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Effects of oxygen and nitrogen conditions on the transformation kinetics of 1,2-dichloroethenes by *Methylosinus trichosporium* OB3b and its sMMO^{C} mutant

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Abstract

Transformation kinetics of *trans*- and *cis*-dichloroethylenes (DCE) by *Methylosinus trichosporium* OB3b wild type (WT) and PP319, a mutant that expresses soluble methane monooxygenase at copper levels up to ~12 μM Cu (sMMO^{C}), were determined to assess the effects of O_2 level and N_2-fixation on degradation capabilities. Two issues were examined: (1) the influence of O_2 level and nitrogen-limitation on DCE degradation kinetics and toxicity in both organisms, and (2) the relative utility of PP319 for contaminant degradation in bioreactors. When both organisms were grown under high O_2 conditions (80% saturation in air), maximum transformation rates (V_{max}) and apparent first-order rate constants (V_{max}/K_M) were lower compared with organisms grown under low O_2 conditions (10% saturation in air) regardless of nitrogen level. Further, V_{max} values were near zero in nitrogen-limited WT cultures when O_2 was high (as expected), whereas PP319 retained moderate V_{max} levels even at high O_2 levels. In general, elevated O_2 conditions reduced DCE degradation rates in OB3b, although the negative effects of O_2 were less in PP319 than in the WT. Given that PP319 retained moderate DCE degradation rates under most O_2 and copper conditions, the mutant appears to have some utility for biodegradation applications.

Introduction

Methanotrophs express non-specific, catalytically active methane mono-oxygenases (MMO) that readily oxidize many contaminants, including trichloroethene (TCE) and dichloroethene (DCE). Some methanotrophs, including the type II strain *Methylosinus trichosporium* OB3b, express two different MMO's depending on the copper-to-cell ratio in the environment (Stanley et al. 1983; Prior & Dalton 1985): a soluble MMO (sMMO) is expressed at low copper-to-cell ratios, whereas a membrane-associated MMO (pMMO) is expressed at higher ratios. sMMO has considerably higher contaminant transformation rates than pMMO (Oldenhuis et al. 1991); however, because sMMO expression is repressed by copper levels as low as 0.25 μM (Brusseau et al. 1990), methanotroph-based biodegradation is not usually optimal.

To overcome the sMMO repression problem, Phelps et al. (1992) developed a set of five stable *M. trichosporium* OB3b mutants (sMMO^{C}) that constitutively express sMMO in the presence of elevated extracellular copper (up to about 12 μM). Subsequent work on these mutants has shown that they are all defective in their ability to acquire copper (Fitch et al. 1993; DiSpirito et al. 1998), resulting in a variety of altered phenotypes, such as general insensitivity to sMMO repression by copper, elevated sMMO activities and expression levels, and consistently high specific growth rates (Phelps et al. 1992; Fitch et al.

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1993; Kim & Graham 2001). Further studies on one of the isolates, PP319, has shown that it can fix N_2 at near atmospheric levels of O_2 (Kim & Graham 2001), which is atypical of all known type II strains (Murrell & Dalton 1983; Lidstrom et al. 1985; Graham et al. 1993). The fact that PP319 can fix N_2 under high O_2 conditions makes it potentially useful for bioremediation applications because it constitutively expresses sMMO independent of Cu level and it might be promoted in bioreactors using low nitrogen-high oxygen operating conditions. However, it first must be shown that PP319 retains high degradation activities under high oxygen conditions, which was one of the goals of this study. Previous work has indicated that the WT can retain elevated TCE transformation rates during N_2 fixation (Chu & Alvarez-Cohen 1996); however, this was only seen under relatively low O_2 conditions ($<$15% saturation in air). Like other aerobic diazotrophs, even moderate dissolved O_2 levels can repress nitrogenase activity in the OB3b WT (Graham et al. 1993). Therefore, if PP319 retains high DCE transformation rates during N_2-fixation when O_2 is high, it may be practically useful.

