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# Methanol Promotes Atmospheric Methane Oxidation by Methanotrophic Cultures and Soils†

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Two methanotrophic bacteria, *Methylobacter albus* BG8 and *Methylosinus trichosporium* OB3b, oxidized atmospheric methane during batch growth on methanol. Methane consumption was rapidly and substantially diminished (95% over 9 days) when washed cell suspensions were incubated without methanol in the presence of atmospheric methane (1.7 ppm). Methanotrophic activity was stimulated after methanol (10 mM) but not methane (1,000 ppm) addition. *M. albus* BG8 grown in continuous culture for 80 days with methanol retained the ability to oxidize atmospheric methane and oxidized methane in a chemostat air supply. Methane oxidation during growth on methanol was not affected by methane deprivation. Differences in the kinetics of methane uptake (apparent  $K_m$  and  $V_{max}$ ) were observed between batch- and chemostat-grown cultures. The  $V_{max}$  and apparent  $K_m$  values (means  $\pm$  standard errors) for methanol-limited chemostat cultures were  $133 \pm 46$  nmol of methane  $10^8$  cells $^{-1}$  h $^{-1}$  and  $916 \pm 235$  ppm of methane (1.2  $\mu$ M), respectively. These values were significantly lower than those determined with batch-grown cultures ( $V_{max}$  of  $648 \pm 195$  nmol of methane  $10^8$  cells $^{-1}$  h $^{-1}$  and apparent  $K_m$  of  $5,025 \pm 1,234$  ppm of methane [6.3  $\mu$ M]). Methane consumption by soils was stimulated by the addition of methanol. These results suggest that methanol or other nonmethane substrates may promote atmospheric methane oxidation in situ.

Despite the contribution of soils to global methane budgets (8, 12, 25, 29), the methane oxidizers active in situ remain unidentified and are considered novel (11, 22, 28). In some respects, soil methanotrophy appears physiologically distinct from methanotrophy in cultures. For example, soil methanotrophs appear capable of oxidizing atmospheric methane indefinitely given suitable temperatures and soil water contents. In contrast, methanotrophs in culture consume atmospheric methane only transiently after growth at high methane concentrations (30). Differences in the kinetics of methane uptake ( $V_{max}$ , apparent  $K_m$ , and threshold) also distinguish soil methanotrophs from known isolates (2, 3). For example, soils typically exhibit a high affinity for methane (approximately 10 nM), whereas pure cultures have a relatively low affinity (>1  $\mu$ M) (18).

Many of the factors that affect soil methane oxidation, including agriculture and disturbances in soil water content and soil nitrogen, have been examined extensively (1, 10, 13, 24, 29, 31). However, relatively little is known about the substrate(s) supporting growth and activity. For example, the extent to which soil methanotrophs depend on atmospheric methane (about 1.7 ppm, equivalent to a dissolved methane concentration in soil water of about 2.5 nM at 22°C) as a sole source of carbon and energy is unknown. Bender and Conrad (4) demonstrated that numbers of soil methanotrophs increase only at methane concentrations of  $\geq 7,000$  ppm, an observation that raises doubts about the role of methane as a sole carbon and energy source. More recently, methanotrophic activity in forest soil was found to be unaffected by methane deprivation, which suggests that nonmethane substrates may support soil methanotrophs (5). However, obligate methanotrophs are restricted to a limited range of C<sub>1</sub> compounds for growth and energy (9),

and use of multicarbon compounds has been reported for only a few presumed facultative methanotrophs (34).

Despite the ability of methanotrophs to grow on some nonmethane substrates, little is known about the role of such compounds as supplementary carbon and energy sources. Methanotrophs have been reported to grow on methanol, methylamine, or trimethylamine (e.g., see references 9 and 14), but the significance of these substrates appears limited at best. Results presented here demonstrate that pure cultures of methanotrophs oxidize atmospheric methane during growth on methanol and that they retain this activity during methanol-limited growth. Soils treated with methanol also showed enhanced methane consumption. On the basis of these collective results, we suggest that methanotrophic activity in soils depends at least partially on nonmethane substrates for maintenance; we also suggest that methanol is the most important nonmethane substrate.

## MATERIALS AND METHODS

**Growth and maintenance of organisms.** *Methylobacter albus* BG8 (formerly *Methylomonas albus* BG8) and *Methylosinus trichosporium* OB3b were obtained from R. S. Hanson. Cultures were maintained on agar slants of nitrate mineral salts medium (NMS) at 30°C (20), under an initial atmosphere of approximately 30 to 70% methane-air; subcultures were transferred every 4 to 5 weeks. To prepare an inoculum for batch and continuous cultivation studies, 4 ml of NMS medium was added to a 3- to 4-day-old slant; the slant was vortexed briefly to resuspend the cells, and the liquid was transferred to a 1-liter flask containing 160 ml of NMS medium. Both cultures were grown on methane (approximately 30 to 70% methane-air) at 30°C with shaking (125 rpm).

**Atmospheric methane consumption during batch growth on methanol.** A volume (100  $\mu$ l) of *M. albus* BG8 or *M. trichosporium* OB3b cultures from mid-exponential growth phase was used to inoculate NMS medium (20 ml) containing methanol (25 mM) in 160-ml serum bottles sealed with butyl rubber stoppers. The cultures were maintained at 30°C with shaking (125 rpm). Atmospheric methane consumption by cultures that had been equilibrated with ambient air at 30°C was determined by measuring the decrease in headspace methane over time by gas chromatography (20). The optical density of cultures at a wavelength of 600 nm during incubation was also determined for a parallel set of bottles. All experiments were performed in triplicate.

