



Methanol oxidation in a spontaneous mutant of *Thiosphaera pantotropha* with a methanol-positive phenotype is catalysed by a dye-linked ethanol dehydrogenase

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Abstract

A spontaneous *Thiosphaera pantotropha* mutant (Tp9002) that is able to grow on methanol has been isolated. With hybridization experiments it has been demonstrated that *mxoF*, the gene encoding the large subunit of methanol dehydrogenase, is absent from *T. pantotropha*. In Tp9002, a dye-linked enzyme activity was found with a substrate specificity similar to that of the dye-linked ethanol dehydrogenase from *Pseudomonas aeruginosa*. The N-terminus of a 26-kDa cytochrome *c*, exclusively synthesized in Tp9002, is homologous to the N-terminus of the electron acceptor of ethanol dehydrogenase. These results suggest that in Tp9002 a dye-linked ethanol dehydrogenase is responsible for methanol oxidation, using a 26-kDa cytochrome *c* as electron acceptor.

Keywords: *Thiosphaera pantotropha*; Ethanol dehydrogenase; *Paracoccus denitrificans*; Methanol dehydrogenase

1. Introduction

Paracoccus denitrificans and *Thiosphaera pantotropha* are closely related bacteria that belong to the α -3 subgroup of the α subdivision of the purple bacteria and have identical 16S rRNA sequences [1]. Although the two organisms are similar in many aspects, they also differ in many others. For example, *P. denitrificans* differs from *T. pantotropha* in its capacity to grow on methanol and methylamine as

sole carbon and energy sources. Both *P. denitrificans* and *T. pantotropha*, however, are able to grow on choline. During the oxidation of one molecule of choline, several molecules of formaldehyde are produced and the oxidation of formaldehyde is therefore not impaired in *T. pantotropha*. Although the wild-type *T. pantotropha* strain is unable to grow on methanol, two groups have succeeded in isolating a *T. pantotropha* strain, designated Mox⁺, which is able to grow on this substrate [2,3].

In *P. denitrificans*, the oxidation of methanol to formaldehyde is catalysed by methanol dehydrogenase (MDH). This enzyme is a periplasmically located quinoprotein which consists of two identical

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large and two identical small subunits [4]. MDH contains pyrroloquinoline quinone (PQQ) as a cofactor non-covalently linked to the large subunit [5]. The enzyme is induced during growth on methanol, methylamine and choline [4], and its substrate specificity is well-defined. Primary alcohols are oxidized, but the efficiency decreases with increasing chain length [6]. Secondary alcohols are not oxidized [7]. The genes encoding the subunits of *P. denitrificans* MDH have been isolated [8,9]. The large subunit is encoded by *mxoF* and the small subunit by *mxoI*, both part of the *mxoFJGIRS* cluster [9,10]. The *mxoG* gene encodes a 17.7-kDa *c*-type cytochrome which is the electron acceptor of MDH. Hybridization studies with *mxoG* as a probe demonstrated that this gene is absent in *T. pantotropha* [11]. Moir and Ferguson [3] identified a 26-kDa cytochrome *c* in the periplasm of the *T. pantotropha* Mox⁺ strain grown on either choline and methanol. They suggested that this cytochrome *c* takes over the role of the *mxoG* gene product as electron acceptor of MDH in the Mox⁺ mutant. Egert et al. [2] suggested that the gene cluster *xoxF-cycB-xoxJ* is involved in methanol oxidation in the Mox⁺ *T. pantotropha* mutant. These genes, present in both *P. denitrificans* [12] and *T. pantotropha* [11], encode proteins that show homology with the gene products of the *mxo* locus.

This report describes the isolation of a spontaneous *T. pantotropha* mutant that is able to grow on methanol. Using hybridization studies, it has been demonstrated that *mxoF*, the structural gene encoding the large subunit of MDH, is absent in *T. pantotropha*. The results obtained here suggest that a dye-linked ethanol dehydrogenase (EDH) is responsible for methanol oxidation in the mutant strain.

2. Materials and methods

2.1. Bacterial strains and growth conditions

P. denitrificans and *T. pantotropha* (wild-type and mutant strain Tp9002) were used. Although it has been proposed that *T. pantotropha* should be renamed *P. denitrificans* because of their identical 16S rRNA sequences [1,13], their physiological differences are sufficient to make this a matter of debate. Until the situation of this group (which would

include *Thiobacillus versutus* and *Rhodobacter capsulatus*) is fully resolved, we consider it less confusing to retain the separate names. *P. denitrificans* and *T. pantotropha* were either grown in brain heart infusion broth with 40 µg of rifampin and 40 µg of gentamycin per ml, respectively, or in aerobic batch cultures at 32°C on mineral salts medium [14] with 50 mM methanol, 30 mM choline chloride or 50 mM ethanol as carbon and energy source.

