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Molecular Genetic Characterization of the L-Lactate Dehydrogenase Gene (*ldhL*) of *Lactobacillus helveticus* and Biochemical Characterization of the Enzyme

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The *Lactobacillus helveticus* L-(+)-lactate dehydrogenase (L-LDH) gene (*ldhL*) was isolated from a lambda library. The nucleotide sequence of the *ldhL* gene was determined and shown to have the capacity to encode a protein of 323 amino acids (35.3 kDa). The deduced sequence of the 35-kDa protein revealed a relatively high degree of identity with other lactobacillar L-LDHs. The highest identity (80.2%) was observed with the *Lactobacillus casei* L-LDH. The sizes and 5′ end analyses of *ldhL* transcripts showed that the *ldhL* gene is a monocistronic transcriptional unit. The expression of *ldhL*, studied as a function of growth, revealed a high expression level at the logarithmic phase of growth. The *ldhL* gene is preceded by two putative −10 regions, but no corresponding −35 regions could be identified. By primer extension analysis, the *ldhL* transcripts were confirmed to be derived from the −10 region closest to the initiation codon. However, upstream of these regions additional putative −10/−35 regions could be found. The L-LDH was overexpressed in *Escherichia coli* and purified to homogeneity by two chromatographic steps. The purified L-LDH was shown to be a nonallosteric enzyme, and amino acid residues involved in allosteric regulation were not conserved in *L. helveticus* L-LDH. However, a slight enhancement of enzyme activity was observed in the presence of fructose 1,6-diphosphate, particularly at neutral pH. A detailed enzymatic characterization of L-LDH was performed. The optimal reaction velocity was at pH 5.0, where the kinetic parameters K_m, and K_{cat} for pyruvate were 0.25 mM and 643 s^{-1}, respectively.

Lactic acid bacteria (LAB) are important in the dairy industry and in the production of other fermented food and silage products. The primary role of LAB is the production of lactic acid, resulting in lowering of the pH, which is required for desirable quality and preservation of food and feed products (23). Depending on the species, LAB synthesize either the L-(+) or D-(−) isomer of lactic acid or both. For several applications L-(+)-lactic acid is the preferred isomer, since it is the normal intermediate in human and animal metabolism. It is advantageous to use L-(+)-lactic acid rather than a mixture of both isomers for some industrial applications as well, including, e.g., manufacturing of biodegradable lactide polymers for the plastic and health care industries (27). Among the lactic acid-producing LAB species, *Lactobacillus helveticus* is considered an ideal host candidate for lactic acid production for several reasons. It is a homofermentative, acid-tolerant, and thermophilic bacterium which is capable of producing high yields of total lactate (28, 37). Furthermore, a D-(−)-lactic acid-negative mutant of *L. helveticus* CNRZ32, producing only L-(+)-lactic acid, has been shown to produce twice as much L-(+)-lactic acid as the wild-type strain (2).

In homofermentative LAB, lactic acid is one of the major end products of carbohydrate fermentation. The glucose is converted to pyruvate via the Embden-Meyerhof-Parnas pathway, and in the final step pyruvate is reduced to two isomeric forms of lactic acid by two distinct NAD-dependent, stereospecific lactate dehydrogenases: L-lactate dehydrogenase (L-LDH) (EC 1.1.1.28) and D-lactate dehydrogenase (D-LDH) (EC 1.1.1.28). The evolutionary relationship between L-LDH and D-LDH is not fully known. It has been suggested that L-LDH and D-LDH descended from distinct evolutionary ancestors, and according to the typical differences of their amino acid sequences, they have been classified into two distinct enzyme families, the L- and D-2-hydroxyacid dehydrogenase families, respectively (1, 16, 17, 19, 36). L-LDH has been found to function as an allosteric enzyme which is activated by fructose 1,6-diphosphate (FBP) or as a nonallosteric enzyme (11). Allosteric L-LDHs have been purified, characterized, and cloned from a variety of eukaryotes and prokaryotes, and their primary and tertiary structures have been extensively studied (5, 6, 14, 29, 42). Much less information is available about the structure and regulation of bacterial nonallosteric L-LDHs. Recently, the genes encoding the nonallosteric L-LDHs of *Pediococcus acidilactici* (10) and *Streptococcus thermophilus* (13), and the closely related *ldhL* genes from *Lactobacillus plantarum* (9) and *Lactobacillus pentosus* (36), have been cloned and sequenced.

In this work, we report the cloning, DNA sequencing, and mRNA analysis of an *ldhL* gene from *L. helveticus*. The overexpression of the *ldhL* gene in *Escherichia coli* and the purification and characterization of the cloned L-LDH are described.

