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Cloning and Characterization of the L-Lactate Dehydrogenase Gene (*ldhL*) from *Lactobacillus reuteri* ATCC 55739

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Abstract The *ldhL* gene encoding the L-(+) lactate dehydrogenase was cloned from *Lactobacillus reuteri* ATCC 55739 chromosomal DNA and characterized. An internal 750-bp fragment of *ldhL* gene was amplified by PCR using primers based on the conserved region of lactobacilli *ldhL* genes. A genomic library of *L. reuteri* ATCC 55739 was constructed using pBR322, and colony hybridization experiments were performed using the 750-bp fragment as a probe. One clone harboring a 4.0-kb *PstI* fragment was identified, and nucleotide sequencing confirmed it as an open reading frame of 972 bp in size in the middle. In addition to *ldhL* gene, an ORF homologous to *Streptococcus pneumoniae* TIGR4 hydrolase gene and 3' part of phosphomevalonate kinase gene (*mvaK2*) were also found on the 4 kb fragment. L-LDH of *L. reuteri* ATCC 55739 showed the highest degree of homology with the L-LDH of *Pediococcus acidilactici* (62.4%), followed by the L-LDH of *Lactobacillus pentosus* (58.7%). The size of *ldhL* transcript determined by Northern blot was 1 kb, indicating the monocistronic nature of *ldhL*.

Key words: *Lactobacillus reuteri*, L-LDH, *ldhL*, gene cloning

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 for food and feed products [28]. Depending on the species, LAB synthesize either the L-(+) or D-(−) isomeric form 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 biodegradable lactide polymers for the plastic and health care industries [31]. In homofermentative LAB, lactic acid is one of the major end products of carbohydrate fermentation. Glucose is converted to pyruvate via the Embden-Meyerhof-Parnas pathway, and pyruvate is reduced to two isomeric forms of lactic acid by two distinct NAD-dependent, stereospecific lactate dehydrogenases; L-lactate dehydrogenase (L-LDH) (EC 1.1.1.27) and D-lactate dehydrogenase (D-LDH) (EC 1.1.1.28). The evolutionary relationship between L-LDH and D-LDH is not fully understood. It has been suggested that L-LDH and D-LDH are originated 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, 19, 20, 21, 39]. L-LDH has been found to function as an allosteric enzyme activated by fructose 1,6-diphosphate (FBP) or as a nonallosteric enzyme [9]. Allosteric L-LDHs have been purified, characterized, and the corresponding genes were cloned from a variety of eukaryotes and prokaryotes, and their primary and tertiary structures have been extensively studied [4, 5, 14, 33, 42]. Much less information is available for bacterial nonallosteric L-LDHs. Recently, the genes encoding the nonallosteric L-LDHs of *Pediococcus acidilactici* [8], *Streptococcus thermophilus* [13], and the closely related *ldhL* genes from *Lactobacillus plantarum* [7], *Lactobacillus pentosus* [39], and *Lactobacillus helveticus* [35] have been cloned and sequenced. In the present work, we report the cloning, DNA sequencing, and mRNA analysis of a *ldhL* gene from *L. reuteri* ATCC 55739, which was initially isolated from intestines of rat, and one of the promising probiotic strains.

Materials and Methods

Bacterial Strains, Plasmids, and Growth Conditions

The bacterial strains and plasmids used in this study are listed in Table 1. *Lactobacillus reuteri* ATCC 55739

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Table 1. Bacterial strains and plasmids used in this study.