Here we grew OB3b WT and PP319 cultures under tightly controlled high and low O_2 conditions at both high and low soluble nitrogen levels. DCE degradation assays were then performed to determine, with statistical confidence, maximum DCE transformation rates ($V_{\max}$), half-saturation constants (K_M), and apparent first-order rate constants (estimated by $V_{\max}/K_M$) for both organisms under four sets of growth conditions (noted above). DCE was chosen for study because it is a contaminant based on its industrial use, it is a breakdown product of the reductive dehalogenation of tetrachloroethylene (PCE) and TCE (Parsons et al. 1984; Bario-Lage et al. 1986), and it provides both *cis*- and *trans*-isomers to assess any stereo-chemical effects on transformation phenomena.

Materials and methods

Microbial cultures and growth conditions

M. trichosporium OB3b WT and PP319 were provided by G. Georgiou from University of Texas, Austin. Cells used for the DCE transformation assays were grown under turbidostatic (see below), continuous-culture conditions in a Bioflo 2.5 L bioreactor controlled by a Bioflo 3000 controller (New Brunswick), using 'copper-free' ($\ll$0.2 μM) nitrate salts medium (Higgins et al. 1981) altered to meet the nitrogen requirements of each experiment.

The appropriate flow rate to maintain turbidostatic conditions in each experiment was determined by initially growing the organisms in batch mode (under the given nitrogen and oxygen conditions), and then measuring the specific growth rate during early log phase. Flow rate was selected such as (1) the cell dilution rate roughly correlated with new cell growth, and (2) medium addition was adequate, relative to specific growth rate, to ensure that nutrients other than fixed nitrogen were not growth limiting. Optical density of the culture at 600 nm ($OD_{600\,nm}$) was monitored and used to regulate flow rate. The reactor was completely mixed at 300 RPM and maintained at 30 °C. Instrument-grade methane was supplied at ~15–20 mL/min. DO level was tightly regulated by a biocontroller coupled to an in-reactor Ingold (Wilmington, MA) O_2 sensor.

Experimental growth conditions

Both high and low oxygen and nitrogen conditions were employed in the experiments, where 'low' and 'high' O_2 were defined as dissolved oxygen (DO) levels of 80% (188 μM) and 10% (24 μM) saturation in air (at 30 °C), respectively, and 'high' and 'low' nitrogen were defined as 10.0 mM (nitrogenase-repression medium) and 40 μM nitrate (nitrogenase-derepression medium), respectively. These DO and nitrate levels were chosen because N_2-fixation patterns are characteristically different between the WT and PP319 under these conditions (Kim & Graham 2001).

Four independent Bioflo reactor cultures were grown for each pair of nitrogen and O_2 conditions for each organism. Typically, the Bioflo cultures were maintained at cell densities between 0.6 and 0.7 OD_{600} for at least three reactor volumes prior to performing the DCE assays, and five separate DCE assays were performed per culture. Therefore, DCE degradation kinetics for each growth condition were based on data from twenty assays – five assays per Bioflo batch with four Bioflo batches per organism. This large number of assays allowed rigorous statistical analysis of the data.

DCE transformation assays

DCE transformation assays were described previously by Kim & Graham (2001). In summary, 5.0 mL of suspended culture was aseptically withdrawn from the bioreactor and sodium formate was added to 10.0 mM

as the reductant for cell activity. This suspension was then transferred to an 8.6-mL Teflon septum-sealed glass vial, flushed with helium for 5 minutes (to purge oxygen from the vial headspace), and a water-saturated solution of DCE (Acros) was added to attain different liquid-phase DCE levels ranging from 5 to 1000 μM. Dimensionless partition coefficients of 0.31 and 0.13 for the *trans*- and *cis*-isomers (Gossett 1987), respectively, were used to account for volatilization into the headspace and in estimating aqueous DCE concentrations. The transformation reaction was initiated by adding 1.0 mL of air to establish a common initial oxygen level in each assay. The vials were then agitated on a rotary shaker table and headspace DCE levels were measured at 3-minute intervals using a Hewlett Packard 5880 gas chromatograph. Initial DCE transformation rates were estimated for each DCE level and kinetic parameters were calculated using the Michaelis–Menten model (Folsom et al. 1990; Oldenhuis et al. 1991; Smith et al. 1997). Enzfitter (Elsevier) was used to fit the data to the model. All rates were normalized to the cell dry weight (CDW) of the different assay cultures (Kim & Graham 2001).

