NOTES

Phylogeny of *Methanopyrus kandleri* Based on Methyl Coenzyme M Reductase Operons

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The *mcrBDCGA* operon that encodes methyl coenzyme M reductase (MR) in the hyperthermophile *Methanopyrus kandleri* was cloned and sequenced. The results of a phylogenetic analysis of the nine MR sequences now available support the position that *M. kandleri* is a separate methanogen lineage. As in other methanogens, the *M. kandleri* *mcr* operon is located immediately upstream of the *mtrE* gene, the promoter-proximal gene in an operon that encodes the *N*⁵-methyltetrahydromethanopterin:coenzyme M methyltransferase that catalyzes the step preceding the MR-catalyzed reaction in methanogenesis. In contrast to other methanogens and hyperthermophilic members of the *Archaea*, CG dinucleotides and CG-containing codons occur frequently in *M. kandleri* DNA. The MR subunit-encoding genes are preceded by sequences consistent with ribosome binding sites, indicating that mRNA-rRNA base pairing can still direct translation initiation in cells growing at temperatures above 100°C.

Methanogens provide a model system for microbial phylogenetics. On the one hand, they are extremely diverse, with genomes whose G+C contents range from 23 to 61 mol%, with bacillary, coccal, and spiral morphologies and a variety of cell envelope structures, and with habitats as different as the rumen and submarine volcanic vents (1, 2, 8, 9, 14, 16). On the other hand, they are unified (and named) by their ability and need to synthesize methane. Facultative methanogens are unknown, and the final reaction in methanogenesis, the reduction of a methyl group bound to coenzyme M, releasing methane, is common to all methanogens (8). Methyl coenzyme M reductase (MR), the enzyme that catalyzes this reaction, has been purified from many methanogens and consistently has an $\alpha_2\beta_2\gamma_2$ polypeptide subunit composition (11). The genes that encode these subunits, *mcrA*, *mcrB*, and *mcrG*, respectively, have been cloned and sequenced from several different methanogens and have always been found to be arranged *mcrBD CGA*, within a single transcriptional unit, designated an MR operon (18, 25, 33). The functions of the *mcrC* and *mcrD* gene products remain unknown (31). Some methanogens contain a second MR isoenzyme (MRII) (27), and two MRII-encoding operons (*mrt* [MR two]) have also been cloned and sequenced (21, 24). These *mrtBDGA* operons differ from the *mcr* operons in lacking a "C" gene but otherwise are very similar and clearly evolved from the same common ancestor as the *mcr* operons. In 1988 Weil et al. (33) proposed that because the basic structure and function of MR are conserved in all methanogens, changes in the sequences of the MR subunits should provide valid estimates of the evolutionary divergence of methanogens, and recently Springer et al. (30) provided experimental support for this concept. A phylogenetic tree for the *Methanosarcinaceae* was constructed on the basis of the sequences of the same 490-bp region amplified from 25 different *mcrA* genes, and this tree was fully consistent with the phylogeny of these

methanogens established previously on the basis of their 16S rRNA sequences.

The position of *Methanopyrus kandleri* is particularly interesting for methanogen phylogenetics (26). *Methanopyrus kandleri* has the most divergent 16S rRNA sequence, genomic DNA with an unusually high G+C content (60 mol%), and the highest known optimum growth temperature (98°C) and grows at temperatures up to 110°C (3, 13, 19). These features suggest that the lineage of this organism diverged very early from the other methanogen lineages, but *Methanopyrus kandleri* is nevertheless a methanogen. It should therefore have a phylogenetically informative MR operon, and in this paper we describe the cloning, sequence, and results of a phylogenetic analysis of the *Methanopyrus kandleri* *mcr* operon. The data obtained in this study also provide evidence that unusual codon usage occurs and that mRNA-tRNA base pairing is used in *Methanopyrus kandleri* cells to direct translation initiation at temperatures up to 110°C.

