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Carbon Catabolite Repression in *Lactobacillus pentosus*: Analysis of the *ccpA* Region

KERSTIN MAHR, WOLFGANG HILLEN, AND FRITZ TITGEMEYER*

Lehrstuhl für Mikrobiologie, Friedrich-Alexander-Universität Erlangen-Nürnberg, D-91058 Erlangen, Germany

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The catabolite control protein CcpA is a central regulator in low-G+C-content gram-positive bacteria. It confers carbon catabolite repression to numerous genes required for carbon utilization. It also operates as a transcriptional activator of genes involved in diverse phenomena, such as glycolysis and ammonium fixation. We have cloned the *ccpA* region of *Lactobacillus pentosus*. *ccpA* encodes a protein of 336 amino acids exhibiting similarity to CcpA proteins of other bacteria and to proteins of the LacI/GalR family of transcriptional regulators. Upstream of *ccpA* was found an open reading frame with similarity to the *pepQ* gene, encoding a prolidase. Primer extension experiments revealed two start sites of transcription for *ccpA*. In wild-type cells grown on glucose, mRNA synthesis occurred only from the promoter proximal to *ccpA*. In a *ccpA* mutant strain, both promoters were used, with increased transcription from the distant promoter, which overlaps a presumptive CcpA binding site called *cre* (for catabolite responsive element). This suggests that expression of *ccpA* is autoregulated. Determination of the expression levels of CcpA in cells grown on repressing and nonrepressing carbon sources revealed that the amounts of CcpA produced did not change significantly, leading to the conclusion that the arrangement of two promoters may ensure constant expression of *ccpA* under various environmental conditions. A comparison of the genetic structures of *ccpA* regions revealed that lactic acid bacteria possess the gene order *pepQ-ccpA*-variable while the genetic structure in bacilli and *Staphylococcus xylosus* is *aroA-ccpA*-variable-*acuC*.

The catabolite control protein CcpA is a global regulator controlling carbon catabolite repression (CCR), glycolysis, fermentative metabolism, and fixation of ammonium in low-G+C-content gram-positive bacteria (42). Thus, bacteria carrying a defect in *ccpA* exhibit deregulated CCR and reduced growth rates (20). The molecular mechanism of CcpA function in bacilli is well understood (for a review, see reference 14). When *Bacillus subtilis* is grown on a preferred carbon source, such as glucose or fructose, the metabolite-activated HPr kinase/phosphatase PtsK phosphorylates the proteins HPr and Crh at a seryl residue via ATP (9, 19, 32). Both seryl-phosphorylated proteins activate CcpA by forming a complex with it, thereby enabling CcpA to bind to catabolite responsive elements (*cre*) found within promoter or coding regions of catabolite-controlled genes (5). This results in a repression of gene expression at the level of mRNA synthesis, as has been demonstrated, for example, for the *gnt* operon of *B. subtilis* and the *xyl* operon of *Bacillus megaterium* (8, 10). Besides its repressor function, CcpA operates as a pleiotropic activator, as has been reported for the *B. subtilis* genes encoding acetate kinase, α-acetolactate synthase, phosphotransacetylase, and glutamate synthase and for the *Lactococcus lactis las* operon, encoding phosphofructokinase, pyruvate kinase, and L-lactate dehydrogenase (7, 11, 26, 33, 36).

Lactic acid bacteria, which are of central importance for the food industry, apparently control utilization of carbon sources via CCR. An internal fragment of the *ccpA* gene of *Lactobacillus pentosus*, an organism involved in fermentation of cucumbers, olives, and cabbage (the latter for sauerkraut production), has been cloned and used to construct a *ccpA* mutant (22). Glucose repression of the *xylAB* operon, encoding D-xylose isomerase and D-xylulose kinase, which are required for xylose fermentation, was relieved in the *ccpA* mutant strain (22). This led to the conclusion that the mechanism of CCR in *L. pentosus* is similar to that found in bacilli. Data supporting this conclusion have been reported for the CcpA proteins of *Lactobacillus casei* and *Lactococcus lactis* and for the CcpA homologue PepR1 of *Lactobacillus delbrueckii* subsp. *lactis* (25, 26, 30, 35). Analysis of the CcpA homologue RegM of *Streptococcus mutans* revealed an opposite effect on CCR (37). When *regM* was inactivated, the mutant strain showed an increase in glucose repression. As in the case of *pepR1* of *L. delbrueckii* subsp. *lactis* and the *ccpA* genes of *L. casei* and *Lactococcus lactis*, *regM* is linked to the gene *pepQ*, encoding a prolidase, suggesting that these genes have similar functions (3, 37, 41; C. Esteban and G. Pérez-Martínez, unpublished data, 1999). Information on the genetic context of *L. pentosus ccpA* is lacking.

In this communication, we report the completion of the determination of the *L. pentosus ccpA* sequence and that of the surrounding genes. We use this information to assess the regulation of *ccpA* itself, showing that it has features distinctly different from those of *L. casei* and *Staphylococcus xylosus*. We inspect CcpA-specific residues among CcpAs and the closely related proteins RegM and PepR1, and we compare the *ccpA* region of *L. pentosus* with *ccpA* regions of other bacteria, providing new insights into the genetic organization of *ccpA* among lactic acid bacteria and other low-G+C-content gram-positive bacteria.

MATERIALS AND METHODS

Bacterial strains. *L. pentosus* MD363 (wild type) was used for isolation of the *ccpA* genomic region (22). *L. pentosus* MD363 and the isogenic *ccpA* mutant *L. pentosus* LPE4 (*ccpA*::*erm*) were used for primer extension and Western blot analyses (22). *Escherichia coli* DH5α was used for standard cloning procedures (34).

Southern blot analysis. Chromosomal DNA of *L. pentosus* MD363 was isolated as described previously (24). Ten-microgram quantities of chromosomal

* Corresponding author. Mailing address: Lehrstuhl für Mikrobiologie, Friedrich-Alexander-Universität Erlangen-Nürnberg, Staudtstrasse 5, D-91058 Erlangen, Germany. Phone: 49-(0)9131-8528095. Fax: 49-(0)9131-8528082. E-mail: ftitgem@biologie.uni-erlangen.de.

