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# Inhibitory Effects of C<sub>2</sub> to C<sub>10</sub> 1-Alkynes on Ammonia Oxidation in Two *Nitrososphaera* Species

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A previous study showed that ammonia oxidation by the *Thaumarchaeota* *Nitrosopumilus maritimus* (group 1.1a) was resistant to concentrations of the C<sub>8</sub> 1-alkyne, octyne, which completely inhibits activity by ammonia-oxidizing bacteria. In this study, the inhibitory effects of octyne and other C<sub>2</sub> to C<sub>10</sub> 1-alkynes were evaluated on the nitrite production activity of two pure culture isolates from *Thaumarchaeota* group 1.1b, *Nitrososphaera viennensis* strain EN76 and *Nitrososphaera gargensis*. Both *N. viennensis* and *N. gargensis* were insensitive to concentrations of octyne that cause complete and irreversible inactivation of nitrite production by ammonia-oxidizing bacteria. However, octyne concentrations ( $\geq 20$   $\mu$ M) that did not inhibit *N. maritimus* partially inhibited nitrite production in *N. viennensis* and *N. gargensis* in a manner that did not show the characteristics of irreversible inactivation. In contrast to previous studies with an ammonia-oxidizing bacterium, *Nitrosomonas europaea*, octyne inhibition of *N. viennensis* was: (i) fully and immediately reversible, (ii) not competitive with NH<sub>4</sub><sup>+</sup>, and (iii) without effect on the competitive interaction between NH<sub>4</sub><sup>+</sup> and acetylene. Both *N. viennensis* and *N. gargensis* demonstrated the same overall trend in regard to 1-alkyne inhibition as previously observed for *N. maritimus*, being highly sensitive to  $\leq$ C<sub>5</sub> alkynes and more resistant to longer-chain length alkynes. Reproducible differences were observed among *N. maritimus*, *N. viennensis*, and *N. gargensis* in regard to the extent of their resistance/sensitivity to C<sub>6</sub> and C<sub>7</sub> 1-alkynes, which may indicate differences in the ammonia monooxygenase binding and catalytic site(s) among the *Thaumarchaeota*.

With only a few isolates of ammonia-oxidizing archaea (AOA) available in pure/axenic culture, very little is known about the physiological and biochemical differences that might exist across the broad genotypic range of AOA (1–3) or how AOA physiology differs from that of ammonia-oxidizing bacteria (AOB). Although it is generally believed that both AOA and AOB initiate the oxidation of NH<sub>3</sub> with ammonia monooxygenase (AMO), there is evidence that suggests some aspects of NH<sub>3</sub> oxidation are probably different between them. For example, the NO scavenger 2-phenyl-4,4,5,5-tetramethylimidazoline-1-oxide-3-oxyl (PTIO) inhibits NH<sub>3</sub> oxidation by AOA and has little effect on AOB activity (4–6), suggesting that the intermediary products of NH<sub>3</sub> oxidation might be fundamentally different in AOA and AOB. Furthermore, whereas NH<sub>3</sub>-oxidizing activity in AOB is inhibited by low concentrations (0.4 to 10  $\mu$ M) of the Cu chelator, allylthiourea (ATU) (4, 7–9), the ATU sensitivity of NH<sub>3</sub> oxidation by AOA is more variable. For example, NH<sub>3</sub>-oxidizing activity of *Nitrososphaera gargensis* is only partially inhibited by 100  $\mu$ M ATU (10), and 500  $\mu$ M ATU is required to completely stop NH<sub>3</sub>-oxidizing activity in *Nitrososphaera viennensis* strain EN76 (4). In contrast, NH<sub>3</sub> oxidation by an obligate acidophilic AOA, *Nitrosotalea devanateri*, is completely inhibited by 50 to 100  $\mu$ M ATU (11).

Recently, Taylor et al. (12) showed that NO<sub>2</sub><sup>−</sup> production by the marine AOA, *Nitrosopumilus maritimus*, was unaffected by 20  $\mu$ M aqueous concentration (C<sub>aq</sub>) of C<sub>8</sub> and C<sub>9</sub> 1-alkynes, whereas NH<sub>3</sub>-oxidizing activity of AOB is completely and quickly ( $\leq$ 30 min) inactivated by 1 to 5  $\mu$ M C<sub>aq</sub> of 1-alkynes with a chain length of C<sub>2</sub> to C<sub>10</sub> (13). A short-term assay, based upon the difference in sensitivity of AOA and AOB to 1-octyne, was subsequently developed to measure the contributions of AOA and AOB to soil nitrifying activity. With the availability of more AOA isolates in pure culture, we deemed it important to confirm if the octyne resis-

tance properties of *N. maritimus* were similar among other AOA isolates, and particularly among the group 1.1b lineage of *Thaumarchaeota*, whose members are widely distributed in terrestrial environments (2, 14). We report here the results of studies carried out to assess the effects of 1-alkynes of various chain lengths on the NH<sub>3</sub>-oxidizing activity of two axenic culture isolates of AOA from group 1.1b, namely, *Nitrososphaera viennensis* strain EN76 (15, 16) and *Nitrososphaera gargensis* (1, 10). Both strains showed similar, albeit not identical 1-alkyne chain length inhibition profiles to *N. maritimus*. However, both strains were partially inhibited by  $\geq 20$   $\mu$ M octyne, which led to additional studies to compare the inhibitory properties of octyne with those of the more extensively studied alkyne, acetylene.

## MATERIALS AND METHODS

**Chemicals.** NH<sub>4</sub>Cl and linear aliphatic 1-alkynes (C<sub>3</sub> to C<sub>10</sub>,  $\geq 97\%$ ) were obtained from Sigma-Aldrich (St. Louis, MO), and acetylene (C<sub>2</sub>H<sub>2</sub>) was obtained from Airgas (Radnor, PA).