MATERIALS AND METHODS

Bacterial strains, plasmids, and growth conditions. *L. helveticus* 53/7 is an industrial starter strain from the collection of Valio Ltd. (Helsinki, Finland). *L. helveticus* was propagated in whey broth at 42°C without shaking for RNA analysis and in MRS containing lactose (2%) at 37°C with slight agitation and without pH control for the protein purification. Bioreactor cultivation was done as described previously (39). *E. coli* DH5αF′ (43) and JM105 (Pharmacia, Uppsala, Sweden) were grown in Luria broth. Erythromycin (300 μg ml^{-1}) or am-

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picillin (50 μg ml^{-1}) was added to the growth medium when the pJDC9 (4) or pKK223-3 (Pharmacia) vector, respectively, was used in *E. coli*.

Determination of the NH_2-terminal amino acid sequences. The L-LDH protein was separated on a sodium dodecyl sulfate (SDS)–12% polyacrylamide gel and transferred electrophoretically onto a polyvinylidene difluoride membrane as described by Matsudaira (22). The NH_2 terminus was sequenced by degrading the protein in an Applied Biosystems Procise Sequencing System. Digestion of the freeze-dried protein, separation of peptides, and peptide sequencing were performed as described previously (41).

Oligonucleotide synthesis, amplification of DNA by PCR, and DNA isolations. The oligonucleotides were synthesized with an Applied Biosystems DNA/RNA synthesizer model 392 and purified by ethanol precipitation or with NAP-10 columns (Pharmacia). PCR was used to synthesize DNA fragments by using reaction conditions recommended by the manufacturer (Finnzymes). Chromosomal DNA from *L. helveticus* was isolated essentially as described earlier (41) without guanidine hydrochloride treatment. Plasmid DNAs from *E. coli* clones were isolated by using the Wizard Minipreps (Promega) or FlexiPrep (Pharmacia) kit.

Screening of an *L. helveticus* genomic library. An *L. helveticus* genomic library (40) was screened by plaque hybridization (30) with an *ldhL*-specific PCR fragment probe of 0.5 kb. For the PCR synthesis of the probe, a degenerate primer pair, 5′TACTGGATCCAA(A/G)AA(C/T)AT(A/C/T)TA(T/C)GC(A/G/C/T)GC(A/G/C/T)GA(C/T)TAC-3′ and 5′TACTGTCGAC(A/G)TT(A/G/T)AT(A/G/T)AT(A/G)TC(A/G)TA(A/C/G/T)GC(A/C/G/T)AC(A/G)TC-3′, was designed according to the NH_2-terminal sequences of the intact L-LDH and an internal tryptic peptide of the purified L-LDH. The labeling of the probe and DNA hybridizations were performed by the protocols of the digoxigenin (DIG) DNA labeling and luminescence detection kits (Boehringer Mannheim), respectively.

DNA sequencing and sequence analysis. The sequencing was performed with an A.L.F. DNA Sequencer (Pharmacia Biotech). The dideoxy sequencing reactions (31) were performed as described in the AutoRead Sequencing Kit manual (Pharmacia). Both DNA strands were sequenced by using pUC19-specific primers and different sequence-specific oligonucleotides. The DNA sequences obtained from the A.L.F. sequencer were assembled and analyzed with the PC/GENE set of programs (release 6.8; IntelliGenetics). The EMBL and SWISS-PROT databases were used for searching for homologous protein and nucleic acid sequences.

RNA isolation, Northern (RNA) blotting, and primer extension. Total RNA was isolated from *L. helveticus* cells as described by Vesanto et al. (40). RNA gel electrophoresis and Northern blotting were performed as described previously (12). For Northern blot analysis, a PCR fragment (1.058 kb) of pKTH2128, carrying the *ldhL* gene, was labeled with DIG-dUTP (Boehringer Mannheim). A DIG luminescence detection kit (Boehringer) was used for hybrid detection. The primer extension was performed with an A.L.F. DNA Sequencer (Pharmacia) as described earlier (40) with total RNA (10 μg) isolated from exponentially growing cells (6 h) (see Fig. 4.) with the antisense fluorescein-labeled oligonucleotide 5′CTGCTGAAGTCCCAGACTTG3′.

Overexpression of *L. helveticus* L-LDH in *E. coli*. The *ldhL* gene was synthesized by PCR with a primer pair (5′AATACCCGGGAGA TTTATTGTTATG GCAAG3′ and 5′AAATCCCGGGGCATCTCTTCCTAAGAAAAG3′) containing *Xma*I sites at their 5′ ends for cloning. The *Xma*I fragment of 1.058 kb, carrying the structural gene of *ldhL* with its ribosome binding site, was ligated with the pKK223-3 vector (Pharmacia) downstream of the inducible *tac* promoter and transferred into the *E. coli* JM105 host (Pharmacia). The transformed cells were propagated to a density of 50 Klett units (filter 60), followed by induction with 1 mM IPTG (isopropyl-β-D-thiogalactopyranoside), and the expression of L-LDH was characterized by measuring product accumulation during 6 h by SDS-polyacrylamide gel electrophoresis (SDS-PAGE) and determining the ability of L-LDH to reduce pyruvate to lactate at pH 5.6 (see "Enzyme assays." below).

