The Genome Sequence of *Methanosphaera stadtmanae* Reveals Why This Human Intestinal Archaeon Is Restricted to Methanol and H₂ for Methane Formation and ATP Synthesis†

Wolfgang F. Fricke,[1]‡ Henning Seedorf,[2]‡ Anke Henne,[1]§ Markus Krüer,[2] Heiko Liesegang,[1] Reiner Hedderich,[3] Gerhard Gottschalk,[1]* and Rudolf K. Thauer[2]

Göttingen Genomics Laboratory, Institute of Microbiology and Genetics, Georg August University, 37077 Göttingen, Germany[1]; and Department of Biochemistry[2] and Department of Ecophysiology,[3] Max Planck Institute for Terrestrial Microbiology, 35043 Marburg, Germany

Methanosphaera stadtmanae has the most restricted energy metabolism of all methanogenic archaea. This human intestinal inhabitant can generate methane only by reduction of methanol with H₂ and is dependent on acetate as a carbon source. We report here the genome sequence of *M. stadtmanae*, which was found to be composed of 1,767,403 bp with an average G+C content of 28% and to harbor only 1,534 protein-encoding sequences (CDS). The genome lacks 37 CDS present in the genomes of all other methanogens. Among these are the CDS for synthesis of molybdopterin and for synthesis of the carbon monoxide dehydrogenase/acetyl-coenzyme A synthase complex, which explains why *M. stadtmanae* cannot reduce CO₂ to methane or oxidize methanol to CO₂ and why this archaeon is dependent on acetate for biosynthesis of cell components. Four sets of *mtaABC* genes coding for methanol:coenzyme M methyltransferases were found in the genome of *M. stadtmanae*. These genes exhibit homology to *mta* genes previously identified in *Methanosarcina* species. The *M. stadtmanae* genome also contains at least 323 CDS not present in the genomes of all other archaea. Seventy-three of these CDS exhibit high levels of homology to CDS in genomes of bacteria and eukaryotes. These 73 CDS include 12 CDS which are unusually long (>2,400 bp) with conspicuous repetitive sequence elements, 13 CDS which exhibit sequence similarity on the protein level to CDS encoding enzymes involved in the biosynthesis of cell surface antigens in bacteria, and 5 CDS which exhibit sequence similarity to the subunits of bacterial type I and III restriction-modification systems.

There are two types of methanogenic archaea, those belonging to the order *Methanosarcinales*, which contain cytochromes and which can use methanol, methyl amines, acetate, and/or CO₂ plus H₂ as methanogenic substrates, and those belonging to the orders *Methanobacteriales*, *Methanomicrobiales*, *Methanococcales*, and *Methanopyrales*, which are devoid of cytochromes and which can use CO₂ plus H₂ and/or formate only to fuel anaerobic growth (95, 102). The energy metabolism of both types of methanogens has been investigated in detail (17). However, there are still a few pertinent questions. For example, why is the growth yield on H₂ and CO₂ of methanogens lacking cytochromes considerably lower (<50%) than that of cytochrome-containing methanogens? The growth yield on H₂ and CO₂ of *Methanobrevibacter arboriphilus* is 1.3 g/mol methane, whereas that of *Methanosarcina barkeri* is 7.3 g/mol (101). Could the reason for this be that in cytochrome-containing methanogens two steps in the reduction of CO₂ to methane, methyl transfer from methyl-tetrahydromethanopterin (methyl-H₄MPT) to coenzyme M and reduction of the heterodisulfide coenzyme M-S-S-coenzyme B (CoM-S-S-CoB) with H₂, are coupled with energy conservation, whereas in methanogens without cytochromes only one step, the methyltransfer reaction, is coupled? Indeed, methanogens with cytochromes contain a heterodisulfide reductase (HdrDE) that is anchored via a cytochrome *b* (HdrE) in the cytoplasmic membrane (35) and is electronically linked via methanophenazine to a cytochrome *b* (VhoC)-containing hydrogenase complex (VhoACG), whose active site faces toward the periplasm (14, 16), whereas in methanogens without cytochromes, the corresponding hydrogenase (MvhADG) is a cytoplasmic enzyme (74, 82) and the HdrABC complex is recovered mainly in the cytoplasmic fraction (35). There is, however, evidence that HdrABC could be anchored via its HdrB subunit in the cytoplasmic membrane (36, 50). Since HdrB lacks recognizable transmembrane helices, this question is still a matter of dispute.

There is only one indication that the Hdr-catalyzed reaction in methanogens without cytochromes is coupled to energy conservation. It comes from the finding that there is one methanogen belonging to the order *Methanobacteriales* that can grow on methanol and H₂. This organism, *Methanosphaera stadtmanae* (64, 65), thrives in the human intestine, where methanol is a product of pectin degradation by *Bacteroides* species and other anaerobic bacteria (18, 40). With cell suspensions of *M. stadtmanae* it has been shown that the reduction of methanol with H₂ to methane is coupled to ADP phosphorylation via the electrochemical proton potential (87). However, correct interpretation of this finding will not be possible as long as the enzymes involved are not known.

M. stadtmanae, a member of the *Methanobacteriales*, is unusual because it is able to grow on methanol and H_2 as energy substrates, a property otherwise restricted to members of the *Methanosarcinales*. It is also remarkable that *M. stadtmanae* can neither oxidize methanol to CO_2 nor reduce CO_2 to methane. This organism is also not capable of autotrophic growth on CO_2, requiring acetate and CO_2 as main carbon sources. Labeling studies have revealed that acetate and CO_2 are assimilated via reductive carboxylation of acetyl-CoA to pyruvate, from which most cell compounds are formed (4, 10, 11, 51, 62). Interestingly, *Methanobrevibacter smithii*, the only other human methanogenic commensal, is also dependent on acetate for biosyntheses. However, this organism grows on H_2 and CO_2 rather than on methanol and H_2 as energy sources (5, 66). These two archaea, both belonging to the order *Methanobacteriales*, also differ significantly in lipid composition (89).

M. stadtmanae has attracted additional interest because it is the first human archaeal commensal whose genome has been sequenced. Such commensals not only are able to survive in the human gastrointestinal tract, which is protected by a highly active immune system, but also stimulate the development of a healthy intestinal epithelium and immune system (58). In Western countries the increasing number of chronic inflammatory diseases of the intestine (referred to as inflammatory bowel disease) indicates that there is perturbation of the precarious commensal relationship in the human intestine. However, the molecular mechanisms of commensalism are still not fully understood. Studying the genetic information encoded in the small genome of *M. stadtmanae* may provide clues that will increase our understanding of adaptations of this organism that allow it to live as a commensal (2, 25, 55).

We describe here the complete genome sequence of *M. stadtmanae* and compare it with the genome sequences of all other methanogenic archaea sequenced so far. The comparison revealed not only why this archaeon is dependent on H_2, methanol, acetate, and CO_2 for growth but also that the archaeon has several unique features that probably reflect its adaptation to the human intestinal environment.

MATERIALS AND METHODS

M. stadtmanae strain DSZM 3091 was obtained from the Deutsche Sammlung von Zellkulturen und Mikroorganismen, Braunschweig, Germany. This methanogenic archaeon was grown on the medium described by Sparling et al. (87, 88), which also contained 0.5 g/liter sodium formate and 10% rumen fluid. The medium was prepared in an anaerobic chamber (95% N_2–5% H_2) and was reduced prior to autoclaving with 0.28 g $Na_2S \cdot 9H_2O$ per liter and 0.14 g L-cysteine-HCl per liter. Methanol was added after autoclaving to a concentration of 0.4% (vol/vol). Cells were routinely grown in 2-liter bottles containing 1 liter of medium and H_2-CO_2 (80:20) at a pressure of 2×10^5 Pa as the gas phase with continuous shaking at 37°C. When an optical density at 578 nm of approximately 2.5 was reached, the cells were harvested by centrifugation.

Sequencing strategy. Total genomic DNA of *M. stadtmanae* was extracted and sheared. Several shotgun libraries were constructed using 3- to 5-kb size fractions. The fragments were cloned into vector pCR4-TOPO (Invitrogen). Insert ends of the recombinant plasmids were sequenced using dye terminator chemistry with MegaBACE 1000 and 4000 (GE Healthcare) and ABI Prism 377 (Applied Biosystems) automated DNA sequencers. Sequences were processed with Phred and assembled into contigs using the Phrap assembly tool (http://www .phrap.org). Sequence editing was done using GAP4, which is part of the Staden software package (90); 8.7-fold coverage was obtained after assembly of 21,555 sequences. In order to solve problems with misassembled regions caused by repetitive sequences and to close remaining sequence gaps, PCR-based techniques and primer walking with recombinant plasmids were used.

Gene prediction and annotation. The initial gene prediction was accomplished using YACOP (93). The output was verified and edited manually using criteria such as the presence of a ribosome binding site, GC frame plot analysis, and similarity to known protein-encoding sequences (CDS). Annotation was done using the ERGO tool (Integrated Genomics) (www.integratedgenomics.com) with a two-step approach. Initially, all proteins were searched against a nonredundant database consisting of all publicly available sequence data by using FASTA3, which resulted in automatic annotation. All predictions were verified and modified manually by comparing the protein sequences with the Swiss-Prot, GenBank, ProDom, COG, and Prosite public databases. All coding sequences were searched for similarities to protein families and domains using CD-search (57). TMpred was used to predict transmembrane helices within the CDS (39). The SIGI tool (59) was used for score-based identification of genomic islands, referred to as alien CDS, and for identification of potentially highly expressed CDS.

Comparative genomics. For comparative analysis, each CDS of one genome was searched against all CDS of another genome by using the BLAST program (1). For these unidirectional BLAST comparisons a strict cutoff value between e^{-15} and e^{-20} was used. BLAST hits were considered an indication of homology. For identification of CDS specific for all methanogens but missing in *M. stadtmanae*, BLAST comparisons (e^{-15}) with the genomes of *Methanocaldococcus jannaschii* (9), *Methanococcus maripaludis* (38), *Methanopyrus kandleri* (85), *Methanosarcina acetivorans* (27), and *Methanosarcina mazei* (15) were carried out using all CDS of *Methanobacterium thermoautotrophicus* (86) as query sequences. CDS of *M. stadtmanae* not present in the genomes of other archaea were identified by searching each CDS encoding more than 100 amino acids against the genomes mentioned above plus those of *Aeropyrum pernix* (44), *Archaeoglobus fulgidus* (48), *Haloarcula marismortui* (3), *Halobacterium* sp. strain NRC-1 (70), *Picrophilus torridus* (26), *Pyrobaculum aerophilum* (24), *Pyrococcus abyssi* (12), *Pyrococcus furiosus* (76), *Pyrococcus horikoshii* (45), *Sulfolobus solfataricus* (83), *Sulfolobus tokodaii* (43), *Thermoplasma acidophilum* (77), and *Thermoplasma volcanium* (46). The CDS without significant BLAST hits (e^{-20}) were searched with the same cutoff against the NCBI protein database (ftp.ncbi.nih .gov/BLAST/db/nr.tar.gz).

Purification of enzymes. Frozen cells (10 g [wet weight]) of *M. stadtmanae* were routinely suspended in 10 ml potassium phosphate (pH 7.0), and the suspension was then passed through a French pressure cell at 10^8 Pa. After removal of cell debris and the membrane fraction by ultracentrifugation, a supernatant was obtained, from which MtaA and the MtaBC complex (methanol:coenzyme M methyltransferase), the HdrABC complex (heterodisulfide reductase), and the MvhADG complex (non-F_{420}-reducing hydrogenase) were purified. Protein concentrations were determined by the Bradford method using bovine serum albumin as the standard.

