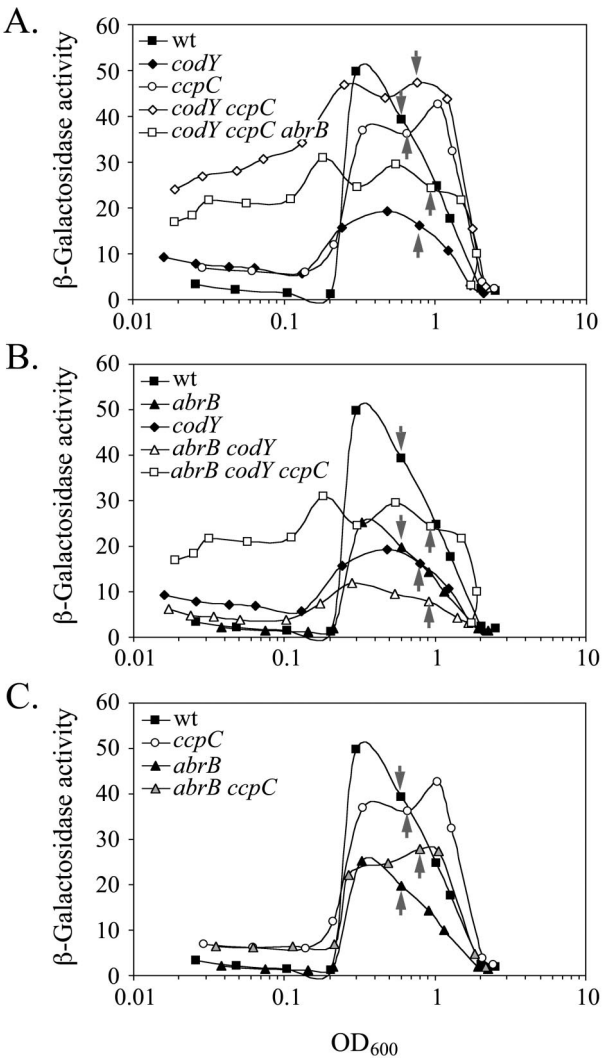

FIG. 1. Growth phase-dependent expression of a *citB-lacZ* fusion in wild-type and *codY*, *ccpC*, and *abrB* mutant strains. β-Galactosidase activity was measured in wild-type and mutant strains sampled at various times during growth in DS medium. Arrows indicate the end of the exponential growth phase. (A) Strains: AF21 (wild type [wt]), HKB95 (*codY*), CJB9 (*ccpC*), HKB98 (*codY ccpC*), and HKB99 (*codY ccpC abrB*). (B) Strains: AF21 (wild-type), HKB94 (*abrB*), HKB95 (*codY*), HKB96 (*abrB codY*), and HKB99 (*codY ccpC abrB*). (C) Strains: AF21 (wild-type), CJB9 (*ccpC*), HKB94 (*abrB*), and HKB97 (*ccpC abrB*).

achieve high-level expression during the exponential growth phase.

Cells lacking AbrB had a lower than normal level of induction during stationary phase (Fig. 1B). Addition of an *abrB* mutation to cells carrying a *ccpC* or *codY* mutation or both also caused a decrease in expression (Fig. 1B and C). An *abrB codY* double mutant had a particularly low level of *citB-lacZ* expression in stationary-phase cells (Fig. 1B and C).

Addition of a high concentration of glucose to cells in DS medium caused a delay in *citB-lacZ* expression. That is, induction occurred at an OD_{600} of 1.0 rather than at an OD_{600} of 0.2 (Fig. 2). Repression by glucose was partly relieved in a *ccpC* mutant strain (Fig. 2), but a *codY* mutation had no detectable

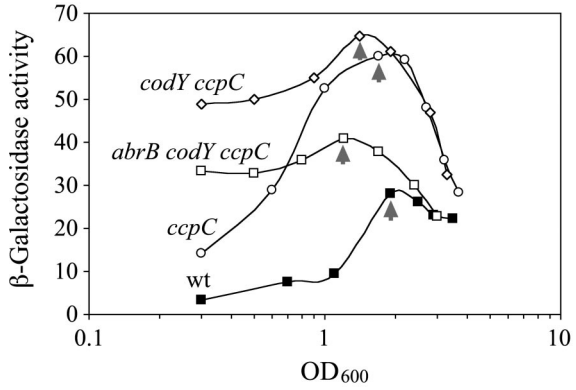

FIG. 2. Effect of glucose on *citB* expression. Strains AF21 (wild type [wt]), CJB9 (*ccpC*), HKB98 (*codY ccpC*), and HKB99 (*abrB codY ccpC*) were grown in DS medium supplemented with 2% glucose, and β-galactosidase activity was measured in samples harvested at various time points during growth. Arrows indicate the end of the exponential growth phase.

