

The Pyrimidine Operon *pyrRPB-carA* from *Lactococcus lactis*

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Received 7 September 2000/Accepted 2 January 2001

The four genes *pyrR*, *pyrP*, *pyrB*, and *carA* were found to constitute an operon in *Lactococcus lactis* subsp. *lactis* MG1363. The functions of the different genes were established by mutational analysis. The first gene in the operon is the pyrimidine regulatory gene, *pyrR*, which is responsible for the regulation of the expression of the pyrimidine biosynthetic genes leading to UMP formation. The second gene encodes a membrane-bound high-affinity uracil permease, required for utilization of exogenous uracil. The last two genes in the operon, *pyrB* and *carA*, encode pyrimidine biosynthetic enzymes; aspartate transcarbamoylase (*pyrB*) is the second enzyme in the pathway, whereas carbamoyl-phosphate synthetase subunit A (*carA*) is the small subunit of a heterodimeric enzyme, catalyzing the formation of carbamoyl phosphate. The *carA* gene product is shown to be required for both pyrimidine and arginine biosynthesis. The expression of the pyrimidine biosynthetic genes including the *pyrRPB-carA* operon is subject to control at the transcriptional level, most probably by an attenuator mechanism in which PyrR acts as the regulatory protein.

The de novo synthesis of pyrimidines is universal. The pathway consists of six enzymatic steps leading to the formation of UMP, which is further converted into UTP, CTP, dCTP, and dTTP. In order to coregulate expression, genes are often found to be organized in operons in procaryotes. The pyrimidine biosynthetic genes (*pyr* genes) have been found to constitute a single operon in a number of different gram-positive organisms including *Bacillus subtilis* (37), *Bacillus caldolyticus* (13), *Enterococcus faecalis* (23), and *Lactobacillus plantarum* (9). In addition a pyrimidine biosynthetic operon is found in *Mycobacterium tuberculosis* (6). The pyrimidine metabolism in *Lactococcus lactis* has been studied for a number of years. Surprisingly it was found that the *pyr* genes of *L. lactis* are scattered on the chromosome in small operons, the *pyrKDbF* operon (2), the *carB* gene (35), the *pyrEC* operon (5), and the *pyrDa* gene (1). This paper describes the genomic organization of the pyrimidine biosynthetic genes of *L. lactis*, since we demonstrate the presence of an operon including *pyrB*, encoding aspartate transcarbamoylase, and *carA*, encoding the small subunit of the carbamoyl-phosphate (CP) synthetase (CPSase). Moreover, the operon includes *pyrR*, the regulatory gene controlling expression of the *pyr* genes, and *pyrP*, encoding the uracil transporter.

Regulation of the expression of the *pyr* genes of *L. lactis* has not been subject to a detailed analysis. It has, however, been shown that the transcription of the *carB* gene is repressed by addition of uracil to the growth medium (35). Moreover, in front of both the *carB* gene (35) and the *pyrKDbF* operon (2) a putative attenuator similar to those found in *B. subtilis* (41) can be identified. These findings suggest that the expression of the *pyr* genes of *L. lactis* is regulated by the same attenuator mechanism as that suggested for *B. subtilis* (41). An RNA binding protein, PyrR, mediates the regulation of the expression of the *pyr* operon in *B. subtilis* by stabilizing the formation

of a transcriptional terminator in the mRNA leader sequence. The binding of PyrR to the mRNA is dependent on the formation of a PyrR-UMP complex. In the absence of a PyrR-UMP complex, an antiterminator structure is preferentially formed (25). In the work presented here, the presence of a protein with a high degree of homology to the PyrR of *B. subtilis* and responsible for repressing expression of the pyrimidine biosynthetic enzymes by exogenous pyrimidines is documented.

The first step in the pyrimidine biosynthetic pathway is the formation of CP utilizing CO₂, ATP, and glutamine. CP is also a precursor for the biosynthesis of arginine. The formation of CP is catalyzed by CPSase. This enzyme consists of a small subunit and a large subunit. The small subunit of the enzyme functions as a glutamine amidotransferase, whereas the other catalytic properties are found in the large subunit. In all procaryotes described so far, two genes, commonly called *carA* and *carB*, encode the CPSase. Procaryotes may contain either a single CPSase encoded by a single set of genes responsible for all CP synthesized or, alternatively, two different sets of CPSase-encoding genes (8). The two sets differ in their regulatory features; the level of pyrimidines in the cell regulates the expression of one set of genes, whereas the other responds to changes in arginine concentration (8). The genes encoding the two subunits have been sequenced for many procaryotes and are almost exclusively transcribed as an operon in the order *carA-carB*. Previously we have been able to show that the *carB* gene in *L. lactis* is transcribed as a monocistronic message and that the *carA* gene is not in the immediate proximity of *carB* (35). This finding was confirmed by the publication of the gene map of another *L. lactis* strain, showing that the *carA* and *carB* genes are separated by approximately 240 kb (5). In this work we show that the *carA* gene is part of an operon including other genes involved in pyrimidine metabolism and regulation.

MATERIALS AND METHODS

Growth conditions. Lactococcal cultures were grown either on M17 glucose broth (39) or on synthetic media based on MOPS (morpholinepropanesulfonic acid) and containing seven vitamins and either 19 (SA) or 8 (BIV) amino acids

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TABLE 1. Strains and plasmids used in this work

Strain or plasmid	Genotype or construction	Source or reference
<i>E. coli</i>		
DH5 α	F80 <i>lacZ</i> DM15 Δ (<i>lacZYA-argF</i>)U169 <i>recA1 endA1 hsdR17 supE44 thi-1 gyrA96 relA1</i>	Laboratory strain
SØ990	<i>pyrB</i>	
BM604	HfrH <i>thi galE</i> Δ (<i>attλ-bio</i>) <i>deoA103 deoC argA cytR upp udp pyrF30</i>	3
<i>L. lactis</i>		
MG1363	Plasmid-free strain	10
PSA001	MG1363 <i>pyrF</i> Em ^r ; uracil requirement	2
MB36	MG1363 <i>carB::lacLM</i> (<i>carB</i> Em ^r); arginine requirement; partial uracil requirement	35
MB400	MG1363 <i>pyrB::pG⁺host9::ISS1</i> (<i>pyrB</i> Em ^r); uracil requirement	This study
MB401	MB36 <i>pyrR::pG⁺host8::ISS1</i> (<i>pyrR</i> Em ^r Tet ^r); partial uracil requirement	This study
MB411	MG1363 <i>pyrR::pAM111</i> (<i>pyrP pyrB carA</i> Em ^r); arginine requirement; partial uridine requirement	This study
MB417	MG1363 <i>pyrP::pAM117</i> (<i>pyrP pyrB carA</i> Em ^r); arginine requirement; partial uridine requirement	This study
MB422	MG1363 <i>pyrB::pAM122</i> (<i>carA</i> Em ^r); arginine requirement; partial uracil requirement	This study
Plasmids		
pRC1	<i>L. lactis</i> integration vector	22
pG ⁺ host9::ISS1	Ori ^{ts} used for ISS1 mutagenesis; Em ^r	30
pG ⁺ host8::ISS1	Ori ^{ts} used for ISS1 mutagenesis; Tet ^r	30
pJS50	<i>SpeI</i> rescue from MB400	This study
pAM111	<i>pyrR</i> open reading frame in pRC1; Em ^r	This study
pAM117	Internal <i>pyrP</i> fragment in pRC1; Em ^r	This study
pAM122	<i>pyrB</i> open reading frame in pRC1; Em ^r	This study

(19) supplied with 1% glucose. *Escherichia 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 μ g/ml, uracil at 20 or 200 μ g/ml, uridine at 40 μ g/ml, erythromycin at 1 μ g/ml for lactococci and 150 μ g/ml for *E. coli*, and ampicillin at 100 μ g/ml. In order to assay for growth on pyrimidines and sensitivity to the toxic analogue 5-fluorouracil, cells were plated on minimal medium containing pyrimidine or the analogue at different concentrations. After incubation at 30°C, the colony sizes were estimated on an arbitrary scale from 0.1 to 1, where the size of the largest colony was set to 1.