Results and discussion

Summary of DCE transformation assays and kinetics evaluation

Michaelis–Menten kinetics and the Lineweaver-Burk double reciprocal plot method were used to estimate $V_{\max}$ and K_m for both organisms and all growth conditions, and the results are summarized in Tables 1 and 2. Typically, initial degradation rates (v_i) were plotted versus DCE concentration for each experimental condition, and classical hyperbolic curves were obtained (see Figures 1 and 2). High quality curve-fits were obtained for most conditions, although some deviation from the Michaelis–Menten model was observed, especially at higher DCE levels. The observed deviation from the model was always related to lower v_i values at higher DCE levels, suggesting DCE toxicity in the assays (similar to Fox et al. 1991; Oldenhuis et al. 1991; Chu & Alvarez-Cohen 1999), although the observed DCE toxicity was different among organisms and growth conditions (see below). For the purpose of kinetic coefficient estimation, individual assays were excluded from the analysis if the assay caused the estimated $V_{\max}$ and K_m values to change by more than 3 standard errors (Johnson 1994).

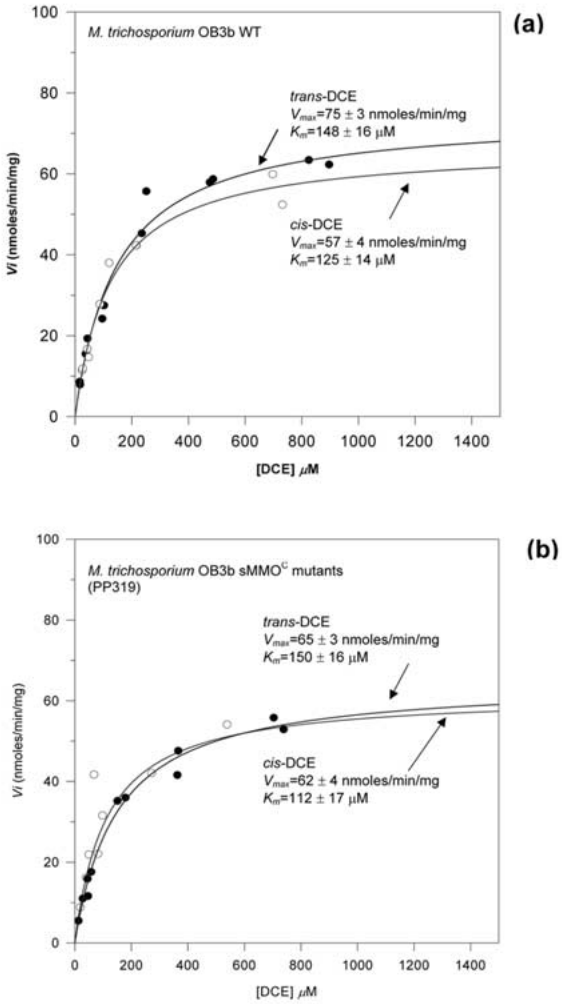

Figure 1. Typical Michaelis–Menten plots of the initial rate of disappearance of DCE from the headspace V_i vs. various *trans*- (filled circles) and *cis*-DCE (unfilled circles) concentrations for *M. trichosporium* OB3b WT (a) and sMMO^C mutants PP319 (b). These plots are for organisms grown under low nitrate conditions in the presence of low O_2 levels.

Effects of growth conditions on DCE toxicity

Apparent DCE toxicity was variable among organisms and conditions. Therefore, to quantitatively compare toxic effects, the DCE concentration at which V_i started to decline relative to increased DCE level were estimated (i.e., the toxicity thresholds, $[DCE]_t$) and are reported in Table 2. Toxicity thresholds ranged from 0.4 to above 5 μmoles DCE mg^{-1} of cell mass, depending upon the conditions. Typically, WT cultures grown under low nitrate – high O_2 conditions were most sensitive to elevated DCE, with reduced DCE

Table 1. V_{max} and K_m values for the transformation of *trans*- and *cis*-DCE with continuous-cultures of *Methylosinus trichosporium* OB3b wild type (WT) and PP319 (an sMMO^C mutant) under various nitrogen (as nitrate) and O_2 conditions

