Molecular Cloning and Characterisation of Alpha Subunit of H^+-ATPase in *Lactobacillus casei* Zhang

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Abstract

Chen X., Yang M., Sun Z., Liu W., Sun T., Meng H., Zhang H. (2009): **Molecular cloning and characterisation of alpha subunit of H^+-ATPase in *Lactobacillus casei* Zhang**. Czech J. Food Sci., **27**: 49–54.

The acid tolerance is an important property of Lactic acid bacteria as potential probiotics. H^+-ATPase is considered a key gene in several bacteria with the ability of acid tolerance. We cloned and sequenced the full length cDNA of alpha subunit of H^+-ATPase gene in *Lactobacillus casei* Zhang, which had been isolated from traditional home-made koumiss in Inner Mongolia of China. The results showed that the respective cDNA sequence is composed of 1530 nucleotides and codes a putative protein including 509 amino acids. In addition, we also reconstructed the phylogenic trees for H^+-ATPase gene based on amino acids sequences of diverse strains of Lactic acid bacteria.

Keywords: H^+-ATPase; clone; sequence; *Lactobacillus casei* Zhang; phylogenic trees

Lactic acid bacteria and other probiotic microorganisms have many documented health effects, so some of them are considered as probiotics which are defined as live microbial food supplements that benefit the health of consumers by maintaining or improving their intestinal microbial balance (Salminen *et al.* 1996; Fuller 1989). Moreover, the research on lactobacilli becomes more and more interesting because of their possible role in the maintenance of gastrointestinal health (Bengmark *et al.* 1998). *Lactobacillus casei* Zhang is a novel potential probiotic which was isolated from the traditional Koumiss widely used in traditional Mongolian medicine in Inner Mongolia of China (Zhang *et al.* 2006). This strain exhibits favourable probiotic properties such as acid tolerance, bile resistance, cholesterol-removing ability and GI colonisation ability (Wu *et al.* 2005; Xu *et al.* 2006; Zhang *et al.* 2006). Moreover, Yun found hypocholesterolemic effect in this strain resulting from its ability to bind and assimilate cholesterol and to suppress the reabsorption of bile acids into the enterohepatic circulation (Yun *et al.* 2006). Zhang found with this strain the regulating function of the cell immunity, the humoural immunity, and

Supported by Natural Science Foundation of China (Grants No. 30560097, 30660135 and 30760156), Hi-tech Research and Development Program of China (863 Program) (Grants No. 2006AA10Z345 and 2007AA10Z353) and New Century Excellent Talent (NCET) Planning of Education Ministry of China.

the intestinal mucous local immunity to mouse (Zhang *et al.* 2006b).

Acid tolerance and tolerance to human gastric juice are considered as important properties in the selection of a preferable probiotic strain (Saarela *et al.* 2000). Studies on the physiology of oral streptococci have led to the view that the cell membrane plays major roles in acid-base regulation. These roles include the extrusion of protons through the membrane and exclusion of the environmental protons (Bender *et al.* 1986). Yokota *et al.* (1995) reported that the acid tolerance of *Lactococcus lactis* subsp. *lactis*, which is used as a starter culture in the dairy industry, depends on the action of cell membrane-bound H^+-ATPase. Miwa *et al.* (1997) found that the H^+-ATPase activity of ruminal acid-tolerant bacteria was higher than that of nonacid-tolerant bacteria, and its activity increased when the bacteria were incubated under acidic conditions. In 2000, Miwa *et al.* (2000) also found that H^+-ATPase has a key role in the acid tolerance of *Streptococcus bovis*. In 2004, Matsumoto suggested that it is necessary for bacteria to increase H^+-ATPase activity quickly and discharge H^+ in order to maintain a constant intracellular pH. The acid-intolerant strains are damaged under acidic conditions since their H^+-ATPase activity cannot be increased, while acid-tolerant strains are less affected and more able to survive because of the rapid increase in their H^+-ATPase activity (Matsumoto *et al.* 2004).

In order to evaluate this strain much more clearly by genomic extent and determine the association between the H^+-ATPase gene and acid tolerance in *Lactobacillus casei* Zhang, the α subunit of H^+-ATPase in *Lactobacillus casei* Zhang was cloned and characterised.

