# Differential inhibition *in vivo* of ammonia monooxygenase, soluble methane monooxygenase and membrane-associated methane monooxygenase by phenylacetylene

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## Summary

**Phenylacetylene was investigated as a differential inhibitor of ammonia monooxygenase (AMO), soluble methane monooxygenase (sMMO) and membrane-associated or particulate methane monooxygenase (pMMO) *in vivo*. At phenylacetylene concentrations $> 1 \mu$M, whole-cell AMO activity in *Nitrosomonas europaea* was completely inhibited. Phenylacetylene concentrations above 100 $\mu$M inhibited more than 90% of sMMO activity in *Methylococcus capsulatus* Bath and *Methylosinus trichosporium* OB3b. In contrast, activity of pMMO in *M. trichosporium* OB3b, *M. capsulatus* Bath, *Methylomicrobium album* BG8, *Methylobacter marinus* A45 and *Methylomonas* strain MN was still measurable at phenylacetylene concentrations up to 1000 $\mu$M. AMO of *Nitrosococcus oceanus* has more sequence similarity to pMMO than to AMO of *N. europaea*. Correspondingly, AMO in *N. oceanus* was also measurable in the presence of 1000 $\mu$M phenylacetylene. Measurement of oxygen uptake indicated that phenylacetylene acted as a specific and mechanistic-based inhibitor of whole-cell sMMO activity; inactivation of sMMO was irreversible, time dependent, first order and required catalytic turnover. Corresponding measurement of oxygen uptake in whole cells of methanotrophs expressing pMMO showed that pMMO activity was inhibited by phenylacetylene, but only if methane was already being oxidized, and then only at much higher concentrations of phenylacetylene and at lower rates compared with sMMO. As phenylacetylene has a high solubility and low volatility, it may prove to be useful for monitoring methanotrophic and nitrifying activity as well as identifying the form of MMO predominantly expressed *in situ*.**

## Introduction

Methanotrophs, bacteria that use methane as their sole source of carbon and energy and autotrophic-nitrifying bacteria that oxidize ammonia for energy, are commonly found at oxic–anoxic interfaces and are widespread in nature. In addition to occupying similar environments, these bacteria show a number of biochemical, morphological and physiological similarities (Bédard and Knowles, 1989; Hanson and Hanson, 1996). In particular, both ammonia oxidizers and methanotrophs can oxidize ammonia to nitrite and methane to methanol, although neither group can grow on the other substrate (Dalton, 1977; O'Neil and Wilkinson, 1977; Hyman and Wood, 1983; Jones and Morita, 1983; Voysey and Wood, 1987; Bédard and Knowles, 1989; Zahn *et al.*, 1994; Bergmann *et al.*, 1998). In autotrophic ammonia-oxidizing bacteria, ammonia is oxidized to hydroxylamine and methane to methanol by a unique membrane-associated enzyme, the ammonia monooxygenase (AMO) (Hollocher *et al.*, 1981; Hyman and Wood, 1983; Jones and Morita, 1983; Hooper *et al.*, 1997). In methanotrophs, the oxidation of ammonia or methane to hydroxylamine or methanol is catalysed by the methane monooxygenase (MMO) (Dalton, 1977; O'Neil and Wilkinson, 1977; Stirling *et al.*, 1983; Prior and Dalton, 1985a,b; Zahn and DiSpirito, 1996). As these cells play significant roles in the global carbon and nitrogen cycles, it is important to understand the relative contribution of methanotrophs and nitrifiers to methane and ammonia oxidation *in situ*.

Monitoring the activity of methanotrophs and nitrifiers in natural systems is further complicated by the existence of two forms of MMO with different characteristics. Most known methanotrophs only express a membrane-

associated or particulate methane monooxygenase (pMMO). pMMO has a great deal of similarity to AMO at the DNA and protein levels (McTavish *et al.*, 1993; Holmes *et al.*, 1995; Semrau *et al.*, 1995). In some methanotrophs, under limiting copper concentrations, a cytoplasmic, soluble methane monooxygenase (sMMO) is expressed that can also oxidize ammonia as well as methane. sMMO, however, has little DNA or protein sequence similarity to either pMMO or AMO (Stainthorpe *et al.*, 1990; Cardy *et al.*, 1991a,b; McTavish *et al.*, 1993; Semrau *et al.*, 1995; Grosse *et al.*, 1999).

An inhibitor that could discriminate between pMMO, sMMO and AMO would allow assessment of the activity of nitrifiers and methanotrophs as well as the relative expression of the two forms of MMO. However, most inhibitors of sMMO and pMMO activity, e.g. acetylene, methyl fluoride and difluoromethane, also inhibit AMO activity (Hooper and Terry, 1974; Hyman and Wood, 1985; Prior and Dalton, 1985a; Ward, 1987; Bédard and Knowles, 1989; Oremland and Culbertson, 1992; Miller *et al.*, 1993; 1998). One study has shown that allylsulphide can distinguish between nitrification by AMO and methane oxidation by methanotrophs expressing sMMO, but it is not known whether allylsulphide can distinguish between AMO and pMMO activity (Roy and Knowles, 1995).

