JOURNAL OF BACTERIOLOGY, Sept. 1998, p. 4380–4386 Vol. 180, No. 17
0021-9193/98/$04.00+0

The *carB* Gene Encoding the Large Subunit of Carbamoylphosphate Synthetase from *Lactococcus lactis* Is Transcribed Monocistronically

JAN MARTINUSSEN* AND KARIN HAMMER

Department of Microbiology, The Technical University of Denmark, DK-2800 Lyngby, Denmark

Received 14 January 1998/Accepted 23 June 1998

The biosynthesis of carbamoylphosphate is catalyzed by the heterodimeric enzyme carbamoylphosphate synthetase. The genes encoding the two subunits of this enzyme in procaryotes are normally transcribed as an operon, but the gene encoding the large subunit (*carB*) in *Lactococcus lactis* is shown to be transcribed as an isolated unit. Carbamoylphosphate is a precursor in the biosynthesis of both pyrimidine nucleotides and arginine. By mutant analysis, *L. lactis* is shown to possess only one *carB* gene; the same gene product is thus required for both biosynthetic pathways. Furthermore, arginine may satisfy the requirement for carbamoylphosphate in pyrimidine biosynthesis through degradation by means of the arginine deiminase pathway. The expression of the *carB* gene is subject to regulation at the level of transcription by pyrimidines, most probably by an attenuator mechanism. Upstream of the *carB* gene, an open reading frame showing a high degree of similarity to those of glutathione peroxidases from other organisms was identified.

In all organisms, pyrimidine metabolism is required in order to supply the cell with building blocks for the synthesis of DNA, RNA, and certain coenzymes needed in central metabolic pathways. In *Lactococcus lactis*, this requirement can be fulfilled either by the use of nucleosides and nucleobases present in the growth medium (36–38) or by means of the pyrimidine biosynthetic pathway, which seems to be universal in all prototrophic organisms investigated so far and consists of six enzymatic steps leading to the formation of UMP.

Many metabolic genes in *L. lactis* such as genes involved in the biosynthesis of amino acids (4, 10, 18) and glycolysis (6, 7, 31) have been identified and sequenced. The amino acid biosynthetic genes were found to be members of large operons, organized like the ones identified in *Bacillus subtilis*. Likewise, the genes encoding the pyrimidine biosynthetic enzymes in different gram-positive bacteria like *Bacillus caldolyticus* (17), *B. subtilis* (40), *Lactobacillus plantarum* (12), and *Enterococcus faecalis* (30) have been identified as members of a single operon. In contrast, it was recently shown that the genes of the pyrimidine biosynthetic pathway in *L. lactis* are organized differently. A *pyr* operon, which consists of only three biosynthetic genes, has been found in *L. lactis* (2). Two of the genes are the well-known *pyr* genes *pyrD* and *pyrF*, which encode dihydroorotate dehydrogenase and OMP decarboxylase, respectively. The third gene, *pyrK*, was identified as a new *pyr* gene encoding a protein which was shown to be necessary for the dihydroorotate dehydrogenase activity encoded by the adjacent *pyrDb* gene (2). The lactococcal *pyrKDbF* operon is highly homologous to the corresponding part of the much larger *pyr* operons found in other gram-positive bacteria. An interesting exception occurs with *Lactobacillus plantarum*, in which the *pyrK* analogue is absent from the operon (12). Another surprising feature of the pyrimidine biosynthesis pathway in *L. lactis* is the presence of two different genes, *pyrDa* and *pyrDb*, both of which encode a dihydroorotate dehydrogenase. The *pyrDb* gene belongs to the same family as the genes encoding dihydroorotate dehydrogenases in other gram-positive bacteria, whereas the *pyrDa* gene is closely related to that of the dihydroorotate dehydrogenase of *Saccharomyces cerevisiae* (1). Only *pyrDb* was shown to be part of the identified *pyr* operon (2).

Carbamoylphosphate is formed from CO_2, ATP, and glutamine and is used in the biosynthesis of both pyrimidine and arginine (Fig. 1). It is synthesized by the heterodimeric enzyme carbamoylphosphate synthetase (CPSase). The small subunit of the enzyme functions as a glutamine amidotransferase, whereas the large subunit has other catalytic properties. In all procaryotes described so far, CPSase activity is encoded by two genes commonly called *carA* and *carB*, and there is no reason to believe that this is not true for *L. lactis*. Procaryotes are characterized by having either a single set of genes that is responsible for all carbamoylphosphate synthesized and that encodes a single CPSase or two different sets of genes that encode CPSase (9). The two sets of genes differ in their regulatory features; one set is regulated by the level of pyrimidines in the cell, whereas the other responds to changes in the concentration of arginine (9). The genes encoding the two subunits have been sequenced for many procaryotes and have been found almost exclusively to be transcribed as an operon in the order *carA-carB*. Exceptions have, however, been reported. In *Pseudomonas aeruginosa* and *Neisseria* spp., sequences between *carA* and *carB* have been found (26, 27).

In this study we have cloned and determined the nucleotide sequence of the *carB* region of the *L. lactis* chromosome. Surprisingly, the gene is shown to be transcribed as a monocistronic unit. Moreover it is shown that *L. lactis* has only one *carB* gene, regulated by the pyrimidine level in the cell.

MATERIALS AND METHODS

Bacterial strains and plasmids. The bacterial strains and the plasmids used in this study are listed in Table 1. Plasmids pJS23 and pSJ24 were made in the following way. pKS2 was digested with *Hin*dIII and ligated into pSMA500 and pRC1, respectively. Competent *L. lactis* MG1363 cells were transformed with the *Escherichia coli* plasmids pSJ23 and pSJ24, which are unable to replicate in *L. lactis* but contain a selectable Em^r marker and cloned pieces of the lactococcal

* Corresponding author. Mailing address: Department of Microbiology, Building 301, The Technical University of Denmark, DK-2800 Lyngby, Denmark. Phone: 45 45 25 24 94. Fax: 45 45 88 26 60. E-mail: jm@im.dtu.dk.

