Effect of oxygen level on simultaneous nitrogenase and sMMO expression and activity in *Methylosinus trichosporium* OB3b and its sMMOC mutant, PP319: aerotolerant N₂ fixation in PP319

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Abstract

Soluble methane monooxygenase (sMMO) expression and activity were monitored under conditions that either promoted or suppressed the expression of nitrogenase in *Methylosinus trichosporium* OB3b wild-type (WT) and in its sMMO-constitutive mutant, PP319. Both WT and mutant cultures had reduced sMMO activity and protein levels under elevated O₂ conditions (188 μM) compared with low O₂ conditions (24 μM). Simultaneous N₂ fixation also reduced sMMO activity in both cultures when O₂ was low. However, when O₂ levels were increased, nitrogenase expression ceased and sMMO activity was reduced by 77% in the WT, whereas sMMO and nitrogenase expression and activity in PP319 were relatively unaffected by the higher O₂ levels. Western immunoblot analysis showed that the nitrogenase Fe protein resolved as two components (apparent molecular mass of 30.5 and 32 kDa) in both the WT and PP319 when O₂ levels were low. When O₂ levels were high, only the 32-kDa form of the Fe protein was present in PP319, whereas neither form was detectable in the WT. Aerotolerant N₂ fixation appears to be associated with the 32-kDa Fe protein in *M. trichosporium* OB3b. © 2001 Published by Elsevier Science B.V. on behalf of the Federation of European Microbiological Societies.

Keywords: Methanotroph; Soluble methane monooxygenase; Nitrogenase; Oxygen; *Methylosinus trichosporium* OB3b

1. Introduction

Despite the O₂-labile nature of nitrogenase in vitro, aerobic diazotrophs possess the ability to fix N₂ in environments where O₂ is present. Maintaining active nitrogenase in the type II methanotrophic bacteria is particularly difficult because O₂ is required both for methane oxidation by methane monooxygenase (MMO) and for aerobic respiration. Understanding the effect of O₂ level in methanotrophs is very important because they have many practical applications, especially when they express soluble MMO (sMMO). For example, Oldenhuis et al. [1] showed that sMMO could transform a variety of environmental contaminants at high rates, and Graham et al. [2] showed that sMMO-bearing type II organisms, which express both sMMO (at low Cu-to-cell ratios) and nitrogenase (under low O₂ conditions), can be enriched over other strains under nitrogen-limited conditions. Our goal here was to further examine the effect of O₂ and nitrogen on the expression and activity of sMMO and nitrogenase in type II methanotrophs to better define limits to these organisms in natural and engineered processes.

To satisfy this goal, we compared sMMO and nitrogenase expression and activity, and contaminant co-oxidation capabilities under different O₂ and nitrogen conditions in *Methylosinus trichosporium* OB3b (wild-type (WT)) and in its sMMO-constitutive (sMMOC) mutant PP319. PP319 is a stable *M. trichosporium* OB3b mutant defective in its ability to uptake copper [3–5] and, as such, expresses sMMO constitutively with < 12 μM Cu [4]. In addition, it does not express particulate MMO (pMMO) even at high copper-to-cell ratios, is minimally impacted by elevated O₂ levels (see later), and has innately high contaminant transformation rates [3–6]. Here we describe experiments assessing the effect of O₂ on nitrogenase and sMMO expression and activity in WT and PP319 cultures. In general, increases in O₂ level negatively affected sMMO and nitrogenase in both organisms; however, surprisingly high levels of nitrogenase expression and activity were...
maintained soluble \( \text{O}_2 \) levels of either 24 or 188 \( \mu \text{M} \) in the media. Low copper media (repression media) contained 10.0 mM nitrate, whereas ‘low’ nitrogen media only 40 \( \mu \text{M} \) nitrate. ‘High’ and ‘low’ \( \text{O}_2 \) were defined as soluble \( \text{O}_2 \) levels of 80% (188 \( \mu \text{M} \)) and 10% (24 \( \mu \text{M} \)) saturation in air (at 30°C), respectively. Dissolved \( \text{O}_2 \) levels in the media were directly measured using an Ingold (Wilmington, MA, USA) \( \text{O}_2 \) sensor probe. Low copper media (<0.2 \( \mu \text{M} \)) were employed to avoid sMMO–pMMO switchover effects in the experiments [8].

