JOURNAL OF BACTERIOLOGY, Feb. 2009, p. 862–872 Vol. 191, No. 3
0021-9193/09/$08.00+0 doi:10.1128/JB.01384-08

CcpC-Dependent Regulation of Citrate Synthase Gene Expression in *Listeria monocytogenes*▽

Meghna Mittal,[1] Silvia Picossi,[2]† and Abraham L. Sonenshein[1,2]*

Program in Molecular Microbiology, Sackler School of Graduate Biomedical Sciences,[1] and Department of Molecular Biology and Microbiology, School of Medicine,[2] Tufts University, Boston, Massachusetts 02111

Received 2 October 2008/Accepted 4 November 2008

Citrate synthase, the first and rate-limiting enzyme of the tricarboxylic acid branch of the Krebs cycle, was shown to be required for de novo synthesis of glutamate and glutamine in *Listeria monocytogenes*. The citrate synthase (*citZ*) gene was found to be part of a complex operon with the upstream genes lmo1569 and lmo1568. The downstream isocitrate dehydrogenase (*citC*) gene appears to be part of the same operon as well. Two promoters were shown to drive *citZ* expression, a distal promoter located upstream of lmo1569 and a proximal promoter located upstream of the lmo1568 gene. Transcription of *citZ* from both promoters was regulated by CcpC by interaction with a single site; assays of transcription in vivo and assays of CcpC binding in vitro revealed that CcpC interacts with and represses the proximal promoter that drives expression of the lmo1568, *citZ*, and *citC* genes and, by binding to the same site, prevents read-through transcription from the distal, lmo1569 promoter. Expression of the lmo1568 operon was not affected by the carbon source but was repressed during growth in complex medium by addition of glutamine.

Listeria monocytogenes is a gram-positive food-borne bacterial parasite of mammals that causes listeriosis, an infection with a 30% mortality rate in susceptible humans that is characterized by fetoplacental and central nervous system infections and gastroenteritis (38). The risk group for listeriosis includes pregnant women, neonates, the elderly, and immunocompromised adults. *Listeria* infection has been an important model system for the study of host-pathogen interactions and mechanisms of intracellular parasitism (38). This bacterium is an intracellular pathogen that induces its own uptake by nonphagocytic cells and spreads from cell to cell via actin-based motility (4). Even though *L. monocytogenes* is able to grow intracellularly in a variety of mammalian cells, it is a facultative pathogen that can adapt to saprophytic growth on decaying soil vegetation (28). It is therefore interesting to examine how this bacterium senses the environment in order to regulate expression of its virulence determinants.

Previous researchers have thoroughly investigated the molecular determinants of *L. monocytogenes* pathogenesis. The genes that encode all the currently known virulence factors are positively regulated by the transcriptional activator PrfA (3). These genes are repressed, however, when *L. monocytogenes* is grown in the presence of fermentable sugars (28). This carbon source-mediated repression of virulence genes does not involve CcpA, the global regulator of catabolite control in many gram-positive bacteria (2, 16, 36), but instead is due to effects of sugar metabolism on PrfA activity. Rapidly metabolized carbon sources alter the phosphorylation state of components of the phosphoenolpyruvate-dependent phosphotransferase system; one or more of these components appear to inhibit PrfA (17, 27). Given that there is some uncertainty about the mechanisms that couple utilization of carbon sources and expression of virulence factors in *L. monocytogenes*, expanding our knowledge about the regulation of central metabolism may help us gain insight into the roles played by carbon sources in virulence factor gene regulation.

One central metabolic pathway, the Krebs citric acid cycle, has the potential to generate ATP, reducing power, and key biosynthetic precursors. *L. monocytogenes*, however, utilizes a split Krebs cycle; it has an oxidative, tricarboxylic acid (TCA) branch (citrate synthase, aconitase, and isocitrate dehydrogenase) leading to 2-ketoglutarate synthesis and a reductive branch (37). This bacterium lacks the 2-ketoglutarate dehydrogenase system, succinyl coenzyme A (succinyl-CoA) synthetase, and succinic dehydrogenase, as well as the enzymes of the glyoxylate shunt. In *L. monocytogenes*, the sole metabolic role of the TCA branch enzymes seems to be to mediate the synthesis of 2-ketoglutarate. 2-Ketoglutarate is a precursor for synthesis of glutamate and glutamine, the cell's most critical nitrogen-containing metabolites.

The *L. monocytogenes* genome has been sequenced completely (14). Hence, it is possible to deduce the presence of some metabolic genes, as well as genes for their transcriptional regulators, by comparison with the genomes of more completely characterized relatives, such as *Bacillus subtilis*. Such an analysis revealed the presence of homologs of the *citZ* (citrate synthase), *citB* (aconitase), and *citC* (isocitrate dehydrogenase) genes. Since carbon metabolism in *B. subtilis* has been extensively analyzed, the information obtained in this research can be useful for gaining a better understanding of *L. monocytogenes* physiology.

In *B. subtilis*, the synthesis and activity of the three enzymes of the TCA branch of the Krebs cycle are regulated in response to the cell's requirement for ATP, reducing power, and 2-

* Corresponding author. Mailing address: Department of Molecular Biology and Microbiology, Tufts University School of Medicine, 136 Harrison Avenue, Boston, MA 02111. Phone: (617) 636-6761. Fax: (617) 636-0337. E-mail: linc.sonenshein@tufts.edu.

† Present address: Instituto de Bioquímica Vegetal y Fotosíntesis, Consejo Superior de Investigaciones Científicas and Universidad de Sevilla, E-41092 Seville, Spain.

▽ Published ahead of print on 14 November 2008.

TABLE 1. Bacterial strains used

Strain	Genotype and/or other characteristics	Source or reference
B. subtilis strains		
SMY	Wild type	P. Schaeffer
LS1002	Δ*amyE*::Φ(*citBp21-lacZ cat*) *ccpC*::*spc ccpA*::Tn*917*(*ble*)	23
SG82	*trpC2 lacA*::*tet*	5
HKB5	*ccpA*::Tn*917*(*ble*)	SMY × LS1002 DNA
BB1888	*lacA*::*tet*	SMY × SG82 DNA
BMM1	Δ*amyE*::Φ(*lmo1569-lmo1568-citZ-lacZ*) Δ*ccpC*::*spc*	BMM6 × LS1002 DNA
BMM3	Δ*amyE*::Φ(lmo1568-*citZ-lacZ*) Δ*ccpC*::*spc*	BMM7 × LS1002 DNA
BMM4	Δ*amyE*::Φ(*citZ-lacZ*) Δ*ccpC*::*spc*	BMM8 × LS1002 DNA
BMM6	Δ*amyE*::Φ(lmo1569-lmo1568-*citZ-lacZ*)	BB1888 × pEMM10
BMM7	Δ*amyE*::Φ(lmo1568-*citZ-lacZ*)	BB1888 × pEMM11
BMM8	Δ*amyE*::Φ(*citZ-lacZ*)	BB1888 × pEMM13
BMM12	Δ*amyE*::Φ(lmo1569-lmo1568-*citZ-lacZ*) Δ*ccpA*::*ble*	BMM6 × HKB5 DNA
BMM13	Δ*amyE*::Φ(lmo1568-*citZ-lacZ*) Δ*ccpA*::*ble*	BMM7 × HKB5 DNA
L. monocytogenes strains		
EGD-e	Animal isolate, genome sequencing strain	14
HKB214	Δ*int*::Φ(*citB-lacZ neo*)	24
LMM16	Δ*int*::Φ(lmo1568-*lacZ neo*), contains 143 bp upstream of lmo1568 fused to the *lacZ* gene	This study
LMM17	Δ*int*::Φ(lmo1569-*lacZ neo*), contains 155 bp upstream of lmo1569 and 36 bp of lmo1569 coding region fused to *lacZ*	This study
LMM18	Δ*int*::Φ(lmo1568-*citZ-lacZ neo*), contains 203 bp upstream of lmo1568, the entire lmo1568 coding sequence, and the region between lmo1568 and *citZ* fused to *lacZ*	This study
LMM19	Δ*int*::Φ(*citZ-lacZ neo*), contains 484 bp upstream of *citZ* fused to *lacZ*	This study
LMM21	Δ*int*::Φ(lmo1568-*lacZ neo*) Δ*ccpC*::*spc*	LMM16 × pHK95
LMM22	Δ*int*::Φ(lmo1569-*lacZ neo*) Δ*ccpC*::*spc*	LMM17 × pHK95
LMM23	Δ*int*::Φ(lmo1568-*citZ-lacZ neo*) Δ*ccpC*::*spc*	LMM18 × pHK95
LMM24	Δ*int*::Φ(*citZ-lacZ neo*) Δ*ccpC*::*spc*	LMM19 × pHK95
LMM33	Δ*int*::Φ(*citB-lacZ neo*) Δ*citZ*	HKB214 × pEMM47
SP121	Δ*int*::Φ(lmo1568-*citZ-lacZ neo*) Δ*ccpA*::*tet*	LMM18 × pSP65
SP122	Δ*int*::Φ(*citZ-lacZ neo*) Δ*ccpA*::*tet*	LMM19 × pSP65
SP124	Δ*int*::Φ(*citZ-lacZ neo*) Δ*ccpC*::*spc* Δ*ccpA*::*tet*	LMM24 × pSP65

ketoglutarate as a precursor for glutamate and glutamine. The *citZ*, *citC*, and *citH* (malate dehydrogenase) genes form an operon (19). The *citC* and *citH* genes also have gene-specific promoters (18, 20). In the presence of a readily utilizable carbon source, such as glucose, and a source of 2-ketoglutarate, such as glutamate or glutamine, the synthesis and activities of the TCA branch enzymes are reduced (9, 11, 15), and the transcription of the *citZCH* operon and the *citB* gene is strongly repressed (20, 31). CcpC, a member of the LysR family, is a major transcriptional regulator of the *citZCH* operon and the *citB* gene (22). CcpC binds with high affinity to the *citZ* and *citB* promoter regions and is a direct repressor of transcription (22, 26, 31). In the presence of citrate, binding of CcpC to the *citZ* and *citB* promoters is altered and both of these genes are derepressed (22).