(i) Methanol:coenzyme M methyltransferase. The MtaBC complex was purified by chromatography on DEAE-Sepharose, Q-Sepharose, and hydroxyapatite (49). Purification was monitored by determining the characteristic absorption spectrum of the corrinoid protein using dicyanocobalamin as the standard ($\varepsilon_{580-640} = 7.3$ mM^{-1} cm^{-1}). Sodium dodecyl sulfate (SDS)-polyacrylamide gel electrophoresis (PAGE) revealed the presence of two polypeptides with apparent molecular masses of 50 kDa and 30 kDa. The N-terminal amino acid sequences of the 50- and 30-kDa subunits were determined by Edman degradation to be SCKYFTKMENASADEMVFG and MD(or S)S(or P)P(or L)L(or E)E(or K)K(or Y)YGKLTL(or A or H)Y, respectively.

MtaA was purified by ammonium sulfate (70%) precipitation, hydrophobic chromatography on Phenyl-Sepharose, gel filtration on Superdex 200, and anion-exchange chromatography on MonoQ (49). The N-terminal amino acid sequence was MDLIENLKAALNGEXVXKVPAISATAAAVEEAFPAANVS.

(ii) Heterodisulfide reductase. The HdrABC complex was purified 40-fold by chromatography on DEAE-Sepharose, Q-Sepharose, Phenyl-Sepharose, and Superdex 200 (75). Purification was monitored by measuring the specific rate of heterodisulfide reduction with reduced methyl viologen. SDS-PAGE revealed the presence of three polypeptides with apparent molecular masses of 71 kDa, 33 kDa, and 21 kDa; the N-terminal amino acid sequences of these subunits were determined by Edman degradation to be XNNEXVVIGVYTHXXXXNV, XAYAYFLGCIMN NXYPGIEKS, and XTLLNESEYITDKDVDPTFK, respectively (75).

(iii) Non-F_{420}-reducing hydrogenase. The MvhADG complex was purified by chromatography on DEAE-Sepharose, hydroxyapatite, and Superdex 200 (75). Purification was monitored by measuring the specific activity of methyl viologen reduction with H_2 at pH 7.6. SDS-PAGE revealed the presence of three polypeptides with apparent molecular masses of 55 kDa, 38 kDa, and 30 kDa. The N-terminal amino acid sequence of the 55 kDa subunit was determined by Edman degradation to be VELTLEPXTXIE (75).

Quantification of corrinoids. Corrinoids were extracted from enzyme and cell samples with KCN as described by Fischer et al. (23). After centrifugation of the

extract, 20 to 80 µl of the supernatant was analyzed by high-performance liquid chromatography using a Phenomenex phenyl-hexyl reverse-phase column. Dicyanocobalamin was used as the standard ($\varepsilon_{580-640}$ = 7.3 mM^{-1} cm^{-1}) The corrinoid was identified as hydroxybenzimidazolyl cobamide (factor III) by matrix-assisted laser desorption ionization—time of flight mass spectrometry (49).

Nucleotide sequence accession number. The *M. stadtmanae* genome sequence has been deposited in the EMBL/GenBank/DDBJ database under accession number CP000102.

RESULTS AND DISCUSSION

M. stadtmanae contains one circular chromosome (Fig. 1), and plasmids were not found. After a short description of the general features, the genome of *M. stadtmanae* is analyzed below mainly with respect to energy metabolism, carbon assimilation into C$_1$ units, and the genes unique to this human intestinal archaeon. The results of the analysis of the carbon and energy metabolism of the C$_1$ units are summarized in Fig. 2.

General features. The circular chromosome consists of 1,767,403 bp and has the lowest G+C content of all archaeal genomes sequenced so far. Other features of the genome are shown in Table 1. The number of protein-encoding sequences (1,534 CDS) is the lowest number obtained for all methanogens (*M. kandleri* has 1,687 CDS). The genome contains open reading frames for 40 tRNAs. One of these, coding for a Met-tRNA, contains a putative intron that is 34 bp long (Msp0230). Selenocysteinyl-tRNAs were not found. Four rRNA operons are present in the genome of *M. stadtmanae*, the highest number found in archaeal genomes (*rrnA* to *rrnD*). Three rRNA operons contain a gene for an alanyl-tRNA between the 16S rRNA and 23S rRNA genes (*rrnB* to *rrnD*). A cumulative GC skew analysis revealed the presence of only one origin of replication in the neighborhood of one of two CDS for Cdc6 (involved in replication initiation) (Msp0001),for the small subunit of DNA polymerase II (Msp1584), for two helicases (Msp0005 and Msp0007), and for two genes for B$_{12}$ biosynthesis (Msp1587 and Msp1588). The genome of *M. stadtmanae* contains four 1,528-bp insertion elements, all of which include either one of three highly homologous CDS (Msp0017, Msp0233, and Msp0471) or a pseudogene (Msp1439), The CDS show up to 29% identity to a CDS identified in an insertion sequence of *Methanobrevibacter smithii* (31), a close relative of *M. stadtmanae*. Additionally, a complete CDS (Msp1478) and a pseudogene (Msp1400) for two putative transposases and a putative integrase gene (Msp1355) were detected, but there is no evidence for the presence of a prophage. A 4.8-kbp genome segment in which a 30-bp sequence is repeated 59 times is noteworthy. This segment includes 10 short CDS (Msp0948 to Msp0957), which may not code for functional proteins.

(i) CDS shared with *Methanothermobacter*. *M. thermoautotrophicus* is the closest relative of *M. stadtmanae* whose genome sequence has been published. A direct BLAST comparison identified gene homologues in *M. thermautotrophicus* for 1,002 CDS of the *M. stadtmanae* genome. Detailed analysis of the shared CDS indicated that the two organisms have very similar machinery for DNA repair, replication, transcription, translation, cell division, and protein folding. The two archaea also have related pathways for biosynthesis of isoprenoid lipids, amino acids, carbohydrates, nucleotides, and cofactors starting from acetyl-CoA. The two archaea do not contain CDS for
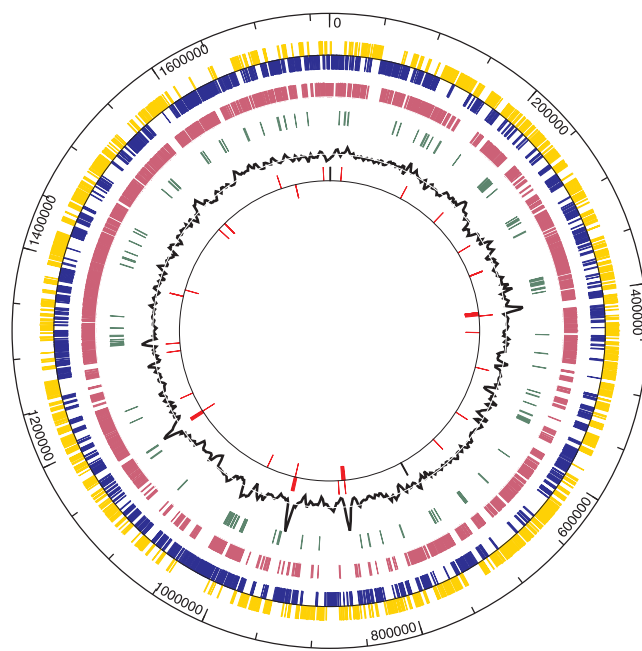

FIG. 1. Circular map of the chromosome of *M. stadtmanae*. Rings from the outside to the inside: scale (in base pairs), protein-encoding sequences (blue or yellow), CDS with gene homologues in *M. thermautotrophicus* (magenta), putative highly expressed CDS (green), G+C content variation (higher values on the outside), rRNA and tRNA coding sequences (red), and *cdc6* genes (black).
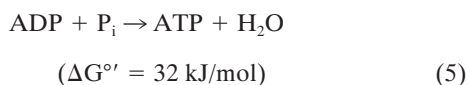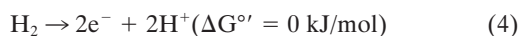
selenoproteins, for cytochromes, for flagellar proteins, for tetrahydrofolate-specific enzymes, and for citrate synthase, aconitase, and isocitrate dehydrogenase. Consistently, archaea belonging to the *Methanobacteriales* are not dependent on selenium for growth, do not contain cytochromes, are nonmotile, do not contain folate, and synthesize 2-oxoglutarate from oxaloacetate via malate, fumarate, and succinate and not via citrate, aconitate, and isocitrate.

(ii) Absence of CDS found in all other methanogens. BLAST comparisons revealed that 37 CDS present in the genomes of all methanogens sequenced so far are absent in *M. stadtmanae*. These CDS include genes for molybdenum cofactor synthesis and CO dehydrogenase/acetyl-CoA synthase (Table 2). Also absent are genes for superoxide dismutase and catalase, which are present in some but not all methanogenic archaea. The two enzymes involved in oxygen detoxification are mentioned since they were shown to be present in *Methanobrevibacter* species (7, 84), which are phylogenetically closely related to *M. stadtmanae* and which thrive in the intestinal tracts of animals and insects (63).

(iii) CDS for membrane-spanning proteins. Approximately 29% (448 CDS) of the 1,534 CDS are predicted to code for transmembrane proteins based on the presence of at least one transmembrane helix. Among these CDS are genes for more than 10 different ABC transporters. They are annotated to be involved in the transport of cobalt, phosphate, other inorganic ions, amino acids, peptides, and polyols. The genome appears to lack a CDS for a molybdate ABC transporter gene, which is consistent with the absence of homologues for molybdopterin biosynthesis genes (Table 2). Examples of genes encoding

FIG. 2. Metabolic pathways involved in methanol reduction to methane with H_2 and in C_1 unit biosynthesis from C-2 of acetate in *M. stadtmanae*. The genes encode the following enzymes (see text): *ehb*, energy-converting hydrogenase; *por*, pyruvate:ferredoxin oxidoreductase; *ppsA*, phosphoenolpyruvate synthase; *eno*, enolase; *apg*, phosphoglycerate mutase; *serA*, phosphoglycerate dehydrogenase; *glyA*, serine:H_4MPT hydroxymethyl transferase; *hps*, hexulose phosphate synthase; *fae*, formaldehyde-activating enzyme; *fwd*, formylmethanofuran dehydrogenase; *ftr*, formylmethanofuran:H_4MPT formyltransferase; *mch*, methenyl-H_4MPT cyclohydrolase; *fprA*, $F_{420}H_2$ oxidase; *fno*, $F_{420}H_2$:NADP oxidoreductase; *mtd*, methylene-H_4MPT dehydrogenase; *thyA*, thymidylate synthase; *frh*, F_{420}-reducing hydrogenase; *mer*, methylene-H_4MPT reductase; *metE*, methionine synthase; *mtr*, methyl-H_4MPT:coenzyme M methyltransferase; *mta*, methanol:coenzyme M methyltransferase; *mvh*, non-F_{420}-reducing hydrogenase; *hdr*, heterodisulfide reductase; and *mrt*, methyl-coenzyme M reductase. PEP, phosphoenolpyruvate; 2-PGA, 2-phosphoglycerate; 3-PGA, 3-phosphoglycerate; FBP, fructose bisphosphate; F6P, fructose-6-phosphate; Hu6P, 3-hexulose-6-phosphate; CHO-MFR, formylmethanofuran; Ru5P, ribulose-5-phosphate.

non-ABC transporters found in the genome of *M. stadtmanae* are CDS for putative subunits of a potassium transport system (Msp0250, Msp0723, Msp0830, Msp1223, Msp1489, and Msp1504), as well as a Co/Zn/Cd transporter (Msp0519), an Mn/Fe transporter (Msp1554), an ammonium transporter (Msp0663), and a ferrous iron transporter (Msp1441 and Msp1440). A sodium-dependent transport system for leucine that has been characterized in *M. stadtmanae* (88) is probably among the six predicted sodium-dependent transporters (Msp0148, Msp0513, Msp0691, Msp0738, Msp0824, and Msp1417). Genes for sodium-proton antiporters found in other methanogens (37) appear to be absent. *M. barkeri* has been shown to grow like *M. stadtmanae* on methanol plus H_2 with acetate as the carbon source and requires sodium ions under these conditions (68). It would be surprising if *M. stadtmanae* could not regulate the intracellular sodium ion concentration by employing a sodium-proton antiporter or a related system.

