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A DNA fragment homologous to F_1-ATPase β subunit was amplified from genomic DNA of *Methanosarcina barkeri*

Indication of an archaebacterial F-type ATPase

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A 490 bp DNA fragment was amplified from *Methanosarcina barkeri* genomic DNA by the polymerase chain reaction (PCR) using oligonucleotide primers designed based on conserved amino acid sequences of the F_1-ATPase β subunits. The amino acid sequence deduced from the DNA sequence of this fragment was highly homologous to a portion of the F_1-ATPase β subunit. This indicates that this archaebacterium has a gene of F-type ATPase in addition to a gene of V-type ATPase.

Archaebacteria; *Methanosarcina barkeri*; ATPase; V-type ATPase; F-type ATPase in archaebacteria

1. INTRODUCTION

The H^+-ATPase synthesizes ATP using an electrochemical proton gradient generated by an electron transport chain and has been considered to play an essential role in energy metabolism. Until recently it was thought that H^+-ATPases from any organisms have the similar structural and functional features and they are designated as F_0F_1-ATPase. However, this concept was modified by the finding that ATPases from archaebacteria [1], such as *Methanosarcina barkeri* [2,3], *Sulfolobus acidocaldarius* [4,5], and *Halobacterium halobium* [6], are only distantly related to F_0F_1-ATPase and, instead, are highly similar to eukaryotic vacuolar H^+-ATPases [7]. It is now generally accepted that F_0F_1-ATPase and vacuolar ATPase are two subclasses of a superfamily of ATPases which do not form phosphorylated enzyme intermediates and that the former, now often called as F-type ATPase, distributes among mitochondria, chloroplasts, and eubacteria and the latter, V-type ATPase, does among archaebacteria and eukaryotic vacuolar systems. Agreement of taxonomic border and distribution of F- and V-type ATPases has stimulated the argument for archaebacterial origin of eukaryotic vacuolar systems. However, it was soon found that distribution of V-type ATPase is not restricted to archaebacteria: a thermophilic eubacterium *Thermus thermophilus* has a V-type ATPase [8,9] and an eubacterium *Enterococcus hirae* has both types of ATPase [10,11]. These facts raise the possibility that restricted distribution of F-type ATPases in eubacteria and V-type ATPases in archaebacteria is not as strict as it used to be thought and that some archaebacteria may have V-type ATPase. In this communication, we report the amplification of a DNA fragment homologous to the F-type ATPase β subunit by the polymerase chain reaction (PCR) from *Methanosarcina barkeri* genomic DNA.

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2. EXPERIMENTAL

2.1. *DNA source and synthetic oligonucleotides*

M. barkeri was a kind gift from Dr. Y. Koga, University of Occupational and Enviromental Health. Genomic DNA from this organism was prepared as described in [12]. Oligonucleotide primers used for PCR and for DNA sequencing were synthesized using a DNA synthesizer (Applied Biosystem 381A). Three oligonucleotide primers (FL1, FL2 and FR) for PCR amplification were designed based on highly conserved regions of reported amino acid sequences of F-type ATPase β subunits. Specific features of their design are given in section 3. The primers for DNA sequencing are FL3 (5′-ATATCTAGAACGTACTCGTGAAGG-3′) and FR3 (5′-ATTGAATTCATCAGCAGCAGGAACATA-3′). Underlined bases are the added restriction sites, *Xba*I and *Eco*RI, respectively, and sequences on the 3′ side of the restriction sites are the same as one of the degenerate sequences of PCR primers, FL2 and FR, respectively.

2.2. *Polymerase chain reaction*

The reaction mixture (100 μl) for PCR contained 200 ng of template (*M. barkeri* genomic DNA), 1 μM of each primer (FR1 and FL), 1 mM $MgCl_2$, 2.5 units *Taq* DNA polymerase (Promega) in the buffer provided by a Promega kit (50 mM KCl, 10 mM Tris-HCl, pH 9.0 at 25°C, 0.1% Triton X-100). PCR reactions were carried out for 1 min at 94°C, 1 min at 60°C, 1 min at 74°C and were repeated for 35 cycles