Transformation, DNA isolation, manipulations, and sequencing. *L. lactis* was transformed by electroporation (17). *E. coli* cells were transformed as described previously (38). Chromosomal lactococcal DNA was prepared as described by Johansen and Kibbenich (20). The methods described by Sambrook et al. (38) were used for general DNA methods in vitro. DNA sequences were determined from plasmid DNA by the dideoxy chain termination method using the Thermo Sequenase radiolabeled terminator cycle sequencing kit (product no. US 79750) from Amersham in accordance with the protocol of the manufacturer.

Southern blot analysis. Southern blot analysis was performed with Gene-Screen nylon membranes (New England Nuclear) and the DIG 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 μ g of DNA in a final volume of 100 μ l containing deoxyribonucleoside triphosphates (0.25 mM each), oligonucleotides (10 μ M), and 2.5 U of AmpliTaq DNA polymerase (Perkin-Elmer). Amplification was performed in one of the following two ways: (i) standard PCR, 30 cycles at 94°C for 1 min and 55°C for 1 min, followed by 3 min at 72°C; easy gene walking, 25 cycles at 94°C for 1 min, and 55°C for 1 min, followed by 3 min at 72°C (10 min for the last cycle).

pGhost9::ISS1 transposon mutagenesis and selection for pyrimidine auxotrophs. A pool of *L. lactis* strain MG1363 pGh9::ISS1 transposition mutants was obtained as previously described (21). After resuspension of the mutant library in SA medium supplemented with glucose and erythromycin but without pyrimidine addition, the cells were grown at 37°C for 30 min to stop the growth of auxotrophs. Then the culture was diluted 100-fold in the same medium, reaching an optical density at 450 nm (OD₄₅₀) of 0.8, and grown for one additional hour. Ampicillin counterselection for the auxotrophs was performed overnight at 37°C, after addition of ampicillin at 100 μ g/ml to the diluted culture. After the washing and resuspension of the cells in 0.9% NaCl solution, aliquots of a 100- μ l culture, both undiluted and 10-fold diluted, were plated on SA-glucose medium containing uracil. After incubation at 37°C overnight, colonies were screened for a

pyrimidine requirement on SA-glucose medium with and without uracil. Pyrimidine-requiring strain MB400 was chosen for further analysis.

Plasmid rescue. Chromosomal DNA was extracted from MB400 and digested with *SpeI*. Following ligation and transformation of *E. coli* strain DH5 α an erythromycin-resistant transformant was obtained. This strain was shown to harbor plasmid pJS50.

Construction of plasmids and strains. The strains and plasmids used in this study are shown in Table 1. The primers used for plasmid constructions are shown in Table 2 and the relevant genetic maps of strains and plasmids are presented in Fig. 1. Plasmid pAM111 was obtained in the following way. With primers *PyrR*-Nterm and *PyrR*_CTny and *L. lactis* MG1363 chromosomal DNA as the template a PCR fragment was obtained. Subsequently, the PCR fragment and pRC1 were digested with *XhoI* and *PstI*, mixed, ligated, and transformed into DH5 α . Plasmid pAM122 was constructed in exactly the same way using primers *PyrB*-Nterm and *PyrB*-Cterm. By the same strategy, plasmid pAM117 was constructed using primers *PyrR*_4F and *PyrB*_14R and restriction enzymes *HindIII* and *EcoRI*. Strains MB411, MB417, and MB422 were constructed by transformations of MG1363 with nonreplicative plasmids pAM111, pAM117, and pAM122, respectively. Integrants were isolated on M17 plates supplied with glucose and 1 μ g of erythromycin/ml and confirmed by PCR analysis of their chromosomal DNA.

RNA extraction. *L. lactis* RNA was harvested from strain MG1363 grown exponentially in SA-glucose medium to a cell density represented by an OD₄₅₀ of approximately 0.8. Total RNA from a 20-ml culture was isolated according to the method of Arnau and coworkers (4).

Primer extension. Synthetic oligonucleotide *PyrR*_3R, complementary to the sense strand, was radioactively labeled in the 5' end using [γ -³²P]ATP and T4 polynucleotide kinase and used for primer extensions on 20 μ g of total RNA isolated from *L. lactis* strain MG1363 as previously described (11). The elongation was performed at 41°C using a SuperScript II reverse transcriptase (RT) (Gibco BRL).

RT-PCR. *L. lactis* RNA was used as the template in the Titan one-tube RT-PCR system from Boehringer Mannheim in accordance with the protocols of the manufacturer.

Enzyme assays. Exponentially growing cells were harvested at an OD₄₅₀ of 0.8, washed, and resuspended in 50 mM Tris-HCl (pH 7.0)–1 mM EDTA–1 mM dithiothreitol, resulting in a 100-fold concentration. The cells were lysed using a French pressure cell press at 18,000 lb/in². Cell debris was removed by centrifugation, and the supernatant was used directly as the enzyme source in the assays. The protein concentration was determined as described by Lowry et al. (24). All assays were performed at 30°C using crude extracts with a final concentration of about 200 μ g of protein/ml. Specific activities are expressed as

TABLE 2. Synthetic oligonucleotides used in PCR amplifications

Primer	Sequence (5'-3')	Usage ^a	Coordinate ^b
PyrR_Ctny	AAACTGCAGCTTAATCTTCACGTTTGATT	Cloning	945
PyrR_F4	CCTTTAAGATTGTCCAGAGAG	RT-PCR	248
PyrR_F5	GACGGACATCTTATTAATGG	RT-PCR	369
PyrR_PintR	GTTGAGCCAAACATGG	RT-PCR	1147
CarA-R6	GCTCTTGTATCTACACCA	RT-PCR	3952
PyrR-Nterm	AGCTCGAGAAATGAAAGGAGCCCC	Cloning	440
PyrB-Nterm	AGCTCGAGGGTAAAGGAGACCTT	Cloning	2603
PyrB-Cterm	AAACTGCAGCTTACTTCGCTTTTTTTTC	Cloning	3518
PyrP_F2	GACAGGGGATGGTTTAG	RT-PCR	1927
PyrR_4F	CAACTTAGAGTTTTAGGAG	Cloning	1057
PyrR_2R	CGGAATGGACGTGTATCC	EGW	639
PyrR_3R	AGTGATCCGTGTAATTGC	Primer ext., EGW, RT-PCR	484
PyrR_4R	CCAAAGAGAATGCAGGTAG	EGW, RT-PCR	309
PyrB_14R	CTCCACCAATAACTGGTG	Cloning	2101
PyrRB_3F	GGATACACGTCCATTCCG	RT-PCR	657
pyrB2	CATATGCTCTTACGCGTG	RT-PCR	3278
pyrB_12R	CACCTTACTGATTGAACTGGC	RT-PCR	2846
PyrRB_2F	GCATTCTCTTTGGAATGCAGC	RT-PCR	336
Prbind-1	TCCAGAGAGGCTNGCAAG	PCR	257
PyrB11	CACGCGTAAGAGCATATG	PCR	3260
EcoRI	NNNNNNNNNNGAATTC	EGW	
HindIII	NNNNNNNNNNAAGCTT	EGW	
Sau3AI	NNNNNNNNNNGATC	EGW	