It is important to find a selective inhibitor of whole-cell AMO, pMMO and sMMO activity for examining the activity of methanotrophs and ammonia oxidizers *in situ*, in part to develop systems for the use of methanotrophs for the biodegradation of priority pollutants, such as trichloroethylene (TCE). Specifically, the maximal uptake rate of TCE ($V_{max}$) by methanotrophs expressing sMMO is typically one or two orders of magnitude faster than for cells expressing pMMO. The affinity of methanotrophs for TCE ($K_s$), however, can be as much as 25 times greater for cells expressing pMMO than for those expressing sMMO, indicating that pMMO-expressing cells may be able to degrade TCE to lower levels than cells expressing sMMO (Oldenhuis *et al.*, 1991; Koh *et al.*, 1993; Lontoh and Semrau, 1998). Owing to differences in the rates of TCE degradation, it may be profitable to have methanotrophs first express sMMO in order to promote rapid rates of TCE removal, and subsequently express the high-affinity pMMO to achieve clean-up standards. An inability to distinguish accurately and easily between sMMO and pMMO activity complicates the development of a system for the use of methanotrophs for hazardous waste remediation.

Although AMO, pMMO and sMMO can oxidize methane and ammonia, these enzymes have very different ranges of substrate specificity. Both sMMO and AMO from *Nitrosomonas europaea* have been shown to oxidize aromatic hydrocarbons, whereas the oxidation of aromatic

compounds has never been observed in cells expressing pMMO (Colby *et al.*, 1977; Burrows *et al.*, 1984; Vannelli and Hooper, 1995; Hooper *et al.*, 1997; Keener *et al.*, 1998) and has not been studied in *Nitrosococcus oceanus*. As mentioned earlier, acetylene is an inhibitor of all three enzymes. Phenylacetylene has been shown to inhibit AMO effectively in *N. europaea* and, based on this result, it has been suggested that phenylacetylene might differentially inhibit whole-cell AMO, pMMO and sMMO activity (Vannelli *et al.*, 1996). In this report, we show that phenylacetylene does indeed differentially inhibit the members of the ammonia and methane monooxygenase family. As phenylacetylene has a higher solubility and lower volatility compared with acetylene, phenylacetylene may be useful for monitoring nitrifying and methanotrophic activity *in situ*.

## Results

### Inhibition of ammonia and methane monooxygenase activity by phenylacetylene

The effect of phenylacetylene on whole-cell AMO, sMMO and pMMO activity is shown in Table 1. In these experiments, as phenylacetylene was not removed before adding substrates, phenylacetylene can reduce enzymatic activity by either preventing other substrates from binding or forming reactive intermediates that could inactivate AMO, sMMO or pMMO. Regardless of how phenylacetylene affected whole-cell enzymatic activity, we use the term inhibition to describe experiments in which phenylacetylene was not removed before measuring whole-cell activity. Ammonia oxidation by *N. europaea*, as measured by nitrite appearance, was severely limited by relatively low concentrations of phenylacetylene, with complete inhibition found at 600 nM phenylacetylene. Methanotrophic activity, as measured via the production of propylene oxide from propylene, was also affected by phenylacetylene, but at different amounts and only at much higher concentrations. For cells expressing sMMO (*Methylosinus trichosporium* OB3b and *Methylococcus capsulatus* Bath), over 95% inhibition was observed at a concentration of 200 μM phenylacetylene. For methanotrophs expressing pMMO, inhibition was much lower at this concentration, varying anywhere between 33% and 52% loss of activity compared with whole cells incubated in the absence of phenylacetylene. At lower concentrations, the amount of pMMO inhibition was much less and, for several methanotrophs, no inhibition was observed in the presence of 100 μM phenylacetylene (compared with at least 90% for cells expressing sMMO). For *Methylomicrobium album* BG8, pMMO activity actually increased in the presence of either 50 or 100 μM phenylacetylene, although higher concentrations did inhibit whole-cell pMMO activity. At the

**Table 1.** Percentage inhibition of ammonia monooxygenase and methane monooxygenase activity by phenylacetylene, as determined by nitrite and propylene oxide production respectively.

| Cell (enzyme) | Phenylacetylene ($\mu$M) | | | | | | | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | 0.1 | 0.3 | 0.6 | 10 | 50 | 100 | 200 | 500 | 800 | 1000 |
| *N. europaea* (AMO) | 15 | 63 | 100 | – | – | – | – | – | – | – |
| *M. capsulatus* Bath (sMMO) | – | – | – | – | 60 | 97 | 100 | – | – | – |
| *M. trichosporium* OB3b (sMMO) | – | – | – | – | – | 90 | 96 | 98 | 97 | 99 |
| *N. oceanus* (AMO) | – | – | – | 1 | – | 29 | – | 86 | – | 98 |
| *M. capsulatus* Bath (pMMO) | – | – | – | – | 0 | 17 | 52 | 64 | – | 96 |
| *M. trichosporium* OB3b (pMMO) | – | – | – | – | – | 26 | 39 | 74 | 74 | 81 |
| *M. marinus* A45 (pMMO) | – | – | – | – | – | 0 | 35 | 78 | – | 82 |
| *M.* sp. MN (pMMO) | – | – | – | – | – | 0 | 39 | – | – | 61 |
| *M. album* BG8 (pMMO) | – | – | – | – | 23[a] | 65[a] | 33 | 84 | 88 | 89 |

–, not determined.
**a.** Stimulation of pMMO activity.

relatively high phenylacetylene concentrations of 1000 $\mu$M, significant inhibition of pMMO activity was observed, often above 80%. Interestingly, complete inhibition of AMO activity in *N. oceanus* was also observed in the concentration range similar to that of methanotrophs expressing pMMO.

