Molecular Characterization of Two Lactate Dehydrogenase Genes with a Novel Structural Organization on the Genome of *Lactobacillus* sp. Strain MONT4

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Two lactate dehydrogenase (*ldh*) genes from *Lactobacillus* sp. strain MONT4 were cloned by complementation in *Escherichia coli* DC1368 (*ldh pfl*) and were sequenced. The sequence analysis revealed a novel genomic organization of the *ldh* genes. Subcloning of the individual *ldh* genes and their Northern blot analyses indicated that the genes are monocistronic.

Lactobacillus sp. strain MONT4 was isolated from high-temperature-fermenting grape musts (2). It is a unique organism due to its capability of fermenting L-arabinose and ribose exclusively to D(–)- and L(+)-lactate via a homolactic fermentation pathway (2). Unlike heterolactic fermentations of pentoses that yield equimolar amounts of lactic and acetic acids, homolactic fermentations of pentoses yield only lactic acid and are unknown among lactobacilli. Our long-range goal is to metabolically engineer *Lactobacillus* sp. strain MONT4 for ethanol production, as it perfectly meets the criteria essential in a commercial biomass-to-ethanol process (25). Metabolic engineering of *Lactobacillus* sp. strain MONT4 for ethanol production requires the introduction of ethanol production genes into this organism. Moreover, inactivation of the lactate dehydrogenase (*ldh*) genes is necessary to eliminate coproduct formation.

Escherichia coli (*pfl ldh*) lacks the ability to grow anaerobically on sugars and thus can be used to clone genes from fermentative pathways (1, 3, 6, 7, 8, 9, 10, 19, 21, 24). In order to clone the *ldh* genes, genomic DNA from *Lactobacillus* sp. strain MONT4 was partially digested with Sau3AI and was ligated to BamHI-digested pJDC9 (5). *E. coli* DC1368 transformants were selected on Luria-Bertani medium containing antibiotics (erythromycin, 1 mg per ml; kanamycin, 60 µg per ml; chloramphenicol, 60 µg per ml) after 48 h of incubation under anaerobic conditions. Four antibiotic-resistant transformants were obtained. One of them, designated *E. coli* DC1368(pUI100), contained a plasmid with a 5-kb insert (Table 1). When the cell extracts from the transformants were subjected to native polyacrylamide gel electrophoresis and lactate dehydrogenase (LDH) activity staining (11), *E. coli* DC1368(pUI100) expressed both L(+)-lactate dehydrogenase (LLDH) and D(–)-lactate dehydrogenase (DLDH) enzymatic activities (Fig. 1).

The DNA sequence of the genomic insert from pUI100 was

determined (GenBank accession no. AY301012). Eleven open reading frames (ORF) were identified. A 1,002-bp ORF that potentially encodes a 334-amino-acid protein was identified as DLDH. BLAST searches revealed that the nucleotide and deduced amino acid sequences for this ORF have identities of 78 to 90% and 43 to 62%, respectively, to the sequences of published DLDH enzymes from other lactic acid bacteria (LAB) (3, 4, 10, 12, 14, 15, 16, 17) (Fig. 2). The nucleic acid and deduced amino acid sequence of the ORF was compared (<http://www.jgi.doe.gov>) to that of unpublished LAB genome sequences (*Bifidobacterium longum* DJO10A, *Brevibacterium linens* BL2 Bio, *Lactobacillus brevis* ATCC 367, *Lactobacillus casei* ATCC 334, *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCCBAA-365, *Lactobacillus gasserii* ATCC 33323, *Lactococcus lactis* subsp. *cremoris* SK11, *Leuconostoc mesenteroides* subsp. *mesenteroides* LA81 [ATCC 8293], *Oenococcus oeni* PSU1 [ATCC BAA-331], *Pediococcus pentosaceus* ATCC 25745, and *Streptococcus thermophilus* LMD-9). At the amino acid level, a 25 to 60% identity and a 45 to 75% similarity within stretches of 244 to 332 amino acids were detected between the deduced amino sequence of the *ldhD* gene and the translated and annotated genome sequences of 11 LAB strains.