**Substrate utilization by *M. albus* BG8.** Substrate utilization by *M. albus* BG8 was determined by using washed cell suspensions of both batch (methane-grown) and chemostat (methanol-limited) cultures. Cultures were serially diluted (ap-

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proximately 500 and 1,000 cells ml<sup>-1</sup> for batch and chemostat cultures, respectively), and samples (100 µl) were plated on NMS agar plates containing various substrates (methane, methanol [vapor], formate, glucose, glucose plus casein hydrolysate, fructose, acetate, succinate, malate, pyruvate, citrate, glutamate, alanine, and serine). Substrates were added at 10 mM unless otherwise indicated. The plates were incubated at 30°C under ambient air, except for the methane treatment, for which an atmosphere of 30% methane in air was used. Colonies were counted after 19 days of incubation. The plates were incubated further to confirm that all colonies had been counted. Control treatments without added substrates were incubated under ambient air as the sole source of carbon and energy.

**Stability of atmospheric methane consumption.** The stability of atmospheric methane consumption by washed cultures of *M. albus* BG8 was determined with methane (1.7 ppm) in the presence or absence of methanol. Cultures of *M. albus* BG8 (160 ml) in mid-exponential growth phase were harvested by centrifugation, and cell pellets were washed and resuspended in a phosphate buffer to an optical density of 4 (equivalent to approximately  $1.4 \times 10^9$  cells ml<sup>-1</sup>). Phosphate buffer was prepared as for NMS medium, but Higgins salts were excluded. Washed cell suspensions (2 ml) were transferred to 120-ml glass jars that were sealed with neoprene rubber stoppers and incubated at 30°C with shaking (125 rpm). Cell suspensions were also sprayed on 10 g of autoclaved sand in 120-ml jars that were sealed with neoprene rubber stoppers and incubated statically. The effect of methanol on the stability of atmospheric methane consumption was examined by injecting methanol (1 µl) with a microsyringe onto the walls of the sealed glass jars at various times. At intervals, atmospheric methane consumption rates were determined by equilibrating the cultures under ambient air and measuring headspace methane. Methane uptake rate constants (*k*) were calculated by regression analysis of logarithmically transformed concentration data. The effects of methane and methanol additions on atmospheric methane uptake by *M. albus* BG8 incubated for 17 days with ambient air as the sole source of carbon and energy were examined by exposing cultures for 1 h to methane (1,000 ppm) or methanol (10 mM), respectively; atmospheric methane consumption rates were determined subsequently.

**Continuous-culture studies.** For continuous cultivation, *M. albus* BG8 was grown under methanol limitation as follows. A 650-ml volume of NMS medium containing methanol (25 mM) in a 1-liter glass vessel was inoculated with mid-exponential-phase cultures. Temperature and pH were maintained at 30°C and 7.3, respectively; pH control was unnecessary. Air continuously sparged the culture at a flow rate of 73 ml min<sup>-1</sup>. Methane concentrations in the inlet and outlet gas streams were measured periodically. After 24-h growth, NMS medium containing methanol (25 mM) was supplied at a flow rate of 10 ml h<sup>-1</sup>, which corresponded to a dilution rate of 0.015 h<sup>-1</sup>, and the culture was stirred with a magnetic stirrer. The purity of the culture was checked regularly by microscopic examination.

Atmospheric methane uptake was monitored frequently by removing samples from the glass vessel via an aseptic sampling port. Cultures (2 ml) were transferred to 120-ml glass jars and maintained at 30°C with shaking (250 rpm). Atmospheric methane consumption by cultures equilibrated with ambient air was determined by measuring the decrease in headspace methane over time. Samples were also removed from the glass vessel for absorbance measurements at 600 nm, cell enumeration, and pH and microscopic analyses. The total number of culturable cells in the chemostat was determined by spread plate counts. The chemostat cultures were serially diluted, and samples were plated on NMS agar plates and incubated at 30°C under an initial atmosphere of approximately 30% methane. Colonies were counted after 7 to 10 days. The plates were further incubated up to 30 days to confirm that all colonies had been counted.

To determine whether methanol or atmospheric methane was responsible for maintaining methane monooxygenase (MMO) activity, the air sparging the culture was replaced with hydrocarbon-free air (<0.03 ppm of methane; Scott Specialty Gases, Inc.) at a flow rate of 73 ml min<sup>-1</sup> between 55 and 85 days. Measurements of the outlet gas streams confirmed removal of traces of methane. The rate of atmospheric methane consumption, absorbance at 600 nm, and cell number were monitored during methane starvation. After 85 days, the hydrocarbon-free air was replaced with ambient air (1.7 ppm of methane).

**Kinetic studies.** The kinetic parameters of methane oxidation (apparent  $K_m$  and  $V_{max}$ ) for *M. albus* BG8 were determined by using washed cell suspensions of both batch (methane-grown) and chemostat (methanol-limited) cultures. Cultures of *M. albus* BG8 were harvested by centrifugation; cell pellets were washed and resuspended in phosphate buffer to an optical density of 0.6 (equivalent to approximately  $2 \times 10^8$  cells ml<sup>-1</sup>). Washed cell suspensions (5 ml) were transferred to 160-ml serum bottles that were sealed with butyl rubber stoppers and incubated at 30°C with shaking (250 rpm). Methane consumption rates (*V*) were measured at various methane concentrations [*S*] ranging between 500 and 30,000 ppm in the headspace. Apparent  $K_m$  and  $V_{max}$  values were obtained from nonlinear fits of the data to the Michaelis-Menten equation  $V = V_{max} [S] / (K_m + [S])$ . Concentrations of dissolved methane were calculated from the gas-phase concentrations by using mole fractions (33).

