XoxF-Type Methanol Dehydrogenase from the Anaerobic Methanotroph “Candidatus Methylomirabilis oxyfera”

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“Candidatus Methylomirabilis oxyfera” is a newly discovered anaerobic methanotroph that, surprisingly, oxidizes methane through an aerobic methane oxidation pathway. The second step in this aerobic pathway is the oxidation of methanol. In Gram-negative bacteria, the reaction is catalyzed by pyrroloquinoline quinone (PQQ)-dependent methanol dehydrogenase (MDH). The genome of “Ca. Methylomirabilis oxyfera” putatively encodes three different MDHs that are localized in one large gene cluster: one so-called MxaFI-type MDH and two XoxF-type MDHs (XoxF1 and XoxF2). MxaFI MDHs represent the canonical enzymes, which are composed of two PQQ-containing large (α) subunits (MxaF) and two small (β) subunits (MxaI). XoxF MDHs are novel, ecologically widespread, but poorly investigated types of MDHs that can be phylogenetically divided into at least five different clades. The XoxF MDHs described thus far are homodimeric proteins containing a large subunit only. Here, we purified a heterotetrameric MDH from “Ca. Methylomirabilis oxyfera” that consisted of two XoxF and two MxaI subunits. The enzyme was localized in the periplasm of “Ca. Methylomirabilis oxyfera” cells and catalyzed methanol oxidation with appreciable specific activity and affinity ($V_{\text{max}}$ of 10 μmol min$^{-1}$ mg$^{-1}$ protein, $K_m$ of 17 μM). PQQ was present as the prosthetic group, which has to be taken up from the environment since the known gene inventory required for the synthesis of this cofactor is lacking. The MDH from “Ca. Methylomirabilis oxyfera” is the first representative of type 1 XoxF proteins to be described.

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rations described to date lack this protein, even though the mxaF and mxaF genes are commonly linked to each other in genomes (8). The mxaACKLD gene products are essential for CaCl2 insertion into the apoprotein (19, 24), whereas the products of the mxaBSEH genes are thought to play a role in further MDH maturation (25–27). MxB is a DNA-binding protein involved in transcriptional regulation, as are the two-component regulators encoded by the mxb and mxc clusters (28). The gene products of the pqqABCDE and pqqEF clusters are involved in PQQ biosynthesis for which the 23- to 24-amino-acid (aa) gene product of pqqA is the proposed precursor of PQQ (29–31).

Genome sequencing projects revealed the presence of MxaF homologs, termed XoxF proteins, that showed, at most, 50% sequence identity to known MxaFs (32–34). XoxF genes can be detected in many genomes, not only of methanotrophic and methylo trophic species, but also of microorganisms that have not been implicated in such a life-style (8, 34). Phylogenetic analysis divides XoxF proteins into at least five different clades (XoxF1 to XoxF5) (8, 34). Despite their widespread occurrence, the function of XoxF proteins has been enigmatic for some time. Unlike mxaF, xoxF genes were hardly expressed during growth under laboratory conditions and their deletion did not result in a clear phenotype (32). In striking contrast, xoxF genes were highly expressed in the plant phyllosphere (35) and by communities of nutrient-limited coastal ocean waters (36). Certain methanotrophs, such as the Verruco microbia (37), lack genes coding for an MxaF MDH, and an XoxF protein would be the only candidate for methanol oxidation. In agreement with this, the deletion of xoxF from Rhodobacter sphaeroides resulted in the loss of this phototroph’s ability to use methanol for photorespiration and aerobic respiration (38). Moreover, the purification of XoxF proteins from different bacterial species established their function as MDHs (39–43). These studies also shed light on their elusive nature. The proteins were specifically induced in the presence of rare earth elements (REEs) like La3+ or Ce4+ (40–42). The recent resolution of the crystal structure of the XoxF MDH from the Verrucomicrobium phyllum member Methylicidiphilum fumariolicum SolV showed that this REE took the position near the PQQ catalytic site usually occupied by calcium in MxaF MDHs (43). Importantly, all of the XoxF MDHs described thus far are homodimeric proteins lacking a small subunit.

