APPLIED AND ENVIRONMENTAL MICROBIOLOGY, Oct. 1986, p. 957–959
0099-2240/86/100957-03$02.00/0

Vol. 52, No. 4

Dinitrogen Production from Nitrite by a *Nitrosomonas* Isolate

MARK POTH

Pacific Southwest Forest and Range Experiment Station, U.S. Department of Agriculture Forest Service, Riverside, California 92507

Received 3 March 1986/Accepted 14 July 1986

A chemolithotrophic ammonium-oxidizing bacterium that was able to reduce $^{15}NO_2^-$ to $^{15}N_2$ (*m/z* 30) while oxidizing ammonium under conditions of oxygen stress was isolated from stream sediments. Energy was derived from ammonium oxidation, as evidence by growth, with CO_2 serving as the sole C source. The organism was a gram-negative, motile, short rod that failed to grow either aerobically or anaerobically in heterotroph media. The organism was identified as a *Nitrosomonas* sp.

Because of the importance of NO and N_2O in atmospheric chemistry (6, 7, 15) and the concern over their rising concentrations (20, 21), much attention has been given to the production of these gasses by chemolithotrophic ammonium oxidizers (nitrifiers). This phenomenon was studied in soils, sediments, and wastewater, with washed cell preparations and in pure culture (2–5, 8, 10–13, 19, 23, 29, 30).

Ritchie and Nicholas (23) presented evidence that N_2O was produced by *Nitrosomonas europaea* from unstable intermediates in ammonia oxidation and by nitrite reduction (denitrification). More recently, a kinetic analysis of ^{15}N and ^{14}N incorporation into N_2O produced from 99% $^{15}NO_2^-$ and 99% $^{14}NH_4^+$ demonstrated that N_2O production by nitrifiers was consistent with the hypothesis that denitrification was the sole mechanism of production under the conditions used (19). Given that certain chemolithotrophic nitrifiers are able to denitrify (i.e., reduce nitrogen oxides to gaseous products), then the possibility exists that some nitrifiers are able to reduce nitrite completely to dinitrogen, the final product in the denitrification process (14, 18). The aim of this work was to isolate a chemolithotrophic ammonium oxidizer capable of producing dinitrogen from nitrite.

Isolations were attempted from soil, sediment, sewage sludge, and water samples. The ammonium oxidizer isolation protocol involved inoculating Soriano and Walker medium (25) at pH 7 (10 ml in a test tube [10 by 150 mm] with a Durham tube) with 1 ml of a field-collected sample. The medium and sample were mixed, and 1 ml was transferred to the next test tube in the rack. This serial dilution was repeated until 10 test tubes were inoculated. The tubes were repeatedly checked at 1-week intervals. Any culture showing a pH change with incubation was transferred to 10 fresh tubes of medium in the same manner as the original sample. At the third such transfer, cultures demonstrating gas production in the Durham tubes were inoculated into tryptic soy broth, nutrient broth, and AC medium (Difco Laboratories, Detroit, Mich.), and after incubation at 25°C for 30 days, they were examined macroscopically (for growth) and microscopically (for organisms with a different morphology from those observed in the Soriano and Walker medium) to check for heterotrophic contaminants.

To assay for dinitrogen production from nitrite, isolates were inoculated into three 250-ml flasks with 50 ml of a $CaCO_3$-buffered ammonium oxidizer medium (1). The ammonium concentration was initially 7.5 mM (375 μmol per flask). $^{15}NO_2^-$ (ICON Inc., Summit, N.J.) was added to this medium to a final concentration of 0.4 mM (20 μmol per flask). Identical flasks were inoculated with *N. europaea* ATCC 19718 and *Nitrosolobus multiformis* ATCC 25196 obtained from the American Type Culture Collection, Rockville, Md. Three flasks were inoculated with sterile Soriano and Walker medium from an uninoculated test tube and analyzed to account for chemical production of N_2O and dinitrogen. Flasks were aseptically sealed with serum stoppers and incubated without shaking (25°C) to induce oxygen stress as the ammonium oxidizers grew and consumed oxygen (19).