Enzyme assays. The standard assay for L-LDH activity was performed spectrophotometrically (A_{340}) at 25°C in phosphate buffer (73 mM KH_2PO_4, 3.5 mM Na_2HPO_4, pH 5.6) containing 1 mM pyruvate and 0.2 mM NADH. One unit of enzyme activity was defined as the quantity of enzyme catalyzing the reduction of 1 μmol of substrate to the end product per min under the reaction conditions used. The protein concentrations were determined with the Bio-Rad protein assay reagent on the basis of the Bradford dye-binding procedure (3). Bovine serum albumin (Sigma) was used as a protein standard.

Purification of L-LDH from *E. coli* JM105. The crude extract of *E. coli* JM105 carrying pKHT2128 was prepared as follows. The cells, grown in 3.0 liters of Luria broth, were harvested 5 h (50 Klett units, filter 60) after induction of *ldhL* expression with 1 mM IPTG. The cells were washed with 200 mM Tris-HCl, pH 8.0. The pellet was resuspended in 60 ml of lysis buffer (20 mM Tris-HCl [pH 8.0], 1 mM EDTA, 0.5 mg of lysozyme ml^{-1}), gently stirred for 45 min at 4°C, and then treated with bovine pancreatic RNase (Sigma) and DNase I (Boehringer) for 30 min at room temperature. The cell debris was removed by centrifugation (10,000 × *g*) at 4°C for 40 min and filtered (0.45-μm-pore-size membrane filter [Millex HA; Millipore, Bedford, Mass.]) prior to Q-Sepharose chromatography. A Q-Sepharose column (gel bed, 255 ml; diameter, 5 cm; Pharmacia LKB) was equilibrated with 50 mM sodium phosphate buffer, pH 6.0. Bound proteins were eluted with a linear gradient of 0 to 0.5 M NaCl in the same buffer (flow rate, 5 ml min^{-1}; gradient volume, 600 ml). Fractions of 10 ml were collected and assayed for pyruvate reduction activity. The fractions containing L-LDH activity (30 ml) were concentrated threefold by ultrafiltration through a 30-kDa-cutoff membrane (Amicon). Half of the concentrated fraction (5 ml) was applied to a phenyl-Sepharose CL-4B chromatography (gel bed, 22 ml; diameter, 1.6 cm; Pharmacia LKB). After the column was washed with 50 mM sodium phosphate buffer (pH 6.0), proteins were eluted in a decreasing gradient of $(NH_4)_2SO_4$ (1.5 to 0 M; flow rate, 1.0 ml min^{-1}; gradient volume, 70 ml; fraction size, 6 ml). The purity of enzyme preparations was monitored by SDS–12% (wt/vol) PAGE as described by Laemmli (18). The gel was stained with Coomassie brilliant blue R250. As marker standards, MWH-SDS proteins (Pharmacia) were used.

Molecular mass determination. The molecular mass of the native L-LDH was determined by gel filtration chromatography (Superdex 200; Pharmacia LKB) with columns equilibrated with 50 mM sodium phosphate (pH 6.0) containing 0.15 M NaCl. The columns were calibrated with catalase (232 kDa), aldolase (158 kDa), bovine serum albumin (67 kDa), and ovalbumin (43 kDa).

Substrate specificity of L-LDH. Activities toward 1 mM β-hydroxypyruvic acid (Sigma), β-phenylpyruvic acid (Sigma), α-ketobutyric acid (Sigma), 3-methyl-2-oxobutanoic acid (Aldrich), and 4-methyl-2-oxopentanoic acid (Aldrich) were analyzed under the same reaction conditions as described in "Enzyme assays." The activity with 1 mM pyruvate as the substrate was taken as 100%.

Inhibition studies. Potential inhibitors were added to final concentrations of 0.1 and 1 mM. L-LDH was incubated in 50 mM sodium acetate buffer (pH 4.5) at room temperature for 30 min. The activities were compared with that of the untreated control, which was taken as 100%.

Kinetic parameters. Kinetic parameters for pyruvate were measured at saturating levels of NADH (0.2 mM) with 50 mM sodium acetate buffers in the pH range of 4.0 to 7.5. The experimental data were evaluated by nonlinear and linear regression analyses with the program GRAFIT (Sigma). Pyruvate concentrations in the assay mixtures varied in the range between 0.05 and 5.0 mM.

Thermal stability. The L-LDH preparations (70 μg/ml) were incubated in 50 mM sodium acetate buffers, pH 4.5 and 5.0, at 40, 45, and 50°C. Aliquots of these preparations were withdrawn at different times and assayed for L-LDH activity. The activities were compared with that of the untreated control, which was taken as 100%.