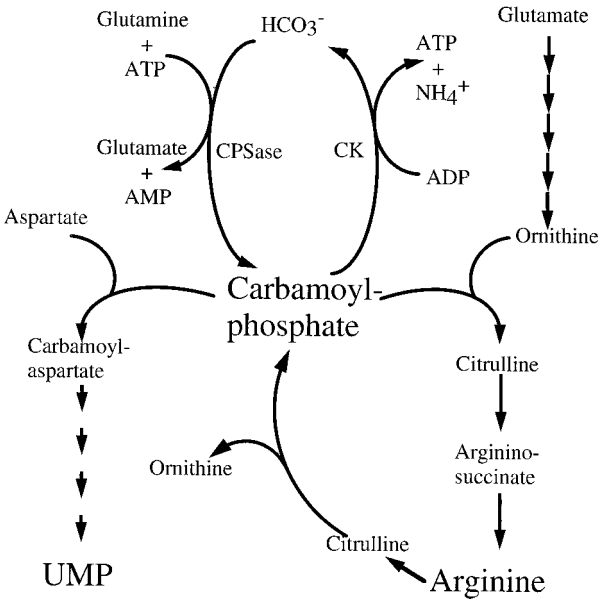

FIG. 1. Carbamoylphosphate pathways in *L. lactis*. Pathways of carbamoylphosphate in the formation of arginine and pyrimidines and its synthesis and degradation to ammonia and carbonate with formation of ATP are shown. CK, carbamate kinase; CPSase, carbamoylphosphate synthetase.

chromosome. Transformants were selected and purified on plates containing 1 μg of erythromycin per ml. Only transformants in which plasmids have recombined into the chromosome will result in Em^r colonies. Chromosomal DNA from strain MB35 was digested with *Spe*I, and the resulting linear molecules were circularized with T4 DNA ligase and transformed into *E. coli* DH5α. A plasmid (pSJ50) conferring erythromycin resistance and containing a 3,200-bp *Spe*I-*Hin*dIII lactococcal DNA fragment was obtained.

Two plasmids fusing the *carB* promoter to *lacLM* were constructed as follows. With pSJ50 as the template and the primers 5′-CCCAAGCTTACAGCCAGT AAATGTGGT-3′ and 3′-CGCGGATCCCATAGTAAAAGCTG-5′, a 1,250-bp PCR product was obtained and subsequently digested with *Hin*dIII and *Bam*HI. This fragment was inserted in the *lacLM* promoter fusion vectors pAK80 (22) and pSMA500 (35), which had been digested with *Hin*dIII and *Bam*HI. The resulting plasmids were termed pSJ60 and pSJ61, respectively.

Plasmid pJM66 was constructed as follows. With pKS2 as the template and the primers 5′-CTTAGGAACTCAAGTCG-3′ and 3′-ACCGGATCCCTTCAAAT ACTTATTAAC-5′, a 1,300-bp PCR product was obtained. After digestion with *Hin*dIII and *Bam*HI, the 1,000-bp fragment was inserted in the *lacLM* vector pSMA500 (35). Competent *L. lactis* MG1363 cells were transformed with plasmid pJM66, which is unable to replicate in *L. lactis* but contains a selectable Em^r marker and a piece of the lactococcal chromosome. Transformants were selected and purified on plates containing 2 μg of erythromycin per ml. Only strains in which homologous recombination between plasmids and chromosomal DNA has occurred after transformation will result in Em^r colonies. The *carB* regions in all the strains used in this work were mapped by Southern blot or PCR analysis.

Growth conditions and enzyme assay. Lactococcal cultures were grown either on M17 glucose broth (48) or on synthetic media that were based on MOPS (morpholinepropanesulfonic acid), contained seven vitamins and either 19 (SA) or 8 (BIV) amino acids (23), and were supplied with 1% glucose. *E. coli* cultures were grown on Luria-Bertani broth. *L. lactis* was cultured at 30°C in filled culture flasks without aeration. *E. coli* in batch cultures was grown at 37°C with vigorous shaking. For all plates, agar was added to 15 g/liter. When needed, the following compounds were added to the different media: arginine at 200 μg/ml, uracil at 20 μg/ml, erythromycin at 1 μg/ml for lactococci and 150 μg/ml for *E. coli*, and ampicillin at 100 μg/ml. For enzyme assays, the cells were grown in SA or BIV glucose medium and aliquots were harvested at different times during exponential growth between optical densities at 450 nm (OD_{450}s) of 0.2 and 0.8. The amount of β-galactosidase in the cells was assayed as previously described (22), but the cell density was measured at 450 nm. Specific enzymatic activity was determined as follows: $OD_{420}/(OD_{450} \times \text{min} \times \text{ml of culture})$.

Transformation. *L. lactis* was transformed by electroporation (21). *E. coli* cells were transformed as described previously (42).

DNA isolation, manipulations, and sequencing. Chromosomal lactococcal DNA was prepared as described by Johansen and Kibenich (24). The methods described by Sambrook et al. (42) were used for general DNA methods in vitro. DNA sequences were determined from plasmid DNA by the dideoxy-chain termination method (44) with a Thermo Sequenase-radiolabelled-terminator cycle sequencing kit (product no. US 79750; Amersham) in accordance with the protocol of the manufacturer.

Southern blot analysis. Southern blot analysis was performed with GeneScreen nylon membranes (New England Nuclear) and the digoxigenin system (Boehringer Mannheim) for colorimetric detection of hybridized products in accordance with the protocols of the manufacturers.

PCR amplification of DNA. *L. lactis* chromosomal DNA was amplified by PCR with 1 μg of DNA in a final volume of 100 μl containing deoxyribonucleoside triphosphates (0.25 mM each), oligonucleotides (10 μM), and 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer). Amplification was performed with 30 cycles of 95°C for 1 min and 55°C for 1 min, followed by 3 min at 72°C.

RNA extraction. *L. lactis* RNA was harvested from strain MG1363 grown exponentially in SA glucose medium to an OD_{450} of approximately 0.8. Total RNA from a 20-ml culture was isolated according to the method of Arnau and coworkers (3).

Primer extension. A synthetic oligonucleotide, 5′-TTTCCTGTTCACAACCT TGC-3′, complementary to the sense strand covering nucleotides 726 to 745 was radioactively labelled at its 5′ end with [γ-^{32}P]ATP and T4 polynucleotide kinase and used for primer extensions on 20 μg of total RNA isolated from *L. lactis* MG1363 as previously described (16). The elongation was performed at 41°C with SuperScript II reverse transcriptase (Gibco BRL).

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to the EMBL data library and assigned the accession no. AJ000109.

RESULTS

Cloning and sequencing of the *carB* region of the chromosome. A part of the *carB* gene of *L. lactis* encoding the large subunit of CPSase (CPSase B) was obtained by chance. During the sequencing of clones obtained from a partial *Sau*3A library in pBR322, plasmid pKS2, which contains two *Sau*3A fragments from the *L. lactis* chromosome (3), turned out to include a 4-kb *Sau*3A fragment which was shown to harbor part of a putative open reading frame showing a high degree of similarity to those encoding the C-terminal parts of the large subunits of CPSases from different organisms. A 1,700-bp *Hin*dIII-*Sau*3A fragment was subcloned in the *E. coli* vector pRC1 (28), thus creating pSJ24. The fragment harbors an internal part of the *carB* open reading frame (Fig. 2). This plasmid was allowed to integrate into the chromosome of MG1363 by homologous recombination, resulting in strain MB35, which carries an insertion in *carB* (Fig. 2).

TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Genotype or description	Source or reference
Strains		
E. coli DH5α	F80*lacZ*DM15 Δ(*lacZYA-argF*)*U169 recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1*	Lab strain
L. lactis		
MG1363	Plasmid-free strain[a]	15
MB35	MG1363 *carB*::pSJ24 (Em^r)[a]	This study
MB36	MG1363 *carB*::*lacLM* (pSJ23) (Em^r)[a]	This study
MB37	MG1363 *carB*::*lacLM* (pSJ61) (Em^r)[a]	This study
MB38	MG1363 *carB*::*lacLM* (pSJ66) (Em^r)[a]	This study
Plasmids		
pRC1	*erm*, *L. lactis* integration vector	28
pAK80	*erm lacLM*, *E. coli* and *L. lactis* shuttle vector	22
pSMA500	*erm lacLM*, *L. lactis* integration vector	35
pKS2	*amp*, *Sau*3A fragments from *L. lactis* in pBR322	3
pSJ23	*erm*, 1,700-bp *Hin*dIII from pKS2 in pSMA500	This study
pSJ24	*erm*, 1,700-bp *Hin*dIII from pKS2 in pRC1	This study
pSJ500	*erm*, *Spe*I rescue from MB35	This study
pSJ60	*erm lacLM*, PCR, *carB* promoter in pAK80	This study
pSJ61	*erm lacLM*, PCR, *carB* promoter in pSMA500	This study
pSJ66	*erm lacLM*, PCR, *carB* terminator in pSMA500	This study

[a] See Fig. 2 for a physical map.

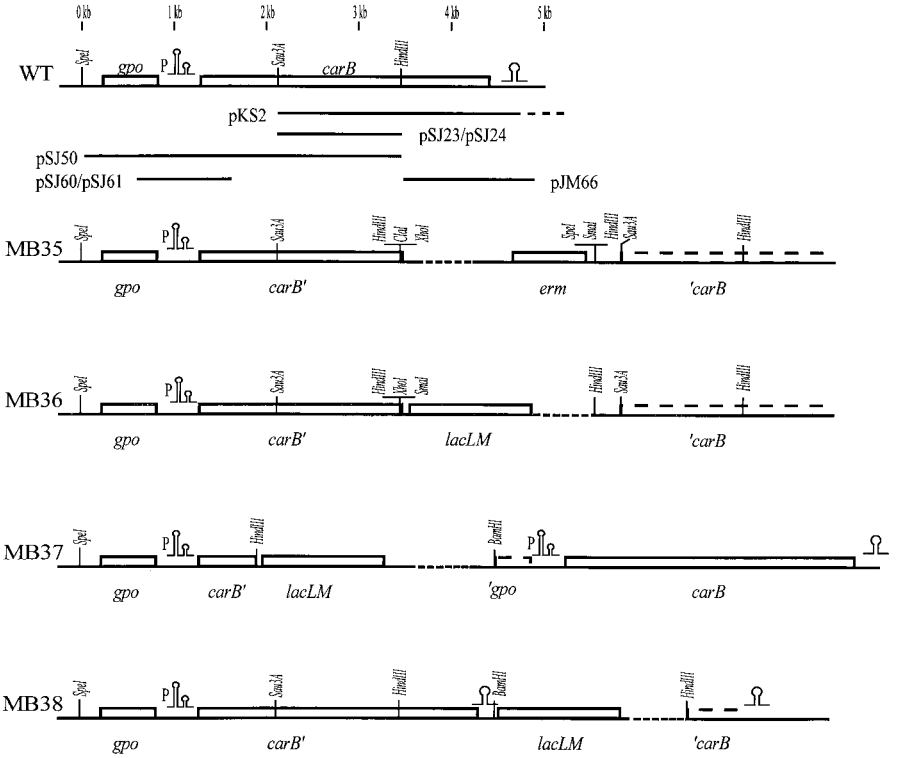

FIG. 2. Genetic maps the *carB* regions of plasmids and strains used in this work. The physical map and the positions of selected restriction endonuclease sites are shown. The *carB* DNAs contained in the different plasmids are shown with lines. The dotted line in pKS2 indicates that the cloned lactococcal DNA extends to a *Sau*3A site at 6.1 kb. A P indicates the position of the *carB* promoter. The maps of the chromosomal DNA in the *carB* regions of the wild type (WT), MB35, MB36, MB37, and MB38 are shown. The broken lines represent the *E. coli* plasmid DNA, which is not drawn to scale. The erythromycin resistance genes of MB36, MB37, and MB38 are not shown. The *carB* terminator and the *carB* attenuator are shown by omega- and double-omega-like structures, respectively.

In order to clone the N-terminal-encoding part of the CPSase B gene by marker rescue, chromosomal DNA was extracted from MB35, digested with different restriction endonucleases, ligated, and transformed to *E. coli* to select for erythromycin resistance. A rescue plasmid (pSJ50) obtained from the *Spe*I digest was subjected to further analysis. By performing Southern blotting experiments on chromosomal DNA isolated from *L. lactis* MG1363 with probes derived from pKS2 and pSJ50, it was shown that the lactococcal DNAs present on the two plasmids overlap (not shown). By combining the sequencing data obtained from pKS2 and pSJ50, the sequence of the *carB* region was determined. Two open reading frames encoding 157 and 1,064 amino acids that are transcribed in the same direction were found by computer analysis of the DNA sequence. The 1,064-amino-acid product of an open reading frame encoding a protein with a theoretical size of 117 kDa showed a high degree of identity (70%) to the CPSase B from *Lactobacillus plantarum* (12) and 66% identity to the same enzyme from *B. subtilis* encoded by the *pyrAB* gene (40). Upstream of the putative *carB* gene, one expects to find the *carA* gene encoding the small CPSase subunit. Surprisingly, the 157-amino-acid product of the open reading frame showed no homology whatsoever to the small CPSase subunits from other organisms. Instead, this open reading frame product showed high degrees of identity to glutathione peroxidases from various organisms: 54% identity to *B. subtilis* (46), 52% identity to *S. cerevisiae* (5), 48% identity to *Synechocystis* spp. (47), and 49% identity to *Chlamydia reinhardtii* (29). Upstream of both reading frames, translational initiation signals can be identified (Table 2).