^a The applications of the different primers used in this study. Cloning, obtaining *L. lactis* DNA used for construction of plasmids; RT-PCR, primers used in the RT-PCR experiments; EGW and PCR, primers used for obtaining DNA templates for sequencing; primer ext., primer used in the primer extension experiment.

^b The coordinates refer to the corresponding 3' end of *pyrRBP-carA* database sequence AJ13262.

milliunits per milligram of protein, and 1 mU is defined as 1nmol of product formed or substrate used per min.

(i) **Aspartate transcarbamoylase (PyrB) activity.** PyrB activity was determined at pH 7.0 in the following way. Potassium aspartate (50 mM), potassium phosphate (25 mM), and enzyme extract were mixed and equilibrated at 30°C. The assay was started by addition of CP (3 mM). Aliquots (150 µl) were extracted between 0 and 20 min, and the formation of carbamoylaspartate was measured using the colorimetric procedure described by Prescott and Jones (36), in which 10⁻³ M carbamoylaspartate corresponds to an absorption of 18 at 560 nm.

(ii) **Dihydroorotase (PyrC) activity.** PyrC activity was determined essentially as described for the aspartate transcarbamoylase (PyrB) assay with the following alterations. Potassium aspartate and potassium phosphate were replaced by Tris-HCl (100 mM)-EDTA (2 mM), and the assay was started by adding dihydroorotate (2 mM) instead of CP.

(iii) **Dihydroorotate dehydrogenase A (PyrDa) activity.** PyrDa activity was determined by monitoring orotate formation in a spectrophotometer at 295 nm ($\epsilon = 3.67 \times 10^3 \text{ M}^{-1}$). The reaction mixture contained 0.1 M sodium phosphate (pH 7.0), 50 mM KCN, and 0.1 mM fumarate. After equilibration the assay was started by addition of dihydroorotate (0.1 mM).

(iv) **Dihydroorotate dehydrogenase B (PyrDb) activity.** PyrDb activity was assayed by the same procedure as that used for dihydroorotate dehydrogenase A, except that fumarate was replaced by 0.1 mM NAD⁺.

(v) **Orotate phosphoribosyltransferase (PyrE) activity.** PyrE activity was measured in a buffer at pH 7.5 containing Tris-HCl (20 mM), EDTA (2 mM), and orotate 300 µM. The reaction was monitored spectrophotometrically at 295 nm after addition of 5-phosphoribosyl-1-pyrophosphate (PRPP). A decrease in absorbance of 3.67 is equivalent to an increase in OMP of 1 mM.

(vi) **OMP decarboxylase (PyrF) activity.** PyrF activity was determined in a buffer (pH 7.5) containing 20 mM Tris-HCl and 2 mM EDTA. After calibration at 30°C, the reaction was initiated by the addition of 50 µM OMP. The activity was monitored in a spectrophotometer at 285 nm. A reduction of the OMP concentration by 1 mM corresponds to a decrease in absorbance of 1.65.

β-Galactosidase. For enzyme assays the cells were grown in SA- or BIV-glucose medium and aliquots were harvested at different times during exponential growth between OD₄₅₀s of 0.2 and 0.8. The amount of β-galactosidase in the cells was assayed as previously described (18), but the cell density was measured at 450 nm. The specific enzymatic activity was determined as OD₄₂₀/(OD₄₅₀ per minute per milliliter of culture).

Preparation and analysis of protein extracts from *E. coli*. *E. coli* cells were grown exponentially in Luria-Bertani broth exponentially in and, at an OD₄₅₀ of

0.7, IPTG (isopropyl-β-D-thiogalactopyranoside) was added, resulting in a final concentration of 1 mM. The cells were incubated at 37°C overnight. The 1-ml cell culture was harvested, washed, and resuspended in a 200-µl solution consisting of 0.5 M NaCl, 1 mM EDTA, and 50 mM Tris-HCl (pH 8.0). The proteins were extracted with 200 µl of phenol equilibrated with the buffer. The proteins were precipitated with 500 µl of ethanol and recovered by centrifugation. The protein pellets were resuspended in 100 µl of sodium dodecyl sulfate loading buffer and analyzed by 12.5% polyacrylamide gel electrophoresis as previously described (38).

Nucleotide sequence accession number. The nucleotide sequence reported in this paper has been submitted to the EMBL data library and assigned accession no. AJ132624.

RESULTS

Cloning and sequencing of the operon. From a library of approximately 6,000 pGh9::ISS1 transposon mutants four pyrimidine auxotrophic mutants were obtained after ampicillin counterselection in medium without pyrimidines added. Marker rescue experiments were conducted in order to obtain the DNA regions flanking the pGh9::ISS1 insertions. Marker rescues from two of the strains resulted in plasmids with chromosomal inserts. The nucleotide sequence of the *Lactococcus* chromosomal DNA was determined. The DNA in one clone was found to be identical to the previously cloned and sequenced *carB* gene from *L. lactis* (35). The other plasmid obtained by an SpeI digestion was termed pJS50 and was shown to contain a 5-kb chromosomal fragment, including part of an open reading frame showing extensive homology to those encoding aspartate transcarbamoylases from a number of different organisms. Consequently, the gene was named *pyrB*. The ISS1 element was inserted in the middle of *pyrB*. Despite numerous attempts using different restriction endonucleases, only clones harboring DNA encoding the C-terminal part of PyrB were obtained.

