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Alpha- and beta-subunits of a V-type membrane ATPase in a hyperthermophilic sulfur-dependent archaeum, *Thermococcus* sp. KI [1]

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Abstract

The genes encoding α- and β-subunits of a V-type ATPase in a sulfur-dependent hyperthermophilic archaeum, *Thermococcus* sp. KI, were cloned and sequenced. The deduced amino acid sequences were approximately 60, 50 and 25% identical to those of other archaeal, eukaryotic V-type and *E. coli* F-type ATPase, respectively. Phylogenetic analysis revealed that *Thermococcus* ATPase was closely related to that of *Thermus*, and those of *Methanosarcina* and *Halobacterium*. © 1997 Elsevier Science B.V.

Keywords: Membrane ATPase; ATPase; Gene cloning; Hyperthermophile; Archaeum; Phylogenetic tree; (*Thermococcus*)

Membrane ATPases are ubiquitous in various organisms from prokaryotes to eukaryotes, and are classified into three groups, P, V and F-types [1,2]. P-type ATPases, which function as a membrane pump like Na^+/K^+-ATPase or Ca^{2+}-ATPase, are sensitive to vanadate and form an acyl phosphate intermediate during ATP hydrolysis. F-type ATPases, which are sensitive to azide, function as ATP synthases in mitochondria, chloroplast and bacteria. V-type ATPases, which are sensitive to nitrate and bafilomycin A1, function as ionic pumps in eukaryotic vacuoles and other organelles. In archaea, membrane ATPases are mostly reported as the V-type in methanogens [3,4], extreme halophiles [5,6] and acidophilic sulfur-dependent autotrophs [7,8]. Occasionally, some of these ATPases are also referred to as archaeal (A)-type [4,5,9]. The archaeal V-type ATPases are thought to function as ATP synthases other than as ionic pumps [10]. Both V-type and F-type ATPases have similar structures such as multiple subunit complexes. Based on a homology analysis of the amino acid sequences of α- and β-subunits, their genes are thought to be derived from common ancestral genes [11]. Molecular phylogenetic analysis based on nucleotide sequences of the gene encoding small subunit (16S) rRNA revealed that hyperthermophiles are in the earliest branches of the phylogenetic tree of all

Abbreviations: CR, polymerase chain reaction; DIG, digoxigenin

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[1] The nucleotide sequence(s) reported in this paper has been submitted to the Gen Bank/EMBL data Bank with accession number(s) D88772.

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organisms on earth [12]. Iwabe et al. [13] used genes for α- and β-subunits of ATPases to assess the phylogenetic position of the origin of life in a phylogenetic tree with the assumption that these subunit genes differentiated before divergence of archaea and bacteria. While the hyperthermophilic archaeal branch is thought to be close to the origin of life, their ATPase genes have remained to be studied.

Membrane ATPase in a hyperthermophilic sulfur-dependent archaeum, *Thermococcus* sp. KI, was purified and was shown to belong to the V-type family [14]. SDS-PAGE analysis revealed that the purified ATPase consisted of 4 subunits with molecular masses of 70, 60, 29 and 15 kDa. The present paper describes nucleotide and deduced amino acid sequences of ATPase α (70 kDa) and β (60 kDa) subunits in this organism, and their phylogenetic relationships are described.