The *carB* gene is transcribed as a monocistronic message. Since the *carB* gene is preceded by the functionally unrelated

TABLE 2. Sequence properties of the *gpo-carB* region

Section of *gpo-carB* region	Nucleotide coordinates[a]	Sequence[b] or no. of codons
gpo translation initiation	150–165	CCAGGAGGTAAACAA**ATG**AA
gpo open reading frame	163–636	157 codons
carB promoter	658–694	TGAGACTTGTAAAAGGTCAATTATTTTGCTAAACTAATAAACA**A**TAA
carB translation initiation		CTACAAAGGAGAAAAACAA**ATG**CC
carB open reading frame		1,064 codons
carB terminator		ACTGTCCCAAATGGGGCTTTTTTTTTTTT

[a] The nucleotide numbers refer to the sequence submitted to the database.

[b] The putative start codons are shown in boldface letters, and the nucleotides of the mRNA complementary to the 3′ end of the 16S rRNA from *L. lactis* (3′-UCUUUCCUCCA-5′) are indicated by underscoring. The −35 and −10 sequences are shown by underscoring, and the first nucleotide to be transcribed is shown in boldface type. The inverted repeats in the putative terminator are shown by double underlining.

TABLE 3. β-Galactosidase activities of strains carrying different *carB*::*lacLM* fusions

Strain	Plasmid	Chromosomal *lacLM* fusion	Relevant genotype	β-Galactosidase activity[a]	
				No supplement	Uracil added
MB36		pSJ23 *carB*	*carB*::*lacLM erm carB*	0.26	0.025
MB37		pSJ61 *carB*	*carB*::*lacLM erm*	0.084	0.033
MB38		pSJ66 *carB* terminator	*erm*	<0.001	ND[b]
MG1363	pSJ60	None	*carB*::*lacLM erm*	0.30	ND
MG1363	pAK80	None	*erm*	<0.001	ND

[a] Cells were grown in BIV minimal medium supplied with arginine. The β-galactosidase activity was determined with the formula $OD_{420}/(OD_{450} \times \text{min} \times \text{ml of culture})$.
[b] ND, activity not determined.

gene *gpo*, which encodes glutathione peroxidase, it is tempting to believe that the *carB* gene is transcribed by a promoter present in the intercistronic region between *gpo* and *carB*. In order to assay promoter activity, a PCR fragment covering the entire intercistronic region and parts of the *gpo* and *carB* open reading frames was amplified and cloned into the promoter probe vector pAK80 (22), thus generating pSJ60 (Fig. 2). After transformation into MG1363, the specific β-galactosidase activity was measured in exponentially growing cells and determined to be 0.3, thus demonstrating the presence of a promoter in the intercistronic region just upstream of *carB*, since the specific activity of cells harboring the pAK80 vector alone is less than 0.001 (Table 3).

In order to map the precise location of the promoter, the 5′ end of the transcript was determined by primer extension on RNA isolated from MG1363. The result is presented in Fig. 3. This experiment mapped the first nucleotide to be transcribed (+1) to position 695 (Fig. 4). This finding is supported by sequence analysis, as an extended −10 sequence (TGCTAAA CT) can be identified. In lactococcal promoters an extended −10 sequence is characterized by a TGN sequence immediately in front of the −10 sequence (TATAAT) (50). Furthermore, by 17 nucleotides upstream of the −10 sequence there is a −35 sequence (TTGTAA) (Fig. 4).

Regarding the 3′ end of the *carB* transcript, a potential transcriptional terminator can be identified at positions 4191 to 4218 (Table 2). In order to show that transcription does not extend past the putative terminator, a promoterless *lacLM* gene was integrated into the chromosome immediately after the stretch of thymine residues following the stem-loop structure (strain MB38) (Fig. 2). The rationale for this was twofold. First, if another pyrimidine- or arginine-biosynthetic gene is expressed from the *carB* promoter, the integration of the plasmid will disrupt transcription, thus resulting in a polar mutation, and the strain will acquire a pyrimidine or arginine requirement. Second, the amount of β-galactosidase produced by this strain will reflect the amount of transcription extending past the putative terminator structure. The phenotype of the resulting MB38 strain was determined by growth on minimal medium in the absence and presence of arginine and/or uracil in the BIV glucose minimal medium. Strain MB38 required neither uracil nor arginine, thus demonstrating that no biosynthetic gene involved in these pathways is located downstream of *carB* and transcribed from the *carB* promoter. As shown in Table 3, no detectable β-galactosidase activity could be identified in BM38, thus showing that transcription from the *carB* promoter is terminated no later than after the stem-loop structure ending at position 4205.

In conclusion, the data presented here unambiguously demonstrate that the identified *carB* gene of *L. lactis* is transcribed as a monocistronic mRNA.

The physiological effect of a *carB* mutation. In order to elucidate the role of the *carB* gene product, the insertion mutant MB35 was subjected to phenotypic analysis. As previously mentioned, MB35 carries a truncated *carB* gene, lacking one third of its coding region for the C terminus (Fig. 2). Carbamoylphosphate is required for the biosynthesis of pyrimidines and arginine. To test whether the *carB* mutation would confer a pyrimidine requirement on the cell, the abilities of the strain to grow in the absence and presence of uracil were tested with the SA glucose medium, which includes arginine. An effect of uracil on growth was observed, since addition of uracil to the SA glucose medium resulted in a slight increase in the growth rate of MB35.

L. lactis has the ability to degrade arginine by the arginine deiminase pathway (8), thus forming carbamoylphosphate as an intermediate. To test whether arginine could serve as carbamoylphosphate donor, which subsequently could be utilized in pyrimidine biosynthesis, MB35 (*carB*) was propagated in the BIV glucose medium in the absence and presence of arginine and/or uracil. Arginine was clearly required for growth, whereas uracil alone was unable to facilitate growth. The addition of a surplus of uracil in addition to arginine resulted in a 60% increase in growth rate. Addition of arginine to the BIV me-

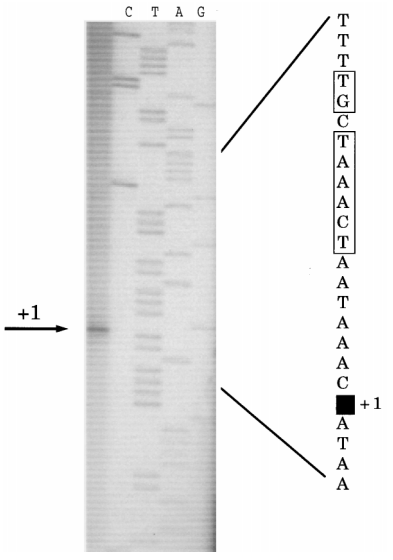

FIG. 3. Primer extension mapping of the 5′ end of the *carB* mRNA. Sequencing ladders generated with the oligonucleotide used for the primer extension were loaded next to the reaction mixture. The DNA sequence of the sense strand around the first nucleotide in the transcript (designated +1) is presented, and the −10 sequence is boxed.

