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Ammonium and Nitrite Inhibition of Methane Oxidation by *Methylobacter albus* BG8 and *Methylosinus trichosporium* OB3b at Low Methane Concentrations†

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Methane oxidation by pure cultures of the methanotrophs *Methylobacter albus* BG8 and *Methylosinus trichosporium* OB3b was inhibited by ammonium choride and sodium nitrite relative to that in cultures assayed in either nitrate-containing or nitrate-free medium. *M. albus* was generally more sensitive to ammonium and nitrite than *M. trichosporium*. Both species produced nitrite from ammonium; the concentrations of nitrite produced increased with increasing methane concentrations in the culture headspaces. Inhibition of methane oxidation by nitrite was inversely proportional to headspace methane concentrations, with only minimal effects observed at concentrations of >500 ppm in the presence of 250 μM nitrite. Inhibition increased with increasing ammonium at methane concentrations of 100 ppm. In the presence of 500 μM ammonium, inhibition increased initially with increasing methane concentrations from 1.7 to 100 ppm; the extent of inhibition decreased with methane concentrations of >100 ppm. The results of this study provide new insights that explain some of the previously observed interactions among ammonium, nitrite, methane, and methane oxidation in soils and aquatic systems.

Although methanotrophic bacteria only grow on a limited array of substrates lacking carbon-carbon bonds, they are metabolically versatile in that a variety of substrates are cometabolized (2, 3, 8, 11, 41). For example, trichlorethylene can be dehalogenated, but this process does not support growth. The numerous secondary reactions characteristic of methanotrophs result from the broad substrate specificity of the particulate and soluble methane monooxygenases (MMOs; see reference 3 for a recent review).

In addition to a variety of aromatic, aliphatic, and halogenated substrates, both of the MMOs oxidize ammonium (2, 11). In fact, the ability of methanotrophic bacteria to oxidize ammonium to nitrite is a well-documented process (3, 10, 12, 17, 44) that has led some to suggest that methanotrophic bacteria dominate nitrification in certain aquatic systems and soils (3, 25). While there is limited direct evidence in support of this proposition (29–31), kinetic analyses indicate that methanotrophs have a lower affinity and maximal uptake velocity for ammonium than ammonia-oxidizing bacteria (3). Thus, the role of methanotrophs in nitrification remains uncertain.

The role of ammonium as an inhibitor of methane oxidation is also uncertain, even though kinetic analyses of cultures and cell extracts indicate that ammonium is a competitive substrate for MMO (10, 34, 44). In contrast to culture data, which suggest minimal effects of ammonium or nitrite on methane uptake in all but extreme cases (e.g., >10 mM ammonium [10, 43, 44]), data from soils indicate that methane consumption is very sensitive to ammonium (1, 32, 33, 40). Even relatively small additions of ammonium fertilizers substantially inhibit atmospheric methane consumption; the inhibition apparently persists even after the added nitrogen is undetectable as either ammonia or other inorganic species (16, 32, 33). The previously reported relative insensitivity of cultures to ammonium may be a function of the high methane concentrations (>14 to 140 μM; >1 to 10% gas phase concentrations) used in most studies. At these concentrations, the responses of pure cultures may not reflect the responses that occur under in situ conditions, when methane concentrations are typically low (<1 to 10 μM) and ammonium levels seldom exceed 1 mM.

The results reported here demonstrate that methane consumption is inhibited by both ammonium and nitrite in pure cultures of two methanotrophs, *Methylobacter albus* BG8 and *Methylosinus trichosporium* OB3b. The extent of inhibition varies nonlinearly as a function of the methane and nitrogen concentrations. The data show enhanced inhibition by ammonium as the methane level increases from atmospheric levels to 100 ppm, with decreased inhibition thereafter. This pattern and the time courses of nitrite production suggest that ammonium inhibition is the result of direct effects due to the interaction of ammonia with MMO and indirect effects resulting from nitrite.