MATERIAL AND METHODS

Strains and culture media. *Lactobacillus casei* Zhang 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 conditions at pH 3.5 and is considered as a potential probiotic. *Lactobacillus casei* Zhang was cultivated in MRS broth without shaking at 37°C.

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

RT-PCR. Using RNA PCR Kit (AMV) Ver. 3.0 (TaKaRa, Japan), reverse transcriptions (RT) were performed with 10 µl reaction volume including 3 µl total RNA(100 ng/µl), 1 µl 10 × 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 conditions were as follows: 30°C for 10 min, 42°C for 30 min, 99°C for 5 min, and 5°C for 5 minutes.

The primers were designed according to the sequence of H^+-ATPase in *Lactobacillus casei* ATCC334 (GenBank accession numbers: NC_008526). The primers were as follows: forward 5'-AGCACCGTTTCGATAAGA-3', reverse 5'-TGGTCGATGCGATCTTGC-3'. The 25 µl PCR reaction mixture was composed of 0.2 µl Taq polymerase (5 U/µl, Takara Tokyo, Japan), 2.5 µl 10 × PCR Buffer (without Mg^{2+}), 2 µl dNTP (2.5mM each), 2 µl $MgCl_2$ (25mM), 0.2 µl forward primer (50pM), 0.2 µl reverse primer (50pM), 1 µl cDNA products and 17.4 µl ddH_2O. The reaction conditions were as follows: 97°C for 5 min, 95°C for 30 s, 55°C for 30 s, 72°C for 1 min, 30 cycles, and then 72°C for 10 min, 4°C for 30 minutes.

Molecular cloning and sequencing. The PCR product of *Lactobacillus casei* Zhang was separated from 1% agarose gel electrophoresis using a Huashun Gel Extraction Kit (Huashun, China). The extracted PCR product was combined with pMD 18-T Vector (Takara, Japan) and cloned. The recombined vector was identified using restriction enzyme digestion with Hind III and BamH I (Takara, Japan) followed by 1% agarose gel electrophoresis, and then the vector was subsequently used for sequencing.

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

The alignments of amino acid sequences of the cloned α subunit of H^+-ATPase and other representatives of Lactic acid bacteria α subunit of H^+-ATPase were used to generate phylogenic trees. Phylogenic trees were constructed utilising DNAMAN software (Ver. 4.0).

RESULTS

Sequence of α subunit of H^+-ATPase in *Lactobacillus casei* Zhang

After RT-PCR and sequencing for confirmation, the cDNA sequence of α subunit of H^+-ATPase was obtained. The cDNA sequence is composed of 1530 bp, and all the nucleotides are ORF. The ORF can be putatively composed of 509 amino acids and the translation start codon (ATG) and stop codon (TAA) were clear and emphasised by boxed (Figure 1). The gene sequence had already been available in GenBank (accession number EU370975).

Homology analysis

To understand the sequence character of α subunit of H^+-ATPase gene, the sequences of CDS and amino acids of some *Lactobacillus* α subunit of H^+-ATPase were compared (Table 1). Then, the phylogenic tree was constructed by DNAMAN software, and is shown in Figure 2. From the table and figure, we can see that the cloned α subunit of H^+-ATPase gene belongs to the group of *Lactobacillus* α subunit of H^+-ATPase. The α subunit of H^+-ATPase gene is conserved in *Lactobacillus*, and is highly conserved in *Lactobacillus casei*.

DISCUSSION

We identified the full-length cDNA sequences of α subunit of H^+-ATPase in *Lactobacillus casei* Zhang. The results were confirmed by sequencing and sequence analysis. The cDNA sequence consists of 1530 nucleotides, and all the nucleotides are ORF which yields a protein of 509 amino acids.

Proton-translocation ATPase (F_1F_0 complex) commonly synthesises ATP in the plasma membranes of bacteria, mitochondria, and chloroplasts. The complete nucleotide sequence of the ATPase genes of *Escherichia coli* was determined (Walker *et al.* 1984). The genes for all eight subunits of the complex reside in a common operon and are transcribed into a single mRNA in bacteria (Gibson *et al.* 1978; Jones *et al.* 1983). A hierarchy was shown wherein pH optima for the enzymes were established for *S. sanguis*, *S. salivarius*, *S. mutans*, and *Lb. casei*, of approximately 7.5, 7.0, 6.0, and 5.0, respectively (Bender *et al.* 1986). The analysis of these data showed that the lower the pH at which the ATPase can function, the more competitive the organism as the end-products of metabolism build up. The central role of ATPase is also seen in the enteric bacteria, with which it was shown that the acid-tolerance response (ATR) does not occur in cells that are defective in the F-ATPase (Foster & Hall 1991). Consequently, it may be presumed that ATPase function is probably a major general component of acid tolerance in bacteria.

