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The effect of oxygen on methanol oxidation by an obligate methanotrophic bacterium studied by *in vivo* ^{13}C nuclear magnetic resonance spectroscopy

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^{13}C NMR was used to study the effect of oxygen on methanol oxidation by a type II methanotrophic bacterium isolated from a bioreactor in which methane was used as electron donor for denitrification. Under high (35–25%) oxygen conditions the first step of methanol oxidation to formaldehyde was much faster than the following conversions to formate and carbon dioxide. Due to this the accumulation of formaldehyde led to a poisoning of the cells. A more balanced conversion of ^{13}C-labelled methanol to carbon dioxide was observed at low (1–5%) oxygen concentrations. In this case, formaldehyde was slowly converted to formate and carbon dioxide. Formaldehyde did not accumulate to inhibitory levels. The oxygen-dependent formation of formaldehyde and formate from methanol is discussed kinetically and thermodynamically. *Journal of Industrial Microbiology & Biotechnology* (2001) **26,** 9–14.

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Introduction

In methanotrophs methane is oxidized to carbon dioxide in a linear pathway (Figure 1) [3,4,7,9–11,18]. Methane is first converted to methanol by means of a NAD(P)H-dependent monooxygenase. Reducing equivalents produced during methanol oxidation are partly used in the methane monooxygenase reaction and partly channeled to the aerobic respiratory chain [11,20]. The relative rates of each of the methane oxidation steps may be balanced when sufficient oxygen and methane are present. However, an unbalance may occur during oxygen limitation. In this case methanotrophs may accumulate and excrete certain intermediates. Methanotrophs have the ability to produce organic compounds like methanol and formaldehyde [15,18], as well as other organic compounds such as polysaccharides and proteins [15,19]. The production of these compounds can lead to a coexistence with other bacteria [1,2,14,24].

Recently, we have analyzed the microbial community of a bioreactor in which methane was used as an electron donor for denitrification under oxygen limitation [9]. In this study no evidence was obtained that the methanotrophs are able to excrete organic C1 compounds under conditions at which oxygen was limiting. However, the most abundant methanotroph was able to form acetate under such conditions. Further studies indicated that a variety of organic compounds can be produced and excreted by the strain [26]. Organic C1 compounds were not formed, neither at a high nor at a low oxygen concentration. The research presented here was undertaken to investigate if organic C1 compounds are formed from methanol instead of methane by the methanotroph. *In vivo* ^{13}C NMR spectroscopy was used because this technique allows the easy detection of intracellular and extracellular organic compounds [21,23,26]. In addition, aerobic formation of formaldehyde and formate from methanol by *Methylosinus trichosporium* OB3b was demonstrated by NMR, using 10–18 mg dry wt ml^{-1} of biomass density, 10 mM ^{13}Cmethanol and an aeration rate of 10 ml min^{-1} [8].

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Materials and methods

The type II methanotroph (strain MTS) has been isolated from the biomass of a denitrifying bioreactor [9,24]. Its rDNA sequence has a 99% sequence similarity with *Methylocystis parvus*. The rDNA sequence has been deposited in the GenBank nucleotide sequence database under accession number AF107461 [9]. The strain was grown in Nitrate Mineral Salts (NMS) medium with 20 mM methanol as a substrate [12,27]. *In vivo* NMR experiments were performed under high and low oxygen conditions.

For the experiment at high oxygen concentration, a fully grown culture (1250 ml) was harvested under sterile conditions. Cells were resuspended in 18 ml of fresh NMS medium and aseptically transferred to a 20-mm NMR tube (total volume 55 ml) supplemented with three 4-mm glass balls to ensure good mixing in the spinning sample. $^{13}CH_3OH$ (Isotec, Miamisburg, Ohio) was added to the cell suspension to give a concentration of 20 mM. The final concentration of biomass in the NMR tube was 15 mg dry wt ml^{-1}. The concentration of oxygen in the O_2–N_2 atmosphere was maintained between 25% and 35% during the experiment. The gas composition in the headspace of the NMR tube was measured every 12 h using a Packard 417 gas chromatograph equipped with a Molsieve 13X (2 m×0.64 mm OD) packed column connected to a thermal conductivity detector with argon as carrier gas (20 ml/min).

