JOURNAL OF BACTERIOLOGY, May 1993, p. 2541–2551
0021-9193/93/092541-11$02.00/0

Vol. 175, No. 9

Identification of a Novel Operon in *Lactococcus lactis* Encoding Three Enzymes for Lactic Acid Synthesis: Phosphofructokinase, Pyruvate Kinase, and Lactate Dehydrogenase

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Received 10 December 1992/Accepted 24 February 1993

The discovery of a novel multicistronic operon that encodes phosphofructokinase, pyruvate kinase, and lactate dehydrogenase in the lactic acid bacterium *Lactococcus lactis* is reported. The three genes in the operon, designated *pfk*, *pyk*, and *ldh*, contain 340, 502, and 325 codons, respectively. The intergenic distances are 87 bp between *pfk* and *pyk* and 117 bp between *pyk* and *ldh*. Plasmids containing *pfk* and *pyk* conferred phosphofructokinase and pyruvate kinase activity, respectively, on their host. The identity of *ldh* was established previously by the same approach (R. M. Llanos, A. J. Hillier, and B. E. Davidson, J. Bacteriol. 174:6956–6964, 1992). Each of the genes is preceded by a potential ribosome binding site. The operon is expressed in a 4.1-kb transcript. The 5′ end of the transcript was determined to be a G nucleotide positioned 81 bp upstream from the *pfk* start codon. The pattern of codon usage within the operon is highly biased, with 11 unused amino acid codons. This degree of bias suggests that the operon is highly expressed. The three proteins encoded on the operon are key enzymes in the Embden-Meyerhoff pathway, the central pathway of energy production and lactic acid synthesis in *L. lactis*. For this reason, we have called the operon the *las* (lactic acid synthesis) operon.

The conversion of carbohydrate to lactic acid is the major energy-producing pathway in many bacteria. With the lactic acid bacteria, the conversion is the basis of a variety of food fermentations that yield products with valuable nutritional and organoleptic properties. One of the best studied of the lactic acid bacteria is *Lactococcus lactis*, which converts lactose to lactic acid during the manufacture of cheese. Analyses of the biochemical regulation of lactic acid synthesis in this organism indicate that phosphoenolpyruvate, fructose-1,6-bisphosphate, and inorganic phosphate act as important metabolic controllers (43). The activity of pyruvate kinase, which converts phosphoenolpyruvate and ADP to pyruvate and ATP, is of critical importance, since it competes directly for phosphoenolpyruvate with the sugar phosphotransferase uptake system. The activities of pyruvate kinase and lactate dehydrogenase are increased markedly by fructose-1,6-bisphosphate, the product of phosphofructokinase (1, 9, 43). Thus, the activities of pyruvate kinase, phosphofructokinase, and lactate dehydrogenase are interdependent and have a major effect on the rates of energy production and lactic acid synthesis in this organism.

Significant progress with the analysis of plasmid-borne genes required for lactic acid synthesis in *L. lactis* has been made in recent years (13, 46). These genes encode proteins responsible for the uptake of lactose as lactose-6-phosphate, its hydrolysis to glucose and galactose-6-phosphate, and the conversion of galactose-6-phosphate via tagatose-6-phosphate to triose phosphates (8). Genes required for the uptake of glucose and its catabolism to lactic acid must be chromosomally encoded, since plasmid-free strains of *L. lactis* can grow on glucose as the sole carbon source (14).

We recently reported the cloning and molecular analysis of the gene (*ldh*) encoding fructose-1,6-bisphosphate-activated L-(+)-lactate dehydrogenase (EC 1.1.1.27), the enzyme responsible for the reduction of pyruvate to lactate (27). A single copy of the gene was found to be present in the lactococcal chromosome. Interestingly, we discovered that *ldh* is part of a 4.1-kbp transcriptional unit. In this article, we present evidence that the other components of the transcriptional unit are genes encoding pyruvate kinase (EC 2.7.1.40) and phosphofructokinase (EC 2.7.1.11). These genes have been designated *pyk* and *pfk*, respectively. The order of the genes in the 5′-to-3′ direction is *pfk pyk ldh*. Because of the important roles in lactic acid synthesis of the enzymes encoded by these three genes, we have called the transcriptional unit the *las* (lactic acid synthesis) operon.

MATERIALS AND METHODS

Bacterial strains, plasmids, phages, and media. The bacterial strains, plasmids, and phages used in this study are listed in Table 1. Unless specified otherwise, *L. lactis* is used throughout to indicate *L. lactis* subsp. *lactis* LM0230. Bacterial cultures were grown as described previously (27). When necessary, media were supplemented with tetracycline (12.5 μg/ml), kanamycin (50 μg/ml), or other antibiotics (27).

DNA techniques. DNA isolations, digestions, electrophoresis, Southern transfers, hybridizations, and DNA sequence determinations using an Applied Biosystems model 373A automated DNA sequencer were performed as described previously (27). When the DNA sequence was derived exclusively from cloned polymerase chain reaction

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TABLE 1. Bacterial strains, plasmids, and phages used in this study

Strain, plasmid, or phage	Characteristics[a]	Source or reference
Strains		
L. lactis subsp. *lactis* LM0230	Plasmid-free derivative of *L. lactis* subsp. *lactis* C2	15
E. coli		
JM107	*endA1 gyrA96 thi-1 hsdR17 supE44 relA1* Δ(*lac-proAB*) (F′ *traD36 proAB lacI*^{q}ZΔM15)	50
SURE	e14$^-$(*mcrA*) Δ(*mcrCB-hsdSMR-mrr*)*171 sbcC recB recJ umuC*::Tn*5*(Km^r) *uvrC supE44 lac gyrA96 relA1 thi-1 endA1*[F′ *proAB lacI*^{q}ZΔM15 Tn*10*(Tc^r)]	Stratagene
Plasmids		
pJDC9	Em^r Δ*lacZ*	5
pUC19	Ap^r Δ*lacZ*	50
pMG36e	Em^r, 3.611-kbp *L. lactis-E. coli* expression vector	24
pMU2901	Em^r, pJDC9 with a 6.0-kbp *Eco*RI fragment from *L. lactis*	27
pMU2908	Em^r, pJDC9 with a 4.8-kbp *Dra*I fragment from λ632 containing 2.54 kbp of *L. lactis* DNA and 2.3 kbp of the right arm of λGEM-11	This study
pMU2909	Em^r, pJDC9 with a 2.05-kbp *Dra*I-*Sac*I region of *L. lactis* DNA containing *pyk*	This study
pMU2910	Em^r, pMU2909 with the *Dra*I-*Sac*I fragment in reverse orientation	This study
pMU2916	Em^r, pMG36e with *pfk* on a 1.4-kbp *Eco*RI fragment made by PCR amplification of *L. lactis* DNA	This study
pMU2917 to pMU2920	Ap^r, pUC19 with *pfk* on a 1.138-kbp *Vsp*I fragment made by PCR amplification of *L. lactis* DNA	This study
pMU2921	Em^r, pJDC9 with 476 bp of *pfk* prepared by PCR amplification of *L. lactis* DNA	This study
pMU2928 to pMU2931	Ap^r, pUC19 with a 431-bp *Vsp*I-*Cla*I fragment made by PCR amplification of *L. lactis* DNA	This study
Phages		
M13mp19		50
λ632	λGEM-11 with a 15-kbp partial *Sau*3AI fragment from *L. lactis* in the *Xho*I site	27

[a] Em^r, Ap^r, Tc^r, and Km^r, resistance to erythromycin, ampicillin, tetracycline, and kanamycin, respectively.

