Cloning, Nucleotide Sequence, Expression, and Chromosomal Location of ldh, the Gene Encoding L-(+)-Lactate Dehydrogenase, from Lactococcus lactis

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A gene (designated ldh) that encodes fructose-1,6-bisphosphate-activated L-(+)-lactate dehydrogenase was cloned from Lactococcus lactis subsp. lactis. Plasmids containing ldh conferred fructose-1,6-bisphosphate-activated L-(+)-lactate dehydrogenase activity on Escherichia coli cells. This activity was conferred only when a promoter had been introduced into the plasmid to express the cloned ldh. The nucleotide sequence of ldh predicted a chain length of 324 amino acids and a subunit molecular weight of 34,910 for the enzyme, after removal of the N-terminal methionine residue. Northern analyses of L. lactis subsp. lactis RNA showed that a 4.1-kb transcript hybridized strongly with ldh and that 1.2- and 1.1-kb transcripts hybridized to much lesser extents. Promoter- and terminator-cloning studies in which we used the vectors pGKV210 and pGKV259 in L. lactis subsp. lactis revealed that the 5' flanking DNA of ldh is devoid of transcription initiation signals and that transcription entering the 3' flanking DNA from either direction is efficiently terminated. These data and the data from Northern analyses led to the conclusion that ldh is expressed as the 3' gene of the 4.1-kb transcript and suggested that posttranscriptional processing yielded the shorter transcripts. We determined that ldh is located on the L. lactis subsp. lactis chromosome between coordinates 1.619 and 1.669 of the previously reported physical map (D. L. Tulloch, L. R. Finch, A. J. Hillier, and B. E. Davidson, J. Bacteriol. 173:2768-2775, 1991).

Although the synthesis of lactic acid is the basis of many important food fermentations, the molecular genetics of bacterial lactic acid synthesis is poorly understood. Lactic acid bacteria that are used to carry out these fermentations include members of the genera Lactococcus, Lactobacillus, Streptococcus, Pediococcus, Leuconostoc, and Propionibacterium. Of these bacteria, the best-studied species in terms of lactic acid synthesis is Lactococcus lactis, which is used to ferment lactose to lactic acid in the manufacture of cheese. In recent years significant progress has been made in the genetic analysis of this organism.

The uptake of lactose and its conversion to lactic acid are essential for normal growth of L. lactis in milk since this process constitutes the major metabolic pathway for energy production. Lactose is brought into a cell by the lactose phosphotransferase system (21, 35) and is then converted by phospho-β-galactosidase to glucose and galactose 6-phosphate. The glucose is metabolized along the Embden-Meyerhoff pathway, and the galactose 6-phosphate is converted via the tagatose-6-phosphate pathway (11) to triose phosphates, which enter the Embden-Meyerhoff pathway. Studies on the regulation of these pathways have shown that phosphoenolpyruvate, fructose 1,6-bisphosphate (FBP), and inorganic phosphate are important in controlling the flux of metabolites by modulating the activity of certain enzymes (48). The role of phosphoenolpyruvate is pivotal, since it is used for ATP (energy) synthesis or to supply energy directly to the phosphotransferase system for the uptake of lactose from the medium. The utilization of phosphoenolpyruvate for ATP production generates lactic acid and requires the action of both pyruvate kinase and L-(+)-lactate dehydrogenase (LDH). Both of these enzymes are regulated positively by the intracellular level of FBP, the product of phosphofructokinase. Therefore, the activities of these three enzymes are interdependent.

Some of the enzymes used in lactic acid synthesis are known to be plasmid encoded. These include two components of the phosphotransferase system, enzyme IIac and enzyme IIIac, phospho-β-galactosidase, and the enzymes of the tagatose-6-phosphate pathway (53, 54). A considerable amount of research has been devoted to the plasmid biology of lacticocci, and this research has resulted in the cloning and characterization of the genes encoding the enzymes mentioned above (12, 52-54). The genes that encode enzymes required for the import of glucose and the Embden-Meyerhoff pathway are believed to be chromosomally located, since plasmid-cured strains of L. lactis subsp. lactis are able to ferment glucose (14). There have been no reports of the cloning of any of these genes, and the mechanisms of their regulation are not known.

As part of a program to study the genetic regulation of lactic acid synthesis in bacterial fermentations, we undertook the isolation of the gene that encodes the FBP-dependent LDH (EC:1.1.1.27) from L. lactis subsp. lactis LM0230. In this paper we describe the structure of this gene, designated ldh, the chromosomal location of ldh, and the expression of ldh in a 4.1-kb transcript.

