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Relative Catalytic Efficiency of *ldhL*- and *ldhD*-Encoded Products Is Crucial for Optical Purity of Lactic Acid Produced by *Lactobacillus* Strains

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NAD-dependent L- and D-lactate dehydrogenases coexist in *Lactobacillus* genomes and may convert pyruvic acid into L-lactic acid and D-lactic acid, respectively. Our findings suggest that the relative catalytic efficiencies of *ldhL*- and *ldhD*-encoded products are crucial for the optical purity of lactic acid produced by *Lactobacillus* strains.

Lactic acid is a building block widely used in the food, pharmaceutical, and chemical industries (6). The use of lactic acid in the synthesis of polylactic acid has grown over the years, and high optical purity is an inevitable prerequisite for lactic acid polymerization (1, 23). Strains of *Lactobacillus*, the largest genus of lactic acid bacteria (LAB), are the most frequently used lactic acid producers (2, 4, 10, 19, 22, 24), but the optical purities of lactic acid produced by various *Lactobacillus* strains are markedly different (Table 1) (3, 15).

The enzymes responsible for L- and D-lactic acid production are NAD-dependent L-lactate dehydrogenases (L-nLDHs) and NAD-dependent D-lactate dehydrogenases (D-nLDHs), respectively, which fall into two different families and are encoded by *ldhL* and *ldhD*, respectively (7, 20). Lactate racemase, which converts L-lactic acid into D-lactic acid, has only been reported in a few DL-type *Lactobacillus* strains. The enzyme was found in only four (*L. curvatus*, *L. paracasei*, *L. plantarum* WCFS1, and *L. sakei* 23k) of hundreds of *Lactobacillus* strains that have been sequenced or reported. However, both *ldhL* and *ldhD* have been shown to be widely distributed in all sequenced or reported *Lactobacillus* strains except *L. sakei* 23k (7, 8, 13) (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). Due to the fact that the optical purities of lactic acid are clearly different and in some cases even completely opposite in various *Lactobacillus* strains (3, 15), studies focusing on the relationship between the *ldhL* and *ldhD* genes and the optical purity of lactic acid are needed.

In this study, *Lactobacillus delbrueckii* subsp. *bulgaricus* ATCC 11842, *L. plantarum* ssp. *plantarum* ATCC 14917, and *Lactobacillus casei* ATCC 334 were selected as representative strains for exhaustive analysis. These three species are lactic acid high-producing strains, and their lactic acid products represent three different types (D, DL, and L, respectively) (see the supplemental material for details of batch fermentation) (Table 1) (5, 9, 14, 16, 17, 18, 23). Moreover, both *ldhL* and *ldhD* but no lactate racemase genes or homologs were found in all three genomes during sequencing and annotation (http://www.ncbi.nlm.nih.gov/genomes/lproks.cgi). First, the enzymatic activities of L- and D-nLDHs in these three *Lactobacillus* strains were determined using whole-cell extracts. Because the substrates for both L- and D-nLDHs are pyruvate and NADH, the reduction activity of these enzymes cannot be measured by monitoring the decrease in NADH. Instead, the reduced products, L- and D-lactic acid, were used to evaluate enzymatic activity (see the supplemental material for details). As shown in Table 2, the relative activities of L- and D-nLDHs were evidently different in the *Lactobacillus* strains. In *L. bulgaricus* ATCC 11842 (D-LAB strain), the specific activity of D-nLDH was much higher than that of L-nLDH, about 111-fold; in *L. plantarum* ATCC 14917 (DL-LAB strain), the specific activity of D-nLDH was similar to that of L-nLDH; and in *L. casei* ATCC 334 (L-LAB strain), the specific activity of L-nLDH was higher than that of D-nLDH, about 39-fold.

