

## Catabolite Repression in *Lactobacillus casei* ATCC 393 Is Mediated by CcpA

VICENTE MONEDERO, MARÍA JOSÉ GOSALBES, AND GASPAR PÉREZ-MARTÍNEZ\*

*Departamento de Biotecnología, Instituto de Agroquímica y Tecnología  
de Alimentos, 46100-Burjassot, Valencia, Spain*

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**The chromosomal *ccpA* gene from *Lactobacillus casei* ATCC 393 has been cloned and sequenced. It encodes the CcpA protein, a central catabolite regulator belonging to the LacI-GalR family of bacterial repressors, and shows 54% identity with CcpA proteins from *Bacillus subtilis* and *Bacillus megaterium*. The *L. casei* *ccpA* gene was able to complement a *B. subtilis* *ccpA* mutant. An *L. casei* *ccpA* mutant showed increased doubling times and a relief of the catabolite repression of some enzymatic activities, such as *N*-acetylglucosaminidase and phospho- $\beta$ -galactosidase. Detailed analysis of CcpA activity was performed by using the promoter region of the *L. casei* chromosomal *lacTEGF* operon which is subject to catabolite repression and contains a catabolite responsive element (*cre*) consensus sequence. Deletion of this *cre* site or the presence of the *ccpA* mutation abolished the catabolite repression of a *lacp::gusA* fusion. These data support the role of CcpA as a common regulatory element mediating catabolite repression in low-GC-content gram-positive bacteria.**

Microorganisms can use a wide range of carbon sources for growth; however, they have developed systems that ensure the preferential use of readily metabolizable carbon sources. One of these systems is carbon catabolite repression (CR), which modulates gene expression in response to the availability of carbon compounds. CR is well known in enteric bacteria, in which the glucose-specific EIIA<sup>Glc</sup> of the phosphotransferase system (PTS) modulates the activity of adenylate cyclase, and it is the level of cyclic AMP which controls the transcriptional regulatory activity of the catabolite-activating protein (CAP) (36). There is also another mechanism of CR that is currently under study for enteric bacteria. This mechanism is cyclic AMP independent and uses the catabolite repressor-activator (Cra) protein, formerly designated FruR, which is a member of the LacI-GalR family of repressor proteins (40, 42).

Among low-GC-content gram-positive bacteria, CR has been best characterized for the genus *Bacillus* (22, 25). In these organisms, CR negatively regulates gene expression and does not involve cyclic AMP. Detailed studies of CR in *Bacillus subtilis* have shown that one of the components of the PTS, the HPr protein, is phosphorylated at a serine residue by an HPr kinase (8, 9). The activity of this HPr kinase is allosterically regulated by the levels of fructose-1,6-bisphosphate and P<sub>i</sub>, which in turn are related to the energy status of the cell. This P-Ser HPr has been shown to mediate inducer exclusion and expulsion effects which can cause CR (41, 51), but it can also interact with CcpA, a protein belonging to the family to which Cra of *Escherichia coli* belongs (7). This interaction stimulates the binding of CcpA to a 14-bp DNA sequence, called the catabolite-responsive element (*cre*), which is found in the 5' region of most genes subject to CR (13, 26). It is believed that CcpA-*cre* binding regulates gene expression by blocking transcription at the level of initiation or elongation. Recent findings also demonstrate P-Ser HPr-independent binding of CcpA complexed with glucose-6-phosphate to other accessory *cre*

sites (16, 32). This would explain why P-Ser HPr is necessary for CR of some genes but not others, such as the *amyE* gene (47). In addition, recent experiments have shown the existence of even other signal transduction pathways that modulate CR, which makes the picture of CR in gram-positive bacteria extremely complex. For example, *Staphylococcus xylosus* mutants defective in a glucokinase gene (*glkA*) still had 75% of the wild-type glucokinase activity but were derepressed for several metabolic activities (48). These findings suggest that GlkA may have a regulatory function in CR similar to that of the CR regulator glucokinase of the high-GC-content *Streptomyces coelicolor* (2).

HPr kinase and *cre* sequences in genes subject to CR have been found in many different gram-positive bacteria. The *ccpA* gene in *B. subtilis* (23), *Bacillus megaterium* (27), and *S. xylosus* (11) has also been characterized; it was also detected by PCR and inactivated in *Lactobacillus pentosus* (30), and antibodies against *B. megaterium* CcpA were shown to react with proteins in cell extracts of several gram-positive bacteria (29). These data suggest that the pathways mediating CR in the genus *Bacillus* could be common to other gram-positive bacteria.

*Lactobacillus casei* is a lactic acid bacterium found in the human intestine and in a wide variety of fermented food products. Preliminary studies of CR and its glucose and lactose transport systems have been reported elsewhere (4, 10, 46), and, recently, two mechanisms controlling the expression of the lactose operon have been suggested: CR mediated by the PTS components and possibly by CcpA and induction by lactose through the antitermination protein LacT (1, 15). However, detailed studies of this microorganism have been difficult because of the nearly complete absence of genetic tools.

We intended to identify a *ccpA* homolog in *L. casei* ATCC 393 and determine its molecular role as mediator of CR. For this purpose, the gene was first cloned and inactivated by a double crossover, the first report of a gene disruption in *L. casei*. In order to monitor the effect of *ccpA*, several enzymatic activities in the wild-type and mutant strains were quantified, and detailed analysis of the promoter region of the lactose operon, containing a *cre* site, was performed.

\* Corresponding author. Mailing address: Departamento de Biotecnología, Instituto de Agroquímica y Tecnología de Alimentos (C.S.I.C.), Polígono de la Coma s/n, Apartado de correos (P.O. Box) 73, 46100-Burjassot, Valencia, Spain. Phone: 34 6 3900022. Fax: 34 6 3636301. E-mail: gaspar.perez@iata.csic.es.

## MATERIALS AND METHODS

**Bacterial strains, plasmids, and culture conditions.** *L. casei* ATCC 393 cured of plasmid pLZ15 was the strain regularly used in all of the experiments, but the absence of the plasmid will not be mentioned hereafter. The strains of *E. coli* chosen were *E. coli* DH5 $\alpha$  for plasmid cloning and *E. coli* LE392 (Promega, Madison, Wis.) for phage propagation. *B. subtilis* GM1199 [*trpC2 pheA*  $\Delta$ (*bgaX*) *amyE*::(*gntRK'*-*lacZ*)] and GM1225 [*trpC2 pheA*  $\Delta$ (*bgaX*) *amyE*::(*gntRK'*-*lacZ*) *ccpA*::Tn917 $\Delta$ (*lacZ-erm*)] (8) were used for complementation studies.

Plasmid pUC19 was used for subcloning and sequencing different fragments of DNA that contained the *ccpA* gene. In order to inactivate the chromosomal copy of the *ccpA* gene from *L. casei*, the suicide vector pCCPA2.6 was constructed as follows. A 2.6-kb DNA fragment with the entire *ccpA* from the selected  $\lambda$ EMBL3 library clone was introduced in pACYC184 (3). The ends of the 1.8-kb *EcoRI*-*Clal* fragment of plasmid pJDC9 (5) that contained the erythromycin resistance gene of pAM $\beta$ 1 were filled in by using Klenow DNA polymerase and the fragment cloned in the *Bst*EII site (made blunt) that corresponded to the amino-terminal region of CcpA (see Fig. 1). We had also prepared plasmid pUCm1 (a pUC19 derivative carrying the chloramphenicol marker of pC194 [24] at the *Sma*I site), in which the 2.6-kb *ccpA* fragment containing the erythromycin determinant was cloned as a *Xba*I-*Sall* fragment to give pCCPA2.6.

