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CcpC-Dependent Regulation of *citB* and lmo0847 in *Listeria monocytogenes*

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In *Bacillus subtilis*, the catabolite control protein C (CcpC) plays a critical role in regulating the genes encoding the enzymes of the tricarboxylic acid branch of the Krebs citric acid cycle. A gene encoding a potential CcpC homolog and two potential target genes were identified in the *Listeria monocytogenes* genome. In vitro gel mobility shift assays and DNase I footprinting experiments showed that *L. monocytogenes* CcpC ($CcpC_{Lm}$) interacts with the promoter regions of $citB_{Lm}$ (the gene that is likely to encode aconitase) and lmo0847 (encoding a possible glutamine transporter) and that citrate is a specific inhibitor of this interaction. To study in vivo promoter activity, a new *lacZ* reporter system was developed. This system allows stable integration into the chromosome of a promoter region transcriptionally fused to a promoterless *lacZ* gene at a nonessential, ectopic locus. Analysis of strains carrying a $citB_{Lm}$-*lacZ* or lmo0847-*lacZ* fusion revealed that $CcpC_{Lm}$ represses $citB_{Lm}$ and lmo0847 in media containing an excess of glucose and glutamine. In addition, regulation of $citB_{Lm}$ expression in rich medium was growth phase dependent; during exponential growth phase, expression was very low even in the absence of $CcpC_{Lm}$, but a higher level of $citB_{Lm}$ expression was induced in stationary phase, suggesting the involvement of another, as yet unidentified regulatory factor.

Listeria monocytogenes is a gram-positive, facultative, intracellular pathogen responsible for listeriosis, a serious infection with a mortality rate of up to 30%, mainly among pregnant women, their fetuses, and immunocompromised persons. Listeriosis is characterized by gastroenteritis and fetoplacental and central nervous system infection, resulting in spontaneous abortion, neonatal death, septicemia, and meningitis (58). *L. monocytogenes* is widespread in nature and is predominantly transferred to humans by ingestion of contaminated foods (13). The bacterium can invade and multiply inside a wide range of phagocytic and nonphagocytic mammalian cells and has served as an important model system for studying the mechanisms of both intracellular parasitism and host cell biology (7, 40, 41).

A great deal of effort has been invested in isolating *L. monocytogenes* virulence genes and understanding mechanisms of pathogenesis (reviewed in reference 58). However, our knowledge of the basic physiology of this important pathogen is very limited, making it difficult to gain a comprehensive understanding of its pathogenesis. There has been increasing evidence that regulation of carbon metabolic pathways plays a critical role in the virulence of pathogenic bacteria. Regulation of the *Salmonella enterica* virulence operon *spv* is controlled by cyclic AMP-cyclic AMP receptor protein complex, the major catabolite regulator, as well as by the positive regulator SpvR (38). Similarly, *Escherichia coli* STb enterotoxin production is repressed by glucose, and repression is relieved by addition of cyclic AMP (4). Leukotoxin production in *Actinobacillus actinomycetemcomitans* is repressed by glucose or fructose (35). A novel catabolite repression system controls expression of hemolytic phospholipase C of *Pseudomonas aeruginosa* (45). In *Staphylococcus aureus*, glycerol and maltose repress enterotoxin A synthesis through the phosphoenolpyruvate phosphotransferase system (50).

In *L. monocytogenes*, most virulence genes identified so far are regulated by the transcriptional activator PrfA (6, 11, 32), which shows significant structural and functional similarity to members of the cyclic AMP receptor protein/Fnr family of proteins (29, 49). Although fermentable carbon sources strongly repress expression of virulence genes in this organism, CcpA, the global regulator of catabolite control in gram-positive bacteria, is not responsible for this form of catabolite repression (2, 33). Instead, PrfA seems to be the relevant regulator, acting by an unknown mechanism (3, 33). When fermentable carbohydrates, such as cellobiose and glucose, are added to support growth, expression of PrfA-dependent virulence genes is strongly repressed (33). However, sugar phosphate can be used as a carbon source for growth without repressing virulence gene expression, and the pathway of sugar phosphate utilization is PrfA dependent (43). Therefore, regulation of carbon metabolism is intimately interrelated with pathogenesis, which is not surprising given that infection may be a mechanism by which a pathogen tries to find useful nutrients. In order to understand pathogenesis of *L. monocytogenes* and to prevent or treat the infection, we need to have a thorough understanding of the physiology of *L. monocytogenes*, including the mechanisms by which it senses nutrients in order to regulate gene expression.

The Krebs citric acid cycle is one of the central pathways of carbon metabolism and couples, through 2-ketoglutarate, the utilization of carbon sources and the assimilation of ammo-

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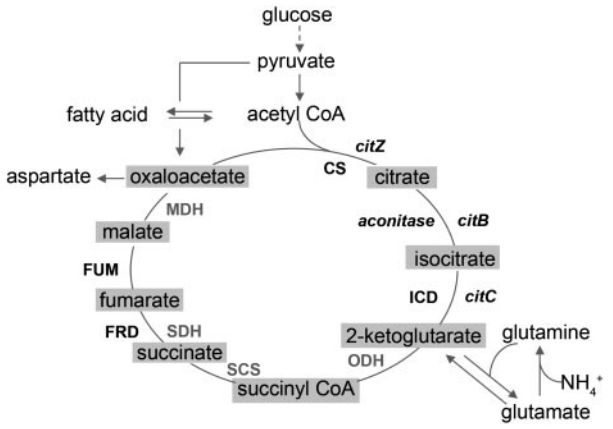


FIG. 1. The Krebs citric acid cycle and its place in central metabolism. In *L. monocytogenes*, the Krebs cycle is incomplete because of the absence of ketoglutarate dehydrogenase, succinyl CoA synthetase, and succinic dehydrogenase. As a result, the enzymes form two independent half-cycles. The right half, corresponding to the tricarboxylic acid branch, synthesizes 2-ketoglutarate from acetyl-CoA and oxaloacetate. 2-Ketoglutarate links central pathways of carbon catabolism and nitrogen assimilation. The left half operates counterclockwise and is used for respiration coupled to fumarate reduction. The source of malate is unknown (see the text). Enzymes are indicated by their names or abbreviations, and genes that encode the enzymes are indicated in italics. Missing enzymes are indicated in light typeface. Abbreviations: CS, citrate synthase; ICD, isocitrate dehydrogenase; ODH, ketoglutarate (oxoglutarate) dehydrogenase; SCS, succinyl CoA synthetase; SDH, succinic dehydrogenase; FRD, fumarate reductase; FUM, fumarate hydratase; MDH, malate dehydrogenase.

nium ion (Fig. 1). Cell extracts of *L. monocytogenes* A4413 appear to show the activity of a noncyclic Krebs pathway, with an oxidative portion (the tricarboxylic acid [TCA] branch [citrate synthase, aconitase and isocitrate dehydrogenase]) and a reductive portion (malate dehydrogenase, fumarate hydratase, and fumarate reductase) (56). The TCA branch of the Krebs citric acid cycle plays an important role in production of ATP, reducing power, and 2-ketoglutarate, as a precursor of glutamate. Moreover, aconitase, one of the enzymes of this branch, is essential for virulence in several bacterial pathogens (51, 52, 61).

Based on analysis of its complete genome sequence (16), *L. monocytogenes* encodes homologs of the *Bacillus subtilis citZ* (citrate synthase), *citB* (aconitase), and *citC* (isocitrate dehydrogenase) genes, and the organization of the genes is similar in the two organisms. However, the Krebs citric acid cycle is incomplete in *L. monocytogenes*, since the genes for 2-ketoglutarate dehydrogenase, succinyl coenzyme A (CoA) synthetase, succinate dehydrogenase, and malate dehydrogenase are missing. (It was reported that cell extracts prepared from *L. monocytogenes* A4413 show malate dehydrogenase activity [56]. We suspect, however, that the enzyme activity measured was that of the decarboxylating malic enzyme of gluconeogenesis, because the *L. monocytogenes* genome contains an apparent *malS* gene (decarboxylating malic enzyme) but no *mdh* (malate dehydrogenase) gene and because the enzyme assay used in the report does not distinguish one from the other.)

When *B. subtilis* cells are grown in an excess of glucose and glutamine (or glutamate), the genes encoding the enzymes of the TCA branch are strongly repressed (9, 22, 44). One of the repressors of the TCA branch genes is CcpC (23), a LysR-type transcriptional regulator (48). CcpC represses expression of the *citZCH* operon (encoding citrate synthase, isocitrate dehydrogenase, and malate dehydrogenase) (22) and *citB* (encoding aconitase) (9, 44) by binding to sequences called Box I (ATAA-N_7-TTAT) and Box II (TTAT, located 28 bp downstream, or ATAA, located 29 bp upstream of Box I) (23). Repression is relieved by citrate, the product of citrate synthase and a substrate of aconitase (23, 28).

We report here that the *L. monocytogenes* gene lmo1010 encodes a functional homolog of *B. subtilis* CcpC. We show further that *L. monocytogenes* CcpC acts on at least two target genes, $citB_{Lm}$ and lmo0847, and is antagonized by citrate. In the course of this work, we created a new transcription reporter system that is useful for studying gene expression in intact *L. monocytogenes* cells.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in Table 1. Preparation of electroporation-competent *E. coli* cells and transformation by electroporation using a TransPoratorPlus (BTX) were as described previously (10). Transformation of *B. subtilis* by chromosomal or plasmid DNA followed the method of Dubnau and Davidoff-Abelson (12). Engineered DNA was introduced into *L. monocytogenes* by conjugation (3) using *E. coli* DW1030/RK231 as the donor strain (17, 55).

E. coli strains were grown at 37°C in Luria (L) broth or on L agar plates with appropriate antibiotics (34). Both kanamycin (50 μg/ml) and ampicillin (100 μg/ml) were added to cultures of *E. coli* strains carrying pHK77 (see below) or its derivatives to prevent loss of intact plasmids. *B. subtilis* strains were grown at 37°C in TSS minimal medium (15) supplemented with 0.2% (wt/vol) glutamine and 0.5% (wt/vol) glucose or 0.5% (wt/vol) succinate. *L. monocytogenes* was grown at 30°C or 37°C in brain heart infusion (BHI) medium (Difco) or HTM (57) supplemented with hemin (0.2 μg/ml) and glutamine (1.6 mg/ml). The following antibiotics were added when necessary: chloramphenicol (2.5 μg/ml), phleomycin (0.25 μg/ml), erythromycin (1 μg/ml), spectinomycin (50 μg/ml), or neomycin (5 μg/ml) for *B. subtilis* and chloramphenicol (10 μg/ml), spectinomycin (100 μg/ml), or neomycin (10 μg/ml) for *L. monocytogenes*.

For measurements of β-galactosidase activity in *B. subtilis*, 1-ml culture samples were harvested and assayed as described previously (27). For β-galactosidase assays with *L. monocytogenes*, cells grown overnight in BHI were either diluted in fresh BHI medium (to give an initial optical density at 600 nm (OD_{600}) of ≅0.05) or washed and diluted in HTM containing hemin and glutamine (to give an initial OD_{600} of ≅0.01). Samples were collected during the subsequent exponential growth and stationary phases, as appropriate.

