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Cloning of *pdh* Genes Encoding Subunits of Pyruvate Dehydrogenase Complex from *Lactobacillus reuteri* ATCC 55739

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Abstract A 2-D gel protein analysis of *Lactobacillus reuteri* ATCC 55739 produced spots corresponding to subunits of the pyruvate dehydrogenase complex, as identified by N-terminal protein sequencing. Oligonucleotide probes specific for the subunits of the pyruvate dehydrogenase complex were synthesized and used to screen a *L. reuteri* genomic library to clone the structural genes. Two positive clones were isolated and identified as having the same 2.2 kb insert. A *pdhB* encoding the β-subunit of E1 subunit (pyruvate dehydrogenase component) of the pyruvate dehydrogenase complex was located in the middle of the insert. Furthermore, a 5' truncated *pdhA* encoding the α-subunit of the E1 subunit and a 3' truncated *pdhC* encoding the E2 subunit (dihydrolipoamide acetyltransferase) were also located upstream and downstream of the *pdhB*, respectively.

Key words: *L. reuteri*, pyruvate dehydrogenase complex, 2-D gel, gene cloning

The pyruvate dehydrogenase (Pdh) complex catalyzes the oxidative decarboxylation of pyruvate with the formation of acetyl-CoA and reduction of NAD^+ to NADH:

$$\text{pyruvate+CoA+NAD}^+ \rightarrow \text{acetyl-CoA+CO}_2\text{+NADH+H}^+.$$

The complex consists of multi-copies of three subunits, pyruvate dehydrogenase component (E1, EC:1.2.4.1), dihydrolipoamide acetyltransferase (E2, EC:2.3.2.12), and dihydrolipoamide dehydrogenase (E3, EC:1.8.1.4) [3]. E1 also consists of two subunits, α and β, in the form of an $\alpha_2\beta_2$ heterotetramer and uses thiamine diphosphate as a coenzyme for the oxidative decarboxylation of pyruvate and reductive acetylation of lipoyl groups covalently attached to E2 lipoyl domains [14]. E2 transfers the acetyl group from the reduced lipoyl group to CoA to form acetyl-CoA, and E3 then uses the reduced E2 lipoyl group as a hydride ion source for the reduction of NAD^+ to NADH [11]. The genes encoding the Pdh complex have already been studied for various microorganisms, such as *Lactococcus lactis* [2], *Bacillus stearothermophilus* [6], *B. subtilis* [7], *Staphylococcus aureus* [1], *Acholeplasma laidlawii* [16], *Mycoplasma pneumoniae*, and *M. genitalium* [5]. *L. reuteri* is the most dominant heterofermentative lactic acid bacterium found in the gastrointestinal tracts of animals and humans [4], however, despite its importance as a probiotic, not much is known about its metabolism and genetics. Accordingly, in the current study, genes for the metabolically important enzymes of *L. reuteri* ATCC 55739 were cloned. 2-D gel analyses were performed on the protein extracts, and protein spots were identified that corresponded to the subunits of the pyruvate dehydrogenase complex. The structural genes of the pyruvate dehydrogenase subunits were then cloned by screening an *L. reuteri* genomic library using oligonucleotide probes based on the N-terminal amino acid sequences.

Bacterial Strains and Growth Conditions

The *L. reuteri* ATCC 55739 was obtained from the American Type Culture Collection (Manassas, U.S.A.). *Escherichia coli* DH5α [*ϕ80dlacZ*DM15, *recA1*, *endA1*, *gyrA96*, *thi-1*, *hsdR17*(r_k^-, m_k^+), *relA1*, *deoR*, Δ(*lacZYA-argF*)U169] was used as the host for the DNA manipulation and transformation experiments. pUC19 was used for the library construction. The *E. coli* was grown in Luria-Bertani (LB) broth with vigorous shaking at 37°C. The *L. reuteri* was grown in MRS broth (Difco Lab, Detroit, U.S.A.) or on MRS plate solidified with 1.5% agar at 37°C anaerobically in a BBLR® GasPak 100™ Anaerobic System jar.