The genome of “Ca. Methyloirabilis oxyfera” codes for three MxaF homologs that have been annotated as MxaF1 to MxaF3 and that are encoded by one long gene cluster (2). However, key genes are commonly linked to each other in genomes (8, 34). Despite their widespread occurrence, the function of XoxF proteins has been enigmatic for some time. Unlike mxaF, xoxF genes were hardly expressed during growth under laboratory conditions and their deletion did not result in a clear phenotype (32). In striking contrast, xoxF genes were highly expressed in the plant phyllosphere (35) and by communities of nutrient-limited coastal ocean waters (36). Certain methanotrophs, such as the Verruc microbia (37), lack genes coding for an MxaF MDH, and an XoxF protein would be the only candidate for methanol oxidation. In agreement with this, the deletion of xoxF from Rhodobacter sphaeroides resulted in the loss of this phototroph’s ability to use methanol for photorespiration and aerobic respiration (38). Moreover, the purification of XoxF proteins from different bacterial species established their function as MDHs (39–43). These studies also shed light on their elusive nature. The proteins were specifically induced in the presence of rare earth elements (REEs) like La3+ or Ce4+ (40–42). The recent resolution of the crystal structure of the XoxF MDH from the Verrucomicrobium phyllum member Methylicidiphilum fumariolicum SolV showed that this REE took the position near the PQQ catalytic site usually occupied by calcium in MxaF MDHs (43). Importantly, all of the XoxF MDHs described thus far are homodimeric proteins lacking a small subunit.

The genome of “Ca. Methyloirabilis oxyfera” codes for three MxaF homologs that have been annotated as MxaF1 to MxaF3 and that are encoded by one long gene cluster (2). However, key genes known to be required for the biosynthesis of PQQ are absent from the genome of “Ca. Methyloirabilis oxyfera” (44). A closer analysis described in this paper affiliates these three “MxaFs” with an MxaF MDH and two different XoxF MDHs. To address the question of which of these proteins is functionally expressed, we purified MDH from the organism. Curiously, the only active MDH that could be isolated was a heterotetrameric enzyme composed of two XoxF1-type large subunits and two small subunits, and it contained PQQ as its prosthetic group. Using specific antibodies raised against the XoxF1 large subunit, we localized the enzyme to the periplasm of “Ca. Methyloirabilis oxyfera” cells.

MATERIALS AND METHODS

“Ca. Methyloirabilis oxyfera” enrichment culture. “Ca. Methyloirabilis oxyfera” strain Ooij was enriched from a ditch draining agricultural land in the Ooij polder, a floodplain of the River Rhine in The Netherlands, by the sequencing batch reactor tech-

“Ca. Methyloirabilis oxyfera” Methanol Dehydrogenase
sulfate (PMS)-mediated reduction of 2,6-dichlorophenol indophenol (DCPIP) at 600 nm ($e_{600}$ of 21,500 M$^{-1}$ cm$^{-1}$) essentially as described by Antony and Zatman (53) with 100 mM Tris-HCl (pH 8.0) as the buffer and increased concentrations of methanol (20 mM) and NH$_4$Cl (45 mM). Reaction mixtures (0.5 ml) contained 1 mM KCN to suppress the reoxidation of reduced DCPIP. Enzyme kinetic parameters were assessed by nonlinear regression analysis with the Origen 8.5.1 program (OrigenLab Corporation, Northampton, MA) by using Michaelis-Menten kinetics.

**Analytical ultracentrifugation.** Analytical equilibrium ultracentrifugation was performed at 20°C in a Beckman XL-I Proteomelab ultracentrifuge (Beckman Coulter, Fullerton, CA) equipped with an An-60 Ti rotor and cells with a 1.2-cm path length. Prior to ultracentrifugation, the protein was equilibrated in 25 mM HEPES-KOH buffer (pH 7.5) containing 25 mM KCl and 1 mM methanol and adjusted to an A$_{280}$ (1 cm) of 0.75. The speed used was 30,000 rpm. Equilibrium data were evaluated for 300 scans with the SEDFIT program (54) and assuming a fractional ratio ($eta_i$) of 1.05 for a slightly ellipsoid protein.

**UV–visible light absorption spectroscopy and ICP-MS.** The absorption spectrum of the purified enzyme was recorded in a cuvette with a 1-cm path at room temperature on a Cary 50 spectrophotometer (Agilent, Santa Clara, CA). The concentration and content of the PQQ prosthetic group were calculated on the basis of a molar absorption coefficient of 9,620 M$^{-1}$ cm$^{-1}$ (Agilent, Santa Clara, CA). The concentration and content of the PQQ prosthetic group were calculated on the basis of a molar absorption coefficient of 9,620 M$^{-1}$ cm$^{-1}$ (Agilent, Santa Clara, CA). The concentration and content of the PQQ prosthetic group were calculated on the basis of a molar absorption coefficient of 9,620 M$^{-1}$ cm$^{-1}$ (Agilent, Santa Clara, CA).

