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27. Draining lymph nodes were harvested and frozen in liquid nitrogen in O.C.T. embedding medium (Miles). Tissue sections (10 μm) were cut on a Cryostat microtome, fixed in acetone, and blocked with goat serum. Sections were stained sequentially with biotinylated KJ1-26, anti-IgM^a, or peanut agglutinin; avidin-biotin complex–labeled alkaline phosphatase; and 5-bromo-4-chloro-3-indolyl phosphate/nitroblue tetrazolium substrate, followed by biotinylated anti-B220, anti-Thy 1.2, or KJ1-26; avidin-biotin complex–labeled peroxidase; and 3,3′-diaminobenzidine substrate (Vector). Development of the first avidin-biotin complex–labeled enzyme destroyed residual avidin binding sites on the primary antibodies, thereby preventing artifactual binding of the second avidin-biotin complex–labeled enzyme. The weak background anti-IgM^a staining mentioned in (*10*) was not detected on tissue sections and thus has no bearing on the immunohistology experiments.
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C_1 Transfer Enzymes and Coenzymes Linking Methylotrophic Bacteria and Methanogenic Archaea

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Methanogenic and sulfate-reducing Archaea are considered to have an energy metabolism involving C_1 transfer coenzymes and enzymes unique for this group of strictly anaerobic microorganisms. An aerobic methylotrophic bacterium, *Methylobacterium extorquens* AM1, was found to contain a cluster of genes that are predicted to encode some of these enzymes and was shown to contain two of the enzyme activities and one of the methanogenic coenzymes. Insertion mutants were all unable to grow on C_1 compounds, suggesting that the archaeal enzymes function in aerobic C_1 metabolism. Thus, methylotrophy and methanogenesis involve common genes that cross the bacterial/archaeal boundaries.

Methylobacterium strains are obligately aerobic bacteria capable of growing on one-carbon (C_1) compounds (methylotrophs) (*1*). They contain a well-known pathway for interconverting C_1 compounds that involves a folate coenzyme, tetrahydrofolate (H_4F), and nicotinamide adenine dinucleotide phosphate [NAD(P)] (Fig. 1A) (*1–3*). It has been postulated that in *Methylobacterium* strains this pathway operates in the oxidative direction to convert formaldehyde to CO_2, generating reduced pyridine nucleotides and serving as a major energy-generating pathway during growth on C_1 compounds (*2*, *3*). The strictly anaerobic Archaea that produce methane (methanogens) or that grow on acetate by sulfate reduction (*Archaeoglobus*) carry out analogous reactions that use a different coenzyme, tetrahydromethanopterin (H_4MPT), and a furan-type cofactor, methanofuran (MFR), and involve either H_2 or the electron acceptor/donor F_{420} rather than NAD(P) (Fig. 1B). In the methanogenic Archaea, these reactions are part of the pathway that reduces CO_2 to methane, the central pathway for energy metabolism in methanogens (*4*). In the sulfate-reducing archaeon *Archaeoglobus*, the H_4MPT- and MFR-linked reactions shown in Fig. 1B operate in the oxidative direction, as part of the energy metabolism for utilizing acetate (*5*). Thus, the H_4MPT/MFR-linked enzymes have until now only been found in methanogenic and sulfate-reducing Archaea, whereas the H_4F-linked enzymes have been found in Bacteria, Eukarya, and some Archaea. This separation has suggested that the two sets of pathways are evolutionarily distinct, with the H_4MPT/MFR-linked pathways arising after the separation of the methanogenic and sulfate-reducing Archaea from other evolutionary branches.

