Microb Ecol (1993) 25:1–17

MICROBIAL ECOLOGY

Factors Affecting Competition Between Type I and Type II Methanotrophs in Two-organism, Continuous-flow Reactors

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Received: March 12, 1992; Revised: August 10, 1992

Abstract. Competition experiments were performed in a continuous-flow reactor using *Methylosinus trichosporium* OB3b, a type II methanotroph, and *Methylomonas albus* BG8, a type I methanotroph. The experiments were designed to establish conditions under which type II methanotrophs, which have significant cometabolic potential, prevail over type I strains. The primary determinants of species selection were dissolved methane, copper, and nitrate concentrations. Dissolved oxygen and methanol concentrations played secondary roles. *M. trichosporium* OB3b proved dominant under copper and nitrate-limited conditions. The ratio of *M. trichosporium* to *M. albus* in the reactor increased ten-fold in less than 100 hours following the removal of copper from the reactor feed. Numbers of *M. albus* declined to levels that were below detection limits ($<10^6$/ml) under nitrogen-limited conditions. In the latter experiment, the competitive success of *M. trichosporium* depended on the maintenance of an ambient dissolved oxygen level below about 7.5×10^{-5} M, or 30% of saturation with air. The ability of *M. trichosporium* to express soluble methane monooxygenase under copper limitation and nitrogenase under nitrate limitation was very significant. *M. albus* predominated under methane-limited conditions, especially when low levels of methanol were simultaneously added with methane to the reactor. The results imply that nitrogen limitation can be used to select for type II strains such as *M. trichosporium* OB3b.

Introduction

Methanotrophic bacteria play a critical role in the global cycling of carbon and in limiting the flux of methane, a greenhouse gas, to the atmosphere [1, 3]. In addition, these organisms are potentially useful in hazardous waste treatment and

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other biotechnical applications [17, 36]. The utility of methanotrophs for technical purposes centers around the initial enzyme in their catabolic pathway, methane monooxygenase (MMO). MMO catalyzes the hydroxylation of n-alkanes, the epoxidation of n-alkenes, and a variety of other cometabolic transformations involving halogenated organic compounds [2, 9, 23, 33].

Methanotrophs are divided into two general classes (type I and type II) based primarily on the structure of their internal membranes [16, 35]. Both classes can express a particulate or membrane-associated MMO (pMMO, 12]). Certain type II strains, however, can also express a soluble MMO (sMMO) that catalyzes the transformation of an extremely broad range of environmentally significant substrates [4, 23, 32]. Type I and type II methanotrophs differ in several other significant respects. Type I organisms typically are unable to fix molecular nitrogen, usually lack a complete tricarboxylic acid (TCA) cycle, utilize the ribulose monophosphate (RuMP) pathway for formaldehyde assimilation, and require copper for growth. Copper is believed to be requisite to pMMO activity [10]. Type II methanotrophs typically can fix nitrogen, possess a complete TCA cycle, use the serine pathway for formaldehyde assimilation, and can express sMMO in the absence of copper [8, 22]. A few methanotrophs, type X, possess a mixture of type I and type II traits [34]. Type X strains, such as *Methylococcus capsulatus*, normally grow at temperatures above 37°C.

The distribution of methanotrophs in aquatic and terrestrial settings has been studied by a number of groups [14, 18, 19, 25, 27]. Methanotroph numbers vary spatially and seasonally. For example, methane oxidation was observed in the metalimnion of a low-dissolved-nitrogen lake in Canada during summer stratification [26, 28]. However, high rates of methane consumption occurred only where oxygen levels were low. Harrits and Hanson [14] also observed that primary methane oxidation in lakes was seasonal and occurred primarily in the metalimnion near the anoxic hypolimnion. Heyer and Suchow [15] found that methane produced by peat bogs was almost completely mineralized in the near surface soils, even at pHs as low as 3.8. They isolated both *Methylosinus sporium* and *Methylosinus trichosporium* from the low-pH zones.

Little work has been aimed at factors that select for type I versus type II strains. Putzer et al. [24] found predominantly type I organisms in high-methane, low-oxygen river water using a Percoll gradient technique for separation by type. Reed and Dugan [25] observed that *Methylomonas methanica*, a type I strain, was present at all water depths in Cleveland Harbor, although the organism was concentrated near the water–sediment interface. In the same study *M. trichosporium*, a type II organism, was found only within 1 meter of the sediment surface. In work performed here, *Methylosinus*-like type II strains were not isolated from surface soils from a methane-producing sanitary landfill despite extensive effort [11]. However, seeded *M. trichosporium* OB3b prevailed over native type I strains in non-sterile, unsaturated columns produced from the same soils. Also, samples collected from the liquid–sediment interface in a nitrogen-limited treatment pond for pulp mill wastes generated predominantly type II strains. From these examples, general patterns of type I and type II selection are discernible. Type II methanotrophs appear to be selected in nitrogen-limited and perhaps low-pH zones. Type I strains appear to be present in almost all methane-enriched locations when other nutrients, such as dissolved nitrogen, are available.

Here we examine competition between type I and type II methanotrophs in continuous-flow systems in order to further define factors leading to type selection. Frederickson and Stephanopolis [10] indicated that when such experiments involve metabolically similar organisms, the faster-growing organism should, in principle, exclude the less competitive strain. The focus of the work was to delineate methanotroph selection patterns that may be used to encourage type II organisms in mixed cultures. The methanotrophs chosen for the experiments were *Methylosinus trichosporium* OB3b, a type II strain, and *Methylomonas albus* BG8, a type I organism. *M. trichosporium* OB3b is well characterized and potentially relevant to hazardous waste treatment [9, 23, 33]. *M. albus* BG8 was selected because of its high growth rate and because it possesses traits common among type I strains [6, 12]. The primary determinants of species selection (independent variables) were concentrations of methane, copper, and nitrogen. The roles of oxygen and a supplementary carbon source (methanol) were also studied.

Materials and Methods

Experimental Procedures

Bacterial Strains. *Methylosinus trichosporium* OB3b, ATCC #35070, was purchased from the American Type Culture Collection (Rockville, MD). *Methylomonas albus* BG8 was obtained from R. Whittenbury at the University of Warwick, U.K.

Growth Media. Unless otherwise noted, the growth medium employed was nitrate-reduced NSM [7]. Components were as follows: $NaNO_3$, 1.0 mM; K_2SO_4, 1.0 mM; $MgSO_4 \cdot 7H_2O$, 0.15 mM; $CaCl_2 \cdot 2H_2O$, 47.6 μM; KH_2PO_4, 3.9 mM; Na_2HPO_4, 6.0 mM; $ZnSO_4 \cdot 7H_2O$, 2.0 μM; $MnSO_4 \cdot 7H_2O$, 1.6 μM; H_3BO_3, 6.0 μM; $NaMoO_4 \cdot 2H_2O$, 0.4 μM; $CoCl_2 \cdot 6H_2O$, 0.4 μM; KI, 1.0 μM; $FeSO_4 \cdot 5H_2O$, 40 μM. Media were sterilized by autoclaving for 20–60 min at 121°C. Following sterilization, iron and copper were added from filter-sterilized stock solutions (10 mM $CuSO_4$; 40 mM $FeSO_4$). For nitrogen-limited cultures, target nitrogen levels were established via the addition of $NaNO_3$ (1.0 M $NaNO_3$ stock solution) to previously prepared, nitrogen-free media.