MATERIALS AND METHODS

Cultures of *M. albus* BG8 and *M. trichosporium* OB3b were grown at approximately 30°C on ultra-high-purity methane in a mineral salts medium (NMM), pH 7, containing sodium nitrate as a nitrogen source (23). The responses of *M. albus* and *M. trichosporium* to nitrite and ammonium were assayed by first growing the cells in batch culture in sealed Erlenmeyer flasks (total volume, 1 to 2 liters), with headspaces initially containing about 30% methane. Cells were harvested during exponential growth (A_{600} = 0.3 to 0.4) by centrifugation at 4°C and 10,000 × *g*. The pellets were washed twice with a phosphate buffer (pH 7) and resuspended to an A_{600} of about 0.3 with either phosphate-buffered (10 mM, pH 7) NMM medium or nitrogen-free medium. Ten milliliters of the final cell suspension was pipetted into 160-ml serum bottles. Ammonium chloride, sodium nitrite, or sodium nitrate was added to the bottles from stock solutions for specific analyses of culture responses. The

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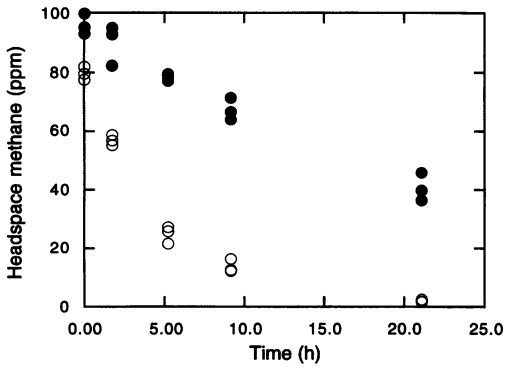

FIG. 1. Time course of methane uptake by triplicate cultures of *M. albus* incubated with 100 ppm of headspace methane and 0 (○) or 2,000 (●) μM ammonium chloride.

bottles were sealed with green neoprene rubber stoppers, and methane was injected into the headspaces as necessary to produce initial concentrations from 1.7 to 1,000 ppm. The serum bottles were incubated at about 30°C with rotary shaking (120 rpm). All treatments were conducted in triplicate.

Methane concentrations in the serum bottle headspaces were analyzed by removing 0.3 cm^3 volumes at intervals by needle and syringe for gas chromatography as described previously (21). Uptake rates or uptake rate constants were estimated from regression analyses of the methane time courses with an exponential model for concentrations of <1,000 ppm. Nitrite production was assayed with a Beckman DU-650 spectrophotometer by the method of Greenberg et al. (13) with 2-ml subsamples obtained at the termination of an experiment in which replicate cultures were incubated with 500 μM ammonium chloride and various methane concentrations.

RESULTS

Methane uptake in most assays was exponential in character (Fig. 1), and errors associated with the means of triplicates were sufficiently low to allow clear distinctions among treatments. Cell biomass, as indicated by absorbance analyses, did not change appreciably during the incubation periods in spite of variations in methane uptake rates due to treatment with ammonium or nitrite (Fig. 2). The stability of the absorbance indicated that the observed variations were due to changes in cell-specific activities, not in cell biomass.

Methane consumption by *M. albus* and *M. trichosporium* was inhibited by ammonium (Fig. 1 to 3). The extent of inhibition varied as a function of the ammonium and headspace methane concentrations. Relative to nitrogen-free controls, inhibition was apparent at 100 μM ammonium with a methane headspace of 100 ppm (equivalent to approximately 140 nM). The greatest effects were observed at between 100 and 500 μM ammonium, with little additional response between 1,000 and 2,000 μM (Fig. 2). The extent of ammonium inhibition was similar for both cultures: 44.3 and 41.2% at 200 μM ammonium and 76.4 and 82.5% at 2,000 μM ammonium for *M. albus* and *M. trichosporium*, respectively. With headspace methane concentrations of 100 ppm, methane uptake was first order in appearance, and the effect of ammonium on first-order uptake rate constants was readily discernible (Fig. 1).

Ammonium inhibition was related nonlinearly to headspace methane concentrations (Fig. 3). The extent of inhibition increased progressively as concentrations were increased from atmospheric levels (1.7 ppm) to 100 ppm in the presence of 500