Nucleotide sequence accession number. The accession number of the 1,350-bp DNA fragment which contains the *ldhL* gene reported in this paper is Z81318 and will appear in the EMBL, GenBank, and DBJ nucleotide sequence databases.

RESULTS AND DISCUSSION

Purification of L-LDH from *L. helveticus*. During an attempt to purify a 29-kDa peptidase from *L. helveticus* by anion-exchange chromatography and hydrophobic interaction chromatography, a proteolysis product derived from L-LDH was copurified. During the DEAE-Sepharose (Pharmacia) chromatography, the proteins were eluted at 0.24 M NaCl in 10 mM sodium phosphate buffer, pH 7.0. This fraction was adjusted to 4.0 M NaCl and applied to a phenyl-Superose HR5/5 (Pharmacia LKB) column equilibrated with the same buffer containing 4.0 M NaCl. The fraction containing the peptidase activity was eluted at 3.3 M NaCl and desalted by being passed through a HITrap DeSalting Sephadex G-25M column (Pharmacia). The protein band of 29 kDa was separated by SDS-PAGE and blotted onto a polyvinylidene difluoride membrane (Immobilon P; Millipore) and analyzed in a gas-pulsed-liquid sequencer. The NH_2-terminal sequence of the purified protein was NH_2-T-S-P-K-N-I-Y-A-A-D-Y-P-D-S-K-D-A-D-L-V-V-I-T-A-G-A-P-Q-K. Homology searches revealed a perfect match of this sequence with various L-LDHs, 58 amino acids downstream of their NH_2 termini (data not shown). Thus, the 29-kDa major protein band on the SDS-polyacrylamide gel did contain, instead of the expected peptidase, the truncated *L. helveticus* L-LDH. Since the fraction analyzed was active against the peptidase substrate used, the amount of this enzyme was most likely below the limit of detection by SDS-PAGE. The specific L-LDH activity in this fraction was less than 0.03% of that of the full-size enzyme (see "Overexpression and purification of L-LDH" below), suggesting that the truncated L-LDH was inactive. Since it is a key enzyme of LAB, the purified L-LDH was subjected to tryptic digestions, and the tryptic peptides were sequenced to develop a PCR primer needed for amplification of a DNA fragment that was

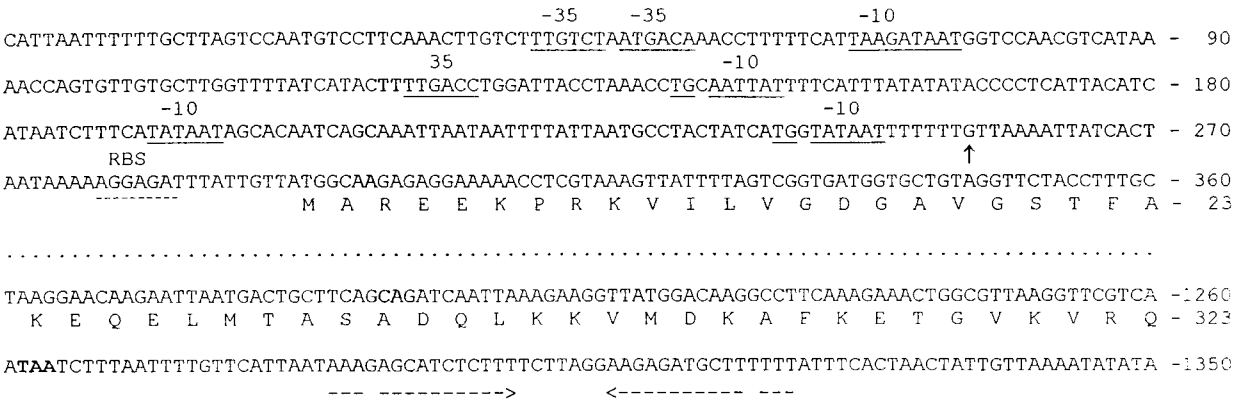

FIG. 1. Partial nucleotide and predicted amino acid sequences of the *L. helveticus* 53/7 *ldhL* gene. Possible −10 and −35 regions of the putative promoters are underlined. The putative ribosome binding site (RBS) and the transcriptional start point are indicated by a dashed line and a vertical arrow, respectively. The translation stop codon is in boldface, and the putative transcription terminator is shown by dashed arrows.

later used as a probe. One of the tryptic peptides was chosen for amino acid sequence analysis, resulting in the N-terminal sequence NH_2-D-M-A-Y-D-I-I-N-K.