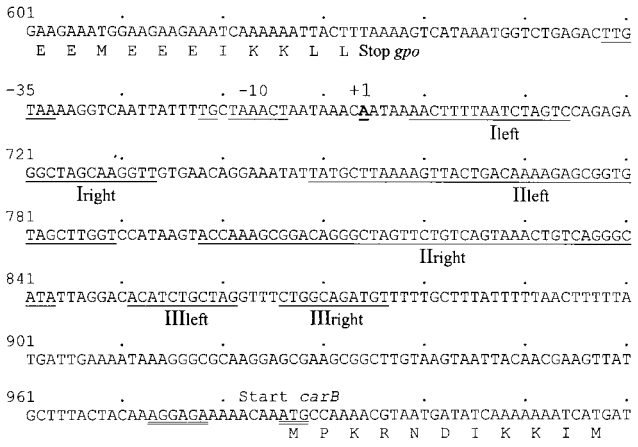

FIG. 4. Sequence of the *gpo-carB* intercistronic region. The numbers refer to the sequences submitted to the database. The amino acids of the glutathione peroxidase and CPSase large subunit derived from the DNA sequence are shown. The translational start site of *carB* is indicated with double underscoring. The −10 and −35 sequences of the *carB* promoter are indicated with underscoring, and the first nucleotide to be transcribed is marked +1. The right and left stems of loops I, II, and III (Fig. 5) are shown.

dium resulted in a slight increase in the growth rate of the wild-type strain MG1363. To further test the effect of the *carB* mutation on growth, strain MB35 was analyzed for its ability to grow on different precursors in the arginine biosynthetic pathway. Citrulline, but not ornithine, was able to support growth of the mutant. Since carbamoylphosphate is required for the conversion of ornithine into citrulline (Fig. 1), the data show that the *carB* mutation results in a shortage of carbamoylphosphate in the cell. This finding suggests that *L. lactis* harbors only one gene encoding the large CPSase subunit. Furthermore, the results imply that *L. lactis* MG1363 has the ability to degrade arginine to carbamoylphosphate, which can subsequently be exploited as a precursor in pyrimidine biosynthesis. An alternative explanation may account for the observations made. If the biosynthesis of arginine and UMP is compartmentalized, meaning that two different CPSases are parts of larger complexes that include either arginine- or pyrimidine-specific biosynthetic enzymes, and carbamoylphosphate is bound to these complexes at all times, then the *carB* gene described in this paper encodes only the arginine-specific CPSase, since strain MB35 carrying the *carB* mutation requires arginine but not uracil.

The expression of *carB* is regulated by pyrimidines. The leader of the *carB* mRNA has the potential to fold into two mutually exclusive structures: a putative terminator and a putative antiterminator (Fig. 5). Furthermore, sequence analysis showed that immediately after the transcriptional start site of the mRNA, the *carB* leader is equipped with a sequence that has extremely high levels of similarity to the three PyrR binding sites of *B. subtilis*, all of which are found at the same position with respect to that of the antiterminator (49). Exactly the same sequence is found in the *pyrKDF* operon of *L. lactis* (2). Moreover, *pyrKDF* mRNA can be folded into a structure similar to the one found in the *carB* leader (Fig. 5B). In order to analyze whether the expression of the *carB* gene is subject to regulation by pyrimidines, the β-galactosidase content of