Extensive information is now available on bacterial genes that encode the subunits of F-ATPase. The structure of F_1F_0-ATPase complexes from different sources are very similar and consist of

Table 1. Comparison of α subunit of H^+-ATPase of *Lactobacillus casei* Zhang with those of other lactobacilli

Species	GenBank accession No.	Length (bp)	Nucleotide identity (%)	Amino acid identity (%)
Lb. casei ATCC334	NC_008526	1530	99.74	100.00
Lb. plantarum WCFS1	NC_004567	1515	71.11	78.98
Lb. johnsonii NCC 533	NC_005362	1512	69.48	78.39
Lb. gasseri ATCC 33323	NC_008530	1521	69.59	77.54
Lb. helveticus DPC4571	NC_010080	1512	70.26	79.57
Lb. acidophilus NCFM	NC_006814	1512	69.42	78.98
Lb. brevis ATCC367	NC_008497	1542	69.71	74.66
Lb. delbrueckii ATCC11842	NC_008054	1512	69.54	77.80
Lb. reuteri 100-23	NZ_APZZ00000000	1530	68.69	75.64
Lb. sakei subsp. *sakei* 23K	NC_007576	1536	72.34	81.02
Lb. salivarius UCC118	NC_007929	1512	71.24	78.59

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1    ATGAGCATCAAGACTGAGGAAATCAGTTCTCTGATCAAAAAACAACTTGAAGGATATCAG
1     M  S  I  K  T  E  E  I  S  S  L  I  K  K  Q  L  E  G  Y  Q

61   GACGATTTGGCGGCTGAAGAAGTCGGCACTGTGACTTACATCGGTGATGGGATCGCACGT
21    D  D  L  A  A  E  E  V  G  T  V  T  Y  I  G  D  G  I  A  R

121  GCGACTGGGCTGGAAAATGCCATGGCCAACGAATTGCTCCAATTTAGCAACGGCTCATAC
41    A  T  G  L  E  N  A  M  A  N  E  L  L  Q  F  S  N  G  S  Y

181  GGGATGGCGTTAAACCTTGAAACGAACGATGTCGGGATCATTATCTTAGGTGACTTCGAT
61    G  M  A  L  N  L  E  T  N  D  V  G  I  I  I  L  G  D  F  D

241  GAGATTCGCGAAGGCGACCAAGTGAAACGCACTGGCCGAATCATGGAAGTTCCTGTCGGG
81    E  I  R  E  G  D  Q  V  K  R  T  G  R  I  M  E  V  P  V  G

301  GATGCCATGATCGGGCGAGTTGTCAATTCTTTGGGTCAGCCGGTCGACGGTTTAGGCGCG
101   D  A  M  I  G  R  V  V  N  S  L  G  Q  P  V  D  G  L  G  A

361  ATTAAGACGGATAAAACCCGTCCGATCGAGTTTAAGGCGCCAGGTGTTATGCAACGCAAA
121   I  K  T  D  K  T  R  P  I  E  F  K  A  P  G  V  M  Q  R  K

421  TCCGTATCAGAACCGTTACAAACCGGTTTGAAAGCGATTGATGCGCTGGTACCGATTGGC
141   S  V  S  E  P  L  Q  T  G  L  K  A  I  D  A  L  V  P  I  G

481  CGTGGTCAGCGTGAGTTGATCATTGGTGACCGGAAAACCGGGAAAACATCAATTGCGATT
161   R  G  Q  R  E  L  I  I  G  D  R  K  T  G  K  T  S  I  A  I

541  GATACCATTTTGAACCAAAAAGATCAAAACATGATCTGTGTGTACGTTGCGATTGGGCAA
181   D  T  I  L  N  Q  K  D  Q  N  M  I  C  V  Y  V  A  I  G  Q