Downstream of *ldhD*, a 963-bp ORF that potentially encodes a 321-amino-acid protein was identified as LLDH. BLAST searches indicated that the nucleotide and deduced amino acid sequences for this ORF have identities of 80 to 85% and 59 to 76%, respectively, to the published LLDH enzymes from other LAB strains (8, 9, 13, 14, 18, 19, 20, 22, 23) (Fig. 3). Asp₁₆₈ and Ile₂₅₀ (amino acids involved in catalysis) and Arg₁₇₁, Gln₁₀₂, Thr₂₄₆, and Arg₁₀₉ (amino acids involved in substrate binding and specificity [8, 13]) were identified. BLAST searches were used to compare the nucleic acid and deduced amino acid sequences of the ORF to the unpublished genome sequences from 11 LAB strains. At the amino acid level, 33 to 75% identity and 52 to 89% similarity were detected, within stretches of 305 to 320 amino acids, between the deduced amino sequence of the *ldhL* gene and the translated and annotated genome sequences of nine LAB strains.

Subcloning of the ORFs that potentially code for LDH enzymes was carried out. Two DNA fragments of 1,674 and 1,297

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TABLE 1. Bacterial strains and plasmids

Strain or plasmid	Relevant characteristics	Source or reference ^a
<i>E. coli</i>		
<i>E. coli</i> DH5 α		Invitrogen Life Technologies
<i>E. coli</i> DC1368		David P. Clark
<i>E. coli</i> DC1368(pUI100)		This study
<i>E. coli</i> DH5 α (pJDC9::ldhD)	<i>thr-1 leu-6 thi-6 lacY tonA22 rspL ldhA::Kan pft::Cam</i>	This study
<i>E. coli</i> DH5 α (pJDC9::ldhL)	Em ^r , LLDH positive, DLDH positive	This study
	Em ^r , DLDH positive	This study
<i>Lactobacillus</i>		
<i>Lactobacillus</i> sp. strain MONT4 (DSMZ 20605)		DSMZ
<i>L. acidophilus</i> NRRL B-4495		USDA-ARS Culture Collection
<i>L. amylovorus</i> NRRL B-4538		USDA-ARS Culture Collection
<i>L. brevis</i> NRRL B-4527		USDA-ARS Culture Collection
<i>L. buchneri</i> NRRL B-1837		USDA-ARS Culture Collection
<i>L. casei</i> subsp. <i>casei</i> NRRL B-1922		USDA-ARS Culture Collection
<i>L. coryniformis</i> subsp. <i>coryniformis</i> NRRL B-4391		USDA-ARS Culture Collection
<i>L. coryniformis</i> subsp. <i>torquens</i> NRRL B-4390		USDA-ARS Culture Collection
<i>L. curvatus</i> subsp. <i>curvatus</i> NRRL B-4562		USDA-ARS Culture Collection
<i>L. curvatus</i> WSU		Stephanie Clark
<i>L. delbrueckii</i> subsp. <i>delbrueckii</i> NRRL B-763		USDA-ARS Culture Collection
<i>L. delbrueckii</i> subsp. <i>lactis</i> NRRL B-4525		USDA-ARS Culture Collection
<i>L. farciminius</i> NRRL B-4566		USDA-ARS Culture Collection
<i>L. fermentum</i> NRRL B-4524		USDA-ARS Culture Collection
<i>L. fructosus</i> NRRL B-1841		USDA-ARS Culture Collection
<i>L. gasseri</i> NRRL B-14168		USDA-ARS Culture Collection
<i>L. helveticus</i> NRRL B-4526		USDA-ARS Culture Collection
<i>L. hilgardii</i> NRRL B-1843		USDA-ARS Culture Collection
<i>L. jensenii</i> NRRL B-4550		USDA-ARS Culture Collection
<i>L. malefermentans</i> NRRL B-1953		USDA-ARS Culture Collection
<i>L. mali</i> NRRL B-4563		USDA-ARS Culture Collection
<i>L. paracasei</i> subsp. <i>paracasei</i> NRRL B-4560		USDA-ARS Culture Collection
<i>L. pentosus</i> NRRL B-227		USDA-ARS Culture Collection
<i>L. plantarum</i> NRRL B-4496		USDA-ARS Culture Collection
<i>L. reuteri</i> NRRL B-14171		USDA-ARS Culture Collection
<i>L. rhamnosus</i> NRRL B-442		USDA-ARS Culture Collection
<i>L. ruminis</i> NRRL B-14853		USDA-ARS Culture Collection
<i>L. salivarius</i> subsp. <i>salicinicus</i> NRRL B-1949		USDA-ARS Culture Collection
Plasmids		
pJDC9	6.95 kb; Em ^r <i>lacZ</i> ; strong transcriptional terminators	5
pUI100	11.9 kb; pJDC9::ldhD-ldhL; Em ^r	This study
pJDC9::ldhD	8.6 kb; Em ^r	This study
pJDC9::ldhL	8.2 kb; Em ^r	This study