**<sup>14</sup>C<sub>4</sub> utilization studies.** *M. albus* BG8, *M. trichosporium* OB3b, and *Methylocystis parvus* OBBP were grown in batch cultures of NMS medium as described above. Late-exponential-phase cultures were harvested by centrifugation (10,000 × g, 10 min at 4°C), washed in phosphate buffer twice, and resuspended in fresh NMS medium. The cultures were then incubated with ambient air at 30°C with

shaking for 4 days. Two-milliliter aliquots with an  $A_{600}$  of about 0.8 were transferred to 30-ml serum bottles containing ambient air immediately after the cultures were resuspended and at intervals of 1, 2, and 4 days. The serum bottles were sealed with neoprene stoppers, after which 50 µl of a [<sup>14</sup>C]methane stock (about 74 kBq cm<sup>-3</sup>; 2.07 GBq mmol<sup>-1</sup>) were injected to create a headspace methane concentration of about 2 ppm. At intervals over a 6- to 8-h period, 1 cm<sup>3</sup> of headspace was sampled by needle and syringe and transferred to a sealed scintillation vial containing 2 ml of 0.1 N KOH to trap <sup>14</sup>CO<sub>2</sub>, which was assayed after addition of Scintiverse BD counting fluid. At the final time point, 2 ml of 1 N HCl was injected to acidify the medium; after degassing of <sup>14</sup>CO<sub>2</sub> and unreacted [<sup>14</sup>C]methane, 0.5 ml of the medium was transferred to scintillation vials to determine the amount of radioactivity incorporated into cells or cell metabolites. *M. albus* BG8 batch culture grown with methanol and continuous culture grown as described above were treated similarly. The continuous culture had been growing for 99 days prior to sampling; the batch culture was harvested from exponential and stationary phases. All assays were conducted in triplicate.

**Effect of methanol additions on soil methane consumption.** The effect of methanol additions on the methane-consuming capacity of forest soil was examined by injecting methanol (1 µl) at various intervals onto the walls of sealed jars containing 10 g (fresh weight) of sieved soil as described previously (5). The soils were incubated at 25°C. At various intervals, atmospheric methane consumption rates were determined.

## RESULTS

**Atmospheric methane oxidation during batch growth on methanol.** Pure cultures of *M. albus* BG8 (group I) and *M. trichosporium* OB3b (group II) were capable of growth on methanol (25 mM). During batch growth on methanol, traces of methane in the bottle headspaces were utilized. For *M. albus* BG8, methane concentrations decreased from an initial concentration of 2.1 ppm to 0.55 ppm over 2 days (Fig. 1A). The specific growth rate of *M. albus* on methanol was 0.11 h<sup>-1</sup>, equivalent to a doubling time of 6.5 h. During batch growth on methanol by *M. trichosporium* OB3b, methane concentrations decreased from an initial concentration of 2 ppm to 0.5 ppm over 7 days (Fig. 1B).

**Stability of atmospheric methane consumption.** *M. albus* BG8 consumed atmospheric methane transiently following growth at high methane concentrations. However, the uptake rate constants for atmospheric methane consumption decreased during subsequent incubations with atmospheric methane as the sole source of carbon and energy; after 9 days, 95% of the original activity was lost. In contrast, cell suspensions supplemented with methanol at intervals retained the ability to oxidize atmospheric methane (Fig. 2A). In addition, an immediate stimulation of atmospheric methane consumption was observed after addition of methanol (10 mM) but not methane (1,000 ppm) to cultures maintained for 17 days on atmospheric methane (data not shown).

The capacity of cell suspensions added to autoclaved sand to consume atmospheric methane was initially lower than that of liquid cultures (Fig. 2B). However, after 6 days of incubation in the presence of methanol, rates of methane oxidation increased twofold compared to those of liquid incubations at the same initial cell density.

**Continuous-culture studies.** *M. albus* BG8 grown in continuous culture (dilution rate, 0.015 h<sup>-1</sup>) for 80 days with methanol retained the ability to oxidize atmospheric methane (Fig. 3). The rate of atmospheric methane consumption and biomass of samples taken from steady-state continuous culture (means ± standard errors) were  $126 \pm 13$  pmol of methane 10<sup>8</sup> cells<sup>-1</sup> h<sup>-1</sup> and  $2.65 \times 10^8 \pm 0.3 \times 10^8$  cells ml<sup>-1</sup>, respectively. The methane-oxidizing activity of *M. albus* BG8 was relatively unaffected by methane starvation during growth on methanol. There were also no significant differences in  $A_{600}$  or consistent trends in the cell number of samples taken from steady-state continuous culture in the presence or absence of atmospheric methane (Fig. 3). However, the ability of *M. albus* BG8 to grow on methane changed during continuous cultivation. Colonies

