Functional investigation of methanol dehydrogenase-like protein XoxF in Methylobacterium extorquens AM1

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Methanol dehydrogenase-like protein XoxF of Methylobacterium extorquens AM1 exhibits a sequence identity of 50% to the catalytic subunit MxaF of periplasmic methanol dehydrogenase in the same organism. The latter has been characterized in detail, identified as a pyrroloquinoline quinone (PQQ)-dependent protein, and shown to be essential for growth in the presence of methanol in this methylorophic model bacterium. In contrast, the function of XoxF in M. extorquens AM1 has not yet been elucidated, and a phenotype remained to be described for a xoxF mutant. Here, we found that a xoxF mutant is less competitive than the wild-type during colonization of the phyllosphere of Arabidopsis thaliana, indicating a function for XoxF during plant colonization. A comparison of the growth parameters of the M. extorquens AM1 xoxF mutant with those of the wild-type during exponential growth revealed a reduced methanol uptake rate and a reduced growth rate for the xoxF mutant of about 30%. Experiments with cells starved for carbon revealed that methanol oxidation in the xoxF mutant occurs less rapidly compared with the wild-type, especially in the first minutes after methanol addition. A distinct phenotype for the xoxF mutant was also observed when formate and CO₂ production were measured after the addition of methanol or formaldehyde to starved cells. The wild-type, but not the xoxF mutant, accumulated formate upon substrate addition and had a 1 h lag in CO₂ production under the experimental conditions. Determination of the kinetic properties of the purified enzyme showed a conversion capacity for both formaldehyde and methanol. The results suggest that XoxF is involved in one-carbon metabolism in M. extorquens AM1.

INTRODUCTION

Methylothrophy is the ability of organisms to use reduced organic compounds without carbon–carbon bonds, such as methane, methanol and methylamine, as their sole sources of carbon and energy. Among these organisms are members of the genus Methylobacterium, which are aerobic facultative methylorophic Alphaproteobacteria. They are well-known ubiquitous plant epiphytes and have been described by cultivation-dependent (Corpe & Rheem, 1989; Hirano & Upper, 1991; Madhaiyan et al., 2009) as well as cultivation-independent methods (Delmotte et al., 2009; Knief et al., 2008, 2010). Methylobacterium has been shown to benefit from its ability to use methanol as a substrate, which is advantageous because the one-carbon compound is produced by plants as a by-product of cell wall synthesis (Abanda-Nkwatt et al., 2006; Fall & Benson, 1996; Sy et al., 2005). The methylorophic metabolism of Methylobacterium has been elucidated over the past 50 years. Detailing of the metabolic processes began after the isolation of strain Methylobacterium extorquens AM1 (Peel & Quayle, 1961), which became an important model to investigate methylothrophy (Chistoserdova et al., 2003; Schrader et al., 2009). When grown in the presence of methanol, the one-carbon substrate is first oxidized to formaldehyde by the periplasmic methanol dehydrogenase (Fig. 1), which is essential for growth in the presence of methanol (Nunn & Lidstrom, 1986a, b). Methanol dehydrogenase has been shown to be a heterotetrameric enzyme ($z_2b_2$) and contains Ca$^{2+}$ and pyrroloquinoline quinone (PQQ) as a prosthetic group (Afolabi et al., 2001; Anthony & Zatman, 1964; Williams et al., 2005). The two subunits are encoded by the mxaFI genes found in the large mxa gene cluster, which also encompasses the gene (mxaG) for the natural electron acceptor, a cytochrome $c_5$, as well as genes for enzymes of unknown functions, such as mxaJ.

Abbreviations: DCPIP, 2,6-dichlorophenol-indophenol; DO, dissolved oxygen; GC-FID, GC-flame ionization detection; PES, phenazine ethosulfate; PQQ, pyrroloquinoline quinone.

Three supplementary figures, showing an amino acid sequence alignment of MxaF and XoxF from M. extorquens AM1 without the predicted N-terminal signal peptides, the response of M. extorquens AM1 and the xoxF::kan/pCM80 mutant to addition of methanol after carbon starvation, and chromatographic separation and a calibration curve of standard proteins including XoxF on a Superdex 200 column, are available with the online version of this paper.
and additional genes for $\text{Ca}^{2+}$ insertion (Anderson et al., 1990; Nunn et al., 1989; Springer et al., 1995). Previous research has demonstrated that a tetrahydromethanopterin ($\text{H}_4\text{MPT}$)-dependent pathway is essential for growth in the presence of methanol and is responsible for the oxidation of formaldehyde to formate in the cytoplasm (Chistoserdova et al., 1998; Pomper et al., 2002; Vorholt, 2002) (Fig. 1). Formate may either be oxidized to $\text{CO}_2$ by formate dehydrogenases or converted via tetrahydrofolate ($\text{H}_4\text{F}$)-dependent enzymes (Chistoserdova et al., 2004, 2007; Crowther et al., 2008). Methylene tetrahydrofolate is used for carbon assimilation via the serine cycle and the recently demonstrated ethylmalonyl-CoA pathway (Anthony, 1982; Chistoserdova et al., 2003; Erb et al., 2007; Peyraud et al., 2009). Other linear oxidation pathways exist in distinct methylotrophic bacteria, e.g. dependent on thiol compounds, as found in the autotrophic Alphaproteobacteria Paracoccus denitrificans and Rhodobacter sphaeroides (Barber et al., 1996; Barber & Donohue, 1998; Ras et al., 1995).