Once the chemolithotrophic ammonium oxidizer had grown and all the ammonium in the flasks was oxidized (approximately 30 days), as determined by reacting aseptically removed samples with Nessler reagent, the headspace gas was analyzed for N_2O via electron-capture gas chromatography. An HP 5790 gas chromatograph (Hewlett-Packard Co., Fullerton, Calif.) equipped with a Porapak Q (80/100 mesh) column (2 m by 3.2 mm) (Waters Associates, Inc., Milford Mass.) operated at 50°C was used for the analysis. The detector temperature was 340°C, and dinitrogen was the carrier gas at 35 ml min^{-1}. Interfaced gas chromatography-mass spectrometry (9, 26) was used to determine the *m/z* 29 and *m/z* 30 headspace dinitrogen concentrations. Isotope content was calculated as follows: moles of ^{15}N excess = moles of ^{15}N in sample − moles of ^{15}N at natural abundance. Atmospheric N_2 was used as the standard for natural abundance measurements (16, 17).

After the headspace gas analysis was completed, the cultures were inoculated into Soriano and Walker medium to test for ammonium oxidation. Subsamples of the cultures were also inoculated into the heterotroph media mentioned above to test for contamination. Final nitrite concentrations were determined colorimetrically with a Technicon autoanalyzer (Technicon Instruments Corp., Tarrytown, N.Y.).

No heterotrophic contamination was detected upon either macroscopic or microscopic (to compare morphologies) examination of the inoculated heterotroph media. All cultures oxidized ammonium to nitrite when reinoculated into sterile Soriano and Walker medium. The isolate met all C requirements by fixing CO_2. The isolated organism was a gram-negative, short, motile rod. On the basis of these characteristics, the isolate was identified as a *Nitrosomonas* sp. (27, 28). It was isolated from stream sediments from a chaparral-covered watershed in the San Gabriel Mountains in southern California. As part of the south coast air basin, which includes the greater Los Angeles metropolitan area, this area receives chronic acidic atmospheric deposition, which includes a significant N component (22).

TABLE 1. Incorporation of $^{15}NO_2^-$ (20 μmol) into gaseous products and NH_4^+ (375 μmol) oxidation to NO_2^- by aseptically sealed, statically incubated, chemolithotrophic ammonium oxidizer batch cultures[a]

Organism	N_2O (μl/liter)	Excess $^{15}N_2$ (nmol)		NO_2 (μmol)
		m/z 29	*m/z* 30	
None	<1.0	−2.2 ± 8.2	1.4 ± 8.1	20.0 ± 1.5
N. europaea	216 ± 75	1.0 ± 8.3	2.3 ± 7.6	392 ± 1.97
Nitrosolobus multiformis	<1.0	2.4 ± 7.3	3.7 ± 8.9	394 ± 3.45
Nitrosomonas sp.	<1.0	81.4 ± 14	87.4 ± 16	394 ± 2.96

[a] All values are the mean (± the standard deviation) of three determinations.

Labeled N from $^{15}NO_2^-$ was incorporated into $^{15}N_2$ by the *Nitrosomonas* sp. isolate under oxygen-stressed (static-incubation) conditions (Table 1). $^{15}NO_2^-$ was not incorporated into N_2 by *N. europaea* or *Nitrosolobus multiformis*. $^{15}N_2$ was not detected at levels above natural abundance in the sterile controls, eliminating the possibility of chemical formation of dinitrogen. N_2O production from the isolated *Nitrosomonas* sp. was undetectable.

The amount of label incorporated into dinitrogen was very small, representing 0.85% of the $^{15}NO_2^-$ added and only 0.045% of the NO_2^- produced by nitrification. This parallels the situation for N_2O production by nitrifiers (4, 12) that Hynes and Knowles (13) pointed out. If N_2 and N_2O formation are minor side reactions of nitrification (23), their production in small amounts would be expected. Alternatively, it may be that only a small fraction of the cells are under the conditions required for N_2 or N_2O formation. If denitrification is responsible for N_2 and N_2O formation, then the cells would require microaerophilic conditions to allow NH_4^+ oxidation and electron production, conserving O_2 for the oxidation of NH_4^+. In statically incubated cell suspensions, the fraction of the cells under aerobic conditions and the fraction under microaerophilic conditions can be controlled by (i) the cell density, owing to the high O_2 consumption rate of chemolithotrophic ammonium oxidizers (19); (ii) the surface area available for O_2 diffusion into the liquid; and (iii) the headspace O_2 concentration. Hence, at high enough cell densities, the aerobic zone is negligible and the rate of gaseous N production can approach that of ammonium (19) or hydroxylamine oxidation (29).