DNA were digested with *Sal*I, *Pst*I, *Spe*I, *Dra*I, or *Eco*RV, and restriction fragments were separated on a 1% agarose gel. Plasmid pEI2 (1 μg) was labelled by nick translation, using a biotin-7-dATP labelling kit (BioNick labelling system; Gibco BRL) according to the recommendations of the manufacturer. DNA fragments from agarose gels were transferred to a nylon membrane (Gibco BRL) and fixed by UV irradiation (UV Stratalinker; Stratagene). Standard aqueous conditions were employed for hybridization and washing of membranes (34). Hybridized DNA fragments on the membrane were visualized by using the PhotoGENE nucleic acid detection system (Gibco BRL). Single *ccpA*-hybridizing DNA fragments of 6.4, 14.0, 10.0, 8.0, and 4.3 kb were obtained after restriction with *Sal*I, *Pst*I, *Spe*I, *Dra*I, and *Eco*RV, respectively.

Inverse PCR. *L. pentosus* MD363 chromosomal DNA (200 ng) was digested with *Sal*I and religated overnight at 14°C in a 200-μl volume of T4 DNA ligase buffer containing 10 U of T4 DNA ligase (Boehringer). The religated DNA was precipitated by addition of 700 μl of ice-cold ethanol (96%), 20 μl of 3 M sodium acetate (pH 4.8), and glycogen (1 μg ml^{-1}) and incubation for 2 h at −70°C. The precipitated DNA was resuspended in 20 μl of Tris-EDTA buffer, pH 8.0. For inverse PCR, the *Tth-Taq* enzyme mixture included in the Sawady-Long PCR Kit from Peqlab was used. The reaction mixture consisted of 3 μl of religated DNA (about 30 ng), 1.75 mM $MgCl_2$, 350 μM each deoxyribonucleoside triphosphate, 2.5 U of the *Tth-Taq* enzyme mixture, and 15 pmol of each primer (LPE1 [5′-CCATTAACCACCCGTGAAACCGTTGCC-3′] and LPE2 [5′-TCCAGCC ATCACCTCAATCACGCAACC-3′]) in a volume of 50 μl. PCR was conducted as follows: 2 min at 93°C; 10 cycles of 10 s at 93°C, 30 s at 55°C, and 5 min at 68°C; 20 cycles of 10 s at 93°C, 30 s at 55°C, and 5 min plus 20 s at 68°C; and a final incubation for 18 min at 68°C. DNA sequencing of inverse-PCR products was performed on an ABI PRISM 310 Genetic Analyzer (Perkin-Elmer) with fluorescence-labelled dideoxyribonucleoside triphosphates provided in the BigDye Terminator Mix (Perkin-Elmer).

Plasmid construction. The *ccpA* gene was cloned in two steps. First, a DNA fragment of 1,260 bp was amplified by PCR from *L. pentosus* MD363 chromosomal DNA, using oligonucleotides LPE9 (5′-AAAAATACAATCTCCGTGT GG-3′) and LPE10 (5′-GATTCCAAACCTAGTATACCGC-3′). This fragment was used as a template for a second PCR with oligonucleotides LPE11 (5′-GG GCTATTTTCATATGGAAAAGC-3′) and LPE12 (5′-ACTTCCCGGATCCG GCGTCTCATTAG-3′) to create an *Nde*I and a *Bam*HI restriction site, respectively (underlined). The *Nde*I-*Bam*HI fragment was cloned into plasmid pET15b (Novagen) which had been cut with the same restriction endonucleases, giving plasmid pWH154 (*ccpA*). The *pepQ-ccpA* intergenic region was amplified by PCR with oligonucleotides LPE17 (5′-CACCGAGGTCGAACAAGACC-3′) and LPE26 (5′-GCCACATCATAAATTGTTACTG-3′). The PCR amplification product of 978 bp, containing 689 bp of *pepQ*, 254 bp of the *pepQ-ccpA* intergenic region, and 35 bp of *ccpA*, was cloned into the *Sma*I restriction site of plasmid pSU2718, giving plasmid pWH156 (27). Nucleotide sequences of inserts were determined by DNA sequencing as described above.

Isolation of RNA. Cells of *L. pentosus* MD363 and LPE4 (*ccpA*::*erm*) were grown overnight at 37°C under static conditions on minimal (M) medium supplemented with 50 mM glucose (23). Cells were harvested by centrifugation and washed twice with 10 ml of M medium. A 50-ml volume of M medium supplemented with 50 mM glucose was inoculated with cells to an optical density at 600 nm (OD_{600}) of 0.1. Cells were grown at 37°C to an OD_{600} of 0.8. A 10-ml volume of the culture was harvested, and the resulting cell pellet was resuspended in 100 μl of Tris-EDTA buffer, pH 8.0. Lysozyme was added to a final concentration of 10 mg ml^{-1}, and the suspension was incubated at 37°C for 1 h. Lysis of the cells and preparation of total RNA were performed with an RNeasy Minikit from Qiagen according to the recommendations of the manufacturer.

Primer extension analysis. Total RNA of *L. pentosus* MD363 and LPE4 was isolated as described above. Primer extension experiments were performed with avian myeloblastosis virus reverse transcriptase (Stratagene) and oligonucleotide LPE27 (5′-AATTGTTACTGTTTGCTTTTCC-3′), which is complementary to positions 24 to 3 of the *ccpA* coding sequence (see Fig. 3A). Oligonucleotides were 5′ labelled by the use of T4 polynucleotide kinase (New England Biolabs). In primer extension reactions, 500 fmol of labelled primer was used with 20 μg of cellular RNA. Reverse transcripts were resolved on 6% urea–polyacrylamide gels. Standard DNA sequencing reactions with Sequenase (U.S. Biochemical Corp.), using the same oligonucleotides, were performed for sizing of the primer extension products. The positions of transcriptional start sites were confirmed by using a second oligonucleotide, LPE26 (5′-GCCACATCATAAATTG-TTACT G-3′), hybridizing to positions 35 to 14 of the *ccpA* coding sequence.