### Oxygen uptake by methanotrophs in the presence of phenylacetylene

To examine further the effect of phenylacetylene on sMMO and pMMO activity in whole cells, oxygen uptake experiments were performed with *M. trichosporium* OB3b. As shown in Fig. 1, cells were incubated in the presence of varying concentrations of phenylacetylene for 5 min, after which 1.5 mM methane was added. For sMMO-expressing cells, the rate of oxygen uptake was noticeably affected at phenylacetylene concentrations as low as 14 $\mu$M, and the rate of oxygen uptake decreased with increasing phenylacetylene concentrations. For *M. trichosporium* OB3b expressing pMMO, however, only a slight reduction was seen in the rates of oxygen uptake at phenylacetylene concentrations up to 140 $\mu$M. As observed in the propylene oxide assays, pMMO inhibition by phenylacetylene was measurable at higher phenylacetylene concentrations.

### Specific mechanism-based inactivation of MMO activity by phenylacetylene

Oxygen consumption by *M. trichosporium* OB3b was measured in the presence of 1 mM phenylacetylene and either 770 $\mu$M methanol or 20 mM formate to determine whether whole-cell inhibition by phenylacetylene was specific to MMO inactivation or if other enzymes were also affected. As shown in Table 2, no reduction in oxygen uptake from the oxidation of methanol or formate

was observed, indicating that phenylacetylene is a specific inhibitor of MMO activity.

From the data in Fig. 1, it is clear that inhibition of sMMO-mediated methane oxidation by phenylacetylene was dependent on the concentration of phenylacetylene. If phenylacetylene is a mechanistic inhibitor of sMMO
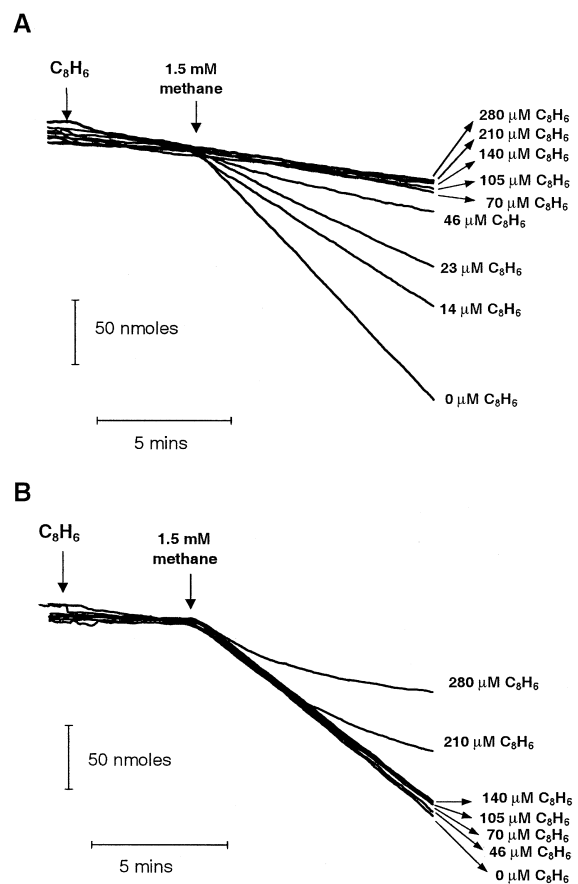


**Fig. 1.** Effect of phenylacetylene on methane-dependent oxygen uptake by *M. trichosporium* OB3b expressing either (A) sMMO or (B) pMMO. Arrows indicate the time at which phenylacetylene and methane were added to the oxygen uptake reactor.

**Table 2.** Whole-cell oxidation of methanol and formate by *M. trichosporium* OB3b expressing either sMMO or pMMO in the presence and absence of phenylacetylene (numbers in parentheses are the range found in duplicate samples).

| Substrate | Phenylacetylene (mM) | Rate of oxygen uptake (nmol min$^{-1}$ mg$^{-1}$ protein) | |
|---|---|---|---|
| | | sMMO | pMMO |
| 20 mM formate | 0 | 82 (1.6) | 67 (1.8) |
| | 1 | 79 (1.8) | 65 (2.0) |
| 770 μM methanol | 0 | 92 (1.0) | 102 (2.9) |
| | 1 | 92 (4.1) | 109 (6.0) |

activity, inactivation should also be first order with respect to time. As the oxygen uptake experiments reported in Fig. 1 were performed with cells incubated for 5 min with phenylacetylene, any time-dependent inactivation would have occurred before adding methane, and thus was not measurable. The time dependence of inactivation, however, can be determined if the order of phenylacetylene and methane addition are reversed. As shown in Fig. 2A, after adding 1.5 mM methane, a constant rate of oxygen uptake was established. Subsequent addition of phenyl-acetylene caused a time-dependent decrease in the rate of oxygen uptake, and the magnitude of decrease increased with increasing phenylacetylene concentration. If the rate of uptake is plotted against time after adding phenylacetylene, as shown in Fig. 2B, a general first-order decay can be fitted to the data, as indicated by the bold lines. The observed first-order rate constants ($k_{obs}$) ranged from 0.06 ± 0.02 to 0.98 ± 0.04 min$^{-1}$ as the phenylacetylene concentration increased from 14 to 280 μM (Fig. 2C). Under the tested concentration range, however, saturation of $k_{obs}$ did not occur.