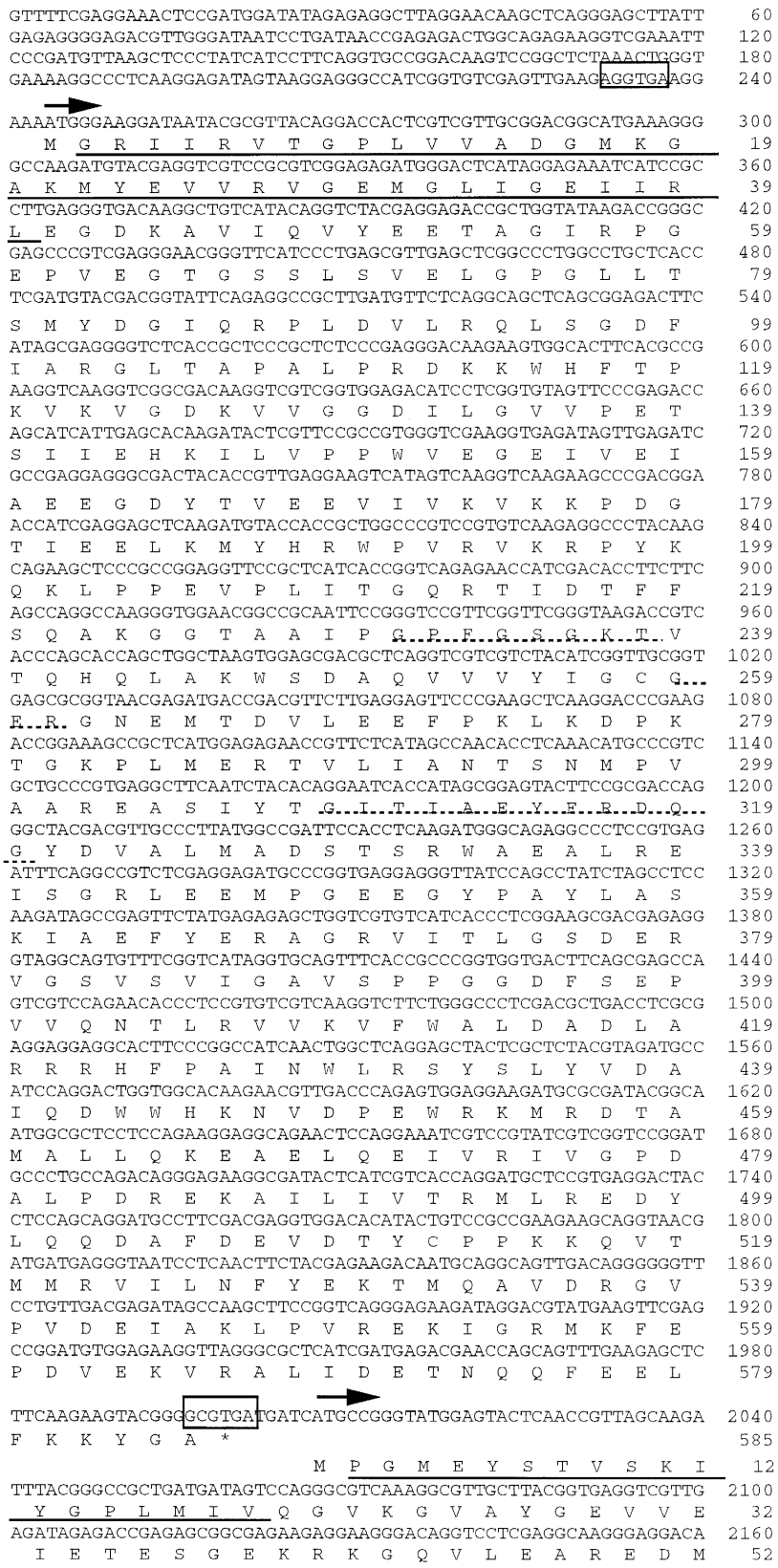
N-terminal amino acid sequences of ATPase 70-kDa and 60-kDa subunits were GRIIRVTGPLVVADGMKGAKMYEVVRXGEMGLIGEIIRL (39 amino acid residues) as described previously [14] and XGMEYSTVSKXYGPLMIV (18 amino acid residues), respectively, where X indicates unidentified amino acid residues.