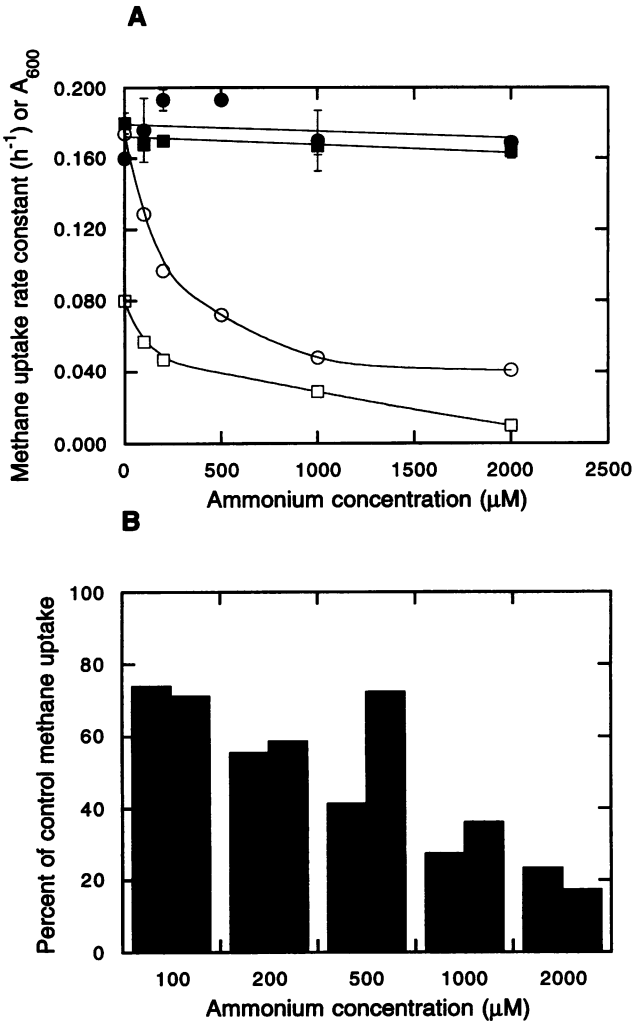

FIG. 2. (A) Methane uptake rate constants (open symbols) and absorbance values (solid symbols) for cultures of *M. albus* (○) and *M. trichosporium* (■) incubated with 100 ppm of headspace methane and various concentrations of ammonium chloride. Values are means of triplicates ± 1 standard error (some error bars are within symbols). (B) Methane uptake as a percentage of the control (no added ammonium) rates for *M. albus* (dark gray bars) and *M. trichosporium* (light gray bars).

μM ammonium; the extent of inhibition decreased with methane concentrations of ±250 ppm. For *M. trichosporium*, ammonium-treated and control rates were equivalent at a methane level of 1,000 ppm. Under the same conditions, *M. albus* proved to be more sensitive to ammonium inhibition, with uptake rates only 61.6% of that of controls at a methane level of 1,000 ppm.

Exogenous nitrite also effectively inhibited methane consumption by whole cells. For incubations with headspace methane concentrations of 100 ppm, 50 μM nitrite decreased uptake by about 28% in *M. albus*, while 250 μM nitrite was required for a similar level of inhibition (25%) in *M. trichosporium* (Fig. 4). At 1,000 μM nitrite, uptake by *M. albus* was inhibited to a much greater extent (71.9%) than was uptake by *M. trichosporium* (41.2%). As observed for ammonium, the sensitivity of both cultures to nitrite inhibition was dependent on headspace methane concentrations. In contrast to the case

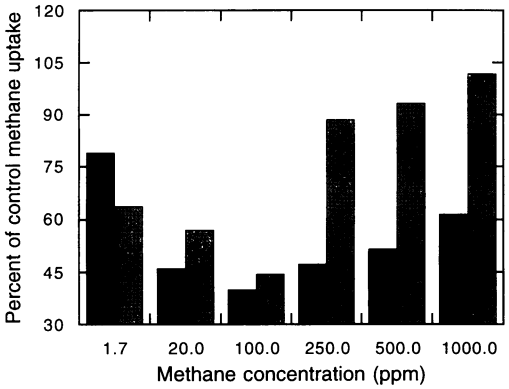

FIG. 3. Methane uptake in cultures of *M. albus* (dark gray bars) and *M. trichosporium* (light gray bars) incubated with various headspace methane concentrations and 500 μM ammonium as a percentage of uptake in controls incubated in nitrogen-free medium.

for ammonium, however, the strongest inhibition for cultures incubated with 250 μM nitrite occurred with atmospheric methane levels; the extent of inhibition decreased progressively with increasing methane concentrations and was negligible at 500 to 1,000 ppm (Fig. 5).