effect (data not shown). A *codY ccpC* double mutant was highly derepressed in DS-glucose medium (Fig. 2). Thus, CodY contributes to repression of *citB* in DS-glucose medium, but the effect of a *codY* deletion is masked by very strong repression by CcpC.

Effect of dyad symmetry element mutation. Deletion of the left arm of the −66 dyad symmetry element, known to be essential for CcpC binding and activity, and upstream DNA had only a slight derepressing effect on *citB-lacZ* expression in cells growing in DS medium (10) (Fig. 3), reinforcing the view that a regulator other than CcpC is active in cells growing in DS medium. When combined with a *codY* mutation, the dyad deletion led to highly derepressed expression during the early exponential growth phase, suggesting again that CodY is the

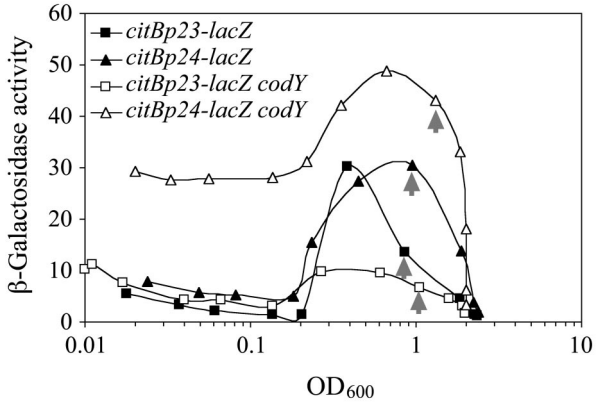

FIG. 3. Effect on *citB* expression of deletion of the CcpC binding site in the *citB* promoter region. Strains AF23 (*citBp23-lacZ*), AF24 (*citBp24-lacZ*), HKB125 (*citBp23-lacZ codY*), and HKB126 (*citBp24-lacZ codY*) were grown in DS medium, and β-galactosidase activity was measured in samples harvested at various time points during growth. Arrows indicate the end of the exponential growth phase. The *citBp23-lacZ* fusion contains the *citB* promoter region from positions −84 to +36 with respect to the transcriptional start site. The *citBp24-lacZ* fusion contains the *citB* promoter region from positions −67 to +36 and is thus missing the left arm of the dyad symmetry element (9).

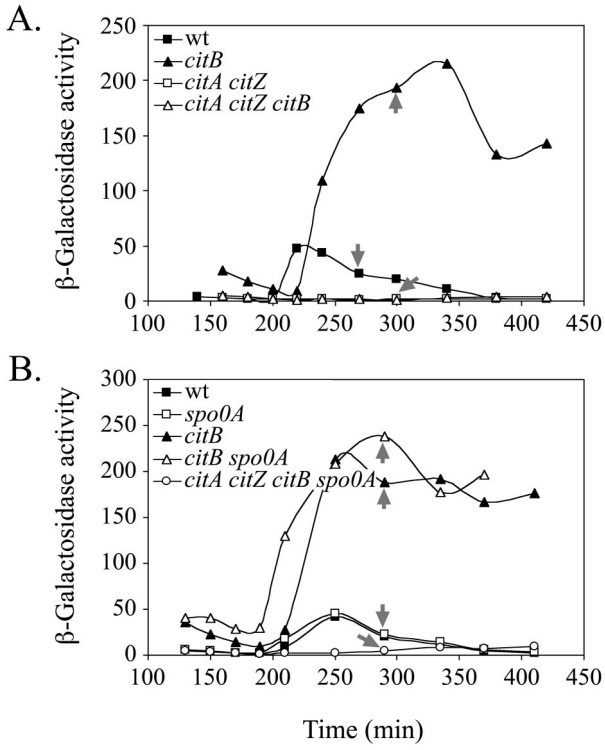

FIG. 4. Effects on *citB* expression of citrate synthase, aconitase, and *spo0A* mutations. Cultures were grown in DS medium, and β-galactosidase activity was measured in samples harvested at various time points during growth. Arrows indicate the end of the exponential growth phase. (A) Strains: AF21 (wild type [wt]), HKB76 (*citA citZ*), HKB165 (*citB*), and HKB166 (*citA citZ citB*). (B) Strains: AF21 (wild type), HKB168 (*spo0A*), HKB165 (*citB*), HKB169 (*citB spo0A*), and HKB170 (*citA citZ citB spo0A*).

