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F- and V-type ATPases in the hyperthermophilic bacterium *Thermotoga neapolitana*

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Abstract Two gene clusters encoding F- or V-type ATPases were found in genomic DNA of the hyperthermophilic bacterium *Thermotoga neapolitana*. The subunit genes of each ATPase formed an operon. While the gene arrangement in the operon of the F-type ATPase resembled those in eukaryotic organelles and bacteria, that of the V-type ATPase was different from those reported for archaea, bacteria, or eukaryotes. Both ATPases were found to be expressed in the cells of *T. neapolitana* by Western blot analysis. Although V-type ATPase could not be rendered soluble, F-type ATPase was solubilized with 1% Triton X-100 and characterized. This is the first report of the coexistence of both F- and V-type ATPases in hyperthermophilic bacteria. It has recently been shown by a genome analysis that *Thermotoga maritima* has no V-type ATPase gene cluster but does have an F-type ATPase gene cluster; however, part of a gene for the D-subunit of the V-type ATPase gene has been reported in the *T. maritima* genome. Evolution of the two types of ATPases in *Thermotoga* is discussed.

Key words Membrane ATPase · Hyperthermophilic bacterium · *Thermotoga* · Gene loss · Gene cloning · Operon · Phylogenetic tree

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Introduction

Ion translocating membrane ATPases are ubiquitous in both prokaryotes and eukaryotes (Grüber et al. 2001). F-type ATPases, which are sensitive to azide, function in ATP synthesis in mitochondria, chloroplasts, and bacteria. V-type ATPases, which are sensitive to nitrate and bafilomycin A1, function as ionic pumps in eukaryotic vacuoles and other organelles. V-type ATPases have also been found in archaea (Konishi et al. 1990) and are sometimes referred to as A-type ATPases (Ihara and Mukohata 1991). Both F- and V-type ATPases are composed of multiple subunit complexes. Homology sequence analyses of the catalytic and noncatalytic subunits of the F- and V-type ATPases have suggested that these genes are derived from a common ancestral gene (Gogarten et al. 1989). It is interesting how the differentiation of these two types of ATPase occurred in their evolution. A phylogenetic analysis based on the DNA sequences of the gene encoding 16S rRNA has revealed that hyperthermophiles are in the deepest branches of the phylogenetic tree of all organisms (Olsen et al. 1994). To understand the evolution of ATPases, it is important to study the phylogenetic distribution of these two types of ATPase in hyperthermophilic archaea and bacteria.

V-type ATPase or its gene has been reported from hyperthermophilic archaea (Iida et al. 1996, 1997; Shibui et al. 1997), while most ATPases of bacteria are F-type. However, it has been reported that the thermophilic bacteria, *Thermus thermophilus* (Tsutsumi et al. 1991) and *Clostridium fervidus* (Höner et al. 1997) have V-type ATPase, while F-type ATPase has also been found in some other *Thermus* species (Radax et al. 1998). The mesophilic bacterium *Enterococcus hirae* has been shown to have both F- and V-type ATPases (Takase et al. 1993). It is interesting to elucidate whether such hyperthermophilic bacteria as *Thermotoga* have F- and/or V-type ATPases. It has recently been shown by a genome sequence analysis that *T. maritima* has no V-type ATPase gene cluster, but it has a gene cluster for F-type ATPase (Nelson et al. 1999).

We report in this paper the coexistence of both F- and V-type ATPases in *Thermotoga neapolitana*. This is the first

report of the presence of both F- and V-type ATPases in hyperthermophilic bacteria.

