Dual role of CcpC protein in regulation of aconitase gene expression in *Listeria monocytogenes* and *Bacillus subtilis*

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The role of the CcpC regulatory protein as a repressor of the genes encoding the tricarboxylic acid branch enzymes of the Krebs cycle (citrate synthase, *citZ*; aconitase, *citB*; and isocitrate dehydrogenase, *citC*) has been established for both *Bacillus subtilis* and *Listeria monocytogenes*. In addition, hyperexpression of *citB–lacZ* reporter constructs in an aconitase null mutant strain has been reported for *B. subtilis*. We show here that such hyperexpression of *citB* occurs in *L. monocytogenes* as well as in *B. subtilis* and that in both species the hyperexpression is unexpectedly dependent on CcpC. We propose a revision of the existing CcpC–*citB* regulatory scheme and suggest a mechanism of regulation in which CcpC represses *citB* expression at low citrate levels and activates *citB* expression when citrate levels are high.

INTRODUCTION

In the Gram-positive bacterium *Bacillus subtilis*, the coordinated expression of the first three enzymes of the Krebs cycle (citrate synthase, *citZ*; aconitase, *citB*; and isocitrate dehydrogenase, *citC*) is controlled by three regulatory proteins: CodY, CcpA and CcpC (reviewed by Sonenshein, 2007). CcpA and CodY are global regulatory proteins that respond to the intracellular pools of fructose-1,6-bisphosphate and ATP for CcpA (Jault *et al.*, 2000) and the combination of GTP and the branched-chain amino acids for CodY (Handke *et al.*, 2008; Villapakkam *et al.*, 2009). CcpA exerts both direct (through *citZ* and *ccpC*) and indirect effects on tricarboxylic acid (TCA) branch enzyme expression (Kim *et al.*, 2002b), while CodY binds to the *citB* promoter region and represses *citB* transcription (Kim *et al.*, 2003a). Unlike these global regulators, CcpC exclusively regulates the TCA branch enzymes by responding to a pathway-specific metabolite, citrate. Citrate-antagonized repression of the *B. subtilis citZ*, *citB* and *citC* genes by CcpC has been described in detail (Jourlin-Castelli *et al.*, 2000; Kim *et al.*, 2003a, b). In the closely related intracellular pathogen *Listeria monocytogenes*, the *citZ* and *citB* genes are regulated by CcpC and CodY, but not by CcpA (Kim *et al.*, 2006; Mittal, 2008; Mittal *et al.*, 2009).

CcpC is a member of the LysR-type transcriptional regulator (LTTR) family of proteins (Jourlin-Castelli *et al.*, 2000; Kim *et al.*, 2003b), a group that includes *B. subtilis* GltC, the regulator of the glutamate synthase genes (Belitsky *et al.*, 1995) and *Escherichia coli* OxyR (Maddocks & Oyston, 2008). *B. subtilis* CcpC dimers bind to two sites within the *citB* promoter region (Fig. 1), a dyad symmetry element centred at position −66 with respect to the transcriptional start site and a half-dyad element located at positions −27 to −33 (Fouet *et al.*, 1990; Fouet & Sonenshein, 1990; Jourlin-Castelli *et al.*, 2000). Both sites are required for repression; interaction between the two CcpC dimers bound at these sites results in bending of the DNA, blocking access of RNA polymerase to the promoter and resulting in repression of *citB* expression (Jourlin-Castelli *et al.*, 2000; Kim *et al.*, 2003b). As with other LTTR proteins, CcpC repression is relieved by the interaction of the protein with a metabolite acting as an inducer; in this case, citrate induces the expression of *citB* (Blencke *et al.*, 2006). Citrate disrupts CcpC binding to the −27 site in the *citB* promoter; while CcpC remains bound to the −66 dyad symmetry element, the loss of binding at the −27 half-dyad element relaxes the bending of the DNA and allows RNA polymerase to interact with the promoter (Kim *et al.*, 2003b). Similar

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Abbreviation: TCA, tricarboxylic acid.

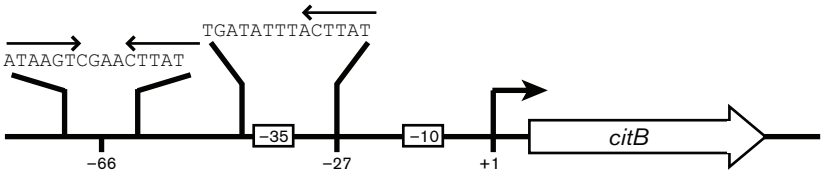

Fig. 1. CcpC binding sites in the *citB* promoter region. Two CcpC binding sites are present in the *citB* regulatory regions of *L. monocytogenes* and *B. subtilis*. The *B. subtilis* −66 dyad symmetry element and −27 half-dyad element are shown with their respective sequences. The −35 and −10 promoter elements are also indicated (distances are not to scale).

citrate-dependent derepression has also been described for the *citB* gene of *L. monocytogenes* (Kim *et al.*, 2006). In *L. monocytogenes*, CcpC represses transcription of *citB* as well as of *citZ* and the *lmo0847* gene, which encodes a putative glutamine transporter (Kim *et al.*, 2006; Mittal *et al.*, 2009). The organization of the CcpC binding sites in the *L. monocytogenes citB* regulatory region is almost identical to that in *B. subtilis*, with a dyad symmetry element centred at position −68 and a half-dyad at positions −28 to −32 (Kim *et al.*, 2006). *In vitro*, in the presence of citrate, binding to the full dyad is maintained, but binding to the half-dyad is reduced (Kim *et al.*, 2006). Therefore, in both species, citrate synthesis is necessary for full expression of the TCA branch enzymes. Because citrate is produced uniquely by the activity of citrate synthase, this regulatory loop provides a mechanism for the sequential expression of these central metabolic enzymes. However, in this report we present data indicating that this model is incomplete.

