# Control of Defined Methanotrophic Populations in Soils by Co-metabolism of Ammonium

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## Summary

Metabolism of inorganic nitrogen (N) by soil microbial communities is heavily impacted by increasing N-loads from anthropogenic sources such as fertilizers and nitrogenous air pollutants. Nitrification, the oxidation of ammonia-N to nitrite/nitrate-N, and denitrification, the reduction of nitrite/nitrate-N to nitrogen oxides and dinitrogen, are well-characterized processes. Likewise, microbial communities performing these processes have been intensively studied. Less well characterized are methane-oxidizing bacteria (MOB), which predominantly convert methane to carbon dioxide, in their capacity to perform both nitrification and partial denitrification in terrestrial ecosystems. In this project we: 1) compared growth kinetics of four methanotrophic bacterial strains in media with ammonia versus nitrate as the N source, 2) examined the capacity of each strain to oxidize ammonia and hydroxylamine (the intermediate of ammonia oxidation) to nitrite, 3) examined the influence of ammonia and nitrite on methane oxidation potential, 4) determined differences in methane-oxidizing enzymes that could account for differences in ammonia oxidation rates, and 5) identified a hydroxylamine oxidoreductase homologue in one strain. The ultimate goals of this project were to: 1) determine the point at which ammonia (or nitrite) becomes a deterrent rather than a benefit to methane oxidation, and 2) characterize the enzymatic components in diverse MOB that oxidize ammonia to nitrite via hydroxylamine. We discovered that MOB respond very differently to ammonia; while the bacteria all grew efficiently with ammonia as an N-source, they had significantly different capacities for oxidizing ammonia to nitrite. This difference was not attributable to differences in *pmoA* gene sequences that encode the catalytic subunit of methane monooxygenase. While three of the four isolates could oxidize ammonia to nitrite via hydroxylamine, only one of the three was found to have a conserved gene encoding hydroxylamine oxidoreductase. This study demonstrated for the first time that not all MOB are capable of dissimilatory ammonia oxidation nor do they all have identifiable gene inventories to carry out ammonia oxidation to nitrite. The capacity for MOB to co-metabolize ammonia rather than assimilate it, especially in N-impacted soils, influences the composition and fitness of the MOB community, which in turn determines the methane oxidizing capacity of soils.

## Objectives

Objective 1: We grew cultivated methanotrophic species in AMS (ammonium mineral salts) and NMS (nitrate mineral salts) media (30% $CH_4$) and monitored methane, carbon dioxide, nitrous oxide, and nitrite concentrations in addition to cell numbers from lag to stationary phase.

Objective 2: We determined the kinetics of ammonia oxidation to nitrite by each species in the absence and presence of reductant. As co-metabolism by methane monooxygenase requires

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electrons (reductant), either methane or formate was included as a reductant source. *pmoA* genes were cloned and sequenced from each strain to determine whether differences in PmoA protein sequences were responsible for differences observed in rates of ammonia co-metabolism.

Objective 3: We determined rates of hydroxylamine oxidation to nitrite and examined the presence of hydroxylamine oxidoreductase homologues in each species.

Objective 4: We determined the influence of ammonia and nitrite on the methane oxidizing capacity of each species.