2.2. Culture conditions and experimental format

Cells were grown as continuous cultures in a Bioflo 2.5-l bioreactor controlled by a Bioflo 3000 controller (New Brunswick Scientific). The cultures were operated under turbidostatic conditions, where the rate of fresh medium addition was sufficiently high, relative to growth rate, to ensure that nutrients other than fixed nitrogen were not growth limiting. The hydraulic flow rate for turbidostat operation was determined for each experiment by initially growing the organisms in batch mode under the desired condition and determining the specific growth rate of that culture. The reactor was then switched to continuous-flow mode using a dilution rate (approximately the maximum growth rate for that treatment) selected to maintain a constant cell density over time under the defined treatment. Optical density of the culture at 600 nm (\( \text{OD}_{600} \text{nm} \)) was constantly monitored, and used to regulate dilution rate to maintain turbidostatic conditions. Culture density typically changed in response to small manipulations in flow rate, indicating turbidostatic operation [9].

Experiments were initiated by adding ~100–200 ml of either WT or PP319 exponential phase overnight cultures to 2.0 l of sterile media in the bioreactor, which was stirred at 300 RPM with temperature and pH maintained at 30°C and 7 (±0.5), respectively. Organisms were provided instrument-grade methane (in excess) at ~15–20 ml per min. Air was supplied and regulated by the biocentrler, in conjunction with the \( \text{O}_2 \) sensor probe, to continuously maintain soluble \( \text{O}_2 \) levels of either 24 or 188 \( \mu \text{M} \). Typically, cultures were grown in batch mode for 2–4 days to develop adequate cell densities for continuous-culture operations (0.5 < \( \text{OD}_{600} \text{nm} < 0.7 \)). Media addition was then commenced, and after the passage of three reactor volumes to achieve a stable culture density, replicate enzyme assays (> five per treatment over a span of 4 days) were performed to quantify enzyme activities under each treatment. When assays were complete, approximately 90% of the reactor’s volume was harvested for protein analyses. This experimental sequence was repeated four times per treatment with independent cultures to permit statistical analysis of the activity data.

2.3. sMMO and nitrogenase assays

Whole-cell sMMO and nitrogenase activity assays were performed only when stable cell densities were achieved in the bioreactor. Each set of assays was initiated by withdrawing a 10-ml sample from the bioreactor into which formate was added to a concentration of 10 mM. Six ml of formate-amended sample was used for the sMMO assay and 3.0 ml for the nitrogenase assay. The sMMO assay was a modified version of the naphthalene oxidation assay [5,10]. Specifically, 50.0 \( \mu \text{l} \) of 5.0 mg ml\(^{-1}\) of o-dianisidine solution (Sigma) was added to 3.0 ml of the formate-amended cell suspension. The reaction was initiated by the addition of 50.0 mg of crushed naphthalene to the sample after which the resulting mixture was hand shaken vigorously 50 times. Color formation was monitored continuously for 20 min by measuring absorbance change at 525 nm compared with 3.0 ml of unamended cell suspension.

The acetylene-ethylene nitrogenase assay used was similar to that described by Graham et al. [2]. Three ml of formate-amended cell suspension was transferred to a 7.4-ml Teflon septum-sealed glass vial (Fisher), which was immediately flushed with helium for 1.0 min to remove headspace gas prior to the assay. The reaction was initiated by the injection of 400 \( \mu \text{l} \) of instrument-grade acetylene into the vial headspace; the vial was then placed onto a rotary shaker table for 0.5 min to allow for diffusion of the gas into the liquid phase. Following stabilization, headspace measurements were made to monitor ethylene formation in the vial (using a Varian 3700 gas chromatograph equipped with a Hayes SepN steel column and a flame ionization detector), typically every 5 min for a period of 35–40 min. Ethylene formation was quantified by comparison to an ethylene standard curve. All enzyme activities were normalized to the cell dry weight (CDW) of the assay sample.

2.4. Cis-dichloroethylene (DCE) transformation assay

DCE transformation rates were determined using procedures similar to the nitrogenase assay. Five ml of formate-amended cell suspension was transferred to an 8.6-ml Teflon septum-sealed glass vial (Kimble). The vial was then flushed with helium for 5 min to remove residual methane, and an appropriate amount of cis-DCE (Acros)
was added to achieve liquid-phase DCE concentrations ranging from 5 to 1000 µM. Reactions were initiated by the addition of 1.0 ml of ambient air. The vials were then agitated on a rotary shaker table and 200 µl headspace samples were analyzed at 3-min intervals, on a Hewlett Packard 5880 gas chromatograph equipped with a J&W DB-1 capillary column (J&W Scientific) and a flame ionization detector. Initial DCE transformation rates were determined for each DCE level, which were used to estimate maximum DCE transformation rates using the classical Monod kinetic model [11]. All transformation rates were normalized to the CDW of the assay culture.