(iv) CDS predicted to be highly expressed. Codon usage analysis with the SIGI tool (59) revealed a group of 116 CDS which are predicted to be highly expressed (Table 3). Among the putative highly expressed CDS are most of the genes for the enzymes involved in energy metabolism in *M. stadtmanae*.

Energy metabolism. The genome of *M. stadtmanae* contains CDS for the following enzymes that are predicted based on their functions in other methanogens to be involved in methanol reduction with H_2 to methane and ATP synthesis: methanol:coenzyme M methyltransferase (MtaABC)(reaction 1), methyl-coenzyme M reductase (MrtABG) (reaction 2), heterodisulfide reductase (HdrABC) (reaction 3), non-F_{420}-reducing hydrogenase (MvhADG) (reaction 4), and proton-translocating ATPase (AhaABCDEFHIK) (reaction 5). Consistent with the proposed catabolic function, the codon usage of most of the genes was that of highly expressed genes (Table 3).

$$CH_3OH + HS\text{-}CoM \rightarrow CH_3\text{-}S\text{-}CoM + H_2O$$

$$(\Delta G°' = -27 \text{ kJ/mol}) \tag{1}$$

$$CH_3\text{-}S\text{-}CoM + HS\text{-}CoB \rightarrow CH_4 + CoM\text{-}S\text{-}S\text{-}CoB$$

$$(\Delta G°' = -30 \text{ kJ/mol}) \tag{2}$$

$$CoM\text{-}S\text{-}S\text{-}CoB + 2e^- + 2H^+ \rightarrow HS\text{-}CoM + HS\text{-}CoB$$

$$(\Delta G°' = -55 \text{ kJ/mol}) \tag{3}$$

TABLE 1. General features of the *M. stadtmanae* genome

Characteristic	Value
Genome length (bp)	1,767,403
% Coding regions	84
G+C content (%)	28
No. of rRNA operons	4
No. of tRNAs	40
No. of tRNAs with introns	1
No. of protein-encoding sequences	1,534
No. of CDS encoding hypothetical proteins	287
No. of CDS encoding conserved hypothetical proteins	281
No. of CDS encoding proteins with functional assignments	966
No. of CDS encoding predicted membrane-spanning proteins	448
No. of highly expressed CDS[a]	116
No. of alien CDS	18
No. of CDS encoding proteins with a twin-arginine motif	0
No. of CDS encoding aminoacyl-tRNA synthetases	19
No. of CDS encoding histones	6
No. of CDS encoding proteins with inteins	0
No. of insertion sequences	4
No. of putative transposases (fragments)	1 (2)
No. of putative recombinases	1
No. of CDS with gene homologues in the genome of *M. thermautotrophicus* (e^{-15})	1,002
No. of CDS in the genome of *M. thermoautotrophicus* with gene homologues in the genomes of all other methanogens except *M. stadtmanae* (e^{-15})[b]	37
No. of CDS (>100 amino acids) without gene homologues in the genomes of other archaea (e^{-20})	323
No. of CDS (>100 amino acids) with gene homologues only in nonarchaeal genomes ($<e^{-20}$)[c]	73

[a] See Table 3.
[b] See Table 2.
[c] See Table 4.

$$H_2 \rightarrow 2e^- + 2H^+ (\Delta G°' = 0 \text{ kJ/mol}) \quad (4)$$

$$ADP + P_i \rightarrow ATP + H_2O$$

$$(\Delta G°' = 32 \text{ kJ/mol}) \quad (5)$$

where HS-CoM is coenzyme M, CH₃-S-CoM is methyl-coenzyme M, HS-CoB is coenzyme B, and P_i is inorganic phosphate. The $E°'$ of the CoM-S-S-CoB/HS-CoM + HS-CoB couple has recently been determined to be −140 mV rather than −200 mV (97). The $\Delta G°'$ of methanol reduction with H_2 to methane and H_2O is −112 kJ/mol (96).

(i) Formation of methyl-coenzyme M from methanol and coenzyme M. In *Methanosarcina* species, the only other methanogens known to grow on methanol and H_2, the initial step in methanol reduction to methane has been shown to be the formation of methyl-coenzyme M from methanol and coenzyme M (reaction 1). This reaction is catalyzed by three cytoplasmatic proteins, a corrinoid protein (MtaC), a methyltransferase (MtaB) that catalyzes the methylation of MtaC with methanol, and a methyltransferase (MtaA) that catalyzes methyl transfer from MtaC to coenzyme M. The corrinoid protein has to be reduced to the cob(I)alamin oxidation state to become active. This reduction is catalyzed by a

TABLE 2. Thirty-seven protein-encoding sequences found in the genome of *M. thermautotrophicus* and all other methanogens except *M. stadtmanae*

CDS or locus	Function according to Smith et al. (function according to our annotations)[a]
NP_276663	Molybdenum cofactor biosynthesis protein MoaA
NP_276967	Molybdenum cofactor biosynthesis protein MoaB
NP_275948	Molybdenum cofactor biosynthesis protein MoaC
NP_276138	Molybdenum cofactor biosynthesis protein MoeA
NP_276820	Carbon monoxide dehydrogenase, alpha subunit (CO dehydrogenase/acetyl-CoA synthase, subunit alpha)
NP_276822	Carbon monoxide dehydrogenase, alpha subunit (CO dehydrogenase/acetyl-CoA synthase, subunit beta)
NP_276825	Corrinoid/iron-sulfur protein, large subunit (CO dehydrogenase/acetyl-CoA synthase, subunit gamma)
NP_276824	Corrinoid/iron-sulfur protein, small subunit (CO dehydrogenase/acetyl-CoA synthase, subunit delta)
NP_276823	Nitrogenase reductase-related protein (CO dehydrogenase/acetyl-CoA synthase, accessory protein)
NP_276732	Thiamine biosynthetic enzyme
NP_276299	Phosphonoacetaldehyde methylase
NP_277018	Possible protein methyltransferase (predicted RNA methylase)
NP_276283	Na⁺/Ca⁺ exchanging protein related
NP_276665	Formate dehydrogenase, alpha subunit homologue
NP_276615	Phenylalanyl-tRNA synthetase alpha subunit
NP_276113	NADP-dependent glyceraldehyde-3-phosphate dehydrogenase (predicted NAD-dependent aldehyde dehydrogenase)
NP_275964	L-Isoaspartyl protein carboxyl methyltransferase[b]
NP_277015	Hypothetical protein (putative RNA 3′-terminal phosphate cyclase)
NP_275838	Hypothetical protein (predicted ABC-type transport system, permease protein)
NP_276300	Possible cation transporter
NP_276301	Cell division inhibitor-related protein
NP_276195	Acetyltransferase
NP_276886	Stomatin-like protein
NP_275744	Conserved protein (contains ferredoxin domain)
NP_276442	Hypothetical protein[b]

[a] All CDS of *M. thermautotrophicus* (86) were searched with the BLAST program against the genomes of all other methanogenic archaea sequenced so far. CDS of *M. thermautotrophicus* with BLAST hits better than e^{-15} in all other genomes except that of *M. stadtmanae* are listed. Only the first CDS is listed when there was more than one hit resulting from different CDS with high levels of sequence similarity in the *M. thermautotrophicus* genome.
[b] Additional hypothetical proteins are encoded by NP_276192, NP_275386, NP_276747, NP_276390, NP_276421, NP_276472, NP_275703, NP_276239, NP_276433, NP_275838, NP_276973, NP_276349, NP_275612, NP_277013, NP_277015, and NP_275894.

methyltransferase that activates the MapAB protein (13). MtaC and MtaB form a tight complex, and the genes that encode them form a transcription unit, *mtaBC*. MtaA is isolated separately and is translated from a monocistronic mRNA (78). The genomes of *M. barkeri*, *M. acetivorans*, and *M. mazei* each contain three sets of *mtaBC* genes and two sets of *mtaA* genes, which are transcribed under different growth conditions (73).

Four *mtaA*, *mtaB*, and *mtaC* homologues were found in the genome of *M. stadtmanae*: *mtaB1C1* (Msp0188 and Msp0189), *mtaB2C2* (Msp0186 and Msp0187), *mtaB3C3* (Msp0184 and Msp0185), *mtaB4C4* (Msp0819 and Msp0818), *mtaA1* (Msp0761), *mtaA2* (Msp0112), *mtaA3* (Msp0774), and *mtaA4* (Msp0182). At the 3′ end of the *mtaB4C4* cluster, the genome harbors a

TABLE 3. CDS in the genome of *M. stadtmanae* predicted to be highly expressed[a]

