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The H^+ ATPase regulatory subunit of *Methanococcus thermolithotrophicus*: amplification of an 800 bp fragment by polymerase chain reaction

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An 800 bp fragment of *Methanococcus thermolithotrophicus* genomic DNA was amplified by the polymerase chain reaction method using primers designed from conserved regions of the V-type H^+ ATPase regulatory subunits from the archaebacterium *Sulfolobus*, and several eukaryotes. Although more than one product was obtained, only one of them had the expected size and was exclusively amplified in the presence of the left and right primers. The DNA and the deduced protein sequences of the putative *Methanococcus* H^+ ATPase subunit revealed homology to the corresponding sequences in *Sulfolobus* and eukaryotes (about 60% identical residues) and a less evident homology to the eubacterial F_1-ATPase α-subunit (22% identical residues with *E. coli*).

Archaebacteria; Methanobacteria; Molecular evolution; Proton pump; Vacuolar type H^+ ATPase (V-type)

1. INTRODUCTION

The catalytic and regulatory subunits of the eukaryotic vacuolar (V-type) H^+ ATPase are currently being explored as phylogenetic markers which allow the resolution of deep branches in the evolutionary tree [1,2]. Sequence analyses have indicated that the archaebacterium, *Sulfolobus acidocaldarius*, has a eukaryotic-type V-ATPase rather than a eubacterial-type F_0F_1-ATPase [1]. The availability of the temperature-resistant DNA polymerase from *T. aquaticus* (Taq polymerase) for use in the polymerase chain reaction (PCR) technique has greatly enhanced the potential for amplifying conserved genes [3,4]. Thus far, the technique has been applied mainly to closely related species. In this report we describe the use of the PCR technique to clone and sequence a DNA fragment from the archaebacterium, *Methanococcus thermolithotrophicus*, using primers based on sequences derived from eukaryotes and *Sulfolobus*.

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2. MATERIALS AND METHODS

2.1. *DNA source*

Genomic DNA from *Methanococcus thermolithotrophicus* was a gift from Dr J. Konisky, University of Illinois.

2.2. *Primer synthesis*

Oligonucleotide primers were designed on the basis of highly conserved regions of the 60 kDa V-type regulatory subunit sequences. Specific features of their design are given in section 3.

2.3. *Polymerase chain reaction*

The PCR technique was performed according to the manufacturer's instructions (Perkin-Elmer, Cetus, Emeryville, CA) using 200 ng of template and 1 μM of primers. The temperature program for the amplification was: 5 min at 94°C (initial melting), 10 cycles of amplification with slow temperature ramps (1 min at 94°C, 13°C/min ramp to 30°C, 2 min at 30°C, 7°C/min ramp to 72°C, 6 min at 72°C), and 25 cycles of amplification (same as above, without the ramps). The final step at 72°C was extended to 20 min. The reaction products were separated in a 1% agarose gel and visualized with ethidium bromide.

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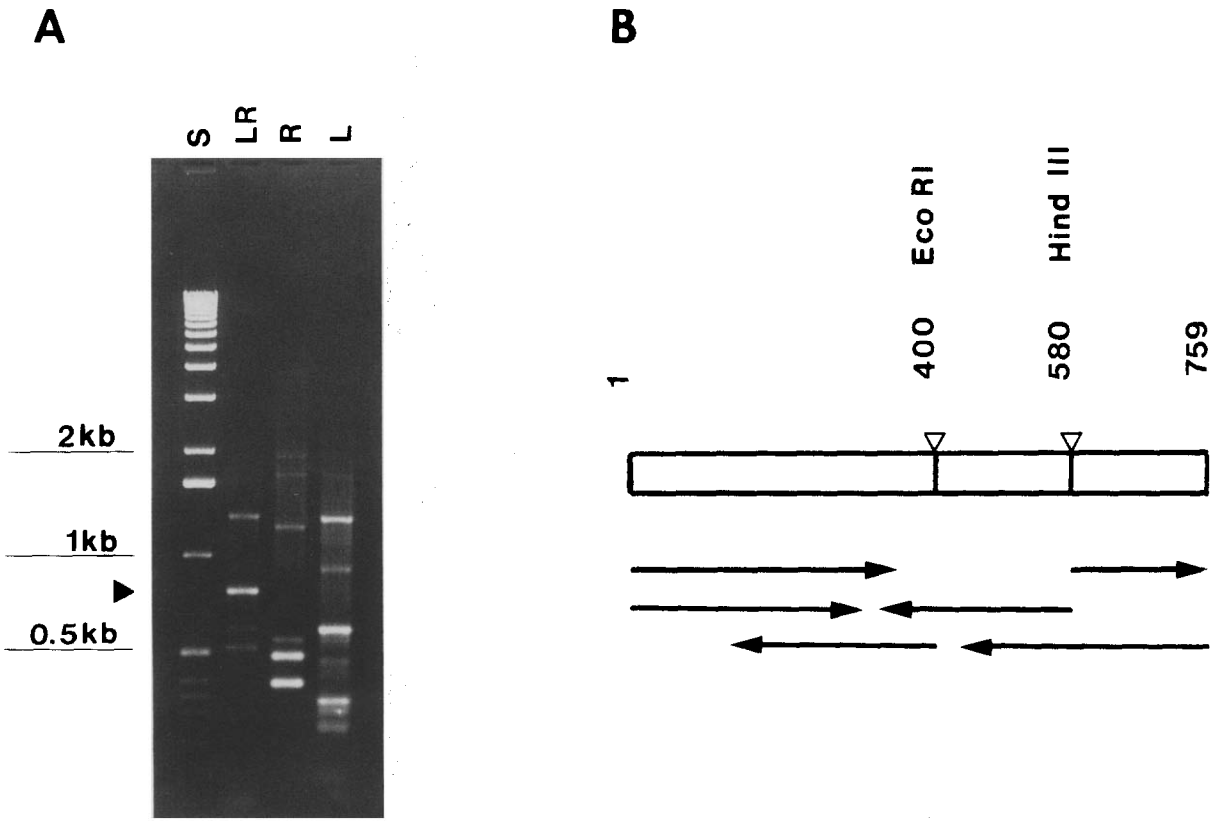