601  AAGGACAGTACGGTTCGAGCTCAAGTTGAAACGTTGAAAAAATATGGTGCGATGGATTAT
201   K  D  S  T  V  R  A  Q  V  E  T  L  K  K  Y  G  A  M  D  Y

661  ACCATCGTGCTGACTGCTGGCCCATCTGAACCTGCACCAATGCTGTATATCGCGCCTTAT
221   T  I  V  L  T  A  G  P  S  E  P  A  P  M  L  Y  I  A  P  Y

721  GCCGGTGCAGCGATGGGTGAAGAGTTCATGTATAACGGCAAGCACGTCTTGATCGTGTAT
241   A  G  A  A  M  G  E  E  F  M  Y  N  G  K  H  V  L  I  V  Y

781  GATGATTTGAGCAAACAGGCAACCTCATATCGTGAGCTGTCCTTGCTGCTCCGTCGTCCG
261   D  D  L  S  K  Q  A  T  S  Y  R  E  L  S  L  L  L  R  R  P

841  CCTGGTCGTGAAGCCTATCCTGGGGATATTTTCTATACCCACAGTCGCTTGTTGGAACGC
281   P  G  R  E  A  Y  P  G  D  I  F  Y  T  H  S  R  L  L  E  R

901  GCTGCTAAATTGAGTGATAAACTCGGCGGCGGTTCTATGACGGCGTTGCCGGTTATTGAA
301   A  A  K  L  S  D  K  L  G  G  G  S  M  T  A  L  P  V  I  E

961  ACTCAGGCAGGCGATATTTCCGCGTACATCCCGACTAACGTTATTTCTATCACGGATGGT
321   T  Q  A  G  D  I  S  A  Y  I  P  T  N  V  I  S  I  T  D  G

1021 CAGATCTTCTTACAAAGCGATCTGTTCTATGCGGGTACACGTCCAGCCATTGATGCCGGT
341   Q  I  F  L  Q  S  D  L  F  Y  A  G  T  R  P  A  I  D  A  G

1081 GCTTCTGTTTCCCGTGTTGGTGGTGATGCGCAGGTCAAGGCGATGAAGAAAGTTGCCGGG
361   A  S  V  S  R  V  G  G  D  A  Q  V  K  A  M  K  K  V  A  G

1141 ACATTGCGGTTGGATCTGGCATCCTTCCGTGAACTGGAAGCCTTCACGCAATTTGGTTCT
381   T  L  R  L  D  L  A  S  F  R  E  L  E  A  F  T  Q  F  G  S

1201 GATTTGGATGCAGCAACGCAAGCTAAGTTGAATCGTGGTCGCCGAACGGTTGAAGTGCTG
401   D  L  D  A  A  T  Q  A  K  L  N  R  G  R  R  T  V  E  V  L

1261 AAGCAGCCTGTTCACAAACCGTTACCGGTTGAAAAGCAGGTTATCATTCTTTACGCATTA
421   K  Q  P  V  H  K  P  L  P  V  E  K  Q  V  I  I  L  Y  A  L

1321 ACCCATGGCTTCCTTGATCCAATTCCGATTGAAGACATTACTCGCTTCCAAGATGAACTG
441   T  H  G  F  L  D  P  I  P  I  E  D  I  T  R  F  Q  D  E  L

1381 TTTGATTTCTTCGATAGCAATGCAGCTGATTTGCTCAAGCAGATTCGTGACACCGGTAAT
461   F  D  F  F  D  S  N  A  A  D  L  L  K  Q  I  R  D  T  G  N

1441 TTACCGGATACCGATAAACTTGATGCACAAATCAAGGCTTTTGCCGGCGGATTCCAAACG
481   L  P  D  T  D  K  L  D  A  Q  I  K  A  F  A  G  G  F  Q  T

1501 AGTAAACAACTTGCTGCAGCGAAAGACTAA
501   S  K  Q  L  A  A  A  K  D  *
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Figure 1. Nucleotide sequence and putative amino acid sequence of α subunit of H^+-ATPase in *Lactobacillus casei* Zhang