Carbon catabolite repression of the *B. subtilis citZ* and *citB* genes is also mediated by CcpA (25). When cells are grown in the presence of glucose, CcpA is activated by interaction with the phosphorylated form of either HPr or Crh proteins (13, 34, 35). In *B. subtilis*, activated CcpA directly represses the transcription of the *citZCH* operon when cells are grown in a medium containing glucose, and it plays an indirect role in *citB* regulation by affecting the activity of CcpC (25); that is, CcpA restricts the synthesis of citrate, keeping CcpC in its active form (25).

L. monocytogenes encodes a homolog of *B. subtilis* CcpC that binds tightly to the *L. monocytogenes citB* promoter region in vitro and represses *citB* transcription in vivo (24). Citrate inhibits the interaction of CcpC with the *citB* promoter region. We report here that the *L. monocytogenes* CcpC protein also represses transcription of *citZ*. Transcription of *citZ* was shown to initiate at promoters for two different genes upstream of *citZ*. CcpC was shown to bind to a dyad symmetry element that is very similar to dyad elements found in other CcpC-binding sites and that overlaps the proximal promoter driving *citZ* expression. In addition to this direct mode of regulation, CcpC also repressed read-through transcription from the more distantly located promoter.

The *L. monocytogenes* homolog of *B. subtilis* CcpA controls the utilization of certain carbon sources (2), but it was found to play no apparent role in regulation of the *citZ* gene.

MATERIALS AND METHODS

Bacterial strains and growth conditions. Bacterial strains used in this study are listed in Table 1. Preparation of electrocompetent *Escherichia coli* cells and transformation by electroporation using a TransPoratorPlus (BTX) were performed as described by other workers (7). A previously described method was used for transformation of *B. subtilis* with chromosomal or plasmid DNA (8). Preparation of electrocompetent *L. monocytogenes* cells with penicillin pretreatment and transformation by electroporation were performed as described previously (29).

E. coli strains were grown at 37°C in Luria broth or on Luria agar plates with appropriate antibiotics. Both kanamycin (50 μg/ml) and ampicillin (100 μg/ml) were added to cultures of *E. coli* strains carrying pHK77 and its derivatives to prevent loss of the plasmids. *B. subtilis* strains were grown at 37°C in TSS minimal medium (12) supplemented with 0.2% (wt/vol) glutamine and 0.5% (wt/vol) glucose. For measurement of β-galactosidase activity in *B. subtilis*, 1-ml culture

TABLE 2. Oligonucleotides used in this work

Oligonucleotide	Sequence (5′ to 3′)[a]	Restriction site(s)
OBB102	CGGAAAGTATGTTGTTTTTACTTC	
OMM014	GACTGAGAATTCGTATATAACGGCAAAACAGC	EcoRI
OMM015	ACGTACGAATTCGGAAATCAAAA GAAACATACGC	EcoRI
OMM016	TCGTCGGAATTCATGTTTACGGAAAGTATGTTG	EcoRI
OMM017	CATGCAAAGCTTGGTACCTTCATTTCCTCCTTTTATCC	HindIII, KpnI
OMM041	CCATTACTTTGCCGTCGAC	
OMM042	GCTACAG CAATGATAAGCG	
OMM046	CCCCAATTTATCCCTTTTGC	
OMM047	GGGGCCAAAAGCCGATTAC	
OMM048	ACTGCAGAATTCCAAGGTGTTGTATATAACGGC	EcoRI
OMM049	TGACTGGGTACCGCCATATAAAGCCCAATAAAG	KpnI
OMM050	GTACGAGAATTCCTATGGGAAAAACTTTCTATAGAGG	EcoRI
OMM051	ACTGCAGGTACCGAAGTAAAAACAACATACTTTCCG	KpnI
OMM064	CGACAGCTACAGCAATGATAAGCG	
OMM070	AAGGCGATTAAGTTGG GTAAC	
OMM071	GCGCAACTGTTGGGAAG	
OMM074	GGTGTTATAACAACTATCCTTGGACTGCTC	
OMM085	GAGAGTTTGATCCTGGCTC	
OMM086	CCTACCAACTAGCTAATGCAC	
OMM087	GTTGCTGAAACGTCCATTAGC	
OMM088	CTGTATCAAATACACCGAGCTTGG	
OMM092	CAACGGCCATATAGCCTACAC	
OMM097	GACGGAACTGGTCCAGACATC	
OMM098	GACCATATCTGTGTCTTCTGGACG	
OMM099	ACTGACGGATCCAATTCGCTTATCATTGCTGTAGCTGTC	BamHI
OMM100	GGCCCGATTTAAGCACACTTATTCAAGCCCTATTCTC	
OMM101	GCTGAGTCTGGCTTTCGGAGAAAATCATTCAGTTTGC	
OMM102	GTCACTCCATGGTGGTGAAGGAACACCTTTGAAATAACG	NcoI
OMM140	ATTTATGTTGGACCTGAAAATAG	
OSP157	AGCGAAAGATCTAAAACTTATTCGTGGTGG	BglII
OSP158	GTAAGTCTTCTCGCAATATCTGGATCCCCACGAGCAAGTTCAGC	BamHI
OSP159	CCAGATATTGCGAGAAGACTTAGTCGACATGACAAGCAACAATACG	SalI
OSP160	AAATTCCCATGGGTGATTAAAGTCCATCGC	NcoI
OSP172	TCCAGACCCGGGAGTTGCATTATCG	SmaI
OSP174	TGCGTACCCGGGCTTCATCTGTTTGCTGG	SmaI

[a] Some of the oligonucleotides contained recognition sites for restriction enzymes, which are indicated by underlining.

samples were harvested and assayed as described previously (25). *L. monocytogenes* was grown at 37 or 30°C in brain heart infusion (BHI) medium (Difco) or IMM (see below), as indicated. 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), and neomycin (5 μg/ml) for *B. subtilis* and chloramphenicol (10 μg/ml), spectinomycin (100 μg/ml), and neomycin (10 μg/ml) for *L. monocytogenes*.

IMM medium (30) was used to analyze the effects of different carbon sources on *citZ-lacZ* expression. *L. monocytogenes* strains were grown overnight in BHI medium, and an overnight culture was used as the inoculum for subsequent growth in IMM medium with 0.5% glucose as the carbon source. When they reached stationary phase, the cultures were diluted in IMM medium with different carbon sources (0.5%) to obtain an initial optical density at 600 nm (OD_{600}) of 0.05. One-milliliter samples were collected in the exponential (OD_{600}, 0.5) and stationary (OD_{600}, 1) phases of growth for β-galactosidase assays.

DNA manipulation. Restriction digestion, DNA ligation, and PCR were performed according to the manufacturer's instructions. *E. coli* plasmids were isolated using a Qiagen miniprep kit (Qiagen Inc.). Agarose gel electrophoresis and polyacrylamide gel electrophoresis were carried out as described by Sambrook et al. (32). Genomic DNA from *B. subtilis* was isolated as described by Fouet and Sonenshein (12) using 1 mg/ml lysozyme. *L. monocytogenes* chromosomal DNA was isolated using mutanolysin and the protocol of Fliss et al. (10).

Construction of *B. subtilis* strains carrying *L. monocytogenes citZ-lacZ* fusions. To construct the *lacZ* fusion plasmids pEMM10, pEMM11, and pEMM13, various regions upstream of the *L. monocytogenes citZ* gene were amplified by PCR from chromosomal DNA of *L. monocytogenes* strain EGD-e using forward primers OMM014, OMM015, and OMM016, respectively, and reverse primer OMM017. These primers and all other primers used in this work are listed in Table 2. The PCR products were digested with EcoRI and HindIII, and the products were cloned independently in similarly digested vector pHK23 (H. J. Kim, personal communication). Transformation of *B. subtilis* strain BB1888 with pEMM10, pEMM11, and pEMM13 resulted in erythromycin-resistant transformants BMM6, BMM7, and BMM8, respectively, in which *citZ-lacZ* fusions were integrated at the chromosomal *amyE* locus by double-crossover recombination. The BMM6, BMM7, and BMM8 strains contain 1,180, 689, and 484 bp, respectively, of sequence upstream of the *citZ* start codon fused to the *lacZ* reporter (Fig. 1).

Strains BMM1, BMM3, and BMM4 were constructed by transforming strains BMM6, BMM7, and BMM8 with chromosomal DNA from LS1002 (*ccpC*::*spc*) and selecting for Spc^r transformants. To create strains BMM12 and BMM13, BMM6 and BMM7 were transformed with chromosomal DNA of strain HKB5 (*ccpA*::*ble*) and phleomycin-resistant transformants were selected.

