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Molecular cloning and characterisation of gamma subunit of H^+-ATPase in *Lactobacillus acidophilus* MG2-9

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Abstract - The acid tolerance is considered as one of the most important properties of lactic acid bacteria as probiotics. H^+-ATPase is one of the genes associated with the ability of acid tolerance. We have cloned and sequenced full length cDNA of γ subunit of H^+-ATPase gene in *Lactobacillus acidophilus* MG2-9. The cDNA sequence consists of 975 nucleotides; the putative protein has 320 amino acids.

Key words: H^+-ATPase gene, acid tolerance, *Lactobacillus acidophilus* MG2-9.

INTRODUCTION

Lactic acid bacteria play vital roles in food and health, although the nature of this role can vary greatly. They include generally-regarded-as-safe organisms used in food fermentations, ubiquitous gastric commensals, and potentially beneficial probiotic bacteria. The gastrointestinal tract is the most densely colonised region of the human body (Savage, 1977; Tannock, 1995); accumulated evidence indicates that gastrointestinal microflora has a powerful influence on the host in which it resides. Comparisons between germ-free and conventional animals have shown that many biochemical, physiological and immunological functions are influenced by the presence of the diverse and metabolically active bacterial community residing in the gastrointestinal tract (Norin *et al.*, 1991; Marteau and Rambaud, 1993; Tannock, 1995). Lactobacilli are important residents of the microflora (Kimura *et al.*, 1997; Ahrne *et al.*, 1998), and have been the subject of intense and growing interest because of their possible role in the maintenance of gastrointestinal health (Bengmark, 1998). Because the gastric pH frequently falls below 2.0 in healthy individuals, acid tolerance is considered as one of the desirable properties used to select potentially probiotic strains. Thus, it is timely to consider the mechanisms used by lactic acid bacteria to protect themselves from the challenge posed by low-pH environments such as food and gastric juice, and comment on the strategies by which they can be aided or impeded.

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In the presence of a low external pH (< 3.5), *Lactobacillus acidophilus* is able to maintain cytoplasmic pH at values close to neutral (Kashket, 1987). However, the information pertaining to acid resistance systems in *Lactobacillus acidophilus* is limited. For several organisms that inhabit the gastrointestinal tract, the F_1F_0-ATPase is an important element in the response and tolerance to low pH. In the bacterium *Enterococcus hirae* and *Streptococcus faecalis*, maintenance of cytoplasmic pH has been shown to occur via amplification of the proton-translocating ATPase (Kobayashi and Murakami, 1982; Kobayashi *et al.*, 1984, 1986; Suzuki and Kobayashi, 1989). Depending on the particular organisms and on the conditions for growth, the enzymes function in the direction of either ATP synthesis or ATP hydrolysis (Futai and Kanazawa, 1983; Cotter and Hill, 2003). In the bacteria which lakes the respiratory chain, the enzyme is involved in the extrusion of protons driven by ATP hydrolysis to generate the necessary driving force for solute transport and to maintain an acceptable intracellular pH value (Kobayashi, 1985).

In order to determine the association of the H^+-ATPase gene with acid stress in *Lactobacillus acidophilus*, the γ subunit of H^+-ATPase in *Lactobacillus acidophilus* MG2-9 isolated from koumiss (Wang *et al*., 2005) was cloned and characterised.

MATERIALS AND METHODS

Strains and culture media. *Lactobacillus acidophilus* MG2-9 was obtained from the Key Laboratory of Dairy Biotechnology and Engineering Ministry of Education, Inner Mongolia Agricultural University, China. This strain can survive in acid condition as pH 3.5. *Lactobacillus acidophilus* MG2-9 was cultured in MRS broth without shaking at 37 °C.

TABLE 1 - Comparison of *Lactobacillus acidophilus* MG2-9 with other *Lactobacillus* γ subunit of H^+-ATPase

Species	GenBank Accession #	Length (bp)	Nucleotide Identity (%)	Amino Acid Identity (%)
L. acidophilus NCFM	NC_006814	975	86.56	92.14
L. acidophillus X	AF098522	963	86.50	92.19
L. johnsonii NCC 533	NP_964794	957	69.25	66.36
L. gasseri ATCC 33323	YP_815046	957	67.62	65.42
L. casei ATCC 334	YP_806395	927	57.42	51.88
L. sakei subsp. *sakei* 23K	YP_395736	939	57.32	51.88