Western blot analysis. *L. pentosus* MD363 and LPE4 (*ccpA*::*erm*) were grown individually overnight at 37°C under static conditions in 10 ml of M medium supplemented with 50 mM glucose (23). Cells were harvested by centrifugation and washed twice with 10 ml of M medium. A 50-ml volume of M medium supplemented with 50 mM glucose or xylose was inoculated with culture to an OD_{600} of 0.1. Cells were grown at 37°C to an OD_{600} of 0.8. After the cells were harvested, the cell pellet was resuspended in 1 ml of starting buffer (20 mM Tris-HCl [pH 7.5], 3 mM dithiothreitol). Crude extracts were prepared by sonification at 45 W (Labsonic U [Braun]; twice, for 20 s each time) and subsequent removal of cell debris by centrifugation. Proteins of cell extracts were separated by sodium dodecyl sulfate-polyacrylamide gel electrophoresis on a 7.5% polyacrylamide gel and transferred to a polyvinylidene difluoride membrane (Fluorotrans) by electroblotting. CcpA was detected with a rabbit polyclonal antiserum raised against CcpA of *B. megaterium* (21). CcpA antibodies on the polyvinylidene difluoride membrane were visualized by using the ECL Western blot analysis system (Amersham).

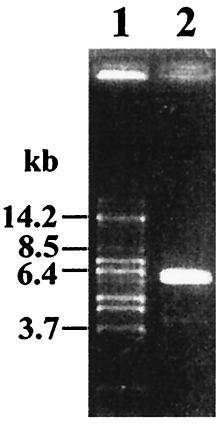

FIG. 1. Amplification product of 5.6 kb of the *ccpA* region of *L. pentosus* on a 1% agarose gel. Sizes of DNA fragments are indicated in kilobase pairs. Lane 1, DNA marker fragments; lane 2, 3 μl of a 50-μl-volume inverse PCR using oligonucleotides LPE1 and LPE2 and religated *L. pentosus* MD363 chromosomal DNA.

Computer analyses. DNA and protein data bank searches were performed with the BLAST server of the National Center for Biotechnology Information at the National Institutes of Health, Bethesda, Md. (URL http://www.ncbi.nlm.nih.gov). The LaserGene workstation software (DNASTAR, Inc.) was used to process DNA and protein sequence data.

Nucleotide sequence accession number. The *pepQ-ccpA* DNA sequence has been submitted to GenBank under accession no. AF176799.

RESULTS AND DISCUSSION

Isolation of the *ccpA* region of *L. pentosus*. The isolation of an internal *ccpA* gene fragment from *L. pentosus* MD353 has been described in a previous publication (22). This fragment was used to construct a *ccpA* mutant in the more highly transformable strain MD363. Thus, we chose MD363 to determine the sequences of the complete *ccpA* gene and flanking genes.

A Southern blot analysis of chromosomal DNA of MD363 was performed with plasmid pEI2, which contains the internal *ccpA* fragment *′ccpA′*, as a probe. Chromosomal DNA restricted with *Sal*I gave a signal of 6.4 kb, indicating the presence of the *ccpA* gene on the chromosomal *Sal*I DNA fragment (data not shown). The 6.4-kb fragment was amplified by inverse PCR with oligonucleotides LPE1 and LPE2, which hybridized to the 5′ end and to the 3′ end of the known *′ccpA′* fragment, respectively. As a result, an amplification product of 5.6 kb was generated (Fig. 1). The PCR fragment was directly subjected to sequencing with the same oligonucleotides. The determined nucleotide sequence comprised the missing ends of *ccpA* and adjacent intergenic sequences, as revealed by analysis of similarity to the *ccpA* gene of *L. casei*. Further sequencing of the PCR product yielded 1,379 bp upstream and 453 bp downstream of *ccpA* (see the section on genetic organization below).

Analysis of CcpA. The *L. pentosus ccpA* gene was amplified by PCR, using chromosomal DNA as a template, as described in Materials and Methods. For further analysis, the fragment was cloned in plasmid pET15b by insertion into *Nde*I and *Bam*HI sites, giving plasmid pWH154. Sequencing of *L. pentosus* MD363 *ccpA* revealed an open reading frame (ORF) of 1,011 bp encoding 336 amino acids with a calculated molecular mass of 36,316 Da. A potential ribosome binding site (RBS; AGAAAGG) is located at positions −12 to −18. At the DNA

TABLE 1. Comparison of contents of CcpA- and LacI/GalR-specific residues

Organism or sequence	Protein	No. of:		% Identity[a]	Accession no.
		CcpA-specific residues	LacI/GalR-specific residues		
Consensus		67	94		
L. pentosus	CcpA	61	79	100	AF176799
L. casei	CcpA	64	79	66	U28137
E. faecalis	CcpA	63	75	62	AJ011113
Listeria monocytogenes	CcpA	62	80	60	AF076520
Streptococcus mutans	RegM	60	78	59	O07329
B. subtilis	CcpA	63	80	56	P25144
B. megaterium	CcpA	61	83	55	P46828
Lactococcus lactis	CcpA	51	75	53	AF106673
L. delbrueckii subsp. *lactis*	PepR1	47	71	50	Q48544
Staphylococcus xylosus	CcpA	55	82	48	Q56194
C. acetobutylicum	RegA	24	71	39	Q45831
E. coli	LacI	0	63	30	P03023
	GalR	0	69	29	P03024

[a] Percent amino acid sequence identity with respect to CcpA of *L. pentosus*.

level, *ccpA* of *L. pentosus* MD363 showed 97% identity to the *'ccpA'* fragment of *L. pentosus* MD353 and 76% identity to the next related *ccpA* gene of *L. casei*. The 20 differences in nucleotides found in the *ccpA* genes of the two *L. pentosus* strains do not affect the amino acid sequence; thus, the gene products are identical.

At the protein level, CcpA of *L. pentosus* shows 66 to 39% identity to CcpA and CcpA-like proteins of *L. casei* (66%), *Enterococcus faecalis* (62%; accession no. AJ011113), *Listeria monocytogenes* (60%), *Streptococcus mutans* (RegM; 59%), *B. subtilis* (56%), *B. megaterium* (55%), *Lactococcus lactis* (53%), *L. delbrueckii* (PepR1; 50%), *Thermoactinomyces* sp. strain E79 (50%; accession no. AF055979), *Staphylococcus xylosus* (48%), and *Clostridium acetobutylicum* (39%) (1, 13, 16, 26, 37, 41).