Fermentor conditions	WT				PP319			
	trans-DCE		*cis*-DCE		*trans*-DCE		*cis*-DCE	
	V_{max}[a]	K_M[b]	V_{max}[a]	K_M[b]	V_{max}[a]	K_M[b]	V_{max}[a]	K_M[b]
Low NO_3 High O_2	4.5 ± 0.2	47 ± 6	5.7 ± 1.1	31 ± 2	29 ± 3	414 ± 87	29 ± 4	397 ± 109
Low NO_3 Low O_2	75 ± 3	148 ± 16	67 ± 4	125 ± 14	65 ± 3	150 ± 16	62 ± 4	112 ± 17
High NO_3 Low O_2	57 ± 7	100 ± 3	41 ± 4	82 ± 10	67 ± 4	190 ± 24	44 ± 2	49 ± 5
High NO_3 High O_2	33 ± 4	106 ± 27	32 ± 2	72 ± 12	26 ± 1	138 ± 14	22 ± 1	50 ± 9

[a] nmoles min^{-1} mg^{-1} of CDW.
[b] μM.

Table 2. V_{max}/K_m and $[DCE]_t$ for the transformation of *trans*- and *cis*-DCE with continuous-cultures of *Methylosinus trichosporium* OB3b wild type (WT) and PP319 (an sMMO^C mutant) under various nitrogen (as nitrate) and oxygen conditions

Fermentor conditions	WT				PP319			
	trans-DCE		*cis*-DCE		*trans*-DCE		*cis*-DCE	
	V_{max}/K_M[a]	$[DCE]_t$[b]	V_{max}/K_M[a]	$[DCE]_t$[b]	V_{max}/K_M[a]	$[DCE]_t$[b]	V_{max}/K_M[a]	$[DCE]_t$[b]
Low NO_3 High O_2	96	0.4	180	0.4	70	3	73	3
Low NO_3 Low O_2	510	3.3	540	3.3	430	>5	550	>5
High NO_3 Low O_2	570	2.5	500	2.5	350	2.5	890	3.3
High NO_3 High O_2	310	2.5	440	2.5	190	3.3	440	3.3

[a] (min^{-1} mg^{-1}).
[b] $[DCE]_t$ is the DCE concentration normalized to cell dry weight (in μmoles mg^{-1} of cell) at which V_{max} was observed in the DCE transformation assays under each experimental condition. It is an approximate measure of the toxicity threshold of DCE under each condition.

transformation rates occurring at DCE levels as low as 200 μM. In contrast, all assays performed under low O_2 conditions for both organisms, and all assays with PP319 had comparatively lower DCE toxicities. PP319 was generally more tolerant to higher DCE than the WT. Further, active N_2-fixation also appeared to result in reduced DCE toxicity, which was also observed with TCE degradation (Chu & Alvarez-Cohen 1999).

Transformation kinetics of the two DCE isomers

The relationship among the estimated kinetic parameters, DCE stereochemistry, nitrogen supply, and O_2 level for the two organisms are summarized in the following sections. Kinetic parameters for the *trans*- and *cis*-DCE isomers will be presented first. Table 1 shows that V_{max} values were similar for the *cis*- and *trans*- isomers for both organisms, although some stereochemical effects were apparent when O_2 was low. This was most evident under high nitrogen-low O_2 conditions, where the *trans*-isomer V_{max} was 40 to 50% higher than the *cis*-isomer for both organisms. Half-saturation constants, K_M, were also higher for the *trans*- isomer compared with the *cis*-isomer, which is consistent with earlier results (Fox et al. 1990; Oldenhuis et al. 1991). In general, transformation patterns for the two isomers were quite similar, and therefore, the results will be pooled for our subsequent comparisons assessing the effects of O_2 conditions and N_2-fixation on DCE transformation.

