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CHAPTER 13

Methanococcales

WILLIAM B. WHITMAN AND CHRISTIAN JEANTHON

Phylogeny

The sequencing of representative genes of the methane-producing archaea in the order Methanococcales suggests that this lineage is ancient and possesses a high degree of genetic diversity. For example, the mesophile *Methanococcus maripaludis* and hyperthermophile *Methanocaldococcus jannaschii* represent the range of diversity within this group. The sequence similarity of their 16S rRNA genes is 88%. Likewise, homologous open reading frames in these two organisms typically possess 60–80% amino acid sequence identity (W. B. Whitman, unpublished observation). For comparison, genes within *Escherichia* and *Yersinia* possess a comparable level of similarity.

In spite of this apparent genetic diversity, the phenotypes of members of the Methanococcales are similar. They have all been isolated from marine habitats and require sea salts for optimal growth. They are all obligately anaerobic methane-producers and use carbon dioxide as the electron acceptor. Hydrogen and sometimes formate are electron donors. Acetate, C-1 compounds such as methanol and methylamines, and alcohols such as isopropanol and ethanol are not utilized as substrates for methanogenesis. They are all irregular cocci with a diameter of 1–3 μm. The cell wall is composed of a protein S-layer, and glycoproteins and cell wall carbohydrates have not been detected. Cells are usually motile by means of flagellar tufts or bundles. However, there is tremendous diversity in the temperature range for growth, which varies from mesophilic to hyperthermophilic. Within each of these temperature ranges, these organisms are among the fastest growing methanogens known, with generation times of 2 hours at 37°C and less than 30 minutes at 85°C. Lastly, many of the Methanococcales require selenium for optimal growth.

Currently, the Methanococcales have been divided into two families and four genera on the basis in part of the temperature optima for growth (Table 1). For the most part this taxonomy reflects the apparent phylogeny of the 16S rRNA gene, but there are some points of ambiguity (Keswani et al., 1996). The two hyperthermophilic genera *Methanocaldococcus* and *Methanotorris* are placed in the family Methanocaldococcaceae, and this grouping appears robust (Fig. 1). It has strong bootstrap support, and it is found by more than one algorithm including the Fitch-Margoliash, neighbor-joining, and maximum likelihood analyses. However, the differences in mol% G+C content of the rRNAs of the hyperthermophiles and mesophiles within this group can bias these computations (Burggraf et al., 1990). When the phylogenetic analyses are performed with only transversions, which ameliorates this bias, the mesophiles appear as a sister group to the hyperthermophile *Methanotorris*, and the Methanocaldococcaceae are no longer a phylogenetic group. To resolve this question, the phylogeny of additional genes should be examined.

Similarly, within the Methanococcaceae, the mesophiles are assigned to the genus *Methanococcus* and the thermophiles to the genus *Methanothermococcus* (Whitman et al., 2001). Within the *Methanococcus*, the deepest phylogenetic group is represented by the patent strain "*Mc. aeolicus*" (Fig. 1). However, upon phylogenetic analyses of the rRNA gene, this strain forms a clade with the thermophile *Methanothermococcus okinawensis* (Takai et al., 2002; Fig. 1). This clade is also supported by transversion analysis. Even though bootstrap support is modest, the common alternative topologies found during the bootstrap analysis do not group the mesophiles to the exclusion of the thermophiles. Although phylogenetic analyses of other molecules are needed to confirm this result, it implies that the genera *Methanococcus* and *Methanothermococcus* are so deeply branched that they include distantly related and phenotypically dissimilar organisms. A taxonomic treatment more consistent with the phylogeny would probably place "*Mc. aeolicus*" and *Mtc. okinawensis* into two novel genera.

Table 1. Taxa in the order Methanococcales[a]

Family	Genus	Species
Methanococcaceae	*Methanococcus*[T]	"*aeolicus*"
		maripaludis
		voltae
		vannielii[T]
	Methanothermococcus	*okinawensis*
		thermolithotrophicus[T]
Methanocaldococcaceae	*Methanocaldococcus*[T]	*fervens*
		infernus
		jannaschii[T]
		vulcanius
	Methanotorris	*igneus*[T]

[a]Nomenclatural types are indicated with a superscript "T." Species that have not been validated are in quotes.

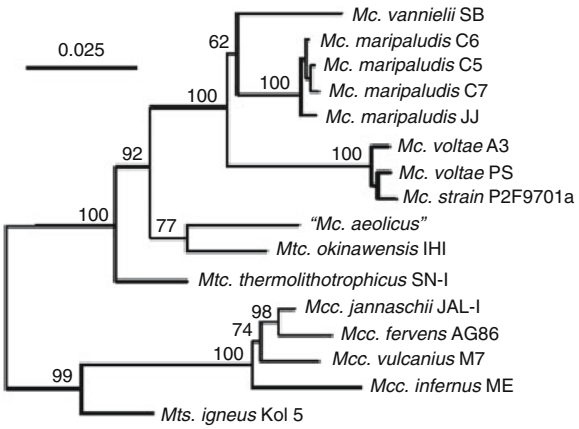

Fig. 1. Phylogeny of the 16S rRNA gene of the Methanococcales. The alignment was manually edited to include 1227 positions of unambiguous alignment. Evolutionary distances were calculated by the Kimura two-parameter model, and the tree was constructed by the Fitch-Margoliash algorithm in PHYLIP (phylogenetic analysis package). Bootstrap analysis was performed with 100 replicates, and values greater than 50 are reported on the nodes. Mc. = *Methanococcus*, Mtc. = *Methanothermococcus*, Mts. = *Methanotorris*, and Mcc. = *Methanocaldococcus*. The scale bar represents evolutionary distance.

Taxonomy

An overview of the current taxonomy of the Methanococcales is given in Table 1. This taxonomy groups organisms with similar temperature optima. All the mesophiles and thermophiles are found within the family Methanococcaceae. The mesophiles are assigned to the genus *Methanococcus* and the thermophiles to the genus *Methanothermococcus*. For the hyperthermophiles, two genera, *Methanocaldococcus* and *Methanotorris* are recognized within the family Methanocaldococcaceae. Although this taxonomy is in general agreement with the phylogeny of the rRNA, some ambiguity remains. To resolve these issues, additional strains and the phylogenies of other molecules will probably be required (see above).

Historically, the genus *Methanococcus* included methane-producing cocci that did not form regular packets (i.e., *Methanosarcina*) or chains (i.e., some species of *Methanobacterium*, now *Methanobrevibacter*; Bryant, 1974). At that time, the type species, *Methanococcus mazei*, was not available in pure culture, and only one other species, *M. vannielii*, was known. Upon analysis of the partial sequence of the 16S rRNAs of methanogens (Balch et al., 1979) and isolation of an archaeon with the phenotype of *M. mazei* (Mah, 1980), it became apparent that *M. mazei* was related to the Methanosarcinaceae and that *M. vannielii* was related to a new species, *Methanococcus voltae*. Thus, it was proposed that *M. vannielii* become the new type species for the genus and *M. mazei* be reclassified as *Methanosarcina mazei* (Balch et al., 1979; Mah and Kuhn, 1984; Judicial Commission, 1986). Likewise, species more closely resembling *M. mazei* were placed in the Methanosarcinaceae. Thus, *Methanococcus halophilus*, which utilizes methylamines for methane synthesis, was not classified with the Methanococcaceae (Zhilina, 1983). Similarly, *Methanococcus frisius* resembles *Methanosarcina mazei* by nutritional and morphological criteria (Blotevogel et al., 1986) and was also classified with the Methanosarcinaceae.