DNA manipulations. Reactions for restriction enzymes, DNA modification enzymes, DNA ligation, and PCR were carried out in accord with instructions provided by suppliers. *E. coli* plasmids were isolated using the QIAprep miniprep kit (QIAGEN). Methods for agarose and polyacrylamide gel electrophoresis were described by Sambrook et al. (46). Extraction of DNA fragments from agarose or polyacrylamide gels made use of the QIAquick gel extraction kit or the QIAEX II gel extraction kit (QIAGEN), respectively. *B. subtilis* chromosomal DNA was isolated as described by Fouet and Sonenshein (15) using lysozyme at 1 mg/ml. Genomic DNA isolation from *L. monocytogenes* was carried out similarly except that addition of 50 μl of 10% sodium dodecyl sulfate to 450 μl of lysozyme-treated cells was followed by incubation at 70°C for 30 min to facilitate cell lysis.

Construction of *B. subtilis ccpC*::*ble* mutants. *B. subtilis* Δ*ccpC*::*ble* mutant strains (HKB186 and HKB187) were created using the technique of long-flanking-homology PCR (60). First, an 887-bp PCR product corresponding to the 5′ end of the *ccpC* gene (the first 41 codons) and upstream DNA was generated using *B. subtilis* chromosomal DNA and the primers HKO46 (5′-GCCTGATT GCGAATTCGTCTTATCG-3′) and HKO74 (5′-**GGCCCGATTTAAGCACA**CT TATTCAAGCCCTATTCTC-3′: sequence for amplification of *ble* in bold). A second PCR product (820 bp) corresponding to the 3′ end of *ccpC* and downstream DNA was generated using the primers HKO75 (5′-**GCTGAGTCTGGCT TTCG**GAGAAAATCATTCAGTTTGC-3′: sequence for amplification of *ble* in bold) and HKO9 (5′-CATGTTAGGATCCCATTAGTTG-3′). Each pair of primers included one with homology to the *ble* gene (for phleomycin resistance).

TABLE 1. Bacteral strains

Strain	Genotype	Source or reference(s)
E. coli		
DH5α	*supE44 ΔlacU* (ϕ80*lacZΔM15*) *hsdR17 recA1 endA1 gyrA96 thi-1 relA1*	Laboratory stock
DW1030/RK231	*ΔlacX74 rpsE gyrA100 recA13 thi*/Kn^r Tc^r	17, 55
JM107	*F′ traD36 lacI*^{q} Δ(*lacZ*)*M15 proA*$^+$*B*$^+$/e14$^-$(*mcrA*) Δ(*lac-proAB*) *thi gyrA96* (Nal^r) *endA1 hsdR17* (r_K^- m_K^+) *relA1 supE44*	62
B. subtilis		
AF21	Δ*amyE*::Φ(*citBp21-lacZ cat*)	15
HKB165	Δ*amyE*::Φ(*citBp21-lacZ cat*) *citB*::*spc*	26
HKB166	Δ*amyE*::Φ(*citBp21-lacZ cat*) Δ*citA*::*neo* Δ*citZ471 citB*::*spc*	26
HKB186	Δ*amyE*::Φ(*citBp21-lacZ cat*) Δ$ccpC_{Bs}$::*ble*	AF21 × Δ$ccpC_{Bs}$::*ble*
HKB187	Δ*amyE*::Φ(*citBp21-lacZ cat*) Δ$ccpC_{Bs}$::*ble citB*::*spc* Δ*citA*::*neo* Δ*citZ471*	HKB166 × Δ$ccpC_{Bs}$::*ble*
HKB199	Δ*amyE*::Φ(*citBp21-lacZ cat*) Δ$ccpC_{Bs}$::*ble citB*::*spc*	HKB186 × DNA HKB165
HKB218	Δ*amyE*::Φ(*citBp21-lacZ cat*) Δ$ccpC_{Bs}$::*ble* Ω(P_{ccpC}-$ccpC_{Lm}$ *erm*)	HKB186 × pHK99
HKB219	Δ*amyE*::Φ(*citBp21-lacZ cat*) Δ$ccpC_{Bs}$::*ble citB*::*spc* Δ*citA*::*neo* Δ*citZ471* Ω(P_{ccpC}-$ccpC_{Lm}$ *erm*)	HKB187 × pHK99
HKB220	Δ*amyE*::Φ(*citBp21-lacZ cat*) Δ$ccpC_{Bs}$::*ble citB*::*spc* Ω(P_{ccpC}-$ccpC_{Lm}$ *erm*)	HKB199 × pHK99
L. monocytogenes		
EGDe	Animal isolate; genome sequencing strain	16
HKB213	Δ*int*::Φ(*lmo0847′-lacZ neo*)	EGDe × pHK85
HKB214	Δ*int*::Φ($citB_{Lm}$′-*lacZ neo*)	EGDe × pHK86
HKB216	Δ*int*::Φ(*lmo0847′-lacZ neo*) Δ*ccpC*::*spc*	HKB213 × pHK95
HKB217	Δ*int*::Φ($citB_{Lm}$′-*lacZ neo*) Δ*ccpC*::*spc*	HKB214 × pHK95

The two initial PCR products were purified, mixed, and incubated with pJPM136 (a source of *ble*) and primers HKO46 and HKO9 in a third PCR (the two initial PCR products functioned as primers for initial amplification of the *ble* gene). The final PCR product contained the *ble* gene surrounded by sequences homologous to the regions flanking *ccpC*, with a 245-bp segment of the *ccpC* gene deleted, and was used to transform strains AF21 and HKB166 to phleomycin resistance to create strains HKB186 and HKB187, respectively. Introduction of the *ccpC* null mutation was confirmed by PCR.

Integration of $ccpC_{Lm}$ at the *B. subtilis ccpC* locus. pHK99 was constructed by ligating XbaI/BamHI-digested pPS34 [pBluescript SK(−) (Stratagene) containing an erythromycin-resistance gene; P. Serror, unpublished] with a 912-bp NdeI/BamHI DNA fragment carrying the $ccpC_{Lm}$ open reading frame (ORF) isolated from pHK67 and a 443-bp XbaI/NdeI DNA fragment corresponding to the region upstream of *B. subtilis ccpC* that was PCR amplified using *B. subtilis* chromosomal DNA as a template and primers, HKO109 (5′-CGGTTCTAGATCCTTCCTACC-3′: XbaI site underlined) and HKO110 (5′-CTTGAAGCTGCATATGGTCCTC-3′: NdeI site underlined). Transformation of *B. subtilis* strains HKB186, HKB187, and HKB199 with pHK99 yielded Erm^r transformants in which $ccpC_{Lm}$ was integrated at the *B. subtilis ccpC* locus under the control of the *B. subtilis ccpC* regulatory region.

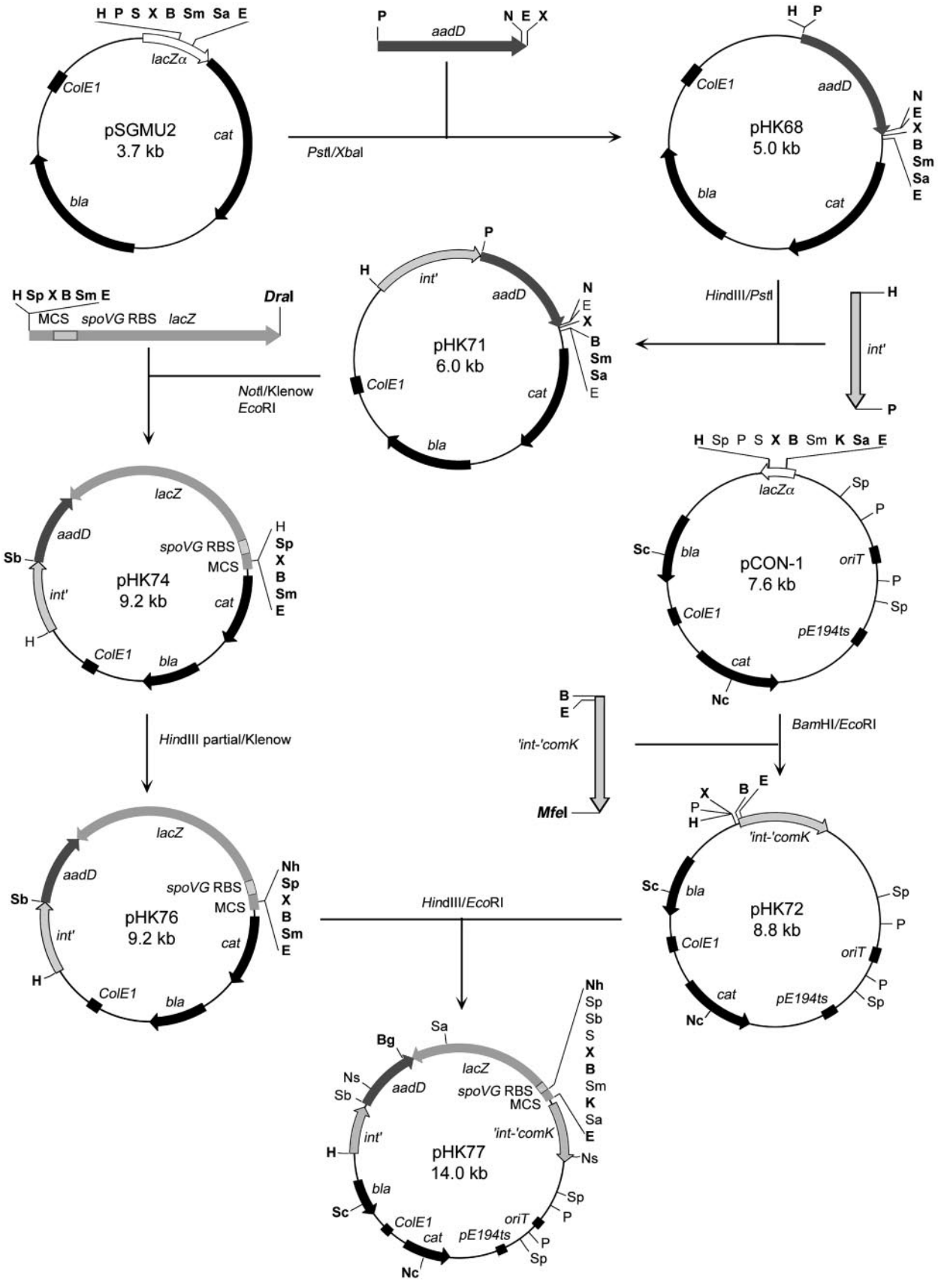
Construction of the integratable *lacZ* fusion vector, pHK77, for *L. monocytogenes*. Plasmid pHK77 was constructed as shown in Fig. 2. A 1.3-kb PstI-XbaI fragment containing the *aadD* gene (encoding neomycin nucleotidyltransferase) was obtained from pBEST501 (20) and cloned in similarly digested pSGMU2 (14), resulting in pHK68. A 1-kb DNA fragment from the N-terminal region of the *L. monocytogenes int* gene (*int′*) was amplified by PCR using chromosomal DNA as a template and HKO90 (5′-CAAGAAAAGAAAAGCTTTCTGATGG-3′: HindIII site underlined) and HKO89 (5′-CAATATACCAATCCTGCAGGACC-3′: PstI site underlined) as primers. The *int′* fragment was digested with HindIII and PstI and cloned in similarly digested pHK68, generating pHK71. The engineered PstI site in pHK71 can also be recognized by SbfI. The *lacZ* gene was derived from pTKlac (24), which was digested with DraI and EcoRI, liberating a 3.2-kb DNA fragment containing the multiple cloning site (MCS), the *B. subtilis spoVG* ribosome binding site, and the *E. coli lacZ* gene. This fragment was cloned in pHK71 that had been digested with NotI, treated with the Klenow fragment to fill in the 5′ overhang generated by NotI, and then digested with EcoRI. The resulting plasmid, pHK74, has two HindIII sites, one at the end of the *int′* fragment and the other in the MCS. In order to have a unique HindIII site, pHK74 was partially digested with HindIII, treated with the Klenow fragment to fill in the 5′ overhang, and religated, creating pHK76, which has a unique HindIII site at the end of the *int′* fragment and a unique NheI site in the MCS. The 1.2 kb *′int-′comK* fragment containing the C-terminal parts of *L. monocytogenes int* and *comK* was amplified from chromosomal DNA by PCR using HKO91 (5′-TTGTCCGGATCCGCTGAATTCGAGCGCGAAAC-3′: BamHI and EcoRI sites underlined) and HKO92 (5′-GTGTGGTTAAAATCCAATTGCATG-3′: MfeI site underlined) as primers. After digestion with BamHI and MfeI, the *′int-′comK* fragment was cloned in pCON-1 (3) that had been digested with BamHI and EcoRI, generating pHK72. The *int′-aadD-lacZ* fragment was then obtained from pHK76 by digestion with HindIII and EcoRI and cloned in similarly digested pHK72, producing pHK77.