CDS	Function	Gene	CDS	Function	Gene
Msp0010	30S ribosomal protein S15P	*rps15p*	Msp0769	Archaeal histone	
Msp0017	Conserved hypothetical protein, encoded in insertion element		Msp0789	Rubrerythrin	
			Msp0818	Methanol corrinoid protein 4	*mtaC4*
Msp0022	Ketol-acid reductoisomerase		Msp0819	Methanol:cobalamin methyltransferase 4	*mtaB4*
Msp0072	Pyridoxine biosynthesis protein Pdx1	*pdx1*	Msp0865	30S ribosomal protein S9P	*rps9p*
Msp0079	Hypothetical protein[b]		Msp0866	50S ribosomal protein L13P	*rpl13p*
Msp0106	30S ribosomal protein S17e	*rps17e*	Msp0869	30S ribosomal protein S11P	*rps11p*
Msp0114	Thermosome, subunit beta	*thsB*	Msp0870	30S ribosomal protein S4P	*rps4p*
Msp0117	Predicted 3-hydroxy-3-methylglutaryl-CoA synthase		Msp0871	30S ribosomal protein S13P	*rps13p*
			Msp0880	50S ribosomal protein L14e	*rpl14e*
Msp0122	Archaeal histone		Msp0882	50S ribosomal protein L34e	*rpl34e*
Msp0130	Thermosome, subunit alpha	*thsA*	Msp0886	50S ribosomal protein L15P	*rpl15p*
Msp0147	Ferredoxin		Msp0887	50S ribosomal protein L30P	*rpl30p*
Msp0182	Methylcobalamin:coenzyme M methyltransferase 4	*mtaA4*	Msp0888	30S ribosomal protein S5P	*rps5p*
			Msp0894	30S ribosomal protein S14P	*rps14p*
Msp0184	Methanol:cobalamin methyltransferase 3	*mtaB3*	Msp0895	50S ribosomal protein L5P	*rpl5p*
Msp0185	Methanol corrinoid protein 3	*mtaC3*	Msp0896	30S ribosomal protein S4e	*rps4e*
Msp0186	Methanol:cobalamin methyltransferase 2	*mtaB2*	Msp0898	50S ribosomal protein L14P	*rpl14p*
Msp0187	Methanol corrinoid protein 2	*mtaC2*	Msp0903	30S ribosomal protein S3P	*rps3p*
Msp0188	Methanol:cobalamin methyltransferase 1	*mtaB1*	Msp0906	50S ribosomal protein L2P	*rpl2p*
Msp0189	Methanol corrinoid protein 1	*mtaC1*	Msp0908	50S ribosomal protein L1e	*rpl1e*
Msp0198	DNA/RNA-binding protein AlbA	*albA*	Msp0960	Predicted ABC-type polar amino acid transport system, periplasmic substrate-binding protein	
Msp0229	Putative preprotein translocase, subunit SecG	*secG*			
Msp0234	Glutamine synthetase				
Msp0251	Putative thiamine biosynthesis protein 2	*thiC2*	Msp0233	Conserved hypothetical protein, encoded in insertion element	
Msp0314	Non-F_{420}-reducing hydrogenase, subunit delta	*mvhD*			
Msp0315	Non-F_{420}-reducing hydrogenase, subunit gamma	*mvhG*	Msp1002	Cysteine desulfurase	*iscS*
Msp0316	Non-F_{420}-reducing hydrogenase, subunit alpha	*mvhA*	Msp1013	Heterodisulfide reductase, subunit B1	*hdrB1*
Msp0318	Methyl-coenzyme M reductase II, subunit B	*mrtB*	Msp1014	Heterodisulfide reductase, subunit C1	*hdrC1*
Msp0320	Methyl-coenzyme M reductase II, subunit G	*mrtG*	Msp1061	Hypothetical protein, related to ferredoxin	
Msp0321	Methyl-coenzyme M reductase II, subunit A	*mrtA*	Msp1134	A_1A_0 ATPase, subunit B	*ahaB*
Msp0325	Predicted peptidyl-prolyl *cis-trans* isomerase 2		Msp1135	A_1A_0 ATPase, subunit A	*ahaA*
Msp0327	50S ribosomal protein L10e	*rpl10e*	Msp1136	A_1A_0 ATPase, subunit F	*ahaF*
Msp0383	Archaeal histone		Msp1139	A_1A_0 ATPase, subunit K	*ahaK*
Msp0409	Conserved hypothetical protein[c]		Msp1141	A_1A_0 ATPase, subunit H	*ahaH*
Msp0444	Rubredoxin		Msp1145	D-3-Phosphoglycerate dehydrogenase	*serA*
Msp0471	Conserved hypothetical protein, encoded in insertion element		Msp1158	Predicted Zn ribbon RNA-binding protein	
			Msp1174	50S ribosomal protein L37e	*rpl37e*
Msp0477	Predicted type I restriction-modification system, restriction subunit		Msp1243	50S ribosomal protein L15e	*rpl15e*
			Msp1253	50S ribosomal protein L37Ae	*rpl37ae*
Msp0478	Hypothetical protein, related to type I restriction-modification system subunit		Msp1256	Partially conserved hypothetical protein, related to prefoldin, subunit beta	
Msp0479	Putative type I restriction-modification system, methyltransferase subunit		Msp1263	50S ribosomal protein L12P	*rpl12p*
			Msp1280	30S ribosomal protein S8	*rps8e*
Msp0518	Archaeal histone		Msp1292	50S ribosomal protein L40e	*rpl41e*
Msp0565	Predicted M42 glutamyl aminopeptidase		Msp1297	30S ribosomal protein S3Ae	*rps30ae*
Msp0594	30S ribosomal protein S19e	*rps19e*	Msp1352	Translation initiation factor 5A (aIF-5A)	*eif5a*
Msp0597	50S ribosomal protein L31e	*rpl31e*	Msp1366	Translation elongation factor 1-alpha (EF-Tu)	*tuf*
Msp0599	50S ribosomal protein LX	*rplX*	Msp1368	30S ribosomal protein S7P	*rps7p*
Msp0600	Conserved hypothetical protein, related to prefoldin, subunit alpha		Msp1369	30S ribosomal protein S12P	*rps12p*
			Msp1416	Glutamate dehydrogenase	*gdhA*
Msp0620	30S ribosomal protein S27Ae	*rps27ae*	Msp1449	Energy-converting hydrogenase B, subunit I	*ehbI*
Msp0628	30S ribosomal protein S6e	*rps6e*	Msp1450	Energy-converting hydrogenase B, subunit H	*ehbH*
Msp0631	50S ribosomal protein L24e	*rpl24e*	Msp1457	Energy-converting hydrogenase B, subunit A	*ehbA*
Msp0632	30S ribosomal protein S28e	*rps28e*	Msp1476	Heterodisulfide reductase, subunit A1	*hdrA1*
Msp0633	50S ribosomal protein L7Ae	*rpl7ae*	Msp1517	Chaperone protein DnaK	*dnaK*
Msp0719	Partially conserved hypothetical protein, related to AAA ATPase		Msp1528	30S ribosomal protein S27e	*rps27e*
			Msp1529	50S ribosomal protein L44e	*rpl44e*
Msp0729	Hypothetical protein, related to nucleic acid-binding protein		Msp1542	50S ribosomal protein L21e	*rpl21e*

[a] Putative highly expressed CDS were obtained by codon usage analysis with the SIGI tool (59).
[b] Additional hypothetical proteins are encoded by Msp0238, Msp0330, Msp0464, Msp0707, Msp0827, Msp1081, Msp1422, and Msp1498.
[c] Additional conserved hypothetical proteins are encoded by Msp0425, Msp0677, and Msp1566.

gene, Msp0190, that codes for a protein with sequence similarity with RamA. The iron-sulfur protein RamA has been shown to be involved in ATP-dependent reductive activation of the methylamine methyltransferase corrinoid protein in *M.*

barkeri (T. Ferguson, T. Lienard, G. Gottschalk, and J. Krzycki, unpublished results). There is no indication that any of these polypeptides are membrane associated and could therefore be involved in proton or sodium ion translocation. Cells of

M. stadtmanae grown on methanol and H_2 in the laboratory were found to contain relatively high concentrations (5%) of MtaBC isoenzymes 2 and 3 and of MtaA isoenzyme 4, as revealed by the N-terminal sequences of purified MtaBC and MtaA (see Materials and Methods). Consistently, the codon usage of the corresponding genes was that of highly expressed genes (Table 3). The MtaBC complex contained 1 mol of 5-hydroxybenzimidazolyl cobamide (factor III) per mol MtaC.

Alignments of the four deduced amino acid sequences encoded by *mtaBC* revealed that the isoenzymes MtaB3C3 and MtaB4C4 are more closely related (>99% sequence identity) than the other isoenzymes (<96%). All four isoenzymes are more closely related to each other than to any of the MtaBC isoenzymes in *Methanosarcina* species (<50%). This relationship indicates that the four MtaBC isoenzymes in *M. stadtmanae* are the result of three gene duplications, two of which occurred more recently. The finding that of the cytochrome-free methanogens only *M. stadtmanae* contains *mtaBC* homologues suggests that a set of *mtaBC* genes was acquired by lateral gene transfer from *Methanosarcina* species. However, this must have occurred a long time ago, as indicated by the low level of sequence identity of MtaBC from *M. stadtmanae* with MtaBC from *Methanosarcina* and by the different order of the genes in the *mta* operons of *M. stadtmanae* (*mtaBC*) and *Methanosarcina* (*mtaCB*). Alignment of the deduced amino acid sequences encoded by *mtaA* revealed a different picture. The four MtaA isoenzymes of *M. stadtmanae* are related to each other (<60%) almost as closely as they are related to the MtaA isoenzymes of *Methanosarcina* (<54%).

The possibility that in *M. stadtmanae* methyl-coenzyme M formation from methanol and coenzyme M could also proceed via methyl-H_4MPT as an intermediate involving a methanol:H_4MPT methyltransferase (MtxXAH) and a methyl-H_4MPT:coenzyme M methyltransferase (MtrABCDEFGH) was considered. The genomes of *Methanosarcina* species harbor the *mtxXAH* genes, which have been proposed to code for a corrinoid-containing enzyme complex that catalyzes the formation of methyl-H_4MPT from methanol and H_4MPT and to be involved in methanol oxidation to CO_2 (32). However, *mtxXAH* homologues are not found in the genome of *M. stadtmanae*. Consistently, in *M. stadtmanae* only 10% of the methyl group of methionine, which is biosynthetically derived from the methyl group of methyl-H_4MPT, is synthesized from methanol. The other 90% is derived from C-2 of acetate via the hydroxymethyl group of serine or from C-1 of 3-hexulose-6-phosphate, as revealed by labeling experiments (62) (Fig. 2). The 10% labeling of the methyl group of methionine by methanol can be explained by the presence of a methyl-H_4MPT:coenzyme M methyltransferase (MtrA-H). The genome of *M. stadtmanae* contains the *mtrEDCBAFGH* gene cluster (Msp0300 to Msp0307), which is predicted to code for the sodium ion-translocating methyl-tetrahydromethanopterin:coenzyme M methyltransferase complex, which like MtaABC is a corrinoid protein. So far, the *mtr* genes have been found in every methanogenic archaeon investigated but not in any other organism. During growth of methanogens on H_2 and CO_2 the Mtr complex catalyzes the exergonic transfer of the methyl group of methyl-H_4MPT to coenzyme M ($\Delta G^{o'} = -30$ kJ/mol), a step in CO_2 reduction to methane, which is coupled to energy conservation. During growth of *Methanosarcina* on methanol the Mtr complex is involved in methanol oxidation to CO_2, and the methyl transfer from methyl-coenzyme M to H_4MPT is driven by the electrochemical sodium ion potential (29). Since *M. stadtmanae* cannot reduce CO_2 to methane or oxidize methanol to CO_2 and synthesizes the methyl group of methionine mainly from C-2 of acetate (Fig. 2), this methanogen probably does not really require the enzyme. Consistently, the specific activity of the Mtr complex in cell extracts of *M. stadtmanae* was found to be below the detection limit (98). However, there is evidence that in *M. stadtmanae* formaldehyde can be reduced with H_2 to methane, which probably proceeds via methylene-H_4MPT, methyl-H_4MPT, and methyl-CoM as intermediates (51), indicating the presence of Mtr complex activity. Mtr complex might not be essential in *M. stadtmanae*. This is indicated by the recent finding that *mtrABCDEFGH* deletion mutants of *M. barkeri* can grow on methanol and H_2 when the medium is supplemented with acetate (100).

Another pathway from methanol to methyl-coenzyme M that was considered starts with the oxidation of methanol to formaldehyde, which spontaneously reacts with H_4MPT to form methylene-H_4MPT. The latter compound is then reduced to methyl-H_4MPT, from which the methyl group is transferred to coenzyme M. In the genome of *M. stadtmanae*, genes for F_{420}-dependent methylene-H_4MPT reductase (Mer) (Msp1128) and (as mentioned above) for methyl-H_4MPT:coenzyme M methyltransferase (Mtr) are found. A gene for a formaldehyde-activating enzyme (Fae) (Msp1498) that catalyzes the formation of methylene-H_4MPT from formaldehyde and H_4MPT (Fig. 2) is also present in the genome. However, a CDS for a formaldehyde dehydrogenase is not apparent. The finding from labeling experiments that only 10% of the methyl group of methionine (62) is synthesized from methanol again indicates that, if present, this pathway has only minor importance. This is even more true as methanol is always contaminated by small amounts of formaldehyde generated from methanol by autoxidation, which could also explain the 10% labeling by methanol.

(ii) Methyl-coenzyme M reduction with coenzyme B to CH$_4$ and CoM-S-S-CoB. In all methanogens that have been investigated for this characteristic methane is generated by reduction of methyl-coenzyme M with coenzyme B (reaction 2). The reaction is catalyzed by methyl-coenzyme M reductase (Mcr or Mrt), which is composed of three subunits, subunits A, B, and G, each of which is present twice. Two moles of nickel porphinoid F_{430} per mol of heterohexamer is tightly but noncovalently bound. Five of the amino acid residues in McrA are post- or cotranslationally modified, and these amino acid positions are highly conserved. Some methanogens contain two methyl-coenzyme M isoenzymes, McrABG and MrtABG, which are formed under different growth conditions. The CDS for the three Mcr or Mrt subunits A, B, and G are organized in transcription units, which generally contain one or two additional open reading frames, subunits C and D, whose functions are unknown (95).