A transcriptional fusion of the *L. casei* *lacTEGF* operon promoter (15) (accession no. Z80834), which lacked the lactose-specific induction region, with the structural gene for  $\beta$ -glucuronidase of *E. coli* was constructed as follows. *lacp* was amplified by PCR with primers lac11 (5'-TAGCACTGATCATTAAA-3') and lac12 (5'-GTCACAATCCACATTGA-3') and the fragment cloned in the *Sma*I site of pUC18. The orientation of the insert was checked by PCR. A clone with the appropriate orientation was digested with *Eco*RI, made blunt with Klenow, and digested with *Bam*HI. The resulting 223-bp fragment was then cloned in *Pvu*II/*Bam*HI-digested pNZ272 (34), giving pNZLac, which contained a *lacp*::*gusA* transcriptional fusion. A deletion in the *cre* site (ATAAAACGTTTACA) of the *lac* promoter in pNZLac was introduced by PCR after amplification of the whole plasmid with primers lac35 (5'-TTATTTTATTTTTTGTGA-3') and lac36 (5'-TTTACATTCTGTTTTCAGA-3'), the resulting fragment was ligated and transformed as described above to give pNZ $\Delta$ lac. This procedure introduced a 4-bp deletion,  $\Delta$ (AACG), in the central part of the *cre* sequence as was checked by sequencing. Plasmid pNZSPO2 (*SPO2p*::*gusA*) was constructed by cloning the 280-bp *Eco*RI fragment from pGAL9 (33), which contained the strong phage SPO2 promoter (*SPO2p*) (44), in the *Eco*RI site of pNZ272.

To express *ccpA* from *L. casei* in *B. subtilis*, the gene was amplified by PCR with primers ccpa8 (5'-TCAGATCTAAGGAGGAAATCAATGG-3') and ccpa9 (5'-CGTTGCACTTATCTAGACAATTCG-3'). These primers changed the ribosome-binding site (RBS) of *ccpA* to a more appropriate sequence for *Bacillus* and the TTG start codon to an ATG and introduced *Bgl*II and *Xba*I sites that were used to clone the gene under the control of SPO2 and AL9 promoters in pGAL9, resulting in pGCCPA.

*L. casei* cells were grown in MRS medium (Oxoid) or MRS fermentation medium (Adsa-Micro, Scharlau S.A., Barcelona, Spain) plus 0.5% carbohydrates at 37°C under static conditions. *E. coli* and *B. subtilis* were grown aerobically at 37°C in Luria-Bertani (LB) medium. When required, the concentrations of antibiotics used were 100  $\mu$ g of ampicillin or 20  $\mu$ g of chloramphenicol per ml to select *E. coli* transformants and 5  $\mu$ g of erythromycin or 5  $\mu$ g of chloramphenicol per ml for *L. casei* and *B. subtilis*. The media were supplemented with 1.5% agar when required. For assays of  $\alpha$ -complementation, 5-bromo-4-chloro-3-indolyl- $\beta$ -D-galactoside (X-Gal) (Sigma Chemical Co., St. Louis, Mo.) was included in the medium at 20 mg per liter.

When required, the sugar utilization patterns of the strains were determined with the API50-CH test series (Biomérieux, Marcy l'Étoile, France).

**Recombinant DNA procedures.** Restriction endonucleases, Klenow DNA polymerase, T4 DNA ligase, and T4 polynucleotide kinase were obtained from Boehringer Mannheim (Boehringer GmbH, Mannheim, Germany) and Amersham (Amersham International, Little Chalfont, Buckinghamshire, United Kingdom) and were used according to the instructions of the manufacturers. Dynazyme thermostable DNA polymerase (Finnzymes Oy, Riihitontuntie, Finland) was used for PCR, and the resulting fragments were cloned with the SureClone Kit (Pharmacia Biotech, Uppsala, Sweden).  $\gamma$ -<sup>32</sup>P- and  $\alpha$ -<sup>35</sup>S-labelled nucleotides were from Amersham. Recombinant DNA, plasmid, and  $\lambda$  DNA isolation techniques were essentially as described by Sambrook et al. (43). Competent *B. subtilis* cells were used for transformation. *L. casei* was transformed by electroporation with a Gene-pulser apparatus (Bio-Rad Laboratories, Richmond, Calif.) as previously described (35).

**Construction of a  $\lambda$ EMBL library of *L. casei* ATCC 393 and screening of recombinant clones.** *L. casei* DNA was isolated as described before (35) and partially digested with *Sau*3AI. The 8- to 20-kb fragments were isolated by sucrose gradient centrifugation, ligated to *Bam*HI-digested EMBL3 arms, and packaged with the Promega Packaging kit. The library was used to infect *E. coli*

LE392, and the initial 10<sup>5</sup> phage were recovered from the agar plates and stored at 4°C. To clone the *ccpA* gene, a PCR screening approach as described by Griffin et al. (17) was used.

**DNA sequencing.** Sets of nested deletions were constructed with the Double Stranded Nested Deletion kit from Pharmacia. Plasmid DNA was sequenced with the Sequenase 2.0 kit from Amersham and  $\alpha$ -<sup>35</sup>S-dATP. Sequence analysis was performed with the Genetics Computer Group (Madison, Wis.) package. Sequence comparisons were performed with the BLAST program (14).

**Southern and Northern hybridization.** Digested *L. casei* DNA was separated in agarose gels, blotted to Hybond nylon membranes (Amersham), and hybridized with digoxigenin-labelled DNA probes (Boehringer) at 60°C in 5 $\times$  SSC (1 $\times$  SSC is 0.15 M NaCl plus 0.015 M sodium citrate) buffer. The DNA probe used in both types of experiments comprised a fragment of 823 bp from nucleotides (nt) 363 to 1186 of the reported sequence (see Fig. 1). The hybridized probe was detected with anti-digoxigenin-alkaline phosphatase conjugate and the chemiluminescent substrate CSPD (Boehringer).

RNA was isolated from cells grown in MRS fermentation medium (Adsa-Micro) with 0.5% appropriate carbohydrates to an optical density at 550 nm (OD<sub>550</sub>) of 0.8. Cells (25 ml) were collected by centrifugation, washed with 20 mM Tris (pH 7.5), and resuspended in 1 ml of 0.2 M ammonium acetate-20 mM magnesium acetate-25% sucrose-5 mg of lysozyme, 10 U of mutanolysin. After 30 min of incubation at 37°C, the cells were centrifuged and lysed with 500  $\mu$ l of 0.2 M sodium acetate (pH 4.8)-10 mM EDTA-1% sodium dodecyl sulfate-500  $\mu$ l of phenol. Nucleic acids were extracted with phenol-chloroform and chloroform-isoamyl alcohol, and RNA was precipitated at 4°C, with 8 M LiCl added to a final concentration of 2 M. The RNA pellet was washed with 70% cold ethanol and resuspended in diethylpyrocarbonate-treated water. RNA (15  $\mu$ g) was separated in 1% agarose-formaldehyde gels, transferred to Hybond nylon membranes, hybridized at 65°C with a DIG-labelled DNA probe of the *ccpA* gene, and detected as described above.

