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

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Abstract

Methylotrophic bacteria possess the ability to use reduced C₁ substrates (i.e. without carbon-carbon bonds) as sole source of carbon and energy, e.g. methanol, methane, or methylamine. Their widespread habitats encompass diverse aquatic and terrestrial ecosystems.

Methylobacterium extorquens, a well-studied representative of this group of microorganisms was chosen as an experimental model strain for the following work. The Gram-negative, facultative methylotrophic bacterium oxidizes methanol and methylamine as well as multicarbon compounds such as succinate. Among other habitats, the pink-pigmented Alphaproteobacterium colonizes plant surfaces where it benefits from methanol produced by the plant. At least three different types of enzymes are known to be involved in the microbial catalysis of the primary C₁ oxidation step from methanol to formaldehyde. The Gram-negative bacterium employs the pyrroloquinoline quinone-dependent methanol dehydrogenase (EC 1.1.99.8) for methanol conversion.

Methanol dehydrogenase-like protein XoxF of *M. 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. It has been identified as a pyrroloquinoline quinone (PQQ)-dependent protein and has been shown to be essential for growth in the presence of methanol in this methylotrophic 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, I 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 and 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 to 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 one-hour 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 obtained data suggest that XoxF is involved in C₁ metabolism in *M. extorquens* AM1 and confers a growth advantage to this organism during plant colonization under competitive conditions.

Zusammenfassung

Methylotrophe Bakterien besitzen die Fähigkeit, reduzierte C₁-Substrate (d.h. ohne Kohlenstoff-Kohlenstoffbindungen) wie z.B. Methanol, Methan oder Methylamin als einzige Kohlenstoff- und Energiequellen zu nutzen. Ihren weitverbreiteten Habitaten gehören verschiedene aquatische und terrestrische Ökosysteme an.

Methylobacterium extorquens AM1, ein gut untersuchter Vertreter dieser Gruppe von Mikroorganismen, diente der folgenden Arbeit als experimenteller Modellorganismus. Das Gram-negative, fakultativ methylotrophe Bakterium oxidiert Methanol und Methylamin sowie Verbindungen mit mehreren Kohlenstoffatomen wie Succinat. Das rosafarbene Alphaproteobakterium besiedelt Pflanzenoberflächen, wo es von dem Methanol profitiert, das Pflanzen freisetzen. Es sind mindestens drei unterschiedliche Enzyme bekannt, die in der mikrobiellen Katalyse des primären C₁-Oxidationsschrittes von Methanol zu Formaldehyd involviert sind. Das Gram-negative Bakterium nutzt die Pyrroloquinolinquinon-abhängige Methanoldehydrogenase (EC 1.1.99.8) für die Umsetzung von Methanol.

Das Methanoldehydrogenase ähnliche Protein XoxF von *M. extorquens* AM1 weist eine 50%ige Identität zu MxaF auf, der katalytischen Untereinheit der periplasmatischen Methanoldehydrogenase im gleichen Organismus. Letztere wurde ausführlich charakterisiert, als Pyrroloquinolinquinon (PQQ)-abhängiges Protein identifiziert und erwies sich in diesem methylotrophen Bakterium als essentiell für das Wachstum in Gegenwart von Methanol. Im Gegensatz dazu wurde die Funktion von XoxF in *M. extorquens* AM1 bislang noch nicht aufgeklärt. In dieser Arbeit konnte gezeigt werden, dass eine *xoxF*-Mutante während der Phyllosphärenkolonisierung von *Arabidopsis thaliana* weniger konkurrenzfähig ist als der Wildtyp. Dies weist auf eine Funktion von XoxF während der Pflanzenkolonisierung hin. Ein Wachstumsparameter-Vergleich der *M. extorquens* AM1 *xoxF*-Mutante und des Wildtyps während des exponentiellen Wachstums liess eine um 30 % reduzierte Methanolaufnahme- und Wachstumsrate für die *xoxF*-Mutante erkennen. Experimente mit Kohlenstoff-hungernden Zellen ergaben, dass die Methanoloxidation in der *xoxF*-Mutante im Vergleich zum Wildtyp langsamer verläuft, insbesondere in den ersten Minuten nach der Methanolzugabe. Es wurde ein deutlicher Phänotyp für die *xoxF*-Mutante beobachtet, als die Formiat- und CO₂-Produktion nach Zugabe von Methanol und Formaldehyd zu den hungernden Zellen gemessen wurde. Der Wildtyp, aber nicht die *xoxF*-Mutante akkumulierte Formiat nach erfolgter Substratzugabe und wies unter den experimentellen Bedingungen eine einstündige Verzögerung in der CO₂-Entstehung auf. Durch die Bestimmung der

kinetischen Eigenschaften des gereinigten Enzyms konnte die Umsetzung beider Substrate, Formaldehyd und Methanol, gezeigt werden. Zusammenfassend lassen diese Ergebnisse vermuten, dass XoxF in dem C₁-Stoffwechselweg in *M. extorquens* AM1 involviert ist und diesem Bakterium unter kompetitiven Bedingungen während der Pflanzenkolonisierung einen Wachstumsvorteil verschafft.

Chapter 1: Introduction

1.1 Aerobic methylotrophic bacteria

Methylotrophy is the ability of organisms to use reduced organic compounds without carbon-carbon bonds, such as methane, methanol, or methylamine, as their sole sources of carbon and energy. Obligate methylotrophic bacteria utilize only reduced carbon compounds without carbon-carbon bonds; however, many methylotrophs are facultative methylotroph and are able to use several multicarbon compounds. Methylotrophs are predominantly strictly aerobic organisms, and a number of them being bacteria and yeasts. Not all methylotrophic bacteria utilize methanol, some oxidize for example only dimethylsulfide, dimethylsulfoxide, dimethyl ether, halomethanes, mono-, di-, and trimethylamine, tetramethylammonium, trimethylsulphonium or formamide (Anthony, 1982; Kolb, 2009). In 1892, *Bacillus methylicus* was described as first aerobic methanol utilizing bacterium (Loew, 1892) and now one counts about 160 described species (approximately 60 genera) belonging to the Alpha-, Beta-, and Gammaproteobacteria, Verrucomicrobia, Cytophagales, Bacteroidetes, Firmicutes, and Actinobacteria (Kolb, 2009). In an alternative classification, M.E. Lidstrom (2006) distinguishes Alpha-, Beta-, and Gammaproteobacteria plus Gram-positive bacteria within the aerobic methylotrophic bacteria. Among these organisms are members of the genus *Methylobacterium* that are aerobic facultative methylotrophic Alphaproteobacteria.

1.2 The habitat of aerobic methylotrophic bacteria

The widely distributed aerobic methylotrophic bacteria colonize diverse aquatic and terrestrial ecosystems covering various physicochemical, climatic, and biogeographic regimes (King, 1992; Lidstrom, 2006). Aerobic methylotrophic bacteria were isolated from lakes, rivers, marine systems, groundwater, biofilters, the human skin, air, wetlands, rice paddies, agricultural or forest soils, grasslands, sediments, and leaves (King, 1992; Kolb, 2009).

A large fraction of aerobic methylotrophic bacteria found in these habitats belong to the genus of *Methylobacterium*, which is also referred to as pink-pigmented facultative methylotrophic bacterium (PPFM) (Corpe, 1985; Kuono & Ozaki, 1975). Kuono and Ozaki reported that this genus represents one quarter of the detected methylotrophic bacteria in soils, compost, raw sewage, and water samples (Kuono & Ozaki, 1975). About 34 different species of the genus *Methylobacterium* have been identified (Euzéby, 2010); the latter in turn is the largest genus of the family Methylobacteriaceae with its genera:

Meganema, *Methylobacterium*, *Microvirga*, and a group of unclassified genera (Bischoff *et al.*, NCBI Taxonomy Browser); Bacteria (superkingdom) → Proteobacteria (phylum) → Alphaproteobacteria (class) → Rhizobiales (order) → Methylobacteriaceae (family) → *Methylobacterium* (genera). A number of researchers have concentrated on studying the importance of *Methylobacteria* on plant surfaces (Corpe & Rheem, 1989; Delmotte *et al.*, 2009; Hirano & Upper, 1991; Knief *et al.*, 2008; 2010b; Madhaiyan *et al.*, 2009). This genus can be found on the plant's surface above and below the ground, and even as endophytes within the plant (Corpe & Rheem, 1989; Idris *et al.*, 2004; Madhaiyan *et al.*, 2007; Poonguzhali *et al.*, 2008; Schmalenberger & Tebbe, 2002; Fig. 1). The term phyllosphere describes the external leaf surface as microbial habitat in analogy with the rhizosphere of roots (Last, 1955; Ruinen, 1956), later defined as aerial above-ground parts of plants colonized by microbes with their inhabitants called epiphytes (Lindow & Brandl, 2003). In an investigation of phyllosphere colonization, Delmotte *et al.* (2009) identified the Methylobacteriaceae as one of the two dominating families of the microbial community on field-grown plant leaves of soybean, clover and *Arabidopsis thaliana* in a culture-independent way.



Fig. 1. Colonization of a plant leaf by methylobacterial bacteria. The figure indicates a leaf imprint of clover (*Trifolium pratense*) on minimal medium.

Methylobacteria are well-known ubiquitous plant epiphytes and were 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; 2010b). *Methylobacterium* has been shown to benefit from its ability to use methanol as a growth substrate, which is advantageous because the C₁ compound is produced by plants as a by-product of cell wall synthesis (Abanda-Nkpwatt *et al.*, 2006; Fall & Benson, 1996; Sy *et al.*, 2005) and during decomposition of fallen woody materials and plant debris (Ander & Eriksson, 1984; Warneke *et al.*, 1999). Methanol is formed mostly from degradation of methylesters and -ethers that occur in plants (pectin and lignin). The methylobacterial metabolism of *Methylobacterium* has been elucidated over the past 50 years. Detailed investigation of the metabolic processes began after the isolation of the strain *M. extorquens* AM1 (Peel & Quayle, 1961) that became an important model to investigate methylobacterial metabolism (Chistoserdova *et al.*, 2003; Schrader *et al.*, 2009). When grown in the presence of methanol, the C₁ substrate is first oxidized to formaldehyde by

the periplasmic methanol dehydrogenase (Fig. 2), which is essential for growth in the presence of methanol (Nunn & Lidstrom, 1986a; Nunn & Lidstrom, 1986b).

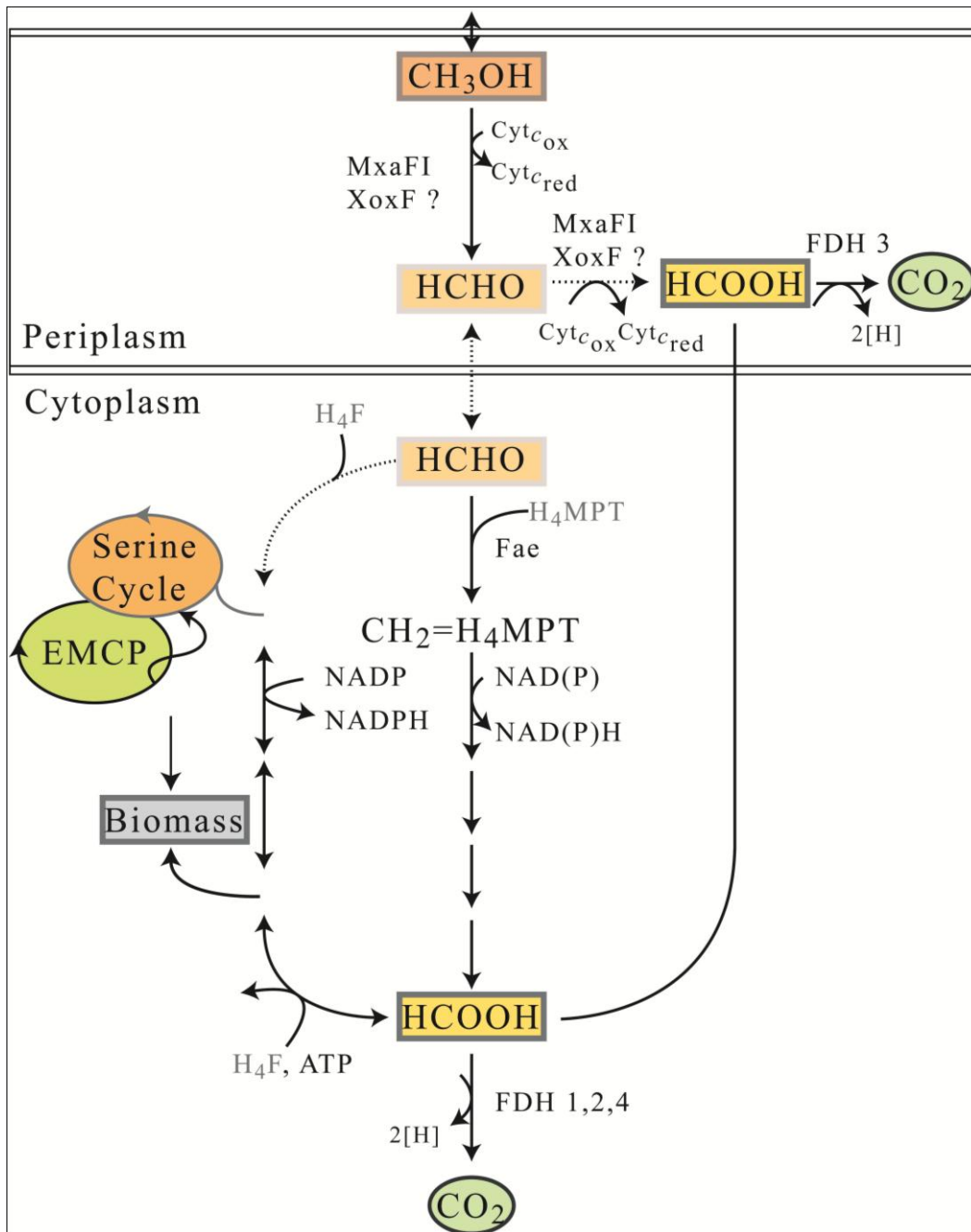


Fig. 2. Schematic view of the one carbon metabolism in *M. extorquens* including XoxF. XoxF is suggested to be involved in periplasmic formaldehyde and/or methanol oxidation. Abbreviations: Cyt_{c_{ox}}, oxidized cytochrome *c*; Cyt_{c_{red}}, reduced cytochrome *c*; FDH, formate dehydrogenase; H₄F, tetrahydrofolate; H₄MPT, tetrahydromethanopterin.

The oxidation of methanol is coupled with reduction of the prosthetic group (pyrroloquinoline quinone) into the corresponding quinol followed by a two step transfer of electrons to the acceptor, which is in case of *M. extorquens* AM1, a highly acidic ferricytochrome c_L , a specific, primary, and physiological electron acceptor for the methanol dehydrogenase; cytochromes c are labeled according to their isoelectric point, with lower (L) $pI = 3-4$, and higher (H) $pI = 8-9$ (Anthony, 1986; Anthony, 1992; DiSpirito, 1990; Nunn & Anthony, 1988). Subsequently electrons are transferred to cytochrome $c_{(H)}$, and to the terminal oxidase (Anthony, 1992).

1.3 The oxidation of methanol to formaldehyde in methylotrophic microorganisms

At least three different types of enzymes have to be distinguished in methylotrophic microorganisms that are responsible for the catalysis of the initial methanol oxidation to formaldehyde (Table 1).

Table 1. The three different types of cofactor-dependent methanol dehydrogenases in methylotrophic microorganisms.

Type	Methylotrophic microorganisms	Cofactor	EC number	Locali- zation	Reference
1	Gram-negative bacteria	PQQ (Quinoprotein)	1.1.99.8 ^a	Periplasmic space	(Anthony, 1986; Duine <i>et al.</i> , 1986)
2	Gram-positive bacteria	NAD(P) ⁺ (Nicotinoprotein)	1.1.1.244 ^b 1.1.1.1 ^c	Cytoplasm	(Bystrykh <i>et al.</i> , 1993a; Hektor <i>et al.</i> , 2000; Reid & Fewson, 1994)
3	Yeasts	FAD (Flavoprotein)	1.1.3.13 ^d	Peroxisome	(Veenhuis & Harder, 1989; Woodward, 1990)

^aPQQ-dependent methanol (alcohol) dehydrogenases, ^bNAD(+) -dependent methanol dehydrogenases, ^cNAD(+) -dependent alcohol dehydrogenases, ^dmethanol (alcohol) oxidases, EC 1.-.-.- oxidoreductases, EC 1.1.-.- acting on the CH-OH group of donors, EC 1.1.1.- With NAD(+) or NADP(+) as acceptor.

Gram-negative bacteria employ a periplasmic pyrroloquinoline quinone (PQQ)-dependent methanol dehydrogenase oxidizing a wide range of primary alcohols (quinoprotein; EC 1.1.99.8, Table 1) that was first investigated in *Pseudomonas* M27 (Anthony & Zatman, 1964b; Anthony & Zatman, 1964c; Anthony & Zatman, 1965; Anthony & Zatman, 1967a;

Anthony & Zatman, 1967b). Methanol oxidation in Gram-negative bacteria appears to be always catalyzed by a NAD⁺-independent, PQQ-dependent enzyme. The periplasmic localization of the PQQ-dependent methanol dehydrogenase of *Hyphomicrobium* sp. strain X could be shown by radiochemical labeling with [¹⁴C] isethionyl acetimidate (Kasprzak & Steenkamp, 1983) and it is known to be periplasmic in other species (Alefounder & Ferguson, 1981; Jones *et al.*, 1982; Quilter & Jones, 1984). The methanol dehydrogenase of the methanotroph *M. album* BG8 was found to be associated to the intracytoplasmic membrane and suggested to be localized in the intracytoplasmic space which is continuous with the periplasm (Brantner *et al.*, 2002).

In general, the methanol dehydrogenase was purified alone (Day & Anthony, 1990; Duine *et al.*, 1978; Liu *et al.*, 2006; Parker *et al.*, 1987), but according to the biochemical and cryo-EM studies of Myronova and coworkers (2006), in methanotrophs it appears as supramolecular protein complex in conjunction with the membrane-bound particulate methane monooxygenase hydroxylase trimer.

Gram-positive methylotrophic bacteria own a cytoplasmic methanol dehydrogenase with NAD(P)(H) as cofactor (nicotinoproteins, EC 1.1.1.244, EC 1.1.1.1, Table 1 (Bystrykh *et al.*, 1993a; Hektor *et al.*, 2000; Reid & Fewson, 1994)). For instance, *Bacillus methanolicus* (Arfman *et al.*, 1989; Arfman *et al.*, 1997), *Brevibacterium methylicum* (Nesvera *et al.*, 1991), the actinomycetes *Amycolatopsis methanolica* (De Boer *et al.*, 1990; Kato *et al.*, 1974), *Mycobacterium gastri* (Kato *et al.*, 1988), and also non-methylotrophic bacteria (*Rhodococcus* and *Desulfovibrio* strains (Nagy *et al.*, 1995; Van Ophem *et al.*, 1991)) possess a methanol dehydrogenase, a methanol:*p* nitroso-N,N'-dimethylaniline oxidoreductase (MNO), or an aldehyde dehydrogenase (ADH). In general, the NAD(P)-dependent alcohol dehydrogenase superfamily (EC 1.1.1.1) is divided into three groups: Group I, zinc-dependent long chain alcohol dehydrogenases with low or no methanol activity; Group II, short chain metal-independent alcohol dehydrogenases with broad substrate specificity; Group III, iron-activated alcohol dehydrogenases, even though iron is not a common feature, rather the sequence similarity and subunit size count as classification parameter (Hektor *et al.*, 2000; Reid & Fewson, 1994). All members of the Group III are microbial and most of them belong to the Gram-positive methylotrophic bacteria with the ability to oxidize methanol *in vivo* (Hektor *et al.*, 2000). The MDH/MNOs of *B. methanolicus*, *A. methanolica*, and *M. gastri* are nicotinoproteins (Arfman *et al.*, 1997; Bystrykh *et al.*, 1993b). It has to be further investigated whether other members of family III are nicotinoproteins as well (Hektor *et al.*, 2002). The purified enzymes of *B. methanolicus* (MDH), *A. methanolica* (MNO), *M. gastri* (MNO), *Desulfovibrio gigas* (ADH), and *Desulfovibrio* HDv (ADH) own a decameric quaternary structure (Arfman *et al.*, 1989; Arfman *et al.*, 1997; Bystrykh *et al.*, 1993b; Hensgens *et*

al., 1993; Hensgens *et al.*, 1995). The well-characterized methanol dehydrogenase of *Bacillus methanolicus*, for instance, contains a tightly non-covalently bound NAD(H) molecule in each subunit (not released during catalysis), one Zn²⁺-ion, one or two Mg²⁺-ions and is strongly stimulated by a specific protein (Kloosterman *et al.*, 2002).

Methylotrophic yeast species are all members of the genera *Pichia*, *Candida* and *Torulopsis* (Van der Klei *et al.*, 2006). The most well-known species are: *P. pastoris*, *P. methanolica*, *C. boidinii* and *Hansenula polymorpha*. In contrast to prokaryotic methylotrophs, eukaryotic methylotrophs are only able to use the C₁ compound methanol as growth substrate and no other C₁ sources (Van der Klei *et al.*, 2006). Yeasts possess a usually octameric alcohol oxidase that contains one non-covalently bound FAD cofactor molecule per subunit (flavoprotein, EC1.1.3.13), and belongs to the family of glucose-methanol-choline oxidoreductases (Ozimek *et al.*, 2005). This enzyme exhibits broad substrate specificity, though it catalyzes *in vivo* only the oxidation of methanol to formaldehyde and hydrogen peroxide using oxygen as electron acceptor (Van der Klei *et al.*, 2006). It is compartmentalized in peroxisomes, where the formed hydrogen peroxide is dismutated directly by a catalase (CAT) to oxygen and water, and where formaldehyde (C₁) and xylulose (C₅) are assimilated by a dihydroxyacetone synthase (DHAS) to form two C₃ molecules dihydroxyacetone and glyceraldehyde 3-phosphate (xylulose-5-phosphate cycle) (Van der Klei *et al.*, 2006; Veenhuis & Harder, 1989; Woodward, 1990).

1.4 Pyrroloquinoline quinone-dependent methanol dehydrogenases

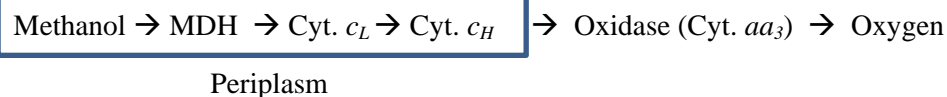
The three different types of methanol dehydrogenases in methylotrophic microorganisms have already been described in detail in this work according to Gram-negative/-positive bacteria and yeasts (1.3; plus Table 1). Anthony further subdivided the PQQ-containing alcohol dehydrogenases in three types: type I includes the "classic" MDH, type II the soluble quinoxinoprotein, and type III the membrane-bound quinoxinoprotein with two additional subunits (Anthony, 2001). In this part the reader's attention should be drawn to the most prevalent methanol dehydrogenase (the "classical" one, type I) in methylotrophic bacteria: the periplasmic pyrroloquinoline quinone-dependent one that is present in Gram-negative bacteria (EC 1.1.99.8, Table 1.). The broad-specificity of this type of methanol dehydrogenase has been first described by Anthony and Zatman (1964c). *In vitro*, methanol dehydrogenase can be coupled to the artificial electron acceptors phenazine (m)ethosulfate or Wurster's blue (NNN'-tetramethyl-*p*-phenylenediamine) in the presence of ammonia or other ammonium compounds, linked to oxygen in an oxygen electrode, or linked to reduction of dichlorophenolindophenol (DCPIP) and analyzed

spectrophotometrically (Day & Anthony, 1990; Frank & Duine, 1990). MDH oxidizes many primary alcohols to the corresponding aldehyde with the release of two protons and two electrons (Table 2). Ghosh and Quayle (1979) advise the assay user to replace phenazine methosulfate (PMS) by phenazine ethosulfate (PES), since the latter exhibits higher stability. However, Frank and Duine observed that dealkylation of PES, especially at high pH leads to aldehyde production and further to endogenous activity (Frank & Duine, 1990).

The cytochrome c_L serves as specific physiological electron acceptor for the methanol dehydrogenase (Anthony, 1986; Anthony, 1992; DiSpirito, 1990; Nunn & Anthony, 1988). The electrons are transferred from PQQ to heme c . Chemical modification studies of Cox and his coworkers (1992) indicated that electrostatic interactions (specific interactions: hydrogen-bonds, salt bridges, ion pairs) exist between methanol dehydrogenase ($\alpha_2\beta_2$) and cytochrome c_L in *M. extorquens*, a facultative methylotrophic bacterium, as well as in *M. methylotrophus*, an obligate methylotrophic bacterium: interaction by means of the carboxyl groups on cytochrome c_L and amino groups of lysyl residues on the α -subunit of MDH. Dales and Anthony confirmed that electrostatic interactions (ionic interactions) are involved in the docking process (Dales & Anthony, 1995). However, Harris and Davidson suggested that hydrophobic interactions (non-specific) prevail between both enzymes in *P. denitrificans* (Harris & Davidson, 1993). One year later Harris *et al.* could prove ionic strength dependence of the same reaction in *P. denitrificans* (Harris *et al.*, 1994). In 2006, Nojiri *et al.* demonstrated that a transient complex between MDH and cytochrome c_L is formed in *Hyphomicrobium denitrificans* during the electron transfer with a pH optimum of 5.5, and they propose that cytochrome c_L reacts transiently as dimer during the predominantly electrostatic binding process (Nojiri *et al.*, 2006).

Crosslinking appeared predominantly with cytochrome c_L and the α -subunit of MDH, but not with the β -subunit (Cox *et al.*, 1992). The cytochrome c_L (core of class I c-type cytochromes, heme-binding motif CSGCH, unusual disulfide bridge, unique calcium-binding site, unusual ligand to heme iron (Williams *et al.*, 2006)) is subsequently oxidized by cytochrome c_H (class I c-type cytochrome, resembles mitochondrial cytochrome c_I rather than that of bacterial cytochromes c_2) (Read *et al.*, 1999). The cross-linking studies of Cox *et al.* showed that ionic interactions between the same carboxylate residues (Asp⁸³ and another aspartate or glutamate) from cytochrome c_L are reacting first with MDH and consecutively with Cyt c_H , and in addition, the interaction takes place via hydrophobic residues around the heme edge of cytochrome c_L (Cox *et al.*, 1992; Dales & Anthony, 1995; Williams *et al.*, 2005). All well-known methylotrophic bacteria possess at least two

soluble cytochromes c that are completely distinct proteins (Beardmore-Gray *et al.*, 1982; Cross & Anthony, 1980a; Cross & Anthony, 1980b; O'Keeffe & Anthony, 1980; Van Verseveld & Stouthamer, 1978). After the electron transfer from cytochrome c_L to cytochrome c_H , and from the latter to a membrane-bound cytochrome oxidase (usually cytochrome aa_3 or o), the electrons reach the final acceptor oxygen (Anthony, 1992). The electrons are transported from electron carriers with a low redox potential to those with a higher one. The mid-point redox potential of the formaldehyde/methanol couple is -0.182 V, it amounts to $+0.090$ V at pH 7.0 for the PQQ/PQQH₂ couple (Duine *et al.*, 1981), and it mounts up to $+0.256$ V for cytochrome c_L , and $+0.294$ V for cytochrome c_H in *M. extorquens* AM1 (Anthony, 1982). The oxidation of methanol to formaldehyde is coupled to an acidification of the external suspending medium (2 H⁺ per mole methanol), and a proton motive force is established by the electron transport chain which is sufficient to drive the synthesis of less than one molecule ATP per mole of methanol oxidized (Williams *et al.*, 2005). Interestingly, no three-protein complex between methanol dehydrogenase (MDH), cytochrome c_L (Cyt c_L), and cytochrome c_H (Cyt c_H) was observed:



The proton and electron steps are kinetically and mechanistically separated (see Mechanism of MDH, 1.4, page 31). The first three soluble enzymes operate in the periplasm and are co-induced together with several other proteins of the *mx*a cluster (Lidstrom, 1990) which are involved e.g. in processing of MDH (Matsushita *et al.*, 1993), in calcium insertion (MxaAKL), in assembly (MxaJ), in regulation, or in PQQ-biosynthesis (Meulenbergh *et al.*, 1992). MDH often constitutes 10 to 20% of the total cell protein e.g. in *Hyphomicrobium X* (Kasprzak & Steenkamp, 1983), *Methylophilus methylotrophus* (0.5 mM) (Jones *et al.*, 1982; Kasprzak & Steenkamp, 1983), and *M. extorquens* strain AM1 (Nunn & Lidstrom, 1986a). The low K_m value of cytochrome c_L (1.2 μ M) suggests that the MDH and cytochrome c_L are mainly found in the associated form (Anthony, 1986; Carver *et al.*, 1984). Although MDH was purified and dialyzed, even without substrate the enzyme catalyzes the reduction of the artificial (PES/PMS) or the natural electron acceptor (Cyt c_L). It was described that cytochrome c is capable of autoreducing the heme iron without added substrate, and this autoreduction is induced by the MDH (Beardmore-Gray *et al.*, 1982; Beardmore-Gray & Anthony, 1983; O'Keeffe & Anthony, 1980). In *Acetobacter methanolicus* MxaJ (32 kDa) has been isolated in association with MDH

($\alpha_2\beta_2\gamma$), but it does not alter its activity and might play a role in assembly of MDH (Matsushita *et al.*, 1993; Van Spanning *et al.*, 1991).

General characteristics (Table 2) of pyrroloquinoline quinone-dependent methanol dehydrogenases are: a high pH optimum for enzyme activity ranging from 9.0 to 10.5, a predominant high isoelectric point on average about 8.0 (exceptions e.g.: MDH of *P. denitrificans* & *Pseudomonas fluorescens* S50 with a *pI* at about 4.0), a total molecular weight (116 to 158 kDa) of usually tetrameric conformation ($\alpha_2\beta_2$) with identical dimers of large (59–76 kDa) and small (8–10 kDa) subunits. The most important feature is the ability to oxidize aldehydes and a wide range of primary alcohols including, for example, crotyl alcohol, bromoethanol, phenylethanol and cinnamyl alcohol. The affinity often decreases with increasing chain length of the alcohol, however, maximum rates are often similar to that for methanol (Anthony, 1986; Duine & Frank, 1980). The highest substrate affinity occurs towards methanol (K_m ranging from 0.003 - 1.530 mM, exception: *Rhodopseudomonas acidophila* with a K_m of 145 mM), and an approximately similar one can be observed for formaldehyde (K_m ranging from 0.01 - 2.3 mM, exception: *Paracoccus denitrificans* with a K_m of 8.59 mM). In contrast, the oxidation of acetaldehyde (*Acetobacter methanolicus*, *Hyphomicrobium* WC, *Methylophilus methylotrophus*, *Rhodopseudomonas acidophila*, *Pseudomonas* TP-1, *Pseudomonas* W1, *Methylophaga marina*), propionaldehyde (*Methylophilus methylotrophus*, *Methylocystis* sp. GB 25, *Rhodopseudomonas acidophila*), secondary alcohols (*Rhodopseudomonas acidophila*, *Methylophaga marina*), aromatic alcohols (*Methylosinus trichosporium*), and alcohols substituted with a methyl group (*Pseudomonas* TP-1, *Pseudomonas* W1, *Pseudomonas* sp. No. 2941, *Hyphomicrobium* WC) is exceptional. By means of chemical models of the active site Itoh *et al.* verified that the substrate affinity is determined by dimensions of the active site (Itoh *et al.*, 1998). Table 2 gives a general overview of the purified and characterized PQQ-dependent methanol dehydrogenases in the last five decades from 26 methylotrophic bacteria. The oxidation pattern for whole cells resembles the one of the purified enzyme with regard to the substrate range (Anthony, 1986). MDH catalyzes formaldehyde not only in the dye-linked system, but also in the cytochrome-coupled system (Beardmore-Gray & Anthony, 1983). It is difficult to make a general statement about the inhibitors of the MDH. The methanol oxidation by whole bacterial cells is often inhibited by EDTA, *p*-nitrophenylhydrazine and high phosphate concentrations, whereas the oxidation measured via the dye-linked assay is sometimes inhibited by PMS, cyclopropanol, cyanide, oxygen, or ammonia at high concentrations (Anthony & Zatman, 1964a; Bamforth & Quayle, 1978a; Beardmore-Gray *et al.*, 1982; Dijkstra *et al.*, 1984; Harris & Davidson, 1993; Harris & Davidson, 1994).

Table 2. Summary of physiological specified and described PQQ-dependent methanol dehydrogenases (EC 1.1.99.8) divided into facultative and obligate methylotrophic bacteria.