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2-D Gel Analysis of *L. reuteri* Proteins

The *L. reuteri* ATCC 55739 cells were lysed by sonication, and the protein were extracts loaded onto an HQ ion-exchange (anion-exchanger) column. After washing the column with 10 ml of 50 mM Tris-HCl (pH 8.0), the bound proteins were eluted with the same buffer plus 1 M NaCl. The fractions were then lyophilized and resuspended in an isoelectric focusing buffer. The isoelectric focusing was performed with commercially available IPG-strips (pH 4-7) and an IPGphor unit (Amersham Pharmacia Biotech, Piscataway, U.S.A.) using various voltage profiles. After consecutive equilibration in solutions containing DTT and iodoacetamide, the gels were overlayed on SDS-polyacrylamide gels of 10% T and 2.6% C and second dimension gel electrophoresis was performed using a Mini Protein II system (Bio-Rad, Hercules, U.S.A.). All the gels were stained with Coomassie blue (Fig. 1). For N-terminal sequencing, the proteins on the 2-D gel were electroblotted onto an Immobilon TM-P transfer membrane (Millipore, Billerica, U.S.A.). After washing with Milli-Q water, the protein spots on the membrane were excised and N-terminal sequencing was carried out at the Korea Basic Science Institute in Seoul. Spots 1 and 2 revealed the same sequence of MAYKFRLPEMGEGLTE, while spots 3 and 4 also revealed the same sequence of AKKTYIKAITEGIIALAE. A Blast search indicated that the sequence of spot 1 had significant homology to the E2 subunits of the pyruvate dehydrogenase complex from Gram-positive bacteria and the highest homology was found with the *Lactobacillus*

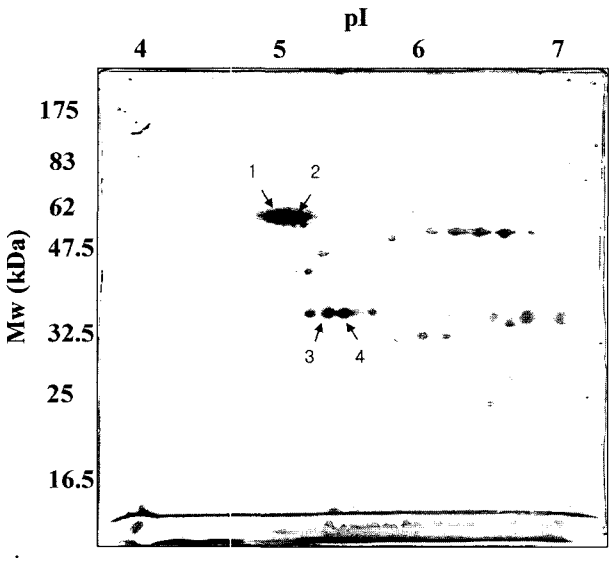

Fig. 1. 2-D gel profile of *L. reuteri* ATCC 55739 proteins.
An IPG-strip (pH 4-7) from Amersham Pharmacia was used for the 1st dimension separation of the proteins eluted from an HQ column. The 2nd dimension separation was performed by SDS-PAGE using a polyacrylamide gel of 10% T and 2.6% C. Spots 1 and 2 corresponded to the E2 subunit of the pyruvate dehydrogenase complex. Spots 3 and 4 corresponded to the β-subunit of the E1 component.

