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Cloning and characterization of the bifunctional alcohol/acetaldehyde dehydrogenase gene (*adhE*) in *Leuconostoc mesenteroides* isolated from kimchi

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Abstract

A bifunctional alcohol/acetaldehyde dehydrogenase (AdhE) gene (*adhE*) was cloned from *Leuconostoc mesenteroides* C7 (LMC7), which is the dominant lactic acid bacterium produced during heterofermentation of kimchi. The nucleotide sequence of the DNA fragment containing putative *adhE*, which is 2685 bp long and encodes an 886 amino acid polypeptide, exhibits 99% homology with *Leu. mesenteroides* sp. *cremoris*. The deduced AdhE comprises two conserved domains: alcohol dehydrogenase (Adh) and acetaldehyde dehydrogenase (Aldh). Moreover, two NAD-binding sites were observed, based on the presence of the GXGXXG motif. A pADHE containing the *adhE* gene expressed AdhE at the translational level in *Escherichia coli* BL21, which was at a higher level than in *E. coli* DH5α and *E. coli* JM109. The AdhE of LMC7 showed Adh and Aldh activities that, when expressed in *E. coli*. BL21, were 7.5 and 5.7 U mg^{-1}, respectively.

Introduction

Leuconostoc is a genus of heterofermentative coccoid lactic acid bacteria (LAB), and is the predominant genus of LAB in the diary industry (Axelsson 1998). This strain has commercial importance, including in spoilage in sugar processing, malolactic fermentation in wine making, and the production of flavor components in dairy- and vegetable-based fermentation (Stiles & Holzapfel 1997). *Leu. mesenteroides* plays a prominent role in the fermentation of vegetables such as sauerkraut, cucumbers, and kimchi (a traditional Korean fermented food), and is responsible for the souring, flavor enhancement, and leavening of other dairy food (Holzapfel & Schillinger 1991, Kim *et al.* 2002). *Leuconostoc* species produce lactate, acetate, ethanol, and CO_2 during fermentation. Among the end products of fermentation, ethanol arises from acetyl-CoA due to the presence of acetaldehyde dehydrogenase (Aldh) and alcohol dehydrogenase (Adh).

The alcohol/acetaldehyde dehydrogenase (AdhE) comprises two conserved domains (Aldh and Adh), is NADH dependent, and is involved in the sequential conversion of acetyl-CoA to ethanol during fermentation (Membrillo-Hernández *et al.* 2000). Few bacteria are known to catalyze these two reactions sequentially (Nair *et al.* 1994, Arnau *et al.* 1998, Asanuma *et al.* 2004) and no bacterium in the *Leuconostoc* family is yet known

to characterize AdhE. The early and intermediate phases of fermentation are considered crucial to the taste of kimchi (Yang *et al*. 2002). When optimally ripened, acidity increases with sourness and a unique flavor with refreshing and coolness components results from ethanol and other products (Kim *et al*. 2002). Elucidating the functions of AdhE should enable the coolness to be controlled, with applications to kimchi and other fermented foods. We have previously isolated *Leu. mesenteroides* C7 (LMC7) and ruled it out as the major LAB in kimchi (Kim *et al*. 2002), and in the present study we cloned the *adhE* gene from the identified LMC7 and characterized it on the basis of enzyme activity of the gene encoding AdhE with a bifunctional enzyme.

Materials and methods

Bacterial strains and growth culture

LMC7 was used as a donor strain of the *adhE* gene. It was grown in MRS (deMan Rogosa Sharpe) broth (Difco, Franklin Lakes, USA) at 30 °C for 18 h. *Escherichia coli* BL21, *E. coli* DH5α, and *E. coli* JM109 were used as hosts for expression; these strains were incubated in LB (Luria–Bertani) broth at 37 °C for 12 h.

Determination of the adhE *nucleotide sequence*

To determine the *adhE* nucleotide sequence, the genomic DNA of LMC7 was purified by a DNeasy tissue kit (Qiagen, Hilden, Germany). A partial (342-bp) sequence of the *adhE* gene was obtained by PCR using primers ADH-S1 and ADH-S2 (Table 1). Primer pairs ADH-6/ADH-8 and ADH-9/ADH-10 amplified the 5′- and 3′-flanking regions of the first 342 bp amplicon. PCR was implemented using 30 cycles of a denaturing step at 94 °C for 2 min, a primer-annealing step at 55 °C for 2 min, and an elongation step at 72 °C for 3 min with a commercial PCR system (GeneAmp 2700, Foster, USA). This nucleotide sequence was analyzed by the chain-termination method according to the procedure described by Sanger *et al*. (1977). We analyzed the homology of the nucleotide sequence and amino acid sequences based on information from NCBI obtained using the BLAST program.