**Protein MS.** SDS-gel slices containing the purified MDH were subjected to tryptic in-gel digestion according to Wilm et al. (56). Peptides were extracted and prepared for matrix-assisted laser desorption ionization–time of flight (MALDI-TOF) MS as described by Farhoud et al. (57). MS of the tryptic digests was performed on a Bruker Biflex III spectrometer (Bruker Daltonics, Fremont, CA) operated in reflectron mode. Spectra (500 to 4,000 m/z) were analyzed with the Mascot Peptide Mass Fingerprint search program (Matrix Science, London, United Kingdom) against the "Ca. Methylo bacterium oxyfera" database, with oxidation (M) as variable modification, 0.2 Da peptide tolerance, and a maximum of one missed cleavage. Molecular masses of the native large and small subunits were determined by MALDI-TOF MS operating in the linear mode. These analyses of the as-isolated enzyme were performed by using the [M + H]$^+$, [2M + H]$^+$, [M + 2H]$^+$, and [M + 3H]$^+$ peaks for determination of molecular masses.

To verify the presence of PQQ, tandem MS was employed. A protein sample was analyzed by C$_3$s reversed-phase nanoflow liquid chromatography (Easy nano-LC; Proxeon, Thermo Fisher Scientific, Waltham, MA) coupled online via a nanoflow electrospray ionization source (Proxeon) to a 7T linear ion trap Fourier transform ion cyclotron resonance (ICR) mass spectrometer (LTQ FT Ultra; Thermo Fisher Scientific). Samples were loaded at a flow rate of 1.2 µl min$^{-1}$ directly onto the analytical column with 5% acetonitrile. After sample application, peptides and PQQ were eluted from the column with a 15-min linear gradient of 5 to 30% acetonitrile at a flow rate of 0.3 µl min$^{-1}$. The mass spectrometer was operated in negative-ion mode and optimized for PQQ detection by direct infusion of 1 µM PQQ (Sigma-Aldrich, St. Louis, MO) in 0.5% acetic acid. The ICR cell was programmed to acquire selected ion monitoring spectra of 314 to 344 m/z. The linear ion trap was set to acquire fragmentation scans of m/z 329 with the following parameters: 3E4 ions, a 3Th isolation width, 30% normalized collision energy, a 30-ms activation time, and an activation q of 0.25. Analysis of the MDH sample was performed first and followed by a blank run. Here, the PQQ standard was analyzed. This order was chosen to prevent carryover effects. A simulated (deprotonated) precursor ion spectrum of PQQ was generated with Thermo Scientific Qual browser software.

**Antiserum production.** Polyclonal antiserum against the "Ca. Methylo bacterium oxyfera" MDH large (α) subunit (α-XoxF1) was raised by injection of rabbits with the synthetic peptide NQYDPELRSGRWDNK (aa 317 to 331). This antiserum target region was selected on the basis of a unique protein surface peptide sequence deduced from BLAST and ClustalW analyses (see Fig. S1A in the supplemental material). Prior to immunization, an extra amino-terminal cysteine was added to the peptide sequence to enable conjugation to keyhole limpet hemocyanin (Eurogentec, Seraing, Belgium). Two rabbits were immunized by using a 3-month immunization protocol. The antisera from both rabbits were pooled and affinity purified (Eurogentec). This affinity-purified antiserum (α-XoxF1) was used as the primary antiserum for immunoblot analysis and immunogold labeling as described below.

**Antiserum specificity.** Antiserum specificity was tested by immunoblot analysis. "Ca. Methylo bacterium oxyfera" cell extracts (30 µg of protein per lane) and purified MDH (10 µg of protein per lane) were separated by SDS–10% PAGE and transferred to a Protran nitrocellulose membrane (Whatman plc, Maidstone, United Kingdom). Immunoblotting and testing of antiserum specificity were performed as described previously (58).

**Immunogold labeling and transmission electron microscopy.** Chemical fixation and gelatin embedding of cells from the "Ca. Methylo bacterium oxyfera" enrichment culture, cryosectioning, and subsequent immunogold labeling were done by established protocols (58, 59). The primary antiserum was diluted 50-fold in phosphate-buffered saline containing 1% BSA. Carbon-Formvar-coated grids (copper, hexagonal 100 mesh) containing ultrathin cryosections of "Ca. Methylo bacterium oxyfera" cells were investigated in a transmission electron microscope at 60 kV (JEOL 1010; JEOL Ltd., Akishima-Tokyo, Japan) operating under the iTEM software (Olympus Soft Imaging Solutions, Münster, Germany). Images were recorded with a charge-coupled device camera ( MegaView; Olympus Soft Imaging Solutions).