Although the remaining methanococcal species were more closely related to each other than other methane-producing bacteria, they were not a closely knit group (Whitman, 1989). In particular, the sequence of the 16S rRNA of *M. jannaschii* was different enough from the other methanococci to justify creation of a new genus (Jones et al., 1983a). The eventual isolation of additional hyperthermophilic methanococci and the sequencing of the 16S rRNAs of most of the methanococci led to the recommendation that the genus be further subdivided into four genera to reduce the genetic diversity (Boone et al.,

1993). This proposal left the mesophilic species in the genus *Methanococcus* and placed the thermophiles and hyperthermophiles in novel genera. The 16S rRNA sequence similarities (>91%) and DNA hybridizations (>3%) among the remaining, mesophilic species were still somewhat lower than those found in many other bacterial and archaeal genera, suggesting that this group was still rather diverse (Keswani et al., 1996). However, in the absence of additional species, there seemed to be little benefit in subdividing this genus at this time.

The more moderate thermophiles, with 16S rRNA sequence similarities of <91% to the mesophiles as well as the hyperthermophiles, were placed in the new genus *Methanothermococcus*. The hyperthermophiles, which possessed <86% rRNA sequence similarity to the mesophiles, were placed in two new genera, *Methanocaldococcus* and *Methanotorris* (originally called "*Methanoignis*," see below). Members of these new genera possessed low 16S rRNA sequence similarity (<93%) to each other as well as differences in nutritional and other phenotypic properties. Within the genus *Methanocaldococcus*, the 16S rRNA sequence similarity was greater than 95%. The genus name "*Methanotorris*" was proposed to replace the original name "*Methanoignis*," which was incorrectly formed (Whitman et al., 2001). The correct latinization was "*Methanignis*." Therefore, *Methanotorris* was proposed to preserve the prefix "*methano-*" in the genus name.

The present descriptions of some of the methanococci are based on the description of a single isolate. Therefore, the known phenotypic characteristics may not be truly representative of the species. For instance, the growth responses of strains of *Methanococcus maripaludis* to NaCl and $MgCl_2$ are variable, and care must be taken when making taxonomic distinctions on this basis (Whitman et al., 1986). More reliable and rapid comparison of strain collections can be made by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) of cellular proteins and restriction fragment length polymorphism (RFLP) analyses of the 16S rRNA (Keswani et al., 1996; Jeanthon et al., 1999a).

Habitat

To date, methanococci have only been isolated from marine environments. *Methanococcus vannielii* was isolated from the shore of the San Francisco Bay (Stadtman and Barker, 1951). The type strain of *M. voltae* was isolated from sediments from the mouth of the Waccasassa River estuary in Florida (Ward, 1970). The type strain of *M. maripaludis* was isolated from salt-marsh sediments near Pawley's Island, South Carolina (Jones et al., 1983b). Additional strains of *M. voltae* and *M. maripaludis* have been isolated from salt-marsh sediments in Georgia and Florida and an estuary in Taiwan (Whitman et al., 1986; Keswani et al., 1996; Lai and Shih, 2001). An unnamed methanococcal isolate has also been obtained from the biofilm of a ship hull (Boopathy and Daniels, 1992).

Methanothermococci have been isolated from coastal geothermally heated marine sediments at Stufe di Nerone near Naples (Italy; Huber et al., 1982) and reservoir water from a North Sea oil field (Nilsen and Torsvik, 1996). A new *Methanothermococcus* species (*M. okinawaensis*) has been recently isolated from a deep-sea hydrothermal vent of the Okinawa Trough (Pacific Ocean; Takai et al., 2002). Environmental clone sequences closely related to this genus have also been obtained from continental high-temperature oil reservoirs (Orphan et al., 2000).

Methanocaldococci are widespread in deep submarine hydrothermal systems. *Methanocaldococcus* species have so far been isolated from chimney material of deep-sea hydrothermal vents at the East Pacific Rise (13°N and 21°N; Jones et al., 1983a; Jeanthon et al., 1999a) and Mid-Atlantic Ridge (14°45′N and 23°N; Jeanthon et al., 1998; Jeanthon et al., 1999a) and hydrothermally heated sediment from Guaymas Basin (Gulf of California; Zhao et al., 1988; Jones et al., 1989; Jeanthon et al., 1999a). Environmental clone sequences closely related to this genus have also been retrieved from hot formation waters of a continental oil reservoir in western Siberia (Nercessian et al., 2000).

Methanotorris was originally isolated from sediments and venting water of a shallow submarine hydrothermal vent at Kolbeinsey Ridge located on the Mid-Atlantic Ridge (north of Iceland; Burggraf et al., 1990). Fifteen additional strains have been isolated from sediments from a Guaymas Basin hydrothermal site (Gulf of California) and chimney samples from the Mid-Atlantic Ridge (23°N; Jeanthon et al., 1999a).

Isolation

The Methanococcales, like other methane-producing archaea, are strict anaerobes that require specialized techniques for their cultivation. This methodology has been reviewed recently by Sowers and Noll (1995) and will not be discussed here. The mesophilic methanococci may be easily isolated after enrichment under H_2 + CO_2 (80:20) in pressurized tubes or bottles (Miller and Wolin, 1974; Balch and Wolfe, 1976). Because of their rapid growth, methanococci frequently outgrow other H_2-utilizing metha-

nogens in marine sediments. Therefore, this enrichment is somewhat specific, and enrichment cultures that take longer than 5 days to develop seldom contain methanococci. The enrichments are transferred to medium containing antibiotics (penicillin G [0.2 mg/ml], erythromycin [0.2 mg/ml] and streptomycin sulfate [0.2 mg/ml]) before plating on agar plates or roll tubes (Jones et al., 1983b; Jones et al., 1983c; Tumbula et al., 1995a). In some cases, it is necessary to include antibiotics in the solid medium to prevent growth of spreading bacteria over colonies of methanococci. Isolated colonies are picked with a syringe needle and transferred to liquid medium. Purity may be demonstrated by microscopic examination, restreaking on agar medium and absence of growth in mineral medium supplemented with 1% casamino acids and glucose under $N_2 + CO_2$ (80:20).

A useful medium for isolation and rapid growth of methanococci consists of:

Glass distilled water	500 ml
General salts solution	500 ml
K_2HPO_4 solution	10 ml
Trace mineral solution	10 ml
Iron stock solution	5 ml
Resazurin solution	1 ml
NaCl solution	75 ml
$NaHCO_3$	5 g

The concentrations of stock K_2HPO_4, NaCl, and resazurin solutions are 14 g/liter, 293 g/liter and 0.1 g/100 ml of glass distilled water, respectively. The iron stock solution is prepared by adding 0.2 g of $Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$ to a small bottle, adding 2 drops of concentrated HCl followed by 100 ml of glass distilled water. This solution is prepared fresh monthly. The oxygen indicator resazurin is optional. These components are combined, and the medium is brought to a boil under a stream of $N_2 + CO_2$ (80:20). After boiling, cysteine or 2-mercaptoethanesulfonate, 0.5 g/liter, is added to reduce the medium. When hot, the medium will form a precipitate that goes back into solution upon cooling. After dispensing the medium anaerobically into crimp seal tubes or serum bottles, the gas is exchanged for $H_2 + CO_2$ (80:20, 100 kPa), and the medium sterilized by autoclaving. The medium may then be stored for several months in an anaerobic chamber (Coy Laboratories, Ann Arbor, Michigan). Within one day of inoculation, one part of sterile 2.5% $Na_2S \cdot 9H_2O$ (w/v) is added to 50 parts of medium. After inoculation, the tubes are pressurized to 200 kPa with $H_2 + CO_2$. Tubes are repressurized periodically throughout growth. During the period of rapid growth immediately following the lag phase, cultures need to be repressurized 3–4 times per day. If the CO_2 in the headspace is allowed to become too low, the pH of the medium will rise. Under alkaline conditions, the cells lyse (Schauer and Whitman, 1989). For growth in vessels that will not maintain a pressure >100 kPa, the $NaHCO_3$ concentration is reduced to 2 g/liter.