Previous results hinted that the nature of the CcpC–*citB* interaction is more complex than a simple repression model. *B. subtilis* cells that lack a functional aconitase (*citB* null mutants) accumulate a vast excess of citrate during growth (Craig *et al.*, 1997). Concomitant with unusually high citrate accumulation is hyperexpression from the *citB* promoter; the expression of a *citB–lacZ* promoter fusion is enhanced in a *citB* null mutant to levels five- to tenfold higher than those seen in wild-type cells, and this overexpression is dependent on a functional citrate synthase (Kim *et al.*, 2003a), reinforcing the notion that citrate is the factor that hyperinduces *citB* expression. We show here that hyperexpression of *citB–lacZ* in a *citB* null mutant is also seen in *L. monocytogenes* and that this phenotype is again correlated with a vast overaccumulation of citrate and is dependent on the synthesis of citrate. In searching for the regulatory mechanism responsible for this citrate-dependent hyperexpression in these related species, we found to our surprise that CcpC, previously known only as a repressor, becomes an activator of *citB* transcription under conditions of high citrate accumulation. Thus, CcpC in both species switches from a negative regulator to a positive regulator when citrate accumulates.

METHODS

Bacterial strains and growth conditions. The bacterial strains and plasmids used in this study are listed in Table 1. *B. subtilis* strains were grown at 37 °C with aeration in DS medium [0.8 % nutrient broth, 0.1 % KCl, 0.025 % $MgSO_4 . 7H_2O$, 1 mM $Ca(NO_3)_2$, 10 μM $MnCl_2$, 1 μM $FeSO_4$ (Fouet & Sonenshein, 1990)] and supplemented with chloramphenicol (2.5 μg ml^{-1}), erythromycin (1.0 μg ml^{-1}), neomycin (2.5 μg ml^{-1}), phleomycin (0.25 μg ml^{-1}) or spectinomycin (50 μg ml^{-1}) when necessary. *L. monocytogenes* strains were grown in brain heart infusion medium (BHI; Difco) at 37 °C. In general, cultures were grown in Erlenmeyer flasks with a medium-to-flask volume ratio of 10 and a circular agitation speed of 200 r.p.m.

Construction of a *citB* null mutant of *L. monocytogenes*. Using oligonucleotide primers specific to each DNA segment, the first 960 bp of the *citB* gene, a tetracycline resistance gene and the last 1019 bp of *citB* were amplified by PCR and ligated sequentially to pPS34, a derivative of pSK- (Stratagene) that was modified to carry an erythromycin-resistance gene active in Gram-positive bacteria (P. Serror, personal communication). The resulting plasmid was digested with *Sac*I and *Kpn*I and the insert (*citB′-tet-′citB*) was ligated to pCON-1 (Behari & Youngman, 1998). The final plasmid, pEMM20, was introduced by transformation into HKB214, a *L. monocytogenes* strain carrying a *citB–lacZ* fusion at the nonessential *int* locus (Kim *et al.*, 2006). Transformants were isolated and passaged to obtain a double-crossover at the *citB* locus, resulting in strain LMM12 [Δ*int*::(Φ *citB–lacZ neo*) Δ*citB*::*tet*]. Plasmid pEMM20 was also introduced into strain HKB217 to create strain LMM25 [Δ*int*::(Φ *citB–lacZ neo*) Δ*citB*::*tet* Δ*ccpC*::*spc*].

***β*-Galactosidase assay.** For β-galactosidase activity assays, samples (1 ml) were removed from *B. subtilis* or *L. monocytogenes* broth cultures during growth after determining the OD_{600} of the culture at that time point, and cell pellets were frozen on dry ice. Cells were permeabilized and assayed as described previously (Belitsky *et al.*, 1995). β-Galactosidase activity (Miller units) was calculated as described previously (Miller, 1972); however, a volume correction factor of 1.25 was used to account for the increase in the reaction volume due to addition of sodium carbonate to stop the reaction.

Assay of intracellular citrate concentrations. Cultures (25 ml) of *L. monocytogenes* strains were grown in BHI at 37 °C until they reached OD_{600} 0.8–1.0. After collection of the cells by centrifugation, the pellet was washed with 20 mM Tris/HCl, pH 8, containing 1 mM EDTA, and resuspended in 4 ml 0.3 M perchloric acid. After incubation on ice for 10 min, the cell debris was removed by centrifugation and the supernatant fluid was mixed with 2 ml 0.75 M K_2CO_3 and kept on ice for 15 min. The concentration of citrate in the supernatant fluid after subsequent centrifugation was determined using a kit (R-Biopharm) in which citrate lyase activity is coupled to malate dehydrogenase, lactate dehydrogenase and oxidation of NADH.