2.5. Protein analysis

Protein concentrations associated with sMMO and nitrogenase expression under each treatment were determined using SDS-PAGE followed by Western blotting. The cells were harvested at the end of each treatment by centrifugation at 9000×g for 20 min at 4°C and re-suspended in a minimal volume of 10 mM Tris–Cl, pH 7.5 buffer and frozen for later analysis. Cells were lysed using a lysis cocktail containing 80% v/v of a mild, non-ionic detergent (Bacterial Protein Extraction Reagent (B-PER), Pierce), 10% v/v of 7.2 mg l⁻¹ of lysozyme (in 10 mM Tris–Cl, pH 7.5), and 10% v/v protease inhibitor cocktail (Sigma). Typically, about 100 mg of previously frozen cells were suspended in 300 µl of lysis cocktail and agitated for 10 min at room temperature. The lysate was centrifuged at 12 000×g for 20 min at 4°C to remove cell debris. This ‘mild’ lysis procedure produced higher nitrogenase Fe protein yields compared with other cell disruption methods assessed.

Proteins were separated on a 4–12% Bis-Tris gradient gel and electrophoretically transferred onto a polyvinylidenefluoride membrane. The membrane was then incubated overnight at 4°C with polyclonal antibodies raised against the α, β, and γ peptides of the hydroxylase subunit of M. trichosporium OB3b sMMO (provided by G. Georgiou) or the nifH gene product (Fe protein) of Rhodospirillum rubrum nitrogenase (provided by P.W. Ludden, University of Wisconsin, Madison, WI, USA). The nifH antisera were pre-absorbed with nitrogenase-repressed lysate at 30°C for 30 min to reduce cross-reactivity. Blotted proteins were visualized using goat anti-rabbit biotinylated secondary antibody attached to horseradish peroxidase with diaminobenzidine as the substrate. Molecular masses were determined by comparing mobilities to biotinylated standards using Kodak Digital Science 1D Image Analysis Software.

3. Results and discussion

3.1. Suppression of sMMO activity by elevated O₂ levels

When steady-state fixed nitrogen levels in the media were high (10.0 mM), nitrogenase was always fully repressed in both the WT and PP319. Under this nitrogen condition, increasing O₂ levels from 24 to 188 µM caused a reduction in sMMO activity of ~30% in the WT and ~22% in PP319 (see Table 1). These reductions were statistically significant (as determined by the Wilcoxon U test at the 95% confidence level (two-way α=0.025)) with P values of 0.04 (96%) and 0.01 (99%) for the WT and PP319, which suggest that elevated O₂ levels can negatively impact sMMO activity in M. trichosporium OB3b. This negative effect of elevated O₂ was also seen with DCE transformation, where increased O₂ levels caused a substantial decline in rates for both organisms (Table 1). Extended exposure (>4 days) to elevated O₂ levels further decreased sMMO activity in both organisms, especially the WT (data not shown). Decreases in sMMO activity were paralleled by decreases in specific growth rates (e.g. from 0.03 to 0.019 h⁻¹ in the WT and from 0.036 to 0.021 h⁻¹ in PP319).

Immunoblot analysis on cells grown under nitrogen-repressed conditions, and harvested before and after the increase in O₂, showed that decreases in the sMMO activity were apparently associated with decreased levels of the α, β, and γ peptides of the sMMO hydroxylase subunit. This implies that the reduction in sMMO activity was due, in part, to reduced levels of sMMO proteins. This was most evident in the WT. Fig. 1a shows that when O₂ level was increased, sMMO protein levels were substantially reduced (lane 2) compared to cultures grown under low O₂ levels (lane 1). Reductions in sMMO protein level in PP319 were smaller, but still apparent (compare lanes 3 and 4). The higher protein level in PP319, relative to the WT, coincided with the generally higher sMMO activities seen in the mutant when O₂ was increased from 24 to 188 µM.

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Table 1: Whole-cell sMMO activity and cis-DCE transformation rates in M. trichosporium OB3b WT and PP319 cultures

<table>
<thead>
<tr>
<th>sMMO¹</th>
<th>Nitrogenaseᵇ WT</th>
<th>PP319</th>
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<tbody>
<tr>
<td></td>
<td>Low O₂</td>
<td>High O₂</td>
</tr>
<tr>
<td>+</td>
<td>0.31 ± 0.03</td>
<td>0.07 ± 0.002</td>
</tr>
<tr>
<td>−</td>
<td>0.40 ± 0.03</td>
<td>0.28 ± 0.04</td>
</tr>
<tr>
<td>cis-DCE²</td>
<td>+</td>
<td>67 ± 4</td>
</tr>
<tr>
<td></td>
<td>−</td>
<td>41 ± 4</td>
</tr>
</tbody>
</table>