MATERIALS AND METHODS

Bacterial strains, plasmids, phases, and media. The bacterial strains, plasmids, and phases used in this study are listed in Table 1. L. lactis was grown in M17 medium (47) supple-
TABLE 1. Bacterial strains, plasmids, and phages used in this study

<table>
<thead>
<tr>
<th>Strain, plasmid, or phage</th>
<th>Characteristics*</th>
<th>Reference or source</th>
</tr>
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<tbody>
<tr>
<td>L. lactis subsp. lactis LM0230</td>
<td>Plasmid-free derivative of L. lactis subsp. lactis C2</td>
<td>15</td>
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<td>L. lactis subsp. cremoris US3</td>
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<td>E. coli strains</td>
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<td>pJDC9</td>
<td>Em′ ΔlacZ</td>
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<tr>
<td>pET3</td>
<td>Ap′; pBR322 containing phage T7 promoter ϕ10</td>
<td>38</td>
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<tr>
<td>pGKV210</td>
<td>Em′; 4.49-kbp promoter-detecting vector</td>
<td>26</td>
</tr>
<tr>
<td>pGKV209</td>
<td>Em′; Cm′; 5.026-kbp terminator-detecting vector</td>
<td>26</td>
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<td>pMU2901</td>
<td>Em′; pJDC9 with a 6.0-kbp EcoRI fragment from L. lactis subsp. lactis LM0230</td>
<td>This study</td>
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<td>pMU2902</td>
<td>Em′; pJDC9 with a 1.32-kbp HaeIII fragment containing ldh from pMU2901</td>
<td>This study</td>
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<td>pMU2903</td>
<td>Em′; pMU2902 with the T7 ϕ10 promoter inserted into the BamHI site, 75 bp 5′ to the ldh start codon</td>
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<td>pMU2904</td>
<td>Em′; pGKV210 with a 621-bp HpaI fragment from pMU2901 in the SalI site</td>
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<td>Em′; same as pMU2904, but with the HpaI fragment in the reverse orientation</td>
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<td>pMU2906</td>
<td>Em′; pGKV259 with a 286-bp PsiI fragment from pMU2901 in the PsiI site</td>
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<td>pMU2907</td>
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<td>Phages</td>
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<td>λGEM-11</td>
<td>λEMBL3 with additional cloning sites forSacI, XhoI, AvrII, XbaI, and SfiI</td>
<td>Promega</td>
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<tr>
<td>A632</td>
<td>λGEM-11 with a 15-kbp partial Sau3AI fragment from L. lactis subsp. lactis LM0230 in the XhoI site</td>
<td>This study</td>
</tr>
</tbody>
</table>

* Em′, Ap′, and Cm′ indicate resistance to erythromycin, ampicillin, and chloramphenicol, respectively.

** BRL, Bethesda Research Laboratories, Gaithersburg, Md.

* See reference 39.

mented with 0.5% (wt/vol) glucose at 30°C without shaking. Escherichia coli was grown in Luria-Bertani medium (39) or in 2YT medium (33) at 37°C with shaking. Antibiotics were used at the following concentrations: erythromycin, 200 μg/ml for E. coli and 5 μg/ml for L. lactis; chloramphenicol, 5 to 200 μg/ml for E. coli and 5 to 20 μg/ml for L. lactis; and ampicillin, 50 μg/ml.

** DNA purification and library construction.** Plasmid DNA from E. coli was purified by using the alkaline lysis method (5) and was then purified on minicolumns (Qiagen, Studio City, Calif.). Plasmid DNA from L. lactis was isolated as described previously (3). Phage DNA was isolated by using the procedure of Helms et al. (20). L. lactis chromosomal DNA was isolated from crude cell lysates (29) as described by Marmur (32). To prepare a lactococcal gene library, L. lactis subsp. lactis LM0230 chromosomal DNA was subjected to partial Sau3AI digestion, and the 15- to 23-kbp fragments were isolated by agarose gel electrophoresis and cloned in λGEM-11, using the Packagene system and purified XhoI half-site arms of the vector (Promega, Madison, Wis.). A library of 780 randomly chosen clones was established by picking pure plaques and storing them individually at 4°C in 50 mM Tris-HCl buffer (pH 7.5) containing 100 mM NaCl, 8 mM MgSO4, and 0.01% (wt/vol) gelatin.

** DNA techniques.** Digestions, electrophoresis, and other DNA manipulations were performed as described elsewhere (39, 50). E. coli cells were transformed by using the method of Hanahan (18), and L. lactis cells were transformed by electroporation (36). Nucleotide sequences were determined by the chain termination method (40), using plasmid DNA as the template, Taq DNA polymerase (Promega), and fluorescent-dye-coupled primers or terminators. The reaction products were analyzed with a model 373A automated DNA sequencer (Applied Biosystems, Foster City, Calif.). All of the sequence reported below was determined for both DNA strands. Oligonucleotides were synthesized in an Applied Biosystems model 381A DNA synthesizer.