Construction of a *citZ* null mutant strain of *L. monocytogenes*. An *L. monocytogenes citZ* null mutant strain (LMM33) was created using the overlap PCR technique (39). First, a 706-bp PCR product corresponding to the 5′ end of the *citZ* gene (the first 100 codons) and upstream DNA including part of the lmo1568 gene was generated by PCR using *L. monocytogenes* chromosomal DNA as the template and primers OMM099 and OMM100. A second PCR product (688 bp) corresponding to the 3′ end of *citZ* and a downstream region including part of the *citC* gene was generated using primers OMM101 and OMM102. The ends of primers OMM100 and OMM101 have extensions that are complementary to each other. The two PCR products were purified, mixed together, and used in a third PCR with primers OMM099 and OMM102 to generate a 1.4-kb product that corresponded to the *citZ* gene with an internal, in-frame deletion of 171 codons and about 827 bp of flanking DNA upstream and downstream. This PCR product was purified, digested with BamHI and NcoI, and ligated to a similarly digested vector, pMAD (1), to create pEMM47. The latter plasmid was used to transform electrocompetent cells of *L. monocytogenes* strain HKB214 to erythromycin resistance. The initial transformants arose by single crossover,

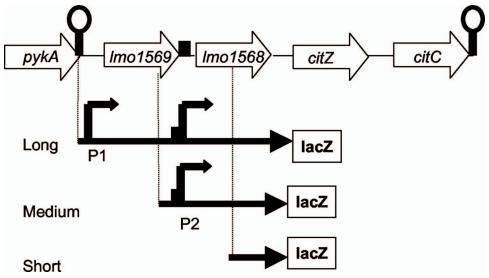

FIG. 1. Organization of the *citZ* locus in *L. monocytogenes* and genetic content of *lacZ* fusions used in *B. subtilis*. *L. monocytogenes* genes are indicated by designations used at the ListiList database site (http://genolist.pasteur.fr/ListiList/). The arrows indicate genes and their orientations, and the balloons indicate the locations of putative transcription termination sites. A putative CcpC-binding site is indicated by a box in the region upstream of lmo1568. The regions fused to *lacZ* and used for assays of gene expression in *B. subtilis* are indicated. The arrowheads indicate the putative positions of transcription start sites. The figure is not drawn to scale.

integrating the entire pEMM47 plasmid. To isolate a clone that had undergone a second crossover event and lost the pMAD-1 sequence, we subcultured an initial transformant three times overnight at 30°C. The presence of the *citZ* null mutation in the resultant strain, LMM33, was confirmed by PCR and by the absence of citrate synthase enzyme activity.

Construction of strains of *L. monocytogenes* carrying *lacZ* fusions. To construct fusions to *lacZ* of various regions from the *L. monocytogenes citZ* locus, the regions between *pykA* and lmo1569, between lmo1569 and lmo1568, and between lmo1568 and *citZ* were amplified by PCR using primer pairs OMM048/OMM049, OMM050/OMM051, and OMM016/OMM017, respectively, and chromosomal DNA of strain EGD-e as the template. To create a *lacZ* fusion to the region between lmo1569 and *citZ*, primer pair OMM015/OMM017 was used. The PCR products were digested with EcoRI and KpnI and ligated independently to pHK77 (24), which had been similarly digested. Plasmid pHK77 does not replicate in *L. monocytogenes* at elevated temperatures but integrates by homologous recombination at the nonessential *int'-'comK* locus (24). After transformation of *E. coli* and isolation of plasmid DNA, the insert was sequenced to confirm that it did not have PCR-generated mutations. Five micrograms of each pHK77 derivative was mixed with electrocompetent cells of strain EGD-e. After electroporation (2 kV, 400 Ω, 25 μF, 5 ms), cells were diluted with 1 ml BHI medium containing 0.5 M sucrose and incubated without shaking at 30°C for 60 min. Transformants were selected at 30°C on BHI medium plates containing neomycin and then grown at 30°C in the presence of neomycin overnight three times to increase the copy number of the plasmid. After subsequent overnight growth at the nonpermissive temperature (41°C), neomycin-resistant clones were tested individually by performing PCR to determine integration into the chromosome. In the last step, strains were grown at 30°C in the absence of neomycin to permit growth of segregants that had lost the plasmid due to excision. A series of PCRs confirmed that the *lacZ* fusions were inserted by double-crossover recombination at the *int-comK* locus and that the strains did not carry any free plasmid.

To introduce a *ccpC*::*spc* mutation into the strains carrying *lacZ* fusions, pHK95 (24) was transferred by electroporation into electrocompetent cells. After incubation at 41°C in the presence of spectinomycin to select for integration of pHK95 into the chromosome, cells were grown at 30°C to allow plasmid excision, and null mutants were purified based on their spectinomycin resistance and chloramphenicol sensitivity. A series of PCRs confirmed that there was allelic replacement of $ccpC^+$ by *ccpC*::*spc*.

ccpA null mutant strains SP121, SP122, and SP124 were created using overlap PCR (39). First, a 617-bp PCR product including the 5′ region of the *ccpA* gene was generated by PCR using *L. monocytogenes* chromosomal DNA as the template and primers OSP172 (with an appended SmaI site at the 5′ end) and OSP158 (with an internal BamHI site). A second PCR product (614 bp) including the 3′ end of *ccpA* was generated using primers OSP159 (with an internal NcoI site) and OSP174 (with an appended SmaI site at the 5′ end). Primers OSP158 and OSP159 have extensions that are complementary to each other. The two PCR products were purified, mixed together, and used in a third PCR with primers OSP172 and OSP174. The PCR product was purified, digested with BamHI and NcoI, ligated to the BamHI-XhoI DNA fragment (1.8 kb) from plasmid pEMM20 (M. Mittal, unpublished) corresponding to the tetracycline resistance cassette, digested again with SmaI, and ligated to SmaI-digested pMAD to create pSP65.

The latter plasmid was used to transform electrocompetent cells of *L. monocytogenes* strains LMM18, LMM19, and LMM24 to erythromycin and tetracycline resistance. The initial transformants arose by a single crossover event, and the entire pSP65 plasmid was integrated. To isolate a clone that had undergone a second crossover event and lost the pMAD sequence, we incubated a culture of an initial transformant overnight three or four times at 30°C in BHI medium to allow plasmid excision and create *ccpA* null strains SP121, SP122, and SP124, respectively. The overnight cultures and plates contained BHI medium with tetracycline, as well as 1.6 mg of glutamate per ml and 1.6 mg of glutamine per ml. The presence of the *ccpA* null mutation was confirmed by PCR.

Citrate synthase enzyme assay. Cells from a 25-ml stationary-phase culture in BHI medium were collected by centrifugation at 10,000 × *g* for 20 min using a refrigerated centrifuge. The pellet was resuspended in 2 ml of 0.05 M Tris-Cl buffer (pH 7.5), and the cells were broken by sonication with a Branson Sonifier cell disrupter (model 200) using five 30-s cycles with 30-s rest periods between the cycles on ice. Cell debris was removed by centrifugation at 5,000 rpm with a refrigerated tabletop centrifuge for 10 min. The total protein concentration in the supernatant was determined using the Bio-Rad protein assay reagent. The activity of citrate synthase was determined by measuring the splitting of 5,5′-dithio-bis(2-nitrobenzoic acid) by CoA liberated from acetyl-CoA using a reaction mixture containing 20 mM Tris-Cl (pH 7.4), 10 mM sodium oxaloacetate, 0.5 mM acetyl-CoA, and 1 mM 5,5′-dithio-bis(2-nitrobenzoic acid). The reaction was monitored at 412 nm and was calibrated with CoA. One unit of enzyme activity was defined as the formation of 1 μmol of product per min. Specific activity was expressed in units per milligram of protein.

Gel mobility shift assays and DNase I footprinting experiments. *L. monocytogenes* CcpC-His_6 was purified as previously described (24). For gel shift assays, a 237-bp DNA fragment corresponding to the lmo1568 promoter region was amplified by PCR using ^{32}P-labeled OMM050 and OMM064 as the forward and reverse primers and genomic DNA from *L. monocytogenes* EGD-e as the template. A 191-bp DNA fragment corresponding to the lmo1569 promoter region was amplified by PCR using ^{32}P-labeled OMM048 and OMM049 as the forward and reverse primers. The primers (50 pmol) were end labeled by incubation with 150 μCi of [γ-^{32}P]ATP (6,000 Ci mmol^{-1}; NEN) and T4 polynucleotide kinase (Invitrogen). The labeled primers were purified using a nucleotide removal kit (Qiagen Inc.). The labeled PCR products (1,000 cpm) and various amounts of CcpC-His_6 were incubated in buffer B for 30 min at room temperature and electrophoresed on nondenaturing polyacrylamide gels as previously described (24).

For DNase I footprinting experiments, primers were labeled as described above. OMM074 and ^{32}P-labeled OMM064 were the primers used to amplify a 548-bp fragment covering the lmo1568 promoter region along with genomic DNA from *L. monocytogenes* strain EGD-e as the template. The ^{32}P-labeled DNA product (20,000 cpm) and various amounts of CcpC-His_6 were incubated in 20-μl reaction mixtures containing buffer B as described above. After 30 min of incubation at room temperature, $MgCl_2$ and $CaCl_2$ were each added to a final concentration of 6 mM, and the reaction mixtures were treated with 0.5 U of RQ1 DNase I (Promega) for 1 min at room temperature. For CcpC-His_6 concentrations of ≥62 nM, the DNase I reaction time was increased to 2 to 3 min. To create a DNA sequencing ladder, plasmid pEMM40 was subjected to the dideoxy chain termination protocol (33) using a Sequenase reagent kit, α-^{35}S-dATP, and primer OMM064.

