Effects of Ammonium and Nitrite on Growth and Competitive Fitness of Cultivated Methanotrophic Bacteria

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The effects of nitrite and ammonium on cultivated methanotrophic bacteria were investigated. Methylomicrobium album ATCC 33003 outcompeted Methylocystis sp. strain ATCC 49242 in cultures with high nitrite levels, whereas cultures with high ammonium levels allowed Methylocystis sp. to compete more easily. M. album pure cultures and cocultures consumed nitrite and produced nitrous oxide, suggesting a connection between denitrification and nitrite tolerance.

The application of ammonium-based fertilizers has been shown to immediately reduce the uptake of methane in a number of diverse ecological systems (3, 5, 7, 8, 11–13, 16, 27, 28), due likely to competitive inhibition of methane monooxygenase enzymes by ammonia and production of nitrite (1). Longer-term inhibition of methane uptake by ammonium has been attributed to changes in methanotrophic community composition, often favoring activity and/or growth of type I Gammaproteobacteria methanotrophs (i.e., Gammaproteobacteria methane-oxidizing bacteria [gamma-MOB]) over type II Alphaproteobacteria methanotrophs (alpha-MOB) (19–23, 25, 26, 30). It has been argued previously that gamma-MOB likely thrive in the presence of high N loads because they rapidly assimilate N and synthesize ribosomes whereas alpha-MOB thrive best under conditions of N limitation and low oxygen levels (10, 21, 23).

Findings from studies with rice paddies indicate that N fertilization stimulates methane oxidation through ammonium acting as a nutrient, not as an inhibitor (2). Therefore, the actual effect of ammonium on growth and activity of methanotrophs depends largely on how much ammonia-N is used for assimilation versus cometabolism. Many methanotrophs can also oxidize ammonia into nitrite via hydroxylamine (24, 29). Nitrite was shown previously to inhibit methane consumption by cultivated methanotrophs and by organisms in soils through an uncharacterized mechanism (9, 17, 24), although nitrite inhibits purified formate dehydrogenase from Methylosinus trichosporium OB3b (15). Together, the data from these studies show that ammonium and nitrite have significant effects on methanotroph activity and community composition and reveal the complexity of ammonia as both a nutrient and a competitive inhibitor. The present study demonstrates the differential influences of high ammonium or nitrite loads on the competitive fitness of a gamma-MOB versus an alpha-MOB strain.

Growth and activity of pure cultures. Methylomicrobium album ATCC 33003 (a gamma-MOB strain) and Methylocystis sp. strain ATCC 49242 (an alpha-MOB strain) were grown in batch cultures (consisting of 100 ml of medium in 250-ml Wheaton bottles sealed with septated screw-top lids) with nitrate mineral salts medium (NMS; ATCC medium 1306) or ammonium mineral salts medium (AMS; ATCC medium 784) containing 10 μM copper at pH 6.8 under a 50% air–50% methane atmosphere. Cultures were initiated with 1 × 10⁶ cells ml⁻¹ and grown in the dark at 30°C with shaking (200 rpm). Although a range of NH₄Cl (25 to 100 mM) and NaNO₂ (0.5 to 5 mM) amendments were tested in both NMS and AMS (data not shown), 50 mM excess ammonium and 2.5 mM excess nitrite (the medium contained a 10 mM concentration of the respective N source) were selected for intensive investigation as these amounts caused differential responses by the bacteria but did not cause measurable osmotic effects. It must be recognized that bacteria in pure cultures have vastly different physiological responses from those operating in diverse natural communities; hence, while these N loads were necessary to stimulate measurable differential responses in the cultivated MOB, they are not directly applicable to MOB in natural environments.

