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Methane and Trichloroethylene Oxidation by an Estuarine Methanotroph, *Methylobacter* sp. Strain BB5.1

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An estuarine methanotroph was isolated from sediment enrichments and designated *Methylobacter* sp. strain BB5.1. In cells grown on medium with added copper, oxidation of methane and trichloroethylene occurred with similar K_s values, but the V_{max} for trichloroethylene oxidation was only 0.1% of the methane oxidation V_{max}. Cells grown on low-copper medium did not oxidize trichloroethylene and showed a variable rate of methane oxidation.

Methanotrophic bacteria are of interest for bioremediation of sites contaminated with trichloroethylene (TCE) (8, 14) because they are able to oxidize TCE by the methane oxidation system, involving methane monooxygenase (MMO). Two types of MMO are known, a membrane-bound copper-containing form, called the particulate MMO (pMMO), and a cytoplasmic iron-containing form, called the soluble MMO (sMMO) (8, 14, 15, 18, 21). All known methanotrophs contain the pMMO, while only a few strains, mainly *Methylosinus* and *Methylococcus* species, contain the sMMO as a second enzyme (8). In strains containing both enzymes, the sMMO is produced only under conditions of copper limitation, when pMMO is not expressed (21). However, it has been shown that a methanotroph containing only pMMO also responds to copper limitation, with decreased growth rates and methane oxidation rates (3). While the sMMO shows a high rate of TCE oxidation, the whole-cell half-saturation constant (K_s) is also relatively high, 145 μM (14). The pMMO has been reported to oxidize TCE, but at rates on the order of 0.1 to 0.2% of the sMMO rates (5), and in some cases TCE oxidation has not been detected in cells expressing only pMMO (22). In marine environments, sMMO-containing methanotrophs have not been detected, either by pure culture isolation or by gene probing (10–12, 20). Therefore, consumption of TCE by in situ populations of methanotrophs in TCE-contaminated marine environments may be restricted to pMMO-mediated cooxidation. Since very little is known about TCE consumption by marine methanotrophs, we have isolated an estuarine marine methanotroph and studied its methane and TCE oxidation kinetics.

Samples of the brown surface layer of estuary sediment were collected at low tide from the Newport Bay Estuary, Calif. Enrichments were prepared by diluting the sediment 10-fold with NMSS medium (nitrate mineral salts medium [24] to which 1.5% [wt/vol] NaCl was added) and incubating in sealed serum vials with 20% (vol/vol) methane (reagent grade; Matheson Gas Co.) at room temperature with shaking. After 12 days, dilutions of the enrichments were spread on NMSS plates containing 10 μM Cu (added after autoclaving as a sterile 0.1 M solution of $CuSO_4 \cdot 5H_2O$) and nystatin (100 mg/liter; Sigma Chemical Co.), and these were incubated with 20% methane. All colonies that showed methane-dependent growth were purified by repeated single-colony isolation under the same growth conditions.

One representative strain, designated BB5.1, was chosen for further characterization. Strain BB5.1 was a motile rod-shaped bacterium that grew best at 30°C, with a doubling time of 3.2 to 3.7 h, and did not grow in the absence of added NaCl. Genomic DNA was isolated from this culture as described previously (19). A DNA fragment encoding the 16S rRNA was amplified from the genomic DNA by PCR using universal primers (23), as described by Holmes and coworkers (10), and sequenced by the Center for AIDS Research DNA Sequencing Facility at the University of Washington using an Applied Biosystems automated sequencer. This sequence was submitted to the Ribosomal Database Project (13) for analysis, and the closest sequences by similarity rank (S_ab = 0.857 to 0.861) were all *Methylobacter* species. Therefore, this strain has been designated *Methylobacter* sp. strain BB5.1. *Methylobacter* strains are found in the γ-proteobacterial group, and so far none of these have been found to contain sMMO (2). To demonstrate the presence of pMMO genes, primers targeted to the 5′ and 3′ ends of the *pmoA* gene encoding one of the pMMO subunits (primer A189GC from reference 9 and primer mb661 [5′ CC GGMCAACGTCYTTACC 3′]) were used to obtain a DNA fragment by PCR. This fragment was sequenced, and the translated sequence showed high identity (70 to 95%) to other *pmoA* genes.

Nucleotide sequence accession numbers. The GenBank accession numbers for these sequences are AF016981 (16S rDNA) and AF016983 (*pmoA*).