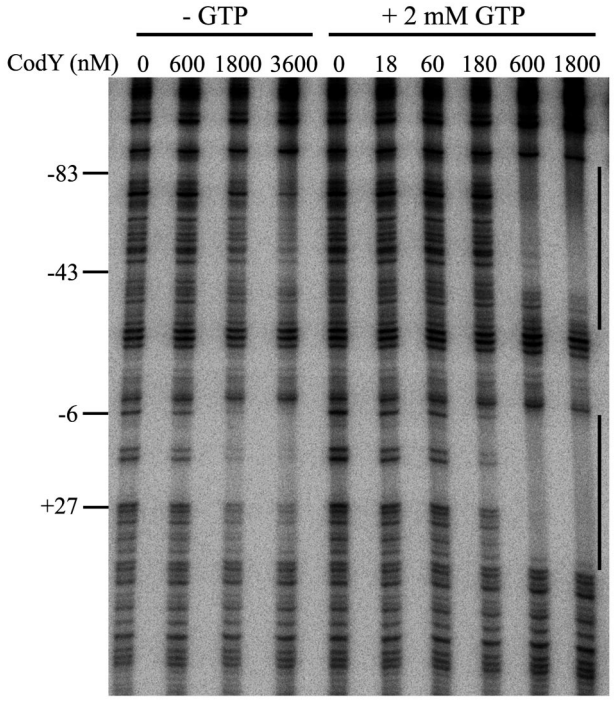

FIG. 5. DNase I footprint assay of CodY interaction with the *citB* regulatory region. A 523-bp PCR product, corresponding to positions −320 to +202 with respect to the *citB* transcriptional start site and labeled at the 5′ end of the template (noncoding) strand, was incubated with increasing amounts of purified CodY-His_6 in the presence or absence of 2 mM GTP. After treatment with DNase I, the DNA was denatured and subjected to electrophoresis. A set of Sanger sequencing reactions (not shown) were primed with the same oligonucleotide used for synthesis of the template strand of the PCR product and used to establish the positions of the protected bands. Vertical lines on the left indicate regions protected by CodY.

second regulator (Fig. 3). The effect of the dyad deletion was similar to that of a *ccpC* null mutation. These results indicate that the site of CodY action is downstream of position −66.

Role of citrate production in *citB* expression. Several lines of evidence indicate that citrate is an inducer of *citB* expression. Blockage of citrate production prevents expression of *citB* in defined medium (7, 11), and addition of citrate to cells in DS medium was reported to lead to hyperexpression of aconitase (32). Direct confirmation that citrate is an inducer that acts by antagonizing the repressing activity of CcpC was obtained by assays of in vitro transcription (23).

In wild-type cells growing in DS medium (Fig. 4A), a double mutation in *citA* and *citZ*, the two genes that encode citrate synthases in *B. subtilis* (22), prevented expression of *citB-lacZ*. Whereas a *citB* mutation led to higher and more prolonged expression of *citB-lacZ*, combining the *citA, citZ,* and *citB* mutations reduced *citB* expression to a an undetectable level, showing that citrate accumulation is necessary for both the normal level of expression and hyperexpression in the *citB* single mutant (Fig. 4A).

Unusually high levels of citrate might cause prolonged and overabundant expression of *citB* by inactivation of the repressing activity of CcpC. However, a *ccpC* null mutant did not show prolonged or unusually high expression of *citB* (Fig. 1). Alternatively, the high intracellular and extracellular accumulations of citrate that occur in a *citB* mutant are known to prevent activation of the transcription factor Spo0A by chelating Mn^{2+} and Fe^{2+} (5). However, deletion of *spo0A* neither prevented the normal shut-off of *citB* expression in an otherwise wild-type cell nor suppressed the hyperexpression of *citB* seen in a *citB* mutant (Fig. 4B). Thus, neither repression by CcpC nor repression by Spo0A is responsible for limiting the extent of *citB* induction in wild-type stationary-phase cells.