# Approach and Procedures

*Methylomicrobium album* ATCC 33003 (*M. album*), *Methylomonas methanica* (*M. methanica*; from J. Gulledge, Univ. Louisville), *Methylosinus sporium* ATCC 35069 (*M. sporium*), and *Methylocystis* sp. ATCC 49242 (*Methylocystis* sp.) were grown in batch cultures (100 or 250 ml) in nitrate mineral salts (NMS) or ammonia mineral salts (AMS) at pH 6.8 under 50-50% air-methane atmosphere in the dark at 30 $^{o}$C with shaking (200 rpm). $CH_4$ consumption and $CO_2$ production was measured over time by GC-TCD (Hayesep Q column). Nitrite (Frear and Burrell 1955) and hydroxylamine (Dalton 1977) were measured colorimetrically. Cell growth was measured by direct microscopic count using a Petroff-Hausser chamber under phase contrast light microscopy. Protein content was measured colorimetrically (Gornall et al.1949). For activity assays, cells were harvested in mid-log phase by centrifugation and washed and resuspended in HEPES buffer (1 ml total vol, 10 mM, pH 6.8). Known numbers of cells were resuspended in HEPES buffer (1 ml final volume, 10 mM, pH 6.8) in 10 ml glass vials, which were sealed with butyl rubber stoppers and aluminum crimp seals and incubated at 30$^{o}$C with shaking (200 rpm). Ammonia oxidation to nitrite, hydroxylamine oxidation to nitrite, and methane consumption in the absence and presence of ammonia or nitrite was measured. $K_m$ and $V_{max}$ for ammonia oxidation kinetics were calculated from Lineweaver-Burke plots. Negative controls for all assays included heat-killed cells. At least three experiments with triplicate assays were conducted using cells grown in separate batch cultures. Universal 16S rRNA primers (Giovannoni et al. 1995) and conserved primers for *pmoA1* and *pmoA2* (Yimga et al. 2003) were used to PCR-amplify genes from each MOB species. Amplification products were TA-cloned (Invitrogen) and bi-directionally sequenced. 16S rRNA gene sequences were assembled and aligned using the RDP server and Greengenes alignment packages (http://rdp.cme.msu.edu/myrdp/help.spr#uploadgrp; http://greengenes.lbl.gov/cgi-bin/nph-NAST_align.cgi). The tree was constructed using the MEGA software package (http://www.megasoftware.net/). Translated *pmoA* sequences were aligned and visualized using GeneDoc (http://www.psc.edu/biomed/genedoc).

# Results

*Identification of isolates.* Phylogenetic analysis of 16S rRNA gene sequences revealed that the Type II MOB, *M. sporium* ATCC 35069 and *Methylocystis* sp. ATCC 49242, were 100% identical to their nearest neighbors, *M. sporium* NSIMB 11126 and *Methylocystis sp.* KS8a, respectively (*fig. 1*). The Type I MOB, *M. album* ATCC 33003 and *M. methanica* had more divergent 16S rRNA gene sequences relative to their nearest neighbors, but were within the species range at >97% sequence identity (*fig. 1*)(Stackebrandt and Goebel 1994).

*Growth of isolates*. All the isolates had similar generation times, but generally grew more slowly in AMS than in NMS media. This was likely due to competition of ammonia with methane for pMMO enzymes, which slows methane oxidation (King and Schnell 1994). The Type I bacteria, *M. album* and *M. methanica*, produced the same or slightly less biomass in AMS than in NMS media, whereas the Type II bacteria, *Methylocystis* sp. and *M. sporium* produced