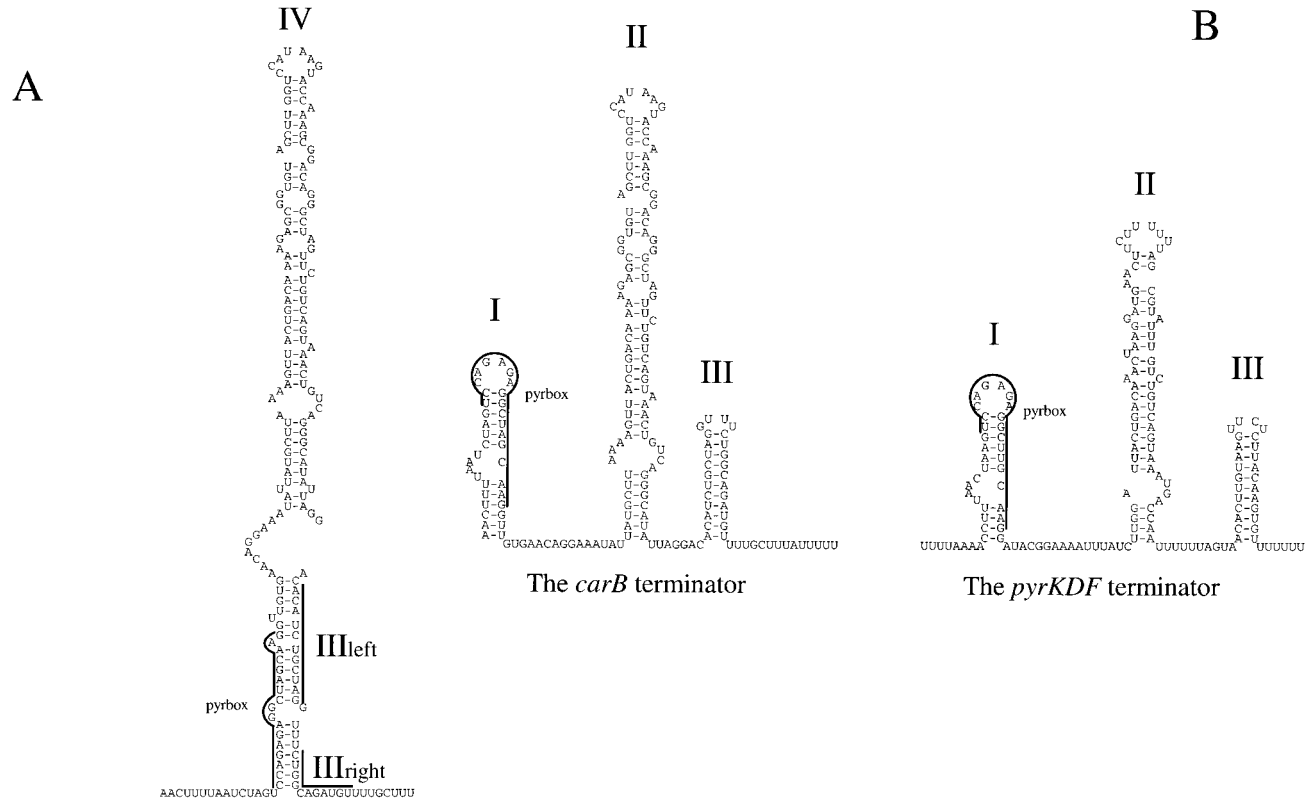

FIG. 5. Sequences of the *carB* and *pyrKDbF* attenuators. (A) *carB* antiterminator structure IV. The putative binding site of the PyrR protein is indicated with a line marked pyrbox. The stems of the terminator are shown as III_{left} and III_{right}. (B) *carB* and *pyrKDF* terminator structures. The putative PyrR binding site in domain I is indicated with a line marked pyrbox. The actual terminator structure is designated III.

L. lactis carrying a *carB*::*lacLM* fusion was monitored. The *Hin*dIII-*Sau*3A fragment from pKS2 harboring an internal part of the *carB* open reading frame and the PCR fragment covering the *carB* promoter used for construction of pSJ60 were subcloned in the *E. coli* vector pSMA500 (35) as 1,700- and 1,250-bp fragments, respectively (Fig. 2). The resulting plasmids were designated pSJ23 and pJS61. These plasmids were allowed to integrate into the chromosome of MG1363 by homologous recombination. The strain obtained by transformation with pSJ23 (MB36) has acquired a *carB* mutation identical to that found in MB35 in addition to the *carB*::*lacLM* fusion (Fig. 2), whereas MB37 which was obtained by transformation with pSJ61 is like the wild type with respect to *carB* despite the fact that this strain carries a *carB*::*lacLM* fusion on the chromosome. MB36 and MB37 were grown in BIV minimal medium supplied with arginine in the absence and presence of uracil, and their levels of β-galactosidase synthesis were assayed. The results are presented in Table 3. The absence of uracil led to a 10-fold induction of expression of the *carB* gene in a *carB* mutant, whereas a wild-type background led to a 3-fold reduction in the induction of expression. It should be noted that since MB36 carries the same *carB* mutation as MB35, in the absence of uracil, strain MB36 must be starved for pyrimidines. In order to investigate whether the expression of the *carB* gene is regulated by arginine, the amounts of β-galactosidase produced by strains growing in BIV minimal medium in the absence and presence of arginine were assayed. This experiment was conducted only with strain MB37, since MB36 is unable to grow in the absence of arginine. The expression of the *carB* gene was not repressed in the presence of exogenous arginine, whereas its expression was repressed threefold by exogenous uracil in the absence of arginine.

DISCUSSION

***L. lactis* harbors only one *carB* allele.** As previously stated, the *carB* strain requires arginine but not uracil for growth. Two different models may account for this observation: either only one *carB* allele is present, and the carbamoylphosphate requirement for pyrimidine biosynthesis is fulfilled by degradation of arginine, or the gene described in this paper is the arginine-specific *carB* allele used in the compartmentalized biosynthesis of arginine. It is highly unlikely that an arginine-specific CPSase is regulated by uracil. However, *carB* expression in *L. lactis* was found to be regulated by uracil. Therefore, the evidence points to the conclusion that *L. lactis* harbors only one *carB* allele.

The *car* genes are usually members of operons. The results presented in this work demonstrate that the *carB* gene from *L. lactis* is transcribed as a monocistronic message. This finding is in contrast to the observation made for most other organisms, namely, that the *carB* gene is part of an operon consisting of either the *carA* and *carB* genes alone or the *carA* and *carB* genes as members of a larger operon that includes other pyrimidine-biosynthetic genes. However, exceptions to this paradigm have been reported; in *P. aeruginosa* the existence of a 216-amino-acid-encoding open reading frame with unknown function between *carA* and *carB* has been demonstrated, although the three genes are part of the same operon transcribed from a promoter upstream of *carA* (26). *Neisseria* seems to be a true exception. Sequences between *carA* and *carB* that vary in size from 2.2 to 3.7 kb were found among different *Neisseria* species. Furthermore, putative transcription terminators in the intergenic DNA were identified by sequence analysis of one species. Whether these structures were of physiological relevance was not indicated by experimental data (27). Recently, the *carA* gene from *L. lactis* has been cloned in our lab, and both Southern blot analysis and PCR failed to demonstrate linkage between *carA* and *carB* in *L. lactis* (45).

Arginine is degraded in *L. lactis*. Originally, *L. lactis* subsp. *lactis* was distinguished from *L. lactis* subsp. *cremoris* by the ability of *L. lactis* subsp. *lactis* to degrade arginine by means of the arginine deiminase pathway (8). The first step in the pathway is the deamination of arginine to citrulline, which subsequently is phosphorolytically cleaved into ornithine and carbamoylphosphate. The latter energy-rich derivative can either be used for pyrimidine biosynthesis as described in this work or be degraded to carbon dioxide and ammonia with formation of ATP, thus generating one energy-rich bond per molecule of arginine. It has been shown that arginine uptake in lactococci is mediated by an energy-independent arginine-ornithine antiporter (11). Therefore, arginine can be used as an energy source. Strain MG1363 has been classified as *L. lactis* subsp. *cremoris* based on genetic evidence (19, 25, 41). Originally MG1363 was considered a strain of *L. lactis* subsp. *lactis* based on its physiological traits, including its capability to degrade arginine. The results presented here further confirm that *L. lactis* MG1363 degrades arginine through the arginine deiminase pathway.

Glutathione peroxidase encoded by the *gpo* gene protects against oxidizing elements. Glutathione is a tripeptide (L-γ-glutamyl-L-cysteinylglycine) and is present in relatively large amounts in *L. lactis* (13). It is important as a scavenger of free radicals and in the control of the redox potential in the cell. In addition, glutathione is involved in transpeptidation and reduction of thiol groups in proteins and it acts as a cofactor in the reduction of ribonucleotides (39). In addition to completely reduced oxygen as found in water, partially reduced forms, such as singlet oxygen, superoxide anions, hydrogen peroxide, and hydroxyl radicals, are present in organisms growing in an aerobic environment. All these compounds are highly reactive, and they can oxidize proteins and damage DNA, and may oxidize membrane fatty acids, leading to peroxidation of the lipids. Aerobic organisms produce these compounds as metabolic by-products, but all oxygen-tolerant organisms, like *L. lactis*, are exposed to these powerful agents and must protect themselves against cell damage (14). In *E. coli* three different activities that protect against the reactive oxygen species noted above have been identified: superoxide dismutase, catalase, and peroxidase (20). In *L. lactis*, a gene encoding superoxide dismutase (*sodA*) has been identified (43). Based on the finding that an *L. lactis* mutant lacking superoxide dismutase is viable in an aerobic environment, Sanders and coworkers (43) concluded that an additional oxygen-protecting mechanism must be present in *L. lactis*. The glutathione peroxidase found in this work may fulfill this role.