Batch Cultures. Pure culture batch experiments were performed with either 165-ml serum vials or 1-liter Erlenmeyer flasks. The serum vials were crimp sealed, with teflon-coated rubber septa (The West Co.). Methane was fed to the vials using sterile 20-ml syringes (Becton Dickerson Co.). The Erlenmeyer flasks were sealed with specially prepared rubber stoppers equipped with two bored entrance ports. These ports were fitted with glass tubes that were capped at one end and open at the other. One glass tube was sealed with a teflon septum to permit syringe access for sample collection. The other was sealed with a plug that could be removed for the exchange of gases. Methane was added by establishing a partial vacuum in the flask and backfilling with 99.0% purity methane (Union Carbide) to the desired methane concentration. Methane was maintained between 15% and 20% of the headspace volume. Liquid volumes were less than 25% of the total flask volume. All glass-

ware was acid washed with 10% H_2SO_4 prior to use. Agitation was provided using a rotary shaker table at 240 rpm (New Brunswick Co.). All experiments were performed at 30°C.

The purpose of the pure culture experiments was to determine appropriate levels of copper and nitrate for the subsequent competition experiments. Cultures of either *M. trichosporium* OB3b or *M. albus* BG8 were pre-grown in the 1000-ml flasks in "copper-free" (typically <0.15 μM) or "nitrate-free" (no nitrate amendment) media to an absorbance of between 0.02 and 0.06 (cm^{-1}; λ = 600 nm; A_{600}). Twenty-milliliter aliquots were then transferred to 165-ml serum vials, and copper or nitrate was added from stock solutions to target concentrations. Methane was added by syringe and growth was monitored spectrophotometrically.

To ensure that reaction kinetics were biologically controlled during batch and competition experiments, methane uptake rates were measured or calculated in both the batch and continuous-flow reactors at various levels of agitation. For the shaker table experiments, *M. albus* BG8 was grown in 1-liter flasks at agitation rates ranging from 130 rpm to 340 rpm. At each speed, growth rate and headspace methane were measured periodically. From these data, methane uptake rates were estimated and compared with theoretically established mass transfer limitations [30]. Similar experiments were conducted in the continuous-flow reactor (see below). Both *M. albus* BG8 and *M. trichosporium* OB3b were used in experiments in which agitation rates ranged from 200 rpm to 600 rpm.

Competition Experiments. These experiments were performed utilizing a Biostat MD, manufactured by B. Braun Biotech, Inc., operated as a continuously stirred tank reactor. The Biostat is capable of simultaneous measurement and control of liquid-phase pH, dissolved oxygen, temperature, and mixing rate. Feed rates for liquid media and methane were maintained by separate flow controllers. Liquid volume was maintained at 2 liters. The steady level of dissolved oxygen was established via manipulation of air flow rate. Methane flow rates were, in general, held constant at each operating condition. Reactor dilution rates were varied between 0.025 h^{-1} and 0.05 h^{-1}. Methane concentrations were measured periodically in the inlet and outlet gas streams and in the reactor contents.

Experiments were initiated by growing 200-ml batch cultures of both *M. trichosporium* OB3b and *M. albus* BG8. During mid-log phase (A_{600} = 0.2 to 0.5, cm^{-1}), 50–150 ml of each culture was transferred to inoculate the Biostat MD. Inoculum size was varied to provide the desired (initial) cell ratios. The Biostat was operated as a batch reactor until cell numbers increased to target levels, then it was switched to continuous-flow mode. For each experiment, the initial reactor conditions were established so as to avoid bias toward either organism. Independent variables were then altered individually over a preestablished range, and culture response was monitored in terms of relative cell numbers of each organism. Typically at least five or six reactor volumes were exchanged between environmental alterations. Dependent variables were measured every 4–12 h.

Analytical Procedures

Culture Enumeration. Absorbance at 600 nm (A_{600}, cm^{-1}; Shimadzu UV-160a recording spectrophotometer) was used to monitor growth in the pure culture

experiments. A_{600} was also used as an approximate measure of total cell density during the competition experiments.

In mixed cultures, individual strains were counted using either fluorescent antibody (FA) probes [33] or acridine orange direct counts (AODC [29]). Fluorescein-labelled antibody probes for *M. trichosporium* OB3b and *M. albus* BG8 were developed at the University of Minnesota and provided by Dr. T.C. Tsien for use in this project. The probes were tested for cross-reactivity and performed satisfactorily.

The basic FA procedure was as follows. One-milliliter samples were collected and diluted to an A_{600} of 0.2. Two 10–15-μl volumes of the diluted sample were then spread over two 1-cm^2 areas on a glass slide that had previously been washed with 70% alcohol. Transferred cells were heat fixed, and 10 μl of the respective antibody solutions was placed on each smear. After applying cover slips, the slide containing the smears was transferred to a Tupperware box, kept humid with moist tissues, and incubated in the dark for 90 min at 30°C. The cover slips were then removed and the slide was washed three times in phosphate buffered saline solution (pH 7.5). The slide was air dried in the dark at room temperature. A drop of mountant (0.01 M phosphate buffer, 0.15 M NaCl, 0.1% w/v *p*-phenylenediamine (Sigma), 90% v/v glycerol, pH 8.0) was placed on the treated smears, the cover slips were replaced, and cells were counted under oil immersion with fluorescence microscopy (1000×; Olympus BH-2 equipped with a Mercury-100 Lamp). Fifteen to 20 fields (0.01 mm^2 in area) were counted per smear. The arithmetic average was used to calculate cell density.

Acridine orange direct counts were also used to determine cell densities in the mixed cultures. With standard procedures, *M. trichosporium* OB3b fluoresces bright green, whereas *M. albus* BG8 fluoresces orange. Given the dramatic difference in color and the fact that the two organisms are morphologically distinct, it was possible to accurately enumerate the two populations with AODC alone. AODC estimates were based on duplicate samples. Throughout the competition experiments, parallel counts were used to correlate values obtained via AODC and FA methods. The two methods were always in agreement within 20%.