**Cultures.** *Nitrososphaera viennensis* strain EN76, here referred to as *N. viennensis*, was cultivated at its optimal growth temperature of 42°C in a

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freshwater medium (FWM) (16, 17), supplemented with 2 mM  $\text{NH}_4^+$  and 1 mM pyruvate. Growth was routinely followed by measuring  $\text{NO}_2^-$  production as described previously (12). *Nitrososphaera gargensis* has now been obtained into pure culture (M. Palatinszky et al., unpublished data) and was cultivated at its optimal growth temperature of 46°C in a carbonate media (18) supplemented with 2 mM  $\text{NH}_4^+$ . During routine growth of *N. gargensis*, further additions of 2 mM  $\text{NH}_4^+$  were made after  $\text{NH}_4^+$  was consumed. Cultures that had consumed a total of 8 to 10 mM  $\text{NH}_4^+$  were used for experimental purposes. The excess insoluble carbonate in the *N. gargensis* medium provides: (i) buffering against the acidification that occurs with 10 mM  $\text{NO}_2^-$  production and (ii) is thought to provide a growth-promoting surface for *N. gargensis*. The presence of the carbonate provides challenges during experimental manipulations. For example, harvesting and concentration of the culture leads to a dense suspension of carbonate that settles immediately upon onset of static conditions. This phenomenon makes it difficult to dispense uniform aliquots of cells to experimental replicates. Because of the challenges posed by the excess carbonate in the media, only a subset of the experiments were performed with *N. gargensis*. As expected, the *N. gargensis* cultures grown with 8 to 10 mM  $\text{NH}_4^+$  yielded higher protein concentrations ( $132.6 \pm 21.0$   $\mu\text{g}$  of protein/ml of culture) than the *N. viennensis* cultures grown with 2 mM  $\text{NH}_4^+$  ( $9.0 \pm 0.3$   $\mu\text{g}$  of protein/ml of culture). However, the *N. gargensis* cultures had much lower rates of  $\text{NO}_2^-$  production when expressed on a milligram protein basis. Protein concentrations were determined by using a Pierce BCA protein assay kit (Thermo Scientific, Waltham, MA) as described by the manufacturer.

**Octyne sensitivity.** Octyne stocks were prepared as described previously (12). *N. viennensis* was cultivated at 42°C in 200-ml volumes of FWM. After accumulation of 1.8 to 2 mM  $\text{NO}_2^-$ , the cultures were harvested by centrifugation ( $10,000 \times g$ , 15 min) and concentrated 100-fold by resuspension in 2 ml of fresh FWM supplemented with 1 mM pyruvate but minus  $\text{NH}_4^+$ . *N. gargensis* was cultivated at 46°C in 200 ml of carbonate medium supplemented with a total addition of 8 to 10 mM  $\text{NH}_4^+$ , harvested by centrifugation ( $10,000 \times g$ , 15 min), and concentrated 100-fold by resuspension in 2 ml of fresh carbonate medium minus  $\text{NH}_4^+$ . Aliquots (100  $\mu\text{l}$ ) of *N. viennensis* or *N. gargensis* cell suspensions were added to 10-ml portions of their respective growth media supplemented with 1 mM  $\text{NH}_4^+$  that had been pre-equilibrated with octyne (0, 10, 20, or 40  $\mu\text{M}$ ) for 30 min at either 42 or 46°C for *N. viennensis* and *N. gargensis*, respectively. Bottles were incubated as described above and sampled periodically over 6 h to quantify the rate of accumulation of  $\text{NO}_2^-$ . Additional incubations were carried out with *N. viennensis* at two different temperatures (35 and 46°C) to evaluate the sensitivity of  $\text{NO}_2^-$  production to 10  $\mu\text{M}$  octyne. An experiment was performed at 42°C in the presence or absence of 1 mM pyruvate to determine whether the latter influenced the rate and sensitivity of  $\text{NO}_2^-$  production to 10  $\mu\text{M}$  octyne.

**Sensitivity of AOA isolates to  $\text{C}_2$  to  $\text{C}_{10}$  alkynes.** A preliminary experiment was conducted in bottles containing alkynes pre-equilibrated at 30, 42, and 46°C. Headspace alkyne concentrations were monitored by using gas chromatography. There was no significant difference in headspace alkyne concentrations between bottles incubated across this temperature range, indicating that temperature shifts over this magnitude did not change the gas/aqueous phase-partitioning in the assay bottles sufficient to modify the aqueous concentration ( $C_{\text{aq}}$ ) of alkynes. *N. viennensis* and *N. gargensis* were grown and harvested as described above, and 100-fold-concentrated cell aliquots (100  $\mu\text{l}$ ) were added to 10-ml portions of their respective growth media supplemented with 1 mM  $\text{NH}_4^+$  that had been pre-equilibrated for 30 min with 10  $\mu\text{M}$   $C_{\text{aq}}$  of each alkyne ( $\text{C}_2$  to  $\text{C}_{10}$ ) in 125-ml bottles sealed with gray butyl stoppers (12). This specific alkyne concentration was chosen after it had been determined that 10  $\mu\text{M}$  octyne had no effect on  $\text{NH}_3$  oxidation by either *N. viennensis* or *N. gargensis* but was previously shown to be more than sufficient to inactivate AOB (12). Bottles were incubated at 42 or 46°C for *N. viennensis* and *N. gargensis*, respectively, and  $\text{NO}_2^-$  accumulation monitored over 8 h.