Materials and methods

PCR amplification of fragments corresponding to catalytic subunits of membrane ATPases

Thermotoga neapolitana DSM 4359 was cultured at 80°C in 1 l of a medium containing 0.6% glucose, 0.2% yeast extract, 0.2% tryptone, 0.1% L-cystine, a trace amount of biotin, 660 ml of seawater, 340 ml of distilled water, and 3 mM piperazine-*N*,*N*′-bis(2-ethanesulfonic acid)-NaOH (pH 7.5). After centrifugation, the cells (0.5 g) were suspended in a 10 mM Tris–HCl buffer (pH 8.0) containing 0.1 M NaCl and 1 mM EDTA. Genomic DNA of *T. neapolitana* was prepared as described previously (Iida et al. 1997). Two sets of primers were used for amplification of the genes for catalytic subunits of the ATPases. The primers for the F-type ATPase, FL2 (5′ GA(AG)(CA)GNACN (CA)GNGA (AG)GGNAA(TC)GA 3′) and FR (5′ TCN GT(TGC) AG(AG)TC(AG)TCNGCNGGNAC(AG)TA 3′), were designed by Sumi et al. (1992) and correspond to the amino acid sequences ERTREGND and YVPADD LTD of the β-subunit of *Escherichia coli* ATPase. The primers for the V-type ATPase, V12F (5′ GGNACNGCNGCN AT(ACT)CCN GG 3′) and V10R (5′-CC(CT)TC(CT)TC NCCNCGCAT (CT)TC-3′), were respectively based on highly conserved sequences in the reported V-type ATPase A-subunits, GTAAIPG and EMPAEEG. A polymerase chain reaction (PCR) analysis was carried out with initial melting (4 min at 94°C) and 30 cycles of amplification (1 min at 94°C, 1.5 min at 50°C, and 2 min at 72°C). The deduced amino acid sequences of the amplified DNA fragments were homologous to the sequences of the corresponding subunits from previously reported F- and V-type ATPases.

Cloning genes for the subunits of F- and V-type ATPases

Genomic DNA from *T. neapolitana* was digested with the restriction endonucleases *Bam*HI, *Eco*RI, *Bgl*II, and *Hin*dIII, and the resulting fragments were ligated into the corresponding sites of the plasmid pUC18. The plasmid was then introduced into *E. coli* JM109. This genomic library was first screened by using the amplified DNA fragments as probes (377 bp and 383 bp for the genes of the F- and V-type ATPases, respectively). These probes were labeled with digoxigenin (DIG)-dUTP, and the positive clones were detected by a DIG DNA labeling and detection kit (Boehringer Mannheim, Germany). Prehybridization and hybridization were carried out at 65°C, and the filters (Hybond N^+, Amersham Pharmacia Biotech, Sweden) were washed with 0.1 × sodium saline citrate (SSC)–0.1% SDS at 65°C. The DNA sequences were determined by using a dye terminator sequencing kit (Perkin Elmer, USA). We used the appropriate portion of the sequenced DNA as the probe to clone the DNA of the flanking region. Each subunit gene was identified from its deduced molecular mass calculated from open reading frames and from its homology to the amino acid sequences of the reported ATPase subunits (A to G of V-type and α to ε of F-type, in decreasing order of molecular mass). Subunits of the F-type ATPase supposed to be embedded in the membrane were named a to c.

The DNA sequences for the F-type and V-type ATPases of *T. neapolitana* have been submitted to the DNA Data Bank of Japan (DDBJ) and GenBank with accession numbers AB004784 and AB004668, respectively.

Preparation, solubilization, and separation of membrane ATPases from *T. neapolitana* cells