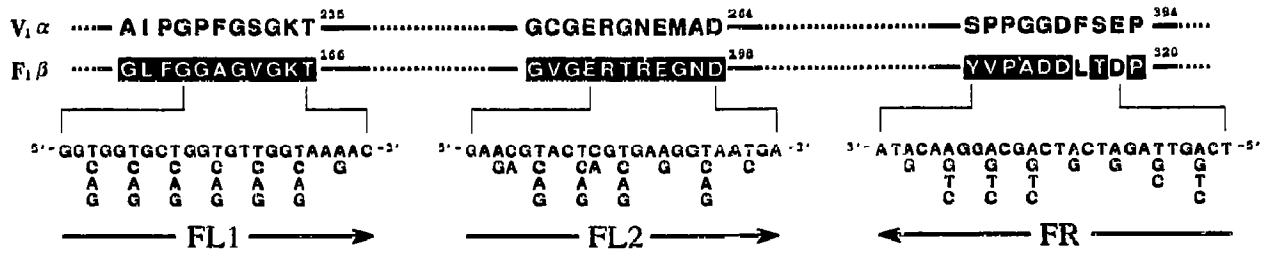

Fig. 1. The amino acid sequences of conserved region of F-type ATPase β subunits and the design of PCR primers. The highly conserved regions among β subunits of F-type ATPases from various sources (bovine mitochondrion, *E. coli*, *R. rubrum*, a thermophilic *Bacillus* strain PS3, spinach chloroplast) are shown by white letters on a black background ($F_1\beta$). The sequence of corresponding regions of the V-type ATPase of *M. barkeri* is also shown above ($V_1\alpha$). To achieve a complete match with the estimated sequence of *M. barkeri* genomic (template) DNA, degenerate sequences were synthesized and used. FL1 and FL2 is left (5'-side, sense) primer and FR is right (3'-side, anti-sense) primer.

(the final step at 74°C was extended to 10 min). The reaction products were separated with a 6% polyacrylamide gel and visualized with ethidium bromide. The DNA fragment of interest was eluted from the gel and used as template DNA for the second amplification which was performed to decrease non-specific products. The reaction mixture (100 μl) for the second amplification contained 2 ng of the template DNA, 1 μM of each primer (FR2 and FL), 2.5 units *Taq* DNA polymerase in the buffer provided by a Promega kit, and was repeated for 30 cycles (1 min at 94°C, 1 min at 60°C, 1 min at 74°C).

2.3. *Sequencing the amplified product*

Single strand DNA templates were prepared with the asymmetric PCR using FR3 and FL3 primers and the sequence was determined by Sequenase version 2.0 (US Biochem. Corp.) using [α-^{35}S]dCTP. All DNA segments were confirmed by sequencing both strands. In order to determine the sequences near the 3' and 5' terminals of amplified fragments, the fragments were cloned into the *Eco*RI and *Xba*I sites of pUC119 vectors and universal sequence primers were used.

2.4. *Southern blotting*

The genomic DNA was digested with *Eco*RI, *Hin*dIII or *Ssp*I, applied on an agarose gel, blotted on a nylon membrane filter and hybridized with the DNA fragment of the second amplification [13]. The biotin-labeling reactions was performed according to the BioNick Labeling System (Bethesda Research Laboratories Life Technologies, Inc.). The DNA fragment corresponding to the amplified region of genomic DNA was detected with the PhotoGene System (Bethesda Research Laboratories Life Technologies, Inc.).

3. RESULTS

3.1. *PCR primers*

The amino acid sequences that are appropriate to design the PCR primers should fulfil two requirements to ensure the specificity of amplification: they should be well conserved among the F-type ATPases, but sufficiently different from the V-type ATPases. We compared F-type ATPase β subunit sequences from various sources including bovine heart mitochondria [14], *Escherichia coli* [15,16], *Rhodospirillum rubrum* [17], a thermophilic *Bacillus* PS3 [18], and spinach chloroplasts [19], and three regions of the sequence were chosen (Fig. 1). The sequences of the selected regions are almost common to any F-type ATPase β subunits but are significantly different from the V-type ATPase α (57K) subunit which is considered to be a corresponding subunit to F-type ATPase β subunit [4].

3.2. *Amplification*

Using FL1 and FR primer, a 490 bp DNA fragment was amplified by PCR from *M. barkeri* genomic DNA (Fig. 2, lane 1). The second PCR reaction was carried out using FR2, instead of FR1 in the first reaction, in order to confirm that the amplified DNA fragment of the first PCR reaction really contained another sequence common to F-type ATPase β subunits. A 380 bp DNA fragment was amplified by the second PCR reaction (Fig. 2, lane 2).

3.3. *DNA and deduced amino acid sequences of the amplified fragment*

The DNA sequence of the 380 bp fragment and the deduced amino acid sequence are given in Fig. 3. The GC content of the fragment is 43% which is close to the overall GC content of this bacterium (36–43%, [20]). The sequence contains an *Ssp*I site (Fig. 3, underlined).