Organism	Optimal pH for maximal enzyme activity	Activator	Substrates	Assay	MW (kDa)	Ref.
<i>Paracoccus denitrificans</i> facultative methylotrophic bacterium	pH 9.0 - 9.5 <i>pI</i> 3.7 Specific activity (μmol/mg/min) =4.2 One single atom of Ca ²⁺ per α ₂ β ₂ tetramer	Ammonium chloride best activator K _a 4 mM, CH ₃ NH ₃ +Cl ⁻ K _a 91 mM; 3 mM KCN was required to give 100% inhibition of the endogenous reaction, but KCN is also an activator	Methanol (K _m 0.56 mM, V _{max} 7.33 U/mg) primary alcohols, formaldehyde (higher V _{max} compared to methanol, V _{max} 12.6 U/mg, K _m 8.59 mM) (Harris & Davidson, 1993)	DCPIP/PMS-dependent assay, less endogenous reaction with Wurster's blue → oxygen competes with PES, but not Wurster's blue, as an electron acceptor for the reduced form of PQQ in methanol dehydrogenase (Davidson <i>et al.</i> , 1992a)	Total molecular mass 153 kDa, hetero-tetramer, subunit sizes 67 kDa and 9.5 kDa	1
<i>Pseudomonas</i> sp. No. 2941 facultative methylotrophic bacterium	pH 9.6 <i>pI</i> 7.38	Ammonium ions, Ammonium chloride K _a 5 mM	Methanol (K _m 20 μM), primary alcohols (C ₁ to C ₇), formaldehyde (K _m 70 μM), methoxy-ethanol and 2-methyl-n-butanol	DCPIP/PMS-dependent assay	128 kDa, subunit molecular weight 62 kDa	2
<i>Pseudomonas</i> M27 facultative methylotrophy	pH 9.0 <i>pI</i> 7 -8	Ammonium chloride, ammonia or methylamine	Methanol (K _m 20 μM)	DCPIP/PMS-dependent assay	120 kDa	3

Organism	Optimal pH for maximal enzyme activity	Activator	Substrates	Assay	MW (kDa)	Ref.
<i>Rhodopseudomonas acidophila</i> , now: <i>Rhodoblastus acidophilus</i> gen. nov., comb. nov. facultative methylotroph	pH 9.0 pI 9.35 Specific activity (μmol/mg/min) =17.9	Ammonium chloride K _a 42 mM, CH ₃ NH ₃ +Cl ⁻ K _a 2.1 mM, nonylamine best activator	Methanol (K _m 145 mM), ethanol K _m 0.03 mM, acetaldehyde (K _m 3.1 mM), secondary alcohols	DCPIP/PMS-dependent assay	116 kDa, subunit molecular weight 63 kDa	4
<i>Methylophilus methylotrophus</i> facultative methylotrophic bacterium	pH 9.8 One single atom of Ca ²⁺ per α ₂ β ₂ tetramer	Ammonium chloride, methylamine hydrochloride	Methanol (K _m 0.020 mM), primary aliphatic alcohols (C ₁ to C ₄), formaldehyde (K _m 1.01 mM), acetaldehyde very slowly	DCPIP/PMS-dependent assay	138 kDa ; hetero-tetramer, large subunit MW 62 kDa	5
			Methanol (K _m 0.02 mM), 1,2-propanediol (K _m 174 mM), formaldehyde (K _m 0.25 mM)	DCPIP/PES-dependent assay		6
<i>Methylophilus methylotrophus</i> W3A1 facultative methylotroph		Ammonium chloride	(Enzymes partially purified) Methanol (K _m 0.02 mM), ethanol (K _m 0.03 mM)	DCPIP/PMS-dependent assay		7

Organism	Optimal pH for maximal enzyme activity	Activator	Substrates	Assay	MW (kDa)	Ref.
<i>Acetobacter methanolicus</i> facultative methylotrophic bacterium	pH 9.5	Ammonium chloride	Methanol & other primary alcohols, formaldehyde, acetaldehyde	DCPIP/PMS-dependent assay	Type I MDH is a dimer (58 to 62 kDa large SU & 8.7 to 10 kDa small SU), type II MDH was suggested to form a trimer (containing MxaJ (32 kDa))	8
<i>Methylobacterium</i> AM1 or <i>Pseudomonas</i> AM1 facultative methylotrophic bacterium	pH 9.0 One single atom of Ca ²⁺ per $\alpha_2\beta_2$ tetramer	Ammonium chloride Ammonium chloride (K _a 2 mM)	Methanol (K _m 0.01 mM), ethanol (K _m 0.01 mM), formaldehyde (K _m 0.01 mM), 1,2-propanediol (K _m 111 mM), 1,3-butanediol (K _m 11 mM), 1,3-propanediol (K _m 0.8 mM) Methanol (K _m 0.003 mM), V _{max} 810 (nmol/min per mg)	DCPIP/PES-dependent assay DCPIP/PES-dependent assay	Tetramer $\alpha_2\beta_2$ conformation, α -subunit 66 kDa, β -subunit 8.5 kDa	9 10
<i>Methylobacterium organophilum</i> Facultative methylotroph (methanotroph)	pH 10.0 - 10.5	Ammonia is not required, but stimulates activity	Primary alcohols (C ₁ to C ₈), methanol (K _m 0.029 mM), formaldehyde (K _m 0.082 mM)	DCPIP/PMS-dependent assay	135 kDa, dimeric, subunits of 62 kDa	11

Organism	Optimal pH for maximal enzyme activity	Activator	Substrates	Assay	MW (kDa)	Ref.
<i>Methylobacterium</i> R6 (only cell suspensions and cell extracts, no purification) facultative methylotrophic bacterium (methanotroph)	pH 9.5	Ammonia	Methanol, formaldehyde, primary alcohols (C ₁ to C ₁₀)	DCPIP/PMS-dependent assay, oxygen electrode		12
<i>Hyphomicrobium</i> WC facultative methylotrophic bacterium		Ammonium ions	Normal aliphatic alcohols, methanol, formaldehyde, and to a limited extent acetaldehyde, glycolamide, 10-hydroxy-decanoic acid, formaldehyde (K _m 0.032 mM), methanol (K _m 0.014 mM), ethanol (K _m 0.016 mM), methyl group on primary alcohol lowers activity	DCPIP/PMS-dependent assay	120 kDa	13

Organism	Optimal pH for maximal enzyme activity	Activator	Substrates	Assay	MW (kDa)	Ref.
<i>Hyphomicrobium</i> X facultative methylotrophic bacterium	pH 9.0 One single atom of Ca ²⁺ per $\alpha_2\beta_2$ tetramer	Ammonium salts	Methanol and other primary alcohols, formaldehyde	Wurster's blue, DCPIP/PMS or PES-dependent assay	120 kDa, heterotetramer	14
<i>Pseudomonas</i> TP-1 facultative methylotrophic bacterium		Ammonium ions	Normal aliphatic alcohols, methanol, formaldehyde, and to a limited extent acetaldehyde, 10- hydroxy-decanoic acid, formaldehyde (K_m 0.039 mM), methanol (K_m 0.017 mM), ethanol (K_m 0.017 mM), methyl group on primary alcohol lowers activity	DCPIP/PMS-dependent assay	120 kDa	13
<i>Pseudomonas</i> W1 obligate methylotrophic bacterium		Ammonium ions	Methanol (K_m 0.015 mM), formaldehyde (K_m 0.033 mM), ethanol (K_m 0.016 mM), to a limited extent acetaldehyde, 10-hydroxydecanoic acid	DCPIP/PMS-dependent assay	120 kDa	13

Organism	Optimal pH for maximal enzyme activity	Activator	Substrates	Assay	MW (kDa)	Ref.
<i>Methylophaga</i> sp. 1 obligate methylotrophic bacterium MDH1	Alkaline pHs, Mn ²⁺ highly inhibitory, Ca ²⁺ and Mg ²⁺ no effect	Ammonium chloride	Primary alcohols (activity decreases with longer chains), methanol (K _m 1.53 mM), ethanol (K _m 5.40 mM), formaldehyde with similar activity to methanol	DCPIP/PES -dependent assay	Two subunits of 59 kDa and 8.8 kDa	15
<i>Methylophaga</i> sp. 1 obligate methylotrophic bacterium MDH2 stronger overall negative charge than MDH1	Alkaline pHs, Mn ²⁺ highly inhibitory, Ca ²⁺ and Mg ²⁺ no effect	Ammonium chloride	Primary alcohols (activity decreases with longer chains), methanol (K _m 1.05 mM), ethanol (K _m 5.19 mM), formaldehyde with similar activity to methanol	DCPIP/PES -dependent assay	Two subunits of 59 kDa and 8.8 kDa	15
<i>Methylococcus</i> <i>capsulatus</i> (Bath) obligate methylotrophic bacterium (methanotroph)	pH 9.0	Ammonium chloride	Methanol (K _m 0.48 mM), formaldehyde (K _m 0.77 mM).	DCPIP/PES-dependent assay	α ₂ β ₂ of 64 and 8 kDa subunits	16

Organism	Optimal pH for maximal enzyme activity	Activator	Substrates	Assay	MW (kDa)	Ref.
Strain 4025 (partially purified) obligate methylotrophic bacterium	pH 9.0	Ammonia, methylamine less efficient	Methanol, ethanol, n-propanol, formaldehyde	PES-linked manometric assay		17
<i>Methylosinus</i> sp. WI 14 obligate methylotrophic bacterium (methanotroph)	pH 9.0, pI 8.3 calcium ions do not have any influence on the activity, Copper (Cu ²⁺) and especially iron (Fe ²⁺) ions are strong inhibitors	Ammonia	Catalyzes the oxidation of methanol (K _m 0.45 mM) to formate directly. Primary alcohols (C ₁ to C ₈) and several aldehydes up to propionalde-hyde were oxidized , highest affinity for sorbic alcohol (K _m 0.18 mM) and the lowest for 1-butanol (K _m 6.4 mM)	DCPIP/PMS-dependent assay	140 kDa, two identical subunits 70 kDa	18
<i>Methylocystis</i> sp. GB 25 obligate methylotrophic bacterium (methanotroph)	pH 9.0 pI 8.3 Copper (Cu ²⁺) and iron (Fe ²⁺) ions are strong inhibitors	Ammonia	Primary alcohols (C ₁ to C ₇), formaldehyde to propion- aldehyde, and glutaraldehyde, formaldehyde (K _m 0.1 mM), methanol (K _m 0.34 mM), ethanol (K _m 0.89 mM)	DCPIP/PMS-dependent assay	120 kDa, two identical subunits of about 60 kDa	19

Organism	Optimal pH for maximal enzyme activity	Activator	Substrates	Assay	MW (kDa)	Ref.
<i>Pseudomonas fluorescens</i> S50	pI 3.82	-	Primary alcohols oxidized	DCPIP/PMS-dependent assay	158,000 Da, subunit molecular weight 76 kDa	20
<i>Methylomonas methanica</i> obligate methylotrophic bacterium (methanotroph)	pH 9.5 high pI	Ammonium salts	Primary alcohols (C ₁ to C ₁₀), 3-chloro-1-propanol, 4-chloro-1-butanol, 6-chloro-1-hexanol, 2-phenoxy-ethanol, 2-methoxy-ethanol, 2-aminoethanol, formaldehyde	DCPIP/PMS-dependent assay	60 kDa (monomeric)	21
<i>Methylomonas</i> J formerly: <i>Pseudomonas</i> sp. J, obligate methylotroph (methanotroph)	pH 9.0 pI 9.3	Ammonium chloride, methylamine hydrochloride	Methanol (K _m 0.33 mM), ethanol (K _m 0.74 mM), formal-dehyde (K _m 2.3 mM), primary alcohols (C ₁ to C ₄), 2-chloro-ethanol, 2-bromo-ethanol	DCPIP/PMS-dependent assay	135 kDa, subunit molecular weight 60 kDa & 10 kDa	22

Organism	Optimal pH for maximal enzyme activity	Activator	Substrates	Assay	MW (kDa)	Ref.
<i>Methylosinus trichosporium</i> obligate methylo-trophic bacterium (methanotroph)	pH 9.0 <i>pI</i> 6 to 7	Ammonium chloride, but not potassium or sodium	Aromatic alcohols (benzyl, vanillyl, veratryl alcohols), with K_m range = 1 to 2 mM, aliphatic primary alcohols (C_1 to C_8), 2-methoxy-ethanol, methanol (K_m 50 μ M)	DCPIP/PMS-dependent assay	60 kDa (identical subunits) Total 120 kDa	23 24
<i>Methylosinus sporium</i> obligate methylotrophic bacterium (methanotroph)	pH 9.0 high <i>pI</i>	Ammonium compounds: Ammonium chloride, ammonium sulfate, sulfamate, phosphate, formate, thiocyanate, molybdate, acetate could replace ammonium chloride as activator, but not potassium or sodium salts	Primary alcohols (C_1 to C_{10}), 3-chloro-1-propanol, 4-chloro-1-butanol, 6-chloro-1-hexanol, 2-phenoxyethanol, 2-methoxy-ethanol, 2-amino-ethanol, phenethyl-alcohol, cinnamyl alcohol, and formaldehyde	DCPIP/PMS-dependent assay with the artificial electron acceptors tested: phenazine ethosulfate, ethylphenazinium ethosulfate, or 5-methyl-phenazinium methyl-sulfate	60 kDa	21

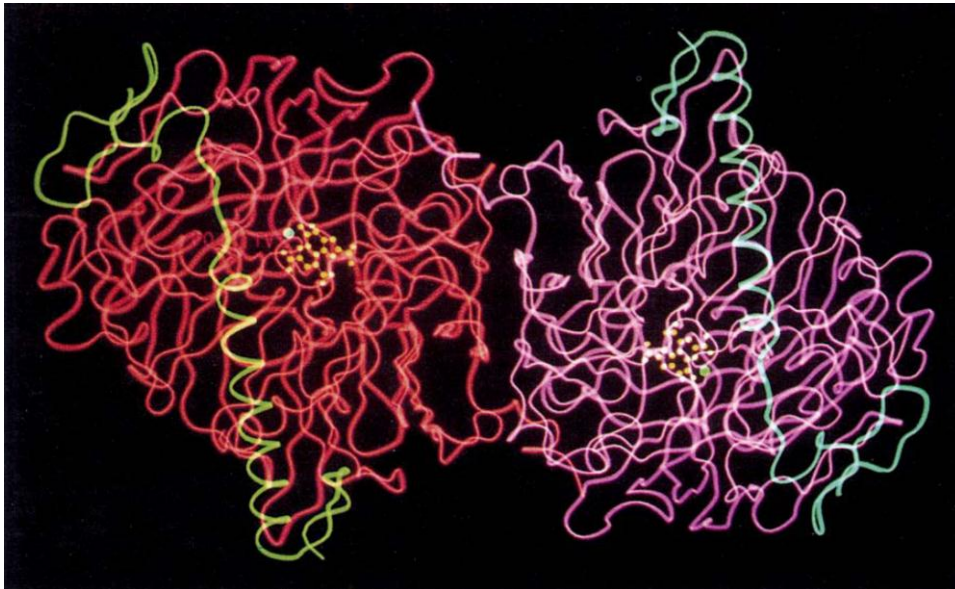
Organism	Optimal pH for maximal enzyme activity	Activator	Substrates	Assay	MW (kDa)	Ref.
<i>Methylococcus capsulatus</i> (Texas) obligate methylotrophic bacterium methanotroph	pH 9.0 high <i>pI</i>	Ammonium ions	Methanol, other primary alcohols (C ₂ to C ₅), formaldehyde	DCPIP/PMS-dependent assay	120 kDa (dimer with 62 kDa subunits)	25
<i>Methylophaga marina</i> obligate methylotrophic bacterium	pH 9.0 pI 6.4	Ammonium chloride	Methanol (K _m 20 μM), multicarbon primary alcohols (C ₁ to C ₈), aldehydes (formaldehyde, acetaldehyde), secondary alcohols (propan-2-ol, butan-2-ol, octan-2-ol)	DCPIP/PMS-dependent assay	145 kDa (dimer, 65 kDa subunit size)	26

DCPIP (2,6-dichlorophenol indophenol); PMS (phenazine methosulfate); PES (phenazine ethosulfate); *pI* (isoelectric point); Ref.(reference); MW (molecular weight).
1 (Bamforth & Quayle, 1978a; Davidson *et al.*, 1992b; Harris & Davidson, 1993; Nunn *et al.*, 1989), 2 (Yamanaka & Matsumoto, 1977b), 3 (Anthony & Zatman, 1964c; Anthony & Zatman, 1967a), 4 (Bamforth & Quayle, 1978b; Bamforth & Quayle, 1979; Sahn *et al.*, 1976), 5 (Ghosh & Quayle, 1981; Xia *et al.*, 1992), 6 (Page & Anthony, 1986), 7 (Kalyuzhnaya *et al.*, 2008), 8 (Matsushita *et al.*, 1993), 9 (Nunn *et al.*, 1989; Page & Anthony, 1986), 10 (Goodwin & Anthony, 1996), 11 (Wolf & Hanson, 1978), 12 (Patel *et al.*, 1978b), 13 (Sperl *et al.*, 1974), 14 (Duine *et al.*, 1978; Frank *et al.*, 1988), 15 (Chang *et al.*, 2002), 16 (Adeosun *et al.*, 2004; Myronova *et al.*, 2006), 17 (Vrdoljak & Froud, 1982), 18 (Grosse *et al.*, 1998), 19 (Grosse *et al.*, 1997), 20 (Yamanaka & Matsumoto, 1977a), 21 (Patel & Felix, 1976; Patel *et al.*, 1978a), 22 (Ohta *et al.*, 1981), 23 (Mountfort, 1990), 24 (Parker *et al.*, 1987), 25 (Patel *et al.*, 1972), 26 (Janvier & Gasser, 1987)

The structure of MDH (Fig. 3a,b) has been solved for *Methylobacterium extorquens* (Afolabi *et al.*, 2001; Anthony & Ghosh, 1998; Blake *et al.*, 1994; Ghosh *et al.*, 1995; Williams *et al.*, 2005) and *Methylophilus* sp. (White *et al.*, 1993; Xia *et al.*, 1996; Xia *et al.*, 1992; Xia *et al.*, 1999; Zheng *et al.*, 2001).

At the active site of methanol dehydrogenase ($\alpha_2\beta_2$ tetramer) one molecule pyrroloquinoline quinone can be found tightly but non-covalently bound per large (α) subunit (2 mol PQQ per mol tetramer), plus one tightly bound divalent calcium cation (*M. extorquens*, *M. methylotrophus*, *P. denitrificans*, *Hyphomicrobium* X.) that is in turn co-ordinated to residues on the protein and also to PQQ (Adachi *et al.*, 1990; Blake *et al.*, 1994; Richardson & Anthony, 1992; White *et al.*, 1993). Additionally, water molecules were found in the structure of *M. extorquens* W3A1 near the active site hydrogen-bonded with Ca^{2+} , PQQ and Glu¹⁷⁷ (Afolabi *et al.*, 2001; Ghosh *et al.*, 1995; White *et al.*, 1993; Williams *et al.*, 2005; Xia *et al.*, 1996; Xia *et al.*, 1992; Xia *et al.*, 1999). The subunits are held very tightly together and cannot be dissociated by high salt concentrations, however, via SDS or guanidinium chloride (Cox *et al.*, 1992; Nunn *et al.*, 1989). The funnel at the entrance to the active site in the α -subunit of MDH mainly consists of hydrophobic residues (Anthony *et al.*, 1994; Ghosh *et al.*, 1995). The small, lysine-rich β -subunit folds partly around the α -subunit. Its function is still unknown, it exhibits no hydrophobic core (Anthony & Ghosh, 1998), it is absent in other quinoproteins, and it does not form a real domain in the MDH molecule (Anthony & Williams, 2003). The small and large subunits cannot be reversibly dissociated and ion-pair interactions are found most often between both. The α -subunit exhibits a propeller fold consisting of eight β -sheets that are radially arranged like propeller blades, and interacting via tryptophan-docking. The PQQ is sandwiched between the indole ring of a tryptophan residue (almost co-planar) and a disulfide ring between two adjacent cysteine residues (Cys¹⁰³-Cys¹⁰⁴), which function is not unequivocally elucidated and which is absent in glucose dehydrogenase. It might be involved in the stabilization of the free radical semiquinone or its protection from solvent at the entrance of the active site in MDH (Avezoux *et al.*, 1995; Blake *et al.*, 1994) or it might be essential for intra-protein electron transfer in all alcohol dehydrogenases (Oubrie *et al.*, 2002). Site-directed mutagenesis of one or both cysteine residues to serine led to production of only small amounts of non-active methanol dehydrogenase in *M. extorquens* (Afolabi *et al.*, 2001; Anthony & Williams, 2003).

a)



b)

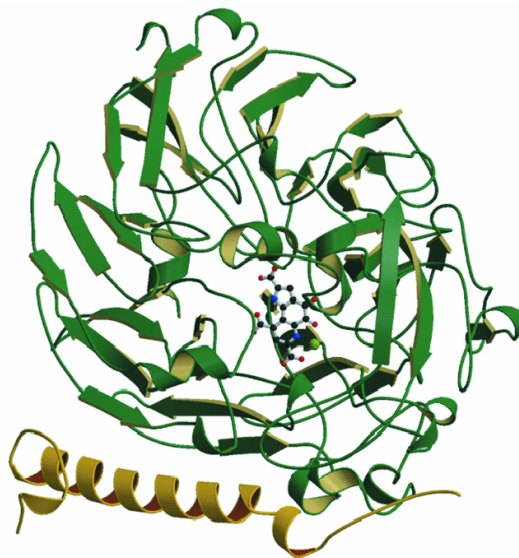


Fig. 3. The structure of methanol dehydrogenase. a) The overall tetrameric structure of methanol dehydrogenase from *M. extorquens* AM1 as ribbon drawing, looking down the molecular symmetry axis with the large, catalytic active α -subunits (red and lilac, 66 kDa each), small β -subunits (green and turquoise, 8.5 kDa each), pyrroloquinoline quinone (PQQ, yellow circles) and calcium ions (blue circles) (Ghosh *et al.*, 1995). b) An $\alpha\beta$ -unit from methanol dehydrogenase. The propeller-structured α -subunit (green, MxaF) exhibits PQQ and a calcium ion (green sphere) at the active site, the small β -subunit (yellow, MxaI) wraps around the side (Williams *et al.*, 2005).

By means of a *Pseudomonas aeruginosa* mutant strain, lacking the disulfide ring in the PQQ-dependent ethanol dehydrogenase, experimental evidence was provided for an essential requirement of the disulfide ring for efficient electron transfer to cytochrome c_{550} (Mennenga *et al.*, 2009). Afore Oubrie *et al.* (2002), Chen *et al.* (2002), Anthony and Williams (2003) and Xia *et al.* (2003) have proposed the involvement of the disulfide ring

in the electron transfer chain based on theoretical calculations with the aid of X-ray structures.

The prosthetic group pyrroloquinoline quinone (2,7,9-tricarboxypyrroloquinoline quinone = PQQ) exhibits a unique heterocyclic *o*-quinone structure (Xia *et al.*, 1996; Fig. 4):

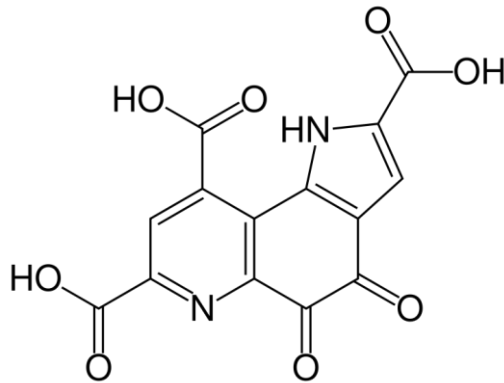


Fig. 4. Chemical structure of PQQ (pyrroloquinoline quinone), $C_{14}H_5O_8N_2$.

PQQ (without the apoenzyme, 249 nm, ϵ 18,400 $M^{-1} cm^{-1}$) is chemically very active, especially the C5 carbonyl group: it catalyzes, for example the oxidation of NADH, amines, basic amino acids, alcohols, aldehydes, ketones, cyanide, and glucose (Dekker *et al.*, 1982; Ohshiro & Itoh, 1988); and it forms a stable free radical under anaerobic conditions. PQQ is able to complex divalent metal ions in solution (affinities: $Ca^{2+} > Sr^{2+} > Ba^{2+}$) (Itoh *et al.*, 1998; Mutzel & Gorisch, 1991). The acidic cofactor pyrroloquinoline quinone (PQQ), former name methoxatin, was first extracted (boiling, acid, or alkali treatment) and purified from alcohol dehydrogenases by Anthony and Zatman (1967b). Later, Salisbury (1979) and Duine plus colleagues (Westerling *et al.*, 1979) characterized the prosthetic group in detail. It is reduced by two electrons at a relatively high midpoint redox potential (+0.090 V, pH 7) in comparison to FAD (-0.045 V) or NAD^+ (-0.320 V) (Matsushita *et al.*, 1989). Three redox forms have been identified to be involved in biological oxidations (PQQ, $PQQH^{\bullet}$ and $PQQH_2$). The PQQ of isolated enzymes can be usually found in the semiquinone free-radical form ($PQQH^{\bullet}$) (de Beer *et al.*, 1983; Dijkstra *et al.*, 1989; Duine *et al.*, 1981; Frank *et al.*, 1988; Salisbury *et al.*, 1979), therefore the enzyme has to be oxidized by phenazine dye or Wurster's Blue, before the reduction by added substrate can be measured. PQQ is also present e.g. in methylamine dehydrogenase from some Gram-negative bacteria, in methylamine oxidase from *Arthrobacter* P1 (Duine & Frank, 1990), or in aldose (glucose) dehydrogenases. The quinoprotein often occurs in its apoform, for instance in *E. coli* (glucose dehydrogenase) or *P. testosteroni* (ethanol dehydrogenase). The apoenzyme of glucose dehydrogenase

can be reconstituted with PQQ to an active enzyme (Anthony, 2000; Cozier *et al.*, 1999; Duine *et al.*, 1980). The water-soluble, reddish PQQ shows in water absorption at 475 nm (PQQ & PQQ-H₂O, hydrated at C₅ position) emitting green light (maximum at low pH, excitation maximum, about 365 nm; emission maximum, about 460 nm), whereas adducts formed by aldehydes, ketones etc. fluoresce blue (Anthony & Zatman, 1967b; Dekker *et al.*, 1982). PQQ-containing quinoproteins show a characteristic absorption at 300-420 nm (peak 345-350 nm, shoulder at about 400 nm); without the PQQ they exhibit a peak at 280 nm and a shoulder at 290 nm (Duine *et al.*, 1981). The methanol dehydrogenase of the purified *M. extorquens mxaA* mutant exhibits no calcium ions in the active site and is characterized by a strongly different spectrum with a decrease of the peak at 345 nm (Goodwin & Anthony, 1996). A similar spectrum change has been observed for the special methanol dehydrogenase of *Methylophaga marina* that sometimes lacks calcium (inactive, red coloured) as a result of a relatively low affinity towards the ion, which might be explained by the organism's natural habitat - the seawater with a calcium content of 10 mM (Chan & Anthony, 1992). In the review "The PQQ story" by J.A. Duine (Duine, 1999), methods of PQQ determination are discussed. It is mentioned that the redox-cycling assay is not reliable (Flueckiger *et al.*, 1995), the GC-MS method detects PQQ-derivatives (Kumazawa *et al.*, 1995), whereas the reconstitution assay works by means of sGDH-apoenzyme detecting only PQQ and no derivatives (Misset-Smits *et al.*, 1997a).

Seven genes (*pqqDGCBA* and *pqqEF*) are required for the 5-step PQQ biosynthesis in *M. extorquens* AM1 and the cofactor is derived from glutamate and tyrosine residues of the PqqA peptide precursor (Morris *et al.*, 1994). The prosthetic group is synthesized independently of the apoenzyme dehydrogenase in the cytoplasm, both are transported into the periplasm (PqqB might be involved in this step (Gomelsky *et al.*, 1996; Velterop *et al.*, 1995)), where the cofactors are incorporated into the enzyme to form the active holoenzyme.

The mechanism of MDH is proposed to work as follows: PQQ is reduced by methanol, formaldehyde is released, afterwards two electrons are transferred successively to the cytochrome *c_L*, during which the PQQH₂ is oxidized back to the quinone via the semiquinone. The rate-limiting step for MDH is the cleavage/breaking of the covalent methyl C-H bond in the substrate (large deuterium isotope effect) (Duine & Frank, 1980; Frank *et al.*, 1988; Leopoldini *et al.*, 2007; Olsthoorn & Duine, 1998). Surprisingly, this step is ammonia dependent in the enzyme assay (Afolabi *et al.*, 2001; Anthony, 2000; Frank *et al.*, 1988; Goodwin & Anthony, 1996). The main effect of ammonia is to increase V_{max}, and it was assumed that different, closely located binding sites exist for ammonia and methanol (Frank *et al.*, 1988; Goodwin & Anthony, 1996). The activator can sometimes be replaced by methylamine or glycine esters (Anthony, 2000).

There is still an ongoing discussion about the reaction mechanism. Three different mechanisms were suggested (Anthony *et al.*, 1994; Anthony, 1996; Frank *et al.*, 1989; Leopoldini *et al.*, 2007):

1. Addition-elimination mechanism: a) base-catalyzed proton abstraction from alcohol group of methanol ("Proton addition to Asp³⁰³" (nomenclature according to Ghosh *et al.*, 1995)) → result: formation of covalent substrate-cofactor complex (hemiketal intermediate with C5 of PQQ) → b) "Proton elimination" from Asp³⁰³ and transfer to O₅ of PQQ → c) Proton transfer from methoxide to the O₄ of PQQ → formaldehyde release
2. Hydride transfer mechanism: base-catalyzed one proton abstraction & direct hydride transfer from methanol to the PQQ C₅ atom → tautomerization of cofactor to PQQH₂
3. Addition-elimination-protonation: first step cleavage of C-H bond, afterwards cofactor protonation.

Mechanism 1 is supported by experimental and theoretical studies of Frank and Itoh (Frank *et al.*, 1988; Frank *et al.*, 1989; Itoh *et al.*, 1998). However, the results of the site-directed mutagenesis of aspartate³⁰³ to glutamate (Afolabi *et al.*, 2001) as well as computational and high resolution crystallographic studies favor a hydride transfer mechanism (Zheng *et al.*, 2001), likewise electron paramagnetic resonance (EPR) studies (Kay *et al.*, 2006), and quantum mechanics/molecular mechanics and molecular dynamics calculations (Reddy & Bruice, 2003; Reddy *et al.*, 2003; Zheng & Bruice, 1997). Additionally, the crystal structure of the enzyme-substrate complex of PQQ-dependent glucose dehydrogenase strongly favors the hydride transfer mechanism (Oubrie *et al.*, 1999). In 2007, Leopoldini *et al.* (2007) proposed a third mechanism based on computational studies. They explained their hypothesis by the incompatibility of kinetic requirements in both mechanisms (too high energy/activation barriers) due to the cleavage of the covalent C-H bond in methanol.

The position of the substrate in the active site of methanol dehydrogenase has not been elucidated yet (Anthony & Williams, 2003), but published data of Hothi *et al.* support the proposal of Reddy and Bruice (Reddy & Bruice, 2003) that close spatial relationships exist in MDH for ligand binding and catalytic sites (Hothi *et al.*, 2005).

Aspartate (Asp³⁰³) was proposed as active site base by site-directed mutagenesis studies from Afolabi *et al.* (2001) (replacement of aspartate by glutamate) and density functional theory calculations from Kay *et al.* (2006). By contrast, the results of molecular dynamics simulations and quantum mechanics/molecular mechanics calculations of Zhang and his coworkers (2006; Zhang *et al.*, 2007) supported Glu¹⁷¹ to be the catalytic base, since the free energy barrier of the endothermic reaction is lower in case of hydrogen

bonding of methanol to glutamate than to aspartate. Additionally, they concluded that the enzyme structure will differ from the former calculated one at lower temperatures (Mazumder-Shivakumar *et al.*, 2004; Zhang & Bruice, 2006; Zhang *et al.*, 2007). Site-directed mutagenesis of glutamate¹⁷⁷ would help to reveal the right base catalyst.

Common similarities of MDH and soluble glucose dehydrogenase (sGDH) were compared by Oubrie & Dijkstra (2000):

- I. PQQ localization in centre near the axis of pseudo-symmetry
- II. Hydrogen-bonding interactions with polar protein side chains in plane of PQQ
- III. Hydrophobic stacking interactions below and/or above PQQ
- IV. Substrate bonding in similar position above PQQ and within hydrogen bond distance of putative catalytic base (Asp²⁹⁷-MDH & His¹⁴⁴-sGDH)

The amino acid sequences and 3D-structures of both enzymes are different, but a similar reaction mechanism has been suggested by the authors based on common similarities.

A calcium ion acts most likely as Lewis acid by means of coordination to the (C₅-) carbonyl oxygen of PQQ. The activity of calcium insertion mutants (e.g. *mxmA* mutant of *M. extorquens*) could be reconstituted by addition of the metal ion (Richardson & Anthony, 1992). Calcium-replacement by barium (Ba²⁺) results in twofold increasing maximum activities, but lower substrate affinities (Goodwin & Anthony, 1996). Since the electron transfer from methanol dehydrogenase to cytochrome *c_L* is very slow *in vitro* compared to the artificial dye-linked assay, other cofactors and proteins might be involved, such as MxaJ (Matsushita *et al.*, 1993), MxaD (Toyama *et al.*, 2003), a small oxygen-labile factor that influences electron transfer (Dijkstra *et al.*, 1988), or a modifier protein (M protein) that increases the affinity of MDH for some substrates and decreases the affinity of MDH for formaldehyde (Ford *et al.*, 1985; Long & Anthony, 1990; Long & Anthony, 1991; Page & Anthony, 1986). However, interaction studies between MDH and cytochrome *c_L* via stopped flow kinetics and studies of isotope effects revealed that the rate-limiting steps might differ *in vivo*, since cytochrome *c_L* worked best at pH 7 and behaved as poor oxidant at pH 9. In contrast, ammonia served as poor activator at pH 7, which results in a slow substrate oxidation (limiting step) and no activation by ammonium has been seen at pH 9 in the presence of cytochrome *c_L*, assuming that an unknown activator exists *in vivo* (Dijkstra *et al.*, 1989).

1.5 Regulation of methanol oxidation in *M. extorquens* AM1

In general the regulation of genes involved in methanol oxidation is not completely elucidated and only some initial attempts have been done to characterize the regulatory proteins of methanol oxidation in detail.

Methanol-grown cells of facultative methylotrophic bacteria have enhanced levels of methanol dehydrogenase activity in comparison to succinate-grown cells (McNerney & O'Connor M, 1980; Weaver & Lidstrom, 1985), MDH polypeptides (Weaver & Lidstrom, 1985), and *mxoF* mRNA (Anderson *et al.*, 1990). Most likely several C₁ sources in addition to methanol serve as inducer for MDH activity since high MDH activities could be also detected for methylamine-grown cells, although MDH is not required for growth on methylamine (Laufer & Lidstrom, 1992; Morris & Lidstrom, 1992). Besides that, according to Anthony and O'Connor, the specific activity of methanol dehydrogenase is not decreased by the presence of both methanol and succinate (Anthony, 1982; O'Connor, 1981). In *M. extorquens* AM1 the *mxoFI* genes are transcriptionally regulated according to C₁ growth substrates (Lidstrom & Stirling, 1990; Marx & Lidstrom, 2001; Okubo *et al.*, 2007), MDH activity is sixfold induced during growth on methanol, however, the activity depends on growth conditions and substrate concentrations (McNerney & O'Connor M, 1980; Weaver & Lidstrom, 1985). According to Anderson *et al.* (1990) methanol-grown *M. extorquens* AM1 cells indicate a 5- to 10-fold higher *mxoF* transcription level than succinate-grown cells. The promoter studies of Morris and Lidstrom showed that this regulation is due to change in transcription (1992). Significant differences in *mxoFI* transcription levels comparing growth on succinate/methanol could additionally be shown by microarray data (Marx & Lidstrom, 2001; Okubo *et al.*, 2007). No significant differences could be observed for the other *mxo* genes of the operon by the studies of Okubo *et al.* (2007). Therefore, it was proposed by the authors that additional internal promoters might exist for *mxoFI* (Okubo *et al.*, 2007)

Up to now five regulatory genes are known that control the expression of genes for enzymes involved in methanol oxidation in *M. extorquens* AM1. Four of them encode two sensor-regulator pairs, which are required for growth on methanol controlling *mxo* gene expression plus *pqq* biosynthesis in a hierarchy, postulated by Springer *et al.* (1997): the two component system *mxoQE* controls the expression of *mxoDM*, which regulates in turn the expression of the *mxoF* promoter and the *pqq* biosynthesis genes. The mentioned sensor kinases (MxoD, MxoQ) are membrane bound equipped with a potential periplasmic loop as receptor domain (Schobert & Gorisch, 2001). MxoB, a response-regulator in *M. extorquens* AM1 with an unidentified sensor kinase, is required for *mxoF* induction and is also involved in *pqq* biosynthesis (Morris & Lidstrom, 1992; Ramamoorthi &

Lidstrom, 1995). No direct binding studies including the *mxoF* promoter and the response regulators (MxbM, MxcE, MxaB) have been published so far. The regulatory genes *mxoB*, *mxoDM*, and *mxoQE* are expressed at a low level during growth on methanol (*xylE* transcriptional fusion studies, (Springer *et al.*, 1997)), and no difference in expression was observed via microarrays in comparison of methanol/succinate-grown cultures (Okubo *et al.*, 2007).

Springer *et al.* suggested that at least two substrates, possibly methanol, formaldehyde, or PQQ, might act as signal during the regulation of genes involved in methanol oxidation (Springer *et al.*, 1997).

1.6 The dissimilation/assimilation of formaldehyde in methylotrophic microorganisms

Formaldehyde is the product of methanol or methylamine oxidation. It is situated at the branch point between the complete oxidation to CO₂ for energy generation (dissimilatory route) or the assimilation into cell biomass. Either formaldehyde is involved in both routes or only in the dissimilatory one (Vorholt, 2002). With regard to carbon fixation in prokaryotes three different pathways have to be distinguished: the serine pathway, the ribulose monophosphate pathway, or the ribulose biphosphate pathway (Murrell & McDonald, 2000), whereas methylotrophic yeasts uniquely exhibit the xylulose monophosphate cycle (Van der Klei *et al.*, 2006).

Linear pathways exist, where formaldehyde is oxidized by a dye-linked formaldehyde dehydrogenase or reacts with a cofactor, such as tetrahydrofolate (H₄F), tetrahydromethanopterin (H₄MPT), glutathione (GSH), or mycothiol (MySH), and cyclic pathways are present, where formaldehyde reacts with sugar phosphates (xylulose monophosphate pathway for yeasts and ribulose monophosphate pathway for methylotrophic bacteria) (Kato *et al.*, 2006; Vorholt, 2002).

The cofactor-dependent, dissimilatory oxidation pathways of formaldehyde contain in general the initial spontaneous or enzyme-catalyzed condensation of formaldehyde with the cofactor to form methylene derivatives (in case of H₄F or H₄MPT), the adduct S-hydroxymethyl-glutathione (in case of glutathione) or S-formyl-mycythiol (in case of mycothiol), followed by the oxidation to formate and then CO₂ (Vorholt, 2002) (Table 3). It is not possible to arrange a precise grouping of methylotrophic bacteria into formaldehyde oxidation pathways, since many methylotrophic bacteria possess more than one strategy to convert formaldehyde. *M. extorquens* AM1 exhibits, for instance, two pterin-dependent pathways: a tetrahydrofolate (H₄F)-linked one for formaldehyde

assimilation and a tetrahydromethanopterin (H₄MPT)-dependent route for formaldehyde dissimilation (Fig. 2, Table 3). Previous research has demonstrated that a tetrahydromethanopterin (H₄MPT)-dependent pathway is essential for growth in *M. extorquens* AM1 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. 2). Formate may either be oxidized to CO₂ by formate dehydrogenases or converted via tetrahydrofolate (H₄F)-dependent enzymes (Chistoserdova *et al.*, 2004; Chistoserdova *et al.*, 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).

Examples of genera for the known cofactor-dependent linear formaldehyde oxidation pathways to CO₂ are mentioned below (Table 3). In addition two cofactor-independent pathways are involved in the formaldehyde dissimilation: the cyclic oxidative ribulose monophosphate pathway (e.g. *Methylobacillus flagellatum*) (Anthony, 1982; Chistoserdova *et al.*, 2000) and the formaldehyde oxidation by the dye-linked formaldehyde dehydrogenase (Klein *et al.*, 1994).

Quayle and Ferenci (1978) divided the ribulose monophosphate cycle into three different phases: phase I (fixation phase) includes the key reaction of the cycle - the condensation of formaldehyde with ribulose 5-phosphate; D-arabinose-3-hexulose-6-phosphate is formed, followed by the isomerization to fructose 6-phosphate; phase II (cleavage phase) covers the cleavage of the hexose phosphate skeleton to form glyceraldehyde-3-phosphate and a C₃ skeleton (dihydroxyacetone phosphate or pyruvate), which is the primary net product of the cycle; and phase III (rearrangement phase) involves the steps for the regeneration of ribulose 5-phosphate.

The key enzymes of the pathway: 3-hexulose-6-phosphate synthase and 6-phospho-3-hexuloisomerase are induced by formaldehyde, function in methylotrophic bacteria plus non-methylotrophic bacteria, and are even expressed in archaeal strains (Kato *et al.*, 2006). The four different variants of the ribulose monophosphate pathway (2 for phase I and 2 for phase II) that possess varying energy budgets have been shown by Quayle and Ferenci (1978) and were described later by Kato *et al.* (2006).

Table 3. Examples of genera involved in the four different cofactor-dependent formaldehyde oxidation pathways (table excerpt from Vorholt (2002), Table 1 with some modifications).