The genome of *M. stadtmanae* was found to harbor a transcription unit, *mrtBDGA* (Msp0318 to Msp0321), and separately an *mcrC* homologue (Msp0299). The *mvhDGA* cluster encoding the non-F_{420}-reducing hydrogenase involved in reduction of methanol to methane with H_2 is located directly upstream of the *mrtBDGA* cluster (see below). The sequence

of *mrtA* is conserved with respect to five posttranslationally modified amino acids, glycine 447, glutamine 402, arginine 274, cysteine 454, and histidine 260. Genes encoding a second methyl-coenzyme M reductase were not found. Based on the deduced amino acid sequence, one of the three subunits (MrtB) is predicted to contain at least one hydrophopic segment that could form a transmembrane helix. Similar predictions have been made for the β subunits (McrB or MrtB) of methyl-coenzyme M reductases from other methanogens. The crystal structures of methyl-coenzyme M reductases from several methanogens have revealed that the hydrophobic segment of the β subunit is buried within the $\alpha_2\beta_2\gamma_2$ complex and that the surface of the complex is completely hydrophilic (22, 30). There is no indication from the biochemical properties of methyl-coenzyme M reductases that the enzyme could be membrane associated and involved in proton or sodium ion translocation.

(iii) CoM-S-S-CoB reduction to coenzyme M and coenzyme B. In methanogens without cytochromes the heterodisulfide reductase complex is generally composed of the subunits HdrA, HdrB, and HdrC. HdrA is an iron-sulfur flavoprotein, and HdrB and HdrC are iron-sulfur proteins. The enzyme contains an active site iron-sulfur cluster located on subunit HdrB (19, 56). The genes coding for the three subunits are organized into two transcription units, *hdrA* and *hdrCB* (36). The physiological electron donor of HdrABC appears to be the iron-sulfur protein MvhD of the MvhADG complex (non-F_{420}-reducing hydrogenase) (see below). HdrB exhibits sequence similarity to subunit GlpC of glycerol phosphate dehydrogenase from *Escherichia coli* (36) and to subunit SdhC of succinate dehydrogenases from archaea belonging to the order *Sulfolobales* (50), which are integral membrane proteins.

The genome of *M. stadtmanae* was found to contain two sets of *hdrA*, *hdrB*, and *hdrC* genes, one set organized in an *hdrC2B2A2* transcription unit (Msp0125 to Msp0127) and the other set organized in two transcription units, *hdrC1B1* (Msp1014 and Msp1013) and *hdrA1* (Msp1476). The HdrABC complex purified from *M. stadtmanae* grown under laboratory conditions is the product of the *hdrC1B1* and *hdrA1* genes, as revealed by the N-terminal sequences (see Materials and Methods). The genome of *M. stadtmanae* lacks a CDS for HdrE (cytochrome *b*), which until now has been found only in *Methanosarcina* species. The *hdrA2* gene in the *hdrA2C2B2* cluster in *M. stadtmanae* is similar to an *hdrA* homologue found in the genome of *Methanosarcina* species since it is extended at the 3′ end by a sequence with similarity to *mvhD* on the protein level. The fusion of the two genes is considered an indication that HdrA and MvhD are also functionally associated in electron transfer from H_2 to CoM-S-S-CoB (91). Another interesting aspect is that in *Methanosarcina* the order of the genes in the putative transcription unit is *hdrACB*, whereas in *M. stadtmanae* it is *hdrC2B2A2*.

(iv) Hydrogen activation for the reduction of CoM-S-S-CoB. Methanogens without cytochromes, which with the exception of *M. stadtmanae* can grow on H_2 and CO_2 as sole energy sources, generally contain three different [NiFe] hydrogenases: a cytoplasmic F_{420}-reducing hydrogenase (FrhABG), a cytoplasmic non-F_{420}-reducing hydrogenase (MvhADG) (92), and a membrane-associated energy-converting hydrogenase (Eha and/or Ehb), which uses ferredoxin as an electron acceptor (33). So far, the

energy-converting hydrogenase has been studied only in *M. barkeri*, in which the homologous enzyme (designated Ech) was shown to be involved in CO_2 reduction to formylmethanofuran (energy metabolism), in reductive carboxylation reactions (e.g., pyruvate synthesis from acetyl-CoA), and in CO_2 reduction to CO (CO_2 assimilation) (60).

FrhABG is involved in methenyl-H_4MPT reduction to methylene-H_4MPT and in methylene-H_4MPT reduction to methyl-H_4MPT (CO_2 reduction to methane), as well as in NADP reduction (biosynthesis). MvhADG appears to be involved only in CoM-S-S-CoB reduction. Some of the methanogens without cytochromes also contain an H_2-forming methylene-H_4MPT dehydrogenase (Hmd), which is a nickel- and iron-sulfur-cluster-free hydrogenase with a function in CO_2 reduction to methane (54).

In the genome of *M. stadtmanae* CDS for three different hydrogenases are found: *frhADGB* (Msp1302 to Msp1305), *mvhDGA* (Msp0314 to Msp0316), and *ehbABCDEFGHIJKLMNOPQ* (Msp1457 to Msp1442 and Msp1436). There is also a second *mvhD* homologue (Msp0638), which is located at the 3′ end of two genes encoding a formate dehydrogenase (*fdhAB*; Msp0640 and Msp0639) and a third gene fused at the 5′ end to *hdrA2* in the *hdrB2C2A2* cluster. The genome lacks CDS for Eha and the nickel-free hydrogenase Hmd. As indicated below, in *M. stadtmanae* the Ehb and Frh hydrogenases probably have a purely anabolic function, Ehb in pyruvate synthesis from acetyl-CoA and Frh in NADP reduction (Fig. 2). Therefore, only the non-F_{420}-reducing hydrogenase Mvh can be involved in methanol reduction to methane. Consistent with a function of Mvh in energy metabolism is the finding that the codon usage of the *mvhDGA* genes rather than that of the *frhADGB* and *ehbA-Q* genes is the codon usage of other highly expressed genes (Table 3).

The non-F_{420}-reducing hydrogenase was purified from *M. stadtmanae* and identified as the *mvhDGA* gene product by N-terminal sequence comparisons (75). From the sequence of the *mvhDGA* genes it was deduced that MvhA is the [NiFe] center-harboring subunit and MvhD and MvhG are iron-sulfur proteins. There is no indication that in *M. stadtmanae* the non-F_{420}-reducing hydrogenase is a membrane-associated enzyme (no predicted transmembrane helices) or is an extracytoplasmic enzyme (no 5′ twin-arginine motif).

In the genomes of most methanogens without cytochromes a *mvhB* gene is present at the 3′ end of the *mvhDGA* transcription unit. MvhB was shown to code for a polyferredoxin whose function is still not known (34, 74). A homologue of *mvhB* was not found in the genome of *M. stadtmanae*. An *mvhB* homologue is also not present in the genome of *M. kandleri*.

(v) ATP synthesis. Methanogenic archaea generally contain a proton-translocating ATPase of the A_1A_0 type composed of 9 or 10 different subunits and encoded by an *ahaHIKECFABD* transcription unit (67). Such a gene cluster is also found in the genome of *M. stadtmanae* (Msp1141 to Msp1133). Based on codon usage the AhaA, AhaB, AhaF, AhaH, and AhaK subunits are predicted to be highly expressed (Table 3), which is consistent with a function of the ATPase in energy metabolism. From the primary structure it was deduced that AhaK is the hydrophobic proteolipid directly involved in proton translocation. AhaK is predicted to contain four transmembrane helices.

(vi) Energy conservation. The genome analysis and biochemical studies revealed that in *M. stadtmanae* only five enzyme complexes are involved in methanol reduction to methane with H_2, a reaction that is coupled to ADP phosphorylation, as shown by growth of the methanogen on H_2 and methanol as sole energy sources. The five complexes are MtaABC, MrtABG, HdrABC, MvhADG, and AhaABCDEFHIK. Transmembrane prediction methods predict that three of these complexes (the Mta, Mvh, and Hdr complexes) are located in the cytoplasm, that the AhaCKI subunits of the A_1A_o complex are membrane integrated, and that the MtrABG complex could be membrane associated via the MrtB subunit. However, as outlined above, the hydrophobic part of MrtB is buried within the enzyme complex rather than exposed to its surface, which is completely hydrophilic. Therefore, methyl-coenzyme M reductases have to be considered cytoplasmic enzymes. Conversely, HdrABC is most probably a membrane-associated enzyme, although none of its three subunits are predicted to contain transmembrane helices. The complex is proposed to be anchored via its HdrB subunit to the membrane, a conclusion based mainly on the observation that SdhC (CAA06782) (50), a protein with 31% sequence identity to HdrB, is a membrane protein and that transmembrane helices are not unequivocally predicted for this protein. In *Methanothermobacter marburgensis* HdrABC and MvhADG have been shown to form a tight complex partially associated with the cytoplasmic membrane (82, 92). In the complex HdrB harbors the active site for CoM-S-S-CoB reduction and MvhA harbors the active site for H_2 oxidation. Electron transfer has been proposed to occur between the MvhD and HdrA subunits (89) based on the finding that in the genomes of *Methanosarcina* and *M. stadtmanae* there is an *hdrA* homologue that is fused to an *mvhD* homologue (see above). The proposed electron transfer from MvhD to HdrA remains to be shown experimentally. With cell suspensions of *M. stadtmanae* it has been shown that methanol reduction with H_2 is coupled to the buildup of an electrochemical proton potential which drives the phosphorylation of ADP (87), most probably via the Aha complex. All the results together suggest that in *M. stadtmanae* the reduction of CoM-S-S-CoB with H_2 is coupled principally with proton translocation, as shown in Fig. 3. In the topology model of the HdrABC/MvhADG complex the anchor function of HdrB is speculative.

The growth yield of *M. stadtmanae* on H_2 and methanol in complex media was found to be approximately 4 g/mol methane (64), and the yield of *M. barkeri* on H_2 and methanol was found to be 4.6 g/mol methane (68). Coupling of methanol reduction to methane with energy conservation thus proceeds in these two organisms with almost identical efficiencies.

(vii) 5-Hydroxybenzimidazolyl cobamide biosynthesis. The corrinoid group is the prosthetic group of the methyltransferase (MtaABC) that catalyzes reaction 1. *M. stadtmanae* has an unusually high 5-hydroxybenzimidazolyl cobamide content, 4 nmol/mg protein; for comparison, *M. marburgensis* has a cobamide content of 0.3 nmol/mg protein. In the genome of *M. stadtmanae* all the genes required for synthesis of the corrin ring from eight glutamate residues (79) were found, which is consistent with the report that growth of the organism is not dependent on vitamin B_{12} (64). The finding that the genome of *M. stadtmanae* appears to lack a gene for a cobalamin-dependent ribonucleotide reductase was unexpected. Only a CDS for a glycyl radical ribonucleotide reductase (ribonucleotide re-
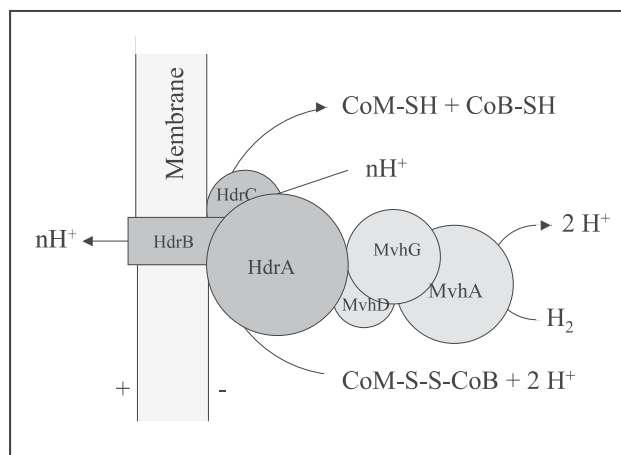

FIG. 3. Topological model of the HdrABC/MvhADG complex catalyzing the reduction of CoM-S-S-CoB with H_2 in a proton-translocating reaction in *M. stadtmanae*. In the model it is assumed that based on indirect evidence, HdrB is an integral membrane protein, although this is not reflected in its primary structure (see text).

ductase III) is present (Msp0254). In the genome of *M. thermoautotrophicus* genes for both a glycyl radical enzyme and a B_{12}-dependent enzyme are found.