TABLE 1 Comparison of enantiomeric excess (ee) values of lactic acid produced by the three representative *Lactobacillus* strains after 24 h of batch fermentation

Strain	Concn of total lactic acid (g l^{-1})	ee value of L-lactic acid (%)	ee value of D-lactic acid (%)
L. bulgaricus ATCC 11842	18.1 ± 0.5		97.2 ± 0.1
L. plantarum ATCC 14917	18.4 ± 0.1		3.3 ± 1.7
L. casei ATCC 334	18.3 ± 0.1	92.0 ± 0.8	

Next, the relative transcriptional levels of *ldhL* and *ldhD* in the three representative strains were examined. Little transcriptional analysis of *ldhL* and *ldhD* has been reported previously (11). In this study, cells of the three representative strains in the middle of the exponential phase were collected for RNA isolation using the E.Z.N.A. bacterial RNA kit (Omega Bio-Tek). The isolated total RNA was treated with DNase I (Fermentas) and then used as a template for cDNA synthesis with TransScript first-strand cDNA synthesis supermix (TransGen Biotech). The resultant cDNA was used for quantitative PCR (qPCR) analysis. Primers for six different genes were designed with Beacon Designer software (see Table S1 in the supplemental material). The qPCRs were performed using the LightCycler 480 Real-Time PCR system with LightCycler 480 SYBR green I master (Roche) according to the manu-

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TABLE 2 The specific activities of L- and D-nLDHs of *Lactobacillus* strains in exponential phase

Strain	Sp act (μmol min^{-1} mg^{-1})		Sp act ratio of L-nLDH to D-nLDH
	L-nLDH	D-nLDH	
L. bulgaricus ATCC 11842	$(1.3 \pm 0.1) \times 10^{-1}$	14.4 ± 0.7	1:111
L. plantarum ATCC 14917	2.4 ± 0.2	4.3 ± 0.2	1:1.8
L. casei ATCC 334	12.4 ± 0.7	$(3.2 \pm 0.3) \times 10^{-1}$	39:1

facturer's instructions. As shown in Fig. 1, the transcriptional levels of *ldhD* were higher than those of *ldhL*, from about 2-fold to 20-fold, in the three representative strains.

The catalytic efficiencies of *ldhL*- and *ldhD*-encoded products were also measured using purified proteins. The *ldhL* and *ldhD* genes of the three strains were heterologously expressed. Six recombinant plasmids containing the various *ldhL* and *ldhD* genes were constructed and transformed separately into *Escherichia coli* Rosetta(DE3) (see Table S1 in the supplemental material). These strains were incubated aerobically in lysogeny broth medium (100 μg ml^{-1} ampicillin) at 37°C to an optical density of 0.6 at 600 nm. Then, 1 mM isopropyl-β-D-thiogalactopyranoside (IPTG) was added to induce protein expression, and cultures were grown at 25°C for a further 5 h. Cells were harvested by centrifugation and washed with 0.85% (wt/vol) sodium chloride solution. Cell pellets were subsequently suspended in a binding buffer (20 mM sodium phosphate, 500 mM sodium chloride, and 20 mM imidazole [pH 7.4]) and then disrupted by sonication. Thereafter, intact cells and cell debris were removed by centrifugation, and the resultant supernatant was filtered and loaded onto a HisTrap HP 5-ml column (GE Healthcare). Purification was performed with gradient elution by using an elution buffer (20 mM sodium phosphate, 500 mM sodium chloride, and 500 mM imidazole [pH 7.4]). All nLDHs were purified to electrophoretic homogeneity for the activity assay. The catalytic efficiencies of recombinant nLDHs were calculated by k_{cat}/K_m (Table 3). For *L. bulgaricus* ATCC 11842 (D-LAB strain), D-nLDH showed a high catalytic efficiency toward

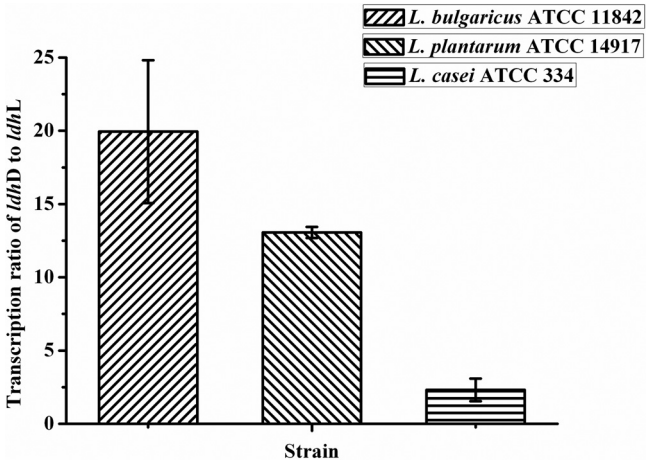

FIG 1 Determination of the relative transcriptional levels of *ldhD* and *ldhL* by using qPCR. Error bars represent the standard deviations of the means of three independent experiments.