Assay of aconitase enzyme activity. *L. monocytogenes* cells were grown in BHI broth and harvested by centrifugation at the beginning of stationary phase, washed with a buffer containing

Table 1. Bacterial strains used in this study

Strain	Genotype	Source or reference
B. subtilis		
SMY	Prototroph	P. Schaeffer
JH642	*trpC2 pheA1*	Brehm *et al.* (1973), Dean *et al.* (1977)
AF21	Δ*amyE*::Φ(*citBp21–lacZ cat*)	SMY × pAF1. Fouet & Sonenshein (1990)
MAB160	*trpC2 pheA1* Ω*citB*::*spc*	Craig *et al.* (1997)
AWS96	*trpC2 pheA1*	MAB160 × JH642 DNA. Serio *et al.* (2006)
PS258	*trpC2* Δ*codY*::*erm*	P. Serror
HKB125	Δ*amyE*::Φ(*citBp23–lacZ cat*) Δ*codY*::*erm*	Kim *et al.* (2003a)
HKB126	Δ*amyE*::Φ(*citBp24–lacZ cat*) Δ*codY*::*erm*	Kim *et al.* (2003a)
HKB165	Δ*amyE*::Φ(*citBp21–lacZ cat*) Ω*citB*::*spc*	Kim *et al.* (2003a)
HKB186	Δ*amyE*::Φ(*citBp21–lacZ cat*) Δ$ccpC_{BS}$::*ble*	Kim *et al.* (2006)
KBP26	*trpC2 pheA1* Δ*amyE*::Φ(*citBp21–lacZ cat*)	AWS96 × AF21 DNA
KBP51	*trpC2 pheA1* Δ*amyE*::Φ(*citBp21–lacZ cat*) Ω*citB*::*spc*	KBP26 × MAB160 DNA
KBP52	*trpC2 pheA1* Δ*amyE*::Φ(*citBp21–lacZ cat*) Δ*ccpC*::*ble*	KBP26 × HKB186 DNA
KBP54	*trpC2 pheA1* Δ*amyE*::Φ(*citBp21–lacZ cat*) Ω*citB*::*spc* Δ*ccpC*::*ble*	KBP51 × HKB186 DNA
KBP56	*trpC2 pheA1* Δ*amyE*::Φ(*citBp23–lacZ cat*)	AWS96 × HKB125 DNA
KBP57	*trpC2 pheA1* Δ*amyE*::Φ(*citBp24–lacZ cat*)	AWS96 × HKB126 DNA
KBP62	*trpC2 pheA1* Δ*amyE*::Φ(*citBp23–lacZ cat*) Ω*citB*::*spc*	MAB160 × KBP56 DNA
KBP63	*trpC2 pheA1* Δ*amyE*::Φ(*citBp24–lacZ cat*) Ω*citB*::*spc*	MAB160 × KBP57 DNA
KBP141	*trpC2 pheA1* Δ*amyE*::Φ(*citBp21–lacZ cat*) Δ*codY*::*erm*	KBP26 × PS258 DNA
KBP142	*trpC2 pheA1* Δ*amyE*::Φ(*citBp21–lacZ cat*) Δ*codY*::*erm* Ω*citB*::*spc*	KBP51 × PS258 DNA
KBP143	*trpC2 pheA1* Δ*amyE*::Φ(*citBp21–lacZ cat*) Δ*codY*::*erm* Δ*ccpC*::*ble*	KBP52 × PS258 DNA
KBP144	*trpC2 pheA1* Δ*amyE*::Φ(*citBp21–lacZ cat*) Δ*codY*::*erm* Ω*citB*::*spc* Δ*ccpC*::*ble*	KBP54 × PS258 DNA
L. monocytogenes		
HKB214	Δ*int*::Φ(*citB–lacZ neo*)	Kim *et al.* (2006)
HKB217	Δ*int*::Φ(*citB–lacZ neo*) Δ*ccpC*::*spc*	Kim *et al.* (2006)
LMM12	Δ*int*::Φ(*citB–lacZ neo*) Δ*citB*::*tet*	HKB214 × pEMM20
LMM25	Δ*int*::Φ(*citB–lacZ neo*) Δ*citB*::*tet* Δ*ccpC*::*spc*	HKB217 × pEMM20
LMM33	Δ*int*::Φ(*citB–lacZ neo*) Δ*citZ*	Mittal *et al.* (2009)
LMM34	Δ*int*::Φ(*citB–lacZ neo*) Δ*citB*::*tet* Δ*citZ*	LMM12 × pEMM47 DNA

20 mM Tris-citrate (pH 7.35), 150 mM KCl and 0.5 mM PMSF and stored frozen at −80 °C. Cell pellets were thawed in the same buffer and treated with mutanolysin as described by Fliss *et al.* (1991). Cell extracts were clarified by centrifugation and stored at 4 °C. Protein concentrations were determined using the Bradford protein assay reagent (Bio-Rad). The aconitase enzyme activity was determined as described previously (Dingman & Sonenshein, 1987). When assaying crude extracts, it is difficult to estimate very low specific activities accurately. One unit of aconitase activity is equal to a change in A_{240} of 0.0033 min^{-1}; the change in absorbance directly measures the production of 1 nmol *cis*-aconitate ml^{-1}. Therefore, when 3 μg of extract is assayed, a change in A_{240} of 0.001 beyond background over 10 min corresponds to a specific activity of 10 units mg^{-1}.