**Characteristics of acetylene and octyne inhibition of  $\text{NO}_2^-$  production by *N. viennensis*.** (i) **Kinetic relationships between  $\text{NH}_4^+$  and alkyne inhibition of *N. viennensis*.** Aliquots of a *N. viennensis* cell suspension were exposed to different concentrations of either octyne (30 or 40  $\mu\text{M}$ ) or acetylene (1, 3, or 6  $\mu\text{M}$ ) in the presence of various concentrations of  $\text{NH}_4^+$  (0.01 to 2 mM) at 42°C. Initial rates of  $\text{NO}_2^-$  production were determined by sampling every 15 min for a total of 90 to 120 min, and the data were plotted in double-reciprocal Lineweaver-Burk plots.

(ii) **Examining the influence of octyne on the relationship between  $\text{NH}_4^+$  concentration and acetylene on the inhibition of  $\text{NO}_2^-$  production by *N. viennensis*.** Aliquots of *N. viennensis* were exposed to 30  $\mu\text{M}$  octyne with increasing concentrations of  $\text{NH}_4^+$  (0.25 to 2 mM) for 15 min, followed by the addition of 1  $\mu\text{M}$  acetylene.  $\text{NO}_2^-$  accumulation was evaluated at 30-min intervals for 120 min at 42°C, and analysis of variance (ANOVA) of rates determined differences in response to acetylene, octyne, or the combination of acetylene and octyne. Controls included treatments without alkyne and a treatment containing 1  $\mu\text{M}$  acetylene minus octyne.

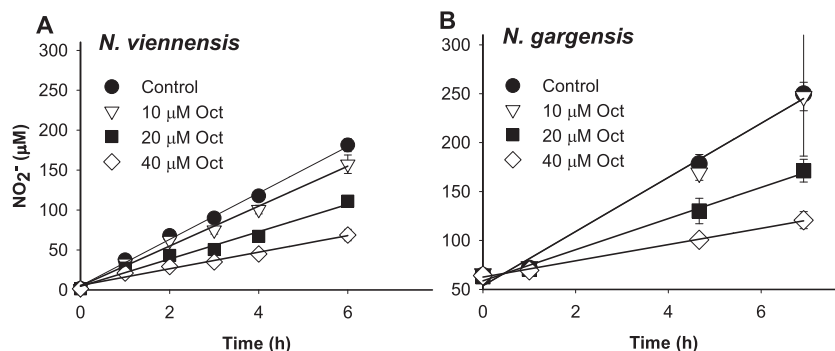
(iii) **Time course of recovery of  $\text{NO}_2^-$  production in *N. viennensis* following inhibition by acetylene or octyne.** Aliquots of a *N. viennensis* cell suspension were exposed to either 40  $\mu\text{M}$  octyne or 6  $\mu\text{M}$  acetylene with an initial  $\text{NH}_4^+$  concentration of 0.5 mM for 2 h, along with a minus-alkyne control also incubated for 2 h. This concentration of  $\text{NH}_4^+$  was chosen to minimize the potential for competition with acetylene. After the alkyne exposure period, bottles were degassed to remove the alkynes (12). Additional  $\text{NH}_4^+$  was added to increase the concentration to 2 mM for optimal growth conditions, and the recovery of  $\text{NO}_2^-$  production was monitored by sampling at 60-min intervals over 4 h.

(iv) **Hydroxylamine-dependent  $\text{NO}_2^-$  production by acetylene- and octyne-treated *N. viennensis*.** It is well established that hydroxylamine-dependent  $\text{NO}_2^-$  production is unaffected by alkyne inactivation of AMO in AOB and has been used on several occasions to infer that alkynes specifically target AMO and have no effect on downstream enzymes in the  $\text{NO}_2^-$  production pathway (19, 20). Aliquots of *N. viennensis* cell suspensions were exposed to 18  $\mu\text{M}$  acetylene or 40  $\mu\text{M}$  octyne, with 2 mM  $\text{NH}_4^+$ , and a range of hydroxylamine concentrations (20 to 400  $\mu\text{M}$ ), and evidence for hydroxylamine-dependent  $\text{NO}_2^-$  accumulation was sought by incubation at 42°C and sampling at 15-min intervals for 2 h. Abiotic controls containing only growth medium plus hydroxylamine were included. We were unable to measure significant hydroxylamine-dependent  $\text{NO}_2^-$  production in octyne-inhibited or acetylene-inactivated cultures ( $P > 0.3$ ). Furthermore, hydroxylamine-dependent  $\text{NO}_2^-$  production could not be measured in *N. viennensis* cultures immediately after total depletion of  $\text{NH}_4^+$  and untreated with either acetylene or octyne.

**Statistics.** SigmaPlot 12.5 (Systat Software, Inc., San Jose, CA) was used to perform statistical analyses. ANOVA was used to determine whether the differences in the mean values among the treatment groups were greater than would be expected by chance. If there was a statistically significant difference the Holm-Sidak method of all pairwise multiple-comparison procedure was used to isolate the group or groups that differed from the others. A nonlinear regression procedure was used to estimate *N. viennensis*  $K_m$  for  $\text{NH}_4^+$  in the presence of 1  $\mu\text{M}$  acetylene. The best-fit slopes of the relationship between  $\text{NO}_2^-$  production and  $\text{NH}_4^+$  concentration in response to the presence of acetylene or octyne were estimated by linear regression of double-reciprocal plots.