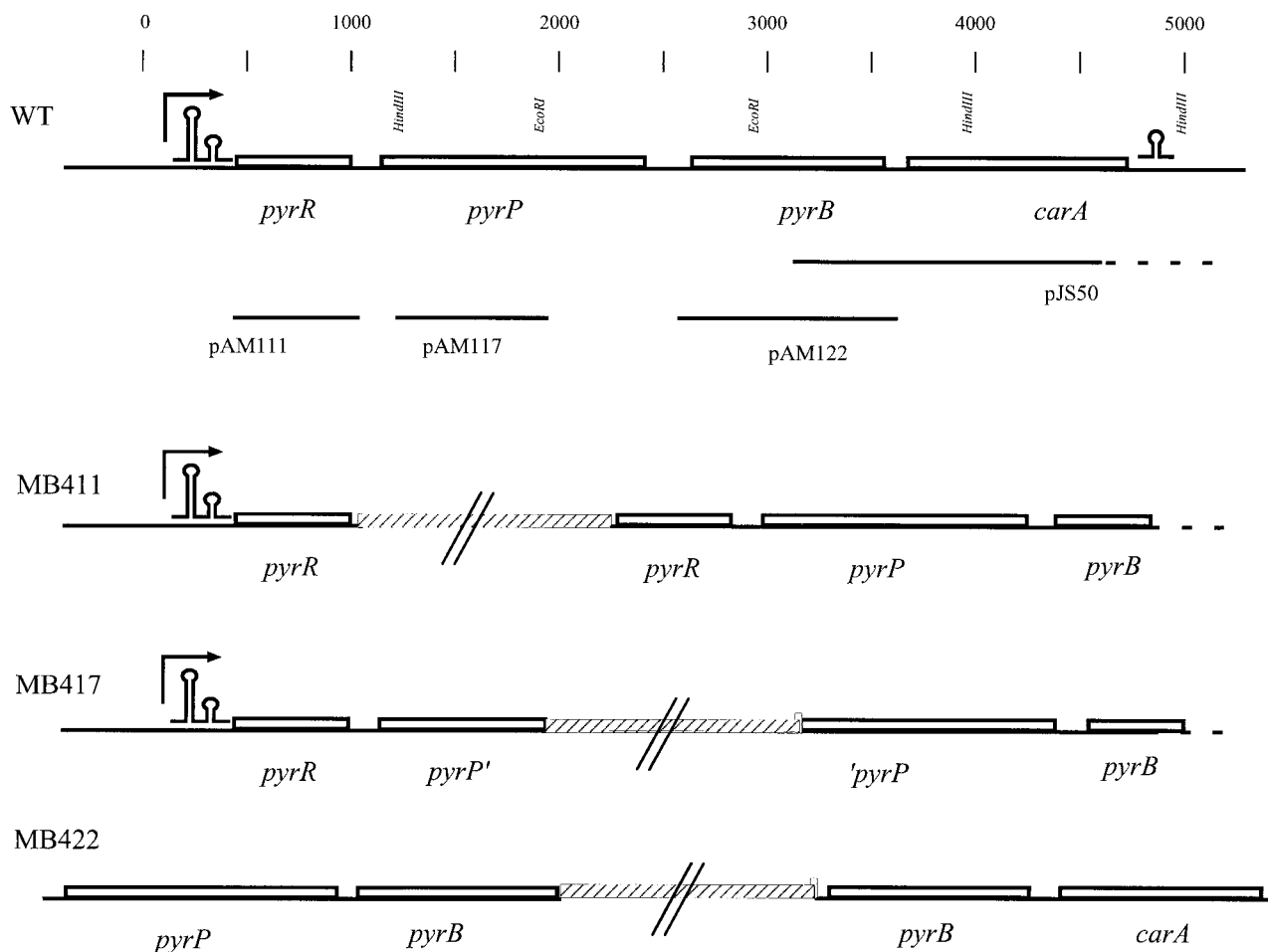


FIG. 1. Genetic maps of the *pyrRBPB-carA* region, the plasmids, and the strains used in this work. The physical map and the positions of selected restriction endonuclease sites are shown in the top line. The scale indicates numbers of base pairs in the sequenced region. Lines below the map show the *L. lactis* DNA contained in the different plasmids. The dotted line in pJS50 indicates that the cloned lactococcal DNA extends to a distal *SpeI* site beyond the sequenced DNA. Arrow, position of the promoter. The maps of the chromosomal DNA in the *pyrRBPB-carA* regions of the wild-type (WT) strain, MB411, MB417, and MB422 are shown. Hatched bars, *E. coli* plasmid DNA (not drawn to scale); omega-like and double-omega-like structures, terminator and attenuator of the operon, respectively.

In order to obtain the upstream sequences, we used a PCR strategy. A *pyrB* primer was used together with a PyrR binding site primer to amplify the upstream DNA. The rationale for doing this was as follows. By assaying the aspartate transcarbamoylase activity in wild-type cells grown in SA-glucose medium in the absence and presence of uracil, we were able to show that the expression of *pyrB* was repressed twofold by addition of uracil. This has previously been shown also to be the case for the *carB* gene (35) and the *pyrKDbF* operon (2) of *L. lactis*. In the leader sequences of these operons, PyrR binding sites very homologous to the PyrR binding sites in *B. subtilis* have been found. Therefore, a similar sequence could very well be present upstream of *pyrB*. Based on the PyrR binding sequences found in *B. subtilis* (14) and *L. lactis* (2, 35), the consensus PyrR binding site (5'-UCCAGAGAGGCUNG CAAG-3') was proposed. A degenerate PyrR binding site primer (Prbind-1) was synthesized (Table 2) and used in a PCR experiment together with a *pyrB*-specific primer (PyrB11) and chromosomal DNA as the template. A 3-kb fragment was

obtained and used as the template for sequencing reactions. By analyzing the sequence obtained, putative open reading frames with homology to those of the PyrB, PyrP, and PyrR proteins from *B. subtilis* could be identified.

Since the PyrR binding sites have been found exclusively in the mRNA leader, the promoter is not expected to be present on the PCR fragment. In order to obtain the upstream sequence, the easy gene walking method was used (16). This method is based on nested PCR. A set of three nested oligonucleotides (PyrR_4R, PyrR_3R, and PyrR_2R) was used together with partly degenerate oligonucleotides containing either an *EcoRI*, *HindIII*, or *Sau3AI* restriction site in the 3' end of the primer (see Table 2 for the sequences of the oligonucleotides). Fragments covering the flanking DNA were obtained in combination with all three degenerate oligonucleotides. These PCR fragments were sequenced without prior cloning. This procedure eliminated errors caused by mutations in individual PCR fragments. The sequence data obtained in the different experiments were merged, resulting in a contin-

TABLE 3. Sequence properties of the *pyrRPB-carA* region

Genetic segment	No. of codons	Predicted mol wt	Nucleotide coordinates ^b	Sequence ^a
<i>pyrRPB-carA</i> promoter PyrR binding site			191–227	TTGACAAAGTTCTAAACTTTGCTATAATCTATCTAA TCCAGAGAGGCAGGCAAG
<i>pyrR</i>	173	19,817	443–961	AAGAATGAAAGGAGCCCCAAATG
<i>pyrP</i>	430	45,232	1063–2352	GAGTTATTTTAGGAGAAAAAGTG
<i>pyrB</i>	310	34,567	2604–3533	GTCAGGGTAAAGGAGAAAAAATG
<i>carA</i>	357	39,647	3624–4694	AAAGCAAAGTTTAAAGGAAATAATTATG
<i>pyrRPB-carA</i> terminator			4763–4792	AAAAAAGAGCCTGTTTATAGCAGGCTCTTTTTTAT

^a The putative start codons are in boldface. Single underlining, nucleotides of the mRNA complementary to the 3' end of the 16S rRNA from *L. lactis* (3'-UCUUUCCUCCA-5'); double underlining, inverted repeats in the putative terminator. The -10 and -35 sequences of the promoter and the first nucleotide in the corresponding mRNA are also in boldface.

^b The nucleotide numbers refers to the sequence submitted to the database.

uous 4.5-kb DNA sequence. Using a probe covering part of *pyrB*, a Southern blot experiment on *Cla*I-, *Hind*III-, and *Eco*RI-digested chromosomal DNA from *L. lactis* MG1363 showed that the DNA originated from *L. lactis* (data not shown).

