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Blockage by Acetylene of Nitrous Oxide Reduction in *Pseudomonas perfectomarinus*

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Suspensions of denitrifying cells of *Pseudomonas perfectomarinus* reduced nitrate and nitrite as expected to dinitrogen; but, in the presence of acetylene, nitrous oxide accumulated when nitrate or nitrite was reduced. When supplied at the outset in place of nitrate and nitrite, nitrous oxide was rapidly reduced to dinitrogen by cells incubated in anaerobic vessels in the absence of acetylene. In the presence of 0.01 atmospheres of acetylene, however, nitrous oxide was not reduced. Ethylene was not produced, nor did it influence the rate of nitrous oxide reduction when provided instead of acetylene. Cells exposed to 0.01 atmospheres of acetylene for as long as 400 min were able to reduce nitrous oxide after removal of acetylene at a rate comparable to that of cells not exposed to acetylene. Acetylene did not affect the production or functioning of assimilatory nitrate or nitrite reductase in axenic cultures of *Enterobacter aerogenes* or *Trichoderma viride*. While exposed to acetylene, bacteria in marine sediment slurries produced measurable quantities of nitrous oxide from glucose- or acetate-dependent reduction of added nitrate. Possible use of acetylene blockage for measurement of denitrification in unamended marine sediments is discussed.

During development of gas exchange assays for possible use in extra terrestrial life detection experiments, Fedorova et al. (2) noted that acetylene blocked reduction of nitrous oxide to nitrogen by soils bacteria. If marine bacteria were similarly affected, application of this method for determination of rates of denitrification in marine sediments and marsh mud (1, 4) seemed promising. The purposes of the current study were therefore to examine the influence of various concentrations of acetylene on the reduction of nitrous oxide by the marine denitrifier *Pseudomonas perfectomarinus* (8) and bacteria in marine sediments, to learn whether the inhibitory effect is reversible, and to determine whether acetylene has any effect on microbial assimilatory nitrate and nitrite reduction.

MATERIALS AND METHODS

P. perfectomarinus was grown anaerobically in STYN broth (0.5% tryptone, 0.15% yeast extract, 0.1% KNO_2 made with 20‰ artificial sea water [ASW]) in 1-liter quantities for 24 h at 30 C. The cells were harvested, washed twice in 0.05 M $MgCl_2$ to prevent lysis, and suspended in 0.02 M potassium phosphate buffer made with 20‰ ASW (pH 6.8). A fresh cell suspension (averaging 44.2 mg [dry weight]/ml) was used for each experiment.

Reaction mixtures were incubated in double-arm Warburg flasks with one arm closed with a rubber serum bottle stopper to permit withdrawal with a hypodermic needle and gas-tight syringe of samples for analysis by gas chromatography of the gaseous atmosphere over the reaction mixtures. The reaction vessels were attached to the manometers of the Warburg apparatus only for monitoring the maintenance of a slight positive gas pressure in each flask and not to measure gas volume changes.

The basal reaction mixture in each flask consisted of 2 ml of 0.02 M potassium phosphate buffer made with 20‰ ASW (pH 6.8), 200 μl of 0.26 M asparagine, and 200 μl of 0.16 M KNO_2 or KNO_3 (as needed). The cell suspension was added to one side arm, and the flasks were gassed with helium for 20 min and closed. Nitrous oxide, acetylene, and ethylene (Matheson Gas Products) were added as required (by hypodermic needle and gas-tight syringe) after gassing and before the cells were tipped in with the other reactants (zero time). Nitric oxide is not reduced by whole cells of *P. perfectomarinus* (1) and was thus not provided. In experiments designed to determine the reversibility of the acetylene inhibition effect, the flasks containing reaction mixtures incubated for various periods of time were opened and then gassed with helium for 10 min and closed. Nitrous oxide was then added (zero time). The reaction mixtures were incubated in a water bath at 30 C.