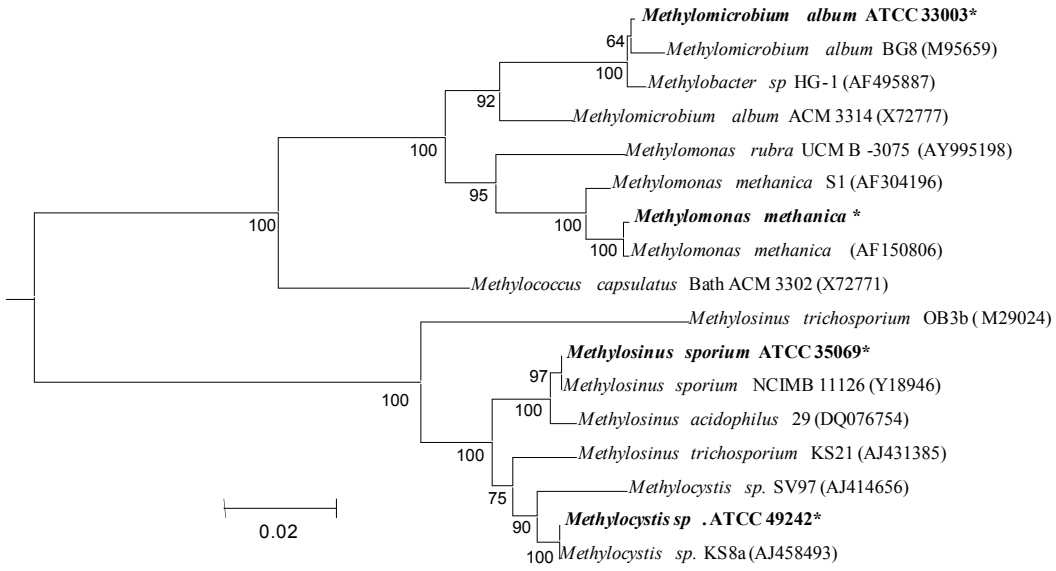


Fig. 1 Phylogenetic tree of 16S rRNA gene sequences of methanotrophic bacteria. The tree is rooted with a 16S rRNA gene sequence from *Bacillus subtilis*.

slightly more biomass, on average, in AMS media. As Type I and Type II MOB have different carbon assimilation pathways (Hanson and Hanson 1996), the Type II strains exhibited higher rates of methane consumption and $CO_2$ production than the Type I MOB (*table 1*). Interestingly, nitrite production was significantly higher for all the isolates in NMS than in AMS media, except for *Methylocystis* sp. This difference may have been due to the escape of nitrite from cells during assimilatory reduction of nitrate relative to the more efficient and direct assimilation of ammonium into biomass.

***Table 1.*** *Growth parameters for methanotrophic isolates.*

| Isolate | Gen. time (h) | Final # (x $10^8$·$ml^{-1}$) | $CH_4$ Cons. (μmol·$h^{-1}$) | $CO_2$ Prod. (μmol·$h^{-1}$) | $NO_2^-$ Prod. (nmol·$h^{-1}$) |
|---|---|---|---|---|---|
| NMS media | | | | | |
| *M. album* | 3.8 (0.2) | 2.2 (0.7) | 22 (11) | 14 (1) | 2 (0.4) |
| *M. methanica* | 3.5 (0.2) | 2.8 (0.3) | 26 (3) | 13 (0.8) | 0.6 (.01) |
| *Methylocystis sp.* | 3.7 (0.5) | 2.6 (0.2) | 53 (10) | 21 (3) | 0.4 (0.1) |
| *M. sporium* | 5.3 (0.2) | 3.5 (1.0) | 58 (1) | 19 (1) | 12 (0.4) |
| AMS media | | | | | |
| *M. album* | 4.8 (1.0) | 1.8 (0.3) | 7 (2) | 9 (0.8) | B.D.L. |
| *M. methanica* | 4.1 (2.8) | 2.0 (0.3) | 12 (2) | 4 (0.9) | 0.1 (.01) |
| *Methylocystis sp.* | 5.0 (1.3) | 3.0 (0.7) | 60 (2) | 23 (1) | 0.5 (0.03) |
| *M. sporium* | 5.7 (0.3) | 4.5 (1.0) | 42 (0.5) | 20 (1) | 5 (0.1) |

*Kinetics of ammonia oxidation.* The differences observed in nitrite production during growth of the isolates led us to examine their kinetics of ammonia oxidation to nitrite. Interestingly, the production of nitrite during growth of MOB in NMS versus AMS media was not reflected in their kinetic constants for ammonia oxidation (*table 2*). For example, *Methylocystis* sp. and *M. methanica* produced the smallest amounts of nitrite during growth, but *Methylocystis* sp. had the lowest $K_s$ and greatest $V_{max}$ for ammonia turnover, whereas *M. methanica* did not oxidize ammonia to nitrite at all (*table 2*). While this result makes sense for *M. methanica*, it leads one to question why *Methylocystis* sp. did not release more nitrite during growth. It was also curious that with formate as the reductant source, the rate of ammonia oxidation by *Methylocystis* sp. was twice the maximum rate when methane provided the reductant. This implies additional enzymatic inventory to deliver reductant to pMMO enzymes in *Methylocystis* sp. that are not present in the other MOB. Furthermore, while *M. sporium* released enormous amounts of nitrite during growth on both NMS and AMS media, it struggled to co-oxidize ammonia.

