Partial Gene Sequences for the A Subunit of Methyl-Coenzyme M Reductase (*mcrI*) as a Phylogenetic Tool for the Family *Methanosarcinaceae*

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Representatives of the family *Methanosarcinaceae* were analyzed phylogenetically by comparing partial sequences of their methyl-coenzyme M reductase (*mcrI*) genes. A 490-bp fragment from the A subunit of the gene was selected, amplified by the PCR, cloned, and sequenced for each of 25 strains belonging to the *Methanosarcinaceae*. The sequences obtained were aligned with the corresponding portions of five previously published sequences, and all of the sequences were compared to determine phylogenetic distances by Fitch distance matrix methods. We prepared analogous trees based on 16S rRNA sequences; these trees corresponded closely to the *mcrI* trees, although the *mcrI* sequences of pairs of organisms had 3.01 ± 0.541 times more changes than the respective pairs of 16S rRNA sequences, suggesting that the *mcrI* fragment evolved about three times more rapidly than the 16S rRNA gene. The qualitative similarity of the *mcrI* and 16S rRNA trees suggests that transfer of genetic information between dissimilar organisms has not significantly affected these sequences, although we found inconsistencies between some *mcrI* distances that we measured and previously published DNA reassociation data. It is unlikely that multiple *mcrI* isogenes were present in the organisms that we examined, because we found no major discrepancies in multiple determinations of *mcrI* sequences from the same organism. Our primers for the PCR also match analogous sites in the previously published *mcrII* sequences, but all of the sequences that we obtained from members of the *Methanosarcinaceae* were more closely related to *mcrI* sequences than to *mcrII* sequences, suggesting that members of the *Methanosarcinaceae* do not have distinct *mcrII* genes.

The taxonomy of methanogens is strongly guided by 16S rRNA sequences, in part because methanogens have very limited catabolic pathways and few easily determined phenotypic characteristics. Also, most methanogenic species have been described since the time that 16S rRNA sequences began to be used as a way to determine phylogenetic relationships. This unusual degree of reliance on 16S rRNA data has yielded a taxonomy that is strongly phylogenetic (6), but there are at least two major concerns or limitations associated with the exclusive use of 16S rRNA trees for taxonomy.

The first concern is that the number of genetic differences between closely related species may be small and may not measure small phylogenetic distances with sufficient precision to define species (13). Organisms that exhibit levels of 16S rRNA sequence similarity of up to 98% (at least 30 differences per 1,500 bp) have been considered too dissimilar to be placed in the same species (6, 8, 45), but Stackebrandt and Goebel (39) have proposed that only when the level of 16S rRNA sequence similarity is less than 97% should strains be considered members of separate species without other phylogenetic data, such as DNA reassociation data. However, two distinct but closely related species may exhibit a level of similarity greater than 98% (6, 8, 13, 39, 45), and no one has proposed a level of 16S rRNA sequence similarity above which microbes must be placed in the same species. An analogous benchmark

of 70% DNA sequence similarity (and a difference between the homologous denaturation temperature and heterologous denaturation temperature of 5°C) has been proposed as the maximum level of similarity (minimum phylogenetic distance) that separates organisms into distinct species, with the stipulation that separate species should have phenotypic differences that are suitable as diagnostic taxonomic tools (41).

Another concern about using comparisons of sequences of a single gene for taxonomy is the possibility of recent transfer of genes between dissimilar species, which would give rise to an organism that appeared to be closely related to the donor of the gene and unrelated to its true close relatives (9, 37). Such a discrepancy might be revealed by a recombination analysis (37) or by a comparison of phenotypic data. Alternatively, independent phylogenetic trees based on DNA reassociation data or genetic sequences unrelated to the ribosomal apparatus can be compared with 16S rRNA phylogenetic trees (23). Such comparisons would likely reveal inaccuracies in the 16S rRNA trees that might be caused by transfer of 16S rRNA genes between dissimilar species.