RNA isolation. *L. monocytogenes* strains grown overnight in BHI medium were diluted to obtain an initial OD_{600} of 0.05 in fresh BHI medium, and the cultures were incubated at 37°C with shaking. Samples used for RNA isolation were collected in mid-exponential phase (OD_{600}, 0.3) and stationary phase (OD_{600}, 1.0), resuspended in 1 ml of ice-cold 50% acetone–50% ethanol, and stored at −80°C. The cells were centrifuged at 4°C, washed twice with 500 μl of TE buffer (10 mM Tris-Cl, 1 mM EDTA; pH 8.0), and resuspended in 1 ml of buffer RLT from a Qiagen RNeasy kit. Silica glass beads (diameter, 0.1 mm) were added, and the cells were disrupted using a Mini-BeadBeater (BioSpec Products Inc.). The silica beads were removed by centrifugation, and total RNA was isolated from the supernatant fluid using a Qiagen RNeasy kit (Qiagen Inc.) according to the manufacturer's instructions.

RT-PCR experiments. To remove contaminating DNA, the RNA samples described above were treated with Turbo DNase I from an Ambion DNA-free kit. Reverse transcription (RT) was performed by using the manufacturer's protocol, SuperScript II reverse transcriptase (Invitrogen) with total RNA (1,000 ng for the P1 read-through transcript, 250 and 500 ng for *citZ* and *citC*, or 50 ng for rRNA), and 2 pmol of a gene-specific primer (OMM086 for rRNA, OMM064

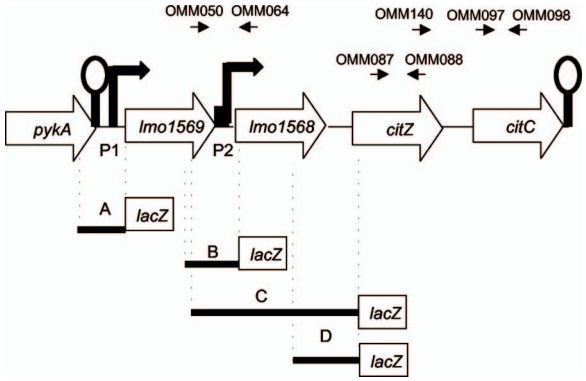

FIG. 2. Genetic content of *lacZ* fusions integrated at the *L. monocytogenes int′-′comK* locus. The regions fused to *lacZ* and used for assays of gene expression in *L. monocytogenes* are indicated below the gene map. The arrows above the gene map indicate the annealing sites and orientations of primers used for RT-PCR experiments. See the legend to Fig. 1 for additional details.

for the P1 read-through transcript, OMM098 for *citC* and the *citZ-citC* read-through transcript, or OMM088 for *citZ*) (Fig. 2) in a 20-μl (total volume) mixture. Two-microliter samples of the RT reaction mixtures were used as templates for PCR with gene-specific primers OMM086 and OMM085 for 16S rRNA, OMM064 and OMM050 for the P1 read-through transcript, OMM087 and OMM088 for *citZ*, OMM097 and OMM098 for *citC*, and OMM140 and OMM098 for the *citZ-citC* read-through transcript. The PCRs were performed using 12 to 27 cycles of denaturation (94°C, 30 s), annealing (45°C, 1 min), and extension (72°C, 30 s). For each gene, the PCR conditions were tested to be sure that the number of PCR cycles and the amount of RNA used were below the saturation limits. To check for chromosomal DNA contamination, 1-μl samples of RNA were used directly for PCRs after Turbo DNase treatment.

To quantify the amount of product in RT-PCR experiments, ImageQuant software was used to capture gel bands, calculate the band intensities (expressed in pixels), subtract the local background, and normalize the data to the areas of the bands. For all experiments, the amount in the experimental sample was normalized to the amount of an RT-PCR product of 16S rRNA. The values obtained for RNA samples from mutant cells were compared to the values obtained for RNA samples from wild-type cells. The values reported below are the averages of two or three biological replicates.

Primer extension experiments. Primer extension experiments were performed with the following oligonucleotides: primer OMM047 for lmo1569 and primers OMM070, OMM071, and OBB102 for lmo1568-*lacZ* fusion mRNA. The latter primers anneal within the *lacZ* gene or the *spoVG* ribosome-binding site of the fusion construct (24). Primers were labeled as described above. A total of 10^6 cpm of primer and 20 μg of total RNA were lyophilized together, resuspended in 20 μl of hybridization buffer containing 80% formamide, 0.5 M NaCl, 1 mM EDTA, and 40 mM piperizine-*N*,*N*′-bis(2-ethanesulfonic acid) (PIPES) (pH 6.8), and incubated at 80°C for 10 min. After slow cooling to 30°C, the primer-RNA mixture was precipitated with ammonium acetate and ethanol and used for an RT reaction catalyzed by SuperScript II (Gibco BRL, Life Technologies). After incubation at 42°C for 50 min, the enzyme was inactivated by heating the mixture at 70°C for 15 min, and then the reaction mixture was extracted with phenol-chloroform, precipitated, and dissolved in 10 μl of formamide-containing loading dye. Extension products were analyzed on 6% polyacrylamide–8 M urea sequencing gels.

RESULTS

Identification of the *L. monocytogenes citZ* gene. In *B. subtilis*, *citZ* is the first gene of the *citZCH* operon (20). The contiguous *L. monocytogenes citZ* and *citC* genes (Fig. 1) were located on the basis of homology with their *B. subtilis* counterparts using the ListiList database (http://genolist.pasteur.fr/ListiList/) that includes the genome sequence data of Glaser et al. (14). In *B. subtilis*, a second citrate synthase is encoded by the *citA* gene (19), but our search of the *L. monocytogenes* genome revealed only a single coding sequence with significant homology to *B. subtilis citZ*. Based on the locations of apparent transcription terminators, the *L. monocytogenes citZ* and *citC* genes seemed to be organized in an operon with the upstream genes lmo1569 and lmo1568, which encode proteins having unknown functions (Fig. 1). No gene similar to *citH* was detected downstream of *citC*. We created a *citZ* in-frame deletion mutant (see Materials and Methods) and confirmed that the *citZ* gene encodes citrate synthase enzyme activity. An extract of the wild-type strain had a citrate synthase specific activity of 0.5 U per mg protein, while the *citZ* mutant had a specific activity of 0.02 U per mg protein.

Use of *lacZ* fusions in *B. subtilis* to locate *citZ* promoters. To study the regulation of *L. monocytogenes citZ*, we first mapped the promoter(s) that drives *citZ* expression using *B. subtilis* as a surrogate host. We created *lacZ* transcriptional fusions with DNA segments corresponding to various lengths of DNA upstream of the *citZ* coding sequence and integrated the fusions in the *B. subtilis* chromosome at the nonessential *amyE* locus (Fig. 1). When *lacZ* expression was measured for the long, medium, and short fusions in a wild-type *B. subtilis* strain, only the long fusion expressed significant β-galactosidase activity (Table 3).

To analyze the potential roles of CcpC and CcpA transcription factors in the regulation of *L. monocytogenes citZ* expression, we tested the three fusions in *B. subtilis ccpC* and *ccpA* mutant strains. A null mutation in *ccpC* caused expression of the long fusion to be derepressed sixfold compared to the expression in the wild-type strain (Table 3). While the medium-length fusion showed a very low level of *citZ-lacZ* expression in a *ccpC*$^+$ background, 38-fold derepression was observed in the *ccpC* null mutant. This result indicated that the medium-length fusion contained a promoter (which we designated P2) that was repressed by *B. subtilis* CcpC. Since with the long fusion there was a high level of β-galactosidase activity both in the presence and in the absence of active CcpC, we deduced that there are probably at least two promoters that drive *citZ-lacZ* expression, at least one of which (P2) is regulated by *B. subtilis* CcpC (see below). The short fusion did not result in significant β-galactosidase activity in either the *cppC*$^+$

TABLE 3. Effects of *ccpC* or *ccpA* mutations on expression of *L. monocytogenes citZ* in *B. subtilis*

Fusion	β-Galactosidase activity (Miller units)[a]		
	Wild type	*ccpC* null mutant	*ccpA* null mutant
Long [(lmo1569-lmo1568-*citZ*)-*lacZ*]	11 ± 0.8	62 ± 0.8	10 ± 2.5
Medium [(lmo1568-*citZ*)-*lacZ*]	0.9 ± 0.2	38 ± 0.2	0.6 ± 0.03
Short (*citZ-lacZ*)	0.7 ± 0.1	0.38 ± 0.01	ND

[a] β-Galactosidase activity was determined for *B. subtilis* wild-type, *ccpC* null mutant, and *ccpA* null mutant strains carrying fusions to *lacZ* of segments of the *citZ* locus (Fig. 1). The organisms were grown at 37°C in TSS medium supplemented with 0.5% glucose and 1.6 mg glutamine per ml, and cells were harvested at several times during exponential growth phase and used for analysis. No growth phase-related regulation was seen. The β-galactosidase activities shown are the averages ± standard deviations of three independent experiments, in each of which samples were removed at multiple time points. ND, not determined.