For *M. voltae*, the mineral medium must be supplemented with either yeast extract, 2 g/liter, or sodium acetate $\cdot 3H_2O$, 0.14 g/liter, L-isoleucine, 0.5 g/liter, L-leucine, 0.5 g/liter, and pantoyllactone, 1.3 mg/liter (Whitman et al., 1986). Similarly, the addition of casamino acids (2 g/liter) and sodium acetate $\cdot 3\ H_2O$ (0.14 g/liter) is stimulatory for *M. maripaludis*.

General Salts Solution (modified from Romesser et al., 1979)

KCl	0.67 g
$MgSO_4 \cdot 7H_2O$	6.9 g
$MgCl_2 \cdot 6H_2O$	5.5 g
NH_4Cl	1.0 g
$CaCl_2 \cdot 2H_2O$	0.28 g
Glass distilled water	1000 ml

Trace Metal Solution (modified from Wolfe's minerals; Balch et al., 1979)

Nitrilotriacetic acid, neutralized with KOH	1.5 g
$MnSO_4 \cdot 2H_2O$	0.1 g
$Fe(NH_4)_2(SO_4)_2 \cdot 6H_2O$	0.2 g
$CoCl_2 \cdot 6H_2O$	0.1 g
$ZnSO_4 \cdot 7H_2O$	0.1 g
$CuSO_4 \cdot 5H_2O$	10 mg
$NiCl_2 \cdot 6H_2O$	25 mg
Na_2SeO_3	0.2 g
$Na_2MoO_4 \cdot 2H_2O$	0.1 g
$Na_2WO_4 \cdot 2H_2O$	0.1 g

Sodium Sulfide Solution

A pellet of NaOH is added to 100 ml of distilled water, and the water is brought to a boil under a stream of N_2 gas and allowed to cool under N_2 in the fume hood. Then a large crystal of about 2.5 g of $Na_2S \cdot 9H_2O$ is briefly washed in a 25-ml beaker of distilled water. The crystal is blotted dry on a paper towel, reweighed and added to the NaOH solution. Because sulfide is toxic, chemically impermeable gloves are worn, and this procedure is performed in the fume hood. The solution is stoppered, brought into the anaerobic chamber, and dispensed into tubes and stoppered. The tubes are pressurized to 100 kPa with N_2, autoclaved, and stored in the anaerobic chamber for up to one month.

Methanothermococci and methanocaldococci may be isolated after enrichment under H_2/CO_2 (80:20, 200 kPa) in 50- or 100-ml serum bottles (Jeanthon et al., 1998).

Methanothermococci-Methanocaldococci Culture Medium

Sea salts (Sigma)	30 g
NH_4Cl	1 g
KH_2PO_4	0.35 g
PIPES	3.46 g
$NaHCO_3$	1 g
Difco yeast extract	2 g
Cysteine $\cdot$HCl	0.5 g
Na_2SeO_4	0.5 mg
$Na_2WO_4 \cdot 2H_2O$	2 mg
Vitamin B_{12}	0.05 mg
Trace element mixture (see below)	1 ml
Vitamin mixture (see below)	1 ml
Thiamine solution (see below)	1 ml
Growth-stimulating factors (see below)	1 ml
Rezasurin	1 mg
Distilled water	1000 ml

Trace Element Mixture (Widdel and Bak, 1992)

HCl (25% = 7.7 M)	12.5 ml
$FeSO_4 \cdot 7H_2O$	2.1 g
H_3BO_3	30 mg
$MnCl_2 \cdot 4H_2O$	100 mg
$CoCl_2 \cdot 6H_2O$	190 mg
$NiCl_2 \cdot 6H_2O$	24 mg
$CuCl_2 \cdot 2H_2O$	2 mg
$ZnSO_4 \cdot 7H_2O$	144 mg
$Na_2MoO_4 \cdot 2H_2O$	36 mg
Distilled water	987 ml

The solution is autoclaved under a N_2 (100%) atmosphere in serum bottles closed with tightly fitting rubber stoppers and fixed with aluminum seals.

Vitamin Mixture (Widdel and Bak, 1992)

Sodium phosphate buffer (10 mM; pH 7.1)	100 ml
4-Aminobenzoic acid	4 mg
D(+)-Biotin	1 mg
Nicotinic acid	10 mg
Calcium D(+)-pantothenate	5 mg
Pyridoxine dihydrochloride	15 mg

The solution is filter-sterilized (pore size, 0.2 µm) and kept in the dark at 4°C under a N_2 atmosphere (100 kPa).

Thiamine Solution (Widdel and Bak, 1992)

Ten mg of thiamine chloride dihydrochloride is dissolved in 100 ml of 25 mM sodium phosphate buffer, pH 3.4. The solution is filter-sterilized and kept at 4°C under a N_2 atmosphere (100 kPa).

Growth-stimulating Factors Solution (Pfennig et al., 1981)

Isobutyric acid	0.5 g
Valeric acid	0.5 g
2-Methyl-butyric acid	0.5 g
3-Methyl-butyric acid	0.5 g
Caproic acid	0.2 g
Succinic acid	0.6 g
Distilled water	100 ml

The solution, adjusted to pH 9 with NaOH, is autoclaved under a N_2 (100%) atmosphere in serum bottles closed with tightly fitting rubber stoppers and fixed with aluminum seals.

The pH of the Methanothermococci-Methanocaldococci culture medium is adjusted to 6.5 using 1 M HCl before autoclaving, and the medium is reduced by adding sodium sulfide $Na_2S \cdot 9H_2O$ to a final concentration of 0.05% (w/v). Solid medium is prepared by the addition of 0.7% (w/v) Phytagel (Sigma Chemical, Co., St. Louis, MO) and reduced with a titanium (III) citrate solution (Zehnder and Wuhrman, 1976). For methanothermococci, agar (2%, w/v) can be used as gelling agent. Medium is dispensed into Petri dishes in an anaerobic glove box. Plates are incubated at 60°C (for methanothermococci) or at 75°C (for methanocaldococci) in anaerobic jars for 3–5 days under a H_2/CO_2 atmosphere (80:20; 250 kPa).

Identification

To identify the mesophilic *Methanococcus* spp., isolates must be first distinguished from a number of morphologically and nutritionally similar species of *Methanogenium* and *Methanoculleus* that are also present in marine environments (Romesser et al., 1979; Rivard and Smith, 1982; Ferguson and Mah, 1983; Rivard et al., 1983). Methanococci may be distinguished from these other species by their faster growth rate, requirement for higher concentrations of NaCl for optimal growth, lack of organic growth requirements (except *M. voltae*) and the lower mol% G+C of their DNA. Unlike some members of these other genera, the methanococci have not been found to use secondary alcohols or ethanol as electron donors for methanogenesis. However, identification of isolates based solely on morphological and growth characteristics is equivocal, and use of salt or mineral requirements has been particularly deceptive (see below). Thus, antigenic crossreactivity (Conway de Macario et al., 1981) and 16S rRNA sequencing (Keswani et al., 1996) are helpful for final identification of new isolates.

Strains of methanococci may also be rapidly screened by one-dimensional SDS-PAGE of cellular proteins (Whitman, 1989; Keswani et al., 1996). Cultures are grown to an absorbance of 1.0 cm^{-1} at 600 nm, and 5-ml cultures are harvested by centrifugation. The cells are resuspended in 0.1 ml of mineral medium or a salt solution prepared without reducing agents. This cell suspension may be stored at –20°C prior to electrophoresis. After thawing, the suspension is vortexed to form an even suspension, and 15 µl are added to 60 µl of sample buffer containing sodium dodecyl sulfate and 2-mercaptoethanol. A portion, 35 µl, of this mixture is subjected to electrophoresis on a 12% polyacrylamide gel. The protein profile on SDS-PAGE is sufficiently distinctive to distinguish species of mesophilic methanococci from each other or from other methanogenic bacteria like *Methanogenium* species (data not shown).