Thermococcus sp. KI was cultured in 5 L medium containing 1% L-cystine as a sulfur source. After centrifugation, cells (0.5 g) were suspended in 10 mM Tris-HCl buffer (pH 8.0) containing 0.1 M NaCl and 1 mM EDTA. Genomic DNA was prepared as described previously [15], and approximately 2 mg of DNA was obtained. Three oligonucleotide primers (20 bases) were used for amplification. Primers, 10F (5′-GT(AGCT)GT(AGCT)GC(AGCT)GA(CT)GG-(AGCT)ATGAA-3′) and 20R (5′-CC(CT)TC(CT)TC-(AGCT)CC(AGCT)CGCAT(CT)TC-3′), were designed from the N-terminal amino acid sequence, corresponding to the amino acid sequence of VVADGMK and MGLIGEI, of α-subunit of *Thermococcus* sp. KI. Another primer, 10R (5′-AT(CT)-TC(AGCT)CC(AGT)AT(AGCT)A(AG)(AGCT)CC-CAT-3′), was designed based on a highly conserved sequence in the reported V-type ATPase α-subunits, EMPAEEG (*Sulfolobus acidocaldarius* α-subunit [7]). Polymerase chain reaction (PCR) was carried out as follows; initial melting (4 min at 94°C) and 30 cycles of amplification (1.5 min at 94°C, 1.5 min at 50°C, 2 min at 72°C). Deduced amino acid sequences of both DNA fragments amplified by using primers 10F and 20R, and primers 10F and 10R, were identical to the N-terminal sequences of the α-subunit of *Thermococcus* ATPase.

Genomic DNA of *Thermococcus* sp. KI was partially digested with a restriction endonuclease *Sau*3AI, and the resultant fragments were ligated into the *Bam*HI site of pUC18. The plasmid was then introduced into *E. coli* strain JM109. This genomic library was screened using the DNA fragment (1.0-kbp) as a probe, which had been amplified by PCR using primers 10F and 10R. This probe was labelled with digoxigenin (DIG)-dUTP and positive clones were detected by a DIG DNA labelling and detection kit (Boehringer Mannheim, Germany). The nucleotide sequence was determined by using a dye terminator sequencing kit (Perkin Elmer, USA).

While the α-subunit gene of *Thermococcus* ATPase had an open reading frame of 1755 bp which encoded 585 amino acid residues, the β-subunit gene had an open reading frame of 1389 bp encoding 463 amino acid residues (Fig. 1). Initiation methionine residues were thought to be removed from both subunits after translation. The sequences, AGGTGA (-11 to -6) and GCGTGA (-11 to -6), located upstream of the ATG codon of the α- and β-subunits, respectively, were similar to Shine-Dalgarno sequence of archaea [3,16,17]. No identical sequence to archaeal promoter sequences [17] was found in the upstream region of the α-subunit gene of *Thermococcus* ATPase. The initiation codon of the β-subunit started six bases downstream of the last letter of the termination codon of the α-subunit. The calculated molecular masses of α- and β-subunits were 65 353 and 52 037, respectively, except for the initiation methionine.

In the middle region of *Thermococcus* ATPase α-subunit, a conserved sequence in a putative ATP binding site, GXXXXGK(T/S) [18], was observed (residues 230–237, Fig. 1). Other conserved sequences, GER (residues 258–260), and GXTX-AEXXRDXG (residues 308–319) in nucleotide binding proteins [19], were found in the α-subunit (Fig. 1). In *E. coli* ATPase, several amino acid residues were revealed to be important [20]. Among them, 6 out of 10 important amino acid residues in the *E. coli* β-subunit were conserved in *Thermococcus* α subunit (G-223, G-230, G-225, K-236, D-327 and D-