The following correlations were used to relate cell count, cell dry weight (CDW), and absorbance data in pure cultures:

M. trichosporium OB3b: $A_{600} = 1.0\ cm^{-1} = 13.5 \times 10^7$ cells/ml = 430 mg CDW/L

M. albus BG8: $A_{600} = 1.0\ cm^{-1} = 15.8 \times 10^7$ cells/ml = 330 mg CDW/L

Enzyme Assays. sMMO activity assays were derived from Oldenhuis et al. [23]. The assay is based on the conversion of cyclohexane to cyclohexanol in an assay buffer. Samples of 2–20-ml (depending on the culture density) were centrifuged at 6000 *g* for 10 min (Beckman J2-21 Centrifuge). The resulting pellet was resuspended in 2 ml sterile formate buffer (10 mM formic acid; 5 mM $MgSO_4$; 5 mM K_2HPO_4; pH adjusted to 7.0) to an absorbance of between 0.4 and 0.6 cm^{-1}. One ml of the suspension was used to measure cell density, and the other was transferred to a 6.5-ml serum vial equipped with a teflon-lined septum cap. Cyclohexane (1.3 μl) (Sigma) was injected into each vial, and cyclohexanol formation was monitored over time by gas chromatography. Enzyme activity was defined as cyclohexanol formed per minute per culture CDW. All assays were performed at 30°C with mild agitation.

The acetylene-ethylene reduction assay was used to measure nitrogenase activity [13]. Modification was necessary since acetylene is a strong inhibitor of methane monooxygenase [2, 16, 22]. One-milliliter culture samples were transferred directly to a 6.5-ml serum vial equipped with a teflon-lined septum cap. The headspace of the serum vial was purged with pure nitrogen gas (>99.8%; Oxygen Co. Ltd.) for 1 min to remove both oxygen and methane from the vial. Air (0.5 ml) was injected into the purged vial to provide a trace of oxygen. Under anoxic conditions, nitrogenase activity in *M. trichosporium* OB3b was observed to be short-lived. The assay was initiated by adding 0.5 ml of acetylene gas (Liquid Air) to the assay vial. Nitrogenase activity was measured as ethylene formation over time using gas chromatography.

Copper Analysis. Copper was measured using a Perkin-Elmer 303 Atomic Absorption Spectrometer with a HGA-400 graphite furnace and a single-element copper lamp. Copper standards were prepared from oven-dried $Cu(NO_3)_{2(s)}$ dissolved in water with 0.1% HNO_3.

Total, soluble, and particulate copper were assessed as follows. Total copper was determined by direct injection of the samples. Soluble copper was measured as the sample centrate after centrifugation at 8000 *g* for 10 min. Particulate copper was assumed to be the difference between the total copper and soluble copper measurements.

Gas Chromatography (GC). All GC analyses (methane, methanol, enzyme activity assays) were conducted using a Hewlett-Packard HP5890A gas chromatograph equipped with a flame ionization detector. A 30 m × 0.53 mm ID capillary column (GSQ, J&W Scientific Ltd.) was used for the measurement of ethylene and gas-phase methane. Injection volume was 200 μl. Ethylene standards were purchased from Aldrich Chemical Co. Methane standards were prepared in serum vials using pure methane gas in air.

For the analysis of liquid-phase methane concentration, 1-ml liquid samples were transferred into 6.5-ml septum-capped vials using methods designed to minimize methane loss. Vial and sample sat idle (without agitation) for 5 min until gas–liquid methane concentrations approached equilibrium. Headspace methane levels were then analyzed in triplicate. The liquid-phase methane concentration was calculated using Henry's law and a mass balance. Low-level methane standards were purchased from Aldrich Chemical Co.

Analyses for methanol, formaldehyde, formate and cyclohexanol were performed using a 30 m × 0.53 ID column (DB-Wax, J&W Scientific Ltd.). Aqueous samples (1.5 μl) were injected directly onto the column. Individual peaks were identified via comparison to authentic standards.

Results and Discussion

Pure Culture/Batch Reactor Experiments

Mixing rate experiments were conducted in the Biostat MD and in flasks on the shaker table. The results of the experiments using *M. albus* BG8 are summarized in

Table 1. Specific growth rate of *Methylomonas albus* BG8 at different mixing rates[1]

Mixing Rate (RPMs)	Shaker Table	Biostat
130	0.099[2]	NT[3]
200	0.144	0.138
240	0.173	0.168
300	0.182	0.182
340	NG[4]	NT
400	NT	0.164
500	NT	0.038
600	NT	NG

[1] Low-nitrate NSM media (7) with copper supplement to 2.0 μM Cu(II).
[2] Specific growth rate in hours^{-1}
[3] NT - Not tested
[4] NG - No growth

Table 1. In the Biostat experiments, growth rates of *M. albus* BG8 were relatively insensitive to agitation rate at speeds between 240 rpm and 400 rpm. At mixing rates below 240 rpm, growth was probably mass-transfer limited (methane or oxygen); at 500 rpm and above, growth may have been inhibited by mechanical shear or by oxygen toxicity resulting from enhanced oxygen transfer. An agitation speed of 300 rpm was adopted for all chemostat competition experiments.

The shaker table experiments showed a similar growth–mixing rate relationship. Optimum growth rate was observed at a mixing rate of 300 rpm (Table 1), although growth was found to be very erratic at that mixing rate. As a result, a mixing rate of 240 rpm was chosen for experiments utilizing the shaker table apparatus.

M. albus BG8 was incapable of growth in "copper-free" medium (as previously defined). When supplemental copper was provided, the specific growth rate of *M. albus* was not influenced by copper concentration in the range $0.5\ \mu M < [Cu(II)] < 5.0\ \mu M$. The maximum specific growth rate for *M. albus* BG8 was 0.182 h^{-1} (minimum generation time, 3.8 h; Table 1). At low cell densities ($A_{600} < 0.1$, cm^{-1}), *M. albus* BG8 was relatively large and oval under phase-contrast microscopy. At higher densities, the average cell size decreased and organisms became more spherical and more opaque. The change in morphology did not appear to affect the specific growth rate as estimated spectrophotometrically.

M. trichosporium OB3b grew in both "copper-free" and "copper-supplemented" media ($[Cu(II)] \leq 5.0\ \mu M$). In "copper-free" medium, its maximum specific growth rate was 0.097 h^{-1} (generation time equal to 7.1 h). In "copper-supplemented" medium, the maximum specific growth rate was 30% higher ($k = 0.126\ h^{-1}$; generation time, 5.5 h).