Copper limitation is known to affect methane oxidation by methanotrophs containing only pMMO (3). However, it is not known whether these effects are specific to copper or are a result of growth limitation, and the effect of altered copper on cometabolism reactions by the pMMO is also unknown. Therefore, the whole-cell kinetics of methane and TCE oxidation were studied in *Methylobacter* sp. strain BB5.1 grown at two different medium copper concentrations, an amount higher than normal (25 μM added) and a low concentration (no copper added). The background copper present in the medium with none added was 3 μM, and the copper present in the medium with copper added was 28 μM, as determined by

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TABLE 1. Kinetics of methane and TCE consumption by cells of *Methylobacter* sp. strain BB5.1 grown with different copper levels

Substrate oxidized	Initial copper concn (μM)	K_s[a]	V_{max}[b]
Methane	28	13	6.6
Methane	28	10	5.4
TCE	28	8	6.1
TCE	28	10	9.0
Methane	3	81	6.1
Methane	3	16	1.7
TCE	3		ND[c]
TCE	3		ND

[a] Micromolar dissolved CH_4 or TCE.
[b] Micromoles of CH_4 or TCE hour^{-1} microgram of total protein^{-1}.
[c] ND, not detectable.

inductively coupled plasma-mass spectrometry analysis on a Perkin-Elmer Sciex Elan 5000 as described previously (1). The amount of copper used in both cases was neither growth limiting nor toxic (data not shown).

Methane consumption rates were determined by measuring the conversion of $^{14}CH_4$ into nonvolatile products over time. For these experiments, late-log-phase cultures of *Methylobacter* sp. strain BB5.1 were evacuated to remove residual methane and were diluted in NMSS to an optical density at 600 nm (OD_{600}) of ≤0.05. This cell concentration was chosen based on mass transfer experiments. In these experiments, methane oxidation rates were measured for a series of cell concentrations. A linear response was obtained up to an OD_{600} of 0.05, but rates leveled off at higher cell densities. These results suggest that with this incubation system, at an OD_{600} at or below 0.05 methane oxidation rates were limited by bacterial consumption rather than by the rate of mass transfer of methane into the liquid phase (4, 16). Aliquots (0.5 ml) of diluted culture were dispensed into serum vials, which were sealed with stoppers and crimp seals. Methane was added by gas-tight syringe to give a range of dissolved methane concentrations. Dissolved concentrations were estimated by using partition coefficients calculated from the model of Duan et al. (6) for the NMSS medium. $^{14}CH_4$ (synthetic; specific activity = 55 mCi/mmol; DuPont NEN) was added to maintain a constant ratio of $^{14}CH_4$ to $^{12}CH_4$ in each vial. The vials were shaken longitudinally at 250 rpm at room temperature. At four intervals over a 3.5-h period, NaOH was added (1 M final concentration) to a triplicate set of vials for each methane concentration. A duplicate set of killed controls, in which the NaOH was added before the methane, was also included. The vials were flushed with air for 5 min, and then the liquid was subsampled for scintillation counting. Methane consumption rates were calculated by linear regression of the scintillation counter time course data converted to moles of methane used and corrected for counts in the killed controls. Protein was determined by the DC Protein Assay (Bio-Rad), performed according to the manufacturer's instructions. The data were fitted to the Michaelis-Menten model by nonlinear regression (TableCurve 3D v.2; Jandel Scientific) to estimate the values of the uptake velocity at substrate saturation (V_{max}) and the whole-cell half-saturation constant (K_s).

TCE oxidation kinetics experiments were performed for cultures grown under the same conditions used for methane oxidation kinetics, except the cultures were used without dilution (OD_{600} = 0.8 to 1.15). Experiments using cultures with an OD_{600} in the range of 0.1 to 1.2 showed roughly linear relationships between TCE oxidation rates and OD_{600}s, which agreed with calculations (4) suggesting that the mass transfer of TCE was not limiting under these conditions. The cultures were dispensed in 1-ml aliquots into headspace sampler vials (Hewlett-Packard), to which TCE was added as a TCE-saturated water solution. Liquid-phase concentrations were calculated according to Henry's law, with a dimensionless partition coefficient of 0.36 (7). The vials were sealed with Teflon-lined septa and aluminum crimp seals immediately after the addition of TCE and shaken longitudinally at 250 rpm at room temperature. Replicate time points were taken as for the methane experiments. TCE oxidation was determined by the disappearance of TCE over time in the gas phase of the vials with a Hewlett-Packard 7694 Headspace Autosampler coupled to a Hewlett Packard (5890 Series II Plus) gas chromatograph equipped with an Electron Capture Detector. Samples were run through a 30-m column (HP 624) with a 0.32-mm inner diameter and a film thickness of 1.8 μm, running at 95°C, with He as the carrier gas. Injector and detector temperatures were 200 and 300°C, respectively. TCE concentrations were determined from the peak areas of the chromatograms by comparing them to a standard curve. Velocities of TCE oxidation were calculated by linear regression of the TCE disappearance time course data, corrected for a small amount of loss in the controls (<5%), and converted to moles of TCE oxidized. The data were fitted to the Michaelis-Menten model as described for the methane consumption experiments.