^a DSMZ, German Collection of Microorganisms and Cell Cultures. USDA-ARS, U. S. Department of Agriculture—Agricultural Research Services.

FIG. 1. Enzymatic activity staining of native polyacrylamide gels after electrophoresis of cell extracts from *E. coli* DC1368 and *E. coli* DC1368 (pUI100) containing the cloned lactate dehydrogenase genes on a 4.687-kb genomic insert from *Lactobacillus* sp. strain MONT4. Three enzymatic staining methods have been employed: LLDH enzymatic activity staining using L(+)-lactic acid as substrate (A); DLDH enzymatic activity staining using D(-)-lactic acid as substrate (B); and LDH enzymatic activity staining using both L(+)-lactic acid and D(-)-lactic acid as substrates (C). Lanes 1, SeeBlue prestained standard (nine polypeptides from 4 to 250 kDa; Invitrogen Life Technologies); lanes 2, purified LDH enzyme (A and C, LLDH; B, DLDH; both from Boehringer Mannheim); lane 2a: purified DLDH; lanes 3, cell extract from *E. coli* DC1368; lanes 4, cell extract from *E. coli* DC1368(pUI100). White and black block arrows indicate the positions of purified LLDH and DLDH enzymes, respectively. Dotted and wavy-lined block arrows indicate the positions of *Lactobacillus* sp. strain MONT4 LLDH and DLDH enzymes, respectively, present in *E. coli* DC1368(pUI100) cell extracts. Thin-line arrows indicate the positions of the top four prestained standards (myosin [250 kDa], bovine serum albumin [98 kDa], glutamic dehydrogenase [64 kDa], and alcohol dehydrogenase [50 kDa]).