## RESULTS

**Sensitivity of *N. viennensis* and *N. gargensis* to octyne.** Initial work focused on the response of  $\text{NO}_2^-$  production by *N. viennensis* and *N. gargensis* to different concentrations of octyne ( $C_8$ ). The rate of  $\text{NO}_2^-$  production by *N. viennensis* in the presence of 10  $\mu\text{M}$  octyne was not significantly different ( $P > 0.05$ ) than the rate in the no-alkyne control ( $175 \pm 20$  nmol/min/mg of protein, Fig. 1A) at the optimal growth temperature of 42°C. However, 20 and 40  $\mu\text{M}$  octyne partially inhibited  $\text{NO}_2^-$  accumulation to 58

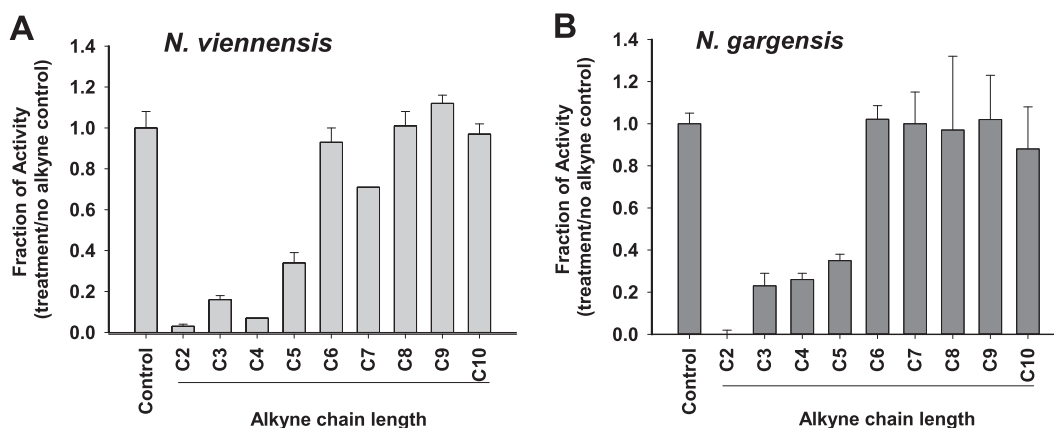


**FIG 1** Response of  $\text{NO}_2^-$  production by *N. viennensis* (A) and *N. gargensis* (B) to different aqueous concentrations of octyne (Oct). Cell suspensions were added to their respective media pre-equilibrated with Oct in the presence of 1 mM  $\text{NH}_4^+$ . Bottles were incubated at 42 or 46°C for *N. viennensis* and *N. gargensis*, respectively, and the accumulation of  $\text{NO}_2^-$  was monitored. Experiments were repeated three times (in triplicate) with similar results. Error bars represent the standard deviations of three replicate bottles of each treatment.

and 33%, respectively, of the activity of the no-alkyne control. Initial rates of  $\text{NO}_2^-$  production (0 to 6 h) by *N. viennensis* were also insensitive to 10  $\mu\text{M}$  octyne at 35 or 46°C ( $P \geq 0.1$ ), and the presence or absence of 1 mM pyruvate had no effect on the inhibitory effects of octyne ( $P \geq 0.1$ ). The rate of  $\text{NO}_2^-$  production by *N. gargensis* was not affected by the presence of 10  $\mu\text{M}$  octyne ( $P > 0.1$ ) compared to the rate in the no-alkyne control ( $3.3 \pm 1.1$  nmol/min/mg of protein, Fig. 1B). As with *N. viennensis*,  $\text{NO}_2^-$  accumulation was partially inhibited by 20 and 40  $\mu\text{M}$  octyne to 56 and 29%, respectively, of the activity of the no-alkyne control. In the case of both AOA isolates,  $\text{NO}_2^-$  production in the presence of 20 and 40  $\mu\text{M}$  octyne continued at a constant rate for at least 24 h (data not shown), suggesting that octyne was not behaving as a turnover-dependent irreversible inactivator of AMO. Octyne concentrations in the headspace of the biological treatments at 24 h were no different than in abiotic controls ( $P > 0.17$ ), providing no evidence of substantial consumption by the AOA. In addition, gas chromatographic analysis provided no evidence of trace contamination of octyne by other volatile compounds that might explain partial inhibition by  $\geq 20$   $\mu\text{M}$  octyne.