**RESULTS**

**Genomic organization of three MDH systems of "Ca. Methylo bacterium oxyfera."** The genome of "Ca. Methylo bacterium oxyfera" codes for three different MDH systems (MDH-1, MDH-2, and MDH-3) that are located on the same strand of a cluster of partly overlapping genes (DAMO_0112 to DAMO_0138) (Fig. 1). The cluster is made up of three subclusters. Each subcluster is preceded by genes encoding proteins (DAMO_0112, DAMO_0124, and DAMO_0134) showing 40 to 74% amino acid sequence identity both to each other and to the large subunits (MxaF, XoxF) of well-defined MxaF and XoxF MDHs (see next). In all three subclusters, genes coding for the putative MDH large subunits are linked to mxaF and mxaG homologs (termed xoxF and xoxG in the
case of XoxF systems). Herein, distinct MxaG/XoxG proteins with the typical CXCH motif for heme c binding would represent the cognate physiological electron acceptor for methanol oxidation, cytochrome c\textsubscript{t}. The function of the MxaI/XoxI-like proteins (DAMO\textsubscript{0113}, DAMO\textsubscript{0125}, DAMO\textsubscript{0136}) is not known, but sequence analysis identified it as a member of the family 3 extracellular solute-binding proteins (COG0834, pfam13533), suggesting a role in the binding of methanol or the release of the toxic reaction product formaldehyde. Only the MDH-1 subcluster harbors a gene (DAMO\textsubscript{0115}) coding for the small (b) subunit of canonical heterotetrameric MxaFI MDHs. Similarly, only the MDH-1 subcluster comprises the nearly complete set of genes coding for proteins involved in PQQ and \textit{Ca}\textsuperscript{2\textsuperscript{+}} insertion (\textit{mxa}-\textit{ACKLD}; DAMO\textsubscript{0118} to DAMO\textsubscript{0121}) and in MDH maturation (\textit{mxaRSE}; DAMO\textsubscript{0116} to DAMO\textsubscript{0117}, DAMO\textsubscript{0122}) (Fig. 1). In \textit{Ca. Methylomirabilis oxyfera}, the \textit{mxaL} and \textit{mxaD} genes are fused (DAMO\textsubscript{0121}). The gene order in the MDH-1 subcluster is the same as that found in \textit{M. extorquens} (19) and in Mxa systems in other organisms (8). However, homologs of \textit{mxaH} and the regulatory \textit{mxaB} are absent, which is not unusual (8).

MDH-2 and MDH-3 subclusters lack nearly all of the genes coding for \textit{Ca}\textsuperscript{2\textsuperscript{+}} insertion and maturation proteins, which seems to be a common property of XoxF systems (8). In this respect, the presence of an \textit{MxaE} homolog (DAMO\textsubscript{0128}) in the MDH-2 subcluster is remarkable. The two subclusters are separated by five genes (DAMO\textsubscript{0129} to DAMO\textsubscript{0133}) (Fig. 1), of which DAMO\textsubscript{0129} and DAMO\textsubscript{0131} encode putative TonB-dependent and TonB-like transporter proteins, respectively. It is interesting that homologs of DAMO\textsubscript{0129} and DAMO\textsubscript{0131} are widely detected in genomes of XoxF-containing methylochromes (data not shown), DAMO\textsubscript{0132} and DAMO\textsubscript{0133} code for \textit{“Ca. Methylomirabilis oxyfera”}-specific exported surface proteins that are structurally characterized by \beta-propeller strands. Deep RNA sequencing indicated that the DAMO\textsubscript{0130} coding region is one of the most highly expressed parts of the genome (Fig. 1), but it remains to be established whether its transcript is translated into an unknown protein (as annotated) or it is a small noncoding RNA that has some regulatory function.

As described below, all of the amino acids involved in the binding of the PQQ prosthetic group are conserved in all three MDH large subunits from \textit{“Ca. Methylomirabilis oxyfera.”} Moreover, in this work, the presence of this cofactor in one of these MDHs remains (43). Sequence comparison showed that Asp\textsubscript{301} was present in the presence of a lanthanide REE (La\textsuperscript{3\textsuperscript{+}}, Ce\textsuperscript{3\textsuperscript{+}}) at its catalytic site instead of Ca\textsuperscript{2\textsuperscript{+}}. This REE is coordinated in close proximity to PQQ by three highly conserved amino acids (Glu\textsubscript{172}, Arg\textsubscript{256}, and Asp\textsubscript{299}) numberings according to that of processed XoxFMf that are also involved in the coordination of Ca\textsuperscript{2\textsuperscript{+}} (8, 43). However, the proper coordination of the REE requires one more amino acid, Asp\textsubscript{301}. Besides other sequence characteristics, the presence of Asp\textsubscript{301} is a highly diagnostic property of XoxF MDHs (8). Next, two amino acid substitutions (Pro\textsubscript{259}→Thr, Ala\textsubscript{171}→Gly) are observed to accommodate the larger REE in the XoxFMf structure (43). Sequence comparison showed that Asp\textsubscript{301} was present in both XoxF\textsubscript{Mo} and XoxF\textsubscript{Mf} (see Fig. S1A in the supplemental material). In addition, XoxF\textsubscript{Mf} displayed the same proline-to-threonine and alanine-to-glutamine substitutions seen in XoxF\textsubscript{Mo} but these substitutions were different in XoxF\textsubscript{Mo} (Pro→Asp, Thr→His) (see Fig. S1A). These observations might indicate that both XoxF MDHs from \textit{“Ca. Methylomirabilis oxyfera”} possess a REE instead of calcium.