Nitrate-limitation experiments are summarized in Figs. 1 and 2. Since *M. albus* BG8 cannot fix nitrogen, growth ceased after nitrate exhaustion. Cell yield was estimated at 24.3 mg CDW/mg N-nitrate. *M. trichosporium* OB3b was also affected by nitrate exhaustion. Maximum growth rates were observed in the presence of nitrate (specific growth rates were between 0.06 h^{-1} and 0.097 h^{-1}). The cell yield prior to nitrogen exhaustion was 26.0 mg CDW/ mg N-nitrate for *M. trichos-*

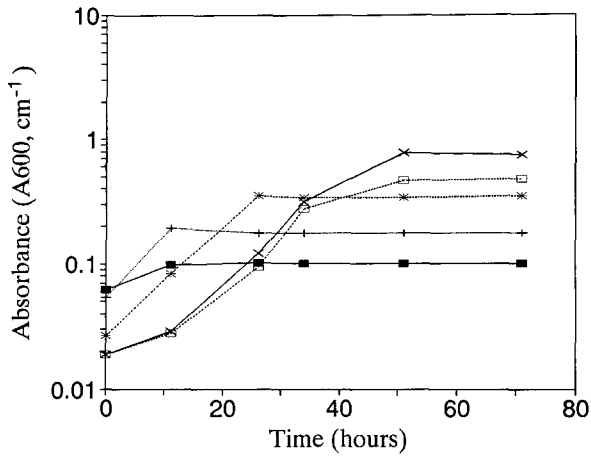

Fig. 1. Batch growth of *Methylomonas albus* BG8 as a function of initial nitrate concentration. ■, 0.0 mM nitrate; +, 0.1 mM nitrate; *, 0.3 mM nitrate; □, 0.5 mM nitrate; and X, 1.0 mM nitrate. Cultures pre-grown in "nitrate-free" media and then supplemented with $NaNO_3$ to specified concentrations.

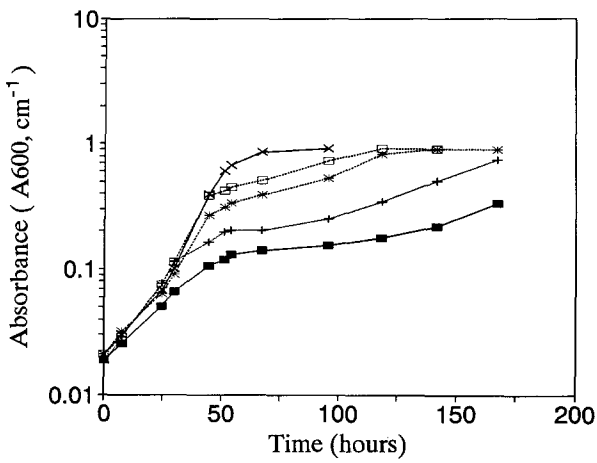

Fig. 2. Batch growth of *Methylosinus trichosporium* OB3b as a function of initial nitrate concentration. ■, 0.0 mM nitrate; +, 0.1 mM nitrate; *, 0.3 mM nitrate, □, 0.5 mM nitrate; and X, 1.0 mM nitrate. Cultures pre-grown in "nitrate-free" media and then supplemented with $NaNO_3$ to specified concentrations.

porium OB3b. Nitrogenase activity was observed in *M. trichosporium* cultures only after nitrate exhaustion. At that point, nitrogenase activity apparently restricted the specific growth rate of *M. trichosporium* ($k < 0.023\ h^{-1}$). Chen and Yoch [5] indicated that the nitrogenase in *M. trichosporium* OB3b is linked through ferredoxin to formate dehydrogenase (as a source of reducing power), and as a consequence, there is a potentially significant bleed on the energy supply when nitrogenase is active.

Fig. 2 also indicates that up to 100 h were required for the recovery of exponential growth following nitrate exhaustion. This period was much shorter or even nonexistent when nitrate exhaustion occurred at cell densities greater than $A_{600} = 0.3$, cm^{-1}. Nitrogenase activity may have been reduced by higher dissolved oxygen concentrations in the low-density cultures, accounting for the interruption in growth.

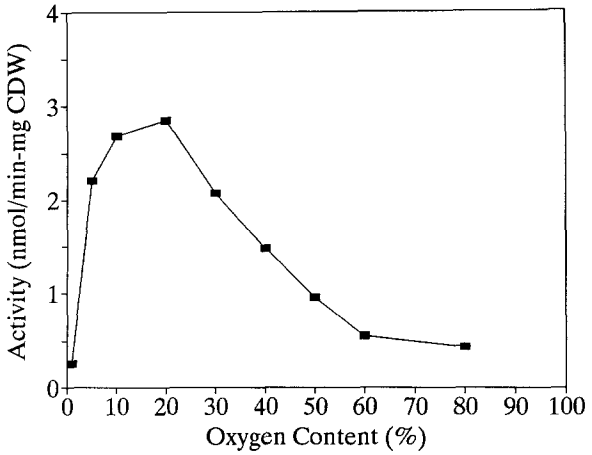

Fig. 3. Nitrogenase activity versus ambient dissolved oxygen level in *Methylosinus trichosporium* OB3b. Nitrogenase activity defined as the ethylene formation rate normalized to cell dry weight. All cells were grown under steady-state conditions in the Biostat MD under nitrate limitation. Oxygen level refers to % saturation in air in the steady-state culture prior to performance of the nitrogenase assay. The nitrogenase assay was performed using a low-oxygen assay procedure (see text).

As indicated in Fig. 3, the nitrogenase of *M. trichosporium* OB3b is sensitive to dissolved oxygen concentrations above about 65 μM (30% saturation in air). Above that concentration, nitrogenase activity decreased with increases in dissolved oxygen. Under anoxic conditions, the absence of nitrogenase activity was probably due to energy depletion. Cultures became noticeably flocculent when nitrogenase-bearing (nitrate-starved) cells were suddenly exposed to O_2 concentrations near saturation with air.

Competition Experiments

Copper Stress. Fig. 4 illustrates the effect of copper stress on competition between *M. trichosporium* OB3b and *M. albus* BG8 when dissolved oxygen was maintained at about 65 μM (30% saturation in air). The type I organism held its own in the two-population experiment at soluble copper concentrations ranging from 0.5 μM to 2.5 μM. At lower copper concentrations, *M. trichosporium* OB3b had a decided advantage, as indicated by a 10-fold shift in the ratio of cell numbers over about 70 h. Expression of sMMO was not apparent until soluble copper concentrations dropped below detection limits (< 0.05 μM).

To obtain the result summarized in Fig. 5, dissolved oxygen was maintained at a considerably higher level (173 μM or 80% of saturation with air). The reactor feed was copper-free throughout this experiment so that liquid-phase copper concentrations dropped precipitously from an initial level of 1.75 μM. Once again, sMMO expression was not apparent until soluble copper levels approached detection limits, after which *M. trichosporium* grew progressively more dominant in the two-organism culture. In this case, complete depletion of soluble copper was not required for the appearance of sMMO activity.