Thermotoga neapolitana cells were anaerobically grown in 5 l of the medium already described above. The cells (2 g in wet weight) were harvested by centrifugation (10,000 *g*, 14 min), washed, and suspended in 10 ml of a 100 mM Tris–HCl buffer (pH 7.2) containing 2 mM $MgCl_2$ and 10% glycerol. They were then sonicated with a type 250 sonifier (Branson Sonic Power, USA) for 5 min, and the debris was removed by centrifugation (5,000 *g*, 20 min). The resulting supernatant was centrifuged (88,000 *g*, 1.5 h). The precipitated membranes were washed twice, suspended in 10 ml of a 20 mM Tris–HCl buffer (pH 7.2), and used as the membrane fraction. The membranes were resuspended in 10 ml of the same buffer containing 1.0% Triton X-100 for solubilization. The suspension was incubated at 30°C for 2 h while shaking, and then centrifuged again (88,000 *g*, 1.5 h). The supernatant was applied to a HiTrap Q column (5 ml, Amersham Pharmacia Biotech) that had been equilibrated with the same buffer. ATPases were eluted with a linear gradient of NaCl from 0 to 0.8 M. Active fractions were collected and dialyzed against the 20 mM Tris–HCl buffer (pH 7.2) containing 1.0% Triton X-100 with a Spectra/Por (Spectrum Laboratories, USA) membrane (cutoff of 12–14 kDa). The precipitate, on the other hand, was suspended in the same buffer and used as the Triton X-100-insoluble membrane fraction.

ATPase assay

ATPase activity was measured at 70°C in a mixture containing 80 mM Tris–HCl (pH 7.2), 2 mM ATP, 2 mM $MgSO_4$, and 8% glycerol. The reaction was started by adding ATP and stopped with trichloroacetic acid (7% final concentration). The ATPase activity was calculated by measuring the amount of inorganic phosphate produced (Fiske and Subbarow 1925). When the effect of inhibitors was tested, ATPase was incubated with an inhibitor at 37°C for 30 min before the analysis. To determine the optimal temperature for the ATPase activity, the reaction mixture containing the purified ATPase was preincubated in the absence of ATP at each temperature for 10 min, and then ATP (2 mM) was added and the mixture was incubated at the same temperature for 10 min. The reaction was stopped with trichloro-

acetic acid (7%), and ATPase activity was measured as described above.

Protein concentration was determined by using a Bio-Rad protein assay kit (Bio-Rad, USA) with bovine serum albumin as the standard.

Western blot analysis

Peptides (14-mer; CAGTIDEVKERAKE and CHFEELE KLYPNKA) corresponding to the C-terminal amino acid sequences of the F-type β- and the V-type A-subunits, respectively, of *T. neapolitana* ATPases with an additional C residue at the N-terminal end were synthesized. They were linked to Keyhole Limpet (Pierce, USA) hemocyanin and used to raise the antiserum in rabbits.

After SDS-PAGE (4%–20% polyacrylamide), the proteins were electroblotted onto a polyvinylidine difluoride membrane (Immobilon-P; Millipore, USA). The blotted membrane was then immunostained with a Vectastain ABC kit (Vector, USA) and an immunostaining HRP-1000 kit (Konica, Japan).

Results

Gene arrangement of both types of ATPases

In the F-type ATPase gene cluster, the order of the genes was I, a, c, b, δ, α, γ, β, and ε, like that of the F-type ATPases in *E. coli* (Walker et al. 1984). Interestingly, the α- and a-subunit genes of *T. neapolitana* F-type ATPase appear to start at a TTG codon.

In the gene cluster of the V-type ATPase of *T. neapolitana*, genes for the F-, G-, E-, A-, B-, and D-subunits were found. In the upstream region of the F-subunit gene, there was a TA-rich sequence and a putative promoter sequence (TTGTG and TAGAAT, –35 and –10 bp from the promoter, respectively; Liao and Dennis 1992). The amino acid sequence of the F-subunit was hydrophobic and similar to that of the K-subunit of *E. hirae* (Takase et al. 1994) and of the c-subunit of *Sulfolobus acidocaldarius* (Denda et al. 1989). This subunit may thus be a proteolipid one. The homology analysis revealed that the order of the genes encoding the *T. neapolitana* V-type ATPase subunits was different from that of other V-type ATPases such as that of *E. hirae* (Takase et al. 1994; F, I, K, E, C, G, A, B, D, H, and J) and *Haloferax volcanii* (Steinert et al. 1997; D, C, E, A, and B). In *Methanosarcina mazei*, a separate gene cluster, which contains genes encoding the H-, I-, and K-subunits, has been found upstream of the cluster of genes encoding the E-, C-, F-, A-, B-, D-, and G-subunits (Ruppert et al. 1998).