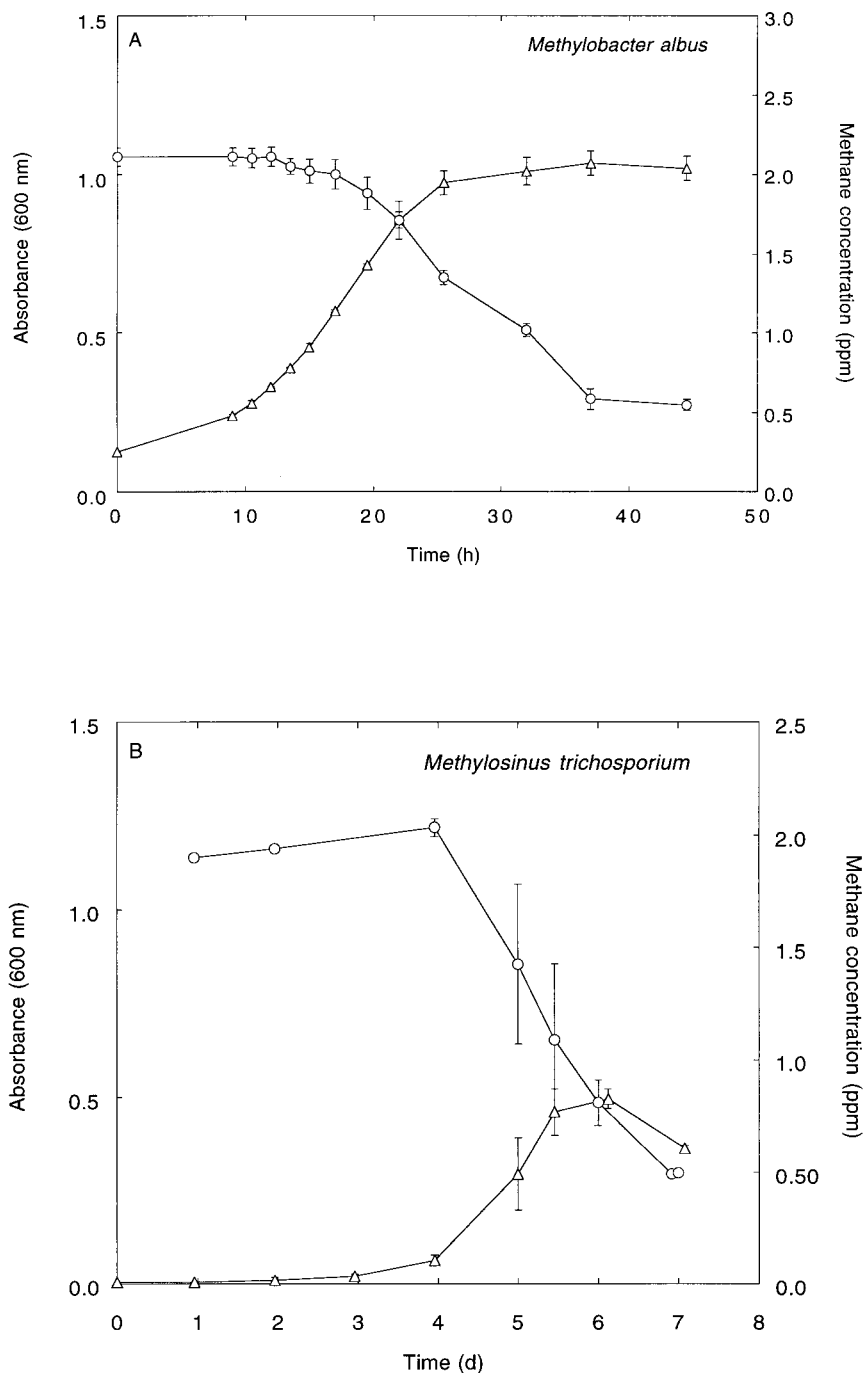


FIG. 1. Atmospheric methane oxidation by pure cultures of methanotrophic bacteria during batch growth on methanol (25 mM). (A) *M. albus* BG8; (B) *M. trichosporium* OB3b.  $\Delta$ , absorbance;  $\circ$ , methane concentration. Each point represents the mean of triplicate determinations  $\pm$  1 standard error. d, days.

enumerated on plates were visible within 10 days of incubation with methane (30% in air) for samples obtained during the first 30 days of chemostat operation. After this time, decreased colony growth was evident, fewer colonies were visible within 10 days, and plates required further incubation (about 20 to 30 days) to count all colonies.

**Kinetics of methane uptake.** Differences in methane oxidation kinetics were observed for washed cell suspensions of batch- and chemostat-grown cultures of *M. albus* BG8 (Fig. 4). The  $V_{\max}$  and apparent  $K_m$  values (means  $\pm$  standard errors)

of methanol-limited chemostat cultures were  $133 \pm 46$  nmol of methane  $10^8$  cells $^{-1}$  h $^{-1}$  and  $916 \pm 235$  ppm of methane (equivalent to a dissolved concentration of 1.2  $\mu$ M), respectively. These values were significantly lower than those determined for batch-grown cultures ( $V_{\max}$  of  $648 \pm 195$  nmol of methane  $10^8$  cells $^{-1}$  h $^{-1}$  and  $K_m$  of  $5,025 \pm 1,234$  ppm of methane [6.3  $\mu$ M]). There were no significant differences in  $V_{\max}$  and apparent  $K_m$  for washed cell suspensions taken from steady-state continuous culture in the presence or absence of atmospheric methane (Table 1).