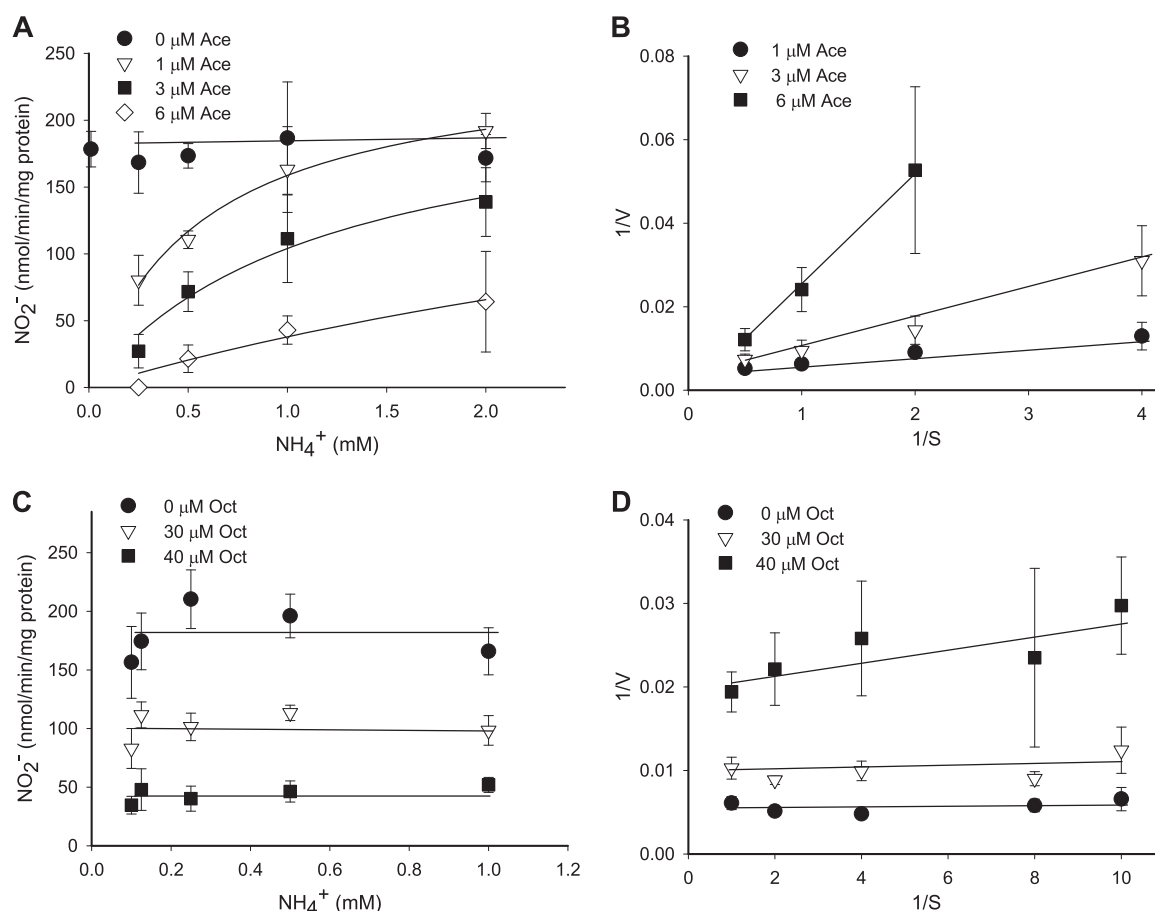
**Sensitivity of AOA isolates to  $\text{C}_2$  to  $\text{C}_{10}$  alkynes.** To determine whether there was differential sensitivity to alkynes of various

chain lengths, we compared the  $\text{NO}_2^-$  production response of *N. viennensis* and *N. gargensis* to  $\text{C}_2$  to  $\text{C}_{10}$  1-alkynes in the presence of 1 mM  $\text{NH}_4^+$  (Fig. 2). There was a significant decrease in  $\text{NO}_2^-$  production compared to the no-alkyne control when *N. viennensis* was exposed to 10  $\mu\text{M}$   $\text{C}_2$  to  $\text{C}_5$  and  $\text{C}_7$  1-alkynes ( $P < 0.05$ ), but there was no significant difference ( $P > 0.05$ ) in  $\text{NO}_2^-$  accumulation when exposed to  $\text{C}_6$ , and  $\text{C}_8$  to  $\text{C}_{10}$  alkynes. The reduced rate of  $\text{NO}_2^-$  production by *N. viennensis* in the presence of 10  $\mu\text{M}$   $\text{C}_7$  ( $69\% \pm 1\%$  of control) continued at the same rate for at least 24 h, showing no sign of a time-dependent reduction of the rate as would be anticipated if  $\text{C}_7$  was behaving as an inactivator of  $\text{NO}_2^-$  production (data not shown). Although 10  $\mu\text{M}$   $\text{C}_7$  was inhibitory, 2.5 and 5.0  $\mu\text{M}$   $\text{C}_7$  did not have a significant effect on the rates of  $\text{NO}_2^-$  accumulation ( $P > 0.07$ ). Furthermore, as described above for octyne, there was no evidence for substantial consumption of  $\text{C}_7$  in the *N. viennensis* treatments after 24 h inhibition ( $P > 0.15$ ), and no evidence of contamination of  $\text{C}_7$  by other alkynes that might explain partial inhibition. In the case of *N. gargensis*, there was a significant inhibitory effect of 10  $\mu\text{M}$   $\text{C}_2$  to  $\text{C}_5$  but no significant effect of  $\text{C}_6$  to  $\text{C}_{10}$  alkynes on  $\text{NO}_2^-$  accumulation ( $P > 0.05$ ). In comparison, our previous work had also shown a significant inhibitory effect of  $\text{C}_2$  to  $\text{C}_5$  1-alkynes on  $\text{NO}_2^-$  production



**FIG 2** Response of  $\text{NO}_2^-$  production by *N. viennensis* and *N. gargensis* when exposed to  $\text{C}_2$  to  $\text{C}_{10}$  1-alkynes. Cultures were harvested and aliquots of cell suspensions were added to treatments containing their respective media with 10  $\mu\text{M}$   $\text{C}_{\text{aq}}$  of  $\text{C}_2$  to  $\text{C}_{10}$  alkyne and 1 mM  $\text{NH}_4^+$ . *N. viennensis* and *N. gargensis* were incubated at 42 and 46°C, respectively, and  $\text{NO}_2^-$  accumulation was measured. Experiments were repeated at least three times with similar results. Bars represent the average activity in three replicate treatments compared to the no-alkyne control. Error bars represent the standard deviations of the average.