Strains and plasmids	Characteristic(s)[a]	Source or reference
Strains		
L. reuteri ATCC 55739	Wild-type strain	ATCC
E. coli DH5α	φ80*dlacZ*ΔM15, *recA1*, *endA1*,*gyrA96*,*thi-1*, *hsdR17*(r_k^-, m_k^+), *relA1*, *deoR*, Δ(*lacZYA-argF*)U169	BRL
Plasmids		
pBR322	4,361 bp Ap^r, Tc^r, general cloning vector	NEB
pBluescriptKS(+)	2,961 bp Ap^r, phagemid derived from pUC19	STRATAGENE®
pJY1	pBluescriptKS(+) containing 750 bp PCR fragment corresponding to the internal region of *ldhL*. 3.7 kb	This study
pJY2	pBR322 containing a 4.0 kb *Pst*I fragment derived from *L. reuteri* ATCC 55739 Ch. DNA. 8.35 kb	This study
pJY3	pBluescriptKS(+) containing a 1.0 kb *Pst*I-*Hin*dIII fragment derived from pJY2. 3.95 kb.	This study
pJY4	pBluescriptKS(+) containing a 0.8 kb *Pst*I-*Cla*I fragment derived from pJY2. 2.75 kb	This study
pJY5	pBluescriptKS(+) containing a 4.0 kb *Pst*I fragment derived from pJY2. 6.95 kb	This study
pJY6	pBluescriptKS(+) containing a 3.0 kb *Pst*I-*Hin*dIII fragment derived from pJY2. 5.95 kb	This study

Ap^r and Tc^r indicate resistance to ampicillin and tetracycline , respectively.

was grown in MRS broth (Difco Laboratories, Detroit, MI, U.S.A.) without shaking or on MRS plates (1.5% agar) at 37°C anaerobically in the BBL® GasPak 100™ Anaerobic System jar. *Escherichia coli* strains were grown in Luria-Bertani (LB) broth at 37°C with vigorous agitation.

5-Bromo-4-chloro-3-indolyl-β-D-galactoside (X-gal) was supplemented at a concentration of 40 μg/ml and 0.5 mM IPTG (isopropyl-β-D-thiogalactopyranoside) when necessary. Antibiotics were used at the following concentrations: ampicillin (Ap), 100 μg/ml; tetracycline (Tc), 10 μg/ml; erythromycin (Em), 200 μg/ml for *E. coli*, 5 μg/ml for *Lactobacillus* strains.

Plasmid DNA Preparation and Transformation

Plasmid DNA from *E. coli* was isolated by using the E.Z.N.A.™ Plasmid Miniprep Kit (Omega Bio-tek, Doraville, U.S.A.) or CsCl-EtBr density gradient ultracentrifugation method [32]. Competent cell preparation and electrotransformation for *E. coli* cells were done according to the method of Dower *et al.* [6]. GENE PULSER(BIO-RAD, Hercules, CA, U.S.A.) apparatus was used for electrotransformation.

Recombinant DNA Techniques

For restriction endonuclease digestions, agarose gel electrophoresis, DNA ligation, and cloning-subcloning related works, standard methods were used as described by Sambrook *et al.* [34]. Restriction enzymes and DNA modifying enzymes were purchased from TaKaRa Shuzo Co. (Otsu, Shiga, Japan) and Promega Co. (Madison, WI, U.S.A.). Other Chemicals were purchased from Sigma Chemical Co. (St. Louis, Mo, U.S.A.).

PCR Cloning of an Internal Region of *ldhL* Gene

A 750-bp fragment corresponding to the internal region of *ldhL* gene of *L. reuteri* ATCC 55739 was obtained by PCR. GeneAmp PCR System 2400 (Perkin-Elmer Biosystems, Foster City, U.S.A.) was used and 3 μg of genomic DNA was used as template [22]. Two oligonucleotide primers (ldh1: 5'-GTYGGYGACGGCGCYGTTGGTTC-3' and ldh2: 5'-CCRTGTTCACCCATGATGTAA-3') were synthesized based on the conserved region of reported *ldhL* genes [16, 26, 37]. Amplification of a genomic DNA was performed in a total volume of 50 μl [30]: 3 μg of template DNA; 20 pmol of each primer; dNTPs, 0.25 mM each; 2 U of Ex-*Taq* DNA polymerase; and 1× Ex-*Taq* DNA polymerase buffer. PCR amplification was carried out under the following conditions; predenaturation for 5 min at 95°C, followed by 35 cycles (denaturation 95°C, 1 min; annealing 50°C, 1 min, extension 72°C, 1 min), and a final extension for 7 min at 72°C. PCR product was recovered from agarose gel using E.Z.N.A.™ Gel Extraction Kit (Omega Bio-tek, Doraville, U.S.A.) and ligated with T-vector. T-vector was prepared from pBlueScript II (+) by the method of Papp *et al.* [29].