*Analysis of pmoA sequences.* We questioned whether these differences in kinetics of ammonia oxidation might be due to differences in the structure of the catalytic subunit of methane monooxygenase, PmoA (Lieberman and Rosenzweig 2004). Like most ammonia-oxidizing bacteria, MOB generally have multiple copies of *pmo* operons (Stolyar et al. 1999). Usually, these operons are nearly identical to one another. However, we found that one of the *pmoA* copies in *M. methanica* encoded a protein that was truncated at the N-terminus and thus lacked three conserved liganding residues (*fig. 2*). The *pmoA* genes from the other strains and the full-length *M. methanica pmoA* encoded highly conserved proteins, and only *M. sporium* had both *pmoA1* and *pmoA2*, which is found only in some Type II MOB (Yimga et al. 2003). Based on these results, we assume that pmoA itself was not responsible for differences in rates of ammonia oxidation, but rather other components of the ammonia-oxidizing pathway. Possible candidates include electron transfer components to the pMMO complex or hydroxylamine-oxidizing enzymes.

***Table 2***. *Kinetics of ammonia oxidation to nitrite by methanotrophic isolates.*

| Isolate | Reductant Source | $K_s$[a] | $V_{max}$[a] |
|---|---|---|---|
| *M. album* | None | 0.56 (0.16)[b] | 4.50 (1.13) |
| | 0.02 mM $CH_4$ | 1.25 (0.29) | 10.22 (3.72) |
| | 0.23 mM $CH_4$ | 1.95 (0.45) | 12.39 (2.92) |
| | 2.26 mM $CH_4$ | 3.90 (2.19) | 12.23 (5.80) |
| | 20 mM sodium formate | 0.69 (0.08) | 11.15 (0.35) |
| *M. methanica* | None | B.D.L. | B.D.L. |
| | 0.02 mM $CH_4$ | B.D.L. | B.D.L. |
| | 0.23 mM $CH_4$ | B.D.L. | B.D.L. |
| | 2.26 mM $CH_4$ | B.D.L. | B.D.L. |
| | 20 mM sodium formate | B.D.L. | B.D.L. |
| *Methylocystis sp.* | None | 0.20 (0.04) | 13.40 (2.55) |
| | 0.02 mM $CH_4$ | 0.26 (0.07) | 14.62 (4.67) |
| | 0.23 mM $CH_4$ | 0.49 (0.17) | 21.75 (8.06) |
| | 2.26 mM $CH_4$ | 1.06 (0.19) | 30.94 (7.80) |
| | 20 mM sodium formate | 0.98 (0.24) | 62.08 (19.24) |
| *M. sporium* | None | B.D.L. | B.D.L. |
| | 0.02 mM $CH_4$ | B.D.L. | B.D.L. |
| | 0.23 mM $CH_4$ | B.D.L. | B.D.L. |
| | 2.26 mM $CH_4$ | 0.79 (0.36) | 5.35 (2.68) |
| | 20 mM sodium formate | 0.19 (0.07) | 3.20 (1.42) |

[a] $K_s$ values are in mM; $V_{max}$ values are in nmol $NO_2^-$ mg protein$^{-1}$ ml$^{-1}$
[b] Standard deviations of three replicate experiments performed on separate days are in parentheses.
[c] B.D.L. = below detection limit