(viii) Coenzyme F_{430} biosynthesis. The nickel porphinoid group is the prosthetic group of methyl-coenzyme M reductase that catalyzes reaction 2. *M. stadtmanae* contains approximately 0.3 nmol F_{430} per mg protein. The biosynthesis of F_{430} has not been elucidated yet. Biosynthesis branches off from the cobalamin biosynthesis pathway at the level of precorrin 2 (71). Since all methanogens synthesize F_{430} and only methanogens synthesize F_{430}, the genes involved in F_{430} biosynthesis must be among the only approximately 40 CDS found in all methanogens and not in any other archaea (data not shown). These CDS include genes for the subunits of methyl-coenzyme M reductase (Mcr and/or Mrt), for methyl-H_4MPT:coenzyme M methyltransferase (Mtr), and for several conserved hypothetical proteins. Some of the latter proteins could be involved in F_{430} biosynthesis and the posttranslational modification and activation of methyl-coenzyme M reductase. Conserved clusters of more than one CDS present in all methanogens could not be identified.
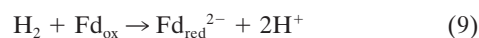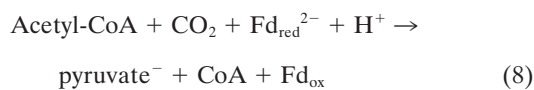
Inability of *M. stadtmanae* to use other methanogenic substrates. Besides the genes encoding the enzymes that catalyze reactions 1 to 5, the genome of *M. stadtmanae* also contains CDS for all the enzymes required for the reduction of CO_2 to methane and for the oxidation of methanol to CO_2 (Fig. 2), as follows: formylmethanofuran dehydrogenase (FwdABCDG) (Msp0241 to Msp0245), which is a molybdopterin-dependent enzyme (FwdB has a putative molybdopterin cofactor binding motif); two formylmethanofuran:H_4MPT formyltransferases (Ftr) (Msp0070 and Msp1502); methenyl-H_4MPT cyclohydrolase (Mch) (Msp1238); F_{420}-dependent methylene-H_4MPT dehydrogenase (Mtd) (Msp0163); F_{420}-dependent methylene-H_4MPT reductase (Mer); and methyl-H_4MPT:coenzyme M methyltransferase (MtrABCDEFGH) (see above). In cell extracts of *M. stadtmanae* only very low activities of these enzymes have been detected (between 20 and 40 mU/mg) (80, 98). As mentioned above, the genome lacks genes for the

biosynthesis of molybdopterin (Table 2), indicating that *M. stadtmanae* cannot synthesize active formylmethanofuran dehydrogenase. Cell suspensions of the organism were shown to slowly oxidize [^{14}C]formaldehyde to $^{14}CO_2$, most probably via methylene-H$_4$MPT and formate (51), which is consistent with the absence of formylmethanofuran dehydrogenase activity. However, low specific activities (40 mU/mg) of this enzyme have been reported to be present in cell extracts of *M. stadtmanae* (80). Therefore, the possibility that *M. stadtmanae* might be able to take up molybdopterin present at trace levels in the yeast extract and/or rumen fluid, which is generally a component of the complex growth medium, has to be considered. It has been reported that methanofuran is absent in *M. stadtmanae* (98); however, the method employed was not sensitive enough to completely exclude the possibility that this coenzyme was present. In this respect it is of interest that in the genome of *M. stadtmanae* a CDS for a tyrosine decarboxylase (*mfnA*; Msp0329) involved in methanofuran biosynthesis (47) has been found. Nonetheless, together the findings can explain why the methanogen cannot reduce CO_2 to methane or oxidize methanol to CO_2.

It is interesting with respect to the function of formylmethanofuran dehydrogenase that the enzyme from *Methylobacterium extorquens* AM1, which lacks a molybdopterin binding site, catalyzes the hydrolysis rather than the dehydrogenation of formylmethanofuran (72). The function of formylmethanofuran dehydrogenase in *M. stadtmanae* could therefore be to catalyze the formation of formate, which is required for the synthesis of C-2 of purines and as an electron donor for ribonucleotide reduction (Fig. 2). It is also noteworthy that the sequences of the subunits of formylmethanofuran dehydrogenase from *M. stadtmanae* are most similar to those of the tungsten-containing isoenzyme present in many methanogens (95). There is one peculiarity: subunits A and G are most closely related to FwdA and FwdG from *M. thermoautotrophicus*, subunits C and D are most closely related to FwdC and FwdD from *M. jannaschii* and *M. maripaludis*, respectively, and subunit B, which harbors the molybdopterin binding site, is most closely related to FwdB from *M. kandleri* (99). The reason for this phylogenetic mixture is not apparent.

As mentioned above, the genome of *M. stadtmanae* lacks genes required for the synthesis of the nickel-containing carbon monoxide dehydrogenase/acetyl-CoA decarbonylase complex (Table 2), which in methanogens is either involved in methanogenesis from acetate or in acetyl-CoA synthesis from CO_2 and a methyl group (95). This finding could explain why *M. stadtmanae* cannot use acetate as a methanogenic substrate and why this archaeon is dependent on acetate as a carbon source for growth.

Carbon assimilation. *M. stadtmanae* is dependent on acetate and CO_2 as main carbon sources for growth. Also, this organism appears to be auxotrophic for isoleucine or leucine and for thiamine (64). Labeling studies have revealed that the methyl group of methionine and positions 2 and 8 of the purines are mainly derived from C-2 of acetate (4, 10, 11, 51, 62) (Fig. 2). The labeling pattern in amino acids is consistent with assimilation of acetate via pyruvate involving reactions 6 to 9.

$$\text{Acetate} + \text{CoA} + \text{ATP} \rightarrow \text{acetyl-CoA} + \text{AMP} + \text{PP}_i \quad (6)$$

$$PP_i + H_2O \rightarrow 2P_i \quad (7)$$

$$\text{Acetyl-CoA} + CO_2 + Fd_{red}^{2-} + H^+ \rightarrow$$
$$\text{pyruvate}^- + \text{CoA} + Fd_{ox} \quad (8)$$

$$H_2 + Fd_{ox} \rightarrow Fd_{red}^{2-} + 2H^+ \quad (9)$$

where Fd is a [4Fe-4S]$_2$-containing ferredoxin assumed to have a redox potential identical to that of the acetyl-CoA + CO_2/pyruvate couple ($E^{\circ\prime} = -500$ mV). All other cell compounds are synthesized from acetyl-CoA and pyruvate.

(i) Pyruvate synthesis from acetate. The genome of *M. stadtmanae* harbors CDS for two predicted acyl-CoA synthetases (AMP forming) (Msp0266 and Msp0447), one inorganic pyrophosphatase (*ppa*; Msp0625), one predicted acyl-CoA synthethase (ADP forming) (Msp0919), one pyruvate:ferredoxin oxidoreductase (PorABCD) (Msp0334 to Msp0337), five ferredoxins with [4Fe-4S]$_2$ (Msp0147, Msp0236, Msp0237, Msp0768, and Msp0861), and one energy-converting hydrogenase (Ehb) (see above). The presence of these gene homologues is consistent with the hypothesis that reactions 6 to 9 are involved in pyruvate synthesis from acetate and CO_2. Gene homologues for acetate kinase and phosphotransacetylase were not found.

The reductive carboxylation of acetyl-CoA to pyruvate ($E^{\circ\prime} = -500$ mV) with H$_2$ ($E^{\circ\prime} = -420$ mV) as the electron donor (reactions 8 and 9) is an endergonic process, which can proceed only when it is pushed by the proton motive force or the sodium motive force. In some methanogens the reductive carboxylation of acetyl-CoA to pyruvate can be coupled to the exergonic oxidation of formylmethanofuran to CO_2 ($E^{\circ\prime} = -530$ mV) or the exergonic oxidation of CO to CO_2 ($E^{\circ\prime} = -520$ mV) (91). In *M. stadtmanae* this is not possible since the organism lacks an active formylmethanofuran dehydrogenase (Fmd) and a CO dehydrogenase (Cdh). However, the genome does contain CDS for an energy-converting hydrogenase, Ehb, which therefore is proposed to catalyze reaction 9. As deduced from the sequence, subunit N (Msp1444) of Ehb harbors the active site [NiFe] center. Interestingly, the *ehbABCDEFGHIJKLMNOPQ* cluster is interrupted between *ehbP* and *ehbQ* by two short open reading frames predicted to encode the iron transport proteins FeoA and FeoB (Msp1441 and Msp1440).

The energy-converting hydrogenase Ehb in *M. stadtmanae* is also required for other reductive carboxylations. The genome contains genes for the synthesis of 2-oxoglutarate:ferredoxin oxidoreductase (Kor) (*korDABC*; Msp1390 to Msp1387) in a conserved cluster together with the gene encoding subunit beta of succinyl-CoA synthetase (*sucC*; Msp1386), indicating that *M. stadtmanae* can generate 2-oxoglutarate by reductive carboxylation of succinyl-CoA. This organism also contains indolepyruvate:ferredoxin oxidoreductase (Ior) (*iorAB*; Msp1043 and Msp1042), indicating that it can assimilate indolacetate and phenylacetate by reductive carboxylation. Genes for 2-oxoisovalerate:ferredoxin oxidoreductase (Vor) present in the genomes of other methanogens were not found (94). Vor is involved in the assimilation of branched-chain fatty acids. Consistent with the absence of *vor* genes in the genome of *M. stadtmanae* is the finding that growth of this intestinal organism appears not to be dependent on the presence of branched-chain fatty acids (64).

(ii) Serine biosynthesis from pyruvate. In the genome of *M. stadtmanae* CDS for pyruvate carboxylase (*pycB*; Msp1173), phosphoenolpyruvate synthase (*ppsA*; Msp0328), enolase (*eno*; Msp0862), two phosphoglycerate mutases (*apgM1* or Msp0485 and *apgM2* or Msp1299), phosphoglycerate dehydrogenase (*serA*; Msp1145), and phosphoserine phosphatase (Msp1096) were found, indicating that serine is synthesized from pyruvate via phosphoenolpyruvate, 2-phosphoglycerate, 3-phosphoglycerate, 3-phosphohydroxypyruvate, and 3-phosphoserine as intermediates (Fig. 2). A CDS for a phosphoserine transaminase appears to be absent. The phosphoserine phosphatase gene, however, contains a long 3′ extension, indicating that in *M. stadtmanae* phosphoserine phosphatase could be a bifunctional enzyme. CDS for pyruvate kinase, pyruvate phosphate dikinase, phosphoenolpyruvate carboxykinase, and phosphoenolpyruvate carboxylase were not detected.