(PCR) products, four different isolates were sequenced to avoid errors introduced by *Taq* polymerase during the PCR. Oligonucleotides used as primers for sequencing and PCR (Table 2) were synthesized in an Applied Biosystems model 381A DNA synthesizer. The locations of hybridization probes are shown in Fig. 1.

DNA sequence analysis. DNA sequences were compiled, analyzed, and compared with data bases by using the ANGIS suite of programs on the University of Sydney SUN computer. The suite includes the Staden (40) and University of Wisconsin Genetics Computer Group programs.

Amplification of DNA by the PCR. The DNA template was present in 100 μl (final volume) of 10 mM Tris-HCl buffer (pH 9.0 at 25°C) containing 50 mM KCl, 1.5 mM $MgCl_2$, 0.01% (wt/vol) gelatin, 0.1% (vol/vol) Triton X-100, deoxyribonucleoside triphosphates (200 μM each), oligonucleotide amplimers (1 μM each), and 2 U of *Taq* DNA polymerase (Perkin-Elmer Cetus, Norwalk, Conn.). The reaction was carried out in a Corbett Research FTS-1C thermal cycler as follows: cycle 1, 95°C for 3 min, 40 to 50°C for 1 min, and 72°C for 2 min; cycles 2 to 40, 95°C for 40 s, 40 to 50°C for 1 min, and 72°C for 2 min; cycle 41, 95°C for 40 s, 40 to 50°C

TABLE 2. Oligonucleotides used as primers for sequencing, PCR, and probe synthesis

Oligonucleotide	Sequence	Position in sequence and/or comment[a]
RL3	GCAAGGGCTGGGAAAGG	1875–1859 (probe C)
RL9	AAACGCATAATTAGGGC	1224–1240 (probe C)
RL10	TCTGCCCTAATTATGCG	1243–1227 (probe D)
RL12	GGTATCGCTGCTGGTGC	710–726 (probe D)
RL13	CCATGCCCGCATATCCG	302–286, from sequence of pMU2921
RL23	CTCTAGAGGATCCCCGGG	From multiple cloning site of M13mp19
RL24	CATTTAAATCTGTCCTCCG	172–154 (probe H)
RL25	GGTCATATCATAGAAGAG	From sequence of a PCR product (Fig. 1) (probe H)
RL26	GGTCCAAGAGTTGAGACG	1317–1300
RL31	GGAATTCATATCATAGAAGAG	RL25 with *Eco*RI site at 5′ end
RL32	GGAATTCCAAGAGTTGAGACG	RL26 with *Eco*RI site at 5′ end
RL33	TTGCGAACAACTGCACGAATAGCC	246–223
RL34	GG(TCAG)ATGAA(TC)GC(TCAG)GC(TCAG)(GA)T	Encodes GMNAA(I/V)
RL35	(CG)(AC)(AG)T(AG)(AG)CG(AT)CCCAT(AT)ACTTC	Encodes EVMGR(Y/H)(C/A) on inverse complement strand[b]

[a] The numbering in base pairs is the same as that in Fig. 1. Probes synthesized with the use of the corresponding oligonucleotides are indicated in parentheses.
[b] The oligonucleotide does not contain some of the codons with C or G in the third position so as to limit the extent of redundancy.

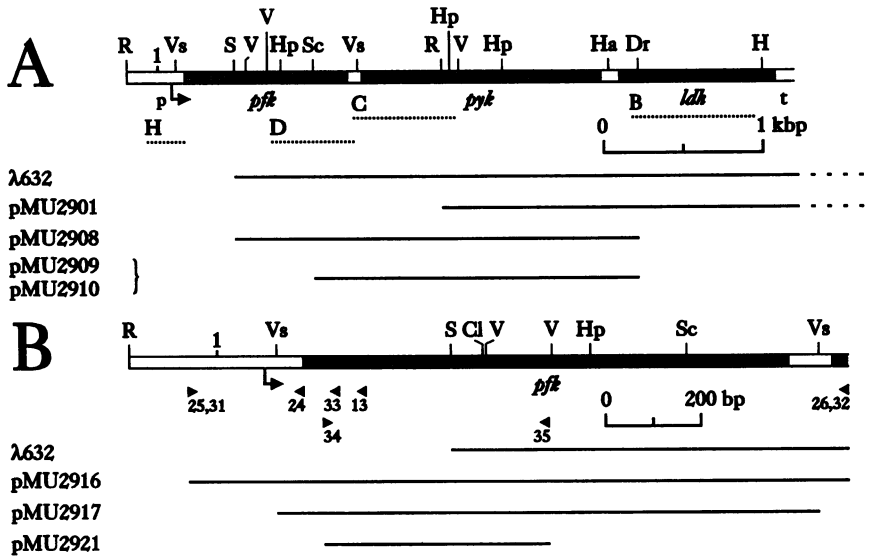

FIG. 1. (A) Restriction map of the *L. lactis las* operon; (B) restriction map of *pfk* and flanking DNA. All of the observed sites are shown for *Eco*RI (R), *Sac*I (Sc), and *Cla*I (Cl), and only relevant sites are shown for *Vsp*I (Vs), *Sau*3AI (S), *Eco*RV (V), *Hpa*I (Hp), *Hae*III (Ha), *Dra*I (Dr), and *Hin*dIII (H). The solid lines under the maps indicate regions that were cloned to yield plasmids (Table 1), the dashed lines indicate fragments that were used as hybridization probes B, C, D, and H, and the arrowheads indicate oligonucleotides (Table 2) used as primers in PCR and primer extension experiments. p and t, *las* promoter and terminator, respectively; 1, the first nucleotide in the sequence shown in Fig. 2; right-angled arrows, start points of transcription.

for 1 min, and 72°C for 10 min. The temperature of the second step in each cycle depended upon the size and base composition of the amplimers.

Amplification of the 5′ end of *pfk* by PCR. Samples of *Eco*RI digests of *L. lactis* DNA (200 ng) and M13mp19 DNA (25 ng) in 10 μl were incubated with DNA ligase in ligation buffer (50 mM Tris-HCl [pH 7.5], 10 mM $MgCl_2$, 10 mM dithiothreitol, 1 mM ATP, and 50 μg of bovine serum albumin per ml) at 4°C for 16 h and then heated to 65°C for 15 min to inactivate the ligase. Samples (1 μl each) of this mixture were amplified by the PCR as described above.