Binding of CodY to the *citB* promoter region. A DNA fragment corresponding to positions −320 to + 202 with respect to the *citB* transcription start site was end labeled and incubated with purified CodY protein. Interaction of the protein and DNA resulted in protection of the DNA against subsequent treatment with DNase I (Fig. 5). Binding of CodY to the *dpp* promoter and repression of *dpp* transcription in vitro are enhanced in the presence of GTP (35). As shown in Fig. 5, GTP (2 mM) enhanced binding of CodY 3- to 10-fold. The concentration of CodY that protected 50% of the DNA molecules in the presence of 2 mM GTP was between 60 and 180 nM.

In the presence of GTP, CodY, at a protein concentration of 180 nM, protected from positions −6 to +27 with respect to the transcription start site (Fig. 5). This localization is consistent with repression of a version of the *citB* promoter region deleted upstream of position −65 (Fig. 3). At higher concen-

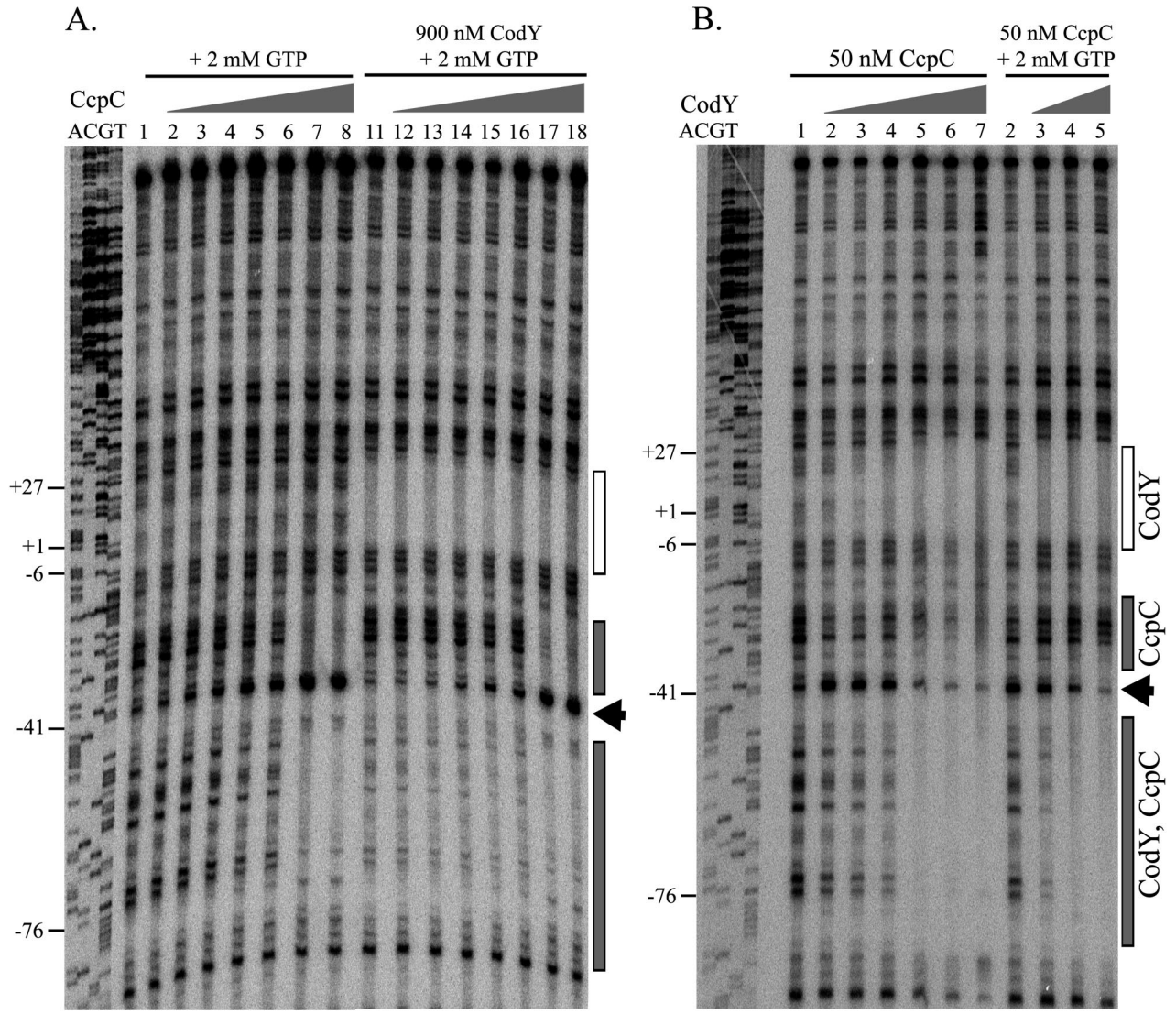

FIG. 6. Simultaneous binding of CcpC and CodY to the *citB* regulatory region. A PCR product encompassing positions −166 to +123 with respect to the *citB* transcription start site was labeled at the 5′ end on the nontemplate (coding) strand. A set of Sanger sequencing reactions (A, C, G, and T) were primed with the same oligonucleotide used for synthesis of the nontemplate strand of the PCR product and used to establish the positions of the protected bands. The vertical bars indicate regions protected by CcpC or CodY, and the arrowhead points to a DNase I-hypersensitive site typically created by binding of CcpC to the *citB* promoter region (22). (A) The PCR product was incubated with increasing amounts of CcpC protein in the presence or absence of 900 nM CodY-His_6. All reaction mixes also contained 2 mM GTP. After DNase I treatment, the DNA fragments were purified as described in Materials and Methods, denatured, and subjected to electrophoresis. CcpC concentrations (nanomolar) were as follows: lanes 1 and 11, 0; lanes 2 and 12, 12; lanes 3 and 13, 25; lanes 4 and 14, 50; lanes 5 and 15, 100; lanes 6 and 16, 200; lanes 7 and 17, 500; and lanes 8 and 18, 1,000. (B) The PCR product was incubated with 50 nM CcpC and increasing amounts of CodY with or without added GTP (2 mM). CodY concentrations (nanomolar) were as follows: lane 2, 0; lane 3, 300; lane 4, 600; lane 5, 1,000; lane 6, 1,800; and lane 7, 3,000. The sample in lane 1 contained neither CcpC nor CodY.