**Primer extension.** A 15- $\mu$ g amount of RNA was annealed for 5 min at 65°C to 0.2 pmol of <sup>32</sup>P-5'-labelled oligonucleotide (ccpa7 [5'-CCATTAACGACTCGA GACTG-3']; nt 377 to 356 of the *ccpA* gene sequence) in 20  $\mu$ l of 1 $\times$  reverse transcriptase buffer (Amersham) containing 0.5 mM deoxynucleoside triphosphates and extended for 1 h at 42°C with 10 U of avian myeloblastosis virus reverse transcriptase (Amersham). The cDNA products were loaded in a 6% polyacrylamide-6 M urea gel, together with a sequencing reaction performed with the same primers, and the bands were detected by autoradiography.

**Enzymatic assays.** Permeabilized *L. casei* cells were obtained as described elsewhere (4). Crude extracts were obtained by vortexing at high speed with glass beads (0.1-mm diameter) for 2 min three times with 1-min interludes during which the cells were kept on ice.

The *N*-acetylglucosaminidase assay was carried out in a 250- $\mu$ l volume containing 10 mM potassium phosphate (pH 6.8), 1 mM MgCl<sub>2</sub>, 5 mM *p*-nitrophenyl-*N*-acetyl- $\beta$ -D-glucosaminide (Sigma), and 5  $\mu$ l of permeabilized cells at 37°C. The reaction was stopped with 250  $\mu$ l of 5% Na<sub>2</sub>CO<sub>3</sub>, and the OD<sub>420</sub> was measured. Phospho- $\beta$ -galactosidase and - $\beta$ -galactosidase were assayed with *o*-nitrophenyl- $\beta$ -D-galactopyranoside-6-phosphate and *o*-nitrophenyl- $\beta$ -D-galactopyranoside as previously described (4).  $\beta$ -Glucuronidase activity was determined as previously described (34). Protein contents were determined by the Bio-Rad dye-binding assay. The production of acetoin in *B. subtilis* grown in LB medium plus 1% glucose was assayed qualitatively by the Voges-Proskauer method.

**Nucleotide sequence accession number.** The nucleotide sequence presented in this paper is available from the EMBL database under accession no. U28137.

## RESULTS

**Cloning and sequence analysis of the *L. casei* *ccpA* gene.** A set of primers (kindly provided by C. Lokman and P. Pouwels, TNO, Rijswijk, The Netherlands) synthesized from the sequence of the *B. megaterium* *ccpA* gene were used in a PCR with *L. casei* DNA as the template. A product of the expected size (800 bp) was obtained, cloned in pUC18, and sequenced, and the sequence showed good homology with the *Bacillus* *ccpA* gene. The DNA sequence obtained was then used to design a pair of specific primers in order to screen a  $\lambda$ EMBL3 *L. casei* library. A total of 1 of 500 lambda clones turned out to be positive. The DNA of one of these clones containing a 13-kb insert was isolated, and the *ccpA*-homologous region was mapped by Southern blotting and subcloned. Sequence analysis of a 3,015-bp fragment revealed two open reading frames

FIG. 1. Sequence of the region containing the *ccpA* gene from *L. casei* ATCC 393 and deduced amino acid sequences. AT-rich stretches in the promoter region of *ccpA* are in boldface. The transcriptional start site is indicated by a vertical arrow. Deduced -10 and -35 sites and the RBS are underlined. A potential *rho*-independent terminator is marked by inverted arrows. The two inverted repeats (IR) flanking the putative transposase gene are in boldface.