** Amplification of DNA by the PCR.** L. lactis subsp. cremoris US3 chromosomal DNA was amplified by the polymerase chain reaction (PCR), using 1 μg of DNA template in 100 μl (final volume) of 10 mM Tris-HCl buffer (pH 8.3 at 25°C) containing 50 mM KCl, 1.5 mM MgCl2, 0.01% (wt/vol) gelatin, deoxyribonucleoside triphosphates (200 μM each), 20 μM oligonucleotide N-LDH, 10 μM oligonucleotide C-LDH, and 2.5 U of Taq DNA polymerase (Perkin Elmer Cetus, Norwalk, Conn.) [oligonucleotide N-LDH was 5′-CA (A/G)GG(A/T/C/G)GA(T/C)GC(A/T/C/G)GA(A/G)GA(T/C) CT-3′, and oligonucleotide C-LDH was 5′-CC(A/G)TA(T/C) TG(A/T/C/G)CC(A/G)TC(T/C)TG(A/G)AA-3′]. The reaction was carried out for 25 cycles in a Hybaid Intelligent heating block as follows: cycle 1, 94°C for 90 s, 37°C for 2 min, and 72°C for 2 min; cycle 2, 94°C for 1 min, 43°C for 2 min, and 72°C for 2 min; cycles 3 to 24, 94°C for 1 min, 50°C for 2 min, and 72°C for 2 min; and cycle 25, 94°C for 1 min, 50°C for 2 min, and 72°C for 13 min. Control mixtures in which template DNA or one of the two primers was absent were incubated at the same time.

** RNA isolation.** Total RNAs were purified from E. coli by using the method of Aiba et al. (1) and from L. lactis by using a modification (6) of the procedure of van Roojen and de Vos (52).

** Transfers and hybridizations.** Phage plaques and bacterial colonies were transferred to Hybond-N+ nylon membranes (Amersham, Amersham, United Kingdom) according to the manufacturer’s instructions. Southern blots of agarose gels containing DNA fragments were prepared with a Vacu gene...
apparatus (Pharmacia, Uppsala, Sweden) by using 0.4 M NaOH as the transfer solution (37). Northern blots of electrophoresed RNA were prepared by using Hybond-N+ membranes and standard procedures (39). Sizes of transcripts were estimated by comparing band mobilities with the band mobilities of standards in an RNA ladder (0.24 to 9.3 kb; Bethesda Research Laboratories, Gaithersburg, Md.). Membrane-bound nucleic acids were hybridized with 32P-labeled, alkali-denatured probes that were prepared by random priming (17). Hybridizations were carried out at 60°C for 18 h in a solution containing 5 × SSPE (1 × SSPE is 150 mM NaCl, 9.7 mM sodium phosphate, and 1 mM EDTA [pH 7.4] [39]), 5 × Denhardt’s solution (39), and 0.5% (wt/vol) sodium dodecyl sulfate. Final washing of the membrane was done at 65°C with 1 × SSC (1 × SSC is 150 mM NaCl plus 15 mM sodium citrate [39]) containing 0.1% (wt/vol) sodium dodecyl sulfate.

Enzyme assays. The cultures used for chloramphenicol acetyltransferase (CAT) assays were grown to an A550 of 0.4. The cells were washed by centrifugation and resuspended in 50 mM Tris-HCl (pH 7.8) containing 30 μM dithiothreitol and then were treated with lysozyme (5 μg/ml) for 10 min at 37°C and sonicated to yield a cell extract. CAT activity was measured by using the method of Shaw (41). Cultures that were used for LDH assays were grown to an A550 of 1.0. The cells were washed by centrifugation and resuspended in 10 mM sodium phosphate buffer (pH 7.0) and then were sonicated to yield a cell extract. L. lactis cells were treated with lysozyme as described above immediately prior to sonication. LDH activity was measured as described previously (22). Unit activities were calculated from the increase in the rate of NADH oxidation following the addition of the final assay component, FBP. The protein contents of cell extracts were determined by using the BCA protein assay reagent (Pierce Chemical Co., Rockford, Ill.). One unit of enzyme activity was defined as the quantity of enzyme which catalyzed the conversion of 1 μmol of substrate to product per min under the reaction conditions used.