Genomic Library Construction and Library Screening

Chromosomal DNA was isolated from *L. reuteri* ATCC 55739 according to the method of Kim *et al.* [18]. Chromosomal DNA was digested with *Pst*I and fragments in sizes between 2 and 8 kb were recovered from the agarose gel, ligated with pBR322 digested with *Pst*I, and then introduced into *E. coli* DH5α competent cells. Genomic library was screened by the colony hybridization method using Zeta-Probe® Blotting Membranes (Bio-Rad Laboratories,

Hercules, CA, U.S.A.) and the 750-bp PCR product was used as a probe [34]. Probe was labeled using a rediprime™ random prime labeling system (Amersham pharmacia biotech, U.K.). Southern blot hybridization was performed according to the established methods [15] using the 750-bp PCR product as a probe.

DNA Sequencing and Sequence Analyses

DNA sequences were determined by the dideoxy-chain terminaton method using the ABI-PRISM BigDye™ Terminator Cycle Sequencing Ready Reaction Kit (PE Applied Biosystems, Foster City, CA, U.S.A.). Primers for sequencing were synthesized at Bioneer (Seoul, Korea). The nucleotide sequence of the *ldhL* gene was desposited into GenBank under the accession number AY046324. Homology of the deduced amino acid sequence was analyzed by the Blast program at NCBI. Sequence alignment was performed with ClustalW program using PAM250 matrix. Amino acids sequence identity matrix was drawn up using BioEdit program version 4.7.8. [10]. Primary structure analysis was performed using ProtParam program.

RNA Isolation and Northern Blot Analysis

L. reuteri ATCC 55739 cells were grown in MRS broth to an optical density of 0.8 at 550 nm. Total RNA was prepared according to the method of Kim *et al.* [17] and the concentration was determined by measuring absorbance at 260 nm. Five µg and 10 µg RNA was loaded and separated in 1.2% agarose-formadehyde gel, respectively, transferred onto a Hybond N+ nylon membrane (Amersham, RPN203B), and hybridized at 65°C with radiolabeled 750-bp *ldhL* specific DNA probe.

RESULTS AND DISCUSSION

Cloning of the *ldhL* Gene from *L. reuteri* ATCC 55739

ldhL gene was cloned by screening *L. reuteri* ATCC 55739 genomic library constructed in *E. coli* DH5α. The 750-bp probe used for library screening was obtained by PCR amplification (results not shown) using a set of primers designed according to the conserved region of lactobacilli *ldhL* genes. The amplified 750-bp fragment was cloned into a T-vector based on pBluescriptII KS(+), generating a recombinant plasmid of 3.65 kb in size, pJY1. Sequencing of the 750-bp fragment confirmed that the fragment was indeed a part of the *ldhL* gene. This 750-bp fragment was used as a probe for screening *L. reuteri* library consisting of 912 *Pst*I clones. Colony hybridization was performed using a P^{32}-labeled 750-bp probe and two positive clones (# 103, 147) were detected by autoradiography (results not shown). Restriction mapping showed that two clones were the same and Southern blot experiment confirmed that the 4.0-kb *Pst*I fragment was really derived from *L. reuteri* ATCC 55739 chromosome (Fig. 1). The resulting recombinant plasmid was 8.3 kb in size and named pJY2. When the 4.0-kb insert was digested with *Hin*dIII, 1.0 and 3.0 kb fragments were generated and each fragment was subcloned into pBluescriptII KS(+), generating pJY3 and pJY6, respectively (Fig. 2). When the 4.0-kb fragment was digested with *Cla*I, 0.8 and 3.2 kb fragments were generated and the smaller 0.8-kb fragment was subcloned into pBluescriptII KS(+), generating pJY4 (Fig. 2). The entire 4.0-kb fragment was subcloned into pBluescriptII KS(+) and the resulting 6.9-kb plasmid was named pJY5 (Fig. 2).