*Hydroxylamine oxidation.* Ammonia oxidation to nitrite involves two enzymatic steps: the oxidation of ammonia to hydroxylamine by a monooxygenase and the oxidation of hydroxylamine to nitrite by an oxidase/reductase (Arp and Stein 2003). Because hydroxylamine is an extremely toxic compound, it must be turned over very rapidly in the cell. Two enzymes have been characterized as hydroxylamine oxidoreductases in MOB: HAO (Bergmann et al. 2005) and cytochrome P460 (Bergmann et al. 1998). Using conserved primer sets for HAO and cytochrome P460 genes, we probed our isolates by Southern blot and PCR to determine whether homologues to these genes were present. We detected a single homologue to HAO only in *M. album* (data not shown). Furthermore, examination of the rates of hydroxylamine oxidation to nitrite by all the MOB isolates essentially mirrored their ammonia oxidation kinetic values – *Methylocystis* sp. had the fastest rate of hydroxylamine turnover followed by *M. album*, followed by *M. sporium* (*table 3*). *M. methanica* could not oxidize hydroxylamine at all, in line with its inactive pathway for ammonia co-metabolism (*table 2*).

***Table 3***. *Rates of hydroxylamine oxidation to nitrite by methanotrophic isolates.*

| Isolate | 0.075 mg protein | 0.15 mg protein |
|---|---|---|
| *M. album* | 4.61[a] (0.61)[b] | 4.00 (0.45) |
| *M. methanica* | B.D.L.[c] | B.D.L. |
| *Methylocystis sp.* | 27.6 (4.91) | 20.68 (3.94) |
| *M. sporium* | 2.73 (0.23) | 2.20 (0.21) |

[a] values are in nmol $NO_2^-$ mg $protein^{-1}$ $ml^{-1}$
[b] Standard deviations of three replicate experiments performed on separate days are in parentheses.
[c] B.D.L. = below detection limit

Methylocystis sp.; MsporA2: M. sporium *pmoA2*; MsporA1: M. sporium *pmoA1*. Other sequences from MOB and AOB were included for comparison. PCR primer sequences are underlined.

*Inhibition of methane oxidation by ammonia or nitrite.* The competitive interaction of ammonia with methane for methane monooxygenase results in a decreased rate of methane consumption with increasing ammonia (Dalton 1977). Furthermore, nitrite is toxic to methane oxidizing activities of some MOB strains resulting in slowed methane-oxidizing potential (King and Schnell 1994). We examined the effects of both ammonia and nitrite on methane oxidizing potential of our isolates to define the inhibitory effects of these compounds to show whether toxicity influenced the kinetics for ammonia oxidation as per table 2. Cells were incubated for 1h in methane plus ammonia or nitrite, at which point the remaining methane was measured and compared to a control incubation containing methane only. As expected, ammonia had a much greater negative effect on the amount of methane oxidized than nitrite due to its competitive interaction with methane monooxygenase enzymes (*figs. 3&4*). Although 2.3 mM methane resulted in significantly more methane consumption than 0.2 mM methane with 2.5 mM and 5 mM ammonia, it did not alter the inhibitory effect of 0.5 mM ammonia in *M. album* or *M. sporium* (*fig. 3*). Conversely, 2.3 mM methane strongly overcame the inhibitory effect of 0.5 mM ammonium, relative to 0.2 mM methane, in *M. methanica* and *Methylocystis* sp. In fact, *Methylocystis* sp. cells incubated with 2.3 mM methane and 0.5 mM ammonia oxidized more methane than the control, likely due to ammonia acting as a nutrient at this lower concentration. These data suggest that *M. album* and *M. sporium* were much more sensitive to ammonia than *M. methanica* and *Methylocystis* sp.