The number of deduced amino acid residues and estimated molecular masses (kDa in parentheses) of the F-, G-, E-, A-, B-, and D-subunits of the *T. neapolitana* V-type ATPase were 143 residues (14.4 kDa), 106 (11.9), 194 (23.0), 586 (66.2), 460 (51.2), and 203 (23.5), respectively. Those of the I-, a-, c-, b-, δ-, α-, γ-, β-, and ε-subunits of the *T. neapolitana* F-type ATPase were 116 residues (12.9 kDa), 280 (31.4), 87 (8.9), 152 (17.9), 183 (21.6), 503 (55.9), 278 (32.5), 468 (51.3), and 107 (12.1), respectively. Those of the I-, a-, c-, b-, δ-, α-, γ-, β-, and ε-subunits of the *T. maritima* F-type ATPase were 116 residues (12.8 kDa), 283 (31.8), 85 (8.6), 164 (19.3), 183 (21.5), 503 (56.1), 273 (31.9), 468 (51.2), and 108 (12.3), respectively. The identities between the deduced amino acid sequences of the subunits of the F-type ATPases of *T. neapolitana* and *T. maritima* were 74.1%, 85.4%, 98.8%, 84.9%, 77.0%, 96.8%, 84.2%, 96.8%, and 86.9%, respectively.

Homology analysis of the ATPase subunits

The amino acid sequences of the α- and β-subunits of the *T. neapolitana* F-type ATPases were 56%–64% and 68%–69% identical to those of the eubacterial F-type ATPases such as that of *E. coli* (Kanazawa et al. 1982). On the other hand, the amino acid sequence of the *T. neapolitana* V-type A-subunit was 51% identical to that of the eubacterial V-type ATPase from *T. thermophilus* (Tsutsumi et al. 1991), 51%–57% identical to those of the V-type ATPases in such archaea as *Methanosarcina barkeri* (Inatomi et al. 1989), *Halobacterium salinarum* (Ihara and Mukohata 1991), and *Thermococcus* sp. KI (Iida et al. 1997), and 48% identical to that of the eukaryotic V-type ATPases in *Neurospora crassa* (Bowman et al. 1988). The identity of the V-type B-subunit to other organisms was slightly higher than that of the A-subunit.

The identity of amino acid sequence of the V-type ATPase A-subunit in *T. neapolitana* to that of the F-type β-subunit was only 25%. The amino acid sequence of the nucleotide binding site, GXXXXGKT, is conserved in the catalytic (V-type A and F-type β) subunits of ATPases (Hsu et al. 1987). In *T. neapolitana*, the sequences for the V-type A- and F-type β-subunits were, respectively, GGFGTGKT (230–237) and GGAGVGKT (154–161).

The identity in amino acid sequence between the *T. neapolitana* ATPase subunits, other than the V-type A/B- and F-type α/β-subunits, and the corresponding reported sequences for other bacteria and archaea was 20%–30%.

Enzymatic properties of the membrane ATPases

In order to characterize both types of ATPase, a membrane fraction was prepared from *T. neapolitana* cells. After being rendered soluble, the supernatant was applied to a HiTrap Q ion-exchange column. The active fractions were pooled and concentrated, and some characteristics of the ATPases were investigated. The optimal temperature for enzyme activity was 80°C. Such anions as HSO_3^-, which are known to stimulate V-type ATPase activity of some archaea (Iida et al. 1996), had no effect. $NaNO_3$ and NH_4VO_3, the respective specific inhibitors of the V- and P-type ATPases, did not affect the ATPase activity at 20 and 1 mM, respectively. Two specific inhibitors for the F-type ATPase, azide (1 mM) and 7-chloro-4-nitrobenzo-2-oxa-1,3-diazole (0.1 mM), inhibited the ATPase activity by 84% and 100%, respec-