<i>Lactobacillus</i> sp. MONT4	(1)	IIIRIVAYGI RAD EKPLVNRWSEKANEPTVSTIGLLEDTTDLAKAGDGVVVYQ--KPTANVNLKASYGVNINSLRVGDNDIDATAVKNPKI	100
<i>Lactobacillus</i> sp.	(1)	--HKIIVYVMDKIPFVWGRGDH--QIVTTITTTTNTTAAAGFDGVSLQAT--EPYSEKFAALQQLCHYLALRVGTDNDIDATAVKNPKI	
<i>L. casei</i>	(1)	--HKIIVYVMDKIPFVWGRGDH--QIVTTITTTTNTTAAAGFDGVSLQAT--EPYSEKFAALQQLCHYLALRVGTDNDIDATAVKNPKI	
<i>L. delbrueckii</i> I	(1)	--HKIIVYVMDKIPFVWGRGDH--QIVTTITTTTNTTAAAGFDGVSLQAT--EPYSEKFAALQQLCHYLALRVGTDNDIDATAVKNPKI	
<i>L. delbrueckii</i> II	(1)	--HKIIVYVMDKIPFVWGRGDH--QIVTTITTTTNTTAAAGFDGVSLQAT--EPYSEKFAALQQLCHYLALRVGTDNDIDATAVKNPKI	
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> I	(1)	--HKIIVYVMDKIPFVWGRGDH--QIVTTITTTTNTTAAAGFDGVSLQAT--EPYSEKFAALQQLCHYLALRVGTDNDIDATAVKNPKI	
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> II	(1)	--HKIIVYVMDKIPFVWGRGDH--QIVTTITTTTNTTAAAGFDGVSLQAT--EPYSEKFAALQQLCHYLALRVGTDNDIDATAVKNPKI	
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> III	(1)	--HKIIVYVMDKIPFVWGRGDH--QIVTTITTTTNTTAAAGFDGVSLQAT--EPYSEKFAALQQLCHYLALRVGTDNDIDATAVKNPKI	
<i>L. gasseri</i>	(1)	--HKIIVYVMDKIPFVWGRGDH--QIVTTITTTTNTTAAAGFDGVSLQAT--EPYSEKFAALQQLCHYLALRVGTDNDIDATAVKNPKI	
<i>L. helveticus</i>	(1)	--HKIIVYVMDKIPFVWGRGDH--QIVTTITTTTNTTAAAGFDGVSLQAT--EPYSEKFAALQQLCHYLALRVGTDNDIDATAVKNPKI	
<i>L. johnsonii</i> I	(1)	--HKIIVYVMDKIPFVWGRGDH--QIVTTITTTTNTTAAAGFDGVSLQAT--EPYSEKFAALQQLCHYLALRVGTDNDIDATAVKNPKI	
<i>L. johnsonii</i> II	(1)	--HKIIVYVMDKIPFVWGRGDH--QIVTTITTTTNTTAAAGFDGVSLQAT--EPYSEKFAALQQLCHYLALRVGTDNDIDATAVKNPKI	
<i>L. paracasei</i>	(1)	--HKIIVYVMDKIPFVWGRGDH--QIVTTITTTTNTTAAAGFDGVSLQAT--EPYSEKFAALQQLCHYLALRVGTDNDIDATAVKNPKI	
<i>L. pentosus</i>	(1)	--HKIIVYVMDKIPFVWGRGDH--QIVTTITTTTNTTAAAGFDGVSLQAT--EPYSEKFAALQQLCHYLALRVGTDNDIDATAVKNPKI	
<i>L. plantarum</i>	(1)	--HKIIVYVMDKIPFVWGRGDH--QIVTTITTTTNTTAAAGFDGVSLQAT--EPYSEKFAALQQLCHYLALRVGTDNDIDATAVKNPKI	
<i>Leu. mesenteroides</i> subsp. <i>cremoris</i>	(1)	--HKIIVYVMDKIPFVWGRGDH--QIVTTITTTTNTTAAAGFDGVSLQAT--EPYSEKFAALQQLCHYLALRVGTDNDIDATAVKNPKI	
<i>Leu. mesenteroides</i> subsp. <i>mesenteroides</i> I	(1)	--HKIIVYVMDKIPFVWGRGDH--QIVTTITTTTNTTAAAGFDGVSLQAT--EPYSEKFAALQQLCHYLALRVGTDNDIDATAVKNPKI	
<i>Leu. mesenteroides</i> subsp. <i>mesenteroides</i> II	(1)	--HKIIVYVMDKIPFVWGRGDH--QIVTTITTTTNTTAAAGFDGVSLQAT--EPYSEKFAALQQLCHYLALRVGTDNDIDATAVKNPKI	
<i>O. oeni</i> I	(1)	--HKIIVYVMDKIPFVWGRGDH--QIVTTITTTTNTTAAAGFDGVSLQAT--EPYSEKFAALQQLCHYLALRVGTDNDIDATAVKNPKI	
<i>O. oeni</i> II	(1)	--HKIIVYVMDKIPFVWGRGDH--QIVTTITTTTNTTAAAGFDGVSLQAT--EPYSEKFAALQQLCHYLALRVGTDNDIDATAVKNPKI	
<i>O. oeni</i> III	(1)	--HKIIVYVMDKIPFVWGRGDH--QIVTTITTTTNTTAAAGFDGVSLQAT--EPYSEKFAALQQLCHYLALRVGTDNDIDATAVKNPKI	
<i>P. acidilactici</i> I	(1)	--HKIIVYVMDKIPFVWGRGDH--QIVTTITTTTNTTAAAGFDGVSLQAT--EPYSEKFAALQQLCHYLALRVGTDNDIDATAVKNPKI	
<i>P. acidilactici</i> II	(1)	--HKIIVYVMDKIPFVWGRGDH--QIVTTITTTTNTTAAAGFDGVSLQAT--EPYSEKFAALQQLCHYLALRVGTDNDIDATAVKNPKI	
<i>P. pentosaceus</i>	(1)	--HKIIVYVMDKIPFVWGRGDH--QIVTTITTTTNTTAAAGFDGVSLQAT--EPYSEKFAALQQLCHYLALRVGTDNDIDATAVKNPKI	
Consensus	(1)	HKIIVYVMDKIPFVWGRGDH--QIVTTITTTTNTTAAAGFDGVSLQAT--EPYSEKFAALQQLCHYLALRVGTDNDIDATAVKNPKI	
<i>Lactobacillus</i> sp. MONT4	(100)	VPAYSPQALAEFTVTEHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	200