Cofactor-linked formaldehyde oxidation pathways	Genera	Phyla	Major assimilation pathway	Formaldehyde activating enzyme	Ref.
H ₄ F-dependent	<i>Methylobacterium</i>	α-Proteobacteria	Serine	-*	1
	<i>Hyphomicrobium</i>	α-Proteobacteria	Serine	-*	2
H ₄ MPT-dependent	<i>Methylobacterium</i>	α-Proteobacteria	Serine	Fae	3
	<i>Hyphomicrobium</i>	α-Proteobacteria	Serine	Fae	4
	<i>Methylococcus</i>	γ-Proteobacteria	RuMP	Fae	5
GSH-dependent	<i>Paracoccus</i>	α-Proteobacteria	RuBP	Gfa	6
	<i>Rhodobacter</i>	α-Proteobacteria	RuBP	Gfa	7
MySH-dependent	<i>Amycolatopsis</i>	Firmicutes (Gram-positive bacteria)	RuMP	-*	8

1 (Chistoserdova & Lidstrom, 1994; Marison & Attwood, 1982; Pomper *et al.*, 1999; Studer *et al.*, 2002; Vannelli *et al.*, 1999; Vorholt *et al.*, 1998), 2 (Goenrich *et al.*, 2002b), 3 (Chistoserdova *et al.*, 1998; Hagemeyer *et al.*, 2000; Kayser *et al.*, 2002; Pomper *et al.*, 1999; Pomper & Vorholt, 2001; Vorholt *et al.*, 1998; Vorholt *et al.*, 2000), 4 (Goenrich *et al.*, 2002b; Vorholt *et al.*, 1999), 5 (Vorholt *et al.*, 1999; Vorholt *et al.*, 2000), 6 (Goenrich *et al.*, 2002a; Harms *et al.*, 1996b; Ras *et al.*, 1995), 7 (Barber *et al.*, 1996; Barber & Donohue, 1998; Wilson *et al.*, 2008), 8 (Misset-Smits *et al.*, 1997b; Norin *et al.*, 1997), Glutathione-dependent formaldehyde-activating (Gfa) enzyme, formaldehyde-activating enzyme (Fae), *unknown.

The assimilatory ribulose monophosphate pathway is more efficient than the serine pathway or the ribulose biphosphate pathway: with formaldehyde as sole input molecule, net biosyntheses are effected with minimum or zero input of energy, or even in some cases, output of energy (Kato *et al.*, 2006; Quayle & Ferenci, 1978). The variant of the ribulose monophosphate pathway with an output of energy was pronounced by Kato *et al.* (2006): in this variant of the pathway are three C₁ molecules (three moles of formaldehyde) converted into one C₃ compound (pyruvate) and one ATP plus one NADH+H⁺ are generated. *Methylophaga marina* is an example of an obligate methylotrophic bacterium assimilating formaldehyde through the ribulose monophosphate pathway (Janvier & Gasser, 1987).

The serine pathway operates in *M. extorquens* AM1 as assimilatory route for formaldehyde incorporation during growth on C₁ compounds. It was elucidated by Quayle and his group almost 50 years ago (Large *et al.*, 1961; Large *et al.*, 1962a; Large *et al.*, 1962b) and closed recently with the glyoxylate regeneration by the ethylmalonyl-CoA

pathway (Erb *et al.*, 2007; Peyraud *et al.*, 2009). The balance of the serine cycle for lyase-negative bacteria such as *M. extorquens* AM1 is as follows: three C₁ molecules (three moles of formaldehyde) plus three of CO₂ are converted into two C₃ compounds (phosphoglycerate), and seven ATP plus four NAD(P)H are consumed (Peyraud *et al.*, 2009). The key enzyme of the pathway is serine hydroxymethyltransferase that catalyzes the addition of formaldehyde to glycine forming the key intermediate serine. To achieve the mentioned balance the first steps of the pathway from glyoxylate to 2-phosphoglycerate have to proceed twice, since two 2-phosphoglycerate molecules are assimilated into cell material and the other one is converted to phosphoenolpyruvate (PEP) that is in turn carboxylized by the PEP carboxylase to oxaloacetate (Peyraud *et al.*, 2009). The latter is further metabolized via malate to malyl-CoA that is cleaved to glyoxylate and acetyl-CoA. The cycle is completed by the ethylmalonyl-CoA pathway that allows the regeneration of two glyoxylate molecules at the same time (Peyraud *et al.*, 2009).

The assimilatory ribulose biphosphate pathway or Calvin cycle operates in a few methylotrophic bacteria, such as *Beijerinckia mobilis* (Dedysh *et al.*, 2005), *Methylovirgula ligni* (Vorob'ev *et al.*, 2009), and the first known *Pseudomonas oxalaticus* (Khambata & Bhat, 1953; Quayle & Keech, 1958; Quayle & Keech, 1959a; Quayle & Keech, 1959b; Quayle & Keech, 1959c; Quayle & Keech, 1960). Interestingly, organisms like *Methylococcus capsulatus* (Bath) exist that possess all three pathways: the ribulose biphosphate-, the ribulose monophosphate-, and the serine pathway (Taylor *et al.*, 1981). The CO₂ derived from the oxidation of reduced C₁ compounds during methylotrophic growth is further assimilated via the ribulose biphosphate pathway with its two unique key enzymes ribulose-1,5-biphosphate carboxylase/oxygenase (RubisCO) and phosphoribulokinase. The balance of the ribulose biphosphate pathway is as follows: one molecule of glyceraldehyde 3-phosphate from 3 molecules of CO₂ (Anthony, 1982).

1.7 MDH-like proteins

The complete genome sequence of *M. extorquens* AM1 (Vuilleumier *et al.*, 2009) revealed the presence of several genes coding for predicted paralogs of the large subunit of methanol dehydrogenase (MxaF, META1_4538), one of which is XoxF (ID ACS39584.1, META1_1740; Chistoserdova & Lidstrom, 1997) of a predicted mass of 65 kDa, another is XoxF2 (ID ACS40517, META1_2757). XoxF and XoxF2 exhibit an amino acid sequence identity to MxaF of 50%, and 90% to each other. The XoxF enzymes possess a predicted signal peptide for periplasmic localization (Bendtsen *et al.*, 2004; Nielsen *et al.*, 1997; SignalIP 3.0 server). The *xoxF* gene META1_1740 is located

adjacent to the two genes *xoxG* coding for a cytochrome *c*, and *xoxJ* coding for XoxJ, a protein of unknown function, which was suggested to be an assembly protein or periplasmic chaperone (Amaratunga *et al.*, 1997; Matsushita *et al.*, 1993). This gene arrangement is similar to the *mxo* operon (*mxoFJGIRSACKLDEHB*) (Fig. 5), and can be even found in orthologous system in *P. denitrificans* and *R. sphaeroides* (Chistoserdova & Lidstrom, 1997; Ras *et al.*, 1991; Wilson *et al.*, 2008). In contrast *xoxF2* is situated as an orphan gene (Fig. 5).

An alignment of both amino acid sequences highlighted conserved residues specific for PQQ and Ca²⁺ interaction with the methanol dehydrogenase (Trp²⁴³, Glu¹⁷⁷, Asn²⁶¹, Arg³³¹; Fig. 6) (Anthony & Williams, 2003). The proposed active site base Asp³⁰³ was present in both sequences as well as the conserved disulfide bridge Cys^{103/104} (C) which is absent in the glucose dehydrogenase (Anthony & Williams, 2003).

A *xoxF* mutant (META1_1740) was able to grow in the presence of methanol as the sole source of carbon and energy and no obvious phenotype was 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. extorquens* strains, other methylotrophic bacteria exhibit paralogs of methanol dehydrogenase, such as *P. denitrificans*. The presence of a *xoxF* gene of *P. denitrificans* was noted earlier. A reduced growth on methanol was observed for an insertion mutant in the neighboring *cycB* gene encoding a cytochrome *c*_{553i} (Ras *et al.*, 1991). The complete genome sequences of other methylotrophic bacteria indicate the presence of *xoxF* but not of *mxoF*, such as *R. sphaeroides* (Wilson *et al.*, 2008), *Methylobium petroleiphilum* PM1 (Kane *et al.*, 2007), and for other strains for which no methylotrophic growth could be 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 MxoF, the physiology of XoxF was investigated in *R. sphaeroides*. The encoding gene of XoxF was 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 documented the induction of *xoxF* expression in the *Methylophaga* strain DMS010 during growth on dimethylsulfide (Schäfer, 2007) and in *Methylotenera mobilis* during growth on methylamine (Bosch *et al.*, 2009; Kalyuzhnaya *et al.*, 2009), which points to an implication in C₁ metabolism in these organisms. XoxF was not detected in 2D protein gels when *M. extorquens* AM1 was grown on methanol minimal medium (Laukel *et al.*, 2003) and was recently shown to be present at about 100-fold lower amounts compared to MxoF in a LC-MS/MS approach (Bosch *et al.*, 2008). On

the contrary, 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) (Fig. 7).

The identification of proteins during phyllosphere colonization of the strain *M. extorquens* PA1 (Knief *et al.*, 2010a) confirmed the expression of *xoxF* (Mext_1809 corresponding to META1_1740; Delmotte *et al.*, unpublished data). This observation suggests an important function of XoxF in a natural environment, such as the phyllosphere.

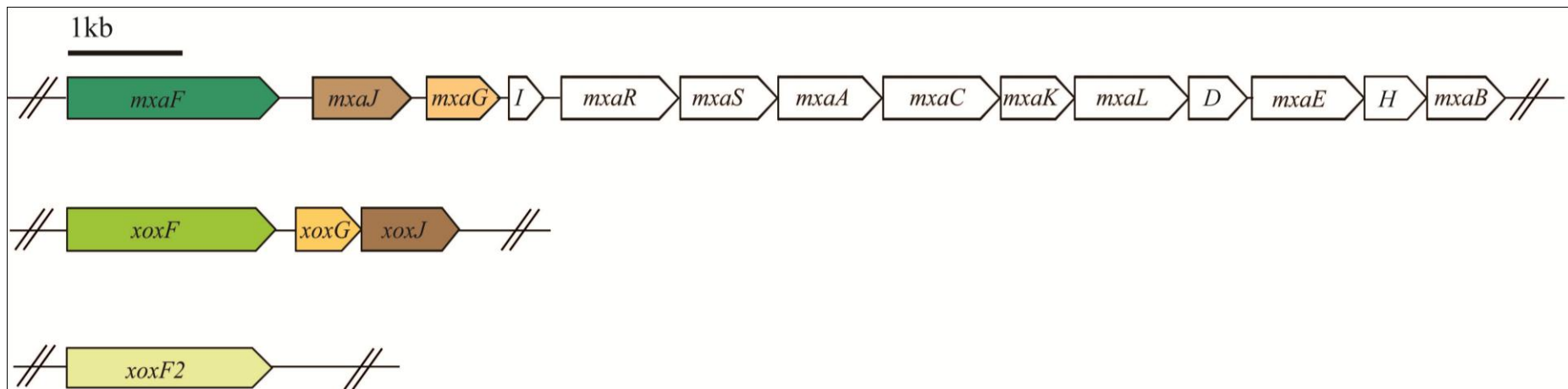
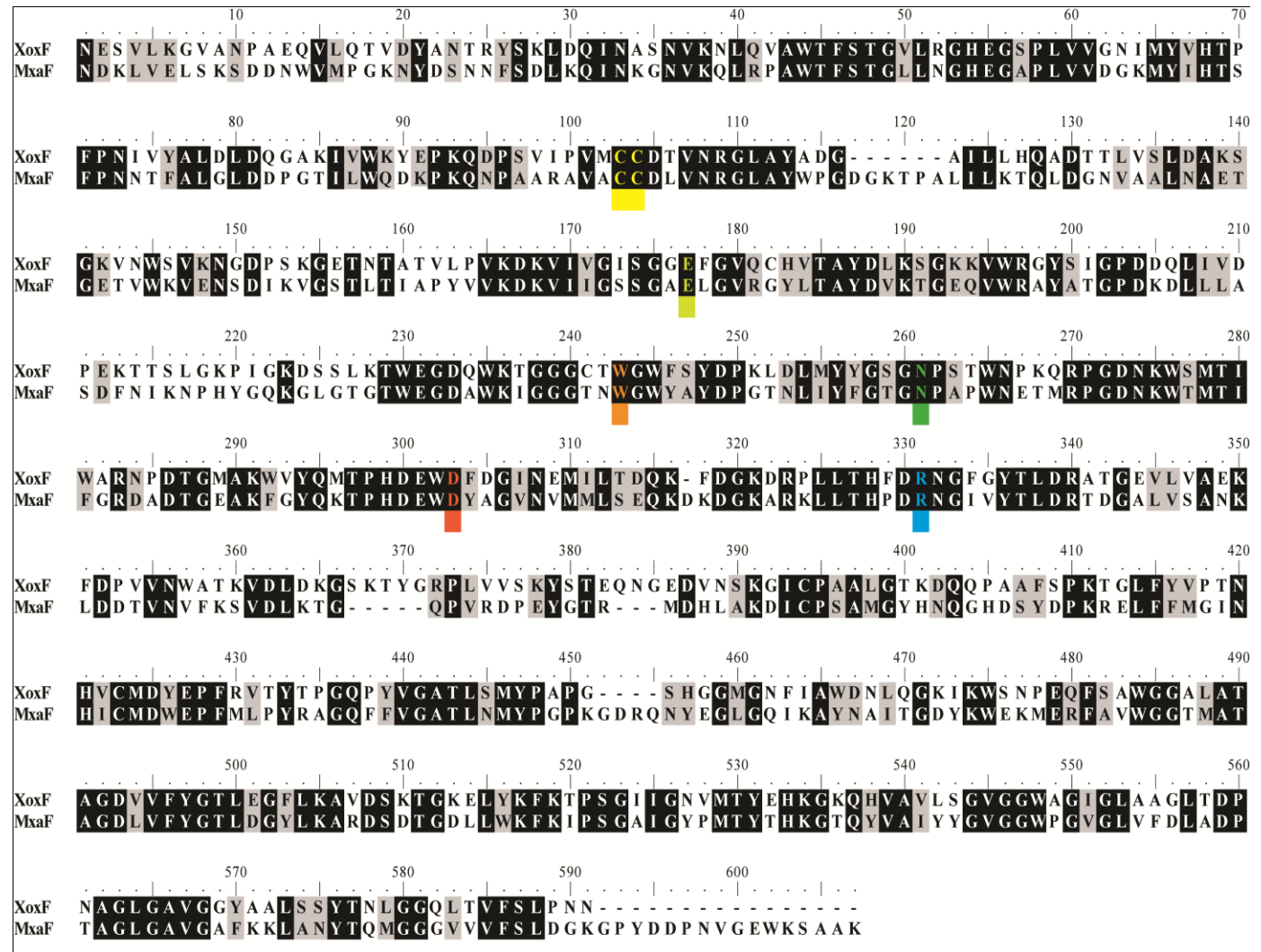


Fig. 5. Comparison of the *mxo* and *xox* operons in *M. extorquens* AM1. The large and small subunit of the methanol dehydrogenase are encoded by *mxoF* and *mxoI*, respectively, *mxoG* encodes the electron acceptor cytochrome c_L (Anderson & Lidstrom, 1988; Nunn & Lidstrom, 1986a; Nunn & Lidstrom, 1986b; Nunn *et al.*, 1989); *xoxF* (META1_1740 (Chistoserdova & Lidstrom, 1997)) and *xoxF2* (META1_2757) encode paralogs of the large subunit (Mxof) of methanol dehydrogenase (50% amino acid sequence identity), *xoxF* (META1_1740) is surrounded by *xoxG* encoding a cytochrome c and *xoxJ* encoding a paralog of Mxoj (unknown function, suggested to be an assembly protein or periplasmic chaperon (Amaratunga *et al.*, 1997; Matsushita *et al.*, 1993)) whereas *xoxF2* (META1_2757) is located alone. The genes *mxoAKL* are required for proper insertion of calcium into the active site (Morris *et al.*, 1995; Richardson & Anthony, 1992), the gene *mxoD* was suggested to be involved in stimulation of the interaction between MDH and cytochrome c_L (Toyama *et al.*, 2003), *mxoB* encodes a response regulator of *mxoF* transcription (Morris & Lidstrom, 1992; Springer *et al.*, 1998), and the function of the other *mxo* genes is unknown. The genes *mxoFJGIRSACKLDEHB* are all transcribed in the same direction and experimental data suggested a transcription of the 14 genes as one single unit (Toyama *et al.*, 1998; Zhang & Lidstrom, 2003), *mxoB* was defined as the end of the *mxo* cluster, the additional upstream gene *mxoW* (not shown) is divergently transcribed (Springer *et al.*, 1998). One promoter was suggested to be present adjacent to *mxoB*, one between *mxoD* and *mxoE* (Springer *et al.*, 1998), the *mxoF* promoter was identified and characterized by Morris and Lidstrom (1992), and a methanol-inducible promoter is located upstream of *mxoW* (Zhang & Lidstrom, 2003).

Fig. 6. Amino acid sequence alignment. The amino acid sequences of MxaF and XoxF from *M. extorquens* AM1 without the predicted N-terminal signal peptides (Bendtsen *et al.*, 2004; Nielsen *et al.*, 1997; SignalIP 3.0 server) were aligned. The conserved amino acid residues involved in PQQ and Ca²⁺ binding (Trp²⁴³, Glu¹⁷⁷, Asn²⁶¹, Arg³³¹) as well as the proposed active site base Asp³⁰³ and the disulfide bridge Cys^{103/104} of methanol dehydrogenase are highlighted.



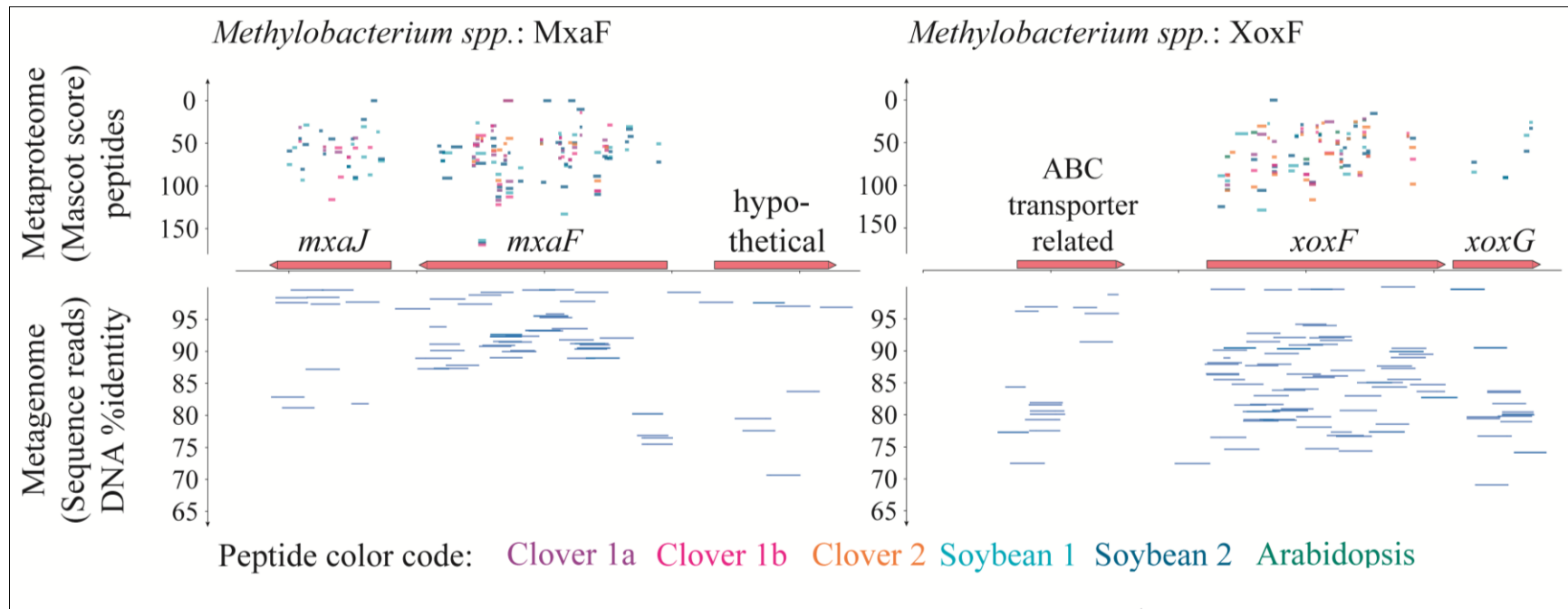


Fig. 7. Fragment recruitment plots for proteins and functional clusters. The plots exhibit a high abundance of XoxF and MxaF in *Methylobacterium* spp. during plant colonization. The relative abundance of both proteins was calculated via metagenomic and metaproteomic data, and correlated (gene length normalized): XoxF: MxaF = 1.41 (Delmotte *et al.*, 2009).

1.8 Specific aims of this work

This work deals with the functional investigations of a methanol dehydrogenase-like protein (XoxF) in the methylotrophic bacterium *Methylobacterium extorquens* AM1. At the beginning of this work, the information about XoxF was limited to the following facts:

- I. XoxF (META1_1740) exhibits about 50% amino acid sequence identity to MxaF, the catalytic subunit of a PQQ-dependent methanol dehydrogenase in *M. extorquens* AM1, and about 90% amino acid sequence identity to XoxF2 (META1_2757), another methanol dehydrogenase-like protein in the same organism.
- II. XoxF belongs to a subgroup of the quinoprotein alcohol dehydrogenase family (Chistoserdova *et al.*, 2009; Kalyuzhnaya *et al.*, 2008; Kane *et al.*, 2007).
- III. The expression level of *xoxF* in minimal medium supplemented with methanol is low, since XoxF was not detected in 2D protein gels (Laukel *et al.*, 2004), and exhibits a 100-fold lower expression level than *mxoF* shown via HPLC-MS/MS studies (Bosch *et al.*, 2008; Laukel *et al.*, 2004).
- IV. No obvious phenotype has been described for a *xoxF* mutant in *M. extorquens* AM1 (Chistoserdova & Lidstrom, 1997).
- V. A reduced growth on methanol was observed for a cytochrome *c_{553i}* insertion mutant, a neighboring gene of *xoxF* in *P. denitrificans* (Ras *et al.*, 1991).

During the course of my Ph.D., the following information became available:

In 2008, Wilson *et al.* demonstrated that XoxF is required for methanol-dependent oxygen uptake and photosynthetic methanol utilization in *R. sphaeroides* (about 80% amino acid sequence identity to XoxF of *M. extorquens* AM1; genome of *R. sphaeroides* lacks the *mxoFI* genes) (Wilson *et al.*, 2008); and just recently, Delmotte *et al.* (2009) could show that *xoxF* is highly expressed in bacterial phyllosphere communities *in situ*, at about the same abundance as *mxoF*. Environmental studies of Bosch as well as Kalyuzhnaya *et al.* documented the induction of *xoxF* expression in *Methylotenera mobilis* during growth on methylamine (Bosch *et al.*, 2009; Kalyuzhnaya *et al.*, 2009).

In order to explore the role of XoxF in C₁ metabolism, three hypotheses were proposed:

- 1) First, it was hypothesized that XoxF is an additional methanol dehydrogenase based on the known facts mentioned above.
- 2) The second hypothesis claimed that XoxF functions as a formaldehyde dehydrogenase due to the results of Page and Anthony (1986) who discovered the

ability of the methanol dehydrogenase (MxaFI) to utilize formaldehyde and methanol with a similar affinity towards both substrates.

- 3) The third hypothesis stated that XoxF plays a role in both, formaldehyde plus methanol conversion of the C₁ metabolism.
- 4) The fourth hypothesis claimed that XoxF functions as regulator of methanol oxidation.

To explore the physiological role of XoxF under *in planta* conditions, it was hypothesized that XoxF is important for plant colonization under competitive conditions.

The goal of this Ph.D. thesis was to test one or more of these three hypotheses and to provide evidence for an involvement of XoxF in methanol or/and formaldehyde oxidation. Possible phenotypes of the *M. extorquens* AM1 *xoxF* mutant in comparison to the wild type were searched: growth parameters for both strains during exponential growth on methanol were determined. The ability of the bacterial strains to convert methanol or formaldehyde upon carbon starvation was studied, and the product formation after substrate addition was analyzed. Additionally, it was investigated whether either XoxF (META1_1740) or XoxF2 (META1_2757) play a role during methanol conversion. Therefore, it was attempted to restore the lacking growth of *mxoF* mutants on methanol by overexpressing *xoxFGJ* (META1_1740-1742), *xoxF* (META1_1740), or *xoxF2* (META1_2757) in the *mxoF* mutant background.

To further characterize XoxF, first enzyme activities of cell extracts from methanol-grown *M. extorquens* AM1 strains were measured and the substrate specificities for the purified enzyme were analyzed. Finally, efforts were made to identify PQQ as potential associated cofactor of XoxF.

Altogether, a series of experiments were conducted, in order to search for a role of XoxF under *in planta* as well as under laboratory culture conditions. The importance of XoxF for plant colonization and the involvement of the enzyme in methanol/formaldehyde oxidation were demonstrated, molecular and catalytic properties of the purified enzyme were determined.

Chapter 2: Experimental procedures

2.1 Bacterial constructs, cultures, and growth conditions

The bacterial strains and plasmids used in this work are listed in Table 4 and 5.

Table 4. Bacterial strains used in this work.

	Characteristics	Source or reference
Strains		
<i>E. coli</i>		
DH5 α	<i>supE44 ΔlacU169 (ϕ80lacZDM15) hsdR17 recA1 endA1 gyrA96 thi-1 relA1</i>	Invitrogen
BL21(DE3)pLysS	<i>ompT hsdS_B(r_B⁻m_B⁻) gal dcm (DE3) pLysS (Cm^R)</i>	Invitrogen
SaS5.5	BL21(DE3)pLysS wild type/pET24b ⁺ - <i>xoxF</i>	This work
<i>M. extorquens</i>		
AM1	Wild type	(Vuilleumier <i>et al.</i> , 2009)
UV26	UV mutant of <i>mxoF</i> gene	Nunn & Lidstrom, (1986b)
<i>mxoF</i> (CM194.1)	Deletion mutant of <i>mxoF</i> gene	Marx <i>et al.</i> (2003)
<i>mxoF</i> -EMS	EMS mutant of <i>mxoF</i> gene (EMS70)	Chistoserdova (unpublished)
<i>xoxF</i>	<i>xoxF::kan</i>	Chistoserdova & Lidstrom, (1997)
CM174.1	<i>kata::loxP-t_{rmB}-P_{mxoF}-gfp-t_{T7}</i>	Marx & Lidstrom, (2004)
UV45	UV mutant of <i>moxC</i> gene (PQQ biosynthesis)	Morris <i>et al.</i> (1994)
SaS1.1	Wild type/pCM80	This work
SaS1.2	Wild type/pCM80- <i>xoxFGJ</i>	This work
SaS1.3	Wild type/pCM80- <i>xoxF-his</i>	This work
SaS1.4	Wild type/pCM80- <i>xoxF</i> (META1_2757)	This work
SaS2.1	<i>mxoF</i> /pCM80	This work
SaS2.2	<i>mxoF</i> /pCM80- <i>xoxFGJ</i>	This work
SaS2.3	<i>mxoF</i> /pCM80- <i>xoxF-his</i>	This work
SaS2.4	<i>mxoF</i> /pCM80- <i>xoxF2</i> (META1_2757)	This work
SaS2.5	<i>mxoF</i> -EMS/pCM80	This work
SaS2.6	<i>mxoF</i> -EMS/pCM80- <i>xoxFGJ</i>	This work
SaS2.7	<i>mxoF</i> -EMS/pCM80- <i>xoxF2</i> (META1_2757)	This work
SaS3.1	<i>xoxF::kan</i> /pCM80	This work
SaS3.2	<i>xoxF::kan</i> /pCM80- <i>xoxFGJ</i>	This work
SaS3.3	<i>xoxF::kan</i> /pCM80- <i>xoxF2</i> (META1_2757)	This work
<i>mxoF-xoxF</i>	Δ <i>mxoF-xoxF::kan</i>	This work
SaS4.1	<i>mxoF-xoxF::kan</i> /pCM80	This work
SaS4.2	<i>mxoF-xoxF::kan</i> /pCM80- <i>xoxFGJ</i>	This work
SaS5.1	UV26/pCM80	This work
SaS5.2	UV26/pCM80- <i>xoxFGJ</i>	This work
SaS5.3	UV26/pCM80- <i>xoxF-his</i>	This work
SaS5.4	UV45/pCM80- <i>xoxF-his</i>	This work

Table 5. Bacterial plasmids used in this work.

	Characteristics	Source or reference
Plasmids		
pAYC61- <i>xoxF::kan</i>	Allelic exchange vector with <i>kan</i> cassette in <i>xoxF</i>	Chistoserdova & Lidstrom, (1997)
pCM80	Tc ^R ; <i>M. extorquens</i> / <i>E. coli</i> shuttle vector for gene expression under the control of the <i>mxoF</i> promoter (P _{lac} -P _{mxoF})	Marx & Lidstrom, (2001)
pCM80- <i>xoxFGJ</i>	Tc ^R ; pCM80 derivative containing <i>xoxF</i> - <i>xoxG</i> - <i>xoxJ</i>	This work
pCM80- <i>xoxF</i> - <i>his</i>	Tc ^R ; pCM80 derivative containing <i>xoxF</i> with a C-terminal hexahistidine tag	This work
pCR2.1-TOPO	Cloning vector	Invitrogen
pET-24b ⁺	DH5 α /pET-24b ⁺ (Kan ^R)	Commercial
pET-24b ⁺ - <i>xoxF</i>	Kan ^R , vector for production of recombinant protein in <i>E. coli</i> adding a N-terminal hexahistidine tag containing <i>xoxF</i>	This work

E. coli DH5 α was grown on Luria-Bertani medium, whereas *E. coli* BL21(DE3)pLysS was cultivated on M9 salts medium with casamino acids (Table 6) supplemented with 0.4% glucose, 0.002% thiamine-HCl, 1 mM MgSO₄, and 0.1 mM CaCl₂ after autoclaving in the presence of antibiotics (10 μ g ml⁻¹ tetracycline, 50 μ g ml⁻¹ kanamycin).

Table 6. M9 salts medium with casamino acids for *E. coli* BL21(DE3)pLysS.

Buffer components	
Na ₂ HPO ₄	6 g l ⁻¹
KH ₂ PO ₄	3 g l ⁻¹
NaCl	0.5 g l ⁻¹
NH ₄ Cl	1 g l ⁻¹
casamino acids	5 g l ⁻¹

Cultures of *M. extorquens* AM1 wild type and *xoxF* insertion mutant (Chistoserdova & Lidstrom, 1997) were grown on minimal medium (Table 7) 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. Partial pressure of dissolved oxygen was monitored using polarographic oxygen sensors (In Pro 6800, Mettler-Toledo). Methanol concentrations were determined by GC-FID measurements (GC6850, Agilent Technologies; column: DB-Wax, J & W Scientific) 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 was supplemented with tenfold increased

cobalt concentration (12.6 μM , final concentration) to recover growth in presence of pCM80 plasmid and tetracycline (Kiefer *et al.*, 2009). The *mxoF*/pCM80-*xoxF*-*his* mutant was grown on minimal media with 30 mM pyruvate. Antibiotics were used at the following concentrations: 10 $\mu\text{g ml}^{-1}$ tetracycline, 50 $\mu\text{g ml}^{-1}$ kanamycin, and 50 $\mu\text{g ml}^{-1}$ rifamycin. The growth characterizations of mutants were tested on plates containing 10 and 120 mM methanol, 20 and 40 mM pyruvate, 30 mM succinate, NH_4Cl (1.62 g l^{-1}) or KNO_3 (3.06 g l^{-1}), none or 1 mM yeast nitrogen base without amino acids and ammonium sulfate (DifcoTM, Chemie Brunschwig).

Table 7. Minimal medium for *M. extorquens* AM1.

Separately sterilized solutions	Buffer components	
Mineral salts	NH_4Cl	1.62 g l^{-1}
	MgSO_4	0.20 g l^{-1}
Buffer pH 6.7 (methanol)	K_2HPO_4	1.59 g l^{-1}
	$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	1.80 g l^{-1}
Buffer pH 7.1 (succinate; succinate plus methanol)	K_2HPO_4	2.40 g l^{-1}
	$\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$	1.08 g l^{-1}
Succinate		8.33 g l^{-1}
Iron solution (1000X, adjust to pH 4.0)	$\text{Na}_2\text{EDTA} \cdot 2\text{H}_2\text{O}$	15 mg l^{-1}
	$\text{FeSO}_4 \cdot 7\text{H}_2\text{O}$	3 mg l^{-1}
Trace element solution (1000X, adjust to pH 1-2)	$\text{ZnSO}_4 \cdot 7\text{H}_2\text{O}$	4.5 mg l^{-1}
	$\text{CoCl}_2 \cdot 6\text{H}_2\text{O}$	0.3 mg l^{-1}
	$\text{MnCl}_2 \cdot 4\text{H}_2\text{O}$	1 mg l^{-1}
	H_3BO_3	1 mg l^{-1}
	CaCl_2	2.5 mg l^{-1}
	$\text{Na}_2\text{MoO}_4 \cdot 2\text{H}_2\text{O}$	0.4 mg l^{-1}
	$\text{CuSO}_4 \cdot 5\text{H}_2\text{O}$	0.3 mg l^{-1}

2.2 Construction of overexpressing strains, cloning techniques

For purification of XoxF from *M. extorquens* AM1, the *xoxF* gene was amplified His-tagged (C-terminal) with specific restriction sites (*Xba*I, *Eco*RI) and cloned into *Xba*I-*Eco*RI sites of pCM80 expression vector (Marx & Lidstrom, 2001). The overexpression vector pCM80 contains the P_{mxoF} promoter of *M. extorquens* AM1. Positive clones were selected by tetracycline resistance and the *lacZ* reporter gene. The forward primer included the *Xba*I site (*XoxF*_forw, Table 8) and started 24 bp upstream of the start codon to include the Shine-Dalgarno sequence. The reverse oligonucleotide (*XoxF*_rev_his, Table 8) contained the *Eco*RI 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 *mxoF*.

The overexpression plasmid pCM80-*xoxFGJ* was created via *xoxFGJ* amplification using the forward oligonucleotide (*META1_1740forw1*, Table 8) including *XbaI* restriction site (primer started 100 bp in front of the *xoxF* start codon) and the reverse oligonucleotide (*META1_1742rev*, Table 8) with *EcoRI* site (oligonucleotide started 30 bp behind the stop codon). The three genes were first subcloned into the pCR®2.1-TOPO® vector (3.9 kb, Invitrogen). The ligation product was control digested with *XbaI*, *EcoRI*, *NcoI*, and sequenced with the oligonucleotides: M13forw (Invitrogen); *META1_1740forw2* (Table 8); *META1_1740forw3* (Table 8); *META1_1740forw4* (Table 8); M13rev (Invitrogen). After verification of the correct sequence, *xoxFGJ* were cloned into pCM80. The pCM80-*xoxFGJ* construct and the empty pCM80 vector were transformed into *M. extorquens* AM1 wild type, *mxoF*, UV26, *xoxF*, and *mxoF-xoxF*.

The overexpression plasmid pCM80-*xoxF2* (*META1_2757*) was created via *xoxF2* amplification using the forward oligonucleotide (*META1_2757forw*, Table 8) including *XbaI* restriction site (primer started 187 bp in front of the *xoxF2* start codon) and the reverse oligonucleotide (*META1_2757rev*, Table 8) with *EcoRI* site (oligonucleotide started 6 bp behind the stop codon). The three genes were first subcloned into the pCR®2.1-TOPO® vector (3.9 kb, Invitrogen). The ligation product was control digested with *XbaI*, *EcoRI*, *NcoI*, and sequenced with the oligonucleotides: M13forw (Invitrogen); *META1_2757forw2* (Table 8); *META1_2757forw3* (Table 8); and M13rev (Invitrogen). After verification of the correct sequence, *xoxF2* was cloned into pCM80. The pCM80-*xoxF2* construct was transformed into *M. extorquens* AM1 wild type, *mxoF*, Δ UV26, *xoxF*, *mxoF-xoxF*, and *mxoF-EMS*.

The double mutant *mxoF-xoxF* was created by means of the suicide vector pAYC61 (Chistoserdov *et al.*, 1994) containing Km^R cassette flanked by *xoxF* sequence parts with insertions at the sites for homologous recombination between the chromosome and the vector (Chistoserdova & Lidstrom, 1997). The transformation into *mxoF* was subjected to tetracycline selection and homologous recombination validated by kanamycin selection, followed by PCR amplification.

The construct pET-24b⁺-*xoxF* was created via *xoxF* (*META1_1740*) amplification using the forward oligonucleotide (pET24bXoxFforwB, Table 8) including *NdeI* restriction site and the reverse oligonucleotide (pETXoxFrev, Table 8) with *NotI* site. The pET-24b⁺-vector contains a C-terminal His-tag. The predicted signal peptide sequence of *xoxF* is located N-terminal (Bendtsen *et al.*, 2004; Nielsen *et al.*, 1997; SignalIP 3.0 server). The ligation product was control digested with *NdeI* and *NotI*, and sequenced with

the oligonucleotides: T7 reverse complement (cuts 308 bp after the multiple cloning site, Microsynth) and T7term (cuts 68 bp before the multiple cloning site, Microsynth). After verification of the correct sequence, the vector construct was transformed into *E. coli* DH5 α and *E. coli* BL21(DE3)pLys.

The detailed PCR conditions (Table 9) with the corresponding PCR programs (Table 10), the protocols for DNA gel electrophoresis, DNA extraction from agarose gel, ligation protocols (Table 11), preparation of chemical and electrocompetent *M. extorquens* and *E. coli* DH5 α cells, and transformation protocols are listed below.

Table 8. Oligonucleotides used in this work.