1 TCCTCAACGTTGTTCACTGGCGCAGTACCATGCAGGTGCTGTGCTATTTAACCAGGACTAGCTCGGATCTGGCTTAAAGTCGTGGCCCA

91 ATCAAGCATCGTGGTAAATAGCACAACTTAAACTTTAGCTTTTAGTATATACCGTGACTAGTGTGATGGGCTTCTGAAAGGGGGT  
-35 -10  
181 TGTGAAATTTTCATGTAATTCAGTAAACGCTTGCATTGCTATTTCTATTCTGTTACTTTTGAATGTAATAGAACTTCGTGATTTCA  
RBS  
271 TCTTTTCACGACTAAGGAGAAAATCAATTGGAAAAGCAAACAATTACAATTTATCGTGTGCGGAGGCTAATGCTCAATGGCGACAGTG  
ccpA ---> M E K Q T I T I Y A C R E A N V S M A T V  
BstEII  
361 TCTCGAGTCGTAAATGGTAACCCAAATGTGAAACAGCTACTCGCAAGAAGTTTGGAAAGTATCGAACGGCTTGAATTCGGCCGAAT  
S R V V N G N P N V K P A T R K K V L E V I E R L D Y R P N  
HindIII  
451 CGAGTTGCTCGTGGTCTGGCAAGCAAGAAGCACAACCTGTGGGGTAAATATCCAGATGTCACGAACATGTTCTTCTCAAGCTTGGCT  
A V A R G L A S K K T T T V G V I I P D V T N M F F S S L A  
541 CGTGGATTTGATGATGTTGCCACGATGTACAAGTACAATATCATCTGGCGAAGCTCTGATGAAAACAATCAAAAAGAAGTCACGGTGTTA  
R G I D D V A T M Y K Y N I I L A N S D E N N Q K E V T V L  
631 AACACATTTGGCTAAACAAGTGGATGGTTGATTTTCATGGGCCATGAATTGACTGACTCAATTCGTGCTGAATTCACGCAAGTAAAG  
N T L L A A K Q V D G L I F M G H E L T D S I R A E F S R S K  
EcoRI  
721 ACCCTGTTGCTGCTGGCTCAATCGATCTGACGAACAGGTGGCAGTGTCAACATCGATTACGTGGCTGCTGCAAGAAGCCACG  
C l a I C l a I  
T P V V L A G S I D P D E Q V G S V N I D Y V A A V E E A T  
811 CGTCAGTTATGGAAAGCGCAACAAGCGGTTGCTCTGCGCACCGGTTCAATTGACGCATCCGATCAATGGGAGTCCGGCTCAAGGGT  
R Q L L E S G N K R V A L A T G S L T H P I N G Q F R L K G  
901 TACAACAGGCTCTGGAAAAGCGGTTGCTTATGACGAAAGCCTGATTTTGAAGTGAAGCAAGTACCAAGCTGGTTAGCCCTG  
Y K Q A L E K A G V A Y D E S L I F E N E P S Y Q A G L A L  
991 TTTGATAAGTTGCAGAAAGTTGGGCAACGGCGGTTATTGACAGTGTGACGAGTTAGCAGTTGGCCTTTGGATGGGCAATTGATAAG  
F D K L Q K V G A T A V I A G D D E L A V G L L D G A I D K  
1081 GGCCTAAGTGGCGACGACTTTGAAATTCACCAGCAATAACACCAAACTGACGGAATGACTCGGCCACAACCTACTTCGATTGAT  
G V K V P D D F E I I T S N N T K L T E M T R P Q L T S I D  
1171 CAGCCATTTGATGATTTGGTGGGTTGCTATGCGCTTGTGACCAAGATGATGAATAAAGAAGAGATCGAGGAAAAGCCGTTATGCTT  
Q P L Y D I G A V A M R L L T K M M N K E E I E E K T V M L  
1261 GGCTTCGATATTTGAAGCGTGGTTCAACGAAATAATGCCAGCTTTTGTACGTTAGCAGACATGTAAGTCATTAGGGTAAAGAAAA  
G F D I L K R G S T K \*  
-----> <----->  
1351 AGGCTTCCAAGCAATTAAGGAGCCTTTTCTTTGCGGCGAATTTGCAAACTAAGT  
AAAGAACCGCGCTTAACAGTTGATTACGTTGCCAAACAGTTTTTTATGAAGTATAC  
IR2 \* V L H L P K D F F V E Y  
1441 CCCAAGTATCAAATTTGAAACGCTCCTGCAACTAAATGACGAAAACCGTCACAAGTTGCAGGACAAGACATATAATAGTTATAGAC  
P T E Y N L V K R P R Q N L Q K Q C H E V D Q E T Y N D I D  
1531 AAGCGAAACCTTAATAAAGGGCTAATTAGGTAAACATAAGAGTAAACAAGGTGCGACAAGTCCGCTTATACCTAGCCCGTTATCTGTC  
T R K P I I E R I L G N T N E N T G R Q E P S Y P D P W Y V  
1621 ATTGTGATTGGAAGCGACTAAAGTAGTTCTACTTCTTAAAGCCAGGTTACTAGACCACATTTACTTGTAAAGAAATCCTTACC  
T V G L R E S I E D L H L F E T G H D P T I S H V F E K P I  
1711 GGGATTGCAAGTTGATTCGGAATAAATAGAGGACGCTCAGGAAAACGTTGGAAAACTATTCGCATTTCGGCTGATTTCGGCTAGCT  
A G L L E V L G K N I E Q S D K Q V V K K I L T L R S L R D  
PvuI  
1801 GTTGGCAATTTTCACCTCACTGGGCAAGCTGGTTAGTGTCTATAGTTAAAGGGTCAAGTGGATGCAACTGCGCAACTATTTGGTC  
V T L L I S H G T R G I V T D I E W D G I R Q R Q N R L  
1891 CCGCGAGTACCTAGCTCTATATCAGCCAGACATGAAGAGCTACAGCAGATCATAAGAACTTACGGCTCAGGCTACAAATACAGACTTA  
P R E H I S I Y D T Q V E R H R R T N K S H R T R H K H R L  
1981 CCGCGACTATGGCCATAGTGGTGCACCTCCTCATGAAGAGTGGATTTAACACAACCTATCTACTGCCATTTAACACAACATCGATTGTG  
H R Q I G T D G R S S Y K E G L N H Q Y I H R Y I T N Y S V  
BstEII  
2071 TGTAACACAAAGGAAATTTTCGCTCGCCATAGACGAGACCTGGTCAAGCCTAGATTCTACATTACTTGTAAACTGGCGTATTAAC  
C W G H E K N F R A T I Q E P S W H P D L I Y H V I Q R W L T  
2161 CACCTACGACAGATTATCGGCAAGAACTGTTAAGACGGCGAAAACCTACTATCAGTAATAGACGAAACGAAACCCCTTATTAATGGAC  
Q P H S D L L R K K H C N Q R K Q H Y D N D A K S P S Y N G  
2251 GTGTTGAAAATTGAGGCACTTTATCAGAACCTAAAGCGGGTAGGCTTGGTGTGCGGCTATCTATTGGGAAATCGAGCATTTT  
A C R K L E R S I T S P N R G M R L A V A R I S L G K A R L  
2341 TGATTTCATAAGTGAAGAAAATATCATTCTACAATAATCGAGTATGGCCTATGGAGCTTAACAAACTAAACACCAATTTGTATAAGA  
F M L I E R E K I T L H K Y S M <--- Tnase  
2431 TGGCCATACCCAAATAAAAAATGAACACAACGTAAGTTAACATTTAAGCGCGCAACTTTACTTTTGGACTAGCACAAACATGA  
IR1  
2521 CTGGCGCGGTCAGCAACAACGTCACCCAGACAACTAACATAATAATCACGGTGGTCATAAGCATGGCGGTGGTCAGCAACATG  
G G G T T T A P P T T T T N T N N T G G T N T G G G T T  
2611 GACACGGCCAAACAGCAGCGTCAAGACACAGCATGGTATGGCGGTGAGGTTGAGGCGGTAACGACGGCGTCAAGGACACC  
G T G T T T G T E T T T G T G G T G G T R D G N S G G T G T  
2701 AAACACCAAGTGAACCGACTTAAAGTCTACAGGGAACAACCAACGACTGAACCTACCGTACAGTGGATCCTCGTGACGATCCAC  
T Q P K V K A P N E S T G Q Q N Q Q S S P V T V S P A S S P  
2791 AGCAGCTCATAGAAGTCAACCGCACTAGCCCCAAGAATCAAGAAAAACCGAAGTCAACTTAGGAGCCAACCGTAGCCCTTTGGACT  
T T A Q T D E T P T I P T E Q T H E K K A E T S D E T P M P F R  
2881 GGTATCGGTAACAAACGTTAGTTCTACAGCTTTCTGTTGACTATTAATCGAAATGACGGTGGCTACCGTTTTAGTAAAAAGCAAC  
V M A M K N A D L H D F F L Q Y N A K S G G I A F D N K T T  
2971 CGACGGTTAGTAACATTTCCGACCACTGCAACTCGGCGTCAAG  
P Q W D N Y L S T V T S G G T <--- ORF