Phylogenetic trees based on 5S rRNA data (16, 17) and 23S rRNA data (24, 36) compare well with 16S rRNA phylogenetic trees. However, 5S rRNA, 23S rRNA, and 16S rRNA genes are all functionally related, and an independent gene tree would be useful for detecting anomalies. More recently, the gene sequences for the beta subunit of ATPase and for elongation factor Tu showed good agreement with each other (23). The organisms included in these studies were phylogenetically diverse, so the studies would not have been expected to detect genetic exchange among closely related strains. A study of the evolution of *nifH* genes included closely related organisms, but discrepancies between the trees may have been due to the gene being located on a plasmid (10).

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TABLE 1. Strains that were analyzed phylogenetically

Taxon	Strain	OCM accession no. ^a	16S rRNA sequence accession no. ^b	<i>mcrI</i> sequence accession no. ^c	Culture medium used
<i>Methanobacterium thermoautotrophicum</i>	ΔH^T		X68720 ^d	U10036 ^e	
<i>Methanobacterium thermoautotrophicum</i>	Marburg	82	X15364 ^f	M18969 ^g	
<i>Methanococcoides burtonii</i>	DSM 6242 ^T	468 ^T	X65537 ^h	U22234	MSH-TMA
<i>Methanococcoides methylutens</i>	TMA-10 ^T	158	M59127 ⁱ	U22235	MSH-TMA
<i>Methanococcus vannielii</i>		148 ^T	M36507 ^j	M16893 ^k	
<i>Methanococcus voltaei</i>	PS ^T	70 ^T	M59290 ^l	X07793 ^j	MG-H2
<i>Methanohalobium</i> sp.	SD-1 ^m	134		U22256	MH-TMA
<i>Methanohalobium evestigatum</i>	Z-7303 ^T	161 ^T	U20149	U22236	MH-TMA
<i>Methanohalophilus</i> sp.	Cas-1 ^m	135		U22254	MH-TMA
<i>Methanohalophilus</i> sp.	HCM6 ⁿ	152		U22255	MH-TMA
<i>Methanohalophilus</i> sp.	Ret-1 ^m	57		U22240	MH-TMA
<i>Methanohalophilus</i> sp.	SF-1 ^o	13	M59132 ^j	U22241	MH-TMA
<i>Methanohalophilus halophilus</i>	Z-7982 ^T	160 ^T		U22259	MH-TMA
<i>Methanohalophilus mahii</i>	SLP ^T	68 ^T	M59133 ^j	U22237	MH-TMA
<i>Methanohalophilus portucalensis</i>	FDF-1 ^T	59 ^T		U22239	MH-TMA
<i>Methanohalophilus portucalensis</i>	FDF-2 ^m	66		U22238	MH-TMA
<i>Methanohalophilus zhilinaeae</i>	WeN5 ^T	62 ^T	— ^p	U22252	MSHA-TMA
<i>Methanobolus bombayensis</i>	B-1 ^T	438 ^T	U20148	U22257	MSH-TMA
<i>Methanobolus oregonensis</i>	WAL1 ^T	99 ^T	U20152	U22242	MSHA-TMA
<i>Methanobolus taylorii</i>	GS-16 ^T	58 ^T	U20154	U22243	MSHA-TMA
<i>Methanobolus tindarius</i>	Tindari 3 ^T	150 ^T	M59135 ^j	U22244	MSH-TMA
<i>Methanobolus vulcani</i>	PL-12/M ^T	157 ^T	U20155	U22245	MSH-TMA
<i>Methanomicrobiaceae</i> species	EBac ^q	550		U22253	MG-H2
<i>Methanosarcina</i> sp.	WH-1 ^r	350	M59136 ^j	U22249	MSCO2-TMA
<i>Methanosarcina acetivorans</i>	C2A ^T	95 ^T	M59137 ^j	U22247	MSH-TMA
<i>Methanosarcina barkeri</i>	Fusaro	83		Y00158 ^s	
<i>Methanosarcina barkeri</i>	227	35	M59144 ⁱ		
<i>Methanosarcina mazei</i>	S-6 ^T	26 ^T	U20151	U22246	MS-TMA
<i>Methanosarcina mazei</i>	C 16 ^t	98	M59138 ⁱ	U22258	MS-TMA
<i>Methanosarcina siciliae</i>	T4/M ^T	156 ^T	U20153	U22248	MSH-TMA
<i>Methanosarcina thermophila</i>	TM-1 ^T	12 ^T	M59140 ^j	U22250	MS-TMA
<i>Methanosarcina vacuolata</i>	Z-761 ^T	85 ^T	U20150	U22251	MS-TMA
<i>Methanothermobacter fervidus</i> ^t	V24S ^T		M32222 ^u	J03375 ^v	