The mechanism proposed for the formation of N_2O according to the nitrification hypothesis (23) fails to account for N_2 formation from NO_2^-. In addition, the ratio of *m/z* 29 to *m/z* 30 N_2 production was 1.87, which approaches the theoretical ratio of two based on denitrification kinetics for N_2O production (19). Since no N_2O was released by this isolate, the kinetics for N_2 production should coincide with those for N_2O production.

The isolation of this dinitrogen-producing chemolithotrophic ammonium oxidizer which could reproducibly reduce NO_2^- to N_2 took 3 years. Nevertheless, nitrifying cultures can be isolated that produce dinitrogen gas (usually pinpoint-size bubbles) in Durham tubes. This ability is apparently very unstable, however, since several presumptive cultures failed a second confirmatory isotope experiment before the *Nitrosomonas* sp. discussed here was isolated. The loss of N_2O-reducing activity by chemolithotrophic ammonium oxidizers with continued culture parallels the loss observed for some heterotrophic denitrifiers with continued culture (14). The *Nitrosomonas* isolate is similar to some heterotrophic denitrifiers (14, 18) in that it produces little or no N_2O.

Denitrification (sensu stricto) is the bacterial reduction of ionic N oxides to gaseous N products in support of anaerobic growth. This definition is the basis of the readily performed diagnostic test for heterotrophs. Denitrification (sensu lato) is the reduction of ionic N oxides to gaseous products when oxygen is limiting (14, 18). This definition implies but does not require anaerobic growth of a denitrifying organism. Indeed, aerobic denitrification (24) is accomplished by bacteria from environments with a niche defined by highly variable oxygen partial pressures over short time intervals. These conditions have selected for constitutively denitrifying organisms. The production of $^{15}N_2$ (*m/z* 30) dinitrogen from $^{15}NO_2^-$ by *Nitrosomonas* sp. meets the sensu lato definition of denitrification. It also supports work showing that the kinetics of N_2O formation by *N. europaea* agrees with denitrification as the mechanism of N_2O production (19) and that it is nitrite reduction and not ammonia oxidation directly that accounts for the production of gaseous N species by nitrifiers. If nitrifiers do denitrify, then the reduction of NO_2^- should be coupled to energy generation (electron transport). Nitrifiers cannot be grown under anaerobic denitrifying conditions with either NH_4^+, because O_2 is required for the initial oxidation to NH_2OH, or NH_2OH, because of inhibitory effects which prevent even aerobic growth on this substrate. The definitive anaerobic, denitrifying growth of nitrifiers might await the advent of a suitable artificial electron donor.

Thanks are due to D. D. Focht for helpful discussions and for making the mass spectrometer available for this study. Valuable comments on the manuscript were provided by M. R. Hyman and two anonymous reviewers.

LITERATURE CITED

1. **Alexander, M., and F. E. Clark.** 1965. Nitrifying bacteria. Agronomy **9**:1477–1483.
2. **Anderson, I. C., and J. S. Levine.** 1986. Relative rates of nitric oxide and nitrous oxide production by nitrifiers, denitrifiers, and nitrate respirers. Appl. Environ. Microbiol. **51**:938–945.
3. **Aulakh, M. S., D. A. Rennie, and E. A. Paul.** 1984. Acetylene and N-serve have effects upon the N_2O emissions from NH_4^+ and NO_3^- treated soils under aerobic and anaerobic conditions. Soil Biol. Biochem. **16**:351–356.
4. **Blackmer, A. M., J. M. Bremner, and E. L. Schmidt.** 1980. Production of nitrous oxide by ammonia-oxidizing chemoautotrophic microorganisms in soil. Appl. Environ. Microbiol. **40**:1060–1066.
5. **Conrad, R., and W. Seiler.** 1985. Localization of microbial activities relevant to the emission of nitrous oxide from the soil into the atmosphere. Soil Biol. Biochem. **17**:893–895.
6. **Crutzen, P. J.** 1981. Atmospheric chemical processes for the oxides of nitrogen including nitrous oxide, p. 17–45. *In* C. C. Delwiche (ed.), Denitrification, nitrification and atmospheric nitrous oxide. John Wiley & Sons, Inc., New York.
7. **Dickinson, R. E., and R. J. Cicerone.** 1986. Future global warming from atmospheric trace gases. Nature (London) **319**:109–115.
8. **Duxbury, J. M., and P. K. McConnaughey.** 1986. Effect of