Construction of plasmids. For the construction of pMU2921 (Fig. 1), the PCR product obtained by using *L. lactis* DNA as a template and RL34 and RL35 (Table 2) as amplimers was gel purified and then incubated at 37°C for 30 min with 2.5 U of T4 DNA polymerase and 2 U of *Escherichia coli* DNA polymerase (Klenow fragment) in 50 mM Tris-HCl buffer (pH 8.0) containing 7 mM $MgCl_2$, 1 mM dithiothreitol, 0.4 mg of bovine serum albumin per ml, and deoxyribonucleoside triphosphates (100 μM each) to generate blunt ends. The DNA was extracted with phenol and cloned in *Sma*I-digested pJDC9 by standard procedures. For the construction of pMU2916 (Fig. 1), the PCR product obtained by using *L. lactis* DNA, RL31, and RL32 (Table 2) was digested with *Eco*RI and ligated with *Eco*RI-digested pMG36e. The ligation mix was transformed into *E. coli* SURE (Stratagene, La Jolla, Calif.), and a transformant that carried the desired insert was identified by colony hybridization with probe H (Fig. 1). For the construction of pMU2917 (Fig. 1), the PCR product obtained by using *L. lactis* DNA, RL25, and RL26 (Table 2) was digested with *Vsp*I and ligated with *Sma*I-digested pUC19. The ligation mix was transformed into *E. coli* SURE, and transformants that carried the desired insert were identified by colony hybridization with probe D (Fig. 1 and Table 2). Four of these (pMU2917 to pMU2920) were chosen for sequence analyses.

Primer extension analysis. Total RNAs were purified from *L. lactis* as described previously (27). The oligonucleotide primer (500 ng of RL24 or RL33 [Table 2]) was annealed with 20 μg of RNA in 3 μl of water by incubating at 90°C for 1 min and then at 42°C for 5 min. Radiolabeled cDNA was synthesized by using [α-^{32}P]dATP by a modification (4) of the procedure of Hudson and Davidson (23). The reaction was terminated by the addition of 5 μl of 95% (vol/vol) formamide containing 20 mM EDTA, 0.05% (wt/vol) bromophenol blue, and 0.05% (wt/vol) Xylene cyanol FF. The products were analyzed on a 6% (wt/vol) polyacrylamide gel containing 7 M urea and detected by autoradiography. The products of the four different dideoxy sequencing reactions obtained with the *fmol* DNA sequencing system (Promega, Madison, Wis.) using pMU2916 as a template and oligonucleotide RL24 or RL33 as a primer served as size standards on the gel.

Pyruvate kinase assays. *E. coli* cultures used for pyruvate kinase assays were grown to an A_{550} of 0.6 to 0.8 in minimal medium (half-strength medium 56 [31]) containing 50 mM acetate as the sole carbon source in the presence or absence of 200 μM isopropyl-β-D-thiogalactopyranoside (IPTG). Cells were washed by centrifugation, resuspended in 5 mM sodium phosphate buffer (pH 7.5), and sonicated to yield a cell extract. Pyruvate kinase activity was measured as described previously (7) in a final volume of 1 ml. Unit activities were calculated from the increase in the rate of NADH oxidation following the addition of the final assay component, phosphoenolpyruvate. One unit of enzyme activity was defined as the amount of enzyme which catalyzed the conversion of 1 μmol of substrate to product per min under the reaction conditions used. To calculate specific activity (in units per milligram of protein), protein concentrations of cell extracts were determined by using the BCA protein assay reagent (Pierce Chemical Co., Rockford, Ill.).

Phosphofructokinase assays. *E. coli* cultures used for phosphofructokinase assays were grown in 2YT medium (30). *L.*

lactis cultures were grown in M17 medium (42) supplemented with 0.5% (wt/vol) glucose. Cells were harvested at an A_{550} of 0.6 to 0.8, washed by centrifugation in 20 mM Tris-HCl (pH 7.5), resuspended in 50 mM Tris-HCl buffer (pH 7.5) containing 10 mM 2-mercaptoethanol, 20% (vol/vol) glycerol, 5 mM $MgSO_4$, and 5 mM EDTA, and disrupted by sonication. Immediately prior to sonication, *L. lactis* cells were incubated with lysozyme (final concentration, 1 mg/ml) for 10 min at 37°C. Phosphofructokinase activity was measured as described previously (17). Unit activities (defined as above) were calculated from the increase in the rate of NADH oxidation following the addition of the final assay component, fructose-6-phosphate. Phosphofructokinase specific activities of *L. lactis* cell extracts were expressed in units per milliliter of culture with an A_{550} of 1.0, because the addition of lysozyme during extract preparation prevented the accurate determination of the lactococcal protein content.

Nucleotide sequence accession number. The sequence of the *las* operon (*pfk* [this study], *pyk* [this study], and *ldh* [27]) from *L. lactis* has been assigned accession number L07920 in the GenBank data base.

RESULTS

Nucleotide sequence of *pyk*. In a previous publication, we reported the isolation of a recombinant λ clone (λ632) carrying the *L. lactis* gene *ldh* and the determination of the *ldh* nucleotide sequence (27). A functioning transcription terminator in the 3′-flanking DNA of *ldh* was detected, but the 5′-flanking DNA was found to be promoterless (Fig. 1). We also found an open reading frame (ORF) in the same orientation as *ldh* that terminated 117 bp upstream of *ldh* (27). We have now determined the nucleotide sequence of this ORF using plasmids pMU2901 and pMU2908 and subclones of them (Fig. 2). The ORF contained 511 codons, and comparison of its amino acid sequence with sequences in the GenBank data base using the FASTA program (26) revealed significant homology with the sequence of *E. coli* pyruvate kinase (Fig. 3). We will refer to the gene in this ORF as *pyk* in view of the observation (see below) that it encoded pyruvate kinase. We concluded that the first ATG codon in the ORF, codon 10, was the most likely site of translation initiation, because it was preceded by a putative ribosome binding site and because this choice maximized homology with the N terminus of the *E. coli* enzyme (Fig. 3). By using this initiation site, the nucleotide sequence predicted a chain length of 502 amino acids and a subunit molecular weight of 54,285 for *L. lactis* pyruvate kinase, assuming no posttranslational cleavage at the N-terminal end. A subunit molecular weight of 59,000 to 62,000 has been reported for this enzyme (9). Sequencing upstream of *pyk* revealed another, colinear ORF that extended to the *Sau*3AI site (bp 480) marking the end of the lactococcal DNA in λ632 (Fig. 2). This ORF was separated from *pyk* by 87 bp. Comparison of its predicted amino acid sequence with sequences in the GenBank data base revealed significant homology with the sequences of various phosphofructokinases.

Cloning and nucleotide sequence of *pfk*. While the sequence of *pyk* was being determined, a lactococcal probe for the gene encoding phosphofructokinase (*pfk*) was isolated by the following strategy. The published amino acid sequences of phosphofructokinases from *E. coli* (22), *Bacillus stearothermophilus* (18), rabbits (35), and *Homo sapiens* (37) were aligned, and two regions of conserved sequence, GMNAA (I/V) and EVMGR(Y/H)(C/A), were selected for PCR primer design. In *B. stearothermophilus*, these regions were separated by 143 residues. Redundant oligonucleotides (RL34 and RL35; Table 2) that encoded these sequences were synthesized and used as PCR primers to amplify lactococcal DNA. Gel electrophoresis showed that the reaction yielded three products, of 800, 450, and 200 bp, in approximately equivalent amounts. The 450-bp fragment, which was closest to the expected size, was extracted from the gel and cloned in pJDC9 to yield a plasmid designated pMU2921 (see Materials and Methods). The nucleotide sequence of the cloned DNA was determined (data not shown). Its encoded amino acid sequence exhibited 65% identity with the sequence of the equivalent portion of the *B. stearothermophilus* phosphofructokinase, providing good evidence that it had been generated by amplification of *L. lactis pfk*. The nucleotide sequence at one end of the pMU2921 insert was the same as that at the end of the lactococcal DNA in λ632 (bp 480 to 687 in Fig. 2). We interpreted this and the sequence data described above to indicate that the ORF 5′ to *pyk* in λ632 was *pfk* and that pMU2921 contained lactococcal DNA extending beyond the *Sau*3AI site at the end of λ632 (bp 480 in Fig. 2).

