The Phylogeny of *Methanopyrus kandleri*

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The phylogenetic position of *Methanopyrus kandleri* has been difficult to determine because reconstructions of phylogenetic trees based on rRNA sequences have been ambiguous. The most probable trees determined by most algorithms place the genus *Methanopyrus* at the base of a group that includes the halobacteria and the methanogens and their relatives, although occasionally some algorithms place this genus near the eocytes (the hyperthermophilic, sulfur-metabolizing prokaryotes), suggesting that it may belong to this lineage. In order to resolve the phylogeny of the genus *Methanopyrus*, we determined the sequence of an informative region of elongation factor 1- α that contains an 11-amino-acid insertion in eocytes and eukaryotes which is replaced by a 4-amino-acid insertion in methanogens, halobacteria, and eubacteria. On the basis of the results of our elongation factor 1- α gene analysis, we concluded that the genus *Methanopyrus* diverged from the eocyte branch before the eukaryotic and eocyte lineages separated and therefore is not an eocyte.

Methanopyrus kandleri is a rod-shaped, gram-positive, methanogenic bacterium that was isolated from a deep thermal vent in the Gulf of California (16). This organism is the first known hyperthermophilic methanogen that grows at temperatures up to 110°C (16). Although extreme thermophily has been found in three phylogenetically different groups of prokaryotes (the eubacteria, the methanogens and their relatives, and the eocytes [hyperthermophilic, principally sulfur-metabolizing prokaryotes]), members of the genus *Pyrodictium* are the only other prokaryotes that are known to grow at a temperature of 110°C or above (37, 38). On the basis of the results of a 16S rRNA and protein synthesis elongation factor 1- α (EF-1 α) sequence analysis, the genus *Pyrodictium* was classified as an eocyte taxon (24, 32, 33, 37); therefore, we wanted to determine whether the genus *Methanopyrus* is also an eocyte taxon.

Methanopyrus kandleri appears to represent a unique lineage within the phylogenetically diverse methanogens (18, 30), and, consistent with this, the results of a Jukes-Cantor distance and parsimony analysis of the 16S rRNA sequence of this organism have shown that it is only distantly related to the other methanogens (8). A suggestion that *M. kandleri* may be related to the eukaryotes resulted from the discovery that a eukaryotic-like topoisomerase I molecule is present in *Methanopyrus* cells (35).

The most probable tree determined in our analysis of small-subunit rRNA sequences in which the Jukes-Cantor (19), Kimura (21) and paralineal distance (27) algorithms were used is shown in Fig. 1; the probabilities of this eocyte tree are 72.5, 60.5, and 63.5%, respectively, and the probabilities of the archaeobacterial tree (data not shown) are 20.5, 29.5, and 20.0%, respectively. The most probable location of the genus *Methanopyrus* on the trees obtained with these three algorithms is at the branch labelled A; branch B is less likely, and branch C is least likely. The trees that have been constructed, the similarity of the maximal growth temperatures of *Methanopyrus* and *Pyrodictium* strains, and the presence of a eukaryotic topoisomerase I-like molecule all raised the possibility that the genus *Methanopyrus* may be more closely related to eukaryotes than previously thought. Given that sequence analysis studies can be biased by unequal rate effects, by site-to-site variation, and by the order of alignment, we sought additional, more reliable

evidence for the phylogenetic position of the genus *Methanopyrus*.

In a previous study, the phyletic distribution of the two variants of EF-Tu and EF-1 α was used to infer an immediate relationship between the eocytes and the eukaryotes (32). Previously, this relationship had been difficult to analyze by direct sequence comparisons because of the extremely long divergence times between the prokaryotes and the eukaryotes. In that study, we showed that the members of the genus *Pyrodictium* and the other eocytes, as well as all of the eukaryotes, contain an 11-amino-acid segment in EF-1 α that is not present in other prokaryotes. In the methanogens, the halobacteria, and the eubacteria this segment is replaced by a 4-amino-acid segment. In light of the questionable phylogenetic position of the genus *Methanopyrus*, determining the sequence of the EF-1 α gene of a member of this taxon could reveal if it is also an immediate eukaryotic relative (containing the 11-amino-acid segment and corresponding to tree branch C) or if it is more closely related to the methanogens and halobacteria (containing the 4-amino-acid segment and corresponding to either tree branch A or tree branch B).

As previously noted (22, 28, 31), EF-1 α appears to be one of the best molecules available for studying deep divergences. This molecule is a slowly evolving protein found in all cells, where it binds, transports, and participates in the selection of the correct aminoacyl-tRNA. In addition, EF-1 α interacts with cellular components encoded by genes dispersed throughout the genome, including aminoacyl-tRNAs, ribosomal proteins, 16S and 18S rRNAs, and elongation factor EF-Ts; therefore, it is unlikely that EF-1 α is transferred laterally between organisms.