For the experiment at low oxygen concentration fully grown cells (600 ml) were harvested, transferred into a 20-mm NMR tube with a total volume of 55 ml as described above. Fresh medium with 20 mM of $^{13}CH_3OH$ was added to obtain a total liquid volume in the NMR tube of 12 ml. The initial gas

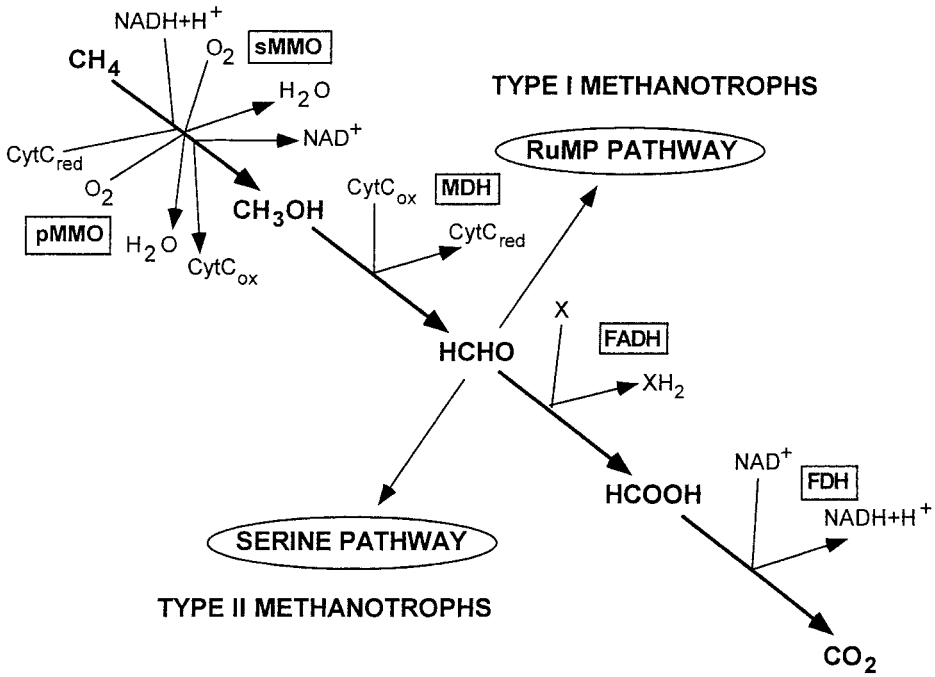

Figure 1 Linear pathway for methane oxidation in methanotrophic organisms [11]. Type II methanotrophs use the serine pathway for formaldehyde assimilation and Type I methanotrophs use the ribulose monophosphate pathway. sMMO (soluble monooxygenase) and pMMO (particulate monooxygenase) are the two methane oxygenases found in methanotrophs. The sMMO is an NADH-dependent enzyme occurring in Type II methanotrophs, and pMMO, which occurs in all methanotrophs, is cytochrome dependent.

atmosphere consisted of 95% N_2 and 5% O_2. Conversion of labeled substrate was analyzed using an AMX-300 NMR wide-bore spectrometer (Bruker, Karlsruhe, Germany) tuned at the wn>13C frequency of 75.47 MHz. ^{13}C NMR spectra were recorded in sequence during runs of 6 h at 30°C; a spectral width of 20.000 Hz was used and 40.000 Free Induction Decays were accumulated for one spectrum. The acquisition time was 0.2 s, the interpulse delay 0.5 s, and the pulse angle 45° (duration 30 μs). Quantification was done by comparison of the 6-h spectra with the spectrum of a reference sample containing the compounds at their natural abundances (1.10%) under investigation (50 mM) in NMS medium. This spectrum was recorded under the same experimental conditions as used for cells. Two-level proton decoupling was applied and a spinning rate of 15–16 Hz was used. The spectra were referenced against TMS by setting the methanol resonance at 50.2 ppm according to Barnard and Sanders [5].

Results

The conversion of ^{13}C-labelled methanol to ^{13}C-labelled formaldehyde, formate and carbon dioxide was studied first at high oxygen concentration. A high oxygen concentration in the gas phase was achieved by introducing 35% O_2 in the headspace of the NMR tube, which was subsequently mixed with the suspension by fast (16 Hz) rotation of the 20-mm NMR tube, which contained three glass balls. All products of methanol oxidation: formaldehyde (83.1 ppm), formate (172.2 ppm) and carbon dioxide (125.7 ppm for dissolved gas and 128.7 ppm for CO_2 gas) were detected within the first 6 h of incubation (Figure 2). However, the conversion of methanol into formaldehyde was much faster than the conversions of formaldehyde to formate and formate to carbon dioxide. As a result of this imbalance, formaldehyde accumulated during methanol conversion whereas the resonances of ^{13}C-labelled formate and carbon dioxide appeared to only a minor extent. Within 6 h about 6% of the added methanol was converted to formaldehyde. This value increased to 33% after 12 h and to 41% after 18 h. Finally, the accumulation of about 7 mM of formaldehyde led to inhibition of all reactions, and within the next 24 h of the experiment no further conversion was observed either of methanol to formaldehyde or of the transformations to formate and carbon dioxide.