Screening of a lactococcal gene library of 780 clones in λGEM-11 (27) with pMU2921 as the probe failed to yield a clone that extended past the *Sau*3AI site delimiting the lactococcal insert in λ632. This library had been used by us to isolate a number of different genes. Hybridization of Southern blots of gel separations of *Eco*RI-digested *L. lactis* DNA with probe D (Fig. 1 and Table 2) indicated that the DNA of interest was contained within a 2.0-kbp *Eco*RI fragment. Attempts to clone this fragment directly in plasmids from *Eco*RI digests of *L. lactis* DNA were unsuccessful, even after using gel electrophoresis to increase the proportion of fragments of the appropriate size. In an alternative approach, PCR was used to analyze this region of the chromosome. The PCR template was a ligated mixture of *Eco*RI-digested DNA from M13mp19 and *L. lactis* (see Materials and Methods), and the amplimers were RL23, which was complementary to the M13mp19 DNA adjacent to the *Eco*RI site (Table 2), and RL13 (Fig. 1). The reaction yielded a DNA fragment of the size expected (525 bp) from amplification of molecules in which the 2.0-kbp *Eco*RI fragment had been ligated into the multiple cloning site of M13mp19. Attempts to clone the 525-bp fragment were unsuccessful, but direct sequencing of it with RL13 as a primer produced a sequence identical to that of portion of pMU2921 (leftward from bp 286 in Fig. 2). This observation established the identity of the 525-bp fragment. Direct sequencing of it from the other end with RL23 as a primer yielded the sequence of the *L. lactis* chromosome near the *Eco*RI site. By using this information, oligonucleotides RL25 and RL31 were synthesized and then used in conjunction with RL26 and RL32, respectively, for PCR amplification of *L. lactis* DNA. The products obtained with these amplifications were used to construct the *pfk*-containing plasmids pMU2916 to pMU2920 (see Materials and Methods), which were then used for determination of the complete nucleotide sequence of *pfk* and its 5′-flanking DNA and for expression studies. Isolation of pMU2916 suggested that the region of DNA which caused problems in isolating molecular clones was located in the 150 to 160 bp between the left end of the pMU2916 insert and the *Eco*RI site (Fig. 1). The reason for the cloning difficulties was not investigated.

The ORF containing *pfk* consisted of 340 codons. The predicted amino acid sequence of the *L. lactis* phosphofructokinase is homologous with other phosphofructokinase

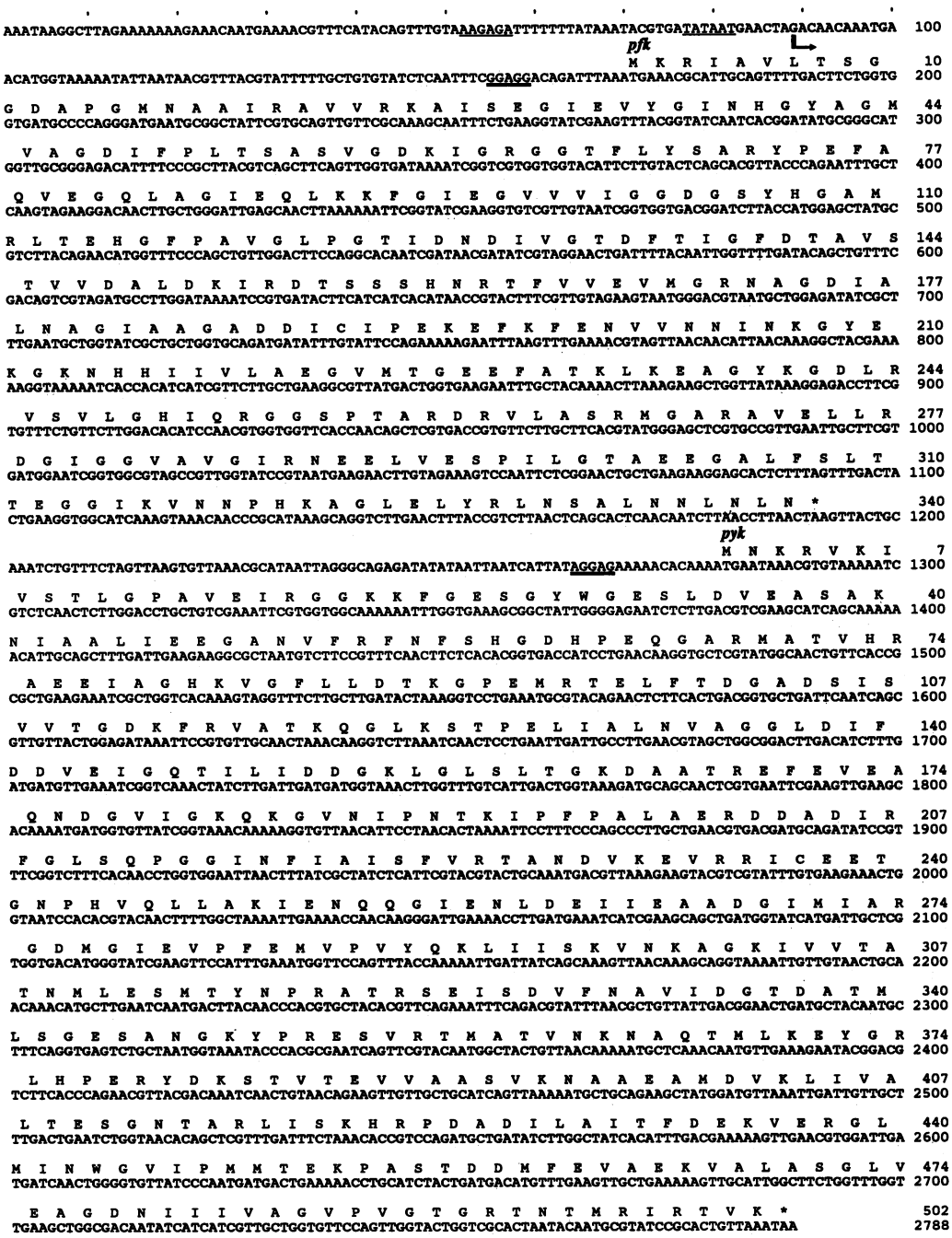

FIG. 2. Nucleotide sequence of *pfk*, *pyk*, and flanking DNA and deduced amino acid sequences of phosphofructokinase and pyruvate kinase from *L. lactis*. Right-angled arrow, start point of transcription determined by primer extension mapping. Likely −35 and −10 hexamers of the promoter upstream from the start point of transcription are underlined with thin lines, and putative ribosome binding sites, which have calculated free energies of −14.4, −11.6, and −14 kcal/mol (−61, −49, and −59 kJ/mol, respectively) (44) for their interactions with the sequence 3′-UCUUUCCUCCA-5′ at the 3′ end of *L. lactis* 16S rRNA (28), are underlined with thick lines. The sequence shown abuts directly to the sequence reported previously (27). *, stop codons.

sequences, especially with that of *B. stearothermophilus* (Fig. 4). We concluded that the first ATG codon in the ORF was the most likely site of translation initiation for reasons similar to those outlined above for *pyk*. Assuming this initiation site and no posttranslational modification, phosphofructokinase was predicted to contain 340 amino acids (Fig. 2) and have a molecular weight of 35,805. The latter value is in agreement with the experimentally determined subunit molecular weight of 33,500 ± 3,500 (17).