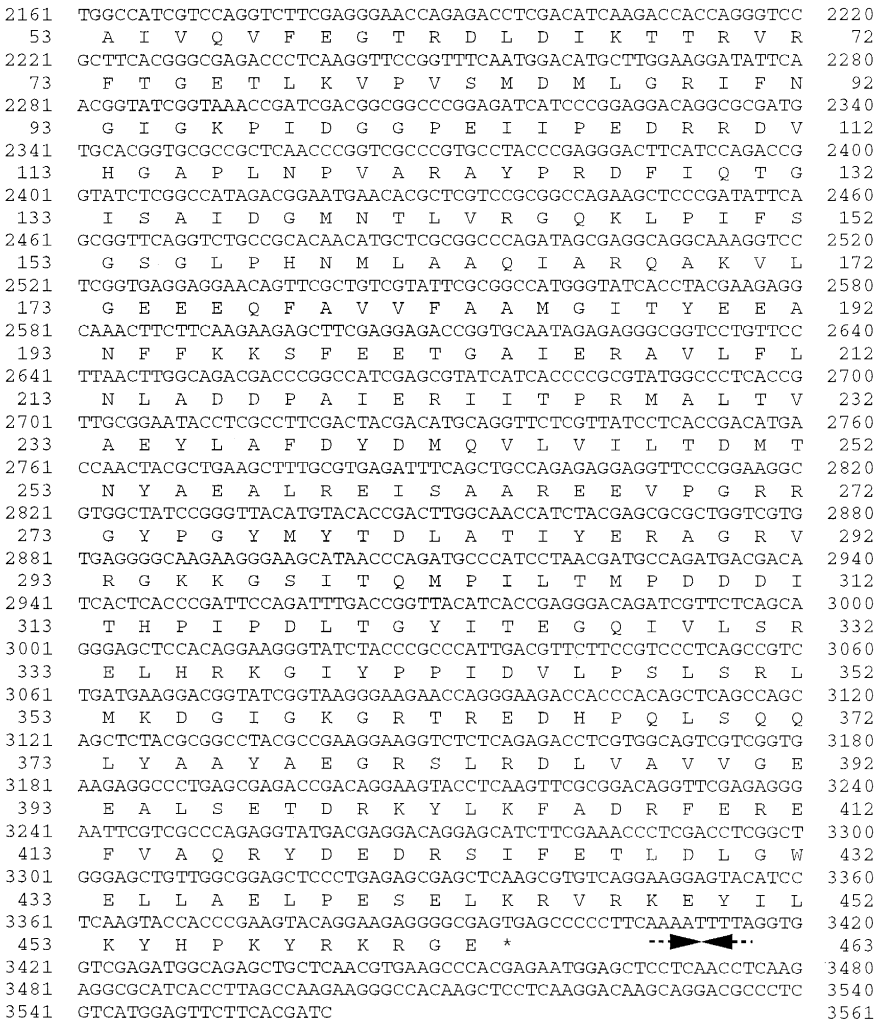


Fig. 1. Nucleotide and deduced amino acid sequences of ATPase α- and β-subunits of *Thermococcus* sp. KI. Arrows above the sequence indicate the initiation codons. The putative Shine-Dalgarno sequences are boxed. N-terminal sequences determined by a protein sequencer are underlined. The homologous sequences of nucleotide binding proteins are underlined with dotted lines. Two arrows with dotted lines after the stop codon of β-subunit gene show an A-T rich, inverted repeat.

331), and 6 of the 7 residues of the *E. coli* α-subunit were conserved in *Thermococcus* β-subunit (P-268, S-318, G-325, S-347, S-349 and R-350). Because the corresponding amino acid residues were also conserved in other V-type ATPases, these residues may be important for the V-type ATPase activity.

Thermococcus ATPase activity was weakly inhibited by nitrate [14], and this inhibition was thought to be caused by the oxidation of cysteine residues in ATPase subunits [21]. In *Thermococcus* ATPase, only two cysteine residues were found in α-subunit (C-217 and C-513) but there was no cysteine in β-subunit. *Thermococcus* ATPase had fewer cysteine residues than other V-type ATPases, and this might be the reason why its sensitivity to nitrate was low.

Table 1 shows the homologies of the amino acid sequences of *Thermococcus* sp. KI ATPase α- and β-subunits with those of other V-type and F-type ATPases. They were more similar to V-type than F-type ATPase.