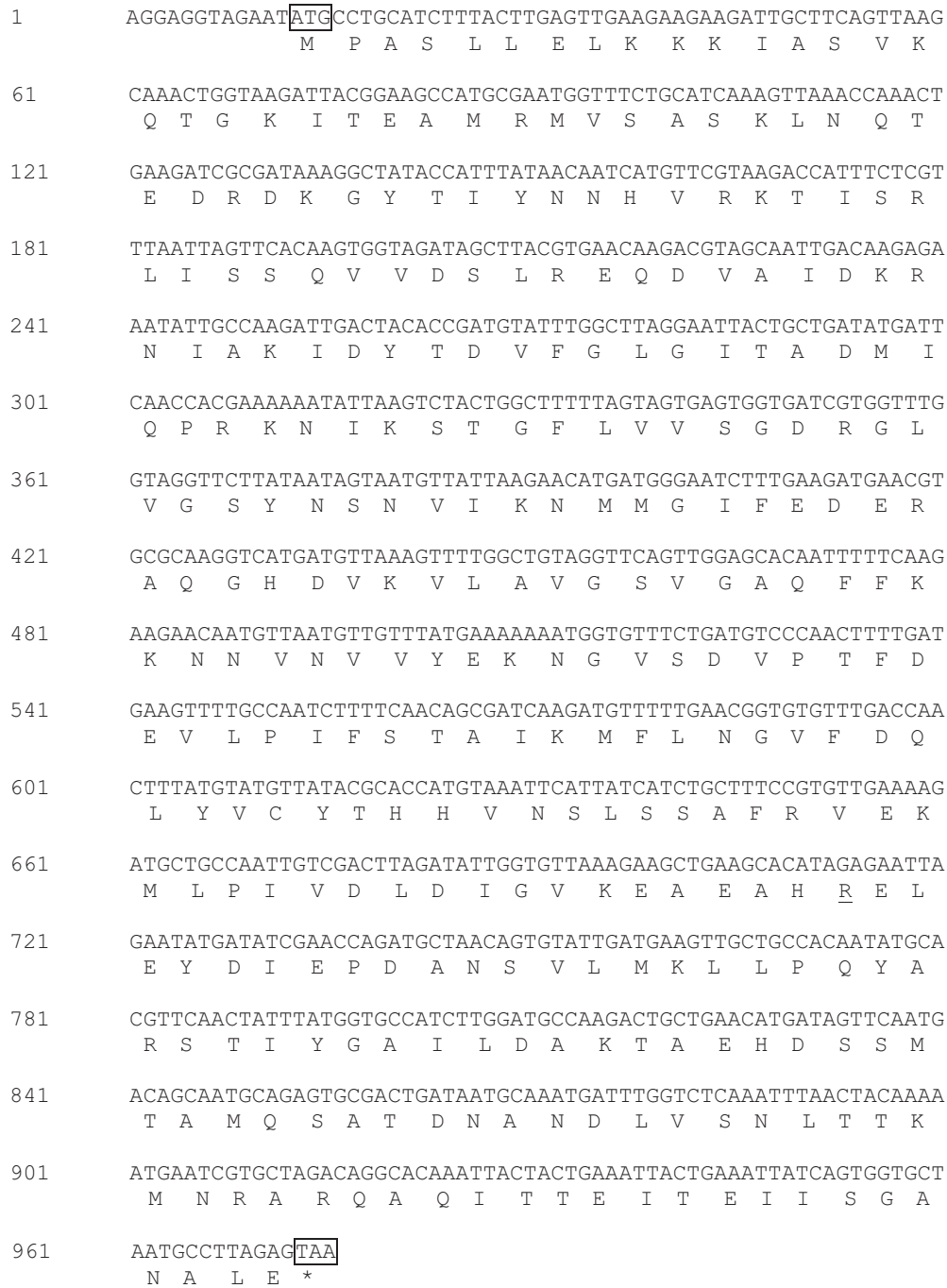

FIG. 1 - Nucleotide sequence and putative amino acid sequence of γ subunit of H^+-ATPase in *Lactobacillus acidophilus* MG2-9.

RNA isolation. The total RNA was extracted using Trizol reagent (Invitrogen, USA) according to the manufacturer's recommendations from overnight culture of *Lactobacillus acidophilus* MG2-9, then concentration of RNA was adjusted to 100 ng/µl by using the Biophotometer (Eppendorf, Germany), and stored at -70 °C until use.

RT-PCR. Using RNA PCR Kit (AMV) Ver. 3.0 (TaKaRa, Japan), reverse transcriptions were performed with 10 µl reaction volume, which includes 3 µl of total RNA (100 ng/µl), 1 µl 10X RT buffer, 2 µl 25 mmol/L $MgCl_2$, 1 µl dNTP mixture, 0.25 µl RNase inhibitor (40 U/µl), 0.5 µl AMV Reverse Transcriptase XL (5 U/µl), 0.5 µl Oligo dT-Adaptor Primer (2.5 pmol/µl), and 1.75 µl RNase Free dH_2O. The RT reaction condition was as follows: 30 °C for 10 min, 42 °C for 30 min, 99 °C for 5 min, 5 °C for 5 min.