2.2. Isolation of the mutant

The wild-type strain *T. pantotropha* GB17 was incubated in methanol-containing medium. After 20 days, growth was observed and an optical density at 660 nm of 0.8 was reached. Cells were isolated from this culture and purified by streaking on methanol minimal medium plates. A single colony was picked and tested further.

2.3. DNA manipulations

DNA was manipulated essentially as described by Maniatis et al. [15]. Chromosomal DNA was isolated as described previously [16].

2.4. Southern analysis

Chromosomal DNA from *P. denitrificans* and *T. pantotropha* was digested with either *Pvu*II, *Eco*RI, or *Bam*HI and subsequently loaded onto a 0.9% agarose gel. After running the gel, DNA was denatured and transferred to a positively charged nylon membrane as specified by the manufacturer (Boehringer GmbH, Mannheim, Germany). A 1.3-kb *Sal*I–*Hind*III fragment containing the *mxoF* gene from *P. denitrificans* was labelled with digoxigenin and used as a probe in the hybridization experiment. After hybridization at 68°C, the blots were washed in 0.1 × SSC (15 mM sodium chloride; 1.5 mM sodium citrate; pH 7) at room temperature. The target DNA was visualized by chemiluminescent detection with Lumigen PPD (Boehringer GmbH, Mannheim, Germany).

2.5. Enzyme activities

Cells were harvested, washed twice in 10 mM Tris · HCl buffer (pH 7.0), and suspended in the

same buffer to an optical density of 50.0 at 660 nm. The assay mixture contained 100 mM Tris · HCl buffer (pH 9.0), 15 mM NH₄Cl, 1 mM KCN, 0.1 mM 2,6-dichlorophenol-indo-phenol (DCPIP), 1.0 mM phenazine methosulfate (PMS) and cell suspension. The mixture was kept anaerobic with argon. Methanol, ethanol, *n*-propanol, *n*-butanol and 2-propanol to a final concentration of 8 mM were used as substrates in the assay. Dye-linked enzyme activities were determined by measuring the changes at 610 nm with the reference wavelength set at 750 nm using an Aminco DW2 UV/VIS spectrophotometer (American Instrument Company) in the dual wave length mode.

2.6. Oxygen consumption rates

The oxygen consumption in whole cells was measured using a Clark-type oxygen electrode. The reaction vessel was filled with air-saturated buffer. The rate of oxygen respiration was measured before and after adding substrate to the cell suspension. Methanol, ethanol, *n*-propanol, *n*-butanol and 2-propanol to a final concentration of 8 mM were used as substrate.

2.7. Isolation of cell extracts

For the isolation of cell extracts, cells were suspended in a 50 mM potassium phosphate buffer (pH 7.5), supplemented with DNase and 1.5 mM magnesium acetate, to an optical density of 50.0 at 660 nm and then broken in a French pressure cell (American Instrument Company, Silver Spring, MD) at 10000 psi. Membranes were removed by centrifuging for 1 h at 100 000 × *g* and 4° C.

2.8. Gel electrophoresis and heme staining

SDS-polyacrylamide gel electrophoresis (PAGE) was performed as previously described [12]. Approximately 400 μg protein was loaded. *c*-Type cytochromes were stained with 3,3',5,5'-tetramethylbenzidine using the method of Thomas et al. [17].

2.9. Protein determination

The concentration of protein was determined by BCA Protein Assay Reagent (Pierce Chemical Com-

pany, Rockford, IL) with bovine serum albumin as a standard [18].

3. Results and discussion

3.1. Characterization of a methanol-utilizing *T. pantotropha* mutant

A spontaneous *T. pantotropha* mutant that was able to grow on methanol was isolated. This mutant, designated Tp9002, showed a growth rate on methanol comparable to the growth rate of *P. denitrificans* on this substrate.