The CcpA proteins comprise a subgroup of the LacI/GalR family of bacterial transcriptional regulators, to which they exhibit about 30% protein sequence identity (43). The CcpA subgroup has been defined by physiological functionality (18). On this basis, 67 CcpA-specific residues have been proposed to be present in all CcpA proteins and predominantly absent from the other members of the family (18). The CcpA-specific residues are clustered in three blocks throughout the amino acid sequence. One block covers the N-terminal helix-turn-helix motif, while most other conserved residues form a continuous patch on the protein surface (18). The contents of LacI/GalR-specific residues among all proteins of the family are similar, ranging between 63 and 83 LacI/GalR-specific positions (Table 1). CcpA of *L. pentosus* possesses 61 of the 67 CcpA-specific amino acid positions (Table 1 and Fig. 2). Thus, CcpA of *L. pentosus* contains the same number of CcpA-specific residues as the well-characterized CcpA of *B. megaterium* (Table 1). An increased number of CcpA-specific residues compared to that of non-CcpA proteins of the LacI/GalR family can be found in the regulator protein RegA of *C. acetobutylicum* (24 of 67). This protein was shown to complement a *B. subtilis ccpA* mutant and must therefore have features necessary for CcpA function (4). The CcpA homologues RegM of *Streptococcus mutans* and PepR1 of *L. delbrueckii* comprise 60 and 47 CcpA-specific residues, respectively, a good indication of a CcpA-like function in these organisms.

Identification of the genes flanking *ccpA*. To determine the genes flanking *ccpA*, parts of the 5.6-kb amplification product were sequenced by primer walking. Upstream of *ccpA*, an ORF of 1,107 bp whose orientation was opposite that of *ccpA* and which exhibited a high degree of similarity to *pepQ* genes of other lactic acid bacteria was identified and found to encode a dipeptidase which specifically cleaves X-prolyl peptide bonds (40). The gene was therefore designated *pepQ*. Multiple-alignment analysis revealed a possible start codon, TTG, which is preceded by a potential RBS (AGGAGTG) located at positions −8 to −14. A potential transcriptional termination site (AATCCGCCCCATCCTGATAAGCACGATGGGGCGGA

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                .         .         .         .         .
CcpA_Lpe    MEKQTVTIYDVAREAAVSMATVSRVVNGNPNVKPATRKKVLAVIERLDYRPNAVARGLAS    60
consensus         tiYdva Ea vsM tvsrv ngN NvK  trK vL     l yRpnAVarGlAS
                .         .         .         .         .
CcpA_Lpe    KRSTTVGVIIPDVTNIYFASLARGIDDIAMMYKYNIILTNSDDAGEQEVNVLNTLMAKQV   120
consensus   K  TtVgVIIp   n  f   ARgi DIa MYKyN     SD     e    NTl  kQv
                .         .         .         .         .
CcpA_Lpe    DGVIFMGNHIDDKLRAEFKRAKAPVVLAGTVDPNNETPSVNIDYAAAVEEAVTNLIGRGH   180
consensus   dg iFMG          F     pvvlA T         vNIdY  a   a   l   gh
                .         .         .         .         .
CcpA_Lpe    KKIALALGSLSQSINAEYRLTGYKRALTKAKIPFDDALVYEAGYSYDAGRKLQPVIADSG   240
consensus   K i    g     IN   r  gyK al             E    Y  g
                .         .         .         .         .
CcpA_Lpe    ATAVFVGDDEMAAGIINASMETGINVPDDLEVVTSNDTIITQITRPAITSITQPLYDIGA   300
consensus    ta     dE a g        g  vp d E     d       Rp  t   qp YD gA
                .         .         .
CcpA_Lpe    VAMRMLTKLMNDKELDEKTVTLPYGIVRRGSTKSAD  332
consensus   VaMR lTK MN                  r
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FIG. 2. Protein sequence of *L. pentosus* CcpA and comparison to a consensus sequence. Lowercase letters of the consensus sequence designate conserved amino acid residues specific for proteins of the LacI/GalR family of regulators, whereas uppercase letters designate amino acid residues specific for CcpA proteins (18).