While nitrite inhibition was clearly lower at elevated methane concentrations, nitrite production from exogenous ammonium was strongly stimulated by increasing methane levels (Fig. 6A). With atmospheric levels of methane and 500 μM ammonium, nitrite reached levels of about 11 and 0.5 μM for *M. albus* and *M. trichosporium*, respectively; with methane at 1,000 ppm and 500 μM ammonium, nitrite accumulated to about 131 and 48 μM for the two respective cultures. The amounts of nitrite produced per unit amount of methane consumption were also substantially higher for *M. albus* than for *M. trichosporium* (Fig. 6B). In addition, total nitrite production was less than or similar to the difference in total methane consumption for *M. trichosporium* cultures incubated with or without ammonium over a range of methane concentrations from 1.7 to 1,000 ppm (Table 1). In contrast, nitrite production greatly exceeded the difference in uptake for *M. albus* cultures incubated with or without ammonium and methane concentrations of ≤20 ppm; however, nitrite production was much lower than this differential for cultures incubated with methane concentrations of from 100 to 1,000 ppm (Table 1).

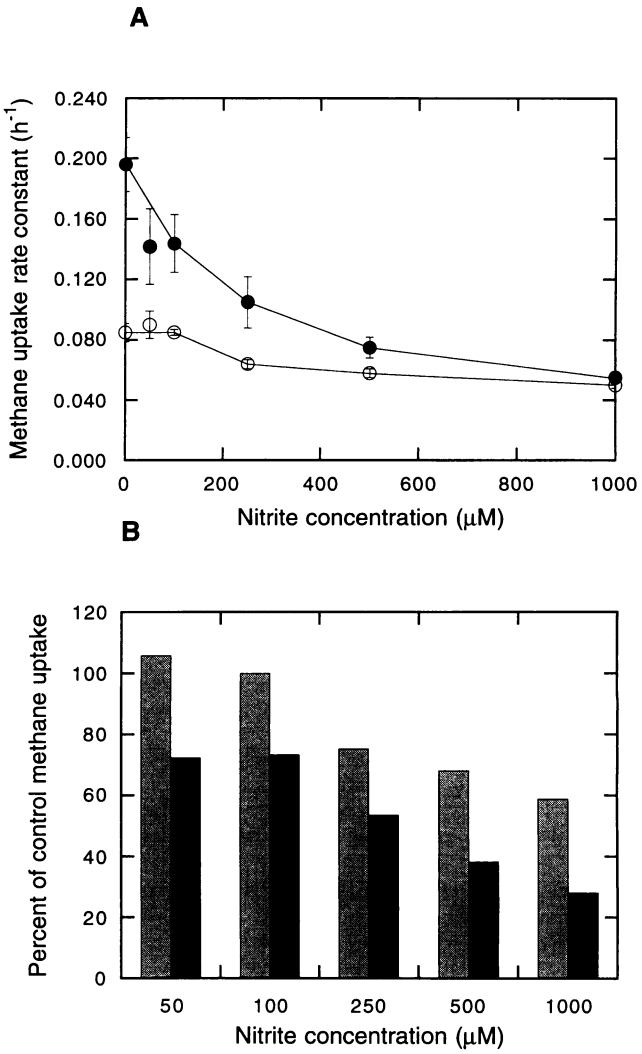

FIG. 4. (A) First-order methane uptake rate constants for cultures of *M. albus* (●) and *M. trichosporium* (○) incubated with headspace methane concentrations of 100 ppm and various concentrations of sodium nitrite. Values are means of triplicates ± 1 standard error. (B) Methane uptake as a percentage of control (no added nitrite) rates for *M. albus* (dark gray bars) and *M. trichosporium* (light gray bars).