fertilizer source on denitrification and nitrous oxide emissions in a maize field. Soil Sci. Soc. Am. J. **50**:644–648.
9. **Focht, D. D., N. Valoris, and J. Lety.** 1980. Use of interfaced gas chromatography-mass spectrometry for detection of concurrent mineralization and denitrification in soils. J. Environ. Qual. **9**:218–223.
10. **Goodroad, L. L., and D. R. Keeney.** 1984. Nitrous oxide production in aerobic soils under varying pH, temperature and water content. Soil Biol. Biochem. **16**:39–43.
11. **Goodroad, L. L., and D. R. Keeney.** 1985. Site of nitrous oxide production in field soils. Bio. Fertil. Soils **1**:3–7.
12. **Goreau, T. J., W. A. Kaplan, S. C. Wofsy, M. B. McElroy, F. W. Valois, and S. W. Watson.** 1980. Production of NO_2^- and N_2O by nitrifying bacteria at reduced concentrations of oxygen. Appl. Environ. Microbiol. **40**:526–532.
13. **Hynes, R. H., and R. Knowles.** 1984. Production of nitrous oxide by *Nitrosomonas europaea*: effects of acetylene, pH and oxygen. Can. J. Microbiol. **30**:1397–1404.
14. **Knowles, R.** 1982. Denitrification. Microbiol. Rev. **46**:43–70.
15. **Levine, J. S., and F. Allario.** 1982. The global troposphere: biogeochemical cycles, chemistry, and remote sensing. Environ. Monit. Assess. **1**:263–309.
16. **Mariotti, A.** 1983. Atmospheric nitrogen is a reliable standard for natural ^{15}N abundance measurements. Nature (London) **303**:685–687.
17. **Mariotti, A.** 1984. Natural ^{15}N abundance measurements and atmospheric nitrogen standard calibration. Nature (London) **311**:251–252.
18. **Payne, W. J.** 1981. Denitrification. John Wiley & Sons, Inc., New York.
19. **Poth, M., and D. D. Focht.** 1985. ^{15}N kinetic analysis of N_2O production by *Nitrosomonas europaea*: an examination of nitrifer denitrification. Appl. Environ. Microbiol. **49**:1134–1141.
20. **Prather, M. J.** 1985. Continental sources of halocarbons and nitrous oxide. Nature (London) **317**:221–225.
21. **Rasmussen, R. A., and M. A. K. Khalil.** 1986. Atmospheric trace gases: trends and distributions over the last decade. Science **232**:1623–1624.
22. **Riggan, P. J., E. N. Lopez, and R. N. Lockwood.** 1985. Deposition and processing of airborne nitrogen pollutants in Mediterranean-type ecosystems of southern California. Environ. Sci. & Technol. **19**:781–789.
23. **Ritchie, G. A. F., and D. J. D. Nicholas.** 1972. Identification of the sources of nitrous oxide produced by oxidative and reductive processes in *Nitrosomonas europaea*. Biochem. J. **126**:1181–1191.
24. **Robertson, L. A., and J. G. Kuenen.** 1984. Aerobic denitrification: a controvery revisited. Arch. Microbiol. **139**:351–354.
25. **Soriano, S., and N. Walker.** 1968. Isolation of ammonium-oxidizing autotrophic bacteria. J. Appl. Bacteriol. **31**:493–497.
26. **St. John, R. T., and T. C. Hollacher.** 1977. Nitrogen 15 tracer studies on the pathway of denitrification in *Pseudomonas aeruginosa*. J. Biol. Chem. **252**:212–218.
27. **Watson, S. W.** 1974. Family I. *Nitrobacteraceae* Buchanan 1917, 349, emend. mut. char. Starkey 1948, 69; Watson 1971, 262, p. 450–456. *In* R. E. Buchanan and N. E. Gibbons (ed.), Bergey's manual of determinative bacteriology, 8th ed. The Williams & Wilkins Co., Baltimore.
28. **Watson, S. W., F. W. Valois, and J. B. Waterbury.** 1981. The family *Nitrobacteraceae*, p. 1005–1022. *In* M. Starr, H. Stolph, H. G. Truper, A. Balous, and H. G. Schlegel (ed.), The prokaryotes. Springer-Verlag, New York.
29. **Yoshida, T., and M. Alexander.** 1970. Nitrous oxide formation by *Nitrosomonas europaea* and heterotrophic microorganisms. Soil Sci. Soc. Am. Proc. **34**:880–882.
30. **Yoshinari, T., and M. Wahlen.** 1985. Oxygen isotope ratios in N_2O from nitrification at a wastewater treatment facility. Nature (London) **317**:349–350.