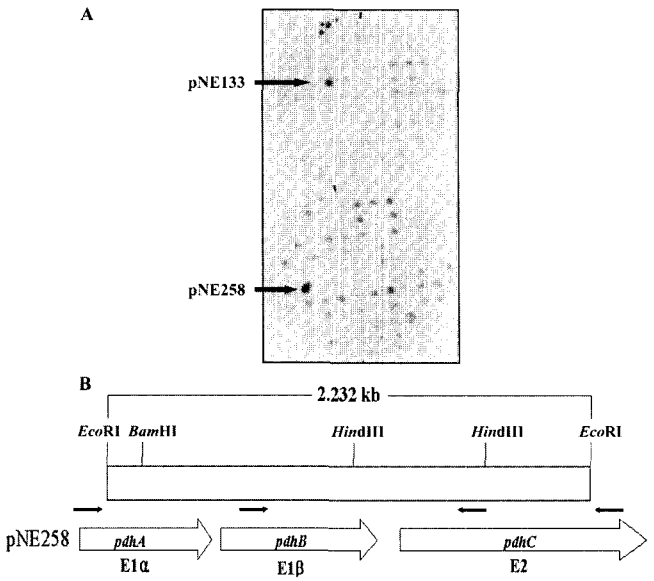

Fig. 2. A. Colony hybridization of constructed genomic library using LI-2 oligonucleotide probe.
Two clones exhibited the strongest signals, and the recombinant plasmids were named pNE133 and pNE258, respectively. B. Genetic organization of *pdh* genes cloned in pNE258. pNE133 and pNE258 revealed the same 2.2 kb *Eco*RI insert with a reverse orientation. A restriction map of pNE258 is shown. The locations and directions of the genes are illustrated with arrows. Also, the positions of the primers used for the primer walking are shown below as short arrows.

plantarum WCFS1 enzyme (NP_785657). Meanwhile, the sequence of spot 3 had the highest homology to the β-subunit of the E1 component of *L. plantarum* WCFS1 (CAD64509). Therefore, these results strongly suggest that the spots 1 and 3 correspond to the E2 and E1β subunits of the pyruvate dehydrogenase complex, respectively.

Genomic Library Construction and Screening

Chromosomal DNA from *L. reuteri* was prepared as described by Park *et al.* [12], and 30 μg were digested with *Eco*RI. Fragments of 1.5-5 kb size were recovered from the agarose gels using a gel extraction kit (Qiagen, Valencia, U.S.A.) and ligated with pUC19. The ligation mixture was introduced into competent *E. coli* DH5α cells by electroporation, then the resulting white colonies on LB plates, containing ampicilin (100 μg/ml), IPTG (Isopropyl-β-D-thiogalactopyranoside, 0.5 mM), and X-gal (5-bromo-4-chloro-3-indolyl-β-D-galactoside, 40 μg/ml) were picked up [8]. About 900 clones were obtained. Colony hybridization, using a Magnacharge nylone transfer membrane (Osmonics, Minnetonka, U.S.A.), was performed to screen the library [9]. Oligonucleotides named LI-2 (5'-TAYAARTTYCGK-YTDCCWGARATGGGYGARGG-3') and LI-4 (5' AAR-AARACNTAYATHAARGCNTAHAC-3') were designed based on the amino acid sequences determined for spots 1 and 3, respectively (Fig. 1). The probes were labeled using γ-^{32}P-ATP and T4 polynucleotide kinase [15]. The

prehybridization and hybridization were carried out at 55°C. Two clones, NE133 and NE258, were identified from the hybridizations with the LI-2 probe, and restriction mapping revealed that both had the same 2.2 kb insert, but with different orientations (Fig. 2). The plasmids were named pNE133 and pNE258. The hybridizations using the LI-4 probe picked up the same clones. Southern blot using the LI-2 probe confirmed that the 2.2 kb insert was derived from the *L. reuteri* ATCC 55739 chromosome (results not shown).

DNA Sequence Analysis

The nucleotide sequence of the 2.2 kb insert was determined by the dideoxy-chain termination method [13] and deposited in the GenBank database under accession number of AY308803. The primers for sequencing were synthesized at Bioneer (Seoul, Korea) and were as follows; pNE133F1 (5'-CTTGGTCCGTGATTACCA-3'), pNE133R1 (5'-CATGCTTAAAGCGGACCT-3'), pNE133F2 (5'-GGGTCACCATCTTTGACT-3'), and pNE133R2 (5'-ATGGTCTAAGAACATGCC-3'). The homologies of the deduced amino acid sequences were analyzed by the Blast program at NCBI (National Center for Biotechnology Information, Bethesda, MD, U.S.A.). As shown in Fig. 3, the LI-2 probe hybridized to the 5' of *pdhC* (nt 1,382-1,413), while LI-4 hybridized to the 5' of *pdhB* (nt 363-388), as expected from the N-terminal protein sequencing data, and these binding sequences are shadowed. The genes for the three