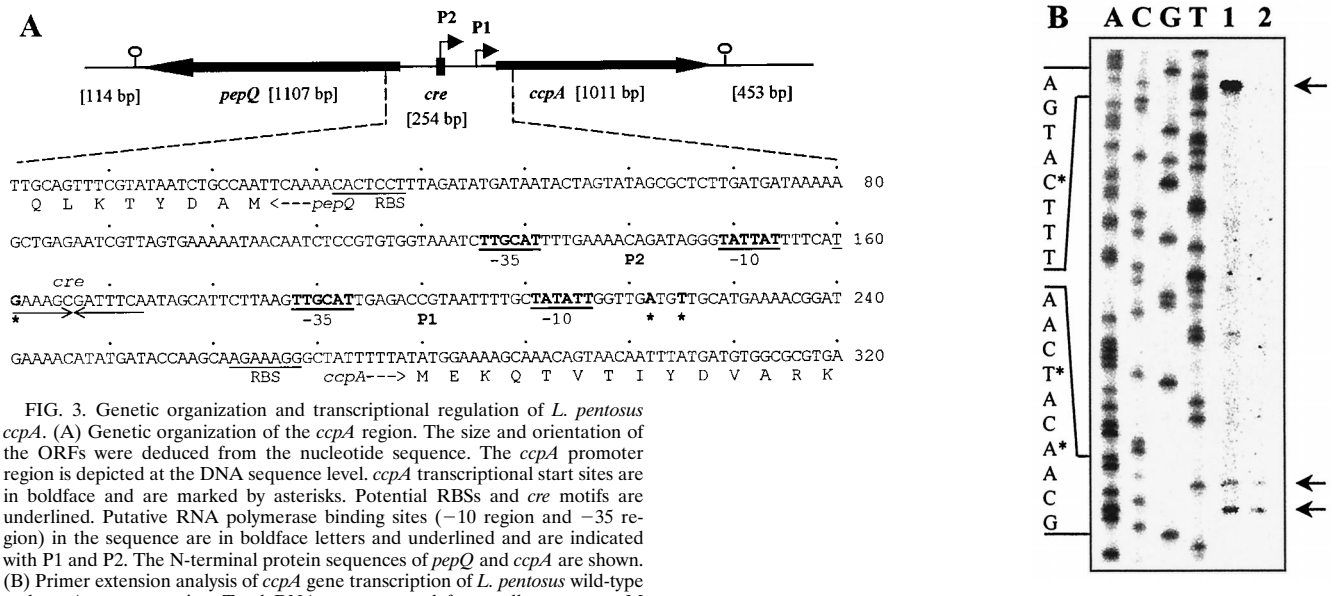

FIG. 3. Genetic organization and transcriptional regulation of *L. pentosus ccpA*. (A) Genetic organization of the *ccpA* region. The size and orientation of the ORFs were deduced from the nucleotide sequence. The *ccpA* promoter region is depicted at the DNA sequence level. *ccpA* transcriptional start sites are in boldface and are marked by asterisks. Potential RBSs and *cre* motifs are underlined. Putative RNA polymerase binding sites (−10 region and −35 region) in the sequence are in boldface letters and underlined and are indicated with P1 and P2. The N-terminal protein sequences of *pepQ* and *ccpA* are shown. (B) Primer extension analysis of *ccpA* gene transcription of *L. pentosus* wild-type and *ccpA* mutant strains. Total RNA was prepared from cells grown on M medium supplemented with 50 mM glucose. Reverse transcription was carried out with end-labelled oligonucleotide LPE27. DNA sequencing reactions were performed with the same oligonucleotide and with pWH156 as template DNA. Primer extension products were analyzed on 6% polyacrylamide–urea gels. Lane 1, RNA (20 μg) from *L. pentosus* LPE4 (*ccpA* mutant); lane 2, RNA (20 μg) from *L. pentosus* MD363 (wild type). The sequence interpretations around the +1 sites (asterisks and arrows) of the two *ccpA* promoters are shown.

TT; the inverted repeat is underlined) was found 33 bp downstream of *pepQ*. PepQ of *L. pentosus* showed 57% identity to PepQ of *Lactobacillus helveticus* (accession no. AK012084) and *L. delbrueckii* subsp. *lactis*, 50% identity to PepQ of *Streptococcus mutans*, and 42% identity to *L. delbrueckii* subsp. *bulgaricus* (31, 37, 40). A catalytical center comprising a zinc-binding motif which is typical of zinc-dependent metallopeptidases has been described (17). PepQ of *L. pentosus* contains this unique signature at amino acid positions 294 to 304.

ccpA and *pepQ* have similar G+C contents (49 and 50%, respectively). The 453-bp sequence downstream of *ccpA* (48% G+C) exhibited no similarity to any known proteins of the data bank. A potential stem-loop structure (underlined), AGG TTTGGAATCTGATTCCAAACCT, could be identified 58 bp downstream of *ccpA*; this might serve as a transcriptional termination site. The *pepQ-ccpA* intergenic region (254 bp) exhibits a decreased G+C content of 32%, typical of promoter-containing regions (39). A fully conserved *cre* site (TGAA AGCGATTTCA) was found to be located at positions −107 to −120 with respect to the *ccpA* gene, suggesting autoregulation of *ccpA*.

Transcriptional regulation of *ccpA*. To determine the transcriptional start point and to examine the potential of *ccpA* for autoregulation, primer extension analyses were performed on RNA extracted from cells of wild-type strain *L. pentosus* MD363 and from cells of *ccpA* mutant strain *L. pentosus* LPE4, respectively, grown on M medium supplemented with 50 mM glucose. In both strains, a transcription start site was found (at positions −55 and −58, respectively), as indicated by a double band in Fig. 3B. Such a double signal can be explained by an alternative use of start sites through the same promoter (P1). Interestingly, the primer extension analysis of mRNA of the *ccpA* mutant strain revealed a second transcription initiation site, indicated by a stronger primer extension signal. It could be assigned to a G at position −119, which represents the second base of the potential *cre* site (P2) (Fig. 3A). Both promoters P1 (TTGCAT-17 bp-TATATT) and P2 (TTGCAT-17 bp-TATT AT) are well conserved compared to the σ^A-dependent promoters of *B. subtilis* (12). The finding that the P2 transcript could be formed only when functional CcpA protein was missing suggests autoregulation of *ccpA*.

CcpA expression levels on various carbon sources. To further determine the effect of transcriptional regulation on the amount of CcpA protein synthesized under different growth conditions, Western blotting experiments were performed (Fig. 4). *L. pentosus* MD363 wild-type cells were grown on M medium under repressing and nonrepressing conditions, using 50 mM glucose and 50 mM xylose, respectively. As can be seen in Fig. 4, CcpA signals of equal intensities were found in cell extracts grown under repressing and nonrepressing conditions (lanes 3 and 4). This finding suggests that nearly constant amounts of cellular CcpA are present independently of the presence or absence of glucose.

Regulation of *ccpA* has been studied in only a few organisms, namely, *B. subtilis*, *B. megaterium*, *Staphylococcus xylosus*, and *L. casei* (6, 16, 29, 30). Western blot analysis and *ccpA*::*lacZ* fusion measurements led to the conclusion that CcpA is constitutively expressed in *B. subtilis* and *B. megate-*

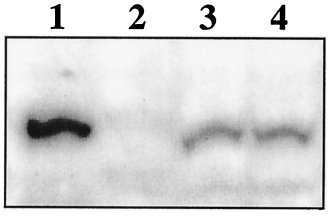

FIG. 4. Western blot analysis of *L. pentosus* grown on various carbon sources. A Western blot of a sodium dodecyl sulfate–7.5% polyacrylamide gel is shown after incubation with polyclonal antibodies derived against CcpA of *B. megaterium*. Lane 1, 50 ng of purified CcpA of *B. megaterium*; lane 2, 0.2 OD_{600} equivalents of protein extract of *L. pentosus* LPE4 (*ccpA* mutant); lanes 3 and 4, 0.2 OD_{600} equivalents of protein extract of *L. pentosus* MD363 grown on M medium with 50 mM glucose and 50 mM xylose, respectively.