Fig.1. PCR amplification of 200 ng samples of *Methanococcus thermolithotrophicus* genomic DNA. (A) Ethidium bromide stained 1% agarose gel. S, molecular mass markers (BRL); LR, PCR amplification using LEFT and RIGHT primers; L, same as LR without RIGHT primer; R, same as LR without LEFT primer. Arrow indicates the selected amplified product. (B) Restriction map and sequencing strategy of the cloned fragment. Numbers indicate the positions from the 5′ end of the coding sequence (without primers). Arrows indicate the direction and the extent of sequencing.

2.4. *Cloning and sequencing*

The product of interest was eluted from the gel (Bio 101, San Diego, CA) and cloned into pBluescript (Stratagene, La Jolla, CA). Subclones were generated using the restriction enzyme sites for *Eco*RI (400) and *Hin*dIII (580, see fig.1). Double-stranded sequencing was performed with Sequenase (US Biochem. Corp.) [5] using [α-^{35}S]thio-dATP (Amersham) [6].

3. RESULTS AND DISCUSSION

Conserved regions of the H^+ ATPase regulatory subunit sequences from yeast [7], *Neurospora* [8], *Arabidopsis* [9] and *Sulfolobus* [10] were used to design the PCR primers. The LEFT primer, derived from a region located 135 bases downstream from the 3′ end of the *Sulfolobus* sequence was CAGTTTTTGAAGGAAC A/T TCTGG C/A AT. The RIGHT primer, 800 bp downstream from the LEFT primer was CCTTC A/T GTAAT A/G TATCCAGTCAAATC. The number of mismatches of the LEFT primer was 5 for *Neurospora* and yeast, 4 for *Sulfolobus* and none for *Arabidopsis*. The RIGHT primer complementary strand had 4 mismatches for *Neurospora* and yeast, 3 for *Arabidopsis* and 2 for *Sulfolobus*. There were no mismatches in the two bases at the 3′ end of the primers. As shown in fig.1, four PCR products were obtained when both primers were used to amplify *Methanococcus* DNA. The size of the products was found to be an insufficient criterion for the selection of the fragment of interest. Thus, parallel amplifications were performed using only one primer at a time in order to identify artifacts. Only the 800 bp piece, close to

the expected size, appeared to be unique to the reaction with both primers.

The complete DNA sequence and translation of the 800 bp fragment are given in fig.2. As a control for possible Taq polymerase errors [11], 300 bp from the ends of the sequence were confirmed by repeating the experiment using an independently amplified sample of *Methanococcus* genomic DNA. Both sequences were identical. Thus, under the conditions used, the error rate of the Taq polymerase is less than 0.3%.