Methanothermococcus is distinguished from the mesophile *Methanococcus* and the hyperthermophiles *Methanocaldococcus* and *Methanotorris* by its moderate thermophily. It differs from most other moderately thermophilic methanogens (such as *Methanothermobacter*) by its coccoid morphology. So far, *Methanoculleus thermophilicum* is the only other moderately thermophilic coccus described (Rivard and Smith, 1982; Ferguson and Mah, 1983). *Methanoculleus thermophilicum* requires acetate for growth, grows in low concentrations of NaCl, has a mol% G+C of 56–60, and is easily distinguished by these properties. Antigenic crossreactivity (Conway de Macario et al., 1981; Bryniok and Trosch, 1989; Nilsen and Torsvik, 1996), 16S rRNA sequencing (Keswani et al., 1996), and SDS-PAGE of cellular proteins (Nilsen and

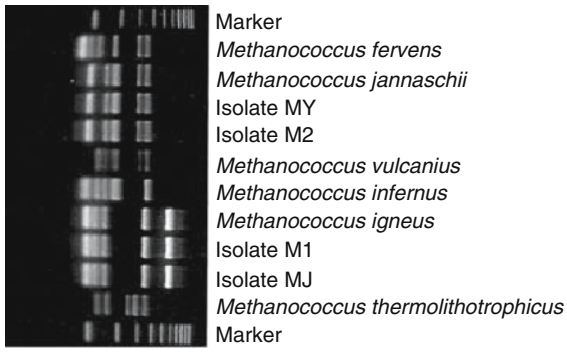

Fig. 2. Restriction patterns of 16S rRNA genes of the type strains of hyperthermophilic methanococci and isolates from deep-sea hydrothermal vents selectively amplified and digested with *Bst*UI. The marker is a 100-bp ladder. From Jeanthon et al. (1999a), and used with permission of the publisher.

Torsvik, 1996) are also helpful for final identification of new isolates.

Methanocaldococcus and *Methanotorris* are distinguished from the mesophile *Methanococcus* and the thermophile *Methanothermococcus* by their hyperthermophily. The hyperthermophiles may be distinguished from each other because *Methanocaldococcus* requires selenium for optimal growth and possesses flagellar tufts. Restriction fragment length polymorphism (RFLP) of the gene encoding the 16S rRNA has proven useful for distinguishing species of *Methanocaldococcus* from each other as well as from strains of *Methanotorris* and *Methanothermococcus* (Jeanthon et al., 1999a). Distinctive RFLP patterns are obtained after restriction of the amplified 16S rRNAs of the species of the three genera with *Hha*I, *Msp*I and *Bst*UI endonucleases (Fig. 2). The profiles obtained with *Hae*III show distinctive patterns for all the type strains except for *M. jannaschii* and *M. fervens*. It is difficult to distinguish species of *Methanocaldococcus* on phenotypic properties alone. Of the four species currently described, only *M. fervens* is resistant to rifampicin, and only *M. jannaschii* is not stimulated by yeast extract.

A number of compounds have also proven to be useful chemotaxonomic markers. The core lipids of *Methanococcus* are composed of archaeol and hydroxyarchaeol (Koga et al., 1998). In *Methanothermococcus*, caldarchaeol is also present. In *Methanocaldococcus* and *Methanotorris*, hydroxyarchaeol is absent and a cyclic derivative of archaeol is found. In all four genera, the polar head groups are composed of glucose, *N*-acetylglucosamine, serine and ethanolamine (in some species). The most abundant polyamine is spermidine in *Methanococcus* and *Methanothermococcus* (Kneifel et al., 1986; Hamana et al., 1998). Spermine is the most abundant polyamine in *Methanocaldococcus*. Unusual compatible solutes have not been detected in *Methanococcus* spp. In *Methanothermococcus*, β-glutamate and *N*-acetyl-β-lysine are abundant, depending on the growth condition (Robertson et al., 1990; Robertson et al., 1992). In *Methanocaldococcus*, β-glutamate is the most abundant compatible solute. *Methanotorris* contains di-*myo*-inositol-1,1′-phosphate in addition to β-glutamate (Ciulla et al., 1994).

Preservation

Because the methanococci lyse shortly after the cessation of growth in liquid media, stock cultures are grown below the temperature optimum (30°C) and stored at room temperature for up to 3 weeks. Strains of all the mesophilic methanococci have been stored with little loss in viability for up to 3 years in 25% glycerol at –70°C (Whitman et al., 1986; Tumbula et al., 1995b). Cultures 10 years or older have been routinely revived. Tube cultures are first concentrated by centrifugation and resuspended in a one-fifth volume of medium containing yeast extract and 25% glycerol (v/v). Portions of the cell suspension are transferred to sterile 1-ml screw-top glass vials in an anaerobic chamber. The vials are then stored at –70°C without anaerobic precautions. To revive the cultures, 0.2 ml of the cell suspension are allowed to thaw in an anaerobic chamber and transferred to fresh medium. Methanococci have also been stored by freeze-drying (Hippe, 1984) and freezing in glycerol (Winter, 1983).

For long-term preservation of the methanothermococci, strains are grown in suspension overnight, repressurized, and stored at 4°C, after which these suspensions can serve as an inoculum for at least 6 months. Using the same procedure, *Methanothermococcus thermolithotrophicus* strain ST22, stored at room temperature, was viable for at least 2 years (Nilsen and Torsvik, 1996). Cultures can also be stored at –80°C in fresh culture medium containing 20% (w/v) glycerol.

Characteristics of Methanococcales

Methanococcus

The genus *Methanococcus* is represented by four species whose properties are summarized in Table 2. Growth occurs at mesophilic temperatures, with the optima near 35–40°C. The pH optima for growth are between 6 and 8. During balanced growth, cells are slightly irregular and uniform in size, between 1 and 2 μm in diameter (Fig. 3). Pairs of cells are common. In stationary

Table 2. Descriptive characteristics of the species of the genus *Methanococcus*.

Characteristic	*vannielii*	*voltae*	*maripaludis*	"*aeolicus*"
Irregular coccus	+	+	+	+
Cell diameter, μm	1.3	1.3–1.7	0.9–1.3	1.7
Motile	+	+	+	+
Substrates for methane synthesis	$H_2 + CO_2$, and formate	$H_2 + CO_2$, and formate	$H_2 + CO_2$, and formate	$H_2 + CO_2$, and formate
Autotrophic growth	+	–	+	+
Growth requirement	None	Ac, Ile, Leu, and Ca^{2+}	None	None
Growth stimulatory	Se	Se, and pantoyllactone	Se, acetate, and amino acids	Se
Sulfur sources	S^{-2}, and S^0	S^{-2}, and S^0	S^{-2}, S^0, and ($S_2O_3^{-2}$)	S^{-2}, and S^0
Nitrogen sources	NH_3, and purines	NH_3	NH_3, N_2, and alanine	NH_3, and N_2
Temperature range, °C	<20–45	<20–45	<20–45	<20–45
pH range	6.5–8	6.5–8	6.5–8	6.5–8
NaCl optimum, %	0.6–2	1–2	0.6–2	1–2
NaCl range, %	0.3–5	0.6–6	0.3–5	1–>5
Mol% G+C[a]	31 (BD) and 33 (LC)	30 (LC) and 31 (BD)	33 (BD and LC)	32 (LC)
Type strain	SB	PS	JJ	PL-15/H[b]
Culture collection	DSM 1224 OCM 148	DSM 1537 OCM 70	DSM 2067 OCM 175	

Symbols: +, property of the species; and –, not a property of the species.
Abbreviations: BD, buoyant density method; LC, liquid chromatography; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen; and OCM, Oregon Collection of Methanogens.
[a]Of the type strain.
[b]Because this species has not been formally described, this strain is not a nomenclatural type.