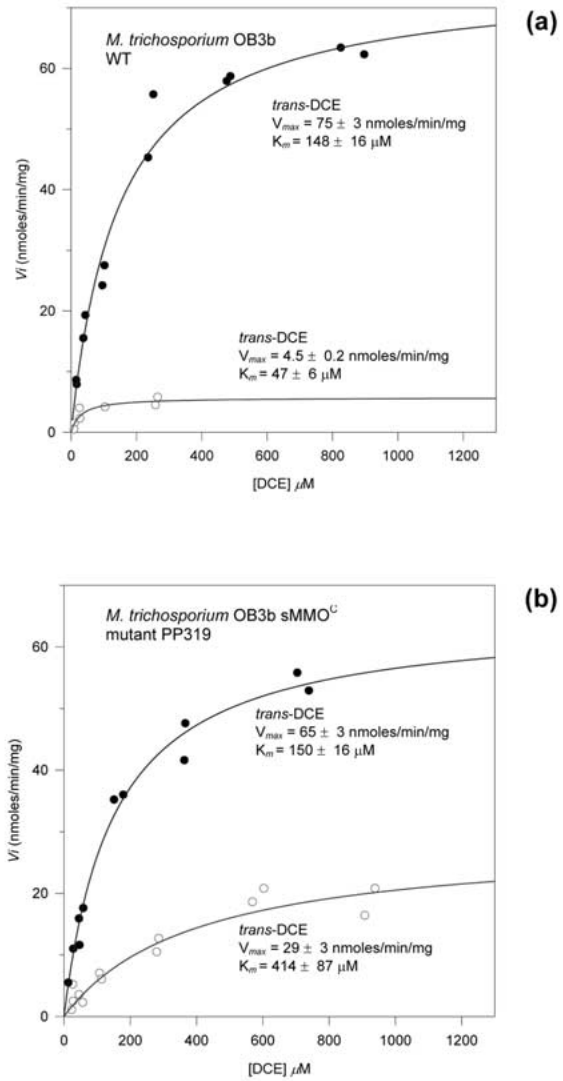

Figure 2. Typical Michaelis–Menten plots of the initial rate of disappearence of DCE from the headspace V_i vs. various *trans*-DCE concentrations for *M. trichosporium* OB3b (a) WT and (b) sMMO^{C} mutants PP319 under conditions of low nitrogen-low O_2 (filled circles) and low nitrogen-high O_2 conditions (open circles).

Effects of nitrogen conditions on $V_{\max}$ *and* $V_{\max}/K_M$

Nitrogen conditions significantly affected DCE degradation kinetics. The highest $V_{\max}$ values were consistently observed in cultures that were actively fixing N_2 under low O_2 conditions. The presence of nitrogenase and its activity was confirmed using immunoblot analysis against the *nifH* protein of nitrogenase and verified using the acetylene-reduction assay, as reported previously (Kim & Graham 2001). Repression of nitrogenase by nitrate in the WT caused $V_{\max}$ to decrease from 75 to 57 nmoles of DCE transformed min^{-1} mg^{-1} of CDW (24% decrease) and from 67 to 41 nmoles min^{-1} mg^{-1} (42%) for the *trans*- and *cis*-isomers, respectively. With PP319, $V_{\max}$ decreased from 62 to 44 nmoles min^{-1} mg^{-1} (29%) for the *cis*- isomer upon nitrate addition, although the *trans*-isomer $V_{\max}$ remained unchanged. Therefore, both WT and PP319 cells were capable of high DCE transformation rates while the cells were actively fixing N_2.

To assess DCE transformation rates when DCE levels were low (the most practical scenario), $V_{\max}/K_M$ values were calculated for the nitrogen conditions. $V_{\max}/K_M$ is the apparent first-order rate constant for DCE transformation when $[\text{DCE}] \ll K_M$. Figure 3 exemplifies DCE degradation patterns when DCE levels are low (i.e., first-order degradation), and Table 2 summarizes $V_{\max}/K_M$ values for the different growth conditions. In general, $V_{\max}/K_M$ values were quite similar between N_2-fixing and nitrate-supplied cultures for both the WT and PP319, although some differences in $V_{\max}/K_M$ were noted under different oxygen conditions (see later).

The most anomalous $V_{\max}/K_M$ estimates were observed under the low nitrogen – high O_2 condition, where the WT retained moderately high $V_{\max}/K_M$ values and PP319 had lower $V_{\max}/K_M$ values, even though the WT grew poorly under this condition and PP319 grew well. These results are surprising because the high $V_{\max}/K_M$ values in the WT corresponded with low $V_{\max}$ values, and the low $V_{\max}/K_M$ values in PP319 were associated with high $V_{\max}/K_M$ values (see Table 2). Although this result may be significant, we feel that it may be misleading. We suspect that the calculated $V_{\max}/K_M$ values under this growth condition may be distorted due to toxic effects, possibly DCE toxicity in the WT assays and O_2 toxicity in the reactor-grown PP319.