The partial sequence obtained for the regulatory subunit of *Methanococcus thermolithotrophicus* clearly shows homology to the corresponding subunits in the archaebacterium *Sulfolobus*, and in the eukaryotes. This is obvious at the nucleic acid level, as depicted in a Pustell matrix comparison (fig.3), and at the amino acid level, as demonstrated in the multiple alignment given in fig.4. At the DNA level, the similarity appeared to be slightly higher with *Sulfolobus*. No regions with 70% or more DNA sequence identity were found

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CAGGTTTTTGAAGGAACTTCTGGCAT      AACACAAACGAAACAAAAGTTAGATTCACAGGAGAAACC   39
   LEFT PRIMER                   N  T  N  E  T  K  V  R  F  T  G  E  T    13

GCAAAAATTGGAGTATCCCTTGAAATGTTAGGTAGGATTTTCAACGGTGCAGGTAAACCAATTGACGGC   108
 A  K  I  G  V  S  L  E  M  L  G  R  I  F  N  G  A  G  K  P  I  D  G     36

GGTCCAGAAATTATCCCTGAAAAGAAATTGGACATTAACGGTTACCCATTAAACCCAGTTTCAAGAAAC   177
 G  P  E  I  I  P  E  K  K  L  D  I  N  G  Y  P  L  N  P  V  S  R  N     59

CCACCTAACGCTTTCGTTCAAACCGGTATCTCAACAATCGACGGTACAAACACACTTGTTAGAGGTCAG   246
 P  P  N  A  F  V  Q  T  G  I  S  T  I  D  G  T  N  T  L  V  R  G  Q     82

AAGTTACCTATCTTCTCAGGTTCAGGTTTACCACACAACACCTTAGCTGCACAAATTGCAAGACAGGCA   315
 K  L  P  I  F  S  G  S  G  L  P  H  N  T  L  A  A  Q  I  A  R  Q  A    105

AAAGTTAGAGGAGAAGGAGAACAGTTTGCGGTAGTATTCGCTGCAATGGGGATTACAAACGAAGAAGCA   384
 K  V  R  G  E  G  E  Q  F  A  V  V  F  A  A  M  G  I  T  N  E  E  A    128

AACTACTTCATGGAAGAATTCAAAAAGCCAGGAGCTCTAGAAAACGCTGTTGTTTTCATCAACTTGGCA   453
 N  Y  F  M  E  E  F  K  K  P  G  A  L  E  N  A  V  V  F  I  N  L  A    151

AACGACCCTGCAATTGAAAGAATTATTACCCCAAGACTTGCTTTAACAACAGCAGAATACCTTGCATAC   522
 N  D  P  A  I  E  R  I  I  T  P  R  L  A  L  T  T  A  E  Y  L  A  Y    174

GAAAAAGATATGCACGTTTTAGTTGTCTTAACTGATATGACAAACTACTGTGAAGCTTTAAGGGAAATT   591
 E  K  D  M  H  V  L  V  V  L  T  D  M  T  N  Y  C  E  A  L  R  E  I    197

GCAGCAGCAAGAAACGAAGTTCCAGGAAGAAGAGGTTACCCTGGTTACATGTATACAGACTTGGCTACA   660
 A  A  A  R  N  E  V  P  G  R  R  G  Y  P  G  Y  M  Y  T  D  L  A  T    220

CTCTACGAAAGAGCAGGTAGAGTTAAAGGTAGAAAAGGAACCGTAACCCAGATTCCAATTTTAACAATG   729
 L  Y  E  R  A  G  R  V  K  G  R  K  G  T  V  T  Q  I  P  I  L  T  M    243

CCTCACGACGATATCACACACCCAATTCCT      GATTTGACTGGATACATTACAGAAGG             759
 P  H  D  D  I  T  H  P  I  P          RIGHT PRIMER                        253
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Fig.2. Nucleotide sequence and translated amino acid sequence. The incorporated LEFT primer and the complementary strand to the RIGHT primer are shown.