Cloning of adhE *gene and SDS-PAGE analysis*

The PCR product amplified by primers ADH-6 and ADH-10 was inserted into plasmid pGEM-T Easy Vector (Promega, Madison, USA) and used to prepare pADHE, which was introduced into BL21, DH5α, and JM109.

The production of AdhE in *E. coli* transformants was analyzed by SDS-PAGE using samples that had been obtained using the same methods of induction for the enzyme assay. Induced cells were harvested, denatured at 100 °C for 3 min, and centrifuged, with the supernatant used subsequently. SDS-PAGE was run in a gel containing 7.5% acrylamide.

Enzyme preparation

The recombinant *E. coli*, cultured until mid-growth in LB broth containing ampicillin (50 μg ml^{-1}), was induced by 1 mM IPTG and incubated for 3.5 h. The cells were harvested by centrifugation, washed, and resuspended in 50 mM potassium phosphate buffer (pH 7.4), then disrupted ultrasonically. The supernatant obtained by

Table 1. PCR primers designed for cloning of *adhE* gene.

Primer	Sequence (5′–3′)	Accession number	Source organism
ADH-S1	CAGTTAAGCCTGACCCAACTTTGTCACAAGC	AF326594	*Leuconostoc mesenteroides* sp. *mesenteroides*
ADH-S2	GTGTTTCATCTGTAATAACGGCAAATGGTG	AF326594	*Leu. mesenteroides* sp. *mesenteroides*
ADH-6	ATGAGCAAGTAAAGGAGCAAAGATTATGGC	ZP_00063848	*Leu. mesenteroides* sp. *mesenteroides* ATCC8293
ADH-8	AAGTCTGCCATTTGGCGAGCAATTTCAACA	This study	*Leu. mesenteroides* C7
ADH-9	AGAAGTCACACCATTTGCCGTTATTACAGA	This study	*Leu. mesenteroides* C7
ADH-10	TTAAAACTGATCTTTCAACAATTGACGAATTT	ZP_00063848	*Leu. mesenteroides* sp. *mesenteroides* ATCC 8293

centrifugation at 145 000 × *g*, 4 °C for 1 h was used as the crude enzyme (Gupta *et al*. 2000).

Enzyme assay

Adh activity was assayed in a reaction mixture comprising 80 mM Tris/HCl buffer (pH 8.8), 2 mM NAD^+, 0.8 mM ethanol, and the enzyme preparation (Blandino *et al*. 1997). Aldh activity was assayed in 800 mM Tris/HCl buffer (pH 8), 2 mM NAD^+, 0.089 mM acetaldehyde, 0.1 mM CoASH, 0.27 mM dithiothreitol, and the enzyme preparation (Gupta *et al*. 2000). The changes in absorbance of the reaction mixture at 340 nm were detected at 37 °C. One unit was defined as the amount of enzyme activity that catalyzed the transformation of 1 μmol NADH per min under these conditions.

Nucleotide sequence accession number

The DNA sequence described in this study is available in the GenBank database under accession number AY804189.

Results and discussion

Sequences determination and homology analysis

DNA fragment containing putative *adhE* was cloned using PCR amplification, which indicated that the nucleotide sequence was 2685 bp long and encoded an 886 amino acid polypeptide with an estimated mass of 96.6 kDa. Identities of the deduced amino acid sequence for AdhE among known Gram-positive bacterial *adhE* gene were over 69% (Table 2), and the homology of nucleotide sequences for *adhE* with *Leu. mesenteroides* sp. *cremoris* was the highest, at 99%. There was only one difference: it converts the amino acid residue 600 glutamic acid to aspartic acid at nucleotide position 1825, whereas at position 1873 the changed base is the third nucleotide of a codon and keeps the same amino acid (isoleucine).

Table 2. Homologies of alcohol/acetaldehyde dehydrogenase (AdhE) from *Leuconostoc mesenteroides* C7 with AdhE of other Gram-positive bacteria.