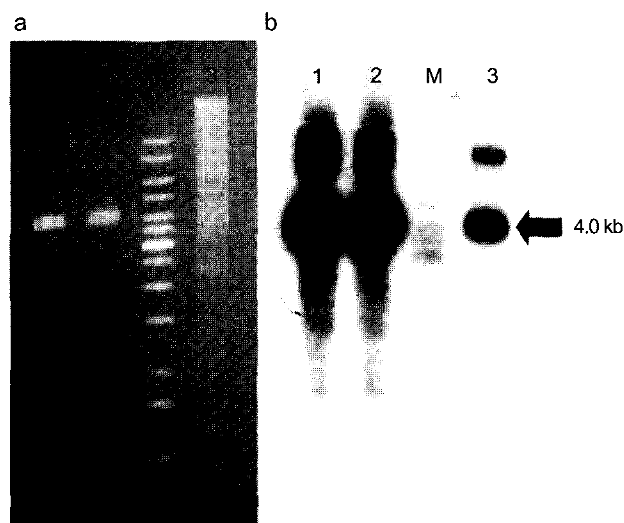

Fig. 1. Southern blot analysis of pJY2.
The 4.0-kb *Pst*I insert DNAs in positive clones were confirmed to be the same and originated from *Lactobacillus reuteri* ATCC 55739 chromosomal DNA. The 750-bp PCR fragment was used as a probe after radiolabeled. a. Agarose gel, b. autoradiogram. M. 1 kb DNA ladder (MBI), 1. *Pst*I digested plasmid DNA from clone no. 103, 2. *Pst*I digested plasmid DNA from clone no. 147, 3. *Pst*I digested *L. reuteri* ATCC 55739 chromosomal DNA.

Sequence Analyses

DNA sequencing for the 4.0-kb insert revealed the presence of two complete open reading frames, ORF1 and ORF2,

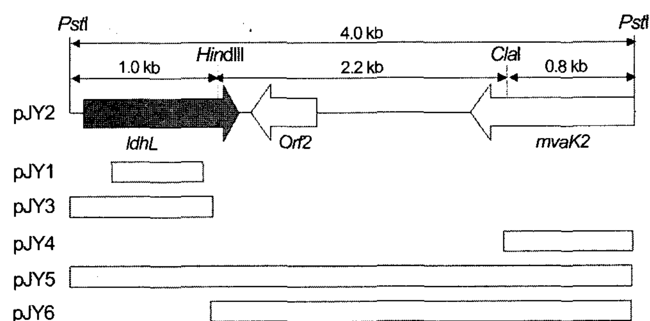

Fig. 2. The restriction map of the 4.0-kb *Pst*I insert in pJY2.
The positions of each fragment subcloned into pBluescriptII KS(+) generating pJY1, pJY3, pJY4, pJY5, and pJY6, respectively, are also shown.

Fig. 3. Nucleotide sequence of the *ldhL* gene and the deduced amino acid sequence.

Putative promoter sequences (−35 and −10 boxes) are underlined and the putative transcriprion start site is shown as a gray colored box. Ribosomal binding site (RBS) is boxed. A possible transcription terminator is indicated by double underline.

Fig. 4. The amino acids sequence alignment of bacterial nonallosteric L-LDH.

The sequence of the *L. reuteri* ATCC 55739 L-LDH (55739) was compared with the sequences of the L-LDH of *L. pentosus* (LDH_ LACPE), *L. plantarum* (LDH_LACPL), *L. helveticus* (LDH_LACHE), *B. subtilis* (LDH_BACSU), *B. caldolyticus* (LDH_BACST), and *P. acidilactici* (PALLDH).