	1				50
CcpA_Lbca	MEKQTITTYA	.CREANVSMA	TVSRVNVGNP	NVKPATRKKV	LEVIERLDYR
CcpA_Sxyl	...MTVTIYD	VAREARVSMA	TVSRVNVGNQ	NVKPETRDKV	NEVIKKLNYR
CcpA_Bmeg	...VNVTIYD	VAREASVSMA	TVSRVNVGNP	NVKPSTRKKV	LETIERLGYR
CcpA_Bsub	...MSNITTYD	VAREANVSMA	TVSRVNVGNP	NVKPTTRKKV	LEAIERLGYR
RegA_Clac	...MATSIKID	VAREAGVSIA	TVSRVNLNDID	VVNEDTKKKV	LDAIKELGYR
	51				100
CcpA_Lbca	PNAVARGLAS	KRTTIVGVII	PDVTNMFSS	LARGIDDVAT	MYKYNIILAN
CcpA_Sxyl	PNAVARGLAS	KRTTIVGVII	PDISNVVYSQ	LARGLEDIAT	MYKYHSIISN
CcpA_Bmeg	PNAVARGLAS	KRTTIVGVII	PDISNIFYAE	LARGLEDIAT	MYKYNIILSN
CcpA_Bsub	PNAVARGLAS	KRTTIVGVII	PDISSIFYSE	LARGLEDIAT	MYKYNIILSN
RegA_Clac	PNIVARSIKT	QRTKTIGILL	PDISNQFYPE	IVRGAEDVSN	IYDNYIILCN
	101				150
CcpA_Lbca	SDENNOKEVT	VLNLTLLAKQV	DGLIFMGHEL	TDISRAEFSS	SKTPVVLGAS
CcpA_Sxyl	SDNDPSKEKE	IFNNLLSKQV	DGIIFLGGTI	SEIIRDLINK	SSVPVVVSGT
CcpA_Bmeg	SDQNQDKELH	LLNNMLGKQV	DGIIFMSSGNV	TEEHVELLKK	SPVPVIVLAAS
CcpA_Bsub	SDQNMKEKELH	LLNTMLGKQV	DGIVFMGGNI	TDEHVAEFKR	SPVPVIVLAAS
RegA_Clac	SDDLIEKEKE	YLRVLKEKVM	DGVIYMSSSL	RDEILELINE	LDLTKVLVET
	151				200
CcpA_Lbca	IDPDEQVGSV	NIDYVA.AVE	EATRQLLESG	NKRVALATGS	LTHFINGQFR
CcpA_Sxyl	NGKDEGISSV	NIDFATLPAK	EITEHLIEKG	AKSFVAVGGD	YSKKAQEDV.
CcpA_Bmeg	IESTNQIPSV	TIDYEQ.AAF	DAVQSLIDSG	HKNIAFVSGT	LEEPINHAKK
CcpA_Bsub	VEEQEETPSV	AIDYEQ.AIY	DAVKLLVDKG	HTDIAFVSGP	MAEPIINSKK
RegA_Clac	RDKDGVLPVS	TIDNIK.GSY	DSTNLLIQKG	IKDIAFIGTK	KDNMNAWGDR
	201				250
CcpA_Lbca	LGKYKQALEK	AGVAYDESLEI	FENEPSYQAG	LALFDKIQKV	G..ATAVIAG
CcpA_Sxyl	LVGLKDVLVQ	HELELDEQLI	FNGNETYKDG	LRAFESL..A	TAKPDAILSI
CcpA_Bmeg	VKGYKRALTE	SGLPVRDSYI	VEGDYTYDSG	IEAVEKLEEE	DEKPTAIFVG
CcpA_Bsub	LQGYKRALEE	ANLPFNEQEV	VEGDYTYDSG	LEALQHLMSL	DKKPTAILS
RegA_Clac	YVGYEKAMNE	AGIKIDPELL	YLDISKVRSG	YEGIQHFLGL	NKKFKGVVCA
	251				300
CcpA_Lbca	DDELAVGLLD	GAIDKGVKVP	DDFEIITSNN	TKLTEMTRPQ	LTSIDQPLYD
CcpA_Sxyl	SHEQAIGLVH	AAQDAGVNVV	NDLQIVSFNN	TRLVEMVRPQ	LSSVIQPLYD
CcpA_Bmeg	TDEMALGVIIH	GAQDRGLNVP	NDLEIIGFDN	TRLSTMVRPQ	LTSVVPQPMYD
CcpA_Bsub	TDEMALGIIH	AAQDQGLSIP	EDLDIIGFDN	TRLSTMVRPQ	LSTVVQPTYD
RegA_Clac	SDDIAMGAIN	ALRDNNMEVP	KDVSVVGFND	NFAASIFYPK	ITTVSQPTYD
	301				338
CcpA_Lbca	IGAVAMRLLT	KMMNKEEIEE	KTVMLGFDIL	KRGSTK..	
CcpA_Sxyl	IGAVGMRLT	KYMNEEDIDE	PNVILPHRIE	YRGTTK..	
CcpA_Bmeg	IGAVAMRLLT	KYMNETVDS	SIVQLPHRIE	FRQSTK..	
CcpA_Bsub	IGAVAMRLLT	KLMNKEPVEE	HIVELPHRIE	LKRSTKS.	
RegA_Clac	MGSVAMRMLI	KLLNKKELDE	PNYVLEHELI	ERESTI..	

FIG. 2. Multiple amino acid sequence alignment of CcpA from *L. casei* (Lbca), *B. subtilis* (Bsub), *B. megaterium* (Bmeg), and *S. xyloso* (Sxyl) and RegA from *Clostridium acetobutylicum* (Clac). The N-terminal helix-turn-helix DNA-binding motifs of the proteins are boxed.

(ORFs) and part of a likely third one (Fig. 1). The first ORF started with a TTG codon which had a potential RBS (AAG GAG) 8 bp upstream. A putative promoter region with characteristic features, such as AT-rich regions and two likely  $-10$  (TG\_TACTTT) and  $-35$  (TTGCAT) regions, was also found. This region coincided with the transcriptional start point determined by primer extension (see below). This first gene encoded a protein of 333 amino acids with an estimated molecular mass of 36 kDa that displayed 54.3 and 54.6% identities with CcpA from *B. subtilis* and *B. megaterium*, respectively, and 51.3% identity with the product of the recently cloned *ccpA* gene from *S. xyloso* (11) (Fig. 2). On the basis of this homology, this gene was designated *ccpA*. The product of this gene contained the DNA-binding helix-turn-helix motif, the putative dimerization, and the inducer-binding domains of proteins of the LacI-GalR family, as suggested by Weickert and Adhya (49).

Downstream of the *ccpA* gene, a potential *rho*-independent terminator was found, followed by two ORFs that could be transcribed in the direction opposite to that of *ccpA*. One was incomplete, and no homology to other sequences in available data bases could be found. The second ORF could encode a 328-residue protein with good homology to transposases of insertion elements of the IS30 family, 46% identity to the transposase of ISAS2 from *Aeromonas salmonicida* (21), and 45.5% identity to the transposase coded by IS1139 from *Streptococcus salivarius* (31). The fact that two imperfect inverted-repeat sequences of 50 nt flank this ORF suggests that it could

be an insertion element, which is present in single copy in the *L. casei* chromosome, as determined by Southern blotting (not shown).

**Transcriptional analysis of *ccpA*.** The transcriptional start point of the *ccpA* gene, which was determined by primer extension experiments, was located at nt  $-47$  (A) relative to the translational start (Fig. 3A). Transcription of *ccpA* seemed to be relatively constant under different growing conditions (0.5% glucose, lactose, and ribose), although the amount of the transcript was slightly smaller in cells grown on glucose.

A unique 1.1-kb RNA was detected by Northern blotting with a *ccpA* probe (Fig. 3B). This result indicates that the *ccpA* gene is transcribed as a single RNA that would terminate at the stem-loop structure.