This can be explained as follows. $V_{\max}$ and especially K_M were very low in the WT under low nitrogen – high O_2 conditions, which resulted in a comparatively high $V_{\max}/K_M$. We feel, however, that both low values result from DCE toxicity; $V_{\max}$ was low due to highly impaired growth under this growth condition, and KM was low (misleading low) due to the fact that a true $V_{\max}$ could not achieved or estimated due to DCE toxicity (see Figure 2; this growth condition had a very low $[\text{DCE}]_t$ value). In contrast, Figure 2 shows that PP319 had a high $V_{\max}$ under this condition, but also a higher K_M, which resulted in a low $V_{\max}/K_M$. Although it is strictly speculation, we suspect that the

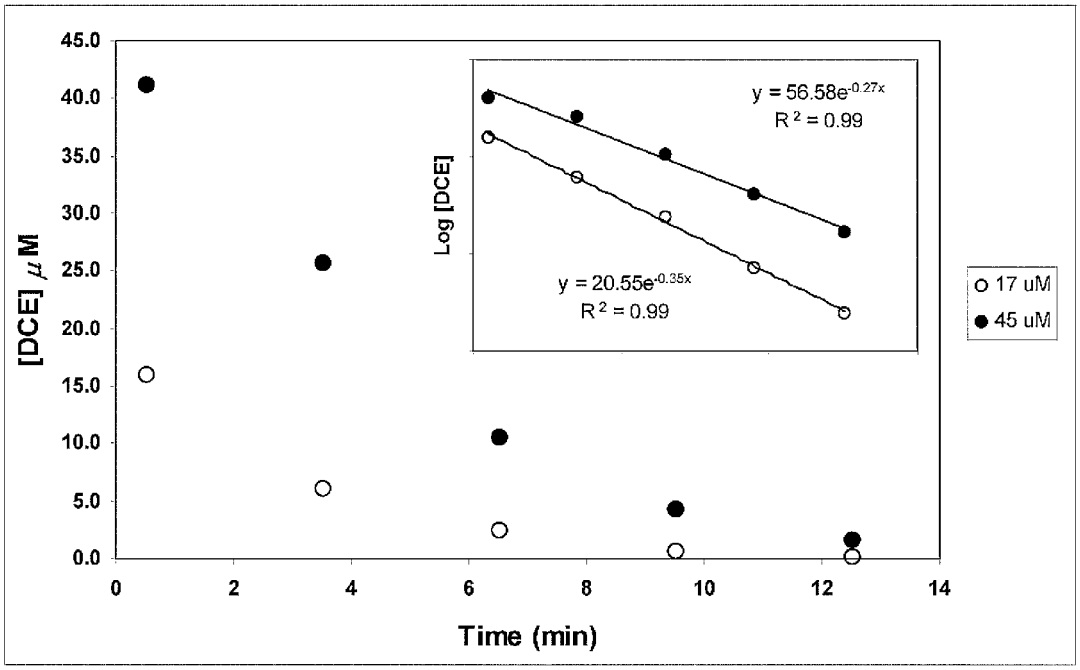

Figure 3. Typical plot of kinetic data for the transformation of *trans*-DCE when concentrations are lower than K_M for *M. trichosporium* OB3b WT grown under low nitrogen-low O_2 conditions. The inset is a transformed log plot illustrating the apparent first-order reaction kinetic when DCE concentrations are below K_M.

high K_M may be a real reflection of a reduced affinity for DCE under the low nitrogen – high O_2 condition. As will be discussed, high O_2 generally reduces DCE transformation rates and it is possible that O_2 might affect affinity, which would be most observable in PP319 during aerotolerant N_2-fixation.

Effects of O_2 level on DCE transformation rates

When WT was grown under low DO conditions and then exposed to high DO levels (with sufficient nitrogen), V_{max} decreased by 42% for the *trans*-isomer and 22% for the *cis*- isomers (see Table 1). Similar decreases were observed with PP319, where V_{max} decreased by 61% and 50%, respectively, for the two isomers. Further, the most pronounced negative effect of O_2 was seen in WT cultures that were actively fixing N_2 – where V_{max} values dropped by >90% for both isomers (see Figure 2). Observed rates also decreased in PP319 (>50%); however, the mutants still retained about six times higher V_{max} rates than the WT for both isomers. The above results clearly indicate that O_2 generally impairs V_{max}, although negative impacts vary from case to case.