To verify further that phenylacetylene acted as a mechanistic-based inhibitor of sMMO, the inactivation of whole-cell MMO activity was monitored in the presence and absence of oxygen. If *M. trichosporium* OB3b expressing sMMO was incubated under anaerobic conditions in the presence of 70 μM phenylacetylene for 10 min, very little loss of methane-dependent oxygen uptake was observed after the phenylacetylene was removed as described in *Experimental procedures* compared with controls in which no phenylacetylene was added (Table 3). If sMMO-expressing cells were exposed to 70 μM phenylacetylene for 10 min in aerobic conditions, however, less than a quarter of the initial oxygen uptake rate was measured after removing phenyl-acetylene, indicating that enzymatic turnover was necessary for phenylacetylene binding and inhibition. This observation is consistent with the catalytic cycle of sMMO, in which the initial reduction of the diiron centre is followed by the binding of oxygen. The heterolytic O–O bond is then cleaved to yield water and an electron-deficient iron species that can attack substrates (Wallar and Lipscomb, 1996). Thus, in the absence of oxygen, a mechanistic-based inactivator such as phenylacetylene is not expected to affect sMMO.
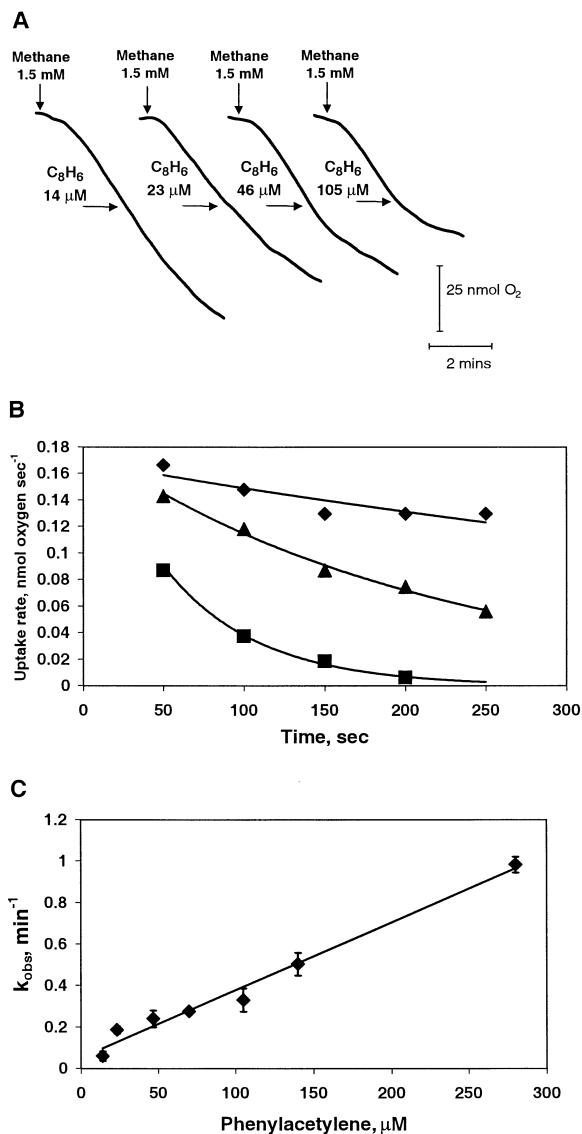
**Fig. 2.** Kinetics of inactivation of methane-dependent oxygen uptake by *M. trichosporium* OB3b expressing sMMO when methane was added before phenylacetylene.
A. Oxygen uptake by cells expressing sMMO.
B. Rate of methane-dependent oxygen consumption versus time for three concentrations of phenylacetylene: (◆) 14 μM; (▲) 70 μM; (■) 280 μM. Solid lines indicate first-order decay model fit to the data.
C. Rate of inactivation ($k_{obs}$) versus phenylacetylene concentration. Solid line indicates the best-fit straight line to the data. Error bars indicate standard deviation of measured $k_{obs}$ values

**Table 3.** Inhibition of *M. trichosporium* OB3b whole-cell sMMO and pMMO activity after incubation with phenylacetylene under aerobic and anaerobic conditions (numbers in parentheses indicate the range in duplicate samples).

| Phenylacetylene ($\mu$M) | Rate of oxygen uptake (nmol min$^{-1}$ mg$^{-1}$ protein) | | | |
|---|---|---|---|---|
| | pMMO | | sMMO | |
| | $-O_2$ | $+O_2$ | $-O_2$ | $+O_2$ |
| 0 | 119 (1.2) | 121 (1.2) | 134 (2.5) | 132 (2.3) |
| 70 | NM | NM | 127 (0.56) | 28.2 (1.36) |
| 140 | 112 (2.6) | 114 (3.2) | NM | NM |

NM, not measured.