The open reading frames in the operon. On the 4.5-kb fragment, four open reading frames can be detected (Fig. 1 and Table 3). The Genemark program (29) predicts the identified open reading frames including ribosome binding sites as coding sequences. In order to assign a function to the open reading frames, Blast searches in protein databases were conducted. The first open reading frame showed homology to pyrimidine regulatory protein PyrR, first identified in *B. subtilis* (41). The second reading frame shows homology to that encoding uracil permease from a number of gram-positive organisms including *B. subtilis* (41). The third open reading frame was the already-identified *PyrB* gene, whereas the last one was homologous to *carA*, which encodes the small subunit of CPSase. The organization of the four open reading frames is shown in Fig. 1. In Table 3, the sizes, positions on the DNA, and translational initiation signals of the four open reading frames are presented. Moreover, the positions and sequences of the putative promoter and terminator are shown.

The four open reading frames are transcribed as an operon.

In order to show whether the four open reading frames constitute an operon, two kinds of experiments were conducted. An RT-PCR experiment was used to define the size of the mRNA in vitro, whereas the phenotypes of strains constructed by gene disruption were used to define the transcriptional units in vivo.

Total RNA was isolated from an exponentially growing culture of the wild-type *L. lactis* MG1363 and treated with DNase. RT-PCR experiments using primers covering the noncoding regions between the open reading frames were conducted. The positions of the primers and the expected PCR products are presented in Fig. 2A. In order to test whether any DNA is present in the samples resulting in false-positive signals, conventional PCR using the same primers and RNA preparation as the template was conducted. No products were observed (not shown). Moreover, in another experiment, the RNA sample was treated with RNase prior to amplification by RT-PCR. One example is shown in Fig. 2B. Only conventional PCR on chromosomal DNA and RT-PCR on RNA templates resulted in products (Fig. 2B). This result shows that the four open reading frames are present on the same message and suggests the presence of a promoter upstream of *pyrR*.

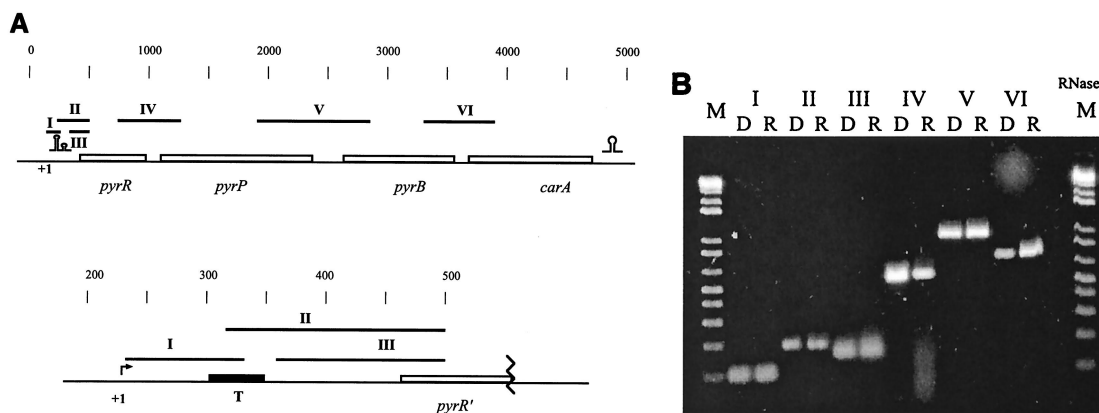


FIG. 2. RT-PCR analysis of the transcription products of the *pyrRPB-carA* region. (A) Genetic map with the expected PCR products. Black box (T), putative terminator in the attenuator. Omega-like and double-omega-like structures are as defined for Fig. 1. (B) Agarose gel electrophoresis of the products obtained by the PCRs. Lanes R, results of the RT-PCR amplifications using RNA extracted from *L. lactis*; lanes D, standard PCRs with chromosomal DNA using the same primers. The following primers were used: I, *PyrR*_F4 and *PyrR*_4R; II, *PyrB*_2F and *PyrR*_3R; III, *PyrR*_F5 and *PyrR*_3R; IV, *PyrB*_3F and *PyrR*_intR; V, *PyrP*_F2 and *PyrB*_12R; VI, *PyrB*_2 and *CarA*_R6. Marker (M), 100-bp DNA ladder; lane RNase, result of RT-PCR amplification with primers used in lanes VI after treatment of the RNA with RNase.

The presence of an operon structure was also demonstrated by data obtained from *in vivo* experiments. Plasmids conferring erythromycin resistance to the cell but unable to replicate in *L. lactis* carrying either the *pyrR* open reading frame without its promoter (pAM111) or an internal part of *pyrP* (pAM117) were transformed into *L. lactis* MG1363. By selecting for erythromycin resistance, strains in which the plasmids were integrated were readily obtained. The two strains were named MB411 and MB417, respectively (Fig. 1). If the genes are part of an operon, integration of the plasmids will disrupt transcription, thus resulting in polar mutations and no expression of *pyrB*. As expected the two mutants were found to have a uridine requirement. Thus, the finding obtained by RT-PCR, that *pyrB* is expressed by a promoter upstream of *pyrR*, was sustained by the uridine requirement of strains MB411 and MB417. Moreover, *pyrB* was shown to be unexpressed in these strains, since no aspartate transcarbamoylase activity could be detected in an enzyme assay performed using cell extracts obtained from strain MB411 or MB417.

Two determine whether *carA* is included in the operon, the growth requirements of strain MB422 were established. If *carA* is part of the operon, the integration of pAM122 would result in an operon disruption (Fig. 1), resulting in an arginine requirement (see below). In a growth experiment it was shown that arginine was required; thus the *carA* gene is no longer expressed in the mutant strains. In conclusion, the *in vivo* experiments confirm the existence of a four-gene operon with a promoter upstream of *pyrR*.

In order to map the precise location of the promoter, the 5' end of the transcript was determined by a primer extension experiment using primer PyrR_3R labeled with ^{32}P on RNA isolated from MG1363 grown in SA-glucose medium in the presence and absence of uracil. The result is presented in Fig. 3. A sequencing reaction with unlabeled PyrR_3R as the primer is included as a size marker. Since the extension product carries an additional charged phosphate compared to the product of the sequencing reaction, it migrates as if it was one nucleotide shorter. Hence, this experiment maps the first nucleotide to be transcribed (+1) to position 226 on the submitted sequence. This finding is supported by sequence analysis since a perfect consensus extended -10 sequence (TGCTATAAT) can be identified. In lactococcal promoters an extended -10 sequence is characterized by a TGN sequence adjacent to the -10 sequence (TATAAT) (42). Furthermore, spaced by 17 nucleotides upstream of the -10 sequence, a consensus -35 sequence (TTGACA) is present. Moreover, there seems to be more transcripts when the cells are grown in the absence of uracil. In order to quantify the amount of RNA, the radioactivity in each band was determined in an instant imager. A 2.9-fold increase in RNA was found when the cells were grown in the absence of RNA, suggesting that the expression of the operon is regulated at the level of transcription.