Expression of *L. lactis pyk* in *E. coli*. The predicted amino acid sequence provided strong evidence that *pyk* encoded pyruvate kinase. To establish this point, we looked for expression of pyruvate kinase activity in *E. coli* cells carrying *pyk*$^+$ plasmids. The two plasmids used, pMU2909 and

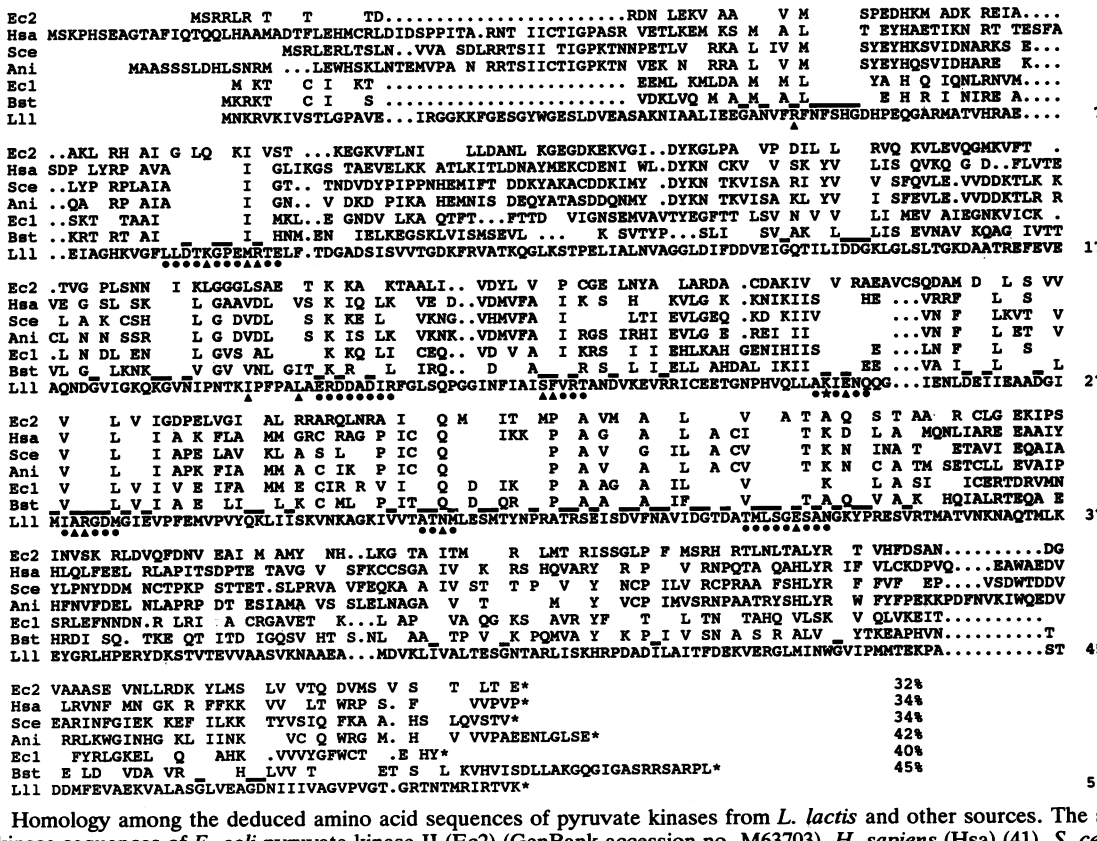

FIG. 3. Homology among the deduced amino acid sequences of pyruvate kinases from *L. lactis* and other sources. The sequences are pyruvate kinase sequences of *E. coli* pyruvate kinase II (Ec2) (GenBank accession no. M63703), *H. sapiens* (Hsa) (41), *S. cerevisiae* (Sce) (3), *Aspergillus nidulans* (Ani) (12), *E. coli* pyruvate kinase I (Ec1) (33), *B. stearothermophilus* (Bst) (47), and *L. lactis* (Lll) (this study). The first amino acid for each pyruvate kinase is the N-terminal amino acid. Spaces indicate positions where sequences are identical to that of *L. lactis*. Amino acids conserved in all of the sequences are overlined in the *L. lactis* sequence. The C-terminal extremity of each sequence (asterisk) and the percentage of identity between each sequence and the *L. lactis* sequence are indicated at the bottom. Symbols below the *L. lactis* sequence indicate amino acids proposed, on the basis of X-ray crystallographic studies with the cat enzyme (32), to have the following properties: residues close to the active site (circles), residues involved in bonding with substrates (triangles), lysine that acts as the acid-base catalyst responsible for the interconversion of pyruvate and enolpyruvate (star).

pMU2910, contained the same region of *L. lactis* DNA (Fig. 1) inserted in opposite orientations in the vector pJDC9. Cells were grown in minimal medium containing acetate as the sole carbon source, because the expression of *E. coli* pyruvate kinase is decreased under these conditions (48). The pyruvate kinase activity of *E. coli* cell extracts was elevated when the cells carried either pMU2909 or pMU2910 (Table 3). The activity conferred by pMU2909, but not by pMU2910, was increased ninefold more by the presence of IPTG in the growth medium. The orientation of *pyk* in pMU2909 would have allowed expression of *pyk* from the inducible *lac* promoter in pJDC9. These observations established that the lactococcal DNA within pMU2909 encoded pyruvate kinase.

Expression studies with *L. lactis pfk* in *E. coli* and *L. lactis*. Expression of enzyme activity was also used as the criterion for establishing that *pfk* encoded phosphofructokinase. Whereas the phosphofructokinase activities of cell extracts of *E. coli* JM107 and *E. coli* JM107(pMU2916) were indistinguishable (data not shown), the presence of pMU2916 in *L. lactis* caused the phosphofructokinase activity of cell extracts to increase from 34 to 180 mU/ml of culture with an A_{550} of 1.0.

Codon usage in the *las* operon. Codon usage in the *las* operon exhibits a strong bias towards certain codons and away from others (Table 4). Thus, 11 amino acid codons are not used in this operon. This bias is most pronounced in the Arg codons, among which only two of the available six are used. While the low G+C content (37%) of *L. lactis* DNA may contribute to nonusage of the seven unused codons which have either C or G in the third position, it cannot be the reason for either the nonusage of the other four or the preference for T compared with A in the third position of the Leu, Ile, and Arg codons.