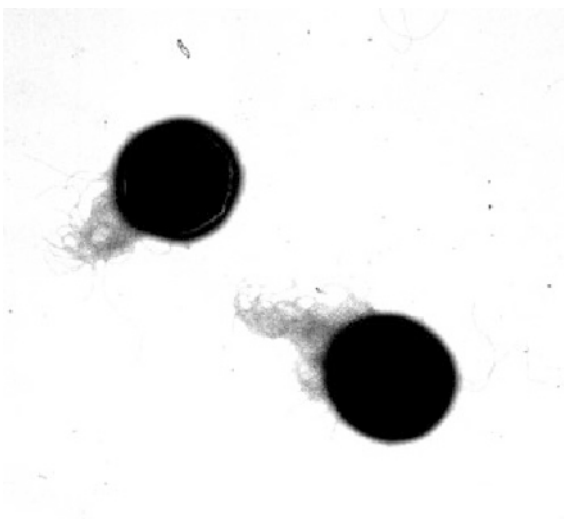

Fig. 3. *Methanococcus voltae* showing the polar flagellar tuft. The cells were negatively stained with 1% phosphotungstate (pH 7) and viewed with a JEOL 1200EX transmission electron microscope at 80 kV. (Electron micrograph courtesy of Shin-Ichi Aizawa and Ken Jarrell.)

cultures, colonies or enrichment cultures, cell shape is very irregular, and large cells up to 10 μm in diameter are observed (Jones et al., 1977b). In wet mounts, a few cells in a preparation may slowly swell and burst (Ward, 1970). Cells on the edge of a slide where drying may occur are much larger, less irregular, and more transparent than cells from the center of the slide. Cells from older cultures are mechanically fragile and rupture during vigorous stirring or upon harvesting by some continuous centrifugation devices. Cells are also osmotically fragile, and they lyse rapidly in distilled water. Cell integrity is maintained in 2% NaCl (w/v). Cells lyse rapidly in 0.01% sodium dodecyl sulfate (SDS) and contain a protein cell wall or S-layer. In *M. vannielii* and *M. voltae*, the outer cell surface is composed of hexagonally ordered structures (Jarrell and Koval, 1989). Cells are motile, and *M. vannielii* contains two tufts or bundles of flagella at the same pole.

Methanococcus spp. are obligate anaerobes and obligate methanogens. Molecular hydrogen (H_2) and formate serve as electron donors. Acetate, methanol and methylamines are not substrates for methanogenesis. All the strains tested are unable to utilize alcohols such as ethanol, isopropanol, isobutanol and cyclohexanol as electron donors for CO_2 reduction (Zellner and Winter, 1987). While an unnamed isolate of *Methanococcus* has also been reported to utilize methylfurfural compounds as a substrate for methanogenesis, the only strain of *Methanococcus maripaludis* tested did not utilize methylfurfural (Boopathy, 1996).

Except for *M. voltae*, the methanococci will grow in mineral medium with sulfide as the sole reducing agent and carbon dioxide as the sole carbon source (Whitman et al., 1986). Autotrophic CO_2 fixation is by the modified Ljungdahl-Wood pathway of acetyl-CoA biosynthesis (Shieh and Whitman, 1987; Shieh and Whitman, 1988; Ladapo and Whitman, 1990).

Methanococcus maripaludis is a facultative autotroph, and acetate and amino acids are stimulatory to growth. The amino acids are incorporated into cellular protein with a high efficiency but not further metabolized (Whitman et al., 1987). In contrast, the growth of *M. vannielii* and "*M. aeolicus*" is not affected by acetate or amino acids. *Methanococcus voltae* requires acetate, isoleucine and leucine for growth (Whitman et al., 1982). Isovalerate and 2-methylbutyrate can substitute for leucine and isoleucine, respectively. Pantoyllactone and pantoic acid, which are formed from pantothenate during autoclaving, can also stimulate growth. These requirements are the same for the type strain as well as other strains isolated from the coastal regions in the southeastern United States (Whitman et al., 1986). However, a strain isolated from Taiwan does not possess an absolute requirement for acetate or amino acids, although acetate and tryptone are stimulatory (Lai and Shih, 2001). The reason for this discrepancy is not known. Since DNA hybridization was not performed, it is possible that this strain may represent a novel but closely related species. Alternatively, the difference in growth responses may be due to different growth conditions employed in different laboratories, or the growth requirements may not be a characteristic of all members of the species. Glycogen has been identified as a storage product in most methanococci (König et al., 1985; Yu et al., 1994).

Nitrogen sources for the methanococci include ammonium, N_2 gas and alanine. Ammonium is sufficient as a nitrogen source for all methanococci and is required by *M. voltae* even during growth with amino acids (Whitman et al., 1982). Molecular nitrogen (N_2 gas) and alanine are additional nitrogen sources for *M. maripaludis*. *Methanococcus vannielii* cannot utilize N_2 gas or amino acids as nitrogen sources, but it will utilize purines (DeMoll and Tsai, 1986; Whitman, 1989).

Sulfide is sufficient as a sulfur source for all methanococci. Elemental sulfur is also reduced to sulfide (Stetter and Gaag, 1983; W. B. Whitman, unpublished data). Cysteine, dithiothreitol and sulfate do not substitute for sulfide (Whitman et al., 1982; Whitman et al., 1987). Some strains of *M. maripaludis* utilize thiosulfate as a sulfur source (Rajagopal and Daniels, 1986).

In addition to NaCl, high concentrations of magnesium salts are stimulatory or required by the methanococci (Whitman et al., 1982; Whitman et al., 1986; Corder et al., 1983; Jones et al., 1983b). Calcium is required by *M. voltae* (Whitman et al., 1982). Selenium is stimulatory to all species tested (Jones and Stadtman, 1977a; Whitman et al., 1982; Jones et al., 1983b). Iron, nickel and cobalt are required or stimulatory for *M. voltae* (Whitman et al., 1982), and tungsten and nickel are required or stimulatory for *M. vannielii* (Jones and Stadtman, 1977a; Diekert et al., 1981).

Like other archaea, methanococci are generally resistant to low concentrations of many common antibiotics (Jones et al., 1977b). Some antibiotics that are inhibitory at low concentrations are: adriamycin, chloramphenicol, efrapeptin, leucinostatin, metonidazole, monensin, pleuromutilin, pyrrolnitrin and virginiamycin (Elhardt and Böck, 1982; Böck and Kandler, 1985). Methanococci are also sensitive to low concentrations of organic tin-containing compounds such as: phenyltin, tripropyltin and triethyltin (Boopathy and Daniels, 1991).

Methanococcus aeolicus This irregular coccus has an average diameter of 1.7 μm and occurs singly or in pairs. For growth, the temperature range is ≥20–45°C, the pH range is 6.5–8.0, the range of NaCl concentration is 1→5%, and the optimum is 1–2%. Ammonium and N_2 can serve as sole nitrogen sources, and selenium but not acetate or amino acids stimulates growth. Cells are susceptible to lysis by 0.01% sodium dodecyl sulfate (SDS) and hypotonic solutions. The mol% G+C is 32.0 (liquid chromatography method [LC]). The type strain has not been designated, and this taxon is currently represented solely by a patent strain (Schmid et al., 1984; Keswani et al., 1996; Whitman, 2001).

Methanococcus deltae Although validly published, this species is a subjective synonym of *M. maripaludis*. Hence, it is not further described here (Corder et al., 1983; Keswani et al., 1996).

Methanococcus maripaludis This irregular coccus has an average diameter of 0.9–1.3 μm and occurs singly or in pairs. For growth, the temperature range is ≥20–45°C, the pH range is 6.5–8.0, the range of NaCl concentration is 0.3–5%, and the optimum is 0.6–2%. Ammonium, alanine and N_2 can serve as sole nitrogen sources. Selenium, acetate and amino acids are stimulatory for growth. Cells are susceptible to lysis by 0.01% SDS and hypotonic solutions. The mol% G+C is 33–35 (LC). The type strain is JJ (= DSMZ 2067 = OCM 175; Jones et al., 1983b; Keswani et al., 1996; Whitman, 2001).