Oligonucleotide	Sequence (5' → 3') and properties (introduced recognition sites for restriction endonucleases are underlined)
Oligonucleotides for overexpression of <i>xoxFGJ</i> (META1_1740-1742) in <i>M. extorquens</i> AM1	
META1_1740forw1	AAATCTAGAGCTGCCGAGCGAATATCATCC
META1_1742rev	AAAGAATTCGGGTTTCGGGCCTTCGCATCACG
META1_1740forw2	AAATCTAGAGCTTGCACATAGAGCCTAAAGG
META1_1740forw3	AAGGTCATCGTCGGCATCTC
META1_1740forw4	AGTACTCGACCGAGCAGAAC
Oligonucleotides for overexpression of <i>xoxF2</i> (META1_2757) in <i>M. extorquens</i> AM1	
META1_2757forw	AAATCTAGACCGACCAACAGGCTCATCCGC
META1_2757rev	AAAGAATTCGGCGCTAGGCTGCCGACAGGCGG
META1_2757forw2	CAAGGACTCCTCGCTGAAAAC
META1_2757forw3	TCACCCACTTCGACCGTAAC
Oligonucleotides for overexpression of <i>xoxF-his</i> (META1_1740) in <i>M. extorquens</i> AM1	
XoxF_forw	ATATATTCTAGAGGATAAGACTTGTCGGAGGAATCC
XoxF_rev_his	TATATAGAATTCCTTAGTGGTGATGGTGATGATGGTTGTTTCGGC AGCGAGAAGACCG
Control of <i>mxoF</i> UV mutant	
MxoF_forw1	ATATATATTCACGATGCGGCACTTCG
MxoF_forw2	ATATATCCTCGTCAACCGCGGCCTCG
MxoF_forw3	ATATATTGAAGCCAAGTTCGGCTACC
MxoF_forw4	ATATATGAGAAGATGGAGCGCTTCGC
Oligonucleotides for overexpression of <i>xoxF-his</i> (META1_1740) in <i>E. coli</i>	
pET24bXoxFforwB	ATATATCATATGAGAGCGGTACATCTCCTCGCACTCG
pETXoxFrev	TATATAGCGGCCGCGTTGTTTCGGCAGCGAGAAGACCG
Commercial available oligonucleotides used in this work (Invitrogen)	
M13forw	TGTAACACGACGGCCAG
M13rev	CAGGAAACAGCTATGACC
Control of pCM80 constructs	
pCM80 F1	GCACTTCGGATGACTTCCGAGCGAGCCTG
pCM80 R1	TCGCCATTCAGGCTGCGCAACTGTTGG

Table 9. PCR approaches.

PCR approach, META1_1740-1742 (<i>xoxFGJ</i>)	1x (total volume 20 µl)
template (genomic DNA, <i>M. extorquens</i> AM1)	1 µl (200 ng)
10 x Tuning buffer (5 Prime)	2 µl
dNTP (10 mM)	1.6 µl
primer forward (10 mM, META1_1740forw1)	1 µl
primer reverse (10 mM, META1_1742rev)	1 µl
Polymerase mix (5 Prime, Extender polymerase mix)	0.2 µl
H ₂ O	13.2 µl

PCR approach, META1_2757 (<i>xoxF2</i>), META1_1740 (<i>xoxF</i>)	1x (total volume 20 µl)
template (genomic DNA, <i>M. extorquens</i> AM1)	1 µl (200 ng)
10 x High fidelity buffer (5 Prime)	2 µl
dNTP (10 mM)	0.4 µl
primer forward (10 mM, META1_1740forw1)	1 µl
primer reverse (10 mM, META1_1742rev)	1 µl
Polymerase mix (5 Prime, Extender polymerase mix)	0.2 µl
H ₂ O	14.4 µl

Table 10. PCR program for gradient PCR.

Temperature (°C)		Time (s)	
META1_1740-1742 (<i>coxFGJ</i>)			
lid temperature 99°C before program start			
1.	95	180	
2.	95	40	Repetition step 2-4 (10 times)
3.	58 (temperature shift 53-63°C)	30	
4.	68	420	
5.	95	40	Repetition step 5-7 (25 times)
6.	53 (temperature shift 48-58°C)	30	plus 20 s ΔT
7.	68	420	
8.	4	pause	
META1_2757 (<i>coxF2</i>)			
1.	95	180	
2.	95	40	Repetition step 2-4 (10 times)
3.	63 (temperature shift 58.2-64.8°C)	30	
4.	72	420	
5.	95	40	Repetition step 5-7 (20 times)
6.	53 (temperature shift 48-58°C)	30	
7.	72	420	
8.	4	pause	
META1_1740 (<i>coxF</i>)			
1.	95	300	
2.	95	30	Repetition step 2-4 (5 times)
3.	59	30	
4.	72	240	
5.	95	30	Repetition step 5-7 (25 times)
6.	54	60	
7.	72	240	
8.	72	300	
9.	4	pause	

DNA gel electrophoresis. Agarose gels (1%) were made using 1 x TAE buffer (50 x TAE containing 57.1 ml acetic acid, 242 g Tris base, and 100 ml 0.5 M EDTA pH 8.0). Onefold TAE buffer was chosen as running buffer. Samples were prepared with sixfold DNA loading dye (Fermentas). A current of 100V (30 min) was applied.

DNA extraction from agarose gel

DNA extractions (15-30 μ l elution volume) from agarose gels were carried out using the NucleoSpin Extract II Kit (Macherey-Nagel). The nanodrop (Thermo Scientific) was used to determine concentrations of DNA.

Ligation of DNA. The approaches of ligation conditions are listed below. First, vectors and inserts were digested with the same restriction enzymes and purified (NucleoSpin Extract II Kit, Macherey-Nagel), afterwards both were applied for ligation.

Table 11. Ligation conditions: 18°C incubation overnight.

Ligation components	μ l
insert	10
final vector	1 (50-100 ng)
T4 ligation buffer (10x, NEB)	1.5
H ₂ O	1.5
T4 ligase (NEB)	1

Production of chemical competent *E. coli* DH5 α cells (Sambrook *et al.*, 1989). *E. coli* DH5 α cells were cultivated on LB media (24 h, 37°C, 160 rpm). 200 ml LB media were inoculated 1:100 with the overnight culture. Cultivation was stopped at an OD₆₀₀ of 0.4 to 0.6. The cells were cooled down (15 min, on ice), harvested (4000 rpm, 10 min, 4°C), resuspended carefully with ice-cold 0.1 M CaCl₂ solution (50% of original volume), incubated on ice (15 min), centrifuged (4000 rpm, 10 min, 4°C), and finally resuspended with ice-cold 0.1 M CaCl₂/15% glycerol solution (1/100 of original volume). Aliquots of 50-100 μ l were immediately frozen in liquid nitrogen and stored at -80°C (stability of 6 months). All working steps were performed on ice. The transformation efficiency (about 1-5 10^6 cfu μ g⁻¹ DNA) was controlled by plasmid transformation of known concentration.

Transformation of plasmids into chemical competent *E. coli* DH5 α cells. After incubation on ice (15 min), DNA (50-100 ng, 2 μ l) was added to the chemical competent *E. coli* DH5 α cells (50-100 μ l), and the mix was incubated on ice again (20 min). A heat shock was carried out at exactly 42°C for 30s in a water bath. LB media (400 μ l) were added, followed by an incubation step (37°C, 1 h, 160 rpm), a plating step on LB-plates (50 μ l, in LB medium resuspended cell pellet), and finally by an incubation step at 37°C overnight. LB/Kan/X-Gal plates were used for Topo cloning, and LB/Tc/X-gal plates for transformation into the pCM80 vector.

Production of electro-competent *M. extorquens* AM1 cells. Hundred ml minimal media were inoculated with an overnight culture (1:5 volume), at exponential growth phase cultures were incubated on ice (15 min) before harvesting the cells (4°C, 8000 rpm, 10 min). Cells were first washed with cold sterile water (100 ml, centrifugation at 4°C, 8000 rpm, 10 min), afterwards with cold glycerol (10% (v/v)), and finally centrifuged and resuspended in 1 ml of 10% glycerol. The electro-competent *M. extorquens* AM1 cells were frozen as 50 µl aliquots in liquid nitrogen (-80°C storage).

Transformation of plasmids into electrocompetent *M. extorquens* AM1 cells (Toyama *et al.*, 1998). Electroporation cuvettes (2 mm diameter) were filled with plasmid DNA (200-400 ng) mixed with electrocompetent *M. extorquens* AM1 cells (50 µl) after short incubation on ice. The BioRad Gene Pulser X-cell was applied for electroporation and the following transformation settings were chosen: 2.5 kV, 400 Ω, 25 µF. 400 µl of nutrient broth media (NB) were added immediately after pulsing, incubation followed (28°C, 4 to 24 h). The plating was carried out in a 1:10 dilution series (undiluted to 1:1000) on plates with minimal media plus tetracycline (incubation 4 to 7 days, 28°C).

Production of electrocompetent *E. coli* DH5α cells. Hundred ml LB media were inoculated (1:100) with an *E. coli* DH5α overnight culture and cultivated (37°C, 160 rpm) until an OD₆₀₀ of 0.4 to 0.6. After incubating the culture on ice for 15 min, cells were centrifuged (4000 rpm, 10 min, 4°C), resuspended carefully in 100 ml ice-cold water followed by three washing steps with sterile water (50 ml), one washing step with sterile glycerol (10 ml, 10% (v/v)), and finally cells were resuspended in glycerol (1 ml, 10% (v/v)), aliquoted, and frozen in liquid nitrogen (stored at -80°C).

2.3 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 cfu ml⁻¹) for inoculation. Sterilized *A. thaliana* ecotype Col-0 seeds were inoculated with a mixture (5 l) of GFP-labeled wild type (CM174.1) and mutant strain (*mxoF*, *xoxF* or *mxoF-xoxF*). The competition GFP-labeled strain against wild type strain served as control. The surface-sterilization of seeds 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 7 times and thereafter incubated for 3 hours in distilled

water before the seeds were deposited. The bottoms of plant full-gas microboxes (Combiness) 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 onto 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-labeled and unlabeled bacteria. All competition experiments with *M. extorquens* AM1 GFP-labeled strain against wild type, and mutant strains were repeated in three independent trials. The loss of competition for each strain mixture was calculated as "percentage of wild type or mutant strain at day 0 (inoculum)" minus "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 pair wise t tests with Bonferroni correction. All statistical analyses were done with the SYSTAT 12 statistical package.

2.4 *M. extorquens* cultures to perform starvation experiments followed by addition of methanol/formaldehyde

M. extorquens AM1 wild type, the *xoxF* mutant, the *xoxF* mutant strain overexpressing *xoxFGJ* (pCM80-*xoxFGJ*), and the *xoxF* mutant with the empty pCM80 vector were grown on methanol to a final OD 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), stepwise added to a final concentration of 10 mM.

The same experimental approach was repeated with *M. extorquens* AM1 wild type and the *xoxF* mutant with succinate as first growth substrate (0.5% v/v) instead of methanol (0.5% v/v) whereat carbon starvation (16h) and methanol/formaldehyde as supplement were retained.

Beforehand, during and after methanol/formaldehyde addition, samples (1 ml) were taken from the cultures, sterile filtrated and used for formate analyses. At the beginning, a time and concentration dependent linear standard curve was established with sodium formate

(Sigma Aldrich). 0.01 to 0.2 ml filtrates (undiluted to 100 fold diluted) were 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 (Cary 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 (standard deviation 10 %).

2.5 Purification of XoxF

Pyruvate-grown cells of *M. extorquens* AM1 *mxoF*/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 x g using a Beckman Avanti J-26XP 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, protease inhibitor tablet (Complete by Roche) and passed through a French pressure cell (SLM Instruments) at 1.2 x 10⁸ Pa. Ultracentrifugation was performed at 200,000 x 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 analyzed on a 12.5% SDS-PAGE. Protein samples containing the desired protein were pooled, desalted, and eluted from imidazole either via a PD-10 column 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 Äkta purifier (Amersham Pharmacia). Apparent molecular masses were estimated by comparison to a low and high molecular mass standard (Amersham Pharmacia).

2.6 Assay for methanol dehydrogenase activity and catalytic characterization methods of XoxF

Methanol dehydrogenase activity was measured using the method by Anthony and Zatman (1967a) with a modification using phenazine ethosulfate (PES)-mediated reduction (Ghosh & Quayle, 1979) of 2,6-dichlorophenol-indophenol (DCPIP, $\epsilon_{600} = 19.1 \text{ mM}^{-1}\text{cm}^{-1}$, (Basford & Huennekens, 1955)). The reaction was catalyzed with the activator NH_4Cl (final concentration 15 mM), which turned out to be essential for XoxF activity in this work. Assays were performed at room temperature in a total volume of 1 ml in a quartz cuvette (1 cm path length). The specific enzyme activity was determined as $\mu\text{mol DCPIP reduction at 600 nm per min (U) per mg protein (U mg}^{-1}\text{)}$. 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 *mxoF*-pCM80*xoxFhis* cells grown on pyruvate. Enzyme kinetics of XoxF were studied using the aforementioned assay conditions and varying substrate concentrations. The endogenous activity (under absence of carbon sources) 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.

2.7 Purification of methanol dehydrogenase

Purification of the methanol dehydrogenase from *M. extorquens* AM1: 300 ml of the *xoxF* mutant cells were cultivated in a bioreactor until $\text{OD}_{600} = 50$. Half of the cells were washed three times, resuspended in 5 ml 50 mM MOPS buffer (pH 7.0) supplemented with one EDTA-free protease inhibitor tablet (Roche) and passed three times through a French pressure cell at $1.2 \times 10^8 \text{ Pa}$. Ultracentrifugation was performed at $229,600 \times g$ (50,000 rpm) for 1 h at 4°C in a Kontron Centrikon T-1180 centrifuge using a Beckman type 70.1 Ti rotor to remove cell debris. The supernatant was filtered and loaded on a self-packed DEAE Sepharose column (XK26 20, 32 ml column volume), after diluting 3:1 with MOPS buffer 50 mM (pH 7.0). The active fractions (flow-through) were merged; 3:1 diluted with 100 mM Mes buffer pH 5.5 and loaded on a Resource Q column (6 ml, Pharmacia). The active fractions (flow through) were finally merged, concentrated and the methanol dehydrogenase assay was performed to determine kinetic parameters. The methanol dehydrogenase was employed for methanol dehydrogenase assays for a

comparison of kinetic parameters with purified XoxF and as a positive control for PQQ extraction/identification methods (2.11).

2.8 Evaluation of XoxF and MxaF concentrations

Protein concentrations were quantified according to Bradford (1976) and BCA test (Smith *et al.*, 1985, PIERCE kit). Calibration curves for the Bradford test were plotted with the measured and analyzed data of a Bradford assay conducted with a dilution series of a BSA standard in 0.9% NaCl (Sigma Aldrich).

Protein identification and quantitative analyses of XoxF and MxaF in cell extracts of *M. extorquens* AM1 wild type, wild type/pCM80-*xoxFGJ*, *xoxF*, *mxoF*, and *mxoF*/pCM80-*xoxFGJ* strains during growth on methanol, succinate, or after starvation were performed via reversed-phase high performance liquid-chromatography coupled to electrospray-ionization tandem mass-spectrometry, HPLC-ESI-MS/MS (LTQ-Orbitrap, Thermo Fisher Scientific) after separation per SDS/PAGE and tryptic digestion. Therefore, protein fractions were first trypsin digested (1 µg trypsin/ 25 µg protein, Promega) overnight. Digestion was quenched with trifluoroacetic acid. Desalting was performed with ZipTip (Millipore) and elution with 60% acetonitrile. Afterwards peptides were separated on a 2 µm C18 80 x 0.75 mm i. d. column. Eluent A was 0.2% aqueous formic acid. Separation and elution were achieved with a linear gradient of acetonitrile (10 to 40%, 50 min).

The MS data were converted to peak lists, analyzed with two search engines, namely Mascot (Matrix Science) and X!Tandem, and validated with Scaffold (Proteome Software Inc.). For protein identification, at least 2 peptide matches were required. In case of XoxF, a rough quantification was performed by spectral counting, whereas spiking of labeled peptides was employed by MxaF.

2.9 SDS gels, Western Blots

The in detail described protocols about the preparation of SDS gels and Western Blots plus the in this context utilized compositions of buffers are described and listed below.

The sodiumdodecylsulfate polyacrylamide gel electrophoresis (SDS-PAGE, Schagger & von Jagow, 1987) was performed with separation gels containing 10-12.5% acrylamide, and stacking gels with 4% acrylamide (Table 12). Gel migration (1 h) was conducted with a current of 150 V.

Table 12. SDS-PAGE (Schagger & von Jagow, 1987)

2 Separation gels	10%	12.5%	2 Stacking gels	4%
Acrylamide 30%	5 ml	6.25 ml	Acrylamide 30%	1 ml
Lower buffer 4x	3.75 ml	3.75 ml	Upper buffer 4x	1.875 ml
H ₂ O	6.25 ml	5 ml	H ₂ O	4.625 ml
APS	0.15 ml	0.15 ml	APS	0.075 ml
Temed	0.015 ml	0.015 ml	Temed	0.0075 ml
<hr/>				
Running buffer	g l⁻¹	Lower buffer (4x)		
Tris base	29	Tris/HCl pH 8.8	1.5 M	
glycine	144	SDS	0.4%	
SDS	10	Upper buffer (4x)		
		Tris/HCl pH 6.8	0.5 M	
		SDS	0.4%	
<hr/>				
Loading buffer (5x), Laemmli				
Tris HCl pH 6.8		312.5 mM		
SDS		1%		
glycerol		50%		
β-mercaptoethanol		12.5%		
Bromophenol blue		0.0625%		

Coomassie staining of SDS-gels. SDS-gels were stained in Coomassie (10% Methanol, 30% acetic acid and 0.25% Coomassie blue) for 30 min, destaining was performed in 10% methanol and 30% acetic acid.

Western Blot. After separating the proteins via SDS-PAGE, we carried out the Western Blot. The protein transfer onto a nitrocellulose membrane was conducted in the following way: one nitrocellulose membrane, and 10 Whatman papers plus the SDS-PAGE gel were equilibrated separately for 10 min in blotting buffer with 20% methanol (v/v) as additive. Tenfold blotting buffer stock included 144 g glycine and 30.3 g Tris-Base per liter. First, five Whatman papers were placed on the anode of the semi-dry blotting instrument, second, the nitrocellulose membrane, third, the SDS-gel, and finally, five Whatman papers covered the whole block on top. Blotting was performed for 1 h at 177 mA (0.8 mA cm⁻¹). Afterwards the nitrocellulose membrane was treated with blocking buffer (5% nonfat, dry milk in PBST, 30 min, with 10 x PBS stock containing 40.03 g NaCl, 10.06 g KCl, 7.09 g Na₂HPO₄, 1.22 g KH₂PO₄, whereat 1 x PBST included 1 x PBS and 0.05% Tween-20). Hybridization with the first antibody was carried out after washing the

membrane twice with PBST, the incubation was performed overnight in blocking buffer containing 1:8000 diluted XoxF polyclonal antibody (BioGenes, animal 4770). Before conducting the second hybridization the membrane was washed twice again. The incubation (1 h) was performed with an anti-rabbit antibody carrying an alkaline phosphatase (1:5000 diluted in blocking buffer). In the end the membrane was washed with PBST again. For development the membrane was incubated in development buffer (100 mM NaCl, 100 mM Tris/HCl pH 9.5, and 10 mM MgCl₂) with 1% NBT (Nitrotetrazolium Blue chloride)-solution (30 mg ml⁻¹ in 70% dimethylformamid) and 1% BCIP (5-Bromo-4-chloro-3-indolyl phosphate)-solution (20 mg ml⁻¹ in 100% dimethylformamide) as additives. The incubation was stopped with water at the moment when bands appeared.

2.10 Formaldehyde sensitivity test

Formaldehyde sensitivity tests of *M. extorquens* AM1 wild type and mutant strains: the formaldehyde sensitivity was determined in a disk diffusion assay (Gourion *et al.*, 2008). The bacterial *M. extorquens* strains: wild type, *xoxF*, *mxoF-xoxF*, *mxoF*, and UV26 were grown as overnight cultures on minimal media supplemented with methanol plus NH₄Cl/KNO₃ as nitrogen source (wild type, *xoxF*) or with succinate (*mxoF-xoxF*, *mxoF*, *mxoFUV*) to an OD₆₀₀ of 1. Hundred ml of prewarmed (42°C) minimal media soft agar (0.75%) were mixed with 5 ml of bacterial cell cultures, 4 ml portions were poured on solid minimal media. Disks were placed at the center of the plates and formaldehyde (5 µl, 1.2 M and 12 M) was deposited on the filter disks. Lower formaldehyde concentrations (0.12, 1.2, 12, 120 mM) did not inhibit growth. The sensitivity towards formaldehyde was measured after three days of incubation at 28°C (diameter of non-overgrown area on the plate/growth inhibition). The inhibition of growth rates (%) was compared for two independent experimental approaches whereby in each case the average of three plates was taken per strain.

2.11 Pyrroloquinoline quinone studies

Extraction and identification methods of pyrroloquinoline quinone

Three different methods were performed for PQQ extraction from XoxF, methanol dehydrogenase (positive control), and BSA spiked with PQQ (described below). The identification of the cofactor after each extraction protocol was conducted by HPLC-MS analyses (HPLC system Rheos 2200, Flux Instruments; coupled to an LTQ Orbitrap mass

spectrometer, Thermo Fisher Scientific; combined with an electrospray ion source). Sample separation (10 μl injection volume) was carried out on a pHILIC column (150 x 2 mm i.d., particle size 5 μm ; Sequant) that is ideal for the separation of the highly polar PQQ and its hydrophilic derivatives. Acetonitrile was chosen as solvent A, solvent B contained formic acid (2 mM) and ammonium (4 mM). During the elution that was carried out at a flow rate of 150 $\mu\text{l min}^{-1}$ the concentration of solvent B (40%) remained constant over 12 min (isocratic elution). Equilibration was carried out at initial conditions (10 min) after each sample. PQQ was analyzed in negative-ion mode (FTMS, resolution of 30,000 at m/z 400). The following parameters were applied as described in Kiefer *et al.* (2008): auxiliary gas flow rate was 5, capillary voltage was -2.5 V, sheath gas flow rate was 10, tube lens was -50 V, and ion spray voltage was -4.4 kV.

The first approach to extract PQQ was performed as described by Davidson *et al.* (Davidson *et al.*, 1985) with some modifications. An incubation step (100°C, 3 min) was performed with the purified, desalted enzymes (PD-10 column, GE Healthcare), followed by a cooling down procedure to 4°C, a centrifugation step (20,000 x g, 20 min, 4°C) to remove the denatured protein, and a cooling down to -80°C. In a second method (based on the protocol of van der Meer *et al.* with several modifications (Van der Meer *et al.*, 1990)) one volume of purified, desalted, and concentrated enzyme (60 μM of methanol dehydrogenase, XoxF, or PQQ (2 μM) labeled BSA; PD-10 column, GE Healthcare; 30 kDa Amicon Ultra Centrifugal Filter Unit, Millipore) was mixed with nine volumes of methanol. The mixture was incubated for 20 min at room temperature, and precipitated material was removed by centrifugation (1 h at 20,000 x g). Afterwards methanol was removed by evaporation in the vacuum centrifuge and the sample was resuspended in double-distilled water. During the third conducted method, PQQ was extracted via trypsin digestion. The purified enzymes (methanol dehydrogenase, XoxF, and PQQ (2 μM)-labeled BSA) were desalted via PD-10 column (GE Healthcare, elution in water), afterwards the protein samples (34 μg protein) were supplemented with ammonium bicarbonate (25 mM final concentration) and trypsin (1 μg per 75 μg protein) in a total volume of 250 - 350 μl . An incubation step was performed overnight (37°C, 500 rpm), peptides were removed via high-performance polymeric solid phase extraction (SPE) using a StrataTMX reversed-phase column (Phenomenex). The reversed phase column was first conditioned with methanol (1 ml) and afterwards equilibrated with distilled water (1 ml). The samples containing 34 μg methanol dehydrogenase, XoxF, or BSA plus PQQ were applied onto the column and eluted with 1 ml distilled water with the cofactor found in the non-bound fraction. All samples were lyophilized immediately for 2 h, redissolved in 60% acetonitrile (40-50 μl) and finally analyzed via a Rheos 2200 HPLC system (Flux

Instruments, Basel, Switzerland) coupled to an LTQ Orbitrap mass spectrometer (Thermo Fisher Scientific) equipped with an electrospray ionization probe as described above.

PQQ studies with XoxF purified from the *M. extorquens* AM1 wild type, *E. coli*/pCM80-*xoxFhis*, and *M. extorquens* AM1 mutant defective in PQQ biosynthesis

XoxF was purified from the wild type/pCM80-*xoxFhis* and the assay was performed without PQQ, as well as with PQQ plus XoxF (5 μ M final concentration per reaction) by equal amounts and with 10-fold and 100-fold excess of PQQ, immediately, and after incubation (after 1 h at room temperature, after 24 h at 4°C) in the presence and absence of magnesium (5, 50, 500 μ M) and calcium (5, 50, 500 μ M) plus/minus EDTA (15 mM, preincubation of 0-30 min, 30°C). Activities were determined as described under 2.6.. *E. coli* BL21(DE3)pLys/pET24b⁺-*xoxF* and *M. extorquens* UV45/pCM80-*xoxF-his* mutant (*moxC* , defect in PQQ biosynthesis) were constructed and analyzed.

Chapter 3: Results

3.1 *In planta* experiments

XoxF confers a growth advantage to *M. extorquens* AM1 during colonization of *Arabidopsis thaliana* under competitive conditions

Under laboratory conditions low levels of XoxF expression are detected in minimal medium supplemented with methanol: XoxF exhibits a 100-fold lower expression level than MxaF (HPLC-MS/MS studies) and was not detected on 2D protein gels (Bosch *et al.*, 2008; Laukel *et al.*, 2004). The still existing problem was strongly diminished by the considerable research findings of Delmotte *et al.* (2009), that highlighted the strong abundance of XoxF during plant colonization of *Arabidopsis thaliana* plants by *Methylobacterium extorquens* under gnotobiotic conditions (Delmotte and others, unpublished data) and of the phyllosphere community (Delmotte *et al.*, 2009). These results prompted the investigations into the importance of XoxF during plant colonization. Concerning the high *xoxF* and *xoxG* expression level in *Methylobacterium* during colonization of plant surfaces (Delmotte *et al.*, Fig. 7), research efforts were concentrated on XoxF (META1_1740). To better understand the involvement of XoxF in bacterial C₁ metabolism during bacterial phyllosphere colonization and to contribute to a broader understanding of the methylophilic adaptation during phyllosphere colonization, plant inoculation experiments were conducted under competitive conditions. *A. thaliana* was chosen as the model plant, because it was used previously for inoculation experiments, and it has the advantage that gnotobiotic conditions can be readily acquired (Gourion *et al.*, 2006). A competitive approach was used because previous inoculation experiments of methylophilic mutants revealed a phenotype only when tested in competition with the wild type strain (Sy *et al.*, 2005). The difference between competitive and single inoculation set-ups might have been caused by the availability of alternative carbon sources, such as organic acids (Sy *et al.*, 2005). To investigate the importance of XoxF, *A. thaliana* seeds were inoculated with a mixture of green fluorescence protein (GFP)-tagged wild type strain (CM174.1) and mutants of *xoxF*, *mxoF*, and *mxoF-xoxF* double mutant; the untagged wild type served as the control (Table 13). The plant inoculation experiments revealed that all these mutant strains were significantly less competitive than the wild type (p-value < 0.001, Table 13). The relative decrease of the *xoxF* mutant in competition was stronger than the relative decrease of the other mutant strains (p-value < 0.001); the *mxoF* mutant and the *mxoF-xoxF* double mutant exhibited comparable losses. The data indicate the importance of XoxF for plant colonization under competitive conditions.

Table 13. Bacterial loss of competition (%) during plant colonization after 21 days. Seeds of *A. thaliana* were inoculated with *M. extorquens* wild type, *mxoF* single mutant, *xoxF* single mutant and *mxoF-xoxF* double mutant in competition with the GFP-labeled strain CM174.1. Results are based on the analysis of 72 plants per mixture combined from three independent trials.

Strain in competition with GFP-wild type	Loss of competition after 21 days (%) (mean +/- SEM*)
Wild type	3** ± 1.3
<i>mxoF</i>	12 ± 1.9
<i>xoxF</i>	23 ± 1.7
<i>mxoF-xoxF</i>	13 ± 1.5

*Standard error of the mean

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

3.2 Growth characterization on methanol

3.2.1 Reduced growth rate

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

With the aim to establish a phenotypic characterization for the *M. extorquens* AM1 *xoxF* mutant, growth parameters for the *M. extorquens* AM1 wild type and the *xoxF* mutant were examined during exponential growth on methanol. A *xoxF* insertion mutant of *M. extorquens* AM1 was previously described to be capable of growth in the presence of methanol as sole source of carbon and energy (Chistoserdova & Lidstrom, 1997) which was confirmed in this work. Although, no differences in growth rates for the *xoxF* mutant relative to the wild type during exponential growth on succinate (data not shown) were noted, the specific growth rate on methanol was reduced for the *xoxF* mutant by 30% in bioreactor experiments relative to the wild type (Table 14). This observation is different to 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 with regard to the phenotype of a *xoxG* (*cycB*) mutant in *P. denitrificans* (Harms *et al.*, 1996a; Ras *et al.*, 1991). Along with the reduced growth rate for the *M. extorquens* AM1 *xoxF* mutant, a decrease in the specific methanol conversion and CO₂ production rate relative to the wild type was observed (Table 14). Inversely, the *xoxF* mutant showed a

comparable biomass yield (Table 14), suggesting that the amount of energy generated from methanol oxidation was unaltered.

On the basis of the data it was presumed that XoxF is involved directly or indirectly in methanol oxidation. By comparing growth parameters of *M. extorquens* AM1 *xoxF* mutant and the wild type, a phenotype was obtained for the *xoxF* mutant during exponential growth on methanol. The growth rate and the specific methanol uptake rate of the *xoxF* mutant strain were significantly reduced compared to the wild type strain, which provides first evidence for a link between XoxF and the C₁ metabolism.

Table 14. Growth parameters of *M. extorquens* AM1 wild type and the *xoxF* mutant during exponential growth on methanol under reference conditions carried out in bioreactors. Methanol specific yields and specific rates were determined from two biological replicates (a, b). μ , growth rate; $Y_{X/S}$, substrate-specific biomass yield; $Y_{CO_2/S}$, substrate specific CO₂ yield; q_S , specific substrate uptake rate, q_{CO_2} , specific CO₂ production rate.

	Wild type		<i>xoxF</i>	
	a	b	a	b
μ [h ⁻¹]	0.14	0.15	0.10	0.10
$Y_{X/S}$ (g g ⁻¹)	0.21	0.20	0.27	0.21
$Y_{CO_2/S}$ (g g ⁻¹)	0.45	0.36	0.43	0.40
q_S [mmol g ⁻¹ h ⁻¹]	20.5	21.3	12.9	13.2
q_{CO_2} [mmol g ⁻¹ h ⁻¹]	9.1	9.4	5.6	5.5

3.2.2 Growth recovery experiments

XoxF does not substitute MxaF: Overexpression of *xoxF* in the *mxoF* mutant background does not restore growth on methanol

To further characterize XoxF and to demonstrate that the protein is involved in methanol oxidation, growth characterizations were carried out by means of *xoxF* overexpressing strains as well as replacement studies of methanol dehydrogenase.

Earlier results indicated that XoxF cannot functionally replace MxaF because a *mxoF* deletion mutant is not able to grow in the presence of methanol (Nunn & Lidstrom, 1986b). In order to investigate whether the low expression level of *xoxF* under laboratory conditions (Bosch *et al.*, 2008) (this work, Table 17) is responsible for the failure to substitute the true methanol dehydrogenase, strains were constructed expressing the *xoxFGJ* genes (META1_1740-1742) and *xoxF2* (META1_2757, 87% amino acid

sequence identity to META1_1740, and 50% amino acid sequence identity to MxaF) under the control of the *mxoF* promoter in *mxoF* mutant backgrounds (deletion mutant, UV and ethylmethanesulfonate (EMS) mutant, Table 4). Different *mxo* mutant strains were chosen with a single mutation in the *mxoF* gene (UV26, *mxoF*-EMS) and with a deletion of the complete gene (*mxoF*), because it was assumed that the whole *mxo* operon *mxoFJGIRSACKLDEHB* is transcribed in the *mxoF*-EMS and *mxoF*-UV mutant, and it might be possible that the transcription is interrupted in case of the *mxoF* deletion mutant (*mxoF*). To explore if a higher expressed *xoxF* can replace the methanol dehydrogenase a series of experiments were conducted.

The rationale for the expression of the *xoxFGJ* cluster in case of *xoxF* META1_1740, rather than *xoxF* alone, was based on the genome arrangement suggesting that the gene products function together and, additionally, on observations made in the orthologous system in *P. denitrificans* and *R. sphaeroides* (Chistoserdova & Lidstrom, 1997; Ras *et al.*, 1991; Wilson *et al.*, 2008). The *xoxF2* gene (META1_2757) was not surrounded by the *xoxG* or *xoxJ* paralogs.

Growth could not be restored for the *mxo* mutant strains from *M. extorquens* AM1 expressing *xoxFGJ* (META1_1740-1742) or *xoxF2* (META1_2757) (Table 15) during tests under several conditions. These encompassed various methanol concentrations, different nitrogen sources (NH₄Cl, KNO₃, data not shown), and shifts from the multi-carbon substrates succinate, pyruvate, and acetate to methanol with and without a period of carbon starvation. The results of the characterization of methanol/succinate-grown mutant strains from *M. extorquens* AM1 on plates are presented in Table 15. The listed *M. extorquens* AM1 strains were investigated: *mxoF* (deletion of the complete *mxoF* gene, whole operon might not be transcribed), *xoxF*, *mxoF*-EMS mutant (transcription of the whole operon *mxoFJGIRSACKLDEHB*), and the wild type carrying the empty vector pCM80, pCM80-*xoxFGJ* (META1_1740-1742), or pCM80-*xoxF2* (META1_2757). All *xoxF* and wild type constructs indicated a comparable growth behavior. XoxF2 (META1_2757) and XoxFGJ (META1_1740-1742) did not substitute for the methanol dehydrogenase, since *mxoF* mutants overexpressing *xoxFGJ* or *xoxF2* could not restore growth on methanol. To rule out that succinate obstructs the adaptation of the *mxoF*/pCM80-*xoxF2* and *mxoF*/pCM80-*xoxFGJ* mutants to methanol-growth, further experiments were carried out with acetate and pyruvate as prior substrates instead of succinate. For this purpose three independent growth characterizations were performed (not shown). For this experimental approach all strains listed in Table 15 were first grown on plates containing acetate, pyruvate, and succinate and afterwards on plates supplemented with different concentrations of methanol. Besides that, different nitrogen sources were tested for the growth studies (KNO₃ and NH₄Cl) to achieve a higher level of

xoxF expression. These conditions were chosen according to studies concerning the induction of *xoxF* expression during growth on methylamine, which might be linked to the nitrogen source (Bosch *et al.*, 2009; Kalyuzhnaya *et al.*, 2009). However, the results of the growth characterizations on plates with acetate and pyruvate as prior growth substrate instead of succinate plus NH₄Cl or KNO₃ as nitrogen source resembled the results described above (data not shown). No growth could be restored on methanol-plates for the *mxoA* mutant strains from *M. extorquens* AM1 expressing *xoxFGJ* (META1_1740-1742) or *xoxF2* (META1_2757).

Table 15. Growth characterization of *M. extorquens* AM1 mutant strains. The growth characterizations of mutants were tested on plates containing 10 and 120 mM methanol, and/or 30 mM succinate, or no carbon source (negative control), NH₄Cl (1.62 g l⁻¹) functioned as nitrogen source. The data refer to three biological replicates (each condition was tested with three technical replicates).

<i>M. extorquens</i> AM1 strains	Carbon sources			
	Succinate	Methanol	Methanol & Succinate	Without carbon source
Wild type/pCM80	+	+	+	-
Wild type/pCM80- <i>xoxFGJ</i> (META1_1740)	+	+	+	-
Wild type/pCM80- <i>xoxF2</i> (META1_2757)	+	+	+	-
<i>mxoA</i> /pCM80	+	-	+	-
<i>mxoA</i> /pCM80- <i>xoxFGJ</i> (META1_1740)	+	-	+	-
<i>mxoA</i> /pCM80- <i>xoxF2</i> (META1_2757)	+	-	+	-
<i>xoxF</i> /pCM80	+	+	+	-
<i>xoxF</i> /pCM80- <i>xoxFGJ</i> (META1_1740)	+	+	+	-
<i>xoxF</i> /pCM80- <i>xoxF2</i> (META1_2757)	+	+	+	-
<i>mxoA</i> - <i>xoxF1</i> /pCM80	+	-	+	-
<i>mxoA</i> -EMS/pCM80	+	-	+	-
<i>mxoA</i> -EMS/pCM80- <i>xoxFGJ</i> (META1_1740)	+	-	+	-
<i>mxoA</i> -EMS/pCM80- <i>xoxF2</i> (META1_2757)	+	-	+	-

To further confirm the results, growth characterizations were conducted in liquid cultures. Therefore, *M. extorquens* AM1 *mxoA*/pCM80-*xoxFGJ* mutant and the *mxoA*/pCM80 mutant strain were cultivated in the bioreactor on succinate plus ¹³C-methanol (10 mM instead of 60 mM), but neither enzyme activities nor methanol consumption could be measured. The methanol decrease equated to the methanol evaporation rate, no

quantifiable $^{13}\text{C-CO}_2$ was formed. Both strains did not differ significantly from each other with regard to oxygen consumption. The relative expression level of *xoxF* was higher for the *M. extorquens* AM1 *mxoF*/pCM80-*xoxFGJ* mutant than for the *mxoF*/pCM80 mutant strain (Table 17, HPLC-ESI-MS/MS data; Figure 8, Western Blot analyses). Additionally, no methanol dehydrogenase activity could be demonstrated for the *M. extorquens* AM1 strains: *mxoF*/pCM80-*xoxFGJ*, *mxoF*/pCM80, *mxoF*-UV/pCM80, and *mxoF*-UV/pCM80-*xoxFGJ* (several primary and secondary alcohols were tested).