PyrR is the *pyr* regulatory protein. As previously discussed, in front of all pyrimidine biosynthetic genes of *L. lactis* identified so far, except for *pyrDa*, a putative PyrR binding site identical to the PyrR boxes in *B. subtilis* has been found. Moreover, the *pyr* leaders of *L. lactis* can be folded in a manner similar to that found in *B. subtilis* forming alternative antiterminator or terminator structures, which suggests the presence of a transcriptional attenuator immediately in front of the

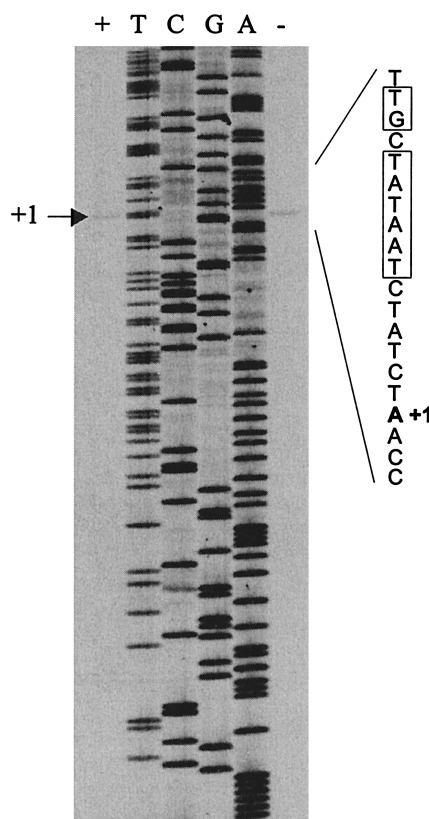


FIG. 3. Primer extension mapping of the 5' end of the *pyrRBP-carA* mRNA. Sequencing ladders generated with the oligonucleotide used for the primer extension were loaded next to the reaction mixture. The DNA sequence of the sense strand around the first nucleotide in the transcript (+1) is presented, and the -10 sequence is boxed. The experiment was conducted on RNA extracted from cells growing exponentially in the presence (+) and absence (-) of uracil at 200 $\mu\text{g/ml}$.

structural genes. These findings strongly predict the presence of a PyrR homologue in *L. lactis*. Indeed, the PyrR open reading frame shows extensive homology to the PyrR open reading frame of *B. subtilis*. In order to identify the gene encoding the regulator of pyrimidine biosynthetic gene expression, the following experiment was conducted. Strain MB36 has a partial pyrimidine requirement due to a disruption of the *carB* gene by an integrative plasmid. Moreover, the truncated *carB* allele is fused to a promoterless *lacLM* gene encoding β -galactosidase. It was previously shown that the expression of β -galactosidase in this strain is repressed by the addition of uracil to the growth medium (35). Plasmid pG⁺host8::ISS1 is a transposon delivery vector conferring tetracycline resistance to the cell and is unable to replicate at 37°C (30). Strain MB36 was transformed with plasmid pG⁺host8::ISS1, and a transposon-induced mutant library comprising several thousand integrants was obtained. In a previous study, we were able to show that *B. subtilis* carrying a deletion of its *pyrR* allele is resistant to the toxic pyrimidine analog 5-fluorouracil at a concentration of 1 $\mu\text{g/ml}$ (32). Therefore, strains were selected for growth in the presence of 5-fluorouracil and screened for high levels of β -galactosidase by plating on SA-glucose medium supplied with 5-fluorouracil (1 $\mu\text{g/ml}$) and 160 μg of X-Gal (5-bromo-

TABLE 4. Specific activities of the pyrimidine de novo biosynthetic enzymes in the *pyrR* mutant compared to wild-type *L. lactis*

Enzyme assayed ^c	Sp act ^a for strain (genotype ^d):			
	MG1363 (wt) in ^b :		MB401 (<i>pyrR</i> ::ISS1) in:	
	No	Uracil	No	Uracil
PyrB	38	19	ND ^e	ND
PyrC	26	11	53	67
PyrDa	22	17	25	20
PyrDb	36	18	50	44
PyrE	190	80	695	560
PyrF	77	36	203	201

^a Specific activity in crude extracts was determined as nanomoles/(minute · milligram of protein) at 30°C.

^b Cells were grown in defined medium in the absence (no) and presence (uracil) of uracil at a concentration of 200 µg/ml.

^c PyrB, aspartate transcarbamoylase; PyrC, dihydroorotase; PyrDa, dihydroorotate dehydrogenase A; PyrDb, dihydroorotate dehydrogenase B; PyrE, orotate phosphoribosyltransferase; PyrF, OMP decarboxylase.

^d wt, wild type.

^e ND, not determined.

4-chloro-3-indolyl-β-D-galactopyranoside)/ml. Two independent mutants with the expected phenotype were isolated, and their chromosomal DNA was extracted. Attempts to amplify the *pyrR* gene by PCR was unsuccessful, suggesting that the integration takes place in the *pyrR* gene (not shown). Furthermore, the presence of an ISS1 element in the *pyrR* gene was clearly shown in a Southern blot experiment (not shown). One of the strains was selected for further analysis and named MB401.

Together with wild-type strain MG1363, MB401 was propagated in the defined SA-glucose medium in the presence and absence of uracil. The activities encoded by the different pyrimidine biosynthetic genes were assayed, and the results are presented in Table 4. The results clearly demonstrate that, upon addition of uracil, the expression of the different genes becomes repressed between two- and fourfold in a wild-type background. An exception is *pyrDa*, which is not regulated by exogenous uracil. On the other hand, the repression vanishes in a strain carrying the *pyrR* mutation. Moreover, the expression of the individual pyrimidine biosynthetic genes is generally enhanced. These findings suggest that *pyrR* is the general regulator of expression of the pyrimidine biosynthetic genes in *L. lactis*.

The PyrR protein of *B. subtilis* has been shown to have the ability to catalyze the formation of UMP from uracil and PRPP, thus being a uracil phosphoribosyltransferase (UPRTase) as well as a regulatory protein. This activity did not seem to have physiological importance, since a true UPRTase encoded by *upp* was identified (32). To test whether the *L. lactis* PyrR protein has catalytic activity, plasmid pAM111 carrying the *L. lactis pyrR* gene expressed from an *E. coli lac* promoter was transformed into *E. coli* BM604 (*upp pyrF*). Transformants were tested on minimal medium in the presence of either uracil or uridine. As controls the previously cloned *L. lactis upp* gene (33) and vector pRC1 were included. The results showed that only the strain harboring the true *upp* gene is able to grow on uracil. Even after 7 days no growth on uracil of the strain harboring *pyrR* plasmid pAM111 was observed, showing that the *L. lactis pyrR* gene is unable to complement the *upp* defect