Methanococcus vannielii Cells are irregular cocci, with an average diameter of 1.3 μm, and occur singly or in pairs. For growth, the temperature range is ≥20–45°C, the pH range is 6.5–8.0, the range of NaCl is 0.3–5%, and the optimum is 0.6–2%. Ammonium and purines, but not alanine and N_2, can serve as sole nitrogen sources. Selenium stimulates growth, whereas acetate

and amino acids do not. Cells are susceptible to lysis by 0.01% SDS and hypotonic solutions. The mol% G+C is 32.5 (LC). *Methanococcus vannielii* is the type species of the genus. The type strain is SB (= ATCC 35089 = DSMZ 1224 = OCM 148; Stadtman and Barker, 1951; Whitman, 2001).

Methanococcus voltae Cells are irregular cocci, with an average diameter of 1.3–1.7 μm, and occur singly or in pairs. For growth, the temperature range is ≥20–45°C, the pH range is 6.5–8.0, the range of NaCl is 0.3–5%, and the optimum is 1–2%. Ammonium but not alanine and N_2 can serve as sole nitrogen source. Selenium is stimulatory for growth. Acetate and amino acids leucine and isoleucine are required or greatly stimulate growth. Cells are susceptible to lysis by 0.01% SDS and hypotonic solutions. The mol% G+C is 29–32 (LC). The type strain is PS (= ATCC 33273 = DSMZ 1537 = OCM 70; Ward, 1970; Whitman, 2001).

Methanothermococcus

The genus *Methanothermococcus* is represented by two species, *Methanothermococcus thermolithotrophicus* and *M. okinawensis*, whose properties are summarized in Table 3. In both cases, cells are Gram-negative irregular cocci and motile by means of a polar tuft of flagella. They lyse immediately in distilled water and in the presence of dilute solutions of SDS. They are thermophilic, with a temperature optimum of 60–70°C. NaCl is required for growth. They grow autotrophically in defined mineral medium with H_2 and CO_2. H_2 and formate are used as electron donors for methanogenesis. Acetate, methanol, and methylamines are not substrates for methane production. Organic carbon sources are not stimulatory for growth. The mol% G+C is 31–32. The type species is *M. thermolithotrophicus*.

Methanothermococcus okinawensis These irregular cocci have an average diameter of 1.0–1.5 μm and occur singly or in pairs. They are vigorously motile by means of a polar bundle of flagella. For growth, the temperature range is 40–70°C, the optimum is 60–65°C, the pH range is 4.5–8.5, optimum is 6–7, the concentration of sea salts is 1.2–9.6%, and the optimum is 2.0–5.0%. Ammonium is the nitrogen source, and selenium and magnetite (Fe_3O_4) are stimulatory for growth. Cells are susceptible to lysis by 0.1% SDS and hypotonic solutions. This species was first isolated from a deep-sea hydrothermal vent chimney at the Iheya Ridge, in the Okinawa Trough. The type strain of the species is IH 1 (= JCM11175 = DSM 14208; Takai et al., 2002).

Methanothermococcus thermolithotrophicus Cells are regular to irregular cocci (diameter, 1.5 μm) that occur singly or in pairs. On agar and in Gelrite, round yellowish colonies around 1 mm in diameter are formed. About 20 flagella are inserted at a distinct area on the cell surface. The cells lyse immediately in the presence of 2% SDS; the cell envelope consists of protein subunits. Their optimum temperature for growth is 60–65°C (range, 17–70°C); optimum pH, 6–7.5 (range, 4.9–9.8); and optimum NaCl concentration, 1.8–4% (range, 0.6–9.4%). Methane is

Table 3. Descriptive characteristics of the species of the genus *Methanothermococcus*.

Characteristic	*thermolithotrophicus*	*okinawensis*
Irregular coccus	+	+
Cell diameter, μm	1.5	1.0–1.5
Motility	+	+
Substrates for methane synthesis	$H_2 + CO_2$, and formate	$H_2 + CO_2$, and formate
Autotrophic growth	+	+
Sulfur sources	S^{-2}, S^0, $S_2O_3^{-2}$, SO_3^{-2}, and SO_4^{-2}	S^{-2}
Nitrogen sources	NH_3, NO_3^-, and N_2	NH_3
Temperature optimum, °C	60–65	60–65
Temperature range, °C	17–70	40–75
pH optimum	5.1–7.5	6–7
pH range	4.9–9.8	4.5–8.5
NaCl optimum, %	2–4	2.5–5.0[a]
NaCl range, %	0.6–9.4	1.2–9.6[a]
Mol% G+C	31 (TM), and 34 (LC)	33.5 (LC)
Type strain	SN1	IH1
Culture collections	DSM 2095, and OCM 138	DSM 14208, and JCM 11175

Symbols: +, property of the species; and –, not a property of the species.
Abbreviations: TM, thermal denaturation; LC, liquid chromatography; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen; and JCM, Japan Collection of Microorganisms.
[a]Sea salts.

formed from H_2/CO_2 and from formate. Organic material does not stimulate growth. Both N_2 and nitrate can serve as sole nitrogen sources (Belay et al., 1984; Belay et al., 1990). Sulfur is reduced to hydrogen sulfide with inhibition of methanogenesis. The DNA base composition is 31–32 mol% G+C. It is the type species of the genus *Methanothermococcus*. Type strain of the species is strain $SN1^T$ (= DSM2095 = ATCC35097 = JCM10549 = OCM 138). The species was first isolated from the sandy geothermally heated sediment of a beach at Stufe di Nerone close to Naples (Italy; Huber et al., 1982; Nilsen and Torsvik, 1996).

Methanocaldococcus

Genus *Methanocaldococcus* is composed of four species (Table 4). Cells are irregular cocci motile by means of polar tufts of flagella and readily lyse in distilled water and in the presence of dilute solutions of SDS. The genus is hyperthermophilic (optimum temperature for growth, 80–85°C). NaCl is required for growth. Cells grow autotrophically in defined mineral medium with H_2 and CO_2. Formate, acetate, methanol, and methylamines are not substrates for methane production. Selenium and tungsten are stimulatory for growth. The G+C content is 31–33 mol%. It is the type genus of the family Methanocaldococcaceae. The type species of the genus is *M. jannaschii*.

Methanocaldococcus fervens These regular to irregular cocci (diameter, 1–2 μm) occur singly and in pairs, and form whitish, translucent, and round colonies about 0.5 mm in diameter on Gelrite plates. Their optimum temperature for growth is 85°C (range, 48–92°C); optimum pH, 6.5 (range, 5.5–7.6); and optimum NaCl concentration, 3% (range, 0.5–5%). Yeast extract, casamino acids, and trypticase are stimulatory for growth. DNA base composition is 33 mol% G+C (thermal denaturation method). The type strain is $AG86^T$ (= DSM4213). The species was first isolated from a deep-sea hydrothermal vent core sample from Guaymas Basin, Gulf of California, at a depth of 2,003 m (Jeanthon et al., 1999b).