The *M. extorquens* AM1 strains *mxoF*/pCM80-*xoxFGJ*, *mxoF*/pCM80, and *mxoF*/pCM80-*xoxF-his* were likewise tested in liquid culture on minimal media with pyruvate as carbon additive, followed by carbon starvation, and methanol addition (60 mM), but no methanol was converted by the mentioned strains.

Finally, it can be concluded from the results of growth characterizations that neither XoxF (META1_1740) nor XoxF2 (META1_2757) substitute for the methanol dehydrogenase under the tested conditions.

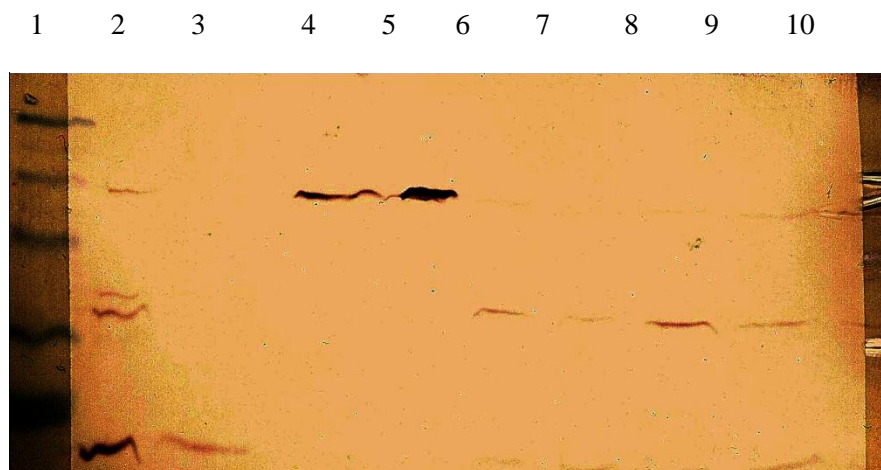


Fig. 8. Western Blot with cell extracts from *M. extorquens* AM1 using a polyclonal antibody against XoxF (BioGenes). Lane 1: prestained protein marker (NEB); lane 2: wild type, exponential growth phase; lane 3: *xoxF*, exponential growth phase; lane 4: *mxoF*/pCM80-*xoxFGJ*; lane 5: purified XoxF; lane 6: wild type, during growth on succinate; lane 7: wild type after growth on succinate plus starvation; lane 8: wild type after growth on succinate, starvation, and methanol addition; lane 9: wild type after growth on succinate, starvation, and formaldehyde addition; lane 10: wild type after growth on succinate, starvation, and after formaldehyde consumption.

3.3 Investigation of XoxF function in C₁ metabolism

The next section deals with the investigation of the XoxF function in the C₁ metabolism. Because of the low level of XoxF expression in minimal media (Bosch *et al.*, 2008) (This work, Table 17), I searched for enhanced expression conditions. Therefore, first different nitrogen sources were examined. Next, *xoxF* expression of methanol-grown cells was compared with carbon-starved cells of *M. extorquens* AM1.

3.3.1 No increase of expression by change of nitrogen source

The level of XoxF expression was not significantly increased when NH₄Cl was replaced by KNO₃ during growth on methanol

In order to establish a phenotype for a *xoxF* mutant of *M. extorquens* AM1, different growth conditions in culture were tested. There is still the need to improve the expression conditions of *xoxF* considering the low expression level during growth on methanol. *M. extorquens* wild type and the *xoxF* mutant were cultivated on methylamine (30 mM) according to the former results of Bosch *et al.* as well as Kalyuzhnaya *et al.*, which demonstrated the induction of *xoxF* expression in *Methylobacterium mobilis* during growth on methylamine (Bosch *et al.*, 2009; Kalyuzhnaya *et al.*, 2009). Furthermore, methanol was chosen as carbon substrate for the cultivation of *M. extorquens* wild type and the *xoxF* mutant with NH₄Cl or KNO₃ as nitrogen source in 250 ml-flasks equipped with an oxygen sensor (PreSens - Precision Sensing) so as to study the impact of an alternative nitrogen source on the expression level of *xoxF* and on the growth behavior in comparison of both strains. The specific growth rates on methanol plus NH₄Cl or KNO₃ were reduced for the *xoxF* mutant by about 30% relative to the wild type in flasks equipped with an oxygen sensor (data not shown). No difference in oxygen response was observable particularly with regard to the different nitrogen source. With methylamine as a growth substrate, the growth rate was, in turn, reduced for the *xoxF* mutant by about 23% relative to the wild type (data not shown).

The level of XoxF expression could be determined by rough quantification of HPLC-ESI-MS/MS data results of the analyzed samples from *M. extorquens* AM1 cell extracts (data not shown). The methanol-grown wild type strains from *M. extorquens* AM1 showed no significant difference in the level of XoxF expression in comparison of the different nitrogen sources (KNO₃ and NH₄Cl). Nevertheless, the values of the XoxF expression have to be considered carefully, since the levels of XoxF expression were close to the detection limit.

KNO₃ appears not to be a suitable replacement for NH₄Cl as nitrogen source in order to increase the XoxF expression level. Further optimizations are required to achieve comparable expression levels of XoxF in methanol-grown cultures with those obtained during plant colonization.

3.3.2 MDH activities of cell extracts

The *M. extorquens* AM1 *xoxF* mutant shows a reduction of methanol dehydrogenase activity after starvation

With the objective of providing further evidence for a XoxF involvement in methanol oxidation, enzyme activities from cell extracts of *M. extorquens* AM1 wild type and the *xoxF* mutant strain were determined by means of the dye-linked methanol dehydrogenase assay (Table 16). During exponential growth phase on methanol, an enzyme activity of 0.2 U mg⁻¹ was observed in crude extracts of the *xoxF* mutant, which was comparable to the wild type. The activity did not increase when cells were taken after carbon starvation, but was found to be 50% lower for the *xoxF* mutant (Table 16). These results suggest an involvement of XoxF in methanol oxidation after methanol starvation. Interestingly, during starvation, no significant alteration in *mxoF* expression between the samples of wild type and *xoxF* mutant was detectable by the estimation and quantification of *mxoF* peptides via HPLC-ESI-MS/MS (data not shown). Despite changes in activities, the level of XoxF expression remained low after starvation: equal amounts of *xoxF* peptides were detected for the wild type after starvation and during exponential growth phase (Table 17). The activity assays were replicated with cell extracts of the *xoxF* mutant strain overexpressing *xoxFGJ* and the *xoxF* mutant containing the empty vector. Both strains exhibited similar results concerning the methanol dehydrogenase activities compared to the values obtained for wild type and *xoxF* mutant (Table 16). It could be shown by HPLC-ESI-MS/MS that the expression level of MxoF in the *xoxF*/pCM80-*xoxFGJ* strain and in the *xoxF*/pCM80 mutant are not significantly different (data not shown). From these data the conclusion may be drawn that XoxF is involved directly or indirectly in methanol oxidation after starvation.

Table 16. Specific enzyme activities were determined for *M. extorquens* AM1 wild type, *xoxF*, *xoxF/pCM80-xoxFGJ*, and *xoxF/pCM80* strains during growth on methanol (exponential growth phase) and after starvation (16 h). The data shown here refer in each case to one biological replicate (one cultivation in the bioreactor), the SEM was calculated from three technical replicates.

	During exponential growth phase on methanol			
<i>M. extorquens</i> AM1 strains	Wild type	<i>xoxF</i>	<i>xoxF/pCM80-xoxFGJ</i>	<i>xoxF/pCM80</i>
Specific enzyme activity (U/mg) (+/- SEM)	0.19 ± 0.02	0.24 ± 0.03	0.32 ± 0.01	0.31 ± 0.03
	After starvation			
<i>M. extorquens</i> AM1 strains	Wild type	<i>xoxF</i>	<i>xoxF/pCM80-xoxFGJ</i>	<i>xoxF/pCM80</i>
Specific enzyme activity (U/mg) (+/- SEM)	0.21 ± 0.01	0.08 ± 0.01	0.12 ± 0.01	0.05 ± 0.00

Table 17. Determination of the XoxF content in cell extracts of *M. extorquens* AM1 strains via HPLC-ESI-MS/MS.

Cell extracts of <i>M. extorquens</i> AM1 strains	XoxF (number of unique peptides detected)
Wild type (exponential growth phase)	2 - 3
Wild type (after starvation)	2 - 3
<i>xoxF</i> (exponential growth phase)	0
<i>xoxF</i> (after starvation)	0
Wild type on succinate	0
<i>xoxF</i> on succinate	0
<i>mxoF</i>	3
<i>mxoF/pCM80-xoxFGJ</i>	10 & 16

3.3.3 Carbon starvation

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

According to the results in the previous sections, there was evidence that XoxF is involved in methanol oxidation. In the following part, some facts are presented supporting the hypothesis of a XoxF-participation in methanol conversion.

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 *Methylobacteria* are subjected to changing concentrations of available carbon under environmental conditions and a generally limited supply of nutrients, a sudden increase in methanol concentration was mimicked after a phase of carbon starvation in a controlled bioreactor. A rather high final concentration of methanol (60 mM) was chosen for being able to monitor effects due to methanol oxidation more easily. The response of the *xoxF* mutant was analyzed in parallel to *M. extorquens* AM1 wild type cells after starving cells for sixteen hours and the eventual 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 to oxygen consumption and relative differences of the latter can be directly seen by DO. At regular time intervals, samples of the culture supernatant were taken and methanol concentration was determined using GC-FID. Very fast oxygen consumption was observed as a response to methanol addition in the wild type but not in the *xoxF* mutant (Fig. 9a). According to the observed differences in oxygen consumption, the mutant strain required twice the time (5 h) to convert the methanol (Fig. 9a). This observation correlated with the measured methanol concentration in the medium (Fig. 9b). Similar results were observed when the *xoxF* mutant strain overexpressing *xoxFGJ* (pCM80-*xoxFGJ*) and the *xoxF* mutant with the empty pCM80 vector (control) were analyzed 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) (Fig. 10). Furthermore, differences were observed in the growth rates for *xoxF* pCM80-*xoxFGJ* ($\mu = 0.18 \text{ h}^{-1}$) and *xoxF* pCM80 ($\mu = 0.13 \text{ h}^{-1}$) 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 ($\mu = 0.14 \text{ h}^{-1}$; (Kiefer *et al.*, 2009)).

A second experimental approach was conducted using the same strains (*M. extorquens* AM1 wild type and *xoxF* mutant) with growth on succinate until exponential growth, followed by starvation (16 h), and methanol addition (60 mM). The *xoxF* mutant did not consume any methanol after starvation (data not shown), whereas the wild type converted the substrate (Fig. 11). However, substrate conversion proceeded with a much slower response towards the substrate compared to methanol as first C₁ compound (Fig. 9a); the adaptation phase occurred within hours and not within the first minutes. In order to compare the values of the succinate-grown cells (optical density of 7) with the ones of the methanol-grown cells (optical density of 9), all data were divided by the cell biomass (data not shown). The strong difference in the velocity of the response was still as obvious as the difference in the height of oxygen consumption. The succinate-grown *M. extorquens* AM1 wild type and *xoxF* mutant upon addition of formaldehyde (stepwise supplemented up to a final concentration of 10 mM) after carbon starvation (16 h) did not differ significantly from each other in the oxygen response (data not shown).

To recapitulate the findings of these experiments: the data demonstrated that the *xoxF* mutant from *M. extorquens* AM1 consumes methanol less rapidly compared to the wild type, particularly in the first minutes after methanol supplementation. Considering the results of the experiments with succinate-grown cells, the data suggest that the multicarbon substrate inhibits or retards the methanol oxidation in the *xoxF* mutant and in the wild type strain from *M. extorquens*.

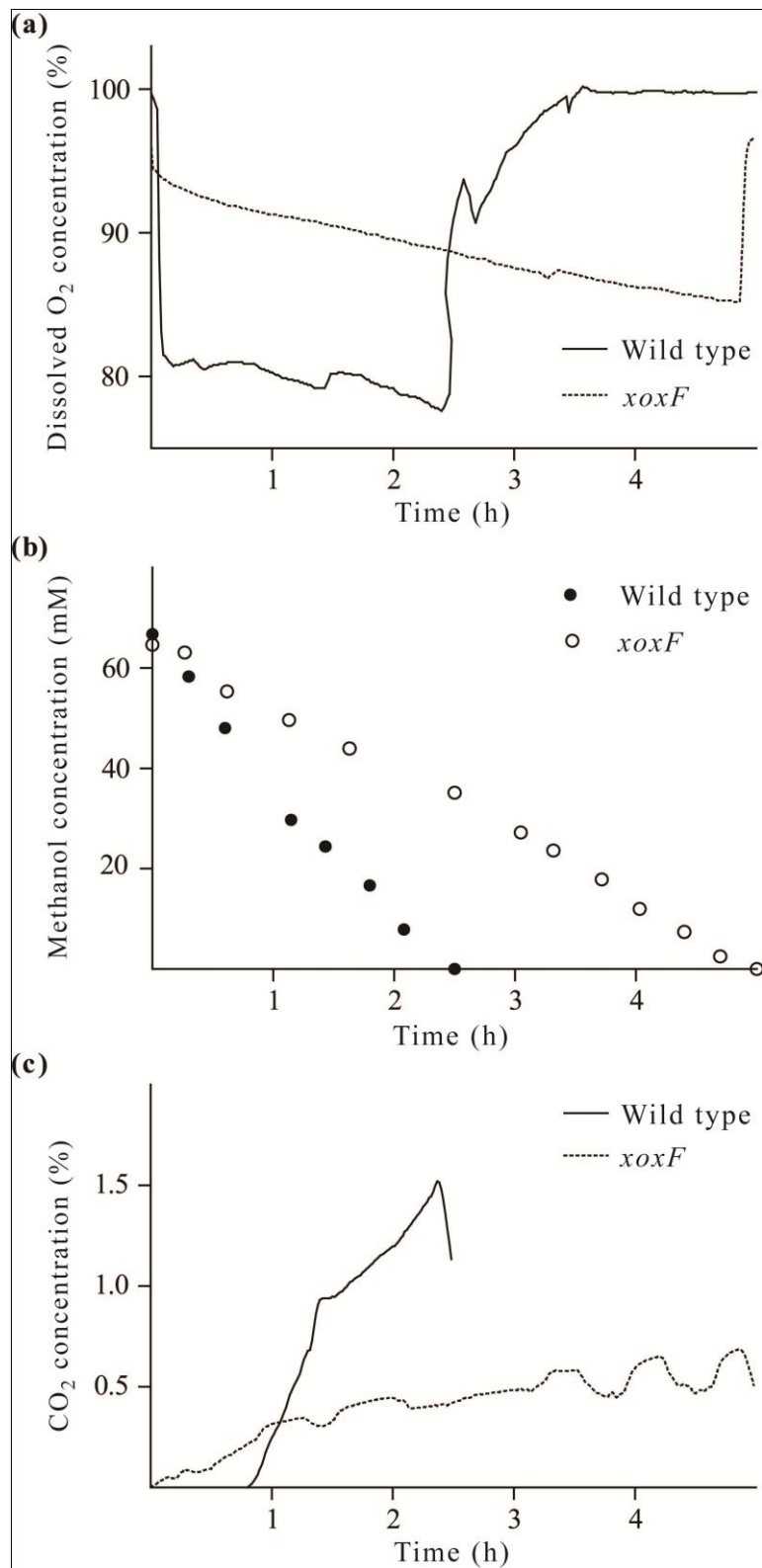


Fig. 9. Response of *M. extorquens* AM1 wild type (solid line/filled circles) and the *xoxF* mutant (dotted line/open circles) upon addition of methanol 0.25% (v/v) after carbon starvation (16 h). The monitored parameters were dissolved oxygen (%) in the liquid phase (a), methanol concentration (b), and CO₂ concentrations in the exhausted gas phase (c). The CO₂ production rate refers to the initial biomass (*). The experiment was confirmed twice with similar results and conducted at an optical density of 9.0.

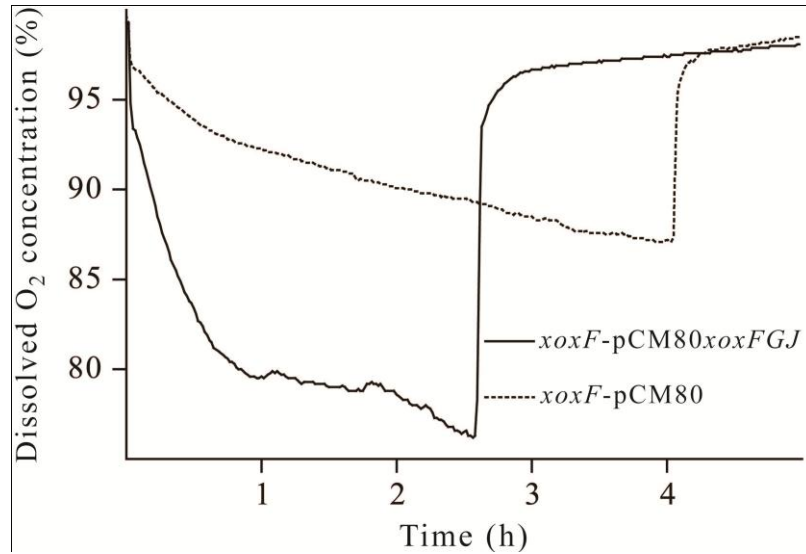


Fig. 10. Response of *M. extorquens* AM1 *xoxF::kan/pCM80-xoxFGJ* (solid line/filled circles) and the *xoxF::kan/pCM80* mutant (dotted line/open circles) upon addition of methanol 0.25% (v/v) after carbon starvation (16 h). Monitored dissolved oxygen (%) in the liquid phase is shown for both strains. The dissolved oxygen correlates directly to the methanol consumption, which occurred faster when *xoxFGJ* was expressed (2.7 h) relative to the mutant strain containing pCM80 (4.1 h).

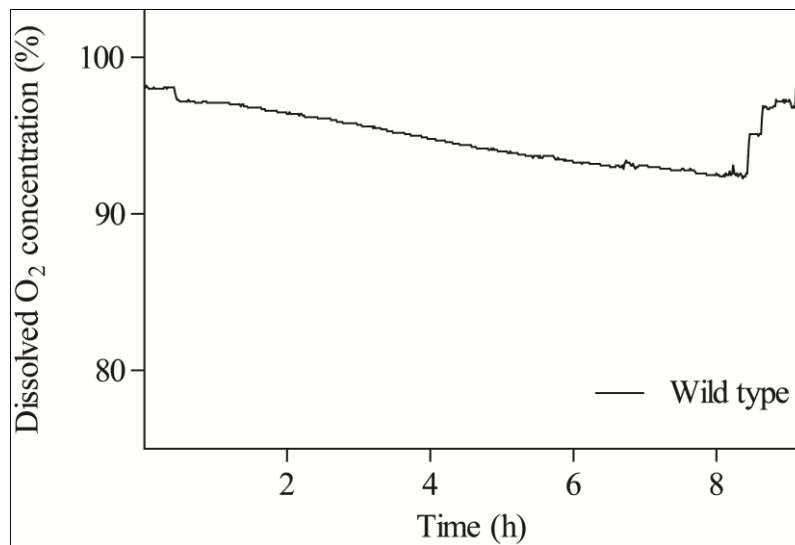


Fig. 11. Response of succinate-grown *M. extorquens* AM1 wild type (solid line/filled circles) upon addition of methanol 0.25% (v/v) after carbon starvation (16 h). The monitored parameter was dissolved oxygen (%) in the liquid phase. The experiment was conducted at an optical density of 7.0. The *xoxF* mutant did not consume any oxygen (data not shown).

With these results evidence was provided to support the hypothesis that XoxF is involved in methanol oxidation.

3.3.4 Formate accumulation

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

Since formate is oxidized to CO₂ in the final step of methanol conversion to CO₂ in *M. extorquens* AM1 (Pomper *et al.*, 2002; Vorholt, 2002), it was worth analyzing the CO₂ production rate in order to gain insights into metabolic defects of the methanol oxidation pathway. In *M. extorquens* AM1 multiple genes were predicted to encode formate dehydrogenases that are involved in the catalysis of formate oxidation to CO₂ (Chistoserdova *et al.*, 2004; Chistoserdova *et al.*, 2007; Laukel *et al.*, 2003), but only one of them, the NAD-dependent formate dehydrogenase (FDH1), was purified and characterized by Laukel *et al.* (Laukel *et al.*, 2003). Interestingly, one formate dehydrogenase exhibits a Tat motif used for periplasmic localization (Palmer *et al.*, 2005). As mentioned in the introduction, methanol might be oxidized to CO₂ in the periplasm of the cell, for example under a situation of sudden increase in formaldehyde concentration. Only very low concentrations of formate were detected in culture supernatants of *M. extorquens* AM1 wild type cells during exponential growth on methanol ((Chistoserdova *et al.*, 2004); or the *xoxF* mutant in this work) suggesting that formate oxidation occurs fast relative to its production.

A one-hour lag phase in CO₂ production occurred in the wild type but not in the *xoxF* mutant (Fig. 9c); 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. It was 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. 12a). This experiment suggests an additional capacity of the wild type to form formate due to the expression of *xoxF*. In a subsequent experiment, it was directly tested 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, 10 times 1 mM formaldehyde was added over a time period of 0.5 h 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 to the wild type (Fig. 12b). 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 oxidation of methanol plus formaldehyde to formate.

Formate concentrations were also measured in supernatants of exponentially growing cells.

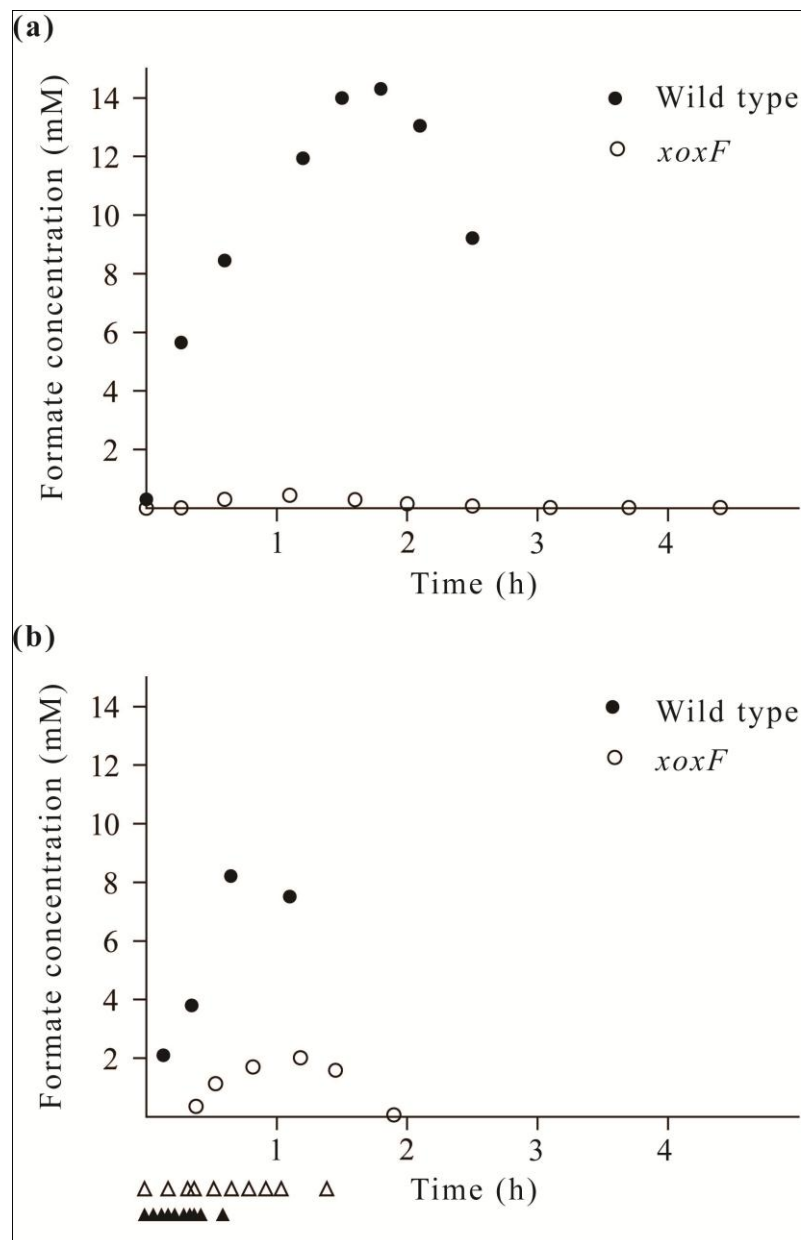


Fig. 12. Formate concentrations in culture supernatants of *M. extorquens* AM1 wild type (filled circles) and the *xoxF* mutant (open circles). Formate was measured upon addition of methanol 60 mM (a), and upon addition of formaldehyde (b). The latter was supplemented stepwise to the wild type (filled triangles) and *xoxF* mutant (open triangles) up to a final concentration of 10 mM. The formate content of the supernatant was determined in a formate assay as described in Materials and Methods. The mean values of three biological replicates are indicated and all measurements agreed within 10%.

Accumulation of only low amounts of formate (below 60 μ M) during growth of *M. extorquens* AM1 in the presence of methanol was determined earlier (Chistoserdova *et*

al., 2004; Chistoserdova *et al.*, 2007). Here, I observed comparably low amounts of formate when the *xoxF* mutant was analyzed in parallel to the wild type. Thus, the phenotype of the *xoxF* mutant for C₁ conversion to formate became evident only upon addition of a substrate after carbon starvation.

3.3.5 Formaldehyde sensitivity tests

The *M. extorquens* AM1 *xoxF* mutant does not show higher sensitivity towards formaldehyde under tested conditions

The previous results of the carbon-starvation experiments suggested an alternative involvement of XoxF in formaldehyde oxidation. In addition, the *M. extorquens* AM1 wild type and the *xoxF* mutant were employed to analyze whether the missing *xoxF* results in a higher sensitivity towards formaldehyde (Fig. 13). The substrate sensitivity was therefore determined in a disk diffusion assay where growth inhibition was used to measure formaldehyde sensitivity. The formaldehyde sensitivities were compared for the *M. extorquens* AM1 wild type and the *xoxF* mutant at the time point of exponential growth on methanol and after starvation with NH₄Cl as nitrogen source. In addition, samples were taken from these strains during exponential growth on methanol with KNO₃ as nitrogen source. Furthermore, succinate-grown *mxoF-xoxF*, *mxoF*, and UV26 (Table 4) mutant strains have been examined. Two different *mxoF* mutant strains were tested (*mxoF*, UV26) in case that the whole *mxoF* operon might be inactive in the *mxoF* deletion mutant. *M. extorquens* AM1 wild type and the *xoxF* mutant showed similar formaldehyde sensitivities at exponential growth phase and after starvation, and were independent from the two nitrogen sources. No significant difference in growth inhibition could be observed for the *mxoF* mutant and for the *mxoF-xoxF* double mutant, neither between each other nor compared to the remaining strains. It can be assumed that the *xoxF* mutant from *M. extorquens* AM1 does not show a higher sensitivity towards formaldehyde than the wild type strain under the tested conditions.

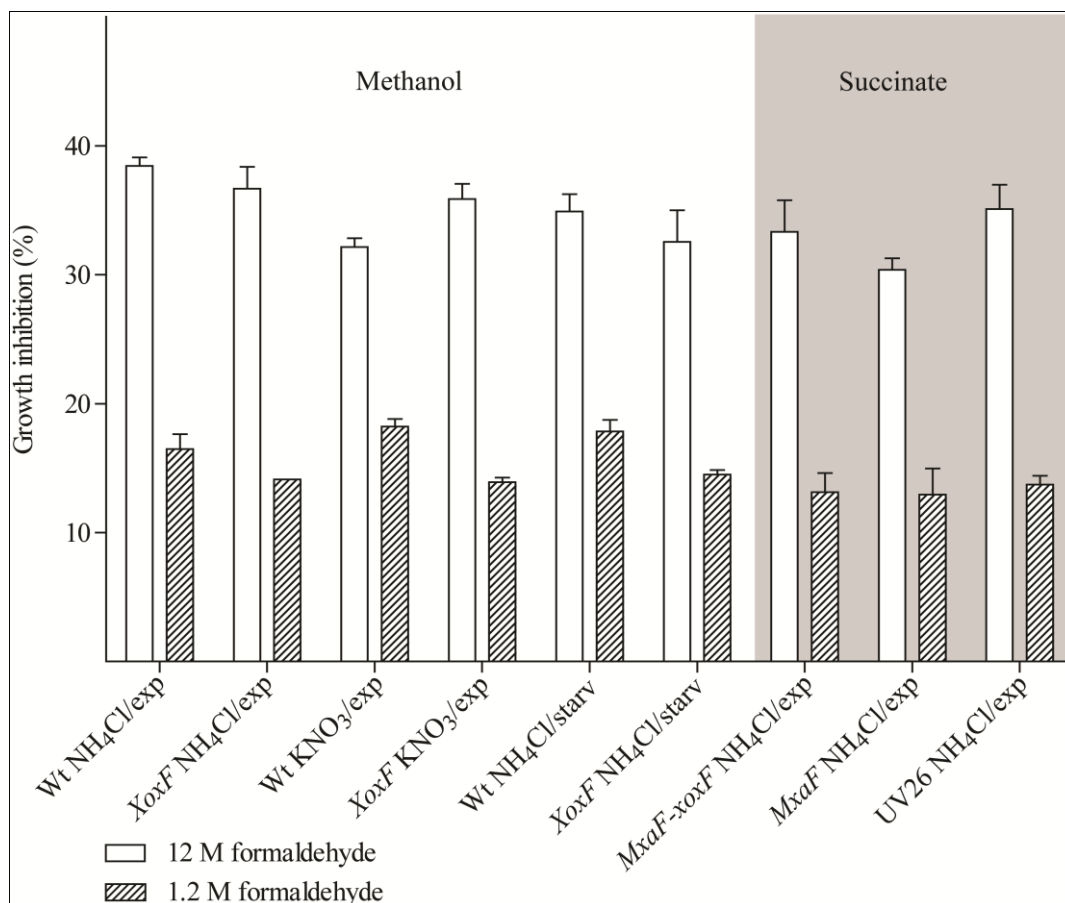


Fig. 13. Formaldehyde sensitivity tests via disk diffusion assays of *M. extorquens* AM1 wild type (Wt) and mutants. Growth inhibition was measured depending on two formaldehyde concentrations (12 M and 1.2 M) for the *M. extorquens* AM1 wild type, and the *xoxF*, *mxoF-xoxF*, *mxoF*, UV26 (*mxoF*-UV) mutant strains. Methanol (wild type, *xoxF*) or succinate (*mxoF-xoxF*, *mxoF*, UV26) served as a carbon source, NH₄Cl/KNO₃ as a nitrogen source. In case of the wild type and *xoxF* strain, samples were taken during starvation (starv) and exponential (exp) growth phase. The shown data were taken from one biological replicate; the standard deviation from three technical replicates varies from 0.7 to 2.4%.

3.4 Enzyme properties

In the previous sections the results of experiments have been reported that were carried out with *M. extorquens* AM1 wild type cells and the *xoxF* mutant. Although enzyme activities have been determined for the crude extracts of *M. extorquens* AM1 wild type cells and the *xoxF* mutant, no MDH assays have been performed with the purified enzyme. In the following sections, the molecular and catalytic properties of purified XoxF will be characterized, and the results of the cofactor analyses will be presented.

3.4.1 Purification and molecular properties

Purification of XoxF from *M. extorquens* AM1 and characterization of molecular properties

In order to purify XoxF from *M. extorquens* AM1, a pCM80-*xoxF*-*his* vector was constructed. The *mxoF* deletion background was used to exclude any contamination with methanol dehydrogenase. Hence, the cells were cultivated on minimal media supplemented with pyruvate as carbon source. XoxF was purified to apparent homogeneity via affinity chromatography and gel filtration (Fig. 14). The apparent molecular mass of the enzyme was determined to be about 60 kDa by gel filtration indicating a monomeric structure of the native enzyme (Fig. 15 a,b).

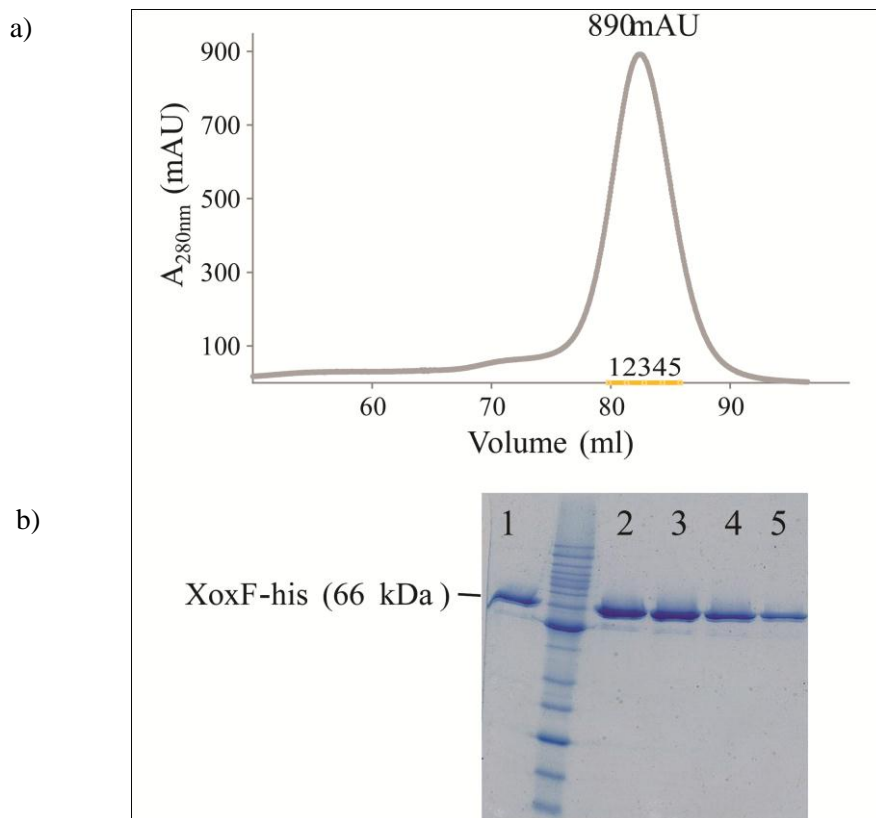


Fig. 14. Purification of XoxF. (a) Histidine labeled XoxF was purified from *M. extorquens* AM1 via gel filtration. Fractions (1 – 5) were merged and concentrated up to a final concentration of 1.3 mg/ml. Absorbance was measured in absorbance units (AU). (b) The merged fractions were controlled via SDS-PAGE (lane 1 – 5).

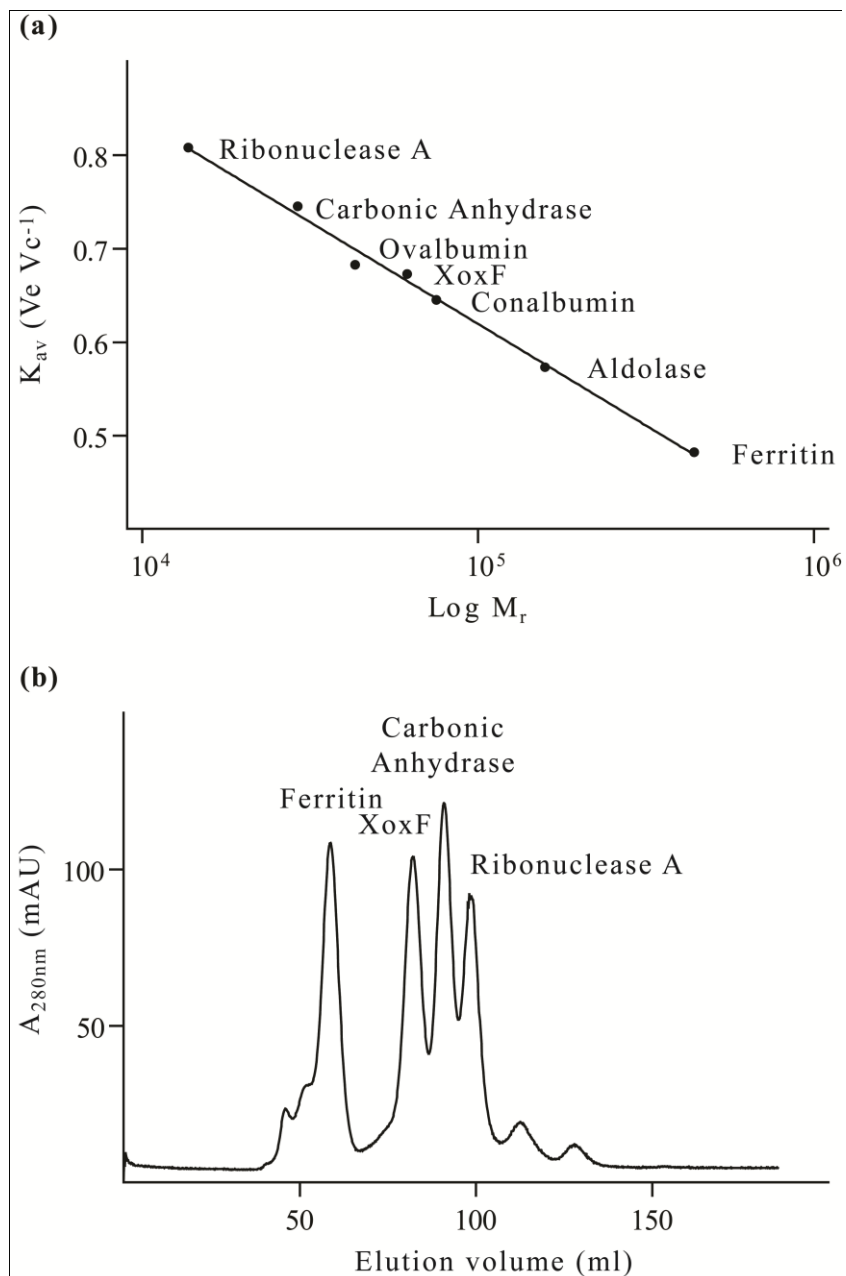


Fig. 15. Chromatographic separation and calibration curve of standard proteins (Amersham Pharmacia) including XoxF on Superdex 200 (HiLoad 16/60) column. These proteins were eluted in the following order: ferritin (440 kDa), aldolase (158 kDa), conalbumin (75 kDa), XoxF (63 kDa), ovalbumin (43 kDa), carbonic anhydrase (29 kDa), ribonuclease A (14 kDa). A 500 μ l sample was loaded onto the column, and the eluate was monitored at 280 nm. The partition coefficients (K_{av}) were calculated for the calibration kit proteins using the equation: $K_{av} = V_e/V_c - 1$ with V_e = elution volume, and V_c = geometric column volume. The calibration curve shows the K_{av} values *versus* log molecular weights.