RESULTS

Hyperexpression of the *citB* promoter in a *L. monocytogenes citB* null mutant

To determine whether the hyperexpression of the *citB* gene previously observed in a *citB* null mutant of *B. subtilis* (Kim *et al.*, 2003a) also occurs in *L. monocytogenes*, a related, medically relevant pathogen, we introduced a *citB* null mutation into a *L. monocytogenes* strain carrying a *citB–lacZ* fusion. The *L. monocytogenes citB* null strain was a glutamate auxotroph; it did not grow in minimal medium unless a source of glutamate (e.g. glutamine) was provided, indicating that it does not possess a complete TCA branch of the Krebs cycle. To confirm the loss of aconitase enzyme activity in the *citB* null strain, cell extracts of wild-type (HKB214) and *citB* null mutant (LMM12) strains were prepared by mutanolysin treatment and assayed for aconitase activity. The wild-type extract had an aconitase specific activity [195 units $(mg\ protein)^{-1}$] that was 10-fold higher than that of the *citB* null mutant extract [18 units $(mg\ protein)^{-1}$]. The apparent presence of residual aconitase activity in the null mutant strain is due to the high background of the assay (see Methods).

To determine the effect of the *citB* null mutation on *citB–lacZ* expression, the wild-type and *citB* null strains grown in BHI were assayed for β-galactosidase activity. Wild-type *L. monocytogenes* yielded a low level of β-galactosidase activity (Fig. 2a). In the *citB* null strain, however, *citB–lacZ* expression was increased 10-fold compared with the wild-type strain (Fig. 2a).

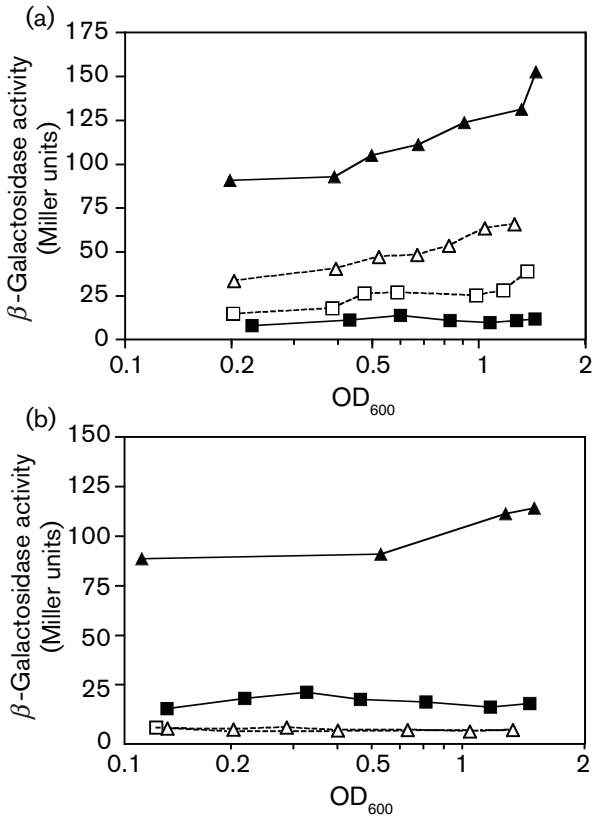

Fig. 2. Expression of a *citB–lacZ* fusion in *citB* and *ccpC* null mutant *L. monocytogenes* strains. β-Galactosidase activity was measured at various points during growth of *L. monocytogenes* cells carrying a *citB′–lacZ* fusion linked to a *neo* resistance marker and integrated at the non-essential *int′–′comK* locus (Kim *et al.*, 2006). For each of the strains, the end of exponential growth phase (OD_{600} ~ 0.8) occurred 2.25–2.75 h after the strains reached OD_{600}=0.2. (a) Strains HKB214 (*citB*$^+$ *ccpC*$^+$; ■), LMM12 (Δ*citB*::*tet ccpC*$^+$; ▲), HKB217 (*citB*$^+$ Δ*ccpC*::*spc*; □) and LMM25 (Δ*citB*::*tet* Δ*ccpC*::*spc;* △) were compared. (b) Strains HKB214 (*citB*$^+$ *citZ*$^+$; ■), LMM12 (Δ*citB*::*tet citZ*$^+$; ▲), LMM33 (Δ*citZ*; □) and LMM34 (Δ*citB*::*tet* Δ*citZ*; △) were compared. The end of exponential growth phase occurred 3–4 h after the strains reached OD_{600} 0.1.

Hyperexpression is dependent on the accumulation of citrate

To verify that citrate synthesis is necessary for hyperexpression of *citB–lacZ* in an *L. monocytogenes citB* null strain, as it is in *B. subtilis*, we created a *citB citZ* double mutant strain. The *citZ* gene encodes the sole citrate synthase in *L. monocytogenes*. A previously described insertion–deletion mutation in the *citZ* gene (Mittal *et al.*, 2009) was utilized; genomic DNA from this strain, LMM33, was introduced into strain LMM12 (*citB* null) by double crossover recombination, producing strain LMM34. Importantly, the *citB citZ* double mutant strain exhibited very low levels of *citB–lacZ* activity compared with the *citB* single mutant (Fig. 2b), indicating that citrate synthesis is required for the hyperexpression of *citB* in *L. monocytogenes* as well as in *B. subtilis*.

To verify that the high level of *citB–lacZ* expression seen in the *citB* mutant strain was correlated with a significant change in the pool of citrate within the cell, we assayed intracellular citrate (see Methods). The concentration of citrate in *citB* mutant cells was about 600-fold higher than that in wild-type cells (Table 2).