Strain	Identities (%)	Positives (%)	Accession number
Leu. mesenteroides sp. *cremoris*	99	100	CAC93842
Oenococcus oeni PSU-1	76	87	ZP_00319746
Lactococcus lactis	75	86	CAA04467
Streptococcus bovis	69	81	BAC87790

The PROSITE algorithm indicated that the deduced AdhE of LMC7 comprised the conserved domains Adh and Aldh (Figure 1). The sequences showed extensive positional identities with other strains, with the exception of only two amino acids in the Adh position. However, NAD-binding sites, which are conserved glycine-rich regions with the GXGXXG motif, are not in every bacterium analyzed (Membrillo-Hernández *et al*. 2000). NAD-binding sites are located close to the *C*-terminal region in Aldh, and upstream of the NH_2-terminal region in Adh. On the other hand, *Oenococcus oeni* PSU-1 and *Streptococcus bovis* have a single NAD-binding site at the NH_2-terminal in Adh. The homology analysis of AdhE with other LAB allowed the locations and identities of Adh and Aldh to be defined. Furthermore, two NAD-binding sites were identified and the conserved sequence showed that AdhE is iron dependent.

Cloning and expression

The AdhE expression in different *E. coli* hosts (BL21, DH5α, and JM109) was studied with SDS-PAGE analysis. pADHE was expressed at the anticipated size of the AdhE protein in BL21 (data not shown). However, in DH5α and JM109, the expression of AdhE was lower than that in BL21; consequently the AdhE was not expressed highly in *E. coli*. AdhE protein is known to be toxic (Membrillo-Hernández *et al*. 2000), and this may either prevent or limit the induction of host bacteria.

Enzyme assay

Protein extracts obtained from the IPTG-induced bacteria harboring pADHE were used to test the activity of AdhE. The results are given in Table 3. Since AdhE is reversible, the activity was measured separately in the reverse reaction.