Copy number of *pyk* and *pfk* in the *L. lactis* chromosome. *Eco*RI, *Hin*dIII, *Hae*III, *Eco*RV, and *Hpa*I digests of *L. lactis* DNA were separated by electrophoresis through agarose (Fig. 5A), and a Southern blot was prepared from the gel. Hybridization of the blot with a ^{32}P-labeled *pyk* probe (probe C; Fig. 1 and Table 2) yielded a single hybridizing band in each lane (Fig. 5B). The measured sizes (in kilobase pairs; expected sizes on the basis of the data in Fig. 2 are given in parentheses) of the hybridizing fragments were as follows: *Eco*RI, 2.05 (2.05 and 6.0); *Hin*dIII, 4.8 (>4.0); *Hae*III, 3.65 (>3.0); *Eco*RV, 1.2 (1.2); *Hpa*I, 1.09 (1.06 and 0.33). These observations indicated the presence of a single copy of *pyk* in *L. lactis*. The failure of the 6.0-kbp *Eco*RI and 0.33-kbp *Hpa*I fragments to hybridize was ascribed to the small extent of the overlap of probe C with each of these fragments.

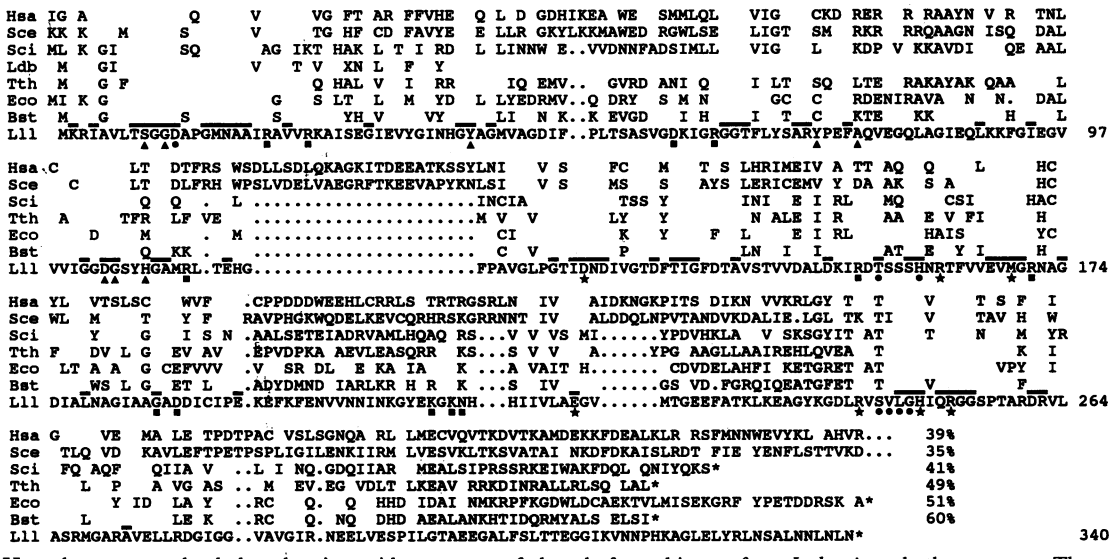

FIG. 4. Homology among the deduced amino acid sequences of phosphofructokinases from *L. lactis* and other sources. The sequences are phosphofructokinase sequences of *H. sapiens* (Hsa) (37), *S. cerevisiae* α-subunit (Sce) (21), *S. citri* (Sci) (6), *Lactobacillus delbrueckii* subsp. *bulgaricus* (Ldb) (25), *Thermus thermophilus* (Tth) (49), *E. coli* phosphofructokinase 1 (Eco) (22), *B. stearothermophilus* (Bst) (18), and *L. lactis* (Lll) (this study). The first amino acids shown for the *H. sapiens* and *S. cerevisiae* enzymes are residues 14 and 204 in their respective published sequences, and only the first 38 residues have been reported for *L. delbrueckii*. Symbols below the *L. lactis* sequence indicate amino acids, identified in X-ray crystallographic studies with the *B. stearothermophilus* and *E. coli* enzymes (16, 22, 36), that are involved in the following functions: fructose-6-phosphate binding (A site) (stars), ATP binding (B site) (triangles), effector binding (C site) (squares), and subunit interfaces (circles). For other details, see the legend to Fig. 3.

Hybridization of the blot with a ^{32}P-labeled *pfk* probe (probe D; Fig. 1 and Table 2) also yielded a single hybridizing band for each digest (Fig. 5C). The sizes of these fragments (in kilobase pairs, with expected sizes in parentheses) were as follows: *Eco*RI, 2.05 (2.05); *Hin*dIII, 4.8 (>4.0); *Hae*III, 3.65 (>3.0); *Eco*RV, 1.2 (1.2); *Hpa*I, 1.09 (1.06 and >0.97). These data indicated the presence of a single copy of *pfk* in *L. lactis*.

Determination of the 5′ end of the *las* operon transcript by primer extension analysis. Primer extension from oligonucleotide RL33 (Fig. 1) using *L. lactis* RNA as a template yielded one major cDNA product, with minor amounts of shorter products (Fig. 6). The mobility of the major product was identical to that of the product in the C track, corresponding to bp 89 in Fig. 2. We concluded that the G at bp 89 was at the 5′ end of the *las* operon transcript. Analysis of the primer extension product from oligonucleotide RL24 (data not shown) led to the same conclusion.

DISCUSSION

In *L. lactis*, phosphofructokinase, pyruvate kinase, and lactate dehydrogenase are key enzymes in the central pathway of energy production, the conversion of carbohydrates to lactic acid. Each enzyme is a target for regulation by metabolites (1, 17, 43), and the levels of their activities play a major role in establishing the rates of energy synthesis, growth, and lactic acid production for the organism. Investigations described above and those reported previously (27) indicate that genes encoding these enzymes are located adjacently to each other, in the order *pfk pyk ldh*, within a 4.0-kbp section of the *L. lactis* chromosome. Hybridization of *pfk*-, *pyk*-, and *ldh*-specific probes with Southern blots of DNA digests gave results indicative of a single chromosomal copy of each gene, although in each case the presence of one or more additional copies having little or no homology with the probe would have been undetected by this approach. In this regard, it is relevant to note that *E. coli* has two phosphofructokinase genes (11, 22) which exhibit no sequence homology (22) and two pyruvate kinase genes (19, 34) that are approximately 40% identical (Fig. 3).