CcpC is necessary for hyperexpression of *citB–lacZ* in a *L. monocytogenes citB* null mutant

Given that high levels of citrate correlate with *citB* hyperexpression in *L. monocytogenes* (Fig. 2b) and that CcpC is known to be inactivated as a repressor by citrate, we attempted to rule out CcpC as the agent of hyperexpression. As previously reported (Kim *et al.*, 2006), a *ccpC* null mutation leads to a slight increase in *citB–lacZ* expression in cells grown in BHI medium. To our surprise, however, the introduction of a *ccpC* null mutation into the *citB* null mutant background resulted in loss of the hyperexpression phenotype (Fig. 2a). The *citB ccpC* null mutant had a level of *citB–lacZ* expression similar to that of the *ccpC* single mutant strain. Thus, *L. monocytogenes* CcpC is required for the hyperexpression of *citB–lacZ* caused by a *citB* null mutation, implying that CcpC acts as a positive regulator of *citB* expression when citrate accumulates to high levels.

In addition, inactivating CcpC overcame the hyperexpression phenotype without reducing the citrate pool. Whereas the concentration of citrate in *ccpC* single mutant cells was below the level of detection, presumably reflecting a high rate of citrate metabolism in the *ccpC* mutant strain, the intracellular pool of citrate in the *ccpC citB* double mutant was high, similar to those levels found in the *citB* single mutant (Table 2). Thus, eliminating CcpC suppresses the high-level *citB–lacZ* expression caused by inactivating *citB* without reducing the accumulation of citrate. This indicates that the high citrate pool alone does not cause *citB* hyperexpression in the absence of CcpC.

Table 2. Intracellular pool of citrate in *L. monocytogenes* mutant and wild-type cells

Cultures of the indicated strains were assayed for intracellular citrate as described in Methods.

Strain	Genotype	Intracellular citrate (mM)
HKB214	Wild-type	0.016 ± 0.007
HKB217	*ccpC* null	Not detectable
LMM12	*citB* null	9.5 ± 0.75
LMM25	*citB ccpC* null	8.9 ± 0.61

CcpC is necessary for hyperexpression of *citB–lacZ* in a *B. subtilis citB* null mutant

To study the role of CcpC in *citB* hyperexpression in a bacterium that is more highly tractable than *L. monocytogenes* and in which our understanding of *citB* regulation is more detailed, we sought to determine whether CcpC is necessary for hyperexpression of a *citB–lacZ* fusion in *B. subtilis*. To do so, we created a *citB ccpC* double null mutant carrying a *citB–lacZ* fusion (KBP54). Wild-type (KBP26), *citB* null (KBP51), *ccpC* null (KBP52) and *citB ccpC* double mutant strains were grown in DS medium and β-galactosidase activity was measured during growth. [DS medium was used because the effect of a *citB* null mutation is more pronounced in that medium than in glucose-minimal medium (data not shown).] As reported previously (Kim *et al.*, 2003a), the *citB* null mutation resulted in hyperexpression of *citB–lacZ*, while the *ccpC* null mutation caused a slight increase in *citB–lacZ* expression (Fig. 3a). As was the case in *L. monocytogenes*, the *ccpC citB* double mutant strain of *B. subtilis* behaved like a *ccpC* null strain; that is, the *ccpC* null mutation suppressed the *citB* hyperexpression phenotype (Fig. 3a). Therefore, CcpC appears to be a positive regulator of *citB* expression in *B. subtilis* as well as in *L. monocytogenes*.

The −66 dyad symmetry element is necessary for the activation of *citB* expression by *B. subtilis* CcpC

As described above, CcpC represses *citB* in *B. subtilis* by binding to a dyad element centred at position −66 and a half-dyad element at position −27 (Jourlin-Castelli *et al.*, 2000). When CcpC interacts with citrate *in vitro*, the protein releases from the −27 half-dyad element, but remains bound to the −66 site (Jourlin-Castelli *et al.*, 2000). To elucidate the mechanism by which CcpC activates *citB* in *B. subtilis* and to provide clues for the potential mechanism in *L. monocytogenes*, we utilized a *citB* promoter fusion lacking the upstream arm of the −66 dyad symmetry element (Fouet & Sonenshein, 1990). This fusion, *citBp24–lacZ*, and a fusion that contains the intact −66 site, *citBp23–lacZ*, were independently introduced into *B. subtilis* wild-type and *citB* null mutant strains, and expression was monitored during growth. In the presence of the intact −66 site, the hyperexpression of *citBp* was seen in a *citB* null strain as expected (Fig. 4). However, the loss of the upstream arm of the dyad resulted in loss of the hyperexpression phenotype; activity levels of the *citBp24–lacZ* fusion in the *citB* null strain were similar to those observed in the wild-type strain. This result indicates that the intact −66 dyad symmetry element is required for CcpC-dependent activation of *B. subtilis citB*, implying a direct role for CcpC in such hyperexpression.

Competition with CodY is not the basis for positive regulation of *citB* by CcpC

In previous work, we have shown that CodY acts as a negative regulator of *citB* and does so by binding to a site that overlaps with the CcpC binding site (Kim *et al.*, 2003a). Therefore, a possible mechanism by which CcpC might act as a positive regulator would be by blocking the binding of CodY. However, the introduction of a *codY* mutation into the various *B. subtilis* strains carrying the *citB–lacZ* fusion had only a small effect, if any, on expression (Fig. 3b). Most tellingly, inactivation of *codY* did not alter the phenotype of the *citB ccpC* double mutant, indicating that the *ccpC* mutation suppresses the *citB* hyperexpression phenotype without causing greater repression by CodY.