The effect of acetylene on the capacity of bacteria and fungi to assimilate nitrate and nitrite nitrogen was tested by using *Enterobacter aerogenes* (CDC 659-66) and *Trichoderma viride* (kindly provided by R. T. Hanlin, University of Georgia). *E. aerogenes* was grown anaerobically at 30 C with and without acetylene (0.01 atmospheres) in a minimal salts medium (each liter contained: K_2HPO_4, 9.28 g; KH_2PO_4, 1.81 g; Na_2SO_4, 0.5 g; $MgSO_4$, 0.01 g; $FeCl_3$,

12.5 mg) with KNO_3 or KNO_2 (0.1%). Under these conditions both assimilatory and dissimilatory nitrate reductases are formed. Additional cultures containing KNO_3 or KNO_2 were supplemented with NH_4Cl (0.05%) to repress the synthesis of the assimilatory nitrate and nitrite reductases (6, 7). *T. viride* was grown aerobically with and without acetylene (0.01 atmospheres) in Westergaard medium (3), using KNO_3 or KNO_2 (0.1%) as the sole nitrogen source. The growth rate of *E. aerogenes* revealed by increasing turbidity was measured spectrophotometrically, whereas the quantity of mycelia accumulated after 3 days of growth at 25 C in the various *T. viride* cultures was observed and compared with the naked eye.

Sediments were collected from the salt marsh at Sapelo Island, Ga., and made into 50% slurries using sterile, 20‰ ASW. Fifty-milliliter portions of slurry were added to 125-ml Erlenmeyer flasks, which were then closed with butyl rubber stoppers and gassed with helium for 30 min. Either glucose or sodium acetate (0.25% final concentration) and KNO_3 (0.1% final concentration) were added to the anoxic slurry in each flask. Control mixtures were not supplemented with acetylene, whereas the atmosphere of experimental flasks was enriched with 0.01 to 0.02 atmospheres of acetylene.

Sampling and gas chromatographic analysis of the gases in the atmosphere over each reaction mixture were conducted as previously described (1, 4).

RESULTS AND DISCUSSION

Incubation in media containing increasing concentrations of acetylene resulted in accumulation of increasing amounts of nitrous oxide by resting cells of *P. perfectomarinus* during reduction of nitrite (Fig. 1). As expected, the only gaseous product of nitrite or nitrate reduction detected in the atmosphere above the reaction mixtures that did not contain acetylene was dinitrogen. Previous studies revealed that growing populations and resting suspensions of this bacterium did not release nitrous oxide during ordinary denitrification (1), although a complex fraction displaying the capacity for reducing nitric oxide to nitrous oxide was separated from crude extracts containing a mixture of the nitrogenous oxide reductases (5). Similar quantities of nitrous oxide accumulated in the reaction vessels containing 0.007 and 0.01 atmospheres of acetylene in their gas space, but a significant amount of dinitrogen "leaked" through the blockage in the vessel containing 0.007 atmospheres of acetylene as evidenced by the relative size of the nitrogen peak in that vessel. The blockage was complete, however, and reduction proceeded no further than nitrous oxide in the reaction mixtures containing 0.01 atmospheres of acetylene in their head space. Cells supplied at the outset with nitrous oxide rapidly reduced the gas to dinitrogen in the absence of acetylene, but increasing quantities of acetylene increasingly blocked reduction of nitrous oxide (Fig. 2). Acetylene was not reduced to ethylene, and when it was supplied

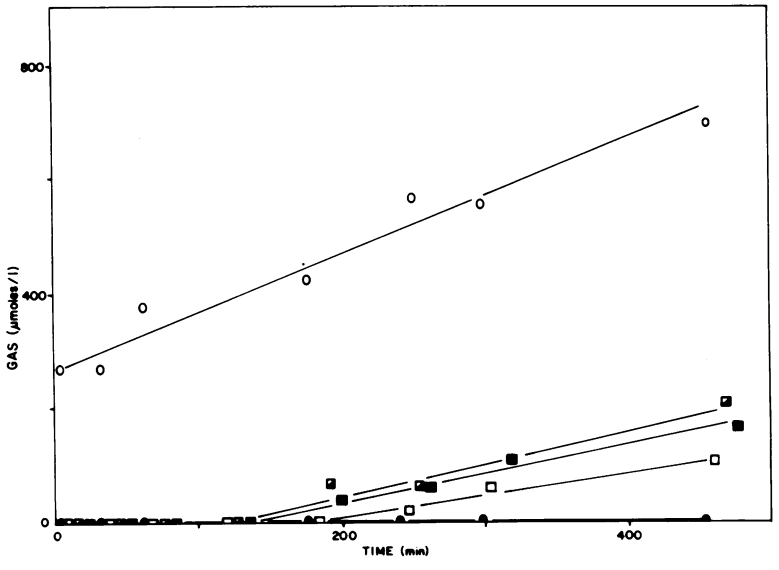

FIG. 1. *Accumulation of nitrous oxide and dinitrogen in whole-cell suspensions of P. perfectomarinus reducing nitrite in the presence of various concentrations of acetylene. Symbols: ○, Dinitrogen produced with no acetylene present; ●, nitrous oxide produced with no acetylene present; □, nitrous oxide produced with 0.003 atmospheres of acetylene present; ◪, nitrous oxide produced with 0.007 atmospheres of acetylene present; ■, nitrous oxide produced with 0.01 atmospheres of acetylene present. Similar results were obtained when nitrate was provided in place of nitrite, but only after an additional 60-min delay.*