The complete genome sequence of $M. \text{extorquens}$ AM1 (Vuilleumier et al., 2009) revealed the presence of several genes encoding predicted paralogues of the large subunit of methanol dehydrogenase (MxaF), one of which is XoxF (ID ACS39584.1, META1_1740; Chistoserdova & Lidstrom, 1997) of a predicted mass of 65 kDa. XoxF exhibits a sequence identity to MxaF of 50%, possesses a predicted signal peptide for periplasmic localization, and conserved amino acid residues involved in PQQ and $\text{Ca}^{2+}$ binding of methanol dehydrogenase (Anthony & Williams, 2003) (Supplementary Fig. S1). A xoxF mutant is able to grow in the presence of methanol as the sole source of carbon and energy, and no obvious phenotype has been described (Chistoserdova & Lidstrom, 1997). XoxF proteins form a distinct subgroup within the family of quinoprotein alcohol dehydrogenases (Chistoserdova et al., 2009; Kalyuzhnaya et al., 2008, Kane et al., 2007).

In addition to $M. \text{extorquens}$ strains, other methylotrophic bacteria exhibit paralogues of methanol dehydrogenase, such as $P. \text{denitrificans}$. The presence of a $xoxF$ gene of $P. \text{denitrificans}$ has been noted earlier. A reduced growth on methanol was observed for an insertion mutant in the neighbouring $\text{cycB}$ gene, encoding a cytochrome $\text{c}553i$ (Ras et al., 1991). The complete genome sequences of other bacteria indicate the presence of $xoxF$ but not of $mxaF$, such as $R. \text{sphaeroides}$ (Wilson et al., 2008) and $\text{Methylibium petroleiphilum}$ PM1 (Kane et al., 2007), and for other strains for which no methylotrophic growth has been fully established so far, e.g. Bradyrhizobium japonicum (Muhlencoert & Muller, 2002; Sudtachat et al., 2009) and Beggiatoa alba (Jewell et al., 2008). Among the organisms possessing XoxF but not MxaF, the physiology of XoxF has been investigated in $R. \text{sphaeroides}$. The gene encoding XoxF has been shown to be essential for methanol-dependent oxygen uptake and required for the utilization of methanol as the sole photosynthetic carbon source (Wilson et al., 2008). Recent environmental studies have documented the induction of $xoxF$ expression in Methylphilosa strain DMS010 during growth on dimethylsulphide (Schäfer, 2007) and in Methylotenera mobilis during growth on methylamine (Bosch et al., 2009; Kalyuzhnaya et al., 2009), which points to its implication in one-carbon metabolism in these organisms.

XoxF was not detected in 2D protein gels when $M. \text{extorquens}$ AM1 was grown on methanol minimal medium (Laukel et al., 2003), and has recently been shown to be present at about 100-fold lower amounts compared with MxaF using a liquid chromatography-MS/MS approach (Bosch et al., 2008). In contrast, XoxF was found to be highly expressed in bacterial phyllosphere communities in situ and was detected approximately as frequently as MxaF in shotgun proteomics (Delmotte et al., 2009). The identification of proteins during phyllosphere colonization of the strain $M. \text{extorquens}$ PA1 has confirmed the expression of $xoxF$ (Mext_1809, corresponding to META1_1740; N. Delmotte and others, unpublished data). This observation suggests an important function of XoxF in a natural environment, i.e. the phyllosphere. In this study, we conducted a series of tests in order to search for a
role for XoxF under in planta as well as under laboratory culture conditions. We demonstrate the importance of XoxF for plant colonization, the involvement of the enzyme in methanol/formaldehyde oxidation, and determine the molecular and catalytic properties of the purified enzyme.

METHODS

Bacterial constructs, cultures and growth conditions. The bacterial strains and plasmids used in this work are listed in Table 1. *Escherichia coli* DH5α was grown on Luria–Bertani medium in the presence of antibiotics (10 μg tetracycline ml⁻¹, 50 μg kanamycin ml⁻¹). Cultures of *M. extorquens* AM1 wild-type and xoxF insertion mutant (Chistoserdova & Lidstrom, 1997) were grown on minimal media (Peyraud et al., 2009), supplemented with 0.5% (v/v) methanol or 0.5% (w/v) succinate. Batch cultivations (400 ml) were carried out in a 500 ml bioreactor (Multifors, Infors-HT) at 28 °C, at a stirring rate of 1000 r.p.m. and an aeration rate of 0.2 l min⁻¹. The pH was maintained at 7.0 by addition of 1 M ammonium hydroxide or 0.5 M sulfuric acid. The partial pressure of dissolved oxygen (DO) was monitored using polarographic oxygen sensors (InPro 6800, Mettler-Toledo). Methanol concentrations were determined by GC-flame ionization detection (GC-FID) (GC 6850, Agilent Technologies; column: DB-Wax, J&W Scientific) measurements, and CO₂ concentrations were monitored in the gas phase using an infrared sensor (BCP-CO₂, BlueSens). In case of the xoxF mutant strain overexpressing xoxFGJ (pCM80-xoxFGJ) and the xoxF mutant with the empty pCM80 vector, the minimal media were supplemented with a 10-fold increased cobalt concentration (12.6 μM final concentration) to recover growth in the presence of the pCM80 plasmid and tetracycline (Kiefer et al., 2009). ΔmxaF/pCM80-xoxF-his was grown on minimal media with 30 mM pyruvate. Antibiotics were used at the following concentrations: 10 μg tetracycline ml⁻¹, 50 μg kanamycin ml⁻¹ and 50 μg rifamycin ml⁻¹. The growth characteristics of mutants were tested on plates containing 10 and 120 mM methanol, 20 and 40 mM pyruvate, 30 mM succinate, NH₄Cl (1.62 g l⁻¹) or KNO₃ (3.06 g l⁻¹), or none or 1 mM yeast nitrogen base without amino acids and ammonium sulfate (Difco, Chemie Brunschwig).