From hybridization studies with *mxgA*, the gene encoding the electron acceptor of MDH from *P. denitrificans*, it was clear that the *mxgA* gene is absent in *T. pantotropha* [11]. Moir and Ferguson [3] demonstrated that an additional cytochrome *c* is present in the periplasm of the Mox⁺ strains grown on either choline and methanol. They suggested that this 26-kDa cytochrome *c* takes over the role of the *mxgA* gene product in the transfer of electrons from MDH to the respiratory chain in the Mox⁺ strains. Fig. 1 shows that this cytochrome was found in cell extracts isolated from choline- and methanol-grown Tp9002. Additionally, it was found in ethanol-grown cells (results not shown). In *P. denitrificans* and other methylotrophs, MDH is encoded by the *mxgF* gene (large subunit) and the *mxgA* gene (small subunit), both part of an operon together with the *mxgG*,

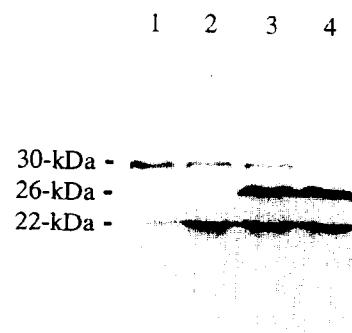


Fig. 1. SDS-PAGE of soluble proteins of *P. denitrificans* (lane 1), *T. pantotropha* (lane 2), Tp9002 (lane 3) and a *cycB* mutant from Tp9002 (lane 4). Cells were grown in batch culture on choline. Covalently bound heme was stained with 3,3',5,5'-tetramethylbenzidine. The relative molecular masses are indicated on the left.

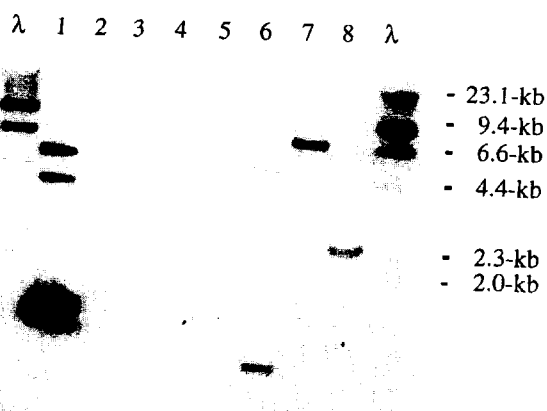


Fig. 2. Autoradiogram of the hybridization experiment of chromosomal DNA of *T. pantotropha* (lanes 2–5) and *P. denitrificans* (lane 6–8) with the labelled 1.3-kb *SalI*–*HindIII* fragment containing the *mxoF* gene from *P. denitrificans*. Control (lane 1); chromosomal DNA digested with *PvuII* (lane 2 and 6), with *SalI* (lane 3 and 7), with *EcoRI* (lane 4 and 8), with *BamHI* (lane 5). Fragment sizes are indicated.

mxoJ and *mxoR* genes in the gene order *mxoFJGIR* [7,18]. Chromosomal DNA of *P. denitrificans* and *T. pantotropha* was isolated and restricted with the enzymes *PvuII*, *SalI*, *EcoRI* and *BamHI*. An 1.3-kb *SalI*–*HindIII* fragment containing a 1.065-kb fragment of *mxoF* from *P. denitrificans* was used as a hybridization probe. As shown in Fig. 2, the *mxoF* probe hybridized with chromosomal *P. denitrificans* DNA fragments. However, no hybridization signal was detected with chromosomal DNA from *T. pantotropha*, indicating that the *mxoF* gene is absent in *T. pantotropha*. Using a *soxF* probe, a hybridization signal was obtained with chromosomal DNA of both *P. denitrificans* and *T. pantotropha* (results not shown). These results indicate that the oxidation of methanol in Tp9002 is not catalysed by MDH.

Egert et al. [2] suggested that the gene cluster *soxF*–*cycB*–*soxJ* is involved in methanol oxidation in the Mox⁺ *T. pantotropha*. This gene cluster is present in both *P. denitrificans* [12] and *T. pantotropha* [11]. The *soxF* gene encodes a protein that shows extensive homology with the large subunit of MDH, and the *soxJ* gene encodes a protein that shows extensive homology with MxoJ. The *cycB* gene codes for a periplasmically located cytochrome *c*_{553i} with a calculated molecular mass of 22.4 kDa. However, on SDS gels this cytochrome has a molecular mass of around 30 kDa [12]. To study the participation of this gene cluster in the ability of Tp9002 to grow on methanol, a mutant was isolated with a kanamycin resistance gene in the *cycB* gene. This mutant was able to grow on methanol and the 26-kDa cytochrome *c* was still present, while cytochrome *c*_{553i} was absent (shown in Fig. 1, lanes 3 and 4). These results indicate that the gene products encoded by the *sox* locus are not involved in the oxidation of methanol in Tp9002.