As mentioned before, only the MDH-1 subcluster contained a gene coding for a small subunit, namely, DAMO\textsubscript{0115} (Fig. 1). DAMO\textsubscript{0115} was translated as a polypeptide (MxaI\textsubscript{Mo} of 94 aa. SignalP analysis (51) suggested a 25-aa leader sequence, pointing to the translocation of the processed protein across the cytoplasmic membrane. The processed protein (69 aa; theoretical molecular mass, 6,075.11 Da) showed 61 to 65% sequence identity to the large subunit of MDHs with known crystal structures (see Fig. S1B in the supplemental material). In these structures, the small subunit tightly binds to the large subunit through a conserved set of large-subunit amino acids (see Fig. S1A). To quite an extent, these amino acids are also conserved in XoxFs, even though the latter may not contain such a small subunit. Like MxaI\textsubscript{Mo}, all three MDH large subunits from \textit{“Ca. Methylomirabilis oxyfera”} were predicted to contain N-terminal leader sequences, again suggesting a periplasmic localization of the processed enzymes. After cleavage of the N-terminal 29 or 30 aa, this would result MxaF\textsubscript{Mo}.
TABLE 1 Purification of MDH from "Ca. Methylomirabilis oxyfera" enrichment culture

<table>
<thead>
<tr>
<th>Purification step</th>
<th>Total protein (mg)</th>
<th>Sp act (μmol min⁻¹ mg⁻¹)</th>
<th>Total activity (μmol min⁻¹)</th>
<th>Yield (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Cell extract</td>
<td>24.5</td>
<td>1.10 ± 0.06</td>
<td>26.4</td>
<td>100</td>
</tr>
<tr>
<td>Soluble fraction</td>
<td>19.7</td>
<td>0.97 ± 0.05</td>
<td>19.2</td>
<td>73</td>
</tr>
<tr>
<td>Membranes</td>
<td>3.4</td>
<td>0.53 ± 0.03</td>
<td>1.8</td>
<td>7</td>
</tr>
<tr>
<td>(NH₄)₂SO₄</td>
<td>5.7</td>
<td>2.94 ± 0.23</td>
<td>16.9</td>
<td>64</td>
</tr>
<tr>
<td>Hydroxyapatite</td>
<td>0.9</td>
<td>9.66 ± 1.52</td>
<td>8.4</td>
<td>32</td>
</tr>
</tbody>
</table>

XoxF₁Moₜ and XoxF₂Moₜ, apoprotein molecular masses of 63,526, 67,660, and 63,713 Da, respectively.

Deep RNA sequencing (2) indicated that mxaF was most highly expressed at the mRNA level (transcriptome coverage: mxaF₁Moₜ, 62-fold; xoxF₁Moₜ, 14-fold; xoxF₂Moₜ, 6-fold) (Fig. 1). Strikingly, XoxF₁Moₜ was most highly expressed in the proteome of "Ca. Methylomirabilis oxyfera" strain Ooij, which was used in the present study, whereas XoxF₂Moₜ was most abundant in the proteome of closely related "Ca. Methylomirabilis oxyfera" strain Twente (2), despite the fact that both strains were enriched and cultured under similar conditions. The above considerations raised the questions of which of the three MDHs is (are) functionally expressed, whether their large subunit contains PQQ or an alternative cofactor, which metal is present at the catalytic site, and where the MDH is localized. To address these questions, we purified the MDH from "Ca. Methylomirabilis oxyfera" and assessed its cellular localization by immunogold labeling.

**Purification of a heterotetrameric XoxF MDH from "Ca. Methylomirabilis oxyfera."** MDH was purified with a 32% yield by the two-step purification procedure summarized in Table 1. The preparation obtained after the hydroxyapatite step represented the predominant MDH activity (>95%); a few other fractions displayed negligible activity and were not further investigated. After ultracentrifugation of the cell extract, 93% of the MDH activity was recovered in the supernatant. Hence, MDH is soluble or only loosely membrane associated. The purification factor (8.4-fold) suggested that MDH is a major protein. The purified enzyme catalyzed methanol oxidation by simple Michaelis-Menten kinetics with apparent Vₘₐₓ and Kₘ values of 10 μmol min⁻¹ mg⁻¹ of protein⁻¹ and 17 μM, respectively. The presence of ammonium strongly stimulated its activity; in the absence of ammonium, it was 10-fold less active.