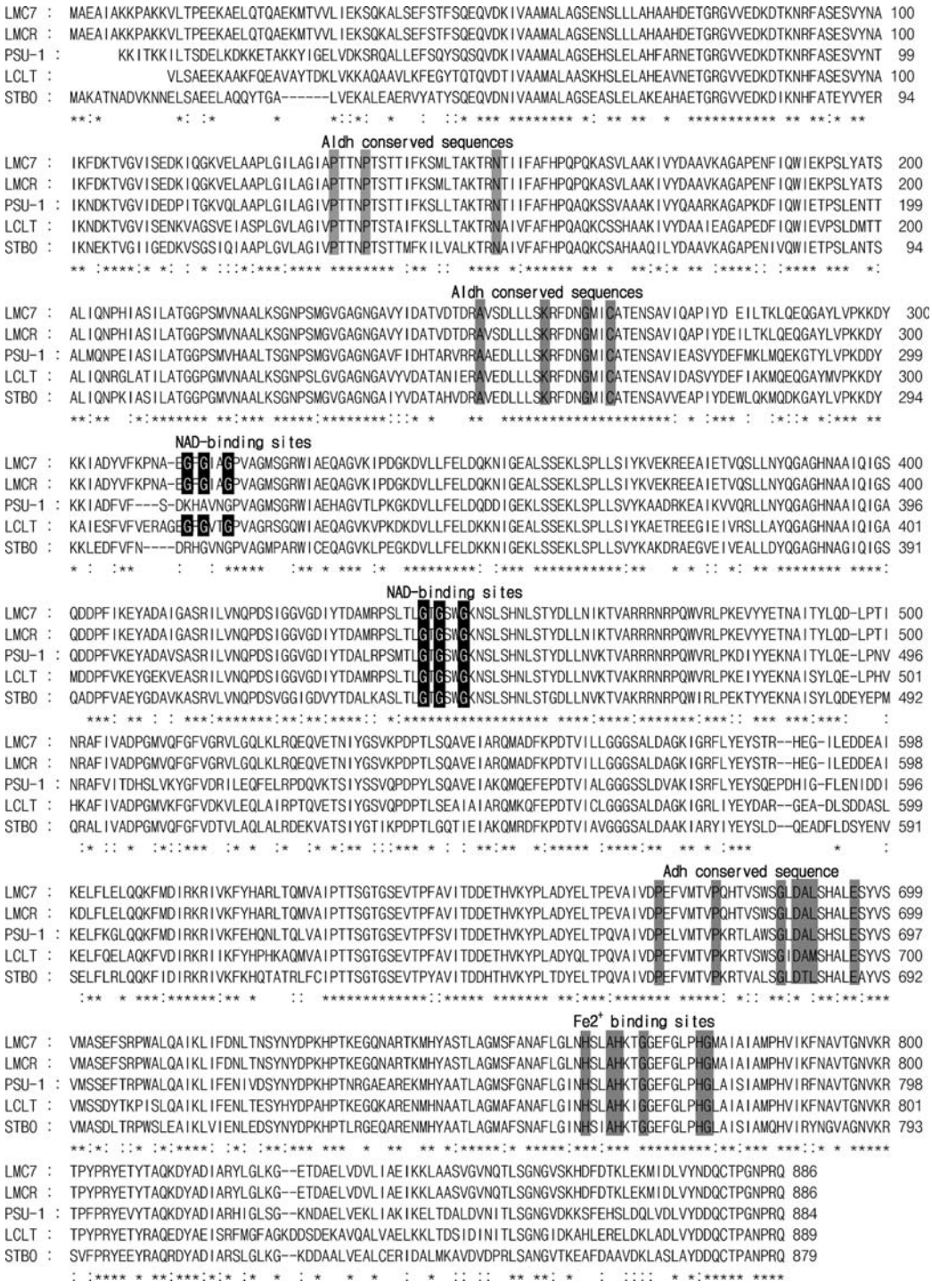

Fig. 1. Alignment of deduced alcohol/aldehyde dehydrogenase of *Leu. mesenteroides* C7 (LMC7) with those of *Leu. mesenteroides* sp. *cremoris* (LMCR), *O. oeni* PSU-1 (PSU-1), *Lacto. lactis* (LCLT), and *S. bovis* (STBO). The conserved domains of aldehyde dehydrogenase, alcohol dehydrogenase and iron-binding sites are marked by gray vertical boxes. The black vertical boxes are NAD-binding sites based on the GXGXXG motif. Identical amino acids are indicated by asterisks, and similar amino acids are indicated by dots.

Table 3. Enzyme activities of alcohol dehydrogenase, acetaldehyde dehydrogenase and alcohol/acetaldehyde dehydrogenase in *E. coli* BL21 and DH5α.

	Alcohol dehydrogenase	Acetaldehyde dehydrogenase	Alcohol/acetaldehyde dehydrogenase
BL21 (pADHE)	7.5 ± 0.2	1.7 ± 0.2	7.8 ± 0
DH5α (pADHE)	5.7 ± 0.2	1.6 ± 0	4.8 ± 0

Protein extracts was induced under the control of T7 promoter, lyzed on ice with ultrasonication, centrifugated and the supernatant was used for the test (Gupta *et al*. 2000). The enzyme activities are reported as units (mg^{-1}) ± standard deviation. One unit was defined as the amount of enzyme activity that catalyzed the transformation of 1 μmol NADH per min under these conditions.

As indicated in Table 3, 4.3- and 3.6-fold more NADH was produced in BL21 and DH5α, respectively, by Adh than by Aldh. Adh is an enzyme involved in the production of end products during heterofermentation, whereas Aldh acts during an intermediate process to give a lower activity. The enzyme activities of Adh and Aldh were 1.3- and 11-fold lower in DH5α than in BL21. This can be attributed to AdhE being easily translated in any bacteria, even when a rate difference is present. Although DH5α harboring pADHE did not exhibit a distinctive level of expression in the SDS-PAGE analysis, the activity of the protein was found to be not directly related to the expression level.

To study the sequential catalysis of AdhE, the AdhE activity level in BL21 was also measured and, as expected, the production of NAD^+ was increased by 1.2%. This activity value does not correspond to the combined activities of Adh and Aldh, but it does indicate that AdhE contains two enzymes and catalyzes sequentially. DH5α can be assumed to exhibit a similar pattern of activity. The enzyme activity of JM109 was close to zero (data not shown), which we can attribute to the toxicity of AdhE.

In conclusion, AdhE obtained from LMC7 has a dual function associated with Adh and Aldh, which produce ethanol and acetaldehyde from acetyl-CoA. This study is the first to employ the cloning of AdhE and analyze its function using enzyme activity at a genetic level in *Leu. mesenteroides*. Our molecular biological approach provides a partial elucidation of sugar metabolism involving the production of ethanol. Further studies will improve fermentation processes, with many applications in dairy-based industries and in the production of foods such as kimchi.

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