DISCUSSION

The patterns of ammonium inhibition observed here agree generally with the data of Whittenbury et al. (43), who reported that ammonium concentrations of >1 to 10 mM inhibited oxidation of 5% methane. Ammonium inhibition has been attributed to competition between methane and ammonia for the active site of MMO (12, 34), but the mechanism appears to be complex. For example, Dalton (10) showed that ammonium oxidation by cell extracts of *Methylococcus capsulatus* required NADH plus H^+ and that the response to both NADH plus H^+ and ammonium could not be analyzed by a simple kinetic model. Dalton (10) has also shown that high concentrations of methane (>5%) inhibited oxidation of >5 mM ammonium, but the mechanism was not discernible. The complexity of ammonium-methane interactions is illustrated further by data from O'Neill and Wilkinson (34), who have reported that ammonium oxidation by *M. trichosporium* is inhibited by 8% methane but stimulated by 0.8% methane. They have also shown that a variety of other substrates linked to NADH plus H^+ formation, e.g., formate, formaldehyde, and 3-hydroxybutyrate, stimulate ammonium oxidation. Similarly, Knowles and Topp (25) have shown that approximately 0.7% methane stimulates nitrite production from ammonium by *M. trichosporium* but that higher concentrations are inhibitory.

The requirement of MMO activity for a reductant provides a basis for understanding the increase in ammonium inhibition with increases in methane concentrations from 1.7 to 100 ppm (Fig. 3). The reductant apparently limits ammonium oxidation over this range. Although rates of methane consumption increase with increasing methane concentrations (Table 1), ammonium co-oxidation decreases cell-specific methane uptake rates. This effect becomes more pronounced as methane concentrations approach 100 ppm. However, at methane concentrations of ≥250 ppm (Fig. 3), the relative extent of ammonium inhibition decreases, presumably because of de-

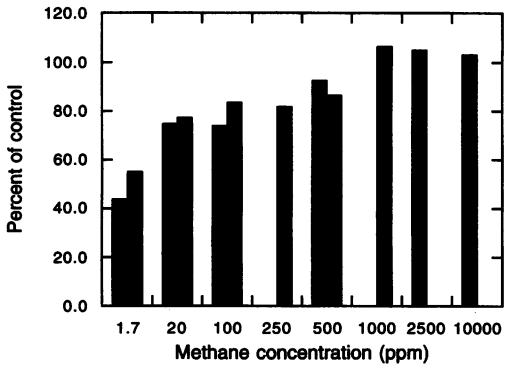

FIG. 5. Methane uptake in cultures of *M. albus* (light gray bars) and *M. trichosporium* (dark gray bars) incubated with various headspace methane concentrations and 250 μM sodium nitrite as a percentage of uptake by controls incubated in nitrogen-free medium.

creased binding of ammonia to MMO and decreases in the toxic effects of ammonium metabolites.

Ammonium inhibition of methane consumption is not simply the result of substrate competition at the level of MMO, however. The first product of ammonium oxidation, hydroxylamine, reversibly inhibits MMO activity in whole cells at <0.1 mM (15). Nitrite, which is readily formed from hydroxylamine, is a known inhibitor of the *M. trichosporium* formate dehydrogenase as well as whole cells of various methanotrophs (19, 34, 43) (Fig. 4 and 5). Inhibition of formate dehydrogenase is particularly significant because this enzyme produces NADH plus H^+. Nitrite might also directly affect MMO by binding reversibly at the active site.

The results from this study show that both nitrite production and the extent of nitrite inhibition of methane consumption depend on methane concentrations (Fig. 5 and 6A). The stimulation of nitrite production with increasing methane concentrations from 1.7 to 1,000 ppm agrees with a model in which ammonium oxidation by MMO is reductant (i.e., methane) limited. Although ammonia is not a particularly potent competitor for MMO relative to methane (3), competitive interactions alone do not determine ammonium oxidation rates at low methane concentrations. If the kinetics of competition were the major determinant, nitrite production would decrease with increasing methane concentrations. In contrast, nitrite production increased progressively from 1.7 to 500 ppm methane, with a plateau observed between 500 and 1,000 ppm (Fig. 6A). Inhibition of nitrite production (ammonium oxidation) might occur at higher methane concentrations (34, 44), but such concentrations have little relevance in situ.