TABLE 3 Kinetic parameters of purified heterologously expressed His-tagged L- and D-nLDHs of the three representative *Lactobacillus* strains

Strain	L-nLDH[a]				D-nLDH				Catalytic efficiency ratio of L-nLDH to D-nLDH
	K_m (mM)	V_{max} (U mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	K_m (mM)	V_{max} (U mg^{-1})	k_{cat} (s^{-1})	k_{cat}/K_m (M^{-1} s^{-1})	
L. bulgaricus ATCC 11842	ND	ND	ND	ND	1.1 ± 0.2	2771.9 ± 307.2	1947.2 ± 215.8	$(1.8 \pm 0.2) \times 10^6$	0
L. plantarum ATCC 14917	1.7 ± 0.2	27.5 ± 2.1	18.0 ± 1.4	$(1.0 \pm 0.0) \times 10^4$	3.1 ± 0.4	371.4 ± 32.2	264.5 ± 22.9	$(8.7 \pm 0.5) \times 10^4$	1:8.7
L. casei ATCC 334	1.0 ± 0.1	969.1 ± 49.4	655.7 ± 33.4	$(6.3 \pm 0.6) \times 10^5$	3.5 ± 0.2	18.7 ± 0.6	13.2 ± 0.4	$(3.8 \pm 0.1) \times 10^3$	166:1

[a] ND, not detected.

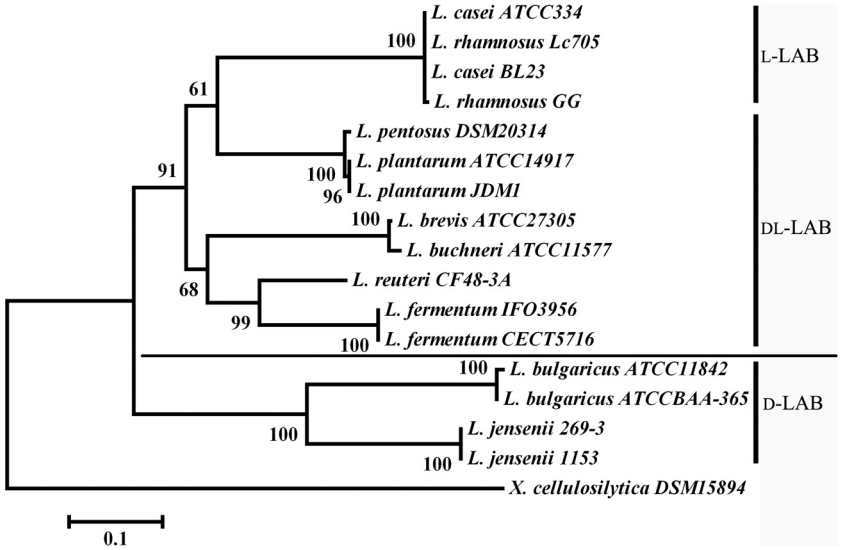

FIG 2 NJ tree based on L-nLDH amino acid sequences from various *Lactobacillus* strains, rooted with *Xylanimonas cellulosilytica*. Numbers at nodes indicate the percentage of NJ bootstrap analyses with 1,000 replicates. The scale bar indicates the level of amino acid sequence divergence.

pyruvate, whereas L-nLDH showed no detectable activity; for *L. plantarum* ATCC 14917 (DL-LAB strain), D- and L-nLDHs showed similar catalytic efficiencies toward pyruvate; for *L. casei* ATCC 334 (L-LAB strain), L-nLDH showed approximately 166-fold higher catalytic efficiency than D-nLDH. On the other hand, D-nLDH of *L. bulgaricus* ATCC 11842 (D-LAB strain) exhibited the highest catalytic efficiency of the three D-nLDHs, and L-nLDH of *L. casei* ATCC 334 (L-LAB strain) exhibited the highest catalytic efficiency of the three L-nLDHs.