On the basis of the highly conserved sequences (33) used as hybridization probes for *mcrB* and *mtrB* transcripts (24), we designed and used GC-rich primers to PCR amplify a 133-bp region from within the *Methanopyrus kandleri* *mcrB* and/or *mtrB* genes. Molecules from the pool of amplified DNA were cloned into pUC19, and several molecules were sequenced (28), which confirmed that they were identical and were from a *mcrB* gene. Southern blot hybridizations (28) with *Methanopyrus kandleri* genomic DNA revealed that the PCR-amplified region was located within an ~15-kbp *EcoRI* fragment, and a λ -gt11 recombinant phage that contained this fragment intact was identified, by plaque hybridization (28), in a *Methanopyrus kandleri* genomic library. This *EcoRI* fragment was subcloned, and from the resulting subclone and additional subclones we sequenced the MR-encoding genes and flanking regions. The sequence obtained (GenBank accession number U57340) contained open reading frames consistent with the presence of a standard *mcrBDCGA* operon located upstream from an *mtrE* gene (Fig. 1A). In other methanogens, the *mtrE*

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FIG. 1. Structure and comparison of the *Methanopyrus kandleri* *mcr* operon and gene products. (A) A 5,914-bp sequence (GenBank accession number U57340) was determined, and this sequence contained the entire *Methanopyrus kandleri* *mcrBDCGA* operon and 231 bp of the *mtrE* gene, organized as shown at the top. The locations of the two sequences used as PCR primers (*mcrB* forward primer BF and *mcrB* reverse primer BR) to amplify the DNA fragment used as the hybridization probe are indicated. Also shown are the calculated molecular masses (in kilodaltons) of the sequenced *mcr* (MRI) and *mtr* (MRII) gene products (gp) and the lengths (in base pairs) of the intergenic regions (ir) (5, 18, 21, 24, 33). The absence of an *mtrC* gene is indicated by asterisks. If the first open reading frame downstream from the α -subunit-encoding gene (*mcrA* or *mtrA*) has been shown to be *mtrE*, this is indicated (Y, yes; N, no) under gpE. nd indicates that an *mtrE* gene has not been detected and no open reading frames have been identified within the ir5' designated regions. (B) Intergenic sequences immediately upstream of *mcr* and the three other *Methanopyrus kandleri* methane genes sequenced (23, 29, 34) aligned, at the arrow, by their translation-initiating codons. Start codons, putative ribosome binding sites, and archaeal TATA-box promoter elements are identified by the boldface type, and the TAA termination codon of the *mcrD* gene is underlined. *M. kandleri*, *Methanopyrus kandleri*; *M. fervidus*, *Methanococcus fervidus*; *M. t. ΔH*, *Methanobacterium thermoautotrophicum* ΔH; *M. t. Marb.*, *Methanobacterium thermoautotrophicum* Marburg; *M. vannielii*, *Methanococcus vannielii*; *M. voltae*, *Methanococcus voltae*; *M. barkeri*, *Methanosarcina barkeri*.

gene is the promoter-proximal gene in an *mtr* operon that encodes N^5 -methyltetrahydromethanopterin:coenzyme M methyltransferase (10). This enzyme catalyzes the penultimate step in methanogenesis, and the same adjacent positioning of the *mcr* and *mtr* operons has also been found in the genomes of *Methanococcus vannielii*, *Methanobacterium thermoautotrophicum*, and *Methanothermobacter fervidus* (22, 24, 33). As growth phase-dependent read-through transcription of the *mtr* operon from the *mcr* promoter has been documented in *Methanobacterium thermoautotrophicum*, this neighborhood arrangement of methane gene operons (24) appears to have functional significance. Some Southern blots adumbrated the existence of a second MR-encoding region in the *Methanopyrus kandleri* genome, but despite a substantial effort, we were unable to clone

directly or to PCR amplify an *mtr* operon from *Methanopyrus kandleri*.