One of the aerobic methylotrophs that has been well studied is the α-proteobacterium *Methylobacterium extorquens* AM1. This bacterium contains nine clusters of genes involved in growth on C_1 compounds (*6*). The largest of these is ~30 kb and contains the gene for NADP-dependent methylene H_4F dehydrogenase (*mtdA*) as well as a number of other methylotrophy genes (*6*). We recently cloned and sequenced a 14-kb region adjacent to one end of this 30-kb gene cluster (*7*) and found that it contains 13 new open reading frames (Fig. 2). The translated products of 12 of these are similar to those of genes in methanogens (*8*) or in *Archaeoglobus fulgidus* (*9*), or both, but not to any other entries in the databases, whereas the amino acid sequence translated from one (*orfX*) is similar to the sequence of *M. extorquens* AM1 MtdA but not to any archaeal polypeptide entries (percent identical amino acids is shown in Table 1). The products of six of these methanogen-like genes (*orfY*, *orf4*, *orf7*, *orf9*, *orf17*, and *orf18*) are similar to unidentified gene products in archaeal genome sequences. The other six are similar to the translated products for known genes of the H_4MPT/MFR pathway in methanogens and *A. fulgidus*, and to another archaeal protein (Fig. 1B and Table 1): FfsA is similar to formyl MFR:H_4MPT formyltransferase; OrfZ to methenyl H_4MPT cyclohydrolase; Orf1, Orf2, and Orf3 to three of the six subunits for formyl MFR dehydrogenase, including the catalytic (B) subunit (*10*); and Orf5 to ribosomal protein S6 modification protein (a methylase) in *Methanococcus jannaschii* (*8*). Alignments of the putative methenyl H_4MPT cyclohydrolase and formyl MFR:H_4MPT formyltransferase sequences with known sequences in the database reveal strong conservation of groups of amino acid residues between all of the sequences (Fig. 3) (*11*).

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Insertion mutations have been generated in the chromosomal copies of all newly identified genes by means of a kanamycin-resistant cassette and allelic exchange with the suicide vector pAYC61 (*12*) (Table 1). Mutants with insertions in *orfY*, *orf5*, and *orf18* were all double-crossover (exchange-type) insertions. These all grew normally on C_1 compounds and were not studied further. Mutants with insertions in the remaining 10 genes were all unable to grow on C_1 compounds but grew normally on succinate, suggesting that these genes were required for C_1 metabolism. Mutants with insertions in three unidentified genes (*orfX*, *orf4*, and *orf7*) were the result of double-crossover recombination events and had a straightforward genotype. However, double-crossover mutants were not obtained for five of the genes that show similarity to known genes (*ffsA*, *orfZ*, *orf1*, *orf2*, and *orf3*) as well as for two unidentified genes (*orf9* and *orf17*), even though 200 or more independent kanamycin-resistant colonies were screened for loss of the vector in each instance. Instead, all insertions in these genes were the result of single-crossover recombination events that produced both a mutated and a normal copy of the targeted genes, separated by vector sequences. We have previously observed a similar phenomenon for *mtdA* mutants, in which single-crossover insertion mutants exhibited a C_1-negative phenotype (*2*). In that instance, the single-crossover event apparently separated the normal gene copy from its native promoter, which resulted in reduced expression of the gene product in the mutant. This low-level constitutive activity was evidently sufficient for growth on succinate but not for growth on C_1 compounds (*2*). For representatives of the mutants described here, hybridization experiments and polymerase chain reaction (PCR) analysis involving specific primers (*13*) suggested that the vector separated the normal gene from upstream chromosomal sequences. Therefore, the C_1-negative phenotype of these mutants may reflect the need for greater expression of these genes during growth on C_1 compounds than on multicarbon compounds.

The inability to obtain null mutants in these genes might be due to polarity effects of the insertions on nearby essential genes. To test this possibility, we generated subclones containing specific genes for complementation studies, for each of the mutants that showed a C_1-negative phenotype (Fig. 2). In each instance these subclones complemented the appropriate mutant. These results demonstrate that the C_1-negative phenotypes were due to alterations in the mutated genes, and not due to polarity effects of the insertions on nearby genes.

Because in methanogens, the methenyl H_4MPT cyclohydrolase and the formyl MFR:H_4MPT formyltransferase are encoded by single genes and corresponding enzyme activities can be detected in *Escherichia coli* containing the methanogen genes