Methanocaldococcus infernus These irregular cocci (diameter, 1–3 μm) occur singly and in pairs. Cells exhibit a tumbling motility by means of at least three tufts of flagella. The cell envelope consists of a hexagonally arranged S-layer with a lattice constant of 12.2 nm (Fig. 4). Pale yellow colonies about 1 mm in diameter form on "Phytagel" plates. Optimum temperature for growth is 85°C (range, 55–91°C); optimum pH, 6.5 (range, 5.25–7.0); and optimum NaCl concentration, 2% (range, 0.8–3.5). Yeast extract is stimulatory for growth. Elemental sulfur is reduced to hydrogen sulfide in the presence of CO_2 and H_2. DNA base composition is 33 mol% G+C (thermal denaturation method). The type strain

Table 4. Descriptive characteristics of the species of the hyperthermophilic genera *Methanocaldococcus* and *Methanotorris*.[a]

Characteristic	*Methanocaldococcus*				*Methanotorris*
	jannaschii	*infernus*	*vulcanius*	*fervens*	*igneus*
Irregular coccus	+	+	+	+	+
Cell diameter (μm)	1.5	1–3	1–3	1–2	1–2
Flagella	2 tufts	3 tufts	3 tufts	Not described	±[a]
Substrates for methane synthesis	$H_2 + CO_2$	$H_2 + CO_2$	$H_2 + CO_2$	$H_2 + CO_2$	$H_2 + CO_2$
Autotrophic growth	+	+	+	+	+
Yeast extract stimulates growth	–	+	+	+	–
Selenium stimulates growth	+	+	+	+	–
Sulfur sources	S^{-2} and S^0	S^{-2} and S^0	S^{-2} and S^0	S^{-2} and S^0	S^{-2} and S^0
Nitrogen sources	NH_3	NH_3 and NO_3^-	NH_3 and NO_3^-	NH_3 and NO_3^-	NH_3
Temperature optimum, °C	85	85	80	85	88
Temperature range, °C	50–91	55–91	49–89	48–92	45–91
pH optimum	6.0	6.5	6.5	6.5	5.7
pH range	5.2–7.0	5.25–7.0	5.2–7.0	5.5–7.6	5.0–7.5
NaCl optimum, %	3.0	2.0	2.5	3.0	1.8
NaCl range, %	1.0–5.0	0.8–3.5	0.6–5.6	0.5–5.0	0.45–7.2
Mol% G+C	31 (BD)	33 (TD)	31 (TD)	33 (TD)	31 (TD)
Type strain	JAL-1	ME	M7	AG86	Kol 5
Culture collections	DSM 2661, and ATCC 43067	DSM 11812	DSM 12094, and ATCC 700851	DSM 4213	DSM 5666

Symbols: +, property of the species; –, not a property of the species; and ( ), not relevant or not tested.
Abbreviations: BD, buoyant density method; TD, thermal denaturation; DSM, Deutsche Sammlung von Mikroorganismen und Zellkulturen; and ATCC, American Type Culture Collection.
[a]Although nonmotile, a few flagella-like structures are observed by electron microscopy.

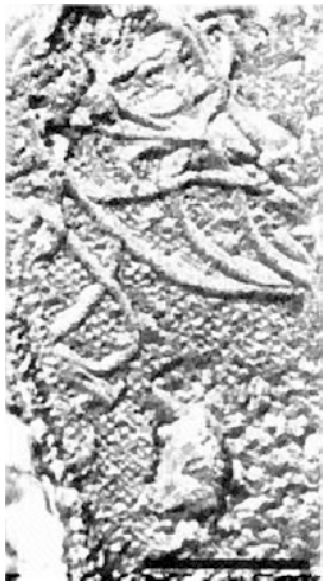

Fig. 4. Freeze-etched and shadowed preparation of cells of *M. infernus* showing the flagella and the S-layer (bar = 100 nm). From Jeanthon et al. (1998), and used with permission of the publisher.

Fig. 5. Freeze-etched and shadowed preparation of cells of *M. vulcanius* showing the three tufts of flagella and the S-layer (bar = 100 nm). Arrows indicate the presence of the S-layer lattice. From Jeanthon et al. (1999b), and used with permission of the publisher.

is strain ME^T (= DSM11812). The species was first isolated from a deep-sea hydrothermal vent chimney in the Mid-Atlantic Ridge (14°45′N; Jeanthon et al., 1998).

Methanocaldococcus jannaschii These irregular cocci measure 1.5 μm in diameter and occur singly or in pairs. The cell surface is composed of hexagonally ordered substructures; two bundles of flagella are inserted close to the same cell pole. Colonies are convex, circular, yellowish in color with a smooth shiny surface. Optimum temperature for growth is 85°C (range, 50–91°C); optimum pH, 6.0 (5.2–7.0); and optimum NaCl concentration, 3%. Acetate, formate, yeast extract, trypticase, and vitamins are not stimulatory for growth. Sulfide is required. DNA base composition is 31 mol% G+C (buoyant density method). The species was first isolated from sedimentary material at the base of a white smoker submarine hydrothermal vent (21°N at 2,600-m depth on the East Pacific Rise). It is the type species of the genus *Methanocaldococcus*. The type strain is strain JAL-1^T (= DSM 2661 = ATCC 43067 = JCM 10045 = OCM 168; Jones et al., 1983a; Jeanthon et al., 1999a).

Methanocaldococcus vulcanius These irregular cocci (diameter, 1–3 μm) occur singly and in pairs. Cells exhibit a tumbling motility by means of three tufts of flagella (Fig. 5). Pale yellow round colonies about 1 mm in diameter form on "Phytagel" plates. Optimum temperature for growth is 80°C (range, 49–89°C); optimum pH, 6.5 (range, 5.25–7); and optimum NaCl concentration, 2.5% (range, 0.6–5.6%). Yeast extract is stimulatory for growth. Sulfur is reduced to hydrogen sulfide in the presence of CO_2 and H_2. DNA base composition is 31 mol% G+C (thermal denaturation method). The type strain is M7^T (= DSM 12094 = ATCC700851). The species was first isolated from a deep-sea hydrothermal vent chimney on the East Pacific Rise (13°N; Jeanthon et al., 1999b).

Methanotorris

Genus *Methanotorris* is represented by a single hyperthermophilic species, *Methanotorris igneus*.

Methanotorris igneus This irregular coccus (diameter, 1.3–1.8 μm) occurs singly and in pairs. Cells are nonmotile, and no tufts of flagella are visible; a few flagella-like filaments are visible on some cells. Pale yellow round smooth colonies are formed on agar. Optimum temperature for growth is 88°C (range, 45–91°C); optimum pH, 5.7 (range, 5–7.5); optimum NaCl, 1.8% (range, 0.45–7.2%). Cells form methane from H_2 and CO_2. Selenium is not required for growth and selenium, yeast extract and peptone are not stimulatory for growth. Formate, acetate, methanol, and methylamines are not substrates for methane production. Sulfur is reduced to hydrogen

sulfide in the presence of CO_2 and H_2. DNA base composition is 31 mol% G+C (thermal denaturation method). It is the type species of the genus *Methanotorris*. The type strain is Kol5^T (= DSM5666). The species was first isolated from a shallow submarine hydrothermal vent system at the Kolbeinsey Ridge (Mid-Atlantic Ridge north of Iceland, at a depth of 106 m; Burggraf et al., 1990; Jeanthon et al., 1999a).

Biochemical and Physiological Properties

Genomic Sequence

Methanocaldococcus jannashii was the first archeaon and hyperthermophile whose genome was sequenced, and it has served as model for other hyperthermophiles and archaea (Bult et al., 1996). While a review of the genome exceeds the scope of this review, an updated annotation that summarizes many of the recent advances is available (Graham et al., 2001). The genomic sequence has also been used to reconstruct the metabolism of this autotroph (Selkov et al., 1997).

Flagellum and Motility

Although the appearance is similar, the flagella of the methanococci and other archaea are very different from those of bacteria (for recent reviews, see Faguy and Jarrell, 1999; Jarrell et al., 1996, and Thomas et al., 2001a). The flagellum itself is somewhat narrower, about 10–14 nm, than bacterial flagella, which are >20 nm. The conspicuous hook and basal structure common on bacterial flagella is not observed; instead, a hook without the basal structure is usually seen (Fig. 6). In some studies, a simple basal body has also been visualized. The archaeal flagellins have sequence similarity to the bacterial type IV pilins and not the bacterial flagellins. They are also biosynthesized with a leader peptide, which implies different mechanisms of assembly and transport across the membrane than are found with the bacterial flagella. Thus, the archaeal and bacterial structures do not appear to be homologous. In support of this conclusion, homologs of genes encoding the bacterial flagellins, basal body and motility apparatus are not found in archaeal genomes.