Molecular cloning of the *ldhL* gene of *L. helveticus* 53/7. The *ldhL* gene was isolated by plaque hybridization from an *L. helveticus* 53/7 genomic library established in λgt10. The 0.5-kb probe used for screening was PCR amplified with the primers designed according to the amino acid sequences of the purified degradation product of L-LDH described in the previous paragraph. The amplified 0.5-kb DNA fragment was cloned into pJDC9 (pKTH2127) and sequenced before labeling to confirm its identity as part of the *ldhL* gene. Screening of 4,500 plaques resulted in 11 hybridization-positive clones. One of these clones (λgt10/cl 5) was determined to contain an insert of 2.8 kb. The 2.8-kb fragment was digested with *Eco*RI, and the resulting fragments of 1.0 and 1.8 kb were subcloned in pJDC9 and designated pKTH2126 and pKTH2125, respectively.

Sequence analyses. The DNA sequence analysis of the 2.8-kb insert revealed three open reading frames, ORF1, ORF2, and ORF3, of 969, 336, and 578 bp, respectively. ORF1 was revealed to encode the complete L-LDH protein of 323 amino acids with a calculated molecular mass of 35,530 Da. ORF2 codes for 112 amino acids of a polypeptide homologous to the *Bacillus subtilis* SpoVC protein (25). The nucleotide and deduced amino acid sequences of ORF3 did not reveal any significant similarity to any known sequences available from the databases used. The nucleotide sequences of the 5′ and 3′ regions of the *L. helveticus ldhL* gene are shown in Fig. 1. *ldhL* starts with ATG at position 293, and a termination codon, TAA, is located at position 1261. A typical prokaryotic ribosome binding site, AGGAGA (33, 35), was identified 9 nucleotides upstream of the initiation codon. The sequence data revealed two highly conserved and several less conserved putative −10 regions of prokaryotic vegetative promoters, whereas no corresponding −35 regions could be identified. Furthermore, upstream of these −10 regions, at least two pairs of relatively well conserved −10/−35 regions could be found (Fig. 1). It is not yet known how the additional putative promoter regions upstream of the conserved −10 regions affect the expression of L-LDH and whether these sequences are used to control the levels of expression under different growth conditions. A review of the promoter regions of the *ldhL* genes from other LAB also indicates the lack of the typical −10/−35 consensus structure, which is an unexpected observation given the high expression levels of *ldhL* genes. Another interesting finding was the adjacent ORF2, which encodes a homolog to the *B. subtilis* SpoVC protein (62% identity), which is involved in coat formation at sporulation stage V (8). A similar arrangement, where the *ldhL* gene is preceded by a divergent SpoVC homolog with overlapping promoters, has also been observed for *Lactobacillus sake* (34). A putative rho-independent-type transcription terminator of the *ldhL* gene was found 40 nucleotides downstream from the stop codon, with a ΔG of −51.9 kJ mol^{-1} (38).

The active site and several other domains of unknown function are highly conserved among all L-LDHs studied, including the *L. helveticus* L-LDH. Two amino acid residues involved in FBP binding and regulation of the enzyme activity, R173 and H188 (11), were, however, replaced by asparagine and glutamine residues, respectively, in the *L. helveticus* enzyme, suggesting a nonallosteric nature. The degree of identity of the *L. helveticus* L-LDH with the allosteric L-LDHs from *Lactobacillus casei* (80.2%) and *L. sake* (79.4%) and the nonallosteric L-LDHs from *L. pentosus* (75.6%) and *L. plantarum* (75.6%) was relative high, indicating that these enzymes have diverged recently in evolution. It is noteworthy that identity was higher with allosteric L-LDHs of LAB than with the known nonallosteric L-LDHs, and thus the identity seemed to correspond to the phylogenetic relationship of the species rather than to the regulation of the enzyme (data not shown).

mRNA analyses and expression of *ldhL*. Expression of the *ldhL* gene in *L. helveticus* 53/7 was studied in a pH-controlled culture in whey broth. The size of the mRNA transcribed from the *ldhL* gene and the steady-state level of *ldhL* transcripts from *L. helveticus* cells were analyzed by Northern blotting with a DIG-labeled *ldhL*-specific hybridization probe. Total RNAs isolated from cell samples taken 4, 6, 8, 12, and 18 h after inoculation were hybridized with a 1.058-kb *ldhL*-specific probe amplified by PCR from the chromosomal DNA of *L. helveticus*. The autoradiogram (Fig. 2) showed a single 1.1-kb transcript, which is in good agreement with the size of the *ldhL* gene predicted from the DNA sequence and thus indicates that *ldhL* forms a monocistronic transcriptional unit. The Northern data also showed a high expression level of the *ldhL* gene at the logarithmic growth phase, whereas at the later stages of growth the amount of *ldhL* transcripts rapidly decreased to below the detection limit (Fig. 2). The primer extension mapping of the 5′ end of the *ldhL* mRNA (data not shown) from exponentially growing cells revealed only one transcription start site (nucleotide G at position 256 [Fig. 1]), located 6 nucleotides down-