trations of CodY, a second binding site, located between positions −43 and −83, was observed (Fig. 5). When GTP was omitted, protection of the weaker binding site was only seen at very high CodY concentrations (Fig. 5).

Simultaneous interaction of *citB* promoter region with CodY and CcpC. The secondary site of binding of CodY in the *citB* promoter region overlaps the dyad symmetry element that is critical for binding of CcpC. Therefore, we tested whether both proteins can bind simultaneously or compete for binding. When CodY was used at 900 nM in the presence of GTP, the addition of increasing amounts of CcpC led to binding of CcpC (as shown by protection of the −30 region and the appearance of a hypersensitive site at position −41) without displacement of CodY from the −6 to +27 site (Fig. 6A). Since virtually all of the DNA molecules were protected in both regions, CcpC and CodY must be binding simultaneously to the same DNA molecules. Formation of the hypersensitive site at position −41 was completely dependent on binding of CcpC to the −66 dyad symmetry element (Kim et al., submitted). Whether binding of CcpC displaces CodY from the −66 region could not be determined from this experiment.

When CcpC was held constant at 50 nM (a concentration

that gives only partial protection of the −30 and −66 regions), CodY (in the presence of GTP) had different effects at different concentrations. When CodY was used at 300 nM, both proteins bound simultaneously, with CodY protecting the −6 to +27 region and CcpC protecting the −66 region (and creating its typical hypersensitive site at position −41) (Fig. 6B). At higher concentrations (500 to 1000 nM), however, CodY caused a reduction in hypersensitivity at position −41, which might be due to displacement of CcpC from the −66 region (Fig. 6B). In the absence of GTP, similar effects were seen at higher CodY concentrations. We estimated the intracellular concentration of CodY to be ≈2.5 μM (R. Shivers and A. L. Sonenshein, unpublished data).

Binding of AbrB to *citB* promoter region. An *abrB* mutation reduced the extent of induction of *citB* as cells approached stationary phase (Fig. 1). To see whether this effect was direct, we tested the ability of purified AbrB protein to interact with the *citB* promoter region in a sequence-dependent manner. As shown in Fig. 7, AbrB protected the *citB* promoter region against DNase I from positions −35 to +14. Surprisingly, this pattern of protection suggests that AbrB binds to a region that overlaps the binding sites for both CodY and CcpC.