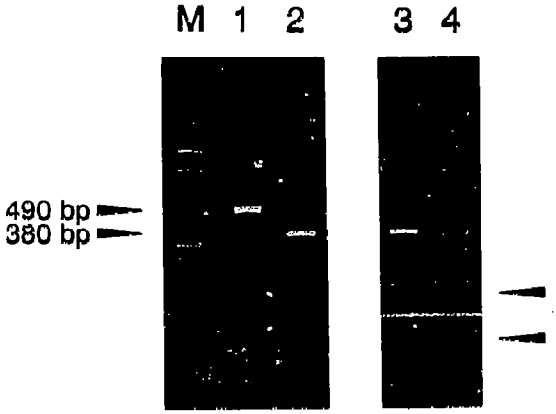

Fig. 2. Polyacrylamide gel electrophoresis of amplified PCR fragment from *M. barkeri*. Primers used for amplification were FL1 and FR (lane 1), FL2 and FR (lane 2), and FL3 and FR3 (lane 3). The 380 bp DNA fragment (lane 3) digested with *Ssp*I is shown in lane 4.

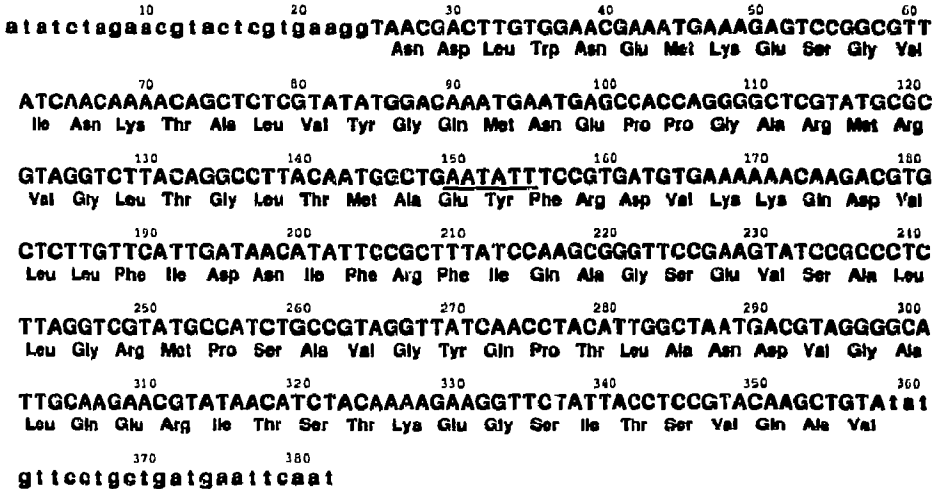

Fig. 3. Partial nucleotide sequence of the amplified 380 kbp DNA fragment and deduced amino acid sequence. The underlined sequence is the restriction site of *Ssp*I.

Consistently, when the amplified fragment was digested with *Ssp*I, it was divided into two fragments (Fig. 2, lane 4). The deduced amino acid sequence shows strong similarity to the equivalent region of F-type ATPase β subunits but not to V-type ATPases 57K subunits (Fig. 4). Therefore, we conclude that the amplified DNA fragment is a part of the F_1-ATPase β subunit gene.

3.4. *Southern blotting*

Since we have repeated the whole procedures using different cultures of *M. barkeri* and obtained the fragment with the same base sequence each time, the possibility that the amplification was due to accidentally contaminating DNA is very small. Furthermore, *M. barkeri* genomic DNA digested with *Eco*RI, *Hin*dIII or *Ssp*I was subjected to Southern blot analysis using the 380 bp DNA fragment as a probe. As shown in Fig. 5, *M. barkeri* genomic DNA digested with *Eco*Ri and *Hin*dIII had a single positive band. Consistent with the determined base sequence of the 380 bp fragment, two positive bands appeared when the DNA was digested with *Ssp*I. Therefore, the possibility that the fragment was amplified from contaminated DNA is very unlikely.