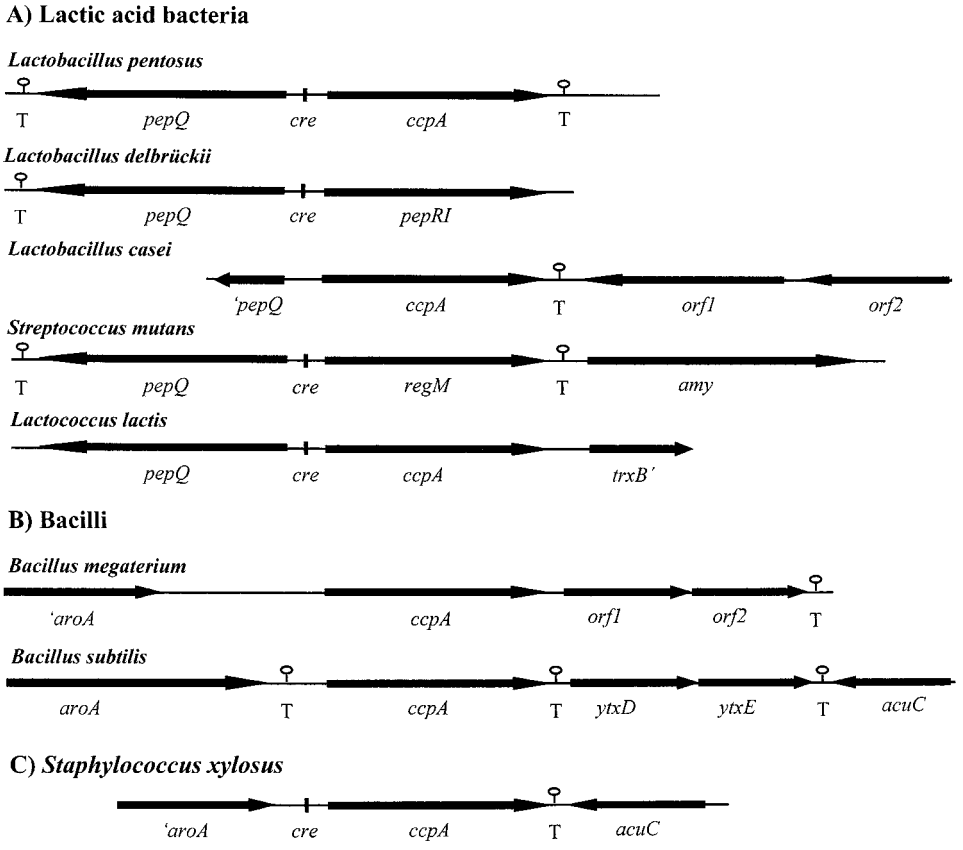

FIG. 5. Comparison of *ccpA* regions of *L. pentosus*, *L. delbrueckii* subsp. *lactis* (40, 41), *L. casei* (30; C. Esteban and G. Pérez-Martínez, unpublished data), *Streptococcus mutans* (37), *Lactococcus lactis* (accession no. AF106673) (3) (A); *B. megaterium* (15), *B. subtilis* (2, 11, 13) (B); and *Staphylococcus xylosus* (6) (C). Orientations of genes are indicated by arrows. Potential transcriptional termination structures (T) and *cre* motifs are indicated.

rium (16, 29). However, in neither case has the promoter been mapped. Constitutive expression of *ccpA* of *L. casei* was also revealed by primer extension and Northern blot analyses (30). The *ccpA* gene of *L. casei* is expressed via only one promoter, which apparently is lacking a *cre* site and is therefore not subject to autoregulation. As in *L. pentosus*, transcription of *ccpA* of *Staphylococcus xylosus* is driven by a tandem promoter and triggered through autoregulation via a *cre* site. However, here the *cre* site overlaps the transcription start site of the promoter proximal to *ccpA* (6). The authors (6) reported that in *Staphylococcus xylosus* there exists a carbon source-dependent autoregulation of *ccpA* mediated by *cre* and resulting in a slight decrease of CcpA expression when cells are grown in the presence of glucose.

In conclusion, we suggest that in all organisms investigated to date, CcpA is produced in more or less constant amounts regardless of growth conditions. Transcriptional regulation of *ccpA* expression may be a means of reaching this goal in *Staphylococcus xylosus* and *L. pentosus*.

Genetic organization. The finding that *pepQ* genes are also located divergently from *ccpA* genes in other lactic acid bacteria led us to compare the genetic organizations of all published *ccpA* regions. As depicted in Fig. 5, the gene order *pepQ-ccpA* (or *pepQ-ccpA* homologue) is found in all lactic acid bacteria, namely *L. pentosus*, *L. delbrueckii*, *L. casei*, *Streptococcus mutans*, and *Lactococcus lactis* (3, 37, 41; C. Esteban and G. Pérez-Martínez, unpublished data, 1999). In contrast, the sequences downstream of *ccpA* differ throughout the lactic acid bacteria. While there are no sequence similarities to other known genes in *L. pentosus*, *orf1* of *L. casei* is homologous to genes encoding transposases of IS*30* family insertion elements (30). The genes downstream of *ccpA* in *Lactococcus lactis* and *Streptococcus mutans* encode homologues of thioredoxin reductase (accession no. AF106673) and α-amylase, respectively (38). Taken together, these findings indicate that *pepQ* is always associated with *ccpA* or a *ccpA* homologue in lactic acid bacteria.

Here, the questions of whether *pepQ* is regulated by CcpA and whether PepR1 and RegM are truly functional equivalents of CcpA of *L. pentosus*, *L. casei*, and *Lactococcus lactis* arise. The contents of CcpA-specific residues support this assumption (Table 1). While the involvement of CcpA in CCR is apparently established for *L. pentosus*, *L. casei*, and *Lactococcus lactis*, recent data suggest that this is also the case for PepR1 of *L. delbrueckii* (35). In the case of *Streptococcus mutans*, inactivation of *regM* resulted in an opposite CCR phenotype; i.e., CCR was enhanced (37). The picture concerning regulation of *pepQ* by CcpA is not clear. While a regulatory effect has been described for *L. delbrueckii*, this was not the case in *Streptococcus mutans* (35, 37).

A very different gene arrangement is found in non-lactic acid gram-positive bacteria. Upstream regions of *ccpA* in *B. megaterium*, *B. subtilis*, and *Staphylococcus xylosus* comprising the *aroA* gene, whose product in *B. subtilis* has been shown to possess chorismate mutase activity, are conserved (2). In *B. megaterium* and *B. subtilis*, two ORFs are found downstream of *ccpA* (*orf1* and *orf2* in *B. megaterium* and *ytxD* and *ytxE* in *B. subtilis*) that are homologous to the *motA* and *motB* genes of *B.*

subtilis (15). MotA and MotB are integral membrane proteins involved in flagellar movement (28). In *Staphylococcus xylosus*, no *motA* or *motB* homologues are linked to *ccpA*; here *acuC* is the gene downstream of *ccpA* encoding a protein involved in acetoin and butanediol metabolism (11). The *acuC* gene of *B. subtilis* is located downstream of *ytxE*, showing that the order of the genes in the *ccpA* region of *B. subtilis* is similar to that of *Staphylococcus xylosus*.