On native PAGE, purified MDH showed one single band with an apparent molecular mass of approximately 260 kDa (Fig. 2A). SDS-PAGE displayed two bands of 67 and 11 kDa (Fig. 2B). Linear-mode MALDI-TOF MS analysis as applied to the isolated MDH revealed an Mᵣ of 8,200 ± 66 (n = 5) for the small subunit, which was significantly less than that judged by SDS-PAGE (see Fig. S3 in the supplemental material). However, this value was close to the calculated molecular mass (8,075.11 Da) of Mxaₐₜ, which is encoded by a gene (DAMO_0115) in the MDH-1 subcluster, after cleavage of the predicted signal peptide. In agreement with this, trypsin cleavage of the MDH small subunit and subsequent reflectron mode MALDI-TOF MS analysis verified the presence of several peptides that were to be expected in DAMO_0115 (see Fig. S1B). The large subunit was resistant to proteolytic cleavage, and only a few peptides were recovered within the 500-to-4,000 m/z frame used for MS analysis. These peptides uniquely mapped to DAMO_0124 (XoxF₁Moₜ) (see Fig. S1A). Linear MALDI-TOF MS gave a molecular mass of 67.3 ± 0.27 kDa (n = 7) for the large subunit (see Fig. S3), which agrees with both that estimated by SDS-PAGE and that expected for N-terminally cleaved XoxF₁Moₜ (DAMO_0124; 67.66 kDa). It should be noted that the molecular masses of the other two large subunits are ~4 kDa less (Mxaₐₜ, 63.526 Da; XoxF₂Moₜ, 63.713 Da), differences that are incompatible with MS. Upon analytical equilibrium ultracentrifugation of native MDH, the dominant 280-nm-absorbing band could be modeled (SEDFIT) to an ~150-kDa protein (Fig. 2D), which is clearly less than that observed by native PAGE. This molecular mass of 150 kDa is consistent with an α₁β₃ protein composed of two 67-kDa large subunits and two 8-kDa small subunits. Besides this major band, three more bands were obtained during analytical ultracentrifugation of the apparently pure protein preparation (Fig. 2B). These bands sedimented at ~67, ~240, and ~310 kDa. The molecular mass of the first band (67 kDa) again agreed with that expected for XoxF₁Moₜ. Both high-molecular-mass bands might represent higher aggregates (α₁β₃, α₁β₁). Taken together, the observations concluded that the MDH purified from "Ca. Methylomirabilis oxyfera" was predominantly a heterotetrameric (α₁β₃) enzyme composed of two Mxaₐₜ, small subunits and two XoxF₁Moₜ, large subunits.

The XoxF MDH from "Ca. Methylomirabilis oxyfera" contains PQQ. The UV-visible light absorption spectrum of the purified MDH from "Ca. Methylomirabilis oxyfera" exhibited the characteristics of a quinoprotein (Fig. 3), having an absorption maximum at 345 to 350 nm and a wide shoulder at 375 to 400 nm (60, 61). The spectrum of the enzyme (6.2 mg ml⁻¹; 82 μM calculated on the basis of an Mᵣ of 75,700 for an αβ heterodimer) had an absorbance at 342 nm of 0.91. Assuming a PQQ molar coefficient of absorption at 342 nm of 9,620 M⁻¹cm⁻¹ (55), the quinone concentration was 95 μM, which indicated that the protein bound PQQ in an about 1:1 stoichiometry. The prominent absorption peak around 345 nm was also indicative of the presence of a metal ion in the active site of the enzyme (62, 63). Incubation of the enzyme preparation with EGTA had no effect on the overall spectrum of the enzyme, indicating that the metal would be tightly bound. To assess the nature of the metal, ICP-MS was used as described before (43). The analysis revealed the presence of calcium only; rare earth metals were below the limit of detection (<0.1 mol%). Since column fractions lacking MDH contained calcium as well, presumably derived from hydroxyapatite [Ca₁₀(PO₄)₆OH] used for purification, it was not possible to determine the content of this compound in the protein.