and a partial ORF (Fig. 2). ORF1 is 972 bp in size, capable of encoding a L-LDH protein of 323 amino acids with a calculated molecular mass of 34,404 Da. The pI value for L-LDH was calculated to be 5.18. ORF2 is 201 bp in size, capable of encoding a polypeptide of 66 amino acids. Gene product of ORF2 is homologous to *Streptococcus pneumoniae* TIGR4 hydrolase (haloacid dehalogenase-like family) [38]. Haloacid dehalogenases are hydrolytic enzymes that cleave the halogen-carbon bond(s) in halogenated aliphatic acids, yielding hydroxy- or oxoalkanoic acids from a substrate with a mono- or di-substitution, respectively [11, 36]. The 3' part of the phosphomevalonate kinase gene (*mvaK2*) was present downstream of *ldhL* in the opposite orientation (Fig. 2). Phosphomevalonate kinase is a key enzyme of the mevalonate pathway for isoprenoid biosynthesis [32]. The nucleotide sequence of *L. reuteri ldhL* gene and deduced amino acid sequence are shown in Fig. 3. The *ldhL* gene starts with ATG at position 161 nc (nucleotide) and ends at a stop codon, TAG at 1,130 nc, capable of encoding a protein of 323 amino acids. A typical prokaryotic ribosome binding site, AGGAGA [36, 38], was present 9 nucleotides upstream of the initiation codon. Putative −10 and −35 promoter sequences were located at 68 and 95 nc, but the sequences were different from typical prokaryotic promoter sequences [41]. Promoter sequences of other *ldhL* genes from LAB also deviate from the typical −10 and −35 consensus sequences [19]. A putative rho-independent transcript terminator was found 5 nc downstream from the stop codon of *ldhL* with a ΔG of 79.29 $kJmol^{-1}$ [26]. The active site and several other domains with unknown functions are highly conserved among all L-LDHs studied, including the *L. reuteri* L-LDH. *L. reuteri* L-LDH has an active site, H176, but lacks a FBP binding site, R173. Thus, *L. reuteri* L-LDH is believed to be a nonallosteric enzyme [9]. Amino acid sequence alignment for *L. reuteri* ATCC 55739 L-LDH and other reported bacterial nonallosteric L-LDHs is shown in Fig. 4. The degree of identity of the *L. reuteri* L-LDH with other bacterial nonallosteric L-LDHs is shown as an amino acid sequence identity matrix (Table 2). The L-LDH of *Pediococcus acidilactici* is the most homologous to that of *L. reuteri* L-LDH (62.4%).

Northern Blot Analysis

The size of the mRNA transcript from the *ldhL* gene was analyzed by northern blot with a radiolabeled *ldhL* specific 750-bp PCR fragment as a probe. The autoradiogram (Fig. 5) showed that only a single 1.0 kb transcript hybridized with the probe, which was in good agreement with the size

Table 2. Amino acid sequence identity matrix of nonallosteric L-LDH from Gram-positive bacteria.

	Amino acid sequence identity (%)						
	55739	[a]LDH_LACPE	LDH_LACPL	LDH_LACHE	LDH_BACSU	LDH_BACST	[b]PALLDH
55739	100	58.7	58.1	47.6	48.3	46.0	62.4
LDH_LACPE	-	100	98.4	60.0	51.2	51.0	82.6
LDH_LACPL	-	-	100	60.0	51.5	51.0	81.7
LDH_LACHE	-	-	-	100	47.6	45.7	59.7
LDH_BACSU	-	-	-	-	1.000	65.1	50.1
LDH_BACST	-	-	-	-	-	100	50.9
PALLDH	-	-	-	-	-	-	100

[a]LDH_LACPE, LDH_LACPL, LDH_LACHE, LDH_BACSU, and LDH_BACST are SWISSPORT locus names.
[b]PALLDH is EMBL ID.

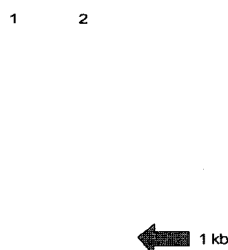

Fig. 5. Northern blot anaysis of *ldhL* gene from *L. reuteri* ATCC 55739.
1. 10 μg total RNA; 2. 5 μg total RNA.

of the *ldhL* transcript predicted from the DNA sequence data. The result confirms that *ldhL* is a monocistronic gene as expected from its sequence data and the transcription terminator downstream of the stop codon of *ldhL* functions *in vivo*. Transcriptional analyses for bacterial *ldhL* genes have been reported for *Lactococcus lactis* [23], *Pediococcus acidolactici* [10], *Bifidobacterium longum* [27], and *Lactobacillus helveticus* [21]. 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. acidolactici*, *B. longum*, and *L. helveticus*, *ldhL* is present as a monocistronic gene. Functional studies for *ldhL* gene are now in progress.

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