Because the L- and D-nLDHs of the different strains exhibited considerable sequence identity (40% to 70%) (see Fig. S1 and S2 in the supplemental material) but various catalytic efficiencies (Tables 2 and 3), multiple alignment of the cloned L- and D-nLDH sequences was undertaken by using Clustal X2 (see Fig. S1 and S2) (12). The analysis revealed that certain pivotal residues are mutated in L-nLDH of *L. bulgaricus* ATCC 11842 (D-LAB) and D-nLDH of *L. casei* ATCC 334 (L-LAB) (see the supplemental material for details). These mutations may affect the activities of L- and D-nLDHs in different *Lactobacillus* strains and result in the different optical purities of lactic acid.

In addition to L- and D-nLDHs from the three representative strains, a series of other L- and D-nLDHs from other *Lactobacillus* strains was selected for phylogenetic analysis by using MEGA4 with the neighbor-joining (NJ) analytic approach (see Table S2 in the supplemental material) (21). The NJ tree showed that L-nLDHs of D-LAB strains (*Lactobacillus bulgaricus* and *Lactobacillus jensenii*) form a monophyletic group, whereas L-nLDHs of DL-LAB and L-LAB strains cluster to another group (Fig. 2). This topology was consistent with the activity assay results shown in Tables 2 and 3, where the L-nLDH catalytic efficiency of D-LAB

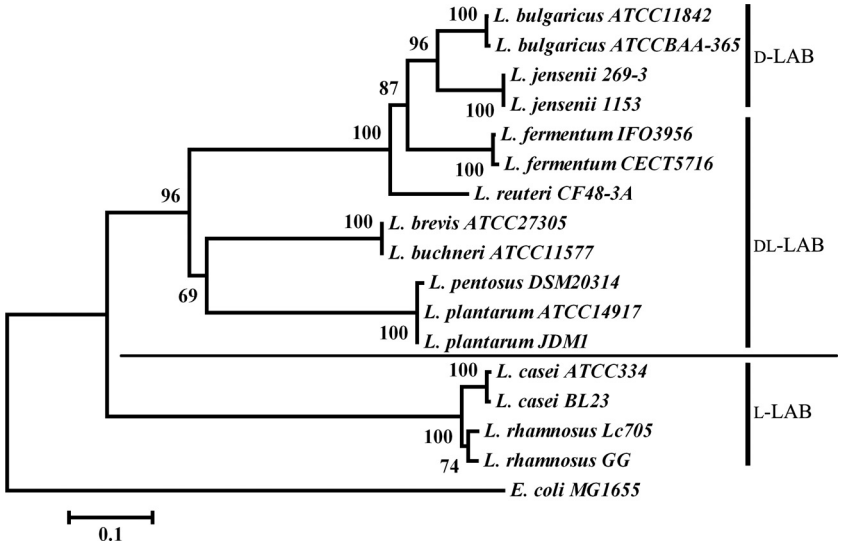

FIG 3 NJ tree based on D-nLDH amino acid sequences from various *Lactobacillus* strains, rooted with *E. coli*. Numbers at nodes indicate the percentage of NJ bootstrap analyses with 1,000 replicates. The scale bar indicates the level of amino acid sequence divergence.

strains was distinctly different from that of DL-LAB and L-LAB strains. Similarly, D-nLDHs of L-LAB strains (*L. casei* and *Lactobacillus rhamnosus*) also form a monophyletic group, whereas D-nLDHs of DL-LAB and D-LAB strains cluster together (Fig. 3). It may be inferred that, because of holistic changes, L-/D-nLDHs of different *Lactobacillus* strains formed separate clades, resulting in different catalytic efficiencies; these different catalytic efficiencies of L- and D-nLDHs resulted in different optical purities of lactic acid and different types of *Lactobacillus* strains.

In summary, the relative catalytic efficiencies of L- and D-nLDHs displayed evident differences, although the transcription of *ldhL* and *ldhD* showed no obvious distinctions among various *Lactobacillus* strains. As the optical purity of the lactic acid monomer is pivotal for polymerization, the production of optically pure lactic acid is of significant importance. The observation that the relative catalytic efficiencies of *ldhL*- and *ldhD*-encoded products are crucial for lactic acid optical purity may provide useful guidance for lactic acid production processes.

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