For cells grown with 28 μM total initial copper, the whole-

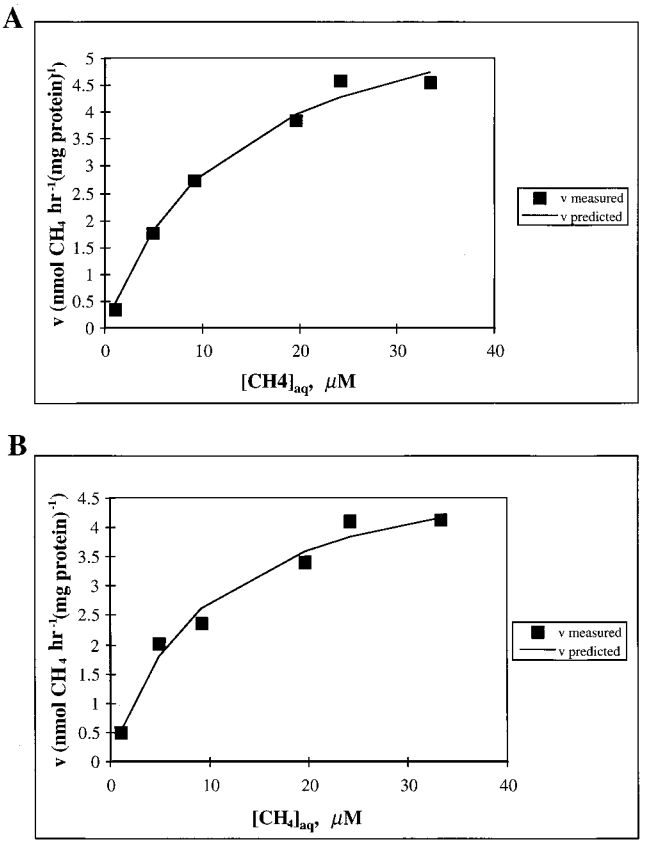

FIG. 1. Kinetics of methane oxidation (v versus concentration) for *Methylobacter* sp. strain BB5.1 grown with 28 μM initial copper in the medium. (A) Experiment 1; (B) experiment 2. The solid lines show the fitted Michaelis-Menten curves. $[CH_4]_{aq}$, concentration of aqueous methane.

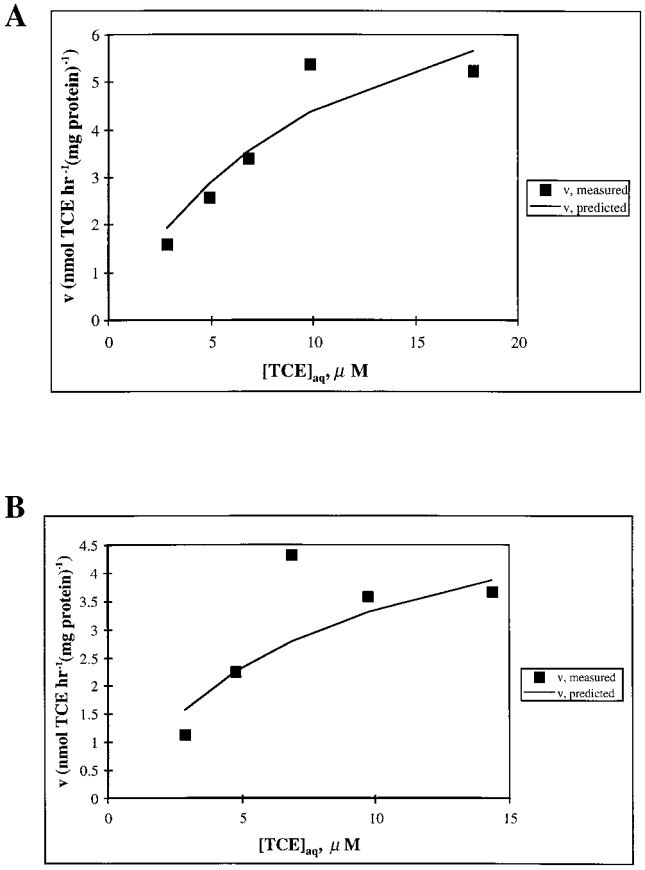

FIG. 2. Kinetics of TCE oxidation (v versus concentration) for *Methylobacter* sp. strain BB5.1 grown with 28 μM copper in the medium. (A) Experiment 1; (B) experiment 2. The solid lines show the fitted Michaelis-Menten curves. $[TCE]_{aq}$, concentration of aqueous TCE.