Construction of overexpressing strains, and oligonucleotides. For purification of XoxF from *M. extorquens* AM1, the xoxF gene was amplified His-tagged (C-terminal) with specific restriction sites (Xhol–EcoRI) and cloned into Xhol–EcoRI sites of the pCM80 expression vector (Marx & Lidstrom, 2001). The overexpression vector pCM80 contains the PmxaF promoter of *M. extorquens* AM1. Positive clones were selected by tetracycline resistance and the lacZ reporter gene. The forward primer included the Xhol site (XoxF_forw, 5’-atatatttaggataagcttgagggagaatctg-3’) and started 24 bp upstream of the start codon to include the Shine–Dalgarno sequence. The reverse oligonucleotide (XoxF_rev_his, 5’-tattataatcttgatgtgttcgattggtctggc-3’). The forward primer included the Xhol site (XoxF_forw, 5’-atatatttaggataagcttgagggagaatctg-3’) and started 24 bp upstream of the start codon to include the Shine–Dalgarno sequence. The reverse oligonucleotide (XoxF_rev_his, 5’-tattataatcttgatgtgttcgattggtctggc-3’).

Table 1. Bacterial strains and plasmids used in this study

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<tr>
<th>Strain or plasmid</th>
<th>Characteristics</th>
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<td>M. extorquens strains</td>
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<tr>
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<td>Wild-type</td>
<td>Laboratory strain</td>
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<td>UV26</td>
<td>UV mutant of mxaF gene</td>
<td>Nunn &amp; Lidstrom (1986a)</td>
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<td>mxaF (CM194,1)</td>
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<td>Marx et al. (2003)</td>
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<td>pCM80</td>
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<td>pCM80-xoxFGJ</td>
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<tr>
<td>pCM80-xoxF-his</td>
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<tr>
<td>pCR2.1-TOPO</td>
<td>Cloning vector</td>
<td>Invitrogen</td>
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http://mic.sgmjournals.org
gatgtagagctgctggcaggaacctg-3') contained the EcoRI site, the stop codon and the His-tag-coding sequence. The correct xoxF sequence was validated by sequencing. Finally, this plasmid, named pCM80-xoxF-his, was transformed into M. extorquens AM1 wild-type and ΔmxaF.

The overexpression plasmid pCM80-xoxFGJ was created via xoxFGJ amplification using the forward oligonucleotide META1_1740forw1 (5'-aaatctagagctgccggagcgaatatcatcc-3') including an XbaI restriction site (primer start 100 bp in front of the xoxF start codon) and the reverse oligonucleotide META1_1742rev (5'-aaaagtgtggctgctgatctgaagctg-3') with an EcoRI site (oligonucleotide start 30 bp behind the stop codon). The three genes were first subcloned into the pCR2.1-TOPO vector (3.9 kb, Invitrogen). The ligation product was validated by kanamycin selection, followed by PCR amplification.

**Seed sterilization, plant growth conditions and harvest.** For plant inoculation experiments, M. extorquens cultures were grown in minimal medium with 30 mM succinate. Exponentially grown cells were washed in 10 mM MgCl₂ and OD₆₀₀ was adjusted to 1.0 (10⁸ c.f.u. ml⁻¹) for inoculation. Sterilized Arabidopsis thaliana ecotype Col-0 seeds were inoculated with a mixture (5 μl) of GFP-labelled wild-type (CM174.1) and mutant strain (mxaF or mxaF-xoxF). The competition of the GFP-labelled strain against the wild-type strain was performed as described by Schlesier et al. (2003). After sterilizing the seeds with 70 % (v/v) ethanol (2 min) and a sodium hypochlorite solution (7 % chlorine) containing 0.2 % (v/v) Triton X-100 (8 min), seeds were washed seven times and thereafter incubated for 3 h in distilled water before the seeds were deposited. The bottoms of plant full-glass microboxes (Combiblot) were filled with Murashige and Skoog media including vitamins (Duchefa), 3 % (w/v) sucrose and 0.55 % (w/v) plant agar (Murashige & Skoog, 1962). Plants were harvested after 3 weeks of growth (1 week at 22 °C, 16 h light/8 h darkness; 2 weeks at 22 °C, 9 h light/15 h darkness). The aerial parts of the plants were individually transferred to 2 ml tubes containing 1 ml phosphate buffer (100 mM, pH 7.0). Bacteria were removed from the plant surfaces by shaking in a Retsch tissue lyser (15 min, 25 Hz; Qiagen) and by ultrasonication (5 min; Branson). Five- and 10-fold dilution series of cell suspensions were plated on minimal media (MM) with 30 mM sodium succinate (5 days, 28 °C). A fluorescence microscope (Zeiss Axioskop 2) was used to distinguish between colonies from GFP-labelled and unlabelled bacteria. All competition experiments with the M. extorquens AM1 GFP-labelled strain against the wild-type and mutant strains were repeated in three independent trials. The loss of competition for each strain mixture was calculated as the percentage of wild-type or mutant strain at day 0 (inoculum) minus the percentage of wild-type or mutant strain at day 21 (after 3 weeks of growth). An analysis of variance was performed to prove significant differences in the performance of the four tested strains based on post-hoc pairwise t tests with Bonferroni correction. All statistical analyses were done with the SYSTAT 12 statistical package.