TABLE 4. Effects of *ccp*C, *ccpA*, and *ccpA ccpC* mutations on *citZ* expression in *L. monocytogenes*

Fusion	β-Galactosidase activity (Miller units)			
	Wild type	*ccpC* mutant	*ccpA* mutant	*ccpC ccpA* mutant
lmo1569-*lacZ* (A)	24 ± 2.0	27 ± 2.4	ND	ND
lmo1568-*lacZ* (B)	5.0 ± 1.5	24 ± 3.2	ND	ND
(lmo1568-*citZ*)-*lacZ* (C)	3.2 ± 1.5	24 ± 5	3.9 ± 0.4	ND
citZ-lacZ (D)	0.5 ± 0.1	0.3 ± 0.05	0.6 ± 0.2	0.7 ± 0.4

[a] β-Galactosidase activity was determined for wild-type, *ccpC* null mutant, *ccpA* null mutant, and *ccpC ccpA* mutant strains of *L. monocytogenes* carrying various *lacZ* fusions. The organisms were grown at 37°C in BHI medium. The results shown are the averages ± standard deviations of three independent experiments. ND, not determined.

or *ccpC* mutant strain (Table 3), indicating that no promoter active under such conditions is located between lmo1568 and *citZ*.

In a *B. subtilis ccpA* null mutant, both the medium-length and long fusions had the same β-galactosidase activities that they had in the wild-type strain, indicating that *B. subtilis* CcpA does not regulate *L. monocytogenes citZ* transcription (Table 3).

Expression of *lacZ* fusions in *L. monocytogenes*. To confirm the CcpC-mediated regulation of *citZ* expression, we cloned four regions (designated fusions A to D [Fig. 2 and Table 4]) upstream of the *citZ* gene in plasmid pHK77 (24) and in this way created *lacZ* transcriptional fusions integrated at the ectopic *int'-'comK* locus on the *L. monocytogenes* chromosome. We compared the promoter activities of these regions by performing β-galactosidase assays with cells grown in BHI medium (Table 4). A strain carrying fusion D had a level of β-galactosidase activity similar to the background level, indicating that the region immediately upstream of the *citZ* gene does not have a promoter that is active under the conditions tested. Fusion A, corresponding to the region upstream of lmo1569 fused to *lacZ*, had a relatively high level of β-galactosidase activity, indicating the presence of a promoter (designated P1) in the region upstream of lmo1569. Fusions B and C were expressed at lower but still significant levels, consistent with the idea that there is a promoter (P2) in the region upstream of lmo1568. These results are also consistent with the results obtained for *B. subtilis* (Table 3).

Repression of *citZ* expression by CcpC in *L. monocytogenes*. To investigate the role of *L. monocytogenes* CcpC in regulation of the *citZ* gene, we introduced a null mutation in the *ccpC* gene (24) into strains carrying fusions A to D (Fig. 2). The β-galactosidase activities in the resultant strains were compared to those in the corresponding wild-type strains (Table 4). The *ccpC* null versions of strains carrying fusions B and C showed approximately four- to eightfold derepression compared to their $ccpC^+$ counterparts, confirming that P2, the promoter upstream of lmo1568, is regulated by CcpC. On the other hand, the *ccpC* null strain carrying the lmo1569-*lacZ* fusion (fusion A) had the same level of *lacZ* expression as its $ccpC^+$ counterpart. These results indicate that the P1 promoter upstream of lmo1569 is CcpC independent (Table 4). A strain carrying fusion D had the same low level of β-galactosidase activity whether the *ccpC* gene was the wild-type gene or a mutant gene, indicating that the region between lmo1568 and *citZ* does not have promoter activity in either *B. subtilis* or *L. monocytogenes*.

Repression of *citZ* expression by CcpA in *L. monocytogenes*. To examine the potential role of CcpA in the regulation of *citZ* in *L. monocytogenes*, we created *ccpA* single mutants and *ccpA ccpC* double mutants of the strains carrying fusions C and D. The *ccpA* single mutants had levels of *lmo1568-citZ-lacZ* and *citZ-lacZ* expression similar to those of the corresponding wild-type strains with these fusions (Table 4). Fusion D did not result in significant β-galactosidase activity even in a *ccpA ccpC* double mutant, reinforcing the idea that the region between lmo1568 and *citZ* does not contain a promoter (Table 4).

To confirm that CcpA does not regulate *citZ* expression in *L. monocytogenes*, we introduced the *ccpA* mutation into wild-type (LMM19) and *ccpC* null (LMM24) strains and assayed *citZ* transcription by RT-PCR (Fig. 3). By quantifying the results shown in Fig. 3 and the results of two additional experiments, we determined that the *citZ* transcript is present at the same level in the wild-type and *ccpA* mutant strains. The *ccpC* and *ccpC ccpA* mutants had similar 9- to 10-fold-higher levels of the *citZ* transcript than the wild-type strain (Fig. 3). Thus, CcpA does not regulate *citZ* expression in *L. monocytogenes*.

Mapping transcription start sites. To map the start sites of *L. monocytogenes citZ* transcripts, we extracted RNA from wild-type and *ccpC* null strains (grown in BHI medium) and performed a primer extension analysis. We mapped the apparent transcription start point (tsp) of lmo1569 to a position located 21 bp upstream of the start codon (data not shown). The abundance of this primer extension product was not affected by a *ccpC* mutation (data not shown). The putative P1 tsp is preceded by sequences that resemble the −10 and −35 recognition sites for the sigma A form of RNA polymerase. We tried to map the tsp of lmo1568 in the same way, but, due to the presence of multiple RT products, the tsp could not be determined conclusively even when we tested several different primers (data not shown). Nonetheless, a comparison of the levels of primer extension products obtained for the wild-type and *ccpC* null strains showed that some products were more abundant when RNA from the *ccpC* mutant strain was used (data not shown).

Since the complexity of the primer extension products observed for lmo1568 might have been due to transcripts originating upstream of P2, we tried to simplify the analysis by using strains in which the region containing the P2 promoter is upstream of the *lacZ* gene at the ectopic *int-comK* locus. In such strains, *lacZ* expression is not influenced by read-through transcription from any upstream promoter. We designed primers that anneal within the *lacZ* part of the fusion and used them to map the P2 tsp. Using three different primers, we found that

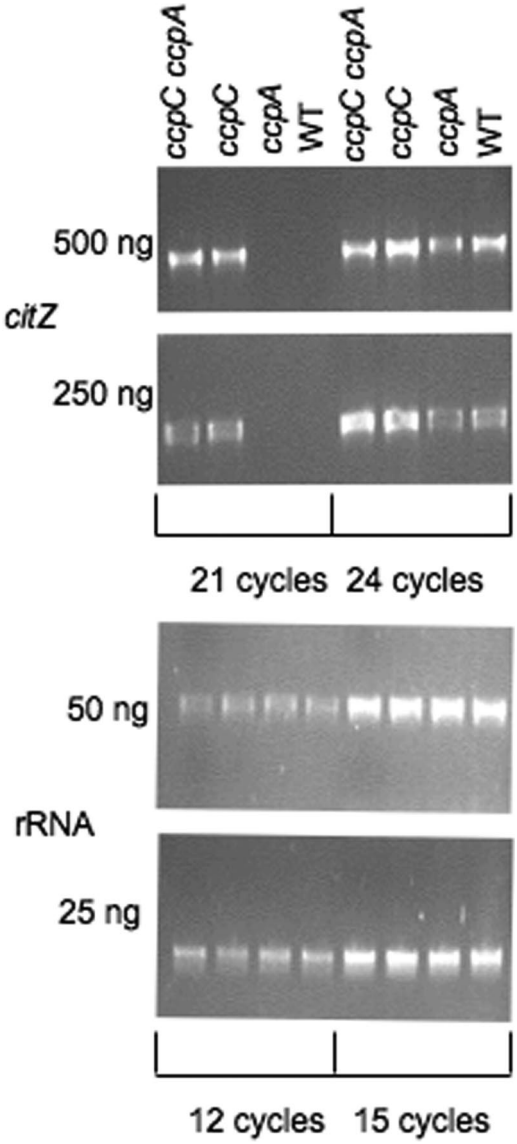

FIG. 3. RT-PCR analysis of the effect of *ccpA* mutation on *citZ* expression. RNA was extracted from wild-type, *ccpC* mutant, *ccpA* mutant, or *ccpC ccpA* double-mutant cells growing exponentially in BHI medium. Primer OMM88 was used for RT, and OMM88 and OMM87 were the primers used in PCR with the cDNA. The upper panels show the results for the *citZ* transcript (two different amounts of RNA and two different numbers of PCR cycles). The lower panels show the results for two different amounts of rRNA used as a control. The intensities of the bands were quantified using ImageQuant software. In two independent experiments, the average values for the *citZ* transcript relative to the wild type, normalized to rRNA, were 0.9 ± 0.1 for the *ccpA* mutant, 10 ± 2 for the *ccpC* mutant, and 9 ± 3 for the *ccpA ccpC* mutant.