**RESULTS**

Cloning of ldh. Amino acid sequences have been reported previously for some of the tryptic peptides of the FBP-activated LDH from L. lactis subsp. cremoris US3 (10, 43). We used homology between these sequences and the amino acid sequence of Bacillus steaerotherophilus LDH (4) to determine the probable location of the peptides in the polyepitope chain of L. lactis LDH. The resulting information was used to design degenerate 20-mer oligonucleotide primers for the preparation of a specific lactococcal DNA probe by PCR amplification of L. lactis subsp. cremoris US3 chromosomal DNA. These primers, designated N-LDH and C-LDH (see Materials and Methods), encoded the amino acid sequences QGDAEDL and FQDGQYG, respectively. The PCR amplification yielded a single DNA fragment of 670 bp, the size expected from the amino acid sequence data. The fragment was not produced in control reaction mixtures that contained no lactococcal DNA or that contained only one of the two primers. A sample of the PCR product was used as a hybridization probe (Fig. 1, probe A) to screen a A/GEM-11 library constructed from L. lactis subsp. lactis LM0230 DNA (see Materials and Methods); 4 of the 780 clones in the library hybridized strongly with probe A in situ plaque hybridization experiments. The restriction endonuclease digestion patterns (data not shown) of DNAs pre
Fig. 2. Nucleotide sequence of ldh and flanking DNA and deduced amino acid sequence of LDH from L. lactis subsp. lactis LM0230. The nucleotide sequence begins with the first ATG codon after the stop codon of the adjacent ORF. Deduced amino acid sequences are shown for LDH (above the nucleotide sequence) and for the ORF 3' to LDH (below the nucleotide sequence). Double underlining indicates a putative ribosome-binding site which extends complemenarity to the sequence 3'-UCUUUCUCCA-5' at the 3' end of L. lactis 16S rRNA (31). The arrows beneath the sequence indicate inverted repeats that could act as transcriptional terminators (see text).

Examined from these clones indicated that three of them were identical and that the fourth carried a smaller portion of the same region of the lactococcal chromosome. One clone, X632, was used for constructing a restriction site map (Fig. 1) and for cloning in pJDC9 of a 6.0-kbp EcoRI restriction fragment that hybridized with probe A. The recombinant plasmid carrying this fragment was designated pMU2901 (Table 1).

DNA sequence of ldh. Sau3AI, HaeIII, HpaII, RsaI, and HpaI fragments from the 6.0-kbp insertion in pMU2901 were cloned in pJDC9, and the nucleotide sequences of these fragments were determined. This approach did not yield the entire sequence, since stable clones could not be obtained for some sections of the region of interest. The sequences of these sections were obtained by using specifically synthesized oligonucleotide primers and pMU2901 as a template for sequencing reactions. The 1,368-bp nucleotide sequence shown in Fig. 2 includes the region that hybridized with probe A and contains an open reading frame (ORF) of 331 codons (from base pair 100 to base pair 1092).

The deduced amino acid sequence near the C terminus of this ORF (codons 246 to 298) (Fig. 3) exhibited 98% identity with the sequence of a 53-residue tryptic peptide from L. lactis subsp. cremoris US3 LDH (10). This observation provided strong evidence that a gene encoding LDH, which we designated ldh, was present in the sequenced DNA. We concluded that the first ORF codon of the ORF (Fig. 2) was the site of translation initiation since the predicted amino acid sequence immediately after this codon was 90% identical to the previously reported N-terminal sequence of the L. lactis subsp. cremoris enzyme (Fig. 3). When this initiation site was used, the nucleotide sequence predicted a chain length of 324 amino acid residues and a subunit molecular weight of 34,910 for L. lactis subsp. lactis LDH, assuming the same posttranslational removal of the N-terminal methionine that was observed with the L. lactis subsp. cremoris enzyme (10).

The adjacent ORF on the 5' side of ldh was 117 bp away (Fig. 1B) and had the same orientation as ldh (data not shown). The only obvious regulatory signal in this 117 bp of DNA was a possible ribosome-binding site 13 bp upstream from the ldh initiation codon (Fig. 2). The nearest ORF on the 3' side of ldh was 122 bp away (Fig. 1B) and was present in the reverse orientation compared with ldh. This 122 bp of DNA contained two inverted repeat sequences (Fig. 2). Transcripts of these repeats could form stem and loop structures with calculated free energy values (49) of -14.2 kcal/mol (−59.4 kJ/mol) (base pairs 1097 to 1140) and -24.0 kcal/mol (−100 kJ/mol) (base pairs 1167 to 1202). The first repeat was preceded by AAA, and the second repeat was followed by TTT.

Expression of L. lactis ldh in E. coli. The predicted amino acid sequence provided strong evidence that the cloned DNA contained ldh. To establish this point, we sought to
Bacillus subtilis mutans (Smu) (13), fragment E. MSSMPNHQ Lc a ASIT DHQ Llc ADKERKKVILVGDGAVGSAYA Lic Bme Bsu M HVN A MKNNGGAR Tca M GI S I GI S

LDH is the Lpl LDH is the unknown Llc Bst Blo extremity pMU2902 (Table 3). Since LDH was isolated from E. lactis, the putative sequence. The symbols indicate amino acids that are conserved in all of the previously reported sequence data are presented and spaces indicate unknown amino acids. Double underlining indicates amino acids that are conserved in all of the sequences. Asterisks indicate the C-terminal extremity of each sequence, and the percentages at the bottom indicate the percentage of identity between each sequence and the L. lactis subsp. lactis sequence. The symbols above the L. lactis sequence indicate amino acids that are involved in various functions, such as: plus signs, catalysis; overlining, substrate binding; solid circles, FBP activation; and solid triangles, the $\beta$ subunit nucleotide-binding fold.