The expression of the *carB* gene is regulated by pyrimidines. In this work we have been able to demonstrate that the *carB* gene is regulated by the presence of uracil in the growth medium. By analyzing the sequence of the *carB* leader, a structure including a *pyrR* binding site similar to the one found in the *pyrKDF* leader can be identified (2). The mechanism by which *B. subtilis* regulates its expression of the *pyr* operon by transcriptional attenuation through the PyrR regulatory protein has been studied in great detail (32–34). The structures that may be formed by the RNA transcribed from the *carB* and *pyrKDF* (2) operons in *L. lactis* are similar to the structures found in the RNA transcribed from the *B. subtilis pyr* operon (33). Figure 5B shows the structures believed to result in termination at the attenuator. Stem-loop structure I, including the PyrR binding site, is highly homologous to a similar structure found in the *B. subtilis pyr* operon designated the anti-

antiterminator by Lu and coworkers (34). These findings strongly suggest the presence of a PyrR homologue in *L. lactis*.

ACKNOWLEDGMENTS

This work was supported by grants from the Danish government program for food science and technology (FØTEK) through the Center for Advanced Food Studies.

We sincerely appreciate the expert technical assistance of Susan Outzen Jørgensen.

REFERENCES

1. **Andersen, P. S., P. J. Jansen, and K. Hammer.** 1994. Two different dihydroorotate dehydrogenases in *Lactococcus lactis*. J. Bacteriol. **176:**3975–3982.
2. **Andersen, P. S., J. Martinussen, and K. Hammer.** 1996. Sequence analysis and identification of the *pyrKDF* operon from *Lactococcus lactis* including a novel gene, *pyrK*, involved in pyrimidine biosynthesis. J. Bacteriol. **178:**5005–5012.
3. **Arnau, J., K. Sørensen, K. Appel, F. K. Vogensen, and K. Hammer.** 1996. Analysis of heat shock gene expression in *Lactococcus lactis* MG1363. Microbiology **142:**1685–1691.
4. **Bardowski, J., S. D. Ehrlich, and A. Chopin.** 1992. Tryptophan biosynthesis genes in *Lactococcus lactis* subsp. *lactis*. J. Bacteriol. **174:**6563–6570.
5. **Budde, E., and U. Stahl.** 1995. Direct submission, EMBL accession no. P40581.
6. **Cancilla, M. R., B. E. Davidson, A. L. Hillier, N. Y. Nguyen, and J. Thompson.** 1995. The *Lactococcus lactis* triosephosphate isomerase gene, *tpi*, is monocistronic. Microbiology **141:**229–238.
7. **Cancilla, M. R., A. L. Hillier, and B. E. Davidson.** 1995. *Lactococcus lactis* glyceraldehyde-3-phosphate dehydrogenase gene, *gap*: further evidence for strongly biased codon usage in glycolytic pathway genes. Microbiology **141:**1027–1036.
8. **Crow, V. L., and T. D. Thomas.** 1982. Arginine metabolism in lactic streptococci. J. Bacteriol. **150:**1024–1032.
9. **Cunin, R., N. Glansdorff, A. Piérard, and V. Stalon.** 1986. Biosynthesis and metabolism of arginine in bacteria. Microbiol. Rev. **50:**314–352.
10. **Delorme, C., S. D. Ehrlich, and P. Renault.** 1992. Histidine biosynthesis genes in *Lactococcus lactis* subsp. *lactis*. J. Bacteriol. **174:**6571–6579.
11. **Driessen, A. J., B. Poolman, R. Kiewiet, and W. N. Konings.** 1987. Arginine transport in *Streptococcus lactis* is catalyzed by a cation exchanger. Proc. Natl. Acad. Sci. USA **84:**6093–6097.
12. **Elagöz, A., A. Abdi, J. C. Hubert, and B. Kammerer.** 1996. Structure and organisation of the pyrimidine biosynthesis pathway genes in Lactobacillus plantarum: a PCR strategy for sequencing without cloning. Gene **183:**37–43.
13. **Fahey, R. C., W. C. Brown, W. B. Adams, and M. B. Worsham.** 1978. Occurrence of glutathione in bacteria. J. Bacteriol. **133:**1126–1129.
14. **Fridovich, I.** 1978. The biology of oxygen radicals. Science **201:**875–880.
15. **Gasson, M. J.** 1983. Plasmid complements of *Streptococcus lactis* NCDO 712 and other streptococci after protoplast-induced curing. J. Bacteriol. **154:**1–9.
16. **Gerdes, K., T. Thisted, and J. Martinussen.** 1990. Mechanism of postsegregational killing by the *hok*/*sok* system of plasmid R1: *sok* antisense RNA regulates formation of a *hok* mRNA species correlated with killing of plasmid-free cells. Mol. Microbiol. **4:**1807–1818.
17. **Ghim, S.-Y., and J. Neuhard.** 1994. The pyrimidine biosynthesis operon of the thermophile *Bacillus caldolyticus* includes genes for uracil phosphoribosyltransferase and uracil permease. J. Bacteriol. **176:**3698–3707.
18. **Godon, J.-J., M.-C. Chopin, and S. D. Ehrlich.** 1992. Branched-chain amino acid biosynthesis genes in *Lactococcus lactis* subsp. *lactis*. J. Bacteriol. **174:**6580–6589.
19. **Godon, J. J., C. Delorme, S. D. Ehrlich, and P. Renault.** 1992. Divergence of genomic sequences between *Lactococcus lactis* subsp. *lactis* and *Lactococcus lactis* subsp. *cremoris*. Appl. Environ. Microbiol. **58:**4045–4047.
20. **Hassan, H. F., and I. Fridovich.** 1977. Enzymatic defenses against the toxicity of oxygen and of streptonigrin in *Escherichia coli*. J. Bacteriol. **129:**1574–1583.
21. **Holo, H., and I. F. Nes.** 1989. High-frequency transformation by electroporation of *Lactococcus lactis* subsp. *cremoris* grown with glycine in osmotically stabilized media. Appl. Environ. Microbiol. **55:**3119–3123.