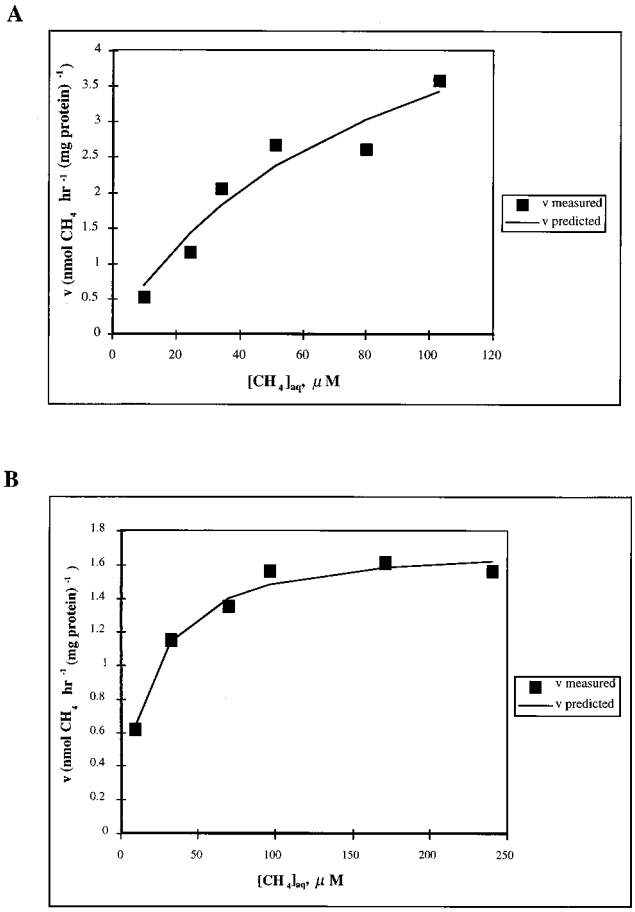

FIG. 3. Kinetics of methane oxidation (v versus concentration) for *Methylobacter* sp. strain BB5.1 grown with 3 μM initial copper in the medium. (A) Experiment 1; (B) experiment 2. The solid lines show the fitted Michaelis-Menten curves. $[CH_4]_{aq}$, concentration of aqueous methane.

cell kinetics of methane oxidation were similar to those reported for methanotrophs expressing pMMO (Table 1 and Fig. 1) (17), suggesting that the pMMO system was the dominant methane oxidation system present. TCE was oxidized at low rates (6 to 9 nmol h^{-1} mg of $protein^{-1}$), corresponding to approximately 0.1% of the methane oxidation rates (Table 1 and Fig. 2). These rates fall within the range reported for other methanotrophs expressing pMMO (3 to 41 nmol h^{-1} mg of $protein^{-1}$), corresponding to 0.1 to 0.3% of the whole-cell methane oxidation rate (5). These cells were clearly not expressing sMMO, as the rate of TCE oxidation by sMMO-expressing cells is similar to the rate of methane oxidation (14). K_s values for TCE oxidation by pMMO have not been previously reported. The values found here (8 to 10 μM) were much lower than those reported for cells expressing sMMO (145 μM [14]) and were similar to the K_s values found for methane oxidation. TCE oxidation was linear over the 4-h period tested, suggesting that TCE was not toxic at the low concentrations used.

Cultures grown with 3 μM total initial copper showed more variable methane oxidation kinetics, with both K_s and V_{max} values varying by factors of 5 from each other and from the results for cells grown with 28 μM copper (Table 1 and Fig. 3). The reasons for this variability are unknown, since the replicate experiments were carried out in the same manner. It is possible that small changes in growth conditions resulted in these effects. However, none of the cultures grown with 3 μM copper exhibited detectable TCE oxidation (Table 1). This result may explain why in some previous studies, TCE oxidation was not detectable by methanotrophs producing pMMO (22).

It has been known for some time that copper concentration is an important variable for TCE oxidation by methanotrophs, as it controls the relative expression of sMMO and pMMO. However, our results suggest that copper concentration is also an important variable for TCE oxidation by methanotrophs that contain only pMMO. In the marine environment, this is an important distinction, since so far no sMMO-containing strains have been detected.

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