Construction of *L. monocytogenes lacZ* fusion strains. To construct the *lacZ* fusion plasmids pHK85 and pHK86, the promoter regions of lmo0847 and $citB_{Lm}$ were PCR amplified from chromosomal DNA of *L. monocytogenes* using primer pairs HKO103 (5′-CGAAAATCTCGAATTCGAAAG-3′: EcoRI site underlined) and HKO98 (5′-CACAAGCGCTAGCCATCATTA-3′: NheI site underlined) or HKO102 (5′-CCACGCGAATTCTTCCTATC-3′: EcoRI site underlined) and HKO97 (5′-GATACGTGCTAGCGTTCAGCTG-3′: NheI site underlined), respectively. The PCR products were digested with EcoRI and NheI, and the products (664 bp for lmo0847 and 537 bp for $citB_{Lm}$) were then cloned independently in pHK77 (see Results), which had been similarly digested.

To create *L. monocytogenes lacZ* fusion strains HKB214 (*citB-lacZ*) and HKB213 (lmo0847-*lacZ*), pHK86 and pHK85, respectively, were transferred by conjugation from cells of *E. coli* DW1030/RK231 to *L. monocytogenes* EGDe, introducing $citB_{Lm}$-*lacZ* or lmo0847-*lacZ* transcriptional fusions at the *int* locus of the *L. monocytogenes* chromosome. A series of PCRs confirmed that the *lacZ* fusions were inserted by double-crossover recombination and that HKB213 and HKB214 did not carry free plasmids (data not shown).

Construction of an *L. monocytogenes ccpC*::*spc* mutant. To create pHK92, a 2.3-kb PstI/EcoRI DNA fragment carrying the $ccpC_{Lm}$ (lmo1010) locus was prepared by PCR amplification with *L. monocytogenes* chromosomal DNA and primers HKO105 (5′-GTAGAAATCTGCAGCGACAATAG-3′: PstI site underlined) and HKO106 (5′-TTAAGTTCACGTGCAGGAATTCC-3′: EcoRI site underlined). After digestion with PstI and EcoRI, the PCR product was cloned in similarly digested pBS(−) (Stratagene). pHK92 was digested with MfeI and XbaI to remove an internal part of $ccpC_{Lm}$ (469 bp) and then ligated with a 1.2-kb EcoRI/SpeI DNA fragment carrying the spectinomycin resistance gene obtained from pJL73 (31), yielding pHK93. A 3-kb EcoRI/HindIII DNA fragment carrying Δ$ccpC_{Lm}$::*spc* was isolated from pHK93 and cloned in similarly digested pCON-1 (3), creating pHK95.

To create a *ccpC* mutant carrying either a $citB_{Lm}$-*lacZ* or lmo0847-*lacZ* fusion, pHK95 was transferred by conjugation from cells of *E. coli* DW1030/RK231 to HKB214 or HKB213. After incubating transconjugants at 41°C in the presence of spectinomycin to select cells with pHK95 integrated into the chromosome, null mutants were purified based on their resistance to spectinomycin and sensitivity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lacZα
ColE1
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Sc
P
ColE1
oriT
cat
pE194ts
P
Sp
Nc

to chloramphenicol. A series of PCRs was performed to confirm that *ccpC*::*spc* was inserted at the *ccpC* locus by double-crossover recombination.

Complementation of an *L. monocytogenes ccpC* mutant. The $ccpC_{Lm}$ coding sequence and 200 bp of upstream sequence were PCR amplified using genomic DNA from *L. monocytogenes* EGDe as a template and primers OMM031 (5′GTCAGCGTCGACGTTGCTCATGTACAGCTTGG 3′: SalI site underlined) and OMM032 (5′ ATCGACGCATGCCCTTCCGATACGTTCTGC 3′: SphI site underlined). A SalI/SphI DNA fragment (1,325 bp), generated from the PCR product, was cloned in similarly digested pAG58 (21), resulting in plasmid pEMM16.

Digestion of pEMM16 with EcoRI and BamHI generated a 3-kb DNA fragment containing the Pspac promoter, *ccpC*, and the *lacI* gene. This fragment was cloned in the similarly digested vector pHP13 (19), resulting in the plasmid pEMM18. The insert was sequenced to confirm that it was free of PCR-generated mutations. Five micrograms of pEMM18 or pHP13 DNA was mixed with 100 μl of electrocompetent cells of strain HKB217 prepared by the method of Camilli et al. (5). After electroporation (2 kV, 400 Ω, 25 μF, 5 ms), cells were diluted in 1 ml BHI containing 0.5 M sucrose and incubated without shaking at 37°C for 60 min. Transformants were selected on BHI plates containing erythromycin.

***L. monocytogenes* CcpC-His$_6$ purification.** The *L. monocytogenes* ORF corresponding to $ccpC_{Lm}$ (lmo1010) was PCR amplified using genomic DNA from *L. monocytogenes* EGDe as a template and primers HKO86 (5′-GGAGGAAAACATATGATTGTAACA-3′: NdeI site underlined) and HKO87 (5′-CCCTTCGGATCCGTTCTGC-3′: BamHI site underlined). An NdeI/BamHI DNA fragment (912 bp), generated from the PCR product, was cloned in similarly digested pET16b (Novagen), resulting in pHK67. The $ccpC_{Lm}$ insert of pHK67 was sequenced to confirm that it was free of PCR-generated mutations. To construct pHK78, a sequence corresponding to the coding sequence and ribosome binding site of $ccpC_{Lm}$ (lmo1010) was PCR amplified using pHK67 as a template, and two primers, HKO95 (5′-GAGAGAATTCATTAAAGAGGAGGAAAATAGATGATTGTAAC-3′: EcoRI site underlined) and HKO96 (5′-GGTTGGTACCTA**ATGATGATGATGATGATG**TTTAACTTGTTCAAGG-3′: KpnI site underlined and six histidine codons in boldface). The PCR product was digested with EcoRI and KpnI, and the 1,026-bp EcoRI/KpnI DNA fragment was cloned in pBAD30 (18), which had been similarly digested.

E. coli DH5α(pHK78) was used to inoculate LB containing ampicillin (50 μg/ml) and incubated overnight at 37°C. The culture was then diluted in fresh medium (1:100) and incubated until the OD_{600} reached 0.5. Arabinose (0.2%) was added to induce expression of $CcpC_{Lm}$-His_6. The culture was incubated for an additional 4 h. Cells were harvested by centrifugation and sonicated in buffer A (50 mM NaH_2PO_4, 300 mM NaCl, 5 mM imidazole, 10% glycerol, 0.05% Nonidet P-40, and 1 mM phenylmethylsulfonyl fluoride). After centrifugation, the supernatant fluid was applied to a column of Ni^+-NTA His-Bind resin (Novagen). The column was washed with buffer A containing increasing amounts of imidazole (10, 20, or 50 mM). $CcpC_{Lm}$-His_6 was eluted with buffer A containing 300 mM imidazole, which was subsequently removed by dialysis using Ultrafree-0.5 centrifugal filter units (Millipore).

Gel mobility shift assays and DNase I footprinting experiments. A 288-bp DNA fragment corresponding to the $citB_{Lm}$ promoter region was amplified by PCR using ^{32}P-labeled HKO82 (5′-CCAAGTTGGATCCAACATTTCC-3′) and HKO83 (5′-GATACGTTTTGGAATTCAGCTG-3′) as forward and reverse primers and genomic DNA from *L. monocytogenes* EGDe as a template. To amplify the promoter region of lmo0847, PCR was conducted using ^{32}P-labeled HKO84 (5′-GCATACGCAAGGATCCAAGAATATG-3′) and HKO85 (5′-CAACACAAGTTCCCATCATTAG-3′) as forward and reverse primers, yielding a 271-bp DNA fragment. For mobility shift assays, the labeled DNA fragments (4,000 cpm) and various amounts of $CcpC_{Lm}$-His_6 were incubated in a 10-μl reaction containing buffer B (20 mM Tris-HCl [pH 8.0], 50 mM NaCl, 10 mM $MgCl_2$, 0.5 mM EDTA, 0.05% [vol/vol] Nonidet P-40, 5% [vol/vol] glycerol, 1 mM dithiothreitol, and calf-thymus DNA [25 μg/ml]) for 15 min at room temperature. The reactions were then loaded on a 6% nondenaturing polyacrylamide gel in Tris-borate-EDTA buffer (89 mM Tris base, 89 mM boric acid, 2 mM EDTA, pH 8.0).

For DNase I footprinting experiments, the ^{32}P-labeled DNA fragments (2×10^4 cpm) and various amounts of the $CcpC_{Lm}$-His_6 protein were incubated in a 20-μl reaction volume containing buffer B as described above. After incubation for 15 min at room temperature, the reactions were supplemented with 6 mM $MgCl_2$ and 6 mM $CaCl_2$ and treated with 0.1 unit of RQ1 DNase I (Promega) for 1 min at room temperature. When citrate or isocitrate was included in the reactions, the concentrations of $MgCl_2$ and $CaCl_2$ were adjusted to 12 mM to compensate for chelation. After addition of EDTA to 20 mM to stop the DNase I reaction, the reaction mixtures were extracted with phenol-chloroform, precipitated with ethanol, and analyzed on 6% polyacrylamide–8 M urea sequencing gels. To create DNA sequence ladders, plasmids pHK85 and pHK86 were subjected to the dideoxy chain termination method (47) using the Sequenase reagent kit (U.S. Biochemical, Inc.), [α-^{35}S]dATP, and primers HKO84 and HKO82, respectively.