When methanol was incubated at a low oxygen concentration, 1–5% in the head space, it was slowly converted to formaldehyde and carbon dioxide (Figure 3). The natural abundance bicarbonate resonance (161.4 ppm) was visible in spectra because of the high HCO_3^- concentration in the dense suspension. The initial oxygen concentration was 5%, but every 6 h the oxygen concentration was approximately 1% less. Therefore, every 24 h, after four NMR spectra were recorded, additional oxygen was added. Addition of new oxygen resulted in rapid formaldehyde formation. The spectra indicate that formaldehyde is produced rapidly at 5% oxygen in the gas phase, but when the concentration of oxygen decreased to lower than 3%, formaldehyde is converted and formate is formed. Both formaldehyde and formate were present in the cell-free spent culture liquid. The highest concentration detected for these two compounds in the experiments with low oxygen was 1.5 mM.

Discussion

The pathway of methane oxidation is intriguing because both oxygen and reducing equivalents have dual functions in the metabolism of methanotrophs [3,4,18]. Oxygen is needed for

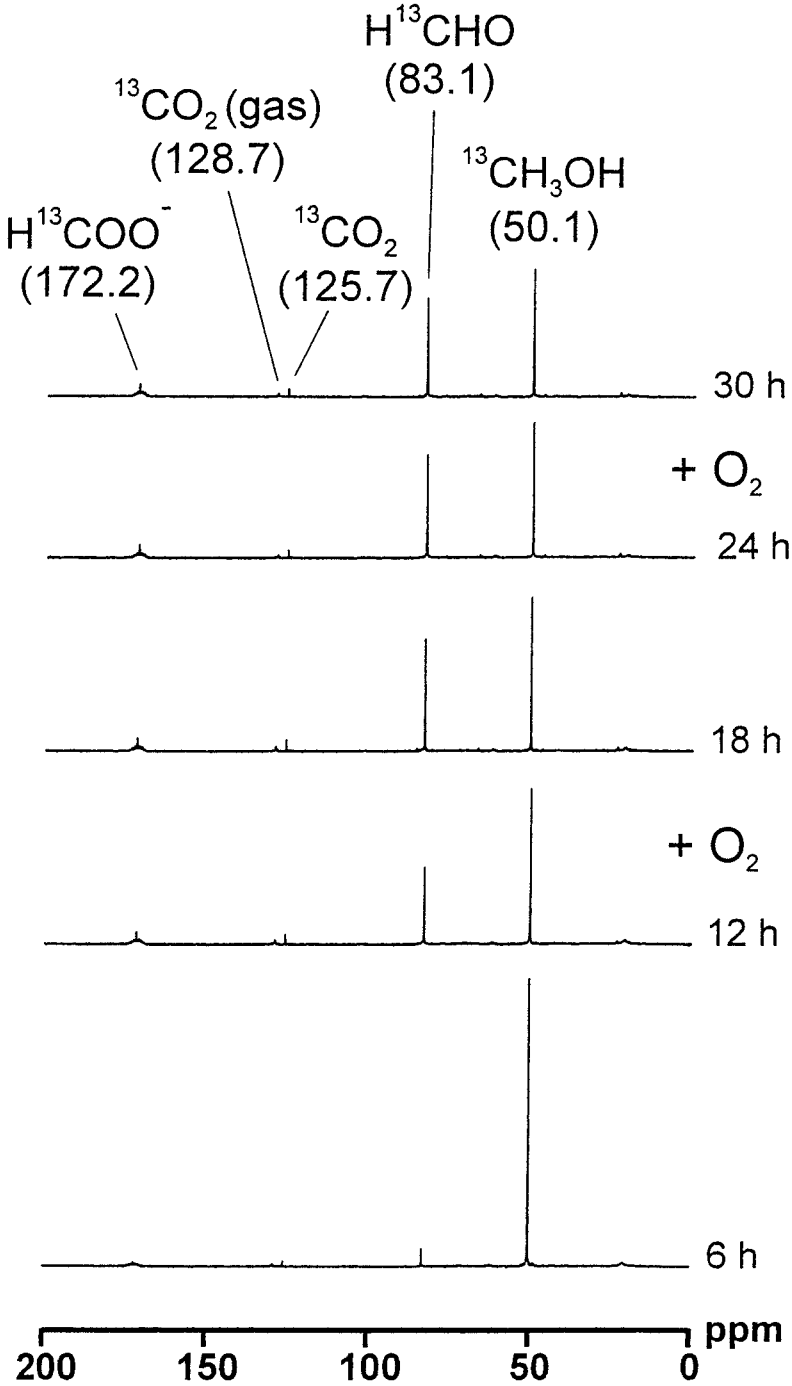

Figure 2 Methanol oxidation at 35% oxygen in the atmosphere (high oxygen conditions). Six-hour spectra were recorded sequentially. Every 12 h extra oxygen was added.