^a OCM, Oregon Collection of Methanogens, the source of all strains except strains ΔH^T and V24S^T.^b Accession number for the Ribosomal Database, University of Illinois (20), except where indicated otherwise. Sequences were determined in this study unless indicated otherwise.^c GenBank accession number for the complete or partial *mcrI* sequence. Sequences were determined in this study unless indicated otherwise.^d Sequence determined by Nöling et al. (29).^e Sequence determined by Pihl et al. (31).^f Sequence determined by Ostergaard et al. (30).^g Sequence determined by Bokranz et al. (1).^h GenBank accession number.ⁱ Sequence determined by Rouvière et al. (33).^j Sequence determined by Jarsch and Böck (18).^k Sequence determined by Cram et al. (7).^l Sequence determined by Klein et al. (19). A partial sequence was confirmed in our laboratory.^m Isolation and characterization have been described previously (5).ⁿ Isolated by Jane E. Boone from the production water of a gas well in Roswell, N.M.^o Isolation and characterization have been described previously (28).^p Sequence determined by Liu et al. (22).^q Isolated by E. Springer and K.-H. Blotvogel.^r Isolation has been described previously (34).^s Sequence determined by Bokranz and Klein (2).^t *Methanosarcina mazei* C 16 may also be classified as *Methanosarcina frisia* C 16^T, but *Methanosarcina frisia* is considered a junior synonym of *Methanosarcina mazei* (25).^u Sequence determined by Haas et al. (14).^v Sequence determined by Weil et al. (42).

We selected the gene for the A subunit (*mcrA*) of the methyl-coenzyme M reductase gene cluster for phylogenetic analysis and comparison with the results of a phylogenetic analysis of 16S rRNA genes. Although this reductase is not present outside the methanogens, it is present in all methanogens. The molecular weights of the subunits are consistent with the 16S rRNA phylogeny at the genus level (34), and this large, complex enzyme might have evolved slowly to yield present-day sequences that exhibit enough similarity to construct accurate evolutionary trees. The *mcrI* sequence of *Methanococcus vol-*

taei is much more similar to the sequence of *Methanococcus vannielii* than it is to the sequences of three other, more phylogenetically distinct species (21). Several complete *mcrI* sequences are available (1, 2, 7, 19, 31, 42) to guide the selection of primers for amplification of sequences by the PCR. Methyl-coenzyme M reductase has three distinct subunits (α_2 , β_2 , γ_2). The genes for these subunits are organized in a single transcription unit, *mcrBDCGA*, that includes two interspersed additional open reading frames (*mcrD* and *mcrC*) whose functions are not known yet. An isoenzyme whose gene sequence

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FIG. 1. Fitch distance matrix tree based on comparisons of a 490-bp segment of the *mcrA* gene. The tree was generated by using *Methanococcus vannielii* as the outgroup, and the significance of each branch is indicated by a bootstrap value (calculated by using 100 subsets). Abbreviation: *Mhp.*, *Methanohalophilus*.

(*mcrII*) is phylogenetically distinct from the sequence of the more common *mcrI* (21, 40) is present in some methanogens (32); this isoenzyme may occur only in members of the order *Methanobacteriales* (40).

We selected a relatively conserved region of the *mcrA* gene from sequences aligned by using their amino acids (43) and determined the corresponding gene sequences for 25 members of the family *Methanosarcinaceae*. These sequences were analyzed to create phylogenetic trees that were compared with 16S rRNA trees and DNA hybridization data for the same organisms.

(Portions of the results have been described previously [3, 38].)