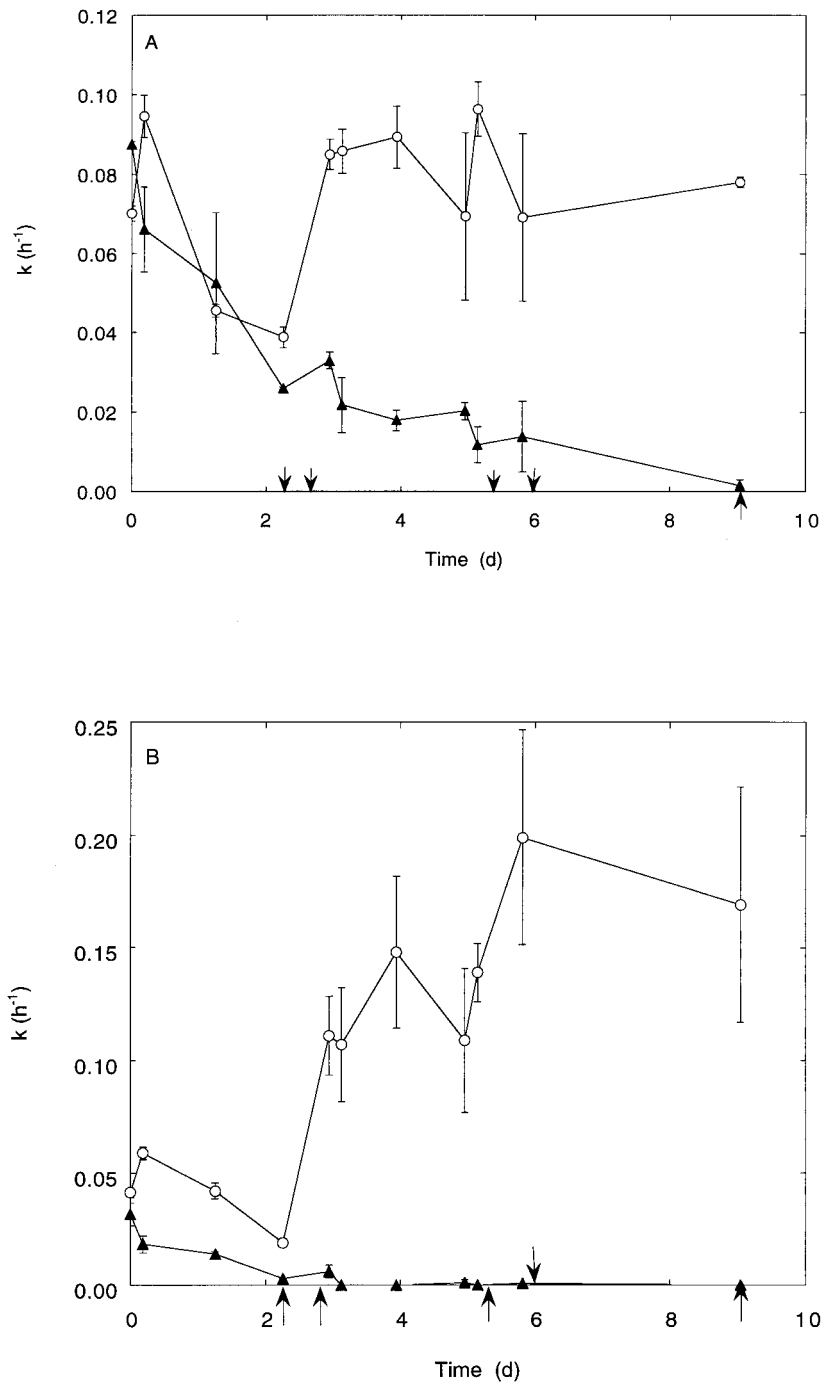


FIG. 2. Stability of atmospheric methane consumption by pure cultures of *M. albus* BG8 incubated without a solid support (A) and added to a solid support (B). ▲, cultures incubated with CH<sub>4</sub> (1.7 ppm); ○, cultures incubated with CH<sub>4</sub> (1.7 ppm) plus methanol. Methanol (25 μmol) was added at the times indicated (arrows). Data are means of triplicate determinations ± 1 standard error. d, days.

**<sup>14</sup>CH<sub>4</sub> utilization studies.** Production of <sup>14</sup>CO<sub>2</sub> in all cultures fit an exponential relationship of the form  $y = 1 - e^{-xt}$  (Fig. 5A), consistent with exponentially decreasing methane as typically observed for cultures at near-ambient methane concentrations. Of the three cultures examined, activity was least for *M. trichosporium* OB3b, though the differences among cultures were not substantial. Activity also decreased with increasing incubation time in all cultures, especially after 2 days, and most dramatically in *M. trichosporium* OB3b (Fig. 5B; note that

these assays involved short-term incubations of replicate treatments rather than continuous incubations with [<sup>14</sup>C]methane). Total radiomethane uptake during 6- to 8-h incubations as measured by <sup>14</sup>CO<sub>2</sub> production and incorporation was initially about 65 to 75% and declined with time to <10% in all cases (Fig. 5B). The percentage of total uptake defined as incorporation (cells and cell metabolites) was generally <10% for *M. parvus* OBBP and *M. trichosporium* OB3b; incorporation was initially low for *M. albus* BG8 but increased with time to