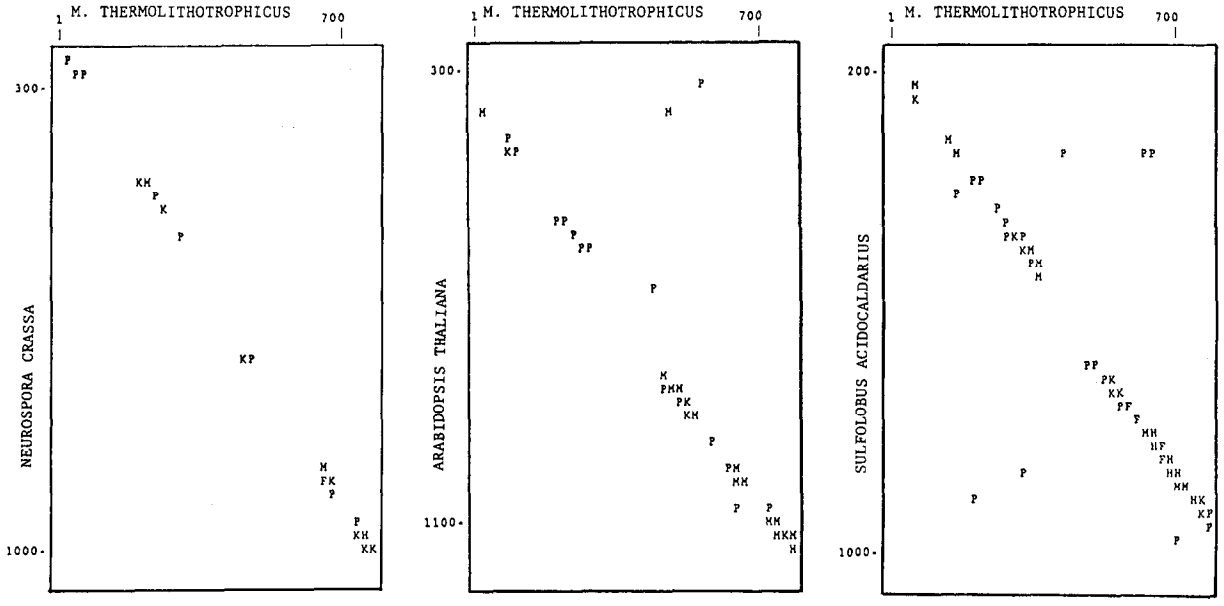

Fig.3. Pustell matrix comparison [13] of the *Methanococcus* sequence with the *Neurospora* [8], the *Arabidopsis* [9] and the *Sulfolobus* [10] sequences. Numbering of the sequences corresponds to the original papers. A 20 base window was used and the letters indicate regions where the homology was 70% (P) to 90% (F).

in the comparison with the eubacterial *E. coli* α-subunit in eubacteria (not shown). The data imply that the *Methanococcus* regulatory subunit belongs to the eukaryotic V-type ATPases. This has recently been confirmed by a similar result for the catalytic subunit [12]. Successful amplification of a methanogenic sequence using primers derived from eukaryotes and *Sulfolobus* suggests that the PCR technique will be a useful tool for evolutionary studies.

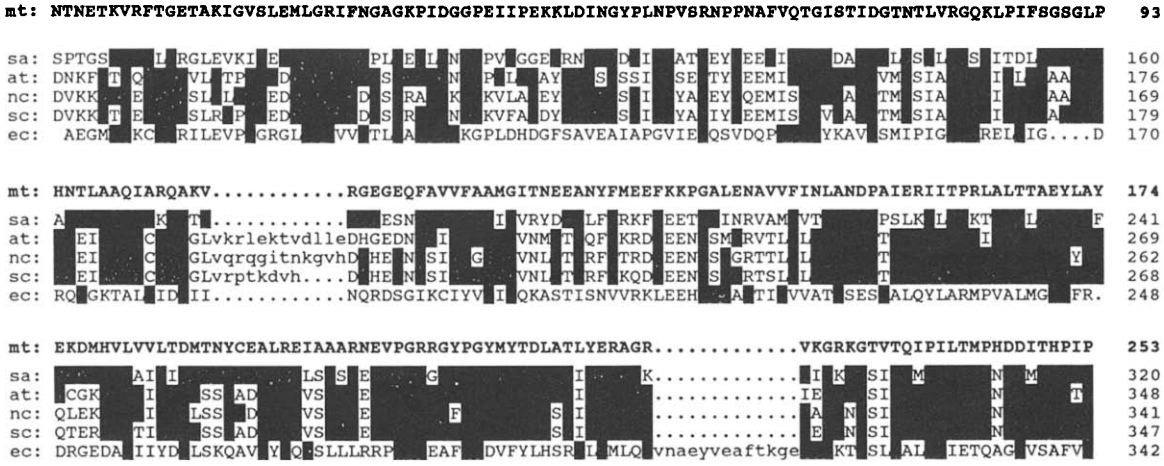

Fig.4. Protein sequences alignment using the Needleman-Wunsch algorithm [14,15] as implemented in [16]. The parameters used were: gap penalty: 2.5, incremental gap penalty: 0.5, Dayhoff's comparison matrix [17]. The *Methanococcus* sequence (mt) was aligned to the *Sulfolobus* (sa), the *Arabidopsis* (at), the yeast (sc), the *Neurospora* (nc) and, for comparison, the *E. coli* α-subunit [18,19] (ec) sequences. Numbering corresponds to the original papers. Identical residues are indicated by black boxes and aligned nonidentical residues by capital letters. Gaps are given as dots and small letters.

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