in place of acetylene at 0.01 atmospheres, ethylene did not influence the rate of reduction of nitrous oxide.

The inhibitory effect of exposure to acetylene was reversible. When vessels containing cells exposed to atmospheres containing various quantities of acetylene for as long as 400 min were flushed with helium and recharged with nitrous oxide (750 μmol/liter), reduction to dinitrogen proceeded to completion at a rate approaching that exhibited by dinitrifying cells that were not exposed to acetylene. Inhibition by 0.02 atmospheres of acetylene was also reversible; however, the activity of cells exposed to 0.03 atmospheres of acetylene for 400 min before flusing and reincubating was slowed, and

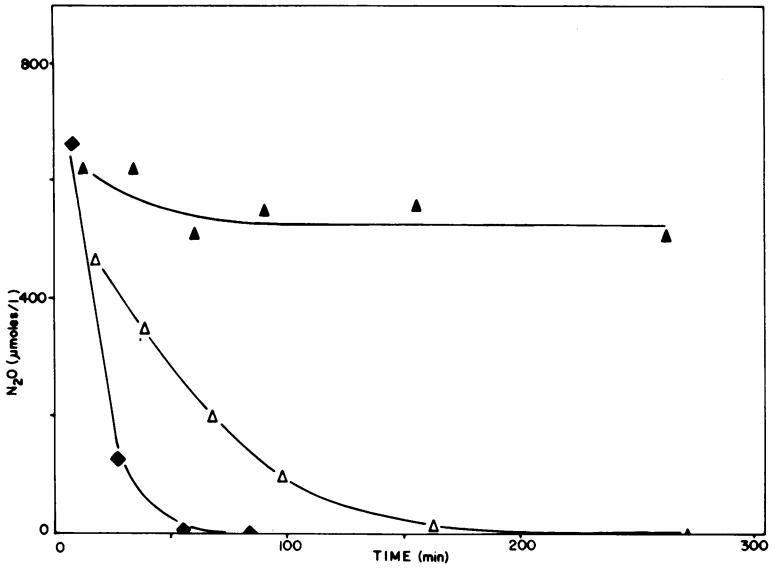

FIG. 2. *Reduction of nitrous oxide in whole-cell suspensions of P. perfectomarinus with various concentrations of acetylene present. Symbols: ▲, 0.01 atmospheres of acetylene; △, 0.002 atmospheres of acetylene; ◆, no acetylene.*

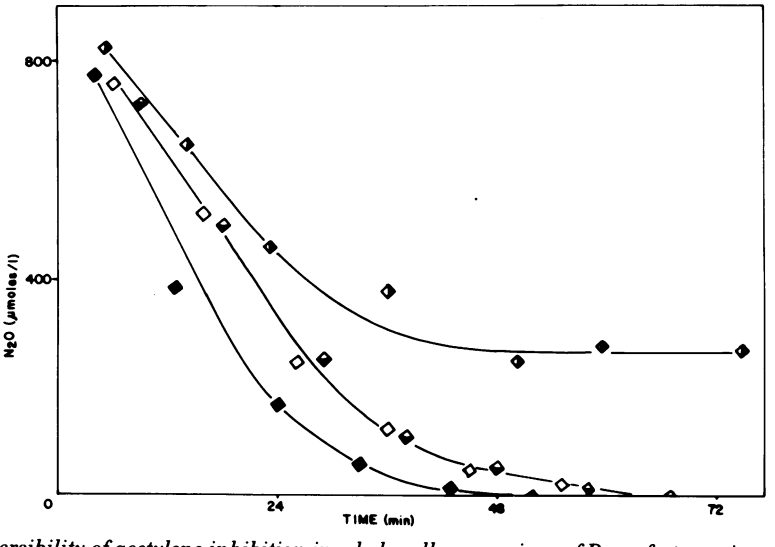

FIG. 3. *Reversibility of acetylene inhibition in whole-cell suspensions of P. perfectomarinus after exposure for 400 min to various concentrations of acetylene. Symbols: ◆, No exposure to acetylene; ◇, after exposure to 0.01 atmospheres of acetylene; ◈, after exposure to 0.02 atmospheres of acetylene; ◈, after exposure to 0.03 atmospheres of acetylene.*

the cells did not complete reduction of the nitrous oxide provided (Fig. 3).