The overall genetic context of *ccpA* and the fact that in most cases a terminator-like structure downstream of *ccpA* is predicted suggest a monocistronic operon organization. This is supported by transcript size mapping of the *ccpA* mRNAs of *L. casei* and *L. pentosus* (22, 30). However, in *L. pentosus*, a second transcript, of 10 kb, has been detected (22). While in RNA of glucose-grown cells the short transcript was the primary product, the 10-kb transcript was predominantly present in RNA of xylose-grown cells. This suggests that transcription of *ccpA* and the genes downstream might be coordinately regulated.

Conclusions. Analysis of the *ccpA* region of *L. pentosus* revealed the gene order *pepQ-ccpA*-variable. This genetic organization is found in all lactic acid bacteria described to date, which may indicate that the *ccpA* genes of *L. pentosus*, *L. casei*, and *Lactococcus lactis*, the *pepR1* gene of *L. delbrueckii*, and the *regM* gene of *Streptococcus mutans* are functional equivalents. The fact that no common gene is linked to *ccpA* throughout the low-G+C-content gram-positive bacteria indicates that none of the flanking genes play a role in CCR. The reported data on the regulation of *ccpA* in *L. pentosus* show that CcpA levels are constant under different environmental conditions. This is a common feature also found in *L. casei*, bacilli, and *Staphylococcus xylosus*. Yet, the mechanisms of *ccpA* transcription differ among these organisms. In *L. pentosus*, *ccpA* transcription is realized by a tandem promoter, the more distant component of which is apparently subject to autoregulation. Further studies are required to elucidate whether CcpA of *L. pentosus* is involved in the regulation of other catabolite-controlled genes; whether CcpA regulates *pepQ*, thereby linking carbon utilization to proteolysis; and whether CcpA triggers gene activation as well.

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REFERENCES

1. **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.
2. **Bolotin, A., V. Khazak, N. Stoynova, K. Ratmanova, Y. Yomantas, and Y. Kozlov.** 1995. Identical amino acid sequence of the *aroA*(*G*) gene products of *Bacillus subtilis* 168 and *B. subtilis* Marburg strain. Microbiology **141:**2219–2222.
3. **Bolotin, A., S. Mauger, K. Malarme, S. D. Ehrlich, and A. Sorokin.** 1999. Low-redundancy sequencing of the entire *Lactococcus lactis* IL1403 genome. Antonie Leeuwenhoek **76:**27–76.
4. **Davison, S. P., J. D. Santangelo, S. J. Reid, and D. R. Woods.** 1995. A *Clostridium acetobutylicum* regulator gene (*regA*) affecting amylase production in *Bacillus subtilis*. Microbiology **141:**989–996.
5. **Deutscher, J., E. Küster, U. Bergstedt, V. Charrier, and W. Hillen.** 1995. Protein kinase-dependent HPr/CcpA interaction links glycolytic activity to carbon catabolite repression in gram-positive bacteria. Mol. Microbiol. **15:** 1049–1053.
6. **Egeter, O., and R. Brückner.** 1996. Catabolite repression mediated by the catabolite control protein CcpA in *Staphylococcus xylosus*. Mol. Microbiol. **21:**739–749.
7. **Faires, N., S. Tobisch, S. Bachem, I. Martin-Verstraete, M. Hecker, and J. Stülke.** 1999. The catabolite control protein CcpA controls ammonium assimilation in *Bacillus subtilis*. J. Mol. Microbiol. Biotechnol. **1:**141–148.
8. **Fujita, Y., Y. Miwa, A. Galinier, and J. Deutscher.** 1995. Specific recognition of the *Bacillus subtilis gnt cis*-acting catabolite-responsive element by a protein complex formed between CcpA and seryl-phosphorylated HPr. Mol. Microbiol. **17:**953–960.
9. **Galinier, A., J. Haiech, M.-C. Kilhofer, M. Jaquinod, J. Stülke, J. Deutscher, and I. Martin-Verstraete.** 1997. The *Bacillus subtilis crh* gene encodes an HPr-like protein involved in carbon catabolite repression. Proc. Natl. Acad. Sci. USA **94:**8439–8444.
10. **Gösseringer, R., E. Küster, A. Galinier, J. Deutscher, and W. Hillen.** 1997. Cooperative and non-cooperative DNA binding modes of catabolite control protein CcpA from *Bacillus megaterium* result from sensing two different signals. J. Mol. Biol. **266:**665–676.
11. **Grundy, F. J., D. A. Waters, T. Y. Takova, and T. M. Henkin.** 1993. Identification of genes involved in utilization of acetate and acetoin in *Bacillus subtilis*. Mol. Microbiol. **10:**259–271.
12. **Helmann, J. D.** 1995. Compilation and analysis of *Bacillus subtilis* σA-dependent promoter sequences: evidence for extended contact between RNA polymerase and upstream promoter DNA. Nucleic Acids Res. **23:**2351–2360.
13. **Henkin, T. M., F. J. Grundy, W. L. Nicholson, and G. H. Chambliss.** 1991. Catabolite repression of α-amylase gene expression in *Bacillus subtilis* involves a *trans*-acting gene product homologous to the *Escherichia coli lacI* and *galR* repressors. Mol. Microbiol. **5:**575–584.
14. **Hueck, C. J., and W. Hillen.** 1995. Catabolite repression in *Bacillus subtilis*: a global regulatory mechanism for the gram-positive bacteria? Mol. Microbiol. **15:**395–401.
15. **Hueck, C. J., A. Kraus, and W. Hillen.** 1994. Sequences of *ccpA* and two downstream *Bacillus megaterium* genes with homology to the *motAB* operon from *Bacillus subtilis*. Gene **143:**147–148.
16. **Hueck, C. J., A. Kraus, D. Schmiedel, and W. Hillen.** 1995. Cloning, expression and functional analyses of the catabolite control protein CcpA from *Bacillus megaterium*. Mol. Microbiol. **16:**855–864.
17. **Jongeneel, C. V., J. Bouvier, and A. Bairoch.** 1989. A unique signature identifies a family of zinc-dependent metallopeptidases. FEBS Lett. **242:**211–214.
18. **Kraus, A., E. Küster, A. Wagner, K. Hoffmann, and W. Hillen.** 1998. Identification of a co-repressor binding site in catabolite control protein CcpA. Mol. Microbiol. **30:**955–963.
19. **Kravanja, M., R. Engelmann, V. Dossonnet, M. Bluggel, H. E. Meyer, R. Frank, A. Galinier, J. Deutscher, N. Schnell, and W. Hengstenberg.** 1999. The *hprK* gene of *Enterococcus faecalis* encodes a novel bifunctional enzyme: the HPr kinase/phosphatase. Mol. Microbiol. **31:**59–66.
20. **Küster, E., T. Hilbich, M. K. Dahl, and W. Hillen.** 1999. Mutations in catabolite control protein CcpA separating growth effects from catabolite repression. J. Bacteriol. **181:**4125–4128.
21. **Küster, E., E. J. Luesink, W. M. de Vos, and W. Hillen.** 1996. Immunological crossreactivity to catabolite control protein CcpA from *Bacillus megaterium* is found in many gram-positive bacteria. FEMS Microbiol. Lett. **139:**109–115.
22. **Lokman, B. C., M. Heerikhuisen, R. J. Leer, A. van den Broek, Y. Borsboom, S. Chaillou, P. W. Postma, and P. H. Pouwels.** 1997. Regulation of expression of the *Lactobacillus pentosus xylAB* operon. J. Bacteriol. **179:**5391–5397.
23. **Lokman, B. C., R. J. Leer, R. van Sorge, and P. H. Pouwels.** 1994. Promoter analysis and transcriptional regulation of *Lactobacillus pentosus* genes involved in xylose catabolism. Mol. Gen. Genet. **245:**117–125.
24. **Lokman, B. C., P. van Santen, J. C. Verdoes, J. Kruse, R. J. Leer, M. Posno, and P. H. Pouwels.** 1991. Organization and characterization of three genes involved in D-xylose catabolism in *Lactobacillus pentosus*. Mol. Gen. Genet. **230:**161–169.
25. **Luesink, E. J., C. M. A. Beumer, O. P. Kuipers, and W. M. De Vos.** 1999. Molecular characterization of the *Lactococcus lactis ptsHI* operon and analysis of the regulatory role of HPr. J. Bacteriol. **181:**764–771.
26. **Luesink, E. J., R. E. van Herpen, B. P. Grossiord, O. P. Kuipers, and W. M. de Vos.** 1998. Transcriptional activation of the glycolytic *las* operon and catabolite repression of the *gal* operon in *Lactococcus lactis* are mediated by the catabolite control protein CcpA. Mol. Microbiol. **30:**789–798.
27. **Martinez, E., B. Bartolome, and F. de la Cruz.** 1988. pACYC184-derived cloning vectors containing the multiple cloning site and *lacZ* alpha reporter gene of pUC8/9 and pUC18/19 plasmids. Gene **68:**159–162.
28. **Mirel, D. B., V. M. Lustre, and M. J. Chamberlin.** 1992. An operon of *Bacillus subtilis* motility genes transcribed by the σ^D form of RNA polymerase. J. Bacteriol. **174:**4197–4204.
29. **Miwa, Y., M. Saikawa, and Y. Fujita.** 1994. Possible function and some properties of the CcpA protein of *Bacillus subtilis*. Microbiology **140:**2567–2575.
30. **Monedero, V., M. J. Gosalbes, and G. Pérez-Martínez.** 1997. Catabolite repression in *Lactobacillus casei* ATCC 393 is mediated by CcpA. J. Bacteriol. **179:**6657–6664.