Similar experiments were performed with *M. trichosporium* OB3b expressing pMMO in which methane was added before phenylacetylene. As shown in Fig. 3A, oxygen uptake from methane oxidation by pMMO *in vivo* was not affected at phenylacetylene concentrations $< 105$ $\mu$M. Interestingly, at a concentration of 140 $\mu$M, substantial inhibition was observed, as noted by the time-dependent decrease in oxygen uptake after adding phenylacetylene. Such inhibition was not apparent if 140 $\mu$M phenylacetylene was added before methane. If the rate of oxygen uptake is plotted with respect to time after adding phenylacetylene, the observed first-order rate constants increased from $0.19 \pm 0.13$ to $0.44 \pm 0.17$ min$^{-1}$ as phenylacetylene concentrations increased from 140 to 280 $\mu$M (Fig. 3B and C). Unlike inhibition of sMMO-expressing cells, the observed rates of inactivation at 140 and 280 $\mu$M phenylacetylene were not statistically different at the 95% confidence interval.

To determine whether phenylacetylene was a mechanistic inhibitor of pMMO *in vivo*, *M. trichosporium* OB3b grown with 20 $\mu$M copper was incubated anaerobically with 140 $\mu$M phenylacetylene for 10 min. As shown in Table 3, pMMO activity was not affected by this concentration of phenylacetylene in the absence of oxygen. Furthermore, as found earlier when cells were incubated with phenylacetylene for 5 min (Fig. 1B), if the cells were exposed to 140 $\mu$M phenylacetylene under aerobic conditions, no loss of pMMO activity was observed after phenylacetylene was removed. From these results, it appears that the order of addition of substrate (methane) and inhibitor (phenylacetylene) was important in inhibiting MMO activity. As shown in Fig. 4, phenylacetylene inhibition of sMMO activity occurred at similar rates, regardless of whether methane was provided before or after phenylacetylene. pMMO activity, however, was affected more substantially if methane was added before phenylacetylene.

## Discussion

Several studies have shown that ammonia oxidizers and methanotrophs are inhibited by the same compounds
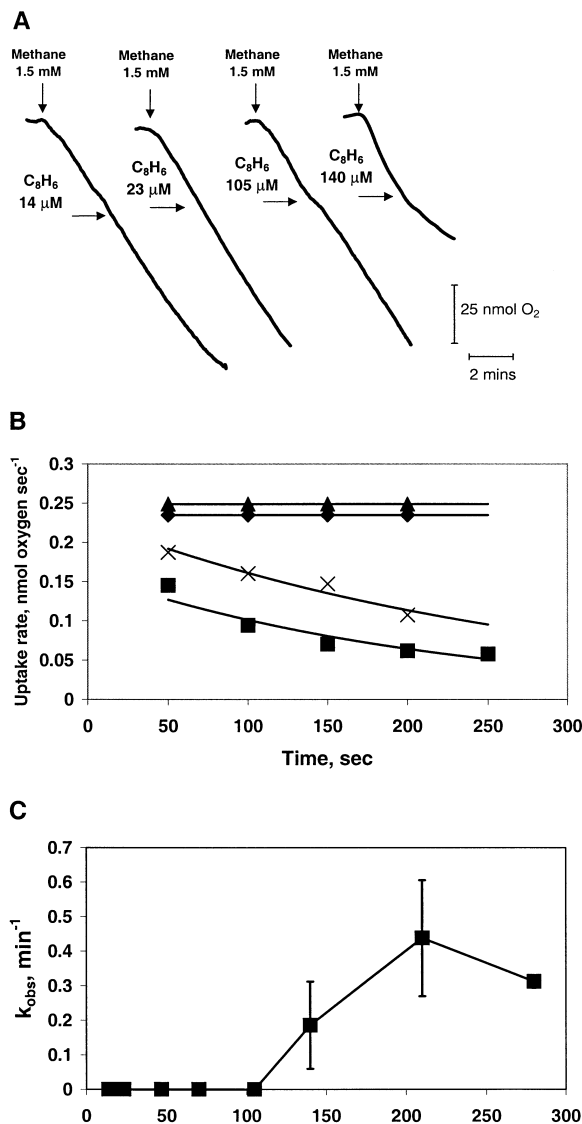


**Fig. 3.** Kinetics of inactivation of methane-dependent oxygen uptake by *M. trichosporium* OB3b expressing pMMO when methane was added before phenylacetylene.
A. Oxygen uptake by cells expressing pMMO.
B. Rate of methane-dependent oxygen consumption versus time for four concentrations of phenylacetylene: ($\blacklozenge$) 14 $\mu$M; ($\blacktriangle$) 105 $\mu$M; (X) 140 $\mu$M; ($\blacksquare$) 280 $\mu$M. Solid lines indicate first-order decay model fit to the data.
C. Rate of inactivation ($k_{obs}$) versus phenylacetylene concentration. Error bars indicate standard deviation of measured $k_{obs}$ values.
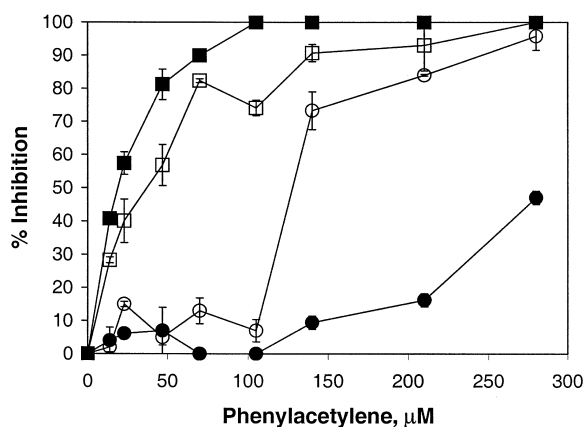
**Fig. 4.** Effect of the order of methane and phenylacetylene addition on inhibition of methane-dependent oxygen uptake by *M. trichosporium* OB3b expressing either sMMO or pMMO. (■) Whole-cell sMMO inhibition when phenylacetylene was added before methane; (□) whole-cell sMMO inhibition when phenylacetylene was added after methane; (●) whole-cell pMMO inhibition when phenylacetylene was added before methane; (○) whole-cell pMMO inhibition when phenylacetylene was added after methane. Error bars indicate range of duplicate samples.