M. album had shorter doubling times in AMS than in NMS (P = 0.03 by the t test), although final cell densities in the two media were equivalent as measured by direct microscopic counting using a Petroff-Hausser chamber under phase-contrast light microscopy (Table 1). Methylocystis sp. had equivalent doubling times and final cell densities when grown in NMS and AMS. Both strains released less nitrite when grown in AMS than in NMS, indicating more efficient uptake and assimilation of ammonium than nitrate as an N source (Table 1). The addition of 2.5 mM nitrite decreased the initial doubling time for M. album in NMS but did not alter the overall growth curves (Fig. 1A and B) or methane consumption rates (Table 1) in either NMS or AMS as measured by gas chromatography (GC)-thermal conductivity detection (TCD) using a GC-8A instrument (Shimadzu) and a Hayesep Q column (Alltech). Final M. album cell densities were 29 and 36% lower in nitrite-
amended than in unamended NMS and AMS, respectively (Table 1). Nitrite-amended cultures also showed net nitrite consumption (measured using a standard colorimetric assay [6]) and production of significantly more nitrous oxide (measured simultaneously with methane) than cultures with unamended medium. Amendment of NMS or AMS with 2.5 mM ammonium significantly increased doubling times by 65% and reduced final cell densities by 46 and 63% relative to those in unamended NMS and AMS, respectively (Table 1).

TABLE 1. Growth and activity measurements for pure cultures and cocultures of *M. album* and *Methylocystis* sp. 

<table>
<thead>
<tr>
<th>Organism(s) and medium formulation</th>
<th>Doubling time (h)</th>
<th>Maximum cell no. ( \times 10^6 )</th>
<th>Rate of CH(_4) consumption (( \mu )mol h(^{-1} ))</th>
<th>Amt (( \mu )mol) of N(_2)O produced</th>
<th>Amt (( \mu )mol) of nitrite produced or consumed</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>M. album</em></td>
<td></td>
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<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NMS</td>
<td>5.01 (0.41)</td>
<td>2.48 (0.28)</td>
<td>25.87 (0.41)</td>
<td>0.17 (0.17)</td>
<td>22.94 (4.3)</td>
</tr>
<tr>
<td>NMS + NO(_3^–)</td>
<td>3.90 (0.06/0.03)</td>
<td>1.77 (0.20/0.05)</td>
<td>25.60 (0.45)</td>
<td>1.41 (0.42/0.03)</td>
<td>-55.32 (6.53/0.002)</td>
</tr>
<tr>
<td>NMS + NH(_4^+)</td>
<td>10.03 (2.67)</td>
<td>1.28 (0.26/0.02)</td>
<td>10.00 (6.01/0.04)</td>
<td>BDL</td>
<td></td>
</tr>
<tr>
<td>AMS</td>
<td>3.94 (0.09)</td>
<td>2.36 (0.35)</td>
<td>23.20 (0.77)</td>
<td>0.90 (0.65)</td>
<td>7.19 (0.05)</td>
</tr>
<tr>
<td>AMS + NO(_3^–)</td>
<td>3.68 (0.21)</td>
<td>1.52 (0.08/0.04)</td>
<td>22.14 (1.23)</td>
<td>4.42 (0.29/0.0002)</td>
<td>-71.99 (10.64/0.01)</td>
</tr>
<tr>
<td>AMS + NH(_4^+)</td>
<td>7.01 (2.34)</td>
<td>1.53 (0.15/0.05)</td>
<td>20.13 (2.27)</td>
<td>BDL</td>
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<tr>
<td><em>Methylocystis</em> sp.</td>
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</tr>
<tr>
<td>NMS</td>
<td>4.28 (0.39)</td>
<td>4.81 (0.90)</td>
<td>23.82 (0.18)</td>
<td>BDL</td>
<td></td>
</tr>
<tr>
<td>NMS + NO(_3^–)</td>
<td>12.40 (1.80/0.006)</td>
<td>2.62 (0.58/0.05)</td>
<td>10.54 (0.39/3.1E-06)</td>
<td>BDL</td>
<td></td>
</tr>
<tr>
<td>NMS + NH(_4^+)</td>
<td>5.92 (0.02/0.007)</td>
<td>3.88 (0.75)</td>
<td>19.57 (0.51/0.0007)</td>
<td>BDL</td>
<td></td>
</tr>
<tr>
<td>AMS</td>
<td>4.57 (0.57)</td>
<td>5.81 (0.90)</td>
<td>20.81 (0.28)</td>
<td>BDL</td>
<td></td>
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<tr>
<td>AMS + NO(_3^–)</td>
<td>9.42 (0.84/0.004)</td>
<td>2.14 (0.26/0.008)</td>
<td>6.75 (1.12/0.0001)</td>
<td>BDL</td>
<td></td>
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<tr>
<td>AMS + NH(_4^+)</td>
<td>6.61 (1.33)</td>
<td>4.01 (0.12)</td>
<td>19.94 (0.37)</td>
<td>BDL</td>
<td></td>
</tr>
<tr>
<td><em>M. album</em> and <em>Methylocystis</em> sp.</td>
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<tr>
<td>NMS</td>
<td>4.24 (0.30)</td>
<td>3.58 (0.85)</td>
<td>23.96 (0.38)</td>
<td>BDL</td>
<td></td>
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<tr>
<td>NMS + NO(_3^–)</td>
<td>6.71 (1.14/0.05)</td>
<td>2.58 (0.31)</td>
<td>23.82 (0.76)</td>
<td>2.54 (0.52)</td>
<td>-0.17 (0.05/0.003)</td>
</tr>
<tr>
<td>NMS + NH(_4^+)</td>
<td>5.69 (0.34/0.02)</td>
<td>2.98 (0.64)</td>
<td>21.44 (0.57/0.01)</td>
<td>0.99 (0.52)</td>
<td>0.027 (0.11)</td>
</tr>
<tr>
<td>AMS</td>
<td>4.16 (0.11)</td>
<td>3.45 (0.67)</td>
<td>22.80 (0.62)</td>
<td>0.12 (0.12)</td>
<td>BDL</td>
</tr>
<tr>
<td>AMS + NO(_3^–)</td>
<td>3.94 (0.33)</td>
<td>2.89 (0.18)</td>
<td>22.57 (0.59)</td>
<td>4.14 (0.47/4.8E-06)</td>
<td>-0.54 (0.11)</td>
</tr>
<tr>
<td>AMS + NH(_4^+)</td>
<td>6.64 (1.00/0.03)</td>
<td>3.16 (0.19)</td>
<td>21.22 (0.72)</td>
<td>0.24 (0.24)</td>
<td>0.007 (0.002)</td>
</tr>
</tbody>
</table>