Fig. 5 also indicates that shifts in the dilution rate between 0.025 h^{-1} and 0.05 h^{-1}, which were made to assess the role of dilution rate on competition, appeared to affect sMMO activity. A sudden drop in dilution rate (at approximately 100 h)

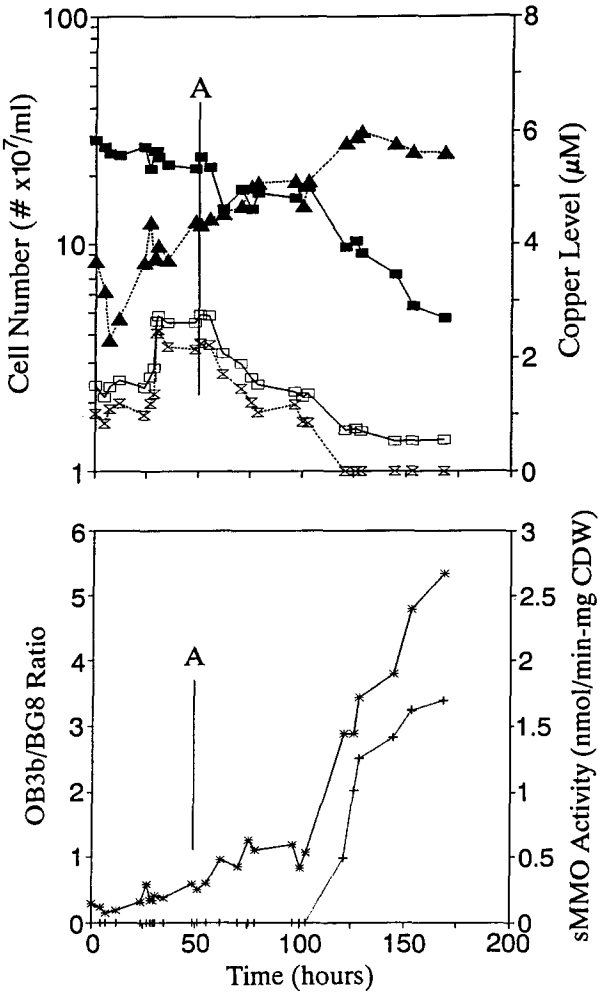

Fig. 4. Copper limitation competition experiment—low oxygen levels. Reactor conditions were as follows: Dilution rate = 0.025 h^{-1}, dissolved oxygen = 30% saturation in air, nitrate = 10.0 mM, and methane = 5.0% v/v in gas feed. Reactor was switched to copper-free media at point A. ■, *M. albus* BG8 cell number; ▲, *M. trichosporium* OB3b cell number; □, total copper; X, dissolved copper; *, OB3b to BG8 cell number ratio; and +, sMMO activity. sMMO activity defined as cyclohexanol formation rate normalized to cell dry weight of *M. trichosporium* in the culture.

resulted in an increase in sMMO activity, whereas a sudden increase in dilution rate caused a transient drop in sMMO activity.

The results in Figs. 4 and 5 suggest that sMMO activity in *M. trichosporium* OB3b and, consequently, competitive success by the type II strain, were dependent on both copper and oxygen concentrations. Rapid appearance of sMMO activity was evident as copper level approached zero in both experiments. The role of oxygen in generating sMMO activity is suggested by comparison of the cell-to-copper ratios at which sMMO activity was first observed under the two oxygen conditions. sMMO activity was first observed at a ratio of 7.25 mg CDW/μg Cu(II) at 30% oxygen saturation in air. At 80% oxygen, however, that ratio was only 0.98 mg CDW/μg Cu(II). Apparently, high oxygen levels promote sMMO activity or expression in *M. trichosporium* OB3b.

The results in Figs. 4 and 5 also suggest that copper speciation affects the bioavailability of copper in these two organisms. In both experiments, copper was

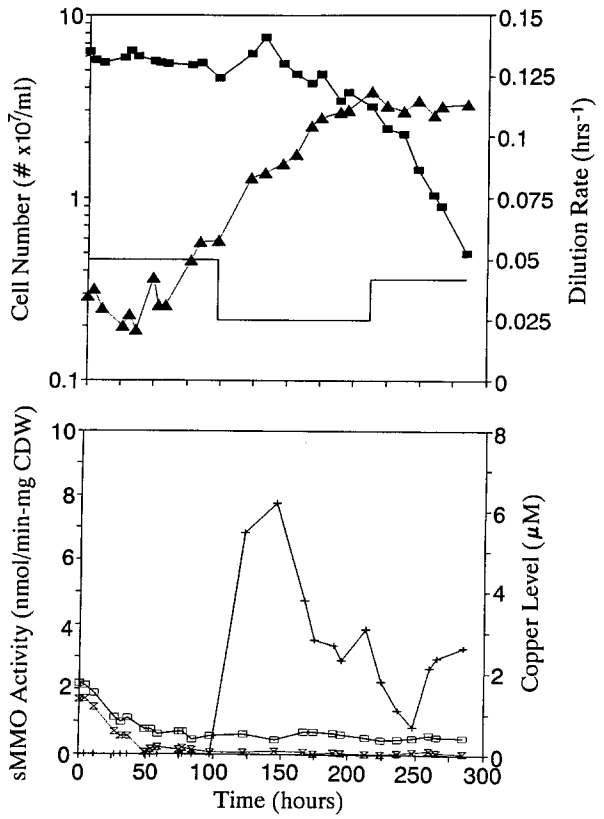

Fig. 5. Copper limitation competition experiment—high oxygen levels. Reactor conditions were as follows: Dissolved oxygen = 80% saturation in air, nitrate = 10.0 mM, and methane = 5.0% v/v in gas feed. ■, *M. albus* BG8 cell number; ▲, *M. trichosporium* OB3b cell number; □, total copper; X, dissolved copper; −, dilution rate; and +, sMMO activity. sMMO activity defined as cyclohexanol formation rate normalized to cell dry weight of *M. trichosporium* OB3b in the culture. Dilution rate was varied between 0.025 h^{-1} and 0.05 h^{-1}, as indicated.

present in both soluble and particulate forms; however, sMMO activity appeared only when the soluble copper concentration approached detection limits, despite the presence of significant levels of particulate copper. The implication is that the availability of a soluble copper source is important for the repression of sMMO in *M. trichosporium* and for the maintenance of pMMO in *M. albus*.

Nitrogen Stress. The results of nitrate-limitation experiments are summarized in Fig. 6. The influent nitrate concentration was maintained at 0.5 mM throughout to provide an initial source of nitrogen for growth of *M. albus* BG8 and to ensure that nitrogen stress would eventually be established. The dissolved oxygen concentration was shifted between levels representing 80% and 10% of saturation with air (175 μM and 22 μM oxygen, respectively).