RESULTS

Identification of a CcpC homolog and potential target genes for CcpC regulation in *L. monocytogenes*. *B. subtilis* CcpC, a member of the LysR family, generally shows about 30% identity to other family members. We could identify proteins with significantly greater similarity to CcpC in only four bacteria in the current microbial genome database. Putative CcpC orthologs in *Bacillus anthracis*, *Bacillus cereus*, *Listeria innocua*, and *L. monocytogenes* are more than 50% identical to *B. subtilis* CcpC and are encoded by genes whose neighbors are similar to those of *B. subtilis ccpC*.

The *L. monocytogenes ccpC* homolog (lmo1010) is the distal gene of a cluster reminiscent of the *B. subtilis ykuJ-ccpC* operon (Fig. 3A). The CcpC proteins of *B. subtilis* and *L. monocytogenes* are 52% identical over their entire length and 80% identical in their N-terminal helix-turn-helix regions. They also have the same residues at many positions suggested to be involved in dimerization and tetramerization (37). Therefore, it seemed likely that the two proteins would recognize similar DNA sequences. In previous work, we have shown that a common feature of *B. subtilis* CcpC binding sites is an interrupted dyad symmetry sequence (Box I: ATAA-N_7-TTAT) (25). Using this CcpC consensus binding site, we searched the *L. monocytogenes* genome and identified 92 potential target sites for CcpC regulation located between positions −300 and +100 with respect to a translational start site.

Among the 92 genes associated with Box I-like sequences, there were 19 genes for which the dyad symmetry element had at an appropriate distance (28 to 29 bp) a repeat of half of the dyad element (AATA or TTAT; Box II), as is found in the CcpC-binding sites of the *B. subtilis citB* and *citZ* genes. Most of these genes are of unknown function. Among those whose function could be deduced by sequence analysis were homologs of *polC* (DNA polymerase α subunit), *fur* (iron metabolism regulator), lmo1568 (a gene just upstream of the Krebs cycle genes *citZ* and *citC*), $citB_{Lm}$ (aconitase), and lmo0847, which appears to encode a glutamine transporter (Fig. 3B).

Direct interaction of *L. monocytogenes* CcpC ($CcpC_{Lm}$) with promoter regions of $citB_{Lm}$ and lmo0847. The potential Ccp-

FIG. 2. pHK77 construction. pHK77 includes the origin of replication of ColE1 to allow replication in *E. coli*, *oriT* for conjugational transfer from *E. coli* to *Listeria*, and the replication system of pE194*ts* for temperature-sensitive replication in gram-positive bacteria. There are three selection markers: *bla* for ampicillin resistance, *cat* for chloramphenicol resistance, and *aadD* for neomycin (kanamycin) resistance. Important restriction sites are shown, and unique sites are in bold: B, BamHI; Bg, BglII; E, EcoRI; H, HindIII; K, KpnI; N, NotI; Nc, NcoI; Nh, NheI; Ns, NsiI; P, PstI; S, SalI; Sa, SacI; Sb, SbfI; Sc, ScaI; Sm, SmaI; Sp, SphI; and X, XbaI.

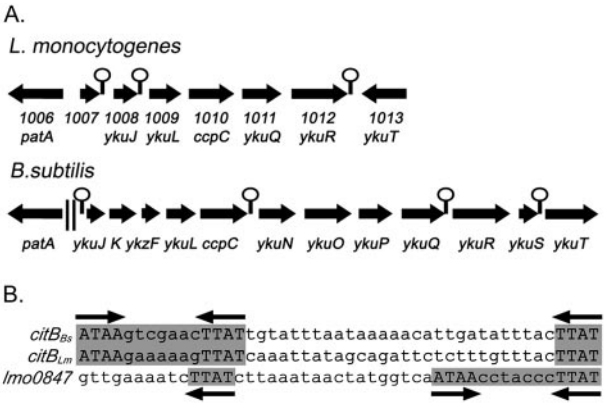

FIG. 3. A. Gene organization of the *ccpC* locus in *L. monocytogenes* and *B. subtilis*. Arrows indicate genes and their orientation, and balloons show locations of putative transcription termination sites. To simplify the figure, the nine *B. subtilis* genes located between *patA* and *ykuJ* have been omitted. *L. monocytogenes* genes are identified by numbers designated at the ListiList database site (http://genolist.pasteur.fr/ListiList/) and by correspondence to *B. subtilis* genes of similar sequence. B. Putative CcpC-binding sites in two *L. monocytogenes* genes, $citB_{Lm}$ and lmo0847, are aligned with the CcpC binding site in *B. subtilis citB* ($citB_{Bs}$). Sequences of Box I and Box II are shaded. Box I contains an interrupted dyad symmetry element, and Box II represents a half-dyad. Consensus sequences are in uppercase, and their orientations are indicated by arrows.

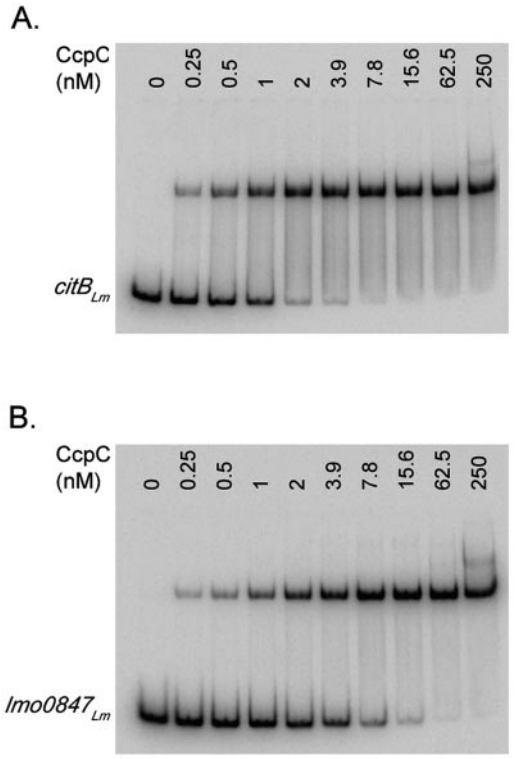

FIG. 4. Gel mobility shift assay of interaction of *L. monocytogenes* CcpC with the $citB_{Lm}$ and lmo0847 promoter regions. $CcpC_{Lm}$-His_6 was incubated with ^{32}P-labeled DNA fragments containing the promoter regions of either $citB_{Lm}$ (A) or lmo0847 (B). The concentration of $CcpC_{Lm}$-His_6 (nM) used in each reaction is shown at the top of each lane.

C_{Lm} target sites of the $citB_{Lm}$ and lmo0847 genes are located upstream of the respective ORFs. In vitro gel mobility shift assays established that *B. subtilis* CcpC recognizes these sites with apparently high affinity (data not shown). To see if Ccp-C_{Lm} is able to interact with these potential target sites, we overexpressed and purified a His_6-tagged version of $CcpC_{Lm}$ (see Materials and Methods) and tested its interaction with the $citB_{Lm}$ and lmo0847 regulatory regions by gel mobility shift assays (Fig. 4). A 288-bp DNA fragment carrying the potential CcpC binding site of $citB_{Lm}$ showed retarded mobility when incubated with the $CcpC_{Lm}$-His_6 protein (Fig. 4A). The concentration of $CcpC_{Lm}$-His_6 needed to retard 50% of the DNA molecules (as an approximation of the K_d) was 1 nM. When a 271-bp DNA fragment encompassing the potential target site of lmo0847 was used, $CcpC_{Lm}$-His_6 bound with an apparent K_d of 3 nM (Fig. 4B). The affinity of *B. subtilis* CcpC for these fragments was similar (data not shown).

To determine whether the Box I-Box II regions are the specific targets for CcpC interaction, DNase I footprinting studies were carried out. The potential target site of $citB_{Lm}$ contains Box I at positions −113 to −99 and Box II at positions −70 to −67, with respect to the translational start codon. In the presence of $CcpC_{Lm}$-His_6, the region from positions −115 to −62 was protected against DNase I digestion (Fig. 5A), confirming that the apparent Box I and Box II elements are indeed part of the target site for the $CcpC_{Lm}$ binding. The potential target site upstream of lmo0847 is comprised of Box I at positions −99 to −85 and two potential Box II sequences, one at positions −56 to −53 and the other at positions −121 to −118 with respect to the start codon. The region protected by $CcpC_{Lm}$-His_6 from DNase I digestion (positions −135 to −83) overlapped with Box I and with the potential Box II sequence at positions −121 to −118 (Fig. 6A). A 29-bp spacer separates the Box II TTAT sequence from the Box I TTAT sequence.

Effect of citrate on the interaction of $CcpC_{Lm}$ with its target sites. *B. subtilis* CcpC represses expression of its target genes, *citZ*, *citB*, and *ccpC*, and citrate specifically relieves repression of *citZ* and *citB* but not *ccpC* by causing dissociation of CcpC from its binding sites (23, 25). The effect of citrate is different at the *citZ* and *citB* promoters, however. For *B. subtilis citB*, citrate inhibits CcpC binding to Box II but not to Box I (23), but for *citZ*, binding to both boxes is affected (23). (Binding of CcpC to the *ccpC* promoter relies on a Box I sequence only and is not affected by citrate [25]). Since the CcpC binding sites in the *L. monocytogenes citB* and lmo0847 regulatory regions have both Box I and Box II motifs, we repeated the DNase I footprinting experiments in the presence of citrate. While Ccp-C_{Lm} protected a region surrounding both Box I and Box II of $citB_{Lm}$ (positions −115 to −62) in the absence of citrate (Fig. 5B, lanes 2 and 7), addition of citrate caused the Box II region (positions −90 to −62) to become sensitive to DNase I digestion, and two positions (−122 and −81) showed hypersensitivity to DNase I digestion (Fig. 5B, lanes 3 to 5). When isocitrate was used instead of citrate, DNase I protection was not affected (Fig. 5B, lanes 8 to 10), indicating that the citrate effect on $CcpC_{Lm}$ binding is specific. A potential promoter region for $citB_{Lm}$ has a −35 region that overlaps with Box II (Fig. 5C). Thus, citrate appears to specifically release $CcpC_{Lm}$ from Box