4. DISCUSSION

The F-type ATPases have been found in a variety of eubacterium and eukaryotic mitochondria and chloroplasts, but, in the archaebacterium kingdom, they have not so far been found. We have shown the amplification

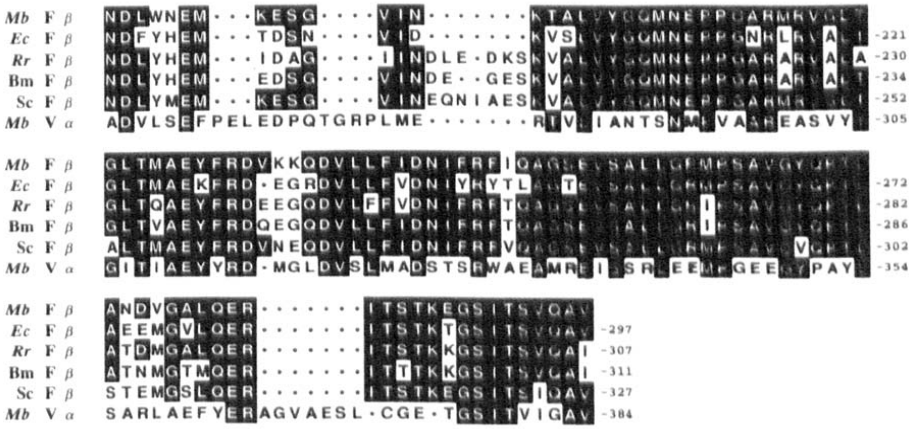

Fig. 4. Alignment of amino acid sequences of F-type ATPase β subunits from various sources and the α (57K) subunit of V-type ATPase from *M. barkeri*. The region corresponding to the amplified 380 kbp DNA fragment from *M. barkeri* (*Mb* Fβ) is shown. Sequence data were taken from the following references: *E. coli* (*Ec* Fβ, [16]), *R. rubrum* (*Rr* Fβ, [17]), bovine mitochondrion (Bm Fβ, [14]), spinach chloroplast (Sc Fβ, [19]). Amino acid residues identical with those from the *M. barkeri* DNA fragment are shown by white letters on a black background. The numbers at the right side of each sequence indicate those of amino acid residues. The corresponding region of *M. barkeri* V-type ATPase α subunit is also shown for reference (*Mb* Vα).

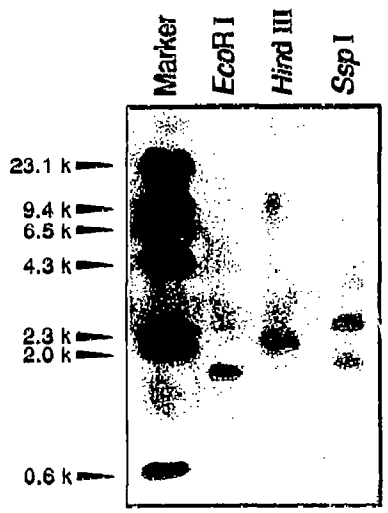

Fig. 5. Genomic southern analysis of *M. barkeri* DNA using the 380 kbp PCR fragment. *M. barkeri* genomic DNA digested with *Eco*RI, *Hin*dIII, or *Ssp*I was electrophoresed, blotted, and hybridized with the biotin-labeled amplified 380 kb DNA fragment. The biotin-labeled λDNA digested with *Hin*dIII was used for the marker lane. The size of the DNA molecular weight markers (bp) are indicated.

of a DNA fragment from *M. barkeri* homologous to typical F-type ATPase β subunit genes. This suggests the strong possibility of the existence of F-type ATPase in the archaebacterium kingdom, although cloning of a full operon or purification of the F-type ATPase from this archaebacterium is necessary to eliminate the small possibility that it has only a part of the F-type ATPase operon and does not synthesize intact F-type ATPase. Isolation of a water-soluble part of V-type ATPase and cloning of genes encoding some of its subunits from *M. barkeri* were reported by Inatomi and his colleagues [3]. Therefore, this archaebacterium appears to have genes of two types of ATPases. Then, two questions arise. What are the physiological roles of the two types of ATPases in the cell, and why has the presence of F-type ATPase not been recognized during procedures of V-type ATPase purification? The recent finding that a eubacterium *Enterococcus hirae* also has two types of ATPase is very interesting in this context. Kakinuma and his colleagues suggested that the F-type ATPase of *E. hirae* is a H^+-ATPase and V-type ATPase is a Na^+-ATPase [11]. The former is constitutively expressed but expression of the latter is induced at high Na^+ concentration in the culture medium. Probably the F-type ATPase of *M. barkeri* pumps a different ion than the V-type ATPase and is synthesized only under the limited condition. Concerning the evolutionary aspect of this finding, it is our view that, since distribution of F-type and V-type ATPases among prokaryote kingdoms turns out to be not as simple as we used to think, exact distribution, sequence comparison, and in vivo function of F- and V-type ATPases in eubacteria and archaebacteria should be clarified before profound argument.

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