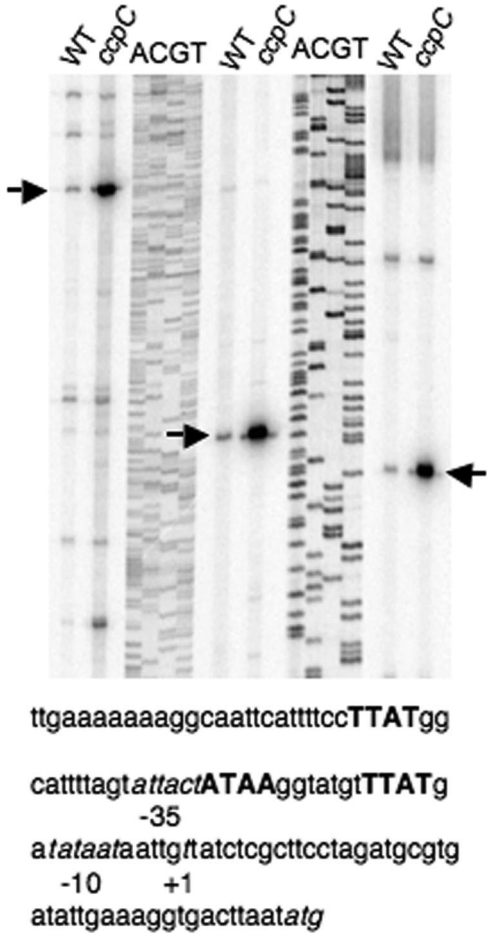

FIG. 4. Primer extension analysis to map the tsp for the lmo1568 gene. Three oligonucleotide primers that were labeled with ^{32}P at the 5′ end and were complementary to *lacZ* mRNA (see Materials and Methods) were annealed to total cellular RNA that was extracted from wild-type strain EGD-e and *ccpC* null mutant cells carrying a lmo1568-*lacZ* fusion and growing exponentially in BHI medium and were extended by using deoxynucleoside triphosphates and reverse transcriptase. The DNA products were separated by electrophoresis on a 6% polyacrylamide–7 M urea gel, and their mobilities were compared with the mobilities of dideoxynucleotide sequencing ladders (lanes A, C, G, and T) generated using two of the same primers used for primer extension and the relevant cloned DNA as the template. (The length of the third reverse transcript was extrapolated from its mobility relative to the mobilities of DNA sequences having known lengths generated with the other primers.) The left and right lanes in each set show products obtained with RNA from the wild-type (WT) and *ccpC* mutant strains, respectively. The following primers were used: for the left panel, OMM070; for the middle panel, OMM071; and for the right panel, OBB102. The arrows indicate the positions of the primer extension products. The sequence of the lmo1568 promoter region is shown below the gels. The consensus recognition sequences for CcpC are indicated by uppercase letters. The putative −35 and −10 regions and transcription start site (+1) of the deduced P2 promoter are also indicated. The start codon (ATG) is italicized.

the apparent tsp is located 42 bp upstream of the start codon of lmo1568 and that transcripts corresponding to this site are derepressed in a *ccpC* mutant strain (Fig. 4).

Read-through transcription from P1 into lmo1568. To test directly whether there is read-through transcription from the P1 promoter into the downstream genes, we carried out a semiquantitative RT-PCR analysis using primers that flank the P2 promoter (Fig. 5). The presence of a PCR product would have indicated that transcription that initiates upstream of the P2 tsp can extend beyond the P2 promoter. By quantifying the amount of this transcript in wild-type and *ccpC* null strains (see Materials and Methods), we found that read-through tran-

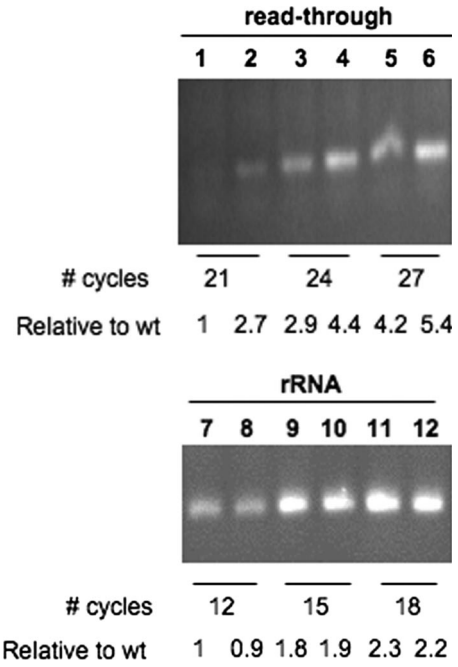

FIG. 5. RT-PCR analysis of read-through transcription from the P1 promoter into lmo1568. RNA was extracted from cells growing exponentially in BHI medium. Lanes 1, 3, 5, 7, 9, and 11 contained RT-PCR products from the wild-type strain, and lanes 2, 4, 6, 8, 10, and 12 contained RT-PCR products from the *ccpC* null strain. For analysis of read-through transcription from P1, the primer used for RT was OMM64; the PCR was primed with OMM50 and OMM64 (Fig. 2). One microgram of RNA was used for RT, and 10% of the product was used as a template for the PCR. To assay for rRNA as a normalization factor, 50 ng of RNA was used for cDNA synthesis and 10% of the product was used as a template for a PCR. The numbers of PCR cycles are indicated below the gels. The band intensities for the *ccpC* mutant strain RNA normalized to rRNA and relative to the wild-type intensities (wt) for the experiment shown were determined by using densitometry and ImageQuant software and are also indicated below the gels. In three independent experiments, the average value for the read-through transcript normalized to rRNA and relative to the wild-type value was 3.5 ± 0.5 for the *ccpC* mutant.

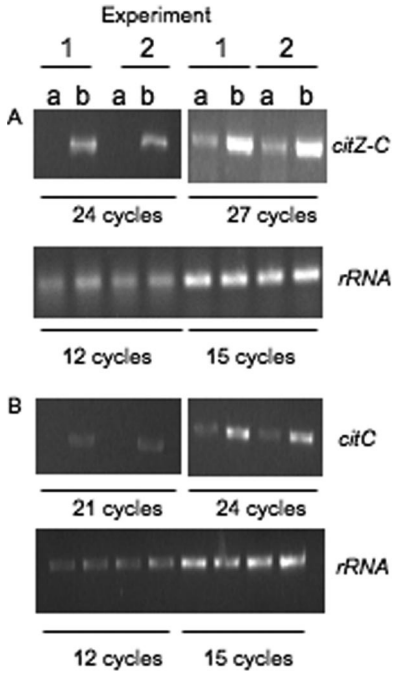

FIG. 6. RT-PCR analysis of read-through transcription from *citZ* into the *citC* gene and regulation of *citC* by CcpC. RNA was extracted from wild-type and *ccpC* mutant cells growing exponentially in BHI medium. To assay for *citZ-citC* read-through transcription or for *citC* transcription, 500 ng of RNA was used for RT and 10% of the product was used as a template for a PCR. To assay for rRNA as a normalization factor, 50 ng of RNA was used for cDNA synthesis and 10% of the product was used as a template for PCR. Data from two independent experiments (experiments 1 and 2) are shown. Each pair of lanes shows the results obtained with RNA from wild-type cells (lanes a) and from *ccpC* mutant cells (lanes b). The numbers of PCR cycles are indicated below the gels. Band intensities were quantified by using densitometry and ImageQuant software. (A) Read-through transcription from *citZ* into the downstream *citC* gene. The primers used were OMM140 and OMM98 (Fig. 2). In two independent experiments, the average amount of read-through transcript (normalized to the amount of rRNA) was 2.5- ± 0.5-fold larger in the *ccpC* mutant than in the wild-type strain. (B) Transcripts of the *citC* gene. The primers used were OMM97 and OMM98 (Fig. 2). In two independent experiments, the amount of *citC* transcript (normalized to the amount of rRNA) was 3- ± 0.2-fold larger in the *ccpC* mutant than in the wild-type strain.

scription was derepressed 3.5-fold by a *ccpC* mutation in cells grown in BHI medium.

Read-through transcription into the *citC* gene and regulation of *citC* by CcpC. In *B. subtilis*, *citC*, the gene downstream of *citZ* in the operon, encodes isocitrate dehydrogenase and is coregulated with *citZ*. About 50% of the *citC* transcripts arise from the *citZ* promoter and are regulated by CcpC (20). A secondary *citC* promoter is located immediately upstream of the *citC* gene but is not subject to carbon source-mediated regulation. To find out if *citC* is coregulated with *citZ* in *L. monocytogenes*, we carried out a semiquantitative RT-PCR analysis using primers within the *citZ* and *citC* genes. We detected a transcript, and it was repressed 2.5- ± 0.2-fold by CcpC in cells grown in BHI medium (Fig. 6A). We also tested the expression of the *citC* gene itself using primers that are within the *citC* gene. The *ccpC* null strain had a 3- ± 0.2-fold-higher level of the *citC* transcript than the wild-type strain (Fig. 6B).

Interaction of CcpC with the P2 promoter region in vitro. To study the interaction of CcpC with the P1 and P2 promoter regions, we purified a hexahistidine-tagged version of the *L. monocytogenes* CcpC protein (24) and tested its binding to these regions by performing gel mobility shift assays. A 237-bp DNA fragment carrying the P2 promoter showed retarded mobility when it was incubated with CcpC-His_6. CcpC was able to shift 50% of the DNA at a protein concentration of 3.9 to 7.8 nM (Fig. 7). By contrast, CcpC did not interact with the P1 promoter region in vitro (Fig. 7). These results are consistent with the hypothesis that there is CcpC-dependent regulation of the P2 promoter and CcpC independence of transcription initiation at the P1 promoter.

The specific site of CcpC binding in the P2 promoter region was determined by performing a DNase I footprinting experiment. In the presence of CcpC-His_6, the region from position −9 to position −61 with respect to the tsp was protected against DNase I digestion (Fig. 8). Within this region there is a dyad symmetry element that matches elements found in the

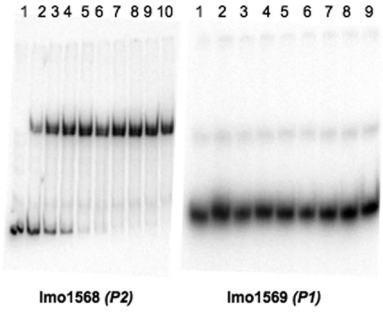

FIG. 7. Gel mobility shift assay of the interaction of *L. monocytogenes* CcpC with the P1 and P2 promoters. CcpC-His_6 was incubated with ^{32}P-labeled DNA fragments containing either the lmo1568 (left panel) or lmo1569 (right panel) promoter region. The concentrations of CcpC-His_6 used in the reactions were as follows: for lmo1568, 0, 3.9, 7.8, 15.6, 31.3, 62.5, 125, 250, 500, and 1,000 nM (lanes 1 to 10, respectively); and for lmo1569, 0, 7.8, 15.6, 31.3, 62.5, 125, 250, 500, and 1,000 nM (lanes 1 to 9, respectively). See Materials and Methods for details.