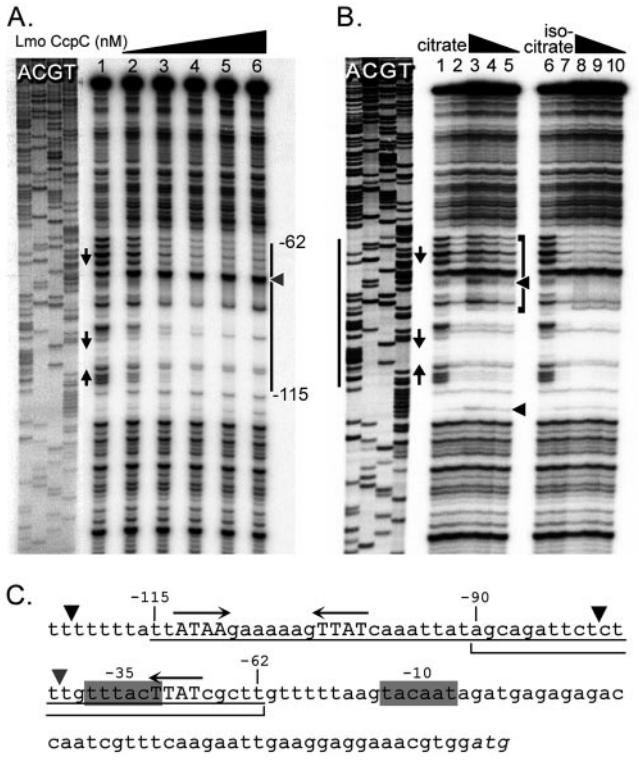

FIG. 5. DNase I footprinting assay of interaction between $CcpC_{Lm}$-His_6 and $citB_{Lm}$. A. A ^{32}P-labeled DNA fragment corresponding to the putative $citB_{Lm}$ promoter region was incubated with different amounts of $CcpC_{Lm}$-His_6 prior to DNase I digestion. Protein concentrations were as follows: 0 (lane 1), 3.9 (lane 2), 15.6 (lane 3), 62.5 (lane 4), 250 (lane 5), and 1,000 nM (lane 6). Sequence ladders obtained by Sanger sequencing of the same DNA fragment are shown to the left. Arrows show the elements of the putative CcpC binding site identified by genome search, and the vertical line indicates a region protected by $CcpC_{Lm}$-His_6 from DNase I digestion. The arrowhead shows a site of hypersensitivity to DNase I digestion in the presence of $CcpC_{Lm}$. B. Effect of citrate on the interaction of $CcpC_{Lm}$-His_6 with $citB_{Lm}$. The same DNA probe as in part A was incubated with $CcpC_{Lm}$-His_6 (0 nM, lanes 1 and 6; 62.5 nM, lanes 2 to 5 and 7 to 10) in the presence of citrate (0.5%, lane 3; 0.25%, lane 4; 0.13%, lane 5) or isocitrate (0.5%, lane 8; 0.25%, lane 9; 0.13%, lane 10). The bracket indicates a region where protection by $CcpC_{Lm}$-His_6 was inhibited specifically by citrate. The black arrowheads indicate sites of hypersensitivity to DNase I that appeared only in the presence of citrate. C. Sequence of the $citB_{Lm}$ promoter region. The region protected by $CcpC_{Lm}$-His_6 from DNase I digestion (positions −115 to −62) is underlined, and the region where $CcpC_{Lm}$-His_6 was released by citrate (positions −90 to −62) is indicated by a bracket. The −35 and −10 regions of the putative promoter are shaded in gray, and the translational start codon is in italics. Arrows show the elements of the putative CcpC binding site identified by genome search. The gray arrowheads show sites of hypersensitivity to DNase I digestion in the presence of $CcpC_{Lm}$.

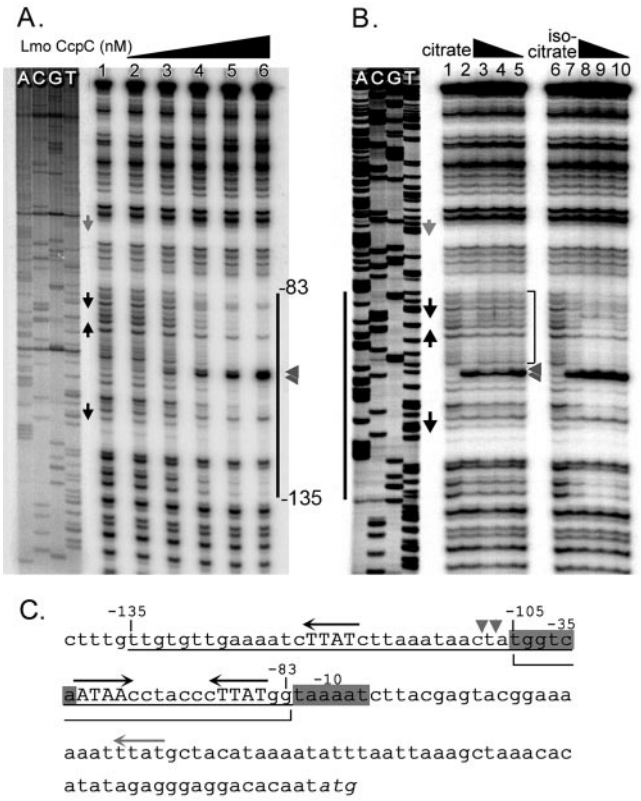

FIG. 6. DNase I footprinting assay of interaction between $CcpC_{Lm}$-His_6 and lmo0847. A. A ^{32}P-labeled DNA fragment corresponding to the putative lmo0847 promoter region was incubated with different amounts of $CcpC_{Lm}$-His_6 prior to DNase I digestion. Protein concentrations were as follows: 0 (lane 1), 3.9 (lane 2), 15.6 (lane 3), 62.5 (lane 4), 250 (lane 5), and 1,000 nM (lane 6). Sequence ladders obtained by Sanger sequencing of the same DNA fragment are shown to the left. Arrows show the elements of the putative CcpC binding site identified by genome search, and the vertical line indicates a region protected by $CcpC_{Lm}$-His_6 from DNase I digestion. The gray arrowheads show sites of hypersensitivity to DNase I digestion in the presence of $CcpC_{Lm}$. B. Effect of citrate on the interaction of $CcpC_{Lm}$-His_6 with lmo0847. The same DNA probe as in part A was incubated with $CcpC_{Lm}$-His_6 (0 nM, lanes 1 and 6; 62.5 nM, lanes 2 to 5 and 7 to 10) in the presence of citrate (0.5%, lane 3; 0.25%, lane 4; 0.13%, lane 5) or isocitrate (0.5%, lane 8; 0.25%, lane 9; 0.13%, lane 10). The bracket indicates a region where protection by $CcpC_{Lm}$-His_6 was inhibited specifically by citrate. The gray arrowheads indicate a site of hypersensitivity to DNase I that was abrogated in the presence of citrate. C. Sequence of the lmo0847 promoter region. The region protected by $CcpC_{Lm}$-His_6 from DNase I digestion (positions −135 to −83) is underlined, and the region where $CcpC_{Lm}$-His_6 was released by citrate (positions −105 to −83) is indicated by a bracket. The −35 and −10 regions of the putative promoter are shaded in gray, and the translational start codon is in italics.

II of the $citB_{Lm}$ promoter and thereby allow RNA polymerase to gain access to the promoter and initiate transcription.

We carried out a parallel experiment with the lmo0847 promoter region to see if citrate causes a similar alteration in the pattern of $CcpC_{Lm}$ binding. While the region including Box I and Box II (positions −135 to −83) was protected by $CcpC_{Lm}$-His_6 in the absence of citrate (Fig. 6B, lanes 2 and 7), the region surrounding Box I (positions −105 to −83) became more sensitive to DNase I digestion upon addition of citrate (Fig. 6B, lanes 3 to 5). In addition, the hypersensitivity to DNase I digestion at positions −108 and −107 caused by $CcpC_{Lm}$-His_6 binding became less obvious when citrate was present, further confirming that citrate causes dissociation of $CcpC_{Lm}$-His_6 from part of the lmo0847 promoter region. Since Box I lies between the putative −10 and −35 sequences of the lmo0847 promoter (Fig. 6C), release of $CcpC_{Lm}$ from this site in the presence of citrate would allow access of RNA polymerase to the promoter site. When isocitrate was used instead of citrate, no dissociation of $CcpC_{Lm}$-His_6 from its binding sites

TABLE 2. β-Galactosidase activities in *L. monocytogenes* strains carrying $citB_{Lm}$-*lacZ* and lmo0847-*lacZ* fusions[a]

Strain	Relevant genotype	Fusion	β-Galactosidase activity[b]
HKB214	$ccpC^+$	$citB_{Lm}$-*lacZ*	17.6
HKB217	Δ$ccpC_{Lm}$::*spc*	$citB_{Lm}$-*lacZ*	40.3
HKB213	$ccpC^+$	*lmo0847-lacZ*	4.0
HKB216	Δ$ccpC_{Lm}$::*spc*	*lmo0847-lacZ*	12.6

[a] Cultures were grown at 30°C in HTM medium supplemented with hemin (0.2 μg/ml) and glutamine (1.6 mg/ml). In this medium, glucose and glutamine serve as the principal carbon and nitrogen sources. Several samples were taken in mid-exponential growth phase (OD_{600} = 0.12 to 0.41) for β-galactosidase assays. Enzyme activities of the various samples were averaged.

[b] Miller units. Values are the averages for at least two experiments, and variation was less than 20%.

was seen. Instead, $CcpC_{Lm}$-His_6 seemed to bind more tightly to the lmo0847 promoter region in the presence of isocitrate (Fig. 6B, lanes 8 to 10).

Construction of pHK77, a new *lacZ* reporter vector for *L. monocytogenes*. We developed a new reporter system in order to study in vivo promoter activity in *L. monocytogenes*. Plasmid pHK77 (Fig. 2) has a promoterless *lacZ* gene downstream of the *B. subtilis spoVG* ribosomal binding site, which is functional in gram-positive bacteria. The multiple cloning sites located upstream of the *spoVG* ribosomal binding site allow cloning of a DNA fragment containing a promoter region of interest, leading to β-galactosidase production.

Plasmid pHK77 carries the ColE1 replicon from pBR322 for replication in gram-negative bacteria and the RP4 origin of transfer (*oriT*) (39) to direct conjugative transfer from *E. coli* to *L. monocytogenes*. pHK77 also contains a temperature-sensitive version of the pE194 origin of replication (*pE194ts*), which allows replication in gram-positive bacteria at temperatures ≤30°C (59). There are three antibiotic selection markers on this plasmid: the *bla* gene codes for ampicillin resistance in *E. coli*; the *aadD* and *cat* genes, respectively, code for kanamycin (neomycin) and chloramphenicol resistance in *E. coli* and *L. monocytogenes*. The *lacZ* expression module and *aadD* are flanked by sequences from the *L. monocytogenes int-comK* locus (30), facilitating single-copy integration of the *lacZ* reporter system at that locus by homologous, double-crossover recombination. Therefore, upon conjugational transfer to *L. monocytogenes*, pHK77 can be maintained as a multicopy plasmid at ≤30°C, with selection for resistance to neomycin and chloramphenicol. By incubating at 41°C in the presence of neomycin, one can select clones in which pHK77 has integrated into the *int-comK* locus on the chromosome. Moreover, clones in which the *lacZ* reporter system has integrated by double-crossover recombination can be distinguished by their resistance to neomycin and sensitivity to chloramphenicol.

CcpC represses expression of $citB_{Lm}$ and lmo0847. To see if $CcpC_{Lm}$ acts as a repressor of $citB_{Lm}$ and lmo0847 in vivo, a *ccpC* null mutation was introduced into strains carrying the $citB_{Lm}$-*lacZ* or lmo0847-*lacZ* fusion. Wild-type (HKB214) and *ccpC* mutant (HKB217) strains were grown at 30°C in HTM defined medium containing glucose as the sole carbon source and supplemented with hemin (0.2 μg/ml) and glutamine (1.6 mg/ml) (57). β-Galactosidase activity was measured in samples collected during exponential growth phase. The $citB_{Lm}$-*lacZ* expression in the *ccpC* mutant was 2.3-fold higher than that in the wild-type strain (Table 2), indicating that $CcpC_{Lm}$ acts as a negative regulator of $citB_{Lm}$.