V_{max}/K_M also generally decreased for both organisms and isomers when O_2 levels were elevated; i.e., the mean V_{max}/K_M seen under low O_2 conditions was 542 ± 131 min^{-1} μM^{-1} (95% confidence interval), whereas V_{max}/K_M was only 224 ± 129 min^{-1} μM^{-1} when O_2 was higher (for both isomers and organisms). Further, when one excludes the low N_2-high O_2 condition from the analysis (i.e., where WT growth was severely inhibited), one can see that the presence or absence of N_2-fixation was not as significant as previously believed to V_{max}/K_M; i.e., the mean V_{max}/K_M during N_2-fixation was 507 ± 87 min^{-1} μM^{-1}, whereas it was 461 ± 209 min^{-1} μM^{-1} under nitrogen sufficient conditions. These data suggest that DCE degradation rates may be more impacted by O_2 conditions than nitrogen conditions and that the previously observed impacts of N_2-fixation on chlorinated ethene degradation kinetics (e.g., Chu & Alvarez-Cohen 1996) may actually be more related to O_2 effects rather than due to N_2-fixation.

Effects of O_2 and nitrogen level, and DCE toxicity on K_M

Although K_M has been discussed previously, a few synoptic comments are useful. First, like with V_{max} and V_{max}/K_M, if one segregates the K_M data for both organisms and isomers between low and high O_2 con-

ditions, one finds that higher O_2 levels produce higher K_M values, although results are not statistically significant; i.e., K_M was 159 $\pm$ 157 μM DCE when O_2 is high versus 119 $\pm$ 44 μM for when O_2 is low (using pooled data). Second, generally lower K_M values are observed when soluble nitrogen was higher for both organisms and isomers; i.e., K_M was 98 $\pm$ 47 μM DCE when nitrate was high and K_M was 178 $\pm$ 147 μM when nitrate was low. These observations in combination suggest that low O_2 – high nitrogen conditions may promote lower K_M values and a higher apparent affinity between DCE and sMMO. It should be noted, however, that this conclusion is biased by data from aerotolerant N_2-fixation in PP319, a condition that appears to reduce the affinity between sMMO and DCE under high O_2 conditions (see Table 2).

Relationships among N_2-fixation, O_2 level, and DCE transformation kinetics

The physiological basis for our observations on N_2-fixation, O_2 level, and DCE transformation rate is unclear. There is some evidence that indicates that low O_2 levels, possibly linked with N_2-fixation, increase the level of poly-β-hydroxybutyrate (PHB) in *M. trichosporium* OB3b cells, which in turn provides energy reserves for the cell that increase contaminant degradation rates (Chu & Alvarez-Cohen 1999; Fitch et al. 1996). However, the specific reason why high O_2 broadly reduces DCE degradation must still be defined; i.e., does O_2 act as broad inhibitor to general cellular function, does it alter cellular resources (e.g., PHB level), or does it specifically inhibit sMMO activity? More work is required. Regardless, our data clearly suggest that O_2 is a key determinant in chlorinated ethene transformation kinetics.

Conclusions and practical observations

We conclude that sMMO^C mutants, like PP319, may have some potential as agents for biotransformation in reactors because (1) they can express sMMO at moderate to high copper levels, and (2) they retain moderate transformation and growth rates even during N_2-fixation when O_2 levels are high. Although observed V_{max}/K_M values for PP319 were not always superior to the WT (e.g., under low nitrogen-high O_2 conditions), PP319 may still be superior to the WT in bioreactor environments because of its ability for aerotolerant N_2-fixation (Kim & Graham 2001). In theory, low nitrogen-high O_2 conditions might be used to select for sMMO^C mutants in methane-enriched reactors because no other known methanotrophs can grow under such conditions.

On a more general level, we conclude that O_2 level rather than N_2-fixation may be more important to chlorinated ethene transformation rate in *M. trichosporium* OB3b. Although the expression and activity of sMMO are clearly regulated by copper conditions (Stanley et al. 1983), O_2 level also appears to affect catalytic activity of this enzyme, especially related to DCE transformation kinetics. Therefore, we suggest that O_2 level be maintained as low as possible in treatment systems that promote biotransformation by methanotrophs like *M. trichosporium* OB3b unless the operational goal is to specifically enrich for sMMO^C mutants like PP319.

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