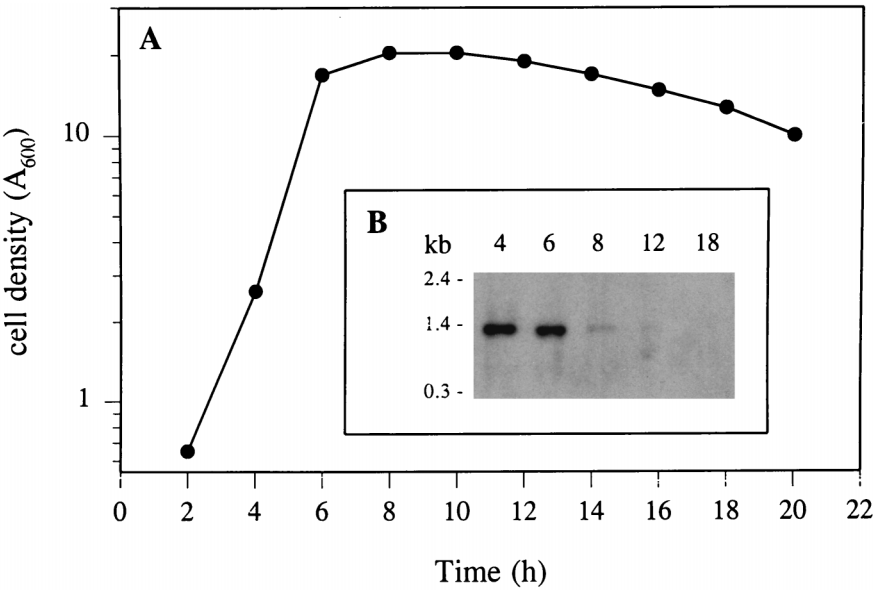

FIG. 2. Expression of the *ldhL* gene. (A) *L. helveticus* 53/7 was cultivated in whey broth at 42°C under pH control (pH 5.6). The cell density is shown as a function of growth. (B) Northern blot analysis of *ldhL* mRNA at different phases of growth. Lanes 1 to 5, samples withdrawn 4, 6, 8, 12, and 16 h after cell inoculation, respectively. The numbers on the left refer to a relevant part of an RNA molecular size marker (Gibco BRL).

stream of the first putative −10 region. The only transcriptional analyses of bacterial *ldhL* genes have been reported for *Lactococcus lactis* (20, 21), *P. acidilactici* (10), and *Bifidobacterium longum* (24). In *L. lactis*, the L-LDH is expressed as part of the *las* operon, where *ldhL* is preceded by the *pfk* and *pyk* genes, encoding phosphofructokinase and pyruvate kinase, respectively. In *P. acidilactici* and *B. longum*, the *ldhL* transcript is monocistronic.

Overexpression and purification of L-LDH. *E. coli* JM105 was transformed with the expression vector pKTH2128 carrying the *ldhL* gene in pKK223-3. After IPTG induction, the amount and activity of L-LDH increased throughout the entire growth phase of 6 h as judged by SDS-PAGE and by determination of the enzyme activity. The yield of L-LDH was approximately 15% of the total cellular proteins. By using two chromatographic steps, L-LDH was purified to homogeneity. The results of the purification are summarized in Table 1 and Fig. 3.

Characterization of the purified L-LDH protein. The electrophoretically pure enzyme preparation migrated as a single band corresponding to a molecular mass of approximately 35 kDa during SDS-PAGE (Fig. 3). The first 20 amino acids of the overexpressed L-LDH were determined to be NH_2-AREEKPRKVILVGDGAVGST, which correspond to the deduced amino acid sequence of the *ldhL* gene lacking the initiation methionine. Analyses of the L-LDH by gel filtration gave calculated molecular masses of 125 to 138 kDa. These results confirm that the native enzyme most probably consists of four subunits.

To confirm the nonallosteric nature of *L. helveticus* L-LDH, which was suggested by the sequence analysis, the enzyme was assayed with 5 mM FBP under acidic conditions (pH 4.5 and 6.0) and with 5 mM FBP and 10 mM $MnSO_4$ under neutral conditions (pH 7.0). At pH 4.5 and 6.0, the enhancement of the enzyme activity with FBP was only 26 and 21%, respectively. Under neutral conditions, the reaction was stimulated fourfold. It has been suggested that the allosteric effect is mainly due to the compensation of the positive charge of H188 by FBP, but in the absence of His, a residual stimulatory effect can also be obtained by interaction of FBP with R173 (32). Although R173 in *L. helveticus* L-LDH also is replaced by a neutral amino acid, it has a Lys residue at position 174, which might have affected the conformation of the substrate binding region and thus could explain the stimulatory effect of FBP at

TABLE 1. Purification of the cloned *L. helveticus* 53/7 L-(+)-LDH from *E. coli* JM105

Purification step	Total protein (mg)	Total activity (U)[a]	Sp act (U/mg)	Purification (fold)	Yield (%)
Crude extract	412.5	127,999.4	310.3	1.0	100
Q-Sepharose	46.9	76,132.8	1,623.3	5.2	59
Phenyl-Sepharose	20.3	43,021.8	2,119.3	6.8	34

[a] One unit corresponds to the reduction of 1 μmol of pyruvate min^{-1} at 25°C.