<i>Lactobacillus</i> sp.	(97)	VPAYSPQALAEFTVTEHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>L. casei</i>	(97)	VPAYSPQALAEFTVTEHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>L. delbrueckii</i> I	(97)	VPAYSPQALAEFTVTEHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>L. delbrueckii</i> II	(97)	VPAYSPQALAEFTVTEHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> I	(97)	VPAYSPQALAEFTVTEHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> II	(97)	VPAYSPQALAEFTVTEHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> III	(97)	VPAYSPQALAEFTVTEHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>L. gasseri</i>	(97)	VPAYSPQALAEFTVTEHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>L. helveticus</i>	(97)	VPAYSPQALAEFTVTEHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>L. johnsonii</i> I	(97)	VPAYSPQALAEFTVTEHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>L. johnsonii</i> II	(97)	VPAYSPQALAEFTVTEHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>L. paracasei</i>	(97)	VPAYSPQALAEFTVTEHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>L. pentosus</i>	(97)	VPAYSPQALAEFTVTEHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>L. plantarum</i>	(97)	VPAYSPQALAEFTVTEHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>Leu. mesenteroides</i> subsp. <i>cremoris</i>	(97)	VPAYSPQALAEFTVTEHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>Leu. mesenteroides</i> subsp. <i>mesenteroides</i> I	(97)	VPAYSPQALAEFTVTEHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>Leu. mesenteroides</i> subsp. <i>mesenteroides</i> II	(97)	VPAYSPQALAEFTVTEHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>O. oeni</i> I	(97)	VPAYSPQALAEFTVTEHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>O. oeni</i> II	(97)	VPAYSPQALAEFTVTEHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>O. oeni</i> III	(97)	VPAYSPQALAEFTVTEHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>P. acidilactici</i> I	(97)	VPAYSPQALAEFTVTEHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>P. acidilactici</i> II	(97)	VPAYSPQALAEFTVTEHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>P. pentosaceus</i>	(20)	VPAYSPQALAEFTVTEHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
Consensus	(101)	VPAYSPQALAEFTVTEHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>Lactobacillus</i> sp. MONT4	(198)	YASDVLSLHVPALNDYHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	300
<i>Lactobacillus</i> sp.	(197)	YASDVLSLHVPALNDYHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>L. casei</i>	(197)	YASDVLSLHVPALNDYHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>L. delbrueckii</i> I	(197)	YASDVLSLHVPALNDYHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>L. delbrueckii</i> II	(197)	YASDVLSLHVPALNDYHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> I	(197)	YASDVLSLHVPALNDYHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> II	(197)	YASDVLSLHVPALNDYHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> III	(197)	YASDVLSLHVPALNDYHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>L. gasseri</i>	(197)	YASDVLSLHVPALNDYHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>L. helveticus</i>	(197)	YASDVLSLHVPALNDYHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>L. johnsonii</i> I	(197)	YASDVLSLHVPALNDYHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>L. johnsonii</i> II	(197)	YASDVLSLHVPALNDYHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>L. paracasei</i>	(197)	YASDVLSLHVPALNDYHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>L. pentosus</i>	(197)	YASDVLSLHVPALNDYHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>L. plantarum</i>	(197)	YASDVLSLHVPALNDYHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>Leu. mesenteroides</i> subsp. <i>cremoris</i>	(197)	YASDVLSLHVPALNDYHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>Leu. mesenteroides</i> subsp. <i>mesenteroides</i> I	(197)	YASDVLSLHVPALNDYHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>Leu. mesenteroides</i> subsp. <i>mesenteroides</i> II	(197)	YASDVLSLHVPALNDYHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>O. oeni</i> I	(197)	YASDVLSLHVPALNDYHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>O. oeni</i> II	(197)	YASDVLSLHVPALNDYHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>O. oeni</i> III	(197)	YASDVLSLHVPALNDYHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>P. acidilactici</i> I	(197)	YASDVLSLHVPALNDYHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>P. acidilactici</i> II	(197)	YASDVLSLHVPALNDYHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>P. pentosaceus</i>	(118)	YASDVLSLHVPALNDYHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
Consensus	(201)	YASDVLSLHVPALNDYHMLNTRKTPFQKLANC--DLMAAPVAAELNQMTCUFATGICGPAITQYKCGAKIAYVFNHPELKEKGIYVDSIDDL	
<i>Lactobacillus</i> sp. MONT4	(298)	PHAFYTTKAVNNHVFAMANDKSLIETGNSDKLWKF----	342
<i>Lactobacillus</i> sp.	(296)	PHAFYTTKAVNNHVFAMANDKSLIETGNSDKLWKF----	
<i>L. casei</i>	(294)	PHAFYTTKAVNNHVFAMANDKSLIETGNSDKLWKF----	
<i>L. delbrueckii</i> I	(296)	PHAFYTTKAVNNHVFAMANDKSLIETGNSDKLWKF----	
<i>L. delbrueckii</i> II	(296)	PHAFYTTKAVNNHVFAMANDKSLIETGNSDKLWKF----	
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> I	(296)	PHAFYTTKAVNNHVFAMANDKSLIETGNSDKLWKF----	
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> II	(296)	PHAFYTTKAVNNHVFAMANDKSLIETGNSDKLWKF----	
<i>L. delbrueckii</i> subsp. <i>bulgaricus</i> III	(296)	PHAFYTTKAVNNHVFAMANDKSLIETGNSDKLWKF----	
<i>L. gasseri</i>	(296)	PHAFYTTKAVNNHVFAMANDKSLIETGNSDKLWKF----	
<i>L. helveticus</i>	(296)	PHAFYTTKAVNNHVFAMANDKSLIETGNSDKLWKF----	
<i>L. johnsonii</i> I	(296)	PHAFYTTKAVNNHVFAMANDKSLIETGNSDKLWKF----	
<i>L. johnsonii</i> II	(296)	PHAFYTTKAVNNHVFAMANDKSLIETGNSDKLWKF----	
<i>L. paracasei</i>	(294)	PHAFYTTKAVNNHVFAMANDKSLIETGNSDKLWKF----	
<i>L. pentosus</i>	(295)	PHAFYTTKAVNNHVFAMANDKSLIETGNSDKLWKF----	
<i>L. plantarum</i>	(295)	PHAFYTTKAVNNHVFAMANDKSLIETGNSDKLWKF----	
<i>Leu. mesenteroides</i> subsp. <i>cremoris</i>	(295)	PHAFYTTKAVNNHVFAMANDKSLIETGNSDKLWKF----	
<i>Leu. mesenteroides</i> subsp. <i>mesenteroides</i> I	(295)	PHAFYTTKAVNNHVFAMANDKSLIETGNSDKLWKF----	
<i>Leu. mesenteroides</i> subsp. <i>mesenteroides</i> II	(295)	PHAFYTTKAVNNHVFAMANDKSLIETGNSDKLWKF----	
<i>O. oeni</i> I	(261)	PHAFYTTKAVNNHVFAMANDKSLIETGNSDKLWKF----	
<i>O. oeni</i> II	(261)	PHAFYTTKAVNNHVFAMANDKSLIETGNSDKLWKF----	
<i>O. oeni</i> III	(261)	PHAFYTTKAVNNHVFAMANDKSLIETGNSDKLWKF----	
<i>P. acidilactici</i> I	(294)	PHAFYTTKAVNNHVFAMANDKSLIETGNSDKLWKF----	
<i>P. acidilactici</i> II	(294)	PHAFYTTKAVNNHVFAMANDKSLIETGNSDKLWKF----	
<i>P. pentosaceus</i>	(153)	PHAFYTTKAVNNHVFAMANDKSLIETGNSDKLWKF----	
Consensus	(301)	PHAFYTTKAVNNHVFAMANDKSLIETGNSDKLWKF----	