The presence of PQQ in the purified MDH from "Ca. Methylomirabilis oxyfera" was confirmed by MS with both precursor ion and fragmentation level data. The acquired precursor ion spectra from the PQQ standard and the purified MDH fully compared the simulated spectrum of PQQ. The monoisotopic mass of the [M-H]-PQQ ion from both the standard MDH (m/z 329.0053; Δ = 4.0 ppm) and the purified MDH (m/z 329.0052; Δ = 3.6 ppm) accurately matched the calculated (deprotonated) monoisotopic mass of PQQ (m/z 329.0040) (see Fig. S4A in the supplemental material). Moreover, the relative abundance of the ²⁵²C isotope peak in precursor spectra from both the PQQ standard (17%) and the purified MDH (16%) was in good agreement with the simulated spectrum (15%). The isotope abundance spectrum was simulated according to de Hoffmann and Stroobant (64). The presence of PQQ in the purified MDH was also confirmed by comparing the collision-induced dissociation fragmenta-
tion spectra of \textit{m/z} 329 from the PQQ standard and the purified enzyme (see Fig. S4B). The two spectra were nearly identical and showed the presence of two abundant fragment ions corresponding to the loss of CO\textsubscript{2} (\textit{m/z} 285 [M-H-CO\textsubscript{2}]) and 2CO\textsubscript{2} (\textit{m/z} 241 [M-H-2CO\textsubscript{2}]) from the precursor ion. The findings agreed with those of Noji et al. (65), except that in neither the standard nor the purified enzyme was a third diagnostic fragment ion (\textit{m/z} 197 [M-H-3CO\textsubscript{2}]) detectable, which was probably due to different instrument characteristics and/or conditions.

**Cellular localization of the XoxF MDH in \textquotedblleft Ca. Methylopirabilis oxyfera\textquotedblright.** The N-terminal signal sequences in XoxF\textsubscript{1Mo} suggested a periplasmic localization of the processed protein (see Fig. S1A in the supplemental material). To investigate this, we generated primary antiserum with a synthetic peptide targeting a sequence of XoxF\textsubscript{1} that was specific for this protein (see Fig. S1A). The specificity of the derived antiserum was con-

*FIG 2* PAGE, immunoblotting, and analytical ultracentrifugation of MDH from \textquotedblleft Ca. Methylopirabilis oxyfera\textquotedblright. (A) Native 10\% PAGE of purified MDH (6 \mu g). (B) SDS–15\% PAGE of the purified MDH (4 \mu g). The upper band corresponds to XoxF\textsubscript{1}, and lower band corresponds to MxaI. Marker proteins and their corresponding molecular masses are shown in the left lanes of panels A to C. (C) Immunoblot analysis of the affinity-purified antiserum (\alpha-XoxF\textsubscript{1}) directed against XoxF\textsubscript{1} of \textquotedblleft Ca. Methylopirabilis oxyfera\textquotedblright. For SDS–10\% PAGE, gels were loaded with cell extract from \textquotedblleft Ca. Methylopirabilis oxyfera\textquotedblright (30 \mu g of protein) or with purified MDH (10 \mu g) and blotted onto a nitrocellulose membrane. Lanes: 1, marker proteins; 2, cell extract blot incubated with only secondary antiserum; 3, cell extract blot incubated with \alpha-XoxF\textsubscript{1} antiserum; 4, purified MDH blot incubated with \alpha-XoxF\textsubscript{1} antiserum. The expected target size (\textasciitilde 67 kDa) is indicated by the arrow. The values to the left are molecular sizes in kilodaltons. (D) Analytical ultracentrifugation of purified MDH. Analytical ultracentrifugation was performed as described in Materials and Methods, and equilibrium data were fitted by the SEDFIT program (54). Concentrations are expressed in arbitrary units (AU) of absorbance at 280 nm.

*FIG 3* UV-visible light absorption spectrum of purified MDH of \textquotedblleft Ca. Methylopirabilis oxyfera\textquotedblright. The spectrum was recorded in 150 mM phosphate buffer (pH 7.0) at 30°C. The protein concentration was 6.2 mg ml\textsuperscript{-1}. 

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firmed with both cell extracts of “Ca. Methylomirabilis oxyfera” and the purified enzyme by SDS-PAGE and immunoblot analysis (Fig. 2C). No bands were detected in blots incubated with the secondary antiserum only. These results showed that the derived α-XoxF1 antiserum was specific and suited for the intracellular localization of affinity-purified antiserum directed against XoxF1 is shown (black and white bars). Longitudinal (A) and cross (B) sections were blocked with 1% BSA and treated with 50-fold-diluted α-XoxF1 serum. Abbreviations: cp, cytoplasm; pp, periplasm; cm, cytoplasmic membrane; om, outer membrane. Scale bars, 200 nm.