* a Cultures were grown in NMS or AMS unamended or amended with nitrite (2.5 mM; 250 \( \mu \)mol for 100 ml of culture) or ammonium (50 mM; 5,000 \( \mu \)mol for 100 ml of culture). Boldface type indicates a significant difference for the parameter between experimental cultures with nitrite or ammonium amendment and control cultures without N amendment. Data for unamended cultures are underlined. The first number in parentheses represents the standard error for three replicated experiments, each with duplicate cultures (\( n = 6 \)). The second number in parentheses is the P value from the two-sample t test for the experimental value and the control value for each significant parameter. P values were not determined when N\(_2\)O was below the detection limit in either the control or the experimental cultures.

b Doubling times were calculated over the interval from h 12 to 24 for all cultures (\( n = 6 \) for each treatment) except those of *Methylocystis* sp. in media with 2.5 mM nitrite, for which the interval from h 48 to 84 was used.

c Average maximum number of cells in stationary phase counted after 60 h of growth, except for cultures of *Methylocystis* sp. in media with 2.5 mM nitrite, in which cells were counted after 96 h of growth. Initial numbers of cells: 1 \( \times 10^6 \) ml\(^{-1} \) for *M. album* cultures, 3 \( \times 10^6 \) ml\(^{-1} \) for *Methylocystis* sp. cultures, and 2 \( \times 10^6 \) ml\(^{-1} \) for mixed cultures (with equivalent numbers of cells of the two species).

d Linear rates of methane consumption were determined over the interval from h 12 to 48 for all cultures except those of *Methylocystis* sp. in media with nitrite amendment, for which the interval from h 12 to 84 was used. \( R^2 \) values for regression lines ranged from 0.88 to 0.97 for *Methylocystis* sp. pure cultures (except those in nitrite-amended NMS and AMS, for which values were 0.75 and 0.5, respectively) and 0.89 to 0.95 for cocultures.

e Levels of N\(_2\)O and nitrite were measured following 72 h of growth. Nitrite values indicate the net amount produced or consumed after subtraction of the 250 \( \mu \)mol from nitrite-amended samples.

f BDL, below the detection limit.
sp. became dominant. Interestingly, nitrite levels did not substantially increase in NMS or AMS cocultures and N2O levels were lower than those in pure cultures of *M. album*, indicating more efficient N assimilation by the coculture than by either pure culture (Table 1).