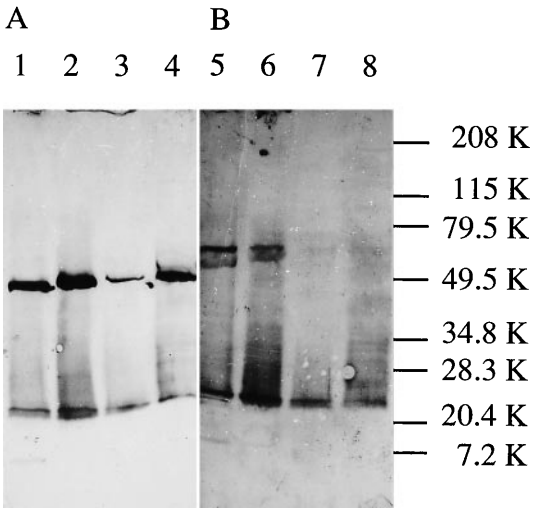

Fig. 1A,B. Western blot analysis of the *T. neapolitana* ATPases. The crude extract, membrane, 1% Triton X-100-soluble membrane, and active fractions separated by HiTrap Q column chromatography were subjected to SDS-PAGE (4%–20% polyacrylamide) and then immunostained. **A** Antiserum for F-type β-subunit. **B** Antiserum for V-type A-subunit. *Lanes 1* and *5*, crude extract (10 μg of protein); *lanes 2* and *6*, membrane fraction (5 μg); *lanes 3* and *7*, 1% Triton X-100-soluble membrane fraction (4 μg); *lanes 4* and *8*, ATPase active fraction (5 μg) by HiTrap Q column chromatography. *Numbers* on the right are the molecular masses of the standards

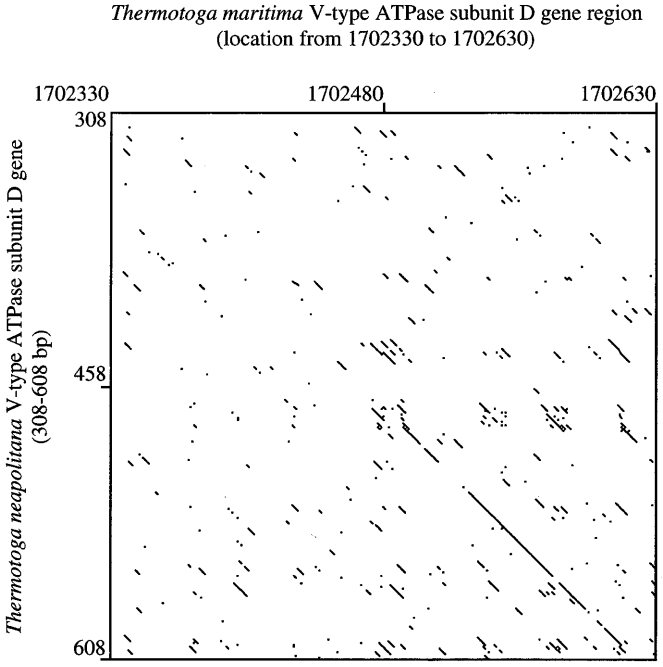

Fig. 2. Homology matrix comparison of the V-type ATPase D-subunit genes of *Thermotoga neapolitana* and *T. maritima*. A *T. maritima* gene fragment (location from 1702330 to 1702630) containing part of TM1724, a spacer, and TM1725 was compared with the D-subunit gene (308–608 bp) of *T. neapolitana* V-type ATPase. The threshold for homology detection was 6 bases in 8 nucleotides. The homology matrix was obtained by using Genetyx Mac version 8.0 and Harrplot version 1.2.2 software (Software Development, Japan)

tively. These results indicate that this ATPase was of the F-type.