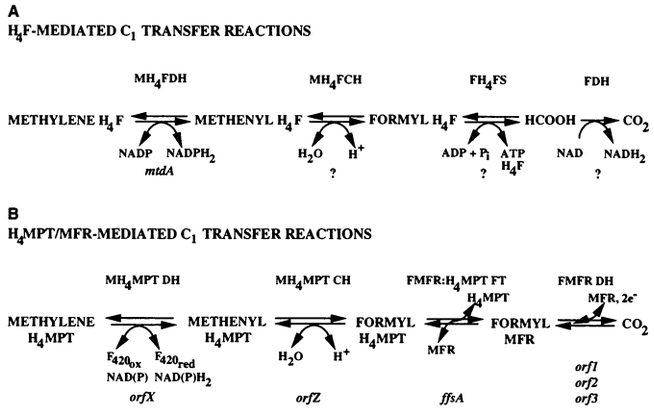

Fig. 1. (**A**) Tetrahydrofolate (H_4F)-mediated C_1 transfer pathway found in Bacteria, some Archaea, and Eukaryotes and (**B**) tetrahydromethanopterin/methanofuran (H_4MPT/MFR)–mediated C_1 transfer pathway found in methanogenic and sulfate-reducing Archaea. MH_4F DH, methylene H_4F dehydrogenase; MH_4MPT DH, methylene H_4MPT dehydrogenase; MH_4F CH, methenyl H_4F cyclohydrolase; MH_4MPT CH, methenyl H_4MPT cyclohydrolase; FH_4FS, formyl H_4F synthase; FMFR:H_4MPT FT, formyl MFR:H_4MPT formyltransferase; FDH, formate dehydrogenase; FMFR DH, formyl MFR dehydrogenase. The two alternative pathways are proposed to operate in *M. extorquens* AM1, except that MH_4MPT DH is NAD(P)-linked. The known genes of *M. extorquens* AM1 for both pathways are shown in italics.

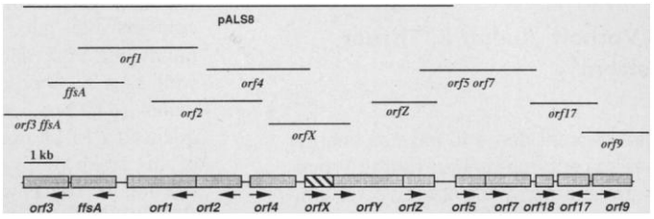

Fig. 2. (**Bottom**) Physical map of the DNA fragment described in this work. Shaded boxes represent putative genes whose translated products are similar to archaeal polypeptides (26 to 44% identical amino acids). Striped box represents a gene similar to another *M. extorquens* AM1 gene, *mtdA* (32% identical amino acids). Arrows show direction of transcription. (**Top**) DNA fragments cloned for complementation studies.

Table 1. Characteristics of insertion mutants described in this study.

Open reading frame	Double-crossover mutants	Growth on C_1 compounds	Percent of amino acid identity with best hit in*		Putative function of the best hit
			A. fulgidus	*M. jannaschii*	
orfY	Yes	Yes	28	19	Unidentified
orf5	Yes	Yes	None	31	Ribosomal protein S6 modification protein
orf18	Yes	Yes	49	32	Unidentified
orfX	Yes	No	None†	None†	Methylene H_4F dehydrogenase
orf4	Yes	No	36	31	Unidentified
orf7	Yes	No	30	26	Unidentified
ffsA	No	No	43	44	Formylmethanofuran: tetrahydromethanopterin formyltransferase
orfZ	No	No	39	40	Methenyl tetrahydromethanopterin cyclohydrolase
orf1	No	No	37	34	Formylmethanofuran dehydrogenase, subunit A
orf2	No	No	26	25	Formylmethanofuran dehydrogenase, subunit B
orf3	No	No	33	27	Formylmethanofuran dehydrogenase, subunit C
orf9	No	No	38	29	Unidentified
orf17	No	No	29	26	Unidentified

*Identities with two of the three archaea whose complete genome sequences are available are shown here. However, identities were also found with other archaeal counterparts present in current databases, and those were in the same range. †Thirty-two percent identity with methylene H_4F dehydrogenase from *M. extorquens* AM1.