For qPCR, primers were designed for *M. album* (Ma455F1, TCTGATGCGGAAATACCCATC, and Ma856R, CACGAATCTTACGAATAAG) and *Methylocystis* sp. (Mcy177F1, GGATACTGCGAGAGCAGA, and Mcy481R1, CCGTCATTATCGTCCCTGGC) by aligning their 16S rRNA genes using ClustalX (14). Primer sets were tested against both strains to ensure specificity. Standard curves were based on a dilution series of 10^2 to 10^8 cell ml⁻¹ for each bacterium and verified by direct cell counting. Total DNA was extracted with a one-step, closed-tube cell lysis and DNA extraction system (ZyGem, New Zealand) with 100% efficiency. qPCRs (with 30-µl reaction mixtures) were performed using a MyIQ optical thermocycler (Bio-Rad, Hercules, CA) with one primer set at a time and with standard reagent concentrations for Taq polymerase, as follows: 95°C for 5 min and 45 cycles at 94°C (10 s), 60°C (20 s), 72°C (20 s), and 85°C (10 s) to measure the fluorescence from Sybr green I (Molecular Probes, Eugene, OR) while avoiding signals from primer-dimer pairs. Threshold cycle (*CT*) values from the standards were used to extrapolate relative cell numbers in the samples.

The addition of 2.5 mM nitrite to the cocultures resulted in essentially no growth of *Methylocystis* sp. until well into the period of zero net change in cell numbers; 98 to 99% of growth through exponential phase was attributable to *M. album*, which also accounted for 94 to 97% of the population after 60 h of growth (Fig. 2B). Methane oxidation rates and final cell densities were unaffected by the addition of nitrite relative to those in unamended cocultures (Table 1). Doubling times increased only in NMS, by 37% relative to those in unamended cocultures. Levels of N2O production in nitrite-amended cocultures were similar to that in nitrite-amended *M. album* pure cultures (Table 1).

Ammonium-amended cocultures showed equivalent growth of the two bacteria up to late log phase (36 h), at which point loss of *M. album* cells and growth of *Methylocystis* sp. resulted in dominance of the latter after 60 h (Fig. 2C). Addition of ammonium increased doubling times for the coculture by 25 and 37% relative to those for unamended NMS and AMS cocultures, respectively (Table 1). Ammonium amendment had no effect on final cell densities in either medium or on methane oxidation rates in AMS, but methane oxidation rates in NMS decreased by 11% relative to those in unamended cocultures. A small amount of nitrous oxide was detected in ammonium-amended cocultures, although far less nitrite was released into the media than in ammonium-amended pure cultures of either bacterium (Table 1).

Results from the cocultures indicate that *M. album* outcompeted *Methylocystis* sp. in the absence of challenge by high N loads (i.e., in unamended cocultures) and in the presence of high nitrite levels and that ammonium-amended cocultures provided greater competitive fitness to *Methylocystis* sp. Findings from prior studies indicate that *Methylocystis* isolates prefer low methane concentrations (18) or low oxygen concentrations (4), which may explain the resurgence of *Methylocystis* sp. following exponential cell growth.

**Conclusions.** It is clear that ammonium and nitrite have strong effects on methanotrophic activity and, in ecological studies, on community composition. The present study demonstrates that competitive fitness of individual methanotrophic...
strains depends upon differential mechanisms to overcome inhibition and toxicity from imposed high N loads, as well as an ability to rapidly respond to and assimilate available nutrients. Consumption of nitrite and production of N₂O by *M. album* suggest that denitrifying ability may be an important mechanism for its relatively high tolerance of nitrite.

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REFERENCES


FIG. 2. Individual growth curves for *M. album* (□, ○) and *Methylocystis* sp. (■, △) in cocultures. Cells of the two isolates were enumerated separately by qPCR. Cocultures were grown in NMS (closed symbols) or AMS (open symbols) without N amendment (A), with the addition of 2.5 mM NaNO₂ (B), or with the addition of 50 mM NH₄Cl (C). Standard errors for replicated experiments (n = 6) are indicated by bars. R² values and PCR efficiency for standard curves with known cell numbers were 0.99 and 107% for *M. album* and 0.94 and 137% for *Methylocystis* sp.