**M. extorquens cultures to perform starvation experiments followed by addition of methanol/formaldehyde.** M. extorquens AM1, the xoxF mutant, the xoxF mutant strain overexpressing xoxFGJ (pCM80-xoxFGJ) and the xoxF mutant with the empty pCM80 vector were grown to a final optical density of 9.5 in batch cultivation. To avoid growth inhibition, methanol was added stepwise during cultivation, maintaining its concentration below 0.5 %. Subsequently, cells were starved for 16 h and thereafter exposed to 60 mM methanol or formaldehyde (methanol-free), added stepwise to a final concentration of 10 mM.

Before, during and after methanol/formaldehyde addition, samples (1 ml) were taken from the cultures, filter-sterilized and used for formate analysis. At the beginning, a time- and concentration-dependent linear standard curve was established with sodium formate (Sigma-Aldrich). A volume of 0.01–0.2 ml of filtrate (undiluted to 100-fold diluted) was mixed with 50 mM Tris/HCl buffer, pH 8.0, 2 mM NAD⁺ and yeast formate dehydrogenase (Sigma-Aldrich; 2 U ml⁻¹) in a total volume of 1 ml. After incubation (60 min, 37 °C), NADH was measured spectrophotometrically (Gary 50, Varian) in plastic cuvettes (1 cm path length) at 340 nm (Chistoserdova et al., 2007). All measurements were done in triplicate with 15 % agreement. A viability test was performed before and after starvation. To this end, three samples of the wild-type and mutant strain were taken before and after starvation. Cell dilutions were plated onto square plates with minimal media supplemented with methanol. The starvation of cells did not result in a significantly reduced viability of the bacterial cultures (SD 10 %).

**Purification of XoxF, determination of the molecular mass, methanol dehydrogenase assay and enzyme kinetics.** Pyruvate-grown cells of M. extorquens AM1 ΔmxaF/pCM80-xoxF-his (10 l, OD₆₀₀ 1.0) and methanol-grown cells of M. extorquens AM1 wild-type/pCM80-xoxF-his (10 l, OD₆₀₀ 4.0) were harvested by centrifugation at 16 000 g using a Beckman Avanti J-26 XP centrifuge (4 °C, 10 min). Cells were resuspended in 25 mM Tris/HCl, pH 8.0, 150 mM NaCl, 5 mM imidazole, pH 8.0, plus a protease inhibitor tablet (Complete, Roche) and passed through a French pressure cell (SLM Instruments) at 1.2 × 10⁶ Pa. Ultranetrifugation was performed at 200 000 g for 1 h at 4 °C in a Kontron Centrikon T-1180 centrifuge to remove cell debris. The supernatant was loaded on an equilibrated 1 ml HisTrap HP column (GE Healthcare). After a wash step with resuspension buffer containing 50 mM imidazole, elution was performed with 400 mM imidazole (1 ml fractions). Fractions were analysed by 12.5 % SDS-PAGE. Protein samples containing the desired protein were pooled, desalted and eluted from imidazole either via a PD-10 column (GE Healthcare) or by concentration (30 kDa Amicon Ultra centrifugal filter unit, Millipore) with buffer exchange (20 mM phosphate, pH 7.0).

Purified XoxF was loaded onto a Superdex 200 (HiLoad 16/60) column (Amersham Pharmacia), equilibrated with 50 mM Tris/HCl buffer, pH 8.0, and 200 mM NaCl at a flow rate of 0.7 ml min⁻¹, using an AKTA purifier (Amersham Pharmacia). Apparent molecular masses were estimated by comparison with low and high molecular mass standards (Amersham Pharmacia). Methanol dehydrogenase activity was measured using the method of Anthony & Zatman (1967), with a modification using phenazine ethosulfate (PES)-mediated reduction (Ghosh & Quayle, 1979) of 2,6-dichlorophenol-indophenol [DCPIP; \( \text{OD}_{600} = 19.1 \text{ mm}^{-1} \text{ cm}^{-1} \)] (Basford & Huennekens, 1955). The reaction was catalysed with the activator \( \text{NH}_4\text{Cl} \) (final concentration 15 mM), which turned out to be essential for XoxF activity in this work. Assays were performed at room temperature (15 °C) at a constant 25 Hz; Qiagen) and by ultrasonication (5 min; Branson). Five- and 10-fold dilution series of cell suspensions were plated on minimal media (MM) with 30 mM sodium succinate (5 days, 28 °C). A fluorescence microscope (Zeiss Axioskop 2) was used to distinguish between colonies from GFP-labelled and unlabelled bacteria. All competition experiments with the M. extorquens AM1 GFP-labelled strain against the wild-type and mutant strains were repeated in three independent trials. The loss of competition for each strain mixture was calculated as the percentage of wild-type or mutant strain at day 0 (inoculum) minus the percentage of wild-type or mutant strain at day 21 (after 3 weeks of growth). An analysis of variance was performed to prove significant differences in the performance of the four tested strains based on post-hoc pairwise t tests with Bonferroni correction. All statistical analyses were done with the SYSTAT 12 statistical package.
temperature in a total volume of 1 ml in a quartz cuvette (1 cm path length). One unit of specific enzyme activity was defined as one micromole DCPIP reduced per minute (determined at 600 nm) and was expressed as units per milligram protein (U mg⁻¹). For all spectrophotometric measurements, a Cary 50 (Varian) spectrophotometer was used.

In order to determine the pH optimum of XoxF in the dye-linked methanol dehydrogenase assay, potassium phosphate (pH 5.8–8.0), Tris/HCl (pH 7.7–10.0) and MES buffers (5.5–6.5) were added, and XoxF was purified from ΔmxaF-pCM80xoxF-his cells grown on pyruvate.