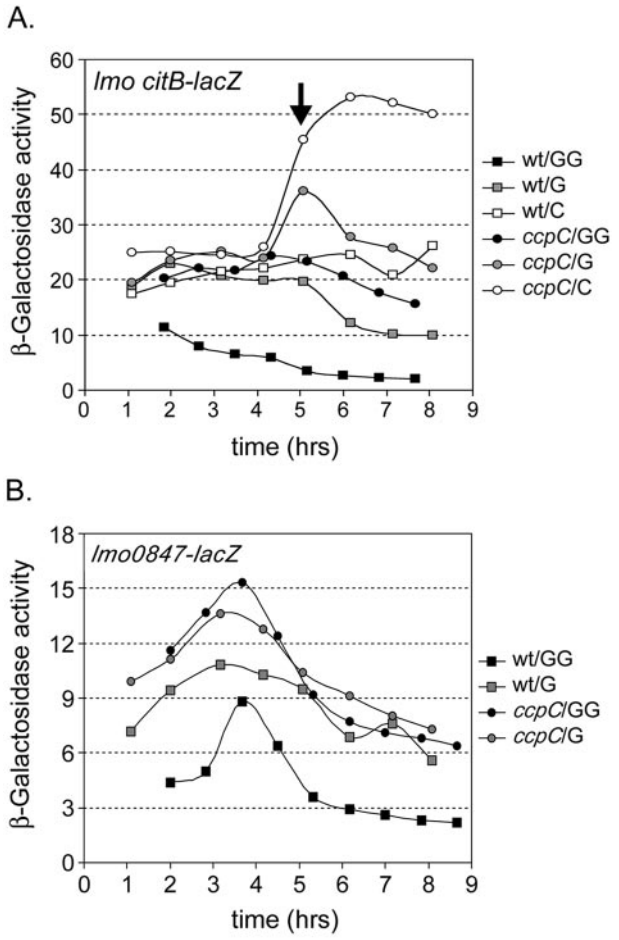

FIG. 7. Effect of a *ccpC* mutation on expression of $citB_{Lm}$ and lmo0847 in BHI medium. β-Galactosidase activity (Miller units) was measured in cells grown at 37°C in BHI medium supplemented with 0.5% glucose and 1.6 mg/ml glutamine (GG), 0.5% glucose (G), or 0.5% citrate (C). A. β-Galactosidase activity was measured in wild-type (HKB214) and *ccpC* mutant (HKB217) $citB_{Lm}$-*lacZ* fusion strains. An arrow indicates the time point when cells entered stationary growth phase. B. β-Galactosidase activity was measured in wild-type (HKB213) and *ccpC* mutant (HKB216) lmo0847-*lacZ* fusion strains.

The activity of $citB_{Lm}$-*lacZ* was also measured in cells grown at 37°C in a complex medium, BHI. When BHI was supplemented with glucose and glutamine, $citB_{Lm}$-*lacZ* expression was maintained at a very low level throughout growth in the wild type (Fig. 7A, wt/GG) and partially derepressed in the *ccpC* mutant (Fig. 7A, *ccpC*/GG). When BHI was supplemented with either glucose or citrate, but not glutamine, $citB_{Lm}$-*lacZ* expression in the wild type (Fig. 7A, wt/G and wt/C) was derepressed to a level similar to that in the *ccpC* mutant grown with both glucose and glutamine (Fig. 7A, *ccpC*/GG), suggesting that $CcpC_{Lm}$ is fully active only when an excess of both glucose and glutamine is available. When the *ccpC* mutant was grown in BHI supplemented either with glucose or citrate but not glutamine (Fig. 7A, *ccpC*/G or *ccpC*/C), $citB_{Lm}$-*lacZ* expression was similar during exponential growth phase to

TABLE 3. Complementation of an *L. monocytogenes ccpC* mutant[a]

Strain	Relevant genotype	Fusion	β-Galactosidase activity[b]
HKB214	$ccpC^+$	$citB_{Lm}$-*lacZ*	8.0
HKB217	Δ$ccpC_{Lm}$::*spc*	$citB_{Lm}$-*lacZ*	18.6
HKB217(pEMM18)	Δ$ccpC_{Lm}$::*spc* (pEMM18 [$ccpC^+$])	$citB_{Lm}$-*lacZ*	11.5
HKB217(pHP13)	Δ$ccpC_{Lm}$::*spc* (pHP13)	$citB_{Lm}$-*lacZ*	19.4

[a] Cultures were grown at 30°C in HTM medium supplemented with hemin (0.2 μg/ml) and glutamine (1.6 mg/ml). Samples were taken in mid-exponential growth phase (OD_{600} = 0.21 to 0.25) for β-galactosidase assays.
[b] Miller units. Values are the averages for at least two experiments, and variation was less than 10%.

that in the *ccpC* mutant grown in BHI with glucose and glutamine (Fig. 7A, *ccpC*/GG). However, as cells entered stationary phase, $citB_{Lm}$ expression in the *ccpC* mutant showed an additional increase in BHI-glucose (Fig. 7A, *ccpC*/G) and an even further increase in BHI-citrate (Fig. 7A, *ccpC*/C). This result suggests that $CcpC_{Lm}$ is a major regulator of $citB_{Lm}$ in complex medium and that there is an additional factor involved in controlling expression of the $citB_{Lm}$ gene whose activity changes as cells enter stationary phase. This unidentified factor might respond to cell population density or nutrient availability.

To verify that the effects attributed to the Δ*ccpC*::*ble* mutation were not due to another, unrecognized mutation, we constructed a low-copy-number plasmid, derived from pHP13 (19), in which the $ccpC_{Lm}$ gene was expressed from the P*spac* promoter (see Materials and Methods). Introduction of this plasmid (pEMM18) into strain HKB217 restored repression of the *citB-lacZ* fusion to nearly the same level as in $ccpC^+$ cells (Table 3). As a control, we showed that pHP13 itself had no effect on *citB-lacZ* expression in strain HKB217 (Table 3). Note that in this series of experiments, β-galactosidase activities were about twofold lower than those in the experiments of Table 2.

To study expression of lmo0847, wild-type (HKB213) and *ccpC* mutant (HKB216) strains were grown in HTM supplemented with hemin and glutamine, and β-galactosidase activity of the lmo0847-*lacZ* fusion was measured in samples taken during exponential growth phase (Table 2). The expression of lmo0847 for the *ccpC* mutant was threefold higher than that for the wild type, indicating that $CcpC_{Lm}$ is a negative regulator of lmo0847. When strains were grown in BHI, lmo0847 expression was repressed in wild-type cells when glucose and glutamine were both supplied and derepressed when only glucose was provided, when citrate was the carbon source added, or when the *ccpC* gene was inactivated (Fig. 7B; also data not shown). The pattern of expression in both wild-type and mutant strains suggests an additional level of growth-phase-responsive regulation (Fig. 7B).

Effect of citrate on the in vivo activity of $CcpC_{Lm}$. Since the binding activity of $CcpC_{Lm}$ was modulated by citrate in vitro, we tested whether citrate availability affects $CcpC_{Lm}$ activity in vivo. Because of the ready availability of mutant strains, *B. subtilis* was used as a surrogate host for these experiments. In *B. subtilis*, a *citB* null mutation causes the cell to accumulate very high amounts of citrate, as a result of which CcpC is completely inactivated as a repressor (26). On the other hand, a *citA citZ* mutant cannot synthesize citrate. In such a mutant strain, CcpC is highly active and *citB* is not expressed (26). We used a $citB_{Bs}$-*lacZ* fusion to monitor the activity of $CcpC_{Lm}$ in strains in which $ccpC_{Lm}$ replaced $ccpC_{Bs}$. $CcpC_{Lm}$ was shown to interact well with the $citB_{Bs}$ promoter region in vitro (data not shown). The $ccpC_{Lm}$ ORF was cloned under the control of the *B. subtilis ccpC* promoter in pHK99 and introduced into the *ccpC* locus in *B. subtilis* strains HKB186 ($ccpC_{Bs}$::*ble*), HKB199 ($ccpC_{Bs}$::*ble citB*), and HKB187 ($ccpC_{Bs}$::*ble citB citA citZ*), yielding strains HKB218, HKB220, and HKB219, respectively.

When *B. subtilis* cells were grown in TSS minimal medium containing glucose and glutamine, expression of the $citB_{Bs}$-*lacZ* fusion was increased more than 30-fold for a *ccpC* mutant (HKB186) compared to that for the wild type (AF21) (Table 4). Synthesis of $CcpC_{Lm}$ repressed *citB* expression sixfold in strain HKB218 (Table 4), indicating that $CcpC_{Lm}$ is active in this condition and that it is possible to analyze its activity in response to citrate availability.

Next, strains HKB218, HKB219, and HKB220 were grown in nutrient broth medium (DSM), and β-galactosidase activity was measured in samples collected throughout growth. The *citB-lacZ* activity in strain HKB218 showed the pattern of *citB* expression typically observed with a *B. subtilis* wild-type strain (AF21) (26), i.e., repression in early exponential growth phase, dramatic derepression in mid-exponential growth phase, and rapid restoration of repression as cells enter stationary growth phase (Fig. 8). When the cells accumulated citrate as a result of a *citB* null mutation (HKB220), *citB* expression was induced as in the wild-type background (HKB218) but remained high during stationary growth phase (Fig. 8). When cells were not able to produce citrate due to the introduction of *citA* and *citZ* mutations (HKB219), *citB* expression was repressed throughout growth. These results indicate that $CcpC_{Lm}$ is active as a

TABLE 4. Complementation of a *B. subtilis ccpC* mutant by $ccpC_{Lm}$[a]

B. subtilis genotype	β-Galactosidase activity of *citB-lacZ* fusion[b]
$ccpC_{Bs}{}^+$	1.4
$ccpC_{Bs}$::*ble*	44.2
$ccpC_{Bs}$::*ble*/$ccpC_{Lm}{}^+$	7.5

[a] *B. subtilis* strains AF21 (wild-type), HKB186 ($ccpC_{Bs}$::*ble*), and HKB218 ($ccpC_{Bs}$::*ble*/$ccpC_{Lm}{}^+$) were grown in TSS minimal medium containing glucose and glutamine, and samples were collected during mid-exponential growth phase (OD_{600} = 0.23 to 0.82) for β-galactosidase assays. Enzyme activities of the various samples were averaged.
[b] Miller units. Values are the averages for at least two experiments, and variation was less than 20%.

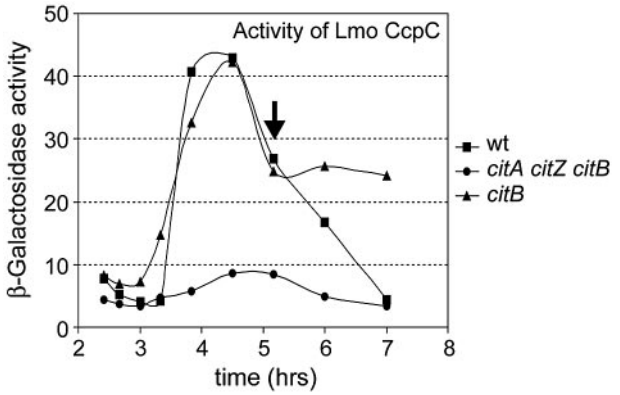

FIG. 8. Effect of citrate on $CcpC_{Lm}$ activity. β-Galactosidase activity (Miller units) of a *B. subtilis citB-lacZ* fusion strain was measured in *B. subtilis ccpC* (*ccpC*$_{Bs}$::*ble*) mutant strains expressing *L. monocytogenes ccpC* (*ccpC*$_{Lm}$). *B. subtilis ccpC*$_{Bs}$::*ble*/*ccpC*$_{Lm}$$^{+}$ strains HKB218 (wild-type), HKB219 (*citA citZ citB*), and HKB220 (*citB*) were grown in DSM, and samples were taken at various times during growth for β-galactosidase assay. An arrow indicates the time at which cells entered stationary growth phase.

repressor in the absence of citrate and inactive when citrate accumulates.