To determine whether the genus *Methanopyrus* is an eocyte genus, we isolated by PCR, cloned, sequenced, and analyzed a fragment in the GDP-binding domain of the gene coding for EF-1 α from several taxa whose sequences had not been determined previously. Total genomic DNAs were isolated from frozen cell pastes of *Methanopyrus kandleri*, *Archaeoglobus fulgidus* (36), and *Thermoplasma maritimum* by using the sodium dodecyl sulfate-proteinase K lysis method (14). Total nucleic acids were extracted from *Dactylococcopsis salina* (40) and purified by CsCl centrifugation (1). Degenerate oligonucleotide primers were designed on the basis of conserved amino acid motifs in all known EF-Tu and EF-1 α sequences (Fig. 2) and used for PCR amplification (34). The primers spanned the

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FIG. 1. Rooted tree showing the three possible positions of the *Methanopyrus* lineage. This tree is the most probable tree derived from an analysis of 16S and 18S rRNA sequences in which the Kimura (21), Jukes-Cantor (19), and parsimony distance (25, 27) algorithms were used. The tree is rooted in the branch leading to the eubacteria, as proposed by other workers (12, 17). The three possible locations of the genus *Methanopyrus* that have finite probabilities are designated branches A, B, and C. The eubacterium used was *Thermotoga maritima*. Abbreviations: *H.volc.*, *Halobacterium volcanii*; *Ms.hun.*, *Methanospirillum hungatei*; *Mc.vann.*, *Methanococcus vannielii*; *Archae.*, *Archaeoglobus fulgidus*.

IGHVD₂₂, DCPGH₈₄, KNMITG₉₄, QTREH₁₁₈, and FAIR₃₇₇ regions of the homologous *Escherichia coli* amino acid sequence (the subscripts indicate the positions of the 5'-most nucleotides of the primers in the *E. coli* EF-Tu sequence [9, 20]). The PCR fragments were cloned into the pT7 Blue vector (Novagen). The sequences of clones containing the PCR insertion were determined by using a Sequenase 2.0 kit (U.S.

Biochemicals) and M13 universal and reverse primers for reading both strands of the amplified fragments.

The nucleotide sequences of the methanogen *Methanopyrus kandleri*, *Archaeoglobus fulgidus*, the halophilic cyanobacterium *Dactylococcopsis salina*, and the eocyte *Thermoplasma maritimum* are shown in Fig. 3. The deduced amino acid sequence of the *Methanopyrus kandleri* EF-1 α fragment was aligned with the sequences of the homologous fragments of eocytes, methanogens and their relatives, a halobacterium, eubacteria, and eukaryotes (Fig. 2). The *Methanopyrus kandleri* sequence contained the 4-amino-acid segment (GVMP) characteristic of eubacteria, halobacteria, and methanogens, and it lacked the 11-amino-acid segment found in eocyte and eukaryotic sequences (Fig. 2).

The absence of the 11-amino-acid segment in the genus *Methanopyrus* is most parsimoniously consistent with branches A and B on Fig. 1. On either branch A or branch B, only a single change from the 4-amino-acid segment to the 11-amino-acid segment (Fig. 1, solid rectangle) is needed to explain the observed distribution of insertions. In comparison, one additional change from the 11-amino-acid segment to the 4-amino-acid segment on branch C would be required to explain the branch C alternative. Therefore, these results strongly indicate that the genus *Methanopyrus* is not an eocyte genus and is less closely related to the eukaryotes. In addition, it should be noted that all of the new sequences described in this paper are consistent with and provide additional support for the eocyte tree, on which the eocytes are the closest relatives of the eukaryotes.

Taxon	Organism	11 amino acid segment	4 amino acid segment
Methanogens & Relatives	Mp.kand.	KNMITGASQADAAVLVVAADD	---GVMP
	T.celer	KNMITGASQADAAVLVVAATD	---GVMP
	P.woes.	KNMITGASQADAAVLVVAATD	---GVMP
	A.fulg.	knmitgASQADAAVLVMDVVE	---KVQP
	Mc.vann.	KNMITGASQADAAVLVNVDD	AKSGIQP
	T.acido.	KNMITGTSQADAAVLVSARD	-GEGVME
Halobacteria	H.maris.	KNMITGASQADNAVLVVAADD	---GVQP
Eubacteria	Th.mar.	KNMITGAAQMDGAILVVAATD	---GPMP
	D.sal.	KNMITGAAQMDGAILVCSAAD	---GPMP
	E.coli	KNMITGAAQMDGAILVVAATD	---GPMP
Eocytes	Td.mari.	KNMITGASQADAAVLVVSARK	GEFEAGMSAEG
	P.occu.	knmitgASQADAAVLVVSARK	GEFEAGMSAEG
	D.muco.	knmitgASQADAAVLVVSARK	GEFEAGMSAEG
	A.infe.	knmitgASQADAAIIVASAKK	GEFEAGMSEEG
	Su.acid.	KNMITGASQADAAVLVVSARK	GEFEAGMSAEG
Eukaryotes	Giardia	KNMITGTSQADVAILLVVAAGQ	GEFEAGISKDG
	Tetrah.	KNMITGTSQADVAILLMIASPD	GEFEAGISKDG
	Yeast	KNMITGTSQADCAILLIAGGV	GEFEAGISKDG
	Tomato	KNMITGTSQADCAVLIIDSTT	GGFEAGISKDG
	Droso.	KNMITGTSQADCAVQIDAAGT	GEFEAGISKND
	Rat	KNMITGTSQADCAVLIIVAGV	GEFEAGISKNG
	Human	KNMITGTSQADCAVLIIVAGV	GEFEAGISKNG
			QTREH
			QTREH
			QTREH