Protein concentrations were quantified according to Bradford (1976) and by the bicinchoninic acid (BCA) test (Pierce) (Smith et al., 1985). Calibration curves were prepared using BSA as standard in 0.9 % NaCl (Sigma-Aldrich). The enzyme kinetics of XoxF were studied using the aforementioned assay conditions and varying substrate concentrations. The endogenous activity (in the absence of a carbon source) was subtracted from measured enzyme activities. Data were fitted with GraphPad Prism version 5.0 according to the Michaelis–Menten equation and Lineweaver and Burk. All alcohols, aldehydes and amines were purchased from Sigma-Aldrich.

### RESULTS

#### XoxF confers a growth advantage upon *M. extorquens* AM1 during colonization of *A. thaliana* under competitive conditions

The expression of xoxF during colonization of *A. thaliana* plants by *M. extorquens* under gnotobiotic conditions (N. Delmotte and others, unpublished data) and of the phyllosphere community (Delmotte et al., 2009) prompted our investigation into the importance of XoxF and of the phyllosphere community (Delmotte et al., 2009) prompted our investigation into the importance of XoxF, *A. thaliana* seeds were inoculated with a mixture of GFP-tagged wild-type strain (CM174.1), xoxF and mxaF mutants, and a mxaF-xoxF double mutant; the untagged wild-type served as control (Table 2). The plant inoculation experiments revealed that all these mutants strains were significantly less competitive than the wild-type (*P* <0.001, Table 2). The relative decrease of the xoxF mutant in competition was stronger than those of the other mutant strains (*P* <0.001); the mxaF mutant and the mxaF-xoxF double mutant showed similar losses. The data indicate the importance of XoxF for plant colonization under competitive conditions.

#### Table 2. Bacterial loss of competition (as a percentage) during plant colonization after 21 days

<table>
<thead>
<tr>
<th>Strain in competition with GFP-wild-type</th>
<th>Loss of competition after 21 days (%) (mean ± SEM)</th>
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<tbody>
<tr>
<td>Wild-type</td>
<td>3 ± 1.3*</td>
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<tr>
<td>mxaF</td>
<td>12 ± 1.9</td>
</tr>
<tr>
<td>xoxF</td>
<td>23 ± 1.7</td>
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<tr>
<td>mxaF-xoxF</td>
<td>13 ± 1.5</td>
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</table>

*The 3 % decrease of the wild-type in comparison with the GFP-tagged strain after 21 days of plant growth was not significant (one-sample *t* test).

#### The *M. extorquens* AM1 xoxF mutant shows a reduced growth rate during exponential growth

A xoxF insertion mutant of *M. extorquens* AM1 has previously been described to be capable of growth in the presence of methanol as sole source of carbon and energy (Chistoserdova & Lidstrom, 1997), and this was confirmed in this study. Although we noted no differences in growth rates for the xoxF mutant relative to the wild-type during exponential growth on succinate (data not shown), in bioreactor experiments we found that the specific growth rate on methanol was reduced for the xoxF mutant by 30 % relative to the wild-type (Table 3). This observation is the converse of the initial description of XoxF in *M. extorquens* AM1, where no difference in growth was noted (Chistoserdova & Lidstrom, 1997). It is, however, similar to the findings described for the phenotype of a xoxG

#### Table 3. Cultivation parameters of *M. extorquens* AM1 wild-type and the xoxF mutant during exponential growth on methanol under the experimental conditions carried out in bioreactors

<table>
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<th>Parameter</th>
<th>Wild-type</th>
<th>xoxF</th>
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<td></td>
</tr>
<tr>
<td><em>μ</em> (h⁻¹)</td>
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<td>0.10</td>
</tr>
<tr>
<td><em>Y</em>&lt;sub&gt;CO₂&lt;/sub&gt; (g g⁻¹)</td>
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<td>0.27</td>
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<td><em>q</em>&lt;sub&gt;CO₂&lt;/sub&gt; (mmol g⁻¹ h⁻¹)</td>
<td>9.1</td>
<td>5.6</td>
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<td><em>q</em>&lt;sub&gt;S&lt;/sub&gt; (mmol g⁻¹ h⁻¹)</td>
<td>20.5</td>
<td>12.9</td>
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http://mic.sgmjournals.org
(cycB) mutant in P. denitrificans (Harms et al., 1996; Ras et al., 1991). Similar to the reduced growth rate for the M. extorquens AM1 xoxF mutant, we observed a decrease in the specific methanol conversion and CO₂ production rate relative to the wild-type (Table 3). Inversely, the xoxF mutant showed a comparable biomass yield (Table 3), suggesting that the amount of energy generated from methanol oxidation was unaltered.

Earlier results indicated that XoxF cannot functionally replace MxaF because a mxaF deletion mutant is not able to grow in the presence of methanol (Nunn & Lidstrom, 1986a). In order to investigate whether the low expression level of xoxF under laboratory conditions (Bosch et al., 2008) is responsible for the failure to substitute the true methanol dehydrogenase, we constructed strains expressing the xoxFGJ genes under the control of the mxaF promoter in mxaF mutant backgrounds (deletion mutant and UV mutant, Table 1). The rationale for the expression of the xoxFGJ cluster, rather than xoxF alone, was based on the genome arrangement, which suggests that the gene products function together, and on observations made in the orthologous systems in P. denitrificans and R. sphaeroides (Chistoserdova & Lidstrom, 1997; Ras et al., 1991; Wilson et al., 2008). No growth could be restored for these strains during tests under several conditions, such as various methanol concentrations, different nitrogen sources (NH₄Cl, KNO₃), and shifts from the multi-carbon substrates succinate and pyruvate to methanol with and without a period of carbon starvation.