![Fig. 3. Homology among the deduced amino acid sequences of LDHs from L. lactis subsp. lactis LM0230 and other bacteria. The sequences are LDH sequences of Bifidobacterium longum (Blo) (34), Thermus caldophilus (Tca) (28), Bacillus steatorrhoeophilus (Bst) (4), Bacillus subtilis (Bsu) (19), Bacillus megaterium (Bme) (42), Lactobacillus plantarum (Lpl) (45), Lactobacillus casei (Lca) (25), Streptococcus mutans (Smu) (13), L. lactis subsp. lactis LM0230 (Lli) (this study), and L. lactis subsp. cremoris US3 (Lco) (10). The first amino acid for each LDH is the N-terminal amino acid. The space indicates positions where sequences are identical to the sequence of L. lactis subsp. lactis, except for the L. lactis subsp. cremoris sequence, where all of the previously reported sequence data are presented and spaces indicate unknown amino acids. Double underlining indicates amino acids that are conserved in all of the sequences. Asterisks indicate the C-terminal extremity of each sequence, and the percentages at the bottom indicate the percentage of identity between each sequence and the L. lactis subsp. lactis sequence. The symbols above the L. lactis sequence indicate amino acids that are involved in various functions, such as: plus signs, catalysis; overlining, substrate binding; solid circles, FBP activation; and solid triangles, the $\beta$ subunit nucleotide-binding fold.](http://jb.asm.org/ Downloaded from)

TABLE 2. LDH activities of cell extracts of L. lactis subsp. lactis and E. coli strains.

<table>
<thead>
<tr>
<th>Strain</th>
<th>Plasmid</th>
<th>Presence of IPTG</th>
<th>LDH sp act (U/mg)</th>
</tr>
</thead>
<tbody>
<tr>
<td>L. lactis subsp. lactis LM0230</td>
<td>pJDC9</td>
<td>–</td>
<td>0.60</td>
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<tr>
<td>E. coli BL21(DE3)</td>
<td>pMU2901</td>
<td>–</td>
<td>&lt;0.045</td>
</tr>
<tr>
<td>E. coli BL21(DE3)</td>
<td>pMU2902</td>
<td>–</td>
<td>&lt;0.045</td>
</tr>
<tr>
<td>E. coli BL21(DE3)</td>
<td>pMU2903</td>
<td>–</td>
<td>2.0</td>
</tr>
<tr>
<td>E. coli BL21(DE3)</td>
<td>pMU2903</td>
<td>+</td>
<td>4.3</td>
</tr>
</tbody>
</table>

* Cells were grown in the absence or presence of 0.4 mM isopropyl-$\beta$-D-thiogalactopyranoside (IPTG), which was added to the culture at an A600 of 0.8.

* FBP-dependent LDH activities were measured as described in Materials and Methods.

observe expression of LDH activity conferred by plasmids carrying this DNA. Since FBP activates lactococcal LDH more than 500-fold (2) but does not affect the activity of E. coli LDH (46), it was possible to analyze E. coli extracts specifically for the lactococcal enzyme by assaying for FBP-stimulated activity. No significant activity was observed in extracts of E. coli cells containing pMU2901 or pMU2902 (Table 2). Because of the possibility that functional transcription initiation signals were not present in the lactococcal insertions of pMU2901 or pMU2902, a 65-bp BglII-BamHI fragment containing the phase T7 promoter $\phi$10 was isolated from pET-3 and inserted 75 bp upstream from the putative $\phi$10 gene in pMU2902, yielding plasmid pMU2903. Initiation of transcription from $\phi$10 requires T7 RNA polymerase, making this promoter functional only in E. coli strains carrying T7 gene 1, the gene encoding the polymerase. To obtain transcription of $\phi$10, pMU2903 was introduced into E. coli BL21(DE3), a strain in which gene 1 was expressed from the lacUV5 promoter (44). Cell extracts of E. coli BL21(DE3)(pMU2903) had high specific activities of FBP-dependent LDH that were increased by growth in the presence of isopropyl-$\beta$-D-thiogalactopyranoside (Table 2). The specific activities of these extracts were approximately sevenfold higher than those of L. lactis subsp. lactis LM0230 extracts. These data established that the ORF in
HindIII, HaeIII, EcoRV, were hybridized with EcoRI (lane 1), HindIII (lane 2), HaeIII (lane 3), EcoRV (lane 4), and HpaI (lane 5) and then separated by electrophoresis through 1.0% (wt/vol) agarose. The numbers on the left show the sizes (in base pairs) of size markers produced by PstI digestion of ADNA (lane λ). Or, location of the origin. (B) Southern blot of the gel in panel A after hybridization with 32P-labeled probe B (Fig. 1).

pMU2903 was ldh, and that the activity of the encoded enzyme was FBP dependent.