FIG. 2. Comparison of the *Methanopyrus kandleri* EF-1 α sequence with the sequences of methanogens, halobacteria, eubacteria, eocytes, and eukaryotes. The 4- and 11-amino-acid segments are underlined. The lowercase letters represent sequences used as the PCR primers. The methanogens and their relatives which we used were *Methanopyrus kandleri* (Mp.kand.) (this study), *Thermococcus celer* (T.celer) (3), *Pyrococcus woesei* (P.woes.) (11), *Archaeoglobus fulgidus* (A.fulg.) (this study), *Methanococcus vannielii* (Mc.vann.) (26), and *Thermoplasma acidophilum* (T.acido.) (39). The halobacterium used was *Halobacterium marismortui* (H.maris.) (6). The eubacterial used were the thermophilic eubacterium *Thermotoga maritima* (Th.mar.) (5), the halophilic cyanobacterium *Dactylococcopsis salina* (D.sal.) (this study), and the enterobacterium *E. coli* (9). The eocytes used were *Thermoplasma maritimum* (Td.mari.) (this study), *Pyrodicticum occultum* (P.occu.) (32), *Desulfurococcus mucosus* (D.muco.) (32), *Acidianus inferus* (A.infe.) (32), and *Sulfolobus acidocaldarius* (Su.acid.) (4). The eukaryotes used were *Giardia lamblia* (Giardia) (13), *Tetrahymena pyriformis* (Tetrah.) (23), *Saccharomyces cerevisiae* (Yeast) (10), *Lycopersicon esculentum* (Tomato) (29), *Drosophila melanogaster* (Droso.) (15), *Rattus norvegicus* (Rat) (2), and *Homo sapiens* (Human) (7).

M. kandleri	AAG AAT ATG ATC ACG GGT GCG AGC CAG GCC GAC GCC GCC ATC CTG GTA GTG GCC GCG GAC GAC
A. fulgidus	--A --C --- --A A-- --C --A TCT --- --G --T CGA --A G-T --C --C A-- --AT --TC --TT --G
D. salina	--A --C --- --A C-- --G --A GCG --T ATG --T --GA --A --T --C --T TGC --CC --T C-- --T
T. maritimus	--A --T --- --A A-- --A --T --- --A --T --T --A --A --A A-A --CA --T T-T --T A-G A-A

M. kandleri	GGT GTG ATG CCG CAG ACC AGA GAA CAC
A. fulgidus	AAG GTG CA- --G --G --- C-- --- ---
D. salina	--T --CG --- --T --A --T C-T --G --T
T. maritimus	GGA GAA TTT GAA GCA GGA ATG AGC GAA GAA GGT ---A --- --- ---

FIG. 3. Nucleotide sequences of the KNMITG₉₅-QTREH₁₁₈ fragments from *Methanopyrus kandleri*, *Archaeoglobus fulgidus*, *Dactylococcopsis salina*, and *Thermoplasma maritimus*. Triplets corresponding to amino acids are separated by spaces. Gaps are represented by solid lines. Dashes represent nucleotides identical to the ones in the *M. kandleri* reference sequence (top row).

Support for the hypothesis that 11-amino-acid segments occur in eukaryotes and eocytes and 4-amino-acid segments or their variants occur in all other prokaryotes was provided by the new sequences described in this paper. These sequences include the sequences of the extremely halophilic eubacterium *Dactylococcopsis salina*, the sulfate reducer *Archaeoglobus fulgidus*, the hyperthermophilic methanogen *Methanopyrus kandleri*, and the eocyte *Thermoplasma maritimus*. Furthermore, the fact that this pattern continues to be found in new sequences from taxa that occupy other critical phylogenetic positions, such as *Giardia lamblia* (13), provides additional evidence that the EF-1 α segment is useful as a phylogenetic marker. We concluded that the genus *Methanopyrus* is either a member of the true archaeobacteria (corresponding to branch A) or a member of the nonarchaeobacterial, paraphyletic clade of organisms (including organisms like *Thermococcus celer* and *Pyrococcus woesei*) which are located before the node which gives rise to the eocytes and eukaryotes (branch B).

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