(iii) C_1 unit biosynthesis from serine. A CDS for serine:H_4MPT hydroxymethyl transferase (*glyA*; Msp1475) is present in the genome of *M. stadtmanae*, indicating that serine reacts with H_4MPT, generating methylene-H_4MPT and glycine (Fig. 2). H_4MPT-dependent hydroxymethyltransferase activity was found in cell extracts of *M. stadtmanae* (52). Methylene-H_4MPT is required for thymidylate synthesis, for synthesis of the methyl group of methionine via methyl-H_4MPT, and for generation of formate. The latter compound could be formed via methenyl-H_4MPT, formyl-H_4MPT, and formylmethanofuran, as described previously for formate synthesis in *M. extorquens* (72). In *M. stadtmanae* formate is required for synthesis of C-2 of purines (10) and as an electron donor for the glycyl radical enzyme ribonucleotide reductase (20, 53). C-8 of purines is labeled by C-2 of acetate even in the presence of formate via an unknown pathway (10). The genome harbors genes for thymidylate synthase (*thyA*; Msp1237) and methionine synthase (*metE*; Msp1236), but a convincing CDS for the enzyme PurT, which in other methanogens catalyzes the ATP-dependent incorporation of formate into C-2 and C-8 of purines, was not found. The only possible homologue present is carbamoyl phosphate synthetase (*carAB*; Msp1201 and Msp1200). The genome also does not contain gene homologues for PurN and PurH, which could catalyze the transfer of the formyl group of N^{10}-formyltetrahydrofolate to positions 2 and 8 of purines. This is in agreement with the findings that *M. stadtmanae* does not contain tetrahydrofolate and that for thermodynamic reasons N^5-formyl-H_4MPT cannot substitute for N^{10}-formyltetrahydrofolate in the two formyltransferase reactions (8). The genome contains a CDS for a molybdopterin-dependent formate dehydrogenase. However, because of the lack of molybdopterin biosynthetic genes, this enzyme should not be functional, although low NAD(P)-dependent formate dehydrogenase activity has been reported for cell extracts of *M. stadtmanae* (51). Also, cell suspensions of the organism were shown to slowly oxidize [^{14}C]formate to $^{14}CO_2$ (51). This is another indication that *M. stadtmanae* might be able to take up molybdopterin present at trace levels in the yeast extract and/or rumen fluid present in the complex growth medium (see above). Growth of *M. stadtmanae* is not dependent on the presence of formate (64), indicating that this archaeon must somehow be able to synthesize this C_1 unit.

Synthesis of C_1 units from C-2 of acetate, as indicated by labeling studies, does not necessarily have to proceed via C-3 of serine (Fig. 2). In methanogenic archaea 3-hexulose-6-phosphate is the precursor of pentose phosphates required for RNA and DNA biosynthesis (28). From hexulose-6-phosphate ribulose-5-phosphate and formaldehyde are formed in a reaction catalyzed by hexulose phosphate synthase (Hps). The formaldehyde is derived from C-1 of the hexulose phosphate, which in turn is derived from C-2 of acetate via C-3 of pyruvate, C-3 of dihydroxyacetone phosphate, C-1 of fructose bisphosphate, and C-1 of fructose-6-phosphate. The formaldehyde reacts with H_4MPT to form methylene-H_4MPT, a reaction catalyzed by the formaldehyde-activating enzyme (Fae) (Fig. 2). All the genes required for operation of this pathway are present in the genome of *M. stadtmanae*. Interestingly, as in other methanogens, the *fae* gene and the *hps* gene encoding hexulose phosphate synthase are fused (Msp1498).

(iv) F_{420}-dependent enzymes. In the genome of *M. stadtmanae* CDS for the following F_{420}-dependent enzymes are present: F_{420}-reducing hydrogenase (FrhABG); F_{420}-dependent methylene-H_4MPT dehydrogenase (Mtd); F_{420}-dependent methylene-H_4MPT reductase (Mer) (see above); $F_{420}H_2$:NADP oxidoreductase (Fno) (Msp0665); and $F_{420}H_2$ oxidase (FprA) (Msp0787) (Fig. 2). F_{420}-reducing hydrogenase activity was not detectable in cell extracts of *M. stadtmanae*, indicating that the *frhADBG* genes were not expressed or were expressed at only low levels in the organism. The *frhD* gene is predicted to code for a protease involved in hydrogenase maturation. *M. stadtmanae* contains relatively low concentrations of F_{420} (0.16 nmol/mg protein, compared to 6 nmol/mg in *M. marburgensis*) (103), indicating that F_{420}-dependent reactions are of minor importance quantitatively, which is in agreement with the finding that F_{420}-dependent methylene-H_4MPT dehydrogenase (Mtd) and F_{420}-dependent methylene-H_4MPT reductase (Mer) are required only for biosynthetic purposes. Likewise, $F_{420}H_2$:NADP oxidoreductase has only an anabolic function, the regeneration of NADPH (Fno) and $F_{420}H_2$ oxidase (FprA), and a function in the detoxification of O_2, the reduction of O_2 to $2H_2O$ (81). Fno from *M. stadtmanae* has been purified and characterized (21).

(v) Thiamine and isoleucine/leucine auxotrophy. In the genome of *M. stadtmanae* at least one CDS required for thiamine biosynthesis appears to be missing (Table 2). However, all of the genes for biosynthesis of leucine and isoleucine were found. The amino acid auxotrophy of this organism is not understood at this time. With respect to the dependence of growth on leucine or isoleucine, it is interesting that *M. stadtmanae* appears to lack a CDS for 2-oxoisovalerate:ferredoxin oxidoreductase (Vor), which in *M. marburgensis* has been shown to exhibit 2-oxoisohexanoate:ferredoxin oxidoreductase activity (94). Therefore, the organism should not be able (as a back-up) to synthesize the two branched-chain amino acids from 2-methylbutyrate and 3-methylbutyrate present in the volatile fatty acid fraction of the rumen fluid generally added to the growth medium of this intestinal inhabitant.

***M. stadtmanae*-specific genes.** Comparisons of whole-genome protein sequences revealed that the genome of *M. stadtmanae* contains 323 CDS without genes in the genomes of other archaea (data not shown). Of these, 73 CDS exhibit high levels of overall homology to genes encoding proteins of bacteria and eukaryotes (Table 4). These 73 CDS include 12 CDS which are predicted to code for a group of large proteins with a conserved N-terminal leader sequence and a repetitive structure

TABLE 4. CDS in the genome of *M. stadtmanae* with close gene homologues only in nonarchaeal genomes[a]

CDS[b]	Function	Length (bp)	Accession no.	Organism
Large CDS with a repetitive structure				
Msp0043	Member of Asn/Thr-rich large protein family	2,451	ZP_00356360	*Chloroflexus aurantiacus*
Msp0046	Member of Asn/Thr-rich large protein family	2,646	CAF23662	*Parachlamydia* sp. strain UWE25
Msp0141	Member of Asn/Thr-rich large protein family	6,363	BAB33965	*Escherichia coli* O157:H7
			YP_189939	*Staphylococcus epidermidis* RP62A
Msp0432	Member of Asn/Thr-rich large protein family	6,135	NP_703779	*Plasmodium falciparum* 3D7 (E)[c]
Msp0590	Member of Asn/Thr-rich large protein family	8,940	CAF23662	*Parachlamydia* sp. strain UWE25
			CAG36249	*Desulfotalea psychrophila* LSv54
Msp0713	Member of Asn/Thr-rich large protein family	6,456	ZP_00356360	*Chloroflexus aurantiacus*
Msp0762	Member of Asn/Thr-rich large protein family	10,071	CAF23662	*Parachlamydia* sp. strain UWE25
			NP_522741	*Ralstonia solanacearum* GMI1000
Msp0765	Member of Asn/Thr-rich large protein family	3,669	ZP_00519573	*Solibacter usitatus* Ellin6076
			CAF23662	*Parachlamydia* sp. strain UWE25
Msp0983	Member of Asn/Thr-rich large protein family	3,936	ZP_00356360	*Chloroflexus aurantiacus*
			CAD71661	*Rhodopirellula baltica* SH 1
Msp1103	Member of Asn/Thr-rich large protein family	2,574	ZP_00356360	*Chloroflexus aurantiacus*
Msp1108	Member of Asn/Thr-rich large protein family	5,766	CAF23662	*Parachlamydia* sp. strain UWE25
			ZP_00525480	*Solibacter usitatus* Ellin6076
Msp1465	Member of Asn/Thr-rich large protein family	6,645	AAG54838	*Escherichia coli* O157:H7 strain EDL933
			CAG76164	*Erwinia carotovora* subsp. *atroseptica* SCRI1043
CDS involved in bacterial surface antigen biosynthesis				
Msp0039	Predicted glycosyltransferase	1,098	AAK80269	*Clostridium acetobutylicum* ATCC 824
Msp0203	Predicted glycosyltransferase	1,959	ZP_00327237	*Trichodesmium erythraeum* IMS101
			ZP_00270340	*Rhodospirillum rubrum*
Msp0207	Predicted glycosyltransferase	870	AAR99614	*Geobacillus stearothermophilus*
			AAK80277	*Clostridium acetobutylicum* ATCC 824
Msp0212	Predicted glycosyltransferase	2,667	AAP76661	*Helicobacter hepaticus* ATCC 51449
			YP_130861	*Photobacterium profundum* SS9
Msp0215	Predicted glycosyltransferase	2,589	YP_086549	*Bacillus cereus* E33L
			YP_022183	*Bacillus anthracis* Ames Ancestor
Msp0220	Predicted glycosyltransferase	2,748	NP_964416	*Lactobacillus johnsonii* NCC 533
			NP_391310	*Bacillus subtilis* subsp. *subtilis* 168
Msp0441	Predicted glycosyltransferase	1,185	ZP_00388322	*Streptococcus thermophilus* LMD-9
			BAB80207	*Clostridium perfringens* 13
Msp0442	Predicted glycosyltransferase	1,077	NP_816137	*Enterococcus faecalis* V583
			BAB80207	*Clostridium perfringens* 13
Msp0495	Predicted glycosyltransferase	2,628	CAB73858	*Campylobacter jejuni* subsp. *jejuni* NCTC 11168
			ZP_00143779	*Fusobacterium nucleatum* subsp. *vincentii* ATCC 49256
Msp0500	Predicted glycosyltransferase	2,211	ZP_00327237	*Trichodesmium erythraeum* IMS101
			ZP_00270340	*Rhodospirillum rubrum*
Msp0581	Predicted UDP-*N*-acetylmuramyl tripeptide synthase	1,344	ZP_00504800	*Clostridium thermocellum* ATCC 27405
			AAO35161	*Clostridium tetani* E88
Msp0921	Putative poly(gamma-glutamate) biosynthesis protein, involved in capsule formation	1,215	YP_080918	*Bacillus licheniformis* ATCC 14580
			NP_391469	*Bacillus subtilis* subsp. *subtilis* 168
Msp1540	Partially conserved hypothetical protein, related to glycosyltransferase	2,910	ZP_00327237	*Trichodesmium erythraeum* IMS101
			ZP_00270340	*Rhodospirillum rubrum*
CDS for restriction-modification systems				
Msp0454	Partially conserved hypothetical protein, related to type I restriction-modification system, restriction subunit	1,350	CAE10219	*Wolinella succinogenes*
			CAH07535	*Bacteroides fragilis* NCTC 9343
Msp0470	Partially conserved hypothetical protein, related to type III restriction-modification system, methylation subunit	1,182	ZP_00504330	*Clostridium thermocellum* ATCC 27405
			ZP_00531584	*Chlorobium phaeobacteroides* BS1
Msp0472	Partially conserved hypothetical protein, related to type III restriction-modification system, methylation subunit	768	ZP_00504330	*Clostridium thermocellum* ATCC 27405
			AAL94619	*Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586
Msp0473	Type III restriction-modification system, restriction subunit	2,988	ZP_00504329	*Clostridium thermocellum* ATCC 27405
			ZP_00143311	*Fusobacterium nucleatum* subsp. *vincentii* ATCC 49256