Copy number of ldh in the L. lactis chromosome. EcoRI, HindIII, HaeIII, EcoRV, and HpaI digests of L. lactis subsp. lactis LM0230 chromosomal DNA were separated by electrophoresis through agarose (Fig. 4A), and a Southern blot was prepared from the gel. Hybridization of the blot with 32P-labeled probe B (Fig. 1) yielded a single hybridizing band in each digest (Fig. 4B). The measured sizes of the hybridizing fragments (6.0, 4.3, 3.32, 2.65, and 0.77 kbp for the EcoRI, HindIII, HaeIII, EcoRV, and HpaI fragments, respectively) were in agreement with the sizes predicted from the sequence and mapping data shown in Fig. 1. These observations indicated that a single chromosomal copy of ldh was present.

Northern analyses of ldh transcripts. Northern blots prepared with RNA isolated from L. lactis subsp. lactis LM0230 were hybridized with the 774-bp HpaI fragment internal to ldh (Fig. 1, probe B). The band that hybridized most strongly with this probe corresponded to a 4.1-kb transcript, while less intense bands corresponding to 1.2- and 1.1-kb transcripts were also observed (Fig. 5, lane 3). Unusually shaped bands were present in the region of the blot containing the front edge of the 23S and 16S rRNA bands. A similar phenomenon, possibly an artifact resulting from the extremely high concentrations of the rRNA species in the gel, has been observed by other investigators (12). To examine the limits of the 4.1-kb transcript, Northern blots of lactococcal RNA were hybridized with probes from the DNA surrounding ldh. Two probes from the 5′ side of ldh (Fig. 1, probes C and D) hybridized with the 4.1-kb transcript (Fig. 5, lanes 1 and 2), whereas a probe from the region that began 900 bp to the 3′ side of ldh (Fig. 1, probe E) hybridized to a slightly smaller transcript (3.9 kbp) (Fig. 5, lane 4). The simplest explanation of these observations is that ldh was expressed at the 3′ end of a 4.1-kb multicistronic operon and that another, slightly smaller transcript was expressed from the DNA immediately 3′ to ldh. The failure to observe hybridization of either of these transcripts with probe F (Fig. 5, lane 5), which contained the DNA 5 kbp 3′ to ldh (Fig. 1), was consistent with this model.

Promoter cloning studies. To search for an ldh promoter, a 621-bp HpaI fragment containing the DNA immediately 5′ to ldh was cloned into the filled-in SalI site of pGKV210, a promoter-detecting E. coli–L. lactis shuttle vector (26). After transformation of E. coli with the ligation mixture, recombinant plasmids carrying the HpaI fragment were identified by colony hybridization with probe G (Fig. 1). Constructions with the fragment inserted in the vector in each orientation (pMU2904 and pMU2905) were then identified by restriction mapping. Assays of L. lactis subsp. lactis LM0230 derivatives carrying these plasmids revealed that neither conferred CAT activity (Table 3). This result showed that the DNA

TABLE 3. CAT activities of plasmid-carrying derivatives of L. lactis subsp. lactis LM0230

<table>
<thead>
<tr>
<th>Plasmid</th>
<th>CAT sp act (mU/mg)</th>
<th>Cm' (μg/ml)*</th>
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<tbody>
<tr>
<td>pGKV210</td>
<td>&lt;3</td>
<td>4</td>
</tr>
<tr>
<td>pMU2904</td>
<td>&lt;3</td>
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<td>5</td>
</tr>
<tr>
<td>pMU2907</td>
<td>13</td>
<td>10</td>
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</table>

* Cm', maximum concentration of chloramphenicol that permitted growth on solid medium.
FIG. 6. Pulsed-field gel electrophoresis of restriction digests of L. lactis LM0230 DNA. (A) High-molecular-weight DNA was digested in agarose with NorI (lane N), NorI plus Smal (lane N+S), and Smal (lane S) and then subjected to pulsed-field gel electrophoresis for 22 h at 200 V. The pulse time was increased linearly from 1 to 30 s. The numbers on the left indicate the sizes in kilobase pairs) of size markers from a HindIII digest of λ DNA (lane 1) and λ DNA concatamers (lane 2). Or, location of the origin. (B) Southern blot of the gel in panel A after hybridization with 32P-labeled λ632 DNA.

immediately 5' to ldh was devoid of transcriptional initiation signals.