3.2. Enzyme activities

To obtain more information about the Tp9002 mutant, MDH and dye-linked alcohol dehydrogenase (ethanol dehydrogenase (EDH)) activities were measured in methanol-, choline- and ethanol-grown cells. MDH of *P. denitrificans* is induced during methylotrophic growth. As shown in Table 1, methanol- and choline-grown *P. denitrificans* cells showed activity with methanol, ethanol, *n*-propanol and *n*-butanol. The activity gradually decreased with increasing chain length. No activity was observed with the secondary alcohol 2-propanol. These findings are consistent with the substrate specificity of MDH of *P. denitrificans* as described earlier [6]. In Tp9002

Table 1

Enzyme activities (nmol of DCPIP min⁻¹ (mg protein)⁻¹) in whole cells of Tp9002, *T. pantotropha* (Tp) and *P. denitrificans* (Pd)

Substrate in assay	Methanol-grown		Choline-grown			Ethanol-grown		
	Tp9002	Pd	Tp9002	Tp	Pd	Tp9002	Tp	Pd
Methanol	172	444	43	0	94	12	0	0
Ethanol	1463	381	282	5	49	1298	103	15
<i>n</i> -Propanol	1404	261	– ^a	–	11	–	109	–
<i>n</i> -Butanol	1004	228	–	–	10	–	–	–
2-Propanol	2571	0	–	–	–	1553	37	26

^a –, not determined.

Table 2
Oxygen consumption rates (nmol O₂ min⁻¹ (mg protein)⁻¹) in whole cells

Substrate in assay	Methanol-grown Tp9002
Methanol	1.7
Ethanol	5.0
<i>n</i> -Propanol	5.2
<i>n</i> -Butanol	5.2
2-Propanol	8.0

grown on methanol, a different substrate specificity was observed. The cells were able to oxidize primary alcohols but the highest activity was observed with 2-propanol. Activity with methanol as a substrate could only be detected when ten times the usual amount of this substrate was added. Similar results were obtained with choline- and ethanol-grown Tp9002, but the activities were lower. These experiments were also done with the *T. pantotropha* Mox⁺ strain isolated by Moir and Ferguson [3] and comparable results were obtained (data not shown). Ethanol-grown *P. denitrificans* showed similar substrate specificity to Tp9002, but the activities were lower and methanol was not oxidized.

In methanol-grown Tp9002, oxygen consumption rates were determined and the same pattern was observed as with the in vitro enzyme activity measurements. Tp9002 showed the highest rate of oxygen consumption with 2-propanol. The oxygen consumption rate with methanol was lower than with ethanol, *n*-propanol, *n*-butanol and *n*-pentanol as substrates. Results are shown in Table 2.

The results presented here indicate the involvement of a dye-linked EDH with a substrate specificity different to MDH. This substrate specificity is similar to that of the dye-linked EDH from *Pseudomonas aeruginosa* [19]. EDH is a periplasmically located, PQQ-containing enzyme that consist of two identical large subunits and two identical small subunits. Cytochrome *c*_{EDH} mediates electron transfer from EDH to the electron transport chain [20]. The

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T. pantotropha Mox+ cytochrome cM   HGDTAP-QAVDTAGLPELPAE
                                     ::   ::   ::   ::   ::
Ps. aeruginosa cytochrome cEDH     AGDWVTTQAVDTRKLEPLGK

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Fig. 3. Homology of the N-terminal amino acid sequence from the 26-kDa cytochrome *c*_M of *T. pantotropha* Mox⁺ [3] with the 14.5-kDa cytochrome *c*_{EDH} of *Ps. aeruginosa* [20].

N-terminus of the 26-kDa cytochrome *c* isolated from the Mox⁺ strain [3] shows homology with the N-terminal amino acid sequence from the 14.5-kDa cytochrome *c*_{EDH} isolated from *Ps. aeruginosa* (see Fig. 3) [20]. This supports our hypothesis that methanol oxidation in Tp9002 is catalysed by a dye-linked EDH, using the 26-kDa cytochrome *c* as electron acceptor. This might be due to an 'up-mutation' in the regulation of EDH, resulting in higher enzyme activities than in the wild-type *T. pantotropha*. *P. denitrificans*, when grown on ethanol, has a dye-linked EDH with a similar substrate specificity to Tp9002. However, starting with a mutant with a large deletion in the *mx* locus, a spontaneous mutant with a methanol⁺ phenotype could not be isolated.

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