TABLE 4—*Continued*

CDS[b]	Function	Length (bp)	Accession no.	Organism
Msp0480	Putative type I restriction-modification subunit, specificity subunit	1,218	YP_185367	*Staphylococcus aureus* subsp. *aureus* COL
			AAP11371	*Bacillus cereus* ATCC 14579
Remaining CDS				
Msp0059	Conserved hypothetical protein[b]	618	ZP_00288946	*Magnetococcus* sp. strain MC-1
Msp0064	Predicted acetyltransferase	609	AAO75579	*Bacteroides thetaiotaomicron* VPI-5482
			ZP_00423781	*Burkholderia vietnamiensis* G4
Msp0093	Predicted flavoprotein	1,242	AAO35881	*Clostridium tetani* E88
			ZP_00510807	*Clostridium thermocellum* ATCC 27405
Msp0128	Predicted helicase	2,196	ZP_00357761	*Chloroflexus aurantiacus*
Msp0140	Predicted 3′-5′ exonuclease	1,338	BAB81107	*Clostridium perfringens* 13
			NP_691978	*Oceanobacillus iheyensis* HTE831
Msp0408	Putative nitroreductase protein	507	CAH08994	*Bacteroides fragilis* NCTC 9343
			NP_661026	*Chlorobium tepidum* TLS
Msp0528	Putative gamma-glutamylcysteine synthetase	1,404	YP_015348	*Listeria monocytogenes* 4b F2365
			NP_466292	*Listeria monocytogenes* EGD-e
Msp0551	Putative RNase HIII	930	NP_078232	*Ureaplasma parvum* serovar 3 strain ATCC 700970
Msp0580	Predicted glutamine amidotransferase	723	AAO35160	*Clostridium tetani* E88
			NP_621720	*Thermoanaerobacter tengcongensis* MB4
Msp0591	Predicted carbonic anhydrase	468	AAT41924	*Fremyella diplosiphon*
			YP_113395	*Methylococcus capsulatus* Bath
Msp0688	Putative secreted RNAse barnase	450	AAB29635	*Bacillus circulans*
			YP_080806	*Bacillus licheniformis* ATCC 14580
Msp0697	Predicted methyladenine glycosylase	555	CAB44429	*Bifidobacterium-longum* biovar Longum
			YP_053518	*Mesoplasma florum* L1
Msp0700	Predicted phosphatase	609	CAH06545	*Bacteroides fragilis* NCTC 9343
			AAO79289	*Bacteroides thetaiotaomicron* VPI-5482
Msp0760	Predicted penicillin V acylase, choloylglycine hydrolase family	981	AAP20760	*Enterococcus faecium*
			NP_465591	*Listeria monocytogenes* EGD-e
Msp0764	Predicted nicotinate phosphoribosyltransferase	1,416	CAB65409	*Suberites domuncula* (E)
			AAH89296	*Xenopus laevis* (E)
Msp0781	D-Tyrosyl-tRNA(Tyr) deacylase (EC 3.1.–.–)	447	AAL94553	*Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586
			NP_622822	*Thermoanaerobacter tengcongensis* MB4
Msp0797	Predicted nitroreductase	558	AAL95419	*Fusobacterium nucleatum* subsp. *nucleatum* ATCC 25586
Msp0837	Predicted glutamylcysteine synthetase	1,413	ZP_00404497	*Streptococcus pneumoniae* TIGR4
			AAN59602	*Streptococcus mutans* UA159
Msp1165	Predicted hydrolase	891	AAB68787	*Fervidobacterium islandicum*
			NP_952175	*Geobacter sulfurreducens* PCA

[a] All CDS of *M. stadtmanae* encoding more than 100 amino acids were compared by BLAST analysis with all CDS of the genomes of all other archaea sequenced so far ($<e^{-20}$). CDS without BLAST hits in this group were compared with the NCBI protein database ($<e^{-20}$) (ftp.ncbi.nih.gov/blast/db/nr.tar.gz). All CDS with significant hits in this database are listed.

[b] Additional conserved hypothetical proteins are encoded by Msp0219, Msp0332, Msp0419, Msp0421, Msp0465, Msp0467, Msp0468, Msp0469, Msp0529, Msp0530, Msp0534, Msp0679, Msp0681, Msp0734, Msp0803, Msp0845, Msp0941, Msp1113, Msp1235, Msp1344, Msp1385, Msp1474, Msp1489, and Msp1555.

[c] E, eukaryote.

(Fig. 4), 13 CDS which code for enzymes involved in the biosynthesis of glycosylated cell wall components in bacteria, and 5 CDS which are predicted to code for three subunits of a putative type III restriction-modification (R-M) system and for two subunits of a type I R-M system.

(i) Large CDS with repetitive structure. The 12 large CDS (>2,400 bp and up to 10,071 bp) identified in the whole-genome comparisons are members of a group of 37 CDS found in the genome of *M. stadtmanae* that share conserved structural features. With the exception of a single CDS (Msp1348) which exhibits partial homology to a CDS from the genome of *M. thermautotrophicus*, the 26 CDS in this group do not have close homologues either in the archaeal domain or in the two other domains. All 37 CDS have an N-terminal leader sequence, which is usually 3 to 8 amino acids long, followed by a

predicted single transmembrane helix consisting of 18 to 21 amino acids and at least one copy of a conserved sequence pattern at the C terminus (Fig. 4). Repetitive peptide sequences that are about 100 amino acids long, whose amino acid compositions and numbers of repeats differ, seem to be a common structural feature of all proteins encoded by the large CDS. In these proteins the amino acids asparagine and threonine are overrepresented by a factor of two. Matches with known signal sequences could not be found. As the large CDS have no counterparts in other archaea sequenced so far but their products exhibit weak fragmentary similarity to proteins with unknown functions from different pathogenic bacteria, functions in the commensal lifestyle are assumed. With respect to the predominance of asparagine and threonine in the proteins encoded by the large CDS, it is interesting that the ge-
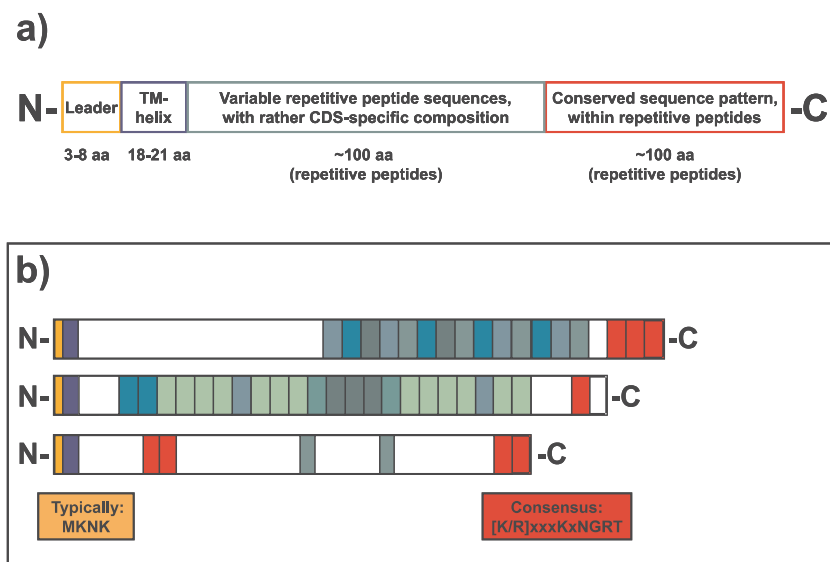
a)

b)

FIG. 4. Schematic representation of the 37 putative proteins encoded by the group of large CDS with a repetitive structure in *M. stadtmanae*. (a) General composition shared by all proteins of this group, including an N-terminal leader sequence (yellow) followed by a predicted transmembrane (TM) helix (blue) and by a variable number of repeated peptide sequences (green and red). (b) Schematic composition of the three largest proteins of this group (Msp0762 protein, 3,357 amino acids [aa]; Msp0568 protein, 2,980 aa; Msp0597 protein, 2,469 aa). Two of these, the Msp0568 and Msp0597 proteins, are unique to *M. stadtmanae*; only the Msp0762 protein exhibits homology to bacterial proteins (Table 4). The short leader sequence is Lys and Asn rich, typically having the form MKNK. At least one copy of a short sequence motif is found at the C terminus of all proteins in this group [G(K/R)XXXKXNGRT]. Sequence variations of repetitive peptide sequences are indicated by green. Repetitive peptides of different large CDS do not necessarily have the same sequence.

nome contains one CDS encoding a predicted protein composed of 91 amino acids with 14 tandem repeats having the form Asn-Asn-Ser-Ser (Msp0474).

(ii) CDS involved in bacterial surface antigen biosynthesis. The genome of *M. stadtmanae* codes for 38 putative glycosyltransferases involved in the biosynthesis of glycosylated cell wall components. Among these glycosyltransferases are 11 proteins that exhibit high levels of homology to bacterial enzymes predicted to be involved in surface antigen biosynthesis. Furthermore, a predicted UDP-*N*-acetylmuramyl tripeptide synthase (Msp0581) and a putative poly(gamma-glutamate) biosynthesis protein involved in capsule formation in *Bacillus* species (Msp0921) indicate that many CDS identified as specific for *M. stadtmanae* among archaea have functions in the biosynthesis of glycosylated cell wall components. These components could represent an adaptation of this organism to the immunologically highly active intestinal growth habitat. The cell wall of *M. stadtmanae* is composed of pseudomurein as the major polymer. The pseudomurein appears to be unique with respect to the presence of serine. An S-layer was not found (42).

(iii) CDS for restriction-modification systems. Quite unusual for archaea, the genome of *M. stadtmanae* harbors CDS for three putative subunits of a type III restriction-modification system (Msp0470, Msp0472, and Msp0473), which are widely distributed among bacteria, and two additional putative subunits of a type I R-M system (Msp0454 and Msp0480). The function of the R-M systems is to protect the organism from nonhost DNA. Additionally, an effect of DNA methylation on gene regulation in bacterial pathogens has been established (41).

Conclusion. Analysis of the genome of *M. stadtmanae* in relation to the well-studied physiology and biochemistry of this organism allowed us to correlate the genotype with the phenotype of this methanogenic archaeon. Thus, the genetic data can explain why *M. stadtmanae* is restricted to H_2 and methanol as energy sources and is dependent on acetate as a carbon source. Both the CDS found and the CDS not found compared to other methanogenic archaea helped to identify the proteins involved in methanol reduction to methane with H_2, to predict how these proteins topologically interact, and to locate the site of energy conservation in subunit B of the HdrABC complex. For methanogens without cytochromes this has not been possible previously. In this respect our decision to determine the genome sequence of *M. stadtmanae* was rewarding. However, this was also true in other respects. The genome of the organism was found to harbor a number of CDS not present in the genome of any other archaeon sequenced so far. Many of these CDS are predicted to be involved in cell surface antigen synthesis. It is tempting to speculate that specific components of the archaeal cell wall are part of the adaptation of *M. stadtmanae* to its special habitat, the human large intestine. Lateral gene transfer between this archaeon and bacteria could be involved, as has been suggested for *M. mazei* and bacteria for another habitat (15). Such adaptations could result in phenotypic variability due to expression and glycosylation of special cell wall components. Similar mechanisms have been found in pathogenic bacteria that use them to avoid the human innate immune response. Large, tandem repeating units of surface proteins have been identified in group B streptococci and other gram-negative pathogens (61, 69). In the gram-negative bacterial pathogen *Anaplasma marginale* variation of cell wall struc-

tures is achieved by recombinatorial gene conversions between whole pseudogenes and between small segments within these genes (6). In analogy to *A. marginale*, short repetitive sequences within the large CDS of the *M. stadtmanae* genome could serve as homologous sites for segmental recombination. In *M. stadtmanae* proteins analogous to enzymes for the biosynthesis of cell wall components in gram-negative and gram-positive bacteria could serve as the machinery to glycosylate proteins encoded by the group of large CDS.

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