31. **Morel, F., J. Frot-Coutaz, D. Aubel, R. Portalier, and D. Atlan.** 1999. Characterization of a prolidase from *Lactobacillus delbrueckii* subsp. *bulgaricus* CNRZ 397 with an unusual regulation of biosynthesis. Microbiology **145:** 437–446.
32. **Reizer, J., C. Hoischen, F. Titgemeyer, C. Rivolta, R. Rabus, J. Stülke, D. Karamata, M. H. J. Saier, and W. Hillen.** 1998. A novel protein kinase that controls carbon catabolite repression in bacteria. Mol. Microbiol. **27:**1157–1169.
33. **Renna, M. C., N. Najimudin, L. R. Winik, and S. A. Zahler.** 1993. Regulation of the *Bacillus subtilis alsS*, *alsD*, and *alsR* genes involved in post-exponential-phase production of acetoin. J. Bacteriol. **175:**3863–3875.
34. **Sambrook, J., E. F. Fritsch, and T. Maniatis.** 1989. Molecular cloning: a laboratory manual, 2nd ed. Cold Spring Harbor Laboratory Press, Cold Spring Harbor, N.Y.
35. **Schick, J., B. Weber, J. R. Klein, and B. Henrich.** 1999. PepR1, a CcpA-like transcription regulator of *Lactobacillus delbrückii* ssp. *lactis*. Microbiology **145:**3147–3154.
36. **Shin, B. S., S. K. Choi, and S. H. Park.** 1999. Regulation of the *Bacillus subtilis* phosphotransacetylase gene. J. Biochem. (Tokyo) **126:**333–339.
37. **Simpson, C. L., and R. R. B. Russell.** 1998. Identification of a homolog of CcpA catabolite repressor protein in *Streptococcus mutans*. Infect. Immun. **66:**2085–2092.
38. **Simpson, C. L., and R. R. B. Russell.** 1998. Intracellular α-amylase of *Streptococcus mutans*. J. Bacteriol. **180:**4711–4717.
39. **Strohl, W. R.** 1992. Compilation and analysis of DNA sequences associated with apparent streptomycete promoters. Nucleic Acids Res. **20:**961–974.
40. **Stucky, K., J. R. Klein, A. Schuller, H. Matern, B. Henrich, and R. Plapp.** 1995. Cloning and DNA sequence analysis of *pepQ*, a prolidase gene from *Lactobacillus delbrueckii* subsp. *lactis* DSM7290, and partial characterization of its product. Mol. Gen. Genet. **247:**494–500.
41. **Stucky, K., J. Schick, J. Klein, B. Henrich, and R. Plapp.** 1996. Characterization of *pepR1*, a gene coding for a potential transcriptional regulator of *Lactobacillus delbrueckii* subsp. *lactis* DSM7290. FEMS Microbiol. Lett. **136:** 63–69.
42. **Stülke, J., and W. Hillen.** 1999. Carbon catabolite repression in bacteria. Curr. Opin. Microbiol. **2:**195–201.
43. **Weickert, M. J., and S. Adhya.** 1992. A family of bacterial regulators homologous to Gal and Lac repressors. J. Biol. Chem. **267:**15869–15874.