3.4.2 Catalytic properties of XoxF

To characterize the catalytic properties of XoxF, various experiments were conducted to determine the optimal conditions for the enzyme assay and to examine the kinetic parameters of the purified XoxF. The substrate ranges and substrate affinities of XoxF were investigated using dye-linked phenazine ethosulphate dependent methanol dehydrogenase assay (Ghosh & Quayle, 1979). The optimum pH of purified XoxF for the oxidation of methanol in the PES/DCPIP-dependent methanol dehydrogenase assay was at a pH of 9.4 (Fig. 16).

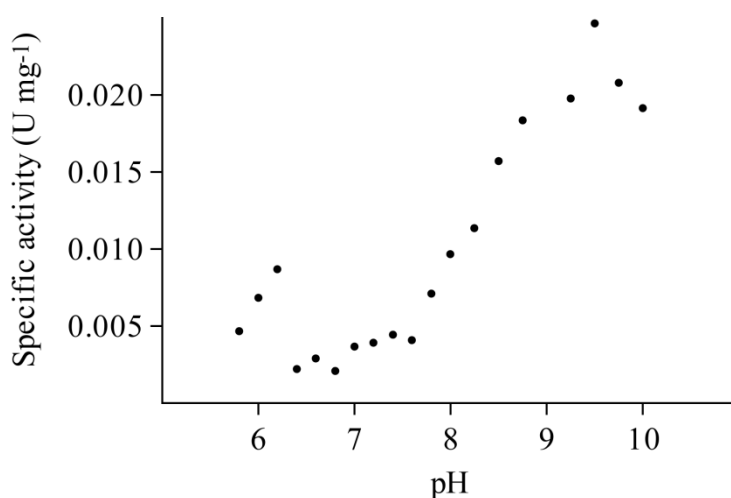


Fig. 16. The pH optimum of purified XoxF in the PES/DCPIP-linked MDH assay. XoxF was purified from the *M. extorquens* AM1 *mxoF*-pCM80*mxoFhis* mutant grown on pyruvate; potassium phosphate buffer (pH 5.8 to 8.0), Tris HCl buffer (pH 7.7 to 10.0), and Mes buffer (pH 5.5 to 6.5) were used.

Ammonium was found to be essential for enzyme activity ($K_a = 8$ Mm, Fig.17), similar to the observation made for methanol dehydrogenase in *M. extorquens* AM1 ($K_a = 2$ mM; (Goodwin & Anthony, 1996). Other amines, i.e. methylamine, dimethylamine, trimethylamine, ethylamine, n-butylamine, hexylamine, n-heptylamine, n-nonylamine were tested as potential activators; however, they could not replace NH_4Cl in the enzyme assays.

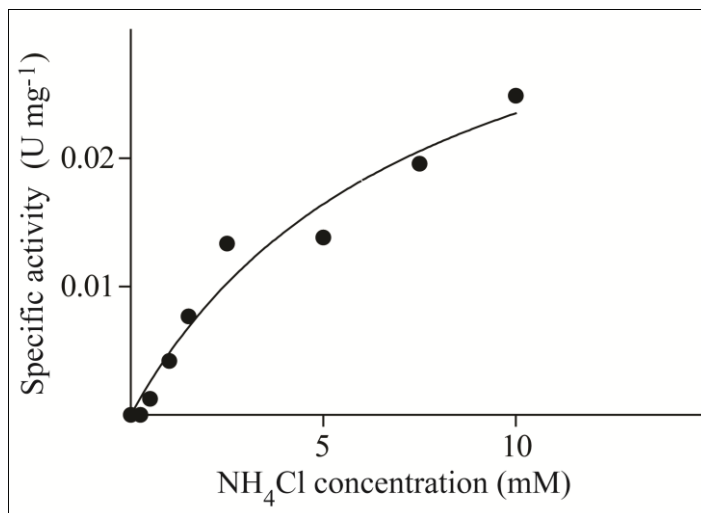


Fig. 17. NH_4Cl serves as essential activator for XoxF activity ($K_a = 8 \text{ mM}$) in the dye-linked assay.

The enzyme oxidized methanol, formaldehyde, and ethanol with high affinity: the K_m for the carbon substrates were $11 \mu\text{M}$ for methanol, $65 \mu\text{M}$ for formaldehyde, and $14 \mu\text{M}$ for ethanol (Fig. 18). 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 (Anthony & Zatman, 1964a; Anthony, 1986; Day & Anthony, 1990; Duine *et al.*, 1978; Duine & Frank, 1980; Ghosh & Quayle, 1981)). Besides that, 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) $0.8\text{-}1.0 \text{ U mg}^{-1}$, (Goodwin & Anthony, 1996; Page & Anthony, 1986), XoxF-his was also purified from methanol grown cells using the wild type containing pCM80-*xoxF-his*. Under these conditions, slightly higher V_{\max} values of 0.08 U mg^{-1} for XoxF were determined. An increased endogenous oxidation was observed rendering accurate substrate affinity determination difficult.

Taken together, purified XoxF oxidizes methanol and formaldehyde with a high affinity.

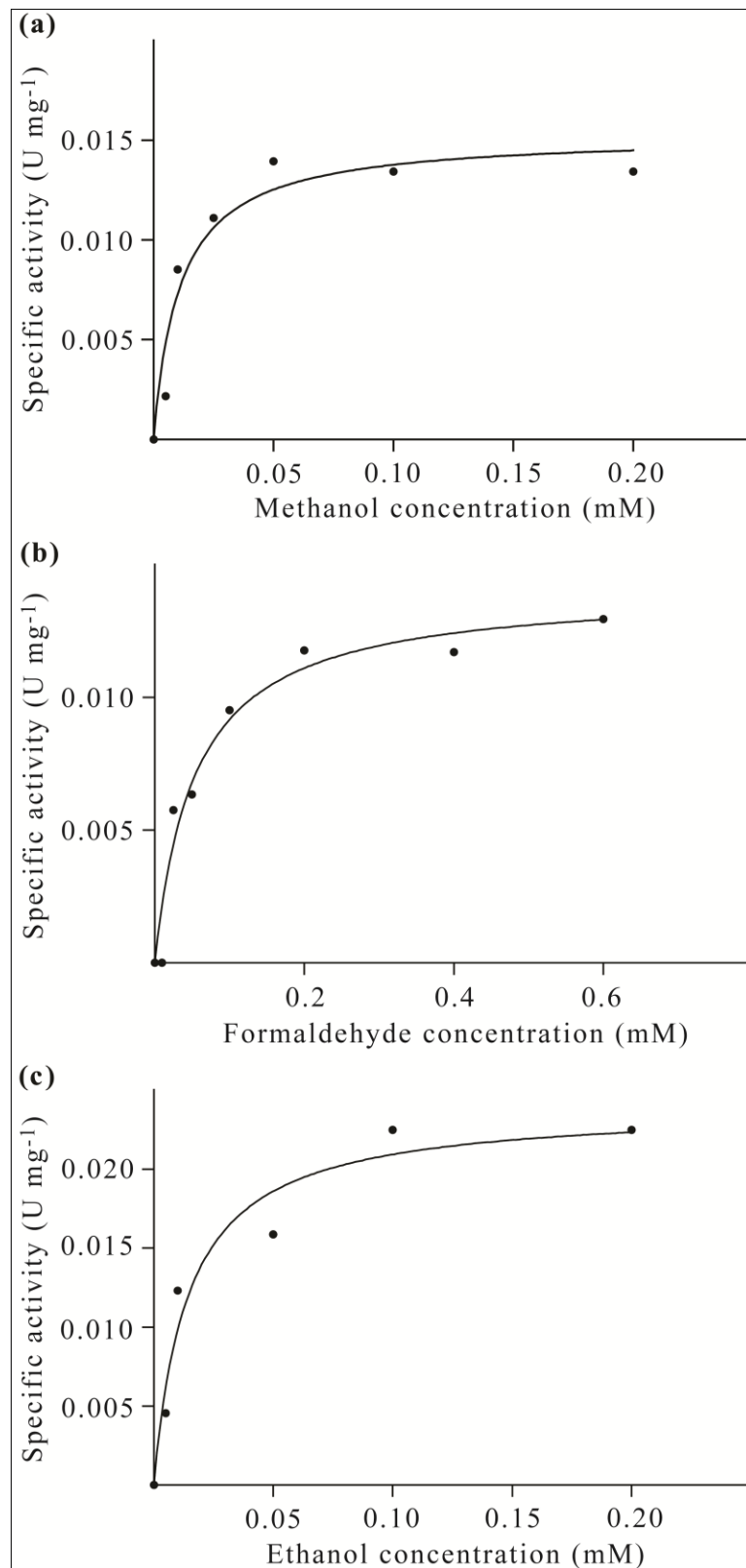


Fig. 18. Kinetics of purified XoxF. Specific XoxF activities (U mg^{-1}) were plotted versus methanol (a), formaldehyde (b) and ethanol (c) concentrations. To measure the activities, the PES/DCPIP-linked methanol dehydrogenase assay was applied. Endogenous activities of the enzyme (without substrate addition) were subtracted. Michaelis-Menten constants were determined by direct fitting of the curves to K_m values and double-reciprocal plots according to Lineweaver and Burk as a control.

3.4.3 Cofactor analyses

The presence of PQQ in XoxF could not be demonstrated

Since methanol dehydrogenase (MxaFI, $\alpha_2\beta_2$) from *M. extorquens* AM1 exhibits one molecule PQQ tightly but non-covalently bound per large subunit (2 mol PQQ per mol tetramer) (Adachi *et al.*, 1990; Blake *et al.*, 1994; Richardson & Anthony, 1992; White *et al.*, 1993), a series of experiments was set up investigating the existence of this potential prosthetic group in XoxF. First, the purified, active enzyme was analyzed by photometric scanning, later it was set out to prove the PQQ-dependence on XoxF activity with *M. extorquens* AM1 mutants lacking the PQQ biosynthesis, and finally, it was aimed to extract and identify PQQ as potential cofactor of XoxF. As mentioned earlier, PQQ-containing quinoproteins show a characteristic absorption peak at 345-350 nm, and a shoulder at about 400 nm. Without the PQQ they exhibit a peak at 280 nm and a shoulder at 290 nm (Duine *et al.*, 1981). To investigate if PQQ is present in XoxF, the enzyme was first purified from *M. extorquens* AM1 wild type, 10-fold concentrated (10 mg/ml) combined with a buffer exchange to remove disturbing imidazole, and subsequently scanned via UV/VIS photometer. In spite of the strong concentration of XoxF no visible peak was detected at about 340 nm, not even when omitting a buffer exchange (1:10 dilution shown in Fig. 19).

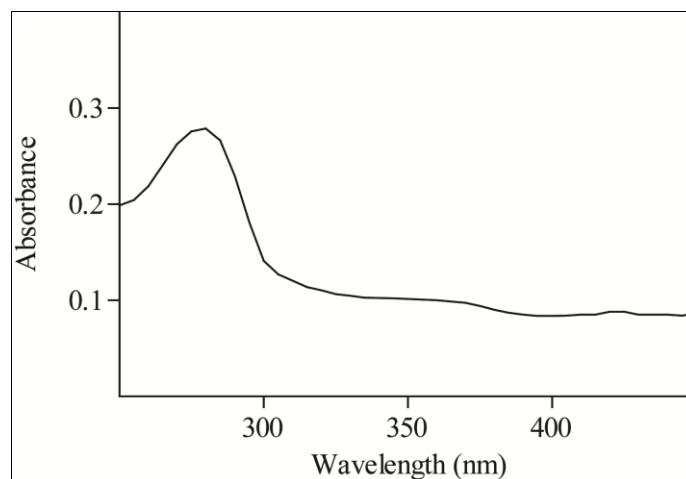


Fig. 19. UV/VIS scanning of purified XoxF from *M. extorquens* AM1 wild type/pCM80-*xoxF-his*. The protein was 1:10 diluted after concentration. The concentration was combined with a buffer exchange (20 mM potassium phosphate buffer, pH 7.0) to remove the imidazole.

To investigate if PQQ titration to the purified XoxF from *M. extorquens* AM1 wild type has any influence on the enzyme activity, incubations were conducted with PQQ plus XoxF under several conditions. However, the activity of XoxF did not increase with an

excess of PQQ, or after addition of divalent ions (magnesium, calcium). To demonstrate the PQQ dependence/independence on XoxF activity, two strains were constructed unable of PQQ biosynthesis: the *E. coli* BL21(DE3)pLysS/pET24b⁺-*xoxF* strain and the *M. extorquens* UV45/pCM80-*xoxF*-*his* strain. However, problems concerning the protein abundance arose, and no significant protein level could be detected for both strains.

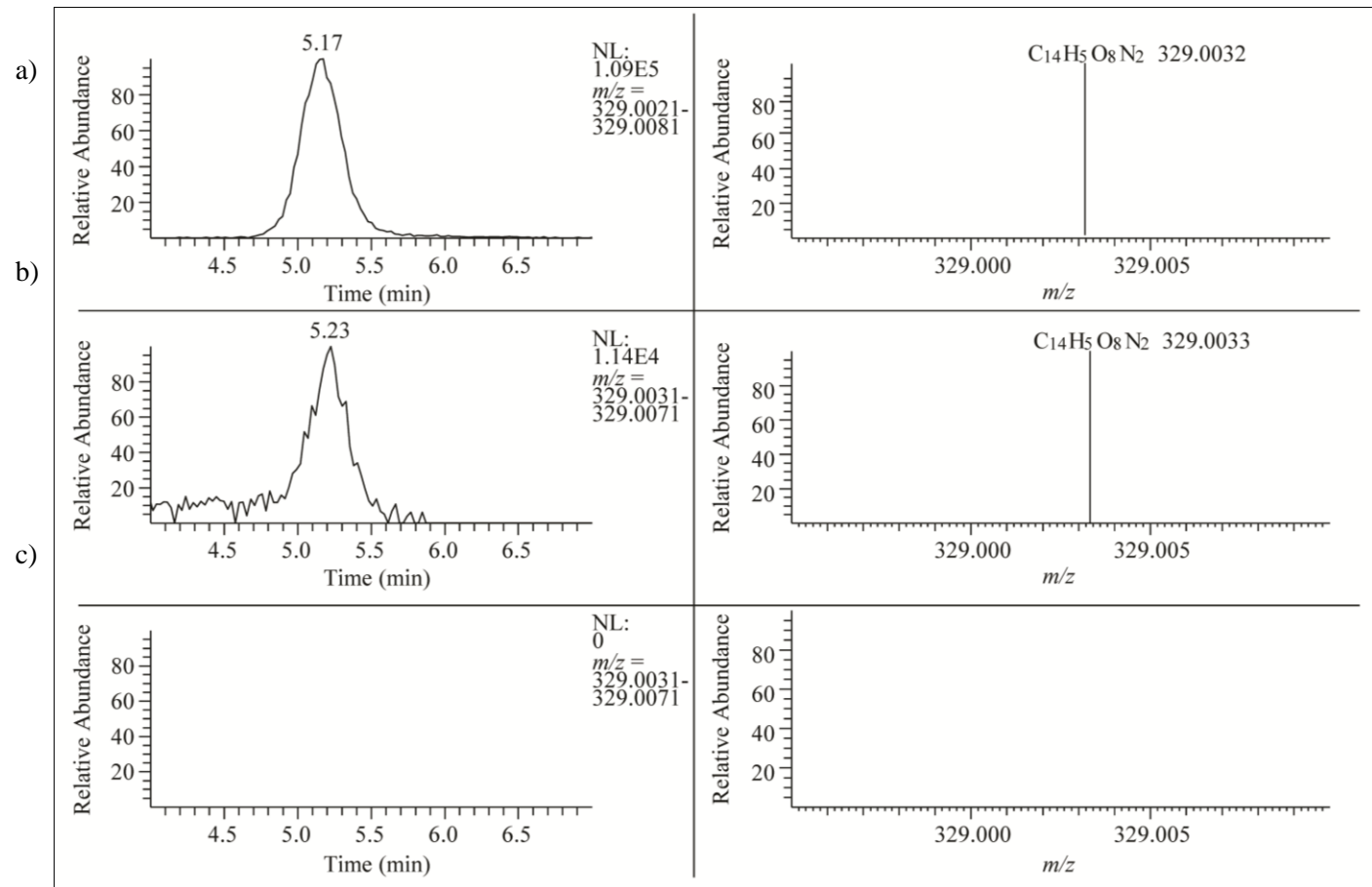
Furthermore, PQQ was extracted from the methanol dehydrogenase (MxaFI) by trypsin digestion, peptides were removed via solid phase extraction, and PQQ was identified via HPLC-MS equipped with an electrospray ionization probe. BSA spiked with PQQ, methanol dehydrogenase, and PQQ standard served as positive control. PQQ (C₁₄H₆N₂O₈) exhibits a molecular weight of 330.21 g mol⁻¹. Analyzed data from HPLC-MS measurements of the PQQ standard, the methanol dehydrogenase sample, and the sample of BSA plus PQQ indicated a retention time of 5.2 min which corresponds to the formula C₁₄H₅O₈N₂ (Fig. 20). No PQQ was detected for the XoxF samples (Fig. 20). Since it is known that PQQ very readily forms adducts at the C₅ position, the data were evaluated carefully with respect to peaks that correspond to several different modifications of the cofactor. However, no PQQ adduct could be found for XoxF.

To recapitulate the findings and to bring the results to a close, I summarize the main points here: I provided evidence that XoxF confers a growth advantage to *M. extorquens* AM1 during colonization of *Arabidopsis thaliana* under competitive conditions. A phenotype could be demonstrated for the *M. extorquens* AM1 *xoxF* mutant, which was characterized by a reduced specific methanol uptake rate and a reduced growth rate during exponential growth on methanol. It could be shown that XoxF does not restore growth on methanol and does not substitute methanol dehydrogenase when *xoxF* or *xoxF2* is overexpressed in the *mxoF* mutant background. The methanol conversion in the *xoxF* mutant occurred less rapidly after carbon starvation compared to the wild type, particularly in the first minutes after substrate addition, which suggests that XoxF plays a role in efficient methanol oxidation. Moreover, only the carbon-starved *M. extorquens* AM1 wild type, but not the *xoxF* mutant strain, accumulated formate during methanol and formaldehyde oxidation and was characterized by a one-hour lag in CO₂ production. The presence of PQQ in the purified XoxF could not be demonstrated by our applied methods. The final point to stress is that the purified XoxF oxidizes methanol and formaldehyde with high affinity.

To conclude, it seems that the function of XoxF could be to provide additional methanol/formaldehyde oxidation capacity, and as one of the dominating proteins in *Methylobacterium extorquens* during plant surface colonization, XoxF confers a growth

advantage to this organism during the process of colonization under competitive conditions.

Fig. 20. PQQ identification via HPLC-MS analyses. The purified enzymes methanol dehydrogenase, and XoxF were trypsin digested, peptides were removed via solid phase extraction, and PQQ was identified via HPLC coupled to an LTQ Orbitrap mass spectrometer equipped with an electrospray ionization probe; the final data are shown in this figure. The PQQ standard is shown in (a), PQQ extracted and identified from methanol dehydrogenase (b), and no PQQ could be extracted and identified from the purified XoxF (c).



Chapter 4: Discussion

In the past, researchers have been unable to elucidate the function of XoxF in *M. extorquens* AM1, in contrast to the in detailed described methanol dehydrogenase. First efforts to describe a phenotype for the *xoxF* mutant of *M. extorquens* AM1 were made by Chistoserdova and Lidstrom (1997). However, no phenotype could be shown for a *xoxF* insertion mutant. A function of XoxF in C₁ metabolism has been suggested - due to the 50% sequence identity to the large subunit of methanol dehydrogenase (Chistoserdova & Lidstrom, 1997) and the conservation of active site residues determined for methanol dehydrogenase in *M. extorquens* AM1 ((Anthony & Williams, 2003); Fig. 6). Additionally, *xoxF* is placed adjacent to *xoxG* and *xoxJ*, which can be found as paralogs in the *mxo* operon (*mxoFJGIRSACKLDEHB*). The genes *xoxG* and *xoxJ* encode for the electron acceptor of methanol dehydrogenase referred to as cytochrome *c_L* (MxaG), and a putative assembly protein or periplasmic chaperon (MxaJ).

This dissertation comprises investigations pointing towards a closer understanding of *M. extorquens* XoxF in methanol and/or formaldehyde conversion. In addition, the article of Delmotte *et al.* (2009) pointed up the strong abundance of XoxF in bacterial phyllosphere communities *in situ*. Hence, another part of this work sets out to focus on the role of XoxF under *in planta* conditions to obtain an accurate picture of the importance of the enzyme during plant colonization.

XoxF of *M. extorquens* AM1 is involved in efficient methanol oxidation.

This work provides experimental evidence that XoxF of *M. extorquens* AM1 is involved in the C₁ metabolism of the organism in addition to MxaF. Growth experiments revealed a 30% reduced specific methanol oxidation rate of a *xoxF* mutant (Table 14). This reduction is in line with the observed reduced growth rate during methylotrophic growth as opposed to that under non-methylotrophic conditions. Experiments of 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. 9ab).

XoxF did not substitute MxaF: Overexpression of *xoxF* in the *mxoF* mutant background did not restore growth on methanol.

The decrease in methanol oxidation might suggest an additional methanol oxidation capacity for XoxF. Such a role would suggest that functional redundancy of MxaF and

XoxF may exist. Since *mxoF* is essential for growth on methanol (Nunn & Lidstrom, 1986b), it was intended to provide evidence for an involvement of XoxF in methanol oxidation or - at best - that XoxF can replace the methanol dehydrogenase by using *M. extorquens* AM1 strains with a defect in the *mxoF* gene, overexpressing *xoxFGJ* (META1_1740 - 1742) or *xoxF2* (META1_2757). However, growth characterizations of these overexpressing strains on methanol under varying conditions did not show any indication for a substitution of MxoF by XoxF. One possible explanation for the lacking growth of the *xoxFGJ* or *xoxF2* overexpressing *mxoF* mutant strains from *M. extorquens* AM1 might be a missing regulation on the protein level, because an increased peptide level of XoxF could be detected via ESI-HPLC-MS/MS for the *mxoF*/pCM80-*xoxFGJ* strain compared to the *mxoF*/pCM80 strain. This might be a missing regulatory factor in absence of the methanol dehydrogenase, a co-regulation of both enzymes on the level of enzyme activity, a missing cofactor that might be present on plants, or a lacking processing of a potential XoxF apoenzyme that inhibits enzyme activity. Activities in cell extracts could not be measured for *mxoF*/pCM80-*xoxFGJ* via the methanol dehydrogenase assay. The sensor-regulator pairs (*mxoQE*, *mxoDM*) which are essential for growth on methanol, control *mxo* gene expression plus *pqq* biosynthesis in a hierarchy postulated by Springer *et al.* (1997) and might also control *xoxF* gene expression. The two component system *mxoDM* might regulate the expression of the *mxoF* and *xoxF* promoter plus the *pqq* biosynthesis genes. MxoB, a response-regulator in *M. extorquens* AM1 might be required not only for *mxoF* (Morris & Lidstrom, 1992; Ramamoorthi & Lidstrom, 1995) but also for *xoxF* induction in addition to the involvement in *pqq* biosynthesis.

XoxF is suggested to be an additional formaldehyde oxidizing enzyme.

The second hypothesis claimed that XoxF functions as a formaldehyde dehydrogenase, which is another possible enzyme activity for XoxF besides the primary role in methanol oxidation. Notably, methanol dehydrogenase of *M. extorquens* AM1 catalyzes not only the oxidation of methanol but also 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) was described earlier that prevents formaldehyde from being oxidized 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; Long & Anthony, 1991) does not have the same effect on 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 cytoplasmic 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. The latter catalyzes the transfer of the formyl group from tetrahydromethanopterin to a presumed methanofuran analog and its subsequent hydrolysis to formate (Pomper & Vorholt, 2001; Pomper *et al.*, 2002) (Fig. 2). 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. 12). This result suggests an additional capacity of the wild type to convert methanol plus formaldehyde or formaldehyde alone. Additional evidence is provided by the one-hour lag phase in CO₂ production of the wild type, which was not observed in the *xoxF* mutant (Fig. 9c). 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; Chistoserdova *et al.*, 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 a situation of sudden increase in the formaldehyde concentration (Fig. 2). During exponential growth, only very low amounts of formate accumulate in culture supernatants of *M. extorquens* AM1 wild type cells ((Chistoserdova *et al.*, 2004); or the *xoxF* mutant this work) suggesting that formate oxidation occurs fast relative to its production. This equilibrium might become imbalanced when cells are starved for carbon and methanol is made suddenly available (Fig. 9). 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. Future approaches are required for a better understanding of the XoxF function in formaldehyde oxidation, e.g. measurements of formaldehyde concentrations in the culture supernatant of *M. extorquens* AM1 wild type and *xoxF* mutant cells. A formaldehyde accumulation in the *xoxF* mutant, but not in the wild type after the methanol pulse would further strengthen the plausibility of a XoxF-involvement in formaldehyde oxidation and could explain an inhibition of methanol dehydrogenase activity when XoxF is missing. A problem might be to establish a sufficiently sensitive assay to determine formaldehyde concentrations in culture

supernatants. It might be possible to follow the colorimetric method described by Nash *et al.* (Nash, 1953) with diacetyl-dihydro-lutidine as product in the presence of acetyl-acetone, ammonia, and formaldehyde or Kato *et al.* with some modifications (Kato, 1990; 1953; Orita *et al.*, 2007). *In vivo* NMR experiments with *xoxF* mutant and the wild type cells with online NMR measurements of methanol, formaldehyde, and formic acid after addition of ^{13}C methanol would be an alternative experimental approach. Formaldehyde sensitivity tests with methanol-grown *M. extorquens* AM1 wild type, *xoxF*, *mxoF*, and *mxoF/xoxF* strains were additionally accomplished to provide evidence that XoxF is involved in formaldehyde oxidation, though resulting in no obvious effect with regard to growth inhibition. This might be explained by a naturally low accumulation of formaldehyde in the periplasm and by a formaldehyde regulation by several proteins, which leads to no visible effect with regard to the growth inhibition.

The monomeric XoxF converted methanol and formaldehyde.

Although it was impossible to compensate the methanol growth defect of the *mxoF* mutant by overexpressing *xoxFGJ*, and the results suggested non-redundant functions of XoxF and MxoF, finally the purified XoxF could be characterized via kinetic analyses. The *in vitro* enzyme assays using purified XoxF from *M. extorquens* AM1 revealed that the enzyme catalyzes with highest affinity the oxidation of methanol, formaldehyde, and ethanol, the three preferred substrates of methanol dehydrogenase (K_m values of 10 μM for methanol, formaldehyde, and ethanol; (Page & Anthony, 1986)). Similarly, the pH optimum of XoxF was found to be 9.0 regarding to the methanol dehydrogenase, and the activity of XoxF *in vitro* was absolutely dependent on the presence of ammonium. The V_{max} values for XoxF of 0.08 U mg^{-1} (determined from methanol grown cells, see results) were 10 times smaller than those measured for methanol dehydrogenase (MxoFI) previously (0.8-1.0 U mg^{-1}) (Goodwin & Anthony, 1996; Page & Anthony, 1986). It should be noted that methanol dehydrogenase is composed of two different subunits, MxoF and MxoI, forming a heterotetrameric enzyme ($\alpha_2\beta_2$) (Afolabi *et al.*, 2001; Anthony & Zatman, 1964a; Williams *et al.*, 2005), whereas XoxF apparently is a monomeric enzyme (Fig. 15). A MxoI orthologous subunit is not encoded in the *xox* cluster. Because the subunit is also absent in the organisms lacking MxoF, such as *R. sphaeroides*, it was already assumed earlier that the enzyme functions without an additional subunit (for discussion see Wilson *et al.*, 2008). For this reason, it cannot be excluded that XoxF may be more fragile, resulting in loss of specific activity during cell disruption and/or purification. No dye-linked XoxF activity could be detected in *R. sphaeroides*, and

methanol activity was determined via oxygen consumption in intact cells (Wilson *et al.*, 2008). A dye-linked alcohol dehydrogenase was characterized, however, from another purple non sulfur bacterium, *Rhodospseudomonas acidophila*, by Quayle's group (Bamforth & Quayle, 1978b; Bamforth & Quayle, 1979; Sahm *et al.*, 1976). Although the 65 kDa protein (denaturing conditions) exhibits similarity with XoxF from *M. extorquens* AM1 regarding a wide substrate specificity range, the substrate affinities are completely different (e.g. K_m for ethanol 30 μ M, K_m for methanol 120 mM) (Sahm *et al.*, 1976). It is currently unclear, whether the described dye-linked alcohol dehydrogenase from *R. acidophila* represents a XoxF ortholog.

No PQQ detection with applied methods

Interestingly, the studies of Goodwin and Anthony (1996) about the *mxmA* mutant of *M. extorquens* AM1 pointed out, that the methanol dehydrogenase purified from the *mxmA* mutant lacks the calcium ion. The missing cofactor led to a strongly different photometric UV/VIS spectrum with a decrease of the characteristic peak at 345 nm, though incorporation of calcium resulted in a normal spectrum. Additionally, a similar spectrum change was observed for the special methanol dehydrogenase of *Methylophaga marina* that sometimes lacks calcium as a result of a relatively low affinity towards the ion (Chan & Anthony, 1992). Since the spectrum scan via UV/VIS photometer of the purified active XoxF from *M. extorquens* AM1 did not indicate the methanol dehydrogenase-typical absorption peak (at 340-350 nm), not even at high concentrations of the purified enzyme (10 mg/ml), the presence/absence of PQQ in XoxF was investigated in detail. Several steps were taken to provide evidence for the presence/absence of PQQ in XoxF.

There was no effect detected by MDH assays on the catalytic activity of XoxF after addition of PQQ and divalent ions. In contrast, the study of Zhao *et al.* (2000) clearly demonstrates the calcium-dependency of methanol dehydrogenase in regard to the enzymatic activity, which significantly enhanced with increasing calcium concentration. The absence of the characteristic 340-350 nm peak during scans of the purified enzyme as well as the independence of PQQ, magnesium, or calcium on XoxF activity may suggest the absence of PQQ in XoxF. In the past, some problems existed concerning the reconstitution of PQQ with the purified methanol dehydrogenase apoprotein. The activity of methanol dehydrogenase from *Methylomonas methanica* could not be retrieved by Patel *et al.* (1978a) and no peak at 340-350 nm could be observed, although they applied an excess of different pteridine derivatives and a high concentration of apo-methanol dehydrogenase. By contrast, Davidson *et al.* (1985) could reassociate the PQQ derived

from the native methanol dehydrogenase of the bacterium W3A1 (*Methylophilus*) with the apoenzyme, as monitored by similar absorption spectra for the reconstituted enzyme and the native one. However, a successful reconstitution of the MDH activity was not reported (Davidson *et al.*, 1985). Likewise, Anthony (2000) mentioned in his review that a reconstitution of active enzyme from PQQ plus the apoenzyme of MDH was impossible. The attempts were more successful in case of glucose dehydrogenases (GDHs) (Cozier *et al.*, 1999; Duine *et al.*, 1980). A smaller amount of equatorial interactions between GDH and PQQ was suggested by Cozier *et al.* as possible reason for the still unrealized reconstitution of MDH activity (Cozier *et al.*, 1999). Because of the mentioned problems to reconstitute the activity of apo-methanol dehydrogenase with PQQ, XoxF activity was analyzed in the absence of PQQ using an *M. extorquens* mutant strain that is defective in PQQ biosynthesis, as well as an *E. coli* strain (no PQQ biosynthesis known), both carrying a plasmid that enables to overexpress *xoxF* and to measure the activity of XoxF in absence of PQQ. However, no expression of *xoxF* could be detected after purification in case of the *pqq* mutant, and an insufficient expression was observed for the purification from *E. coli*-pET24b⁺-*xoxF*. This might be explained e.g. by missing chaperons, regulatory proteins, or cofactors in *E. coli*, and by an instability of the translated peptide in absence of PQQ.

Additionally, PQQ was extracted from the purified methanol dehydrogenase as well as from BSA plus PQQ (trypsin digestion protocol, solid phase extraction with reversed-phase column), and identified after analyses of ESI HPLC-MS/MS data. No PQQ could be identified from the analyzed data of the XoxF samples. PQQ might be absent in XoxF; no cofactor might be bound to the enzyme; the enzyme might be regulated by another cofactor or an unknown PQQ derivative, since PQQ is chemically highly active. The PQQ concentration might have been under the detection limit, as the leakage of PQQ during the extraction from BSA spiked with PQQ was relatively high. A cofactor might be better protected inside the tetrameric ($\alpha_2\beta_2$) methanol dehydrogenase (each α -subunit contains one non-covalently bound PQQ) than inside the active center of the monomeric XoxF. Interestingly, all important amino acid residues (Trp²⁴³, Glu¹⁷⁷, Asn²⁶¹, Arg³³¹), which are involved in PQQ and Ca²⁺ binding in MDH of *M. extorquens* AM1, are also present in XoxF. Further efforts have to be made to identify a potential cofactor of XoxF.

XoxF conferred a growth advantage to *M. extorquens* AM1 during colonization of *Arabidopsis thaliana* under competitive conditions.

In the context of plant colonization, only very recently the research interest was focused on XoxF, particularly with regard to the high abundance of XoxF within the detected bacterial proteins in the phyllosphere of natural grown soybean (*Glycine max*), clover (*Trifolium repens*), and *A. thaliana* (Delmotte *et al.*, 2009) (Fig. 7). The high abundance of XoxF and XoxG in *Methylobacterium* during plant colonization has been completely uninvestigated before. No experiments have been carried out so far to elucidate the function of XoxF during bacterial colonization of plant surfaces, and still it is unknown how XoxF is involved in the bacterial C₁ metabolism during this colonization process. However, the observation suggests an important role for XoxF in a natural environment, e.g. the phyllosphere. The results of the conducted plant inoculation experiments indicate the importance of XoxF for plant colonization under competitive conditions (Table 13). The *mxoF*, *xoxF* and *mxoF-xoxF* mutations resulted in significant loss of competitive fitness, which is similar to earlier results showing that *M. extorquens* AM1 benefits from methanol dehydrogenase during colonization of *Medicago truncatula* (Sy *et al.*, 2005). Here, a significantly greater loss of the *xoxF* insertion mutant was observed when compared to that of the *mxoF* mutant in competition with the wild type. This points clearly to a primary role of XoxF under environmental conditions. The high abundance of XoxF during plant colonization may induce effects that cannot be mimicked under "in vivo" laboratory conditions currently. The higher abundance of XoxF under natural conditions may reveal additional phenotypes and 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* in dependence of the *mxoF* promoter (pCM80-*xoxFGJ*) compared to the wild type with empty plasmid. It was expected 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 might become available.

Additional investigations will be required to further elucidate the biochemical function of XoxF in methanol and/or formaldehyde oxidation. Furthermore, the molecular basis for the regulation of the expression level of XoxF remains to be uncovered. The mentioned results contribute to the understanding of bacteria in the phyllosphere regarding

the C₁ metabolism - especially with the advantage to convert methanol and to occupy ecological niches.

Concluding remarks

This work demonstrated that XoxF of *M. extorquens* AM1 plays a role in C₁ metabolism, probably in the primary oxidation steps. The enzyme is not capable of substituting the function of the methanol dehydrogenase, which might be explained by missing regulations on the protein level. Besides that, XoxF was suggested to be a bifunctional enzyme with formaldehyde-oxidizing activities - similar to the methanol dehydrogenase. The presumption was based on the two different phenotypes, which were characterized for the *M. extorquens xoxF* mutant: on the one hand the *xoxF* mutant showed a reduced growth rate and a reduced specific methanol conversion rate during the exponential growth on methanol, on the other hand the mutant did not accumulate formate during growth on methanol after starvation as observed for the wild type strain and consumed methanol less rapidly than the wild type strain.

Although PQQ could not be detected with the applied methods, further investigations have to be carried out in this direction. Moreover, it can be concluded that XoxF is important during plant colonization, as it confers a growth advantage to *M. extorquens* AM1 under competitive conditions.

Outlook

In the following section I would like to list various ideas for future experiments:

For a better understanding of the XoxF function, conditions have to be found, where XoxF is highly abundant during growth on methanol at levels comparable to those of MxaF and to those of XoxF observed in *Methylobacteria* during plant colonization in nature (Delmotte *et al.*, 2009). Further investigations are necessary to determine whether XoxF might act as a regulator of methanol oxidation. Site-directed mutagenesis of the *xoxF* gene, for instance by exchange of the cysteines 103 and 104, might be a reasonable approach to elucidate the mechanism of the XoxF function. The formaldehyde content in the supernatant of carbon-starved *M. extorquens* AM1 *xoxF* and wild type cells during methanol or formaldehyde oxidation has to be examined to provide further evidence of the XoxF involvement in methanol/formaldehyde oxidation. Moreover, further attention has to be drawn to the analyses of a modified PQQ or other cofactors that might be present in XoxF. It would be interesting to know whether there might be a difference in XoxF activity in case of a XoxFJ copurification.