Consumption of methanol after carbon starvation indicates that XoxF is involved in efficient methanol oxidation

The rate of methanol emission by plant leaves fluctuates depending on different parameters, such as stomatal conductance, stomata opening and leaf growth, and alternates in a diurnal manner (Fall & Benson, 1996; Hüve et al., 2007). Because methylbacteria are subjected to changing concentrations of available carbon under environmental conditions and a generally limited supply of nutrients, we mimicked a sudden increase in methanol concentration after a phase of carbon starvation in a controlled bioreactor. We chose a relatively high final concentration of methanol (60 mM) to enable us to monitor effects due to methanol oxidation more easily. The response of the xoxF mutant was analysed in parallel to M. extorquens AM1 wild-type cells after starving cells for 16 h followed by the addition of methanol. Dissolved oxygen (DO) was measured to monitor substrate oxidation by the cultures. Since biomass concentrations and cultivation conditions were identical for both strains, DO is directly correlated with oxygen consumption, and differences in the latter can be directly monitored by DO. At regular time intervals, samples of the culture supernatant were taken and methanol concentration was determined using GC-FID. We observed very fast oxygen consumption as a response to methanol addition in the wild-type but not in the xoxF mutant (Fig. 2a). According to observed differences in oxygen consumption, the mutant strain required twice the time (5 h) to convert the methanol (Fig. 2a). This observation correlated with the measured methanol concentration in the medium (Fig. 2b). Similar results were observed when the xoxF mutant strain over-expressing xoxFGJ (pCM80-xoxFGJ) and the xoxF mutant with the empty pCM80 vector (control) were analysed after addition of methanol: methanol consumption occurred faster when xoxFGJ was expressed (2.7 h) relative to the mutant strain containing pCM80 (4.1 h) (Supplementary Fig. S2). Furthermore, we observed differences in the growth rates for xoxF pCM80-xoxFGJ (μ=0.18 h⁻¹) and xoxF pCM80 (μ=0.13 h⁻¹) of about 30 %. Interestingly, the growth rate found for the xoxF-overexpressing strain was significantly higher than the recently described growth rate of M. extorquens AM1 wild-type strain with the empty pCM80 plasmid grown under the same cultivation conditions (μ=0.14 h⁻¹; Kiefer et al., 2009).

Carbon-starved M. extorquens AM1 wild-type, but not the xoxF mutant, accumulates formate during methanol and formaldehyde oxidation

A 1 h lag phase in CO₂ production occurred in the wild-type but not in the xoxF mutant (Fig. 2c), a result that might be indicative of an accumulation of formate after the addition of methanol to the wild-type. This observation prompted us to investigate the formate concentration in culture supernatants of the wild-type and the xoxF mutant. We found that the wild-type accumulated up to 14 mM formate after starvation plus methanol addition, whereas the formate concentration in the xoxF mutant did not rise beyond 400 μM (Fig. 3a). This experiment suggests an additional capacity of the wild-type to form formate due to the expression of xoxF. In a subsequent experiment, we tested directly whether the oxidation of formaldehyde to formate was affected in the xoxF mutant by adding formaldehyde instead of methanol to carbon-starved cells. To avoid poisoning the cells, we added 1 mM formaldehyde 10 times over a time period of 0.5 and 1.3 h (wild-type and xoxF mutant, respectively). Wild-type cells converted the additional formaldehyde into formate stoichiometrically, whereas the xoxF mutant accumulated only up to one-fourth of the formate compared with the wild-type (Fig. 3b). The accumulation of formate in the medium after the addition of formaldehyde in the wild-type, but not in the xoxF mutant, indicates that XoxF is involved in formaldehyde oxidation or, alternatively, in the oxidation of methanol plus formaldehyde to formate.

Formate concentrations were also measured in supernatants of exponentially growing cells. Accumulation of only low amounts of formate (below 60 μM) during growth of M. extorquens AM1 in the presence of methanol has been demonstrated earlier (Chistoserdova et al., 2004, 2007). Here, we observed comparably low amounts of
formate when the \textit{xoxF} mutant was analysed in parallel to the wild-type. Thus, the phenotype of the \textit{xoxF} mutant for one-carbon conversion to formate became evident only upon addition of a substrate after carbon starvation.

\begin{figure}
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\includegraphics[width=\textwidth]{fig2.png}
\caption{Response of \textit{M. extorquens} AM1 wild-type (solid line/filled circles) and the \textit{xoxF} mutant (dotted line/open circles) upon addition of 0.25\% (v/v) methanol after carbon starvation (16 h). The monitored parameters were DO in the liquid phase (a), methanol concentration (b) and CO\textsubscript{2} concentration in the exhausted gas phase (c). The experiment was confirmed twice with similar results and conducted at an optical density of 9.0.}
\end{figure}

\begin{figure}
\centering
\includegraphics[width=\textwidth]{fig3.png}
\caption{Formate concentrations in culture supernatants of \textit{M. extorquens} AM1 wild-type (filled circles) and the \textit{xoxF} mutant (open circles) (a) upon addition of methanol (60 mM final concentration) and (b) upon addition of formaldehyde. The latter was supplemented stepwise to the wild-type (filled triangles) and \textit{xoxF} mutant (open triangles) up to a final concentration of 10 mM. The formate content of the supernatant was determined as described in Methods. The mean values of three biological replicates are indicated and all measurements agreed within 10\%.}
\end{figure}

**Purification of XoxF from \textit{M. extorquens} AM1, determination of the apparent molecular mass, and substrate range**