Based on amino acid sequences of the α- and β-subunits, a phylogenetic tree was constructed by the neighbor-joining method [22] (Fig. 2). This phylogenetic tree indicated that these subunits were

Table 1
Sequence homology of the *Thermococcus* ATPase α- and β-subunits with corresponding subunits in other species

Source	Type of ATPase	% Identity		Domain	Ref.
		α-subunit	β-subunit		
Halobacterium halobium	V	57	62	Archaea	[9]
Methanosarcina barkeri	V	61	66	Archaea	[3]
Sulfolobus acidocaldarius	V	60	63	Archaea	[7,8]
Thermus thermophilus	V	60	64	Bacteria	[27]
Neurospora crassa	V	50	54	Eucarya	[28,29]
Carrot	V	54		Eucarya	[30]
Escherichia coli	F	26	26	Bacteria	[31,32]

evolved by duplication of a single gene, as reported elsewhere [9,11,13]. Both subunits of *Thermococcus* ATPase were in V-type family, as expected. In archaea, *Thermococcus* ATPase was more closely related to those of *Methanosarcina* and *Halobacterium* than that of *Sulfolobus* (Fig. 2). Based on DNA sequences coding for 16S rRNA, the genus *Thermococcus* was placed in a branch, Euryarchaeota, with *Methanosarcina* and *Halobacterium*. On the other hand, *Sulfolobus* was in another branch, Crenarchaeota. The present tree topology was consistent with that based on 16S rDNAs. Interestingly, *Thermus thermophilus* (thermophilic bacterium) ATPase was closer to *Thermococcus* ATPase than eukaryotic and *Sulfolobus* ATPases. In the analysis of noncatalytic subunits, *Thermus* was most closely related to *Thermococcus* (Fig. 2). These results differed from the tree based on DNA sequences encoding 16S rRNA, and might be due to evolution for adaptation to environments requiring high thermostability.

Recently, an F-type ATPase has also been reported in addition to V-type ATPase in methanogens [23,24] and *Enterococcus hirae* [25,26]. The gene encoding F-type ATPase was not found in *Thermococcus* sp. KI genomic DNA by PCR using primers designed by Sumi et al. [23]. Perhaps *Thermococcus* sp. KI has

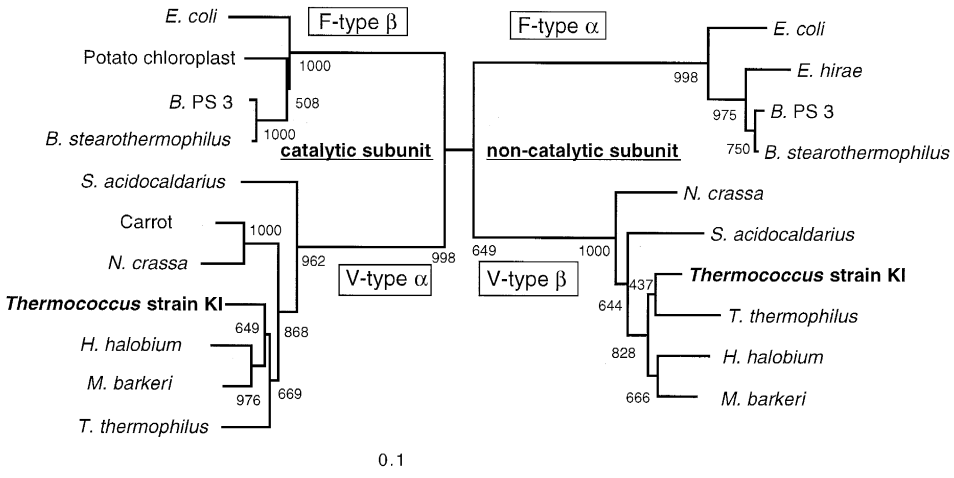


Fig. 2. A phylogenetic tree based on amino acid sequences of ATPase α- and β-subunits. The amino acid sequences of highly conserved regions, fundamentally similar to those as described by Iwabe et al. [13], were aligned by Clustal W Ver. 1.4, with some manual adjustment after removal of gaps. A phylogenetic tree was constructed by neighbor-joining method [22] using PHYLIP Ver. 3.57C and Tree View. Sequence data were taken from the GenBank data base (release 95). The scale bar represents 0.1 amino acid substitutions per site. The bootstrap resamplings [33] were repeated 1000 times to obtain the reliability of the phylogenetic tree, and the probabilities are indicated in the diverging points of the tree.

only the V-type ATPase, which in these organisms plays a role resembling that of F-type ATPases in energy production.

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