Terminator cloning studies. The results of the Northern blot analyses suggested that the terminator for the ldh transcript was located immediately 3' to the gene. To investigate this further, a 286-bp PsrI fragment containing the 122 bp of DNA between the 3' ends of ldh and the adjacent gene (Fig. 1) was cloned in a terminator-detecting vector, pGKV25 (26). For this cloning it was necessary to transform L. lactis subsp. lactis LM0230 cells directly with the ligation mixture, since major rearrangements of the recombinant plasmid were observed in E. coli transformants. In one of the plasmids which we isolated (pMU2906) the inserted DNA was transcribed from left to right (as drawn in Fig. 1), enabling this plasmid to be used to detect a terminator of ldh transcription. Another isolate (pMU2907) contained the PsrI fragment in the other orientation. Both pMU2906 and pMU2907 conferred significantly lower levels of CAT activity than the level conferred by pGKV25 (Table 3), indicating that transcription entering the PsrI fragment from either direction was terminated efficiently.

Mapping of ldh on the L. lactis subsp. lactis chromosome. A physical map of the chromosome of L. lactis subsp. lactis DL11 was constructed recently by using pulsed-field gel electrophoresis (50). To place ldh on this map, samples of high-molecular-weight DNA from L. lactis subsp. lactis DL11 were digested with NorI, SmaI, and NorI plus SmaI, the products were separated by pulsed-field gel electrophoresis (Fig. 6A), and Southern blots of the resulting gels were hybridized with 32P-labeled λ632 DNA; in the three separations, the bands that hybridized with the probe were bands containing fragments of 310, 50, and 50 kb, respectively (Fig. 6B). The 310-kbp band corresponded to fragment NtD, while the 50-kbp bands corresponded to fragment SmJ3, which has been shown previously to contain three fragments (fragments SmJ1, SmJ2, and SmJ3) (50). Since only fragment SmJ3 is within fragment NtD (50), we concluded that ldh is located on the L. lactis subsp. lactis DL11 chromosome in fragment SmJ3, between coordinates 1.619 and 1.669 on the physical map (50).

### DISCUSSION

A molecular clone containing ldh, the gene encoding FBP-activated LDH, was isolated from L. lactis subsp. lactis LM0230. Proof of the identity of the gene was obtained from a comparison of the predicted amino acid sequence of the product with the partial amino acid sequence of LDH from L. lactis subsp. cremoris US3 and from the expression of high levels of LDH activity from the gene in E. coli. The availability of a molecular clone of ldh enabled the chromosomal origin of ldh to be confirmed and its location on the physical map of the chromosome to be determined.

The derived amino acid sequence of L. lactis subsp. lactis LDH exhibits considerable homology with the sequences of other bacterial LDHs (Fig. 3). This homology is strongest with the LDH of Streptococcus mutans (78% identity) and weakest with the LDH of Bifidobacterium longum (36% identity). At the DNA level the L. lactis and S. mutans genes exhibit 75% sequence identity. Extensive structural and mutagenic studies with LDHs from muscle and Bacillus stearothermophilus have resulted in the identification of a number of the amino acids involved in catalysis, ligand binding, and activation by FBP (8, 9, 23). These amino acids can be readily identified in L. lactis LDH by using the amino acid sequence homology data (Fig. 3 and Table 4). One of the amino acids, Ile-250, which lines the coenzyme-binding site, is replaced in the L. lactis enzyme by a valine, Val-236. It is not known whether this substitution (which is also present in Bacillus subtilis LDH) causes any alteration in the kinetic or binding properties of the enzyme. The sequence GXXGXG, which is characteristic of a β8p fold involved in the binding of NAD (55), is also conserved in the L. lactis enzyme near the N terminus (Fig. 3).

The 5' flanking DNA of ldh did not function as a promoter in either L. lactis subsp. lactis or E. coli, but contained signals for the initiation of translation in each of these organisms. A putative ribosome-binding site, which had a calculated free energy of −14 kcal/mol (−59 kJ/mol) (49) for its interaction with the 3' end of L. lactis 16S rRNA, was identified 13 bp upstream from the ATG start codon of ldh (Fig. 2). This site resembled other putative lactococcal