To understand the complete function of XoxF in C₁ metabolism, experiments have to be conducted to elucidate the functions of XoxF2 (META1_2757) and of the proteins encoded by the *mx*a cluster. To gain more insights into the function of XoxF (META1_1740) and XoxF2 (META1_2757) it would be helpful to examine the single (*xoxF*, *xoxF2*) and double (*xoxF-xoxF2*) mutant in comparison, as well as the purified proteins XoxF and XoxF2. In order to enhance the activity of XoxF, it might be interesting to study the regulatory proteins involved in methanol oxidation more in detail and further efforts have to be made to discover new regulatory enzymes.

Furthermore, it would be worth to identify the Modifier protein (M-protein) that prevents formaldehyde from being oxidized by methanol dehydrogenase by lowering its affinity to formaldehyde (Bolbot & Anthony, 1980; Ford *et al.*, 1985; Page & Anthony, 1986). In addition, it would be interesting to know if the M-protein has the same effect on XoxF or XoxF2 as on the methanol dehydrogenase (Long & Anthony, 1990; Long & Anthony, 1991).

In general, the remaining gaps of the C₁ metabolism have to be closed. Regarding the function of XoxF, it would be especially of interest to study the presence of an alternative, periplasmic route of formaldehyde oxidation to formate. In this context, it would be interesting to examine the formate dehydrogenase that contains the Tat motif used for periplasmic localization (Palmer *et al.*, 2005).

With this Ph.D. work I have tried to draw the microbial methanol oxidation pathway to the attention of the reader, which operates for example in aerobic methylotrophic

bacteria during plant colonization. I hope that the present work serves as a baseline for further excursions into the regulation of microbial methanol oxidation.

References

- Abanda-Nkpwatt, D., Krimm, U., Coiner, H. A., Schreiber, L. & Schwab, W. (2006).** Plant volatiles can minimize the growth suppression of epiphytic bacteria by the phytopathogenic fungus *Botrytis cinerea* in co-culture experiments. *Environ Exp Botany* **56**, 108-119.
- Adachi, O., Matsushita, K., Shinagawa, E. & Ameyama, M. (1990).** Calcium in quinoprotein methanol dehydrogenase can be replaced by strontium. *AgricBiol Chem* **54**, 2833-2837.
- Adeosun, E. K., Smith, T. J., Hoberg, A. M., Velarde, G., Ford, R. & Dalton, H. (2004).** Formaldehyde dehydrogenase preparations from *Methylococcus capsulatus* (Bath) comprise methanol dehydrogenase and methylene tetrahydromethanopterin dehydrogenase. *Microbiology* **150**, 707-713.
- Afolabi, P. R., Mohammed, F., Amaratunga, K. & other authors (2001).** Site-directed mutagenesis and X-ray crystallography of the PQQ-containing quinoprotein methanol dehydrogenase and its electron acceptor, cytochrome *c_L*. *Biochemistry* **40**, 9799-9809.
- Alefounder, P. R. & Ferguson, S. J. (1981).** A periplasmic location for methanol dehydrogenase from *Paracoccus denitrificans*: implications for proton pumping by cytochrome *aa₃*. *Biochem Biophys Res Commun* **98**, 778-784.
- Amaratunga, K., Goodwin, P. M., OConnor, C. D. & Anthony, C. (1997).** The methanol oxidation genes *mxoFJGIR(S)ACKLD* in *Methylobacterium extorquens*. *FEMS Microbiol Lett* **146**, 31-38.
- Ander, P. & Eriksson, K.-E. (1984).** Methanol formation during lignin degradation by *Phanerochaete chrysosporium*. *Appl Microbiol Biotechnol* **21**, 96-102.
- Anderson, D. J. & Lidstrom, M. E. (1988).** The *moxFG* region encodes four polypeptides in the methanol-oxidizing bacterium *Methylobacterium* sp. strain AM1. *J Bacteriol* **170**, 2254-2262.

Anderson, D. J., Morris, C. J., Nunn, D. N., Anthony, C. & Lidstrom, M. E. (1990). Nucleotide sequence of the *Methylobacterium extorquens* AM1 *moxF* and *moxJ* genes involved in methanol oxidation. *Gene* **90**, 173-176.

Anthony, C. & Zatman, L. J. (1964a). The methanol-oxidizing enzyme of *Pseudomonas* sp. M 27. *Biochem J* **92**, 614-621.

Anthony, C. & Zatman, L. J. (1964b). Microbial oxidation of methanol. 1. Isolation and properties of *Pseudomonas* sp. M 27. *Biochem J* **92**, 609-614.

Anthony, C. & Zatman, L. J. (1964c). Microbial oxidation of methanol. 2. Methanol-oxidizing enzyme of *Pseudomonas* sp. M 27. *Biochem J* **92**, 614-621.

Anthony, C. & Zatman, L. J. (1965). The alcohol dehydrogenase of *Pseudomonas* sp. M27. *Biochem J* **96**, 808-812.

Anthony, C. & Zatman, L. J. (1967a). The microbial oxidation of methanol: purification and properties of the alcohol dehydrogenase of *Pseudomonas* sp. M27. *Biochem J* **104**, 953-959.

Anthony, C. & Zatman, L. J. (1967b). The microbial oxidation of methanol: The prosthetic group of the alcohol dehydrogenase of *Pseudomonas* sp. M27: a new oxidoreductase prosthetic group. *Biochem J* **104**, 960-969.

Anthony, C. (1982). *The biochemistry of methylotrophs*, 1st edition. Academic Press, London, 431 pp.

Anthony, C. (1986). The bacterial oxidation of methane and methanol. *Adv Microb Physiol* **27**, 113-210.

Anthony, C. (1992). The c-type cytochromes of methylotrophic bacteria. *Biochim Biophys Acta* **1099**, 1-15.

Anthony, C., Ghosh, M. & Blake, C. C. (1994). The structure and function of methanol dehydrogenase and related quinoproteins containing pyrroloquinoline quinone. *Biochem J* **304**, 665-674.

- Anthony, C. (1996).** Quinoprotein-catalysed reactions. *Biochem J* **320**, 697-711.
- Anthony, C. & Ghosh, M. (1998).** The structure and function of the PQQ-containing quinoprotein dehydrogenases. *Progr Biophys Mol Biol* **69**, 1-21.
- Anthony, C. (2000).** Methanol dehydrogenase, a PQQ-containing quinoprotein dehydrogenase. *Subcell Biochem* **35**, 73-117.
- Anthony, C. (2001).** Pyrroloquinoline quinone (PQQ) and quinoprotein enzymes. *Antioxid Redox Signal* **3**, 757-774.
- Anthony, C. & Williams, P. (2003).** The structure and mechanism of methanol dehydrogenase. *Biochim Biophys Acta* **1647**, 18-23.
- Arfman, N., Watling, E. M., Clement, W., van Oosterwijk, R. J., de Vries, G. E., Harder, W., Attwood, M. M. & Dijkhuizen, L. (1989).** Methanol metabolism in thermotolerant methylotrophic *Bacillus* strains involving a novel catabolic NAD-dependent methanol dehydrogenase as a key enzyme. *Arch Microbiol* **152**, 280-288.
- Arfman, N., Hektor, H. J., Bystrykh, L. V., Govorukhina, N. I., Dijkhuizen, L. & Frank, J. (1997).** Properties of an NAD(H)-containing methanol dehydrogenase and its activator protein from *Bacillus methanolicus*. *Eur J Biochem* **244**, 426-433.
- Avezoux, A., Goodwin, M. G. & Anthony, C. (1995).** The role of the novel disulphide ring in the active site of the quinoprotein methanol dehydrogenase from *Methylobacterium extorquens*. *Biochem J* **307**, 735-741.
- Bamforth, C. W. & Quayle, J. R. (1978a).** Aerobic and anaerobic growth of *Paracoccus denitrificans* on methanol. *Arch Microbiol* **119**, 91-97.
- Bamforth, C. W. & Quayle, J. R. (1978b).** The dye-linked alcohol dehydrogenase of *Rhodospseudomonas acidophila*. Comparison with dye-linked methanol dehydrogenases. *Biochem J* **169**, 677-686.
- Bamforth, C. W. & Quayle, J. R. (1979).** Structural aspects of the dye-linked alcohol-dehydrogenase of *Rhodospseudomonas acidophila*. *Biochem J* **181**, 517-524.

Barber, R. D., Rott, M. A. & Donohue, T. J. (1996). Characterization of a glutathione-dependent formaldehyde dehydrogenase from *Rhodobacter sphaeroides*. *J Bacteriol* **178**, 1386-1393.

Barber, R. D. & Donohue, T. J. (1998). Function of a glutathione-dependent formaldehyde dehydrogenase in *Rhodobacter sphaeroides* formaldehyde oxidation and assimilation. *Biochemistry* **37**, 530-537.

Basford, R. E. & Huennekens, F. M. (1955). Studies on thiols .1. Oxidation of thiol groups by 2,6-dichlorophenol indophenol. *J Am Chem Soc* **77**, 3873-3877.

Beardmore-Gray, M., O'Keeffe, D. T. & Anthony, C. (1982). The autoreducible cytochromes *c* of the methylotrophs *Methylophilus methylotrophus* and *Pseudomonas* AM1. *Biochem J* **207**, 161-165.

Beardmore-Gray, M. & Anthony, C. (1983). The absence of quinoprotein alcohol dehydrogenase in *Acinetobacter calcoaceticus*. *J Gen Microbiol* **129**, 2979-2983.

Bendtsen, J. D., Nielsen, H., von Heijne, G. & Brunak, S. (2004). Improved prediction of signal peptides. *J Mol Biol* **340**, 783-795.

Bischoff, J., Domrachev, M., Federhen, S., Hotton, C., Leipe, D., Soussov, V., Sternberg, R., Turner, S.: NCBI Taxonomy Browser;
(<http://www.ncbi.nlm.nih.gov/Taxonomy>)

Blake, C. C., Ghosh, M., Harlos, K., Avezoux, A. & Anthony, C. (1994). The active site of methanol dehydrogenase contains a disulphide bridge between adjacent cysteine residues. *Nat Struct Biol* **1**, 102-195.

Bolbot, J. A. & Anthony, C. (1980). The metabolism of 1,2-propanediol by the facultative methylotroph *Pseudomonas* AM1. *J Gen Microbiol* **120**, 245-254.

Bosch, G., Skovran, E., Xia, Q. W., Wang, T. S., Taub, F., Miller, J. A., Lidstrom, M. E. & Hackett, M. (2008). Comprehensive proteomics of *Methylobacterium extorquens* AM1 metabolism under single carbon and nonmethylotrophic conditions. *Proteomics* **8**, 3494-3505.

- Bosch, G., Wang, T., Latypova, E., Kalyuzhnaya, M. G., Hackett, M. & Chistoserdova, L. (2009).** Insights into the physiology of *Methylothera mobilis* as revealed by metagenome-based shotgun proteomic analysis. *Microbiol* **155**, 1103-1110.
- Bradford, M. M. (1976).** Rapid and sensitive method for quantitation of microgram quantities of protein utilizing principle of protein-dye binding. *Anal Biochem* **72**, 248-254.
- Brantner, C. A., Remsen, C. C., Owen, H. A., Buchholz, L. A. & Perille Collins, M. L. (2002).** Intracellular localization of the particulate methane monooxygenase and methanol dehydrogenase in *Methylomicrobium album* BG8. *Arch Microbiol* **178**, 59-64.
- Bystrykh, L., Arfman, N. & Dijkhuizen, L. (1993a).** The methanol-oxidizing enzyme systems in Gram-positive methylotrophic bacteria, pp. 245-251. In: *Microbial growth on C₁ compounds* (Murrell, C. & Kelly, D.P., editors), Intercept Ltd., Andover.
- Bystrykh, L. V., Vonck, J., van Bruggen, E. F., van Beeumen, J., Samyn, B., Govorukhina, N. I., Arfman, N., Duine, J. A. & Dijkhuizen, L. (1993b).** Electron microscopic analysis and structural characterization of novel NADP(H)-containing methanol: N,N'-dimethyl-4-nitrosoaniline oxidoreductases from the gram-positive methylotrophic bacteria *Amycolatopsis methanolica* and *Mycobacterium gastri* MB19. *J Bacteriol* **175**, 1814-1822.
- Carver, M. A., Humphrey, K. M., Patchett, R. A. & Jones, C. W. (1984).** The effect of EDTA and related chelating agents on the oxidation of methanol by the methylotrophic bacterium, *Methylophilus methylotrophus*. *Eur J Biochem* **138**, 611-615.
- Chan, H. T. & Anthony, C. (1992).** The mechanism of inhibition by EDTA and EGTA of methanol oxidation by methylotrophic bacteria. *FEMS Microbiol Lett* **75**, 231-234.
- Chang, A. K., Lim, C. Y., Kim, S. W., You, H. J., Hahm, K. S., Yoon, S. M., Park, J. K. & Lee, J. S. (2002).** Purification and characterization of two forms of methanol dehydrogenases from a marine methylotroph. *J Basic Microbiol* **42**, 238-245.
- Chen, Z. W., Matsushita, K., Yamashita, T., Fujii, T. A., Toyama, H., Adachi, O., Bellamy, H. D. & Mathews, F. S. (2002).** Structure at 1.9 angstrom resolution of a quinoxinoprotein alcohol dehydrogenase from *Pseudomonas putida* HK5. *Structure* **10**, 837-849.

Chistoserdov, A. Y., Chistoserdova, L. V., McIntire, W. S. & Lidstrom, M. E. (1994). Genetic organization of the *mau* gene cluster in *Methylobacterium extorquens* AM1: complete nucleotide sequence and generation and characteristics of *mau* mutants. *J Bacteriol* **176**, 4052-4065.

Chistoserdova, L. & Lidstrom, M. E. (1997). Molecular and mutational analysis of a DNA region separating two methylotrophy gene clusters in *Methylobacterium extorquens* AM1. *Microbiology* **143**, 1729-1736.

Chistoserdova, L., Vorholt, J. A., Thauer, R. K. & Lidstrom, M. E. (1998). C₁ transfer enzymes and coenzymes linking methylotrophic bacteria and methanogenic Archaea. *Science* **281**, 99-102.

Chistoserdova, L., Gomelsky, L., Vorholt, J. A., Gomelsky, M., Tsygankov, Y. D. & Lidstrom, M. E. (2000). Analysis of two formaldehyde oxidation pathways in *Methylobacillus flagellatus* KT, a ribulose monophosphate cycle methylotroph. *Microbiology* **146**, 233-238.

Chistoserdova, L., Chen, S. W., Lapidus, A. & Lidstrom, M. E. (2003). Methylotrophy in *Methylobacterium extorquens* AM1 from a genomic point of view. *J Bacteriol* **185**, 2980-2987.

Chistoserdova, L., Laukel, M., Portais, J. C., Vorholt, J. A. & Lidstrom, M. E. (2004). Multiple formate dehydrogenase enzymes in the facultative methylotroph *Methylobacterium extorquens* AM1 are dispensable for growth on methanol. *J Bacteriol* **186**, 22-28.

Chistoserdova, L., Crowther, G. J., Vorholt, J. A., Skovran, E., Portais, J. C. & Lidstrom, M. E. (2007). Identification of a fourth formate dehydrogenase in *Methylobacterium extorquens* AM1 and confirmation of the essential role of formate oxidation in methylotrophy. *J Bacteriol* **189**, 9076-9081.

Chistoserdova, L., Kalyuzhnaya, M. G. & Lidstrom, M. E. (2009). The expanding world of methylotrophic metabolism. *Ann Rev Microbiol* **63**, 477-499.

- Chistoserdova, L. V. & Lidstrom, M. E. (1994).** Genetics of the serine cycle in *Methylobacterium extorquens* AM1: identification of *sgaA* and *mtdA* and sequences of *sgaA*, *hprA*, and *mtdA*. *J Bacteriol* **176**, 1957-1968.
- Corpe, W. A. (1985).** A method for detecting methylotrophic bacteria on solid surfaces. *J Microbiol Methods* **3**, 215-221.
- Corpe, W. A. & Rheem, S. (1989).** Ecology of the methylotrophic bacteria on living leaf surfaces. *FEMS Microbiol Ecol* **62**, 243-249.
- Cox, J. M., Day, D. J. & Anthony, C. (1992).** The interaction of methanol dehydrogenase and its electron acceptor, cytochrome *c_L* in methylotrophic bacteria. *Biochim Biophys Acta* **1119**, 97-106.
- Cozier, G. E., Salleh, R. A. & Anthony, C. (1999).** Characterization of the membrane quinoprotein glucose dehydrogenase from *Escherichia coli* and characterization of a site-directed mutant in which histidine-262 has been changed to tyrosine. *Biochem J* **340**, 639-647.
- Cross, A. B. & Anthony, C. (1980a).** The electron-transport chains of the obligate methylotroph *Methylophilus methylotrophus*. *Biochem J* **192**, 429-439.
- Cross, A. R. & Anthony, C. (1980b).** The purification and properties of the soluble cytochromes *c* of the obligate methylotroph *Methylophilus methylotrophus*. *Biochem J* **192**, 421-427.
- Crowther, G. J., Kosály, G. & Lidstrom, M. E. (2008).** Formate as the main branch point for methylotrophic metabolism in *Methylobacterium extorquens* AM1. *J Bacteriol* **190**, 5057-5062.
- Dales, S. L. & Anthony, C. (1995).** The interaction of methanol dehydrogenase and its cytochrome electron acceptor. *Biochem J* **312**, 261-265.
- Davidson, V. L., Neher, J. W. & Cecchini, G. (1985).** The biosynthesis and assembly of methanol dehydrogenase in bacterium W3A1. *J Biol Chem* **260**, 9642-9647.

Davidson, V. L., Kumar, M. A. & Wu, J. Y. (1992a). Apparent oxygen-dependent inhibition by superoxide dismutase of the quinoprotein methanol dehydrogenase. *Biochemistry* **31**, 1504-1508.

Davidson, V. L., Wu, J., Miller, B. & Jones, L. H. (1992b). Factors affecting the stability of methanol dehydrogenase from *Paracoccus denitrificans*. *FEMS Microbiol Lett* **73**, 53-58.

Day, D. J. & Anthony, C. (1990). Methanol dehydrogenase from *Methylobacterium extorquens* AM1. *Methods Enzymol* **188**, 210-216.

de Beer, R., Duine, J. A., Frank, J. & Westerling, J. (1983). The role of pyrroloquinoline semiquinone forms in the mechanism of action of methanol dehydrogenase. *Eur J Biochem* **130**, 105-109.

De Boer, L., Dijkhuizen, L., Grobben, G., Goodfellow, M., Stackebrandt, E., Parlett, J. H., Whitehead, D. & Witt, D. (1990). *Amycolatopsis methanolica* sp. nov., a facultatively methylotrophic actinomycete. *Int J Syst Bacteriol* **40**, 194-204.

Dedysh, S. N., Smirnova, K. V., Khmelenina, V. N., Suzina, N. E., Liesack, W. & Trotsenko, Y. A. (2005). Methylotrophic autotrophy in *Beijerinckia mobilis*. *J Bacteriol* **187**, 3884-3888.

Dekker, R. H., Duine, J. A., Frank, J., Verwiel, P. E. & Westerling, J. (1982). Covalent addition of H₂O, enzyme substrates and activators to pyrroloquinoline quinone, the coenzyme of quinoproteins. *Eur J Biochem* **125**, 69-73.

Delmotte, N., Knief, C., Chaffron, S., Innerebner, G., Roschitzki, B., Schlapbach, R., von Mering, C. & Vorholt, J. A. (2009). Community proteogenomics reveals insights into the physiology of phyllosphere bacteria. *Proc Natl Acad Sci U S A* **106**, 16428-16433.

Dijkstra, M., Frank, J., Jongejan, J. A. & Duine, J. A. (1984). Inactivation of quinoprotein alcohol dehydrogenases with cyclopropane-derived suicide substrates. *Eur J Biochem* **140**, 369-373.

Dijkstra, M., Frank, J. & Duine, J. A. (1988). Methanol oxidation under physiological conditions using methanol dehydrogenase and a factor isolated from *Hyphomicrobium X*. *FEBS Lett* **227**, 198-202.

Dijkstra, M., Frank, J., Jr. & Duine, J. A. (1989). Studies on electron transfer from methanol dehydrogenase to cytochrome *c_L*, both purified from *Hyphomicrobium X*. *Biochem J* **257**, 87-94.

DiSpirito, A. A. (1990). Soluble cytochromes *c* from *Methylomonas A4*. *Meth Enzymol* **188**, 289-297.

Duine, J. A., Frank, J. & Westerling, J. (1978). Purification and properties of methanol dehydrogenase from *Hyphomicrobium x*. *Biochim Biophys Acta* **524**, 277-287.

Duine, J. A. & Frank, J., Jr. (1980). Studies on methanol dehydrogenase from *Hyphomicrobium x*. Isolation of an oxidized form of the enzyme. *Biochem J* **187**, 213-209.

Duine, J. A., Frank, J. & Verwiël, P. E. (1980). Structure and activity of the prosthetic group of methanol dehydrogenase. *Eur J Biochem* **108**, 187-192.

Duine, J. A., Frank, J. & Verwiël, P. E. J. (1981). Characterization of the second prosthetic group in methanol dehydrogenase from *Hyphomicrobium X*. *Eur J Biochem* **118**, 395-399.

Duine, J. A., Jzn., J. F. & Jongejan, J. A. (1986). PQQ and quinoprotein enzymes in microbial oxidations. *FEMS Microbiol Rev* **32**, 165-178.

Duine, J. A. & Frank, J. (1990). The role of PQQ and quinoproteins in methylotrophic bacteria. *FEMS Microbiol Rev* **87**, 221-225.

Duine, J. A. (1999). The PQQ story. *J Biosci Bioeng* **88**, 231-236.

Erb, T. J., Berg, I. A., Brecht, V., Muller, M., Fuchs, G. & Alber, B. E. (2007). Synthesis Of C₅-dicarboxylic acids from C₂-units involving crotonyl-CoA carboxylase/reductase: The ethylmalonyl-CoA pathway. *Proc Natl Acad Sci U S A* **104**, 10631-10636.

Euzéby, J. P. (2010). List of prokaryotic names with standing in nomenclature (LPSN). www.bacterio.net, last complete update: August 05, 2010

Fall, R. & Benson, A. A. (1996). Leaf methanol - the simplest natural product from plants. *Trends Plant Sci* **1**, 296-301.

Flueckiger, R., Paz, M. A. & Gallop, P. M. (1995). Redox-cycling detection of dialyzable pyrroloquinoline quinone and quinoproteins. *Methods Enzymol* **258**, 140-149.

Ford, S., Page, M. D. & Anthony, C. (1985). The role of a methanol dehydrogenase modifier protein and aldehyde dehydrogenase in the growth of *Pseudomonas* AM1 on 1,2-propanediol. *J Gen Microbiol* **131**, 2173-2182.

Frank, J. & Duine, J. A. (1990). Methanol dehydrogenase from *Hyphomicrobium* X. *Methods Enzymol* **188**, 202-209.

Frank, J., Jr., Dijkstra, M., Duine, J. A. & Balny, C. (1988). Kinetic and spectral studies on the redox forms of methanol dehydrogenase from *Hyphomicrobium* X. *Eur J Biochem* **174**, 331-338.

Frank, J., Jr., van Krimpen, S. H., Verwiél, P. E., Jongejan, J. A., Mulder, A. C. & Duine, J. A. (1989). On the mechanism of inhibition of methanol dehydrogenase by cyclopropane-derived inhibitors. *Eur J Biochem* **184**, 187-195.

Ghosh, M., Anthony, C., Harlos, K., Goodwin, M. G. & Blake, C. (1995). The refined structure of the quinoprotein methanol dehydrogenase from *Methylobacterium extorquens* at 1.94 Å *Structure* **3**, 177-187.

Ghosh, R. & Quayle, J. R. (1979). Phenazine ethosulfate as a preferred electron-acceptor to phenazine methosulfate in dye-linked enzyme assays. *Anal Biochem* **99**, 112-117.

Ghosh, R. & Quayle, J. R. (1981). Purification and properties of the methanol dehydrogenase from *Methylophilus methylotrophus*. *Biochem J* **199**, 245-250.

Goenrich, M., Bartoschek, S., Hagemeyer, C. H., Griesinger, C. & Vorholt, J. A. (2002a). A glutathione-dependent formaldehyde-activating enzyme (Gfa) from

Paracoccus denitrificans detected and purified via two-dimensional proton exchange NMR spectroscopy. *J Biol Chem* **277**, 3069-3072.

Goenrich, M., Bursy, J., Hubner, E., Linder, D., Schwartz, A. C. & Vorholt, J. A. (2002b). Purification and characterization of the methylene tetrahydromethanopterin dehydrogenase MtdB and the methylene tetrahydrofolate dehydrogenase FOLD from *Hyphomicrobium zavarzinii* ZV580. *Arch Microbiol* **177**, 299-303.

Gomelsky, M., Biville, F., Gasser, F. & Tsygankov, Y. D. (1996). Identification and characterization of the *pqqDGC* gene cluster involved in pyrroloquinoline quinone production in an obligate methylotroph *Methylobacillus flagellatum*. *FEMS Microbiol Lett* **141**, 169-176.

Goodwin, M. G. & Anthony, C. (1996). Characterization of a novel methanol dehydrogenase containing a Ba²⁺ ion at the active site. *Biochem J* **318** 673-679.

Gourion, B., Rossignol, M. & Vorholt, J. A. (2006). A proteomic study of *Methylobacterium extorquens* reveals a response regulator essential for epiphytic growth. *Proc Natl Acad Sci U S A* **103**, 13186-13191.

Gourion, B., Francez-Charlot, A. & Vorholt, J. A. (2008). PhyR is involved in the general stress response of *Methylobacterium extorquens* AM1. *J Bacteriol* **190**, 1027-1035.

Grosse, S., Wendlandt, K. D. & Kleber, H. P. (1997). Purification and properties of methanol dehydrogenase from *Methylocystis* sp. GB 25. *J Basic Microbiol* **37**, 269-279.

Grosse, S., Voigt, C., Wendlandt, K. D. & Kleber, H. P. (1998). Purification and properties of methanol dehydrogenase from *Methylosinus* sp. WI 14. *J Basic Microbiol* **38**, 189-196.

Hagemeier, C. H., Chistoserdova, L., Lidstrom, M. E., Thauer, R. K. & Vorholt, J. A. (2000). Characterization of a second methylene tetrahydromethanopterin dehydrogenase from *Methylobacterium extorquens* AM1. *Eur J Biochem* **267**, 3762-3769.

Harms, N., Ras, J., Koning, S., Reijnders, W. N. M., Stouthamer, A. H. & Van Spanning, R. J. M. (1996a). Genetics of C₁ metabolism regulation in *Paracoccus*

denitrificans, pp. 126-132. In: *Microbial growth on C₁ compounds* (Lidstrom, M.E. & Tabita, F.R., editors), Kluwer Academic Publishers, Dordrecht.

Harms, N., Ras, J., Reijnders, W. N., van Spanning, R. J. & Stouthamer, A. H. (1996b). S-formylglutathione hydrolase of *Paracoccus denitrificans* is homologous to human esterase D: a universal pathway for formaldehyde detoxification? *J Bacteriol* **178**, 6296-6299.

Harris, T. K. & Davidson, V. L. (1993). A new kinetic model for the steady-state reactions of the quinoprotein methanol dehydrogenase from *Paracoccus denitrificans*. *Biochemistry* **32**, 4362-4368.

Harris, T. K. & Davidson, V. L. (1994). Replacement of enzyme-bound calcium with strontium alters the kinetic properties of methanol dehydrogenase. *Biochem J* **300**, 175-182.

Harris, T. K., Davidson, V. L., Chen, L., Mathews, F. S. & Xia, Z. X. (1994). Ionic strength dependence of the reaction between methanol dehydrogenase and cytochrome *c_{551i}*: evidence of conformationally coupled electron transfer. *Biochemistry* **33**, 12600-12608.

Hektor, H. J., Kloosterman, H. & Dijkhuizen, L. (2000). Nicotinoprotein methanol dehydrogenase enzymes in Gram-positive methylotrophic bacteria. *J Mol Cat* **8**, 103-109.

Hektor, H. J., Kloosterman, H. & Dijkhuizen, L. (2002). Identification of a magnesium-dependent NAD(P)(H)-binding domain in the nicotinoprotein methanol dehydrogenase from *Bacillus methanolicus*. *J Biol Chem* **277**, 46966-46973.

Hensgens, C. M., Vonck, J., Van Beeumen, J., van Bruggen, E. F. & Hansen, T. A. (1993). Purification and characterization of an oxygen-labile, NAD-dependent alcohol dehydrogenase from *Desulfovibrio gigas*. *J Bacteriol* **175**, 2859-2863.

Hensgens, C. M., Hagen, W. R. & Hansen, T. A. (1995). Purification and characterization of a benzylviologen-linked, tungsten-containing aldehyde oxidoreductase from *Desulfovibrio gigas*. *J Bacteriol* **177**, 6195-6200.

Hirano, S. S. & Upper, C. D. (1991). Bacterial community dynamics, pp. 271-294. In: *Microbial ecology on leaves* (Andrews, J.H. & Hirano, S. S., editors), Springer-Verlag, New York.

Hothi, P., Sutcliffe, M. J. & Scrutton, N. S. (2005). Kinetic isotope effects and ligand binding in PQQ-dependent methanol dehydrogenase. *Biochem J* **388**, 123-133.

Hüve, K., Christ, M. M., Kleist, E., Uerlings, R., Niinemets, U., Walter, A. & Wildt, J. (2007). Simultaneous growth and emission measurements demonstrate an interactive control of methanol release by leaf expansion and stomata. *J Exp Bot* **58**, 1783-1793.

Idris, R., Trifonova, R., Puschenreiter, M., Wenzel, W. W. & Sessitsch, A. (2004). Bacterial communities associated with flowering plants of the Ni hyperaccumulator *Thlaspi goesingense*. *Appl Environ Microbiol* **70**, 2667-2677.

Itoh, S., Kawakami, H. & Fukuzumi, S. (1998). Model studies on calcium-containing quinoprotein alcohol dehydrogenases. Catalytic role of Ca²⁺ for the oxidation of alcohols by coenzyme PQQ (4,5-dihydro-4,5-dioxo-1H-pyrrolo[2,3-f]quinoline-2, 7,9-tricarboxylic acid). *Biochemistry* **37**, 6562-6571.

Janvier, M. & Gasser, F. (1987). Purification and properties of methanol dehydrogenase from *Methylophaga marina*. *Biochimie* **69**, 1169-1174.

Jewell, T., Huston, S. L. & Nelson, D. C. (2008). Methylophony in freshwater *Beggiatoa alba* strains. *Appl Env Microbiol* **74**, 5575-5578.

Jones, C. W., Kingsbury, S. A. & Dawson, M. J. (1982). The partial resolution and dye-mediated reconstitution of methanol oxidase activity in *Methylophilus methylophilus*. *FEMS Microbiol Lett* **13**, 195-200.

Kalyuzhnaya, M. G., Hristova, K. R., Lidstrom, M. E. & Chistoserdova, L. (2008). Characterization of a novel methanol dehydrogenase in representatives of *Burkholderiales*: Implications for environmental detection of methylophony and evidence for convergent evolution. *J Bacteriol* **190**, 3817-3823.

Kalyuzhnaya, M. G., Martens-Habbena, W., Wang, T., Hackett, M., Stolyar, S. M., Stahl, D. A., Lidstrom, M. E. & Chistoserdova, L. (2009). *Methylophilaceae* link

methanol oxidation to denitrification in freshwater lake sediment as suggested by stable isotope probing and pure culture analysis. *Env Microbiol Reports* **1**, 385-392.

Kane, S. R., Chakicherla, A. Y., Chain, P. S. G. & other authors (2007). Whole-genome analysis of the methyl tert-butyl ether-degrading *Betaproteobacterium Methylibium petroleiphilum* PM1. *J Bacteriol* **189**, 1931-1945.

Kasprzak, A. A. & Steenkamp, D. J. (1983). Localization of the major dehydrogenases in two methylophs by radiochemical labeling. *J Bacteriol* **156**, 348-353.

Kato, N., Tsuji, K., Tani, Y. & Ogata, K. (1974). A methanol-utilizing actinomycete. *J Ferment Technol* **52**, 917-920.

Kato, N., Miyamoto, N., Shimao, M. & Sakazawa, C. (1988). 3-Hexulose phosphate synthase from a new facultative methyloph, *Mycobacterium gastri* MB19. *AgricBiol Chem* **52**, 2659-2661.

Kato, N. (1990). 3-Hexulose-6-phosphate synthase from *Mycobacterium gastri* MB19. *Methods Enzymol* **188**, 397-401.

Kato, N., Yurimoto, H. & Thauer, R. K. (2006). The physiological role of the ribulose monophosphate pathway in bacteria and archaea. *Biosci Biotechnol Biochem* **70**, 10-21.

Kay, C. W. M., Mennenga, B., Gorisch, H. & Bittl, R. (2006). Substrate binding in quinoprotein ethanol dehydrogenase from *Pseudomonas aeruginosa* studied by electron-nuclear double resonance. *Proc Natl Acad Sci U S A* **103**, 5267-5272.

Kayser, M. F., Ucurum, Z. & Vuilleumier, S. (2002). Dichloromethane metabolism and C₁ utilization genes in *Methylobacterium* strains. *Microbiology* **148**, 1915-1922.

Khambata, S. R. & Bhat, J. V. (1953). Studies on a new oxalate-decomposing bacterium, *Pseudomonas oxalaticus*. *J Bacteriol* **66**, 505-507.

Kiefer, P., Portais, J. C. & Vorholt, J. A. (2008). Quantitative metabolome analysis using liquid chromatography-high-resolution mass spectrometry. *Anal Biochem* **382**, 94-100.

Kiefer, P., Buchhaupt, M., Christen, P., Kaup, B., Schrader, J. & Vorholt, J. A. (2009). Metabolite profiling uncovers plasmid-induced cobalt limitation under methylotrophic growth conditions. *PLoS One* **4**, e7831.

King, G. M. (1992). Ecological aspects of methane oxidation, a key determinant of global methane dynamics. *Adv Microb Ecol* **12**, 431-468.

Klein, C. R., Kessler, F. P., Perrei, C., Frank, J., Duine, J. A. & Schwartz, A. C. (1994). A novel dye-linked formaldehyde dehydrogenase with some properties indicating the presence of a protein-bound redox-active quinone cofactor. *Biochem J* **301**, 289-295.

Kloosterman, H., Vrijbloed, J. W. & Dijkhuizen, L. (2002). Molecular, biochemical, and functional characterization of a Nudix hydrolase protein that stimulates the activity of a nicotinoprotein alcohol dehydrogenase. *J Biol Chem* **277**, 34785-34792.

Knief, C., Frances, L., Cantet, F. & Vorholt, J. A. (2008). Cultivation-independent characterization of *Methylobacterium* populations in the plant phyllosphere by automated ribosomal intergenic spacer analysis. *Appl Environ Microbiol* **74**, 2218-2228.

Knief, C., Frances, L. & Vorholt, J. A. (2010a). Competitiveness of diverse *Methylobacterium* strains in the phyllosphere of *Arabidopsis thaliana* and identification of representative models, including *Methylobacterium extorquens* PA1. *Microb Ecol* **6**, 440-452.

Knief, C., Ramette, A., Frances, L., Alonso-Blanco, C. & Vorholt, J. A. (2010b). Site and plant species are important determinants of the *Methylobacterium* community composition in the plant phyllosphere. *ISME J* **4**, 719-728.

Kolb, S. (2009). Aerobic methanol-oxidizing *Bacteria* in soil. *FEMS Microbiol Lett* **300**, 1-10.

Kumazawa, T., Sato, K., Seno, H., Ishii, A. & Suzuki, O. (1995). Levels of pyrroloquinoline quinone in various foods. *Biochem J* **307**, 331-333.

Kuono, K. & Ozaki, A. (1975). Distribution and identification of methanol utilizing bacteria, pp. 11-21. In: *Microbiol growth on C₁ compounds* (Organizing Committee, editors), Soc. Ferm. Tech., Japan.

Large, P. J., Peel, D. & Quayle, J. R. (1961). Microbial growth on C₁ compounds. II. Synthesis of cell constituents by methanol- and formate-grown *Pseudomonas* AM 1, and methanol-grown *Hyphomicrobium vulgare*. *Biochem J* **81**, 470-480.

Large, P. J., Peel, D. & Quayle, J. R. (1962a). Microbial growth on C₁ compounds. 3. Distribution of radioactivity in metabolites of methanol-grown *Pseudomonas* AM1 after incubation with [¹⁴C]methanol and [¹⁴C]bicarbonate. *Biochem J* **82**, 483-488.

Large, P. J., Peel, D. & Quayle, J. R. (1962b). Microbial growth on C₁ compounds. 4. Carboxylation of phosphoenolpyruvate in methanol-grown *Pseudomonas* AM1. *Biochem J* **85**, 243-250.

Last, F. T. (1955). Seasonal incidence of *Sporobolomyces* on cereal leaves. *Trans Brit Mycol Soc* **38**, 221-239.

Laufer, K. & Lidstrom, M. E. (1992). Regulation and expression of bacterial quinoproteins, pp. 193-222. In: *Quinoproteins* (Davidson, V., editor), Marcel Dekker, Inc., New York.

Laukel, M., Chistoserdova, L., Lidstrom, M. E. & Vorholt, J. A. (2003). The tungsten-containing formate dehydrogenase from *Methylobacterium extorquens* AM1: purification and properties. *Eur J Biochem* **270**, 325-333.