(*14*), assays were carried out for these enzymes in both *M. extorquens* AM1 and in *E. coli* containing the *M. extorquens* AM1 genes (Table 2). The formyltransferase was assayed with MFR and H_4MPT isolated from the methanogen *Methanobacterium thermoautotrophicum* and the cyclohydrolase with methenyl H_4MPT isolated from the same methanogen (*15*). Activities similar to those found in methanogens (*16*) were present in *M. extorquens* AM1 grown on methanol, and lower activities were found in succinate-grown cells. *Methylobacterium extorquens* AM1 cells containing pALS8, a plasmid containing both *ffsA* (predicted to encode the formyltransferase) and *orfZ* (predicted to encode the cyclohydrolase), showed increased levels of the two enzyme activities. *Escherichia coli* containing the *orfZ* fragment (Fig. 2) transcribed by the *lac* promoter showed significant activities of the cyclohydrolase and no detectable formyltransferase. *Escherichia coli* containing the *ffsA* fragment (Fig. 2), also transcribed by the *lac* promoter, demonstrated formyltransferase activity and no cyclohydrolase activity. These results show that genes encoding these enzymes were present on the expected DNA fragments. These results also strongly suggest that the products of *ffsA* and *orfZ* in *M. extorquens* AM1 must be carrying out reactions with H_4MPT and MFR or with unknown cofactors that are readily interchangeable with H_4MPT and MFR. It seems likely that FfsA and OrfZ function in an oxidative pathway similar to the H_4F-linked pathway for formaldehyde oxidation (Fig. 1).

To substantiate these results we purified methenyl H_4MPT cyclohydrolase from *M. extorquens* AM1 and examined whether the methylotroph contains H_4MPT. Cell extracts of *M. extorquens* AM1 were found to catalyze the hydrolysis of both methenyl H_4MPT (3 U/mg) and methenyl H_4F (0.03 U/mg). The two activities were separated by chromatography on DEAE Sephacel and Q-Sepharose yielding a more than 100-fold purified methenyl H_4MPT cyclohydrolase preparation that did not catalyze the hydrolysis of methenyl H_4F. SDS–polyacrylamide gel electrophoresis revealed the presence of a 33-kD polypeptide. By determination of its mass and NH_2-terminal amino acid sequence, the polypeptide was identified as the *orfZ* gene product (*17*).

The cell extracts were also found to contain the coenzyme H_4MPT. Because of higher stability of the methenyl form (*18*), H_4MPT was first converted to methenyl H_4MPT (*19*), which was then purified by reversed-phase high-performance liquid chromatography (*5*). The compound obtained showed an ultraviolet (UV)/visible spectrum identical to that of methenyl