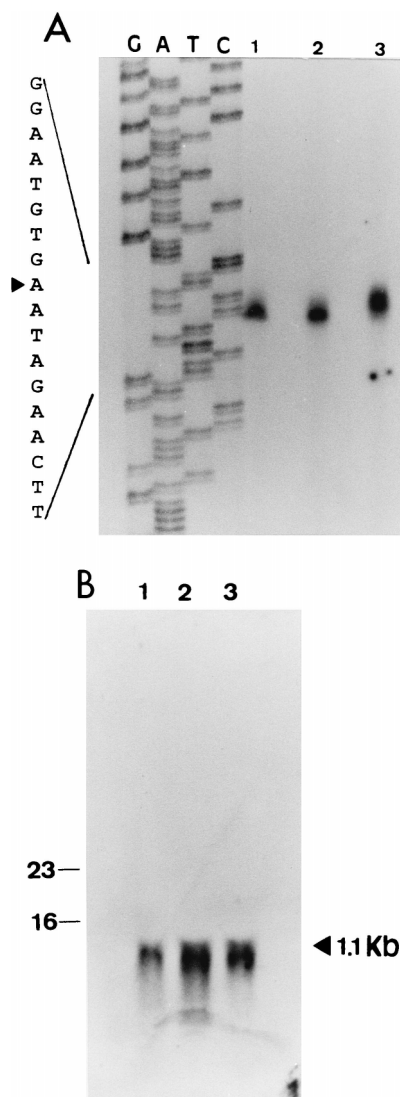


FIG. 3. Transcriptional analysis of *ccpA*. (A) Primer extension of the *ccpA* gene with avian myeloblastosis virus reverse transcriptase and a  $^{32}$ P-labelled oligonucleotide complementary to the RNA strand. Fifteen micrograms of total RNA from cells grown in MRS basal medium with 0.5% glucose (lane 1), lactose (lane 2), or ribose (lane 3) to an  $OD_{550}$  of 0.8 was used in each reaction. (B) Northern blot of *L. casei* RNA (15  $\mu$ g) separated in a 1% agarose-formaldehyde gel and hybridized with a DIG-labelled *ccpA* probe. Migration of the 23S and 16S rRNAs is indicated by the blot. Lanes 1, 2, and 3 are the same as in panel A.

**Disruption of the *L. casei ccpA* gene.** In order to inactivate *ccpA* by Campbell-like recombination, *L. casei* was transformed with pCCPA2.6. The first *Erm<sup>r</sup> Cm<sup>r</sup>* transformants contained one or several amplified tandem copies of the integrated vector (Fig. 4A). One of the colonies with the plasmid integrated in single copy was grown for 200 generations on erythromycin-containing medium. Strains that had undertaken the second recombination event could be selected as *Erm<sup>r</sup> Cm<sup>s</sup>* colonies on MRS agar plates. That process favored the selection of clones with the excision of the integrated plasmid and disruption of the *ccpA* gene (Fig. 4B). The strain constructed contained a stable integration of an *erm* determinant in *ccpA*.

The fermentation pattern of the mutant selected was exactly the same as that of the wild type, as determined by the API50-CH (Biomerieux) series, but its doubling times (*t*<sub>2</sub>) on different sugars were clearly affected (Table 1). The mutant showed *t*<sub>2</sub> values higher than that of the wild type on all of the eight sugars tested, and the values were notably greater on ribose or mannitol.

**Enzymatic activities in the wild type and the *ccpA* mutant.**

The effect of the *ccpA* mutation on CR in *L. casei* was investigated by measuring *N*-acetylglucosaminidase and phospho-β-galactosidase, two enzymatic activities that are subject to CR. The level of *N*-acetylglucosaminidase activity was low in the presence of glucose or fructose and progressively increased in lactose and ribose (Table 2). Glucose repressed this activity by a factor of 9.2 compared to ribose. In the *ccpA* mutant, CR of *N*-acetylglucosaminidase was abolished.

Phospho-β-galactosidase activity was clearly induced by lactose in both the wild type and the *ccpA* mutant, since very low levels of activity were found when the cells were grown on glucose, fructose, or ribose (Table 2). The presence of glucose or fructose in addition to lactose repressed the activity in the wild type by a factor of 73, regardless of whether the inoculum was pregrown on lactose (Table 2). In the *ccpA* mutant, glucose and fructose in addition to lactose still repressed the phospho-β-galactosidase, but not as strongly as in the wild type (10- and 2.8-fold, respectively). This partial relief of CR in the *ccpA* mutant was found only when the cells had previously been pregrown on lactose (Table 2). When a glucose-grown inoculum of the *ccpA* mutant was used in the assays, it showed phospho-β-galactosidase activities similar to those of the wild type (3.0 and 3.3 nmol/min/mg of protein for lactose plus glucose and lactose plus fructose, respectively).

**Influence of *ccpA* on the *lacTEGF* operon expression.** Phospho-β-galactosidase in *L. casei* ATCC 393 is encoded by the *lacG* gene, which is located in the *lacTEGF* operon, together with the genes for the lactose-specific PTS (*lacE* and *lacF*) and the regulator, *lacT* (15). The strong CR of the phospho-β-galactosidase led us to study in more detail the expression of this operon, which transcribed as a single mRNA (1). The promoter region of the *lac* operon has a putative *cre* site, with only one deviation from the consensus sequence (ATAAAAC GTTACA); the *cre* overlaps the -35 sequence and a ribonucleic antiterminator sequence (RAT) plus a *rho*-independent terminator located downstream, both possibly involved in lactose induction by an antitermination mechanism mediated by LacT (1, 15).

In order to determine if the observed CR was due to the binding of CcpA to the putative *cre* site, wild-type *L. casei* and the *ccpA* mutant were transformed with pNZlac. This plasmid carries a transcriptional fusion of the *E. coli gusA* gene to the *cre*-containing *lac* promoter. In this fusion, the RAT terminator area was not cloned to exclude the effect of lactose regulation. In spite of the high copy number of the plasmid, glucose repressed expression of the fused β-glucuronidase gene by