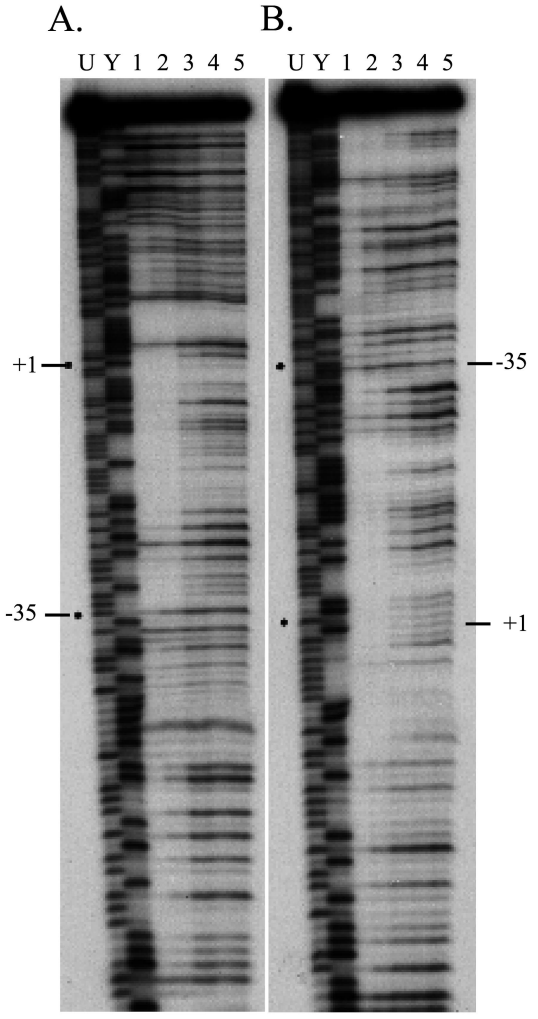

FIG. 7. DNase I footprint of AbrB binding to the *citB* regulatory region. (A) The template strand was labeled at its 3′ end. (B) The nontemplate strand was labeled at its 3′ end. Lanes: 1, 10 μM AbrB; 2, 5 μM AbrB; 3, 1 μM AbrB; 4 and 5, no AbrB. Maxam-Gilbert purine (U) and pyrimidine (Y) sequencing ladders for each strand are shown for reference. The positions of the +1 and −35 nucleotides of the promoter are indicated.

DISCUSSION

Many genes that are induced as *B. subtilis* cells make the transition from rapid exponential-phase growth to stationary phase do so in response to multiple signals. These signals may be gene specific or global or both. The accumulation of citrate, acting on CcpC, serves as a selective signal for genes that encode tricarboxylic acid branch enzymes. The gene for the major citrate synthase, *citZ*, is directly repressed by both CcpC and CcpA (24). As a result, when rapidly metabolizable carbon sources become limiting, CcpA loses repressing activity, allowing partial derepression of *citZ*. The resulting synthesis of citrate then inactivates CcpC, leading to high-level expression of *citZ* and *citB*. In a defined medium containing glucose and glutamine as the sole carbon and nitrogen sources, this mode of regulation appears to be the primary mechanism for controlling *citB* expression (23, 24). CcpA has no direct role in *citB* expression, since the protein does not bind to any sequence in the *citB* regulatory region (24).

In cells growing in a complex medium, however, at least one additional factor is implicated in *citB* regulation. Elimination of CcpC or its primary binding site yields only a slight derepression of *citB* expression in this case. In cells deleted for both *ccpC* and *codY*, however, *citB* expression is almost fully derepressed during rapid exponential growth, yet deletion of *codY* alone leads to only a slight derepression of *citB*. These results imply that either CcpC or CodY alone is sufficient to provide substantial repression of *citB* in cells growing in nutrient broth medium. In other words, induction of *citB* in such cells only occurs when the cells accumulate enough citrate to inactivate CcpC and become sufficiently depleted of GTP that CodY becomes inactivated. Since recent studies indicate that CodY responds to branched-chain amino acids as well as to GTP (18; R. Shivers and A. L. Sonenshein, unpublished data), the induction of *citB* presumably depends on the depletion of certain amino acid pools as well.