**FIG 3** Response of initial velocities of  $\text{NO}_2^-$  production by *N. viennensis* to acetylene (A and B) and octyne (C and D) at increasing concentrations of  $\text{NH}_4^+$ . (A) Inhibition by 1  $\mu\text{M}$  acetylene is overcome by increasing the  $\text{NH}_4^+$  concentration. (B) Lineweaver-Burk double-reciprocal plot shows that the slopes for each acetylene concentration differ, indicating competitive inhibition. (C)  $V_i$  decreases with higher octyne concentrations, but octyne inhibition cannot be overcome by increasing  $\text{NH}_4^+$ . (D) Lineweaver-Burk double-reciprocal plots intercept the  $1/V_0$  axis at different values indicating noncompetitive inhibition. Error bars represent the standard deviations of three replicates.

by the marine AOA *N. maritimus* whereas 20  $\mu\text{M}$   $\text{C}_6$  and  $\text{C}_7$  1-alkynes were partially inhibitory ( $56\% \pm 2\%$  and  $58\% \pm 4\%$ , respectively), and 20  $\mu\text{M}$   $\text{C}_8$  and  $\text{C}_9$  1-alkynes had no effect (12).

**Characteristics of acetylene and octyne inhibition of  $\text{NO}_2^-$  production by *N. viennensis*.** Because  $\text{NO}_2^-$  production rates in both strains were partially inhibited by  $\geq 20 \mu\text{M}$  octyne but showed no sign of time-dependent rate reduction, several experiments were conducted to compare in more detail the characteristics of acetylene and octyne inhibition in *N. viennensis*.

**(i) Kinetic relationships between  $\text{NH}_4^+$  and alkyne inhibition of *N. viennensis*.** The first experiment compared the concentration-dependent inhibition of  $\text{NO}_2^-$  production by acetylene and octyne. In the absence of acetylene or octyne the rate of  $\text{NO}_2^-$  production by *N. viennensis* was saturated with  $\text{NH}_4^+$  concentrations from 0.01 to 2.0 mM (Fig. 3A). In the presence of 1  $\mu\text{M}$  acetylene and 0.25 mM  $\text{NH}_4^+$ , the initial rate of  $\text{NO}_2^-$  production ( $V_i$ ) was inhibited to 47% of the control, but the inhibition could be completely overcome by increasing the  $\text{NH}_4^+$  concentration to 1 mM (Fig. 3A). When acetylene concentrations were increased to 3 and 6  $\mu\text{M}$ , inhibition could not be completely overcome by increasing the  $\text{NH}_4^+$  concentration to 2 mM. These results suggest that acetylene and  $\text{NH}_4^+$  are competitive substrates for binding at

the active site. Although  $V_{\text{max}}$  was achieved in the presence of 1  $\mu\text{M}$  acetylene by raising the  $\text{NH}_4^+$  concentration, the apparent  $K_m$  increased from  $\leq 0.01$  mM to  $0.55 \pm 0.08$  mM  $\text{NH}_4^+$ . Although there are no published values of the  $K_s$  for  $\text{NH}_4^+/\text{NH}_3$  in these particular AOA isolates, a maximum velocity of  $\text{NO}_2^-$  production was achieved with 10  $\mu\text{M}$   $\text{NH}_4^+/\text{NH}_3$  in the absence of acetylene (Fig. 3A). When the responses of the rates of  $\text{NO}_2^-$  production to a range of acetylene and  $\text{NH}_4^+$  concentrations were plotted in a double-reciprocal plot (Fig. 3B), the lines representing each inhibitor concentration had a different slope, also diagnostic of competitive inhibition. In contrast, there was no significant effect of  $\text{NH}_4^+$  concentration ( $P > 0.07$ ) on  $V_i$  at any inhibitory octyne concentration, indicating that octyne inhibition could not be overcome with the addition of higher concentrations of  $\text{NH}_4^+$  (Fig. 3C). When the responses of  $\text{NO}_2^-$  production to a range of octyne and  $\text{NH}_4^+$  concentrations were plotted in a double-reciprocal plot the intercepts on the  $y$  axis are distinctively different (Fig. 3D). This result would be expected if inhibition by octyne was noncompetitive with respect to  $\text{NH}_4^+$ .

**(ii) Examining the influence of octyne on the relationship between  $\text{NH}_4^+$  concentration and acetylene on the inhibition of  $\text{NO}_2^-$  production by *N. viennensis*.** An additional experiment

**TABLE 1** Comparison of the response of rates of  $\text{NO}_2^-$  production by *N. viennensis* in a minus-alkyne control to treatments exposed to 1  $\mu\text{M}$  acetylene, 30  $\mu\text{M}$  octyne, or a combination of acetylene and octyne at different concentrations of  $\text{NH}_4^+$ <sup>a</sup>

$\text{NH}_4^+$ concn (mM)	$\text{NO}_2^-$ production (nmol/ml/h) after exposure to various alkyne treatments			
	Control	Acetylene	Octyne	Acetylene + octyne
2.0	13.1 (1.9) <sup>A</sup>	11.5 (2.5) <sup>A</sup>	6.5 (2.6) <sup>A</sup>	6.7 (1.1) <sup>A</sup>
1.0	15.5 (3.8) <sup>A</sup>	11.7 (2.4) <sup>A</sup>	4.9 (1.3) <sup>A</sup>	4.9 (0.6) <sup>A</sup>
0.5	14.0 (2.3) <sup>A</sup>	7.2 (1.7) <sup>AB</sup>	5.1 (1.2) <sup>A</sup>	1.6 (0.7) <sup>B</sup>
0.25	16.9 (0.8) <sup>A</sup>	4.1 (1.3) <sup>B</sup>	4.9 (1.5) <sup>A</sup>	1.0 (0.3) <sup>B</sup>

<sup>a</sup> The acetylene-octyne samples were exposed to 30  $\mu\text{M}$  octyne for 15 min before the addition of 1  $\mu\text{M}$  acetylene. Values in parentheses represent the standard deviations of three replicates. Different superscript letters indicate significant differences ( $P \leq 0.03$ ) between different  $\text{NH}_4^+$  concentrations within a specific alkyne treatment. Values with superscript letters in common are not significantly different.