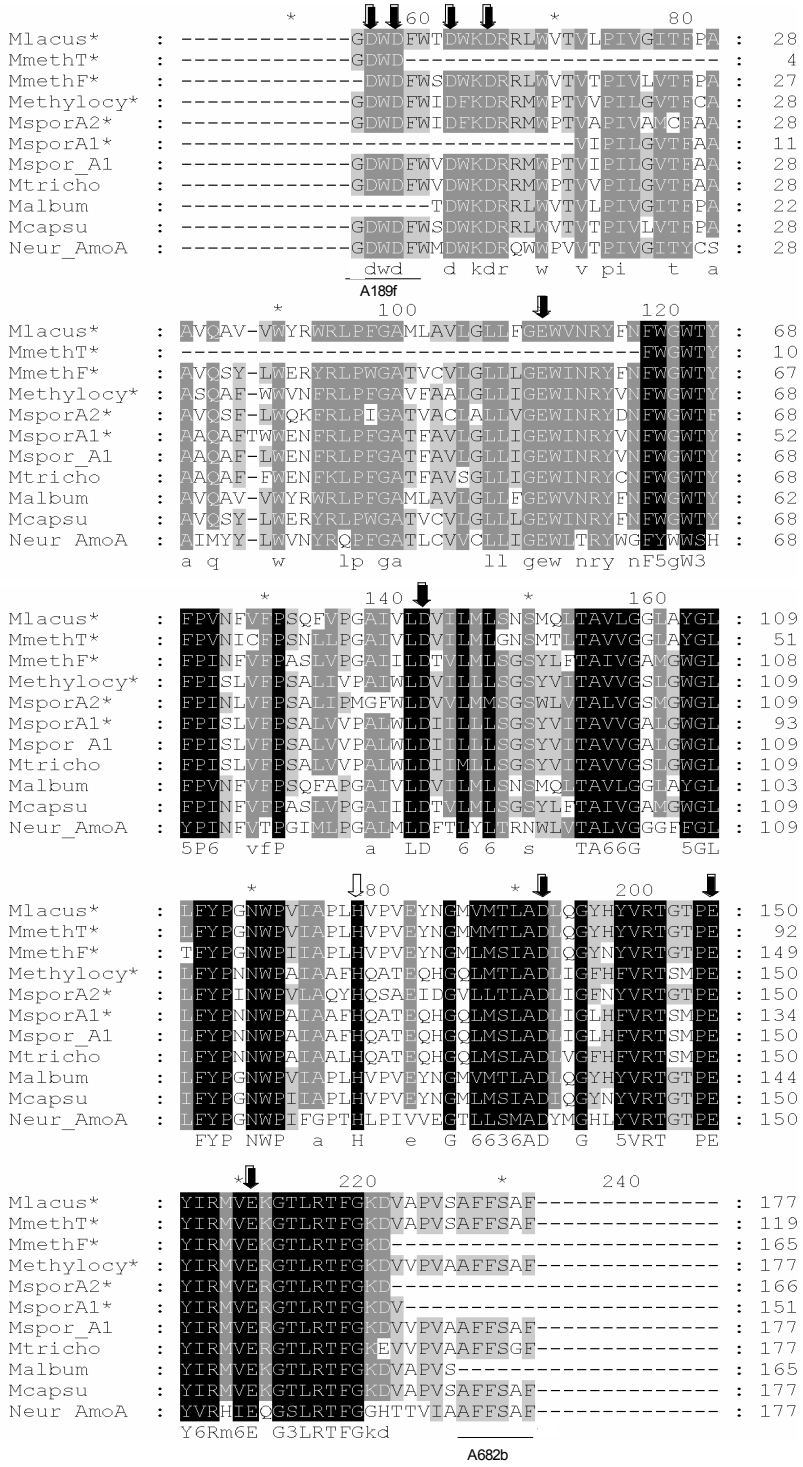


**Figure. 2** Alignment of pmoA sequences from MOB. Black arrow indicates conserved potential liganding residues and the white arrow designates a conserved histidine residue, which is part of the enzyme active site (Lieberman and Rosenzweig 2004). Names marked by asterisks are from this study and are designated as follows: Mlacus: M. album; MmethT: M. methanica, truncated; MmethF: M. methanica, full; Methylocy.