methane monooxygenase activity and it serves as the terminal electron acceptor. Reducing equivalents are used in the methane monooxygenase reaction and in the respiratory chain [6,11,20]. Therefore, it can be envisaged that the metabolism of methanotrophs is strongly affected by the oxygen level [16,17,22]. At present it is not known if the methane monooxygenase or the electron transport chain has the highest affinity for oxygen. If the monooxygenase has the highest affinity the accumulation and possible excretion of organic intermediates can be expected under conditions of low oxygen. As the formation of methanol, formaldehyde and formate has been reported [8,15,18], one may assume that the methane monooxygenase has a higher affinity for oxygen than the electron transport chain. However, in previous experiments with strain MTS we were not able to demonstrate the formation of organic C1 compounds from methane at high or low oxygen concentration, while we could detect the formation of a variety of other organic compounds [9,26].

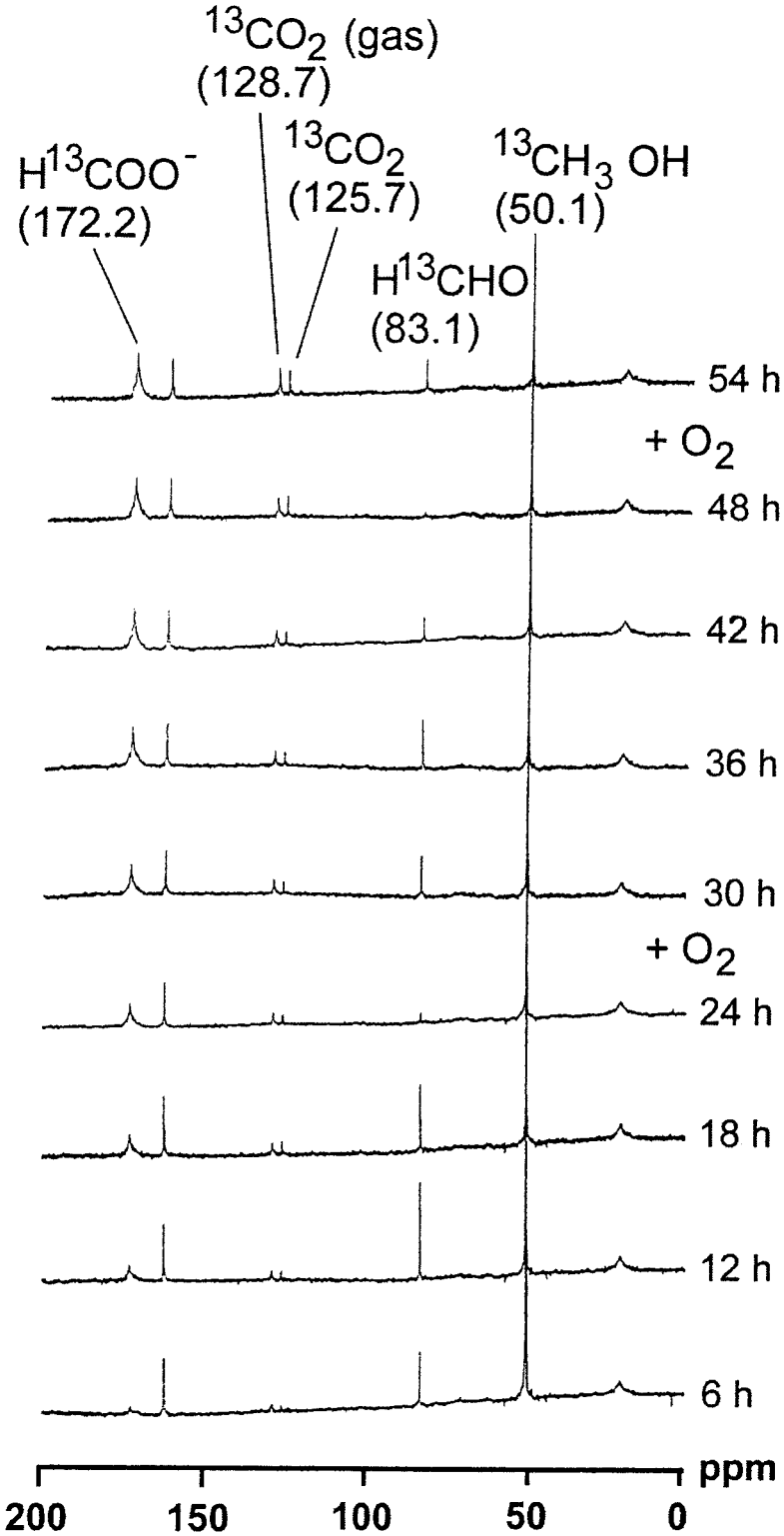

Figure 3 Methanol oxidation at 5% oxygen in the atmosphere (oxygen limitation). Six-hour spectra were recorded sequentially. After 24 and 48 h of incubation extra oxygen was added.

By means of *in vivo* NMR we were able to follow the fate of methanol in cells of strain MTS at two different oxygen levels. At a high oxygen level, methanol was rapidly converted to formaldehyde, which resulted in an inhibition of cell metabolism. However, by applying 5% of oxygen in the gas phase, conversion of methanol was more balanced. Two different situations were observed. At 3–5% of oxygen in the gas phase formaldehyde is produced rapidly whereas the

Table 1 K_m and V_{max} values for oxygen uptake by *Methylobacterium* (former *Pseudomonas*) *extorquens* [13]

Electron donor	K_m [μM]	V_{max} [mmol g^{-1} h^{-1}]
Methanol	20.4	10.5
Formaldehyde	104	6.1
Formate	228	10.0

production of formate was slow. Under these conditions, formate production apparently is a rate-limiting step. At 1–3% oxygen in the gas phase, formaldehyde no longer accumulated, whereas the accumulation of formate continued. Apparently, the conversion of methanol to formaldehyde is more strongly affected by low oxygen concentrations than formaldehyde conversion to formate. This is in accordance with the Gibbs free energy changes ($\Delta G^{\circ\prime}$) of these conversions, which can be calculated from data of Thauer *et al* [25]:

$$\text{methanol} + 0.5O_2 \rightarrow \text{formaldehyde} + H_2O \qquad \Delta G^{\circ\prime} = -192 \text{ kJ}$$
$$\text{formaldehyde} + 0.5O_2 \rightarrow \text{formate}^- + H^+ \qquad \Delta G^{\circ\prime} = -260 \text{ kJ}$$
$$\text{formate}^- + 0.5O_2 \rightarrow HCO_3^- \qquad \Delta G^{\circ\prime} = -236 \text{ kJ}$$

The Gibbs free energy change of formaldehyde conversion to formate is most negative. Therefore, this reaction may proceed till lower oxygen concentrations are reached.

Kinetically, the affinity for oxygen can be compared by means of the K_m value for oxygen uptake with methanol, formaldehyde and formate as electron donors. At present, we do not have information about the kinetic parameters of strain MTS. However, for a methanol-oxidizing methylotrophic bacterium (*Methylobacterium* (former *Pseudomonas*) *extorquens*) K_m and V_{max} values for oxygen uptake using methanol, formaldehyde and formate as electron donors are reported [13] (Table 1). The affinity of oxygen for methanol is about five times higher than that with formaldehyde and about 10 times higher than that with formate. If these data are also valid for our methanotrophic strain, one might expect that under oxygen-limiting conditions formate is the first organic compound of the linear methanol oxidation pathway to accumulate, followed by formaldehyde. However, formaldehyde is also the precursor for cell carbon synthesis. As shown in previous studies with strain MTS, intermediates of the carbon fixation pathway are synthesized and excreted [9,26]. More research is needed to get insight into how oxygen affects the flux of carbon through assimilatory and dissimilatory pathways in methanotrophs.

It is still not known which enzymes are involved in the oxidation of methanol in strain MTS. Methanol conversion by a type II methanotroph, *M. trichosporium* strain OB3b, was studied in detail by Cornish *et al* [8]. Accumulation of formaldehyde was observed only in suspensions grown under conditions that yield particulate, membrane-bound, methane monooxygenase. Cells grown under conditions that yield soluble methane monooxygenase or cells grown on methanol never showed detectable formation of formaldehyde. Our strain MTS most likely is a *Methylocystis parvus* strain. *M. parvus* contains only particulate methane monooxygenase [11]. However, we do not know if this enzyme is also induced when cells are grown on methanol.

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