and, in some cases, differential inhibition is apparent (Bédard and Knowles, 1989; Roy and Knowles, 1995). Previous research, however, has not identified any substance(s) that can be used selectively to inactivate AMO, sMMO and pMMO activity in whole cells. Phenylacetylene, already shown completely to inhibit AMO of *N. europaea* at 100 μM (Vannelli *et al.*, 1996), is shown here to have the same effect at 1 μM. Furthermore, over 90% of sMMO activity, as measured using propylene oxide assays, was lost when *M. trichosporium* OB3b and *M. capsulatus* Bath were exposed to 100 μM phenylacetylene. For the five methanotrophs examined expressing pMMO, < 25% inactivation was observed at 100 μM phenylacetylene, with some residual activity remaining at 1000 μM phenylacetylene. Interestingly enough, small amounts of AMO activity in *N. oceanus* were also observed at 1000 μM phenylacetylene. The DNA-derived protein sequence of AMO of *N. oceanus* is more similar to that of pMMO than to that of AMO expressed by *N. europaea* (Holmes *et al.*, 1995). Although it is known that AMO of *N. europaea* can oxidize aromatic compounds, we found that AMO of *N. oceanus* is unable to oxidize styrene or ethylbenzene (data not shown). This is consistent with the ability of AMO in *N. oceanus* to oxidize the smaller substrates ethylene and methylbromide, but not 1,2 dibromochloropropane or 1,2 dichloropropane (Rasche *et al.*, 1990). It is surprising, however, that AMO in *N. oceanus* is inhibited by 4 μM 2-chloro-6-trichloromethylpyridine (Salvas and Taylor, 1984; Ward, 1987), as is MMO activity in *M. trichosporium* OB3b and *M. capsulatus* Bath (Topp and Knowles, 1982).

The effect of phenylacetylene on nitrifying and methanotrophic activity can be separated into three general groups: (i) *in vivo* inhibition of *N. europaea* AMO at phenylacetylene concentrations < 1 μM; (ii) *in vivo* inhibition of sMMO activity at concentrations of the order of 100 μM; and (iii) *in vivo* inhibition of pMMO activity and of closely related AMO from *N. oceanus* at 1000 μM. As *N. oceanus* AMO apparently does not oxidize aromatic compounds, the naphthalene assay developed for monitoring sMMO activity (Brusseau *et al.*, 1990) may prove useful as an initial screening tool to determine which class of AMO is expressed in environmental nitrifying isolates. The use of phenylacetylene as a selective inhibitor is facilitated by the fact that it is a liquid at 25°C, has a low volatility (dimensionless Henry's constant, $K_H$, of 0.0244) and a relatively high solubility in water (4.46 mM) (Howard and Meylan, 1997). Therefore, its ease of use compared with other inhibitors, coupled with the differential inhibition of monooxygenase activity, offers a promising technique for monitoring AMO, sMMO and pMMO activity *in situ*.

After determining that phenylacetylene effectively inhibited whole-cell MMO activity, phenylacetylene was examined extensively to determine how it inhibited sMMO activity. As outlined earlier (Silverman, 1988), there are several criteria that must be satisfied when considering a substrate to act as a mechanism-based inactivator. First, the inhibitor must act only upon the enzyme of interest in the cell, i.e. sMMO. As shown in Table 2, oxygen uptake by *M. trichosporium* OB3b expressing sMMO in the presence of formate and methanol, but in the absence of methane, was not affected by 1 mM phenylacetylene. Secondly, sMMO activity must be irreversibly inhibited by phenylacetylene, as shown in Table 3. Thirdly, decrease in sMMO activity in the presence of phenylacetylene should be first order with respect to time. As shown in Fig. 2, the rates of oxygen uptake by whole cells expressing sMMO decreased with first-order decay at phenylacetylene concentrations as low as 14 μM. Fourthly, inhibition must require catalytic turnover of phenylacetylene. As shown in Table 3, no inhibition of whole-cell sMMO activity was observed after incubating *M. trichosporium* OB3b with 70 μM phenylacetylene for 10 min under anaerobic conditions, but substantial inactivation was observed in aerobic conditions. Collectively, these results indicate that phenylacetylene acted as a specific mechanism-based inhibitor of sMMO activity in *M. trichosporium* OB3b.