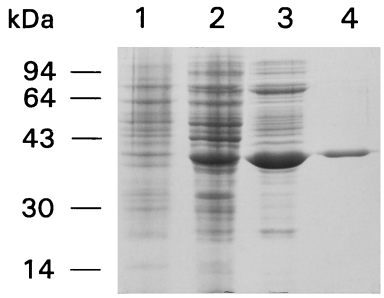

FIG. 3. SDS-PAGE analysis of L-LDH expression and purification in *E. coli*. Lane 1, molecular mass markers; lanes 2 and 3, crude extracts isolated 5 h after IPTG induction from *E. coli* JM105 with pKK223-3 vector and from *E. coli* JM105 with pKK223-3 carrying the *ldhL* gene (pKTH2128), respectively; lanes 4 and 5, L-LDH fractions after anion-exchange chromatography (Q-Sepharose) and hydrophobic interaction chromatography (phenyl-Sepharose), respectively. Gel electrophoresis was performed in an SDS–12% polyacrylamide gel and was followed by staining with Coomassie brilliant blue R-250.

TABLE 2. Effect of chemical reagents on L-LDH activity

Reagent	L-LDH activity (%) at reagent concn of:	
	0.1 mM	1 mM
Control	100	100
DTT	100	108
2-Mercaptoethanol	103	115
pHMB	0	0
pHMB + DTT	0	0
pHMB + 2-mercaptoethanol	0	0

neutral pH. While the activities of the allosteric L-LDHs are strongly repressed in the absence of FBP and they show a sigmoidal saturation curve for pyruvate under these conditions, the *L. helveticus* L-LDH had high constitutive enzyme activity and showed a hyperbolic saturation curve for pyruvate in the absence of FBP. Thus, these results clearly suggest that the *L. helveticus* L-LDH is a nonallosteric enzyme. The sodium phosphate and ammonium acetate buffers increased the enzyme specific activity twofold compared to the sodium acetate buffer, whereas the L-LDH activity was strongly inhibited by Tris-HCl, MES (morpholineethanesulfonic acid), and HEPES buffers.

The substrate specificity assays showed that L-LDH was considerably less active against 2-ketocarboxylic acids with longer aliphatic side chains than against pyruvate, whereas β-hydroxypyruvate and pyruvate were reduced with an equal efficiency. The activities were 3, 0, 28, and 0% for α-ketobutyrate, 3-methyl-2-oxobutanoic acid, β-phenylpyruvate, and 4-methyl-2-oxopentanoic acid, respectively, when compared to that against pyruvate taken as 100%. By mutating *Bacillus stearothermophilus* L-LDH, it has been demonstrated that substrates with large and more-hydrophobic side chains require an enlarged substrate binding pocket compared to that required by pyruvate (15, 29).

Examination of the effects of various metal ions on the L-LDH activity revealed that Ca^{2+} and Zn^{2+} ions at 1 mM stimulated the activity almost twofold. The enzyme was weakly inhibited by 0.1 mM and 1.0 mM Cd^{2+}, Co^{2+}, and Cu^{2+} ions, while Mg^{2+} and Mn^{2+} did not markedly affect the enzyme activity. The effect of chemical reagents on L-LDH activity is shown in Table 2. The enzyme activity was not inhibited by the reducing agents dithiothreitol (DTT) and 2-mercaptoethanol, suggesting that intact disulfide groups are not essential for the enzyme activity or that they are inaccessible to these reagents. The L-LDH was strongly inhibited by *p*-hydroxymercuribenzoic acid (pHMB), a thiol group-modifying reagent. *L. helveticus* L-LDH is a tetramer consisting of four subunits, and each of these has one cysteine residue (C72) (data not shown), which is apparently adjacent to a structurally important loop region of the active site (26). It has been observed that the replacement of the Cys residue by Gly induces some structural changes that affect the catalytic properties of the L-LDH (15). It is thus probable that the total inactivation by thiol modifying was due to a steric hindrance rather than to a direct role of the Cys residue in catalysis. However, the unexpected finding that the activity could not be restored by the reducing agents, DTT and 2-mercaptoethanol may suggest an irreversible change in the enzyme structure caused by pHMB.