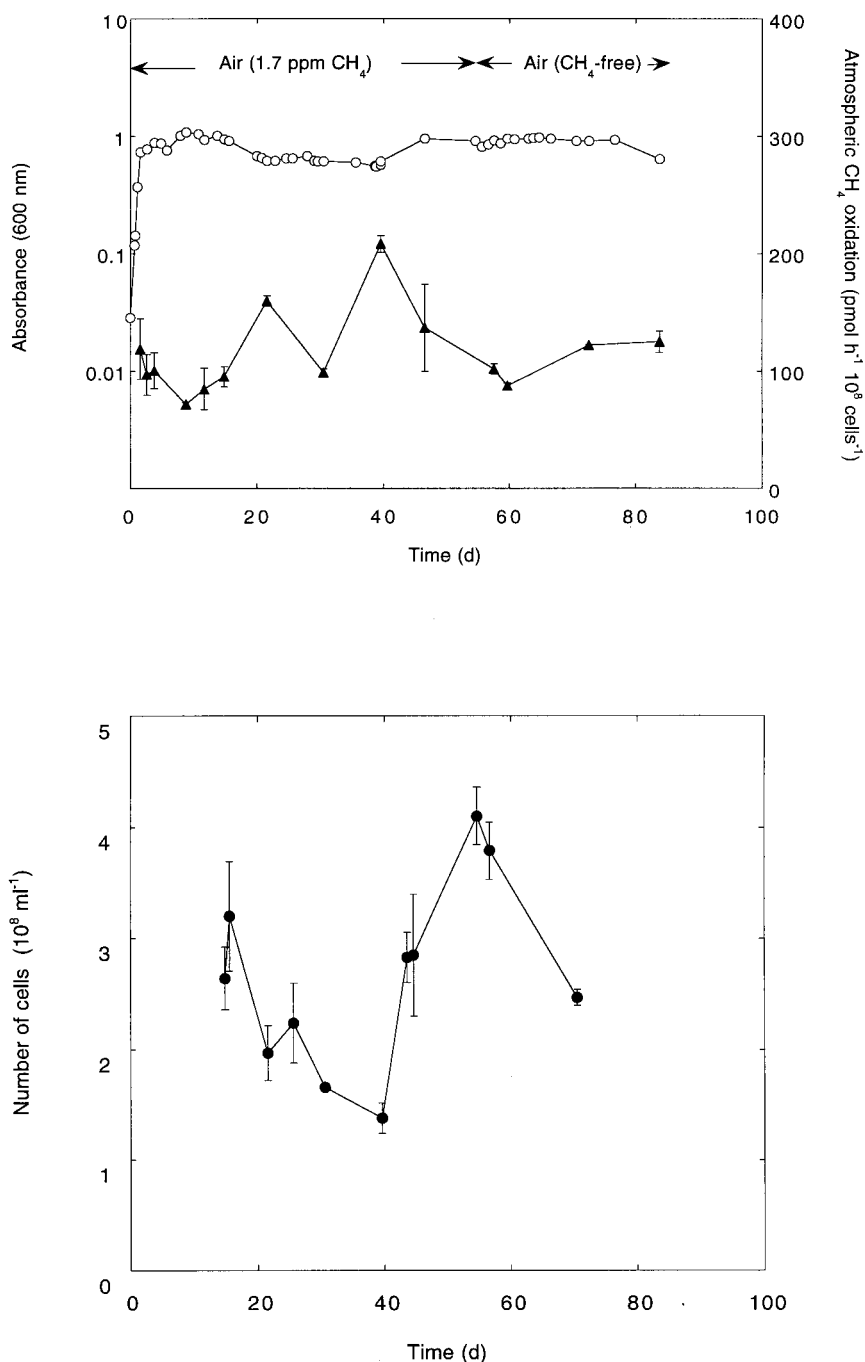


FIG. 3. Atmospheric methane oxidation during continuous cultivation of *M. albus* BG8 under methanol limitation. ○, absorbance; ▲, methane oxidation; ●, cell number. Data are means of triplicate determinations  $\pm$  1 standard error. d, days.

about 45% (Table 2). Incorporation was relatively high for both methanol-limited continuous culture of *M. albus* BG8 and *M. albus* BG8 grown in batch on methanol; higher values were obtained from the latter during exponential growth (Table 2).

**Effect of methanol additions on soil methane consumption.** The addition of methanol ( $2.5 \mu\text{mol g}^{-1}$  [fresh weight] of soil<sup>-1</sup>) resulted in a rapid inhibition of soil methane consumption (Fig. 6). However, 5 days after treatment, the rate constants for atmospheric methane consumption were significantly higher than those for the control treatments (Student's *t* test;  $P <$

0.05). No stimulatory effect on methane consumption was observed after the addition of  $0.05 \mu\text{mol}$  of methanol g (fresh weight) of soil<sup>-1</sup> (Fig. 6).

## DISCUSSION

The ability to maintain atmospheric methane consumption for extended periods is one of the primary traits distinguishing soil methanotrophs from known methanotrophic cultures. The latter can consume atmospheric methane only transiently after

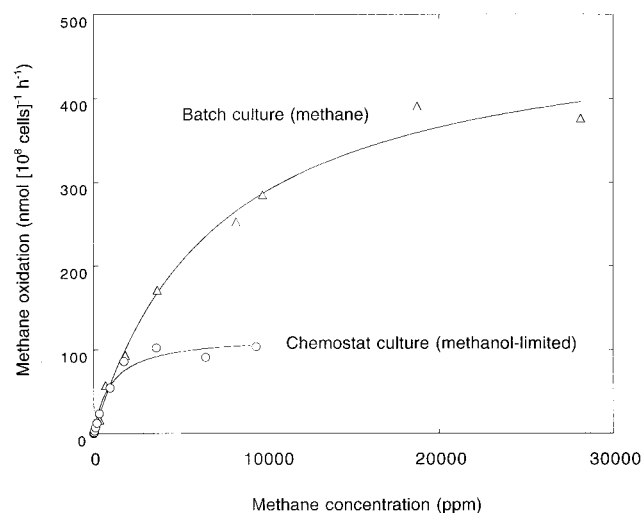


FIG. 4. Rates of methane consumption by *M. albus* BG8 at concentrations between 500 and 30,000 ppm.

growth at high methane concentrations and are unable to grow with atmospheric methane as a sole source of carbon and energy (30). However, results presented here demonstrate that *M. albus* BG8 (a group I methanotroph) retained the ability to oxidize atmospheric methane when grown in continuous culture with methanol. Such cultures may serve as models for soil methane consumption and provide predictive insights regarding the behavior of soil methanotrophy with respect to controls and responses to methane deprivation. Although physiologically novel methanotrophs growing solely on atmospheric methane may yet be proven to exist in soils, long-term atmospheric methane consumption by *M. albus* BG8 when using methanol suggests as an alternative that methanotrophic activity in soils could be supported by substrates other than methane.

Of the various nonmethane substrates, methanol is perhaps the most important as a supplement for methanotrophs. It can be derived from methoxylated sugars (e.g., pectins) and various aromatics in lignin (19). These sources likely dwarf terrestrial sources for other  $C_1$  substrates, such as methylamines. The significance of other substrates, especially multicarbon substrates, appears minimal (30). A few reports have indicated that select multicarbon substrates (e.g., glucose) support growth of facultative methanotrophs (e.g., *Methylobacterium* spp. [26, 34]). However, these organisms are not considered dominant in soils, although this may be a reflection of the conditions used for enrichments and isolation procedures (14). In addition, *M. albus* BG8 grown on methanol does not subsequently grow on common multicarbon substrates, suggesting that the inability to use multicarbon substrates is not a function of repression by methane.

TABLE 1. Kinetics of methane uptake by *M. albus* BG8<sup>a</sup>

Treatment	Methane concn	$K_m$ (ppm)	$V_{max}^b$
Batch (methane)	20%	$5,025 \pm 1,234$	$648 \pm 195$
Chemostat (methanol limited)	1.7 ppm	$916 \pm 235$	$133 \pm 46$
	<0.03 ppm	$1,119 \pm 42$	$133 \pm 45$

<sup>a</sup> Results are the means for triplicate cultures  $\pm$  standard errors of the means.

<sup>b</sup> Nanomoles of  $CH_4$   $10^8$  cells<sup>-1</sup> h<sup>-1</sup>.