was conducted to confirm that the mechanisms of octyne and acetylene inhibition differed. We reasoned that if octyne does not interact at the same site as acetylene it should not affect the competitive behavior between acetylene and  $\text{NH}_4^+$ . *N. viennensis* was treated with octyne in some treatments before adding acetylene (Table 1). In the no-alkyne control  $V_{\text{max}}$  was saturated by all  $\text{NH}_4^+$  concentrations (0.25 to 2 mM  $\text{NH}_4^+$ ,  $P > 0.3$ ). When *N. viennensis* was treated with 1  $\mu\text{M}$  acetylene, there was a statistically significant effect of  $\text{NH}_4^+$  concentration on  $V_{\text{max}}$  ( $P \leq 0.005$ ), as shown earlier (Fig. 3). In contrast, the addition of 30  $\mu\text{M}$  octyne decreased  $V_{\text{max}}$  ( $P < 0.01$ ), but there was no significant protective effect of increasing  $\text{NH}_4^+$  concentration on the rate of  $\text{NO}_2^-$  production (0.25 to 2 mM  $\text{NH}_4^+$ ,  $P > 0.3$ ). When *N. viennensis* was treated with octyne prior to acetylene, there was a statistically significant protective effect of increasing  $\text{NH}_4^+$  concentrations on the rate of octyne-insensitive, acetylene-sensitive  $\text{NO}_2^-$  production ( $P \leq 0.005$ ), indicating that the competitive behavior between  $\text{NH}_4^+$  and acetylene was not modified by the presence of octyne. This result gives additional support, suggesting that octyne is not functioning akin to acetylene as a competitive inhibitor of  $\text{NH}_4^+/\text{NH}_3$  oxidation.

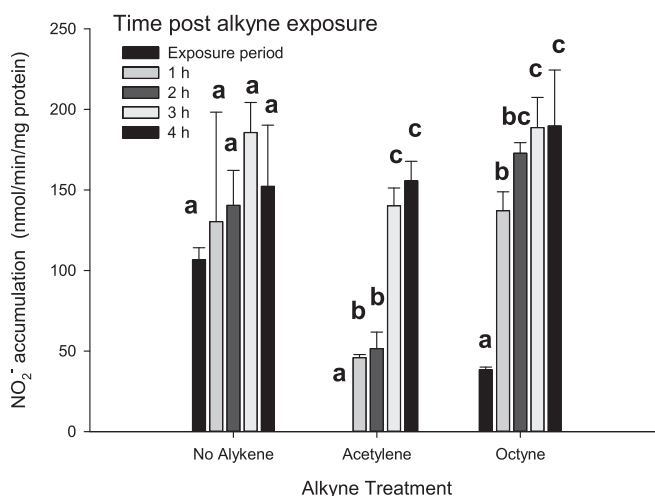
**(iii) Time course of recovery of  $\text{NO}_2^-$  production in *N. viennensis* following inhibition by acetylene or octyne.** It is known that acetylene is an irreversible inactivator of AMO of AOA (12, 21) and AOB (22). Recovery from inactivation requires new AMO protein synthesis resulting in a lag of several hours for resumption of maximum  $\text{NO}_2^-$  production. Previously, Vajjala et al. (21) showed that the eukaryotic protein synthesis inhibitor, cycloheximide, prevented recovery of  $\text{NO}_2^-$  production in *N. maritimus* after acetylene inhibition. In the case of *N. viennensis*, the same concentration range of cycloheximide slowed but did not prevent recovery of  $\text{NO}_2^-$  production after inactivation with acetylene (data not shown). The lack of an unambiguous protein synthesis inhibitor for *N. viennensis* made us reliant upon the comparative time course of recovery of  $\text{NO}_2^-$  production after exposure to acetylene or octyne. After exposure of *N. viennensis* to either 6  $\mu\text{M}$  acetylene or 40  $\mu\text{M}$  octyne,  $\text{NO}_2^-$  production was inhibited to 0 or 21%, respectively, of the control (Fig. 4). After the alkynes were removed by degassing, the initial rates of  $\text{NO}_2^-$  production during the first hour of the recovery period averaged 32% (acetylene) and 96% (octyne) of the no-alkyne control, respectively, indicat-

ing that the majority of octyne inhibition was quickly reversible. In the case of acetylene, 3 h were required for the  $\text{NO}_2^-$  production rate to reach the rate of the control treatment. A “starved” control incubated without  $\text{NH}_4^+$  for 2 h immediately accumulated  $\text{NO}_2^-$  at the same rate as the minus alkyne control upon the addition of  $\text{NH}_4^+$  (data not shown).

## DISCUSSION

In an earlier study, we showed that  $\text{NO}_2^-$  production by the marine AOA, *N. maritimus* (*Thaumarchaeota* group 1.1a) was very resistant to inhibition by  $\geq C_7$  1-alkynes in comparison to the AOB, *N. europaea* and *Nitrosospora multiformis* (12). In the present study, we confirm that  $\text{NO}_2^-$  production by pure culture isolates of *N. viennensis* strain EN76 (15, 16) and *N. gargensis* (10), both members of *Thaumarchaeota* group 1.1b, show the same overall trend, being highly sensitive to  $\leq C_5$  and much more resistant to longer-chain-length alkynes. They are also insensitive to concentrations of the  $C_8$  alkyne, octyne, that cause complete and irreversible inactivation of  $\text{NH}_3$  oxidation in AOB. These observations provide direct evidence that octyne-resistant  $\text{NO}_2^-$  production is a property of members of the phylogenetically distinct *Thaumarchaeota* group 1.1b and strengthens the idea that octyne might be exploited to differentiate between the relative activities of AOA and AOB in different natural environments where groups 1.1a and 1.1b are found.