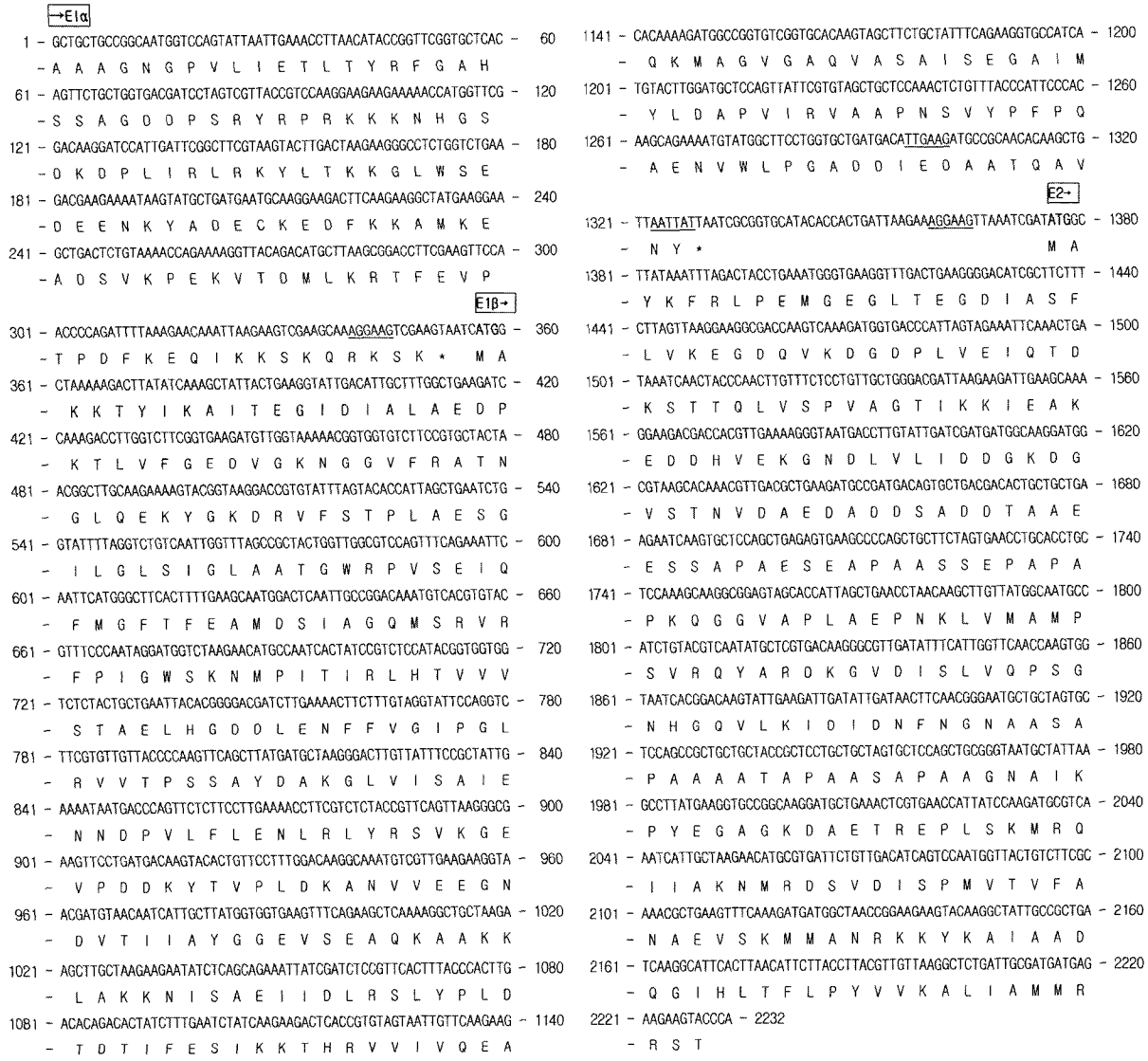

Fig. 3. Nucleotide sequence and predicted amino acid sequence of the 2.2 kb *Eco*RI insert containing *pdh* genes.
The putative promoter regions (-35 and -10) and ribosome-binding site of the *pdhC* gene are underlined. The LI-2 and LI-4 oligonucleotide binding sequences are shadowed. The start codons are in bold. Also, the start site is marked above the sequence. The termination sites are marked by asterisks.

open reading frames (ORFs) were designated *pdhA*, *pdhB*, and *pdhC*, based on their homologies to other known genes.