¹sMMO activity in ng of naphthol formed min⁻¹ (mg CDW)⁻¹.
²Continuous cultures where nitrogenase was either expressed (+) or repressed (−) when O₂ levels were either low (24 µM) or high (188 µM).
³Activity values are means of multiple runs (≥10) with independently grown cultures; 95% confidence intervals are noted.
⁴Maximum cis-DCE transformation rate activity estimated using the Monod model (in nmol of DCE transformed min⁻¹ (mg CDW)⁻¹).
3.2. Effects of O₂ on the interrelationship between sMMO and nitrogenase

Due to the fact that methanotrophs are O₂-dependent diazotrophs, O₂ is required for respiration, which, in turn, is required for energy production to support N₂ fixation and other cellular activities. In methanotrophs, these energy-generating processes are linked to the sMMO-catalyzed oxidation of methane and the formation of its fueling intermediates. Thus, we examined sMMO activity in cultures that simultaneously expressed sMMO and nitrogenase. These conditions, theoretically, create higher energy demands on the organisms, even though the cellular ATP and NADH pools remain unchanged.

Both WT and PP319 cultures actively fixed N₂ when grown under low nitrogen (40 μM nitrate)-low O₂ (24 μM) conditions. Specifically, sMMO activity under this treatment was lower for both the WT (22%) and PP319 (18%) compared with organisms grown under higher nitrogen conditions (see Table 1). When O₂ levels were increased to 188 μM, during nitrogen fixation, nitrogenase activity in the WT reduced dramatically from 0.47 to less than 0.02 nmol min⁻¹ mg⁻¹ (Table 2). Fig. 2 (lane 1) shows that nitrogenase Fe protein disappeared in the WT in conjunction with this loss of activity. Similarly, sMMO activity in the WT decreased considerably from 0.31 to 0.07 ng min⁻¹ mg⁻¹ (77%) under this treatment (Table 1), apparently associated with the large reduction in sMMO hydroxylase peptide levels (Fig. 1b, lane 2).

Thus, it appears that the utilization of O₂ by sMMO is not adequate to fully reduce O₂ stress during N₂ fixation in the WT. Furthermore, WT cells grown under low nitrogen-high O₂ conditions became notably flocculent, less motile, and had ~35% lower specific growth rates. These signs indicate stressed growth in the WT, presumably due to nitrogen starvation resulting from nitrogenase inhibition by O₂.

In contrast, PP319 cultures, grown under N₂-fixing conditions, and then exposed to higher O₂ levels, showed minimal indications of cell stress. In fact, nitrogenase activity in PP319 was similar to that observed under the lower O₂ conditions (see Table 2). Additionally, sMMO activity in the mutant decreased only from 0.4 to 0.29 ng min⁻¹ mg⁻¹ (see Table 1) and, unlike the WT, retained high levels of all three bands of the sMMO hydroxylase.

Table 2

<table>
<thead>
<tr>
<th></th>
<th>WT</th>
<th>PP319</th>
</tr>
</thead>
<tbody>
<tr>
<td>High O₂</td>
<td>0.02 ± 0.01b</td>
<td>0.33 ± 0.04</td>
</tr>
<tr>
<td>Low O₂</td>
<td>0.47 ± 0.01</td>
<td>0.33 ± 0.03</td>
</tr>
</tbody>
</table>

*Continuous cultures grown under high and low O₂ conditions; 188 and 24 μM, respectively.

*Activity values in nmol acetylene reduced min⁻¹ (mg CDW)⁻¹ are averages of multiple runs (n > 12) with independently grown organisms; 95% confidence intervals are noted.

Fig. 1. Effect of O₂ level on sMMO protein levels in M. trichosporium OB3b WT and in PP319. Cells grown under either low O₂ (24 μM) or high O₂ (188 μM) conditions, harvested and analyzed for sMMO protein using Western blot analysis with antisera raised against the α, β, and γ peptides of the hydroxylase subunit. (a) sMMO protein levels in cells when nitrogenase was repressed. (b) sMMO protein levels in cells when nitrogenase was derepressed.
subunit (Fig. 1b, lane 2 vs. lane 4). Moderately high DCE transformation rates were also retained in PP319, with rates decreasing by 53% compared with 91% in the WT (Table 1). These data suggest that PP319 is more tolerant than the WT to elevated O2 levels (especially during N2 fixation) with respect to both sMMO activity and expression, and that it has some significant advantages over the WT for contaminant transformation.