Western blot analysis

The Western blot analysis was carried out to determine whether both ATPase genes were expressed and translated to proteins. A band in the lanes of the crude extract, the membrane fraction, and the active fraction from HiTrap Q column chromatography reacted positively with the antiserum against the β-subunit of F-type ATPase (Fig. 1). Its apparent molecular mass was approximately 50 kDa, which is similar to the molecular mass of 51.3 kDa that was calculated from the deduced amino acid sequence of the β-subunit. It is therefore concluded that the active HiTrap Q fraction contained the F-type ATPase.

The A-subunit of V-type ATPase could not be detected in the 1.0% Triton X-100-soluble membrane fraction of *T. neapolitana* by the Western blot analysis with its specific antiserum, but it was detected in the crude extract and membrane fraction (Fig. 1). This indicates that the *T. neapolitana* membrane contained both F- and V-type ATPases.

Discussion

We found in this study that *T. neapolitana* had both the F- and V-type ATPase genes and that both genes were expressed in the cells. In the hyperthermophilic archaeon, *Thermococcus* sp. KI, only the V-type ATPase gene (Iida et al. 1997) has been found by PCR and Southern blot analyses. In the complete genomic DNA sequences of hyperthermophilic archaea such as *Methanococcus jannaschii* (Bult et al. 1996), only the V-type ATPase gene cluster is present. On the other hand, the hyperthermophilic bacterium *Aquifex aeolicus* has been shown to have only an F-type ATPase gene cluster (Deckert et al. 1998). Some bacteria and archaea have been shown to have the genes of both types of ATPase. Among bacteria, *E. hirae* has been shown to have both types of ATPase (Takase et al. 1993), and the methanogenic archaeon *M. barkeri* has separate gene clusters for both the F- and V-type ATPases (Sumi et al. 1997), although the expression of the F-type ATPase gene has not yet been shown.

It has recently been reported that the genome sequence of *T. maritima* contains only an F-type ATPase gene cluster (Nelson et al. 1999). The identity of the deduced amino acid sequences of its subunits to those of *T. neapolitana* F-type ATPase was 74%–99%. It has no V-type ATPase genes, except for a gene fragment of 198 bp that is homologous to that of the D-subunit of V-type ATPase [gene TM1725 with accession number AE001812 at The Institute for Genomic Research (TIGR) database; http://www.tigr.org/tigr-scripts/CMR2/GenomePage3.spl?database=btm]. The presence of a stop codon in the sequence and a frame shift suggests that this gene is silent in the present genome of *T. maritima*.

The possibly vestigial D-subunit fragment between two stop codons was thought to code a 48-amino-acid peptide that showed an identity of 77% to the corresponding region of the V-type ATPase of *T. neapolitana*. A homology-matrix comparison of this gene fragment with the *T. neapolitana* V-type ATPase D-subunit gene showed that approximately 120 bp of the *T. maritima* fragment was highly homologous to the gene fragment corresponding to the C-terminal region of the *T. neapolitana* V-type ATPase D-subunit gene product (Fig. 2). This strongly suggests that the ancestral *Thermotoga* species had the V-type gene cluster. The gene cluster including the 5′ half of the D-subunit gene has disappeared in *T. maritima* with the species divergence from *T. neapolitana*.

A phylogenetic analysis of the F-type α/β- and V-type A/B-subunits of ATPases in bacteria, archaea, and eukaryotic organelles was carried out by the neighbor-joining method (Fig. 3). The β-subunit of F-type ATPase formed a clade with the A-subunit of the V-type, as did the B-subunit of the V-type ATPase and the α-subunit of the F-type ATPase (Gogarten et al. 1989). The α- and β-subunits of the F-type ATPase of *T. neapolitana* are most closely related to those of *T. maritima*, while the V-type ATPase of *T. neapolitana* was most closely related to one of two distinct V-type ATPases of *Treponema pallidum* (Fraser et al. 1998). It is widely accepted that the catalytic (V-type A- and F-type β-) and noncatalytic (V-type B and F-type α) subunits of the ancestral ATPase originated from gene duplication prior to the divergence of archaea and bacteria, and that in archaea the ATPase evolved to the V-type, and in bacteria to the F-type (Gogarten et al. 1989). However, it has also been proposed that F- and V-type ATPases differentiated before the divergence of archaea and bacteria (Forterre et al. 1993). After the divergence of the two domains, most archaea may have lost the F-type ATPase genes, and most of the bacteria, the V-type ATPase genes. If this hypothesis is plausible,