Nitrogen limitation was established about one day into the experiment, after which numbers of *M. albus* BG8 were stable or declined slightly. The population of *M. trichosporium* OB3b was steady or slightly increasing, but nitrogenase expression was not apparent until the dissolved oxygen concentration was lowered to about 10% of saturation with air. Beyond that point, nitrogenase activity was immediately apparent. The numbers of *M. trichosporium* OB3b increased, and *M. albus* BG8 fell below measurable numbers over the next 50 h. The speed with

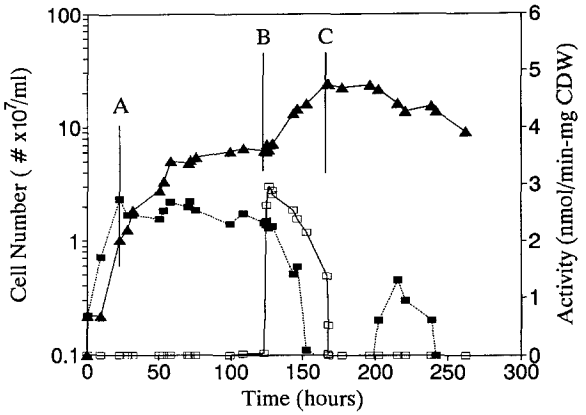

Fig. 6. Nitrate limitation competition experiment. Reactor conditions were as follows: Dilution rate = 0.025 h^{-1}, initial dissolved oxygen = 80% saturation in air, nitrate = 0.5 mM, copper = 2 μM, and methane = 5.0% v/v in gas feed. Reactor was switched to continuous-flow mode at point A. At point B, dissolved oxygen reduced to 10% saturation in air. At point C, dissolved oxygen returned to 80% saturation in air. ■, *M. albus* BG8 cell number; ▲, *M. trichosporium* OB3b cell number; and □, nitrogenase activity. Nitrogenase activity defined as ethylene formation rate normalized to estimated *M. trichosporium* OB3b cell dry weight.

which nitrogenase activity developed suggests that nitrogenase was present in the cells prior to the change in oxygen status (i.e. nitrate limitation appears to induce nitrogenase in *M. trichosporium*). The *M. trichosporium* nitrogenase may be inhibited by oxygen or activated when oxygen tension is reduced. Such speculation is supported by pure culture data (Fig. 3). Nitrogenase activity was soon lost (<30 min) after the dissolved oxygen concentration was returned to 80% of saturation with air, and thereafter numbers of *M. trichosporium* OB3b dropped towards previous levels.

It is apparent that the increase in cell numbers associated with nitrogen fixation (at low nitrate and dissolved oxygen concentrations) enabled *M. trichosporium* to outcompete *M. albus* in the reactor, presumably by making additional demands on an already-limited supply of nitrate. Although *M. albus* BG8 again reached countable numbers near the end of the experiment, the recovery was short-lived.

The dilution rate in this experiment was 0.025 h^{-1}, or slightly higher than the specific growth rate of *M. trichosporium* in nitrogen-free media (<0.023 h^{-1}). *M. trichosporium* OB3b probably avoided washout and eventually extended its numbers by obtaining a greater proportion of the influent nitrate (when *M. albus* BG8 was washed out of the reactor). *M. albus*'s precipitous decline illustrates the consequences of nitrogen limitation on a type I organism. Because their nitrogen requirements are significant, a shortage of nitrogen can have dramatic consequences [31].

Methane Stress. The results of methane-limitation experiments are presented in Figs. 7and 8. The rate of methane addition was varied by manipulating the methane feed rate of between 9.0 ml/min and 4.4 ml/min. The ratio of *M. trichosporium* to *M. albus* numbers declined following the establishment of methane limitation,

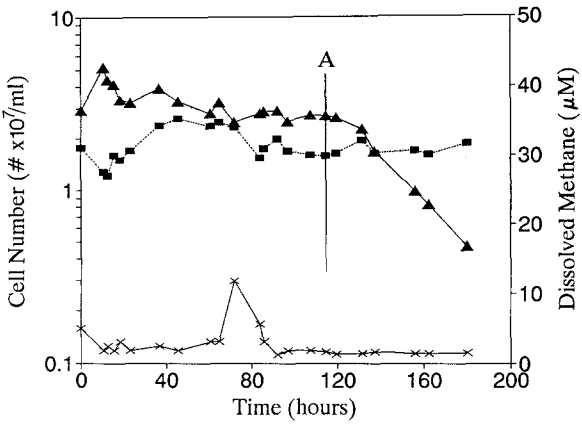

Fig. 7. Methane limitation—vary methane feed rate. Reactor conditions were as follows: Dilution rate = 0.05 h^{-1}, dissolved oxygen = 80% saturation in air, nitrate = 1.0 mM, copper = 2 μM, initial methane feed rate = 3.5% v/v. Methane feed rate reduced to 1.7% v/v at point A. ■, *M. albus* BG8 cell number; ▲, *M. trichosporium* OB3b cell number; and X, dissolved methane.

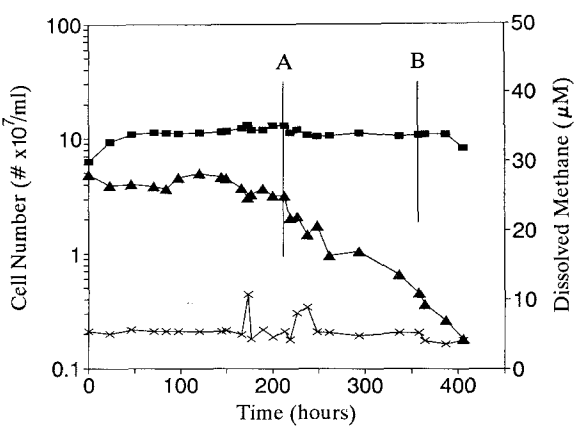

Fig. 8. Methane limitation—vary dissolved oxygen level and methane feed rate. Reactor conditions were as follows: Dilution rate = 0.05 h^{-1}, initial dissolved oxygen = 10% saturation in air, nitrate = 1.0 mM, copper = 2 μM, initial methane feed rate = 3.4% v/v. Methane feed rate reduced to 1.7% v/v at point A. Dissolved oxygen level reduced to 80% saturation in air at point B. ■, *M. albus* BG8 cell number; ▲, *M. trichosporium* OB3b cell number; and X, dissolved methane.

reaching near-steady conditions after about two days (Fig. 7). The transient increase in liquid-phase methane concentration at about 75 h was due to operator error and did not significantly alter the results of the competition experiments. After methane flux was further restricted at 108 h, the ratio of cell numbers dropped again by almost an order of magnitude, although changes in liquid-phase methane concentration were barely discernible. Except for the brief loss of control, methane concentration was maintained below 6 μM. The dramatic success of *M. albus* after the reduction in methane flux is difficult to explain based on Michaelis-Menten kinetics alone. Dissolved methane levels were much lower than the Michaelis or half-saturation constant for the two organisms (estimated at 25 μM for each [11]). Differences in yield and cell maintenance parameters between the two organisms probably explain the competitive success of the type I organism.

In methane stress experiments, dissolved oxygen concentration was varied between 10% and 80% of saturation with air. In the range tested, dissolved oxygen played no role in the outcome of competition experiments. *M. albus* BG8 always had an advantage when competition was based on methane limitation. This advantage was most evident when methane stress was severe (Figs. 7 and 8).