MATERIALS AND METHODS

Source and growth of cultures. Cultures were obtained from the Oregon Collection of Methanogens and were grown at 37°C in the media indicated in Table 1. *Methanococcoides burtonii* was grown at room temperature, and *Methanosarcina thermophila* was grown at 50°C. Strain EBac (=OCM 550 = DSM 8220) was isolated by E. Springer and K.-H. Blotvogel. The culture media used were based on MS medium (4). MS-TMA was MS medium supplemented with 40 mM trimethylamine; MG-H2 was MS medium supplemented with 2.5 g of NaCl per liter and 5 mM acetate and pressurized to 100 kPa with H₂; MSCO₂-TMA was MS-TMA with a gas phase consisting of pure CO₂; MH-TMA was MS-TMA supplemented with an additional 2 g of NaOH per liter, 1.5 mol of NaCl per liter, 5 g of MgCl₂ · 6H₂O per liter, and 20 mmol of KCl per liter (the final pH was 7.3); MSH-TMA was a 2:1 mixture of MS-TMA and MH-TMA; and MSHA-TMA was MSH-TMA equilibrated with an N₂ gas phase (final pH, 8.0).

Purification of DNA. Cells were lysed by resuspending pellets in buffer and adding 20% (wt/vol) sodium dodecyl sulfate (final concentration, approximately 0.1 g per liter), and DNA was extracted (26) with chloroform-phenol (1:1).

PCR amplification and cloning. Degenerate primers (17 bases) were constructed for two conserved amino acid regions of the *mcrA* gene. These regions were YDQIWL (amino acids 337 to 343) and NYAMNV (amino acids 503 to 509) (43). The primers were obtained from Oligos Etc., Inc., Wilsonville, Ore. The nucleotide sequences were TAYGAYCARATHGGYT (sense) and ACR TTCATNGCARTATT (antisense). The intervening sequences were amplified with VENT-Polymerase (New England Biolabs, Beverly, Mass.) in an ERICO MP EasyCycler apparatus (Ericomp, Inc., San Diego, Calif.) by using a low-temperature program (annealing at 37°C for 30 s, extension at 55°C for 60 s, and denaturation at 94°C for 60 s, repeated for 35 cycles). The reaction volume was 100 µl with 300 ng of genomic DNA. No amplification occurred if the extension temperature was above 60°C or if the extension step was omitted. The low-temperature program usually yielded a single major band that was visible on agarose gels.

PCR products were extracted once with a phenol-chloroform mixture (1:1), precipitated with ethanol, and treated with kinase (T4-PolyNucleotide kinase; US Biochemicals, Cleveland, Ohio). The DNA was precipitated with ethanol and dissolved in 15 µl of H₂O; 5 µl of the resulting preparation was added to 10 µl of a ligation mixture containing gel-purified, *EcoRV*-cut pBluescriptIIISK- (Stratagene, La Jolla, Calif.) and T4-Ligase (New England Biolabs) as recommended by the manufacturer (polyethylene glycol was not used). This solution was incubated at 15°C overnight, 1 µl was added to 40 µl of competent cells (DH5alpha or XL-1 Blue), and the preparation was electroporated. The cells were then spread on Luria-Bertani plates containing ampicillin (100 mg/liter) and X-Gal (5-bromo-4-chloro-3-indolyl-β-D-galactopyranoside)-IPTG (isopropyl-β-D-thiogalactopyranoside) for blue-white selection. Clones containing DNA fragments of the expected size were selected by using ultrarapid plasmid minipreps and the boiling method (35). The fragments were released by brief digestion with *EcoRI* and *HindIII*.

Plasmid preparation and sequencing. DNA was obtained from 30-ml overnight cultures by alkaline lysis (35) followed by a final polyethylene glycol precipitation step. The DNA yield from each culture was usually sufficient for sequencing both strands of the whole clone. Sequencing reactions were performed with Sequenase 2.0 (US Biochemicals) with the enzyme concentration reduced to 60% of the recommended concentration and with the protocol modified for use with 10-µl microtiter dishes. The dishes were heated at 92°C for 6.5

FIG. 2. Comparison of Fitch trees based on a 490-bp segment of the *mcrA* gene (right side) and on 16S rRNA sequences (left side). Abbreviation: *M.*, *Methanosarcina*.

min, and the DNAs in the samples were separated by electrophoresis in 6% acrylamide gels. Fixation in methanol-acetate was omitted. For each methanogenic strain, two clones from different PCR preparations were sequenced in most cases; the exceptions were strains OCM 66, OCM 68^T, (T = type strain), OCM 70^T, OCM 150^T, OCM 156, and OCM 158. At least 90% (average, 98%) of all sequence information for each clone was confirmed by studying both strands. Three clones of strain OCM 550 were sequenced, but mostly on just one strand (see GenBank entry for details). We also confirmed the previously published sequence (19) of the analogous fragment in *Methanococcus voltaei* OCM 70^T (=DSM 1537^T).