TABLE 3. Pyruvate kinase activities of cell extracts of *E. coli* JM107 carrying different plasmids

Plasmid	Sp act (U/mg)	
	Without IPTG	With 200 μM IPTG
None	0.38	0.27
pJDC9	0.38	0.32
pMU2909	1.8	16.7
pMU2910	1.2	1.33

A number of observations provide clear evidence that the three genes form a single transcriptional unit. First, specific probes for *ldh*, *pyk*, and *pfk* (probes B, C, and D; Fig. 1) hybridized with a 4.1-kb transcript on Northern (RNA) blots of *L. lactis* RNA (27). Second, an efficient transcription terminator in the DNA immediately 3′ to *ldh* was identified (27). This DNA contains two palindromes, each characteristic of a rho-independent transcription terminator. Third, the 5′ end of a transcript has been mapped to the G nucleotide situated 81 bp 5′ of *pfk*. A transcript that was initiated at this G and terminated at one of the two palindromes would be 3.85 or 3.91 kb long, depending on which of the above-mentioned palindromes was the site of termination. In view of the central role of these three enzymes in the synthesis of lactic acid in *L. lactis*, we have called the transcriptional unit the *las* (lactic acid synthesis) operon.

To our knowledge, the presence of a multicistronic operon containing these genes has not been reported for another

TABLE 4. Codon usage in *L. lactis* genes

Codon	Amino acid	No. of codons used in[a]: *las* operon	Chromosomal genes[b]	*lac* operon[c]
UUU	F	21 (47)	367 (78)	52 (50)
UUC	F	24 (53)	101 (22)	53 (50)
UUA	L	1 (1)	300 (30)	24 (13)
UUG	L	31 (38)	246 (25)	56 (31)
CUU	L	43 (53)	254 (26)	73 (41)
CUC	L	6 (7)	65 (7)	10 (6)
CUA	L	0 (0)	63 (6)	14 (8)
CUG	L	0 (0)	57 (6)	1 (1)
AUU	I	37 (43)	589 (69)	75 (56)
AUC	I	49 (57)	187 (22)	54 (41)
AUA	I	0 (0)	80 (9)	4 (3)
AUG	M	28 (100)	236 (100)	50 (100)
GUU	V	70 (65)	351 (50)	81 (46)
GUC	V	7 (7)	120 (17)	29 (16)
GUA	V	29 (27)	147 (21)	50 (28)
GUG	V	1 (1)	88 (12)	16 (9)
UCU	S	18 (30)	170 (26)	27 (35)
UCC	S	0 (0)	30 (5)	1 (1)
UCA	S	35 (57)	206 (31)	46 (59)
UCG	S	1 (2)	21 (3)	4 (5)
CCU	P	11 (31)	117 (33)	27 (36)
CCC	P	0 (0)	30 (9)	1 (1)
CCA	P	23 (64)	172 (49)	40 (53)
CCG	P	2 (6)	33 (9)	8 (11)
ACU	T	41 (65)	236 (40)	40 (36)
ACC	T	0 (0)	56 (10)	9 (8)
ACA	T	21 (33)	224 (38)	52 (46)
ACG	T	1 (2)	67 (11)	11 (10)
GCU	A	75 (59)	362 (42)	88 (46)
GCC	A	9 (7)	108 (12)	21 (11)
GCA	A	40 (31)	321 (37)	69 (36)
GCG	A	3 (2)	81 (9)	13 (7)
UAU	Y	4 (17)	288 (71)	57 (66)
UAC	Y	20 (83)	120 (29)	30 (34)
UAA	*[d]	3 (100)	22 (69)	6 (86)
UAG	*	0 (0)	5 (16)	1 (14)
CAU	H	7 (33)	172 (74)	24 (52)
CAC	H	14 (67)	60 (26)	22 (48)
CAA	Q	28 (100)	365 (85)	42 (84)
CAG	Q	0 (0)	66 (15)	8 (16)
AAU	N	19 (15)	428 (78)	55 (58)
AAC	N	35 (65)	124 (22)	40 (42)
AAA	K	68 (96)	681 (83)	114 (78)
AAG	K	3 (4)	139 (17)	32 (22)
GAU	D	43 (60)	517 (76)	92 (71)
GAC	D	29 (40)	159 (24)	37 (29)
GAA	E	83 (97)	714 (86)	129 (83)
GAG	E	3 (3)	119 (14)	26 (17)
UGU	C	2 (100)	68 (84)	13 (87)
UGC	C	0 (0)	13 (16)	2 (13)
UGA	*	0 (0)	5 (16)	0 (0)
UGG	W	5 (100)	85 (100)	22 (100)
CGU	R	47 (89)	152 (38)	32 (58)
CGC	R	6 (11)	27 (7)	9 (16)
CGA	R	0 (0)	61 (15)	7 (13)
CGG	R	0 (0)	29 (7)	0 (0)
AGU	S	2 (3)	173 (26)	17 (17)
AGC	S	5 (8)	62 (9)	6 (6)
AGA	R	0 (0)	110 (28)	6 (11)
AGG	R	0 (0)	19 (5)	1 (2)
GGU	G	73 (62)	274 (34)	98 (59)
GGC	G	11 (9)	115 (14)	14 (8)
GGA	G	28 (24)	340 (42)	46 (28)
GGG	G	5 (4)	73 (9)	9 (5)
Total		1,170	11,070	2,096

[a] Percent of all codons used for the amino acid is given in parentheses.
[b] On the basis of the nucleotide sequences for the following *L. lactis* chromosomal genes in the GenBank data base (accession no. in parentheses): *his* (M90760), *leu* (M90761), and *ilv* (M90761) operons; *recA* (M88106); *pepN* (M65867); *adk*, *infA*, *rpmJ*, and *rpsM* (X59250); *lap* (X61230); *usp45* (M35374); *thyA* (M33770); *pepXP* (M8315); *pabB* (M64860); and *rpmG* (X62621).
[c] On the basis of the nucleotide sequences for *lacABCDFEG* (13, 46).
[d] *, stop.

bacterium. In *E. coli*, the equivalent genes, *pfkA*, *pfkB*, *pykF*, *pykA*, and *lct* (lactate dehydrogenase), are unlinked (2, 19, 22). A recent report described a chromosomal DNA fragment from the mollicute *Spiroplasma citri* as having a *pfk* homolog alongside the 5′ end of a *pyk* homolog (6). The 3′ end of the *pyk* homolog was not present on the fragment. It was suggested that these two genes belonged to a 4-kb transcript. In *B. stearothermophilus*, the 3′ end of a *pfk* homolog has been found 26 bp upstream of *pyk* (47). These data encourage the viewpoint that homolog of the *las* operon are present in other bacteria, at least those in the gram-positive lineage and its mollicute side branch (29, 39).