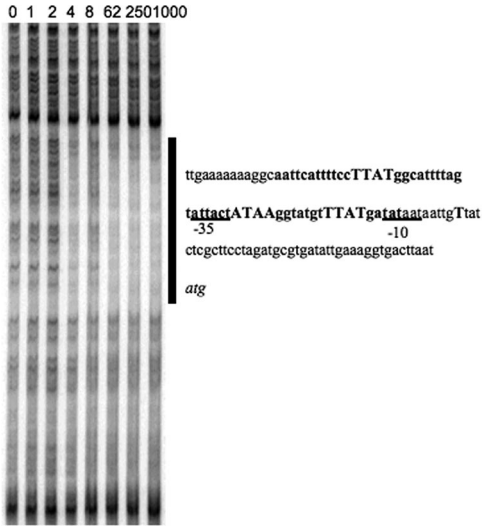

FIG. 8. DNase I footprinting assay of the interaction between CcpC-His_6 and the P2 promoter region. A ^{32}P-labeled DNA fragment corresponding to the lmo1568 promoter region was incubated with various amounts of CcpC-His_6 prior to DNase I digestion. The concentrations of CcpC-His_6 (in nM) used in the reactions are indicated above the lanes. The vertical bar indicates the region protected from DNase I digestion by CcpC-His_6. In the sequence of the lmo1568 promoter region shown on the right, the region protected by CcpC-His_6 from DNase I digestion (positions −9 to −61 with respect to the transcription start site, as deduced from other experiments in which a DNA sequence ladder was generated) is indicated by bold type. The consensus recognition sequences for CcpC are indicated by uppercase letters. The transcription start site is indicated by a single uppercase T, and the −10 and −35 regions are also indicated. The start codon of lmo1568 is indicated by italics.

CcpC-binding site of the *L. monocytogenes citB* promoter and in *B. subtilis* binding sites for CcpC. A half-dyad element is located 17 bp from the upstream end of the dyad element. Similar orientations of full and half sites have been found in other CcpC-binding sites (23).

Regulation of *citZ* expression by different carbon sources. It is possible that we did not observe any promoter activity in the region immediately upstream of the *citZ* gene because it is masked by CcpA-independent catabolite repression. BHI medium, in which the cells were grown for these experiments, contains glucose at a concentration of 0.2% (6). To address this issue, we tested the strains carrying fusion C or D in defined medium with different carbon sources. These carbon sources included the catabolite-repressing sugars glucose and mannose, as well as glycerol and citrate, which do not cause catabolite repression (21). In defined medium containing any carbon source tested except citrate, the strains grew with similar doubling times and reached stationary phase (OD_{600}, 1.0). The strains grew poorly in citrate medium, and the maximum OD_{600} was 0.1 to 0.2 even after prolonged incubation (48 h). The poor growth in citrate probably reflected poor uptake of citrate by the cells. Moreover, due to the split nature of the Krebs cycle in *L. monocytogenes*, citrate cannot be used to generate oxaloacetate, which is used in gluconeogenesis as a source of hexose and pentose as precursors of nucleic acids and cell envelope polymers. *L. monocytogenes* also appears to lack citrate lyase and isocitrate lyase enzymes, which could be used alternatively for synthesizing oxaloacetate. The strains containing the D fusion had the same low levels of β-galactosidase activity in all media tested and in both null backgrounds, reinforcing the idea that the region immediately upstream of the *citZ* gene does not contain a promoter that is masked by the repressive effect of glucose (Table 5). In addition, we found that CcpC-mediated repression of the C fusion occurred at the same level under all the conditions tested, suggesting that no other carbon source-responsive regulator acts along with CcpC to regulate *citZ* expression.

Regulation of *citZ* expression by glutamine. We measured the expression of the lmo1568-*lacZ* fusion in strain LMM16 grown at 37°C in BHI medium supplemented with glutamine, glucose, or citrate or combinations of nutrients. Neither glucose (which is present at a concentration of 0.2% in unsupplemented BHI medium [6]) nor citrate altered the expression of β-galactosidase (Fig. 9). When BHI medium was supplemented with glutamine or combinations of glutamine and glucose or citrate, *lacZ* expression was repressed about fivefold (Fig. 9). Repression in glutamine-containing medium was overcome by a *ccpC* mutation (Fig. 9).

DISCUSSION

Our work showed that the *L. monocytogenes citZ* gene is part of a complex operon along with the upstream genes lmo1569 and lmo1568 and the downstream gene *citC*. While the RT-PCR data indicate that *citZ* and *citC* are cotranscribed, it was not possible to deduce from our results whether *citC* also has an independent promoter and whether, if there is a promoter, it is a target of CcpC. (The fact that a *ccpC* mutation derepressed *citZ* transcription ninefold but derepressed *citC* transcription only threefold suggests that the *citC* gene may have a second, CcpC-independent promoter.) lmo1569 and lmo1568 are annotated as genes with unknown functions (14). A homology search revealed that they are similar only to other genes with unknown functions. Why these genes are coregulated with *citZ* remains unknown, but it is interesting that *ytwI*, the gene immediately upstream of *citZ* in *B. subtilis*, is a close

TABLE 5. Effects of different carbon sources on *citZ* expression in *L. monocytogenes*

Strain	Fusion	β-Galactosidase activity (Miller units) with the following carbon sources:			
		Glucose	Mannose	Glycerol	Citrate
LMM18	(lmo1568-*citZ*)-*lacZ* (C)	4.6	4.1 ± 0.2	5.2 ± 1.2	4.1 ± 0.2
LMM19	*citZ-lacZ* (D)	0.6 ± 0.02	0.7 ± 0.3	0.7 ± 0.3	0.8 ± 0.3
LMM23	(lmo1568-*citZ*)-*lacZ* (C) in *ccpC* null mutant	28 ± 1.9	31 ± 2.2	29 ± 2.2	26 ± 2.2
LMM24	*citZ-lacZ* (D) in *ccpC* null mutant	0.9 ± 0.3	0.9 ± 0.2	0.6 ± 0.3	0.9 ± 0.2

[a] β-Galactosidase activity was determined for wild-type and *ccpC* null mutant strains of *L. monocytogenes* carrying various *lacZ* fusions (Fig. 2). The organisms were grown at 30°C in IMM medium. Samples were collected in mid-exponential phase (OD_{600}, 0.5) for all of the sugars tested except citrate. Samples were collected at an OD_{600} of 0.1 or 0.2 when citrate was used as the sole carbon source. The results are the averages ± standard deviations of two independent experiments (except for the strain with fusion C in glucose medium). In all experiments, multiple samples were taken during the exponential growth phase.

homolog (57% identity) of lmo1568 (Fig. 10). The closest homolog of lmo1569, *ytzA*, is the third gene upstream of *citZ* in *B. subtilis* (Fig. 10). The possibility of cotranscription of *ytzA* or *ytwI* with *citZ* has not been addressed, but the region between *ytwI* and *citZ* contains an apparent transcription terminator (Fig. 10).

CcpC binds to a single site but acts as a direct repressor of the lmo1568, *citZ*, and *citC* genes in two different ways. Two promoters drive the expression of these genes; CcpC represses P2, presumably by competition with RNA polymerase for access to the promoter, and aborts transcripts from P1 by acting as a roadblock to RNA polymerase molecules that initiate transcription at P1. By performing a footprinting analysis we located the CcpC-binding site, which includes a dyad symmetry element ($ATAAN_7TTAT$) and a half-copy of the dyad symmetry element (TTAT). This pattern of dyad and half-dyad elements is typical of CcpC-binding sites in *B. subtilis* and *L. monocytogenes* (23, 24).

Transcription of *citZ* is partially constitutive during growth in BHI medium, because even when CcpC is active enough to repress P2, some transcripts from P1 pass through the CcpC roadblock. As a result, the cell is primed to synthesize some citrate and to fully derepress the TCA branch when the substrates for citrate synthase, oxaloacetate, and acetyl-CoA become available.

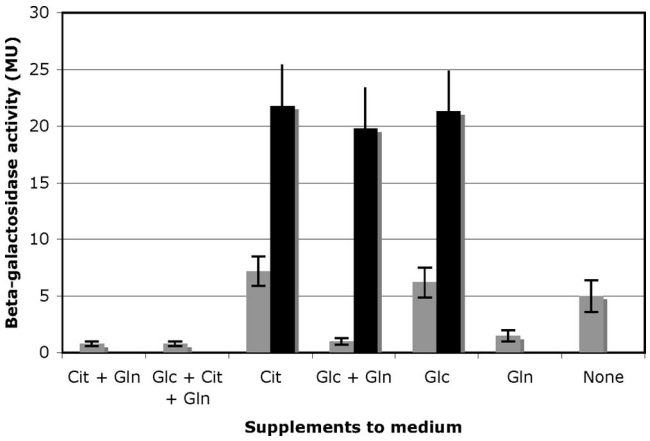

FIG. 9. Effect of medium composition on expression of a *lacZ* fusion in *L. monocytogenes*. Strains LMM16 (*ccpC*$^+$) (gray bars) and LMM21 (Δ*ccpC*::*spc*) (black bars), both of which carried an lmo1568-*lacZ* fusion, were grown at 37°C in BHI medium supplemented with 0.5% glucose, 1.6 mg glutamine per ml, or 0.5% citrate or with combinations of these supplements. β-Galactosidase activity was measured using samples taken at various points during the exponential phase of growth. The values are the averages of two experiments.