Some clones were shortened with *ExoIII* and *S1* nuclease to obtain full double-stranded sequence information. Most clones were sequenced without subcloning by using degenerate primers that annealed to a conserved amino acid sequence near the center of the cloned 490-bp sequence. The conserved amino acid sequence was FGGSQR, and we used one of two degenerate primers, TTYG GWGNTCMCARAG (sense) and CTYTGNAGNCWCRAA (antisense), in combination with promoter primers T3 and T7 from pBluescript- (Stratagene) and the appropriate primer for amplification. For larger overlaps, the PCR primers were replaced with two other degenerate primers that were based on the DIATES region (GAYATHGCWACHGARTC [sense]) and the DLQDQC region (GCNCCRCAYTGRTCYTG [antisense]) (obtained from the Center for Gene Research and Biotechnology, Oregon State University, Corvallis). Sequences were edited with the MacVector program (International Biotechnologies, Inc., New Haven, Conn.).

Determination of 16S rRNA sequences. We determined 16S rRNA sequences by the reverse transcriptase method (44).

Phylogenetic analysis. Sequences of 16S rRNA genes were aligned with the program Clustal V (15) called from the GDE editor (20) and checked manually; the *mcrI* sequences were aligned by hand after the inferred amino acid sequences were aligned. Bootstrap subsets (100 sets) and phylogenetic trees were constructed with the PHYLIP package (11, 12).

RESULTS AND DISCUSSION

Sequence alignment. Most of the partial *mcrI* sequences of members of the *Methanosarcinaceae* were 490 bp long; the only

exception was the *Methanosarcina* sp. strain WH-1 sequence, which was 487 bp long. The latter sequence was confirmed with a third clone and was aligned by using a 3-base deletion. The alignment is available in electronic form from our FTP site (<ftp.esf.edu>) as pub/OCM/metmcr.

***mcrI* isogenes.** The sequences of the primers used for amplification of *mcrI* were identical (antisense primer) or very similar (sense primer) to the sequences of the only previously described isogenes of *mcrI*, *mcrII* of *Methanothermobacter* (21) and *Methanobacterium thermoautotrophicum* ΔH (31), so the degenerate primers should have amplified an *mcrII* gene if it had been present. However, none of the *mcrI* genes that we sequenced appeared to be *mcrII*, which is phylogenetically distinct from *mcrI* (21, 40). Also, no major differences between clones of the same organism were found. Although it is possible that an isogene of *mcrI* was present, we did not find one. It is possible that *mcrII* or some other isogene was present but that it was so distinct that our primers did not recognize it. It is also possible that *mcrII* or some other distinct isogene was present but rare among members of the *Methanosarcinaceae*, so that although we sequenced multiple clones, we never selected one containing the alternate sequence. Thus far, *mcrII* has been detected only in members of the *Methanobacteriales*, despite attempts to find it in other methanogens, including *Methanosarcina barkeri* (40).

Phylogenetic tree based on partial *mcr* sequences of members of the *Methanosarcinaceae*. Figure 1 shows the phylogenetic relationships among members of the *Methanosarcinaceae* based on their partial *mcrI* sequences. The division of type

FIG. 3. Scatter plot of 16S rRNA distances versus reductase distances (based on a 490-bp segment). Symbols: ○, distances between pairs of strains belonging to the same genus; □, distances between pairs of strains belonging to different genera but the same family; △, distances between pairs of strains belonging to different families. *M. fervidus*, *Methanothermobacter fervidus*; *M. thermoautotrophicum*, *Methanobacterium thermoautotrophicum*; *M. voltaei*, *Methanococcus voltaei*; *M. vannielii*, *Methanococcus vannielii*.

strains into genera is consistent with a recently proposed taxonomy based on 16S rRNA sequences (6).