The inhibition of methane consumption by exogenous nitrite also depends on methane concentrations. However, unlike nitrite production, nitrite inhibition decreases with increasing methane levels (Fig. 5). The effect is more pronounced in *M. trichosporium* than in *M. albus*, which generally shows greater sensitivity to nitrite inhibition. Differences in sensitivity are particularly evident in comparisons of nitrite formation and the decrease in methane consumed by cultures incubated with ammonium relative to control cultures (Table 1). For *M. trichosporium*, nitrite accumulation is largely equivalent to the decrease in methane consumption by ammonium-treated cells. This indicates that nitrite per se has minimal secondary toxic effects relative to direct inhibition by ammonium. Limited nitrite toxicity agrees with the results of Yoshinari (44), who found no evidence for inhibition of methane oxidation or growth by *M. trichosporium* incubated with 0.1 to 10 mM

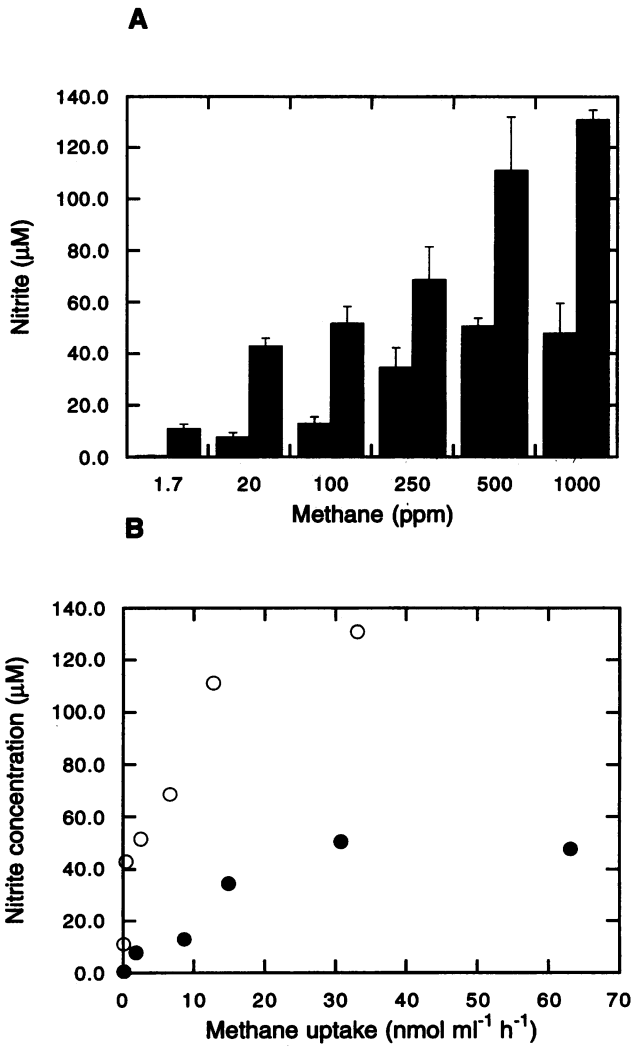

FIG. 6. (A) Nitrite production by cultures of *M. albus* (light gray bars) and *M. trichosporium* (dark gray bars) incubated with various headspace methane concentrations and 500 μM ammonium chloride; values are means ± 1 standard error for triplicate determinations. Cultures were incubated for 18 to 19 h prior to the nitrite assay. (B) Nitrite concentrations from panel A versus volume-specific methane uptake rates for cultures of *M. albus* (○) and *M. trichosporium* (●); for both cultures, increasing methane uptake rates correspond to increasing methane concentrations, as indicated for the ordinate of panel A.

ammonium chloride and headspace methane concentrations of about 2.5%. However, for *M. albus*, nitrite accumulation only partially accounts for the difference between methane consumed by cells incubated with and without ammonium (Table 1), suggesting that secondary, toxic effects of nitrite contribute significantly to inhibition in these organisms. The mechanism of nitrite toxicity is at present unclear, as is the source of differences between *M. albus* and *M. trichosporium*. However, the amelioration of nitrite inhibition by methane (Fig. 5) indicates that energy- or reductant-dependent transformations or transport is involved.

The sensitivity of methanotrophs to ammonium and nitrite has important ecological consequences. Low methane concentrations often occur in the zones of greatest methanotrophic activity. Soils typically contain ≤2.5 nM dissolved methane

TABLE 1. Methane consumption and nitrite production in cultures of *M. albus* BG8 and *M. trichosporium* OB3b incubated with various methane concentrations and nitrogen-free medium or medium with 500 μM ammonium chloride