The sizes of the MR α , β , and γ subunits are very similar in all methanogens (11), including *Methanopyrus kandleri* (Fig. 1A), whereas the intergenic regions within and downstream of the different MR operons vary in length and have divergent sequences (Fig. 1A) (18, 33). Methanogens synthesize large amounts of MR (8, 11, 27), and sequences consistent with the presence of very strong ribosome binding sites have been identified upstream of the previously sequenced *mcrA*, *mcrB*, and *mcrG* genes (2, 25). In Fig. 1B, all of the sequences so far determined upstream of *Methanopyrus kandleri* methane genes are aligned, and the data demonstrate that in every case there is at least one 5'-GGGG and/or 5'-GGAGG sequence, appropriately positioned for a ribosome binding site, that could base pair with the sequence HO-UCCUCCACU-5' at the 3' terminus of the *Methanopyrus kandleri* 16S rRNA (3). The consistency and locations of these sequences argue that they do function as ribosome binding sites (2, 25) which, in turn, argues that rRNA-mRNA base pairing must be possible inside *Methanopyrus kandleri* cells growing at 110°C. These sequences are, however, somewhat shorter and offer fewer opportunities for consecutive base pairing with the 16S rRNA than do their counterparts in other methanogens, suggesting that rRNA-mRNA interactions in *Methanopyrus kandleri* must also be facilitated and stabilized by mechanisms in addition to spontaneous Watson-Crick base pairing.

Codon usage in the MR subunit-encoding genes is also consistent with the high levels of expression of these genes (18, 33). Each of the synonymous codon pairs AAC/U, GAC/U, AUC/U, UUC/U, and UAC/U is translated by one tRNA, and all of these tRNAs have a G in the first anticodon position. The previously sequenced *mcrA/mtrA*, *mcrB/mtrB*, and *mcrG/mtrG* genes preferentially use the codons with the C residue in the wobble position, which results in G·C rather than G·U codon·anticodon base pairs. This is expected to maximize the rate of translation of the transcripts (32). The *mcrC* and *mcrD* gene products, in contrast, are not synthesized in large amounts (31), and the *mcrC* and *mcrD* genes do not exhibit this preferential use of the C-containing codons from these synonymous pairs (18, 33). The overall G+C content of *Methanopyrus kandleri* genomic DNA is 60 mol%; therefore, codons with a C in the wobble position would be expected to occur frequently, and this is the case. The *Methanopyrus kandleri* *mcrC* and *mcrD* genes contain 44 members of the synonymous codon pairs, and 40 of these have a C in the wobble position. There is therefore a 10:1 ratio in favor of the C-containing codons over the U-containing codons in these genes. In the MR subunit-encoding genes there are 228 C-containing codons and only 7 U-containing codons from these pairs; i.e., the ratio in these genes is 32:1 in favor of the C-containing codons. Thus, the increased use of the C-containing codons in the *mcrA*, *mcrB*, and *mcrG* genes compared with the *mcrC* and *mcrD* genes in *Methanopyrus kandleri* is 3.2-fold, which is consistent with the range of 2.2- to 5.7-fold increased usage of C-containing codons found in other methanogens (33).

The dinucleotide 5'CG occurs very frequently in *Methanopyrus kandleri* DNA. This codon is the most abundant dinucleotide in the *mcr* operon, accounting for ~13% of all dinucleotides and contributing to the presence of 238 CG-containing codons. In contrast, the CG dinucleotide and CG-containing codons occur only rarely in other methanogens and nonmethanogenic hyperthermophiles (6). There are only 26, 31, 46, 26, 20, and 12 CG-containing codons in the *mcr* operons of *Methanobacterium thermoautotrophicum* ΔH and Marburg, *Methanosarcina barkeri*, *Methanothermobacter fervidus*, *Methanococcus*

FIG. 2. Phylogenetic trees constructed by parilinear distance analysis of the sequences of the α , β , and γ subunits of MRI and MR II in *Methanosarcina barkeri* (*M. barkeri*), *Methanococcus voltaei* (*M. voltaei*), *Methanococcus vannielii* (*M. vannielii*), *Methanobacterium thermoautotrophicum* (*M. t. ΔH*), *Methanobacterium thermoautotrophicum* Marburg (*M. t. Marburg*), *Methanothermobacterium fervidus* (*M. fervidus*), and *Methanopyrus kandleri* (*M. kandleri*). The branch lengths, in number of nucleotide substitutions per position (20), are indicated. (A) Tree rooted by assuming that the rates of nucleotide substitution were the same in the *mcr* and *mrt* genes in all of the methanogen lineages. (B) Tree rooted on the basis of the conclusion from 16S rRNA studies that *Methanopyrus kandleri* is the most divergent methanogen (3, 13, 19). The most probable unrooted trees generated from the sequences by maximum-parsimony (94% probability), Jukes-Cantor (96.5% probability), and Kimura (96% probability) distance analyses (15, 17) had the same branching topologies.