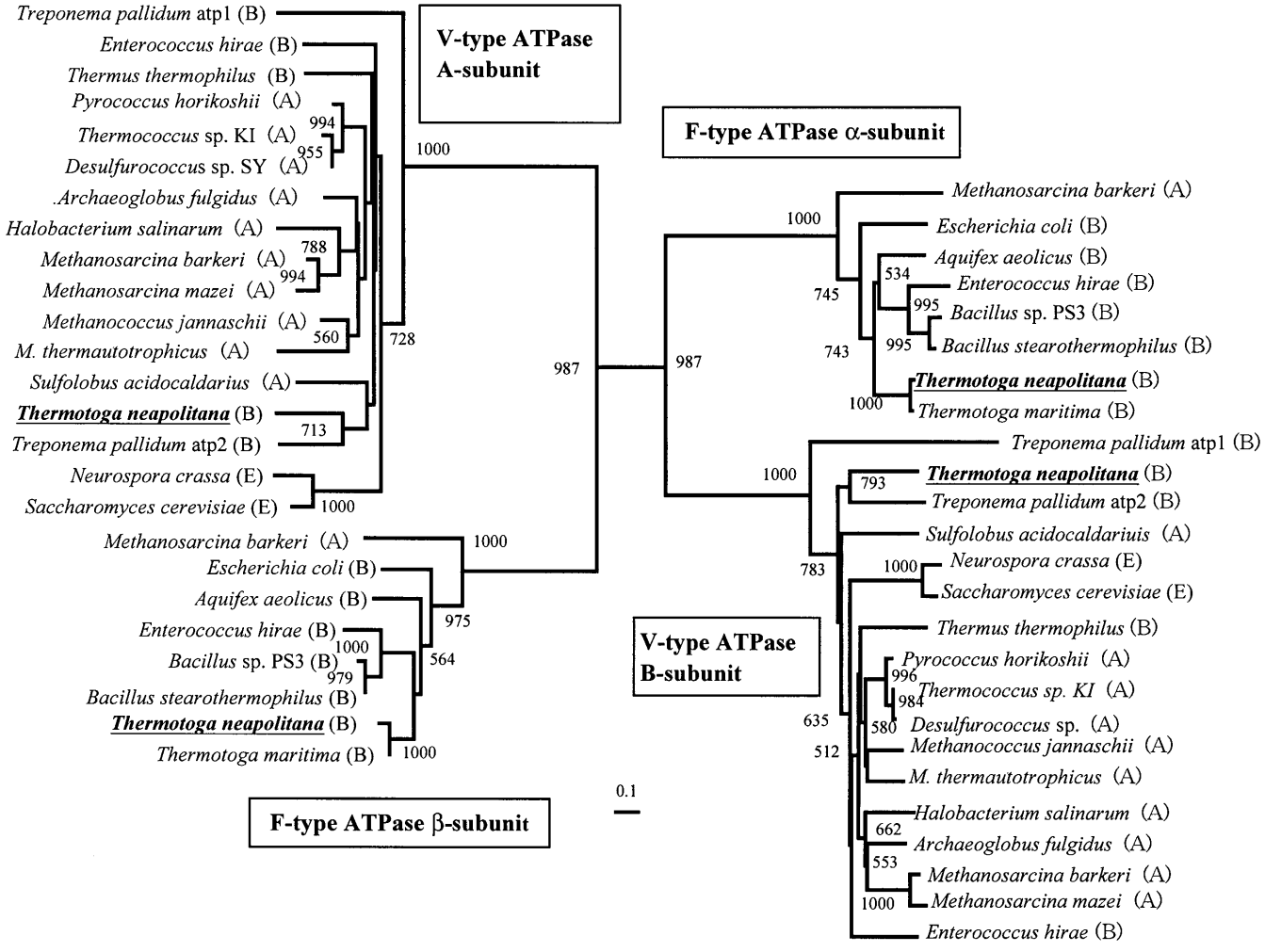

Fig. 3. Phylogenetic tree constructed by the neighbor-joining method (Saitou and Nei 1987). The amino acid sequences of the ATPase catalytic and noncatalytic subunits were aligned, the gaps were removed, and the sequences analyzed by Clustal W version 1.7. The analysis employed 389 sites. The phylogenetic tree was constructed by Tree View, and sequence data were taken from the DNA Data Bank of Japan and GenBank databases. *Treponema pallidum* has two distinct V-type ATPases, which are designated as atp1 and atp2 (Fraser et al. 1998). Archaea, bacteria, and eukaryotes are shown as *(A)*, *(B)*, and *(E)*, respectively. *M. thermautotrophicus* indicates *Methanothermobacter thermautotrophicus*, formerly named *Methanobacterium thermautotrophicum* strain ΔH. *Numbers* at the nodes indicate the bootstrap values for the clades with 1,000 replications; bootstrap values higher than 500 are shown. The *bar* indicates 0.1 substitution per site

bacterial F- and archaeal V-type ATPase genes must be monophyletic. However, it is not clear whether the A- and B-subunits of archaeal and bacterial V-type ATPases are monophyletic (Fig. 3). The coexistence of F- and V-type ATPases in *T. neapolitana* may be explained by the latter hypothesis that ancestors of archaea and bacteria had both F- and V-type ATPases. In the genus *Thermotoga*, *T. neapolitana* retains both F- and V-type ATPases, while *T. maritima* lost the V-type during evolution. It is interesting that some *Thermus* species have V-type ATPase and some others of the genus have F-type ATPase (Radax et al. 1998). This coexistence of both types of ATPase may also be explained by horizontal or lateral gene transfer (Hilario and Gogarten 1993), which has been proposed to explain the evidence that a