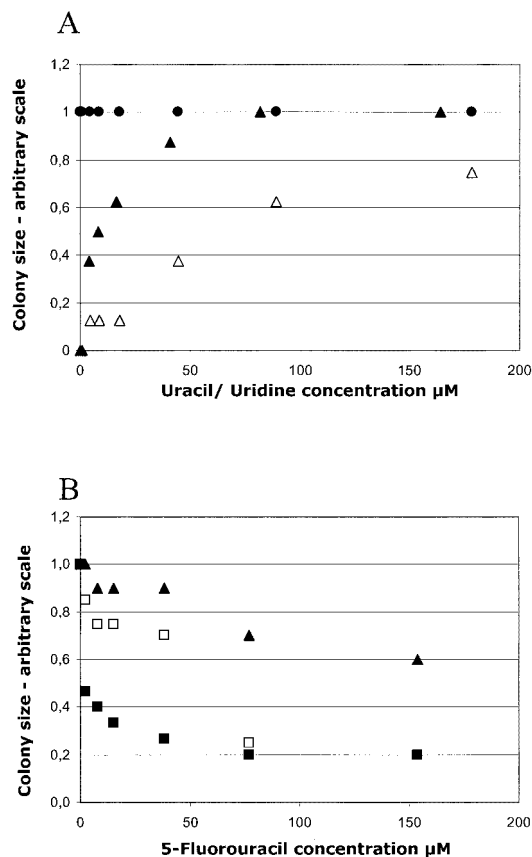


FIG. 4. Growth dependence of an *L. lactis pyrP* mutant on different pyrimidine compounds. The growth was monitored by determining colony size after 40 h of incubation at 30°C on SA-glucose plates with the indicated additions. The size of the largest colonies was arbitrarily set to 1. (A) Growth of MB417 (*pyrP pyrB*) on uracil (△) and uridine (▲). The *pyrF* mutant PSA001 gave the same result with uracil and uridine as MB417 with uridine. Wild-type strain MG1363 was included as a control (●). (B) Growth of MB417 on uridine (16.4 µM) and increasing concentrations of 5-fluorouracil (▲). As controls *pyrF* mutant PSA001 (■) and wild-type strain MG1363 (□) were included. All curves intercept the y axis at 1.0.

and suggesting that the *L. lactis* PyrR protein does not contain a UPRTase activity. Crude extracts from *E. coli* BM604 cells harboring either vector pRC1 or pAM111 were prepared and analyzed by sodium dodecyl sulfate polyacrylamide gel electrophoresis. A distinct band corresponding to the expected size of the PyrR protein could be identified only in cells harboring pAM111 (data not shown).

The *pyrP* gene encodes a uracil transporter. As previously stated PyrP shows extensive similarity to the uracil permease from *B. subtilis*, shown to be encoded by *pyrP* (41). To test whether *pyrP* from *L. lactis* encodes a uracil permease, strain MB417 (*pyrP*::pAM117) was tested on SA-glucose plates with increasing concentrations of uracil and uridine. This strain carries *pyrP* and has a pyrimidine requirement due to an interrupted transcription of the downstream genes, including *pyrB*. A wild-type strain and a strain carrying a mutated *pyrF* gene (PSA001) were included as controls. The results are presented in Fig. 4A, and it is clearly seen that a strain carrying a mutation in *pyrP* is unable to exploit uracil present at low

concentrations, whereas no effect is seen at higher concentrations.

Pyrimidine analog 5-fluorouracil is toxic to *L. lactis* only after entering the cell and after metabolic conversion to phosphorylated derivatives (34). Therefore, strain MB417 was tested for its sensitivity to 5-fluorouracil. Uridine at a low concentration (16 μ M) was used as the pyrimidine source in order to minimize competition between the drug and the required pyrimidine. As a control pyrimidine-requiring strain PSA001 (2) was used. At a concentration of 2 μ M 5-fluorouracil strain PSA001 is clearly affected whereas only a concentration beyond 50 μ M inhibited the growth of a strain carrying a defective *pyrP* allele (Fig. 4B). These findings define *pyrP* as the gene encoding the sole, high-affinity uracil transporter in *L. lactis*.

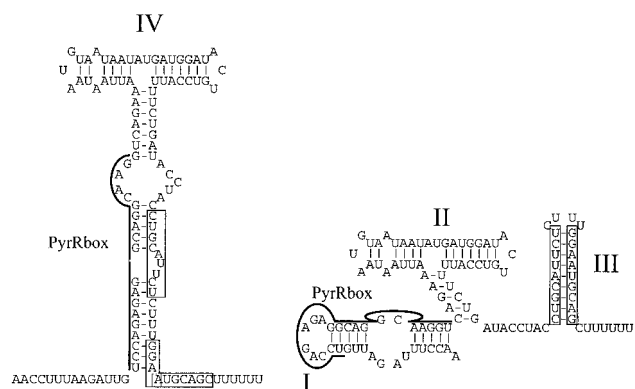
The *pyrB* allele of *L. lactis* MG1363 encodes a functional aspartate transcarbamoylase. In order to show the functional properties of the protein, a complementation experiment was conducted. The *pyrB* open reading frame was amplified from the chromosome of *L. lactis* MG1363 and cloned into *E. coli* vector pRC1, thus creating pAM122. Plasmid DNA was propagated in an *E. coli* wild-type strain and transformed into pyrimidine-requiring *E. coli* strain SØ990, which carries a defective *pyrB* allele. Transformed strains carrying either pAM122 or vector pRC1 were screened for their ability to grow in the absence of uracil, and only strains harboring pAM122 were able to grow, thus demonstrating that the *pyrB* allele of *L. lactis* cloned in this work complements the *pyrB* defect of SØ990.

The *carA* gene encodes the small subunit of CPSase. In a previous study it was concluded that, since no *carA* gene could be identified in the immediate proximity of the *carB* gene, it must be present somewhere else on the chromosome (35). Moreover it was shown that a *carB* mutation results in an arginine requirement, in accordance with CP being a precursor for both pyrimidine and arginine biosynthesis. In order to show that the *carA* gene actually encodes the small CPSase subunit, the ability of strain MB422 (*carA*) to grow in the absence of arginine was tested. Addition of arginine ensures the formation of CP for pyrimidine synthesis through the degradation of arginine by the arginine deiminase pathway (7). Together with MG1363 (wild type) and MB35 (*carB*), MB422 was plated on BIV-glucose plates without additional nutrients or BIV-glucose plates supplied with uracil, arginine, or uracil and arginine simultaneously. Strain MB422 had the same growth pattern as MB35, i.e., it requires arginine for growth, whereas uracil is unable to support growth of the strain. This finding suggests that *carA* is the sole gene encoding a functional small subunit in the CPSase of *L. lactis*.

DISCUSSION

Genetic organization of the pyrimidine biosynthetic genes.

The pyrimidine biosynthetic genes in *L. lactis* are scattered on the chromosome in at least five different transcriptional units. Four of these, *pyrKDbF* (2), *pyrDa* (1), *carB* (35), and *pyrRPB-carA* (this work), have been described, whereas it is presently unknown whether *pyrC* and *pyrE* are cotranscribed or are members of two different transcriptional units. The *carB* gene encoding the large subunit of CPSase is transcribed as a mono-



A: The antiterminator B: The terminator

FIG. 5. Sequence of the *pyrRPB-carB* attenuator. Boxes, sequences constituting the stems of the terminator. (A) *pyrRPB-carA* antiterminator including stem IV. (B) Structure of the terminator. Stem III indicates the putative terminator. PyrRbox (stem I), putative PyrR binding site.

cistronic message and does not have the same message as *carA*, as is normally found (35). This, in conjunction with the observation that *L. lactis* harbors two different *pyrD* alleles (1), makes *L. lactis* a unique organism with respect to the organization of the *pyr* genes.