22. **Israelsen, H., S. M. Madsen, A. Vrang, E. B. Hansen, and E. Johansen.** 1995. Cloning and partial characterization of regulated promoters from *Lactococcus lactis* Tn*917-lacZ* integrants with the new promoter probe vector pAK80. Appl. Environ. Microbiol. **61:**2540–2547.
23. **Jensen, P. R., and K. Hammer.** 1993. Minimal requirements for exponential growth of *Lactococcus lactis*. Appl. Environ. Microbiol. **59:**4363–4366.
24. **Johansen, E., and A. Kibenich.** 1992. Characterization of leuconostoc isolates from commercial mixed strain mesophilic starter cultures. J. Dairy Sci. **75:**1186–1191.
25. **Klijn, N., A. H. Weerkamp, and W. M. de Vos.** 1991. Identification of mesophilic lactic acid bacteria by using polymerase chain reaction-amplified variable regions of 16S rRNA and specific DNA probes. Appl. Environ. Microbiol. **57:**3390–3393.
26. **Kwon, D. H., C. D. Lu, D. A. Walthall, T. M. Brown, J. E. Houghton, and A. T. Abdelal.** 1994. Structure and regulation of the *carAB* operon in *Pseudomonas aeruginosa* and *Pseudomonas stutzeri*: no untranslated region exists. J. Bacteriol. **176:**2532–2542.
27. **Lawson, F. S., F. M. Billowes, and J. A. Dillon.** 1995. Organization of carbamoyl-phosphate synthase genes in *Neisseria gonorrhoeae* includes a large, variable intergenic sequence which is also present in other *Neisseria* species. Microbiology **141:**1183–1191.
28. **Le Bourgeois, P., M. Lautier, M. Mata, and P. Ritzenthaler.** 1992. New tools for the physical and genetic mapping of *Lactococcus* strains. Gene **111:**109–114.
29. **Leisinger, U., A. J. B. Zehnder, and R. I. F. Eggen.** 1997. Direct submission, GenBank accession no. AF014927.
30. **Li, X., G. M. Weinstock, and B. E. Murray.** 1995. Generation of auxotrophic mutants of *Enterococcus faecalis*. J. Bacteriol. **177:**6866–6873.
31. **Llanos, R., C. J. Harris, A. J. Hillier, and B. E. Davidson.** 1993. Identification of a novel operon in *Lactococcus lactis* encoding three enzymes for lactic acid synthesis: phosphofructokinase, pyruvate kinase, and lactate dehydrogenase. J. Bacteriol. **175:**2541–2551.
32. **Lu, Y., and R. L. Switzer.** 1996. Evidence that the *Bacillus subtilis* pyrimidine regulatory protein PyrR acts by binding to *pyr* mRNA at three sites in vivo. J. Bacteriol. **178:**5806–5809.
33. **Lu, Y., and R. L. Switzer.** 1996. Transcriptional attenuation of the *Bacillus subtilis pyr* operon by the PyrR regulatory protein and uridine nucleotides in vitro. J. Bacteriol. **178:**7206–7211.
34. **Lu, Y., R. J. Turner, and R. L. Switzer.** 1996. Function of RNA secondary structures in transcriptional attenuation of the *Bacillus subtilis pyr* operon. Proc. Natl. Acad. Sci. USA **93:**14462–14467.
35. **Madsen, S. M., B. Albrechtsen, E. B. Hansen, and H. Israelsen.** 1996. Cloning and transcriptional analysis of two threonine biosynthetic genes from *Lactococcus lactis* MG1614. J. Bacteriol. **178:**3689–3694.
36. **Martinussen, J., P. S. Andersen, and K. Hammer.** 1994. Nucleotide metabolism in *Lactococcus lactis*. Salvage pathways of exogenous pyrimidines. J. Bacteriol. **176:**1514–1516.
37. **Martinussen, J., and K. Hammer.** 1994. Cloning and characterization of *upp*, a gene encoding uracil phosphoribosyltransferase from *Lactococcus lactis*. J. Bacteriol. **176:**6457–6463.
38. **Martinussen, J., and K. Hammer.** 1995. Powerful methods to establish chromosomal markers in *Lactococcus lactis*: an analysis of pyrimidine salvage pathway mutants obtained by positive selections. Microbiology **141:**1883–1890.
39. **Meister, A., and M. E. Anderson.** 1983. Glutathione. Annu. Rev. Biochem. **52:**711–760.
40. **Quinn, C. L., B. T. Stephenson, and R. L. Switzer.** 1991. Functional organization, nucleotide sequence and regulation of the *Bacillus subtilis* pyrimidine biosynthetic operon. J. Biol. Chem. **266:**9113–9127.
41. **Salama, M., W. Sandine, and S. Giovannoni.** 1991. Development and application of oligonucleotide probes for identification of *Lactococcus lactis* subsp. *cremoris*. Appl. Environ. Microbiol. **57:**1313–1318.
42. **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.
43. **Sanders, J. W., K. J. Leenhouts, A. J. Haandrikman, G. Venema, and J. Kok.** 1995. Stress response in *Lactococcus lactis*: cloning, expression analysis, and mutation of the lactococcal superoxide dismutase gene. J. Bacteriol. **177:**5254–5260.
44. **Sanger, F., S. Nicklen, and A. R. Coulson.** 1977. DNA sequencing with chain-terminating inhibitors. Proc. Natl. Acad. Sci. USA **74:**5467–5473.
45. **Schallert, J., and J. Martinusssen.** 1997. Unpublished data.
46. **Sorokin, A. V., V. Azevedo, E. Zumstein, N. Galleron, S. D. Ehrlich, and P. Serror.** 1996. Direct submission, Swiss-Prot accession no. P52035.
47. **Tabata, S.** 1996. Direct submission, DDBJ accession no. D90913.
48. **Terzaghi, B. E., and W. E. Sandine.** 1975. Improved medium for lactic streptococci and their bacteriophages. Appl. Microbiol. **29:**807–813.
49. **Turner, R. J., Y. Lu, and R. L. Switzer.** 1994. Regulation of the *Bacillus subtilis* pyrimidine biosynthetic gene cluster by an autogenous transcriptional attenuation mechanism. J. Bacteriol. **176:**3708–3722.
50. **van de Guchte, M., J. Kok, and G. Venema.** 1992. Gene expression in *Lactococcus lactis*. FEMS Microbiol. Rev. **88:**73–92.