FIG 4 Transmission electron micrographs of chemically fixed and cryosectioned “Ca. Methylomirabilis oxyfera” cells. The immunogold localization of affinity-purified antiserum directed against XoxF1 is shown (black and white bars). Longitudinal (A) and cross (B) sections were blocked with 1% BSA and treated with 50-fold-diluted α-XoxF1 serum. Abbreviations: cp, cytoplasm; pp, periplasm; cm, cytoplasmic membrane; om, outer membrane. Scale bars, 200 nm.

DISCUSSION

The genome of the anaerobic methanotroph “Ca. Methylomirabilis oxyfera” codes for three different MDH systems that are localized in one large gene cluster, which is highly unusual: one MxaFI MDH and two XoxF MDHs (Fig. 1). Here, we purified the predominant MDH that is functionally expressed under the growth conditions used (Fig. 2 and Table 1). The enzyme was composed of two small and two large subunits, as is common among MxaFI MDHs (5–8). MALDI-TOF MS identified the small subunit as the N-terminally cleaved gene product of DAMO_0115 (MxaI_{Mo}). Five lines of evidence (SDS-PAGE, MALDI-TOF MS of the native and trypsin-cleaved protein, analytical ultracentrifugation, antibody specificity) consistently indicated that the large subunit with a size of 67 kDa was XoxF1Mo (DAMO_0124). Immunogold labeling localized the protein in the periplasm (Fig. 4), as is also known for MDHs from other methanotrophs and methylo trophs. In the established (PMS and DCPIP) dye-coupled assay (6, 53), “Ca. Methylomirabilis oxyfera” MDH catalyzed the oxidation of methanol with high affinity (K_{m} of 17 μM) and a high V_{max} of 10 μmol min⁻¹ mg⁻¹. The apparent K_{m} for methanol is comparable to those of well-investigated MxaFI MDHs (2 to 20 μM), while the V_{max} of the “Ca. Methylomirabilis oxyfera” enzyme was substantially higher than those of known MxaFI MDHs (0.8 to 1.0 μmol min⁻¹ mg⁻¹) (60, 63, 66). In addition, MDH was a major enzyme in “Ca. Methylomirabilis oxyfera.” Taking into account that the very slowly growing bacterium “Ca. Methylomirabilis oxyfera” oxidizes methane at an extremely low rate (1.7 nmol min⁻¹ mg⁻¹ of protein⁻¹) (2, 46), the abundance of MDH and its favorable catalytic efficiency (V_{max}/K_{m}) allow the conversion of methanol, the product of methane activation, down to low concentrations.

“Ca. Methylomirabilis oxyfera” MDH contains PQQ as its prosthetic group. While the unambiguous presence of this cofactor in an XoxF-type large subunit was established only quite recently (43), the finding was expected. Except for nicotinoprotein methanol/alcohol dehydrogenase (3, 4), all of the MDHs known to date rely on PQQ as the catalytic center and PQQ-binding motifs have been identified before in XoxF MDHs (see Fig. S1A) (8, 32). However, the genome of “Ca. Methylomirabilis oxyfera” lacks known PQQ biosynthesis machinery. So, either the organism has evolved a novel biosynthetic pathway, which is unlikely considering the highly specific chemistry of the (oxygen-dependent) reactions involved (67), or the microorganism derives the cofactor from producers in the enrichment culture. Our finding that the supply of boiled cells of M. fumarriolicum strongly stimulated “Ca. Methylomirabilis oxyfera” culture activity and growth supports the latter view. Otherwise, the dependence on exogenous PQQ is not without precedent. For example, Escherichia coli and other enteric bacteria are incapable of PQQ synthesis, but the organisms readily incorporate the cofactor present in growth medium into their glucose dehydrogenase quinoproteins (68, 69). In addition, it has been known for quite some time that microorganisms that are capable of PQQ biosynthesis may excrete the compound, thereby stimulating the growth of other organisms (70). Still, it would be astonishing if “Ca. Methylomirabilis oxyfera,” with its unique life-style, depended on other microorganisms in the environment for the supply of an essential cofactor of one of its key enzymes.

The large subunit of purified MDH from “Ca. Methylomirabilis oxyfera” belongs to the XoxF MDHs. XoxF MDHs likely represent an ecologically highly relevant but grossly overlooked group of MDHs (8). Genes coding for these proteins are widely found in the genomes of methylo trophic and methanotrophic bacteria and can be phylogenetically differentiated into at least five lineages (see Fig. S2 in the supplemental material) (8, 34). While hardly expressed under laboratory growth conditions (32), xoxF genes are among the most highly expressed genes in natural sys-
tems (35, 36). The reason for their long-time elusiveness is that XoxF apparently harbors lanthanide REEs, compounds that are rarely included in laboratory trace element solutions, at its catalytic site (40–43). The presence of a REE may confer on the pro-

REFERENCES