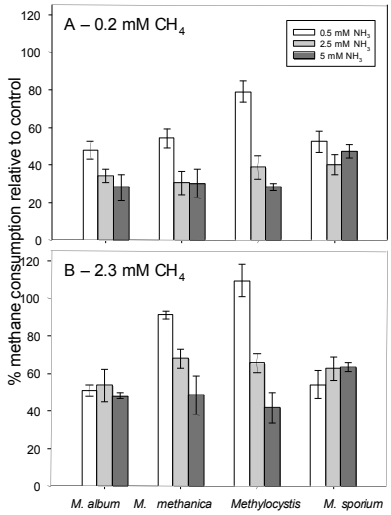


Fig. 3 Effect of ammonia on amount of methane consumed by MOB strains. Control incubation contained methane only.

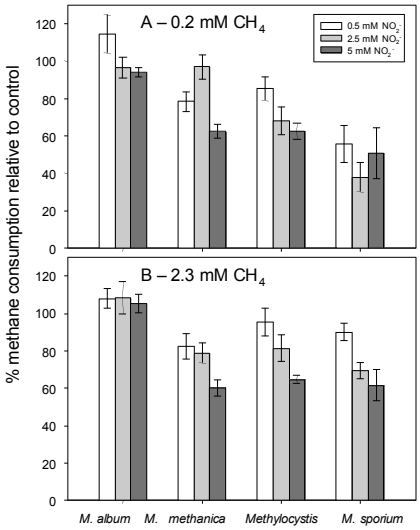


Fig. 4 Effect of nitrite on amount of methane consumed by MOB strains. Control incubations contained methane only.

Interestingly, *M. album* was the least sensitive to nitrite toxicity among the strains and *M. sporium* was the most sensitive (*fig .4*). Nitrite, especially at low concentrations (0.5 mM), stimulated methane oxidation above the control in *M. album*. Increased methane significantly protected methane-oxidizing enzymes in *Methylocystis* sp. and *M. sporium* from nitrite toxicity at 0.5 and 2.5 mM nitrite, but not at 5 mM nitrite. Increased methane did not have a significantly protective effect on methane-oxidizing enzymes of either *M. album* or *M. methanica.*

# Discussion

MOB are extremely important organisms to both the carbon and nitrogen cycles in soils. This Kearney project investigated inorganic nitrogen metabolism in MOB at the physiological and genetic levels to understand how N-loads impact both ammonia- and methane-oxidizing potentials of these important soil microorganisms. In our first objective, we showed that four strains of MOB were capable of growth on both ammonia and nitrate as an N-source and that their production of nitrite during growth was quite different. Characterization of their ammonia oxidation kinetics showed no linkage between the production of nitrite during growth and their ability to co-metabolize ammonia to nitrite. In fact, the strain with the highest ammonia-oxidizing potential, *Methylocystis* sp., produced only a very small amount of nitrite during growth relative to *M. sporium*, which only inefficiently oxidized ammonia. It is intriguing that *Methylocystis* sp. was so efficient at oxidizing ammonia with relatively low $K_m$ and high $V_{max}$ values and high rates of hydroxylamine turnover. Yet, this organism released only a small amount of nitrite during growth, and we could not detect a homologue to HAO or cytochrome P460. Our results with ammonia oxidation kinetics of *Methylocystis* sp. and *M. album* differ from a previous study on *Methylosinus trichosporium* OB3b and *M. album* BG8 in which the latter organism was the more efficient ammonia-oxidizer (King and Schnell 1994). Thus, much remains to be identified in the ammonia-oxidizing pathway of *Methylocystis* sp., particularly in

the reductant delivery systems to methane monooxygenase (e.g., cytochrome *c* or quinol-linked enzymes), to determine the underlying basis for its efficient oxidation of ammonia.