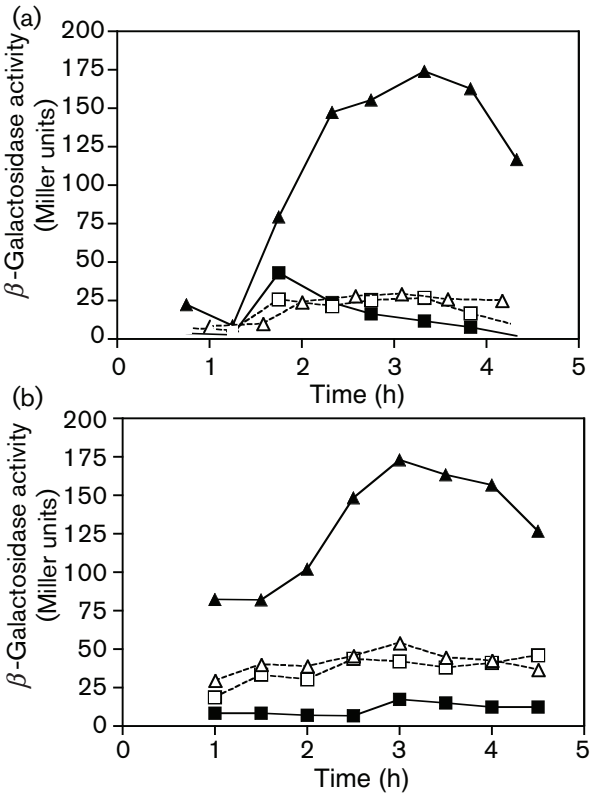

Fig. 3. Expression of a *citB–lacZ* fusion in *citB*, *ccpC* and *codY* mutant strains of *B. subtilis*. β-Galactosidase activity was measured at various points during growth of *B. subtilis* strains carrying a *citB′–lacZ* fusion integrated at the non-essential *amyE* locus. (a) Strains KBP26 (*citB*$^+$; ■), KBP51 (*citB* null; ▲), KBP52 (*ccpC* null; □) and KBP54 (*citB ccp*C null; △) strains were compared. The end of exponential growth phase was reached at 3.3 h for strains KBP26, KBP51 and KBP52 and at 2.67 h for strain KBP54. (b) Strains KBP141 (*codY* null; ■), KBP142 (*codY citB* null; ▲), KBP143 (*codY ccpC* null; □) and KBP144 (*codY citB ccpC* null; △) were compared. The end of exponential growth phase was reached at 3.5 h for strains KBP141, KBP142 and KBP144 and at 4.0 h for strain KBP143.

DISCUSSION

We report here evidence that CcpC acts as both a negative and a positive regulator of the *citB* promoter in the related

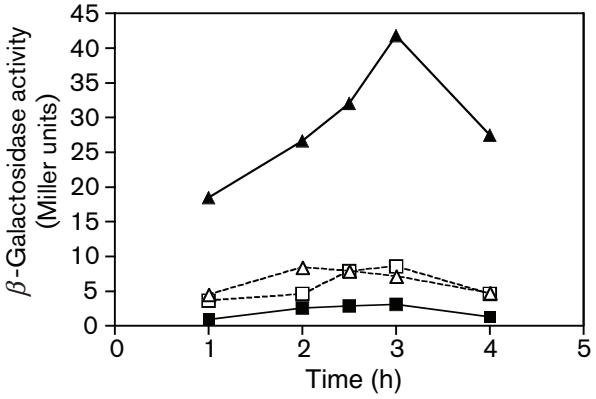

Fig. 4. The −66 dyad symmetry element is necessary for CcpC-dependent hyperexpression of *citB–lacZ* in *B. subtilis*. β-Galactosidase activity was measured at various times during growth of *B. subtilis* cells carrying two different *citB′–lacZ* fusions: one containing the intact −66 dyad symmetry element on the *citB* promoter (*citBp23–lacZ*) and the other missing the upstream arm of the dyad (*citBp24–lacZ*) (Fouet & Sonenshein, 1990). Expression of the two fusions was compared in *citB*$^+$ and *citB* null cells: for *citBp23–lacZ,* strains KBP56 (*citB*$^+$; ■) and KBP62 (*citB* null; ▲); for *citBp24–lacZ*, strains KBP57 (*citB*$^+$; □) and KBP63 (*citB* null; △). The end of exponential growth phase was reached between 3 and 4 h of growth for all strains.

bacteria *B. subtilis* and *L. monocytogenes*. In both organisms, the high citrate levels found in *citB* null mutants result in CcpC-dependent activation of a *citB–lacZ* reporter gene. Further exploration in *B. subtilis* revealed that this activation is independent of CodY. In addition, in *B. subtilis* (and presumably in *L. monocytogenes*) this activation is dependent on the CcpC binding site centred at position −66 with respect to the *citB* transcriptional start point. The evidence of CcpC-dependent activation presented here was generated using a *citB* null mutant strain, and thus we were unable to assess the contribution of CcpC activation of the *citB* promoter to aconitase protein levels. However, recent results shed light on this issue. Two *B. subtilis citB* point mutants (*citB2*, *citB7*) that result in increased citrate levels lead to the CcpC-dependent activation of the *citB* promoter; this effect is accompanied by overaccumulation of aconitase protein (K. B. Pechter and others, unpublished data).