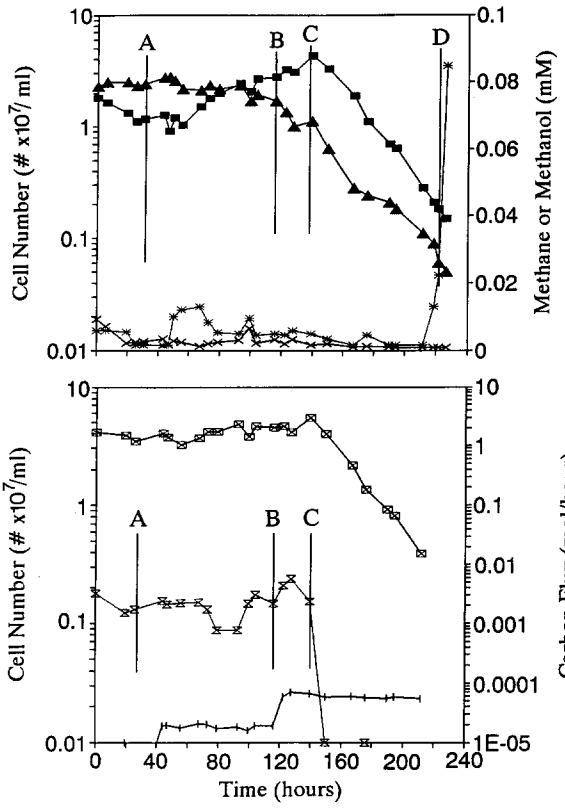

Fig. 9. Methane limitation—methanol supplementation experiment. Reactor conditions were as follows: Dilution rate = 0.05 h^{-1}, dissolved oxygen = 80% saturation in air, nitrate = 1.0 mM, copper = 2 μM, methane feed rate = 3.4% v/v, and initial methanol feed concentration = 0.0 mM. At point A, 0.2 mM methanol feed was initiated. Methanol feed concentration was increased to 0.75 mM at point B. Methane feed was shut off at point C. At point D, 3.0 mM NaN_3 was added to the reactor and the reactor feed. ■, *M. albus* BG8 cell number; ⊠, total cell number; ▲, *M. trichosporium* OB3b cell number; X, dissolved methane; *, dissolved methanol; X, methane flux; and +, methanol flux.

Methanol Accumulation. Under conditions that lead to unbalanced metabolism, the intermediates of methane oxidation (methanol, formaldehyde, and formate) can accumulate to very high levels in methanotrophic cultures [20, 21]. In preliminary experiments, methanol accumulated to millimolar levels under low O_2 conditions (1–8% of saturation with air) in cultures of both *M. albus* and *M. trichosporium* OB3b. Under the conditions of these experiments, growth was not limited by methane concentration, copper level (2.5 μM total copper), or nitrate (10 mM initial concentration). Formaldehyde, formate, and acetone were below the limits of detection.

To explore the effects of metabolic intermediates on methanotroph competition, *M. trichosporium* OB3b and *M. albus* BG8 were grown to mid-exponential phase (A_{600} = 0.3, cm^{-1}) in pure batch cultures. Cells were removed via centrifugation, and the residual cell-free medium was cross-inoculated (*M. trichosporium* into *M. albus* medium and the reciprocal cross). If inhibitory metabolic intermediates accumulated in solution, there should have been growth inhibition after crossing; however, the recovered media had no effect on subsequent growth of either organism (results not shown).

Methanol effects were tested directly by supplementing the Biostat feed stream with methanol in competition experiments (Fig. 9). The feed concentration of

methanol was increased in increments from 0 mM to 0.5 mM. Influent methane was established at a level that should lead to methane limitation and was eliminated halfway through the experiment. Copper and nitrate were provided in excess.

The initiation of methanol addition to the reactor was accompanied by a 4-fold increase in the numbers of *M. albus* BG8. Numbers of *M. trichosporium* OB3b decreased by 50% during the same period. Methanol consumption was evident following a transient increase in the liquid-phase methanol concentration to a maximum value of about 0.01 mM. When methane was eliminated, it became apparent that methanol could not support the continued growth of either population at the level provided. This is not surprising since the rate of methanol addition was a small fraction (1:50 to 1:170; molar ratio) of the original methane flux to the reactor (Fig. 9). At the conclusion of the experiment, the reactor was poisoned with 3.0 mM NaN_3 to verify that microbial activity, as opposed to physical processes, provided the primary sink for methanol in the reactor.

Whittenbury et al. [35] showed that both *M. albus* BG8 and *M. trichosporium* OB3b can grow in the presence of the methanol levels utilized here. They also indicated that *M. trichosporium* OB3b would preferentially use methanol for growth when methane and methanol were provided simultaneously. *M. albus* uses both methane and methanol concurrently under similar conditions. The poor competitive success of *M. trichosporium*, therefore, may have been due to a deregulating effect of methanol on methane utilization. That is, *M. albus* thrived because: (1) methane utilization by *M. trichosporium* OB3b was inhibited by the low levels of methanol present, and (2) there was insufficient methanol present to maintain a large population of *M. trichosporium*.

Summary. The results of two-organism competition studies generally followed predictions based on physiological principles. A number of points can be made in summary.

1. Because copper is required for pMMO activity, it was essential for growth by *M. albus* BG8 but not by *M. trichosporium* OB3b. Copper limitation resulted in the derepression of sMMO in *M. trichosporium* OB3b and promoted the selection of the type II organisms in competition experiments.

2. It was possible to establish copper-limited conditions without exhausting the soluble copper concentration and without inducing sMMO in *M. trichosporium* OB3b. Copper limitation favored *M. trichosporium* under all conditions tested; the advantage of the type II organism was overwhelming under conditions that result in sMMO expression.

3. In the high-oxygen-tension experiment, sMMO activity appeared at measurable soluble copper levels (0.15 μM). At lower dissolved oxygen concentrations (<30% saturation in air), sMMO expression could not be detected until the soluble copper concentration fell below the limits of detection (<0.05 μM). The cell-to-copper ratio at which sMMO activity initially appeared was inversely related to dissolved oxygen concentrations. Results suggest that oxygen tension is among the determinants of sMMO expression.

4. *M. albus* BG8 was unable to grow following nitrate exhaustion in pure cultures. *M. trichosporium* OB3b was capable of fixing nitrogen and continued to grow, albeit at a much lower rate than when nitrate was present.

5. Nitrogenase activity was acutely dependent on dissolved oxygen concentration, peaking at concentrations that were about 20% of saturation with air. Consequently, the competitive advantage experienced by *M. trichosporium* OB3b under nitrate-limitation was dependent on the dissolved oxygen concentration.

6. *M. albus* BG8 enjoyed a competitive advantage over the type II organism when growth was carbon limited, perhaps owing to its superior specific growth rate. The competitive success of *M. albus* BG8 appeared to be accelerated when low levels of methanol were provided simultaneously with methane.

7. Results suggest that reactor conditions can be manipulated to select for type II methanotrophs. This may be of advantage in situations where unique metabolic features of those microorganisms are to be encouraged. Selection under field conditions may be less straightforward, as witnessed by the difficulties in finding natural settings in which type II methanotrophs are dominant.

Acknowledgments. This work was funded in part by grants from the National Science Foundation Nos. BCS8658002 and BCS9008145. The manuscript and figures were prepared with the help of Ms. Beth Marlatt and Ms. Alison Habel, respectively.