Strain	Methane headspace concn (ppm)	Mean methane uptake (nmol/ml of culture/h) ± 1 SE		Δ[a] (nmol)	Total nitrite produced[b] (nmol)
		Control	Ammonium treated		
BG8	1.7	0.09 ± 0.03	0.07 ± 0.04	4	111
	20	0.9 ± 0.1	0.4 ± 0.02	92	430
	100	6.3 ± 0.5	2.5 ± 0.3	684	517
	250	14.0 ± 1.3	6.6 ± 0.7	1,320	687
	500	24.7 ± 1.8	12.7 ± 0.7	2,163	1,112
	1,000	53.7 ± 3.3	33.1 ± 2.2	3,740	1,309
OB3b	1.7	0.11	0.07	8	5
	20	1.8 ± 0.1	1.0 ± 0.3	146	78
	100	8.6 ± 0.3	3.8 ± 0.3	912	130
	250	14.9 ± 0.5	13.2 ± 0.6	321	346
	500	30.8 ± 0.8	28.7 ± 1.9	390	507
	1,000	63.1 ± 0.7	64.3 ± 2.7	−222	479

[a] Δ, difference between total methane uptake by control and ammonium-treated cultures during the incubation interval (about 19 h).
[b] Total nitrite produced during the incubation interval.

(equivalent to equilibrium with an atmosphere containing ≤1.7 ppm [1, 4]); dissolved methane in the water column of freshwater systems often occurs at 1 to 2 μM concentrations (equivalent to equilibrium with a gas phase containing about 1,000 ppm [14, 27, 37]); dissolved methane in the marine water column is usually ≤10 to 15 nM (equivalent to equilibrium with a gas phase containing <10 ppm methane [35, 39, 42]). Even in the active methanotrophic zones of sediments, dissolved methane concentrations of <10 μM have been reported (21, 27, 36). In contrast, ammonium concentrations may be relatively high; values of ≥1 to 100 μM are reported often (14). As a result, ammonium may constrain methane oxidation, thereby enhancing fluxes from aquatic systems or decreasing uptake by soils. The potential for ammonium inhibition has been shown previously for lake, wetland, and rice paddy sediments (5, 9, 21) and has been documented for soils (1, 32, 33, 38, 40). Although inhibition of atmospheric methane consumption in soil has been attributed to ammonium-related shifts in methane consumption by ammonia-oxidizing bacteria (32, 40), Jones and Morita (20) have reported that ammonium stimulated methane oxidation by ammonia-oxidizing bacteria when both substrates were present at low concentrations. The similarity between the responses of soils (24, 38) and cultures of methanotrophs to ammonium suggests that inhibition of soil methane consumption is explained better by the physiology of methanotrophs than by the activities of ammonia oxidizers.

The results of this study also indicate that group I and group II methanotrophs (e.g., *M. albus* and *M. trichosporium*, respectively) might differ with respect to ammonium and nitrite sensitivity. These differences, along with other well-known physiological and biochemical differences (6, 11, 22), may affect the outcome of competitive interactions between the two groups and determine their relative distributions in natural systems. For example, when nitrate but not ammonium is available as a nitrogen source, group I methanotrophs may predominate because of their higher growth yields; if ammonium is abundant, group II methanotrophs could be favored. Of course, these simplistic predictions do not consider variations among each group with respect to ammonium or nitrite sensitivity. For example, Krämer et al. (26) have found higher rates of nitrite production by *M. trichosporium* than by *Methylosinus agile* for cultures incubated with about 10% methane and 1.9 mM ammonium chloride. Although Krämer et al. did not examine the inhibitory effects of either ammonium or nitrite (26), no such effects would be expected on the basis of the culture conditions. Nonetheless, the patterns of nitrite production observed by Krämer et al. (26) and reported here might indicate that *M. agile* is less sensitive to ammonium or nitrite inhibition than is *M. albus*. In this context, it is also important to note that Lees et al. (28) have described two group I marine isolates that apparently were unable to transport ammonium efficiently, a characteristic unusual among known methanotrophs (28). Though unusual, a limited capacity for ammonium uptake would have selective advantages in systems where the potential for ammonium inhibition is significant, e.g., systems with low methane concentrations relative to ammonium. Some agricultural soils also show an apparently ammonium-insensitive capacity for atmospheric methane uptake (7, 16). This could be attributed to methanotrophic populations with a limited capacity for ammonium transport. Clearly, a greater understanding of the physiology and biochemistry of methanotrophic nitrogen transformations will contribute to a better understanding of the distribution of methanotrophs and methane oxidation in situ.

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