Combining these new data with previously published work, we can propose a model for CcpC as a complex, citrate-responsive regulator of *citB* (Fig. 5). When the citrate pool is low, CcpC dimers are unliganded and they are able to interact with both the full −66 dyad symmetry element and the −27 half-dyad element to repress *citB* transcription (Fig. 2b) (Kim *et al.*, 2006, 2003b). As citrate levels rise, citrate binds to the CcpC dimer and alters its conformation, leading to release from the −27 site. This is supported by *in vitro* data; when citrate is present at 7–17 mM (0.2–0.5 % Na_2-citrate . $2H_2O$), equivalent to the high concentration of citrate in *citB* mutant cells, CcpC binding to the −27 site is reduced and a new hypersensitive band is created at the upstream end of the element, suggesting an alteration in the bending of the DNA (Kim *et al.*, 2003b). However, binding to the −66 dyad symmetry element is not affected by citrate, and due to the interaction between the two dimers, they remain tethered to the −66 site as a tetramer. This orientation is likely to permit interaction of CcpC with RNA polymerase to increase the efficiency of transcription from the *citB* promoter.

Mechanistically, there are two possibilities for how citrate converts CcpC to an activator. First, binding of the citrate ligand to a single site on CcpC could convert CcpC from a repressing to an activating conformation, as depicted in Fig. 5. This would result in an on/off mechanism of regulation, and in this case the moderate level of induction of *citB* expression seen in wild-type cells in the late-exponential growth phase would be the consequence of modest accumulation of citrate and conversion of only a fraction of the CcpC molecules to the activating conformation. In addition, the level of *citB* expression seen in a *ccpC* null mutant would reflect the intrinsic activity of the *citB* promoter in the absence of both repression and activation. (The *citB* promoters of *B. subtilis* and *L. monocytogenes* have only 8/12 and 9/12 matches, respectively, with the −10 and −35 consensus sequences.) The second possibility is that CcpC has two binding sites for citrate with different affinities and that CcpC interacts with the *citB* promoter in three ways: repression, derepression and activation. CcpC bound in the unliganded state would repress *citB*, as described above. When citrate accumulates to a moderate level, it would bind to a relatively high affinity site on CcpC, changing the CcpC conformation to cause release from the −27 half-dyad element along with derepression of *citB*. When citrate accumulates to a very high level, as in a *citB* null strain or in conditions when aconitase is inactive (see below), citrate would bind to both the high affinity site and a second, lower affinity site on CcpC, converting the protein to a transcriptional activator and causing *citB* to be hyperexpressed. The effect of citrate on CcpC binding to the *citB* promoters of *B. subtilis* and *L. monocytogenes in vitro* is consistent with both models. It is important to note, however, that it is also possible that CcpC influences *citB* transcription indirectly by regulating the synthesis of another factor.

We also note that hyperactivation of *B. subtilis citB–lacZ* expression in a *citB* null mutant seems to overcome repression by CodY. In fact, introduction of a *codY* mutation does not increase *citB–lacZ* expression substantially in a *citB* null mutant. One interpretation of these results is that binding of CcpC in its activating conformation interferes with CodY binding. This would not be surprising inasmuch as the two proteins bind to overlapping sequences in the *citB* promoter region (Kim *et al.*, 2003a). However, if this interpretation were correct, the level of β-galactosidase activity in the *citB ccpC codY* triple mutant would be considerably higher than in the *citB ccpC*