FIG. 2. Alignment of the deduced amino acid sequence of *ldhD* from *Lactobacillus* sp. strain MONT4 and the published amino acid sequences of the *ldhD* genes from other LAB and LAB-like organisms. *L.*, *Lactobacillus*; *Leu.*, *Leuconostoc*; *O.*, *Oenococcus*; *P.*, *Pediococcus*. I, II, and III represent multiple amino acid sequences from the same species. Letter colors: black, dissimilar sequence; black with gray background, similar sequence; blue, conservative sequence; red with gray background, identical sequence; green, weakly similar sequence.

FIG. 3. Alignment of the deduced amino acid sequence of *ldhL* from *Lactobacillus* sp. strain MONT4 and the published amino acid sequences of *ldhL* genes from other LAB and LAB-like organisms. *B.*, *Bifidobacterium*; *Lc.*, *Lactococcus*; *L.*, *Lactobacillus*; *P.*, *Pediococcus*; *S.*, *Streptococcus*. I, II, and III represent multiple amino acid sequences from the same species. Letter colors: black, dissimilar sequence; black with gray background, similar sequence; blue, conservative sequence; red with gray background, identical sequence; green, weakly similar sequence.

bp that include the *ldhD* and *ldhL* genes, respectively, were amplified, ligated to pJDC9, introduced into *E. coli* DH5 α , and screened by blue-white screening. Two erythromycin-resistant white-colored transformants containing the subcloned *ldhD* and *ldhL* genes were designated *E. coli* DH5 α (pJDC9::*ldhD*) and *E. coli* DH5 α (pJDC9::*ldhL*), respectively (Table 1). *E. coli* DH5 α (pJDC9::*ldhD*) expressed only DLDH activity, while *E. coli* DH5 α (pJDC9::*ldhL*) expressed only LLDH activity.

As determined by DNA sequencing, the *ldhD* gene from *Lactobacillus* sp. strain MONT4 is located upstream of the *ldhL* gene. Such structural organization of the *ldh* genes on genomes of LAB strains has not been reported until now. When the unpublished genomes of 11 LAB strains were investigated, no *ldhD* or *ldhL* gene was found to be located upstream or downstream from each other in any of the 8 organisms that appeared to contain both *ldhD* and *ldhL* genes. Our goal is to understand the biological significance of this novel organization by generating and characterizing isogenic mutants.

Northern blots were performed to study the expression of the *ldh* genes as a function of growth using three probes (663-bp probe for *ldhD*, 819-bp probe for *ldhL*, and 1,755-bp probe for *ldhD*-*ldhL*). Low and high levels of transcription were observed at late lag and late exponential stages of growth, respectively. Expression of the *ldhL* genes in *Lactobacillus* spp. seems to be species dependent, as the *ldhL* from *Lactobacillus helveticus* revealed a high level of expression at the exponential phase of growth (22). The *ldh* genes from *Lactobacillus* sp. strain MONT4 were found to be monocistronic, with a transcript size of 1.1 kb each. The only polycistronic *ldh* gene from LAB strains is the one from *Lactococcus lactis*. It is part of an operon along with the genes encoding phosphofructokinase and pyruvate kinase enzymes (18).

Based on a neighbor-joining tree generated with 16S rRNA gene alignments, 27 strains representing 24 *Lactobacillus* species (Table 1) were selected for Southern blots. Low- and high-stringency Southern blots of the PstI-digested total genomic DNA were conducted using the *ldhD*, *ldhL*, and *ldhD*-*ldhL* probes. Unlike low-stringency conditions, no hybridization under high-stringency conditions was detected between the *ldh* probes and the genomic DNA from 24 *Lactobacillus* species, suggesting that differences exist among LDHs at the nucleotide level.

Our present research deals with the inactivation of the *ldh* genes and metabolic engineering of the *ldh*-deficient strains for ethanol production.

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