vannielii, and *Methanococcus voltaei*, respectively. CG-containing sequences are frequently targets for DNA cytosine methylations, and methyl-cytosines have a high propensity for spontaneous deamination, especially at high temperatures (7), so that nonessential CG-containing sequences in cells containing DNA cytosine-methylating enzymes should eventually become TG-containing sequences (4). It appears, therefore, that *Methanopyrus kandleri* cells must lack such DNA cytosine-methylating enzymes or contain mechanisms that prevent or correct methyl-cytosine deaminations.

To construct phylogenetic trees based on the MR subunit data, the amino acid sequences of all of the available α , β , and γ subunits were aligned, and after regions of uncertain alignment were discarded, the remaining sequences were back-translated into nucleotide sequences. Third codon positions were then eliminated, and only uniquely defined first- and second-position nucleotides were used to calculate the parilinear distances (20) that were used to generate the trees shown in Fig. 2. Unrooted trees with the same branching topology were also obtained when the alignments were subjected to maximum-parsimony, Jukes-Cantor, and Kimura distance analyses (15, 17). Because MR occurs only in methanogens, it was impossible to root these trees with an outgroup, and therefore we constructed two trees, one rooted by assuming that nucleotide substitution rates in the *mcr-mrt* genes were the

same in all lineages (Fig. 2A) and one rooted in the *Methanopyrus* lineage, as indicated by the 16S rRNA studies (Fig. 2B) (3). Both trees contain *Methanopyrus kandleri* in a separate and very divergent lineage, but if the tree is rooted, as in Fig. 2A in the *Methanosarcina barkeri* lineage, then the *Methanopyrales* and *Methanobacteriales* are related as neighbors, which is consistent with the similar cell wall structures of these groups (16).

Surprisingly, the *mrt* genes (MR II) in the members of the *Methanobacteriales* are more similar to the methanococcal *mcr* genes (MRI) than they are to their own *mcr* genes (21, 24). The methanococcal *mcr* operons could therefore be *mrt* operons that still contain an *mrtC* gene; however, this is difficult to reconcile with the neighborhood organization. Immediately downstream from the *Methanococcus vannielii* *mcr* operon is an *mtr* operon (22), as is also the case in the genomes of *Methanopyrus kandleri*, *Methanobacterium thermoautotrophicum* and *Methanothermobacterium fervidus* (Fig. 1A). Alternatively, the *mrt* operons could have evolved after lateral gene transfer of an *mcr* operon from a member of the *Methanococcales* into the ancestor of the extant *Methanobacteriales* (24), or the operon duplication that gave rise to the *mcr* and *mrt* operons could have occurred before the divergence of the *Methanococcales* and *Methanobacteriales* (21). If the latter explanation is correct, then the evidence against the presence a second MR-encoding region in *Methanococcus vannielii* (12) suggests that members of the *Methanococcales* subsequently lost one MR-encoding region. As a phylogenetic study of 25 amplified *mcrA* sequences (30) also failed to find any evidence for a second MR-encoding region in the members of the *Methanosarcinaceae*, this lineage may also have lost the second MR-encoding region. The *Methanosarcinaceae* could, alternatively, have diverged from all of the other methanogen lineages before the operon duplication occurred, which would be consistent with the phylogenetic tree shown in Fig. 2A. If there is also an *mrt* operon in *Methanopyrus kandleri*, this would provide additional parsimonious support for the phylogeny shown in Fig. 2A, but it would still not eliminate the possibility that the original methanogen contained both an *mcr* operon and an *mrt* operon.

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