To detect possible functions of XoxF, enzyme activities with crude extracts of \textit{M. extorquens} AM1 wild-type cells and the \textit{xoxF} mutant were determined using the dye-linked methanol dehydrogenase assay. During the exponential growth phase, an enzyme activity of 0.2 U mg\textsuperscript{-1} was observed in crude extracts of the \textit{xoxF} mutant, which was comparable with the wild-type. The activity was not increased when cells were taken after carbon starvation. Moreover, no methanol dehydrogenase activity was demonstrated in crude extracts of the \textit{mxaF} deletion strain and the \textit{ΔmxaF/pCM80-xoxFGJ} strain. We therefore
constructed a pCM80-\textit{xoxF}-\textit{his} vector for purification of XoxF via affinity chromatography from \textit{M. extorquens} AM1. We used a \textit{mxaF} deletion background to exclude any contamination with methanol dehydrogenase and grew the cells on pyruvate. XoxF was purified to apparent homogeneity. The apparent molecular mass of the enzyme was determined to be \textasciitilde60 kDa by gel filtration (Supplementary Fig. S3), indicating a monomeric structure of the native enzyme. The substrate ranges and substrate affinities of XoxF were investigated using a dye-linked PES-dependent methanol dehydrogenase assay (Ghosh & Quayle, 1979). The optimum pH for the oxidation of methanol was pH 9.0. The enzyme oxidized methanol, formaldehyde and ethanol with high affinity: the $K_m$ for the carbon substrates were 11 $\mu$M for methanol, 65 $\mu$M for formaldehyde and 14 $\mu$M for ethanol (Fig. 4). The $V_{\max}$ values were 0.015 U mg$^{-1}$ for methanol, 0.014 U mg$^{-1}$ for formaldehyde and 0.024 U mg$^{-1}$ for ethanol. The indicated $K_m$ and $V_{\max}$ values were calculated based on activities from which the endogenous activity of 0.01 U mg$^{-1}$ was subtracted [i.e. without substrate addition, also observed for purified methanol dehydrogenase \textit{MxaFI} (Anthony & Zatman, 1964; Anthony, 1986; Day & Anthony, 1990; Duine \textit{et al.}, 1978; Duine & Frank, 1980; Ghosh & Quayle, 1981)]. In addition, other primary alcohols and aldehydes, including acetaldehyde, propionaldehyde, propan-1-ol, butan-1-ol and decan-1-ol, and the secondary alcohol propan-2-ol were converted by XoxF. However, the $K_m$ values were in the millimolar range. Since the specific enzyme activities with methanol, formaldehyde and ethanol were lower than the ones determined for methanol dehydrogenase purified from methanol-grown cells [$V_{\max}$ methanol dehydrogenase (MxaFI) 0.8–1.0 U mg$^{-1}$; Goodwin & Anthony, 1996; Page & Anthony, 1986], we also purified XoxF-his from methanol-grown cells using the wild-type containing pCM80-\textit{xoxF}-\textit{his}. Under these conditions, a slightly higher $V_{\max}$ value of 0.08 U mg$^{-1}$ for XoxF was determined. An increased endogenous oxidation was observed, rendering accurate substrate affinity determination difficult. Ammonium was found to be essential for enzyme activity ($K_a$=8 mM), similar to the observation made for methanol dehydrogenase in \textit{M. extorquens} AM1 ($K_a$=2 mM; Goodwin & Anthony, 1996). Other amines, i.e. methylamine, dimethylamine, trimethylamine, ethylamine, n-butylamine, hexylamine, n-heptylamine and n-nonylamine, were tested as potential activators; however, they could not replace NH$_4$Cl in the enzyme assays.

**DISCUSSION**

In this work, we investigated the role of \textit{M. extorquens} XoxF in methanol and formaldehyde conversion. A possible function of XoxF in one-carbon metabolism of the organism has been previously considered, due to the 50% sequence identity to the large subunit of methanol dehydrogenase of the organism (Chistoserdova & Lidstrom, 1997) and the conservation of active site residues determined for the methanol dehydrogenase of the organism (Anthony & Williams, 2003; Supplementary Fig. S1). However, no phenotype had been established.
earlier for the *xoxF* mutant from *M. extorquens* AM1. Here, we provide experimental evidence that XoxF of *M. extorquens* AM1 is involved in the one-carbon metabolism of the organism in addition to MxaF. Growth experiments revealed a 30% reduced specific methanol oxidation rate of a *xoxF* mutant (Table 3). This reduction is in agreement with the observed reduced growth rate during methylothrophic growth as opposed to that under non-methylothrophic conditions. Experiments involving carbon starvation followed by methanol addition also indicated that XoxF is required for efficient methanol oxidation because the *xoxF* insertion mutant showed a reduced ability to convert methanol (Fig. 2a, b). The decrease in methanol oxidation might suggest an additional methanol oxidation capacity of XoxF. Such a role would suggest that a functional redundancy of MxaF and XoxF exists. However, a *mxaF* mutant is unable to grow in the presence of methanol (Nunn & Lidstrom, 1986a), and we were unable to compensate the methanol growth defect of the *mxaF* mutant by overexpressing *xoxFGJ*. Although we cannot exclude that the failure to restore growth on methanol might be attributed to a coregulation of both enzymes through shared cofactors or other enzymes, these results suggest non-redundant functions of XoxF and MxaF.