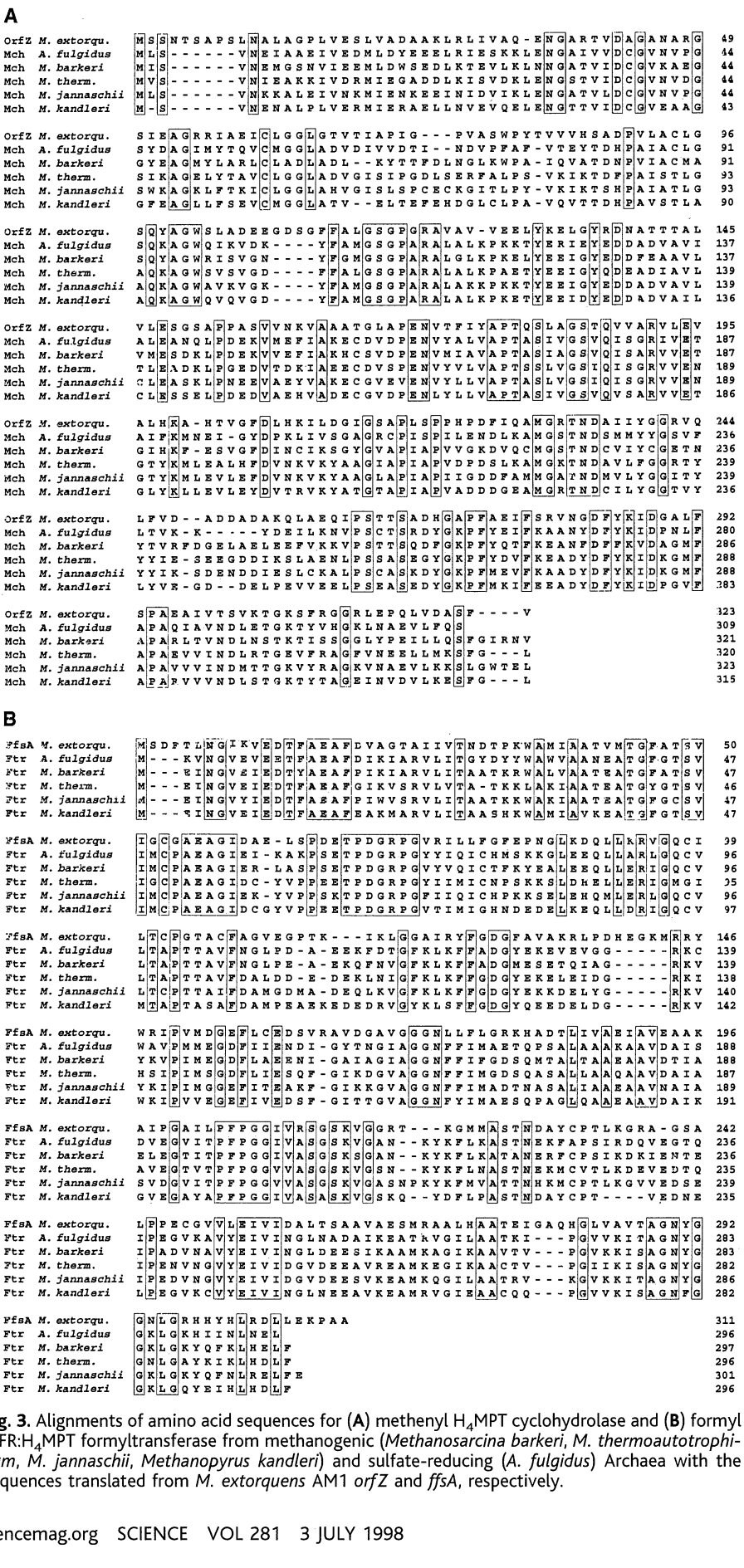

Fig. 3. Alignments of amino acid sequences for (**A**) methenyl H_4MPT cyclohydrolase and (**B**) formyl MFR:H_4MPT formyltransferase from methanogenic (*Methanosarcina barkeri*, *M. thermoautotrophicum*, *M. jannaschii*, *Methanopyrus kandleri*) and sulfate-reducing (*A. fulgidus*) Archaea with the sequences translated from *M. extorquens* AM1 *orfZ* and *ffsA*, respectively.

H_4MPT from *M. thermoautotrophicum* and significantly different from that of methenyl H_4F. Matrix-assisted laser desorption/ionization–time-of-flight mass spectrometry (MALDI/TOFMS) revealed that the compound had a mass of 577 daltons, identical to that of methenyl dephospho H_4MPT, a form of H_4MPT also present in some methanogens (*20*) and exhibiting the same UV/visible spectrum as H_4MPT (*21*). Using an extinction coefficient $\varepsilon_{335} = 21.6\ mM^{-1}\ cm^{-1}$ for methenyl H_4MPT (*18*), we estimated the amount of dephospho H_4MPT in *M. extorquens* AM1 to be 1.6 nmol per milligram of protein, equal to an intracellular concentration of 0.2 mM. This concentration is lower than in most methanogens (*22*) but is the same order of magnitude as in *Archaeoglobus* species (*23*).

The formyltransferase (FfsA) and the cyclohydrolase (OrfZ) are not the only two enzymes present in *M. extorquens* AM1 that use H_4MPT as coenzyme. Cell extracts of the methylotroph catalyze the dehydrogenation of methylene H_4MPT with NADP (7.3 U/mg) and NAD (0.6 U/mg) as electron acceptors. Two enzymes were separated by chromatography on Phenyl Sepharose and Q-Sepharose (*13*). The NADP-dependent enzyme did not show activity with NAD, but exhibited activity with both H_4MPT and H_4F. However, the H_4MPT-dependent specific activity of the NADP-specific enzyme was 10 times greater than the activity with H_4F. The other enzyme was specific for H_4MPT and showed highest activity with NAD. However, it also showed some activity with NADP. This NAD-specific enzyme appears to be encoded by *orfX*, one of the genes for which double crossover C_1-negative insertion mutants were obtained (Table 1) and the translated product of which is similar to MtdA. The NAD-dependent enzyme activity was below detection level in the *orfX* insertion mutant, and it was increased in *M. extorquens* AM1 carrying the corresponding gene in pALS8 (Table 2).