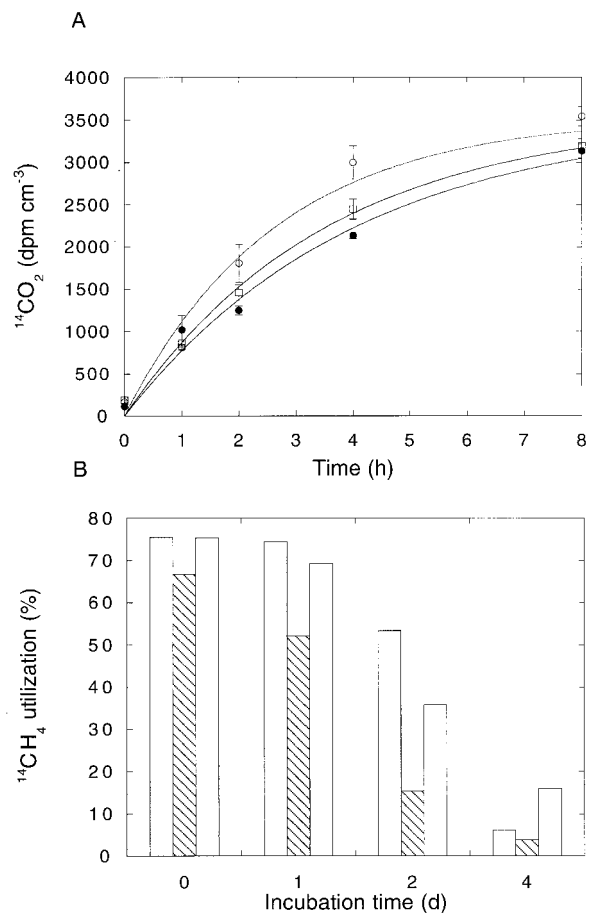


FIG. 5.  $^{14}CH_4$  utilization by pure cultures of methanotrophic bacteria incubated with ambient air. (A)  $^{14}CO_2$  production.  $\circ$ , *M. parvus*;  $\square$ , *M. albus* BG8;  $\bullet$ , *M. trichosporium* OB3b. (B)  $^{14}CH_4$  utilization.  $\square$ , *M. parvus*;  $\text{hatched}$ , *M. albus* BG8;  $\blacksquare$ , *M. trichosporium* OB3b. Data are means of triplicate determinations  $\pm$  1 standard error. d, days.

Methanol can act as a sole source of carbon and energy for methanotrophs (9). Group I methanotrophs (e.g., *Methylobacter* and *Methylomonas* spp.) appear to grow readily on it. Although methanol can be toxic to group II methanotrophs (e.g., *Methylosinus* and *Methylocystis* spp.), they have been preadapted to growth on high methanol concentrations (15). The ability of group II taxa to use methanol is significant because

TABLE 2. Percent incorporation of radiolabelled  $^{14}CH_4$  into biomass and cell metabolites by various cultures of methanotrophs<sup>a</sup>

Time (days)	% Incorporation of $^{14}CH_4$				
	<i>M. parvus</i>	<i>M. trichosporium</i>	<i>M. albus</i>	<i>M. albus</i> Ch	<i>M. albus</i> BM
0	$4.5 \pm 0.8$	$6.1 \pm 0.4$	$2.0 \pm 0.0$	$38.8 \pm 2.2$	$29.4 \pm 1.1$
1	$5.4 \pm 0.2$	$5.1 \pm 0.1$	$6.9 \pm 1.6$		
2	$5.0 \pm 0.7$	$27.1 \pm 4.9$	$23.8 \pm 1.6$		
4	$8.5 \pm 0.3$	$10.1 \pm 0.6$	$45.3 \pm 0.8$		

<sup>a</sup> *M. parvus*, *M. trichosporium*, and *M. albus* were grown under batch conditions with about 30% methane and then subjected to an incubation with atmospheric methane concentrations (see text for details). *M. albus* Ch was grown in a methanol-limited chemostat, and *M. albus* BM was grown in a batch culture with methanol; samples of the latter were obtained from stationary-phase growth. Values are means of triplicates  $\pm$  1 standard error.

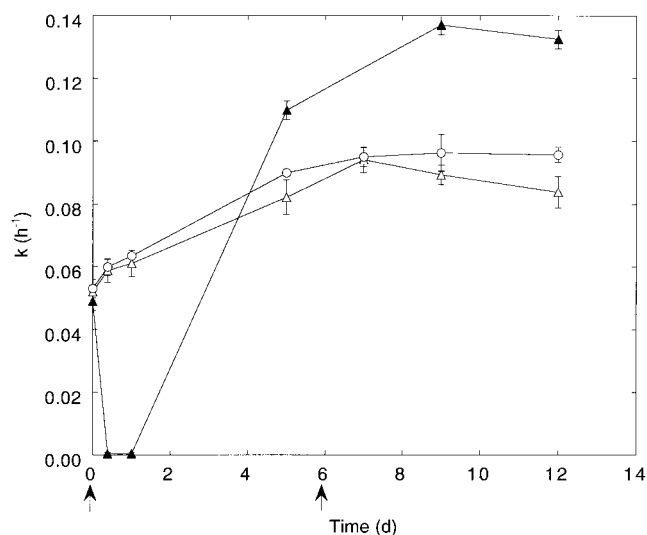


FIG. 6. Effect of methanol addition on soil methane consumption. ▲, 25  $\mu\text{mol}$ ; ○, 0.5  $\mu\text{mol}$ ; △, control treatments. Methanol additions were made at the times shown (arrows). Experiments were carried out with sieved soils. Each point represents the mean of triplicate determinations  $\pm$  1 standard error. d, days.

they have been suggested as the dominant populations in soils (14).

Though methanotrophs can use methanol, the expression and activity of MMO during growth on methanol are uncertain. For example, Hou et al. (16) have concluded that MMO was induced by methane and that growth of *M. trichosporium* OB3b, *Methylococcus capsulatus*, and *Methylobacterium organophilum* on methanol resulted in a loss of MMO activity. Other reports have shown that MMO activity was retained during growth of methanotrophs on methanol (7, 17, 23, 27). In agreement with the latter studies, *M. albus* BG8 has continuously expressed MMO during growth in a methanol-limited chemostat in the presence of atmospheric methane, in spite of the fact that methane was at most a secondary carbon source. In addition, MMO expression was observed for a 30-day period during which the chemostat was sparged with hydrocarbon-free air. Contrary to the findings of Hou et al. (16), this indicates that MMO expression is constitutive and not methane dependent.