The presence of 0.01 atmospheres of acetylene did not alter the rates of anaerobic growth of *E. aerogenes* dependent on assimilatory reduction of either nitrate or nitrite. Similarly, growth of *T. viride* at the expense of assimilatory nitrate and nitrite reduction was not affected by acetylene. The growth of either organism in media containing ammonium ion was not affected by the presence of acetylene.

In preliminary experiments (Fig. 4), we found that the presence of 0.01 atmospheres of acetylene above the glucose-nitrate-supplemented marine marsh sediment slurries consistently permitted the accumulation of quantities of nitrous oxide significantly greater than those produced in the absence of acetylene. Bacteria in slurries supplemented with acetate and nitrate and exposed to acetylene also consistently produced significant quantities of nitrous oxide. However, nitrous oxide production was not detected in the atmosphere of these acetate-nitrate-supplemented reaction mixtures in the absence of acetylene.

The denitrifying potential of the bacterial population of a marine marsh mud sample may be estimated by enumeration of denitrifying bacteria or by adding nitrate or nitrite to the system and measuring the output of dinitrogen (4). In contrast, estimation of actual ongoing rates of release of dinitrogen from naturally generated nitrate or nitrite is difficult because quantities liberated in the short term are too small for reliable quantitation and because the extent of simultaneous nitrogen fixation may not be known. Blockage of the type displayed here by acetylene is needed to permit more rapid accumulation of a measurable sample of a gas that is undeniably the product of denitrification.

The results of the present preliminary experiments indicate that the blockage does occur when the technique is applied to marine sediment. Even though electron donor and nitrate supplementation was used in these experiments, the results are promising. It appears likely that in situ experiments could detect nitrous oxide buildup without supplementation with nitrogenous oxides and electron donors in the presence of 0.01 atmospheres of acetylene. The unknown quantity of nitrogen fixation would not be a problem in the short-term type of assay currently envisioned because nitrous oxide is not fixed.

Measurement of transient quantities of nitrous oxide is an accepted method of qualitatively assaying for denitrification in soil and water. No other biological source of nitrous oxide is known. Because this gas is ordinarily an unreliable quantity subject to continual reduction in systems that are not blocked, and because the relatively small amounts of dinitrogen released by denitrification over a small area of the earth's surface are diluted by such a large reservoir as the earth's atmosphere, quantitation of actual daily and yearly rates of dinitrogen release in nature has not been achieved. The results presented by Fedorova et al. (2) and those reported here indicate that acetylene may be useful as a barrier that can be used, relieved, and reapplied repeatedly in natural systems to provide quantifiable amounts of nitrous oxide over prolonged intervals of time without significantly altering denitrifying populations. Studies testing this hypothesis are currently underway.

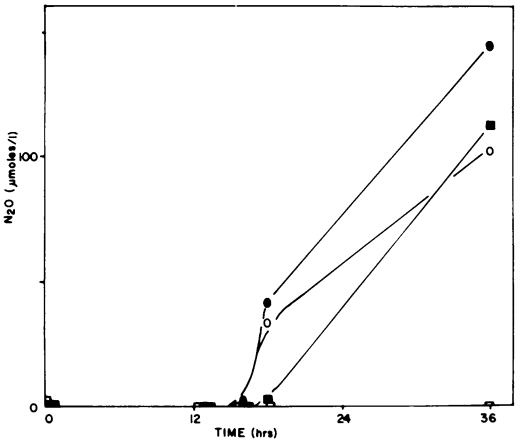

FIG. 4. *Accumulation of nitrous oxide above supplemented marine sediment slurries with and without acetylene. Symbols: ○, Glucose and nitrate; ●, glucose, nitrate, and 0.014 atmospheres of acetylene; □, acetate and nitrate; ■, acetate, nitrate, and 0.014 atmospheres of acetylene.*

ACKNOWLEDGMENTS

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