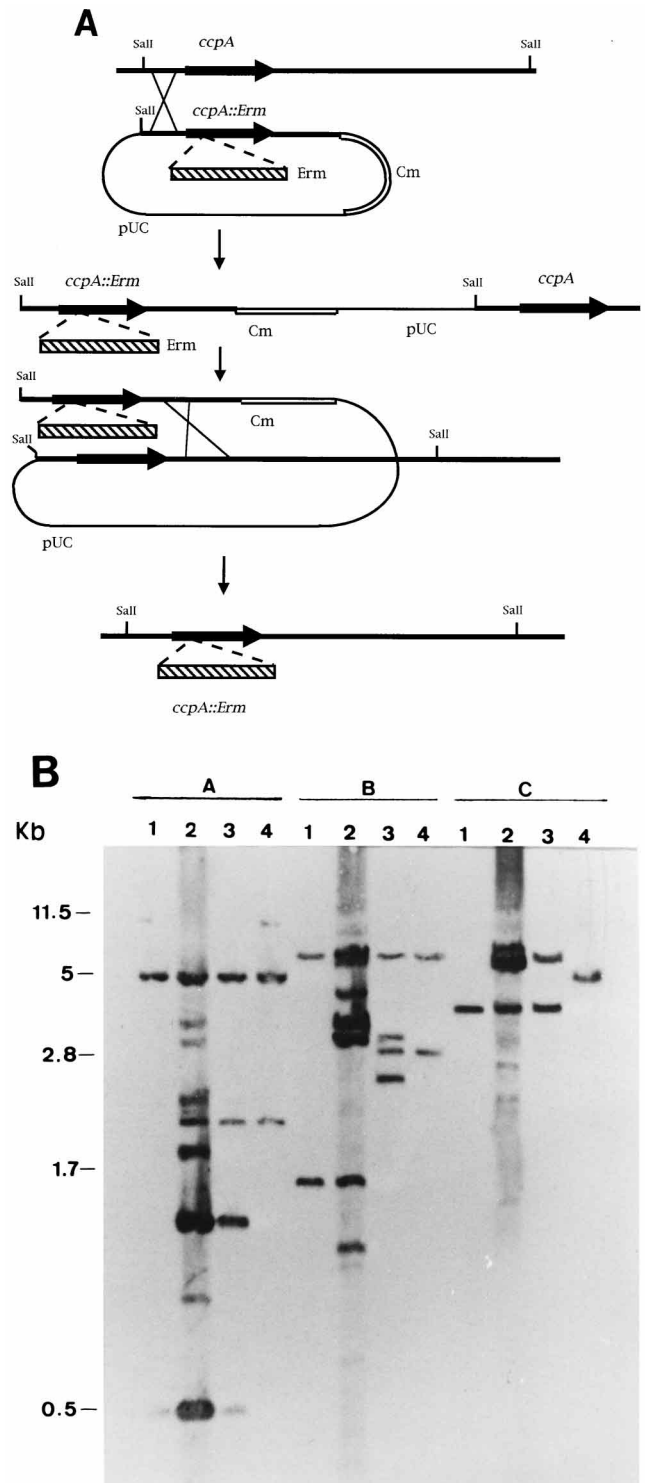


FIG. 4. Construction of a *ccpA* mutant. (A) Strategy for inserting the *erm* gene of pAMβ1 in the *ccpA* gene in two consecutive recombination events: *Erm*, erythromycin; *Cm*, chloramphenicol. (B) Southern blot of digested *L. casei* DNA hybridized with a *ccpA* probe. Lanes 1, wild type; lanes 2, integrant with multiple tandem copies of the integrated vector; lanes 3, single-copy integrant of the vector; lanes 4, *ccpA::erm* recombinant after the second crossover. A, *Hind*III; B, *Eco*RI; C, *Sal*I.

TABLE 1.  $t_2$  of wild-type *L. casei* ATCC 393 and the *ccpA* mutant

Growth in	$t_2$ (min) <sup>a</sup>	
	Wild-type	<i>ccpA</i> mutant
Glucose	94.0 ± 6.8	118.2 ± 9.6
Fructose	95.6 ± 10.0	122.0 ± 10.4
Mannose	104.1 ± 4.6	135.7 ± 5.2
Lactose	147.7 ± 9.0	169.6 ± 18.6
Ribose	146.1 ± 15.3	238.3 ± 10.2
Maltose	158.6 ± 13.9	172.9 ± 15.3
Galactose	106.8 ± 13.2	138.6 ± 8.4
Mannitol	133.7 ± 4.5	195.0 ± 3.1

<sup>a</sup>  $t_2$  values. Growth was measured as OD<sub>550</sub> on MRS fermentation medium plus 0.5% appropriate sugars at 37°C. Data from three growth curves are presented.

ninefold compared to the activity found on ribose-grown cells (Table 3).

In the *ccpA* mutant, the transcription turned out to be sugar independent; however, its level was lower than that of the wild type. After deletion of the central AAGC sequence of the *cre* site (pNZΔlac [Table 3]), the glucose control of the fusion was lost. The SPO2 constitutive promoter (44) was used as a control. In pNZSPO2 (*SPO2p::gusA*), the expression of *gusA* was not sugar dependent and β-glucuronidase activities in the wild type and in the *ccpA* mutant were comparable (Table 3). These results indicate that the *cre* sequence of the *lac* promoter is a target for CR and proves the involvement of the cloned *ccpA* gene in the CR of the *lac* operon in *L. casei*.

**Complementation of a *B. subtilis* *ccpA* mutant.** The similarities between *ccpA* from *Bacillus* species and *L. casei* prompted us to test the complementation of a *B. subtilis* *ccpA* mutant with the *L. casei* gene. *B. subtilis* GM1225 carries a fusion of the gluconate kinase promoter with the *lacZ* gene (*gntRK'-lacZ*), as well as a *ccpA* mutation (8). In this strain, the expression of *lacZ* is induced by gluconate and is not repressed by glucose, while in GM1199, containing wild-type *ccpA*, the presence of a *cre* site in the reading frame of *gntR* causes a strong repression of β-galactosidase in the presence of glucose (Table 4). Initial attempts to complement GM1225 expressing *L. casei*

TABLE 2. Enzymatic activities in wild-type *L. casei* ATCC 393 and the *ccpA* mutant

Enzyme	Sugar	Enzymatic activity <sup>a</sup>	
		Wild type	<i>ccpA</i> mutant
N-acetylglucosaminidase	Glucose	3.2 ± 0.2	26.7 ± 3.2
	Fructose	4.1 ± 0.2	26.0 ± 2.0
	Lactose	14.1 ± 2.1	31.8 ± 2.1
	Ribose	29.5 ± 1.2	24.2 ± 3.5
Phospho-β-galactosidase	Glucose	0.9 ± 0.1	2.7 ± 0.1
	Fructose	1.0 ± 0.1	2.8 ± 0.4
	Ribose	8.9 ± 0.4	10.3 ± 1.3
	Lactose	103.4 ± 8.4	105.0 ± 7.6
	Lactose + glucose <sup>b</sup>	1.4 ± 0.3	10.3 ± 1.8
	Lactose + fructose <sup>b</sup>	1.4 ± 0.4	37.8 ± 0.2

<sup>a</sup> The assays were performed with cells grown with 0.5% of each sugar to an OD<sub>550</sub> of 0.8. *p*-nitrophenyl-*N*-acetyl-β-D-glucosaminide was used as the substrate for *N*-acetylglucosaminidase, and *o*-nitrophenyl-β-D-galactopyranoside-6-phosphate was used as the substrate for phospho-β-galactosidase. Activities are given in nanomoles per minute per milligram of dry weight and of protein, respectively. Means and standard deviations of at least three experiments are given.

<sup>b</sup> The inoculi for these assays were grown in lactose.

TABLE 3. Expression of different transcriptional fusions in wild-type *L. casei* ATCC 393 and the *ccpA* mutant

Plasmid	Sugar	Glucuronidase activity (nmol/min/mg of protein) <sup>a</sup>	
		Wild type	<i>ccpA</i> mutant
pNZlac	Glucose	13.1 ± 2.4	31.0 ± 3.0
	Lactose	93.9 ± 1.0	29.7 ± 2.1
	Ribose	121.3 ± 7.8	36.6 ± 4.0
pNZΔlac	Glucose	71.2 ± 3.5	66.6 ± 4.1
	Lactose	64.2 ± 8.0	110.9 ± 8.2
	Ribose	51.0 ± 1.1	96.7 ± 5.3
pNZSPO2	Glucose	21.7 ± 3.3	20.7 ± 3.2
	Lactose	19.6 ± 1.4	34.7 ± 4.0
	Ribose	10.0 ± 0.1	18.3 ± 0.6

<sup>a</sup> Activities were measured with *p*-nitrophenyl-β-D-glucuronic acid in extracts of cells grown with 0.5% of the different sugars to an OD<sub>550</sub> of 0.8. Means and standard deviations of at least three experiments are given.

*ccpA* from its own initiation signals failed. However, placing *L. casei* *ccpA* under the control of vegetative gram-positive promoters and changing the RBS and start codon to adapt it to its new host (pGCCPA [see Materials and Methods]) allowed expression of the gene in *B. subtilis*. With this latter construct, the glucose-specific regulation of the *gntRK* operon was restored, although wild-type repression could not be reached (7-fold repression for the wild type and 3-fold repression for the complemented mutant, while in the control mutant it was 0.9-fold [Table 4]). The presence of the *L. casei* *ccpA* gene also restored the capacity of GM1225 to produce acetoin in the presence of excess glucose as determined qualitatively by the Voges-Proskauer method, indicating that CcpA from *L. casei* could also act as an activator of the *ackA* gene in *B. subtilis*, which indirectly leads to the production of acetoin (22).