### Table 4. Assigned roles of some amino acids in L. lactis LDH

<table>
<thead>
<tr>
<th>Amino acid</th>
<th>Function</th>
<th>Reference</th>
<th>Corresponding amino acid in L. lactis</th>
</tr>
</thead>
<tbody>
<tr>
<td>His-195</td>
<td>Active-site proton donor or acceptor</td>
<td>23</td>
<td>His-178</td>
</tr>
<tr>
<td>Arg-109</td>
<td>Active site</td>
<td>23</td>
<td>Arg-91</td>
</tr>
<tr>
<td>Asp-168</td>
<td>Active site</td>
<td>23</td>
<td>Asp-151</td>
</tr>
<tr>
<td>Arg-171</td>
<td>Substrate binding</td>
<td>23</td>
<td>Arg-154</td>
</tr>
<tr>
<td>Ile-250</td>
<td>NADH binding</td>
<td>8</td>
<td>Val-236</td>
</tr>
<tr>
<td>Gln-102</td>
<td>Substrate binding</td>
<td>8</td>
<td>Gln-85</td>
</tr>
<tr>
<td>Thr-246</td>
<td>Substrate binding</td>
<td>8</td>
<td>Thr-232</td>
</tr>
<tr>
<td>His-188</td>
<td>FBP binding</td>
<td>9</td>
<td>His-171</td>
</tr>
<tr>
<td>Arg-173</td>
<td>FBP activation</td>
<td>9</td>
<td>Arg-156</td>
</tr>
</tbody>
</table>

* Amino acids were numbered according to the commonly used N system for LDH, as defined previously (16).

* The amino acid numbers for L. lactis LDH are from Fig. 2.
ribosome-binding sites (51) in its calculated free energy value and in its distance from the start codon. The 3' flanking DNA of \( \text{lhd} \) acted as an efficient terminator of transcription when it was cloned in either orientation in a terminator-detecting plasmid. The precise sites of termination were not established. There are two inverted repeats in the 3' flanking DNA of \( \text{lhd} \) (Fig. 2), and transcription of either should yield a stable stem and loop structure that could function as a transcription terminator. The closer of these two repeats to \( \text{lhd} \) is preceded by AAA, and the other is followed by TTT. It is tempting to postulate that transcription through \( \text{lhd} \) is terminated at one of these repeats (perhaps the distal one because of the following TTT sequence) and that transcription in the reverse direction is terminated at the other. The only other previously published identification of a lactococcal terminator known to us is that of the \( L. \ \text{lactis} \) subsp. \( \text{cremoris} \) \( \text{Wg2} \) protease gene terminator, which has an inverted repeat whose size is similar to the sizes shown in Fig. 2 (27, 51).

Northern analyses indicated that \( \text{lhd} \) is expressed in a 4.1-kb transcript and is therefore likely to be a component of a multistronic operon. Since the 3' end of this transcript is produced from the DNA near the end of \( \text{lhd} \), the 3' end must be transcribed from approximately 3.2 kbp upstream of \( \text{lhd} \). The observations that probes consisting of DNA from up to 2.4 kbp 5' to \( \text{lhd} \) also hybridized with a 4.1-kb transcript and that DNA 3' distal to the \( \text{lhd} \) terminator hybridized to a slightly smaller 3.9-kb transcript are consistent with this conclusion. The adjacent ORF on the 3' side of \( \text{lhd} \) is in the reverse orientation compared with \( \text{lhd} \). Therefore, it is likely that the 3.9-kb transcript is produced by transcription of the chromosome toward \( \text{lhd} \). The observation that there are shorter transcripts (1.2 and 1.1 kbp) that hybridize with \( \text{lhd} \) is interesting. The bands containing these transcripts were less intense than the band containing the 4.1-kb species. Because there is no promoter in the \( \text{lhd} \) 5' flanking DNA, these transcripts were probably produced by posttranscriptional processing of the 4.1-kb transcript.

A complete transcriptional analysis of the gene encoding LDH has not been reported for any other bacterium. The best-defined examples are \( \text{Lactobacillus casei} \) and \( \text{Bifidobacterium longum} \), in which the promoter is close to the 5' end of the gene and the 3' DNA contains a putative transcriptional terminator, suggesting that a monocistronic transcriptional unit occurs in these organisms (25, 34). In \( \text{Bacillus steatorrhophilus} \) the LDH gene may be at the 5' end of a multistrionic operon (4). The regulatory implications of the expression of \( \text{lhd} \) in a multistrionic operon in \( L. \ \text{lactis} \) subsp. \( \text{lactis} \) are of interest. The full complement of the operon is not defined; however, the predicted amino acid sequences of two ORFs 5' to \( \text{lhd} \) exhibit homology with the sequences of pyruvate kinase and phosphofructokinase (30). Like LDH, these two enzymes are required for the conversion of lactose to lactic acid. The activities of these three enzymes are interdependent and significantly affect the rate of synthesis of lactic acid (48). Therefore, the characterization of the operon and its regulation should provide useful insights into the control of lactic acid synthesis and energy production in \( L. \ \text{lactis} \).

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