Not all criteria for the demonstration of mechanism-based inhibition of sMMO by phenylacetylene have been satisfied, however. An additional criterion is that the rate of inactivation of sMMO activity should be proportional to phenylacetylene at low concentrations, but become saturated at high concentrations. As seen in studies of mechanism-based inhibitors of AMO and toluene-2-

monooxygenase (Keener *et al.*, 1998; Yeager *et al.*, 1999), inhibition of sMMO activity increased proportionally with phenylacetylene, but saturation of the observed inactivation rate was not found over the tested concentration range. It is unclear why saturation was not observed, although it may occur at higher concentrations. As suggested for the inactivation of toluene-2-monooxygenase by 1-butyne (Yeager *et al.*, 1999), it is possible that the maximal rate of inactivation is faster than the rate at which phenylacetylene binds to sMMO, or that a conventional enzyme–inactivator complex might not be formed before inactivation. Finally, a 1:1 stoichiometry of labelled phenylacetylene to the active site of sMMO must also be demonstrated to verify phenylacetylene as a mechanistic inactivator of sMMO. [$^{14}$C]-Phenylacetylene is not commercially available; therefore, these experiments were not performed.

Similar studies on whole-cell pMMO activity were not conclusive as to the manner in which phenylacetylene inhibits pMMO activity. As for sMMO-expressing cells, formate and methanol oxidation was not affected by phenylacetylene in pMMO-expressing cells (Table 2). Whole-cell inhibition of pMMO activity was apparent, but only at much higher phenylacetylene concentrations than those observed for sMMO. As shown in Fig. 3, oxygen uptake rates by pMMO did not exhibit first-order decay until the phenylacetylene concentration increased to at least 140 $\mu$M. Interestingly, inhibition at this concentration was only observed if *M. trichosporium* OB3b was first exposed to both methane and oxygen. It appears that, for phenylacetylene to inhibit pMMO activity effectively, methane must first be bound by pMMO. Such a result, although surprising, is not unprecedented, as inhibition of AMO activity by several compounds in *N. europaea* has been seen to be enhanced by the presence of ammonia (Arp *et al.*, 1996). For AMO, it has been suggested that the addition of ammonia promotes the production of some reactive species that activated the inhibitors (Keener *et al.*, 1998). A similar mechanism may be occurring in pMMO-expressing cells. For example, methane may act as a precursor to a biological electron donor, e.g. methanol or formate, which creates a reduced oxygen species that activates phenylacetylene. Alternatively, radical species may be formed during methane oxidation that react with phenylacetylene, causing pMMO inactivation. Although it is unclear how methane stimulates phenylacetylene inhibition of pMMO, the finding that methane must be present before adding phenylacetylene for effective inhibition of pMMO, as shown in Fig. 4, provides another methodology for determining which form of MMO is expressed by natural communities of methanotrophs. Also, as sMMO activity was strongly affected over a range of phenylacetylene concentrations, whereas pMMO was not, varying the concentration of phenylacetylene added

to environmental samples and monitoring the resulting effect on methane oxidation may provide another mechanism for determining which form of MMO is expressed *in situ*.

The finding that sMMO was much more sensitive than pMMO to phenylacetylene may prove useful for monitoring methanotrophic activity during *in situ* bioremediation. As the two forms of MMO oxidize priority pollutants such as chlorinated ethylenes at different rates, it is imperative to know what form of MMO is expressed during methanotrophic bioaugmentation or biostimulation so that the time frame for clean up can be determined more accurately. By measuring the rates of methane oxidation before and after the addition of phenylacetylene to environmental samples, it should be possible to understand better which form of MMO is expressed by methanotrophic communities under different conditions, allowing for the optimization of methanotrophic bioremediation.

In conclusion, selective inhibition of AMO, sMMO and pMMO activity by phenylacetylene was observed in pure cultures of nitrifiers and methanotrophs. As such, phenylacetylene promises to be a useful tool for the study of methanotrophic and nitrifying activity *in situ*. Future work should extend these experiments to consider soil microcosms in which microbial activity can be verified independently (e.g. through the use of reverse transcription–polymerase chain reaction) so that the phenylacetylene assay can be calibrated for more widespread use.

## Experimental procedures

### Materials

All chemicals used in media preparation were of reagent grade or better. Highest purity methane ($>$ 99.99%) was obtained from the Matheson Gas Co. Phenylacetylene (98%), methanol (99.9%), formate (90%) and propylene ($>$ 99%) were obtained from Aldrich. Distilled deionized water was used for all experiments.

### Growth and preparation of nitrifying bacteria

*N. europaea* was grown in continuous culture as described previously (Logan *et al.*, 1995) Cells were collected and resuspended to a concentration of 20% (wet w/v) in 50 mM potassium phosphate buffer (pH 7.8) and used as the stock culture for phenylacetylene inhibition experiments. *N. oceanus* was grown in 1 l batch culture as described previously (Watson, 1965), except that the concentration of MgSO$_4$ was increased to 1.45 mM, and the ammonium sulphate concentration was decreased to 20 mM. *N. oceanus* was collected by centrifugation and resuspended to 20% (wet w/v) in growth medium lacking ammonium sulphate and used as the stock culture for the phenylacetylene inhibition experiments.