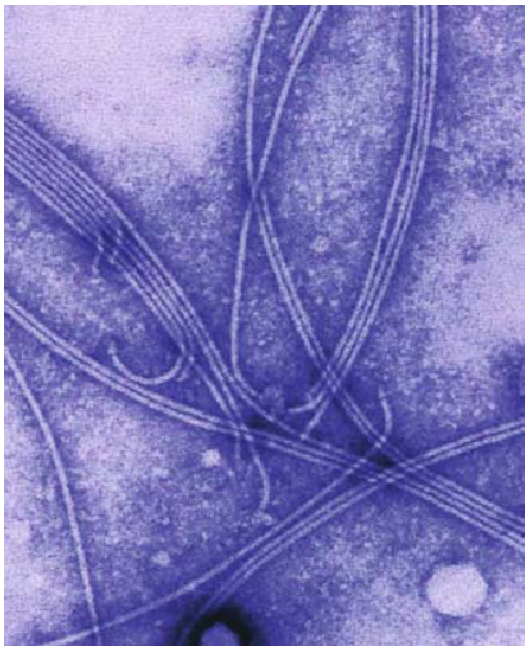

Fig. 6. Flagella of *Methanococcus voltae*. A hook region similar to that found on bacterial flagella is present on the flagella from methanococci. While a basal body has not been observed in these preparations, it is possible that the bodies are present in the cells but lost upon purification. Whole cells were concentrated and solubilized with 1% of the detergent OP-10 in the presence of DNase and RNase. Following removal of the membranes by centrifugation, the flagella were precipitated using 2% polyethylene glycol and 100 mM NaCl. The flagella were then further purified on a KBr gradient. (Electron micrograph courtesy of Shin-Ichi Aizawa and Ken Jarrell.)

Effects of Pressure on Thermophilic Methanogens

Methanocaldococcus jannaschii was the first hyperthermophilic organism isolated from deep-sea hydrothermal vents. Owing to the depth of these vents, the in situ pressure is generally between 20 and 30 MPa (hydrostatic pressure increases by about 10 MPa or 100 atm for every kilometer of seawater). *Methanocaldococcus jannaschii* has therefore served as a model for the effects of pressure on the growth and metabolism of deep-sea thermophiles and for comparison with organisms isolated from the surface at low pressure (i.e., *M. thermolithotrophicus*). High hyperbaric pressures of helium (up to 75 MPa) increase the growth rate of *M. jannaschii* at 86 and 90°C but do not to extend its upper temperature limit for growth (Miller et al., 1988). However, increased pressures extend the upper temperature limit for methanogenesis from less than 94°C at 0.78 MPa to 98°C at 25 MPa. In contrast, following growth at 50 MPa, cells of *M. thermolithotrophicus* are anomalously large and elongated, and the cellular amino acid composition and protein pattern are dramatically changed (Jaenicke et al., 1988). Its growth rate is also enhanced without extending its temperature range (Bernhardt et al., 1988).

Understanding the mechanisms that stabilize proteins, especially at extreme temperatures, is a challenging problem in both biochemistry and biotechnology. The initial findings raised the question of how pressure could extend the thermal stability of thermophilic enzymes. Hei and Clark (1994) compared the pressure effect on the thermal stability of a partially purified hydrogenase from *M. jannaschii* to that from *M. igneus*, *M. thermolithotrophicus* and *M. maripaludis*, all isolated from shallow marine sites. Application of 50 MPa increased the thermal half-life of hydrogenase from *M. jannaschii* 4.8-fold at 90°C. Since hydrogenase from the other hyperthermophile, *M. igneus*, was also substantially stabilized by pressure whereas the hydrogenases from *M. thermolithotrophicus* and *M. maripaludis* were destabilized by pressure, it was suggested that pressure stabilization of enzymes was related to their thermophilicity. In further studies, the thermostability of a protease from *M. jannaschii* also increased at high pressure (Michels and Clark, 1997). However, the destabilization by pressure of the adenylate kinase from *M. jannaschii* demonstrated that hyperbaric stabilization was not an intrinsic property of all enzymes from deep-sea thermophiles (Konisky et al., 1995). In studying the pressure effects on lipids from *M. jannaschii*, Kaneshiro and Clark (1995) showed that pressure had a lipid-ordering effect over the full range of growth temperatures for this organism. The shift toward macrocyclic archaeol from archaeol and caldarchaeol was shown to decrease membrane fluidity.

Genetics

Genetic systems have been utilized extensively in the mesophiles *Methanococcus voltae* and *M. maripaludis* (for a review, see Tumbula and Whitman, 1999). Methods that have become available include efficient transformation systems; antibiotic resistance markers for puromycin and neomycin; reporter genes such as those for β-galactosidase, β-glucuronidase and trehalase; and shuttle and expression vectors. Evidence for a phage transduction system has also been described for *M. voltae* strain PS (Bertani, 1999). This system depends upon VTA (or *voltae* transfer agent) and allows for low frequencies of genetic exchange with a wide variety of markers. The VTA appears to be a small polyhedral phage that carries 4.4-kb fragments of circular DNA that are derived from the host genome (Eiserling et al., 1999). This phage-like particle is similar to the gene transfer agent described for *Rhodobacter capsulata* but different from another phage discovered in *M. voltae* strain A3 (Wood et al., 1985).

Genetic methods have been utilized to examine the expression of the hydrogenase and motility genes of *M. voltae* and the nitrogen fixation genes of *M. maripaludis*. *Methanococcus voltae* contains two sets of hydrogenase genes. One set encodes the selenium-dependent enzymes that contain selenocysteinyl residues at the active site. The second set encodes the selenium-free enzymes. Both sets include both the coenzyme F_{420}-reducing and coenzyme F_{420}-independent hydrogenases (reviewed in Sorgenfrei et al., 1997). The genes encoding the selenium-free enzymes are divergently transcribed from a 453-bp intergenic region that contains the *cis* elements for transcriptional regulation. Expression is only observed in the absence of selenium in the growth medium and appears to be both negatively and positively regulated. Negative regulation results from a silencer region in the middle of the intergenic region (Noll et al., 1999). Positive regulation results from binding of a transcriptional activator immediately upstream of both promoters (Müller and Klein, 2001).

The flagellar biosynthetic genes have been extensively studied in *M. voltae* (reviewed in Thomas et al., 2001a). These genes are arranged into two transcriptional units. The first unit encodes only *flaA*, one of the four flagellin genes present. The second unit encodes the remaining three flagellin genes as well as a number of other genes whose functions are not fully known. Homologs to these other genes are found in the motility gene clusters of other archaea, so they are probably required for flagellar assembly or motility. Although the functions of these genes are not known, mutants with an insertional inactivation of at least one of these genes are no longer flagellated (Thomas et al., 2001b). This experiment provides direct evidence for a role in motility.

Methanococcus maripaludis fixes nitrogen, and the regulation of the *nif* operon, which encodes the nitrogenase genes as well as other genes important for nitrogen fixation, has been studied in detail (for a review, see Leigh, 1999). Expression of the *nif* operon is under control of a repressor (Cohen-Kupiec et al., 1997). In addition, cellular nitrogenase activity is further regulated by two GlnB homologs. In various bacteria, GlnB homologs posttranslationally regulate the activity of a number of key enzymes in nitrogen assimilation, including nitrogenase and glutamine synthetase. Although the specific mechanism is not known, the methanococcal GlnB homologs are responsible for the rapid and reversible "switching off" of nitrogenase activity (Kessler et al., 2001).

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