The highly conserved *pmoA* genes in each strain suggested that differences observed in ammonia oxidation kinetics were more likely due to differences in enzymatic pathways that deliver reductant to pMMO than to pMMO itself. This hypothesis makes sense in light of the significantly increased velocity of ammonia oxidation by *Methylocystis* sp. in the presence of formate relative to methane. For the velocity of ammonia turnover by pMMO to double, the delivery of reductant would have to be much more efficient. Thus, electrons from formate oxidation must be delivered to pMMO via different carriers than electrons from methane oxidation. The only major difference observed in pmoA sequences among the strains was the truncated copy in *M. methanica*. As this protein was likely inactive due to the absence of several liganding sites, it is doubtful that it contributed to either ammonia or methane oxidation. However, further investigations of its expression and activity are necessary to confirm this hypothesis.

Results of ammonia and nitrite inhibition of methane oxidation in *M. album* and *Methylocystis* sp. was consistent with results from a previous study on *M. album* BG8 and *Methylocystis trichosporium* OB3b, respectively (King and Schnell 1994). This study showed greater sensitivity of *M. album* BG8 to ammonia than *M. trichosporium* OB3b, and the inverse result for sensitivity to nitrite. A recent study suggested that *Methylocystis* sp. are particularly sensitive to ammonia addition to soils (Mohanty et al. 2006), and that *Methylomicrobium* sp. are insensitive to ammonia addition. Our data support this notion for *Methylocystis* sp. as our strain is quite sensitive to ammonia at high concentrations. However, our results with *M. album* and those of *M. album* BG8 (King and Schnell 1994) suggest that these bacteria should also be negatively affected by the addition of ammonium, and perhaps even more so than *Methylocystis* sp. Further ecological work will need to be conducted to understand strain-specific responses of MOB to ammonia additions. Alternatively, the nitrite insensitivity of *M. album* could be supportive of its survivial in N-impacted soils in which nitrite toxicity is more a factor than ammonia inhibition. The dual sensitivity of *M. sporium* to both ammonia and nitrite explains its relatively slow turnover of ammonia. The connection between this physiology and niche specialization of *M. sporium* strains has not yet been fully elucidated.

The largest remaining mystery in this study is the behavior of *M. methanica*, which is incapable of turning over both ammonia and hydroxylamine. Yet, its pMMO enzyme binds to and is significantly inhibited by ammonia. This organism grows perfectly well with ammonia as an N-source, suggesting that it takes up ammonia, but produces next to zero nitrite during growth. Perhaps given the right environment, *M. methanica* is an excellent competitor for ammonia, but becomes more sensitive as ammonia increases. Indeed, *M. methanica* is not widely found in managed soils, and cultivated representatives have been collected from coal mine drainage water, soil, and freshwater sediments (Bowman et al. 1993). Our future competition experiments between *M. album* and *M. methanica* will determine which is best at surviving in high-ammonia and/or nitrite environments.

The nitrogen cycle has been wildly altered from anthropogenic inputs, especially from agriculture and industry. A long-term goal for my laboratory is to determine how soil bacteria involved in nitrogen transformations are impacted by human activities, specifically in their production and consumption of greenhouse gases. Our research indicates that the ammonia-oxidizing inventory of methane-oxidizing species translates into altered rates of methane-

consuming activity. Thus, the creation of unifying assumptions regarding populations of these bacteria and their activities in the environment will require investigations of their roles in both carbon and nitrogen metabolism. We are narrowing the possibilities for how MOB metabolize nitrogen in N-impacted soils and are rapidly developing a suite of traditional and molecular techniques to correlate identity, abundance and function of these bacteria. So far, our results have changed our initial assumptions regarding how these microbes actually function in nature. Together, the results from this study showed that even closely related methanotrophic strains have evolved different mechanisms and pathways with different efficiencies for metabolizing inorganic nitrogen. Future studies investigating the relative fitness of individual MOB species in the presence of increasing N-loads (i.e., ammonia, nitrite, or nitrate) will help determine how niche specialization occurs and how nitrogen impacts the overall methane-oxidizing capacity of a soil.

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