Comparison of phylogenetic trees based on *mcrI* data and 16S rRNA data. The phylogenetic trees generated from partial *mcrI* sequences were similar to those generated from 16S rRNA sequences (Fig. 2). Analyses of the two types of sequences resulted in the same division of strains into genera, although there were some minor discrepancies in the relative distances between species of the same genus and some discrepancies in the relative distances of the various genera from each other. We compared the 16S rRNA distance (i.e., the number of differences per nucleotide site) with the *mcrI* distance for each pair of strains and found that the *mcrI* distances were 3.01 ± 0.541 times larger (Fig. 3). A linear least-squares fit of the *mcrI* distance data to the 16S rRNA distance data gave a slope of 2.12 ($R^2 = 0.78$) when the line was forced through the origin. However, pairs of organisms belonging to different families had comparatively smaller ratios of *mcrI* distance to 16S rRNA distance (Fig. 3). A least-squares analysis of the distances between organisms belonging to the same family revealed a slope of 3.03 ($R^2 = 0.71$). Figure 3 also shows that the distances between members of different species of the same genus were smaller (the *mcrI* distances were 0.0937 ± 0.0313 , and the 16S rRNA distances were 0.0214 ± 0.0065) than the distances between members of different genera belonging to the same family (the *mcrI* distances were 0.245 ± 0.038 , and the 16S rRNA distances were 0.0807 ± 0.0096). The lack of overlap in these values suggests that within the family *Methanosarcinaceae*, the assignments of species to genera and genera to family are consistent with evolutionary distances.

Comparison of *mcrI* distance data with DNA reassociation values. The evolutionary distances computed by using *mcrI*

sequences were also compared with DNA-DNA reassociation data. The *mcrI* tree is only partially consistent with the DNA reassociation tree for the genus *Methanohalophilus* (5). The high levels of DNA sequence similarity among FDF-1, FDF-2, Ret-1, and SF-1 were supported by similar *mcrI* sequences. DNA reassociation values were not always consistent with *mcrI* distances, however. The *mcrI* distances indicated that *Methanohalophilus halophilus* was more similar to *Methanohalophilus portucalensis* than to *Methanohalophilus mahii*, whereas DNA reassociation data indicated the opposite (5). DNA reassociation data indicated that strain Cas-1 is distantly related to other *Methanohalophilus* strains (5), whereas the *mcrI* sequence data for this strain indicated that it is similar to *Methanohalophilus* sp. strains Ret-1 and SF-1. Also, according to DNA reassociation data, strains CAS-1 and SD-1 are closely related (5); however, on the *mcrI* sequence tree, strain SD-1 is phylogenetically related to a different genus, the genus *Methanohalobium*.

DNA reassociation data were the basis for the proposal to consider *Methanosarcina frisia* a junior subjective synonym of *Methanosarcina mazei* (25). This proposal is supported by 16S rRNA data but not by *mcrI* data (Fig. 2). The phylogenetic distance between the type strains of these two species may beg the definition of species level phylogenetic distance. Two of the three measures of phylogenetic distance indicate a distance small enough that the two species should be considered synonymous, but the third suggests that they could be considered separate species.

Inconsistencies between 16S rRNA distances and DNA reassociation values also occur in the genus *Aeromonas* (27). Some strains that have nearly identical 16S rRNA sequences exhibit low levels of DNA reassociation, and in one case two strains that exhibited a high level of DNA reassociation were separated on the 16S rRNA tree (27).

Both 16S rRNA trees and *mcrI* trees separate species belonging to the *Methanosarcinaceae* into genera with a high degree of certainty on distance matrix trees (with bootstrap values of 99% or higher). A parsimony analysis of the same 16S rRNA sequences also gave bootstrap values of 100%, and most of the *mcrI* bootstrap values were 100%; the only exceptions were the genera *Methanococcoides* (95%) and *Methanobolus* (87%). When the bootstrap analyses included additional strains (such as those shown in Fig. 1), the bootstrap values were slightly lower.

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