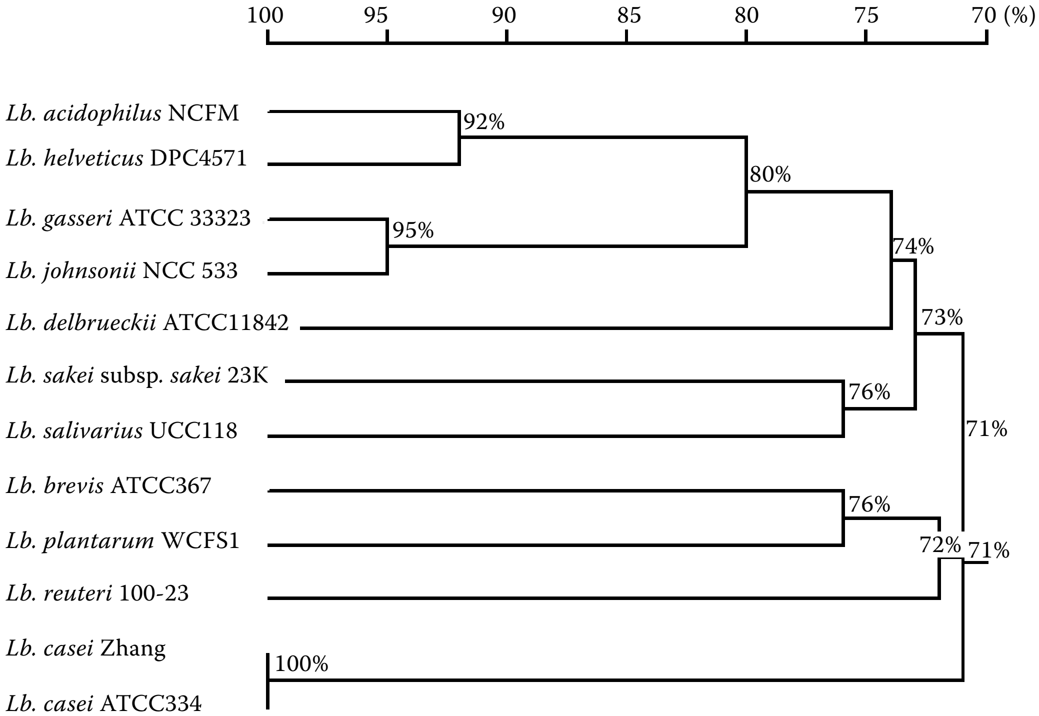

Figure 2. Homology tree of amino acid sequence of α subunit of H^+-ATPase in some lactobacilli. The percent numbers represent identical degree

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 (Koebmann *et al.* 2000). In general, the subunits in the cytoplasmic F_1 domain (consisting of the δ, α, γ, β, and ε subunits) showed a far higher level of homology as compared with the membrane-bound F_0 domain (consisting of the a, c, and b subunits) (Quivey *et al.* 2001).

Lactobacillus casei Zhang is a natural lactobacillus that was isolated from traditional home-made koumiss (Zhang *et al.* 2006a), while *Lactobacillus casei* ATCC334 was isolated from emmental cheese. Moreover, a comparative sequence analysis of the genes encoding 16S rRNA of *Lactobacillus casei* ATCC334 and *Lactobacillus casei* Zhang had the same 16S rRNA sequence with homology of 100% (Wang *et al.* 2008). However, when we checked the physiological and biochemical characteristics of these two strains, we found that *Lb. casei* ATCC334 can ferment rhamnose, melibiose, raffinose, and lactose, but *Lb. casei* Zhang can not. As we have described before, *Lactobacillus casei* Zhang is a potential probiotics, which can survive in artificial gastric juice. Moreover, the phylogenic tree can illustrate the relations between various *Lactobacillus* species. From the tree we can find that the sequences of α subunit of H^+-ATPase of various *Lactobacillus* species are conserved. Moreover, *Lb. acidophi*lus NCFM and *Lb. helveticus* DPC4571, *Lb. gasseri* ATCC33323 and *Lb. johnsonii* NCC533 are highly similar. *Lb. casei* ATCC334 proved not to have so highly conserved species. However, from the above results, we can see that α subunit of H^+-ATPase in *Lactobacillus casei* is amazingly highly conserved, even if they come from different sources and countries. Thus the molecular cloning and characterisation of α subunit of H^+-ATPase in *Lactobacillus casei* Zhang makes it possible for further research to identify the association of the H^+-ATPase gene with acid tolerance.

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Received for publication July 26, 2008

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