References

1. Andreae MO, Crutzen PJ (1985) Atmospheric chemistry. In: Malon TF, Roederer JG (eds) Global change. ICSU Press/Cambridge University Press, Cambridge, pp 75–113
2. Anthony C (1982) The biochemistry of methylotrophs. Academic Press, London
3. Blake DR, Rowland FS (1988) Continuing worldwide increase in tropospheric methane, 1978 to 1987. Science 239:1129–1131
4. Brusseau GA, Tsien HC, Hanson RS, Wackett LP (1990) Optimization of trichloroethylene oxidation by methanotrophs and the use of a colorimetric assay to detect soluble methane monooxygenase activity. Biodegradation 1:19–29
5. Chen YP, Yoch DC (1988) Reconstitution of the electron transport system that couples formate oxidation to nitrogenase in *Methylosinus trichosporium* OB3b. J Gen Microbiol 134:3123–3128
6. Collins MLP, Buchholz LA, Remsen CC (1991) Effect of copper on *Methylomonas albus* BG8. Appl Environ Microbiol 57:1261–1264
7. Cornish A, Nicholls KM, Scott D, Hunter BK, Aston WJ, Higgins IJ, Sanders JKM (1984) In vivo 13C NMR investigations of methanol oxidation by the obligate methanotroph *Methylosinus trichosporium* OB3b. J Gen Microbiol 130:2565–2575
8. Dalton H, Higgins IJ (1987) Physiology and biochemistry of methylotrophic bacteria. In: Van Verseveld MW, Duine JA (eds) Microbiol growth on C_1 compounds. Martinus Nijhoff Publishers, Dordrecht, The Netherlands, pp 75–82
9. Fox BG, Borneman JG, Wackett LP, Lipscomb JD (1991) Haloalkene oxidation by the soluble methane monooxygenase from *Methylosinus trichosporium* OB3b: mechanistic and environmental implications. Biochemistry 29:6419–6427
10. Frederickson AG, Stephanopolis G (1981) Microbial competition. Science 213:972–974
11. Graham DW, Korich D, LeBlanc RP, Sinclair NA, Arnold RG (1992) Applications of a colorimetric plate assay for soluble methane monooxygenase activity. Appl Environ Microbiol 58:2231–2236
12. Hanson RS, Netrusov AI, Tsuji K (1991) The obligate methanotrophic bacteria: *Methylococcus*, *Methylomonas*, and *Methylosinus*. In: Balows A, Trüper HG, Dworkin M, Harder W, Schleifer KH (eds) The Prokaryotes. Springer-Verlag, New York, pp 661–684
13. Hardy RW, Holsten RD, Jackson EK, Burns RC (1968) The acetylene-ethylene assay for N_2 fixation: Laboratory and field evaluation. Plant Physiol 43:1185–1207

14. Harrits S, Hanson RS (1980) Stratification of aerobic methane-oxidizing organisms in Lake Mendota, Madison, Wisconsin. Limnol Oceanogr 25:412–421
15. Heyer J, Suchow R (1985) Okologishe Untersuchungen der Methanoxidation in einem sauren Moorsee. Limnologica 6:267–276
16. Higgins IJ, Best DJ, Hammond RC, Scott D (1981) Methane oxidizing microorganisms. Microbiol Rev 45:556–590
17. Hou CT (ed) (1984) Methylotrophs: Microbiology, biochemistry, and genetics. CRC Press Inc., Boca Raton, Florida
18. Kramer M, Baumgartner M, Bender M, Conrad R (1990) Consumption of NO by methanotrophic bacteria in pure culture and in soil. FEMS Microbiol Ecol 73:345–350
19. Megraw SR, Knowles R (1987) Active methanotrophs suppress nitrification in a humisol. Biol Fertil Soils 4:205–212
20. Mehta PK, Mishra S, Ghosh TK (1991) Methanol biosynthesis by covalently immobilized cells of *Methylosinus trichosporium*: Batch and continuous studies. Biotechnol Bioeng 37:551–556
21. Mountfort DO, Pybus V, Wilson R (1990) Metal-ion mediated accumulation of alcohols during alkane oxidation by whole cells of *Methylosinus trichosporium*. Enzyme Microb Technol 2:343–348
22. Murrell JC, Dalton H (1983) Nitrogen fixation in obligate methanotrophs. J Gen Microbiol 129:3481–3486
23. Oldenhuis R, Vink R, Janssen DB, Witholt B (1989) Degradation of chlorinated hydrocarbons by *Methylosinus trichosporium* OB3b expressing soluble methane monooxygenase. Appl Environ Microbiol 55:2819–2826
24. Putzer KP, Buchholz LA, Lidstrom ME, Remsen CC (1991) Separation of methanotrophic bacteria by using percoll and its application to isolation of mixed and pure cultures. Appl Environ Microbiol 57:3656–3659
25. Reed WM, Dugan PR (1978) Distribution of *Methylomonas methanica* and *Methylosinus trichosporium* in Cleveland Harbor as determined by an indirect flourescent antibody-membrane filter technique. Appl Environ Microbiol 35:422–430
26. Rudd JWM, Hamilton RD (1978) Methane cycling in eutrophic shield lake and its effects on whole lake metabolism. Limnol Oceanogr 23:337–348
27. Rudd JWM, Taylor CD (1980) Methane cycling in aquatic environments. Adv Aquat Microbiol 2:77–150
28. Rudd JWM, Hamilton RD, Campbell NER (1974) Measurement of microbial oxidation of methane in lake water. Limnol Oceanogr 19:519–524
29. Strugger S (1948) Fluorescence microscope examination of bacteria. Can J Res Series C 26:188–193
30. Thibodeaux LJ (1979) Chemodynamics: Environmental movements of chemicals in air, water, and soil John Wiley & Sons, New York
31. Topp E, Knowles R (1984) Effects of nitrapyrin [2-chloro-6 (trichoromethyl)pyridine] on the obligate methylotroph *Methylosinus trichosporium* OB3b. Appl Environ Microbiol 47:248–262
32. Tsien HC, Brusseau GA, Hanson RS, Wackett LP (1989) Biodegradation of trichloroethylene by *Methylosinus trichosporium* OB3b. Appl Environ Microbiol 55:3155–3161
33. Tsien HC, Bratina BJ, Tsuji K, Hanson RS (1990) Use of oligodeoxynucleotide signiture probes for identification of physiological groups of methylotrophic bacteria. Appl Environ Microbiol 56:2858–2865
34. Whittenbury R, Krieg NR (1984) *Methylococcaceae* fam. nov. In: Krieg NR, Holt JG (eds) Bergey's manual of determinative bacteriology, vol. 1. Williams and Wilkins, Baltimore, pp 256–262
35. Whittenbury R, Phillips KC, Wilkenson JF (1970) Enrichment, isolation and some properties of methane utilizing bacteria. J Gen Microbiol 61:205–218
36. Wilson JT, Wilson BH (1985) Biotransformation of trichloethylene in soil. Appl Environ Microbiol 49:242–243