quarter of *T. maritima* genomic genes are most similar to archaeal genes (Nelson et al. 1999). Thus, the ancestor of the *Thermotoga* species might have gained V-type ATPase genes by lateral gene transfer. The V-type ATPase gene cluster, except the part of the D-subunit gene, was lost in *T. maritima* after its divergence from the ancestor of *T. neapolitana*.

The function of the F-type ATPase in *T. neapolitana* remains to be studied. *Thermotoga* is an anaerobic heterotroph that fermentatively synthesizes ATP (Adams 1994), and it is unclear whether this organism possesses a respiratory chain. *Thermotoga neapolitana* produced H_2S after the addition of sulfur to the culture medium (Belkin et al. 1986). Although sulfur reduction is not coupled with the growth yield of *Thermotoga* species, the yield increased in the presence of thiosulfate (Ravot et al. 1995). Respiratory growth with Fe(III) has been suggested in *T. maritima* (Vargas et al. 1998). Thus, the reduction of these electron acceptors may be coupled with ATP production by F-type ATP synthase (ATPase) activity as reported for *Salmonella typhimurium* (Sasahara et al. 1997). In *E. hirae*, the F-type ATPase is H^+-translocating, and the V-type ATPase is Na^+-translocating (Takase et al. 1994; Murata et al. 1999). On the other hand, the V-type ATPase translocates H^+ in such methanogens as *M. mazei* (Becker and Müller 1994). The ion specificity of the ATPases in *T. neapolitana* is currently unknown, and further physiological work is necessary to determine this. An investigation of the function of V-type ATPase in *T. neapolitana* may provide a clue to solve the problem of how this gene cluster was lost from the *T. maritima* genome and how its function was complemented.

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