### Phenylacetylene inactivation of whole-cell AMO activity

Inactivation assays were run in duplicate. Five millilitres of

50 mM potassium phosphate buffer (pH 7.8) was transferred aseptically to 10 ml hypovials and then crimp sealed with butyl rubber septa (Chrom-Tech). Phenylacetylene was then added from a 100 mM DMSO stock solution to the appropriate final concentration. Control experiments were performed using the same volumes of DMSO alone. *N. europaea* was added from a stock culture to a final concentration of 0.08% (wet w/v) and preincubated for 20 min at room temperature with orbital shaking at 100 r.p.m. Assays of AMO activity were initiated by the addition of ammonium sulphate to a final concentration of 5 mM from a stock solution of 1.25 M $(NH_4)_2SO_4$. Rates of inhibition relative to the DMSO control were monitored by the production of nitrite over 1 h as described previously (Hooper and Terry, 1973). To determine the effect of phenylacetylene on AMO activity in *N. oceanus*, the same procedure was used as that described for *N. europaea*, except that 50 mM phosphate buffer was exchanged for the buffer used for resuspension of *N. oceanus*.

### Growth of methanotrophs

Methanotrophs from all three categories, types I, II and X, were used in this study. Two freshwater type I strains that can only express pMMO, *Methylomonas* sp. MN and *Methylomicrobium album* BG8, were grown in nitrate mineral salts (NMS) medium with 20 μM copper added as $Cu(NO_3)_2$ $2.5(H_2O)$, as described previously (Lontoh and Semrau, 1998). A third type I marine methanotroph, *Methylobacter marinus* A45, which can also only express pMMO, was grown in NMS medium plus 1.5% (w/v) NaCl (Lidstrom, 1988) at 37°C. Two strains that can express both forms of MMO, *Methylosinus trichosporium* OB3b (type II) and *Methylococcus capsulatus* Bath (type X), were grown in NMS medium either in the absence of copper to promote sMMO expression or with 20 μM copper for pMMO expression. To monitor the expression of sMMO, the naphthalene assay specific for sMMO activity was used for all cell suspensions (Brusseau *et al.*, 1990).

### Inhibition of whole-cell pMMO and sMMO activity by phenylacetylene

The effect of phenylacetylene on whole-cell methanotrophic activity was measured by two different methods to determine the effectiveness of this compound as a selective inhibitor of sMMO and pMMO activity. First, propylene oxide production, an assay commonly used to monitor MMO activity (Prior and Dalton, 1985b), was used. In these assays, cells were grown in NMS medium to an $OD_{600}$ of 0.45 with either 0 or 20 μM copper. Cells were collected by centrifugation, washed and resuspended in 10 mM PIPES buffer to a protein concentration of 0.15 mg ml$^{-1}$. For cells expressing pMMO, 20 μM copper was also added to the PIPES buffer. Cell suspension (3 ml) was then transferred aseptically to 20 ml serum vials and crimp sealed with butyl rubber septa (Wheaton). Phenylacetylene was added from a stock DMSO solution to obtain aqueous concentrations ranging from 0 to 1 mM, and each concentration was run in duplicate. Propylene was then added after shaking the vials for 15 min at 30°C and

270 r.p.m. MMO activity was determined by measuring the amount of propylene oxide production after 1 h of incubation. Headspace samples (100 μl) were injected into an HP 6890 gas chromatograph with a flame ionization detector and a 30 m DB5 column (J and W Scientific). The helium carrier gas flow rate was 36.8 ml min$^{-1}$. The oven temperature was set initially at 60°C for 3 min and then increased at a rate of 45°C min$^{-1}$ to 160°C and held at that temperature for 2 min. The FID and inlet temperatures were both 250°C. Under these conditions, the retention time of propylene oxide was 2.45 min.

The effect of phenylacetylene on whole-cell methanotrophic activity was also measured for *M. trichosporium* OB3b expressing either sMMO or pMMO using an oxygen uptake measurement system described previously (Lontoh *et al.*, 1999). The voltage output from the oxygen electrode was smoothed by applying a moving average trend line using Microsoft Excel 97 for Windows. Cells were grown, collected by sedimentation and then added to the oxygen uptake reactor at a concentration range of 0.13–0.14 mg of protein ml$^{-1}$ in NMS medium. Methane was added using a Dynatech A-2 gas-tight syringe giving an aqueous concentration of 1.5 mM. Phenylacetylene was added to obtain aqueous concentrations up to 280 μM using a Hamilton 1700 series gas-tight syringe. Similar experiments were performed with methanol and formate as substrates at aqueous concentrations of 0.77 and 20 mM respectively. At phenylacetylene concentrations $> 140$ μM, phenylacetylene itself caused a probe effect in which a slight constant decrease in oxygen concentration was observable over time in NMS medium without cells. This effect was considered by subtracting it from cell oxygen uptake measured in the presence of methane and phenylacetylene.

To determine whether catalytic turnover was necessary for MMO inhibition, 10 ml of a 0.13 mg of protein ml$^{-1}$ *M. trichosporium* OB3b suspension expressing either sMMO or pMMO was aseptically transferred to 20 ml serum vials and crimp sealed with rubber butyl rubber stoppers. In some vials, the headspace was evacuated by applying a vacuum for 5 min and replaced with nitrogen. For sMMO- and pMMO-expressing cells, phenylacetylene was added using Hamilton series 1700 gas-tight syringes to provide 70 and 140 μM in solution respectively. The cells were incubated in the presence of phenylacetylene under either aerobic or anaerobic conditions for 10 min. The vials were then vacuumed again for 5 min to remove the phenylacetylene and backfilled with sterile air. The oxygen uptake rate was measured in the presence of 1.5 mM methane. Controls were also performed in which the entire procedure was followed but without adding phenylacetylene.

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