Our observations that higher concentrations of octyne partially inhibit AOA activity in a manner that is (i) time independent, (ii) fully reversible, (iii) not competitive with  $\text{NH}_4^+$ , and (iv) without effect on the competitive interaction between  $\text{NH}_4^+$  and acetylene raises the possibility that kinetic and catalytic properties of AMO from AOA and AOB might differ. For example, the observation that octyne inhibition of  $\text{NO}_2^-$  production in *N. viennensis* is noncompetitive with respect to  $\text{NH}_4^+$  concentration



**FIG 4** Response of  $\text{NO}_2^-$  production by *N. viennensis* after acetylene or octyne exposure. Cells were exposed for 2 h to either 6  $\mu\text{M}$  acetylene or 40  $\mu\text{M}$  octyne in media with 0.5 mM  $\text{NH}_4^+$ . Alkyne and no-alkyne treatments were then degassed and  $\text{NH}_4^+$  increased to 2 mM.  $\text{NO}_2^-$  accumulation was monitored after alkyne exposure for 4 h. Bars represent the average activity in three replicate treatments. Error bars represent the standard deviations of the average. Lowercase letters indicate the significance ( $P \leq 0.02$ ) of sampling time within a specific treatment. Values that have lowercase letters in common are not significantly different.

(23), suggests that octyne binds to an alternate (non-NH<sub>3</sub>) binding site on AMO. This observation relates to earlier kinetic work of Keener et al. (24), who showed that, whereas NH<sub>4</sub><sup>+</sup> and acetylene competed for the same binding site on AMO of *N. europaea*, NH<sub>4</sub><sup>+</sup> concentration did not affect the rate of inactivation of *N. europaea* AMO by either 1- or 3-hexyne. These authors suggested that *N. europaea* AMO contained different binding sites or different access channels to the active site for small substrates (e.g., NH<sub>3</sub>, CH<sub>4</sub>, and acetylene) versus larger substrates (e.g., 1- and 3-hexyne). Indeed, Keener and Arp (25) also showed that most alternate substrates for *N. europaea* AMO are noncompetitive inhibitors of NH<sub>3</sub> oxidation. The specific reason(s) why octyne inhibits rather than inactivates AOA AMO are unknown and require further study.

Our data on the inhibitory properties of alkynes of different chain length on AOA AMO are worthy of discussion in context with the topic of substrate range of the membrane-bound, Cu-containing methane monooxygenase (pMMO). Whereas it makes intuitive sense to compare characteristics of AMO from AOA and AOB, a suggestion has been made recently that AOA *amo* sequences are more closely related to the particulate methane monooxygenase (*pmo*) of the *Verrucomicrobia* phylum than to AOB *amo* sequences (26). It has been known for many years that pMMOs of the well-studied methanotrophs, *Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* (Bath), exhibit a hydrocarbon substrate range that extends beyond CH<sub>4</sub> and yet, are restricted to short-chain alkanes (C<sub>2</sub> to C<sub>5</sub>) and alkenes (C<sub>2</sub> to C<sub>4</sub>) (27–29). It has been suggested that the chain-length-limited substrate range of pMMO is due to the active site of the enzyme being located in a hydrophobic pocket that cannot accommodate alkanes of >C<sub>5</sub>, and there is speculation that the pocket might have separate binding sites for CH<sub>4</sub> versus the larger substrates (28–31). Perhaps, it is not unreasonable to link our own data with the above-mentioned properties of pMMO to suggest that AOA AMO might also contain a binding pocket resembling pMMO that is either too small to freely accommodate ≥C<sub>6</sub> 1-alkynes or constrained sufficiently to allow binding but does not allow turnover of the longer 1-alkynes. Clearly, since AMO of *N. europaea* oxidizes C<sub>2</sub> to C<sub>8</sub> alkanes and is also inactivated by C<sub>2</sub> to C<sub>8</sub> 1-alkynes, it is reasonable to conclude that the substrate range and catalytic properties of the two AMOs are different.

Furthermore, the reproducible differences in alkyne-chain-length sensitivity among the three isolates of *Thaumarchaeota* may also indicate differences in the properties of the AMO among the AOA. Whether such differences have ecological significance awaits further study.

Acetylene specifically and irreversibly inactivates AMO of *N. europaea* and under that inactivated condition *N. europaea* demonstrates hydroxylamine-dependent NO<sub>2</sub><sup>−</sup> production at a rate identical to NH<sub>3</sub>-driven NO<sub>2</sub><sup>−</sup> production (19, 20). *N. maritimus* also demonstrates hydroxylamine-dependent NO<sub>2</sub><sup>−</sup> production when inactivated with acetylene (32), suggesting that acetylene inhibition is AMO specific, and led those authors to suggest that hydroxylamine is an intermediate in NH<sub>4</sub><sup>+</sup> oxidation in AOA, as well as AOB. However, we cannot rule out that partial inhibition by octyne occurs at a site other than AMO since we were unable to measure hydroxylamine-dependent NO<sub>2</sub><sup>−</sup> production in cultures of *N. viennensis* either partially inhibited by octyne, inactivated with acetylene, or under NH<sub>4</sub><sup>+</sup>-depleted conditions. Much remains to be resolved regarding the mechanism of NH<sub>3</sub>/NH<sub>4</sub><sup>+</sup> ox-

idation in AOA (33), and further work is needed to determine whether there are fundamental differences among the AOA.

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