The results presented here indicate that the coenzyme H_4MPT, in a dephospho form, and H_4MPT-dependent enzymes previously thought to be unique to anaerobic methanogenic and sulfate-reducing Archaea are present and functionally required in the aerobic proteobacterium *M. extorquens* AM1. Our preliminary results show that these activities are also present in other methylotrophs (*13*). The methanogenic and sulfate-reducing Archaea and methylotrophic bacteria have in common an energy metabolism based on the interconversion of C_1 compounds. It may therefore not be unexpected that for the same catabolic interconversion they also use closely related coenzymes and enzymes, even with the large evolutionary distance between Archaea and Proteobacteria. The genes encoding these enzymes have either been conserved, because these organisms evolved from a common ancestor, or they have been transferred horizontally between more recent ancestors. The latter possibility is especially intriguing in light of the recent hypothesis that eukaryotes may have arisen as a result of a symbiotic association between a methanogenic archaeon and a proteobacterium (*24*). It is possible that the presence of these archaeal-like genes in Proteobacteria reflects processes that were involved in such an event. Therefore, the detailed functional analysis of all genes involved in C_1 metabolism in *M. extorquens* AM1 and other methylotrophic bacteria is of significant interest to understanding the evolutionary history of C_1 oxidation and reduction pathways in all organisms.

Table 2. Activities of tetrahydromethanopterin-linked enzymes in *M. extorquens* AM1, the double-crossover mutant ORFX, and *E. coli* transformed with *orfZ* or *ffsA*. Activities are shown in units per minute per milligram of protein and were determined with H_4MPT and MFR isolated from *M. thermoautotrophicum* as coenzymes. The activity of methylene H_4F dehydrogenase is given as a control.

Strain*	Growth and induction substrate†	Methenyl H_4MPT CH	FMFR: H_4MPT formyl transferase	Methylene H_4MPT DH (NAD)	Methylene H_4MPT DH (NADP)	Methylene H_4F DH (NADP)
AM1	Succinate	1.3	0.02	0.5	1.4	0.04
	Methanol	2.9	0.06	0.6	7.3	0.44
	Methanol-induced	1.3	0.03	0.5	1.8	0.07
AM1 (pALS8)	Methanol	14.5	0.2	4.2	7.7	0.42
ORFX	Succinate	0.6	ND	<0.01	0.7	0.03
	Methanol-induced	0.6	ND	<0.01	1.3	0.06
E. coli (orfZ)		14.5	<0.01	<0.01	<0.01	0.01
E. coli (ffsA)		<0.01	0.06	<0.01	<0.01	0.01

*AM1, wild-type *M. extorquens*, ORFX is a mutant defective in a putative gene for NAD-specific methylene H_4MPT dehydrogenase (*orfX*). pALS8 is a plasmid containing *ffsA*, *orfX*, and *orfZ* (Fig. 2). †Succinate: grown on succinate; methanol: grown on methanol; methanol-induced: grown on succinate, cells pelleted and then incubated with methanol for about 24 hours.

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7. Molecular cloning procedures were done as described by J. Sambrook *et al.* [*Molecular Cloning: A Laboratory Manual*, C. Nolan, Ed. (Cold Spring Harbor Laboratory Press, Cold Spring Harbor, NY, ed. 2, 1989)]. Sequencing was done by the Sequencing Facility of the Department of Biochemistry at the University of Washington. The sequence described here has been deposited with GenBank under accession numbers L43136 (*ffsA* and *orf3*) and AF032114 (the remainder).
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11. These are clearly authentic *M. extorquens* AM1 genes. No identity exists at the DNA level for these genes with any archaeal genes. In addition, the GC content (68%) and the codon usage for the new genes are in perfect agreement with the values known for *M. extorquens* AM1. Finally, the DNA region described here overlaps the region containing genes specific for aerobic methylotrophy not found in Archaea characterized to date or in the available archaeal genome sequences, and it is possible to isolate *M. extorquens* AM1 mutants in all of these genes by allelic exchange techniques.
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