Expression of the three genes from a single promoter in *L. lactis* raises the interesting possibility that they are subjected to coordinated regulation at the transcriptional level. At present, we have no information on factors that might modulate transcription of the *las* operon or, indeed, whether it is modulated at all. Given the fact that lactococcal promoters also function in *E. coli* (45), the observation that a plasmid (pMU2916) containing the *las* promoter and *pfk* expressed elevated phosphofructokinase levels in *L. lactis* but not in *E. coli* raises the possibility that a factor required for transcription from this promoter was not present in the *E.*

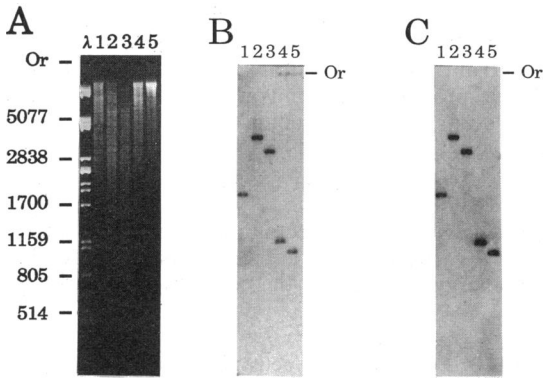

FIG. 5. Electrophoretic separation of restriction digests of *L. lactis* DNA. (A) DNA was digested with *Eco*RI (lane 1), *Hin*dIII (lane 2), *Hae*III (lane 3), *Eco*RV (lane 4), or *Hpa*I (lane 5), separated by electrophoresis through 1.0% (wt/vol) agarose, and then visualized by staining with ethidium bromide. The numbers on the left show the sizes (in base pairs) of size markers produced by *Pst*I digestion of λ DNA (lane λ). Or, location of the origin. (B and C) Southern blot of the gel shown in panel A after hybridization with ^{32}P-labeled probe C or D, respectively (Fig. 1).

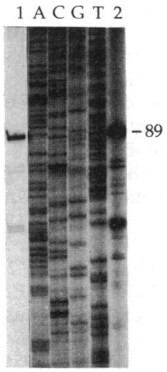

FIG. 6. Primer extension analysis of the 5′ end of the *las* operon. Lanes 1 and 2, autoradiographs (2 and 16 h, respectively) showing electrophoretic separation of samples of primer extension products after oligonucleotide RL33 (Table 2) was annealed with *L. lactis* RNA; lanes A, C, G, and T, electrophoretic separation of the products of dideoxynucleotide sequencing using oligonucleotide RL33 as a primer and plasmid pMU2916 as a template. The number on the right refers to the nucleotide sequence numbering in Fig. 1.

coli host. On the other hand, mRNA instability and/or poor translation initiation in *E. coli* can equally well explain this observation. Experiments to distinguish between these possibilities and to explore the genetic regulation of the *las* operon are in progress in our laboratory.

The three enzymes encoded by the operon have been purified from *L. lactis* C10 (9, 10, 17). The phosphofructokinase and pyruvate kinase preparations were homogeneous or nearly so, while the lactate dehydrogenase contained small amounts of contaminants. Using the reported specific activities of the purified enzymes (assuming 50% purity for the lactate dehydrogenase), we calculated the numbers of picomoles of enzyme subunit per milligram of protein in the original cell extracts to be 75, 300, and 140 for phosphofructokinase, pyruvate kinase, and lactate dehydrogenase, respectively. The magnitudes of these values suggest that the rates of expression of the individual genes of the *las* operon are dissimilar, but not greatly so, although direct experimental comparisons are obviously required to verify this suggestion. Different rates of translational initiation on a single transcript could provide fine tuning in the relative amounts of the three enzymes. All three ATG start codons have a good putative ribosome binding site similar distances upstream (Fig. 2) (27). Prediction of the secondary structures in the RNA around each site (51) revealed no thermodynamically significant structure in which the sites for either *pfk* or *pyk* were blocked. However, the *pyk-ldh* intergenic region (the 187 nucleotides downstream from bp 2738 in Fig. 2) was predicted to form a structure (ΔG = −33.4 kcal/mol [−141 kJ/mol]) in which the *ldh*-binding site base paired with the nucleotides immediately downstream of the *pyk* stop codon. Formation of this structure in vivo would block *ldh* translation initiation. Conversely, movement of a translating ribosome through the 3′ end of *pyk* would disrupt the base pairing and enable the initiation of *ldh* translation. The end result of such a mechanism would be that the rate of synthesis of lactate dehydrogenase would not exceed that of pyruvate kinase.

Codon usage in the three *las* operon genes was found to exhibit a strong bias (Table 4). Biased codon usage in *E. coli* and *Saccharomyces cerevisiae* genes is well documented (20, 38). In those organisms, a positive correlation between the degree of codon bias and the level of gene expression has been demonstrated; the most highly expressed genes exhibit the greatest degree of synonymous codon bias. Little information concerning codon usage in *L. lactis* is available. A compilation of codon usage patterns in *L. lactis* genes was made by van de Guchte et al. (45), but the available data base was too small to enable any conclusions to be reached. Sequences for 27 *L. lactis* chromosomal genes, containing a total of 11,070 codons, have now been reported in the GenBank data base. Analysis of the codon usage pattern in these 27 genes reveals that it is significantly less biased than that in the *las* operon (Table 4). The presence of the strong bias in the *las* operon, therefore, argues that *pfk*, *pyk*, and *ldh* are highly expressed genes. In view of the central role of the *las* gene products in energy metabolism in *L. lactis*, this is not surprising. On the other hand, the genes of the plasmid-borne *lac* operon, which also encode a number of the proteins required for the utilization of lactose as an energy source, do not exhibit the same amount of bias. The pattern of codon usage in the *las* operon may, therefore, be the best available paradigm for the codon usage pattern in highly expressed *L. lactis* chromosomal genes.

The three-dimensional structure of the *B. stearothermophilus* phosphofructokinase in both conformational states (the R and T states) has been determined, and amino acids involved in the binding of ligands and subunit interactions have been identified (16, 22, 36). The homology with the *B. stearothermophilus* enzyme enables these amino acids to be recognized readily in the *L. lactis* phosphofructokinase, since the majority are identical in the two enzymes (Fig. 4). Three differences in the residues in the ATP-binding site are unlikely to have a significant effect on the binding properties of the enzyme. They involve Cys-73 in *B. stearothermophilus* (Tyr-73 in *L. lactis*), which interacts through the main-chain amide and carbonyl groups, Lys-77 (Ala-77 in *L. lactis*), which makes hydrophobic contacts, and Gln-107 (His-107 in *L. lactis*), which possibly makes an H bond (22). There are four changes in residues at the effector-binding site, the site for binding of ADP: Lys-111 (Arg-111 in *L. lactis*), which makes hydrophobic contacts, Glu-187 (Asp-187 in *L. lactis*), in which the carboxyl H-bonds with Mg^{2+}, Arg-211 (Lys-211 in *L. lactis*), which makes H bonds to α-P and adenine N7, and Lys-214 (Asn-214 in *L. lactis*), which interacts through the main-chain amide with a ribose hydroxyl. Whether these substitutions change the effector-mediated properties of the *L. lactis* enzyme is not known.

The predicted amino acid sequence of the *L. lactis* pyruvate kinase is also homologous to the sequences of pyruvate kinases from other organisms (Fig. 3). Elucidation of the three-dimensional structure of cat muscle pyruvate kinase enabled Muirhead et al. to identify amino acids close to the active site and to propose the probable involvement of some of them in binding (32). Many of these amino acids are conserved in the *L. lactis* pyruvate kinase (Fig. 3). The lysine residue suggested to act as an acid-base catalyst in the active site (Lys-250) is also conserved.

ACKNOWLEDGMENTS

We are grateful to Phil Arnold for synthesizing oligonucleotides.

R.M.L. is the recipient of a Postgraduate Studentship from the Dairy Research and Development Corporation. This study was supported financially by the Australian Research Council and the Dairy Research and Development Corporation.

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