Is there a second mechanism that couples TCA branch expression to the physiological state of the cell? In *B. subtilis*, expression of *citZ* is regulated by the carbon source through the activity of CcpA (25). We found no effect of the carbon source on *citZ* expression, no role for CcpA in *L. monocytogenes citZ* regulation, and no sequence similar to the consensus CcpA-binding site in the region upstream of or within the *L. monocytogenes citZ* coding sequence.

Instead, *L. monocytogenes citZ* expression is repressed in a CcpC-dependent manner in BHI medium containing glutamine. Given the split Krebs cycle in this bacterium, 2-ketoglutarate is the only end product of the TCA branch, and the principal role of 2-ketoglutarate is to provide the carbon skeleton for glutamate and glutamine. Hence, repression of the lmo1569-lmo1568-*citZ* operon by CcpC in the presence of excess glutamine (which would presumably be reflected in high levels of 2-ketoglutarate) would allow efficient use of these enzymes. In *B. subtilis*, citrate synthase enzyme activity is feedback inhibited by 2-ketoglutarate (H. J. Kim, unpublished). If 2-ketoglutarate has the same effect on *L. monocytogenes* citrate synthase, its accumulation would affect the activity of CcpC indirectly by reducing the synthesis of citrate.

The dual regulation of *citZ* expression by CcpC presumably helps *L. monocytogenes* fine-tune the expression of TCA branch enzymes according to the metabolic state of the cell. Citrate synthase, the first enzyme in this pathway, controls the downstream enzymes aconitase and isocitrate dehydrogenase in two ways. First, citrate synthase produces citrate, the sub-

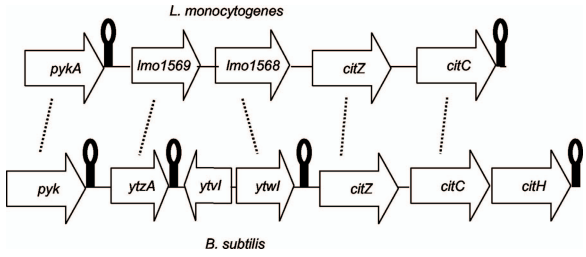

FIG. 10. Synteny of the *citZ* loci in *L. monocytogenes* and *B. subtilis*. The designations of the *L. monocytogenes* and *B. subtilis* genes are the designations used in the ListiList (http://genolist.pasteur.fr/ListiList/) and SubtiList (http://genolist.pasteur.fr/SubtiList/) databases. The arrows indicate genes and their orientations, and the balloons indicate the locations of putative transcription termination sites. Dotted lines connect genes whose products share sequence similarity.

strate for aconitase. Second, citrate antagonizes the interaction of CcpC with the *citB* promoter, thus inducing *citB* transcription (24). If it is cotranscribed with *citZ*, *citC* would also be under CcpC control. Hence, CcpC is likely to coordinate the regulation of all three enzymes of the oxidative branch of the Krebs cycle in response to the intracellular concentration of citrate.

ACKNOWLEDGMENTS

We thank H. J. Kim for providing plasmid pHK23 and B. R. Belitsky for helpful discussions and a critical review of the manuscript.

This work was supported by research grant GM036718 from the Public Health Service.

REFERENCES

1. **Arnaud, M., A. Chastanet, and M. Debarbouille.** 2004. New vector for efficient allelic replacement in naturally nontransformable, low-GC-content, gram-positive bacteria. Appl. Environ. Microbiol. **70:**6887–6891.
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. **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.
4. **Dabiri, G. A., J. M. Sanger, D. A. Portnoy, and F. S. Southwick.** 1990. Listeria monocytogenes moves rapidly through the host-cell cytoplasm by inducing directional actin assembly. Proc. Natl. Acad. Sci. USA **87:**6068–6072.
5. **Daniel, R. A., J. Haiech, F. Denizot, and J. Errington.** 1997. Isolation and characterization of the *lacA* gene encoding beta-galactosidase in *Bacillus subtilis* and a regulator gene, *lacR*. J. Bacteriol. **179:**5636–5638.
6. **Difco Laboratories.** 1998. Difco manual. Difco Laboratories, Detroit, MI.
7. **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.
8. **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.
9. **Flechtner, V. R., and R. S. Hanson.** 1969. Coarse and fine control of citrate synthase from Bacillus subtilis. Biochim. Biophys. Acta **184:**252–262.
10. **Fliss, I., E. Emond, R. E. Simard, and S. Pandian.** 1991. A rapid and efficient method of lysis of Listeria and other gram-positive bacteria using mutanolysin. BioTechniques **11:**453, 456–457.
11. **Fortnagel, P.** 1970. The regulation of aconitase and isocitrate dehydrogenase in sporulation mutants of Bacillus subtilis. Biochim. Biophys. Acta **222:**290–298.
12. **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.
13. **Galinier, A., J. Haiech, M. C. Kilhoffer, M. Jaquinod, J. Stulke, J. Deutscher, and I. Martin-Verstraete.** 1997. The Bacillus subtilis crh gene encodes a HPr-like protein involved in carbon catabolite repression. Proc. Natl. Acad. Sci. USA **94:**8439–8444.
14. **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.
15. **Hanson, R. S., and D. P. Cox.** 1967. Effect of different nutritional conditions on the synthesis of tricarboxylic acid cycle enzymes. J. Bacteriol. **93:**1777–1787.
16. **Henkin, T. M.** 1996. The role of CcpA transcriptional regulator in carbon metabolism in Bacillus subtilis. FEMS Microbiol. Lett. **135:**9–15.
17. **Herro, R., S. Poncet, P. Cossart, C. Buchrieser, E. Gouin, P. Glaser, and J. Deutscher.** 2005. How seryl-phosphorylated HPr inhibits PrfA, a transcription activator of Listeria monocytogenes virulence genes. J. Mol. Microbiol. Biotechnol. **9:**224–234.
18. **Jin, S., M. De Jesus-Berrios, and A. L. Sonenshein.** 1996. A *Bacillus subtilis* malate dehydrogenase gene. J. Bacteriol. **178:**560–563.
19. **Jin, S., and A. L. Sonenshein.** 1994. Identification of two distinct *Bacillus subtilis* citrate synthase genes. J. Bacteriol. **176:**4669–4679.
20. **Jin, S., and A. L. Sonenshein.** 1994. Transcriptional regulation of *Bacillus subtilis* citrate synthase genes. J. Bacteriol. **176:**4680–4690.
21. **Joseph, B., S. Mertins, R. Stoll, J. Schar, K. R. Umesha, Q. Luo, S. Muller-Altrock, and W. Goebel.** 2008. Glycerol-metabolism and PrfA activity in *Listeria monocytogenes*. J. Bacteriol. **190:**5412–5430.
22. **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.
23. **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.
24. **Kim, H. J., M. Mittal, and A. L. Sonenshein.** 2006. CcpC-dependent regulation of *citB* and lmo0847 in *Listeria monocytogenes*. J. Bacteriol. **188:**179–190.
25. **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.
26. **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.
27. **Mertins, S., B. Joseph, M. Goetz, R. Ecke, G. Seidel, M. Sprehe, W. Hillen, W. Goebel, and S. Muller-Altrock.** 2007. Interference of components of the phosphoenolpyruvate phosphotransferase system with the central virulence gene regulator PrfA of *Listeria monocytogenes*. J. Bacteriol. **189:**473–490.
28. **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.
29. **Park, S. F., and G. S. Stewart.** 1990. High-efficiency transformation of Listeria monocytogenes by electroporation of penicillin-treated cells. Gene **94:**129–132.
30. **Phan-Thanh, L., and T. Gormon.** 1997. A chemically defined minimal medium for the optimal culture of Listeria. Int. J. Food Microbiol. **35:**91–95.
31. **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.
32. **Sambrook, J., E. F. Fritsch, and T. J. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory, Cold Spring Harbor, NY.
33. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5463–5467.
34. **Schumacher, M. A., G. Seidel, W. Hillen, and R. G. Brennan.** 2006. Phosphoprotein Crh-Ser46-P displays altered binding to CcpA to effect carbon catabolite regulation. J. Biol. Chem. **281:**6793–6800.
35. **Schumacher, M. A., G. Seidel, W. Hillen, and R. G. Brennan.** 2007. Structural mechanism for the fine-tuning of CcpA function by the small molecule effectors glucose 6-phosphate and fructose 1,6-bisphosphate. J. Mol. Biol. **368:**1042–1050.
36. **Titgemeyer, F., and W. Hillen.** 2002. Global control of sugar metabolism: a gram-positive solution. Antonie van Leeuwenhoek **82:**59–71.
37. **Trivett, T. L., and E. A. Meyer.** 1971. Citrate cycle and related metabolism of *Listeria monocytogenes*. J. Bacteriol. **107:**770–779.
38. **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.
39. **Wach, A.** 1996. PCR-synthesis of marker cassettes with long flanking homology regions for gene disruptions in S. cerevisiae. Yeast **12:**259–265.