An alternative enzyme activity of XoxF could be a primary role in formaldehyde oxidation. Notably, methanol dehydrogenase of *M. extorquens* AM1 catalyses not only the oxidation of methanol but also that of formaldehyde (Anthony & Zatman, 1965). However, the significance of the latter activity in vivo is currently unclear. Interestingly, a so-called Modifier protein (M-protein) has been described that prevents the oxidation of formaldehyde by methanol dehydrogenase by lowering its affinity for formaldehyde (Bolbot & Anthony, 1980; Ford et al., 1985; Page & Anthony, 1986). It is thus tempting to speculate that the homotrimeric or tetrameric 45 kDa subunit Modifier protein (Long & Anthony, 1990, 1991) does not have the same effect on the methanol dehydrogenase-like protein XoxF, resulting in formaldehyde oxidation by XoxF in vivo. If XoxF acted as a bifunctional enzyme or was more specifically involved in periplasmic formaldehyde oxidation, it would represent an alternative route to the cytoplasmatic formation of formate via the tetrahydromethanopterin-dependent formaldehyde oxidation pathway, which includes formaldehyde-activating enzyme (Vorholt et al., 2000), methylene tetrahydromethanopterin dehydrogenase (Hagemeier et al., 2000), methenyl tetrahydromethanopterin cyclohydrolase (Pomper et al., 2002) and formyltransferase/hydrolase, which catalyses the transfer of the formyl group from tetrahydromethanopterin to a presumed methanofuran analogue and its subsequent hydrolysis to formate (Pomper & Vorholt, 2001; Pomper et al., 2002) (Fig. 1). An indication for XoxF as a periplasmic formaldehyde dehydrogenase arises from the observed accumulation of formate after the addition of methanol or formaldehyde in the wild-type but not in the *xoxF* insertion mutant (Fig. 3). This result suggests an additional capacity of the wild-type to convert methanol plus formaldehyde or formaldehyde alone. Additional evidence is provided by the 1 h lag phase in CO₂ production of the wild-type, which was not observed in the *xoxF* mutant (Fig. 2c). Because formate accumulation reached a maximum after 1.8 h under the experimental conditions, the additional capacity to oxidize formate to CO₂ might have been induced after methanol addition. Multiple formate dehydrogenases have been previously identified (Chistoserdova et al., 2004, 2007). One of these formate dehydrogenases contains a Tat motif used for periplasmic localization (Palmer et al., 2005). A complete oxidation of methanol to CO₂ in the periplasm of the cell might be envisioned under the situation of a sudden increase in the formaldehyde concentration (Fig. 1). During exponential growth, only very low amounts of formate accumulates in culture supernatants of *M. extorquens* AM1 wild-type cells (Chistoserdova et al., 2004) or the *xoxF* mutant (this study), suggesting that formate oxidation is fast relative to its production. This equilibrium might become unbalanced when cells are starved for carbon and methanol is suddenly made available (Fig. 3). Under such conditions, additional oxidation capacity in the periplasm with concomitant reduction of a specific cytochrome for transfer of electrons to the terminal oxidase appears to be a plausible option for the role of XoxF as an additional formaldehyde-oxidizing enzyme.

In *vitro* enzyme assays using purified XoxF from *M. extorquens* AM1 revealed that the enzyme catalyses with highest affinity the oxidation of methanol, formaldehyde and ethanol, the three preferred substrates of methanol oxidation. Because the subunit is also absent in the organisms lacking dye-linked alcohol dehydrogenase has been characterized,
however, from another purple non-sulfur bacterium, *Rhodopseudomonas acidiphila*, by Quayle’s group (Bamforth & Quayle, 1978, 1979; Sahm et al., 1976). Although the 65 kDa protein (denaturing conditions) exhibits similarity to XoxF from *M. extorquens* AM1 regarding a wide substrate specificity range, the substrate affinities are completely different (e.g. $K_m$ for ethanol 30 μM, $K_m$ for methanol 120 mM) (Sahm et al., 1976). It is currently unclear whether the described dye-linked alcohol dehydrogenase from *Rhodopseudomonas acidiphila* represents a XoxF orthologue.

Our results from the plant inoculation experiments indicate the importance of XoxF for plant colonization under competitive conditions (Table 2). The *mxaF*, *xoxF* and *mxaF-xoxF* mutations resulted in a significant loss of competitive fitness, which was similar to earlier results that show that *M. extorquens* AM1 benefits from methanol dehydrogenase during colonization of *Medicago truncatula* (Sy et al., 2005). Here, we observed a significantly greater loss in the xoxF insertion strain when compared with the *mxaF* mutant in competition with the wild-type, pointing to a primary role for XoxF under environmental conditions. The high expression level of XoxF during the colonization of plants such as *A. thaliana* (Delmotte et al., 2009) may have effects that cannot currently be mimicked under ‘in vivo’ laboratory conditions. The higher expression level of XoxF under natural conditions may reveal additional phenotypes and the capacity to oxidize methanol, as well as formaldehyde, in the periplasm of the cell. This assumption is supported by the observed increase in growth rate of a strain expressing xoxFGJ under the control of the *mxaF* promoter (pCM80-xoxFGJ) relative to the wild-type with an empty plasmid.

We expect that the faster methanol conversion rate of the wild-type relative to the xoxF mutant applies under natural conditions and is of physiological importance, given that the methanol emission rates of plants vary during the course of the day, such as upon the opening of stomata (Fall & Benson, 1996). Under these environmental conditions, XoxF may help to capture the volatile plant product and consequently diminish its evaporation into the atmosphere. It may also prevent formaldehyde poisoning in the periplasm of the cell if a suddenly elevated amount of methanol becomes available. Additional investigation will be required to further elucidate the biochemical function of XoxF in methanol or formaldehyde oxidation. Furthermore, the molecular basis for the regulation of the expression level of XoxF remains to be uncovered.

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