The N-terminal truncated PdhA (pyruvate dehydrogenase E1 subunit) was the first ORF. Since the PdhA from *L. plantarum* consists of 370 aa and that from *B. subtilis* consists of 371 aa, the 5' part encoding the N-terminal fragment of ca 255 aa was missing in the 2.2 kb insert. The *pdhA* was linked closely to the *pdhB* encoding the E1 β-subunit, and the latter ORF started just 2 bp downstream from the stop condon of the *pdhA*, indicating an operon structure. The *pdhB* was 975 bp (nt 357-1,331) in length and capable of encoding a protein of 324 aa. The calculated pI and molecular weight of the PdhB was 4.97 and 35037.04 Da, respectively. The ribosome binding site (RBS, AGGAAG, nt 340-345) preceded the start codon by 18 bp. The C-terminal truncated *pdhC* ORF followed the *pdhB*. The start codon of the *pdhC* (ATG) was located 44 nt downstream of the stop codon of the *pdhB* and the RBS (AGGAAG, nt 1,360-1,365) was present 10 nt upstream of the start codon. The size of the PdhCs from other related bacteria is around 430 aa (*L. plantarum*, 431 aa; *B. subtilis*, 442 aa), thus the sequence shown in Fig. 3 indicates lack of a C-terminal fragment of ca 150 aa. Possible promoter sequences are located upstream of the *pdhC*, -35 box (TTGAAG, nt 1,297-1,302) and -10 sequence (AATTAT, nt 1,323-1,328). The spacing between the -35 and -10 sequences is 20 nt. However, further experiments are necessary to verify whether a transcription started from this promoter.

Comparison of Deduced Amino Acid Sequences of *pdh* Complex Homologues

Table 1 shows the sequence homologies between the *L. reuteri* Pdh proteins and the Pdh subunits from other organisms. The PdhB from *L. plantarum* WCSF1 was the most homologous to the 35 kDa PdhB from *L. reuteri* ATCC 55739 (59% identity, 75% similarity). Also, the PdhBs from *E. faecalis* (58% identity, 73% similarity) and *B. stearothermophilus* (56% identity, 72% similarity) were significantly homologous. The PdhA and PdhC from *L. reuteri* exhibited weaker homologies with corresponding proteins from other organisms. One reason may have been the smaller number of amino acids due to the truncation. Lysine residues, known as the lipoyl binding sites in the E2 subunit and conserved among other organisms, were also present in the PdhC from *L. reuteri* (Lys-43, Lys-151) [10]. Therefore, based on the N-terminal amino acid sequences and homology data on the translated products from the cloned genes, it was concluded that the 2.2 kb insert contained the complete *pdhB* and parts of the *pdhA* and *pdhC* genes from *L. reuteri* ATCC 55739.

Acknowledgments

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Table 1. Homologies of Pdh complex from *L. reuteri* with Pdh complexes from other Gram (+) bacteria.

L. reuteri	Enzyme	Organisms or source	% Identity	% Similarity	Protein sequence number
PdhA	(117 aa)[1]	*Lactobacillus plantarum*	52	73	NP_785659
		Listeria monocytogenes	47	68	NP_464577
		Listeria innocua	47	68	CAC96275
		Geobacillus stearothermophilus	47	70	CAA37628
PdhB	(324 aa)	*Lactobacillus plantarum*	59	75	NP_785658
		Enterococcus faecalis	58	73	NP_815075
		Bacillus stearothermophilus	56	72	S14230
		Geobacillus stearothermophilus	56	72	CAA37629
PdhC	(285 aa)[2]	*Lactobacillus plantarum*	40	54	CAD64508
		Geobacillus stearothermophilus	36	52	P11961
		Bacillus stearothermophilus	36	52	CAA37630

[1]N-terminal part truncated.
[2]C-terminal part truncated.

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