The observed high rate of N2 fixation in PP319 under high O2 levels is in contrast to the generally observed sensitivity of nitrogenases to O2. Elevated O2 conditions clearly eliminated nitrogenase activity in the WT; however, PP319 consistently fixed N2 at elevated rates under both high and low O2 conditions. This apparent insensitivity of PP319 to high O2 levels suggests that the apparent copper uptake defect in PP319, which results in constitutive expression of sMMO [5,6], provides some fortuitous protection against the negative effects of O2. This is plausible because many antioxidant enzymes, including superoxide dismutase (SOD), are also copper-associated. The nitrogenase and O2-defense systems in M. trichosporium OB3b are currently under investigation.

3.3. Alteration of the Fe protein composition of nitrogenase

Immunoblot analysis on WT and PP319 cultures that were actively fixing N2 revealed the existence of two distinct bands that immunologically corresponded to the nifH gene product (Fig. 2): a slower migrating form with an apparent molecular mass of 32 kDa and a faster migrating form of about 30.5 kDa. Both Fe proteins were present in the WT and PP319 when O2 levels were low (Fig. 2, lanes 2 and 4). However, only the slower migrating form of the nifH gene product was seen in N2-fixing PP319 cultures grown under high O2 conditions (Fig. 2, lane 4). This observation suggests that either the faster migrating Fe protein is an inactive degradation product (and that O2-mediated breakdown of this product occurs under high O2 conditions), or the slower migrating form is associated with nitrogenase activity in M. trichosporium OB3b under higher O2 conditions. In fact, similar observations have been made on Anabaena sp. strain CA where high O2 conditions caused a shift in the expressed Fe protein from a faster to a slower migrating form (also with an apparent molecular mass difference of 1.5 kDa) that then retained elevated nitrogenase activity [12]. This implies that the apparent alteration in Fe protein may facilitate N2 fixation at higher O2 levels.

Fe protein modification may not be the only strategy employed by M. trichosporium OB3b to provide protection for nitrogenase against O2 inactivation. Indirect evidence suggests that nitrogenase in this organism may also be membrane-associated due to the fact that the Fe protein was only found in whole-cell, crude extracts during protein characterization. When only soluble fractions were used for protein assessment, both Fe proteins were barely detectable (data not shown). The possibility that the nitrogenase in M. trichosporium OB3b might be membrane-associated is unusual, but not unique. Azotobacter sp. also has a membrane-associated nitrogenase [13] and, interestingly, this organism is also relatively unaffected by higher O2 levels. Additionally, the membrane association of the pMMO, which is O2 sensitive [14], suggests that a localization of nitrogenase within the membranes may not be coincidental. More detailed studies are required to establish the cellular location and the nature of the two Fe protein components in M. trichosporium OB3b.

3.4. Practical considerations

There are two practical considerations that arise from our results; one related to future nitrogenase research and the other related to contaminant transformation with methanotrophs. First, a unique new system has been identified that can be used to assess the relative role(s) of the two forms of the nitrogenase Fe protein in response to altered O2 conditions. We have recently discovered and purified an SOD from M. trichosporium OB3b, and are currently sequencing and raising antibodies against the new protein. Once the antibodies are produced, we can study the relationship(s) among SOD, MMO, and nitrogenase expression and activity in the WT and PP319, and potentially better explain aerotolerant N2 fixation in the sMMO6 mutant.

Second, the ability of PP319 to retain high levels of sMMO expression and activity under nitrogen-limited, high oxygen conditions provides a potentially useful new tool for contaminant transformation. For example, our results show that the mutant was able to not only fix N2, but was also capable of relatively high cis-DCE transformation rates, even at O2 levels that impaired sMMO activity in WT organisms (Table 1). These results have clear practical implications for using O2 and nitrogen pro-

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Fig. 2. Fe protein expression under low nitrogen conditions at low (24 μM) or high (188 μM) O2 levels in M. trichosporium OB3b WT and in PP319. Cells grown under either low O2 (24 μM) or high O2 (188 μM) conditions, harvested and analyzed for nitrogenase protein using Western blot analysis with antisera raised against purified Fe protein (anti-nifH) of R. rubrum.
cess control to select for sMMO<sup>C</sup> mutants in treatment systems.

In summary, we have shown that O<sub>2</sub> level clearly modulates sMMO activity and its biotransformation potential under both nitrogen-fixing and nitrate-sufficient conditions. Additionally, we have further demonstrated that the sMMO<sup>C</sup> mutant has advantages over the WT for biotechnical applications because it retains high sMMO activity under all O<sub>2</sub> conditions and, in theory, can be selectively enriched in treatment systems using combined O<sub>2</sub> and nitrogen control. The characteristics of this interesting and unique mutant must continue to be examined to ensure that its biotechnical potential is fully realized.

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References