All genes known to be repressed by CodY are also regulated by other factors. For the *dpp* operon, the second regulator is AbrB (41); for the *gabP* gene, it is TnrA (8); for *srfAA*, it is ComA~P (31, 36); for the *ure* operon, it is PucR and GlnR (3, 51); for the *bkd* operon, it is BkdR (6); and for the *roc* operons, it is RocR (16). Thus, CodY seems to impose a general nutrient-responsive repression that is fine-tuned by regulators that sense other kinds of metabolic signals.

The different roles of CodY in cells grown in defined and complex media have been verified by microarray analysis. Several hundred genes are derepressed by a *codY* mutation in cells growing in DS medium or in defined medium containing glucose and a mixture of 17 amino acids, but very few genes are affected by a *codY* mutation during growth in minimal-glucose-glutamine medium (30a).

Curiously, a *codY* deletion mutant was unable to induce the *citB* gene in stationary phase to the same level as in wild-type

cells. Two general hypotheses could explain this phenomenon. First, the absence of CodY may permit another regulator, such as CcpC, to repress expression more effectively by eliminating a competitor for binding to the *citB* regulatory region. The highly derepressed expression of *citB* in a *codY ccpC* double mutant is consistent with this hypothesis, as are in vitro studies that show that CodY, at high concentrations, interferes with the binding of CcpC. Alternatively, CodY may normally repress, during exponential growth phase, the expression of a gene whose product represses *citB* during stationary phase. This idea is consistent with the observation that *citB* expression at the end of the exponential growth phase is transient. These two hypotheses are neither exhaustive nor mutually exclusive.

Cells in DS medium sporulate efficiently after they enter stationary phase, and CodY appears to be the primary monitor of nutrient availability for sporulation. That is, a *codY* null mutant sporulates in a rich medium in which sporulation would normally be repressed (35). Since aconitase is required for sporulation, it is not surprising that the *citB* gene is under the control of CodY. The role of aconitase in sporulation is not completely understood. Part of the answer is that, without an active Krebs cycle, the cell is unable to make the ATP, reducing power, and biosynthetic intermediates needed for spore formation. The specific absence of aconitase, however, causes at least one additional defect. Failure to metabolize citrate leads to its accumulation, to a consequent drop in extracellular pH, and to chelation of divalent cations, such as Mn^{2+} and Fe^{2+}, needed for critical enzymatic activities (5). In addition, *B. subtilis* aconitase protein appears to have a second, nonenzymatic function as an RNA binding protein (1). Whether this second activity is necessary for sporulation is unknown.

AbrB also appears to be implicated in *citB* regulation. AbrB binds to the *citB* promoter region, and an *abrB* null mutation causes a reduction in *citB* expression both in stationary-phase cells and in rapidly growing cells in defined medium. This result is surprising, since the binding site for AbrB overlaps the presumed RNA polymerase interaction site. Either AbrB, like MerR (2), stimulates transcription while binding to the promoter region, or the in vivo effect of AbrB is indirect. That is, AbrB may block binding of an as yet unidentified repressor of *citB* or may control the expression of a gene whose product participates in regulating *citB*. Since the effect of an *abrB* mutation was seen in cells carrying mutations in the *ccpC* and *codY* genes, the hypothetical target of AbrB cannot be *ccpC* or *codY*. Since Spo0A represses AbrB, we might have anticipated that a *spo0A* mutant strain, which overproduces AbrB (48), would have a higher level of *citB* expression than a wild-type strain. In fact, such an effect occurs in cells growing in minimal medium (M. Strauch, unpublished data), but we saw no such effect in DS medium.

The factor(s) responsible for reestablishing repression of *citB* in stationary-phase cells in DS medium remains unknown. Inactivation of this factor may be responsible for the hyperexpression of *citB* seen when citrate accumulates. Our results rule out CcpC, CodY, AbrB, and any protein whose synthesis depends on Spo0A. Aconitase itself can also be ruled out as a negative autoregulator, since *citB* hyperexpression also occurs in a *citC* (isocitrate dehydrogenase) mutant. (Aconitase protein is overproduced in a *citC* mutant but fails to block expression of a *citB-lacZ* fusion [data not shown]. Aconitase also cannot be a positive autoregulator, since hyperexpression was seen in a *citB* null mutant.) If the putative additional factor acts alone, it should be possible to isolate mutant strains in which *citB* expression is abnormally high during the late stationary phase in DS medium.

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