Laukel, M., Rossignol, M., Borderies, G., Volker, U. & Vorholt, J. A. (2004). Comparison of the proteome of *Methylobacterium extorquens* AM1 grown under methylotrophic and nonmethylotrophic conditions. *Proteomics* **4**, 1247-1264.

Leopoldini, M., Russo, N. & Toscano, M. (2007). The preferred reaction path for the oxidation of methanol by PQQ-containing methanol dehydrogenase: addition-elimination versus hydride-transfer mechanism. *Chemistry* **13**, 2109-2117.

Lidstrom, M. E. (1990). Genetics of carbon metabolism in methylotrophic bacteria. *FEMS Microbiol Rev* **7**, 431-436.

Lidstrom, M. E. & Stirling, D. I. (1990). Methylotrophs: genetics and commercial applications. *Annu Rev Microbiol* **44**, 27-58.

Lidstrom, M. E. (2006). Aerobic methylotrophic prokaryotes, pp. 618-634. In: *The prokaryotes* (Falkow, S.; Rosenberg, E.; Schleifer, K.H.; Stackebrandt, E. & Dworkin, M., editors), Springer-Verlag, New York.

Lindow, S. E. & Brandl, M. T. (2003). Microbiology of the phyllosphere. *Appl Environ Microbiol* **69**, 1875-1883.

Liu, Q., Kirchhoff, J. R., Faehnle, C. R., Viola, R. E. & Hudson, R. A. (2006). A rapid method for the purification of methanol dehydrogenase from *Methylobacterium extorquens*. *Protein Expr Purif* **46**, 316-320.

Loew, O. (1892). Über einen Bacillus, welcher Ameisensäure und Formaldehyd assimilieren kann. *Zentralblatt für Bakteriologie, Parasitenkunde, Infektionskrankheiten und Hygiene* **12**, 462-465.

Long, A. R. & Anthony, C. (1990). Modifier protein for methanol dehydrogenase of methylotrophs. *Methods Enzymol* **188**, 216-222.

Long, A. R. & Anthony, C. (1991). The periplasmic modifier protein for methanol dehydrogenase in the methylotrophs *Methylophilus methylotrophus* and *Paracoccus denitrificans*. *J Gen Microbiol* **137**, 2353-2360.

Madhaiyan, M., Poonguzhali, S. & Sa, T. (2007). Influence of plant species and environmental conditions on epiphytic and endophytic pink-pigmented facultative methylotrophic bacterial populations associated with field-grown rice cultivars. *J Microbiol Biotechnol* **17**, 1645-1654.

Madhaiyan, M., Poonguzhali, S., Kwon, S. W. & Sa, T. M. (2009). *Methylobacterium phyllosphaerae* sp. nov., a pink-pigmented, facultative methylotroph from the phyllosphere of rice. *Int J Syst Evol Microbiol* **59**, 22-27.

Marison, I. W. & Attwood, M. M. (1982). A possible alternative mechanism for the oxidation of formaldehyde to formate. *J Gen Microbiol* **128**, 1441-1446.

Marx, C. J. & Lidstrom, M. E. (2001). Development of improved versatile broad-host-range vectors for use in methylotrophs and other Gram-negative bacteria. *Microbiology* **147**, 2065-2075.

Marx, C. J., Chistoserdova, L. & Lidstrom, M. E. (2003). Formaldehyde-detoxifying role of the tetrahydromethanopterin-linked pathway in *Methylobacterium extorquens* AM1. *J Bacteriol* **185**, 7160-7168.

Marx, C. J. & Lidstrom, M. E. (2004). Development of an insertional expression vector system for *Methylobacterium extorquens* AM1 and generation of null mutants lacking *mtdA* and/or *fch*. *Microbiology* **150**, 9-19.

Matsushita, K., Shinagawa, E., Adachi, O. & Ameyama, M. (1989). Quinoprotein D-glucose dehydrogenase of the *Acinetobacter calcoaceticus* respiratory chain: membrane-bound and soluble forms are different molecular species. *Biochemistry* **28**, 6276-6280.

Matsushita, K., Takahashi, K. & Adachi, O. (1993). A novel quinoprotein methanol dehydrogenase containing an additional 32-kilodalton peptide purified from *Acetobacter methanolicus*: identification of the peptide as a *moxJ* product. *Biochemistry* **32**, 5576-5582.

Mazumder-Shivakumar, D., Kahn, K. & Bruice, T. C. (2004). Computational study of the ground state of thermophilic indole glycerol phosphate synthase: structural alterations at the active site with temperature. *J Am Chem Soc* **126**, 5936-5937.

McNerney, T. & O'Connor M, L. (1980). Regulation of enzymes associated with C₁ metabolism in three facultative methylotrophs. *Appl Environ Microbiol* **40**, 370-375.

Mennenga, B., Kay, C. W. & Gorisch, H. (2009). Quinoprotein ethanol dehydrogenase from *Pseudomonas aeruginosa*: the unusual disulfide ring formed by adjacent cysteine residues is essential for efficient electron transfer to cytochrome *c*₅₅₀. *Arch Microbiol* **191**, 361-367.

Meulenbergh, J. J. M., Sellink, E., Riegman, N. H. & Postma, P. W. (1992). Nucleotide sequence and structure of the *Klebsiella pneumoniae* pqq operon. *Mol Gen Genet* **232**, 284-294.

- Misset-Smits, M., Oltshoorn, A. J., Dewanti, A. & Duine, J. A. (1997a).** Production, assay, and occurrence of pyrroloquinoline quinone. *Meth Enzymol* **280**, 89-98.
- Misset-Smits, M., van Ophem, P. W., Sakuda, S. & Duine, J. A. (1997b).** Mycothiol, 1-O-(2'-[N-acetyl-L-cysteinyl]amido-2'-deoxy- α -D-glucopyranosyl)-D- *myo*-inositol, is the factor of NAD/factor-dependent formaldehyde dehydrogenase. *FEBS Lett* **409**, 221-222.
- Morris, C. J. & Lidstrom, M. E. (1992).** Cloning of a methanol-inducible *moxF* promoter and its analysis in *moxB* mutants of *Methylobacterium extorquens* AM1rif. *J Bacteriol* **174**, 4444-4449.
- Morris, C. J., Biville, F., Turlin, E., Lee, E., Ellermann, K., Fan, W. H., Ramamoorthi, R., Springer, A. L. & Lidstrom, M. E. (1994).** Isolation, phenotypic characterization, and complementation analysis of mutants of *Methylobacterium extorquens* AM1 unable to synthesize pyrroloquinoline quinone and sequences of *pqqD*, *pqqG*, and *pqqC*. *J Bacteriol* **176**, 1746-1755.
- Morris, C. J., Kim, Y. M., Perkins, K. E. & Lidstrom, M. E. (1995).** Identification and nucleotide sequences of *mxmA*, *mxnC*, *mxnK*, *mxnL*, and *mxnD* genes from *Methylobacterium extorquens* AM1. *J Bacteriol* **177**, 6825-6831.
- Mountfort, D. O. (1990).** Oxidation of aromatic alcohols by purified methanol dehydrogenase from *Methylosinus trichosporium*. *J Bacteriol* **172**, 3690-3694.
- Muhlencoert, E. & Muller, P. (2002).** A novel two-component system of *Bradyrhizobium japonicum*: ElmS and ElmR are encoded in diverse orientations. *DNA Seq* **13**, 93-102.
- Murashige, T. & Skoog, F. (1962).** A revised medium for rapid growth and bioassays with tobacco tissue cultures. *Physiol Plantarum* **15**, 473-497.
- Murrell, J. C. & McDonald, I. R. (2000).** Methylo-trophy, pp. 245-255. In: *Encyclopedia of Microbiology* (Lederberg, J., editor), Academic Press, London.

Mutzel, A. & Gorisch, H. (1991). Quinoprotein ethanol dehydrogenase - preparation of the apoform and reconstitution with pyrroloquinoline quinone and Ca^{2+} or Sr^{2+} ions. *Agr Biol Chem Tokyo* **55**, 1721-1726.

Myronova, N., Kitmitto, A., Collins, R. F., Miyaji, A. & Dalton, H. (2006). Three-dimensional structure determination of a protein supercomplex that oxidizes methane to formaldehyde in *Methylococcus capsulatus* (Bath). *Biochemistry* **45**, 11905-11914.

Nagy, I., Verheijen, S., De Schrijver, A., Van Damme, J., Proost, P., Schoofs, G., Vanderleyden, J. & De Mot, R. (1995). Characterization of the *Rhodococcus* sp. NI86/21 gene encoding alcohol: N,N'-dimethyl-4-nitrosoaniline oxidoreductase inducible by atrazine and thiocarbamate herbicides. *Arch Microbiol* **163**, 439-446.

Nash, T. (1953). The colorimetric estimation of formaldehyde by means of the Hantzsch reaction. *Biochem J* **55**, 416-421.

Nesvera, J., Pátek, M., Hochmannova, J., Chibisova, E., Serebrijski, I., Tsygankov, Y. & Netrusov, A. (1991). Transformation of a new Gram-positive methylotroph, *Brevibacterium methylicum*, by plasmid DNA. *Appl Microbiol Biotechnol* **35**, 777-780.

Nielsen, H., Engelbrecht, J., Brunak, S. & von Heijne, G. (1997). Identification of prokaryotic and eukaryotic signal peptides and prediction of their cleavage sites. *Protein Eng.* **10**, 1-6.

Nojiri, M., Hira, D., Yamaguchi, K., Okajima, T., Tanizawa, K. & Suzuki, S. (2006). Crystal structures of cytochrome c_L and methanol dehydrogenase from *Hyphomicrobium denitrificans*: structural and mechanistic insights into interactions between the two proteins. *Biochemistry* **45**, 3481-3492.

Norin, A., Van Ophem, P. W., Piersma, S. R., Persson, B., Duine, J. A. & Jornvall, H. (1997). Mycothiol-dependent formaldehyde dehydrogenase, a prokaryotic medium-chain dehydrogenase/reductase, phylogenetically links different eukaryotic alcohol dehydrogenases: Primary structure, conformational modelling and functional correlations. *Eur J Biochem* **248**, 282-289.

Nunn, D. N. & Lidstrom, M. E. (1986a). Phenotypic characterization of 10 methanol oxidation mutant classes in *Methylobacterium* sp. strain AM1. *J Bacteriol* **166**, 591-597.

- Nunn, D. N. & Lidstrom, M. E. (1986b).** Isolation and complementation analysis of 10-methanol oxidation mutant classes and identification of the methanol dehydrogenase structural gene of *Methylobacterium* sp. strain AM1. *J Bacteriol* **166**, 581-590.
- Nunn, D. N. & Anthony, C. (1988).** The nucleotide sequence and deduced amino acid sequence of the cytochrome *c_L* gene of *Methylobacterium extorquens* AM1, a novel class of c-type cytochrome. *Biochem J* **256**, 673-676.
- Nunn, D. N., Day, D. & Anthony, C. (1989).** The second subunit of methanol dehydrogenase of *Methylobacterium extorquens* AM1. *Biochem J* **260**, 857-862.
- O'Connor, M. L. (1981).** Regulation and genetics in facultative methylotrophic bacteria, pp. 294-300. In: *Microbial growth on C₁ compounds* (Dalton, H., editor), Heyden, London, Philadelphia, Rheine.
- O'Keeffe, D. T. & Anthony, C. (1980).** The two cytochromes *c* in the facultative methylotroph *Pseudomonas* AM1. *Biochem J* **192**, 411-419.
- Ohshiro, Y. & Itoh, S. (1988).** *PQQ and Quinoproteins*. Kluwer, Dordrecht, 195 pp.
- Ohta, S., Fujita, T. & Tobari, J. (1981).** Methanol dehydrogenase of *Methylomonas* J: purification, crystallization, and some properties. *J Biochem* **90**, 205-213.
- Okubo, Y., Skovran, E., Guo, X., Sivam, D. & Lidstrom, M. E. (2007).** Implementation of microarrays for *Methylobacterium extorquens* AM1. *OMICS* **11**, 325-340.
- Olsthoorn, A. J. & Duine, J. A. (1998).** On the mechanism and specificity of soluble, quinoprotein glucose dehydrogenase in the oxidation of aldose sugars. *Biochemistry* **37**, 13854-13861.
- Orita, I., Sakamoto, N., Kato, N., Yurimoto, H. & Sakai, Y. (2007).** Bifunctional enzyme fusion of 3-hexulose-6-phosphate synthase and 6-phospho-3-hexuloisomerase. *Appl Microbiol Biotechnol* **76**, 439-445.

Oubrie, A., Rozeboom, H. J., Kalk, K. H., Olsthoorn, A. J., Duine, J. A. & Dijkstra, B. W. (1999). Structure and mechanism of soluble quinoprotein glucose dehydrogenase. *EMBO J* **18**, 5187-5194.

Oubrie, A. & Dijkstra, B. W. (2000). Structural requirements of pyrroloquinoline quinone dependent enzymatic reactions. *Protein Sci* **9**, 1265-1273.

Oubrie, A., Rozeboom, H. J., Kalk, K. H., Huizinga, E. G. & Dijkstra, B. W. (2002). Crystal structure of quinoxinoprotein alcohol dehydrogenase from *Comamonas testosteroni*: structural basis for substrate oxidation and electron transfer. *J Biol Chem* **277**, 3727-3732.

Ozimek, P., Veenhuis, M. & van der Klei, I. J. (2005). Alcohol oxidase: a complex peroxisomal, oligomeric flavoprotein. *FEMS Yeast Res* **5**, 975-983.

Page, M. D. & Anthony, C. (1986). Regulation of formaldehyde oxidation by the methanol dehydrogenase modifier proteins of *Methylophilus methylotrophus* and *Pseudomonas* AM1. *J Gen Microbiol* **132**, 1553-1563.

Palmer, T., Sargent, F. & Berks, B. C. (2005). Export of complex cofactor-containing proteins by the bacterial Tat pathway. *Trends Microbiol* **13**, 175-180.

Parker, M. W., Cornish, A., Gossain, V. & Best, D. J. (1987). Purification, crystallisation and preliminary X-ray diffraction characterisation of methanol dehydrogenase from *Methylosinus trichosporium* OB3b. *Eur J Biochem* **164**, 223-227.

Patel, R. N., Bose, H. R., Mandy, W. J. & Hoare, D. S. (1972). Physiological studies of methane- and methanol-oxidizing bacteria: comparison of a primary alcohol dehydrogenase from *Methylococcus capsulatus* (Texas strain) and *Pseudomonas species* M27. *J Bacteriol* **110**, 570-577.

Patel, R. N. & Felix, A. (1976). Microbial oxidation of methane and methanol: crystallization and properties of methanol dehydrogenase from *Methylosinus sporium*. *J Bacteriol* **128**, 413-424.

- Patel, R. N., Hou, C. T. & Felix, A. (1978a).** Microbial oxidation of methane and methanol: crystallization of methanol dehydrogenase and properties of holo- and apomethanol dehydrogenase from *Methylomonas methanica*. *J Bacteriol* **133**, 641-649.
- Patel, R. N., Hou, C. T. & Felix, A. (1978b).** Microbial oxidation of methane and methanol: isolation of methane-utilizing bacteria and characterization of a facultative methane-utilizing isolate. *J Bacteriol* **136**, 352-358.
- Peel, D. & Quayle, J. R. (1961).** Microbial growth on C₁ compounds. 1. Isolation and characterization of *Pseudomonas* AM 1. *Biochem J* **81**, 465-469.
- Peyraud, R., Kiefer, P., Christen, P., Massou, S., Portais, J. C. & Vorholt, J. A. (2009).** Demonstration of the ethylmalonyl-CoA pathway by using ¹³C metabolomics. *Proc Natl Acad Sci U S A* **106**, 4846-4851.
- Pomper, B. K., Vorholt, J. A., Chistoserdova, L., Lidstrom, M. E. & Thauer, R. K. (1999).** A methenyl tetrahydromethanopterin cyclohydrolase and a methenyl tetrahydrofolate cyclohydrolase in *Methylobacterium extorquens* AM1. *Eur J Biochem* **261**, 475-480.
- Pomper, B. K. & Vorholt, J. A. (2001).** Characterization of the formyltransferase (Ftr) from *Methylobacterium extorquens* AM1. *Eur J Biochem* **268**, 4769-4775.
- Pomper, B. K., Saurel, O., Milon, A. & Vorholt, J. A. (2002).** Generation of formate by the formyltransferase/hydrolase complex (Fhc) from *Methylobacterium extorquens* AM1. *FEBS Lett* **523**, 133-137.
- Poonguzhali, S., Madhaiyan, M., Yim, W. J., Kim, K. A. & Sa, T. M. (2008).** Colonization pattern of plant root and leaf surfaces visualized by use of green-fluorescent-marked strain of *Methylobacterium suomiense* and its persistence in rhizosphere. *Appl Microbiol Biotechnol* **78**, 1033-1043.
- Quayle, J. R. & Keech, D. B. (1958).** Carbon dioxide and formate utilization by formate-grown *Pseudomonas oxalaticus*. *Biochim Biophys Acta* **29**, 223-225.

Quayle, J. R. & Keech, D. B. (1959a). Carbon assimilation by *Pseudomonas oxalaticus* (OX 1). 1. Formate and carbon dioxide utilization during growth on formate. *Biochem J* **72**, 623-630.

Quayle, J. R. & Keech, D. B. (1959b). Carbon assimilation by *Pseudomonas oxalaticus* (OX 1). 2. Formate and carbon dioxide utilization by cell-free extracts of the organism grown on formate. *Biochem J* **72**, 631-637.

Quayle, J. R. & Keech, D. B. (1959c). Carboxydismutase activity in formate- and oxalate-grown *Pseudomonas oxalaticus* (strain OX1). *Biochim Biophys Acta* **31**, 587-588.

Quayle, J. R. & Keech, D. B. (1960). Carbon assimilation by *Pseudomonas oxalaticus* (OX1). 343. Oxalate utilization during growth on oxalate. *Biochem J* **75**, 515-523.

Quayle, J. R. & Ferenci, T. (1978). Evolutionary aspects of autotrophy. *Microbiol Rev* **42**, 251-273.

Quilter, J. A. & Jones, C. W. (1984). The organization of methanol dehydrogenase and c-type cytochromes on the respiratory membrane of *Methylophilus methylotrophus*. *FEBS Lett* **174**, 167-172.

Ramamoorthi, R. & Lidstrom, M. E. (1995). Transcriptional analysis of *pqqD* and study of the regulation of pyrroloquinoline quinone biosynthesis in *Methylobacterium extorquens* AM1. *J Bacteriol* **177**, 206-211.

Ras, J., Reijnders, W. N., Van Spanning, R. J., Harms, N., Oltmann, L. F. & Stouthamer, A. H. (1991). Isolation, sequencing, and mutagenesis of the gene encoding cytochrome *c_{553i}* of *Paracoccus denitrificans* and characterization of the mutant strain. *J Bacteriol* **173**, 6971-6979.

Ras, J., Van Ophem, P. W., Reijnders, W. N., Van Spanning, R. J., Duine, J. A., Stouthamer, A. H. & Harms, N. (1995). Isolation, sequencing, and mutagenesis of the gene encoding NAD- and glutathione-dependent formaldehyde dehydrogenase (GD-FALDH) from *Paracoccus denitrificans*, in which GD-FALDH is essential for methylotrophic growth. *J Bacteriol* **177**, 247-251.

Read, J., Gill, R., Dales, S. L., Cooper, J. B., Wood, S. P. & Anthony, C. (1999). The molecular structure of an unusual cytochrome c_2 determined at 2.0 angstroms; the cytochrome c_H from *Methylobacterium extorquens*. *Protein Sci* **8**, 1232-1240.

Reddy, S. Y. & Bruce, T. C. (2003). *In silico* studies of the mechanism of methanol oxidation by quinoprotein methanol dehydrogenase. *J Am Chem Soc* **125**, 8141-8150.

Reddy, S. Y., Mathews, F. S., Zheng, Y. J. & Bruce, T. C. (2003). Quinoprotein methanol dehydrogenase: a molecular dynamics study and comparison with crystal structure. *J Mol Struct* **655**, 269-277.

Reid, M. F. & Fewson, C. A. (1994). Molecular characterization of microbial alcohol dehydrogenases. *Crit Rev Microbiol* **20**, 13-56.

Richardson, I. W. & Anthony, C. (1992). Characterization of mutant forms of the quinoprotein methanol dehydrogenase lacking an essential calcium ion. *Biochem J* **287**, 709-715.

Ruinen, J. (1956). Occurrence of *Beijerinckia* species in the 'Phyllosphere'. *Nature* **177**, 220-221.

Sahm, H., Cox, R. B. & Quayle, J. R. (1976). Metabolism of methanol by *Rhodopseudomonas acidophila*. *J Gen Microbiol* **94**, 313-322.

Salisbury, S. A., Forrest, H. S., Cruse, W. B. & Kennard, O. (1979). A novel coenzyme from bacterial primary alcohol dehydrogenases. *Nature* **280**, 843-844.

Sambrook, J., Fritsch, E. F. & Maniatis, T. (1989). *Molecular cloning: a laboratory manual*, 2nd edition. Cold Spring Harbor Laboratory Press, New York, 1659 pp.

Schäfer, H. (2007). Isolation of *Methylophaga* spp. from marine dimethylsulfide-degrading enrichment cultures and identification of polypeptides induced during growth on dimethylsulfide. *Appl Environ Microbiol* **73**, 2580-2591.

Schlesier, B., Bréton, F. & Mock, H. P. (2003). A hydroponic culture system for growing *Arabidopsis thaliana* plantlets under sterile conditions. *Plant Mol Biol Rep* **21**, 449-456.

Schmalenberger, A. & Tebbe, C. C. (2002). Bacterial community composition in the rhizosphere of a transgenic, herbicide-resistant maize (*Zea mays*) and comparison to its non-transgenic cultivar *Bosphore*. *FEMS Microbiol Ecol* **40**, 29-37.

Schobert, M. & Gorisch, H. (2001). A soluble two-component regulatory system controls expression of quinoprotein ethanol dehydrogenase (QEDH) but not expression of cytochrome *c₅₅₀* of the ethanol-oxidation system in *Pseudomonas aeruginosa*. *Microbiology* **147**, 363-372.

Schrader, J., Schilling, M., Holtmann, D., Sell, D., Filho, M. V., Marx, A. & Vorholt, J. A. (2009). Methanol-based industrial biotechnology: current status and future perspectives of methylotrophic bacteria. *Trends Biotechnol* **27**, 107-115.

Smith, P. K., Krohn, R. I., Hermanson, G. T. & other authors (1985). Measurement of protein using bicinchoninic acid. *Anal Biochem* **150**, 76-85.

Sperl, G. T., Forrest, H. S. & Gibson, D. T. (1974). Substrate specificity of purified primary alcohol dehydrogenases from methanol-oxidizing bacteria. *J Bacteriol* **118**, 541-550.

Springer, A. L., Chou, H. H., Fan, W. H., Lee, E. & Lidstrom, M. E. (1995). Methanol oxidation mutants in *Methylobacterium extorquens* AM1: identification of new genetic complementation groups. *Microbiology* **141** 2985-2993.

Springer, A. L., Morris, C. J. & Lidstrom, M. E. (1997). Molecular analysis of *mxbD* and *mxbM*, a putative sensor-regulator pair required for oxidation of methanol in *Methylobacterium extorquens* AM1. *Microbiology* **143**, 1737-1744.

Springer, A. L., Auman, A. J. & Lidstrom, M. E. (1998). Sequence and characterization of *mxkB*, a response regulator involved in regulation of methanol oxidation, and of *mxwW* a methanol-regulated gene in *Methylobacterium extorquens* AM1. *FEMS Microbiol Lett* **160**, 119-124.

Studer, A., McAnulla, C., Buchele, R., Leisinger, T. & Vuilleumier, S. (2002). Chloromethane-induced genes define a third C₁ utilization pathway in *Methylobacterium chloromethanicum* CM4. *J Bacteriol* **184**, 3476-3484.

Sudtachat, N., Ito, N., Itakura, M., Masuda, S., Eda, S., Mitsui, H., Kawaharada, Y. & Minamisawa, K. (2009). Aerobic vanillate degradation and C₁ compound metabolism in *Bradyrhizobium japonicum*. *Appl Env Microbiol* **75**, 5012-5017.

Sy, A., Timmers, A. C., Knief, C. & Vorholt, J. A. (2005). Methylotrophic metabolism is advantageous for *Methylobacterium extorquens* during colonization of *Medicago truncatula* under competitive conditions. *Appl Environ Microbiol* **71**, 7245-7252.

Taylor, S. C., Dalton, H. & Dow, C. S. (1981). Ribulose-1,5-bisphosphate carboxylase/oxygenase and carbon assimilation in *Methylococcus capsulatus* (Bath). *J Gen Microbiol* **122**, 89-94.

Toyama, H., Anthony, C. & Lidstrom, M. E. (1998). Construction of insertion and deletion *mx*A mutants of *Methylobacterium extorquens* AM1 by electroporation. *FEMS Microbiol Lett* **166**, 1-7.

Toyama, H., Inagaki, H., Matsushita, K., Anthony, C. & Adachi, O. (2003). The role of the MxaD protein in the respiratory chain of *Methylobacterium extorquens* during growth on methanol. *Biochim Biophys Acta* **1647**, 372-375.

Van der Klei, I. J., Yurimoto, H., Sakai, Y. & Veenhuis, M. (2006). The significance of peroxisomes in methanol metabolism in methylotrophic yeast. *Biochim Biophys Acta* **1763**, 1453-1462.

Van der Meer, R. A., Groen, B. W., van Kleef, M. A., Frank, J., Jongejan, J. A. & Duine, J. A. (1990). Isolation, preparation, and assay of pyrroloquinoline quinone. *Methods Enzymol* **188**, 260-283.

Van Ophem, P. W., Euverink, G. J., Dijkhuizen, L. & Duine, J. A. (1991). A novel dye-linked alcohol dehydrogenase activity present in some Gram-positive bacteria. *FEMS Microbiol Lett* **80**, 57-63.

Van Spanning, R. J., Wansell, C. W., De Boer, T., Hazelaar, M. J., Anazawa, H., Harms, N., Oltmann, L. F. & Stouthamer, A. H. (1991). Isolation and characterization of the *moxJ*, *moxG*, *moxI*, and *moxR* genes of *Paracoccus denitrificans*: inactivation of

moxJ, *moxG*, and *moxR* and the resultant effect on methylotrophic growth. *J Bacteriol* **173**, 6948-6961.

Van Verseveld, H. W. & Stouthamer, A. H. (1978). Electron-transport chain and coupled oxidative phosphorylation in methanol-grown *Paracoccus denitrificans*. *Arch Microbiol* **118**, 13-20.

Vannelli, T., Messmer, M., Studer, A., Vuilleumier, S. & Leisinger, T. (1999). A corrinoid-dependent catabolic pathway for growth of a *Methylobacterium* strain with chloromethane. *Proc Natl Acad Sci U S A* **96**, 4615-4620.

Veenhuis, M. & Harder, W. (1989). Metabolism of one-carbon compounds, pp. 289-316. In: *The Yeasts* (Rose, A.H. & Harrison, J.S., editors), Academic Press, London.

Velterop, J. S., Sellink, E., Meulenber, J. J., David, S., Bulder, I. & Postma, P. W. (1995). Synthesis of pyrroloquinoline quinone *in vivo* and *in vitro* and detection of an intermediate in the biosynthetic pathway. *J Bacteriol* **177**, 5088-5098.

Vorholt, J. A., Chistoserdova, L., Lidstrom, M. E. & Thauer, R. K. (1998). The NADP-dependent methylene tetrahydromethanopterin dehydrogenase in *Methylobacterium extorquens* AM1. *J Bacteriol* **180**, 5351-5356.

Vorholt, J. A., Chistoserdova, L., Stolyar, S. M., Thauer, R. K. & Lidstrom, M. E. (1999). Distribution of tetrahydromethanopterin-dependent enzymes in methylotrophic bacteria and phylogeny of methenyl tetrahydromethanopterin cyclohydrolases. *J Bacteriol* **181**, 5750-5757.

Vorholt, J. A., Marx, C. J., Lidstrom, M. E. & Thauer, R. K. (2000). Novel formaldehyde-activating enzyme in *Methylobacterium extorquens* AM1 required for growth on methanol. *J Bacteriol* **182**, 6645-6650.

Vorholt, J. A. (2002). Cofactor-dependent pathways of formaldehyde oxidation in methylotrophic bacteria. *Arch Microbiol* **178**, 239-249.

Vorob'ev, A. V., de Boer, W., Folman, L. B., Bodelier, P. L., Doronina, N. V., Suzina, N. E., Trotsenko, Y. A. & Dedysh, S. N. (2009). *Methylovirgula ligni* gen. nov., sp. nov.,

an obligately acidophilic, facultatively methylotrophic bacterium with a highly divergent *mxoF* gene. *Int J Syst Evol Microbiol* **59**, 2538-2545.

Vrdoljak, M. & Froud, S. (1982). Characterisation of the electron transport chain of an obligate methylotroph, strain 4025. *Int J Biochem* **14**, 1019-1023.

Vuilleumier, S., Chistoserdova, L., Lee, M. C. & other authors (2009). *Methylobacterium* genome sequences: a reference blueprint to investigate microbial metabolism of C₁ compounds from natural and industrial sources. *PLoS One* **4**, e5584.

Warneke, C., Karl, T., Judmaier, H., Hansel, A., Jordan, A., Lindinger, W. & Crutzen, P. J. (1999). Acetone, methanol, and other partially oxidized volatile organic emissions from dead plant matter by abiological processes: Significance for atmospheric chemistry. *J Geophys Res* **13**, 9-17.

Weaver, C. A. & Lidstrom, M. E. (1985). Methanol dissimilation in *Xanthobacter* H4-14: activities, induction and comparison to *Pseudomonas* AM1 and *Paracoccus denitrificans*. *J Gen Microbiol* **131**, 2183-2197.

Westerling, J., Frank, J. & Duine, J. A. (1979). The prosthetic group of methanol dehydrogenase from *Hyphomicrobium* X: electron spin resonance evidence for a quinone structure. *Biochem Biophys Res Commun* **87**, 719-724.

White, S., Boyd, G., Mathews, F. S., Xia, Z. X., Dai, W. W., Zhang, Y. F. & Davidson, V. L. (1993). The active site structure of the calcium-containing quinoprotein methanol dehydrogenase. *Biochemistry* **32**, 12955-12958.

Williams, P., Coates, L., Mohammed, F., Gill, R., Erskine, P., Bourgeois, D., Wood, S. P., Anthony, C. & Cooper, J. B. (2006). The 1.6 angstrom X-ray structure of the unusual c-type cytochrome, cytochrome *c_L*, from the methylotrophic bacterium *Methylobacterium extorquens*. *J Mol Biol* **357**, 151-162.

Williams, P. A., Coates, L., Mohammed, F., Gill, R., Erskine, P. T., Coker, A., Wood, S. P., Anthony, C. & Cooper, J. B. (2005). The atomic resolution structure of methanol dehydrogenase from *Methylobacterium extorquens*. *Acta Crystallogr D Biol Crystallogr* **61**, 75-79.

Wilson, S. M., Gleisten, M. P. & Donohue, T. J. (2008). Identification of proteins involved in formaldehyde metabolism by *Rhodobacter sphaeroides*. *Microbiology* **154**, 296-305.

Wolf, H. J. & Hanson, R. S. (1978). Alcohol dehydrogenase from *Methylobacterium organophilum*. *Appl Environ Microbiol* **36**, 105-114.

Woodward, J. R. (1990). Biochemistry and applications of alcohol oxidase from methylotrophic yeasts, pp. 193-225. In: *Advances in autotrophic microbiology and one-carbon metabolism* (Codd, G.A., editor), Kluwer Academic Publishers, Dordrecht.

Xia, Z., Dai, W., Zhang, Y., White, S. A., Boyd, G. D. & Mathews, F. S. (1996). Determination of the gene sequence and the three-dimensional structure at 2.4 angstroms resolution of methanol dehydrogenase from *Methylophilus* W3A1. *J Mol Biol* **259**, 480-501.

Xia, Z. X., Dai, W. W., Xiong, J. P., Hao, Z. P., Davidson, V. L., White, S. & Mathews, F. S. (1992). The three-dimensional structures of methanol dehydrogenase from two methylotrophic bacteria at 2.6-angstrom resolution. *J Biol Chem* **267**, 22289-22297.

Xia, Z. X., He, Y. N., Dai, W. W., White, S. A., Boyd, G. D. & Mathews, F. S. (1999). Detailed active site configuration of a new crystal form of methanol dehydrogenase from *Methylophilus* W3A1 at 1.9 Å resolution. *Biochemistry* **38**, 1214-1220.

Xia, Z. X., Dai, W. W., He, Y. N., White, S. A., Mathews, F. S. & Davidson, V. L. (2003). X-ray structure of methanol dehydrogenase from *Paracoccus denitrificans* and molecular modeling of its interactions with cytochrome *c_{551i}*. *J Biol Inorg Chem* **8**, 843-854.

Yamanaka, K. & Matsumoto, K. (1977a). Comparative studies of three types of crystalline methanol dehydrogenases of bacteria, pp. 72-74. In: *Abstracts of international symposium on microbial growth on C₁-compounds*, Puschino.

Yamanaka, K. & Matsumoto, K. (1977a). Comparative studies of three types of crystalline methanol dehydrogenases of bacteria, pp. 72-74. In: *Abstracts of international symposium on microbial growth on C₁-compounds*, Puschino.

Yamanaka, K. & Matsumoto, K. (1977b). Purification, crystallization and properties of primary alcohol-dehydrogenase from a methanol-oxidizing *Pseudomonas* sp no. 2941. *Agric Biol Chemistry* **41**, 467-475.

Zhang, M. & Lidstrom, M. E. (2003). Promoters and transcripts for genes involved in methanol oxidation in *Methylobacterium extorquens* AM 1. *Microbiology* **149**, 1033-1040.

Zhang, X. & Bruice, T. C. (2006). Temperature dependence of the structure of the substrate and active site of the *Thermus thermophilus* chorismate mutase ES complex. *Biochemistry* **45**, 8562-8567.

Zhang, X., Reddy, S. Y. & Bruice, T. C. (2007). Mechanism of methanol oxidation by quinoprotein methanol dehydrogenase. *Proc Natl Acad Sci U S A* **104**, 745-749.

Zhao, Y., Wang, G., Cao, Z., Wang, Y., Cheng, H. & Zhou, H. M. (2000). Effects of Ca^{2+} on the activity and stability of methanol dehydrogenase. *J Protein Chem* **19**, 469-473.

Zheng, Y. J. & Bruice, T. C. (1997). Conformation of coenzyme pyrroloquinoline quinone and role of Ca^{2+} in the catalytic mechanism of quinoprotein methanol dehydrogenase. *Proc Natl Acad Sci U S A* **94**, 11881-11886.

Zheng, Y. J., Xia, Z. X., Chen, Z. W., Mathews, F. S. & Bruice, T. C. (2001). Catalytic mechanism of quinoprotein methanol dehydrogenase: A theoretical and X-ray crystallographic investigation. *Proc Natl Acad Sci U S A* **98**, 432-434.

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

(Focus on protein biochemistry, enzyme assays, enzyme activities, plant-microbe interactions)

01/2006 – 10/2006 University of Cologne, Institute of Biochemistry, Diploma thesis with Prof. Dr. Günter Schwarz, (final grade: 1.0; on a scale from 1 to 6 with 1 = best grade)

Negative and positive influence of copper on molybdenum cofactor biosynthesis in human liver cells

(Focus on bioanalytical methods such as cofactor analyses via HPLC, anion/cation exchange chromatography and gel filtration at the FPLC, sulfite oxidase assay, detection of Moco/MPT via *nit-1* complementation, enzyme activity tests and kinetics, protein crystallography)

11/2005 – 12/2005 TU Braunschweig, Department of Microbiology, Research assistant in the laboratory of Prof. Dr. Dieter Jahn
The expression of the recombinant aerobic Mg-Protoporphyrin-IX-monomethylester-cyclase from T. elongates

(Focus on purification of recombinant proteins)

- 03/2005 – 04/2005 TU Braunschweig, Department of Plant Biology, Research assistant in the laboratory of Prof. Dr. Ralf Mendel
Protein expression and purification of GABA-loops and the expression and purification of Cnx1 ligands
(Focus on protein expression and purification, neurobiological background)
- 03/2004 – 04/2004 Research assistant at the Department of German Collection of Microorganisms and Cell Cultures GmbH (DSMZ), Braunschweig
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Overexpression, purification and crystallization of recombinant Glutamyl-tRNA Synthetase (GluRS) from M. kandleri
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Teaching experience

- 2006 - 2009 Practical course assistant "Microbiology for Pharmacy and Food Scientists" Course organizer "Microbiology for Pharmacy and Food Scientists"
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- 2004 - 2006 Teaching assistant, Institute of Genetics, TU Braunschweig

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Publications/ Conferences

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MIM retreat in Fiesch 2008, Switzerland

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Languages

German (mother tongue)
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IT Skills

Microsoft Office, MATLAB R2008a, Accelrys Gene 2.5, CLoneManager 9, Prism 5, SigmaPlot 11, SPSS 16.0, SYSTAT 12, Adobe Illustrator CS3, Adobe Photoshop CS3, Corel PHOTO-PAINT X4, EndNote Program 12, R 2.9, BioRad Quantity One 4.6.7, UNICORN 5.11, Chromeleon

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Presentation skills course for scientists
Organization of the Microbiology and Immunology retreat 2008

**Extracurricular
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Karate, First DAN (black belt)
Trainer license C of the German Karate Association
Karate trainer at the Technical University of Braunschweig (2001 - 2005)
Karate Junior-/Senior master of Schleswig-Holstein, Germany
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