In contrast to expression, maximum levels of *M. albus* BG8 MMO activity varied during continuous cultivation on methanol. This was evident from a decrease in  $V_{\text{max}}$  per cell compared to that of batch-grown cultures (Table 1). A decrease in  $V_{\text{max}}$  can be attributed to regulation of MMO by methane concentration (or flux). A decrease can also be explained in part by the diminution in size (about 25%) over time of methanol-grown cells relative to that of the initial inoculum. The effect of size is illustrated by an expression for diffusion-limited substrate uptake for a spherical cell, in which  $V_{\text{max}} = 4\pi r^2 k D C_0$ , where  $r$  is cell radius,  $k$  is a transport coefficient related to the number of uptake sites and time the sites are occupied by substrate,  $D$  is the diffusion coefficient, and  $C_0$  is the concentration of substrate in the bulk medium (6). Regardless of the mechanism, changes in  $V_{\text{max}}$  observed for *M. albus* BG8 suggest that cell-specific activities for soil methanotrophs are probably lower than for methanotrophs in culture; further isolates obtained by enrichment with high methane concentrations may exhibit characteristics that differ from those expressed in situ.

The affinity for methane (apparent  $K_m$ ) of pure cultures of

methanotrophic bacteria can also vary as a function of growth conditions: copper concentration affects apparent  $K_m$  (22), and apparent  $K_m$  for *M. albus* BG8 in batch culture exceeds that for continuous methanol-limited culture fivefold (Table 1). The explanation for this difference is unclear. Although  $K_m$  can be related to cell radius ( $K_m = krD^{-1}$  [6]), changes in cell size account for only a fraction of the change in  $K_m$ . This suggests the possibility of either posttranscriptional modifications of a single MMO or expression of a higher-affinity enzyme.

Alternatively, a model recently proposed by Koch (21) suggests that apparent  $K_m$  may change in response to the dynamics of substrate utilization as determined by the coupling between transport, growth, and internal substrate pools. In Koch's model, apparent  $K_m$  can change even if the amounts of transport proteins and their reaction rate constants are unaltered. The general model may be particularly useful when applied to methanotrophs because the first product of methane oxidation, methanol, is sometimes excreted and affects the kinetics of MMO. These properties indicate that the coupling of methanol to growth and regulation of intracellular methanol concentrations may be key components of a control system for methane uptake kinetics.

In any case, the ability of a methanotroph to display different kinetic properties as a function of growth conditions raises questions about the meaning of previous observations showing distinct high- and low-affinity methane uptake systems in soils (2). Do the former represent novel methanotrophs (for example, see references 2, 11, and 28), or "typical" methanotrophs adapted to growth regimens differing from those that promote significant cell proliferation? Might the low-affinity populations also express a high-affinity MMO under suitable growth conditions? If expression of a high-affinity MMO requires prolonged exposure to low (e.g., atmospheric) methane concentrations and a cosubstrate (e.g., methanol), comparisons of the properties of batch-grown cultures and soils may be misleading.

$^{14}\text{CH}_4$  metabolism illustrates further the need for examining cultures prior to drawing conclusions about either atmospheric methane consumption capacities or differences relative to soils.  $^{14}\text{CH}_4$  incorporation is generally low (<10% [Table 2]) for pure cultures incubated with near-ambient methane. Although  $^{14}\text{CH}_4$  incorporation increases somewhat over time for *M. albus* BG8, activity overall decreases and the capacity for atmospheric methane consumption is greatly diminished (Fig. 5B). In contrast, the level of  $^{14}\text{CH}_4$  incorporation by *M. albus* BG8 grown in a methanol-limited chemostat and incubated with ambient methane is significantly higher than that of either *M. trichosporium* OB3b or *M. parvus* OBBP incubated with ambient methane only (Table 2). Although  $^{14}\text{CH}_4$  incorporation by batch-grown *M. albus* BG8 after incubation for 4 days with atmospheric methane is comparable to that of cells from a methanol-limited chemostat, total methane consumption by the former is less than one-third that of the latter, and the uptake capacity for chemostat-grown cells appears to be stable indefinitely, while that of the batch culture is not.  $^{14}\text{CH}_4$  incorporation by *M. albus* BG8 from a methanol-limited chemostat is comparable to  $^{14}\text{CH}_4$  incorporation by soils, which also have a relatively stable capacity for atmospheric methane consumption (28, 30, 32). This similarity may indicate that soil methanotrophs are supported at least in part by methanol or other nonmethane substrates.

In a recent analysis, forest soil methanotrophy was unaffected by methane deprivation (5). This suggests a possible supporting role for nonmethane substrates, e.g., methanol. However, despite the ability of methanotrophs to grow on methanol, little is known about its utilization in soils. Previous



attempts to stimulate soil methanotrophic activity with a variety of C<sub>1</sub> substrates (methanol and formate) and various organics (glucose, acetate, starch, malate, and β-hydroxybutyrate) have proven unsuccessful (5, 30). In the present study, methane consumption was stimulated in soils for which no enhancement had been observed before (5, 30). The difference between this study and earlier studies may reflect differences in the mass and timing of methanol additions or differences in the physiological status of the methanotrophs. With respect to the latter point, rate constants for methane consumption in the present study were about fourfold lower than in earlier studies. A role for methanol in soil methane consumption is particularly interesting since the potential for growth of soil methanotrophs on atmospheric methane alone is limited (28). Methanol may help support the growth and activity of methanotrophs in soils by two mechanisms: (i) as a substrate for growth and energy production and (ii) as a source of reducing equivalents that are required for the continued oxidation of atmospheric methane.

In conclusion, results presented here demonstrate that methanol facilitates continued atmospheric methane oxidation by methanotrophic cultures. Consequently, isolates obtained by traditional enrichment may be representative of populations active in situ. Involvement of nonmethane substrates in soil may also explain why enrichments using high concentrations of methane as a sole carbon and energy source have apparently failed to produce high-affinity methanotrophs (30). This hypothesis should be tested by evaluating methanotrophic activity of cultures under conditions which reflect the soil environment (e.g., nutrient limitation and mixed-substrate utilization).

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