DISCUSSION

Our results show that $CcpC_{Lm}$ acts as a repressor of *citB*$_{Lm}$ and lmo0847 in vivo and binds to the regulatory regions of these genes in vitro. DNA binding in vitro is inhibited by citrate. Citrate may also be the in vivo effector of $CcpC_{Lm}$ activity, based on studies of the activity of $CcpC_{Lm}$ when expressed in *B. subtilis*. In *B. subtilis*, citrate production is controlled by repression of *citZ* by CcpA and CcpC (23, 27) and by feedback inhibition of citrate synthase by 2-ketoglutarate (H.-J. Kim, unpublished data). In *L. monocytogenes*, it is not known at present how the citrate concentration in vivo is controlled. The *L. monocytogenes* homolog of *citZ* may be regulated by CcpC, as it is in *B. subtilis*, since the gene immediately upstream of *citZ* (lmo1568) has an apparent CcpC binding site in its putative promoter region. No apparent transcription terminator separates lmo1568 and *citZ*. Because *L. monocytogenes* does not have an intact Krebs citric acid cycle and cannot use intermediates of the pathway, such as succinate or citrate, as the sole carbon source for growth, it has been suggested that the function of the cycle is strictly anabolic (56), i.e., to provide 2-ketoglutarate as a precursor to glutamate and glutamine (and all compounds derived from them). It would then be logical for the cell to use 2-ketoglutarate (or glutamine) as a key factor for the regulation of citrate concentration and $CcpC_{Lm}$ activity. Our finding that $CcpC_{Lm}$ is most active as a repressor when the growth medium contains excess glutamine is consistent with such a mechanism. It will be interesting to analyze regulation of *citZ* expression at both the transcriptional and posttranslational levels.

lmo0847 encodes a protein with high similarity to a component of ABC-type glutamine transporters from many gram-positive bacteria. Generally an ABC transporter system is comprised of three types of proteins, a solute-binding protein, a permease, and an ATP-binding protein. The Lmo0847 protein has an apparent solute-binding domain in the N-terminal region and a potential permease domain in the C-terminal region. lmo0847 and the immediate downstream gene (lmo0848), which is highly similar to genes for ATP-binding proteins, seem to be organized in a single transcription unit that is surrounded by potential transcription terminators. Thus, $CcpC_{Lm}$ appears to control glutamine transport into the cells, in direct or indirect response to glutamine and citrate availability. Thus, $CcpC_{Lm}$ regulates the synthesis of glutamate and 2-ketoglutarate via both anabolic and catabolic pathways.

It would not be surprising to find that enzymes of central metabolism, such as those that catalyze the reactions of the tricarboxylic acid branch of the Krebs cycle, play a critical role in virulence. These enzymes form the only de novo pathway of glutamate biosynthesis. Glutamate is usually the most abundant amino acid in bacterial cells and the cell's major anion. Hence, regulating the level of glutamate biosynthesis in response to nutritional availability inside a host cell should be a critical concern for the bacterium. Moreover, metabolism of citrate must be carefully controlled. Citrate is an important carrier of cations (e.g., magnesium and iron). Excess citrate accumulation, however, can have deleterious effects by lowering the environmental pH and by chelating, both extracellularly and intracellularly, cations needed for enzymatic reactions (8). Finally, the aconitase protein has both enzymatic and nonenzymatic functions. In addition to this protein's role in metabolism, aconitase proteins from *E. coli* and *B. subtilis* have been shown to bind to RNA (1, 53, 54), suggesting that these proteins play a role in posttranscriptional control of gene expression. Whether the effects of aconitase mutations on virulence of *Staphylococcus aureus* (52), *Xanthomonas campestris* (61), and *Pseudomonas aeruginosa* (51) are due to interrupting enzyme activity or RNA-binding activity remains to be determined.

β-Galactosidase assays of the *citB*$_{Lm}$-*lacZ* fusion in cells grown in BHI show that regulation of *citB*$_{Lm}$ is both $CcpC_{Lm}$ dependent and growth phase dependent. An additional factor is involved in the regulation, because *citB*$_{Lm}$ expression in a *ccpC* mutant increases when excess glutamine is removed from the growth medium and increases further when citrate replaces glucose in the medium. The lmo0847-*lacZ* fusion also showed growth-phase-dependent expression. In preliminary experiments, *citB*$_{Lm}$ expression was induced in BHI supplemented with excess glucose but not glutamine, a condition in which $CcpC_{Lm}$ is inactive, upon addition of decoyinine (H. J. Kim, unpublished data). Since decoyinine inhibits synthesis of GTP, we suggest that the second regulator is a GTP-sensing protein, such as CodY, a protein known to be activated as a repressor in *B. subtilis* by GTP (42). *L. monocytogene*s CodY shares 79% overall identity with *B. subtilis* CodY and 97% identity in the putative GTP-binding and DNA-binding residues.

In order to study regulation of gene expression in any organism, it is very useful to have a reporter system to monitor promoter activity in vivo. The *E. coli lacZ* gene has been previously used to study expression of *L. monocytogenes* genes (36). It has been general practice, however, to fuse the promoter region of an *L. monocytogenes* gene to a promoterless *lacZ* gene and introduce the entire construct into the *L. monocytogenes* chromosome through single-crossover recombination

between the promoter region of the fusion and its corresponding region on the chromosome. This method has several limitations. First, since homologous recombination seems to be a very rare event in *L. monocytogenes*, the DNA fragment fused to the *lacZ* gene has to be at least 1 kb in size. This size limitation makes it difficult to map regulatory elements precisely. Second, the activity of a fusion introduced at the homologous locus can be affected by transcription originating from upstream regions even if those regions are not included in the original fusion construct. Third, single-crossover recombination leaves vector sequences in the chromosome, which may make it difficult to introduce another fusion or mutation into the same strain. These problems can be eliminated by using a vector such as pHK77. Because pHK77 integrates into an ectopic region of the chromosome, there is no minimal size for the promoter-containing DNA fragment used for the fusion. In addition, pHK77 was designed in such a way that the activity of the fusion is not affected by the region upstream of the fusion. Upon integration by double-crossover recombination, the vector part of the plasmid is lost, allowing introduction into the same strain of another version of the same plasmid, if necessary. As we have shown here, this new reporter system can be used effectively to study gene expression in *L. monocytogenes*.