## DISCUSSION

CcpA belongs to the LacI-GalR family of repressors, which includes several proteins that act as specific regulators of operons (49). This protein has been shown to be responsible for the main mechanism of CR in all of the low-GC-content gram-positive bacteria in which it has been described (8, 12, 18, 23). Other members of the LacI-GalR family with significant homology to CcpA have been described in these microorganisms, but their role in CR was not clearly established. For example, the *regA* gene from *Clostridium acetobutylicum* (6) was able to repress α-amylase synthesis in *B. subtilis*, but this effect was independent of the presence of glucose in the medium; the *pepR1* gene from *Lactobacillus delbrueckii* subsp. *lactis* could activate the expression in *E. coli* of a promoter of a prolidase gene which contained a putative *cre* site (45). In the present paper, we have tried to determine the mechanism underlying CR in *L. casei*. For this purpose, we have isolated a *ccpA* homolog from this organism and studied the pleiotropic effects of its mutation.

The genetic structure of the *ccpA* genes and surrounding region in *Bacillus* is quite conserved. In *B. subtilis* and *B. megaterium*, the *ccpA* gene is part of an operon with two other ORFs with a great homology to *motA* and *motB*, two genes encoding membrane integral proteins involved in flagellar motility (20, 27); however, disruption of these ORFs has no apparent phenotype in motility or CR. The *ccpA* gene from *S. xylosum* was found to be monocistronic, although the presence of *acuC* and

TABLE 4. Complementation of a *B. subtilis ccpA* mutant with the *ccpA* gene from *L. casei* ATCC 393

Plasmid <sup>a</sup>	Sugar <sup>b</sup>	β-Galactosidase activity (nmol/min/mg of protein)	
		GM1199	GM1225
pGAL9	Gluconate	31.9 ± 1.7	76.3 ± 3.0
	Gluconate + glucose	4.5 ± 0.3	83.1 ± 0.2
pGCCPA	Gluconate	ND <sup>c</sup>	51.4 ± 2.8
	Gluconate + glucose	ND	17.6 ± 2.1

<sup>a</sup> *B. subtilis* GM1199 [*amyE::(gntRK'-lacZ)*] and GM1225 [*amyE::(gntRK'-lacZ) ccpA::Tn917Δ(lacZ-erm)*] were transformed with pGAL9 (control) and pGCCPA expressing the *L. casei ccpA* gene.

<sup>b</sup> The strains were grown in LB medium with 0.5% of the indicated sugars to an OD<sub>550</sub> of 0.7 to 0.8. The values and standard deviations are from three independent experiments.

<sup>c</sup> ND, not determined.

*acuA* homologs downstream of *ccpA* suggests an operon structure similar to that in *Bacillus* (11). In the case of the *L. casei ccpA* gene, the organization was also monocistronic. Transcriptional analysis with the *L. casei ccpA* gene indicated that transcription takes place from a promoter located immediately upstream and terminates at the end of the *ccpA* reading frame, in which a putative IS element (IS30-like) is located.

Regulation of CcpA synthesis has been studied for *B. megaterium*, in which it is expressed constitutively (27). However, autoregulation of CcpA synthesis would not be a rare phenomenon, since this has been reported for other members of the LacI-GalR family (39, 50). In *S. xylosum*, the *ccpA* gene is transcribed from two promoters; one is weak and constitutive, and the other possesses a *cre* sequence and is repressed by CcpA itself. This leads to a reduction in CcpA levels in cells grown with glucose; possibly this could be an adaptation to regulate the levels of available CcpA depending on the growth conditions (11). In relation to the *L. casei ccpA* gene, no *cre* sequences with fewer than two mismatches to the consensus could be found in its promoter region and differences in the amounts of mRNA or reverse transcripts in cells grown with different sugars were not significant, indicating a constitutive transcription.

In this study, we have tried to determine the putative regulatory role of CcpA in *L. casei*. We could show that CR of the *N*-acetylglucosaminidase activity found in the wild type was completely released in the *ccpA* mutant. Phospho-β-galactosidase was also partially released from CR in the *ccpA* mutant. The residual CR effect in phospho-β-galactosidase activity could be explained by other control mechanisms involved in the regulation of the *lac* operon, such as inducer exclusion or expulsion (4), and an additional, CcpA-independent CR mechanism. Such a mechanism has been proposed for the *bglPH* operon in *B. subtilis*, a system with homologies to the *lac* operon of *L. casei*, and involves the RAT terminator region, the antitermination protein LicT, and PTS components (28). This tight regulation would explain the need for using lactose pregrown cells (fully induced for the *lac* operon) to detect the effect of the *ccpA* mutation (see results).

At present, molecular genetic data are available only for phospho-β-galactosidase, which is encoded within the *lac* operon. In experiments involving *lacp::gusA* fusions, we were able to show that both a *cre* sequence in the *lac* promoter and the *ccpA* gene were necessary for glucose control. The *L. casei ccpA* gene could also restore glucose regulation of a *gntRK'-lacZ* fusion and production of acetoin in a *B. subtilis ccpA*

mutant, suggesting that the sequence conservation of *L. casei* CcpA was paralleled by a similar function of CcpA in this organism. These data clearly demonstrate that the cloned *ccpA* gene is actually involved in CR in *L. casei*. Although previously reported data suggested the presence of CcpA-like elements in other lactic acid bacteria (29, 30, 45) and their likely regulatory role, this is the first report in which a *ccpA* gene from a lactic acid bacterium has been conclusively shown to be involved in CR.

As in the *B. megaterium ccpA* mutants (27), *t*<sub>2</sub> values were increased on all carbon sources in the *L. casei ccpA* mutant. This relates CcpA to other metabolic pathways and suggests that, in addition to its role in CR, it could also be involved in other regulatory processes. Indeed, in *B. subtilis* CcpA has been proved to mediate glucose activation of carbohydrate metabolism genes such as the acetate kinase gene (*ackA*) (19).

Two different DNA-binding regulatory proteins responsible for cyclic AMP-independent CR have been detected in bacteria thus far: one mediated by Cra in enteric bacteria and the CcpA system in low-GC-content gram-positive bacteria (40). Although Cra and CcpA belong to the same family of bacterial regulators and share a similar core sequence for DNA binding, they are clearly different in their mechanisms of action. Fructose phosphates are the effectors of Cra, and they displace it from DNA (38), while P-Ser HPr and glucose-6-P are the effectors of CcpA and promote its DNA binding (8, 16, 32). It is noteworthy that Cra has the ability not only to repress genes involved in catabolism but also to activate anabolic genes, thus controlling the carbon flow of the cell (37). The fact that at least one gene (*ackA*) has been shown to be positively regulated by CcpA raises the possibility that similar carbon flow-controlling mechanisms are present in both gram-negative and gram-positive bacteria. Supporting this idea, *cre* sites have been found in genes encoding central glycolytic enzymes in several gram-positive bacteria (26). Future research will be needed to answer this question and establish a more-accurate picture of the carbon regulatory pathways in gram-positive organisms, especially lactic acid bacteria.

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