L. lactis harbors only one *carA* gene. The first step in the pyrimidine biosynthetic pathway is the formation of CP catalyzed by the CPSase complex comprising large and small subunits encoded by *carB* and *carA*, respectively. The small subunit is responsible for the binding of glutamine and the transfer of its amide nitrogen group to an ammonia binding site on the large subunit. The other activities in the formation of CP from ammonia, bicarbonate, and ATP are located on the large subunit (8). CP is not only used in the biosynthesis of pyrimidines but is also required for arginine biosynthesis. In a previous paper we presented evidence for the presence of only one CPSase activity in *L. lactis*, since a *carB* mutant required arginine for growth (35). In the work presented here, we were able to show that a *carA* mutant also acquired arginine, thus supporting the theory that only one CPSase is present in *L. lactis*.

The expression of the *pyrRPB-carA* operon is regulated by pyrimidines. The presented data show that the *pyrRPB-carA* operon is regulated by the presence of uracil in the growth medium. At the enzymatic level aspartate transcarbamoylase (PyrB) is reduced twofold by the addition of uracil, whereas the mRNA level is repressed threefold as judged by primer extension. Note that the primer binds within the *pyrR* open reading frame. By analyzing the sequence of the mRNA leader a structure including a PyrR binding site similar to the one found in the *pyrKDbF* (2) and *carB* (35) leaders can be identified. 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 (14, 15, 25–28). The structures that may be formed by the RNA in the *carB* and *pyrKDbF* leaders in *L. lactis* are similar to the structures found in the RNA transcribed from the *B. subtilis* *pyr* operon. Figure 5 shows the two mutually exclusive structures believed to result in antitermination (stem IV) or termination

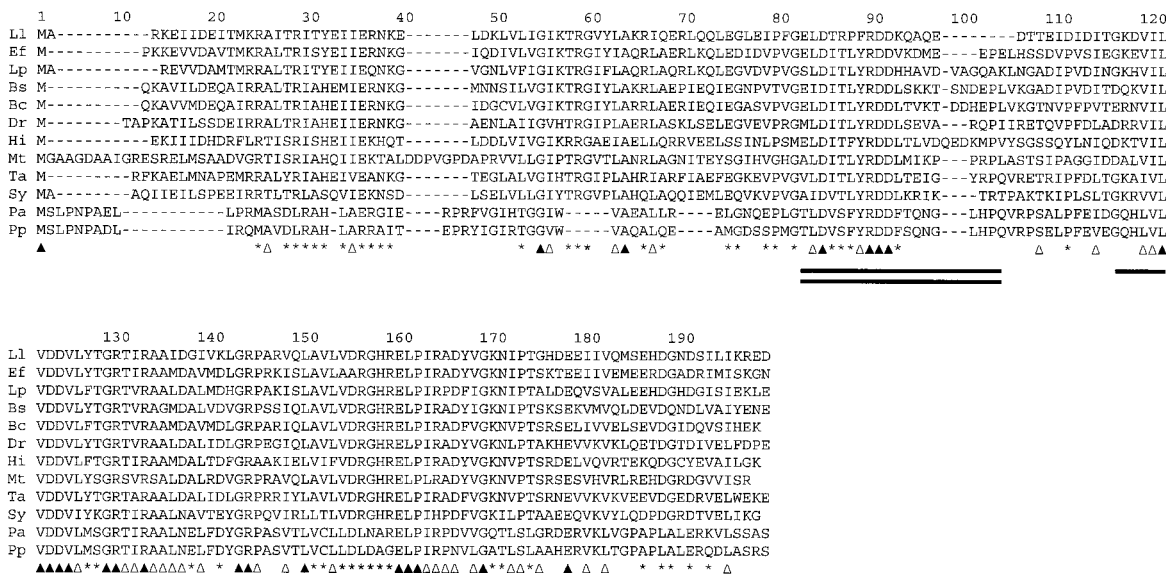


FIG. 6. Clustal W alignment of amino acid sequences deduced from *pyrR* homologues of *L. lactis* (Ll), *E. faecalis* (Ef), *L. plantarum* (Lp), *B. subtilis* (Bs), *B. caldolyticus* (Bc), *Deinococcus radiodurans* (Dr), *Haemophilus influenzae* (Hi), *Mycobacterium tuberculosis* (Mt), *Thermus aquaticus* (Ta), *Synechocystis* sp. (Sy), *Pseudomonas aeruginosa* (Pa), and *Pseudomonas putida* (Pp). The sequences were retrieved from protein databases after a Blast search using the search engine at the National Center for Biotechnology Information. Solid triangles, amino acid residues conserved in all sequences; open triangles, positions where only conservative substitutions have occurred; asterisks, positions where the amino acid residues are conserved in more than 66% of sequences; single line, putative binding site for PRPP; double line, flexible-loop domain.

at stem III. Although the potential structures that may be formed by the *pyrR* leader are somewhat different from those found in the other *pyr* attenuators in *L. lactis* and *B. subtilis*, the functionality is preserved. A potential functional terminator (stem III) can be identified, and stem-loop structure I, including the PyrR binding site (Fig. 5), is highly homologous to a similar structure found in the *B. subtilis pyr* operon designated the antiantiterminator by Lu and coworkers (28).

In this work we show that the uracil regulation of the *L. lactis pyr* genes is mediated by the PyrR protein encoded by the first open reading frame in the *pyrRPB-carA* operon. PyrR shows extensive homology to the PyrR protein from *B. subtilis*. Since PyrR binding sites identical to the ones found in *B. subtilis* (14) can be identified in the antiterminator structures of *L. lactis*, it is very likely that *pyr* gene expression is regulated by the same mechanism in *L. lactis* and *B. subtilis*.

The PyrR protein of *L. lactis* does not possess UPRTase activity. Unlike the finding for *B. subtilis* (32), the PyrR protein of *L. lactis* was shown not to encode UPRTase activity. The Blast search using the *L. lactis* PyrR amino acid sequence as the query revealed the presence of a class of proteins with a size of 170 to 193 amino acid residues with the highest similarity to PyrR from *L. lactis*. The different sequences are aligned in Fig. 6. The overall similarity among the different PyrR proteins is extended over the entire sequence, but especially the C-terminal part is highly conserved (Fig. 6). This part includes the binding site for PRPP. The most-pronounced deviation of the *L. lactis* PyrR sequence from those of the other PyrRs is the eight-amino-acid-residue deletion around amino acid residue 80 of the *L. lactis* PyrR sequence, corresponding to position 100 of the arbitrary scale in Fig. 6. The PyrR protein from *E. faecalis* has only a four-amino-acid-residue

deletion at the same position (Fig. 6), and it has been shown that the PyrR protein from *E. faecalis* retains its catalytic activity (12). Moreover, the amino acid residues immediately before the 8-bp deletion (Fig. 6) are less conserved in the *L. lactis pyrR*. Assuming that the three-dimensional structure of PyrR from *L. lactis* is similar to that of PyrR from *B. subtilis*, this region is part of the “phosphoribosyltransferase flexible loop,” important for enzyme activity (40). Moreover, flexible-loop motif LDITLYRDD, which is fully conserved in *B. subtilis*, *B. caldolyticus*, and *E. faecalis*, in which the enzymatic activity is known to be retained, is changed in *L. lactis* to LDTRPFRDD. Whether this change accounts for the loss of enzyme activity remains to be solved.

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.

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