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REFERENCES

1. **Alen, C., and A. L. Sonenshein.** 1999. *Bacillus subtilis* aconitase is an RNA-binding protein. Proc. Natl. Acad. Sci. USA **96:**10412–10417.
2. **Behari, J., and P. Youngman.** 1998. A homolog of CcpA mediates catabolite control in *Listeria monocytogenes* but not carbon source regulation of virulence genes. J. Bacteriol. **180:**6316–6324.
3. **Behari, J., and P. Youngman.** 1998. Regulation of *hly* expression in *Listeria monocytogenes* by carbon sources and pH occurs through separate mechanisms mediated by PrfA. Infect. Immun. **66:**3635–3642.
4. **Busque, P., A. Letellier, J. Harel, and J. D. Dubreuil.** 1995. Production of *Escherichia coli* STb enterotoxin is subject to catabolite repression. Microbiology **141:**1621–1627.
5. **Camilli, A., L. G. Tilney, and D. A. Portnoy.** 1993. Dual roles of *plcA* in *Listeria monocytogenes* pathogenesis. Mol. Microbiol. **8:**143–157.
6. **Chakraborty, T., M. Leimeister-Wachter, E. Domann, M. Hartl, W. Goebel, T. Nichterlein, and S. Notermans.** 1992. Coordinate regulation of virulence genes in *Listeria monocytogenes* requires the product of the *prfA* gene. J. Bacteriol. **174:**568–574.
7. **Cossart, P., J. Pizarro-Cerda, and M. Lecuit.** 2003. Invasion of mammalian cells by *Listeria monocytogenes*: functional mimicry to subvert cellular functions. Trends Cell Biol. **13:**23–31.
8. **Craig, J. E., M. J. Ford, D. C. Blaydon, and A. L. Sonenshein.** 1997. A null mutation in the *Bacillus subtilis* aconitase gene causes a block in Spo0A-phosphate-dependent gene expression. J. Bacteriol. **179:**7351–7359.
9. **Dingman, D. W., and A. L. Sonenshein.** 1987. Purification of aconitase from *Bacillus subtilis* and correlation of its N-terminal amino acid sequence with the sequence of the *citB* gene. J. Bacteriol. **169:**3062–3067.
10. **Dower, W. J., J. F. Miller, and C. W. Ragsdale.** 1988. High efficiency transformation of *E. coli* by high voltage electroporation. Nucleic Acids Res. **16:**6127–6145.
11. **Dramsi, S., C. Kocks, C. Forestier, and P. Cossart.** 1993. Internalin-mediated invasion of epithelial cells by *Listeria monocytogenes* is regulated by the bacterial growth state, temperature and the pleiotropic activator *prfA*. Mol. Microbiol. **9:**931–941.
12. **Dubnau, D., and R. Davidoff-Abelson.** 1971. Fate of transforming DNA following uptake by competent *Bacillus subtilis*. I. Formation and properties of the donor-recipient complex. J. Mol. Biol. **56:**209–221.
13. **Farber, J. M., and P. I. Peterkin.** 1991. *Listeria monocytogenes*, a food-borne pathogen. Microbiol. Rev. **55:**476–511.
14. **Fort, P., and J. Errington.** 1985. Nucleotide sequence and complementation analysis of a polycistronic sporulation operon, *spoVA*, in *Bacillus subtilis*. J. Gen. Microbiol. **131:**1091–1105.
15. **Fouet, A., and A. L. Sonenshein.** 1990. A target for carbon source-dependent negative regulation of the *citB* promoter of *Bacillus subtilis*. J. Bacteriol. **172:**835–844.
16. **Glaser, P., L. Frangeul, C. Buchrieser, C. Rusniok, A. Amend, F. Baquero, P. Berche, H. Bloecker, P. Brandt, T. Chakraborty, A. Charbit, F. Chetouani, E. Couve, A. de Daruvar, P. Dehoux, E. Domann, G. Dominguez-Bernal, E. Duchaud, L. Durant, O. Dussurget, K. D. Entian, H. Fsihi, F. Garcia-del Portillo, P. Garrido, L. Gautier, W. Goebel, N. Gomez-Lopez, T. Hain, J. Hauf, D. Jackson, L. M. Jones, U. Kaerst, J. Kreft, M. Kuhn, F. Kunst, G. Kurapkat, E. Madueno, A. Maitournam, J. M. Vicente, E. Ng, H. Nedjari, G. Nordsiek, S. Novella, B. de Pablos, J. C. Perez-Diaz, R. Purcell, B. Remmel, M. Rose, T. Schlueter, N. Simoes, A. Tierrez, J. A. Vazquez-Boland, H. Voss, J. Wehland, and P. Cossart.** 2001. Comparative genomics of Listeria species. Science **294:**849–852.
17. **Guiney, D. G., and D. R. Helinski.** 1979. The DNA-protein relaxation complex of the plasmid RK2: location of the site-specific nick in the region of the proposed origin of transfer. Mol. Gen. Genet. **176:**183–189.
18. **Guzman, L. M., D. Belin, M. J. Carson, and J. Beckwith.** 1995. Tight regulation, modulation, and high-level expression by vectors containing the arabinose P_{BAD} promoter. J. Bacteriol. **177:**4121–4130.
19. **Haima, P., S. Bron, and G. Venema.** 1987. The effect of restriction on shotgun cloning and plasmid stability in *Bacillus subtilis* Marburg. Mol. Gen. Genet. **209:**335–342.
20. **Itaya, M., K. Kondo, and T. Tanaka.** 1989. A neomycin resistance gene cassette selectable in a single copy state in the *Bacillus subtilis* chromosome. Nucleic Acids Res. **17:**4410.
21. **Jaacks, K. J., J. Healy, R. Losick, and A. D. Grossman.** 1989. Identification and characterization of genes controlled by the sporulation-regulatory gene *spo0H* in *Bacillus subtilis*. J. Bacteriol. **171:**4121–4129.
22. **Jin, S., and A. L. Sonenshein.** 1994. Transcriptional regulation of *Bacillus subtilis* citrate synthase genes. J. Bacteriol. **176:**4680–4690.
23. **Jourlin-Castelli, C., N. Mani, M. M. Nakano, and A. L. Sonenshein.** 2000. CcpC, a novel regulator of the LysR family required for glucose repression of the *citB* gene in *Bacillus subtilis*. J. Mol. Biol. **295:**865–878.
24. **Kenney, T. J., and C. P. Moran, Jr.** 1991. Genetic evidence for interaction of sigma A with two promoters in *Bacillus subtilis*. J. Bacteriol. **173:**3282–3290.
25. **Kim, H. J., C. Jourlin-Castelli, S. I. Kim, and A. L. Sonenshein.** 2002. Regulation of the *Bacillus subtilis ccpC* gene by CcpA and CcpC. Mol. Microbiol. **43:**399–410.
26. **Kim, H. J., S. I. Kim, M. Ratnayake-Lecamwasam, K. Tachikawa, A. L. Sonenshein, and M. Strauch.** 2003. Complex regulation of the *Bacillus subtilis* aconitase gene. J. Bacteriol. **185:**1672–1680.
27. **Kim, H. J., A. Roux, and A. L. Sonenshein.** 2002. Direct and indirect roles of CcpA in regulation of *Bacillus subtilis* Krebs cycle genes. Mol. Microbiol. **45:**179–190.
28. **Kim, S. I., C. Jourlin-Castelli, S. R. Wellington, and A. L. Sonenshein.** 2003. Mechanism of repression by *Bacillus subtilis* CcpC, a LysR family regulator. J. Mol. Biol. **334:**609–624.
29. **Lampidis, R., R. Gross, Z. Sokolovic, W. Goebel, and J. Kreft.** 1994. The virulence regulator protein of *Listeria ivanovii* is highly homologous to PrfA from *Listeria monocytogenes* and both belong to the Crp-Fnr family of transcription regulators. Mol. Microbiol. **13:**141–151.
30. **Lauer, P., M. Y. Chow, M. J. Loessner, D. A. Portnoy, and R. Calendar.** 2002. Construction, characterization, and use of two *Listeria monocytogenes* site-specific phage integration vectors. J. Bacteriol. **184:**4177–4186.
31. **LeDeaux, J. R., and A. D. Grossman.** 1995. Isolation and characterization of *kinC*, a gene that encodes a sensor kinase homologous to the sporulation sensor kinases KinA and KinB in *Bacillus subtilis*. J. Bacteriol. **177:**166–175.
32. **Mengaud, J., S. Dramsi, E. Gouin, J. A. Vazquez-Boland, G. Milon, and P. Cossart.** 1991. Pleiotropic control of *Listeria monocytogenes* virulence factors by a gene that is autoregulated. Mol. Microbiol. **5:**2273–2283.
33. **Milenbachs, A. A., D. P. Brown, M. Moors, and P. Youngman.** 1997. Carbon-source regulation of virulence gene expression in *Listeria monocytogenes*. Mol. Microbiol. **23:**1075–1085.
34. **Miller, J. H.** 1972. Experiments in molecular genetics, Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
35. **Mizoguchi, K., H. Ohta, A. Miyagi, H. Kurihara, S. Takashiba, K. Kato, Y. Murayama, and K. Fukui.** 1997. The regulatory effect of fermentable sugar levels on the production of leukotoxin by *Actinobacillus actinomycetemcomitans*. FEMS Microbiol. Lett. **146:**161–166.
36. **Moors, M. A., B. Levitt, P. Youngman, and D. A. Portnoy.** 1999. Expression of listeriolysin O and ActA by intracellular and extracellular *Listeria monocytogenes*. Infect. Immun. **67:**131–139.
37. **Muraoka, S., R. Okumura, N. Ogawa, T. Nonaka, K. Miyashita, and T. Senda.** 2003. Crystal structure of a full-length LysR-type transcriptional regulator, CbnR: unusual combination of two subunit forms and molecular bases for causing and changing DNA bend. J. Mol. Biol. **328:**555–566.
38. **O'Byrne, C. P., and C. J. Dorman.** 1994. The *spv* virulence operon of *Sal-*

monella typhimurium LT2 is regulated negatively by the cyclic AMP (cAMP)-cAMP receptor protein system. J. Bacteriol. **176:**905–912.
39. **Pansegrau, W., E. Lanka, P. T. Barth, D. H. Figurski, D. G. Guiney, D. Haas, D. R. Helinski, H. Schwab, V. A. Stanisich, and C. M. Thomas.** 1994. Complete nucleotide sequence of Birmingham *IncP* alpha plasmids. Compilation and comparative analysis. J. Mol. Biol. **239:**623–663.
40. **Portnoy, D. A., V. Auerbuch, and I. J. Glomski.** 2002. The cell biology of *Listeria monocytogenes* infection: the intersection of bacterial pathogenesis and cell-mediated immunity. J. Cell Biol. **158:**409–414.
41. **Portnoy, D. A., T. Chakraborty, W. Goebel, and P. Cossart.** 1992. Molecular determinants of *Listeria monocytogenes* pathogenesis. Infect. Immun. **60:** 1263–1267.
42. **Ratnayake-Lecamwasam, M., P. Serror, K. W. Wong, and A. L. Sonenshein.** 2001. *Bacillus subtilis* CodY represses early-stationary-phase genes by sensing GTP levels. Genes Dev. **15:**1093–1103.
43. **Ripio, M. T., K. Brehm, M. Lara, M. Suarez, and J. A. Vazquez-Boland.** 1997. Glucose-1-phosphate utilization by *Listeria monocytogenes* is PrfA dependent and coordinately expressed with virulence factors. J. Bacteriol. **179:**7174–7180.
44. **Rosenkrantz, M. S., D. W. Dingman, and A. L. Sonenshein.** 1985. *Bacillus subtilis citB* gene is regulated synergistically by glucose and glutamine. J. Bacteriol. **164:**155–164.
45. **Sage, A. E., and M. L. Vasil.** 1997. Osmoprotectant-dependent expression of *plcH*, encoding the hemolytic phospholipase C, is subject to novel catabolite repression control in *Pseudomonas aeruginosa* PAO1. J. Bacteriol. **179:**4874–4881.
46. **Sambrook, J., E. F. Fritsch, and T. J. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, N.Y.
47. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
48. **Schell, M. A.** 1993. Molecular biology of the LysR family of transcriptional regulators. Annu. Rev. Microbiol. **47:**597–626.
49. **Sheehan, B., A. Klarsfeld, R. Ebright, and P. Cossart.** 1996. A single substitution in the putative helix-turn-helix motif of the pleiotropic activator PrfA attenuates *Listeria monocytogenes* virulence. Mol. Microbiol. **20:**785–797.
50. **Smith, J. L., M. M. Bencivengo, and C. A. Kunsch.** 1986. Enterotoxin A synthesis in *Staphylococcus aureus*: inhibition by glycerol and maltose. J. Gen. Microbiol. **132:**3375–3380.
51. **Somerville, G., C. A. Mikoryak, and L. Reitzer.** 1999. Physiological characterization of *Pseudomonas aeruginosa* during exotoxin A synthesis: glutamate, iron limitation, and aconitase activity. J. Bacteriol. **181:**1072–1078.
52. **Somerville, G. A., M. S. Chaussee, C. I. Morgan, J. R. Fitzgerald, D. W. Dorward, L. J. Reitzer, and J. M. Musser.** 2002. *Staphylococcus aureus* aconitase inactivation unexpectedly inhibits post-exponential-phase growth and enhances stationary-phase survival. Infect. Immun. **70:**6373–6382.
53. **Tang, Y., and J. R. Guest.** 1999. Direct evidence for mRNA binding and post-transcriptional regulation by *Escherichia coli* aconitases. Microbiology **145:**3069–3079.
54. **Tang, Y., M. A. Quail, P. J. Artymiuk, J. R. Guest, and J. Green.** 2002. *Escherichia coli* aconitases and oxidative stress: post-transcriptional regulation of *sodA* expression. Microbiology **148:**1027–1037.
55. **Thompson, J. S., and M. H. Malamy.** 1990. Sequencing the gene for an imipenem-cefoxitin-hydrolyzing enzyme (CfiA) from *Bacteroides fragilis* TAL2480 reveals strong similarity between CfiA and *Bacillus cereus* beta-lactamase II. J. Bacteriol. **172:**2584–2593.
56. **Trivett, T. L., and E. A. Meyer.** 1971. Citrate cycle and related metabolism of *Listeria monocytogenes*. J. Bacteriol. **107:**770–779.
57. **Tsai, H. N., and D. A. Hodgson.** 2003. Development of a synthetic minimal medium for *Listeria monocytogenes*. Appl. Environ. Microbiol. **69:**6943–6945.
58. **Vazquez-Boland, J. A., M. Kuhn, P. Berche, T. Chakraborty, G. Dominguez-Bernal, W. Goebel, B. Gonzalez-Zorn, J. Wehland, and J. Kreft.** 2001. Listeria pathogenesis and molecular virulence determinants. Clin. Microbiol. Rev. **14:**584–640.
59. **Villafane, R., D. H. Bechhofer, C. S. Narayanan, and D. Dubnau.** 1987. Replication control genes of plasmid pE194. J. Bacteriol. **169:**4822–4829.
60. **Wach, A.** 1996. PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in *S. cerevisiae*. Yeast **12:**259–265.
61. **Wilson, T. J., N. Bertrand, J. L. Tang, J. X. Feng, M. Q. Pan, C. E. Barber, J. M. Dow, and M. J. Daniels.** 1998. The *rpfA* gene of *Xanthomonas campestris* pathovar campestris, which is involved in the regulation of pathogenicity factor production, encodes an aconitase. Mol. Microbiol. **28:**961–970.
62. **Yanisch-Perron, C., J. Vieira, and J. Messing.** 1985. Improved M13 phage cloning vectors and host strains: nucleotide sequences of the M13mp18 and pUC19 vectors. Gene **33:**103–119.