The primers were designed according to the sequence of H^+-ATPase in *Lactobacillus acidophilus* NCFM (GenBank accession number: NC_006814). The primers were as follows: forward 5′-TCATCTGCTTTCCGTGTT-3′, reverse 5′-TTCGGACTTGATTACACG-3′. The 25 µl PCR reaction mixture comprised with 0.2 µl Taq polymerase (5 U/µl, Takara Tokyo, Japan), 2.5 µl 10X PCR Buffer (without Mg^{2+}), 2 µl dNTP (2.5 mM each), 2 µl $MgCl_2$ (25 mM), 0.2 µl forward primer (50 pM), 0.2 µl reverse primer (50 pM), 1 µl cDNA products (100 ng/µl) and 17.4 µl ddH_2O. The reaction conditions were as follows: 97 °C for 5 min, 95 °C for 30 s, 51 °C for 30 s, 72 °C for 1 min, 31 cycles, and then 72 °C for 10 min, 4 °C for 30 min.

Molecular cloning and sequencing. The PCR products were separated from 1% agarose gel electrophoresis using a Huashun Gel Extraction Kit (Huashun, China). The extracted PCR product was connected with pMD 18-T Vector (Takara) and cloned. The recombined plasmids that cut by Hind III and BamH I (Takara) were verified with 1% agarose gel electrophoresis. The recombined plasmids were used to sequencing.

Characteristic analysis. The γ subunit of H^+-ATPase gene sequence was entered into the EditSeq program of the DNASTAR software package to search the biggest open reading frame (ORF) and translated into amino acid sequences using standard genetic code.

The alignments of amino acid sequences of the cloned γ subunit of H^+-ATPase and other representatives of lactic acid bacteria γ subunit of H^+-ATPase were used to generate homology trees. Homology trees were constructed utilising DNAMAN software (version 4.0).

RESULTS

Sequence of the γ subunit of H^+-ATPase in *Lactobacillus acidophilus* MG2-9

After RT-PCR and sequencing for confirmation, the cDNA sequence of γ subunit of H^+-ATPase was obtained. The cDNA sequence is composed of 975 bp, which include an ORF of 963 bp. The ORF can be putative to compose of 320 amino acids and the translation start codon (ATG) and stop codon (TAA) were clear and emphasized by boxed (Fig. 1). The gene sequence had already been available in GenBank (accession number DQ409083).

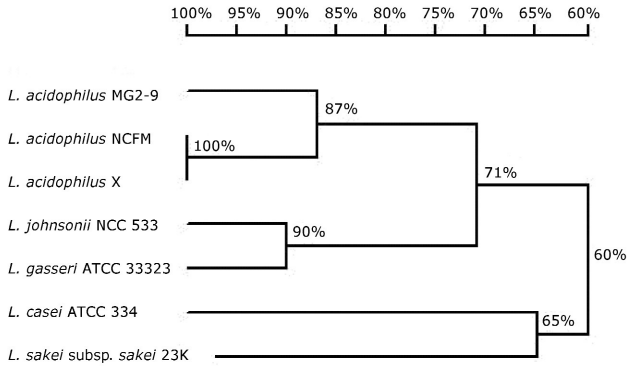

FIG. 2 - Homology tree of amino acid sequence γ subunit of H^+-ATPase in some *Lactobacillus*. The percent numbers represent identical degree.

Homology analysis

To understand the sequence character of γ subunit of H^+-ATPase gene, the sequences of CDS and amino acid of some *Lactobacillus* γ subunit of H^+-ATPase were compared (Table 1). Then, the homology tree is constructed by DNAMAN software (version 4.0), which is shown in Fig. 2. From the table and figure, we can see that the cloned γ subunit of H^+-ATPase gene belongs to the group of *Lactobacillus* γ subunit of H^+-ATPase. The γ subunit of H^+-ATPase gene is conserved in *Lactobacillus*, and is highly conserved in *Lactobacillus acidophilus*.

DISCUSSION

We identified the full-length cDNA sequences of γ subunit of H^+-ATPase in *Lactobacillus acidophilus* MG2-9. The results were confirmed by sequencing and sequence analysis. The cDNA sequence is consisted by 975 nucleotides, including 963 bp ORF that yields a protein of 320 amino acids.

The (F_1F_0) H^+-ATPase complex plays an important role in the free energy metabolism of virtually all living cells. The structures of F_1F_0-ATPase complexes from different sources are very similar and consist of two parts: a membrane integral part, F_0, which forms a proton channel, and a soluble part, F_1, which contains the catalytic site for ATP hydrolysis. There are eight open reading frames with putative ribosome binding sites within the 7-kb region, in which c, a, b belong to F_0, δ, α, γ, β, ε belongs to F_1. Among the ATPase subunits, the α, β and γ subunits from the cytoplasmic domain, F_1, were especially highly conserved between *Lactococcus lactis*, *Streptococcus mutans*, and *Streptococcus bovis* (Koebmann *et al.*, 2000).

The results of homology analysis indicate that γ subunit of H^+-ATPase in *Lactobacillus* is conversed, but not highly. The molecular cloning and characterisation of γ subunit of H^+-ATPase in *Lactobacillus acidophilus* MG2-9 make it possible for further research of the association of the H^+-ATPase gene with acid stress of *Lactobacillus acidophilus* isolated from the koumiss.

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