The reaction rate was found to be strongly dependent on pH, with a maximum at pH 5.0 (Fig. 4), where the K_m, K_{cat}, and K_{cat}/K_m values for pyruvate reduction were 0.25 mM, 643 s^{-1}, and 2,593 mM^{-1} s^{-1}, respectively. For the nonallosteric L-LDHs from several other LAB, K_m values in the range of 0.37 to 8.0 mM have been reported, whereas for the allosteric enzymes, the reported K_m values were generally lower (11). It has been demonstrated that the binding of pyruvate in L-LDH, to form a stable ternary complex, requires the protonated form of H195 (5, 6) and that the pK value of the His residue determines the pH dependence of K_m (7). The effect of pH on the thermal stability of the L-LDH is shown in Fig. 5. The enzyme was found to be quite unstable under the assay conditions used, since even 10 min of incubation at 50°C decreased the activity down to 10% when the enzyme treatment and the reactions were performed at pH 4.5. Enzyme incubation performed at a higher pH (5.0) resulted in a more rapid inactivation at all temperatures tested, indicating that the lower pH probably stabilizes the protonated form of the enzyme-coenzyme and/or apoenzyme during the catalytic cycle. The low thermostability of the *L. helveticus* L-LDH was unexpected considering the high growth temperature of the strain. Thus, factors not present in the assay must contribute to its stability within the cell. The potential protective effect of Zn^{2+} ion, Ca^{2+} ion, phos-

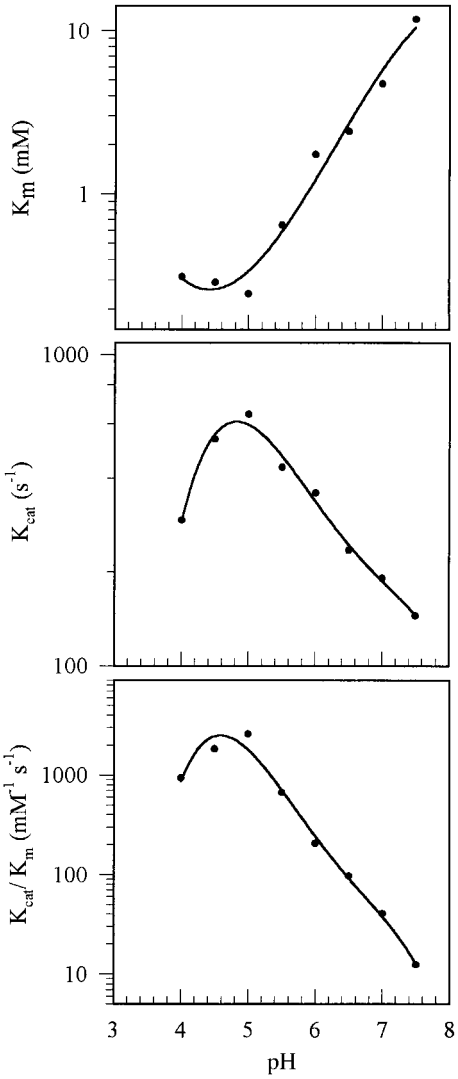

FIG. 4. pH-dependent reduction of pyruvate by the purified L-LDH. K_m and K_{cat} values were calculated by nonlinear regression analyses for different concentrations of pyruvate at saturating levels of NADH in 50 mM sodium acetate at 25°C.

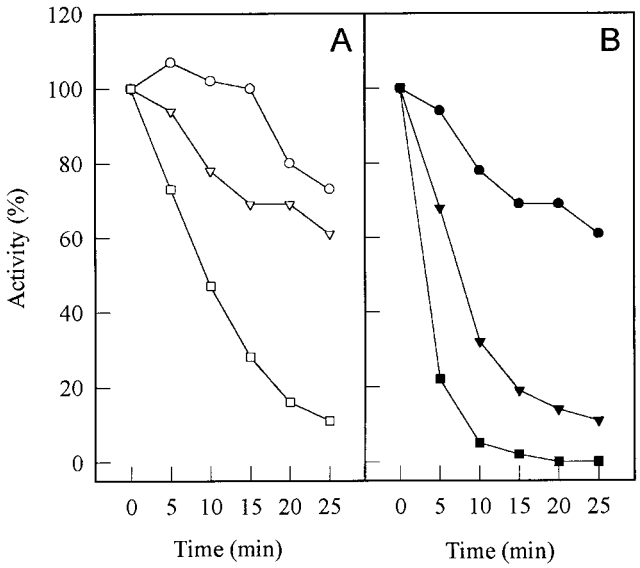

FIG. 5. Stability of the purified L-LDH at different temperatures as a function of time. The L-LDH activity was determined in 50 mM sodium acetate buffers, pH 4.5 (A) and 5.0 (B), at 40°C (circles), 45°C (triangles), or 50°C (squares).

phate buffer, or the allosteric factors on the L-LDH during the heat treatment was not studied.

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