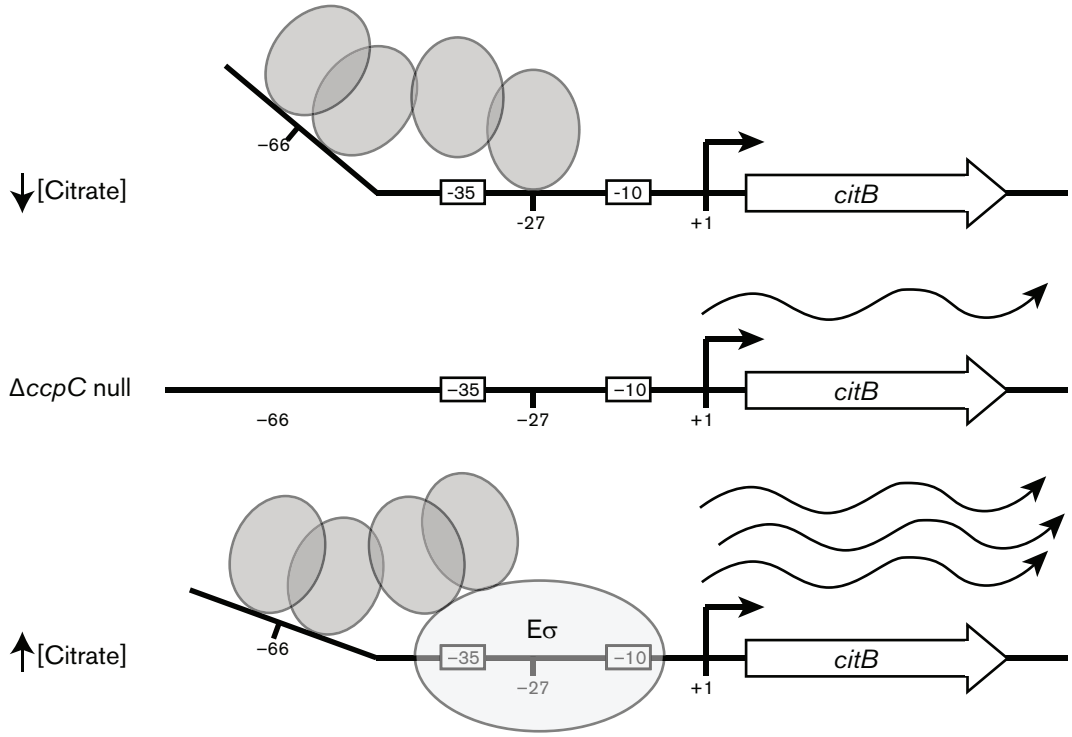

Fig. 5. An updated model of CcpC binding to the *citB* promoter. CcpC binds to the *citB* promoter and acts as a repressor or an activator in response to citrate. In the presence of no or very low levels of citrate, CcpC (grey circles) binds as a dimer to both the −66 and −27 binding sites, blocking access of RNA polymerase to the promoter and resulting in repression of *citB* expression. In the case of a *ccpC* null mutant, derepression of *citB* occurs and a low level of transcript is made. In the presence of high levels of citrate (as in a *citB* null mutant), binding to the −27 site is lost; however, the CcpC complex remains bound at the −66 site. We hypothesize that a direct interaction between CcpC and RNA polymerase allows CcpC to activate *citB* gene expression, resulting in the production of high levels of transcript.

double mutant. It is not, indicating that the contribution of CodY to *citB* regulation under the conditions tested is relatively small.

CcpC is not the only LTTR family member capable of switching between multiple regulatory modes. The *B. subtilis* GltC protein has two metabolite ligands and each induces opposing effects; GltC activates the *gltAB* locus when bound to 2-oxoglutarate and represses *gltAB* when complexed with glutamate (Picossi *et al.*, 2007). The unliganded and ligand-bound forms of GltC bind differentially to three sites within the *gltAB* promoter region to effect this regulatory switch. In addition, the glutamate dehydrogenase enzyme, RocG, also acts as a negative regulator of *gltAB* transcription, apparently by interacting with GltC (Gunka *et al.*, 2010). In other cases, LTTR family proteins (such as NahR from *Pseudomonas putida* and TrpI from *P. aeruginosa*) bind to a primary site in the absence of effector, but, in the presence of effector, binding is extended to a second site and transcription is activated (Chang & Crawford, 1990; Huang & Schell, 1991). In yet another group of LTTR proteins (Toledano *et al.*, 1994; van Keulen *et al.*, 2003; Wang & Winans, 1995), the presence of the inducer shifts the protein from one binding site on the promoter to another, causing activation. In the case of the CbbR regulatory protein from *Xanthobacter flavus*, which controls the expression of Calvin cycle genes, three regulatory sites are found at the *cbb* promoter, two of which (IR2 and IR3) are overlapping. CbbR binds to IR1 and IR3 in the absence of inducer, but, in the presence of the inducer, NADPH, CbbR shifts from IR3 to IR2, activating expression of the *cbb* operon (van Keulen *et al.*, 2003).

From a physiological point of view, the dual regulatory role of CcpC at the *citB* promoter creates an efficient system to control the intracellular abundance of citrate. Importantly, the action of CcpC as an activator is specific to aconitase; in other work we have shown that CcpC acts solely as a repressor of the *citZ* operon (K. B. Pechter and others, unpublished data). As is the case for the *E. coli lac* operon, which is induced by allolactose (Meiss *et al.*, 1969), and the *Salmonella typhimurium hut* operon, which is induced by urocanate (Jobe & Bourgeois, 1972), expression of the TCA branch of the Krebs cycle is induced by the product of the first enzyme of the pathway. When citrate synthase substrates acetyl-CoA and oxaloacetate are present, any leaky *citZ* expression, despite repression by CcpC, will

allow the accumulation of a small amount of citrate that will alleviate some CcpC repression and increase the levels of citrate synthase. If citrate levels rise faster than they can be reduced by aconitase, as would occur in cells stressed by iron limitation or exposure to reactive oxygen species, CcpC will convert to an activator, increasing aconitase expression dramatically. Moreover, the relief of autorepression of the *ccpC* gene as citrate levels increase ensures that the concentration of CcpC will also increase (Kim *et al.*, 2002a). This mechanism not only allows the cell to dissipate internally produced citrate and to prevent the hyperaccumulation of citrate acquired from the environment but also endows the cell with a high concentration of CcpC that is available to reimpose repression when the citrate pool decreases. Citrate is not only a key metabolic intermediate but also a carrier for iron uptake and a chelator of iron and other cations. To balance these roles, the cell needs a robust mechanism to avoid excessive intracellular citrate accumulation. While other bacterial species can metabolize citrate via citrate lyase, aconitase is the only citrate-metabolizing enzyme in *B. subtilis* and *L. monocytogenes*; thus, its activity is critical to maintaining this fine balance. In addition, it is noteworthy that the metabolism of citrate to 2-oxoglutarate by the combined activities of aconitase and isocitrate dehydrogenase leads to conversion of $NADP^+$ to NADPH; high-level citrate metabolism would potentially exhaust the $NADP^+$ pool. Such an outcome would be deleterious to the cell, but could be attenuated by rapid conversion of 2-oxoglutarate and NADPH (the products of the isocitrate dehydrogenase reaction) to glutamate and $NADP^+$ by glutamate synthase.

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